Search for *Pseudomonas aeruginosa*

Immune-modulatory but Non-inducing agents

by

GOPAL P. JADHAV, M. Pharm. (Pune)

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To my beloved wife Madhuri

&

To my parents

This thesis is gratefully dedicated.

Their constant encouragement and inspiration made this work possible.

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ABSTRACT

Dysfunctional immunity which is the most common antecedent of immunological diseases is central to the common disorders like rheumatoid arthritis, psoriasis, thyroid disease, type-1 diabetes mellitus, multiple sclerosis and other autoimmune conditions that are leading causes of chronic morbidities and disabilities. The currently employed therapies suffer from serious side effects. For example, steroid therapy, being nonselective is seldom used. Myleotoxicity limits azathioprine while cyclosporine-A (CsA) and FK506 do show myleo-, nephro and neuro-toxicities. Antibody therapies cause immune mediated toxicity. Therefore, the need of developing less toxic and specific immune modulatory agents is a top priority.

Recently, *Pseudomonas aeruginosa* quorum sensing signal molecules (QSSMs), *N*-(3-oxododecanoyl)-L-homoserine lactone (3O, C_{12}-HSL) and 2-*n*-heptyl-3-hydroxy-4-(1*H*)-quinolone (PQS) have been found to modulate eukaryotic immune processes via different targets. The structure activity relationship (SAR) study of 3O, C_{12}-HSL by Chhabra et.al, showed that L-homoserine lactone ring, 3-oxo or hydroxyl group and 12 to14-carbons long acyl chain, apparently conferring optimum lipophilicity and flexibility, are important for immune modulation. However, the new analogues including 3O, C_{12}-HSL negated clinical usefulness as these also participated in bacterial quorum-sensing (QS) activity thus promoting bacterial growth. Also analogues with more than 12-carbons alkyl chain could not be explored by *in vitro* immune assays due to their limited solubility in the solvents employed.

The present study addresses these issues by making further judicial structural changes in the 3O, C_{12}-HSL in order to optimize its immune modulatory activity while losing its intrinsic QS activity. Accordingly, a series of new ring variants, 3-acetoxy, aza, thia, oxa and fluorine substituted analogues were developed. The later were largely confined in the 1, 3-dicarbonyl segment of the 3O, C_{12}-HSL structure in order to modulate the *3-enolic* content of the molecule. Also tetramic acid analogue, a non-enzymatic degradative product of 3O, C_{12}-HSL was synthesized. After purity and identity characterization the new analogues were evaluated for their immune modulation (with murine cells proliferation assay), cytotoxicity (with *Trypan* dye

exclusion assay) and auto-inducing or QS activity (with a specifically designed *lux* bacterial bio-reporter).

The splenocyte proliferation assay demonstrated that immune suppression in 4-aza analogues increased with increasing alkyl chain (up to C_{15}) yet without any solubility problems in the standard solvents used. Similar trend was observed when N^4 was alkyl substituted (*n*-propyl, being most potent). At least one of the C^2 hydrogens was found to be essential for activity since, difluoro substitution lost activity. Retention of immune suppression by N^3-OH and increased potency by N^3-NH_2 (EC_{50}=0.99 µM) demonstrated that C^3=O can be replaced by an appropriate H-bond donor/acceptor groups. Complete loss of activity with 3-thia substitution (3-thia C_{12}-HSL) and then regain of potency by 3-sulfoxide/sulfone derivatives further demonstrated the importance of 3-oxo (suitable H-bond acceptor/donor) in immune suppression. Insertion of oxygen at C^4 retained activity but additional oxygen(s) in the side chain yielded analogues that were devoid of immune suppression. Analogue with thiazole ring instead of HSL ring retained activity. In case of hetero ring altered analogues of 3O, 4-aza C_{12}-HSL, almost all retained immune suppressive activities except their oxa analogues.

In conclusion, all the above strategies have delivered potent immune modulatory agents yet gratifyingly devoid of QS activity. Some of the most potent and promising non-inducing immune modulatory agents are 3O, 4-aza-C_{15}-HSL (EC_{50} 0.65 µM), 3-NH_2-3-aza-C_{12}-HSL (EC_{50} 0.99 µM) and *N*, *N*'-dimethyl-2-(3-oxo-4-azadodecanoyl) aminobenzamide (EC_{50} 0.7 µM) and are worthy of further study to be developed as therapeutic agents.

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List of abbreviations

Δ	Heat
°C	Degrees centigrade
μM	Micromolar
3O, C_{12}-HSL	*N*-(3-Oxododecanoyl)-L-homoserine lactone
5HT	5-Hydroxytryptophan
ABMT	Autologous bone marrow transplant
Ac	Acetyl
ADCC	Antibody dependant cell mediated cytotoxicity
AHL	Acylated homoserine lactone
AI	Auto-inducer
AIDS	Acquired immunodeficiency syndrome
ALG	Anti-lymphocyte immunoglobulin
Anal.	Elemental analysis
APCs	Antigen presenting cells
aq	Aqueous
ATG	Anti-thymocyte immunoglobulin
AZT	Zidovudine
B. subtilis	*Bacillus subtilis*
BALT	Bronchial associated lymphoid tissue
B-Cells	B-lymphocytes
BCG	Bacille-Calmette and Guirin
BCR	B-Cell receptors
BHL	*N*-Butanoyl-L-homoserine lactone
BMDM	Bone-marrow derived macrophages
Boc	*tert*-Butoxy carbonyl
BPO	Benzoyl peroxide
C_4-HSL	*N*-Butyryl-L-homoserine lactone
C_6-HSL	*N*-Hexanoyl-L-homoserine lactone
$CDCl_3$	Deuterated chloroform
CDI	1,1'-Carbonyldiimidazole
CF	Cystic fibrosis
CFTR	Cystic fibrosis trans-membrane conductance regulator
CLL	Chronic lymphocytic leukemia
ConA	Concanavalin A
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CPGES	Cytosolic prostaglandin E synthase
CRTH-2	Chemo-attractant receptor homologous expressed in T_H2 cells
CsA	Cyclosporine A
CSF	Colony-stimulating factor
CTL or T_c	Cytotoxic lymphocytes
d	Doublet (NMR)
DBU	1,8-Diazabicyclo[5.4.0]-undec-7-ene
DC	Dendritic cells
DCCI	1,3-Dicyclohexylcarbodiimide
DCE	Dichloroethane
DCM	Dichloromethane
DCU	*N*,*N*'-Dicyclohexylurea
DHEA	Dehydroepiandrosterone
DIEA/DIPEA	Diisopropylethylamine
DKP	Diketopiperazine
DMAP	4-Dimethylaminopyridine
DMARDs	Disease modifying anti-rheumatic drugs
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid

DSG	15-Deoxyspergualine
DTH	Delayed type hypersensitivity
EC_{50}	Effective concentration to inhibit 50 % proliferating cells
EDC	1-Ethyl-3-(dimethylaminopropyl)-carbodiimide
eIF2α	Eukaryotic translation initiation factor-2α
ER	Endocyclic reticulum
Et	Ethyl
Fig	Figure
FK506	Tracolimus
g	Gram(s)
GALT	Gut associated lymphoid tissue
G-CSF	Granulocyte-colony-stimulating factor
gem	Geminal
GM-CSF	Granulocyte-macrophages-colony-stimulating factor
GvHD	Graft versus host disease
h	Hour(s)
HAQ	4-Hydroxy-2-alkylquinoline
HHQ	4-Hydroxy-2-heptylquinoline
HIV	Human immunodeficiency virus
HOBt	1-Hydroxybenzotriazole
i.p.	Intra peritoneal
IDDM	Insulin-dependent (Type I) diabetes mellitus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
InPx	Inosine pranobex
IR	Infrared
Itk	Interleukin-2-inducible T-cell kinase
IVIGs	Intravenous immune globulins
L-Asn	L-Aspargine
L-Gln	L-Glutamine
L-HSL	L-Homoserine lactone
LMS	Levamisole
LPS	Lipo-polysaccharides
m	Multiplet
M	Molar
m.p.	Melting point
m/z	Mass to charge ratio
mAb	Monoclonal antibody
MAC	Membrane attack complex of complement
MALT	Mucosa associated lymphoid tissue
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemolactic protein
MDP	*N*-Acetyl muramyl dipeptide
Me	Methyl
MeCN	Acetonitrile
MetAP	Methionine aminopeptide
MHC	Major histocompatibility complex
min	Minute(s)
MIP	Macrophage inflammatory protein
mL	Milliliters
mm	Millimeter
mmol	Millimole
mol	Moles
mPGES	Membrane-associated prostaglandin E synthase
mRNA	messenger Ribonucleic acid
MS	Mass spectrometry
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NMR	Nuclear magnetic resonance

NO	Nitrous oxide
NOD	Non obese diabetic
n-Pr	*n*-Propyl
NSAID	Non-steroidal anti-inflammatory drug
NT	Not toxic
OD_{495}	Optical density at 495 nm
OHHL	*N*-(3-Oxohexanoyl)-L-homoserine lactone
OVA	Ovalbumin
P. aeruginosa	*Pseudomonas aeruginosa*
PAMPs	Pathogen associated membrane patterns
PARP	Poly(ADP-ribose)polymerase
PBS	Phosphate-buffer saline
Ph	Phenyl
PLC	Preparative thin-layer chromatography
PQS	*Pseudomonas* quinolone signal
PRRs	Pattern recognition receptors
PTK	Protein tyrosine kinase
q	Quartet (NMR)
QS	Quorum sensing
QSSM	Quorum sensing signal molecule
RBCs	Red blood corpuscles
R*f*	Retention factor
rhIFN-α	Human recombinant interferon-alpha
RNA	Ribonucleic acid
s	Singlet (NMR)
S	Cytostatic
S. aureus	*Staphylococcus aureus*
SALT	Skin associated lymphoid tissue
SAR	Structure activity relationship
SCID	Severe combined immunodeficiency disease
SD	Standard deviation
SLE	Systemic lupus erythematosus
t	Triplet (NMR)
TBAF	Tetrabutylammonium fluoride
$TBAHSO_4$	Tetrabutylammonium hydrogensulphate
TCA-3	T-cell activation gene-3
TCR	T-cell receptors
TEA	Triethylamine
TFA	Trifluoroacetic acid
TGF	Tumor growth factor
T_H	T-helper cells
THF	Tetrahydrofuran
TIR	Toll/Interleukin receptor
TRIF	TIR domain-containing adaptor protein inducing interferon β
TLC	Thin-layer chromatography
TMC	Tetramic acid
TMS	Tetramethylsilane
TNF	Tumor necrosis factor
TOF	Time of flight
TP	Thromboxane type prostanoid receptor
TPP	Triphenylphosphine
WBCs	White blood corpuscles
WM	Waldenstrom's macroglobulinemia

1.0 INTRODUCTION

Many people believe that we as a human beings have special obligation to preserve the beauty, complexity, and balance of the world around us since we have greater power than any other species over the fate of the planet. Throughout history the human beings have always put members of the biological world to use in ingenious ways as food, fiber, and medicines. This biodiversity is one of the Earth's greatest treasures. However, the human species has been profoundly affected by microorganisms. These have decimated humanity countless times with high rates of mortality. Moreover, nothing was known until late in the 19th century that infectious agents cause diseases. It was Robert Koch who proved that microorganisms are responsible for infectious diseases. We now distinguish four broad categories of disease-causing microorganisms, or pathogens: (i) viruses (ii) bacteria (iii) pathogenic fungi and (iv) parasites (relatively large and complex eukaryotic organisms). Great microbiologists of 19th century coupled with Koch's discoveries then stimulated the extension of Jenner's strategy of vaccination to other diseases. In the 1880s, Louis Pasteur devised a vaccine against cholera in chickens and also developed a rabies vaccine. In 1890, Emil von Behring and Shibasaburo Kitasato discovered antibodies from the serum of vaccinated individuals. Those discoveries were metamorphosed into other aspects of the immune systems.

The immune system is miraculous. Some sort of cut-and-paste, shuffle-and-deal technique has definitely exists in our immune system that enables it to generate billions of kinds of antibodies in comparison to only about 30,000 human genes. Until 1960's, the molecular-level details of such things were unknown. However, when observed they found to be extremely complicated.[1,2]

Immune systems are generally characterized by their ability to distinguish between self and non-self cells, tissues, or molecules, and to eradicate the non-self. The mammalian immune system is an exceptionally complex and intricate system that can recognize non-self and provide protection from a wide variety of pathogens. Although there is intrinsic bond between its components, the immune system can be divided into two subsystems, the innate and the adaptive immune systems. In general, innate immunity is a non-specific, inducible response to pathogens. It is immediate in action, yet short-

lived. On the other hand, the adaptive immune system is much more specific, but takes longer to activate. It also features immunological memory. Both systems work together to provide protection against a diverse and rapidly-evolving array of pathogens.

However, it is now realized that the immune system can be overactive, as well as underactive, and that balance is the answer. On the one hand, the immune system needs macrophages, natural killer cells and other "soldiers" to stay alert to attack invading organisms before these can infect the body. On the other hand, autoimmune diseases like rheumatoid arthritis, type-1 diabetes, etc. where the body attacks itself, afflict millions of people. Immune overactivity causes unpleasant side effects, such as allergies and inflammation, and can lead to immune exhaustion and collapse. The answer to maintaining health, therefore, is to balance the body's immune system so that it is neither overactive nor underactive. Balancing the immune system, however, is more challenging than simply stimulating it and requires thorough knowledge of the human immune system.

1.1 IMMUNE SYSTEM

The immune system comprises of a diverse army of cells and organs that work together in harmony to protect the human body against attacks by "foreign" invaders, like microbes (germs) e.g. infection-causing organisms such as bacteria, viruses, parasites, and fungi. Because the human body makes available an ideal environment for many microbes, thus making it prone to microbial infection. However, it is the immune system's job to keep them out or, to seek out and ward them off. The immune system is astonishingly complex. It can recognize and remember several different enemies and can generate secretions and cells to wipe out each one of them. The secret to its success is an active and sophisticated communications network. A large numbers of cells, organized into sets and subsets, co-operate and pass information among themselves like clouds of bees swarming around a hive. Once immune cells receive the alarm, they undergo strategic changes and start generating dominant chemicals. These substances allow the cells to regulate their own growth and behavior and recruit new cells to harm foreign invaders.[3]

Immune system cells in particular, white blood corpuscles (WBC's) or leukocytes roam in the body to detect localized infections. Organs on the other hand focus where leukocytes mature and where they effectively interact with antigens to become effector or memory cells. Effector cells eliminate antigens whereas memory cells more effectively response to repeated antigen exposure.[4] The organs of the immune system are situated throughout the body. They can be divided as primary and secondary *lymphoid organs* as shown in **Fig 1.1**.

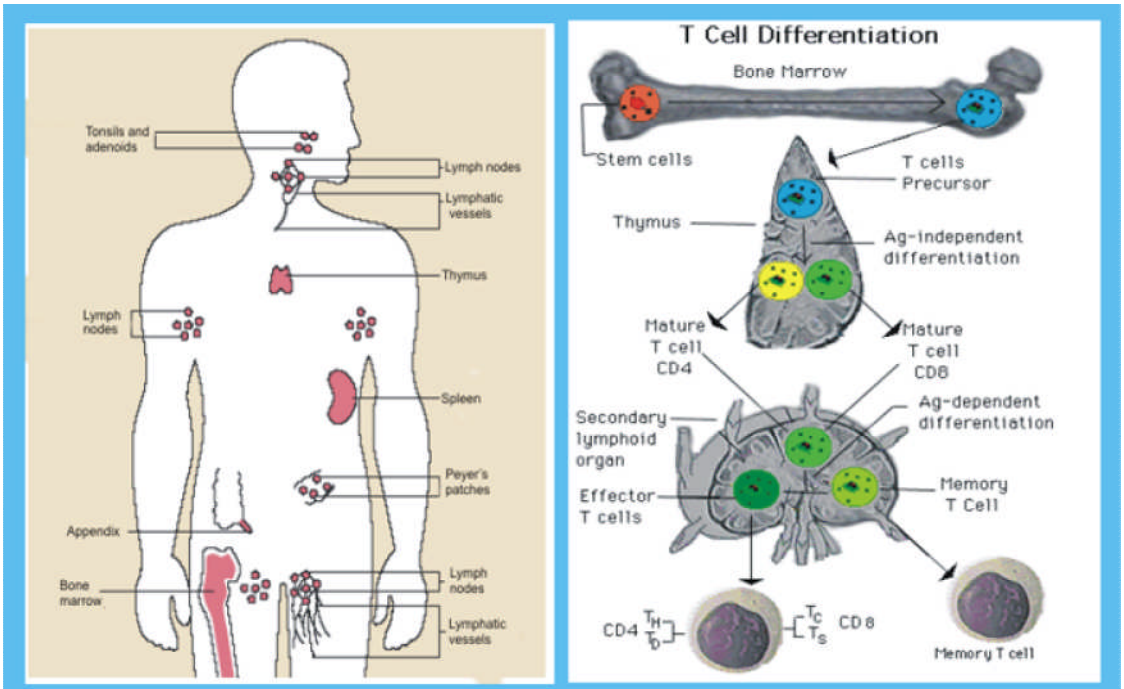


Fig 1.1 Locations of the primary and secondary lymphoid organs in the body

1.1.1 Primary (Central) Lymphoid Organs

The bone marrow and the thymus are the primary lymphoid organs, where the lymphocytes (responsible for specific immunity) originate. The bone marrow, the soft tissue in the hollow center of bones, is the vital source of all blood cells, including white blood cells destined to become immune cells. Both B-lymphocytes and T-lymphocytes are produced from stem cells in the bone marrow. B-lymphocytes however, mature in the bone marrow whereas T-lymphocytes migrate to the thymus (an organ that lies behind the breastbone) and mature there.

Lymphocytes can travel throughout the body using the blood vessels as well as lymphatic vessels that closely parallels the body's veins and arteries. The exchange of cells and fluids between blood and lymphatic vessels enables the lymphatic system to monitor the body for invading microbes. The lymphatic vessels carry a clear fluid called lymph that bathes the body's tissues. During haematopoiesis, lymphocytes gain specific antigen receptors, cytokine receptors, co-receptors and carrier molecules that take cells to a particular immune organ. Haematopoiesis is regulated by growth factors, growth factor receptors and apoptosis. After maturation both lymphocytes circulate in blood until they accumulate in one of the secondary lymphoid organs.[3,4]

1.1.2 Secondary (Peripheral) Lymphoid Organs

Peripheral lymphoid tissues are available throughout the body and are responsible for adaptive immune response. Secondary lymphoid organs comprise of (a) highly organized **lymph nodes** and **spleen** and (b) less organized accumulation of lymphocytes scattered throughout body such as tonsils and adenoids, the appendix, the Peyer's patches in the lining of the small intestines (gut associated lymphoid tissues-GALT), lymphoid tissues beneath the bronchial mucous membranes (bronchial associated lymphoid tissue or BALT) and lymphoid tissues aggregate throughout mucosal membrane (Mucosa associated lymphoid tissues or MALT), and beneath the skin (Skin associated lymphoid tissues or SALT).[5-7]

Small, bean-shaped **lymph nodes** are laced along the lymphatic vessels, with clusters in the neck, armpits, abdomen, and groin. Each lymph node contains specialized compartments where immune cells assemble, and where they can come across antigens. The **spleen** is a flattened organ at the upper left of the abdomen. Like the lymph nodes, the spleen contains specialized compartments where immune cells accumulate and work. It also serves as a meeting point where immune defenses strike antigens.

Adaptive immune responses demand antigen-presenting cells such as macrophages, dendritic cells (DCs), and ever changing population of B-lymphocytes and T-lymphocytes. Antigen-presenting cells collectively detect antigens in the secondary lymphoid organs. Lymphatic clusters and **M-cells** (specialized antigen collecting

epithelial cells) accumulate at the mucosal membranes of respiratory, digestive and urogenital systems where pathogen contact is highest. Reticular fibers that support antigen-presenting cells are present in both peripheral organs (lymph nodes and spleen). When microorganisms or other antigens enter the tissues, they are carried into secondary organs, where they encounter the ever changing B-lymphocyte population, thereby filtered out and phagocytosed by fixed macrophages and dendritic cells and finally exposed to changing T-lymphocyte population.[3,4]

1.1.3 Lymphatic Vessels

Lymphatic vessels regulate the flow of lymph within lymphoid system. Fluid (lymphocyte) exits the blood circulation through high endothelium venules, and bathes the tissues, supplying nutrients and washing away waste products. Antigen-presenting cells, entering through afferent lymphatic vessels, in lymph nodes, activate the lymphocytes. Antigens in paracortex generally activate T-cells. In germinal centers of the lymphoid follicles activated B-cells become antibody producing plasma cells.[1] Activated lymphocytes leave the nodes through efferent lymphatics and carried back to blood stream through fluid in lymphatic vessels.[3-6]

1.1.4 Products of Immune Cells

The immune system reserves a huge arsenal of immune cells, not only lymphocytes but also cell-engulfing phagocytes and their family members as shown in **Fig 1.2**. To be effective in their work, most immune cells require the cooperation of other cells of the immune system. Sometimes immune cells communicate by direct physical contact, sometimes by releasing chemical messengers. The immune system stores specific cells needed to recognize millions of enemies. When an antigen appears, those matching cells multiply into full-scale soldiers. On completeing their task, these lie dormant leaving behind an army to watch for future attacks. All immune cells begin as immature stem cells in the bone marrow. They respond to different cytokines and other signals to grow into specific immune cell types, such as T-cells, B-cells, or phagocytes. Before maturity, stem cells however, are an interesting possibility for treating some immune system disorders. It is under investigation whether, a person's own stem cells

can be used to regenerate damaged immune responses in autoimmune diseases and immune deficiency diseases.[8]

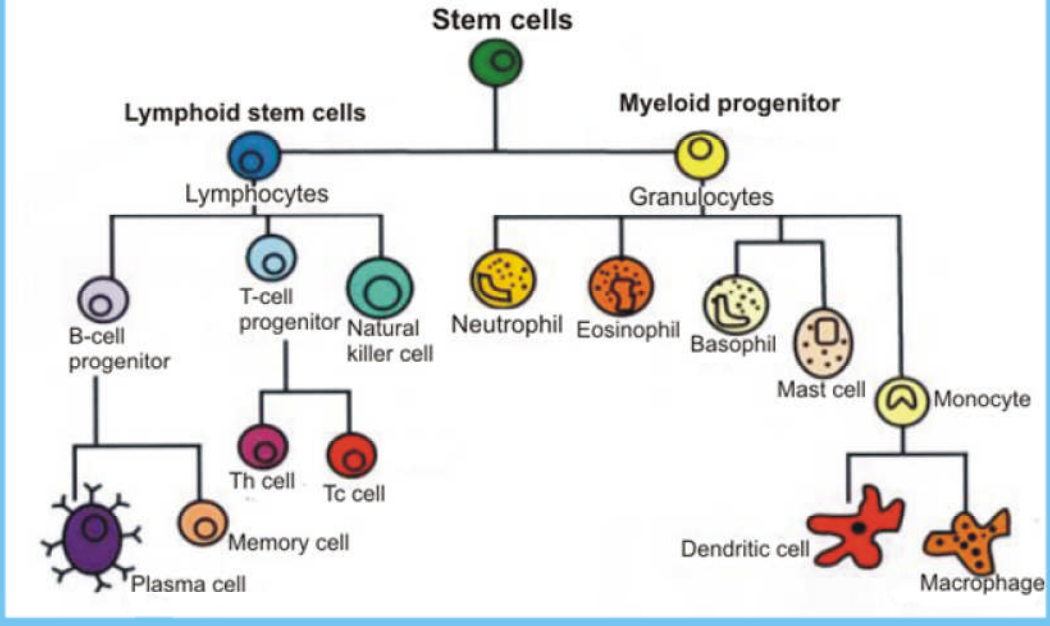


Fig 1.2 Cells of immune system

1.1.4.1 Lymphocytes

B-cells and T-cells are the main types of lymphocytes. These lymphocytes are responsible for adaptive immunity.

1.1.4.1.1 B-Lymphocytes

B-cells work chiefly by secreting substances called antibodies into the body's fluids. Antibodies ambush antigens circulating the bloodstream. The function of attacking target cells is to deport infected cells to T-cells or other immune cells. Each B-cell is programmed to make one specific antibody.

Antibodies: Antibodies are plasma glycoproteins. Because of their mobility in an electric field and in immunity, they are respectively called gamma globulins and

immunoglobulin (Ig). Antibodies which are identical with each other at every amino acid are called monoclonal antibodies (mAb).

Each antibody "monomer" as shown in **Fig 1.3** is composed of two identical heavy (H) polypeptide chains and two identical light (L) chains, covalently bonded via inter chain disulfide (S-S) linkages between cysteine residues. All antibodies have one of two kinds of L chain, kappa (κ) or lambda (λ). Furthermore, each antibody has two identical κ chains or two identical λ chains. Five different H chains have been observed: alpha (α), gamma (γ), delta (δ), epsilon (ε), and mu (μ).

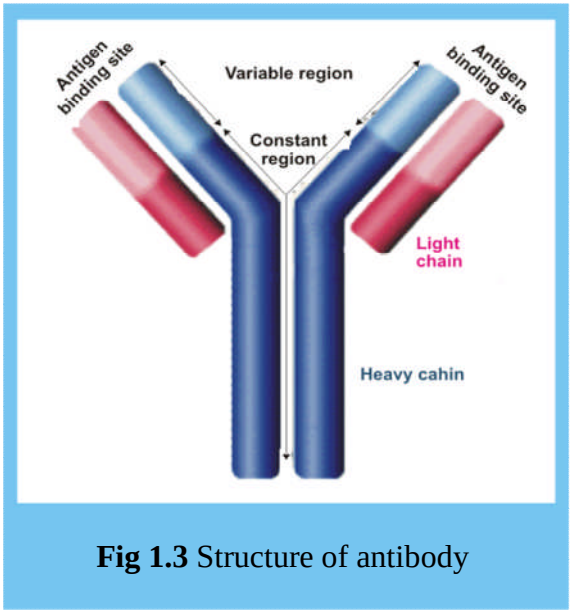


Fig 1.3 Structure of antibody

When a B-cell encounters its triggering antigen, it gives rise to many large cells known as plasma cells. Every plasma cell is essentially a factory for producing an antibody. Each of the plasma cells obtained from a given B-cell manufactures millions of identical antibody molecules and diffuses them into the bloodstream.

An antigen matches an antibody like a key matches a lock. Some match exactly; others fit more like a skeleton key. Once antigen and antibody interlock, the antibody marks the antigen for destruction.

Antibody isotypes (classes) belong to a family of large molecules known as immunoglobulins and are named IgA, IgG, IgD, IgE, and IgM to correspond to their H

chain types. Different types play different roles in the immune defense strategy, as shown in **Table 1.1**[9]

Table 1.1 Properties of antibody isotypes.

Isotype	% of total Ig (adult serum)	Biological half-life (days)	Biological Functions
IgA_1	11-14	5.9	Pathogen neutralization in mucosal secretions
IgA_2	1-4	4.5	
IgD	0.2	2-8	Membrane BCR
IgE	0.004	1-5	Mast cell histamine release
IgG_1	45-53	21-24	Pathogen neutralization in tissues Classical complement activation Opsonization, NK cell ADCC Transplacental transfer
IgG_2	11-15	21-24	Pathogen neutralization in tissues
IgG_3	0.03-0.06	7-8	Pathogen neutralization in tissues, Classical complement activation Opsonization, NK cell ADCC, Transplacental transfer
IgG_4	0.015-0.045	21-24	Pathogen neutralization in tissues Transplacental transfer
IgM	10	5-10	Classical complement activation Membrane BCR (monomer)

1.1.4.1.2 T-Lymphocytes

In contrast to B-cells, T-cells does not remember free-floating antigens. To a certain extent, presence of specialized antibody-like receptors on their surfaces makes them to see fragments of antigens on the surfaces of infected or cancerous cells. There are two major ways through which T-cells contribute in immune defences: (i) by directing and regulating immune responses and (ii) directly attacking infected or cancerous cells. Their active participation in variable immune defences is because they exist in several forms as shown in **Fig 1.4**.

Helper T-cells, (T_H cells) synchronize by communicating with other cells. Type-1 T-helper cells (T_H1) call in microbe-swallowing cells called phagocytes, Type-2 T-helper cells (T_H2) stimulate nearby B-cells to produce plasma cell and eventually an antibody, still others activate other T-cells. Third type, Killer T-cells also called cytotoxic T-lymphocytes (CTLs or T_C) directly attacks especially viruses because viruses often hide from other parts of the immune system while they grow inside infected cells.

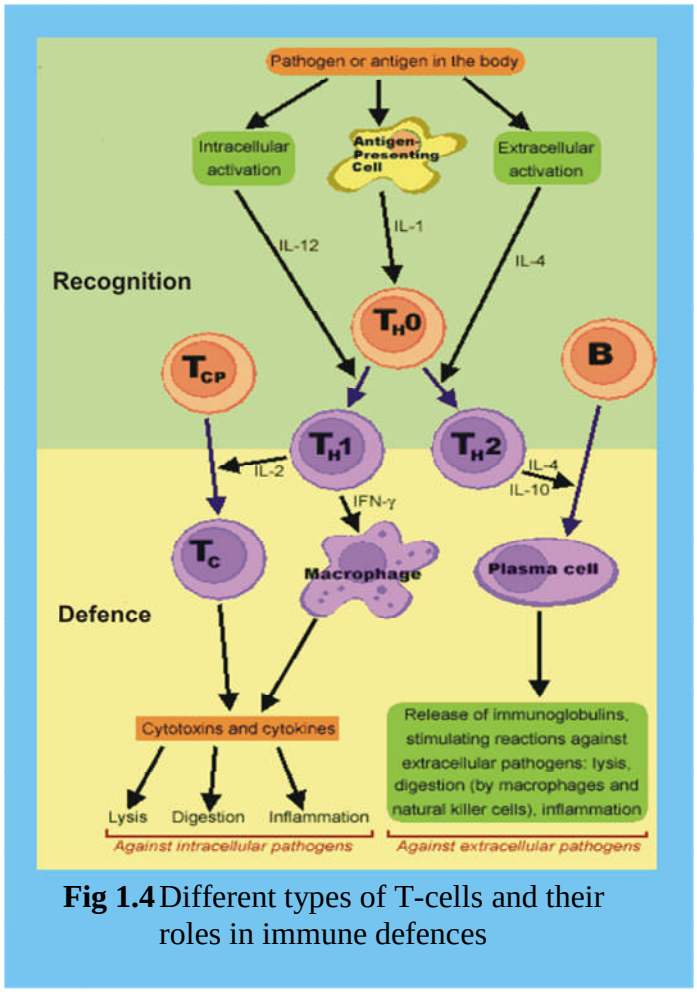


Fig 1.4 Different types of T-cells and their roles in immune defences

1.1.4.1.3 Natural killer (NK) cells

NK cells (**Fig 1.2**) are another kind of lethal lymphocyte that has the potential to assail many types of foreign invaders. Because they are equipped with dedicated potent chemicals they can recognize cells lacking self-MHC molecules while killer T-cells look for antigen fragments bound to self-MHC molecules.

1.1.4.2 Phagocytes

These are large white cells that can engulf and digest microbes and other foreign particles. ***Monocytes*** are phagocytes that circulate in the blood. When monocytes migrate into tissues, they develop into ***macrophages***. Macrophages show many functions. They scavenge dead cells and debris as well as expel them out of the body. They also provide monokines, which are vital to the immune responses.

1.1.4.3 Granulocytes

These are another kind of immune cell containing granules containing potent chemicals capable of destroying microorganisms. Some of these chemicals, such as histamine, also contribute to inflammation and allergy. The **neutrophil**, is a also granule containing phagocyte; it ingests the microbes and sprays its pre-stored chemicals to kill them. **Eosinophils** and **basophils** are granulocytes that "degranulate," showering their chemicals onto harmful cells or microbes in proximity.

The ***mast cell*** is not a blood cell; however, it is a twin of the basophils. It is located in the lungs, skin, tongue, and linings of the nose and intestinal tract, and is responsible for the allergic symptoms. Cell fragments, the blood ***platelets***, too, contain granules. They not only promote blood clotting and wound repair, but also activate some of the immune defences.

1.1.4.4 Cytokines

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and haematopoiesis. Cytokines are components of the immune system that communicate with one another by exchanging chemical messengers.

Cytokine is a general term; other names include **lymphokine** (cytokines made by lymphocytes), **monokine** (cytokines made by monocytes), **chemokine** (cytokines with chemotactic activities), and **interleukin** (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (**autocrine action**), on cells in proximity (**paracrine action**), or in some instances on distant cells **(endocrine action**). The various cytokines with their sources, targets and functional activities are listed in **Table 1.2**. A diverse range of cytokines with different activities contributes mutually in their actions and together in harmony. For example; they show **pleiotropy** (different cells may secrete the same cytokine or a single cytokine may act on several different cells) and **redundancy** (similar functions can be stimulated by different cytokines) in their activity. They are often produced in a cascade and can act either synergistically or antagonistically. Although many cell populations make cytokines, but T_H cells and macrophage are the predominant (**Fig 1.4**).

Table 1.2 Immune cytokines and their activities.			
Cytokine	**Producing cell**	**Target cell**	**Function****
GM-CSF	T_H cells	progenitor cells	growth and differentiation of monocytes and DC
IL-1α IL-1β	monocytes macrophages B-cells DC	T_H cells	co-stimulation
		B-cells	maturation and proliferation
		NK cells	activation
		Various	inflammation, acute phase response, fever
IL-2	T_H1 cells	activated T-and B-cells, NK cells	growth, proliferation, activation
IL-3	T_H cells NK cells	stem cells	growth and differentiation
		mast cells	growth and histamine release
IL-4	T_H2 cells	activated B-cells	proliferation and differentiation IgG_1 and IgE synthesis
		macrophages	MHC Class II
		T-cells	Proliferation
IL-5	T_H2 cells	activated B-cells	proliferation and differentiation IgA synthesis
IL-6	monocytes macrophages T_H2 cells stromal cells	activated B-cells	differentiation into plasma cells
		plasma cells	antibody secretion
		stem cells	Differentiation
		Various	acute phase response
IL-7	marrow stroma thymus stroma	stem cells	differentiation into progenitor B- and T-cells
IL-8	macrophages endothelial cells	Neutrophils	Chemotaxis
IL-10	T_H2 cells	macrophages	*cytokine production*
		B-cells	Activation
IL-12	macrophages B-cells	activated T_C cells	differentiation into CTL (with IL-2)
		NK cells	Activation
IFN-α	leukocytes	various	*viral replication* MHC I expression
IFN-β	fibroblasts	various	*viral replication* MHC I expression
IFN-γ	T_H1 cells, T_C cells, NK cells	various	*Viral replication*
		macrophages	MHC expression
		activated B-cells	Ig class switch to IgG_{2a}
		T_H2 cells	*Proliferation*
		macrophages	pathogen elimination
MIP-1α	macrophages	monocytes, T-cells	Chemotaxis
MIP-1β	lymphocytes	monocytes, T-cells	Chemotaxis
TGF-β	T-cells, monocytes	monocytes, macrophages	Chemotaxis
		activated macrophages	IL-1 synthesis
		activated B-cells	IgA synthesis
		various	*Proliferation*
TNFα	macrophages, mast cells, NK cells	macrophages	CAM and cytokine expression
		tumor cells	cell death
TNF-β	T_H1 and T_C cells	phagocytes	phagocytosis, NO production
		tumor cells	cell death

** CTL: cytotoxic T-lymphocytes; DC: dendritic cells; GM-CSF: Granulocyte-Monocyte Colony Stimulating Factor; IL: interleukin; IFN: Interferon; TGF: Tumor Growth Factor; TNF: Tumor Necrosis Factor. ** Italicized activities are inhibited.*

1.2 HOW DOES THE IMMUNE SYSTEM DEFEND THE BODY?

The immune system can be pictured as a huge cellular and molecular army working in concert to protect the body. The main targets of all immune responses are foreign agents such as bacteria or other invaders, termed as **antigens**. Antigen-presenting cells (macrophages) wander throughout the body, ingesting the antigens they find and breakdown those into fragments called antigenic peptides. These fragmented peptides are then fixed to major histocompatibility complex (***MHC***) molecules and are displayed on the cell surface. Receptors present on T-lymphocytes make them to recognize different peptide-MHC combinations. This recognition activates T-cells, which then divide and secrete lymphokines or chemical signals that further trigger other components of immune system. Set of cells comprising of B-lymphocytes that also contain receptor molecule of single specificity on their surface responds to those signals. However, receptors of B-cells can recognize fragments of antigens free in solution without MHC molecules. When activated, B-cells are converted to plasma cells that secrete antibodies (soluble forms of their receptors). Antibodies can neutralise antigens or precipitate their destruction by complement enzymes or scavenging cells. Some T-cells and B-cells are stored as memory cells in circulation that eliminate same antigens if appear in future. It should be noted that antibody response improves after repeated immunisation this is because genes for antibodies in B-cells mutate frequently.[3,4] This defence phenomenon is termed as **immunity** which can be defined as body's protective mechanism against foreign agents such as microorganisms, food, drugs or pollens[1] and is conveniently divided into two types; **Innate and Adaptive**

1.2.1 Innate Immunity

This is a non-specific immunity comprising of elements that are unable to recognize antigens but can distinguish foreign organisms.[6,10] This immunity is devoid of immunological memory and it responds in the same manner every time the host is exposed to the same antigens. Innate immunity consists of (a) physical and chemical barriers and (b) cellular defences. These are summarised in **Fig 1.5**.

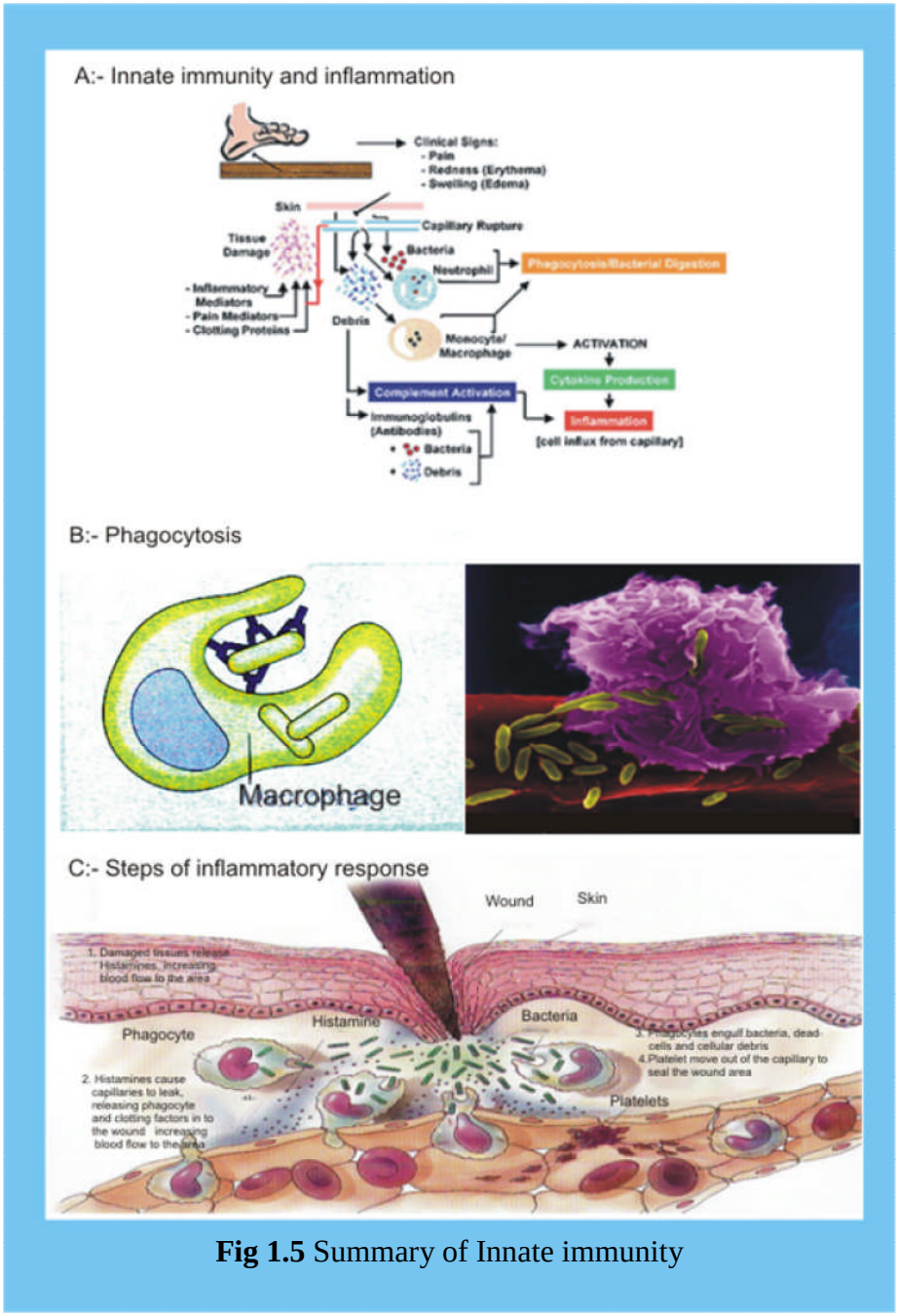


Fig 1.5 Summary of Innate immunity

1.2.1.1 Physical and chemical barriers

The main physical barriers are mucous membrane and skin, which provide mechanical obstructions to the invading organisms[11] and thus prevent their access to the host. However, an invading organism can gain access through the damages or cuts.[6] Epithelial layers, in addition produce chemicals such as secretion of HCl and proteolytic enzymes in the stomach, antibacterial proteins in the gut and respiratory tract.[3,12]

1.2.1.2 Cellular defences

Cellular barriers include "professional" and "non-professional" phagocytes. "Professional phagocytes" are those polymorphonuclear neutrophils such as monocytes, macrophages and dendritic cells (DCs), which have antigen-presenting capacity. "Non-professional" phagocytes include endothelial cells and Kupffer cells. Phagocytes ingest, destroy and digest the invading microorganisms intracellularly, by producing (for example through enzymatic cascade) oxygen free radicals and nitrous oxide (NO), which are toxic for intracellular bacteria. Cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) play an important role in triggering and activating this mechanism.[7,12,13] Acidification of lysosomal enzymes[13] also involved in killing intracellular bacteria. Kupffer cells, the spleen and the lymph nodes provide an antibacterial defence if microbial invasion occur through blood or lymphatic circulation. Macrophages interacting with certain bacterial components produce a range of cytokines, which non-specifically amplify immunological and inflammatory responses.[14]

Other cells showing innate immune defences are natural killer (NK) cells. These release perforin, which upon damaging cell membrane lead to the death of the target cells. Cytokines such as interleukin-12 (IL-12) and IFN-α activate the NK cells thus enhancing their activity against invading organisms.[15-17]

1.2.2 Acquired Immunity

Microbes devise specific system to circumvent inherited eukaryotic immune defences. To overcome this vertebrates have an adaptive immune power, also known as antigen specific defence mechanism. Adaptive immunity cannot protect against all infections but is more specific defence mechanism that enables the body to recognize and respond to any microbe, even if it had never been exposed to the invader before. The process of clonal selection operates it. Adaptive immunity retains the immunologic memory and makes us able to resist infections we have faced before, more efficiently and forcefully than we do face them for the first time. The body stores the lymphocytes that are exposed in previous infection by microbes. When the same microbes again

enter the body, these lymphocytes are rapidly reactivated and form antibodies against microbes and remove them, thus recurrence of disease is avoided.[3,18] This immunity is developed throughout life. As shown in **Fig 1.6,** acquired immunity is divided into two types, namely humoral and cell mediated immunities.[3,5]

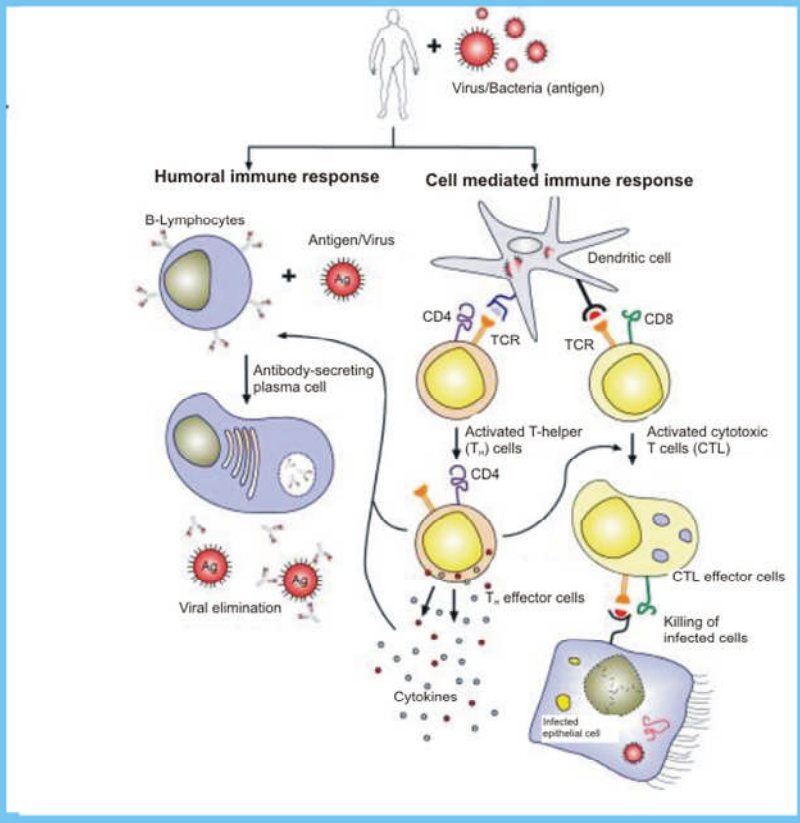


Fig 1.6 Acquired immunity
(Humoral and cell mediated)

1.2.2.1 Humoral immunity

This is mediated by B-lymphocytes and involves production of antibodies, specific in recognising and eliminating antigens. B-lymphocytes (plasma cells derived from B-cells) secrete antigen-specific antibodies, immunoglobulins,[19] that specifically bind to antigens and deactivate them either by neutralising their activity or by eliminating them via activating complement cascade through type-2 receptors[20] or phagocytose them by acting as opsonins or killing them via stimulating NK cells.

1.2.2.2 Cell mediated immunity

This is mediated by T-lymphocytes and involves the production of cytotoxic T-lymphocytes, activated macrophages, activated NK cells and cytokines in response to antigens. Recent research suggested that, bacteria are capable to down regulate antibacterial responses of T-helper1 (T_H1), whereas parasite helminths down regulate responses of T-helper2 (T_H2).[21] Two major histocompatibility complexes (*MHCI* and *MHCII*) can bind to a range of antigenic peptides or protein fragments in forming respective complexes (*Peptide-MHCI* and *Peptide-MHCII*). *Peptide-MHCI* complex activates CD8+ T-cells (T-killer) whereas *Peptide-MHCII* complex activates CD4+ T-cells (T-helper). The antigen processing by MHC is shown in **Fig 1.6**.

MHCII molecules produced by macrophages bind to antigenic peptides (from invading organisms) and carry them to the cell surface in the form of *peptide-MHCII* complex. This complex coupled with *B7* on the surface of macrophages activates CD4+ T-cells containing complementary receptor molecule. *B7* provides an additional signal to T-cells in order to develop immunologic response. *B7* is recognised by separate protein, CD28 on T-cell surface.

Activated T_H1 cells release interferon-gamma which enhances the production of other cytokines (TNF) and chemicals like NO and toxic form of oxygen, which lead to the destruction of microbes. Whereas, activated T_H2 cells secrete IL-4 and IL-10 that enhance antibodies production by B-cells and thereby destroy microbes.[3,22]

The target of T-killer cells are mainly cells infected by viruses. *Peptide-MHCI* complexes activate T-killer cells that express CD8+. Activated Tc cells then kill infected cells directly or indirectly. They destroy infected targets by apoptosis thereby secreting perforin and other proteins that lyse cellular membranes (**Fig 1.6**). Additionally, activated CD8+ cells release potent cytokines (IFN-α and TNF) that inhibit the replication of viruses and other cytokines that kill the infected cells.[3,21,23]

1.3 DISORDERS OF THE IMMUNE SYSTEM

The immune system helps the body defend itself against various microbes and pollutants. However, the immune system itself can be futile and in some cases dysfunctional. This leads to the development of immune disorders. An impaired immune system is also known as immuno-compromised and can make the body susceptible to various viral, bacterial, or fungal opportunistic infections. Immune deficiency can cause various illnesses such as viral infections, chronic illnesses, or immune system illnesses (like AIDS). The other type of immune disorder involves an over-active immune response. This leads to several different classes of diseases: hypersensitivities, autoimmune diseases, etc.

1.3.1 Hypersensitivity

The damage made by immune response to the body gives rise to hypersensitivity. Many immune responses damage the body during antigen removal, causing swelling and pain from inflammation or lysis of virus-infected cells by cytotoxic T-cells. However, hypersensitivity can become life-threatening if the damage is too great. Four types hypersensitivity are observed depending upon the mechanisms by which immune responses damage cells.

1.3.1.1 Type-I hypersensitivity

This is usually called "allergy" in which IgE is produced in response to an antigen called an allergen. This immediate hypersensitivity includes hay fever, skin inflammation (urticaria), food allergies, asthma, and systemic anaphylaxis. The risk of developing a Type-I hypersensitivity (atopy) is linked to family history and IgE levels.

1.3.1.2 Type-II hypersensitivity

It is mediated by IgG (or occasionally IgM) and occurs when an antibody binds to a cell surface antigen. The surface antigen-antibody complexes activate complement or bind to FcγRI on cells that can perform antibody-dependent cell-mediated cytotoxicity

(ADCC). Both processes result in lysis of the target cell. Examples are hyper acute graft rejection, acetylcholine receptor in myasthenia gravis, thyroid hormone receptor in Graves' disease, and erythrocyte membrane proteins in autoimmune hemolytic anemia.

1.3.1.3 Type-III hypersensitivity

It is also mediated by IgG (or occasionally IgM) antibody and caused by immune complex deposition in the tissues, where the classical complement cascade results in tissue damage. Frequently, deposition and tissue damage are observed in blood vessel walls, kidney, and joints. Immediately after cell damage, an inflammatory response is initiated. In this process, a release of cytoplasmic enzymes and influx of inflammatory cells prolong the hypersensitivity. Examples include serum sickness and occupational diseases (i.e., Farmer's Lung) in which antibodies are produced to soluble environmental antigens that are encountered repeatedly. Autoantibodies produced in Systemic Lupus Erythematosis cause tissue damage and infectious diseases with persistent pathogens (including malaria, some virus infections, leprosy, and Lyme disease).

1.3.1.4 Type-IV hypersensitivity

It is also called delayed-type hypersensitivity (**DTH**) because it occurs 48-72 hours after antigen contact, is mediated by antigen-specific T_H1 cells and activated macrophages. Examples of DTH include local skin reactions to proteins in insect venom and injected *Mycobacterial* proteins used in skin testing. Other include contact sensitivities to poison ivy, latex, nickel in coins and jewelry, and cleaning products which come in contact with the skin.

1.3.2 Immunodeficiency Disorders

The missing of one or more of components of the immune system during its response results with an immunodeficiency disorder. Immunodeficiency disorders can be inherited, acquired through infection, or produced unintentionally by drugs such as those used to treat people with cancer or those who have received transplants.

Temporary immune deficiencies can develop a susceptibility to common virus infections, including influenza, infectious mononucleosis, and measles. Moreover, blood transfusions, surgery, malnutrition, smoking, and stress can also depress immune responses.

Some children are born with poorly functioning immune systems. Some have disabilities in the B-cells to produce antibodies. Infants born with none of all major immune defenses are seldom. However, this condition is known as severe combined immunodeficiency disease or SCID.

1.3.2.1 Acquired immunodeficiency syndrome (AIDS)

This is an immunodeficiency disorder caused by a human immunodeficiency virus (HIV) that infects immune cells. HIV can destroy or disable vital T-cells, paving the way for a variety of immunologic flaws. HIV also can lie dormant for long periods in immune cells. As the HIV abates immune defenses it makes a person vulnerable to unusual, often life-threatening infections and rare cancers. AIDS is a contagious disease spread by intimate sexual contact, transfer of the virus from mother to infant during pregnancy, or direct blood contamination. There is no cure for AIDS, but several developed antiviral drugs have been developed that can slow the advance of the disease, at least for a time. Moreover, HIV vaccines are in clinical studies.

1.3.3 Cancers of the Immune System

Like other cells, the immune system cells can also grow uncontrollably, resulting in cancer. The proliferation of white blood cells, or leukocytes cause leukaemias. Multiple myeloma is due to the unrestricted growth of antibody-producing plasma cells. Whereas, Hodgkin's disease includes cancers of the lymphoid organs called lymphomas.

1.3.4 Autoimmune Diseases[10,24,25]

The immune system is a complicated cell and cell component network set to defend the body and eliminate infections caused by bacteria, viruses, and other invading

microbes. In an autoimmune disease, the immune system mistakenly attacks self, targeting the cells, tissues, and organs of a person's own body.

Table 1.3 Human autoimmune diseases

Disease	Autoantigen	Symptoms	Extent*
Type II: antibodies to cell surface molecules			
Autoimmune hemolytic anemia	Rh blood group antigens, I antigen	Lysis of RBC by complement and FcR^+ cells, anemia	Organ specific
Autoimmune thrombo-cytopenic purpura	Platelet integrin GpIIb:IIIa	Abnormal bleeding	Organ specific
Goodpasture's syndrome	Basement membrane Type IV collagen	Glomerulonephritis, pulmonary hemorrhage	Organ specific
Graves' disease	Thyroid-stimulating hormone receptor	Thyroid over-activity	Organ specific
Hashimoto's thyroiditis	Thyroglobulin, thyroid peroxidase	Thyroid under-activity	Organ specific
Hypoglycemia	Insulin receptor (agonist)	Low blood glucose	Organ specific
Insulin-resistant diabetes	Insulin receptor (antagonist)	High blood glucose, ketoacidosis	Organ specific
Myasthenia gravis	α chain of nicotinic acetylcholine receptor	Progressive weakness	Organ specific
Pemphigus vulgaris	Epidermal cadherin	Skin blisters	Organ specific
Pernicious anemia	Intrinsic factor, gastric parietal cells	Anemia	Organ specific
Rheumatic fever	Streptococcal cell wall antigens; antibodies cross-react with heart muscle	Arthritis, myocarditis, heart valve scars	Organ specific
Spontaneous infertility	Sperm antigens	Infertility	Organ specific
Type III: Immune complex disease			
Ankylosing spondylitis	Immune complexes	Damage to vertebrae	Systemic
Mixed essential cryoglobulinemia	Rheumatoid factor IgG complexes	Systemic vasculitis	Systemic
Rheumatoid arthritis	Rheumatoid factor IgG complexes	Arthritis	Systemic
Systemic lupus erythematosus (**SLE**)	DNA, histones, ribosomes, snRNP, scRNP	Glomerulonephritis, vasculitis, rash	Systemic
Type IV: T-cell-mediated disease			
Experimental autoimmune encephalomyelitis (**EAE**), multiple sclerosis	Myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein	Brain invasion by CD4 T-cells, weakness	Systemic
Hashimoto's thyroiditis	Thyroid antigen(s)	Thyroid under-activity	Organ specific
Insulin-dependent (Type I) diabetes mellitus (**IDDM**)	Pancreatic β cell antigen(s)	β cell destruction	Organ specific
Rheumatoid arthritis	Unknown synovial joint antigen	Joint inflammation and destruction	Systemic

Autoimmunity is caused by an adaptive immune response against "self" antigen. The random generation of many diverse T-cell receptors (TCR) and B-cell receptors (BCR) makes autoimmunity possible. Clonal deletion and anergy of self-specific lymphocytes greatly reduces but does not eliminate the possibility of low affinity self-specific responses. Transient autoimmune responses are common but usually cause no long-term damage. Prolonged autoimmune responses resulting in tissue damage can be serious, because self-antigens are continually present in the body. Autoimmune diseases can be caused by antibodies or T-cells and may be organ-specific or systemic. **Table 1.3** summarizes some information about some human autoimmune diseases. Frequently observed autoimmune diseases are summarised below.

1.3.4.1 Rheumatoid arthritis

In rheumatoid arthritis the immune system predominantly targets the synovium that covers various joints resulting in the symmetrical inflammation of the synovium thereby causes pain, swelling, and stiffness of the joints. Sometimes it may associate with other autoimmune diseases such as Sjogren's syndrome (chronic inflammatory disease), itching, and scaling.

1.3.4.2 Multiple sclerosis

In multiple sclerosis the immune system targets nerve tissues of the central nervous system, intermittently damaging the central nervous system and eventually preventing a person from leading a normal life. Multiple sclerosis is the most common disabling disease of the nervous system in young adults.

1.3.4.3 Immune-mediated or type-1 diabetes mellitus

Autoimmune destruction of the insulin-producing cells of the pancreas results in Type-1 diabetes mellitus, which is manifested by fatigue, frequent urination, increased thirst, and possible sudden confusion. Insulin keeps the blood sugar (glucose) level under control. High levels of blood glucose are responsible for the symptoms and the complications of the disease. It is observed that most of the insulin-producing cells are destroyed before the patient develops symptoms of diabetes.

1.3.4.4 Inflammatory bowel diseases

Two diseases namely Crohn's disease and Ulcerative colitis are of inflammatory bowel type. In those, the immune system attack the gut (intestine), resulting in diarrhoea, nausea, vomiting, abdominal cramps, and uncontrolled pain.

1.3.4.5 Systemic lupus erythematosus

Patients with systemic lupus erythematosus commonly experience profound fatigue, rashes, and joint pains. In severe cases, the immune system attacks and damages the kidneys, the brain, or the lungs and the patient needs close monitoring.

1.3.4.6 Psoriasis

This immune system disorder affects the skin, and occasionally the eyes, nails, and joints. Psoriasis may affect small areas of the skin or cover the entire body, building up red scales called plaques.

1.3.4.7 Scleroderma

This autoimmune disease results in thickening of the skin and blood vessels. Almost every patient with scleroderma has Raynaud's disease, which is a spasm of the blood vessels of the fingers and toes.

1.3.4.8 Autoimmune thyroid diseases

The two main types of autoimmune thyroid diseases are Hashimoto's thyroiditis and Grave's disease. These destruct the immune system or stimulate the thyroid tissue. Symptoms include fatigue, nervousness, cold or heat intolerance, weakness, and changes in hair texture or weight gain or loss.

Scientists searching for ways to prevent and treat autoimmune diseases are studying disease pathogenesis and investigating new ways of modifying the immune system. To

this end a large number of immune modulatory drugs have been developed to correct the dysfunctional immune system.

1.4 MODULATION OF THE IMMUNE SYSTEM

Immune modulation may involve either the immune system's attenuation as in the case of organ transplantation or its stimulation, for example to combat viral infections. Clinically immune suppressants are used to withhold the harmful effects of immune response, primarily in organ transplantation thus avoiding organ rejection. Other clinical applications involve autoimmune diseases or hypersensitivity. The immune modulatory agents can be divided as either immunosuppressants or immunostimulants. Other agents such as tolerogens and disease modifying anti-rheumatic drugs (DMARDs) are also responsible for immune modulation to some extent.

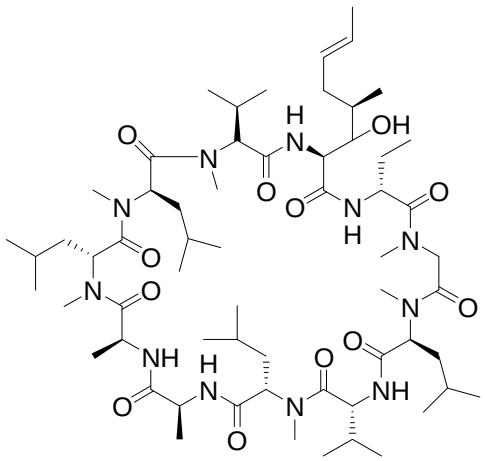
1.4.1 Immunosuppressants

Immunosuppressants react differently to different immune reactions. For example, they can suppress the primary immune response more readily than the secondary (memory). Immunosuppressants are conveniently differentiated into flour classes; helper T-cell blockers, Glucocorticostroids (glucocorticoids), cytotoxic drugs and antibody reagents.

1.4.1.1 Helper T-cell blockers

1.4.1.1.1 Ciclosporin

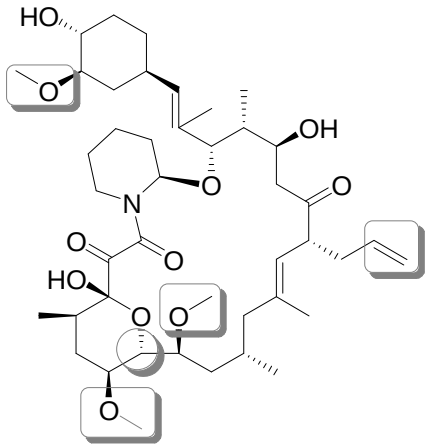
Ciclosporin **1.1** is also known as cyclosporin or cyclosporine. It was discovered by Borel *et al* from the fungal species *Tolypocladiumv inflaturm Gams.*[26,27] It is a cyclic peptide mainly used as prophylaxis and in the treatment of graft-versus-host disease (GvHD). This immunosuppressant is a calcineuurin inhibitor. It inhibits antibody synthesis against T-lymphocyte-dependent antigens and lymphokines (IL-2) synthesis by activated T-lymphocytes. Unfortunately nephrotoxicity and hypertension are major side effects.[27,28]



1.1

1.4.1.1.2 Tacrolimus

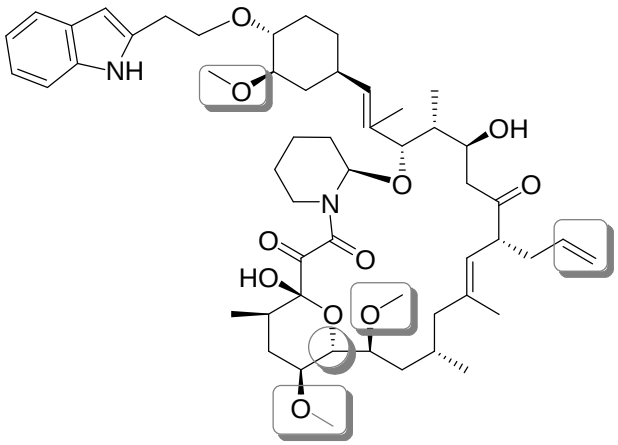
Tacrolimus also termed as FK506 **1.2** is a macrolide triene, discovered in Japan from the soil fungus *Streptomyces tsukubaensis* and acts in the same way as that of ciclosporin but binds to a different immunophilin. Its binding forms a complex that interferes with T-cell actvation through its action on calcineurin.[29-31]



1.2
Boxed groups are essential for immune suppression

It is used to prevent rejection of liver transplants and in the treatment of rheumatoid arthritis and causes few side effects. Its use in dermatological diseases through systemic route is associated with its side effects where as topical applications have shown positive therapeutic effects. Major side effects are neurotoxicity, diarrhoea, upset stomach, vomiting, stomach pain, loss of appetite and insomnia.[32,33]

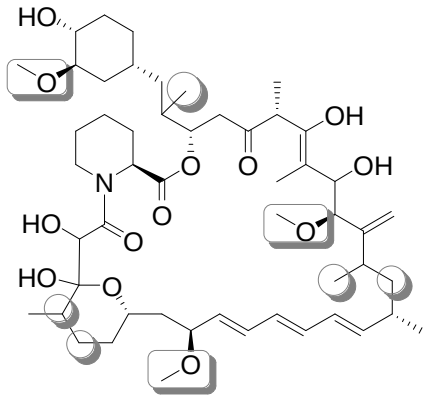
Recently, SAR of tracolimus has developed the semi-synthetic analogue indolyl-ascomycin, **1.3**. This compound contains 2-(3-indolyl)ethoxy group at C^{32} position that alters its drug-receptors interactions. This drug retains immunosuppression whilst improving its therapeutic index. It has also shown reduction in nephrotoxicity and neurotoxicity respectively by 2 and 3 folds.[34]



1.3
Boxed groups are essential for immune suppression

1.4.1.1.3 Sirolimus (Rapamycin)

Sirolimus is also one of the potent compounds of the macrocyclic triene series and is isolated from *Streptomyces hygroscopicus*. Like tracolimus, sirolimus **1.4** binds to immunophilins, with no calcineurin inhibitory activity. It inhibits T-cell proliferation by acting at tow kinases.[26,35]

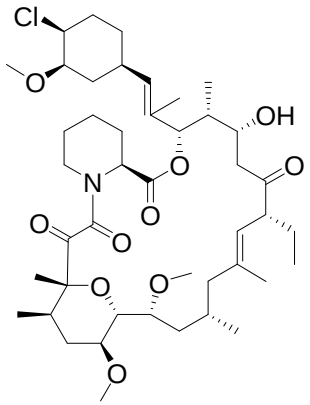


1.4
Boxed positions are sites for metabolism and are essential for immune suppression

Its synergism with ciclosporin makes it possible to reduce doses of ciclosporin still within the therapeutic index and thus abates cyclosporine related side effects. Like other agents, sirolimus also maintains toxicity like hyperlipidemia, leukopenis and thrombocytopenia whereas, unlike other structurally related agents, it is devoid of nephrotoxicity.[26,36] Hepatic metabolism of sirolimus greatly reduces immunosuppressive effect. This suggests that sites of metabolism shown in **1.4** are important features for immune suppression.[33]

1.4.1.1.4 Pimecrolimus

Pimecrolimus **1.5** is an ascomycin derivative isolated from *Streptomyces hygroscopius* var. *ascomycetes*. It mimics the tracolimus chemically with somewhat different biological behaviour. Pimecrolimus binds to immunophilin before it can affect T-cell proliferations induced by calcineurin phosphatase.

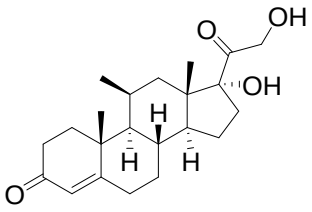


1.5

It is choice of drug for dermatitis preferably by topical route at comparatively 3 times larger doses than that of cyclosporine. It has also been used systemically for graft rejection as well as for contact and atopic dermatitis. Pimecrolimus at well tolerated systemic doses have shown exciting results in reduction of psoriasis area and its severity. A comparatively high lipophilic character lowers its permeation through skin and also reduces its systemic uptake in blood through topical application. The poor systemic absorption of topical pimecrolimus however, makes it a choice drug for treatment of immune mediated skin disorders.[37] To a lesser extent it also shows spurious effects like burning sensation, pruritus, erythema, etc.[38]

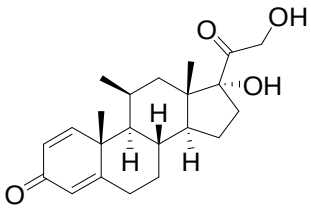
1.4.1.2 Glucocorticoids (Glucocorticostroids)

Hench's *et al* first discovered Glucocorticoids in 1949 and in that they showed that cortisone had beneficial acitivities in patients with rheumatoid arthritis.[39,40] A range of glucocorticoids is available right from the short acting natural hydrocortisone **1.6** through the intermediate acting synthetic prednisolone **1.7** to the long acting dexamethasone **1.8**.



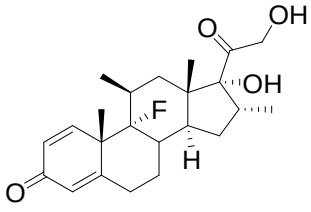
Hydrocortisone

1.6



Prednisolone

1.7



Dexamethasone

1.8

These glucocorticoids show a wide range of immune modulating effects. These effects include decreasing vasodilatation, delayed macrophage activation,[41] slowing down of the prostaglandin synthesis and decreased lymphocyte levels.[42] Glucocorticoids also lower cytokine production.[43] Synthetic derivatives cause more severe side effects than those of hydrocortisone. They are used in autoimmune diseases such as active chronic hepatitis, rheumatoid arthritis, myasthenia gravis and inflammatory diseases.[44]

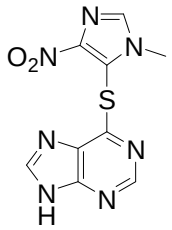
1.4.1.3 Cytotoxic agents

Cytotoxic agents are mainly antineoplastic drugs used in prevention of rapid division of cells and clonal expansion of T- and B-cells.

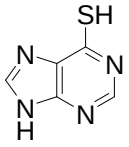
1.4.1.3.1 Azathioprine

Azathioprin, **1.9** is a prodrug approach of purine analogue, which, upon metabolism is converted to 6-mercaptopurine **1.10** and inhibits the production of purine nucleotides

thus inhibiting DNA replication. It also affects cell-mediated immune responses and antibody production.[43]



1.9

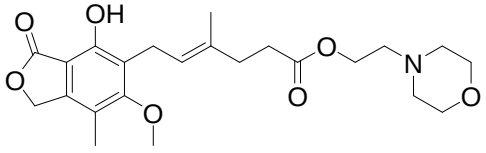


1.10

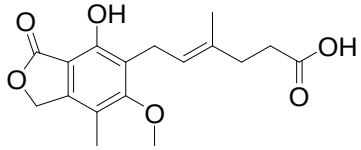
It is used in severe rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, myasthenia gravis etc. Toxicity includes GI disturbances, hair loss and hepatotoxicity.[44]

1.4.1.3.2 Mycophenolate Mofetil

Mycophenolate Mofetil (MMF) **1.11** is a semi-synthetic prodrug of mycophenolic acid **1.12** derived from *Penicillium* fungi. Mycophenolic acid acts by inhibiting inosine monophosphate dehydrogenase enzyme involved in the *de novo* synthesis of xanthine monphosphate.[45]



1.11

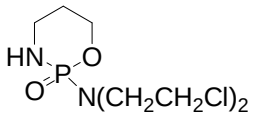


1.12

It also prevents T-cell proliferation and antibody formation by B-lymphocytes, which are vital in a *de novo* synthesis of purine nucleotides.[43] MMF is mainly used in prevention of rejection of allogenic kidney, heart and liver transplants. It is usually given in combination with ciclosporin and corticosteroids. Being more selective in action, it causes only mild side effects of gastrointestinal tract.[44]

1.4.1.3.3 Cyclophosphamide

Chemically **1.13** is known as prodrug of alkylating agents that is enzymatically converted to cytotoxic phosphoramide mustard by cytochrome P_{450} oxidases in liver.[28] The resulting alkylation of nitrogen 7 of guanidine causes DNA cross-linking thus inhibiting DNA replication and transcription.[26,43]

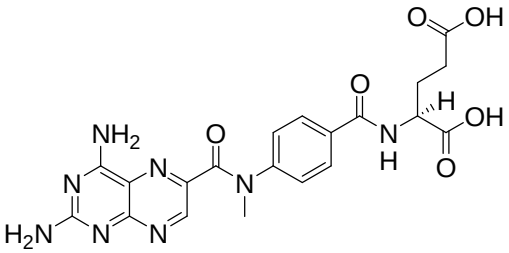


1.13

It also triggers apoptosis. It is mainly useful in severe rheumatoid arthritis not adequately controlled by standard disease modifying antirheumatic drugs (DMARDs). Its toxicity includes myelosuppression, sterility, especially in men and risk of leukaemia.[44]

1.4.1.3.4 Methotrexate

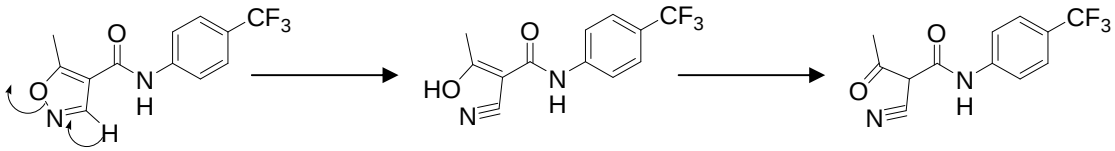
Methotrexate **1.14** is a competitive inhibitor of dihydrofolate reductase enzyme, which is involved in synthesising thymidine nucleotides by converting dihydrofolate to tetrahydrofolic acid. The latter is essential in DNA replication and thus proliferation of lymphocytes.[26] It also inhibits protein synthesis.[43] It is primarily used to treat rheumatoid arthritis, severe psoriasis, Crohn's disease, and ulcerative colitis. Hepatotoxicity, teratogenicity and pulmonary toxicity in rheumatoid arthritic patients, are common side effects.[44]



1.14

1.4.1.3.5 Leflunomide

Leflunomide **1.15** is completely metabolised by hydrolytic cleavage in the cytoplasm to its active constituent A177 1726 **1.17 (Scheme 1.1)**. The metabolite of this isoxazole derivative may be involved in inhibiting dihydroorotate dehydrogenase in *de novo* pyrimidine synthesis thus avoiding T-cell proliferation.[26] It also blocks phosphorylation of tyrosine, and inhibits activity of cyclo-oxygenase-2 (COX-2). It is mainly used as disease modifying antirheumatic drug (DMARD) and may show cumulative toxicity.[44]



1.15 **1.16** **1.17**

Scheme 1.1 Leflunomide **1.15** and its active metabolite A77 1726 **1.17**

1.4.1.4 Antibody reagents (cytokines therapy)

Antibodies eliminate invading molecules by phagocytosis. Various antibodies specific for cytokines, lymphocytes are used as immunosuppressant. These are prepared by immunising human lymphocytes with specific animal (horses, rabbits or mice) cells. Polyclonal antibodies such as **ALG** (Antilymphocyte immunoglobulin) and **ATG** (Antithymocyte immunoglobulin) reduce activity of circulating T-cells interacting with surface proteins.[46] These antibodies therefore, can be made to target different cytokine receptors.

1.4.1.4.1 Targeting IL-2 receptors

Humanized monoclonal antibodies created by recombinant DNA technology **daclizumab** and an achimeric monoclonal antibody containing both murine[47,48] and human antibody sequences **basiliximab** bind to the alpha-chain of the IL-2 receptor and prevent proliferation blocking signal transduction.[49,50]

1.4.1.4.2 Targeting TNF

A significant role of TNF in inflammatory bowel disease and rheumatoid arthritis has highlighted that targeting the TNF might lead to favourable immune modulation[51] Infliximab is an achimeric IgG antibody to TNF-∝ that dose dependently neutralises the cytotoxic effects of the TNF.[52] **Etanercept** is synthesized by fusion process and contains two chains of recombinant human TNF-receptor ρ75 monomers and F_C domain of the human IgG, which binds to and inactivates TNF. It has been successfully used in rheumatoid arthritis with negligible side effects.[52]

1.4.1.4.3 Targeting CD cells

Some antibodies **mAbs** target CD3 or CD4 protein on T-cells.[49] **MuromonabCD3** (also called OKT3) is the first monoclonal antibodies marketed for the treatment of organ rejection and inhibit T-cell functions by binding to CD3. **Alemtuzumab** acts against cluster of CD52 that is located on most immune cells. It is used in the treatment of B- cell lymphocytic leukaemia. **Rituximab** selectively targets CD20 on B-cells and leads to the disappearance of B-cells within the circulation after treatment. It is used for the treatment of B-cell non Hodgkin's lymphoma and also has been evaluated in autoimmune diseases.[43]

1.4.2 Immunostimulants

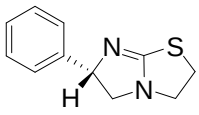
Immunostimulants are agents employed to encourage or excite the immune system and enable the body to recognise and respond to foreign agents. It can be broadly categorised as synthetic agents, natural agents such as bacterial derived agents, intravenous immune globulins (IVIGs) and cytokines (interferons, interleukins and colony-stimulating factors), and finally endogenous hormones.

Vaccines are used as prophylactic against infections. They stimulate adaptive immunity. Antisera like Immunoglobulins (IgG), antivenoms provide protection against poisons from snakes and antitoxins provide protection against poisons from microbes.

1.4.2.1 Synthetic agents

1.4.2.1.1 Levamisole and Imuthiol

Both the sulfur containing compounds Levamisole (LMS) **1.18** and Imuthiol **1.19** act by similar mode of action. Levamisole is primarily used as an antihelmintic. It increases NK cells and activated T-cells with consistent effect on production of tumor necrosis factor and no effect on monocyte cytotoxicity.



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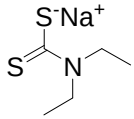
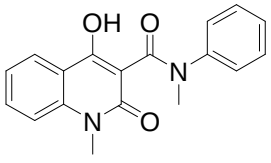


1.19

It is used in the UK as the drug of choice in the treatment of *Ascaris lumbricoides* (roundworm) infections.[44] Common side effects are diarrhoea, metallic taste and nausea. LMS may cause a temporary loss of hair in some people. Imuthiol is less toxic than levamisole and has also shown radio and chemo protective and antifungal properties.[53] Both these drugs have also been evaluated for their activity in the treatment of rheumatic arthritis.[54,55]

1.4.2.1.2 Linomide

Linomide **1.20** (*N*-methyl-*N*-phenyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxo-quinoline -3-carboxamide) has been shown to be an immunomodulator.



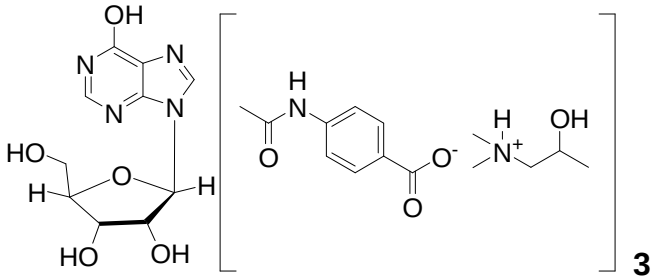
1.20

In clinical trials, it has been shown to have potential in the treatment of autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosis and multiple sclerosis. Linomode is a quinoline derivative with pleiotropic immunomodulatory

activity. It has been found to enhance natural killer (NK) cell number and activity after autologous bone marrow transplant (ABMT) in patients with acute myeloid leukemia (AML).

1.4.2.1.3 Isoprinosine

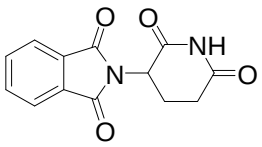
Isoprinosine (inosine pranobex (InPx)) **1.21** is a synthetic compound formed from the *p*-acetamido benzoate salt of 3-(*N*-*N*-dimethylamino)-2-propanol and inosine in a 3:1 molar ratio. It increases natural killer (NK) cell activity, total T-cells, and T-helper cells. Some of its effect may persist for months after completion of the treatment period. It is used in therapy of HIV-1 and rhinoviruse infections[56,57]



1.21

1.4.2.1.4 Thalidomide

Thalidomide **1.22** is 2-(2,6-dioxo-3-piperidinyl)-isoindoline-1,3-dione available as two stereo isomers. The (*R*)-stereoisomer has sedative and antinausea effects, while the (*S*)-stereoisomer has teratogenic effect.



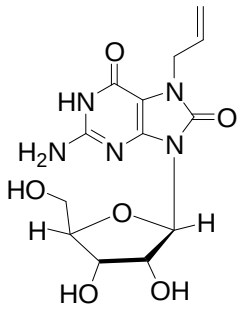
1.22

Thalidomide affects variety of cytokines and inflammatory mediators including TNF-a, ILi1b, IFN-γ, IL-6, IL-10, IL-12 and COX-2 and angiogenesis factors. However, its mechanism of action has not yet been elucidated. It is used in the treatment of

Waldenstrom's macroglobulinemia (WM), as well as multiple myeloma, myelodysplastic syndrome, myelofibrosis, chronic lymphocytic leukemia (CLL), and B-cell lymphomas. Major side effects include neuropathy and teratogenicity (phocomelia) when used as antiemetic or sedative in pregnant women.

