Part I: Chemical functionalization of peptides and proteins. Part II: Synthetic studies towards the total synthesis of Platensimycin

Jenefer Alam

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PART I
CHEMICAL FUNCTIONALIZATION OF PEPTIDES AND
PROTEINS

PART II
SYNTHETIC STUDIES TOWARDS THE TOTAL
SYNTHESIS OF PLATENSIMYCIN

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TABLE OF CONTENTS

Acknowledgements	i
Table of Contents	ii
Summary	viii
Abbreviations	xii
Part I:	
Chapter 1: Chemical Functionalization of Peptides and Proteins	
1.1 Introduction.....	1
1.2 Posttranslational modifications (PTMs): Enzymatic Functionalization.....	2
1.3 Methodologies for Chemical modifications of Amino Acids at its Specific Functional Groups	3
1.3.1 Lysine Amino Group Selective Modification.....	5
1.3.2 Amino Acid Specific Modification.....	7
A. Selective Modification of Native Amino Acid.....	8
B. Selective Modification by Creation of Unique Site	11
B.1 Creation of Unique Site by Unnatural Amino Acid Incorporation	11
B.2 Creation of Unique Site by N-terminal Modification	13
B.2.1 N-terminal Modification by Acylation	13
B.2.2 N-terminal Modification by Oxidation	15
(I) N-terminal Oxidation by Periodate	15
(II) N-terminal Oxidation by Biomimetic Transamination	16
1.4 Secondary Modification of Introduced Sites	18
A. Azide Modification	18
A.1 Staudinger Ligation	19
A.2 Copper Catalyzed Azide-Alkyne [3+2] Cycloaddition	
(CuAAC)-“Click” Chemistry	20
A.3 Strain Promoted [3+2] Cycloaddition	20

B. Alkene Modification	21
C. Modification of Halogenated Amino Acid	22
1.5 Challenges in Developing Reactions for Selective Protein Modification	22
1.5.1 Aqueous Media.	23
1.5.2 Temperature.	24
1.5.3 Solution pH.	25
1.5.4 Salts & Surfactants.....	26
1.5.5 Analytics.	26
1.6 Summary	27
Chapter 2: Functionalization of Peptides	
2.1 Aza Diels Alder	30
2.1.1 Introduction: Literature Overview	30
2.1.2 Activation in aza Diels-Alder Reaction.	31
2.1.3 Hetero Diels-Alder Reaction in Aqueous Solvent.....	32
2.1.4 Synthetic Applications of aza Diels-Alder reaction.....	33
2.1.5 Aza Diels-Alder: Our Extension.....	35
A. Selection of Lewis Acid	37
B. Selection of Diene	38
2.1.7 Experimental.	39
A. Synthesis of Starting Material	39
B. Optimizing Conditions for aza Diels-Alder Reaction	41
2.1.8 Summary	48
2.2 Indium Mediated Allylation	49
2.2.1 Introduction: Literature Overview	49
2.2.1 Indium-Mediated Allylation Reaction in Aqueous Media.....	51
2.2.3 Synthetic Applications of Indium Mediated Allylation.....	52

2.2.4	Allylation in Peptides.....	56
2.2.5	Indium Mediated Allylation: Our Extension	57
2.2.6	Optimizing Conditions for Allylation of peptides.	58
2.2.7	Applications.	61
2.2.8	Summary	64
2.3	Mukaiyama-Aldol Reaction	65
2.3.1	Introduction: Literature Overview	65
2.3.2	Lewis acid catalysis in asymmetric Mukaiyama-aldol reaction.	66
2.3.3	Aqueous Mukaiyama-aldol reaction.	67
2.3.4	Synthetic Applications of Mukaiyama-Aldol Reaction.....	68
2.3.5	Mukaiyama-Aldol Reaction: Our Extension	69
2.3.6	Optimizing Conditions for Mukaiyama-aldol Reaction of Peptides.....	71
2.3.7	Applications.	74
2.3.8	Summary	80

Chapter 3: Functionalization of Proteins

3.1	Introduction.	82
3.2	Indium Mediated Allylation	84
3.2.1	Introduction: Literature Overview	84
	A. Introduction of Olefinic Group by Unnatural Amino Acid Incorporation	84
	B. Introduction of Olefinic Group by Chemical Modification of Amino Acids	85
	C. Applications	86
3.2.2	Allylation Reaction: Our Extension	86
	A. Periodate Oxidation and Allylation of Peptides	85
	B. Oxidation and Allylation of Myoglobin	87
3.2.3	Summary	95

3.3 Mukaiyama Aldol reaction	96
3.3.1 Introduction.....	96
3.3.2 Mukaiyama-Aldol Reaction : Our Work	97
A. Periodate Oxidation and Mukaiyama-Aldol	
Reaction of Peptides	97
B. Oxidation and Mukaiyama-Aldol Reaction of Myoglobin	97
3.3.3 Applications	106
3.3.4 Summary	121

Part II:

Chapter 4: Synthetic Studies Towards the Total Synthesis of Platensimycin

4.1 BACKGROUND	123
4.1.1 Structural and Biological Aspects of Platensimycin.....	123
4.2 Reported Synthesis of Platensimycin	124
4.2.1 First synthesis.....	124
4.2.2 Total Synthesis.....	125
4.2.3 Syntheses of oxatetracyclic core.....	127
4.3 Cascade Reaction	132
4.4 Radical Cascade Reaction	133
4.5 Retrosynthetic Analysis of Oxatetracyclic Core	135
4.5.1 First Generation	135
4.6 Synthesis.....	136
4.6.1 α -bromination	137
4.6.2 Synthesis of Intermediate 78	139
4.6.3 Elimination.....	140

4.6.4	Hydrobromination.....	141
4.6.5	Vinylation of alcohol	141
4.6.6	Attempts towards radical cascade reaction	142
4.7	Summary	143
 Chapter 5: Methodology Development for 1,4-Dicarbonyls and its Application		
5.1	Second Generation Retrosynthesis	145
5.2	Synthesis of Intermediate 87	146
5.3	Methodology Development Towards Intermediate 88	150
5.4	Radical Approaches to 1,4-Dicarbonyl Compounds	152
5.5	Synthesis of Compound 93 for Development of Radical Methodology	153
5.6	Radical Initiators	154
5.6.1	Tin Based Initiator.	154
5.6.2	Application of Tin Initiator in Our Methodology	155
5.6.3	Et ₃ B Based Initiator	157
5.6.4	Application of Et ₃ B to Our Methodology	157
5.6.5	Conclusion.	160
5.7	Synthesis of Iodo Intermediate 104	160
5.8	Application of Et ₃ B Initiation to Platensimycin	160
5.9	Cyclization Attempts of Intermediate 93	162
5.10	Xanthate Based Radical Group Transfer Reaction	163
5.10.1	Synthesis of Xanthate Precursor 107	165
5.10.2	Application of Xanthate Based Approach to Platensimycin.....	166
5.11	Summary	167

Chapter 6: Experimental Section170

SUMMARY

Part I

The last decade has seen an abundance of research in the area of protein conjugation. Labeling of proteins at a specific site with small molecules can advance our understanding of biochemical events. With this novel technology, investigation of protein expression, localization, protein-protein interaction and their conformational changes during the cell cycle can be studied. Herein, we will report the methodology developed for the site specific functionalization of peptides and protein through the formation of carbon-carbon bond.

This was achieved by performing reactions at the *N*-terminal aldehyde of peptides and protein in aqueous media at room temperature. Aldehyde moiety in peptides was introduced through periodate attack on their *N*-terminal serine. While pyridoxal phosphate mediated biomimetic transamination at the *N*-terminal glycine was used to introduce aldehyde functionality in protein.

Next, various reactions at the aldehyde of the glyoxylate were performed for the direct introduction of an appropriate chromophore or a tether with functional group that will allow the easy attachment of the chromophore in the next step. These reactions include; *Aza Diels-Alder*, Indium Mediated *Allylation* and *Mukaiyama Aldol*.

Chapter 2, demonstrates methodology development and application of the above reactions in the selective *N*-terminal functionalization of peptides from easily accessible starting materials.

The obtained results illustrate that the developed methodologies could be used for the targeted delivery of a wide variety of functional groups into specific peptide site through the formation of a stable carbon-carbon bond.

In chapter 3, the methodology of indium mediated *Allylation* and *Mukaiyama* aldol reaction in aqueous media were further developed and applied in the functionalization of protein *N*-terminus aldehyde. They were used for the targeted introduction of terminal alkene moiety into the *N*-terminal of protein under very mild conditions.

For the *Mukaiyama* aldol condensation it has been demonstrated that in the case of Myoglobin, the functionalization of the protein can be carried out without disturbing the tertiary structure and more importantly the enzymatic activity of the protein. The *Mukaiyama* aldol reaction of proteins is very fast and highly efficient and as a consequence may be of particular interest when the protein stability is a concern.

Part II

In Part II of this thesis, synthetic studies towards the total synthesis of Platensimycin is described. Our initial retrosynthetic analysis for Model Study of Oxatetracyclic core of Platensimycin had two key features 1) construction of the oxatetracyclic core with an allyl handle *via* an efficient intramolecular radical cascade approach from intermediate **I**; and 2) keep in the sequence short. Although the intermediate **I** was obtained successfully, our attempts towards intramolecular radical cascade failed to give us the desired product. This led us to pursue an intermolecular radical approach instead. Along the line we developed a radical based methodology for the formation of 1,4-dicarbonyl intermediates and demonstrated its application in the radical step (from **87** to **88**).

INDEX OF ABBREVIATIONS

α	alpha
μ	micro
δ	chemical shift
Δ	reflux
$^{\circ}\text{C}$	degree centigrade
Ac	acetyl
ACCN	<i>azo-bis</i> -cyclohexane carbonitrile
AIBN	<i>azo-bis</i> -isobutyronitrile
Ala	Alanine
aq.	aqueous
BOC	tert-butoxycarbonyl
brs	broad singlet
BuLi	butyl lithium
BuOH	butanol
Bu_3SnH	tri-n-butyl tin hydride
<i>t</i> -bu	tert-butyl
calcld	calculated
cat	catalytic
Cbz	benzyloxycarbonyl
CD	circular dichroism
CDCl_3	deuterated chloroform
CuSO_4	copper sulfate
CH_2Cl_2	dichloromethane

CHCl ₃	chloroform
cm ⁻¹	inverse centimeter
cyc	cyclohexane; cyclohexenyl
d	doublet
dba	dibenzylidene acetone
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	1,3-dicyclohexylcarbodiimide
dd	doublets of doublet
dr	diastereomeric ratio
DIA	diisopropylamine
DIEA	diisopropylethylamine
DMAP	4-(<i>N,N</i> -dimethylamino)pyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
dt	doublets of triplet
dq	doublets of quartet
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
<i>ee</i>	enantiomeric excess
EI	electron impact ionization
equiv.	equivalent
ESI	electrospray ionization
Et	ethyl
ether	diethyl ether
Et ₃ N	triethylamine
EtOAc	ethyl acetate

EtOH	ethanol
FAB	fast atomic bombardment
FTIR	Fourier transform infrared spectroscopy
g	gram
D, Glu	Glutamate
G, Gly	Glycine
h	hour
H	hydrogen
^2H	deuterium
HATU	<i>O</i> -(7-azabenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HCl	hydrogen chloride
Hex	hexane
H, His	histidine
HMPA	hexamethylphosphoramide
HMQC	heteronuclear multiple quantum correlation
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
Hz	Hertz
I, Ile	Isoleucine
In	indium
InCl ₃	indium trichloride
IR	infrared
<i>i</i> -Pr	isopropyl

J	coupling constants
K_2CO_3	potassium carbonate
kg	kilogram
LDA	lithium diisopropylamide
L, Leu	Leucine
LiHMDS	Lithium hexamethyl disilazide
LiOH	Lithium hydroxide
LRMS	Low resolution mass spectroscopy
K, Lys	Lysine
M	concentration (mol/dm^{-3})
M^+	parent ion peak (mass spectrum)
m	multiplet
<i>m</i> -CPBA	<i>meta</i> -chloroperoxybenzoic acid
MA	Mukaiyama-Aldol
Me	methyl
MeCN	acetonitrile
MeOH	methanol
M, Met	methionine
mg	milligram
$MgSO_4$	magnesium sulfate
MHz	Megahertz
min	minute
mmol	millimoles
mol	moles
MS	mass spectrum

Ms	methanesulfonyl
MWCO	molecular weight cut off
n	nano
N	concentration (normality)
N ₂	nitrogen
NaHCO ₃	sodium bicarbonate
NaHMDS	sodium hexamethyl disilazide
NaIO ₄	sodium metaperiodate
NaPi	sodium phosphate buffer
NBS	<i>N</i> -bromosuccinimide
<i>n</i> -Bu	<i>n</i> -butyl
NMR	nuclear magnetic resonance
N.R.	no reaction
Na ₂ SO ₄	sodium sulfate
O ₂	oxygen
obsd.	Observed
OMe	O-methoxy
OTf	trifluoromethanesulfonate
p	page
PBr ₃	phosphorus tribromide
Pd/C	palladium on carbon
Ph	phenyl
P, Phe	phenyl alanine
PhMe	Toluene
PLP	pyridoxal 5'-phosphate

ppm	parts per million
Py	pyridine
q	quartet
qd	quartets on doublet
quint.	quintet
rt.	room temperature
RBF	round bottom flask
R _f	retention factor
s	singlet
<i>sat.</i>	saturated
sec	secondary
S, Ser	Serine
t	triplet
<i>t</i> -BOC	<i>tert</i> -butoxycarbonyl
<i>t</i> -Bu	<i>tert</i> -butyl
td	triplets of doublet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
THP	tetrahydropyran
TIPS	triisopropyl silyl
<i>p</i> TsCl	para-toluene sulfonyl chloride
TiBr ₄	titanium tetrabromide
TLC	thin layer chromatography
TMSCl	trimethylsilyl chloride
TMSBr	trimethylsilyl bromide

TMSOTf trimethylsilyl trifluoromethane sulfonate

Ts *p*-toluenesulfonyl

T.S. transition state

UV Ultra violet

vol volume

PART I

Chapter 1

Chemical Functionalization of Peptides & Proteins

Chapter 1

1.1 Introduction

Advances in scientific research have provided us with opportunities to tackle illnesses. Despite the progress made to date, challenges still remain. Even though treatments for a number of diseases exist, the exact biological mechanisms involved in the process are often unclear. In addition, there are instances where an existing drug is no longer effective as the organisms involved have become resistant to the therapeutics. Thus, discovery of new and innovative medicinal agents is crucial. This necessitates a better understanding of the basic biology in the cellular environment. A detailed study of biomolecular mechanism not only facilitates the design of novel medicinal agents, it can also offer new technologies for genetic manipulations and diagnostic studies.

A majority of the available treatments target proteins that are essential for the disease process. Hence development of new methods that aid in the study of protein function is essential.

In principle there are three ways to study proteins in the cellular environment. First, genetic manipulation of protein expression can be used to regulate the levels of a protein. As a second, more difficult option, modulators of protein function can be identified and their influence on the cellular phenotype can be studied.¹ Finally studies of biomolecular function can be carried out through covalent modification of proteins. This latter methodology is very attractive as it has the potential to enable scientist to study the fate of proteins inside the cells in real time. The work described in the first part of this thesis is concerned with novel methodology for the covalent

¹ (a) Sletten, E. M. and Bertozzi, C. R. *Angew. Chem. Int. Ed.* **2009**, *48*, 6974-6998; (b) Qi, D.; Tann, C. M.; Haring, D.; Distefano, M. D. *Chem. Rev.* **2001**, *101*, 3081-3112; (c) Walsh, D. P; Chang, Y. -T. *Chem. Rev.* **2006**, *106*, 2476-2530.

Chapter 1

modification of proteins and the following chapters of the introduction will review the state of the art in this area.

1.2 Posttranslational modifications (PTMs): Enzymatic Functionalization

Posttranslational modifications (PTMs) are enzyme-catalyzed protein modifications that occur on the backbone or side chains of newly formed, folded proteins under physiological conditions. PTMs modulate protein activity through their roles in protein folding, localization, signaling, protein stability and enzyme activation.²

The five main covalent additions to proteins are phosphorylation, acylation, alkylation, glycosylation, and oxidation (Scheme 1-1).

O-phosphorylation at serine residue

ϵ -*N*-acylation at lysine residue

² (a) Walsh, C. T. *Posttranslational Modification of Proteins: Expanding Nature's Inventory*, Roberts and Co., Englewood, Colorado, **2005**; (b) Wold, F. *Annu. Rev. Biochem.* **1981**, 50, 783-814.

Chapter 1

 ϵ -N-alkylation at lysine residue

O-glycosylation at serine residue

Oxidation at proline residue

Scheme 1-1: Representative examples of five major types of PTMs.

1.3 Methodologies for Chemical modifications of Amino Acids at its Specific Functional Groups

Chemical modification is the process by which amino acid residues in the protein can be modified through the action of residue specific or functional group specific

Chapter 1

reagents. As opposed to PTM's which are under cellular control, we are talking here about protein modifications using synthetic chemistry or by the use of natural or engineered enzymes. The modifications that are introduced by this process are unique and are close mimic of those incorporated by nature itself. The selective modification of proteins at specific residues that is achieved through this process provides us with modified proteins in amounts and form that are desirable for their studies. Because of its uniqueness, scientists have used this chemical alteration to introduce certain desired functions to proteins. The most recurrent use in this regard has been fluorescent and affinity tagging.³

In their therapeutic applications, protein conjugates⁴ have been used with much success in fighting cancer,⁵ HIV,⁶ malaria⁷ and pathogenic bacteria.⁸ A glycoconjugate vaccine for *Haemophilus influenzae* type b (Hib) was generated by covalently coupling synthetic capsular polysaccharide to a carrier protein.¹⁰

In a representative example of diagnostic use, artificial sugars were introduced into living cells biosynthetically through a process called metabolic oligosaccharide engineering.⁹ Mice treated with azido sugar precursor, *N*- α -azidoacetylmannosamine (ManNAz) could incorporate azide containing sialic acid residues (SiaNAz) in their membrane glycoconjugates. In altered physiological states, such as those arising from cancers or other immunological changes, more of SiaNAz will be incorporated into

³ (a) Carrico, I. S. *Chem. Soc. Rev.* **2008**, 37, 1423-1431; (b) Foley, T. L.; Burkart, M. D. *Curr. Opin. Chem. Biol.* **2007**, 11, 12-19.

⁴ (a) Dube, D. H.; Bertozzi, C. R. *Nat. Rev. Drug Discovery* **2005**, 4, 477-488; (b) Doores, K. J.; Gamblin, D. P.; Davis, B. G. *Chem. Eur. J.* **2006**, 12, 656-665.

⁵ Krug, L. M.; Ragupathi, G.; Ng, K. K.; Hood, C.; Jennings, H. J.; Guo, Z.; Kris, M. G.; Miller, V.; Pizzo, B.; Tyson, L.; Baez, V.; Livingston, P. O. *Clin. Can. Res.* **2004**, 10, 916-923.

⁶ Astronomo, R. D.; Lee, H. -K.; Scanlan, C. N.; Pantophlet, R.; Huang, C. -Y.; Wilson, I. A.; Blixt, O.; Dwek, R. A.; Wong, C. -H.; Burton, D. R. *J. Virol.* **2008**, 82, 6359-6368.

⁷ Seeberger, P. H.; Werz, D. B. *Nature* **2007**, 446, 1046-1051.

⁸ Varez-Bencomo, V. *Science* **2004**, 305, 522-525.

⁹ Prescher, J. P.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, 430, 873-877.

Chapter 1

the cells. One can detect the altered state by exposing the azide modified cells to phosphine functionalized imaging agents under Staudinger ligation.

Use of chemical modification provides us with means for introducing wide-range of functionalities into proteins that do not occur naturally. Thus it has become an important tool for protein engineering in the emerging field of chemical biology. As a consequence novel methodologies aiming at protein modifications are always sought after. Our present research aims to enhance the tool kit for protein chemists through the development and application of new C-C bond forming reactions in protein functionalization.

1.3.1 Lysine Amino Group Selective Modification.

In the early days of research into chemical modification strategies, the functional groups of natural amino acids were the preferred targets.

Lysines can be modified by reaction with isocyanates, isothiocyanates, sulfonyl chloride and activated esters by taking advantage of electrophilic nature of these reagents (Scheme 1-2). Water soluble carbodiimides can be used to activate the acidic side chains of aspartate and glutamate to give active esters which can yield stable bioconjugates through nucleophilic addition.

Chapter 1

Scheme 1-2: Modification of Lysine residues.

Ir(III)-complex **1** (Scheme 1-3) can be used as a mild reducing agent for reductive alkylation of amino groups of lysine.¹⁰ In typical reactions, the imines formed from the exposure of proteins to aldehydes are reduced by NaBH_3CN , a large excess of which can denature proteins. But the Ir system is comparatively milder in this respect. Ir-hydride **2** is generated from Ir(III)-complex **1** and HCO_2Na in buffer (pH 7.4) at room temperature, this then efficiently reduces the generated imine in proteins at low concentrations (10 μM).

¹⁰ McFarland, J. M. & Francis, M. B. *J. Am. Chem. Soc.* **2005**, 127, 13490-13491.

Scheme 1-3: Iridium catalyzed reduction of imines.

Although this process can label proteins with a number of different chemical entities, the major disadvantage of this strategy is that depending on the targeted amino acid several modification events are possible, because of the frequency with which surface exposed amino acids appear on proteins. Some of these reactions can also modify the *N*-termini of proteins. Thus this method lacks the selectivity that is crucial for further studies with the modified proteins. To address this problem protein scientist have developed and applied the concept of site selective modification.

1.3.2 Amino Acid Specific Modification.

For successful protein modification, the most important aspect of the new reaction is its selectivity. It should be able to selectively modify the residue of interest through covalent coupling of mutually and uniquely reactive functional groups among a multitude of potentially reactive functionalities.¹¹ The design of human immune system is based on exactly this principle. Among a plethora of biological molecules, an antibody molecule manages to find its target and form a highly specific noncovalent complex. This concept has a wide scope of application like engineering

¹¹ Chen, I.; Ting, A. Y. *Curr. Opin. Biotechnol.* **2005**, *16*, 35-40.

Chapter 1

novel reactivity in biopolymers. Complex biomolecules can be assembled from simpler components without going through elaborate protection schemes. Immobilization of biopolymers for high-throughput genomic and proteomic studies can be undertaken using this idea. Studying cellular processes in their endogenous environment is possible.

The two prominent ways by which this is achieved is:

(1) By taking advantage of the reactivity of the 20 natural amino acids present in proteins and modifying the native functionality.

(2) By incorporation of synthetic amino acid with a unique chemical handle that reacts specifically to a desired functionality during modification. The modification should not alter the conformation of the biomolecule or the biological function of the biological system through unintended interaction with any other residue or reagents.¹² These events are described in detail in the following sections.

A. Selective Modification of Native Amino Acids. Cysteine can serve as a convenient target for selective modification. The low pK_a (~ 8) of thiolate anion together with its strong nucleophilicity, and low natural abundance of the amino acid itself can provide high specificity. Cysteines can be selectively modified by maleimides and α -halo reagents to yield stable adducts (Scheme 1-4a).¹³

¹² (a) Xie, J.; Schultz, P. G. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 775-782; (b) Link, A. J.; Mock, M. L.; Tirrell, D. A. *Curr. Opin. Biotechnol.* **2003**, *14*, 603-609.

¹³ (a) Fodge, M. N.; Al-Karadaghi, S. *Protein Eng.* **2002**, *15*, 353-358; (b) Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G. *Chem. Asian. J.* **2009**, *4*, 630-640.

Chapter 1

Scheme 1-4: Modification of cysteine and tyrosine residues.

Tyrosine residues can be selectively modified through electrophilic aromatic substitution in two different ways. In the first method, electron deficient diazonium salts couple to the *ortho* position of tyrosine residues to generate azobenzene functionalities at 4 °C in high coupling efficiencies (> 90%) and rate (15 min) at pH 9 (Scheme 4b).¹⁴ In the second method, the tyrosine residues undergo Mannich modification with activated imines, generated from anilines with electron donating

¹⁴ Schlick, T. L.; Ding, Z. B.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, 127, 3718-3723.

Chapter 1

substituents and specific aldehydes (formaldehyde, pyruvaldehyde and glyoxylic acid) at near physiological pH (6.5) (Scheme 1-4b).¹⁵

Indole functionality of tryptophan residues in proteins can be selectively modified by rhodium carbenoids, where the active species **4** are generated through catalytic degradation of diazo compound **3** *in situ* in aqueous solution by $\text{Rh}_2(\text{OAc})_4$ (Scheme 1-5).¹⁶ $\text{H}_2\text{NOH.HCl}$ was used as an additive for the reaction to be successful in low concentrations (10 μM). The fact that a mixture of *N*-alkylated and *C*-alkylated products are formed was confirmed through small-molecule model studies. The authors attributed the high selectivity of the reaction to the ‘leveling effect’ provided by water. One of the limitations of this method is that, the pH of the reaction medium is acidic (<3.5).

Scheme 1-5: Rhodium catalyzed Tryptophan modification.

A new tyrosine modification by electrophilic organometallic complexes involves the use of *pi*-allylpalladium intermediates **6** generated from allylic acetate and carbamate **5** in aqueous solution by $\text{Pd}(\text{OAc})_2$ and water soluble ligand triphenylphosphinetris(sulfonate) (TPPTS). The activated complexes thus generated

¹⁴ Joshi, N. S.; Whitaker, L. R.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 15942-15943.

¹⁶ Antos, J. M. & Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 10256-10257.

Chapter 1

were able to alkylate phenolate oxygen of surface-accessible tyrosine residues of proteins at a low concentration of (5 μ M) at pH 9 (Scheme 1-6).¹⁷

Scheme 1-6: Palladium catalyzed Tyrosine modification.

Although the method of selectively labeling native amino acid is quite unique in providing the scientist with the idea of probable sites of functionalization, the drawback it faces is the multiple copy of modification that it delivers. This greatly reduces the number of proteins available for modification. Introduction of unique reactive handles in proteins under such instances affords one with the luxury of choosing the position for functionalization.

B. Selective Modification by Creation of Unique Site. Creation of unique site in proteins for labeling studies has gained immense popularity over the years. This is because in this process, one can predict and plan the site of functionalization. It was initiated with the incorporation of artificial amino acids into proteins through molecular biology techniques. Later on chemical techniques were introduced to simplify the overall process.

B.1 Creation of Unique Site by Unnatural Amino Acid Incorporation. There are two ways of incorporating unnatural amino acids into proteins.

¹⁷ Tilley, S. D. & Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128*, 1080-1081.

Chapter 1

The older and more easily accessible method is the **residue-specific** incorporation, which is based upon the competition between the natural amino acid and its unnatural analog during their incorporation into growing peptide chains by *t*RNA.¹⁸ Unnatural amino acids with structural and electronic similarity to the natural analogs can be easily incorporated into proteins by this method. Residues like ketones (7), azides (8-11), alkenes (12-18), alkynes (19-20) and halogenated amino acids (21-22) have been successfully introduced (Scheme 1-7). The natural amino acids that have been replaced are phenylalanine, leucine, isoleucine, proline and methionine.¹⁹ Even though this method provides one with the ease of accessibility, it lacks the control over site-specific introduction of the new residue.

This problem was addressed through **biosynthetic site-specific** incorporation of unnatural amino acids, where *t*RNAs are charged with stop codons that dictate the placement of unnatural analogs into growing polypeptide chains. As opposed to the residue-specific incorporation which is amenable to gram scale quantities of proteins, this method is only applicable when small amount of protein is involved.²⁰

Scheme 1-7: Example of unnatural amino acids incorporated into proteins.

¹⁸ Connor, R. E and Tirrell, D. A. *Polym. Rev.* **2007**, 47, 9-28.

¹⁹ Kirshenbaum, K.; Carrico, I. S.; Tirrell, D. A. *ChemBioChem.* **2002**, 3, 235-237.

²⁰ (a) Wang, L.; Schultz, P. G. *Angew. Chem. Intl. Ed.* **2005**, 44, 34-66; (b) Dougherty, D. A. *Curr. Opin. Chem. Biol.* **2000**, 4, 645-652; (c) Nowak, M. W.; Gallivan, J. P.; Silverman, S. K. Labarca, C. G.; Dougherty, D. A.; Lester, H. A. *Ion. Channels, Part B*, **1998**, 293, 504-529.

Chapter 1

The method of introducing uniquely reactive handles in proteins has revolutionized the field of proteomics. A lot of elegant work has surfaced that targeted these unique handles to further functionalize proteins in the study of their mechanisms. Yet, the process can be very tedious, and even after successful incorporation of the unique chemical entity through extensive research, the newly formed proteins might not be able to fold properly or lose the function that we intend to study. This necessitated the development of easier and faster methods of functionalization. Introduction of unique site by chemical modification was the solution to these challenges.

B.2 Creation of Unique Site by *N*-terminal Modification. As the title implies, in this method the specific *N*-terminal site is introduced into the protein by chemical reactions at particular amino acid. This is a developing field and much work still needs to be done to make these methods generally useful. So far the most useful methods are concerned with the modification of the *N*-terminus of the proteins.

The significantly lower pK_a of *N*-terminal amine (~ 8) compared to lysine (~ 10) makes it an attractive target for selective electrophilic modification at a near neutral pH. *N*-terminal modification thus can deliver a modifying group uniquely to a preselected site in peptide or protein. The reaction is performed using a reagent that is specific for a single type of functional group that may exist in a protein. The result is that there can be only one modification event.

B.2.1 *N*-terminal Modification by Acylation. Several strategies targeting the *N*-terminus to achieve site-selective protein modifications have been developed because

Chapter 1

of its uniquely reactive properties.²¹ *N*-terminal amino group has been used for directing acylation at this site by controlling the reaction pH,

- *N*-terminal cysteines form thiazolidines in the presence of aldehydes, these thiazolidines in turn are quite stable under physiological conditions (Scheme 1-8(a)).
- *N*-terminal tryptophan residues can be modified through Pictet-Spengler reaction (Scheme 1-8(b)).
- Fusion proteins have been obtained by native chemical ligation (NCL) through the transesterification of *C*-terminal thioesters by *N*-terminal cysteines (Scheme 1-8(c)). The coupling partners can be generated either synthetically or biosynthetically.

Scheme 1-8: Modification of *N*-terminal cysteine and tryptophan residues.

²¹ (a) Baker, D. P.; Lin, E. Y.; Lin, K.; Pellegrini, M.; Petter, R. C.; Chen, L. L.; Arduini, R. M.; Brickelmaier, M.; Wen, D.; Hess, D. M.; Chen, L.; Grant, D.; Whitty, A.; Gill, A.; Lindner, D. J.; Pepinsky, R. B. *Bioconj. Chem.* **2006**, *17*(1), 179-188; (b) Li, X.; Zhang, L.; Hall, S. E.; Tam, J. P. *Tetrahedron Lett.* **2000**, *41*, 4069-4073; (c) Bang, D.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem. Int. Ed.* **2006**, *45*, 3985-3988; (d) Lu, W.; Gong, D. Q.; Bar-Sagi; Cole P. A. *Mol. Cell.* **2001**, *8*, 759-769.

Chapter 1

B.2.2 N-terminal Modification by Oxidation. Another very useful method of *N*-terminal modification is based upon the strategy of directing the modification to a group that is not naturally present in the peptides and proteins and whose reactivity distinguishes it from the rest. One such pair of unique groups is ketones and aldehydes. Aldehydes and ketones are powerful electrophiles possessing unique reactivity among the exclusively nucleophilic native amino acid side chains. Protein chemists have developed two different methodologies for the introduction of these groups at the protein *N*-terminus. Initially a reactive aldehyde or ketone group is introduced at the *N*-terminal, followed by chemoselective labeling with a unique entity.

(I) N-terminal Oxidation by Periodate. In this method *N*-terminal serine or threonine is the target of periodate attack. The method is based on the very rapid oxidation of 1,2-amino alcohol of these two amino acids to aldehyde by periodate as follows.²² In proteins this generates a glyoxylyl residue at the site of attack (Scheme 1-9).

Scheme 1-9: Periodate Oxidation of 1,2-amino alcohols

²² (a) Geoghegan, K. F. And Stroh, J. G. *Bioconj. Chem.* **1992**, 3(2), 138-146; (b) Chelius, D. and Shaler, T. A. *Bioconj. Chem.* **2003**, 14(1), 205-211.

Chapter 1

Once generated, the aldehyde has been functionalized in numerous ways. Formation of hydrazones using Biocytin hydrazide for specific removal of proteins containing *N*-terminal serine or threonine from a mixture,²³ attachment of polyethylene glycol (PEG) to protein *N*-terminus to prolong circulatory life time of proteins in vivo via an oxime linkage,²⁴ synthesis of large protein like molecules from natural peptides,²⁵ and double fluorescent tagging of peptide substrates for studying proteases through energy transfer²⁶ are a few examples among the wide applications available for aldehyde ligation.

The advantage of using this method is that, the reaction occurs under mild aqueous condition. The oxidation is an exceptionally fast reaction that proceeds best at neutral pH. It is also highly selective when a low molar excess of periodate over peptide is used, as the desired oxidation is faster than a number of the potentially competing side reactions. Possible side reactions include oxidation of the side chain of Methionine, Trptophan and Histidine, with the conversion of Methionine to Methionine sulfoxide [Met(O)] presenting the gravest danger, thus making completely selective oxidation difficult to achieve.^{21a} To circumvent this problem the second alternative method that has been developed by Francis group is based upon the modification of protein *N*-terminal through a Biomimetic Transamination reaction.²⁷

(II) *N*-terminal Oxidation by Biomimetic Transamination. This methodology developed by Francis group²¹ is based on the oxidation of *N*-terminal amino group to an imine, followed by hydrolysis to afford either a ketone or an aldehyde depending

²³ Gaertner, H. F.; Rose, K.; Cotton, R.; Timms, D.; Camble, R.; Offord, R. E. *Bioconj. Chem.* **1992**, *3*, 262-268.

²⁴ Gaertner, H. F. and Offord, R. E. *Bioconj. Chem.* **1996**, *7*, 38-44.

²⁵ Rose, K.; Zeng, W.; Regamey, P. -O.; Chernushevich, I. V.; Standing, K. G.; Gaertner, H. F. *Bioconj. Chem.* **1996**, *7*, 552-556.

²⁶ Geoghegan, K. F.; Emery, M. J.; Martin, W. H. McColl, A. S.; Daumy, G. O. *Bioconj. Chem.* **1993**, *4*, 537-544.

²⁷ (a) Gilmore, J. M.; Scheck, R. A.; Esser-Hahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem. Intl. Ed.* **2006**, *45*, 5307-5311; (b) Scheck, R. A.; Dedeo, M. T.; Lavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 11762-11770.

Chapter 1

on the type of amino acid at the site of attack. Here, PLP **23** (Pyridoxal-5-phosphate), a biological cofactor is used to carry out the transamination. The reaction is initiated with the formation of an imine **24** from the condensation of amino groups and PLP aldehyde. The imine **24** having an acidic α proton tautomerizes rapidly to a glyoxyl imine **25** which then undergoes hydrolysis to yield aldehyde or ketone **26** (Scheme 1-10).

Scheme 1-10: Biomimetic Transamination of N-terminal amino acid

Detailed study of the *N*-terminal residues have revealed that, Ala, Gly, Asp, Glu, or Asn are the best amino acids for this transformation; with the rest of the amino acids the yields vary. The authors have used this methodology for fluorophore sensing applications and modification of protein termini for device integration.²⁸ In a demonstration of the effectiveness, they first introduced a ketone functionality at the *N*-terminus through pyridoxal phosphate (PLP) mediated oxidative deamination. following which the protein was fluorescent labeled by conjugation with an alkoxyamine-appended fluorophore. Subsequently, the labeled protein was separated from the unlabeled protein by affinity chromatography using cyclodextrin resin (Scheme 1-11).

²⁸ Nguyen, T.; Joshi, N. S.; Francis, M. B. *Bioconj. Chem.* **2006**, *17*, 869-872.

Chapter 1

Scheme 1-11: Fluorescent labeling of proteins and their purification by affinity chromatography.

The advantage of this methodology is that, it does not depend on the identity of the amino acid side chain. Also it seems that, it is milder than periodate oxidation and would not undergo side reactions that might interfere with enzymes activity.

1.4 Secondary Modification of Introduced Sites.

Once the unique chemical handles are successfully incorporated into proteins, their distinctive reactivity is explored through the attachment of complementary reactive probes by a number of well-designed chemically renowned reactions described below.

A. Azide Modification. For intracellular functionalizations, azides serve as a convenient handle, compared to ketones and aldehydes which have the drawback of relatively high natural abundance. Because of their ease of synthesis, relatively small size and kinetic stability under physiological conditions, biological systems can

Chapter 1

tolerate azides pretty well. They are usually metabolically incorporated into biological systems through unnatural amino acids and monosaccharides. Several reactions have evolved that utilize the uniquely selective properties of azides in further functionalization.

A.1 Staudinger Ligation. In biocompatible variation of Staudinger ligation the azides undergo nucleophilic attack by triarylphosphines to give azaylide intermediate **II** through the loss of nitrogen from intermediate **I** under physiological conditions. The proximal ester from the phosphine reagent traps the azaylide thus generated to give a stable amide bond in good yields (Scheme 1-12 (a)).²⁹ In a native chemical ligation variation of this reaction known as the traceless Staudinger ligation, phosphonothioester replaces traditional triarylphosphine ester to trap the azaylide intermediate (Scheme 1-12 (b)).³⁰ This derivation has the advantage of expulsion of oxidized phosphine reagent during the loss of nitrogen while forming the amide bond.

Scheme 1-12: Staudinger ligations. (a) Traditional Staudinger ligation. (b) Traceless Staudinger ligation.

This reaction has been used with much success for tagging azides on live cells.

²⁹ (a) Saxon, E. and Bertozzi, C. R. *Science*. **2000**, 287, 2007-2010; (b) Hang, H. C.; Yu, C.; Kato, D. L.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A* **2003**, 100, 14846-14851.

³⁰ Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, 2, 2141-2143.

Chapter 1

A.2 Copper Catalyzed Azide-Alkyne [3 + 2] Cycloaddition (CuAAC)-“Click”

Chemistry. In the bio adaptable variation, of Cu(I) –promoted Huisgen azide-alkyne [3 + 2] electrocyclizations,³¹ the reaction can be performed in water, with optimal kinetics attainable in a pH range of 7 and 9. The active Cu(I) catalyst is generated in situ from Cu(II) salts and a reductant like ascorbic acid or tris(2-carboxyethylphosphine) (TCEP) or with the help of trivalent triazole ligands **27** & water soluble bathophenanthroline **28** (Scheme 1-13).

Scheme 1-13: “Click” reaction and Cu(I) ligands.

This particular chemistry has been successfully applied in detecting azides in protein samples. But the toxicity of the reagents involved has made it unsuitable for labeling live cells.

A.3 Strain Promoted [3 + 2] Cycloaddition. To make this reaction more biocompatible by overcoming the hurdles of Cu related cytotoxicity, insolubility of ligands in water or requirement of oxygen-free condition, Bertozzi and coworkers introduced a strain promoted copper free click chemistry, where the alkyne counterpart is activated by ring strain instead of metal.³² Cyclooctyne derivatives

³¹ (a) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem. Intl. Ed.* **2002**, *41*, 2596-2599; (b) Tornøe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057-3064; (c) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686-4687.

³² Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046-15047.

Chapter 1

(Scheme 1-14) serve as the alkyne and has been successfully used for labeling azides on cell surfaces and living systems with no apparent cytotoxicity.³³

Scheme 1-14: Cyclooctyne reagents for strain-promoted copper-free click reactions.

B. Alkene Modification. Introduction of olefins to proteins opens up a wide avenue for their orthogonal modifications through addition reactions or ruthenium-catalyzed metathesis.³⁴ In their recent works Davis and co-workers have successfully transformed *O*-mesitylenesulfonylaminehydroxylamine functionalized cysteine into Dha **16** on protein surface without any side reaction on methionine.³⁵ The formed Dha served as the site of attack for several thiol functionalized derivatives for further important modification of the proteins. By using S-allylcysteine (Sac, **29**) as the thiol component, the authors were able to produce S-allyl functionalized protein, that later served as the substrate for cross-metathesis reactions with a variety of other important functionalities (Scheme 1-15).

³³ (a) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486-11493; (b) Agard, N. J.; Baskin, J. M.; Prescher, J. A.; Lo, A.; Bertozzi, C. R. *ACS Chem. Biol.* **2006**, *1*, 664-648 (c) Sletten, E. M.; Bertozzi, C. R. *Org. Lett.* **2008**, *10*, 3097-3099; (d) Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. *Science* **2008**, *320*, 664-667.

³⁴ Blackwell, H. E.; Grubbs, R. H. *Angew. Chem. Int. Ed.* **1998**, *37*, 3281-3284.

³⁵ (a) Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 15, 5052-5053; (b) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 30, 9642-9643.

Chapter 1

Scheme 1-15: Addition and cross-metathesis reactions with alkenes.

C. Modification of Halogenated Amino Acids. Palladium has been used to link a biotin carrying alkene to protein containing *p*-iodophenylalanine through Heck coupling. The *p*-iodophenylalanine was incorporated into the protein by biological means. The efficiency of labeling achieved was 2% (Scheme 1-16 (a)).³⁶ Very recently, a palladium-pyrimidine catalyst system has been used to couple a variety of aryl and vinyl boronic acids to protein surfaces by Suzuki-Miyaura cross-coupling (Scheme 1-16 (b)).³⁷

Scheme 1-16: Palladium catalyzed phenylalanine modifications.

1.5 Challenges in Developing Reactions for Selective Protein Modification

Use of chemical transformations in protein studies has led scientists to explore and

³⁶ Kodama, K.; Fukuzawa, S.; Nakayama, H.; Kigawa, T.; Sakamoto, K.; Yabuki, T.; Matsuda, N.; Shirouzu, M.; Takio, K.; Tachibana, K. *ChemBioChem* **2006**, *7*, 134-139.

³⁷ Chalker, J. M.; Wood, C. S. C.; Davis, B. G. *J. Am. Chem. Soc.* **2009**, *130*, 1080-1081.

Chapter 1

develop new reactions for selective protein modification in recent years. Developing such reactions poses certain challenges which we need to address first before we embark on our own journey of developing more simple and practical reactions for chemical modifications of proteins.

1.5.1 Aqueous Media.

Organic reactions are usually performed in organic solvents, as it can better dissolve the substrates involved and result in homogeneous solutions. In homogeneous solutions the reaction can proceed faster, monitoring by thin layer chromatography (TLC) and workup with aqueous solutions becomes easier, leading to straightforward purifications with chromatographic techniques.

Proteins are entities that are very sensitive towards their surrounding environment. A small change in the immediate proximity can cause the tertiary structure to become perturbed. This results in protein unfolding, aggregation and precipitation. To maintain protein structure and prevent its denaturation, having aqueous reaction media is essential.

The requirement of using aqueous reaction media in protein studies imposes a number of challenges. When organic reactants are used to modify proteins, heterogeneous solutions result. This in turn slows down the reaction rate and certain reactants might become unstable and decompose under aqueous conditions. Continuous monitoring by TLC becomes ineffective; regular workup to remove side products becomes impossible; special purification and analytical techniques need to be adopted. All these issues need to be addressed while developing new methods for protein modifications.

Chapter 1

Although a lot of challenges exist, aqueous media also have considerable advantages over reactions performed in conventional organic solvents.³⁸

They are of special significance in dealing with reactions of biological molecules like carbohydrates, low molecular weight aldehydes, peptides and proteins which are sometimes commercially available as aqueous solution. Being able to manipulate them in water allows their easier handling.

Aqueous media can effect the reactivities and stereoselectivity of reactions carried out in it due to its high cohesive energy density and high interfacial energy. Salt in buffers can also alter the selectivity of reactions compared to anhydrous organic solvent media.

Organic reactions performed in aqueous media will open up an even more sophisticated field of chemistry under the most desirable environmentally friendly condition.

1.5.2 Temperature.

Temperature can play a key role in designing organic reactions. Usually organic reactions are performed at room temperature. Conditions where the reactions proceed rather slowly, necessitates heating under reflux conditions to increase its conversion rate. On the other hand, reactions where stereoselectivity is a concern, very low temperatures (-78 °C to -40 °C) are usually applied.

Equilibrium between folded and unfolded states of a protein is closely related to its thermodynamic stability which in turn is dependent on its external temperature.³⁹ At both high (e.g., 50-100 °C) and low (e.g., less than -10 °C)

³⁸ Li, C. -J.; Chen, T. -H. *Organic Reactions in aqueous Media*, John Wiley and Sons, Inc. New York, 1997, p 2.

³⁹ Chi, E. Y.; Krishnan, S.; Randolph, T. W.; Carpenter, J. F. *Pharm. Res.* **2003**, 20, 1325-1336.

Chapter 1

temperatures the native protein conformation can be perturbed to a sufficient degree to promote unfolding and aggregation leading to denaturation. This effect is more pronounced at higher temperatures. Although in most reactions the rate constants increase exponentially with temperature, protein aggregation also increases simultaneously. It is important to achieve near quantitative conversion at a fast rate since proteins are usually available in low concentrations. Achieving a fast rate is useful in addressing instability issues if this becomes a concern in our developing reactions.

Hence, when developing a new reaction for protein modification we need to strike a balance between the reaction rate and protein stability by choosing appropriate temperature.

1.5.3 Solution pH.

Solution pH can strongly influence protein aggregation rates over a broad range. This is because, electrostatic interactions of proteins that arises from nonspecific repulsions is dependent on total and type of charge on protein and is determined by solution pH. When the surrounding pH is far from isoelectric point (pI), because of an increase in the acidity or basicity of solution, the unfolded protein becomes more stable than the folded form resulting from lesser charge density and electrostatic free energy in it.⁴⁰ In contrast, specific charge interactions resulting from ion pairing or salt bridges can stabilize folded state of proteins. Here also we need to establish an optimum pH for the developing reaction to maintain proteins in folded state.

⁴⁰ Dill, K. A. *Biochemistry*. **1990**, 29, 7133-7155.

Chapter 1

1.5.4 Salts & Surfactants.

Certain solutes and salts like sugars, polyols, and ammonium sulfate in high concentrations (≥ 1 M) can affect protein equilibrium solubility and conformational stability by stabilizing the native folded state, whereas solutes like urea and guanidine hydrochloride denature proteins by binding to the unfolded state to a greater extent over folded state thus favoring unfolding.¹⁴ Solution pH and salt effects are closely related to each other and in designing a proper reaction we need to take these effects into consideration.

1.5.5 Analytics.

Usually, organic reactions are monitored through a combination of methods. TLC, nuclear magnetic resonance spectroscopy (NMR) and mass spectrometric (MS) analyses helps monitor the reaction. Once the desired product has been purified by chromatographic techniques, it is analyzed by NMR and MS.

Proteins are large and complex molecules. It is impracticable to monitor their reactions in aqueous media, through conventional TLC and NMR analysis. They are mostly analyzed by electron spray ionization (ESI)-MS. Sometimes due to their structure or high heterogeneity, proteins do not ionize properly and make analytics difficult. Under such circumstances more complicated analytical techniques like matrix assisted laser desorption-ionization time-of-flight (MALDI-TOF) has to be adopted. Other indirect approaches towards monitoring protein reactions are Ultra-violet spectroscopy (UV) (provided an established assay system exists), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (when the modification functionalizes proteins with biotin or fluorescent tags). All these are quite complicated and time consuming processes. Hence, when designing our

Chapter 1

methodology for protein modifications we have to keep the complexity of analysis in mind.

1.6 Summary

The last decade has seen an abundance of research in the area of protein conjugation. Labeling of proteins at a specific site with small molecules can advance our understanding of biochemical events. With this novel technology investigation of protein expression, localization, protein-protein interaction and their conformational changes during the cell cycle can be studied. This in turn will facilitate the investigation of protein function in the development of phenotype in vivo and in vitro. There is still a need for significant expansion in new and sophisticated chemoselective reactions. Herein, we will report the methodology developments for the site specific functionalization of peptides by performing carbon-carbon bond forming reactions at their *N*-terminal aldehyde in aqueous media and its application in protein functionalization. The result will be discussed in more detail in the next section.

PART I

Chapter 2

Functionalization of Peptides

Chapter 2

In developing water based reactions for the selective functionalization of *N*-terminal peptide aldehydes, we explored three specific reactions.

- 1) *Aza Diels-Alder* Reaction,
- 2) Indium mediated *Allylation* Reaction &
- 3) *Mukaiyama Aldol* Reaction

This chapter will elaborate on the methodology development for each of these reactions beginning with an introduction, followed by rationalizing their selection, methodology development, applications and finally discussion.

2.1 *Aza Diels Alder*

2.1.1 Introduction: Literature Overview

The *aza Diels-Alder* reaction is one of the most powerful methods for constructing six membered nitrogen based heterocycles. These heterocycles in turn serve as important building blocks in the synthesis of natural products. The *aza Diels-Alder* reaction is an extension of the *Diels-Alder* reaction itself.

The two reactive partners of this reaction are termed diene and dienophile. Diene is the electron rich component and dienophile is the electron poor component. For a successful *Diels-Alder* reaction the diene needs to be in a *s-cis* conformation.

When one or more of the carbon atoms in the six membered ring is replaced with a heteroatom, it gives rise to the *hetero Diels-Alder* reaction.⁴¹ It was Alder who first disclosed this concept in 1943 through a serendipitous discovery.⁴² In his investigation he found that an imine tautomer could react with a number of dienes to

⁴¹ (a) Daly, J. W.; Spande, T. F. *Alkaloids: Chemical and Biological Perspectives*, Pelletier, S. W., Ed.; New York, 1986; Vol. 4, pp 1-254. (b) Fodor, G. B.; Colasanti, B. *Alkaloids: Chemical and Biological Perspectives*, Pelletier, S. W., Ed.; New York, 1985; Vol. 3, pp 1-91.

⁴² Alder, K. *Neuere Methoden der Preparativen Organischen Chemie*. Verlag Chemie, Berlin, 1943, pp. 251-442.

Chapter 2

give the [4+2] cycloaddition product. Among the *hetero Diels-Alder* reactions the *aza Diels-Alder* or *imino Diels-Alder* reaction having nitrogen as the hetero atom has been extensively studied.⁴³

This reaction can be classified into 3 basic categories based upon the position of the imine component. It can either be in the dienophile or 1 (1-azadiene) or 2 (2-azadiene) position of diene (Scheme 2-1).

Scheme 2-1: Categories of *aza-Diels-Alder* reaction.

Among them the first variation is more common than the other two. Imine dienophiles are generated from the reaction between aldehydes and ketone with amine through some activation or it can be used together with some activated diene.

2.1.2 Activation in aza Diels-Alder Reaction.

Imino dienophiles **1** are usually generated through Lewis acid activation. Triflates of rare earth metals (eg. Yb(OTf)₃, Sc(OTf)₃ and In(OTf)₃) are effective in this regard (Eq. 1).⁴⁴

⁴³ (a) Boger, D. L. *Tetrahedron* **1983**, 39, 2869-2939. (b) Buonora, P.; Olsen, J. C.; Oh, T. *Tetrahedron* **2001**, 57, 6099-6138.

⁴⁴ (a) Kobayashi, S.; Araki, M.; Ishitani, H.; Nagayama, S.; Hachiya, I. *Synlett*. **1995**, 233-234; (b) Ali, T.; Chauhan, K. K.; Frost, C. G. *Tetrahedron Lett.* **1999**, 40, 5621-5624; (c) Kobayashi, S.; Ishitani, H.; Nagayama, S. *Synthesis* **1995**, 1195-120; (d) Kobayashi, S.; Ishitani, H.; Nagayama, S. *Chem. Lett.* **1995**, 423-424; (e) Yu, L.; Chen, D.; Wang, P. G. *Tetrahedron Lett.* **1996**, 37, 2169-2172.

Chapter 2

Cycloaddition reactions involving imines **1** and Danishefsky's diene **2** demonstrated good yields under Bronsted acid activation.⁴⁵ Bronsted acids like HBF₄, TsOH and CF₃CO₂H were used in solvents that included acetonitrile, methanol and water to afford dihydro-4-pyridone derivatives (Eq. 2). Acetonitrile and methanol proved to be the solvents of choice.

2.1.3 Hetero Diels-Alder Reaction in Aqueous Solvent.

Breslow and Rideout demonstrated the first *Diels-Alder* reaction in aqueous media about two decades ago.⁴⁶ They credited the 700 fold rate enhancement of the reaction of cyclopentadiene and butanone in water (Eq. 3) compared to non polar solvents to the “hydrophobic effect” or “salt out” effect (in the presence of salts). Due to this effect, non-polar components when mixed with polar solvents associate with each other densely to reduce hydrocarbon-water interfacial area. This in turn results in rate acceleration from bringing the nonpolar reaction partners together in transition state.

Few years later Grieco and coworkers introduced the *aza Diels-Alder* in water,⁴⁷ from the reaction of an unactivated iminium salt (generated in situ in water from benylamine and formaldehyde) and cyclopentadiene (Scheme 2-2). They

⁴⁵ (a) Akiyama, T.; Takaya, J.; Kagoshima, H. *Tetrahedron Lett.* **1999**, 40, 7831-7834.

⁴⁶ (a) Breslow, R.; Rideout, D. C. *J. Am. Chem. Soc.* **1980**, 102, 7816-7817; (b) Breslow, R. *Acc. Chem. Res.* **1991**, 24, 159-164.

