

SYNTHESIS OF BIOACTIVE AMINO ACID BUILDING BLOCKS AND THEIR APPLICATIONS  
TOWARDS THE PEPTIDES AND GLYCOPEPTIDES

KALYAN KUMAR PASUNOOTI

2011



**NANYANG  
TECHNOLOGICAL  
UNIVERSITY**

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**KALYAN KUMAR PASUNOOTI**

School of Physical and Mathematical Sciences

A thesis submitted to the Nanyang Technological University  
in partial fulfillment of the requirement for the degree of  
Doctor of Philosophy

**2011**

## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Assistant Professor Liu Xue-Wei for his strong encouragement, scientific mentorship, extraordinary enthusiasm, optimism and great understanding during my PhD studies. I especially appreciate his immense support for my graduate studies throughout my PhD at NTU.

I would like to express my sincere appreciation to Associate Professor Liu Chuan Fa for his kind instruction, valuable advice and great help for peptide and protein related works during our collaboration. My appreciation is also expressed to Mr. Yang Renliang and Mr. Li Fupeng for their great help during our project discussions.

I greatly acknowledge Assistant Professor Zhang Dawei, Associate Professor Park Cheol-Min, Assistant Professor Liang Zhao-Xun and Associate Professor Yoon Ho Sup Joe for their valuable suggestions and comments.

I am thankful to Nanyang Technological University for providing valuable financial support and sophisticated environment to do my research.

I am grateful to Mr. Kishan Gorityala, who is my colleague stood behind me like my brother during my four years of PhD studies. And I am thankful to my senior Dr. Ma Jimei and Mr. Siming Wang for their coordination and teamwork with me during our new lab establishment in CBC-Level 4. And I would like to thank my lab-mates, Dr. Biswajit Maji, Mr. Liu Jiehua, Mr. Ding Feiqing, Ms. Leow Minli, Mr. Seenuvasn Vedachalam, Ms. Cai Shuting, Mr.Zeng Jing, Ms. Chai Hua, Ms. Chantel Nixon Jensen, Mrs.Weizhi Xiangfeng, Ms. Ji Li and all other lab members.

Finally, I wish to thank my family, especially my wife Swathi, my parents Sambaiah & Sarojana, my son Nimish Kumar, and my in-laws Srinivas & Jamuna for their constant support and encouragement.

## TABLE OF CONTENTS

	<b>Page</b>
<b>ACKNOWLEDGEMENTS</b>	i
<b>TABLE OF CONTENTS</b>	ii
<b>SUMMARY</b>	iv
<b>INDEX OF ABBREVIATIONS</b>	vi
<b>CHAPTER 1</b> <i>Synthesis of <math>\gamma</math>-mercapto lysine derivatives: Application for dual native chemical ligation</i>	
- INTRODUCTION	1
- RESULTS AND DISCUSSION	22
- CONCLUSION	36
- EXPERIMENTAL	37
- REFERENCES	66
<b>CHAPTER 2</b> <i>Synthesis of K48-linked diubiquitin using dual native chemical ligation at lysine</i>	
- INTRODUCTION	70
- RESULTS AND DISCUSSION	80
- CONCLUSION	88
- EXPERIMENTAL	89
REFERENCES	110

**CHAPTER 3** *Native chemical ligation on aspartic Acid (Asp): Application towards on-resin convergent synthesis of N-linked glycopeptides*

- INTRODUCTION	112
- RESULTS AND DISCUSSION	125
- CONCLUSION	141
- EXPERIMENTAL	142
- REFERENCES	161

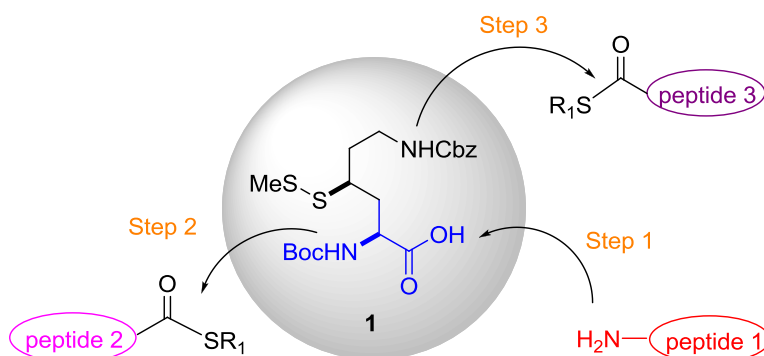
**CHAPTER 4** *Additional studies: Synthesis of bioactive compounds for medicinal chemistry applications*

- <i>Microwave-assisted copper catalyzed one-pot four-component synthesis of multi-functionalized dihydropyridines</i>	164
- <i>A microwave-assisted, copper-catalyzed three-component synthesis of dihydropyrimidinones under mild conditions</i>	211
- <i>A general and mild copper-catalyzed three-component synthesis of protected homoallyl amines</i>	245

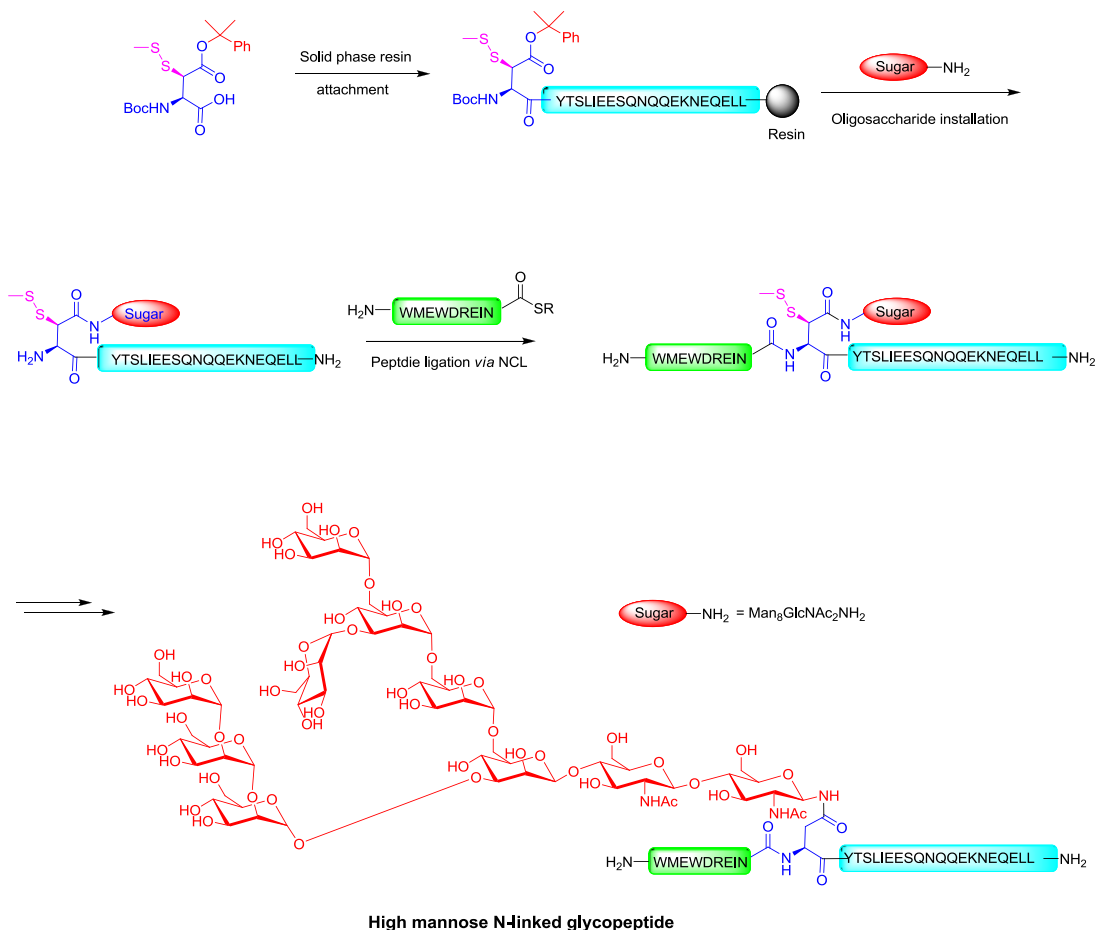
<b>LIST OF PUBLICATIONS &amp; CONFERENCES</b>	259
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## SUMMARY

Native chemical ligation (NCL), developed by Dawson and Kent in 1994, allows the combination of two unprotected amino acids in aqueous media. This phenomenal discovery sets new direction for bioactive peptides and proteins to be assembled in an efficient way. As cysteine is an uncommon amino acid in naturally occurring proteins, several other strategies have been developed to expand the application of NCL. Adopting these investigations, considerable efforts have been made by various research groups including our group, in order to extend the applicability of NCL on amino acid residues utilizing alanine, phenylalanine, valine, leucine and threonine. Based on previous methods, we have successfully demonstrated the NCL concept to lysine and efficiently synthesized ubiquitin and diubiquitin proteins. As shown in below figure, the  $\gamma$ -mercapto lysine residue **1** could facilitate ligation at  $\alpha$ - and  $\epsilon$ -amines of lysine. We successfully synthesized key building block **1** using Zn-mediated diastereoselective Reformasky reaction. The basic advantage of our strategy that a single thiol group introduced on the  $\gamma$ -carbon of lysine would mediate ligation at both the  $\alpha$ - and  $\epsilon$ -amines, to form native and isopeptide bonds.



Native chemical ligation (NCL) concept is not only useful for peptide chemistry, but also useful for glycopeptides chemistry. Previously, our group has applied NCL concept at lysine and successfully synthesize complex ubiquitin and diubiquitin proteins. Based on our previous investigations, we wish to extend NCL strategy on aspartic acid (Asp) residue which could be utilized in *N*-linked glycopeptide synthesis. *N*-linked glycopeptides and their analogues are useful building blocks for glycopeptide based vaccines (HIV-1 entry inhibitor) and other therapeutics. With this approach, oligosaccharide glycan and the peptide segments of backbone polypeptide can be introduced in a more convergent and high-yielding method.



**INDEX OF ABBREVIATIONS**

$\delta$	chemical shift
$\Delta$	reflux or heat
$^{\circ}\text{C}$	degree centigrade
aq	aqueous
Bn	benzyl
Boc	tert-butoxycarbonyl
br	broad
brs	broad singlet
<i>t</i> -Bu	tert-butyl
cat.	catalytic
Cbz	Benzyloxycarbonyl
$\text{cm}^{-1}$	inverse centimeter
Cy	cyclohexane; cyclohexyl
d	doublet
dd	doublet of doublets
DBU	1,8-diazabicycloundec-7-ene
DIBAL-H	diisobutylaluminum hydride
DIPEA	diisopropylethylamine
DMAP	4-( <i>N,N</i> -dimethylamino)pyridine
DMF	<i>N,N</i> -dimethylformamide
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EI	electron impact ionization
equiv	equivalent

ESI	electrospray ionization
Et	ethyl
ether	diethyl ether
Fmoc	Fluorenylmethoxycarbonyl
FTIR	Fourier transfer infrared spectroscopy
GalNAc	galactosamine
GlcNAc	glucosamine
h	hour (time)
Hex	hexane
HRMS	high resolution mass spectroscopy
Hz	Hertz
IR	infrared
J	coupling constants
K	Lysine
LiHMDS	lithium hexamethyldisilazide
M <sup>+</sup>	parent ion peak (mass spectrum)
m	multiplet
Me	methyl
min	minute
MESNa	sodium mercaptoethanesulfonate
Ms-Cl	Methanesulfonyl chloride
MS	molecular sieve
N	concentration (normality)
NCL	native chemical ligation
NMR	nuclear magnetic resonance

NVOC	<i>o</i> -nitroveratryloxycarbonyl
Nu	nucleophile
Pd/C	palladium on carbon
Ph	phenyl
PMB	<i>p</i> -methoxybenzyl
ppm	parts per million
Py	pyridine
q	quartet
rt	room temperature
RBF	round bottom flask
s	singlet
sat	saturated
SPPS	solid phase peptide synthesis
t	triplet
TBAF	<i>tetra-n</i> -butylammonium fluoride
TBDMS	<i>tert</i> -butyldimethylsilyl
TBDPS	<i>tert</i> -butyldiphenyl silyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Ub	Ubiquitin

# CHAPTER 1

## Synthesis of $\gamma$ -mercapto lysine derivatives: Potential building blocks for dual native chemical ligation

1. Synthesis of 4-mercapto-L-lysine derivatives: Potential building blocks for sequential native chemical ligation, *Bioorg. Med. Chem. Lett.* **2009**, *50*, 6268-6271.

Pasunooti, K. K.;<sup>#</sup> Yang, R.;<sup>#</sup> Vedachalam, S.; Gorityala, B. K.; Liu, C. F.;\* Liu, X.-W.\* (# these authors contributed equally)

2. Dual native chemical ligation at lysine, *J. Am. Chem. Soc.* **2009**, *131*, 13592-13593.

Yang, R.; Pasunooti, K. K.; Li, F.; Liu, X.-W.\* Liu, C. F.\*

- Featured in *Chem. Eng. News*, Vol 87, 46, Nov16, **2009**.
- Featured in *Chem. Eng. News*, Sep18, **2009**.

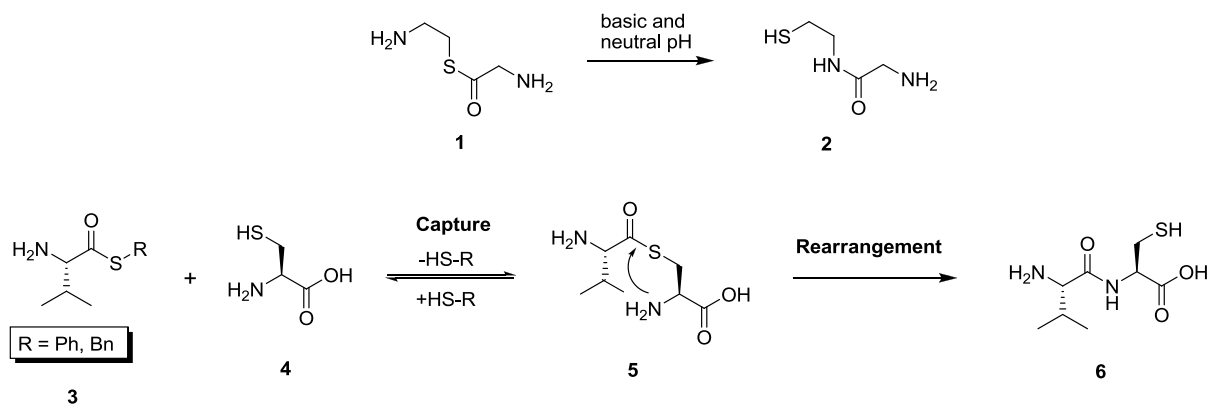
## 1. INTRODUCTION

Since the discovery of native chemical ligation (NCL) by Dawson and Kent in 1994, the field of peptide synthesis has advanced significantly over the last two decades.<sup>1,2</sup> During this time the NCL technique has played a significant role in peptide, protein and glycopeptide chemistry. This technique relies on the combination of a N-terminal cysteine residue with a C-terminal thioester to form a native peptide bond. One year following its discovery, Tam and co-workers expanded this concept using orthogonal coupling method for unprotected peptides in 1995.<sup>3</sup> This concept was further extended to glycopeptide chemistry by other research groups for construction of complex glycoproteins.<sup>4,5</sup> As cysteine is an uncommon amino acid in naturally occurring proteins, several other strategies have been developed to expand the application of NCL. Simultaneously, auxiliary mediated native chemical ligation<sup>6-10</sup> and whilst another strategy employs native chemical ligation followed by desulfurization,<sup>11,12</sup> were introduced and applied for complex protein synthesis. Adopting these investigations, considerable efforts have been made by various research groups including our group, in order to extend the applicability of NCL on amino acid residues utilizing alanine,<sup>11</sup> phenylalanine,<sup>13</sup> valine,<sup>14,15</sup> lysine,<sup>16-18</sup> leucine<sup>19</sup> and threonine.<sup>20</sup> Based on previous methods, we have successfully demonstrated the NCL concept to lysine and efficiently synthesized ubiquitin and diubiquitin proteins.<sup>16-18</sup> Furthermore, we have applied this concept to glycopeptides chemistry.

The term “peptide” was first introduced by Professor Emil Fischer in 1901. His scientific discoveries began with the first account on peptide composition in chemical synthesis. The chemical synthesis of proteins is now possible because of the remarkable advances in peptide synthesis since last century. In 1901, Fischer reported the foremost synthesis of dipeptide (glycyl

glycine) which was followed by the origination of the term “peptide” used to refer to a polymer of amino acids.<sup>21</sup> Later, in 1907 major breakthrough in the synthesis of an octadecapeptide consisting of 15 glycine and 3 leucine residues considered to be a phenomenal achievement.<sup>22</sup> These discoveries paved a platform for modern peptide and protein chemistry.

In 1953, Wieland and co-workers were initially investigated the chemical properties of amino acid thioesters.<sup>23</sup> First, they described that thioesters can undergo intermolecular aminolysis in the presence of amines to yield amides. However, in contrast to this observation, a glycine thioester of cysteamine **1** could not be synthesized under neutral pH conditions (Scheme 1).<sup>23</sup> This observation could be attributed to the additional amino group required with the thiol moiety. Consequently, a rapid intramolecular *S*→*N* shift furnished the corresponding amide **2**. This report infers that this reaction could be used for peptide synthesis. For this purpose the previously described intramolecular rearrangement was combined with an intermolecular thiol-thioester exchange. Similarly, a valine (Val) thioester **3** was treated with cysteine (Cys) **4** allowing the highly reactive thioester to rapidly exchange with the thiol moiety of cysteine in the so-called ‘capture step’. The combined Val-S-Cys **5** subsequently rearranged to form a dipeptide (Val-Cys) **6**, which is linked to a native peptide bond.

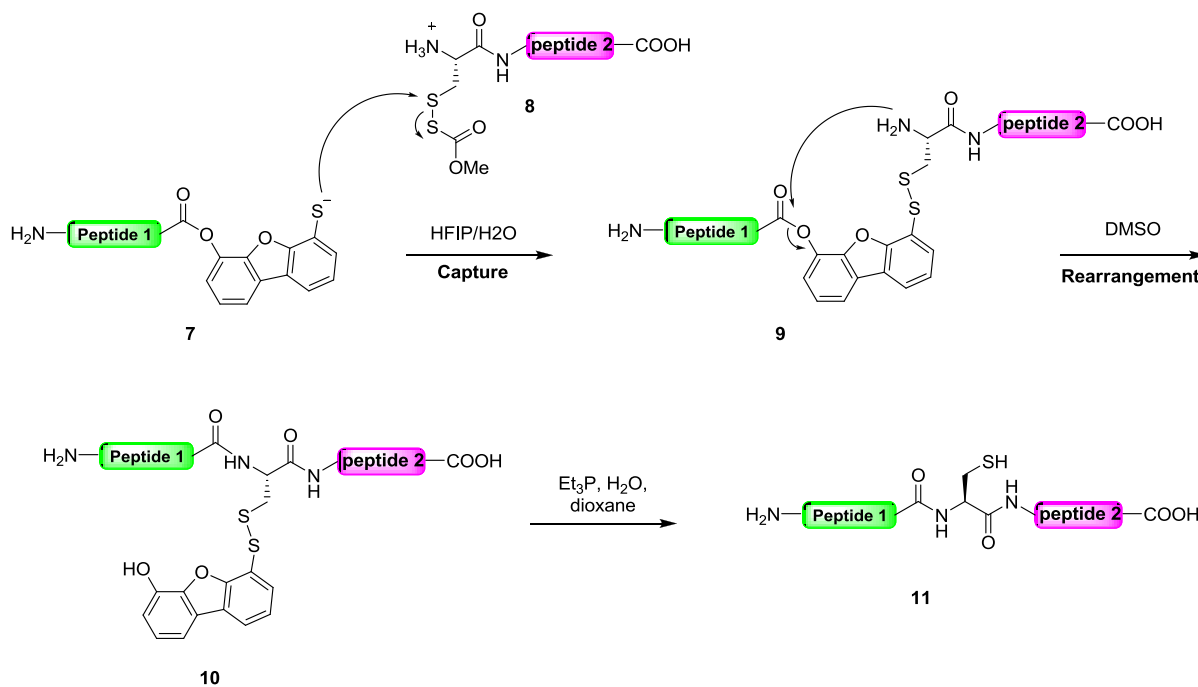


**Scheme 1** Early experiments on aminolysis of thioesters by Wieland method.

Later, in 1963 Professor Merrifield introduced the concept of solid-phase peptide synthesis (SPPS) and successfully synthesized a tetrapeptide.<sup>24,25</sup> The advent of this technique heralded a revolution in peptide and protein chemistry. The synthesis proceeded *via* an attachment of an amino acid to an insoluble solid support through its carboxyl group and then coupled the next protected amino acid *via* an activated carboxyl group. The protecting group of amine was then removed, and the next amino acid was introduced in a similar way. Within a short time, Merrifield invented an instrument for the automated synthesis of peptides which was allowed for the first chemical synthesis of the enzyme, Ribonuclease A (RNase A).<sup>26,27</sup>

Later on, during 1970s Kemp and co-workers described the ligation technique called ‘prior thiol capture strategy’ for peptides.<sup>28-32</sup> Initially, they attached an auxiliary template to the C-terminal peptide, such as 6-hydroxy-4-mercaptodibenzofuran through an ester bond using solid phase peptide synthesis (Scheme 2).<sup>33</sup> The capture step is mediated by a disulfide exchange between a cysteine residue **8** and the free thiol of the auxiliary attached peptide **7**. Similarly, an intramolecular O→N shift takes place to produce **9** to give peptide product **10** as a native bond between two peptides. Upon completion of the acyl transfer reaction, triethylphosphine reduction produces the ligation product **11**.<sup>34</sup> The usefulness of this approach was demonstrated by the

ligation of a variety of peptide fragments. Kemp's prior thiol capture strategy is an important contribution to the development of peptide ligation concepts. It represents the first demonstration of the chemoselective ligation using unprotected peptide fragments. This concept was well optimized and utilized for various unprotected peptides to produce polypeptides containing up to 39 amino acids. In addition, it demonstrates that acyl transfer occurs *via* an intramolecular reaction.

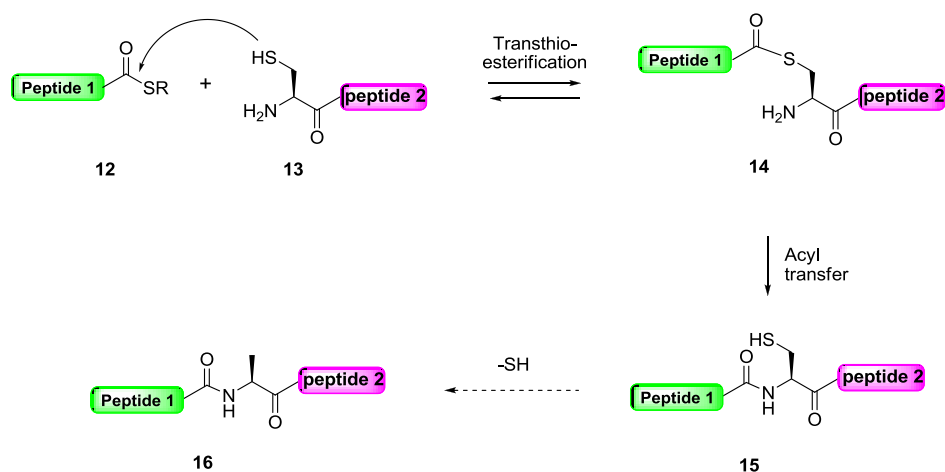


**Scheme 2** Kemp's Prior thiol captures strategy. DMSO = dimethylsulfoxide, HFIP = hexafluoroisopropanol.

These important investigations were stimulated to set the basic landmarks for further research activities in this field. After this era, chemoselective peptide ligation strategy became a powerful tool in peptide and protein chemistry. This method involves a ‘capture step’ that links two peptides in a chemoselective way followed by an intramolecular rearrangement. The “capture/rearrangement” concept was introduced and broadly used in native chemical ligation (NCL) in peptide and protein chemistry during 90’s.<sup>35</sup>

## 1.1 Native chemical ligation

Over the past two decades, native chemical ligation (NCL) technique has played a significant role in peptide, protein and glycopeptide chemistry. Since the discovery of NCL concept by Dawson and Kent in 1994, the field of peptide and protein synthesis has achieved significant advancement over the past two decades.<sup>1</sup> This technique relies on the combination of a N-terminal cysteine residue with a C-terminal thioester to form a native peptide bond. This study demonstrated that the reaction undergoes a chemoselective ligation with unprotected peptides and proteins. At present, the NCL technique is a widely used protocol based on a “capture/rearrangement” concept.<sup>23</sup> The first chemoselective capture step is mediated by a reversible thiol-thioester exchange between the C-terminus thioester **12** and the nucleophilic thiol of a cysteine (Cys) located at the N-terminus of the C-terminal peptide fragment **13** (Scheme 3). In the rearrangement step, the thioester intermediate **14** undergoes a rapid intramolecular  $S \rightarrow N$  shift *via* a favorable five-membered transition state to form a native peptide bond between the two peptides (**15**). More importantly, an additional cysteine residue of peptide does not interfere in the overall reaction process.



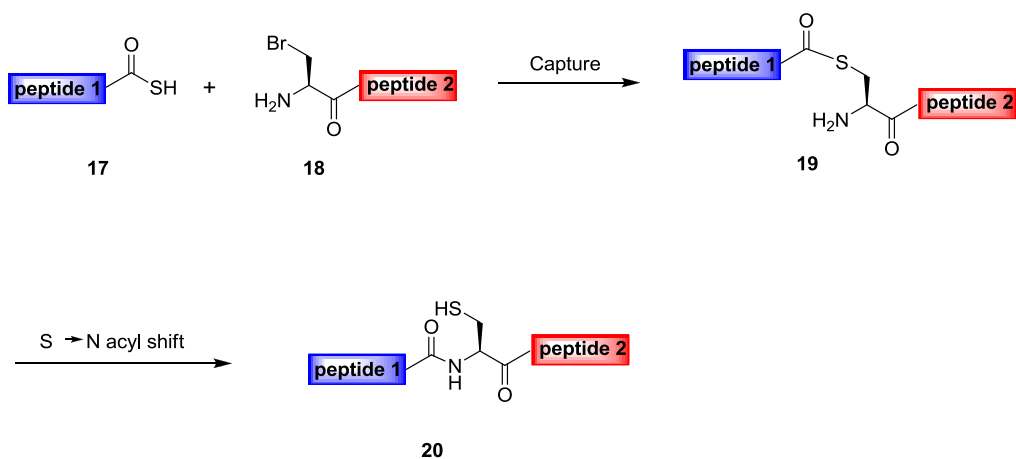
**Scheme 3** Native chemical ligation (NCL) by Dawson and Kent.

A major advantage of NCL is the mild reaction conditions, which allow the ligation to be carried out in aqueous conditions under neutral pH values.<sup>36</sup> The strong basic conditions make other amino acids such as Lys amenable to react with thioesters. Similarly, strong acidic conditions do not favor the ligation reaction due to the low reactivity of the thiol functionality of Cys and the N-terminal amine of the Cys. The second major advantage of NCL is the nature of the amino acid located at the C-terminal thioester during the ligation. Dawson's research group has studied this observation in detail using various amino acids at the C-terminal thioester.<sup>37</sup> These studies concluded that all amino acid thioesters can undergo the ligation reaction, but the side chains affect the ligation rates significantly. The Gly thioesters react very fast taking only four hours to complete. In contrast, thioesters containing sterically hindered and branched amino acids such as leucine and proline undergo reaction at very slow rate. Finally, the nature of the thioesters has a great impact on the NCL reaction. In addition, it has been found that alkyl thioesters are less reactive than aryl thioesters, thus, peptide thioesters are synthesized as alkyl thioesters and converted into the corresponding aryl thioesters *in situ* by the addition of excess aryl thiols.<sup>38</sup> The addition of aryl thiol allows the reaction to proceed rapidly.

Native chemical ligation (NCL) is a robust approach for the total chemical synthesis of proteins.<sup>39</sup> The innovative tool, NCL is currently being used to synthesize many bioactive proteins from two synthetic peptide fragments. The practical utility of NCL in protein chemistry is tremendously expanding. Some recent examples includes, synthesis of the human matrix Gla protein (84 residues),<sup>40</sup> the anticoagulant microprotein S (116 residues),<sup>41</sup> and the human neutrophil pro  $\alpha$ -defensin-1 (75 residues).<sup>41</sup> In addition, glycoproteins such as diptercin (58 residues)<sup>42</sup> and lymphotactin (93 residues)<sup>41</sup> were synthesized using this approach. One further example is the total chemical synthesis of crambin (46 residues) synthesized in a one-pot reaction by the sequential coupling of three synthetic polypeptide chains.<sup>43</sup>

A powerful aspect of chemically synthesizing proteins using NCL is the ability to introduce non-natural residues as probes for structural and functional determination. Nowadays, there are several research groups utilizing the NCL strategy for various purposes such as (a) the selective introduction of protein modifications or tags, (b) the total chemical synthesis of post-translationally modified and unmodified proteins, and (c) isotopic labeling of proteins.

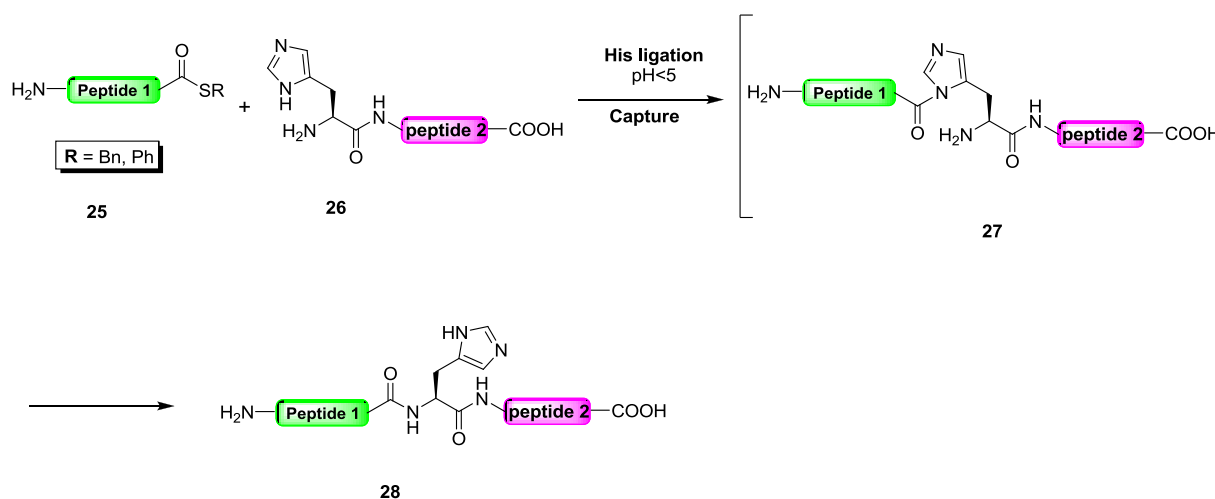
Later on, in 1995 Liu CF and Tam J expanded this concept using orthogonal coupling methods for unprotected peptides.<sup>3</sup> In this method, peptide bearing a C-terminal thioacid reacts with a N-terminal peptide with  $\beta$ -bromoalanine to generate native peptide bond. Initially, the peptide thioacid **17** reacts with the second peptide  $\beta$ -bromoalanine **18** to generate a thioester analogue **19** during the ligation process (Scheme 4). This precursor further undergoes  $S \rightarrow N$  acyl transfer to generate a native amide bond with a cysteine residue at the ligation site to form polypeptide **20**.



**Scheme 4** Orthogonal coupling method by Tam and co-workers.

## 1.2 Peptide ligation with Histidine (His)

In 1953 Wieland and co-workers had proposed the first example for peptide ligation at Histidine.<sup>44</sup> Histidine contains nitrogen that acts as a nucleophile, which proceeds through acyl transfer under acidic conditions (Scheme 5). However, this approach gives low yields when histidine peptide **26** ligates with the C-terminal peptide thioester **25**. This approach could not become popular due to low reactivity of histidine.

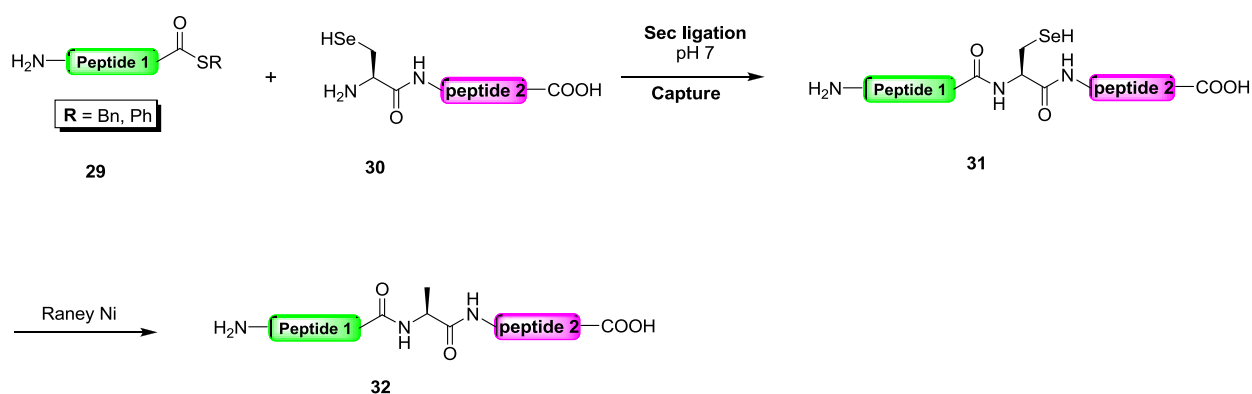


**Scheme 5** ligation at Histidine (His).

## 1.3 Peptide ligation with Selenium

Another strategy for native chemical ligation involves the use of a selenol as a nucleophile, instead of a thiol group to form a peptide ligation product.<sup>45</sup> Selenocysteine (Sec) is a naturally occurring amino acid and the feasibility of using selenocysteine as a ligation product in NCL has been well studied with different model systems (Scheme 6). Ligation with a selenocysteine residue occurs much faster than that of cysteine at lower pH values. The selenocysteine (Sec) group is more acidic and highly nucleophilic than a thiol and Sec-mediated ligations are very fast

and furnish high yields. Selenocysteines have been widely used as ligation products in the synthesis of various peptides and proteins. Selenol act as a nucleophile in selenocysteine **30**, instead of a thiol group for NCL and reacts to give the peptide ligation product **31** with selenocysteine. This functionality is further converted into an alanine residue **32** when using Raney nickel employed as a reducing catalyst.



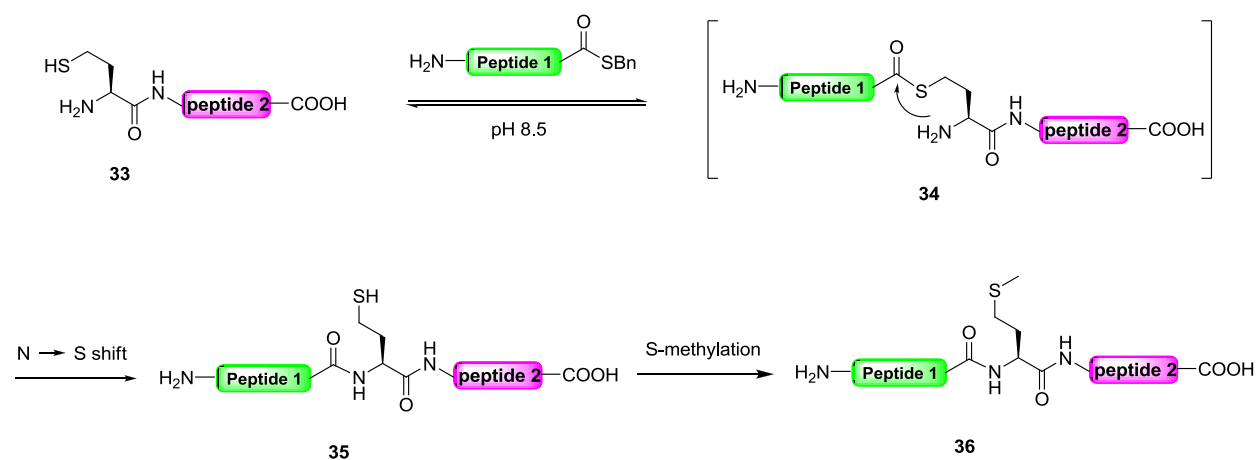
**Scheme 6** Ligation at Selenocysteine (Sec).

#### 1.4 Methionine (Met) ligation

Methionine ligation pathway proceeds in a similar manner to NCL.<sup>46</sup> Homocysteine also undergoes  $S \rightarrow N$  acyl transfer efficiently due to the additional methylene group in its side chain. After the ligation process, a methyl group was attached on the sulfur using *p*-nitrobenzenesulfonate (Scheme 7).

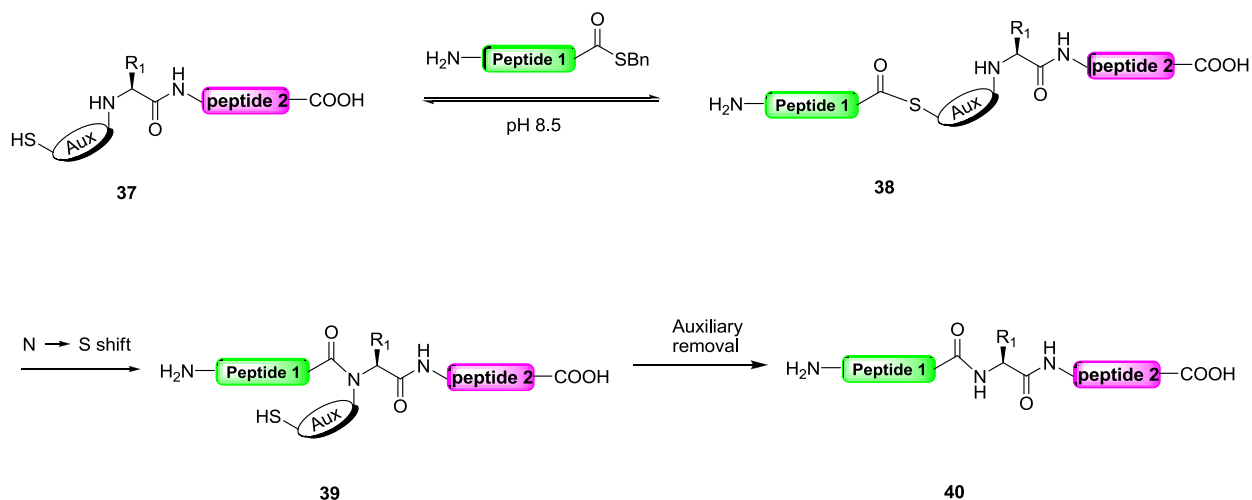
This intermediate leads to acyl migration to form an amide bond at the ligation junction. The latent thiol functionality of methionine can be exploited using homocysteine peptide **33** for transthioesterification with another  $\alpha$ -thioester peptide to give an *S*-acyl intermediate **34**. Subsequently,  $S \rightarrow N$  acyl migration of this acyl precursor spontaneously rearranges to form

homocysteinyl amide bond **35**. S-methylation performs with excess *p*-nitrobenzenesulfonate to produce Met at the ligation site (**36**). Under slightly basic pH conditions, methionine ligation is selective and orthogonal and moreover furnished in short reaction time without any side reactions.



**Scheme 7** Orthogonal coupling with Methionine (Met) at the ligation site.

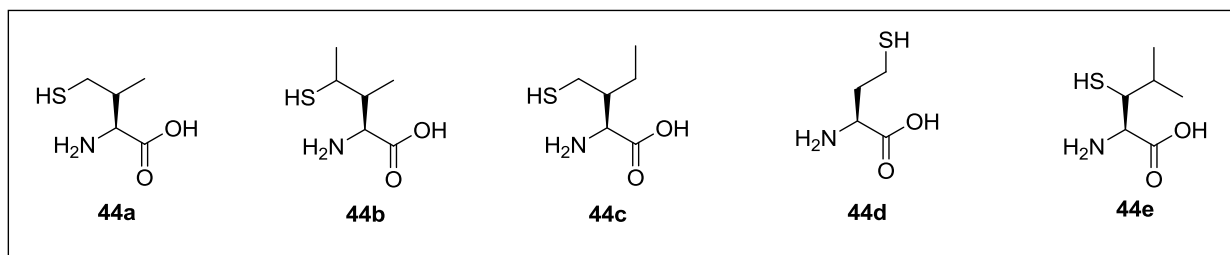
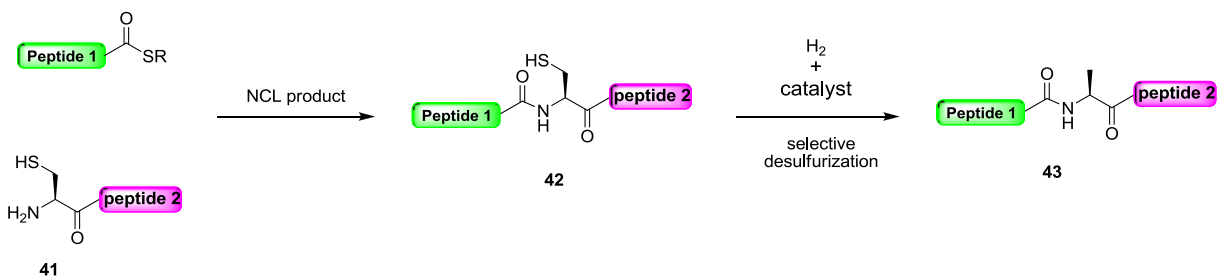
### 1.5 Auxiliary mediated native chemical ligation

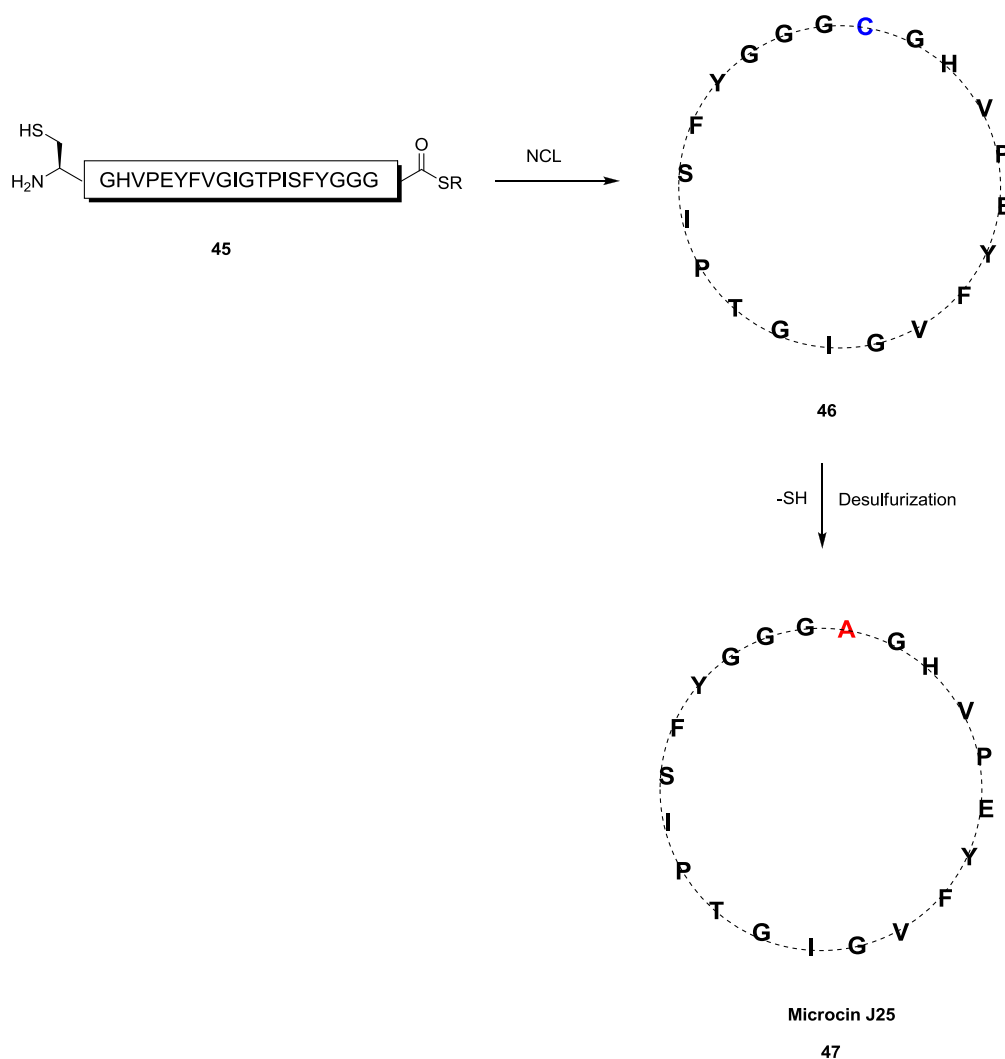


**Scheme 8** Auxiliary mediated ligation.

Several strategies have been performed to extend the native chemical ligation (NCL) concept utilizing auxiliary mediated approaches to combine unprotected peptide segments. Initially, Dawson<sup>6,7,47</sup> and Kent<sup>8-10</sup> research groups independently exploited their investigation on auxiliary mediated NCL reaction and applied to protein chemistry. As shown in Scheme 8, auxiliaries act as cysteine residues to initiate the chemical ligation of peptide fragments. These auxiliaries are attached to the N-terminal peptides. The C-terminal peptide thioester is activated to allow capture of the auxiliary peptide **37**. After the capture step, a  $\text{S} \rightarrow \text{N}$  acyl migration reaction produces to form an amide bond (**39**). The final step is removal of the auxiliary, which produces the desired product **40**. Among all other auxiliaries, Dawson's 4,5,6-trimethoxy-2-mercaptobenzyl (Tmb) auxiliary functions as a cysteine for the ligation and easy to remove in final step under mild conditions.<sup>47</sup> In 2008, Muir's group chose photo-cleavable compound<sup>48</sup> for ubiquitylated histone synthesis,<sup>49</sup> which is based on a ligation auxiliary described previously by Dawson<sup>47</sup> and Aimoto.<sup>50</sup>

## 1.6 Chemical modification of native chemical ligation products





**Scheme 9** Dawson's synthesis of peptide antibiotic Microcin J25.

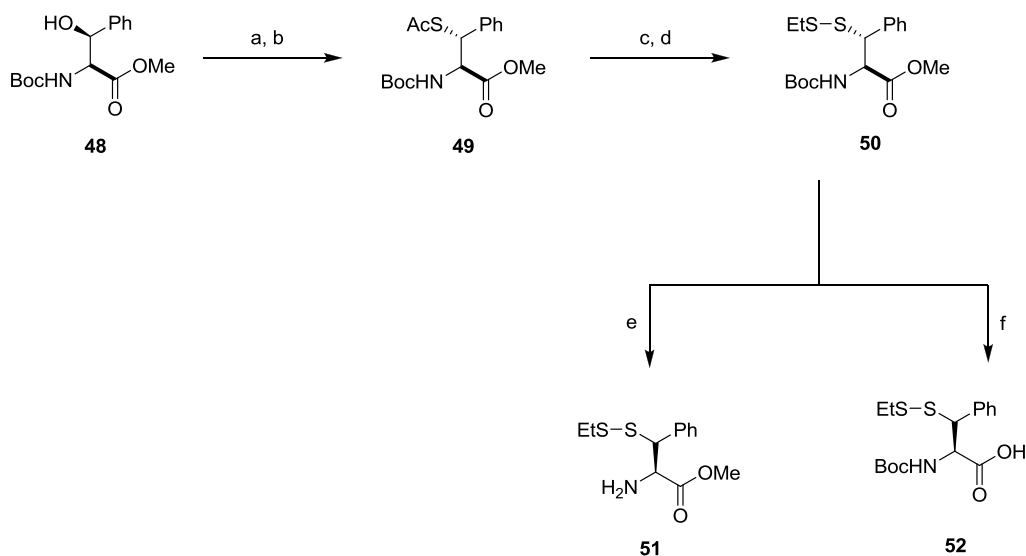
Application of the NCL method is often limited due to the shortage of cysteine residues in naturally occurring proteins. The chemical modification of ligated peptides can be achieved *via* native chemical ligation followed by desulfurization.<sup>11</sup> This strategy employs standard NCL strategy using a cysteine residue. After the ligation step, chemical desulfurization of the cysteine residue gives alanine at the ligation junction. The utility of this approach has been expanded by chemical modification of the peptide and protein.