1.4.2.1.5 loxoribine

Loxoribine (7-allyl-8-oxoguanosine) **1.23** is used as primary adjuvant for antibody responses to a wide variety of antigen types in a variety of species. It involves the augmentation of CTL-mediated, NK cell-mediated, macrophage mediated, and LAK cell-mediated cytotoxicities.

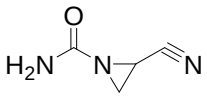


1.23

It is an inducer of cytokines. Main side effects noted resemble those of interferon and are transient. It up-regulates humoral immune responses in immunodeficiency and acts as a surrogate T_H signal

1.4.2.1.6 Azidiridine derivatives

2-Cyanoaziridine-1-carboxamide **1.24** had been successfully used by Bicker against PIE 2-3 sarcoma in Wister rats. Since it suffered from side effects, the original structure was thus use as a start point to develop more promising drug candidates. Eventually, azimexone **1.25** and ciamexone **1.26** were discovered to have anti-tumour activity with relatively low toxicity.



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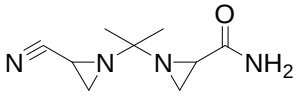

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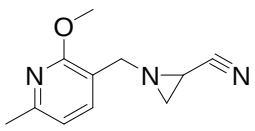
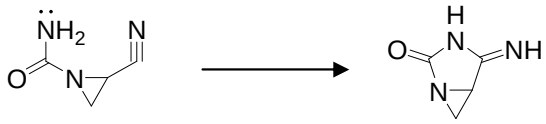


1.26

Azimexone acts through increasing serum levels, secretion of CSF and increasing peripheral macrophages.[58] Whereas, ciamexone alters the balance between T_H2 and T_H1, lymphocytes and is responsible for delayed hypersensitivity reaction in mice. However, both these compounds gave disappointing results in clinical trials. Further research on this lead molecule, let to the development of imexone **1.27** (**scheme 1.2**).



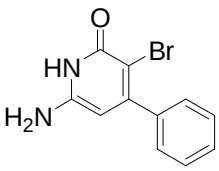
1.24 **1.27**

Scheme 1.2 Synthesis of imexone **1.27**

Imexone acts by augmenting T-cell function without effecting NK cells at relatively tolerated doses with only nausea and vomiting side effects. This compound has been found active against wide range of cancers and also recently it has undergone clinical trials in USA for the treatment of AIDS.[59]

1.4.2.1.7 Bropirimine

Bropirimine **1.28** is a nucleic acid derivative having significant immunostimulatory effects through induction of IFN-∝, TNF-∝ and IL-2, B-lymphocytes. It also stimulates IL-2 receptors, macrophages and NK cells. It is currently undergoing clinical trials for the treatment of bladder cancer as a sole therapeutic agent and also in combination with BCG vaccine.[60,61]



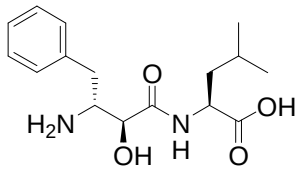
1.28

1.4.2.2 Natural immunostimulants

Among several bacterial derived agents Bacille-Calmette-Guerin (BCG) is most extensively studied and approved worldwide in intravesical therapy of superficial

bladder cancer. It is an attenuated live strain of *Mycobacterium bovis.* It stimulates the release of TNF-α from macrophages. However, hypersensitivity, chills, fever and immune complex disease are major side effects.

The Dipeptide **Amastatin 1.29** and the tetrapeptide **Bestatin 1.30** derived from *Streptomyces olivoreticuli* and *Streptomyces ME98-M3* respectively possesses a diverse range of activities including anti-tumour and immunomodulatory activities.



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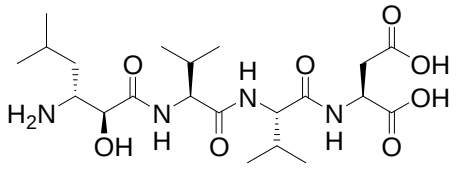
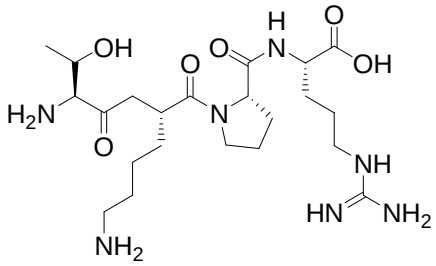


1.30

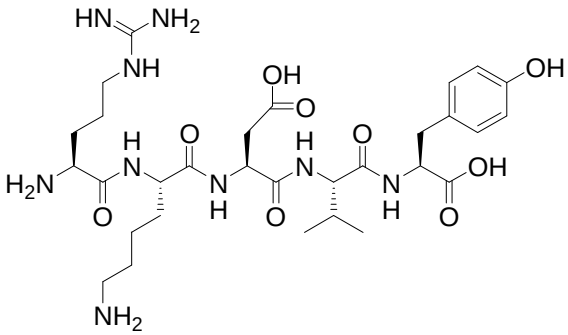
Bestatin also known as Ubenimex is an orally active non-toxic peptide and is believed to activate NK cells and macrophages through binding to amino peptidase.[62,63] Another non toxic tetrapeptide peptide **Tuftsin 1.31** derived from the F_C portion of the immunoglobulin molecule is considered to enhance phagocytosis and the bactericidal and tumourcidal activities of macrophages.



1.31

It enhances IL-1 and TNF release. Aberrant behaviour of thymus, one of the important organs of the immune system might be due to the disruption in its biochemical integrity.[64] More recently, immunologists have expressed interest in **thymic peptides** owing to their reduced toxicity. Currently, a few thymic peptides such as **thymopentin, thymosin $\propto_1$, thymulin and thymic humoral factor** are under study although little is known about their roles in the endocrinology of the thymus.[54,55]

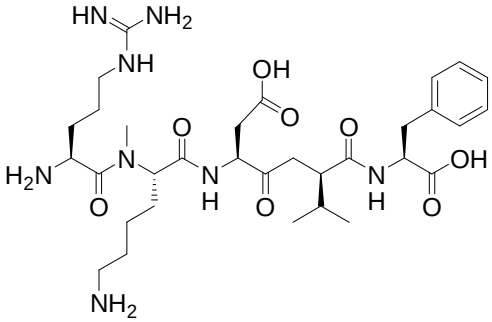
Thymopentin 1.32 is a pentapeptide of 32-36 residues of thymopoietin. It promotes T-cell proliferation and interleukin production. It is licensed in Italy for the treatment of cancer and immunodeficiency disorders. Currently, it is under clinical trials in the USA for the HIV combination therapy with zidovudine (AZT).[54,55]



H-Arg-Lys-Asp-Val-Tyr-OH

1.32

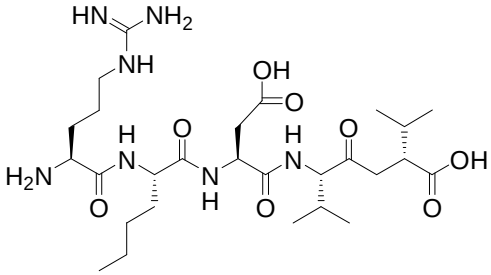
Various derivatives of thymopentin e.g. **1.33** and **1.34** have also been synthesized.[65]



H-Arg-NMeLys-Asp(k)Val-Phe-OH

Thymopentin analogue

1.33



H-Arg-Nle-Asp-Val(k)Val-OH

Thymopentin analogue

1.34

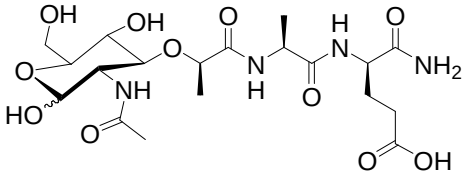
Thymosin $\propto_1$ is a 28 residue recombinant peptide that acts by enhancing the production of IL-2 and displays IL-2 receptors in T-lymphocytes. It is also undergoing clinical trials in the USA for the treatment of cancer and hepatitis. An octapeptide of **thymus thymic humoral factor 1.35** has shown increased T-cell responses in HIV patients in clinical trials in Israel.[54,55]

H-Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu-OH

Thymic humoral factor

1.35

***N*-Acetyl muramyl dipeptide (MDP) 1.36** derivatives have been found to have adjuvant activities.[66] **Romuritide 1.37** has been widely accepted in the Japanese Pharmaceutical market for the treatment of cancer patients undergoing chemotherapy or radiotherapy. It recovers and maintains WBC's and platelet counts by inducing cytokines.



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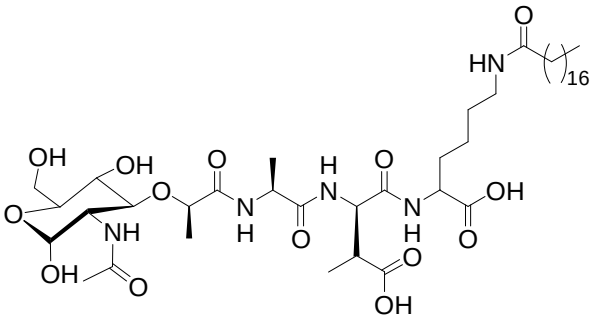
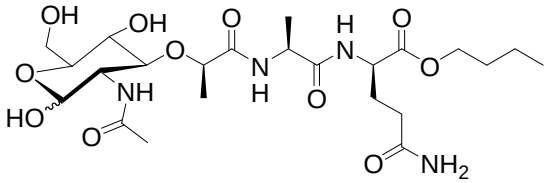


1.37

Murabutide 1.38 and **Temurtide 1.39** are improved MDPs with reduced side effects. Among these temurtide has been successfully used as vaccine adjuvant in animal studies against several antigens including influenza virus haemagglutinin. [60]



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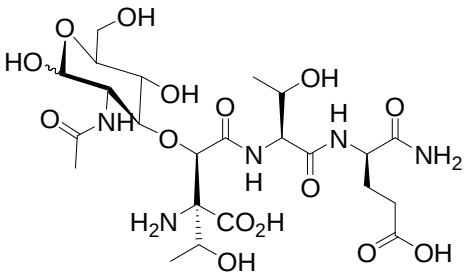
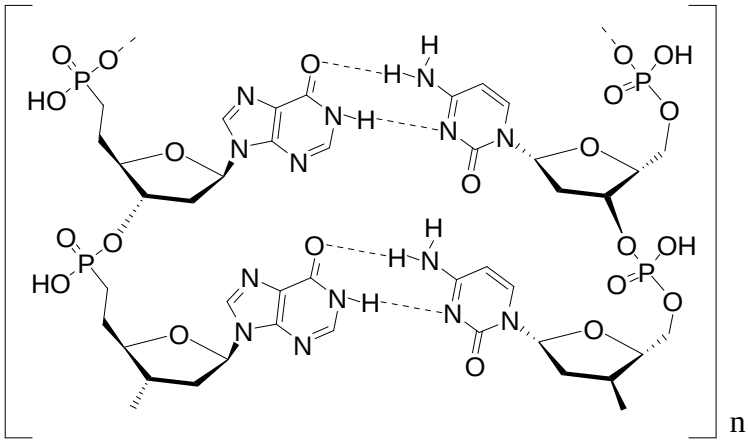
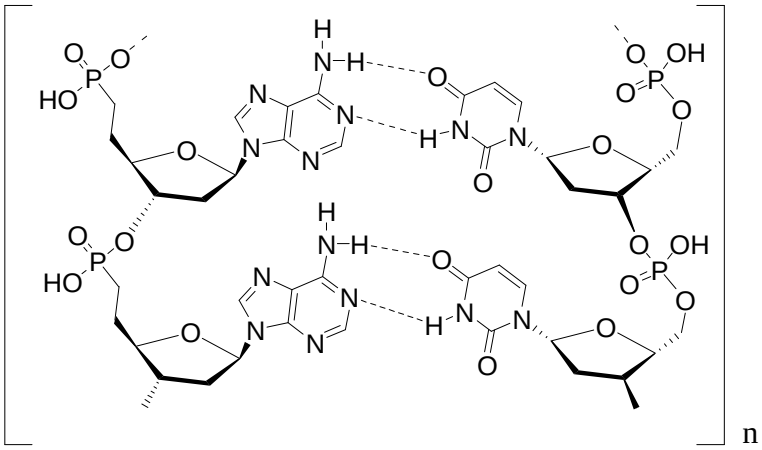


1.39

Similar to MDP, **Polynucelotides** can also be explored for their adjuvants activity. Poly(I).poly(C) **1.40** and Poly(A).poly(U) **1.41** have been observed to have potential for increasing antibody-forming cell numbers.[67]



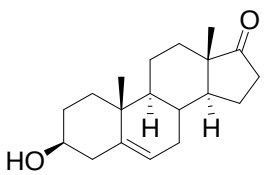
1.40



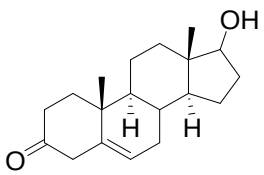
1.41

1.4.2.3 Androgenic Steroids

Unlike immunosuppressants, androgenic steroids such as endogenous hormones provide indispensable elements that improve the immune system by controlling the production of some lymphokines.[67] Dehydroepiandrosterone (DHEA) **1.42** and Dihydrotestosterone (DTH) **1.43** have been extensively evaluated for their immunomodulatory roles.



1. 42



1.43

DHEA is produced from DHEA sulphate (DHEAS) through the action of a sulfatase enzyme where as DTH is obtained from testosterone through the action of 5∝-reductase. DHEA has been found to improve T-cells function by accelerating the secretions of IL-2 and IFN-γ, whereas DTH influence T-cell function by decreasing secretions of IL-4, IL-5 and IFN-γ.[68,69]

1.4.2.4 Intravenous Immunoglobulins

Intravenous immuno-globulins (IVIGs) are semi-purified entities obtained from pooled plasma from many donors containing variable IgA, normal IgGs (major subclasses) and antibodies to common pathogens (antigens). IVIGs are mainly used as replacement therapy in primary immune deficiency diseases.

1.4.2.5 Cytokines as Immunostimulants

Cytokines such as interferons (IFN), interleukins (IL) and colony stimulating factors (CSF) have been observed as vital immune stimulating agents. IFN-α, IFN-β and IFN-γ modulate immune responses by interfering with viral replications. IFN-α in low dose acts as immune stimulator and activates NK cells, macrophages and cytotoxic T-cells. It is used in the prevention of infections in chronic granulomatous disease. Human recombinant IFN-α (rhlFN-α) is employed in the treatment of hairy cell leukaemia, AIDS related Kaposi's sarcoma, human papillomavirus and hepatitis C infections. An analogue of IFN-β (IFN-β-1b) has been shown to be capabile of reducing the number or the severity of attacks in relapsing-remitting multiple sclerosis. Major side effects of IFNs include fever, headache, mylagias and GI disturbances.

Interleukins (ILs) bind to IL-2 receptor in immune cells, induce proliferation and differentiation of helper T-cells and cytotoxic T-cells. They have been found to elevate serum IL-1, TNF-α, and IFN-γ levels. Human recombinant IL-2 (rhIL-2) is used to treat metastatic renal cancer. It is highly toxic and may lead to capillary leak syndrome (oedema, reduce organ perfusion and hypotension), cardiac arrhythmias, myocardial infraction and changes in mental status.

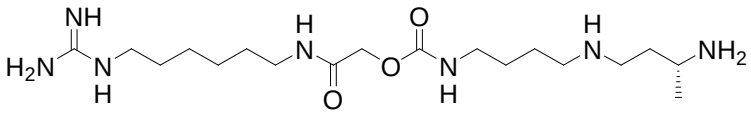
Granulocyte-colony-stimulating factor (G-CSF) induces the maturation of neutrophils and is used after chemotherapy to lower the incidence of infections in patients with non-myeloid malignancies. Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces the development of neutrophils, eosinophils and macrophages and is used in Hodgkin's disease and acute lymphoblastic leukaemia. Side effects of G-CSF include bone pain and splenomegaly whereas with short-term therapy whilst GM-CSF leads to fluid accumulation.

1.4.3 Selective Tolerogens

15-Deoxyspergualine (DSG) derivative LF 15-0195

Selective tolerogens are used along with immunosuppressants especially in the prevention of immune responses against viral vectors used in gene therapy.

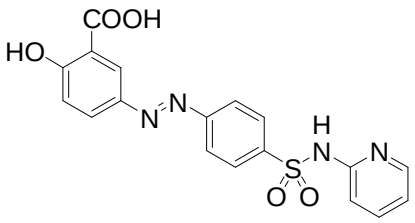
DSG **1.44** a derivative of spergualine, decreases antibody production, inhibits production of IL-6 and TNF-α, inhibits B-cell development and inhibits the growth of native CD4+ T-cells after activation and production of IFN-γ by T_H1 lymphocytes.



1.44

1.4.4 Antirheumatic Drugs as Immune Modulators

Some antirheumatic drugs especially slow acting ones (e.g. Sulfasalazine **1.45**, chloroquine **1.46**, and gold compounds) are used as immune modulators. Sulfasalazine in autoimmune conditions inhibits leukotriene synthesis thus lymphocyte function. Chloroquine, is mainly an anti-malarial but also possesses anti-inflammatory and immunomodulating potential. Sodium aurothiomalate **1.47** has been found to modify cellular and humoral immune responses.[57]



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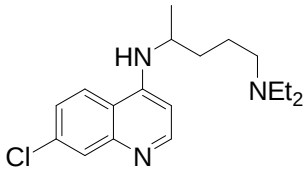

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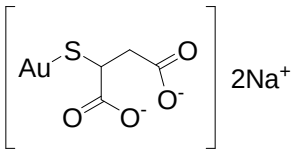


1.47

Modulation of the immune system as an approach to treat autoimmune diseases has been explored for the past 50 years. However, efficacy of this treatment has been achieved in a limited number of patients. A major obstacle of immune therapy is the toxicity related to the non-specific nature of immune activation. Therefore, continued efforts are needed to improve the specificity of the immune responses. To this end it has been recently shown that active components excreted by certain bacteria can modulate the host's immune system. This is quite logical since, in order to survive, microbes must reproduce and spread to other host. While doing this, they learn and adopt skills to override the host's immune responses. Microbes usually subvert immune responses by modulating the immune system. The development of strategies for therapeutic intervention therefore, require an extensive understanding of how microbes can escape the immune system.

1.4.5 Evasion of immune responses by microbes: can it be a better strategy for immune modulation?[70]

Pathogens (invaders and parasites) begin the rapport with host species to sustain their own chances for survival, proliferation, and subsequent dissemination. Their stable interaction with the host helps them predominate among the host population and ensures their lifelong persistence with minimal symptoms of disease. At this stage, pathogens would have sufficient time for reproduction and spread to new hosts. The

host immune system, however, mobilizes its molecular arsenal to thwart multiplication and eliminate the infectious agent. Infectious organisms in response have evolved many ways of evading the host immune system.

Pathogens can evade the innate immune system by circumventing phagocytosis. It frustrates binding (as seen in capsules, M protein, and fibrin coats) to the components of macrophages and neutrophils. To avoid destruction in the phagosome, some pathogens inhibit fusion with the lysosome and drop in pH. Other pathogens avoid complement-mediated lysis by blocking insertion of the membrane attack complex of complement (MAC) into their cell walls.

Pathogens have also evolved strategies to avoid adaptative immunity. Some form sheath of host proteins around their cell walls. Many pathogens employ antigenic variation, in which multiple antigenic variants of a pathogen occur and immunity to one variant is not protective against another, (examples: *Influenza virus* and HIV). Both are RNA viruses whose error-prone RNA-copying enzymes result in antigenically distinct mutants. Influenza undergoes small changes each year (antigenic drift), and the changing serotypes require new vaccines. Occasionally two influenza viruses from different species co-infect an animal, usually a pig or a fowl, and exchange RNA; when this happens, large antigenic shifts occur which may result in pandemics of influenza and may give rise to high mortality rate. The parasite *Trypanosome cruzii* undergoes programmed antigen variation. As the host makes antibodies to one surface antigen, it is quickly replaced by expression of a different one.

Exogenous pathogens block antibody function by making surface molecules that bind the IgG Fc regions to inhibit antibody effector functions. Endogenous pathogens however, remain inside host cell, thereby avoiding antibodies and complements. Viruses can remain latent in the host cell, do not replicate and therefore do not express and present viral proteins on Class-I MHC. Other viruses avoid CTL, antigen processing and presentation. Some of these microbial strategies for immune evasion are summarised in **Table 1.4**.

Table 1.4 Examples of Microbial Evasion of Immune Defenses		
Pathogen		
Streptococcus pneumoniae	Capsule	Avoids phagocytosis
Gram positive bacteria, some Gram negative bacteria	Resist insertion of complement MAC	Avoid complement-mediated lysis
Mycobacterium tuberculosis	Blocks lysosome fusion with phagosome	Avoids antibody and complement opsonization, macrophage killing
Listeria monocytogenes	Escapes phagosome into cytoplasm	Avoids macrophage killing, presentation on Class II MHC
Toxoplasma gondii	Escapes phagosome into own cytoplasmic vesicle	Avoids macrophage killing, presentation on both Class I and Class II MHC
Treponema pallidum	Covers membrane with host proteins	Avoids immune system recognition
Herpes viruses	Persist in host cells without dividing (**latency**)	Avoid immune system recognition
Streptococcus pneumoniae Influenza virus, HIV	Antigenic variation	Avoids memory response
Herpes viruses	Infect cells with little Class I MHC expression (CNS)	Avoid presentation on Class I MHC
Staphylococcus aureus Cytomegalovirus Herpes simplex virus	Expresses membrane Fc-binding protein	Avoids IgG opsonization
Neisseria meningitidis *Neisseria gonorrhoeae* *Haemophilus influenzae*	Expresses IgA protease	Avoids IgA neutralization
Herpes simplex virus	Expresses membrane complement receptor	Blocks complement function
Vaccinia virus	Expresses complement control protein on infected cell	Blocks complement-mediated lysis of infected cell
Vaccinia virus	Expresses soluble cytokine receptor	Blocks inflammation
Epstein Barr virus (EBV)	Inhibits host cell expression of LFA-3, ICAM-1	Blocks adhesion of CTL to infected cells
Herpes simplex virus Cytomegalovirus	Inhibits host cells Class I expression	Blocks CTL recognition
Herpes simplex virus	Blocks TAP function	Blocks CTL recognition
Epstein Barr virus	Expresses homolog of IL-10	Inhibits T_H1 response, IFN-γ production, cellular immunity
Staphylococcus aureus	Secretes superantigens	Suppresses immune response
Hepatitis B Virus HIV	Peptides act as antagonists	T-cell activation is blocked
Mycobacterium leprae	Stimulates T_H2 response	Suppresses T_H1 response
Measles virus	Suppresses T-and B-cell immunity	Suppresses immune response to many pathogens
Pseudomonas aeruginosa	**Secretes elastase that inactivates C3a and C5a**	**Blocks inflammation**

Not surprisingly, the immune evasion strategies are interrelated and important for reasons outlined below.

First, it gives valuable information regarding the host-pathogen relationship i.e. how a particular pathogen can subvert the immune system in order to survive and reproduce.

Second, the identification of microbial immunomodulatory proteins occasionally precedes identification of its host counterpart. This offers opportunities to discover new immunity-associated molecules and to further our understanding of the immune response.

Lastly, the function and sites of activity of immunomodulatory proteins suggest potential agents of and targets for immunotherapy, respectively.

Thus, more recently, a strategy for specific immune modulation has been explored involving the use of selective microbial species. As shown in **Table 1.4** the ability of bacteria to evade or co-exist with the host's immune system suggests that they can alter the immune system in a manner that is beneficial to them. How bacteria manipulate the host responses is of great interest. Lately it has been realised that one way bacteria did modulate host immune system is by Quorum sensing, a mechanism where immunomodulatory effect is dependant on bacterial population size. Among Gram-negative bacteria, *Pseudomonas aeruginosa* inactivates complement cascades C3a and C5a thus block inflammation. *Pseudomonas aeruginosa*, an opportunistic hospital pathogen has the ability to cause cross infections right from harmless colonisation to serious septicaemia. It also establishes chronic colonisation in patients of chronic obstructive pulmonary disease (COPD) and chronic lung infections in cystic fibrosis (CF). Whether *Pseudomonas aeruginosa,* in common with other microorganism has immune modulation through quorum sensing is thus of high interest to researchers in the field.

1.5 QUORUM SENSING AND IMMUNE MODULATION

1.5.1 Regulators of virulence factor producing genes

Bacteria have evolved multiple integrated sensory systems in order to facilitate adaptation to environmental challenges at both the individual cell and population levels. The perception and processing of chemical information form a pivotal

component of the regulatory mechanisms necessary for such population-dependent adaptive behaviour. Many bacteria employ self-generated small diffusible signal molecules (***autoinducers***) to control gene expression as a function of cell population density. In this process, termed "quorum-sensing" (QS), the concentration of a signal molecule which accumulates in the extracellular environment reflects the cell number. The perception of a threshold concentration of a signal molecule determines when the population is 'quorate' and thus ready to make a collective behavioural adaptation.[71-74]

1.5.2 The quorum-sensing paradigm

In Gram negative bacteria, the autoinducers are usually synthesised by enzymes belonging to the LuxI family of AHL synthases. Once synthesised, AHLs accumulate extracellularly and diffuse into neighbouring bacterial cells where they usually interact with members of the LuxR family of transcriptional regulators.[73-75] AHLs bind to, and activate LuxR homologous proteins and the resulting LuxR protein/AHL complex activates or represses the relevant target structural gene(s).[73,74,76] LuxR proteins bind to a region within the promoter/operator of the target genes.

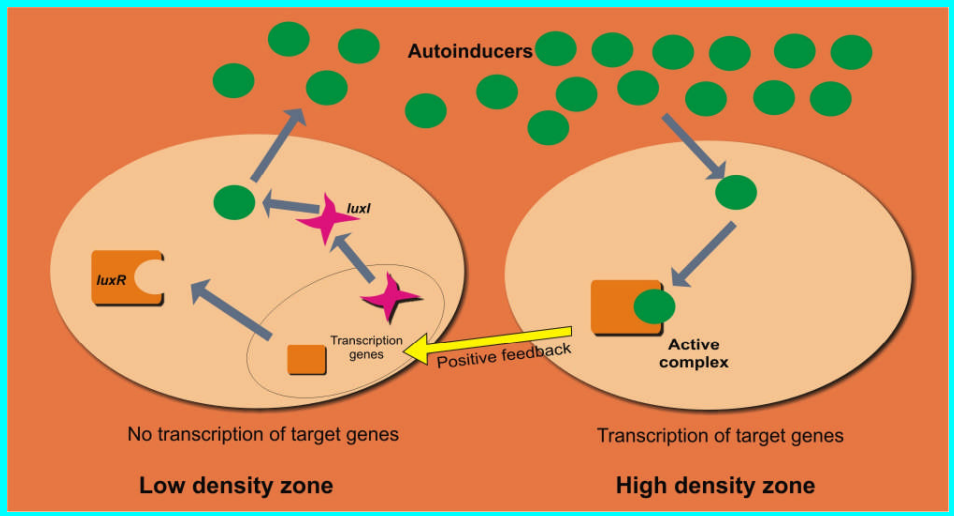
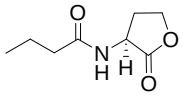
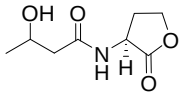
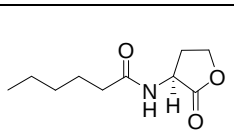
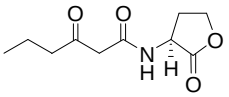
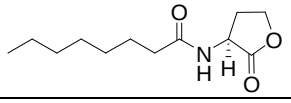
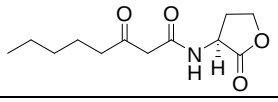
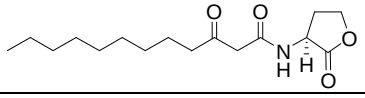
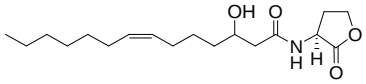


Fig 1.7 Mechanism of quorum sensing

1.5.3 The diversity of signal molecules

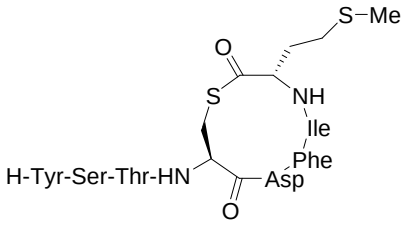
To date a large number of Gram-negative bacteria have *luxI luxR*-like QS systems which generate a range of AHLs which control a variety of processes, many of which are associated with virulence. Many Gram-negative bacteria have been found to utilize *N*-acylhomoserine lactones (AHLs) as signalling molecules[77] **1.48-1.55** (**Table 1.5**) in which the homoserine lactone (HSL) ring is a common structural unit with a side chain which varies in length and can be 3-oxo or 3-hydroxy substituted, saturated or unsaturated.

Table 1.5 AHL signalling molecules produced by Gram-negative bacteria

Microorganisms	QS system	AHL Produced	Abbreviations
Serratia liquefaciens, *Pseudomonas aeruginosa*	*swrI, sweR* *rhlI/R*		C_4-HSL **(1.48)**
Vibrio harveyi	*LuxM*		3(OH), C_4-HSL **(1.49)**
Serratia liquefaciens, *Chromobacterium violaceum* *Pseudomonas aureofaciens*	*SurI* *CviI* *PhzI*		C_6-HSL **(1.50)**
Vibrio fischeri *Erwinia stevartii* *Erwinia caratovora* *Yersinia enterocolitica*	*LuxI* *EsaI* *ExpI/carI* *YenI*		3O, C_6-HSL **(1.51)**
Vibrio fischeri	*AinS*		C_8-HSL **(1.52)**
Aerobacterium tumefaciens	*TarI*		3O, C_8-HSL **(1.53)**
Pseudomonas aeruginosa	*LasI*		3O, C_{12}-HSL **(1.54)**
Rhodobacter sphaeroides	*CerI*		3(OH)-Δ^{7Z}-C_{14}-HSL **(1.55)**

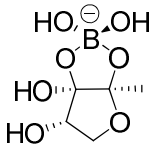
Among the vast majority of Gram-negative bacteria have been studied thus far, *Pseudomonas aeruginosa* is perhaps the best understood in terms of quorum sensing versus pathogenisity mechanisms.

Gram positive bacteria such as *B. subtilis* and *S. aureus* make use of oligopeptide (**1.56**) mediated QS to sense their environment[78] while the boron containing signaling molecule, AI-2 has been identified as a second class of signal molecule which is involved in quorum sensing in *V. harveyi* (**1.57**).[79,80]



1.56

S. aureus (AIP-I)



1.57

AI-2 *Vibrio harveyi* (LuxS)

1.5.4 Quorum sensing in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (family *Pseudomonadaceae*) is an opportunistic human pathogen capable of causing a variety of infections ranging from harmless colonisation to life threatening septicaemia. It is a particular problem in patients suffering from chronic obstructive pulmonary disease (COPD) and is the main cause of morbidity and mortality in patients with cystic fibrosis (CF).[81,82] Cystic fibrosis is a genetic disorder affecting the gene which codes for the cystic fibrosis transmembrane conductance regulator (CFTR). Patients with abnormalities in CFTR have decreased salt and fluid secretions, resulting in abnormally viscous mucous which eventually makes patients susceptible to lung infections.[83] *Pseudomonas aeruginosa* is also a common cause of nosocomial infections including ventilator associated pneumonia,[84] bloodstream infections[85] and infections in burn-wound patients.[86]

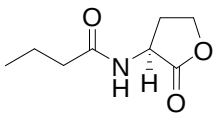
The potential of *Pseudomonas aeruginosa* to cause these infections is due to its ability to synthesize an armoury of virulence factors[87] such as (i) cell-associated factors (e.g. flagella and pili) that adhere to bacteria surfaces, (ii) extra-cellular virulence factors (proteases e.g. alkaline protease and elastase)[88,89] that interfere with the host defense systems and (iii) some secreted exotoxins (toxin A)[89] that cause local tissue damage.

Pyocyanin, a pigment has been observed to disrupt normal Ca^{2+} regulated homeostasis in human airway epithelial cells thus resulted in abnormal mucus secretions and ciliary beats.[90] Pyocyanin has also been shown to affect IL-8 expression in lung epithelia.[91].

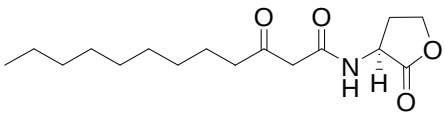
The expression of many *Pseudomonas aeruginosa* exoproducts is dependent on the cell population density and is regulated by AHL mediated quorum signalling.[92-94] *Pseudomonas aeruginosa* has two quorum sensing circuits ***rhlI/R*** and ***lasI/R***.[95]

The transcriptional activator RhlR and AHL synthase *RhlI* direct *N*-butyryl-L-homoserine lactone **(C_4-HSL) 1.48** synthesis.[96,97] A **RhlR-C_4-HSL complex** regulates the expression of *rhlAB* (required for the production of rhamnolipid), *lasB, aprA, RpoS* (stationary phase sigma factor) and for secondary metabolites (pyocyanin and cyanide) production.[77,92,97-101]

The second QS system in *Pseudomonas aeruginosa* comprises *lasI* and *lasR. lasI* directs the sythesis of *N*-(3-oxododecanoyl)-L-homoserine lactone **(3O, C_{12}-HSL) 1.54** which, when complexed with LasR, co-ordinates the expression of a number of virulence genes such as elastase *lasA*,[102] *lasB*,[88,103] and alkaline protease *aprA* as well as exotoxin *toxA*[89,92-94,104] **LasR-3O, C_{12}-HSL** also regulates the transcription of *lasI*[105] and *rsaL*,[95] the products of which regulate *Pseudomonas aeruginosa* negatively inhibiting *lasI* gene expression.[106]

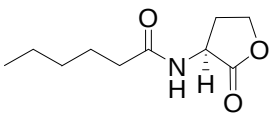


1.48

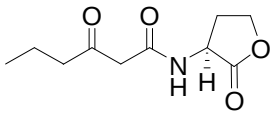


1.54

Pseudomonas aeruginosa also produces other acyl homoserine lactones in small quantities.[107,108] These are *N*-hexanoyl-L-homoserine lactone (**C_6-HSL**) **1.50**, *N*-(3-oxohexanoyl)-L-homoserine lactone (**3O, C_6-HSL**) **1.51** and *N*-(3-oxooctanoyl)-L-homoserine lactone (**3O, C_8-HSL**) **1.53**. The exact role of these signalling molecules is unknown however.[109]



1.50



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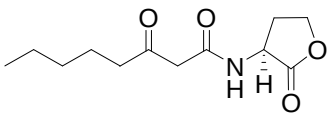
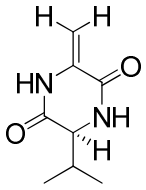


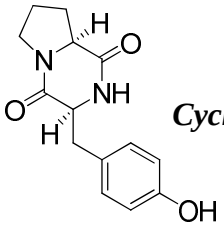
1.53

Furthermore, a class of diketopiperazines (DKP) **1.58** and **1.59** have been identified from *Pseudomonas aeruginosa* supernatants that were found to antagonize 3O, C_6-HSL based induction of bioluminescence in a LuxR based biosensor and also C_4-HSL based swarming motility in *Serratia liquefaciens*.[110] Similar DKP have been isolated from an antarctic sponge associated strain of *Pseudomonas aeruginosa* and have shown to inhibit the growth of many Gram-positive bacteria.[111]



***Cyclo*(Δ-Ala-L-Val)**

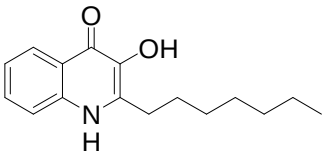
1.58



***Cyclo*(L-Pro-L-Tyr)**

1.59

In addition to various AHLs another autoinducer belonging to 4-quinolone family, namely **2-*n*-heptyl-3-hydroxy-4(*1H*)-quinolone 1.60,** termed PQS (***P**seudomonas **Q**uorum **S**ensing*) was also discovered in *Pseudomonas aeruginosa.*[112,113]



1.60

The *las* system has been found to regulate the synthesis, and the *rhl* system bioactivity of PQS molecule. The transcriptional activator *las*R regulates PQS synthesis that in turn, induces expression of *lasB* (this codes, *rhl* and *rhlR*) and *rhlI*. This suggests that there exists a regulatory link between PQS, *las* and *rhl*.[112,114] It is now clear that PQS does not participate in sensing cell density since it is produced substantially in the late stationary phase of growth in the absence of *lasR*. It is involved in stimulation of LasR independent activation of *rhl* system.[115]

In spite of similarities between LasR and RhlR, both respond to structurally different AHLs for their activation. This in turn indicates that, specific recognition sequences are a must for targeted genes when expressing quorum sensing systems.[77] Despite there being high specificity of these systems for their regulatory molecules and gene targets, there exists a link, in that the ***las*** system positively regulates expression of both ***rhlR*** and ***rhlI***.[114] Also **3O, C_{12}-HSL** competes with **C_4-HSL** for RhlR binding and given that this situation can arise in *Pseudomonas aeruginosa*, 3O, C_{12}-HSL is therefore an ideal target to examine the antagonistic effects of newly synthesised AHL analogues. This clarifies that, in *Pseudomonas aeruginosa* quorum sensing is arranged in hierarchical manner with ***las*** being dominant regulator and exerts transcriptional control over both *rhlR* and *rhlI* (**Fig 1.8**).[95,116]

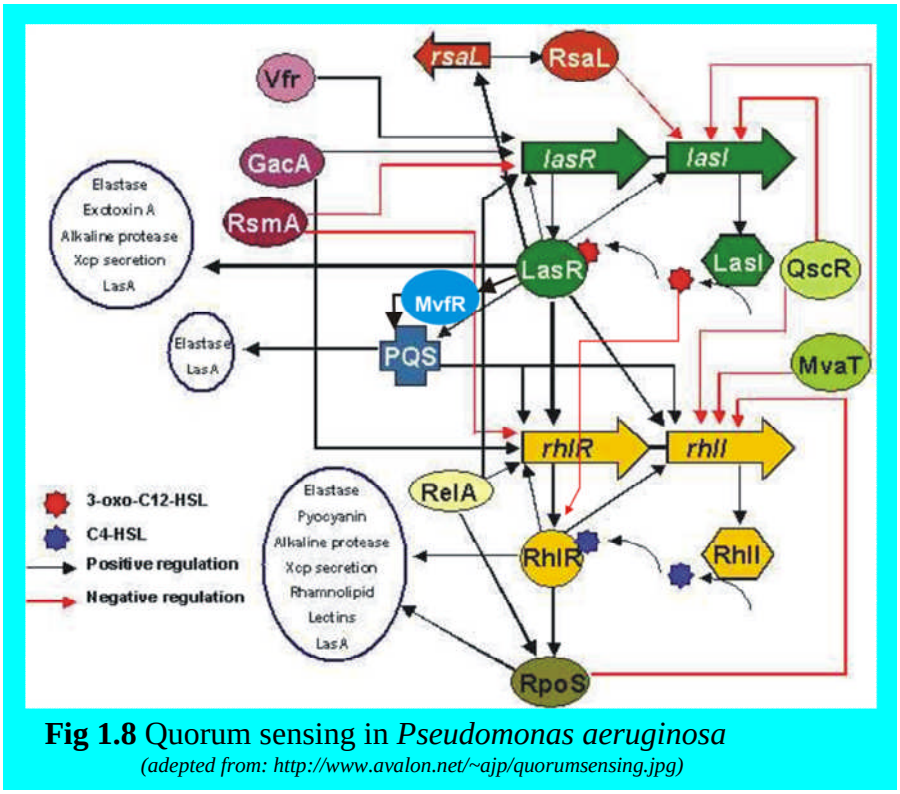
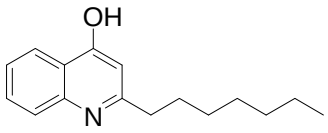


Fig 1.8 Quorum sensing in *Pseudomonas aeruginosa*
(adepted from: http://www.avalon.net/~ajp/quorumsensing.jpg)

Recently the biosynthesis of PQS, a 4-hydroxy-2-alkylquinolines (HAQ) family of secondary metabolite from *Pseudomonas species* has been carried out.[117-121] The biosynthesis of HAQ requires the *pqsABCDE* genes and is regulated by MvfR. The 4-hydroxy- 2-heptyl-quinoline (HHQ) **1.61,** which is congener of HAQ and precursor of PQS is converted to PQS through PqsH. However, LasR regulates PqsH. PQS induces the expression of *lasB* elastase and *rhlI*.[97,116]

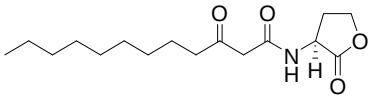
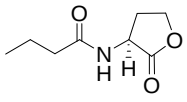
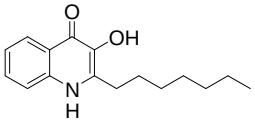


1.61

In *Pseudomonas aeruginosa* quorum sensing system, other regulators have also been identified. Positive regulator such as Vfr;[122] which regulates the expression of *lasR* and GacA[123,124] regulate the expression of both *lasR, rhlR* as well as *rhlI*. On the other hand negative regulator such as QscR,[125] a homolog of LasR and RhlR act as repressors for the expression of *lasI* and Rsal.[106] It is also known to repress *lasI*, probably at lower cell densities.

QS in *Pseudomonas aeruginosa* represents a very complex network of regulatory systems. A summary of the QS associated virulence genes and transcription factors employed by *Pseudomonas aeruginosa* is shown in **Table 1.5**.

Table 1.5 Quorum sensing regulation in *Pseudomonas aeruginosa*

Signaling molecule	R protein	I protein	Genes regulated	Virulence Factors
 **1.54**	LasR	LasI	*lasI, lasR, rhlR, lasB, lasA, toxA, aprA, xcpR, xcpP*	Elastase, Exotoxin A, Alkaline protease
 **1.48**	RhlR	RhlI	*rhlI, rhlAB, lasB, lasA,aprA*	Elastase, Alkaline protease, Pyocyanin
 **1.60**	NA	PqsH	*rhlI, lasB*	Elastase

Interestingly, the *Pseudomonas aeruginosa* quorum-sensing system not only coordinates virulence gene expression through cell-to-cell intercommunication but also modulates the host immune response.

1.5.5 *Pseudomonas aeruginosa* and immune modulation

Quorum sensing signals support bacteria to minimise host immune defence by delaying tissue damaging virulence factor synthesis until sufficient bacteria are accumulated over time and alert them to overwhelm host defence mechanisms in establishing infections.[126]

It is clear that upon infection *Pseudomonas aeruginosa* coordinates virulence gene expression through cell-to-cell inter-communication. There is also a substantial body of evidence which suggests that *Pseudomonas aeruginosa* can modulate the host immune response to an infection.[92,127,128] *Pseudomonas aeruginosa* in combination with fixed immunoglobulins have been found to eliminate *in vitro* immune suppression triggered by hydrocortisone[129] whilst **3O, C_{12}-HSL [OdDHL]** can modulate the inflammatory response by inducing the production of cyclooxygenase-2 (COX-2) and prostaglandin-E_2 in human lung fibroblasts [127,130] and has also been shown to down regulate both human and murine lymphocyte proliferation as well as LPS-induced secretion of TNF-α and interleukin-12. **3O, C_{12}-HSL [OdDHL]** also promotes immunoglobulinG-1 (IgG-1) production in antigen challenged splenocyte cultures and can induced apoptosis of leukocytes.[92,128] It is interesting to note that 3O, C_{12}-HSL has shown its potential in stimulating gamma interferon (IFN-γ) in T-cells as well as accelerating apoptosis in macrophages and neutrophils.[127]

C_4-HSL was not active in any of the above-mentioned systems, although it has been found be effective with protein tyrosine kinase (PTK) albeit at very high doses. However, recently more C_4-HSL than 3O, C_{12}-HSL have been isolated from the sputum of CF patients, suggesting its importance in biofilm formation but it has lacked immune modulation *in vitro*.[126]

PQS has been isolated in substantial amounts from the lungs of CF patients infected with *Pseudomonas aeruginosa.* This indirectly showed that, the *in vitro* presence of PQS might have helped *Pseudomonas aeruginosa* to develop to a chronic state.[115,131] PQS acts as an immune modulator by a distinctly different mechanism to that of 3O, C_{12}-HSL. Both PQS and 3O, C_{12}-HSL prevent leukocyte proliferation in the production of interleukin-2, and 3O, C_{12}-HSL is also capable of down regulating TNF secretion although this is not the case for PQS.[128] This thus suggested the presence of two separate targets for PQS and 3O, C_{12}-HSL binding for the same response to be initiated.[132]

The ability of these bacterial pheromones to affect the immune responses of eukaryotic cells in addition to their role in effecting prokaryotic genes expression, lead to their investigation as immune modulators. Telford *et al,* revealed that one of the lead structures 3O, C_{12}-HSL inhibited lymphocyte proliferation in a ConA mitogen stimulated murine spleen cell proliferation assay **(Appendix-I)**. This suggested that 3O, C_{12}-HSL exhibits anti-proliferative activity by modulating T-lymphocyte responses and not by depressing immune responses.[128] These results by Telford *et al* led Chhabra et al. to consider 3O, C_{12}-HSL as the lead structure for chemical modifications to generate synthetic analogues as novel immune modulators. However, structural modifications made to 3O, C_{12}-HSL.[131] were limited in their activity because some analogues of 3O, C_{12}-HSL have been found to possess comparable prokaryotic autoinduction in addition to eukaryotic immune modulation. Additionally, some analogues have not been evaluated *in vitro* because they showed no or poor solubility in a range of solvents and several analogues which were synthesized by replacing the L-HSL ring by other rings could not confirm that L-HSL ring is essential for immune modulation.[131]

1.6 PROJECT AIMS

The 3O, C_{12}-HSL, of *Pseudomonas aeruginosa* has sophisticated dual mechanisms in that it co-operates with bacterial cell-to-cell communication thus inducing a virulence gene expression. It also mediates the immune modulation in eukaryotes.[92] As mentioned previously, various analogues of 3O, C_{12}-HSL synthesized to date also possess comparable prokaryotic autoinduction in addition to eukaryotic immune

modulation.[131] In this research we sought to tackle the above issues to search for new immune modulating drug candidates devoid of any prokaryotic autoinduction. Therefore, the principal aim of the research programme was to synthesise and assay analogues of 3O, C_{12}-HSL that are immune modulatory but non-inducing agents.

This could be achieved by further alterations in 3O, C_{12}-HSL structure through replacement of, C^1 or C^3 carbonyl functionality by other functional group; C^4 carbon with hetero atom (nitrogen with or without substitution or oxygen); one or both the C^2 hydrogen by electron withdrawing fluorine and replacement of hetero ring with other rings as well as insertion of hetero atom(s) in the side chain A, as shown in **Fig 1.7**.

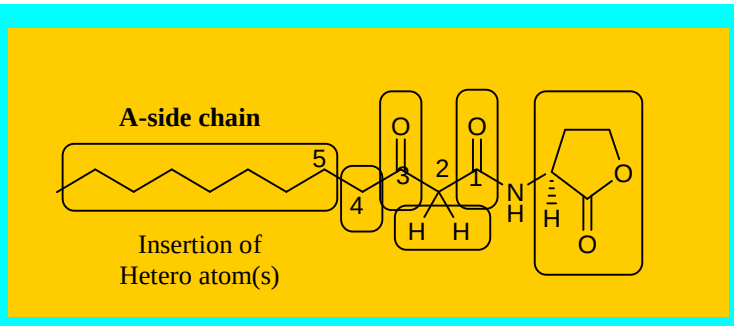


Fig 1.7 Structure of 3O, C_{12}-HSL showing points where changes can be made.

These analogues will be taken forward to evaluate for their immunological and microbiological (quorum sensing agonistic/antagonistic) assays. Newly developed analogues might show promising immune modulation even better than 3O, C_{12}-HSL as shown in **Fig 1.8**.

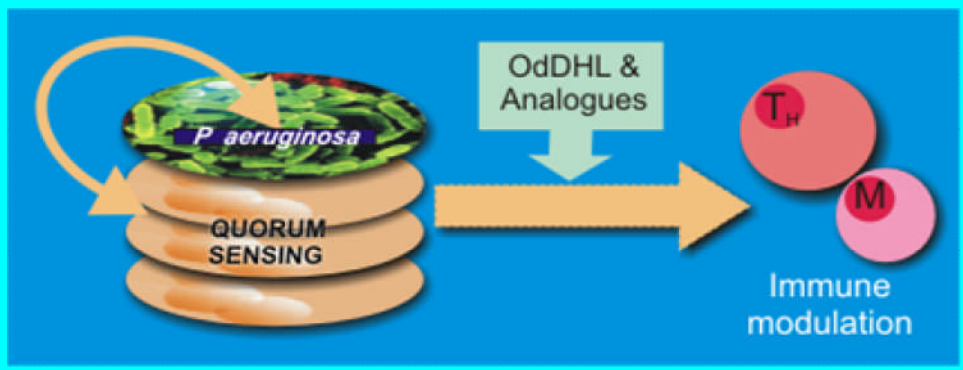


Fig 1.8 Immune modulations by quorum sensing molecules
T_H: Helper T-cells type1; M: Monocyte/macrophage
(Adepted from Pritchard et'al)

2.0 SEARCH FOR IMMUNE-MODULATORY BUT NON-INDUCING AGENTS: Design and synthesis of analogues of the *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-oxododecanoyl)-L-homoserine lactone

As already discussed in the previous chapter, the opportunistic pathogen *Pseudomonas aeruginosa* can actively monitor the host immune system through quorum sensing (QS). When a sufficient bacterial cell density is achieved to overwhelm host defense, the harmony within individual processes such as virulence factor release, biofilm formation and antimicrobial resistance by quorum sensing is considered to promote bacterial evasion to host immune responses thereby upregulating virulence factors.[77] Moreover, it is now understood that the immune system has evolved ways of sensing viral or bacterial products e.g. lipopolysaccharides. Among these toll-like receptors which activate antigen presenting cells, are perhaps the best studied ones.[133]

A recent study by Wu et al. has indicated that the opportunistic pathogen *Pseudomonas aeruginosa* elicits production of virulence factor in response to IFN-γ and thus actively monitors the host immune status.[134] The interaction between OprF, an outer membrane protein of *Pseudomonas aeruginosa* and host cytokine IFN-γ was found to upregulate production of virulence factors by OprF specifically binding to the cytokine IFN-γ. This exciting new element of bacteria-host interactions exemplifies that *Pseudomonas aeruginosa* QS system, both senses and modulates the host immune state.[135]

Wu et al., while monitoring expression of the *rhl*-regulated virulence factors, type-I *Pseudomonas aeruginosa* lectin (lecA) and pyocyanin, observed that exogenous IFN-γ substantially enhanced upregulation of lecA. This effect was revoked in mutations of rhlR or rhlI strains of *Pseudomonas aeruginosa* PAO1. This suggests the importance of *rhl* QS system in mediating the bacterial response to exogenous IFN-γ. An extended examination showed that the major outer-membrane protein OprF specifically binds to IFN-γ and that this activates the *rhl* QS system in an unknown manner.[134] Moreover, how does the *las* system transduce the signal(s) responsible for IFN-γ enhancement of the *rhl* system is still an important unresolved question. Thus the observed activation of the *rhl* system by exogenous cytokines that is caused by stimulation of the *las* system or by another regulatory pathway (or pathways) remains to be established.

As already discussed above that the immunomodulatory effects observed with autoinducer molecules 3O, C_{12}-HSL and C_4-HSL suggest that *Pseudomonas aeruginosa* not only actively senses but also tailors the host immune response.[134,136] 3O, C_{12}-HSL stimulates various host signaling pathways to inhibit or activate immune cell responses, whereas C_4-HSL does not[127,131,136]

Therefore, a cyclic network as shown in **Fig 2.1** can be proposed, from Wu et al. research data to exist between the *Pseudomonas aeruginosa* QS system and T-cells. The *las* regulon synthesizes the 3O, C_{12}-HSL (green ovals) which is actively transported outside of the cellular membrane by the MexAB-OprM efflux pump.[77] The 3O, C_{12}-HSL then stimulates T-cells and produces IFN-γ (pink circles).[136] IFN-γ then binds to OprF and this interaction then transduces a signal that might upregulate the *rhl* QS system by (a) activation of the PQS system; (b) upregulation of the *las* system through unknown regulator(s); or (c) activation through a currently unknown pathway. Two or more pathways may contribute to mediate the observed effect on the *rhl* system. Positive regulation is shown by plus symbol (+) and red arrows.

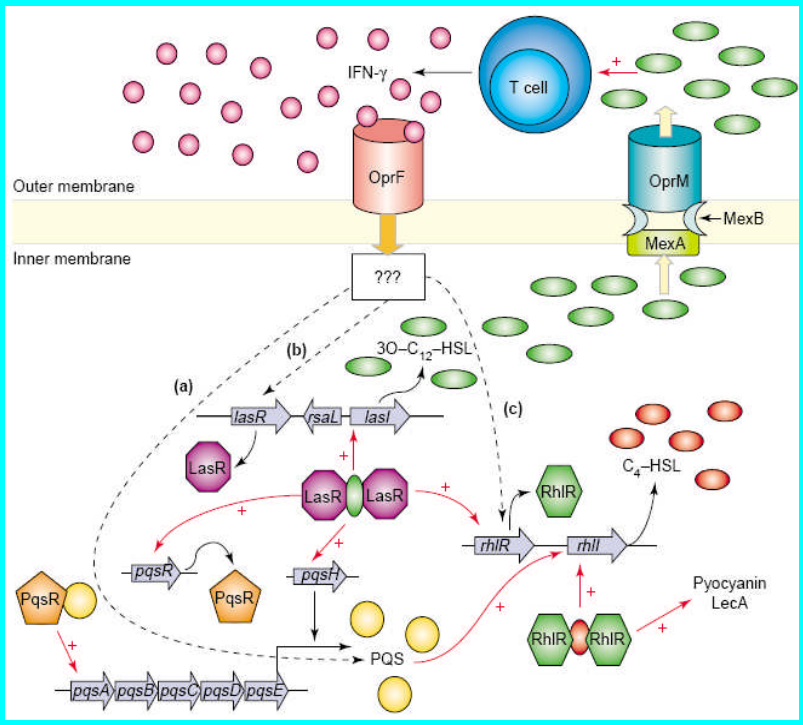


Fig 2.1 Current model of QS and proposed mechanisms of IFN-γ mediated QS activation.
Adepted from: Wagner et al., Trends in Microbiology **2006**, *14*, 55-58.

2.1 The diverse immunological bioactivity of 3O, C_{12}-HSL

2.1.1 Effect of 3O, C_{12}-HSL on T_H1-T_H2 balance

In the beginning of research in this field, the long chain AHL, 3O, C_{12}-HSL but not C_4-HSL, was shown to modulate immune system. Here 3O, C_{12}-HSL showed suppression of IL-12 and TNF-α secretion by bacterial lipopolysaccharide (LPS)-stimulated macrophages, and also inhibited T-cell proliferation. In contrast, *in vitro* immune evaluation in murine and human leukocytes showed that 3O, C_{12}-HSL at high concentrations ($>7\times10^{-5}$ M) inhibits antibody production by keyhole limpet hemocyanin-stimulated spleen cells; but at lower concentrations ($<7\times10^{-5}$ M), it stimulates antibody production, apparently by increasing the proportion of the immunoglobulin G1 (IgG1) isotype. It also promotes IgE production by IL-4-stimulated human peripheral blood mononuclear cells. These data suggest that 3O, C_{12}-HSL may influence the T_H1–T_H2 balance in the infected host in a dose dependant manner.[128]

2.1.2 Effect of 3O, C_{12}-HSL on insulitis and diabetes

Demolition of β-cell in the islets of Langerhans leads to loss of insulin production by the pancreas and symptoms of hyperglycaemia, polyuria, polydipsia and weight loss.[137] This β-cell destruction is preceded insulitis and infiltration of mononuclear cells is characterized by a T_H1-type CD4 T-cell response.[138] The work was therefore taken forward to evaluate the efficacy of 3O, C_{12}-HSL *in vivo* for the alleviation of insulitis and diabetes in non-obese diabetic mice (NOD). In this, groups of 20 mice were administered with 3O, C_{12}-HSL intraperitoneally (i.p.) at a dose of 30 milligram per kilogram (mg/kg) from weeks 4-8 of the lifespan. This treatment with 3O, C_{12}-HSL showed significant reduction in insulitis and also inhibited the infiltration of mononuclear cells into pancreatic islets, thus resulted in a complete and statistically significant reversal of the incidence of cumulative diabetes in the NOD mouse. Solvent controls did not show any protective effect in this model of diabetes.[139]

2.1.3 Effect of 3O, C_{12}-HSL on inflammatory chemokines and cytokines

The immunological effects of 3O, C_{12}-HSL have been further explored by other groups, who showed the induction of dermal inflammation *in vivo* by autoinducer. To determine how 3O, C_{12}-HSL did induce inflammation in the skin of mice, the research was carried out further in detail to evaluate effect of 3O, C_{12}-HSL on the individual components of the inflammatory process such as chemokines, cytokines, etc. Induction of chemokines and cytokines was determined from RNA extracted from skin samples of mice injected with 3O, C_{12}-HSL. A rise of five-to-six fold was found with the chemokines macrophage inflammatory protein-1β (MIP-1β), MIP-2, and monocyte chemotactic protein-1 (MCP-1). Similarly for the chemokines inducible protein-10 (IP-10) and T-cell activation gene-3 (TCA-3) as well as for proinflammatory cytokines IL-1α and IL-6, a three-to-four fold induction in the mRNA levels was found when stimulated with 3O, C_{12}-HSL *in vivo*. Altogether it suggests potency of 3O, C_{12}-HSL as an inducer of both inflammatory cytokine and chemokine genes. Among these, chemokines are known to encourage the migration of both activated and naive T-cells.[127] This known fact then motivated Smith et al. further to determine what effects, if any, 3O, C_{12}-HSL would have on these cells. Although not substantial, but significant induction in the production of the inflammatory cytokine IFN-γ was observed when naive T-cells previously activated with antigen and antigen-presenting cells (APCs) were cultured with 3O, C_{12}-HSL. The IFN-γ levels were analogous to those induced by the cytokine IL-12, a potent stimulator of IFN-γ production in T-cells. It is noteworthy to mention that 3O, C_{12}-HSL did not stimulate T-cells to produce IL-4, a cytokine generated by T_H2 cells. These data suggest that 3O, C_{12}-HSL potentially activates T-cells through induction of the inflammatory T_H1 phenotype. Not only as an inducer of inflammatory chemokines and cytokines, but 3O, C_{12}-HSL has also induced additional inflammatory mediators *in vivo* such as Nuclear Factor-kappa B (NF-κB), Cox and prostaglandins.[130]

The chemokines MIP-2, MIP-1β, IP-10, and MCP-1, induced by 3O, C_{12}-HSL, contain in their promoter regions specific binding sites for NF-κB.[140-143] 3O, C_{12}-HSL induced NF-κB was found to regulate the production of the chemokine IL-8. This enabled Smith et al. to hypothesize that the inflammation stimulated by 3O, C_{12}-HSL is

mediated through the induction of NF-κB. This hypothesis was supported by model where mouse keratinocytes were purified and stained with an anti-NF-κB antibody to visualize the localization of NF-κB in the cell. Cells stimulated with 3O, C_{12}-HSL showed translocation of NF-κB into the nucleus, where it could then potentially induce the transcription of multiple genes. Whereas, in cells without stimulation, NF-κB remain sequestered in an inactive form.[130]

2.1.4 Effect of 3O, C_{12}-HSL on Cox enzymes

Cox enzymes available in two isofoms, Cox-1 and Cox-2 are essential in biosynthesis of prostaglandins (PG) from arachidonic acid. Prostaglandins are involved in both normal and pathophysiological processes, such as inflammation, edema, platelet aggregation, fever, and hyperalgesia. Cox-1 favors normal physiological functions whereas, inflammatory stimulation is thought to induce Cox-2. Moreover, Cox-2 is identified as an immediate-early gene product due to the rapid induction of mRNA after stimulation.[94] The skin samples from normal mice (injected with phosphate-buffered saline (PBS) articulated no Cox-2 mRNA but constitutively expressed Cox-1 mRNA. In contrast, high levels of both Cox-1 and Cox-2 mRNAs were observed in samples from mice treated with 3O, C_{12}-HSL. Similarly protein extracts from mice skin injected with 3O, C_{12}-HSL, expressed a significant induction in Cox-2 protein highlight that Cox-2 is localized to the cells of inflammatory infiltrate as well as isolated cells, such as fibroblasts, lower in the dermis. Moreover, it demonstrates that 3O, C_{12}-HSL is a potent inducer of Cox-2 expression and not of Cox-1 *in vivo*.[144]

2.1.5 Effect of 3O, C_{12}-HSL on Prostaglandin E_2

Prostaglandin E_2 (PGE_2) is a potent molecule that influences multiple functions in the body. Its production thus may have profound effects on the pathogenesis of *Pseudomonas aeruginosa* because it is anti-inflammatory in the lung and inhibits the release of mediators from mast cells as well as migration of eosinophils and induces bronchodilation in asthmatic patients.[145] Moreover, it has been demonstrated that PGE_2 is induced by *Pseudomonas aeruginosa* in mouse pneumonia and thermal injury models.[146-148] Therefore, it was of interest to see if *Pseudomonas aeruginosa* quorum sensing molecule 3O, C_{12}-HSL has any effect on PGE_2 production in human lung

fibroblasts. A novel finding by Smith et al. revealed that the expression of membrane-associated prostaglandin E synthase (mPGES) in human lung fibroblasts was increased with 3O, C_{12}-HSL stimulation in sequential manner with Cox-2. This sequential regulation of these Cox-2 and mPGES enzymes is most likely a method of controlling the levels of PGE_2 production. The sequential induction of both Cox-2 and mPGES significantly increased the production of PGE_2.[144] However, Cox-2 induction was quicker and short-lived than that of mPGES. Similar sequential expression of Cox-2 and mPGES was induced by stimulation of orbital fibroblasts with IL-1β.[149] It was also demonstrated that 3O, C_{12}-HSL stimulation did not induce cytosolic PGES (cPGES) and Cox-1. This supports the previous finding that cPGES is functionally associated to Cox-1 while mPGES is bonded with Cox-2.[150,151] This indicated that 3O, C_{12}-HSL predominately induces Cox-2 and mPGES for the induction of PGE_2.

2.1.6 Effect of 3O, C_{12}-HSL on biochemical signaling events of innate immunity

Because the airways are the main point of entry of *Pseudomonas aeruginosa* into the human body, a diverse range of cells including alveolar macrophages, lung fibroblasts, and bronchial epithelial cells is therefore most likely to be exposed to this pathogen and eventually to 3O, C_{12}-HSL. Very recently, Kravchenko[152] et al., have demonstrated that 3O, C_{12}-HSL induce the phosphorylation of mitogen-activated protein kinase (MAPK) p38 and eukaryotic translation initiation factor 2α (eIF2α) in these diverse cell types. This phosphorylation event is one of the important innate immune responses observed when immune cells encounter the pathogen-associated molecular patterns (PAMPs).[153] Moreover, they have clearly demonstrated that 3O, C_{12}-HSL mediated signaling events are independent on MyD88, TRIF, TLR2, TLR4, Nod1, and Nod2, key molecules of pattern recognition receptors (PRRs).[133,153-155] These observations suggested that 3O, C_{12}-HSL might represent a new class of PAMP that signals through pathways different from those occupied by TLR and Nod1/Nod2 proteins.[152]

2.1.7 Contradictory effects of 3O, C_{12}-HSL

It is noteworthy that in contrast to the findings of Telford et al., findings of Smith et al., led later authors to postulate that 3O, C_{12}-HSL was in fact encouraging a T_H1 environment. However, one cannot deny the effects observed by Smith et al., though these were at high dose (100 μM) of 3O, C_{12}-HSL than those of Telford et al., which were at low dose (<10 μM).[144,156,157] Very recently Prichard et al., found that the doses used by Smith et al., have in fact been shown to cause programmed cell death (apoptosis) in eukaryotic cells. This perhaps raises question related to the physiological relevance of the high doses of 3O, C_{12}-HSL.[158] Moreover, an independent research conducted by Tateda et al., confirmed the pro-apoptotic effect of high-dose exposure.[127]

Until now, several reports although controversial, have suggested that 3O, C_{12}-HSL has immune modulatory effect and promoted either humoral (T_H2-mediated) or inflammatory (T_H1-mediated) responses. Ritchie et al., have tried to investigate this phenomenon in detail in order to resolve confliction of previous reports. They studied a series of experiments using *in vivo* and *in vitro* animal models; T_H1 biased C57BL/6, T_H2 biased BALB/c and T-cell receptor (TCR) transgenic. Ritchie et al., were the first to demonstrate a direct effect of 3O, C_{12}-HSL on *in vivo* antigen-specific immune response i.e. the production of antibody to the protein antigen OVA (ovalbumin). *In vivo* administration of BALB/c mice with 3O, C_{12}-HSL at an equivalent dose of 0.3 mg/kg was found to raise the T_H2-associated antibodies, OVA-specific (immunoglobulinG1) IgG1, while having no significant effect on the levels of T_H1-associated antibodies, OVA-specific IgG2a. The 3O, C_{12}-HSL was also found to inhibit *in vitro* cytokines production i.e. inhibition of both IFN-γ and IL-4 in splenocytes of C57BL/6 and BALB/c mice stimulated with ConA and also in TCR transgenic splenocytes, where IFN-γ being more inhibited than IL-4. Interestingly, in the case of antigen-specific immune responses, *in vivo* administration of 3O, C_{12}-HSL in T_H1-biased C57BL/6 mice favored the expression of the T_H1 cytokine IFN-γ over the T_H2 cytokine IL-4, thus temporarily accentuated the T_H1 response. Whereas, mitogen and antigen induced responses in T_H2-biased BALB/c mice favored enhancement in level of T_H2 cytokine IL-4 over IFN-γ, thus accentuated T_H2 response.

In summary, unlike earlier findings of Telford et al., this effect proposed that action of 3O, C_{12}-HSL on T-cell cytokine production is likely to be a relatively nonspecific, not promoting either T_H1 or T_H2 responses but is dependant on the model being used.[159]

Further research by Ritchie et al., selected a well-described TCR-transgenic mouse model to explore the effects of 3O, C_{12}-HSL in a defined *in vitro* antigen-specific T-cell response.[160] In this model the transgenic TCR expressed on the majority of CD4 T-cells recognizes a defined peptide antigen from moth or pigeon cytochrome-c.[161] Using this model, Ritchie et al., have demonstrated that the effects of 3O, C_{12}-HSL on $CD4^+$ T-cell was to inhibit the differentiation of both T_H1 and T_H2 cells, that 3O, C_{12}-HSL must be present within the first 2 h after antigen or mitogen stimulation. In addition, in contrast to Telford et al., and Smith et al., they have showed that the cytokine production was not due to toxicity or death of T-cells. Similarly its site of action was found likely to be cytoplasmic and did not appear to depend on its insertion into the cell membrane as explained by Chhabra et al.[131]

In summary, recent research has now established that quorum sensing signal molecule (QSSM) hierarchical cascades is biologically linked to the presence of chemically diverse and differentially active immune modulating molecules in *Pseudomonas aeruginosa*.[162] These findings have played a pivotal role in understanding of bacterial pathogenesis, and may be useful in the development of better future tactics for disease management. For example, QSSM inhibitors and conventional antibiotics combination have been recently recommended.[163,164] In addition, novel therapeutics for a multitude of immunological diseases through a potentially valuable natural source of chemical templates has been identified. For example, 3O, C_{12}-HSL has been found to act not only as a eukaryotic immune modulator but also a prokaryotic autoinducer.[165] Recently, Kravchenko et al., have classified this 3O, C_{12}-HSL as separate type of PAMP which modulates immune system via mechanism distinct from those by TLR and Nod1/Nod2 proteins.[152]

2.2 Biological targets for 3O C_{12}-HSL

Since the immune modulatory capabilities of 3O, C_{12}-HSL have been discovered on various cellular patterns of host immune system, its precise binding target in mammalian cells has remained to be identified. Smith et al. have observed that 3O, C_{12}-HSL enhances activation of NF-κB[130] and COX-2[144] in respiratory epithelial cells. Ritchie et al., have further narrowed down the possible modes of action in T-cells by strongly suggesting modulation of some aspect of the TCR-dependent or costimulatory signal transduction pathways by 3O, C_{12}-HSL. However, researchers were unable to identify where in the pathway 3O, C_{12}-HSL was bound to lead this activation.

Hooi et al., on the other hand proved that 3O, C_{12}-HSL in eukaryotes mediate fluctuations in immunological behaviours by targeting specific receptor(s) different from another class of QSSM namely PQS targets. 3O, C_{12}-HSL and PQS exhibited aberrant effects on the release of IL-2. Administration of 3O, C_{12}-HSL has inhibited cell proliferation and IL-2 release in previously activated human T-cells by the T-cell receptor and CD28, whereas PQS administration has inhibited cell proliferation without disturbing IL-2 release. In addition, as mentioned by Tateda et al., in human monocytes, 3O, C_{12}-HSL has found to inhibit release of TNF-α in the presence of *Escherichia coli* LPS, whereas PQS unable to inhibit TNF-α.[132,166]

As discussed by Kravchenko et al., the effects of 3O, C_{12}-HSL imitate the effects of adaptor proteins, MyD88 and TIR-domain-containing protein inducing INF-*β* (TRIF). Whether 3O, C_{12}-HSL requires MyD88 or TRIF for phosphorylation of p38 and eIF2α as well as induction of cleavage of poly(ADP-ribose) polymerase (PARP) was of interest. Effects in BMDM of control mice and mice with genetic defects in MyD88 or TRIF functions were compared. Biochemical analysis of BMDM from the adaptor protein-deficient mice showed that 3O, C_{12}-HSL induction of PARP cleavage, P-p38, and P-eIF2α was identical to that of control mice. Thus, neither MyD88 nor TRIF was expressed in the 3O, C_{12}-HSL mediated biochemical signalling events. Eventually, these findings suggested that the recognition and transduction of the 3O, C_{12}-HSL signal did not require Toll-like receptors (TLRs) such as TLR-3, -5, -7, -8, and TLR-9. These TLRs in specific pattern are essential for MyD88 and TRIF. Other receptors

TLR-2 and TLR-4 that are able to recognize long-chain fatty acid moieties containing molecules were also observed unimportant for 3O, C_{12}-HSL mediated biochemical changes. It is also known that the intracellular proteins Nod1 and Nod2 also participate in innate immune responses distinct from those intervened by the TLRs.[133,153,167] Examination of BMDM from mice missing both Nod1 and Nod2 revealed that 3O, C_{12}-HSL induced phosphorylation of p38 and eIF2α and PARP cleavage was similar as that of control mice. This suggested that initial recognition and signalling events of 3O, C_{12}-HSL in macrophages do not require Nod1 and Nod2 proteins.[152]

Mitochondrial stresses activate caspase-9 and caspase-3 proapoptotic signals which eventually lead to inevitable proteolytic cleavage of PARP.[168,169] Similarly, with local stress stimuli the endoplasmic reticulum (ER) changes the rates of translation and transduction processes through phosphorylation of eIF2α.[170] Kravchenko et al., showed with evidence the ability of 3O, C_{12}-HSL to induce mitochondrial damage depends on the activation of caspase-9, a biochemical event indicative of mitochondria-mediated apoptosis[169] as well as its ability to induce ER stress and phosphorylate eIF2α. Additionally diverse cell types of either human or murine origin were found to be affected by 3O, C_{12}-HSL in a qualitatively similar manner. Electron microscopic analysis of 3O, C_{12}-HSL treated BMDM fibroblasts and epithelial cells showed dramatic morphological alterations including mitochondrial swelling and distension of the ER. However, less pronounced mitochondrial alterations in fibroblasts and epithelial cells suggested that macrophages are more sensitive to the proapoptotic effects of 3O, C_{12}-HSL. In contrast, identical ER distention in macrophages and non-myeloid cells, suggested that the ER is involved centrally in the response of different cell types to 3O, C_{12}-HSL. In total, these observations suggested that 3O, C_{12}-HSL affects signaling pathways in a manner distinct from TLR-specific PAMPs.[152]

Overall, explanation of the binding target(s) of 3O, C_{12}-HSL therefore, demands analysis of effects of 3O, C_{12}-HSL at proteomics level and/or modification of its structure into new analogue(s) with high affinity to specific immune receptor(s). This still raises question: ‘Is the 3O, C_{12}-HSL a good lead molecule to aim at in order to alleviate aberrant immune system?’

2.3 Is the 3O, C_{12}-HSL an ideal molecule to aim at in order to combat dysfunctional immune system?

The first question to address is whether the 3O, C_{12}-HSL is an ideal molecule to select in the fight to correct abnormal immune behaviours. Consensus is generally positive with some immune modulatory results of 3O, C_{12}-HSL being brought forward. The healthy immune system is apt to combat infections and diseases, whereas an unbalanced immune system hampers good health and quality of life. As mentioned already 3O, C_{12}-HSL has the potential to modulate immune system. However, it must be noted that the 3O, C_{12}-HSL is utilised by *Pseudomonas aeruginosa* not only to co-ordinate its population density but also to control the expression of numerous virulence factors. Therefore, while explaining 3O, C_{12}-HSL to target the immune system, care must be taken to avoid the expression of virulence factors.

A wise approach therefore, is to alter 3O, C_{12}-HSL chemically for its structural modifications and to generate a series of new synthetic congeners that would selectively enable eukaryotic immune modulation without promoting prokaryotic quorum sensing as shown in **Fig 2.2**.

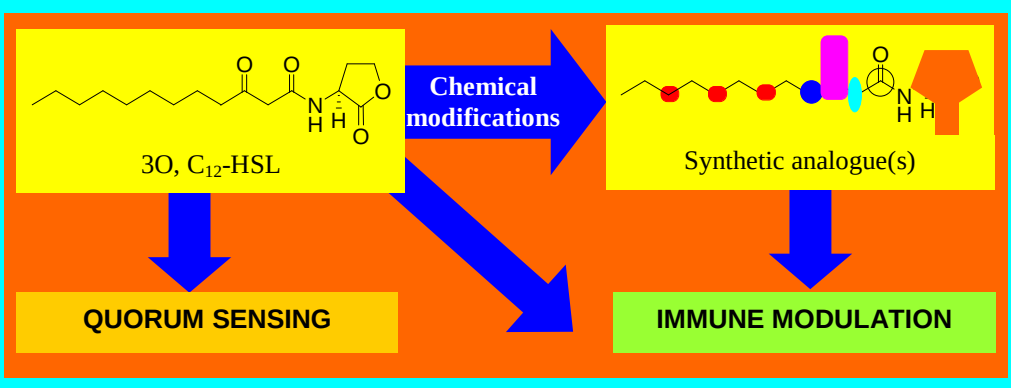


Fig. 2.2 New synthetic analogues of 3O, C_{12}-HSL as selective immune modulators.

To this end recently Chhabra et al., have synthesized a series of synthetic analogues through variations within the structural components of the native autoinducer 3O, C_{12}-HSL with some success in the improvement of eukaryotic immune modulation.[131,171]

2.4 First generation immune modulatory analogues of 3O, C_{12}-HSL: SAR studies to date

As mentioned earlier among all QSSMs of *Pseudomonas aeruginosa*, 3O, C_{12}-HSL has been thoroughly investigated because of its ability to mediate immune processes of eukaryotic cells in addition to its role in effecting prokaryotic genes expression.[126] The preferred anti-proliferative activity observed by Telford et al., prompted Chhabra et al., to explore 3O, C_{12}-HSL as a 'lead structure' for SAR studies and developed a large number of immune modulatory analogues of 3O, C_{12}-HSL.[128,131,156,171]

The SAR is an approach which can be used to relate the effect of a chemical moiety (drug or toxic chemical or bioactive component of natural or synthetic origin) on an animal, plant or the environment to its molecular structure. The SAR may be assessed by considering a lead molecule, making gradual changes in its structure to obtain a series of new molecules and finally noting the effect of each change upon their biological activity. With this principle, synthetic analogues of 3O, C_{12}-HSL were prepared introducing single or combined structural variations within the structural components A, B and C (as shown in Fig. 2.3)[131,156] and were subjected to immunological assays.

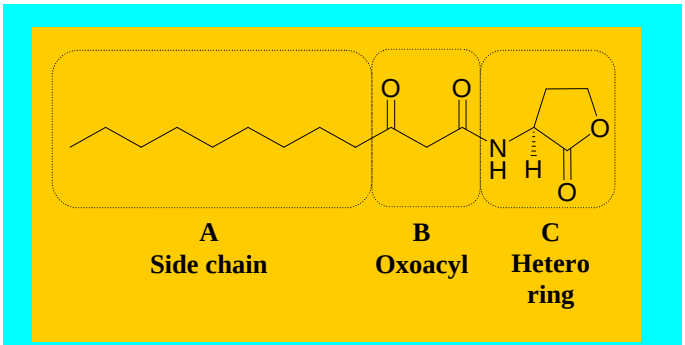


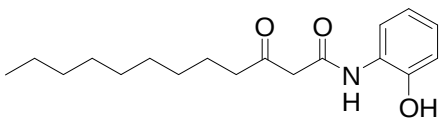
Fig 2.3 3O, C_{12}-HSL structure showing A, B and C fragment, where changes have been made.

Biological screening of analogues synthesised by Chhabra *et al*., showed that the L-HSL ring acylated with 3-oxo or hydroxy, substituted 12-14 carbon chain is optimal for immune suppressive activity. Terminal carbon halogenation (bromide) and/or hydroxylation as well as unsatutation in acyl chain 'A' retained activity. Product

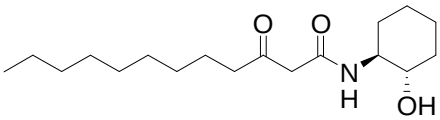
formed from the conversion of 3-oxo to hydroxy group was equi-potent to that of parent compound in immune suppression. Long acyl chains have found to maintain lipid solubility that is important for immune suppression by these compounds. However, compounds with more than 12 carbon acyl chain gave solubility problems in the solvents used for bioassays. Opening of L-HSL ring 'C' and its replacement by other ring such as thiolactone have shown no immune suppression.[131] Interestingly, replacement of 'C' with cyclohexanol, caprolactam have resulted compounds with agonistic behaviour in prokaryotes and replacement with quinoline ring showed strong agonism, though this might be due to its structural similarity with the autoinducer PQS.[172]

Tateda et al, in their previous study of the small number of compounds without 3-oxo functionality and/or with (*D*)-HSL found no apoptosis activity.[127] Same was reconfirmed later by Horikawa et al., when activity was measured on the macrophage P388D1 cell line.[173] This profoundly supported Chhabra et al., research and proved the significance of the 3-oxo acyl group and of the homoserine lactone moiety to be of (**L**)-configuration.[131]

Moreover, Horikawa et al, have also synthesized an array of 3O, C_{12}-HSL analogues containing different acyl side chains as well as *Pseudomonas* QS agonist (**2.1**) and an antagonist (**2.2**)[172] and subjected them for the induction of apoptosis in macrophages that is, the P388D1 cells.