⁴⁷ (a) Larsen, S. D.; Grieco, P. A. *J. Am. Chem. Soc.* **1985**, 107, 1768-1769; (b) Grieco, P. A.; Larsen, S. D. *Org. Syn.* **1990**, 68, 206; (c) Grieco, P. A.; Larsen, S. D.; Fobare, W. F. *Tetrahedron Lett.* **1986**, 27, 1975-1978; (d) Grieco, P. A.; Parker, D. T.; Fobare, W. F.; Ruckle, R. *J. Am. Chem. Soc.* **1987**, 109, 5859-5861; (e) Grieco, P. A.; Bahsas, A. *J. Org. Chem.* **1987**, 52, 5746-5749; (f) Grieco, P. A.; Clark, J. D. *J. Org. Chem.* **1990**, 55, 2271-2272.

Chapter 2

expanded the scope of this reaction by including different types of dienes and also showed intramolecular version of the reaction. In their reactions no Lewis acid activation was necessary. In their studies, replacing water with alcohols resulted in the reduction of reaction rate.

Scheme 2-2: Aza Diels-Alder in water.

This work was followed by the development of Lewis acid activated version, where water stable triflates of rare earth metals (eg. Yb(OTf)₃, Sc(OTf)₃ and In(OTf)₃) and lanthanide bis(trifluoromethane sulfonyl) amide were used as the Lewis acid component.⁴⁸ The presence of these Lewis acid complexes in water not only improved the enantioselectivity of the reactions but also raised their chemical yields.

2.1.4 Synthetic Applications of aza Diels-Alder reaction.

Natural product synthesis. Although numerous examples of *hetero Diels-Alder* based natural product synthesis exist, a select few will be discussed to illustrate the most important aspects of the reaction.

The most general and widely used application of this methodology is in the synthesis of bridgehead nitrogen containing alkaloids through its intramolecular variant (Eq 4).⁴⁹

⁴⁸ (a) Mikami, K.; Kotera, O.; Motoyama, Y.; Sakaguchi, H. *Synlett*. **1995**, 34, 975-977; (b) Sasai, H.; Suzuki, T.; Itoh, N.; Tanaka, K.; Date, T.; Okamura, K.; Shibasaki, M. *Tetrahedron Lett.* **1993**, 34, 851-854; (c) Yu, L.; Chen, D.; Wang, P. G.; *Tetrahedron Lett.* **1996**, 37, 2169-2172; (d) Kobayashi, S.; Hachiya, I.; Araki, M.; Ishitani, H. *Tetrahedron Lett.* **1993**, 34, 3755-3758; (e) Kobayashi, S.; Araki, M.; Ishitani, H.; Nagayama, S.; Hachiya, I. *Synlett*. **1995**, 233-234; (f) Ali, T.; Chauhan, K. K.; Frost, C. G. *Tetrahedron Lett.* **1999**, 40, 5621-5624; (g) Kobayashi, S.; Ishitani, H.; Nagayama, S. *Synthesis* **1995**, 1195-1202; (h) Kobayashi, S.; Ishitani, H.; Nagayama, S. *Chem. Lett.* **1995**, 423-424; (i) Yu, L.; Chen, D.; Wang, P. G. *Tetrahedron Lett.* **1996**, 37, 2169-2172.

⁴⁹ Oppolzer, W. *Angew. Chem., Int. Ed. Engl.* **1972**, 11, 1031-1032.

Chapter 2

In its first diastereoselective application, it was used in the synthesis of swainsonine analogs (Scheme 2-3).⁵⁰ Diacetal protected form of *D*-arabinose was reacted with benzylamine to give Schiff's base, which then reacted with Danishefsky's diene to give the cycloaddition product in the diastereomeric ratio of 4.7:1.

Scheme 2-3: *Aza Diels-Alder* reaction in the synthesis of Swainsonine analogs.

Nicolaou's group demonstrated an elegant application of the *aza Diels-Alder* reaction in the synthesis of core structure of thiopeptide antibiotics GE2270A, GE2270T and GE2270C1 (Scheme 2-4).⁵¹ These antibiotics are very potent against methicillin and vancomycin resistant bacterial strains. The common thiazole pyridine fragment in these molecules was obtained through brilliantly designed *aza Diels-Alder* based [4 + 2] dimerization.

⁵⁰ Herczegh, P.; Kovacs, I.; Szilagyi, L.; Sztaricskai, F. *Tetrahedron* **1994**, *50*, 13671-13686.

⁵¹ Nicolaou, K. C.; Dethe, D. H.; Leung, G. Y. C.; Zou, B.; Chen, D. Y. -K. *Chem. Asian. J.*, **2008**, *3*, 413-429.

Chapter 2

GE2270A : $R^1 = \text{CH}_2\text{OMe}$; $R^2 = \text{CH}_3$; $R^3 = \text{CH}_3$; $R^4, R^5 = \text{H}$
 GE2270T : $R^1 = \text{CH}_2\text{OMe}$; $R^2 = \text{CH}_3$; $R^3 = \text{CH}_3$; $R^4, R^5 = \text{C}=\text{C}$
 GE2270C1 : $R^1 = \text{H}$; $R^2 = \text{CH}_3$; $R^3 = \text{CH}_3$; $R^4, R^5 = \text{H}$

Scheme 2-4: *Aza Diels-Alder* in the synthesis of thiopeptides.

2.1.5 Aza Diels-Alder: Our Extension

Glyoxylic acid and derivatives have proved to be suitable dienophiles in *hetero Diels-Alder* reaction when water was used as solvent.⁵² This is because of their inherent solubility in water (Eq. 5). α -hydroxyl- γ -lactones can be obtained from the rearrangement of the intermediate arising from the *hetero Diels-Alder* reaction of aqueous solutions of glyoxylic acids and cyclopentadiene in aqueous media. These

⁵² (a) Lubinéau, A.; Augé, J.; Lubin, N. *Tetrahedron Lett.* **1991**, 32, 7529-7530; (b) Grieco, P. A.; Henry, K. J.; Nunes, J. J. Matt. J. E.; Jr. *J. Chem. Soc., Chem. Commun.* **1992**, 368-370.

Chapter 2

5,5-fused systems generated through *hetero Diels-Alder* have been used in the total synthesis of therapeutic agents like (-)-carbivor,⁵³ an anti-HIV agent and intermediate of mevinic acids.⁵⁴ The Authors emphasized several points; 1) in this reaction aqueous solution of glyoxylic acid was used, 2) the relative rate of the *hetero Diels-Alder* reaction to cyclopentadiene dimerization was enhanced in the aqueous media, and 3) acid catalysis lead to a rate improvement.

In another application, glyoxylic acid was used as a dienophile by Lubineau and coworkers in an *oxo Diels-Alder* reaction in the synthesis of sialic acids 3-deoxy-D-manno-2-octulosonic acid (**KDO**) and 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (**KDN**) (Eq. 6).⁵⁵ When glyoxylic acid was used as dienophile, the skeleton of KDO was obtained from a *si* face attack of the diene. While *re* face attack gave the skeleton of KDN.

All of the above examples using aqueous solution of glyoxylate in *hetero Diels-Alder* reaction inspired us in choosing this reaction for peptide

⁵³ MacKeith, R. A.; McCague, R.; Olivo, H. F.; Palmer, C. F.; Roberts, S. M. *J. Chem. Soc., Perkin Trans. 1*. **1993**, 313-314.

⁵⁴ McCague, R.; Olivo, H. F.; Roberts, S. M. *Tetrahedron Lett.* **1993**, 34, 3785-3786.

⁵⁵ Lubineau, A.; Auge, J.; Lubin, N. *Tetrahedron* **1993**, 49, 4639-4650.

Chapter 2

be kept in mind are; i) speed of the coordination-dissociation equilibrium; ii) a high coordination number and iii) re-usability of the Lewis acid. Indium trichloride, being able to meet these requirements was a promising activator. Together with its unique properties, the sheer lack of scientific work in this field at that time raised our curiosity in the use of this special Lewis acid in *Diels-Alder* reaction. Our group had demonstrated the efficient use of indium trichloride (InCl_3) as Lewis acid in *Diels-Alder* reaction.⁵⁸ Cyclic and acyclic dienes react with different dienophiles in water at room temperature in the presence of indium trichloride (20 mol %) to give the desired product in good yield and selectivity (Eq. 7).

B. Selection of Diene. For our peptide based *hetero Diels-Alder* reaction we decided to use Danishefsky's Diene **4** as the diene component. Danishefsky's diene is a reactive diene that has been used with much success over the years since its first introduction.⁵⁹

Most of the reactions utilizing Danishefsky's diene are based on amino acid derived imines. Having a reactive diene in hand eliminates the possibility of reaction failure from not having a reactive diene component.

⁵⁸ (a) Loh, T. P.; Pei, J.; Lin, M. *J. Chem. Soc., Chem. Commun.* **1996**, 2315-2316. (b) Loh, T. P.; Wei, L. L. *Synlett.* **1998**, 975-976.

⁵⁹ Kerwin, J. F.; Danishefsky, S. *Tetrahedron Lett.* **1982**, 23, 3739-5986.

Chapter 2

2.1.6 Experimental.

Present Work.

A. Synthesis of Starting Materials. Before applying *Hetero-Diels Alder* reaction to proteins, test reactions with dipeptide aldehyde **5** (Serine-Alanine-*O*-methyl ester, Ser-Ala-OMe, Scheme 2-6) and tetrapeptide aldehyde **6** (Serine-Phenylalanine-Leucine-Glutamic acid-*O*-methyl ester, Ser-Phe-Leu-Glu-OMe, Scheme 2-7) were carried out to determine the optimum conditions. We decided to use protected peptides as they are easy to handle during work up and purifications. Solution phase peptide synthesis was carried out for both the starting materials. HATU was used as the coupling reagent, hydrolysis of methyl esters was carried out under basic conditions and the final deprotection to expose the free *N*-terminal was achieved through treatment with trifluoroacetic acid. The final products were purified by High Performance Liquid Chromatography (HPLC) using C18 column and reversed phase system.

Synthesis of dipeptide **5**. Scheme 2-6: Synthesis of dipeptide Ser-Ala-OMe, **5**

The synthesis of dipeptide **5** was carried out as shown in scheme 2-6, starting from commercially available, Boc-Serine-OH. Reaction of Boc-serine-OH with *L*-Alanine-*O*-methyl ester and HATU in dichloromethane in presence of Et₃N at room temperature for 18 hours furnished the dipeptide **7** in 95% isolated yield.

Chapter 2

Subsequently, the protected dipeptide **7** was treated with a 10% solution of trifluoroacetic acid in dichloromethane for 18 hours to furnish the desired dipeptide **5** in 90% yield after HPLC purification. We chose this particular combination of amino acids, because having alanine at the *C*-terminus of the peptide facilitated the determination of diastereoselectivity by NMR.

Synthesis of tetrapeptide **6**.

Scheme 2-7: Synthesis of tetrapeptide Ser-Phe-Leu-Glu(OMe)-OMe, **6**

Synthesis of tetrapeptide **6.** Tetrapeptide Boc-Ser-Phe-Leu-Glu(OMe)-OMe is a combination of four amino acids. Phenyl alanine in the second position was chosen to provide us with an UV active handle and aid us in purifications. Leucine helped to make it less polar. And acidic Glutamate at the *C*-terminal was randomly chosen. The synthesis started with the coupling of commercially available Boc-Serine-OH with a solution of *L*-Phenylalanine-*O*-methyl ester in dichloromethane in the presence of HATU and Et₃N at room temperature for 18 hours to give the dipeptide **8** in 90% isolated yield. Subsequently, the protected dipeptide **8** underwent basic hydrolysis to furnish free carboxy terminus for the next coupling event. Similar series of coupling with *L*-Leucine-*O*-methyl ester, basic hydrolysis followed by coupling with *L*-Glutamate-(OMe)-OMe ester furnished the protected tetrapeptide **12** through the

Chapter 2

intermediates **9** (quantitative yield), **10** (80% yield) and **11** (quantitative yield) respectively. Treatment of tetrapeptide **12** with 20% solution of trifluoroacetic in dichloromethane for 18 hours produced the desired tetrapeptide with unmasked *N*-terminus **6** in 60% yield after HPLC purification.

B. Optimizing Conditions for Periodate Oxidation & *aza* Diels-Alder Reaction of Peptides. In our initial studies, a solution of dipeptide **5** in 25 mM sodium phosphate buffer of pH 7.0 was treated with sodium metaperiodate (NaIO_4 , 2 Equivalent) in the dark and reaction monitored by LC-ESI-MS. Upon complete conversion of the starting material **5** (expected mass $[\text{M}+\text{H}]^+$ 191.99, observed mass 192, retention time 2.27 min, Figure 2-1, b & d) to product **13** ($[\text{M}+\text{H}]^+$ 160.94, observed mass 160.4, retention time 0.77 min, Figure 2-1, a & c) the solution was freeze dried and used for the next step, *aza* Diels-Alder reaction.

Figure 2-1. HPLC-ESI-MS analysis of periodate reaction with dipeptide **5**. **a)** Mass spectrum of peptide aldehyde **13** retention time 0.77 min. **b)** Mass spectrum of dipeptide **5** retention time 2.27 min.

Chapter 2

Figure 2-1. ESI-MS analysis of periodate reaction with dipeptide **5**. **c)** TIC of peptide aldehyde **13** ($[M+H]^+$ 160.94, observed mass 160.4). **d)** TIC of dipeptide **5** (expected mass $[M+H]^+$ 191.99, observed mass 192).

In the process, the aldehyde was redissolved in 25 mM sodium phosphate buffer at pH 7.0 and treated with indium trichloride (InCl_3 , 10%), aniline (1 Equivalent) and 2-trimethylsiloxy-4-methoxy-1,3-butadiene (2.5 Equivalent) at 0 °C (refer to scheme for table 2-1). The reaction was then stirred at room temperature for 20 h. TLC analysis of the resulting reaction mixture, revealed no consumption of starting material or formation of new product. While this was a disappointing outcome of the reaction, we thought it was likely that the very polar reaction medium was responsible for the lack of conversion to the Diels-Alder product. The reaction was repeated with the freeze dried peptide aldehyde, in acetonitrile instead of buffer and keeping all the other ingredients constant. Acetonitrile was chosen as the solvent because literature references have shown it to be one the best solvent choice for *Diels-Alder* type cycloaddition.⁶⁰ We were still unsuccessful in getting the desired product (Table 2-1).

⁶⁰ Han, B.; Li, J. -L.; Ma, C.; Zhang, S. -J.; Chen, Y. -C. *Angew. Chem., Int. Ed. Engl.* **2008**, *120*, 10119-10122.

Chapter 2

Table 2-1 *Aza Diels-Alder* trials following aldehyde generation by Periodate reaction^a

Entry	Solvent	Yield	Remarks
1	25 mM Sodium Phosphate buffer, pH 7.0	-	Acidic (pH 4-5) from excess iodic acid
2	CH ₃ CN	-	

^aPeriodate oxidation reactions were run with 2 equiv of NaIO_4 , 1 equiv of peptide **5** in 25 mM sodium phosphate (NaPi) buffer (pH 7.0) in the dark till completion as judged by LC-ESI-MS analysis. Following which the solvent was removed by freeze drying and taken for the next step of *aza Diels-Alder*. *Aza Diels-Alder* reactions were run with 1 equiv of peptide aldehyde **13**, 10% InCl_3 powder, aniline (1 equiv) and Danishefsky's diene **4** (2.5 equiv) in different solvents.

A careful look at the periodate oxidation of the peptide, suggested that an excess of periodate might be interfering with the *aza Diels-Alder* step.

To address this issue a number of screening studies were performed to obtain the optimum condition (Table 2-2). In all these studies, the *aza Diels-Alder* reaction was performed in CH_3CN .

The results showed that, the *aza Diels-Alder* reaction would not proceed if **13** was contaminated with NaIO_4 (entries 1-2). As a consequence Na_2SO_3 was added during the work up. Furthermore the best results were obtained if the inorganic impurities are removed by solid phase extraction (entries 5, 6 and 8). Then N_2 gas was passed through for further purification to remove HCHO and gaseous impurity such

Chapter 2

as NH_3 . After removal of water by freeze drying, the peptide aldehyde **13** was obtained as a white powder, ready for the next reaction.

Table 2-2 Optimization of Periodate work up for *aza Diels-Alder* reaction^a

Entry	Neutralization of IO_3^-	Sample Extraction ^b	Freeze Drying	Yield ^c	Remarks
1	-	-	-	-	Acidic (pH 4-5)
2	-	+	+	-	
3	$\text{HOCH}_2\text{CH}_2\text{OH}$	+	+	2%	Neutral
4	(3 equiv over NaIO_4)	+	-	-	pH
5	Na_2SO_3 (0.74 equiv over NaIO_4)	+	+	26%	Neutral pH
6	Na_2SO_3	+	+	30%	Clean product
7	(0.5 equiv over NaIO_4)	-	-	-	
8	NaIO_4	+	+	47%	Clean product

^aPeriodate oxidation reactions were run with 2 equiv of NaIO_4 , 1 equiv of peptide **5** in 25 mM sodium phosphate (NaPi) buffer, pH 7.0 in the dark till completion as judged by LC-ESI-MS analysis. Following which different workup conditions were accessed and taken for the next step of *aza Diels-Alder* reaction. Reactions were run with 1 equiv of peptide aldehyde **13**, 10% InCl_3 powder, 3 equiv of MgSO_4 , aniline (1 equiv) and Danishefsky's diene **4** (2.5 equiv) in acetonitrile. ^bC18 Seppak sample extraction cartridges were used for (E 2-6) and HLB Oasis sample extraction cartridge used for (E-8).

^cIsolated yield.

Chapter 2

From the above results the best possible conditions for the periodate oxidation followed by *aza Diels-Alder* reaction was established. Under the optimum conditions the generated peptide aldehyde could undergo successful *aza-Diels-Alder* reaction when the reaction was followed by a simple workup of the peptide aldehyde. The *aza Diels-Alder* product was obtained in 47% yield as an inseparable mixture of diastereomers with a diastereoselectivity of 58:42. The diastereomeric ratios were determined by integration of the ^{13}C signals (61.7 and 61.9 ppm) of the carbon attached to quarternary proton of heterocyclic ring (Figure 2-2).

Figure 2-2. ^{13}C NMR of **14**. The diastereomeric ratios were determined by integration of the ^{13}C signals (61.7 and 61.9 ppm) of the carbon attached to quarternary proton of heterocyclic ring

Next a number of amines were screened to ascertain the substrate scope for this reaction (Table 2-3).

Chapter 2

Table 2-3 Amine screening for *aza Diels-Alder* reaction^a

Entry	R	Yield ^b	dr ^c
1		47	58:42
2		50	61:39
3		23	47:53
4	CH ₃ CH ₂ CH ₂ -	-	-
5		26	60:40

^aPeptide aldehydes were generated by periodate oxidation and taken for *aza Diels-Alder* reaction. *Aza Diels-Alder* reactions were run with 1 equiv of peptide aldehyde **13**, 10% InCl₃ powder, 3 equiv of MgSO₄, amine (1 equiv) and Danishefsky's diene **4** (2.5 equiv) in acetonitrile. ^bIsolated yield. ^cDiastereomeric ratios were based on ¹³C NMR analysis. The diastereomeric ratios were determined by integration of the ¹³C signals (61.8 and 61.9 ppm) of the carbon attached to quarternary proton of heterocyclic ring.

The results showed that aromatic amines in general performed better as amine component with the yields in the range of 50% (entries 1 & 2). One important information obtained from this screening study is that, we could also apply cyclic aliphatic amine (entry 3) as the amine component; but reactions with open chain aliphatic amines were not successful. This information could be used to couple a second peptide to the parent peptide. An attempt to improve the diastereoselectivity by using chiral amine (entry 5) to induce stereoselectivity did not give promising results. Next, we moved on to screen solvents and other indium based Lewis acids for further improvements (Table 2-4).

Chapter 2

Table 2-4 Solvent and Lewis acid screening for *aza Diels-Alder* reaction^a

Entry	Solvent	Lewis Acid	Product	Yield ^b	dr ^c
1	CH ₃ CN	InCl ₃	14	47	58:42
2	CH ₃ CN	InBr ₃	-	-	-
3	CH ₃ CN	Yb(OTf) ₃	-	-	-
4	H ₂ O	InCl ₃	-	-	-
5	25 mM NaPi, pH 7.0	InCl ₃	-	-	-
6	CH ₃ CN: H ₂ O	InCl ₃	15	13	-
7	DCM	InCl ₃	14	25	50:50
8	MeOH	InCl ₃	14	19	50:50

^aPeptide aldehydes **13** were generated by periodate oxidation and taken for *aza Diels-Alder* reaction. Reactions were run with 1 equiv of peptide, 10% Lewis acid, 3 equiv of MgSO₄, aniline (1 equiv) and Danishefsky's diene **4** (2.5 equiv) in solvent (2.5 mL). ^bIsolated yield. ^cDiastereomeric ratios were based on ¹³C NMR analyses. The diastereomeric ratios were determined by integration of the ¹³C signals (61.8 and 61.9 ppm) of the carbon attached to quaternary proton of heterocyclic ring.

In an attempt to raise selectivity and yield, Lewis acids InBr₃ and Yb(OTf)₃ were introduced (entries 2-3). Interestingly, when InCl₃ was replaced with InBr₃, no product was obtained while reactions catalyzed by Yb(OTf)₃ gave a complex mixture of products. So we decided to keep InCl₃ as our source of Lewis acid.

To make this reaction more biocompatible, it was necessary to demonstrate its application in aqueous media. Thus the reaction was run in H₂O and buffer (entries 4 and 5), but to our disappointment no product was obtained. Only when a 1:1 mixture of CH₃CN: H₂O was used as the solvent (entry 6) a new product was obtained, however majority of the starting material remained even after 20 h. This new

Chapter 2

compound was the non-cyclized Mannich type addition product **15**. Changing solvent to either MeOH or CH₂Cl₂ (entries 7 & 8) failed to provide improved results.

When the *Diels-Alder* reaction under optimized condition was applied to the tetrapeptide aldehyde **16** generated from *N*-terminal oxidation of tetrapeptide **6**, the desired cycloaddition product was obtained in 57% yield with a diastereoselectivity of 73:27 (Table 2-5). The increase in selectivity could be attributed to the increase in peptide chain length.

Table 2-5 *aza Diels-Alder* reaction with peptidic substrates^a

Entry	R	Yield ^b	<i>dr</i> ^c
1		47	58:42
2		57	73:27

^a*Aza Diels-Alder* reactions were run with 1 equiv of peptides, 10% lewis acid, 3 equiv of MgSO₄, aniline (1 equiv) and Danishefsky's diene **4** (2.5 equiv) in solvent (2.5 mL). ^bIsolated yield. ^cDiastereomeric ratios were based on ¹³C NMR analysis. The diastereomeric ratios were determined by integration of the ¹³C signals (61.8 and 61.9 ppm) of the carbon attached to quaternary proton of heterocyclic ring.

2.1.7 Summary

In conclusion, we have demonstrated an important extension of *aza Diels-Alder* reaction by its application in the functionalization of peptides. The notable

Chapter 2

feature of this reaction is the selective *N*-terminal site modification that is obtained. While conventional *aza Diels-Alder* reaction is carried out under high temperature or pressure conditions, in our method the reaction proceeds efficiently at room temperature with easily accessible amine and diene.

We have also shown that, this reaction can provide Mannich type addition product in aqueous environment.

These interesting results raised our hopes of applying this reaction to proteins. However, the failure to apply it in aqueous media and a strict requirement for CH₃CN as the solvent together with the formation of Mannich product in mixed aqueous solvent deterred us from pursuing this reaction on protein level. A biocompatible metal free Mannich reaction on proteins has been successfully demonstrated by Francis and coworkers.⁶¹

Next we moved on to develop methodology for functionalization of peptides through Indium mediated *allylation*.

2.2 Indium Mediated *Allylation*

2.2.1 Introduction: Literature Overview

Allylation reaction is one of the most successful nucleophilic addition reactions to carbonyl groups. Allylation reactions furnish homoallylic alcohols or amines from the reaction between organometallic reagents and aldehyde, ketone or imines. These homoallylic alcohols and amines can serve as versatile intermediates. . The resulting products can be further functionalized by epoxidation or ozonolysis of the terminal alkene moiety (Scheme 2-8).

⁶¹ Joshi, N. S.; Whitaker, L. R.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 15942-15943.

Chapter 2

Scheme 2-8: Functionalization of aldehydes and imines by allylation

The history of allylation reaction dates back to 1898, when Barbier replaced zinc with magnesium in the organic reactions of carbonyl compounds with alkyl halides. This was followed by the use of magnesium in a stepwise manner in similar reactions by Grignard which gained a lot of popularity due to the stability and ease with which the prepared “Grignard reagent” could be handled. After 1970, the tremendous momentum gained in the reactions of carbonyl group led to the development of methods using many other metals like lithium, aluminium, sodium, manganese, lead, platinum, cerium, copper and iron and more recently tin, and titanium. Today, all the one-step and two-step processes that use organometallic reagents as nucleophiles to react with carbonyls are frequently referred to as Barbier-Grignard type reaction.⁶² The success of this reaction is credited to the high reactivity of allyl halides. Various metals have been found to mediate such a reaction in organic solvents under anhydrous conditions.⁶³ Developing such reactions in aqueous media poses a lot of challenges as, for such a reaction to be successful, the metal needs to be

⁶² Blomberg, C. *The Barbier Reaction and Related One-Step processes*. Springer-Verlag, Berlin Heidelberg, 1993.

⁶³ (a) Buse, C. T.; Heathcock, C. H. *Tetrahedron Lett.* **1978**, 1685-1688; (b) Hoffmann, R. W.; Zeiss, H. –J. *Angew. Chem., Int. Ed. Engl.* **1979**, 18, 306-307; (c) Yamamoto, Y.; Yatagai, H.; Naruta, Y.; Maruyama, K. *J. Am. Chem. Soc.* **1980**, 102, 7107-7109.

Chapter 2

inert towards water and also should not form aqueous insoluble oxides in the reaction media. Zinc, tin and indium belong to the special class of metals that have been successfully used in aqueous media.⁶⁴

2.2.1 Indium-Mediated Allylation Reaction in Aqueous Media.

The fact that metal mediated allylation could be carried out in aqueous media was first demonstrated by the use of zinc in 1977, in an allylation reaction in ethanol/water (95:5) system (Eq. 8).⁶⁵ Although the product was obtained in low yield, it encouraged further research in this field.

The use of indium in Barbier-Grignard type reactions in water was first demonstrated in 1991, during the development of an aqueous chemical strategy in the synthetic efforts towards sialic acids, namely (+)-3-deoxy-*D*-glycero-*D*-galactonulosonic acid (**KDN**), 3-deoxy-*D*-manno-octulosonic acid (**KDO**) and *N*-acetylneuraminic acid (Eq. 9).⁶⁶ In the coupling of a sugar with ethyl (2-bromomethyl) acrylate that was needed during this synthesis, indium was used to replace zinc and tin owing to the harsh conditions required for zinc or tin mediated coupling. Because of its low first ionization potential (ev 5.79) compared to other metals used for this reaction, e.g. [tin (ev 7.43), magnesium (ev 7.65) and zinc (ev 9.39)] indium is more reactive.⁶⁷ On the other hand, indium is one of the most stable metals in aqueous

⁶⁴ (a) Li, C. -J.; Chen, T. -H. *Organic Reactions in aqueous Media*, John Wiley and Sons, Inc. New York, 1997, p 2; (b) Reviews: Lubineau, A.; Augé, J.; Queneau, Y. *Organic Synthesis in water*, Grieco, P. A. ed., Blackie A & P, London, 1998, pp 102; (c) Li, C. -J. *Chem. Rev.*, **1993**, 93, 2023-2035; (d) Li, C. -J. *Tetrahedron*. **1996**, 52, 5643-5668.

⁶⁵ Killinger, T. A.; Boughton, N. A.; Runge, T. A.; Wolinsky, J. *J. Organomet. Chem.*, **1977**, 124, 131-134.

⁶⁶ Li, C. -J.; Chan, T. -H. *Tetrahedron Lett.* **1991**, 32, 7017-7020.

⁶⁷ Li, C. -J.; Chan, T. -H. *Tetrahedron* **1999**, 55, 11149-11176.

Chapter 2

environment and does not form oxides easily in air. Due to these special properties indium is one of the most popular metals for allylation reactions.

Compared to zinc and tin mediated allylations that require acid catalysis, heat or sonication for activation, the indium mediated allylation occurs quite smoothly at room temperature in water without the need for any promoter or organic co-solvent. In fact, the indium mediated allylation is so mild that, it has been used to allylate a ketone **17** containing acid sensitive acetal functional group (Eq 10).

2.2.3 Synthetic Applications of Indium Mediated Allylation.

Sialic Acids and Carbohydrate Homologations. As previously mentioned, indium mediated allylation in water has been used for the synthesis of (+)-3-deoxy-*D*-glycero-*D*-galacto-nonulosonic acid (**KDN**, **18**) (Scheme 2-9), 3-deoxy-*D*-manno-octulosonic acid (**KDO**, **19**), *N*-acetyl-neuraminic acid (**20**) and other sialic acid derivatives (**21** and **22**).⁶⁸

⁶⁸ (a) Gordon, D. M.; Whitesides, G. M. *J. Org. Chem.* **1993**, 58, 7937-7938; (b) Gao, J.; Harter, R.; Gordon, D. M.; Whitesides, G. M. *J. Org. Chem.* **1994**, 59, 3714-3715.

Chapter 2

Scheme 2-9: Sialic acid and Carbohydrate Homologation

Higher analogs of carbohydrates and deoxy sugars (**23-26**) have been synthesized through indium-mediated allylation reaction by several groups.⁶⁹

Our group has also applied the indium mediated allylation on glucose-derived aldehydes. The major product was found to be the non-chelation controlled product and organic co-solvents were found to increase the diastereoselectivity. A screen of

⁶⁹ (a) Kim, E.; Gordon, D. M.; Schmid, W.; Whitesides, G. M. *J. Org. Chem.* **1993**, *58*, 5500-5507; (b) Schmid, W.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 6674-6675; (c) Binder, W. H.; Prenner, R. H.; Schmid, W. *Tetrahedron*. **1994**, *50*, 749-758; (d) Carda, M.; Castillo, E.; Rodriguez, S.; Marco, J. A. *Tetrahedron: Asym.* **1998**, *9*, 1117-1120.

Chapter 2

Lewis acids, demonstrated ytterbium trifluoromethanesulfonate [Yb(OTf)₃] as the best means of increasing reactivity as well as achieving good diastereoselectivity (Scheme 2-12).⁷⁰

Scheme 2-10: Indium mediated allylation of glucose derived aldehyde

β-Lactams. Indium mediated allylation with allyl bromide and indium in water/THF mixture has been applied to azetidine-2,3-diones to form β-Lactams. The same reaction in the presence of zinc was obtained in a very poor yield and 1:1 mixture of diastereomers whereas the indium mediated reaction gave better yields providing a single diastereomer.⁷¹

Carbocycles. Cyclopentane derivatives have been synthesized by Li. *et al.* through indium mediated allylation of 1,3-dicarbonyls in water.⁷² The authors have also used

⁷⁰ (a) Wang, R.; Lim, C. M.; Tan, C. H.; Lim, B. K.; Sim, K. Y.; Loh, T. P. *Tetrahedron: Asym.* **1995**, *6*, 1825-1828; (b) Loh, T. P.; Diana, S. C. H.; Sim, K. Y. *Synlett.* **1996**, 263-264.

⁷¹ (a) Jayaraman, M.; Manhas, M. S.; Bose, A. K. *Tetrahedron: Lett.* **1997**, *38*, 709-712; (b) Paquette, L. A.; Rothhaar, R. R.; Isaac, M.; Rogers, L. M.; Rogers, R. D. *J. Org. Chem.* **1998**, *63*, 5463-5472.

⁷² Li, C. -J.; Lu, Y. Q. *Tetrahedron Lett.* **1996**, *37*, 471;

Chapter 2

this reaction for carbocycle ring enlargements in water (Scheme 2-11).⁷³ Here, the reactions were found to be successful only when water was used as the solvent and were unsuccessful in organic solvents.

Scheme 2-11: *In* mediated carbocycle ring enlargement

Our group has used indium mediated allylation of aldehyde in the synthesis of an advanced intermediate of ichthyotoxic compound antillatoxin.⁷⁴ The advanced intermediate **27** was obtained in 80% yield and 93:7 *syn:anti* selectivity through indium mediated allylation of methyl (*Z*)-2-(bromomethyl)-2-butenate to aldehyde **28** in saturated NH_4Cl under sonication in the presence of lanthanide triflate (Scheme 2-12).

Scheme 2-12: Synthesis of intermediate of antillatoxin

We have also demonstrated the use of aqueous media for Indium mediated *allylation* in the stereoselective synthesis of 1,3-amino alcohol intermediate **29**

⁷³ (a) Li, C. -J.; Chen, D. L. *Synlett*. **1999**, 37, 471; (b) Li, C. -J.; Chen, D. L.; Lu, Y. Q.; Haberman, J. X.; Mague, J. T. *J. Am. Chem. Soc.* **1996**, 118, 4216; (c) Li, C. -J.; Chen, D. L.; Lu, Y. Q.; Haberman, J. X.; Mague, J. T. *Tetrahedron*. **1998**, 54, 2347.

⁷⁴ (a) Loh, T. P.; Cao, G. Q.; Pei, J. *Tetrahedron Lett.* **1998**, 39, 1457.; (b) Lee, K. -C.; Loh, T. P. *Chem. Commun.* **2006**, ?, 4209-4211.

Chapter 2

(Scheme 2-13).⁷⁵ These 1,3-amino alcohol units can serve as key building blocks in the synthesis of important biologically active compounds (e.g Dysiherbaine **30** neurotransmitters).

Scheme 2-13: Stereoselective synthesis of 1,3-amino alcohol units.

2.2.4 Allylation in Peptides.

To our knowledge, not much work has been done on the straightforward introduction of allylic units to peptides. Crich and coworkers⁷⁶ have shown the formation of allyl alkyl sulfides **31** from primary allylic selenosulfides **32** through allylic rearrangement and loss of selenium in the presence of PPh_3 . The allylic selenosulfides were in turn obtained from primary allylic selenosulfates and selenocyanates **33** through the transfer of allylic selenide moiety to thiols (Eq 11). Alkyl allylic secondary and tertiary disulfides, obtained from thiols through the transfer of sulfide from allylic heteroallyl disulfides also underwent allylic desulfurative rearrangement in the presence of PPh_3 temperature in methanolic acetonitrile (Eq 12). Both of these rearrangements have been applied for the functionalization of amino acids with lipids and are compatible with the functionalities found in the full range of proteinogenic amino acids. The fact that these methods can be applied to unprotected peptides was demonstrated by its application in aqueous media.

⁷⁵ (a) Loh, T. P.; Huang, J. -M.; Xu, K. -C.; Goh, S. -H.; Vittal, J. J. *Tetrahedron Lett.* **2000**, *41*, 6511-6515; (b) Huang, J.-M.; Xu, K.-C.; Loh, T.-P. *Synthesis* **2003**, 755-764.

⁷⁶ Crich, D.; Krishnamurthy, V.; Brebion, F.; Karatholuvhu, M.; Subramanian, V.; Hutton, T. K. *J. Am. Chem. Soc.* **2007**, *129*, 10282-10294.

2.2.5 Indium Mediated Allylation: Our Extension

In our group, indium has been extensively used for water based allylation reaction of glucose derived aldehydes and synthesis of homoallylic alcohols as intermediates in pursuit of natural products.⁷⁷ Among all the applications, the allylation of glyoxylates **34** (Scheme 2-14),⁷⁸ inspired us to apply this reaction in the allylation of peptides.

Scheme 2-14: Indium mediated allylation of glyoxylic acid

Similar glyoxylyl product could be synthesized by periodate oxidation of 1,2-amino alcohol in the *N*-terminus of serine or threonine containing peptides and proteins to aldehydes and ketones **3** (Scheme 2-14).⁷⁹ We chose to use *N*-terminal Serine containing peptides, as they generate aldehyde functionalities upon periodate oxidation which are more reactive than ketones.

⁷⁷ (a) Loh, T. P.; Chua, G. L. *Chem. Commun.* **2006**, 2739. (b) Chua, G. L.; Loh, T. P. *Yuki Gosei Kagaku Kyokaishi* **2005**, *63*, 1137-1146.

⁷⁸ Loh, T. P.; Xu-Ran, L. *Eur. J. Org. Chem.* **1999**, 8, 1893-1899.

⁷⁹ (a) Geoghegan, K. F. And Stroh, J. G. *Bioconj. Chem.* **1992**, 3(2), 138-146; (b) Chelius, D. and Shaler, T. A. *Bioconj. Chem.* **2003**, 14(1), 205-211.

Chapter 2

Scheme 2-14: Periodate Oxidation of 1,2-amino alcohols

We envisaged that the indium mediated allylation could be used to form new carbon-carbon bond at the specific *N*-terminal aldehyde once they were generated. If this is successful, the use of different allylic bromides would give rise to different groups on to the *N*-terminal through allylation and extend its use in the functionalization of proteins at a single specific site.

Present Work.

Like the previous section, we used a protected dipeptide (Serine-Alanine-*O*-methyl ester, Ser-Ala-OMe, **5**) and a protected tetrapeptide (Serine-Phenylalanine-Leucine-Glutamic acid-*O*-methyl ester, Ser-Phe-Leu-Glu-OMe, **6**) as the peptide substrates (Scheme 2-15).

Scheme 2-15: Peptide substrates for indium mediated allylation reaction.**2.2.6 Optimizing Conditions for Allylation of peptides.**

The optimum condition developed for periodate oxidation in the *aza Diels-Alder* based approach was applied to generate the *N*-terminal peptide aldehyde and taken for the next step of allylation. In the process, the aldehyde **13** was redissolved in

Chapter 2

25 mM sodium phosphate buffer (pH 7.0) followed by addition of indium powder (2 equivalent) and cooling to 0 °C. To this was added allyl bromide (3 Equivalent) and stirred at room temperature overnight. Next day TLC analysis revealed the formation of a clear new spot. The product was extracted with ether from the aqueous phase after filtering out excess indium. The combined organic phase was dried and upon column purification the desired allylation product **35** was obtained in 56% isolated yield as an inseparable mixture of diastereomers with a diastereoselectivity of 50:50 (Scheme 2-16). The diastereomeric ratios were determined by integration of the ^{13}C signals (70.7 and 70.8 ppm) of the alcoholic carbon (Scheme 2-16, Figure 2-3).

Scheme 2-16: Peptide functionalization by Indium mediated allylation.

Figure 2-3: ^{13}C NMR of **35**. The diastereomeric ratios were determined by integration of the ^{13}C -NMR signals (70.7 and 70.8 ppm) of the alcoholic carbon.

With the optimum reaction condition for periodate oxidation and allylation in hand, a number of allylic bromide species were screened to ascertain the substrate scope for this efficient reaction (Table 2-6).

Chapter 2

Table 2-6 Allylation reactions with dipeptide aldehyde^a

Entry	R ¹	R ²	R ³	Yield ^b	Dr ^c
1	H	H	H	56	50:50
2	H	CH ₃	H	50	21:24:22:23
3	CH ₃	CH ₃	H	45	51:49
4	H	COOMe	H	34	50:35:15
5	H	Ph	H	25	45:44:6:5
6	H	H	COOMe	19	65:35

^aAllylation reactions were run with 1 equiv of peptide aldehyde **13**, 2 equiv of Indium powder and 3 equiv of allylic bromide reagents in 25 mM sodium phosphate (NaPi) buffer (pH 7.0) for 18 h. ^bIsolated yield. ^cDiastereomeric ratios were based on ¹³C NMR analysis. The diastereomeric ratios were determined by integration of the ¹³C signals (70.7 and 70.8 ppm) of the alcoholic carbon.

The information obtained from screening experiments gave allyl bromide (Table 2-6, entry 1) as the preferred allylic halide partner for our reactions. The low yielding yet successful reaction of allylic halide substrates bearing carboxylate ester group (entries 4 and 6) opened up more areas of possible expansion of this reaction in labeling studies with fluorescent tags and peptides which will be discussed in due course.

To ascertain the effect of increasing peptide chain length on the yield and selectivity of the indium based *allylation* reaction, tetrapeptide aldehyde **16** was subjected to optimized conditions of indium mediated allylation in buffer. The desired allylation product was obtained in 53% yield with a diastereoselectivity of 60:40 (Table 2-7). Similar to the results of *aza Diels-Alder* reaction, we could observe an

Chapter 2

improvement in the selectivity with the increase in peptide chain length. This could be the result of the steric bulk of the peptide side chain. The success of the reaction in functionalizing longer peptides encouraged us in its application in the functionalization of proteins. These results will be discussed in a separate chapter.

Table 2-7 Aldehyde screening for *Allylation* reaction^a

Allylation Product

Entry	R	Yield ^b	dr ^c
1		56	50:50
2		53	60:40

^aAllylation reactions were run with 1 equiv of peptide aldehyde, 2 equiv of Indium powder and 3 equiv of allyl bromide in 25 mM sodium phosphate (NaPi) buffer (pH 7.0) for 18 h. ^bIsolated yield. ^cDiastereomeric ratios were based on ¹³C NMR analysis. The diastereoselectivities were determined by integration of the ¹³C signals (70.7 to 70.8 ppm) of the alcoholic carbon.

2.2.7 Applications.

The successful incorporation of methyl acrylate group into peptide through indium mediated allylation reaction (Table 2-6, entry 6), encouraged to further explore this reaction. We contemplated that different groups (peptides, amino acids, fluorescent tags) could be attached to the peptides by manipulations at the carboxylic ester site of

Chapter 2

acrylate species. With this plan in mind we synthesized three different substrates; a chiral amine derivative **36**, an amino acid derivative **37**, and a rhodamine based fluorescent derivative **38**.

All three of them were synthesized following the same strategy, where bromomethyl acrylic acid was first activated to give the acryloyl chloride derivative by refluxing with thionyl chloride followed by amide bond formation in the presence of *N,N'*-dimethylamino pyridine (DMAP) (Scheme 2-17).

Scheme 2-17: Acrylate based bromide substrates for allylation

The rhodamine amine **38a** was synthesized from rhodamine B following literature procedure. Upon synthesis, these substrates were subjected to indium

Chapter 2

mediated allylation reaction with aldehyde **13** (Table 2-8). We could successfully label the peptides at *N*-terminal selectively with amine, amino acid and fluorescent tag through our developed methodology.

Table 2-8 Application of indium mediated *Allylation* in selective peptide labeling^a

Entry	R	Yield ^b	<i>dr</i> ^c
1	36	42	63:37
2	37	46	60:40
3 ^d	38	47	51:49

^aAllylation reactions were run with 1 equiv of peptide aldehyde, 2 equiv of Indium powder and 3 equiv of allyl bromide in 25 mM sodium phosphate (NaPi) buffer (pH 7.0) for 18 h. ^bIsolated yield. ^cDiastereomeric ratios were based on ¹³C NMR data. The diastereomeric ratios were determined by integration of the ¹³C signals (between 70 to 72 ppm) of the alcoholic carbon. ^dReaction run in THF:buffer 1:1, *t*-BuOH:buffer 1:1 system did not give the desired product after 24 h, due to solubility problem of starting bromide in polar solvent system.

Chapter 2

2.2.8 Summary

In conclusion, we have demonstrated a key extension of indium mediated *Allylation* reaction in water in the functionalization of peptide *N*-terminal aldehydes. Like the previously described *aza Diels-Alder* reaction, this reaction also provides us with a single, site selective modification. The reaction proceeds efficiently at room temperature with easily accessible allylic bromide substrates.

The application of this reaction in the selective functionalization of peptides with amine, amino acids and fluorescent tag is of particular interest. These results illustrate that this method could be used for the targeted delivery of a wide variety of functional groups into specific peptide site through the formation of a stable carbon-carbon bond. The C-C bond generated in our method would be stronger. It also equips the peptide with a terminal olefin handle that opens up a wide avenue for their orthogonal modifications through addition reactions or ruthenium-catalyzed metathesis.⁸⁰

Although the results from allylation reaction was encouraging for protein functionalization studies, the requirement of metal (indium) for their progress can sometimes lead to unwanted side reactions if the concentration is not properly controlled. A probable alternative to this issue was the development of a metal free water based carbon-carbon bond forming reaction.

In searching for a metal free C-C bond forming reaction we came across *Mukaiyama aldol* reaction which will be discussed in detail in the following section.

⁸⁰ (a) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem. Int. Ed.* **1998**, 37, 3281-3284; (b) Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, 130, 15, 5052-5053; (c) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, 130, 30, 9642-9643

Chapter 2

2.3 Mukaiyama-Aldol Reaction

2.3.1 Introduction: Literature Overview

The *Mukaiyama*-aldol reaction was developed as a Lewis acid mediated aldol addition of enol silanes to carbonyl compounds in the early 1970's (Scheme 2-18) by T. Mukaiyama and K. Narasaka.⁸¹ The *Mukaiyama*-aldol reaction is one of the most important and highly efficient C-C bond forming reactions. This reaction affords synthetically useful β -hydroxy ketones and esters.^{82,83} The sheer volume of publications on this methodology testifies to its utility. Lewis acids such as TiCl_4 , SnCl_4 , AlCl_3 , $\text{BCl}_3 \cdot \text{OEt}_2$, and ZnCl_2 were required as catalysts for this transformation. The diastereoselectivity of this reaction can be controlled if substrates and conditions are carefully chosen. The asymmetric *Mukaiyama*-aldol reaction has also been extensively investigated with both chiral Lewis acid complexes and Lewis bases.⁸⁴

Scheme 2-18 Mukaiyama-Aldol reaction

⁸¹ Mukaiyama, T.; Narasaka, K.; Banno, K. *Chem. Lett.* **1973**, 1011; (b) Mukaiyama, T.; Banno, K.; Narasaka, K. *J. Am. Chem. Soc.* **1974**, 96, 7503.

⁸² (a) Saigo, K.; Osaki, M.; Mukaiyama, T. *Chem. Lett.* **1975**, 989; (b) Mukaiyama, T. *Org. React.* **1982**, 28, 203; (c) Mukaiyama, T. *Angew. Chem., Int. Ed. Engl.* **1977**, 16, 817; (d) Mukaiyama, T. *Aldrichimica Acta* **1996**, 29, 59; (e) Gröger, H.; Vogl, E. M.; Shibasaki, M. *Chem. Eur. J.* **1998**, 4, 1137-1141; (f) Nelson, S. G. *Tetrahedron: Asym.* **1998**, 9, 35-389; (g) Bach, T. *Angew. Chem., Int. Ed. Engl.* **1994**, 33, 417-419.

⁸³ (a) Evans, D. A.; Takacs, J. M.; McGee, L. R.; Ennis, M. D.; Mathre, D. J.; Bartroli, J. *Pure Appl. Chem.* **1981**, 53, 1109; (b) Heathcock, C. H. in *Comprehensive Carbanion Chemistry*; Buncl, E., Durst, T., Eds.; Elsevier: Amsterdam, **1984**; Part B, Chapter 4; (c) Heathcock, C. H. in *Asymmetric Synthesis*; Morrison, D. J., Ed.; Chapter 2, Academic: New York, **1984**; Vol. 3, Part. B, p. 111; (d) Evans, D. A.; Nelson, J. V.; Taber, T. R. *Top. Stereochem.* **1982**, 13, 1; (e) Masamune, S.; Choy, W.; Petersen, J. S.; Sita, L. R. *Angew. Chem., Int. Ed. Engl.* **1985**, 24, 1; (f) Braun, M.; Sacha, H. *J. Prakt. Chem.* **1993**, 335, 653; (g) Heathcock, C. H. In *Comprehensive Organic Synthesis*; Trost, B. M. Ed.; Pergamon: Oxford, **1993**; Vol. 2, Chapter 1, 6; (h) Heathcock, C. H. *Mod. Synth. Meth.* **1992**, 1; (i) Heathcock, C. H. *Aldrichimica Acta* **1990**, 23, 99; (j) Heathcock, C. H. *Science* **1981**, 214, 395; (k) Mukaiyama, T. *Org. React.* **1982**, 28, 203.

⁸⁴ (a) Carreira, E. M. *Mukaiyama aldol reaction: Comprehensive Asymmetric Catalysis I-III* **1999**, 3, 997; (b) Kazuaki, I.; Yamamoto, H. *Modern Aldol Reactions*, **2004**, 2, 25.

Chapter 2

2.3.2 Lewis acid catalysis in asymmetric *Mukaiyama*-aldol reaction.

Kobayashi and coworkers demonstrated the use of Lewis acid together with chiral ligands to achieve asymmetric induction in *Mukaiyama*-aldol reaction in ethanolic solvent. They used a combination of $\text{Cu}(\text{OTf})_2$ /chiral bis(oxazoline) ligand, $\text{Pb}(\text{OTf})_2$ /chiral crown ether, and $\text{Ln}(\text{OTf})_3$ /chiral bis-pyridino-18-crown-6 for catalysis (Eq. 13).⁸⁵

Natural products Febrifugine and Isofebrifugine have been synthesized from the key intermediate **39**. Intermediate **39** in turn is obtained through tin(II)-catalyzed asymmetric *Mukaiyama*-aldol reaction on substrate **40**, followed by lanthanide-catalyzed three-component reaction in aqueous media (Scheme 2-19).⁸⁶

Scheme 2-19. Asymmetric *Mukaiyama*-aldol reaction in the synthesis of natural products.

⁸⁵ (a) Kobayashi, S.; Nagayama, S.; Busujima, T. *Chem. Lett.* **1999**, 71; (b) Kobayashi, S.; Mori, Y.; Nagayama, S.; Manabe, K. *Green Chem.* **1999**, 1, 175; (c) Kobayashi, S.; Nagayama, S.; Busujima, T. *Tetrahedron* **1999**, 55, 8739; (d) Benaglia, M.; Cinquini, M.; Cozzi, F.; Celentano, G. *Org. Biomol. Chem.* **2004**, 2, 3401; (e) Nagayama, S.; Kobayashi, S. *J. Am. Chem. Soc.* **2000**, 122, 11531; (f) Kobayashi, S.; Hamada, T.; Nagayama, S.; Manabe, K. *Org. Lett.* **2001**, 3, 165.

⁸⁶ Kobayashi, S.; Ueno, M.; Suzuki, R.; Ishitani, H. *Tetrahedron Lett.* **1999**, 40, 2175.

Chapter 2

2.3.3 Aqueous *Mukaiyama*-aldol reaction.

Lubineau first reported the aqueous *Mukaiyama*-aldol reaction in 1986.⁸⁷ Without any catalyst, the reaction took several days to complete. Kobayashi and coworkers later pioneered the development of water-tolerant Lewis acids for *Mukaiyama*-aldol reaction.⁸⁸ They introduced lanthanide triflates ($\text{Yb}(\text{OTf})_3$, $\text{Gd}(\text{OTf})_3$ and $\text{Lu}(\text{OTf})_3$) as the catalyst species which greatly improved the rate and yield of this reaction, with moderate diastereoselectivities.⁸⁹ They also used water soluble aldehydes and were able to recover and reuse the catalyst. Other metal catalysts such as $\text{Bi}(\text{OTf})_3$, $\text{Cu}(\text{OTf})_2$, and FeCl_3 has also been used as Lewis acids to promote *Mukaiyama*-aldol reaction with much success.⁹⁰ One noteworthy point for water based *Mukaiyama*-aldol reaction is that, in majority of the cases *syn* isomer is the major product while under anhydrous conditions *anti* isomer predominates.

In our group we have used InCl_3 as the Lewis acid to promote *Mukaiyama*-aldol reaction.⁹¹ We used a glucose derived silyl enol ether for carbon chain elongation using the InCl_3 catalyzed *Mukaiyama*-aldol reaction (Eq. 14).

⁸⁷ (a) Lubineau, A. *J. Org. Chem.* **1986**, *51*, 2143; (b) Lubineau, A.; Meyer, E. *Tetrahedron*. **1988**, *44*, 6065.

⁸⁸ (a) Kobayashi, S.; Hachiya, I. *Tetrahedron. Lett.* **1992**, *33*, 1625; (b) Hachiya, I.; Kobayashi, S. *J. Org. Chem.* **1993**, *58*, 6958; (c) S. Kobayashi, M. Sugiura, H. Kitagawa, W. L. Lam, *Chem. Rev.* **2002**, *102*, 2227.

⁸⁹ (a) Kobayashi, S.; Hachiya, I. *J. Org. Chem.* **1994**, *59*, 3590; (b) Kobayashi, S. *Synlett.* **1994**, 689.

⁹⁰ (a) Le Roux, C.; Ciliberti, L.; Laurent-Robert, H.; Laporterie, A.; Dubac, J. *Synlett.* **1998**, 1249; (b) Kobayashi, S. Nagayama, S.; Busujima, T. *Chem. Lett.* **1997**, 959; (c) Aoyama, N.; Manabe, K.; Kobayashi, S. *Chem. Lett.* **2004**, *33*, 312.

⁹¹ (a) Loh, T. -P.; Chua, G. -L.; Vittal, J. J.; Wong, M, -W. *Chem. Commun.* **1998**, 861. (b) Loh, T. -P.; Pei, J.; Cao, G. -Q. *Chem. Commun.* **1996**, 1819. (c) Loh, T. -P.; Pei, J.; Hoh, K. S. -V.; Cao, G. -Q.; Li, X. -R. *Tetrahedron Lett.* **1997**, *38*, 3465. (d) Loh, T. P.; Feng, L. -C.; Wei, L. -L. *Tetrahedron Lett.* **2000**, *56* (37), 7309-7312.