Since cysteine is an uncommon amino acid among naturally occurring proteins, several strategies have been developed to expand the application of NCL. It was proposed that the extension of NCL to amino acids other than cysteine could be achieved most directly by post-desulfurization of modified cysteine residue. Cysteine-containing peptides can be converted to alanine derivatives through desulfurization. The NCL followed by desulfurization strategy was further extended to homo cysteine (hCys) residues, which demonstrate the utility of this method to other amino acids. This strategy was first proposed by Dawson and co-workers in 2001.<sup>11</sup> In the first step, the peptide thioester reacts with the cysteinyl peptide **41** in a reversible thiol exchange reaction to furnish the native peptide bond (Scheme 9). Subsequently the cysteine containing peptide **42** can be converted to alanine **43** through selective desulfurization. The NCL followed by desulfurization strategy further extend to various other natural and unnatural amino acids **44a-44e** (Val, Ile, Ile, Abu, Leu).<sup>11</sup> Their method was demonstrated in the synthesis a barnase analogue, a streptococcal protein G B1 domain and Microcin J25. Microcin J25 **47** is a 21 amino acid containing cyclic peptide, which is naturally produced by a strain of *Escherichia coli*. Alanine was replaced by cysteine in the linear thioester **45** to perform native chemical ligation step. The intramolecular native chemical ligation of **46** involves N-terminal cysteine residue and the C-terminal glycine thioester. Further, metal mediated desulfurization provides the Gly-Ala junction of the cyclic Microcin J25 **47**.<sup>11</sup>

## 1.7 Native chemical ligation at phenylalanine

Inspired by Dawson's NCL followed by desulfurization strategy, several other research groups explored the native chemical ligation concept. In 2007, Crich and co-workers demonstrated "native chemical ligation at phenylalanine".<sup>13</sup> They reported that the  $\beta$ -thiol group of phenylalanine precursor facilitate as a sulfur source for ligation.

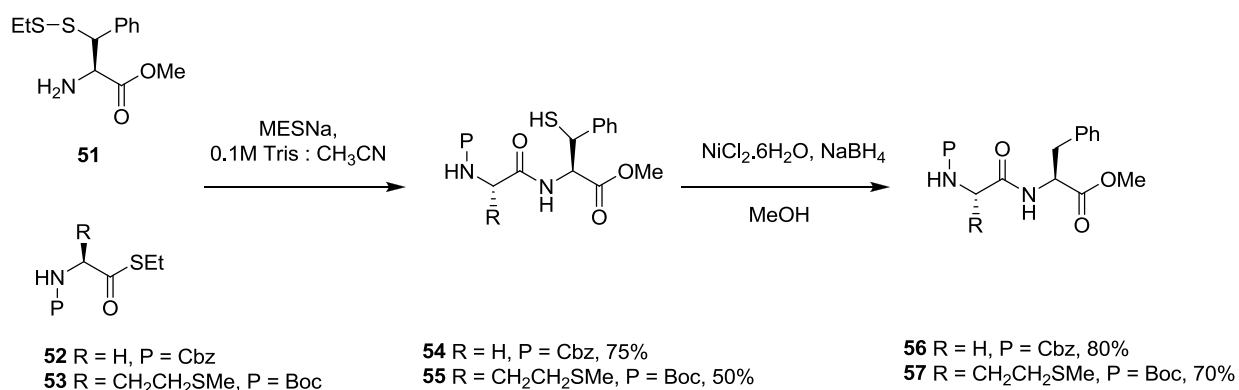
$\beta$ -mercaptophenylalanine, a key building block for native chemical ligation was synthesized from readily available L-phenylalanine. *Threo*- $\beta$ -hydroxy-L-phenylalanine derivative **48** was conveniently synthesized from L-phenylalanine using Easton's bromination protocol<sup>51</sup> (Scheme 10). The alcohol **48** was then converted in to the thiol derivative **49** by using well established methods. Then the thiol was protected in disulfide form using *S*-ethyl ethanethiosulfonate to furnish compound **50** in good yields. Treatment of compound **50** with trifluoroacetic acid (TFA) afforded amino ester **51** and saponification of compound **50** gave the acid compound **52**.



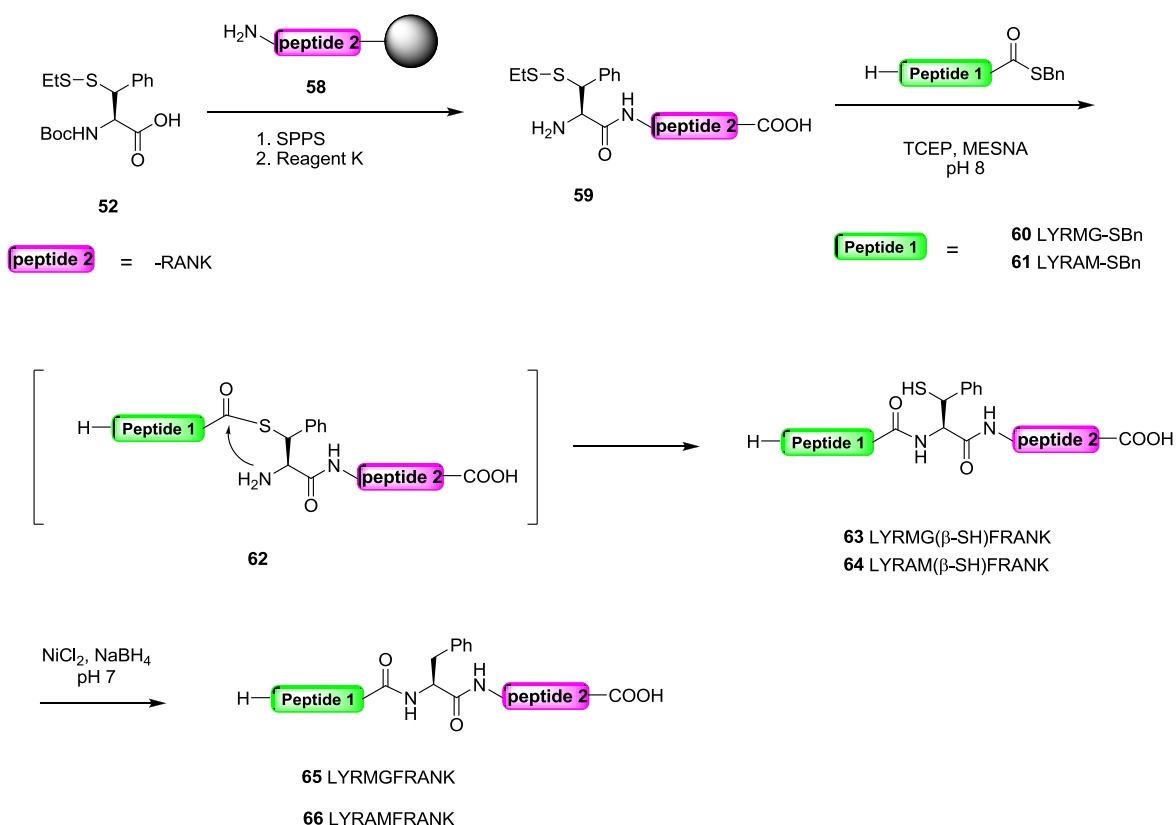
**Scheme 10** Synthesis of  $\beta$ -mercaptophenylalanine: (a) MsCl, Et<sub>3</sub>N, DCM; (b) AcSH, DBU, DMF; (c) 1N NaOH, MeOH; (d) *S*-ethyl ethanethiosulfonate, Et<sub>3</sub>N, DCM, 3 steps combine yield 55-60%; (e) TFA, DCM, 76%; (f) LiOH, THF, 40%.

The *N*-Cbz glycine and *N*-Boc-L-methionine thioesters **52** and **53** were chosen as a model system for ligation (Scheme 11). Thioesters **52** and **53** were ligated with the  $\beta$ -mercaptophenylalanine derivative **51** in the presence of sodium 2-mercaptophenylalanine (MESNa) in a mixture of acetonitrile and Tris buffer at pH 7.5-8.0. Under these conditions, the glycine based dipeptide **54** and methionine analogue **53** were obtained in good yields. Successful desulfurization of dipeptides **54** and **55** in the presence of nickel boride (in situ by sodium borohydride reduction of nickel chloride) furnished *N*-Cbz-Gly-L-Phe-OMe **56** and *N*-Boc-Met-L-Phe-OMe **57** in good yields, respectively. With the proof of preliminary results, they successfully applied this method to other small chain peptides.

$\beta$ -(SSEt)-FRANK peptide **59** was successfully synthesized by using solid phase peptide synthesis (SPPS) conditions from acid compound **52** (Scheme 12). Ligation of both thioesters **60** and **61** with  $\beta$ -(SSEt)-FRANK peptide **59** was achieved in the presence of MESNa and tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl) in buffer solution. After purification of ligated products **63** and **64** treated with reducing reagent ( $\text{NiCl}_2/\text{NaBH}_4$ ) in the presence of buffer solution to provide the target peptides LYRMGFRANK (**65**) and LYRAMFRANK (**66**) in very good yields.



**Scheme 11** Model ligation studies.

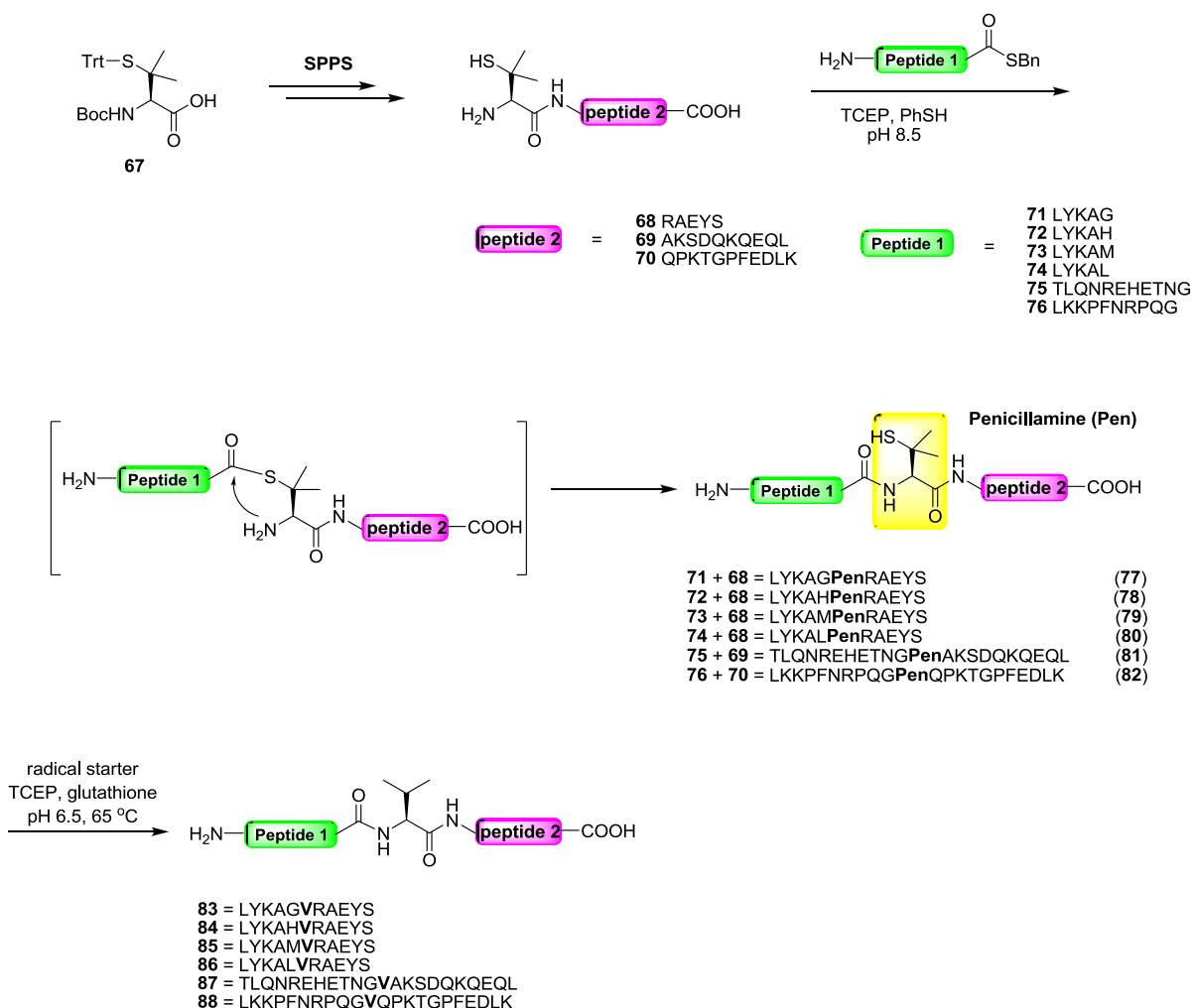


**Scheme 12** Native chemical ligation at Phenylalanine (Phe).

### 1.7 Native chemical ligation at valine

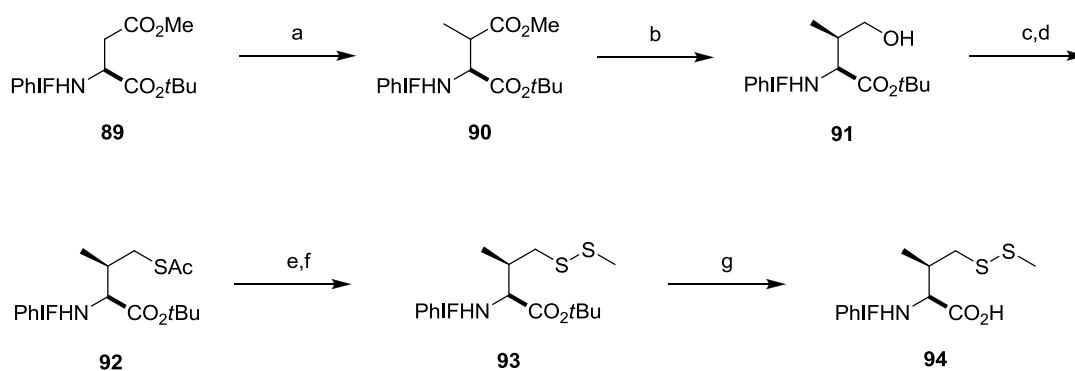
Another successful native chemical ligation/desulfurization strategy was demonstrated at the same time by Seitz<sup>14</sup> and Danishefsky<sup>15</sup> groups in 2008. These two groups extended the scope of the native chemical ligation strategy using thiol containing valine as a sulfur source. Seitz's group utilized Penicillamine (Pen) as a key precursor of valine in the ligation and desulfurization strategy.<sup>14</sup> Valine is an abundant amino acid in nature compared to cysteine (valine 6.6%, cysteine 1.3%). Boc/Tri-protected penicillamine **67** was treated with various N-terminal peptides using solid-phase peptide synthesis (SPPS) (Scheme 13). The sterically hindered penicillamine

peptides **68-70** were treated with peptide thioesters (**71-76**) under high pH (7.5-8.5) conditions. The penicillamine-mediated ligation reaction was performed with Gly-, His-, Met-, and Leu-thioesters yielding up to 87% (**77-82**). The resulting penicillamine ligation products were successfully converted into the valine peptides (**83-86**) by using an optimized desulfurization method. Furthermore, the generality of the penicillamine-mediated ligation/desulfurization was demonstrated in the synthesis of two long chain peptides (signal transduction proteins STAT 1 **87** and Syk-kinase **88**).



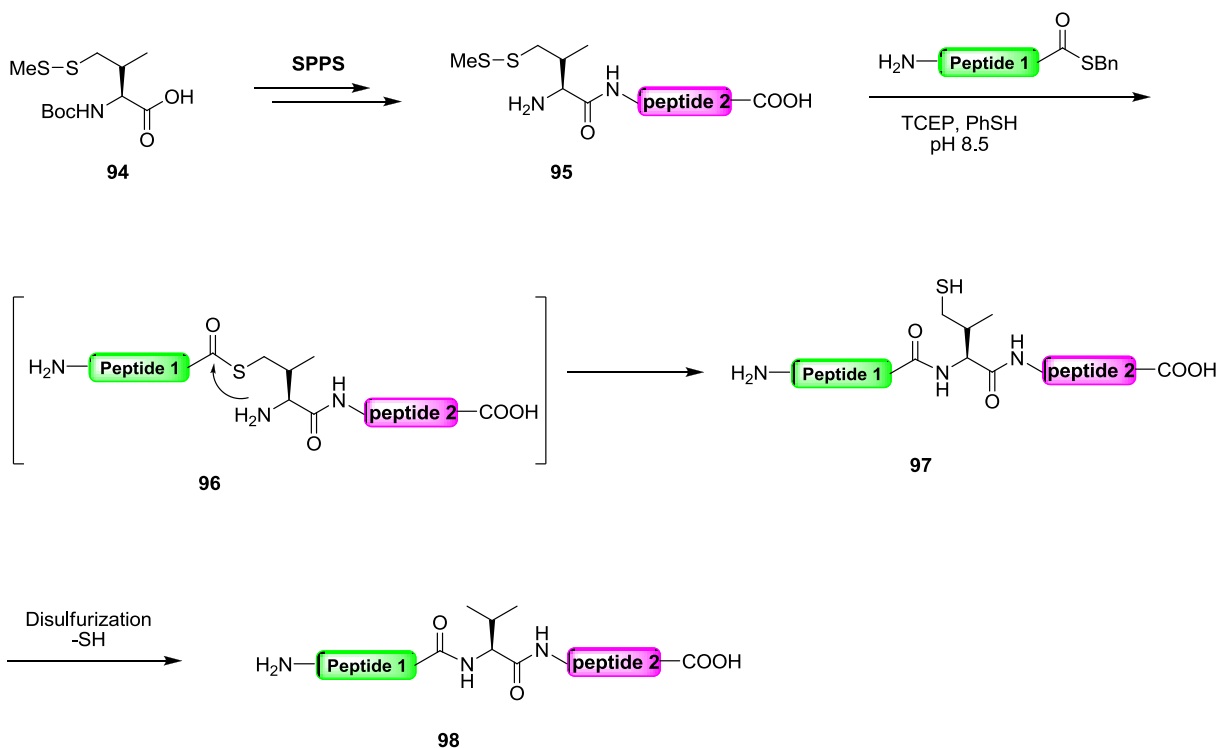
**Scheme 13** Native chemical ligation at Valine (Val).

At the same time, Danishefsky and co-workers reported the use of another valine precursor for native chemical ligation.<sup>15</sup> A  $\gamma$ -thiolated valine building block **94** facilitate the ligation/desulfurization reaction (Scheme 14). Synthesis of the key intermediate  $\gamma$ -hydroxy valine **91** was obtained from L-aspartic acid using the Rapoport and Wolf method.<sup>52</sup> The valine alcohol **91** was successfully converted in to the target compound **94** using known literature conditions.



**Scheme 14** Native chemical ligation at Valine (Val): (a) KHMDS, MeI, THF, 97%; (b) DIBAL-H, THF, 83%; (c) MsCl, Et<sub>3</sub>N, DCM; (d) AcSH, DBU, DMF, 73% in 2 steps (e) 1N NaOH, MeOH; (f) MMTS, Et<sub>3</sub>N, DCM, 86% in 2 steps; (g) HCl in EtOAc, 82%.

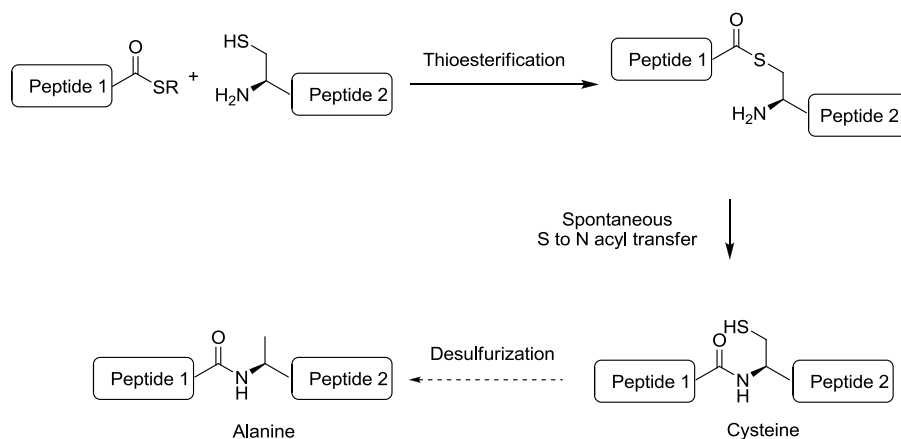
This  $\gamma$ -thiolated valine building block **94** was treated with various N-terminal peptides using solid-phase peptide synthesis (SPPS). The ligation of peptide **95** with various peptide thioesters proceeded faster than the pencillamine-mediated ligation because of the high reactivity of the primary amine (Scheme 15). Finally, desulfurization of peptides using free-radical method furnishes the desired products (**98**).



**Scheme 15** Native chemical ligation at valine (Val).

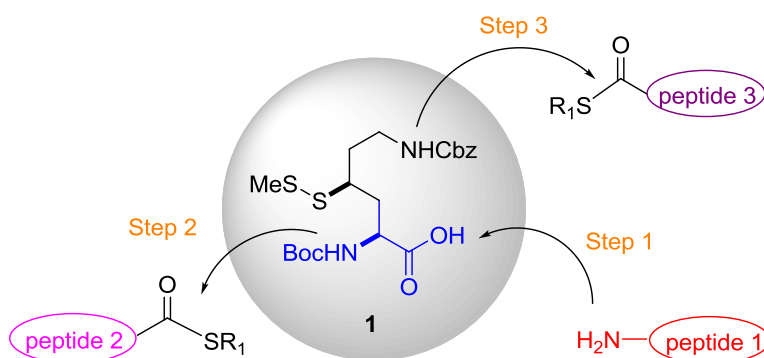
## 2. RESULTS AND DISCUSSION

Amino acids like lysine are the important building blocks of many proteins. The substituted lysine derivatives are useful intermediates for many biological applications such as Post-translational modifications. Post-translational modifications play a significant role in regulating protein function. Among all other modifications, ubiquitination is one of the most important post-translational modifications. Accordingly, we adopted native chemical ligation (NCL) strategy which was developed by Dawson and Kent in 1994 for novel peptides and protein domains.<sup>1</sup> Since its discovery it has become a powerful tool for the chemical synthesis of cyclic and linear peptides. This technique relies on the combination of a N-terminal cysteine residue with a C-terminal thioester to form a peptide bond (Figure 1). Unfortunately, very few natural thiol-containing amino acids like cysteine are available for NCL to form peptide bond. So, synthesis of 4-mercapto L-lysine derivatives serves as a pivotal building block for assemble branched peptides.



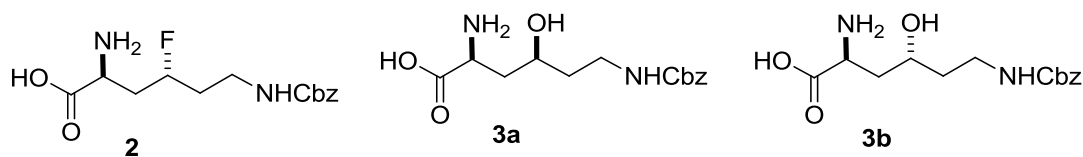
**Figure 1** Native chemical ligation (NCL)

Inspired by native chemical ligation (NCL), we hypothesized that the  $\gamma$ -mercapto lysine residue **1** could facilitate ligation at  $\alpha$ - and  $\epsilon$ -amines of lysine (Figure 2). We chose a dual native chemical ligation strategy through the use of a  $\gamma$ -mercapto lysine residue. The basic advantage of our strategy that a single thiol group introduced on the  $\gamma$ -carbon of lysine would mediate ligation at both the  $\alpha$ - and  $\epsilon$ -amines, to form native and isopeptide bonds. Therefore, by using a proper protecting group, one can perform two consecutive ligation steps *via* the same thiol to synthesize proteins containing specifically modified lysine residues (e.g., ubiquitinated or biotinylated). In addition, the  $\epsilon$ -amino group of lysine provides a platform for post-translational protein modifications, such as methylation, acetylation, and ubiquitination. The side-chain amine of lysine is also used as an anchor point for labeling proteins with a biophysical or biochemical tag. Therefore, synthesis of these functionalized lysine derivatives is a challenging task due to the multiple functionality of lysine.



**Figure 2**  $\gamma$ -Mercapto lysine mediated dual native chemical ligation.

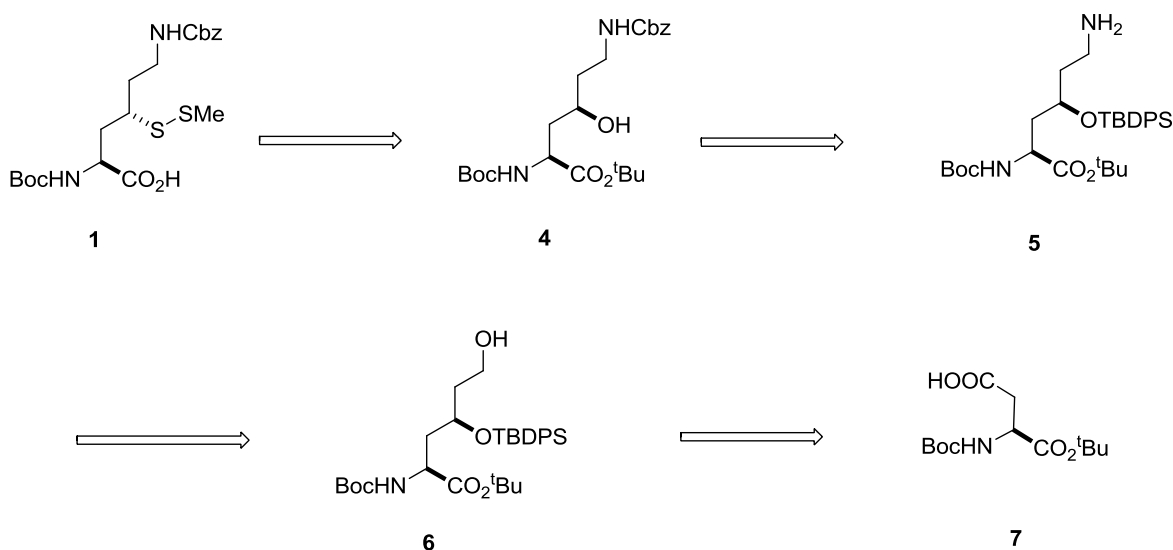
First, the thiol group lies between the  $\alpha$ -amino group and the side chain amine of a lysine **1**, which allows double native chemical ligation and furnishes branched peptides, as shown in Figure 2. Likely, this new branched structure endows the peptides or proteins with new property and capacity. Second, lysine is an essential amino acid and a necessary building block for all proteins in the body. The substituted lysine derivatives, such as (2S, 4R)-4-fluoro L-lysine **2** is a bioactive molecule which enhances the biological activity relative to the parent molecule.<sup>53</sup> The hydroxyl analogue **3a** and **3b** are also useful precursors in many biological active components (Figure 3).<sup>54</sup> Synthesis of these substituted L-lysine derivatives is challenging task due to the multi-functionality of L-lysine.



**Figure 3** Some of 4-substituted L-lysine derivatives.

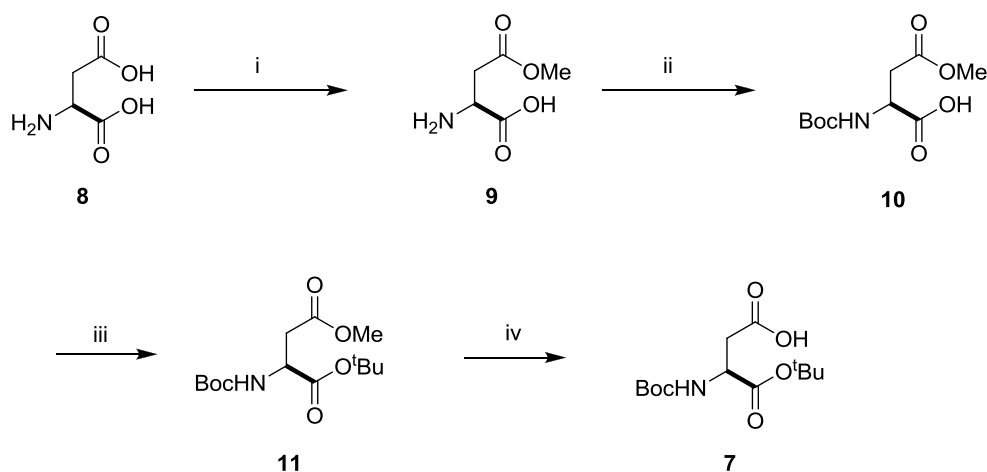
We chose  $\gamma$ -mercapto lysine **1** derivative as a key building block for expanding native chemical ligation technique. The retro-synthetic pathway for protected  $\gamma$ -mercapto lysine **1** was shown in Scheme 1. We envisioned that the chirally pure target molecule, (2S, 4S)-4-mercapto L-lysine could be assembled from protected L-aspartic acid (**7**), which is clearly the most inexpensive starting material. The side chain homology, stereoselective introduction of thio

group and installation of amino groups are the key steps to obtain target molecule. The method of Guichard and co-workers was adopted to prepare a 4-hydroxylysine derivative **6** from readily available aspartic acid **7**.<sup>56</sup> In our preliminary studies on dual ligation, we chose Guichard's protocol for synthesis of our target precursor. Finally, the  $\gamma$ -hydroxy group in compound **4** was easily converted to thiol functionality to get desired compound **1**.



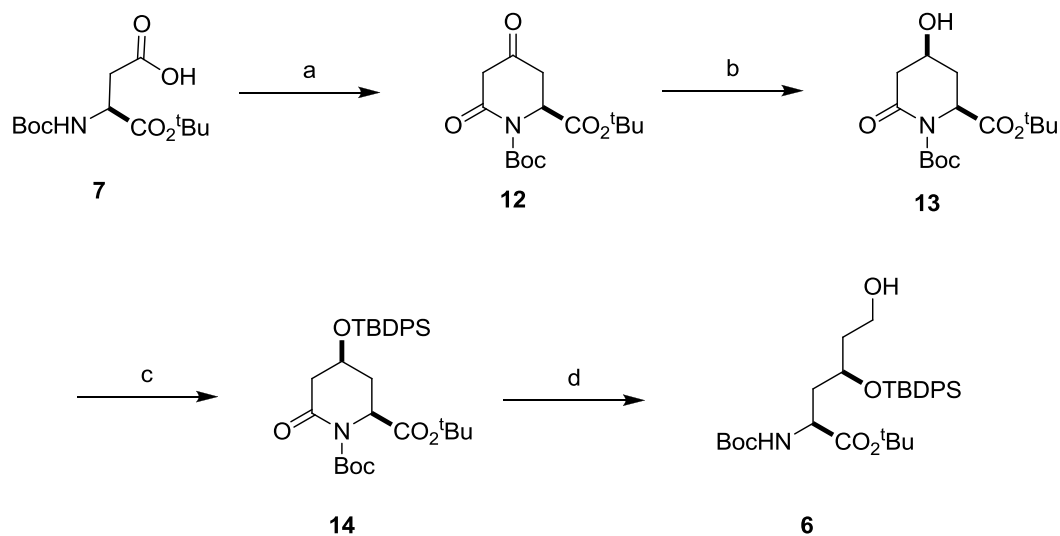
**Scheme 1** Retro-synthetic analysis for 4-mercapto lysine **1**.

At first, we investigated the synthesis of L-aspartic acid (**8**) to  $\alpha$ -*tert*-butyl-(*S*)-*N*-*tert*-butoxycarbonyl aspartate (**7**) using the known Woodard's protocol as outlined in Scheme 2.<sup>55</sup> The esterification of aspartic acid **8** to its corresponding  $\gamma$ -methyl ester **9** was obtained using methanolic HCl. Further, the methyl ester **9** was converted into the Boc protected derivative **10** in 70% yield by treating with di-*tert*-butyl dicarbamate and sodium carbonate in aq dioxane. Then the  $\alpha$ -carboxylic acid was esterified with *tert*-butyl alcohol, dicyclohexylcarbodiimide (DCC), and 4-(dimethylamino)pyridine (DMAP) to give the aspartate **11**. Finally, selective partial hydrolysis of the diester with aq 1 N NaOH in acetone afforded the monoester **7** with good yields.



**Scheme 2** Reagents and conditions: (i) MeOH, HCl, reflux, 80%; (ii) Boc<sub>2</sub>O, Na<sub>2</sub>CO<sub>3</sub>, dioxane/water, 70%; (iii) DCC, DMAP, <sup>t</sup>BuOH, DCM, 75%; (iv) aq NaOH, acetone, rt, 1 h, 85%.

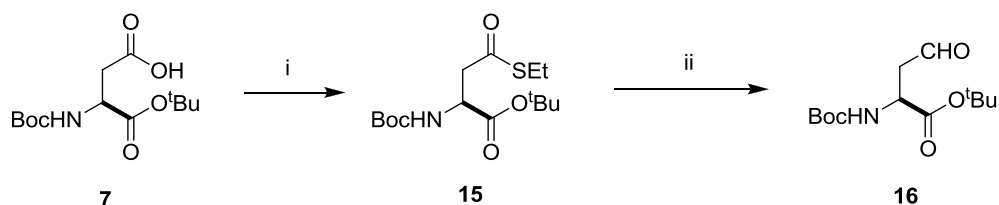
Further, we examine the synthesis of 4-hydroxylysine derivative (**6**) from  $\alpha$ -*tert*-butyl-(*S*)-*N*-*tert*-butoxycarbonyl aspartate (**7**) adopting the Guichard's method (Scheme 3).<sup>56</sup> Aspartate **7** was treated with Meldrum's acid in the presence of EDC and DMAP, followed by cyclization at 80 °C in ethylacetate, provides enantiopure *N*-protected 4,6-dioxopiperidine **12**. *N*-protected dioxopiperidine **12** can be selectively reduced to the corresponding *cis*-4-hydroxy derivative **13** by treatment with NaBH<sub>4</sub> in DCM and acetic acid. Further protection of secondary alcohol **13** by a TBDPS prior to the reduction with NaBH<sub>4</sub>, the protected 1,3 diol **6** could be obtained in excellent yields.



**Scheme 3** Guichard's protocol for synthesis of  $\gamma$ -hydroxy lysine derivative **6**: (a) Meldrum's acid, EDC·HCl, DMAP, and DCM then ethylacetate, reflux, 70%; (b) NaBH<sub>4</sub>, acetic acid, DCM, 72 h, 82%; (c) TBDPSCl, Imidazole, DCM, 85%; (d) NaBH<sub>4</sub>, EtOH, 90%.

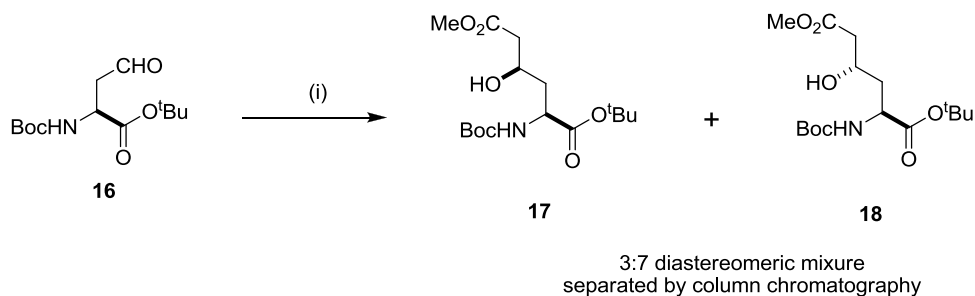
Alternatively, we developed a new methodology to introduce a hydroxy group onto the  $\gamma$ -position of lysine side chain by exploiting Reformatsky reaction as a key step. In this work, we developed a new methodology to introduce a hydroxy group onto the 4-position of L-lysine side chain by using Zn-mediated diastereoselective Reformatsky reaction as a key step.<sup>57</sup> With this precursor, we have synthesized 4-mercapto lysine derivative for native chemical ligation. This methodology allows us to develop various 4-substituted lysine derivatives (Figure 3). The chirally pure target molecule,  $\gamma$ -hydroxy lysine could be assembled from (*S*)-aspartate semi-aldehyde **16**. The side chain homology, stereoselective introduction of thiol and amino groups are the key steps in our strategy.

Further, we would like to synthesize (*S*)-aspartate semi-aldehyde **16** derivative for Zn-mediated diastereoselective Reformatsky reaction (Scheme 4).<sup>58</sup> Adopting the known literature conditions, the aspartic acid **7** was transformed to the corresponding thioester **15** in an excellent yield (95%), using DCC, ethanethiol and DMAP at room temperature. The resulting thioester was reduced with triethylsilane and 10% Pd/C to give (*S*)-aspartate semi-aldehyde **16** in 85% yield.



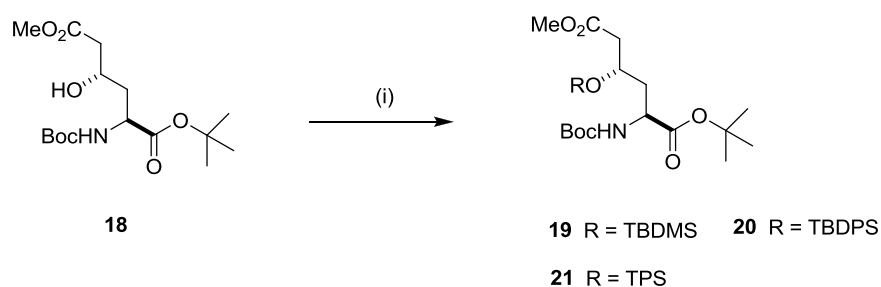
**Scheme 4** Reagents and conditions: (i) EtSH, DCC, DMAP, DCM, rt, 95%; (ii) Et<sub>3</sub>SiH, 10% Pd/C, DCM, rt, 85%.

As shown in Scheme 5, the homologation of the side chain was installed *via* the diastereoselective Reformatsky reaction of (*S*)-aspartate semi-aldehyde (**16**) and methyl bromoacetate.<sup>57</sup> The resulting Reformatsky product has the same carbon skeleton as in the target molecule, and installation of a hydroxyl group is set, which allows easy access to a series of 4-substituted lysine analogues by nucleophilic substitution. Optimization of reaction conditions identified Zinc and trimethylsilylchloride in THF at 0 °C to provide the best substrate conversion rate and product yield.<sup>59</sup> A reasonable diastereoselectivity of erythro/threo (**17/18**, 3/7) was also obtained for this reaction. The selectivity toward diastereomer **18** was quite favorable. It was noted that these two diastereomers could be easily separated by column chromatography.



**Scheme 5** Reagents and conditions: (i) methyl bromoacetate, Zn, TMSCl, THF, 0 °C, 92%.

The major product of Reformatsky reaction **18** was protected with various protecting groups. The selective reduction of methyl ester to the corresponding terminal alcohol was attempted with NaBH<sub>4</sub> in ethanol giving the best results. TBDPS-protected substrate was further converted in to amine functionality using the standard conditions.