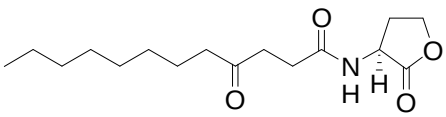


2.1

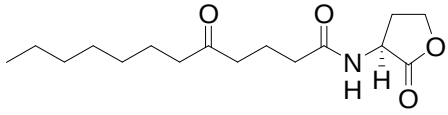


2.2

The lack of apoptotic activity in the analogues **2.1** and **2.2** further confirmed the role of the L-HSL in immune modulation. Analogues with 3-oxo-acyl chains of 12-14 carbons retained potent apoptosis-inducing activities. The shifting of the 3-oxo group to the 4-(**2.3**) or 5- (**2.4**) position greatly reduced the activity.



2.3



2.4

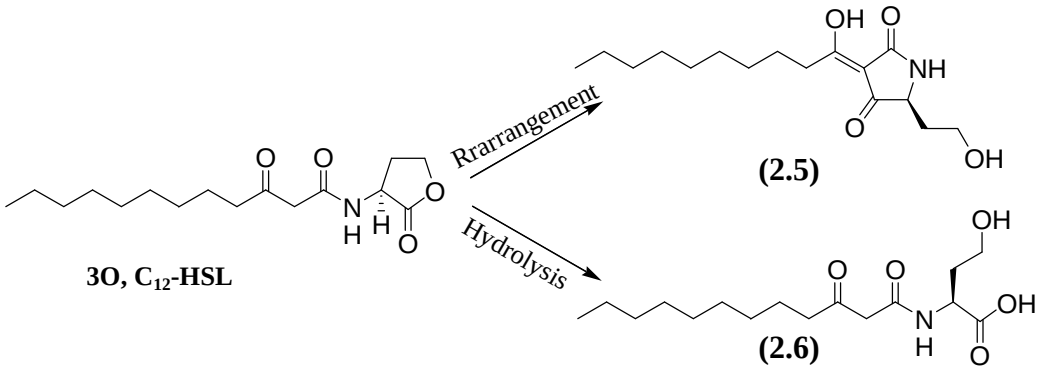
Terminal substitution of acyl chain with hydrophilic functional groups, (such as hydroxy or succinyl group), reduced the apoptosis inducing ability whereas terminal lipophilic (phenyl, benzyl, cyclopropyl or cyclopropylethyl groups) substitution gave potent apoptosis inducing compounds.[173]

As discussed previously, Kravchenko et al, have also suggested that the structural integrity of the homoserine lactone ring motif in 3O, C_{12}-HSL was required for induction of apoptotic pathways as well as phosphorylation of MAPK p38 and eIF2α. A variety of structurally highly conserved microbial products also referred as PAMPs when encounter to key cells of the innate immune system such as macrophages, it induces several signalling events including the phosphorylation of MAPK p38, and/or of eIF2α. MyD88- and TRIF-dependent signalling pathways control the activation of the transcription factor NF-κB and phosphorylation of MAPK including p38. Whereas, TRIF-dependent signalling attenuate protein synthesis via phosphorylation of the eukaryotic eIF2α. Both MyD88 and TRIF are known as Toll/interleukin-1 receptor (TIR) domain-containing adaptors and among these TRIF induces IFN-γ. Kravchenko et al., therefore, decided to determine effects of 3O, C_{12}-HSL and its analogues on the phosphorylation state of p38 and eIF2α to investigate possible PAMP-like signaling in macrophages.[152]

Bone marrow derived macrophages (BMDM) when exposed to 3O, C_{12}-HSL exhibited phosphorylation of p38 and eIF2α in time- and dose-dependent manner. The specific L-stereochemistry of natural analogue was observed to be essential, because unnatural D-stereoisomer lacked activity. Interestingly, induction of phosphorylation was retained by other members of the 3-oxo-AHL family (C_{10} and C_{14}). However, C_4-HSL not containing 3-oxo group has no activity. Additionally, the lactam congener of the 3O, C_{12}-HSL also failed to induce phosphorylation. Taken together, these data have

suggested the importance of integrity of the homoserine lactone ring motif to induce distinct cellular events leading to P-p38 and P-eIF2α.[152]

Moreover, at experimental cell culture pH~7.4, 3O, C_{12}-HSL through two non-enzymatic reactions is converted into tetramic acid derivative (**2.5**) and its ring opened hydrolysed structure (**2.6**) as shown below.



The product **2.6** was rapidly generated and predominantly distributed in cellular fractions whereas 3O, C_{12}-HSL predominantly distributed in the supernatant. Association of 3O, C_{12}-HSL, **2.6** or both with the cells was found to be essential to induce P-p38 as well as P-eIF2α. However, administration of **2.5** and **2.6** separately with macrophages has not shown the induction of P-p38 and P-eIF2α. This thus supports importance of integrity of 3O, C_{12} HSL structure.[152]

As already shown by Li et al., that 3O, C_{12}-HSL on prolonged incubation with transformed human cells[174] have induced the cleavage of PARP, a biochemical marker suggestive of apoptosis.[168] Whereas, **2.5**, **2.6** or lactam analogue, have not induced the cleavage of PARP. Thus, induction of apoptotic pathways as well as P-p38 and P-eIF2α requires structural integrity of the homoserine lactone ring moiety.[152]

2.4.1 Limitations in the SAR studies and conflicting reports in the immune modulatory behaviour of 3O C_{12}-HSL

Unfortunately, the analogues including 3O, C_{12}-HSL do suffer from a main drawback that besides mediating eukaryotic immune responses, these also participate in bacterial

intercellular communication inducing virulence gene expressions. This later trait promotes bacterial growth and thus obliviously negates the clinical usefulness of these analogues. Additionally, synthetic analogues with an alkyl chain more than 12-carbons possess low solubility in routine solvents employed for *in vitro* testing and thus, could not be explored for their immunomodulatory effects. Furthermore, opening of L-HSL ring and thiolactone ring containing analogues developed by Chhabra et al., as well as lactam congener of Kravchenko et al, do not strongly convince that the L-HSL ring is essential for immune modulation. This would have been better supported by synthesizing a few more other ring variants of 3O, C_{12}-HSL and then correlating their immune responses.

As explained, the acyl chain which maintains lipophilicity of the molecule was important for immune suppression. This suggested that 3O, C_{12}-HSL must be acting by disruption of the association of the TCR molecules possibly by entering within the lipid bilayer. However, research data of the *in vitro* investigation of TCR-transgenic CD4 T-cells model obtained by Ritchie et al., have contradicted this where the effect of 3O, C_{12}-HSL on activation and cytokine production did not depend on its insertion in the cell membrane, instead was likely at an intracellular site.

Furthermore, as already discussed earlier, inherent immune modulatory capability of 3O, C_{12}-HSL has been extensively studied by several groups, indicating a range of contradictory effects on mammalian cells. Chhabra et al., Ritchie et al. and Telford et al., discovered *in vitro* suppression of lymphocytes by 3O, C_{12}-HSL. Telford group further observed more pronounced inhibition of preferably T_H1 type responses by 3O, C_{12}-HSL. Smith et al. however, produce contradictory data to support up-regulation of proinflammatory cytokines such as IL-8 at higher concentrations of 3O, C_{12}-HSL. Ritchie et al., revealed that the action of 3O, C_{12}-HSL on T-cell cytokine production was likely to be a relatively nonspecific and dependant on the model being used. Recently, Ritchie and group came up with other contradictory results that 3O, C_{12}-HSL inhibits the differentiation of T_H1 and T_H2 $CD4^+$ T-cell within the first 2 h of after antigen or mitogen stimulation. They also showed that immunosuppressive effect of 3O, C_{12}-HSL does not depend on its insertion in the cell membrane.

In spite of the contradictory results observed by different groups, what is undoubted is the immune modulatory potential of 3O, C_{12}-HSL. It would therefore be desirable to introduce further modifications in the structure of 3O, C_{12}-HSL molecule in order to improve its immunological profile while losing prokaryotic QS activity and also to alleviate solubility problem. Based on this, the aim of the current project was to generate a second generation analogues of 3O, C_{12}-HSL and then carry out a detailed SAR studies.

2.5 Second-generation immune modulatory analogues of 3O, C_{12}-HSL and its SAR studies

Being highly amenable to chemical modifications, 3O, C_{12}-HSL provides immense scope for the generation of potential immune modulatory drug candidates. We considered introducing further structural changes in 3O, C_{12}-HSL molecule by applying the following strategies and expected to generate new analogues with better biological profile.

2.5.1 Strategies for new SAR studies of 3O C_{12}-HSL

To generate synthetic analogues of 3O, C_{12}-HSL, which would retain immunological modulation in the absence of any deleterious bacterial growth, 3O, C_{12}-HSL molecule was divided into three regions A, B and C as shown in **Fig 2.4**.

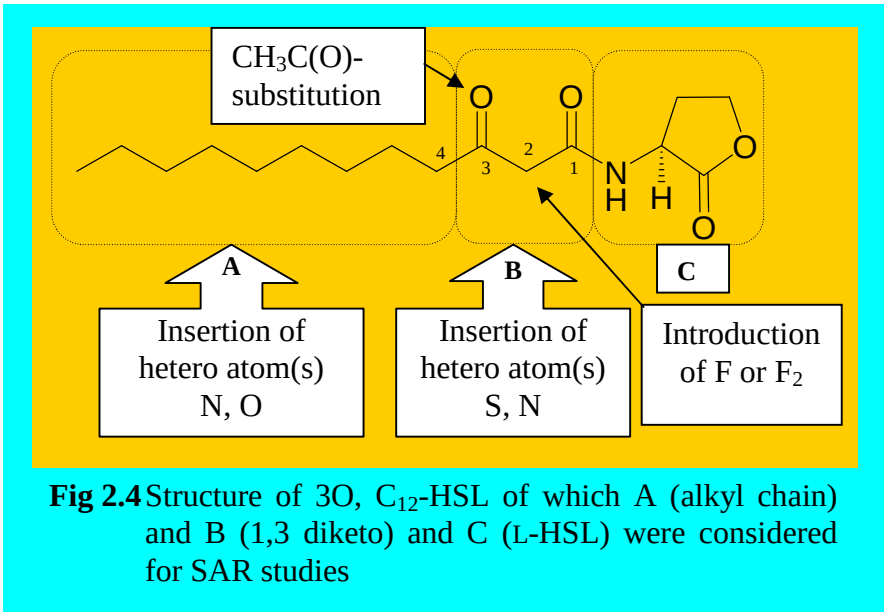


Fig 2.4 Structure of 3O, C_{12}-HSL of which A (alkyl chain) and B (1,3 diketo) and C (L-HSL) were considered for SAR studies

Chhabra and coworkers[13] have already explored the SAR of this molecule by synthesizing a number of analogues and showed that 3-oxo functionality is important for its immunomodulatory activity.[131] As the 3-hydroxy compound was equipotent, we wondered whether the activity was elicited through 3-oxo structure or its enolic form. We thus, decided to extend investigation of this molecule by modulating its *3-enolic* content (**Fig 2.4**) by using the following strategies

Strategy-1: *Aza (4-aza and 3-Aza) analogues of 3O, C_{12}-HSL*

C^3 or C^4 carbon atom may be replaced by heteroatom nitrogen and nitrogen may be optionally substituted.

Strategy-2: *Fluorinated 4-aza analogues of 3O, C_{12}-HSL*

One or both the C^2 hydrogen(s) in 4-aza analogues of 3O, C_{12}-HSL may be replaced by electronegative fluorine(s).

Strategy-3: *1-Thia and 3-thia analogues of 3O, C_{12}-HSL*

Here alteration of carbonyl group at C^1 or C^3 position by sulphide, sulfonyl or sulfone group with or without insertion of hetero atom at C^4 carbon may be performed.

Strategy-4: *4-Oxa analogue of 3O, C_{12}-HSL*

C^4 carbon may be replaced by another heteroatom, oxygen.

Strategy-5: *Other ring altered analogues of 3O, C_{12}-HSL and its 4-aza analogues*

L-Homoserine lactone ring of 3O, C_{12}-HSL may be replaced by other aromatic or hetero aromatic ring with or without substitution.

Strategy-6: *Insertion of hetero atom(s) in the acyl chain of 3O, C_{12}-HSL or its 4-aza analogues*

Hetero atom(s) oxygen may be introduced at C^8 position individually or C^7 and C^{10} positions in combination to increase hydrophilicity.

Strategy-7: *3-Acetoxy analogue of 3O, C_{12}-HSL*

C^3 oxo group may be reduced to hydroxyl group and then acetylated.

Strategy-8: *Tetramic acid (TMC) analogue derived from 3O, C_{12}-HSL*

3O, C_{12}-HSL may be transformed using strong base to obtain a TMC derivative via an intramolecular cyclisation.

The purpose of these alterations was to establish the optimal pharmacophores in the A, B and C regions of the nolecule which could alleviate problems associated with (a) QSSM activity without losing immunomodulatory activity and (b) solubility of the molecule so that longer chain length can be accommodated in the A region of the molecule.

The new 3O, C_{12}-HSL analogues will be screened for activity using established immunological assays and are expected to have selective human immune modulation without any prokaryotic autoinduction.

2.6 4-Aza analogues of 3O, C_{12}-HSL

Introduction of nitrogen in bioactive organic molecules has become increasingly popular recently in drug design due to its ability to confer electronic and steric effects. This in turn has effect on the biological potency of a drug molecule. One of the objectives of synthesizing 4-aza analogues of 3O, C_{12}-HSL as shown in **Fig 2.5** was in part to evaluate its immune modulation compared to 3O, C_{12}-HSL.

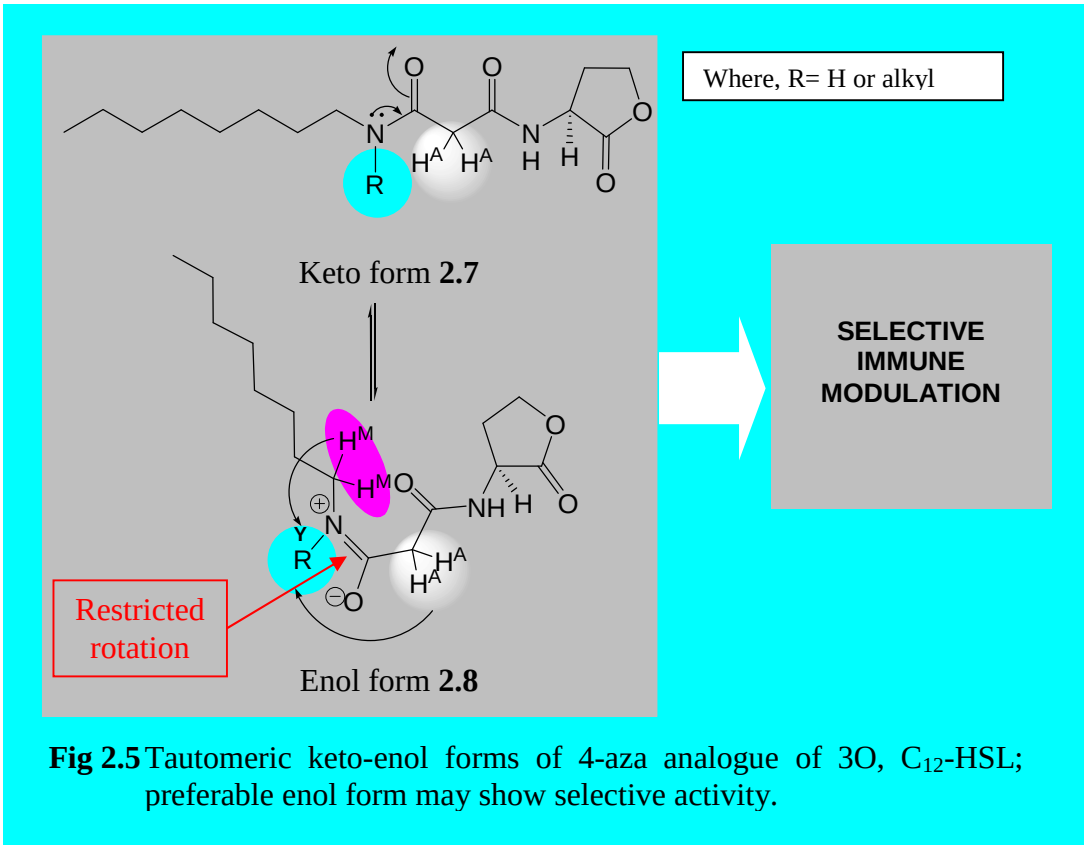


Fig 2.5 Tautomeric keto-enol forms of 4-aza analogue of 3O, C_{12}-HSL; preferable enol form may show selective activity.

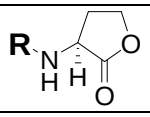
The electronegative nitrogen was expected to provide an additional site for receptor binding and thus may contribute towards selective immune modulation in eukaryotes. If active, 4-aza analogues will then be N^4-substituted to see if the activity is retained and this in turn would be useful information to confirm whether N^4-H is a site of interaction with the receptor.

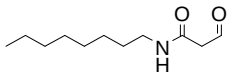
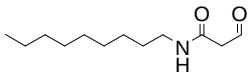
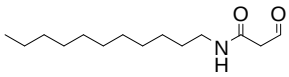
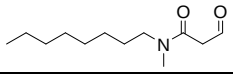
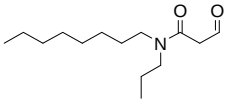
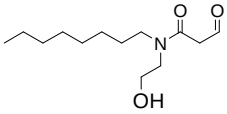
Furthermore, the new C^3-N^4 amide bond can give rise to keto **2.7** -enol **2.8** form tautomerism.[175] Also alkyl substituted 4-aza derivatives **2.11-2.13** are expected to exist

preferably as *E*-isomer (**Fig 2.5**) due to the restricted rotation of the N^4-C^3. The later attribute may influence the auto-inducing activity of 3O, C_{12}-HSL.

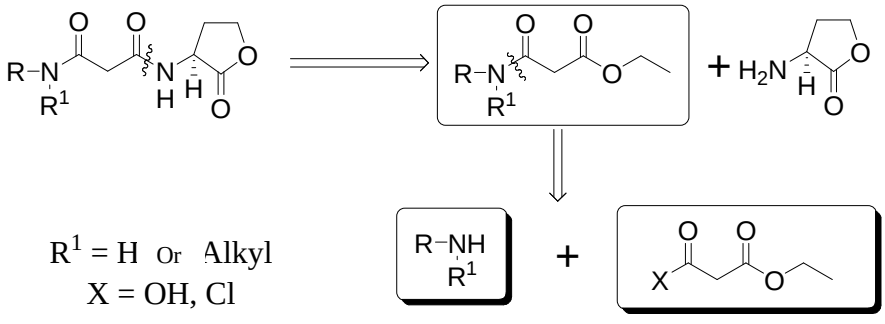
Moreover, the electronegative N^4 would be expected to improve solubility of new analogues through hydrogen bonding. This would in turn maintain the hydrophilic-lipophilic balance and may allow alkyl chains of the larger length to be accommodated. Various 4-aza analogues **2.8-2.13** of 3O, C_{12}-HSL were consequently synthesized as outlined in **Table 2.1**.

Table 2.1 4-Aza analogues of 3O, C_{12}-HSL



Comp.	R	Chemical name	Abbreviation
2.8		*N*-(3-Oxo, 4-azadodecanoyl)-L-HSL	3O, 4-aza C_{12}-HSL
2.9		*N*-(3-Oxo, 4-azatridecanoyl)-L-HSL	3O, 4-aza C_{13}-HSL
2.10		*N*-(3-Oxo, 4-azapenta decanoyl)-L-HSL	3O, 4-aza C_{15}-HSL
2.11		*N*-(3-Oxo, 4-methyl, 4-azadodecanoyl)-L-HSL	3O, 4-Me, 4-aza C_{12}-HSL
2.12		*N*-(3-Oxo, 4-*n*-propyl, 4-azadodecanoyl)-L-HSL	3O, 4-Pr^n, 4-aza C_{12}-HSL
2.13		*N*-(3-Oxo, 4-(2-hydroxyethyl), 4-azadodecanoyl)-L-HSL	3O, 4-(2-hydroxyEt), 4-aza C_{12}-HSL

The retro-synthetic approach for the generation of these analogues of 3O, C_{12}-HSL is shown in **Scheme 1**.



R^1 = H Or Alkyl
X = OH, Cl

Scheme 1 Retrosynthesis of 4-aza analogues of 3O, C_{12}-HSL

As seen in **Scheme 1,** the starting material required for the generation of these analogues are either primary amines **2.14-2.16** or secondary amines **2.17-1.19** (**Table 2.2)**.

Table 2.2. Amines required as starting materials

H R–N–R^1			
Comp.	**R**	**R^1**	**Chemical name**
2.14	-C_8H_{17}	H	*n*-Octylamine
2.15	-C_9H_{19}	H	*n*-Nonylamine
2.16	-$C_{11}H_{23}$	H	*n*-Undecylamine
2.17	-C_8H_{17}	-CH_3	*N*-Methyloctylamine
2.18	-C_8H_{17}	-*n*-C_3H_7	*N*-Propyloctylamine
2.19	-C_8H_{17}	-C_2H_5-OH	*N*-(Hydroxyethyl)octylamine

The primary amines **2.14-2.16** were commercially available but the secondary amines **2.18** and **2.19** except *N*-methyl octylamine **2.17**, needed to be synthesized.[176-179]

2.6.1 Synthesis of secondary amines (2.18 and 2.19)

Secondary amines due to their role in physiological activities become core pharmacophores in many bioactive compounds and thus play exemplary role in drug discovery.[180] Designing of expedient synthetic routes for these compounds has been the focus of many research groups over the years. A brief survey of the major traditional methods of secondary amine syntheses[180] is shown in **Fig 2.6**.

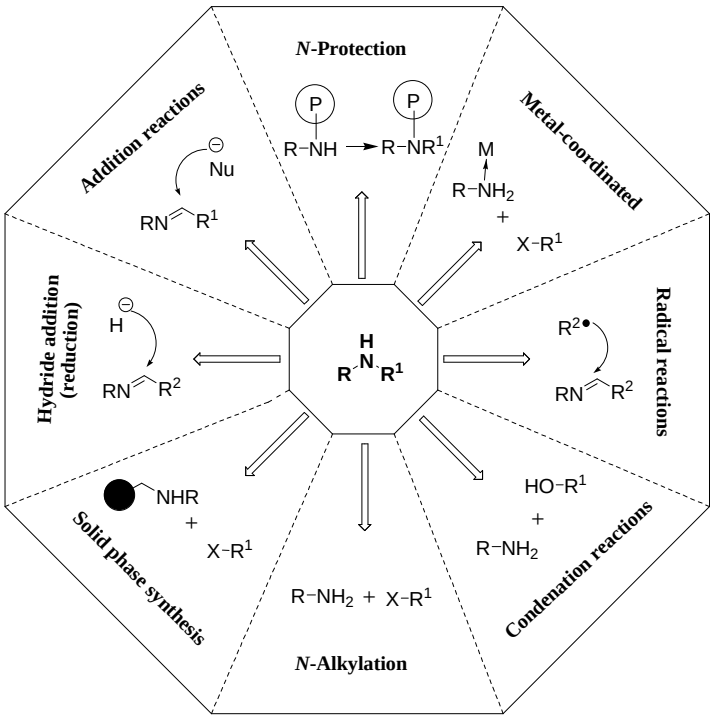
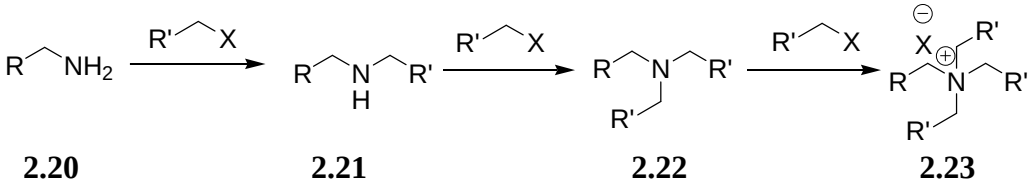


Fig 2.6 Methods for the synthesis of secondary amines (Where, R, R^1, R^2, R^3 = Alkyl or aromatic)

From a methodological standpoint, alkylation of primary amines with alcohols using metal catalyst thorium oxide (ThO_2) and transition metal catalysts such as nickel (Ni), rhodium (Rh) or ruthenium (Ru) are although selective for mono-*N*-alkylation, have main drawback that they prefer homogeneous phase reaction conditions where separation and recycling of catalyst is rather a difficult task. However, cesium hydroxide promoted *N*-alkylation of primary amines has been found to give predominant or exclusive secondary amines.[181] Direct alkylation of primary amine **2.20** with alkyl halides have been somewhat limited due to the concomitant further alkylation of the desired secondary amine **2.21** to tertiary amines **2.22** and the quaternary ammonium salt (**2.23**) as shown in **Scheme 2**.

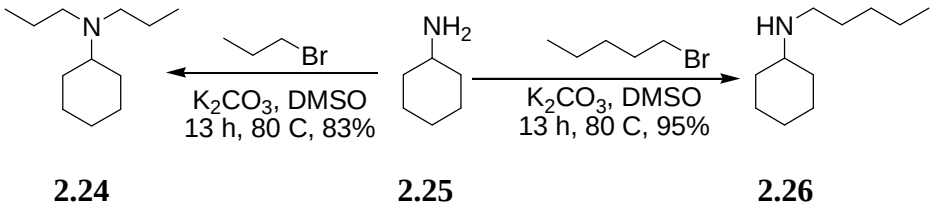


2.20 **2.21** **2.22** **2.23**

Scheme 2 Overalkylation of amine

However, secondary amine **2.21** can be obtained predominantly using excess of **2.20** in the reaction. Therefore, by controlling reaction conditions such as appropriate

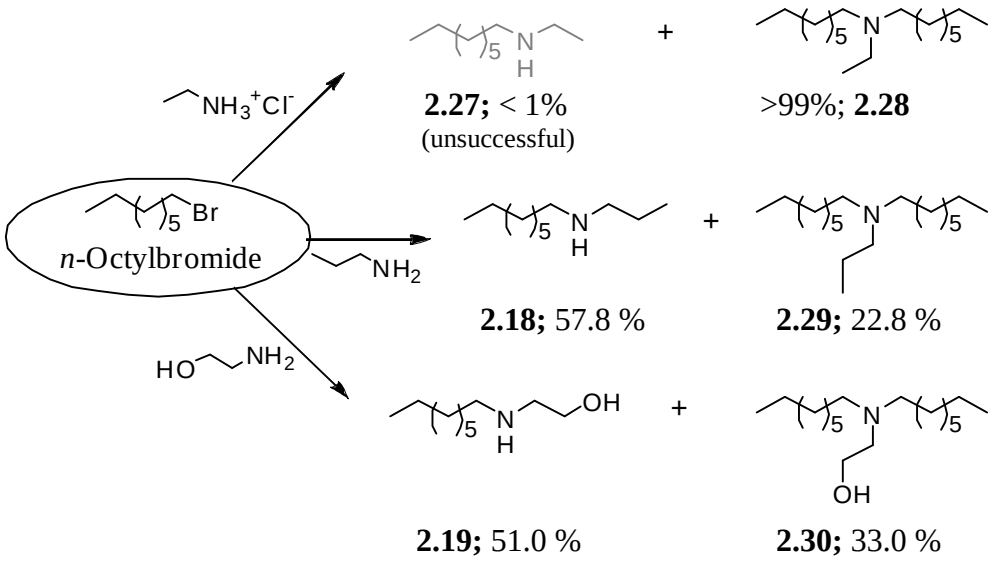
proportion in reactants, temperature, time and other reagents, it is possible to obtain desired secondary amine. Shrivastava et al., (**Scheme 3**) have observed that selective amine can be obtained by using inorganic base such as K_2CO_3 in a polar aprotic solvent (DMF, DMSO, or MeCN) at appropriate temperature (depending upon nature of starting material).[182]



2.24 **2.25** **2.26**

Scheme 3 Selective mono and di-*N*-alkylation of primary amine

As mentioned in **Scheme 3**, here alkylation was strongly dependant on a type of electrophile (alkyl bromide) used. Long chain alkyl bromide afforded exclusively mono-*N*-alkylation **2.26** where as short chain alkyl bromide gave rise exclusively to *N*, *N*-dialkyl product **2.24**. Similarly, Maeda et al., obtained selectively a range of secondary amines by mono alkylation of excess of appropriate primary amines with alkyl halides in the presence of Na_2CO_3.[183] The ready availability of *n*-octylbromide prompted us to apply the aforementioned methods for the synthesis of *N*-propyloctylamine **2.18** and *N*-(hydroxyethyl)octylamine **2.19** as shown in **Scheme 4**.

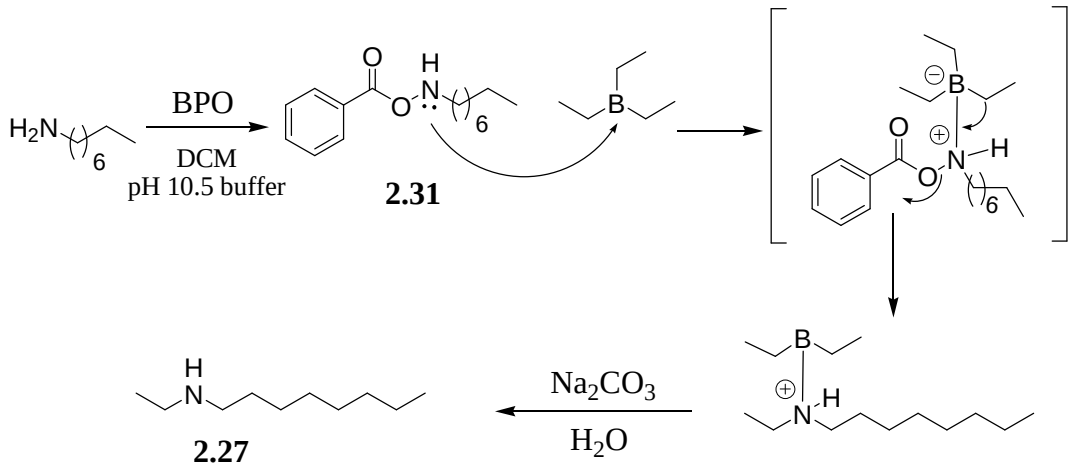


Reaction conditions: K_2CO_3, Acetonitrile (MeCN), rt., 18 h

Scheme 4 Synthesis of secondary amines

The *N*-alkylation of commercially available *n*-propylamine in excess amounts (2 mole equivalents) with a mole equivalent of *n*-octylbromide in a polar aprotic solvent, MeCN using K_2CO_3 as the base yielded a mixture of **2.18** and **2.29** in a proportion of 2.5:1. Similar alkylation of hydroxyethylamine gave a mixture of **2.19** and **2.30** in a proportion of 1.5:1. The required products *N*-Propyloctylamine **2.18** and *N*-(hydroxyethyl)octylamine **2.19** were separated from their respective mixtures by chromatography in methanol-ammonia (9.7:0.3).

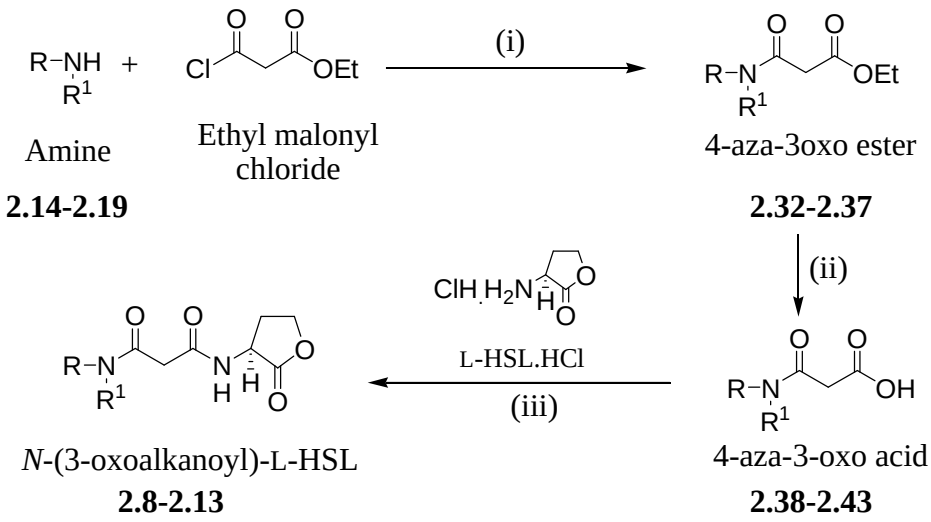
Unfortunately, in several attempts using 2.0, 2.5, 3.0 and 5.0 moles of ethylamine (used as HCl salt) failed to give the expected secondary amine **2.27**; instead the tertiary amine **2.28** was isolated in nearly a quantitative yield. However, the amine **2.27** can be selectively obtained by a route developed by Phanstiel et al., where they used benzoyl peroxide (BPO), amines and organoborane as reactants in dichloromethane (DCM)[181] (**Scheme 5)**.



Scheme 5 Synthesis of *N*-ethyloctylamine[179]

2.6.2 Synthesis of 3-oxo-4-azaalkanoyl-L-homoserine lactones (compounds 2.8-2.13)

Newly synthesized aforementioned amines **2.18** and **2.19** as well as commercially available amines **2.14-2.17** were utilized for the synthesis of 4-aza analogues of 3O, C_{12}-HSL **2.8-2.13** as shown in **Scheme 6**.

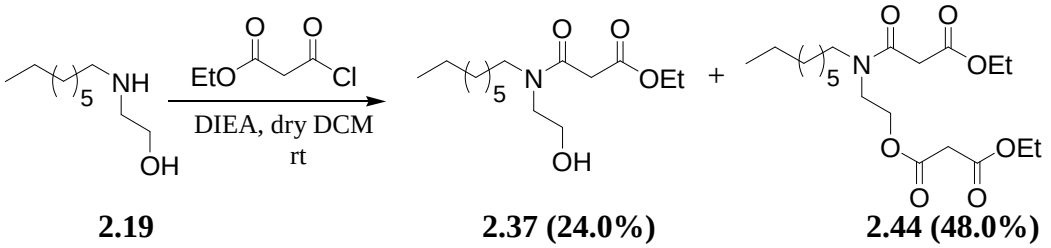


Reaction conditions: (i) dry DCM, 0 °C to rt., 2 h; (ii) $NaOH_{aq}$., EtOH, rt., 4 h; (iii) CDI, TEA, dry DCM, rt., 16 h (for comp **2.11**) and EDC, TEA, Water-Dioxane, rt., 16 h. (for comp. **2.8-2.10** and **2.12-2.13**)

Compounds	R	R^1	Chemical name
2.32	$-C_8H_{17}$	H	Ethyl 3-oxo-4-azadodecanoate
2.33	$-C_9H_{19}$	H	Ethyl 3-oxo-4-azatridecanoate
2.34	$-C_{11}H_{23}$	H	Ethyl 3-oxo-4-azapentadecanoate
2.35	$-C_8H_{17}$	$-CH_3$	Ethyl 3-oxo-4-methyl-4-azadodecanoate
2.36	$-C_8H_{17}$	$-n-C_3H_7$	Ethyl 3-oxo-4-propyl-4-azadodecanoate
2.37	$-C_8H_{17}$	$-C_2H_5$-OH	Ethyl 3-oxo-4(2-hydroxyethyl)-4-azadodecanoate
2.38	$-C_8H_{17}$	H	3-Oxo-4-azadodecanoic acid
2.39	$-C_9H_{19}$	H	3-Oxo-4-azatridecanoic acid
2.40	$-C_{11}H_{23}$	H	3-Oxo-4-azapentadecanoic acid
2.41	$-C_8H_{17}$	$-CH_3$	3-Oxo-4-methyl-4-azadodecanoic acid
2.42	$-C_8H_{17}$	$-n-C_3H_7$	3-Oxo-4-propyl-4-azadodecanoic acid
2.43	$-C_8H_{17}$	$-C_2H_5$-OH	3-Oxo-4(2-hydroxyethyl)-4-azadodecanoic acid

Scheme 6 General synthetic route of *N*-(3-oxo, 4-azaalkanoyl)-L-HSL

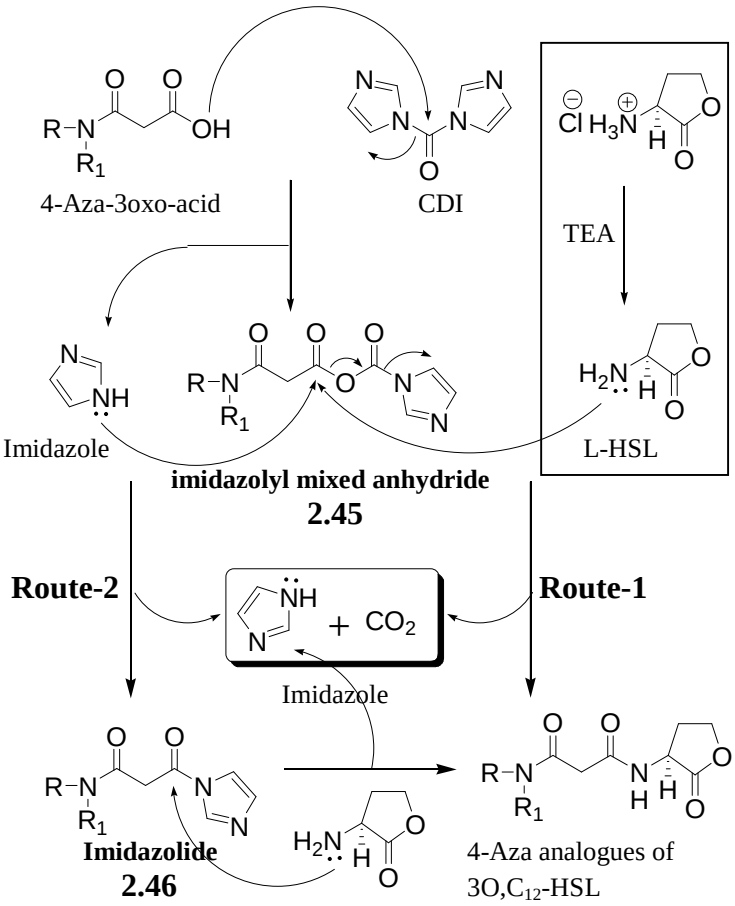
Amines **2.14-2.18** were readily acylated with ethyl malonyl chloride in the presence of diisopropylethylamine (DIEA) to yield 4-aza-3-oxo esters **2.32-2.36**. However, acylation of *N*-(hydroxyethyl)octylamine **2.19** with ethyl malonyl chloride under similar conditions gave a mixture of the desired *N*-acylated product **2.37** and *N*-, *O*-bis acylated derivative **2.44** in a proportion of 1:2 as shown in **scheme 7**.



Scheme 7 Formation of *N*-acylated and *N*-, *O*-bis acylated products

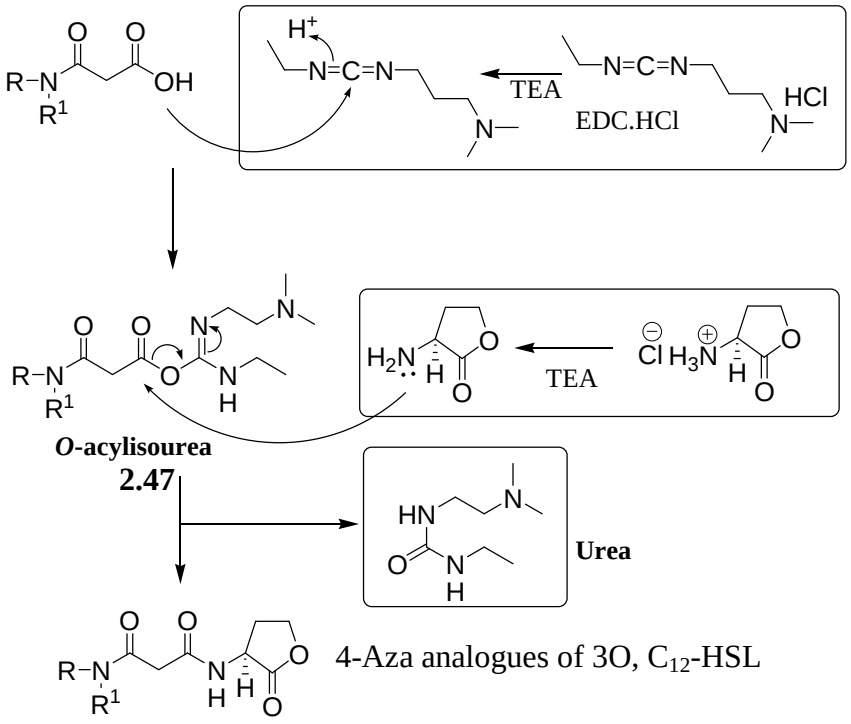
Saponification of esters **2.32-2.37** and **2.44** with NaOH gave the corresponding 4-aza acids **2.38-2.43**. Aza acid **2.41** was coupled to L-homoserine lactone using 1,1'carbonyldiimidazole (CDI) and triethylamine (TEA) in DCM and furnish the expected product **2.11** in somewhat lower yield. Low product yield was in part due to lower solubility of the resultant product in DCM. Therefore, other including higher chain length aza acids **2.38-2.40** and **2.42-2.43** were coupled to L-HSL in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in dioxane-water and obtained the desired products **2.8-2.10** and **2.12-2.13** respectively in better yields.

Mechanistically, during CDI coupling, the critical formation of imidazolyl mixed anhydride **2.45** can proceed via two routes to yield the desired amide products. In one route direct reaction between **2.45** and the free amine takes place while in other route, **2.45** is converted to imidazolide **2.46** before it reacts with the free amine (**Scheme 8**).



Scheme 8 Reaction mechanism for the coupling of 4-aza acid and L-HSLin the presence of CDI

The formation of an amide using a water soluble carbodiimide (EDC) is straightforward, as shown in **scheme 9**. The acid first reacts with the carbodiimide to produce the activated intermediate the *O*-acylisourea **2.47**. The latter then acylates the L-HSL to deliver the desired 4-aza analogues.



Scheme 9 EDC catalysed coupling of acid and L-HSL

Final products **2.14-2.19** were easily purified by preparative thin layer chromatography using an appropriate hexane - ethyl acetate solvent system. ^{1}HNMR spectrum of **2.11** as shown in **Fig 2.5** suggested that due to restricted rotation between C^3-N^4 bond the product exists as a mixture of *E* and *Z* isomers and consequently *N*-Me appears as two singlets at δ 2.96 and 3.03. Other analogues were also duly characterised by their ^{1}HNMR spectra, which displayed N^4-*H* and N^4-C*H*$_2$- signals at δ values as shown in **Table 2.3**.

Table 2.3 Analytical characteristics of analogues

Compound	Chromatography solvent system	Proton(s)	δ
2.8	Hexane-Ethyl acetate (2:8)	N^4-H	s, 7.14
2.9	Hexane-Ethyl acetate (1:4)	N^4-H	s, 6.49
2.10	Hexane-Ethyl acetate (1:4)	N^4-H	s, 6.48
2.12	Ethyl acetate	N^4-CH_2-	m, 3.31
2.13	Ethyl acetate	N^4-CH_2-	m, 3.50

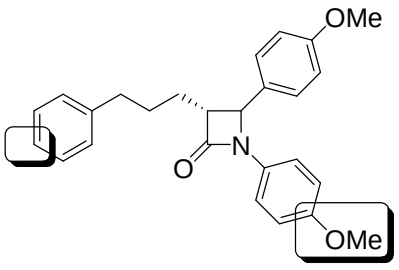
Where: s – singlet, m – multiplet

Biologically all 4-aza analogues **2.8-2.13** showed immune suppressive activity in murine monocyte prolifereation assays *in vitro*. They were also found to be non-inducers of quorum sensing with no or partial agonist activities (Chapter 3).

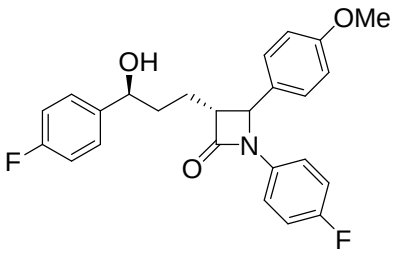
2.7 Fluorinated 4-aza-3O, C_{12}-HSL analogues

Over the last 30 years, introduction of fluorine atoms in established pharmaceutical drug candidate has become routine and occasionally improved their therapeutic activity. Being comparatively smaller atom (Van der waals radius 1.47A°), its covalent bonded form occupies smaller volume than methyl, amino or hydroxyl groups while slightly larger volume than hydrogen.[184,185] Therefore, it was of interest to replace these groups with fluorine atom. This will introduce changes in molecular properties and consequently will modulate biological behaviour.

Presence of fluorine within the structure modulates its metabolic stability and thus bioavailability as well. Lipophilic molecules are usually labile to oxidative liver enzymatic (Cytochrome P_{450}) degradation. Replacing oxidative C-H group of compound by C-F group can block this degradative site without affecting its target protein binding. Well known example of this is the discovery of Ezetimibe **2.49**, the potent cholesterol-absorption inhibitor. In this molecule introduction of fluorine blocks two metabolically labile sites preventing oxidation of phenyl ring to phenol and dealkylation of the methoxy group of the lead molecule SCH 48461 **2.48** (**Fig 2.7**).[186,187]



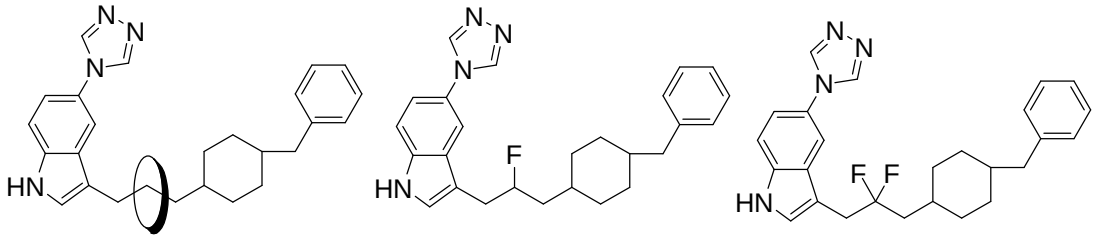
SCH 48461 **2.48**



Ezetimib **2.49**

Fig 2.7 Development of Ezetimib by optimization of SCH 48461

Being most electronegative atom, fluorine influences acidity and basicity strongly of the functional group present in its vicinity in a molecule. The pKa profoundly affects the pharmacokinetic properties and binding affinity of the molecules. Successive fluorine incorporation in the earlier 5-hydroxytryptophan (5HT) receptor agonist **2.50** was found to reduce its basicity (**Fig 2.8**) and thus reduced pKa of the molecules **2.51** and **2.52** that in turn increased their membrane permeability and substantial bioavailability.[188]



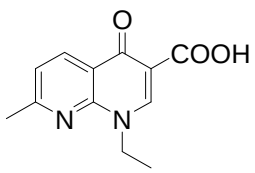
2.50
pKa 9.7
(very low bioavailability)

2.51
pKa 8.7
(medium bioavailability)

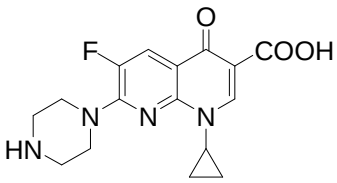
2.52
pKa 6.7
(substantial bioavailability)

Fig 2.8 Effect of fluorine on the bioavailability of 5HT agonists

Also fluorination of the weak and narrow spectrum first generation antibacterials **2.53** (e.g. Nalidixic acid) resulted in the discovery of novel broad-spectrum fluoroquinolone antibacterials **2.54** (e.g. Ciprofluoxacin).[184,188]

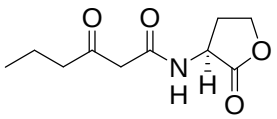


Nalidixic acid **2.53**

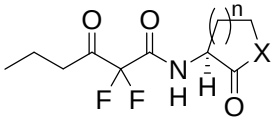


Ciprofluoxacin **2.54**

Overall, the fluorine substituents have been found to modulate the molecular properties. Very recently Welch et al. had synthesized difluoro derivatives **2.55-4.58** of 3-oxo-hexanoyl-L-homoserine lactone (OHHL), one of the quorum sensing signalling molecules of *Erwinia carotovora* and tested them to see how they affected protease and carbapenem production in the Ecc ATTn10 *CarI* mutant.[189]



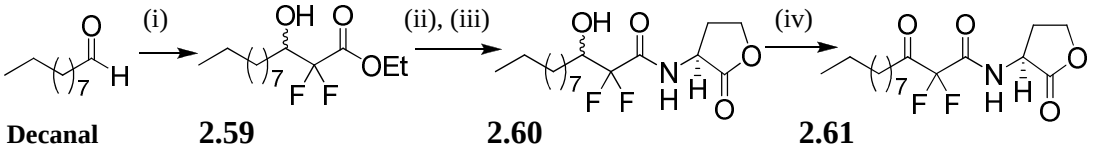
OHHL



2.55 n = 1; X = CH_2
2.56 n = 2; X = CH_2
2.57 n = 3; X = CH_2
2.58 n = 1; X = O

Biological results showed that though the parent compound OHHL substantially induced carbapenem biosynthesis and exoprotease activity, among fluorinated analogues, only **2.58** elicited some carbapenem production at high concentration, displayed tighter binding to EccR, a LuxR homologue and induced 100 fold less protease activity than OHHL.[189]

Kline et al., have synthsized geminal (*gem*) difluoro analogues of 3O, C_{12}-HSL as shown in **scheme 10** to determine their agonist or antagonist autoinducing activity in both PAI-1-LasR and PAI-2-RhlR systems of *Pseudomonas aeruginosa*.



Decanal **2.59** **2.60** **2.61**

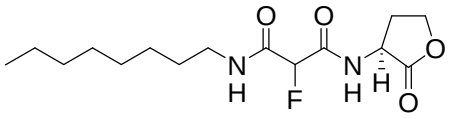
Reaction conditions: (i) ethyl bromodifluoroacetate, Zn dust, THF, reflux (ii) KOH, THF-EtOH-H_2O (iii) L-HSL, EDC. HOAc, DIEA, DMF (iv) Dess-Martin periodinane, DCM.

Scheme 10 Synthesis of difluoro analogues of 3O, C_{12}-HSL

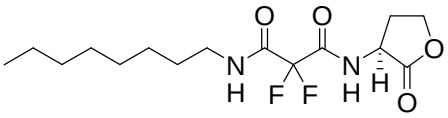
A Reformatsky reaction between decanal and ethyl bromodifluoroacetate gave hydroxy ester **2.59**. Saponification of **2.59** followed by amidation with L-HSL using EDC, acetic acid (HOAc) and DIEA yielded **2.60** as a mixture of diastereomers. Oxidation of **2.60** with Dess-Martin periodinane readily produced difluoro analogue **2.61**. In autoinducing assays both analogues **2.60** and **2.61** were agonists at LasR with **2.60** being less potent.[190]

As mentioned in previous section, 4-aza analogues showed immune modulatory behaviour, We intended to modulate 4-aza-3O, C_{12}-HSL at C^2 position by replacing one and both hydrogens with fluorine isostere(s) to generate respectively 4-aza-2-fluoro-3-oxododecanoyl-L-homoserine lactone (4-aza-2-F-3O, C_{12}-HSL) **2.62** and 4-

aza-2, 2-difluoro-3-oxododecanoyl-L-homoserine lactone (4-aza-2-F_2-3O, C_{12}-HSL) **2.63**. These new molecules were expected to have better lipid solubility and possibly with preferred molecular conformations for appropriate protein binding interactions and also with better pharmacokinetic profile. Moreover, fluorine substitution at C^2 position would eventually shed light on the importance of C^2 hydrogens in immune modulation.

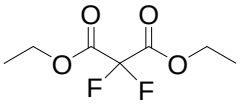


4-Aza-2-F-3O, C_{12}-HSL **2.62**

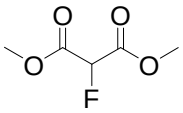


4-Aza-2-F_2-3O, C_{12}-HSL **2.63**

Commercially available diethyl difluoromalonate **2.64** and dimethyl fluoromalonate **2.65** were used as starting materials for the synthesis of **2.62** and **2.63** respectively.

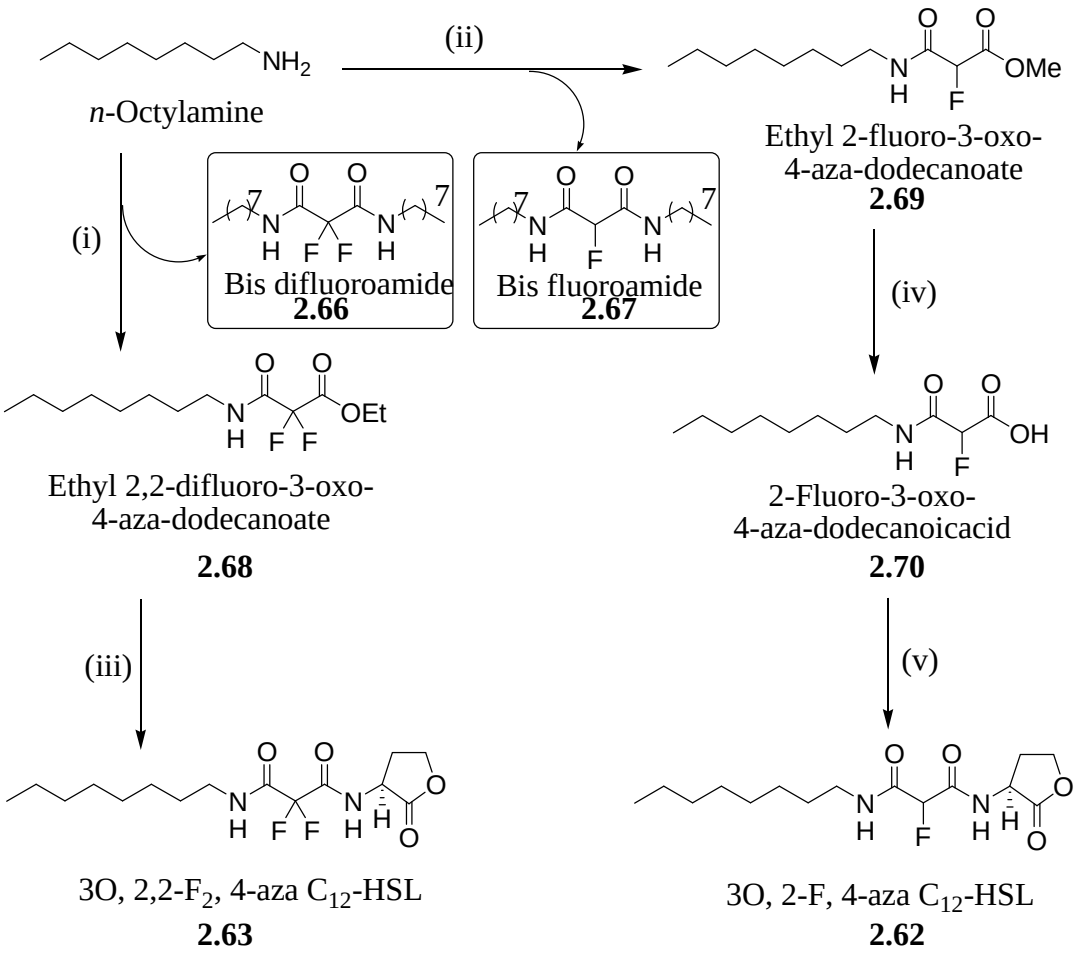


Diethyl difluoromalonate **2.64**



Dimethyl fluoromalonate **2.65**

Difluoromalonate **2.64** and monofluoromalonate **2.65** esters were each reacted with *n*-octylamine to generate their respective amides, which were then further processed as shown in **Scheme 11** to obtain the desired products 4-aza-2-F-3O, C_{12}-HSL and 4-aza-2-F_2-3O, C_{12}-HSL. Here the formation of the undesired difluorobisamide **2.66** and monofluorobisamide **2.67** could not be avoided but these could be easily removed via chromatography.



Reaction conditions: (i) Diethyl difluoromalonate, ethanol, rt., 2 h; (ii) dimethyl fluoromalonate, methanol, rt., 2 h; (iii) L-HSL.HCl, TEA, acetonitrile, reflux, 16 h; (iv) $NaOH_{aq.}$, methanol, rt., 3 h; (v) L-HSL.HCl, DCCI, 1-hydroxy benzotriazole (HOBt), dry DCM, 16 h.

Scheme 11 Synthesis of fluorinated 4-aza analogues of 3O, C_{12}-HSL.

2.7.1 Synthesis of 4-aza-2-F_2-3O, C_{12}-HSL

In the first attempt, direct reaction between diethyl difluoromalonate **2.64** and *n*-octylamine in anhydrous ethanol gave mainly undesired bisamide **2.66,** the desired product **2.68** was only isolated in 23 % yield after thin layer chromatography. Therefore, in the next attempt a *n*-octylamine was added dropwise to the solution of diethyl difluoromalonate in dry ethanol over a period of 2 h. With this procedure the yield of the desired product **2.68** was increased to 50%. However, bisamide was still produced as a major side product.

Finally, the direct coupling of the ethyl 2, 2-difluoro-3-oxo-4-azadodecanoate **2.68** with L-homoserine lactone in acetonitrile gave 4-aza-2-F_2-3O, C_{12}-HSL **2.63**. The product was purified by preparative layer chromatography (PLC) in ethyl acetate-

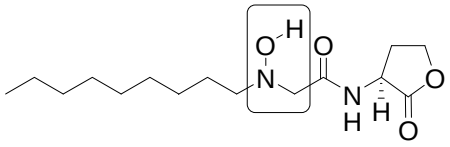
hexane (1:1) in a low yield of 15%. The ^{1}H NMR spectrum of the product did not display C^2 protons signal present at δ 3.28 in 4-aza-3O, C_{12}-HSL thus indicating that replacement of C^2 hydrogens by fluorines has taken place. The molecular ion peak at *m/z* 335 due to MH^+ in the mass spectrum (MS) confirmed the structure.

2.7.2 Synthesis of 4-aza-2-F-3O, C_{12}-HSL

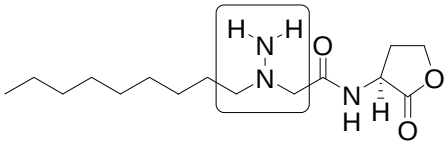
As above the dropwise addition of *n*-octylamine to the dimethyl fluoromalonate **2.65** solution in anhydrous methanol resulted in the formation of methyl 2-fluoro-3-oxo-4-azadodecanoate **2.69** in a yield of 47% and monofluorobisamide **2.67** as a side product. Both **2.69** and **2.67** were separated by PLC using diethyl ether - petroleum ether (3:2). Direct coupling of **2.69** with L-homoserine lactone did not give any product. Therefore, **2.69** was subjected to base saponification to yield 2-fluoro-3-oxo-4-azadodecanoic acid **2.70** in a quantitative yield. The free amine was generated from L-homoserine lactone HCl using HOBt and this was coupled to the acid **2.70** using DCCI (1, 3-dicyclohexylcarbodiimide) as a coupling agent to yield the desired product **2.62**. Purification by PLC in ethyl acetate afforded **2.62** in a highly pure form in 11 % yield. ^{1}H NMR of 4-aza-2-F-3O, C_{12}-HSL showed C^2 proton signal at δ 5.3 as dd with typical coupling constants $J_{H, NH}$ 10.2 Hz and $J_{H, F}$ 38.0 Hz.

2.8 3-Aza analogues of 3O, C_{12}-HSL

As already seen in 4-aza analogues, how modulation of the 3-*enolic* content of 3O, C_{12}-HSL through the N^4 insertion had influenced eukaryotic immune modulation (chapter 3). Therefore, it was of interest to further modulate 3-*enolic* content of the lead molecule 3O, C_{12}-HSL by replacing C^3=O functional group by N^3-OH (hydroxamic acid) and N^3-NH_2 (hydrazine) isosteres. Thus *N*-(3-hydroxy-3-azadodecanoyl)-L-HSL (3-OH, 3-aza-C_{12}-HSL) **2.71** and *N*-(3-amino-3-azadodecanoyl)-L-HSL (3-amino, 3-aza-C_{12}-HSL) **2.72** structures were elaborated.

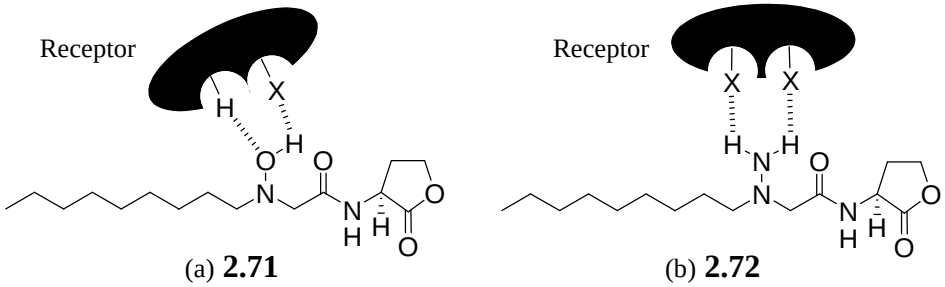


3-OH, 3-aza-C_{12}-HSL **2.71**



3-NH_2, 3-aza-C_{12}-HSL **2.72**

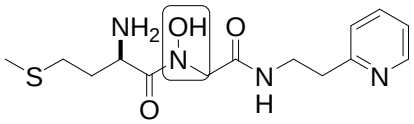
In one study, Li et al. demonstrated that hydrogen bonding between an inhibitor and S_1' subsite of calpain is important for potent inhibition of the enzyme.[191] Calpain is calcium-dependent proteolytic enzyme involved in cell motility and have been implicated in apoptotic cell death.[192,193] With this report in mind, we envisioned that N^3-OH insertion would lower p*Ka* of **2.71** relative to that of 3O, C_{12}-HSL and may improve the binding affinity through H-bonding to the active site of enzyme (**Fig 2.9-a**) whereas, N^3-NH_2 substitution **2.72** may explore the receptor and identify new binding interactions (**Fig 2.9-b**). The whole process may render the molecule metabolically more stable to enzymatic degradation.[194-196] Overall, an increased strength of hydrogen bonding of **2.71** and new binding interactions of **2.72** with the enzymes may potentiate selective immune modulation.



(a) **2.71** (b) **2.72**

Fig 2.9 Proposed hydrogen bonding interactions between the compounds (a & b) and the active sites of enzyme

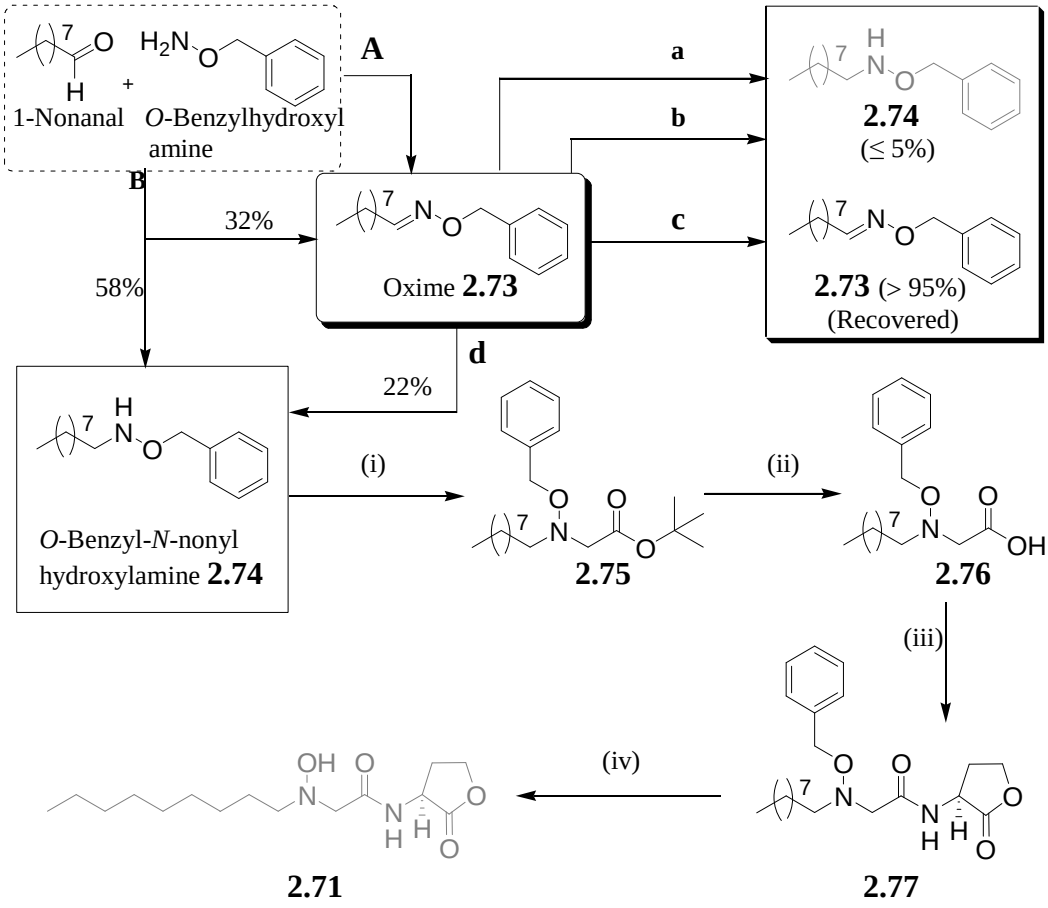
Hydroxamic acid motif containing molecules have been investigated as inhibitors of amino peptidases whereas aza-peptide substrates are reported to function as inhibitors of serine proteases.[194,195] Compounds with hydroxamic acid motif possess a potential to target tumors and bacteria. To this end, compound **MetAP-I** has been observed as the most potent inhibitor of methionine aminopeptidase (MetAP). MetAP is an essential enzyme of both bacteria (e.g. *Escherichia coli*) and human. Eukaryotes contain two different enzymes, MetAP-1 and MetAP-2 the latter stimulates angiogenesis.[194,197-201]



MetAP-I

2.8.1 Synthesis of 3-OH, 3-aza-C_{12}-HSL

Synthesis of 3-OH, 3-aza-C_{12}-HSL **2.71** is shown in **Scheme 12.** Condensation of 1-nonanal and *O*-benzylhydroxylamine in methanol at pH ≅ 4.7 gave oxime **2.73** which was a mixture of *E* and *Z* isomers in a proportion of 4:1 as estimated from its proton nuclear magnetic reaonance (NMR).[202]



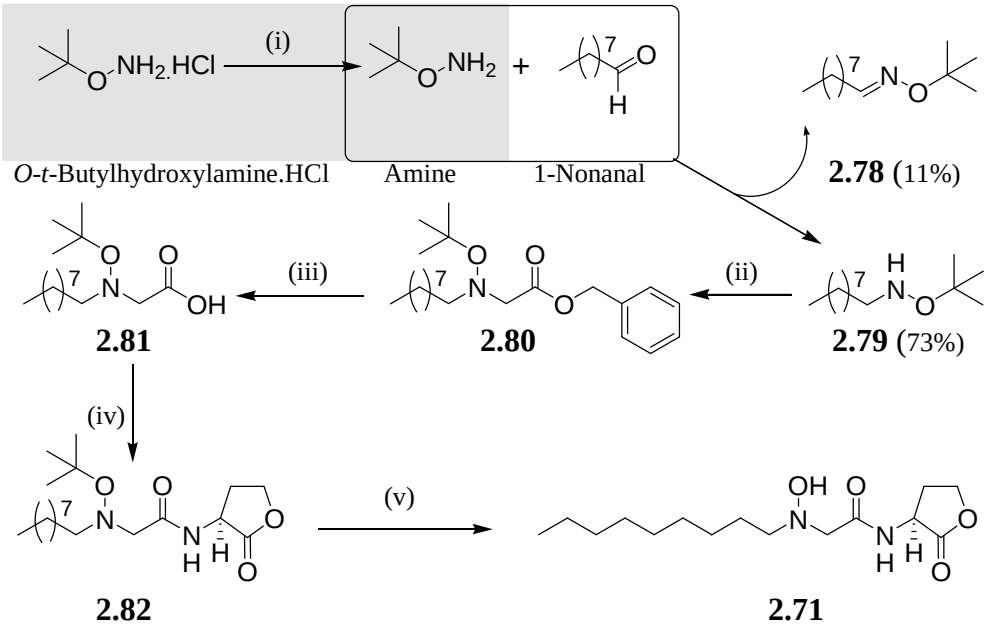
Reaction conditions: (A) Methanol 0 °C to rt, p*H*≅4.7, 4 h; (a) sodium borohydride, methanol, rt, 2 h; (b) triacetoxyborohydride-acetic acid, dichloroethane (DCE), rt, 6 h; (c) sodium cyanoborohydride (1M solution in THF)-methanolic HCl, methanol, p*H*≅3.4, rt, 6 h; (d) sodium cyanoborohydride-acetic acid, DCE, rt, p*H*≅4.2, 4 h; (B) sodium cyanoborohydride-acetic acid, methanol, 0 °C to rt, p*H*≅4.2, 16 h (i) *t*-butyl bromoacetate, DIEA, THF, reflux, 18 h; (ii) TFA, ethyl acetate, rt, 3 h; (iii) L-HSL.HCl, EDC.HCl, TEA, dioxane-water, rt, 6 h (iv) H_2, Pd/C, ethanol or ethyl acetate

Scheme 12 Attempted synthesis of 3-OH, 3-aza-C_{12}-HSL

Attempts to reduce **2.73** by sodium borohydride (a), triacetoxyborohydride-acetic acid (b) and 1M sodium cyanoborohydride in THF (c) were unsuccessful.[202-204] As shown in **Scheme 12**, the reagent (a) did not effect any reduction, while reagents (b) and (c)

delivered the reduced product, *N*-nonyl derivative of *O*-benzylhydroxyamine **2.74** only in poor yield.[205] Gratifyingly, the reductive amination of 1-nonanal with *O*-*t*-butyl-hydroxylamine in the presence of sodium cyanoborohydride at acidic pH afforded the desired product **2.74** though substantial amount of the oxime **2.73** was also isolated as a side product.[202,205,206] The reduced product **2.74** was purified by chromatography in ethyl acetate-hexane (1:9).

Alkylation of the key interemediate **2.74** with *t*-butyl bromoacetate gave *N, N*-dialkylated *O*-benzylhyxroxylamine derivative **2.75**.[194,207] Deprotection of *t*-butyl ester with TFA afforded free acid **2.76**, which was then coupled with L-HSL and obtained 3-3-benzyloxy, 3-aza-C_{12}HSL **2.77**. Although Dupont et al.[208] have achieved it successfully, even after several trials in different reaction conditions, we were not able to remove benzyl group of **2.77** by catalytic hydrogenation. Thus, an alternative protecting group strategy as shown in **scheme 13** was employed for the synthesis of **2.71**. However, it is noteworthy to mention that compound **2.77** was found as non-QS immunosuppressive agent.



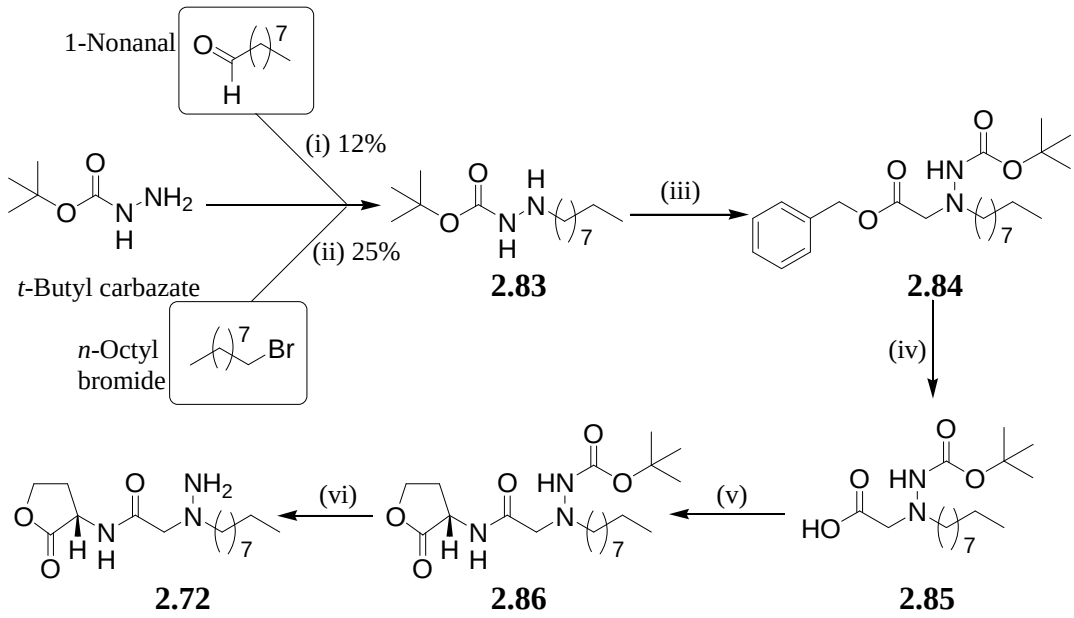
Reaction conditions: (i) *O*-*t*-butylhydroxylamine.HCl, NaOMe, Sodium cyanoborohydride-acetic acid, methanol, 0 °C to rt., 16 h; (ii)benzyl 2-bromoacetate, DIEA, THF, reflux, 16 h; (iii) H_2, Pd/C, EtOH, rt., 8 h; (v) L-HSL.HCl , EDC.HCl, TEA, water-dioxane, rt., 16 h; (vi) 50% v/v TFA/DCM, rt., 6 h.

Scheme 13 Synthesis of 3-OH, 3-aza C_{12}-HSL

Here free amine obtained by the neutralisation of *O*-*t*-butylhydroxlamine.HCl with sodium methoxide, was used for the reductive amination of 1-nonanal to obtain a mixture of **2.78** and **2.79** in a proportion of 1:7. The alkylation of purified **2.79** with benzyl 2-bromoacetate followed by deprotection of benzyl ester **2.80** under catalytic conditions afforded the free acid **2.81**. Water soluble carbodiimide (EDC) mediated coupling of this acid with L-HSL yielded **2.82**. Finally the deprotection of *O*-*t*-butyl group with TFA gave the desired product **2.71**. The ^{1}H NMR spectrum of the product displayed N^3-OH proton signal at δ 10.38 and *m/z* 301 of MH^+ by MS confirmed final structure.

2.8.2 Synthesis of 3-amino, 3-aza C_{12}-HSL

The synthesis of 3-amino, 3-aza-C_{12}-HSL **2.72** required the production of a key compound *N*-octylcarbazate **2.83**. The reductive amination of 1-nonanal with *t*-butyl carbazate gave **2.83** in poor yield whereas, direct alkylation of *t*-butyl carbazate with *n*-nonyl bromide gave **2.83** in an increased yield of 25%. The overall synthesis of **2.72** is shown in **scheme 14**.



Reaction conditions: (i) Sodium cyanoborohydride-acetic acid, methanol, 0 °C to rt, p*H*≅4.2, 16 h; (ii) THF, reflux, 18 h; (iii) benzyl-2-bromoacetate, DIEA, THF, reflux, 16 h; (iv) H_2, Pd/C, EtOH, rt, 16 h; (v) L-HSL.HCl, EDC.HCl, TEA, water-dioxane, rt., 16 h; (vi) 50% v/v TFA/DCM, rt., 6 h

Scheme 14 Synthesis of 3-amino, 3-aza C_{12}-HSL

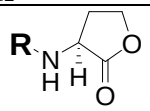
Compound **2.83** was further alkylated with benzyl *2*-bromoacetate to obtain the corresponding *N*, *N*-dialkylated carbazate derivative **2.84** which upon catalytic hydrogenation gave deprotected acid **2.85**. Product **2.85** was coupled with L-HSL using EDC and obtained **2.86**. Lastly *Boc* group deprotection by TFA yielded the desired compound 3-amino, 3-aza C_{12}-HSL **2.72** in a quantitative yield. In the ^1H NMR spectrum N^3-NH_2 protons appeared as broad singlet at δ 8.66 whereas, in MS MH^+ appeared at *m/z* 300.

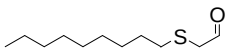
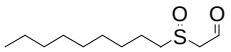
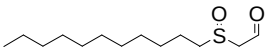
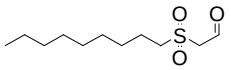
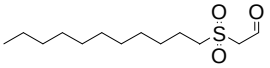
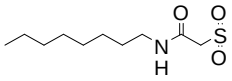
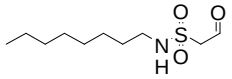
2.9 Thia analogue of 3O, C_{12}-HSL

Although native sulphur has limited success as a medicinal agent, there is hardly a class of drugs which does not contain compounds having sulphur in their structure. Sulphur occurs in drug molecules in all its oxidative states and in all its forms of organic combination such as sulfone, sulfoxide and sulfonamide. Moreover, introduction of sulfur in the form of sulfone or sulfoxide imparts antibiotic properties to the otherwise inactive organic compounds. Sulphur-containing dyes have produced many clinically useful substances (sulfonamides as antibacterial), others, such as the lactam antibiotics, have been developed from naturally occurring molecules. Thus, organo-sulphur compounds unquestionably form a major therapeutic resource however, their potential remains to be further exploited.

Incorporating several defined bioactive structural units to form a more potent pharmacophore is well known approach in medicinal chemistry. We surmised that the introduction of sulfur at the C^1 and C^3 position in 3O, C_{12}-HSL structure would alter the *3-enolic* content due to its stereoelectronic properties. Thus, a range of 1-thia and 3-thia analogues as shown in **Table 2.4** were elaborated and screened for their immunological and auto-induction capabilities.

