3-21-2011

The 4-aza-S-ribosyl-L-homocysteine Derivatives and the Related Gamma-lactam and Azahemiacetal Analogs: Synthesis, Inhibition and Quorum Sensing Activity

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DOI: 10.25148/etd.FI11042104

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

THE 4-AZA-*S*-RIBOSYL-L-HOMOCYSTEINE DERIVATIVES AND THE RELATED
GAMMA-LACTAM AND AZAHEMIACETAL ANALOGS:
SYNTHESIS, INHIBITION AND QUORUM SENSING ACTIVITY

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Venkata Malladi

2011

To: Dean Kenneth Furton
College of Arts and Sciences

This dissertation, written by Venkata Malladi, and entitled The 4-aza-*S*-ribosyl-L-homocysteine Derivatives and the Related Gamma-lactam and Azahemiacetal Analogs: Synthesis, Inhibition and Quorum Sensing Activity, have been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Venkata Malladi is approved.

Dean Kenneth Furton
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Interim Dean Kevin O'Shea
University Graduate School

Florida International University, 2011

DEDICATION

I dedicate this work to my beloved ‘SWAMI’ who is my guiding light and source of happiness.

Also to my parents and my husband for their unconditional love and support.

ACKNOWLEDGMENTS

I would like to extend my heartfelt gratitude to my mentor, Dr. Wnuk for teaching me valuable lessons in chemistry and beyond. His remarkable enthusiasm, knowledge and motivation guided me to become an independent researcher. I also am thankful for his care and help beyond professional development. I am grateful to my committee members for their valuable suggestions and intellectual support. I would like to acknowledge the chemistry department for the financial assistance. I am also grateful to all my lab members for all the joyful moments we shared and for giving me moral and intellectual support whenever I needed. I am indebted to my family and friends for their unconditional love and care through out my studies. Last but not the least, I would like to thank GOD for this life and the experiences that came with it.

ABSTRACT OF THE DISSERTATION

THE 4-AZA-S-RIBOSYL-L-HOMOCYSTEINE DERIVATIVES AND THE RELATED
GAMMA-LACTAM AND AZAHEMIACETAL ANALOGS:
SYNTHESIS, INHIBITION AND QUORUM SENSING ACTIVITY

by

Venkata Malladi

Florida International University, 2011

Miami, Florida

Professor Stanislaw F. Wnuk, Major Professor.

Quorum sensing (QS) is a population-dependent signaling process bacteria use to control multiple processes including virulence, critical for establishing infection. There are two major pathways of QS systems. Type 1 is species specific or intra-species communication in which *N*-acylhomoserine lactones (Gram-negative bacteria) or oligopeptides (Gram-positive bacteria) are employed as signaling molecules (autoinducer one). Type 2 is inter-species communication in which *S*-4,5-dihydroxy-2,3-pentanedione (DPD) or its borate esters are used as signaling molecules. The DPD is biosynthesized by LuxS enzyme from *S*-ribosylhomocysteine (SRH).

Recent increase in prevalence of bacterial strains resistant to antibiotics emphasizes the need for the development of new generation of antibacterial agents. Interruption of QS by small molecules is one of the viable options as it does not affect bacterial growth but only virulence, leading to less incidence of microbial resistance. Thus, in this work, inhibitors of both *N*-acylhomoserine lactone (AHL) mediated intra-species and LuxS enzyme, involved in inter-species QS are targeted.

The γ -lactam and their reduced cyclic azahemiacetal analogs, bearing the additional alkylthiomethyl substituent, were designed and synthesized targeting AHL mediated QS systems in *P. aeruginosa* and *Vibrio harveyi*. The γ -lactams with nonylthio or dodecylthio chains acted as

inhibitors of *las* signaling in *P. aeruginosa* with moderate potency. The cyclic azahemiacetal with shorter propylthio or hexylthio substituent were found to strongly inhibit both *las* and *rhl* signaling in *P. aeruginosa* at higher concentrations. However, lactam and their azahemiacetal analogs were found to be inactive in *V. harveyi* QS systems.

The 4-aza-*S*-ribosyl-L-homocysteine (4-aza-SRH) analogs and 2-deoxy-2-substituted-*S*-ribosyl-L-homocysteine analogs were designed and synthesized targeting *Bacillus subtilis* LuxS enzyme. The 4-aza-SRH analogs in which oxygen in ribose ring is replaced by nitrogen were further modified at anomeric position to produce pyrrolidine, lactam, nitron, imine and hemiaminal analogs. Pyrrolidine and lactam analogs which lack anomeric hydroxyl, acted as competitive inhibitors of LuxS enzyme with K_i value of 49 and 37 μM respectively. The 2,3-dideoxy lactam analogs were devoid of activity. Such findings attested the significance of hydroxyl groups for LuxS binding and activity. Hemiaminal analog of SRH was found to be a time-dependent inhibitor with IC_{50} value of 60 μM .

TABLE OF CONTENTS

CHAPTER	PAGE
1. INTRODUCTION	1
1.1. Quorum sensing	1
1.1.1. Intraspecies quorum sensing	1
1.1.1.1. Gram negative bacteria	1
1.1.1.2. Gram positive bacteria	4
1.1.2. Interspecies quorum sensing	5
1.1.2.1. The signaling pathway for auto inducer of type 2 (AI-2)	7
1.2.2.2. S-ribosylhomocysteinase (LuxS)	10
1.2. Applications of QS modulation	14
1.2.1. Modulation of intraspecies communication mediated by acylhomoserine lactones (AHLs)	15
1.2.1.1. Inhibitors targeting the synthases	15
1.2.1.2. Inhibitors targeting the ligand	16
1.2.1.3. Inhibitors targeting the receptor	16
1.2.2. Modulation of interspecies communication	26
1.2.2.1. Inhibition of LuxS enzyme	26
1.2.2.2. 5'-Methylthioadenosine/AdoHcy nucleosidase (MTAN) inhibitors	29
1.3. Aza sugars	38
1.3.1. Transition state analogs of glycoside hydrolases	39
1.3.1.1. Cleavage mechanism of glycosidases	40
1.3.1.2. Aza sugars as transition state analogs	41
1.3.2. Hemiaminal sugar chemistry	45
2. RESEARCH OBJECTIVE	48
3. RESULTS AND DISCUSSION	52
3.1. Design and synthesis of 4-aza-S-ribosyl-L-homocystiene analogs	52
3.1.1. Pyrrolidine analog	52
3.1.2. Lactam analog	53
3.1.3. Hemiaminal analog	55
3.1.4. Nitron analog	56
3.1.5. Imine analog	56
3.1.6. γ -lactams with alkylthiomethyl substitution at carbon γ and their N,O-acetal counterparts	58
3.1.7. (5S)-(hexyl- or nonylthiomethyl)-3,4-dihydropyrrolidin-2-ones and their hemiaminal congeners	59
3.2. Design and synthesis of S-ribosyl-L-homocysteine and its C2 modified analogs	60
3.2.1. S-Ribosyl-L-homocysteine	60
3.2.2. 2-Deoxy S-ribosyl-L-homocysteine	62
3.2.3. S-Arabinosyl-L-homocysteine	63
3.2.4. 2-Fluoro-2-deoxy-S-arabinosyl-L-homocysteine	63
3.3. Biological evaluation of targeted quorum sensing modulators	65
3.3.1. Quorum sensing assays of γ -lactams with alkylthiomethyl substitution at carbon γ and their N,O-acetal counterparts	65
3.3.1.1. Screening against <i>las</i> and <i>rhl</i> signaling in <i>P. aeruginosa</i>	65

3.3.1.2. Screening against bioluminescence in <i>V. harveyi</i>	70
3.3.2. Inhibition studies of 4-aza-SRH analogs against LuxS enzyme	71
3.3.3. Complexation studies with aza-DPD	83
4. EXPERIMENTAL PROCEDURES	86
4.1. General procedures	86
4.2. Synthesis of the inhibitors	87
4.3. Borate complexation study	115
4.4. Biological screening methods	116
4.4.1. Antiquorum sensing assay	116
4.4.1.1. β -galactosidase assay	116
4.4.1.2. Growth and luminescence measurement drop assay	116
4.4.2. LuxS enzyme inhibition studies with 4-aza-SRH analogs	117
4.4.2.1. LuxS activity assay	117
4.4.2.2. LuxS inhibition assay	117
5. CONCLUSION	119
BIBLIOGRAPHY	124
APPENDIX : ^1H NMR spectra of the selected compounds	135
VITA	141

LIST OF TABLES

TABLE		PAGE
1.	Representative AHLs along with their regulators and corresponding functions	3
2.	Effect of lactam and and cyclic hemiaminal analogs on <i>rhl</i> signaling.....	70
3.	Effect γ -lactam and hemiaminal analogs on <i>V. harveyi</i> bioluminescence and growth	71
4.	Rate of LuxS catalysis at various concentrations of natural substrate	72
5.	Rate and % activity of LuxS at different concentrations of analogs 129 and 141	76
6.	Rate and % activity of LuxS at different concentrations of analogs 143 and 160a	80

LIST OF FIGURES

FIGURE	PAGE
1. Model of AHL biosynthesis and quorum sensing in a bacterial cell.	2
2. Representative structures of autoinducers.....	5
3. A. <i>Vibrio harveyi</i> signal transduction at low cell density: B. <i>Vibrio harveyi</i> signal transduction at high cell density.....	7
4. Crystal structure of <i>V. harveyi</i> LuxP with AI-2 at the binding site (Figure 4A) and crystal structure of <i>Salmonella typhimurium</i> LsrB with AI-2 at the binding site (Figure 4B).	10
5. Crystal structure of LuxS _{Bs} in complex with 2-keto intermediate.....	12
6. AHL mimics modified at the acyl side chain.....	18
7. (A) AHL antagonists identified across <i>A. tumefaciens</i> , <i>E. coli</i> and <i>V. fischeri</i> strains (B) Isothiocyanate analogs as irreversible inhibitors of LasR.....	20
8. <i>P. aeruginosa</i> AHLs and their ring modified analogs.	22
9. Cyclopentanone, phenol analogs (43-44b) and thiolactone and caprolactam analogs (45- 46).	22
10. <i>P. aeruginosa</i> AHLs analogs with modification at lactone and acyl side chain.	23
11. Inhibitors of LasR identified through high-throughput-cell based screening.	24
12. Triphenyl, nonnative agonists of 3-oxo-C ₁₂ -AHL mediated QS.....	24
13. Halogenated bromofuranones as AHL mediated QS modulators	25
14. Butenolide analogs (61-63) and inhibitors from garlic (64-65)	25
15. Reported LuxS enzyme inhibitors.....	28
16. Transition state analogs of 5'-Methylthioadenosine/AdoHcy nucleosidase	31
17. Various DPD agonists resembling DPD, furanosyl DPD or its borate ester AI-2	33
18. Pyrogallols and heterocyclic sulfones as DPD analogs.	36
19. 3-Amino-3-deoxy sugar and nucleoside inhibitors.....	37
20. Fatty acids as AI-2 mediated QS inhibitors in <i>V. harveyi</i>	37

21. Representative examples of naturally occurring aza sugars.	39
22. Examples of aza sugars with proven biological applications.....	39
23. Cleavage mechanism of (a) retaining and (b) inverting glycosidases. (c) Resonance forms of oxocarbenium ion in half chair confirmation.....	41
24. Aza sugar based transition state analogs of glycosylases.	43
25. Novel 4-aza-SRH analogs proposed in this study.....	50
26. Effect of lactam and hemiaminal analogs on P_{lasI} - <i>lacZ</i> expression in <i>E. coli</i>	67
27. Effect of lactam and cyclic hemiaminal analogs on P_{rhlA} - <i>lacZ</i> expression in <i>E. coli</i>	69
28. Lineweaver-Burk plot of Co-LuxS enzyme with SRH.....	73
29. Dose response curve of pyrrolidine analog 129 against LuxS enzyme	74
30. Activity of the selected 4-aza-SRH analogs against LuxS enzyme	75
31. Dose response curve of lactam analog 141 against LuxS enzyme	76
32. Dose response curve of hemiaminal analog 143 against LuxS enzyme	78
33. Dose response curve of propylthio lactam analog 160a against LuxS enzyme.....	82
34. ^{11}B NMR spectrum of D-ribose in saturated borate solution in D_2O	84
35. DFT calculations for borate esters of 203b and <i>S</i> -THMF.....	85
36. Two systems of nomenclatures used in the dissertation	87

LIST OF SCHEMES

SCHEME	PAGE
1. Biosynthesis of 4,5-dihydroxy- 2,3-pentanedione (DPD) (precursor to AI-2).....	8
2. Formation of AI-2 from 4,5-dihydroxy-2,3-pentanedione (DPD).	9
3. LuxS catalyzed conversion of SRH into Homocysteine and DPD as proposed by Pei <i>et al.</i>	14
4. (A) 5'-Methylthioadenosine/AdoHcy nucleosidase catalyzed hydrolysis of 5'-methylthioadenosine and S-adenosylmethionine (B) Proposed transition state of 5'-Methylthioadenosine/AdoHcy nucleosidase along with aza TS analogs.....	30
5. Structures of ribose, 4-thioribose and 4-azaribose in their respective open (aldehyde)	45
6. 4-Amino-4-deoxyribofuranoside in its equilibrium forms at variable pH.	46
7. 1-N-imino sugar of galacturonic acid 122b in its equilibrium forms at pH 5.0.....	47
8. Synthesis of pyrrolidine analog of 4-aza-SRH	53
9. Synthesis of lactam and hemiaminal analogs of 4-aza-SRH	54
10. Benzyl oxime derivative of 4-azaribose	56
11. Synthesis of nitron analog of 4-aza-SRH.....	57
12. Synthesis of imine analog of 4-aza-SRH	58
13. Synthesis of γ -lactams and corresponding hemiaminal analogs with thioalkyl side chain...	59
14. Synthesis of ribolactams and their hemiaminal congeners	61
15. Synthesis of S-ribosyl-L-homocysteine	61
16. Synthesis of 2-deoxy-S-ribosyl-L-homocysteine.....	62
17. Synthesis of S-arabinosyl-L-homocysteine (SAH).....	64
18. Synthesis of 2-deoxy-2-fluoro-S-arabinosyl-L-homocysteine.....	65
19. LuxS activity assay for detection of the released homocysteine employing DTNB.....	73
20. Plausible mechanism of interaction of hemiaminal analog of SRH 143 with LuxS. Production and complexation of aza-DPD analogs.	79
21. Plausible mechanism of interaction of cyclic imine analog of SRH 158 with LuxS.....	81
22. Borate binding study of ethanediol and aminoethanol.....	84

LIST OF ABBREVIATIONS

α	alpha
A	absorbance
Ac	acetyl
AHL	acylhomoserine lactone
AI	autoinducer
APCI	atmospheric pressure chemical ionization
aq	aqueous
β	beta
Bn	benzyl
Boc	<i>tert</i> -butyloxycarbonyl
br	broad (NMR)
<i>t</i> -Bu	<i>tert</i> -butyl
°C	degrees Celsius
cm	centimeter
conc	concentration
δ	delta chemical shift in parts per million (NMR)
d	doublet (NMR)
dA	change in absorbance
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	dichloromethane
dd	double doublet (NMR)
DFT	density functional theory
DIBAL	diisobutylaluminum hydride
DMAP	4-(<i>N,N</i> -dimethylamino)pyridine

DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethylsulfoxide
DPD	4,5-dihydroxy-2,3-pentanedione
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
ϵ	epsilon
ESI	electrospray ionization
Et	ethyl
γ	gamma
g	gram(s)
<i>gem</i>	geminal
h	hour(s)
Hcy	homocysteine
HEPES	<i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -(2-ethanesulfonic acid)
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
ICP-MS	inductively coupled plasma mass spectroscopy
IPTG	isopropyl β -D-thiogalactoside
<i>J</i>	coupling constant in Hz (NMR)
L	liter
LU	light units
LuxS	<i>S</i> -ribosylhomocysteinase
LuxR _{vh}	LuxR present in <i>V. harveyi</i>
m	milli; multiplet (NMR)
μ	micro
M	moles per liter

Me	methyl
MHz	megahertz
min	minute(s)
mol	mole(s)
Ms	mesyl
MS	mass spectrometry
MTAN	5'-methylthioadenosine/AdoHcy nucleosidase
MTA	5'-methylthioadenosine
<i>m/z</i>	mass to charge ratio (MS)
NMR	nuclear magnetic resonance
nM	nano molar
OD	optical density
o.d.	outer diameter
<i>p</i>	para
Ph	phenyl
ppm	parts per million
%	percentage
q	quartet (NMR)
QS	quorum sensing
rt	room temperature
rel	relative
s	singlet (NMR); second(s)
SAdH	<i>S</i> -adenosylhomocysteine
SAH	<i>S</i> -arabinosylhomocysteine
SAM	<i>S</i> -adenosylmethionine

sat	saturated
SRH	<i>S</i> -ribosylhomocysteine
soln	solution
RP	reverse phase
<i>t</i>	tertiary (tert)
t	triplet (NMR)
TBDMS	<i>t</i> -butyldimethylsilyl
TCEP	tris(2-carboxyethyl)phosphine
TEA	triethylamine
TFA	trifluoroacetic acid
tf	trifluoromethyl
<i>t_R</i>	retention time
THF	tetrahydrofuran
TIPS	triisopropylsilyl
TLC	thin layer chromatography
TMS	trimethylsilyl
TNB	5-thio-2-nitrobenzoate
TOF	time of flight
TS	transition state
UV	ultraviolet
<i>V</i>	reaction rate
v	volume
VIS	visible
vs	versus

1. INTRODUCTION

1.1. Quorum sensing

Quorum sensing (QS) is a cell density dependent bacterial communication phenomenon. The term, ‘quorum’ is defined as “the minimum bacterial behavioral unit required inducing this communication”.¹ Observation of autoinduction of luminescence in *V. fischeri* was the first example of involvement of QS.² Later, many examples of bacteria employing QS (both Gram positive and Gram negative) for interspecies and intraspecies communication were reported. Such conversations allow bacteria which are single-celled organisms to control crucial functions in united communities such as symbiosis, motility, virulence, antibiotic production, biofilm formation, exchange of DNA and many other processes; and thereby receive some of the benefits of multicellular organisms. Bacteria secrete chemical molecules called autoinducers (AIs) to induce QS.³⁻⁵ At low cell density, the concentration of this chemical is also low. When cell density increases, the concentration increases to a point critical for induction of genes responsible for community behaviors.³⁻⁵

There are two major pathways of QS systems. Type 1 is species specific or intra-species communication in which acyl homoserine lactones (Gram-negative bacteria) and oligopeptides (Gram-positive bacteria) are employed as autoinducers (referred as AI-1s). On the other hand type 2 is inter-species signaling system in which borate diester of cyclized dihydroxypentanedione or *R*-tetrahydroxytetrahydrofuran (*R*-THMF) are used as autoinducers (referred as AI-2) (Figure 2).⁶

1.1.1. *Intraspecies quorum sensing*

1.1.1.1. Gram negative bacteria

Acyl homoserine lactones (AHLs)

The major intraspecies signaling molecules among Gram negative bacteria are AHL analogs.⁶ QS in marine luminescent *V. fischeri* was the first report of AHL involvement as

autoinducer molecules.^{7,8} These sensor molecules can be distinct to each species or could be shared among other bacteria.

The AHLs contain a long fatty acid chain connected to a homoserine lactone through an amide bond. The AHLs are synthesized from SAM and acylated acyl carrier protein (acyl-ACP) by inducer synthases (LuxI like proteins) at low basal levels.⁹ At high cell densities, as the AHL reaches the threshold concentration, it binds to the cognate receptors (LuxR-like proteins) which reside in cytoplasm. Thereafter the AHL:LuxR complex undergoes homodimerization followed by multimerization. Subsequently, the multimer binds to 20 bp-element called *lux*-type-box and activates transcription of target genes required for community behaviors (Figure 1).^{10,11} Details of different LuxI/LuxR type of systems present in various Gram negative bacteria along with their functions are included in Table 1.⁶

Figure 1. Model of AHL biosynthesis and quorum sensing in a bacterial cell.¹⁰

The LuxR type proteins share 18-23% sequence homology. The conserved residues required for binding were identified to be Ala38 (Gly in LasR and VanR), Tyr53 (Cys in AhyR), Tyr57, Asp70 and Thr129 (Ser in AhyR, LasR, LuxR, RhlR and VanR).¹² Recently, the crystal structure of *Agrobacterium tumefaciens* TraR in complex with its inducing ligand, 3-oxo-C₈-AHL bound to target DNA was solved by Allesandro *et al.*¹³ From the crystallographic studies, the binding pocket of TraR was found to have hydrophobic and aromatic residues to accommodate the fatty acid side chain. Also, hydrogen bonding between Trp57 and carbonyl of lactone, Asp70 and amide nitrogen and Tyr53 and side chain amide carbonyl were observed.¹³

Table 1. Representative AHLs along with their regulators and corresponding functions

Bacterium	Signal ^a	LuxI/LuxR	QS phenotype
<i>Agrobacterium hydrophilia</i>	C ₄ -AHL	AhyI/AhyR	Exoprotease production
<i>A. tumefaciens</i>	3-oxo-C ₈ -AHL	TraI/R	Ti plasmid conjugation
<i>Burkholderia cenocepacia</i>	C ₈ -AHL, C ₆ -AHL	CciI/R;CepI/R	Biofilm, swarming motility
<i>Erwinia carotovora</i>	3-oxo-C ₆ -AHL	ExpI/R; CarI/R	Carbapenem, exoenzymes,virulence
<i>Psuedomonas aeruginosa</i>	3-oxo-C ₁₂ -AHL	LasI/R	Exoenzymes, pyocyanin and biofilm
	3-oxo-C ₄ -AHL	RhlI/R	formation
<i>Serratia liquefaciens</i>	C ₄ -AHL	SwrI/R	Extracellular proteases, swarming
<i>Vibrio fischeri</i>	3-oxo-C ₆ -AHL	LuxI/R	bioluminescence
<i>Vibrio harveyi</i>	3-hydrdoxy-C ₄ -AHL	LuxLM/N	bioluminescence

^aAHL abbreviations/nomenclature used in the entire text

Recently, a third chemical class of QS signal (CAI-1as AI-1) in *V. cholerae* was also discovered. It is identified as (S)-3-hydroxytridecane-4-one (Figure 2). While other bacteria produce biofilms and virulence factors at high cell density through QS, *V. cholerae*, in contrast at

high population of bacteria, represses the factors regulating their production by employing CAI-1 and AI-2.¹⁴

A fourth type of QS system was observed in *P. aeruginosa* known as 4QS pathway (4-quinolone signal). It is modulated by using 2-heptyl-3-hydroxy-4-quinolone as an autoinducer (Figure 2) and detected by QscR receptor. Three signaling pathways exist in *P. aeruginosa*. Two QS mediated systems (*lasR* and *rhlR*) use AHL and the third uses a 4-quinolone derivative. The LasI/R synthesizes and detects *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C₁₂-AHL) while RhlI/R synthesizes and detects *N*-butanoyl-L-homoserine lactone (C₄-AHL) (Figure 2). These two signaling systems regulate 6% of the gene expression in *P. aeruginosa*. The QscR is an orphan receptor which lacks LuxI of its own and recognizes AHL produced by LasI and RhlI systems. It represses QS activity.¹⁵ Together, the three QS systems act in a complex hierarchical partially redundant manner to control virulence, secondary metabolite production and various host immune responses.¹⁶

1.1.1.2. Gram positive bacteria

Gram positive bacteria use oligopeptides as autoinducers to mediate two component QS regulatory system.¹⁷ These two components are: (i) receptor for binding to AI-1s and (ii) response-regulatory protein. The latter is involved in gene regulation upon activation along with AI-1 production through a positive feedback loop. When AI-1 binds to the sensor receptor, a histidine sensor kinase protein is activated which auto phosphorylate the histidine residue.¹⁸ The phosphorylated protein transfers the phosphoryl group to the conserved aspartate residue in the cognate response regulator leading to the activation of several genes responsible for bacterial QS.^{18,19}

A. Acylhomoserine lactones (AI-1)

R group

P. aeruginosa (LasI)

P. aeruginosa (RhII)

A. tumefaciens (TraI)

V. harveyi (LuxLM)

B. Oligopeptides (AI-1)

ADPITROWGD

B. subtilis (ComX)

ERGMT

B. subtilis (CSF)

EMRLSKFRDFILQRKK

S. pneumoniae (CSP)

S. aureus (AgrD)

AIP-I

AIP-II

AIP-III

AIP-IV

C. Furanones (AI-2)

AI-2 in *V. harveyi* (LuxS)

AI-2 in *S. typhimurium* (LuxS)

D. Tridecane (AI-1)

CAI-1 in *V. cholera* (CqsA)

E. 4-Quonilone (AI-1)

PQS in *P. aeruginosa* (PqsABCD)

Figure 2. Representative structures of autoinducers (respective synthases mentioned in parenthesis) A. AHLs used by Gram negative bacteria B. Oligopeptides used by Gram positive bacteria. C. AI-2s recognized by *V. harveyi* and *S. typhimurium*. D. AI-1 autoinducer used in *V. cholerae* E. PQS autoinducer used by *P. aeruginosa*.

1.1.2. Interspecies quorum sensing

While AHLs are the chemical lexicon of Gram negative bacteria and oligo peptides for Gram positive bacteria, for communication within the species, derivatives of 4,5-dihydroxy-2,3-

pentanedione (DPD), referred as AI-2 is the common signaling molecule for both Gram positive and negative bacteria.²⁰⁻²³

Vibrio harveyi, a Gram negative bioluminescent marine bacterium, is the paradigm for the interspecies communication, as involvement of AI-2 was extensively studied and demonstrated in this organism. It uses AHL mediated QS for regulation of bioluminescence. In a series of biochemical and genetic experiments conducted by Bassler *et al.* to study QS in *V. harveyi*, the bacterial mutant cultures, in spite of lacking the AHL synthase (LuxM) and cognate receptor (LuxN),^{24,25} were observed to induce bioluminescence. Such findings suggested a second class of signaling molecules, apart from AHL. That was the first example of association of AI-2 as QS mediator. However, apart from AHLs and AI-2 QS systems, another new class of QS molecule called CAI-1 (whose structure is unknown but speculated to be related to CAI-1 in *V. cholerae* ((*S*)-3-hydroxytridecane-4-one)) was also shown to mediate QS (Figure 2).¹⁴ These three QS systems work convergently to control production of bioluminescence²⁶ virulence factors such as metalloprotease production, type III secretion²⁷ and siderophore production.²⁸

An overview of QS system of *V. harveyi*

V. harveyi recognizes and secretes three types of autoinducers: (1) the AI-1 (*N*-(3-hydroxybutyryl)-L-homoserine lactone) referred as HAI-1, is synthesized by LuxM, (2) the AI-2 (borate ester of 4,5-dihydroxy-2,3-pentanedione²⁷ (DPD) is synthesized by LuxS (it will be discussed in detail in later sections), and (3) cholera autoinducer 1 (CAI-1) is synthesized by CqsA enzyme.¹⁴ Membrane bound histidine kinases detect the autoinducers (Figure 3).²⁹ The HAI-1 is detected by the LuxN histidine kinase.^{27,30} The AI-2 is detected by the periplasmic protein LuxP in complex with the LuxQ histidine kinase,^{24,27,31} while CAI-1 is detected by the CqsS histidine kinase.^{14,27,32} These proteins are bifunctional as they can act as kinases and phosphatases depending on cell density.²⁸ In the absence autoinducers, i.e., at low cell density, these proteins get phosphorylated and subsequently transfer the phosphate to LuxO via LuxU.

Phosphorylated LuxO along with σ^{54} activates production of five sRNAs.^{33,34} These in turn bind with chaperon Hfq to destabilize mRNA encoding the transcriptional regulator, LuxR_{vh}.³⁵ Therefore no LuxR_{vh} is produced at low cell density. At high cell density, when AIs are released, the cognate receptors turn themselves into phosphatases and reverse the sequence of phosphorylation leading to up regulation of LuxR_{vh} which regulates for more than 50 genes including those encoding luciferase²⁶ and virulence factors (Figure 3).^{36,37}

1.1.2.1. The signaling pathway for auto inducer of type 2 (AI-2)

In order to study the enzymes involved in the AI-2 signaling, Schaudre *et al.* made mutants of *V. harveyi*, *E. coli* and *S. typhimurium*. In all the mutants of bacteria, *luxS* gene was observed to be highly conserved.³⁸ The involvement of *luxS* in AI-2 signaling was confirmed by observation of the fact that bacterial cultures lacking *luxS* gene failed to produce AI-2 activity.³⁸ The *luxS* gene was found to be present in more than 60 bacterial species (both Gram positive and negative) supporting the hypothesis of AI-2 as inter species signal.

Figure 3. A. *Vibrio harveyi* signal transduction at low cell density: B. *Vibrio harveyi* signal transduction at high cell density.²⁹

Biosynthesis of AI-2

The key to identification of biosynthetic pathway of AI-2 was unraveled when *luxS* gene was observed to be located downstream of *pfs* and *metk*.³⁹ The gene *metk* encodes for enzyme, S-

adenosyl-L-methionine synthetase which catalyzes production of *S*-adenosyl-L-methionine (SAM) from ATP and methionine.⁴⁰ The SAM acts as a methyl donor in the biosynthesis and/or modification of DNA, RNA and various proteins and produces *S*-adenosylhomocysteine (SAdH). Through negative feedback inhibition, SAdH inactivates SAM-dependent methyltransferase. Therefore, SAdH should be transformed into a nontoxic metabolite for the successful operation of SAM dependent methylation reactions.⁴¹ Eukaryotes employ the enzyme, SAdH hydrolase to convert *S*-SAdH to adenine and homocysteine (Hcy) whereas prokaryotes follow a two-step strategy using Pfs enzyme and LuxS.⁴² In the first step Pfs catalyzes depurination of SAdH to give adenine and *S*-ribosyl-L-homocysteine (SRH) (Scheme 1). In the second step, LuxS converts SRH to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) by a nonredox pathway. Subsequently, homocysteine undergoes methylation and reenters the catalytic cycle.⁴³ It was hypothesized that LuxS catalyzes the final step of converting SRH into DPD which ultimately leads to production of AI-2 activity. This hypothesis was proved by conducting series of experiments with the use of cell lysates, purifies Pfs, LuxS and substrates like SAM, SAdH and SRH.⁴⁴

Scheme 1. Biosynthesis of 4,5-dihydroxy-2,3-pentanedione (DPD) (precursor to AI-2)

The structure of AI-2

The DPD is a highly unstable molecule. It undergoes dehydration and cyclization reactions to give a mixture of interconvertible furanones. But none of these sugar derivatives, including DPD, showed bioluminescence comparable to AI-2 activity in *V. harveyi* bioassay. Such result clearly shows that LuxS *in vitro* mixtures contain an elusive chemical (AI-2) which is distinct from the rest of the furanones.⁴⁴ Bassler *et al.* successfully solved the structure of AI-2 from the crystal structure of *V. harveyi* AI-2 sensing protein, LuxP in complex with AI-2 (depicted in Figure 4 along with network of hydrogen bonds) and ¹¹B NMR studies. It was found to be borate diester of *S*-THMF (*S*)-THMF (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran). The proposed formation of AI-2 from DPD involves (i) cyclization and hydration of DPD to give the furanone derivative, *S*-THMF (ii) complexation of *S*-THMF with borate to form AI-2 (Scheme 2).²²

Scheme 2. Formation of AI-2 from 4,5-dihydroxy-2,3-pentanedione (DPD).⁴⁵

The crystal structure of *Salmonella typhimurium* LsrB (AI-2 binding protein) bound AI-2, revealed a chemically different form of AI-2 compared to *V. harveyi* (Scheme 2).⁴⁵ While *V. harveyi* uses *S*-THMF-borate as AI-2, *S. typhimurium* uses *R*-THMF ((2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran). *S. typhimurium* being a gut pathogen has no access to boron, so it is conceivable that it uses nonboron compound as a sensing molecule. These findings support the validity of DPD as a precursor to AI-2 sensor and establish its role in interspecies communication.