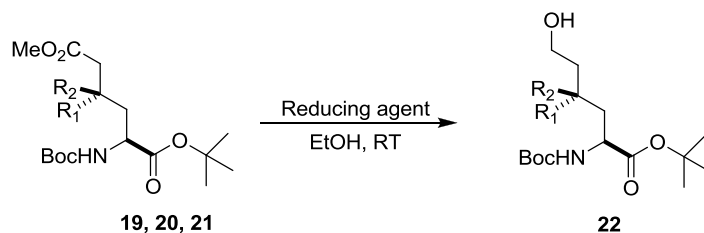


**Scheme 6** Reagents and conditions: (i) general procedure for **20**, TBDPSCl, imidazole, DCM, rt, 89%.

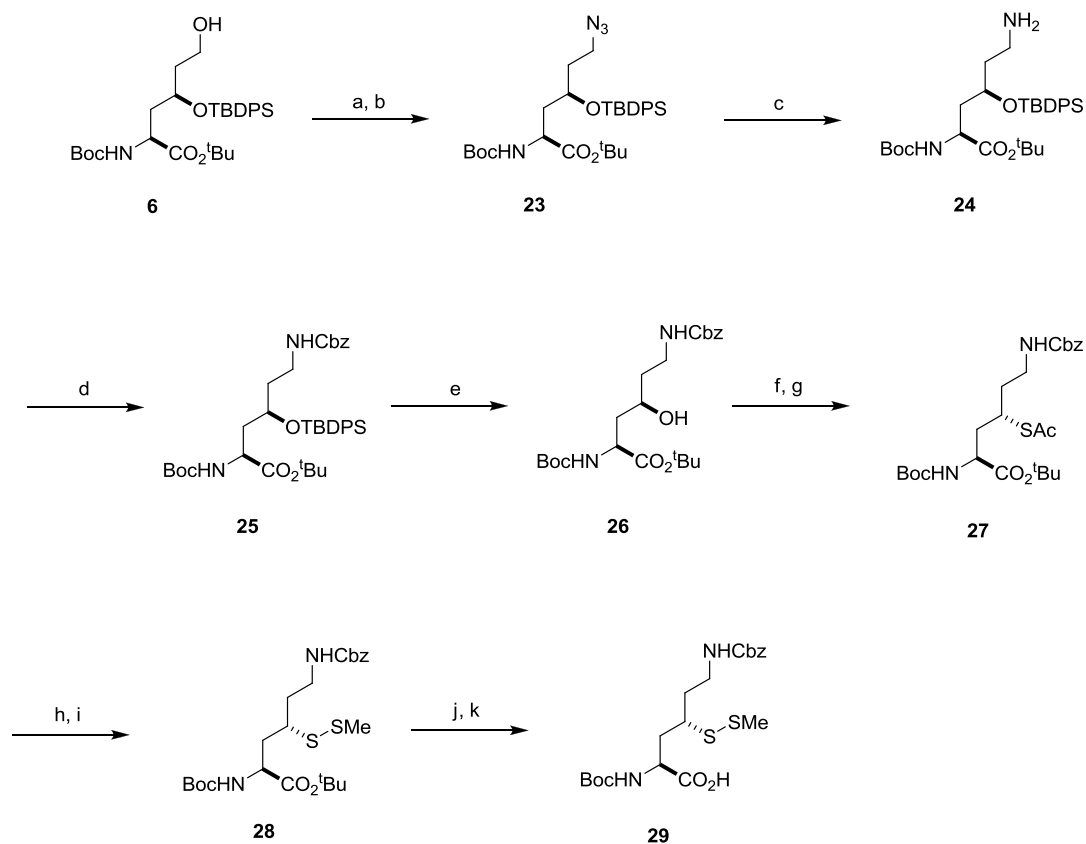
The Reformatsky product **18** was protected with various protecting groups like for instance, TBDMS (**19**, 80%), TBDPS (**20**, 89%) and TPS (**21**, 75%), as shown in Scheme 6. Reduction of methyl ester functionality to terminal alcohol was initially attempted using LiAlH<sub>4</sub> in ether. There was unfortunately no desired product obtained (Table 1, entry 1). To our delight, DIBAL-H in THF afforded the desired product but in a low yield of only 20% (entry 2). To further

improve the yield of the reduction of **21** to **22** with a view to synthesize 4-hydroxy lysine derivatives, other reducing reagents,  $\text{NaBH}_3\text{CN}$  and  $\text{NaBH}_4$  in alcoholic solvents were examined (entries 3-6). Among them,  $\text{NaBH}_4$  in ethanol at room temperature was found to be superior to other reducing reagents in terms of yields (entry 6). With the optimized reaction conditions in hand, we decided to investigate the influence of reducing reagent on the different protecting groups. Thus, the reaction of protected amino acids with  $\text{NaBH}_4$  in ethanol at room temperature was studied (entries 6-10). In all cases TBDPS protected amino acid treated with  $\text{NaBH}_4$  in ethanol (entry 6, Table 1) gave the best result with 92% yield (entry 6). At this stage, the stereochemistry of this compound was assigned by analogy to the similar substrate.<sup>56</sup>

**Table 1** Optimized conditions for selective reduction of methyl ester **19-21**.



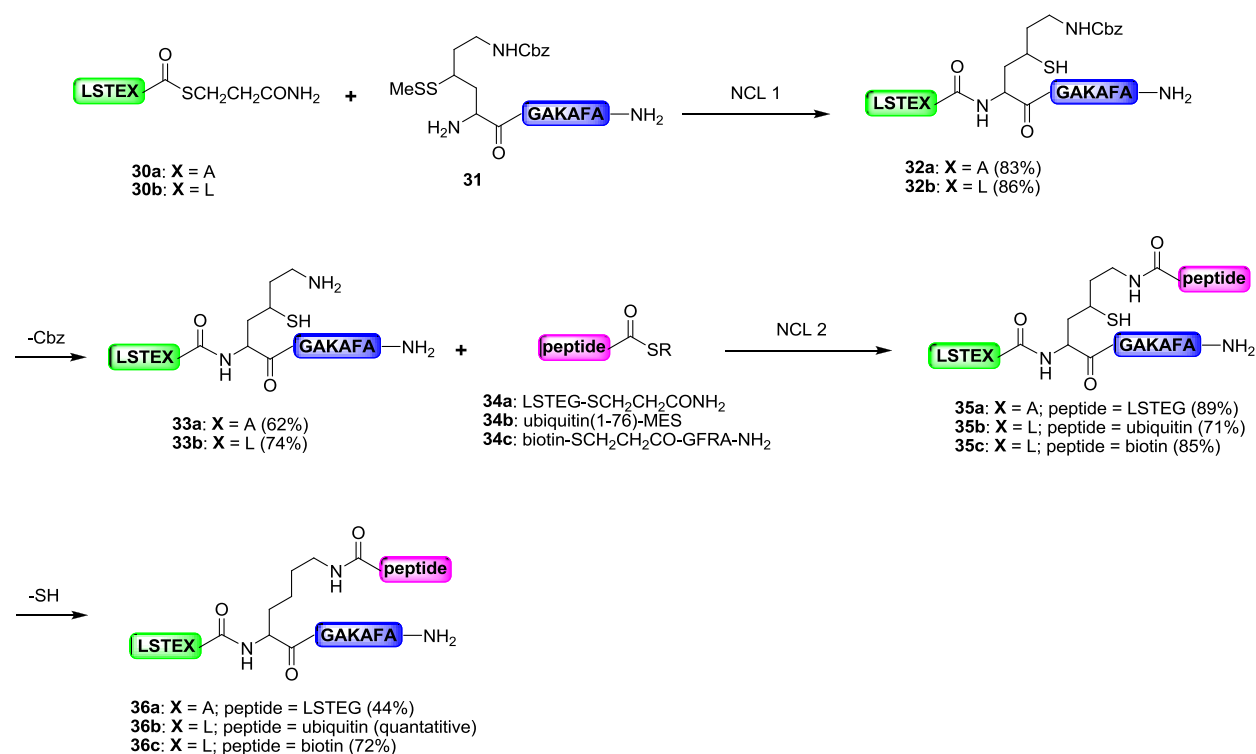
No.	R <sub>1</sub>	R <sub>2</sub>	Reducing agent	Solvent	Yield (%)
1	OTBDMS	H	$\text{LiAlH}_4$	$\text{Et}_2\text{O}$	-
2	OTBDMS	H	DIBAL-H	THF	23
3	OTBDMS	H	$\text{NaBH}_3\text{CN}$	EtOH	36
4	OTBDMS	H	$\text{NaBH}_4$	MeOH	53
5	OTBDMS	H	$\text{NaBH}_4$	EtOH	70
<b>6</b>	<b>OTBDPS</b>	<b>H</b>	<b><math>\text{NaBH}_4</math></b>	<b>EtOH</b>	<b>92</b>
7	OTPS	H	$\text{NaBH}_4$	EtOH	65
8	OH	H	$\text{NaBH}_4$	EtOH	70
9	H	OTBDMS	$\text{NaBH}_4$	EtOH	68
10	H	OTBDPS	$\text{NaBH}_4$	EtOH	85



**Scheme 7** Reagents and conditions: (a) Methanesulfonyl chloride, diisopropyl ethylamine (DIPEA), 0 °C; (b) NaN<sub>3</sub>, DMF, 80 °C, two steps 83% yield; (c) Pd/C, ethylacetate, quantitative yield, rt; (d) CbzCl, NaHCO<sub>3</sub>, dioxane:water (2:1), 0 °C, 81%; (e) TBAF, THF, 0 °C, 77%; (f) Methanesulfonyl chloride, diisopropyl ethylamine (DIPEA), 0 °C; (g) Potassium thioacetate, DMF, 40 °C, two steps 70% yield; (h) NaOH, MeOH, rt; (i) MMTS, Triethylamine, CH<sub>2</sub>Cl<sub>2</sub>, rt; (j) 95% TFA, H<sub>2</sub>O, rt; (k) Boc<sub>2</sub>O, MeOH, Triethylamine, rt.

The last part of the synthesis of protected 4-thiol-lysine consists of introducing thiol unit on lysine side chain (Scheme 7). Having the optimized conditions from us and Guichard's protocol,<sup>56</sup> TBDPS-protected alcohol **6** in DCM was treated with methanesulfonyl chloride and

DIPEA using standard procedure to obtain mesylated product which was converted into azide **23** using  $\text{NaN}_3$  in DMF at  $80\text{ }^\circ\text{C}$ . The azide **23** was subjected to Pd/C catalysed hydrogenation in ethyl acetate under  $\text{H}_2$  atmosphere at room temperature to produce amine **24**. Subsequently, the terminal amine of lysine was easily protected with Cbz group using the standard procedure. The last part of the synthesis of protected 4-mercapto L-lysine consisted of introducing thiol unit into lysine chain. After deprotection of the silyl ether **25** with tetra-butylammonium fluoride (TBAF), the secondary alcohol of amino acid **26** was mesylated followed by nucleophilic substitution of thioacetate to afford **27** in good yield. Substrate **26** was useful intermediate for sequential native chemical ligation.



**Scheme 8** Demonstration of dual native chemical ligation at lysine.

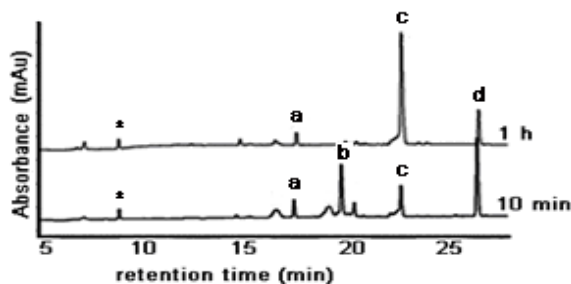
First, the 4-mercaptolysine derivative was introduced as the N-terminal residue of the small peptide **31** with Cbz remaining on its  $\epsilon$ -amine. Peptide **31** was directly in ligation reactions, as the free-thiol form could be generated in situ under the reducing conditions of the ligation reaction (Scheme 8). The next stage of peptide ligation *via* dual native chemical ligation was carry out by our collaborator, Prof. Liu Chuan-Fa group from School of Biological Sciences, NTU.

Robust ligation was observed when **31** was subjected to reaction with the thioester peptide H-LSTEА-COSR (**30a**). The 4-mercaptolysyl peptide reactant was completely consumed in excellent yield (90%), and the ligation product **32a** based on HPLC analysis. Ligation with another thioester peptide with a large C-terminal Leu residue, H-LSTEL-COSR (**30b**), also proceeded efficiently and producing the ligation product **32b** in 92% yield by HPLC analysis after reaction for 1 h, indicating that the ligation reaction was not affected by the bulkiness of Leu (Figure 4). The Cbz group in **32** was then removed using cocktail containing TFMSA in order to expose the  $\epsilon$ -NH<sub>2</sub> of 4-mercaptolysine for the second ligation. First, the peptide **33a** was reacted with a small peptide thioester **34a**, H-LSTEG-COSR. Similar to the First ligation at  $\alpha$ -amine, the second ligation proceeded very efficiently to give the product **35a** in 92% yield. This indicating the vital role of the  $\gamma$ -thiol group is mediating ligation at both  $\alpha$ - and  $\epsilon$ -amino groups of the 4-mercaptolysine residue. Further, we performed our method to site-specific peptide ubiquitination. Peptide **33b** was reacted with large (76 amino acids) ubiquitin thioester, ubi(1-76)-CO-SCH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub><sup>-</sup> [ubi(1-76)-76] which was prepared by thiolysis of a ubiquitin-intein fusion protein.<sup>60</sup> As shown in Figure 5, ligation with the ubiquitin thioester was complete within only 45 min, giving a clean product **35b** in 90% yield based on HPLC analysis. The efficiency of the reaction in our dual NCL scheme makes it a particular viable method for the synthesis of

complex protein conjugates such as ubiquitinated proteins for the functional elucidation of such post-translational modifications on lysine.

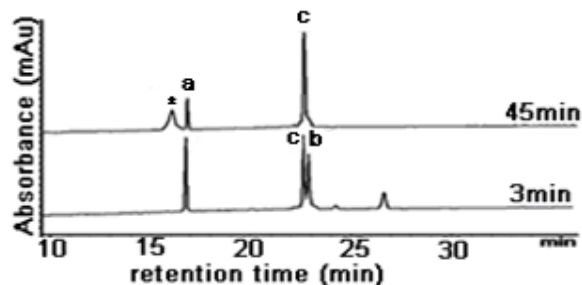
To generate native Lys at the ligation junction, we first tried the Raney nickel-mediated desulfurization method which developed by Dawson's research group.<sup>11</sup> Desulfurization of **35a** gave only a moderate yield of 44% based on HPLC analysis. Moreover, this metal-based method did not work for **35b**. We then tried the recently developed free-radical desulfurization approach.<sup>61</sup> Desulfurization of **35b** using VA-044 reached completion to give the final ubiquitinated peptide **36b**, and the conversion was near quantitative based on MS analysis. To further test whether our method can be used for specific biotinylation on the lysine side chain, peptide **33b** was reacted with a biotin thioester **34c**. The reaction was complete in 3 h and gave the biotinyl ligation product **36c** in 90% yield by HPLC analysis. Free-radical desulfurization afforded the final biotinylated peptide **36c** in 80% yield based on HPLC analysis.

The above results show that  $\gamma$ -SH group on the N-terminal lysine mediates facile chemical ligation at both  $\alpha$ - and  $\epsilon$ -amines. If used without the second ligation step, our method would allow conventional linear NCL at lysine. Through the use of our dual ligation can produce complex protein structures.



**Figure 4** HPLC monitoring of ligation between H-LSTEL-COSR (**30b**) and peptide **31** at 10 min and 1 h, respectively. Gradient: 0-60% buffer B in 30 min. Peak a, H-LSTEL-COSR; peak b,

peptide **31**; peak c, ligation product peptide **32b**; peak d, H-LSTEL-COSBz; peak \*, H-LSTEL-OH. Ligation conditions: 28 mM H-LSTEL-COSR, 20 mM **31**, 6 M Gdn-HCl, 0.2 M phosphate, 60 mM TCEP, 1% benzyl mercaptan, pH 8.0, 37 °C.



**Figure 5** HPLC monitored ligation between peptide **33b** and ubiquitin(1-76)-MES at 3 min and 45 min, respectively. Gradient: 0-36% buffer B in 18 min, 36-45% in 18 min. Peak a, peptide **33b**; peak b, ubiquitin(1-76)-MES; peak c, ligation product **34b**; peak \*, unidentified. Ligation conditions: 6.6 mM **33b**, 1.2 mM ubiquitin(1-76)-MES, 6 M Gdn-HCl, 0.2 M phosphate, 60 mM TCEP, pH 8.0, 37 °C.

### 3. CONCLUSION

A practical and concise synthesis of the fully protected amino acid (2S, 4S)-4-mercapto L-lysine has been developed starting from commercially available L-aspartic acid. The synthesis involves diastereo-selective Reformatsky reaction to form hydroxy group at 4-position of L-lysine and subsequent reduction of methyl ester to get amine functionality at L-lysine side chain. We found that this approach allows a direct access to (2S, 4R)-4-hydroxy L-lysine derivatives that offer some interesting biologically active molecules. The (2S, 4S)-4-mercapto L-lysine derivative is a key precursor for our dual native chemical ligation (NCL) strategy. Moreover, the above results show that a  $\gamma$ -SH group on an N-terminal lysine mediates facile chemical ligation at both its  $\alpha$ - and  $\epsilon$ -amines. Through the use of the dual ligation technique, it is possible to synthesize complex proteins that are functionalized on lysine side chains.

## 4. EXPERIMENTAL

Unless otherwise noted, all reactions were carried out in oven dried glassware under an atmosphere of nitrogen and distilled solvents were transferred by syringe. Solvents and reagents were purified according to the standard procedure prior to use. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 nm). Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on 400 MHz Bruker DPX 400 and 500 MHz Bruker AMX 500 NMR spectrometers, respectively. The residual solvent signals were taken as the reference (7.26 ppm for  $^1\text{H}$  NMR spectroscopy and 77.0 ppm for  $^{13}\text{C}$  NMR spectroscopy). Chemical shift ( $\delta$ ) is referred in terms of ppm, coupling constants ( $J$ ) are given in Hz. Following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal. Infrared spectra were recorded on a Bio-RAD FTS 165 FT-IR Spectrometer and reported in  $\text{cm}^{-1}$ . Samples were prepared in thin film technique. HRMS (ESI) spectra were recorded on a Finnigan/MAT LCQ quadrupole ion trap mass spectrometer, coupled with the TSP4000 HPLC system and the Crystal 310 CE system.

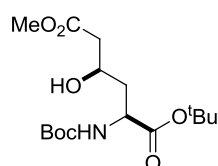
Amino acid derivatives, coupling reagents and resins were purchased from GL Biochem (Shanghai, China) or Novabiochem. All the other chemical reagents were purchased from Alfa Aesar, Sigma-Aldrich Chemical Company, Fisher Scientific, Acros Organics. PCR kit, restriction enzymes, and chitin beads for construction of ubiquitin-intein expression vector and the purification of intein-CBD fusion proteins were obtained from New England Biolabs. All the analytic HPLC analyses were performed by using an Agilent 1100 series instrument equipped with a Jupiter C18 (5  $\mu\text{m}$ , 4.6 x 250 mm) reverse-phase column with a flow rate of 1.0 ml/min.

Detection was achieved with a UV-VIS-detector at wavelength  $\lambda = 220$  nm. A typical gradient used for analysis was buffer B rising 2% every minute starting from 0%. The purification was performed using a semi-preparative HPLC column on a Shimadzu system equipped with a vydac C18 column (5  $\mu$ m, 10 x 250 mm) with a flow rate of 2.5 ml/min. The buffer system for all the analysis was buffer A H<sub>2</sub>O (containing 0.045% TFA) and buffer B, 90% acetonitrile in H<sub>2</sub>O (containing 0.04% TFA). Peptide masses were measured using a Thermo FINNIGAN LCQ Deca XP MAX equipped with ESI ion source.

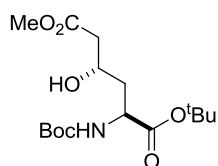
### Experimental procedure and Spectral Data

**(2S,4R)-1-tert-Butyl 6-methyl 2-(tert-butoxycarbonylamino)-4-hydroxyhexanediote (17) /**

**(2S,4S)-1-tert-butyl 6-methyl 2-(tert-butoxycarbonylamino)-4-hydroxyhexanediote (18) :**



17



18

To a cooled (0 °C) solution of aldehyde (0.50 g, 1.83 mmol) in THF (7.32 mL, 0.25 M) was added dropwise a solution of reformatsky reagent (4.57 mL, 4.57 mmol) (The experimental procedure for reformatsky reagent given below) over a period of 15 min and the reaction mixture was stirred for 2 h. The reaction was quenched by the addition of saturated, aqueous ammonium chloride solution and extracted three times with ethyl acetate. The combined organic layers were then washed with saturated sodium bicarbonate solution and brine, dried over sodium sulphate, then concentrated in vacuo. The residue was purified by silica gel flash column chromatography

using ethylacetate/hexane (20:80) as eluent.  $R_f$  (**17**) [2S,4R] = 0.28;  $R_f$  (**18**) [2S,4S] = 0.34. Combined Yield 92% (Colorless solid).

**17** [2S,4R] diastereomer:  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 5.38 (bs, 1H, NH), 4.20-4.18 (m, 2H,  $\text{CH-NH}$ ,  $\text{CH-OH}$ ), 3.68 (s, 3H,  $\text{CH}_3$ ), 3.24 (bs, 1H, OH), 2.54-2.44 (m, 2H,  $\text{CH}_2\text{-CO}_2\text{Me}$ ), 1.94-1.86 (m, 2H,  $\text{CH}_2$ ), 1.44 (s, 9H,  $3\text{CH}_3$ ), 1.41 (s, 9H,  $3\text{CH}_3$ ).  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 172.8 (C=O), 171.5 (C=O), 155.5 (C=O), 82.0 (C), 79.8 (C), 65.2 ( $\text{CH-OH}$ ), 51.9 ( $\text{CH}_3$ ), 51.7 (C-NH), 41.0 ( $\text{CH}_2$ ), 38.8 ( $\text{CH}_2$ ), 28.3 ( $3\text{CH}_3$ ), 27.9 ( $3\text{CH}_3$ ). **IR** ( $\text{CHCl}_3$ ):  $\nu = 3429, 3018, 2980, 2401, 1724, 1367 \text{ cm}^{-1}$ . **HRMS** (ESI):  $m/z$ : calcd for  $\text{C}_{16}\text{H}_{30}\text{NO}_7$ : 348.2022 [ $M$ ] $^+$ ; found: 348.2021.

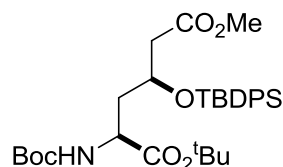
**18** [2S,4S] diastereomer: mp: .  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 5.42 (d,  $J = 7.9 \text{ Hz}$ , 1H, NH), 4.37-4.33 (m, 1H,  $\text{CH-OH}$ ), 4.21 (d,  $J = 3.5 \text{ Hz}$ , 1H, CH-NH), 4.07 (bs, 1H, OH), 3.68 (s, 3H,  $\text{CH}_3$ ), 2.55 (dd,  $J = 15.7 \text{ \& } 8.1 \text{ Hz}$ , 1H, CH-CO<sub>2</sub>Me), 2.40 (dd,  $J = 15.7 \text{ \& } 4.7 \text{ Hz}$ , CH-CO<sub>2</sub>Me), 1.89-1.84 (m, 1H), 1.61-1.54 (m, 1H), 1.44 (s, 9H,  $3\text{CH}_3$ ), 1.41 (s, 9H,  $3\text{CH}_3$ ).  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 172.1 (C=O), 171.6 (C=O), 156.7 (C=O), 82.3 (C), 80.4 (C), 64.4 ( $\text{CH-OH}$ ), 51.7 ( $\text{CH}_3$ ), 51.2 ( $\text{CH-NH}$ ), 41.2 ( $\text{CH}_2$ ), 40.6 ( $\text{CH}_2$ ), 28.2 ( $3\text{CH}_3$ ), 27.9 ( $3\text{CH}_3$ ). **IR** ( $\text{CHCl}_3$ ):  $\nu = 3425, 3018, 2981, 2399, 1730, 1369 \text{ cm}^{-1}$ . **HRMS** (ESI):  $m/z$ : calcd for  $\text{C}_{16}\text{H}_{30}\text{NO}_7$ : 348.2022 [ $M$ ] $^+$ ; found: 348.2033.

#### Preparation of Reformatsky reagent (1 M solution in THF):

A flame dried two-necked flask fitted with a reflux condenser and septum was charged with zinc dust (0.47 g, 7.18 mmol), trimethylchlorosilane (TMSCl) (0.11 mL, 0.98 mmol), and dry THF (3.50 mL), then heated to reflux with stirring under nitrogen for 15 min. The flask was then removed from the heat and methyl bromoacetate (0.60 mL, 6.53 mmol) in dry THF (3.50 mL) was added via syringe at such a rate as to maintain gentle reflux. Stirring was continued for

5 min then stopped and the suspension allowed to settle leaving a green solution of the reformatsky reagent ( 1M solution in THF).

**(2S,4S)-1-tert-Butyl 6-methyl 2-(tert-butoxycarbonylamino)-4-(tert butyl diphenyl silyloxy) hexanedioate (20):**

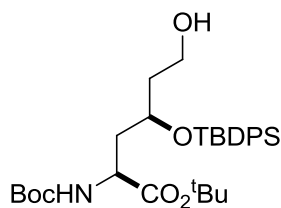


**20**

Imidazole (0.157 g, 2.30 mmol) and TBDPSCl (0.30 mL, 1.15 mmol) were added to a solution of 0.20 g (0.58 mmol) of **18** in 4 mL (0.15M) of dichloromethane at (0 °C). The reaction mixture was allowed to reach room temperature and stirred for overnight. The reaction mixture was quenched by saturated ammonium chloride solution and diluted by dichloromethane. The layers were separated, the aqueous layer was extracted with dichloromethane two times, and the combined organic extract was washed with water and brine. Drying over anhydrous sodium sulphate and removal of solvent under vacuum resulted in a colorless oily residue, which was purified by column chromatography on silica gel to give desired product **20** (0.3 g, 89 %) as a colorless semi solid. **<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 7.70-7.65 (m, 4H, Ph), 7.44-7.36 (m, 6H, Ph), 5.18 (d,  $J = 7.8$  Hz, 1H, NH), 4.25 (t,  $J = 5.9$  Hz, 1H, CH-NH), 4.13 (dd,  $J = 13.0$  & 8.2 Hz, 1H, CH-OH), 3.49 (s, 3H, CH<sub>3</sub>), 2.48 (d,  $J = 6.3$  Hz, 2H, CH<sub>2</sub>-CO<sub>2</sub>Me), 1.99-1.73 (m, 2H, CH<sub>2</sub>), 1.42 (s, 9H, 3CH<sub>3</sub>), 1.37 (s, 9H, 3CH<sub>3</sub>), 1.03 (s, 9H). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 171.6 (C=O), 171.1 (C=O), 155.3 (C=O), 81.5 (C), 79.4 (C), 68.2 (CH-OTBDPS), 51.8 (CH<sub>3</sub>), 51.4 (CH-NH), 41.3 (CH<sub>2</sub>), 38.9 (CH<sub>2</sub>), 28.3 (3CH<sub>3</sub>), 27.8 (3CH<sub>3</sub>), 26.8 (3CH<sub>3</sub>), 19.2 (C-

Si). **IR (CHCl<sub>3</sub>):**  $\nu$  = 3427, 3018, 2981, 2399, 1714, 1215 cm<sup>-1</sup>. **HRMS (ESI):**  $m/z$ : calcd for C<sub>32</sub>H<sub>48</sub>NO<sub>7</sub>Si: 586.3200 [ $M$ ]<sup>+</sup>; found: 586.3201.

**(2S,4S)-tert-Butyl 2-(tert-butoxycarbonylamino)-4-(tert-butyldiphenylsilyloxy) 6-hydroxyhexanoate (6)**<sup>56</sup>

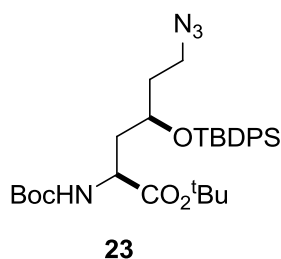


**6**

The amino acid **20** (0.5 g, 1.10 mmol) was placed in a round-bottom flask and dissolved in (5 mL, 0.2M) of ethanol and cooled to (0 °C). After the portionwise addition of sodium borohydride (0.20 g, 5.40 mmol), the mixture was allowed to reach room temperature and stirred for 8 h. After being quenched by water, the mixture was diluted with ethyl acetate. The solution was washed with water and brine. Drying over anhydrous sodium sulphate and removal of solvent under vacuum resulted in a colorless oily residue, which was purified by column chromatography on silica gel to give desired product **6** (0.50 g, 92 %) as a colorless oil. **<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 7.70-7.69 (m, 4H, Ph), 7.44-7.36 (m, 6H, Ph), 5.31 (d,  $J$  = 7.4 Hz, 1H, NH), 4.25 (t,  $J$  = 5.9 Hz, 1H, CH-NH), 4.13 (dd,  $J$  = 13.0 & 8.2 Hz, 1H, CH-OH), 3.49 (s, 3H, CH<sub>3</sub>), 2.48 (d,  $J$  = 6.3 Hz, 2H, CH<sub>2</sub>-CO<sub>2</sub>Me), 1.99-1.73 (m, 2H, CH<sub>2</sub>), 1.42 (s, 9H, 3CH<sub>3</sub>), 1.37 (s, 9H, 3CH<sub>3</sub>), 1.03 (s, 9H). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 171.7 (C=O), 155.3 (C=O), 81.6 (C), 79.5 (C), 69.6 (CH-OTBDPS), 59.0 (CH<sub>3</sub>), 51.9 (CH-NH), 38.4 (CH<sub>2</sub>), 38.3 (CH<sub>2</sub>-OH), 28.3 (3CH<sub>3</sub>), 27.8 (3CH<sub>3</sub>), 26.9 (3CH<sub>3</sub>), 19.2 (C-Si). **IR (CHCl<sub>3</sub>):**  $\nu$  = 3427, 3018,

2981, 2399, 1714, 1215  $\text{cm}^{-1}$ . **HRMS (ESI):**  $m/z$ : calcd for  $\text{C}_{31}\text{H}_{47}\text{NO}_6\text{SiNa}$ : 580.3070  $[\text{M}+\text{Na}]^+$ ; found: 580.3088.

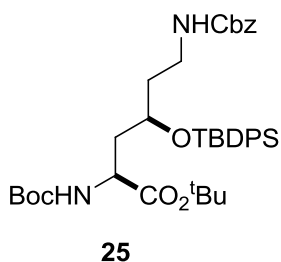
**(2S,4R)-tert-Butyl 6-azido-2-(tert-butoxycarbonyl amino)-4-(tert-butyl diphenyl silyloxy) hexanedioate (23):**



0.13 mL (0.72 mmol) of diisopropyl ethylamine (DIPEA) was added to a solution of 0.20 g (0.36 mmol) of compound **6** in 3.5 mL (0.1M) of dichloromethane at room temperature. The solution was cooled to 0 °C, and 0.04 mL (0.54 mmol) of methanesulfonyl chloride was added. The reaction mixture was allowed to reach room temperature and stirred for 2 h. The reaction mixture was quenched with saturated ammonium chloride solution at 0 °C and diluted with dichloromethane. The layers were separated, the aqueous layer was extracted with dichloromethane two times, and the combined organic extract was washed with water and brine. Drying over anhydrous sodium sulphate and removal of solvent under vacuum resulted in a colorless oily residue which was dissolved in DMF (4 mL, 0.1M). A total of 0.07 g (1.08 mmol) of sodium azide was added to the solution, which was heated to 80 °C for 5 h. After allowed to reach room temperature, water was added to the solution, which was extracted twice with ethyl acetate. The combined organic layer was washed with water and brine, dried over sodium sulphate and evaporated. The crude product was purified by column chromatography on silica

gel to give desired product **23** (0.174 g, 83 %) as a colorless oil.  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 7.69 (d,  $J = 6.8$  Hz, 4H, Ph), 7.45-7.37 (m, 6H, Ph), 5.22 (d,  $J = 7.3$  Hz, 1H, NH), 4.12 (d,  $J = 5.7$  Hz, 1H,  $\text{CH-NH}$ ), 3.95 (d,  $J = 5.4$  Hz, 1H,  $\text{CH-OH}$ ), 3.16 (t,  $J = 5.7$  Hz, 2H,  $\text{CH}_2\text{-CO}_2\text{Me}$ ), 1.96-1.90 (m, 1H,  $\text{CH}_2$ ), 1.85 (d,  $J = 6.6$  Hz, 1H,  $\text{CH}_2$ ), 1.74-1.69 (m, 2H,  $\text{CH}_2$ ), 1.43 (s, 9H,  $3\text{CH}_3$ ), 1.37 (s, 9H,  $3\text{CH}_3$ ), 1.06 (s, 9H).  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 171.6 (C=O), 155.2 (C=O), 135.8 (Ph), 135.7 (Ph), 133.6 (Ph), 133.1 (Ph), 129.9 (Ph), 129.8 (Ph), 127.7 (Ph), 127.7 (Ph), 81.6 (C), 79.5 (C), 69.0 ( $\text{CH-OH}$ ), 51.8 ( $\text{CH-NH}$ ), 47.5 ( $\text{CH}_2\text{-N}_3$ ), 38.6 ( $\text{CH}_2$ ), 35.2 ( $\text{CH}_2$ ), 28.3 ( $3\text{CH}_3$ ), 27.9 ( $3\text{CH}_3$ ), 26.9 ( $3\text{CH}_3$ ), 19.3 (C-Si). **IR** ( $\text{CHCl}_3$ ):  $\nu$  = 3427, 3018, 2399, 2098, 1647, 1215  $\text{cm}^{-1}$ . **HRMS (ESI)**:  $m/z$ : calcd for  $\text{C}_{31}\text{H}_{47}\text{N}_4\text{O}_5\text{Si}$ : 583.3316 [ $M$ ] $^+$ ; found: 583.3317.

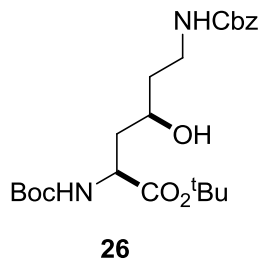
**(2S,4R)-tert-Butyl 6-(benzyloxycarbonylamino)-2-(tert-butoxycarbonylamino)-4-(tert-butyl diphnyl silyloxy) hexanedioate (25):**



To a solution of **23** (100 mg) in ethyl acetate was added 10% Pd/C (30 mg) at room temperature. The reaction mixture was stirred for 6 h under an  $\text{H}_2$  atmosphere at room temperature then filtered through a short pad of celite and washed with chloroform-methanol (1:1). The solvent removed under reduced pressure (quantitative yield). This compound was directly taken for the next step without further purification.

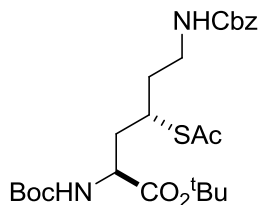
Above amine **24** (0.50 g, 0.89 mmol) was dissolved in 2:1 ratio of dioxane-water (10 mL-5 mL) and cooled to 0 °C. Sodium bicarbonate (0.19 g, 2.22 mmol) was introduced portions wise at the same temperature. Then CbzCl (0.19 mL, 1.3 mmol) was added slowly to the reaction mixture. The reaction mixture was allowed to reach room temperature and stirred for 4 h. After being cooled to 0 °C, the reaction mixture was quenched by the addition of 0.5 N HCl (2 mL) and stirred for a further 5 min. The solution was dissolved in ethyl acetate and washed with saturated sodium bicarbonate solution, water, and brine. The organic layer was dried over sodium sulphate and concentrated in vacuo, which was purified by column chromatography on silica gel to give desired product **25** (0.5 g, 81 %) as a colorless oil. **<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 7.69 (d,  $J$  = 6.2 Hz, 4H, Ph), 7.39-7.31 (m, 11H, Ph), 5.46 (d,  $J$  = 6.3 Hz, 1H, NH), 5.04-4.97 (m, 2H, CH<sub>2</sub>-Ph), 4.35 (bs, 1H, CH-NH), 4.19 (bs, NH), 3.90 (bs, 1H, CH-OH), 3.12 (t,  $J$  = 6.5 Hz, 2H, CH<sub>2</sub>-NH), 2.96 (m, 1H, CH<sub>2</sub>-NH), 1.94 (bs, 2H, CH<sub>2</sub>), 1.62-1.60 (m, 2H, CH<sub>2</sub>), 1.44 (s, 9H, 3CH<sub>3</sub>), 1.40 (s, 9H, 3CH<sub>3</sub>), 1.06 (s, 9H). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 171.9 (C=O), 156.3 (C=O), 155.5 (C=O), 136.6 (Ph), 135.9 (Ph), 133.9 (Ph), 133.1 (Ph), 129.9 (Ph), 129.8 (Ph), 128.4 (Ph), 128.0 (Ph), 127.7 (Ph), 127.6 (Ph), 81.4 (C), 79.3 (C), 69.0 (CH-OH), 66.5 (CH<sub>2</sub>-Ph), 51.9 (CH-NH), 38.0 (CH<sub>2</sub>-NH), 37.0 (CH<sub>2</sub>), 36.5 (CH<sub>2</sub>), 28.4 (3CH<sub>3</sub>), 27.9 (3CH<sub>3</sub>), 26.9 (3CH<sub>3</sub>), 19.3 (C-Si). **IR (CHCl<sub>3</sub>):**  $\nu$  = 3392, 3018, 2399, 2088, 1645, 1215 cm<sup>-1</sup>. **HRMS (ESI):**  $m/z$ : calcd for C<sub>39</sub>H<sub>55</sub>N<sub>2</sub>O<sub>7</sub>Si: 691.3779 [M]<sup>+</sup>; found: 691.3785.

**(2S,4R)-tert-Butyl 6(benzyloxycarbonylamino)-2-(tert-butoxycarbonylamino)-4-hydroxyhexanedioate (26) :**



Compound **25** (0.05 g, 0.07 mmol) was dissolved in dry THF (1.5 mL, 0.05M) and cooled to 0 °C. 1M solution of TBAF (0.11 ml, 0.11 mmol) was introduced via syringe at the same temperature. The reaction mixture was stirred for 8 h at 0 °C and quenched with saturated ammonium chloride solution. Evaporation of the solvent gave a residue, which was dissolved in ethyl acetate. The solution was washed with water, and brine. The organic layer was dried over sodium sulphate and concentrated in vacuo, which was purified by column chromatography on silica gel to give desired product **26** (0.025 g, 77 %) as a colorless oil. **<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 7.34-7.27 (m, 5H, Ph), 5.46-5.44 (m, 2H, NH & NH), 5.07 (dd,  $J = 18.0$  & 12.3 Hz, 2H, CH<sub>2</sub>-Ph), 4.47 (bs, 1H, OH), 4.35 (dt,  $J = 8.2$  & 2.4 Hz, 1H, CH-NH), 3.68 (bs, 1H, CH-OH), 3.48 (dt,  $J = 13.1$  & 6.2 Hz, 1H, CH<sub>2</sub>-NH), 3.23 (dd,  $J = 12.5$  & 5.9 Hz, 1H, CH<sub>2</sub>-NH), 1.86 (dt,  $J = 10.9$  & 2.7 Hz, 1H, CH<sub>2</sub>), 1.63-1.47 (m, 3H, CH<sub>2</sub>, CH), 1.45 (s, 9H, 3CH<sub>3</sub>), 1.43 (s, 9H, 3CH<sub>3</sub>). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 171.7 (C=O), 156.3 (C=O), 156.7 (C=O), 136.6 (Ph), 128.4 (Ph), 128.0 (Ph), 128.0 (Ph), 82.4 (C), 80.5 (C), 66.5 (CH<sub>2</sub>-Ph), 66.3 (CH-OH), 51.1 (CH-NH), 41.7 (CH<sub>2</sub>-NH), 38.9 (CH<sub>2</sub>), 36.0 (CH<sub>2</sub>), 28.2 (3CH<sub>3</sub>), 27.9 (3CH<sub>3</sub>). **IR (CHCl<sub>3</sub>):**  $\nu$  = 3423, 3018, 2399, 1645, 1215 cm<sup>-1</sup>. **HRMS (ESI):**  $m/z$ : calcd for C<sub>23</sub>H<sub>37</sub>N<sub>2</sub>O<sub>7</sub>: 453.2601 [M]<sup>+</sup>; found: 453.2590.

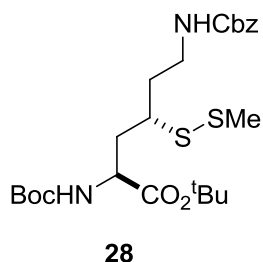
**(2S,4S)-tert-Butyl 4-(acetylthio)-6-(benzyloxycarbonyl amino)-2-(tert-butoxycarbonylamino) hexane dioate (27) :**

**27**

0.08 mL (0.44 mmol) of diisopropyl ethylamine (DIPEA) was added to a solution of 0.10 g (0.22 mmol) of compound **26** in 2.2 mL (0.1M) of dichloromethane at room temperature. The solution was cooled to 0 °C, and 0.02 mL (0.27 mmol) of methanesulfonyl chloride was added. The reaction mixture was allowed to reach room temperature and stirred for 2 h. The reaction mixture was quenched with saturated ammonium chloride solution at 0 °C and diluted with dichloromethane. The layers were separated, the aqueous layer was extracted with dichloromethane two times, and the combined organic extract was washed with water and brine. Drying over anhydrous sodium sulphate and removal of solvent under vacuum resulted in a colorless oily residue which was dissolved in DMF (4.4 mL, 0.05 M). A total of 0.75 g (0.66 mmol) of potassium thioacetate was added to the solution, which was heated to 40 °C for 8 h. After allowed to reach room temperature, water was added to the solution, which was extracted twice with ethyl acetate. The combined organic layer was washed with water and brine, dried over sodium sulphate and evaporated. The crude product was purified by column chromatography on silica gel to give desired product **27** (0.04 g, 70 %) as a pale brown oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ in ppm = 7.34-7.28 (m, 5H, Ph), 5.46 (bs, 1H, NH), 5.23 (d, *J* = 8.0 Hz, 1H, NH), 5.07 (dd, *J* = 14.8 & 12.5 Hz, 2H, CH<sub>2</sub>-Ph), 4.26 (dd, *J* = 13.6 & 7.8 Hz, 1H, CH<sub>2</sub>-NH), 3.66-3.59 (m, 1H, CH<sub>2</sub>-S), 3.39 (dd, *J* = 13.0 & 5.4 Hz, 1H, CH<sub>2</sub>-NH), 3.15-3.10 (m, 1H, CH<sub>2</sub>-NH), 2.31 (s, 3H, CH<sub>3</sub>), 2.03 (dd, *J* = 13.4 & 6.5 Hz, 2H, CH<sub>2</sub>), 1.90-1.82 (m, 1H, CH<sub>2</sub>), 1.68-1.59 (m, 1H, CH<sub>2</sub>) 1.44 (s, 9H, 3CH<sub>3</sub>), 1.40 (s, 9H, 3CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ

in ppm = 195.9 (COCH<sub>3</sub>), 171.1 (C=O), 156.4 (C=O), 155.6 (C=O), 136.7 (Ph), 128.4 (Ph), 128.1 (Ph), 127.9 (Ph), 82.4 (C), 80.1 (C), 66.5 (CH<sub>2</sub>-Ph), 51.9 (CH-NH), 39.7 (CH<sub>2</sub>-NH), 38.2 (CH<sub>2</sub>), 38.2 (CH<sub>2</sub>), 33.7 (CH-S), 33.7 (CH<sub>3</sub>) 28.2 (3CH<sub>3</sub>), 27.9 (3CH<sub>3</sub>). **IR (CHCl<sub>3</sub>):**  $\nu$  = 3431, 3018, 2399, 1645, 2088, 1637, 1215 cm<sup>-1</sup>. **HRMS (ESI):** *m/z*: calcd for C<sub>25</sub>H<sub>39</sub>N<sub>2</sub>O<sub>7</sub>S: 511.2478 [M]<sup>+</sup>; found: 511.2480.

**(2S,4S)-tert-Butyl-6-(benzyloxycarbonyl)-2-(tert-butoxycarbonyl)-4-(2-methyldisulfanyl)hexanedioate (28) :**

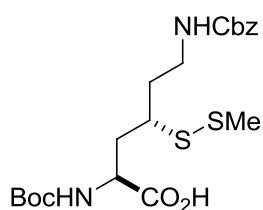


0.020 g of Boc-lys(SAc, Cbz)-OtBu (**27**) was dissolved in 0.6 mL of methanol. 0.175 mL of 1N NaOH was added. After 30 min at room temperature, the mixture was neutralized carefully with 1 N HCl at 0 °C. The mixture was concentrated and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulphate and evaporated.

The residue was dissolved in 0.4 mL of DCM and added dropwise to a mixture containing 0.4 mL of DCM, 0.006 mL of Et<sub>3</sub>N and 0.015 mL of S-methyl methanethiosulfonate (MMTS) while stirring at room temperature. The mixture was continuously stirred for 30 min.

The solvent was removed and the residue was dissolved in methanol and purified by C18 semi-preparative HPLC followed by lyophilization to give desired product Boc-lys(SSMe, Cbz)-OtBu **28** (10 mg) as a white solid. **<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 7.36-7.28 (m, 5H, Ph), 5.47 (bs, 1H, NH), 5.25 (d,  $J = 7.6$  Hz, 1H, NH), 5.10 (s, 2H, CH<sub>2</sub>-Ph), 4.26 (s, 1H, CH-NH), 3.47 (m, 1H, CH-S), 3.37 (d,  $J = 4.5$  Hz, 1H, CH<sub>2</sub>-NH), 2.82 (bs, 1H, CH), 2.40 (s, 3H, CH<sub>3</sub>), 2.07-1.97 (m, 2H, CH<sub>2</sub>), 1.85-1.80 (m, 2H, CH<sub>2</sub>) 1.49 (s, 9H, 3CH<sub>3</sub>), 1.39 (s, 9H, 3CH<sub>3</sub>). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 171.2 (C=O), 156.5 (C=O), 155.9 (C=O), 136.7 (Ph), 128.4 (Ph), 128.2 (Ph), 127.9 (Ph), 82.6 (C), 80.3 (C), 66.5 (CH<sub>2</sub>-Ph), 51.9 (CH-NH), 44.7 (CH<sub>2</sub>-NH), 40.4 (CH<sub>3</sub>-S), 38.4 (CH<sub>2</sub>), 32.0 (CH<sub>2</sub>), 28.2 (3CH<sub>3</sub>), 28.0 (3CH<sub>3</sub>), 24.0 (CH-S). **IR (CHCl<sub>3</sub>):**  $\nu$  = 3425, 3025, 1640, 2090, 1650 cm<sup>-1</sup>. **HRMS (ESI):**  $m/z$ : calcd for C<sub>24</sub>H<sub>38</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>Na: 537.2069;  $[M+Na]^+$  found: 537.2066.

**(2S,4S)-tert-Butyl-6-(benzyloxycarbonyl)-2-(tert-butoxycarbonyl)-4-(2-methyldisulfanyl)hexanoic acid (29) :**



**29**

The white powder of Boc-lys(SSMe, Cbz)-OtBu **28** was desolved in 0.5 mL 95% TFA. After 1 h at room temperature, the TFA was removed by evaporation.

The residue was dissolved in 1 mL methanol/H<sub>2</sub>O mixture (3:1). Adjust pH to about 8 with Et<sub>3</sub>N. 0.02 mL of Boc<sub>2</sub>O was added. After 3 h at room temperature, the sample was subjected to C18 semi-preparative HPLC. After lyophilization 7 mg of Boc-Lys(SSMe, Cbz)-OH

**29** was isolated.  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 7.34-7.33 (m, 5H, Ph), 5.33 (bs, 1H, NH), 5.29 (d,  $J = 7.2$  Hz, 1H, NH), 5.08 (s, 2H,  $\text{CH}_2\text{-Ph}$ ), 4.43 (b, 1H,  $\text{CH-NH}$ ), 3.38 (b, 2H,  $\text{CH}_2$ ), 2.92-2.86 (m, 1H,  $\text{CH}$ ), 2.38 (s, 3H,  $\text{CH}_3$ ), 2.17-2.03 (m, 2H,  $\text{CH}_2$ ), 1.87-1.83 (m, 2H,  $\text{CH}_2$ ) 1.39 (s, 9H,  $3\text{CH}_3$ ).  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 175.3 (C=O), 156.7 (C=O), 156.0 (C=O), 136.4 (Ph), 128.4 (Ph), 128.2 (Ph), 128.1 (Ph), 80.8 (C), 66.8 ( $\text{CH}_2\text{-Ph}$ ), 51.4 ( $\text{CH-NH}$ ), 44.7 ( $\text{CH}_2\text{-NH}$ ), 39.1 ( $\text{CH}_3\text{-S}$ ), 38.5 ( $\text{CH}_2$ ), 32.6 ( $\text{CH}_2$ ), 28.2 ( $3\text{CH}_3$ ), 24.0 ( $\text{CH-S}$ ). **IR** ( $\text{CHCl}_3$ ):  $\nu = 3440, 3022, 2399, 1650, 1640$   $\text{cm}^{-1}$ . **HRMS (ESI)**:  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{30}\text{N}_2\text{O}_6\text{S}_2\text{Na}$ : 481.1443;  $[M+\text{Na}]^+$ ; found 481.1446.

### Solid-phase peptide synthesis:

Synthesis of thioester peptides: H-**LSTEA**- $\text{SCH}_2\text{CH}_2\text{CONH}_2$ , H-**LSTEG**- $\text{SCH}_2\text{CH}_2\text{CONH}_2$  and H-**LSTEL**- $\text{SCH}_2\text{CH}_2\text{CONH}_2$  were manually synthesized employing standard *t*-Boc chemistry. First, Trt- $\text{SCH}_2\text{CH}_2\text{COOH}$  was coupled onto MBHA resin. The trityl group was removed by treatment with a cocktail containing TFA/TIS/ $\beta$ -mercaptoethanol/DCM (5:2.5:2.5:90). For the coupling of amino acids, Boc-amino acid (4 eq) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (4 eq) were dissolved in DCM. DIEA (12 eq.) was added in the solution. After 2 min of activation, the mixture was mixed with resin. The reaction was undertaken for 1.5 h. The coupling efficiency was checked with Kaiser test. The Boc group was removed by treatment with 30% TFA in DCM for 10 min, followed by 15 min. The side chain protected amino acid derivatives used were Boc-Ser(Bzl)-OH, Boc-Thr(Bzl)-OH, Boc-Glu(OBzl)-OH. After sequence assembly, peptide thioesters were cleaved from the resin with a cocktail consisting TFMSA/TFA/*p*-cresol/methyl phenyl sulfide (1:7:1:1) for 1 h. The crude peptides were purified with C18 semi-preparative HPLC.