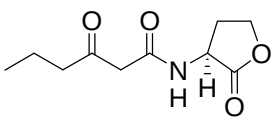
Table 2.4 Thia analogues of 3O, C_{12}-HSL



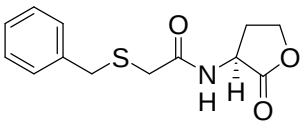
Comp.	R	Chemical name	Abbreviation
A. 3-Thia analogues of 3O, C_{12}-HSL			
2.87		*N*-(3-Thiadodecanoyl)-L-HSL	3-Thia C_{12}-HSL
2.88		*N*-(3-Oxo, 3-thia dodecanoyl)-L-HSL	3O, 3-Thia C_{12}-HSL
2.89		*N*-(3-Oxo, 3-thia tetradecanoyl)- L-HSL	3O, 3-Thia C_{14}-HSL
2.90		*N*-(3, 3-Dioxo, 3-thia dodecanoyl)-L-HSL	3, 3-Dioxo, 3-thia C_{12}-HSL
2.91		*N*-(3, 3 Dioxo, 3-thia tetradecanoyl)-L-HSL	3, 3-Dioxo, 3-thia C_{14}-HSL
B. 1-or 3-Sulfonamide motif containing analogues of 3O, C_{12}-HSL			
2.92		*N*-(1, 3-Dioxo, 4-aza, 1-thia dodecanoyl)-L-HSL	1, 3-Dioxo, 4-aza, 3-thia C_{12}-HSL
2.93		*N*-(3, 3-Dioxo, 4-aza, 3-thia dodecanoyl)-L-HSL	3, 3-Dioxo, 4-aza, 3-thia C_{12}-HSL

2.9.1 3-Thia analogues of 3O, C_{12}-HSL

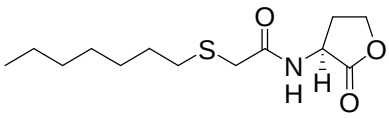
Reverchon et al., although not in context with the long chain 3O, C_{12}-HSL, were the first to synthesize 3-thia derivative **2.94** of the shorter chain 3O, C_6-HSL **1.51**. Among a range of analogues bearing ramified cycloalkyl or aryl substituent at C^4, **2.94** was found as a quorum sensing inhibitor.[209]



1.51

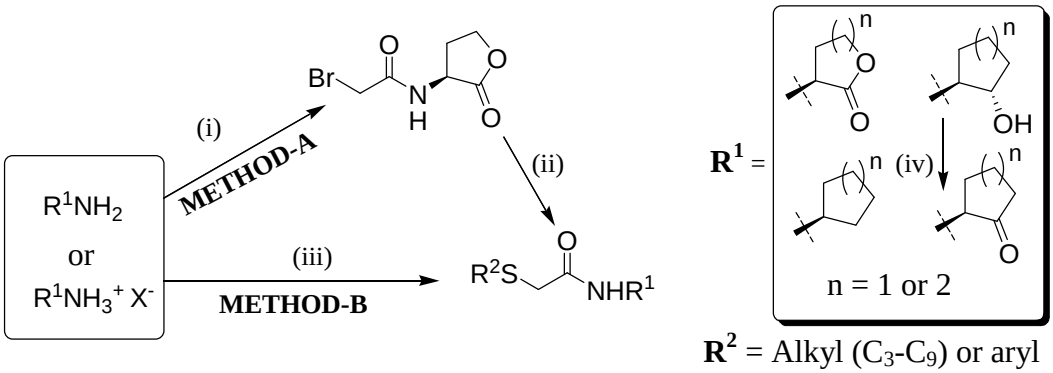


2.94



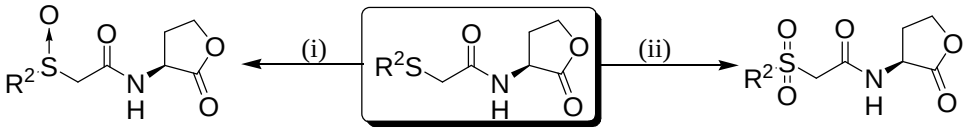
2.95

This exciting bioactivity of **2.94** prompted Persson et al. to synthesize few more 3-thia analogues of **1.51** with an extended or aryl substituted acyl chains (**Scheme 15a)** as well as their corresponding sulfoxide and sulfone derivatives (**Scheme 15b**). [207] In this study *N*-(heptylsulfanylacetyl)-L-HSL **2.95** was found to be most potent quorum sensing inhibitor.



Reaction conditions: (i) bromoacetyl brmide, TEA, DCM, -78 °C to rt, 70%; (ii) R^2SH, TEA, ethanol, rt; (iii)) bromoacetyl brmide, TEA, DCM, -20 °C to rt, then R^2SH, TEA; (iv) $(COCl)_2$, dimethyl sulfoxide (DMSO), DCM, -78 °C.

Scheme 15a Synthetic scheme of 3-thia analogues as quorum sensing inhibitors

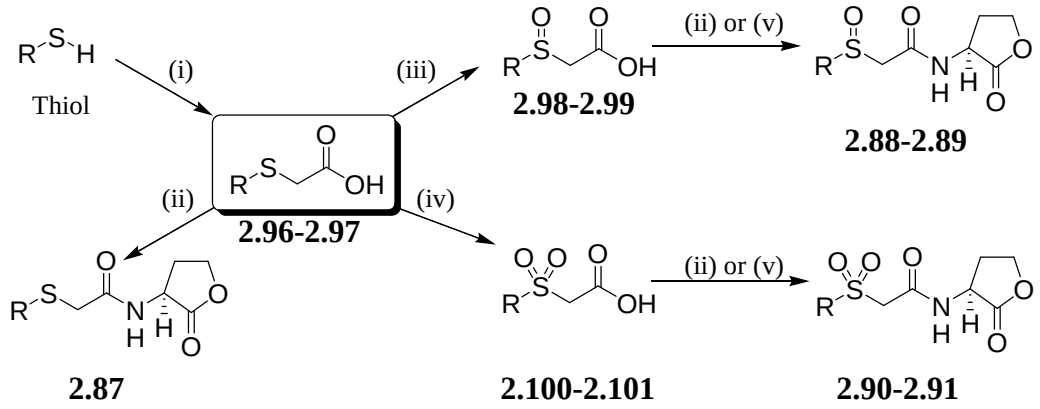


R² = Alkyl or aryl

Reaction conditions: (i) 3-chloroperbenzoic acid (*m*-CPBA), DCM, -10 °C; (ii) *m*-CPBA, DCM, rt.

Scheme 15b Synthetic scheme of 3-sulfoxide and 3-sulfone analogues

The synthetic sequences utilized for the preparation of derivatives, **2.87-2.91** are outlined in **Scheme 16**. The key thioether intermediates, **2.96-2.97** were obtained by alkylating appropriate thiols with *t*-butyl bromoactetate.[210] Extreme basic conditions and elevated reaction temperature afforded *in situ* deprotection of *t*-butyl group.[211] Alternatively, these thioethers can also be prepared by Mitsunobu reaction of the appropriate alcohols with thiolacetic acid or by reaction between alkyl bromides and methyl thioglycolate to give the corresponding thioacetates which can then be easily hydrolyzed.[212]



Reaction conditions: (i) *t*-butyl bromoacetate, 18% w/v NaOH, 90 ^{0}C, 14 h; (ii) L-HSL.HCl , CDI, TEA, dry DCM, rt., 16 h (for compounds **2.87**, **2.88** and **2.90**); (iii) 0.5 *N* bromate-bromide, HCl, acetic acid, 0 ^{0}C, 45 min.; (iv) 4% w/v $KMnO_4$, acetic acid, 50 ^{0}C to rt., 40 min.; (v) L-HSL.HCl, EDC.HCl, TEA, water-dioxane, rt., 16 h (for compounds **2.89** and **2.91**).

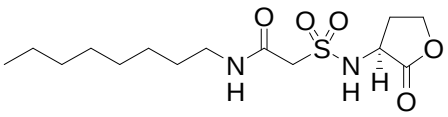
3-thia acids		R	Chemical name
Sulphide	**2.96**	$-C_9H_{19}$	3-Thiadodecanoic acid
	2.97	$-C_{11}H_{23}$	3-Thiatetradecanoic acid
Sulfoxide	**2.98**	$-C_9H_{19}$	3-Oxo-3-thiadodecanoic acid
	2.99	$-C_{11}H_{23}$	3-Oxo-3-thiatetradecanoic acid
Sulfone	**2.100**	$-C_9H_{19}$	3,3,-Dioxo-3-thiadodecanoic acid
	2.101	$-C_{11}H_{23}$	3, 3-Dioxo-3-thiatetradecanoic acid

Scheme 16 Synthesis of 3-thia analogues of 3O, C_{12}-HSL

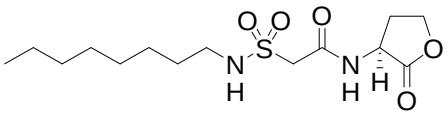
Oxidation of **2.96-2.97** to obtain sulfoxides, **2.98-2.99** and sulfones, **2.100-2.101** was carried out using bromate-bromide/acid[211] and potassium permanganate/acid[213-215] solutions respectively. Oxidation using cerric (III) ammonium nitrate and sodium bromate was not effective.[216] No attempts were made at this stage to resolve the enantiomeric mixtures of **2.98** and **2.99**. The enantiomeric ratio in these mixtures, as estimated from their ^{1}H NMR spectra, was approximately 1:1. Compounds **2.96**, **2.98** and **2.100** were coupled to L-HSL using CDI to furnish expected derivatives **2.87**, **2.88** and **2.90**. Adducts **2.99** and **2.101** were successfully coupled with L-HCL using EDC to produce desired analogues **2.89** and **2.91**. Analogues **2.88** and **2.89** were obtained as a mixture of their diastereomers. The absolute stereochemical identies of these analogues **2.88** and **2.89** were not established because their separation by PLC proved unsuccessful. The diastereomeric ratio in their mixtures, as estimated from ^{1}H NMR spectra, were approximately 3:1 and 1:1 respectively.

2.9.2 Sulfonamide motif containing analogues of 3O, C_{12}-HSL

The sulfonamide moiety, a direct isostere of the amide bond is almost a common constituent of especially small medicinal molecules because it exhibits ubiquitous biological activity and provides a relative ease of further derivatization as well. The motif imparts the tetrahedral SO_2NH junction that may alter the conformation and may provide a hydrolytically stable new analogue. The incorporation of a sulfonamide into novel 3O, C_{12}-HSL was thus of interest and accordingly new analogues **2.92** and **2.93** with sulfonamide moieties were synthesized.

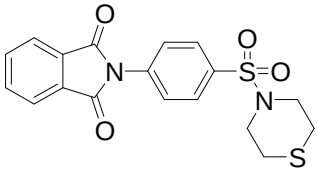


2.92

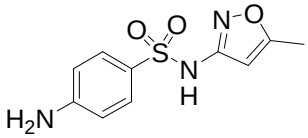


2.93

Several sulfonamides with immune modulatory potential have been reported. For example, *N*-phenyl-phthalimide sulfonamide, LASSBio 468 **2.102**, a compound having a sulfonyl-thiomorpholine moiety, inhibited LPS-induced neutrophil recruitment in Male BALB/c mice.[217] In HIV-1-infected patients with tuberculosis, daily co-trimoxazole (sulfamethoxazole **2.103** and trimethoprim) prophylaxis was found to be well tolerated and has significantly decreased mortality rates.[218]

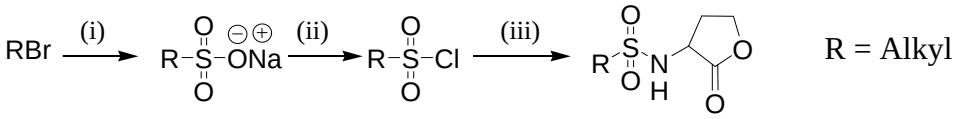


LASSBio 468 **2.102**



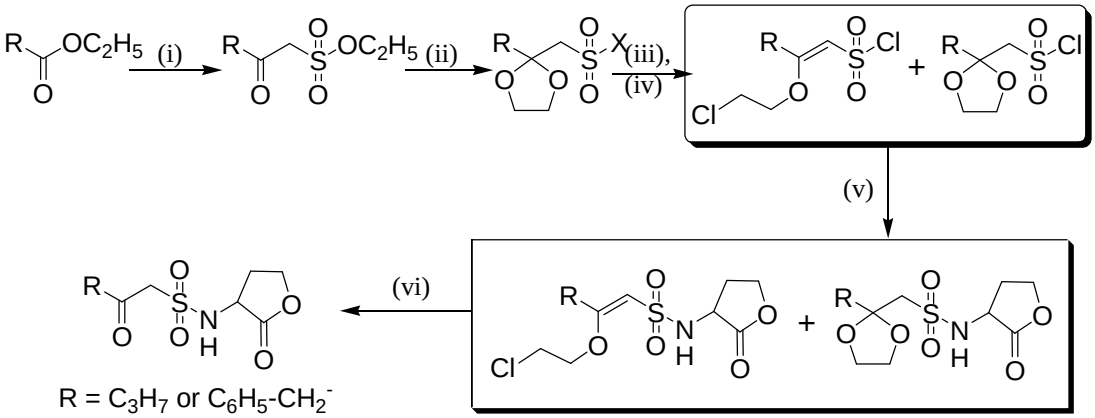
Sulfamethoxazole **2.103**

A series of *N*-acylhomoserine lactone analogues by replacement of the carboxamide bond by a sulfonamide group, were synthesized by Casting el al. as shown in **Schemes 17a and 17b**. These compounds were evaluated for their ability to inhibit the action of 3O, C_6-HSL, which in turn activates expression of bioluminescence in *Vibrio fischeri*. Several compounds displayed antagonist activity.[219]



Reaction conditions: (i) sodium sulfite, water, 24 h reflux; (ii) thionyl chloride, benzene, 4h reflux; (iii) L-HSL.HBr, 1,8-diazabicyclo[5.4.0]-undec-7-ene, DMAP, DCM, 24 h, rt.

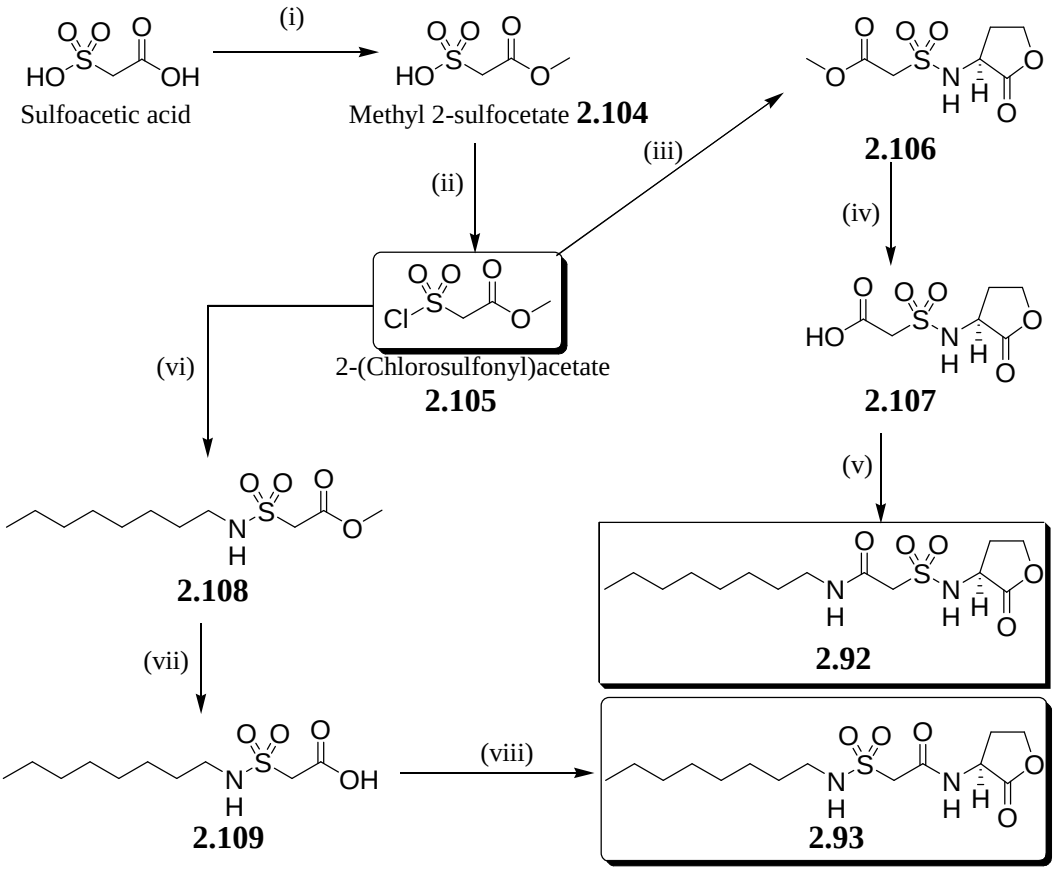
Scheme 17a Synthetic route for *N*-alkyl sulfonyl-HSL



Reaction conditions: (i) $LiCH_2SO_3C_2H_5$, THF, 1.5 h 60 °C, then 2 h rt ; (ii) ethylene glycol, *p*-toluenesulfonic acid, benzene, 24 h, reflux,; (iii) NaI, acetone, 24 h rt; (iii) triphenylphosphine, sulfuryl chloride, DCM, 16 h, rt; (iv) L-HSL.HBr, 1,8-diazabicyclo[5.4.0]-undec-7-ene, DMAP, DCM, 24 h, rt; (vi) aqueous 95% TFA, 2 h, rt.

Scheme 17b Synthetic route for 3-oxo-alkyl sulfonyl-HSL

We aimed to produce new analogues **2.92** and **2.93** as outlined in **scheme 18** by replacing ^1C=O and ^{3}C=O in our 4-aza analogues with S=O group. Synthesis of methyl 2-(chlorosulfonyl)actetate **2.105** was accomplished in two steps.[220] Firstly, esterification of sulfoacetic acid to yield methyl ester **2.104** was achieved by refluxing it in a mixture of methanol-benzene (20:80) with azeotropic distillation of water. Refluxing **2.104** with phosphoryl chloride ($POCl_3$) afforded sulfonyl chloride **2.105**. The amidation of sulfonyl chloride **2.105** with L-HSL in presence of 1, 8-diazabicyclo [5.4.0]undec-7-ene (DBU) and DMAP gave the sulfonamide **2.106** while its amidation with refluxing octylamine in presence DBU and K_2CO_3 gave sulfonamide **2.108**. Acid hydrolysis of methyl ester, **2.106** was preferred to preserve the integrity of L-HSL ring and successfully obtained the free acid, **2.107**. Attempts to couple **2.107** with octylamine using CDI or EDC were unsuccessful. However, it was successfully coupled using DCCI and DMAP and afforded **2.92**. Simple saponification of **2.108** yielded **2.109**, which was successfully coupled to L-HSL using EDC to furnish **2.93**.

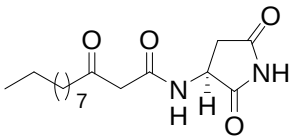


Reaction conditions: (i) Methanol-benzene (2:8), distillation, 8 h; (ii) $POCl_3$, reflux, 4 h; (iii) L-HSL.HCl, DMAP, DBU, dry DCM, 0 ^{0}C to rt., 16 h; (iv) 1.0 M HCl, reflux, 4 h; (v) *n*-octylamine, DCCI, DMAP, dry DCM, rt., 16 h.; (vi) *n*-octylamine, DBU, K_2CO_3, dry DCM, reflux, 3 h; (vii) $NaOH_{aq.}$, methanol, rt., 4 h; (viii) L-HSL.HCl , EDC.HCl, TEA, water-dioxane, rt., 16 h

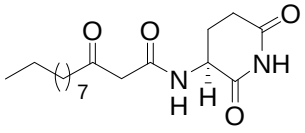
Scheme 18 Synthesis of 1- or 3-sulfonamide motif containing analogues of 3O, C_{12}-HSL.

2.10 Hetero ring altered analogues of 3O, C_{12}-HSL

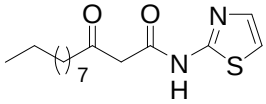
In a limited number of analogues examined, it was found that homoserine ring was essential for immune modulation activity.[131,156] This is borne out from the observation that the ring opened derivative is devoid of any activity.[221] Thus the analogues with changes in the hetero ring are relatively less explored. Hence, one of the aims of this project was to prepare 3O, C_{12}-HSL analogues by introducing structural changes within the homoserine lactone ring and to evaluate their immune modulation effects in eukaryotes. The three molecules *N*-(3-oxododecanoyl)-L-aspartimide (NOdDA) **2.110**, *N*-(3-oxododecanoyl)-L-glutarimide (NOdDG) **2.111** and *N*-(3-oxododecanoyl)-2-aminothiazole (NOdAT) **2.112** as shown below were designed.



2.110



2.111



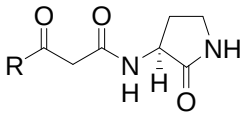
2.112

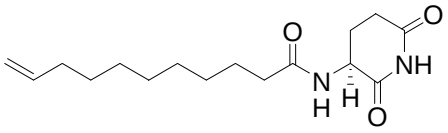
Here the designed analogues retain the same acyl side chains as that of 3O, C_{12}-HSL, but with an aspartimide, glutarimide or thiazole ring instead of the homoserine lactone ring.

Chemokines signalling orchestrate an inappropriate inflammation in a range of human diseases including autoimmune diseases, atherosclerosis, osteoporosis, asthma and cancer. Because chemokines involve in the regulation of human systems both in inflammation and during host defence, they represent an important target for immune modulation therapy.[222-224]

The modifications in both **2.110** and the higher ring structure **2.111** are the replacement of the hetero-atom oxygen within the ring of L-HSL with a nitrogen and incorporation of an additional carbonyl at position 5 and 6 respectively.

Pyrrolidinone based hetero-ring analogues **2.113**, but not containing 5-oxo substituent have been synthesized in the past[131,225] but were found to be inactive as immuno-modulators. However, the **NR58-4** (*N*-undec-10-enoyl-3-aminoglutarimide) **2.114** has been found as most potent broad-spectrum chemokine inhibitor *in vitro*.[226,227]



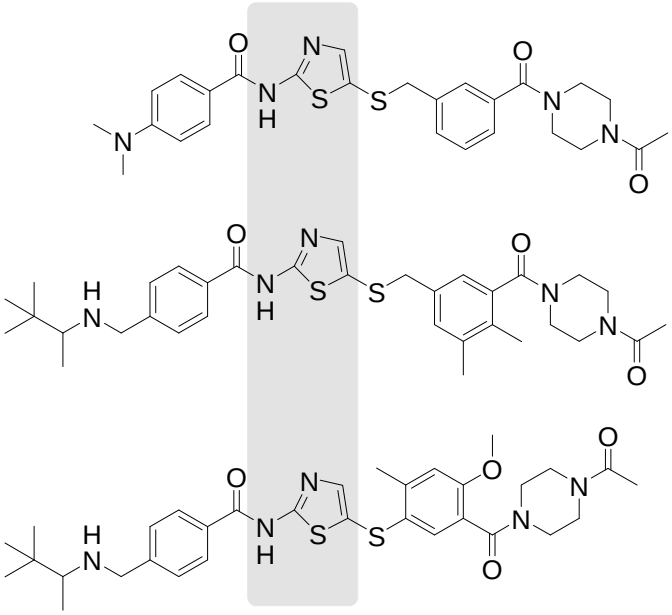


3-Acylamino-L-2-pyrrolidinone **2.113**

NR58-4 **2.114**

Therefore, further modifications by incorporation of oxo group next to the nitrogen within the expanded or non-expanded ring will change the overall chemical properties of the molecules, which may overcome their inefficacy possibly by making them non-inducing immune modulators. Probably the additional carbonyl substituent might increase the molecule's affinity for the receptor through hydrogen bonding, mapping larger area within the receptor via the delocalisation of the lone pair of electrons of nitrogen onto the neighbouring carbonyls. The improved binding to the receptors might lead to better immune modulation.

Immune modulatory potential of 2-aminothiazole has already been established by Das et al., by synthesizing a novel aminothiazole based small molecule inhibitors of Interleukin-2-inducible T-cell kinases (Itk)[228]. Itk is expressed mainly in T, natural killer and mast cells.[229,230] Itk is implicated in activation of T-cells through TCR and CD28 engagement.[231,232] Optimization of the **2.115** led to the discovery of **2.116** as a potent and selective Itk inhibitor (**Fig 2.10**).[233] Screening of 2-amino-5-thioaryl-thiazole series led to the identification of **2.117** as a highly potent and selective Itk inhibitor. It also reduced T-cell proliferation *in vitro*, IL-2 production in mice *in vivo* and lung inflammation in a mouse model of ovalbumin induced allergy/asthma.[234]



2.115
Itk IC_{50} = 1 μM
Jurkat IL-2 I_{50} = 22 μM

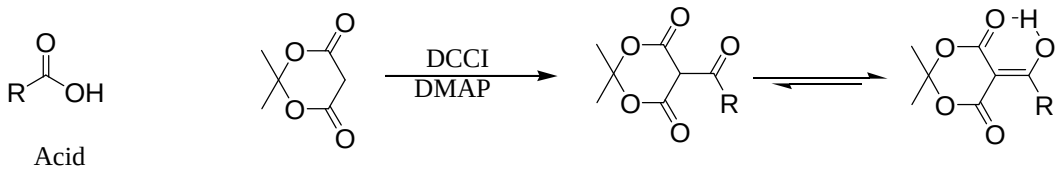
2.116
Itk IC_{50} = 96 nM
Jurkat IL-2 I_{50} = 700 nM

2.117
Itk IC_{50} = 19 nM
Jurkat IL-2 I_{50} = 250 nM

Fig 2.10 Itk inhibitory activity of aminothiazoles

Therefore, replacement of pH sensitive L-HSL ring of 3O, C_{12}-HSL with immunologically active 2-aminothaizaole ring may enhance its immune modulatory behaviour and may overcome auto-induction.

Chemical synthesis of **2.110-2.112** required the initial formation of 5-decanoyl Meldrum's acid **2.118** which was prepared almost in quantitative yield by the *N*, *N*'-dicyclohexylcarbodiimide (DCCI) and 4-dimethylaminopyridine (DMAP) catalysed acylation of Meldrum's acid with *n*-decanoic acid in dry DCM (**Scheme 19**).[131,235]



Acid
R = C_9H_{19}

Meldrum's acid

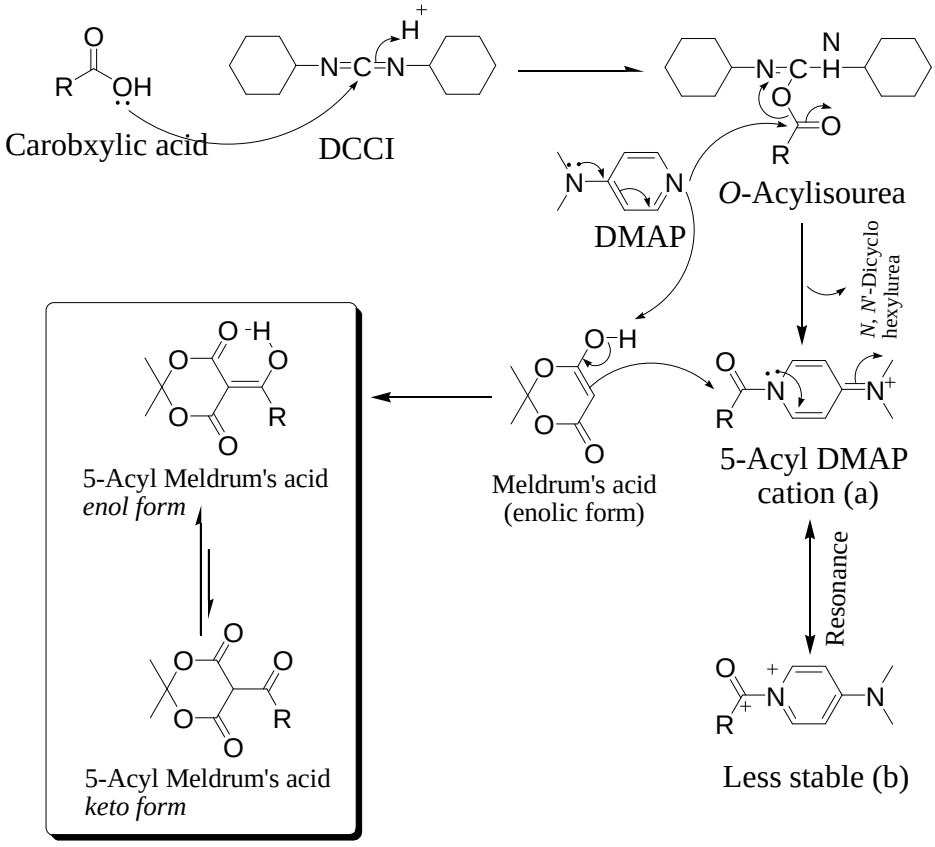
keto form

enol form

5-Decanoyl Meldrum's acid
2.118

Scheme 19 Synthesis of 5-decanoyl Meldrum's acid

The reaction mechanism for DMAP catalysed acylation is shown in **Scheme 20**.

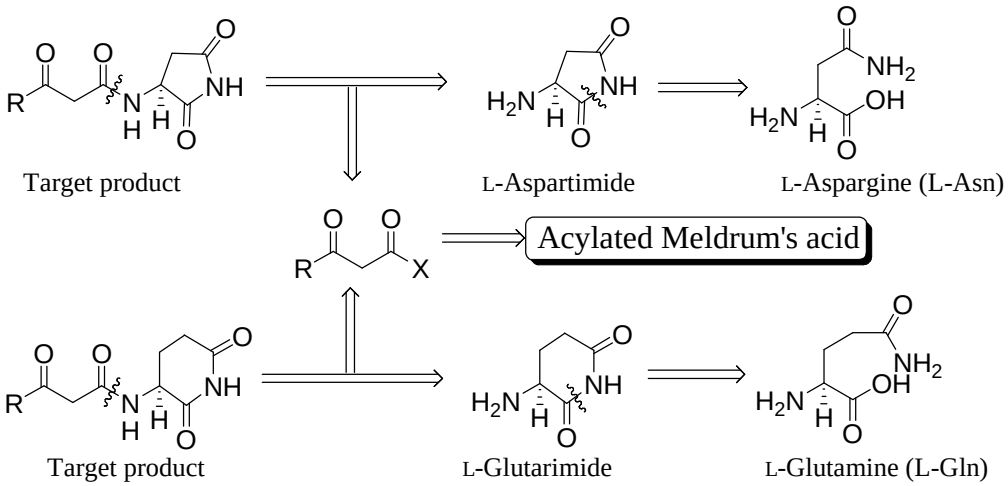


Scheme 20 The reaction mechanism for DMAP catalysed acylation

The intermediate *N*-acylpyridinium cation formed exists in two resonance forms (**a** & **b**). The **b** form is less favoured because the positive charge on the pyridinium nitrogen is destabilised by the adjacent carbonyl's δ^+ charge. The final product **2.118** exists in the enolic form rather than the keto form as evidenced by their ^{1}H NMR, in which the presence of the H-bonded *enolic* proton resonates at a downfield δ value of 15.3.

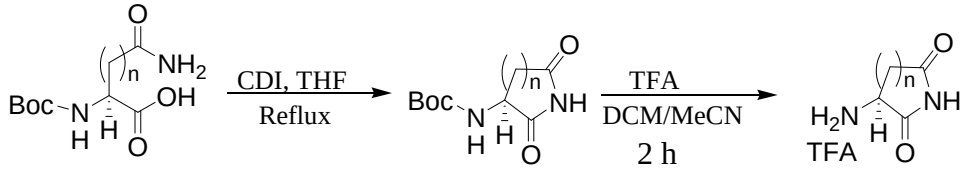
2.10.1 Retro-synthesis of NOdDA and NOdDG

The retro-synthetic approach to the construction of these molecules is shown in **Scheme 21**.



Scheme 21 Retro-synthesis of NOdDA and NOdDG.

The hetercycles L-aspartimide and L-glutarimide required for the analogues **2.110** and **2.111** respectively were elaborated from L-Asn and L-Gln as shown in **Scheme 22**.

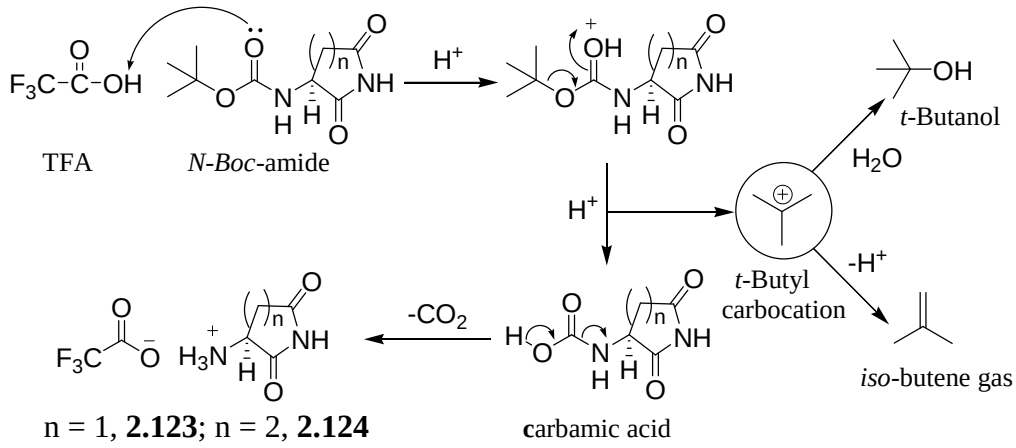


n = 1; *N-Boc*-L-Asn **2.119** — *N-Boc*-aspartimide **2.121** — TFA salt **2.123**
n = 2; *N-Boc*-L-Gln **2.120** — *N-Boc*-glutarimide **2.122** — TFA salt **2.124**

Scheme 22 Synthesis of aspartimide and glutarimide

Commercially available *N-Boc*-L-Asn **2.119** and *N-Boc*-L-Gln **2.120** were used for cyclisation to yield *N-Boc*-L-aspartimide **2.121** and *N-Boc*-L-glutarimide **2.122** respectively. Intramolecular cyclisation was achieved by activating COOH group with the coupling agent 1,1'-carbonyldiimidazole (CDI) in dry tetrahydrofuran (THF). The product **2.121** was easily purified by chromatography using ethyl acetate. The pure product was obtained as a white solid in a yield of 57%. The product **2.122** was pure by TLC and was recrystallised from ethyl acetate as a white solid in a yield of 81%.

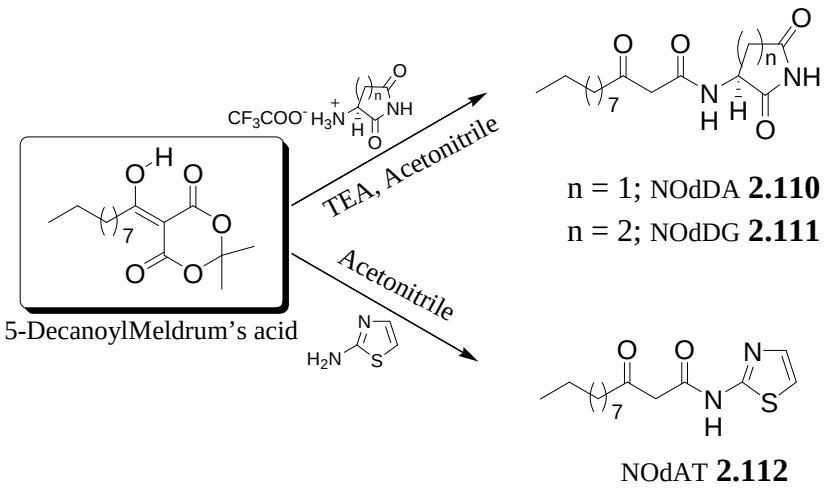
Removal of the *Boc* group from **2.121** and **2.122** was achieved by acidolysis with trifluroacetic acid (TFA) in DCM and acetonitrile respectively. This resulted in the formation of the trifluroacetate salts of aspartimide **2.123** and glutarimide **2.124**. Along with the desired product in each reaction other by products from the generated *t*-butyl carbocation were expected to be produced are shown in **Scheme 23**. These are either *iso*butene gas by loss of proton or *t*-butanol via quenching of the *t*-butyl carbocation with water. The *t*-butanol was expected to be produced rather than the *iso*butene gas due to the presence of water in DCM and acetonitrile.



Scheme 23 Removal of *Boc* by acidolysis

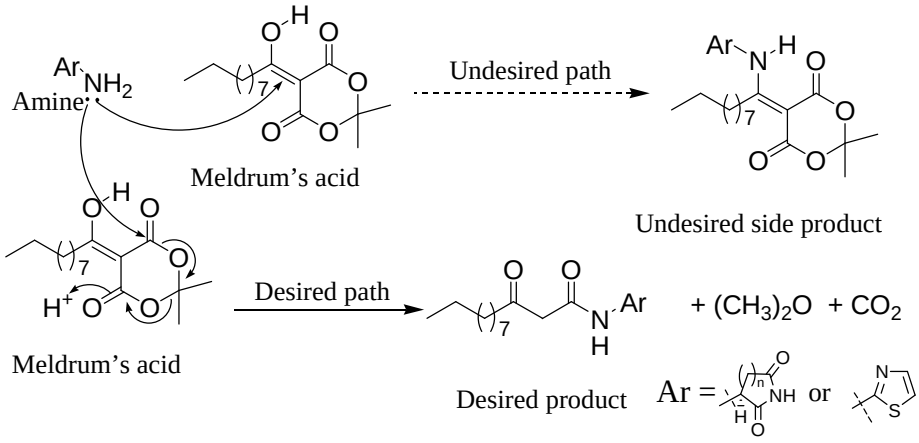
2.10.2 Synthesis of NOdDA, NOdDG and NOdAT

The condensation of L-aspartimide TFA salt **2.123** and L-glutarimide TFA salt **2.124** with **2.118** and two molar equivalents of TEA in acetonitrile under reflux conditions gave the desired products NOdDA **2.110** and NOdDG **2.111**. Similar condensation of 2-aminothiazole with **2.118** in acetonitrile gave NOdAT **2.112.** The syntheses and mechanism of their reactions are shown in **Scheme 24**.



Scheme 24 Synthesis of NOdDA, NOdDG and NOdAT

Condensation of acylated Meldrum's acid with amines does produce small amounts of undesirable side products (**Scheme 25**). This is because amidation could also occur on the exocyclic carbonyl instead of the endocyclic carbonyls, which is the desired route. We tried to minimise this problem by leaving both reaction mixtures overnight at room temperature first to discourage the Schiff's base formation with the exocyclic carbonyl and hence increase the chances for the more reactive endocyclic carbonyls to be favourably attacked by the amine.



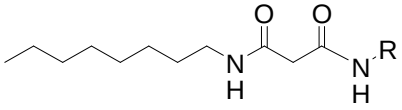
Scheme 25 Amidation of acylated Meldrum's acid

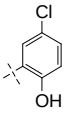
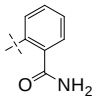
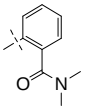
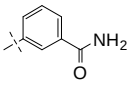
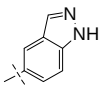
The crude NOdDA, NOdDG and NOdAT were successfully purified using PLC in yields of 58%, 51% and 31% respectively. The pure NOdDA was a pale-yellow solid whereas NOdDG and NOdDAT were off white solids. These were further characterised using IR and ^{1}H NMR. Analogues **2.110**-**2.112** showed reduction in QS activity but only **2.112** displayed an equipotent immune modulation behaviour as that of 3O, C_{12}-HSL whereas, **2.110**-**2.111** did not.

2.11 Hetero ring altered analogues of 4-aza 3O, C_{12}-HSL

As discussed previously, the hetero ring moiety of the 3O, C_{12}-HSL was considered to be essential for immune modulation. However, this statement now stands to be corrected by looking at immune modulatory profile shown by **2.112**. Additionally, on observing the preservation of immune modulation and loss of QS behaviour by 4-aza analogues prompted us to use these analogues to investigate the effect of replacement of L-HSL by other aryl rings on immune modulation. Thus new analogues of 4-aza-3O, C_{12}-HSL **2.125-2.135** as shown in **Table 2.5** were conceived with an expectation that they may display selective immune modulation.

Table 2.5 Hetero ring altered 4-Aza analogues of 3O, C_{12}-HSL

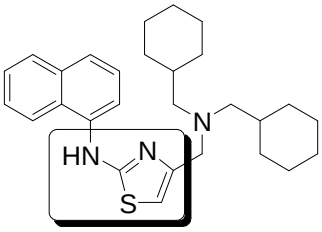


Comp.	R	Chemical name
2.125		2-(3-Oxo-4-azadodecanoyl)aminothiazole
2.126		(1*R*,2*S*)-2-(3-Oxo-4-azadodecanoyl)aminocyclopentanol
2.127		(1*R*,2*S*)-2-(3-Oxo-4-azadodecanoyl)aminocyclohexanol
2.128		*N*-(3-Oxo-4-azadodecanoyl)cycloheptylamine
2.129		2-(3-Oxo-4-azadodecanoyl)aminophenol
2.130		2-(3-Oxo-4-azadodecanoyl)amino-4-chlorophenol
2.131		2-(3-Oxo-4-azadodecanoyl)aminobenzamide
2.132		*N, N*-Dimethyl-2-(3-oxo-4-azadodecanoyl)aminobenzamide
2.133		3-(3-Oxo-4-azadodecanoyl)aminobenzamide
2.134		2-(3-Oxo-4-azadodecanoyl)aminopyridine
2.135		5-(3-Oxo-4-azadodecanoyl)amino-1*H*-indazole

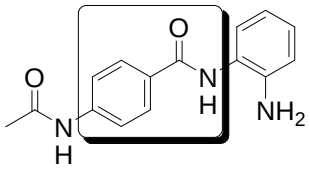
2.11.1 Selection of R-group for the hetero-ring replacement

An active component of either existing immune modulatory or antibacterial agents or both was chosen as R-group. This might either potentiate immune modulation or restrain QS by the new analogues.

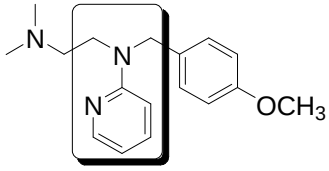
In addition to already mentioned utilization of 2-aminothiazole in Itk inhibitors **2.115-2.117**, recently it has also been explored by Wang et al. who discovered a novel series of 2-aminothiazole-derived CCR4 antagonists. This led to the invention of a compound **2.136**, with adequate potency and pharmacokinetic properties.[236] CCR4 is a G-protein-coupled receptor from the C-C motif containing family of chemokine receptors that is involved in the migration of inflammatory cells into inflamed tissue and predominantly expressed on T-lymphocytes, mainly on those of the T_H2 phenotype.[237,238] The 2-aminocyclohexanol has been used to make QS agonist where as 2-amino cycloheptanol, cycloheptylamine, 2- and 3-aminobenzamides, 2-aminophenols and 5-aminoindazole have been used to develop QS antagonists.[172] Moreover, aminobenzamide derivative CI-994, *N*-acetyldinaline-[4-(acetylamino)-*N*-(2-aminophenyl)benzamide] **2.137** is an antitumor cytostatic agent currently undergoing clinical trial[239-242], the 3-aminobenzamide as PARP inhibitor[243], phenols in analgesic-antipyretic (e.g. paracetamol) drugs and a 2-aminopyridine containing pyrilamine **2.138** as antihistamine.[244]



2.136



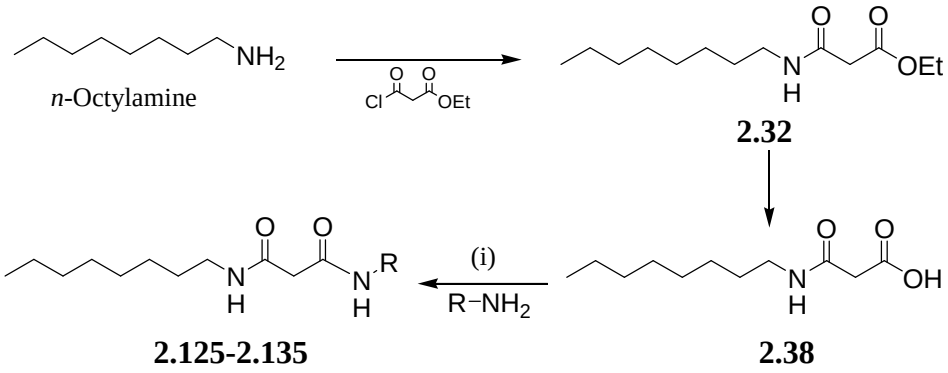
CI994 **2.137**



Pyrilamine **2.138**

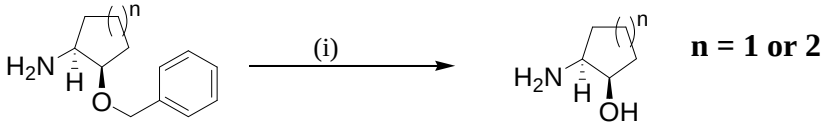
Synthetic route to **2.125-2.135** analogues was as similar to that of 4-aza-3O, C_{12}-HSL (**Scheme 6**). Except coupling of 3-oxo-4-azadodecanoinc acid **2.38** with L-HSL, here various aryl, heteroaryl and cycloalkyl amines (R-groups as shown in **Table 2.5**) were coupled separately with **2.38** as shown in **Scheme 26-a** to generate the desired

analogues **2.125-2.135**. Moreover, benzyl protected R groups (**2.126-a** and **2.127-a)** were deprotected by catalytic hydrogenation as shown in **Scheme 26-b**.



Reaction conditions: (i) EDC.HCl, TEA, Dioxane-water/DCM, 8-16 h. rt.

Scheme 26-a Synthesis of hetero ring altered analogues of 4-aza-3O, C_{12}-HSL



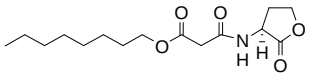
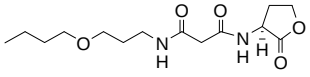
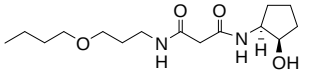
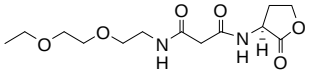
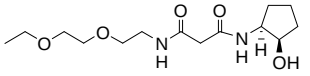
Reaction conditions: (i) H_2, Pd/C, EtOH, rt., 6 h.

Scheme 26-b Removal of benzyl group of **2.126-a (***n=1***)** and **2.127-a** (*n=2*) by catalytic hydrogenation

2.12 Oxa analogues of 3O, C_{12}-HSL

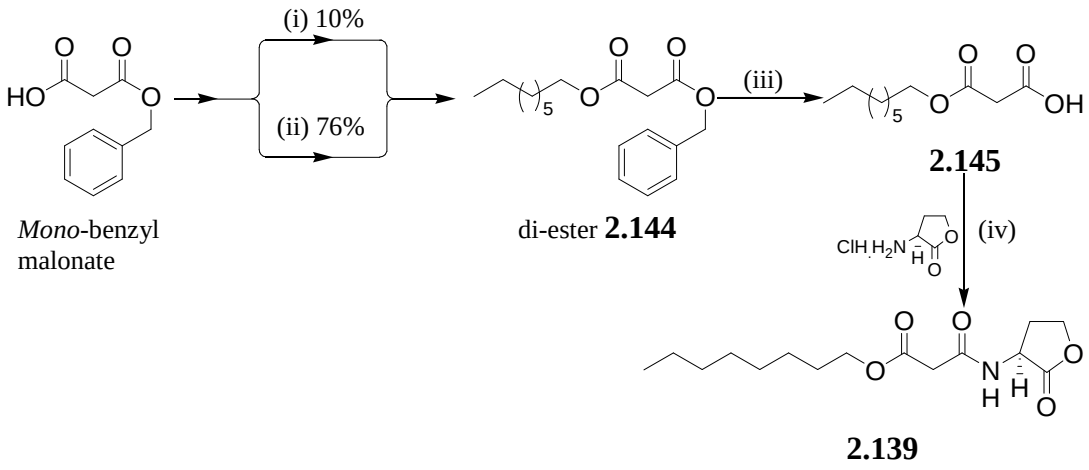
Besides showing immune modulatory activity, lower solubility of the acyl chain larger than 12 carbons containing 3O, C_{12}-HSL analogues (ref. 'A-part' in **Fig 2.4**, page 72) in the solvents used for their *in vitro* bioassays have restricted these derivatives to be studied furher. We therefore, decided to introduce in the 'A' side chain a hetero atom(s) 'oxygen(s)' in 3O, C_{12}-HSL at C^4 position to modulate its *3-enolic* content[245,246] and in 4-aza-3O, C_{12}-HSL at C^7 and C^{10} or at C^8 position(s) to modulate its hydrophilic-lipophilic balance. Moreover, introduction of oxygen at C^4 might provide an additional site for the target proteins to form hydrogen bonding and show potent biological response whereas; oxygen(s) introduction at C^7 and C^{10} or C^8 position(s) might enhance its solubility in appropriate solvents. Thus three types of analogues **2.139-2.143** as shown in **Table 2.6** were developed and evaluated for immune and microbiological responses.

Table 2.6 Oxa analogues of 3O, C_{12}-HSL

Comp.	Structure	Chemical name	Abbreviation
2.139		*N*-(3-Oxo, 4-oxadodecanoyl)-L-HSL	3O, 4-oxa C_{12}-HSL
2.140		*N*-(3-Oxo, 8-oxa 4-aza dodecanoyl)-L-HSL	3O, 8-oxa, 4-aza C_{12}-HSL
2.141		(1*R*,2*S*)-2-(3-Oxo- 8-oxa-4-azadodecanoyl) aminocyclopentanol	
2.142		*N*-(3-Oxo, 7, 10-dioxa, 4-aza dodecanoyl)-L-HSL	3O, 7, 10-dioxa, 4-aza C_{12}-HSL
2.143		(1*R*,2*S*)-2-(3-Oxo- 7, 10-dioxa- 4-azadodecanoyl) aminocyclopentanol	

2.12.1 Synthesis of 3O, 4-oxa C_{12}-HSL

For the preparation of 4-Oxa analogue **2.139** (**Scheme 27**), esterification of *mono*-benzyl malonate with octyl bromide in the presence of tetrabutylammonium fluoride (TBAF) gave di-ester **2.144**. The catalyst TBAF was generated *in situ* from tetrabutylammoniun hydrogen sulfate ($TBAHSO_4$) and potassium fluoride dihydrate ($KF.2H_2O$) in THF.[245,246] However, this reaction gave the desired di-ester **2.144** in a very low yield of only 10%. Therefore, *mono*-benzyl malonate was esterified with 1-octanol in the presence of DCCI and DMAP to furnish di-ester **2.144**, in an excellent yield of 76%. The di-ester **2.144** was then deprotected by catalytic hydrogenation to afford the free acid **2.145**. Compound **2.145** was activated by CDI and coupled with L-HSL to furnish the desired 3O, 4-oxa C_{12}-HSL **2.139**.



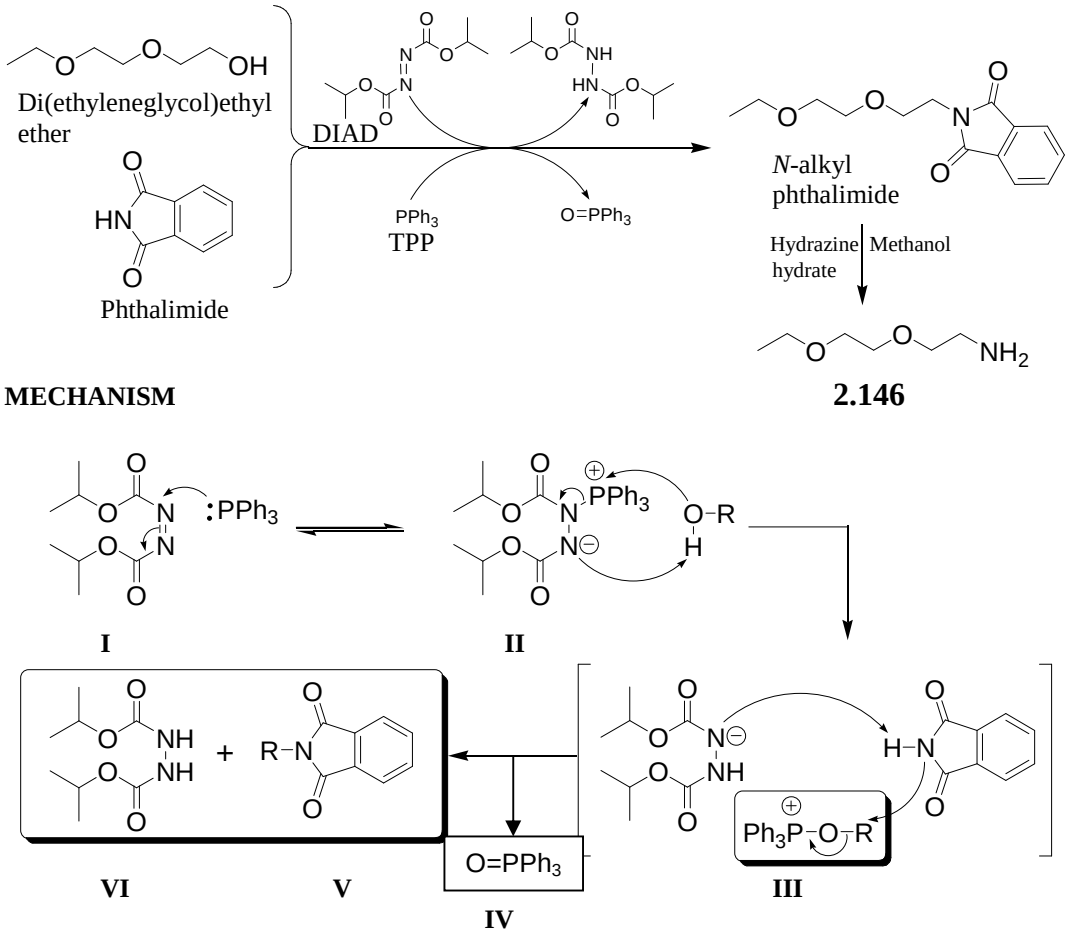
Reaction conditions: (i) *n*-octylbromide, $TBAHSO_4$, $KF.2H_2O$, THF, rt., 18 h; (ii) *n*-octyl alcohol, DCCI, DMAP, DCM, rt., 16 h; (iii) H_2, Pd/C, EtOH, rt., 6 h; (iv) CDI, TEA, DCM, rt., 16 h.

Scheme 27 synthesis of 3O, 4-oxa C_{12}-HSL

The identity of the final product **2.139** was confirmed by its ^{1}H NMR which showed all protons at the expected δ values and the MS which displayed exact mass of *m/z* 300 for MH^{+}.

2.12.2 Synthesis of 8-oxa and 7, 10-dioxa analogues of 3O, 4-aza C_{12}-HSL

The starting amine 3, 5-dioxa-octylamine **2.146** required for the syntheses of **1.242** and **1.243** was prepared by Mitsunobu reaction between di(ethyleneglycol)ethyl ether and phtahlimide using triphenylphosphine (TPP) to generate *N*-alkylphtalimide.[247] The phthalimide derivative was then deprotected by hydrazinolysis to afford **2.146**.[248] The Mitsunobu reaction is reported to proceed via the path as shown in **Scheme 28**.

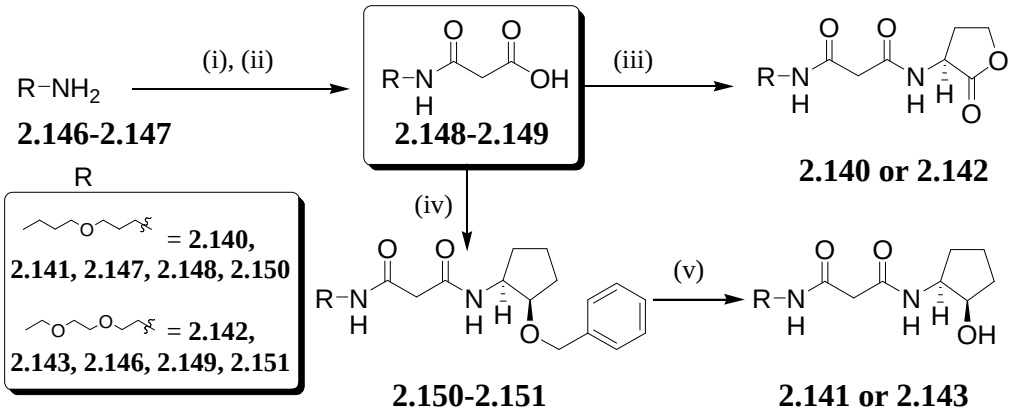


Scheme 28 synthesis of amine **2.146** by Mitsunobu reaction

Initially, the nucleophilic attack of TPP upon DIAD (**I**) produces a betaine intermediate **II**, which deprotonates the alcohol (ROH) to form the hydrazo anion and

alkoxyphosphonium cation **III**. The hydrazo anion then deprotonates phthalimide which in turn attacks cation **III** to form the desired product **V** along with triphenylphosphine oxide (**IV**) and *dihydro*-DIAD (**VI**).

Commercially available 3-butoxypropylamine **2.147** was used as the starting material for the syntheses of 8-oxa analogues **1.240** and **1.241**. The synthetic route used for the synthesis of **2.140-2.143** is shown in **scheme 29**. The acylation of amines **2.146-2.147** with ethyl malonyl chloride followed by base hydrolysis afforded 4-aza acids **2.148-2.149**.



Reaction conditions: (i) ehyl malonyl chloride, DCM, 0 °C to rt., 16 h; (ii) $NaOH_{aq}$., EtOH, rt., 6 h; (iii) L-HSL.HCl, EDC.HCl, TEA, water-dioxane, rt., 16 h. (iv) (1*S*, 2*S*)-2-benzyloxy cyclopentylamine, EDC.HCl, TEA, dry DCM, rt., 16 h. (v) H_2, Pd/C, EtOH, rt., 8 h.

Scheme 29 synthesis of oxa analogues of 3O, 4-aza C_{12}-HSL

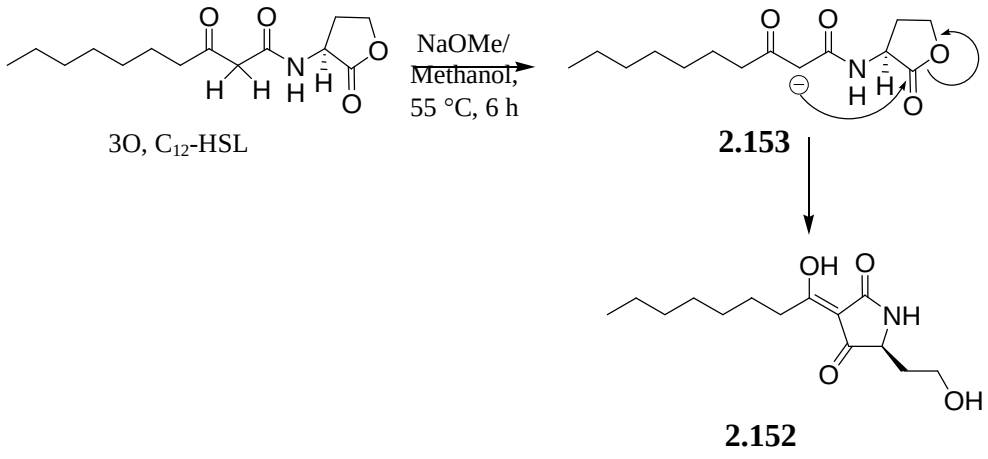
The acids **2.148-2.149** were coupled separately with L-HSL and (1*R*, 2*S*)-2-benzyloxycyclopentylamine to generate desired products **2.140, 2.142** and bezyloxy analogues **2.150-2.151** respectively. Deprotection of benzyl group in **2.150-2.151** was achieved by catalytic hydrogenation to afford other target products **2.141** and **2.143**.

2.13 Miscellaneous analogues of 3O, C_{12}-HSL

2.13.1 Synthesis of tetramic acid derivative: Rearranged isomeric product of 3O, C_{12}-HSL

The kauffmaun et al., discovered a previously undescribed nonenzymatically formed product, the 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione, **2.152** a tetramic acid. The degradation product **2.152** is derived from 3O, C_{12}-HSL via an unusual intramolecular Claisen-like condensation reaction and showed bactericidal activity against Gram negative. We considered that **2.152** if displays immune modulatory activity, it might be a breakthrough in the development of a new set of immune modulatory drugs.[249]

Abstraction of C^2 acidic protons of 3O, C_{12}-HSL by a strong base sodium methoxide (NaOMe) in methanol at 55°C afforded carbanion **2.153** which underwent intramolecular Claisen like condensation and yielded a desired product **2.152** as shown in **scheme 30**.



3O, C_{12}-HSL

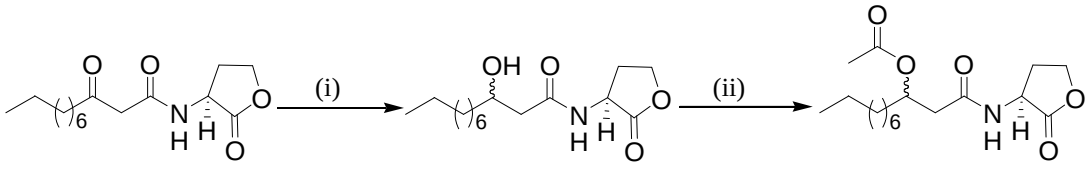
2.153

2.152

Scheme 30 synthesis of 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl) pyrrolidine-2,4-dione

2.13.2 Synthesis of 3-acetoxy, C_{12}-HSL

Acetylation of hydroxyl groups is a frequently used transformation method in medicinal chemistry to protect hydroxyl group from conversion by enzymes *in vivo* or chemical reactions *in vitro* to other undesired functional group. Chemically acetyl is the most convenient group in view of its easy introduction, being stable to the acidic reaction conditions, and also easily removable by mild alkaline hydrolysis.[250,251] Due to the importance of acetylation, we designed and synthesized *N*-(3-acetoxy-dodecanoyl)-L-HSL **2.154** as shown in **scheme 31**.



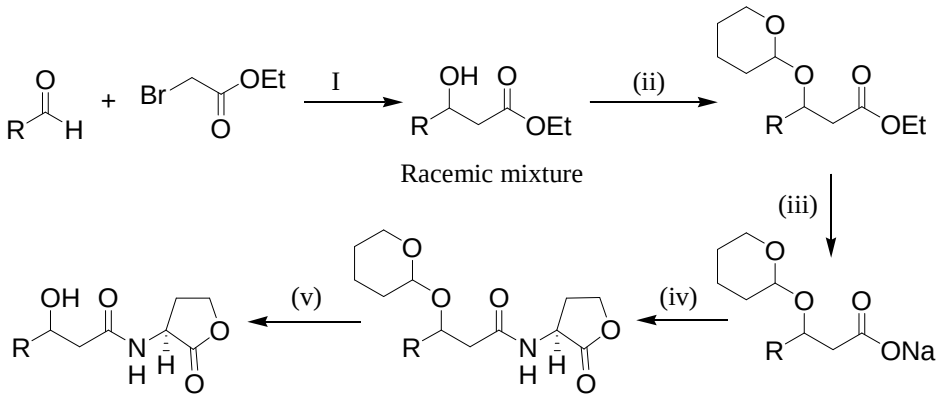
3O, C_{12}-HSL (racemic mixture) **2.155** **2.154**

Reaction conditions: (i) sodium cyanoborohydride, methanol-HCl, rt, p*H*≅4.0, 16 h; (ii) acetic anhydride, reflux, 2 h.

Scheme 31 synthesis of 3O-Ac,C_{12}-HSL

The key intermediate required for the synthesis of **2.154** was the *N*-(3-hydroxy-dodecanoyl)-L-HSL **2.155** which was synthesized from 3O, C_{12}-HSL by reduction method suggested by Chhabra et al.[225] who were able to reduce *N*-3-oxo analogues to *N*-3-hydroxy analogues (e.g. **2.155**) as a mixture of diastereomers without the protection and deprotection of the β-hydroxy moiety using sodium cyanoborohydride reducing agent. The sodium cyanoborohydride reducing agent was preferred over sodium borohydride because it required acidic conditions in which both the stability of the L-HSL ring and the chiral integrity of the molecule are retained.

The *N*-(3-hydroxyacyl) derivatives have been synthesized by Zhu et al.[252] by coupling protected β-hydroxy alkanoic acid with L-HSL as shown in **scheme 32**.



Reaction conditions: (i) Zinc, ethanol, H_3O^+; (ii) dihydropyran, tosic acid; (iii) $NaOH_{aq}$, dioxane; (iv) L-HSL.HCl, EDC.HCl, TEA, water; (v) aqueous perchloric acid.

Scheme 32 General synthesis *N*-(3-hydroxyacyl)-L-HSL

Here β-hydroxy alkanoic acid was prepared by the Reformatsky reaction between an aldehyde and ethyl bromoacetate in the presence of zinc. Prior to saponification and coupling with L-HSL, the β-hydroxy group was protected with dihydropyran to obtain the tetrahydropyran (THP) ether.

Alternatively, **2.153** can be obtained from 3O, C_{12}-HSL using suitable reducing agents. The nature of the reducing agents have been found to determined the enantiomeric excess of the resulting mixture of β-hydroxy derivatives (**Table 2.7**).[253-256]

Table 2.7 List of reducing agents showing yields obtained with their use and enantiomeric excess

Reagents	**Stereo-chemistry**	**Yield (%)**	**Enantiomeric excess**
$RuCl_3$/(*R*)-(MeO-BIPHEP) 1 mol % H_2 (6 bar), methanol, 80 °C, 23 h.	*R*	96	99
$NaBH_4$, THF, 0 °C.	*R/S*	74	-
R-BINAP (2 mol %), H_2 (1 bar), 50 °C, 48 h.	*R*	100	97
$Zn(BH_4)/ZnCl_2$, toluene, -78 °C, 3 h.	*R*	94	84
DIBAL/BHT, toluene, 78 °C, 3 h	*S*	82	92

Lastly, the desired product **2.154** was obtained by acetylation of the 3-hydroxyl group of **2.155** by refluxing it with acetic anhydride. Moreover, commonly used reaction condition for this conversion uses acetic anhydride in the presence of an acid/base

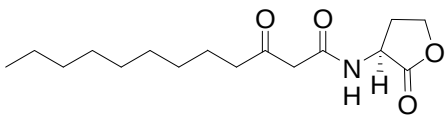
catalyst.[257] Various metal salts such as $CoCl_2$,[258] $TiCl_4$-$AgClO_4$,[259] Me_3SiCl,[260] $LiClO_4$,[261] $Mg(CLO_4)_2$,[262] $CuSO_4.5H_2O$[263-265] and some metaltriflates such as $Se(OTf)_3$,[266] MeSiOTf,[267] $In(OTf)_3$,[268] $Cu(OTf)_2$,[269] and $Bi(OTf)_3$,[270] have been investigated to meet the demands for more efficient and selective methods. The purity of the final product was confirmed from its ^1H NMR.

3.0 BIOLOGICAL ASSAYS

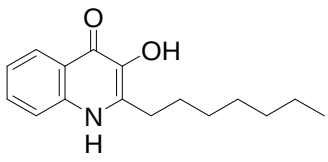
3.1 Immunology

Immune modulation is the means of equilibrating immune system via external stimuli. Balancing immune system is the only solution to combat disorders due to its overactivity as well as underactivity. The macrophages, natural killer cells and other "soldiers" of the immune system attack invading organisms and shield the body from their infections. Whereas, the immune system when attacks the body itself leads to the autoimmune diseases like rheumatoid arthritis, type-1 diabetes, etc. Immune overactivity causes unpleasant side effects, such as allergies and inflammation, and can lead to immune exhaustion and collapse. Therefore, balancing the body's immune system so that it is neither overactive nor underactive is the real answer to maintain good health. The existing agents used to balance the immune system have been reviewed in Chapter 1. A number of drugs, which modulate immune system, have been developed directly or indirectly from the natural resources. For example, the potent immunosuppressive agents derived from fungi or bacteria such as CsA, FK506, and rapamycin, which have a degree of selective action on the immune system, has improved the success rate of transplant surgery indicating the value of natural products to clinical medicine.[26] The recent observation that the bacterial signal molecules participating in their cellular communication (QS), also possesses immune modulatory activity[128] in animals had motivated researchers to study these signal molecules biologically for better understanding of their mechanism of action and chemically for the development of novel immuno therapeutic agents.[131]

The *Pseudomonas aeruginosa* quorum sensing signal molecules (QSSMs), 3O, C_{12}-HSL **1.54** and PQS **1.60** have been observed to modulate immune system apparently by targeting different receptors. However, details of locations and exact structure of immunophilin targets are still unknown.[132]



1.54



1.60

Recently, detailed structure activity relationship (SAR) study using PQS as a lead molecule has been undertaken with successful development of its novel analogues.[271] However, initial SAR study using 3O, C_{12}-HSL as a lead molecule had delivered its analogues that though exhibit the desired immune suppressive behaviour[131] but also have undesired quorum sensing effects (unpublished data). Moreover, some of the analogues of 3O, C_{12}-HSL suffered with solubility problems in the routine solvents. The 3O, C_{12}-HSL structure thus demanded further investigations to overcome these problems of solubility and unwanted quorum sensing behaviour. To address these issues 3O, C_{12}-HSL was subjected to further structural modifications for the development of further analogues with improved immunomodulatory and optimum solubility behaviors as well as with no auto-induction or QS activity.

3.1.1 Structural modifications in 3O, C_{12}-HSL

Biologically active compounds impart their effects by interacting with their target receptors to which they selectively bind through one or all of the binding interactions such as ionic bonding, *Van der Waal's* interactions, dipole-dipole interactions and/or hydrogen bonding. The functional group(s) of the active molecule(s) can be perhaps prone to one or all of these interactions, or even this may not be happening at all in practice. Importance of these potential interactions can be established by partially altering the lead structure and comparing their activity. Similar activity to that of lead molecule reveals that the altered group does not participate in interactions whereas, enhanced or reduced activity compare to the lead concludes influence of group on interactions. The SAR studies quite often employed to ascertain biologically important part(s) of the molecule that eventually confirm final structure of a lead molecule. In initial SAR studies of 3O, C_{12}-HSL, Chhabra et al, have been successful to ascertain few functional units amenable for binding to immunophilin targets but none of the analogues were devoid of molecular interactions responsible for unwanted QS effect.[131] In order to identify functional units within the structure of 3O, C_{12}-HSL that are important for selective binding to immunophilin targets, it was essential to carry out further structural modifications in it. As already discussed**,** the areas that might have potential for binding to a selective receptor are as shown in **Fig 2.4**.

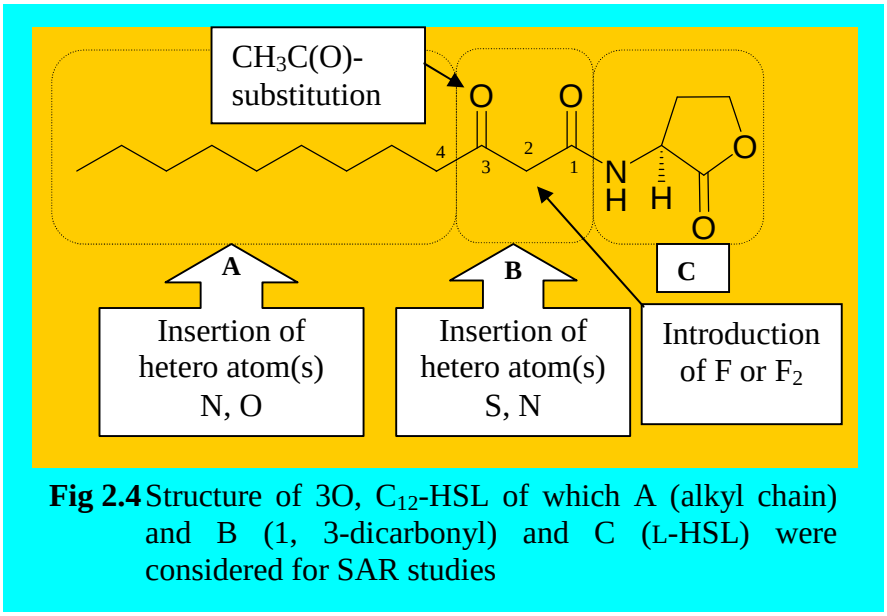


Fig 2.4 Structure of 3O, C_{12}-HSL of which A (alkyl chain) and B (1, 3-dicarbonyl) and C (L-HSL) were considered for SAR studies

Further alterations in 3O, C_{12}-HSL structure were achieved by replacing; C^1 or C^3 carbonyl functionality by other functional group; C^4 carbon with hetero atom (nitrogen with or without substitution or oxygen); one or both the C^2 hydrogen(s) by electron withdrawing fluorine and replacement of hetero ring with other rings as well as insertion of hetero atom(s) in positions other than C^4 in the side chain A. Newly synthesized analogues of the 3O, C_{12}-HSL were evaluated for immunological activity via inhibition of T-cell proliferation by them (murine leukocyte proliferation assay) and cytotoxicity effects (dye exclusion assay). Murine leukocyte proliferation assay and toxicity assay are outlined in Appendix-I and Appendix-II respectively.[128] The results of the murine splenocyte proliferation assay are expressed as EC_{50} (μM) and are an indicator of compound's pharmacological activity. The term EC_{50} is a dose (μM) of compound required to inhibit 50% of a proliferating population of murine splenocytes, measured using tritiated-thymidine ([^{3}H]-thymidine) incorporation as a marker of proliferation and determined by nonlinear regression analysis. Splenocytes stimulated with concanavalin A (ConA) are stimulated to incorporate [^{3}H]-thymidine into newly synthesized DNA, indicated as counts per minute (cpm).

All analogues were assayed over a concentration range of 0.3 to 1000 μM in duplicates ($n = 2$) and EC_{50} was assigned from the dose-response curve of murine splenocytes proliferation (cpm) marked as incorporation of [^{3}H]-thymidine into newly synthesized DNA versus concentrations (μM). The DMSO was used as solvent control for all

analogues and it was found to display little or no inhibitory effect on cell proliferation. The results are presented to illustrate stimulations to ConA compared with positive control (cells + ConA with no compounds or DMSO), negative control (cells only) and reference standard (cells + ConA with dexamethasone). The effect of 3O, C_{12}-HSL in the proliferation of murine leukocytes in ConA stimulated assay is shown in **Fig 3.1**.

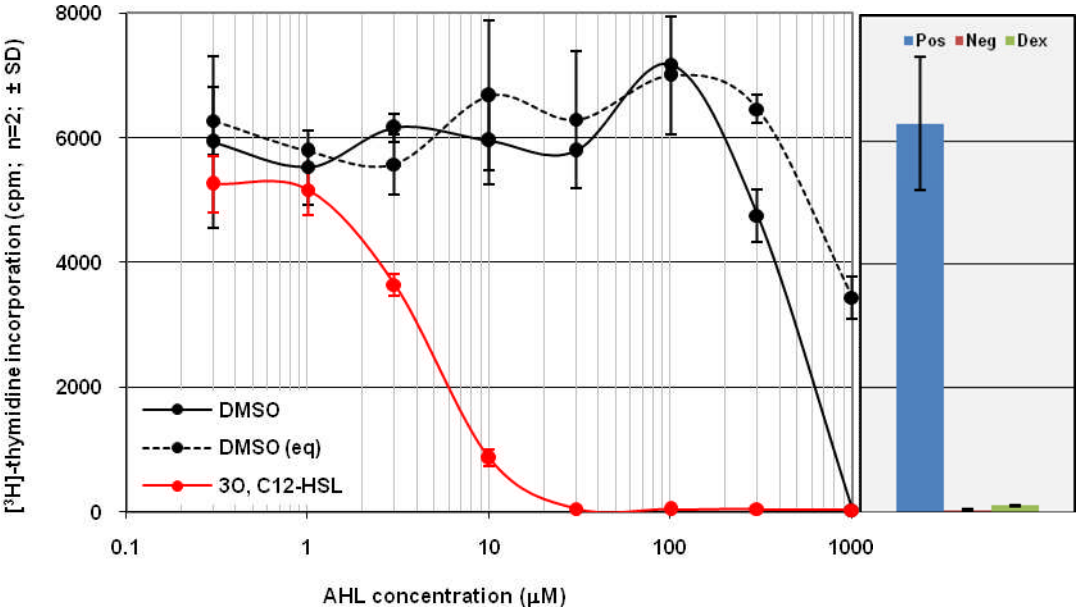


Fig- 3.1 Dose-response curves of 3O, C_{12}-HSL and DMSO on ConA stimulated murine leukocytes proliferation.

3.1.2 SAR studies to develop second generation immune modulatory analogues of 3O, C_{12}-HSL

New analogues as described in Chapter 2 developed by systematic alterations in the structure of 3O, C_{12}-HSL within the indicated areas as shown in **Fig 2.4.**

3.1.2.1 Insertion of N at position 4 in the side chain A of 3O, C_{12}-HSL:

4-Aza analogues

As evidenced from previously reported data, lipophilicity of the alkyl chain A of 3O, C_{12}-HSL was found to be essential for its immuno suppressive effect with an optimum length between 9 to 11 carbons.[131,171] However, these analogues also exhibited unwanted QS effect and analogue(s) with alkyl chain length of 11 or more carbons

were insoluble in solvent employed for bioassays. Thus insertion of N^4 in alkyl chain was expected to modulate *3-enolic* content of the parent compound which is reported to be must for immune modulation.[131,171] It would also provide an additional site for binding interactions with immune specific receptors, thus may display a selective immune modulation. Whether N^4-H is, a site of interaction with the receptor was further confirmed by developing N^4-substituted analogues. These 4-aza analogues were obtained via the synthetic methods discussed in chapter 2. The results of analogues **2.8-2.13** in the proliferation of murine leukocytes in ConA stimulated assay are shown in **Fig 3.2**.

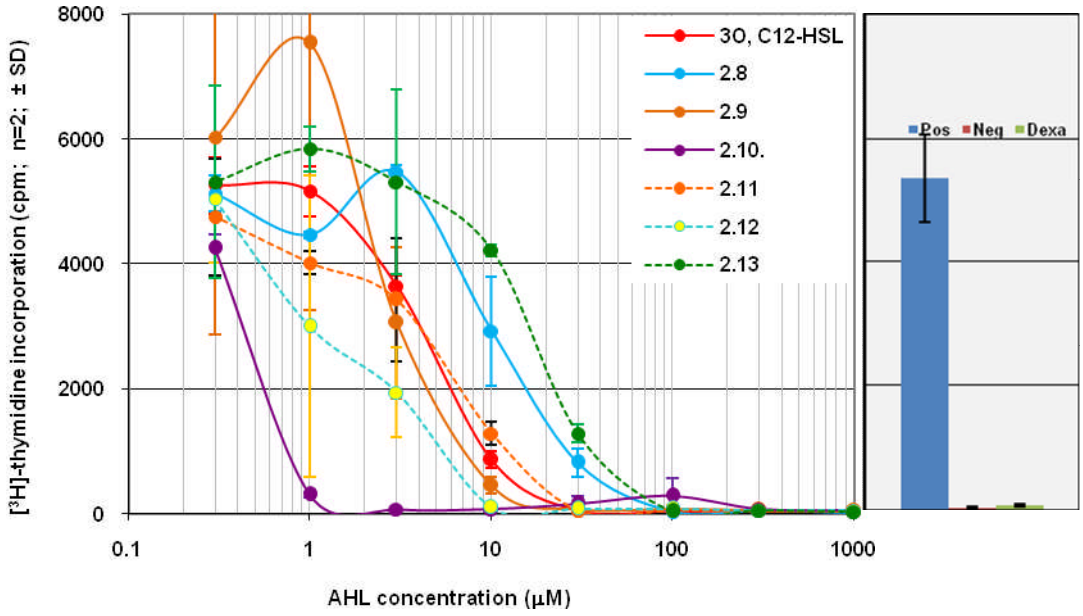


Fig- 3.2 Dose-response curves of 4-aza analogues of 3O, C_{12}-HSL on ConA stimulated murine leukocytes proliferation

From **Fig 3.2** it can be deduced that insertion of N^4 in the side chain A is tolerated as it has retained immune suppression. The dose-response curves were extrapolated and EC_{50} obtained from these curves were compared with 3O, C_{12}-HSL as summarised in **Table 3.1**

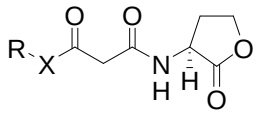


Table 3.1 Immunological results of 4-aza/substituted 4-aza analogues of 3O, C_{12}-HSL

Compound(s)	***R***	***X***	***EC_{50} (μM)***
1.54 (3O, C_{12}-HSL)	$CH_3(CH_2)_7$-	CH_2	4.30-4.50
2.8	$CH_3(CH_2)_7$-	NH	15.10
2.9	$CH_3(CH_2)_8$-	NH	2.90
2.10	$CH_3(CH_2)_{10}$-	NH	0.652
2.11	$CH_3(CH_2)_7$-	NCH_3	6.10
2.12	$CH_3(CH_2)_7$-	$N(CH_2)_2CH_3$	2.90
2.13	$CH_3(CH_2)_7$-	$N(CH_2)_2OH$	17.10

The three-fold drop in immune suppressive activity observed with analogue **2.8** as compared to 3O, C_{12}-HSL However, increased chain length to C_9 and C_{11} (compounds **2.9** and **2.10**) enhanced the immune suppression potency when compared to the parent compound **1.54**. Thus, it further confirmed that a longer alkyl chain length (*R*) is important for activity. The alkyl substitution on N^4 also contributes towards potency with optimum level reached with *n*-propyl substitution (**2.12)**. Insertion of N^4 not only retains lipophilic character of resultant analogues but also improves their solubility as evidenced from observation that all analogues were completely soluble in the solvents employed for their bioassays. However, hydrophilic hydroxylethyl substitution on N^4 (**2.13)** had slightly reduced potency thus supporting the importance of lipophilicity[131] in immune modulation. These data also support that N^4 may be interacting with an additional site on the immunophilin receptors in order to elicit selective immune suppression. Gratifyingly, 4-aza analogues did not show any auto-induction rather they were found to as QS antagonists (will be discussed in microbiology study).