Chapter 2

It was found by Munoz and his group that *i*-PrOH/H₂O (95:5) was the best solvent for InCl₃ reactions and the reaction was unsuccessful in pure water. Later on Munoz-Muniz and coworkers attributed this effect to the decomposition of starting silyl enol ether in the presence of water.⁹²

To circumvent this problem a Lewis acid free *Mukaiyama*-aldol reaction was developed, where a reactive ketene silyl acetal reacted with an activated aldehyde to give the corresponding aldol product in good yields (Eq. 15).⁹³

2.3.4 Synthetic Applications of *Mukaiyama*-Aldol Reaction.

Application to Peptides. Yb(OTf)₃ catalyzed *Mukaiyama*-aldol reaction was used to form C-C bond at the *N*-terminal aldehyde of a peptide on solid support in a mixed solvent system (CH₃CN:H₂O 4:1).⁹⁴ The authors proposed that this discovery could lend itself useful in combinatorial library based approaches.

Oligopeptide motifs with an oxirane ring **41** are well known for their inhibitory activity against cysteine proteases,⁹⁵ HIV-1 proteases⁹⁶ and metalloproteases.⁹⁷ These could be obtained from the oxidative conversion of β-hydroxyselenide **42** synthesized via *Mukaiyama*-aldol reaction of β-selenyl aldehyde **43** (Scheme 2-20).⁹⁸

⁹² Munoz-Muniz, O.; Quintanar-Audelo, M.; Juaristi, E. *J. Org. Chem.* **2003**, *68*, 1622.

⁹³ Loh, T. -P.; Feng, L. C.; Wei, L. L. *Tetrahedron* **2000**, *56*, 7309-7312.

⁹⁴ Graven, A.; Grotli, M.; Meldal, M. *J. Chem. Soc., Perkin 1* **2000**, 955.

⁹⁵ (a) Albeck, A.; Persky, R.; Kliper, S. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1767; (b) Giordano, C.; Gallina, C.; Consalvi, V.; Scandurra, R. *Eur. J. Med. Chem.* **1990**, *25*, 479.

⁹⁶ Park, C.; Koh, J. S.; Son, Y. C.; Choi, H.; Lee, C. S.; Choy, N.; Moon, K. Y.; Jung, W. H.; Kim, S. C. Yoon, H. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1843.

⁹⁷ Kim, D. H.; Kim, K. B. *J. Am. Chem. Soc.* **1991**, *113*, 3200.

⁹⁸ Mann, A.; Quaranta, L.; Reginato, G.; Taddei, M. *Tetrahedron Lett.* **1996**, *37*, 2652-2654.

Chapter 2

Scheme 2-20. Mukaiyama-aldol reaction in the synthesis of protease inhibitors.

2.3.5 Mukaiyama-Aldol Reaction: Our Extension

Our group has demonstrated that various reactive aldehydes can react with silyl ketene acetals in water at ambient temperature without any Lewis acid activation to afford the corresponding aldol product in moderate to good yields.⁹⁹ Of special interest was the aldol reaction of methyl glyoxylate **44** with silyl ketene acetal, that proceeded smoothly in water at room temperature to afford the desired product in 70% yield after 24 hrs (Eq. 15). This particular example together with the potential of using small molecules in a mild and environmentally friendly solvent system for labeling studies led us to further explore the scope of this reaction by its application in the functionalization of peptides in site specific manner.

This in turn is initiated from the generation of reactive glyoxylyl product **17** (Scheme 2-21) through the very rapid oxidation of 1,2-amino alcohol of *N*-terminal serine or threonine containing peptides (Scheme 2-21).¹⁰⁰

⁹⁹ Loh, T. P., Feng, L. C.; Wei, L. L. *Tetrahedron* **2000**, 56(37), 7309-7312.

¹⁰⁰ (a) Geoghegan, K. F. And Stroh, J. G. *Bioconj. Chem.* **1992**, 3(2), 138-146; (b) Chelius, D. and Shaler, T. A. *Bioconj. Chem.* **2003**, 14(1), 205-211.

Chapter 2

Scheme 2-21: Periodate Oxidation of 1,2-amino alcohols

We envisaged that catalyst free aqueous *Mukaiyama*-aldol reaction could be used to form new carbon-carbon bond at the specific *N*-terminal aldehyde once they were generated. If this is successful, we would like to make use of different silyl ketene acetal substrates to install different functionalities on to the *N*-terminal through *Mukaiyama*-aldol reaction and extend its application to functionalize proteins at a single specific site.

Present Work.

Like the previous section, we used a protected dipeptide (Serine-Alanine-*O*-methyl ester, Ser-Ala-OMe, **5**) and a protected tetrapeptide (Serine-Phenylalanine-Leucine-Glutamic acid-*O*-methyl ester, Ser-Phe-Leu-Glu(OMe)-OMe, **6**) as the peptide substrates (Scheme 2-22). To extend the scope further, we also added an unprotected heptapeptide (Isoleucine-Glycine-Serine-Leucine-Alanine-Lysine-OH, Ile-Gly-Ser-Leu-Ala-Lys-OH, **45**) purchased from commercial suppliers.

Scheme 2-22: Peptide substrates for *Mukaiyama*-aldol reaction.

Chapter 2

2.3.6 Optimizing Conditions for *Mukaiyama*-aldol Reaction of Peptides.

The optimum conditions developed for periodate oxidation in the *aza Diels-Alder* based approach was applied to generate the *N*-terminal peptide aldehyde **13** and taken for the next step of *Mukaiyama*-aldol reaction. In the process, the aldehyde was redissolved in 25 mM sodium phosphate buffer (pH 7.0) followed by vigorous stirring with 2 equivalent of silyl ketene acetal **46** for 24 h at rt. TLC analysis next day revealed clean conversion of the starting aldehyde to *Mukaiyama*-aldol product. The product was extracted with ether from the aqueous phase. The combined organic phase was dried and upon column purification, the desired *Mukaiyama*-aldol product **47** was obtained in 55% isolated yield as an inseparable mixture of diastereomers with a diastereoselectivity of 57:43 (Scheme 2-23). The diastereomeric ratios were determined by integration of the ^{13}C signals (74.8 and 75.2 ppm) of the alcoholic carbon (Figure 2-4).

Scheme 2-23: Peptide functionalization by *Mukaiyama*-aldol reaction.

Figure 2-4: ^{13}C NMR of **47**. The diastereomeric ratios were determined by integration of the ^{13}C -NMR signals (74.8 and 75.2 ppm) of the alcoholic carbon.

Chapter 2

Following similar conditions, a number of silyl ketene acetal reagents were screened to ascertain the substrate scope for this mild reaction (Table 2-9).

Table 2-9. *Mukaiyama*-aldol reactions with dipeptide aldehyde **13**^a

$ \begin{array}{c} \text{H} \\ \parallel \\ \text{C} \\ \\ \text{C}=\text{O} \\ \\ \text{R} \end{array} \xrightarrow[\text{pH 7.0, rt, 24 h}]{\text{Silyl Ketene acetal, 10 mM NaPi}} \text{Product} $ <p>R = Ala-OMe, 13</p>				
Entry	Ketene Acetal	Product	Yield ^b	Dr ^c
1	46	47	55	57:43
2 ^d	46	47	71	57:43
3	48	49	11	52:48
4	50	51	11	55:45
5	52	53	5	50:50
6	54	nr	0	0

^aConditions: Peptide Aldehyde **13**, 2 Equivalent of Ketene, 10 mM sodium phosphate buffer (pH 7.0), RT, 24 h. ^b Yield refers to column purified yield of *Mukaiyama*-aldol product after 2 steps. ^c Diastereomeric ratios were determined by integration of the ¹³C signals (74 to 75 ppm) of the alcoholic carbon. ^d 70 Equivalent of silyl ketene acetal used instead of 2.

Chapter 2

These experiments gave *Mukaiyama*-aldol peptide products in yields ranging from 5 to 55%, with 1-methoxy-2-methyl-1-(trimethylsiloxy)propene **46** giving the highest yield (Table 2-9, entry 1). In general, alkyl substituted ketene silyl acetals proved to be more suitable for this application, yielding results comparable to compound **46** (Table 2-9, entry 1).

In an attempt to increase the amount of product formed (Table 2-9, entry 2), when the dipeptide aldehyde was stirred with increased equivalent (70 equiv) of the silyl ketene acetal **46**, an increase in the *Mukaiyama* aldol product yield (71% from 55%) was observed. But the high volume of ketene acetal needed for such transformation makes this option less attractive and hence for all the peptide based studies, 2 equivalent of ketene acetal was used.

Further model reactions of this particular silyl ketene acetal with protected tetrapeptide aldehyde **16** and unprotected peptide aldehyde (from oxidation of **45**) in buffer showed that the reaction proceeds efficiently with the yield of product improving to 80% with longer and unprotected peptides (Table 2-10, entry 2 & 3).

Table 2-10 *Mukaiyama*-aldol reactions with peptide aldehydes^a

Entry	R	Yield ^b	Dr ^c
1	Ala-OMe, 5	55	57:43
2	Phe-Leu-Glu(OMe)-OMe, 6	71	60:40
3	Ile-Gly-Ser-Leu-Ala-Lys-OH, 45	80	53:47

^aConditions: Periodate oxidation reactions were run with 2 equiv of NaIO₄, 1 equiv of peptide **5/6/45** in 25 mM sodium phosphate (NaPi) buffer (pH 7.0) in the dark till completion as judged by LC-ESI-MS analysis. Following which after optimized workup taken for the next step of *Mukaiyama*-aldol. Peptide Aldehyde, 2 Equivalent of Ketene, 25 mM sodium phosphate buffer (pH 7.0), RT, 24 h. ^b Yield refers to column purified yield of *Mukaiyama*-aldol product after 2 steps. ^cIsomer ratios were determined by integration of the ¹³C signals (between 74 to 75 ppm) of the alcoholic carbon.

Chapter 2

These results proved that site selective *Mukaiyama*-aldol functionalization of peptides could be achieved under suitably tuned conditions. Keeping in mind that the reaction seems to perform better on more complex peptides, these results were very encouraging for the functionalization of proteins.

2.3.7 Applications.

With these screening results in hand, our next step was to apply the *Mukaiyama*-aldol reaction in the functionalization of peptide *N*-terminus with fluorescent tags and biotin. Our plan was to synthesize a silyl ketene acetal with a biotin or fluorescent group attached to it. If this was successful, it would aid us in separation and biological activity evaluation of modified proteins *via* immobilization mechanisms based on the strong interaction of biotin with avidin or streptavidin.¹⁰¹ We designed the biotin silyl ketene acetal **55** and fluorescent silyl ketene acetal **56**, isopropylester derivative was chosen as screening results indicated that having isopropyl group increases yield because of the inherent reactivity of this group (Scheme 2-24).

Scheme 2-24: Design of biotin and fluorescent tagged silyl ketene acetal.

¹⁰¹ Bayer, E. A. And Wilchek, M. *Methods Enzymol.* **1990**, 184, 138-160.

Chapter 2

Scheme 2-25: Synthetic scheme for biotin tagged silyl ketene acetal. **Scheme 2-26:** Synthetic scheme for fluorescent tagged silyl ketene acetal.

The biotin and fluorescent ester intermediates **57** and **59** were synthesized according to Scheme 2-25 and 2-26.¹⁰² However in both cases, the final step to the desired silyl ketene acetal derivatives was unsuccessful.

¹⁰² G. Loving, B. Imperiali, *J. Am. Chem. Soc.* **2008**, 130, 13630-13638.

Chapter 2

This led us to explore an indirect approach towards the attachment of labeling groups to peptide *N*-terminus. We envisaged the use of trimethyl silyl ketene acetals with functionalized tethers such as alkene, alkyne and thioesters for subsequent coupling with probes through different orthogonal techniques (eg. Olefin metathesis, Azide-alkyne cycloaddition or Native Chemical Ligation respectively). Thus we synthesized several unique silyl ketene acetals (Scheme 2-28), **60** and **61** with terminal alkene, **62** containing terminal alkyne, **63** with terminal thioester and **64** with a polyethylene glycol (PEG) group.

Scheme 2-28: Silyl ketene acetals with functionalized tethers

Upon synthesis, these substrates were subjected to *Mukaiyama*-aldol conditions with dipeptide aldehyde **13** (Table 2-11). We could successfully label the peptide's *N*-terminal selectively with alkene, alkyne, thioester and polyethylene glycol (PEG) group through our developed methodology.

Chapter 2

Table 2-11 *Mukaiyama-aldol* reactions with dipeptide aldehyde **13**^a

Entry	Ketene Acetal	Product	Yield ^b	Dr ^c
1	 60	 65	45	60:40
2	 61	 66	41	60:40
3	 62	 67	12	60:40
4	 63	 68	52	58:42
5	 64	 69	42	53:47

^aConditions: Peptide Aldehyde **13**, 2 Equivalent of Ketene, 10 mM sodium phosphate buffer (pH 7.0), RT, 24 h. ^bYield refer to column purified yield of *Mukaiyama-aldol* product after 2 steps.

^cDiastereomeric ratios were determined by integration of the ¹³C signals (between 74 to 76 ppm) of the alcoholic carbon.

The results in Table 2-11 suggest that this method is well suited for the introduction of substituents containing alkenes, alkynes and thioester (Table 2-11, entries 1-4) functional groups which can be further modified using olefin

Chapter 2

metathesis,¹⁰³ Cu(I)-promoted Huisgen [3+2] cycloadditions,¹⁰⁴ or native chemical ligation (NCL) respectively.¹⁰⁵ Furthermore entry 5 in Table 2-11 indicates that polyethylene glycol conjugated proteins¹⁰⁶ could be synthesized using this method.

In order to demonstrate the utility of the *Mukaiyama* adducts, thioallyl fluorescent probe **70** and biotin azide **71** were synthesized (Scheme 2-29 and 2-30).

The fluorescent alcohol **58** was converted to bromide by reacting with phosphorus tribromide followed by substitution of the bromide with thioallyl group to give the fluorescent tag **70** ready to undergo olefin cross-metathesis (Scheme 2-29).

Scheme 2-29: Synthetic scheme for fluorescent tagged thioallyl substrate **70**.

¹⁰³ a) Hong, S. H.; Grubbs, R. H. *J. Am. Chem. Soc.* **2006**, *128*, 3508-3509; b) Lin, Y. A.; Chalker, J. M. J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. I. *J. Am. Chem. Soc.* **2008**, *130*, 9642-9643.

¹⁰⁴ a) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192-3193; b) Beatty, K. E.; Xie, F.; Wang, Q.; Tirrell, D. A. *J. Am. Chem. Soc.* **2005**, *127*, 14150-14151.

¹⁰⁵ a) Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776; b) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923; c) Bang, D.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem. Intl. Ed.* **2006**, *45*, 3985. Parveen, S.; Sahoo, S. K. *Clin. Pharmacokinet.* **2006**, *45*, 965-988

¹⁰⁶ Parveen, S.; Sahoo, S. K. *Clin. Pharmacokinet.* **2006**, *45*, 965-988

Chapter 2

Biotin azide **71** on the other hand was prepared by coupling biotin with aminopropyl azide in the presence of EDC.HCl followed by purification by reversed phase HPLC (Scheme 2-30).

Scheme 2-30: Synthetic scheme for biotin azide.

When **66** was coupled to thioallyl fluorescent probe **70** under the cross-metathesis conditions reported by Davis using Hoveyda-Grubbs 2nd generation catalyst, compound **72** was obtained in 68% yield (Scheme 2-31).

Scheme 2-31: Olefin cross-metathesis of **70** with Mukaiyama-aldol product **66**.

Under click reaction conditions, Mukaiyama adduct **67** underwent [3 + 2] cycloaddition with biotin azide **71** to give **73** in 76% yield (Scheme 2-32).

Scheme 2-32: Click reaction of azide **71** with Mukaiyama-aldol product **67**.

Chapter 2

With the success of labeling *Mukaiyama*-aldol modified peptides with fluorescent tag and biotin, as a next step, we sought to apply the *Mukaiyama*-aldol functionalization to proteins. This will be described in detail in the following chapter.

2.3.8 Summary

In conclusion, we have developed a new extension to *Mukaiyama*-aldol reaction by its application in the selective functionalization of peptide *N*-terminus. If we compare all the three reactions (*aza Diels-Alder*, *Allylation* and *Mukaiyama*-aldol) we studied, we would argue that *Mukaiyama*-aldol is the best reaction for our intended purpose of selective peptide functionalization. This is because; this reaction can be performed in aqueous media without any catalyst at ambient temperature. Depending upon the silyl ketene acetal used, it can label peptides with terminal alkenes, alkynes, thioesters and polyethylene glycol (PEG) chains. The newly formed *Mukaiyama*-aldol products can be further tagged with biotin and fluorescent probes through orthogonal techniques. These reactions have provided us with simple and practical methods for the functionalization of biomolecules. To assess our hypothesis we embarked on the journey of functionalizing protein *N*-terminus through *allylation* and *Mukaiyama*-aldol condensation as elaborated in the following chapter.

PART I

Chapter 3

Functionalization of Proteins

Chapter 3

3.1 Introduction.

With our success of selective functionalization of peptide *N*-terminal aldehyde through the mild water based indium mediated *allylation* and *Mukaiyama*-aldol condensation, we sought as a next step to elaborate the substrate scope for these reactions by including proteins. This idea was further bolstered by the fact that the reactions seemed to perform better on more complex peptides.

We planned to apply the *Allylation* and *Mukaiyama*-aldol functionalizations to Horse heart myoglobin.

We chose this protein for several reasons;

- Myoglobin was one of the several examples mentioned by Francis and coworkers in their PLP mediated transamination.¹⁰⁷
- It has an *N*-terminal glycine residue with a primary sequence of 153 amino acids.
- PLP mediated oxidative transamination would be able to convert the *N*-terminal glycine residue to an aldehyde group. While having any other amino acid residue will give rise to ketones. Since, our peptide functionalizations are based upon aldehyde modifications; for proteins we wanted to retain the same target functionality.
- It is commercially available and gives us the advantage of testing our hypothesis straight away without going through elaborate and time consuming processes of protein cloning and purification.
- Structural stability and integrity of the protein can be studied from characteristic heme absorbance at 410 nm using ultraviolet (UV)

¹⁰⁷ (a) Gilmore, J. M; Scheck, R. A.; Esser-Hahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem. Intl. Ed.* **2006**, *45*, 5307-5311; (b) Scheck, R. A.; Dedeo, M. T.; Lavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 11762-11770.

Chapter 3

spectroscopy, secondary structure through circular dichroism spectroscopy (CD) and peroxidase assay based mechanistic studies.

With the protein in hand we went on to test whether the reactions developed for the peptides could be used for protein modifications.

In general, HPLC-MS was used for protein sample analysis. The analyses were performed at the Novartis facilities in Basel, Switzerland. Only data, with positive results were reported, while results showing in heterogeneity or complex mixtures were assumed to be unsuccessful and noted in tables and discussions as complex mixtures.

Chapter 3

3.2 Indium Mediated Allylation

3.2.1 Introduction: Literature Overview

To date several avenues have been explored for the study of proteins through site-selective chemical functionalization. One approach utilizes olefins as the modification site. Introduction of olefins offers a wide range of modifications based on the use of cross-coupling metathesis reactions.¹⁰⁸ Although there has been no report of direct incorporation of allylic side chains into proteins, olefins have been introduced into proteins in two ways as discussed in Chapter 1 (Page 11-12):

A Introduction of Olefinic Group by Unnatural Amino Acid Incorporation. Unnatural amino acids can be incorporated into proteins in two ways.

- The **Residue-specific incorporation** is based on the competition between the natural amino acid and its unnatural analog during their incorporation into growing peptide chains by *t*RNA.¹⁰⁹ Unnatural amino acids with structural and electronic similarity to the natural analogs can be easily incorporated into proteins by this method. Alkenes (**1-6**) have been successfully introduced (Scheme 3-1). The natural amino acids that have been replaced are phenylalanine, leucine, isoleucine, proline and methionine.¹¹⁰ The lack of control in the site-specific introduction of the new residue makes this process less attractive.
- In the **Biosynthetic site-specific incorporation where** *t*RNA's are charged with stop codon's that dictate the placement of unnatural analogs into growing polypeptide chains. As opposed to the residue-specific incorporation which is

¹⁰⁸ Blackwell, H. E.; Grubbs, R. H. *Angew. Chem. Int. Ed.* **1998**, 37, 3281-3284.

¹⁰⁹ (a) Connor, R. E and Tirrell, D. A. *Polym. Rev.* **2007**, 47, 9-28; (b) Kirshenbaum, K.; Carrico, I. S.; Tirrell, D. A. *ChemBioChem.* **2002**, 3, 235-237.

¹¹⁰ Kirshenbaum, K.; Carrico, I. S.; Tirrell, D. A. *ChemBioChem.* **2002**, 3, 235-237.

Chapter 3

amenable to gram scale quantities of proteins, this method is only applicable when small amount of protein is required.¹¹¹

Scheme 3-1: Unnatural amino acids with alkene functionality incorporated into proteins.

B Introduction of Olefinic Group by Chemical Modification of Amino Acids. In this method either prevailing natural amino acids in the protein are chemically modified or unnatural amino acids are first incorporated through molecular biology followed by chemical modification to generate olefinic residues at the desired site. In their recent works Davis and co-workers have successfully transformed *O*-mesitylenesulfonylaminehydroxylamine functionalized cysteine into Dha 7 on protein surface without any side reaction on methionine (Scheme 3-2).¹¹²

Scheme 3-2: Introduction of alkene functionality by chemical modification.

Unwanted side reactions to sensitive amino acid side chains resulting from harsh oxidation conditions limit the use of these strategies.

¹¹¹ (a) Wang, L.; Schultz, P. G. *Angew. Chem. Intl. Ed.* **2005**, *44*, 34-66; (b) Dougherty, D. A. *Curr. Opin. Chem. Biol.* **2000**, *4*, 645-652; (c) Nowak, M. W.; Gallivan, J. P.; Silverman, S. K. Labarca, C. G.; Dougherty, D. A.; Lester, H. A. *Ion. Channels, Part B*, **1998**, *293*, 504-529.

¹¹² (a) Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 15, 5052-5053; (b) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 30, 9642-9643.

Chapter 3

C Applications. After their incorporation into proteins, the alkenes served as the substrate for cross-metathesis reactions with a variety of other important functionalities.¹¹³ As an illustration S-allyl functionalized protein generated through chemical means has been used to attach different entities to proteins through olefin-metathesis (Scheme 3-3).¹¹⁴

Scheme 3-3: Cross-metathesis reactions with alkenes.

3.2.2 Allylation Reaction: Our Extension

A Periodate Oxidation and Allylation of peptides. As described in chapter 2, under optimized conditions for indium mediated *allylation*, after generating *N*-terminal aldehydes through the periodate reaction on serine at the site, the desired allylation product was obtained in moderate yield when the reaction was mediated by indium in aqueous media at room temperature (Scheme 3-4).

Scheme 3-4: Peptide functionalization by Indium mediated allylation.

¹¹³ (a) Clark, T. D.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1995**, *117*, 12364; (b) Chatterjee, C.; Paul, M.; Xie, L. L.; van der Donk, W. A. *Chem. Rev.* **2005**, *105*, 633; (c) Cotter, P. D.; Hill, C.; Ross, R. P. *Nat. Rev. Microbiol.* **2005**, *3*, 777; (d) Galonic, D. P.; van der Donk, W. A.; Gin, D. Y. *Chem. Eur. J.* **2003**, *9*, 5997; (e) Zhu, Y.; van der Donk, W. A. *Org. Lett.* **2001**, *3*, 1189; (f) Marcaurelle, L. A.; Bertozzi, C. R. *Chem. Eur. J.* **1999**, *5*, 1384.

¹¹⁴ (a) Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 15, 5052-5053; (b) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 30, 9642-9643.

Chapter 3

A screening experiment with a number of allylic bromide reagents revealed allyl bromide **8** (Scheme 3-4) as the best allylic halide counterpart. With this information in hand we went on to apply the reaction to myoglobin.

B Oxidation and Allylation of Myoglobin. Myoglobin *N*-terminal aldehyde was generated by pyridoxal 5'-phosphate (PLP, **9**) mediated biomimetic transamination reaction. A 75 μ M solution of the protein was incubated with 10 mM **9** for 20 h at 37 °C in 25 mM sodium phosphate buffer (pH 6.5). Following removal of excess **9** with NanoSep 10 filtration devices, the product solution was divided into two portions. One half was incubated with 25 mM Biotin-X-hydrazide (**10**) overnight to confirm the formation of the aldehyde, while another half was incubated at room temperature with indium (1 equiv.) and 7.5 mM allyl bromide **8** (200 equiv.) in 25 mM phosphate buffer (pH 7.0) after pipette mixing (Scheme 3-4).

After overnight reaction, 60% conversion to the biotinylated product **11** (Scheme 3-5) was observed by LC-ESI-MS [Biotinylated protein **11**, expected mass for (myoglobin aldehyde + 352 – H₂O = 17302, observed mass 17303) (Figure 3-1, **a** and **b**); 352 is the expected mass increase for myoglobin aldehyde after modification with biotinamidohexanoic acid hydrazide **10** to form the hydrazone **11**]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE analysis) (Figure 3-1, **c**) and Western blotting (Figure 3-1, **d**) of SDS-PAGE gel was used to further confirm biotinylation. While the biotinylation result was encouraging, no allylation product was detected in the LC-ESI-MS analysis.

Chapter 3

Scheme 3-5: Myoglobin functionalization attempt by indium mediated *allylation*.

Figure 3-1: LC-ESI-MS and Western Blotting analysis of modified horse heart Myoglobin. **a)** Unmodified Myoglobin. **b)** Biotinylated Myoglobin **11**. **c)** SDS-PAGE analysis of modified products upon coomassie staining. **d)** Western blot analysis of the biotinylated product using Avidin-AP.

A likely explanation for the unsatisfactory result could be the poor solubility of allyl bromide in water, together with lack of vigorous stirring. In the peptide based allylation reactions we did not come across such complications because the vigorous stirring that was applied could resolve the immiscibility associated with allyl bromide.

Chapter 3

While in protein reactions stirring was not used as it has been reported that proteins will loose their stability upon stirring.¹¹⁵ To address these problems two additional reaction conditions were performed.

- 1) In the first, the reaction was repeated under same conditions but shaken gently on a laboratory rotisserie.
- 2) In the second, the use of a co-solvent was explored. So, in the second reaction the aqueous buffer was replaced with 50% *t*-BuOH/buffer and shaken like condition 1 and after removing excess of the reagents through filtration subjected to LC-ESI-MS analysis (Table 3-1).

Table 3-1 Allylation trials following aldehyde generation by transamination^a

Entry	Solvent	%Conversion	Shaking
1	25 mM sodium phosphate buffer, pH 6.5	-	+
2	50% <i>t</i> -BuOH/buffer	Trace amount of product	+

^aOxidation reactions were run with 133 equiv of PLP 9, (10 mM final concentration in 25 mM sodium phosphate buffer, pH 6.5), 1 equiv of protein (75 μ M final protein concentration) in 25 mM sodium phosphate (NaPi) buffer (pH 6.5) at 37 °C for 20 h. Following removal of excess 9 with NanoSep 10 filtration devices the concentrate was redissolved in the same buffer and added to a pre-vortex mixed suspension of 1 equiv of indium and allylbromide 8 (200 equiv) either in buffer or *t*-BuOH-buffer mix. After shaking in the laboratory rotisserie for 20 h, the reaction was freeze dried and taken for LC-ESI-MS analysis.

No allylation product was detected in reaction performed in buffer alone (Table 3-1, entry 1), however when the reaction media was changed to 50% *t*-BuOH/buffer system trace amount of product was detected in LC-ESI-MS analysis [Allylated protein 12, expected mass for (myoglobin aldehyde + 41 = 16990,

¹¹⁵ Chi, E. Y., Krishnan, S., Randolph, T. W., Carpenter, J. F. *Pharm. Res.* **2003**, 20, 1325-1336.

Chapter 3

observed mass 16991); 41 is the expected mass increase for myoglobin aldehyde after undergoing allylation with allyl bromide in the presence of indium] (Table 3-1, entry 2). While this was not the successful outcome that we had expected, it still showed that the reaction could be applied on protein level and further optimization studies could lead us to the desirable effect. Hence we next tried to optimize the condition further by changing solvent and amount of allyl bromide added (Table 3-2).

Table 3-2 Optimization of *Allylation* reaction^a

Entry	Solvent	Allyl Bromide Eq	% Conversion ^b	Remarks
1	<i>t</i> -BuOH:25 mM NaPi (pH 6.5) (1:1)	200	trace	Complex mixture
2	<i>t</i> -BuOH:H ₂ O (1:1)	100	50	Clean product
3	<i>t</i> -BuOH:H ₂ O (1:1)	67	50	Clean product
4	<i>t</i> -BuOH:H ₂ O (1:1)	50	35	Clean Product
5	<i>t</i> -BuOH:H ₂ O (4:6)	67	50	Clean product
6	<i>t</i> -BuOH:H ₂ O (3:7)	67	0	No Product
7	<i>t</i> -BuOH:H ₂ O (1:4)	67	0	No product
8	Ethylene glycol:H ₂ O (1:4)	67	0	No product
9	Ethylene glycol:H ₂ O (1:1)	67	0	No product

^aOxidation reactions were run with 133 equiv of PLP **2** (10 mM final concentration in 25 mM sodium phosphate buffer, pH 6.5), 1 equiv of protein (75 μ M final protein concentration) in 25 mM sodium phosphate (NaPi) buffer (pH 6.5) at 37 °C for 20 h. Following removal of excess **2** with NanoSep 10 filtration devices the concentrate was redissolved in either buffer (E 1) or H₂O (E 2-9) and added to a pre-vortex mixed suspension of 1 equiv of indium and allylbromide either in organic solvent-buffer/H₂O mix. After shaking in the laboratory rotisserie for 20 h, the reaction was purified, freeze dried and taken for LC-ESI-MS analysis. ^bBased on area under the peak values for unmodified and modified myoglobin obtained from LC-ESI-MS chromatograms.

Chapter 3

These screening experiments identified *t*-BuOH/water (4:6) as the best solvent choice reducing the percentage of organic solvent needed to 40% and resulting in 50% conversion to the desired product **12** (Table 3-2, entry 5; Figure 3-2) after 20 h based on LC-ESI-MS analysis. % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], myoglobin dihydrate **14** [expected mass, (myoglobin aldehyde **13** + H₂O = 16967, observed mass 16968)], allylated protein **12**, [expected mass for (myoglobin aldehyde + 41 = 16990, observed mass 16991); 41 is the expected mass increase for myoglobin aldehyde after undergoing allylation with allyl bromide in the presence of indium]; allylated protein **14** + H₂O, [expected mass for (allylated protein **14** + H₂O = 17008, observed mass 17008)]. Having successfully demonstrated that the Allylation reaction can be applied to proteins, the amount of allyl bromide needed for the reaction was optimized. The best conversion (53 %) to the desired product could be achieved with 67 equivalents (Table 3-1, entry 5) of the allyl bromide reagent.

Figure 3-2: Myoglobin oxidation and *allylation*.

With the successful conversion to the desired *Allylation* product established, we performed a control reaction on the native protein to demonstrate that the current reaction conditions do not alter the protein in the absence of an *N*-terminal aldehyde

Chapter 3

or through non covalent binding of the reagent. Thus in this experiment the aldehyde generation was inhibited by performing reaction without PLP under identical conditions. After spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) and eluting with water, an aliquot of the protein (100 μ L, 37.5 μ M final protein concentration) was added to a pre-vortexed suspension of allylbromide (260 μ L, 11.5 mM in *t*-BuOH), Indium (0.0005 g, 0.004 μ mol) and water (40 μ L); after brief vortex mixing and sonication the reaction mixture was incubated on a laboratory rotisserie for 20 h at room temperature. Upon spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) and freeze drying LC-ESI-MS analysis of freeze dried sample revealed only unmodified protein and further confirmed the success of Allylation reaction.

To confirm the site specificity of the modification, products **11** and **12** were subjected to tryptic digestion. Analysis of the resulting peptide fragments by LC-ESI-MS revealed an increase in the molecular weight of the *N*-terminal peptide fragment [GLSDGEWQQVLNVWGK (expected mass = 1815, observed mass = 908.4 (M/2+1), entry **a**, Figure 3-3] by [352 + GLSDGEWQQVLNVWGK (expected mass = 1815 + 352 = 2167, observed mass = 1084, (M/2+1), entry **b**, Figure 3-3], + 352 is the expected mass increase for myoglobin after biotinylation and MS-MS of Allylation modified and unmodified *N*-terminal peptide **12**. MS-MS of unmodified *N*-terminal fragment (**zoom region 150-350**) GL [expected mass = 171, observed mass = 171.5, entry **d**, Figure 3-3] 213 (+42)). MS-MS of modified *N*-terminal fragment (**zoom region 150-350**) GL [expected mass [M + H] = 171 + 41 + 1 = 213, observed mass = 213, entry **c**, Figure 3-3] 41 is the expected mass increase for myoglobin after modification with allyl bromide though indium mediated allylation a proof that the reactions had successfully modified the protein *N*-terminus.

Chapter 3

Figure 3-3. HPLC-ESI-MS analysis of trypsin digest of myoglobin. **a)** MS of unmodified protein, *N*-terminal fragment (**residues 1-16**, GLSDGEWQQVLNVWGK) (expected mass = 1815, observed mass = 908.4, ($M/2 + 1$)). **b)** MS of Biotinylated protein, *N*-terminal fragment (**residues 1-16**, GLSDGEWQQVLNVWGK + 352) (expected mass 1815 + 352 = 2167, observed mass = 1085, ($M/2 + 1$)); + 352 is the expected mass increase for myoglobin after biotinylation **c)** MS-MS of allylation modified *N*-terminal fragment (**zoom region 150-350**) GL [expected mass [$M + H$] = 171 + 41 + 1 = 213, observed mass = 213, entry **c**, Figure 3-3] 41 is the expected mass increase for myoglobin after modification with allyl bromide though indium mediated allylation. **d)** MS-MS of unmodified *N*-terminal fragment (**zoom region 150-350**) GL (expected mass = 171, observed mass = 171).

Our next step was to study whether the modified purified myoglobin (obtained by filtering through Nanosep filtration device) retained its tertiary structure. For this purpose two separate studies were performed.

In the first study, the modified protein and unmodified controls (obtained by subjecting a sample of native protein to allylation conditions followed by filtration

Chapter 3

without going through the initial aldehyde generation step) were analyzed by ultraviolet spectroscopy for the 409 nm absorbance characteristic of the heme moiety bound to myoglobin.¹¹⁶ A decrease in absorbance indicates dissociation of heme and suggests that the protein tertiary structure has changed. The UV-Vis spectrum revealed a strong absorbance at $\lambda_{\text{max}} = 409$ nm for the unmodified sample, but we noticed a moderate decrease of absorbance in the modified sample suggesting partial loss of heme moiety.

The most important advantage associated with the indium mediated allylation is the stability of the newly formed carbon-carbon bond. Hence the modified protein was taken for reconstitution experiment, in which the modified sample was incubated with chlorohemin under basic condition, at ambient temperature on a laboratory rotisserie overnight. Upon removal of excess hemin by filtration we gained back the characteristic UV absorption at 409 nm. The ultraviolet (UV) spectroscopy traces of the modified reconstituted protein and unmodified protein were identical showing the success of reconstitution experiments (Scheme 3-6).

Scheme 3-6. UV-Vis spectra of the modified reconstituted and unmodified myoglobin.

¹¹⁶ Makinen, M. W.; Churg, A. K. In *Iron Porphyrins*; Lever, A. B. P.; Gray, H. B., Eds.; Addison-Wesley: Reading, 1983; Vol. 1, pp 141-236.

Chapter 3

2) In the second study the modified reconstituted myoglobin and unmodified myoglobin solutions were taken for circular-dichroism spectroscopy and an average of three scans from 190 to 250 nm was collected for each sample. The ellipticity values were plotted against wavelength using prizm software. The CD spectra exhibited by the modified reconstituted sample suggested that the secondary structure of the modified reconstituted protein remained essentially unperturbed relative to that of unmodified myoglobin thus supporting the success of reconstitution experiment (Scheme 3-7).

Scheme 3-7. CD spectra of the modified reconstituted and unmodified myoglobin.

3.2.3 Summary

In conclusion, we describe the first indium mediated allylation in the functionalization of *N*-terminal aldehydes of proteins.

We illustrate that this method could be used for the targeted delivery of a terminal alkene functionality into specific protein site through the formation of a stable carbon-carbon bond.

The C-C bond generated in our method is more robust compared to imines and oximes, facilitating biological characterization studies and even allowing the protein to be refolded without loss of activity. It equips the protein with a terminal olefin handle that opens up a wide avenue for their orthogonal modifications through

Chapter 3

addition reactions or ruthenium-catalyzed metathesis.¹¹⁷ It complements existing methods of introducing olefins to proteins which face the challenges of selectivity and side product formation.¹¹⁸

The use of indium as the metal mediator adds to the growing repertoire of metal mediated reaction in protein functionalization. We hope that this demonstration will encourage the protein chemists to further explore into the untapped elements of the periodic table. We believe that this method has the potential to be used for the efficient labeling of proteins, with an array of functionalities thus aiding in the study and manipulation of biological functions. The application of this method to more classes of proteins is currently in progress in our lab.

3.3 Mukaiyama Aldol reaction

3.3.1 Introduction

While the indium mediated allylation is a useful addition to the armamentarium of biological chemistry, there is a desire in the field of protein modification chemistry to develop metal free reactions.

The peptide based *Mukaiyama*-aldol reaction that we developed (Chapter 2) seemed to have potential in this regard. Thus we went onto explore the possibility of applying *Mukaiyama*-aldol reaction to myoglobin. To the best of our knowledge there have been no reports of *Mukaiyama*-aldol mediated functionalization of proteins.

¹¹⁷ (a) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem. Int. Ed.* **1998**, *37*, 3281-3284; (b) Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 15, 5052-5053; (c) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 30, 9642-9643.

¹¹⁸ (a) Connor, R. E and Tirrell, D. A. *Polym. Rev.* **2007**, *47*, 9-28; (b) Kirshenbaum, K.; Carrico, I. S.; Tirrell, D. A. *ChemBioChem.* **2002**, *3*, 235-237; (c) Van-Hest, J. C. M.; Tirrell, D. A. *FEBS Lett.* **1998**, *428*, 68-70; (d) Wang, L.; Schultz, P. G. *Angew. Chem. Intl. Ed.* **2005**, *44*, 34-66; (e) Dougherty, D. A. *Curr. Opin. Chem. Biol.* **2000**, *4*, 645-652; (f) Nowak, M. W.; Gallivan, J. P.; Silverman, S. K. Labarca, C. G.; Dougherty, D. A.; Lester, H. A. *Ion. Channels, Part B*, **1998**, *293*, 504-529.

Chapter 3

3.3.2 Mukaiyama-Aldol Reaction : Our Work

A Periodate Oxidation and Mukaiyama-aldol Reaction of Peptides. As illustrated in chapter 2, under optimized conditions for the *Mukaiyama*-aldol reaction, *N*-terminal peptide aldehydes generated by periodate oxidation were treated with 2 equivalent of silyl ketene acetal in (pH 7.0) buffer for 24 hrs at room temperature to give the desired *Mukaiyama*-aldol product in moderate yield (Scheme 3-8).

Scheme 3-8: Peptide functionalization by *Mukaiyama*-aldol reaction.

A screening experiment with a number of silyl ketene acetal reagents revealed 1-methoxy-2-methyl-propenyloxy)-trimethylsilane (**15**) as the best ketene acetal counterpart. With this information in hand we next moved on to apply the reaction to myoglobin.

B Oxidation and Mukaiyama-aldol reaction of myoglobin. Myoglobin *N*-terminal aldehyde was generated by pyridoxal 5'-phosphate (PLP, **9**) mediated biomimetic transamination reaction and the success of aldehyde generation established through biotinylation (Chapter 3, page 88, **3.2.2 B**, Scheme 3-5). A 75 μM solution of the protein was incubated at room temperature with 5 M silyl ketene acetal **15** in 25 mM phosphate buffer (pH 7.0) after pipette mixing (Scheme 3-9).

After overnight reaction only a trace amount of the *Mukaiyama* aldol product **16** was formed under the conditions used for the peptides (Scheme 3-9).

Chapter 3

Scheme 3-9: Myoglobin functionalization attempt by *Mukaiyama*-aldol reaction.

As discussed in the *allylation* reaction, a likely explanation for the unsatisfactory result is the poor solubility of the silyl ketene acetal in water, together with lack of stirring. Hence the use of a co-solvent was explored.

Ethylene glycol being a suitable solvent for protein functionalizations was introduced first (entries 2 & 3, Table 3-4). However in the case of the *Mukaiyama*-aldol reaction water/ethylene glycol mixtures were not suitable. When buffer/ethanol (1:1) was used, the desired product was obtained in 76% yield (Table 3-4, entry 4). As the high ethanol concentration used could perturb the protein structure.¹¹⁹ So the amount of ethanol was reduced. Although we could see 50% conversion to the desired product in low ethanol concentrations (Table 3-4, entry 5), complications associated with the MS-spectrum (complex spectrum indicating multiple reaction products or degradation), made the analysis difficult and we went onto explore other solvent mixtures. After a number of experiments *t*-BuOH/water (17:83) was identified as the best solvent (Table 3-4, entry 19), with 90% conversion to the desired product (Scheme 3-10). % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], *Mukaiyama*-aldol modified protein **16**, [expected mass for (myoglobin aldehyde + 101 = 17051, observed mass 17052); 101 is the expected mass increase for myoglobin aldehyde after undergoing *Mukaiyama*-aldol reaction.

¹¹⁹ Khmel'nitsky, Y. L.; Mozhaev, V. V.; Belova, A. B.; Sergeeva, M. V.; Martinek, K. *Eur. J. Biochem.* **1991**, *198*, 31-41.

Chapter 3

Table 3-4. Solvent study for the conversion to *Mukaiyama*-aldol product.^a

Entry	Solvent System	% Conversion ^b
1	Buffer (25mM NaPi, pH 7.0)	0
2	H ₂ O : Ethylene glycol (1:1)	0
3	H ₂ O : Ethylene glycol (4:1)	0
4	Buffer : EtOH (1:1)	76
5	Buffer : EtOH (4:1)	50*
6	Buffer : <i>t</i> -BuOH (1:1)	0
7	Buffer : <i>t</i> -BuOH (4:1)	90*
8	Buffer : DMSO (4:1)	0
9	Buffer : DMSO (1:1)	0
10	Buffer : DMF (4:1)	20*
11	Buffer : DMF (1:1)	0
12	Buffer : Glycerol (4:1)	trace
13	Buffer : Glycerol (1:1)	0
14	H ₂ O	0
15	H ₂ O : EtOH (1:1)	90
16	H ₂ O : EtOH (4:1)	60*

^aOxidation reactions were run with 133 equiv of PLP 9 (10 mM final concentration in 25 mM sodium phosphate buffer, pH 6.5), 1 equiv of protein (75 μ M final protein concentration) in 25 mM sodium phosphate (NaPi) buffer (pH 6.5) at 37 °C for 20 h. Following removal of excess 9 with NanoSep 10 filtration devices the concentrate was redissolved in either buffer (E 1 & 4-13) or H₂O (E 2, 3 & 14-24) and treated with 1-Methoxy-2-methyl-1-trimethylsiloxy propene (15, 0.3 μ L, 5 M, Technical grade 95%) and (100 μ L) different solvent system. After proper pipette mixing they were incubated at ambient temperature for 5 h. Upon freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column). ^bBased on LC-ESI-MS analysis. % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], *Mukaiyama*-aldol modified protein 16, [expected mass for (myoglobin aldehyde + 101 = 17051, observed mass 17052); 101 is the expected mass increase for myoglobin aldehyde after undergoing *Mukaiyama*-aldol reaction. *Complex mixture in the Mass Spec analysis.

Chapter 3

Table 3-4. Solvent study for the conversion to *Mukaiyama*-aldol product.^a

Entry	Solvent System	% Conversion ^b
17	H ₂ O : <i>t</i> -BuOH (1:1)	90
18	H ₂ O : <i>t</i> -BuOH (1:1)	90
19	H ₂ O : <i>t</i> -BuOH (5:1)	90
20	H ₂ O : <i>t</i> -BuOH (6:1)	90*
21	H ₂ O : Acetonitrile (1:1)	90
22	H ₂ O : Acetonitrile (4:1)	50
23	H ₂ O : Acetonitrile (5:1)	0
24	H ₂ O : Acetonitrile (6:1)	0

^aOxidation reactions were run with 133 equiv of PLP 9 (10 mM final concentration in 25 mM sodium phosphate buffer, pH 6.5), 1 equiv of protein (75 μM final protein concentration) in 25 mM sodium phosphate (NaPi) buffer (pH 6.5) at 37 °C for 20 h. Following removal of excess 9 with NanoSep 10 filtration devices the concentrate was redissolved in either buffer (E 1 & 4-13) or H₂O (E 2, 3 & 14-24) and treated with 1-Methoxy-2-methyl-1-trimethylsiloxy propene (15, 0.3 μL, 5 M, Technical grade 95%) and (100 μL) different solvent system. After proper pipette mixing they were incubated at ambient temperature for 5 h. Upon freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column). ^bBased on LC-ESI-MS analysis. % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], *Mukaiyama*-aldol modified protein 16, [expected mass for (myoglobin aldehyde + 101 = 17051, observed mass 17052); 101 is the expected mass increase for myoglobin aldehyde after undergoing *Mukaiyama*-aldol reaction. *Complex mixture in the Mass Spec analysis.

Scheme 3-10: Myoglobin oxidation and *Mukaiyama*-aldol reaction.

Chapter 3

With a near quantitative conversion to the desired *Mukaiyama*-aldol product, we ran a control reaction without PLP under otherwise identical conditions. After spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) eluting with water, an aliquot of the mixture (100 μ L, 37.5 μ M final protein concentration) was treated with (64 μ L) water, 1-Methoxy-2-methyl-1-trimethylsiloxy propene (**15**, 0.3 μ L, 5 M, Technical grade 95%) and (36 μ L) *t*-butanol. After pipette mixing, this reaction was incubated at ambient temperature for 5 h without further agitation. Upon freeze drying the reaction mixture was analyzed by LC-ESI-MS (Poros R1/H column) revealing only unmodified myoglobin. This suggested that the *Mukaiyama*-aldol reaction had successfully modified myoglobin in the presence of *N*-terminal aldehyde and there was no non-covalent interaction between the reagent and the myoglobin.

To confirm the site specificity of modification, product **16** was subjected to tryptic digestion. Analysis of the resulting peptide fragments by LC-ESI-MS revealed an increase in the molecular weight of the *N*-terminal peptide fragment by GLSDGEWQQVLNVWGK (expected mass = 1815, observed mass = 908.4 (M/2+1), entry **a**, Figure 3-4] by [87 (when terminal methyl ester OMe is hydrolyzed to OH)+ GLSDGEWQQVLNVWGK (expected mass = 1815 + 87 = 1902, observed mass = 1902, entry **b**, Figure 3-3]. Although [101 (when terminal methyl ester OMe is intact) + GLSDGEWQQVLNVWGK (expected mass = 1815 + 101 = 1916)] is the expected mass increase for myoglobin after modification with ketene silyl acetal to form the *Mukaiyama*-aldol product. This [-14 (OCH₃ – OH = 14)] difference in the observed mass results from the hydrolysis of the *N*-terminal methyl ester to corresponding acid from exposure to basic conditions during tryptic digestion. Nevertheless the result obtained from tryptic digestion is a clear proof that the reaction had successfully modified the protein *N*-terminus.

Chapter 3

Figure 3-2. HPLC-ESI-MS analysis of trypsin digest of myoglobin. **a)** Mukaiyama aldol modified protein **16** (residues 1-16) GLSDGEWQQVLNVWGK [(expected mass = 1815 + 101 = 1916, observed mass = 1902]; (-14) difference is due to the hydrolysis of methyl ester to corresponding acid from exposure to basic conditions during tryptic digest. +101 is the expected mass increase for myoglobin after modification with ketene silyl acetal to form the Mukaiyama aldol product. **b)** MS-MS of Mukaiyama aldol modified protein, *N*-terminal fragment (residues 1-16) (molecular weight 1902.9).

Having successfully demonstrated that the *Mukaiyama*-aldol reaction can be applied to proteins, the amount of silyl ketene acetal needed for the reaction was optimized. Near quantitative conversion could be achieved with 190 equivalents (0.3 μ L) of the substrate (Table 3-5, entry 6). % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], *Mukaiyama*-aldol modified protein **16**, [expected mass for (myoglobin aldehyde + 101 = 17051, observed mass 17052)]; 101 is the expected mass increase for myoglobin aldehyde after undergoing

Chapter 3

Mukaiyama-aldol reaction. Optimized conditions for the *Mukaiyama* aldol reaction with myoglobin use 190 equivalents of silyl ketene acetal. The greater efficiency of the reaction compared to the peptides (55% conversion, Chapter 2, Table 2-9, entry 1, pp 72) is likely due to the greater excess of ketene acetal. We have shown that the yield of the *Mukaiyama* aldol reaction with peptides can be improved (Chapter 2, Table 2-9, entry 2, pp 72, 55% to 71%), if a larger excess of ketene acetal is used (70 equivalent instead of 2 equivalent, Chapter 2, Table 2-9, entry 2 vs entry 1, pp 72).

Table 3-5. Silyl ketene acetal quantitation for *Mukaiyama*-aldol reaction.^a

Entry	Amount Silyl Ketene Acetal (3 M) (X equiv.)	% Conversion ^b
1	4 μL (2560)	90
2	3 μL (1900)	90
3	2 μL (1290)	90
4	1 μL (650)	90
5	0.5 μL (320)	90
6	0.3 μL (190)	90

^aOxidation reactions were run with 133 equiv of PLP 9 (10 mM final concentration in 25 mM sodium phosphate buffer, pH 6.5), 1 equiv of protein (75 μM final protein concentration) in 25 mM sodium phosphate (NaPi) buffer (pH 6.5) at 37 °C for 20 h. Following removal of excess 9 with NanoSep 10 filtration devices the concentrate was redissolved in H_2O and treated with varying amounts of 1-Methoxy-2-methyl-1-trimethylsiloxy propene (15, 5 M, Technical grade 95%) and (64 μL H_2O and 36 μL *t*-BuOH). After proper pipette mixing they were incubated at ambient temperature for 5 h. Upon filtration and freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column).

^bBased on area under the curve for LC-ESI-MS analysis.

An investigation into the speed of the reaction revealed near quantitative conversion (90 %) to the desired product in one hour. We observed a 76% conversion

Chapter 3

to the desired product within 5 minutes (Scheme 3-11). Maximum conversion to the *Mukaiyama-aldol* product (90%) is achieved within 1 h. Reaction times of longer at 5 h or 20 h did not show any significant increase in product conversion. % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], *Mukaiyama-aldol* modified protein **16**, [expected mass for (myoglobin aldehyde + 101 = 17051, observed mass 17052)]; 101 is the expected mass increase for myoglobin aldehyde after undergoing *Mukaiyama-aldol* reaction.

Scheme 3-11: Time study for *Mukaiyama-aldol* product formation.

With all the optimized conditions established, our next step was to study the structural integrity of the *Mukaiyama-aldol* modified protein. For this purpose three separate studies were performed.

1) In the first study, the modified and unmodified samples were analyzed by ultraviolet spectroscopy for the 409 nm absorbance characteristic of the heme moiety bound to myoglobin.¹²⁰ The UV-Vis spectrum revealed a strong absorbance at $\lambda_{\text{max}} = 409$ nm for both the samples signifying the retention of heme moiety (Scheme 3-12) and protein structural stability. In comparison to Allylation reaction, this result is very promising. In Allylation reaction we notice a decrease in heme absorbance, an

¹²⁰ Makinen, M. W.; Churg, A. K. In *Iron Porphyrins*; Lever, A. B. P.; Gray, H. B., Eds.; Addison-Wesley: Reading, 1983; Vol. 1, pp 141-236.

Chapter 3

indication that the metal free Mukaiyama aldol reaction is more suitable for functionalization studies.

Heme Stability in Mukaiyama Aldol Reaction

Scheme 3-12. UV-Vis spectra of the modified and unmodified myoglobin.

2) In the second study modified and unmodified myoglobin solutions were taken for Circular-Dichroism spectroscopy and an average of three scans from 190 to 250 nm was collected for each sample. The ellipticity values were plotted against wavelength using Prizm software. The ultraviolet CD spectra exhibited by the modified sample suggested that the secondary structure of the modified protein remained essentially unperturbed relative to that of unmodified myoglobin (**Scheme 3-13**).

Scheme 3-13. CD spectra of the modified and unmodified myoglobin.

Chapter 3

3) In the third study, rate constants k_1 and k_3 for the ping-pong mechanism of peroxidase defined by Dunford were determined for both unmodified and modified myoglobin under steady-state conditions.¹²¹ The results showed that, at saturating concentrations of peroxide the modification did not have any effect on the reaction of Myoglobin with the substrate ABTS (Scheme 3-14).