Synthesis of Biotinyl-SCH<sub>2</sub>CH<sub>2</sub>CO-GFRA-NH<sub>2</sub>: The amino acid sequence **GFRA** in the biotin thioester was assembled manually on rink-amide MBHA resin employing standard Fmoc chemistry, followed by the coupling of Trt-SCH<sub>2</sub>CH<sub>2</sub>COOH. After removal of trityl by repeated treatment with TFA/TIS/ $\beta$ -mercaptoethanol/DCM (2.5:2.5:2.5:92.5), biotin was coupled onto the thiol-derived resin (4 eq. of biotin, 4 eq. of PyBOP, 12 eq. of DIEA in DMF). The biotin thioester was cleaved from the resin with a cocktail consisting TFA/TIS/H<sub>2</sub>O (95:2.5:2.5) for 2.5 h. The crude thioester was purified with C18 semi-preparative HPLC.

Synthesis of H-K( $\gamma$ -SSMe,  $\epsilon$ -Cbz)GAKAFA-NH<sub>2</sub> (peptide **31**): All the amino acids except Boc-K( $\gamma$ -SSMe,  $\epsilon$ -Cbz)-OH in peptide **31** were coupled employing standard Fmoc-based chemistry on rink amide MBHA resin. The peptide was cleaved from the resin with a cocktail consisting of TFA/TIS/H<sub>2</sub>O (95:2.5:2.5) for 20 min. The crude peptide was purified with C18 semi-preparative HPLC.

### **Preparation of ubiquitin(1-76)-MES**

Construction of ubiquitin expression plasmid pTYB1-Ubi. The plasmid containing human ubiquitin gene was obtained from Dr. Cheung Ching For, Peter's lab in Nanyang Technological University. Ubiquitin gene was amplified by PCR using the primers: Ubi\_F: 5'-GGTGGTCATATGCAGATCTTTGTGAAG-3' and Ubi\_R: 5'-GGTGGTTGCTCTTCCGCAGCCACCTCGCAGGCG-3'. The PCR condition was 95 °C for 3 min, 30 cycles of 95 °C 30 s, 54 °C 30 s, 72 °C 30 s, and 72 °C 10 min for final extension. The PCR product was purified and ligated into the T-easy vector (Promega). The T-Ubi vector was transformed into *E. coli* cell and amplified. The extracted and purified vector T-Ubi was digested with *Nde*I and *Sap*I restriction enzymes. The digested segment was purified by PCR purification

kit (Qiagen) and ligated into the identically digested pTYB1 vector (New England Biolabs). The correct insert was confirmed by DNA sequencing.

Overexpression and purification of ubiquitin (1-76) -MES: The plasmid pTYB1-Ubi was transformed into *E. coli* BL21(DE3) CaCl<sub>2</sub> competent cells. The cells were grown in LB medium containing 100 µg/mL ampicillin at 37 °C with shaking at 250 rpm to an OD<sub>600</sub> of 0.6-0.8. The cells were induced by 50 µM IPTG at 15 °C for 18 h. After centrifugation at 6000 rpm for 10 min, cell pellets from 1 L culture were suspended in 50 mL lysis buffer (20 mM HEPES, 0.5 M NaCl, 1 mM EDTA, pH 7.0). Cells were lysed with a microfluider (Microfluidics, Newton, USA) at a chamber pressure of 12K/50 psi. Debris was removed by centrifugation at 20,000g for 30 min. The supernatant was mixed with 3 mL chitin beads (New England Biolabs) pre-equilibrated by the lysis buffer at 37 °C for 2 h. The beads were poured into a column and washed with 40 mL of the lysis buffer. The fusion protein was cleaved by adding 2.5 mL of the cleavage buffer (100 mM MESNa, 20 mM HEPES, 0.5 M NaCl, 1 mM EDTA, pH 8.0) and incubating at 37 °C overnight. The Ubiquitin(1-76)-MES was eluted with 10 mL of the lysis buffer. The cleaved product was purified by C8 semi-prep RP-HPLC. The identity of the purified Ubiquitin(1-76)-MES was identified with C18 analytic HPLC and ESI-MS.

#### **General procedures for native chemical ligation at the $\gamma$ -mercaptolysine residue:**

0.2 – 2 mg of peptides involved in ligation were dissolved in 50 – 100 µL ligation buffer containing 6 M guanidine, 0.2 M phosphate, 60 mM *tris*(2-carboxyethyl)phosphine (TCEP), pH 8.0. Except the ligation involved ubiquitin(1-76)-MES, 1% v/v benzyl mercaptan was added into the ligation mixture. All the reactions were performed at 37 °C. The time course of the ligation was monitored with C18 analytic HPLC.

**General procedures for cbz removal:**

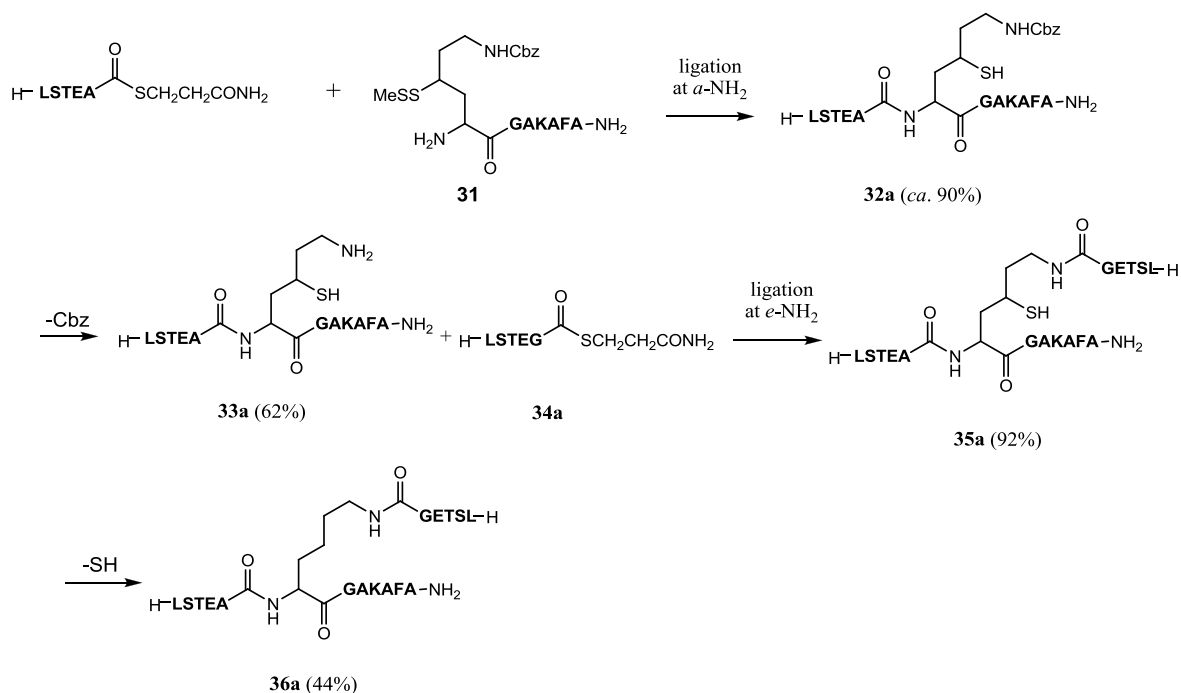
50 or 100  $\mu\text{L}$  of chilled cocktail containing TFMSA/TFA/p-cresol/methyl phenyl sulfide (1:7:1:1) was added into  $\sim 12$  mg of Cbz containing peptide on ice. The mixture was kept on ice for 2 min and in room temperature for another 20 min.  $0.5 \sim 1$  mL diethyl ether was added and the mixture was centrifuged. The pellet was dissolved in 50% ACN/ $\text{H}_2\text{O}$  and purified with C18 semi-preparative HPLC.

**Raney nickel-mediated desulfurization of peptide 11a to give peptide 12a**<sup>47</sup>

0.5 mg of peptide **11a** was dissolved in 200  $\mu\text{L}$  of buffer containing 6 M guanidine, 0.2 M phosphate, 100 mM TCEP, pH 6.0. Raney nickel was prepared by dissolving 90 mg of  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$  in 2 mL water. 5 mg of  $\text{NaBH}_4$  was added into the solution slowly. After 5 min, the pellet was collected by centrifugation and washed extensively with water. The peptide solution was added into the pellet. The reaction was stirred at room temperature. After 2 h, fresh raney nickel prepared from 180 mg of  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$  and 20 mg of  $\text{NaBH}_4$  was added into the mixture. After another 6 h, analytic HPLC showed that the desulfurization was completed.

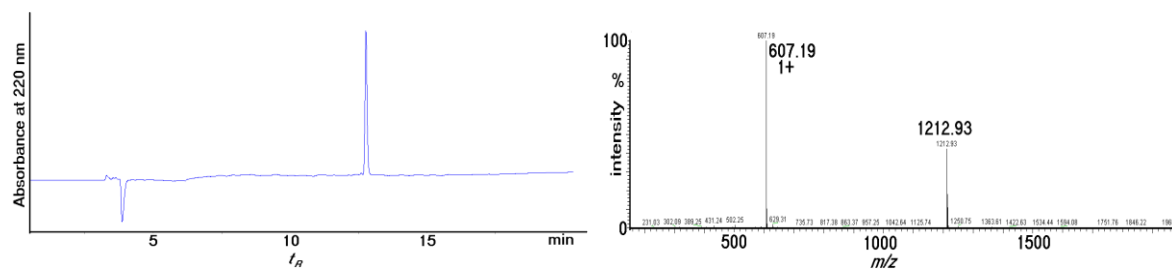
**General procedure for free radical-mediated desulfurization**<sup>61</sup>

All the following steps were performed under nitrogen. All the solutions were prepared immediately before being used. 0.2 mg peptide was dissolved in 150  $\mu\text{L}$  buffer (6 M Gdn.HCl, 0.1 M phosphate, pH  $\sim 6.5$ ). 100  $\mu\text{L}$  of 0.5 M TCEP (pH adjusted to 6-7 with 5 M NaOH) was added in the solution. 25  $\mu\text{L}$  of 10 mM glutathione was added. 10  $\mu\text{L}$  0.2 M desulfurization agent VA-044 was added. The mixture was stirred at 37  $^\circ\text{C}$  for 5 h.



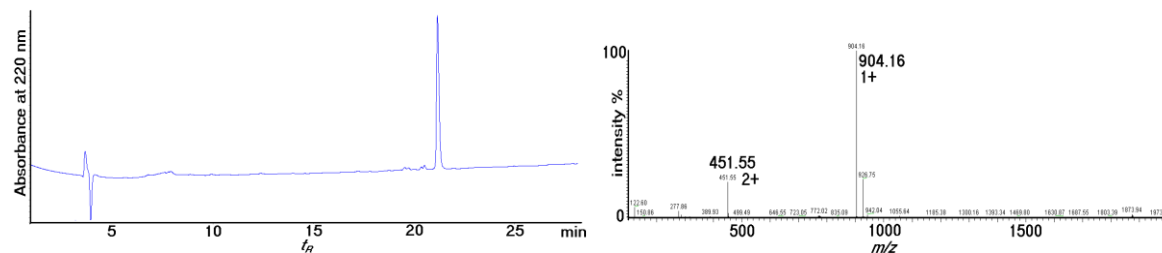
**Scheme 1** Demonstration of dual peptide ligation with two small peptide thioesters

Peptide thioester H-LSTEA-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>:



**Figure 1.** C18 analytic HPLC analysis and ESI-MS of H-LSTEA-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>. Gradient: Buffer B 0-40% in 20 min. ESI-MS:  $m/z$  = 607.19, calculated  $[\text{M}+\text{H}]^+ = 607.28$ .

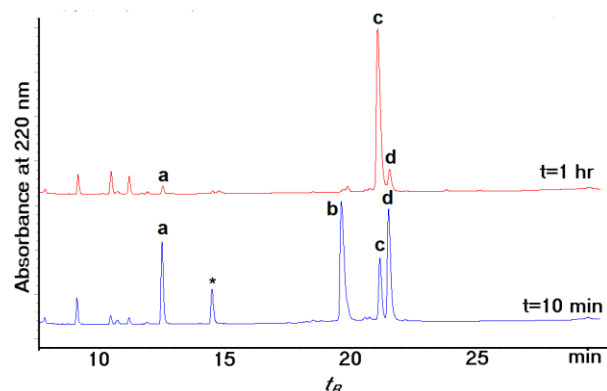
Peptide **31**: H-**K**( $\gamma$ -SSMe,  $\epsilon$ -Cbz)**GA**K**A**F**A**-NH<sub>2</sub>.



**Figure 2** C18 analytic HPLC and ESI-MS analysis of purified peptide **31**. Gradient: Buffer B 0-60% in 30 min. ESI-MS observed  $m/z = 904.16$ , calculated  $[M+H]^+ = 903.42$ .

Ligation between H-LSTEASCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> and peptide **31**:

2 mg of H-LSTEASCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> and 2 mg of peptide **31** were dissolved in 100  $\mu$ L of ligation buffer (6 M Gdn.HCl, 0.2 M phosphate, 60 mM TCEP, pH 8.0, 1% v/v benzyl mercaptan) and incubated at 37 °C. The ligation progression was monitored with C18 analytic HPLC which showed over 90% ligation yield after 1 h reaction. The ligation product was purified with C18 semi-preparative HPLC. 2.5 mg ligation product **32a** was isolated.



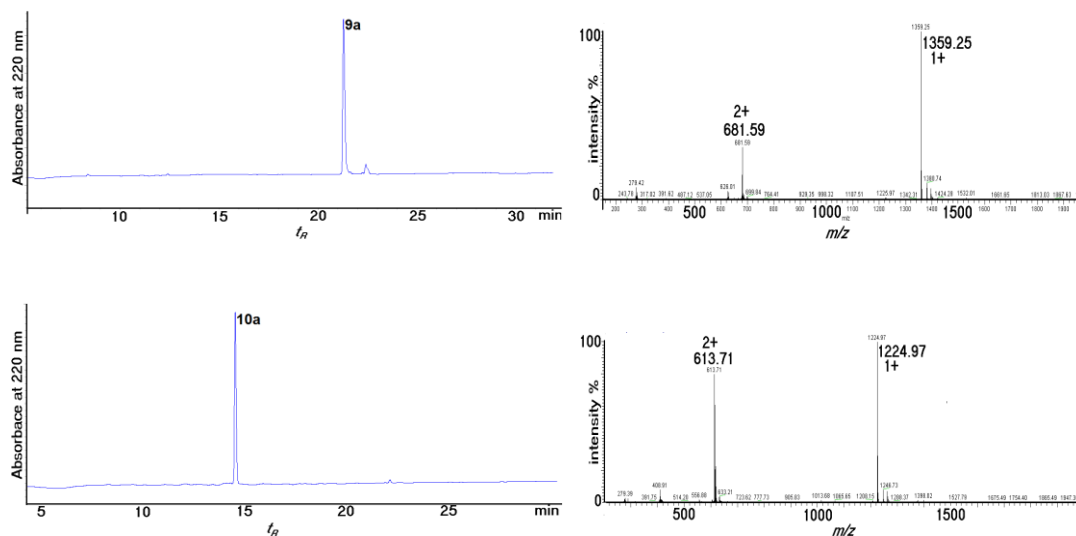
**Figure 3** C18 Analytic HPLC monitored ligation between peptide thioester H-LSTEASCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> and peptide **7** at 10 min and 1h, respectively. Gradient: Buffer B 0-60% in 30

min. Peak a, H-**LSTE**A-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>, ESI-MS observed  $m/z = 607.3$ , calculated  $[M+H]^+ = 607.28$ ; peak b, reduced peptide **31**, K( $\gamma$ -SH,  $\epsilon$ -Cbz)GAKAFA-NH<sub>2</sub>, ESI-MS observed  $m/z = 858.1$ , calculated  $[M+H]^+ = 857.44$ ; peak c, ligation product **32a**, ESI-MS observed  $m/z = 1359.2$ , calculated  $[M+H]^+ = 1358.68$ ; peak d, H-**LSTE**A-SBz, ESI-MS observed  $m/z = 626.6$ , calculated  $[M+H]^+ = 626.29$ ; peak \*, H-**LSTE**A-SMe, ESI-MS observed  $m/z = 550.2$ , calculated  $[M+H]^+ = 550.26$ .

We have also performed the ligation between H-**LSTE**A-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> and peptide **31** at different pH and/or temperature. At pH 8 and at 23 °C, the reaction gave *ca.* 78% of ligation product after 1.5 h; At pH 7 and at 37 °C, the reaction gave *ca.* 70% ligation product after 1.5 h; At pH 7 and 23°C, the yield of the ligation product was *ca.* 57 % after 1.5 h reaction. When H-**LSTE**A-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> was reacted with the cysteinyl peptide H-**CGAKAFA**-NH<sub>2</sub> at pH 7 and 23°C, a ligation yield of *ca.* 73% was obtained after 1.5 h reaction. All yields are based on HPLC analysis.

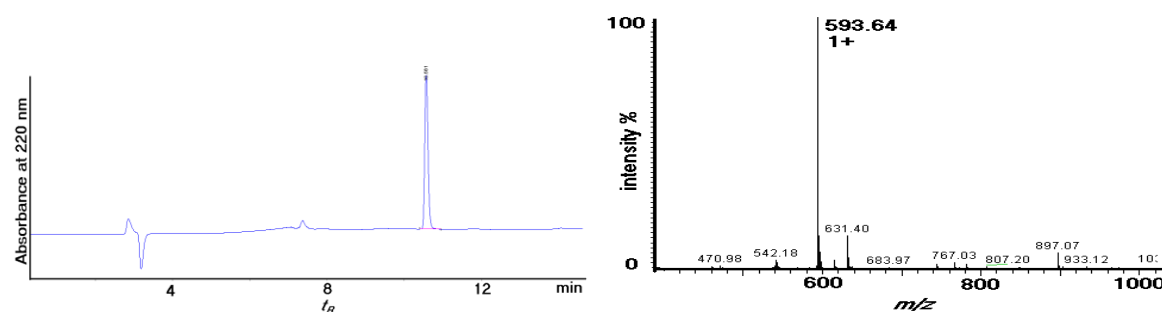
#### Removal of Cbz from peptide **32a** to give peptide **33a**:

100  $\mu$ L of chilled cocktail containing TFMSA/TFA/p-cresol/methylphenyl sulfide (1:7:1:1) was added into 0.9 mg of peptide **32a** on ice. The mixture was kept on ice for 2 min and in room temperature for another 20 min. 1 mL diethyl ether was added and the mixture was centrifuged. The pellet was dissolved in 50% ACN/H<sub>2</sub>O and purified with C18 semi-preparative HPLC. After lyophilization, 0.5 mg of peptide **33a** was isolated (yield 62%).



**Figure 4** C18 analytic HPLC analysis and ESI-MS of purified peptide **32a** (upper trace) and its purified Cbz removed product **33a** (bottom trace). Gradient: Buffer B 0-60% in 30 min. ESI-MS observed for peptide **32a**, observed  $m/z = 1359.25$ , calculated  $[M+H]^+ = 1358.68$ ; ESI-MS observed for peptide **33a**, observed  $m/z = 1224.97$ , calculated  $[M+H]^+ = 1224.64$ .

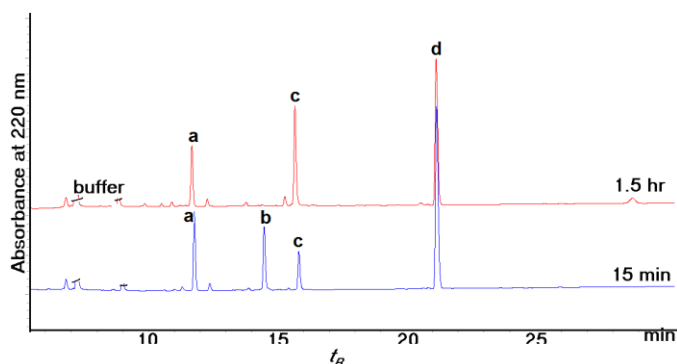
Peptide thioester H-LSTEG-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>:



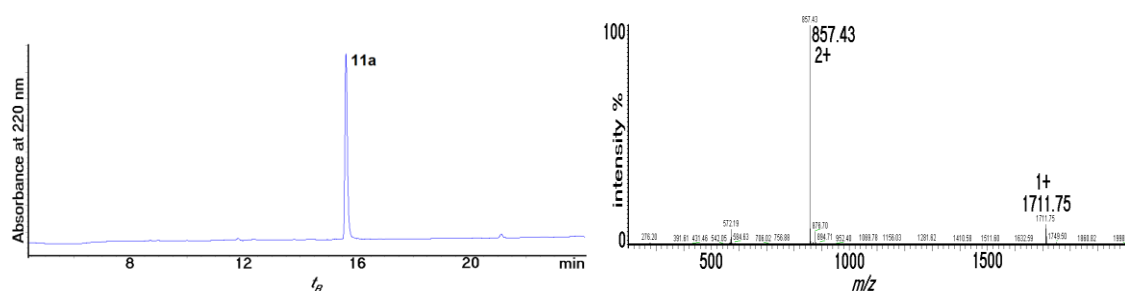
**Figure 5.** HPLC analysis and ESI-MS of H-LSTEG-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>. Gradient Buffer B 0-40% in 20 min. ESI-MS observed  $m/z = 593.64$ , calculated  $[M+H]^+ = 593.26$ .

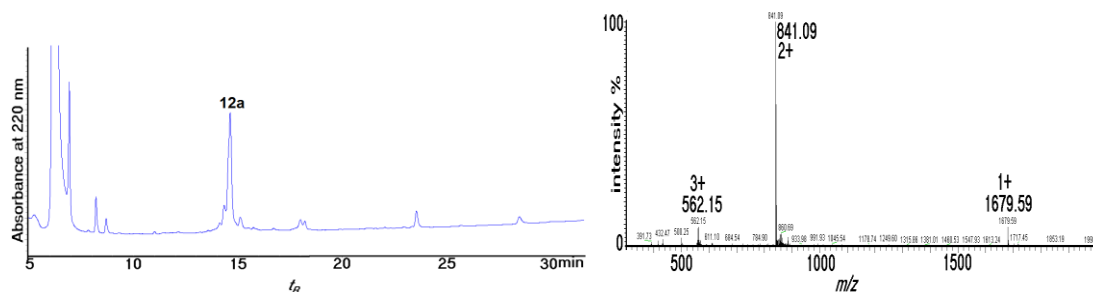
Ligation between peptide **33a** and H-LSTEG-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> **34a**:

0.4 mg of peptide **33a** and 0.4 mg of H-LSTEG-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> (**34a**) were dissolved in 50  $\mu$ L of ligation buffer and incubated at 37°C. The reaction was monitored with C18 analytic HPLC which showed 92% ligation yield after 1.5 h. The ligation product was purified with multiple runs of analytic HPLC. 0.5 mg ligation product **35a** was isolated.

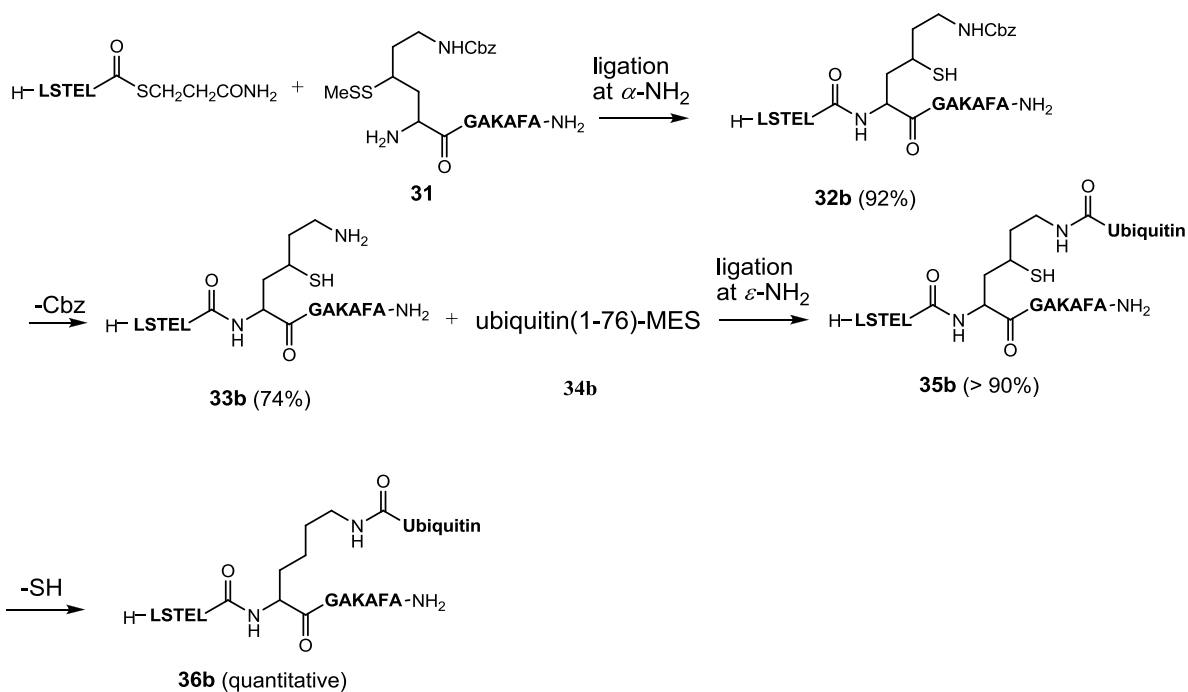


**Figure 6.** C18 analytic HPLC monitored ligation between peptide **33a** and H-LSTEG-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> at 15 min and 1.5 h, respectively. Gradient: Buffer B 0-60% in 30 min. Peak a, H-LSTEG-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>, ESI-MS observed  $m/z = 593.6$ , calculated  $[M+H]^+ = 593.26$ ; peak b, peptide **33a**, ESI-MS observed  $m/z = 1225.0$ , calculated  $[M+H]^+ = 1224.64$ ; peak c, ligation product **35a**, ESI-MS observed  $m/z = 1711.8$ , calculated  $[M+H]^+ = 1711.87$ ; peak d, H-LSTEG-SBz, ESI-MS observed  $m/z = 612.3$ , calculated  $[M+H]^+ = 612.27$ .

Desulfurization of peptide **35a** to give peptide **36a**:

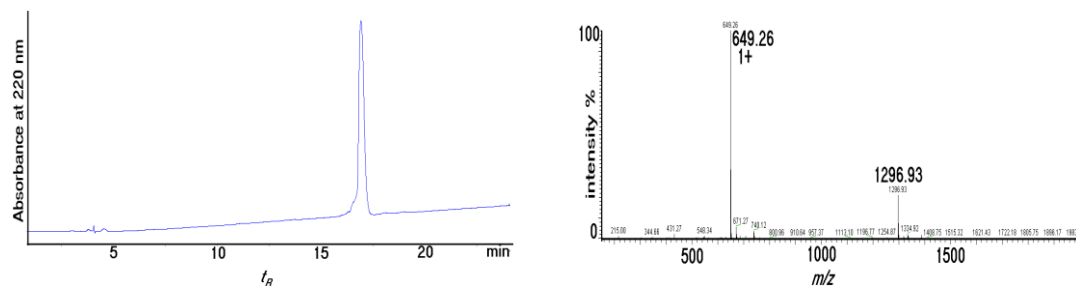


**Figure 7** C18 analytic HPLC and ESI-MS of peptide **35a** (upper trace) and its crude desulfurization product **36a** (lower trace). Gradient: Buffer B 0-60% in 30 min. ESI-MS observed for peptide **35a**  $m/z = 1711.75$ , calculated  $[M+H]^+ = 1711.87$ ; ESI-MS observed for peptide **36a**  $m/z = 1679.59$ , calculated  $[M+H]^+ = 1679.90$ .



**Scheme S2** Demonstration of dual chemical ligation for the synthesis of ubiquitinated peptides

Peptide thioester H-**LSTEL**-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>:



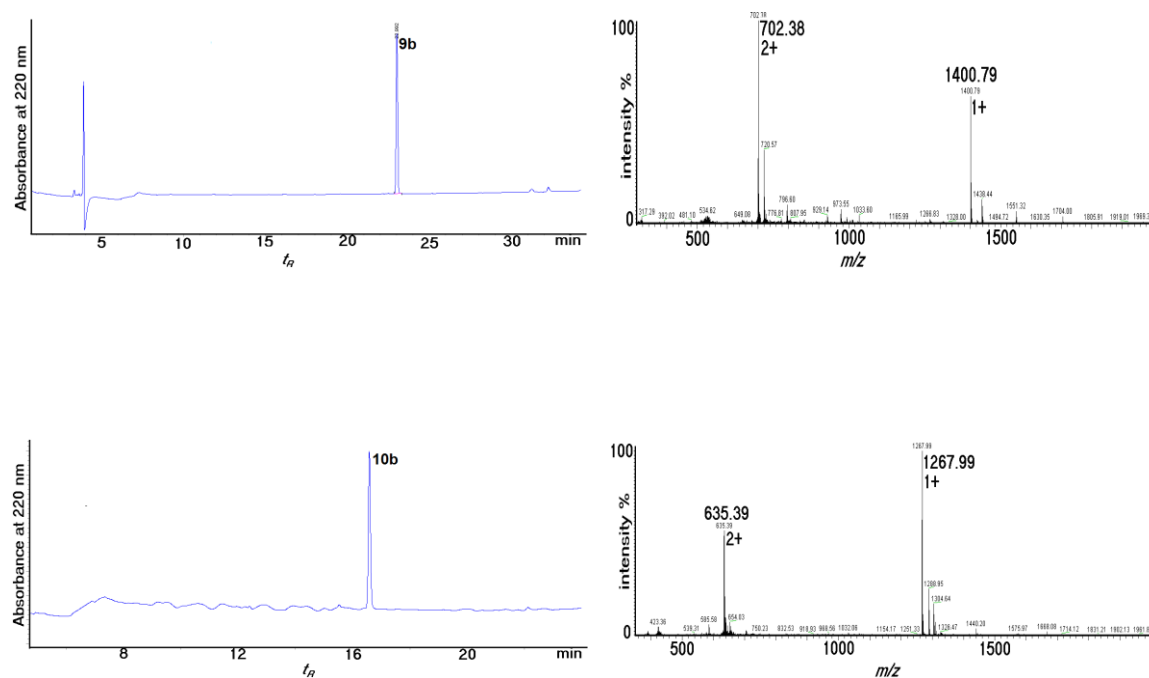
**Figure 8** C18 analytic HPLC analysis and ESI-MS of H-**LSTEL**-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub>. Gradient: Buffer B 0-40% in 20 min. ESI-MS observed  $m/z = 649.26$ , calculated  $[M+H]^+ = 649.33$ .

Ligation between H-**LSTEL**-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> and peptide **31**:

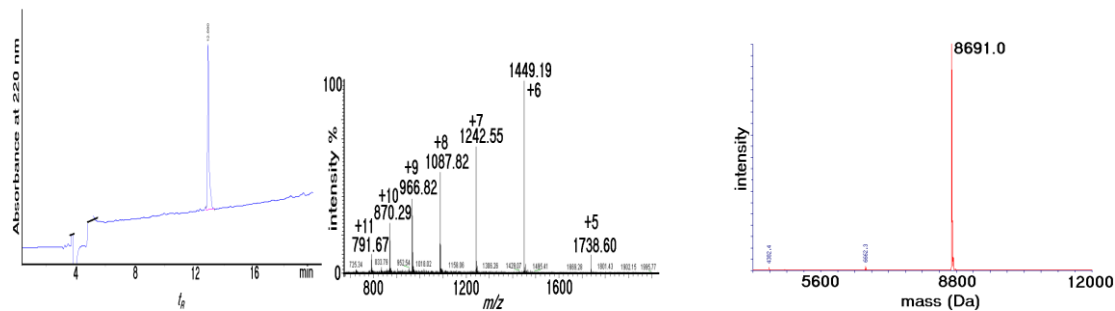
0.9 mg of H-**LSTEL**-SCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> and 0.9 mg of peptide **31** were dissolved in 50  $\mu$ L of ligation buffer and incubated at 37°C. After 1 h, the reaction was almost completely finished. The yield was 92 % based on analytic HPLC. The ligation product was purified with C18 semi-preparative HPLC. 1.2 mg of ligation product **32b** was isolated. The HPLC profile of the ligation is shown in main text (Figure 1).

Removal of Cbz from peptide **32b**:

50  $\mu$ L of chilled cocktail containing TFMSA/TFA/*p*-cresol/methyl phenyl sulfide (1:7:1:1) was added into 1.2 mg of peptide **32b** on ice. The mixture was kept on ice for 2 min and in room temperature for another 20 min. 0.5 mL diethyl ether was added and the mixture was centrifuged. The pellet was dissolved in 50% ACN/H<sub>2</sub>O and purified with C18 semi-preparative HPLC. After lyophilization, 0.8 mg of peptide **33b** was isolated (yield 74%).



**Figure 9** C18 analytic HPLC analysis and ESI-MS of purified peptide **32b** (upper trace) and its purified Cbz removed product peptide **33b** (bottom trace). Gradient: Buffer B 0-60% in 30 min. ESI-MS observed for peptide **32b**,  $m/z = 1400.79$ , calculated  $[M+H]^+ = 1400.73$ ; ESI-MS observed for peptide **33b**,  $m/z = 1267.99$ , calculated  $[M+H]^+ = 1266.69$ .

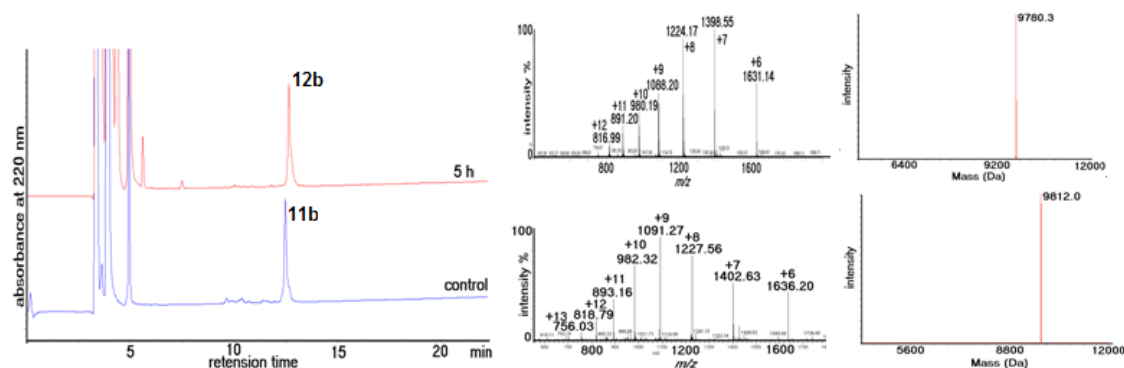
Ligation of peptide **33b** with ubiquitin(1-76)-MES:

**Figure 10** C18 analytic HPLC and ESI-MS of purified ubiquitin(1-76)-MES. Gradient: Buffer B 20-60% in 20 min. Deconvoluted ESI-MS observed 8691.0, calculated molecular weight 8689.

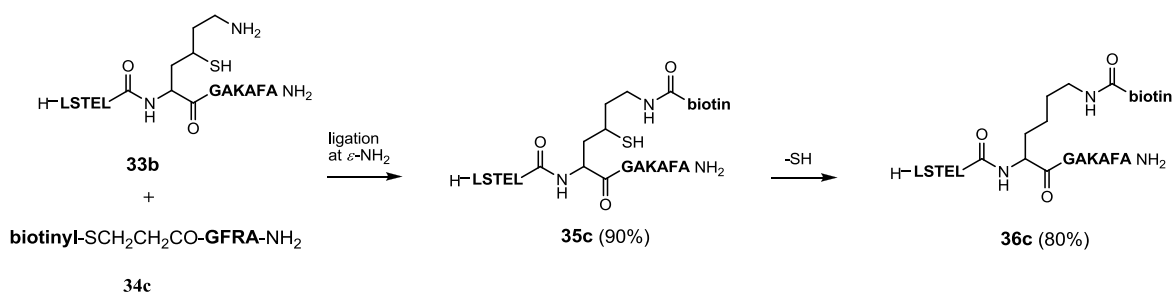
The HPLC profile of the ligation between **33b** and ubiquitin(1-76)-MES is shown in main text (Figure 5).

Free radical desulfurization of peptide **35b** to give peptide **36b**:

0.2 mg peptide **35b** was desulfurized according to the general procedure of free radical-mediated desulfurization described previously. The yield was quantitative based on HPLC and MS analysis.



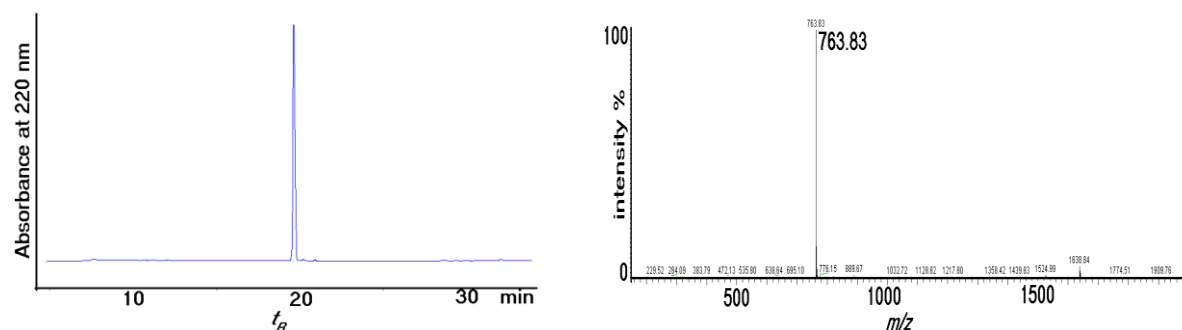
**Figure 11** C18 analytic HPLC and ESI-MS monitored free radical-mediated desulfurization of peptide **35b**. The lower trace was the purified peptide **35b** dissolved in desulfurization buffer. The upper trace showed the crude desulfurization after 5 h. Peak **36b** was the desulfurization product. HPLC gradient: buffer B 20 - 60% in 20 min. Peptide **35b**: deconvoluted ESI-MS observed molecular weight 9812.0, calculated molecular weight 9811.6; peptide **36b**: Deconvoluted ESI-MS observed molecular weight 9780.3, calculated molecular weight 9779.6.



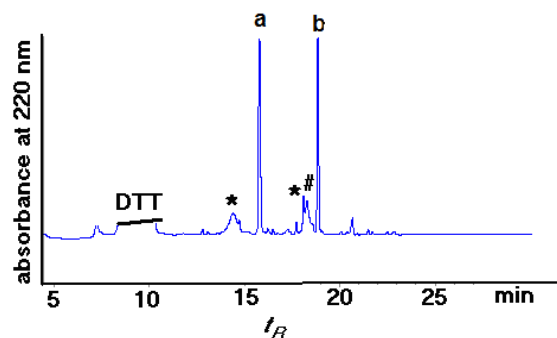
**Scheme 3** Demonstration of dual chemical ligation for the synthesis of biotinylated peptides.

Ligation of peptide **33b** with Biotinyl-SCH<sub>2</sub>CH<sub>2</sub>CO-GFRA-NH<sub>2</sub>:

0.3 mg of peptide **33b** and 0.3 mg of Biotinyl-SCH<sub>2</sub>CH<sub>2</sub>CO-GFRA-NH<sub>2</sub> were dissolved in 50  $\mu\text{L}$  ligation buffer and incubated at 37°C. After 3 h, excess of DTT was added into the mixture. Let the mixture be reduced for 10 min. An aliquot was taken out for C18 analytic HPLC analysis. The yield was about 90% based on analytic HPLC. The rest was purified by 2 runs of C18 analytic HPLC. About 0.3 mg biotinylated peptide **35c** was isolated.



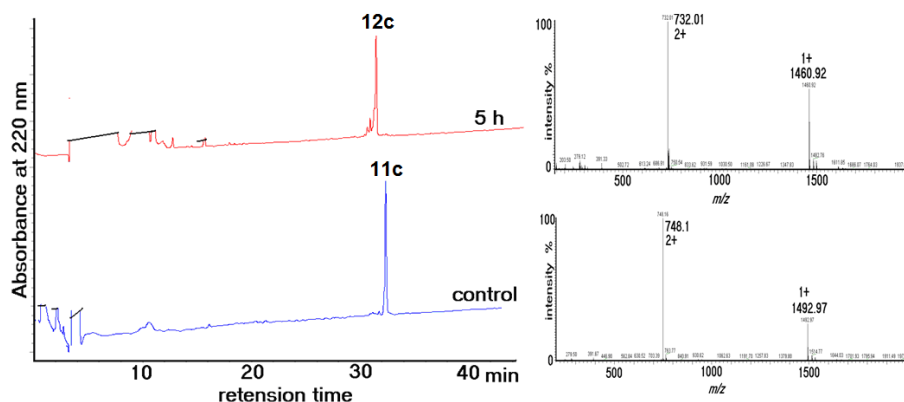
**Figure 12** C18 analytic HPLC and ESI-MS of Biotinyl-SCH<sub>2</sub>CH<sub>2</sub>CO-GFRA-NH<sub>2</sub>. Gradient: Buffer B 0-60% in 30 min. ESI-MS observed  $m/z = 763.83$ , calculated  $[\text{M}+\text{H}]^+ = 763.34$ .



**Figure 13** C18 analytic HPLC analysis of ligation between peptide **33b** and Biotinyl-SCH<sub>2</sub>CH<sub>2</sub>CO-GFRA-NH<sub>2</sub> after 3h reaction. Gradient: Buffer B 0-60% in 30 min. Peak a, HS-CH<sub>2</sub>CH<sub>2</sub>CO-GFRA-NH<sub>2</sub>, ESI-MS observed  $m/z = 537.6$ , calculated  $[M+H]^+ = 537.26$ ; peak b, peptide **35c**, ESI-MS observed  $m/z = 1492.94$ , calculated  $[M+H]^+ = 1492.77$ ; peaks \*, unidentified; peak #, incomplete reduction of oxidative adduct between HS-CH<sub>2</sub>CH<sub>2</sub>CO-GFRA-NH<sub>2</sub> and peptide **35c**.

### Free radical desulfurization of **35c**:

0.3 mg peptide **35c** was desulfurized according to the general procedure of free radical-mediated desulfurization. The yield was 80% based on quantitative HPLC analysis.



**Figure 14** C18 analytic HPLC monitored free radical-mediated desulfurization of peptide **35c**. The lower trace was the purified peptide **35c** for desulfurization. The upper trace showed the desulfurization reaction mixture after 5 h. Peak **36c** was the desulfurization product. HPLC gradient: buffer B 0-40% in 40 min. ESI-MS observed for peptide **35c**  $m/z = 1492.97$ , calculated  $[M+H]^+ = 1492.77$ ; ESI-MS observed for peptide **36c**  $m/z = 1460.92$ , calculated  $[M+H]^+ = 1460.80$ .

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# CHAPTER 2

## Synthesis of K48-linked diubiquitin using dual native chemical ligation at lysine

Synthesis of K48-linked diubiquitin using dual native chemical ligation at lysine, *Chem. Commun.* **2010**, 46, 7199-7201.

Yang, R.; # Pasunooti, K. K.; # Li, F.; Liu, X.-W.\*; Liu, C. F.\* (# these authors contributed equally)

- Featured in *Chem. Eng. News (C&EN)*, Vol 88, 41, Oct11, **2010**, 2.
- *Carpe Diubiquitin*, by L. Martin & R. T. Raines, *Angewandte Chemie Int. Ed.* **2010**, 49, 9042-9044.
- Featured in *Chem. Eng. News (C&EN)*, Dec20, **2010**, issue: Chemical Year in Review **2010**.

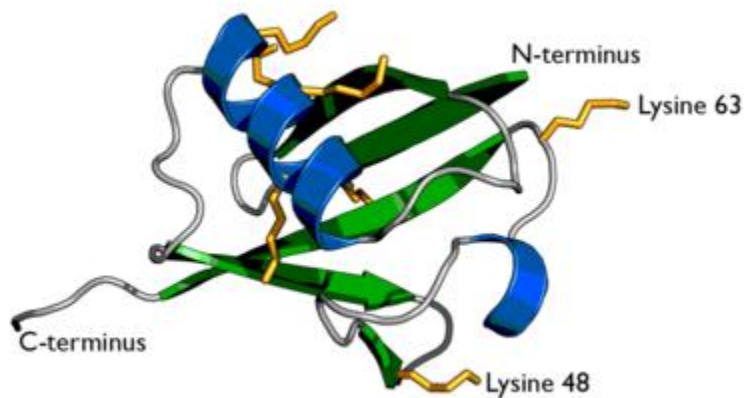
## 1. INTRODUCTION

Ubiquitin is a small regulatory protein which is found in tissues of eukaryotic organisms.<sup>1</sup> It was first identified in 1975 as a protein with unknown function in eukaryotic cells. Later in 1980s the basic functions and the major components for ubiquitination pathway were elucidated, in which one of the major function of ubiquitin is recycling of protein. Eukaryotic cells are made more diverse and complex by an intricate assembly of protein post-translational modifications.<sup>1</sup> It plays a significant role in regulating protein structure, shape and its function. Post-translational modification is a chemical modification of a protein after its translation and it is one of the later steps in protein biosynthesis for many proteins. Post-translational modifications may involve the formation of disulfide bridges and attachment of any of a number of biochemical functional groups such as acetate, phosphate, various lipids and carbohydrates. Depending on the addition of a functional group there are several types of modifications occurs which include Glycosylation, Disulfation, Proteolytic cleavage, Phoshorylation, Acetylation, Methylation, Prenylation, Tyrosine sulfation, Sumoylation and Ubiquitination.