3.1.2.2 Replacement of C^2 hydrogen(s) of 4-aza 3O, C_{12}-HSL by fluorine(s):

Fluorinated 4-aza analogues

Being most electronegative atom, fluorine greatly influences acidity or basicity of the nearby functional groups and thus the pK_a of the molecule. The pK_a has control over

the pharmacokinetic properties of the molecule and its binding affinity with target proteins. As discussed before, lipophilicity is a key molecular parameter in medicinal chemistry as functional groups of substantial lipophilicity within the structure demonstrate good binding affinity to the target proteins. Moreover, being smallest, fluorine does not encroach on the spatial requirements of the molecule. Its introduction in the molecule also imparts metabolic stability.[184] Thus C^2 acidic hydrogens of 3O, C_{12}-HSL were replaced by fluorine(s) and analogues **2.62** and **2.63** were developed (**Table 3.2**).

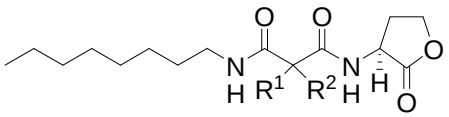


Table 3.2 Immunological results of fluorinated 4-aza-3O, C_{12}-HSL analogues

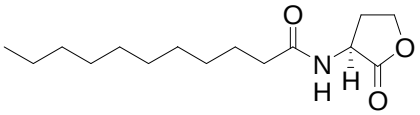
Compound(s)	***R^1***	***R^2***	***EC_{50} (µM)***
1.54 (3O, C_{12}-HSL)	H	H	4.30-4.50
2.62	F	H	21.40
2.63	F	F	82.30

Although at higher EC_{50} compared to 3O, C_{12}-HSL, monofluoro analogue **2.62** retained inhibitory effect on proliferated murine leukocyte cells. Interestingly, distinct drop in immune suppression activity observed with difluoro analogue **2.63** suggesting that minimum one hydrogen at C^2 is must for immune modulation. When exposed to target proteins within the biological system, the C^2 proton(s) of the analogue(s) might be contributing either via *3-enolic* content of the molecule or by binding to the active site via H-bonding in order to show selective immune modulation. Monofluoro analogue also did not show QS effect.

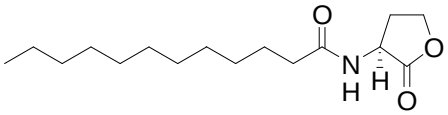
3.1.2.3 Insertion of N at position 3 in 3O, C_{12}-HSL: *3-Aza analogues*

Carbonyl groups present in a drug molecule are often involved in hydrogen bonding and ionic bonding interactions with target receptors. Here carbonyl group acts as hydrogen bond acceptor. Conversion of hydrogen bond acceptor carbonyl group to hydrogen bond donator hydroxyl group, its bulkier congener benzyloxy group or its replacement with lone pair containing amino group would alter the nature of bonding

to the receptor. It had been seen that C^3=O group of parent compound 3O, C_{12}-HSL was essential for immune modulation as analogues lacking C^3=O such as C_{11}-HSL **3.1** and C_{12}-HSL **3.2** were weakly active in murine splenocyte proliferation assay.[131,171]



3.1



3.2

This observation proposes that there exist an interaction between C^3=O group of the active molecule and hydrogen donor group of the receptor. We therefore, decided to further modulate 3-*enolic* content of the lead molecule 3O, C_{12}-HSL by replacing C^3=O functional group with N^3-OH (hydroxamic acid), more dense N^3-OBn and N^3-NH_2 (hydrazine). These replacements resulted in the production of **2.71, 2.77** and **2.72** as shown in **Table 3.3**.

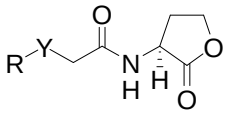


Table 3.3 Immunological results of 3-aza analogues of 3O, C_{12}-HSL

Compound(s)	***R***	***Y***	***EC_{50} (μM)***
1.54 (3O, C_{12}-HSL)	$CH_3(CH_2)_8$-	C=O	4.30-4.50
2.71	$CH_3(CH_2)_8$-	N-OH	13.10
2.77	$CH_3(CH_2)_8$-	N-OBn	1.30
2.72	$CH_3(CH_2)_8$-	N-NH_2	0.999

Murine splenocytes proliferation assay showed that activity was reduced by three folds when free N^3-OH was inserted to produce analogue **2.71** and newly added functional group failed to improve the binding affinity through H-bonding to the active sites of receptors. The hydroxamic acid motif in **2.71** might have lowered its p*Ka* and have slightly increased its polarity than the parent compound. This was further supported by potent immune suppression by analogues **2.77** and **2.72** where N^3 was substituted by lipophilic -OBn and more basic -NH_2 groups respectively. Benzyl group would have balanced appropriate lipophilic content in **2.77**. Moreover, through aromatic interactions, structure in benzyl group might have mapped lager surface of receptor.

Apart from hydrogen bonding, the N^3-NH_2 substitution would have identified new binding interactions through its lone pair electrons with receptor.

These results further suggest that by selective modulation of *3-enolic* content of parent compound 3O, C_{12}-HSL, it is possible to discover new active candidates of improved immune modulatory properties. This denies previous finding that 3-oxo or hydroxyl group is must for immune modulation.[131] Thus suitably substituted compounds specifically with hydrogen bonding donating moiety at C^3 can exhibit better immune modulation. However, further investigations in this direction warranted to support this hypothesis.

3.1.2.4 Insertion of *S* in 3O, C_{12}-HSL

Insertion of sulphur as such or its oxidative forms such as sulfone and sulfoxide or sulphonamide motifs in the medicinal drugs have been achieved successfully to improve their biological responses. Introduction of sulphur in the form of sulfone or sufoxide imparts antibiotic properties and its sulphonamide form provides strength against enzymatic degradation to the otherwise inactive compounds.[210,212,272] It is now rarely possible to find a class of drugs, which does not possess sulphur in their structure.

3.1.2.4.1 3-Thia analogues

In order to investigate SAR of 3-thia (**2.87**-**2.91)** analogues of 3O, C_{12}-HSL, evaluation of inhibition of cell proliferation by them was undertaken in the murine leukocytes. Immune modulatory activity was lost totally in **2.87** where S alone inserted at C^3 in the parent structure 3O, C_{12}-HSL. Expectedly, introduction of oxo group on S^3 of **2.87** gave rise to compound **2.88** having very high potency of immune suppression compared to parent compound 3O, C_{12}-HSL. Almost same level of immune suppression observed when S^3 of **2.87** replaced by sulfone **2.90**. These exciting results are in accordance with the previous statement that 3-oxo group is must for immune modulation. Around 10 folds further increased immune suppressive potency of analogues was observed when alkyl chain length A was increased to C_{11} (**2.89** and **2.91**). The results of 3-thia analogues are summarised in **Table 3.4**.

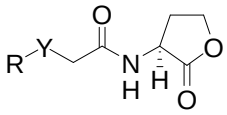


Table 3.4 Immunological results of 3-thia analogues of 3O, C_{12}-HSL

Compound(s)	***R***	***Y***	***EC_{50} (μM)***
1.54 (3O, C_{12}-HSL)	$CH_3(CH_2)_8$-	C=O	4.30-4.50
2.87	$CH_3(CH_2)_8$-	S	Inactive
2.88	$CH_3(CH_2)_8$-	S=O	0.547
2.89	$CH_3(CH_2)_{10}$-	S=O	0.152
2.90	$CH_3(CH_2)_8$-	SO_2	0.932
2.91	$CH_3(CH_2)_{10}$-	SO_2	0.450

Moreover, retention of immune modulation by sulfone analogues (**2.90**-**2.91**) suggests that the immunophilin receptors possibly possess considerably bigger size pocket having more interacting sites to accommodate larger size functional groups like sulfoxide and sulfone.

3.1.2.4.2 ***Sulphonamide motif containing analogues***: Analogues **2.91** and **2.92**

In medicinal chemistry, it is common approach to incorporate several defined bioactive structural units within single molecule to achieve a more potent pharmacophore.[273] We successfully employed this concept where active constituents from 4-aza and thia analogues were utilized in the generation of potent sulphonamide derivatives **2.91** and **2.92** as shown in **Table 3.5**.

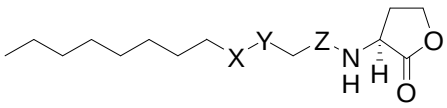


Table 3.5 Immunological results of 3O, C_{12}-HSL analogues with sulphonamide motif

Compound(s)	***X***	***Y***	***Z***	***EC_{50} (μM)***
1.54 (3O, C_{12}-HSL)	NH	C=O	C=O	4.30-4.50
2.91	NH	C=O	SO_2	9.10
2.92	NH	SO_2	C=O	2.00

Interestingly, slightly less immune suppression was obtained with analogue **2.91** where C^1=O was replaced by S^1=O to generate S^1(O)NH whereas intense immune suppression was observed with **2.92** in which S^3(O)N^4H was generated by replacing C^3=O by S^3=O followed by insertion of N^4. This favours our belief that modulation of *3-enolic* content of 3O, C_{12}-HSL is more important than modulating C^1=O function. Furthermore, in microbiological assay these S containing analogues were found to be non-quorum sensing.

3.1.2.5 Replacement of L-HSL by other hetero ring

In previous studies, it was found that homoserine ring of 3O, C_{12}-HSL was essential for immune modulation activity.[131,156] This was further strengthened by the observation that the ring opened derivative was devoid of any activity.[171,221] However, this finding was based on immune assays of very few analogues of the altered hetero ring and thus they were relatively less explored. Hence, one of the aims of this project was to re-evaluate importance of L-HSL ring of 3O, C_{12}-HSL in eukaryotic immune modulation. To address this, we synthesized new analogues in which 3-oxo-C_{12} chain was retained and other groups replaced the L-HSL ring.

3.1.2.5.1 Hetero ring altered analogues

Introduction of additional heteroatom as well as expansion of active constituents in the drug molecule can bring about changes in its physiochemical properties.[274] Consequently, a series of hetero ring altered analogues **2.110**-**2.112** was developed where the hetero atom *O* of the L-HSL ring is replaced with N, additional C=O group is inserted and ring is expanded. A non-chiral thiazole ring replaces the L-HSL. As shown in **Table 3.6** incorporation of N, additional C=O group and ring expansion by one carbon unit in the hetero ring altered analogues demonstrated complete loss of immune suppression activity indicating that receptor must be having very specific pocket to interact with C trait (**ref. Fig 2.4)** of 3O, C_{12}-HSL. Surprisingly, analogue with thiazole ring **2.112** retained equipotent immune suppression as that of parent 3O, C_{12}-HSL.

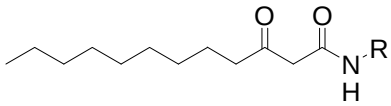


Table 3.6 Immunological results of hetero ring altered analogues of 3O, C_{12}-HSL

Compound(s)	***R***	***EC_{50} (µM)***
1.54 (3O, C_{12}-HSL)		4.30-4.50
2.110		Inactive
2.111		Inactive
2.112		12.50

This is not only contradictory to the previous finding that HSL ring and its chirality are important for immune suppression, but also opened an avenues for the development of new series of immune modulatory agents. The dose-response effect of **2.112** on the murine cell proliferation is as shown in **Fig 3.3**.

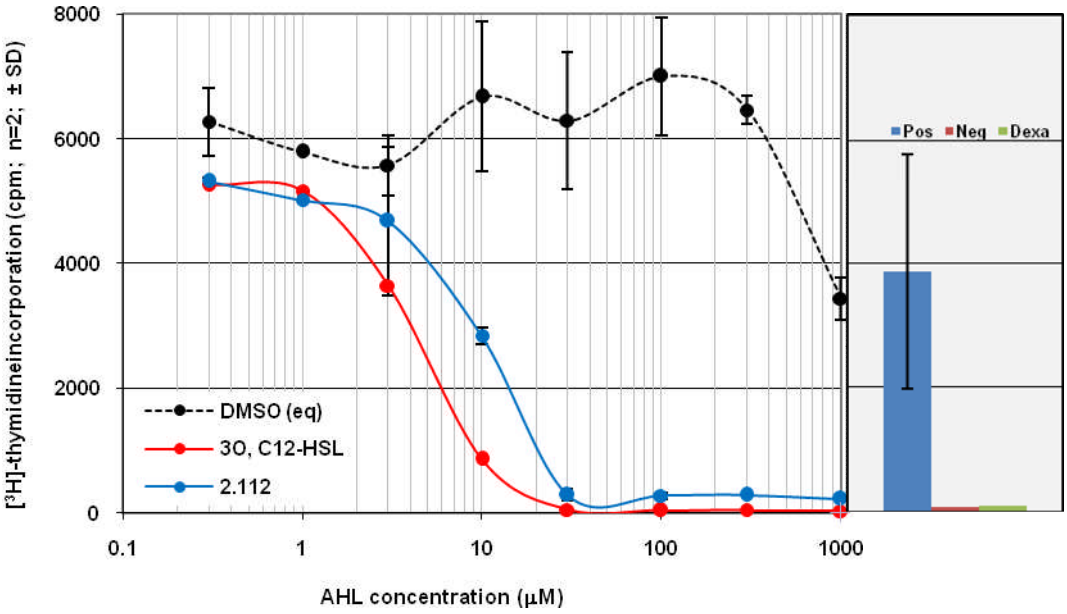


Fig- 3.3 Dose-response curves of DMSO, **2.112** and 3O, C_{12}-HSL on ConA stimulated murine leukocytes proliferation

Moreover, these analogues proved to be non-inducers of QS and very good antagonists.

3.1.2.5.2 Hetero ring altered analogues of 3O, 4-aza-C_{12}-HSL

Immunosuppressive results obtained with analogue **2.112** prompted us to further optimise immunomodulatory potential of existing 3O, 4-aza-C_{12}-HSL **2.8**. This will further explore whether 5-membered ring with a keto or hydroxyl group (or other hydrogen bond acceptor) adjacent to the amine functionality is essential structural requirement for binding and activation of immune receptors. Accordingly, types of cyclic structure that can be constructed are 5- or 6 membered mono or fused rings; (i) where amino and hydroxyl groups are superimposable with amino and C=O groups in HSL e.g. 2-aminocyclopentanol, 2-aminocyclohexanol, 2-aminophenols, 2-aminobenzamides etc., (ii) with amino group but without adjacent hydroxyl (or keto) group e.g., amino cycloheptanol, 2-aminothiazole, 2-aminopyridine, etc., and (iii) with more complex structure e.g., 5-aminoindazole. We thus decided to explore immune modulation of **2.8** by replacing its L-HSL ring by a series of other rings and developed hetero ring altered analogues of 3O, 4-aza-C_{12}-HSL. Interestingly, all these analogues when assayed for their inhibitory effects on ConA stimulated proliferative murine leukocytes showed immune inhibition. Inhibitory effects of few active molecules are as shown in **Fig. 3.4** and **Fig 3.5**.

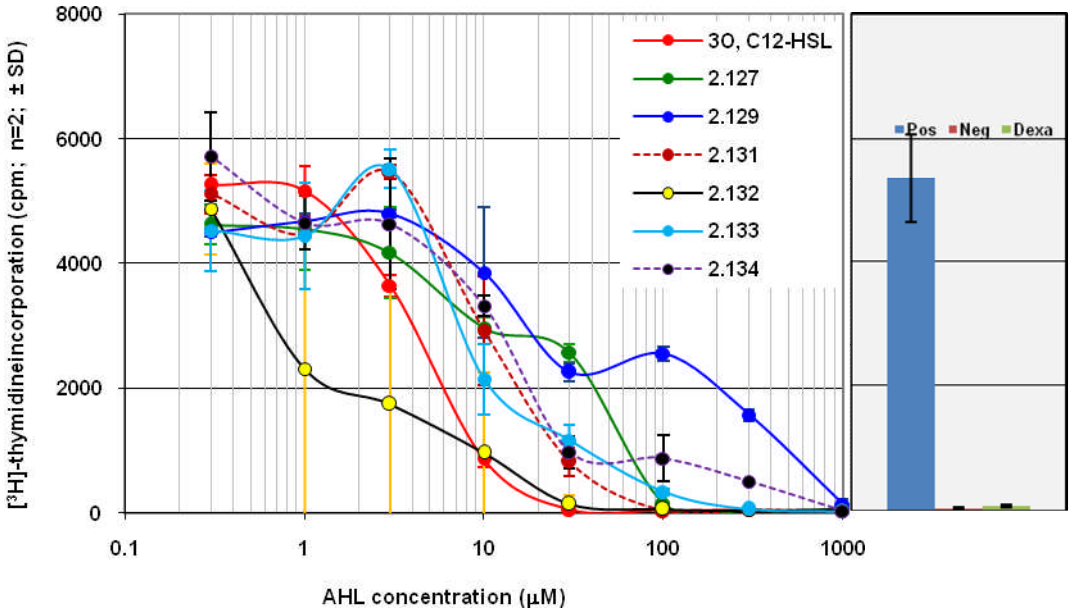


Fig- 3.4 Dose-response curves of hetero ring altered 4-aza analogues and 3O, C_{12}-HSL on ConA stimulated murine leukocytes proliferation.

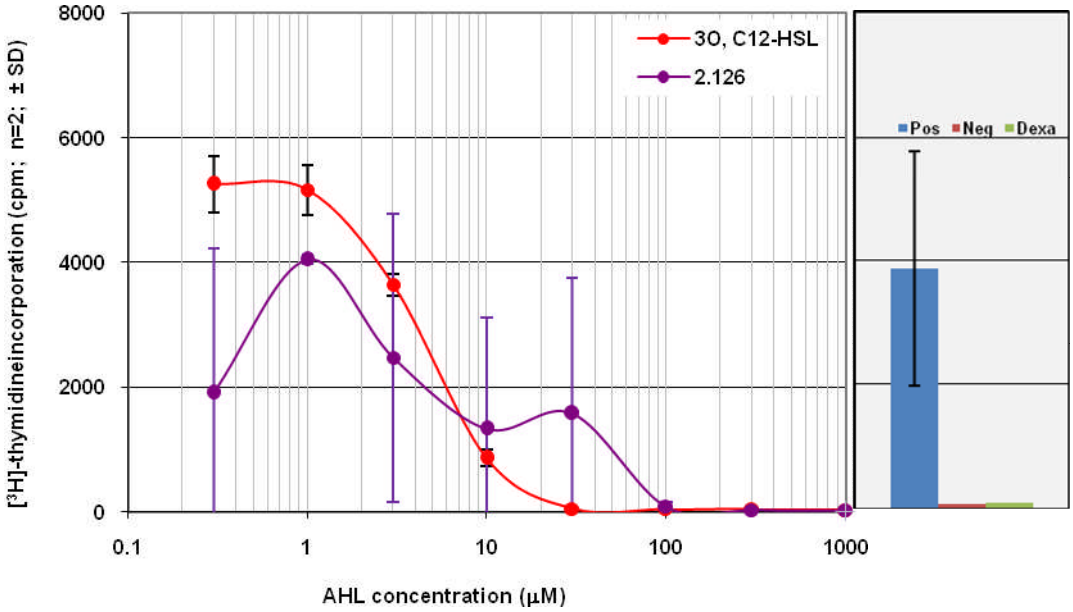


Fig- 3.5 Dose-response curves of **2.126** and 3O, C_{12}-HSL on ConA stimulated murine leukocytes proliferation.

As expected, insertion of thiazole hetero ring **2.125** profoundly increased immune suppressive potential even more than **2.112** and 3O, C_{12}-HSL. This along with immune suppression by **2.128** (cycloheptyl) and **2.134** (2-pyridinyl) further indicate that replacement of L-HSL with other suitable ring without containing keto group (C=O) retains immune suppression as shown in **Table 3.7**. As mentioned above, we initially assumed that since 2-aminocyclopentanol (**2.126**), 2-aminocyclohexanol (**2.127**) and 2-aminophenol (**2.129**) have hydroxyl group, they would better mimic L-HSL ring analogy. In fact immunomodulatory activity was retained but at considerably larger doses than the parent 3O, C_{12}-HSL. This shows the serious participation of ketone in hydrogen bonding and presumably, hydroxyl substitution seems to maintain less efficient binding with receptors. The most intriguing observation was that a subtle structural change from 2-aminophenol (**2.129**) to 2-amino-4-cholorophenol (**2.130**) drastically increased immunosuppressive activity. It is unlikely that such a small structural difference would completely influence binding to the respective receptors. A more likely, possible explanation is that 2-aminocyclopentanol (**2.126**), 2-aminocyclohexanol (**2.127**) and 2-aminophenol (**2.129**) compounds can still bind immunophilin receptors with similar affinity but are unable to activate them.

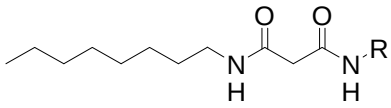
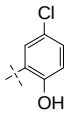
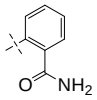
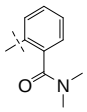
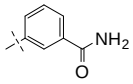
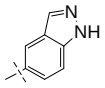


Table 3.7 Immunological results of hetero ring altered 4-aza-3O, C_{12}-HSL

Compound(s)	***R***	***EC_{50} (μM)***
1.54 (3O, C_{12}-HSL)		4.30-4.50
2.125		2.40
2.126		18.80
2.127		26.10
2.128		2.70
2.129		20.00
2.130		2.50
2.131		12.00
2.132		0.70
2.133		9.40
2.134		11.70
2.135		6.27

Among these analogues, **2.129-2.133** and **2.130** are aniline derivatives. This set of molecules also has an *ortho*- or *meta*- substituent of a hydroxyl, carboxyamide, or pyridyl group, which can act as H bond acceptors. The position of these substituents seems important, and depends on the particular substituent i.e., *ortho* for hydroxyl or pyridyl and *ortho*/*meta* for carboxyamide. We speculate that the hydroxyl in **2.129** and **2.130**, carbonyl group in **2.131, 2.132** and **2.133** as well as the pyridyl group in **2.134** maintains the putative hydrogen bond interaction observed with receptors. In case of the *ortho* and *meta* carboxamide substituents almost similar potency was retained however, *ortho* substitution with more lipophilic dimethyl carboxamide group, greatly

increased (5-folds) immune suppression by **2.132**. We therefore, conclude that the combination of an aniline structure and hydrogen bond acceptor dictates the immune suppression by this set of molecules. We also identified analogue **2.135** as equipotent as parent 3O, C_{12}-HSL molecule. To our surprise this does not has obvious structural connection with the aniline or other analogues discussed above.

3.1.2.6 Insertion of *O* in 3O, C_{12}-HSL: *Oxa analogues*

Introduction of hetero atom *O* in the medicinal compounds has been widely favored to modify their biological and physical characteristics. Being larger that carbon, oxygen can provide an additional site(s) not only to interact with target proteins but also to form hydrogen bonds with solvent molecules or biological fluids. As discussed already by Chhabra et al., that lipophilicity is must for immune modulation. However, more lipophilic analogues exhibited solubility issue.[131] We envisioned addressing this issue by introducing hetero oxygen(s) within the structure of 3O, C_{12}-HSL not to disrupt its lipophilicity but to maintain proper hydrophilic-lipophilic balance. Moreover, it can also modulate *3-enolic* content of parent structure. Introduction of oxygen was successfully achieved with the generation of analogues **2.139-2.143** as shown below in **Table 3.8**.

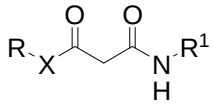


Table 3.8 Immunological results of oxa-analogues of 3O, C_{12}-HSL

Compound(s)	***R***	***X***	***R*^{1}**	***EC*$_{50}$ *(μM)***
1.54 (3O, C_{12}-HSL)	$CH_3(CH_2)_7$-	CH_2		4.30-4.50
2.139	$CH_3(CH_2)_7$-	O		4.80
2.140	$CH_3(CH_2)_3O(CH_2)_3$-	NH		200.00
2.141	$CH_3(CH_2)_3O(CH_2)_3$-	NH		154.00
2.142	$CH_3CH_2O(CH_2)_2O(CH_2)_2$-	NH		240.00
2.143	$CH_3CH_2O(CH_2)_2O(CH_2)_2$-	NH		47.80

Murine splenocyte proliferation assays of these analogues demonstrated the importance of lipophilicity in exhibiting biological effects. The oxygen insertion next to C^3=O was well tolerated as 4-oxa analogue **2.139** retained equipotent activity as that of parent compound. Immune suppression activity was profoundly dropped down when hetero oxygen was positioned 3 carbons away from C^3=O as can be seen in analogues **2.140** and **2.141**. Moreover, insertion of two additional oxygens also resulted in almost inactive analogues **2.142** and **2.143**. Overall, oxygen introduction greatly enhanced hydrophobicity of the resultant molecules without enhancing immune modulation. The dose-response curves of **2.140** and **2.143** are compared with parent 3O, C_{12}-HSL in **Fig 3.6.** However, these molecules did not show QS behaviour.

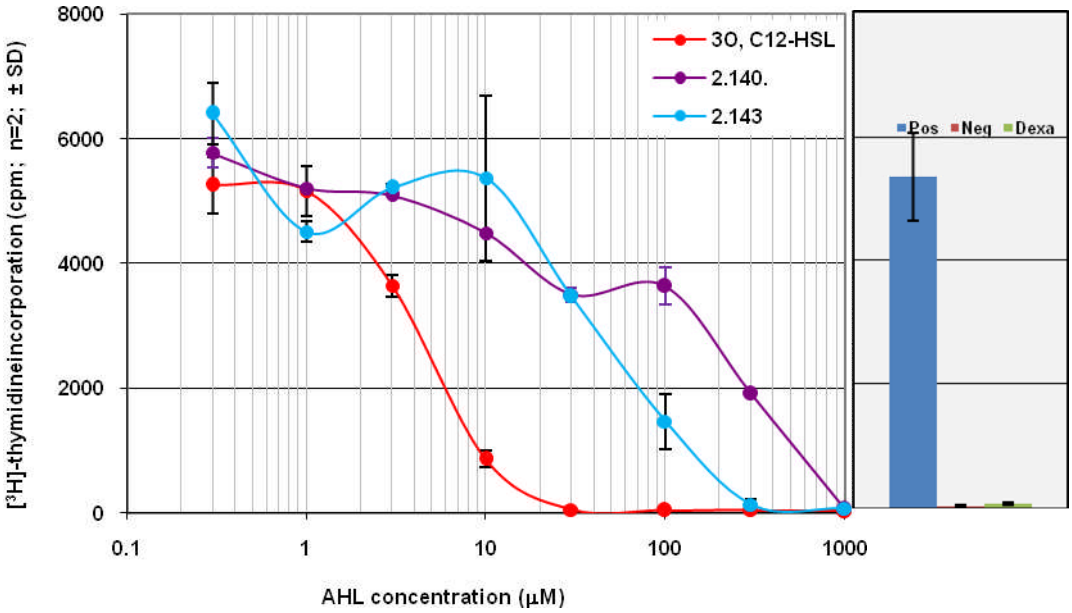


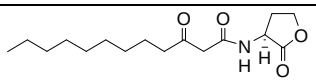
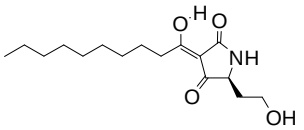
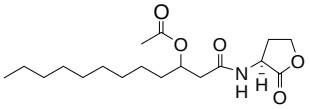
Fig- 3.6 Dose-response curves of Oxa analogues and 3O, C_{12}-HSL on ConA stimulated murine leukocytes proliferation.

3.1.2.7 Miscellaneous analogues: *Tetramic acid motif containing analogue of 3O, C_{12}-HSL and 3-acetoxy, C_{12}-HSL*

The previously undescribed non-enzymatically formed product tetramic acid **2.152** was discovered by kauffmaun et al. Because this is non-enzymatically degraded product of parent compound, it was of interest to verify whether immune modulation by parent 3O, C_{12}-HSL is exhibited via formation of this tetramic acid analogue. We

therefore considered to verify if **2.152** displays immune modulatory activity.[249] Also acetylation of hydroxyl groups is a frequently used protection in medicinal chemistry to protect hydroxyl group from conversion by enzymes *in vivo* or chemical reactions *in vitro* to other undesired functional group.[250,251] It can also modulate 3-keto group of parent compound. We designed and synthesized **2.152** and **2.154** and checked them for their immune suppressive behaviors as shown in **Table 3.9**

Table 3.9 Immunological results of tetramic acid motif containing and 3-*O*-acetyl analogues of 3O, C_{12}-HSL

Compound(s)	***Structure***	***EC_{50} (µM)***
1.54 (3O, C_{12}-HSL)		4.30-4.50
2.152		5.30
2.154		0.520

It is noteworthy to mention here that tetramic acid analogue **2.152** demonstrated equipotent immune suppression behaviour as that of 3O, C_{12}-HSL. Whereas, 3*O*-acetylation on the other hand incredibly increased 10 times more immune suppression than the parent compound once again indicating the importance of C^3=O functionality. The both analogues also found to be non-QS.

3.2 Cytotoxicity assay

It is necessary to assess the cytotoxic effects of all the newly developed analogues of 3O, C_{12}-HSL before further *in vitro* tests will be carried out. The cytotoxicity data are useful information (i) for the prediction of influence of compounds on the cell proliferation in the murine splenocyte assay and (ii) for the exploitation of the molecules as drug candidate. Ideally, result of the cytotoxicity assay is for a compound to be non-toxic (NT) but if this is not the case; a large gap also called therapeutic window exists between the EC_{50} and the concentration at which the compound is toxic. If the EC_{50} and cytotoxicity concentration are very close, there are more possibilities of toxic effects being observed when compound is used as drug candidate.

The cytotoxicity can be quantitated *in vitro* by several methods e.g. *Trypan* blue exclusion, ^{51}Cr release, inhibition of colony formation and lactate dehydrogenase (LDH) release.[275] One of the earliest means of assessing cell damage was the uptake of trypan blue by dead cells.[276] This technique is still widely used because it is simple, rapid, involves little manipulation of cells and advantageous over LDH release. We therefore, employed *Trypan blue exclusion assay* (see **Appendix-II**) technique to measure cytotoxicity. In this murine spleen cells were incubated with 3O, C_{12}-HSL analogues for 24 h at 37 °C in 5 % CO_2/air. The 0.4 % Trypan blue dye which is incorporated in to dead cells leaving viable cells unstained was then added to cells. The viable cells were then counted with a counting chamber.

Sometimes, toxicity reports are expressed as 'cytostatic' (S), concentration of compounds sufficient to stop cell growth but do not kill them. The toxicity concentrations of solvent, parent compound 3O C_{12}-HSL and few analogues required to kill viable cells were extrapolated from the graph between viable cell counts and concentration in µM as shown in **Fig 3.7** and **Fig 3.8**

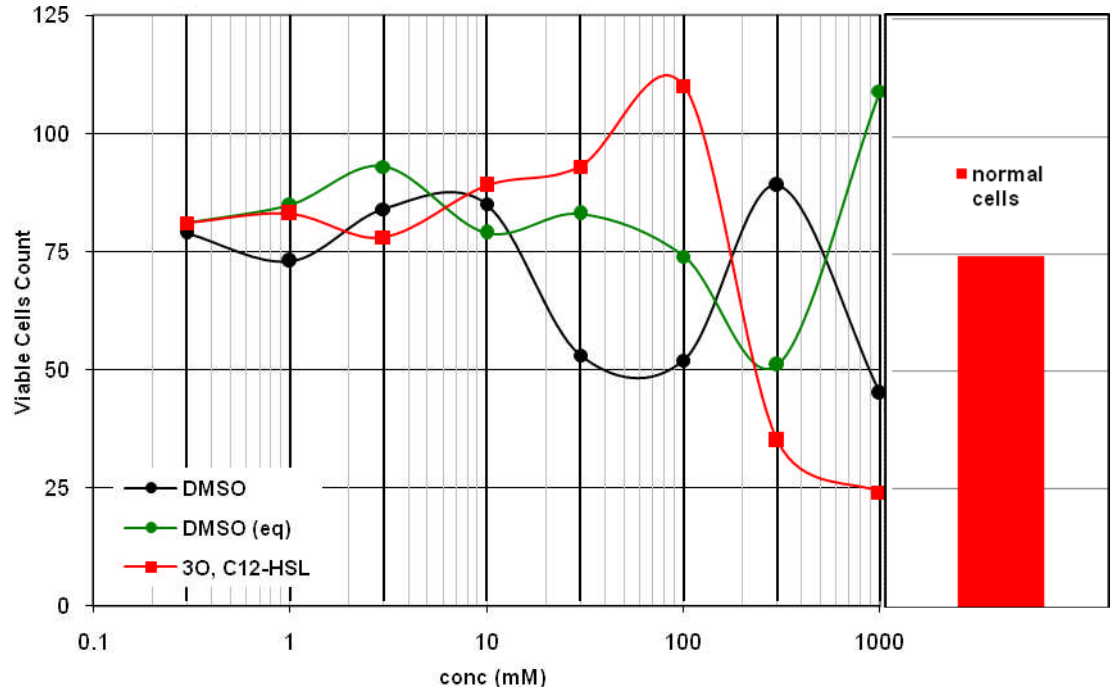


Fig 3.7 Toxicity analysis of solvent DMSO and 3O, C_{12}-HSL

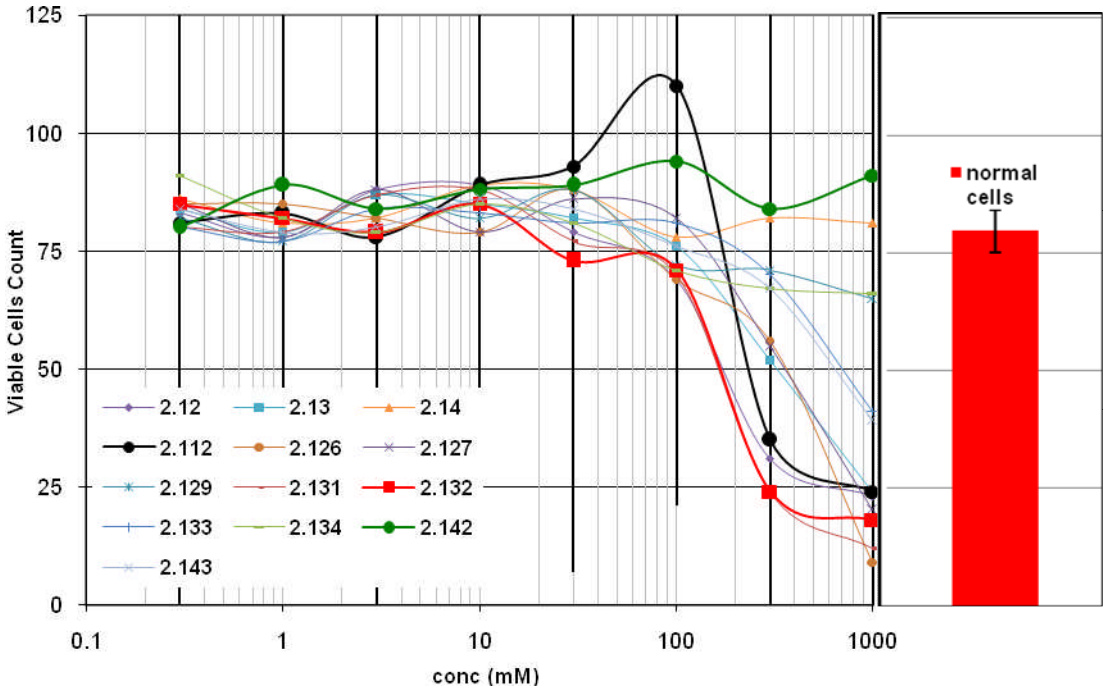


Fig 3.8 Toxicity analysis of 3O, C_{12}-HSL analogues

The results of analogues using this assay are depicted in **Table 3.10** and compared with their EC_{50} values.

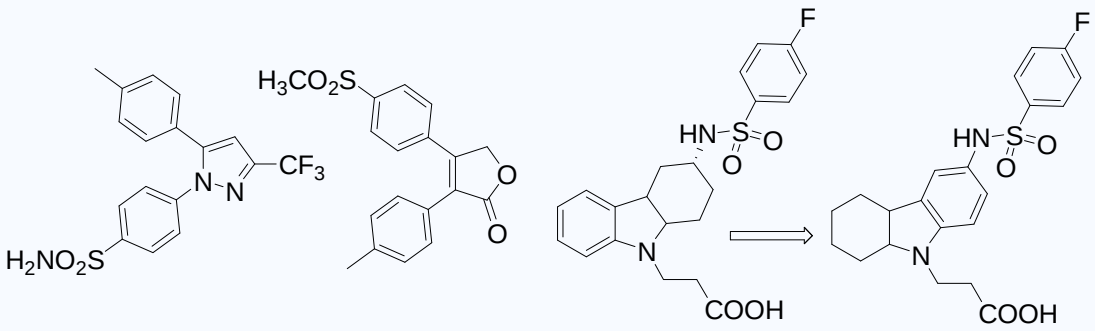
Table 3.10 Toxicity data for DMSO, DMSO(eq), 3O, C_{12}-HSL and its analogues.

Compound(s)	*EC_{50} (μM)*	*Toxicity (μM)*
1.54 (3O, C_{12}-HSL)	4.30-4.50	250
DMSO	-	30
DMSO (eq)	-	300S
2.8	15.10	200
2.9	2.90	530
2.10	0.652	140
2.11	6.11	>1000
2.12	2.90	200
2.13	17.10	300
2.62	21.40	800
2.63	82.30	>1000
2.71	13.10	400
2.72	0.999	410
2.77	1.30	63
2.87	Not active	>1000
2.88	0.547	720
2.89	0.152	45
2.90	0.932	200
2.91	0.450	170
2.92	9.10	220
2.93	2.00	900
2.110	Not active	*
2.111	Not active	*
2.112	12.50	800
2.125	2.40	600
2.126	18.80	400
2.127	26.10	400
2.128	2.70	78
2.129	21.00	>1000
2.130	2.50	>1000
2.131	12.00	120
2.132	0.70	160
2.133	9.40	700
2.134	11.70	>1000
2.135	6.27	>1000
2.139	4.80	500
2.140	200.00	>1000
2.141	154.00	>1000
2.142	240.30	>1000
2.143	47.80	700
2.152	5.30	52
2.154	0.520	120

*S-cytostatic effect; *-activity not performed*

3.3 Microbiology (*Quorum-sensing induction and antagonism*)

Manipulation of dual biological functional molecules to block one effect over the other is most widely accepted approach in the medicinal chemistry. For example, celecoxib **3.3** and rofecoxib **3.4** are non-steroidal anti-inflammatory drugs (NSAIDs) but directly target COX-2, an enzyme responsible for inflammation and pain. Selectivity for COX-2 reduces the risk of peptic ulceration.[277] Robarge et al. recently synthesized a ramatroban isostere **3.6** that is a selective and potent antagonist of the chemo-attractant receptor-homologous molecule expressed on T_H^2 cells (CRTH-2). Ramatroban **3.5** is marketed in Japan for allergic rhinitis as a selective thromboxanetype prostanoid receptor (TP) antagonist. However, recently ramatroban also identified as a CRTH-2 antagonist. [278]



3.3 **3.4** **3.5** **3.6 (**Isostere**)**

As noted earlier *Pseudomonas aeruginosa* signal molecule 3O, C_{12}-HSL has dual biological function in that it imparts QS activity and also modulates host's immune system.[128,131] Developing selective immune modulatory 3O, C_{12}-HSL analogues which were devoid of QS behaviour was the main objective of our research. Since newly discovered analogues shared some structural similarities with the parent 3O, C_{12}-HSL molecule therefore, it was of interest to see if these analogues also exhibited auto-induction or QS phenomenon in *Pseudomonas aeruginosa*. In case, no auto-induction was observed, these analogues could be used as safe immune modulators. To verify this, we investigated agonist and antagonist activities of these analogues.

Auto-induction or QS activity can be measured by the ability of the compound to induce bioluminescence in a specifically designed bacterial bio-reporter system. Here

analogues were tested for their ability to induce bioluminescence in *Pseudomonas aeruginosa* reporter strain, PAO1(Δ*las*I-P*lasB*::*lux*)[279] where QS system can be exogenously activated by the addition of the synthetic 3O, C_{12}-HSL or its analogues. Values are expressed as a percentage of the bioluminescence measured after exposure of the bacterial reporter cells to the analogues for 5 h at 37 °C relative to positive control 3O, C_{12}-HSL (100%).

3.3.1 Screening of analogues for auto-induction (agonist) activity

The ability of the signal molecule to activate LasR was determined by following the luminescence of the *Pseudomonas aeruginosa* PAO1 harbouring in to chromosomal transcriptional fusion Δ*lasI*-P*lasB*::*lux*. Since this biosensor strain lacks the ability to produce natural auto-inducer 3O, C_{12}-HSL due to the disruption of *LasI* synthase genes, activation of LasR relies on the addition of exogenous signal molecule 3O, C_{12}-HSL. Thus, maximum expression of bioluminescence by exogenous addition of a range of 3O, C_{12}-HSL concentrations was used as a marker to determine its optimal concentration required for maximum bacterial growth to be observed as shown in **Fig. 3.8**.

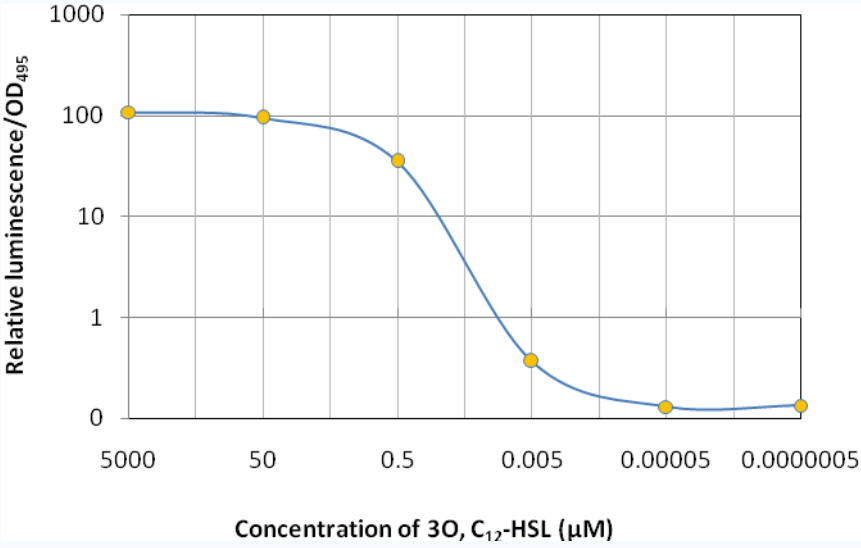


Fig.3.8 Optimum concentration of 3O, C_{12}-HSL (5 µM) required for maximum growth of *Pseudomonas aruginosa*

Screening of individual molecules for their ability to activate LasR was conducted using 3O, C_{12}-HSL as a **positive control** and the reporter strain (Δ*lasI*-P*lasB*::*lux*) as a **control**. The results are as shown in **Fig 3.9**.

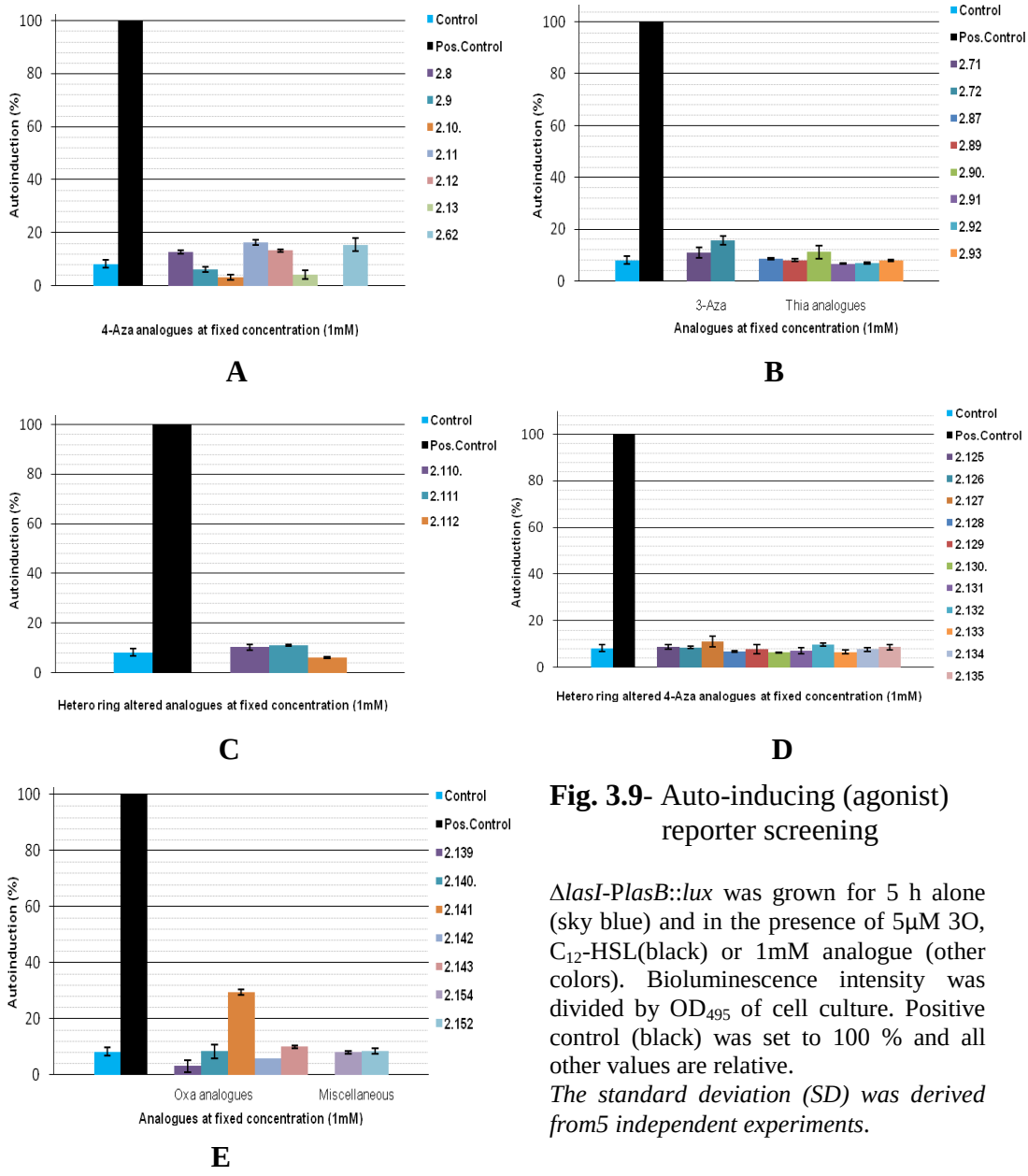


Fig. 3.9- Auto-inducing (agonist) reporter screening

Δ*lasI*-P*lasB*::*lux* was grown for 5 h alone (sky blue) and in the presence of 5µM 3O, C_{12}-HSL(black) or 1mM analogue (other colors). Bioluminescence intensity was divided by OD_{495} of cell culture. Positive control (black) was set to 100 % and all other values are relative.
The standard deviation (SD) was derived from5 independent experiments.

Surprisingly, out of 39 new analogues tested only (*1R,2S*)-2-(3-oxo-8-oxa-4-azadodecanoyl)aminocyclopentanol **2.141** (**Fig 3.9-E**) displayed partial auto-inducing activity whereas, all other analogues showed no auto-induction. Notably, **2.141** also exhibited very weak immune suppression activity. Non-inducing results are quite encouraging and possible explaination for this behaviour is that they still bind LasR but not with appropriate affinity required to activate it. Therefore, we wonder that

these compounds might act as QS antagonists. Thus these compouns were taken forward for antagonist assays.

3.3.2 Antagonist assays

Based on the above hypothesis, we extended our efforts to perform competition assay of new analogues using same reporter strain ((Δ*lasI*-P*lasB*::*lux*). The results were calculated as % inhibition of relative bioluminescence (QS) by an analogue compared to the parent 3O, C_{12}-HSL (**positive control**) and cell culture (**control**). The antagonistic activities of analogues are shown below.

3.3.2.1 Antagonistic behavoir of 4-aza, fluorinated 4-aza and 3-aza analogues

As shown in **Fig 3.10**, 4-aza analogues **2.8**, **2.9** and **2.10** showed potent QS inhibition to about 20 %, 5 % and 15 % respectively at 20-fold excess concentration over 3O, C_{12}-HSL. The N^4-alkyl subtituted analogues **2.11** and **2.12** showed lesser inhibitory acitivity (40 % and 24 % reduction respectiviely) under the same conditions. However, polar hydroxylethyl substitution at N^4 **2.13** exhibited potent inhibition to 20 %. 2-Monofluoro analogue **2.62** also retained antagonistic behaviour. Since it is non-immune suppressive, difluoro analogue **2.63** was not assayed.

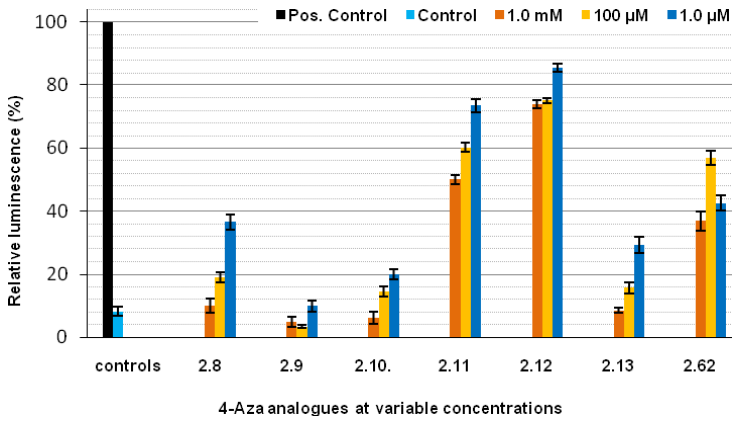


Fig. 3.10 Antagonist activity of 4-aza and fluoro-4-aza analogues
Black bar (positive control) represents expression of gene proteins in presence of 5 µM 3O, C_{12}-HSL. *Sky blue* bar (control) represents background expression in absence of 3O, C_{12}-HSL or any analogs. *Orange, yellow and blue bars*; gene proteins expression in presence of 5 µM of 3O, C_{12}-HSL and 1.0 mM, 100 µM and 1.0 µM of analogues. *The standard deviation (SD) was derived from 5 independent experiments.*

Similarly, the significant activity was exihibited by 3-aza analogues at 100 µM concentrations as shown in **Fig 3.10**.

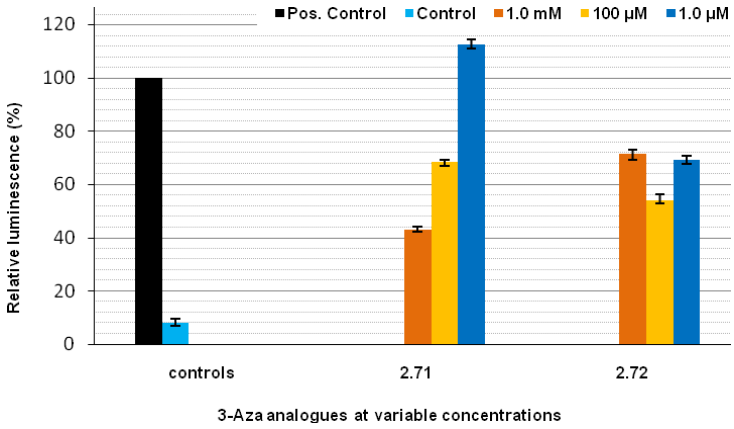


Fig. 3.10 Antagonist activity of 3-aza analogues

3.3.2.2 Antagonistic behavoir of 3-Thia and sulfonamide motifs containing analogues

Among 3-thia analogues, only **2.87** and sulfoxide **2.89** retained inhibition The sulfone **2.90** showed QS inhibition at high magnitude whereas with increased carbon chain in **2.91** inhibitory potential was dropped down to certain extent as shown in **Fig 3.11**. Moreover, sulfonamide analogues **2.92** and **2.93** showed partial antagonistic activity, presumably because of steric hinderence possibly by large atomic size of sulphur.

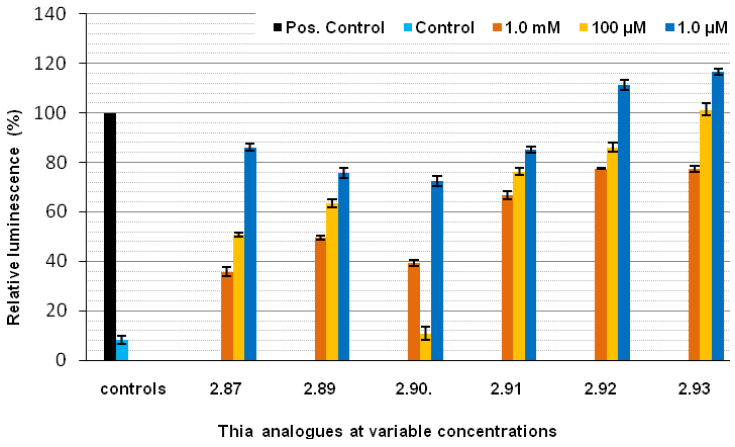


Fig. 3.11 Antagonist activity of Thia-analogues

3.3.2.3 Antagonism by hetero ring altered analogues of 3O, C_{12}-HSL and 4-aza 3O, C_{12}-HSL

Replacement of L-HSL ring of parent compound with aspartimide **2.110**, glutarimide **2.111** and 2-aminothiazole **2.112** heterocycles gave rise to considerable QS inhibition. Among these, **2.112** was found to be most potent one. In **2.110** and **2.111** position of additional carbonyl group and presence of sulphur and nitrogen in **2.112** seems to maintain putative H bond intractions similar to those osevrved between TraRTrp57 and the *A. tumefaciens* autoinducer.[280] Results are shown below in **Table 3.12**.

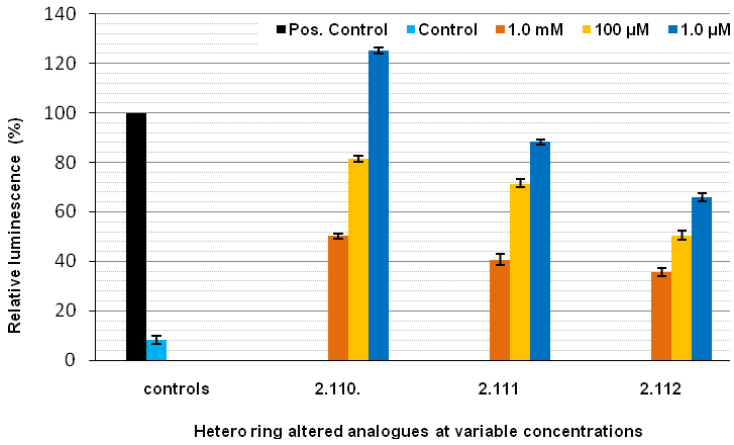


Fig. 3.12 Antagonist activity of hetero ring altered analogues

In case of hetero ring altered 4-aza analogues **2.125-2.135** as expected we identified eight compounds that inhibited more than or almost 50 % QS. As shown in **Table 3.13** analogues **2.128, 2.129, 2.131** and **2.132** are most potent antagonists.

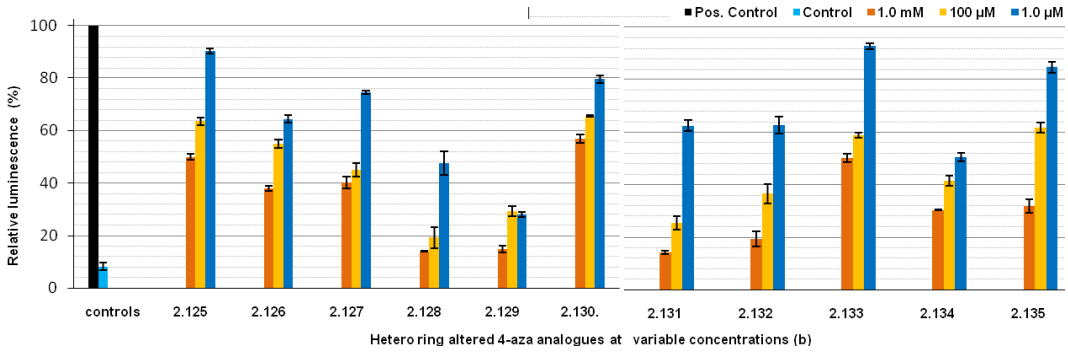


Fig. 3.13 Antagonist activity of hetero ring altered 4-aza analogues

Among these are aniline derivatives **2.129-2.133** and **2.130** with substitution of hydroxyl, carboxyamide, or pyridyl group, in the *ortho* or *meta* position that can act as H bond acceptors. The position of substituents is also important; carboxyamide substituent at *meta* position **2.133** retained considerably lesser activity than *ortho* substituted **2.131-2.132**. Similarly introduction of an electronegative Cl in the *para* position in **2.129** gave rise to **2.130** with 2-fold drop in activity. Possibly hydroxyl, carbonyl and the pyridyl groups might be involved to maintain putative hydrogen bond interaction with receptor proteins. We also analyzed structurally dissimilar compound **2.135** and it was observed to possess antagonist activity.

3.3.2.4 Antagonisic behaviour of Oxa-analogues and miscellaneous analogues

The 4-oxa analogue **2.140** was found to be as potent antagonist whereas, compounds with introduction of oxa additional group in the side chain (**2.140, 2.141** and **2.143**) were deprived of activity. However, di-oxa analogue of 4-aza-C_{12}-HSL retained inhibitory activity.

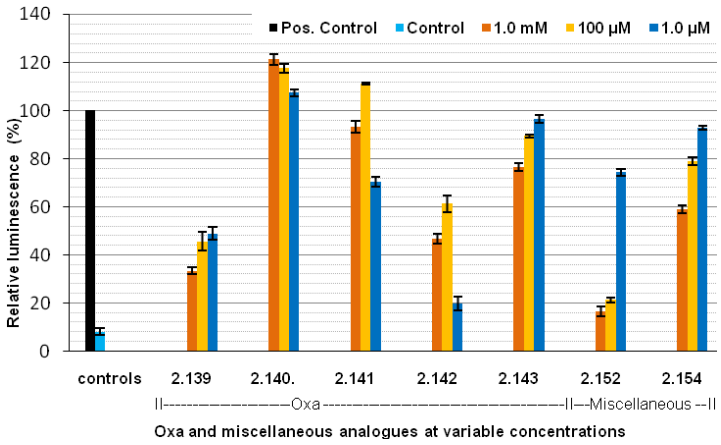


Fig. 3.14 Antagonist activity of oxa and miscellaneous (*3-O-acetyl and tetramic acid*) analogues

As predicted tetramic acid analogue **2.152** was also found to be most potent QS inhibitor. Acetoxy derivative **2.154** retained weak antagonist behaviour.

4.0 SUMMARY, DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary

In the present study we have successfully developed a number of novel synthetic analogues of 3O, C_{12}-HSL, which retain immunological modulation in the absence of any deleterious bacterial growth. For this 3O, C_{12}-HSL molecule was divided in to three regions A, B and C (**Fig 2.4**).

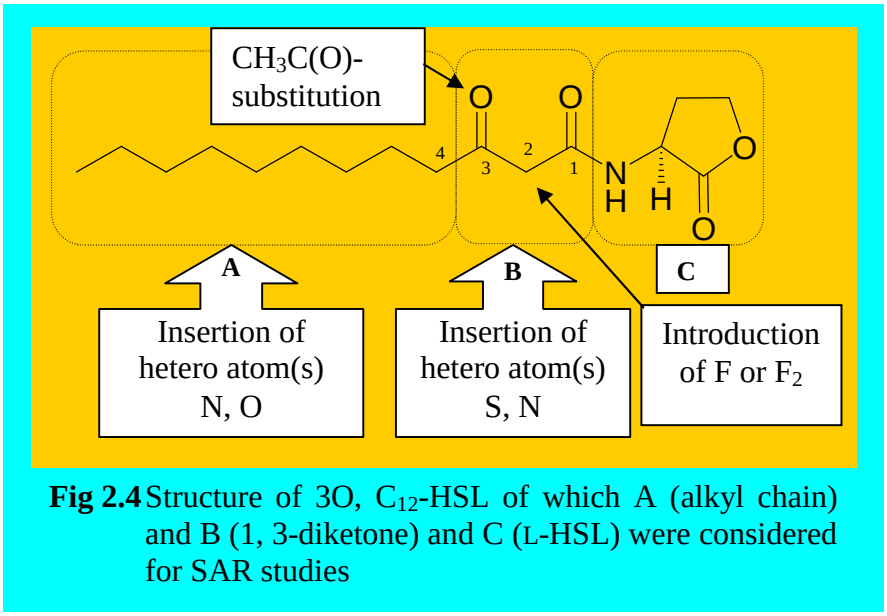


Fig 2.4 Structure of 3O, C_{12}-HSL of which A (alkyl chain) and B (1, 3-diketone) and C (L-HSL) were considered for SAR studies

Chhabra and coworkers have already explored the SAR of this molecule by synthesizing a number of analogues and showed that 3-oxo functionality of this molecule is important for its immunomodulatory activity.[131] We wondered whether the activity was elicited through 3-oxo structure or its *enolic* form. We thus, decided to extend investigation of this molecule to modulate its *3-enolic* content (**Fig 2.4**) by introducing hetero atom(s) in its structure at C^1, C^3 and/or C^4 individually or in combinations. Alternatively, to modulate *3-enolic* content 3O, C_{12}-HSL was reduced to hydroxyl group first and then acetylated. We also replaced C^2 hydrogen(s) by fluorine(s) and L-HSL ring by other aromatic or hetero aromatic ring with or without substitution. In order to enhance hydrophilicity we introduced oxygen(s) at C^7 position individually or C^8 and C^{10} positions within the alkyl chain. Finally, 3O, C_{12}-HSL was transformed using strong base to a tetramic acid derivative via an intra-molecular

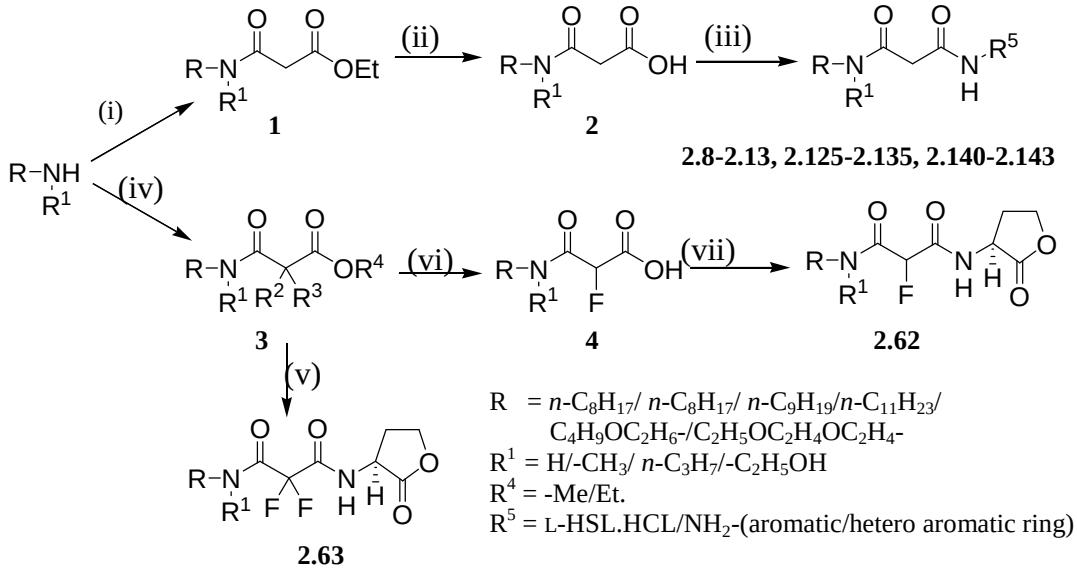
cyclisation. The purpose of these alterations was to establish the optimal A and B regions which could (a) alleviate problems associated with QSSM activity without losing immunomodulatory activity and solubility of the molecule so that longer chain length can be accommodated in the A region of the molecule (b) establish importance of C^3=O group and L-HSL ring in immune modulation.

Modifications of biologically active lead molecule by introduction of hetero atoms such as nitrogen, oxygen, sulfur and the electronegative atom fluorine is common for drug development. These strategies alter molecular properties and thus, biological activity. We envisioned that presence of nitrogen in 4-Aza[175] compounds (**2.8-2.13, 2.125-2.135, 2.140-2.143** and **2.62-2.63**) as well as oxygen in 4-oxa[245,246] compound (**2.139**) might provide an additional site for target proteins to form hydrogen bonding and thus would show potent biological response. Also replacing oxidative 2-CH_2 group of 3-oxoamide compound by –CHF or –CF_2 in **2.62** and **2.63** might block oxidative liver enzymatic degradation of 2-CH_2 site without affecting its target protein binding.[184,185] In 3-aza compounds N^3-substitution with the hydroxyl (compound **2.72**) would be expected to increase the strength of H-bond with the receptor whereas, N^3-amino substitution (compound **2.73**) would explore receptor to identify new binding interactions and may render the molecule metabolically more stable to enzymatic degradation.[191] Replacement of L-HSL of 3O, C_{12}-HSL (compounds **2.110-2.112**) and 4-aza-C_{12}-HSL (compounds **2.125-2.135** and **2.140-2.143**) by aromatic or hetero-aromatic ring would not only explore receptor to identify new binding interactions but also verify the importance of L-HSL ring in immune modulation.[172] These in turn may result in potentiation of immune modulation. Moreover, insertion of additional oxygen(s) in the side chain (analogue **2.140-2.143**) might improve hydrophilic-lipophilic balance. The substantial structural alterations planned and availability of most of starting materials made us to use rather diverse synthetic schemes as summarized below.

4.1.1 4-aza, fluorinated-4-aza 7,10-dioxa-4aza, 8-oxa-4aza and hetero ring altered 4-aza analogues

As described in **Scheme 4.1**, malonamic acid derivatives (**1),** were obtained through acylation of an appropriate amines with ethyl malonyl chloride whereas, fluoro esters

(**3**) were synthesized by direct condensation of *n*-octylamine with diethyl difluoromalonate/dimethyl fluoromalonate. Saponification of esters gave corresponding acids (**2** and **4**). Acids were coupled to L-HSL using either EDC in dioxane-water or CDI in dichloromethane or DCCI with HOBt to give final products, **2.8-2.13, 2.125-2.135, 2.140-2.143**, and **2.62**. Compound **2.63** was obtained by direct reaction of acid **3** with L-HSL in refluxing acetonitrile.

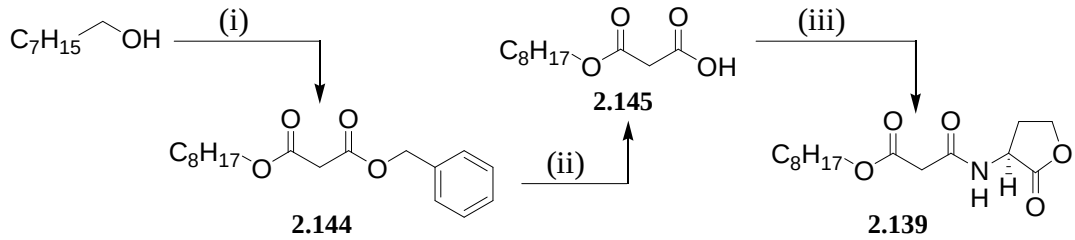


Scheme 4.1 Facile synthesis of fluorinated, hetero ring altered and 4-aza analogues

Reaction conditions: (i) $ClCOCH_2COOEt$, dry DCM 0 ^0C to rt., 2 h; (ii) $NaOH_{aq}$, EtOH, rt., 4 h; (iii) L-HSL.HCl (or amino-aromatic or hetero aromatic ring with or without substitution), EDC, TEA, Water-Dioxane, rt., 16 h or L-HSL.HCl , CDI, TEA, dry DCM, rt., 16 h; (iv) $EtCOCF_2COEt$, EtOH, rt., 2 h or MeCOCH(F)COMe, MeOH, rt., 2 h; (v) L-HSL.HCl , TEA, CH_3CN, reflux, 16 h.; (vi) $NaOH_{aq}$, MeOH, rt., 3h; (vii) L-HSL.HCl , DCCI, HOBT, dry DCM, rt., 16 h.

4.1.2 4-Oxa analogue

For the preparation of 4-Oxa analogue **2.139**, *mono*-benzyl malonate was esterified with *n*-octanol in the presence of DCCI and DMAP to furnish di-ester **2.144.** Di-ester **2.144** was then deprotected by catalytic hydrogenation to afford the free acid **2.145**. Compound **2.145** was activated by CDI and coupled with L-HSL to furnish 4-oxa analogue **2.139** (**Scheme 4.3**).

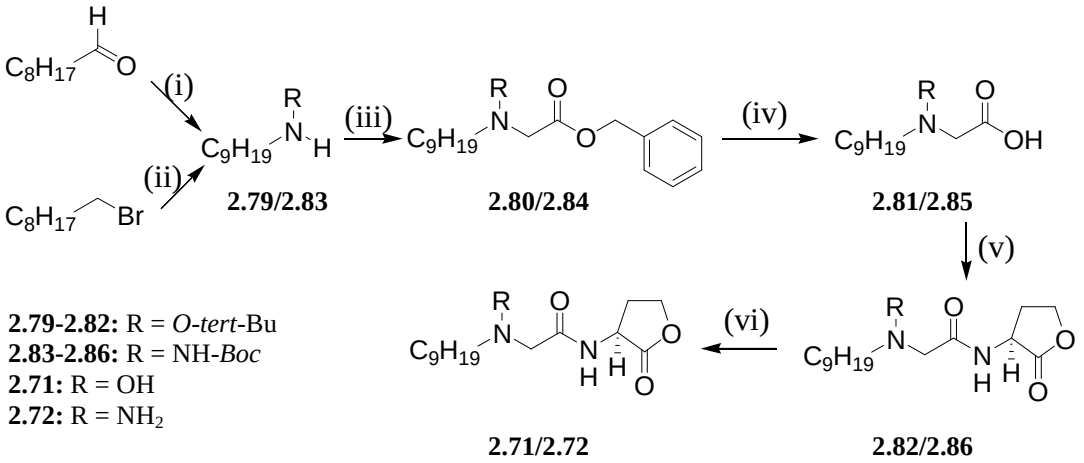


Scheme 4.3 Facile synthesis of 4-oxa analogue

Reaction conditions: (i) $PhCH_2OCOCH_2COOH$, DCCI, DMAP, dry DCM, rt., 16 h; (ii) H_2, Pd/C, EtOH, rt., 8 h; (iii) L-HSL.HCl , CDI, TEA, dry DCM, rt., 16 h.

4.1.3 3-aza derivatives:

The introduction of N-OH and N-NH_2 motifs to modify the drug candidates is very much familiar in medicinal chemistry. The syntheses of compounds with a nitrogen atom at C^3 position (**Scheme 4.2**), began with the reductive amination of nonyl aldehyde with *O-tert*-butylhydroxylamine in the presence of a reducing agent, sodium cyanoborohydride to obtain *N*-octyl derivative **2.79**.



Scheme 4.2 Facile synthesis of 3-aza analogues

Reaction conditions: (i) *O-tert*-butylhydroxylamine HCl, NaOMe, CH_3COOH, Sodium cyanoborohydride/AcOH, MeOH, 0 ^{0}C to rt., 16 h; (ii) *tert*-butyl carbazate, THF, reflux, 18 h; (iii) $BrCH_2COOCH_2Ph$, DIPEA, THF, reflux, 16 h; (iv) H_2, Pd/C, EtOH, rt., 8 h; (v) L-HSL.HCl , EDC. HCl, TEA, Water-Dioxane, rt., 16 h; (vi) 50% v/v TFA/DCM, rt., 6 h.

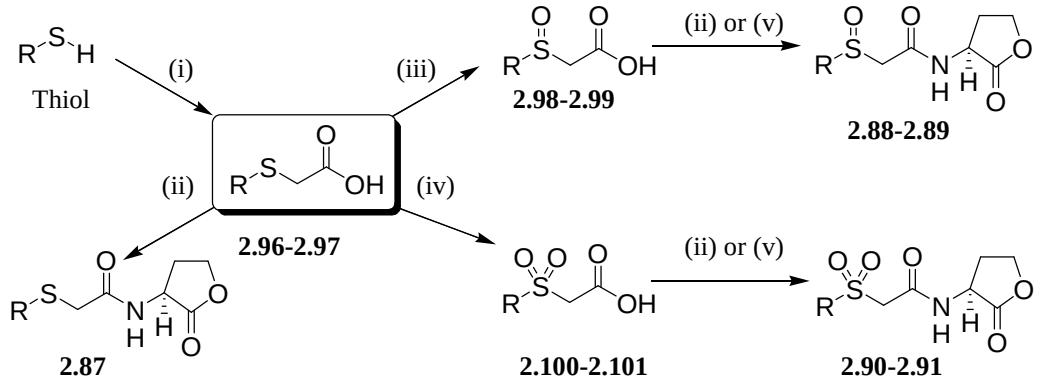
Alkylation of *tert*-butyl carbazate with *n*-octyl bromide gave *N*-octyl carbazate **2.83**. Compounds **2.79** and **2.83** were further alkylated with benzyl bromoacetate to obtain corresponding alkylated hydroxylamine and carbazate derivatives **2.80** and **2.84**

respectively. Deprotection of benzyl ester under catalytic hydrogenation conditions afforded the free acids **2.81** and **2.85**. Water soluble carbodiimide (EDC) mediated coupling of these acids with L-HSL yielded **2.82** and **2.86**. Finally the deprotection of *O-tert*-butyl and *Boc* groups with TFA yielded **2.71** and **2.72** respectively.

4.1.4 1-Thia and 3-thia derivatives

Incorporating several defined bioactive structural units to form a more potent pharmacophore is well known approach in medicinal chemistry. In medicinal drugs insertion of sulfur, sulfone, sulfoxide and sulfonamide motifs have been achieved successfully to improve their biological responses. For example, introduction of sulfur in the form of sulfone or sulfoxide imparts antibiotic properties to the otherwise inactive organic compounds. We surmised that the introduction of sulfur at the C^1 and C^3 position in 3O, C_{12}-HSL structure would alter the *3-enolic* content due to its stereoelectronic properties. Thus, a range of thia analogues **2.87-2.93** were elaborated and screened for their immunological and auto-induction capabilities. The synthetic sequences utilized for the preparation of derivatives, **2.87-2.91** are outlined in **Scheme 4.4**.

The key intermediates, **2.96-2.97** were obtained by alkylating appropriate thiols with *tert*-butyl bromoactetate. Extreme basic conditions and elevated reaction temperature afforded *in situ* deprotection of *tert*-butyl group. Oxidation of **2.96-2.97** to obtain monosulfoxides, **2.98-2.99**, as a mixture of diastereomers and sulfones, **2.100-2.101** were carried out respectively using bromate-bromide/acid and potassium permanganate/acid solutions. No attempts were made at this stage to resolve the enantiomeric mixtures of **2.98** and **2.99.** Compounds **2.96**, **2.98** and **2.100** were coupled to L-HSL using CDI to furnish desired derivatives, **2.87**, **2.88** and **2.90**. Acids **2.99** and **2.101** were successfully coupled with L-HSL using EDC to produce analogues **2.89** and **2.91**. The analogues **2.88** and **2.89** were obtained as a mixture of their diastereomers. The precise stereochemical identity of these derivatives was not established as the diastereomers were not separable by PLC.

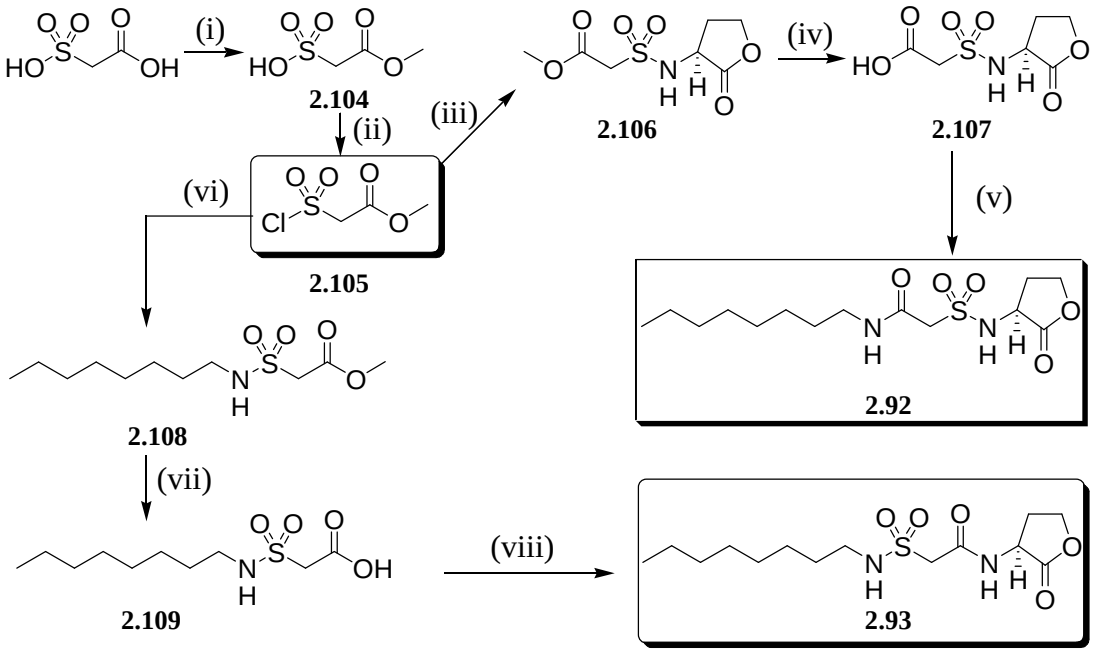


Scheme 4.4 Facile synthesis of 3-thia analogues of 3O, C_{12}-HSL

Reaction conditions: (i) *t*-butyl bromoacetate, 18% w/v NaOH, 90 ^{0}C, 14 h; (ii) L-HSL.HCl , CDI, TEA, dry DCM, rt., 16 h (for compounds **2.87**, **2.88** and **2.90**); (iii) 0.5 *N* bromate-bromide, HCl, acetic acid, 0 ^{0}C, 45 min.; (iv) 4% w/v $KMnO_4$, acetic acid, 50 ^{0}C to rt., 40 min.; (v) L-HSL.HCl, EDC.HCl, TEA, water-dioxane, rt., 16 h (for compounds **2.89** and **2.91**).