Figure 4. Crystal structure of *V. harveyi* LuxP with AI-2 at the binding site (Figure 4A)²² and crystal structure of *Salmonella typhimurium* LsrB with AI-2 at the binding site (Figure 4B).⁴⁵

1.2.2.2. S-ribosylhomocysteinase (LuxS)

Currently four homologues of LuxS (*Deinococcus radiodurans*, *Haemophilus influenzae*, *Helicobacter pylori* and *Bacillus subtilis*) have been resolved by X-ray crystallography at resolution of 1.2 to 2.4 Å.⁴⁶⁻⁴⁸ All LuxS proteins are homodimers. Each subunit is a single polypeptide chain which share a novel α - β fold consisting of four-stranded antiparallel β -sheet bordered by 3 α helices on one side and a short 3_{10} helix on the other side.⁴⁶⁻⁴⁸ The LuxS sequence

alignment from 26 species, revealed 23 repeated amino acid residues, some of which are part of the active center. In the crystal, as well as in solution, LuxS is present as symmetric homodimer with two symmetric active sites. The dimeric interface contains a complex network of H bonding, ionic and hydrophobic interactions.⁴⁷

The LuxS_{Bs} (LuxS present in *Bacillus subtilis*) is a single polypeptide with 157 amino acids per each monomer.⁴⁶ Initially, the structure of LuxS was determined with Zn²⁺ at the active site, coordinated by three conserved residues *viz* His-54, His-58, Cys-126 and water molecule (Figure 5). Because of the presence of zinc, a molecule of water and the motif HXXEH, similar to the protease-binding motif found in thermolysin and other zinc dependent hydrolases, LuxS was proposed to perform a similar enzymatic role. The function of the metal as a Lewis acid was established from the crystal structure of LuxS with the 2-keto intermediate, **3** (Scheme 3).⁴⁹ The proposed metal mediated catalysis was confirmed by the dramatic change in electronic absorption spectrum of cobalt ion when the natural substrate, SRH was added to the Co-LuxS_{Bs} (from peaks at 530, 570, 650 nm to none).⁵⁰

Interestingly, Cys84 which is highly conserved in the active site, was found oxidized to cysteine sulfonic acid and cysteine sulfinic acids leading to rapid inactivation of LuxS, suggesting complexation of metal ion to Cys84 in catalysis.^{46,47} A ligand combination (two His, one Cys, one water molecule and Fe²⁺) similar to LuxS was also reported in peptide deformylase which also underwent inactivation by an identical route.^{51,52} These findings along with the studies done by Pei *et al.* on purified LuxS enzyme, suggested that native metal ion in LuxS was Fe²⁺ instead of Zn²⁺.⁵⁰ However, the Zn and the Co coordinated proteins were found to be more stable to aerobic oxidation compared to native iron bound LuxS. Substitution of Co²⁺ or Fe²⁺ for Zn²⁺ gave a variant with a 10-fold increase in catalytic activity *in vitro*.⁵⁰

Figure 5. Crystal structure of LuxS_{Bs} in complex with 2-keto intermediate.⁴⁹

Active site of LuxS (Figure 5)

The crystal structure of LuxS_{Bs} in complex with SRH at 2.2 Å° and homocysteine at 2.3 Å° respectively, was determined by Baker *et al.*⁴⁶ “The homocysteine moiety of SRH is bound in an extended conformation with the amino and carboxyl groups stabilized by hydrogen bonds with five neighboring residues from helix α1 and strand β3 (Arg65, Asp78, Ile79, Ser80 and Gly127 and Lys35 of neighboring subunit). The ribose moiety appears to be in the C2'-endo conformation and the ribose O2 and O3 hydroxyl groups can be seen involved in long ion-dipole interactions with the Zn²⁺ ion.²³ In addition, the O2 and O3 ribose hydroxyl groups are stabilized by hydrogen bonds with the oxygen atoms of oxidized Cys84”.^{46,53}

Mechanism of S-ribosylhomocysteinase (LuxS)

The proposed catalytic mechanism of LuxS involves consecutive aldose-ketose and ketose-ketose isomerizations and subsequent β-elimination to release Hcy and DPD (Scheme 3).⁵⁴ In the

first catalytic step, the aldehyde form of SRH binds to the metal ion, to increase the acidity at C2 so that, Cys84 in LuxS_{Bs} acting as acid/base, could abstract the hydrogen at C2 to form enediolate, **2**. Assisted by the second base /acid (probably Glu57), the metal ion migrates from C1 to C2 position, followed by reprotonation at C1 by Cys84 to give 2-keto intermediate **3** (Scheme 3). The second step is repetition of the isomerization reactions as in first step, to give 3-keto intermediate, **5**. In the third step, base/acid abstracts proton at C4 with elimination of homocysteine to give an enol intermediate which ultimately tautomerizes to DPD.^{49,50,53,54} The LuxS-catalyzed cleavage of the C5–S thioether bond in SRH is dramatically different from that proposed in the reversible mechanism for SAdHase. While SAdHse cleaves the thioether bond in SAdH by using enzyme-bound NAD⁺ (redox reaction), LuxS cleaves thioether bond in a nonredox fashion using enzyme bound water, acid-base pairs and a metal ion via a series of isomerization steps.^{41,55}

The proposed mechanism was validated by conducting various studies: (i) existence of proton transfer reactions were confirmed by studies with D₂O,⁵⁰ (ii) involvement of keto intermediates, **3** and **5** was established using real time by ¹³C NMR spectroscopy.⁵⁴ Involvement of keto intermediates as chemically and kinetically competent substrates was further proved by the synthesis of 2 and 3-keto intermediates, **3** and **5** and testing against LuxS,^{54,56} (iii) the intermediacy of **3** in catalysis and the function of metal ion as Lewis acid was proved by the crystal structure of 2-ketone intermediate **3** bound to a catalytically inactivated LuxS mutant (Cys84),⁴⁹ and (iv) the stereochemical and regiochemical specificity of the proton transfer reactions catalyzed by Cys84 and Glu57 was elucidated by deuterium labeled SRH studies in LuxS_{Bs} and the conversion of intermediate **3** to **5** was found to be partially rate limiting,^{53,54} and (v) the role of Glu57 and Cys84 as general acids/bases (Scheme 3) was confirmed by the site-directed mutagenesis.⁵⁰

Recent studies done by Pei *et al.* using [3-F]SRH and [3-Br]SRH as substrates for LuxS_{BS} suggested involvement of Tyr-89 and His-11 along with Glu57 and Cys84 in keto-isomerization steps (part of proton relay system).⁵⁶

Scheme 3. LuxS catalyzed conversion of SRH into Homocysteine and DPD as proposed by Pei *et al.*⁵⁴

1.2. Applications of QS modulation

Recent increase in prevalence of bacterial strains resistant to antibiotics emphasizes the need for the development of new generation of antibacterial agents. Since some of the pathogenic bacteria use QS to regulate virulence, antibiotic resistance and biofilm formation, blocking bacterial QS offers a novel therapeutic strategy.^{6,19,57-63} Over the past decade, several new components in QS signal transduction pathways (both intra and interspecies) were identified expanding the range of new approaches available to modulate QS. Interruption of QS, which regulates virulence or biofilm formation, does not result in either bacteriostatic or bactericidal

effect. It only attenuates the virulence but does not affect bacterial growth. Such behavior has the advantage of putting less selective pressure on bacteria resulting in lower incidence of microbial resistance. An important bacterial behavior regulated by QS, is biofilm formation, which is one of the causes of antibiotic therapy failure. Biofilm can be described as “an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material”⁶⁴ which makes bacterial cells less permeable to antibiotics. *P. aeruginosa*, one of the human opportunistic pathogen survives in the host by formation of biofilms causing hindrance to the entry of antibiotics into the cells and makes therapy ineffective. Thus, inhibition of biofilm formation should make the bacteria more susceptible to antibiotics. Therefore, QS inhibitors can act as adjuvants to antibiotic therapy.^{65,66} The importance of QS inhibition in regulating bacterial pathogenicity and other community related behaviors can be further underscored by the natural evolution of bacterial quorum quenching enzymes to prevent these population based activities of bacteria competing for energy and nutrients.^{67,68}

General strategies for manipulation of QS include:

(1) inhibition of synthesis of autoinducer molecules, (2) inhibition of binding of autoinducer to sensor proteins, (3) inhibition of molecular targets downstream of autoinducer synthesis, (4) sequestration of autoinducer *via* molecular traps,⁶¹ (5) degradation of autoinducer,⁶⁷⁻⁶⁹ and (6) inhibition of autoinducer transport and release.⁶

1.2.1. Modulation of intraspecies communication mediated by acylhomoserine lactones (AHLs)

Interception of the AHL (LuxI/LuxR) pathway can be achieved by three general approaches: (1) Inhibition of AHL synthases (LuxI), (2) degradation of AHLs, (3) inhibition of ligand-receptor binding (AHL-LuxR)

1.2.1.1. Inhibitors targeting the synthases

The AHLs are synthesized by proteins called LuxIs or its homologues from SAM and

acyl carrier proteins. Therefore inhibition of enzymes which utilize SAM can also inhibit AHL mediated QS. The SAM is also the substrate for methyl transferases which are involved in crucial biochemical processes. Consequently, AHL synthases which alter the SAM metabolism can have undesired effects on the ubiquitous SAM pathway. Hence, inhibitors of synthases were not developed until recently. With the recent availability of crystal structures of LuxI type of proteins, design of ligands which specifically target AHL synthases without interfering with other SAM requiring enzymes might be undertaken.⁷⁰⁻⁷²

1.2.1.2. Inhibitors targeting the ligand

The second method to interrupt AHL pathway is degradation of the AHL ligand itself by using several methods such as, specific enzymes (acylases or lactonases), oxidizing agents and pH (lactones are hydrolyzed at basic pH). So far the quorum quenching approach has been utilized in protecting plants from infectious bacterium.^{73,74}

1.2.1.3. Inhibitors targeting the receptor

The final method of interruption of AHL mediated QS, is inhibiting the AHL binding to its transcriptional regulators or LuxR type proteins. Significant research has been done in designing AHL analogs as LuxR inhibitors.⁷⁵ According to the review published by Wang *et al.*⁶ AHL inhibitors can be classified based on the structural modification on the AHL signal as: inhibitors (1) with alteration of the acyl side chain, (2) with modification on the γ -lactone ring, (3) with simultaneous modification on both acyl side chain and lactone ring, and (4) inhibitors structurally unrelated to AHL.⁶

(1) Inhibitors with modification on the acyl side chain of AHLs

As there is 70-80% sequence homology at the ligand binding domain of the LuxR-type of proteins and some species share identical AHLs, cross QS communication from one species to another is expected. The AHLs with short acyl chain length tend to act as antagonists in species

which use long chain AHL. The C₄-AHL, an agonist for RhIR (cognate receptor for *P. aeruginosa*), AhvR (cognate receptor for *A. hydrophilia*) and SwrR (cognate receptor for *S. liquefaciens*) is an antagonist for LuxR (receptor for *V. fischeri*).⁷⁶ Winan *et al.* studied activities of AHL mimics against TraR in *A. tumefaciens* WCF47Δ-*traI* derivative harbouring *lacZ* fusion as their reporter strain. The C₈-AHL, a natural ligand for CciR in *Burkholderia cenocepacia*, was the most potent inhibitor identified in this study (Table 1).⁷⁷

Eberhard *et al.*⁷⁶ and Greenberg *et al.*⁷⁸ independently studied the effect of synthetic AHLs having various side chain and lactone ring modifications of 3-Oxo-C6-AHL which regulates LuxR system in *V. fischeri*. Compound **7** with chain length of C7 and lacking carbonyl at C3 was found to be an inhibitor. From these studies, the authors suggested: (1) the 3-oxo group is required for agonistic activity, (2) chain length longer than C5 results in inhibitory activity, (3) introducing unsaturation into acyl chains results in only moderate agonistic activity, suggesting the requirement of flexibility for binding (Figure 6).

Douthett and co-workers have synthesized a series of AHL analogs by introducing steric bulk at the side chain⁷⁹ and replacing the amide bond with either sulfonamide⁸⁰ or urea⁸¹ functionality. They tested the compounds in a strain harboring LuxR and *lux* box from *V. fischeri* and the luciferase gene cluster *luxCDABE* from *Photobacterium luminescens* which produces luminescence upon addition of exogenous AHL. In the first study, the effect of steric bulk on binding to LuxR was studied. The cyclopentyl analogue **8** with ED₅₀ = 0.25 μM (equal to the natural substrate) was the most potent agonist while the phenyl substituted **9** and **10** with IC₅₀ = 2 μM were the most potent antagonists identified in the study (Figure 6).⁷⁹ Among sulfonamide group and urea series, none had agonistic activity. The most active inhibitors from sulfonamide series are **11** (IC₅₀ = 2 μM) and **12** (IC₅₀ = 3 μM) and from urea series are **13** (IC₅₀ = 1 mM) and **14** (IC₅₀ = 1.1 mM).

Figure 6. AHL mimics modified at the acyl side chain.⁶

The data from Douthea and co-workers studies suggest: (1) addition of bulky substituent is well tolerated by LuxR leading to inhibition, but more than one ring can render the molecule inactive (naphthyl is inactive), (2) replacement of amide with urea or sulfonamide results in loss of agonism and can confer antagonism, (3) reduction of acyl side chain close to C6 or less can render antagonistic properties, (4) presence of amide isosters, sulfonyl and urea groups are tolerated as they make hydrogen bond contacts at the LuxR binding domain, and (5) flattening of lactone ring (**15**) by introducing unsaturation results in loss of activity (Figure 6).^{80,81}

Neilson *et al.* reported a series of AHL mimics containing sulfonyl, sulfoxide and thioether groups on the acyl chain (Figure 6).⁸² As LuxR system in *V. fischeri* accepts ligands with wider

structural diversity than *P. aeruginosa* LasR, initial screening was performed in *V. fischeri* using a quorum sensing inhibitor selector system (QSIS) constructed by Rasmussen *et al.*⁷³ Compounds active in this screening were later tested in *P. aeruginosa* *las* system in which *lasB* promoter is fused to *gfp* (encodes for green fluorescence protein). Compounds with the thioether group were found to be potent inhibitors while sulfoxide analogs were weak inhibitors and sulfonyls were inactive. Among the synthesized thioether AHL mimics, compounds **16** and **17** displayed strong antagonism with $IC_{50} = 6 \mu M$ and $IC_{50} = 50 \mu M$ (Figure 6).

Blackwell *et al.* prepared a library of AHL derivatives having various long chain and phenyl substituents and tested in *V. fischeri* ES114 Δ -*luxI* reporter strain. Of the compounds tested, 3-nitro-phenyl acetyl lactone, **18** was found to be a super agonist with $EC_{50} = 0.3 \mu M$ in comparison to the natural ligand, 3-oxo-C12 with $EC_{50} = 3 \mu M$ (Figure 6). Interestingly phenyl acetyl lactones, **19** ($IC_{50} = 0.6 \mu M$), **20** ($IC_{50} = 0.9 \mu M$), **21** ($IC_{50} = 1.0 \mu M$), **22** ($IC_{50} = 1.1 \mu M$), **24** ($IC_{50} = 1.4 \mu M$) and **23** ($IC_{50} = 3.7 \mu M$) with electron-withdrawing groups (EWG) at the para position were identified to be inhibitors.⁸³ They also reported indole derivative of AHL, **25** as antagonist of TraR in *A. tumefaciens* and LasR in *P. aeruginosa*, reduced biofilm formation in *P. aeruginosa* (Figure 6).⁶³

On the basis of these results, Blackwell *et al.* prepared various AHL analogs with diverse structural modifications in order to understand structure activity relationship of AHL analogs in QS systems of reporter strains viz., *A. tumefaciens* WCF47 (lacks *traI*) harboring a plasmid-born *PtraI-lacZ* fusion (pCF372), *E. coli* DH5R harboring the LasR expression vector pJN105L and a plasmid-born *PlasI-lacZ* fusion (pSC11) and *V. fischeri* ES114 lacking *luxI*.⁸⁴ Compounds **26** to **31** are the most potent antagonists, identified from this study (Figure 7A).⁸⁴ The structure and activity correlations observed can be summarized as: (1) TraR ligand binding site is most compact and lipophilic of the three tested. Hence subtle changes in the ligand structure results in dramatic change in activities, (2) AHLs with acyl chain length of not more than eight atoms and

aromatic group with electron withdrawing groups or long chains can antagonize all three receptors, (3) carbon spacer length between lactone moiety and aromatic ring should not be less than one, to confer inhibitory activity, (4) D-AHL **31** was also capable of inducing antagonism in LasR (as the natural ligand was L-AHL), and (5) LuxR antagonists derived from phenyl AHLs are agonists at higher concentrations (compound **21**).⁸⁴

Figure 7. (A) AHL antagonists identified across *A. tumefaciens*, *E. coli* and *V. fischeri* strains and their corresponding IC₅₀ values.⁸⁴ (B) Isothiocyanate analogs as irreversible inhibitors of LasR.⁸⁵

Meijler *et al.* designed and synthesized electrophilic AHL analogs as probes to covalently modify Cys79, found at the active site of LasR. Among the analogs tested isothiocyanate analogs (**32-34**) were found to modify the binding pocket covalently and inhibit QS controlled activities

(Figure 7B). When tested for inhibition in reporter strain PAO-JP2 (*lasI/rhlI* deleted) harboring plasmid pKD201 containing a *lasI* coupled to *luxCDBE* luminescence system⁸⁶, compound **33** displayed partial agonism.⁸⁵

(2) Inhibitors with modification of the lactone ring

In order to explore the effect of modification of the lactone ring functionality on the AHL mediated LuxR type of receptors, analogs with replacement of the lactone ring with saturated and unsaturated rings of various sizes were designed.

Suga *et al.* reported a library of antagonists of LasR and RhlR in *P. aeruginosa* fluorescent strain PAO-JP2 which lacks genes encoding AHL synthases (LasI for 3-oxo-C₁₂-AHL and RhlI for 3-oxo-C₄-AHL).⁸⁷ All of the inhibitors had acyl side chain identical to the natural ligand (3-oxo-C₁₂-AHL in case of LasR and 3-oxo-C₄-AHL in case of RhlR) and lactone carbonyl group replaced with amine, alcohol and ketones groups which are capable of hydrogen bonding (Figure 8). The 3-oxo-C₁₂-AHL cyclopentanone derivative **35** had agonistic activity against LasR signaling protein, comparable to native substrate at 400 μ M and its C₄ side chain counterpart **36** activated RhlR at 100 μ M. The cyclopentanol analog **37** had agonistic activity comparable to 3-oxo-C₁₂-AHL at 50 μ M when tested against LasR. The 3-oxo-C₁₂-cyclohexanone derivative **39** inhibited LasR at 100 μ M by 35% and its C₄ counterpart, **40** inhibited RhlR systems by 60% at 50 μ M when competing with 1 μ M 3-oxo-C₁₂-AHL and 10 μ M 3-oxo-C₄-AHL respectively. Cyclohexanone analog **39** also inhibited QS mediated pyocyanin, elastase B and biofilm production.⁸⁷ Based on the initial results, eight non-AHL analogs with lactone ring replaced by aromatic group were reported. Compound **43** (Figure 9) was found to inhibit LasR system and also elastase B production in *P. aeruginosa*.⁸⁸

Figure 8. *P. aeruginosa* AHLs and their ring modified analogs.⁸⁷

Moroshi and co-workers reported acyl cyclopentane derivatives as inhibitors of *Serratia marcescens* QS.⁸⁹ *Serratia marcescens* is a Gram negative pathogen which regulates prodigiosin production, swarming motility and biofilm formation using AHL mediated QS.^{90,91} It uses *N*-hexanoyl homoserine lactone (C₆-AHL) and *N*-(3-oxo-hexanoyl) homoserine lactone (3-oxo-C₆-AHL) as QS signals.⁹² The *N*-nonanoyl-cyclopentylamide **44a** lacking the lactone ring was found to inhibit prodigiosin production at four fold concentration relative to the natural agonist (Figure 9). Kato *et al.* reported *N*-decanoyl cyclopentyl amide, **44b** as inhibitor of *P. aeruginosa* QS. It inhibited 3-oxo-C₁₂-AHL mediated *lasB-lacZ* expression with IC₅₀ value of 80 μM and 3-oxo-C₄-AHL mediated *rhlA-lacZ* expression with 90 μM (Figure 9).⁹³

Figure 9. Cyclopentanone, phenol analogs (**43-44b**)⁸⁹ and thiolactone and caprolactam analogs (**45-46**).⁷⁸

Eberhard *et al.*⁷⁶ and Greenberg *et al.*⁷⁸ independently studied the effect of synthetic AHLs having various side chain and lactone ring modifications in *V. fischeri* LuxR system. The AHL mimics with replacement of lactone ring with thiolactone **45** and caprolactam **46** were prepared (Figure 9). Thiolactone **45** had only moderate activity but caprolactam **46** was inactive, probably because of the lack of sensor binding. However, the detailed studies on lactam analogs with various ring sizes were not explored in this study

(3) Inhibitors with modifications on the lactone ring and acyl side chain

In an effort to find more potent QS inhibitors, AHL analogs modified at both lactone and acyl side chain were also prepared. *Pseudomonas aeruginosa* produces 3-oxo-C₁₂-AHL as the AI-1 to coordinate QS related behaviors like virulence factor production and also host immunomodulatory responses. Spring and co-workers described the total synthesis of 3-oxo-C₁₂-AHL and also reported four 3-oxo-C₁₂-AHL analogs **47-48b** having various nonhydrolysable cyclic ketone and lactone rings along with changes at the acyl side chain as LasR inhibitors.^{94,95} Cyclic ketones, **48a**, **48b** were found to reduce immunomodulatory responses (for e.g., TNF α factor and nitric oxide production) (Figure 10).

Figure 10. *P. aeruginosa* AHLs analogs with modification at lactone ring and acyl side chain.⁹⁵

Muh *et al.* screened a library of 200,000 compounds using ultra-high-throughput-cell based screening and identified two LasR inhibitors having a phenyl (**49**) and tetrazole ring (**50**) connected to 12 carbon aliphatic chain similar to 3-oxo-C₁₂-AHL (Figure 11).⁹⁶ Phenyl analog **49** and tetrazole analog **50** inhibited both production of pyocyanin and elastase B in strain PAO-1-MW1 (which lacks *lasI* and *rhII* genes) with IC₅₀ value of 10 μ M and 30 nM, respectively.

Figure 11. Inhibitors of LasR identified through high-throughput-cell based screening.⁹⁶

4. Inhibitors structurally unrelated to AHL

Muh *et al.* discovered the triphenyl compound, **51** and its analog **52** (which is structurally distinct to the natural ligand) mimicking the activity of 3-oxo-C₁₂-AHL against LasR protein (**51** had EC₅₀ of 0.014 μM and **52** had EC₅₀ of 0.054 μM) from the ultra-high-throughput-cell based screening (Figure12).⁹⁷

Figure 12. Triphenyl, nonnative agonists of 3-oxo-C₁₂-AHL mediated QS.⁹⁷

De Nys *et al.* isolated halogenated furanones **53-60** from red alga *Delisia pulchra* (Figure 13).⁹⁸ These furanones were the first example of small molecules identified to inhibit AHL mediated QS.⁹⁹ Compounds **53** and **54** were found to reduce QS-mediated behaviors like swarming motility in *S. liquefaciens* mutants lacking AHL synthase activity.⁹⁹ Bromofuranones were proposed to inhibit QS by destabilization of LuxR protein.¹⁰⁰ Compound **56** displayed concentration-dependent inhibition of bioluminescence production in *E. coli* (pSB403) by interrupting binding of its auto inducer, 3-oxo-C₆-AHL to LuxR. Compound **58** and **60** were able to reduce *P. aeruginosa* infection in pulmonary model.^{101,102}

Figure 13. Halogenated bromofuranones as AHL mediated QS modulators.⁹⁸

Several butenolides found in *Streptomyces antibioticus* demonstrated antimicrobial activity against *P. aeruginosa* and weak inhibition of chitinase in *Serratia marcescens*. On the basis of preliminary biological results and their structural resemblance to furanones, Nielsen *et al.* prepared synthetic butenolides and tested for anti QS activity.¹⁰³ From the library of compounds tested on GFP-fused *E. coli* (pJBA89) QS (that responds to 3-oxo-C₁₂-AHL to induce QS-mediated fluorescence), compounds with short side chain displayed maximum inhibition compared to long chain derivatives. Compound **61** was the most effective inhibitor with IC₅₀ = 2.4 μ M, while **62** had IC₅₀ = 3.5 μ M and **63** had IC₅₀ = 3.1 μ M (Figure 14).

Using QS inhibitor selector system (QSISI), Nielsen *et al.* discovered anti-QS properties of garlic extracts. Garlic components **64** and **65** were shown to have LuxR antagonistic activities (Figure 14).⁸²

Figure 14. Butenolide analogs (**61-63**)¹⁰³ and inhibitors from garlic (**64-65**).⁸²

1.2.2. Modulation of interspecies communication

Inhibition of AHL mediated intraspecies communication among Gram negative bacteria have been studied over two decades. With recent discovery of AI-2 pathway which was found in more than half of the bacterial species, interspecies communication can also be inhibited giving unique advantage of simultaneous inhibition of communication among multiple species. Moreover, bacteria which are devoid of AI-2 pathway, can still recognize the foreign AI-2 and induce QS.¹⁰⁴ Interspecies regulation of QS can be achieved by: (1) inhibiting LuxS which synthesizes DPD, (2) inhibiting the sensor receptor for AI-2 (LuxP in case of *V. harveyi* and LsrB in case of *S. typhimurium* and *E. coli*), (3) inhibiting enzymes downstream of AI-2 biosynthesis (e.g., 5'-Methylthioadenosine/AdoHcy nucleosidase (MTAN) inhibitors).

1.2.2.1. Inhibition of LuxS enzyme

The *luxS* gene is conserved in more than 60 microbial species including many Gram positive and negative human pathogenic bacteria such as *Vibrio cholerae* (causative agent of cholera), *Salmonella typhimurium* (salmonellosis), *Shigella flexneri* (shigellosis), *Streptococcus pneumonia* (pneumonia, meningitis and arthritis), *Neisseria meningitidis* (meningitis), *Clostridium perfringens* (food poisoning) and *Borrelia burgdorferi* (lyme disease).⁶ The LuxS enzyme is reported to be involved in cross talk among bacteria for regulating various community related behaviors like virulence factor production, biofilm formation, toxin production etc.⁵ Many examples or cases of involvement of LuxS/AI-2 in bacterial pathogenicity are reported recently and the list is growing. Mutations of *luxS* gene resulted in interference in expression of virulence factors and in some cases rendering the bacteria avirulent.¹⁰⁵⁻¹⁰⁷ As LuxS enzyme is a bacterial enzyme inhibition of which might cause less toxicity in humans. All of these factors/facts validate LuxS inhibitors as viable targets, to serve as broad spectrum antibacterial agents.

Reported LuxS Inhibitors

As SRH is a most important intermediate in the synthesis of AI-2 and LuxS is present in many species, researchers aimed at making SRH analogs as inhibitors of LuxS. The crystal structure and mechanism of LuxS catalysis was identified recently. However, the inhibitors of LuxS are limited. Recently Zhou *et al.* reported the first examples of LuxS inhibitors, *S*-(anhydribose)-L-homocysteine (**66**) and *S*-(homoribose)-L-cysteine (**67**).⁵⁹ The 1-deoxySRH **66** was reported to act by inhibiting the first mechanistic step in enzyme catalysis and compound **67** by inhibiting the final step of β -elimination (Figure 15). Pei *et al.* prepared a series of structural analogs with planar hydroxamate group which mimic the unstable enediolate intermediate formed during the isomerizations. Hydroxamates **68** and **69** were the most potent inhibitors reported to date with K_i values of 0.72 and 0.37 μ M respectively.⁵⁸ These results also proposed that homocysteine moiety is essential for binding and activity. On the basis of the similarities between substrates of mammalian SAHase and bacterial LuxS enzyme, as well as differences in mechanism of the two enzymes, our lab designed and synthesized SRH analogs with 6-(fluoro)vinyl unit in place of the C5 and sulfur atoms which acted as weak/moderate inhibitor of LuxS enzyme.¹⁰⁸ Compound **70** acted as a competitive inhibitor with K_i value of 96 μ M. Recently our lab in collaboration with Pei lab reported [3-F]SRH, **71** and [3-Br]SRH, **72** as time-dependent reversible inhibitors of LuxS. It was rationalized that covalent inhibition will be observed by nucleophilic attack of active site cysteine at the C3 position, with removal of halide. But time-dependent inhibition was observed because of the enzyme catalyzed elimination of halide ions *via* E1cb mechanism (Figure 15).⁵⁶ Recent studies in our laboratory focused on design and synthesis of 3-deoxy and 3-deoxy-3-substituted SRH analogs. These 3-fluoro and 3-*O*-methyl SRH analogs (lacking enolizable hydroxyl group at C3) were designed as mechanism based LuxS inhibitors.¹⁰⁹ Among the 3-modified SRH analogs, xylo analog, **73** was found to be most potent slow binding inhibitor with K_i value of 4.2 and K_i^* value of 0.43, while compound **74** and **75**

were moderately potent slow binding inhibitors with K_i * value of 2.8 and 8.8, respectively. These findings are consistent with the time-dependent inhibition proposed for [3-F] SRH, **71** and [3-Br] SRH, **72** (Figure 15).¹⁰⁹

Figure 15. Reported LuxS enzyme inhibitors

Halogenated furanones isolated from red alga *D. pulchra* were reported to clear *P. aeruginosa* infection in mouse pulmonary infection models and inhibit *B. anthracis* growth and expression of virulence genes. However the mechanism of QS antagonism was elusive.¹¹⁰ Recently Zhou *et al.* reported the mechanism of their action to be the time-dependent modification of LuxS enzyme. On the basis of experimental findings, an addition elimination mechanism was proposed for the covalent inhibition of LuxS by bromofuranone **53** (Figure 15).¹¹¹

1.2.2.2. 5'-Methylthioadenosine/AdoHcy nucleosidase (MTAN) inhibitors

The 5'-Methylthioadenosine/AdoHcy nucleosidase (MTAN) is a dual substrate-specific microbial enzyme encoded by *pfs* gene.⁷⁰ It catalyzes deadenylation of SAH to form adenine and SRH, the substrate for LuxS and also deadenylation of 5'-methylthioadenosine (MTA) to 5'-methylthioribose (MTR) (Scheme 4). The MTA is synthesized from *S*-adenosylmethionine and acylated acyl carrier protein by AHL synthase. It is a feedback inhibitor of AHL synthase.⁷⁰ Thus MTAN plays a vital role in both AI-1 and AI-2 biosynthesis. According to the MTAN catalytic mechanism as proposed by Schramm group, it undertakes catalysis through an early and late transition states (TS) involving formation of ribosyl oxacarbenium ions.¹¹² Based on the oxacarbenium ion transition state of MTAN catalyzed reactions, early and late TS analogs were synthesized. The 5'-thio-substituted immucillin (ImmA) mimic the early TS where the glycosylic bond is partially broken whereas the second generation DADMe-immucillin analogs mimic a late TS in which ribose moiety is completely broken from adenine (Scheme 4).¹¹²

Schramm and coworkers¹¹³ designed and synthesized a series of TS analogs (Scheme 4). The TS analogs **76-84** inhibit MTAN with K_i in nM range when tested in *Streptococcus pneumoniae* (Figure 16). Early TS analogs **76** and **77** inhibited with K_i of 60 nM and 40 nM, respectively. The DADMe-Immucillin analogs **80** and **84**, mimicking the late TS inhibited with K_i values of 24 nM and 2.3 nM, respectively.¹¹³ In order to improve the potency of TS analogs, the Schramm group synthesized a series of azetidine analog resembling late TS. Methylthio azetidine **85** inhibits *E. coli* and *S. pneumonia* MTAN with K_i value of 0.84 nM and 150 nM, respectively.

Scheme 4. (A) 5'-Methylthioadenosine/AdoHcy nucleosidase catalyzed hydrolysis of 5'-methylthioadenosine and *S*-adenosylmethionine (B) Proposed transition state of 5'-Methylthioadenosine/AdoHcy nucleosidase along with aza TS analogs.¹¹³

The compound **86** was shown to have K_i of 0.45 and 84 nM with *E. coli* and *S. pneumonia* MTAN, respectively.¹¹⁴ When **80** (MT-DADMe-Immucillin-A), **81** (EtT-DADMe-Immucillin-A) and **82** (BuT-DADMe-Immucillin-A) were tested in *V. cholerae* MTAN, they were found to be slow-onset, tight-binding inhibitors of with IC₅₀ values of 27, 31 and 6 nM, respectively. The DADMe-Immucillins **80** and **82** also inhibited *E. coli* MTAN with IC₅₀ in nM range. These results clearly validated the use of MTAN inhibitors as potential drug molecules.⁵⁷

Figure 16. Transition state analogs of 5'-Methylthioadenosine/AdoHcy nucleosidase

1.2.2.3. Modulation of AI-2 receptor (LuxP/LsrB)

As described earlier, many bacteria use AI-2 as interspecies signaling molecule but different bacteria recognize different forms of AI-2. For example, *V. harveyi* recognizes borate ester of *S*-THMF while *S. typhimurium* recognizes *R*-THMF as AI-2.⁴⁵ The sensor protein for *V. harveyi* is LuxP and LsrB in case of *S. typhimurium*. The crystal structures of the binding proteins along with their AI-2s revealed a substantial difference in the residues at the binding pocket between LuxP and LsrB explaining the selective preference of LuxP for the anionic borate ester and LsrB for the neutral *R*-THMF).⁴⁵ As AI-2 binding proteins play a significant role in QS cascade,

compounds which can inhibit LuxP or LasrB receptors or which can mimic the structures of AI-2 will provide substantial information on the binding site. This might open new doors for development of novel QS inhibitors.

AI-2 agonists

The crystal structure of LuxP with AI-2 was solved by Bassler *et al.*⁴⁴ Certain degree of flexibility within the binding pocket of LuxP was observed. Encouraged by such results, the Janda group synthesized eight DPD structural analogs **87-94** and tested in *V. harveyi* strain MM30 (a LuxS mutant that is unable to synthesize DPD) (Figure 17).¹¹⁵ The results of the activity studies suggested a cleft in the binding pocket responsible for accommodation of structurally diverse molecules. Boron binding, presence, position and stereochemistry of hydroxyl group were suggested to be critical for optimum activity.