Scheme 3-14. Peroxidase Steady state kinetics for modified and unmodified myoglobin

All the above three experiments showed that, in comparison to indium mediated Allylation reaction, the developed *Mukaiyama*-aldol based method of protein functionalization could successfully modify protein while keeping its structural integrity and catalytic efficiency intact.

3.3.3 Applications

After establishing the efficiency, mildness and speed of the *Mukaiyama*-aldol reaction in protein functionalization, we next sought to explore the scope of this novel reaction. First a number of different silyl ketene acetals with important functional groups were used to broaden the substrate scope (Table 3-6). A 95% conversion to the

¹²¹ Dunford, H. B. (1991) in *Peroxidases in Chemistry and Biology*, Vol II, eds. Everse, K. E. & Grisham, M. B. (CRC, Boca Raton), pp. 1-24.

Chapter 3

desired product **17** was seen (Table 3-6, entry 3) (Scheme 3-15) suggesting that this newly developed reaction is well suited for the functionalization of proteins. Complex mixtures arising from MS-analysis failed to confirm the success of rest of the modifications (Table 3-6, entry 2 & 4-6).

Table 3-6: Screening of Silyl Ketene Acetals for Myoglobin.^a

(0.3 μ L, 190 Equivalent) Silyl ketene acetal,
16% t-BuOH in H₂O, RT, 5 h
R' = Myoglobin

Entry	Substrate	Product	[%] ^b Conversion
1	<p>15</p>	<p>16</p>	90
2	<p>18</p>	Complex mixture	-
3	<p>19</p>	<p>17</p>	95
4	<p>20</p>	Complex mixture	-
5	<p>21</p>	Complex mixture	-

^aReactions were carried out on a 200 μ L scale (37.5 μ M, final protein concentration). ^b % conversion determined by LC-ESI-MS analysis % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], *Mukaiyama*-aldol modified protein **17**, [expected mass for (myoglobin aldehyde + 101 = 17051, observed mass 17052); 101 is the expected mass increase for myoglobin aldehyde after undergoing *Mukaiyama*-aldol reaction.

Chapter 3

Scheme 3-15: LC-ESI-MS of *N*-terminal alkene functionalized myoglobin **17**.

After the success obtained with *Mukaiyama*-aldol modification of proteins, we decided to focus our attention towards exploring the application of our basic *Mukaiyama*-aldol method to other proteins.

We chose two proteins for study.

1. Dengue NS3 Serine Protease; a 28.5 KDa, *N*-terminal glycine containing protein.
2. Dengue 3 Methyl Transferase; a 30 kDa, *N*-terminal glycine containing protein.

When both of these proteins were subjected to PLP oxidation followed by biotinylation and *Mukaiyama*-aldol reaction no product was obtained.

The failure of Biotinylation pointed to the fact that, probably the first step of PLP mediated transamination was ineffective. This was unexpected, since Francis et al¹²² had shown that the PLP methodology was quite widely applicable.

¹²² (a) Gilmore, J. M; Scheck, R. A.; Esser-Hahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem. Intl. Ed.* **2006**, *45*, 5307-5311; (b) Scheck, R. A.; Dedeo, M. T.; Lavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 11762-11770.

Chapter 3

We know that Dengue NS3 is a robust protein and can tolerate higher temperatures as well as denaturing conditions. It has the ability to refold back to its native form if the tertiary structure is perturbed. Since it was possible that the *N*-terminal glycine was inaccessible for steric reasons, we employed more forcing conditions for the oxidation. However even at elevated temperatures (Table 3-8, entries 3 and 4), and in the presence of denaturing agents (Table 3-8, entries 2 and 4), that would be expected to unfold the protein, completely exposing the *N*-terminus, we failed to see any conversion to the aldehyde.

Table 3-8: PLP mediated oxidation of Dengue NS3^a

Entry	Temperature	Denaturing agent	Results ^b
1	37 °C	none	No reaction
2	37 °C	urea	No reaction
3	65 °C	none	No reaction
4	65 °C	urea	No reaction

^a Reactions were carried out on a 200 μL volume with a final [protein] of 50 μM . Incubation was done with (200 equiv.) PLP **9** in each case for 20 h. After 20 h, excess PLP was removed by filtration through NanoSep 10 filtration devices. The concentrate was redissolved in H_2O and treated with Biotin reagent **10** (100 equiv.) overnight. Upon freeze drying they were taken for analysis. ^b analysis done by LC-ESI-MS.

Chapter 3

Disappointed with the above results, we sought to find an explanation for the observed outcome. Upon a careful examination of the protein sequence we noted a similarity between the two proteins. Both the proteins had a serine residue at the 2nd position after the *N*-terminal glycine.

Dengue NS3 *N*-terminal pentapeptide : GSHML

Dengue 3 Methyl transferase *N*-terminal pentapeptide: GSGTG

In our specific case we were interested in investigating proteins which had been purified by His-tag technology followed by thrombin cleavage, which leaves the Gly in the nonprime site followed by serine at the 2nd position from the *N*-terminal. This method is common for many bacteria expression systems and is found in our expression vector pET15b.

We thought that a possible explanation was that the aldehyde was forming but a lactol **22** could be generated from the attack by neighboring serine hydroxyl which might lead to a very low concentration of the free aldehyde (Scheme 3-16) to undergo the next step of Biotinylation.

Scheme 3-16: Possible lactol generation from neighboring serine hydroxyl

To test this hypothesis three studies were performed;

In order to examine this issue further we decided to study a simple model system that is amenable to analysis by NMR and HPLC. Several dipeptides having protected and

Chapter 3

unprotected serine at the 2nd position to *N*-terminus were subjected to PLP conditions followed by *Mukaiyama*-aldol (Table 3-9).

Table 3-9: Screening of *N*-terminal dipeptides for *Mukaiyama*-aldol.

Reaction scheme showing the conversion of a dipeptide (H₂N-CH₂-C(=O)-NH-R) to an aldehyde intermediate (O=CH-C(=O)-NH-R) using 2.6 equiv. PLP, 25 mM NaPi (pH 6.5) at 37 °C, 22 h. The aldehyde intermediate then reacts with 5 equiv. of a Mukaiyama reagent (3-methoxy-4-(trimethylsilyl)but-3-en-2-one) in 25 mM NaPi (pH 6.5) at RT, 3-18 h to form the Mukaiyama-aldol product.

Entry	Peptide	R	Yield ^a	Remarks
1	GA-OMe	-Ala-OMe	65%	
2	GL-OMe	-Leu-OMe	69%	
3	GS(OtBu)-OMe	-S(OtBu)-OMe	58%	Recovered starting material 29%
4	GS-OMe	-S-OMe	-	Recovered starting material 82%

^a Yields are based on isolated compounds.

We did not isolate any lactol like product **22** for reaction with dipeptide containing unprotected serine in the second position (entry 4, Table 3.9). We recovered the starting material in 82% yield after HPLC purification confirming that the glycine had not been oxidized by the PLP. NMR and LC-ESI-MS were used to confirm the identity of the recovered material. The reaction with peptide where the serine residue was protected gave the desired *Mukaiyama*-aldol product in 58% isolated yield (Table 3-9, entry 3) with recovery of 29% of starting material.

This study suggested that the presence of the unprotected serine at the second position from the *N*-terminus somehow inhibited one of the steps and therefore no *Mukaiyama*-aldol product could be isolated (Table 3-9, entry 4).

These experiments suggest that under the currently employed conditions, the aldehyde is not formed in appreciable amounts when a serine is adjacent to the *N*-

Chapter 3

terminal glycine. This is an unexpected result and prompted us to examine the reaction in more detail (Scheme 3-17)

Scheme 3-17: PLP mediated transamination.

It is unlikely that the formation of imine B is influenced by an adjacent serine. However the rearrangement B to C or the elimination of PLP may be subject to perturbation by the peptide sequence. The literature suggest that the PLP mediated oxidation is reversible,¹²³ leaving us with two hypotheses:

- 1 The rearrangement of B to C is inhibited by the serine.
- 2 Serine may influence the rate of the reaction which may lead to very low levels of aldehyde after 22 h of reaction time.

These hypotheses were tested through isotope studies (Scheme 3-18).

¹²³ Metzler, D. E. and Snell, E. E. *J. Am. Chem. Soc.* **1952**, 74, 979-983.

Chapter 3

Scheme 3-18: Isotope loss in PLP mediated transamination.

We used α -deuterium labelled Gly-Ser-OMe dipeptides with or without serine protection as the substrates. They were subjected to PLP conditions (Table 3-10) followed by *Mukaiyama-aldol*. Exchange of deuterium in the recovered starting material (entry 3, Table 3-10) will be an indication that the intermediates B' and C' are formed during the reaction. The reaction with the protected serine (entry 2, Table 3-10) will serve as a positive control, since we have established previously that the Mukaiyama aldol proceeds as expected with this substrate.

Chapter 3

Table 3-10: Study for deuterium exchange.

Entry	Peptide	R	Yield ^a	Remarks
1	² H ₂ GS(OtBu)-OMe	-S(OtBu)-OMe	-	Control, reaction run in buffer without PLP. No ² H ₂ exchange.
2	² H ₂ GS(OtBu)-OMe	-S(OtBu)-OMe	54%	Complete ² H ₂ exchange in the product and starting material. 29% recovered.
3	² H ₂ GS-OMe	-S-OMe	-	Complete ² H ₂ exchange in the recovered starting material. 79% recovered.

^a Yields are based on isolated compounds.

As expected, (Table 3-10, entry 1) which was run without PLP led to the isolation of unchanged starting material, i.e. the two deuterium atoms in the glycine were not exchanged. (Table 3-10, entry 2) the reaction with protected serine proceeded to the *Mukaiyama*-aldol product with complete replacement of the deuterium atoms with hydrogen in the glycine. The recovered starting material also showed deuterium exchange. (Table 3-10, entry 3) did not produce any *Mukaiyama*-aldol product but instead 79% of the starting material was recovered. However the starting material had lost both of the deuterium atoms.

The results suggest that hypothesis 1 is not correct. The reaction proceeds as shown in Scheme 3.18 at the very least to intermediate C', but at this point for unknown reason the aldehyde cannot be trapped by the *Mukaiyama* reagent.

Chapter 3

Unfortunately we were unable to follow the reaction by HPLC/MS, since we were unable to find conditions that can separate the aldehyde from the starting peptide.

After some experimentation we found that this was feasible with longer peptides, so the study was repeated with three pentapeptides (Table 3-11). The reactions were monitored for aldehyde formation over time, without conversion to the aldol product. The first in the series was the *N*-terminal pentapeptide of Myoglobin (GLSAG-OH), we chose this sequence to study the reaction which we already have positive results of. The second pentapeptide was the *N*-terminal sequence of NS3 with a protected serine (GS(OtBu)HML-OMe). This was selected to answer our questions on the unsuccessful PLP mediated transamination on NS3 by comparing with a third pentapeptide where the serine was not protected (GSHML-OMe) (Table 3-11, Scheme 3-19, 3-20 and 3-21). In each study, to a buffered solution of peptide (5 mg, pH 6.5) at 37 °C was added a solution of PLP (2.6 equiv, in pH 6.5 buffer). At each time point 50 µL aliquots of the reaction was removed, diluted with water and taken for LC-ESI-MS analysis.

Table 3-11: Time Study for PLP mediated transamination.^a

Entry	Peptide	R	Remarks
1	GLSAG-OH	-LSAG-OH	Myoglobin <i>N</i> -terminal pentapeptide
2	GS(OtBu)HML-OMe	-S(OtBu)HML-OMe	NS3 <i>N</i> -terminal serine protected pentapeptide
3	GSHML-OMe	-SHML-OMe	NS3 <i>N</i> -terminal pentapeptide

^aReactions were run with 5 mg of starting peptides. Monitored by HPLC-ESI-MS. % conversion determined based upon the area under the curve values.

Chapter 3

% conversion was determined based upon the area under the peak values for extracted ion chromatogram. For Myoglobin N-terminal pentapeptide GLSAG-OH (retention time of starting material 1.5 min, [expected mass 447, $[M + H]^+$ observed mass 447.9]), for GLSAG-OH aldehyde (retention time of aldehyde 1.71 min, [expected mass 446, $[M + H]^+$ observed mass 446.9]), -1 is the mass decrease for conversion to aldehyde (Figure 3-3, Scheme 3-19).

Figure 3-3. HPLC-ESI-MS analysis of myoglobin N-terminal pentapeptide reaction with PLP. **a)** MS spectrum of the reaction. Pentapeptide GLSAG-OH rt 1.5 min. GLSAG-OH aldehyde rt 1.71 min. **b)** TIC of GLSAG-OH aldehyde (expected mass = 446, observed mass = 446.9, $(M + H)$). **c)** TIC of GLSAG-OH (expected mass 447, observed mass = 447.9, $(M + H)$).

Scheme 3-19: Time study with Myoglobin *N*-terminal pentapeptide

For NS3 *N*-terminal serine protected pentapeptide GS(OtBu)HML-OMe (retention time of starting material 1.62 min, [expected mass 613, $[M + H]^+$ observed mass 614]), for GS(OtBu)HML-OMe aldehyde (retention time of aldehyde 1.88 min, [expected mass 6304, $[M + H]^+$ observed mass 631]) (Figure 3-4, Scheme 3-20).

Chapter 3

Figure 3-4. HPLC-ESI-MS analysis of NS3 *N*-terminal serine protected pentapeptide reaction with PLP. **a)** MS spectrum of the reaction. Pentapeptide GS(OtBu)HML-OMe rt 1.62 min. GS(OtBu)HML-OMe aldehyde rt 1.88 min. **b)** TIC of GS(OtBu)HML-OMe aldehyde (expected mass = 612, observed mass = 613, ($M + H$)). **c)** TIC of GS(OtBu)HML-OMe (expected mass 613, observed mass = 614, ($M + H$)).

Scheme 3-20: Time study with NS3 *N*-terminal serine protected pentapeptide

For NS3 *N*-terminal pentapeptide GSHML-OMe (retention time of starting material 1.36 min, [expected mass 557, ($M + H$)⁺ observed mass 558.10]), for GSHML-OMe aldehyde (retention time of aldehyde 1.50 min, [expected mass 556, ($M + H$)⁺ observed mass 557.09]), for GSHML-OMe dihydrate (retention time of aldehyde 1.54 min, [expected mass 574, ($M + H$)⁺ observed mass 575.29]) (Figure 3-5, Scheme 3-21).

Chapter 3

Figure 3-5. HPLC-ESI-MS analysis of NS3 N-terminal pentapeptide reaction with PLP. **a)** MS spectrum of the reaction. Pentapeptide GSHML-OMe rt 1.36 min. GSHML-OMe aldehyde rt 1.50 min. GSHML-OMe dihydrate rt 1.54 min. **b)** TIC of GSHML-OMe aldehyde (expected mass = 556, observed mass = 557, (M + H)⁺). **c)** TIC of GSHML-OMe dihydrate (expected mass 574, observed mass = 575, (M + H)⁺). **d)** TIC of GSHML-OMe (expected mass 557, observed mass = 558, (M + H)⁺).

Chapter 3

Scheme 3-21: Time study with NS-3 *N*-terminal pentapeptide

The results from time study suggested that the speed of PLP mediated transamination is very much dependent on the amino acid sequence at the *N*-terminal. With a free serine at the 2nd position the rate of the reaction seems to be much faster than the one with a protected serine (Scheme 3-21 vs 3-19 & 3-20).

For the myoglobin and protected serine based pentapeptides, (Scheme 3-19 and 3-20) we noticed, a gradual increase in aldehyde formation over time in the first 22 h. However, when allowed to run longer for further conversion to product, we noticed, the regeneration of starting material associated with a decrease in product aldehyde concentration. For the pentapeptide without any serine protection, a similar trend was observed (Scheme 3-21) with a much faster inter conversion rate (maximum aldehyde concentration reached within 14 h) suggesting the involvement of a reversible transamination reaction. This is in agreement with the literature precedence's of reversibility of PLP mediated reactions.¹²⁴ This study pointed to the importance of monitoring reaction time in PLP mediated non enzymatic

¹²⁴ (a) Metzler, D. E. and Snell, E. E. *J. Am. Chem. Soc.* **1952**, 74, 979-983. (b) Voet, D.; Voet, J. G. in *Biochemistry*, 2nd edition, **1994**, pp 728-730.

Chapter 3

transamination. If the reaction time is carefully controlled, it is possible to vary the reaction yield to give maximum amount of the desired product. To ascertain this concept, *Mukaiyama aldol* reaction was run with NS-3 *N*-terminal pentapeptide (unprotected) **GSHML-OMe**, after two different lengths of PLP mediated transamination (Table 3-12). As expected, we could see a noticeable difference in *Mukaiyama-aldol* product yield (72% yield at 14 h versus 29% yield at 23 h) depending on the length of the PLP mediated transamination reaction (Table 3-12).

Table 3-12: *Mukaiyama-aldol* yield with varying length of PLP mediated transamination.

Entry	Transamination reaction time	Yield ^a
1	24 h	29%
2	14 h	72%

^a Yields are based on isolated compounds.

3.3.4 Summary

In conclusion, we describe a novel method for site directed functionalization of proteins using the *Mukaiyama aldol* condensation in aqueous media. This reaction furnishes a carbon-carbon bond and therefore leads to a stable product.

Chapter 3

The method can be used for the targeted introduction of a wide variety of functional groups into specific *N*-terminal protein under very mild conditions as illustrated by the introduction of terminal alkene moiety.

It has been demonstrated that in the case of Myoglobin, the functionalization of the protein can be carried out without disturbing the tertiary structure and more importantly the enzymatic activity of the protein. The *Mukaiyama aldol* reaction of proteins is very fast and highly efficient and as a consequence may be of particular interest when the protein stability is a concern.

We have also demonstrated through time study that by carefully controlling the reaction time for PLP mediated transamination the yield for the *Mukaiyama aldol* reaction can be improved for peptides.

Furthermore we have learnt from our studies, that the amino acid sequence at the *N*-terminal can also play a crucial role in determining the success of PLP mediated transamination which eventually affects the yield of *Mukaiyama aldol* reaction following it.

PROJECT II

Chapter 4

Synthetic Studies Towards the Total Synthesis of Platensimycin

Chapter 4

4.1 BACKGROUND

4.1.1 Structural and Biological Aspects of Platensimycin

Platensimycin was discovered in 2006 by a group of researchers at Merck.^{125, 126} The compound was obtained from the fermentation extracts of a soil bacterium *Streptomyces platensis* collected in South Africa.

It shows high antibacterial activity against gram-positive bacteria with IC_{50} values in the range of 48 to 160 nM. It exerts its activity by a novel mode of action which involves inhibition of cellular lipid biosynthesis.¹²⁷ The activity is brought about by the binding of Platensimycin to the acyl-enzyme intermediate of the elongation-condensing enzyme β -ketoacyl-acyl carrier protein synthase I/II (FabF/B) in the fatty acid biosynthetic pathway.

Figure 4-1

The molecule has two structural components, a polar 3-amino-2,4-dihydroxybenzoic acid head group which is linked to a unique lipophilic oxatetracycle by a flexible propionamide chain.

¹²⁵ J. Wang, J.; Soisson, S. M.; Young, K.; Shoop, W.; Kodali, S.; Galgoci, A.; Painter, R.; Parthasarathy, G.; Tang, Y. S.; Cummings, R.; Ha, S.; Dorso, K.; Motyl, M.; Jayasuriya, H.; Ondeyka, J.; Herath, K.; Zhang, C.; Hernandez, L.; Allocco, J.; Basilio, A.; Tormo, J. R.; Genilloud, O.; Vicente, F.; Pelaez, F.; Colwell, L.; Lee, S. H.; Michael, B.; Felcetto, T.; Gill, C.; Silver, L. L.; Hermes, J. D.; Bartizal, K.; Barrett, J.; Schmatz, D.; Becker, J. W.; Cully, D.; Singh, S. B. *Nature* **2006**, *441*, 358–361.

¹²⁶ Singh, S. B.; Jayasuriya, H.; Ondeyka, J. G.; Herath, K. B.; Zhang, C.; Zink, D. L.; Tsou, N. N.; Ball, R. G.; Basilio, A.; Genilloud, O.; Diez, M. T.; Vicente, F.; Pelaez, F.; Young, K.; Wang, J. *J. Am. Chem. Soc.* **2006**, *128*, 11916–11920.

¹²⁷ Häbich, D.; Nussbaum, F. V. *ChemMedChem* **2006**, *1*, 951–954.

Chapter 4

Low mammalian cell toxicity, lack of cross resistance with other antibiotics together with its challenging chemical structure makes this molecule an unusually interesting drug target for total synthesis.

4.2 Reported Synthesis of Platensimycin

K. C. Nicolaou and co-workers reported the first racemic¹²⁸ total synthesis of the molecule. Although a number of research groups have worked on this molecule, only two groups have reported a total synthesis.¹²⁹ All the other efforts were concerned with the development of methods for the construction of the platensimycin core.¹³⁰

4.2.1 First synthesis

The synthesis of the oxatetracyclic core **1** poses the major challenge in the synthetic scheme. In their first racemic synthesis, Nicolaou's group overcame this obstacle through the samarium(II)iodide-mediated ketyl radical cyclization of a spirocyclic aldehyde **5**. The scheme started with the double alkylation of ketone **2** to install the first quaternary center, which after four steps gave **3**. A ruthenium-

¹²⁸ Nicolaou, K. C.; Li, A.; Edmonds, D. J. *Angew. Chem.* **2006**, *118*, 7244-7248; *Angew. Chem. Int. Ed.* **2006**, *45*, 7086-7090.

¹²⁹ (a) Nicolaou, K. C.; Edmonds, D. J.; Li, A.; Tria, G. S. *Angew. Chem.* **2007**, *119*, 4016-4019; *Angew. Chem. Int. Ed.* **2007**, *46*, 3942-3945; (b) Ghosh, A. K.; Xi, K. *J. Org. Chem.* **2009**, *74*, 1163-1170.

¹³⁰ (a) Nicolaou, K. C.; Pappo, D.; Tsang, K. Y.; Gibe, R.; Chen, D. Y. -K. *Angew. Chem.* **2008**, *120*, 958-960; *Angew. Chem. Int. Ed.* **2008**, *47*, 944-946; (b) Zou, Y.; Chen, C. -H.; Taylor, C. D.; Foxman, B. M.; Snider, B. B. *Org. Lett.* **2007**, *9*, 1825-1828; (c) Li, P.; Payette, J. N.; Yamamoto, H. *J. Am. Chem. Soc.* **2007**, *129*, 953-954; (d) Lalic, G.; Corey, E. J.; *Org. Lett.* **2007**, *9*, 4921-4923; (e) Tiefenbacher, K.; Mulzer, J. *Angew. Chem.* **2007**, *119*, 8220-8221; *Angew. Chem. Int. Ed.* **2007**, *46*, 8074-8075; (f) Nicolaou, K. C.; Tang, Y.; Wang, J. *Chem. Commun.* **2007**, 1922-1923; (g) Kim, C. H.; Jang, K. P.; Choi, S. Y.; Chung, Y. K.; Lee, E. *Angew. Chem.* **2008**, *120*, 4073-4075; *Angew. Chem. Int. Ed.* **2008**, *47*, 4009-4011; (h) Matsuo, J. -I.; Takeuchi, K.; Ishhibashi, H. *Org. Lett.* **2008**, *10*, 4049-4052; (i) Yun, S. Y.; Zheng, J. -C.; Lee, D.; *J. Am. Chem. Soc.* **2009**, *131*, 8413-8415; (j) McGrath, N. A.; Bartlett, E. S.; Sittihan, S.; Njardarson, J. T. *Angew. Chem.* **2009**, *120*, 2582-2590; *Angew. Chem. Int. Ed.* **2009**, *48*, 8543-8546; (k) Kaliappan, K. P.; Ravikumar, V. *Org. Lett.* **2007**, *9*, 2417-2419; (l) Nicolaou, K. C.; Lister, T.; Denton, R. M.; Montero, A.; Edmonds, D. J. *Angew. Chem. Int. Ed.* **2007**, *46*, 4712-4714; (m) Nicolaou, K. C.; Tong, Y.; Wang, J.; Stepan, A. F.; Li, A.; Montero, A. *J. Am. Chem. Soc.* **2007**, *129*, 14850-14851; (n) Nicolaou, K. C.; Tria, G. S.; Edmonds, D. J. *Angew. Chem. Int. Ed.* **2008**, *47*, 1780-1783; (o) Nicolaou, K. C.; Lee, A.; Ellery, S. P.; Edmonds, D. J. *Angew. Chem.* **2009**, *121*, 6411-6413; *Angew. Chem. Int. Ed.* **2009**, *48*, 6293-6295; (p) Recent Review of synthetic approaches: Palanichamy, K.; Kaliappan, K. P. *Chem. Asian J.* **2010**, *5*, 668-703.

Chapter 4

catalyzed cycloisomerization of **3** gave the spiro-cyclopentanone derivative **4** in a 1:1 mixture of diastereomers. These compounds were converted to the desired aldehyde intermediate **5** in another three steps, which upon ketyl radical cyclization and treatment with trifluoroacetic acid provided the desired tetracyclic core **1** (Scheme 4-1).

4.2.2 Total Synthesis

Two different asymmetric approaches were later published by the Nicolaou group.^{129,130a} In the enantioselective cycloisomerization strategy (Scheme 4-1) intermediate **3** was converted to **6** in four steps, which gave the spiro compound **7** by asymmetric chiral induction through the use of chiral rhodium catalyst. After four additional steps, aldehyde intermediate **8** was obtained, which through the ketyl radical cyclization, similar to the racemic synthesis gave the desired diastereoisomer selectively.

Scheme 4-1. Synthesis of the oxatetracyclic core by Nicolaou et al.^{129, 130a}

In the same paper, the authors reported an auxiliary-based approach (Scheme 4-2), where Myers' asymmetric alkylation was used to induce the chirality via

Chapter 4

intermediate amide **9**. After five steps through intermediate **10**, **9** gave the common intermediate **5**. The rest of the steps were similar.

Scheme 4-2. Synthesis of the oxatetracyclic core through diastereoselective alkylation.^{130a}

Nicolaou et al. also reported another new asymmetric synthesis of **1** (Scheme 4-3) from readily available and inexpensive (*R*)-Carvone **11**.^{131a} After a sequence of Grignard reaction, oxidation, oxymercuration / reductive-alkylation and dehydration alkene **12** was obtained, which after three more steps furnished the dienone aldehyde **13**. SmI₂-mediated radical cyclization followed by Mitsunobu inversion and base catalyzed epimerization rendered **14** and after several more steps the tetracyclic core **1** was obtained.

Scheme 4-3. Synthesis of optically active Oxatetracyclic core by Nicolaou et al.^{131a}

Ghosh et al. reported an asymmetric synthesis of the oxatetracyclic core from commercially available (+)-carvone **15**. They used an intramolecular Diels-Alder based approach to construct the oxatetracyclic core **17**, through the intermediate **16** (Scheme 4-4).^{130b}

Chapter 4

Scheme 4-4. Synthesis of optically active Oxatetracyclic core by Diels-Alder reaction.^{130b}

4.2.3 Syntheses of oxatetracyclic core.

B.B. Snider and his team reported the formal synthesis of racemic **1** through intermediate dione **18**, which was obtained from 5-methoxy-1-tetralone **19** in 5 steps by a radical cyclization of key intermediate **20**. A series of L-Selectride reduction, acid-catalyzed cyclization, alcohol dehydration and allylic oxidation gave **1** (Scheme 4-5).^{131b}

Scheme 4-5. Racemic synthesis of the oxatetracyclic core by Snider et al.^{131b}

Chapter 4

Yamamoto et al. published one of the most elegant enantioselective synthesis of Platensimycin,^{131c} in which the quaternary center and the six-membered rings were constructed in a single step using diastereoselective Robinson annulation (Scheme 4-6, **24** to **1**). Another special feature of this approach is the use of a Bronsted acid supported chiral Lewis acid in Diels-Alder reaction of methyl acrylate **21** and methyl cyclopentadiene **22** to give the product **23** after nitrosoaldol reaction and oxidative decarboxylation in a highly-efficient manner (92% yield, 99% *ee*). The intermediate **23** went through several more chemical manipulations to give the key intermediate **24**. Intermediate **24** furnished the Michael addition product under the catalysis of L-Proline in 5 days which was converted to **1** in situ under base catalysis.

Scheme 4-6. Synthesis of optically active oxatetracyclic core by Yamamoto et al.^{131c}

Corey et al. utilized a dearomatizing alkylation step (Scheme 4-7, **28** to **29**) to give the known intermediate **29** in their efforts in the synthesis of enantioselective **1**.^{131d} Starting from compound **25** which can be prepared from commercially available starting material, compound **26** can be obtained in 94% *ee* and high yield after oxidative ketalization and enantioselective addition of the isoprenyl group. A series of reduction, protection and deprotection steps furnished intermediate **27** in good yield. Change of protecting group and bromination yielded the key intermediate **28** which was converted to the tetracyclic structure **29** after a TBAF reaction in sealed vessel. Later three more steps furnished the core **1**.

Chapter 4

Scheme 4-7. Synthesis of optically active oxatetracyclic core by Corey et al.^{131d}

Mulzer et al described the racemic synthesis of **1** starting from the tricyclic intermediate **30**,^{131e} which could be synthesized following a previously known literature method. Regio- and stereoselective addition of methylmagnesium iodide followed by stereoselective bromination yielded the intermediate **31**, which upon base-induced cyclization installed the tetrahydrofuran ring in **29**. Intermediate **29** upon catalytic hydrogenation and selective mono-oxidation gave the oxatetracyclic core **1** (Scheme 4-8).

Scheme 4-8. Synthesis of racemic oxatetracyclic core by Mulzer et al.^{131e}

Nicolaou et al published a synthetic route which has a similar key step as Snider's work (Scheme 4-5).^{131g} Following the same strategy as their first synthesis (Scheme 4-1) dienone **32** was obtained in five steps. Intermediate **32** went through a series of deprotection, oxidation and Stetter reaction to yield the bicyclic intermediate **33**. From there the key radical step as Snider's synthesis (Scheme 4-5) was reached in another 3 more steps to form the tricycle **34** (Scheme 4-9).

Chapter 4

Scheme 4-9. Racemic synthesis of the oxatetracyclic core by Nicolaou et al.^{131g}

Recently Lee et al. reported the asymmetric synthesis of **1** (Scheme 4-10)^{131g} by a Rhodium (II) catalyzed decomposition of α -diazoketone **36** through [3 + 2] cycloaddition approach to give **37**. Reduction of **37** gave the tricycle **38** which was subsequently converted to **1** through four additional steps. α -diazoketone **36** in turn can be prepared from isopropyl cyanoacetate **35** by five steps.

Scheme 4-10. Synthesis of optically active oxatetracyclic core by Lee et al.^{131g}

Matsuo et. al demonstrated a synthesis of oxatetracyclic core with three key findings.^{131h} They used a diastereoselective Diels-Alder reaction to construct the bicyclic intermediate **41**, from enone **39** and siloxy diene **40** (Scheme 4-11). Intramolecular alkenyl ether formation to give intermediate **42** was achieved by subjecting intermediate **41** to palladium-catalysis. Intermediate **42** lead to α -alkoxy radical precursor **43** which underwent transannular radical cyclization to give **1**.

Chapter 4

Scheme 4-11. Synthesis of oxatetracyclic core by Matsuo et al.^{131h}

Lee et al used a C-H insertion method of alkylidene carbenes as a key step in the formation of oxatetracyclic core **1** (Scheme 4-12).¹³¹ⁱ They used the commercially available (*S*)-carvone as the starting material, which after several steps furnished the key intermediate **44**. **44** underwent C-H insertion to give **45**, which after two more steps and an intramolecular aldol reaction gave **1**.

Scheme 4-12. Synthesis of oxatetracyclic core by Lee et al.¹³¹ⁱ

Few other approaches that have furnished unfinished intermediates and analogues of Platensimycin are the attempts by Njardarson et al. in the synthesis of **46**,^{131j} **47** by Kaliappan et al.,^{131k} Adamantaplatensimycin **48**,^{131l} Carbaplatensimycin **49**^{131m} and Platencin **50**¹³¹ⁿ by Nicolaou et al. (Scheme 4-13).

Chapter 4

Scheme 4-13. Synthesis of Platensimycin analogues and intermediates.

Although radical based approaches have played a vital role in many of the synthetic routes to oxatetracyclic core of Platensimycin, there have been no reports of the synthesis where cascade type radical approach has been used. In our initial synthetic route to Platensimycin we had planned to approach the core by a radical based cascade reaction to perform three steps in one pot.

4.3 Cascade Reaction

Cascade reaction is the process in which two or more reactions take place sequentially one after another in one pot from the starting substrates without the addition of any new reagent throughout the process. Nature has provided its very own cascade reaction products, for example, in the presence of enzyme, four bonds are constructed in one-pot manner to give lanosterol **51** through a highly efficient and stereoselective cascade reaction starting from (*S*)-2,3-oxidosqualene **52** (Scheme 4-14).¹³¹

¹³¹ Wendt, K. U.; Schulz, G. E.; Corey, E. J.; Liu, D. R. *Angew. Chem. Int. Ed.* **2000**, 39, 2812.

Scheme 4-14. Cascade reaction in the biosynthesis of Lanosterol **51**

4.4 Radical Cascade Reaction

Although a number of different types of cascade reactions exist, here we will concentrate on radical type cascade reactions. Radical based transformations have drawn much attention from organic chemists as in most cases they proceed in the presence of free hydroxyl and amino groups, as well as keto and ester functionalities. It has established itself as an important alternative to nonpolar methods for the connection of carbon atoms. In marked contrast to polar processes, radical transformations can proceed without protecting group manipulations and they are equally feasible in adding to inactivate double and triple bonds as to substrates bearing polarizing groups.¹³²

Nature has efficiently used radical cascade reaction in the biosynthesis of natural products. In the biosynthesis of morphine, a radical cascade reaction plays an important role. Starting from (-)-reticuline the di-phenolic radical **53** undergoes an *o,p*-coupling to give salutaridine, from the tautomerization of the initial coupling product **54** to phenol (Scheme 4-15).

¹³² For general reviews of radical chemistry, see: (a) Giese, B. *Radicals in Organic Synthesis: Formation of Carbon-Carbon Bonds*, Pergamon, Oxford, 1986, p. 294; (b) Giese, B. *Radicals in Organic Synthesis, Vols. 1&2* (Eds.: P. Renaud, M. P. Sibi), Wiley-VCH, Weinheim, 2001, p. 1110; (c) Zard, S. Z. *Radical Reactions in Organic Synthesis*, Oxford University Press, Oxford, 2003, p. 256; (d) Albert, M.; Fensterbank, L.; Lacôte, E.; Malacria, M. *Top. Curr. Chem.* **2006**, 264, 1; (e) McCarroll, A. J.; Walton, J. C. *Angew. Chem. Int. Ed.* **2001**, 40, 2224.

Scheme 4-15. Cascade radical reaction in the biosynthesis of Morphine

Over the year's chemist have also taken advantage of radical cascade reaction in the synthesis of natural products in laboratory settings. Curran's total synthesis of Hirsutene **58** is a prominent example in the field of radical cascade cyclization strategy (Scheme 4-16).¹³³ The tri-*n*-butyltin radical generated *in-situ* reacts with iodide **55** to give the transient radical **56** which participates in a facile 5-*exo*-trig radical cyclization, followed by a 5-*exo*-dig radical cyclization to give the tricyclic vinyl radical **57**. **57** then undergoes radical termination by abstraction of a hydrogen atom from tri-*n*-butyltin hydride to afford Hirsutene **58**.

¹³³ (a) Curran, D. P.; Rakiewicz, D. M. *J. Am. Chem. Soc.* **1985**, *107*, 1448; (b) Curran, D. P.; Rakiewicz, D. M. *Tetrahedron*, **1985**, *41*, 3943.

Chapter 4

Scheme 4-16 Radical cascade cyclization in the total synthesis of Hirsutene

4.5 Retrosynthetic Analysis of Oxatetracyclic Core

4.5.1 First Generation

Our retrosynthetic analysis for Oxatetracyclic core is delineated in Scheme 4-17. The two key features of our strategy are 1) construction of the oxatetracyclic core **59** with an allyl handle *via* an efficient cascade approach from intermediate **60**; and 2) keep in the sequence short. This would provide us with a key advantage over existing syntheses since most of them require several steps, resulting in a poor overall yield.

Ring C and D would be constructed in a single step starting from intermediate **60**. We hypothesize that the allyl tri-*n*-butyltin radical generated *in-situ* will react with the bromide to give the transient radical **66** which will participate in a facile 5-*endo*-trig radical cyclization to **67**, followed by a 6-*exo*-trig radical cyclization to give the tetracyclic radical **68**. **68** would then undergo radical termination by abstraction of an allyl group from allyl tri-*n*-butyltin to afford the oxatetracyclic core **59**.

Retrosynthetic cleavage of the indicated vinylic bond in **60** furnished alcohol **61** *via* a vinylation reaction. Alcohol **61** could in turn be obtained through a regioselective alkylation at the ketone carbonyl of ring B in contrast to the enone

Chapter 4

carbonyl in ring A from the intermediate **62**. **62** can be envisioned from regioselective α -bromination of ring B (intermediate **63**). Friedel-Crafts acylation furnishes the spiro diketone intermediate **63** from commercially available **64** and ethylene gas **65** following literature procedure.¹³⁴ We envisaged that except for the vinylation step, all the rest will pose a considerable level of difficulty. Steps leading to intermediates **61** and **62** will have regioselectivity issues. While to obtain **61** from **62** the alkylation has to be selective, where alkylation of ketone has to go in preference to alkylation at enone.

Scheme 4-17 The first generation of retrosynthetic analysis

4.6 Synthesis

Model study

To make sure that our key step of cascade reaction is functional, we decided to do model study where we eliminated the regioselective alkylation step (give numbers). Our idea was that if the key cascade radical step was successful, we could

¹³⁴ Adam, M. F.; Stanford, R. S. *Syn. Commun.* **1996**, 26, 1623-1627.

Chapter 4

come back to this step and pursue the alkylation. We designed the following synthetic route for model studies (Scheme 4-18).

Scheme 4-18 Retrosynthetic analysis for model study

Starting from 4-Methoxy phenyl acetyl chloride **64** the bicyclic diketone **63** was obtained in 55% isolated yield through Friedel Crafts reaction using ethylene gas **65**.¹³⁵

4.6.1 α -bromination

The regioselective α -bromination was thought to be synthetically challenging (Scheme 4-18). Since in the α -bromination of intermediate **63** to give **62**, there are two possible sites of attack. In fact in our trials to synthesize intermediate **62** through the intermediacy of lithium enolate followed by the trapping of enolate as trimethylsilyl ether under varied reaction conditions was unsuccessful or resulted in undesired enolate formation (Table 4-1).¹³⁶ It was evident that undesired enolate **76** was the kinetic product formed at low temperature of $-78\text{ }^{\circ}\text{C}$.

¹³⁵ Adam, M. F.; Stanford, R. S. *Syn. Commun.* **1996**, 26, 1623-1627.

¹³⁶ House H.; Czuba, L. J.; Gall, M.; Olmstead, H. D. *J. Org. Chem.* **1969**, 34, 2324-2336.

Chapter 4

Table 4-1 Attempts towards regioselective α -enolization

Entry	Base	Temperature (°C)	Time (h)	76 ^{a,b}
1	a. DIA, n-BuLi	-78	1	90%
	b. TMSCl	-78 to rt	1	
2	a. Et ₃ N, DMF	rt	4	-
	b. TMSCl			
3	a. LiHMDS	-78 to rt	2	50%
	b. TMSCl			
4	TIPSOTf, Et ₃ N	0	3	-

^a Conversion judged based upon ¹H NMR. ^b 75 was never observed.

Direct bromination attempts using different kinds of brominating reagents were also unsuccessful (Table 4-2).¹³⁷

Table 4-2 Attempts towards direct α -bromination

Entry	Base	Temperature (°C)	Time (h)	Yield
1	NBS, NH ₄ OAc, Et ₂ O, THF	rt	1	-
2	CuBr ₂ , CHCl ₃ /EtOAc 1:1	85	24	-
3	Ph ₃ P ⁺ CH ₂ CH ₂ COOHBr ₃ ⁻	0	2	-

¹³⁷ (a) Tanemura, K.; Suzuki, T.; Nishida, Y.; Satsumabayashi, K.; Horaguchi, T. *Chem. Commun.* **2004**, 470-471. (b) Armstrong, V. W.; Chishti, N. H.; Ramage, R. *Tetrahedron. Lett.* **1975**, 6, 373-376. (c) Bauer, D. P.; Macomber, R. S. *J. Org. Chem.* **1975**, 40, 1990-1992.

Chapter 4

When none of the bromination attempts were successful, an indirect approach to the intermediate **70** was explored (Scheme 4-19).

Scheme 4-19 Revised synthetic route for model study

4.6.2 Synthesis of Intermediate **78**

In the event the spiroketone underwent regioselective Meerwein-Ponndorf-Verley reduction to the alcohol **78** (yield 60%) in the presence of aluminium tert-butoxide and 2-butanol (Scheme 4-20).¹³⁸

Scheme 4-20 Preparation of alcohol **78**.

¹³⁸ Iwata, C.; Yamada, M.; Ida, Y.; Imao, T.; Miyagawa, H.; Miyashita, K. *Chem. Pharm. Bull.* **1988**, *36*, 2864-2871.

Chapter 4

4.6.3 Elimination

The alcohol was then reacted with mesylate chloride to give **79** (Table 4-3, entry 3). Initial attempts to activate it as a tosylate were unsuccessful. Upon further explorations the desired olefinic intermediate **80** was obtained (**80:85** ratio 1:2) as an inseparable mixture with its regioisomer **85** under DBU conditions (Table 4-3, entry 7). This mixture was taken to the next step, with the hope of being able to separate the isomers after bromohydrin formation.

Table 4-3 Generation of internal olefin

Entry	Conditions A	Temperature (°C)	Time (h)	79 ^a
1	<i>p</i> TsCl, pyridine	-15	6	-
2	<i>p</i> TsCl, pyridine, DMAP	0	6	-
3	MsCl, pyridine	-15 to 0	3	98%
Entry	Conditions B	Temperature (°C)	Time (h)	80 + 85 ^a
4	Pyridine	rt	6	-
5	Pyridine, Et ₃ N	0	6	-
6	Pyridine, Et ₃ N	rt	6	-
7	DBU, toluene	110	20h	80% (1:2) ^b

^a Yield refers to column purified yield. ^b The mesylate intermediate **79** was column purified before taking it for elimination.

Chapter 4

4.6.4 Hydrobromination

Next, we subjected the olefinic mixture (1:2) of **80** and **85** to hydrobromination conditions with *N*-bromosuccinimide in H₂O:Et₂O 1:1 system. To our advantage the reaction not only performed successfully, but we were able to separate the two regioisomers (Scheme 4-21). The yield was quantitative with the undesired product being the major isomer (**81**:**86** in a ratio of 1:2).

Scheme 4-21 Hydrobromination of olefinic mixture.

4.6.5 Vinylation of alcohol

With ample amounts of bromohydrin **81** in hand, we screened various vinylation conditions as summarized in Table 4-4.¹³⁹ In most cases, only trace or no desired product was obtained. Only when the method A was followed and the catalyst was preactivated the desired product **70** was obtained in 64% yield (Table 4-4, entry 6).

¹³⁹ Bosch, M. and Schlaf, M. *J. Org. Chem.* **2003**, 68, 5225-5227.

Chapter 4

Table 4-4 Vinylation of alcohol

Entry	Catalyst mol%	Method	Time (h)	70 ^a
1	-	B in THF	14	-
2	-	B in DCM	14	-
3	0.5	A, 3 equiv. Et ₃ N	14	-
4	10	A, 3 equiv. Et ₃ N	14	6
5	20	A, 3 equiv. Et ₃ N	14	8
6 ^b	20	A, 0.4 equiv. Et ₃ N	3	64

^a Yields refer to column purified yield of product **70**. ^b The Pd-catalyst was preactivated by mixing the Pd(II)TFA and bathophenanthroline in butyl vinyl ether for 30 min under N₂, following slow addition of C₂H₄Cl₂ solution of alcohol and Et₃N into the catalyst mix under reflux condition.

4.6.6 Attempts towards radical cascade reaction

With the intermediate vinyl alcohol **70** in hand we went on to test our hypothesis by subjecting it to the key step of radical cascade conditions (Table 4-5). We were disappointed to find that, under radical conditions we only obtained the reduced product and the desired product was not obtained.

Chapter 4

Table 4-5 Key step^a

Entry	Radical Initiator	Time (h)	Product ^b
1	ACCN	18	SM recovered
2	ACCN, HSn(Bu) ₃	18	
3	ACCN, 	18	

^a The reactions were run in deuterated solvent, the radical initiator was added over 18 h using a syringe pump. ^b Product identified based upon ¹H NMR of the crude reaction.

4.7 Summary

We have successfully synthesized the key intermediate **70** for radical cascade reaction trials. But our attempts towards radical cascade failed to give us the desired product. There might be several reasons behind the undesired outcome.

- The loss of bromine demonstrates that the intermediate radical **82** (Scheme 4-19) is formed.
- After the formation of radical we did not notice the formation of intermediate **83**. This may be explained by the fact that, the formation of intermediate **83** is a 5-*endo*-trig radical cyclization process, which is disfavored by Baldwin's rule.¹⁴⁰ But examples exist where there have been reports of successful 5-*endo*-trig radical cyclizations in the formation of 4-

¹⁴⁰ (a) Baldwin, J. E. *J. Chem. Soc., Chem. Commun.* **1976**, 734. (b) Baldwin, J. E. *J. Chem. Soc., Chem. Commun.* **1976**, 736.

Chapter 4

hydrindanone, oxazines etc.¹⁴¹ These examples led us to pursue this unfavoured pathway in the first place.

- A careful look at the molecular model, demonstrated that, although the first ring formation (intermediate **83**) seems feasible the next step to close the system by a favored 6-*exo*-trig radical cyclization, needs to overcome a very strong ring strain and seems almost impossible. Lack of driving force under such circumstances might have rendered the reaction unfeasible.
- We know that in successful radical reactions the selection of appropriate reactive partner is very important.¹⁴² A closer look into our design, revealed that, in our reaction the secondary radical that is generated (intermediate **82**) is electron rich, by having an electron donating group nearby. It would be easier for this radical intermediate to undergo coupling with an electron poor/ electron withdrawing system. But in our synthesis the partner it needs to combine with is an electron rich vinyl alcohol group. Hence, most probably in our scenario, it prefers to undergo easy quenching with an allyl or hydrogen radical instead.

However, this setback did not discourage us from continuing the synthesis of Platensimycin. Instead the points mentioned above inspired us to plan the second generation retrosynthetic strategy proposed in the next section based on the development of a radical based 1,4-dicarbonyl strategy.

¹⁴¹ Gao, J. and Rusling, J. F. *J. Org. Chem.*, **1998**, 63,218-219; (b) Johnson, C. D. *Acc. Chem. Res.* **1993**, 26, 476-482; (c) Fife, T. H. *et al. J. Org. Chem.* **1992**, 57, 1295-1298; (d) Johnson, C. D. *Tetrahedron* **1985**, 41, 5919-5928.

¹⁴² Trost, B. M; Fleming, I.; Semmelhack, M. E. *Comprehensive Organic Synthesis*, Vol 4, Pergamon Press, New York, 1993, pp 715-833.

PROJECT II

Chapter 5

Methodology Development for 1,4-Dicarbonyls and its Application

Chapter 5

5.1 Second Generation Retrosynthesis

Similar to the first generation retrosynthesis, we adopted a radical mediated cascade reaction for our second generation synthesis. While our initial strategy was based upon intramolecular radical cascade; here we decided to pursue an intermolecular radical approach instead (Scheme 5-1). We envisaged that the intermediate **63** from our previous strategy could be used to give bromo intermediate **87**, which would then serve as our radical precursor. Having an electron withdrawing ketone group nearby, will make the radical intermediate **90** electrophilic. Under radical coupling conditions, it should couple with electron rich olefinic substrate to give intermediate **91**. We assumed that the formed electron rich radical intermediate **91** will instantaneously undergo favored 6-*exo*-trig radical cyclization on to the ring **A**, through the formation of ring **C** at the same time giving the intermediate **88**. If this could be successfully performed, regioselective olefination would give the intermediate **89**.

Scheme 5-1: 2nd generation synthetic scheme.

When the X group in intermediate **89** is an oxygen atom it resembles an intermediate from Nicolaou's synthetic route (Scheme 4-1). In Nicolaou's route this compound is obtained by SmI_2 mediated radical cyclization of aldehyde **5** (scheme 5-

Chapter 5

2). From there on we could follow the same procedure as Nicolaou's synthetic scheme to form the tetrahydrofuran ring (D ring).

Scheme 5-2: Comparison of Nicolaou's and our intermediate.

Since **92** and its derivatives would be key intermediates in our strategy, we focused our initial work on the development of radical based methodology for the formation of 1,4-dicarbonyl intermediates and demonstrate its application in the radical step (from **87** to **88**).

Before that we needed to synthesize intermediate **87**.

5.2 Synthesis of Intermediate **87**

From our initial unsuccessful attempts through silyl enol ether based indirect bromination, regioselective bromination through enamine intermediate seemed more promising.¹⁴³ We pursued enamine formation reactions with different amines and obtained morpholine and pyrrolidine enamines as the best choice, giving the desired and undesired enamine in the ratio of 1:3 and 1:5 respectively (Table 5-1). It was evident from these studies that the desired enamine regioisomer **A** was the

¹⁴³ (a) Stork, G.; Brizzolara, A.; Landesman, H.; Szmuszkovicz, J.; Terrel, R. *J. Am. Chem. Soc.* **1963**, *85*, 207-222. (b) Whitesell, J. K. and Whitesell, M. A. *Synthesis*. **1983**, 517-537. (c) Hearn, B. R.; Zhang, D.; Li, Y.; Myles, D. C. *Org. Lett.* **2006**, *8*, 3057-3059.

Chapter 5

thermodynamic product formed at room temperature compared to undersired regioisomeric enolate formed at -78 °C (Chapter 4, sec 4.6.1, pg 133 and 134). We noticed that, with decreasing ring size the amount of the desired enamine **A** seemed to increase. Product ratios and % conversions were determined from the ¹H NMR peak integrations for the peaks in the aromatic region (2H, ds, 5.97 for **B**, 6.05 for **A** and 6.17 for starting material, Figure 5-1).

Table 5-1. Amine screening for the formation of enamine.

Entry	Amine	Product ratio (A:B) ^a	Conversion
1		1:3	90%
2		1:5	quantitative
3		1:5	50%
4		1:15.4	quantitative
5		-	No reaction
6		-	No reaction
7		1:9.5	90%

^a Product ratios were determined from ¹H NMR peak integrations for the peaks in aromatic region (2H, d at 5.97, 6.05 and 6.17 ppm) after the evaporation of solvent from the reaction mix and taking it directly for NMR.

Chapter 5

Figure 5-1: Reaction monitoring for the formation of enamine intermediate.

Next a series of screening experiments were performed to optimize the conditions for enamine reaction (Table 5-2 and 5-3). The best solvent for the enamine formation was found to be Et₂O/CH₂Cl₂ (4:1) (Table 5-2, entry 1).

Table 5-2. Solvent screening for the formation of enamine.

Entry	Solvent	Product ratio (A:B) ^a	% Conversion
1	Et ₂ O/CH ₂ Cl ₂ (4:1)	1:3	90
2	MeOH	1:3.5	90
3	THF	1:3	70
4	CH ₂ Cl ₂	1:3	90
5	Isopropanol	1:2.6	50
6 ^b	Isopropanol	1:2.6	70
7	Toluene	1:2.7	10
8 ^c	Toluene	1:8.4	quantitative
9	<i>t</i> -BuOH	1:9.5	50

^a Product ratios were determined from ¹H NMR peak integrations for the peaks in aromatic region (2H, d at 5.97, 6.05 and 6.17 ppm) after the evaporation of solvent from the reaction mix and taking it directly for NMR. ^b Molecular sieve used. ^c Azeotropic distillation.

Chapter 5

The best amine equivalent for the quantitative conversion to enamine was 2.0 equivalents (Table 5-3, entry 2).

Table 5-3. Amount of base screening for the formation of enamine.

Entry	Amine equivalent	Product ratio (A:B) ^a	% Conversion
1	1	1:3.5	61
2	1.5	1:5	Quantitative
3	2	1:5	Quantitative
4	2.5	1:5	Quantitative
5	3	1:5	Quantitative

^a Product ratios were determined from ¹H NMR peak integrations for the peaks in aromatic region (2H, d at 5.97, 6.05 and 6.17 ppm) after the evaporation of solvent from the reaction mix and taking it directly for NMR.

In our bromination trials, successful bromination was only possible when pyrrolidine enamine was used (Scheme 5-3). Under optimum conditions, the desired bromo intermediate was obtained in 60% yield based upon the recovered starting material. The fortuitous decomposition of the undesired bromo-compound together with the recovered starting material and obtained yield for the desired compound pointed to the reversibility of the reactions.

Scheme 5-3. Direct route to bromo intermediate from ketone.

Chapter 5

With the success of obtaining the bromo intermediate **87** in a two step one-pot manner, starting from the diketone **63**, it was crucial to perform a model study before executing the radical step (from **87** to **88**). We chose bromo-indanone **93** for the model study as the aromatic ring would help us in tracing the reactions by the UV-254 absorption and the cyclopentanone part would give us a feel of the reactions on the B ring.