3-thia acids		R	Chemical name
Sulphide	**2.96**	-C_9H_{19}	3-Thiadodecanoic acid
	2.97	-$C_{11}H_{23}$	3-Thiatetradecanoic acid
Sulfoxide	**2.98**	-C_9H_{19}	3-Oxo-3-thiadodecanoic acid
	2.99	-$C_{11}H_{23}$	3-Oxo-3-thiatetradecanoic acid
Sulfone	**2.100**	-C_9H_{19}	3,3,-Dioxo-3-thiadodecanoic acid
	2.101	-$C_{11}H_{23}$	3, 3-Dioxo-3-thiatetradecanoic acid

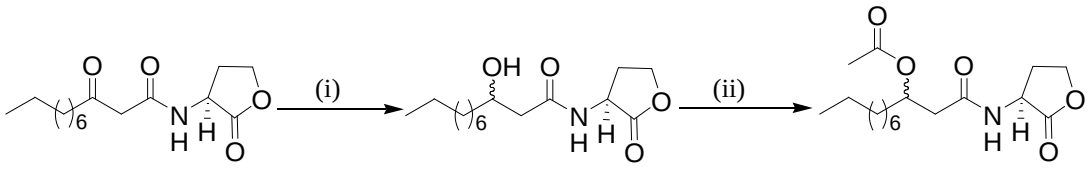
As outlined in **scheme 4.5**, firstly, esterification of sulfoacetic acid to give methyl ester **2.104** was carried out by refluxing it in a mixture of methanol-benzene (1:4) with azeotropic distillation of water. Refluxing **2.104** with $POCl_3$ afforded sulfonyl chloride **2.105.** The amidation of **2.105** with L-HSL in the presence of DBU and DMAP gave the sulfonamide **2.106** while its amidation with refluxing octylamine in the presence DBU and potassium carbonate gave sulfonamide **2.108**. Acid hydrolysis of methyl ester, **2.106** delivered the free acid, **2.107** which was successfully coupled with octylamine using DCCI and DMAP to afford **2.92**. Simple saponification of **2.108** yielded **2.109**, which was successfully coupled to L-HSL using EDC to furnish **2.93**.



Scheme 4.5 Facile synthesis of sulfonamide motif containing analogue

Reaction conditions: (i) MeOH-Benzene (20:80), distillation, 4 h; (ii) $POCl_3$, reflux, 4 h; (iii) L-HSL.HCl , DMAP, DBU, dry DCM, 0 ^{0}C to rt., 16 h; (iv) 1.0 *M* HCl, reflux, 4 h; (v) *n*-Octylamine, DCCI, DMAP, dry DCM, rt., 16 h. (vi) *n*-Octylamine, DBU, K_2CO_3, dry DCM, reflux, 3 h; (vii) NaOH/water, MeOH, rt., 4 h; (viii) L-HSL.HCl, EDC.HCl, TEA, Water-Dioxane, rt., 16 h.

In another approach to modulate the 3-enolic structure of 3O, C_{12}-HSL **2.155** (3-OH) and **2.154** (3-OAc) derivatives were synthesized as shown in **Scheme 4.6**. Acid catalyzed reduction of 3-oxo of 3O, C_{12}-HSL, using sodium cyanoborohydride gave the 3-OH derivative albeit as a racemic mixture. Acetylation of **2.155** to obtain **2.154** was achieved in quantitative yield by refluxing it with acetic anhydride.



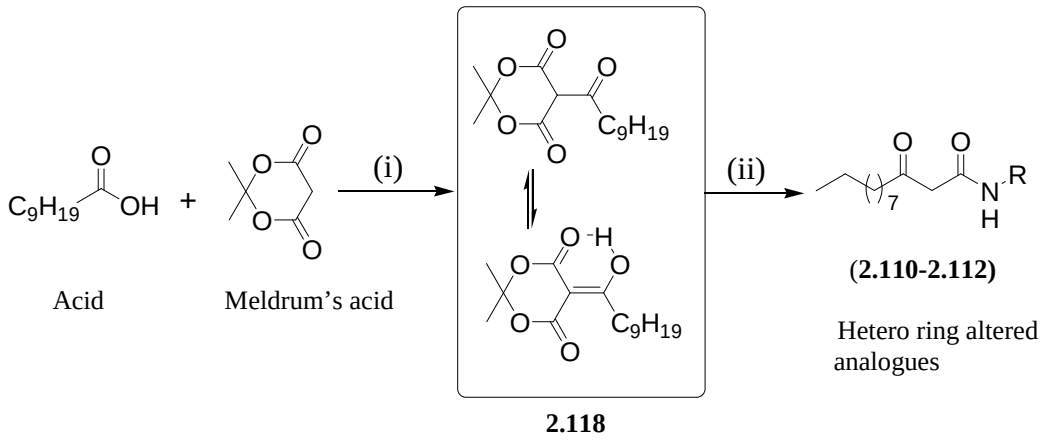
3O, C_{12}-HSL (racemic mixture) **2.155** **2.154**

Reaction conditions: (i) sodium cyanoborohydride, methanol-HCl, rt, p*H*≅4.0, 16 h; (ii) acetic anhydride, reflux, 2 h.

Scheme 4.6 Synthesis of 3-hydroxy and 3-acetoxy derivatives of 3O, C_{12}-HSL

4.1.5 Hetero ring altered derivatives of 3O, C_{12}-HSL

As shown in **Scheme 4.7** acylated Meldrum's acid **2.118** was synthesized from *n*-decanoic acid and Meldrum's acid using DCCI and DMAP. Condensation of **2.118** with an appropriate amine under reflux yielded corresponding compounds (**2.110-2.112)**.

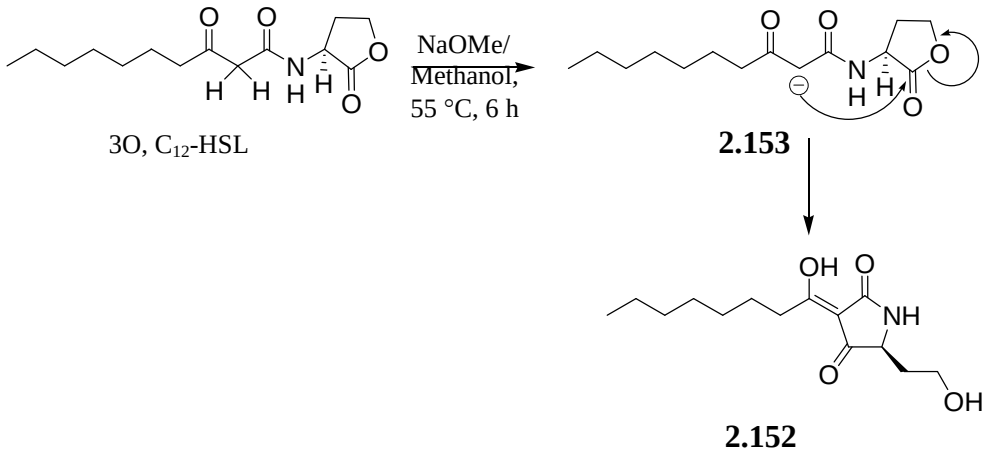


Scheme 4.7 Facile synthesis of hetero ring altered analogue of 3O, C_{12}-HSL

Reaction conditions: (i) DCCI, DMAP dry DCM, 0 ^{0}C to rt., 16 h; (ii) RNH_2, TEA, CH_3CN, rt., 16 h.

4.1.6 Tetramic acid derivative

Treatment of 3O, C_{12}-HSL with a strong base NaOMe in methanol at 55 °C afforded **2.153,** which underwent intramolecular Claisen like condensation to yield a **2.152** (**Scheme 4.8)**



Scheme 4.8 Facile synthesis of 3-(1-hydroxydecylidene)-5-(2-hydroxy-ethyl) pyrrolidine-2,4-dione (*Tetramic acid*)

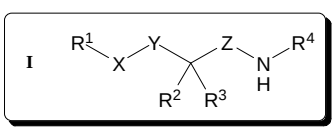
All the newly synthesized analogues were analyzed first for their purities and identities and then taken forward to assay verify them for their immune modulation, cytotoxicity, non-auto-inducing or non-QS properties.

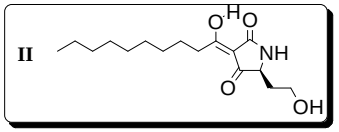
4.2 Discussion

The present study was undertaken to determine the optimal structural requirements for the immune modulatory activity without promoting any deleterious auto-inducing activity of new 3O, C_{12}-HSL analogues. The parent compound 3O, C_{12}-HSL isolated from the spent culture of the Gram-negative bacterium *Pseudomonas aeruginosa* and its synthetic analogues suppress murine and human leucocytes proliferation[13,19] however; these also promote deleterious bacterial growth. Our investigation dealt with structure alterations on the A, B and C parts (**Fig 2.4**; page 146) of 3O, C_{12}-HSL molecule to verify its molecular basis for immunomodulatory activity. Our goal was to get potent immunomodulatory compounds that were easily accessible but devoid of auto-induction. A total of 39 compounds were synthesized and characterized. Their purity was established by chromatography (TLC) and spectroscopic analyses such as IR, NMR and MS. These compounds were assayed for their ability to modulate murine leukocyte proliferation stimulated with concanavalin A (ConA), cytotoxicity and also for their auto-inducing (or QS) activity.

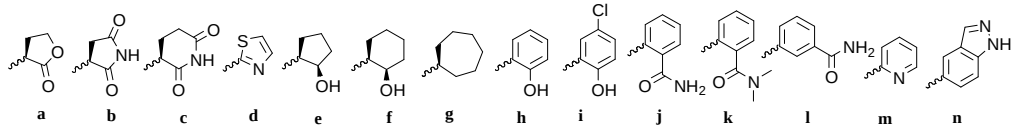
Quantitative and comparative results presented in **Table 4.1** are generated from the types of data and plots as illustrated in Chapter 3. The EC_{50} is a dose (μM) of compound required to inhibit 50% of a proliferating population of murine splenocytes, measured using [^{3}H]-thymidine incorporation as a marker of proliferation and determined by nonlinear regression analysis. Splenocytes stimulated with Con A are stimulated to incorporate [^{3}H]-thymidine into newly synthesized DNA, indicated as counts per minute (cpm). An auto-inducer (or QS) activity is a measure of the ability of the compound to induce light production in a specifically designed bacterial bio-reporter system. Values are expressed as a percentage of the bioluminescence measured after exposure of the bacteria to the analogues for 5 hours at 37°C relative to control 3O, C_{12}-HSL (100 %). In this data EC_{50} for 3O, C_{12}-HSL would be calculated to be in between 4.30 and 4.50 μM.

Table 4.1 Quantitative and comparative immune modulatory and quorum-sensing activities of 3O, C_{12}-HSL and various analogues.





R^4=



No.		Structure							Immune modulation activity* EC_{50} (μM)		Quorum-sensing activity** ± SD		
		X	Y	Z	R^1	R^2	R^3	R^4	Inhibition of murine cells proliferation	Cytotoxicity	Auto-induction	Antagonism in presence of 5 μM 3O, C_{12}HSL at variable analogue conc.	
Compounds											(fold)***	1 mM	100μM
1.54	**I**	CH_2	CO	CO	$CH_3(CH_2)_7$	H	H	a	4.4 ± 0.1	250	12.3	100	100
2.8	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	a	15.1	200	0.92	20 ± 2	20 ± 2
2.9	**I**	NH	CO	CO	$CH_3(CH_2)_8$	H	H	a	2.9	530	0.70	4.8 ± 1.5	3.4 ± 0.4
2.10	**I**	NH	CO	CO	$CH_3(CH_2)_{10}$	H	H	a	0.65	140	0.37	6.1 ± 1.8	14 ± 2
2.11	**I**	NMe	CO	CO	$CH_3(CH_2)_7$	H	H	a	6.1	>1000	1.3	70 ± 2	60 ± 2
2.12	**I**	NPr^n	CO	CO	$CH_3(CH_2)_7$	H	H	a	2.9	200	1.9	104 ± 2	101 ± 1
2.13	**I**	$N(CH_2)_2OH$	CO	CO	$CH_3(CH_2)_7$	H	H	a	17.1	300	0.95	8.5 ± 0.8	16 ± 2
2.62	**I**	NH	CO	CO	$CH_3(CH_2)_7$	F	H	a	21.4	800	1.7	37 ± 3	57 ± 2
2.63	**I**	NH	CO	CO	$CH_3(CH_2)_7$	F	F	a	82.3	>1000	0.003	2.7± 2.3	6.0 ± 1
2.71	**I**	CH_2	NOH	CO	$CH_3(CH_2)_7$	H	H	a	13.1	400	1.1	43 ± 1	69 ± 1
2.72	**I**	CH_2	NNH_2	CO	$CH_3(CH_2)_7$	H	H	a	1.0	410	1.1	71 ± 2	55 ± 2
2.77	**I**	CH_2	NOBn	CO	$CH_3(CH_2)_7$	H	H	a	1.3	63	****	****	****
2.87	**I**	CH_2	S	CO	$CH_3(CH_2)_7$	H	H	A	Not active	>1000	0.89	36 ± 2	51 ± 1
2.88	**I**	CH_2	SO	CO	$CH_3(CH_2)_7$	H	H	a	0.55	720	NA	NA	NA
2.89	**I**	CH_2	SO	CO	$CH_3(CH_2)_9$	H	H	a	0.15	45	0.90	50 ± 1	63 ± 2
2.90	**I**	CH_2	SO_2	CO	$CH_3(CH_2)_7$	H	H	a	0.932	200	1.3	39 ± 1	72 ± 2
2.91	**I**	CH_2	SO_2	CO	$CH_3(CH_2)_9$	H	H	a	0.45	170	0.74	67 ± 2	76 ± 1
2.92	**I**	NH	CO	SO_2	$CH_3(CH_2)_7$	H	H	a	9.1	220	0.96	77 ± 1	86 ± 2
2.93	**I**	NH	SO_2	CO	$CH_3(CH_2)_7$	H	H	a	2.0	900	1.1	77 ± 1	101 ± 2
2.110	**I**	CH_2	CO	CO	$CH_3(CH_2)_7$	H	H	b	Not active	****	1.4	125 ± 1	82 ± 1
2.111	**I**	CH_2	CO	CO	$CH_3(CH_2)_7$	H	H	c	Not active	****	1.1	41 ± 2	71 ± 2
2.112	**I**	CH_2	CO	CO	$CH_3(CH_2)_7$	H	H	d	12.5	800	0.84	36 ± 2	51 ± 2
2.125	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	d	2.4	600	0.91	50 ± 1	64± 1
2.126	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	e	18.8	400	1.5	38 ± 1	55 ± 2
2.127	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	f	26.1	400	1.2	40 ± 2	45 ± 3
2.128	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	g	2.7	78	1.1	14 ± 1	19 ± 4
2.129	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	h	20.0	>1000	1.0	15 ± 1	29 ± 2
2.130	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	i	2.5	>1000	0.70	57 ± 2	66 ± 1
2.131	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	j	12.0	120	0.93	14 ± 1	23 ± 2
2.132	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	k	0.70	160	1.5	19 ± 3	37± 4
2.133	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	l	9.4	700	0.69	50 ± 1	59 ± 1
2.134	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	m	11.7	>1000	1.0	30 ± 1	41 ± 2
2.135	**I**	NH	CO	CO	$CH_3(CH_2)_7$	H	H	n	6.3	>1000	1.5	32 ± 3	62 ± 2
2.139	**I**	CH_2	O	CO	$CH_3(CH_2)_7$	H	H	a	4.8	500	0.48	33 ± 1	46 ± 4
2.140	**I**	NH	CO	CO	$CH_3(CH_2)_3O(CH_2)_3$	H	H	a	200	>1000	1.2	121 ± 2	118 ± 2
2.141	**I**	NH	CO	CO	$CH_3(CH_2)_3O(CH_2)_3$	H	H	e	154	>1000	3.8	93 ± 3	111 ± 1
2.142	**I**	NH	CO	CO	$CH_3CH_2O(CH_2)_2O(CH_2)_2$	H	H	a	240	>1000	1.3	47 ± 2	61 ± 3
2.143	**I**	NH	CO	CO	$CH_3CH_2O(CH_2)_2O(CH_2)_2$	H	H	e	48	700	1.1	77 ± 2	89 ± 1
2.152	**II**	-	-	-	-	-	-	-	5.3	52	1.1	17 ± 2	21 ± 1
2.154	**I**	CH_2	CHOAc	CO	$CH_3(CH_2)_7$	H	H	a	0.52	120	1.1	59 ± 2	70 ± 2

***** Independent observations n=2; * independent readings n=5; SD = standard deviation; *** fold- calculated as ratio of (relative luminescence of cell culture alone)/(relative luminescence of cell culture with analogue); ****-not performed; NA-not available***

Compound activity in the murine splenocyte proliferation assay demonstrated that introduction of nitrogen at C^4 in the alkyl chain (compounds **2.8**-**2.10**) had retained immuno-suppressive activity as that of parent compound. Gratifyingly, activity was increased considerably with the alkyl chain length of 13 and 15 C, (analogues **2.9** and **2.10**) as well as when nitrogen at position 4 was substituted with methyl **2.11 (**EC_{50} 6.1 μM) and propyl **2.12** (EC_{50} 2.9 μM). The activity although low, was retained when N^4 was substituted with hydrophilic hydroxyethyl **2.13 (**EC_{50} 17.1 μM), suggesting that appropriate lipophilicity is important for activity. The 4-aza analogues also showed no auto-induction and profound reduction in biosensor activity. Interestingly, with the insertion of nitrogen we were able to accommodate longer alkyl chains (compounds **2.9**-**2.11**) with no loss of solubility for *in vitro* testing.

The anti-proliferation activity maintained, albeit slightly less when one of the C^2-hydrogens in alkyl chain was replaced with fluorine (compound **2.62**). Unfortunately, replacement of both hydrogens by fluorines (compound **2.63**) showed no activity against murine splenocyte proliferation. It thus appears that minimum of one hydrogen at C^2 is essential for activity supporting our belief that the immune response is possibly elicited via *3-enolic* tautomer. Moreover, difluoro substitution in compound **2.63**, dramatically improved quorum sensing inhibition activity whereas, it is improved comparatively less but substantial with monofluoro substitution in monofluoro compound. The replacement C^3=O with N^3-OH (compound **2.71**) maintained equipotent immune suppression whereas, its replacement with N^3-NH_2 (compound **2.72**) and N^3-OBn (compound **2.77**) displayed profound increased in the activity. This demonstrated that suitably substituted compounds specifically with hydrogen bonding donating entity at C^3 can show potent immune suppression. However, it needs further investigations to favour this hypothesis. Moreover, compounds **2.71** and **2.72** did not exhibit auto-induction activity rather were found to have QS antagonist activity.

Among 3-thia compounds, **2.87** showed no activity against splenocyte proliferation. Substitution of sulfoxide group at C^3 position in 3O, C_{12}-HSL gave compounds **2.88** and **2.89**. Surprisingly, compounds **2.88** (EC_{50} 0.55 μM) and **2.89** (EC_{50} 0.15 μM) showed potent immune suppression, supporting the initial findings of Chhabra et al. that the presence of a 3-oxo substituent is essential to retain immune suppression. The

3-sulfoxide derivatives were assayed as mixtures of the diastereoisomers because they could not be resolved by chromatography. In view of these observations, sulfonyl derivatives **2.90** and **2.91** were synthesized. As predicted **2.90** (EC_{50} 0.932 μM) and **2.91** (EC_{50} 0.45 μM) also exhibited potent anti-proliferation activity. Furthermore, no auto-induction was observed with 3-thia analogues **2.87**, and **2.89-2.91** as well as they all exhibited competitive reduction (antagonists) in biosensor activity compared to parent compound. The C^1 sulfonamide analogues **2.92** (EC_{50} 9.1μM) displayed immuno-suppression at almost double dose than that of 3O, C_{12}-HSL (EC_{50} 4.5 μM) whereas C^3 sulphonamide analogue **2.93 (**EC_{50} 2.0 μM) displayed at equivalent of half dose of parent molecule. This suggested that modulation at C^3=O is more important than the C^1=O for better immune suppressive activity. These were also found to be non-QS. Additionally all thia analogues offered improved *in vitro* solubility.

Among the ring altered analogues, the replacement of L-HSL by L-aspartimide (compound **2.110**) and L-glutarimide (compound **2.111**) units demonstrated complete loss of immune suppression activity while surprisingly, L-HSL replacement with 2-aminothiazole ring **2.112** (EC_{50} 12.5 μM) retained immune suppression yet without deleterious auto-induction. This finding was surprising as the previous reports emphasized the importance of HSL ring and its chirality. This observation opens door for the development of a new series of immune modulatory agents via replacement of HSL ring by other suitable ring(s).

To this end, immune modulatory potential of existing 3O, 4-aza-C_{12}-HSL **2.8** was successfully metamorphosed by developing analogues **2.125-2.135** where L-HSL ring was replaced with other 5- or 6 membered mono or fused aromatic rings. As expected analogue **2.125** (EC_{50} 2.4 μM) exhibited potent immune inhibition. The 2-aminocyclopentanol (**2.126**), 2-aminocyclohecanol (**2.127**) and 2-aminophenol (**2.129**) containing compounds retained activity but did not show any improvement over **2.8**. The 2-aminocyclohexane ring analogue **2.128** on the other hand showed potent activity. Among the aniline derivatives *ortho* chloro substitution in **2.129** gave compound more potent **2.130** (EC_{50} 2.5 μM) than **2.8**. The *ortho* and *meta* carboxamide substitution (compounds **2.131** and **2.133**) also led to retention of immune suppression whereas, *ortho* substitution with more bulky *N*,*N*-

dimethylcarboxamide yielded most potent analogue **2.12** (EC_{50} 0.7 μM). Analogues with 2-aminopyridine (**2.134**) and 5-aminoindazole (**2.135**) were also found to retain equipotent immune suppressive activity. Overall, improvment of immune suppression by above analogues demonstrated that L-HSL ring can be replaced by other appropriate ring(s). Moreover, all these hetero-ring altered analogues showed no microbiological auto-induction.

In case of tetramic acid analogue **2.152** equipotent immune suppression as that of 3O, C_{12}-HSL was observed whereas, 3-*O*-acetylation **2.154** on the other hand unbelievably increased 10 times more immune suppression than the parent compound once again indicating the importance of C^3=O functionality. The both analogues also found to be non-QS.

Insertion of oxygen at C^4 (compound **2.139**) retained anti-proliferation activity at comparatively equivalent dose (EC_{50} 4.8 μM) and only also inhibition in the bioluminescence activity was observed with this. However, insertion of additional oxygen(s) in the side chain at 7^{th} and 10^{th} positions or at 8^{th} position resulted in almost inactive analogues **2.140-2.143**, demonstrating that appropriate lipophilicity is must for immune modulation. Notably analogue **2.141** was found to be partial auto-inducer (fold 3.8) and almost all of them were inactive as QS antagonists.

4.3 Conclusions

To investigate structural necessity for the 1, 3-dicarbonyl moiety of the parent molecule in terms of immunomodulatory activity and alkyl chain towards solubility, we have synthesized a series of new 3O, C_{12}-HSL analogues. Here we have shown that introduction of nitrogen and oxygen at C^4 position can be compatible. 4-aza exhibited equipotent immunomodulation with great degree of reduction in bacterial bioluminescence. *N*-Alkylation at this site also retained activity. At least, single hydrogen at C^2 position also appears to be important for activity since difluoro analogue showed no activity. The replacement C^3 keto group by hydroxyl aza (N^3-OH, **2.71**), its benzyl substituted dervative (N^3-OBn, **2.77**) and amino aza (N^3-NH_2, **2.72**) group was successfully achieved and these analogues also exhibited potent immune modulation without detrimental QS effect. Moreover, C^3=O of 1, 3-dicarbonyl moiety can be favorably replaced by sulfoxide and sulfones. However, its replacement with thia group led to inactive compound thus, it is apparent that the 3-oxo substitution is important for immune suppressive activity. 3-sulfoxide derivatives, even as a mixture of diastereoisomers, were potent immune suppressive. The replacement 3-keto function by sulfonamide (compound **2.93**) as well as C^1 keto group by sulfone (compound **2.92**) groups also preserved immune suppressive activity. The L-HSL ring was found not essential for immune modulation as appropriate ring containing hetero ring altered analogues (compounds **2.110**-**2.112** and **2.125**-**2.135**) revealed improved immune suppression. Insertion of *O* at C^4 can be better tolerated whereas its insertion at C^8 as well as with additional oxygen at C^7 and C^{10} resulted in complete loss of activity. The simple 3-*O*-acetylation also was found to be tolerated and yielded potent analogue **2.154.** Moreover structurally unrelated tetramic acid analogue also retained activity.

All these structural modifications also lowered the lipophilic character of the compounds and thus showed improved *in vitro* solubility. The preliminary quorum sensing cellular assays of all these analogues have exhibited great degrees of reduction in bacterial bioluminescence compared to 3O, C_{12}-HSL.

4.4 Future directions

4.4.1 Further biological investigations of active analogues in higher animals

Following, the results of extended SAR and primary biological (immunological and microbiological) assays in well-established models, a number of active analogues (**2.9, 2.10, 2.12, 2.72, 2.88-2.91, 2.93, 2.125, 2.128, 2.130, 2.132** and **2.139)** can be selected as promising candidate and need to be taken further for immunological investigations in higher animal/human models. For example, inhibition of mitogen-driven proliferation of human peripheral blood monocyte cells (HPMC) and inhibition TNF-α secretion in HPMC stimulated by bacterial lipopolysaccharide (LPS).[131] If successful, these compounds would establish the basis for further molecular mechanistic studies to identify molecular targets in eukaryotic cells.

4.4.2 Synthesis of new series of 3-aza analogues of 3O, C_{12}-HSL

In an attempted synthesis of 3-(N-OH) C_{12}-HSL (compound **2.71**) as shown in **scheme-12** (page 91), its precursor 3-(N-OBn) C_{12}-HSL (compound **2.77)** was first synthesized. It was just serendipity that when investigated, this compound showed potent immune suppression with EC_{50} = 1.3 μM, even more than its congener molecule **2.71** (EC_{50} = 13.1 μM). These results further suggest that by hydrophobic interacting site might be available in the immunophilin receptors to interact with aromatic benzene ring of analogue **2.77**. With this logic it would be possible to design and develop a new series of 3-aza analogues by replacing benzyl group by various hydrophobic 5 or 6 membered heterocyclic mono or fused (substituted or un-substituted) or other aromatic rings as shown below in **Fig 4.1-A.** In addition, new analogues would also be obtained by replacing L-HSL with other rings used in the synthesis of analogues **2.125-2.135** as shown in **Fig 4.1-B**. Similar approach could also be applicable for other active analogues containing L-HSL ring e.g. active analogues from thia series and 3-O-aceyl analogue **2.154**.

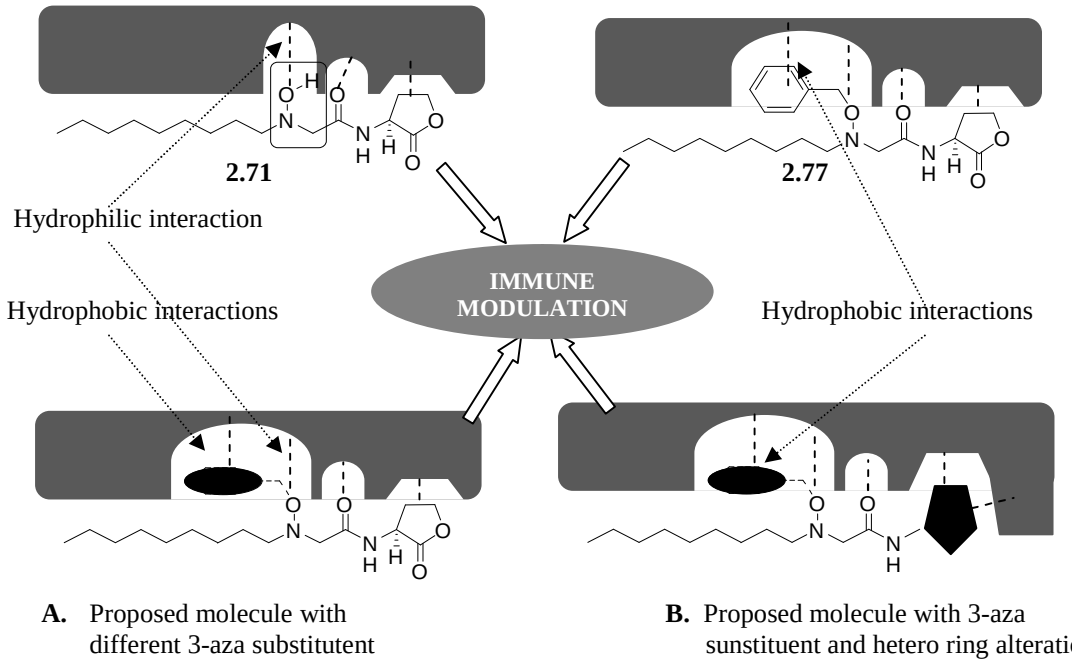


Fig 4.1 Proposed new 3-aza analogues showing possible interactions with receptor

4.4.3 Synthesis of bivalent ligands

A bivalent ligand is a composite structure that comprises of two pharmacophores linked through an appropriate spacer.[281,282] It serves as the conceptual basis for the design of congener with dual function and thus may be with better potency. Thorough immunological evaluations of 3O, C_{12}-HSL at cellular and molecular levels in eukaryotic cells have made it clear that there exist(s) specific **receptor(s)** for its binding in eukaryotes to mediate fluctuations in immunological behaviours. Similar studies in PQS showed the presence of an **additional target** that is different from 3O, C_{12}-HSL targets in eukaryotes for its binding to mediate immune modulation. Surprisingly combination of 3O, C_{12}-HSL and PQS exhibited synergism in suppressing leukocyte proliferation. This demonstrates that both 3O, C_{12}-HSL and PQS have separate receptors for their binding in eukaryotes in showing their synergistic effects in immune modulation. However, exact location and structures of these targets have not yet been identified and need further investigation. It is possible to maximize this synergistic behaviour by design and synthesis of bivalent ligands by linking these two structures through an appropriate spacer as shown below in **Fig 4.2**.

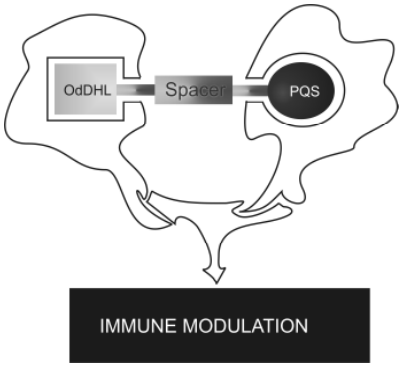


Fig 4.2 Synergistic behaviour of divalent ligands

Active analogues of newly synthesized compounds would be used to develop their homo- and/or hetero- bivalent dimers with PQS molecule or its active analogues in order to synergize immune modulatory effect. The possible combinations of this approach are shown below in **Fig 4.3**

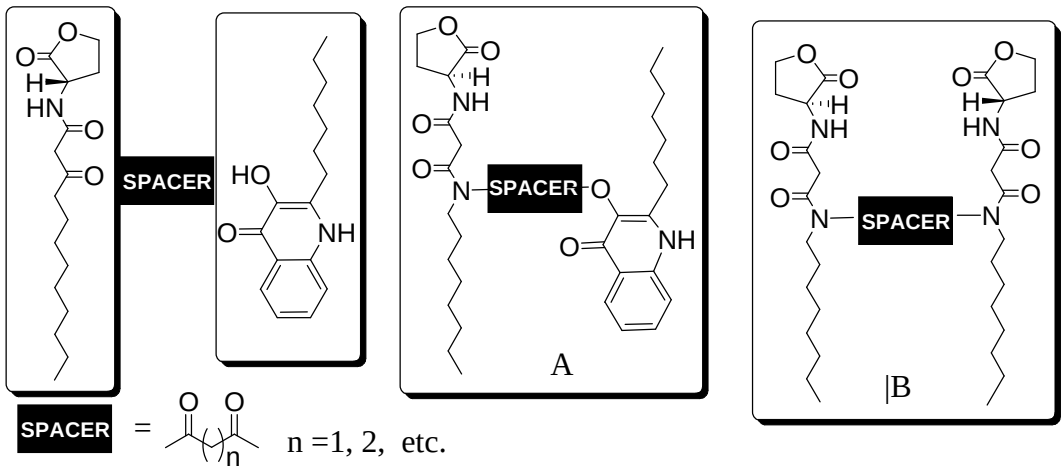


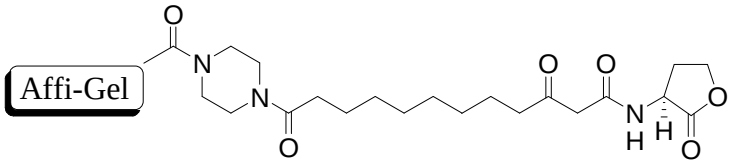
Fig 4.3 (A) Hetero-ligands of 3O, C_{12}-HSL; (B) homo-dimer of 3O, 4-aza C_{12}-HSL.

The new dimeric molecules with a spacer of optimal length would be expected to exhibit a potency that is greater than that derived from the sum of its two monovalent pharmacophores i.e. 3O, C_{12}-HSL and PQS.

4.4.4 Isolation and identification of target proteins (immunophillins)

The understanding of immune system really means our ability to understand how drugs act to modulate immune system. To understand how drugs act within the immune system, the isolation and the characterization of the target proteins is imperative. This has been exploited by employing the affinity chromatography, this involves loading the drug molecule on the biological fluids for participatory targets.[283]

In the previous study Harty et al. have developed terminal chain linked affinity matrix **4.1** to isolate targeted proteins responsible for immune activity. Here, **4.1** was treated with mouse splenocyte and human granulocytes extracts and two proteins of molecular weight 14 KDa (Kilo Daltons) and 8 KDa were isolated from each extract. These proteins were identified as calgranulin A and B respectively. Subsequent research has demonstrated calprotectin as the putative target for the 3O, C_{12}-HSL. How 3O, C_{12}-HSL interacts with calprotectin to exhibit immunological activity is yet to be understood.



4.1

As 3O, C_{12}-HSL has dual activities that are QS and immune modulation. It is thus possible that it in this form it might not have strong affinity for the appropriate target proteins. Also, it is understood that lipophilic character of the alkyl side chain is must for activity. This further suggests that possibly free alkyl chain may be interacting to hydrophobic pocket in the immunophilin. This raises the possibility, that binding proteins must be linked to other linkage point in alkyl chain and not terminally.

Therefore, if newly developed selective immune modulatory analogues are presented to the binding proteins through any linkage except the terminal linkage and then are treated with mouse splenocyte or human granulocyte extracts, these might target selectively to putative proteins responsible for immune modulation. Representative structures of analogue(s) linked Affi-Gel matrices are shown below in **Fig 4.4**.

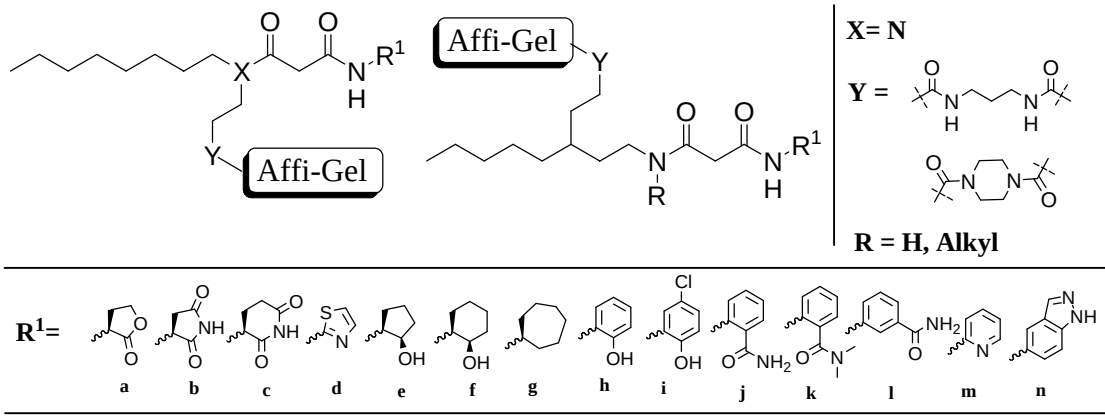


Fig 4.4 Proposed analogue loaded Affi-Gel matrices via 1,3-diaminopropane or piperazine linker

Isolation of the correct target immunophilins would further facilitate the development of novel immune modulatory agents for therapeutic intervention.

5.0 EXPERIMENTAL

5.1 Chemicals and reagents

L-Homoserine obtained from NovaBiochem (Nottingham, U.K.), was used to make L-homoserine lactone hydrochloride. Carboxylic acids and other starting materials were purchased from Aldrich Chemical Co. and Lancaster chemicals. The solvents used were of HPLC grade. Dichloromethane was dried by storing it over anhydrous calcium chloride and distillation.

5.2 General analytical methods

Melting points were recorded on Kofler Hot Stage melting point apparatus and are reported uncorrected.

FT-Infrared spectra were taken on Avatar 360 Nicolet FT-IR spectrophotometer through the range 4000-600 cm^{-1} and measured in KBr pellets or as thin films.

^{1}H NMR spectra were recorded in deuteriochloroform ($CDCl_3$) or DMSO-d_6 solvent as specified on Bruker AMX-250 or Bruker Avance-400 spectrophotometer operating at 250 and 400 MHz respectively.
^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 with a Bruker Avance-400 instrument operating at 100 MHz.
Chemical shifts are referenced to an internal standard Tetramethylsilane (TMS) or residual protic solvents, on a broad band decoupled mode, and assignments were made using a DEPT pulse sequence. Chemical shifts are expressed as δ units and *J* values are in Hz.

Electrospray mass spectra (ES-MS) were recorded using Micromass VG platform and Micromass LCT spectrometers for low- and high-resolution spectra, respectively.

Thin-layer chromatography (TLC) was performed using Merck silica gel 60 GF_{254} precoated (0.2 mm) aluminium plates. **Preparative thin-layer chromatography**

(PLC) was performed using Merck silica gel 60 GF_{254} coated (1.0 mm) glass plates (20 cm × 20 cm). **Flash chromatography** was performed using Merck Kieselgel 60 (230-400 mesh) silica. All products during chromatography were visualized using either UV light (λ=254 nm) or by staining with dilute potassium permanganate or phosphomolybdic acid solution.

5.3 Synthesis of analogues of 3O, C_{12}-HSL

5.3.1 Method-A

To a stirred solution of an appropriate acid (1 mmol) in a minimum volume of dioxane (4-6 mL) was added a solution of L-HSL.HCl (1 mmol) in a minimum amount of water (about 2 mL). To this was added sequentially TEA (3 mmol) followed by EDC.HCl (1.5 mmol) and stirring was continued for 16 h at room temperature. Solvents were removed under reduced pressure and the residue redissolved in warm ethyl acetate (20 mL). Organic solution was sequentially washed with water (3 × 10 mL), saturated sodium bicarbonate (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain desired products.

5.3.2 Method-B

To a stirred solution of an appropriate acid (1 mmol) in dry DCM (20 ml) was added sequentially CDI (1 mmol), TEA (1.5 mmol) and L-HSL.HCl (1 mmol). The solution was stirred at room temperature for 16 h and then filtered to remove the precipitated *N, N'*-dicyclohexylurea. The filtrate was evaporated in vacuo. The residue was redissolved in ethyl acetate (25 mL). Organic solution was washed with saturated sodium bicarbonate (3 × 10 mL), 1 M HCl (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain product.

5.3.3 Method-C: Synthesis of 3-oxo-4-azaalkanoic acids (*Saponification of ethyl esters of 3-oxo-4-azaalkanoic acids*)

To a stirred solution of ethyl or methyl, 3-oxo-4azalkanoates (5 mmol) in ethanol or methanol (25 mL), was added dropwise NaOH (6 mmol) solution in water (15 mL). The stirring was continued at room temperature for 4 h. The mixture was rotary evaporated and the residue redissolved in water (20 mL) and washed with ethyl acetate (2 × 10 mL). Aqueous layer was acidified with 2 M HCl to pH 2 and the free acid was extracted with ethyl acetate or DCM (3×15 mL), dried over $MgSO_4$, filtered and solvent rotary evaporated to afford the title acids.

5.3.4 Ethyl esters of 3-oxo-4-azaalkanates

5.3.4.1 Method-D

To a stirred solution of an appropriate amine (10 mmol) in dry DCM (25 mL) at 0 ^{0}C, ethyl malonyl chloride (5 mmol) was added dropwise over a period of 20 min. The stirring was continued at room temperature overnight. An additional portion of DCM (15 mL) was added, and the solution was sequentially washed with saturated sodium bicarbonate (3 × 20 mL), 2 M HCl (3 × 15 mL) and brine (1 × 25 mL) solution. The organic layer was separated, dried over $MgSO_4$ and rotary evaporated to give the corresponding ethyl 3-oxo-4-azaalkanoate. Final product was recrystallized from ether.

5.3.4.2 Method-E

To a stirred solution of an appropriate amine (5 mmol) and diisopropylethylamine (5.5 mmol) in dry DCM (20 mL) at 0 ^{0}C, a solution of ethyl malonyl chloride (5.5 mmol) in DCM (5 mL) was added dropwise over a period of 20 min. The mixture was stirred overnight at room temperature. The solvent was rotary evaporated and the residue was redissolved in ethyl acetate (25 mL). The solution was sequentially washed with saturated sodium bicarbonate (3 × 20 mL), 2 M HCl (3 × 15 mL) and brine (1 × 25 mL) solution. The organic layer was separated, dried over $MgSO_4$ and rotary evaporated to give the corresponding ethyl 3-oxo-4-azaalkanoate.

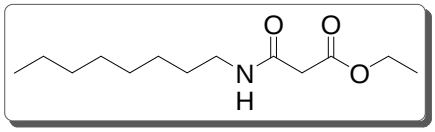
5.3.5 Method-F: *N*-substituted octylamines

To a solution of appropriate amine (4 mmol) in acetonitrile (20 mL) was added potassium carbonate (4.4 mmol) and stirred for 10 min. To this suspension was added *n*-octylbromide and stirring was continued for 18 h at room temperature. Solvent was removed by rotary evaporation and the residue was redissolved in ethyl acetate (25 mL). This solution was washed with water (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was removed under vacuum to obtain an oily product that was a mixture of the desired product and the tertiary amine as a side product. The desired product was isolated by column chromatography in methanol-ammonia (9.6:0.4) solvent system.

5.4 Synthesis of 4-Aza analogues of 3O, C_{12}-HSL

5.4.1 3O, 4-aza C_{12}-HSL [2.8]

Ethyl 3-oxo-4-azadodecanoate [2.32]



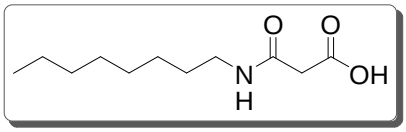
Ethyl 3-oxo-4-azadodecanoate was synthesized as white crystals in 95 % yield from *n*-octylamine using Method- D.

TLC-R_f = 0.43 in ethyl acetate-hexane (1:1)

FT-IR (KBr) cm^{-1}: 3297m, (*ν*NH); 1731s (*ν*C=O, ester); 1650s(*ν*C=O, amide); 1568s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.93 (3H, t, *J* = 6.0 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.07-1.77 (13H, m, CH$_3$(C*H*$_2$)$_5$- and C*H*$_3$CH$_2$-), 1.43-1.52 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.23 (2H, t, *J* = 7.5, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.30 (2H, s, -COC*H*$_2$CO-), 4.23 (2H, q, *J* = 7.5 Hz, CH$_3$C*H*$_2$-), 7.71 (1H, s, broad, -(CH$_2$)$_7$N*H*-).

3-Oxo-4-azadodecanoic acid [2.38]



3-Oxo-4-azadodecanoic acid was synthesized as white needles in 85 % yield from saponification of ethyl, 3-oxo-4-azadodecanoate using Method-C.

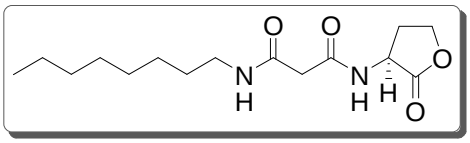
mp = 78-80 °C.

FT-IR (KBr) cm^{-1}: 3325s, (*ν*NH); 1717s (*ν*C=O, acid); 1623s (*ν*C=O, amide); 1571s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.9 (3H, t, *J* = 7.2 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.23-1.32 (10H, m, CH$_3$ (C*H*$_2$)$_5$-), 1.53-1.58 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.33 (2H, t, *J* = 6.8, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.37 (2H, s, -COC*H*$_2$CO-), 6.69 (1H, s, -(CH$_2$)$_7$N*H*-), 9.86 (1H, s, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 168.68, 168.59, 90.71, 40.16, 38.19, 31.76, 29.15, 29.11, 26.82, 22.63, 14.11.

3O, 4-aza C_{12}-HSL [2.8]



3O, 4-aza C_{12}-HSL was synthesized as white crystals in 38 % yield from 3-oxo-4-azadodecanoic acid using Method-A.

TLC-R_f = 0.38 in ethyl acetate-hexane (4:1)

mp = 126-128 °C.

FT-IR (KBr) cm^{-1}: 3293s, br. (νNH); 1771s (νC=O, lactone); 1685s (νC=O, 1, 3-diamide); 1645s (νC=O, amide); 1557s (δNH, amide).

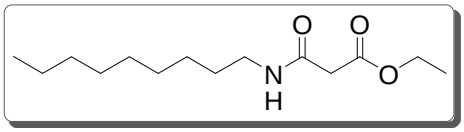
^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, *J* = 6.5 Hz, $CH_3(CH_2)_7$-), 1.27-1.48 (10H, m, $CH_3(CH_2)_5$-), 1.51 (2H, s, broad, $CH_3(CH_2)_5CH_2$-) 2.34 (1H, m, ring, 4α-*H*), 2.66 (1H, m, ring, 4β-*H*), 3.22 (2H, t, *J* = 6.6, $CH_3(CH_2)_6CH_2$-), 3.28 (2H, s, -$COCH_2CO$), 4.23 (1H, m, ring, 3-*H*), 4.49 (1H, m, ring, 5α-*H*), 4.60 (1H, m, ring, 5β-*H*), 7.14 (1H, s, -$(CH_2)_7NH$-), 8.20 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 172.21, 168.13, 167.12, 65.92, 49.28, 42.62, 39.79, 31.81, 29.31, 29.18(3), 26.94, 22.62, 14.11.

(ES-MS/FAB-MS); *m/z* 299.1960 ($M+H^+$) $C_{15}H_{27}N_2O_4$, requires *m/z* 299.1971(Δ = 3.68 ppm).

5.4.2 3O, 4-aza C_{13}-HSL [2.9]

Ethyl 3-oxo-4-azatridecanoate [2.33]



Ethyl 3-oxo-4-azatridecanoate was synthesized as pink crystals in 66 % yield from *n*-nonylamine using Method-D.

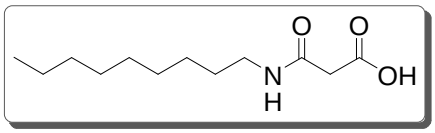
TLC-R_f = 0.45 in ethyl acetate-hexane (1:1)

mp = 185-187 °C.

FT-IR (KBr) cm^{-1}: 3294m, (νNH); 1744s (νC=O, ester); 1654s(νC=O, amide); 1560s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.21-1.32 (15H, m, CH$_3$(C*H*$_2$)$_6$- and C*H*$_3$CH$_2$-), 1.50-1.55 (2H, m, broad, *J* = 7.2 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.29 (2H, t, *J* = 7.2, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 3.31 (2H, s, -COC*H*$_2$CO-), 4.21 (2H, q, *J* = 7.2 Hz, CH$_3$C*H*$_2$-), 7.13 (1H, s, -(CH$_2$)$_7$N*H*-).

3-Oxo-4-azatridecanoic acid [2.39]



3-Oxo-4-azatridecanoic acid was synthesized as white crystals in 94 % yield from saponification of ethyl, 3-oxo-4-azatridecanoate using Method-C.

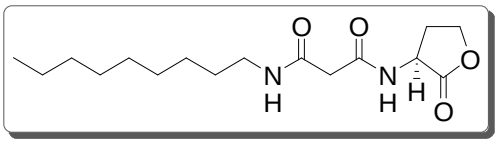
mp = 82-84 °C.

FT-IR (KBr) cm^{-1}: 3300s, (*ν*NH and OH); 1708s (*ν*C=O, acid); 1638s(*ν*C=O, amide); 1561s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.0 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.24-1.38 (12H, m, CH$_3$(C*H*$_2$)$_6$-), 1.53-1.58 (2H, m, broad, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.32 (2H, s, -COC*H*$_2$CO-), 3.35 (2H, t, *J* = 7.2, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 6.30 (1H, s, -N*H*-), 11.21 (1H, s, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 168.66, 168.18, 40.14, 38.22, 31.82, 29.43, 29.20, 29.17, 29.14, 26.80, 22.64, 14.08.

3O, 4-aza C$_{13}$-HSL [2.9]



3O, 4-aza C$_{13}$-HSL was synthesized as pinkish white crystals in 31 % yield from 3-oxo-4-azatridecanoic acid using Method-A.

TLC-R$_f$ = 0.70 in ethyl acetate-hexane (4:1)

mp = 122-124 °C.

FT-IR (KBr) cm^{-1}: 3301s, br. (*ν*NH); 1778s (*ν*C=O, lactone); 1679m (*ν*C=O, 1, 3-diamide); 1642s (*ν*C=O, amide); 1550s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.27-1.38 (12H, m, CH$_3$(C*H*$_2$)$_6$-), 1.51-1.56 (2H, m, broad, CH$_3$(CH$_2$)$_6$C*H*$_2$-) 2.26-2.31 (1H, m, ring, 4α-*H*), 2.72-2.79 (1H, m, ring, 4β-*H*), 3.24 (2H, s, -COC*H*$_2$CO), 3.28 (2H, t, *J* = 7.0 Hz,

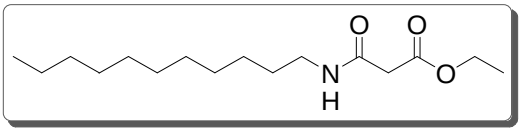
$CH_3(CH_2)_7CH_2$-), 4.26-4.33 (1H, m, ring, 5α-*H*), 4.48-4.53 (1H, m, ring, 5β-*H*), 4.55-4.61 (1H, m, ring, 3-*H*), 6.49 (1H s, broad, -N*H*), 7.78 (1H, d, *J* = 6.0 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.67, 167.67, 166.73, 65.81, 49.05, 42.46, 39.86, 31.85, 29.56, 29.48, 29.31, 29.25(2), 26.89, 22.67, 14.12.

(ES-MS/FAB-MS); *m/z* 313.2138 ($M+H^+$) $C_{16}H_{29}N_2O_4$, requires *m/z* 313.2127(Δ = 3.5 ppm).

5.4.3 3O, 4-aza C_{15}-HSL [2.10]

Ethyl 3-oxo-4-azapentadecanoate [2.34]



Ethyl 3-oxo-4-azapentadecanoate was synthesized as pale yellow crystals in 93 % yield from *n*-undecylamine using Method- D.

TLC-R_f = 0.41 in ethyl acetate-hexane (1:1)

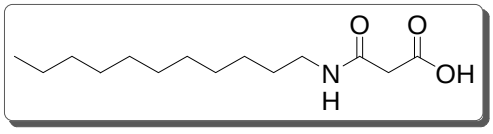
mp = 196-198 °C.

FT-IR (KBr) cm^{-1}: 3292s, (*ν*NH and OH); 1745s (*ν*C=O, ester); 1636s(*ν*C=O, amide); 1548s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_{10}$-), 1.25-1.37 (19H, m, $CH_3(CH_2)_8$- and CH_3CH_2-), 1.50-1.56 (2H, m, broad, *J* = 7.2 Hz, $CH_3(CH_2)_8CH_2$-), 3.28 (2H, t, *J* = 7.2, $CH_3(CH_2)_9CH_2$-), 3.31 (2H, s, -$COCH_2CO$-), 4.21 (2H, q, *J* = 7.2 Hz, CH_3CH_2-), 7.12 (1H, s, -$(CH_2)_7NH$-).

^{13}C NMR ($CDCl_3$) δ 168.13, 166.87, 61.52, 41.05, 39.60, 31.90, 29.57, 29.51(2), 29.36, 29.32, 29.27, 26.90, 22.68, 14.10, 14.05.

3-Oxo-4-azapentadecanoic acid [2.40]



3-Oxo-4-azapentadecanoic acid was synthesized as white crystals in 87 % yield from saponification of ethyl, 3-oxo-4-azapentadecanoate using Method-C.

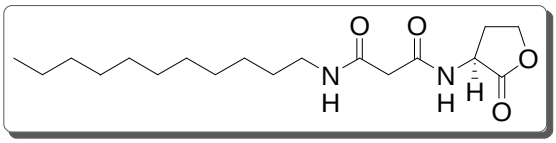
mp = 92-94 °C.

FT-IR (KBr) cm^{-1}: 3317s, (νNH and OH); 1708s (νC=O, acid); 1638s(νC=O, amide); 1553s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.0 Hz, C*H*$_3$(CH_2)$_{10}$-), 1.25-1.38 (16H, m, CH_3(C*H*$_2$)$_8$-), 1.53-1.59 (2H, m, broad, $CH_3(CH_2)_8CH_2$-), 3.31 (2H, s, -COC*H*$_2$CO-), 3.34 (2H, t, *J* = 7.2, $CH_3(CH_2)_9CH_2$-), 6.22 (1H, s, -N*H*-), 12.6 (1H, s, broad, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 171.68, 166.67, 40.17, 38.10, 31.89, 29.57, 29.54(2), 29.46, 29.30, 29.15, 26.80, 22.67, 14.10.

3O, 4-aza C_{15}-HSL [2.10]



3O, 4-aza C_{15}-HSL was synthesized as pinkish white crystals in 55 % yield from 3-oxo-4-azapentadecanoic acid using Method-A.

TLC-R_f = 0.52 in ethyl acetate-hexane (4:1)

mp = 123-125 °C.

FT-IR (KBr) cm^{-1}: 3293s, br. (νNH); 1771s (νC=O, lactone); 1686m (νC=O, 1, 3-diamide); 1644s (νC=O, amide); 1554s (δNH, amide).

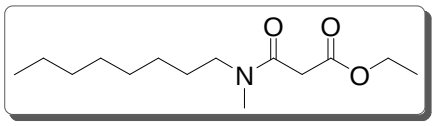
^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.0 Hz, C*H*$_3$(CH_2)$_{10}$-), 1.27-1.33 (16H, m, CH_3(C*H*$_2$)$_8$-), 1.50-1.58 (2H, m, broad, $CH_3(CH_2)_8CH_2$-) 2.25-2.30 (1H, m, ring, 4α-*H*), 2.72-2.78 (1H, m, ring, 4β-*H*), 3.25 (2H, s, -COC*H*$_2$CO), 3.27 (2H, t, *J* = 7.0 Hz, $CH_3(CH_2)_9CH_2$-), 4.26-4.33 (1H, m, ring, 5α-*H*), 4.48-4.53 (1H, m, ring, 5β-*H*), 4.55-4.62 (1H, m, ring, 3-*H*), 6.48 (1H s, -N*H*), 7.75 (1H, d, *J* = 6.0 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 175.17, 167.67, 166.13, 65.78, 54.12, 43.05, 41.46, 32.26, 30.25(2), 29.38, 29.19, 26.82, 22.74, 14.12.

(ES-MS/FAB-MS); *m/z* 341.2434 ($M+H^+$) $C_{18}H_{33}N_2O_4$, requires *m/z* 341.2440 (Δ = 1.76 ppm).

5.4.4 3O, 4-Me, 4-aza C_{12}-HSL [2.11]

Ethyl 3-oxo-4-methyl-4-azadodecanoate [2.35]

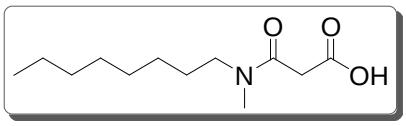


Ethyl 3-oxo-4-methyl-4-azadodecanoate was synthesized in 97 % yield from *N*-methyloctylamine using Method-D. Final product was recrystallized from ether.

TLC-R_f = 0.46 in ethyl acetate-hexane (1:1)

^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, *J* = 6.7 Hz, ($CH_3(CH_2)_7NCH_3$-), 1.26-1.32 (13H, m, $CH_3(CH_2)_5$- and OCH_2CH_3), 1.55 (2H, s, broad, $CH_3(CH_2)_5CH_2$-), 2.96 (3H, dd, *J* = 2.9, 8.6 Hz, -NCH_3), 3.24 (1H, *J* = 7.6 Hz, $CH_3(CH_2)_6CH$(H)-), 3.39 (1H, quint, *J* = 2.9, 7.6 Hz, $CH_3(CH_2)_6CH$(*H*)-), 3.44 (2H, d, *J* = 2.9 Hz, -$COCH_2CO$-), 4.2 (2H, 2 × q, - OCH_2CH_3).

3-Oxo-4-methyl-4-azadodecanoic acid [2.41]

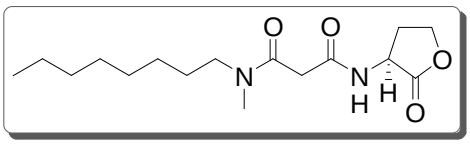


Saponification of ethyl, 3-oxo-4-methyl-4azadodecanoate afforded 3-oxo-4-methyl-4-azadodecanoic acid as white crystals in 93 % yield using Method-C.

FT-IR (KBr) cm^{-1}: 3296s, (*ν*OH); 1718s (*ν*C=O, acid); 1649(*ν*C=O, amide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.0 Hz, ($CH_3(CH_2)_7$-), 1.10-1.45 (10H, m, $CH_3(CH_2)_5$-), 1.58 (2H, s, broad, $CH_3(CH_2)_5CH_2$-), 3.03 (3H, s, -NCH_3), 3.24 and 3.44, (2H, t, *J* = 7.6 Hz, $CH_3(CH_2)_6CH_2$-*(Z and E-form)*), 3.37 and 3.39 (2H, 2 × s, -$COCH_2CO$-, *(Z and E –isomers)*), 10.15 (1H, s, -COO*H*).

3O, 4-Me, 4-aza C_{12}-HSL [2.11]



3O, 4-Me, 4-aza C_{12}-HSL was synthesized as a white solid from 3-oxo-4-methyl-4-azadodecanoic acid using Method-B. The product was purified as crystalline solid in 20 % yield by PLC using ethyl acetate-hexane (1:1).

TLC-R_f = 0.36 in ethyl acetate-hexane (1:1)

mp = 71-73 °C.

FT-IR (KBr) cm^{-1}: 3300s, br. (*ν*NH); 1777s (*ν*C=O, lactone); 1684m (*ν*C=O, 1, 3-diamide); 1655s and 1640s, (*ν*C=O, amide); 1557s (δNH, amide).

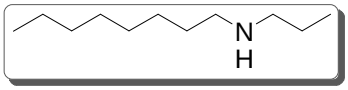
^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, *J* = 5.6 Hz, ($CH_3(CH_2)_7$-), 1.10-1.31 (10H, m, $CH_3(CH_2)_5$-), 1.53 (2H, s, broad, $CH_3(CH_2)_5CH_2$-), 2.29 (1H, m, ring, 4α-*H*), 2.66 (1H, m, ring, 4β-*H*), 2.96 and 3.03 (3H, 2 × s, NCH_3-*(E and Z isomers*), 3.30 (2H, t, *J* = 7.5 Hz, $CH_3(CH_2)_6CH_2$-), 3.38 (2H, dd, *J* = 4.9, 8.2 Hz, -$COCH_2CO$-), 4.28 (1H, m, ring, 3-*H*), 4.48 (1H, t, *J* = 9.1 Hz, ring, 5α-*H*), 4.6 (1H, m, ring, 5β-*H*), 8.84 (1H, d, *J* = 7.4 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.2, 166.62 (2), 65.88, 53.82, 42.97, 39.76, 31.92, 30.16, 29.72(2), 29.42(2), 26.81, 22.82, 14.02.

(ES-MS/FAB-MS); *m/z* 313.2134 ($M+H^+$) $C_{16}H_{29}N_2O_4$, requires *m/z* 313.2127 (Δ = 2.24 ppm).

5.4.5 3O, 4-*n*-Pr, 4-aza C_{12}-HSL [2.12]

N-Propyloctylamine [2.18]



N-Propyloctylamine was synthesized and purified as colorless oil in 58 % yield from *n*-propylamine using Method-F.

TLC-R_f = 0.43 in methanol-ammonia (9.6:0.4)

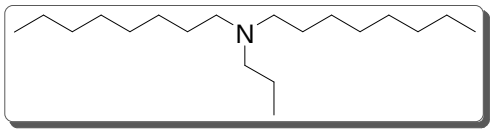
FT-IR (KBr) cm^{-1}: 3386m, br., (*ν*NH); 1460s (δNH); 1131s (*ν*C-N).

^{1}H NMR ($CDCl_3$) δ 0.86-0.94 (6H, m, *J* = 7.2 Hz, $CH_3(CH_2)_7$- and $CH_3(CH_2)_2$-), 1.20-1.35 (10H, m, $CH_3(CH_2)_5$-), 1.42 (1H, s, broad, -N*H*-), 1.47 (2H, m, broad, $CH_3(CH_2)_5CH_2$-), 1.53 (2H, quint., *J* = 7.2 Hz, $CH_3CH_2CH_2$-) 2.57 (2H, t, *J* = 7.4 Hz, $CH_3(CH_2)_6CH_2$-), 2.6 (2H, t, *J* = 7.6 Hz, $CH_3(CH_2)CH_2$-).

^{13}C NMR ($CDCl_3$) δ 51.99, 50.08, 31.85, 30.14, 29.56, 29.29, 27, 43, 23.19, 22.67, 14.11, 11.82.

(ES-MS/FAB-MS): *m/z* 172.2132 ($M+H^+$) $C_{11}H_{25}N$, requires *m/z* 172.2065 (Δ = 39 ppm).

N,N-Dioctylpropylamine [2.29]

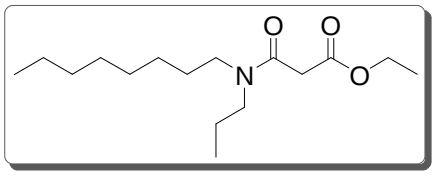


The side product was purified as colorless oil in 23 % yield.

TLC-R_f = 0.70 in methanol-ammonia (9.6:0.4)

^{1}H NMR ($CDCl_3$) δ 0.894 (9H, t (overlap), *J* = 7.0 Hz, 2C*H*$_3$(CH_2)$_7$- and C*H*$_3$(CH_2)$_2$-), 1.28-1.33 (20H, m, broad, 2CH_3(C*H*$_2$)$_5$-), 1.44-1.52 (6H, m, broad, 2CH_3(CH_2)$_5$C*H*$_2$- and CH_3C*H*$_2$$CH_2$-) 2.39-2.46 (6H, m, 2$CH_3$($CH_2$)$_6$C*H*$_2$- and CH_3CH_2C*H*$_2$-).

Ethyl 3-oxo-4-n-propyl-4-azadodecanoate [2.36]



Ethyl 3-oxo-4-*n*-propyl-4-azadodecanoate was synthesized as yellowish oil in 78 % yield from *N*-propyloctylamine using Method-E.

TLC-R_f = 0.60 in ethyl acetate-hexane (1:1)

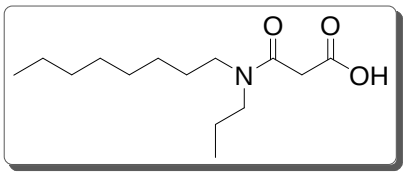
FT-IR (KBr) cm^{-1}: 1741s (*ν*C=O, ester); 1649s(*ν*C=O, amide).

^{1}H NMR ($CDCl_3$) δ 0.89-0.96 (6H, m, C*H*$_3$(CH_2)$_7$- and C*H*$_3$(CH_2)$_2$-), 1.24-1.31 (13H, m, CH_3(C*H*$_2$)$_5$- and C*H*$_3$$CH_2$O-), 1.52-1.62 (4H, m, broad, CH_3(CH_2)$_5$C*H*$_2$- and CH_3C*H*$_2$$CH_2$-) 3.20 (2H, q, *J* = 8.0 Hz, CH_3(CH_2)$_6$C*H*$_2$-), 3.32 (2H, t, *J* = 7.8 Hz, CH_3(CH_2)C*H*$_2$-), 3.45 (2H, s, -COC*H*$_2$CO-), 4.21 (2H, q, *J* = 7.2 Hz, CH_3C*H*$_2$O-).

^{13}C NMR ($CDCl_3$) δ 167.88, 165.74, 61.40, 50.16, 48.63, 41.27, 31.81, 29.38, 29.25, 27.47, 26.98, 22.64, 20.71, 14.12, 11.32.

(ES-MS/FAB-MS): *m/z* 286.2413 ($M+H^+$) $C_{16}H_{32}NO_3$, requires *m/z* 286.2382 (Δ = 10.8 ppm).

3-Oxo-4-propyl-4-azadodecanoic acid [2.42]



Saponification of ethyl, 3-oxo-4-propyl-4-azadodecanoate afforded 3-oxo-4-*n*- propyl-4-azadodecanoic acid as oil in 55 % yield using Method-C.

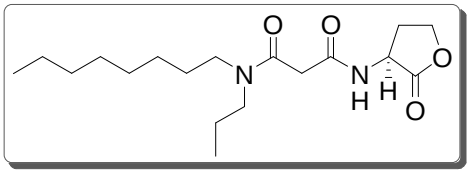
FT-IR (KBr) cm^{-1}: 3319s, (*ν*OH); 1738s (*ν*C=O, acid); 1629(*ν*C=O, amide).

^{1}H NMR ($CDCl_3$) δ 0.91-0.99 (6H, m, C*H*$_3$(CH$_2$)$_7$- and C*H*$_3$(CH$_2$)$_2$-), 1.29-1.35 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.56-1.67 (4H, m, broad, CH$_3$(CH$_2$)$_5$C*H*$_2$- and CH$_3$C*H*$_2$CH$_2$N-) 3.22 (2H, q, *J* = 7.8 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.34-3.40 (4H, m, CH$_3$(CH$_2$)C*H*$_2$- and -COC*H*$_2$CO-), 14.20 (1H, s, broad, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 172.13, 165.71, 88.41, 48.92, 47.19, 31.95, 29.41(2), 27.96, 27.13, 22.85, 21.53, 14.1, 11.58.

(ES-MS/FAB-MS): *m/z* 256.1906 (M - H^+) $C_{14}H_{26}NO_3$, requires *m/z* 256.1913 (Δ = 2.70 ppm)

3O, 4-n-Pr, 4-aza C_{12}-HSL [2.12]



3O, 4-Pr, 4-aza C_{12}-HSL was synthesized as yellowish semisolid product in 54 % yield from 3-oxo-4-*n*-propyl-4-azadodecanoic acid using Method-A.

TLC-R_f = 0.28 in ethyl acetate

mp = 47-49 °C.

FT-IR (KBr) cm^{-1}: 3288m, br. (*ν*NH); 1772s (*ν*C=O, lactone); 1654s and 1632s, (*ν*C=O, amide); 1552m (δNH, amide).

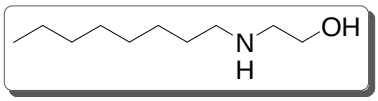
^{1}H NMR ($CDCl_3$) δ 0.87-0.98 (6H, m, C*H*$_3$(CH$_2$)$_7$- and C*H*$_3$(CH$_2$)$_2$N-), 1.24-1.36 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.50-1.67 (4H, m, broad, CH$_3$(CH$_2$)$_5$C*H*$_2$- and CH$_3$C*H*$_2$CH$_2$-) 2.23-2.43 (1H, m, ring, 4α-*H*), 2.68-2.75 (1H, m, ring, 4β-*H*), 3.23 (2H, m, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.31 (2H, m, CH$_3$(CH$_2$)C*H*$_2$-), 3.38 (2H, s, COC*H*$_2$CO), 4.23 (1H, m, ring, 5α-*H*), 4.48 (1H, m, ring, 5β-*H*), 4.64 (1H, m, ring, 3-*H*), 8.89 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.92, 166.67, 165.69, 65.81, 54.12, 48.71, 46.49, 41.97, 31.97, 30.29, 29.41(2), 27.93, 27.17, 22.80, 21.04, 14.12, 11.53.

(ES-MS/FAB-MS); *m/z* 341.2449 (M+H^+) $C_{18}H_{33}N_2O_4$, requires *m/z* 341.2440 (Δ = 2.64 ppm).

5.4.6 3O, 4-(2-hydroxyethyl), 4-aza C_{12}-HSL [2.13]

N-(2-Hydroxyethyl)octylamine [2.19]



N-(*2*-Hydroxyethyl)octylamine was synthesized and purified as an oil in 51 % yield from *2*-hydroxyethylamine using Method-F.

TLC-R_f = 0.44 in methanol-ammonia (9.6:0.4)

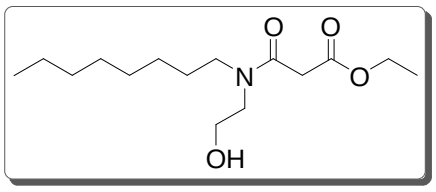
FT-IR (KBr) cm^{-1}: 3301s, broad, (νNH and OH); 1467s (δNH); 1121m and 1060m (νC-N).

^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_7$-), 1.25-1.29 (10H, m, $CH_3(CH_2)_5$-), 1.45-1.50 (2H, m, broad, $CH_3(CH_2)_5CH_2$-), 1.26 (2H, t, *J* = 7.2 Hz, $CH_3(CH_2)_6CH_2$-) 2.75 (2H, t, *J* = 5.2 Hz, -$CH_2CH_2CH_2OH$), 3.00 (2H s, broad, -N*H*- and -O*H*), 3.65 (2H, t, *J* = 5.2 Hz, $CH_2CH_2CH_2OH$).

^{13}C NMR ($CDCl_3$) δ 60.56, 51.24, 49.61, 31.81, 29.90, 29.49, 29.26, 27.30, 22.64, 14.08.

(ES-MS/FAB-MS): *m/z* 174.1892 ($M+H^+$) $C_{10}H_{24}NO$, requires *m/z* 174.1858 (Δ = 19 ppm).

Ethyl 3-oxo-4-(2-hydroxyethyl)-4-azadodecanoate [2.37]



Ethyl 3-oxo-4-(2-hydroxyethyl)-4-azadodecanoate was synthesized as a colorless viscous oil in 24 % yield from *N*-(hydroxyethyl)octylamine using Method-E. This reaction also yielded a compound ethyl, 3-oxo-4-(2-ethoxycarbonylacetyloxyethyl)-4-azadodecanoate **(2.44)** in 48 % yield. Column chromatographic separation in ethyl acetate-hexane (1:1) gave **2.37** and **2.44** in their pure forms.

TLC-R_f = 0.14 in ethyl acetate-hexane (1:1)

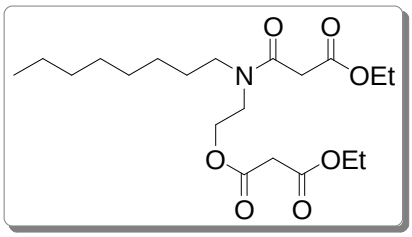
FT-IR (KBr) cm^{-1}: 3418s, (νOH); 1740s (νC=O, ester); 1633s(νC=O, amide).

^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_7$-), 1.28-1.32 (13H, m, $CH_3(CH_2)_5$- and CH_3CH_2O-), 1.59 (2H, m, broad, $CH_3(CH_2)_5CH_2$-), 2.47 (1H, s,

broad, -O*H*), 3.29 and 3.38 (2H, t, *J* = 7.6 Hz, $CH_3(CH_2)_6CH_2$-(*Z* and *E*-isomers)), 3.46 and 3.56 (2H, 2 × s, *J* = 5.6 Hz, $COCH_2CONH$- (*E* and *Z* isomers)), 3.48 and 3.61 (2H, 2 × s, $COCH_2CO$- (*Z* and *E* isomers)), 3.78 and 3.81 (2H, 2 × t, *J* = 5.0 Hz, -CH_2CH_2OH-(*E* and *Z*-isomers)), 4.22 (2H, q, *J* = 7.2 Hz, CH_3CH_2-).

(ES-MS/FAB-MS): *m/z* 288.2189 (M + H^+) $C_{15}H_{30}NO_4$, requires *m/z* 288.2175 (Δ = 4.85 ppm).

Ethyl, 3-oxo-4-(2-ethoxycarbonylacetyloxyethyl)-4-azadodecanoate [2.44]

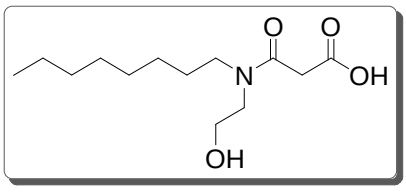


TLC-R_f = 0.43 in ethyl acetate-hexane (1:1)

^{1}H NMR ($CDCl_3$) δ 0.87-0.92 (3H, m, *J* = 6.8 Hz, $CH_3(CH_2)_7$-), 1.21-1.32 (16H, m, $CH_3(CH_2)_5$- and $2CH_3CH_2O$-), 1.56-1.59 (2H, m, broad, $CH_3(CH_2)_5CH_2$-), 3.28 and 3.37 (2H, t, *J* = 8.0 Hz, $CH_3(CH_2)_6CH_2$-(*Z* and *E*-isomers)), 3.39 and 3.42 (2H, 2 × s, $COCH_2CONH$- (*Z* and *E* isomers)), 3.45 and 3.51 (2H, 2 ×s, $COCH_2COO$- (*Z* and *E* isomers)), 3.57 and 3.62 (2H, 2 ×t, *J* = 5.8 Hz, -$CH_2CH_2CH_2O$-(*E* and *Z*-isomers)), 4.23 (4H, q, *J* = 7.2 Hz, $2CH_3CH_2$-), 4.28 and 4.33 (2H, 2 × t, *J* = 6.0 Hz, -CH_2CH_2O-(*E* and *Z*-isomers)).

(ES-MS/FAB-MS): *m/z* 424.2337 (M + Na) $C_{20}H_{35}NO_7Na$, requires *m/z* 424.2311 (Δ = 6.12 ppm).

3-Oxo-4-(2-hydroxyethyl)-4-azadodecanoic acid [2.43]



3-Oxo-4-(2-hydroxyethyl)-4-azadodecanoic acid was synthesized as white crystals in 83 % yield from saponification of ester **2.44** (0.5 mmol) with NaOH (1.2 mmol) using Method-C.

mp = 68-70 °C.

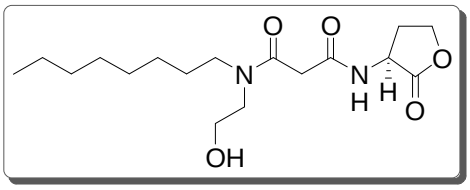
FT-IR (KBr) cm^{-1}: 3392s, br., (*ν*NH and OH); 1727s (*ν*C=O, acid); 1629(*ν*C=O, amide).

^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_7$-), 1.28-1.33 (10H, m, $CH_3(CH_2)_5$-), 1.60 (2H, s, broad, $CH_3(CH_2)_5CH_2$-), 2.52 (1H, s, broad, -O*H*), 3.35 and 3.43 (2H, 2 × t, *J* = 8.0 Hz, $CH_3(CH_2)_6CH_2$-(*Z* and *E*-isomers)), 3.44 and 3.58 (2H, 2 × s, $COCH_2CO$ (*Z* and *E* isomers)), 3.47 and 3.62 (2H, 2 × t, -CH_2CH_2OH (*Z* and *E* isomers)), 3.83 and 3.85 (2H, 2 × t, *J* = 5.2 Hz, -CH_2CH_2OH-(*E* and *Z*-isomers)), 10.23 (1H, s, broad -COO*H*).

^{13}C NMR ($CDCl_3$) δ 171.23, 165.27, 60.93, 59.71, 49.47, 35.01, 31.76, 29.21, 28.53, 27.31, 26.91, 22.60, 14.08.

(ES-MS/FAB-MS): *m/z* 258.1702 ($M - H^+$) $C_{13}H_{24}NO_4$, requires *m/z* 258.1705 (Δ = 1.16 ppm)

3O, 4-(2-hydroxyethyl), 4-aza C_{12}-HSL [2.13]



3O, 4-(2-hydroxyethyl), 4-aza C_{12}-HSL was synthesized as white crystals in 44 % yield from 3-Oxo-4(2-hydroxyethyl)-4-azadodecanoic acid using Method-A.

mp = 54-56 °C.

FT-IR (KBr) cm^{-1}: 3309s, br. (*ν*NH and OH); 1775s (*ν*C=O, lactone); 1684m (*ν*C=O, 1, 3-diamide); 1655s and 1637s, (*ν*C=O, amide); 1542s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.85-0.91 (3H, m, $CH_3(CH_2)_7$-), 1.24-1.30 (10H, m, $CH_3(CH_2)_5$-), 1.55-1.60 (2H, m, broad, $CH_3(CH_2)_5CH_2$-) 2.25-2.36 (1H, m, ring, 4α-*H*), 2.40 (1H, s, broad, -O*H*), 2.68-2.75 (1H, m, ring, 4β-*H*), 3.37 (2H, m, $CH_3(CH_2)_6CH_2$-), 3.47 (2H, s, $COCH_2CO$), 3.56 (2H, m, -CH_2CH_2OH), 4.29 (1H, m, ring, 5α-*H*), 4.49 (1H, m, ring, 5β-*H*), 4.64 (1H, m, ring, 3-*H*), 8.43 and 8.59 (1H, 2 × d, *J* = 6.8 Hz, -N*H*-HSL (*E* and *Z* isomers).

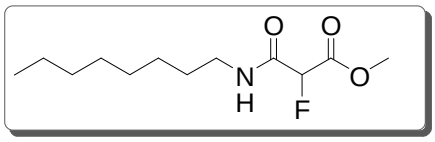
^{13}C NMR ($CDCl_3$) δ 175.13, 166.6, 165.6, 65.79, 59.27, 54.87, 49.64, 46.51, 40.79, 31.72, 30.3, 29.43(2), 27.85, 27.15, 22.80, 14.1.

(ES-MS/FAB-MS): *m/z* 343.2219 ($M+H^+$) $C_{17}H_{31}N_2O_5$, requires *m/z* 343.2233 (Δ = 4.08 ppm); *m/z* 365.2037 (M+Na) $C_{17}H_{30}N_2O_5Na$, requires *m/z* 365.2052 (Δ = 4.1ppm).

5.5 Synthesis of fluorinated 4-aza analogues of 3O, C_{12}-HSL

5.5.1 3O, 2-F, 4-Aza C_{12}-HSL [2.62]

Methyl, 2-fluoro-3-oxo 4-azadodecanoate [2.69]



To a stirred solution of dimethyl fluoromalonate **2.65** (1 mmol, 0.1501 g) in anhydrous methanol (10 mL) at room temperature was added dropwise a solution of *n*-octylamine (1 mmol, 166 μl) in methanol (5 mL) over a period of 1 h and stirring was continued for further 2 h. The solution was rotary evaporated and the residue redissolved in ethyl acetate (20 mL). The ethyl acetate solution was sequentially washed with 2 M HCl (2 × 10 mL) and brine (1 × 15 mL) solution. The organic layer was separated, dried over $MgSO_4$, and rotary evaporated to yield a mixture of bisamide **2.67** and methyl, 2-fluoro-3-oxo 4-azadodecanoate. The PLC separation in petroleum ether b.p. 40-60 ^{0}C-diethyl ether (3:2) gave the titled product **2.69** in 47 % yield.