The DPD is an unstable molecule and difficult to isolate and purify. Because of this reason studies on AI-2 QS were performed using LuxS mutants as the source of DPD. Employment of mutants lacks the accurate control on concentrations of DPD used. To overcome these practical limitations and to study binding of AI-2 at the receptor, many groups have obtained DPD and its analogs by synthetic route.¹¹⁶⁻¹¹⁹ Recently Doutheau *et al.* synthesized bis-(*O*)-acetylated DPD (Ac₂-DPD), a precursor to DPD, **96** (Figure 17). The Ac₂-DPD is stable at physiological pH and also in concentrated solutions. The esterases present *in vivo* hydrolyze **96** to provide DPD. However, Ac₂-DPD was found to be less potent than DPD in BB170 strain of *V. harveyi* bioluminescence assay and MET844 strain of *S. typhimurium* β -galactosidase assay, presumably because of the difference in rates of hydrolysis to release DPD. It has comparable activity to DPD in biofilm formation when assayed in the *B. cereus* strain 407.¹²⁰ As replacement of hydrogen atoms with fluorine atom often results in modulation of biological and metabolic properties, the Doutheau lab also reported a trifluoro methyl analog of DPD, **95** (Figure 17). However, **95** was found to be less potent than the natural substrate.¹²¹

Figure 17. Various DPD agonists resembling DPD, furanosyl DPD or its borate ester AI-2

Janda *et al.* found that *S*-THMF-carbonate, **97** produced from the complexation of furanosyl form of *S*-DPD with carbonate, acts as AI-2 agonists by inducing bioluminescence in *V. harveyi* strain MM30 (Figure 17). Among the several metal salts tested to identify the potential of metal

ions complexes of furanosyl DPD other than boron to act as QS modulators, cesium carbonate was able to induce bioluminescence. Interestingly, carbonate anion rather than the metal ion was found responsible for the QS modulatory activity. The agonistic activity of S-THMF-carbonate was conceivable, as it fits perfectly in the binding pocket of LuxP mimicking the borate complex.¹²²

Steinberg *et al.* synthesized several oxaborazolidine analogs resembling the structure of AI-2. Compounds **98** and **99** (Figure 17) displayed co-agonistic activity in *V. harveyi* strain BB170 (which lacks *luxN*, the sensor for AI-1) and also in strain BB77 (which lacks *luxS* and *luxM*, the synthases for AI-1 and 2, respectively). These results demonstrated the involvement of oxaborazolidines specifically in AI-2 signal transduction by their ability to act synergistically with DPD or AI-2.¹²³

Recently Janda *et al.* reported C1-Alkyl substituted DPD analogs as modulators of AI-2 QS (Figure 17). These compounds were evaluated in *S. typhimurium* strain Met844 with a *lacZ-lsr* fusion (lacks *luxS*, the DPD synthase) for the induction of β -galactosidase activity, with and without external DPD (*lacZ* encodes for the enzyme β -galactosidase which is under the control of promoter *lsr*. The *lsr* activation is, in turn influenced by AI-2). Propyl-DPD, **101** was found to be an antagonist while ethyl derivative, **100** has no activity. Interestingly when tested in *V. harveyi* MM32 (lacks AI-1 sensor, LuxN and DPD synthase, LuxS) all the derivatives showed agonistic activity only in presence of DPD by inducing luminescence. Propyl-DPD, **101** and azido-DPD, **103** (Figure 17) were found to be active by seven fold when compared to DPD.¹²⁴ Meijler *et al.* and Sintim *et al.* also synthesized alkyl DPDs independently and also observed similar agonistic activity in *V. harveyi* assays, supporting the results observed by the Janda group.^{125,126} However, no definite trend was observed with respect to the effect of C1-chain length or ring size on synergism with AI-2 induced bioluminescence.¹²⁶

The Janda group also performed a short and efficient synthesis of natural product fimbrolide, a known QS inhibitor (**53**) and studied its inhibitory effects on AI-2 mediated QS in comparison with the C1-substituted DPD analogs.¹²⁷ Hexyl-DPD, **102** was found to be more potent than fimbrolide **53**, in inhibiting bioluminescence of *V. harveyi* (Figure 17)

AI-2 antagonists

Recently Wang *et al.* reported para-substituted phenylboronic acid derivatives, **104-108** (Figure 18) as antagonists of AI-2 mediated QS in *V. harveyi* strain MM32 which shows bioluminescence only with addition of external DPD. Only para substituted phenyl boronic acids were found to exhibit strong antagonism (IC_{50} in single digit μM range) whereas derivatives substituted with amino, carboxyl and alkyl groups possessed no comparable activity. Analogs with no ionizable functional groups and low pK_a values were found to possess strong antagonism for LuxP receptor. It was proposed that, analogs which existed as tetrahedral borates at neutral pH, mimic the natural substrate and exhibit antagonism.⁶⁰

Wang group also reported pyrogallols as antagonists to AI-2 signal protein in *V. harveyi* strain MM32.¹²⁸ Since the natural substrate, AI-2 in *V. harveyi* is a diol-borate complex, it was rationalized that diols which could bind to borate tightly and mimic the structural geometry of AI-2 and LuxP complex, could be antagonists.¹²⁹⁻¹³¹ Among the fifteen aromatic polyols tested, pyrogallols, **109-113** possessing nonionizable functional groups were found to be more potent inhibitors with IC_{50} values in single digit μM range (Figure 18). From the structure based virtual screening of a library of compounds against the crystal structure of LuxP heterocyclic sulfones, **114** and **115** were identified as AI-2 QS inhibitors.¹³²

Figure 18. Pyrogallols and heterocyclic sulfones as DPD analogs.¹²⁹⁻¹³¹

Coenye *et al.* reported nucleoside analogs as AI-2 signal transduction inhibitors.¹³³ The most active compound found from their screening was an adenosine derivative substituted at C3 by *p*-methoxyphenylpropionamide moiety, **116** and its deadenylated derivative **117** (Figure 19). Interestingly, though structurally **116** resembles SAdH, the microbiological assays performed on *V. harveyi* mutant strains revealed the molecular target of **116** to be LuxPQ which is involved in AI-2 signal transduction (which is upstream of AI-2 production). Adenosine derivative, **116** also

reduced the AI-2 mediated QS behaviors like starvation response, biofilm formation, pigment and protease production in various *Vibrio* species.

Figure 19. 3-Amino-3-deoxy sugar and nucleoside inhibitors.¹³³

Using *V. harveyi* strain BB170 which lacks the sensor for AI-1, Pillai *et al.* discovered a series of fatty acids obtained from poultry meat wash to be inhibitors of AI-2 mediated QS.¹³⁴ Linoleic acid, **118a** oleic acid, **118b** palmitic acid, **118c** and stearic acid, **118d** (Figure 20) at 0.1, 1, and 10 mM concentrations resulted in AI-2 inhibition ranging from approximately 25% to 99%.

Figure 20. Fatty acids as AI-2 mediated QS inhibitors in *V. harveyi*.¹³⁴

Recently Bentley *et al.* investigated inhibition of interspecies QS by sequestration of AI-2- using LsrK. The LsrK is a AI-2 kinase enzyme which phosphorylates AI-2 into phosphoAI-2 which in turn modulates QS by activating *lsr* regulated gene expression in *S. typhimurium* and

E. coli.⁶¹ It was rationalized that by providing LsrK externally, AI-2 could be phosphorylated *ex vivo* and should be incapable of entering the cells to induce QS. Quorum quenching was observed, when LsrK was added to cultures of *E. coli* which generates AI-2 and *S. typhimurium* which detects AI-2, suggesting the utility of LsrK as broad range quorum quencher.

1.3. Aza sugars

As part of my dissertation deals with aza sugar analogs of SRH in which ribosyl oxygen is replaced with nitrogen atom, I would like also to summarize recent developments in the chemistry of biologically active aza sugars. Synthetic strategies pertaining to the synthesis of 4-aza-SRH analogs will be discussed (Detailed synthesis of aza sugars was reviewed by Pearson *et al.*¹³⁵)

Aza sugars are polyhydroxy alkaloids that mimic natural sugars. They are carbohydrate analogs in which the ring oxygen is replaced by a nitrogen atom. They are also called the imino sugars.¹³⁶ Naturally available aza sugars identified so far are classified based on the type of ring structure as: (1) polyhydroxy piperidines (e.g., nojirimycin), (2) pyrrolidines (e.g., CYB 3, nectricin), (3) indolizidines, (e.g., swaianosonine) (4) pyrrolizidines, (e.g., alexin) and (5) nortropanes (e.g., calystegine A3). Representative structures belonging to each class are depicted in Figure 21.¹³⁵

Aza sugars are found to act as glycosidase inhibitors because of their resemblance to natural sugars. Glycosidase and glycotransferases are set of enzymes which control metabolism and biosynthesis of oligosaccharides and glycoconjugates. Such carbohydrate derivatives are essential for vital biological functions like cell adhesion, cell-cell recognition, cell growth, maintenance of immune system, oncogenesis, tumor metastasis etc. Therefore, inhibitors of glycosidases are widely sought after, as they can provide novel antiviral, antibacterial, anticancer, antidiabetic, antiHIV-AIDS agents.

Figure 21. Representative examples of naturally occurring aza sugars belonging to different structural class.^{135,137}

For example, *N*-hydroxyethyl-1-deoxynojirimycin (Miglitol)¹³⁸ and *N*-butyl-1-deoxynojirimycin (Miglustat)¹³⁹ are approved drugs against type-2 diabetes and Gaucher's disease, respectively (Figure 22). Furthermore, castanospermine is found to show potent antiviral activity,¹⁴⁰ swainsonine¹⁴¹ displays anticancer activity, and the bicyclic indoline derivative, lentiginosine¹⁴² is an amyloglucosidase inhibitor.

Figure 22. Examples of aza sugars with proven biological application.^{138-140,142}

1.3.1. Transition state analogs of glycoside hydrolases

According to TS theory, the catalytic proficiency of an enzyme is derived from its capacity to lower activation energy and stabilization of the TS. Altered substrates which mimic the TS of the enzyme bind to the enzyme with greater affinity than the natural substrate as they are proposed to

catch all the binding interactions available.¹⁴³ Such non-natural substrates also assist in understanding the mechanism of enzyme catalysis and thereby facilitate the development of potent inhibitors. As a result of the wide utility of glycoside hydrolases in vital biological functions, there has been huge interest in developing inhibitors.

Glycoside hydrolases catalyze cleavage of glycosidic bond in sugar *via* formation of an oxacarebenium ion which is in the half chair conformation at the anomeric center (at the TS) leading to either retention (retaining glycosidases) or inversion of configuration (inverting glycosidases) of anomeric center. Two carboxylic residues at the active site acting as acid/base and a molecule of water catalyze hydrolysis.

1.3.1.1. Cleavage mechanism of glycosidases

Retaining glycosidases

The proposed mechanism of catalysis for retaining glycosidases of sugars (**119a**) involves: (i) protonation of anomeric oxygen with acidic groups present at the active site (**119b**, Figure 23), (ii) generation of oxocarbenium ion **119c** after removal of ROH from the anomeric carbon (the glycosyl cation **119c** is proposed to exist as oxocarbenium ion **119h** in half chair conformation), (iii) subsequent formation of an ester bond at the anomeric center with the active site carboxylate (**119d** from Figure 23), and (iv) formation of hemiacetal product **119e** with the retention of anomeric configuration by nucleophilic displacement of ester group with the enzyme bound water molecule (Figure 23).¹³⁷

Inverting glycosidases

The first step of catalysis is similar to the retaining glycosidases where general acid protonates the anomeric oxygen. In the later step, however inverting glycosidases differ from the retaining glycosidases. In case of retaining glycosidases, ester intermediate precedes nucleophilic attack by naked water molecule generating the product, while inverting glycosidases act by

nucleophilic attack of the carboxylate directed water molecule at the anomeric center to generate the hemiacetal product **119f**, hence inversion of configuration (Figure 23).

Figure 23. Cleavage mechanism of (a) retaining and (b) inverting glycosidases. (c) Resonance forms of oxocarbenium ion in half chair confirmation.¹³⁷

1.3.1.2. Aza sugars as transition state analogs

The design of TS inhibitors focuses on either emulating the supposed half chair geometry or the charge at the TS or both. Since the charge build up occurs in different places at the TS,

mimics with charge at various positions were designed. TS analogs are broadly categorized according to the charge build up and geometry.¹³⁷

(a) Inhibitors mimicking positive charge at anomeric oxygen

Since the proposed glycosidases mechanism of catalysis was shown to have protonated exocyclic oxygen in early TS (**119b** in Figure 23), analogs mimicking the charge at the anomeric oxygen were synthesized. Most potent inhibitors in this category are sugars which possess a nitrogen atom in place of anomeric oxygen.¹³⁷ The protonated amine can mimic the early TS. Trehalase inhibitor, trehazolin **120a** and chitinase inhibitor, allosimidine **120b** (Figure 24) are examples of this class of inhibitors.¹⁴⁴

(b) Inhibitors mimicking positive charge at ring oxygen

As late TS involves formation of glucosyl oxacarbenium ion in half chair conformation with charge either at anomeric carbon (**119g**) or at ring oxygen (**119h**), TS analogs mimicking either geometry or charge at the ribocarboxenium ion were designed. Most potent α -glucosidase inhibitor, 1-deoxynojirimycin¹⁴⁵ **120c** ($K_I = 18 \mu\text{M}$) which contains nitrogen in place of ring oxygen (resembles oxacarbenium ion upon protonation) and mannosidase inhibitor, imidazolo pentose¹⁴⁶ **120d** (Figure 24) (resembles half chair geometry) are examples of this class of inhibitors.

(c) Inhibitors mimicking positive charge at anomeric carbon

The TS analogs resembling intermediates **119c** or **119a** which possess charge at the anomeric carbon were also developed.¹³⁷ The most potent inhibitors of glycoside hydrolases fall into the category of **119c** mimics. For example, β -glycosidase inhibitor, isofagomine **120e** ($K_I = 0.11 \mu\text{M}$) contains nitrogen at the psuedo anomeric position (Figure 24). The difference in selectivity of isofagomines and 1-deoxynojirimycin by α and β -glucosidases was attributed to the location of their active site residues. In case of α -retaining glucosidase TS, active site carboxylate group is at the top of the sugar ring and charge is located at the endocyclic oxygen where as in case of β -

retaining TS, active site carboxylate group is present beneath the sugar ring and charge is located at the anomeric carbon.¹⁴⁷

Figure 24. Aza sugar based transition state analogs of glycosylases.¹³⁷

(d) Inhibitors mimicking positive charge at multiple places

Inhibitors with charge distributed over to multiple atoms at the TS (Figure 23) combining the electronic properties of the charged TS intermediates **119b**, **119c** and **119h** as well as half chair confirmation were also developed. Such TS analogs mimic TS more closely because of the charge at various positions:

(i) The amidine analog **120f** resembling both endo cyclic and exocyclic charge on oxygen (**119b** and **119h**) was found to be strong β -glucosidase inhibitor with K_I of 8 μ M.¹⁴⁸

(ii) The imiadazole derivative **120g** which mimic both half chair confirmation and charge at the anomeric center was also developed. As tetrazoles have pK_a value of -4 and cannot be expected to be fully protonated in the active site. Compound **120g** inhibited α -glucosidase and α -galactosidase with a K_I of about 1.5 μ M.¹⁴⁹

(iii) The 1-azafagomine **120h** possessing endocyclic nitrogen similar to 1-deoxynojirimycin and exocyclic nitrogen at the pseudo anomeric position like isofagomine displayed strong inhibition of both α - and β -glucosidases (α -glucosidase with K_I = 6.9 μ M and β -glucosidase with K_I = 0.32 μ M, respectively) (Figure 24).¹⁵⁰

(iv) The hydrazine analog **120i**, a hybrid of isogalactofogamine and aminogalactose was found to be inhibitor of galactosidase (α -galactosidase with K_I = 73 μ M and β -galactosidase with K_I = 410 μ M) (Figure 24).¹⁵¹ Such analog mimics glucosyl cation **119c** and intermediate with protonated anomeric oxygen **119b**.

(e) Inhibitors which does not imitate charge

From the kinetic isotope effects and TS structure, it was suggested that the anomeric carbon is sp^2 -hybridized.¹⁵² Moreover such observation was attested by potent inhibition observed by lactam and lactone analogs although they only mimic the hybridization but not the charge. However except for lactam analog **120j**¹⁵³ and **120k**¹⁵⁴ which inhibit xylanase with K_I = 0.34 μ M and with K_I = 18 nM, respectively, in general, this class of inhibitors are the weakest of all the

classes of inhibitors described earlier. The weak inhibition was attributed to the mimicry of geometry alone rather than the charges at the TS (Figure 24).

Recently Schramm *et al.* prepared various femtomolar and picomolar TS mimics of 5'-methylthioadenosine nucleosidase (MTAN), which also bear ring nitrogen in place of oxygen and mimic the oxacarebenium ion formed at the TS.¹¹³ For more description of these MTAN inhibitors refer to section 1.2.2.2.

1.3.2. Hemiaminal sugar chemistry

As the proposed 4-aza-SRH analogs are structurally hemiaminal congeners (*N,O*-acetal or azahemiacetal) of ribose sugar (polyhydroxylated pyrrolidine), in this section I will discuss in brief, the chemistry of the biologically relevant hemiaminal sugars. Hemiaminals as hemiacetals and hemithiols exist in equilibrium as a mixture of open chain aldehyde and closed ring forms whose individual percentage varies depending on pH, conformation of sugar, leaving group strengths, anomeric effect and ionic strength of the solvent medium (Scheme 5). The chemistry of *N,O* acetals of sugars is found to be complex.

Scheme 5. Structures of ribose, 4-thioribose and 4-azaribose in their respective open (aldehyde) and closed ring forms

4-amino-4-deoxyribofuranoside (4-*epi*-nectricine)

Nectricine (Figure 21) is a fungal metabolite isolated from *Nectria lucida*. It exists solely as an imine sugar while its ribose epimer 4-*epi*-nectricine exist as a complex equilibrium mixture consisting of dimer, imine and hemiaminal forms.¹⁵⁵ These isomers transform interchangeably depending on the pH and concentration of the aqueous solution (Scheme 6). At pD 7-7.5, in 1M solution, it exists as a mixture of 4-aza-4-deoxyribopyranoside **121a** and its dimeric form **121d** in equal ratio. At pD 9-11, dimer **121d** was observed as a single entity. While at more basic pD (13-14) dimer **121d** and dehydrated hemiaminal **121c** (imine) forms coexist in equilibrium. Interestingly when the 1 M solution was diluted by 20 times at pD 7-7.5, hemiaminal **121b** appeared along with imine **121c** and pyranoside **121a**.¹⁵⁶

Scheme 6. 4-Amino-4-deoxyribofuranoside in its equilibrium forms at variable pH.¹⁵⁶

Nojircimycin

The naturally occurring hemiaminal sugar nojircimycin is a highly potent glycosidase inhibitor relative to its 1-deoxy analog (Figure 21 and 24). It is weakly basic with pK_a value of 5.3 in water.¹⁴⁵ As it exists purely in its closed ring form (hemiaminal), it does not react with

ninhydrin reagent. The chemical shifts and J values of anomeric proton signals of nojirimycin in D₂O are 5.3 (*J* = 2.5) for α-anomer and 5.79 (*J* = 10.0) for β-anomer. Because of the complex ¹H NMR, nojirimycin is characterized by converting it into its 1-deoxy analog using catalytic reduction.

gem-Diamine 1-*N*-imino sugars

Siastatin B (*gem*-diamine, 1-*N*-imino sugar) (**122a**, Scheme 7) is a glycosidase inhibitor in which anomeric hydroxyl is replaced by nitrogen.¹⁵⁷ The protonated form of siastatin B **122a** was proposed to mimic the hexopyranosyl cation formed at the TS. As a result of the potent inhibitory activity of siastatin B, trifluoromethyl derivative of siastatin B, **122b** was synthesized and tested for enzymatic inhibition at various pH.¹⁵⁸ A time-dependent alteration of **122b** was observed at pH 5. From the spectral studies, the hemiaminal **122c**, imine **122d**, enolate **122e** and ketone **122f** formation was revealed (Scheme 7). The similar equilibrium mixture was also observed previously in case of hemiaminal 4-*epi*-nectricine.

Scheme 7. 1-*N*-imino sugar of galacturonic acid **122b** in its equilibrium forms at pH 5.0.¹⁵⁷

2. RESEARCH OBJECTIVE

The overall aim of this study is the design, synthesis and biological evaluation of (a) novel 4-aza-*S*-ribosyl-L-homocysteine (4-aza-SRH) analogs and (b) 2-deoxy-2-substituted-*S*-ribosyl-L-homocysteine analogs as potential inhibitors of LuxS enzyme which is involved in interspecies QS. Also targeted are γ -lactam and their reduced cyclic hemiaminal analogs, bearing the additional alkylthiomethyl substituent as AHL mediated intraspecies QS antagonists in *P. aeruginosa* and *V. harveyi*.

QS is a population dependent bacterial communication phenomenon utilized by number of pathogenic bacteria to direct symbiosis, virulence and biofilm formation. The recent increase in prevalence of bacterial strains resistant to antibiotics emphasizes the need for the development of new generation of antibacterial agents. Interruption of QS, as a strategy to combat pathogenesis is a viable option as it does not affect bacterial growth but only virulence, leading to less incidence of microbial resistance. The LuxS is a bacterial enzyme present in more than 60 species and responsible for biosynthesis of AI-2, a sensor molecule utilized for intraspecies bacterial communication. As a result, inhibitors of LuxS enzyme should inhibit AI-2 synthesis and consequently AI-2 mediated QS, giving rise to a new generation of antibacterial agents. Intraspecies communication in Gram negative bacteria is mediated largely by AHLs using “R” type sensor proteins. Human opportunistic Gram negative pathogen, *Pseudomonas aeruginosa* regulates virulence factor production, biofilm formation via *las* and *rhl* mediated QS. Thus, inhibition of these QS systems can result in attenuation of virulence. For these reasons, inhibition of AHL sensors protein, LasR, can serve as attractive target for the design of antimicrobial therapy.

Initially, I have taken effort to synthesize 4-aza-*S*-ribosyl-L-homocysteine (SRH) analogs with sugar ring oxygen replaced by nitrogen atom (e.g., **143**). The nitrogen atom in 4-aza-SRH compound should have the ability to mimic the interactions created by oxygen atom present in

natural substrate with the LuxS enzyme. The 4-aza-SRH will allow to study of the effect of *N,O*-acetal function vs *O,O*-acetal present in the natural substrate. Since the hemiaminal sugar analogs are weak bases at physiological pH, they exist predominantly in cyclic hemiaminal form. I envisaged that upon enzyme mediated protonation of anomeric hydroxyl group and dehydration, the resulting imine derivatives might act as suicide inhibitors by reacting with proximal nucleophiles in the protein. The 4-aza-SRH may also undergo LuxS mediated catalysis and release aza analogs of 4,5-dihydroxy-2,3-pentanedione (DPD), a precursor to the novel aza-AI-2 analog.

In order to interfere with the enzyme mediated sugar ring opening, 4-aza-SRH is further modified at the anomeric carbon to afford analogs bearing pyrrolidine (e.g., **129**), lactam (e.g., **141**), endocyclic imine (e.g., **158**), and cyclic nitron (e.g., **153**) functionality (Figure 24). I rationalized that 1-deoxy-4-aza-SRH **129**, could inhibit the enzyme mediated ring opening because of the lack of hydroxyl group at the anomeric carbon and hence can act as a competitive inhibitor. In the case of the lactam analog of SRH **141** in which C1-hydroxyl is replaced by carbonyl group, I rationalized that its interaction with LuxS might also result in competitive inhibition because of the increase in stability of amide bond towards the hydrolysis. Moreover since carbonyl group has the ability to act as good hydrogen bond acceptor and can tautomerize into the enolic form, new insights into LuxS binding should be unraveled. In order to attest the hypothesis of the covalent adduct formation between 4-aza-SRH derived imine intermediates and the enzyme, an imine analog of 4-aza-SRH bearing methyl group at C1 (**158**) is also targeted. Since the nitron group is a better electrophile than imine, the proposed covalent adduct formation is also investigated using the nitron analog of SRH (**153**).

Figure 25. Novel 4-aza-SRH analogs proposed in this study

In my second approach towards design of LuxS inhibitors, I aimed at designing and synthesizing SRH analogs modified at C2. Such analogs might prevent the first aldo-keto isomerization and hence inhibit formation of 2-keto intermediate. In this series, I plan to target 2-deoxy-SRH, 2-deoxy-2-fluoro-S-arabinosyl-L-homocysteine ([2-F]SAH) **194** and S-arabinosyl-L-homocysteine (**187**, SAH) (Figure 25). The [2-F]SAH might also undergo nucleophilic attack at C2 by the active site Cys-84 residue, to form a covalent adduct with the enzyme, leading to irreversible inhibition. In case of SAH, because of the reversal of stereochemistry at C2-hydroxyl, proton abstraction from C2 by Cys84 might be forbidden since proton transfers were proved to occur stereochemically. Moreover, such mechanism based inhibitors might also provide information on structural features necessary for inhibition and further explain the role of active site residues in catalysis.

The next goal of my research was to develop modulators of intraspecies QS regulated by AHLs. Though several AHL mimics were discovered to be potent QS inhibitors, the utility of such compounds to act as drug targets is limited because of the instability of lactone ring. For this reason, the development of non-native AHL ligands has emerged as a promising new strategy to regulate QS in Gram-negative bacteria. Only a few examples of inhibitors with the altered lactone ring structure of AHL have been reported. Though the effect of caprolactam analogs were explored before, detailed studies on γ -lactams were not conducted. In order to study effect of nonnative γ -lactam ring on QS and also to find common structural requirements for inhibition of LuxS and AHL-mediated QS, I undertook an effort to synthesize γ -lactam and their reduced cyclic hemiaminal analogs, bearing the additional alkylthiomethyl substituent (from C₃-C₁₂) (e.g., **160**). I envisioned that lactam ring being an isoster of lactone ring, will be more stable to basic environments when compared to AHLs. Thus, these analogs are expected to act as potential QS inhibitors in Gram negative bacteria.

3. RESULTS AND DISCUSSION

3.1. Design and synthesis of 4-aza-S-ribosyl-L-homocystiene analogs

3.1.1. Pyrrolidine analog

The first targeted azasugar analog of SRH was pyrrolidine analog **129** lacking the hydroxyl group at C1 (Scheme 8). Since the analog **129** could be protonated but could not undergo ring opening, I predicted that the initial step of the LuxS-catalyzed reaction could be precluded. The protected 1,4-dideoxy-1,4-imino-D-ribitol **123** served as a convenient starting material which was prepared from commercially available D-gulonic- γ -lactone.¹⁵⁹ However, attempted mesylation of the *N*-benzyl protected **123** instead of providing 5-*O*-mesyl precursor for coupling with homocysteine resulted in the formation of piperidine byproduct **130** as a mixture of two diastereomers at C5 (~3:1). Analogous rearrangements of pyrrolidines into piperidines through ring enlargement via aziridine type intermediates are known.^{160,161} We found that either replacement of the benzyl protection group with the Boc group at ring nitrogen or oxidation of the iminoribitol to ribonolactam (e.g., **131**, *vide infra*) reduced basicity of nitrogen enough to prevent ring enlargement providing stable 5-*O*-mesyl derivatives. Thus, silylation of **123** with *tert*-butyldimethylsilyl chloride (TBDMSCl) afforded **124** and catalytic hydrogenolysis (5% Pd/C) of **124** in the presence of (Boc)₂O affected removal of benzyl group and protection with *Boc* in a one-pot reaction^{161,162} yielding **125** (99% from **123**). Desilylation of **125** produced **126** (70%), which upon mesylation gave stable **127** (96%). Nucleophilic displacement of mesylate with thiolate generated from protected L-homocysteine⁵⁴ with tris(2-carboxyethyl)phosphine hydrochloride afforded **128** (86%). Using water soluble tris(2-carboxyethyl)phosphine for the generation of homocysteine reagent not only improved the yields but also simplified purification as compared to the previously described analogous substitution which employed tributyl⁵⁴ and triethylphosphines⁵⁹ for the reduction of homocysteine. Treatment of **128** with TFA effected simultaneous removal of the *N*-Boc, acetonide and *t*-butyl ester protection groups to give 4-azaSRH analog **129** (66%).

Reagents and conditions (a) TBDMSCl/imidazole/DMAP/CH₂Cl₂/rt; (b) H₂/Pd-C/(Boc)₂O/Et₃N/EtOH/rt; (c) MsCl/Et₃N/CH₂Cl₂/rt; (d) TBAF/THF/rt; (e) BocNHCH(CH₂CH₂SH)CO₂*t*-Bu/LDA/DMF/rt; (f) (i) TFA/0 °C (ii) TFA/H₂O/0 °C.

Scheme 8. Synthesis of pyrrolidine analog of 4-aza-SRH

3.1.2. Lactam analog

Next, lactam-type SRH analog **141** with the amide oxygen at C1 was targeted. As the flat planar carbonyl group in amides might mimic the keto intermediate and also fail to undergo ring opening, lactam **141** can act as competitive inhibitor. I also speculated that the lactim tautomer can mimic the anomeric hydroxyl resulting in binding affinity comparable to the natural substrate. Thus, regioselective oxidation of the 5-*O*-TBDMS-azasugar **124** (hydroxypyrrolidine) at C1 with RuO₂/NaIO₄ under EtOAc/H₂O biphasic conditions¹⁶³ produced lactam **131** (65%). Minor quantity (18%) of the corresponding *N*-benzoylpyrrolidinone byproduct was also formed because of the additional oxidation of the benzylic protons (Scheme 9). Desilylation of **131** with TBAF produced **132** (97%). Treatment with MsCl and replacement of 5-mesylate group from **135** with protected Hcy gave **137** (70%). Treatment of **137** with TFA effected removal of all acid-labile

protection groups to yield **140** (48%). Attempts to remove *N*-benzyl protection [e.g., H₂/Pd-C or Pd(OH)₂-C, Na/NH₃(liq), BCl₃] from **137** were unsuccessful.

Reagents and conditions (a) NaIO₄/Hydrated RuO₂/EtoAc/H₂O/rt; (b) TBAF/THF/rt; (c) MsCl/Et₃N/CH₂Cl₂/rt; (d) BocNHCH(CH₂CH₂SH)CO₂*t*-Bu/LDA/DMF/rt; (e) (i) TFA/0 °C (ii) TFA/H₂O/0 °C; (f) (Boc)₂O/Et₃N/CH₂Cl₂/rt (g) LiEt₃BH/THF/-78 °C.

Scheme 9. Synthesis of lactam and hemiaminal analogs of 4-aza-SRH

To overcome this setback the synthetic approach was undertaken with acid-labile Boc protection group at nitrogen. Thus, RuO₂-catalyzed oxidation of **125** gave **133** (60%) as a sole product and subsequent desilylation with TBAF afforded **134** (77%). Although mesylation of **134** to provide

136 was unsuccessful we were able to prepare **136** by oxidation of **127** with $\text{RuO}_2/\text{NaIO}_4$. Coupling of **136** with thiolate generated from L-Hcy resulted in **138** with loss of Boc group at sugar nitrogen. Deprotection with TFA followed by TFA/ H_2O yielded lactam **141** (58%).

3.1.3. Hemiaminal analog

The hemiaminal **143** (Scheme 9) which possess ring nitrogen in place of oxygen in SRH, was synthesized to explore its effect on LuxS binding, LuxS catalyzed ring opening and degradation. Thus, direct reduction of the unprotected amide **138** (or **141**) with super hydride¹⁶⁴ failed to yield **143**. However, treatment of **138** with $(\text{Boc})_2\text{O}/\text{DMAP}$ gave fully protected lactam **139** which upon treatment with LiEt_3H produced hemiaminal **142**. Deprotection with TFA and TFA/ H_2O gave desired 4-azaSRH **143** as a mixture of complex mixture of several isomers. Interestingly, analogous *N,O*-acetals were reported to be stable under conditions required for coupling with nucleoside bases.¹⁶³

3.1.3.1. Benzyloxime derivative of hemiaminal sugar

In order to support our hypothesis that the hemiaminal analog **143** could be converted to the open aldamine form during the first step of LuxS catalysis, limited model studies were performed with hemiaminal 4-amino-4-deoxy- α/β -D-ribofuranose **146a**¹⁵⁶ (Scheme 10). Thus, previously described *N*-Boc protected lactam **133** was reduced with LiEt_3H to afford protected hemiaminal **144**. Desilylation with TBAF yielded **145**, which was treated with TFA to give deprotected hemiaminal **146a** as a mixture of α/β anomers. Subsequent treatment of **146a** with *O*-benzylhydroxylamine gave desired oxime derivative **147** as a single product. The formation of oxime **147** suggested that though hemiaminal **146a** is the major isomer at neutral pH, equilibrium could be shifted towards open aldehyde form **146b** by derivatization. Such results clearly supported the plausible conversion of hemiaminal analog **143** to its open aldehyde form.

Reagents and conditions (a) $\text{LiEt}_3\text{BH/THF}$, $\text{THF/-78 } ^\circ\text{C}$; (b) TBAF/THF/rt ; (c) (i) $\text{TFA/0 } ^\circ\text{C}$ (ii) $\text{TFA/H}_2\text{O/0 } ^\circ\text{C}$; (d) $\text{BnO-NH}_2/\text{Pyridine/rt}$.