5.3 Methodology Development Towards Intermediate **88**.

Literature Reference

There has always been an interest in 1,4-dicarbonyl compounds as they have served as starting materials and intermediates in the syntheses of many natural products including perfumery constituents, insecticides and hormones (Scheme 5-4).

Scheme 5-4: Representative 1,4-dicarbonyl compounds.

Chapter 5

The formation of such structures through direct intermolecular coupling by classical synthetic reactions has always been a challenge.

One of the most common approaches to these systems is based on the conjugate addition of acyl anions and their equivalents **94** to conjugated enones **95** (Scheme 5-5).¹⁴⁴ The acyl anions were generated in a number of ways, including; lithiation of acylcarbonylnickelate (Eq. 1); lithiated acylcuprate reagents (Eq. 2); and electrochemical (Eq. 3) and light assisted B12 catalyzed addition of (Eq. 4) acid anhydrides to activated olefins.¹⁴⁵

Scheme 5-5. Synthesis of 1,4-dicarbonyl compounds by conjugate addition.

¹⁴⁴ (a) Corey, E. J.; Hegedus, L. S. *J. Am. Chem. Soc.* **1969**, *91*, 4926-4928. (b) Seyferth, D.; Hui, R. C. *J. Am. Chem. Soc.* **1985**, *107*, 4551-4553. (c) Kubota, Y.; Nemoto, H.; Yamamoto, Y. *J. Org. Chem.* **1991**, *56*, 7195-7196.

¹⁴⁵ (a) Shono, T.; Nishiguchi, I.; Ohmizu, H. *J. Am. Chem. Soc.* **1977**, *99*, 7396-7397. (b) Scheffold, R.; Orlinski, R. *J. Am. Chem. Soc.* **1983**, *105*, 7200-7202. (c)

Chapter 5

One of the competing reactions in these systems are the 1,2 addition to the enone moiety.

1,4-dicarbonyl systems have also been accessed from furans in a number of ways,¹⁴⁶

- Acid-catalyzed ring opening (Eq. 5)

- From 2,5-dialkoxy-2,5-dihydrofuran (Eq. 6)

- Via oxidative ring fission (Eq. 7)

- Through Marckwald reaction (Eq. 8)

5.4 Radical Approaches to 1,4-Dicarbonyl Compounds

In radical procedures, α -(phenylseleno) carbonyl compounds were used as radical precursor to react with acetonyltributylstannane to give 1,4-dicarbonyl

¹⁴⁶ Piancatelli, G.; D'Auria, M.; D'Onofrio, F. *Synthesis*. **1994**, 867-889.

Chapter 5

compounds (Eq. 9).¹⁴⁷ Although this methodology furnished addition compounds in reasonable yields, the use of toxic tin and selenium compounds was a drawback. Also only diketones were available through this method.

Phenyl selenoester based method has also given access to a range of 1,4-dicarbonyl compounds (Eq. 10).¹⁴⁸

Oshima et al reported a triethyl borane based radical method for the synthesis of 1,4-dicarbonyl compounds using gallium enolates (Eq. 11).¹⁴⁹ They were able to form ester through this method. One of the drawbacks of this method is that the enolates are not commercially available and hence need to be synthesized from the corresponding silyl enolates through lithium exchange.

5.5 Synthesis of Compound 93 for Development of Radical Methodology

In the development of our method we first synthesized the model starting material **93** in two different ways (Scheme 5-6). We decided to pursue the second, one step route to the desired starting material for its simplicity and easy handling.

¹⁴⁷ Watanabe, Y.; Yoneda, T.; Ueno, Y.; Toru, T. *Tet. Lett.* **1990**, 31, 6669-6672.

¹⁴⁸ Bennasar, M.-L.; Roca, T.; Griera, R.; Bosch, J. *Org. Lett.* **2001**, 3, 1697-1700.

¹⁴⁹ Usugi, S.-I.; Yorimitsu, H.; Shinokubo, H.; Oshima, K. *Bull. Chem. Soc. Jpn.* **2002**, 75, 2049-2052.

Chapter 5

Scheme 5-6: Synthesis of starting material for model study.

From our previous synthetic scheme it was evident that, finding a proper coupling partner and optimized reaction conditions to give high yields of the desired product was the key point for a successful radical based coupling reaction.

We approached this task with several radical initiator based reactions.

5.6 Radical Initiators.

5.6.1 Tin Based Initiator.

Organotin hydrides are the most commonly used initiators for radical chain reactions. They can be used to generate a wide variety of carbon and heteroatom-centered radicals under mild conditions and the resulting radicals can be efficiently captured by reactive partners. A small amount of diazo derivative AIBN is used to generate isobutyronitrile radicals that react with the tin hydride to give the initiator tributylstannyl radical to generate the radical in starting material (eq. 1) (Scheme 5-7). Upon generation of radical $\text{R}^\cdot$ a number of competing pathways come into effect in the presence of reactive alkene partners. Although the desired pathway is addition to alkene to give **A**, there are two competing pathways at this point; (1) to give the desired product **B** by hydrogen atom abstraction (eq. 2) or (2) addition to another

Chapter 5

alkene to give undesired oligomers (eq. 3). One of the most common undesired side reactions in radical methods is the reduction of the starting material to give **C** (eq. 4).

The concentration of reagents plays an important role in determining the pathway. Keeping the concentration of the stannane low (by adding it slowly to the reaction mixture) and increasing the concentration of the olefin partner will favor the formation of **A** over **C**, but at the same time we will risk increased formation of oligomers. On the other hand increasing the amount of stannane might lead to increased formation of **C**. In intermolecular radical based addition reactions one of the ways to tackle this problem is to design the reactive partners appropriately.

Scheme 5-7. Reaction mechanism for intermolecular radical reactions.

5.6.2 Application of Tin Initiator in Our Methodology

To develop tin mediated radical reactions we used bromo indanone **93** as the starting material and screened a number of alkenes for coupling reactions (Table 5-4). ACCN was used as the radical initiator and the addition was done slowly through a syringe pump.

Chapter 5

Table 5-4. Tin based radical approach.

Entry	Substrate	Solvent	Product ^a	97
1		toluene	27%	63%
2		THF	-	Quantitative
3		toluene	10%	83%
4		toluene	52% 98	43%

^a Yields are based upon isolated yield.

Initial model studies demonstrated that, the electrophilic radical generated under tin and ACCN conditions could give coupling products from low to moderate yields with toluene as the solvent. Having sulfur as the heteroatom gave products in better yield (Table 5-2, entry 4), most probably because of the stabilizing effect of nearby sulfur on the radical intermediate.¹⁵⁰ Although the results from tin initiated radical reactions were very encouraging, the formation of reduced product **97** as the major component in most of these cases was the biggest drawback for this method.

We suspected that the radical initiator combination used (ACCN and Bu_3SnH) was

¹⁵⁰ Viehe, H. G.; Janousek, Z.; Mereyni, R. *Substituent Effects in Radical Chemistry, Series C*, 1986, p 252, Mathematical & Physical Sciences Vol 189, NATO ASI Series.

Chapter 5

serving as a powerful reducing agent (by providing the hydrogen radical easily) and gave the reduced product in large amounts.

Furthermore, the difficulty of working with stannanes from its nauseating stench, the well-known toxicity, to the inability to remove side products completely from product during purification and the formation of **97**, led us to pursue triethylborane based radical initiator.

5.6.3 Et₃B Based Initiator.

When lower reaction temperatures are required for radical processes usually a combination of triethylborane and oxygen is used.¹⁵¹ In the presence of oxygen these conditions generate ethyl radicals at temperatures as low as -78 °C. The ethyl radicals can be exploited to produce and capture radicals from iodides (Scheme 5-8).

Scheme 5-8. Reaction mechanism for triethylborane mediated reaction.

5.6.4 Application of Et₃B to Our Methodology

For triethylborane mediated reaction the required starting material was the iodo derivative **99**. This compound was obtained from indanone **97** through an enolate

¹⁵¹ (a) Kitagawa, O.; Fujiwara, H.; Taguchi, T. *Tetrahedron Lett.* **2001**, 42, 2165-2167; (b) Nozaki, K.; Oshima, K.; Utimoto, K. *J. Am. Chem. Soc.* **1987**, 109, 2547-2549.

Chapter 5

intermediate followed by trapping of the enolate with *N*-iodosuccinimide (Scheme 5-9).

Scheme 5-9: Synthesis of starting material for model study.

In developing our triethylborane mediated radical reactions iodo-indanone **99** was used as the starting material and screened a number of alkenes were screened for coupling reactions (Table 5-5). A combination of Et₃B/air was used as the radical initiator.

With iodo-indanone and vinyl acetate as the coupling partners in toluene, under triethylborane initiation the desired coupling product was not obtained (Table 5-5, entry 1). Instead we only obtained a dimer of the starting material **100** and hydroxylated product **101** together with the reduced compound **97**. Dimerization shows that the radical was successfully generated; while hydroxylation is a common side reaction for triethylborane mediated radical reaction.¹⁵² Vinyloxy trimethyl silane, and vinyloxy triisopropyl silane on the other hand mostly gave aldol addition product **102** with reduction product **97** as the major product (Table 5-5, entries 2-7). However, we were not discouraged by these results and went on to pursue other coupling partners. Our first positive results came with dimethoxy ketene as the coupling partner to give **103** (Table 5-5, entry 8). One vital information that we obtained from these experiments was that having sulfur as the substituent in the alkene partner gave the desired 1,4-dicarbonyl coupling products **98** in acceptable yields (Table 5-5, entries 9-10). In all the cases we noticed the formation of common triethylborane mediated reaction side product **101** together with reduced product **97**.

¹⁵² Kihara, N.; Olivier, C.; Renaud, P. *Org. Lett.* **1999**, *1*, 1419-1422.

Chapter 5

Table 5-5. Triethylborane based radical approach.^a

Entry	Substrate	Solvent	100 ^c	102 ^c	101 ^c	97 ^c
1 ^b		toluene	26%	-	18%	37%
2 ^b		toluene	-	12%	22%	55%
3 ^b		CH ₂ Cl ₂	-	25%	9%	53%
4 ^b		THF	-	17%	21%	58%
5 ^b		DMSO	-	-	-	Quantitative
6 ^b		CH ₂ Cl ₂	15%	18%	15%	42%
7 ^b		CH ₂ Cl ₂	-	-	21%	69%
8		CH ₂ Cl ₂	 103, 34%		15%	43%
9		CH ₂ Cl ₂	 98, 69%		7%	19%
10		CH ₂ Cl ₂	 98, 75%		6%	14%

^a The reactions were run in deuterated solvent, the radical was generated over 18 h using a syringe pump. ^b No base was added. ^c Yield refers to isolated yield.

Chapter 5

5.6.5 Conclusion.

We have developed a successful extension of radical coupling reactions in the synthesis of 1,4-dicarbonyl compounds. Through this method we were able to obtain 1,4-dicarbonyl products in the form of ester **103** and thioester **98** (Table 5-5, entries 8-10). The ester or thioester could be further reduced to aldehyde under literature conditions. The products were obtained in moderate to good yields. The utility of this method lies in the fact that, the reaction uses commercially available enol ether coupling partners, which has not been demonstrated before to the best of our knowledge.

5.7 Synthesis of Iodo Intermediate **104**

The iodo-intermediate **104** for this application was obtained from the bromo intermediate **87** through halogen exchange using sodium iodide (Scheme 5-10).

Scheme 5-10: Conversion of bromo intermediate to iodo intermediate

5.8 Application of Et₃B Initiation to Platensimycin

Although triethylborane mediated reactions gave the desired reaction products, we were not able to completely suppress the formation of dehalogenated product. We ignored this problem for the time being and explored its application for the synthesis of platensimycin (Table 5-6).

Chapter 5

Table 5-6. Application of triethylborane based radical approach to platensimycin.^a

Entry	Substrate	Product ^b	106 ^b	63 ^b
1		-	-	92%
2		 105 , 65%	3%	25%
3		 105 , 75%	4%	15%
4		 93 , 32%	3%	60%

^a The radical was generated over 18 h using a syringe pump. ^b Yield refers to isolated yield.

When the newly developed methodology was applied to the iodo intermediate **104**, successful coupling reactions were obtained with alkene substrates containing sulfur to give the thioester **105** (Table 5-6, entries 2 & 3). The most interesting aspect was the formation of intermediate **93** with ethyl vinyl sulfide (Table 5-6, entry 4) which is our desired intermediate. Although we were delighted with the initial results, there were two factors that were of concern.

Chapter 5

The fact that the radical cascade did not proceed further was something that raised questions. No matter what substrate was used, the reaction was always terminated by reaction with oxygen. So we repeated the reactions under three different conditions; (a) using degassed reagents, (b) in the presence of molecular sieves and (c) in the presence of MgSO_4 . However none of these variations gave the desired cascade product **88**. The insertion of oxygen atom could not be prevented even under anhydrous conditions. This was probably due to the air that we introduced to generate the radical under triethylborane conditions.

5.9 Cyclization Attempts of Intermediate **93**.

With the intermediate **93** in hand, we explored possibilities to cyclize the intermediate to give **88**. Under UV_{254} and SmI_2 conditions we observed extensive decomposition of the starting material and none of the desired product could be detected.

With these results, we decided to explore other radical based group transfer conditions in search for cascade reaction using ethyl vinyl sulfide as the coupling partner. To make this method more practical it was necessary to minimize the formation of reduced product.

For this we looked at alternative atom transfer radical processes where proton ($\text{Bu}_3\text{SnH}/\text{ACCN}$ method) and oxygen ($\text{Et}_3\text{B}/\text{O}_2$) sources could be avoided. We also wanted to use a method where we could utilize existing intermediates from the synthetic pathway.

Upon a careful search, Kharasch-type Xanthate based group transfer method seemed as a promising alternative. It could be easily obtained from the bromo intermediate **87** in a single step (Scheme 5-14).

Chapter 5

5.10 Xanthate Based Radical Group Transfer Reaction

Xanthate groups can be successfully transferred by designing the reaction sequence in such a way that C-S bond is cleaved in preference to other bonds in generating the radical intermediate. Once the radical $R^\cdot$ is generated, it could add to the starting xanthate because of the high radicophilic effect of thiocarbonyl group (Scheme 5-12). This reversible reaction can be prevented by having a group on the oxygen atom that gives radical of high energy, thus not favored (**I** to **II**, Scheme 5-12). Ethyl group is one of the most widely used group for such purpose. Therefore the only alternative route left for the generated radical is to take the desired addition to olefin pathway, minimizing commonly encountered routes to generate side products.

Scheme 5-12. Reactions mechanism for xanthate transfer process.

There are certain advantages to using xanthate based radical reactions;¹⁵³

- Their ready availability from commercially available and inexpensive potassium *O*-ethyl xanthate.

¹⁵³ Zard, S. Z. *Radical Reactions in Organic Synthesis*, Oxford University Press, 2003.

Chapter 5

- Their strong nucleophilicity leading to the displacement of halides to give the precursors in a generally straightforward manner.
- The comparatively long lifetime of the intermediates due to the stability of the xanthates.
- The experimental simplicity and possibility of operating under high concentrations.
- Not needing tin derivatives for reaction termination and also incorporation of functionality in the end product.
- The reversibility of the reaction ensures that the formation of side products and dehalogenated products are minimized.

We envisaged that, when the xanthate precursor **107** (Scheme 5-13) would be applied to radical conditions it would give rise to the radical intermediate **108**, which will couple to the ethyl vinyl sulfide substrate and give intermediate **109**. Intermediate **109** can either form product **110**, which itself is another radical precursor or it could cyclize to give intermediate radical **111** and give our desired final product **112**.

Chapter 5

Scheme 5-13. Proposed reaction scheme for xanthate reaction.

5.10.1 Synthesis of Xanthate Precursor **107**

To test the proposed reaction scheme the bromo intermediate **87** was converted to the xanthate precursor **107** by reaction with potassium *O*-ethyl xanthate in acetonitrile, providing the desired product in 70% isolated yield (Scheme 5-14).

Scheme 5-14. Synthesis of xanthate precursor **107**.

Chapter 5

5.10.2 Application of Xanthate Based Approach to Platensimycin

When the intermediate **107** was subjected to radical conditions in dichloroethane solvent in the presence of 2,6-lutidine using lauroyl peroxide as the radical generator intermediate **110** and aldehyde **93** were obtained in 15% and 75% yield respectively with no detectable cyclized product **112** (Scheme 5-15).

Scheme 5-15. Radical reaction with xanthate intermediate

Although we failed to initiate the radical cascade, the great benefits of this strategy is the complete absence of reduced side product **63** and also the high yield of aldehyde **93** (yield 75%) obtained (Scheme 5-15). We added 2,6-lutidine in this reaction to protect the vinyl thio ether from decomposition by adventitious acid. The formation aldehyde **93** is still not clear.

Our various cyclization attempts with the intermediate **110** were unsuccessful and in all cases we obtained the aldehyde **93** (Table 5-8).

Table 5-8. Cyclization attempts with intermediate **110**.

Entry	Condition	Result ^a
1	Visible light	SM decomposed
2	Lauroyl peroxide, reflux, ClCH ₂ CH ₂ Cl	93 (72%)
3	Lauroyl peroxide, reflux, ClCH ₂ CH ₂ Cl, InCl ₃	93 (81%)

^aYields refer to column purified yield.

Chapter 5

Since all the attempts to synthesize platensimycin through either intra or intermolecular radical cascade cyclization reactions were unsuccessful we decided to stop further work on the radical cascade at this point.

5.11 Summary.

We have developed a mild triethylborane based intermolecular radical reaction for the formation of 1,4-dicarbonyls. The reaction can be carried out at low temperature and results in a moderate yield of the coupling product. We were able to obtain different esters, thioesters and aldehydes through this method using commercially available alkene partners. This is an additional advantage over existing radical based coupling methods to 1,4-dicarbonyl which require separate synthetic steps to form the starting material.

In our application of this method along the formal synthesis attempts towards Platensimycin core we successfully inhibited the formation of reduced products by utilizing xanthate based group transfer process. A high yield was obtained for the radical coupling step.

Along the similar attempts towards the oxatetracyclic core we also developed an enamine based methodology for the regioselective bromination of ketone **63** to give the desired bromo intermediate **87** in a two-step one pot manner with moderate yield. This particular development makes the overall synthetic route to the aldehyde intermediate **5** concise with fewer steps.

Although radical cascade methods in synthesizing the oxatetracyclic core of Platensimycin were not successful, we could still intercept Nicolaou's intermediate **5** through regioselective olefination of ketone moiety of intermediate **114** (Scheme 5-16).

Chapter 5

Scheme 5-16. Alternate synthetic route to Nicolaou's intermediate **5**.

SUPPORTING INFORMATION

PART I

**CHEMICAL FUNCTIONALIZATION OF PEPTIDES
AND PROTEINS**

PART II

**SYNTHETIC STUDIES TOWARDS THE TOTAL
SYNTHESIS OF PLATENCIMYSIN**

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2010

EXPERIMENTAL SECTION

GENERAL EXPERIMENTAL INFORMATION

All moisture and/or sensitive experiments were performed under a positive pressure of nitrogen or argon in flame-dried glassware equipped with a rubber septum inlet. Solvents and liquid reagents were transferred by oven-dried syringes cooled in a dessicator or via double-tipped cannular needles. Reaction mixtures were stirred with teflon-coated magnetic stirring bars unless otherwise stated. Moisture in non-volatile reagents/compounds was removed by the addition of dry THF or dry toluene, followed by the removal of the solvent and traces of moisture *in vacuo* by means of an oil pump (~30 mmHg, 23-50 °C) and subsequent purging with nitrogen or argon. Solvents were removed in *vacuo* at 23 °C using Buchi rotary evaporator equipped with cooling circulator (cooled with circulating ethylene glycol / water mixture (1:1)) at -5 °C under ~30 mmHg.

Materials

Reagents were purified prior to use unless otherwise stated following the guidelines of Armarego and Chai.¹⁵⁴ Solvents such as hexane, ethyl acetate, dichloromethane and water were freshly distilled prior to use. Anhydrous THF and ether were obtained by distillation under nitrogen atmosphere from a deep purple solution resulting from sodium benzophenone ketyl. Anhydrous dichloromethane and diisopropylethylamine were distilled over calcium hydride under nitrogen atmosphere. Azeotropic drying of starting materials or reagents was performed by the addition of the stated amount of anhydrous tetrahydrofuran, ensued by azeotropic

¹⁵⁴ Armarego, W. L. F. and Chai, C. L. L. *Purification of Laboratory Chemicals*; 5th ed., Butterworth-Heinemann, Britain. 2003.

removal of tetrahydrofuran with traces of moisture *in vacuo* followed by subsequent purging with nitrogen.

Triethylamine and toluene were distilled over calcium hydride and stored over molecular sieves to maintain dryness. DMF was distilled over Linde type 4A molecular sieve prior to usage. 1M hydrochloric acid was diluted from concentrated 37% solution using deionised water. 3M lithium hydroxide solution was prepared from lithium hydroxide pearls. Saturated solutions of ammonium chloride, sodium chloride, sodium bicarbonate, and sodium carbonate were prepared from their respective solids.

Water used in biological procedures was distilled and deionized using Millipore purification system (Bedford, MA, USA).

Chromatography

Analytical thin layer chromatography was performed using Merck 60 F₂₅₄ pre-coated silica gel plates (0.25 mm thickness). Subsequent to elution, visualization was accomplished with UV light (254 nm) on Spectroline Model ENF-24061/F 254 nm. Further visualization was possible by staining with acidic solution of ceric molybdate, ninhydrin, potassium permanganate and developing on a hot plate. It could also be visualized by staining in Iodine.

Flash column chromatography was performed using Merck Silica Gel 60 (0.010-0.063 mm) and freshly distilled solvents. Columns were packed as slurry of silica gel in hexane/CH₂Cl₂ and equilibrated with the appropriate solvent/solvent mixture prior to use. Eluting system was determined by TLC analysis. All organic solvents were removed using a rotary evaporator under reduced pressure. The solute was loaded neat or as a concentrated solution using the appropriate solvent system. The elution was assisted by applying pressure with an air pump.

Instruments & Equipments

Infrared Spectroscopy

Infrared spectra were recorded on a Shimadzu IR Prestige-21 FT-IR Spectrometer. Solid samples were analyzed as a KBr pressed-disk while liquid samples were examined neat between NaCl salt plates.

UV/Vis and CD Spectra

UV-VIS spectroscopic measurement was conducted on a Infinite Tecan M1000 benchtop spectrophotometer. Circular dichroism spectra were obtained in a J-810 spectropolarimeter (JASCO, Tokyo, Japan). The data were processed using Microsoft Excel and Prizm software.

Optical Rotation

Optical rotation was measured using a JASCO P-1030 Polarimeter equipped with a sodium vapor lamp at 589 nm. Concentration is denoted as c and was calculated as grams per milliliters ($\text{g} / 100 \text{ mL}$) whereas the solvent was indicated in parentheses (c , solvent).

Mass Spectroscopy

Mass spectra (EI) were recorded on a Thermo Finnigan Polaris Q GCMS. MS (ESI) spectra were recorded on a Thermo Finnigan LCQ Deca XP Max. Peptide mass spectrometry (ESI, electrospray ionization) spectra was recorded on a Thermo Finnigan LCQ Deca XP Max (San Jose, CA) ultra high sensitivity quadrupole ion trap mass spectrometer fitted with Surveyor LC Auto Sampler and MS Pump. Acquisition mass range was typically m/z 500-2000. Data were recorded and processed using X-Calibur software (Thermo Scientific, MA, USA). High Resolution Mass Spectrometry (HRMS) (ESI) spectra were recorded on a Q-ToF Premier mass spectrometer (Micromass, MS Technologies, UK) fitted with Acquity Ultra Performance Auto

sampler and LC (Waters, Manchester, UK). Acquisition mass range was typically m/z 100-1000. Calibration was achieved by using the multiple-charged ion peaks of sodium formate. Data was processed using MassLynx™ software (Waters, Manchester, UK). MS and HRMS were reported in units of mass to charge ratio (m/z).

Nuclear Magnetic Resonance Spectroscopy

Proton nuclear magnetic resonance (^1H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectroscopy were performed on a Bruker Avance 300, 400, 500 NMR and Varian 300 NMR spectrometers as noted. Chemical shifts were reported as δ in units of parts per million (ppm) downfield from tetramethylsilane (δ 0.00), using the residual solvent signal as an internal standard: deuterio chloroform- d , CDCl_3 (^1H NMR, δ 7.26, singlet; ^{13}C NMR, δ 77.20, triplet); deuterio acetone- d_6 , CD_3COCD_3 (^1H NMR, δ 2.05, quintet; ^{13}C NMR, δ 29.8, septet); deuterio methanol- d_4 , (^1H NMR, δ 3.31, septet; ^{13}C NMR, δ 49.0, septet); deuterio water- d_2 , D_2O (^1H NMR, δ 4.67, singlet; ^{13}C NMR, δ 67.4, dioxane standard).

Multiplicities were given as: s (singlet), d (doublet), t (triplet), m (multiplets), br (broad), dd (doublet of doublets), dt (doublet of triplets), ddd (doublet of doublet of doublets) and ddt (doublet of doublet of triplets). Coupling constants (J) were recorded in Hertz (Hz). The number of protons (n) for a given resonance was indicated by $n\text{H}$.

High Performance Liquid Chromatography

Peptide purification was performed on an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA). Chromatographic separation of **12** and **13** were achieved on 19 mm x 280 mm Atlantis C18 dC OBC™ 10 μm (Waters, Manchester, UK) reversed-phase column at room temperature. Sample volumes of

500 μ l (250 mg) were typically injected onto the column manually. Eluent A was water containing 0.1% TFA. Eluent B was acetonitrile containing. A gradient from 0% B to 50% B was run in 30 minutes. HPLC was controlled by MassLynx software (Waters, Manchester, UK). UV detection was performed at 214 nm. The flow rate was 20 ml/min. Upon purification, the solvent was removed by freeze drying. Protein Purification and Mass Spectrometry

Separation of proteins was performed on a HP1100 HPLC system (Hewlett Packard, Palo-Alto, CA) employing a 1 mm x 150 mm column packed with POROS R1/H (Perspective Biosystems, Foster City, CA). The column was kept at 80 °C. Sample volumes of 3-25 μ l were typically injected onto the column using a CTC PAL autosampler (CTC, Zwingen, Switzerland) fitted with a Valco model C6UW HPLC valve (Valco, Houston, TX) and a 25 μ l injection loop. HPLC was controlled by MassLynx software (Waters, Manchester, UK). UV detection was performed at 214 nm. Eluent A was water containing 0.05% TFA. Eluent B was a 1:9 mixture of water : acetonitrile containing 0.045% TFA. A gradient from 20% B to 90% B was run in 12 minutes. The flow rate was typically 80 μ l/min. The total flow from the LC system was introduced into the UV detection cell prior to introduction in the ESI source. The HPLC system was controlled and the signal from the UV detector was processed using MassLynx™ software (Waters, Manchester, UK). Mass spectrometry was carried out using a Q-ToF (Waters, Manchester, UK) quadrupole time-of-flight hybrid tandem mass spectrometer equipped with a Waters Z-type electrospray ionization source. Acquisition mass range was typically m/z 500-2000. Data were recorded and processed using OpenLynx™ software and MaxEnt option (Waters, Manchester, UK). Calibration of the 500-2000 m/z scale was achieved by using the multiple-charged ion peaks of horse heart myoglobin (MW 16951.5 Da)

Trypsin digested protein fragments were analyzed using Vydak C18 reversed phase column (1 mm X 150 mm) with acetonitrile : water (1:9) isocratic mobile phase (80 μ l/min) containing 0.05% trifluoroacetic acid at 40 °C. Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD).

Gel Analyses

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished on a Mini-Protean apparatus (Bio-Rad, USA) with 12% gradient polyacrylamide gels under reducing conditions following the general protocol of Laemmli.¹⁵⁵ All electrophoresis protein samples were mixed with SDS loading buffer in the presence of dithiothreitol (DTT) and heated to 100 °C for 10 min to ensure reduction of disulfide bonds and complete denaturation of protein. Commercially available markers (Bio-Rad, USA) were applied to at least one lane of each gel for calculation of apparent molecular weights. Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, USA). Biotinylated proteins were detected with Avidin-Alkaline Phosphatase Conjugate (Avidin-AP) using Western Blotting Detection System (GE Healthcare, USA).

Nomenclature

Systematic nomenclature for the compounds would follow the numbering system as defined by IUPAC. Compounds were named with assistance from ISIS draw software.

¹⁵⁵ Laemmli, U. K. *Nature* **1970**, 227, 680.

CHAPTER 2

Synthesis of Dipeptide 5.

(S)-2-((S)-2-tert-Butoxycarbonylamino-3-hydroxy-propionylamino)-propionic acid methyl ester (7). To a solution of Boc-L-serine (513 mg, 2.5 mmol, 1.0 equiv.) in 30 mL CH₂Cl₂ was added L-alanine-methyl ester hydrochloride (419 mg, 3 mmol, 1.2 equiv.) and stirred. To this stirring solution was added Et₃N (0.52 mL, 3.75 mmol, 1.5 equiv.) followed by *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate HATU (1140 mg, 3.0 mmol, 1.2 equiv.). The reaction was stirred overnight under nitrogen gas. Solvent was evaporated and the residue was dissolved in EtOAc (30 mL). The organic phase was washed with 10% citric acid (2 x 10 mL) and sat. NaHCO₃ (2 x 20 mL) and then dried over Na₂SO₄, filtered, and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane – ethyl acetate, gradient, 0 to 70%) to afford the product **7** as a pale transparent gum (0.69 g, 95% yield). *R*_f = 0.05 (hexane – ethyl acetate, 1:1). ¹H NMR (400 MHz, CD₃OD) δ 1.41 (d, *J* = 7.3 Hz, 3H, *J* = 7.3 Hz), 1.47 (s, 9H), 3.73 (s, 3H), 3.76-3.79 (m, 2H), 4.17 (brs, 1H), 4.45-4.50 (m, 1H) ppm. ¹³C NMR (100 MHz, CD₃OD) δ 28.3, 37.7, 52.5, 53.4, 55.0, 62.9, 80.5, 127.3, 128.7, 129.2, 135.7, 155.9, 171.1, 171.8 ppm. IR (neat, cm⁻¹): ν 3400, 3220, 2978, 1745, 1630, 1500, 1430, 1355, 1156. LRMS (ESI) *m/z* calculated for C₁₂H₂₃N₂O₆ [M+H]⁺ 291.51 found 291.88. HRMS (ESI) *m/z* calculated for C₁₂H₂₃N₂O₆ [M+H]⁺ 291.1556, found 291.1559. [α]_D²⁰ -36.9 (c = 1.0 g/100 mL, MeOH).

(S)-2-((S)-2-Amino-3-hydroxy-propionylamino)-propionic acid methyl ester (5).

To a solution of **7** (690 mg, 2.37 mmol) in dichloromethane (18 mL) at 0 °C was added trifluoroacetic acid (2 mL) to give a 10% solution. The reaction was stirred overnight at room temperature. After removing the solvent in rotary evaporator, the product was purified by reversed phase HPLC. **5** (405 mg, 90%) was obtained as white solid after freeze drying: ^1H NMR (400 MHz, CD_3OD) δ 1.43 (d, $J = 7.3$ Hz, 3H), 3.74 (s, 3H), 3.80-3.84 (m, 1H), 3.97-4.0 (m, 2H), 4.49-4.53 (m, 1H) ppm. ^{13}C NMR (75 MHz, CD_3OD) δ 15.9, 51.5, 54.8, 60.3, 166.6, 172.8 ppm. IR (neat, cm^{-1}): ν 3406, 3018, 2700, 1664, 1521, 1498, 1215, 775. LRMS (ESI) m/z calculated for $\text{C}_7\text{H}_{15}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 191.10 found 191.99. HRMS (ESI) m/z calculated for $\text{C}_7\text{H}_{15}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 191.1032 found 190.1129. $[\alpha]_D^{20}$ -34.5 ($c = 0.58$ g/100 mL, MeOH).

Syntheses of Tetrapeptide 6.

(S)-2-((S)-2-tert-Butoxycarbonylamino-3-hydroxy-propionylamino)-3-phenyl-propionic acid methyl ester (8). To a solution of Boc-L-serine (513 mg, 2.5 mmol, 1.0 equiv.) in 30 mL CH_2Cl_2 was added L-phenylalanine methyl ester hydrochloride (650 mg, 3 mmol, 1.2 equiv.) and stirred. To this stirring solution was added Et_3N (0.52 mL, 3.75 mmol, 1.5 equiv.) followed by *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate HATU (1140 mg, 3.0 mmol, 1.2 equiv.).

The reaction was stirred overnight under nitrogen gas. Solvent was evaporated and the residue was dissolved in EtOAc (30 mL). The organic phase was washed with 10% citric acid (2 x 10 mL) and saturated NaHCO₃ (2 x 20 mL) and then dried over Na₂SO₄, filtered, and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane – ethyl acetate, gradient, 0 to 70%) to afford the product **8** as a pale transparent gum (0.83 g, 90% yield). R_f = 0.17 (hexane – ethyl acetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.45 (s, 9H), 3.07 (dd, 1H, J = 6.9, 13.9 Hz), 3.20 (dd, 1H, J = 5.5, 13.9 Hz), 3.59-3.63 (m, 1H), 3.75 (s, 3H), 4.02 (d, 1H, J = 10.2 Hz), 4.11-4.16 (m, 1H), 4.84-4.89 (m, 1H), 5.46-5.48 (m, 1H), 6.99 (brs, 1H), 7.13-7.15 (m, 2H) 7.26-7.33 (m, 3H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 28.3, 37.7, 52.5, 53.4, 55.0, 62.9, 80.5, 127.3, 128.7, 129.2, 135.7, 155.9, 171.1, 171.8 ppm. IR (neat, cm⁻¹): ν 3400, 3321, 3086, 3062, 2978, 1745, 1693, 1645, 1519, 1487, 1444, 1367, 1166. LRMS (ESI) m/z calculated for C₁₈H₂₇N₂O₆ [M+H]⁺ 367.19 found 367.34. HRMS (ESI) m/z calculated for C₁₈H₂₇ N₂O₆ [M+H]⁺ 367.1864 found 367.1854. $[\alpha]_D^{20}$ -13.8 (c = 0.65 g/100 mL, CH₂Cl₂).

(S)-2-((S)-2-tert-Butoxycarbonylamino-3-hydroxy-propionylamino)-3-phenylpropionic acid (9). To a stirred solution of **8** (0.64 g, 1.75 mmol) in THF (10 mL) and water (10 mL) was added 3 N LiOH solution (1 mL) at 0 °C. The reaction mixture was stirred for 18 hours. Then the solvent was evaporated and the residue was acidified to (pH 4.0) with 1 N HCl at 0 °C. The aqueous phase was extracted with EtOAc (3 x 20 mL). Combined organic phase was dried over Na₂SO₄. After filtration and solvent evaporation the desired compound **9** was obtained as a pale oil (0.61 g,

quantitative yield). ^1H NMR (400 MHz, CDCl_3) δ 1.45 (s, 9H), 3.03 (dd, 1H, $J = 7.3$, 13.9 Hz), 3.22 (d, 1H, $J = 4.7$, 13.9 Hz), 3.61 (dd, 1H, $J = 5.9$, 10.9 Hz), 3.86-3.88 (m, 1H), 4.24 (brs, 1H), 4.81-4.85 (m, 1H), 5.68-5.70 (m, 1H), 7.18-7.33 (m, 7H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 28.3, 37.3, 53.6, 55.2, 63.0, 80.7, 127.1, 128.6, 129.3, 135.9, 156.0, 171.3, 171.4, 174.5, 176.4 ppm. IR (neat, cm^{-1}): ν 3400, 3086, 3062, 2978, 1730, 1714, 1651, 1643, 1633, 1519, 1392, 1165. LRMS (ESI) m/z calculated for $\text{C}_{17}\text{H}_{25}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$ 353.16 found 353.37. HRMS (ESI) m/z calculated for $\text{C}_{17}\text{H}_{25}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$ 353.1707 found 353.1710. $[\alpha]_D^{20}$ -59.0 ($c = 0.66$ g/100 mL, CH_2Cl_2).

(S)-2-[(S)-2-((S)-2-tert-Butoxycarbonylamino-3-hydroxy-propionylamino)-3-phenyl-propionyl amino]-4-methyl-pentanoic acid methyl ester (10). To a solution of **9** (710 mg, 2.0 mmol, 1.0 equiv.) in 20 mL CH_2Cl_2 was added L-Leucine-methyl ester hydrochloride (436 mg, 2.4 mmol, 1.2 equiv.) and stirred. To this stirring solution was added Et_3N (0.42 mL, 3.0 mmol, 1.5 equiv.) followed by HATU (913 mg, 2.4 mmol, 1.2 equiv.). The reaction was stirred overnight under nitrogen gas. Solvent was evaporated and the residue was dissolved in EtOAc (30 mL). The organic phase was washed with 10% citric acid (2 x 10 mL) and saturated NaHCO_3 (2 x 20 mL) and then dried over Na_2SO_4 , filtered, and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane – ethyl acetate, gradient, 0 to 70%) to afford the product **10** as a pale transparent gum (0.77 g, 80% yield). $R_f = 0.20$ (hexane – ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 0.89-

0.92 (m, 6H), 1.43-1.58 (m, 12H), 3.12-3.14 (m, 2H), 3.35 (bs, 1H), 3.60-3.65 (m, 1H), 3.71 (s, 3H), 3.93-3.96 (m, 1H), 4.17 (brs, 1H), 4.54-4.61 (m, 1H), 4.72-4.77 (m, 1H), 5.46-5.56 (m, 1H), 6.70-6.72 (m, 1H), 6.70-6.95 (m, 1H), 7.21-7.32 (m, 5H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 21.8, 22.7, 24.7, 28.3, 37.5, 41.3, 50.9, 52.3, 54.5, 55.5, 63.0, 80.5, 127.1, 128.7, 129.3, 136.2, 155.8, 170.5, 171.1, 173.1 ppm. IR (neat, cm^{-1}): ν 3307, 3290, 3064, 3030, 2956, 1743, 1716, 1653, 1606, 1489, 1456, 1436, 1161. LRMS (ESI) m/z calculated for $\text{C}_{24}\text{H}_{38}\text{N}_3\text{O}_7$ $[\text{M}+\text{H}]^+$ 480.27 found 480.44. HRMS (ESI) m/z calculated for $\text{C}_{24}\text{H}_{38}\text{N}_3\text{O}_7$ $[\text{M}+\text{H}]^+$ 480.2704 found 480.2693. $[\alpha]_D^{20}$ -68 ($c = 0.84$ g/100 mL, MeOH).

(S)-2-[(S)-2-((S)-2-tert-Butoxycarbonylamino-3-hydroxy-propionylamino)-3-phenylpropionylamino]-4-methyl-pentanoic acid (11). To a stirred solution of **10** (370 mg, 0.77 mmol) in THF (5 mL) and water (5 mL) was added 3 N LiOH solution (0.5 mL) at 0 °C. The reaction mixture was stirred for 18 hours. Then the solvent was evaporated and the residue was acidified to (pH 4.0) with 1 N HCl at 0 °C. The aqueous phase was extracted with EtOAc (3 x 10 mL). Combined organic phase was dried over Na_2SO_4 . After filtration and solvent evaporation the desired compound **11** was obtained as a white foam (355 mg, quantitative yield). ^1H NMR (400 MHz, CDCl_3) δ 0.88-0.89 (m, 6H), 1.22-1.75 (m, 12H), 3.11-3.17 (m, 2H), 3.67 (brs, 1H), 3.84-3.89 (m, 1H), 4.11-4.18 (m, 1H), 4.45-4.56 (m, 2H), 4.74 (bs, 1H), 5.83-5.86 (m, 0.6H), 6.55-6.57 (m, 0.4H), 7.18-7.28 (m, 8H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 22.0, 24.4, 27.3, 37.5, 40.2, 50.7, 54.2, 57.0, 61.9, 79.5, 126.3, 128.0, 128.1, 129.0,

136.9, 156.3, 171.7, 171.9, 174.2 ppm. IR (neat, cm^{-1}): ν 3390, 3285, 3060, 3035, 2950, 1740, 1718, 1653, 1606, 1485, 1459, 1434, 1159. LRMS (ESI) m/z calculated for $\text{C}_{23}\text{H}_{36}\text{N}_3\text{O}_7$ $[\text{M}+\text{H}]^+$ 466.25 found 466.90. HRMS (ESI+) m/z calculated for $\text{C}_{23}\text{H}_{36}\text{N}_3\text{O}_7$ $[\text{M}+\text{H}]^+$ 466.2553 found: 466.2538. $[\alpha]_D^{20}$ -59 ($c = 1.65$ g/100 mL, MeOH).

(S)-2-[(S)-2-[(S)-2-((S)-2-tert-Butoxycarbonylamino-3-hydroxy-propionylamino)-3-phenyl-propionylamino]-4-methyl-pentanoylamino}-pentanedioic acid dimethyl ester (12). To a solution of **11** (350 mg, 0.75 mmol, 1.0 equiv.) in 15 mL CH_2Cl_2 was added L-glutamic acid dimethyl ester hydrochloride (190.5 mg, 0.9 mmol, 1.2 equiv.) and stirred. To this stirring solution was added Et_3N (0.16 mL, 1.2 mmol, 1.5 equiv.) followed by HATU (342 mg, 0.9 mmol, 1.2 equiv.). The reaction was stirred overnight under nitrogen gas. Solvent was evaporated and the residue was dissolved in EtOAc (20 mL). The organic phase was washed with 10% citric acid (2 x 5 mL) and saturated NaHCO_3 (2 x 10 mL) and then dried over Na_2SO_4 , filtered, and concentrated in vacuo. The residual crude product was purified by flash column chromatography (dichloromethane – methanol, gradient, 0 to 4%) to afford the product **12** as a transparent gum (350 mg, 75% yield). $R_f = 0.20$ TLC (dichloromethane – methanol, 9:1). ^1H NMR (300 MHz, CDCl_3) δ 0.85-0.86 (m, 6H), 1.36-1.42 (m, 12H), 1.95-2.05 (m, 1H), 2.16-2.25 (m, 1H), 2.37-2.42 (m, 2H), 2.56 (bs, 1H), 3.07-3.15 (m, 2H), 3.63, 3.67, 3.73 (m, 7H), 3.90 (bs, 1H), 4.10-4.18 (m, 1H), 4.51-4.53 (m, 2H), 4.73-4.77 (m, 1H), 5.70 (m, 0.6H), 7.15-7.50 (m, 8H) ppm. ^{13}C NMR (300 MHz, CDCl_3) 21.7, 22.8, 24.5, 26.7, 28.3, 36.9, 41.2, 51.8, 52.4, 54.8,

55.5, 63.2, 80.3, 127.2, 128.7, 129.1, 136.0, 156.2, 170.9, 171.2, 171.9, 172.6, 173.3 ppm. IR (neat, cm^{-1}): ν 3406, 3018, 2400, 1735, 1664, 1521, 1498, 1215, 775. LRMS (ESI) m/z calculated for $\text{C}_{30}\text{H}_{47}\text{N}_4\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 623.33 found 623.02. HRMS (ESI) m/z calculated for $\text{C}_{30}\text{H}_{47}\text{N}_4\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 623.3292, found 623.3292. $[\alpha]_D^{20}$ -31 ($c = 1.13$ g/100 mL, MeOH).

(S)-2-[(S)-2-[(S)-2-((S)-2-Amino-3-hydroxy-propionylamino)-3-phenyl-propionylamino]-4-methyl-pentanoylamino]-pentanedioic acid dimethyl ester

(6). To a solution of **12** (350 mg, 0.56 mmol) in dichloromethane (8 mL) at 0 °C was added trifluoroacetic acid (2 mL) to give a 20% solution. The reaction was stirred overnight at room temperature. After removing the solvent in rotary evaporator, the product was purified by reversed phase HPLC. **6** (175 mg, 60%) was obtained as white solid after freeze drying: ^1H NMR (400 MHz, CD_3OD) δ 0.94 (d, $J = 6.2$ Hz, 3H), 0.98 (d, $J = 6.2$ Hz, 3H), 1.56-1.70 (m, 3H), 1.93-2.02 (m, 1H), 2.16-2.24 (m, 1H), 2.25-2.48 (m, 2H), 2.91-2.97 (m, 1H), 3.19-3.28 (m, 1H), 3.68 (s, 4H), 3.74 (s, 4H), 4.41-4.50 (m, 2H), 4.68-4.72 (m, 1H), 7.20-7.31 (m, 5H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 20.7, 21.9, 24.3, 26.1, 29.5, 37.0, 40.3, 50.8, 51.4, 51.5, 51.7, 54.7, 55.3, 62.3, 126.4, 128.1, 128.8, 128.9, 136.8, 171.9, 173.2, 173.3 ppm. IR (neat, cm^{-1}): ν 3406, 3018, 2400, 1735, 1664, 1521, 1498, 1215, 775. LRMS (ESI) m/z calculated for $\text{C}_{25}\text{H}_{39}\text{N}_4\text{O}_8$ $[\text{M}+\text{H}]^+$ 523.27 found 523.14. HRMS (ESI) m/z calculated for $\text{C}_{25}\text{H}_{39}\text{N}_4\text{O}_8$ $[\text{M}+\text{H}]^+$ 523.2768 found: 523.2775. $[\alpha]_D^{20}$ -67 ($c = 0.57$ g/100 mL, MeOH).

Representative experimental procedure for periodate oxidation of peptides

(S)-2-(2-Oxo-acetyl-amino)-propionic acid methyl ester/ (S)-2-(2,2-Dihydroxy-acetyl-amino)-propionic acid methyl ester (13). To a solution of peptide **5** (0.12 g, 0.64 mmol) in 25 mM sodium phosphate buffer (pH 7.0) (0.2 mL) was added a solution of sodium metaperiodate (0.27 g, 1.28 mmol) in the same buffer (0.8 mL) and stirred in the dark till the HPLC-ESI-MS analysis revealed complete conversion of starting material to product. After the completion of reaction, excess periodate was neutralized by sodium sulfite (0.8 g, 0.64 mmol) solution in the same buffer (0.4 mL) till (pH 7.0). The neutralized reaction was loaded onto a solid phase extraction cartridge (OASIS HLB, Waters) equilibrated with water and after an initial wash with water eluted with methanol. After evaporating the solvent in vacuo the product aldehyde was freeze dried to yield **13** as a transparent gum (100 mg, quantitative) and carried forward to next step. $R_f = 0.5$ (dichloromethane – methanol, 93:7). ^1H NMR (400 MHz, CD_3OD) δ 1.42 (d, 3H, $J = 7.7$ Hz), 3.74 (s, 3H), 4.44-4.50 (m, 1H) ppm. ^{13}C NMR (75 MHz, CD_3OD) δ 16.0, 48.0, 51.4, 93.6, 170.1, 172.8 ppm. IR (neat, cm^{-1}): ν 1725, 1688, 1437 cm^{-1} . LRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{10}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 160.05 found 160.94. HRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{10}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 161.0610 found 161.0613. $[\alpha]_D^{20} -6.0$ ($c = 1.1$ g/100 mL, CH_2Cl_2).

Representative experimental procedure for aza *Diels-Alder* reaction.

(S)-2-[(4-Oxo-1-phenyl-1,2,3,4-tetrahydro-pyridine-2-carbonyl)-aminol]propionic acid methyl ester (14**).** To a suspension of InCl₃ (5.5 mg, 0.025 mmol) and MgSO₄ (0.194 g, 1.61 mmol) in acetonitrile (0.25 mL) was added aldehyde **13** (0.04 g, 0.25 mmol) and aniline (23.3 μ L, 0.023 g, 0.25 mmol) in acetonitrile (0.75 mL) followed by 2-trimethoxysiloxy-4-methoxy-1,3-butadiene (119 μ L, 0.011 g, 0.625 mmol) at 0 °C and allowed to warm up to room temperature over 20 hours. Acetonitrile was evaporated before adding water (5 mL) and ethylacetate (10 mL). After filtration, the aqueous phase was extracted with ethylacetate (3 x 10 mL), the combined organic phase dried over Na₂SO₄, filtered and then solvent evaporated in vacuo. The crude product was purified by flash column chromatography (ethyl acetate – methanol, 9:1) to afford the hetero-diels alder product **14** (0.035 g, 47%) as yellow oil. The diastereoselectivity was found to be in the ratio of 54:46. R_f = 0.12 (hexane – ethyl acetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.36 (dd, 3H, J = 2.8, 7.1 Hz), 2.99-3.07 (m, 2H), 3.71 (s, 3H), 4.54-4.59 (m, 1H), 4.65-4.70 (m, 1H), 5.31 (dd, 1H, J = 3.2, 7.8 Hz), 6.85 – 6.95 (m, 1 H), 7.09-7.22 (m, 4H), 7.38-7.43 (m, 2H), 7.54-7.58 (m, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 17.9, 18.0, 38.2, 38.4, 48.4, 48.5, 52.5, 52.6, 61.6, 61.8, 103.7, 103.8, 118.3, 118.7, 124.9, 125.2, 144.1, 144.2, 147.3, 147.4, 168.6, 168.7, 172.9, 189.6, 189.7 ppm. IR (neat, cm⁻¹): ν 2092, 1734, 1656, 1601, 1568, 1456, 1436, 1343, 1327, 1302, 1279, 1218, 1158, 893, 759,

732, 694. LRMS (ESI) m/z calculated for $C_{16}H_{18}N_2O_4$ $[M+H]^+$ 303.13 found 303.03.

HRMS (EI, m/z) calculated for $C_{16}H_{18}N_2O_4$ $[M+H]^+$ 303.1345 found 303.1345.

(S)-2-[[1-(4-Methoxy-phenyl)-4-oxo-1,2,3,4-tetrahydro-pyridine-2-carbonyl]-amino]-propionic acid methyl ester. (0.034 mg, 50%) as brown gum. The diastereoselectivity was found to be in the ratio of 61:39. R_f = 0.2 (hexane : ethyl acetate, 1:1); 1H NMR (400 MHz, $CDCl_3$) δ 1.37 (dd, 3H, J = 2.7, 7.1 Hz), 2.94-3.09 (m, 2H), 3.74 (s, 3H), 3.82 (s, 3H), 4.54-4.62 (m, 2H), 5.28 (dd, 1H, J = 5.4, 7.6 Hz), 6.74 – 6.81 (m, 1 H), 6.92-6.95 (m, 2H), 7.06-7.09 (m, 1H), 7.14-7.16 (m, 1H), 7.45-7.49 (m, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.0, 18.2, 38.0, 38.2, 48.3, 48.4, 52.6, 52.7, 62.3, 62.5, 102.8, 102.9, 115.0, 120.5, 121.0, 137.7, 137.8, 148.0, 148.1, 157.3, 157.4, 168.7, 168.7, 172.9, 173.0, 189.3, 189.4 ppm. IR (neat, cm^{-1}): ν 2958, 2919, 2836, 1740, 1685, 1570, 1513, 1456, 1410, 1336, 1288, 1247, 1216, 1181, 1029, 836. LRMS (ESI) m/z calculated for $C_{17}H_{20}N_2O_5$ $[M+H]^+$ 333.35 found 333.05. HRMS (EI, m/z) calculated for $C_{17}H_{20}N_2O_5$ $[M+H]^+$ 333.1450, found 333.1351.

(S)-2-[(1-Cyclohexyl-4-oxo-1,2,3,4-tetrahydro-pyridine-2-carbonyl)-amino]-propionic acid methyl ester. (0.030 g, 23%) as pale brown oil. The diastereoselectivity was found to be in the ratio of 53:47. R_f = 0.1 (hexane - ethyl acetate, 1:1); 1H NMR (400 MHz, $CDCl_3$) δ 1.30-1.46 (m, 7H), 1.60-1.95 (m, 6H), 2.81-2.83 (m, 2H), 3.02-3.15 (m, 2H), 3.75 (s, 3H), 4.14-4.19 (m, 1H), 4.48-4.61 (m,

1H), 4.93-5.06 (m, 1H), 6.78-6.85 (m, 1H), 7.11-7.24 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 18.2, 18.3, 25.0, 25.5, 25.5, 25.6, 31.6, 32.1, 32.2, 32.4, 38.3, 48.1, 48.4, 52.6, 52.7, 58.9, 59.0, 64.1, 64.2, 64.4, 99.3, 99.4, 149.6, 149.8, 169.4, 169.7, 173.2, 173.3, 189.2, 189.3 ppm. IR (neat, cm^{-1}): ν 2933, 2856, 1740, 1670, 1623, 1564, 1456, 1405, 1345, 1299, 1227, 1158. LRMS (ESI) m/z calculated for $\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 309.37 found 309.14. HRMS (EI, m/z) calculated for $\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 309.1814 found 309.1814.