Ubiquitin (Ub) is a small, robust and highly conserved protein. The ubiquitin protein consists of 76 amino acid residues with molecular mass about 8.5 kDa. Key features include C-terminal chain with 7 lysine amino acid residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) shown in Figure 2.1, and is conserved among Eucaryotes.

Ubiquitin plays a significant role in key cellular processes such as the proteosomal degradation of cytosolic and membrane proteins, endocytosis of membranes and the regulation

of gene transcription. Physiologically, ubiquitination is involved in protein degradation, signal transduction and so on.<sup>2</sup>



**Figure 2.1** Three dimensional structure of ubiquitin (Ub).

Ubiquitin (Ub) is a 76 amino acid protein residue that is post-translationally conjugated to achieve a site-specific tagging of the lysine residue in the target proteins with three distinct ubiquitinating enzymes, known as the E1, E2, and E3. The E1 enzyme uses the cleavage of ATP into AMP and PP for its activation.<sup>3</sup> E2 is a ubiquitin conjugating enzyme and each cell has several E2 enzymes. E3 is a ubiquitin ligase which either transiently form a thiolester with ubiquitin or allow the E2 enzyme to transfer the ubiquitin to the substrate.

Ubiquitination is an enzymatic protein post translational modification in which the carboxylic acid of the C-terminal glycine from the di-glycine motif in the activated ubiquitin forms an amide bond to the  $\epsilon$ -amine of the lysine in the modified protein. It refers to the linking of one or more ubiquitins through its C-terminal glycine to the lysine side chain of another ubiquitin or the other protein.<sup>4</sup> Since ubiquitin (Ub) itself consist seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63), its polymers have distinct linkages with different properties, all possible linkages regulate many cellular processes like proteasomal degradation, cell-cycle progression, and signal transduction.<sup>5</sup> Among seven lysine residues, K48-linked polyubiquitin is the best understood polyubiquitination and is shown to serve as a signal for targeting the protein for proteasomal degradation.<sup>6</sup> This process is quite challenging due to the requirement of isolation of the specific ligase (E3) from recombinant protein expression. Moreover, this method provides limited quantities of the ubiquitinated proteins.

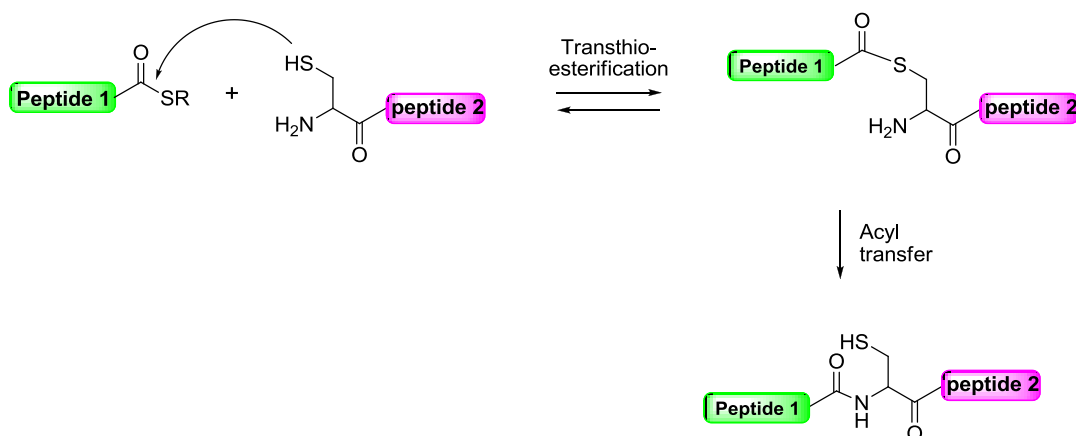
Ubiquitination consists of a series of steps:

1. The first step is the activation of Ubiquitin which is a two step reaction done by E1 ubiquitin activating enzyme which requires ATP as an energy source. The initial step involves the formation of ubiquitin – adenylate intermediate. In the second step the ubiquitin is transferred to the E1 active site cysteine residue by releasing AMP. This step results the formation of thioester linkage between the C-terminal carboxy group of ubiquitin and the E1 cysteine sulfhydryl group.
2. The second step is the transfer of ubiquitin from E1 to the active site cysteine of a ubiquitin – conjugating enzyme E2 through a trans (thio) esterification reaction.

- The final step of the ubiquitination cascade forms an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. This step requires many E3 enzymes (ubiquitin ligases).

Generally, the existing chemical strategies (solid-phase peptide synthesis-SPPS and the native chemical ligation-NCL)<sup>7,8</sup> for the preparation of post-translationally modified proteins could overcome these challenges and allow for site-specific ubiquitination of the modified protein in sufficient quantities for biochemical, structural and analytical studies.

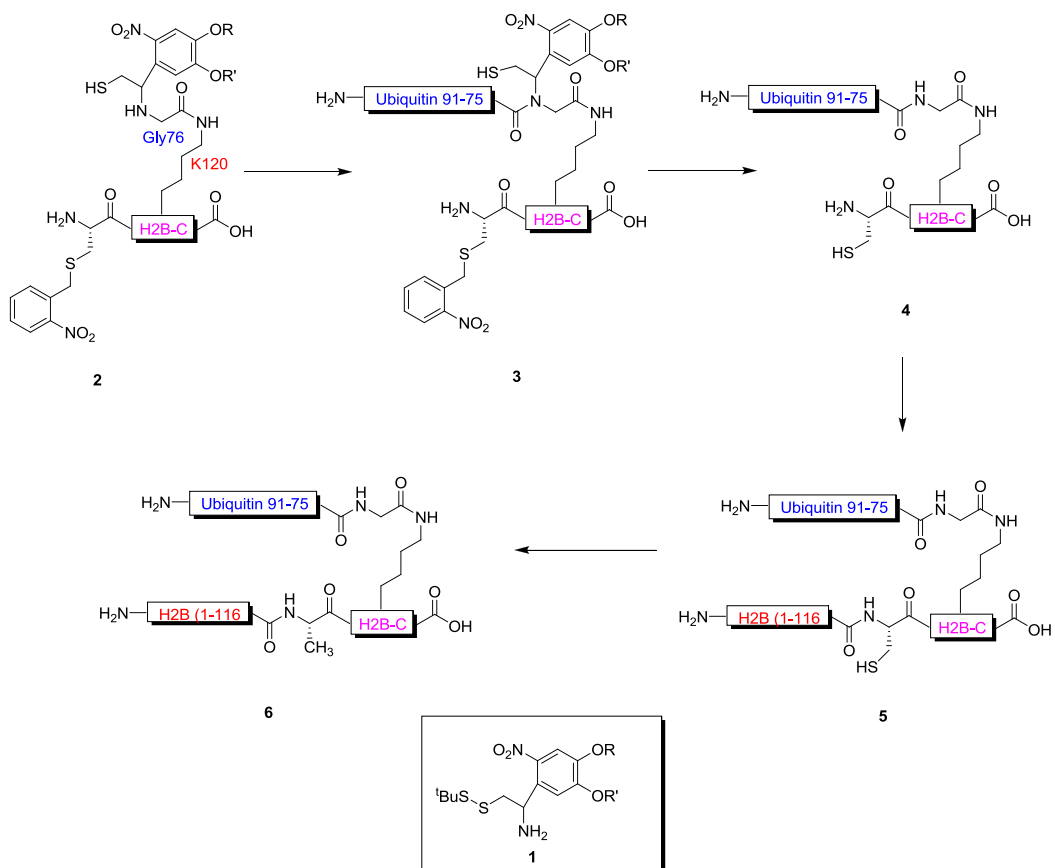
Protein chemical synthesis through chemoselective peptide ligation is becoming an increasingly important enabling technology in the study of proteins. A ligation method known as Native chemical ligation (NCL), which involves the reaction of a thioester peptide with another cysteinyl peptide to form a native bond at the ligation junction is also an useful method (Figure 2.2).<sup>9</sup> Previously, the total chemical synthesis of ubiquitin has been synthesized using combine solid-phase peptide synthesis (SPPS) and the native chemical ligation (NCL).



**Figure 2.2** General Native chemical ligation (NCL) mechanism.

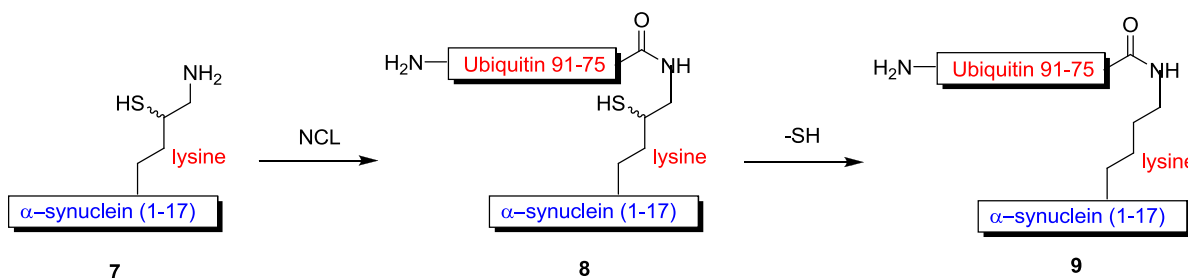
For the first time Ploegh and co-workers investigated the semi-synthesis of ubiquitin analogues with C-terminal electrophiles by direct aminolysis of recombinant Ub- $\alpha$ -thioesters generated from the corresponding Ub-intein fusions by thiolysis.<sup>10</sup> Adopting these preliminary results, Muir research group investigated an elegant approach for site-specific peptide and protein mono ubiquitination.<sup>11</sup> Their fascinating discovery relies on lysine residue consist with photo-removable auxiliary to mediate isopeptide bond formation with the ubiquitin thioester (Ub- $\alpha$ -thioester). The recombinant Ub- $\alpha$ -thioesters facilitate as a ligation partner to the  $\epsilon$ -amine group of lysine residue in a peptide using expressed protein ligation (EPL).

This phenomenon was well standardized and further applied to synthesize a homogeneous ubiquitylated histone H2B.<sup>12</sup> As shown in Scheme 1, they chose photo-labile protecting group **1** as auxiliary<sup>11</sup> which was described previously by the groups of Dawson<sup>13</sup> and Aimoto.<sup>14</sup> These auxiliaries are sterically less demanding which can be use under mild reaction conditions and make it compatible for fold the proteins. Peptide **2** (residues 117-125 of H2B) was synthesized using the ligation auxiliary **1** attached to the  $\epsilon$ -NH<sub>2</sub> of lysine120. Photo-labile auxiliary containing peptide **2** was treated with ubiquitin (1-75)- $\alpha$ -thioester which produced by thiolysis of a corresponding intein fusion protein, to give a ubiquitinated peptide **3**. Subsequent UV irradiation of peptide **3** provides to efficient removal of both the auxiliaries on the peptide to give a branched protein **4**. This precursor was further subjected to recombinant H2B (1-116)- $\alpha$ -thioester to give ubiquitin (Ub) containing human H2B **5**. Final step, Raney nickel mediated desulfurization<sup>15</sup> was used to convert the single cysteine residue in branched protein **6** to the native alanine residue present in uH2B.



**Scheme 2.1** The strategy used for the synthesis of K48-linked diubiquitin.

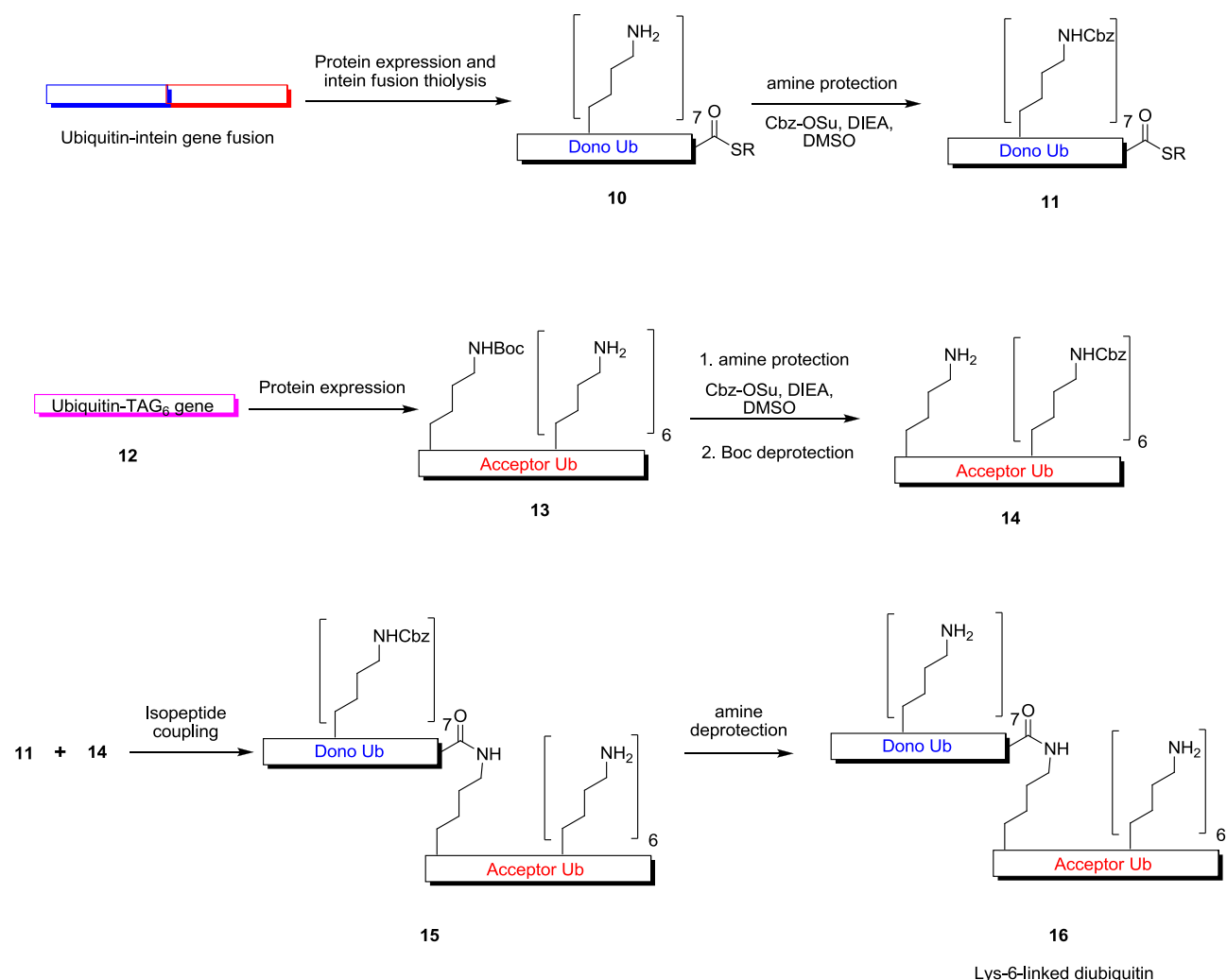
Recently our group investigated dual native chemical ligation strategy through the use of a  $\gamma$ -mercapto lysine residue for diubiquitin synthesis.<sup>16</sup> In parallel to our research, Brik and co-workers reported native chemical ligation on lysine by positioning thiol functionality at  $\delta$ -position of the side chain (Scheme 2).<sup>17</sup> This thiol containing lysine incorporated in the  $\alpha$ -synuclein peptide (1-17) **7** and resulted peptide was treated with ubiquitin thioester using standard native chemical ligation. Finally, applying the desulfurization strategy on ligation product **8** led to ubiquitinated  $\alpha$ -synuclein **9** in good yields.<sup>15,18</sup>



**Scheme 2.2** The strategy used for the synthesis of mono ubiquitination.

More recently, three research groups including our group have independently investigated the chemical synthesis of diubiquitin chains.<sup>16,19-21</sup> The specific formation of an isopeptide bond between two Ubiquitins requires the C-terminal thioester ubiquitin (donor) and specific lysine residue in another ubiquitin with N-terminal  $\epsilon$ -amine (acceptor). Firstly, Chin and co-workers reported a new approach for the synthesis of homogeneously linked diubiquitin chain for preparing Lys6 and Lys29 linked ubiquitin chains (Scheme 3).<sup>19</sup> Their approach contains powerful combination of genetic code expansion and chemoselective protein chemistry. They used the generality of GOPAL (genetically encoded orthogonal protection and activated ligation) for couple two selectively protected ubiquitins. A donor Ubiquitin **11** was synthesized as a C-

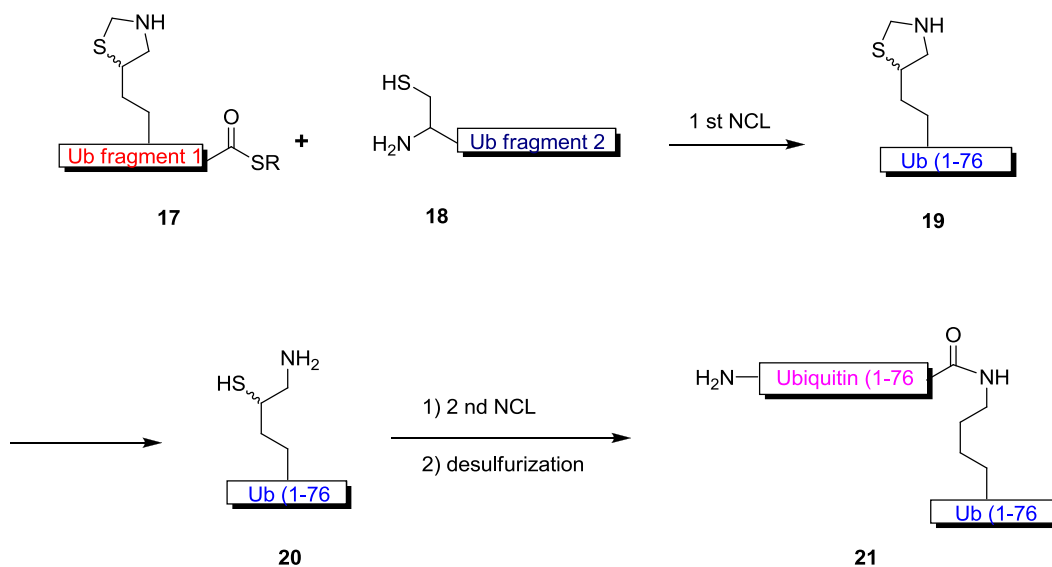
terminal thioester **10** by thiolysis of an intein gene fusion. To protect the seven free amines including N-terminal amine in Ub thioester of **10** used eight equivalents of Cbz-OSu in the presence of mild base (di-isopropyl ethylamine-DIEA). Next, the selectively protected lysine residue in acceptor ubiquitin **13** synthesized from recombinant protein expression. To protect rest of the amine functionalities on lysine and the N-terminal amine with Cbz group, they used Cbz-OSu reagent in basic DMSO. Further, acceptor Ub **14** was obtained by using deprotection step with TFA in water. Then thioester **11** can be activated and converted in to protected diubiquitin **15** in the presence of silver salt, which allowing selective acylation with amine **14**. Finally, the deprotected diubiquitin was obtained after Cbz removal using TFMSA/TFA.



**Scheme 2.3** Synthesis of Lys6-linked diubiquitin.

Secondly, our research group synthesized Lys48 diubiquitin using a combination of solid phase peptide synthesis and native chemical ligation (NCL).<sup>16</sup> Our key precursor is a lysine analogue containing a photo-labile protecting group on the  $\epsilon$ -nitrogen and a thiol group on the  $\gamma$ -carbon of the lysine chain (see in results and discussion part).<sup>22,23</sup> Same time, Brik and co-workers synthesized all diubiquitin chains by using native chemical ligation technique.<sup>20</sup> The overall strategy for the diubiquitin synthesis was shown in Scheme 4. First, Ub fragment 1 **18** was treated with the thiazolidine protected  $\delta$ -mercaptolysine (Ub fragment 1) **17** to obtain ligation product **19**. Further, deprotection step produces the  $\delta$ -mercaptolysine **20** which mediates the thioesterification

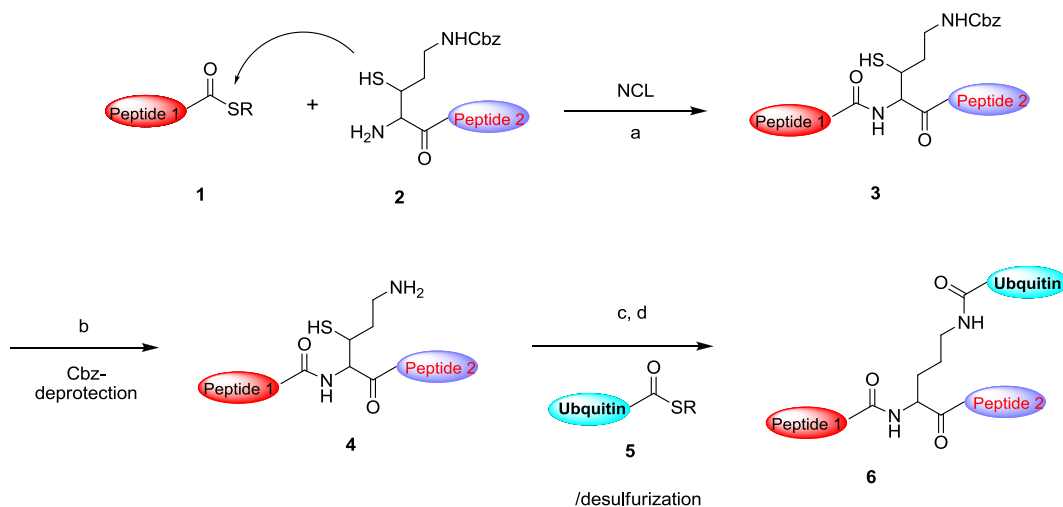
step of ubiquitin thioester and followed by S→N acyl transfer to form the isopeptide bond. Finally, the thiol groups of polypeptide can be easily removed by using the desulfurization strategy to get the diubiquitin **21**.



**Scheme 2.4** Brik's diubiquitin synthesis.

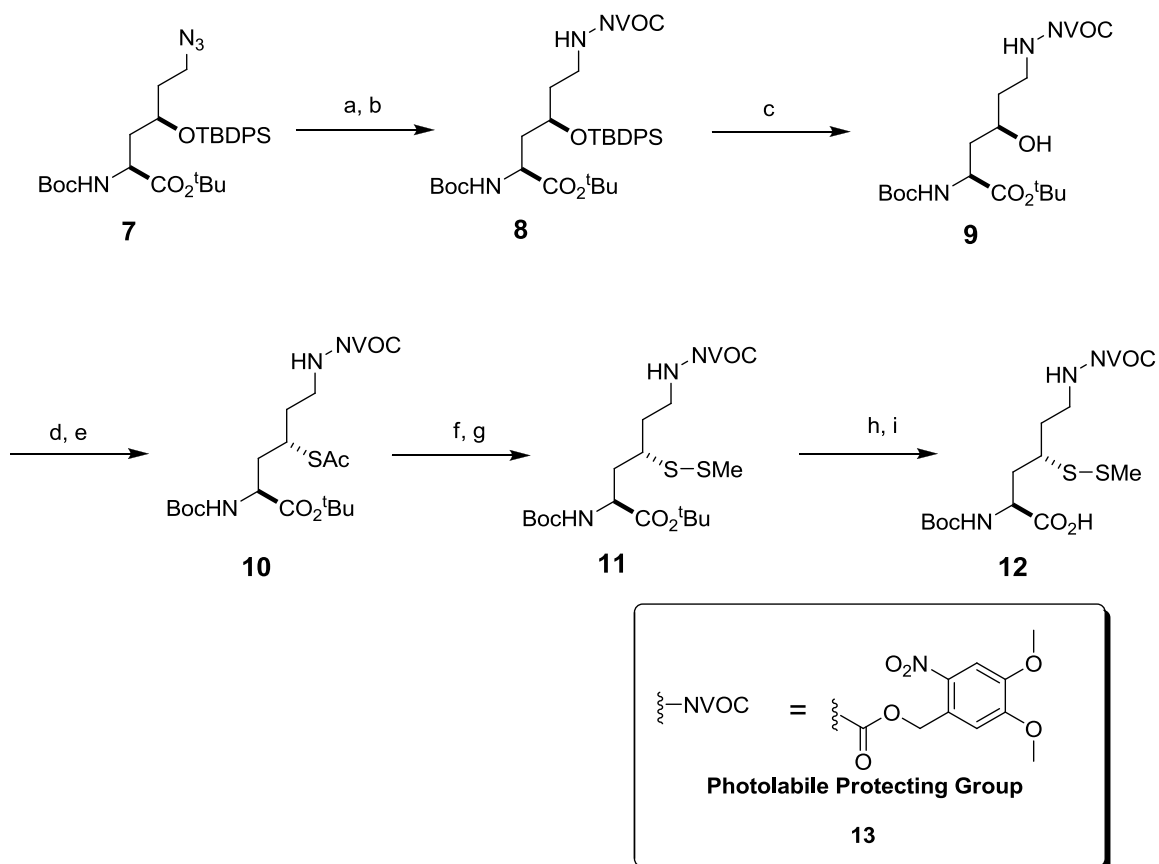
## 2. RESULTS AND DISCUSSION

Recently, our group reported dual native chemical ligation at lysine by positioning thiol group at  $\gamma$ -position of the side chain.<sup>22,23</sup> We have demonstrated that the thiol group of  $\gamma$ -mercaptolysine can mediate dual chemical ligation at both  $\alpha$ - and  $\epsilon$ - amines of lysine. With this approach, we successfully synthesized an ubiquitinated peptide (Scheme 2.1). The  $\gamma$ -mercapto lysine derivative **2** was used as the N-terminal amino acid precursor with thioester **1** for native chemical ligation. Further, Cbz-group of peptide **3** was deprotected with trifluoromethanesulfonic acid (TFMSA) to obtain the  $\epsilon$ -amine functionality on  $\gamma$ -mercapto lysine for the second ligation. Using the similar conditions like first ligation, post-translationally modified tags **5** (Ubiquitin and biotin thioesters) were attached to the key precursor **4** using second native chemical ligation. Finally, free radical-mediated desulfurization was used to generate the branched protein **6**. Recently, several other research groups including our group reported the synthesis of a monoubiquitinated peptide through native chemical ligation strategy.<sup>11,17,24</sup> In our previous study, we employed benzyloxycarbonyl (Cbz) as the orthogonal protecting group for the  $\epsilon$ -amino group of  $\gamma$ -mercaptolysine to make sure that the ligation first selectively occurred at the  $\alpha$ -amine.<sup>23</sup> Then the Cbz group was removed with a strong acid to free the  $\epsilon$ -amine for the side chain ligation. We realize that the harsh conditions for Cbz removal may limit the application scope of our methodology for large protein ubiquitination. We revise our orthogonal protection strategy by replacing Cbz with a photolabile protection group, *o*-nitroveratryloxycarbonyl (NVOC, **13**) (Scheme 2.2). We then apply our revised approach for the synthesis of K48-linked diubiquitin.



**Scheme 2.1.** General scheme for dual native chemical ligation (a) 1<sup>st</sup> ligation with **Peptide 1**-COSR; (b) Deprotection; (c) 2<sup>nd</sup> ligation with **Ubiquitin**-COSR; (d) Desulfurization.

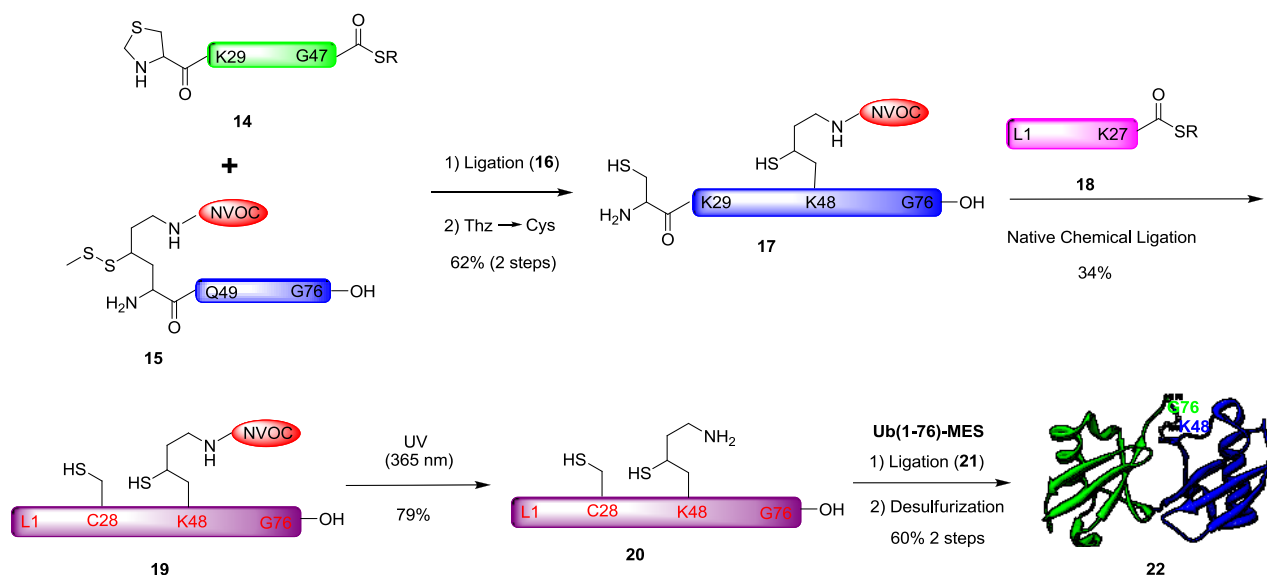
The N $\epsilon$ -NVOC protected 4-mercaptolysine derivative was synthesized in a similar way to that previously reported by using NVOC-Cl instead Cbz-Cl as the side chain protecting group.<sup>22,23</sup> The strategy of synthesizing the key precursor **12** as shown in Scheme 2. The azide **7** was reduced by catalytic hydrogenation to amine which was further protected with *o*-nitroveratryloxycarbonyl chloride (NVOC-Cl) to afford **8**. After deprotection of the silyl ether in **8** with TBAF, the secondary alcohol was mesylated, followed by nucleophilic substitution of thioacetate to afford **9** in good yields. Following saponification, the thiol was protected in disulfide form using MMTS to furnish **11** according to literature methods.<sup>25</sup> Acidolytic deprotection with TFA and further protection with Boc anhydride yielded **12**, the protected form of 4-mercaptolysine **12** ready for use in peptide synthesis.



Scheme 2.2: Reagents and conditions: (a)  $\text{H}_2$ , Pd/C, ethyl acetate, rt, 95%; (b) NVOC-Cl,  $\text{Na}_2\text{CO}_3$ , dioxane:water (2:1), 0 °C, 85%; (c) TBAF, THF, 0 °C, 75%; (d) Ms-Cl, DIPEA, 0 °C; (e)  $\text{CH}_3\text{COSK}$ , DMF, 40 °C, two steps 70% yield; (f) NaOH, MeOH, rt, ; (g) *S*-methyl methanethiosulfonate (MMTS), triethylamine,  $\text{CH}_2\text{Cl}_2$ , rt, two steps 60%; (h) TFA,  $\text{H}_2\text{O}$ , rt; (i)  $\text{Boc}_2\text{O/T-EA}$ , MeOH, rt, two steps 73% yield.

The next stage of peptide ligation and diubiquitin synthesis *via* dual native chemical ligation was carried out by our collaborator, Prof. Liu Chuan-Fa group from School of Biological Sciences, NTU. The overall strategy of synthesizing the diubiquitin is shown in Scheme 2.3. To synthesize K48-linked diubiquitin (**22**) using dual NCL at lysine method, the crucial step is the installation of 4-mercaptolysine at position 48 of the monoubiquitin. The synthesis of ubiquitin and its

mutants through stepwise synthesis or sequential chemical ligation/desulfurization has been reported. Here, we synthesized the K48(4-SH)-containing ubiquitin (**19**) using C-to-N sequential ligation with Ala28Cys and K48(4-SH) as the ligation junctions. After we got **19**, the NVOC group was removed by 365 nm UV irradiation. Monoubiquitin **20** with free  $\epsilon$ -NH<sub>2</sub> on K48 was then reacted with ubiquitin thioester Ub(1-76)-MES which was generated by thiolysis of ubiquitin-intein fusion protein with sodium mercaptoethanesulfonate (MESNa). To generate the native K48-linked diubiquitin, free radical mediated desulfurization<sup>18</sup> was performed for the ligation product to convert Cys28 to Ala and K48(4-SH) to Lys.

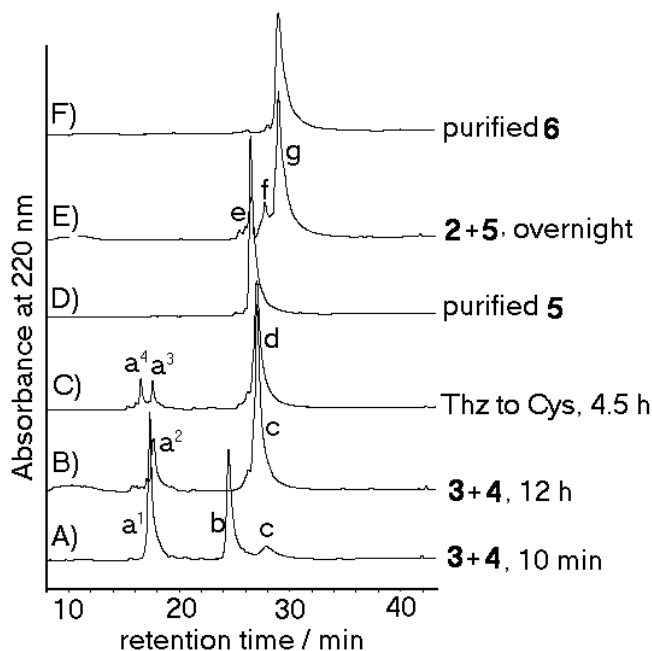


**Scheme 2.3** The overall synthetic strategy for diubiquitin.

HPLC data for the synthesis of **19** was shown in Figure 2.1. The middle segment of ubiquitin, peptide  $\alpha$ thioester **14**, was reacted with C-terminal segment **15** through 4-mercaptolysine mediated ligation. 8 mg of peptide  $\alpha$ thioester of **14** and 10 mg of C-terminal peptide **15** were dissolved in ligation buffer (6 M Gdn•HCl, 0.2 M phosphate, 20 mM TCEP, pH 7.5). In the presence of TCEP, the disulfide in **15** was immediately reduced and gave the free

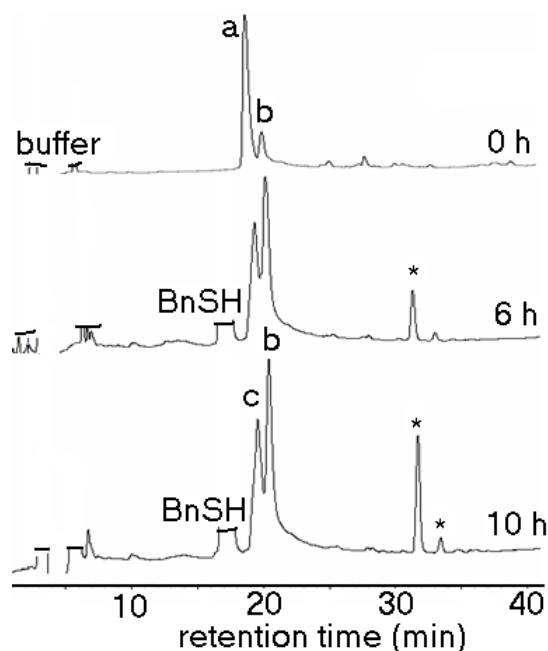
thiol. Upon addition of the thiol additive MESNa (0.2 M), **14** was converted to its MES thioester. The ligation proceeded efficiently. After 12 h, the reaction was completed. The conversion of Thz (1,3-thiazolidine-4-carboxo group) to Cys was directly performed in the ligation mixture. 0.4 M MeONH<sub>2</sub>•HCl (final concentration) was added into the mixture and pH was adjusted to 4.0. After 4.5 h at room temperature, the deprotection was completed (Figure 2.1C).<sup>26</sup> Product **17** was purified by C18 semi-preparative HPLC and about 62% of yield was obtained for two steps after lyophilization. For the next ligation step, 7.3 mg of peptide thioester **18** and 10 mg of peptide **17** were dissolved in the ligation buffer (6 M Gdn•HCl, 0.2 M phosphate, 0.2 M MESNa, 20 mM TCEP, pH 7.5). The reaction was completed overnight. The major side reaction was the self-cyclization of **18** involving possibly the C-terminal lysine residue. The full-length ubiquitin **19** was obtained in 34% of yield after purified by C18 semi-preparative HPLC.

To remove the photolabile protection group, full length peptide **19** was dissolved in 60% acetonitrile aqueous solution (containing 0.045% trifluoroacetic acid). The solution was irradiated with 365 nm UV light. The deprotection process was monitored with electrospray ionization mass spectrometry (ESI-MS). After 2 h, ESI-MS confirmed that the NVOC group was completely removed to afford product **20**. The solution was diluted and subjected to C18 semi-preparative HPLC purification. Compound **20** was obtained in 79% yield after lyophilization.



**Figure 2.1** C8 analytic HPLC data for the synthesis of **19**. A) and B): Ligation between **14** and **15** at 10 min and 12 h, respectively. Peak  $a^1$ , the 1:10 mixture of **14** and its MES thioester; peak b, reduced **15**; peak c, ligation product **Thz28-K48(4-SH, N $\epsilon$ -NVOC)-G76-OH**; peak  $a^2$ , about 1:1 mixture of **14** and its MES thioester. C): The *in situ* conversion of Thz to Cys for 4.5 h. peak  $a^3$ , peptide **14**; peak  $a^4$ , Cys28-G47-NHOCH<sub>3</sub>; peak d, product **17**. D): Purified **17**. E): Ligation between **18** and **17** overnight. Peak e, the MES thioester of **18**; peak f, cyclization product of **18**; peak g, ligation product **19**. F): Purified **19**.

To synthesize K48-linked diubiquitin, ubiquitin **20** was reacted with Ub(1-76)-MES (Figure 2.2). 1.3 mg of Peptide **20** and 1.5 mg of Ub(1-76)-MES were dissolved in buffer solution (6 M Gdn•HCl, 0.1 M phosphate, 40 mM TCEP, 1 % v/v benzyl mercaptan, pH 8.0). After 6 h, the ligation product was formed with a yield of about 65 % based on analytic HPLC. After another 4 h, the yield of ligation product increased slightly.



**Figure 2.2** C4 analytic HPLC monitored ligation between **20** and Ub(1-76)-MES at 0 h, 6 h and 10 h, respectively. Peak a: mixture of **20** and ubi(1-76)-MES. Peak b: ligation product. Peak c: mixture of ubi(1-76)-OH and small amount of remaining **20** and ubi(1-76)-SBn. Peak \*: nonproteinous product. Note: 0 h HPLC was run before adding benzyl mercaptan. Bn = benzyl.

To get the native K48-linked diubiquitin, free radical mediated desulfurization was performed to convert cysteine 28 and 4-mercaptolysine 48 at the ligation junctions to alanine and lysine, respectively. After 9 h of treatment with the free radical initiator VA-044, both sulphur atoms on the two residues were removed, as confirmed by ESI-MS. Deconvoluted ESI-MS showed the correct molecular weight of the final native K48-linked diubiquitin in 55% yield. For the characterization of the final product, the diubiquitin was checked with 18% SDS-PAGE. Coomassie blue staining showed a single band. Western blot with antibody FK2H, which is an HRP-conjugated antibody against mono- and polyubiquitinated conjugates but not free ubiquitin, detected the same band as the one in coomassie blue staining. To test whether the synthesized

diubiquitin can be folded to its native form, circular dichroism (CD) was measured with dialyzed diubiquitin **22**. CD spectrum indicated that the diubiquitin was well folded after dialysis. Next we performed the cleavage assay using ubiquitin C-terminal hydrolase. It was found that the diubiquitin can be hydrolyzed by the hydrolase, UCH-L3 (Figure 10, Experimental).

### 3. CONCLUSION

In summary, the dual native chemical ligation at lysine strategy was revised by replacing the strong acid-labile Cbz with the photolabile NVOC protecting group for the side-chain amine of 4-mercaptolysine. We have optimized and successfully installed the *o*-nitroveratryloxycarbonyl (NVOC, **13**) protecting group on  $\epsilon$ -NH<sub>2</sub> of lysine for our ligation approach. The synthesis of K48-linked diubiquitin using the improved protocol demonstrates the practical utility of this ligation strategy in synthetic protein chemistry.

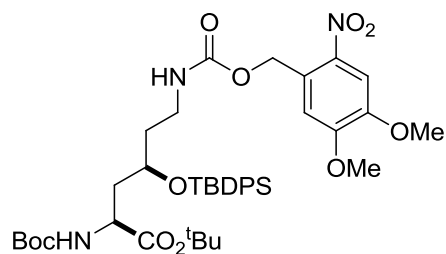
## 4. EXPERIMENTAL

Unless otherwise noted, all reactions were carried out in oven dried glassware under an atmosphere of nitrogen and distilled solvents were transferred by syringe. Solvents and reagents were purified according to the standard procedure prior to use. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 nm). Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on a 300 MHz Bruker ACF 300, 400 MHz Bruker DPX 400 and 500 MHz Bruker AMX 500 NMR spectrometers, respectively. The residual solvent signals were taken as the reference (7.26 ppm for  $^1\text{H}$  NMR spectroscopy and 77.0 ppm for  $^{13}\text{C}$  NMR spectroscopy). Chemical shift ( $\delta$ ) is referred in terms of ppm, coupling constants ( $J$ ) are given in Hz. Following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal. Infrared spectra were recorded on a Bio-RAD FTS 165 FT-IR Spectrometer and reported in  $\text{cm}^{-1}$ . Samples were prepared in thin film technique. HRMS (ESI) spectra were recorded on a Finnigan/MAT LCQ quadrupole ion trap mass spectrometer, coupled with the TSP4000 HPLC system and the Crystal 310 CE system.

Amino acid derivatives, coupling reagents and resins were purchased from GL Biochem (Shanghai, China), Novabiochem and Chemimpex. All the other chemical reagents were purchased from Alfa Aesar, Sigma-Aldrich Chemical Company, Fisher Scientific, Acros Organics. PCR kit, restriction enzymes, and chitin beads for construction of ubiquitin-intein expression vector and the purification of intein-CBD fusion proteins were obtained from New England Biolabs. The antibody FK2H was purchased from Enzo life sciences. C18 and C8

Analytic HPLC analyses were performed by using an Agilent 1100 series instrument equipped with a Jupiter C18 (5  $\mu$ , 4.6 x 250 mm) and a Vydac MS C8 (5  $\mu$ , 4.6 x 250 mm) reverse-phase column with a flow rate of 1.0 mL/min. C4 Analytic HPLC analyses were performed by using an Shimadzu UFLC system equipped with a Vydac 214MS C4 (5  $\mu$ , 4.6 x 250 mm) reverse-phase column with a flow rate of 1.0 mL/min. Detection was achieved with a UV-VIS-detector at wavelength  $\lambda = 220$  nm. Semi-preparative purifications were performed with a Shimadzu system equipped with a Jupiter C18 column (5  $\mu$ , 10 x 250 mm) with a flow rate of 2.5 mL/min. Preparative purifications were performed with a Waters system equipped with a Prosphere C18 column (10  $\mu$ , 22 x 250 mm) with a flow rate of 10 mL/min. The buffer system for all the analysis was buffer A, H<sub>2</sub>O (containing 0.045% TFA) and buffer B, 90% acetonitrile in H<sub>2</sub>O (containing 0.04% TFA). Peptide masses were measured using a Thermo FINNIGAN LCQ Deca XP MAX equipped with ESI ion source.

**(7R,9S)-tert-butyl-7-(tert-butyldiphenylsilyloxy)-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-3,11-dioxo-2,12-dioxo-4,10-diazatetradecane-9-carboxylate (8):**



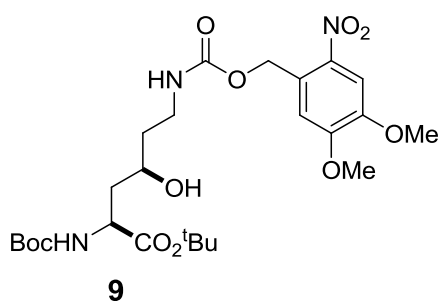
**8**

To a solution of azide **7** (0.5 g) in ethyl acetate (4.5 mL, 0.2M) was added 10% Pd/C (0.15 g) at room temperature. The reaction mixture was stirred for 6 h under an H<sub>2</sub> atmosphere at room temperature then filtered through a short pad of celite and washed with chloroform\methanol (1:1). The solvent removed under reduced pressure (quantitative yield). This compound was direct taken for the next step without further purification.

Amine (0.45 g, 0.8 mmol) was dissolved in 2:1 ratio of dioxane\water (10 mL□5 mL) and cooled to 0 °C. Sodium carbonate (0.57 g, 1.76 mmol) was introduced portions wise at the same temperature. Then NVOC-Cl (0.24 g, 0.88 mmol) was added slowly to the reaction mixture. The reaction mixture was allowed to reach room temperature and stirred for 4 h. After being cooled to 0 °C, the reaction mixture was quenched by the addition of 0.5 N HCl (2 mL) and stirred for a further 5 min. The solution was dissolved in ethyl acetate and washed with water, and brine. The organic layer was dried over sodium sulphate and concentrated *in vacuo*, which was purified by column chromatography on silica gel to give disired product **8** (0.64 g, 85 %) as a pale yellow solid.