Scheme 10. Benzyl oxime derivative of 4-azaribose

3.1.4. Nitron e analog

A nitron e analog of SRH was also targeted. As nitrones are more electrophilic than imines such analog might act as a suicide inhibitor by forming a covalent adduct with proximal nucleophilic groups in the protein. Thus, treatment of the 1,4-iminoribitol **148** with $\text{SeO}_2/\text{H}_2\text{O}_2$ gave nitron e **149**¹⁶⁵ (73%) as a white crystalline solid (Scheme 11). Desilylation and subsequent mesylation yielded **151** (56%). Treatment of the mesylated sugar **151** with protected Hcy afforded a nitron e-type SRH analog **152** (43%). Deprotection of **152** with TFA produced unstable homocysteine derivative **153** (40%).

3.1.5. Imine analog

To explore possibility of the LuxS-mediated addition of water across carbon-nitrogen double bond an imine-type analog of SRH **158** (Scheme 12) was targeted. It is noteworthy that addition of water to imine **158** might result in the *in situ* generation of the hemiaminal of type **143**. Thus,

the precursor, 1-methyl iminocyclitol **154** was prepared by the Moriarty rearrangement¹⁶⁶ of L-lyxo sugar to give the D-ribozasugar.

Reagents and conditions (a) $\text{H}_2/\text{Pd-C}/\text{EtOH}/\text{rt}$; (b) $\text{SeO}_2/\text{H}_2\text{O}_2/\text{Me}_2\text{CO}/-4\text{ }^\circ\text{C}$; (c) $\text{TBAF}/\text{THF}/-4\text{ }^\circ\text{C}$; (d) $\text{MsCl}/\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2/-4\text{ }^\circ\text{C}$; (e) $\text{BocNHCH}(\text{CH}_2\text{CH}_2\text{SH})\text{CO}_2t\text{-Bu}/\text{LDA}/\text{DMF}/-20\text{ }^\circ\text{C}$; (f) (i) $\text{TFA}/-4\text{ }^\circ\text{C}$; (ii) $\text{TFA}/\text{H}_2\text{O}/-4\text{ }^\circ\text{C}$.

Scheme 11. Synthesis of nitrone analog of 4-aza-SRH

The imine **154**¹⁶⁶ was converted into 5-*O*-mesyl derivative **155** (85%), which was coupled with protected L-Hcy to give **156** (Scheme 12, 85%). Treatment of **156** with TFA gave isopropylidene protected **157**. The protons at the C1-methyl group were found to be exchangeable with deuterium within a few hours when compound **157** was dissolved in D_2O . Treatment of **157** with aqueous TFA (9:1) yielded fully deprotected **158** in quantitative yield. As in the case of **157**, protons at C1-methyl group of **158** were exchangeable with deuterium. These exchange indicate that 1,4-ketimine-SRH analog **158** might undergo enzyme-catalyzed hydrolysis to generate a 4-aza-SRH analog with a methyl ketone rather than an aldehyde at C1. This change might affect the regioselectivity and rate of the first isomerization step in the LuxS-catalyzed reaction. I also

showed that the methyl group protons in **154** are not susceptible to exchange even if **154** was dissolved in D₂O for several hours.

Scheme 12. Synthesis of imine analog of 4-aza-SRH

3.1.6. γ -lactams with alkylthiomethyl substitution at carbon γ and their *N,O*-acetal counterparts

As part of the second objective of my dissertation, I have designed and synthesized γ -lactams and cyclic azahemiacetals, bearing alkylthiomethyl substituent with different length of carbon chain (C₃-C₁₂) as AHL mediated QS inhibitors. Although, previously ϵ -lactam (caprolactam) analogs were reported to lack LuxR binding,⁷⁶ studies on γ -lactams were not performed. In order to further investigate the effects of non-native lactam scaffold on QS, novel γ -lactam ligands were designed. As lactam rings are more stable to basic conditions than lactones, these nonnative lactam analogs might prove to be potent inhibitors. Moreover, the γ -lactam and cyclic azahemiacetal ligands were further modified in a way that they resemble SRH.

Initially, 5-*S*-alkylthio derivatives of lactam **160** differing in alkyl chain length were prepared (Scheme 13). The key substrate (*S*)-5-(bromomethyl)-2-pyrrolidone (**159**) was conveniently derived from L-pyrroglutamic acid¹⁶⁷ (Scheme 13). Displacement of bromide in **159** with sodium

propanethiolate produced γ -lactam with propylthiomethyl substitution at carbon γ (**160a**, 96%). As it was demonstrated previously that the length of side chain is also critical for determining the agonistic and antagonistic activity,¹⁶⁸ I also synthesized analogs containing various chain lengths. Higher homologated analogs **160b-d** with C6, C9 and C12 alkyl straight chains were prepared analogously to **160a**. A set of reduced form of lactam analogs which contain a hydroxyl group instead of a keto group at C2 were also prepared. Attempted reduction of lactams **160** with LiEt_3H were unsuccessful, however reduction of the *N*-Boc protected lactams **161a-d** proceeded smoothly to afford hemiaminals (*N,O*-acetals) **162a-d**. Subsequent deprotection with trifluoroacetic acid gave *N,O*-acetal counterparts of γ -lactams (**163a-d**) as a mixture of at least four isomers along with the open chain aldehyde form (25%; ^1H NMR). The existence of hemiaminal moiety is proved by treatment of this mixture with *O*-benzylhydroxylamine which produced the desired oxime derivative.

Reagents: (a) $\text{R'SH}/\text{NaH}/\text{DMF}$; (b) $(\text{Boc})_2\text{O}/\text{DMAP}/\text{CH}_2\text{Cl}_2$; (c) $\text{LiEt}_3\text{BH}/\text{THF}/\text{CH}_2\text{Cl}_2$; (d) TFA

Scheme 13. Synthesis of γ -lactams and the corresponding hemiaminal analogs with thioalkyl side chain

3.1.7. (5S)-(hexyl- or nonylthiomethyl)-3,4-dihydroxypyrrolidin-2-ones and their hemiaminal congeners

To increase the polarity/solubility of the lactam and azahemiacetal analogs in the testing media, aza analogs with hydroxyl groups at C3 and C4 were also prepared. The *N*-Boc protected 1,4-dideoxy-1,4-imino-D-ribitol **126**, readily prepared from the commercially available D-gulonic- γ -lactone, served as a convenient starting material for the synthesis of dihydroxy lactams

164/165 (Scheme 14).^{159,162,169} Mesylation of compound **126** with MsCl and triethylamine gave **127**. Coupling of the mesylate **127** with sodium hexane- and nonanethiolate produced 5-*S*-alkyl thioethers **164** and **165** in high yields which were deprotected with TFA to give (5*S*)-(hexyl- or nonylthiomethyl)-3,4-dihydropyrrolidin-2-ones **166** and **167** (Scheme 14). Reduction of **164** and **165** with LiBEt₃H afforded hemiaminals **168** and **169** and subsequent deprotection with TFA afforded 5-*S*-(hexyl or nonyl)-3,4-dihydropyrrolidin-2-ols **170** and **171** as a complex mixture of azahemiacetals existing in equilibrium with dehydrated form (imine) as well as with open aldehyde and dimeric forms, as reported for such class of 4-azaribofuranoses.¹⁵⁶

3.2. Design and synthesis of *S*-ribosyl-*L*-homocysteine and its C2 modified analogs

3.2.1. *S*-Ribosyl-*L*-homocysteine

The SRH, required for our enzymatic study, was synthesized according to the methods described previously with few modifications that made the synthesis more efficient and bench friendly.¹⁷⁰ First of all, I have used ribose sugar derivative (required for coupling to homocysteine) with bromide as a leaving group instead of the activated hydroxyl. Secondly homocysteine thiolate was generated by using our previously published method¹⁰⁹ employing triscarboxyethylphosphine hydrochloride (TCEP) as water soluble reducing agent of disulfide bond rather than *in situ* generated homocysteine thiolate by Mitsunobu reaction between sugar and homocysteine.¹⁷⁰ Thus, bromo ribose **172**¹⁷¹ upon treatment with protected Hcy afforded **173** in 72% yield (Scheme 15). Subsequent standard deprotection yielded natural substrate SRH **174** in 50% yield.

Reagents: (a) MsCl/NEt₃/CH₂Cl₂/rt; (b) NaIO₄/hydrated RuO₂/EtOAc/H₂O/rt; (c) RSH/NaH/DMF; (d) TFA/H₂O; (e) LiEt₃BH/THF/CH₂Cl₂

Scheme 14. Synthesis of ribolactams and their hemiaminal congeners

Reagents and conditions (a) BocNHCH(CH₂CH₂SH)CO₂*t*-Bu/LDA/DMF/rt; (b) (i) TFA/0 °C (ii) TFA/H₂O/0 °C.

Scheme 15. Synthesis of *S*-ribosyl-L-homocysteine

3.2.2. 2-Deoxy *S*-ribosyl-*L*-homocysteine

The 2-deoxySRH **180** (Scheme 16), because of the lack of hydroxylic group at the second position, should not undergo the first aldose-ketose isomerization step and hence block 2-keto intermediate formation and DPD release. Thus, the 2-deoxyribose was converted to methyl 2-deoxy- α/β -D-*erythro*-pentofuranoside, **175** with methanol and H₂SO₄.¹⁷² Treatment of **175** with MsCl and NEt₃ gave compound **176** (43%) which upon protection with the benzoyl group at C3 afforded **177** (95%) (Scheme 16). Coupling of mesylated derivative **177** with protected Hcy gave 2-deoxySRH derivative **178**. However, attempted removal of of Boc protection under acidic conditions failed to produce the desired 2-deoxySRH. Nonetheless, the methyl 2-deoxySRH analog, **179** (37%) was obtained by coupling of the mesylated sugar **175** with racemic D/L-homocysteine in aqueous NaOH.¹⁰⁹

Reagents and conditions (a) MsCl/Et₃N/CH₂Cl₂/ 0 °C; (b) BzCl,/2,6-lutidine/DMAP/CH₂Cl₂/ 35 °C; (c) BocNHCH(CH₂CH₂SH)CO₂*t*-Bu/LDA/DMF/rt; (d) NH₂CH(CH₂CH₂SH)CO₂H/NaOH/H₂O/65 °C (e) TFA/H₂O/0 °C or AcOH, HCl or TMSOTf

Scheme 16. Synthesis of 2-deoxy-*S*-ribosyl-*L*-homocysteine

3.2.3. *S*-Arabinosyl-*L*-homocysteine

I next explored synthesis of *S*-arabinosyl-*L*-homocysteine (SAH) **187** (Scheme 17). As it was found earlier that the xylo analog **73**,¹⁰⁹ with the inversion of stereochemistry at C3 was the most potent of C3 modified sugars (as the proton shuttle is stereospecific), it will be interesting to explore the effect of inversion of stereochemistry at C2 (SAH). Thus, selective mesylation of acetonide protected **181**¹⁷³ gave **182** (33%). Subsequent benzylation of the free hydroxyl at C3 afforded **183** in 63% yield. However, coupling of the mesylated sugar **183** with protected homocysteine was unsuccessful. In another approach, the mesylated sugar **183** was converted to bromo compound **184** (47%) by treatment with NaBr/DMF¹⁷¹ (Scheme 17). Subsequently, bromo sugar **184** was coupled to the thiolate of Hcy to afford protected SAH intermediate **185** in a very low yield. However, such reaction was not reproducible and the targeted SAH **187** was not obtained in desired purity to perform enzymatic study.

3.2.4. 2-Fluoro-2-deoxy-*S*-arabinosyl-*L*-homocysteine

As it is well known that introduction of fluorine atom brings change in physical, chemical and biological properties of a molecule, I next focused on synthesis of [2-F]SAH **194** (Scheme 18). Such a compound can potentially act as competitive inhibitor as it could bind to LuxS but because of the lack of hydroxyl group at C2, should not undergo carbonyl shift from C1 to C2 to form 2-keto intermediate. Alternatively [2-F]SAH might undergo nucleophilic attack by Cys-84 forming a covalent adduct between inhibitor and protein, resulting in irreversible inhibition of the enzyme. Thus, benzylation of the triisopropylsilyl (TIPS) protected 2-deoxy-2-fluoroarabinose **188**¹⁷⁴ at anomeric hydroxyl, produced **189** (77%).

Reagents and conditions (a) $\text{MsCl}/\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2/0^\circ\text{C}$; (b) $\text{BzCl}/2,6\text{-lutidine}/\text{DMAP}/\text{CH}_2\text{Cl}_2/35^\circ\text{C}$; (c) $\text{NaBr}/\text{DMF}/\text{CH}_2\text{Cl}_2/120^\circ\text{C}$; (d) $\text{BocNHCH}(\text{CH}_2\text{CH}_2\text{SH})\text{CO}_2t\text{-Bu}/\text{LDA}/\text{DMF}/\text{rt}$; (e) $\text{TFA}/\text{H}_2\text{O}/0^\circ\text{C}$; (f) NH_3/MeOH

Scheme 17. Synthesis of *S*-arabinosyl-L-homocysteine (SAH)

Desilylation of **189** with NH_4F gave the corresponding 2-deoxy-2-fluoroarabinose **190** (37%) which upon mesylation afforded **191** (65%) (Scheme 18). However, attempted coupling of mesylated sugar **191**, under various conditions failed to produce desired protected 2-fluoro analog of SRH **192**. In another approach, selective acid mediated mono desilylation of **189** with $\text{TFA}/\text{H}_2\text{O}$ and subsequent mesylation afforded 5-*O*-mesyl-3-*O*-TIPS protected mesyl sugar. Unfortunately, attempts to couple this sugar with Hcy were unsuccessful. Therefore, desired [2-F]SAH **194** was not obtained.

Reagents and conditions (a) BzCl/2,6-lutidine/DMAP/CH₂Cl₂/ 35 °C; (b) NH₄F/MeOH/60 °C; (c) MsCl/Et₃N/CH₂Cl₂/0 °C; (d) BocNHCH(CH₂CH₂SH)CO₂t-Bu/LDA/DMF/rt; (e) TFA/H₂O/0 °C; (f) NH₃/MeOH.

Scheme 18. Synthesis of 2-deoxy-2-fluoro-S-arabinosyl-L-homocysteine

3.3. Biological evaluation of targeted quorum sensing modulators

3.3.1. Quorum sensing assays of γ -lactams with alkylthiomethyl substitution at carbon γ and their *N,O*-acetal counterparts

3.3.1.1. Screening against *las* and *rhl* signaling in *P. aeruginosa*

To determine the effect of the γ -lactam (**160**, **166** and **177**) (Figure 26) and their corresponding hemiaminal analogs (**163**, **170** and **171**) (Figure 26) on the *P. aeruginosa las* and *rhl* AHL-mediated pathways, *las*- and *rhl*-dependent β -galactosidase reporters were independently expressed with their respective receptor proteins in *E. coli* as previously described by Dr. Kalai Mathee's research group at FIU.¹⁵ As expected and in agreement with published data, exogenous 3-oxo-C₁₂-AHL and C₄-AHL activated the *las* and *rhl* β -galactosidase reporters, respectively. The AHL treated controls (3-oxo-C₁₂-AHL in case of *las* and C₄-AHL in case of *rhl*)

in absence of inhibitor are considered as 100% active (Figure 26). Also, no activity was observed in absence of exogenous AHL. The effect of the synthesized γ -lactam and their corresponding hemiaminal analogs on the *las* and *rhl* reporter activity was also compared with the effect of addition of the DMSO as a solvent (Figure 26).

Screening against *las* signaling

Compounds **160a**, **163a** were initially screened at a concentration of 100 μ g/mL (0.57 mM) for their activity against the *las* system (Figure 26). Hemiaminal **163a** was found to enhance *las* reporter activity by 15% while lactam **160a** inhibited *las* activity approximately by 28%. Inhibition was dependent upon the addition of 2 μ M of 3-oxo-C₁₂-AHL. However, hemiaminal **163a** significantly stimulated (approximately 2.3-fold) *las* reporter activity at 50 μ g/mL and inhibited *las* reporter activity by 69% and 89% at 150 and 200 μ g/mL, respectively. In contrast, lactam **160a** inhibited *las* activity at all concentrations tested. Cell growth was not inhibited by the addition of lactam and hemiaminal compounds at the tested concentrations.

Among other lactam analogs tested, percent inhibition increased in a concentration dependent manner. Inhibition potency also increased as the alkylthio chain length increased. Specifically, nonylthio lactam **160c** and dodecylthio lactam **160d** were found to possess greatest inhibition at all concentrations tested. At the lowest concentration tested (50 μ g/mL), **160c** inhibited 28% while **160d** inhibited 48%. On the contrary, among the cyclic hemiaminals analogs, no general trend was observed between chain length and percent inhibition. As with **163a**, hemiaminal **163b** also stimulated QS at 50 μ g/mL but with much lesser potency, however inhibited 100% at all higher concentrations tested. Hemiaminals containing nonyl side chain (**163c**) and dodecyl chain (**163d**) showed moderate activity. The ribolactam analogs (**166** and **167**) and their cyclic hemiaminal counterparts (**170** and **171**) were found to inhibit *las* activity at all concentrations tested but only with moderate potency with hemiaminals being slightly more active.

Figure 26. Effect of lactam and hemiaminal analogs on P_{lasI} - $lacZ$ expression in *Escherichia coli*. The compounds were tested at 50 -200 $\mu\text{g/mL}$ concentrations against 2 μM of 3-oxo-C12-AHL. P_{lasI} - $lacZ$ activity is depicted as percent activity relative to the DMSO treated control in presence of AHL. Significance was determined by a paired two-tailed Student t -test and is denoted as follows: * p -value < 0.05, † p -value < 0.02, ‡ p -value < 0.01. The p value < 0.05 correspond to 5% (< 0.02 corresponds to 2% and, < 0.01 corresponds to 1%) chance of rejecting the null hypothesis when it is true. Rejection of null hypothesis infers that the result is statistically significant. Each trial was performed as a triplicate.

Screening against *rhl* signaling

The hexylthio lactam **160b** and nonylthio lactam **160c** stimulated *rhl* QS activities at higher concentrations with moderate potency with hexylthio lactam **160b** being most active (Figure 27; Table 2). Propylthio lactam **160a** and dodecylthio lactam **160d** were inactive at the concentrations tested. In contrast, cyclic hemiaminals with shorter alkylthio chain (**163a** and **163b**) inhibited *rhl* activity, while analogs with longer alkyl chain (**163c** and **163d**) were inactive. The hexylthio hemiaminal **163b** completely inhibited *rhl* signaling at concentrations of 100 $\mu\text{g/mL}$ and higher. The strong inhibition observed with propylthio **163a** and hexylthio **163b** hemiaminal analogs having the side chain lengths similar to C_4 -AHL is in agreement with the structure activity relationship reported for various synthetic AHL mimetics targeting RhlR.^{75,175} Except for ribolactam **166** which stimulated *rhl* signaling, all the ribolactam analogs (**167**) and their cyclic hemiaminal counterparts (**170/171**) were inactive in *rhl* signaling system (Figure 27; Table 2)

Thus, in this dissertation novel lactam and the corresponding hemiaminal analogs as *las* and *rhl* signaling modulators were reported. Studies of structural features other than the AHL scaffold as tools to understand the R type protein interaction with AHLs are limited, only a few examples of inhibitors with the altered lactone ring structure of AHL have been reported (for more discussion on AHL mimics with altered lactone ring, refer to section 1.2.1.3.). For example, Smith *et al.* reported 3-oxo- C_{12} -(2-aminocyclohexanone) (Figure 8, **39**) as a strong antagonist of LasR system,⁸⁷ while Muh *et al.* identified two LasR inhibitors having a phenyl and tetrazole ring (Figure 11, **49** and **50**, respectively), with IC_{50} in nM range (Figure 11).⁹⁶ It is noteworthy that γ -thiolactone analogue of **7b** (Figure 9) showed inhibition of LuxR while the corresponding ϵ -lactam (caprolactam) analog was reported to lack LuxR binding.⁷⁸ The targeted optically pure γ -lactams and cyclic hemiaminals were capable of either inhibiting or, in some cases, inducing *P. aeruginosa* QS pathways. Though, such analogues had moderate antagonism (dodecylthio lactam **160d** had 48% inhibition at 0.16 mM and hexylthio hemiaminal **163b** had 100% inhibition at 0.38

mM) when compared to previously reported inhibitors (with IC₅₀ values in submicromolar), the novel lactam scaffold might serve as stable alternative to lactone present in AHL and further facilitate future generation of AHL mimics.

Figure 27. Effect of lactam and cyclic hemiaminal analogs on P_{rhlA} - $lacZ$ expression in *E. coli*. The compounds were tested at 50-200 µg/mL concentrations against 2 µM of C₄-AHL. P_{rhlA} - $lacZ$ activity is depicted as percent activity relative to the DMSO treated control in presence of AHL. Significance was determined by a paired two-tailed Student *t*-test and is denoted as follows: *p-value < 0.05, †p-value < 0.02, ‡p-value < 0.01. The p-value < 0.05 corresponds to 5%, (< 0.02 corresponds to 2% and < 0.01 corresponds to 1%) chance of rejecting the null hypothesis when it is true. Rejection of null hypothesis infers that the result is statistically significant.

Table 2. Effect of lactam and cyclic hemiaminal analogs on *rhl* signaling

Concentration ($\mu\text{g}/\text{mL}$)	50	100	150	200
Compound	Percent Activity ^{a,b} $\pm$ Standard Deviation			
No inhibitor	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
No AHL	0.29 $\pm$ 0.4 ^c			
<i>C.viminalis</i>	33 $\pm$ 6 ^d			
160a	104 $\pm$ 24	118 $\pm$ 0.8	104 $\pm$ 13	108 $\pm$ 17
160b	126 $\pm$ 8	126 $\pm$ 29	130 $\pm$ 31	145 $\pm$ 19 [‡]
160c	95 $\pm$ 3	115 $\pm$ 30	108 $\pm$ 12	137 $\pm$ 46
160d	103 $\pm$ 7	106 $\pm$ 6	109 $\pm$ 8	106 $\pm$ 5
163a	121 $\pm$ 6	138 $\pm$ 3	41 $\pm$ 1 [†]	9 $\pm$ 1 [†]
163b	26 $\pm$ 0.2	0.2 $\pm$ 0.3 [†]	0 $\pm$ 0 [†]	0 $\pm$ 0 [*]
163c	93 $\pm$ 12	80 $\pm$ 13	100 $\pm$ 11	99 $\pm$ 3
163d	98 $\pm$ 13	93 $\pm$ 27	91 $\pm$ 8	96 $\pm$ 5
166	132 $\pm$ 36	129 $\pm$ 10	148 $\pm$ 9	161 $\pm$ 7 [†]
167	106 $\pm$ 3	117 $\pm$ 8 [†]	115 $\pm$ 11	135 $\pm$ 5 [†]
170	94 $\pm$ 8	105 $\pm$ 3	109 $\pm$ 12	116 $\pm$ 18
171	98 $\pm$ 5	127 $\pm$ 14 [*]	116 $\pm$ 9	121 $\pm$ 14

^a Effect of lactam and cyclic hemiaminal derivatives on P_{rhlA} -*lacZ* expression in *E. coli* tested at 50-200 $\mu\text{g}/\text{mL}$ concentrations against 2 μM of C_4 -AHL. P_{rhlA} -*lacZ* activity is depicted as percent activity relative to the DMSO treated control in presence of AHL. ^b *p-value < 0.05, †p-value < 0.02, ‡p-value < 0.01 according to a paired, two-tailed Student *t*-test. ^c Activity in absence of AHL was considered negligible. ^d 20 μL water extract of *Callistemon.viminalis* was shown to have anti QS activity as expected and reported previously.¹⁹¹

3.3.1.2. Screening against bioluminescence in *V. harveyi*

The lactam (**160**, **166** and **167**) and hemiaminal derivatives (**163**, **170** and **171**) were also tested for their effect on QS mediated luminescence of wild type *V. harveyi* MAV. None of the analogs had any effect on growth (Table 3). In case of luminescence assay, γ -lactams and their hemiaminal counterparts displayed two patterns showing (i) inhibition of luminescence as found for lactam compounds **160b**, **166** and **167** and hemiaminals **163b**, **163c** and **170**), and (ii) no effect at the concentrations tested, as observed with propylthio γ -lactam **160a** as well as with **160d**, **163c**, **163d** and nonylthio ribohemiaminal **171** (Table 3). In order to identify the pathway

of inhibition of luminescence (as three QS systems operate in *Vibrio harveyi*), the synthetic ligands were also tested against *V. harveyi* strains BB170 and BB152 lacking AI-1 (AHL) sensor. Such tests revealed similar luminescence data as found for wild type. The analogs which showed bioluminescence inhibition (**160b**, **160c**, **163b**, **166**, **167** and **170**) when tested against light producing enzyme, luciferase displayed identical trend in reduction of bioluminescence (personal communication of Dr. Makemson at FIU). In conclusion, luminescence and luciferase assays revealed that the inhibition of luminescence was not mediated by QS but by luciferase.

Table 3. Effect γ -lactam and hemiaminal analogs on *V. harveyi* MAV bioluminescence and growth

Aza analogs	% Inhibition Luminescence	% Inhibition of Growth	Conc X 10 ⁻⁴ M
160a	0	0	3.5
160b	99.8	0	3.5
160c	64	0	3.1
160d	0	0	2.7
163a	75	0	2.2
163b	99.7	0	1.2
163c	0	0	3.1
163d	0	0	2.7
166	29	0	3.2
167	80	0	2.8
170	52	0	3.2
171	0	0	2.7

^aCompounds dissolved in water or DMSO served as a control and % inhibition is calculated relative to the solvent control. Bioluminescence was recorded in light units (1 LU = 1.08 x 10⁸ q/s/mL) and all measurements were obtained in a single trial.

3.3.2. Inhibition studies of 4-aza-SRH analogs against LuxS enzyme

Bacillus subtilis LuxS preparation and preliminary inhibition studies of selected 4-aza-SRH analogs against LuxS_{Bs} were performed by Dr. Pei's research group at Ohio State University using published protocols.^{54,58} The LuxS activity assay was based on quantitative detection of the

liberated homocysteine released from the natural substrate SRH which is readily detected by Ellman's reagent (DTNB).¹⁷⁶ The DTNB reacts with free thiol (homocysteine) rapidly and quantitatively to form a chromophore, 5-thionitrobenzoic acid (TNB) whose absorbance can be measured at 412 nm ($\epsilon_{412} = 14,150 \text{ M}^{-1}\text{cm}^{-1}$) (Scheme 19). From activity assay, LuxS was found to show saturation kinetics toward SRH with a K_M value of 1.05 μM and a V_{max} value of $1.8 \times 10^{-4} \mu\text{M}/\text{min}$ (fitted into Michaelis-Menten equation $V = (V_{\text{max}}[\text{S}]/(K_M + [\text{S}]))$ (Table 4; Figure 28).

Table 4. Rate of LuxS catalysis at various concentrations of natural substrate

[SRH] μM	dA/min (rate)
44.5	0.0185
26.7	0.0178
8.9	0.0153
4.4	0.0141
2.2	0.0126

Inhibition of 4-Aza-SRH analogs was determined in a manner similar to the activity assay except for addition of various concentrations of inhibitors to the reaction mixture besides DTNB, SRH and LuxS_{Bs}. Rate of the reaction and K_M values in presence of aza analogs were calculated and mechanism of inhibition was determined. Percentage activity of LuxS in presence of inhibitors was reported relative to the percentage activity of SRH alone (i.e., without inhibitor activity percentage was considered 100%). From the 4-aza-SRH derivatives tested, pyrrolidine analog **129**, lactam analog **140**, and propylthio γ -lactam analog **160a** were found to act as competitive inhibitors of moderate potency.

Scheme 19. LuxS activity assay for detection of the released homocysteine employing DTNB

Figure 28. Lineweaver-Burk plot of Co-LuxS enzyme with SRH (data were fitted into the Michaelis-Menten equation $V = V_{\text{max}}[S]/(K_M + [S])$)

Because of the absence of the hydroxylic group at C1, 1-deoxy-4-aza-SRH **129** (Figure 30) should not undergo enzyme mediated ring opening which is the very first step in the LuxS-mediated catalysis, but might still be able to bind to LuxS (because of the ability to bind at homocysteine pocket and chelate to the metal ion via C2 and C3 hydroxyls) hence act as competitive inhibitor. Supporting our hypothesis, pyrrolidine analog **129** acted as a competitive inhibitor with K_I value of 49 μM and IC_{50} value of 0.8 mM (Figure 29). Weak inhibition of **129** was probably the result of loss of the binding energy derived from C1-hydroxyl. This hydroxylic group was originally shown to interact with several active site residues (Ser-6, His-11 and Arg-39) *via* hydrogen bonding in the co-crystal structure of C84A-LuxS_{Bs} with 2-ketone intermediate.⁴⁹ Because of the positively charged ring nitrogen ($\sim\text{p}K_a = 6.6$), the conformational change in sugar ring might have also resulted in improper fit into the active site.

Figure 29. Dose response curve of pyrrolidine analog **129** against LuxS enzyme

Figure 30. Activity of the selected 4-aza-SRH analogs against LuxS enzyme

Lactam-type SRH analog **141** (Figure 30) with the amide oxygen at C1 was tested next as LuxS inhibitor. Apart from its inability to open the sugar ring, the lactam ring was chosen mainly because the planar amide group resembles the carbonyl group existing in aldose form of SRH or the C2 keto group present in 2-keto intermediate. Secondly the carbonyl of lactam might also form new interactions at the active site *via* hydrogen bonding. Thirdly, the carboxamide moiety

has the potential to tautomerize to a lactim form so as to generate a hydroxylic group at C1 which mimics the natural substrate.^{177,178} Lactam **141** was found to be a competitive inhibitor with K_i value of 37 μM and IC_{50} value of 0.66 mM (Figure 31; Table 5). As expected, the corresponding lactam analog **140** with benzylic group at the ring nitrogen was found to be inactive. This may be the result of the steric bulkiness caused by benzylic group preventing access to LuxS active site.

Table 5. Rate and percentage activity of LuxS at different concentrations of analogs **129** and **141**

Compound	Concentration (μM)	dA/min (rate)	% Activity
129	0	0.01012	100
129	200	0.00865	85.5
129	400	0.00659	65.1
129	800	0.00500	49.4
129	1600	0.00392	38.7
129	3200	0.00261	25.8
141	0	0.00936	100
141	80	0.00833	89
141	160	0.00728	78
141	320	0.00578	62
141	655	0.00462	49
141	1310	0.00306	33

Figure 31. Dose response curve of lactam analog **141** against LuxS enzyme

As the lactam analog **141** was found to inhibit LuxS, further modification of the lactam analog by removing hydroxylic groups at C2 and C3 was performed to provide 2,3-dideoxy lactam analog of SRH (e.g., **195**; Figure 30).¹⁷⁹ Lactam **195** was found to be inactive. The lack of the vicinal hydroxyl groups might have totally eradicated LuxS binding ability, probably because of the large difference in polarity and absence of metal chelation. The hemiaminal analog **196** (Figure 30) was also devoid of activity confirming the significance of C2 and C3 hydroxylic groups for LuxS binding. These findings are consistent with the structural requirements for LuxS binding and activity as reported in literature.⁵⁸

Hemiaminal analog of 4-aza-SRH **143** (Figure 30) was tested to study the effect of *N,O*-acetal function vs *O,O*-acetal present in natural substrate on the LuxS catalyzed reaction. As only open aldehyde form of SRH is catalytically active, ring opening of SRH should serve as an important step to study catalysis of LuxS enzyme. The LuxS was reported to bind to cyclic ribofuranose form of SRH (*O,O*-hemiacetal) and catalyze ring opening. I envisaged that because of the intrinsic difference in leaving group property of amine when compared to hydroxylic group (existing in natural substrate), the hemiaminal analog **143** can interfere with enzyme mediated ring opening and therefore act as LuxS inhibitor. In case of natural substrate SRH, (*O,O*-hemiacetal), the enzyme catalyzes protonation of the ring oxygen, leading to the sugar ring opening. On the other hand, as the pK_a of hemiaminal **143** is around 5.8¹⁸⁰ at physiological pH, the enzyme mediated protonation of **143** is speculated to occur preferentially at anomeric hydroxyl as opposed to ring nitrogen, resulting in loss of water to give imine **197**. The imine intermediate **197** might act as suicide inhibitor (path (a) in Scheme 20) by forming a covalent adduct with proximal nucleophilic groups (for example Cys84). Alternatively it can also tautomerize to give 2-keto-4-aza-SRH **200** (path (b) in Scheme 20) *via* formation of enamine **199** (Amadori rearrangement).¹⁵⁸ The keto byproduct **200** might also serve as competitive inhibitor because of its structural resemblance to the 2-keto SRH intermediate. The LuxS-mediated *N,O*-

acetal ring opening leading to the open chain aldamine and ultimately to the formation of aza analog of DPD **201** ((S)-1,2-dihydroxy-4-iminopentan-3-one) has to be also considered (path (c) in Scheme 20). The acyclic imine analog of DPD **201** may cyclize to give furan derivative **203a** (aza analog of pro-AI-2). The imine analog of pro-AI-2 might also tautomerize to give enamine **202** or can hydrolyze to provide DPD. Because of the electron withdrawing nature of α -substituents, it is speculated that the imine tautomer is less favorable when compared to enamine.¹⁸¹ A time-dependent inhibition of this *N,O*-acetal analog with IC₅₀ value of 60 μ M was observed with 4-aza-SRH **143** (Figure 32; Table 6). Chemical equilibration among hemiaminal, open aldehyde, imine and enamine forms of **143** might be responsible for time-dependent inhibition. The complex kinetics of this analog precluded determination of K_i value. Further work is necessary to determine the exact mechanism of inhibition and to support the formation of the aza DPD analog as well as the detection of enzyme substrate complex.