(S)-2-[[4-Oxo-1-((R)-1-phenyl-ethyl)-1,2,3,4-tetrahydro-pyridine-2-carbonyl]-amino]-propionic acid methyl ester. (0.043 g, 26%) as pale brown gum. The diastereoselectivity was found to be in the ratio of 60:40. R_f = 0.15 (hexane - ethyl acetate, 1:1); ^1H NMR (400 MHz, CDCl_3) δ 1.32-1.37 (m, 3H), 1.65-1.69 (m, 3H), 2.62-2.77 (m, 2H), 3.74, 3.75 (s, 3H), 4.17 (ddd, 1H, J = 3.2, 7.2, 62.0 Hz), 4.43-4.72 (m, 2H), 4.48-4.61 (m, 1H), 5.04 (dd, 1H, J = 7.7, 37.5 Hz), 6.80-6.98 (m, 1H), 7.17 (d, 0.5H, J = 7.6 Hz), 7.25-7.42 (m, 5H), 7.50 (d, 0.5H, J = 7.7 Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 17.9, 18.0, 18.1, 21.1, 38.1, 48.3, 48.4, 52.6, 52.7, 58.1, 59.8, 62.5, 62.7, 99.3, 99.7, 126.3, 127.2, 128.5, 128.7, 129.1, 129.2, 139.4, 141.2, 148.9, 151.3, 168.9, 169.5, 173.1, 173.2, 189.2, 189.5 ppm. IR (neat, cm^{-1}): ν 2977, 1738, 1639, 1566, 1449, 1373, 1349, 1296, 1278, 1206, 1156, 1134, 1065, 1027, 973, 757, 696, 609, 541. LRMS (ESI) m/z calculated for $\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 331.38 found 331.02. HRMS (ESI, m/z) calculated for $\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 331.1658 found 331.1658.

(S)-2-((E)-6-Methoxy-4-oxo-2-phenylamino-hex-5-enoylamino)-propionic acid methyl ester (15). A suspension of InCl_3 (5.5 mg, 0.025 mmol) and aldehyde **13** (40 mg, 0.25 mmol) were mixed and stirred at room temperature in water (0.75 mL) for 10 minutes before the addition of aniline (23.3 μL , 22.8 mg, 0.25 mmol). The resulting mixture was stirred at room for another 30 minutes. 2-trimethylsiloxy-4-methoxy-1,3-butadiene (119 μL , 108 mg, 0.625 mmol) was then added in acetonitrile (0.75 mL) and stirred the reaction for 20 h at room temperature. Acetonitrile was evaporated before adding water (5 mL) and ethyl acetate (10 mL). The suspension was filtered and the filtrate was extracted with ethyl acetate (3 x 10 mL), the combined organic phase dried over sodium sulfate, filtered and then solvent evaporated in vacuo. The crude product was purified by flash chromatography (hexane - ethyl acetate, 3:7) to afford the Michael product **15** (10.9 mg, 13%) as yellow oil. R_f = 0.08 (hexane - ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.44 (d, 2H, J = 7.2 Hz), 1.51 (d, 1H, J = 7.2 Hz), 2.44 (d, 2H, J = 5.13 Hz), 3.69 (d, 3H, J = 6.8 Hz), 3.77 (d, 3H, J = 5.9 Hz), 4.49-4.64 (m, 1H), 4.69-4.74 (m, 1H), 5.35 (d, 1H, J = 1.83 Hz), 6.66-6.70 (m, 2H), 6.78-7.34 (m, 4H), 7.88 (dd, 1H, J = 3.3, 12.7 Hz), 9.43-9.50 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 17.6, 17.7, 18.1, 24.5, 24.6, 27.6, 27.7, 47.8, 48.2, 48.3, 52.3, 52.4, 52.5, 54.6, 54.7, 60.5, 61.0, 61.2, 107.2, 110.5, 110.6, 113.5, 113.8, 116.1, 116.2, 116.7, 116.8, 119.3, 119.5, 119.7, 123.5, 123.6, 123.9, 129.3, 129.4, 129.5, 129.6, 129.7, 129.8, , 140.0, 140.1, 144.0, 144.1, 144.2, 144.3, 146.0, 146.1, 146.5, 171.0, 171.2, 171.6, 171.7, 172.5, 172.7, 172.8, 173.4, 196.2, 196.4, 198.7 ppm. IR (neat, cm^{-1}): ν 1647, 1634, 1602, 1581, 1568,

1558, 1495, 1314, 1279, 1220, 801, 754, 732, 693, 665. LRMS (ESI) m/z calculated for $C_{17}H_{22}N_2O_5$ [M] 334.16 found 334.83. HRMS (ESI) m/z calculated for $C_{17}H_{22}N_2O_5$ [M+H]⁺ 335.1607 found 335.1607.

(S)-2-[(S)-4-Methyl-2-[(S)-2-(2-oxo-acetyl-amino)-3-phenyl-propionyl-amino]-pentanoyl-amino]-pentanedioic acid dimethyl ester/ (S)-2-[(S)-2-[(S)-2-(2,2-Dihydroxy-acetyl-amino)-3-phenyl-propionyl-amino]-4-methyl-pentanoyl-amino]-pentanedioic acid dimethyl ester pentanedioate. A mixture of the aldehyde and hydrated form. Obtained as yellow oil (0.030 g, quantitative). R_f = 0.25 (ethyl acetate – methanol, 4:1). ¹H NMR (400 MHz, CDCl₃) δ 0.87-0.91 (m, 6H), 1.43-1.48 (m, 2H), 1.48-1.67 (m, 1H), 1.97-2.04 (m, 1H), 2.18-2.23 (m, 1H), 2.35-2.47 (m, 2H), 3.04-3.15 (m, 2H), 3.67-3.68 (s, 3H), 3.71-3.76 (s, 3H), 4.42-4.49 (m, 1H), 4.53-4.60 (m, 1H), 4.72-4.83 (m, 1H), 6.69-6.93 (m, 2H), 7.18-7.29 (m, 6H), 9.24 (s, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 22.0, 22.2, 22.6, 22.7, 24.6, 27.0, 29.9, 30.0, 37.9, 38.0, 38.1, 40.8, 41.1, 41.2, 51.6, 51.9, 52.0, 52.5, 54.2, 54.3, 54.5, 54.7, 93.4, 93.6, 127.1, 127.2, 127.3, 128.6, 128.7, 128.8, 129.2, 129.3, 135.6, 135.9, 136.0, 159.7, 169.4, 169.6, 169.7, 170.4, 170.5, 171.4, 171.6, 171.7, 171.9, 172, 173.3, 173.6, 187.3 ppm. IR (neat, cm⁻¹): ν 3424, 2999, 1736, 1663, 1534, 1437, 1028 cm⁻¹. LRMS (ESI) m/z calculated for $C_{24}H_{34}N_3O_8$ [M+H]⁺ 492.23, found 492.67. HRMS (ESI) m/z calculated for $C_{24}H_{34}N_3O_8$ [M+H]⁺ 492.2346 found 492.2360. $[\alpha]_D^{20}$ -11.0 (c = 0.60 g/100 mL, CH₂Cl₂).

(S)-2-((S)-4-Methyl-2-((S)-2-[(4-oxo-1-phenyl-1,2,3,4-tetrahydro-pyridine-2-carbonyl)-amino]-3-phenyl-propionylamino)-pentanoylamino)-pentanedioic acid dimethyl ester. By the same procedure as the formation of **14**. Purified the crude with flash chromatography (hexane - ethyl acetate, gradient, 0 to 100%) to produce yellow oil (0.020 g, 57%). R_f = 0.17 (hexane – ethyl acetate, 1:1). ^1H NMR (400 MHz, CD_3OD) δ 0.73-0.94 (m, 6H), 1.31-1.72 (m, 4H), 1.88-2.00 (m, 1H), 2.00-2.23 (m, 2H), 2.03-2.40 (m, 2H), 2.92-3.17 (m, 2H), 3.54 (s, 1H), 3.63 (d, 2H), 3.72 (d, 3H), 4.29-4.37 (m, 1H), 4.44-4.53 (m, 1H), 4.53-4.77 (m, 2H), 5.03-5.07 (m, 1H), 6.57-6.62 (m, 1H), 6.66-6.85 (m, 1H), 6.89-6.99 (m, 2H), 7.09-7.41 (m, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 21.7, 22.0, 22.8, 23.0, 24.5, 24.6, 26.7, 26.9, 29.7, 30.0, 36.8, 39.9, 40.9, 51.5, 51.7, 51.8, 51.9, 52.4, 54.5, 54.7, 55.0, 109.5, 113.9, 114.2, 116.2, 116.3, 119.4, 123.8, 126.8, 127.0, 128.6, 128.7, 128.9, 129.1, 129.2, 129.4, 129.6, 129.7, 136.0, 136.3, 139.9, 144.4, 146.0, 170.8, 171.4, 171.8, 172.8, 173.2, 196.7 ppm. IR (neat, cm^{-1}): ν 3300, 2954, 2927, 1732, 1645, 1598, 1583, 1568, 1504, 1454, 1435, 1294, 1267, 1215, 1178, 752, 694, 663 cm^{-1} . LRMS (ESI) m/z calculated for $\text{C}_{34}\text{H}_{42}\text{N}_4\text{O}_8$ $[\text{M}+\text{H}]^+$ 635.31 found 634.91. HRMS (ESI) m/z calculated for $\text{C}_{34}\text{H}_{42}\text{N}_4\text{O}_8$ $[\text{M}+\text{H}]^+$ 635.31 found 634.91. $[\text{M}-\text{H}]^-$ 635.3081 found: 635.3081.

Representative experimental procedure for *Allylation* reaction.

(S)-2-(2-Hydroxy-pent-4-enoylamino)-propionic acid methyl ester (35). The peptide aldehyde **13** (0.1 g, 0.63 mmol) was dissolved in sodium phosphate buffer, pH 7.0, (3.0 mL) and indium powder (0.15 g, 1.26 mmol) was added to it and cooled to 0 °C followed by the addition of allyl bromide (0.16 mL, 1.89 mmol). The mixture was allowed to run at room temperature for 16 h. White suspension had formed during the reaction. The reaction was filtered through a filter paper to get rid of the unreacted metal. The aqueous suspension was extracted with diethyl ether (3 X 10 mL). The combined organic phase was dried over MgSO₄. After filtration and solvent evaporation, the crude was purified by flash chromatography using 0-70% ethyl acetate in hexane as the eluent.

Yield 61% (0.77 g). Transparent gum. ¹³C NMR analysis showed that the compound is in a diastereomeric ratio of 57:43. *R*_f = 0.17 (hexane - ethyl acetate, 1:1). ¹H NMR (CDCl₃, 300 MHz) δ 1.40 (dd, 3H, *J* = 3.0, 7.2 Hz), 2.35-2.48 (m, 1H), 2.57-2.65 (m, 1H), 3.73 (s, 3H), 4.13-4.18 (m, 1H), 4.52-4.62 (m, 1H), 5.12-5.20 (m, 2H), 5.73-5.88 (m, 1H), 7.20-7.23 (m, 1H) ppm. ¹³C NMR (CDCl₃, 75 MHz) δ 18.1, 18.3, 38.9, 39.0, 47.6, 52.4, 52.5, 70.7, 70.8, 119.0, 119.3, 133.2, 172.9, 173.0, 173.2, 173.4 ppm. IR (neat, cm⁻¹): ν 3077, 2955, 1770, 1455, 1301, 1259, 1225, 1159. LCMS calculated for C₉H₁₅NO₄ [M+H]⁺ 202.10 found 202.4. HRMS calculated for C₉H₁₅NO₄ [M+H]⁺ 202.1079 found 202.1079.

(S)-2-(2-Hydroxy-3-phenyl-pent-4-enoylamino)-propionic acid methyl ester.

Yield 58% (0.10 g). White powder. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 4:6:32:58. $R_f = 0.28$ (hexane – ethyl acetate, 1:1). ^1H NMR (CDCl_3 , 300 MHz) δ 1.06 (d, 1H, $J = 7.0$ Hz), 1.36 (dd, 2H, $J = 7.0, 6.0$ Hz), 3.66, 3.69, 3.74 (s, 3H), 3.88-3.94 (m, 1H), 4.36-4.49 (m, 2H), 5.17-5.25 (m, 2H), 6.15-6.27 (m, 1H), 6.80-6.87 (m, 1H), 7.21-7.35 (m, 5H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz) δ 18.1, 18.3, 18.4, 18.5, 47.5, 47.9, 52.6, 52.6, 52.7, 52.7, 53.0, 53.1, 53.2, 74.9, 75.0, 75.5, 75.7, 117.2, 117.5, 118.7, 118.9, 127.0, 128.3, 128.4, 128.5, 128.6, 128.7, 128.8, 129.1, 129.5, 135.1, 137.5, 137.9, 138.5, 138.6, 140.8, 171.7, 171.9, 172.0, 172.0, 172.9, 173.2, 173.5 ppm. IR (neat, cm^{-1}): ν 3395, 3331, 2341, 2318, 1740, 1720, 172.9, 173.2, 173.5 ppm. IR (neat, cm^{-1}): ν 3395, 3331, 2341, 2318, 1740, 1529, 1493, 1451, 1381, 1340, 1303, 1210, 1157, 1130, 1057, 999, 976, 847, 749, 722, 700, 666, 591, 553, 490, 417. LCMS calculated for $\text{C}_{15}\text{H}_{19}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 278.32 found 278.30. HRMS calculated for $\text{C}_{15}\text{H}_{19}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 278.1392 found 278.1392.

2-[Hydroxy-((S)-1-methoxycarbonyl-ethylcarbamoyl)-methyl]-but-3-enoic acid methyl ester.

Yield 34% (0.56 g). Yellow oil. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 48:28:13:11. $R_f = 0.23$ (hexane – ethyl acetate, 1:1). ^1H NMR (CDCl_3 , 300 MHz) δ 1.39 (dd, 3H, $J = 7.5, 12.6$ Hz), δ 3.71 (s, 1.9H), δ 3.72 (s, 1.7H), 3.73 (s, 3H), 4.17-4.20 (m, 0.7H), 4.29-4.32 (m, 0.7H), 4.53-4.59 (m, 1.1H), 5.25-5.31 (m, 2H), 5.80-6.0 (m, 0.93H), 7.35 (s, 1H) ppm. ^{13}C NMR (CDCl_3 , 50 MHz) δ 18.0, 18.1, 18.3, 47.6, 47.8, 47.9, 52.0, 52.2, 52.3, 52.4, 52.5,

71.9, 72.0, 72.5, 72.6, 120.0, 120.1, 120.9, 129.6, 129.7, 131.4, 170.3, 171.3, 171.4, 172.9, 173.1, 173.6 ppm. IR (neat, cm^{-1}): ν 2956, 1734, 1663, 1533, 1457, 1437, 1217, 1162, 1101, 1061, 996, 931. LCMS calculated for $\text{C}_{11}\text{H}_{17}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 260.26 found 260.92. HRMS calculated for $\text{C}_{11}\text{H}_{17}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 260.1134 found 260.1134.

(S)-2-(2-Hydroxy-3,3-dimethyl-pent-4-enoylamino)-propionic acid methyl ester.

Yield 45% (0.65 g). Transparent gum. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 50:50. $R_f = 0.41$ (hexane – ethyl acetate, 1:1). ^1H NMR (CDCl_3 , 300 MHz) δ 1.09 (s, 6H), 1.40 (dd, 3H, $J = 4.0, 7.1$ Hz), 3.20-3.22 (m, 0.4H), 3.47 (brs, 0.4H), 3.74, 3.80 (s, 3H), 3.81 (s, 1H), 4.54-4.58 (m, 1H), 5.09-5.18 (m, 2H), δ 5.87-5.95 (m, 1H), 6.79-6.90 (m, 1H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz) δ 18.2, 18.3, 22.3, 22.4, 22.5, 22.7, 41.4, 41.6, 47.8, 52.4, 52.5, 76.7, 77.0, 114.2, 114.4, 144.5, 144.7, 171.4, 171.7, 173.1, 173.3 ppm. IR (neat, cm^{-1}): ν 2968, 1742, 1668, 1520, 1455, 1266, 1217, 1159, 738, 704 cm^{-1} . LCMS calculated for $\text{C}_{11}\text{H}_{19}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 230.27 found 230.99. HRMS calculated for $\text{C}_{11}\text{H}_{19}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 230.1392 found 230.1392.

(S)-2-(2-Hydroxy-3-methyl-pent-4-enoylamino)-propionic acid methyl ester.

Yield 50% (0.68 g). Transparent gum. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 23:24:25:28. $R_f = 0.35$ (hexane – ethyl acetate, 1:1). ^1H NMR (CDCl_3 , 300 MHz) δ 0.97 (dd, 1.5H, $J = 7.0, 10.4$ Hz), 1.14 (dd, $J = 0.8, 7.0$ Hz, 1.4H), 1.39-1.44 (m, 3H), 2.75-2.82 (m, 1H), 3.0-3.5 (m, 1H), 3.74-3.75 (m, 3H), 4.02-4.14 (m, 1H), 4.55-4.63 (m, 1H), 5.09-5.18 (m, 2H), 5.73-5.96 (m, 1H), 7.00-

7.28 (m, 1H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz) δ 11.7, 11.9, 15.4, 15.8, 18.1, 18.2, 18.3, 18.4, 40.6, 40.7, 41.2, 41.3, 47.6, 47.7, 47.7, 52.4, 52.5, 52.5, 52.5, 74.2, 74.3, 74.9, 75.1, 115.8, 116.0, 116.7, 117.0, 137.6, 137.7, 140.0, 140.1, 172.2, 172.3, 172.5, 172.6, 173.0, 173.1, 173.3, 173.4 ppm. IR (neat, cm^{-1}): ν 3059, 2985, 1745, 1662, 1524, 1450, 1440, 1423, 1395, 1343, 1264, 1217, 1155, 1058, 1017, 924, 736. LCMS calculated for $\text{C}_{10}\text{H}_{17}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 216.25 found 216.9. HRMS calculated for $\text{C}_{10}\text{H}_{17}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 216.1236 found 216.1236.

4-Hydroxy-4-((S)-1-methoxycarbonyl-ethylcarbamoyl)-2-methylene-butylric acid methyl ester. Yield 19% (0.31 g). Yellow oil. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 65:35. R_f = 0.23 (hexane – ethyl acetate, 1:1). ^1H NMR (CDCl_3 , 300 MHz) δ 1.41 (dd, 3H, J = 7.2, 11.8 Hz), 2.64-2.71 (m, 1H), 2.91-2.96 (m, 1H), 3.74 (s, 3.3H), 3.82 (s, 2.5H), 4.24-4.25 (m, 1H), 4.43 (bs, 0.4H), 4.54-4.60 (m, 1.4H), 5.81-5.82 (m, 1H), 6.28-6.29 (m, 1H), 7.30-7.4 (m, 1H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz) δ 18.2, 18.5, 37.3, 47.6, 47.7, 52.4, 52.5, 52.6, 52.7, 71.9, 72.2, 129.7, 130.0, 135.8, 135.9, 169.7, 169.9, 172.3, 173.0 ppm. IR (neat, cm^{-1}): ν 3150, 2955, 1722, 1658, 1652, 1527, 1454, 1439, 1339, 1312, 1213, 1155, 1091, 1060, 955, 735. LCMS calculated for $\text{C}_{11}\text{H}_{17}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 260.26 found 260.94. HRMS calculated for $\text{C}_{11}\text{H}_{17}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 260.1134 found 260.1134.

(S)-2-[(S)-2-[(S)-2-(2-Hydroxy-pent-4-enoylamino)-3-phenyl-propionylamino]-4-methyl-pentanoylamino]-pentanedioic acid dimethyl ester. Yield 72% (0.24 g). Yellow oil. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 65:35. $R_f = 0.475$ (dichloromethane – methanol, 93:7). ^1H NMR (400 MHz, CDCl_3) δ 0.88-0.92 (m, 7H), 1.25-1.27 (m, 3H), 1.46-1.72 (m, 4H), 1.95-2.61 (m, 7H), 3.01-3.19 (m, 2H), 3.68 (s, 3H), 3.75 (s, 3H), 4.12-4.14 (m, 1H), 4.39-4.47 (m, 1H), 4.52-4.59 (m, 1H), 4.66-4.77 (m, 1H), 5.07-5.16 (m, 2H), 5.52-5.78 (m, 1H), 6.74-6.83 (m, 1H), 7.18-7.32 (m, 7H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 22.0, 22.1, 22.7, 22.8, 24.6, 26.9, 27.0, 29.6, 30.0, 37.5, 37.7, 38.7, 38.9, 40.7, 40.8, 51.7, 51.9, 52.0, 52.1, 52.5, 53.4, 53.9, 54.4, 68.8, 70.8, 70.9, 119.2, 119.4, 127.0, 127.1, 128.7, 129.2, 132.9, 136.2, 170.8, 171.0, 171.9, 173.2, 173.3, 173.5, 173.6 ppm. IR (neat, cm^{-1}): ν 3700, 3273, 3081, 2957, 1738, 1635, 1539, 1436, 1265, 1211, 1174, 715. LCMS calculated for $\text{C}_{27}\text{H}_{39}\text{N}_3\text{O}_8$ $[\text{M}+\text{H}]^+$ 534.61 found 534.07. HRMS calculated for $\text{C}_{27}\text{H}_{39}\text{N}_3\text{O}_8$ $[\text{M}+\text{H}]^+$ 534.2815 found 534.2816.

Representative experimental procedure for the synthesis of allylic bromides 36, 37 and 38.

Scheme 2-26: Acrylate based bromide substrates for allylation

To a solution of 2-bromomethyl-acrylic acid (0.29 g, 1.77 mmol) in dry dichloromethane (5 mL) at 0 °C under N_2 was added thionyl chloride (0.4 mL, 5.34 mmol) and heated to reflux for 2h. After removing the excess thionyl chloride in vacuo the formed acyl chloride was redissolved in dry dichloromethane (10 mL) and cooled to 0 °C in an ice bath. To this stirring solution was added a solution of *N,N*-dimethylamino pyridine (0.02 g, 0.18 mmol) and respective amine component (1.6 mmol) in dry dichloromethane (10 mL). The reaction was stirred for 18 h while the temperature was allowed to rise to room temperature. Upon reaction completion the solvent was evaporated under reduced pressure. The residue was redissolved in ethyl acetate (50 mL) and washed successively with water (20 mL), sat. NaHCO_3 (20 mL) and brine (20 mL), dried over MgSO_4 , filtered and solvent evaporated in vacuo under reduced pressure. The crude product thus obtained was purified by flash silica gel column chromatography.

2-Bromomethyl-N-((R)-1-phenyl-ethyl)-acrylamide (36a). Yield 45% (0.19 g). R_f = 0.6 (hexane – ethyl acetate, 1:1). ^1H NMR (CDCl_3 , 300 MHz) δ 1.55 (d, 3H, J = 7.0 Hz), 4.25-4.35 (m, 2H), 5.13-5.23 (m, 1H), 5.66 (s, 1H), 5.83 (s, 1H), 7.25-7.35 (m, 6H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz) δ 21.8, 43.7, 49.3, 122.1, 126.3, 127.6, 128.9, 141.3, 142.3, 165.4 ppm. IR (neat, cm^{-1}): ν 3301, 2981, 1721, 1653, 1616, 1537, 1493, 1434, 1359, 1265, 1228, 1154, 1121, 1074, 1015, 968, 947, 928, 802, 746, 697, 584, 533, 442. LCMS calculated for $\text{C}_{12}\text{H}_{14}\text{BrNO}$ $[\text{M}+\text{H}]^+$ 269.15 found 269.15. HRMS calculated for $\text{C}_{12}\text{H}_{14}\text{BrNO}$ $[\text{M}+\text{H}]^+$ 268.0337 found 268.0337. $[\alpha]_D^{20}$ -33.38 (c = 1 g/100 mL, CH_2Cl_2).

(S)-2-[2-Hydroxy-4-((R)-1-phenyl-ethylcarbamoyl)-pent-4-enoylamino]propionic acid methyl ester. Yield 37% (0.80 g). White solid. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 63:37. R_f = 0.13 (hexane – ethyl acetate, 1:1). ^1H NMR (CDCl_3 , 400 MHz) δ 1.31 (d, 1H, J = 7.2 Hz), 1.40 (d, 2H, J = 7.2 Hz), 1.52 (d, 3H, J = 7.2 Hz), 2.60-2.68 (m, 1H), 2.75-2.80 (m, 1H), 3.71 (s, 3H), 4.13-4.16 (m, 1H), 4.46-4.58 (m, 1H), 5.07-5.14 (m, 1H), 5.47 (d, 1H, J = 8.3 Hz), 5.64 (d, 1H, J = 5.5 Hz), 6.60-6.69 (m, 1H), 7.24-7.35 (m, 5H), 7.44-7.52 (m, 1H) ppm. ^{13}C NMR (CDCl_3 , 100 MHz) δ 17.9, 18.2, 21.4, 21.5, 37.1, 37.2, 47.4, 47.5, 49.3, 49.4, 52.1, 52.2, 72.3, 72.6, 122.0, 122.1, 125.9, 127.4, 128.6, 140.6, 140.7, 142.4, 170.1, 170.3, 172.7, 172.8, 172.8, 172.9 ppm. IR (neat, cm^{-1}): ν 3400, 2900, 1741, 1654, 1608, 1529, 1452, 1436, 1232, 1159, 700. LCMS calculated for

$C_{18}H_{24}N_2O_5$ $[M+H]^+$ 349.17 found 349.06. HRMS calculated for $C_{18}H_{24}N_2O_5$ $[M+H]^+$ 349.1763 found 349.1764.

(R)-2-(2-Bromomethyl-acryloylamino)-4-methyl-pentanoic acid methyl ester

(37a). Obtained as white solid. Yield 58% (0.27 g). R_f = 0.26 (hexane – ethyl acetate, 1:1); 1H NMR ($CDCl_3$, 300 MHz) δ 0.93-0.95 (m, 6H), 1.56-1.71 (m, 3H), 3.74 (s, 3H), 4.25-4.35 (m, 2H), 4.67-4.74 (m, 1H), 5.13-5.23 (m, 1H), 5.72 (s, 1H), 5.90 (s, 1H), 6.45-6.51 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 22.1, 22.9, 25.0, 41.7, 43.5, 51.1, 52.6, 122.8, 140.7, 166.0, 173.5 ppm. IR (neat, cm^{-1}): ν 3400, 2900, 1741, 1654, 1608, 1529, 1452, 1436, 1232, 1159, 700. LCMS calculated for $C_{11}H_{18}BrNO_3$ $[M+H]^+$ 292.17 found 292.05. HRMS calculated for $C_{11}H_{18}BrNO_3$ $[M+H]^+$ 292.0548 found 292.0548. $[\alpha]_D^{20}$ -33.1 (c = 1 g/100 mL, CH_2Cl_2).

(R)-2-[4-Hydroxy-4-((S)-1-methoxycarbonyl-ethylcarbamoyl)-2-methylene

butyrylamino]-4-methyl-pentanoic acid methyl ester. Yield 46% (0.10 g). White solid. ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 60:40. R_f = 0.13 (hexane – ethyl acetate, 1:1). 1H NMR ($CDCl_3$, 400 MHz) δ 0.94-0.96(m, 6H), 1.39 (dd, 3H, J = 7.2, 23.4 Hz), 1.57-1.72 (m, 3H), 2.52-2.93 (m, 2H), 3.71, 3.72, 3.74, 3.75 (s, 6H), 4.16-4.25 (m, 1H), 4.56-4.62 (m, 2H), 5.52 (d, 1H, J = 16 Hz), 5.66 (d, 1H, J = 8 Hz), 6.80-6.82 (m, 0.3H), 7.12-7.14 (m, 0.5H), 7.57-7.64

(m, 1H) ppm. ^{13}C NMR (CDCl_3 , 100 MHz) δ 18.0, 18.7, 22.0, 22.1, 22.9, 23.0, 25.2, 37.7, 37.2, 38.0, 47.6, 47.7, 51.4, 51.5, 52.5, 52.6, 52.7, 52.8, 72.2, 73.0, 122.5, 122.6, 140.5, 140.7, 142.4, 171.0, 171.9, 172.7, 172.9, 173.5, 173.6, 173.7, 173.9 ppm. IR (neat, cm^{-1}): ν 3400, 2900, 1741, 1654, 1608, 1529, 1452, 1436, 1232, 1159, 700. LCMS calculated for $\text{C}_{17}\text{H}_{28}\text{N}_2\text{O}_7$ $[\text{M}+\text{H}]^+$ 373.41 found 373.01. HRMS calculated for $\text{C}_{17}\text{H}_{28}\text{N}_2\text{O}_7$ $[\text{M}+\text{H}]^+$ 373.1975 found 373.1975.

To a solution of rhodamine B (1.2g, 2.5 mmol) in ethanol (30 mL) in a round bottom flask was added ethylenediamine (0.22 mL, 3.25 mmol) dropwise at room temperature with vigorous stirring. The stirred mixture was then heated to reflux overnight while the solution became clear. Upon reaction completion as judged from LCMS analysis, the reaction was cooled and solvent evaporated under reduced pressure. 1 M HCl (50 mL) was added to the residue to remove any unreacted amine followed by slow addition of 1M NaOH with stirring until the solution pH was around 9-10. The aqueous phase was extracted with dichloromethane (3 X 50 mL), the combined organic phase washed with water (30 mL), dried over Na_2SO_4 , filtered and the solvent evaporated under reduced pressure. The crude product thus obtained was purified by flash silica gel column chromatography (ethyl acetate – methanol, gradient, 95:5) to give desired product as a pink foam (0.9 g, 75%). R_f = 0.15 (ethyl acetate - methanol, 95:5). ^1H NMR (300 MHz, CDCl_3) δ 1.12 (t, 12H, J = 7.2 Hz), 2.65 (t, 2H, J = 5.3 Hz), 3.22-3.32 (m, 10H), 6.23 (dd, 2H, J = 2.6, 8.9 Hz), 6.33 (d, 2H, J = 2.4 Hz), 6.37 (s, 1H), 6.40 (s, 1H), 7.01-7.04 (m, 1H), 7.38-7.41 (m, 2H),

7.81-7.84 (m, 1H) ppm. ^{13}C NMR (75 MHz, CDCl_3) δ 12.7, 41.0, 41.7, 44.4, 53.6, 65.9, 97.9, 104.7, 108.5, 123.0, 124.0, 128.3, 128.6, 130.6, 132.9, 149.0, 153.4, 153.7, 169.7 ppm. IR (neat, cm^{-1}): ν 3400, 2970, 1680, 1633, 1614, 1514, 1467, 1427, 1357, 1265, 1232, 1118, 680. LCMS calculated for $\text{C}_{30}\text{H}_{36}\text{N}_4\text{O}_2$ $[\text{M}+\text{H}]^+$ 484.63 found 484.42. HRMS calculated for $\text{C}_{30}\text{H}_{36}\text{N}_4\text{O}_2$ $[\text{M}+\text{H}]^+$ 485.2917 found 485.2917.

Obtained as a purple solid (0.82 g, 78%). R_f = 0.68 (ethyl acetate - methanol, 95:5).

^1H NMR (300 MHz, CDCl_3) δ 1.14 (t, 12H, J = 7.0 Hz), 3.02-3.07 (m, 2H), 3.27-3.43 (m, 10H), 4.29 (s, 2H), 5.69 (s, 1H), 5.92 (s, 1H), 6.25 (dd, 2H, J = 2.4, 8.9 Hz), 6.35 (d, 2H, J = 2.4 Hz), 6.40 (s, 1H), 6.43 (s, 1H), 7.05-7.07 (m, 1H), 7.40-7.46 (m, 2H), 7.87-7.90 (m, 1H) ppm. ^{13}C NMR (75 MHz, CDCl_3)

δ 12.7, 39.9, 41.4, 43.7, 44.6, 66.1, 98.0, 104.7, 108.6, 122.1, 123.1, 124.1, 128.4, 128.6, 130.4, 133.1, 140.8, 149.1, 153.4, 154.0, 166.0, 170.5 ppm. IR (neat, cm^{-1}): ν 3444, 2970, 2927, 2378, 1666, 1631, 1614, 1546, 1514, 1467, 1427, 1398, 1355, 1267, 1118, 949. LCMS calculated for $\text{C}_{34}\text{H}_{39}\text{BrN}_4\text{O}_3$ $[\text{M}+\text{H}]^+$ 632.60 found 633.17. HRMS calculated for $\text{C}_{34}\text{H}_{39}\text{BrN}_4\text{O}_3$ $[\text{M}+\text{H}]^+$ 631.2335 found 632.2335.

Obtained as a purple gum (0.32 g, 72%). $R_f = 0.34$ (ethyl acetate - methanol, 95:5). ^1H NMR (300 MHz, CDCl_3) δ 1.16 (t, 12H, $J = 7.0$ Hz), 1.28-1.41 (m, 3H), 2.57-2.68 (m, 1H), 2.74-2.81 (m, 1H), 2.99-3.04 (m, 2H), 3.30-3.37 (m, 10H), 3.66, 3.71, 3.75, 3.76, 3.77 (s, 3H), 4.13-4.18 (m, 1H), 4.50-4.63 (m, 1H), 5.54, 5.57 (s, 1H), 5.78, 5.87 (s, 1H), 6.26-6.30 (m, 2H), 6.38-6.44 (m, 4H), 7.08-7.10 (m, 1H), 7.45-7.48 (m, 2H), 7.88-7.91 (m, 1H), 8.02-8.05 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 12.6, 18.1, 18.6, 37.3, 37.4, 41.6, 44.3, 44.6, 47.5, 47.6, 52.2, 52.3, 66.0, 72.5, 72.9, 97.7, 104.4, 108.3, 122.7, 122.8, 122.9, 123.9, 128.3, 130.0, 130.2, 133.0, 140.2, 149.0, 153.2, 153.7, 170.8, 170.9, 171.17, 172.9, 173.0, 173.1 ppm. FTIR (neat, cm^{-1}): ν 3725, 3316, 2969, 2925, 2171, 2157, 1712, 1669, 1633, 1613, 1513, 1467, 1427, 1375, 1355, 1328, 1304, 1265, 1217, 1153, 1117, 1091, 1016, 819, 788, 758, 733, 701, 670, 576, 537. LCMS calculated for $\text{C}_{40}\text{H}_{49}\text{N}_5\text{O}_7$ $[\text{M}+\text{H}]^+$ 712.85 found 712.43. HRMS calculated for $\text{C}_{40}\text{H}_{49}\text{N}_5\text{O}_7$ $[\text{M}+\text{H}]^+$ 712.3710 found 712.3710

Synthesis of silyl enol ethers.

(1-Furan-2-yl-vinyloxy)-trimethyl-silane (50). To a solution of 2-acetylfuran (5.5 g, 50 mmol) and Et_3N (16.7 mL, 124 mmol) in anhydrous DMF (50 mL) was added

TMSCl (7.6 mL, 60 mmol) and stirred under reflux for 18 h. After overnight stirring the reaction was cooled to room temperature, diluted with hexane (60 mL) and washed with ice cold sat. NaHCO₃. The aqueous phase was extracted with hexane (2 X 60 mL). The combined organic layer was washed consecutively with water and brine, dried over MgSO₄, filtered and concentrated. The crude product was purified by vacuum distillation (47 °C, 3 mm Hg). Yield 50% (5.45 g). ¹H NMR (400 MHz, CDCl₃) δ 0.28 (s, 9H), 4.39 (d, 1H, *J* = 1.4 Hz), 4.92 (d, 1H, *J* = 1.3 Hz), 6.38-6.42 (m, 2H), 7.36 (s, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 0.01, 90.3, 106.9, 111.0, 142.3, 147.6, 152.1 □ppm. LRMS (EI) *m/z* calculated for C₉H₁₄O₂Si [M] 182.29 found 182.18. HRMS (EI) *m/z* calculated for C₉H₁₄O₂Si [M] 182.0758 found: 182.0753.

2-Trimethylsilanyloxy-acrylic acid methyl ester (54). Prepared according to literature procedure.¹⁵⁶ To a solution of methyl pyruvate (1.8 mL, 20 mmol) and TMSCl (2.68 mL, 22.5 mmol) in anhydrous THF (40 mL) was added a solution of Et₃N (3.76 mL, 27 mmol) in anhydrous THF (26 mL) under N₂ and stirred at room temperature for 6 h. The reaction was diluted with hexane (60 mL) and filtered. The solution was washed with water (2 X 20 mL) and brine (1 X 20 mL), dried over MgSO₄, filtered and concentrated. The crude product was purified by vacuum distillation (47 °C, 3 mm Hg). Yield 70% (2.5 mL). ¹H NMR (300 MHz, CDCl₃) δ 0.16 (s, 9H), 3.72 (s, 3H), 4.83 (d, 1H, *J* = 1.02 Hz), 5.45 (d, 1H, *J* = 1.02 Hz) ppm. ¹³C NMR (75 MHz, CDCl₃) δ 0.1, 51.9, 103.7, 146.9, 164.7 □ppm.

¹⁵⁶ *J. Org. Chem.* **1997**, 62 (8), 2458-2465.

Representative experimental procedure for Mukayama aldol reaction of peptides:

3-Hydroxy-*N*-((*S*)-1-methoxycarbonyl-ethyl)-2,2-dimethyl-succinamic acid methyl ester (47**).** To a suspension of the peptide aldehyde **13** (0.078 g, 0.49 mmol) in (pH 7.0) sodium phosphate buffer, (2.5 mL) was added the silyl ketene acetal (0.98 mmol) and stirred for 1 day. The aqueous solution was then extracted with EtOAc (5 x 5 mL). The combined organic phase was dried over MgSO₄. After filtration and solvent evaporation, the crude was purified by flash chromatography using (hexane - ethyl acetate, gradient, 0 to 70%) as the eluent.

Obtained as pale yellow transparent oil (0.025 g, 55% yield). $R_f = 0.27$ (hexane – ethyl acetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.2 (d, 3H, $J = 7.3$ Hz), 1.29 (d, 3H, $J = 5.3$ Hz), 1.42 (dd, 3H, $J = 7.2, 2.1$ Hz), 3.74 (s, 3H), 3.75 (s, 3H), 4.40-4.41 (m, 1H), 4.53-4.62 (m, 1H), 7.10-7.18 (m, 1H) ppm.

¹³C NMR (100 MHz, CDCl₃)

δ 18.1, 18.2, 19.5, 19.9, 21.6, 22.1, 46.8, 46.9, 47.7, 47.8, 52.3, 52.3, 52.5, 74.8, 75.2,

171.0, 171.3, 173.0, 173.2, 177.7, 177.8 ppm. IR (neat, cm⁻¹): ν 3408, 3019,

1740, 1674, 1522, 1452, 1215. LRMS (ESI) m/z calculated for C₁₁H₂₀NO₆ [M+H]⁺

262.12 found 262.86. HRMS (ESI) m/z calculated for C₁₁H₂₀NO₆ [M+H]⁺ 262.1285

found 262.1288.

(S)-2-(2-Hydroxy-4-oxo-4-phenyl-butyrylamino)-propionic acid methyl ester.

Transparent oil (0.015 g, 11% yield). ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 52:48. $R_f = 0.25$ (hexane – ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.47 (dd, 3H, $J = 7.2, 1.6$ Hz), 3.36 (ddd, 1H, $J = 18.3, 8.9, 8.3$ Hz), 3.69 (ddd, 1H, $J = 18.3, 3.3, 2.9$ Hz), 3.76, 3.78 (s, 3H), 4.06 (dd, 1H, $J = 19.0, 4.5$ Hz), 4.60-4.67 (m, 1H), 4.67-4.72 (m, 1H), 7.42-7.44 (m, 1H), 7.47-7.51 (m, 2H), 7.60-7.64 (m, 1H), 7.97-7.99 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 18.2, 18.3, 42.0, 42.1, 47.7, 47.8, 52.5, 68.4, 68.6, 76.6, 77.0, 77.4, 128.2, 128.8, 133.9, 136.0, 136.1, 171.9, 172.0, 173.0, 200.3, 200.4 ppm. IR (neat, cm^{-1}): ν 3404, 3018, 1741, 1670, 1525, 1450, 1217, 769, 665. LRMS (EI) m/z calculated for $\text{C}_{14}\text{H}_{17}\text{NO}_5$ $[\text{M}]^+$ 279.11 found 279.83. HRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{17}\text{NO}_5$ $[\text{M}+\text{H}]^+$ 280.1179 found 280.1168.

(S)-2-(4-Furan-2-yl-2-hydroxy-4-oxo-butyrylamino)-propionic acid methyl ester.

Transparent oil (0.015 g, 11% yield). ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 55:45. $R_f = 0.18$ (hexane - ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.44 (dd, 3H, $J = 7.2, 2.3$ Hz), 3.17-3.27 (m, 1H), 3.49 (ddd, 1H, $J = 17.7, 3.5, 3.2$ Hz), 3.74, 3.75 (s, 3H), 4.16-4.20 (m, 1H), 4.56-4.61 (m, 1H), 4.63-4.68 (m, 1H), 6.55-6.57 (m, 1H), 7.27-7.28 (m, 1H), 7.41-7.43 (m, 1H), 7.62-7.63 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3)

δ 18.1, 18.2, 41.7, 41.8, 47.7, 47.8, 52.5, 68.2, 68.3, 112.5, 118.7, 118.8, 147.3, 147.4, 151.8, 151.9, 171.9, 172.0, 173.1, 188.3, 188.4 ppm. IR (neat, cm^{-1}): ν 3404, 3017, 1742, 1667, 1570, 1525, 1467, 1396, 1348, 1215, 1159, 1085, 883, 769, 667. LRMS (ESI) m/z calculated for $\text{C}_{12}\text{H}_{15}\text{NO}_6$ [M] 269.25 found 269.81. HRMS (ESI) m/z calculated for $\text{C}_{12}\text{H}_{15}\text{NO}_6$ [M+H]⁺ 270.0972 found 270.0983.

(S)-2-[2-Hydroxy-2-(2-oxo-2,3-dihydro-furan-3-yl)-acetyl-amino]-propionic acid methyl ester. Transparent oil (0.005 g, 4% yield). ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 50:50. R_f = 0.16 (hexane – ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.48 (dd, 3H, J = 7.2, 2.1 Hz), 3.78 (s, 3H), 4.46-4.48 (m, 1H), 4.55-4.69 (m, 2H), 5.48-5.51 (m, 1H), 6.21-6.23 (m, 1H), 7.18-7.35 (m, 1H), 7.43-7.63 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3)

δ 17.5, 18.0, 47.8, 48.0, 52.6, 52.7, 70.4, 70.5, 83.8, 84.4, 123.1, 123.2, 152.9, 153.4, 169.0, 169.1, 172.7, 172.8, 173.2, 173.6 ppm. IR (neat, cm^{-1}): ν 3404, 3017, 1742, 1661, 1531, 1456, 1348, 1215, 1159, 1128, 1095, 1047, 883, 769, 667. LRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{13}\text{NO}_6$ [M] 243.21 found 243.84. HRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{13}\text{NO}_6$ [M+H]⁺ 244.0861 found 244.0804.

((S)-2-[(S)-2-[(S)-2-(2-Hydroxy-3-methoxycarbonyl-3-methyl-butyl-amino)-3-phenyl propionyl amino]-4-methyl-pentanoyl-amino]-pentanedioic acid dimethyl

ester. Obtained as pale yellow oil (0.028 g, 71% yield). $R_f = 0.30$ (hexane - ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 0.90-0.91 (m, 7H), 1.08 (d, 3H, $J = 4.1$ Hz), 1.17 (s, 1H), 1.27-1.30 (m, 2H), 1.46-1.56 (m, 2H), 1.65-1.71 (m, 1H), 1.84 (s, 2H), 1.97-1.20 (m, 1H), 2.18-2.28 (m, 1H), 2.33-2.45 (m, 2H), 3.01-3.22 (m, 2H), 3.48-3.50 (m, 1H), 3.67, 3.68, 3.69, 3.75 (s, 8H), 3.92-3.93 (m, 0.6H), 4.11-4.13 (m, 0.4H), 4.31-4.33 (m, 0.6H), 4.36-4.46 (m, 1H), 4.48-4.60 (m, 1H), 4.60-4.70 (m, 1H), 6.61-6.63 (m, 0.6H), 6.84-6.86 (m, 0.4H), 6.95-7.01 (m, 1H), 7.10-7.33 (m, 7H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 20.0, 21.3, 21.5, 21.7, 21.5, 21.9, 24.6, 26.9, 27.0, 29.9, 30.0, 37.1, 37.5, 40.2, 40.6, 46.7, 47.1, 51.6, 51.8, 51.9, 52.0, 52.1, 52.3, 52.5, 52.6, 54.2, 54.4, 75.5, 76.0, 127.1, 128.8, 129.1, 129.2, 136.1, 136.2, 170.7, 170.9, 171.6, 171.8, 171.9, 172.0, 172.4, 173.2, 173.4, 177.3, 177.7 $\square$ ppm. $\square\square$ IR (neat, cm^{-1}): ν 3408, 3020, 2954, 1732, 1645, 1525, 1452, 1436, 1263, 1209, 1082, 788, 752. LRMS (ESI) m/z calculated for $\text{C}_{29}\text{H}_{44}\text{N}_3\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 594.29 found 594.01. HRMS (ESI) m/z calculated for $\text{C}_{29}\text{H}_{44}\text{N}_3\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 594.3021 found 594.3007.

6-Amino-2-((S)-2-[(S)-2-((S)-3-hydroxy-2-{2-[(S)-3-methyl-2-(2-oxo-acetyl-amino)-pentanoylamino]-acetyl-amino}-propionyl-amino)-4-methyl-pentanoylamino]-propionyl-amino}-hexanoic acid. A mixture of the aldehyde and

hydrated form. Obtained as white powder (0.109 g, quantitative). ^1H NMR (400 MHz, CD_3OD) δ 0.94-0.98 (m, 15H), 1.24-1.92 (m, 20H), 2.97 (brs, 2H), 3.36-3.44 (m, 2H), 3.84-4.02 (m, 5H), 4.32 (brs, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 10.0, 14.5,

15.8, 20.2, 22.1, 22.3, 24.5, 24.8, 26.5, 30.7, 36.6, 39.3, 42.7, 48.5, 49.8, 52.7, 53.0, 56.6, 56.7, 58.2, 61.0, 93.6, 165.0, 170.7, 171.1, 172.1, 172.9, 173.0, 173.7, 175.5, 181.0 ppm. IR (neat, cm^{-1}): ν 3424, 2999, 1736, 1663, 1534, 1437, 1028. LRMS (ESI) m/z calculated for $\text{C}_{28}\text{H}_{50}\text{N}_7\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 644.35 found 644.57. HRMS (ESI) m/z calculated for $\text{C}_{28}\text{H}_{50}\text{N}_7\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 644.5680 found 644.5676. $[\alpha]_D^{20}$ -51.0 ($c = 1.0$ g/100 mL, MeOH).

6-Amino-2-((S)-2-[(S)-2-((S)-3-hydroxy-2-{2-[(S)-2-(2-hydroxy-3-methoxycarbonyl-3-methyl butyryl amino)-3-methyl-pentanoylamino]-acetyl-amino}-propionyl-amino)-4-methyl-pentanoylamino] propionyl amino)-hexanoic acid. Obtained as yellow gum (0.100 g, 80% yield). ^1H NMR (400 MHz, CD_3OD) δ 0.92-0.98 (m, 17H), 1.12 (s, 3H), 1.24 (s, 4H), 1.29-1.41 (m, 6H), 1.46-1.48 (m, 2H), 1.57-1.94 (m, 13H), 2.9 (bs, 2.5H), 3.69, 3.70 (s, 3H), 3.77-3.99 (m, 6H), 4.13-4.30 (m, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 11.5, 11.6, 16.1, 16.2, 17.3, 17.4, 19.5, 20.3, 21.7, 21.8, 23.1, 23.5, 23.7, 23.8, 23.9, 24.0, 25.9, 26.0, 26.2, 26.4, 28.0, 32.3, 38.1, 38.5, 40.8, 40.9, 41.0, 44.2, 44.5, 47.9, 48.0, 50.0, 51.4, 51.6, 52.7, 52.8, 54.3, 54.4, 54.5, 54.7, 57.9, 58.5, 59.2, 59.6, 62.5, 62.6, 76.7, 76.9, 170.5, 172.5, 173.0, 173.7, 173.9, 174.5, 174.9, 175.0, 175.3, 175.4, 175.5, 175.7, 175.9, 177.0, 177.1 ppm. LRMS (ESI) m/z calculated for $\text{C}_{33}\text{H}_{60}\text{N}_7\text{O}_{12}$ $[\text{M}+\text{H}]^+$ 746.42 found 746.48. HRMS (ESI+) m/z calculated for $\text{C}_{33}\text{H}_{60}\text{N}_7\text{O}_{12}$ $[\text{M}+\text{H}]^+$ 746.4300 found 746.4303.

Synthetic attempt towards biotinylated silyl ketene acetal (55) **4-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoyl]-piperazine-1-**

carboxylic acid tert-butyl ester. To a solution of biotin (0.56 g, 2.3 mmol) and boc-piperazine (0.67 g, 3.6 mmol) in methanol (7.5 mL) and acetonitrile (22.5 mL) was added *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium-hexa-fluoro-

phosphate HATU (0.65 g, 1.7 mmol) followed by triethylamine (1.2 mL, 6.9 mmol) and stirred for 18 h at room temperature. The solvent was evaporated in vacuo and redissolved in MeOH and taken for HPLC purification using 0-50% acetonitrile in water as the eluent. Yield 50% (0.47 g). White powder. ^1H NMR (400 MHz, CD_3OD) δ 1.29–1.92 (m, 15H), 2.42–2.51 (m, 3H), 2.71 (d, 1H, $J = 12.7$ Hz), 2.93 (dd, 1H, $J = 12.7, 5.0$ Hz), 3.21–3.31 (m, 6H), 3.41–3.60 (m, 4H), 4.31 (dd, 1H, $J = 7.8, 4.4$ Hz), 4.49–4.52 (m, 1H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 25.9, 26.2, 28.6, 29.5, 29.7, 29.8, 33.2, 33.7, 41.0, 42.6, 43.6, 44.4, 44.5, 49.8, 56.7, 61.6, 63.4, 81.7, 156.3, 166.1, 174.1, 174.2 ppm. IR (neat, cm^{-1}): ν 2938, 1698, 1651, 1462, 1433, 1365, 1323, 1257, 1184, 1132, 1083, 1014, 985, 837, 795, 722, 602, 516, 445, 409.

. LRMS (ESI) m/z calculated for $\text{C}_{19}\text{H}_{32}\text{N}_4\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 413.55 found 413.5. HRMS (ESI) m/z calculated for $\text{C}_{19}\text{H}_{32}\text{N}_4\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 413.2223 found 413.2223.

4-(5-Oxo-5-piperazin-1-yl-pentyl)-tetrahydro-thieno[3,4-d]imidazol-2-one. To a solution of Boc-piperazine biotin (0.82 g, 2.0 mmol) in dichloromethane (18 mL) was added trifluoroacetic acid (8 mL) to give a 30% solution and the reaction stirred for 18 h at room temperature. The solvent was evaporated in vacuo and the residue redissolved in MeOH and taken for HPLC purification using 0-50% acetonitrile in water as the eluent. Yield 80% (0.50 g). White powder. ^1H NMR (400 MHz, CD_3OD) δ 1.37–1.45 (m, 2H), 1.50 – 1.74 (m, 4H), 2.40 (t, 2H, $J = 7.3$ Hz), 2.65 (d, 1H, $J = 12.7$ Hz), 2.87 (dd, 1H, $J = 12.7, 4.9$ Hz), 3.14 - 3.25 (m, 6H), 3.74-3.76 (m, 4H), 4.25 (dd, 1H, $J = 7.8, 4.4$ Hz), 4.43 (dd, 1H, $J = 7.6, 4.9$ Hz), 4.84 (m, 1H, hidden under neath solvent peak) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 26.0, 29.6, 29.8, 33.3, 39.5, 41.1, 43.6, 44.4, 44.5, 57.0, 61.7, 63.4, 166.1, 174.1 ppm. IR (neat, cm^{-1}): ν 3400, 2856, 2740, 2520, 1687, 1681, 1500, 1203, 1115. LRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{24}\text{N}_4\text{O}_2\text{S}$ $[\text{M}+\text{H}]^+$ 313.43 found: 313.82. HRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{24}\text{N}_4\text{O}_2\text{S}$ $[\text{M}+\text{H}]^+$ 313.1698 found 313.1702.

Isobutyric acid 3-{4-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoyl]-piperazin-1-yl}-propyl ester. To a solution of biotin piperazine (0.24 g, 0.77 mmol) in DMF (2 mL) was added 3-bromopropyl isobutyrate (0.7 g, 3.4 mmol) followed by

diisopropyl ethyl amine (0.5 mL, 2.4 mmol) and an additional portion of DMF (2 mL) and stirred for 18 h at room temperature. Upon complete conversion of starting material to product as judged from ESI-LC-MS analysis, the solvent was evaporated in vacuo and the crude redissolved in MeOH and taken for HPLC purification using 0-50% acetonitrile in water as the eluent. Yield 89% (0.30 g). White powder. ^1H NMR (400 MHz, CDCl_3) δ 1.47 (s, 6H), 1.60-1.94 (m, 6H), 1.82-1.89 (m, 2H), 2.32 (t, 2H, $J = 7.4$ Hz), 2.46 – 2.58 (m, 6H), 2.71 (d, 1H, $J = 12.8$ Hz), 2.93 (dd 1H, $J = 12.8, 4.9$ Hz), 3.10 - 3.15 (m, 1H), 3.53-3.64 (m, 4H), 4.07 (t, 2H, $J = 6.3$ Hz), 4.26-4.29 (m, 1H), 4.46 – 4.49 (m, 1H), 6.02 (s, 1H), 6.52 (s, 1H), 8.14 (s, 1H) ppm.