**<sup>1</sup>H NMR (500 MHz, THF-D<sub>8</sub>):**  $\delta$  in ppm = 7.61-7.55 (m, 5H, H-Ph), 7.29-7.21 (m, 6H, H-Ph), 6.93 (s, 1H, H-Ph), 6.16-6.15 (m, 1H, NH), 6.07 (d,  $J = 8.1$  Hz, 1H, NH), 5.26-5.19 (m, 2H, CH<sub>2</sub>-Ph), 4.09-4.04 (m, 1H, CH-NH), 3.75 (bs, 1H, CH-OH), 3.73 (s, 6H, OCH<sub>3</sub>), 2.91-2.86 (m, 2H, CH<sub>2</sub>-NH), 1.86-1.65 (m, 2H, CH<sub>2</sub>), 1.56-1.50 (m, 2H, CH<sub>2</sub>), 1.30 (s, 9H, 3CH<sub>3</sub>), 1.26 (s, 9H, 3CH<sub>3</sub>), 0.93 (s, 9H, 3CH<sub>3</sub>). **<sup>13</sup>C NMR (100 MHz, THF-D<sub>8</sub>):**  $\delta$  in ppm = 171.7 (C=O), 155.7 (C=O), 155.4 (C=O), 153.4 (Ph), 148.1 (Ph), 139.8 (Ph), 135.8 (Ph), 135.8 (Ph), 133.7 (Ph), 133.1 (Ph), 129.9 (Ph), 129.8 (Ph), 128.1 (Ph), 127.7 (Ph), 127.7 (Ph), 110.4 (Ph), 108.1 (Ph), 81.6 (CMe<sub>3</sub>), 79.5 (CMe<sub>3</sub>), 69.2 (CH-OTBDPS), 63.3 (CH<sub>2</sub>-Ph), 56.3 (OCH<sub>3</sub>), 51.9 (CH-NH), 38.2 (CH<sub>2</sub>-NH), 37.1 (CH<sub>2</sub>), 36.0 (CH<sub>2</sub>), 28.3 (3CH<sub>3</sub>), 27.8 (3CH<sub>3</sub>), 26.9 (3CH<sub>3</sub>), 19.3 (C-Si). **IR (CHCl<sub>3</sub>):**  $\nu_{\max} = 3018, 1710, 1521, 1215, 669$  cm<sup>-1</sup>. **HRMS (ESI):**  $m/z$ : calcd for C<sub>41</sub>H<sub>58</sub>N<sub>3</sub>O<sub>11</sub>Si: 796.3841; [ $M$ ]<sup>+</sup> found: 796.3835.

**(7R,9S)-tert-butyl-1-(4,5-dimethoxy-2-nitrophenyl)-7-hydroxy-13,13-dimethyl-3,11-dioxo-2,12-dioxo-4,10-diazatetradecane-9-carboxylate (9):**

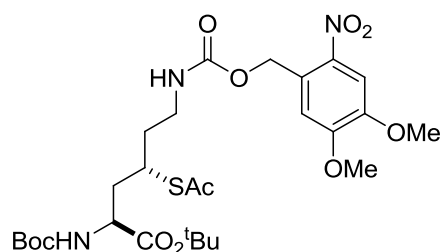


Compound **8** (0.1 g, 0.12 mmol) was dissolved in dry THF (3 mL, 0.04M) and cooled to 0 °C. 1M solution of TBAF (0.19 ml, 0.19 mmol) was introduced via syringe at the same temperature. The reaction mixture was stirred for 10 h at 0 °C and quenched with saturated ammonium chloride solution. Evaporation of the solvent gave a residue, which was dissolved in ethyl acetate. The solution was washed with water, and brine. The organic layer was dried over sodium

sulphate and concentrated *in vacuo*, which was purified by column chromatography on silica gel to give desired product **9** (0.053 g, 75 %) as a colorless oil.

**<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 7.70 (s, 1H, Ph), 7.02 (s, 1H, Ph) 5.71 (bs, 1H, NH), 5.50 (s, 2H, CH<sub>2</sub>-Ph), 5.42 (d,  $J$  = 7.6, 1H, OH), 4.60 (bs, 1H, CH-OH), 4.34 (d,  $J$  = 8.8, 1H, CH-NH), 3.98 (s, 3H, OCH<sub>3</sub>), 3.94 (s, 3H, OCH<sub>3</sub>), 3.71 (bs, 1H, CH<sub>2</sub>-NH), 3.55-3.49 (m, 1H, CH<sub>2</sub>-NH), 3.25-3.24 (m, 1H, CH<sub>2</sub>), 1.63 (d,  $J$  = 11.2, 1H, CH<sub>2</sub>), 1.67-1.65 (m, 2H, CH<sub>2</sub>), 1.46 (s, 9H, 3CH<sub>3</sub>), 1.44 (s, 9H, 3CH<sub>3</sub>). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 171.6 (C=O), 157.0 (C=O), 156.1 (C=O), 153.5 (Ph), 147.9 (Ph), 139.6 (Ph), 128.6 (Ph), 109.9 (Ph), 108.0 (Ph), 82.5 (C), 80.6 (C), 66.8 (CH<sub>2</sub>-Ph), 63.3 (CH-OH), 56.4 (OCH<sub>3</sub>), 56.3 (OCH<sub>3</sub>), 51.0 (CH-NH), 41.9 (CH<sub>2</sub>), 39.3 (CH<sub>2</sub>), 35.7 (3CH<sub>3</sub>), 28.2 (3CH<sub>3</sub>), 27.9 (3CH<sub>3</sub>). **IR (CHCl<sub>3</sub>):**  $\nu_{\max}$  = 3421, 3018, 1710, 1508, 1215, 669 cm<sup>-1</sup>. **HRMS (ESI):**  $m/z$ : calcd for C<sub>25</sub>H<sub>40</sub>N<sub>3</sub>O<sub>11</sub>: 558.2654; [ $M$ ]<sup>+</sup> found: 558.2663.

**(7S,9S)-tert-butyl-7-(acetylthio)-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-3,11-dioxo-2,12-dioxo-4,10-diazatetradecane-9-carboxylate (10) :**

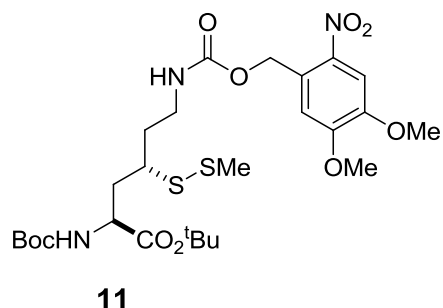


**10**

0.05 mL (0.27 mmol) of diisopropyl ethylamine (DIPEA) was added to a solution of 0.1 g (0.18 mmol) of alcohol in 3 mL (0.06M) of dichloromethane at room temperature. The solution was cooled to 0 °C, and 0.017 mL (0.21 mmol) of methanesulfonyl chloride was added. The reaction

mixture was allowed to reach room temperature and stirred for 2 h. The reaction mixture was quenched with saturated ammonium chloride solution at 0 °C and diluted with dichloromethane. The layers were separated, the aqueous layer was extracted with dichloromethane two times, and the combined organic extract was washed with water and brine. Drying over anhydrous sodium sulphate and removal of solvent under vacuum resulted in a colorless oily residue which was dissolved in DMF (3 mL, 0.05 M). A total of 0.06 g (0.5 mmol) of potassium thioacetate was added to the solution, which was heated to 40 °C for 12h. After allowed to reach room temperature, water was added to the solution, which was extracted twice with ethyl acetate. The combined organic layer was washed with water and brine, dried over sodium sulphate and evaporated. The crude product was purified by column chromatography on silica gel to give desired product **10** (0.077 g, 70 %) as a pale brown oil. **<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):** δ in ppm = 7.66 (s, 1H, Ph), 7.01 (s, 1H, Ph), 5.64 (bs, 1H, NH), 5.51-5.40 (m, 2H, CH<sub>2</sub>-Ph), 5.24-5.22 (m, 1H, NH), 4.23-4.21 (m, 1H, CH-NH), 3.94 (s, 3H, OCH<sub>3</sub>), 3.90 (s, 3H, OCH<sub>3</sub>), 3.63-3.61 (m, 1H, CH-S), 3.37-3.34 (m, 1H, CH<sub>2</sub>-NH), 3.14 (bs, 1H, CH<sub>2</sub>-NH), 2.28 (s, 3H, CH<sub>3</sub>), 2.04-2.01 (m, 2H, CH<sub>2</sub>), 1.84-1.80 (m, 1H, CH<sub>2</sub>), 1.67-1.57 (m, 1H, CH<sub>2</sub>) 1.41 (s, 9H, 3CH<sub>3</sub>), 1.40 (s, 9H, 3CH<sub>3</sub>). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):** δ in ppm = 195.8 (COCH<sub>3</sub>), 171.0 (C=O), 155.9 (C=O), 155.7 (C=O), 153.5 (Ph), 147.9 (Ph), 128.7 (Ph), 109.8 (Ph), 108.0 (Ph), 82.5 (C), 80.1 (C), 63.2 (CH<sub>2</sub>-Ph), 56.4 (OCH<sub>3</sub>), 56.3 (OCH<sub>3</sub>), 52.0 (CH-NH), 39.8 (CH<sub>2</sub>-NH), 38.3 (CH<sub>2</sub>), 38.0 (CH<sub>2</sub>), 33.5 (CH-S), 30.7 (CH<sub>3</sub>) 28.2 (3CH<sub>3</sub>), 27.9 (3CH<sub>3</sub>). **IR (CHCl<sub>3</sub>):** ν<sub>max</sub> = 3427, 3018, 1689, 1521, 1215, 669 cm<sup>-1</sup>. **HRMS (ESI):** m/z: calcd for C<sub>27</sub>H<sub>41</sub>N<sub>3</sub>O<sub>11</sub>SNa: 638.2360; [M+Na]<sup>+</sup> found: 638.2350.

**(7S,9S)-tert-butyl-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-7-(methyldisulfanyl)-3,11-dioxo-2,12-dioxa-4,10-diazatetradecane-9-carboxylate (11) :**



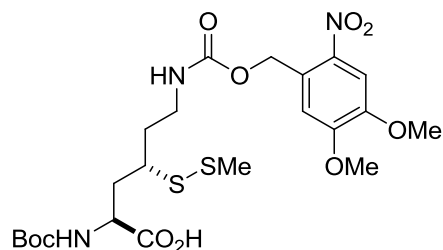
0.020 g of compound **10** was dissolved in 0.6 mL of methanol. 0.175 mL of 1 N NaOH was added. After 30 min at room temperature, the mixture was neutralized carefully with 1 N HCl at 0 °C. The mixture was concentrated and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulphate and evaporated. The residue was dissolved in 0.4 mL of DCM and added dropwise to a mixture containing 0.4 mL of DCM, 0.006 mL of Et<sub>3</sub>N and 0.015 mL of S-methyl methanethiosulfonate (MMTS) while stirring at room temperature. The mixture was continuously stirred for 30 min. The solvent was removed and the residue was dissolved in methanol/H<sub>2</sub>O (3:1) and purified by C18 semi-preparative HPLC followed by lyophilization to give desired product **11** (12 mg) as a white solid.

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):** δ in ppm = 7.59 (s, 1H, Ph), 7.03 (s, 1H, Ph), 6.43-6.42 (m, 1H, NH), 6.27 (d, *J* = 7.6 Hz, 1H, NH), 5.30 (s, 2H, CH<sub>2</sub>-Ph), 4.09-4.04 (m, 1H, CH-NH), 3.80 (s, 3H, OCH<sub>3</sub>), 3.76 (s, 3H, OCH<sub>3</sub>), 3.47 (bs, 1H, CH-S), 3.21-3.05 (m, 1H, CH<sub>2</sub>-NH), 3.02-2.97 (m, 1H, CH), 2.17 (s, 3H, CH<sub>3</sub>), 2.00-1.85 (m, 2H, CH<sub>2</sub>), 1.74-1.62 (m, 2H, CH<sub>2</sub>) 1.32 (s, 9H, 3CH<sub>3</sub>), 1.31 (s, 9H, 3CH<sub>3</sub>).

**<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):** δ in ppm = 193.9, 171.0 (C=O), 155.5 (C=O), 153.8 (C=O), 148.3 (Ph), 139.7 (Ph), 128.5 (Ph), 110.1 (Ph), 107.9 (Ph), 80.6 (C), 78.2 (C), 62.4 (CH<sub>2</sub>-Ph), 55.5 (OCH<sub>3</sub>), 55.4 (OCH<sub>3</sub>), 52.1 (CH-NH), 33.8 (CH<sub>3</sub>-S), 29.5 (CH<sub>2</sub>), 27.6

(3CH<sub>3</sub>), 27.1 (3CH<sub>3</sub>), 24.8 (CH-S). **IR (CHCl<sub>3</sub>):**  $\nu_{\max}$  = 3423, 3018, 1215, 758, 669 cm<sup>-1</sup>. **HRMS (ESI):**  $m/z$ : calcd for C<sub>26</sub>H<sub>41</sub>N<sub>3</sub>O<sub>10</sub>S<sub>2</sub>Na: 642.2131; [ $M+Na$ ]<sup>+</sup> found: 642.2121.

**(7S,9S)-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-7-(methylsulfanyl)-3,11-dioxo-2,12-dioxo-4,10-diazatetradecane-9-carboxylic acid (12) :**



**12**

The white powder of **5** was dissolved in 0.5 mL 95% TFA. After 1.5 h at room temperature, the TFA was removed by evaporation. The residue was dissolved in 1 mL methanol/H<sub>2</sub>O mixture (3:1). Adjust pH to about 8 with Et<sub>3</sub>N. 0.02 mL of Boc<sub>2</sub>O was added. After 3 h at room temperature, the sample was subjected to C18 semi-preparative HPLC. After lyophilization 8 mg of **12** was isolated. **<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 10.72 (bs, 1H, COOH), 7.58 (s, 1H, Ph), 7.02 (s, 1H, Ph), 6.54 (bs, 1H, NH), 5.34 (d,  $J$  = 7.2 Hz, 1H, NH), 5.34-5.25 (m, 2H, CH<sub>2</sub>-Ph), 4.25-4.21 (m, 1H, CH-NH), 3.79 (s, 3H, OCH<sub>3</sub>), 3.75 (s, 3H, OCH<sub>3</sub>), 3.28-3.22 (m, 1H, CH<sub>2</sub>), 3.15-3.06 (m, 1H, CH), 2.78-2.76 (m, 1H), 2.29 (s, 3H, CH<sub>3</sub>), 2.29-1.87 (m, 1H, CH<sub>2</sub>), 1.85-1.83 (m, 2H, CH<sub>2</sub>), 1.73-1.61 (m, 1H, CH<sub>2</sub>), 1.32 (s, 9H, 3CH<sub>3</sub>). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 172.8 (C=O), 155.6 (C=O), 153.8 (C=O), 148.3 (Ph), 139.7 (Ph), 128.2 (Ph), 128.5 (Ph), 110.1 (Ph), 107.9 (Ph), 78.2 (C), 62.4 (CH<sub>2</sub>-Ph), 55.5, 55.4, 51.1 (CH-NH), 45.3 (CH<sub>2</sub>-NH), 38.5 (CH<sub>3</sub>-S), 32.8 (CH<sub>2</sub>), 27.6 (3CH<sub>3</sub>), 23.2 (CH-S). **IR (CHCl<sub>3</sub>):**  $\nu_{\max}$  =

3444, 3427, 3018, 1708, 1635, 1215  $\text{cm}^{-1}$ . **HRMS (ESI):**  $m/z$ : calcd for  $\text{C}_{22}\text{H}_{34}\text{N}_3\text{O}_{10}\text{S}_2$ : 564.1686;  $[M]^+$ ; found 564.1678.

### Solid phase peptide synthesis:

Synthesis of Ub(L1-K27)-COSCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> (**18**) and Ub(Thz28-G47)-COSCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> (**14**). **18** and **14** were synthesized manually employing standard *tert*-Butyloxycarbonyl (Boc) chemistry. The synthesis was started with 0.5 g of MBHA resin (0.9 mmol/g). *S*-trityl mercaptopropanoic acid (4 eq. PyBOP, 4 eq. acid, 12 eq. DIEA, preactivated in DCM for 2 min) was coupled onto the resin and served as the thiol linker. Trityl group with removed by repeated treatment with TFA/ $\beta$ -mercaptoethanol/TIS/DCM (5/2.5/2.5/90). The amino acids were then coupled one by one in a way similar to the loading of *S*-trityl mercaptopropanoic acid. The coupling was monitored with ninhydrin test. The amino acid derivatives used were Boc-Lys(2Cl-Z)-OH, Boc-Asn(Xan)-OH, Boc-Glu(OcHx)-OH, Boc-Thr(Bzl)-OH, Boc-Asp(OcHx)-OH, Boc-Ser(Bzl)-OH, Boc-Gln(Trt)-OH, Boc-Arg(Tos)-OH, Boc-Thz-OH. After sequence assembly, the Boc group was removed by treated with 30 % TFA before cleavage. The peptide thioesters were cleaved by HF/*p*-cresol/anisole/ (9:0.5:0.5) for 1 h at 0 °C. The crude was harvested by ether precipitation and centrifugation. The peptides were purified by C18 preparative HPLC. The desired products were characterized with C18 analytic HPLC and ESI-MS.

Synthesis of K48(4-SSMe, NVOC)-G76-OH (**15**). The peptide was synthesized manually employing standard fluorenylmethyloxycarbonyl (Fmoc) chemistry started with 1 g of Wang resin (0.44 mmol/g). The C-terminal Gly was loaded by using 8 eq. DCC, 0.8 eq DMAP, 8 eq. Fmoc-Gly-OH in dry DCM/DMF overnight. The loading was repeated for another 5 h and the

resin was then capped with Ac<sub>2</sub>O for 1 h. Fmoc group was removed with 20 % piperidine in DMF. The following amino acids were coupled using 4 eq. PyBOP, 4. eq. amino acid, 8 eq. DIEA preactivated in DMF. On average, each coupling reaction lasted for 1.5 h. The coupling was monitored with ninhydrin test. The *N*α-Fmoc protected amino acids used were Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH. The Gly at the AspGly junction was coupled using Fmoc-(Hmb)Gly-OH (2 eq. PyBOP, 2 eq. amino acid, 4 eq. DIEA). The N-terminal Lys was coupled using Boc-Lys(4-SSMe, NVOC)-OH (**12**). For the coupling of **12**, 40 mg of **12**, 10.6 mg of HOBt and 16 mg of DCC were dissolved in minimum amount of dry DCM/DMFA. The mixture was reacted with 300 mg of peptide resin for 3 h. After sequence assembly, the resin was treated with 20 % piperidine in DMF for 20 min to hydrolyze any possible acylation at hydroxyl group of Hmb group of (Hmb)Gly residue. The resin was then cleaved with TFA/TIS/H<sub>2</sub>O (95/2.5/2.5) for 2.5 h. The crude peptide was harvested by ether precipitation and purified with C18 preparative HPLC. The desired product was analyzed with C18 analytic HPLC and ESI-MS.

### **Preparation of Ub(1-76)-MES :**

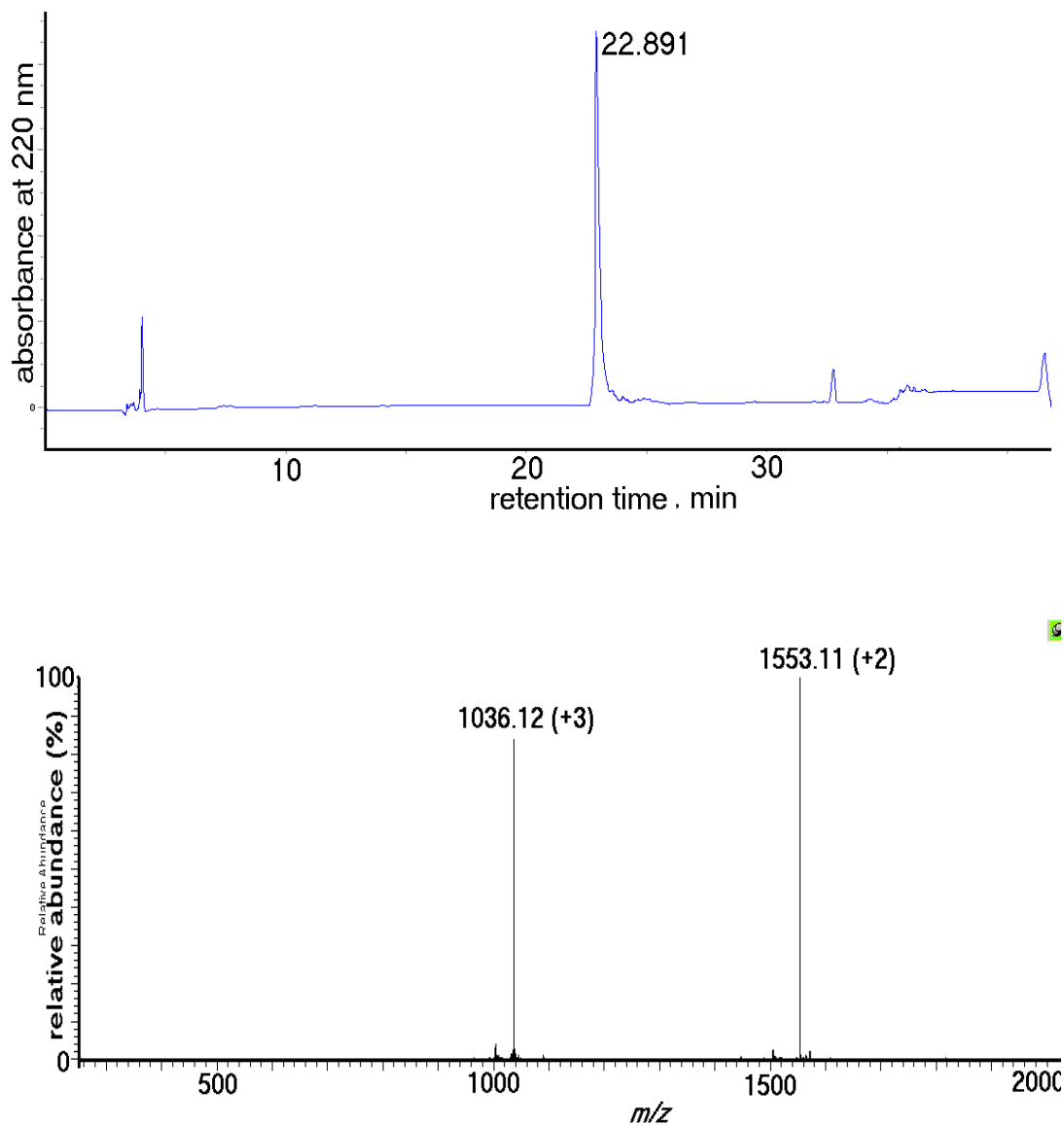
The construction of ubiquitin-intein fusion protein expression plasmid pTYB1-Ubi, the expression and MESNa hydrothiolysis of ubiquitin-intein were the same as we reported in the previous work. The purified product was analyzed with C4 analytic HPLC and ESI-MS.

**Free radical mediated desulfurization:**

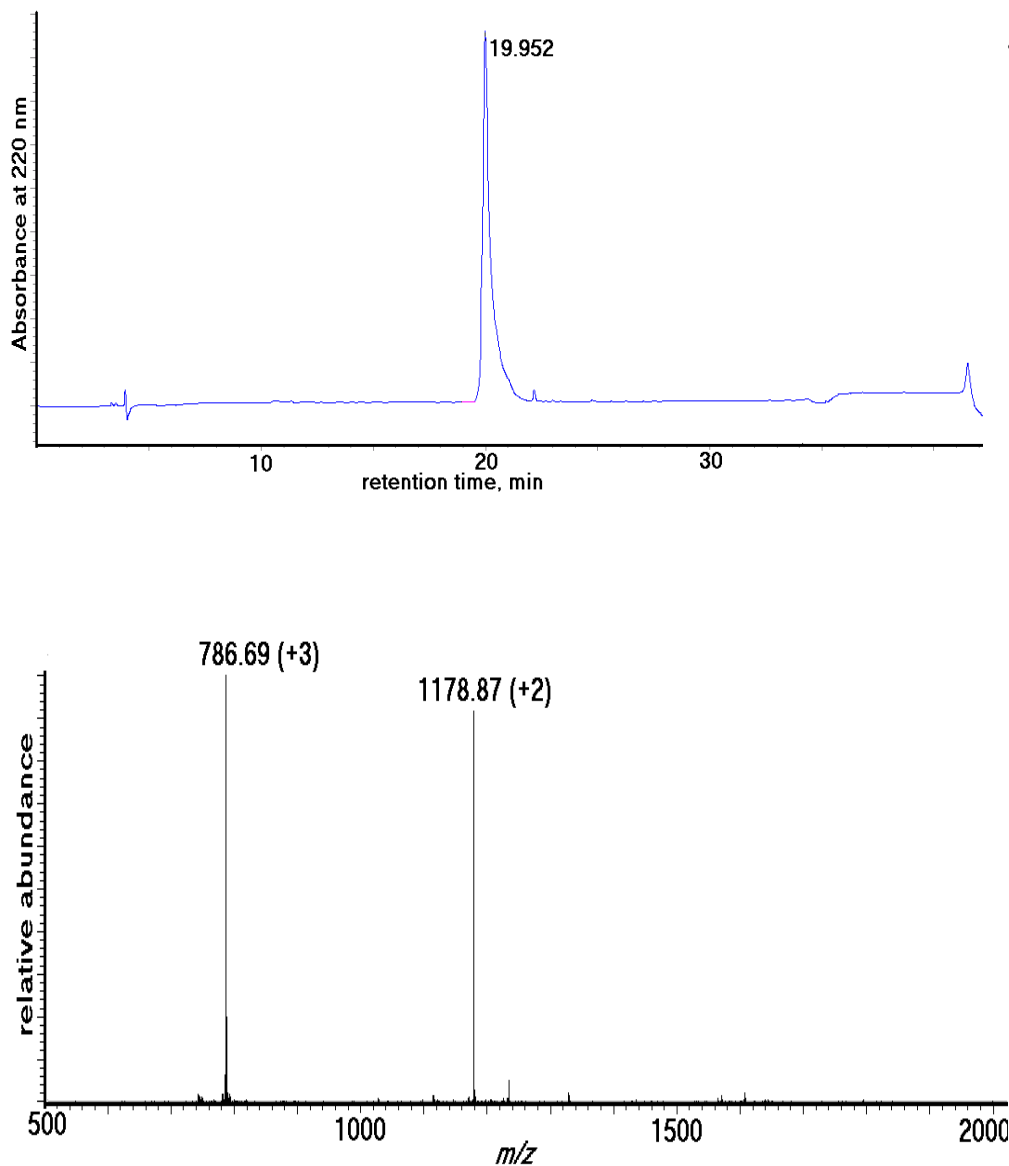
The desulfurization was performed under N<sub>2</sub>. All the solutions were prepared under N<sub>2</sub> immediately before use. 0.8 mg of sulfur containing diubiquitin was dissolved in 300 μL buffer containing 6 M Gdn•HCl, 0.1 M phosphate, pH 6.5. 100 μL of 0.5 M TCEP solution (neutralized with NaOH) was added. 25 μL of 10 mM glutathione was added. 10 μL of 0.2M VA-044 was added. The solution was stirred at 37 °C for 6 h. 10 μL of 0.2M VA-044 was added to the mixture and the solution was continuously stirred for another 3 h. The final K48-linked diubiquitin (**22**) was purified by C18 semi-preparative HPLC.

**Western blot analysis of K48-linked diubiquitin (22):**

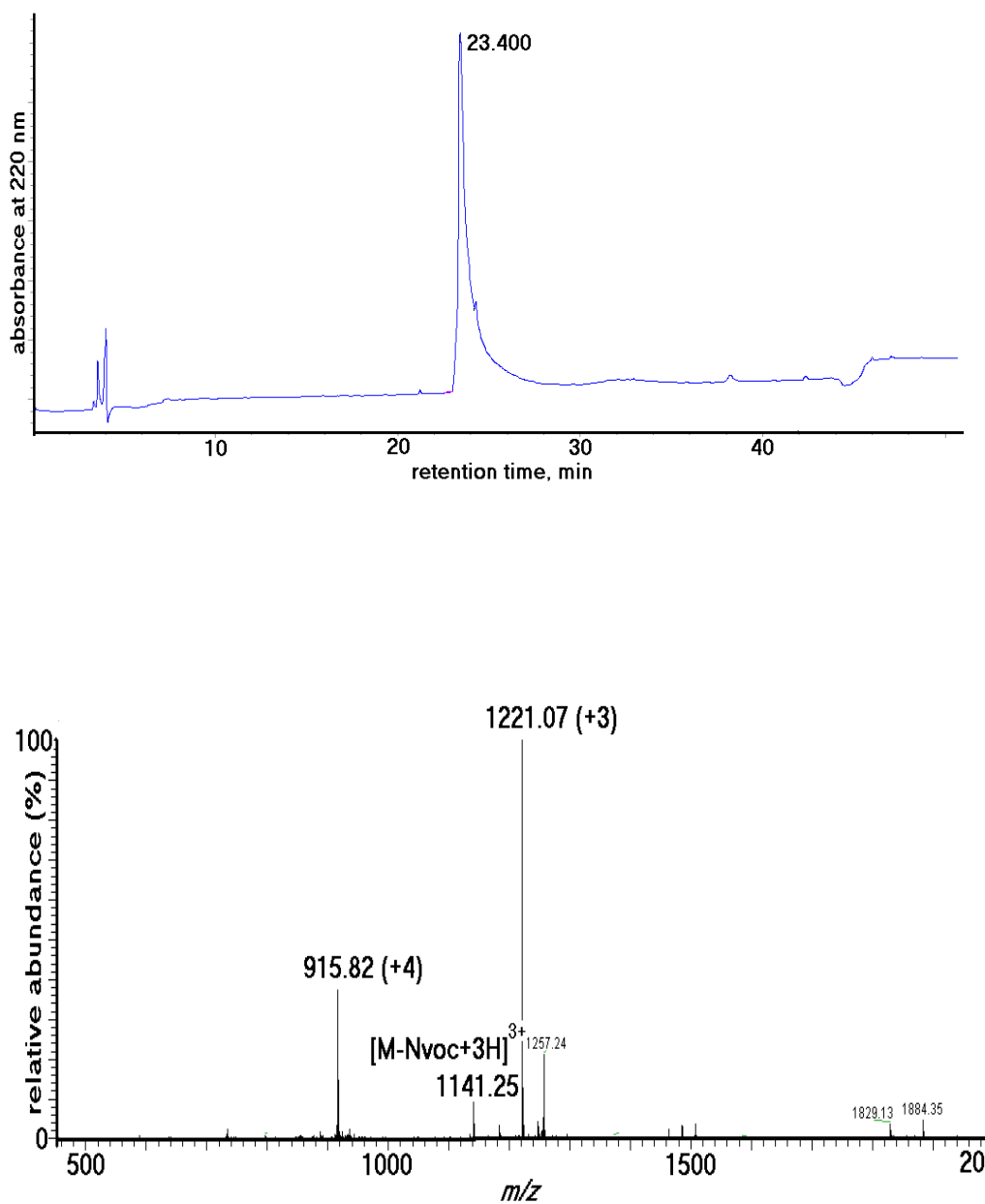
**22** was dissolved in 8 M urea and analyzed with 15 % SDS-PAGE. The samples on the gel were then electrotransferred to PVDF (polyvinylidene difluoride) membrane. The membrane was blocked by 5 % w/v non-fat milk powder in TBS buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.1 % Tween-20. A 1: 3000 diluted FK2H was used to detect **13** as visualized by chemilluminescence (SuperSignal West Dura Trial Kit, Pierce, USA).

**Ub(L1-K27)-COSCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> (18)**

**Figure 1** C18 analytic HPLC and ESI-MS of purified Ub(L1-K27)-COSCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> (18). HPLC gradient: 0-60% B in 30 min. Calculated  $[M+H]^+ = 3105.6$ , found  $[M+2H]^{2+} = 1553.11$ ,  $[M+3H]^{3+} = 1036.12$ .

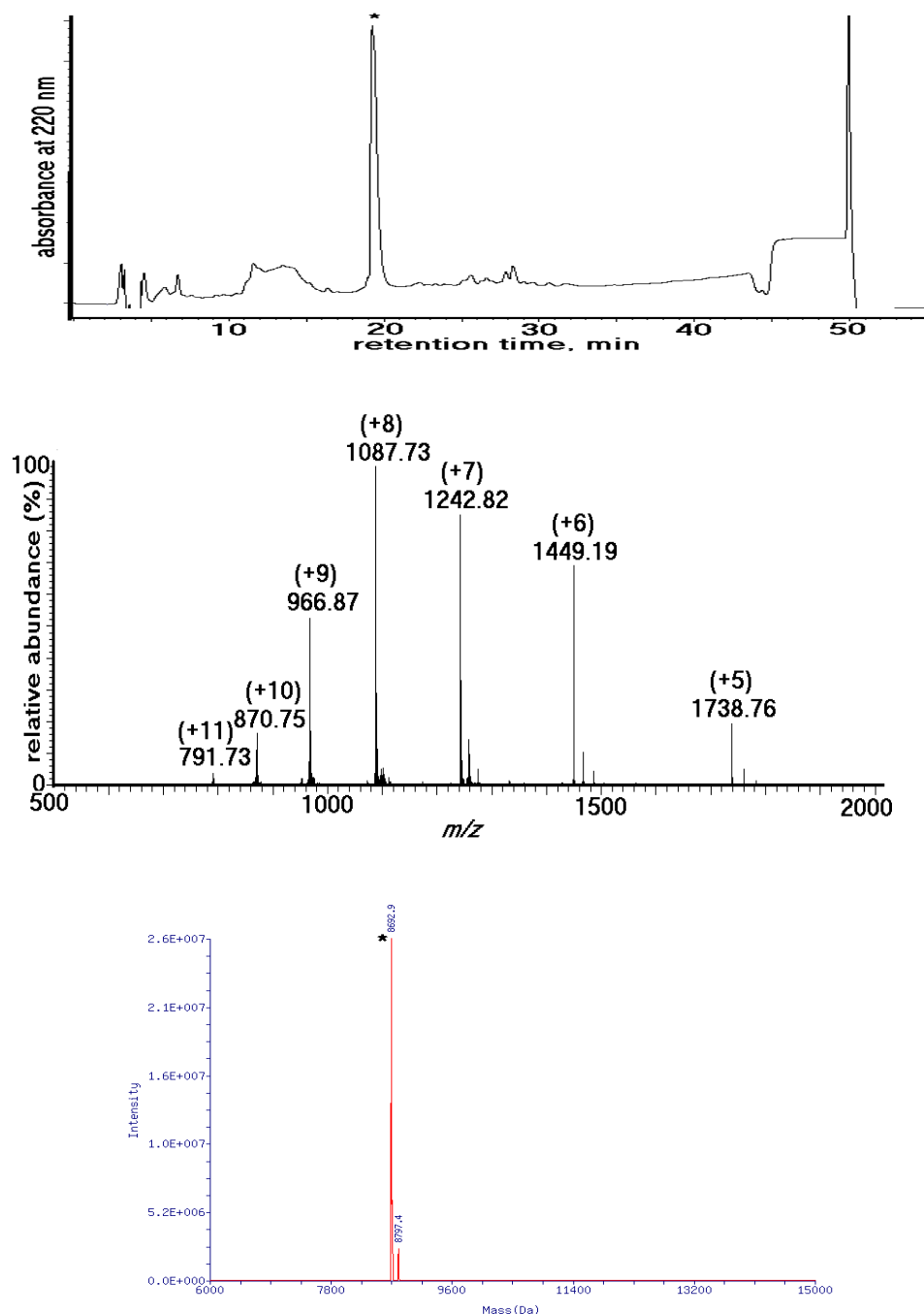
**Ub(Thz28-G47)-COSCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> (16):**

**Figure 2** C18 analytic HPLC and ESI-MS of purified Ub(Thz28-G47)-COSCH<sub>2</sub>CH<sub>2</sub>CONH<sub>2</sub> (16). HPLC gradient: 0-60% B in 30 min. Calculated  $[M+H]^+ = 2356.7$ , found  $[M+2H]^{2+} = 1178.87$ ,  $[M+3H]^{3+} = 786.69$ .

**K48(4-SSMe, NVOC)-G76-OH (17):**

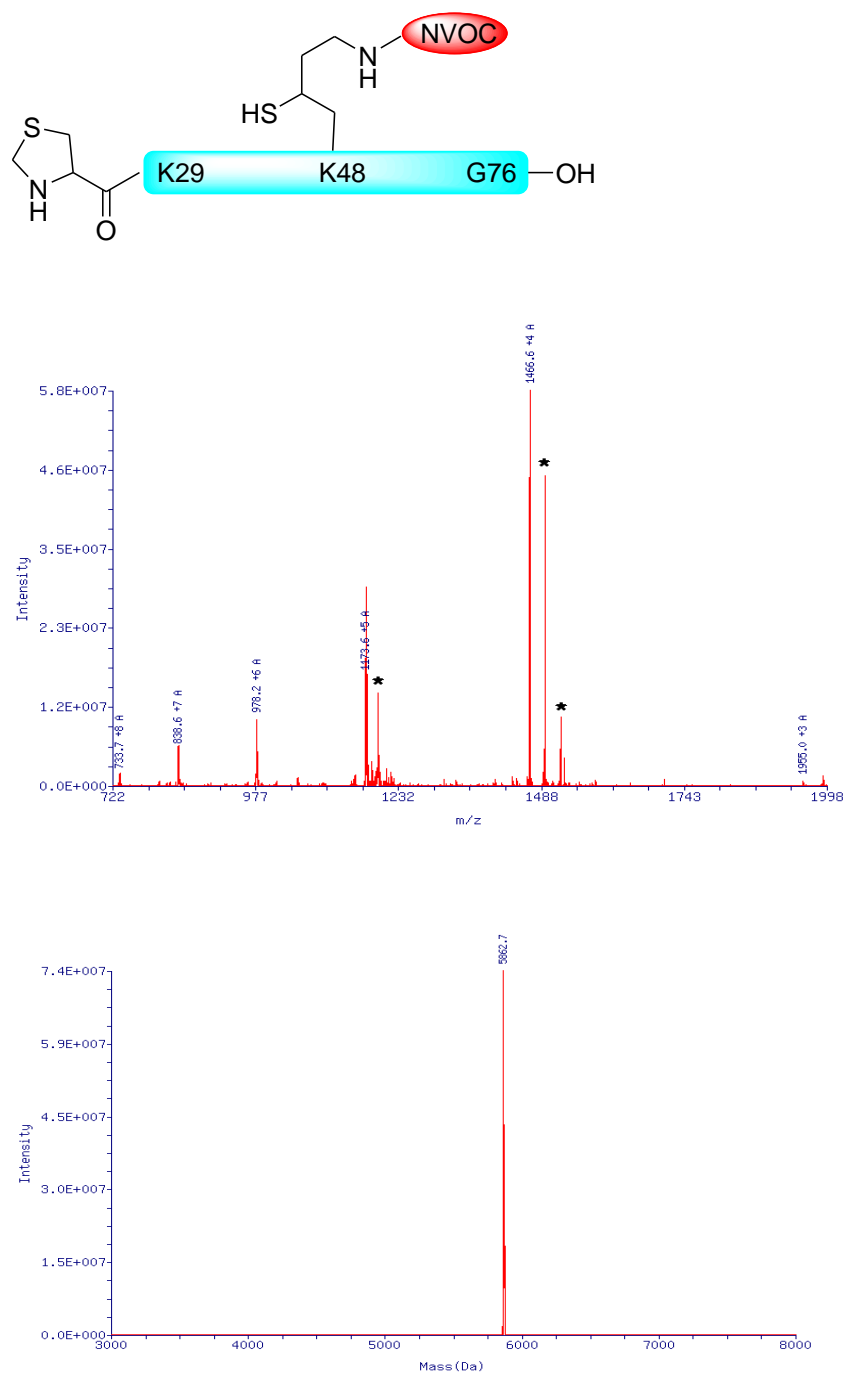
**Figure 3** C18 analytic HPLC and ESI-MS of purified K48(4-SSMe, NVOC)-G76-OH (17). HPLC gradient: 0-80% B in 40 min. Calculated  $[M+H]^+ = 3659.0$ , found  $[M+3H]^{3+} = 1221.07$ ,  $[M+4H]^{4+} = 915.82$ ,  $[M-NVOC+3H]^{3+} = 1141.25$ . The peak with MS of 1257.24 is the artificial adduct resulted from ESI-MS.

## Ub(1-76)-MES:

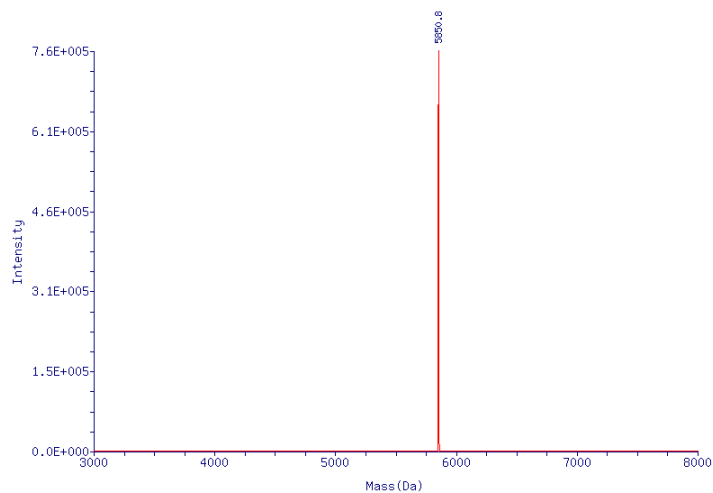
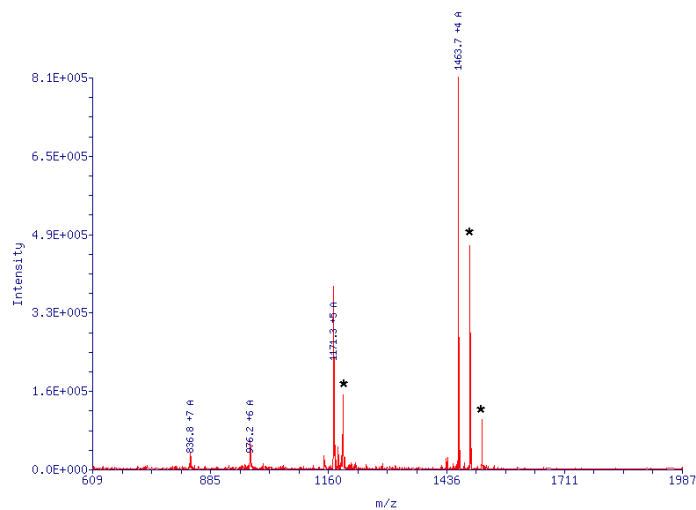
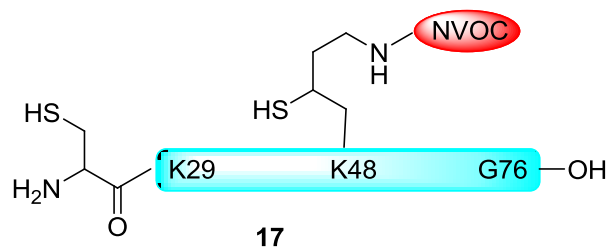


**Figure 4** C4 analytic HPLC and ESI-MS of purified Ub(1-76)-MES. HPLC gradient: 0-80% B in 40 min. “\*” indicates the desired product. Calculated M = 8689.1 Da, deconvoluted M = 8692.9 Da. The 8792.4 peak is an artificial adduct resulted from ESI-MS.

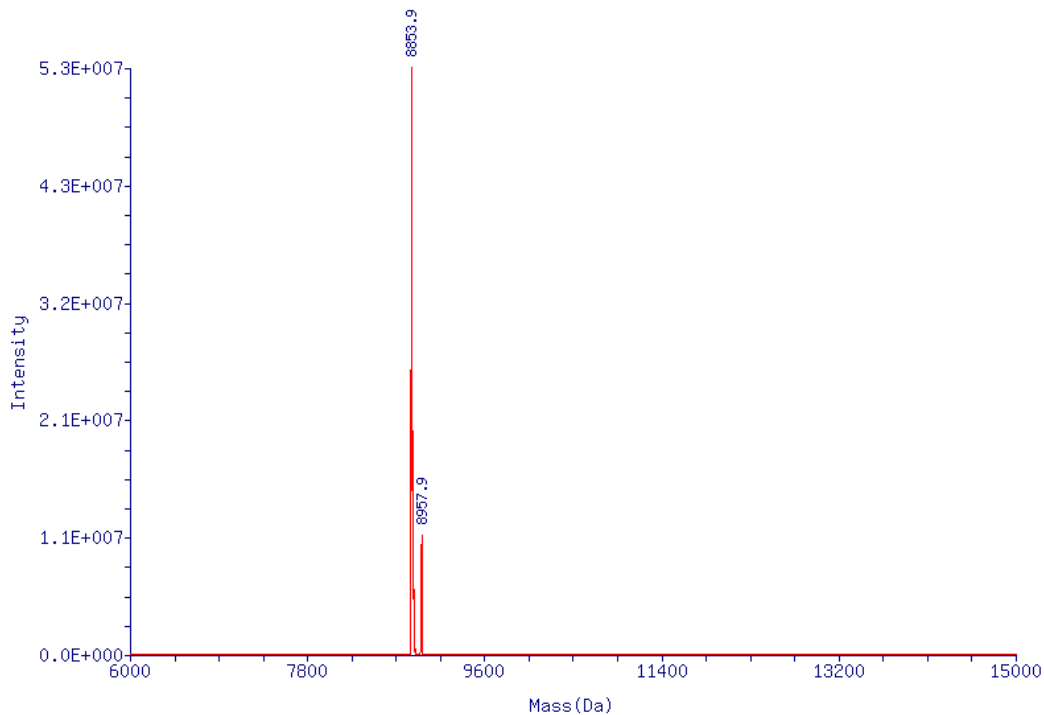
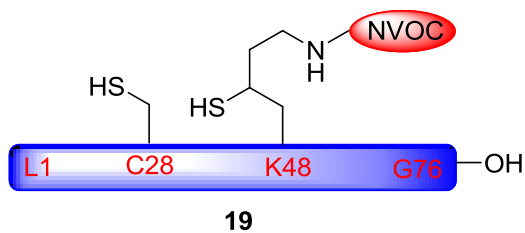
ESI-MS of some key intermediates during the synthesis of **16**:



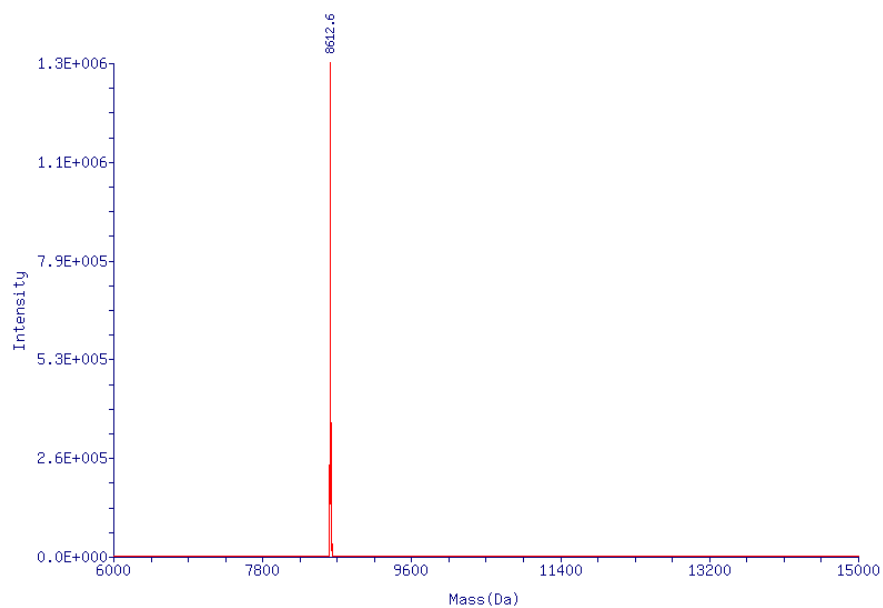
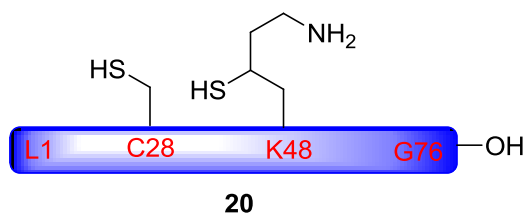
**Figure 5** ESI-MS of Thz28-K48(4-SH, NVOC)-G76-OH, the ligation product of **14** and **15**. calculated M = 5862.6 Da, deconvoluted M = 5862.7 Da. The peaks with “\*” are the artificial adducts resulted from ESI-MS.