Figure 32. Dose response curve of hemiaminal analog **143** against LuxS enzyme

Table 6. Rate and percentage activity of LuxS at different concentrations of analogs **143** and **160a**

Compound	Concentration (μM)	dA/min (rate)	% Activity
143	0	0.01028	100
143	20	0.00917	89
143	40	0.00777	76
143	50	0.00665	65
143	60	0.00548	53
143	80	0.00395	38
143	100	0.00322	31
143	200	0.00196	19
160a	0	0.00967	100
160a	100	0.00935	97
160a	200	0.00896	93
160a	400	0.00906	94
160a	800	0.00862	89
160a	1600	0.00830	86
160a	3200	0.00720	74
160a	6069	0.00638	66
160a	12138	0.00457	47

In order to further investigate the effect of imine group on the inhibition of LuxS, I also synthesized 1-methyl imino SRH analog **158**. Imino sugars with endocyclic C=N structural motif were described to act as potential suicide inhibitors because of the strong electrophilic nature of imine bond.¹⁸⁶ Three plausible ways of interaction of this analog with LuxS are speculated. Firstly, nucleophilic residues at the active site might covalently bond to imine carbon, resulting in irreversible inhibition (pathway (a) in Scheme 21). Secondly, imine analog **158** can also tautomerize to give methyl 2-keto-4-aza-SRH **208** analogous to path (b) described for hemiaminal **143** in Scheme 20. As a third plausible pathway, **158** can transform *in situ* into the ketohemiaminal **209** by nucleophilic attack of the enzyme bound water ($pK_a = 10$) across imine bond. Ring opening of the hemiaminal should generate ketone **208** which might be coordinated by Co^{+2} and converted to an aza analog of DPD **211** with an additional methyl group at C1 (path

(c) in Scheme 20). The imine **209** might also tautomerize to enamine **212** or cyclize to give furan derivative **213**. Unfortunately, inhibition assay determined no activity for the imine **158**. This may be because of its misfit into the polar active site of LuxS owing to hydrophobic CH₃ at C1.

Scheme 21. Plausible mechanism of interaction of cyclic imine analog of SRH **158** with LuxS

In order to find structural requirements for inhibition of both intra species (AHL mediated) and inter species QS (AI-2 mediated), the propylthio γ -lactam analog **160a** and its hemiaminal counterpart **163a**, which were earlier found to be AHL mediated QS inhibitors in *P. aeruginosa* (Section 3.4.1.2), were tested for LuxS inhibition as well. The propylthio-lactam analog **160a** displayed very weak inhibition with K_i value of 0.71 mM and IC_{50} value of 11 mM (Figure 33). The reduced hemiaminal counterpart **163a** was devoid of inhibition (Table 6). These data were consistent with the LuxS inhibition results found for 2,3-dideoxy SRH analogs, **195** and **196** which were also inactive. Depletion of inhibition might be because of the loss of binding energy derived from: (i) binding of C1-hydroxylic group at the active site, (in case of lactam analog), (ii) homocysteine moiety, and (iii) metal ligation to hydroxylic groups owing to missing C1, C2 and C3 hydroxylic groups and homocysteine. However, no common structural motif was found as propylthio compounds were inactive, probably owing to the large intrinsic differences in the active sites of LasR and LuxS.

Figure 33. Dose response curve of propylthio lactam analog **160a** against LuxS enzyme

The LuxS inhibition studies with 4-aza-SRH analogs were also attempted in our laboratory with histidine-tagged LuxS protein. *E. coli* BL21(DE3)/pLuxS strain was kindly provided by Dr. Zhou from Northeastern University. The protein was isolated and purified by Dr. Wang's

research group at FIU by following published methods.⁴⁷ Such purified HTCo-LuxS_{Bs} protein showed characteristics identical to the properties described in the literature.⁵⁰ For example, presence of purple color and UV-VIS absorption bands at 630, 580 and 530 nm.⁵⁰ Moreover cobalt incorporation into apo-enzyme was confirmed by determination of cobalt concentration (12.87 mM) using ICP-MS analysis in the laboratory of Dr.Cai at FIU. However, testing results of this protein are still not conclusive. The inhibition studies with 2-deoxy analog **179** and nitrone analog **153** will be investigated in future.

3.3.3. Complexation studies with aza-DPD

With ¹¹B NMR

Since 4-aza-SRH **143** was proposed to release aza analog of DPD **201** upon LuxS mediated catalysis (pathway (c), Scheme 20), an additional model study was performed to investigate the possibility of formation of aza-DPD analog **201** and its plausible cyclisation and binding with borate to form aza-AI-2, **203c** or diol complex **203d** (Scheme 22). The differential affinity of borate binding towards amino alcohol (as in pro-aza-AI-2 **203b**) vs *cis*-diols (as in pro-AI-2, *S*-THMF, Scheme 2) was measured employing ¹¹Boron NMR binding studies. The aminoethanol **205**, *cis*-2-aminocyclopentanol and ethanediol **204** in borate solution were used as model compounds, to simulate the complexation of **203b**. Treatment of either 2-aminoethanol or *cis*-2-aminocyclopentanol with saturated borate solution in D₂O at pH 7.3 or in benzene solution showed broad peaks on ¹¹B NMR spectra which were inconclusive of complexation as reported for the related *vicinal* aminoalcohols with borate.¹⁸² On the contrary, ¹¹B NMR data for D- ribose was consistent with literature values for 2:1 and 1:1 sugar/borate complexes (Figure 34).¹⁸³ Taylor *et al.* showed that NH₂C(CH₂OH)₃ has less tendency to form borate complexes than the corresponding triols with alkyl substituents (instead of amino group), although mono-chelate and bis-chelate borate complexes with vicinal hydroxyl groups were formed.¹⁸⁵

Scheme 22. Borate binding study of ethanediol and aminoethanol

Figure 34. ^{11}B NMR spectrum of D-ribose in saturated borate solution in D_2O at pH 7.8. Peaks at 18.92 ppm (C), 12.92 ppm (D) and 1.01 (E) ppm correspond to borates $\text{B}(\text{OH})_4^-$, $\text{B}_3\text{O}_3(\text{OH})_4^-$ and $\text{B}_5\text{O}_6(\text{OH})_4^-$, respectively.¹⁸⁴

With DFT calculations

Energy minimization calculations [density functional theory (DFT)] were performed between 2,3-borate ester (AI-2) and 3,4-borate ester of the cyclic DPD by Dr.Mebel from FIU (Figure 35). The relative energy of 2,3-diester (AI-2) was found to be lower than that of the corresponding 3,4-diester. However, in case of aza analogues, DFT calculations between aza-AI-2 **203c** and 3,4-borate ester **203d** showed that complex **203** had lower energy than aminoalcohol complex **203c**. Such results indicate once more the preferential affinity of boron for diols as compared to aminoalcohols.

Figure 35. DFT calculations for borate esters of **203b** (Figure 35A) and *S*-THMF (Figure 35B)

4. EXPERIMENTAL PROCEDURES

4.1. General procedures

The ^1H (400 or 600 MHz) and ^{13}C (100 MHz) spectra were determined with solutions in CDCl_3 unless otherwise noted. The ^{11}B (192.6 MHz) NMR spectra were recorded in D_2O . Mass spectra (MS) were obtained with atmospheric pressure chemical ionization (APCI) or ESI technique and HRMS in AP-ESI or TOF-ESI mode. The TLC was performed with Merck kieselgel 60-F₂₅₄ sheets products were detected with 254 nm light or by visualization with $\text{Ce}(\text{SO}_4)_2/(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}/\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ reagent. Merck kieselgel 60 (230-400 mesh) was used for column chromatography. The HPLC purifications were performed using XTerra® preparative RP₁₈ OBD™ column (5 μm 19 x 150 mm) with gradient program using $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ as a mobile phase. Reagent grade chemicals were used, and solvents were dried by reflux over and distillation from CaH_2 (except THF/potassium) under argon. Using SPSS analytical software two-tailed Student *t*-test was performed and *p* values were generated.

The aza sugars synthesized in this dissertation are treated as SRH analogs throughout the manuscript and therefore numbering of carbons starts at *anomeric* position. However, in the experimental section, the numbering of carbons for aza sugars either follows sugar nomenclature or heterocyclic nomenclature depending on the nature of the sugar (Figure 36).

Figure 36. Two systems of nomenclatures used in the dissertation

4.2. Synthesis of the inhibitors

***N*-Benzyl-5-*O*-*tert*-butyldimethylsilyl-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-D-ribose (124).** To a stirred solution of **123**¹⁵⁹ (150 mg, 0.57 mmol) in anhydrous CH₂Cl₂ (5 mL) at rt under Ar atmosphere was added DMAP (7 mg, 0.05 mmol) and imidazole (93 mg, 1.36 mmol) followed by TBDMSCl (0.68 mmol, 103 mg). The mixture was then stirred for 10 h and partitioned (CH₂Cl₂//NaHCO₃/H₂O). The organic layer was washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (15% EtOAc/hexane) to give **124**¹⁸⁷ (204 mg, 98%) as a colorless oil. ¹H NMR δ -0.13 (s, 3, CH₃), 0.00 (s, 3, CH₃), 0.83 (s, 9, *t*-Bu), 1.26 (s, 3, CH₃), 1.49 (s, 3, CH₃), 2.65 (dd, *J* = 2.7, 10.3 Hz, 1, H1), 2.94 ('q', *J* = 2.2 Hz, 1, H4), 3.04 (dd, *J* = 5.5, 10.3 Hz, 1, H1'), 3.57 (dd, *J* = 4.1, 10.6 Hz, 1, H5), 3.64 (d, *J* = 13.4 Hz, 1, Bn), 3.71 (dd, *J* = 10.6, 4.3 Hz, 1, H5'), 3.94 (d, *J* = 13.4 Hz, 1, Bn), 4.49 (dd, *J* = 2.0, 6.5 Hz, 1, H3), 4.58 ('dt', *J* = 2.7, 6.2 Hz, 1, H2), 7.13-7.23 (m, 5, Bn); ¹³C NMR δ -5.50, (CH₃), -5.43, (CH₃), 18.16 (*t*-Bu), 25.20 (CMe₂), 25.72 (CH₃), 25.91 (CH₃), 27.20 (CMe₂), 56.91 (Bn), 59.28 (C1), 63.15 (C5), 68.93 (C4), 79.44 (C2), 83.33 (C3), 111.86 (CMe₂), 128.22, 128.47, 126.83, 139.41 (Bn); MS (APCI) *m/z* 378 (100, MH⁺).

***N*-*tert*-Butoxycarbonyl-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-5-*O*-*tert*-butyldimethylsilyl-D-ribose (125).** A solution of **124** (145 mg, 0.38 mmol), triethylamine (76.7 mg, 0.106 mL, 0.76 mmol), di-*tert*-butyldicarbonate (126 mg, 0.57 mmol) and 5% Pd/C (300 mg) in ethanol (6 mL) was stirred under an atmosphere of hydrogen at room temperature for 6 h. The reaction mixture was filtered through Celite to remove the catalyst. The Celite was washed with ethanol (5 mL) and washings and the filtrate were combined and evaporated. The residue was partitioned (EtOAc//NaHCO₃/H₂O). The organic layer was washed (brine), dried (MgSO₄) and evaporated. The crude product was column chromatographed (20 → 30% EtOAc/hexane) to give **125** (147 mg, 99%) with data as reported.¹⁶⁴

***N*-tert-Butoxycarbonyl-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-D-ribitol (126).**

TBAF (1 M/THF, 0.25 mL, 0.25 mmol) was added to a stirred solution of **125** (66 mg, 0.17 mmol) in THF (5 mL) at ambient temperature. After stirring for 30 min, the reaction mixture was partitioned (EtOAc//NaHCO₃/H₂O). The organic layer was washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (50→ 60% EtOAc/hexane) to give **126** (32 mg, 70%) with data as reported.¹⁶⁹

***N*-tert-Butoxycarbonyl-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-5-*O*-**

methanesulfonyl-D-ribitol (127). Procedure A. Triethylamine (66 mg, 92 μ L, 0.66 mmol) and MsCl (37.6 mg, 25 μ L, 0.33 mmol) were added drop wise to stirred solution of **126** (60 mg, 0.22 mmole) in anhydrous CH₂Cl₂ (6 mL) at 0°C (ice-bath). After 5 min, ice-bath was removed and the reaction mixture was allowed to stir at ambient temperature for 30 min. The reaction mixture was quenched with saturated NaHCO₃/H₂O and was extracted with CH₂Cl₂. The organic layer was washed (brine), dried (MgSO₄) and evaporated to give **127** (73 mg, 96%) as a mixture (~3:2) of two rotamers of sufficient purity to be directly used in next step: ¹H NMR δ 1.28 (s, 3, CH₃), 1.42 (s, 12H, *t*-Bu, CH₃), 2.96 (s, 1.2, Ms), 2.98 (s, 1.8, Ms), 3.39 (dd, *J* = 4.8, 12.5 Hz, 0.4, H1), 3.46 (dd, *J* = 4.8, 12.5 Hz, 0.6, H1), 3.69 (d, *J* = 12.5 Hz, 0.6, H1'), 3.82 (d, *J* = 12.5 Hz, 0.4, H1'), 4.10-4.14 (m, 0.4, H4), 4.22-4.30 (m, 0.6, H4), 4.22-4.29 (m, 1.4, H5,5'), 4.45 (dd, *J* = 4.1, 10.1 Hz, 0.6, H5), 4.65 ('d', *J* = 5.9 Hz, 1, H3); 4.72 ('t', *J* = 5.3 Hz, 1, H2); ¹³C NMR (major rotamer) δ 24.93 (*CMe*₂), 26.93 (*CMe*₂), 29.59 (*t*-Bu), 37.09 (Ms), 52.51 (C1), 62.39 (C4), 68.94 (C5), 79.20 (C2), 80.43 (*t*-Bu), 81.68 (C3), 112.07 (*CMe*₂), 154.24 (CO); ¹³C NMR (minor rotamer) δ 24.93 (*CMe*₂), 26.93 (*CMe*₂), 29.59 (*t*-Bu), 37.48 (Ms), 53.11 (C1), 62.64 (C4), 68.59 (C5), 78.54 (C2), 80.64 (*t*-Bu), 82.53 (C3), 112.07 (*CMe*₂), 153.63 (CO); MS (APCI) *m/z* 252 [100, (MH₂-Boc)⁺], 352 (10, MH⁺)

***S*-(*N*-tert-Butoxycarbonyl-1,4-imino-2,3-*O*-isopropylidene-1,4,5-trideoxy-D-ribitol-5-yl)-**

***N*-tert-butoxycarbonyl-L-homocysteine *tert*-butyl ester (128). Procedure B. Step a.** H₂O (0.4

mL) and tris(2-carboxyethyl)phosphine hydrochloride (140 mg, 0.5 mmol) were added to a stirred solution of *N,N'*-di(*tert*-butoxycarbonyl)-L-homocystine di(*tert*-butyl) ester⁵⁴ (250 mg, 0.4 mmol) in anhydrous DMF (4 mL) at ambient temperature under Ar atmosphere. After 24 h, the reaction mixture [TLC (EtOAc/hexane, 2:8) showed conversion of disulfide (*R_f* 0.55) into thiol (*R_f* 0.65)] was partitioned between EtOAc, and saturated NaHCO₃/H₂O. Aqueous layer was extracted with EtOAc, and the combined organic layer was washed with brine, dried (MgSO₄), and concentrated to give *N-tert*-butoxycarbonyl-L-homocysteine *tert*-butyl ester⁵⁴ (240 mg, 99%) as colorless oil of sufficient purity to be directly used in next step. *Step b.* LDA (2.0 M/THF and heptanes; 85 μ L, 0.17 mmol) was added drop wise (10 min) to a stirred solution of freshly prepared thiolate from step a (200 mg, 0.6 mmol) in anhydrous DMF (5 mL) under a vigorous stream of argon at 0 °C (ice-bath). After an additional 10 min, **127** (100 mg, 0.2 mmol) in anhydrous DMF (5 mL) was added via a syringe. After 15 min ice-bath was removed and the reaction mixture was stirred for 24 h at ambient temperature. Ice-cold saturated NH₄Cl/H₂O was added and the resulting suspension was diluted with EtOAc. The organic layer was separated and the aqueous layer was extracted with EtOAc. The combined organic layer was washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (40→ 50% EtOAc/hexane) to give **128** (130 mg, 86%) as a mixture of rotamers (~1:1): ¹H NMR δ 1.29 (s, 3, CH₃), 1.41 (s, 12H, *t*-Bu, CH₃), 1.44 (s, 18H, *t*-Bu), 1.79-1.92 (m, 2, H8,8'), 2.39-2.80 (m, 4, H5,5',7,7'), 3.37 (dd, *J* = 4.2, 11.7 Hz, 0.5, H1), 3.43 (dd, *J* = 4.5, 11.7 Hz, 0.5, H1), 3.70 (d, *J* = 12.6 Hz, 0.5, H1'), 3.84 (d, *J* = 12.8 Hz, 0.5, H1'), 3.99-4.05 (m, 0.5, H4), 4.11-4.17 (m, 0.5, H4), 4.18-4.29 (m, 1, H9), 4.56 (dd, *J* = 5.6, 10.4 Hz, 0.5, H3), 4.60 (dd, *J* = 5.6, 10.4 Hz, 0.5, H3), 4.69 (d, *J* = 4.8 Hz, 0.5, H2), 4.71 (d, *J* = 4.8 Hz, 0.5, H2), 5.06 (br. d, *J* = 7.3 Hz, 0.5, NH), 5.29 (br. d, *J* = 6.1 Hz, 0.5, NH); ¹³C NMR δ 24.97 (*CMe*₂), 26.93 (*CMe*₂), 27.91 (C7), 28.01 (*t*-Bu), 28.34 (*t*-Bu), 28.43 (*t*-Bu), 32.18 (C5), 32.57 (C8), 32.92 (C8), 33.22 (C5), 51.73 (C1), 52.42 (C1), 53.48 (C9), 62.76 (C4), 63.16 (C4), 78.47 (C2), 78.50 (*t*-Bu), 79.21 (C2), 79.23 (*t*-Bu), 80.00 (*t*-Bu), 80.10 (*t*-Bu), 83.49

(C3), 84.07 (C3), 111.85 (CMe₂), 154.09 (CO), 154.93 (CO), 155.36 (CO), 171.25 (CO), 171.46 (CO); MS (APCI) *m/z* 547 (100, MH⁺).

S-(1,4,5-Trideoxy-1,4-imino-D-ribitol-5-yl)-L-homocysteine (129). *Procedure C. Step a.* Compound **128** (39 mg, 0.07 mmol) in TFA (4.0 mL) was stirred at 0 °C for 3 h. Volatiles were evaporated and coevaporated with toluene to give an oily residue, which was used in next step. *Step b.* Product from step *a* was treated with TFA/H₂O (9:1, 4.0 mL) for 1 h at 0 °C. Volatiles were evaporated and the crude product was purified on RP-HPLC (5% CH₃CN/H₂O for 50 min at 2.5 mL/min; *t_R* = 12 min) to give **129** (12 mg, 66%) as a colorless oil: ¹H NMR (D₂O) δ 2.00-2.15 (m, 2, H8,8'), 2.61-2.71 (m, 2, H7,7'), 2.77 (dd, *J* = 10.4, 14.5 Hz, 1, H5), 3.05 (dd, *J* = 4.3, 14.5 Hz, 1, H5'), 3.24 (dd, *J* = 1.9, 13.0 Hz, 1, H1), 3.43 (dd, *J* = 4.0, 13.0 Hz, 1, H1'), 3.59 (ddd, *J* = 4.3, 8.7, 10.4 Hz, 1, H4), 3.76 (t, *J* = 6.2 Hz, 1, H9), 4.04 (dd, *J* = 4.1, 8.7 Hz, 1, H3), 4.29 (ddd, *J* = 1.9, 4.0, 4.1 Hz, 1, H2); ¹³C NMR (D₂O) δ 26.81 (C8), 30.06 (C7), 30.62 (C5), 49.16 (C1), 53.55 (C9), 59.48 (C4), 69.48 (C2), 74.34 (C3), 174.02 (COOH); MS *m/z* 251 (100, MH⁺). HRMS (TOF MS-ESI) *m/z* calculated for C₉H₁₈N₂O₄S [M+H]⁺ 251.106; found 251.1063.

1-Benzyl-5-chloro-3,4-dihydroxy-3,4-O-isopropylidene piperidine [130(3S,4S,5R/S)].

Treatment of **122** (50 mg, 0.19 mmol) with MsCl (31.9 mg, 21.9 μL, 0.28 mmol) by Procedure A [column chromatography (20 → 30% EtOAc/hexane)] gave **130** (25 mg, 46%) as a 3:1 mixture of diastereomers: The major isomer had: ¹H NMR δ 1.30 (s, 3, CH₃), 1.50 (s, 3, CH₃), 2.13-2.19 (m, 1, H2), 2.49 (dd, *J* = 3.7, 13.3 Hz, 1, H6), 2.87 (dd, *J* = 1.7, 12.0 Hz, 1, H2'), 3.05 ('dt', *J* = 2.1, 13.3 Hz, 1, H6'), 3.50 (d, *J* = 13.4 Hz, 1, Bn), 3.61 (d, *J* = 13.4 Hz, 1, Bn), 3.93-3.99 (m, 2, H3,5), 4.19 (dd, *J* = 3.7, 7.5 Hz, 1, H4), 7.20-7.25 (m, 5, Bn); ¹³C NMR δ 26.18 (CMe₂), 28.43 (CMe₂), 53.54 (C6), 56.53 (C2), 58.48 (C5), 61.52 (Bn), 73.77 (C4), 79.52 (C3), 112.80 (CMe₂), 127.26, 128.40, 128.92, 137.16 (Bn); MS *m/z* 282 (100, MH⁺[³⁵Cl]), 284 (40, MH⁺[³⁷Cl]); HRMS (TOF MS-ESI) *m/z* calculated for C₁₅H₂₀³⁵ClNO₂ [M+H]⁺ 282.1255; found. 282.1259.

***N*-Benzyl-5-(*tert*-butyldimethylsiloxymethyl)-3,4-dihydroxy-3,4-*O*-isopropylidenepyrrolidin-2-one [131(3*R*,4*R*,5*S*)]. Procedure D.** RuO₂·xH₂O (4.3 mg, 0.032 mmol) was added to a stirred solution of NaIO₄ (83 mg, 0.39 mmol) in H₂O (1 mL) at ambient temperature. After 5 min, a solution of **124** (50 mg, 0.13 mmol) in EtOAc (1 mL) was added drop wise and the reaction mixture was continued to stir for 12 h. Water (10 mL) and EtOAc (10 mL) were added and the separated aqueous layer was furthermore extracted with EtOAc (2 × 10 mL). The combined organic layers were washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (50→ 60% EtOAc/hexane) to give **131** (33 mg, 65%) and *N*-benzoylpyrrolidinone as a byproduct (10 mg, 18%). Compound **131** had: ¹H NMR δ 0.01 (s, 6, CH₃), 0.84 (s, 9, *t*-Bu), 1.35 (s, 3, CH₃), 1.42 (s, 3, CH₃), 3.51 (t, *J* = 2.1 Hz, 1, H5), 3.62 (dd, *J* = 2.0, 10.9 Hz, 1, H6), 3.69 (dd, *J* = 2.3, 10.9 Hz, 1, H6'), 3.93 (d, *J* = 15.2 Hz, 1, Bn), 4.50 (d, *J* = 5.6 Hz, 1, H4), 4.69 (d, *J* = 5.6 Hz, 1, H3), 5.00 (d, *J* = 15.2 Hz, 1, Bn), 7.22-7.32 (m, 5, Bn); MS *m/z* 392 (100, MH⁺). ¹³C NMR δ -5.67, (CH₃), -5.56, (CH₃), 18.11 (*t*-Bu), 25.79 (*t*-Bu), 25.81 (CMe₂), 27.26 (CMe₂), 44.19 (Bn), 60.22 (C6), 62.02 (C5), 76.66 (C4), 78.01 (C3), 111.69 (CMe₂), 127.71, 128.18, 128.73, 135.59 (Bn), 171.89 (CO). The *N*-Benzoyl-5-(*tert*-butyldimethylsiloxymethyl)-3,4-dihydroxy-3,4-*O*-isopropylidene-2-pyrrolidinone byproduct had: ¹H NMR δ 0.01 (s, 3, CH₃), 0.05 (s, 3, CH₃), 0.87 (s, 9, *t*-Bu), 1.40 (s, 3, CH₃), 1.51 (s, 3, CH₃), 3.83 (dd, *J* = 1.5, 10.7 Hz, 1, H6), 4.19 (dd, *J* = 2.2, 10.7 Hz, 1, H6'), 4.58 (t, *J* = 1.8 Hz, 1, H5), 4.65 (d, *J* = 5.5 Hz, 1, H4), 4.76 (d, *J* = 5.5 Hz, 1, H3), 7.38-7.43 (m, 2, Bn), 7.51 (dt, *J* = 1.3, 6.7 Hz, 2, Bn), 7.54-7.58 (m, 1, Bn), ¹³C NMR δ -5.72, (CH₃), -5.58, (CH₃), 18.16 (CH₃), 25.34 (CMe₂), 25.82 (*t*-Bu), 27.22 (CMe₂), 61.68 (C5), 62.12 (C6), 76.30 (C4), 78.67 (C3), 112.09 (CMe₂), 127.87, 128.73, 132.15, 134.11 (Bn) 170.56 (CO), 171.66 (CO); MS (APCI) *m/z* 406 (100, MH⁺).

***N*-Benzyl-3,4-dihydroxy-5-(hydroxymethyl)-3,4-*O*-isopropylidenepyrrolidin-2-one [132b(3*R*,4*R*,5*R*)].** TBAF (1 M/THF, 0.18 mL, 0.18 mmol) was added drop wise to a stirred

solution of **131** (49 mg, 0.12 mmol) in THF (10 mL) at 0 °C. After 5 min, the ice-bath was removed and reaction mixture was allowed to stir at ambient temperature for 2 h. The reaction mixture was quenched with water and volatiles were evaporated. The residue was partitioned (EtOAc//NaHCO₃/H₂O). The organic layer was washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (80 → 90% EtOAc/hexane) to give **132**¹⁸⁸ as a white solid (33 mg, 97%): ¹H NMR δ 1.37 (s, 3, CH₃), 1.47 (s, 3, CH₃), 3.35 (s, 1, OH), 3.54 (t, *J* = 1.8 Hz, 1, H5), 3.64 (d of m, *J* = 5.0, 11.8 Hz, 1, H6), 3.85 (br. d, *J* = 12.0 Hz, 1, H6'), 4.08 (d, *J* = 15.2 Hz, 1, Bn), 4.64 (d, *J* = 5.6, Hz, 1, H4), 4.77 (d, *J* = 5.2 Hz, 1, H3), 5.04 (d, *J* = 15.2 Hz, 1, Bn), 7.29-7.37 (m, 5, Bn); MS (APCI) *m/z* 310 (100, [MH+MeOH]⁺).

***N*-(*tert*-Butoxycarbonyl)-5-(*tert*-butyldimethylsiloxymethyl)-3,4-dihydroxy-3,4-*O*-isopropylidene-pyrrolidin-2-one [133(3*R*,4*R*,5*R*)].** Oxidation of **125** (90 mg, 0.23 mmol) with NaIO₄ (126 mg, 0.7 mmol) and RuO₂·xH₂O (8 mg, 0.05 mmol) by Procedure D [column chromatography (50 → 60% EtOAc/hexane)] gave **133** (56 mg, 60%) as a colorless oil with data as reported.¹⁶⁴

***N*-(*tert*-Butoxycarbonyl)-3,4-dihydroxy-5-(hydroxymethyl)-3,4-*O*-isopropylidene-pyrrolidin-2-one [134(3*R*,4*R*,5*R*)].** Desilylation of **133** (50 mg, 0.12 mmol) with TBAF (1 M/THF, 0.14 mL, 0.14 mmol), as described for **132**, [column chromatography (70 → 80% EtOAc/hexane)] gave **134** (27 mg, 77%) as a colorless oil: ¹H NMR δ 1.36 (s, 3, CH₃), 1.46 (s, 12, *t*-Bu, CH₃), 3.88 (dt, *J* = 3.9, 5.3 Hz, 1, H5), 4.03 (dd, *J* = 4.1, 11.4 Hz, 1, H6), 4.19 (dd, *J* = 3.8, 11.4 Hz, 1, H6'), 4.57 (dd, *J* = 5.3, 5.7 Hz, 1, H4), 4.63 (d, *J* = 5.7 Hz, 1, H3); ¹³C NMR δ 25.66 (CMe₂), 26.98 (CMe₂), 27.60 (*t*-Bu), 56.68 (C5), 66.63 (C6), 76.70 (C3), 77.02 (C4), 83.28 (*t*-Bu), 112.37 (CMe₂), 152.98 (CO), 173.71 (CO); MS (APCI) *m/z* 320 (100, [MH+MeOH]⁺).

***N*-Benzyl-3,4-dihydroxy-3,4-*O*-isopropylidene-5-[(methanesulfonyloxy)methyl]pyrrolidin-2-one [135(3*R*,4*R*,5*R*)].** Mesylation of **132** (67 mg, 0.24 mmol) with MsCl (41.0 mg, 27 μL, 0.36 mmol) by Procedure A gave **135** (83 mg, 97%) as a

colorless oil of sufficient purity to be used directly in next step: ^1H NMR δ 1.30 (s, 3, CH_3), 1.36 (s, 3, CH_3), 3.07 (s, 3, Ms), 3.65 (t, $J = 2.7$ Hz, 1, H5), 4.06 (d, $J = 15.2$ Hz, 1, Bn), 4.16 (dd, $J = 2.2, 11.0$ Hz, 1, H6), 4.22 (dd, $J = 3.0, 11.0$ Hz, 1, H6'), 4.51 (d, $J = 5.6$ Hz, 1, H4), 4.69 (d, $J = 5.6$ Hz, 1, H3), 4.92 (d, $J = 15.2$ Hz, 1, Bn), 7.18-7.30 (m, 5, Bn); MS (APCI) m/z 388 (100, $[\text{MH} + \text{MeOH}]^+$), 356 (40, MH^+).

***N*-(*tert*-Butoxycarbonyl)-3,4-dihydroxy-3,4-*O*-isopropylidene-5-**

[(methanesulfonyloxy)methyl]pyrrolidin-2-one [136(3*R*,4*R*,5*R*)]. Oxidation of **127** (80 mg, 0.32 mmol) with NaIO_4 (172 mg, 0.96 mmol) and $\text{RuO}_2 \times \text{H}_2\text{O}$ (8.5 mg, 0.064 mmol) by procedure D [column chromatography (EtOAc)] gave **136** (78 mg, 95%) as a colorless oil: ^1H NMR δ 1.37 (s, 3, CH_3), 1.44 (s, 3, CH_3), 1.54 (s, 9, *t*-Bu), 3.01 (s, 3, Ms), 4.39-4.43 ('m', 2, H5,6), 4.58 (d, $J = 5.4$ Hz, 1, H4), 4.64 (dd, $J = 3.1, 11.2$ Hz, 1, H6'), 4.70 (d, $J = 5.5$ Hz, H3); ^{13}C NMR δ 25.62 (CMe_2), 27.04 (CMe_2), 27.97 (*t*-Bu), 37.70 (Ms), 59.18 (C5), 67.03 (C6), 74.54 (C4), 77.54 (C3), 84.72 (*t*-Bu), 112.75 (CMe_2), 149.66 (CO), 170.20 (CO); MS (APCI) m/z 297 [100, $(\text{MH}_2 - \text{Boc} + \text{MeOH})^+$].