^{13}C NMR (75 MHz, CDCl_3)

δ 18.9, 25.0, 25.1, 28.0, 28.2, 32.4, 33.8, 40.4, 40.6, 44.8, 52.2, 52.7, 54.4, 55.5, 60.1, 61.8, 62.0, 164.2, 165.4, 171.5, 177.0 ppm.

IR (neat, cm^{-1}): ν 2938, 1698, 1651, 1462, 1433, 1365, 1323, 1257, 1184, 1132, 1083, 1014, 985, 837, 795, 722, 602, 516, 445, 409.

LRMS (ESI) m/z calculated for $\text{C}_{21}\text{H}_{36}\text{N}_4\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 441.60 found 441.60. HRMS

(ESI) m/z calculated for $\text{C}_{21}\text{H}_{36}\text{N}_4\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 441.2536 found 441.2538.

4-(5-{4-[3-(2-Methyl-1-trimethylsilyloxy-propenyloxy)-propyl]-piperazin-1-yl}-5-oxo-pentyl)-tetrahydro-thieno[3,4-d]imidazol-2-one. To a solution of

diisopropylamine (0.025 mL, 0.18 mmol) in THF (0.5 mL) at 0 °C was added n-Butyllithium dropwise and the resulting mixture stirred at this temperature for 30 minutes. It was the cooled down to -78 °C and a solution of biotin ester **82** (0.040 g,

0.092 mmol) in THF (0.5 mL) was added dropwise. The resulting solution was stirred at that temperature for 1.5 h followed by the addition of trimethylsilyl chloride (0.024 mL, 0.19 mmol) dropwise. After stirring at -78 °C for 30 minutes, the reaction was allowed to warm up to room temperature over 1 h and the suspension was filtered through filter paper. The solvent was evaporated in vacuo and the crude taken for NMR. Desired product was not obtained and only unreacted starting material was observed.

Synthetic attempt towards fluorescent tagged silyl ketene acetal (56)

4-*N,N*-dimethylamino-1,8-naphthalic anhydride.¹⁵⁷ A solution of 4-bromo-1,8-naphthalic anhydride (2.77 g, 10 mmol) in 3-methyl-1-butanol (70 mL) was heated to reflux in a two neck round bottom flask at 130 °C under argon. 3-dimethylaminopropionitrile (4.5 mL, 40 mmol) was added and the stirring continued overnight until HPLC-MS analysis revealed complete consumption of starting material. The solution was allowed to cool to room temperature. The product precipitated as orange crystals which were filtered and washed with cold hexane (3 X 100 mL) and dried to afford the desired product (2.36 g, quantitative). ¹H NMR (500 MHz, CDCl₃) δ 8.57 (d, 1H, *J* = 7.3 Hz), 8.49-8.46 (m, 2H), 7.68 (dd, 1H, *J* = 7.4, 8.4 Hz), 7.11 (d, 1H, *J* = 8.3 Hz), 3.18 (s, 6H) ppm. ¹³C NMR (125 MHz, CDCl₃) δ 44.8, 109.8, 113.4, 125.2, 132.9, 133.3, 135.2, 160.9, 167.8 ppm. IR (neat, cm⁻¹): ν 3443, 2924, 2852, 1755, 1730, 1585, 1494, 1454, 1394, 1342, 1309, 1020, 999. LRMS (ESI) *m/z* calculated for

¹⁵⁷ G. Loving, B. Imperiali, *J. Am. Chem. Soc.* **2008**, 130, 13630-13638.

$\text{C}_{14}\text{H}_{11}\text{NO}_3$ $[\text{M}]^+$ 241.24 found 241.48. HRMS (ESI, m/z) calculated for $\text{C}_{14}\text{H}_{11}\text{NO}_3$ $[\text{M}+\text{H}^+]$ 242.0817 found 242.0814.

6-Dimethylamino-2-(2-hydroxy-ethyl)-benz[de]isoquinoline-1,3-dione.¹⁵⁸ 4-*N,N*-dimethylamino-1,8-naphthalic anhydride (1.89 g, 7.8 mmol) was taken in a two-necked round bottom flask equipped with stirring bar, septum and connected to a reflux condenser. The air inside was replaced by applying vacuum and refilling with argon consecutively three times. Anhydrous ethanol (50 mL) was added by a syringe and the resulting suspension heated to reflux at 80 °C. At this point 1-amino ethanol (0.54 mL, 8.7 mmol) was added and a clear orange solution formed. The stirring was continued for another 2 hours until HPLC-ESI-MS analysis revealed complete consumption of starting material. The solution was allowed to cool to room temperature. The solvent was evaporated in a vacuo and allowed to dry further under high vacuum overnight. The desired product was obtained as an orange solid **58** (2.18 g, quantitative). ^1H NMR (500 MHz, CDCl_3) δ 8.51 (d, 1H, $J = 7.1$ Hz), 8.43-8.39 (m, 2H), 7.63-7.59 (m, 1H), 7.06 (d, 1H, $J = 8.5$ Hz), 4.41 (t, 2H, $J = 5.4$ Hz), 3.95-3.93 (m, 2H), 3.09 (s, 6H) ppm. ^{13}C NMR (125 MHz, CDCl_3) δ 42.9, 44.9, 53.4, 62.3, 113.4, 114.5, 122.9, 125.0, 125.3, 130.6, 131.5, 131.8, 133.2, 157.4, 165.2, 165.7 ppm. IR (neat, cm^{-1}): ν 3797, 3450, 2536, 2308. LRMS (ESI) m/z calculated for

¹⁵⁸ Kollar, J.; Hrdlovic, P.; Chmela, S.; Sarakha, M.; Guyot, G. *J. Photochem. Photobiol.*, **2005**, *170*, 151-159.

$C_{16}H_{16}N_2O_3$ $[M+H]^+$ 285.31 found 285.00. HRMS (ESI, m/z) calculated for $C_{16}H_{16}N_2O_3$: $[M+H]^+$ 285.1239 found 285.1237.

Isobutyric acid 2-(6-dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-ethyl ester. To a solution of the alcohol **58** (0.4 g, 1.3 mmol) and isobutyric acid (0.13 mL, 1.4 mmol) in CH_2Cl_2 at 0 °C was added dicyclohexylcarbodiimide DCC (0.4 g, 1.3 mmol) followed by N,N-dimethylamino pyridine DMAP (0.005 g, 0.12 mmol) and allowed the temperature to rise to room temperature. Upon reaction completion as judged by TLC, the reaction was filtered and the solvent evaporated in vacuo. The crude was purified by flash chromatography using (hexane – ethyl acetate, gradient, 0-70%) as the eluent. Yield 75% (0.77 g). Orange solid. R_f = 0.5 (hexane – ethyl acetate, 1:1). 1H NMR (500 MHz, $CDCl_3$) δ 1.07 (dd, 6H, J = 6.9, 2.5 Hz), 2.44-2.50 (m, 1H), 3.06 (d, 6H, J = 2.4 Hz), 4.3 (brs, 2H), 4.4 (brs, 2H), 7.05-7.07 (m, 1H), 7.58-7.62 (m, 1H), 8.38-8.42 (m, 2H), 8.49-8.51 (m, 1H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.7, 33.8, 38.7, 44.6, 61.5, 113.1, 114.5, 122.7, 124.7, 125.1, 130.1, 130.9, 131.2, 132.6, 156.9, 163.8, 164.5, 176.8 ppm. IR (neat, cm^{-1}): ν 2873, 2850, 2796, 1337, 1650, 1321, 1242, 1190, 1151, 1064, 1022. LRMS (ESI) m/z calculated for $C_{20}H_{22}N_2O_4$ $[M+H]^+$ 355.40 found 355.097. HRMS (ESI, m/z) calculated for $C_{20}H_{22}N_2O_4$ $[M+H]^+$ 355.1658 found 355.1657.

transparent concentrate was sufficiently pure for subsequent reactions (10.20 g, 94 % yield). ^1H NMR (400 MHz, CDCl_3) δ 0.19 (s, 9H), 1.47-1.52 (m, 5H), 1.57-1.65 (m, 5H), 2.05-2.10 (m, 2H), 3.69 (t, 2H, $J = 6.7$ Hz), 4.93-5.02 (m, 2H), 5.74-5.84 (m, 1H) ppm.

^{13}C NMR (100 MHz, CDCl_3): 0, 16.3, 16.9, 25.4, 28.9, 33.5, 68.7, 91.3, 114.5, 138.5, 148.2 ppm.

(1-(ethylthio)vinyl)oxy)trimethylsilane. According to literature procedures.¹⁵⁹

^1H NMR (300 MHz, CDCl_3) δ 0.19 (s, 9H), 1.52 (s, 3H), 1.57 (s, 3H), 2.32-2.39 (m, 2H), 3.74 (t, 2H, $J = 6.7$ Hz), 5.03-5.14 (m, 2H), 5.77-5.90 (m, 1H) ppm.

^{13}C NMR (75 MHz, CDCl_3) δ 0.3, 16.6, 17.1, 34.0, 68.2, 91.9, 116.8, 135.2, 148.2 ppm.

(1-(ethylthio)vinyl)oxy)trimethylsilane. A stirred solution of 3-butyne-2-one (2.4 mL, 30 mmol, 1 equiv.) in dichloromethane (50 mL) was added with trimethylsilyl trifluoromethanesulfonate (6.0 mL, 36 mmol, 1.2 equiv.). The mixture was cooled down to 0 °C. 2,6-lutidine (6.2 mL, 45 mmol, 1.5 equiv.) was added dropwise and the reaction stirred at 0 °C for 2 hours. Dichloromethane was removed in vacuo and the two layers were separated. The upper layer was subjected to bulb to bulb distillation

¹⁵⁹ Zhu, Z.; Rider, J.; Yang, C. Y.; Gilmartin, M. E.; Wnek, G. E. *Macromolecules* **1992**, 25, 7330-

(40–60 °C, 2 mmHg) to give the desired product as a transparent liquid (2.5 mL, 50 % yield).

^1H NMR (300 MHz, CDCl_3) δ 0.19 (s, 9H), 2.8 (s, 1H), 4.6 (s, 1H), 4.7 (s, 1H) ppm.

^{13}C NMR (75 MHz, CDCl_3) δ 0, 14.4, 75.4, 103.6, 138.2 ppm.

(1-(ethylthio)vinyl)trimethylsilyl ether.¹⁶⁰ A stirred solution of ethyl thioacetate (5.3 mL, 50 mmol, 1 equiv.) in dichloromethane (50 mL) was added with triethylamine (8.4 mL, 60 mmol, 1.2 equiv.). The mixture was cooled down to 0 °C. Trimethylsilyl trifluoromethanesulfonate (9.0 mL, 50 mmol, 1 equiv.) was added dropwise and the reaction stirred at 0 °C for 2 hours. Dichloromethane was removed in vacuo and the two layers were separated. The upper layer was subjected to bulb to bulb distillation (82 °C, 5 mbar) to give the desired product as a transparent liquid (7.40 g, 84% yield).

^1H NMR (300 MHz, CDCl_3) δ 0.26 (s, 9H), 1.28 (t, 3H, $J = 7.4$ Hz), 2.69 (q, 2H, $J = 7.4$ Hz), 4.42 (d, 1H, $J = 1.7$ Hz), 4.36 (d, 1H, $J = 1.7$ Hz) ppm.

^{13}C NMR (75 MHz, CDCl_3) δ 0, 14.4, 25.6, 93.1, 153.5 ppm.

^1H NMR (300 MHz, CDCl_3) δ 0.12 (s, 9H), 1.58 (s, 6H), 3.38 (s, 3H), 3.53–3.59 (m, 4H), 3.63–3.66 (m, 2H), 3.73–3.77 (m, 2H) ppm.

^{13}C NMR (75 MHz, CDCl_3) δ 0, 14.0, 59.0, 61.9, 67.7, 70.4, 71.9, 147.7 ppm.

¹⁶⁰ G. Simchen, W. West, *Synthesis*, **1977**, 247–248.

3-Hydroxy-*N*-((*S*)-1-methoxycarbonyl-ethyl)-2,2-dimethyl-succinamic acid hex-5-enyl ester (65). Transparent oil (0.074 g, 45% yield). ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 60:40. $R_f = 0.43$ (hexane - ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.18 (d, 3H, $J = 3.8$ Hz), 1.26 (d, 3H, $J = 5.9$ Hz), 1.39-1.48 (m, 6H), 1.62-1.69 (m, 2H), 2.03-2.1 (m, 2H), 3.72, 3.73 (s, 3H), 4.11-4.14 (m, 2H), 4.39 (s, 1H), 4.50-4.58 (m, 1H), 4.93-5.02 (m, 2H), 5.72-5.82 (m, 1H), 7.14-7.24 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 18.1, 18.2, 19.7, 19.9, 21.5, 21.9, 25.1, 27.8, 33.2, 46.9, 47.7, 47.8, 52.5, 65.0, 74.8, 75.2, 114.8, 114.9, 138.2, 138.3, 171.1, 171.4, 173.0, 173.2, 177.3 ppm. IR (neat, cm^{-1}): ν 3421, 3018, 1734, 1653, 1521, 1456, 1215, 756, 667. LRMS (ESI) m/z calculated for $\text{C}_{16}\text{H}_{27}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 330.18 found 330.12. HRMS (ESI) m/z calculated for $\text{C}_{16}\text{H}_{27}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 330.1911 found 330.1749.

(3-Hydroxy-*N*-((*S*)-1-methoxycarbonyl-ethyl)-2,2-dimethyl-succinamic acid but-3-enyl ester (66). Transparent oil (0.034 g, 41% yield). ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 60:40. $R_f = 0.15$ (cyclohexane - ethyl acetate, 4:6). ^1H NMR (300 MHz, CDCl_3) δ 1.18 (d, 3H, $J = 3.2$ Hz), 1.25 (d, 3H, $J = 5.0$ Hz), 1.39 (dd, 3H, $J = 7.0, 1.5$ Hz), 2.36-2.43 (m, 2H), 3.72, 3.73 (s, 3H), 4.11-4.21 (m, 2H), 4.36 (bs, 1H), 4.49-4.60 (m, 1H), 5.05-5.14 (m, 2H), 5.70-5.84 (m, 1H), 7.08-7.18 (m, 1H) ppm. ^{13}C NMR (75 MHz, CDCl_3)

δ 18.3, 18.4, 20.0, 20.4, 21.6, 22.0, 33.1, 47.1, 47.2, 47.9, 48.0, 52.6, 64.1, 64.1, 75.0, 75.3, 117.5, 117.6, 134.1, 134.2, 171.2, 171.4, 173.1, 173.3, 177.3, 177.3 ppm. IR (neat, cm^{-1}): ν 3398, 2981, 2938, 1734, 1655, 1210, 1156, 1130, 1080, 989, 913, 735. LRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{23}\text{NO}_6$ $[2\text{M}+\text{H}]^+$ 603.68 found 603.21. HRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{23}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 302.1604 found 302.1604.

(S)-2-(2-Hydroxy-4-oxo-hex-5-ynoylamino)-propionic acid methyl ester (67).

Transparent oil (0.014 g, 12% yield). ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 62:38. R_f = 0.28 (hexane - ethyl acetate, 3:7). ^1H NMR (300 MHz, CDCl_3) δ 1.43 (dd, 3H, J = 5.8, 0.9 Hz), 2.97-3.04 (m, 1H), 3.28-3.34 (m, 1H), 3.35 (brs, 1H), 3.40-3.42 (m, 1H), 3.74, 3.75 (s, 3H), 4.55-4.60 (m, 2H) ppm. ^{13}C NMR (75 MHz, CDCl_3)

δ 18.2, 18.3, 47.7, 47.8, 49.1, 49.2, 52.5, 67.6, 67.7, 80.5, 80.6, 170.9, 171.0, 172.9, 173.0, 185.9, 186.1 ppm. IR (neat, cm^{-1}): ν 3365, 2954, 2924, 2852, 1738, 1707, 1533, 1399, 1384, 1357, 1312, 1217, 1159, 1092, 974. LRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{13}\text{NO}_5$ $[\text{M}+\text{H}]^+$ 228.21 found 228.20. HRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{13}\text{NO}_5$ $[\text{M}+\text{H}]^+$ 228.0872 found 228.0872.

(S)-2-(3-Ethylsulfanylcarbonyl-2-hydroxy-propionylamino)-propionic acid methyl ester (68). Transparent oil (0.014 g, 12% yield). ^{13}C NMR analysis showed

that the compound is in a diastereomeric ratio of 58:42. $R_f = 0.28$ (hexane - ethyl acetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.22 (t, 3H, $J = 7.42$ Hz), 1.40 (d, 3H, $J = 7.11$ Hz), 2.85 (q, 2H, $J = 7.68$ Hz), 3.12 - 3.17 (m, 1H), 3.71, 3.72 (s, 3H), 4.49 - 4.50 (m, 2H), 7.30 - 7.34 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 14.4, 14.5, 18.0, 18.1, 23.5, 47.2, 47.3, 47.7, 52.4, 68.7, 68.8, 171.8, 171.6, 172.9, 173.1, 198.9, 199.2 ppm. IR (neat, cm^{-1}): ν 3392, 2958, 2931, 1741, 1654, 1529, 1454, 1261, 1157, 1097, 1058, 1004, 975. LRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{15}\text{NO}_5\text{S}$ [M] 263.31 found 263.87. HRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{15}\text{NO}_5\text{S}$ $[\text{M}+\text{H}]^+$ 264.0906 found 264.0906.

3-Hydroxy-*N*-((*S*)-1-methoxycarbonyl-ethyl)-2,2-dimethyl-succinamic acid 2-(2-methoxy-ethoxy)-ethyl ester (69). Transparent oil (0.073 g, 42% yield). ^{13}C NMR analysis showed that the compound is in a diastereomeric ratio of 53:47. $R_f = 0.05$ (cyclo hexane - ethyl acetate, 4:6). ^1H NMR (300 MHz, CDCl_3) δ 1.22 (d, 3H, $J = 12.9$ Hz), 1.30 (d, 3H, $J = 6.0$ Hz), 1.41 (d, 3H, $J = 7.0$ Hz), 3.36 (d, 2H, $J = 6.5$ Hz), 3.39 (s, 3H), 3.52-3.56 (m, 3H), 3.73, 3.74 (s, 3H), 3.57-3.62 (m, 4H), 4.08-4.18 (m, 1H), 4.42-4.61 (m, 2H) ppm. ^{13}C NMR (75 MHz, CDCl_3) δ 18.2, 18.3, 19.8, 19.9, 21.3, 21.4, 47.7, 47.8, 47.8, 47.9, 52.6, 59.0, 59.1, 59.2, 62.0, 63.4, 68.8, 68.9, 70.2, 70.5, 71.9, 72.2, 72.7, 75.1, 75.4, 171.4, 171.5, 173.1, 173.3, 176.4, 176.5 ppm. IR (neat, cm^{-1}): ν 3369, 2932, 1738, 1658, 1525, 1453, 1213, 1156, 1091, 1057, 970. LRMS (ESI) m/z calculated for $\text{C}_{15}\text{H}_{27}\text{NO}_8$ $[\text{M}+\text{Na}]^+$ 372.38 found

372.08. HRMS (ESI) m/z calculated for $C_{15}H_{27}NO_8$ $[M+H]^+$ 350.1815 found 350.1815.

Synthesis of thioallyl tagged fluorescent tag (70).

2-(2-Bromo-ethyl)-6-dimethylamino-benz[de]isoquinoline-1,3-dione. To a solution of the alcohol (0.85 g, 3.0 mmol) in $CHCl_3$ at $0\text{ }^{\circ}C$ was added phosphorus tribromide (1.0 mL, 10.6 mmol) dropwise and allowed the temperature to rise to room temperature while stirring. After 3 h of stirring, the solvent was evaporated in vacuo. Ice was added to the crude and the aqueous phase was basified with 2M K_2CO_3 . The aqueous phase was extracted with $CHCl_3$ (3 X 20 mL), the combined organic phase was dried over Na_2SO_4 , filtered and concentrated in vacuo. The crude was purified by flash chromatography using (cyclohexane - ethyl acetate, gradient, 0 to 25%) as the eluent. Yield 80% (0.83 g); R_f = 0.57 (cyclohexane – ethyl acetate, 1:1). 1H NMR (300 MHz, $CDCl_3$) δ 3.05 (s, 6H), 3.58 (t, 2H, J = 7.3 Hz), 4.49-4.53 (m, 2H), 7.05 (d, 1H, J = 8.2 Hz), 7.56-7.62 (m, 1H), 8.37-8.43 (m, 2H), 8.49-8.51 (m, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.2, 41.2, 45.0, 113.5, 122.9, 125.1, 125.4, 130.2, 131.5, 131.7, 133.2, 157.3, 164.6, 165.4 ppm. IR (neat, cm^{-1}): ν 3452, 2951, 2870, 2349, 1915, 1681, 1641, 1566, 1525, 1450, 1386, 1342, 1255, 1203, 1122, 1095, 1080, 1062. LRMS (ESI) m/z calculated for $C_{16}H_{15}BrN_2O_2$ $[M+H]^+$ 349.21 found 349.90. HRMS (ESI, m/z) calculated for $C_{16}H_{15}BrN_2O_2$ $[M+H]^+$ 347.0395 found 347.0395.

2-(2-Allylsulfanyl-ethyl)-6-dimethylamino-benz[de]isoquinoline-1,3-dione. To a solution of 2-propene-1-thiol (0.02 mL, 0.3 mmol) in DMF (5 mL) was added a solution of K_2CO_3 (0.04 g, 0.3 mmol) in H_2O (5 mL). After stirring for 10 minutes, a solution of the bromide (0.1 g, 0.3 mmol) in DMF (1 mL) was added. After 3 h all the starting material was consumed. The reaction was poured over ice and extracted with EtOAc (3 X 5 mL), the combined organic phase was dried over Na_2SO_4 , filtered and concentrated in vacuo. The crude was purified by flash chromatography using (cyclohexane – ethyl acetate, gradient, 0 to 15%) as the eluent. Yield 87% (0.086 g). R_f = 0.29 (hexane – ethyl acetate, 8:2). 1H NMR (300 MHz, $CDCl_3$) δ 2.78-2.83 (m, 2H), 3.08 (s, 6H), 3.25 (d, 2H, J = 7.3 Hz), 4.31-4.36 (m, 2H), 5.09-5.26 (m, 2H), 5.74-5.88 (m, 1H), 7.09 (d, 1H, J = 8.2 Hz), 7.63 (dd, 1H, J = 8.50, 7.3 Hz), 8.41-8.47 (m, 2H), 8.55 (dd, 1H, J = 7.3, 1.2 Hz) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.1, 28.3, 34.6, 39.4, 45.0, 113.5, 115.0, 117.7, 123.1, 125.1, 125.5, 130.5, 131.3, 131.5, 132.9, 134.3, 157.2, 164.1, 164.7 ppm. IR (neat, cm^{-1}): ν 2828, 2852, 2795, 1688, 1643, 1582, 1517, 1453, 1422, 1380, 1349, 1316, 1255, 1210, 1130, 1077, 1023, 993, 780, 756, 578. LRMS (ESI) m/z calculated for $C_{19}H_{20}N_2O_2S$ $[M+H]^+$ 341.44 found 341.07. HRMS (ESI, m/z) calculated for $C_{19}H_{20}N_2O_2S$ $[M+H]^+$ 341.1324 found 341.1321.

Olefin cross-metathesis of fluorescent tag with Mukaiyama-aldol product.

3-Hydroxy-N-((S)-1-methoxycarbonyl-ethyl)-2,2-dimethyl-succinamic acid (Z)-5-[2-(6-dimethylamino-1,3-dioxo-1H,3H-benz[de]isoquinolin-2-yl)-ethylsulfanyl]-pent-3-enyl ester (72). To a solution of fluorescent thio allyl (0.002 g, 0.005 mmol) and alkene peptide (0.0065 g, 0.02 mmol) in *t*-BuOH:H₂O (1:2, 0.065 mL) at 32 °C was added a Hoveyda-Grubbs 2nd generation catalyst solution (0.026 mL, 0.0002 mmol, of a 0.9 mg solution of the catalyst in 0.15 mL *t*-BuOH) and stirred. After 1 h another batch of catalyst solution (0.026 mL, 0.0002 mmol) was added followed by H₂O (0.026 mL). Likewise two more batches of catalyst and H₂O were added and stirred overnight. After overnight stirring taken 10 µL out and diluted with (90 µL) MeOH and taken the crude reaction for LC-ESI-MS analysis. Upon completion the solvent was evaporated in vacuo. The crude was purified by flash chromatography using (cyclohexane – ethyl acetate, gradient, 20 to 100%) as the eluent. Yield 68% (0.0021 g). *R_f* = 0.29 (ethyl acetate – hexane, 1:4). ¹H NMR (500 MHz, CDCl₃) 1.26 (m, 3H), 1.27-1.28 (m, 3H), 1.40-1.42 (m, 4H), 2.34-2.45 (m, 2H), 2.79-2.88 (m, 2H), 3.13 (s, 6H), 3.71-3.75 (m, 5H), 4.15-4.21 (m, 3H), 4.33-4.39 (m, 2H), 4.55-4.59 (m, 2H), 5.53-5.60 (m, 1H), 5.64-5.69 (m, 1H), 7.12-7.18 (m, 2H), 7.67-7.70 (m, 1H), 8.47-8.50 (m, 2H), 8.57 (d, 1H, *J* = 7.0 Hz) ppm. ¹³C NMR (75 MHz, CDCl₃) δ 18.3, 20.1, 20.5, 21.3, 21.8, 27.1, 28.3, 31.7, 33.7, 39.5, 45.0, 47.2, 47.9, 48.0, 52.6, 64.3,

75.0, 75.4, 113.6, 123.0, 125.2, 125.4, 128.7, 129.3, 130.5, 131.5, 131.6, 133.0, 157.2, 164.2, 164.7, 171.2, 173.2, 177.2 ppm. IR (neat, cm^{-1}): ν 3398, 2981, 2938, 2828, 2852, 2795, 1734, 1688, 1655, 1643, 1582, 1517, 1453, 1422, 1380, 1349, 1316, 1255, 1210, 1156, 1130, 1077, 1023, 993, 913, 780, 756, 735, 578. LRMS (ESI) m/z calculated for $\text{C}_{31}\text{H}_{39}\text{N}_3\text{O}_8\text{S}$ $[\text{M}+\text{H}]^+$ 614.72 found 614.01. HRMS (ESI, m/z) calculated for $\text{C}_{31}\text{H}_{39}\text{N}_3\text{O}_8\text{S}$ $[\text{M}+\text{H}]^+$ 614.2536 found 614.2540.

Synthesis of biotin azide (71).

N-(3-azidopropyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-

yl)pentanamide (71). To a solution of biotin (0.12 g, 0.5 mmol) and EDC.HCl (0.12 g, 0.6 mmol) in methanol (1 mL) and acetonitrile (3 mL) was added 3-azidopropan-1-amine (0.1 g, 1.0 mmol) and stirred for 18 h. The solvent was evaporated in vacuo and redissolved in MeOH and taken for HPLC purification using 0-50% acetonitrile in water as the eluent. Yield 70% (0.77 g). White powder. ^1H NMR (400 MHz, CD_3OD): δ 1.26–1.86 (m, 10H), 2.18 (t, 0.6H, $J = 7.4$ Hz), 2.32 (t, 2H, $J = 7.4$ Hz), 2.67 (d, 1H, $J = 12.7$ Hz), 2.90 (dd, 1H, $J = 12.7, 5.0$ Hz), 3.15–3.29 (m, 4H), 4.27 (dd, 1H, $J = 7.8, 4.5$ Hz), 4.46 (dd, 1H, $J = 7.8, 4.9$ Hz), 4.84 (m, 1H, hidden under neath solvent peak) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 25.9, 29.5, 29.7, 34.6, 41.0, 52.0, 57.0, 60.5, 61.7, 63.4, 166.1, 175.9 ppm. FTIR (neat, cm^{-1}): ν 3400, 2953, 2104, 1645, 1265, 1116, 1014, 810, 688. LRMS (ESI) m/z

calculated for $C_{13}H_{22}N_6O_2S$ $[M+H]^+$ 327.42 found 327.31. HRMS (ESI) m/z calculated for $C_{13}H_{22}N_6O_2S$ $[M+H]^+$ 327.1603 found 327.1552.

Click reaction of azide **71 with *Mukaiyama*-aldol product.**

To a solution of peptide **67** (0.003 g, 0.013 mmol) and biotin azide **71** (0.009 g, 0.026 mmol) in $H_2O:t$ -BuOH 1:1 (0.05 mL) was added Na-ascorbate (0.003 mL, 0.5 M solution in ddH_2O) followed by $CuSO_4 \cdot 5H_2O$ (0.0003 g, 0.0001 mmol) and stirred vigorously. Upon reaction completion as judged from HPLC-ESI-MS, the solvent was evaporated and product purified by reversed phase HPLC purification using 0-50% acetonitrile in water as the eluent. Yield 76% (0.005 g). White powder.

1H NMR (500 MHz, CD_3OD) δ 1.16–1.30 (m, 3H), 1.41–1.79 (m, 6H), 2.14 (t, 0.7H, $J = 7.5$ Hz), 2.20 (t, 1H, $J = 7.4$ Hz), 2.70 (d, 1H, $J = 12.5$ Hz), 2.92 (dd, 1H, $J = 12.5$, 5.0 Hz), 3.16–3.24 (m, 4H), 3.39–3.45 (m, 1H), 3.72, 3.74, 3.75 (s, 3H), 4.29–4.73 (m, 4H), 6.49 (s, 1H), 8.42 (s, 2.4 H), 8.58 (s, 0.6H) ppm (some peaks hidden under the solvent). LRMS (ESI) m/z calculated for $C_{23}H_{35}N_7O_7S$ $[M+H]^+$ 554.63 found 553.96. HRMS (ESI) m/z calculated for $C_{23}H_{35}N_7O_7S$ $[M+H]^+$ 554.2397 found 554.2402.

CHAPTER 3

General Procedure for Protein Oxidation.

Scheme 3-5: Myoglobin functionalization attempt by indium mediated Allylation

To a solution of myoglobin (120 μL of 250 μM solution in 25 mM sodium phosphate buffer, pH 6.5) and sodium phosphate buffer (180 μL , 25 mM, pH 6.5) in a 1.6 mL eppendorf tube was added a solution of pyridoxal 5'-phosphate (**9**, 300 μL of a 20 mM solution in 25 mM phosphate buffer, pH adjusted to 6.5 with 2 M NaOH). After brief agitation for proper mixing, the mixture was incubated at 37 $^{\circ}\text{C}$ for 18 h without further agitation. The PLP was removed from the reaction mixture via spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) eluting with water. An aliquot of this purified protein (100 μL) was treated with biotinamidohexanoic acid hydrazide (**3**, 80 μL , 25 mM solution in 16% EtOH-water system) and water (20 μL). The reaction was allowed to sit without agitation for another 18 h (Scheme 3-5). After freeze drying the reaction mixture was analyzed by LC-ESI-MS (Poros R1/H column), SDS-PAGE and Western Blotting. LC-ESI-MS [Biotinylated protein **11**, expected mass for (myoglobin aldehyde + 352 – H_2O) = 17302, observed mass 17303] (Figure 3-1, **a** and **b**); 352 is the expected mass increase for myoglobin aldehyde after

modification with biotinamido hexanoic acid hydrazide **10** to form the hydrazone **11**], gel analysis and western blotting confirmed biotinylation and hence generation of *N*-terminal aldehyde (**Figure 6-1, a and b**).

Figure 6-1: LC-ESI-MS and Western Blotting analysis of modified horse heart Myoglobin. **a)** Unmodified Myoglobin. **b)** Biotinylated Myoglobin **4**. **c)** Allylation modified myoglobin **12**. **d)** SDS-PAGE analysis of modified products upon coomassie staining. **e)** Western blot analysis of the biotinylated product using Avidin-AP.

General Procedure for Protein Functionalization by Indium-mediated Allylation reaction.

After preparing the aldehyde containing myoglobin by the procedure described above, an aliquot of the purified mixture (100 μL , 37.5 μM final protein concentration) was added to a pre-vortexed suspension of allylbromide (260 μL , 11.5 mM in *t*-BuOH), Indium (0.0005 g, .004 μmol) and water (40 μL); after brief vortex mixing and sonication the reaction mixture was incubated on a laboratory rotisserie for 20 h at room temperature. Upon spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) and freeze drying the reaction mixture was analyzed by LC-ESI-MS (Poros R1/H column). LC-ESI-MS confirmed 50% conversion to desired allylation product **12** (m/z 16991) % conversion was determined from the area under the peak values in the respective LC-ESI-MS chromatograms for; unmodified myoglobin

[expected mass 16950, observed mass 16951], myoglobin dihydrate **14** [expected mass, (myoglobin aldehyde **13** + H₂O = 16967, observed mass 16968)], allylated protein **12**, [expected mass for (myoglobin aldehyde + 41 = 16990, observed mass 16991); 41 is the expected mass increase for myoglobin aldehyde after undergoing allylation with allyl bromide in the presence of indium]; allylated protein **14** + H₂O, [expected mass for (allylated protein **14** + H₂O = 17008, observed mass 17008)]. (**Figure 6-1, c**).

General Procedure for Protein Control Reaction. In the control reaction the aldehyde generation was inhibited by performing the reaction without PLP under identical conditions. After spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) eluting with water, an aliquot of the mixture (100 µL, 37.5 µM final protein concentration) was added to a pre-vortexed suspension of allylbromide (260 µL, 11.5 mM in *t*-BuOH), Indium (.0005 g, .004µmol) and water (40 µL); after brief vortex mixing and sonication the reaction mixture was incubated on a laboratory rotisserie for 20 h at room temperature. Upon spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) and freeze drying the reaction mixture was analyzed by LC-ESI-MS (Poros R1/H column) revealing only unmodified myoglobin.

Procedure for Trypsin Digestion of Myoglobin. Proteolytic protein digests by trypsin were performed by incubating the desalted and concentrated protein (40 µg) with 8M urea (5 µL in 0.4M NH₄HCO₃), water (44 µL), tris (1 µL, pH 10) and trypsin (Promega, 1 µL, 1 µg/µL) at 37 °C overnight. The resulting peptide fragments were then analyzed by LC-ESI-MS using Vydac C18 Column (1 X 150 mm), at 40 °C and at a flow rate of 0.08 mL/min with eluent containing 90% acetonitrile (with 0.045% trifluoroacetic acid) and 0.05% trifluoroacetic acid (**Figure 6-2**).

Figure 6-2. HPLC-ESI-MS analysis of trypsin digest of myoglobin. **a)** Unmodified protein, the *N*-terminal fragment (rt 17.8 min) (**residues 1-16**) GLSDGEWQQVLNVWGK (expected mass = 1815, observed mass = 1814.9). **b)** Biotinylated protein **11** (rt 19.2 min) (**residues 1-16**) (expected mass = 1815 + 352 = 2167, observed mass = 2167.0). +352 is the expected mass increase for myoglobin after modification with biotinamido hexanoic acid hydrazide to form the hydrazone. **c)** MS-MS of Allylation modified and unmodified *N*-terminal peptide. **d)** MS-MS of unmodified *N*-terminal fragment (**zoom region 150-350**) GL [expected mass = 171, observed mass = 171.5, entry **d**, Figure 3-3] 213 (+42)). MS-MS of modified *N*-terminal fragment (**zoom region 150-350**) GL [expected mass $[M + H] = 171 + 41 + 1 = 213$, observed mass = 213, entry **c**, Figure 3-3] 41 is the expected mass increase for myoglobin after modification with allyl bromide though indium mediated allylation **e)** MS of unmodified protein, *N*-terminal fragment (**residues 1-16**) (expected mass = 1815, observed mass =

908.4, ($M/2 + 1$)). **f**) MS of Biotinylated protein, *N*-terminal fragment (**residues 1-16**) (expected mass + 352 = 2167, observed mass = 1085, ($M/2 + 1$)).

Allyl Bromide Quantity Screening. Samples of purified aldehyde containing myoglobin, (100 μ L, 37.5 μ M, final protein concentration in reaction) were treated with different concentrations of pre-vortexed suspension of Allyl Bromide **1**, (80 μ L, X mM, in *t*-BuOH) and (20 μ L) of H₂O. After brief vortex mixing and sonication the reaction mixture was incubated on a laboratory rotisserie for 20 h at room temperature. Upon spin concentration and freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column). 50% conversion to allylation product is achieved with as low as (67 equivalent) of allyl bromide (Table 3-2, entries 1-4).

Solvent Studies. Samples of purified aldehyde containing myoglobin, (100 μ L, 37.5 μ M) was added to pre-vortexed suspension of allyl bromide (40 μ L, 34.6 mM, in *t*-BuOH) and (60 μ L) different solvent system. After brief vortex mixing and sonication the reaction mixture was incubated on a laboratory rotisserie for 20 h at room temperature. Upon spin concentration and freeze drying the reaction mixtures were analyzed by ESI-LCMS (Poros R1/H column). As seen in (Table 3-2, entry 5) highest conversion to allylation modified product is achieved in water:*t*-BuOH (6:4) system.

Reconstitution of Modified Horse Heart Myoglobin. A 500 μ L of aliquot of spin concentrated (Nanosep centrifugal filter, 10,000 MWCO (PALL)) allylation modified myoglobin (30 nmol) was diluted with 1900 μ L of 100 mM Na₂HPO₄ (pH 7.0). A solution of chlorohemin (20 μ L, 1.0 mg/mL, 30 nmol) in 100 mM NaOH was then added and the reaction mixture incubated on a laboratory rotisserie for 20 h at room temperature. After filtration the modified, reconstituted sample was taken for analysis.

UV-Vis Spectroscopy. The modified reconstituted sample was analyzed by UV-Vis spectrum that revealed a strong absorbance at $\lambda_{\text{max}} = 409$ nm. This 409 nm

absorbance is characteristic of the heme moiety bound to myoglobin.¹⁶¹ The obtained UV-Vis spectrum was in good agreement with data obtained from a sample of unmodified myoglobin (Scheme 3-6).

Circular Dichroism Spectroscopy. Myoglobin samples (3 μM) were prepared in 100 mM sodium phosphate buffer (pH 7.0). Circular dichroism spectra were recorded with Jasco Model J-810 spectrophotometer. Protein solutions were placed in a cylindrical, water-jacketed quartz cell (1-cm path length, volume 3.0 mL, 25 $^{\circ}\text{C}$), and the average of three scans from 190 to 250 nm was collected. The ellipticity values were plotted against wavelength using prizm software. The ultraviolet CD spectra exhibited by the modified reconstituted sample suggested that the secondary structure of the modified protein remained essentially unperturbed relative to that of unmodified myoglobin (Scheme 3-7).

General Procedure for Protein Functionalization by Mukaiyama aldol Condensation. After preparing the aldehyde containing myoglobin by the procedure described above, an aliquot of the purified mixture (100 μL , 37.5 μM final protein concentration) was treated with (64 μL) water, 1-Methoxy-2-methyl-1-trimethylsiloxy propene (**15**, 0.3 μL , 5 M, Technical grade 95%), and (36 μL) *t*-BuOH. After proper pipette mixing this reaction was incubated at ambient temperature for 5 h without further agitation. Upon spin concentration and freeze drying the reaction mixture was analyzed by LC-ESI-MS (Poros R1/H column). LC-ESI-MS confirmed 90% conversion to desired mukaiyama aldol (**16**, m/z 17052) product (**Figure 6-3**). % conversion was determined from the area under the peak values in the respective LC-

¹⁶¹ Makinen, M. W.; Churg, A. K. In *Iron Porphyrins*; Lever, A. B. P.; Gray, H. B., Eds.; Addison-Wesley: Reading, 1983; Vol. 1, pp 141-236.

ESI-MS chromatograms for; unmodified myoglobin [expected mass 16950, observed mass 16951], *Mukaiyama*-aldol modified protein **16**, [expected mass for (myoglobin aldehyde + 101 = 17051, observed mass 17052); 101 is the expected increase for myoglobin aldehyde after undergoing *Mukaiyama*-aldol reaction.

Figure 6-3: LC-ESI-MS of modified horse heart *Mukaiyama* aldol modified myoglobin **16**, the TFA adduct is generated from the HPLC-MS method.

Solvent Studies. Samples of purified aldehyde containing myoglobin, (100 μ L, 37.5 μ M, final protein concentration in reaction) were treated with 1-Methoxy-2-methyl-1-trimethylsiloxy propene (**15**, 0.3 μ L, 5 M, Technical grade 95%) and (100 μ L) different solvent system. After proper pipette mixing they were incubated at ambient temperature for 5 h. Upon spin concentration and freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column). As seen in (**Table 3-4**), highest conversion to *Mukaiyama* aldol modified product is achieved in water : *t*-BuOH (5:1) system.

General Procedure for Protein Control Reaction. In the control reaction the aldehyde generation reaction was inhibited by performing the reaction without PLP under identical conditions. After spin concentration (Nanosep centrifugal filter 10,000 MWCO (PALL)) eluting with water, an aliquot of the mixture (100 μ L, 37.5 μ M final protein concentration) was treated with (64 μ L) water, 1-Methoxy-2-methyl-1-trimethylsiloxy propene (**15**, 0.3 μ L, 5 M, Technical grade 95%) and (36 μ L) *t*-BuOH. After proper mixing with pipette, this reaction was incubated at ambient temperature for 5 h without further agitation. Upon spin concentration and freeze drying the reaction mixture was analyzed by LC-ESI-MS (Poros R1/H column) revealing only unmodified myoglobin.

Procedure for Trypsin Digestion of Myoglobin. Proteolytic protein digests by trypsin were performed by incubating the desalted and concentrated protein (40 μ g) with 8M urea (5 μ L in 0.4M NH_4HCO_3), water (44 μ L), tris (1 μ L, pH 10) and trypsin (Promega, 1 μ L, 1 μ g/ μ L) at 37 °C overnight. The resulting peptide fragments were then analyzed by LC-ESI-MS using Vydac C18 Column (1 X 150 mm), at 40 °C and at a flow rate of 0.08 mL/min with eluent containing 90% acetonitrile (with 0.045% trifluoroacetic acid) and 0.05% trifluoroacetic acid (**Figure 6-4**).

a

Figure 6-4. HPLC-ESI-MS analysis of trypsin digest of myoglobin. **a)** Mukaiyama aldol modified protein **16** (**residues 1-16**) GLSDGEWQQVLNVWGK (expected mass of unmodified peptide = 1815) + [87 (when terminal methyl ester OMe is hydrolyzed to OH). GLSDGEWQQVLNVWGK (expected mass = 1815 + 87 = 1902, observed mass = 1902. Although [101 (when terminal methyl ester OMe is intact) + GLSDGEWQQVLNVWGK (expected mass = 1815 + 101 = 1916)] is the expected mass increase for myoglobin after modification with ketene silyl acetal to form the *Mukaiyama*-aldol product. This [-14 ($\text{OCH}_3 - \text{OH} = 14$)] difference in the observed mass results from the hydrolysis of the *N*-terminal methyl ester to corresponding acid from exposure to basic conditions during tryptic digestion. **b)** MS of unmodified *N*-terminal fragment (**residues 1-16**) GLSDGEWQQVLNVWGK (expected mass = 1815, observed mass = 1814.9). **c)** MS/MS of Mukaiyama aldol modified protein, *N*-terminal fragment (**residues 1-16**) (molecular weight 1902.9).

Silyl ketene acetal Quantity Screening. Samples of purified aldehyde containing myoglobin, (100 μL , 37.5 μM , final protein concentration in reaction) were treated with different quantities of 1-Methoxy-2-methyl-1-trimethylsiloxy propene (**15**, X μL ,

5 M, Technical grade 95%) and water (64 μL) and *t*-BuOH (36 μL). After proper pipette mixing they were incubated at ambient temperature for 5 h. Upon freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column). High conversion (90%) to Mukaiyama aldol modified product is achieved with as low as (0.3 μL , 5 M, Technical grade 95%, 190 equivalent) of ketene silyl acetal (Table 3-5).

Time Studies. Samples of purified aldehyde containing myoglobin, (100 μL , 37.5 μM , final protein concentration in reaction) were treated with 1-Methoxy-2-methyl-1-trimethylsiloxy propene (**15**, 0.3 μL , 5 M, Technical grade 95%), water (64 μL) and *t*-BuOH (36 μL). After proper pipette mixing they were incubated at ambient temperature for varying lengths of time. Upon freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column). As seen in (**Figure 3-11 and 6-5**) high conversion to Mukaiyama aldol modified product is achieved within 5 mins (76%) and reaches maximum to 90% in 1 h and stays constant henceforth.

Figure 6-5. LC-ESI-MS of Time study for the conversion to Mukaiyama Aldol product. **a)** At 5 min (76% conversion to product). **b)** At 1 h (90% conversion to product). **c)** At 20 h (90% product).

UV-Vis Spectroscopy. The modified sample was analyzed by UV-Vis spectrum that revealed a strong absorbance at $\lambda_{\text{max}} = 409 \text{ nm}$. This 409 nm absorbance is characteristic of the heme moiety bound to myoglobin.⁵ The obtained UV-Vis

spectrum was in good agreement with data obtained from a sample of unmodified myoglobin (**Figure 3-12**).

Circular Dichroism Spectroscopy. Myoglobin samples (3 μM) were prepared in 100 mM sodium phosphate buffer (pH 7.0). Circular dichroism spectra were recorded with Jasco Model J-810 spectrophotometer. Protein solutions were placed in a cylindrical, water-jacketed quartz cell (1-cm path length, volume 3.0 mL, 25 $^{\circ}\text{C}$), and the average of three scans from 190 to 250 nm was collected. The ellipticity values were plotted against wavelength using prizm software. The ultraviolet CD spectra exhibited by the modified sample suggested that the secondary structure of the modified protein remained essentially unperturbed relative to that of unmodified myoglobin (**Figure 3-13**).

Peroxidase Kinetics of Modified Horse Heart Myoglobin.¹⁶² The steady state kinetics of unmodified and Mukaiyama aldol modified horse heart myoglobin were analyzed with benchtop spectrophotometer fitted with microtiter plate reader (Infinite Tecan M1000). Varying concentrations (0 to 273.5 μM) of 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, St. Louis, MO) was added to an assay mixture containing (0.1 μM) Mb-H preincubated with (0.3 μM) of H_2O_2 in 50 mM sodium phosphate buffer, pH 6.5, for 2 min 30 sec. The initial rates for the oxidation of ABTS were measured by the change in absorbance at 412 nm over 3 min, due to the generation of radical cation oxidation product. Each concentration was analyzed in replicates of four. The rate constants k_1 and k_3 were calculated from the equation defined by Dunford⁹ ($2[\text{E}]_0/V = 1/k_1[\text{H}_2\text{O}_2] + 1/k_3[\text{ABTS}]$) for the peroxidase ping-pong mechanism. The rate constant values k_1 (1.4 vs 1.7 $\times 10^4$) and k_3 (2.6 vs 2.8

¹⁶² Dunford, H. B. (1991) in *Peroxidases in Chemistry and Biology*, Vol II, eds. Everse, K. E. & Grisham, M. B. (CRC, Boca Raton), pp. 1-24.

$\times 10^4$) obtained under steady state condition are almost similar for both modified and unmodified myoglobin respectively suggesting that the *N*-terminal modification has relatively no effect on the reaction of myoglobin with its substrates ABTS and H_2O_2 (Figure 3-14).

Screening with different types of silyl ketene acetal. Samples of purified aldehyde containing myoglobin, (100 μL , 37.5 μM) were treated with different silyl ketene acetals (0.3 μL each), water (64 μL) and *t*-BuOH (36 μL). After proper pipette mixing they were incubated at ambient temperature for 5 h. Upon freeze drying the reaction mixtures were analyzed by LC-ESI-MS (Poros R1/H column). Successful incorporation of an alkene group was achieved through reaction of silyl ketene acetal **19**, (95% conversion) (Table 3-6, Figure 6-6). However, high heterogeneity in the MS data, was unable to confirm the reaction between the protein and other silyl ketene acetals.

Figure 6-6: LC-ESI-MS of *N*-terminal alkene functionalized horse heart myoglobin **25**.

Representative experimental procedure for the synthesis of protected dipeptide: **(S)-2-(2-tert-Butoxycarbonylamino-acetylamino)-propionic acid methyl ester.**

To a solution of Boc-L-glycine (0.44 g, 2.5 mmol, 1.0 equiv.) in 30 mL CH₂Cl₂ was added L-alanine methyl ester hydrochloride (0.42 g, 3 mmol, 1.2 equiv.) and stirred. To this stirring solution was added Et₃N (0.52 mL, 3.75 mmol, 1.5 equiv.) followed by *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate HATU (1.140 g, 3.0 mmol, 1.2 equiv.). The reaction was stirred overnight under nitrogen gas. Solvent was evaporated and the residue was dissolved in EtOAc (30 mL). The organic phase was washed with 10% citric acid (2 x 10 mL) and saturated NaHCO₃ (2 x 20 mL) and then dried over Na₂SO₄, filtered, and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane – ethyl acetate, gradient, 0 to 70%) to afford the desired product as pale transparent gum (0.62 g, 95% yield). *R*_f = 0.5 (hexane – ethyl acetate, 1:1).

¹H NMR (400 MHz, CDCl₃) δ 1.36 (d, 3H, *J* = 7.2 Hz), 1.41 (s, 9H), 3.71 (s, 3H), 3.72-3.81 (m, 2H), 4.53-4.57 (m, 1H), 5.09 (brs, 1H), 6.58-6.59 (m, 1H) ppm.

¹³C NMR (100 MHz, CDCl₃) δ 18.4, 28.3, 41.6, 49.7, 53.0, 80.1, 156.0, 168.9, 174.5 ppm.

IR (neat, cm⁻¹): ν 2976, 2929, 2885, 2615, 2351, 1730, 1714, 1693, 1514, 1454, 1382, 1367, 1249, 1165, 1060, 1029, 914, 852.

LRMS (ESI) *m/z* calculated for C₁₁H₂₀N₂O₅ [2M+H]⁺ 521.28 found 521.18.

HRMS (ESI) *m/z* calculated for C₁₁H₂₀N₂O₅ [M+H]⁺ 261.1451 found: 261.1450.

[α]_D²⁰ -28.39 (c = 1.0 g/100 mL, MeOH).

Representative experimental procedure for *N*-terminal deprotection of dipeptide:

(*S*)-2-(2-Amino-acetyl-amino)-propionic acid methyl ester. To a solution of protected dipeptide (0.62 g, 2.37 mmol) in dichloromethane (18 mL) at 0 °C was added trifluoroacetic acid (2 mL) to give a 10% solution. The reaction was stirred overnight at room temperature. After removing the solvent in rotary evaporator, the product was purified by reversed phase HPLC. The desired product (0.34 g, 90%) was obtained as white solid after freeze drying.

^1H NMR (400 MHz, CD_3OD) δ 1.42 (d, J = 7.32 Hz, 3H), 3.74 (s, 3H), 3.76 (s, 2H), 4.47-4.52 (m, 1H) ppm.

^{13}C NMR (100 MHz, CD_3OD) δ 17.6, 41.6, 49.7, 53.0, 167.4, 174.5 ppm.

IR (neat, cm^{-1}): ν 2963, 1745, 1673, 1598, 1497, 1471, 1425, 1329, 1293, 1269, 1232, 1197, 1174, 1134, 1012, 991, 959, 938, 905, 838, 805, 780, 760, 718, 667.

LRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 161.08 found 161.92. HRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 161.0926 found 161.0926.

$[\alpha]_D^{20}$ -27.7 (c = 1.0 g/100 mL, MeOH).

(*S*)-2-(2-tert-Butoxycarbonylamino-acetyl-amino)-4-methyl-pentanoic acid

methyl ester. Obtained as white foam, 0.74 g, Yield 98%. R_f = 0.38 (ethyl acetate - hexane, 1:1).

^1H NMR (400 MHz, CDCl_3) δ 0.90-0.93 (m, 6H), 1.44 (s, 9H), 1.52-1.66 (m, 3H), 3.72 (s, 3H), 3.74-3.87 (m, 2H), 4.60-4.65 (m, 1H), 5.22 (brs, 1H), 6.56-6.57 (m, 1H) ppm.

^{13}C NMR (100 MHz, CDCl_3)

δ 22.0, 22.9, 24.9, 28.4, 41.7, 44.5, 50.8, 52.5, 80.5, 156.2, 169.5 173.4 ppm.

IR (neat, cm^{-1}): ν 3298, 2979, 1661, 1521, 1455, 1366, 1213, 1153, 1051, 1030, 984, 941, 862, 512, 460.

LRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$: 303.37 Found: 303.20.

HRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$: 303.3712 Found: 330.3683.

$[\alpha]_D^{20}$ -31.86 ($c = 1.0$ g/100 mL, MeOH).

(S)-2-(2-Amino-acetyl-amino)-4-methyl-pentanoic acid methyl ester. Obtained as white solid, 0.47 g, Quantitative.

^1H NMR (400 MHz, CD_3OD) δ 0.90-0.94 (m, 6H), 1.58-1.70 (m, 3H), 3.72-3.73 (s, 5H), 4.49-4.53 (m, 1H) ppm.

^{13}C NMR (100 MHz, CD_3OD) δ 21.8, 23.2, 25.9, 41.1, 41.5, 52.3, 52.8, 167.5, 174.3.

IR (neat, cm^{-1}): ν 2963, 1745, 1673, 1598, 1497, 1471, 1425, 1329, 1293, 1269, 1232, 1197, 1174, 1134, 1012, 991, 959, 938, 905, 838, 805, 780, 760, 718, 667.