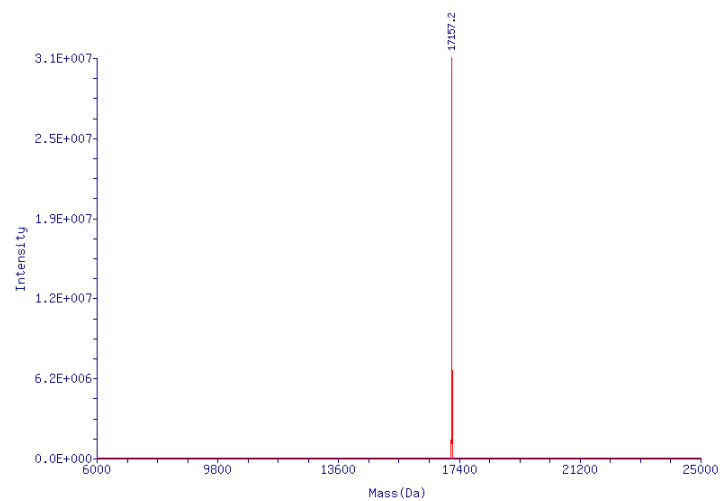
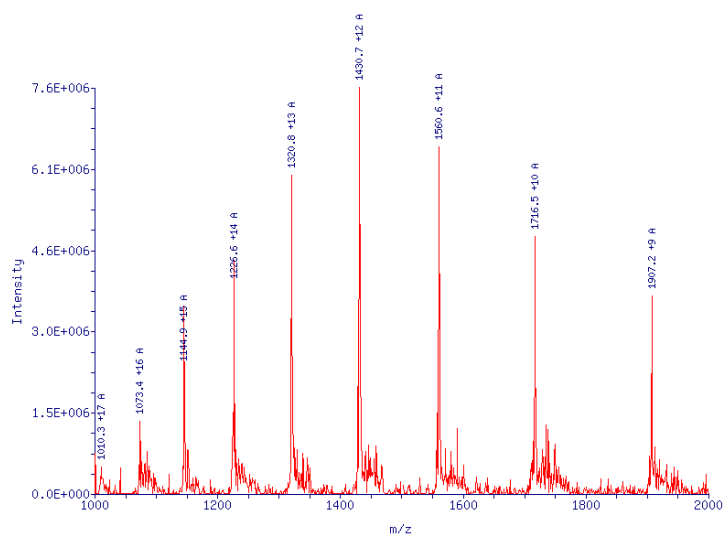
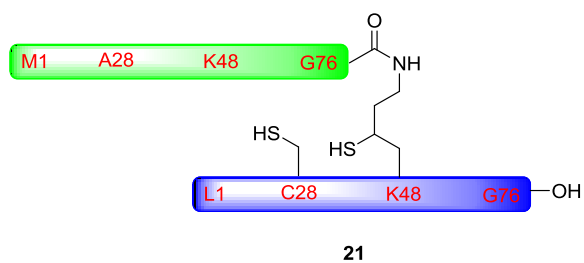
**Figure 6** ESI-MS of C28-K48(4-SH, NVOC)-G76-OH (**17**), the Thz deprotection product. Calculated M = 5850.6 Da, deconvoluted M = 5850.8 Da. The peak with “\*” are the artificial adducts resulted from ESI-MS.



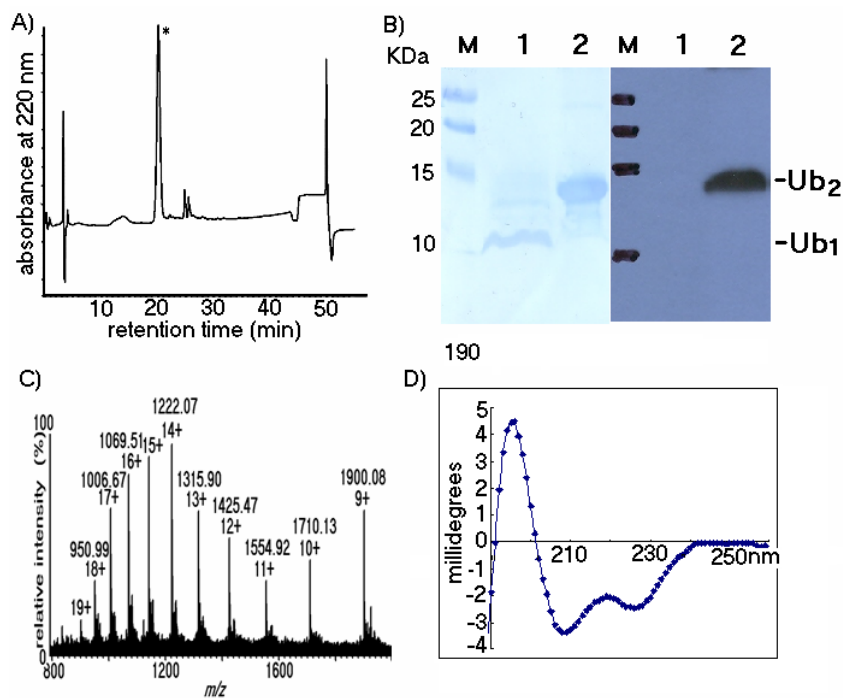
**Figure 7** The deconvoluted ESI-MS profile of **19**. Calculated M = 8850.1 Da, deconvoluted M = 8853.9 Da. The peak with mass of 8957.9 Da is the artificial adduct resulted from ESI-MS. The similar adduct has been detected in Ub(1-76)-MES as well as other intermediates. The ESI-MS before deconvolution was shown in main text.



**Figure 8** The deconvoluted ESI-MS profile of **20**. Calculated M = 8610.9 Da, deconvoluted M= 8612.6 Da.



**Figure 9** ESI-MS of sulfur containing diubiquitin (before desulfurization). Calculated  $M = 17157.8$  Da, deconvoluted  $M = 17157.2$  Da.



**Figure 10** Characterization of the synthesized K48-linked diubiquitin. A) C4 analytic HPLC. B) 18% SDS-PAGE analysis stained with coomassie blue (left) and western blot (right). M, protein marker; Lane 1, monoubiquitin; Lane 2, K48-linked diubiquitin **22**. C) ESI-MS profile. MW calculated 17093.4 Da, found 17094.8 Da. D) CD spectrum of folded **22**.

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# **CHAPTER 3**

**Native chemical ligation on aspartic acid (Asp):  
Application towards on-resin convergent synthesis of  
N-linked glycopeptides**

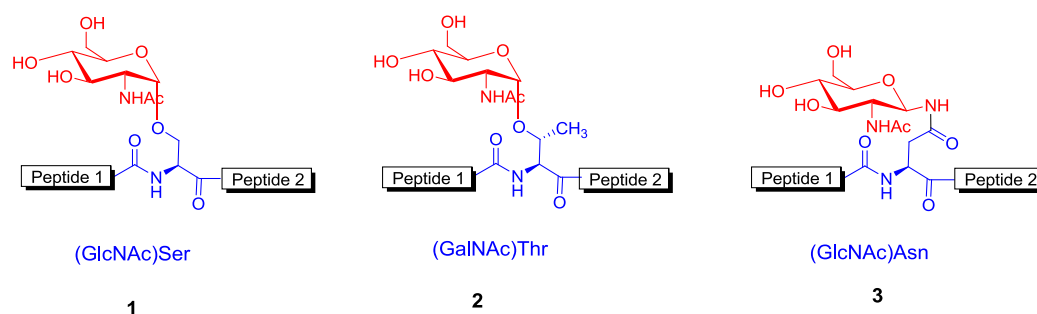
## 1. INTRODUCTION

Native chemical ligation (NCL), developed by Dawson and Kent in 1994, allows the combination of two unprotected amino acids in aqueous media.<sup>1</sup> This phenomenal discovery sets new direction for bioactive peptides and proteins to be assembled in an efficient way. In addition, considerable efforts have been made to extend the generality and applicability of NCL on various amino acid residues including alanine (Ala),<sup>2</sup> phenylalanine (Phe),<sup>3</sup> valine (Val),<sup>4,5</sup> lysine (Lys),<sup>6,7</sup> leucine (Leu)<sup>8,9</sup> and threonine (Thr).<sup>10</sup> Based on these previous approaches, non-cysteine residues are synthesized and successfully applied in peptide and protein chemistry. In contrast to the earlier findings, there have been no reports available on NCL at aspartic acid (Asp). Previously, our group has applied NCL concept at lysine and successfully synthesize complex ubiquitin and diubiquitin proteins.<sup>6,11</sup> Based on our previous investigations, we hope to extend NCL strategy on aspartic acid (Asp) residue which could be utilized in *N*-linked glycopeptide synthesis. *N*-linked glycopeptides and their analogues are useful building blocks for glycopeptide based vaccines (HIV-1 entry inhibitor) and other therapeutics.<sup>12,13</sup>

Protein glycosylation is a ubiquitous yet the most complex post-translational modifications that usually present at Asn, Ser and Thr residues. The significance of glycoproteins in biological system is well-recognized due to their inherent complexity and variability of oligosaccharides which allows the introduction of enormous structural diversity to proteins.<sup>14-16</sup> These carbohydrate chains introduced from glycosylation play an essential role in various biological activities such as protein folding, cell adhesion, cell differentiation, cell

growth and tumor metastasis.<sup>17-20</sup> Consequently, with the development of glycopeptide-based vaccines, diagnostics and therapeutics has gained enormous interest and as a result, has further stimulated the studies of glycopeptide synthesis.<sup>12,13</sup>

Glycoproteins are naturally presented as mixtures of glycoforms that possess the same polypeptide backbone but differ in both the nature and site of glycosylation. The isolation of homogeneous glycopeptides from natural sources is virtually impossible. As a result, numerous strategies have been developed for the chemical synthesis of both *O*-linked (Ser **1**, or Tyr **2**  $\alpha$ -glycosides) and *N*-linked (**3**, Asn  $\beta$ -glycosides) homogeneous glycopeptides over the past decades (Figure 3.1). Compared to *O*-glycopeptides, which are mostly prepared by the introduction of glycosylated amino acids during solid phase peptide synthesis (SPPS), *N*-glycopeptides can be prepared in several other approaches with each strategy having its own advantages and drawbacks.

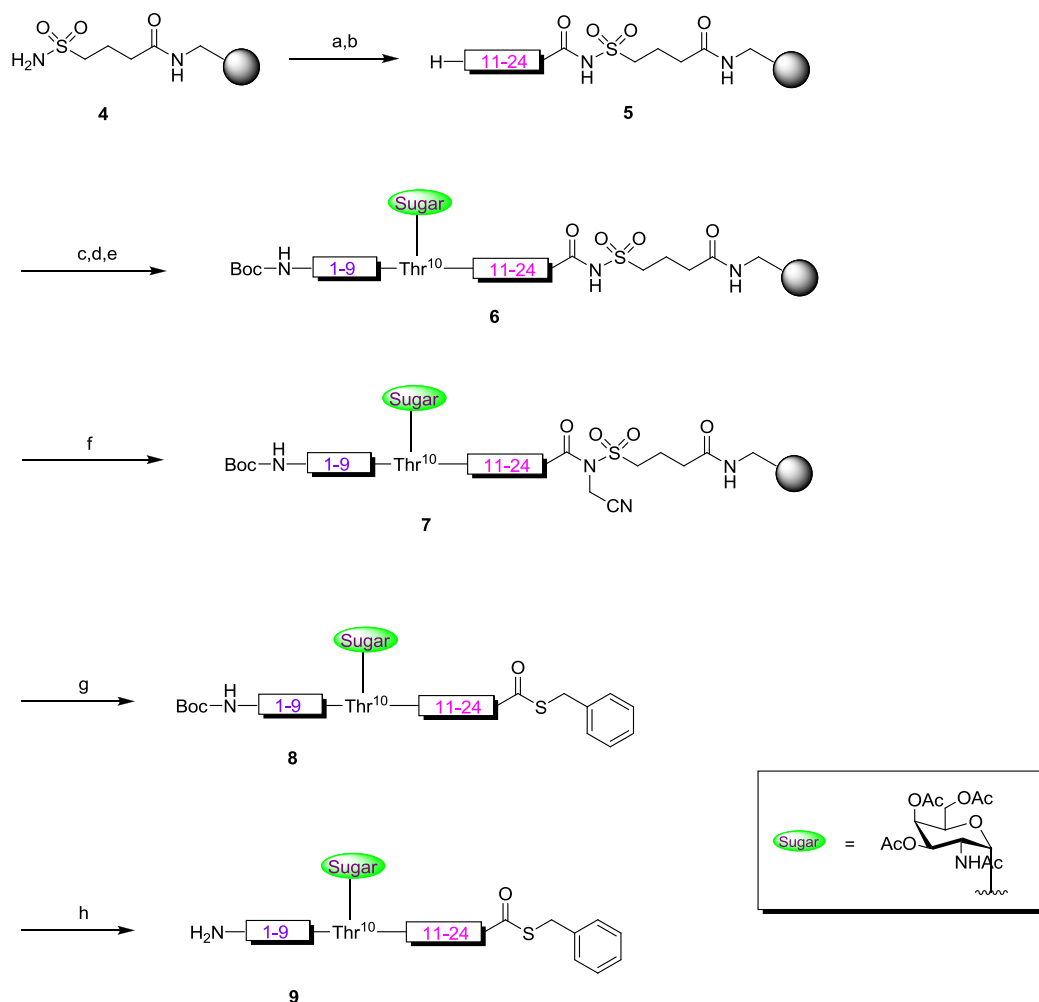


**Figure 3.1** Common *O*-linked and *N*-linked glycosides in protein glycosylation.

### 1.1 Bertozzi's *O*-linked glycoprotein (diptericin) synthesis by native chemical ligation

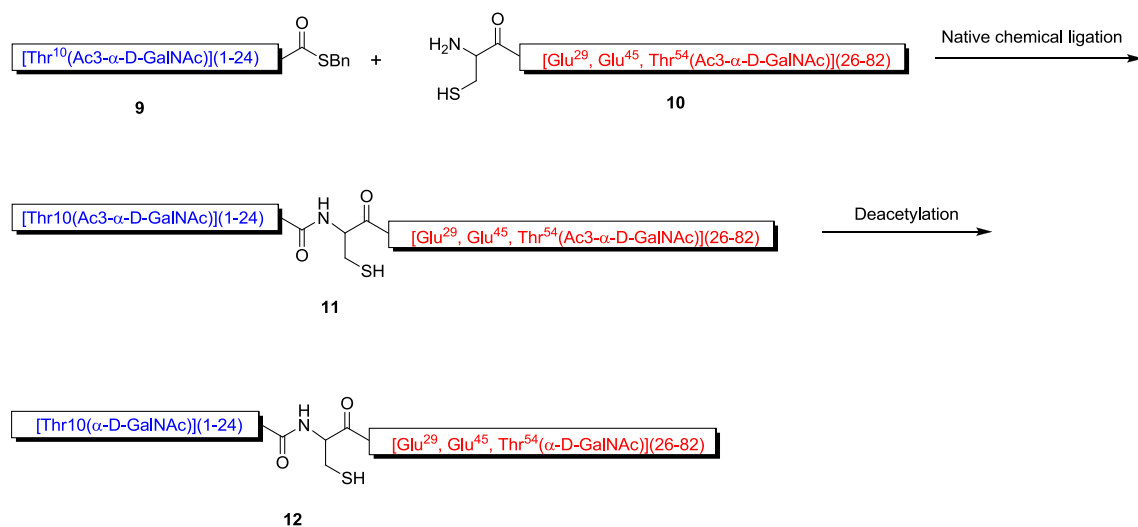
Till date, several research groups have exploited novel methodologies for the synthesis of *O*-linked and *N*-linked glycosides by using ligation techniques. Firstly, Bertozzi and co-workers extended the synthesis of *O*-linked glycopeptide by applying NCL technique.<sup>21</sup> Adopting this strategy, they first achieved the total chemical synthesis of diptericin, an 82 residue antimicrobial glycoprotein of insects. However, the major challenge in their synthesis was the preparation of glycopeptide- $\alpha$ thioester, which was complicated due to the incompatibility of sugar linkage with Boc chemistry and glycopeptide- $\alpha$ thioesters with Fmoc chemistry. Fortunately, this problem was resolved by the development of a modified Kenner sulfonamide safety-catch linker, reported by Backes and Ellman.<sup>22</sup>

The synthesis of benzyl glycopeptide- $\alpha$ thioester [(1-24)- $\alpha$ COSBn] **9**, using the sulfonamide safety-catch linker was depicted in Scheme 3.1. Firstly, *O*-linked sugar installed in peptide chain **6** was obtained by reacting with resin **4**, using standard Fmoc and Boc chemistry. Consequently, peptide containing resin **6** was converted to **7** by using alkylation by iodoacetonitrile. Lastly, the resin **7** was treated with benzylmercaptan followed by Boc deprotection to yield the desired glycosylated peptide thioester **9**.



**Scheme 3.1** Synthesis of the glycopeptide-<sup>α</sup>thioester using the sulfonamide “safety-catch linker”.

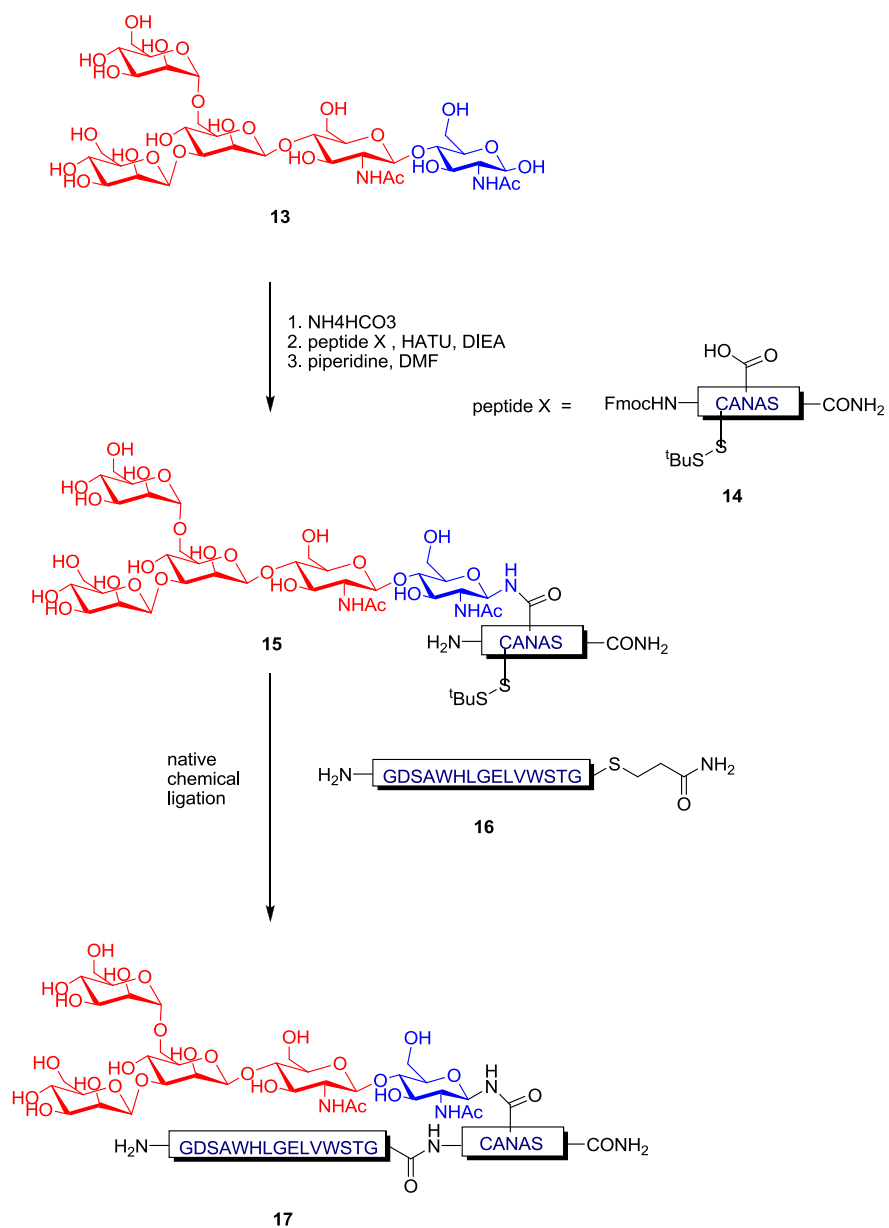
This successful total chemical synthesis of diptercin was summarized in Scheme 3.2. Peptide thioester **9** was treated with cysteine rich peptide **10** under standardized conditions to get the ligation product **11** which was followed by deacetylation by hydrazine to produce the diptercin with two GalNAcs **12**.



**Scheme 3.2** Total chemical synthesis of diptercin using native chemical ligation.

## 1.2 *N*-glycoprotein synthesis by using native chemical ligation

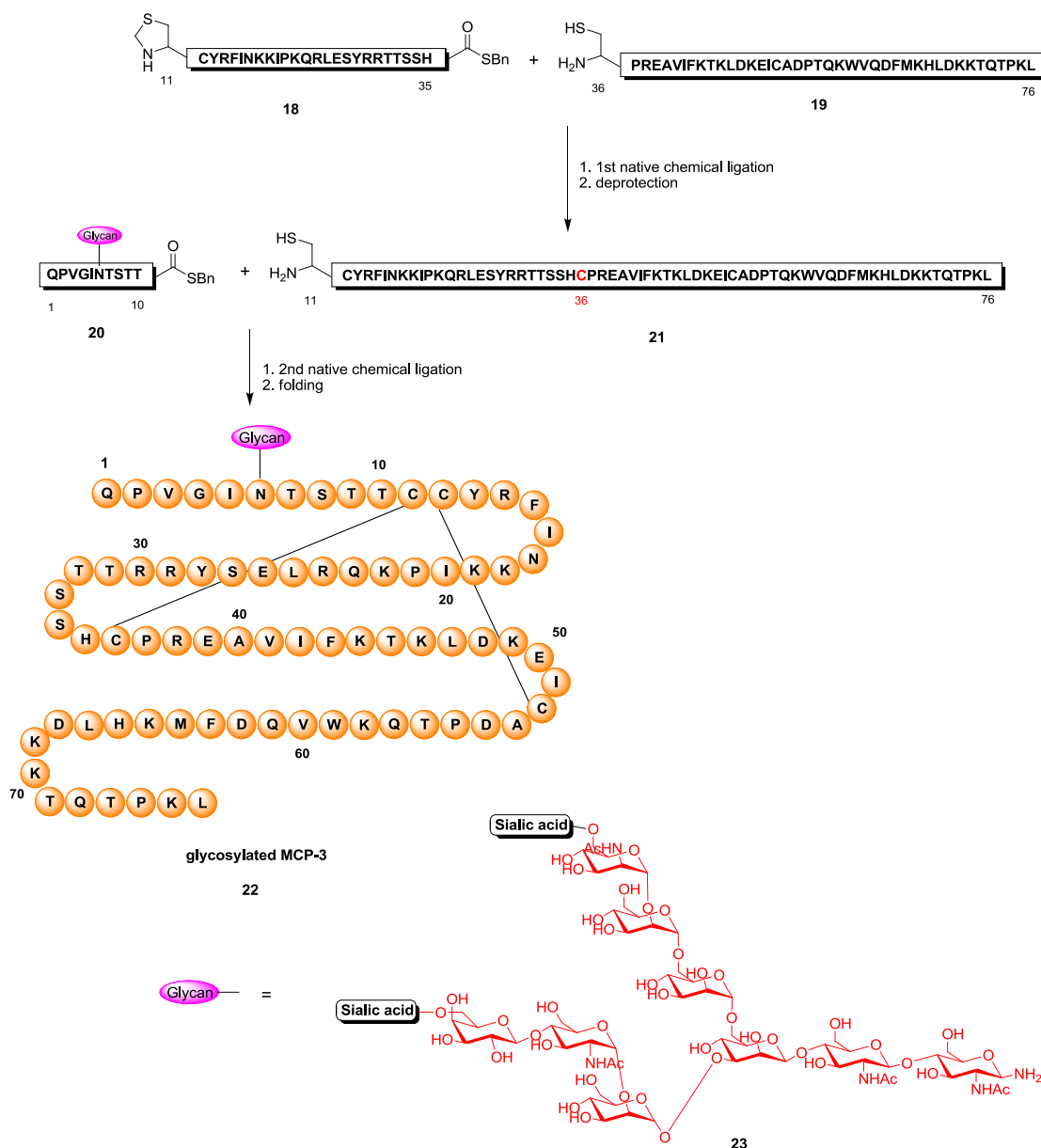
Danishefsky and co-workers reported a highly efficient synthesis of *N*-linked glycoprotein adopting native chemical ligation.<sup>23</sup> In their work, they prepared a GP120 residue of HIV carrying high mannose type glycan to generate efficient HIV vaccines.<sup>24</sup> This high mannose type glycopeptide was synthesized by the selective introduction of unprotected synthetic carbohydrates on the side chain carboxylic acid of a peptide. This highly efficient synthetic protocol for glycoprotein synthesis was summarized in Scheme 3.3. Pentasaccharide core **13** a key precursor for glycopeptide synthesis, was prepared by chemical synthesis. Amination of sugar **13** followed by coupling with Fmoc-protected peptide **14**, further removal of Fmoc-protecting group gives the pentasaccharide glycopeptide **15** as a single isomer. Further, NCL of glycopeptide **15** with peptide thioester **16** afforded the desired glycopeptide **17** in excellent yields.



**Scheme 3.3** Danishefsky's high mannose type glycoprotein synthesis.

### 1.3 Chemical synthesis of monocyte chemotactic protein-3 (MCP-3)

With the help of NCL technique, Dawson and Kajihara groups synthesized a single glycoform of monocyte protein-3 (MCP-3), which contains 76 amino acid residues and one sialyloligosaccharide.<sup>25</sup> In this synthesis, the main feature includes a three-segment NCL strategy using 3 peptide fragments. While for the synthesis of sialylglycopeptide- $\alpha$ thioester, they successfully used Fmoc and Boc-protection from the SPPS. This efficient synthesis of glycoprotein **22** was described in Scheme 3.4. In this scheme, glycoprotein MCP-3 was synthesized from three peptide fragments, **20**(1-10), **18**(11-35), **19**(36-76) using NCL strategy. Firstly, sialylglycopeptide- $\alpha$ thioester **20** was synthesized by using Boc chemistry. Next, the protected peptide fragment **18** was treated with *N*-terminal cysteine containing peptide fragment **19** to afford ligation product **21** under standard NCL conditions. Lastly, the second NCL reaction performed between segments **20** and **21** to afford glycosylated MCP-3.

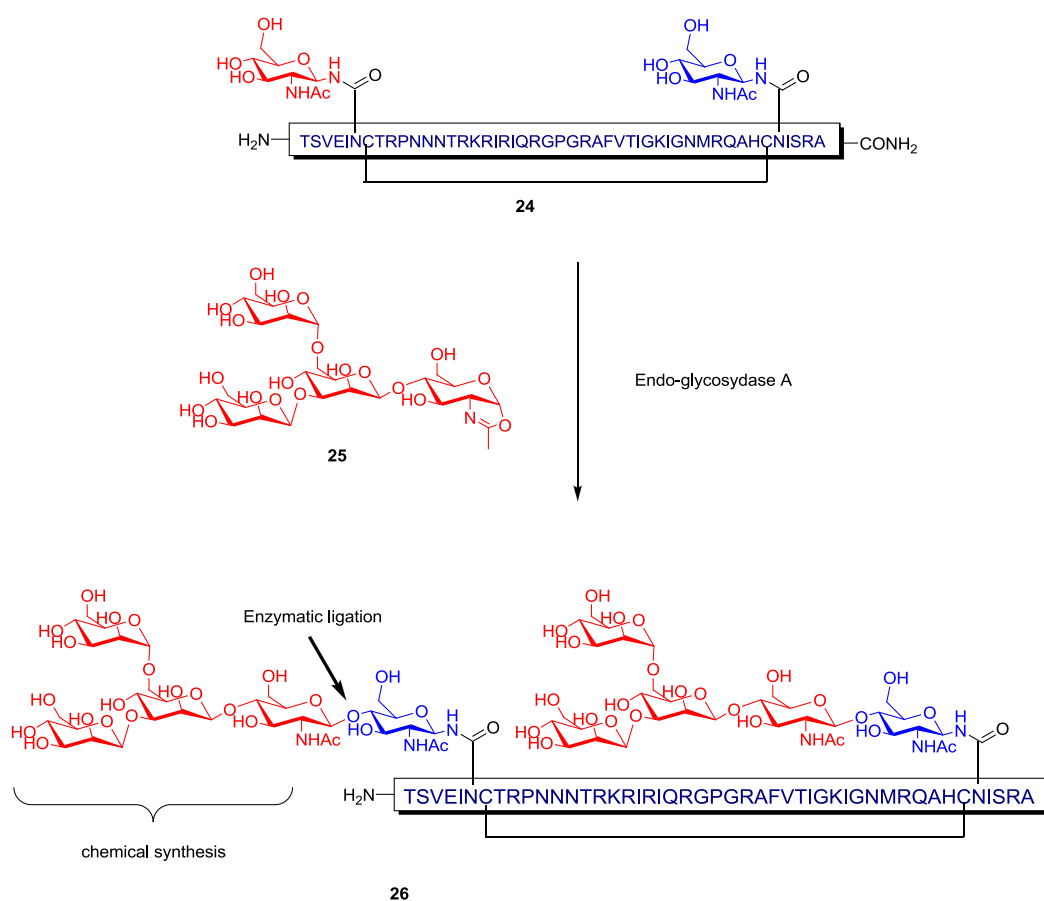


**Scheme 3.4** The chemical synthesis of complex glycoprotein (MCP-3) by native chemical ligation (NCL).

#### 1.4 Wang's transglycosylation method

L.-X.Wang research group reported the transglycosylation reaction using endo-glycosylase A to obtain *N*-linked glycopeptides.<sup>26-28</sup> The key feature of this method is that the

purified glycan can be directly used without protection of a sugar donor. The synthetic pathway for transglycosylation was shown in Scheme 3.5. It was found that chemically synthesized oligosaccharide oxazoline **25** is a good substrate for this reaction. HIV-1 V3 domain linking with two *N*-linked pentasaccharides was prepared by using this method. The glycopeptide carrying two *N*-linked GalNAc **24** was synthesized by using standard SPPS. The glycopeptides **24** was further treated with Endo A for transglycosylation in the presence of oxazoline **25** to obtain the glycopeptide **26** in good yields. However, the major disadvantage of this method is the low efficiency of the transglycosylation.

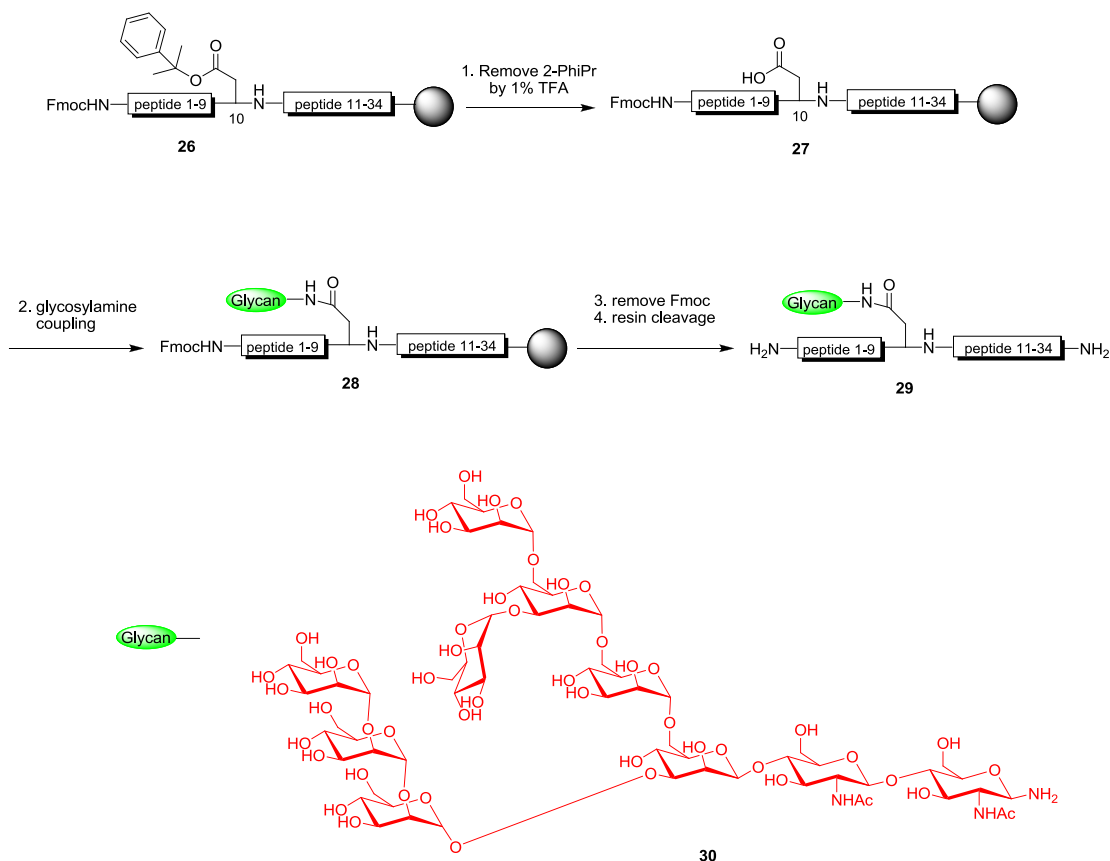


**Scheme 3.5** Wang's transglycosylation protocol for *N*-linked glycoproteins

Conventional synthetic approaches to *N*-glycopeptides involve the coupling of pre-formed *N*-glycosylated asparagine building blocks to the peptide chain *via* sequential SPPS.<sup>29-31</sup> Various glycopeptides have been successfully synthesized which utilizes the well-established Fmoc SPPS chemistry.<sup>29-31</sup> However, this strategy bears a major disadvantage that excess amount of the Fmoc-protected oligosaccharide building blocks must be used to drive the coupling to completion. In addition, another problem arises when the glycosyl moiety of the Fmoc-Asn (glycosyl)-OH building block contains free hydroxy groups, as this would cause *O*-acylation formation in all following coupling steps.<sup>32</sup> An alternative to stepwise glycopeptide synthesis, the convergent synthesis of *N*-linked glycosylamines proceeds through Lansbury aspartylation.<sup>33</sup> This in-solution convergent strategy allows the introduction of *N*-linked oligosaccharides at a later stage which maximizes the yield of glycopeptides produced based on the amount of *N*-linked oligosaccharide used. On the other hand, the in-solution strategy introduces additional purification steps that eliminates the advantages of SPPS, and in most cases, formation of persistent by-products, such as peptide aspartimides and peptide rearrangement products, is inevitable.<sup>23,34</sup>

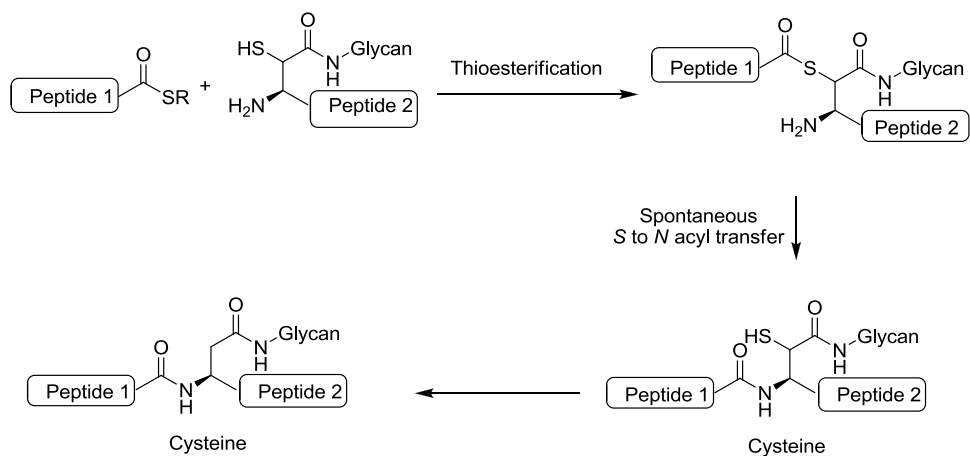
A recent on-resin convergent strategy has been established by Tolbert *et al.* employing on-resin Lansbury aspartylation to couple glycosylamines to peptides on the solid phase (Scheme 3.6).<sup>35</sup> This strategy combines the benefits of SPPS and convergent synthesis where the on-resin peptide chain is first synthesized by SPPS with orthogonally protected aspartic acid residue. Selective deprotection of Asp residue allows the coupling of glycosylamine to the Asp side chain and a subsequent resin cleavage to form the desired glycopeptide. Nevertheless, the on-resin

coupling reaction cannot be achieved to 100% completion but the unreacted glycan is recovered. In addition, this strategy suffers from the drawbacks caused by Fmoc-SPPS chemistry, when longer peptide chains are to be synthesized prior to the on-resin Lansbury aspartylation.



**Scheme 3.6** Tolbert's *N*-linked glycopeptides synthesis using on-resin Lansbury aspartylation.

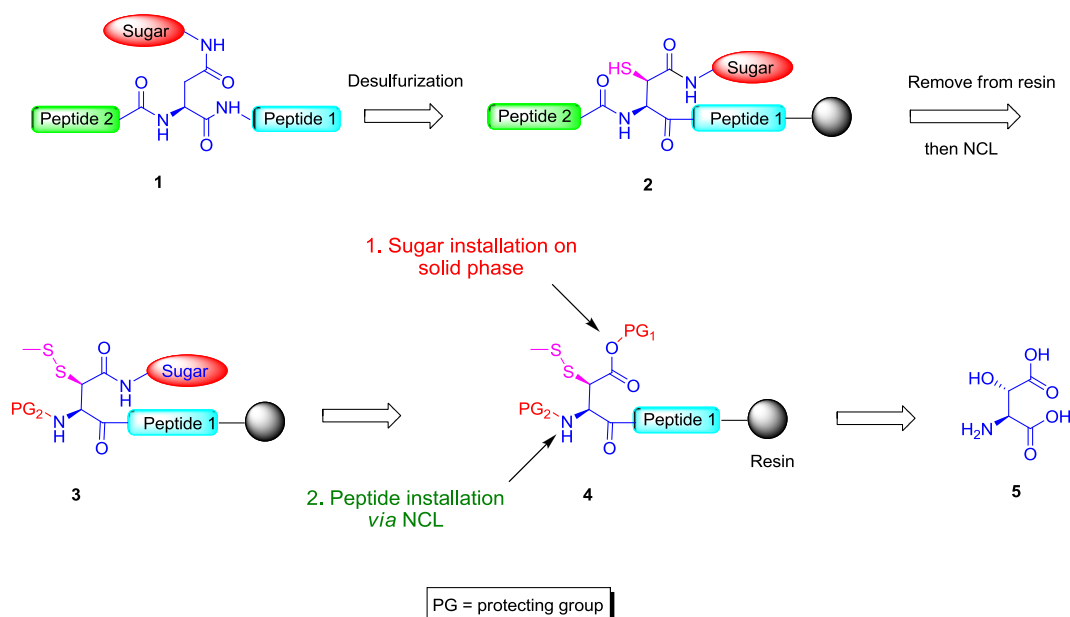
To surpass the size limit inherent in linear SPPS, the convergent condensation of unprotected peptide segments, known NCL, has emerged as an effective approach. NCL enables the amide bond formation of an *N*-terminal cysteine residue with a *C*-terminal thioester through a chemoselective thioesterification followed by a spontaneous *S* to *N* acyl transfer (Figure 3.2). To overcome above drawbacks, we wish to extend NCL concept on glycopeptide chemistry.



**Figure 3.2** Native chemical ligation (NCL) on aspartic acid.

## 2. RESULTS AND DISCUSSION

Our approach focuses on the aspartic acid (Asp) residue which is the central and fundamental scaffold of N-glycopeptides. Herein, we have proposed a new synthetic strategy which combines the benefits of SPPS and convergent on-resin peptide synthesis to N-glycopeptides as shown in Figure 1. Starting from  $\beta$ -hydroxy aspartic acid **5**, thiol functionality can be introduced to the  $\beta$ -position making it eligible for a subsequent native chemical ligation with a thioester. In the meantime, its carboxyl functionality enables it to undergo amination with an oligosaccharide-amine to afford the glycopeptides bond. In this case, the polypeptide backbone of desired N-glycopeptide is disconnecting into more manageable small peptides. Resin attachment together with installation of small peptide fragment makes the subsequent amination easier to manipulate, and the native chemical ligation that follows will anneal this construct to a larger glycopeptide backbone. The major advantage of NCL is that it enables the ligation of unprotected peptide chains, in aqueous solution at neutral pH, to form a chemoselective long peptide chain in near quantitative yield. With this in mind, a new approach to N-glycopeptides can be proposed by combining the existing strategy with NCL. Though our protocol is slightly less convergent as compared to one-grand acylation or glycosylation, it is certainly more practical and flexible.

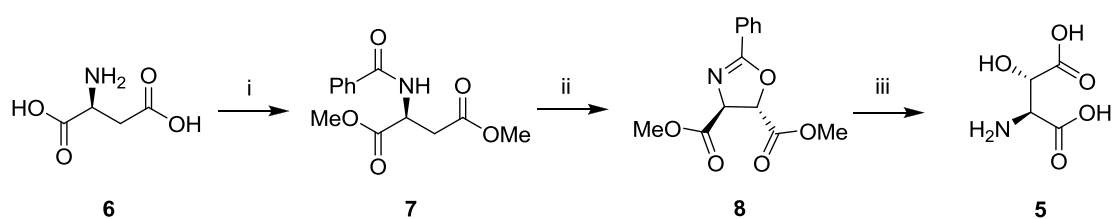


**Figure 1** Retro-synthetic approach to N-glycopeptides utilizing NCL strategy.

Starting from (2S, 3S)-3-hydroxyaspartic acid **5**, an orthogonally protected 3-thioaspartate derivative **4** has been synthesized as a fundamental structure for N-glycopeptides. The carboxyl groups and amine group were protected orthogonally by allyl, 2-PhiPr and Boc after careful manipulation and optimization, which as a result, has facilitate the resin attachment and the subsequent oligosaccharide installation for N-glycopeptide synthesis. The disulfide functionality of this compound enables a chemoselective ligation on the amine side which assembles the rest of polypeptide backbone of the N-glycopeptide desired. The major advantage of our strategy is that it enables the ligation of unprotected peptide chains **3** after careful removal of resin, in aqueous solution at neutral pH, to form a chemoselective long peptide chain **2**. Finally, successful desulfurization of peptide **2** yields the desired glycopeptide target **1**. We would like to apply this approach to the solid phase synthesis of glycosylated

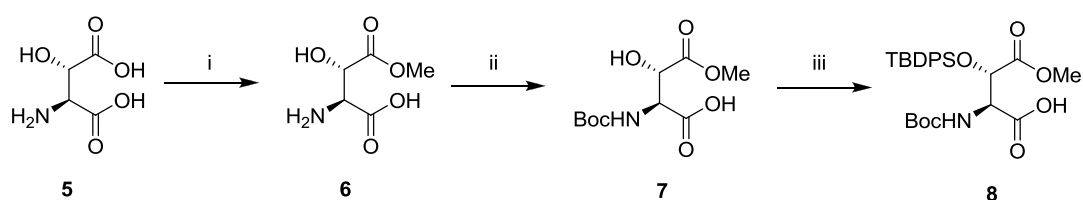
forms of the 34 amino acid HIV-1 gp41 C34 glycopeptide, which is an HIV-1 entry inhibitor. This concept further extending to bioactive polysaccharide synthesis (mannose Man<sub>8</sub>GlcNAc<sub>2</sub>-C34, HIV entry inhibitor).<sup>35</sup>

To accomplish our synthetic strategy, we first synthesized optically pure (2S,3S)-3-hydroxyaspartic acid **5** from L-aspartic acid **6** in a four-step which was reported by Cardillo *et al.* (Scheme 1).<sup>36</sup> L-Aspartic acid **6** was first converted to (2S)-N-benzoyl-dimethyl aspartate **7** in two steps by esterification of both carboxyl groups followed by acylation at nitrogen with benzoyl chloride. The subsequent regioselective deprotonation of **7** at C-3 followed by quenching the resulting dienolate with I<sub>2</sub> afforded oxazoline **8**. As reported by Cardillo *et al.*, the cyclization was completely stereoselective and as a result, oxazoline **8** was obtained in exclusively *trans* form, with no traces of the *cis* oxazoline being detected. Hydrolysis that followed by refluxing **8** in 6N HCl afforded our desired product **5**.