***S*-[*N*-Benzyl-3,4-dihydroxy-3,4-*O*-isopropylidene-pyrrolidin-2-one-5-yl)methyl]-*N*-*tert*-butoxycarbonyl-L-homocysteine *tert*-butyl ester [137(3*R*,4*R*,5*S*)].** Treatment of **135** (85 mg, 0.24 mmol) with lithium homocysteinate (104 mg, 0.36 mmol) by Procedure B [column chromatography (60 $\rightarrow$ 70% EtOAc/hexane)] gave **137** (94 mg, 70%) as a colorless oil. ^1H NMR δ 1.28 (s, 3, CH_3), 1.37 (s, 12, *t*-Bu, CH_3), 1.39 (s, 9, *t*-Bu), 1.74-1.86 (m, 1, H8), 1.95-2.17 (m, 1, H8'), 2.46-2.57 (m, 2, H7,7'), 2.69-2.73 (m, 2, H6,6'), 3.59-3.71 (m, 1, H5), 3.86 (d, $J = 15.0$ Hz, 1, Bn), 4.17-4.18 (m, 1, H9), 4.43 (d, $J = 5.7$ Hz, 1, H4), 4.79 (d, $J = 5.5$ Hz, 1, H3), 4.97 (d, $J = 15.0$ Hz, 1, Bn), 5.05 (br. d, $J = 7.1$ Hz, 1, NH); 7.17-7.27 (m, 5, Bn); ^{13}C NMR δ 25.63 (CMe_2), 27.04 (CMe_2), 27.99 (*t*-Bu), 28.31 (*t*-Bu), 29.02 (C7), 33.13 (C6), 33.18 (C8), 44.40 (Bn), 53.08 (C9), 60.34 (C5), 77.19 (C4), 77.48 (C3), 79.90 (*t*-Bu), 82.35 (*t*-Bu), 112.09 (CMe_2), 127.94,

128.16, 128.80, 135.18 (Bn), 152.98 (CO), 171.11 (CO), 171.35 (CO); MS m/z 551 (100, MH^+).
HRMS (AP-ESI) m/z calculated for $C_{28}H_{43}N_2O_7S$ [MH] $^+$ 551.2785; found 551.2792.

***S*-[*(3,4-Dihydroxy-3,4-O-isopropylidenepyrrolidin-2-one-5-yl)methyl*]-*N-tert-butoxycarbonyl-L-homocysteine tert-butyl ester* [138(3*R*,4*R*,5*S*)].** Treatment of **136** (70 mg, 0.19 mmol) with lithium homocysteinate (83 mg, 0.28 mmol) by Procedure B [column chromatography (70% → 80% EtOAc/hexane)] gave **138** (40 mg, 45%) as a light yellow oil. 1H NMR δ 1.36 (s, 3, CH_3), 1.43 (s, 9, *t*-Bu), 1.45 (s, 12, *t*-Bu, CH_3), 1.82-1.90 (m, 1, H8), 1.97-2.06 (m, 1, H8'), 2.58 (t, $J = 7.4$ Hz, 2, H7,7'), 2.64-2.72 (m, 2, H6,6'), 3.83 (t, $J = 5.8$ Hz, 1, H5), 4.23-4.24 (m, 1, H9), 4.48 (d, $J = 4.4$ Hz, 1, H4), 4.69 (d, $J = 4.6$ Hz, 1, H3), 5.21 (br. d, $J = 7.9$ Hz, 1, NH); ^{13}C NMR δ 25.55 (CMe_2), 26.98 (CMe_2), 27.98 (*t*-Bu), 28.43 (*t*-Bu), 29.16 (C7), 32.88 (C8), 33.78 (C6), 53.15 (C9), 60.38 (C5), 75.90 (C4), 77.52 (C3), 79.98 (*t*-Bu), 82.36 (*t*-Bu), 112.41 (CMe_2), 149.88 (CO), 155.94 (CO), 170.37 (CO), 171.15 (CO); MS (APCI) m/z 461 (100, MH^+).

***S*-[*N-(tert-Butoxycarbonyl)-3,4-dihydroxy-3,4-O-isopropylidenepyrrolidin-2-one-5-yl)methyl*]-*N-tert-butoxycarbonyl-L-homocysteine tert-butyl ester* [139(3*R*,4*R*,5*S*)].**
Procedure E. DMAP (18.8 mg, 0.15 mmol) and (Boc) $_2$ O (46.4 mg, 0.21 mmol) were added to a stirred solution of compound **138** (27 mg, 0.06 mmol) in CH_2Cl_2 (2 mL) at ambient temperature under Ar atmosphere. After 48 h, the reaction mixture was quenched with H_2O (5 mL) and partitioned (CH_2Cl_2 // $NaHCO_3$ / H_2O). The organic layer was washed (brine), dried ($MgSO_4$) and evaporated. The residue was column chromatographed (30 → 40% EtOAc/hexane) to give **139** (30 mg, 93%) as a colorless oil: 1H NMR δ 1.36 (s, 3, CH_3), 1.43 (s, 9, *t*-Bu), 1.45 (s, 3, CH_3), 1.46 (s, 9, *t*-Bu), 1.54 (s, 9, *t*-Bu), 1.82-1.87 (m, 1, H8), 2.02-2.04 (m, 1, H8'), 2.49-2.55 (m, 1, H7), 2.58-2.62 (m, 1, H7'), 2.70 (dd, $J = 7.1, 14.2$ Hz, 1, H6), 2.95 (dd, $J = 2.5, 14.2$ Hz, 1, H6'), 4.22-4.24 (m, 1, H9), 4.23 (dd, $J = 2.5, 7.1$ Hz, 1, H5), 4.46 (d, $J = 5.5$ Hz, 1, H4), 4.80 (d, $J = 5.5$ Hz, 1, H3), 5.08 (br. d, $J = 7.9$ Hz, 1, NH); ^{13}C NMR δ 25.56 (CMe_2), 26.93 (CMe_2), 27.98 (*t*-Bu),

28.33 (*t*-Bu), 28.42 (*t*-Bu), 28.91 (C7), 33.21 (C8), 36.58 (C6), 53.15 (C9), 60.39 (C5), 77.52 (C4), 75.90 (C3), 82.28 (2x *t*-Bu), 84.11 (*t*-Bu), 112.41 (CMe₂), 149.88 (CO), 155.50 (CO), 170.37 (CO), 171.15 (CO); MS (ESI) *m/z* 583 (100, [M+Na]⁺).

***S*-[*N*-Benzyl-3,4-dihydroxypyrrolidin-2-one-5-yl)methyl]-L-homocysteine**

[140(3*R*,4*R*,5*S*)]. Treatment of **137** (40 mg, 0.07 mmol) with TFA by Procedure C (step *a*, 3 h) gave crude **140** as colorless oil. RP-HPLC purification (5% CH₃CN/H₂O for 30 min followed by gradient 5 → 90% CH₃CN/H₂O for 30 min at 2.5 mL/min; *t_R* = 45 min) gave **140** (12 mg, 48%): ¹H NMR (D₂O) δ 1.88-2.01 (m, 2, H8,8'), 2.47-2.51 (m, 2, H9,9'), 2.61 (dd, *J* = 8.4, 14.0 Hz, 1, H6), 2.72 (dd, *J* = 3.9, 14.0 Hz, 1, H6'), 3.47 (dd, *J* = 3.6, 8.3 Hz, 1, H5), 3.68 ('t', *J* = 5.8 Hz, 1, H9), 4.26 (dd, *J* = 5.3 Hz, 1, H4), 4.27 (d, *J* = 14.7 Hz, 1, Bn), 4.62 (d, *J* = 5.2 Hz, 1, H3), 4.68 (d, *J* = 14.7 Hz, 1, Bn), 7.23-7.36 (m, 5, Bn); ¹³C NMR (D₂O) δ 27.71 (C7), 30.31 (C8), 30.75 (C6), 45.18 (Bn), 53.58 (C9), 63.87 (C5), 70.03 (C4), 70.14 (C3), 128.03, 128.07, 128.02, 135.12 (Bn), 173.88 (CO), 174.73 (CO); MS (APCI) *m/z* 355 (50, MH⁺). HRMS (AP-ESI) *m/z* calculated for C₁₆H₂₂N₂O₅S [M+Na]⁺ 377.1147; found 377.1156.

***S*-[(3,4-Dihydroxypyrrolidin-2-one-5-yl)methyl]-L-homocysteine** **[141(3*R*,4*R*,5*S*)].**

Treatment of **138** (30 mg, 0.065 mmol) with TFA by Procedure C (step *a*, 3 h) gave crude **141** as colorless oil. RP-HPLC purification (5% CH₃CN/H₂O at 2.5 mL/min; *t_R* = 16 min) gave **141** (10 mg, 58 %): ¹H NMR (D₂O) δ 2.00-2.18 (m, 2, H8,8'), 2.65-2.67 (m, 3, H6,7,7'), 2.73-2.74 (m, 1, H6'), 3.63 ('t', *J* = 6.5 Hz, 1, H5), 3.85 ('t', *J* = 5.8 Hz, 1, H9), 4.19 ('d', *J* = 5.2 Hz, 1, H4), 4.47 (d, *J* = 5.2 Hz, 1, H3); ¹³C NMR (D₂O) δ 26.21 (C7), 28.53 (C8), 32.70 (C6), 58.83 (C5), 51.07 (C9), 69.71 (C3), 70.46 (C4), 170.87 (CO), 176.10 (CO); MS (ESI) *m/z* 265 (100, MH⁺).

***S*-[*N*-(*tert*-Butoxycarbonyl)-3,4-dihydroxy-3,4-*O*-isopropylidenepyrrolidin-2-ol-5-yl)methyl]-*N*-*tert*-butoxycarbonyl-L-homocysteine *tert*-butyl ester** **[142(3*R*,4*R*,5*S*)].**

Procedure F. LiEt₃BH (1 M/THF; 125 μL, 0.12 mmol) was added to a stirred solution of **139** (28 mg, 0.05 mmol) in anhydrous THF (1 mL) at -78°C under N₂ atmosphere. After 30 min, the

solution was quenched with water and volatiles were evaporated. The residue was partitioned (EtOAc//NaHCO₃/H₂O), washed (brine) and dried (MgSO₄). The resulting oil was chromatographed (40% EtOAc/hexane) to give **142** [26 mg, 92%; mixture of anomers (α/β , 3:2) which appear as a set of rotamers (1:1)] as colorless oil: ¹H NMR δ 1.30 (s, 3, CH₃), 1.43 (s, 12, *t*-Bu, CH₃), 1.46 (s, 9, *t*-Bu), 1.48 (s, 9, *t*-Bu), 1.83-1.94 (m, 1, H8), 2.03-2.10 (m, 1, H8'), 2.51-2.62 (m, 3, H7,H7',6), 2.83 (dd, J = 3.7, 13.5 Hz, 0.6, H6'), 2.92 (dd, J = 3.5, 13.7 Hz, 0.4, H6'), 3.45 (dd, J = 3.5, 10.6 Hz, 0.3, H5), 3.99-4.28 (m, 2.7, H5), 4.57 (d, J = 5.8 Hz, 0.6, H4), 4.59 (d, J = 5.9 Hz, 0.4, H4), 4.66 (d, J = 6.7 Hz, 0.4, H3), 4.72 (d, J = 5.8 Hz, 0.6, H3), 5.09 (br. d, J = 7.2 Hz, 0.6, NH); 5.32 (br. d, J = 7.8 Hz, 0.4, NH), 5.39 (s, 0.4, H2), 5.50 (s, 0.6, H2); ¹³C NMR δ (major isomer) 24.82 (CMe₂), 26.68 (CMe₂), 27.99 (*t*-Bu), 28.31 (*t*-Bu), 28.38 (*t*-Bu), 29.68 (C7), 32.85 (C8), 34.68 (C6), 53.21 (C9), 63.86 (C5), 82.76 (C3), 84.44 (C4), 87.13 (C2), 81.22 (2 $\times$ *t*-Bu), 82.31 (*t*-Bu), 112.65 (CMe₂), 154.31 (CO), 155.39 (CO), 171.25 (CO); MS (ESI) m/z 585 (100, [M+Na]⁺).

S-[3,4-Dihydroxypyrrolidin-2-ol-5-yl)methyl]-L-homocysteine 143(3*R*,4*R*,5*S*). Treatment of **142** (24 mg, 0.04 mmol) with TFA by Procedure C (step *a*, 1 h; step *b*, 10 h at 0 °C) gave crude **143**. The RP-HPLC (5% CH₃CN/H₂O at 2.5 mL/min; t_R = 14 min) yielded **143** [8 mg, 72%; as mixture of anomers, (α/β , 3:2)] as a slightly yellow oil: ¹H NMR (D₂O) δ 2.15-2.24 (m, 1, H8), 2.26-2.37 (m, 1, H8'), 2.75-2.82 (m, 2, H7,H7'), 2.84-2.98 (m, 1, H6,6'), 3.09-3.17 (m, 1, H6,6'), 3.55-3.69 (m, 0.6, H5), 3.76-3.83 (m, 0.4, H5), 4.15-4.21 (m, 2, H3,4,9), 4.23-4.25 (m, 0.4, H3), 4.34 (dd, J = 4.8, 6.3 Hz, 0.6, H4), 5.27 (d, J = 2.6 Hz, 0.6, H2); 5.41 (d, J = 2.2 Hz, 0.4, H2); ¹³C NMR δ (major isomer) 25.83 (C7), 28.66 (C8), 30.72 (C6), 51.28 (C9), 58.76 (C5), 71.98 (C4), 72.96 (C3), 86.54 (C2), 171.15 (CO); MS (ESI) m/z 267 (50, MH⁺), 249 (100, [M-17]⁺)

N-(tert-Butoxycarbonyl)-5-(tert-butyldimethylsiloxy)-2,3-O-isopropylidene-4-amino-4-deoxy- α/β -D-ribofuranose (144). Reduction of **133**¹⁶⁴ (68 mg, 0.17 mmol) with LiEt₃BH (1

M/THF; 0.43 mL, 0.43 mmol) in anhydrous THF (2 mL) at -78 °C by the procedure F gave **144**¹⁶⁴ (68 mg, 100%) as a colorless oil with data as reported.

***N*-(*tert*-Butoxycarbonyl)-2,3-*O*-isopropylidene-4-amino-4-deoxy- α/β -D-ribofuranose**

(145). Desilylation of **144** (55 mg, 0.13 mmol) with TBAF (1 M/THF, 0.19 mL, 0.19 mmol), as described for **132**, [column chromatography (50 $\rightarrow$ 60% EtOAc/hexane)] gave **145** (α/β , 3:2; 39 mg, 96%) as a colorless oil: ^1H NMR δ 1.30 (s, 3, CH₃), 1.40 (s, 3, CH₃), 1.46 (s, 5.4, *t*-Bu), 1.49 (s, 3.6, *t*-Bu), 2.87 (br. s, 0.4, OH), 3.15 (br. s, 0.6, OH), 3.6-3.77 (m, 1.6, H5,5'), 3.84-3.90 (m, 0.8, H5',OH), 4.10 ('s', 0.6, H4), 4.27 ('s', 0.4, H4), 4.30 (br. s, 0.6, OH), 4.55 (d, J = 5.9 Hz, 1, H3), 4.73 (d, J = 5.9 Hz, 0.4, H2), 4.77 (d, J = 5.9 Hz, 0.6, H2), 5.36 (d, J = 5.7 Hz, 0.4, H1), 5.51 (s, 0.6, H1); ^{13}C NMR (α anomer) δ 24.58 (*CMe*₂), 26.58 (*CMe*₂), 28.33 (*t*-Bu), 62.61 (C5), 65.84 (C4), 81.87 (C2), 81.34 (*t*-Bu), 85.31 (C3), 86.40 (C1), 111.39 (*CMe*₂), 154.29 (NHCO); ^{13}C NMR (β anomer) δ 24.69 (*CMe*₂), 26.66 (*CMe*₂), 28.33 (*t*-Bu), 62.61 (C5), 65.84 (C4), 81.21 (C2), 81.34 (*t*-Bu), 86.40 (C3), 86.53 (C1), 111.39 (*CMe*₂), 153.53 (CO); MS (APCI) m/z 272 (50, [M-17]⁺), 213 (100, [MH-Boc-OH+CH₃CN]⁺).

4-Amino-4-deoxy- α/β -D-ribofuranose (146a). Treatment of **145** (27 mg, 0.09 mmol) with TFA by Procedure C (step *a*, 5 h; step *b*, 6 h at 0°C) gave crude **146a** (13 mg, 92% as a light yellow oil of a mixture of anomers (α/β , 0.65:0.35): ^1H NMR (D₂O) 3.37-3.38 (m, 0.35, H4), 3.41-3.44 (m, 0.65, H4), 3.61-3.63 (m, 0.65, H3), 3.70 (dd, J = 6.0, 13.1 Hz, 0.35, H5), 3.71 (dd, J = 2.1, 12.8 Hz, 0.65, H5), 3.83 (m, 0.35, H3), 3.91 (dd, J = 3.0, 4.0 Hz, 0.35, H2), 3.95 (dd, J = 2.8, 13.5 Hz, 0.35, H5'), 4.01 (dd, J = 3.0, 12.8 Hz, 0.65, H5'), 4.05 ('t', J = 3.4 Hz, 0.65, H2), 4.69 (d, J = 1.3 Hz, 0.35, H1), 4.97 (d, J = 4.0 Hz, 0.65, H1); ^{13}C NMR (major rotamer) δ 50.13 (C4), 58.86 (C5), 65.61 (C2), 69.84 (C3), 94.05 (C1); ^{13}C NMR (minor rotamer) δ 49.85 (C4), 61.50 (C5), 63.56 (C2), 69.79 (C3), 94.05 (C1); MS (APCI) m/z 150 (100, MH⁺).

4-Amino-4-deoxy-D-ribose *O*-Benzyloxime (147). A solution of **146a** (13 mg, 0.09 mmol) and *O*-benzylhydroxylamine hydrochloride (43 mg, 0.27 mmol) in anhydrous pyridine (4 mL)

was stirred under an atmosphere of nitrogen at room temperature for 12 h. Pyridine was evaporated to give **147** of sufficient purity for spectroscopic analysis: ^1H NMR (MeOD) δ 3.52 ('dt', $J = 4.1, 8.4$ Hz, 1, H4), 3.79 (dd, $J = 3.8, 11.5$ Hz, 1, H5), 3.85 (dd, $J = 4.4, 8.7$ Hz, 1, H3), 3.94 (dd, $J = 8.4, 11.5$ Hz, 1, H5'), 4.13 (dd, $J = 6.8, 8.7$ Hz, 1, H2), 4.92-5.16 (Bn, 2H, signal within the envelope of the solvent peaks but cross peaks to each other protons observed in COSY and confirmed by HETCOR), 7.41 (H1, signal within the envelope of Bn protons but cross peaks to H2 observed in COSY), 7.35-7.47 (m, 5H, Bn); ^{13}C NMR δ 56.20 (C4), 58.81 (C5), 71.22 (C3), 71.56 (C2), 77.01 (Bn), 128.91, 129.30, 129, 139.07 (Bn); MS (ESI) m/z 255 (60, MH^+).

5-*O*-tert-Butyldimethylsilyl-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-D-ribitol (148).

To a solution of **124** (109 mg, 0.28 mmol) in EtOH (6 mL) was added 5% Pd/C (300 mg) and stirred under an atmosphere of H_2 at room temperature for 6 h. The mixture was filtered through celite to remove the catalyst. The celite bed was washed with ethanol (5 mL) and washings and the filtrate were combined and evaporated. The residue was column chromatographed (30% EtOAc/hexane) to give **148**¹⁸⁷ as a colorless oil (70 mg, 80%): ^1H NMR δ 0.04 (s, 3, CH_3), 0.04 (s, 3, CH_3), 0.87 (s, 9, *t*-Bu), 1.32 (s, 3, CH_3), 1.46 (s, 3, CH_3), 2.33 (s, 1, NH), 2.98 ('d', $J = 2.6$ Hz, 2, H1,1'), 3.20 ('dt', $J = 0.6, 5.8$ Hz, 1, H4), 3.52 (dd, $J = 5.9, 10.3$ Hz, 1, H5), 3.62 (dd, $J = 5.1, 10.3$ Hz, 1, H5'), 4.63 (dd, $J = 0.8, 5.8$ Hz, 1, H3), 4.68 ('dt', $J = 2.6, 5.8$ Hz, H2) MS (APCI) m/z 288 (100, MH^+).

5-*O*-tert-Butyldimethylsilyl-1,*N*-didehydro-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-D-ribitol *N*-Oxide (149). A stirred solution of **148** (70 mg, 0.24 mmol) and SeO_2 (0.01 mmol, 1.1 mg) in acetone (3 mL) was cooled to -4°C under N_2 atmosphere and H_2O_2 (25%) was added slowly (3-4 h) until the reaction was completed (monitored by TLC). Volatiles were evaporated and the residue was partitioned (EtOAc// $\text{NaHCO}_3/\text{H}_2\text{O}$). The organic layer was collected, washed (brine) and dried (MgSO_4). The resulting solid was chromatographed (50% EtOAc/hexane) to give **148** (54 mg, 73%) as a white crystalline solid with data as reported.¹⁶⁵

1,*N*-Didehydro-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-D-ribitol *N*-Oxide (150).

Desilylation of **149** (155 mg, 0.51 mmol) with TBAF (1 M/THF, 0.77 mL, 0.77 mmol) at -4 °C as described for **132** [column chromatography (10%→ 20% MeOH/CHCl₃)] gave **150** (40 mg, 42%) as a white crystalline solid: ¹H NMR δ 1.34 (s, 3, CH₃), 1.41 (s, 3, CH₃), 3.87 (dd, *J* = 2.1, 19.6 Hz, 1, H5), 4.02-4.03 (m, 1, H4), 4.14 (dd, *J* = 2.3, 11.9 Hz, 1, H5'), 4.96 (d, *J* = 6.2 Hz, 1, H3), 5.21 ('dt', *J* = 1.4, 6.2 Hz, 1, H2), 6.90 (br. s, 1, H1); ¹³C NMR δ 25.66 (CMe₂), 27.15 (CMe₂), 58.87 (C5), 77.16 (C3), 78.85 (C4), 80.78 (C2), 111.50 (CMe₂), 134.63 (C1); MS (ESI) *m/z* 186 (100, [M-1]⁺).

1,*N*-Didehydro-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-5-*O*-methanesulfonyl-D-ribitol *N*-Oxide (151). Treatment of **150** (40 mg, 0.21 mmol) at -4 °C with MsCl (26 μL, 0.32 mmol) by Procedure A [column chromatography (80% → 90% EtOAc/hexane)] gave **151** (24 mg, 56%) as a colorless oil: ¹H NMR δ 1.37 (s, 3, CH₃), 1.46 (s, 3, CH₃), 3.04 (s, 3, Ms), 4.23-4.24 (m, 1, H4), 4.55 (dd, *J* = 1.8, 11.1 Hz, 1, H5), 4.83 (dd, *J* = 2.4, 11.1 Hz, 1, H5'), 4.91 (dd, *J* = 1.1, 6.3 Hz, 1, H3), 5.25 ('dt', *J* = 1.5, 6.4 Hz, 1, H2), 6.99 (s, 1, H1); ¹³C NMR δ 25.56 (CMe₂), 27.07 (CMe₂), 37.42 (Ms), 65.54 (C5), 75.78 (C3), 77.75 (C4), 78.43 (C2), 112.64 (CMe₂), 134.10 (C1); MS (APSI) *m/z* 266 (100, MH⁺).

***S*-(1,*N*-Didehydro-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-D-ribitol-5-yl *N*-Oxide)-*N*-tert-butoxycarbonyl-L-homocysteine *tert*-butyl ester (152).** Treatment of **151** (24 mg, 0.09 mmol) with L-homocysteinate (38 mg, 0.13 mmol) by Procedure B (step *a* and *b*, 8 h at -20°C) and purification by column chromatography (80% → 90% EtOAc/Hexane) gave **152** as a colorless oil (24 mg, 43%): ¹H NMR δ 1.44 (s, 9, *t*-Bu), 1.45 (s, 3, CH₃), 1.46 (s, 9, *t*-Bu), 1.81-1.90 (m, 1, H8), 1.96-2.09 (m, 1, H8'), 2.52-2.70 (m, 2, H7,H7'), 3.07 (dd, *J* = 3.5, 14.4 Hz, 1, H5), 3.14 (dd, *J* = 5.2, 14.4 Hz, 1, H5'), 4.20-4.31 (m, 2, H4,9), 4.71 (d, *J* = 6.2 Hz, 1, H3), 5.06 (br. s, 1, NH), 5.31 ('dt', *J* = 1.4, 6.4 Hz, 1, H2), 6.97 (s, 1, H1); ¹³C NMR δ 25.64 (CMe₂), 26.07 (CMe₂), 28.00 (*t*-Bu), 28.32 (*t*-Bu), 29.07 (C7), 32.00 (C5), 32.81 (C8), 53.07 (C9), 77.94 (C3),

78.96 (C2), 79.13 (C4), 79.82 (*t*-Bu), 82.24 (*t*-Bu), 111.96 (CMe₂), 133.43 (C1), 155.39 (CO), 171.09 (CO); MS (APCI) *m/z* 461 (100, MH⁺).

***S*-(1,*N*-Didehydro-1,4-dideoxy-1,4-imino-D-ribitol-5-yl *N*-Oxide)-L-homocysteine (153).**

Treatment of **152** (72 mg, 0.15 mmol) with TFA by Procedure C (step *a*, 5 h; step *b*, 6 h at 0 °C) gave crude **152**. Purification by HPLC (5% CH₃CN/H₂O for at 2.5 mL/min; *t_R* = 10-14 min) afforded **153** (16 mg, 40 %) as a white solid: ¹H NMR (D₂O) δ 2.01-2.17 (m, 2, H8,8'), 2.83-2.89 (m, 2, H7,7'), 2.97 (dd, *J* = 6.3, 14.4 Hz, 1, H5), 3.06 (dd, *J* = 3.8, 14.4 Hz, 1, H5'), 3.73-3.77 (m, 1, H9), 4.08-4.22 (m, 1, H4), 4.40 (dd, *J* = 3.2, 6.0 Hz, 1, H3), 4.89-4.96 (m, 1, H2), 7.25 (s, 1, H1); ¹³C NMR δ 27.63 (C7), 29.87 (C5), 30.51 (C8), 53.73 (C9), 70.42 (C3), 78.26 (C4), 80.71 (C2), 141.80 (C1), 173.92 (CO); MS (APCI) *m/z* 265 (50, MH⁺). HRMS (AP-ESI) *m/z* calculated for C₉H₁₆N₂NaO₅S [MH+Na]⁺ 287.0678; found 287.0664.

1,*N*-Didehydro-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-5-*O*-methanesulfonyl-1-methyl-D-ribitol (155). Treatment of **154**¹⁶⁶ (48.5 mg, 0.26 mmol) with MsCl (0.031 mL, 45 mg, 0.39 mmol) in the presence of Et₃N (0.11 mL, 80 mg, 0.79 mmol) by Procedure A [3 h; column chromatography (EtOAc → 10% MeOH/EtOAc)] gave **155** (59 mg, 85%) as a colorless oil: ¹H NMR δ 1.38 (s, 3, CH₃), 1.39 (s, 3, CH₃), 2.16 (d, *J* = 1.0 Hz, 3, N=CMe), 3.00 (s, 3, Ms), 4.38 (s, 1, H4), 4.39 (dd, 1, *J* = 3.6, 11.2 Hz, 1, H5), 4.53 (dd, *J* = 4.2, 11.3 Hz, 1, H5'), 4.64 (d, *J* = 5.8 Hz, 1, H3), 4.95 ('q', *J* = 5.6 Hz, 1, H2); ¹³C NMR δ 17.08 (N=CMe), 25.70 (CMe₂), 26.84 (CMe₂), 37.37 (Ms), 69.68 (C5), 74.97 (C4), 79.77 (C3), 87.42 (C2), 112.37 (CMe₂), 177.04 (C=N); MS (APCI) *m/z* 264 (100, MH⁺).

***S*-(1,*N*-Didehydro-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-1-methyl-D-ribitol-5-yl)-*N*-(*tert*-butoxycarbonyl)-L-homocysteine *tert*-butyl ester (156).** Treatment of **155** (49.5 mg, 0.19 mmol) with protected L-Hcy (82 mg, 0.284 mmol) by Procedure B (step *a* and *b*, 36 h) gave 124 mg of yellowish oily residue. Crude product was column chromatographed (50 → 60% EtOAc/hexane) to give **156** as a colorless oil (73 mg, 85%): ¹H NMR δ 1.35 (s, 3, CH₃), 1.35 (s,

3, CH₃), 1.43 (s, 9, *t*-Bu), 1.46 (s, 9, *t*-Bu), 1.84 ('sx', *J* = 7.3 Hz, 1, C8), 1.98-2.07 (m, 1, H8'), 2.11 (d, *J* = 1.1 Hz, 3, N=CMe), 2.54 ('t', *J* = 7.6 Hz, 2, H7,7'), 2.65 (dd, *J* = 6.4, 13.5 Hz, 1, H5), 2.87 (dd, *J* = 4.4, 13.5 Hz, 1, H5'), 4.17-4.27 (m, 1, H9), 4.33 (br. 't', *J* = 4.6 Hz, 1, H4), 4.48 (d, *J* = 5.6 Hz, 1, H2) 4.96 (d, *J* = 5.6 Hz, 1, H3) 5.13 (br. d, *J* = 7.7 Hz, 1, NH); ¹³C NMR δ 16.97 (N=CMe), 25.72 (CMe₂), 26.79 (CMe₂), 27.99 (*t*-Bu), 28.30 (*t*-Bu), 29.16 (C7), 33.12 (C8), 35.32 (C5), 53.34 (C9), 76.46 (C4), 79.75 (*t*-Bu), 81.80 (C2), 82.11 (*t*-Bu), 87.27 (C3), 111.91 (CMe₂), 155.32 (CO), 171.23 (CO), 174.67 (N=C); MS (APCI) *m/z* 459 (100, [MH]⁺); HRMS (TOF MS-ESI) *m/z* calculated for C₂₂H₃₈N₂O₆S [MH]⁺ 459.2523; found 459.2523.

***S*-(1,*N*-Didehydro-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-1-methyl-D-ribitol-5-yl)-L-homocysteine (157).** Treatment of **156** (62 mg, 0.136 mmol) with TFA by Procedure C (step *a*, ambient temperature) gave **157** (41 mg, 99%) as a colorless oil: ¹H NMR (D₂O) δ 1.38 (s, 3, CH₃), 1.41 (s, 3, CH₃), 2.10-2.20 (m, 1, H8), 2.22-2.31 (m, 1, H8'), 2.57 (br. s, 3 → 0, exchanged with D₂O within few hours, N=CMe), 2.75 (t, *J* = 7.3 Hz, 2, H7,7'), 2.99 (d, *J* = 6.2 Hz, 2, H5,5'), 4.17 (t, *J* = 6.4 Hz, 1, H9), 4.73 ('t', *J* = 6.3 Hz, 1, H4), 4.91 (d, *J* = 5.4 Hz, 1, H2), 5.64 (d, *J* = 5.3 Hz, 1, H3); ¹H NMR (DMSO) δ 1.28 (s, 3, CH₃), 1.30 (s, 3, CH₃), 1.95-2.07 (m, 2, H8,8'), 2.08 (s, 3, N=CMe), 2.60 (dd, *J* = 7.4, 13.6 Hz, 1, H5) 2.65 ('t', *J* = 7.9 Hz, 2, H7,7'), 2.82 (dd, *J* = 5.2, 13.7 Hz, 1, H5'), 4.01 (br. s, 1, H9), 4.21 ('t', *J* = 5.8 Hz, 1, H4), 4.51 (d, *J* = 5.5 Hz, 1, H2), 5.10 (d, *J* = 5.5 Hz, 1, H3), 7.28 (br. s, 3, ⁺NH₃); ¹³C NMR (D₂O) δ 15.45 ('q', *J* = 20 Hz, N=CCD₃), 23.99 (CMe₂), 25.44 (CMe₂) 27.41 (C7), 29.46 (C8), 31.63 (C5), 51.36 (C9), 71.15 (C4), 78.97 (C2), 83.97 (C3), 114.23 (CMe₂), 171.45 (COOH), 191.73 (N=CCD₃); ¹³C NMR (DMSO) δ 16.53 (N=CCD₃), 25.38 (CMe₂), 26.59 (CMe₂), 27.47 (C7), 30.07 (C8), 33.24 (C5), 50.94 (C9), 74.75 (C4), 80.76 (C2), 86.05 (C3), 111.13 (CMe₂), 170.67 (CO), no signal (N=CCD₃); MS (APCI) *m/z* 303 (100, MH⁺).

***S*-(1,*N*-Didehydro-1,4-dideoxy-1,4-imino-1-methyl-D-ribitol-5-yl)-L-homocysteine (158).** Treatment of **157** (41 mg, 0.136 mmol) with TFA/H₂O by Procedure C (step *b*, TFA/H₂O 4:1,

ambient temperature, 12 h) gave homogenous **158** (36 mg, 98%) as a colorless oil: ^1H NMR (D_2O) δ 2.05-2.16 (m, 1, H8), 2.17-2.27 (m, 1, H8'), 2.45 (br. s, 3 $\rightarrow$ 0, exchanged with D within few hrs, N=CMe), 2.73 (dt, J = 3.3, 7.3 Hz, 2, H7,7'), 2.78 (dd, J = 8.3, 14.4 Hz, 1, H5), 2.92 (dd, J = 6.0, 14.3 Hz, 1, H5') 4.14 (t, J = 6.4 Hz, 1, H9), 4.35 ('t', J = 7.1 Hz, 1, H4), 4.37 (d, J = 5.3 Hz, 1, H2), 5.12 (d, J = 5.5 Hz, 1, H3); ^{13}C NMR (D_2O) δ 15.36 ('q', J = 21 Hz, N=CCD₃), 26.95 (C7), 29.32 (C8), 30.73 (C5), 51.31 (C9), 71.62 (C2), 71.92 (C4), 76.75 (C3), 171.36 (CO), 195.79 (N=CCD₃); MS (APCI) m/z 263 (100, MH⁺).