TLC-R_f - 0.23 in petroleum ether-diethyl ether (2:3)

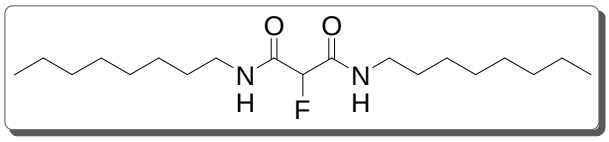
mp = 38-40 °C.

FT-IR (KBr) cm^{-1}: 3293s, (*ν*NH); 1762s (*ν*C=O, ester); 1668s(*ν*C=O, amide); 1576w (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, *J* = 6.4 Hz, $CH_3(CH_2)_7$-), 1.26-1.34 (10H, m, $CH_3(CH_2)_5$-), 1.50-1.58 (2H, m, broad, $CH_3(CH_2)_5CH_2$-), 3.32-3.38 (2H, m, $CH_3(CH_2)_6CH_2$-), 3.87 (3H, s, CH_3O-), 5.38 (1H, d, *J* = 31.2, COCF(*H*)CO), 6.41 (1H, s, broad, -N*H*-).

(ES-MS/FAB-MS): *m/z* 248.1648 ($M+H^+$) $C_{12}H_{23}FNO_3$, requires *m/z* 248.1662 (Δ = 5.64 ppm).

N^1, N^3-dioctylfluoromalonamide [2.67]



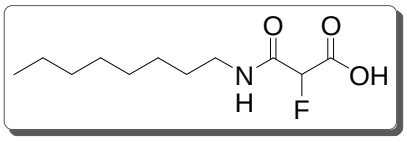
Bisamide N^1, N^3-dioctylfluoromalonamide was isolated by PLC as a white solid.

TLC-R_f - 0.08 in petroleum ether-diethyl ether (2:3)

^{1}H NMR ($CDCl_3$) δ 0.89 (6H, t, *J* = 7.0 Hz, $CH_3(CH_2)_7$-), 1.3 (20H, m, 2 × $CH_3(CH_2)_5$-), 1.53 (4H, s, broad, 2 × $CH_3(CH_2)_5CH_2$-), 3.3 (4H, t, *J* = 6.7 Hz, 2 × $CH_3(CH_2)_6CH_2$-), 5.18 (1H, d, *J* = 38.0 Hz, CF(*H*)), 6.9 (2H, s, 2 × (-N*H*-)).

(ES-MS/FAB-MS): *m/z* 345.2938 (M +H^+) $C_{19}H_{38}FN_2O_2$, requires *m/z* 345.2917 (Δ = 6.1 ppm)

2-Fluoro-3-oxo-4-azadodecanoic acid [2.70]

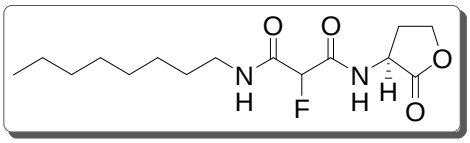


To a stirred solution of methyl, 2-fluoro-3-oxo-4-azadodecanoate **2.69** (0.2 mmol, 0.047 g) in methanol (15 mL), was added dropwise cold NaOH (0.2 mmol, 0.08 g) solution in water (5 mL). The stirring was continued at room temperature for 2 h. The mixture was rotary evaporated to remove solvents. The residue was redissolved in water (15 mL) and washed with ethyl acetate (2 × 5 mL). Aqueous layer was acidified to pH 2 with 4 M HCl and the free acid was extracted with ethyl acetate (3×10 mL), dried over $MgSO_4$, filtered and solvent rotary evaporated to give the titled monofluoro acid **2.70** in a 98 % yield.

FT-IR (KBr) cm^{-1}: 3323s, br., (*ν*NH and OH); 1762s (*ν*C=O, acid); 1668s (*ν*C=O, amide); 1576w (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.4 Hz, $CH_3(CH_2)_7$-), 1.26-1.33 (10H, m, $CH_3(CH_2)_5$-), 1.54-1.62 (2H, m, broad, $CH_3(CH_2)_5CH_2$-), 3.37-3.42 (2H, m, $CH_3(CH_2)_6CH_2$-), 5.35 (1H, d, *J* = 47.2, COCF(*H*)CO), 6.71 (1H, broad s, -N*H*-), 7.60 (1H, s, broad, -COO*H*).

3O, 2-F, 4-Aza C_{12}-HSL [2.62]



To a stirred solution of 2-fluoro-3-oxo 4-azadodecanoic acid **2.70** (1 mmol, 0.233 g) in dry DCM (15 ml) was added sequentially L-HSL HCl (1 mmol, 0.1375 g), HOBt (1.2 mmol, 0.162 g) and finally DCCI (1.1 mmol, 0. 227 g). The stirring was continued for 16 h and solvent was removed under high vacuo. The residue was redissolved in ethyl acetate (25 mL). The organic solution was washed with saturated sodium bicarbonate (3 × 10 mL), 1 M $KHSO_4$ (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated. The residue was redissolved in cold acetone (2-4 mL) and left aside. When the remaining DCU crystallized out, this was carefully removed to obtain oil. The product **2.62** was isolated by PLC in ethyl acetate as a white solid in 11 % yield.

mp = 53-53 °C.

TLC-R_f = 0.32 in ethyl acetate

FT-IR (KBr) cm^{-1}: 3292s, (*ν*NH); 1765s (*ν*C=O, lactone); 1669s (*ν*C=O, 1, 3-diamide); 1557m (δNH, amide).

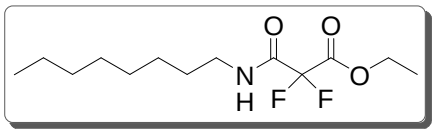
^{1}H NMR ($CDCl_3$) 0.89 (3H, t, *J* = 6.9 Hz, $CH_3(CH_2)_7$-), 1.3 (10H, m, $CH_3(CH_2)_5$-), 1.56 (2H, s, broad, $CH_3(CH_2)_5CH_2$-), 2.27 (1H, m, ring, 4α-*H*), 2.80 (1H, m, ring, 4β-*H*), 3.33 (2H, m, $CH_3(CH_2)_6CH_2$-), 4.28 (1H, m, ring, 3*H*), 4.53 (1H, m, ring, 5α-*H*), 4.64 (1H, m, ring, 5β-*H*), 5.3 (1H, dd, *J* = 10.2, 38.0 Hz, -COCF(*H*)CO-), 6.63 (1H, s, $CH_3(CH_2)_6CH_2NH$-), 7.5 (1H, dd, *J* = 13.3, 6.4 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.87, 167.86(2), 106.36, 58.18, 56.12, 41.61, 31.79, 28.87(2), 27.82, 26.12, 22.80, 14.17.

(ES-MS/FAB-MS): *m/z* 317.1887 ($M+H^+$) $C_{15}H_{26}FN_2O_4$, requires *m/z* 317.1877 (Δ = 3.15 ppm).

5.5.2 3O, 2-F_2, 4-Aza C_{12}-HSL [2.63]

Ethyl 2, 2-difluoro-3-oxo-4-azadodecanoate [2.68]



To a stirred solution of diethyl difluoromalonate **2.64** (2 mmol, 0.3923 g) in ethanol (30 mL) was added dropwise *n*-octylamine (2 mmol, 331 µl) over a period of 2 h at room temperature. The solution was rotary evaporated and the residue redissolved in ethyl acetate (30 mL). The ethyl acetate solution was sequentially washed with 2 M HCl (2 × 15 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$ and rotary evaporated to obtain a mixture of ethyl, 2, 2-difluoro-3-oxo-4-azadodecanoate **2.68** and bisamide **2.66**. Desired product was isolated as a white solid by PLC in 50 % yield using petroleum ether b.p. 40-60 ^{0}C-diethyl ether (1:1).

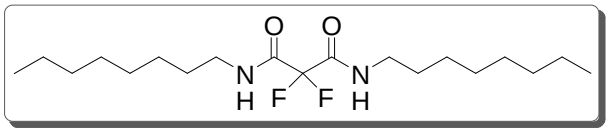
TLC-R_f = 0.46 in petroleum ether-diethyl ether (1:1)

mp = 39-41 °C.

^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.26-1.1.36 (13H, m, CH$_3$(C*H*$_2$)$_5$- and C*H*$_3$CH$_2$O-), 1.50-1.60 (2H, s, broad, *J* = 7.0 Hz, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.36 (2H, q, *J* = 6.8 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 4.38 (2H, q, *J* = 7.0, CH$_3$ C*H*$_2$O-), 6.40 (1H, s, broad, -N*H*-).

(ES-MS/FAB-MS): *m/z* 280.1720 (M +H$^+$) $C_{13}H_{24}F_2NO_3$, requires *m/z* 280.1724 (Δ = 1.42 ppm).

N^1, N^3-Dioctyldifluoromalonamide [2.66]



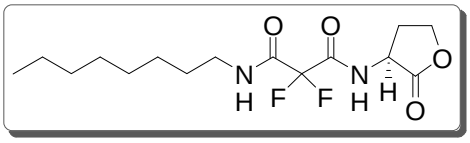
Bisamide N^1, N^3-dioctyldifluoromalonamide was isolated by PLC as a white solid.

TLC-R_f = 0.08 in petroleum ether-diethyl ether (1:1)

^{1}H NMR ($CDCl_3$) δ 0.88 (6H, t, *J* = 6.5 Hz, 2 × C*H*$_3$(CH$_2$)$_7$-), 1.28 (20H, m, 2 × CH$_3$(C*H*$_2$)$_5$-), 1.56 (4H, s, broad, 2 × CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.33 (4H, t, *J* = 6.71 Hz, 2 × CH$_3$(CH$_2$)$_6$C*H*$_2$-), 6.72 (2H, s, 2 × (-N*H*-)).

(ES-MS/FAB-MS): *m/z* 363.2902 (M +H^+) $C_{19}H_{37}F_2N_2O_2$, requires *m/z* 363.2823 (Δ = 21.7 ppm)

3O, 2-F_2, 4-Aza C_{12}-HSL [2.63]



To a stirred solution of ethyl 2, 2-difluoro-3-oxo-4-azadodecanoate **2.68** (0.1 mmol, 0.028 g) in ethanol (5 mL) was added L-HSL HCL (0.11 mmol, 0.014 g) and TEA (0.15 mmol, 21 μl). It was then refluxed overnight. The solution was cooled to room temperature and solvent was rotary evaporated under vacuo. The residue was redissolved in ethyl acetate (15 mL). The ethyl acetate solution was sequentially washed with saturated sodium bicarbonate (2 × 10 mL), 2 M HCl (2 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain a crude product which was purified by PLC in ethyl acetate-hexane (1:1) in 15 % yield as white crystals.

TLC-R_f = 0.27 in ethyl acetate-hexane (1:1)

mp = 66-68 °C.

FT-IR (KBr) cm^{-1}: 3304s, br. (*ν*NH); 1778m (*ν*C=O, lactone); 1697s (*ν*C=O, 1, 3 diamide); 1550s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.5 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.27-1.32 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.52-1.55 (2H, m, broad, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.26-2.32 (1H, m, ring, 4α-*H*), 2.79-2.93 (1H, m, ring, 4β-*H*), 3.35 (2H, q, *J* = 6.8 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 4.27-4.35 (1H, m, ring, 5α-*H*), 4.49-4.56 (1H, m, ring, 5β-*H*), 4.58-4.65 (1H, m, ring, 3-*H*), 6.6 (1H s, -N*H*), 7.41 (1H, d, *J* = 6.0 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 173.97, 168.79(2), 137.61, 68.24, 57.23, 43.17, 32.91, 29.97(2), 28.42(2), 27.02, 23.10, 14.10.

(ES-MS/FAB-MS): *m/z* 335.1769 (M +H^+) $C_{15}H_{25}F_2N_2O_4$, requires *m/z* 335.1782 (Δ = 3.88 ppm).

5.6 Synthesis of 3-aza analogues of 3O, C_{12}-HSL

5.6.1 Method-G: *N-substituted nonylamine by reductive amination*

O-Benzylhydroxylamine (2 mmol, 0.2503 g) was mixed with methanol (20 mL) at 0 °C, whereas *O-tert*-butylhydroxylamine was released from its hydrochloride salt (2 mmol, 0.252 g) in methanol (20 mL) at 0 °C by adding sodium methoxide (sodium metal- 2 mmol, 0.051 g). The pH was adjusted below 4.5 with gacial acetic acid. The 1-nonanal (2 mmol, 344 µL) was added and the mixture was allowed to warm to room temperature. The pH was maintained by the addition of further gacial acetic acid. After 1 h stirring, the reaction mixture was cooled to 0 °C, and first portion of sodium cyanoborohydride (2.2 mmol, 0.1382 g) was added. The pH was maintained by addition of glacial acetic acid and the reaction was warmed to room temperature. After intervals of 1 h at pH 4 second and third portions of sodium cyanoborohydride (1.1 mmol, 0.063 g, each time) were added. The reaction mixture was then stirred for 4 h at room temperature and solvent was removed to dryness under high vacuo. The residue was redissolved in ethyl acetate (40 mL) and sequentially washed with water (3 × 10 mL), saturated sodium bicarbonate (3 × 15 mL) and brine (1 × 20 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain the title product as an oil.

5.6.2 Method-H: *Benzyl or tert-butyl ester(s) of N-substituted 3-azadodecanoic acid(s)*

To a stirred solution of an appropriate *N*-substituted nonylamine (4 mmol) in THF (30 mL) containing DIPEA (8 mmol) was added dropwise benzyl 2-bromoacetate or *tert*-butyl bromoacetate (8 mmol) and the mixture refluxed overnight. The solution was cooled to room temperature and rotary evaporated under vacuo. The residue was redissolved in ethyl acetate (40 mL). The ethyl acetate solution was sequentially washed with water (3 × 10 mL), saturated sodium bicarbonate (3 × 15 mL), 1 M $KHSO_4$ (2 × 15 mL) and brine (1 × 20 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain the title products as oils.

5.6.3 **Method-I:** *Removal of benzyl group by catalytic hydrogenation.*

An appropriate *O*-benzyl or benzyl ester product (2 mmol) was dissolved in absolute ethanol (10 mL). To this was added Pd/C (10 % w/w) and the heterogeneous mixture was stirred for 16 h under an atmosphere of hydrogen gas. The catalyst was filtered off using celite as a filter aid. The filtrate was evaporated under vacuo to give the de-benzylated product.

5.6.4 **Method-J:** *Coupling of L-HSL to N-substituted 3-azadodecanoic acids*

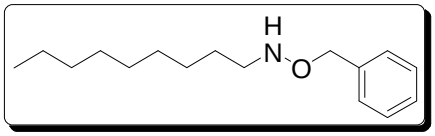
To a stirred solution of an appropriate 3-azadodecanoic acid (0.5 mmol) in dioxane (3 mL) was added a solution of L-HSL.HCl (0.5 mmol) in a water (about 1 mL). To this was added sequentially TEA (1.5 mmol) and EDC.HCl (0.75 mmol) and stirring was continued for 16 h (6 h for **2.71**) at room temperature. Solvent was removed under high vacuo and residue was extracted with warm ethyl acetate (20 mL). Organic solution was sequentially washed with saturated sodium bicarbonate (2 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain desired products.

5.6.5 **Method-K:** *Deprotection of tert-butyl or Boc group*

The acid-catalyzed deprotection of the *tert*-butyl group or *Boc* group was carried out by using excess of trifluoroacetic acid (TFA). Solution of TFA (50 % v/v) in DCM (3 mL) was added to *O-tert*-butyl or *N-Boc* group containing compound (0.1 mmol). The mixture was stirred for 2 h. The excess TFA was flushed out under vacuo by repeatedely dissolving in acetonitrile (3 × 10 mL) and finally under as stream of nitrogen. The residue was redissolved in ethyl acetate (15 mL) and filtered. The filtrate was concentrated under vacuo to obtain the deprotected product.

5.6.6 3-BnO, 3-aza C_{12}-HSL [2.77]

N-(Benzyloxy)nonylamine [2.74]



Reductive amination (Method-G) of 1-nonanal gave mixture of *N*-(benzyloxy)nonylamine and the intermediate oxime **2.73** (32 % yield). The product

was isolated as an oil by column chromatography using ethyl acetate-hexane (1:9) in 58 % yield.

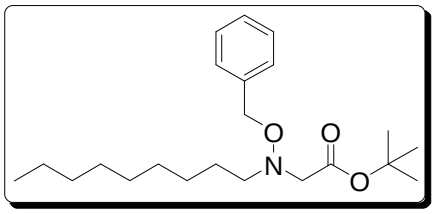
TLC-R_f = 0.25 in ethyl acetate-hexane (1:9)

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH_2)$_8$-), 1.25-1.38 (12H, m, CH_3(C*H*$_2$)$_6$-), 1.49-1.58 (2H, quint, *J* = 7.2, CH_3(CH_2)$_6$C*H*$_2$-), 2.95 (2H, t, *J* = 7.2 Hz, CH_3(CH_2)$_7$C*H*$_2$-), 4.73 (2H, s, -C*H*$_2$C$_6$H$_5$), 7.30-7.35 (5H, br, m, -C$_6$*H*$_5$).

^{13}C NMR ($CDCl_3$) δ 138.02, 128.38(2), 128.36(2), 127.78, 76.20, 52.24, 31.89, 29.54(2), 29.28, 27.33, 27.20, 22.69, 14.13.

(ES-MS/FAB-MS): *m/z* 250.2159 (M+H$^+$) $C_{16}H_{28}NO$, requires *m/z* 250.2171 (Δ = 4.8 ppm).

tert-Butyl 3-benzyloxy-3-azadodecanoate [2.75]



N-Alkylation of *N*-(benzyloxy)nonylamine **2.74** with *tert*-butyl bromoacetate gave desired product *tert*-butyl, 3-benzyloxy-3-azadodecanoate as a viscous oil in 89 % yield (Method-H).

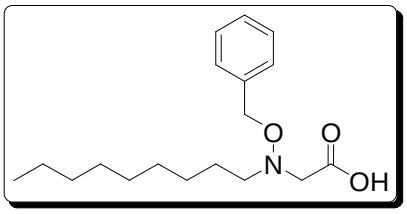
TLC-R_f = 0.35 in ethyl acetate-hexane (1:9)

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH_2)$_8$-), 1.25-1.36 (12H, m, CH_3(C*H*$_2$)$_6$-), 1.51 (9H, s, -C(C*H*$_3$)$_3$), 1.61-1.64 (2H, m, CH_3(CH_2)$_6$C*H*$_2$-), 2.80 (2H, t, *J* = 7.6 Hz, CH_3(CH_2)$_7$C*H*$_2$-), 3.47 (2H, s, -COC*H*$_2$CO-), 4.88 (2H, s, -C*H*$_2$C$_6$H$_5$), 7.34-7.35 (5H, m, broad, -C$_6$*H*$_5$).

^{13}C NMR ($CDCl_3$) δ 168.57, 137.37, 128.79(2), 128.31(2), 127.86, 81.07, 75.34, 61.25, 59.48, 31.91, 29.54(2), 29.30(2), 28.13(3), 27.23, 22.70, 14.15.

(ES-MS/FAB-MS): *m/z* 364.2867 (M -H$^+$) $C_{22}H_{38}NO_3$, requires *m/z* 364.2852 (Δ = 4.1 ppm)

3-Benzyloxy-3-azadodecanoic acid [2.76]

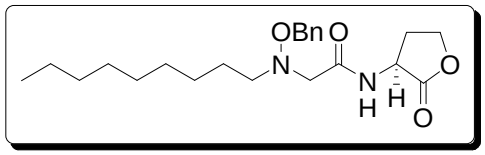


3-Benzyloxy-3-azadodecanoic acid was synthesized from *t*-butyl, 3-benzyloxy-3-azadodecanoate **2.75** by the acid-catalyzed deprotection of the *tert*-butyl group using TFA. The desired 3-azadodecanoic acid was obtained in 73 % yield.

^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_8$-), 1.23-1.39 (12H, m, $CH_3(CH_2)_6$-), 1.57-1.64 (2H, quint, *J* = 7.2, $CH_3(CH_2)_6CH_2$-), 2.88 (2H, t, *J* = 7.6 Hz, $CH_3(CH_2)_7CH_2$-), 3.61 (2H, s, -$COCH_2CO$-), 4.83 (2H, s, -$CH_2C_6H_5$), 7.31-7.36 (5H, m, broad, -C_6H_5), 10.38 (1H, s, broad, COO*H*).

(ES-MS/FAB-MS): *m/z* 306.2078 ($M-H^+$) $C_{18}H_{28}NO_3$, requires *m/z* 306.2069 (Δ = 2.93 ppm)

3-BnO, 3-aza C_{12}-HSL [2.77]



The 3-BnO, 3-aza C_{12}-HSL was obtained from 3-BnO-3-azadodecanoic acid **2.76** using Method-J. The column chromatography in ethyl acetate gave pure solid product in 27 % yield.

TLC-R_f = 0.48 in ethyl acetate

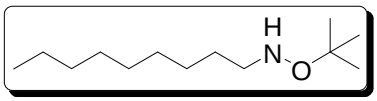
mp = 82-84 °C.

FT-IR (KBr) cm^{-1}: 3403m, 3378m (*ν*NH, amide); 1778s (*ν*C=O, lactone); 1678s (*ν*C=O, amide); 1664s (*ν*C=C, aromatic); 1519m (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_8$-), 1.23-1.37 (12H, m, $CH_3(CH_2)_6$-), 1.54-1.59 (2H, m, $CH_3(CH_2)_6CH_2$-), 1.94-2.05 (1H, m, ring, 4α-*H*), 2.75-2.81 (1H, m, ring, 4β-*H*), 2.82-2.87 (2H, dt, *J* = 7.2, 2.0 Hz, $CH_3(CH_2)_7CH_2$-), 3.49 (2H, s, -$COCH_2CO$-), 4.24-4.31 (1H, m, ring, 5α-*H*), 4.43-4.47 (1H, m, ring, 5β-*H*), 4.48-4.55 (1H, m, ring, 3-*H*), 4.75 (2H, dd, *J* = 28.4, 11.2 Hz, -$CH_2C_6H_5$), 7.17 (1H, d, *J* = 6.4 Hz, -N*H*-HSL), 7.31-7.36 (5H, m, broad, -C_6H_5),

5.6.7 3-OH, 3-aza C_{12}-HSL [2.71]

N-(tert-Butoxy)nonylamine [2.79]



This was synthesized using Method-G, the obtained oil was found as a mixture of desired amine and the intermediate oxime **2.78** (11 % yield). The desired product was isolated by column chromatography using ethyl acetate-hexane (1:9) in 72 % yield.

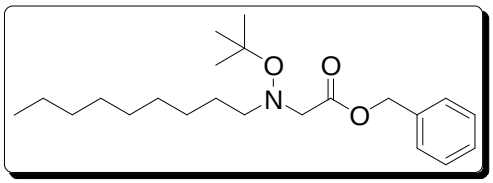
TLC-R_f = 0.51 in ethyl acetate-hexane (1:9)

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH_2)$_8$-), 1.19 (9H, s, -C(C*H*$_3$)$_3$), 1.92-1.34 (12H, m, CH_3(C*H*$_2$)$_6$-), 1.45-1.48 (2H, m, CH_3(CH_2)$_6$C*H*$_2$-), 2.86 (2H, t, *J* = 7.2 Hz, CH_3(CH_2)$_7$C*H*$_2$-), 4.88 (1H, s, broad, -N*H*-).

^{13}C NMR ($CDCl_3$) δ 79.10, 42.83, 32.14, 29.52, 28.87(2), 27.38(3), 27.10, 26.92, 22.58, 14.13.

(ES-MS/FAB-MS): *m/z* 216.2341 ($M+H^+$) $C_{13}H_{30}NO$, requires *m/z* 216.2327 (Δ = 6.47 ppm)

Benzyl 3-tert-butoxy-3-azadodecanoate [2.80]

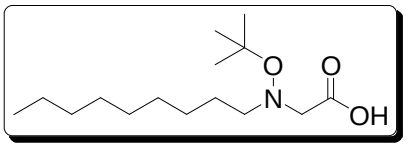


Benzyl, 3-*tert*-butoxy-3-azadodecanoate was synthesized by *N*-alkylation of *N*-(*tert*-butyl-oxy)nonylamine **2.79** with benzyl 2- bromoacetate to obtain the title compound as an oil in 85 % yield (Method-H).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH_2)$_8$-), 1.19 (9H, s, -C(C*H*$_3$)$_3$), 1.27-1.35 (12H, m, CH_3(C*H*$_2$)$_6$-), 1.38-1.43 (2H, m, CH_3(CH_2)$_6$C*H*$_2$-), 2.86 (1H, t, *J* = 7.2 Hz, CH_3(CH_2)$_7$C(H)*H*-), 2.94 (1H, s, broad, CH_3(CH_2)$_7$C(*H*)H-), 3.62 (2H, d, *J* = 12.8 Hz, -NC*H*$_2$CO-), 5.17 (2H, s, -C*H*$_2$$C_6H_5$), 7.32-7.39 (5H, m, broad, -C_6*H*$_5$).

(ES-MS/FAB-MS): *m/z* 364.2878 ($M-H^+$) $C_{22}H_{38}NO_3$, requires *m/z* 364.2852 (Δ = 7.1 ppm)

3-t-BuO-3-azadodecanoic acid [2.81]



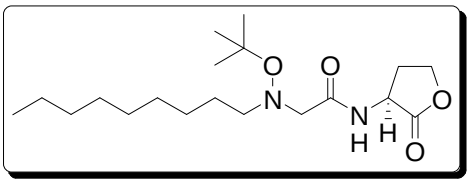
3-*t*-BuO-3-azadodecanoic acid was obtained from its benzyl ester **2.80** by catalytic hydrogenation in 85 % yield using Method-I.

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 7.0 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.19-1.28 (21H, m, -C(C*H*$_3$)$_3$ and CH$_3$(C*H*$_2$)$_6$-), 1.35-1.46 (2H, m, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.86 (2H, t, broad, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 3.68 (2H, s, -NC*H*$_2$CO-), 10.23 (1H, s, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 171.18, 75.17, 61.21, 59.60, 31.84, 29.49, 29.46, 29.24, 27.23(3), 26.99, 26.81, 22.68, 14.10.

(ES-MS/FAB-MS): *m/z* 272.2238 (M-H$^+$) $C_{15}H_{30}NO_3$, requires *m/z* 272.2226 (Δ = 4.40 ppm)

3-t-BuO, 3-aza C_{12}-HSL [2.82]



The 3-*t*-BuO, 3-aza C_{12}-HSL was obtained from 3-*t*-BuO-3-azadodecanoic acid **2.81** using Method-J. The column chromatography in acetone gave pure desired product as a yellow solid in 55 % yield.

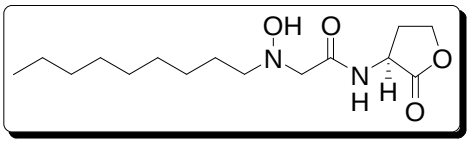
TLC-R_f = 0.47 in acetone

FT-IR (KBr) cm^{-1}: 3372m (*ν*NH, amide); 1775s (*ν*C=O, lactone); 1761s (*ν*C=O, amide); 1519m (δNH, amide); 1368m (δC-H, *tert*-butyl).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.20 (9H, s, -C(C*H*$_3$)$_3$), 1.23-1.27 (12H, s, CH$_3$(C*H*$_2$)$_6$-), 1.38-1.51 (2H, s, broad, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.10-2.21 (1H, m, ring, 4α-*H*), 2.79 (2H, s, broad, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 2.84-2.91 (1H, m, ring, 4β-*H*), 3.59(2H, s, broad, -NC*H*$_2$CO-), 4.28-4.34 (1H, m, ring, 5α-*H*), 4.47-4.52 (1H, m, ring, 5β-*H*), 4.48-4.55 (1H, m, ring, 3-*H*), 7.54 (1H, d, *J* = 5.6 Hz, -N*H*-HSL).

(ES-MS/FAB-MS): *m/z* 357.2762 (M +H$^+$) $C_{19}H_{37}N_2O_4$, requires *m/z* 357.2753 (Δ = 2.52 ppm)

3-OH, 3-aza C_{12}-HSL [2.71]



The 3-OH, 3-aza C_{12}-HSL was synthesized as white crystals in 97 % yield from 3-*t*-BuO, 3-aza C_{12}-HSL **2.82** using Method-K.

FT-IR (KBr) cm^{-1}: 3480 w, broad, (*ν*N-OH); 1769s (*ν*C=O, lactone); 1678s (*ν*C=O, amide); 1520s (δNH, amide).

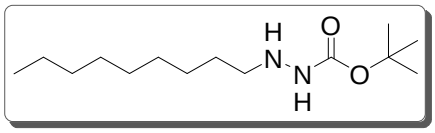
^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_8$-), 1.21-1.37 (12H, m, $CH_3(CH_2)_6$-), 1.74 (2H, s, broad, $CH_3(CH_2)_6CH_2$-), 1.94 (2H, t, *J* = 7.2 Hz, $CH_3(CH_2)_7CH_2$-), 2.33-2.38 (1H, m, ring, 4α-*H*), 2.76-2.81 (1H, m, ring, 4β-*H*), 3.89(2H, t, *J* = 7.2 Hz, -NCH_2CO-), 4.28-4.34 (1H, m, ring, 5α-*H*), 4.50-4.54 (1H, m, ring, 5β-*H*), 4.65-4.71 (1H, m, ring, 3-*H*), 7.13 (1H, s, -N-O*H*), 10.37 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 175.63, 171.53, 74.23, 59.17, 57.31, 49.03, 31.80(2), 29.34, 29.15, 29.11, 26.51, 22.64(2), 14.08.

(ES-MS/FAB-MS): *m/z* 301.2140 ($M + H^+$) $C_{15}H_{29}N_2O_4$, requires *m/z* 301.2127 (Δ = 4.31 ppm)

5.6.8 3-NH_2, 3-aza-C_{12}-HSL [2.72]

tert-Butyl N^2-nonyl carbazate [2.83]



A THF solution (25 mL) containing *n*-nonyl bromide (3 mmol, 571 µL) and *t*-butyl carbazate (2 mmol, 0.2643 g) was refluxed over 18 h. Solvent was removed to dryness under high vacuo to obtained yellow a oil that was purified by column chromatography using ethyl acetate-hexane (1:3) to obtain the desired product as a yellow oil in 25 % yield.

TLC-R_f = 0.50 in ethyl acetate-hexane (1:3)

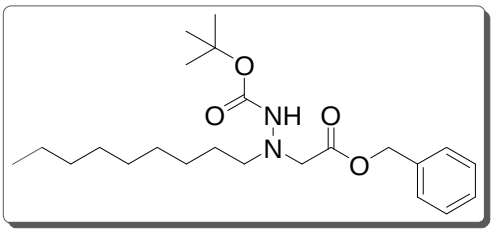
FT-IR (KBr) cm^{-1}: 3315m, br. (*ν*NH); 1713s (*ν*C=O, *Boc*); 1458m (δNH); 1367m (δC-H, *tert*-butyl).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.20-1.34 (12H, m, CH$_3$(C*H*$_2$)$_6$-), 1.42-1.45 (2H, m, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 1.47 (9H, s, -C(C*H*$_3$)$_3$), 2.83 (2H, t, *J* = 7.2 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 3.92 (1H, s, broad -CH$_2$N*H*-), 6.09 (1H, s, broad, -N*H*-*Boc*).

^{13}C NMR ($CDCl_3$) δ 158.68, 80.37, 52.18, 31.88, 29.57, 29.52, 29.27, 28.37(3), 27.86, 27.09, 22.68, 14.12.

(ES-MS/FAB-MS): *m/z* 259.2392 (M+H$^+$) $C_{14}H_{31}N_2O_2$, requires *m/z* 259.2386 (Δ = 2.31ppm).

Benzyl, 3-Boc-amino-3-azadodecanoate [2.84]



Benzyl, 3-*Boc*-amino-3-azadodecanoate was synthesized by *N*-alkylation of *N*-(*Boc*-amino)nonylamine **2.83** with benzyl 2- bromoacetate to obtain the title product as a yellow oil in 82 % yield (Method-H).

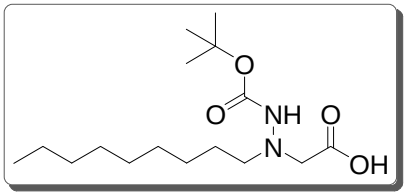
FT-IR (KBr) cm^{-1}: 3364m (*ν*NH); 1739s (*ν*C=O); 1456m (δNH, amide); 1366m (δC-H, *tert*-butyl).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 7.0 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.24-1.31 (12H, m, CH$_3$(C*H*$_2$)$_6$-), 1.46 (9H, s, -C(C*H*$_3$)$_3$), 1.51 (2H, s, broad, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.88 (2H, t, *J* = 7.4 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 3.77 (2H, s, -NC*H*$_2$CO-), 5.18 (2H, s, -C*H*$_2$C$_6$H$_5$), 6.56 (1H, s, broad, N*H*-Boc), 7.34.-7.40 (5H, m, broad, -C$_6$*H*$_5$).

^{13}C NMR ($CDCl_3$) δ 168.15, 158.71, 135.21, 128.67(2), 128.50, 128.35(2), 81.73, 75.32, 66.40, 62.52, 31.89, 29.52(2), 29.29, 28.34(3), 27.60, 26.99, 22.68, 14.13.

(ES-MS/FAB-MS): *m/z* 407.2889 (M+H$^+$) $C_{23}H_{39}N_2O_4$, requires *m/z* 407.2910 (Δ = 5.15 ppm)

3-Boc-NH- 3-azadodecanoic acid [2.85]



3-*Boc*-NH- 3-azadodecanoic acid was obtained from its benzyl ester **2.84** by catalytic hydrogenation as white solids in 74 % yield using Method-I.

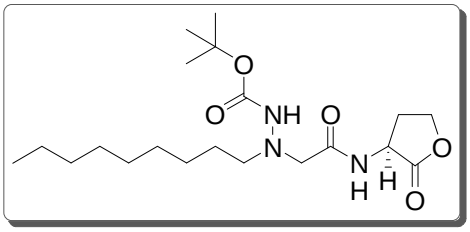
mp = 76-78 °C.

FT-IR (KBr) cm^{-1}: 3342m (*ν*NH); 2578w (*ν*OH); 1724s (*ν*C=O); 1503m (δNH, amide); 1368w (δC-H, *tert*-butyl).

^1H NMR ($CDCl_3$) δ 0.91 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_8$-), 1.24-1.35 (12H, m, $CH_3(CH_2)_6$-), 1.47 (9H, s, -$C(CH_3)_3$), 1.50 (2H, s, broad, $CH_3(CH_2)_6CH_2$-), 2.86 (2H, t, *J* = 7.2 Hz, $CH_3(CH_2)_7CH_2$-), 3.58 (2H, s, -NCH_2CO-), 5.92 (1H, s, borad, N*H*-Boc), 10.5 (1H, s, borad, -COO*H*).

(ES-MS/FAB-MS): *m/z* 315.2272 ($M-H^+$) $C_{16}H_{31}N_2O_4$, requires *m/z* 315.2284 (Δ = 3.80 ppm)

3-Boc-NH, 3-aza C_{12}-HSL [2.86]



3-*Boc*-NH, 3-aza C_{12}-HSL was obtained from 3-*Boc*-amino, 3-azadodecanoic acid **2.85** (Method-J). The desired compound was purified by column chromatography using acetone solvent in 60 % yield as a yellow solid.

TLC-R_f = 0.62 in acetone

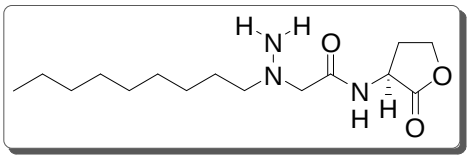
mp = 60-62 °C.

FT-IR (KBr) cm^{-1}: 3339w, (*ν*NH, *from NH-Boc*); 3288m (*ν*NH, amide); 1779s (*ν*C=O, lactone); 1705s, 1692s (*ν*C=O, *Boc*); 1661s (*ν*C=O, amide); 1529s (δNH).

^1H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_8$-), 1.35-1.43 (12H, m, $CH_3(CH_2)_6$-), 1.45 (9H, s, -$C(CH_3)_3$), 1.47-1.52 (2H, s, broad, $CH_3(CH_2)_6CH_2$-), 2.34-2.39 (1H, m, ring, 4α-*H*), 2.52-2.61 (1H, m, ring, 4β-*H*), 2.75-2.80 (2H, dt, *J* = 6.8, 2.8 Hz, $CH_3(CH_2)_7CH_2$-), 3.46 (2H, dd, *J* = 37.6, 8.8 Hz, -NCH_2CO-), 4.25-4.28 (1H, m, ring, 5α-*H*), 4.47-4.52 (1H, m, ring, 5β-*H*), 4.61-4.74 (1H, m, ring, 3-*H*), 5.51 (1H, s, N*H*-Boc), 8.75 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

(ES-MS/FAB-MS): *m/z* 400.2798 ($M+H^+$) $C_{20}H_{38}N_3O_5$, requires *m/z* 400.2811 (Δ = 3.24 ppm)

3-NH_2, 3-aza-C_{12}-HSL [2.72]



The 3-NH_2, 3-aza C_{12}-HSL was synthesized as light yellow crystals in 99 % yield from 3-*Boc*-amino, 3-aza C_{12}-HSL **2.86** using Method-K.

TLC-R_f = 0.38 in ethyl acetate

mp = 99-101 °C.

FT-IR (KBr) cm^{-1}: 3396w, (*ν*NH, *from N-NH_2*); 3287m (*ν*NH, amide); 1778s (*ν*C=O, lactone); 1704s (*ν*C=O, amide); 1530s (δNH).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH_2)$_8$-), 1.26-1.34 (12H, m, CH_3(C*H*$_2$)$_6$-), 1.69 (2H, s, broad, CH_3(CH_2)$_6$C*H*$_2$-), 2.33-2.44 (1H, m, ring, 4α-*H*), 2.61-2.67 (1H, m, ring, 4β-*H*), 3.16 (2H, t, *J* = 7.8, Hz, CH_3(CH_2)$_7$C*H*$_2$-), 3.90 (2H, dd, *J* = 23.6, 16.0 Hz, -NC*H*$_2$CO-), 4.28-4.35 (1H, m, ring, 5α-*H*), 4.49-4.53 (1H, m, ring, 5β-*H*), 4.62-4.67 (1H, m, ring, 3-*H*), 5.50 (2H, s, broad, -N*H*$_2$), 8.66 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 175.63, 171.53, 72.89, 62.37, 57.31, 49.03, 31.80(2), 29.34, 29.15, 29.11, 26.51, 22.64(2), 14.08.

(ES-MS/FAB-MS): *m/z* 300.2279 ($M+H^+$) $C_{15}H_{30}N_3O_3$, requires *m/z* 300.2287 (Δ = 2.66 ppm)

5.7 Synthesis of thia analogue of 3O, C_{12}-HSL

5.7.1 Method-L: *n-Alkylmercaptoacetic acid (3-thia-alkanoic acid)*[ref]

To an 18 % (w/v) solution of NaOH in water (20 mL) containing *n*-alkylmercaptan (10 mmol) was added 18 % (w/v) solution of NaOH in water (10 mL) containing *tert*-butyl bromoacetate (11 mmol, 1.624 mL). The mixture was heated to 90 °C and stirring was continued for 14 h. The solution was cooled to room temperature and acidified to pH 2 using 5 M HCl. Alkylmercaptoacetic acid was extracted in ethyl acetate (3 × 15 mL). Ethyl acetate was washed with brine (1 × 20 mL), dried over $MgSO_4$ and rotary evaporated under vacuo to the desired white product which was recrystallized from ether.

5.7.2 **Method-M:** *Alkylsulfinylacetic acids (3-oxo-3-thiaalkanoic acid)*[ref]

The alkylmercaptoacetic acid (3 mmol) was dissolved in glacial acetic acid (20 mL) containing conc. HCl (1.5 mL) and 0.5 *N bromate-bromide solution** (30 mL) was added dropwise (reddish/yellow color of the reaction mixture persists for longer than 15 min). The mixture was poured into stirring ice water to produce crystals of the alkylsulfinylacetic acid. They were separated by filtration. The filtrate was extracted with warm chloroform (35 °C, 2 × 10 mL) to furnish some more desired product. The crude product was washed quickly with warm petroleum ether and dried.

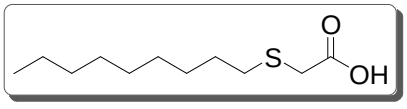
* 0.5 *N Bromate-bromide solution*: Dissolve potassium bromate (1.4 g) and potassium bromide (5.96 g) in sufficient water to make 100 mL.

5.7.3 **Method-N:** *Alkylsulfonylacetic acids (3, 3-dioxo-3-thiaalkanoic acid)*[ref]

The alkylmercaptoacetic acid (3 mmol) was dissolved in glacial acetic acid (20 mL) and temperature was raised to 50 °C. To this 26 mL of 30 % aqueous $KMnO_4$ solution (5 mmol, 0.79 g) was added dropwise over a period of 30 min and stirring was continued further 30 min at 50 °C. The mixture was cooled to room temperature and was poured into ice water with stirring to produce crystals of the alkylsulfonylacetic acid. They were extracted with warm chloroform (35 °C, 3 × 15 mL) and the chloroform extract was washed with 5 % sodium thiosulphate (3 × 15 mL) to remove black colour MnO_2, 1 M HCl (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain the title product as white crystals.

5.7.4 **3-Thia C_{12}-HSL [2.87]**

3-Thia-dodecanoic acid or n-nonylmercaptoacetic acid [2.96]



3-Thia-dodecanoic acid was synthesized as white crystals in 97 % yield from *n*-nonylmercaptan using Method-L.

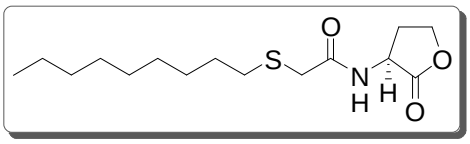
mp = 54-56 °C.

FT-IR (KBr) cm^{-1}: 3199w, (νOH); 1685s (νC=O).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.4 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.27-1.32 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.40 (2H, m, broad, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 1.59-1.67 (2H, quint, , *J* = 7.6 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.67 (2H, t, *J* = 7.6 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 3.29 (2H, s, -SC*H*$_2$CO), 10.70 (1H, s, broad, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 173.68, 44.62, 33.27, 32.12, 30.49, 29.81, 29.38, 29.21, 28.67, 22.77, 14.11.

3-Thia C$_{12}$-HSL [2.87]



3-Thia C_{12}-HSL was synthesized as a white crystalline product in 34 % yield from 3-thiadodecanoic acid **2.96** using Method-B.

TLC-R_f = 0.31 in ethyl acetate-acetone (7:3)

mp = 90-92 °C.

FT-IR (KBr) cm^{-1}: 3312s, (*ν*NH); 1777s (*ν*C=O, lactone); 1648s (*ν*C=O, amide); 1554s (δNH).

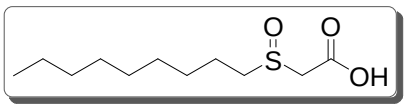
^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.17-1.34 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.39 (2H, m, broad , CH$_3$(CH$_2$)$_5$C*H*$_2$-), 1.58-1.63 (2H, quint, , *J* = 7.6 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.18-2.23 (1H, m, ring, 4α-*H*), 2.59 (2H, t, *J* = 6.8 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 2.79-2.82 (1H, m, ring, 4β-*H*), 3.29 (2H, d, *J* = 2.0 Hz, -SC*H*$_2$CO), 4.30-4.35 (1H, m, ring, 5α-*H*), 4.48-4.53 (1H, m, ring, 5β-*H*), 4.60-4.62 (1H, m, ring, 3-*H*), 7.41 (1H, d, *J* = 8.0 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 175.13, 172.11, 66.17, 54.20, 42.23, 32.36, 30.37(2), 29.79(2), 29.13, 28.97, 28.37, 22.82, 14.11.

(ES-MS/FAB-MS): *m/z* 302.1792 (M+H$^+$) $C_{15}H_{28}NO_3S$, requires *m/z* 302.1790 (Δ = 0.60 ppm)

5.7.5 3O, 3-thia C$_{12}$-HSL [2.88]

3-Oxo-3-thiadodecanoic acid or n-nonylsulfoacetic acid [2.98]



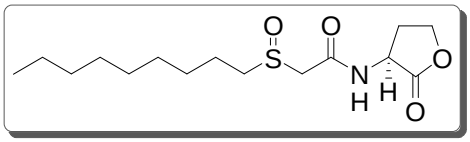
3-Oxo-3-thiadodecanoic acid was prepared as a white crystalline mixture of diastereomers (1:1) in 70 % yield from *n*-nonylmercaptoacetic acid using Method-M.

mp = 69-71 °C.

FT-IR (KBr) cm^{-1}: 3398s, br. (νOH); 1726m, 1685s (νC=O); 1049m (νS=O, sulfoxide).

^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 7.0 Hz, $CH_3(CH_2)_8$-), 1.28-1.35 (10H, m, $CH_3(CH_2)_5$-), 1.41-1.52 (2H, m, $CH_3(CH_2)_5CH_2$-), 1.75-1.79 (2H, m, $CH_3(CH_2)_6CH_2$-), 2.81-2.91 (1H, m, $CH_3(CH_2)_7CH_2$-), 3.03-3.06 (1H, m, $CH_3(CH_2)_7CH_2$-), 3.45 (1H, d, J = 14.8 Hz, -S(O)CH_2CO), 3.86 (1H, d, J = 14.8 Hz, -S(O)CH_2CO), 5.13 (1H, s, broad, -COO*H*).

3O, 3-thia C_{12}-HSL [2.88]



3O, 3-thia C_{12}-HSL was synthesized as a white crystalline mixture of diastereomers (3:1) in 50 % yield from 3-oxo-3-thiadodecanoic acid **2.98** using Method-B.

TLC-R_f = 0.21 in ethyl acetate-acetone (7:3)

mp = 132-134 °C.

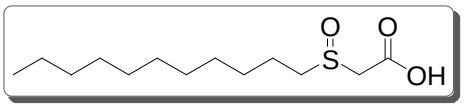
FT-IR (KBr) cm^{-1}: 3317m, (νNH); 1773s (νC=O, lactone); 1649s (νC=O, amide); 1543m (δNH); 1037m (νS=O, sulfoxide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, J = 6.8 Hz, $CH_3(CH_2)_8$-), 1.27-1.36 (10H, m, $CH_3(CH_2)_5$-), 1.45-1.51 (2H, m, $CH_3(CH_2)_5CH_2$-), 1.72-1.78 (2H, m, $CH_3(CH_2)_6CH_2$-), 2.21-2.35 (0.3H, m, ring, 4α-*H*), 2.38-2.51 (0.7H, m, ring, 4α-*H*), 2.61-2.72 (1H, m, $CH_3(CH_2)_7CH_2$-), 2.78-2.89 (1H, m, $CH_3(CH_2)_7CH_2$-), 3.02-3.09 (0.3H, m, ring, 4β-*H*), 3.11-3.18 (0.7H, m, ring, 4β-*H*), 3.29 (0.6 H, d, J = 14.4 Hz, -S(O)CH_2CO), 3.79 (1.4 H, d, J = 14.4 Hz, -S(O)CH_2CO), 4.27-4.32 (1H, m, ring, 5α-*H*), 4.42-4.59 (0.7H and 1H, m, ring, 5β-*H* and 3-*H*), 4.80-4.87 (0.3H, m, ring, 5β-*H*), 7.80 (1H, d, J = 6.4 Hz, -N*H*-HSL).

(ES-MS/FAB-MS): *m/z* 318.1725 ($M+H^+$) $C_{15}H_{28}NO_4S$, requires *m/z* 318.1739 (Δ = 4.4 ppm)

5.7.6 3O, 3-thia C_{14}-HSL [2.89]

3-Oxo-3-thiatetradecanoic acid or n-undecylsulfoacetic acid [2.99]



3-Oxo-3-thiatetradecanoic acid was prepared as a white crystalline mixture of diastereomers (1:1) in 86 % yield from *n*-undecylmercaptoacetic acid using Method-M.

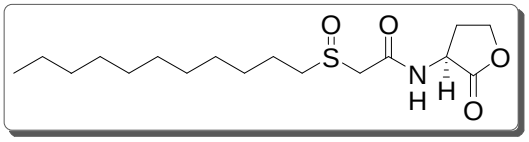
mp = 81-82 °C.

FT-IR (KBr) cm^{-1}: 3404s, br. (νOH); 1711m, 1685s (νC=O); 1034m (νS=O, sulfoxide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, J = 7.0 Hz, C*H*$_3$(CH$_2$)$_{10}$-), 1.26-1.37 (14H, m, CH$_3$(C*H*$_2$)$_7$-), 1.47-1.52 (2H, m, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 1.78-1.83 (2H, m, CH$_3$(CH$_2$)$_8$C*H*$_2$-), 2.82-2.90 (1H, m, CH$_3$(CH$_2$)$_9$C*H*$_2$-), 3.04-3.11 (1H, m, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 3.45 (1H, d, J = 14.8 Hz, -S(O)C*H*$_2$CO), 3.88 (1H, d, J = 14.8 Hz, -S(O)C*H*$_2$CO), 10.23 (1H, s, broad, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 172.93, 51.668, 49.73, 31.87, 29.53, 29.48(2), 29.27, 29.07, 28.58, 22.71, 22.67, 14.10.

3O, 3-thia C_{14}-HSL [2.89]



3O, 3-thia C_{14}-HSL was synthesized as off white crystalline mixture of diastereomers (1:1) in 18 % yield from 3-oxo 3-thiatetradecanoic acid **2.99** using Method-A.

TLC-R_f = 0.22 in ethyl acetate-acetone (7:3)

mp = 114-116 °C.

FT-IR (KBr) cm^{-1}: 3316m, (νNH); 1773s (νC=O, lactone); 1648s (νC=O, amide); 1543m (δNH); 1037m (νS=O, sulfoxide).

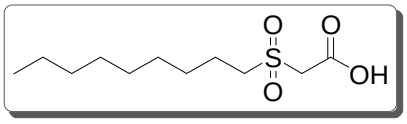
^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.8 Hz, C*H*$_3$(CH$_2$)$_{10}$-), 1.26-1.37 (14H, m, CH$_3$(C*H*$_2$)$_7$-), 1.45-1.51 (2H, m, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 1.73-1.78 (2H, m, CH$_3$(CH$_2$)$_8$C*H*$_2$-), 2.22-2.34 (0.5H, m, ring, 4α-*H*), 2.41-2.50 (0.5H, m, ring, 4α-*H*), 2.64-2.73 (1H, m, CH$_3$(CH$_2$)$_9$C*H*$_2$-), 2.79-2.90 (1H, m, CH$_3$(CH$_2$)$_9$C*H*$_2$-), 3.01-3.11 (0.5H, m, ring, 4β-

H), 3.12-3.19 (0.5H, m, ring, 4β-*H*), 3.27 (1H, dd, *J* = 16.4, 14.4 Hz, -S(O)C*H*$_2$CO), 3.77 (1H, dd, *J* = 14.4, 2.4 Hz, -S(O)C*H*$_2$CO), 4.28-4.33 (1H, m, ring, 5α-*H*), 4.39-4.48 (0.5H, m, ring, 5β-*H*), 4.49-4.54 (1H, m, ring, 3-*H*), 4.81-4.89 (0.5H, m, ring, 5β-*H*), 7.63 (0.5H, d, *J* = 6.4 Hz, -N*H*-HSL), 7.70 (0.5H, d, *J* = 6.4 Hz, -N*H*-HSL).

(ES-MS/FAB-MS): *m/z* 346.2037 ($M+H^+$) $C_{17}H_{32}NO_4S$, requires *m/z* 346.2052 (Δ = 4.33 ppm)

5.7.7 3, 3-Dioxo, 3-thia C_{12}-HSL [2.90]

3, 3-Dioxo-3-thiadodecanoic acid or n-nonylsulfonylacetic acid [2.100]



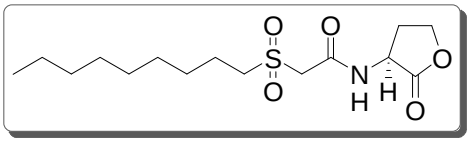
3, 3-Dioxo-3-thiadodecanoic acid was prepared as a white crystalline solid in 33 % yield from *n*-undecylmercaptoacetic acid using Method-N.

mp = 104-106 °C.

FT-IR (KBr) cm^{-1}: 3147m, br. (*ν*OH); 1699s, (*ν*C=O); 1321s, 1148s (*ν*S=O, sulfone).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.28-1.35 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.41-1.51 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 1.79-1.91 (2H, m, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.28 (2H, m, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 4.03 (2H, s, -S(O)$_2$C*H*$_2$CO), 10.12 (1H, s, broad, -COO*H*).

3, 3-Dioxo, 3-thia C_{12}-HSL [2.90]



3, 3-Dioxo, 3-thia C_{12}-HSL was synthesized as white crystals in 32 % yield from 3, 3-dioxo-3-thiadodecanoic acid **2.100** using Method-B.

TLC-R_f = 0.23 in ethyl acetate-hexane (9:1)

mp = 102-104 °C.

FT-IR (KBr) cm^{-1}: 3307s, (*ν*NH); 1778s (*ν*C=O, lactone); 1662s (*ν*C=O, amide); 1557m (δNH); 1327s, 1130s (*ν*S=O, sulfone).

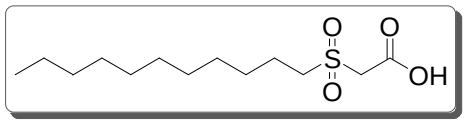
^{1}H NMR ($CDCl_3$) δ 0.81 (3H, t, *J* = 7.0 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.17-1.27 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.36-1.39 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 1.75-1.81 (2H, m, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.22-2.33 (1H, m, ring, 4α-*H*), 2.63-2.71 (1H, m, ring, 4β-*H*), 3.14 (2H, t, *J* = 8.0 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 3.85 (2H, s, -S(O)$_2$C*H*$_2$CO), 4.21-4.31 (1H, m, ring, 5α-*H*), 4.41-4.53 (1H, m, ring, 5β-*H*), 4.56-4.61 (1H, m, ring, 3-*H*), 7.05 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.73, 167.21, 66.61, 57.42, 52.42, 50.23, 32.07, 30.23, 29.68, 28.88(2), 28.32, 22.94, 20.86, 14.11.

(ES-MS/FAB-MS): *m/z* 334.1691 (M+H$^+$) $C_{15}H_{28}NO_5S$, requires *m/z* 334.1688 (Δ = 0.98 ppm)

5.7.8 3, 3-Dioxo, 3-thia C_{14}-HSL [2.91]

3, 3-Dioxo-3-thiatetradecanoic acid or n-undecylsulfonylacetic acid [2.101]



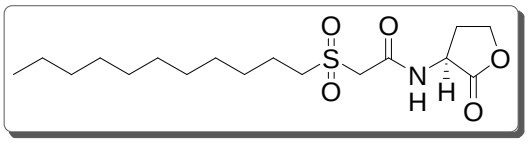
3, 3-Dioxo-3-thiatetradecanoic acid was prepared as a white crystalline solid in 21 % yield from *n*-undecylmercaptoacetic acid using Method-N.

mp = 110-112 °C.

FT-IR (KBr) cm^{-1}: 3154m, br. (*ν*OH); 1699s, (*ν*C=O); 1321s, 1148s (*ν*S=O, sulfone).

^{1}H NMR ($CDCl_3$) δ 0.83 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_{10}$-), 1.15-1.25 (14H, m, CH$_3$(C*H*$_2$)$_7$-), 1.37-1.41 (2H, m, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 1.69-1.82 (2H, m, CH$_3$(CH$_2$)$_8$C*H*$_2$-), 3.22 (2H, m, CH$_3$(CH$_2$)$_9$C*H*$_2$-), 3.88 (2H, s, -S(O)$_2$C*H*$_2$CO), 9.20 (1H, s, broad, -COO*H*).

3, 3-Dioxo, 3-thia C_{14}-HSL [2.91]



3, 3-Dioxo, 3-thia C_{14}-HSL was synthesized as off white crystals in 86 % yield from 3, 3-dioxo-3-thiatetradecanoic acid **2.101** using Method-A.

TLC-R_f = 0.25 in ethyl acetate-hexane (9:1)

mp = 110-112 °C.

FT-IR (KBr) cm^{-1}: 3306s, (*ν*NH); 1777s (*ν*C=O, lactone); 1662s (*ν*C=O, amide); 1556m (δNH); 1328s, 1131s (*ν*S=O, sulfone).

^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, *J* = 7.0 Hz, $CH_3(CH_2)_{10}$-), 1.21-1.37 (14H, m, $CH_3(CH_2)_7$-), 1.41-1.52 (2H, m, $CH_3(CH_2)_7CH_2$-), 1.82-1.91 (2H, m, $CH_3(CH_2)_8CH_2$-), 2.34-2.41 (1H, m, ring, 4α-*H*), 2.70-2.74 (1H, m, ring, 4β-*H*), 3.23 (2H, t, *J* = 8.8 Hz, $CH_3(CH_2)_9CH_2$-), 3.96 (2H, s, -$S(O)_2CH_2CO$), 4.28-4.35 (1H, m, ring, 5α-*H*), 4.50-4.55 (1H, m, ring, 5β-*H*), 4.57-4.63 (1H, m, ring, 3-*H*), 7.23 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

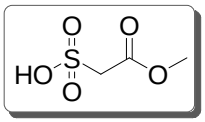
^{13}C NMR ($CDCl_3$) δ 175.47, 165.72, 65.66, 55.74, 53.24, 51.02, 31.92, 30.31, 29.72(3), 29.38(2), 28.83, 22.79, 21.08, 14.11.

(ES-MS/FAB-MS): *m/z* 362.2012 ($M+H^+$) $C_{17}H_{32}NO_5S$, requires *m/z* 362.2001 (Δ = 3.04 ppm).

5.8 Synthesis of sulphonamide analogues of 3O, C_{12}-HSL

5.8.1 1, 3-Dioxo, 4-aza, 1-thia C_{12}-HSL [2.92]

Methyl 2-sulfoacetate [2.104]

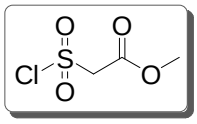


To a 250 mL long neck round bottom flask containing a 60 mL mixture of methanol-benzene (2:8) was added sulfacetic acid (30 mmol, 3.84 g). The solution was refluxed for 8 h to remove traces of water by azeotropic distillation. Solution was cooled to room temperature and filtered. Solvent was rotary evaporated under vacuo to obtain the desired product in 98 % yield.

FT-IR (DCM) cm^{-1}: 3417s, br. (*ν*OH); 1657s (*ν*C=O); 1203s, 1066s, 640m (*ν*S=O, sulfonic acid)

^{1}H NMR (DMSO) δ 3.47 (2H, s, -$COCH_2S(O)_2$-), 3.55 (3H, s, CH_3O-).

Methyl 2-(chlorosulfonyl)acetate [2.105]

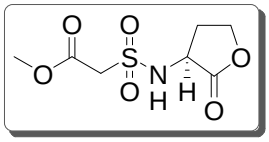


Methyl 2-sulfoaceate **2.104** (10 mmol, 1.42 g) was refluxed in phosphorus oxychloride or $POCl_3$ (3 mL) for 4 h. Excess $POCl_3$ was removed under high vacuo. The residue was redissolved in DCM (25 mL) and filtered. Solvent was rotary evaporated to obtain the title product in a 85 % yield.

FT-IR (DCM) cm^{-1}: 1751s (*ν*C=O); 1383s, 1169s (*ν*S=O, sulfonyl chloride)

^{1}H NMR (DMSO) δ 3.92 (3H, s, C*H*$_3$O-), 4.63 (2H, s, -COC*H*$_2$S(O)$_2$-).

N-(Methoxycarbonylmethylsulfonyl)-L-HSL [2.106]



A dry DCM solution (15 mL) containing L-HSL.HCl (2.5 mmol, 0.344 g), 1, 8-diazabicyclo [5.4.0]undec-7-ene or DBU (2.5 mmol, 374 µL) and DMAP (2.5 mmol, 0.306 g) was stirred at 0 °C for 10 min. To this 2-(chlorosulfonyl)acetate **2.105** (3.5 mmol, 0.618 g) was added and whole mixture was stirred further 16 h at room temperature. The solvent was removed by rotary evaporation under vacuo and residue was redissolved in hot ethyl acetate (30 mL). Ethyl acetate solution was quickly extracted with 1 M HCl (2 × 10 mL) and brine (1 × 15 mL) and then it was dried over $MgSO_4$. Removal of ethyl acetate under vacuo gave an impure oily product. Desired compound was isolated by column chromatography in 47 % yield using ethyl acetate-acetonitrile (1:1) solvent system.

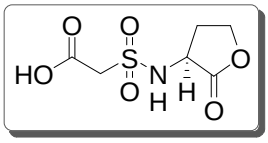
FT-IR (KBr) cm^{-1}: 3309s, (*ν*NH, sulfonamide); 1782s (*ν*C=O, lactone); 1756s (*ν*C=O, ester); 1457w (δNH); 1348s, 1143s (*ν*S=O, sulfonamide).

^{1}H NMR ($CDCl_3$) 2.29-2.40 (1H, m, ring, 4α-*H*), 2.71-2.84 (1H, m, ring, 4β-*H*), 3.84 (3H, s, C*H*$_3$O-), 4.23 (1H, d, *J* = 16 Hz, -COC(H)*H*S(O)$_2$-), 4.27-4.34 (1H, m, ring, 5α-*H*), 4.80 (1H, d, *J* = 16 Hz, -COC(*H*)HS(O)$_2$-), 4.46-4.52 (1H, m, ring, 5β-*H*), 4.57-4.63 (1H, m, ring, 3-*H*), 5.65 (1H, d, *J* = 7.2 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.71, 164.99, 65.83, 57.23, 53.24, 52.82, 30.46.

(ES-MS/FAB-MS): *m/z* 236.0209 ($M-H^+$) $C_7H_{10}NO_6S$, requires *m/z* 236.0229 (Δ = 8.84 ppm)

N-(Carboxymethylsulfonyl)-L-HSL [2.107]



N-(Methoxycarbonylmethylsulfonyl)-L-HSL **2.106** (1 mmol, 0.237 g) was refluxed in 1 M HCl solution (15 mL) for a period of 4 h. Solution was concentrated in vacuo. The residue was redissolved in ethyl acetate (20 mL), washed with brine (1 × 15 mL) and dried over $MgSO_4$. Removal of ethyl acetate under vacuo gave the desired product as white crystals in 77 % yield.

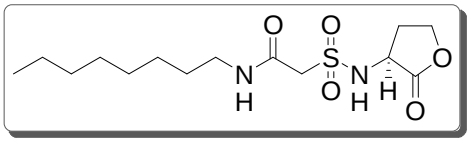
FT-IR (KBr) cm^{-1}: 3395s, (*ν*NH, sulfonamide); 2658w (*ν*OH); 1771s (*ν*C=O, lactone); 1636s (*ν*C=O, carboxylate); 1456w (δNH); 1340s, 1143s (*ν*S=O, sulfonamide).

^1H NMR ($CDCl_3$) 2.29-2.40 (1H, m, ring, 4α-*H*), 2.71-2.84 (1H, m, ring, 4β-*H*), 4.16 (1H, d, *J* = 15.6 Hz, -COC(H)*H*S$(O)_2$-), 4.19-4.26 (1H, m, ring, 5α-*H*), 4.32 (1H, d, *J* = 15.6 Hz, -COC(*H*)HS$(O)_2$-), 4.38-4.43 (1H, m, ring, 5β-*H*), 4.45-4.48 (1H, m, ring, 3-*H*), 6.86 (1H, s, broad, -N*H*-HSL), 10.56 (1H, s -COO*H*).

^{13}C NMR ($CDCl_3$) δ 174.87, 166.813, 65.69, 52.51, 49.36, 30.24.

(ES-MS/FAB-MS): *m/z* 222.0038 ($M-H^+$) $C_6H_8NO_6S$, requires *m/z* 222.0072 (Δ = 15.3 ppm)

1, 3-Dioxo, 4-aza, 1-thia C_{12}-HSL [2.92]



To a stirred solution of *N*-(carboxymethylsulfonyl)-L-HSL **2.107** (2 mmol) in dry DCM (15 ml) was added sequentially *n*-octylamine (2 mmol), DMAP (3 mmol) and finally DCCI (2.2 mmol). The stirring was continued for 16 h and solvent was removed under high vacuo. The residue was redissolved in hot ethyl acetate (25 mL). The organic solution was washed with saturated sodium bicarbonate (3 × 10 mL), 1 M HCl (3 × 10 mL) and brine (1 × 15 mL). The ethyl acetate solution was dried over $MgSO_4$, filtered and rotary evaporated to obtain product. The product was kept aside in

the cold. When the remaining DCU crystallized out this was carefully removed by passing it through small pipette containing tissue paper plug. A chilled acetone (2-4 mL) was used for rinsing. Final product was isolated as white crystals by PLC using ethyl acetate in 22 % yield.

TLC-R_f = 0.44 in ethyl acetate

mp = 94-96 °C.

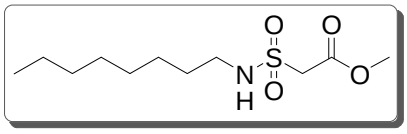
FT-IR (KBr) cm^{-1}: 3386s (*ν*NH, amide); 3323w, (*ν*NH, sulfonamide); 1761s (*ν*C=O, lactone); 1683s (*ν*C=O, amide); 1560m (δNH); 1327s, 1168s (*ν*S=O, sulfonamide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH_2)$_8$-), 1.27-1.33 (10H, m, CH_3(C*H*$_2$)$_5$-), 1.52-1.57 (2H, m, $CH_3(CH_2)_5$C*H*$_2$-), 2.33-2.46 (1H, m, ring, 4α-*H*), 2.71-2.79 (1H, m, ring, 4β-*H*), 3.28 (2H, m, $CH_3(CH_2)_6$C*H*$_2$-), 4.07 (1H, s, *J* = 14.2 Hz, -COC(H)*H*S(O)$_2$-), 4.26 (1H, s, *J* = 14.2 Hz, -COC(*H*)HS(O)$_2$-), 4.27-4.34 (1H, m, ring, 5α-*H*), 4.45-4.52 (1H, m, ring, 3-*H*), 4.46-4.51 (1H, m, ring, 5β-*H*), 6.21 (1H, d, *J* = 7.6 Hz, -N*H*-HSL), 6.69 (1H, t, -CON*H*-).

(ES-MS/FAB-MS): *m/z* 335.1638 ($M+H^+$) $C_{14}H_{27}N_2O_5S$, requires *m/z* 335.1641 (Δ = 0.89 ppm)

5.8.2 3, 3-Dioxo-4-aza-3-thia C_{12}-HSL [2.93]

Methyl 3, 3-dioxo-3-thia-4-azadodecanoate [2.108]



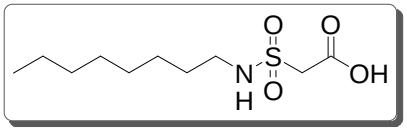
To a dry DCM solution (15 mL) containing 1, 8-diazabicyclo [5.4.0]undec-7-ene or DBU (1 mmol, 150 µL), *n*-octylamine (1 mmol, 165 µL) and K_2CO_3 (1 mmol, 0.138g) at 0 °C was added 2-(chlorosulfonyl)acetate **2.105** (1 mmol, 0.1765 g). The mixture was refluxed for 3 h. The solvent was removed by rotary evaporation under vacuo and residue was redissolved in hot ethyl acetate (20 mL). Ethyl acetate solution was washed with 1 M HCl (3 × 10 mL) and brine (1 × 15 mL) and then it was dried over $MgSO_4$. Removal of ethyl acetate under vacuo gave an impure dark brown colored liquid product. This product was used in the next step without purification.

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.0 Hz, C*H*$_3$(CH_2)$_8$-), 1.34-1.39 (10H, m, CH_3(C*H*$_2$)$_5$-), 1.59-1.62 (2H, m, $CH_3(CH_2)_5$C*H*$_2$-), 3.16 (2H, dd, *J* = 13.6, 7.2 Hz,

$CH_3(CH_2)_6CH_2$-), 3.83 (3H, s, CH_3O-), 4.02 (2H, s, -$S(O)_2CH_2CO$-), 4.71 (1H, t, -$NHS(O)_2$-).

(ES-MS/FAB-MS): *m/z* 266.1382 ($M+H^+$) $C_{11}H_{24}NO_4S$, requires *m/z* 266.1426 (Δ = 16.53 ppm)

3, 3-Dioxo-3-thia-4-azadodecanoic acid [2.109]



To a stirred solution of *crude** methyl 3, 3-dioxo-3-thia-4-azadodecanoate **2.108** (2 mmol, 0.530 g) in methanol (10 mL), was added dropwise cold NaOH (2.5 mmol, 0.1 g) solution in water (5 mL). The stirring was continued at room temperature for 4 h. This mixture was rotary evaporated to remove solvent. The residue was redissolved in water (20 mL) and washed with ethyl acetate (2 × 10 mL). Aqueous layer was acidified with 2 M HCl to pH 2 and the free acid was extracted with ethyl acetate (3 × 10 mL), dried over $MgSO_4$, filtered and solvent rotary evaporated to give 3, 3-dioxo-3-thia-4-azadodecanoic acid in 72 % yield.

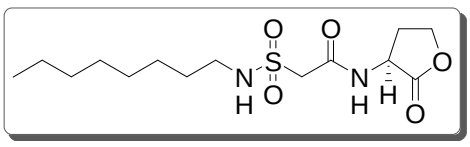
(* the product was not purified in the previous step)

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_8$-), 1.27-1.35 (10H, m, $CH_3(CH_2)_5$-), 1.54-1.62 (2H, m, $CH_3(CH_2)_5CH_2$-), 3.13 (2H, dd, *J* = 12.8, 6.8 Hz, $CH_3(CH_2)_6CH_2$-), 3.96 (2H, s, -$S(O)_2CH_2CO$-), 5.02 (1H, s, broad, -$NHS(O)_2$-), 9.87 (1H, s, broad, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 172.15, 54.67, 43.77, 31.73, 29.19, 29.12, 29.07, 26.54, 22.60, 14.06.

(ES-MS/FAB-MS): *m/z* 250.1098 ($M-H^+$) $C_{10}H_{20}NO_4S$, requires *m/z* 250.1113 (Δ = 12.79 ppm)

3, 3-Dioxo-4-aza-3-thia C_{12}-HSL [2.93]



3, 3-dioxo-3-thia-4-azadodecanoic acid **2.109** (1 mmol, 0.251 g) was coupled with L-HSL.HCl (1 mmol, 0.1375 g) using Method-A to obtain an impure 3, 3-Dioxo, 4-aza,

3-thia C_{12}-HSL. Final compound was isolated as white crystals in 64 % yield by PLC using ethyl acetate.

TLC-R_f = 0.43 in ethyl acetate

mp = 132-134 °C.

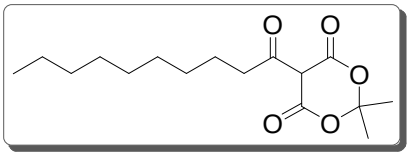
FT-IR (KBr) cm^{-1}: 3325s (*ν*NH, amide); 3252s, (*ν*NH, sulfonamide); 1773s (*ν*C=O, lactone); 1671s (*ν*C=O, amide); 1542m (δNH); 1320s, 1147s (*ν*S=O, sulfonamide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 7.2 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.28-1.37 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.54-1.61 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 2.39-2.49 (1H, m, ring, 4α-*H*), 2.65-2.74 (1H, m, ring, 4β-*H*), 3.16 (2H, dd, *J* = 13.2, 7.2 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 4.01 (2H, s, -S(O)$_2$C*H*$_2$CO-), 4.30-4.36 (1H, m, ring, 5α-*H*), 4.56-4.61 (1H, m, ring, 5β-*H*), 4.62-4.66 (1H, m, ring, 3-*H*), 5.26 (1H, t, *J* = 6.0 Hz, -N*H*S(O)$_2$-), 7.01 (1H, d, *J* = 7.6 Hz, -N*H*-HSL).

(ES-MS/FAB-MS): *m/z* 335.1628 (M+H$^+$) $C_{14}H_{27}N_2O_5S$, requires *m/z* 335.1641 (Δ = 3.88 ppm)

5.9 Synthesis of hetero ring altered analogues of 3O, C_{12}-HSL

5.9.1 5-Docecanoyl Meldrum's acid [2.118]



To a dry dichloromethane solution (40 mL) containing *n*-decanoic acid (10 mmol, 1.723 g) was added sequentially Meldrum's acid (10 mmol, 1.442 g), DMAP (15 mmol, 1.833 g) and finally DCCI (11mmol, 2.27 g). This solution was stirred at room temperature for 16 h and then filtered to remove the precipitated *N, N'*-dicyclohexylurea (DCU). The filtrate was washed with 2 M HCl (2×20 mL) and dried over $MgSO_4$. Solvent was rotary evaporated to afford yellow-orange oil contaminated with small DCU. It was kept aside to allow residual DCU to separate, mixed with acetone (8 mL) and chilled in a freezer. The solution was then filtered through tissue paper using pipette and washed with 4 mL chilled acetone. The filtrate after rotary evaporating gave pure 5-decanoyl Meldrum's acid as yellow-orange oil in 86 % yield.

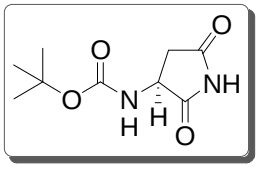
TLC: R_f = 0.26 in ethyl acetate

^{1}H NMR ($CDCl_3$) δ 0.9 (3H, t, *J* = 6.9 Hz, C*H*$_3$(CH$_2$)$_8$-), 1.29 (10H, m, CH$_3$-(C*H*$_2$)$_5$(CH$_2$)$_3$C(OH)-), 1.38 (2H, quart, *J* = 7.3 Hz, CH$_3$(CH$_2$)$_5$C*H*$_2$(CH$_2$)$_2$C(OH)-), 1.71 (2H, quart, *J* = 7.0 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$ CH$_2$C(OH)-), 1.75 (6H, m, (C*H*$_3$)$_2$COO)), 3.09 (2H, t, *J* = 7.15 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$C(OH)), 15.31 (1H, s, enolic -O*H*).

5.9.2 *N-Boc*-L-aspartimide and *N-Boc*-L-glutarimide

To a stirred dry tetrahydrofuran solution (60 mL) containing *Boc*-L-asparagine (5 mmol, 1.16 g)/Boc-L-glutamine (5 mmol, 1.23 g) was added 1,1'-carbonyldiimidazole (5.5 mmol, 0.895 g). This reaction mixture was stirred and refluxed for 7h. The resulting mixture was then cooled to room temperature and evaporated to dryness in vacuum. The residue was redissolved in excess ethyl acetate (150 mL), as the desired product was less soluble in it. The organic solution was sequentially washed with 1 M potassium hydrogen sulphate (2 × 25 mL) and brine (1×25 mL). After drying over $MgSO_4$, the organic solvent was rotary evaporated.

5.9.2.1 *N-Boc-L-aspartimide [2.121]*

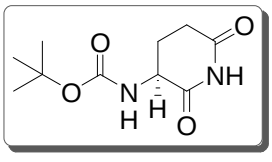


The residue was purified by column chromatography in ethyl acetate. Purification provided *N-Boc*-L-aspartimide as a white solid in 57 % yield.

TLC-R_f = 0.74 in ethyl acetate

^{1}H NMR ($CDCl_3$) δ 1.44 (9H, s, -OC(C*H*$_3$)$_3$), 2.8 (1H dd, ring, 4α-*H*), 3.1 (1H, dd, ring, 4β-*H*), 4.4 (1H, m, ring, 3-*H*), 5.7 (1H, d, *J*=8.0 -OCON*H*-), 9.5 (1H, s, ring, imide –N*H*-).

5.9.2.2 *N-Boc-L-glutarimide [2.122]*



Recrystallization of crude product from ethyl acetate yielded pure *N-Boc*-L-glutarimide in 81 % yield.

TLC: R_f = 0.26 in ethyl acetate-hexane (1:1).

^{1}H NMR ($CDCl_3$) δ 1.41 (9H, s, -OC(CH_3)$_3$), 1.83 (1H m, ring, 4α-*H*), 2.45 (1H, m, ring, 4β-*H*), 2.63 (1H m, ring, 5α-*H*), 2.73 (1H, m, ring, 5β-*H*), 4.3 (1H, m, ring, 3*H*), 5.46 (1H, d, *J*=8.0, -OCON*H*-), 8.85 (1H, s, ring, imide –N*H*-)

5.9.3 L-Aspartimide [2.123] and L-glutarimide [2.124] trifluoroacetates

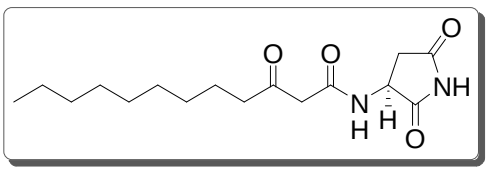
To a solution of acetonitrile (5 mL) containing *Boc*-L-aspartimide (1.5 mmol)/*Boc*-L-glutaimide (1.5 mmol) was added trifluoroacetic acid (5 mL). The mixture was stirred at room temperature for about 3 h. The solvent was rotary evaporated. Then the resulting yellow oil was redissolved in acetonitrile (15 mL) and rotary evaporated to dryness. This step was done thrice to obtained constant weight product.

Both L-aspartimide trifluoroacetate **2.123** and L-glutarimide trifluoroacetate **2.124** were obtained as yellow oils with 95 % and 97 % yields respectively. These were utilized for next step without any further characterization or purification.

5.9.4 Method-O: *Synthesis of hetero ring altered analogues of 3O, C_{12}-HSL*

To a stirred solution of decanoyl Meldrum's acid (1 mmol, 300 mg) in dry acetonitrile (30 mL) was added an appropriate amine (1 mmol) and TEA (1.1 mmol, 155 µl) was also added when L-aspartimide/L-glutarimide trifluroacetate salt was used. The mixture was stirred for 16 h at room temperature and then refluxed for 3 h. Solvent was rotary evaporated and the residue was redissolved in ethyl acetate. The organic layer was washed with 1 M potassium hydrogen sulphate (2×20 mL), dried over MgSO4 and solvent rotary evaporated to obtain the desired product.

5.9.5 *N*-(3-Oxododecanoyl)-L- aspartimide [2.110]



The product was purified by PLC in ethyl acetate. The product *N*-(3-oxododecanoyl)-L-aspartimide appeared as a pale yellow solid in 58 % yield (Method-O).

TLC-R_f = 0.62 in ethyl acetate

mp = 114-115 °C.