LRMS (ESI) m/z calculated for $\text{C}_9\text{H}_{18}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 203.25 found 203.00.

HRMS (ESI) m/z calculated for $\text{C}_9\text{H}_{18}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 203.1396 found 203.1401.

$[\alpha]_D^{20}$ -30.90 ($c = 1.0$ g/100 mL, MeOH).

Representative experimental procedure for N-terminal oxidation and***Mukaiyama-Aldol reaction:***

To a solution of peptide (0.045 g, 0.22 mmol, in 25 mM sodium phosphate buffer, pH 6.5, 2 mL) was added a solution of pyridoxal 5'-phosphate (0.146 g, 0.55 mmol in 25 mM phosphate buffer, pH adjusted to 6.5 with 2 M NaOH, 0.5 mL). The reaction was stirred at 37 °C for 22 h. This was followed by the addition of 1-Methoxy-2-methyl-1-trimethylsiloxy propene (0.45 mL, 2.2 mmol) and stirred for another 8 h. After freeze drying the reaction mixture was purified by HPLC-MS.

(S)-2-(2-Hydroxy-3-methoxycarbonyl-3-methyl-butrylamino)-4-methyl-pen

tanoic acid methyl ester. Obtained as pale transparent oil, 0.054 g, Yield 72%. R_f = 0.29 (ethyl acetate - hexane, 1:1).

^1H NMR (400 MHz, CDCl_3) δ 0.93-0.95 (m, 6H), 1.19 (s, 3H), 1.29 (s, 3H), 1.61-1.67 (m, 3H), 3.55 (d, 1H, J = 6.0 Hz), 3.72 (s, 3H), 3.75 (s, 3H), 4.41 (d, 1H, J = 6.0 Hz), 4.57-4.63 (m, 1H) ppm.

^{13}C NMR (100 MHz, CDCl_3)

δ 19.7, 21.9, 22.5, 23.0, 25.0, 41.4, 47.1, 50.8, 52.5, 52.6, 74.8, 171.7, 173.3, 178.4.

IR (neat, cm^{-1}): ν 3250, 2976, 2931, 2885, 2625, 2351, 1907, 1730, 1714, 1693, 1514, 1454, 1382, 1367, 1249, 1165, 1060, 1029, 914, 852, 781, 756 cm^{-1} .

LRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{25}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 304.35 found 304.4.

HRMS (ESI) m/z calculated for $\text{C}_{14}\text{H}_{25}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 304.3521 found 304.3471.

(S)-3-tert-Butoxy-2-(2-tert-butoxycarbonylamino-acetylamino)-propionic acid methyl ester. Obtained as transparent gum, 0.77 g, Yield 93%. R_f = 0.42 (ethyl acetate - hexane, 1:1).

^1H NMR (400 MHz, CDCl_3) δ 1.11 (s, 9H), 1.44 (s, 9H), 3.54 (dd, 1H, J = 9.1, 3.2 Hz), 3.72 (s, 3H), 3.79-3.82 (m, 2H), 3.88-3.89 (m, 1H), 4.68-4.71 (m, 1H), 6.82-6.84 (m, 1H) ppm.

^{13}C NMR (100 MHz, CDCl_3)

δ 27.4, 28.4, 44.3, 52.6, 52.9, 62.0, 73.7, 80.3, 160.4, 169.4, 170.9 ppm.

IR (neat, cm^{-1}): ν 3297, 2976, 2878, 1668, 1507, 1392, 1365, 1248, 1167, 1087, 930, 882, 721, 460, 411.

LRMS (ESI) m/z calculated for $\text{C}_{15}\text{H}_{28}\text{N}_2\text{O}_6$ $[2\text{M}+\text{H}]^+$ 665.78 found 665.35.

HRMS (ESI) m/z Calcd for $\text{C}_{15}\text{H}_{28}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$ 333.2026 found 333.2026.

$[\alpha]_D^{20}$ -31.99 (c = 1.0 g/100 mL, MeOH).

(S)-2-(2-Amino-acetylamino)-3-tert-butoxy-propionic acid methyl ester. Obtained as transparent gum, 0.43 g, Yield 80%.

^1H NMR (400 MHz, CDCl_3) δ 1.1 (s, 9H), 3.54-3.56 (m, 1H), 3.71 (s, 3H), 3.76-3.89 (m, 3H), 4.70 (brs, 1H), 7.84-7.86 (m, 1H) ppm.

^{13}C NMR (100 MHz, CDCl_3) δ 27.2, 41.2, 52.7, 53.6, 61.7, 73.8, 167.2, 171.2 ppm.

IR (neat, cm^{-1}): ν 3435, 2978, 1643, 1633, 1440, 1203, 1141, 891, 837, 802, 723, 665.

LRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{20}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 233.38 found 233.16.

HRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_{20}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 233.1501 found 233.1505.

$[\alpha]_D^{20}$ -29.87 ($c = 1.0$ g/100 mL, MeOH).

(S)-2-(2-Amino-acetylamino)-3-hydroxy-propionic acid methyl ester. Obtained as slightly yellow oil, 0.33 g, Yield quantitative.

^1H NMR (300 MHz, CD_3OD) δ 3.75 (s, 3H), 3.79 (s, 2H), 3.80-3.85 (m, 1H), 3.90-3.95 (m, 1H), 4.59-4.62 (m, 1H) ppm.

^{13}C NMR (75 MHz, CD_3OD) δ 41.5, 52.9, 56.3, 62.7, 167.8, 171.9 ppm.

IR (neat, cm^{-1}): ν 3234, 2890, 1735, 1667, 1556, 1438, 1361, 1182, 1132, 1048, 838, 799, 722, 517, 421.

LRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 177.17 found 177.16.

HRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 177.0875 found 177.0874.

$[\alpha]_D^{20}$ -32.13 ($c = 1.0$ g/100 mL, MeOH).

(N-((S)-2-tert-Butoxy-1-methoxycarbonyl-ethyl)-3-hydroxy-2,2-dimethyl-succinamic acid methyl ester. Obtained as transparent gum, 0.046 g, Yield 56%.

^1H NMR (300 MHz, CDCl_3) δ 1.2 (d, 3H, $J = 7.2$ Hz), 1.29 (d, 3H, $J = 5.5$ Hz), 1.42 (dd, 3H, $J = 7.2, 2.1$ Hz), 3.74 (s, 3H), 3.75 (s, 3H), 4.40-4.41 (m, 1H), 4.55-4.60 (m, 1H), 7.10-7.18 (m, 1H) ppm.

^{13}C NMR (100 MHz, CDCl_3)

δ 18.1, 18.2, 19.5, 19.9, 21.6, 22.1, 46.8, 46.9, 47.7, 47.8, 52.3, 52.3, 52.5, 74.8, 75.2, 171.0, 171.3, 173.0, 173.2, 177.7, 177.8 ppm.

IR (neat, cm^{-1}): ν 3413, 2977, 2879, 1739, 1661, 1520, 1472, 1437, 1392, 1364, 1193, 1134, 1083, 1022, 984, 868, 734, 702, 458, 423.

LRMS (ESI) m/z calculated for $\text{C}_{15}\text{H}_{27}\text{NO}_7$ $[\text{M}+\text{H}]^+$ 334.38 found 334.12.

HRMS (ESI) m/z calculated for $\text{C}_{15}\text{H}_{27}\text{NO}_7$ $[\text{M}+\text{H}]^+$ 334.1866 found 334.1877

(S)-methyl 4-(3-tert-butoxy-1-methoxy-1-oxopropan-2-ylamino)-3-hydroxy-2,2-dimethyl-4-oxobutanoate. (S)-3-tert-Butoxy-2-(2-tert-butoxycarbonylamino-acetylamino)-propionic acid methyl ester. Obtained as transparent gum, 0.79 g, Yield 95%.

^1H NMR (300 MHz, CDCl_3) δ 1.11 (s, 9H), 1.44 (s, 9H), 3.52-3.56 (m, 1H), 3.73 (s, 3H), 3.79-3.83 (m, 1H), 4.67-4.72 (m, 1H), 6.82-6.84 (m, 1H) ppm.

^{13}C NMR (75 MHz, CDCl_3)

δ 27.4, 28.4, 45.8, 52.6, 52.9, 62.0, 73.7, 80.3, 166.8, 169.5, 170.9 ppm.

IR (neat, cm^{-1}): ν 3311, 2976, 2878, 1668, 1507, 1392, 1365, 1248, 1167, 1093, 931, 882, 721, 460, 411.

LRMS (ESI) m/z calculated for $C_{15}H_{26}D_2N_2O_6$ $[M+Na]^+$ 335.21 found 335.25

HRMS (ESI) m/z calculated for $C_{15}H_{26}D_2N_2O_6$ $[M+H]^+$ 335.2151 found 335.2147.

$[\alpha]^{20}_d$ -32.55 ($c = 1.0$ g/100 mL, MeOH).

(S)-methyl 4-(3-tert-butoxy-1-methoxy-1-oxopropan-2-ylamino)-3-hydroxy-2,2-dimethyl-4-oxobutanoate. Obtained as transparent gum, 0.49 g, Yield 88%.

1H NMR (400 MHz, $CDCl_3$) δ 1.10 (s, 9H), 3.53-3.57 (m, 1H), 3.71 (s, 3H), 3.75-3.79 (m, 1H), 4.67-4.70 (m, 1H), 7.86-7.89 (m, 1H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ 27.3, 52.7, 53.6, 61.8, 73.8, 167.2, 169.5, 171.2 ppm.

IR (neat, cm^{-1}): ν 2977, 2879, 1744, 1670, 1542, 1437, 1394, 1365, 1182, 1133, 1100, 1053, 1022, 835, 799, 722, 519, 441, 411.

LRMS (ESI) m/z calculated for $C_{10}H_{18}D_2N_2O_4$ $[M+H]^+$ 235.29 found 235.35

HRMS (ESI) m/z calculated for $C_{10}H_{18}D_2N_2O_4$ $[M+H]^+$ 235.1627 found 235.1627.

$[\alpha]^{20}_d$ -32.27 ($c = 1.0$ g/100 mL, MeOH).

(S)-methyl 4-(3-tert-butoxy-1-methoxy-1-oxopropan-2-ylamino)-3-hydroxy-2,2-dimethyl-4-oxobutanoate. Obtained as transparent gum, 0.49 g, Yield quantitative.

1H NMR (400 MHz, CD_3OD): δ , 3.71 (s, 3H), 3.76-3.80 (m, 1H), 3.86-3.90 (m, 1H), 4.55-4.57 (m, 1H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ , 41.0, 53.0, 56.2, 62.6, 167.7, 172.0 ppm.

IR (neat, cm^{-1}): ν 3255, 2886, 2829, 1735, 1667, 1557, 1439, 1361, 1182, 1132, 1048, 902, 839, 799, 722, 517, 443.

LRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{10}\text{D}_2\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 179.18 found 179.21

HRMS (ESI) m/z calculated for $\text{C}_6\text{H}_{10}\text{D}_2\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 179.1627 found 179.1638.

$[\alpha]_D^{20}$ -34.21 ($c = 1.0$ g/100 mL, MeOH).

(8S,11S,14S)-14-(carboxymethyl)-5-hydroxy-11-(hydroxymethyl)-8-isobutyl-4,4-dimethyl-3,6,9,12,15-pentaoxo-2-oxa-7,10,13,16-tetrazaoctadecan-18-oic acid.

Obtained as transparent oil, 0.075 g, Yield 42%.

^1H NMR (400 MHz, CDCl_3) δ 0.89 (d, 3H, $J = 6.4$ Hz), 0.94 (d, 3H, $J = 6.4$ Hz), 1.09 (s, 3H), 1.20 (s, 3H), 1.55-1.70 (m, 5H), 1.83-1.92 (m, 2H), 2.04-2.08 (m, 6H), 2.38-2.48 (m, 3H), 3.04-3.14 (m, 3H), 3.46-3.47 (m, 1H), 3.65-3.71 (m, 6H), 3.86 (dd, 1H, $J = 5.3, 11.0$ Hz), 4.30 (t, 1H, $J = 5.4$ Hz), 4.41-4.46 (m, 3H), 4.58 (dd, 1H, $J = 5.0, 6.7$ Hz), 6.96 (s, 1H), 8.34 (s, 1H) ppm.

^{13}C NMR (75 MHz, D_2O)

δ 15.2, 19.7, 21.9, 23.4, 26.0, 29.2, 31.1, 32.5, 41.3, 47.8, 48.0, 50.1, 51.9, 52.4, 52.5, 52.6, 52.7, 52.8, 52.9, 53.9, 56.7, 63.1, 76.8, 115.5, 128.3, 136.4, 169.2, 172.5, 173.9, 174.1, 174.5, 174.7, 175.1, 175.3 ppm. □

IR (neat, cm^{-1}): ν 3297, 2979, 2875, 2830, 1672, 1615, 1542, 1472, 1436, 1367, 1266, 1203, 1140, 1087, 866, 799, 736, 701, 648, 519, 470, 458.

LRMS (ESI) m/z calculated for $\text{C}_{28}\text{H}_{46}\text{N}_6\text{O}_{10}\text{S}$ $[\text{M}+\text{H}]^+$ 659.76 found 659.6.

HRMS (ESI) m/z calculated for $\text{C}_{28}\text{H}_{46}\text{N}_6\text{O}_{10}\text{S}$ $[\text{M}+\text{H}]^+$ 659.3074 found 659.3064.

CHAPTER 4

Spiro[4.5]deca-6,9-diene-2,8-dione.¹⁶³ A solution of anhydrous aluminium chloride (15.3 g, 115 mmol) in dry dichloromethane (220 mL) was cooled to -40 °C under nitrogen. Ethylene gas was bubbled into the solution for 20 minutes. A solution of 4-methoxy-phenylacetyl chloride (7.6 mL, 50 mmol) in dry dichloromethane (25 mL) was added dropwise while the flow of ethylene gas was maintained. After addition of the acid chloride was complete, ethylene gas flow was maintained for another 10 min and the temperature allowed to raise to -10 °C over 1 hr and 30 min. The temperature was maintained at -10 °C for an additional 1 hr. The solution turned to a nice red color during this time. When TLC monitoring showed that all the starting material had been consumed, the mixture was poured over ice and stirred for 1 hr. The two layers were separated and the organic layer was washed with saturated sodium bicarbonate (3 X 50 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane - ethylacetate, gradient, 0 to 70%) to afford the desired product as a brown solid (3.5 g, 43% yield). R_f = 0.31 (hexane - ethylacetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 2.08 (t, 2H, J = 8.0 Hz), 2.33 (s, 2H), 2.43 (t, 2H, J = 8.0 Hz), 6.18 (d, 2H, J = 10.0 Hz), 6.85 (d, 2H, J = 10.0 Hz) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 33.5, 35.9, 45.1, 48.3, 128.9, 151.5, 185.3, 214.6 ppm. IR (neat, cm⁻¹): ν 2938, 1732, 1654, 1619, 1402, 1288, 1255, 1155, 1130, 1068, 1044, 1000, 858, 771, 742, 683, 574, 495, 461, 412. GCMS m/z calculated for C₁₀H₁₀O₂ [M] 162.19 found 162.02. HRMS (ESI+) m/z calculated for C₁₀H₁₀O₂ [M+H]⁺ 163.0759 found 163.0766.

¹⁶³ Adam, M. F. and Stanford, R. S. *Syn. Commun.* **1996**, 26, 1623-1627.

2-hydroxyspiro[4.5]deca-6,9-dien-8-one.¹⁶⁴ A solution of Spiro diketone (0.63 g, 3.87 mmol), sec-BuOH (40 mL), dry toluene (8 mL) and aluminium-tri-tert-butoxide (1.4 g, 5.67 mmol) were stirred under reflux condition at 85 °C under nitrogen till all the starting material was converted to product. The reaction was cooled to room temperature and poured on ice water and extracted with (3 X 50 mL) ethyl acetate. The combined organic solution was dried over Na₂SO₄, filtered and concentrated. The residual crude product was purified by flash column chromatography (hexane - ethylacetate, gradient, 0 to 70%) to afford the desired product as white needle (0.48 g, 75% yield). *R_f* = 0.20 (hexane - ethylacetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.80-1.88 (m, 2H), 1.97-2.18 (m, 5H), 2.74 (s, 1H), 4.62 (brs, 1H), 6.80-6.84 (m, 1H), 7.19-7.22 (m, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 35.4, 35.5, 46.5, 47.7, 73.8, 126.3, 126.9, 155.3, 156.6, 186.6 ppm. IR (neat, cm⁻¹): ν 3331, 2932, 2370, 2338, 1650, 1605, 1428, 1415, 1323, 1267, 1149, 1178, 1054, 997, 962, 945, 862, 686, 639, 597, 469, 455, 428. GCMS *m/z* calculated for C₁₀H₁₂O₂[M] 164.20 found 164.06. HRMS (ESI+) *m/z* calculated for C₁₀H₁₂O₂[M+H]⁺ 165.0916 found 165.0916.

¹⁶⁴ Iwata, C.; Yamada, M.; Ida, Y.; Imao, T.; Miyagawa, H.; Miyashita, K. *Chem. Pharm. Bull.* **1988**, *36*, 2864-2871.

Methanesulfonic acid 8-oxo-spiro[4.5]deca-6,9-dien-2-yl ester. To a solution of Spiro alcohol (0.28 g, 1.74 mmol) in dry pyridine (20 mL) stored over molecular sieve overnight under nitrogen, at -10 °C was added methane-sulfonyl chloride (0.64 mL, 8.24 mmol) dropwise. The temperature was raised to room temperature over 2 hours. Upon completion the reaction was poured over a mixture of ice and saturated sodium bicarbonate solution (20 mL). After separation of the two phases the aqueous phase was extracted with (3 X 25 mL) ethyl acetate. The combined organic phase was successively washed with water, saturated CuSO₄, water, saturated NaHCO₃, water, and brine and dried over Na₂SO₄, filtered and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane - ethylacetate, gradient, 0 to 70%) to afford the desired product as transparent oil (0.32 g, quantitative yield). R_f = 0.24 (hexane - ethylacetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.92-1.95 (m, 1H), 2.09-2.42 (m, 5H), 3.07 (s, 3H), 5.37-5.41 (m, 1H), 6.23-6.26 (m, 2H), 6.76-6.80 (m, 1H), 7.02-7.06 (m, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 33.4, 35.6, 38.6, 44.3, 46.9, 83.2, 127.6, 127.9, 152.9, 153.6, 185.6 ppm. IR (neat, cm⁻¹): ν 2924, 2370, 2348, 2324, 1711, 1611, 1498, 1420, 1335, 1140, 1038, 779, 535. GCMS m/z calculated for C₁₁H₁₄O₄S [M] 242.29 found 242.30. HRMS (ESI+) m/z calculated for C₁₁H₁₄O₄S [M+H]⁺ 243.0691 found 243.0691.

Spiro[4.5]deca-2,6,9-trien-8-one. To a solution of mesylate (0.23 g, 1.25 mmol) in dry toluene (10 mL) stored over molecular sieve overnight under nitrogen, was added DBU (0.56 mL, 3.74 mmol) and heated to reflux at 110 °C till all the starting material was consumed. Upon completion the solvent was evaporated and residue redissolved in ethylacetate. The organic phase was successively washed with saturated NaHCO_3 , 1 N HCl and brine and dried over Na_2SO_4 , filtered and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane - ethyl acetate, gradient, 0 to 20%) to afford the desired product as a transparent oil and (1:2) inseparable mixture with its regioisomer (0.17 g, yield 94%). $R_f = 0.75$ (hexane : ethylacetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.98-2.04 (m, 2H), 2.49 (s, 2H), 2.55-2.59 (m, 2H), 5.16-5.18 (m, 1H), 5.73 (s, 1H), 6.02-6.04 (m, 1H), 6.15 (d, 3H, $J = 10.0$ Hz), 6.73 (d, 2H, $J = 10.0$ Hz), 6.94 (d, 1H, $J = 10.0$ Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 32.4, 34.8, 42.8, 47.1, 54.3, 60.2, 127.0, 127.5, 128.8, 131.4, 135.5, 153.3, 154.7, 186.0, 186.1 ppm. IR (neat, cm^{-1}): ν 2954, 2925, 2857, 1726, 1461, 1379, 1274, 1123, 1072, 743. GCMS m/z calculated for $\text{C}_{10}\text{H}_{10}\text{O}$ [M] 147.19 found 146.98. HRMS (ESI+) m/z calculated for $\text{C}_{10}\text{H}_{10}\text{O}$ [M+H] $^+$ 147.0810 found 147.0784.

2-Bromo-3-vinyloxy-spiro[4.5]deca-6,9-dien-8-one. A solution of Palladium (II) trifluoroacetate (0.04 g, 0.120 mmol) and bathophenanthroline (0.038 g, 0.120 mmol) in 8 mL of butyl vinyl ether was stirred under N₂ for 30 minutes. To this stirring solution was added a solution of bromohydrin (0.14 g, 0.58 mmol) in dichloroethane (2 mL) and triethylamine (0.033 mL, 0.24 mmol) and heated to reflux to 81-82 °C till the TLC showed consumption of starting material. The catalyst was removed by running the reaction through a short plug of activated carbon and silica gel and washed successively with dichloromethane (30 mL), ethylacetate (30 mL) and concentrated the combined organic phase in vacuo. The crude product was purified by flash column chromatography (hexane - diethylether, gradient, 0 to 12%) to afford the desired product as transparent oil (0.100 g, yield 64%). R_f = 0.40 (hexane - diethylether, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 2.04-2.08 (m, 1H), 2.34 (dd, 1H, J = 2.4, 13.1 Hz), 2.63 (dd, 1H, J = 5.0, 14.4 Hz), 2.74 (dd, 1H, J = 5.0, 14.4 Hz), 4.16 (dd, 1H, J = 2.3, 4.4 Hz), 4.31 (dd, 1H, J = 2.4, 11.9 Hz), 4.46-4.48 (m, 1H), 4.68-4.70 (m, 1H), 6.13-6.20 (ddd, 2H, J = 1.8, 7.8, 9.9 Hz), 6.30-6.35 (m, 1H), 6.97 (dd, 1H, J = 2.9, 12.0 Hz), 7.10 (dd, 1H, J = 2.9, 12.0 Hz) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 40.5, 45.1, 46.9, 51.2, 86.6, 90.3, 126.7, 127.5, 149.4, 154.7, 154.9, 185.7 ppm. FTIR (neat, cm⁻¹): ν 3404, 2956, 2928, 1724, 1656, 1614, 1409, 1376, 1260, 1074, 862, 669. GCMS m/z calculated for C₁₂H₁₃BrO₂ [M] 269.13 found 267.17. HRMS (ESI+) m/z calculated for C₁₂H₁₃BrO₂ [M+H]⁺ 269.0177 found 269.0177.

2-Allyl-3-vinyloxy-spiro[4.5]deca-6,9-dien-8-one. To a degassed refluxing solution of starting material (0.02 g, 0.074 mmol) in $^2\text{H}_6$ -benzene (2 mL) at 85 °C was added a degassed $^2\text{H}_6$ -benzene solution of initiator (0.024 mL, 0.0895 mmol) and ACCN (4.33 mg, 0.018 mmol) through a syringe pump over 18 h. The crude reaction was taken for purified by flash column chromatography (hexane - diethylether, gradient, 0 to 20%) to afford the dehalogenated reduced product as transparent oil (0.011 g, yield 80%). R_f = 0.30 (hexane - diethyl ether, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.93-2.05 (m, 3H), 2.11-2.17 (m, 1H), 2.17-2.22 (m, 1H), 2.32-2.38 (m, 1H), 2.48-2.55 (m, 1H), 4.06-4.08 (m, 1H), 4.18-4.26 (m, 2H), 5.05-5.11 (m, 2H), 5.73-5.83 (m, 1H), 6.17-6.22 (m, 2H), 6.30-6.35 (m, 1H), 6.73 (dd, 1H, J = 2.9, 10.0 Hz), 7.20-7.23 (m, 1H) ppm.

2-Vinyloxy-spiro[4.5]deca-6,9-dien-8-one. (0.011 g, yield 76%). R_f = 0.26 (hexane - diethyl ether, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.81-1.87 (m, 1H), 2.00-2.08 (m, 3H), 2.10-2.14 (m, 1H), 2.17-2.27 (m, 1H), 4.07-4.09 (m, 1H), 4.19-4.23 (m, 1H), 4.61-4.65 (m, 1H), 6.19-6.23 (m, 2H), 6.31-6.36 (m, 1H), 6.78-6.81 (m, 1H), 7.08-7.11 (m, 1H) ppm. IR (neat, cm^{-1}): ν 3727, 3404, 2956, 1724, 1656, 1614, 1409, 1200,

1074, 862, 669, 482, 464, 442, 409. GCMS m/z calculated for $C_{12}H_{14}O_2$ [M] 190.24 found 190.05. HRMS (ESI) m/z calculated for $C_{12}H_{14}O_2$ [M]⁺ 191.1072 found 191.1072.

CHAPTER 5

To a solution of diketone (0.10 g, 0.61 mmol) in (ether/ CH_2Cl_2 4:1) (20 mL) was added pyrrolidine (0.10 mL, 1.23 mmol) under nitrogen followed by MgSO_4 (0.08 g) and stirred for 23 h. The solvent was evaporated in vacuo and redissolved in THF (20 mL) followed by addition of NaHCO_3 (0.10 g) and stirred at -78°C . To this was added a solution of NBS (0.13g, 0.73 mmol) in THF (7 mL) dropwise. After stirring for 3 hrs at this temperature, the reaction was quenched by dropwise addition of sat. NaHCO_3 solution (10 mL) and addition of ($\text{EtOAc}/\text{H}_2\text{O}$ 1:1) (10 mL) and allowed the temperature to rise to room temperature while stirring. The two phases were separated and the organic phase extracted with ethyl acetate (3 X 20 mL). The combined organic phase was dried over Na_2SO_4 filtered and concentrated. The crude residue was purified by flash column chromatography (hexane - ethyl acetate, gradient, 0 to 50%) to give the desired product as a brown solid (0.025g, 60% yield based on recovered starting material of 0.07 g). Fortunately the undesired bromo isomer was not isolated, pointing to its spontaneous decomposition.

^1H NMR (400 MHz, CDCl_3) δ 2.07 (t, 2H, $J = 8.0$ Hz, **B**), 2.17 (t, 2H, $J = 8.0$ Hz, **SM**), 2.20-2.24 (m, 1H, **A**), 2.43 (s, 2H, **SM**), 2.43 (s, 1H, **B**), 2.52-2.56 (m, 4H, **A** & **SM**), 2.62 (t, 2H, $J = 8.0$ Hz, **B**), 2.87 (t, 4H, $J = 10.0$ Hz, **B**), 2.93-2.96 (t, 4H, $J = 5.0$ Hz, **A**), 3.87 (s, 1H, **B**), 4.21 (t, 1H, $J = 5.0$ Hz, **A**), 4.45 (s, 1H, **B**), 6.12 (d, 2H, $J = 10.0$ Hz, **B**), 6.20 (d, 2H, $J = 10.0$ Hz, **A**), 6.32 (d, 2H, $J = 10.0$ Hz, **SM**), 6.82 (d, 2H, $J = 10.0$ Hz, **B**), 6.89 (d, 2H, $J = 10.0$ Hz, **SM**) 7.03 (d, 2H, $J = 10.0$ Hz, **A**) ppm.

2-Bromo-3-hydroxy-spiro[4.5]deca-6,9-dien-8-one. Light brown solid. $R_f = 0.3$ (hexane - diethylether, 1:2). ^1H NMR (400 MHz, CDCl_3) δ 2.49 (dd, 1H, $J = 7.9, 14.5$ Hz), 2.59 (d, 1H, $J = 18.7$ Hz), 2.70 (d, 1H, $J = 18.7$ Hz), 2.77 (ddd, 1H, $J = 1.1, 8.4, 14.5$ Hz), 4.54 (t, 1H, $J = 8.1$ Hz), 6.34 (ddd, 2H, $J = 1.8, 4.9, 10.2$ Hz), 6.82 (dd, 1H, $J = 3.1, 10.1$ Hz), 7.08 (dd, 1H, $J = 3.1, 10.1$ Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 43.4, 43.7, 43.8, 45.9, 129.4, 149.9, 151.4, 184.8, 207.5 ppm. IR (neat, cm^{-1}): ν 3490, 1633, 1163, 621, 586, 528. GCMS m/z calculated for $\text{C}_{10}\text{H}_9\text{BrO}_2$ [M] 241.08 found 241.11. HRMS (ESI) m/z calculated for $\text{C}_{10}\text{H}_9\text{BrO}_2$ $[\text{M}+\text{H}]^+$ 240.9864 found 240.9864.

2-Bromo-indan-1-one.¹⁶⁵ A solution of 1-Indanone (0.13 g, 1 mmol) in dry tetrahydrofuran (6 mL) was cooled to $-78\text{ }^\circ\text{C}$ under nitrogen. A (1.2 mL, 1.2 mmol) 1 M solution of sodium hexamethyldisilazide in tetrahydrofuran was added and stirred at $-78\text{ }^\circ\text{C}$ for 1 hour. Trimethyl silyl chloride (0.18 mL, 1.4 mmol) was introduced and stirring continued for additional 30 min with warming to room temperature over another 30 minute. The reaction was quenched with saturated NaHCO_3 and extracted with diethylether (3 X 15 mL). The combined organic layer was dried over Na_2SO_4 , filtered and concentrated. The crude product was redissolved in a mixture of THF and

¹⁶⁵ Hartung, R.; Paquette, L. A. *J. Org. Chem.*, **2005**, 70(5), 1597-1604.

water (5:1, 12 mL), at which point *N*-bromosuccinimide (0.5 g, 3 mmol) was added and the solution stirred for another 4 hrs. Upon completion the mixture was quenched with saturated NaHCO_3 and extracted with diethylether (3 X 15 mL). The combined organic solution was dried over Na_2SO_4 , filtered and concentrated in vacuo. The residual crude product was purified by flash column chromatography (hexane - diethylether, gradient, 0 to 70%) to afford the desired product as a brown solid (0.15 g, 70% yield).

¹⁶⁶A solution of 1-Indanone (0.13 g, 1 mmol) in chloroform (4 mL) and ethylacetate (4 mL) was treated with portion wise addition of copper bromide (0.45 g, 2 mmol) over 16 hours under reflux at 85 °C overnight during which time all the purple solids disappeared and white precipitate formed. When TLC monitoring showed that all the starting material had been consumed, the reaction was filtered and the precipitate washed with chloroform (30 mL) and the combined organic phase evaporated to dryness. The residue was redissolved in ether (10 mL) and washed with saturated NaHCO_3 (5 mL), brine (5 mL) and water (5 mL), dried over Na_2SO_4 , filtered and concentrated.

(3H-Inden-1-yloxy)-trimethyl-silane. ^1H NMR (400 MHz, CDCl_3) δ 0.38 (s, 9H), 3.33 (d, 2H, $J = 2.29$), 5.50 (t, 1H, $J = 2.29$ Hz), 7.27 (t, 1H, $J = 7.9$ Hz), 7.36 (t, 1H,

¹⁶⁶ Bauer, D. P.; Macomber, R. S. *J. Org. Chem.*, **1975**, *40*, 1990-1992.

$J = 7.5$ Hz), 7.45 (d, 2H, $J = 8.0$ Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 0, 33.9, 106.1, 118.0, 123.7, 125.0, 125.9, 141.8, 142.7, 153.5 ppm.

$R_f = 0.49$ (hexane - diethylether, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 3.34 (dd, 1H, $J = 3.0, 18.0$ Hz), 3.76 (dd, 1H, $J = 7.5, 18.0$ Hz), 4.58 (dd, 1H, $J = 3.1, 7.5$ Hz), 7.4 (t, 2H, $J = 7.4$ Hz), 7.59 (t, 1H, $J = 7.5$ Hz), 7.76 (d, 1H, $J = 7.6$ Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 37.9, 44.0, 125.0, 127.9, 128.2, 135.9, 151.0, 199.5 ppm. IR (neat, cm^{-1}): ν 2924, 1737, 1716, 1603, 1585, 1461, 1422, 1348, 1325, 1297, 1272, 1232, 1206, 1183, 1171, 1125, 1097, 1046, 1025, 974, 949, 931, 846, 832, 800, 773, 707, 700, 666, 627, 596, 573, 558, 539, 504, 464. $\text{C}_9\text{H}_7\text{BrO}$ [M] 211.06 found 211.85. HRMS (ESI) m/z calculated for $\text{C}_9\text{H}_7\text{BrO}$ $[\text{M}+\text{H}]^+$ 213.0625, found 213.0559.

Representative Experimental Procedure for tin mediated coupling.

Acetic acid 2-(1-oxo-indan-2-yl)-ethyl ester. To a degassed refluxing solution of bromo indanone (0.5 g, 0.24 mmol), vinyl trimethoxy silane (0.350 mL, 2.4 mmol) and 1,1'-azobis (cyclohexane carbonitrile) (0.002 g, 0.01 mmol) in toluene (3 mL) and ethylacetate (3 mL) at 85 °C was added a degassed toluene (1 mL) solution of tributyl tin hydride (0.064 mL, 0.24 mmol) through a syringe pump over 10 hours. When all the starting material had been consumed, the solvent was evaporated in vacuo and the crude product was purified by flash column chromatography (hexane :

diethyl ether, gradient, 0 to 50%) to afford the coupled product as a transparent oil (0.015 g, yield 15%) together with the dehalogenated product (0.011 g, 27%). $R_f = 0.23$ (hexane - diethylether, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.27-1.38 (m, 2H), 1.60-1.67 (m, 1H), 1.80-1.88 (m, 1H), 2.05-2.13 (m, 1H), 2.84-2.89 (m, 1H), 3.39-3.46 (m, 1H), 3.80-3.93 (m, 1H), 7.38 (t, 1H, $J = 7.4$ Hz), 7.46 (d, 1H, $J = 7.6$ Hz), 7.60 (t, 1H, $J = 7.1$ Hz), 7.76 (d, 1H, $J = 7.5$ Hz) ppm. FTIR (neat, cm^{-1}): ν 3424, 2928, 1714, 1605, 1510, 1465, 1266, 1213, 1155, 1081, 928, 830, 735, 700, 669, 650, 521, 465, 436, 420. GCMS m/z calculated for $\text{C}_{11}\text{H}_{12}\text{O}_2$ $[\text{M}+\text{H}]^+$ 177.21 found 177.04. HRMS (ESI+) m/z calculated for $\text{C}_{11}\text{H}_{12}\text{O}_2$ $[\text{M}+\text{H}]^+$ 177.0916 found 177.0916.

Representative Experimental Procedure for $\text{Et}_3\text{B}/\text{O}_2$ mediated coupling.

To a solution of iodo indanone (0.05 g, 0.24 mmol), alkene (2 mmol) and 2,6-lutidine (0.02 mL, 0.04 mmol) in toluene (0.5 mL) at 0 °C was added triethyl borane (0.04 mL, 0.04 mmol) (1 M solution in toluene) and injected air (10 mL) through a syringe over an hour. Upon completion of the reaction the solvent was evaporated in the vacuo and the crude product was purified by flash column chromatography.

2-(1-Hydroxy-ethyl)-indan-1-one. $R_f = 0.16$ (hexane - diethylether, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.30 (d, 3H, $J = 6.3$ Hz), 2.65-2.70 (m, 1H), 2.81 (dd, 1H, $J = 4.5, 17.2$ Hz), 3.30 (dd, 1H, $J = 8.0, 17.1$ Hz), 3.93-4.07 (m, 1H), 4.42 (brs, 1H), 4.56 (brs, 1H), 7.39 (t, 1H, $J = 7.3$ Hz), 7.48 (d, 1H, $J = 7.6$ Hz), 7.62 (t, 1H, $J = 7.1$ Hz), 7.77 (d, 1H, $J = 7.6$ Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 21.4, 29.7, 53.3, 65.8, 69.0, 124.0, 126.5, 127.7, 135.4, 136.4, 153.8, 209 ppm. IR (neat, cm^{-1}): ν 3424,

2928, 1714, 1605, 1510, 1465, 1266, 1213, 1155, 1081, 928, 830, 735, 700, 669, 650, 521, 465, 436, 420. GCMS m/z calculated for $C_{11}H_{12}O_2$ [M] 176.21 found 176.9. HRMS (ESI⁺) m/z calculated for $C_{11}H_{12}O_2$ [M+H]⁺ 177.0916 found 177.0921.

(1-Oxo-indan-2-yl)-thioacetic acid S-ethyl ester. R_f = 0.46 (hexane - diethyl ether, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.25 (t, 3H, J = 7.4 Hz), 2.79 (dd, 1H, J = 9.5, 16.2 Hz), 2.90 (q, 2H, J = 7.4 Hz), 3.02-3.12 (m, 1H), 3.25 (dd, 1H, J = 3.7, 16.1 Hz), 3.42 (dd, 1H, J = 8.0, 17.1 Hz), 7.38 (t, 1H, J = 7.4 Hz), 7.45 (d, 1H, J = 7.8 Hz), 7.45-7.63 (m, 1H), 7.76 (d, 1H, J = 7.82 Hz) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 14.9, 23.7, 32.9, 44.1, 44.8, 124.3, 126.7, 127.7, 135.2, 136.4, 153.4, 198.0, 206.7 ppm. IR (neat, cm⁻¹): ν 2924, 1743, 1661, 1620, 1511, 1377, 1217, 1009, 862, 832, 670, 465, 424. GCMS m/z calculated for $C_{13}H_{14}O_2S$ [M] 234.31 found 234.04. HRMS (ESI⁺) m/z calculated for $C_{13}H_{14}O_2S$ [M+H]⁺ 235.0793 found 235.0793.

2-Iodo-indan-1-one. R_f = 0.29 (hexane - diethylether, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 3.49 (dd, 1H, J = 2.0, 18.4 Hz), 3.89 (dd, 1H, J = 7.3, 18.0 Hz), 4.96 (dd, 1H, J = 2.5, 7.4 Hz) 7.40-7.48 (m, 2H), 7.67 (t, 1H, J = 7.5 Hz), 7.87 (d, 1H, J = 7.9 Hz) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 19.5, 39.7, 125.2, 126.6, 128.3, 132.9, 135.7, 151.4, 201.6 ppm. IR (neat, cm⁻¹): ν 2921, 1605, 1463, 1429, 1323, 1297, 1272, 1230, 1205, 1176, 1151, 1095, 1025, 961, 927, 831, 816, 789, 747, 734, 667, 591,

555, 521, 457, 437, 420, 403. GCMS m/z calculated for C_9H_7IO [M] 259.06 found 259.19. HRMS (ESI+) m/z calculated for C_9H_7IO [M+H]⁺ 258.9620 found 258.9620.

R_f = 0.69 (hexane - diethylether, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 2.80 (dd, 2H, J = 5.0, 8.0 Hz), 2.97 (dd, 2H, J = 4.4, 18.0 Hz), 3.26 (dd, 2H, J = 8.4, 17.8 Hz) 7.38 (t, 2H, J = 7.5 Hz), 7.48 (d, 2H, J = 7.5 Hz), 7.59-7.63 (m, 2H), 7.74 (d, 2H, J = 7.5 Hz) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 21.4, 25.9, 29.9, 32.1, 36.5, 57.2, 74.0, 123.9, 126.7, 127.7, 135.4, 154.3, 202.5, 202.7 ppm. IR (neat, cm⁻¹): ν 3734, 2933, 1698, 1606, 1275, 1055, 1033, 843, 830, 799, 785, 752, 669, 537, 522, 508, 466, 451, 436, 420, 404. GCMS m/z calculated for $C_{18}H_{14}O_2$ [M] 263.30 found 263.96. HRMS (ESI) m/z calculated for $C_{18}H_{14}O_2$ [M+H]⁺ 263.1072 found 263.1072.

(1-Oxo-indan-2-yl)-acetic acid methyl ester. R_f = 0.42 (hexane - diethylether, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 2.46-2.63 (m, 1H), 2.85-2.97 (m, 1H), 3.0-3.06 (m, 1H), 3.16-3.57 (m, 2H), 3.66, 3.69, 3.71, 3.74 (s, 3H), 7.33-7.47 (m, 2H), 7.54-7.62 (m, 1H), 7.75-7.81 (m, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 32.9, 34.9, 43.5, 47.0, 51.8, 123, 124, 126.5, 126.6, 127.5, 127.6, 134.9, 135.0, 136.2, 153.2, 172.5, 206.7 ppm. IR (neat, cm⁻¹): ν 2951, 1709, 1608, 1464, 1435, 1365, 1327, 1276, 1203, 1147, 1095, 1052, 995, 931, 844, 830, 817, 799, 753, 683, 669, 651, 571, 537, 521, 508, 465, 452, 437, 420, 403. GCMS m/z calculated for $C_{12}H_{12}O_3$ [M] 204.22 found 204.07. HRMS (ESI) m/z calculated for $C_{12}H_{12}O_3$ [M+H]⁺ 205.0865 found 205.0865.

2-Hydroxy-indan-1-one. R_f = 0.16 (hexane - diethylether, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 3.02 (dd, 1H, J = 4.7, 16.5 Hz), 3.59 (dd, 1H, J = 7.9, 16.5 Hz), 4.55 (dd, 1H, J = 5.11, 7.9 Hz), 7.41 (t, 1H, J = 7.4 Hz), 7.47 (d, 1H, J = 7.6 Hz), 7.64 (t, 1H, J = 7.5 Hz), 7.77 (d, 1H, J = 7.6 Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 35.1, 74.3, 116.1, 124.4, 126.8, 128.0, 135.9, 150.8, 206.3 ppm. IR (neat, cm^{-1}): ν 3424, 2928, 1714, 1605, 1510, 1465, 1266, 1213, 1155, 1081, 928, 830, 735, 700, 669, 650, 521, 465, 436, 420. GCMS m/z calculated for $\text{C}_9\text{H}_8\text{O}_2$ [M] 148.16 found 148.04. HRMS (ESI+) m/z calculated for $\text{C}_9\text{H}_8\text{O}_2$ [M+H] $^+$ 149.0603 found 149.0603.

To a solution of bromo diketone (0.022 g, 0.09 mmol) in toluene (1 mL) was added sodium iodide (0.082 g, 0.546 mmol) followed by ethanol (1 mL) and stirred at room temperature overnight. Upon completion as judged by TLC water was added to the reaction and aqueous phase extracted with ethyl acetate (3 X 5 mL). The combined organic layer was washed successively with $\text{Na}_2\text{S}_2\text{O}_3$ (5 mL), brine (5 mL) dried over Na_2SO_4 , filtered and concentrated in vacuo. The crude product was purified by flash column chromatography (hexane - ethylacetate, gradient, 0 to 50%) to afford the desired product as a white solid (0.015 g, 65%). R_f = 0.53 (hexane - ethylacetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 2.57 (s, 2H), 2.56 (d, 1H, J = 8.5 14.4 Hz), 2.76 (dd, 1H, J = 8.4, 14.4 Hz), 4.83 (t, 1H, J = 8.5 Hz), 6.33 (ddd, 2H, J = 1.8, 5.5, 10.1 Hz), 6.82 (dd, 1H, J = 3.3, 10.1 Hz), 7.10 (dd, 1H, J = 3.1, 10.1 Hz) ppm. ^{13}C NMR (100

MHz, CDCl₃) δ 29.9, 45.1, 45.2, 45.4, 129.3, 129.5, 149.7, 151.5, 184.0, 209.4 ppm.

IR (neat, cm⁻¹): ν 2310, 1653, 1651, 1645, 1633, 1516, 1454, 1392, 621, 586, 528.

GCMS m/z calculated for C₁₀H₉IO₂ [M] 288.97, found: 288.63. HRMS (ESI+) m/z calculated for C₁₀H₉IO₂ [M+H]⁺ 288.9726 found 288.9726.

(0.041 g, 65%). R_f = 0.56 (hexane - ethylacetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.27 (t, 3H, J = 7.2 Hz), 2.08-2.14 (m, 1H), 2.26 (ddd, 1H, J = 2.0, 8.2, 12.9 Hz), 2.46 (d, 1H, J = 18.6 Hz), 2.70 (d, 1H, J = 18.5 Hz), 2.85-2.95 (m, 4H), 3.12 (dd, 1H, J = 3.0, 15.9 Hz) 6.34 (ddd, 2H, J = 1.8, 10.0, 20.8 Hz), 6.86 (dd, 1H, J = 3.0, 10.0 Hz), 6.98 (dd, 1H, J = 3.0, 10.1 Hz) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 14.6, 23.6, 39.0, 43.0, 43.4, 43.6, 47.3, 128.4, 129.5, 150.0, 153.3, 185.2, 197.3, 214.1 ppm. IR (neat, cm⁻¹): ν 3728, 2924, 2367, 2340, 1743, 1661, 1620, 1511, 1376, 1216, 1009, 862, 832, 670, 519, 466, 424, 408. LCMS (ESI) m/z calculated for C₁₄H₁₆SO₃ [M+]⁺ 265.34 found 265.06. HRMS (ESI) m/z calculated for C₁₄H₁₆SO₃ [M+H]⁺ 265.0898 found 265.0898.

R_f = 0.22 (hexane - ethylacetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 2.02-2.10 (m, 1H), 2.21-2.29 (m, 1H), 2.42-2.49 (m, 1H), 2.73 (d, 1H, J = 18.7 Hz), 2.83-2.87 (m, 1H), 2.89-2.91 (m, 1H), 2.99-3.08 (m, 1H) 6.29-6.36 (m, 2H), 6.85 (dd, 1H, J = 3.0, 9.8 Hz), 6.99 (dd, 1H, J = 3.0, 10.1 Hz), 9.76 (s, 1H) ppm. ¹³C NMR (100 MHz,

CDCl₃) δ 39.0, 41.5, 43.2, 43.7, 47.2, 128.4, 129.5, 150.0, 153.3, 187.2, 198.8, 214.5 ppm. IR (neat, cm⁻¹): ν 2922, 2853, 1740, 1660, 1623, 1455, 1406, 1261, 1183, 1009, 913, 860, 830, 816, 800, 785, 742, 701, 669, 553, 537, 521, 493, 464, 437, 420, 404. LCMS (ESI) m/z calculated for C₁₂H₁₂O₃ [M+H]⁺ 205.22 found 205.09. HRMS (ESI) m/z calculated for C₁₂H₁₂O₃ [M+H]⁺ 205.0865 found 205.0865.

R_f = 0.53 (hexane - ethylacetate, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 2.10 (dd, 1H, J = 3.2, 10.0 Hz), 2.48 (ddd, 1H, J = 1.5, ?, 13.6 Hz), 2.57-2.65 (m, 2H), 4.41-4.47 (m, 1H), 6.33 (ddd, 2H, J = 1.9, 3.9, 10.0 Hz), 6.84-6.94 (m, 2H), ppm. ¹³C NMR (100 MHz, CDCl₃) δ 30.0, 41.0, 41.1, 45.1, 73.2, 129.0, 129.3, 150.8, 152.7, 187.2, 213.9 ppm. IR (neat, cm⁻¹): ν 3401, 2923, 2173, 1752, 1660, 1275, 1055, 1032, 909, 858, 843, 830, 799, 785, 746, 682, 669, 652, 569, 537, 521, 508, 492, 466, 452, 437, 420, 404. GCMS m/z calculated for C₁₂H₁₂O₃ [M+H]⁺ 205.22 found 205.55. HRMS (ESI) m/z calculated for C₁₂H₁₂O₃ [M+H]⁺ 205.0865 found 205.0865.

Dithiocarbonic acid S-(3,8-dioxo-spiro[4.5]deca-6,9-dien-2-yl) ester O-ethyl ester.

A solution of potassium ethyl xanthogenate (0.46 g, 2.9 mmol) in acetone (10 mL) was added to a solution of bromo intermediate (0.57 g, 2.4 mmol) in acetone (10 mL) dropwise. The reaction was stirred at room temperature till TLC monitoring revealed complete consumption of starting material. Upon completion the solvent was evaporated and the residue redissolved in ethyl acetate (20 mL) and the organic phase

washed with brine and dried over Na_2SO_4 , filtered and concentrated. The crude was purified by flash column chromatography (hexane - ethylacetate, gradient, 0 to 40%) to afford the desired product as a white solid (0.57 g, yield 70%). $R_f = 0.23$ (hexane - diethylether, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.41 (t, 3H, $J = 7.0$ Hz), 2.46 (t, 1H, $J = 12.5$ Hz), 2.53-2.66 (m, 2H), 2.66-2.75 (m, 1H), 4.44-4.53 (m, 1H), 4.64 (q, 2H, $J = 7.0$ Hz), 6.34 (dd, 2H, $J = 9.0, 10.3$ Hz), 6.85 (dd, 1H, $J = 2.9, 10.0$ Hz), 7.03 (dd, 1H, $J = 3.0, 10.3$ Hz) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 13.7, 29.6, 39.6, 42.9, 47.2, 52.6, 71.1, 128.7, 129.6, 149.4, 151.9, 184.8, 208.0, 211.3 ppm. IR (neat, cm^{-1}): ν 3431, 2063, 1643, 1361, 1193, 1147, 1085, 1047, 665. LC-ESI-MS m/z calculated for $\text{C}_{13}\text{H}_{14}\text{O}_3\text{S}_2$ $[\text{M}+\text{H}]^+$ 283.38 found 283.92. HRMS (ESI) m/z calculated for $\text{C}_{13}\text{H}_{14}\text{O}_3\text{S}_2$ $[\text{M}+\text{H}]^+$ 283.0643 found 283.0643.

A solution of xanthate (0.1 g, 0.4 mmol), 2,6-lutidine (0.04 mL, 0.38 mmol) and vinyl ethyl thio ether (0.2 mL, 1.9 mmol) in 1,2-dichloroethane (20 mL) was degassed for 10 minutes and then heated to reflux under nitrogen at 80 °C. To the refluxing solution was added lauroyl peroxide (0.02 g, 0.05 mmol) portion wise. Upon completion of reaction the solvent was evaporated and the crude purified by flash column chromatography (hexane - ethyl acetate, gradient, 0 to 50%) to afford aldehyde (0.061 g, 75% yield) and thio acetal intermediate (0.033 g, yield 22%).

$R_f = 0.44$ (hexane - ethylacetate, 1:1). ^1H NMR (400 MHz, CDCl_3) δ 1.24-1.31 (m, 3H), 1.42 (t, 3H, $J = 7.0$ Hz), 1.94-2.16 (m, 1H), 2.34-2.52 (m, 4H), 2.55-3.0 (m, 4H), 4.59-5.03 (m, 3H), 6.28-6.37 (m, 2H), 6.79-6.86 (m, 1H), 6.93-7.01 (m, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3) δ 13.9, 14.0, 14.6, 14.7, 25.9, 26.1, 31.1, 36.8, 36.9, 39.9, 40.7, 43.2, 43.3, 45.4, 47.7, 47.8, 54.0, 54.0, 70.3, 70.4, 128.6, 128.7, 129.6, 129.7, 150.3, 150.4, 153.4, 153.5, 185.4, 213.8, 214.3, 214.6, 214.8 ppm. IR (neat, cm^{-1}): ν 3458, 2974, 2926, 1743, 1708, 1640, 1625, 1450, 1408, 1257, 1220, 1182, 1145, 1111, 1045, 1002, 860, 734, 704, 555, 521. LC-ESI-MS m/z calculated for $\text{C}_{17}\text{H}_{22}\text{O}_3\text{S}_3$ $[\text{M}+\text{H}]^+$ 371.55 found 371.25. HRMS (ESI) m/z calculated for $\text{C}_{17}\text{H}_{22}\text{O}_3\text{S}_3$ $[\text{M}+\text{H}]^+$ 371.0809 found 371.0809.

PUBLICATIONS

1. Functionalization of Peptides and Proteins by Mukaiyama Aldol Reaction; Jenefer Alam, Thomas, H. Keller and Teck-Peng Loh; *Journal of the American Chemistry Society*, **2010**, 132, 9546-9548.
2. Indium in Peptide and Protein Functionalization; Jenefer Alam, Thomas, H. Keller and Teck-Peng Loh. Manuscript in preparation.

CONFERENCES

1. Functionalization of Peptides & Proteins by Mukaiyama Aldol Reactions. Jenefer Alam, Thomas Hugo Keller and Teck-Peng Loh, 6th Asian-European Symposium on Metal Mediated Efficient Reactions, 7-9th Jun 2010, Singapore. (poster presentation)
2. Chemical Functionalization of Peptides & Proteins by Mukaiyama Aldol Reactions in Aqueous Solution. Jenefer Alam, Thomas Hugo Keller and Teck-Peng Loh, 238th American Chemical Society National Meeting, 16-20th Aug 2009, Washington, DC, USA. (poster presentation)
3. Functionalization of Peptides & Proteins by Mukaiyama Aldol Reactions in Aqueous Solution. Jenefer Alam, Thomas Hugo Keller and Teck-Peng Loh, 2nd Waseda-NTU Joint Chemistry Symposium, 13th Mar 2009. (poster presentation)
4. Chemical Functionalization of Peptides and Proteins. Jenefer Alam, Thomas Hugo Keller and Teck-Peng Loh, International Symposium on Catalysts and Fine Chemicals 2007, 16-21 Dec 2007 (poster presentation).

5. Chemical Functionalization of Peptides. Jenefer Alam, Thomas Hugo Keller and Teck-Peng Loh, NTU-Warwick Symposium 2006, 8-10th Dec 2006 (poster presentation).