5-(Propylthiomethyl)pyrrolidin-2-one [160a(5S)]. Procedure G. Propanethiol (50 μL , 42 mg, 0.55 mmol) was added dropwise to a stirred suspension of NaH (35 mg, 0.875 mmol, 60% mineral oil) in dry DMF (1 mL) under Ar atmosphere at 0°C. After 10 min (till gas evolution has ceased), solution of compound **159**¹⁶⁷ [(5S), 82 mg, 0.46 mmol] in dry DMF (1 mL) was added dropwise, and after 15 min the reaction mixture was allowed to warm to ambient temperature. After 12 h the resulting mixture was quenched with water at 0 °C, volatiles were evaporated, and the residue was column chromatographed (EtOAc $\rightarrow$ 10% MeOH/EtOAc) to give **160a(5S)** (77 mg, 96%) as a colorless oil: ^1H NMR 0.98 (t, J = 7.3 Hz, 3, H9), 1.60 (sx, J = 7.3 Hz, 2, H8,8'), 1.76-1.87 (m, 1, H4), 2.25-2.34 (m, 1, H4'), 2.34-2.45 (m, 2, H3,3'), 2.52 (t, J = 7.3 Hz, 2, H7,7'), 2.54 (dd, J = 7.7, 13.2 Hz, 1, H6), 2.68 (dd, J = 5.5, 13.2 Hz, 1, H6'), 3.80 ('q', J = 5.5 Hz, 1, H6), 6.73 (br. s, 1, NH); ^{13}C NMR δ 13.39 (C9), 23.05 (C8), 26.64 (C4), 30.16 (C3), 34.65 (C7), 38.56 (C6), 53.90 (C5), 177.95 (CO); MS (APCI) m/z 174 (MH⁺). HRMS (AP-ESI) m/z calculated for C₈H₁₅NNaOS [M+Na]⁺ 196.0772; found 196.0779.

5-(Hexylthiomethyl)pyrrolidin-2-one [160b(5S)]. Treatment of **159**¹⁶⁷ [(5S), 823 mg, 4.62 mmol] in dry DMF (6 mL) with a thiolate soln in dry DMF (6 mL) generated from hexanethiol (682 μL , 573 mg, 4.86 mmol), and NaH (204 mg, 5.09 mmol, 60%/mineral oil) by Procedure G [column chromatography (80% EtOAc/hexane $\rightarrow$ 5% MeOH/EtOAc)] gave **160b(5S)** (932 mg, 94%) as a colorless oil. ^1H NMR δ 0.90 (t, J = 7.0 Hz, 3, H12), 1.24-1.33 (m, 4, H10,H11), 1.33-

1.42 (m, 2, H9), 1.58 ('q', $J = 7.4$ Hz, 2, H8,8'), 1.78-1.87 (m, 1, H4), 2.27-2.46 (m, 3, H4',H3,3'), 2.53 (dd, $J = 8.0, 13.4$ Hz, 1, H6), 2.54 (t, $J = 7.3$ Hz, 2, H7,7'), 2.70 (dd, $J = 5.3, 13.2$ Hz, 1, H6'), 3.81 ('q', $J = 6.6$ Hz, 1, H5), 6.47 (br. s, 1, NH); ^{13}C NMR δ 14.00 (C12), 22.51 (C11), 26.75 (C4), 28.47 (C9), 29.72 (C8), 30.10 (C3), 31.37 (C10), 32.67 (C7), 38.74 (C6), 53.77 (C5), 177.65 (CO); MS (APCI) m/z 248 (75, $[\text{MH}+\text{MeOH}]^+$), (ESI) m/z 216 (100, MH^+).

5-(Nonylthiomethyl)pyrrolidin-2-one [160c(5S)]. Treatment of **159**¹⁶⁷ [(5S), 458 mg, 2.58 mmol] in dry DMF (3 mL) with thiolate soln in dry DMF (7 mL) generated from nonanethiol (433 mg, 510 μL , 2.71 mmol) and NaH (114 mg, 2.84 mmol, 60%/mineral oil) in dry DMF (7 mL) by Procedure G [column chromatography (80% EtOAc/hexane $\rightarrow$ EtOAc)] gave **160c(5S)** (652 mg, 98%) as a colorless oil. ^1H NMR δ 0.86 (t, $J = 7.0$ Hz, 3, H15), 1.19-1.29 (m, 10, H10-H14), 1.29-1.38 (m, 2, H9), 1.54 ('q', $J = 7.3$ Hz, 2, H8,8'), 1.75-1.85 (m, 1, H4), 2.23-2.43 (m, 3, H4',H3,3'), 2.51 (t, $J = 7.5$ Hz, 2, H7,7'), 2.54 (dd, $J = 7.4, 13.2$ Hz, 1, H6), 2.65 (dd, $J = 5.8, 13.2$ Hz, 1, H6'), 3.78 ('q', $J = 6.5$ Hz, 1, H5), 6.97 (br. s, 1, NH); ^{13}C NMR δ 14.05 (C15), 22.61 (C14), 26.59 (C3), 28.78 (C9), 29.17, 29.20, 29.43 (C11-C13), 29.76 (C8), 30.14 (C3), 31.81 (C10), 32.70 (C7), 38.63 (C6), 53.93 (C5), 177.97 (CO); MS (ESI) m/z 258 (100, MH^+).

5-(Dodecylthiomethyl)pyrrolidin-2-one [160d(5S)]. Treatment of **159**¹⁶⁷ [(5S), 448 mg, 2.52 mmol] in dry DMF (3 mL) with thiolate soln in dry DMF (7 mL) generated from dodecanethiol (634 μL , 535 mg, 2.65 mmol), and NaH (110 mg, 2.77 mmol, 60%/mineral oil) in dry DMF (7 mL) by Procedure G [column chromatography (80% EtOAc/hexane $\rightarrow$ EtOAc)] gave **160d** (5S) (637 mg, 85%) as a colorless oil. ^1H NMR δ 0.88 (t, $J = 7.0$ Hz, 3, H18), 1.21-1.32 (m, 16, H10-H17), 1.32-1.40 (m, 2, H9), 1.57 ('q', $J = 7.4$ Hz, 2, H8,8'), 1.77-1.87 (m, 1, H4), 2.26-2.45 (m, 3, H4',H3,3'), 2.53 (t, $J = 7.6$ Hz, 2, H7,7'), 2.54 (dd, $J = 7.8, 13.2$ Hz, 1, H6), 2.68 (dd, $J = 5.4, 13.2$ Hz, 1, H6'), 3.80 ('q', $J = 6.6$ Hz, 1, H5), 6.64 (br. s, 1, NH); ^{13}C NMR δ 14.08 (C18), 22.66 (C17), 26.70 (C3), 28.81 (C9), 29.19, 29.32, 29.50, 29.57, 29.60, 29.62 (C11-

C16), 29.77 (C8), 30.11 (C3), 31.89 (C10), 32.69 (C7), 38.71 (C5), 53.83 (C5), 177.74 (CO); MS (ESI) m/z 300 (100, MH^+).

N-tert-Butoxycarbonyl-5-(propylthiomethyl)pyrrolidin-2-one [161a(5S)]. **Procedure E:** DMAP (114 mg, 0.93 mmol), and (Boc)₂O (398 mg, 1.82 mmol) were added to a stirred solution of compound **160a** (77 mg, 0.445 mmol) in CH₂Cl₂ (2 mL) at ambient temperature under Ar atmosphere. After 48 h, the reaction mixture was quenched with H₂O (5 mL) and partitioned between CH₂Cl₂/NaHCO₃/H₂O. The organic layer was washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (30 → 40% EtOAc/hexane) to give **161a** (5S) (107 mg, 88%) as a colorless oil : ¹H NMR δ 0.95 (t, J = 7.3 Hz, 3, H9), 1.50 (s, 9, *t*-Bu), 1.58 (sx, J = 7.3 Hz, 2, H8,8'), 1.96-2.04 (m, 1, H4), 2.06-2.17 (m, 1, H4'), 2.40 (ddd, J = 2.6, 9.6, 17.9 Hz, 1, H3), 2.50 ('dt', J = 4.9, 7.3 Hz, 2, H7,7'), 2.58-2.67 (m, 1, H3'), 2.60 (dd, J = 9.2, 13.5 Hz, 1, H6), 2.86 (ddd, J = 0.5, 2.8, 13.5 Hz, 1, H6'), 4.20-4.27 (m, 1, H5); ¹³C NMR δ 13.31 (C9), 21.91 (C3), 23.12 (C8), 28.03 (*t*-Bu), 31.17 (C2), 34.80 (C7), 35.35 (C6), 57.50 (C5), 83.06 (*t*-Bu), 149.75 (CO), 174.17 (CO); MS (ESI) m/z 274 (10, MH^+), 215 (100, [$MH-59$]⁺).

Structure of compound **160a** was additionally confirmed by conversion to the corresponding *O*-benzyloxime derivative with benzylhydroxylamine hydrochloride (6 equiv.) in anhydrous pyridine: MS (ESI) m/z 281 (60, MH^+), 158 (100, [$M-BnOH$]⁺), (APCI) m/z 281 (100, MH^+).

N-tert-Butoxycarbonyl-5-(Hexylthiomethyl)pyrrolidin-2-one [161b(5S)]. Treatment of **160b** (311 mg, 1.45 mmol) in CH₂Cl₂ (6 mL) with DMAP (185 mg, 1.52 mmol), and (Boc)₂O (746 mg, 3.42 mmol) by procedure E [column chromatography (20 → 40% EtOAc/hexane)] gave **161b** (5S) (429 mg, 94%) as a colorless oil. ¹H NMR δ 0.89 (t, J = 7.0 Hz, 3, H12), 1.25-1.33 (m, 4, H10,H11), 1.34-1.42 (m, 2, H9), 1.55 (s, 9, *t*-Bu), 1.59 ('q', J = 7.4 Hz, 2, H8,8'), 2.01-2.08 (m, 1, H4), 2.10-2.21 (m, 1, H4'), 2.45 (ddd, J = 2.5, 9.6, 17.9 Hz, 1, H3), 2.56 ('dt', J = 2.9, 7.3 Hz, 2, H7,7'), 2.62-2.72 (m, 1, H3'), 2.63 (dd, J = 9.3, 13.5 Hz, 1, H6), 2.91 (dd, J = 2.7, 13.5 Hz, 1, H6'), 4.24-4.31 (m, 1, H5); ¹³C NMR δ 13.99 (C12), 21.97 (C3), 22.51 (C11), 28.07 (*t*-Bu), 28.42

(C9), 29.80 (C8), 31.21 (C3), 31.37 (C10), 32.93 (C7), 35.53 (C6), 57.53 (C5), 83.10 (*t*-Bu), 149.82 (CO), 174.10 (CO); MS (APCI) m/z 316 (5, $M-1^+$), (ESI) m/z 315 (15, M^+), 256 (100, $[M-59]^+$).

***N*-tert-Butoxycarbonyl-5-(nonylthiomethyl)pyrrolidin-2-one [161c(5S)].** Treatment of **160c** (250 mg, 0.97 mmol) in CH_2Cl_2 (5 mL) with DMAP (125 mg, 1.02 mmol), and (Boc)₂O (712 mg, 3.27 mmol) by procedure E [column chromatography (20 → 25% EtOAc/hexane)] gave **161c(5S)** (345 mg, 99%) as a colorless oil. ¹H NMR δ 0.85 (t, J = 7.0 Hz, 3, H15), 1.19-1.29 (m, 10, H10-H14), 1.29-1.38 (m, 2, H9), 1.51 (s, 9, *t*-Bu), 1.55 ('q', J = 7.3 Hz, 2, H8,8'), 1.96-2.04 (m, 1, H4), 2.06-2.17 (m, 1, H4'), 2.40 (ddd, J = 2.5, 9.6, 17.8 Hz, 1, H3), 2.52 ('dt', J = 3.0, 7.4 Hz, 2, H7,7'), 2.58-2.67 (m, 1, H3'), 2.60 (dd, J = 9.3, 13.5 Hz, 1, H6), 2.86 (dd, J = 2.6, 13.5 Hz, 1, H6'), 4.21-4.27 (m, 1, H5); ¹³C NMR δ 14.04 (C15), 21.94 (C4), 22.60 (C14), 28.03 (*t*-Bu), 28.71 (C9), 29.14, 29.18, 29.40 (C11-C13), 29.80 (C8), 31.16 (C3), 31.80 (C10), 32.87 (C7), 35.50 (C6), 57.48 (C5), 82.99 (*t*-Bu), 149.77 (CO), 174.01 (CO); MS (APCI) m/z 358 (10, MH^+), (ESI) m/z 357 (10, M^+), 299 (100, $[MH-59]^+$).

***N*-tert-Butoxycarbonyl-5-(dodecylthiomethyl)pyrrolidin-2-one [161d(5S)].** Treatment of **160d** (234 mg, 0.78 mmol) in CH_2Cl_2 (5 mL) with DMAP (100 mg, 0.82 mmol), and (Boc)₂O (600 mg, 2.75 mmol) by procedure E [column chromatography (15 → 20% EtOAc/hexane)] gave **161d(5S)** (302 mg, 97%) as a solidifying oil. ¹H NMR δ 0.87 (t, J = 7.0 Hz, 3, H18), 1.21-1.31 (m, 16, H10-H17), 1.31-1.40 (m, 2, H9), 1.53 (s, 9, Boc), 1.58 ('q', J = 7.5 Hz, 2, H8,8'), 1.99-2.06 (m, 1, H4), 2.08-2.20 (m, 1, H4'), 2.43 (ddd, J = 2.5, 9.6, 17.9 Hz, 1, H3), 2.54 ("dt", J = 2.9, 7.4 Hz, 2, H7,7'), 2.60-2.70 (m, 2, H3'), 2.62 (dd, J = 9.2, 13.5 Hz, 1, H6), 2.89 (dd, J = 2.6, 13.5 Hz, 1, H6'), 4.23-4.29 (m, 1, H5); ¹³C NMR δ 14.08 (C18), 21.96 (C4), 22.65 (C17), 28.06 (*t*-Bu), 28.75 (C9), 29.18, 29.31, 29.48, 29.56, 29.59, 29.61 (C11-C16), 29.83 (C8), 31.19 (C3), 31.88 (C10), 32.91 (C7), 35.52 (C6), 57.51 (C5), 83.04 (*t*-Bu), 149.81 (*t*-Bu), 174.04 (CO); MS (APCI) m/z 300 (25, $[M-Boc]^+$), (ESI) m/z 400 (10, MH^+), 341 (100, $[MH-59]^+$).

5-(Propylthiomethyl)pyrrolidin-2-ol [162a(5S)]. Procedure F. LiEt₃BH (1M soln in THF, 0.98 mL, 0.98 mmol) was added to a stirred solution of **161a** (107 mg, 0.39 mmol) in CH₂Cl₂ (3 mL) at -78 °C under N₂ atmosphere. After 30 min, the solution was quenched with MeOH (4 mL) and allowed to warm to ambient temperature. Volatiles were evaporated. The residue was partitioned (EtOAc// NaHCO₃/H₂O), washed (brine) and dried (MgSO₄). The resulting oil was chromatographed (~~30~~ 40% EtOAc/hexane) to give *N*-*tert*-butoxycarbonyl-5-(propylthiomethyl)pyrrolidin-2-ol **162a(5S)**; 104 mg, 96%] as a colorless oil of the mixture of 4 anomers/rotamers: MS (APCI) *m/z* 274 (10, [M-1]⁺), 258 (90, [M-17]⁺), (ESI) *m/z* 258 (65, [M-17]⁺). **Procedure D.** Compound **162a** (104mg, 0.37 mmol) in TFA (4.0 mL) was stirred at rt for 2 h. Volatiles were evaporated to give **163a** as light yellow oil having spectral properties of a complex mixture [4 isomers of aza-furanose forms accompanied by 25% of aldehyde (chain form), as indicated by ¹H and ¹³C NMR spectra]: MS (ESI) *m/z* 158 (10, [M-17]⁺).

5-(Hexylthiomethyl)pyrrolidin-2-ol [163b(5S)]. Treatment of **161b** (178 mg, 0.56 mmol) in CH₂Cl₂ (3 mL) with LiEt₃BH (1 M soln in THF, 1.41 mL, 1.41 mmol), by procedure F [column chromatography (~~30~~ 40% EtOAc/hexane)] gave *N*-*tert*-butoxycarbonyl-5-(hexylthiomethyl)pyrrolidin-2-ol [**162b(5S)**; 170 mg, 95%] as a colorless oil as a mixture of 4 isomers: MS (ESI) *m/z* 316 (100, [M-1]⁺), 300 (20, [M-17]⁺). Treatment of **162b** with an excess of TFA by Procedure D (step *a*, 2 h at rt) gave crude **163b** as light yellow oil as a mixture of 4 isomers of the aza-furanose form accompanied by 25% of aldehyde (chain form); MS (APCI) *m/z* 200 (100, [M-17]⁺) (ESI) *m/z* 200 (10, [M-17]⁺).

5-(Nonylthiomethyl)pyrrolidin-2-ol [163c(5S)]. Treatment of **161c** (227 mg, 0.64 mmol) in CH₂Cl₂ (4 mL) with LiEt₃BH (1 M soln in THF, 1.59 mL, 1.59 mmol), by procedure F [column chromatography (~~20~~ 30% EtOAc/hexane)] gave *tert*-Butoxycarbonyl-5-(nonylthiomethyl)pyrrolidin-2-ol [**162c(5S)**; 220 mg, 96%; as a colorless oil of a complex mixture of 4 isomers]: MS (ESI) *m/z* 358 (10, [M-1]⁺), 342 (100, [M-17]⁺). Treatment of **162c**

with an excess of TFA by Procedure D (step *a*, 2 h at rt) gave crude **163c** as light yellow oil as a mixture of 4 isomers of the aza-furanose forms accompanied by 25% of the aldehyde (chain form): MS (APCI) *m/z* 258 (15, [M-1]⁺), 242 (100, [M-17]⁺).

5-(Dodecylthiomethyl)pyrrolidin-2-ol [163d(5S)]. Treatment of **162d** (224 mg, 0.56 mmol) in CH₂Cl₂ (4 mL) with LiEt₃BH (1 M soln in THF, 1.4 mL, 1.4 mmol), by procedure F [column chromatography (20–30% EtOAc/hexane)] gave *N*-*tert*-butoxycarbonyl-5-(dodecylthiomethyl)pyrrolidin-2-ol [**162d(5S)**] (219 mg, 97%) as a solidifying oil of the mixture of 4 isomers]: MS (APCI) *m/z* 400 (50, [M-1]⁺), 384 (100, [M-17]⁺). Treatment of **162d** with an excess of TFA by Procedure D (step *a*, 2 h at rt) gave crude **163d** as light yellow oil as a mixture of 4 isomers of aza-furanose form accompanied by 25% of aldehyde (chain form): MS (APCI) *m/z* 301 (5, M⁺), 300, (20, [M-1]⁺), 284 (100, [M-17]⁺).

***N*-(*tert*-Butoxycarbonyl)-3,4-dihydroxy-3,4-*O*-isopropylidene-5-(hexylthiomethyl)pyrrolidin-2-one [164 (3*R*,4*R*,5*S*)]**. Treatment of **136** (60 mg, 0.16 mmol) in dry DMF (0.5 mL) with sodium hexathiolate (generated from hexanethiol (46.8 μL, 0.33 mmol)/NaH (14mg, 0.35 mmol, 60%/mineral oil) in dry DMF (0.5 mL)) by Procedure G [column chromatography (5% → 10% MeOH/EtOAc)] gave **164** (25 mg, 40%) as a colorless oil and *N*-Boc deprotected **164** (24 mg, 38%) as a white crystalline solid. Compound **164** had: ¹H NMR δ 0.81 (t, *J* = 7.0 Hz, 3, H12), 1.16-1.27 (m, 6, H9, 10, 11), 1.30 (s, 3, CH₃), 1.39 (s, 3, CH₃), 1.44-1.59 (m, 11, H8,8', *t*-Bu), 2.36-2.50 (m, 2, H7,7'), 2.76 (dd, *J* = 6.2, 14.4 Hz, 1, H6), 2.82 (dd, *J* = 2.7, 14.4 Hz, 1, H6'), 4.31 (dd, *J* = 2.7, 6.2 Hz, 1, H5), 4.38 (d, *J* = 5.5 Hz, 1, H4), 4.78 (d, *J* = 5.5 Hz, 1, H3); ¹³C NMR δ 14.00 (C12), 22.49 (C10), 24.38 (CMe₂), 25.46 (CMe₂), 27.97 (*t*-Bu), 28.30 (C9), 29.63 (C8), 31.33 (C11), 33.67 (C7), 33.85 (C6), 60.84 (C5), 76.09 (C4), 77.63 (C3), 83.94 (*t*-Bu), 112.27 (CMe₂), 170.59 (CO) 149.80 (CO); MS (APCI) *m/z* 288 (100, [MH-Boc]⁺). *N*-Boc deprotected **164** had: ¹H NMR δ 0.88 (t, *J* = 7.0 Hz, 3, H12), 1.25-1.36 (m, 6, H9,10,11), 1.38 (s, 3, CMe₂), 1.48 (s, 3, CMe₂), 1.56-1.62 (m, 2, H8,8'), 2.52-2.75 (m, 3, H6,7,7'), 2.73 (dd,

$J = 5.9, 13.4$ Hz, 1, H6'), 3.81 (t', $J = 6.1$ Hz, 1, H5), 4.50 (d, $J = 5.9$ Hz, 1, H4), 4.69 (d, $J = 5.9$ Hz, 1, H3), 5.94 (s, 1, NH); ^{13}C NMR δ 14.01 (C12), 29.66 (C8), 22.52, 28.49, 31.36 (C9, C10, C11), 25.57 (CMe_2), 26.94 (CMe_2), 33.19 (C7), 33.66 (C6), 58.01 (C5), 76.60 (C4), 79.15 (C3), 112.73 (CMe_2), 173.23 (CO); MS (APCI) m/z 288 (100, $[\text{MH}^+]$).

***N*-(*tert*-Butoxycarbonyl)-3,4-dihydroxy-3,4-*O*-isopropylidene-5-(nonylthiomethyl)pyrrolidin-2-one [165(3*R*,4*R*,5*S*)].** Treatment of **136** (60 mg, 0.16 mmol) in dry DMF (0.5 mL) with nonathiolate [generated from nonanethiol (62 μL , 0.33 mmol)/NaH (14 mg, 0.35 mmol, 60% mineral oil) in dry DMF (0.5 mL)] by Procedure G (column chromatography (5% $\rightarrow$ 10% MeOH/EtOAc) gave **165** (30 mg, 42%) as a colorless oil: ^1H NMR δ ; 0.87 (t, $J = 7.0$ Hz, 3, H15), 1.25-1.31 (m, 12, H9-H14), 1.36 (s, 3, CMe_2), 1.45 (s, 3, CMe_2), 1.56-1.57 (m, 11, H8,8', *t*-Bu), 2.44-2.54 (m, 2, H7,7'), 2.82 (dd, $J = 6.2, 14.4$ Hz, 1, H6), 2.91 (dd, $J = 2.7, 14.4$ Hz, 1, H6'), 4.38 (dd, $J = 2.7, 6.2$ Hz, 1, H5), 4.45 (d, $J = 5.5$ Hz, 1, H4), 4.85 (d, $J = 5.5$ Hz, 1, H3); ^{13}C NMR δ 14.07 (C15), 22.64, 28.68, 29.15, 29.22, 29.43, 31.83 (C9-14), 29.66 (C8), 24.38 (CMe_2), 25.46 (CMe_2), 26.99 (C9), 27.97 (*t*-Bu), 33.67 (C7), 33.87 (C6), 60.84 (C5), 76.09 (C4), 77.61 (C3), 83.93 (*t*-Bu), 112.27 (CMe_2), 149.80 (CO), 170.58 (CO); MS (APCI) m/z 330 (100, $[\text{MH}_2\text{-Boc}]^+$).

3,4-Dihydroxy-5-(hexylthiomethyl)pyrrolidin-2-one [166(3*R*,4*R*,5*S*)]. TFA/H₂O (1 mL, 9:1) was added to *N*-Boc deprotected **164** (22 mg, 0.07 mmol) or **164** and the resulting solution was stirred at 0 °C 3 h. Evaporation of volatiles gave light yellow oil that was column chromatographed (5 $\rightarrow$ 10% MeOH/EtOAc) to give **166** (12 mg, 63%) as a colorless oil: ^1H NMR δ 0.87 (t, $J = 7.0$ Hz, 3, H12), 1.24-1.39 (m, 6, H9, 10, 11), 1.52-1.59 (m, 2, H8,8'), 2.50-2.55 (m, 3, H5,7,7'), 2.73 (dd, $J = 5.5, 13.6$ Hz, 1, H6'), 3.71 (t', $J = 6.4$ Hz, 1, H5), 4.21 (d, $J = 5.0$ Hz, 1, H4), 4.44 (d, $J = 5.0$ Hz, 1, H3), 7.11 (br. s, 1, NH); ^{13}C NMR δ 14.01 (C12), 29.63 (C8), 14.11, 22.55, 31.42 (C9,C10,C11), 32.73 (C7), 35.27 (C6), 59.85 (C5), 69.77 (C4), 71.75 (C3), 175.97 (CO); MS (APCI) m/z 248 (100, $[\text{MH}]^+$).

3,4-Dihydroxy-5-(nonylthiomethyl)pyrrolidin-2-one [167(3*R*,4*R*,5*S*)]. Treatment of **165** (20 mg, 0.04 mmol) with TFA/H₂O (1 mL, 9:1) as described for **164** gave **167** (10 mg, 85%) as a colorless oil. ¹H NMR 0.88 (t, *J* = 6.8 Hz, 3, H15), 1.20-1.38 (m, 12, H9-H14), 1.52-1.59 (m, 2, H8,8'), 2.49-2.54 (m, 3, H 6,7,7'), 2.70 (dd, *J* = 5.4, 13.5 Hz, 1, H6'), 3.71 (t, *J* = 5.6 Hz, 1, H5), 4.25 (d, *J* = 4.9 Hz, 1, H4), 4.49 (d, *J* = 4.6 Hz, 1, H3), 7.15 (br. s, 1, NH); ¹³C NMR δ 14.07 (C15), 22.66, 28.84, 29.24, 29.27, 29.65, 31.86 (C9-14), 29.50 (C8), 26.99 (C9), 32.61 (C7), 34.99 (C6), 60.66 (C5), 69.74 (C4), 72.50 (C3), 176.24 (CO); MS (APCI) *m/z* 290 (100, [MH]⁺).

3,4-Dihydroxy-5-(hexylthiomethyl)pyrrolidin-2-ol [170(3*R*,4*R*,5*S*)]. Treatment of **164** (40 mg, 0.1 mmol) in THF (1 mL) with LiEt₃BH (1 M/THF, 0.26 mL, 0.26 mmol), by procedure F [column chromatography (10→ 20% EtOAc/hexane)] gave *N*-(*tert*-butoxycarbonyl)-3,4-dihydroxy-3,4-*O*-isopropylidene-5-hexylthiomethyl-pyrrolidine-2-ol [**168** (3*R*,4*R*,5*S*)]; 39 mg, 97%) as a colorless oil of the mixture of 4 isomers: MS (ESI) *m/z* 389 (100, [M-17]⁺). Treatment of **168** (39 mg, 0.1 mmol) with TFA/H₂O (0.9:0.1 mL) at 0 °C for 3 h gave a light yellow oil that was column chromatographed (5→ 10% MeOH/EtOAc) to give **170** (22 mg, 88%) as a light yellow oil. ¹H NMR showed a complex mixture of four isomers of the aza-furanose form also accompanied by aldehyde (chain form); MS (APCI) *m/z* 250 (20, [MH]⁺), 232 (60, [M-17]⁺).

3,4-Dihydroxy-5-(nonylthiomethyl)pyrrolidin-2-ol [171(3*R*,4*R*,5*S*)]. Treatment of **165** (40 mg, 0.09 mmol) in THF (1 mL) with LiEt₃BH (1 M/THF, 0.22 mL, 0.22 mmol), by procedure F [column chromatography (10→ 20% EtOAc/hexane)] gave *N*-(*tert*-butoxycarbonyl)-3,4-dihydroxy-3,4-*O*-isopropylidene-5-nonylthiomethyl-pyrrolidine-2-ol [**168**(3*R*,4*R*,5*S*)]; 30 mg, 75%) as a colorless oil as a mixture of 4 isomers: MS (APCI) *m/z* 414 (100, [M-17]⁺), 314 (95 [MH-Boc-H₂O]⁺). Treatment of **169** (28 mg, 0.06 mmol) with an excess of TFA/H₂O (0.9:0.1 mL) at 0 °C for 3 h gave a light yellow oil that was column chromatographed (10→ 10% MeOH/EtOAc) to give **171** (18 mg, 96%) as a light yellow oil. ¹H NMR showed a complex

mixture of four isomers of the aza-furanose form also accompanied by aldehyde (chain form); MS (APCI) m/z 292 (80, $[MH]^+$), 274 (60, $[M-17]^+$).

S-(Methyl 2,3-*O*-Isopropylidene- β -D-ribofuranos-5-yl)-*N*-(*tert*-butoxycarbonyl)-L-homocysteine *tert*-butyl ester (173**).** Treatment of **172** (170 mg, 0.64 mmol) (prepared according to reported methods¹⁷¹ with NMR data as reported¹⁸⁹ with lithium homocysteinate (260 mg, 0.89 mmol) by Procedure B [column chromatography (15 $\rightarrow$ 20% EtOAc/hexane)] gave **173** (220 mg, 72%) as a light yellow oil: 1H NMR δ 1.32 (s, 3, CH₃), 1.45 (s, 9, *t*-Bu), 1.47 (s, 12, *t*-Bu, CH₃), 2.07-2.21 (m, 2, H8,8'), 2.54-2.59 (m, 3, H5,7,7'), 2.77 (dd, J = 6.1, 13.4 Hz, 1, H5'), 4.21-4.25 (m, 1, H9), 4.23 (dd, J = 6.1, 9.5 Hz, 1, H4), 4.60 (d, J = 5.9 Hz, 1, H3), 4.70 (d, J = 5.9 Hz, 2, H2), 5.10 (s, 1, H1), 5.30 (br. s, 1, NH); ^{13}C NMR δ 24.94 (CMe_2), 26.42 (CMe_2), 27.99 (*t*-Bu), 28.31 (*t*-Bu), 27.73 (C7), 33.18 (C8), 35.58 (C5), 53.77 (C9), 54.93 (OCH₃), 83.32 (C2), 85.26 (C3), 85.78 (C4), 109.59 (C1), 82.17 (*t*-Bu), 82.21 (*t*-Bu), 112.44 (CMe_2), 155.32 (CO), 171.05 (CO); MS m/z 500 (100, $[M+Na]^+$).

S-Ribosyl-L-homocysteine. Treatment of **173** (30 mg, 0.06 mmol) with TFA/H₂O (9:1, 3 mL) by Procedure C at 0 °C for 12 h gave SRH **174** (8.4 mg, 50%) as a colorless oil with data as reported.¹⁷⁰

Methyl 2-Deoxy-5-*O*-methanesulfonyl- α/β -D-erythro-pentofuranoside (176**).** Treatment of **175**¹⁷² (550 mg, 3.73 mmol) with MsCl (0.290 mL, 316 mg, 4.1 mmol) in the presence of Et₃N (1.56 mL, 1.13 g, 11.19 mmol) by Procedure A [3 h; column chromatography (EtOAc $\rightarrow$ 10% MeOH/EtOAc)] gave **176** (α/β , 3:2; 367 mg, 43%): 1H NMR δ 1.99-2.18 (m, 2, H2), 3.06 (s, 1.8, Ms), 3.09 (s, 1.2, Ms), 3.39 (s, 1.2, OMe), 3.41 (s, 1.8, OMe), 4.19-4.25 (m, 0.6, H3), 4.25-4.32 (m, 2.6, H4,5,5'), 4.08-4.14 (m, 0.4, H4), 4.44 (dt, J = 4.5, 5.6 Hz, 0.4, H3), 5.09 (dd, J = 1.6, 5.2 Hz, 0.4, H1), 5.11 (d, J = 4.6 Hz, 0.6, H1); ^{13}C NMR δ 37.50 (Ms), 37.53 (Ms), 41.05 (C2), 41.31 (C2), 55.06 (OCH₃), 55.16 (OCH₃), 69.31 (C5), 70.21 (C5), 71.87 (C4), 72.30 (C4), 83.14 (C3), 83.99 (C3), 105.49 (C1), 105.55 (C1); MS (APCI) m/z 240 (100, $[M+H_2O]^+$).

Methyl 3-*O*-Benzoyl-2-deoxy-5-*O*-methanesulfonyl- α/β -D-erythro-pentofuranoside (177).