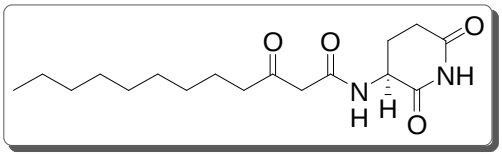
FT-IR (KBr) cm^{-1}: 3302m (νNH, amide and imide), 1786 (νC=O, imide), 1719, 1657(νC=O, amide); 1552s (δNH).

^{1}H NMR ($CDCl_3$ with a drop of DMSO) δ 9.4 (1H, s, imide -N*H*), 8.1 (1H, d, amide -N*H*), 4.6 (1H, m, ring 3-*H*), 3.5 (2H, s, OCH_2O) 3.1 (1H, dd, ring 4-*H*), 2.8 (1H, dd, ring 4-*H*), 2.5 (2H, t, -CH_2CO), 1.6 (2H, m, CH_2CH_2O) 1.2 (12H, m, -CH_2(CH_2)$_6$-), 0.8 (3H, t, -CH_3).

^{13}C NMR ($CDCl_3$) δ 206.67, 179.17, 174.73, 166.81, 47.95, 47.36, 41.86, 38.51, 31.29, 29.78, 29.41, 29.25(2), 22.89, 22.21, 14.12.

(ES-MS/FAB-MS); *m/z* 311.1985 (M+H^+) $C_{16}H_{27}N_2O_4$, requires *m/z* 311.1971 (Δ = 4.5 ppm).

5.9.6 *N*-(3-Oxododecanoyl)-L-glutarimide [2.111]



The product was purified by PLC in ethyl acetate. The product N-(3-oxododecanoyl)-L-glutarimide obtained as off-white solids in 51 % yield (Method-O).

TLC-R_f = 0.27 in ethyl acetate

mp = 96-98 °C.

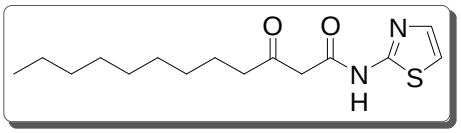
FT-IR (KBr) cm^{-1}: 3339s (νNH, amide and imide), 1723 (νC=O, imide), 1703, 1639(νC=O, amide); 1533s (δNH).

^{1}H NMR ($CDCl_3$) δ 0.9 (3H, t, *J* = 7.0 Hz, CH_3(CH_2)$_8$-), 1.21-1.3 (12H, m, CH_3(CH_2)$_6$-), 1.62 (2H, m, broad, CH_3(CH_2)$_6$CH_2-), 1.94 (1H, m, ring, 4α-*H*), 2.51 (1H, m, ring, 4β-*H*), 2.57 (2H, quart, *J* = 7.2, 4.2 Hz, CH_3(CH_2)$_7$CH_2-), 2.75 (1H, m, ring, 5α-*H*), 2.82 (1H, m, ring, 5β-*H*), 3.5 (2H, s, -COCH_2CO-), 4.64 (1H, m, ring, 3-*H*), 7.85 (1H, d, *J* = 6.0, -$COCH_2CON$*H*-), 8.0 (1H, s, ring, 1-N*H*-).

^{13}C NMR ($CDCl_3$) δ 206.52, 172.77, 169.12, 166.66, 57.45, 47.93, 41.62, 31.91, 29.79, 29.78, 29.51, 29.39, 29.28(2), 23.97, 22.18, 14.12.

(ES-MS/FAB-MS); *m/z* 325.2113 (M+H^+) $C_{17}H_{29}N_2O_4$, requires *m/z* 325.2127 (Δ = 4.3 ppm).

5.9.7 2-(3-Oxododecanoyl-2-amino)thiazole [2.112]



The product was purified by PLC in ethyl acetate-hexane (1:1). The product 2-(3-oxododecanoyl-2-amino)thiazole was obtained as a white solid in 30 % yield (Method-O).

TLC-R_f = 0.41 in ethyl acetate

mp = 137-139 °C.

FT-IR (KBr) cm^{-1}: 3427s (*ν*NH, amide), 1719 (*ν*C=O, amide), 1679 (*ν*C=O, diamide); 1587s (*δ*NH).

^{1}H NMR ($CDCl_3$) δ 0.9 (3H, t, *J* = 7.0 Hz, $CH_3(CH_2)_8$-), 1.22-1.29 (12H, m, $CH_3(CH_2)_6$-), 1.62 (2H, m, broad, $CH_3(CH_2)_6CH_2$-), 2.57 (2H, quart, *J* = 7.2, 4.2 Hz, $CH_3(CH_2)_7CH_2$-), 3.34 (2H, s, -$COCH_2CO$-), 7.12 (1H, d, *J* = 3.6 Hz, ring, 5-*H*), 7.56 (1H, d, *J* = 3.6 Hz, ring, 4-*H*), 8.21 (1H, s, -CH_2CONH-)

^{13}C NMR ($CDCl_3$) δ 207.14, 164.4, 162.92, 138.6, 107.95, 47.93, 42.6, 32.17, 29.72, 29.38(2), 29.18, 23.2, 22.79, 14.12.

(ES-MS/FAB-MS); *m/z* 297.1624 ($M+H^+$) $C_{15}H_{25}N_2O_2S$, requires *m/z* 297.1637 (Δ = 4.3 ppm).

5.10 Synthesis of hetero ring altered analogues of 4-aza 3O, C_{12}-HSL

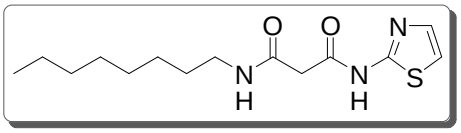
5.10.1 Method-P

To a stirred solution of 3-oxo-4-azadodecanoic acid **2.38** (1 mmol, 0.215 g) in DCM (15 mL) was added sequentially TEA (1.5 mmol, 210 µL), EDC.HCl (1.2 mmol, 0.23 g), and an appropriate amine (1.2 mmol). Stirring was continued for 16 h at room temperature. Solvent was removed under high vacuo and residue redissolved in hot ethyl acetate/DCM (20 mL). Organic solution was sequentially washed with saturated sodium bicarbonate (3 × 10 mL) 1 M HCl (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain desired product.

5.10.2 Method-Q

To a stirred solution of 3-oxo-4-azadodecanoic acid **2.38** (1 mmol, 0.215 g) in a minimum volume of dioxane (4-6 mL) was added a solution of appropriate amine (1.2 mmol) in a minimum amount of water (about 2-4 mL). To this was added sequentially TEA (1.5 mmol, 210 µL) and EDC.HCl (1.2 mmol, 0.23 g) and stirring was continued for 16 h at room temperature. Solvent was removed under high vacuo and residue redissolved in hot ethyl acetate/DCM (20 mL). Organic solution was sequentially washed with water (3 × 10 mL), saturated sodium bicarbonate or1 M $KHSO_4$ (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to obtain desired products.

5.10.3 2-(3-oxo-4-azadodecanoylamino)thiazole [2.125]



2-(3-oxo-4-azadodecanoylamino)thiazole was synthesized from 2-aminothiazole (1.2 mmol, 0.12 g) as off-white crystals in 34 % yield using Method-P. The product was purified by washing it with ether.

TLC-R_f = 0.6 in ethyl acetate

mp = 137-139 °C.

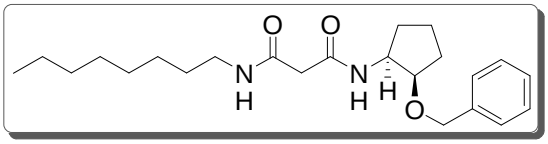
FT-IR (KBr) cm^{-1}: 3314s (νNH, amide), 1701 (νC=O, amine), 1637 (νC=O, diamide); 1558s (δNH).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.4 Hz, $CH_3(CH_2)_7$-), 1.23-1.39 (10H, m, $CH_3(CH_2)_5$-), 1.52-1.57 (2H, m, $CH_3(CH_2)_5CH_2$-), 3.29-3.34 (2H, dd, *J* = 13.2, 7.2 Hz, $CH_3(CH_2)_6CH_2$-), 3.50 (2H, s, -$COCH_2CO$), 6.64 (1H, t, *J* = 5.8 Hz, -N*H*-), 7.03 (1H, d, *J* = 3.6 Hz, ring, 5-*H*), 7.61 (1H, d, *J* = 3.6 Hz, ring, 4-*H*), 12.05 (1H, s, ring, 2-N*H*-)

^{13}C NMR ($CDCl_3$) δ 167.12, 164.27, 162.93, 139.11, 109.47, 43.51, 39.76, 32.92, 30.11, 29.53(2), 26.73, 22.91, 14.18.

(ES-MS/FAB-MS): *m/z* 298.1579 ($M+H^+$) $C_{14}H_{24}N_3O_2S$, requires *m/z* 298.1589 (Δ = 3.35 ppm)

(1S, 2R)-1-O-Benzyl 2-(3-oxo-4-azadodecanoylamino)cyclopentanol **[2.126-a]**



(1*S*, 2*R*)-*O*-Benzyl-2-(3-oxo-4-azadodecanoylamino)cyclopentol was synthesized from (1*S*,2*R*)-1-*O*-benzyl-2-aminocyclopentanol (1.2 mmol, 0.229 g) as pink crystals in 53 % yield using Method-Q.

TLC-R_f = 0.39 in ethyl acetate

mp = 55-57 °C.

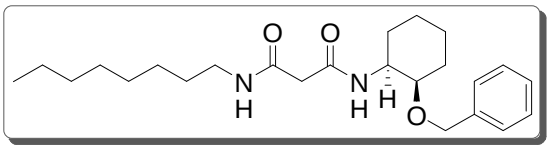
FT-IR (KBr) cm^{-1}: 3280m (*ν*NH, amide), 1655s (*ν*C=O, amide), 1637s (*ν*C=O, diamide); 1560s (*δ*NH), 1064s (*ν*C-OPh, ring).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_7$-), 1.24-1.38 (10H, m, $CH_3(CH_2)_5$-), 1.48-1.56 (3H, m, sequentially, $CH_3(CH_2)_5CH_2$-, and ring, 4α-*H*), 1.70-1.77 (2H, m, sequentially ring, 5α-*H*, 4β-*H*), 1.80-1.85 (1H, m, ring, 3α-*H*), 1.91-1.98 (1H, m, ring, 5β-*H*), 2.11-2.20 (1H, m, ring, 3β-*H*), 3.27 (2H, m, $CH_3(CH_2)_6CH_2$-), 3.35 (2H, s, -$COCH_2CO$), 3.85 (1H, m, ring 2-*H*), 4.25 (1H, m, ring, 1-*H*), 4.59 (2H, dd, *J* = 20.4, 12.0 Hz, -$CH_2C_6H_5$), 7.29-7.34 (5H, m, -C_6H_5), 7.70 (1H, s, broad, -CH_2NH-), 7.78 (1H, broad, ring, 2-N*H*-).

^{13}C NMR ($CDCl_3$) δ 166.60, 166.35, 137.51, 128.71(2). 127.93, 127.51(2), 79.10, 73.17, 51.03, 43.29, 39.79, 31.89, 30.21, 30.09, 29.41(2), 29.1, 27.4, 23.2, 19.4, 14.10.

(ES-MS/FAB-MS); *m/z* 389.2814 ($M+H^+$) $C_{23}H_{37}N_2O_3$, requires *m/z* 389.2804 (Δ = 2.5 ppm).

(1S, 2R)-1-O-Benzyl 2-(3-oxo-4-azadodecanoylamino)cyclohexanol **[2.127-a]**



(1*S*, 2*R*)-1-*O*-Benzyl 2-(3-oxo-4-azadodecanoylamino)cyclohexanol was synthesized from (1*S*,2*R*)-1-*O*-benzyl-2-aminocyclohexanol (1.2 mmol, 0.2464 g) as glassy-white crystals in 56 % yield using Method-Q.

TLC-R_f = 0.4 in ethyl acetate

mp = 77-79 °C.

FT-IR (KBr) cm^{-1}: 3284m (*ν*NH, amide), 1661s (*ν*C=O, amide and diamide); 1559s (*δ*NH), 1101s (*ν*C-OPh, ring).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_7$-), 1.21-1.35 (10H, m, $CH_3(CH_2)_5$-), 1.36-1.44 (4H, m, sequentially, ring, 4α-*H*, 5α-*H*, 6α-*H*, 4β-*H*), 1.51 (2H, m, $CH_3(CH_2)_5CH_2$-), 1.67 (1H, m, 5β-*H*), 1.78 (1H, m, ring, 3α-*H*), 2.06-2.12 (2H, m, sequentially, ring, 5β-*H*, 3β-*H*), 3.20 (2H, s, -COCH_2CO), 3.24 (2H, t, 8.0 Hz, $CH_3(CH_2)_6CH_2$-), 3.29 (1H, m, ring 2H), 3.80-3.85 (1H, m, ring, 1*H*), 4.48 (1H, d, *J* = 12.0 Hz, -C(H)*H*C_6H_5), 4.66 (1H, dd, *J* = 12.0, 1.6 Hz, -C(*H*)HC_6H_5), 7.16 (1H, s, broad, -CH_2N*H*-), 7.28-7.31 (5H, m, -C_6H_5), 7.61 (1H, broad, ring, 2-N*H*-).

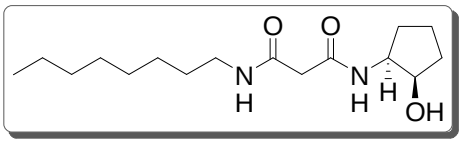
^{13}C NMR ($CDCl_3$) δ 166.20, 166.15, 138.71, 128.43(2). 127.67(2), 127.62, 79.42, 70.67, 53.83, 39.89, 31.80(2), 30.88, 30.21(2), 29.22, 29.19, 26.94, 24.02, 23.69, 22.64, 14.10.

(ES-MS/FAB-MS); *m/z* 403.2944 ($M+H^+$) $C_{24}H_{39}N_2O_3$, requires *m/z* 403.2961 (Δ = 4.2 ppm).

5.10.4 Catalytic hydrogenation

An appropriate (1*S*, 2*R*) *O*-benzyl-2-(3-oxo-4-azadodecanoyl)aminocyclopentanol or (1*S*, 2*R*) *O*-benzyl-2-(3-oxo-4-azadodecanoyl)aminocycloxexanol (1 mmol) was dissolved in absolute ethanol (10 mL). To this was added Pd/C (10 % w/w) and the heterogeneous mixture was stirred for 4 h under atmosphere of hydrogen gas. The catalyst was filtered off using celite as a filter aid. The filtrate was evaporated under vacuo to obtain the desired product **2.126** or **2.127** in 98 % yield.

5.10.5 (1*S*, 2*R*)-2-(3-oxo-4-azadodecanoylamino)cyclopentanol [2.126]



mp = 103-105 °C.

FT-IR (KBr) cm^{-1}: 3273s (*ν*NH, amide and *ν*OH, ring), 1668 (*ν*C=O, amide), 1633 (*ν*C=O, diamide); 1562s (*δ*NH), 1348m (*δ*OH).

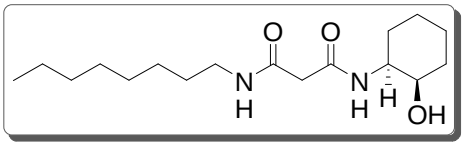
^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_7$-), 1.25-1.38 (10H, m, $CH_3(CH_2)_5$-), 1.08 (2H, s, broad, $CH_3(CH_2)_5CH_2$-), 1.65-1.85 (4H, m, sequentially ring, 4α-*H*, 5α-*H*, 4β-*H*, 3α-*H*), 2.00-2.06 (1H, m, ring, 5β-*H*), 2.12-2.19 (1H, m, ring, 3β-*H*), 3.27 (2H, t, *J* = 6.4 Hz, $CH_3(CH_2)_6CH_2$-), 3.36 (1H, s, -COCH_2CO), 3.87 (1H,

m, ring 2-*H*), 4.04 (1H, m, ring, 1-*H*), 4.50 (1H, s, broad, ring, 1-O*H*), 7.55 (1H, s, -CH_2N*H*-), 8.16 (1H, d, *J* = 4.4 Hz, ring, 2-N*H*-).

^{13}C NMR ($CDCl_3$) δ 166.62, 166.35, 75.42, 53.81, 43.89, 39.80, 32.46, 30.88, 30.12, 29.42(2), 28.79, 26.84, 22.84, 19.64, 14.10.

(ES-MS/FAB-MS); *m/z* 299.2329 ($M+H^+$) $C_{16}H_{31}N_2O_3$, requires *m/z* 299.2335 (Δ = 2.0 ppm).

5.10.6 (1*S*, 2*R*)-2-(3-oxo-4-azadodecanoylamino)cyclohexanol [2.127]



mp = 102-104 °C.

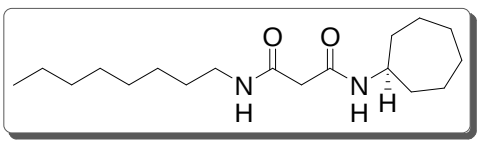
FT-IR (KBr) cm^{-1}: 3279s (*ν*NH, amide and *ν*OH, ring), 1660 (*ν*C=O, amide), 1624 (*ν*C=O, diamide); 1567s (*δ*NH), 1379m (*δ*OH, ring); 1074s (*ν*C-OH, ring).

^{1}H NMR ($CDCl_3$) δ 0.92 (3H, t, *J* = 7.2 Hz, C*H*$_3$(CH_2)$_7$-), 1.21-1.32 (10H, m, CH_3(C*H*$_2$)$_5$-), 1.34-1.43 (4H, m, sequentially, ring, 4α-*H*, 5α-*H*, 6α-*H*, 4β-*H*), 1.47 (2H, m, $CH_3(CH_2)_5$C*H*$_2$-), 1.67 (1H, m, 5β-*H*), 1.78 (1H, m, ring, 3α-*H*), 2.06-2.12 (2H, m, sequentially, ring, 5β-*H*, 3β-*H*), 3.20 (2H, s, -COC*H*$_2$CO), 3.24 (2H, t, 8.0 Hz, $CH_3(CH_2)_6$C*H*$_2$-), 3.29 (1H, m, ring 2H), 4.21 (1H, s, broad, ring, 1-*H*), 4.66 (1H, ring 1-O*H*), 7.16 (1H, s, broad, -CH_2N*H*-), 7.61 (1H, broad, ring, 2-N*H*-).

^{13}C NMR ($CDCl_3$) δ 166.76, 166.32, 67.21, 62.21, 44.19, 39.71, 32.06, 31.48, 30.23, 29.47(2), 27.69, 26.91, 23.22, 22.83, 21.86, 14.10.

(ES-MS/FAB-MS); *m/z* 313.2480 ($M+H^+$) $C_{17}H_{33}N_2O_3$, requires *m/z* 313.2491 (Δ = 3.51 ppm).

5.10.7 *N*-(3-oxo-4-azadodecanoyl)cycloheptylamine [2.128]



N-(3-oxo-4-azadodecanoyl)cycloheptylamine was synthesized from cycloheptylamine (1.2 mmol, 153 µL) as off white crystals in 39 % yield using Method-P.

TLC-R_f = 0.34 in ethyl acetate

mp = 53-55 °C.

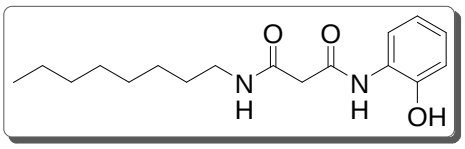
FT-IR (KBr) cm^{-1}: 3299s (νNH, amide), 1662s (νC=O, amide and diamide); 1552s (δNH).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 5.2 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.21-1.39 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.43-1.61 (8H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$- and ring, 4-*2H*, 5-*2H*, 3α-*H*, 6α-*H*), 1.63-1.67 (4H, m, ring, 3β-*H*, 6β-*H*, 2α-*H*, 7α-*H*), 1.89-1.95 (2H, m, ring, 2β-*H*, 7β-*H*), 3.13 (2H, s, -COC*H*$_2$CO), 3.24-3.29 (2H, m, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.95 (1H, m, ring, 1-*H*), 6.72 (1H, s, broad, -CH$_2$N*H*-), 6.88 (1H, d, ring, 1-N*H*-) .

^{13}C NMR ($CDCl_3$) δ 167.18, 166.10, 50.69, 43.37, 39.68, 34.82(2), 31.79, 29.36, 29.23, 29.19, 27.97(2), 26.91, 24.00(2), 22.64, 14.09.

(ES-MS/FAB-MS); *m/z* 311.2687 (M+H$^+$) $C_{18}H_{35}N_2O_2$, requires *m/z* 311.2699 (Δ = 3.85 ppm).

5.10.8 2-(3-Oxo-4-azadodecanoyl)aminophenol [2.129]



2-(3-Oxo-4-azadodecanoyl)aminophenol was synthesized from 2-aminophenol (1.2 mmol, 0.1309 g) as a shiny white crystal in 82 % yield using Method-Q.

TLC-R_f = 0.43 in ethyl acetate-hexane (9:1)

mp = 84-86 °C.

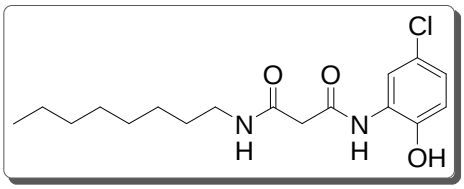
FT-IR (KBr) cm^{-1}: 3327m, sharp, (νOH, phenol); 3261s (νNH, amide); 3095m, (νCH, aromatic); 1676s, 1643s, (νC=O, amide and diamide); 1614s, 1601s, 1455s (aromatic skeletal); 1546s (δNH); 744s (γCH, *O*-disubtituted benzene).

^{1}H NMR ($CDCl_3$ with a drop of DMSO) δ 0.82 (3H, t, *J* = 6.4 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.18-1.31 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.47 (2H, s, broad, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.20 (2H, t, *J* = 6.4 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.39 (1H, s, -COC*H*$_2$CO), 6.78 (1H, t, *J* = 7.6 Hz, ring 6-*H*), 6.90 (1H, d, *J* = 8.0 Hz, ring, 4-*H*), 6.98 (1H, t, *J* = 7.6 Hz, ring, 5-*H*), 7.47 (1H, d, *J* = 8.0, 1.2, Hz, ring, 3-*H*), 7.65 (1H, s, -N*H*-), 8.0 (1H, s, broad, ring, -N*H*-), 9.97 (1H, s, ring , 1-O*H*).

^{13}C NMR (DMSO) δ 166.64, 165.07, 150.1, 128.1. 126.46, 123.16, 121.98, 116.12, 42.31, 40.1, 32.18, 29.99, 29.51, 29.27, 27.21, 23.22, 14.13.

(ES-MS/FAB-MS); *m/z* 307.2034 (M+H$^+$) $C_{17}H_{27}N_2O_3$, requires *m/z* 307.2022 (Δ = 3.9 ppm).

5.10.9 2-(3-Oxo-4-azadodecanoyl)amino-4-chlorophenol [2.130]



2-(3-Oxo-4-azadodecanoyl)amino-4-chlorophenol was synthesized from 2-amino-4-chlorophenol (1.2 mmol, 0.1722 g) as a white solid in 36 % yield using Method-Q.

TLC-R_f = 0.82 in ethyl acetate

mp = 80-82 °C.

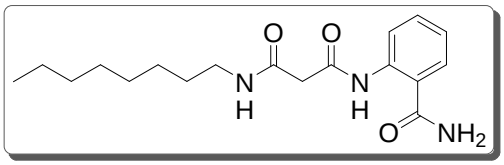
FT-IR (KBr) cm^{-1}: 3373m, sharp, (νOH, phenol); 3253s (νNH, amide); 3100m, (νCH, aromatic); 1677s, 1644s, (νC=O, amide and diamide); 1608m, 1598m, 1422s (aromatic skeletal); 1544s (δNH); 809w (γCH, adjacent 2H of benzene).

^{1}H NMR (DMSO) δ 0.85 (3H, t, *J* = 6.8 Hz, $CH_3(CH_2)_7$-), 1.21-1.26 (10H, m, $CH_3(CH_2)_5$-), 1.39-1.42 (2H, m, $CH_3(CH_2)_5CH_2$-), 3.08 (2H, dd, *J* = 12.8, 6.8 Hz, $CH_3(CH_2)_6CH_2$-), 3.35 (1H, s, -$COCH_2CO$), 6.86 (1H, d, *J* = 8.4 Hz, ring 6-*H*), 6.93 (1H, dd, *J* = 8.4, 2.4 Hz, ring, 5-*H*), 8.15 (1H, d, *J* = 2.8 Hz, ring, 3-*H*), 8.21 (1H, t, *J* = 5.6 Hz, -N*H*-), 9.97 (1H, s, broad, ring, 2-N*H*-), 10.27 (1H, s, ring , 1-O*H*).

^{13}C NMR (DMSO) δ 167.64, 166.07, 145.99, 128.18. 123.56, 122.56, 119.96, 116.25, 43.93, 39.15, 31.68, 29.35, 29.15, 29.12, 26.83, 22.55, 14.41.

(ES-MS/FAB-MS); *m/z* 341.1625 ($M+H^+$) $C_{17}H_{26}N_2O_3Cl$, requires *m/z* 341.1632 (Δ = 2.05 ppm).

5.10.10 2-(3-Oxo-4-azadodecanoyl)aminobenzamide [2.131]



2-(3-Oxo-4-azadodecanoyl)aminobenzamide was synthesized from anthranilamide (1.2 mmol, 0.1634 g) as a white solid in 52 % yield using Method-P.

TLC-R_f = 0.28 in ethyl acetate

mp = 120-121 °C.

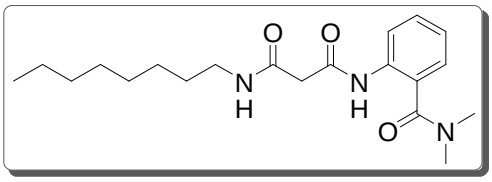
FT-IR (KBr) cm^{-1}: 3402w, 3306m, (νNH, amides); 3201m, (νCH, aromatic); 1685s (νC=O, benzamide); 1634s, (νC=O, doamide); 1592m, 1449w (aromatic skeletal); 1521s (δNH); 752s (γCH, adjacent 4H of benzene).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 7.2 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.26-1.31 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.52-1.59 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.34 (2H, dd, *J* = 12.8, 6.8 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.46 (1H, s, -COC*H*$_2$CO), 6.04 (1H, d, ring, 1-N(H)*H*), 6.26 (3H, s, ring, 1-N(*H*)H), 7.14-7.18 (1H, dt, *J* = 8.0, 1.2 Hz, ring 5-*H*), 7.50 (1H, s, broad, -N*H*-) , 7.52-7.60 (2H, m, ring, 4-*H*, 3-*H*), 8.57 (1H, d, *J* = 8.0 Hz, ring, 6-*H*), 11.59 (1H, s, ring , 2-N*H*-).

^{13}C NMR ($CDCl_3$) δ 167.61, 165.52, 165.01, 138.38. 133.25, 127.37, 124.67, 123.53, 121.81, 44.36, 39.75, 31.80, 29.35, 29.25, 29.19, 26.95, 22.65, 14.11.

(ES-MS/FAB-MS); *m/z* 334.2123 (M+H$^+$) $C_{18}H_{28}N_3O_3$, requires *m/z* 334.2131 (Δ = 2.39 ppm); *m/z* 317.1853 (M-NH$_2$), requires *m/z* 317.1865.

5.10.11 *N*, *N*-Dimethyl-2-(3-oxo-4-azadodecanoyl)aminobenzamide [2.132]



N, *N*-Dimethyl-2-(3-oxo-4-azadodecanoyl)aminobenzamide was synthesized from *N*, *N*-dimethyl anthranilamide (1.2 mmol, 0.180 g) as a pink solid in 66 % yield using Method-P.

TLC-R$_f$ = 0.14 in ethyl acetate-hexane (9:1)

mp = 59-60 °C.

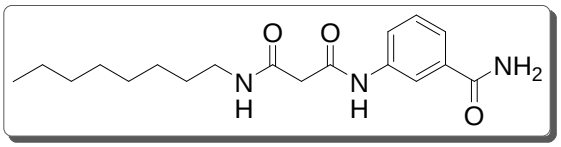
FT-IR (KBr) cm^{-1}: 3304m, (*ν*NH, amides); 3160m, (*ν*CH, aromatic); 1685s, 1660w (*ν*C=O, benzamide, diamide); 1605s, 1484w (aromatic skeletal); 1530m (*δ*NH); 722s (*γ*CH, adjacent 4H of benzene).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.24-1.34 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.49-1.55 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.03 (3H, s, ring, -N(C*H*$_3$)CH$_3$), 3.15 (3H, s, ring, -N(CH$_3$)C*H*$_3$), 3.28 (2H, dd, *J* = 13.2, 7.2 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.36 (1H, s, -COC*H*$_2$CO), 7.14-7.18 (1H, dt, *J* = 8.0, 1.2 Hz, ring 5-*H*), 7.21 (1H, s, broad, -N*H*-), 7.27-7.29 (1H, dd, *J* = 8.0, 1.2 Hz, ring, 4-*H*), 7.38-7.43 (1H, dt, *J* = 8.2, 1.2 Hz, ring, 3-*H*), 9.71 (1H, s, ring , 2-N*H*-).

^{13}C NMR ($CDCl_3$) δ 168.76, 166.55, 164.51, 139.98. 130.35, 127.71, 124.31(2), 121.43, 42.43, 39.59, 37.71(2), 31.98, 30.09, 29.45(2), 26.82, 22.86, 14.11.

(ES-MS/FAB-MS); *m/z* 362.2459 (M+H$^+$) $C_{20}H_{32}N_3O_3$, requires *m/z* 362.2444 (Δ = 4.14 ppm); *m/z* 317.1866 (M-*N(CH$_2$)$_3$*), requires *m/z* 317.1865.

5.10.12 3-(3-Oxo-4-azadodecanoyl)aminobenzamide [2.133]



3-(3-Oxo-4-azadodecanoyl)aminobenzamide was synthesized from 3-aminobenzamide (1.2 mmol, 0.1634 g) as a pink solid in 33 % yield using Method-Q.

mp = 185-187 °C.

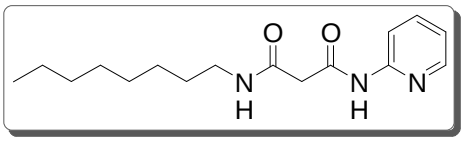
FT-IR (KBr) cm^{-1}: 3374m, sharp, 3307m (νNH, amides); 3171w, (νCH, aromatic); 1659s, 1637s (νC=O, benzamide, diamide); 1586s, 1452m (aromatic skeletal); 1541s (δNH); 791s (γCH, adjacent 3H of benzene).

^{1}H NMR (DMSO) δ 0.87 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.24-1.27 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.35-1.41 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.06 (2H, dd, *J* = 12.8, 6.8 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.23 (1H, s, -COC*H*$_2$CO), 7.34 (1H, s, ring, 1-N(H)*H*), 7.37 (1H, t, *J* = 8.0 Hz, ring 5-*H*), 7.53-7.55 (1H, dt, *J* = 8.0, 1.2 Hz, ring, 6-*H*), 7.74-7.76 (1H, dd, *J* = 8.0, 1.2 Hz, ring, 4-*H*), 7.94 (1H, s, broad, -N*H*-), 8.02 (1H, t, *J* = 2.0 Hz, ring, 1-N(*H*)H), 8.05 (1H, d, *J* = 8.0 Hz, ring, 3-*H*), 10.21 (1H, s, ring, 3-N*H*-).

^{13}C NMR ($CDCl_3$) δ 168.16, 166.62, 164.15, 138.63. 134.45, 129.17, 125.17, 123.63, 117.28, 42.46, 39.71, 31.90, 30.15, 29.42(2), 26.81, 22.83, 14.10.

(ES-MS/FAB-MS); *m/z* 334.2146 (M+H$^+$) $C_{18}H_{28}N_3O_3$, requires *m/z* 334.2131 (Δ = 4.4 ppm).

5.10.13 2-(3-Oxo-4-azadodecanoyl)aminopyridine [2.134]



2-(3-Oxo-4-azadodecanoyl)aminopyridine was synthesized from 2-aminopyridine (1mmol, 0.0901 g) as purple crystals in 17 % yield using Method-P.

(*Note: Here washing of ethyl acetate extract with HCl was excluded to avoid formation of water soluble pyridinium chloride salt*).

TLC-R_f = 0.41 in ethyl acetate

mp = 134-136 °C.

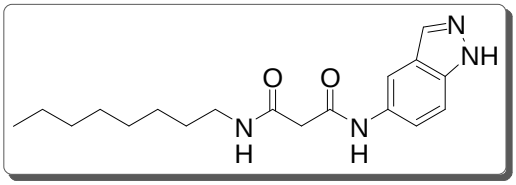
FT-IR (KBr) cm^{-1}: 3306m, (νNH, amides); 3117w, (νCH, aromatic); 1645s (νC=O, diamide); 1679m, 1578s, 1438s (aromatic skeletal); 1555s (δNH); 778s (γCH, adjacent 4H of pyridine).

^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.8 Hz, $CH_3(CH_2)_7$-), 1.27-1.30 (10H, m, $CH_3(CH_2)_5$-), 1.52-1.58 (2H, m, $CH_3(CH_2)_5CH_2$-), 2.86 (1H, s, -NH-), 3.28 (2H, dd, J = 9.6, 6.8 Hz, $CH_3(CH_2)_6CH_2$-), 3.57 (1H, s, -$COCH_2CO$), 7.16 (1H, s, ring, 2-NH-), 7.23 (1H, t, J = 6.4 Hz, ring 5-*H*), 7.93 (1H, t, J = 8.0 Hz, ring, 4-*H*), 8.19 (1H, t, J = 8.8 Hz, ring, 3-*H*), 8.44 (1H, d, J = 4.8 Hz, ring, 6-*H*).

^{13}C NMR ($CDCl_3$) δ 167.1, 164.10, 150.16, 146.15, 139.1, 120.21, 116.21, 42.58, 39.76, 31.89, 30.1, 29.37, 29.32, 26.81, 22.76, 14.1.

(ES-MS/FAB-MS); *m/z* 292.2016 ($M+H^+$) $C_{16}H_{26}N_3O_3$, requires *m/z* 292.2025 (Δ = 3.1 ppm).

5.10.14 5-(3-Oxo-4-azadodecanoyl)amino-1*H*-indazole [2.135]



To a stirred solution of 3-oxo-4-azadodecanoic acid **2.38** (1 mmol, 0.215 g) in a minimum volume of dioxane (4 mL) was added a solution of 5-aminoindazole (1.2 mmol, 0.1598 g) in water (about 2-4 mL). To this was added sequentially TEA (1.5 mmol, 210 µL) and EDC.HCl (1.2 mmol, 0.23 g) and stirred for 16 h at room temperature. Because product was insoluble in solvent used it was deposited in reaction mixture as crystals. Solvent was removed under high vacuo and the residue redissolved in warm ethyl acetate (10 mL). The crystals settled down were then filtered and collected. Product was sequentially washed with water (1 × 5 mL), 1 M $KHSO_4$ (1 × 5 mL) and finally with methanol (10 mL) to obtained desired product as off-white crystals in 62 % yield.

mp = 45-47 °C.

FT-IR (KBr) cm^{-1}: 3283s, (νNH, amides, ring); 1632s (νC=O, diamide); 1663s, 1597w, (aromatic skeletal); 1565w (δNH); 966m (γCH, adjacent 1H of indazole); 816m (γCH, adjacent 2*H* of indazole).

^{1}H NMR (DMSO) δ 0.85 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.24-1.29 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.40-1.44 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.08 (2H, dd, *J* = 8.8, 6.8 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 3.23 (1H, s, -COC*H*$_2$CO), 7.39-7.41 (1H, dd, *J* = 8.8, 2.0 Hz, ring 7-*H*), 7.48 (1H, d, *J* = 8.8 Hz, ring, 6-*H*), 8.01 (1H, s, ring, 3-*H*), 8.05 (1H, t, *J* = 5.6 Hz, -N*H*-), 8.13 (1H, s, ring, 4-*H*), 10.09 (1H, s, ring, 5-N*H*-), 12.98 (1H, s, ring, 1-N*H*-).

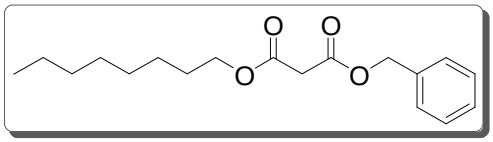
^{13}C NMR (DMSO) δ 166.90, 166.00, 137.36, 133.85, 132.48, 123.61, 120.58, 110.60, 109.97, 45.08, 39.16, 31.70, 29.47, 29.18, 29.14, 26.83, 22.56, 14.43.

(ES-MS/FAB-MS); *m/z* 331.2146 (M+H$^+$) $C_{18}H_{27}N_4O_2$, requires *m/z* 331.2134 (Δ = 3.6 ppm).

5.11 Synthesis of oxa analogues of 3O, C_{12}-HSL

5.11.1 3O, 4-oxa C_{12}-HSL [2.139]

O-Benzyl-O-octanyl malonate [2.144]

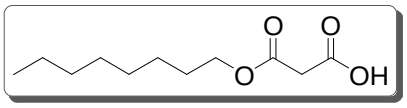


To a stirred solution of monobenzyl malonate (2 mmol, 388 g) in a dry DCM (15 ml) was added sequentially *n*-octyl alcohol (2 mmol, 318 µL), DMAP (3 mmol, 0.366 g) and DCCI (2.2 mmol, 0. 454 g). The mixture was stirred for 16 h and solvent rotary evaporated under vacuo. The residue was redissolved in ethyl acetate (25 mL) and was washed with saturated sodium bicarbonate (3 × 10 mL), 1 M HCl (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and rotary evaporated to get the residue was kept aside in the cold acetone (4-6 mL). When the remaining DCU separated this was carefully removed. The final product **2.144** was purified by column chromatography in ether-hexane (3:7) as colorless oil in 76 % yield.

TLC-R_f = 0.4 in ether-hexane (3:7)

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.25-1.33 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.61-1.64 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.44 (2H, s, -COC*H*$_2$CO-), 4.14 (2H, t, *J* = 6.8 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 5.20 (2H, s, -C*H*$_2$C$_6$H$_5$), 7.35-7.39 (5H, broad, m, -C$_6$*H*$_5$).

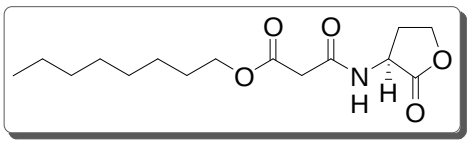
3-Oxo-4-oxadodecanoic acid [2.145]



O-Benzyl-*O*-octanyl malonate **2.144** (1 mmol) was dissolved in absolute ethanol (10 mL). To this was added Pd/C (10 % w/w) and the heterogeneous mixture was stirred for 6 h under atmosphere of hydrogen gas. The catalyst was filtered off using celite as a filter aid. The filtrate was evaporated under vacuo and gave the desired product in 94 % yield.

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.25-1.32 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.63-1.68 (2H, m, CH$_3$(CH$_2$)$_5$C*H*$_2$-), 3.45 (2H, s, -COC*H*$_2$CO-), 4.19 (2H, t, *J* = 6.8 Hz, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 10.80 (1H, s, -COO*H*).

3O, 4-oxa C$_{12}$-HSL [2.139]



3O, 4-oxa C_{12}-HSL was synthesized from 3-oxo-4-oxadodecanoic acid **2.145** as a white solid in 55 % yield using Method B.

TLC-R_f = 0.31 in ethyl acetate-hexane (1:1)

mp = 57-59 °C.

FT-IR (KBr) cm^{-1}: 3300m (*ν*NH, amide); 1790s (*ν*C=O, lactone); 1737s (*ν*C=O, β-diketoester); 1650s (*ν*C=O, amide); 1535s (*δ*NH); 1194s, 1165s (*ν*O-C, 2-bands of ester).

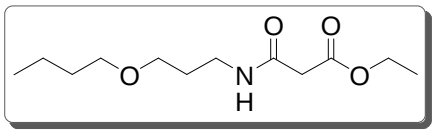
^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.8 Hz, C*H*$_3$(CH$_2$)$_7$-), 1.26-1.35 (10H, m, CH$_3$(C*H*$_2$)$_5$-), 1.63-1.69 (2H, m, broad, CH$_3$(CH$_2$)$_6$C*H*$_2$-), 2.18-2.31 (1H, m, ring, 4α-*H*), 2.75-2.83 (1H, m, ring, 4β-*H*), 3.40 (2H, s, COC*H*$_2$CO), 4.17 (2H, t, *J* = 6.8 Hz, CH$_3$(CH$_2$)$_7$C*H*$_2$-), 4.29-4.34 (1H, m, ring, 5α-*H*), 4.48-4.56 (1H, m, ring, 5β-*H*), 4.58-4.65 (1H, m, ring, 3-*H*), 7.80 (1H, d, *J* = 5.8 Hz, -N*H*-HSL).

^{13}C NMR (DMSO) δ 174.97, 168.0, 167.16, 89.15, 66.18, 65.32, 54.2, 32.23, 30.37, 29.47, 29.18, 29.14, 26.32, 23.16, 14.43.

(ES-MS/FAB-MS); *m/z* 300.1818 (M+H$^+$) $C_{15}H_{26}NO_5$, requires *m/z* 300.1811 (Δ = 2.33 ppm).

5.12 Synthesis of 8-oxa analogues of 3O, 4-aza C_{12}-HSL

Ethyl 3-oxo-8-oxa-4-azadodecanoate

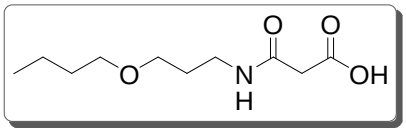


Ethyl 3-oxo-8-oxa-4-azadodecanoate was synthesized as yellow viscous oil in 99 % yield from 3-butoxypropylamine **2.147** using Method-D.

^{1}H NMR ($CDCl_3$) δ 0.93 (3H, t, *J* = 7.2 Hz, C*H*$_3$(CH$_2$)$_3$-), 1.30 (3H, t, *J* = 7.2 Hz, C*H*$_3$CH$_2$CO-), 1.36-1.43 (2H, m, CH$_3$C*H*$_2$(CH$_2$)$_2$-), 1.54-1.61 (2H, m, CH$_3$CH$_2$ C*H*$_2$CH$_2$-), 1.78-1.84 (2H, m, -OCH$_2$C*H*$_2$CH$_2$NH-), 3.29 (2H, s, -COC*H*$_2$CO-), 3.31-3.45 (4H, m, -C*H*$_2$O(CH$_2$)$_2$C*H*$_2$NH-), 3.49-3.52 (2H, t, *J* = 5.8 Hz, -OC*H*$_2$ (CH$_2$)$_2$NH-), 4.21 (2H, q, *J* = 7.2 Hz, CH$_3$C*H*$_2$CO-), 7.36 (1H, s, broad, -N*H*-).

(ES-MS/FAB-MS); *m/z* 246.1709 (M+H$^+$) $C_{12}H_{24}NO_4$, requires *m/z* 246.1705 (Δ = 1.62 ppm).

3-Oxo-8-oxa-4-azadodecanoic acid **[2.148]**



Saponification of ethyl, 3-oxo-8-oxa-4-azadodecanoate afforded 3-oxo-8-oxa-4-azadodecanoic acid as colorless viscous oil in 79 % yield.

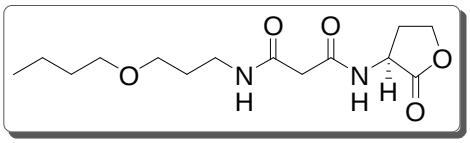
FT-IR (CH_3CN) cm^{-1}: 3319m, br, (*ν*NH and *ν*OH); 1731s (*ν*C=O, acid); 1634s (*ν*C=O, amide); 1562s (*δ*NH, amide); 1115m (*ν*C-O, ether).

^{1}H NMR ($CDCl_3$) δ 0.96 (3H, t, *J* = 7.2 Hz, C*H*$_3$(CH$_2$)$_3$-), 1.34-1.43 (2H, m, CH$_3$C*H*$_2$(CH$_2$)$_2$-), 1.54-1.61 (2H, m, CH$_3$CH$_2$C*H*$_2$CH$_2$-), 1.81-1.87 (2H, m, -OCH$_2$C*H*$_2$ CH$_2$NH-), 3.28 (2H, s, -COC*H*$_2$CO-), 3.44-3.53 (4H, m, -C*H*$_2$O(CH$_2$)$_2$C*H*$_2$NH-), 3.58-3.60 (2H, m, -OC*H*$_2$(CH$_2$)$_2$NH-), 7.20 (1H, s, broad, -N*H*-), 10.23 (1H, s, -COO*H*).

^{13}C NMR ($CDCl_3$) δ 169.32, 168.03, 91.21, 71.81, 70.32, 38.13, 31.74, 28.18, 19.40, 13.89.

(ES-MS/FAB-MS); *m/z* 216.1245 (M-H$^+$) $C_{10}H_{18}NO_4$, requires *m/z* 216.1236 (Δ = 4.1 ppm).

5.12.1 3O, 8-oxa-4-aza C_{12}-HSL [2.140]



3O, 8-oxa-4-aza C_{12}-HSL was synthesized from 3-oxo-8-oxa-4-azadodecanoic acid **2.148** using Method-A. Desired compound was purified by column chromatography using ethyl acetate-methanol (4:1) as a white solid in 68 % yield.

TLC-R_f = 0.56 in ethyl acetate-methanol (4:1)

mp = 83-85 °C.

FT-IR (KBr) cm^{-1}: 3282s, (*ν*NH); 1770s (*ν*C=O, lactone); 1684m (*ν*C=O, diamide); 1647s (*ν*C=O, amide); 1556s (*δ*NH, amide); 1118m (*ν*C-O, ether).

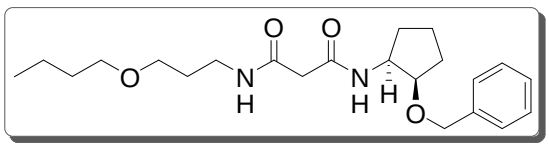
^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, *J* = 7.4 Hz, C*H*$_3$(CH$_2$)$_3$-), 1.33-1.43 (2H, m, CH$_3$C*H*$_2$(CH$_2$)$_2$-), 1.54-1.61 (2H, quint, *J* = 9.6, 6.8 Hz, CH$_3$CH$_2$C*H*$_2$CH$_2$-), 1.77-1.83 (2H, quint, *J* = 6.0 Hz, -OCH$_2$C*H*$_2$CH$_2$NH-), 2.24-2.35 (1H, m, ring, 4α-*H*), 2.68-2.75 (1H, m, ring, 4β-*H*), 3.23 (2H, s, -COC*H*$_2$CO-), 3.38-3.45 (4H, m, -C*H*$_2$O(CH$_2$)$_2$C*H*$_2$NH-), 3.52-3.55 (2H, m, -OC*H*$_2$(CH$_2$)$_2$NH-), 4.25-4.32 (1H, m, ring, 5α-*H*), 4.47-4.52 (1H, m, ring, 5β-*H*), 4.56-4.60 (1H, m, ring, 3-*H*), 7.05 (1H, s, broad, -N*H*-), 8.21 (1H, d, *J* = 6.4 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.71, 167.41, 167.09, 71.08, 69.86, 65.81, 48.93, 42.24, 38.79, 31.78, 29.48, 28.67, 19.39, 13.93.

(ES-MS/FAB-MS); *m/z* 323.1574 (M+Na) $C_{14}H_{24}N_2O_5Na$, requires *m/z* 323.1583 (Δ = 2.78 ppm) and *m/z* 301.1748 (M+H$^+$) $C_{14}H_{25}N_2O_5$, requires *m/z* 301.1763 (Δ = 4.9 ppm).

5.12.2 (1*S*, 2*R*)-2-(3-Oxo, 8-oxa-4-azadodecanoyl)aminocyclopentanol [2.141]

(1S, 2R)-1-O-Benzyl-2-(3-oxo-8-oxa-4-azadodecanoyl)aminocyclopentanol [2.150]



(1*S*, 2*R*)-1-*O*-Benzyl-2-(3-oxo-8-oxa-4-azadodecanoyl)aminocyclopentanol was isolated as an oil from 3-oxo-8-oxa-4-azadodecanoic acid **2.148** in 45 % yield using Method-P.

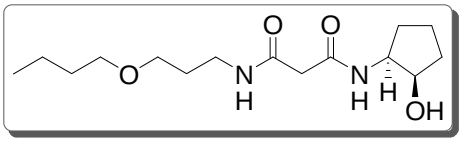
TLC-R_f = 0.55 in ethyl acetate-methanol (4:1)

^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, *J* = 7.4 Hz, C*H*$_3$(CH$_2$)$_3$-), 1.34-1.43 (2H, m, CH$_3$C*H*$_2$(CH$_2$)$_2$-), 1.44-1.52 (1H, m, ring, 4α-*H*), 1.54-1.61 (2H, m, CH$_3$CH$_2$C*H*$_2$CH$_2$-) , 1.67-1.75 (3H, m, ring, 5α-*H*, 4β-*H*, 3α-*H*), 1.76-1.84 (2H, m, -OCH$_2$C*H*$_2$CH$_2$NH-), 1.85-1.98 (1H, m, ring, 5β-*H*), 2.13-2.22 (1H, m, ring, 3β-*H*), 3.12 (2H, s, -COC*H*$_2$CO-), 3.37-3.47 (4H, m, -C*H*$_2$O(CH$_2$)$_2$C*H*$_2$NH-), 3.51-3.54 (2H, m, -OC*H*$_2$(CH$_2$)$_2$NH-), 3.80-3.84 (1H, m, ring, 2-*H*), 4.24-4.28 (1H, m, ring, 1-*H*), 4.62 (2H, dd, *J* = 12.0, 28.8 Hz, -C*H*$_2$C$_6$H$_5$), 7.03 (1H, d, *J* = 4.8 Hz, ring-N*H*-), 7.31 (1H, s, broad, -N*H*-), 7.33-7.51 (5H, m, -C$_6$*H*$_5$).

^{13}C NMR ($CDCl_3$) δ 166.38, 166.13, 138.66, 128.34(2), 127.64(2), 127.46, 84.75, 71.12, 71.05, 69.69, 55.79, 42.53, 38.57, 31.78, 30.56, 30.47, 28.79, 21.72, 19.38, 13.93.

(ES-MS/FAB-MS); *m/z* 413.2399 (M+Na) $C_{22}H_{35}N_2O_4Na$, requires *m/z* 413.2416 (Δ = 4.1 ppm); *m/z* 391.2575 (M+H$^+$) $C_{22}H_{35}N_2O_4$, requires *m/z* 391.2597 (Δ = 3.9 ppm).

(1S, 2R)-2-(3-Oxo, 8-oxa-4-azadodecanoyl)aminocyclopentanol [2.141]



(1*S*, 2*R*)-2-(3-Oxo, 8-oxa-4-azadodecanoyl)aminocyclopentanol was synthesized as a white solid by catalytic hydrogenation of (*1S*, *2R*)-1-*O*-benzyl-2-(3-oxo-8-oxa-4-azadodecanoyl)aminocyclopentanol **2.150** in 88 % yield.

mp = 49-51 °C.

FT-IR (KBr) cm^{-1}: 3320m (*ν*NH, amide and *ν*OH, ring), 1627 (*ν*C=O, amide, diamide); 1560s (*δ*NH), 1347m (*δ*OH); 1114m (*ν*C-O, ether).

^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t, *J* = 7.2 Hz, C*H*$_3$(CH$_2$)$_3$-), 1.34-1.43 (2H, m, CH$_3$C*H*$_2$(CH$_2$)$_2$-), 1.48-1.54 (1H, m, ring, 4α-*H*), 1.56-1.62 (2H, m, CH$_3$CH$_2$C*H*$_2$CH$_2$-) , 1.64-1.76 (3H, m, ring, 5α-*H*, 4β-*H*, 3α-*H*), 1.78-1.84 (2H, m, -OCH$_2$C*H*$_2$CH$_2$NH-), 2.03-2.08 (1H, m, ring, 5β-*H*), 2.13-2.18 (1H, m, ring, 3β-*H*), 3.16 (2H, s, -COC*H*$_2$CO-), 3.38-3.46 (4H, m, -C*H*$_2$O(CH$_2$)$_2$C*H*$_2$NH-), 3.53-3.56 (2H, m, -OC*H*$_2$(CH$_2$)$_2$NH-), 3.82-3.85 (1H, m, ring, 2-*H*), 3.97-4.03 (1H, m, ring, 1-*H*), 4.34 (1H, s, broad, ring, -O*H*-), 6.92 (1H, s, broad, -N*H*-), 7.77 (1H, s, ring-N*H*-).

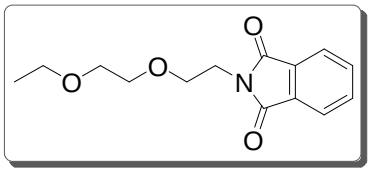
^{13}C NMR ($CDCl_3$) δ 166.47, 166.22, 74.75, 71.23, 70.25, 53.19, 45.29, 36.67, 32.48, 29.96, 28.97, 19.28(2), 14.13.

(ES-MS/FAB-MS); *m/z* 301.2119 ($M+H^+$) $C_{15}H_{29}N_2O_4$, requires *m/z* 301.2127 (Δ = 2.6 ppm).

5.13 Synthesis of 7, 10-dioxa analogues of 3O, 4-aza C_{12}-HSL

5.13.1 3O, 7, 10-dioxa, 4-aza C_{12}-HSL [2.142]

N-(3, 6-Dioxaoctyl)phthalimide



Diisopropyl azadicarboxylate (14.1 mmol, 2.85 g) was added to a pre-cooled (with a salt-ice bath) solution of toluene (40 mL) containing triphenylphosphine (13 mmol, 3.41 g), di(ethyleneglycol)ethylether (13 mmol, 1.75 g) and phthalimide (12 mmol, 1.766 g). The reaction mixture was stirred for 50 minutes and then cooling bath was removed. The reaction mixture was allowed to warm to 15-25 °C and stirred for 1 h. To this methanol (10 mL) was added and stirred further 16 h at room temperature.

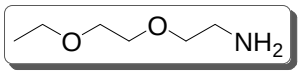
The mixture was filtered and filtrate concentrated under vacuum to obtain more precipitate. The crude precipitate was washed with methanol (5 mL) and dried to give white solids in 75 % yield.

mp = 47-49 °C.

^{1}H NMR ($CDCl_3$) δ 1.14 (3H, t, *J* = 6.8 Hz, C*H*$_3$-), 1.46 (2H, q, *J* = 7.2 Hz, CH_3CH_2-), 1.54 (2H, q, *J* = 6.4, 5.2 Hz, $CH_3CH_2OCH_2$-), 3.65 (2H, q, *J* = 6.4, 5.2 Hz, $CH_3CH_2OCH_2CH_2$-), 3.75 (2H, t, *J* = 5.8 Hz, $CH_3CH_2O(CH_2)_2OCH_2$-), 3.91 (2H, t, *J* = 5.8 Hz, $CH_3CH_2O(CH_2)_2OCH_2CH_2$-), 7.71 (2H, q, *J* = 5.6, 3.2 Hz, ring, 4'-*H*, 7'-*H*), 7.85 (2H, m, ring, 5'-*H*, 6'-*H*).

(ES-MS/FAB-MS); *m/z* 264.1238 ($M+H^+$) $C_{14}H_{18}NO_4$, requires *m/z* 264.1236 (Δ = 0.76 ppm).

3, 6-Dioxaoctyl amine [2.146]



To a solution of *N*-(3, 6-dioxaoctyl)phthalimide (8 mmol, 2.104 g) in methanol (35 mL) added hydrazine hydrate (16 mmol, 498 µL). After 2 h sodium borohydride (8 mmol, 0.303 g) and a second lot of hydrazine hydrate (16 mmol, 498 µL) were added and stirred for further 16 h. Suspension was filtered to remove white precipitate and filtrate was concentrated under vacuo and then under N_2 gas. Residue was redissolved in ethyl acetate (20 mL) heated to 50 °C, sonicated and then cooled to room temperature. Suspension was kept in liquid nitrogen and filtered. Ethyl acetate rotary evaporated to get oil. Oil was dissolved in ether (20 mL) filtered and rotary evaporated under vacuo to obtain desired yellow viscous oil in 80 % yield.

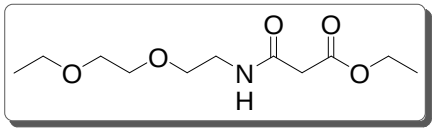
TLC-R_f = 0.2 in methanol-ammonia (9.7:0.3)

FT-IR (CH_3CN) cm^{-1}: 3398m, 3479m (*ν*NH, primary amine); 1677m (δNH, primary amine); 1265s (*ν*C-O, ether); 1108s (*ν*C-N).

^{1}H NMR ($CDCl_3$) δ 1.21 (3H, t, *J* = 7.2 Hz, C*H*$_3$-), 1.75 (2H, s, -N*H*$_2$), 2.87 (2H, t, *J* = 5.2 Hz, -C*H*$_2$NH$_2$), 3.50-3.55 (4H, m, $CH_3CH_2O(CH_2)_2OCH_2$-), 3.57-3.63 (4H, m, -O(C*H*$_2$)$_2$O-).

^{13}C NMR ($CDCl_3$) δ 73.24, 70.35, 69.78, 66.66, 65.84, 41.67, 15.15.

Ethyl 3-oxo-7, 10-dioxa-4-aza-dodecanoate



Ethyl 3-oxo-7, 10-dioxa-4-azadodecanoate was synthesized as yellowish oil from 3, 6-dioxaoctyl amine **2.146** using Method-D. Product was purified as colorless oil in 62 % yield by column chromatography using ethyl acetate-hexane (9:1).

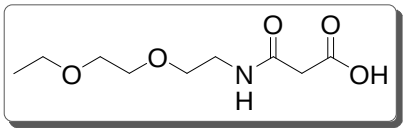
TLC-R_f = 0.2 in ethyl acetate-hexane (9:1)

FT-IR (KBr) cm^{-1}: 3379m, br, (*ν*NH); 1738s (*ν*C=O, ester); 1660s (*ν*C=O, amide); 1556s (*δ*NH, amide); 1106s, (*ν*C-O, ether).

^{1}H NMR ($CDCl_3$) δ 1.21 (3H, t, *J* = 7.2 Hz, C*H*$_3$CH$_2$OCH$_2$-), 2.27 (3H, t, *J* = 7.2 Hz, C*H*$_3$CH$_2$CO-), 3.29 (2H, s, -COC*H*$_2$CO-), 3.46-3.51 (2H, m, -C*H*$_2$NH-), 3.50-3.59 (6H, m, CH$_3$C*H*$_2$O(C*H*$_2$)$_2$O-), 3.61-3.64 (2H, m, -C*H*$_2$CH$_2$NH-), 4.19 (2H, q, *J* = 7.2 Hz, CH$_3$C*H*$_2$CO-), 7.32 (1H, s, broad, -N*H*-).

(ES-MS/FAB-MS); *m/z* 248.1494 ($M+H^+$) $C_{11}H_{22}NO_5$, requires *m/z* 248.1498 (Δ = 1.6 ppm).

3-Oxo-7, 10-dioxa-4-azadodecanoic acid [2.149]



Saponification of ethyl, 3-oxo-7, 10-dioxa-4-azadodecanoate afforded 3-oxo-7, 10-dioxa-4-azadodecanoic acid as a white hygroscopic solid in 89 % yield.

mp = 68-60 °C.

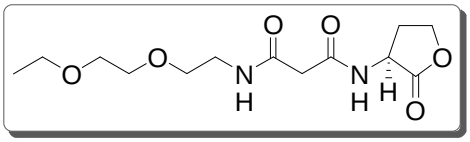
FT-IR (KBr) cm^{-1}: 3317m, (*ν*NH and *ν*OH); 2681w, (δOH); 1735s (*ν*C=O, acid); 1639s (*ν*C=O, amide); 1578s (*δ*NH, amide); 1104s, 1093s (*ν*C-O, ether).

^{1}H NMR ($CDCl_3$) δ 1.24 (3H, t, *J* = 7.2 Hz, C*H*$_3$CH$_2$OCH$_2$-), 3.34 (2H, s, COC*H*$_2$CO), 3.52-3.55 (2H, m, -C*H*$_2$NH-), 3.54-3.64 (6H, m, CH$_3$C*H*$_2$O(C*H*$_2$)$_2$O-), 3.65-3.68 (2H, m, -C*H*$_2$CH$_2$NH-), 7.17 (1H, s, broad, -N*H*-), 10.37 (1H, s, -COO*H*)

^{13}C NMR ($CDCl_3$) δ 169.06, 167.13, 70.32, 69.73, 69.03, 66.76, 39.69, 38.26, 15.14.

(ES-MS/FAB-MS); *m/z* 218.1037 ($M-H^+$) $C_9H_{16}NO_5$, requires *m/z* 218.1028 (Δ = 4.1 ppm).

3O, 7, 10-dioxa, 4-aza C_{12}-HSL [2.142]



To a stirred solution of 3-oxo-7, 10-dioxa-4-azadodecanoic acid **2.149** (1 mmol) in a minimum volume of dioxane (4-6 mL) was added a solution of L-HSL.HCl (1 mmol) in a minimum amount of water (about 2 mL). To this was added sequentially TEA (3 mmol) and EDC.HCl (1.5 mmol) stirred 16 h at room temperature. Solvent was removed under vacuo and residue redissolved in warm ethyl acetate (2 × 15 mL). Solvent was rotary evaporated to obtain crude product that was dissolved in minimum ethyl acetate (2 mL) and was loaded on small (5 cm length) column containing 2 cm silica gel. Impurities were eluted with ethyl acetate and desired product with methanol. Methanol was rotary evaporated to obtain pure desired product as a white semisolid in 58 % yield.

mp = 44-46 °C.

FT-IR (KBr) cm^{-1}: 3457m, 3259s, (*ν*NH, amides); 1773s (*ν*C=O, lactone); 1665s (*ν*C=O, diamide); 1636s (*ν*C=O, amide); 1567s (*δ*NH, amide); 1116s (*ν*C-O, ether).

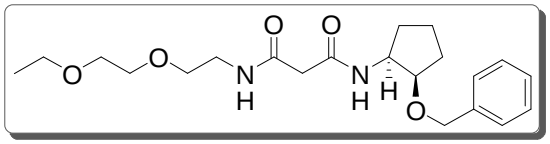
^{1}H NMR ($CDCl_3$) δ 1.24 (3H, t, *J* = 7.2 Hz, $CH_3CH_2OCH_2$-), 2.27-2.35 (1H, m, ring, 4α-*H*), 2.66-2.73 (1H, m, ring, 4β-*H*), 3.27 (2H, s, -$COCH_2CO$-), 3.46-3.51 (2H, m, -CH_2NH-), 3.52-3.60 (6H, m, $CH_3CH_2O(CH_2)_2O$-), 3.62-3.65 (2H, m, -CH_2CH_2NH-), 4.24-4.31 (1H, m, ring, 5α-*H*), 4.46-4.51 (1H, m, ring, 5β-*H*), 4.56-4.63 (1H, m, ring, 3-*H*), 7.21 (1H, s, broad, -N*H*-), 8.21 (1H, d, *J* = 6.8 Hz, -N*H*-HSL).

^{13}C NMR ($CDCl_3$) δ 174.84, 167.51, 167.53, 70.34, 69.74, 69.33, 66.71, 65.85, 48.94, 42.18, 39.49, 29.37, 15.16.

(ES-MS/FAB-MS); *m/z* 303.1562 ($M+H^+$) $C_{13}H_{23}N_2O_6$, requires *m/z* 303.1556 (Δ = 1.98 ppm).

5.13.2 (1*S*, 2*R*)-2-(3-Oxo, 7, 10-dioxa-4-azadodecanoyl)aminocyclopentanol [2.143]

(1S, 2R)-1-O-Benzyl-2-(3-oxo-7, 10-dioxa-4-azadodecanoyl)aminocyclopentanol [2.151]

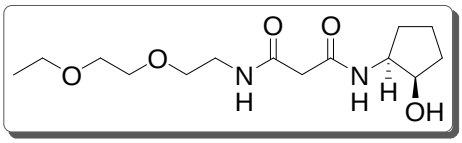


(1*S*, 2*R*)-1-*O*-Benzyl-2-(3-oxo-7, 10-dioxa-4-azadodecanoyl)aminocyclopentanol was synthesized from 3-oxo-7, 10-dioxa-4-azadodecanoic acid **2.149** (1 mmol, 0.219 g) and (1*S*,2*R*)-1-*O*-benzyl-2-aminocyclopentanol (1.2 mmol, 0.229 g) as a yellow liquid using Method-P. The product was purified by column chromatography in 70 % yield using ethyl acetate-methanol (4:1) solvent system.

TLC-R_f = 0.5 in ethyl acetate-methanol (4:1)

(ES-MS/FAB-MS); *m/z* 393.2376 ($M+H^+$) $C_{21}H_{33}N_2O_5$, requires *m/z* 393.2389 (Δ = 3.3 ppm).

(1S, 2R)-2-(3-Oxo, 7, 10-dioxa-4-azadodecanoyl)aminocyclopentanol [2.143]



(1*S*, 2*R*)-2-(3-Oxo, 7, 10-dioxa-4-azadodecanoyl)aminocyclopentanol was synthesized as a colorless liquid in 93 % yield by catalytic hydrogenation of (1*S*, 2*R*)-1-*O*-benzyl-2-(3-oxo-7, 10-dioxa-4-azadodecanoyl)aminocyclopentanol **2.151**.

FT-IR (CH_3CN) cm^{-1}: 3326m (*ν*NH, amide and *ν*OH, ring), 1657 (*ν*C=O, amide); 1632s (*ν*C=O, diamide); 1553s (*δ*NH), 1338m (*δ*OH); 1109m (*ν*C-O, ether).

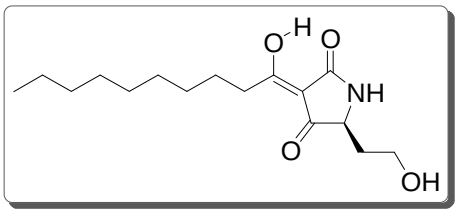
^{1}H NMR ($CDCl_3$) δ 1.25 (3H, t, *J* = 7.2 Hz, C*H*$_3$CH$_2$OCH$_2$-), 1.45-1.57 (1H, m, ring, 4α-*H*), 1.62-1.68 (1H, m, ring, 5α-*H*), 1.69-1.75 (1H, m, ring, 4β-*H*), 1.77-1.87 (1H, m, ring, 3α-*H*), 1.99-2.09 (1H, m, ring, 5β-*H*), 2.12-2.19 (1H, m, ring, 3β-*H*), 2.31 (1H, s, broad, ring, -O*H*), 3.22 (2H, s, -COC*H*$_2$CO-), 3.47-3.50 (2H, m, -C*H*$_2$NH-), 3.53-3.61 (6H, m, CH$_3$C*H*$_2$O(C*H*$_2$)$_2$O-), 3.63-3.67 (2H, m, -C*H*$_2$CH$_2$NH-), 3.81-3.89 (1H, m, ring, 2-*H*), 3.97-4.02 (1H, m, ring, 1-*H*), 7.06 (1H, s, broad, -N*H*-), 7.80 (1H, s, ring, -N*H*-).

^{13}C NMR ($CDCl_3$) δ 168.53, 167.89, 79.31, 70.39, 69.75, 69.31, 66.74, 60.62, 42.21, 39.49, 32.48, 30.10, 21.21, 15.17.

(ES-MS/FAB-MS); *m/z* 301.1773 (M-H$^+$) $C_{14}H_{25}N_2O_5$, requires *m/z* 301.1763 (Δ = 3.32 ppm).

5.14 Miscellaneous analogues of 3O, C_{12}-HSL

5.14.1 3-(1-Hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione [2.152]



The Tetramic acid derivative was prepared essentially by the procedure described by Kaufmann et al. A solution of sodium methoxide in methanol (0.5 M, 1.12 mmol, 2.24 mL) was added to a stirred solution of 3-oxo-C_{12}-HSL (1.12 mmol, 0.333 g) in methanol (3 mL) under nitrogen. The reaction mixture was stirred for 3 h at 55 °C and then overnight at 50 °C. The mixture was cooled to room temperature and then passed

through an acidic ion exchange resin (DowexWX2-200). The resin was further eluted with MeOH (30 mL), the eluants were combined and concentrated in vacuum to obtain the tetramic acid derivative as a white solid product in 88 % yield.

mp = 84-86 °C.

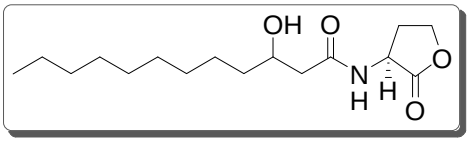
^{1}H NMR ($CDCl_3$) δ 0.895 (3H, t, J=6.8 Hz, ($CH_3(CH_2)_8$-), 1.28 (10H, m, $CH_3CH_2(CH_2)_5$-), 1.38 (2H, m, broad, CH_3CH_2-), 1.68 (2H, m, $CH_3(CH_2)_6CH_2$-), 1.78 (1H, m, OH CH_2CH_2- α), 2.13 (1H, m, $OHCH_2CH_2$-β), 2.85 (2H, m, $CH_3(CH_2)_7CH_2$-), 3.86 (1H, s, ring *H*), 3.97 (2H, m, $OHCH_2CH_2$-), 6.6 (1H, s, broad -N*H*- ring).

^{13}C NMR ($CDCl_3$) δ 14.1, 22.6, 25.9, 29.2, 29.4, 31.8, 32.9, 34.0, 61.3, 62.0, 100.5, 175.0, 190.0, 195.3.

(ES-MS/FAB-MS): *m/z* 298.2023 ($M+H^+$) $C_{16}H_{28}NO_4$, requires *m/z* 298.2018 (Δ = 1.7 ppm).

5.14.2 3-Acetyloxy, C_{12}-HSL [2.154]

3-Hydroxy, C_{12}-HSL [2.155]



To a stirred solution of 3O, C_{12}-HSL (2 mmol, 0.594 g) in methanol (20 mL) was added first portion of sodium cyanoborohydride (2.2 mmol, 0.1382 g). The pH was maintained by dropwise addition of 3 % HCl in methanol. After intervals of 2 h at pH=4 second and third portions of sodium cyanoborohydride (1.1 mmol, 0.063 g) were added, each time pH was maintained below 4 with 3 % HCl in methanol. The reaction mixture was then stirred for 12 h at room temperature and solvent was removed to dryness under high vacuo. The residue was extracted with hot ethyl acetate (3 × 15 mL). Ethyl acetate was rotary evaporated to obtain crude product which was purified in 54 % yield by column chromatography using ethyl acetate solvent.

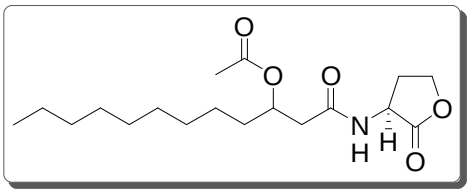
FT-IR (KBr) cm^{-1}: 3297m, (*ν*NH, amide); 1778s (*ν*C=O, ring); 1643s(*ν*C=O, amide); 1547s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_8$-), 1.27-1.38 (14H, m, $CH_3(CH_2)_7$-), 1.46-1.54 (2H, m, $CH_3(CH_2)_7CH_2$-) 1.55 (1H, s, broad, -C(H)O*H*), 2.21-2.25 (1H, m, ring, 4α-*H*), 3.32-2.40 (1H, m, -C(OH)HC(H)*H*CO-), 2.44-2.50 (1H, m, -

C(OH)HC(*H*)HCO-), 2.79-2.86 (1H, m, ring, 4β-*H*), 3.40 (1H, s, broad, -C(*H*)OH), 4.27-4.34 (1H, m, ring, 3*H*), 4.47-4.55 (1H, m, ring, 5α-*H*), 4.56-4.64 (1H, m, ring, 5β-*H*), 6.64 (0.5H s, -N*H*-HSL), 6.69 (0.5H s, -N*H*-HSL).

(ES-MS/FAB-MS): *m/z* 300.2187 ($M+H^+$) $C_{16}H_{30}NO_4$, requires *m/z* 300.2175 (Δ = 3.98 ppm).

3-Acetyloxy, C_{12}-HSL [2.154]



A solution of 3-hydroxy, C_{12}-HSL **2.155** (2mmol, 0.598 g) in acetic anhydride (5 mL) was refluxed for 2 h. An excess of acetic anhydride was removed under high vacuum and residue was redissolved in ethyl acetate (20 mL). Ethyl acetate solution was washed with saturated sodium bicarbonate (3 × 10 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was removed under vacuum to obtain desired product as white solids in 97 % yield.

mp = 65-67 °C.

FT-IR (KBr) cm^{-1}: 3284m, (*ν*NH, amide); 1793s (*ν*C=O, ring); 1735s(*ν*C=O, acetoxy); 1645s (*ν*C=O, amide); 1538s (δNH, amide).

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 7.2 Hz, $CH_3(CH_2)_8$-), 1.27-1.39 (14H, m, $CH_3(CH_2)_7$-), 1.61 (2H, m, $CH_3(CH_2)_7CH_2$-), 2.09 (1.5H, s, -COC*H*$_3$), 2.10 (1.5H, s, -COC*H*$_3$), 2.11-2.19 (1H, m, ring, 4α-*H*), 2.53 (1H, d, *J* = 6.0 Hz, -C(OH)HC(H)*H*CO-) , 2.54 (1H, d, *J* = 6.0 Hz, -C(OH)HC(*H*)HCO-), 4.26-4.32 (1H, m, ring, 5α-*H*), 4.46-4.51 (1H, m, ring, 5β-*H*), 4.50-4.56 (1H, m, ring, 3*H*), 5.16-5.21 (1H, m, -C(*H*)OCOCH$_3$), 6.25 (1H t, *J* = 6.4 Hz, -N*H*-HSL).

(ES-MS/FAB-MS): *m/z* 342.2292 ($M+H^+$) $C_{18}H_{32}NO_5$, requires *m/z* 342.2280 (Δ = 3.5 ppm).

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Appendix-I

Immunological Assay

Optimization of cell culture conditions

In the cell culture assays, the number of mouse splenocytes used was initially optimized to 100 000 cells per well. The optimal dose of ConA, 1 μg/mL, used in the cell proliferation assays was determined from ConA titration curves.

Concanavalin A (ConA) mitogen-stimulated proliferation of murine splenocytes

ConA stimulated cell proliferation assay was used to assess the effect of *N*-acylhomoserine lactones (AHLs) on T-cell stimulated proliferation. Proliferation was assessed by the incorporation of [^{3}H]-thymidine into DNA. Eight-week-old female BALB/c mice were obtained from Harlan (Bicester, Oxon, U.K.) and given food and water ad libitum. Splenocyte suspensions were prepared by removing the spleen and placing them into RPMI 1640 medium (Sigma, Poole, U.K.). Spleens were forced through 70 μm pore size wire gauzes using the plunger from a 5 mL syringe to produce a single cell suspension. Cells were pelleted by centrifugation, and erythrocytes were lysed with 0.017 M Tris, 0.144 M ammonium chloride buffer, pH 7.2. Leukocyte pellets were washed twice with RPMI 1640 medium with 2% (v/v) fetal calf serum (FCS) and resuspended in complete T-cell culture medium (CTCM) consisting of RPMI 1640 medium with 5% FCS, 2 mM L-glutamine, and 5×10^{-5}M 2-mercaptoethanol (Sigma, Poole, U.K.). Test compounds were studied using doubling-down dilutions ranging from 1 mM to 0.1 μM in a final volume of 200 μL of CTCM containing ConA (Sigma, Poole, U.K.) at 1 μg/mL and 100 000 spleen cells. Following incubation for 48 h at 37 °C in 5%CO_2/air, 0.25 μCi [^{3}H]-thymidine (Amersham Pharmacia Biotech, U.K.) in 10 μL of RPMI 1640 medium was added and cells were incubated for a further 24 h. Cells were collected onto fiberglass filters with a filtermate harvester (Packard Bioscience, Pangbourne, U.K.). After the addition of 25 μL of MicroScint-O (Packard Bioscience, Pangbourne, U.K.) to each well, these filters were counted with a Packard Top Count scintillation counter.

Appendix-II

Cytotoxicity

Trypan blue exclusion assay

Trypan blue exclusion assay is a standard method used to assess cell viability because only viable cells can exclude the dye added to the medium. The 100 µl of murine splenocyte cells were exposed to a concentration range 0.3-1000 µM of analogues or physiological saline (control) at 37 °C in 5 % CO_2/air for 24 h. Tissues were thoroughly washed with physiological saline and then stained by 0.4% Trypan blue dye. The number of viable cells and damaged cells stained in blue were counted in hemocytometer (counting chamber) in 3 microscopic fields. Cell viability was determined as the ratio of viable cells to all cells counted in the fields. The experiment was performed in triplicates.

Appendix-III

QS-induction and QS-inhibition assays

Strain and culture media

Strain used in this study was *Pseudomonas aeruginosa* strain, PAO1 (Δ*lasI*-P*lasB*::*lux*) which was cultured overnight at 37 °C in Luria-Bertani (LB) broth medium (5 mL) containing 100 μg/ml tetracycline.

QS-induction and -inhibition assays with *Pseudomonas aeruginosa* PAO1(Δ*lasI*-P*lasB::lux)*

The reporter strain PAO1***(ΔlasI-PlasB::lux)*** was grown overnight as explain above in LB broth medium media containing 100 μg/mL tetracycline at 37º C. Overnight culture of *Pseudomonas aeruginosa* (1 mL) was centrifuged, supernatant was discarded and the residue was diluted to 1 mL with fresh LB medium (40 μL of this in 1mL of LB medium is equivalent to an OD_{495} of 1.0) To the sample tubes (2 mL capacity) each containing 40μL cell culture was added analogue and/or 3O, C_{12}-HSL and diluted to 1 mL with fresh LB medium as explain below.

QS-induction assay:

To each sample tube containing 40μL cell culture added analogues of concentration 1mM and diluted to 1mL with fresh LB medium. Only cell culture was used as control and 3O, C_{12}-L-HSL (5 μM) was added in the positive control. An aliquot of 200-μl was added to separate well in 96 wells plate. The bioluminescence of the PAO1 strain was determined as a function of cell population density with a combined, automated luminometer-spectrometer (Anthos Labtech LUCYI) set at 37 °C. The luminescence and turbidity of the cultures (optical density at λ=495 nm) were automatically determined every 30 min. Luminescence is given in relative light units per unit of optical density at 495 nm. Experiments were repeated five times with similar results.

QS-inhibition (Antagonist) assay

To each sample tube containing 40µL cell culture and 5 µM of 3O, C_{12}-L-HSL, individual analogues were added in a concentrations range of 1µM, 100µM and 1 mM. Only cell culture was used as control and 3O, C_{12}-L-HSL (5 µM) was added in the positive control. An aliquot of 200-µl was added to separate well in 96 wells plate. The bioluminescence of the PAO1 strain was determined as a function of cell population density with a combined, automated luminometer-spectrometer (Anthos Labtech LUCYI) set at 37 °C. Experiments were repeated five times with similar results.

If you have any queries, don’t hesitate to contact:

Gopal P Jadhav	gopal.jadhav@nottingham.ac.uk
Dr. SiriRam Chhabra	mrzrc3@nottingham.ac.uk
Prof. Peter Fischer	peter.fischer@nottingham.ac.uk
Prof. David Pritchard	david.pritchard@nottingham.ac.uk