Procedure H. A mixture of **176** (300 mg, 1.3 mmol), 2,6-lutidine (0.30 mL, 278 mg, 2.6 mmol) and catalytic amount of DMAP (7.5 mg, 0.65 mmol) in CH₂Cl₂ was heated to 35 °C and BzCl (0.180 mL, 218 mg, 1.56 mmol) was added to this mixture and stirred overnight. The mixture was cooled to room temperature and partitioned (CH₂Cl₂/NaHCO₃/H₂O). The organic layer was washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (25% EtOAc/hexane) to give **177** (α/β , 3:2; 440 mg, 95%) isolated by column chromatography. The α -Anomer had: ¹H NMR δ 2.36 (dt, J = 5.3, 10.7, 14 Hz, 1, H₂), 2.50 (ddd, J = 2.1, 7.3, 14 Hz, 1, H_{2'}), 3.10 (s, 3, Ms), 3.41 (s, 3, OMe), 4.35-4.44 (m, 3, H_{4,5}), 4.49 (dd, J = 4.4, 9.5 Hz, 1, H_{5'}), 5.22 (dd, J = 2.1, 5.4 Hz, 1, H₁), 5.45-5.49 (m, 1, H₃), 7.45-7.96 (m, 5, H_{arom}); ¹³C NMR δ 37.72 (Ms), 39.02 (C₂), 55.52 (OCH₃), 69.80 (C₅), 75.01 (C₃), 81.73 (C₄), 105.49 (C₁), 105.87 (C₁), 128.51, 129.70, 133.49 (C_{arom}), 166.45 (OCOPh); MS (APCI) m/z 147 ([MH-Bz-Ms]⁺). β -Anomer had: ¹H NMR δ 2.21 (dd, J = 1.2, 14.4 Hz, 1, H₂), 2.48 (ddd, J = 5.3, 8.3, 14.4 Hz, 1, H_{2'}), 3.08 (s, 3, Ms), 3.41 (s, 1.0, OMe), 4.37 (q, 1, H₄), 4.54 (d, J = 3.4 Hz, 2, H₅), 5.18 (d, J = 5.0 Hz, 1, H₁), 5.30-5.33 (m, 1, H₃), 7.45-8.06 (m, 5, H_{arom}).

S-(Methyl 3-*O*-Benzoyl-2-deoxy- β -D-erythro-pentofuranos-5-yl)-*N*-(*tert*-butoxycarbonyl)-L-homocysteine *tert*-butyl ester (178). Treatment of β -anomer of **177** (427 mg, 1.29 mmol) with protected L-Hcy (530 mg, 1.8 mmol) by Procedure B (step *a* and *b*, 36 h) gave 950 mg of oily residue. The crude was column chromatographed (20 $\rightarrow$ 30% EtOAc/hexane) to give **178** as a colorless oil (440 mg, 64%). β -Anomer had: ¹H NMR δ 1.39 (s, 9, *t*-Bu), 1.42 (s, 9, *t*-Bu), 1.84-1.89 (m, 1, H₈), 2.03-2.09 (m, 1, H_{8'}), 2.10 (dd, J = 1.5, 14.5, 1, H₂), 2.51 (ddd, J = 5.3, 8.4, 14.3 Hz, 1, 2'), 2.63 ('t', J = 7.8 Hz, 2, H_{7,7'}), 2.84 (dd, J = 5.7, 14.0 Hz, 1, H₅), 2.92 (dd, J = 4.4, 13.8 Hz, 1, H_{5'}), 3.36 (s, 3, OMe), 4.17-4.26 (m, 1, H₉), 4.35 (dt, J = 4.3, 5.4 Hz, 1, H₄), 5.10 (d, J = 5.0 Hz, 1, H₁), 5.13 (br. d, J = 7.9 Hz, 1, NH), 5.20 (ddd, J = 2.3, 4.0, 8.3 Hz, 1, H₃), 7.42-8.02 (m, 5, H_{arom}); ¹³C NMR δ 27.88 (*t*-Bu), 28.31 (*t*-Bu), 29.03

(C7), 33.15 (C8), 34.86 (C5), 39.25 (C2), 54.96 (C9), 55.48 (OCH₃), 79.73 (C3), 82.53 (C4), 104.79 (C1), 82.06 (*t*-Bu), 83.68 (*t*-Bu), 128.36, 129.75, 129.86, 133.15 (C_{arom}), 155.36 (CO), 166.45 (COPh), 171.12 (CO); MS (APCI) *m/z* 426 (60, [MH₂-Boc]⁺). Treatment of **178** with TFA/H₂O resulted in a complex mixture indicating decomposition of sugar. Therefore, 2-deoxy-SRH could not be synthesized from **178**.

S-(Methyl 2-Deoxy- α/β -D-erythro-pentofuranos-5-yl)homocysteine (179) Compound **176** (65 mg, 0.28 mmol) was added to 1 M NaOH/ H₂O (5 mL) and degassed with N₂ for 30 min, followed by addition of D/L-Homocysteine (56.7 mg, 0.42 mmol) and the suspension was heated to 60 °C. After 12 h, the reaction mixture was cooled to room temperature, neutralized with dilute HCl to pH 7 and washed with EtOAc (3 $\times$ 10 mL).¹⁰⁹ The water layer was evaporated and purified by HPLC (5% CH₃CN/H₂O for 35 min at 2.5 mL/min; *t*_R = 13.6 min) to give **179** (α/β , 3:1; 30 mg, 37%). β -Anomer had: ¹H NMR (D₂O) δ 1.8-1.84 (m, 1, H2), 2.30-2.37 (m, 1, H2'), 2.02-2.15 (m, 2, H8), 2.61-2.78 (m, 4, H5,5',7,7'), 3.76 (t, *J* = 5.8 Hz, 1 H9), 4.05-4.09 (m, 1, H4), 4.11-4.15 (m, 1, H3), 3.28 (s, 3, OMe), 5.10 (d, *J* = 5.0 Hz, 1, H1), 5.13 (br. d, *J* = 7.9 Hz, 1, NH), 5.06 (d, *J* = 4.7 Hz, 1, H1); ¹³C NMR δ 27.49 (C7), 30.38 (C8), 39.85 (C5), 39.25 (C2), 53.78 (C9), 54.79 (OCH₃), 79.60 (C4), 84.20 (C3), 105.48 (C1), 174.10 (COOH); MS (APCI) *m/z* 266 (100, MH⁺).

1,2-O-Isopropylidene-5-O-methanesulfonyl- β -D-arabinofuranoside (182). Treatment of **181**¹⁷³ (85 mg, 0.44 mmol) with MsCl (0.03mL, 37.7 mg, 0.49 mmol) in the presence of Et₃N (0.186 mL, 135 mg, 1.34 mmol) by Procedure A [3 h; column chromatography (60 $\rightarrow$ 70% EtOAc/hexane) gave **182** (40 mg, 33%) as a colorless oil: ¹H NMR δ 1.32 (s, 3, CH₃), 1.55 (s, 3, CH₃), 3.12 (s, 3, Ms), 4.30 (br. s, 1, H3), 4.25 (ddd, *J* = 1.9, 5.4, 7.3 Hz, 1, H4), 4.36 (dd, *J* = 5.4, 10.9 Hz, 1, H5), 4.47 (dd, *J* = 7.3, 10.9 Hz, 1, H5'), 4.60 (d, *J* = 3.8 Hz, 1, H2), 5.98 (d, *J* = 3.8 Hz, 1, H1); ¹³C NMR δ 25.81 (CMe₂), 26.59 (CMe₂), 37.94 (Ms), 68.81 (C5), 75.50 (C3), 85.10 (C4), 105.49 (C1), 86.38 (C2), 106.13 (C1), 113.0 (CMe₂).

3-*O*-Benzoyl-1,2-*O*-isopropylidene-5-*O*-methanesulfonyl- β -D-arabinofuranoside (183).

Treatment of **182** (35 mg, 0.13 mmol) with 2,6-lutidine (0.030 mL, 27.8 mg, 2.6 mmol), DMAP (0.75 mg, 0.065 mmol) and BzCl (0.018 mL, 21.8 mg, 0.156 mmol) in CH₂Cl₂ by Procedure H [12 h; column chromatography (50 $\rightarrow$ 60% EtOAc/hexane) afforded **183** (52 mg, 63%): ¹H NMR δ 1.32 (s, 3, CH₃), 1.62 (s, 3, CH₃), 3.13 (s, 3, Ms), 4.45-4.56 (m, 3, H_{4,5,5'}), 4.80 (d, J = 3.8 Hz, 1, H₂), 5.30 (s, 1, H₃), 6.05 (d, J = 3.8 Hz, 1, H₁), 7.46-8.01 (m, 5, H_{arom}); ¹³C NMR δ 25.75 (CMe₂), 26.49 (CMe₂), 38.05 (Ms), 68.63 (C₅), 77.43 (C₃), 84.14 (C₂), 83.32 (C₄), 106.34 (C₁), 113.23 (CMe₂), 128.48, 128.84, 129.81, 133.80 (C_{arom}), 165.41 (OCOPh); MS (APCI) m/z 373 (10, MH⁺).

3-*O*-Benzoyl-5-bromo-1,2-*O*-isopropylidene- β -D-arabinofuranoside (184). Compound **183** (40 mg, 0.107 mmol) and NaBr (12 mg, 0.117 mmol) were heated in DMF (2 mL) under reflux for 12 h. The reaction mixture was poured into ice water (20 mL). The mixture was partitioned (CH₂Cl₃/NaHCO₃/H₂O). The organic layer was washed (brine), dried (MgSO₄) and evaporated. The residue was column chromatographed (5 $\rightarrow$ 10% EtOAc/hexane) to give **184** (18 mg, 47%): ¹H NMR δ 1.34 (s, 3, CH₃), 1.59 (s, 3, CH₃), 3.62 (dd, J = 6.3, 10.2 Hz, 1, H₅), 3.71 (dd, J = 8.4, 10.3 Hz, 1, H_{5'}), 4.48 (t, J = 7.6 Hz, 1, H₄), 4.74 (d, J = 3.8 Hz, 1, H₂), 5.49 (br. s, 1, H₃), 6.03 (d, J = 3.8 Hz, 1, H₁), 7.46-8.03 (m, 5, H_{arom}); ¹³C NMR δ 25.83 (CMe₂), 26.85 (CMe₂), 31.35 (C₅), 78.43 (C₃), 83.32 (C₄), 84.47 (C₂), 85.39 (C₄), 106.32 (C₁), 113.14 (CMe₂), 128.54, 129.08, 129.83, 133.64 (C_{arom}), 165.30 (OCOPh).

***S*-(3-*O*-Benzoyl-1,2-*O*-isopropylidene- β -D-arabinofuranos-5-yl)-*N*-(*tert*-butoxycarbonyl)-L-homocysteine *tert*-butyl ester (185).** Treatment of **184** (7 mg, 0.019 mmol) with protected L-Hcy (6.6 mg, 0.02 mmol) by Procedure B (step *a* and *b*, 36 h) [column chromatographed (20 $\rightarrow$ 25% EtOAc/hexane)] gave **185** as a colorless oil (5 mg, 18%): ¹H NMR δ 1.32 (s, 3, CH₃), 1.37 (s, 9, *t*-Bu), 1.38 (s, 9, *t*-Bu), 1.80-1.87 (m, 1, H₈), 1.88-2.02 (m, 1, H_{8'}), 2.52-2.63 (m, 2, H_{7,7'}), 2.86 (dd, J = 6.9, 13.8 Hz, 1, H₅), 2.95 (dd, J = 8.0, 13.8 Hz, 1, H_{5'}), 4.29

(br. s, 1, H9), 4.30 (t, $J = 6.6$ Hz, 1, H4), 4.66 (d, $J = 3.9$ Hz, 1, H2), 5.13 (br. d, $J = 7.6$, 1, NH), 5.29 (br. s, 1, H3), 5.93 (d, $J = 3.9$ Hz, 1, H1), 7.37-7.96 (m, 5, H_{arom}); MS m/z 590 (45, [M+Na]⁺).

1-*O*-Benzoyl-2-deoxy-2-fluoro-3,5-di-*O*-triisopropylsilyl- α/β -D-arabinofuranoside (189).

Procedure G. Treatment of **188**¹⁷⁴ (540 mg, 1.16 mmol) with 2,6-lutidine (0.26 mL, 248 mg, 2.32 mmol), DMAP (6.7 mg, 0.58 mmol) and BzCl (0.16 mL, 194 mg, 1.39 mmol) by Procedure H [column chromatographed (3 $\rightarrow$ 5% EtOAc/hexane)] gave **189** (α/β , 1:1; 500 mg, 77 %): ¹H NMR δ 0.78-1.21 (m, 42H, TIPS), 3.77-3.93 (m, 2, H5,5'), 4.26-4.35 (m, 1, H4), 4.78-4.86 (m, 1, H3), 5.16-4.96 (m, 1, H2), 6.46-6.53 (m, 0.5, H1), 6.56-6.62 (m, 0.5, H1), 7.40-7.96 (m, 5, H_{arom}); ¹⁹F NMR δ -188.58 (ddd, $J = 11.2, 20.8, 44.7$ Hz, 1, F2), -203.26 (dd, $J = 15.5, 52.2$ Hz, 1, F2); (APCI) m/z 569 (100, MH⁺).

1-*O*-Benzoyl-2-deoxy-2-fluoro- α/β -D-arabinofuranoside (190). A mixture of **189** (100 mg, 0.17 mmol) and NH₄F (51.8 mg, 1.4 mmol) was refluxed in methanol for 12 h at 60 °C. The reaction mixture was evaporated and column chromatographed to give (75 $\rightarrow$ 80% EtOAc/hexane) **190** (α/β , 1:1; 17 mg, 37 %): β -Anomer had: ¹H NMR δ 3.85 (dd, $J = 3.4, 12.4$ Hz, 1, H5), 3.69 (dd, $J = 4.1, 12.6$ Hz, 1, H5'), 3.99 (dt, $J = 3.8, 7.4$ Hz, 1, H4), 4.71 (dt, $J = 7.2, 17.0$ Hz, 1, H3), 5.12 (dd, $J = 1.7, 50.7$ Hz, 1, H2), 6.43 (d, $J = 1.3$ Hz, 1, H1), 7.37-8.01 (m, 5, H_{arom}); ¹³C NMR δ 62.09 (C5), 72.31 (d, $J = 21.5$ Hz, 1, C3), 82.42 (d, $J = 9.6$ Hz, 1, C4), 99.83 (d, $J = 38.5$ Hz, 1, C1), 100.55 (d, $J = 200.9$ Hz, 1, C2), 128.60 (d, $J = 3.5$ Hz), 129.21 (d, $J = 7$ Hz), 129.90 (d, $J = 7.1$ Hz), 133.72 (d, $J = 4.3$ Hz) (C_{arom}), 171.32 (OCOPh); ¹⁹F NMR δ -190.63 (ddd, $J = 11.3, 23.2, 50.6$ Hz, 1, F2). α -Anomer had ¹H NMR δ 3.81 (ddd, $J = 1.74, 3.7, 12.5$ Hz, 1, H5), 3.73 (dd, $J = 4.0, 12.6$ Hz, 1, H5'), 4.22 (dt, $J = 3.8, 5.8$ Hz, 1, H4), 4.45 (ddd, $J = 1.7, 5.8, 23.3$ Hz, 1, H3), 5.07 (ddd, $J = 5.0, 7.3, 52.6$ Hz, 1, H2), 6.49 (d, $J = 4.5$ Hz, 1, H1), 7.37-8.01 (m, 5, H_{arom}); ¹³C NMR δ 61.77 (C5), 75.35 (d, $J = 22$ Hz, 1, C3), 86.00 (d, $J = 4$ Hz, 1, C4),

93.26 (d, $J = 18.1$ Hz, 1, C1), 94.53 (d, $J = 184.5$ Hz, 1, C2), 128.46, 129.49, 130.16, 133.61 (C_{arom}), 165.1 (OCOPh); ^{19}F NMR δ -190.63 07 (ddd, $J = 11.3, 23.2, 50.6$ Hz, 1, F2).

1-*O*-Benzoyl-2-deoxy-2-fluoro-5-*O*-methanesulfonyl- α/β -D-arabinofuranoside (191**).**

Treatment of **190** (15 mg, 0.05 mmol) with MsCl (5.0 μL , 7.3 mg, 0.06 mmol) in the presence of Et_3N (21 μL , 15.1 mg, 0.15 mmol) by Procedure A [3 h; column chromatography (60 $\rightarrow$ 70% EtOAc /hexane) gave **191** (α/β , 1:1; 13 mg, 65%) as a crystalline solid : ^1H NMR δ 2.98 (s, 3, Ms), 3.13 (s, 3, Ms), 4.41 ('br. s', 4, H5,5'), 4.46 (dd, $J = 4.1, 6.8$ Hz, 1, H4), 4.60 ('q', $J = 4.0$ Hz, 1, H4), 5.13 (dd, $J = 4.2, 19.6$ Hz, 1, H3), 5.26-5.40 (m, 3, H2,3), 6.55 (d, $J = 8.0$ Hz, 1, H1), 6.56 (d, $J = 5.8$ Hz, 1, H1), 7.39-8.10 (m, 10, H_{arom}); ^{13}C NMR δ 37.82 (Ms), 38.31 (Ms), 66.40 (C5), 67.28 (C5), 77.77 (d, $J = 8$ Hz, 1, C4), 78.90 (d, $J = 23$ Hz, 1, C3), 79.35 (d, $J = 31$ Hz, 1, C3), 91.79 (d, $J = 204$ Hz, 1, C2), 92.57 (d, $J = 18$ Hz, 1, C1), 97.05 (d, $J = 37$ Hz, 1, C1), 97.07 (d, $J = 186$ Hz, 1, C2), 128.31, 128.71, 128.90, 129.97, 130.08, 130.55, 134.06, 134.57 (C_{arom}), 165.41 (d, $J = 31$ Hz, 1, OCOPh), 171.13 (OCOPh); ^{19}F NMR δ -190.14 (ddd, $J = 10.0, 19.7, 48.8$ Hz, 1, F2).

4.3. Borate complexation study

The ^{11}B NMR spectra were recorded following the published methods described for the study of DPD binding to borate.¹⁸³ The ^{11}B NMR spectra were recorded at 25 $^{\circ}\text{C}$ using Bruker ultra shield™ spectrophotometer equipped with TBI probe $^1\text{H}/^{13}\text{C}/\text{D}$ -BB Z-GRD at 192.6 MHz and referenced to $\text{Et}_2\text{O}.\text{BF}_3$. The NMR sample tubes (10 mm o.d.) made of poly(tetrafluoroethylene) (PTFE) were used for all measurements. All the spectra were recorded by collecting 16 scans. The boron binding affinities of aminoalcohols and *cis*-diols were estimated using ^{11}B NMR either by taking compounds of interest and borate in 1:1, 1:2 and 2:1 molar ratios in 0.5 mL of NaHCO_3 in D_2O solution at pH 7.8 or 5 mg of compound in saturated borate at pH 7.8 in 0.5 mL of D_2O solution.

4.4. Biological screening methods

4.4.1. *Antiquorum* sensing assay

4.4.1.1. β -galactosidase assay

An overnight culture of *E. coli* DH5 α harboring the plasmids pSC11, which contains a P_{lasI}-*lacZ* translational fusion¹⁵ and pJN105L, which contains a P_{BAD}-*lasR* expression plasmid¹⁹⁰ grown in LB media (10 g tryptone, 5 g yeast extract, 5 g sodium chloride per liter) supplemented with ampicillin (100 μ g/ ml) and gentamycin (15 μ g/ ml), was diluted to an OD₆₀₀ of 0.150. At this time, arabinose (0.2 % w/v), *N*-3-(oxododecanoyl)homoserine lactone (3-oxo-C₁₂-AHL; 2 μ M), and either the compound under analysis, water extract from *Conocarpus erectus* or *Callistemon viminalis*¹⁹¹ or solvent (water or DMSO), were added to the culture (1.5 mL). A negative control containing only solvent and arabinose (0.2% w/v) was also assayed. The cultures were incubated with shaking for three hours at 37 °C.

The conditions for the *rhl* biomonitor *E. coli* DH5 α harboring pECP61.5 plasmid¹⁹², were essentially same except that the LB medium was only supplemented with ampicillin (100 μ g/ ml), the overnight culture was diluted to an OD₆₀₀ of 0.150, induced with 1 mM isopropyl β -D-thiogalactoside (IPTG), 2 μ M C₄-AHL and the compounds or the controls added when the OD₆₀₀ reached 1.0. After incubation at 37 °C for 4 hours with shaking, β -galactosidase activity was assayed as described previously.¹⁹³ Assays were repeated at least twice. For each biological replicate, experimental triplicates were performed and the average percent activity calculated by dividing the average Miller units from the samples containing compound or extract by the average Miller units from the sample containing solvent and multiplying by 100. Significance of inhibition was determined using a paired two-tailed Student *t*-test.

4.4.1.2. Growth and luminescence measurement drop assay

Growth and luminescence measurement drop assays were conducted by Professor Makemson at FIU. The *V. harveyi* strain B392 (MAV) (or strain BB170 (AI-1⁻, AI-2⁺) or strain BB152 (AI-1⁻

, AI-2⁺), was grown in AB medium (75% artificial sea water, 0.3% glycerol, 0.2% casamino acids, 0.02 % glycerol-phosphate, 25 mM HEPES buffer) at a pH 7.5¹⁹⁴ Growth was measured in a Spectronic 20 as OD_{660nm} and luminescence was measured in a calibrated photometer and recorded in light units (1 LU = 1.08 x 10⁸ q/s/mL). An overnight culture of *V. harveyi* was added to 45 mL of AB medium to an OD_{660nm} of ~0.03, When the culture was rapidly growing (OD_{660nm} ~0.05 to 0.07), the culture was split (2.5 mL/ 13x100mm culture tubes) and small amounts (2.5 µL and 10 µL) of each compound were added to each tube. The compound, dissolved in water or DMSO served as a control. Each measurement was obtained from a single trial and luminescence without inhibitor was considered 100%.

4.4.2. *LuxS* enzyme inhibition studies with 4-aza-SRH analogs

4.4.2.1. *LuxS* activity assay

The DTNB assay was performed in a buffer containing 50 mM HEPES (pH 7.0), 150 mM NaCl and 150 µM DTNB. The concentration of substrate (SRH) varied from 2.2 to 50 µM. The reaction was initiated by the addition of 0.8 µM of Co-BsLuxS and monitored continuously at 412 nm ($\epsilon = 14150 \text{ M}^{-1} \text{ cm}^{-1}$) in a Perkin-Elmer λ 25 UV-VIS spectrophotometer at room temperature. The initial rates were recorded from the early regions of the progress curves (3 min for BsLuxS reactions) and fitted against Michaelis-Menten equation $V = (k_{\text{cat}}[E]_0 [S]/(K_M + [S]))$ to obtain K_M and k_{cat} values.

4.4.2.2. *LuxS* inhibition assay

The LuxS inhibition assay was carried out in a similar manner as the activity assay but with varying concentrations of inhibitors and 17.8 µM of SRH (to a total volume of 1.0 mL). The inhibitors were dissolved in DMSO and the final DMSO concentration was kept less than 5% as to not to interfere with the enzyme activity. Kinetics was started by the addition of 0.8 µM *B. subtilis* LuxS coordinated with cobalt (Co-BsLuxS) and monitored at 412 nm. Slope was determined from 0-3 min. To calculate the K_I values, the relative activity (V/V_0) (V and V_0 are the

reaction rates in the presence and absence of inhibitors, respectively) was plotted against inhibitor concentrations $[I]$ and fitted against the equation: $V/V_0 = (K_M + [S]) / \{K_M(1 + [I]/K_I) + [S]\}$.¹⁹⁵ In case of hemiaminal **143** which displayed time-dependent inhibition, Co-BsLuxS (1.6 μM) was incubated with **143** in buffer (without DTNB) for 30 min at room temperature. The reaction was then started by adding SRH and DTNB. The K_I^* values were obtained in the same way from a similar equation: $V/V_0 = (K_M + [S]) / K_M(1 + [I]/K_I^*) + [S]\}$.

5. CONCLUSION

I have designed and synthesized various LuxS inhibitors and LasR QS modulators. The common synthetic approach involved activation of C5 hydroxyl group of the suitable sugar precursor and coupling with either thiolate derived from homocysteine (in case of LuxS inhibitors) or alkyl thiols (in case of γ -lactam and hemiaminal analogs). The *N*-Boc-L-Hcy-CO₂*t*-Bu was synthesized from commercially available L-homocystine. Reduction of the protected homocystine with water soluble tris(2-carboxyethyl)phosphine hydrochloride generated protected Hcy thiol. The *N*-*tert*-butoxycarbonyl-1,4-dideoxy-1,4-imino-2,3-*O*-isopropylidene-D-ribitol **124** served as the common sugar precursor for the synthesis of 4-aza-SRH analogs. Thus, the synthesis of pyrrolidine analog **129** was achieved by coupling of the mesylated *N*-Boc protected iminoribitol **127** with thiolate of *N*-Boc-L-Hcy-CO₂*t*-Bu and subsequent deprotection of acid labile groups with TFA. For the synthesis of lactam analog **141**, the mesylated ribolactam **136** required for coupling to Hcy was produced by selective oxidation of the mesylated aza sugar **127** with RuO₂/NaIO₄. The hemiaminal analog **143** was accessed conveniently by the super hydride® promoted reduction of the protected lactam analog **139** and subsequent TFA treatment. Oxidation of TBDMS protected imino ribitol **148** with SeO₂/H₂O₂ produced nitrone based sugar **149** which upon subsequent standard desilylation and mesylation yielded desired activated nitrone sugar **151**. In case of methyl imino analog **158**, the key sugar substrate, D-riboiminocyclitol **154** was synthesized from L-lyxo sugar by *exo*-imino to *endo* iminocyclitol rearrangement. Such imino sugar was subsequently mesylated and coupled to the thiolate of *N*-Boc-L-Hcy-CO₂*t*-Bu to give protected methyl imine analog **157** which upon TFA treatment gave imine analog **158**. The 2-deoxy-SRH analog **179** was synthesized by condensation of the mesylated methyl 2-deoxy- α/β -D-ribose **176** with Hcy. The mesylated-arabinose intermediate **183**, required for coupling to Hcy was conveniently accessed from D-arabinose. However, coupling of the mesyl sugar **183** with protected Hcy provided protected *S*-arabinosyl-L-homocysteine (SAH) **187** in very low yields. In

case of [2-F]SAH **194**, the key sugar precursor 5-*O*-mesyl-2-fluoroarabinose **188** was synthesized from reported 2-fluoroarabino lactone. However coupling of the -*O*-mesyl derivative **188** with Hcy was unsuccessful.

The γ -lactams with alkylthiomethyl substitution at carbon γ and their *N,O*-acetal counterparts were prepared from the key substrate (*S*)-5-(bromomethyl)-2-pyrrolidone **159** (derived from L-pyrroglutamic acid). Displacement of bromide in **159** with sodium alkylthiolate produced 5-*S*-alkylthio lactams **160** with different lengths of alkyl chain (C_3 - C_{12}). Reduction with super hydride® and subsequent deprotection gave hemiaminal analogs **163**. The hexylthio ribolactam **166** and nonylthio ribolactam **167** were synthesized analogously. Reduction of the protected ribolactams with super hydride and subsequent deprotection with TFA provided the hemiaminal counterparts **170** and **171**.

The optically pure γ -lactams and corresponding hemiaminals were evaluated for their effect on *P. aeruginosa* AHL-dependent *las* and *rhl* QS pathways isolated in *E. coli*. Lactam analogs **160** showed selectivity between two QS systems, acting as inhibitors against *lasR* and moderate activators against *rhlR*, presumably because of the differences in the active sites of their cognate R proteins. Antagonism of *lasR* activity increased with the length of the alkylthio chain. Interestingly, the cyclic hemiaminal derivatives with shorter alkylthio chain (**163a** and **163b**) were found to stimulate *lasr* QS systems at lower concentrations while strongly inhibit both QS systems at higher concentrations. The ribolactam **166/167** and the corresponding cyclic hemiaminal **170/171** analogs inhibited *lasR* and stimulated *rhlR* moderately. Although, the mechanism of inhibition is still unknown, it is plausible that the compounds act as competitive inhibitors by binding to the QS sensor or affect events downstream, such as the binding of LasR (or in some cases, RhlR) to the promoter. Affecting downstream events would require that the compound enter the cells, which is unknown. Alternatively, it is established that although the *rhl* signaling molecule, C_4 -AHL diffuses freely, transport of the *las* signaling molecule is more

complex, involving partitioning into the membrane and transport out of the cell by the MexAB-OprM efflux pump. Thus, it is also possible that the compounds with longer side chains affect the membrane and that the *las* pathway is more sensitive to these changes.

The pyrrolidine analog **129**, lacking C1-hydroxyl exhibited weak inhibition against LuxS enzyme with an IC_{50} value of 0.8 mM and K_I of 49 μ M. Similarly, lactam analog **141**, which also lacked anomeric hydroxyl displayed weak competitive inhibition with IC_{50} value of 0.6 mM and K_I of 37 μ M, suggesting the significance of anomeric hydroxyl for binding or the requirement of substrate to exist in open form. This data were also in agreement with the weak inhibition observed previously for 1-deoxy-SRH. However, as the inhibitors **129** and **141** possess K_I values in similar range (40-60 μ M), the inhibition might be because of the binding of the homocystiene moiety alone. As expected, the *N*-benzyl lactam analog **140** had no measurable activity. The large nonpolar benzylic group at ring nitrogen might have prevented access of **140** into the active site. Similarly 2,3-dideoxy lactam analogs of SRH, **195** and **196** were devoid of activity at tested concentrations, underscoring the importance of hydroxyl groups for metal chelation and binding at the active site.

The 4-aza-SRH **143** was found to be a time-dependent inhibitor of moderate potency with an IC_{50} value of 60 μ M. However, because of the complex kinetics of **143** observed with the enzyme, the K_I value could not be determined and the mechanism of time-dependent inhibition was not identified. Nonetheless, in order to support the hypothesis of covalent inhibition by 4-aza-SRH **143** via the dehydrated imine **197**, methyl imine analog of 4-aza-SRH **158** was independently synthesized and tested. Unfortunately, the 1-methyl imine analog of SRH **158** was found to be inactive. Lack of anomeric hydroxyl or misfit of iminocyclitols at the active site or lack of stability of the compound at the testing conditions might have contributed to the loss of activity. However, without further enzymatic studies, the precise effect of imine functionality on

LuxS activity could not be concluded at the present time. Also, despite the attempts from various research groups, correlation between LuxS enzymatic activity and QS was not found so far.

In this dissertation, two approaches for identification of QS inhibitors in both interspecies (LuxS inhibitors) as well as intraspecies (human pathogen, *P. aeruginosa*) were addressed. The structural and biological data provided by these analogs might contribute to the development of more effective QS inhibitors which in turn might impact drug discovery and health care.

Future directions

As a result of this dissertation, a future work towards understanding the function of *S*-ribosylhomocysteinase (LuxS) should mainly concentrate on synthesis of the aza-DPD analogs as well as novel inhibitors of LuxS with modifications at C2 or C4 of the ribose ring of the natural substrate.

Aza-DPD analogs should be prepared to evaluate their modulation of QS activity and might also allow study of their complexation with borate. Borate complexation experiments with aminoalcohols related to aza-AI-2 should shed the light on understanding the binding preferences of borate and might further facilitate the understanding of the mechanism of action of hemiaminal

143.

Synthesis and evaluation of C2 modified SRH derivatives as potential LuxS inhibitors might assist in understanding the role of acid/base residues at the active site. As [3-F]SRH and [3-Br]SRH were previously found to be time-dependent LuxS inhibitors due to the LuxS catalyzed elimination of halide ion, it will be interesting to study if the mechanism of interaction of [2-F]SRH and [2-Br]SRH with LuxS follows the nucleophilic substitution at C2 resulting in covalent inhibition or elimination mechanism as observed with C3 analogs. So far, SRH analogs with modifications at C1 and C2 and C3 were targeted as LuxS inhibitors. Such substrate analogs were proposed to inhibit sugar ring opening, first and second keto-enol tautomerization steps associated with LuxS catalysis, respectively. Design and synthesis of C4 modified SRH analogs

should permit to study the β -elimination reaction in LuxS catalysis (which is the last step leading to the release of DPD and Hcy). Modification of SRH with long alkyl side chains either at C4 or C7 might inhibit homodimerization of LuxS and thereby prevent LuxS catalysis. As it is known from literature that Hcy binding pocket is far from the sugar binding active site, such SRH analogs with hydrophobic alkyl chains should fit into the binding pockets for both sugar and Hcy moieties.⁴⁹ However, the presence of hydrophobic alkyl chain might prevent dimerization and hence LuxS catalysis.

Moreover, structural basis for the time-dependent inhibition observed by **143** can be estimated by cocrystallizing LuxS with **143**. As this analog exists as a complex mixture of several isomers, such study will also reveal the identity of the isomer responsible for inhibition. Conformational changes, if any occurred due to the replacement of ring oxygen of SRH with nitrogen (as in hemiaminal **143**) can also be studied using crystal structural data.

To confirm the LasR inhibition observed by lactam/hemiaminal analogs, inhibition of LasR regulated extracellular virulence factors (pyocyanin and elastase production) in culture fluids of *P. aeruginosa* can be estimated. Lactam/hemiaminal analogs with alkylthiomethyl substitution at carbon γ were targeted as QS modulators in this study. As a step forward, analogs with alkylthiomethyl substitution at α position can also be synthesized. Since similar α linkage of side chain to the lactone ring is found in the native AHL, such analogs might prove to be better QS inhibitors than γ substituted analogs. Similar thiolactam analogs can also be prepared and targeted as QS modulators.

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APPENDIX : ^1H NMR spectra of the selected compounds. For peak assignments refer to the experimental section.

140

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