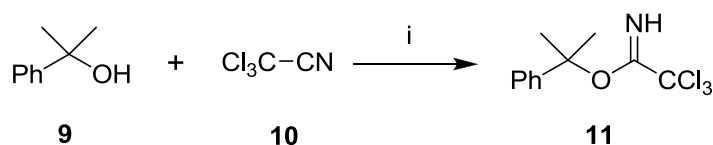
**Scheme 1** Synthesis of (2S,3S)-3-hydroxyaspartic acid from L-aspartic acid. Reagents and conditions: (i) a. SOCl<sub>2</sub>, MeOH, 0 °C to rt, 12h, 97%. b. PhCOCl, Et<sub>3</sub>N, DCM, rt, 2h, 95%. (ii) LiHMDS, I<sub>2</sub>, THF, 0 °C -78 °C, 2.5 h, 65%; (iv) 6N HCl, reflux, 6 h, 85%. LiHMDS = lithium bis(trimethylsilyl)amide.

With (2S,3S)-3-hydroxyaspartic acid **5** prepared, we then moved on to the next stage of synthesizing 3-thioaspartic acid derivative. In order to facilitate the final installation of solid phase resin at C-1, installation of oligosaccharide at C-4 and the coupling of peptide segment *via* NCL at C-2 amine, the two carboxyl groups and amine group of 3-thioaspartic acid derivative must be orthogonally protected. Following similar protocol as we did on dual native chemical ligation at lysine (Lys),<sup>14</sup> we chose *tert*-butyloxycarbonyl (Boc) group to protect the C-2 amine as hydrolysis under basic condition would be necessary when performing orthogonal protection on the two carboxyl groups, making 9-fluorenylmethyloxycarbonyl (Fmoc) an inappropriate protecting group. The proper protecting group on C-1 carboxyl was then examined by first protecting **5** with Boc on amine and methyl (Me) on C-4 carboxyl, allowing different protecting groups to be introduced on C-1 carboxyl (Scheme 2).

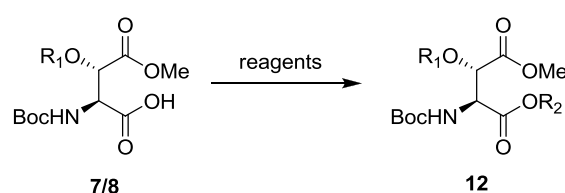


**Scheme 2** Reagents and conditions: (i) HCl, MeOH, reflux, 3 h, 85%. (ii) Boc<sub>2</sub>O, 10% Na<sub>2</sub>CO<sub>3</sub>, dioxane, rt, 12 h, 90%. (iii) TBDPSCl, imidazole, DMF, 80%. Boc<sub>2</sub>O = di-*tert*-butyl dicarbonate; TBDPSCl = *tert*-butyl diphenylchlorosilane.

As listed in Table 1, various protecting groups were attempted including *tert*-butyl (<sup>t</sup>Bu), benzyl (Bn), allyl, *p*-methoxybenzyl (PMB) and 2-phenylisopropyl (2-PhiPr). 2-Phenylisopropyl protection was performed with **11**, which was synthesized by the reaction shown in Scheme 3. Within these protecting groups, <sup>t</sup>Bu failed to be introduced using different reagents and additives (Entries 1 to 4), Bn and PMB were introduced successfully with similar yields (Entries 5 and 7) whereas allyl and 2-PhiPr appeared to be the most appropriate protecting groups with a same yield of 85% (Entries 6 and 8). Moreover, protecting the C-3 hydroxyl group with *tert*-butyldiphenylsilyl (TBDPS) (Scheme 2, **7** to **8**) showed no significant increase in the yields (Entries 9 to 14).



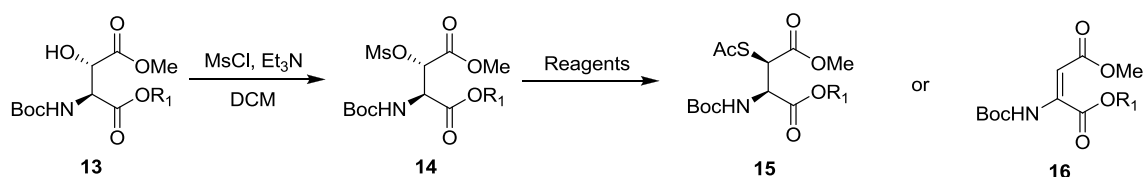
**Scheme 3** Synthesis of **11** as the reagent for 2-PhiPr protection. Reagents and conditions: (i) NaH, Et<sub>2</sub>O, rt, 1.5 h, 98%.

**Table 1.** Protecting group introduction on C-1 carboxyl of (2*S*,3*S*)-3-hydroxyaspartic acid.

Entry	R <sub>1</sub> <sup>a</sup>	R <sub>2</sub>	Reagents <sup>b</sup>	Result (Yield) <sup>c</sup>
1	H	<sup>t</sup> Bu-	<sup>t</sup> BuOH, DCC, DMAP, DCM	No reaction
2	H	<sup>t</sup> Bu-	<sup>t</sup> BuOH, EDC.HCl, HOBT, DCM	No reaction
3	H	<sup>t</sup> Bu-	<sup>t</sup> BuOH, EDC.HCl, DMAP, DCM	No reaction
4	H	<sup>t</sup> Bu-	<sup>t</sup> BuOH, EDC.HCl, HOBT, DMAP, DCM	No reaction
5	H	Bn	BnBr, NaHCO <sub>3</sub> , DMF	80%
6	H	Allyl	Allyl bromide, NaHCO <sub>3</sub> , DMF	85%
7	H	PMB	PMBCl, NaHCO <sub>3</sub> , DMF	78%
8	H	2-PhiPr	11, DCM	85%
9	TBDPS	<sup>t</sup> Bu-	<sup>t</sup> BuOH, DCC, DMAP, DCM	No reaction
10	TBDPS	<sup>t</sup> Bu-	<sup>t</sup> BuOH, EDC.HCl, HOBT, DCM	No reaction
11	TBDPS	Bn	BnBr, NaHCO <sub>3</sub> , DMF	82%
12	TBDPS	Allyl	Allyl bromide, NaHCO <sub>3</sub> , DMF	85%
13	TBDPS	PMB	PMBCl, NaHCO <sub>3</sub> , DMF	80%
14	TBDPS	2-PhiPr	7, DCM	85%

(a) R<sub>1</sub> = H, **7**; R<sub>1</sub> = TBDPS, **8**. (b) Reagents, additives and solvents used in the reaction. (c) Yield of isolated product, before the subsequent Me deprotection. DCC = N,N'-dicyclohexylcarbodiimide; DMAP = 4-dimethylaminopyridine; EDC.HCl = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBT = hydroxybenzotriazole. PMBCl = *p*-methoxybenzyl chloride.

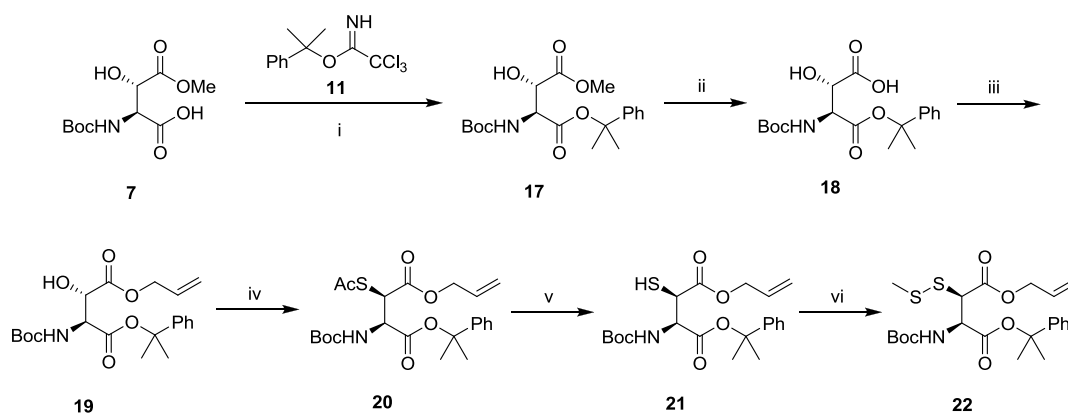
Considering that Bn, PMB, allyl and 2-PhiPr gave similar yields on the protection of C-1 carboxyl group (ranged from 78% to 85%), the reaction conditions and yields of the latter stage thioacetylation was examined then (Table. 2). The C-3 hydroxyl group was first mesylated by methanesulfonyl chloride (MsCl) to be converted into a better leaving group, thioacetylation was then followed using either potassium thioacetate (AcSK) or thioacetic acid (AcSH). We first tried the thioacetylation with AcSK salt as we did for the dual NCL on lysine (Lys),<sup>6</sup> however, an unexpected dehydration occurred forming a carbon-carbon double bond between C-2 and C-3 (Entries 1 to 4). The reagents were then changed to AcSH as used by Danishelfsky *et. al* for NCL on valine (Val),<sup>5</sup> the thioacetylated products were successfully obtained (Entries 5 to 8). The yields of all the four reactions did not differ by much (65% to 77%), with the 2-PhiPr protected aspartic acid derivative having high yield.

**Table 2.** Thioacetylation on C-3 hydroxyl of (2S,3S)-3-hydroxyaspartic acid.

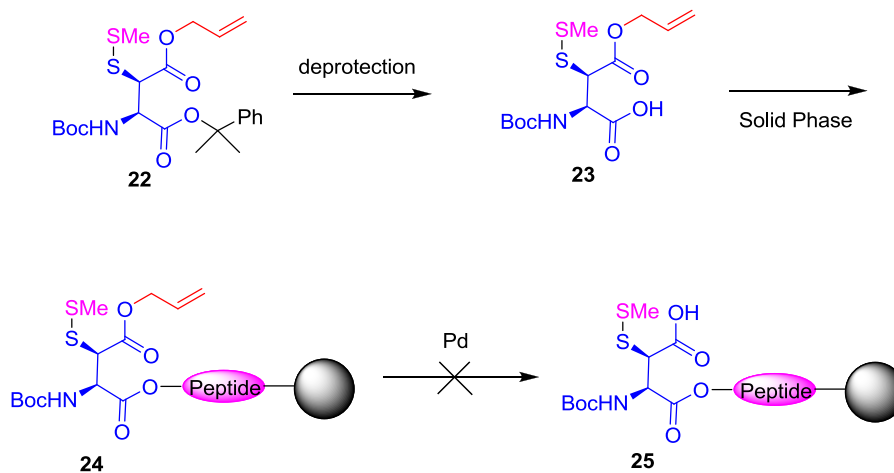
Entry	R <sub>1</sub>	Reagent <sup>a</sup>	Solvent	Temp & Rxn time	Product <sup>b</sup>	Yield <sup>c</sup>
1	Bn	AcSK salt	DMF	40 °C, 8h	<b>16</b> only	60%
2	Allyl	AcSK salt	DMF	40 °C, 8h	<b>16</b> only	65%
3	PMB	AcSK salt	DMF	40 °C, 8h	<b>16</b> only	62%
4	2-PhiPr	AcSK salt	DMF	40 °C, 8h	<b>16</b> only	72%
5	Bn	AcSH, DBU	DMF	rt, 12h	<b>15</b> only	65%
6	Allyl	AcSH, DBU	DMF	rt, 12h	<b>15</b> only	70%
7	PMB	AcSH, DBU	DMF	rt, 12h	<b>15</b> only	65%
8	2-PhiPr	AcSH, DBU	DMF	rt, 12h	<b>15</b> only	77%

(a) Reagents and additives used in the reaction. (b) Only one of the products, either **15** or **16**, was formed in all eight entries. (c) The isolated yields of products based on only the thioacetylation step, regardless of the previous mesylation. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

According to the above protecting group manipulations, 2-PhiPr appeared to be the most suitable protecting group for C-1 carboxyl group, with Bn, PMB and allyl groups being potential candidates as well. The next step was to introduce an orthogonal protecting group to the C-4 carboxyl. To fulfill that, the methyl protected C-4 carboxyl was first treated with 1N LiOH to reform the free carboxylic acid. However, an unfortunate circumstance occurred that except for 2-PhiPr, all the other three protecting groups were removed by LiOH, making 2-PhiPr the only possible protecting group for C-1 carboxyl group. Considering 2-PhiPr is labile to acid hydrolysis, the possible protecting groups on C-4 carboxyl were limited to acid stable protecting groups. Herein, we chose allyl group which was stable towards both acidic and basic conditions and was very commonly used as orthogonal protecting groups in peptide synthesis. The introduction of allyl group to C-4 carboxyl underwent smoothly as well as the following mesylation with MsCl and thioacetylation by AcSH and DBU additive (Scheme 4). The subsequent deacetylation by 1N NaOH followed by disulfide introduction by methyl methanethiosulfonate (MMTS) afforded the fundamental acceptor compound **22** that was readily for peptide and oligosaccharide installation.

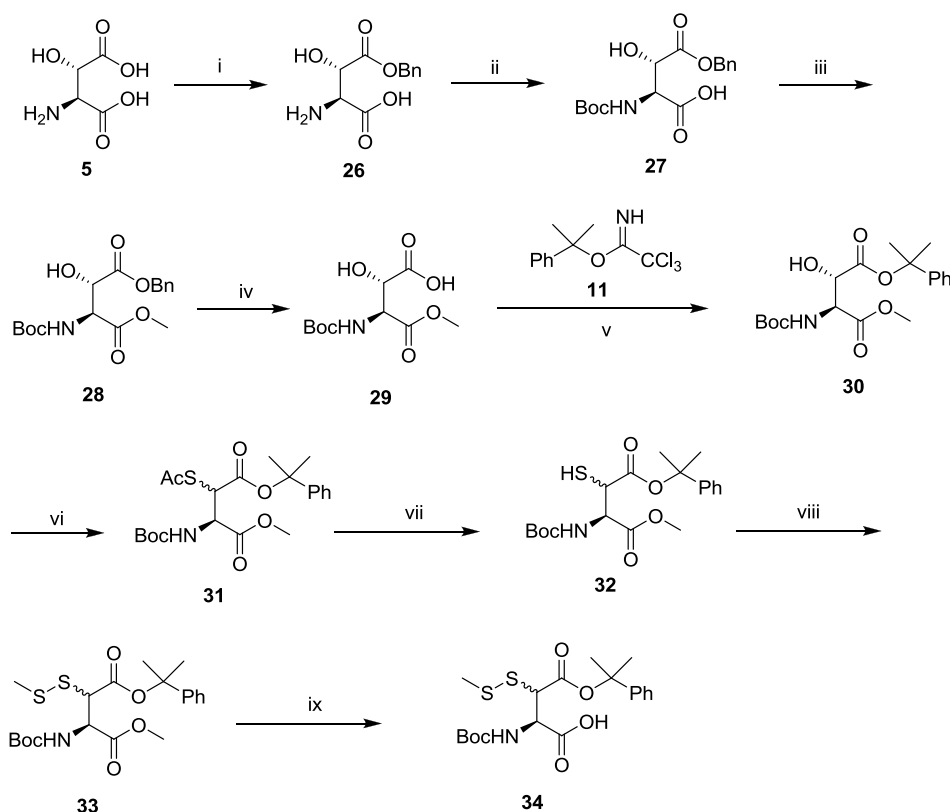


**Scheme 4** Reagents and conditions: (i) **7**, DCM, rt, 12h, 85%. (ii) 1N LiOH, THF, rt, 12 h, 75%. (iii) Allyl bromide, NaHCO<sub>3</sub>, DMF, rt, 24 h, 75%. (iv) a. MeSO<sub>2</sub>Cl, Et<sub>3</sub>N, DCM, 0 °C, 1 h. 95%. b. AcSH, DBU, DMF, rt to 60 °C, 25 h, 76%. (v) 1N NaOH, MeOH, 0 °C, 10 min, 92%. (vi) MMTS, Et<sub>3</sub>N, rt, 30 min, 95%. MMTS = methyl methanethiosulfonate.



**Scheme 5** Convergent synthetic route to N-glycopeptides.

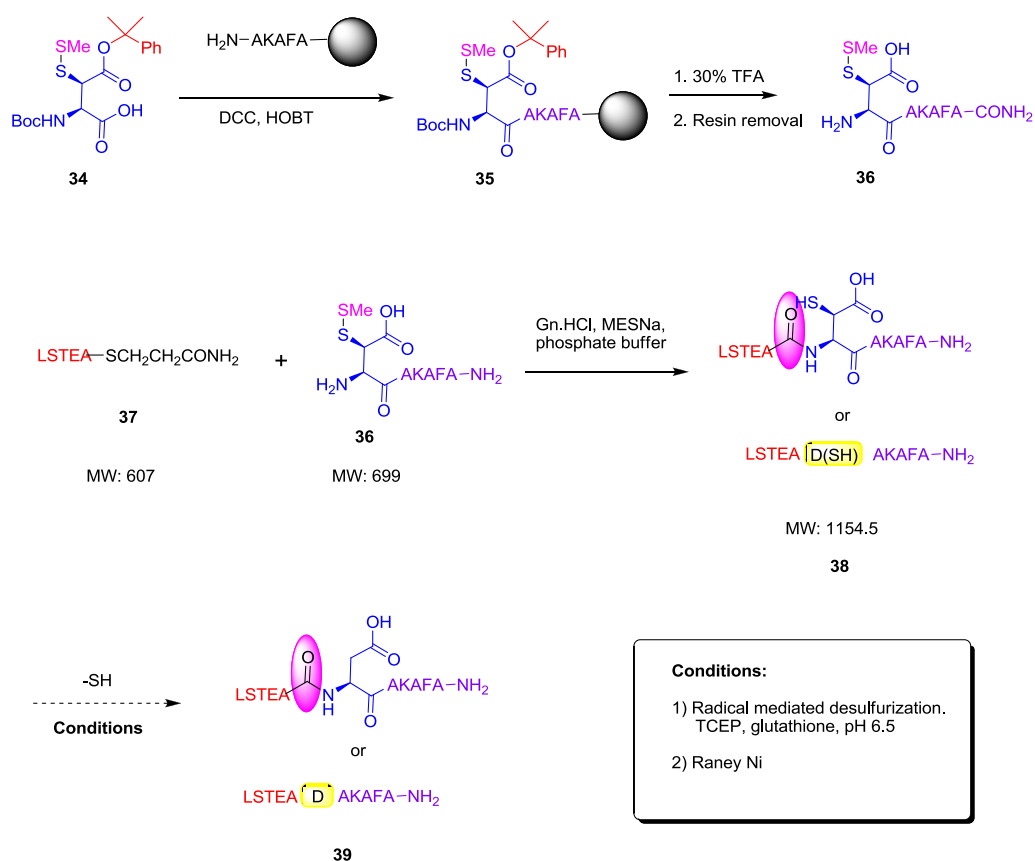
The synthetic plan for solid phase resin attachment, oligosaccharide installation and peptide ligation as described in Scheme 5. First, deprotection step of the Boc and 2-PhiPr functional groups using 30% TFA in DCM yielded the free amino acid. Next, amine functionality re-protected with Boc group with  $\text{Boc}_2\text{O}$  in the presence of 10%  $\text{Na}_2\text{CO}_3$  in dioxane to obtain compound **23**. Further, installation of peptide resin and Pd mediated deprotection of allyl group doesn't work under known protocols. So, we felt that allyl protecting group is not a good orthogonal protecting group for this reaction. Then, we turned our attention to change allyl to 2-PhiPr group as an orthogonal protection for aspartic acid.



**Scheme 6** Reagents and conditions: (i) HCl, BnOH, 70 °C, 3 h, 90%. (ii) Boc<sub>2</sub>O, 10% Na<sub>2</sub>CO<sub>3</sub>, dioxane, rt, 12 h, 95%. (iii) MeI, KHCO<sub>3</sub>, DMF, rt, 24 h, 75%. (iv) Pd/C, H<sub>2</sub>, ethyl acetate, rt, 96%. (v) **11**, DCM, rt, 12h, 95%. (vi) a. MeSO<sub>2</sub>Cl, Et<sub>3</sub>N, DCM, 0 °C, 1 h. b. AcSH, DBU, DMF, rt to 60 °C, 25 h, 65% (two steps). (vii) 1N NaOH, MeOH, 0 °C, 10 min, 90%. (viii) MMTS, Et<sub>3</sub>N, rt, 30 min, 92%. (ix) 1N LiOH, THF, H<sub>2</sub>O, 0 °C to rt, 45%. MMTS = methyl methanethiosulfonate.

The revised strategy for selective protection of aspartic acid as depicted in Scheme 6. Initially, orthogonal protection of (2S,3S)-3-hydroxyaspartic acid **5** was performed with benzyl alcohol in the presence of HCl. The amine functionality of benzyl protected  $\beta$ -hydroxy aspartic acid **26** protected with *tert*-butyloxycarbonyl (Boc) group. Following similar experimental conditions as we did before,<sup>14</sup> we chose Methyl group to protect the  $\alpha$ -carboxylic acid followed by deprotection of benzyl (Bn) group under known literature conditions. Then, the orthogonal protection of amino acid **29** performs with appropriate protecting group, which tolerate standard SPPS conditions. Here, we chose 2-PhiPr group as an ideal orthogonal protecting group to obtain amino acid **30** for our glycopeptide synthesis. We then further move on to the next stage of synthesizing thiol installation on aspartic acid backbone. Under the optimized reaction conditions which are developed in Scheme 4 for thiol installation, we performed similar conditions to compound **30** to obtain the desired protected aspartic acid **33** (Scheme 6, four steps with good yields). Finally, hydrolysis of amino acid **33** under aq LiOH obtain the aspartic acid derivative **34** in low yield (45%).

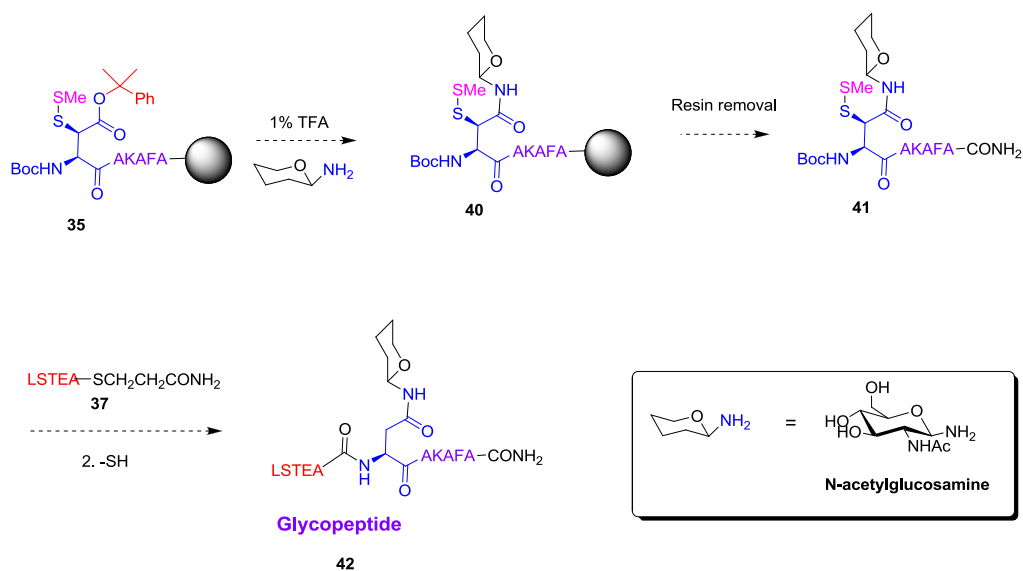
The synthetic plan for solid phase resin attachment and peptide ligation as described in Scheme 7. Initially, we anticipated that the orthogonal protected aspartic acid **34** could be assembling with small peptide (AKAFA) using SPPS. Then, deprotection step of the Boc and 2-PhiPr functional groups using 30% TFA in DCM followed by resin cleavage yielded the free amino acid **36**. Having the thiol containing aspartic acid **36** in hand, we investigated the reactivity of native chemical ligation with small peptide **37**. Initially, Peptide **36** treated with thioester **37** in the presence of 2-mercaptoethanesulfonate (MESNa) under standardized NCL conditions. The ligation reaction proceeds very smooth under above conditions. Further, optimization of NCL reaction with various substrates, desulfurization of ligated products and sugar installation is in under way.



**Scheme 7** Demonstration of peptide ligation on aspartic acid (Asp).

Scheme 8 described the synthetic method from the 3-thioaspartic acid derivative **35** to N-linked glycopeptides containing a sugar moiety. The process involves first the attachment of a solid phase resin with a small peptide segment. The process involves first the deprotection of 2-PhiPr group on  $\beta$ -carboxylic acid which allows the attachment of a sugar template on a peptide segment. This provides us an N-terminal thiol-containing on-resin N-linked glycopeptide, which after the Boc deprotection can undergo a native chemical ligation with a C-terminal thioester. After the final step of desulfurization and cleavage from resin, a sugar containing N-linked glycopeptide can be obtained.

The next stage of solid phase resin attachment, oligosaccharide installation and peptide ligation *via* NCL was carry out by our collaborator, Prof. Liu Chuan-Fa group from School of Biological Sciences, NTU.



**Scheme 8** Future direction for N-linked glycopeptide synthesis.

### 3. CONCLUSION

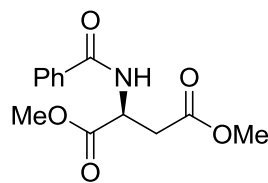
We have expanding the scope of native chemical ligation to aspartic acid which can be utilized in convergent on-resin N-glycopeptide synthesis. An orthogonally protected 3-thioaspartic acid derivative, (*R*)-2-(*tert*-butoxycarbonylamino)-3-(methylsulfanyl)-4-oxo-4-(2-phenylpropan-2-yloxy) butanoic acid **34** have been synthesized after careful optimization and protecting group manipulation. This compound can serve as a core scaffold for *N*-linked glycopeptide synthesis where the oligosaccharide glycosylamine can be installed to C-4 carboxyl and two peptide segments can be introduced by solid phase resin as well as native chemical ligation with a C-terminal thioester. This concept has not only extended NCL to aspartic acid, but also furnished us with a novel synthetic approach to *N*-linked glycopeptides and glycoproteins. With this approach, oligosaccharide glycan and the peptide segments of backbone polypeptide can be introduced in a more convergent and high-yielding method.

## 4. EXPERIMENTAL

Unless otherwise noted, all reactions were carried out in oven dried glassware under nitrogen atmosphere and all the distilled solvents used were transferred by syringes. Solvents and reagents were purified according to standard procedures prior to use. Evaporation of organic solutions was achieved by rotary evaporation, with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 mm). Technical grade solvents were used for column chromatography and distilled prior to use. Mass spectra were recorded on a The Thermo LCQ Deca Ion Trap mass spectrometer fitted with an electrospray ionization source. NMR spectra were recorded at room temperature on 300 MHz Bruker ACF 300 and 400 MHz Bruker DPX 400 spectrometers. The residual solvent signals were taken as the reference (7.26 ppm for  $^1\text{H}$  NMR spectra and 77.0 ppm for  $^{13}\text{C}$  NMR spectra in  $\text{CDCl}_3$ , 4.79 ppm for  $^1\text{H}$  NMR spectra in  $\text{D}_2\text{O}$ ). Chemical shift ( $\delta$ ) is reported in ppm, coupling constants ( $J$ ) are reported in Hz. The following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quarter, m = multiplet or unresolved, br = broad signal.

## Experimental Procedure and Spectroscopic Data

### (*S*)-*N*-Benzoyl-dimethylaspartate (**7**)<sup>37,38</sup>



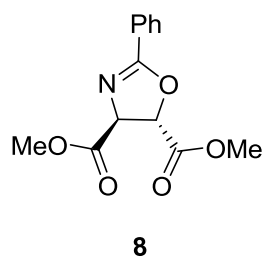
**7**

To a suspension of L-aspartic acid (**6**, 10 g, 75.1 mmol) in methanol (50 mL), thionyl chloride (7.1 mL, 97.6 mmol) was added dropwise at 0 °C. The mixture was stirred at 0 °C for 30 min and then allowed to room temperature. After stirring at room temperature for another 12 h, the reaction mixture was concentrated and triturated with diethyl ether. The resulting white crystalline solid was filtered, washed with cold ether and dried to give the dimethyl L-aspartate hydrochloride (14.4 g, 97%).

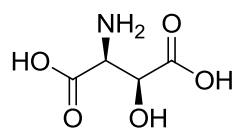
Dimethyl L-aspartate hydrochloride (14.4 g, 73.2 mmol) was then dissolved in CH<sub>2</sub>Cl<sub>2</sub> (150 mL) without further purification. Triethylamine (40 mL, 0.29 mol) was added to the solution at room temperature, followed by dropwise addition of benzoyl chloride (9.3 mL, 80.1 mmol). The reaction mixture was stirred at room temperature for 2 h. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and washed with 1N HCl (100 mL), saturated NaHCO<sub>3</sub> solution (100mL) and brine (100 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to afford **2** (18.3 g, 95%) as a white solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ in ppm = 7.80 (m, 2H, Ph), 7.50-7.42 (m, 3H, Ph), 7.23 (d, *J* = 8 Hz, 1H, NH), 5.03 (m, 1H, CH-NH), 3.76 (s, 3H, Me), 3.67 (s, 3H,

Me), 3.12 (dd,  $J_1 = 17$  Hz,  $J_2 = 4$  Hz, 1H,  $\underline{\text{C}}\text{H}_2\text{-COOMe}$ ), 2.96 (dd,  $J_1 = 17$  Hz,  $J_2 = 4$  Hz, 1H,  $\underline{\text{C}}\text{H}_2\text{-COOMe}$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 171.7, 171.3, 166.8, 133.5, 131.8, 128.6, 127.0, 52.9, 52.1, 48.8, 36.1; MS:  $[\text{M}+1]^+$   $m/z$  found 266.10.

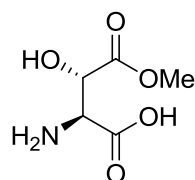
**(4S,5S)-2-Phenyl-4,5-dimethoxycarbonyloxazoline (8)**<sup>38</sup>



To a stirred solution of **7** (2 g, 7.54 mmol) in anhydrous THF (80 mL), LiHMDS (15.1 mL 1M solution in THF, 15.1 mmol) was added at 0 °C. After stirring for 30 min, the reaction mixture was cooled to -78 °C, and a solution of iodine (3.84 g, 15.1 mmol) in anhydrous THF (40 mL) was added dropwise. The reaction was stirred at -78 °C for 2 h, and quenched with saturated  $\text{NH}_4\text{Cl}$  solution. The solvent was removed and then dissolved in ethyl acetate, washed twice with saturated  $\text{Na}_2\text{S}_2\text{O}_3$  solution, and separated. The organic layer was dried over  $\text{Na}_2\text{SO}_4$  and concentrated. The resulting oil was purified by flash column chromatography (10% EA-hexane) to afford oxazoline **8** as a pale brown oil (1.29 g, 65%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 8.01 (m, 2H, Ph), 7.30-7.51 (m, 3H, Ph), 5.45 (d, 1H,  $J = 6.6$  Hz,  $\underline{\text{C}}\text{H-COOMe}$ ), 5.01 (d, 1H,  $J = 6.6$  Hz,  $\underline{\text{C}}\text{H-COOMe}$ ), 3.85 (s, 3H, Me), 3.83 (s, 3H, Me);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 170.2, 169.5, 165.4, 132.1, 128.6, 128.3, 126.2, 78.0, 72.6, 53.1, 52.9; MS:  $[\text{M}+1]^+$   $m/z$  found 264.13.

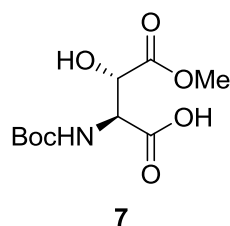
**(2S,3S)-3-Hydroxyaspartic acid (5)**<sup>36</sup>**5**

A solution of **8** (2 g, 7.6 mmol) in 6N HCl (60 mL) was refluxed for 6 h. The acid aqueous solution was then washed twice with ethyl acetate, concentrated under reduced pressure and dissolved with water (12 mL). The mixture was adsorbed on cation exchange resin. The resin was washed with distilled water until the washing came out neutral, then with 1.5N aqueous NH<sub>4</sub>OH to recover product **5** (0.96 g, 85%). **<sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O):**  $\delta$  in ppm = 4.71 (br, 1H, CH-OH), 4.24 (br, 1H, CH-NH<sub>2</sub>); **MS:** [M+1]<sup>+</sup> *m/z* found 150.01.

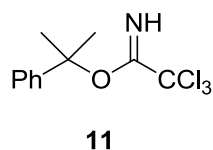
**(2S,3S)-2-Amino-3-hydroxy-4-methoxy-4-oxobutanoic acid (6)**<sup>38</sup>**6**

(2S,3S)-3-Hydroxyaspartic acid **5** (2 g, 13.4 mmol) was dissolved in a solution of concentrated HCl (2.2 mL, 26.8 mmol) and MeOH (45 mL) at 0 °C. The reaction was then heated to reflux, after stirring for 3 h at reflux, the reaction mixture was cooled to room temperature and concentrated in vacuo. The crude product **5** (1.86g, 85%) was triturated with diethyl ether, filtered and used for the next step without further purifications.

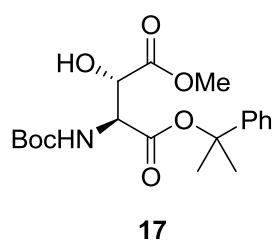
**(2S,3S)-2-((tert-butoxycarbonyl)amino)-3-hydroxy-4-methoxy-4-oxobutanoic acid (7)<sup>39</sup>**



The crude compound **6** (1.86 g, 11.4 mmol) was dissolved with an aqueous solution of 10% Na<sub>2</sub>CO<sub>3</sub> (45 mL) at 0 °C. A solution of di-*t*-butyl dicarbonate (7.46 g, 34.2 mmol) in dioxane (45 mL) was added dropwise to the reaction mixture and the resulting solution was stirred for 12 h at room temperature. The reaction mixture was then concentrated, dissolved with ethyl acetate (150 mL) and washed three times with 1N HCl solution (3 × 100 mL). The separated organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated in vacuo. Flash column chromatography (30% EA-hexane followed by 10-25% MeOH-CHCl<sub>3</sub>) provided **7** (2.7 g, 90%) as a white solid. **<sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD):** δ in ppm = 4.79 (s, 1H, CH-NHBoc), 4.51 (s, 1H, CH-OH), 3.79 (s, 3H, Me), 1.46 (s, 9H, <sup>t</sup>Bu); **<sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD):** δ in ppm = 174.3, 170.1, 155.1, 80.4, 73.1, 58.9, 52.6, 28.8; **MS:** [M+1]<sup>+</sup> *m/z* found 264.09.

**1-Methyl-1-phenylethyl trichloroacetimidate (11)**<sup>40</sup>

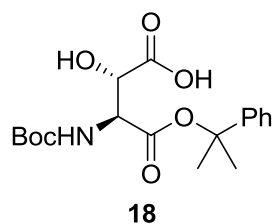
To a suspension of NaH (50 mg, 2.1 mmol) in diethyl ether (2 mL), a solution of 1-methyl-1-phenylethanol **9** (2.5 g, 19 mmol) in diethyl ether (2 mL) was added dropwise at room temperature. The mixture was stirred at room temperature for 20 min and cooled to 0 °C. Trichloroacetonitrile **10** (2 mL, 19 mmol) was then slowly added in 15 min, and the reaction mixture was again warmed to room temperature. After stirring at room temperature for 1 h, the mixture was concentrated, dissolved with pentane (2 mL) and filtered. The filtrate was evaporated under vacuo to give crude imidate **7** (5.3 g, 98%) as a reddish oil. The imidate was used for the next step without further purification.

**(2S,3S)-4-methyl 1-(2-phenylpropan-2-yl) 2-((tert-butoxycarbonyl)amino)-3-hydroxysuccinate (17)**<sup>40</sup>

The crude imidate **11** (0.43 g, 1.52 mmol) was added to a solution of (2S,3S)-2-NHBoc-3-OH-4-Me-aspartate **7** (0.2 g, 0.76 mmol) in DCM (2 mL). The reaction mixture was stirred at room temperature for 12 h, and filtered. The filtrate was evaporated and the resulted crude product was purified by flash column

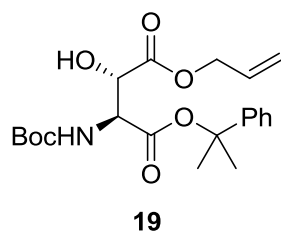
chromatography (1% MeOH-CHCl<sub>3</sub>) to yield compound **17** (246 mg, 85%) as a colorless oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ in ppm = 7.40-7.25 (m, 5H, Ph), 5.29-5.26 (m, 1H, CH-NH), 4.78-4.73 (m, 1H, CH-OH), 3.81 (s, 3H, OMe), 3.30 (s, 1H, OH), 1.82 (s, 3H, Me), 1.81 (s, 3H, Me), 1.42 (s, 9H, <sup>t</sup>Bu); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ in ppm = 172.6, 167.9, 155.4, 144.9, 128.4, 128.3, 127.3, 124.4, 124.3, 84.1, 80.2, 71.3, 68.0, 56.7, 53.1, 28.2; MS: [M+23]<sup>+</sup> m/z found 404.33.

**(2S,3S)-3-((tert-butoxycarbonyl)amino)-2-hydroxy-4-oxo-4-((2-phenylpropan-2-yl)oxy)butanoic acid (**18**)<sup>41</sup>**



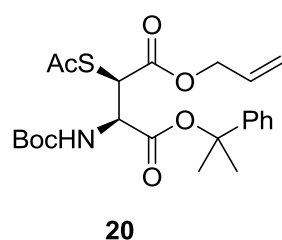
To a solution of **17** (347 mg, 0.91 mmol) in anhydrous THF (1 mL), LiOH (24 mg, 1 mmol) in H<sub>2</sub>O (1mL) was added slowly at room temperature. The reaction mixture was stirred at room temperature for 12 h when it was then acidified to pH 4 with 1M HCl. The solution was extracted three times with diethyl ether, and the combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The crude product **18** (250 mg, 75%) was used for the next step without further purification.

**(2S,3S)-1-allyl 4-(2-phenylpropan-2-yl) 3-((tert-butoxycarbonyl)amino)-2-hydroxysuccinate (19)**



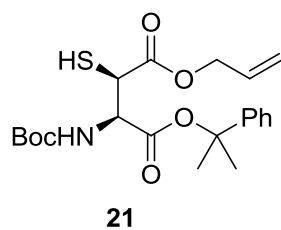
To a solution of **18** (250 mg, 0.68 mmol) in anhydrous DMF (2 mL) were added powdered NaHCO<sub>3</sub> (114 mg, 1.36 mmol) and allyl bromide (0.12 mL, 1.36 mmol). The reaction mixture was stirred at room temperature for 24 h and the solvent was removed under reduced pressure. The residue was quenched with water and extracted three times with ethyl acetate. The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated. The product was purified by flash column chromatography (10% EA-hexane) to afford **19** (208 mg, 75%) as a colorless oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ in ppm = 7.40-7.24 (m, 5H, Ph), 6.00-5.93 (m, 1H, CH=CH<sub>2</sub>), 5.40-5.24 (m, 3H, CH=CH<sub>2</sub>, CH-NH), 4.80-4.70 (m, 3H, CH<sub>2</sub>-CH=CH<sub>2</sub>, CH-OH), 3.27 (s, 1H, OH), 1.82 (s, 3H, Me), 1.81 (s, 3H, Me), 1.42 (s, 9H, <sup>t</sup>Bu); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ in ppm = 171.9, 167.9, 155.3, 144.9, 131.3, 128.4, 128.3, 127.3, 124.4, 124.3, 119.4, 84.1, 80.1, 71.4, 67.0, 56.6, 28.2; MS: [M+23]<sup>+</sup> m/z found 429.86.

**(2R,3R)-1-allyl 4-(2-phenylpropan-2-yl) 2-(acetylthio)-3-((tert-butoxycarbonyl)amino) succinate (20)**



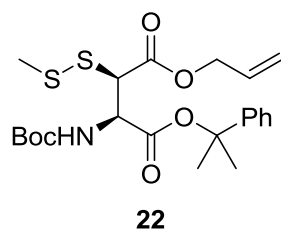
To a stirred solution of **19** (200 mg, 0.49 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (2 mL), Et<sub>3</sub>N (0.11 mL, 0.74 mmol) and MsCl (50 μL, 0.64 mmol) were added slowly at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and quenched with saturated NH<sub>4</sub>Cl solution. The organic layer was washed with water, brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The mesylated product (226 mg, 0.47 mmol, 95%) was then dissolved in DMF (2 mL). A solution of DBU salt in AcSH was prepared by adding thioacetic acid (0.17 mL, 2.35 mmol) to DBU (0.25 mL, 1.65 mmol) in DMF (1 mL). The DBU solution was added slowly to the reaction at room temperature and stirred for 20 h. The reaction mixture was then heated to 60 °C for 5 h and concentrated. The residue was dissolved with ethyl acetate and washed with saturated NH<sub>4</sub>Cl solution, water, and brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The product was purified by flash column chromatography (5% EA-hexane) to yield **20** (166 mg, 76%) as a pale yellow oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ in ppm = 7.36-7.25 (m, 5H, Ph), 5.93-5.82 (m, 1H, CH=CH<sub>2</sub>), 5.47-5.23 (m, 3H, CH=CH<sub>2</sub>, CH-NH), 4.86-4.60 (m, 3H, CH<sub>2</sub>-CH=CH<sub>2</sub>, CH-OH), 2.39 (s, 3H, S(=O)Me), 1.79 (s, 3H, Me), 1.76 (s, 3H, Me), 1.44 (s, 9H, <sup>t</sup>Bu); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ in ppm = 192.3, 168.9, 155.6, 144.6, 131.4, 128.3, 127.4, 124.5, 119.0, 118.7, 84.3, 80.2, 66.8, 55.4, 30.1, 28.3; MS: [M+23]<sup>+</sup> m/z found 487.78.

**(2R,3R)-1-allyl 4-(2-phenylpropan-2-yl) 3-((tert-butoxycarbonyl)amino)-2-mercaptosuccinate (21)**

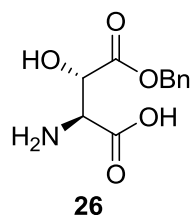
**21**

To a solution of **20** (150 mg, 0.32 mmol) in methanol (2 mL) was treated with 1N NaOH solution (0.48 mL) at 0 °C. The mixture was stirred at 0 °C for 10 min and then carefully neutralized by the addition of 1N HCl at 0 °C. The reaction was diluted with ethyl acetate and washed with water, brine. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The crude product obtained (125 mg, 92%) was directly used for the next step without further purifications.

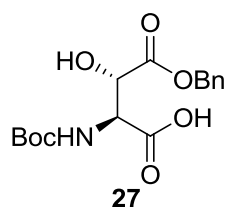
**(2R,3R)-1-allyl 4-(2-phenylpropan-2-yl) 3-((tert-butoxycarbonyl)amino)-2-(methylsulfanyl)succinate (22)**



A solution of crude product **21** (125 g, 0.3 mmol) in  $\text{CH}_2\text{Cl}_2$  (1 mL) was added dropwise to a solution of S-methyl methanethiosulfonate (42  $\mu\text{L}$ , 0.45 mmol) and  $\text{Et}_3\text{N}$  (0.08 mL, 0.6 mmol) in  $\text{CH}_2\text{Cl}_2$  (2 mL). The mixture was stirred at room temperature for 30 min and then concentrated under reduced pressure. The product was purified by flash column chromatography (3% EA-hexane) to afford **22** (134 mg, 95%) as a pale yellow oil.  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 7.34-7.24 (m, 5H, Ph), 5.93-5.83 (m, 1H,  $\text{CH}=\text{CH}_2$ ), 5.66-5.64 (m, 1H,  $\text{CH}=\text{CH}_2$ ), 5.37-5.24 (m, 2H,  $\text{CH}=\text{CH}_2$ ,  $\text{CH}-\text{NH}$ ), 4.86-4.82 (m, 1H,  $\text{CH}-\text{OH}$ ), 4.63-4.61 (m, 2H,  $\text{CH}_2-\text{CH}=\text{CH}_2$ ), 3.12 (s, 3H, SMe), 1.76 (s, 3H, Me), 1.75 (s, 3H, Me), 1.44 (s, 9H,  $^t\text{Bu}$ );  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 170.0, 168.3, 156.1, 144.8, 131.2, 128.3, 127.3, 124.4, 119.3, 84.1, 80.2, 66.5, 55.6, 55.3, 28.2, 23.7; **MS**:  $[\text{M}+23]^+$   $m/z$  found 492.59.

**(2S,3S)-2-amino-4-(benzyloxy)-3-hydroxy-4-oxobutanoic acid (26)**

(2S,3S)-3-Hydroxyaspartic acid **5** (2 g, 13.4 mmol) was dissolved in a solution of concentrated HCl (2.3 mL, 26.8 mmol) and benzyl alcohol (20 mL) at 0 °C. The reaction was then heated to 70 °C for 3 h. The reaction mixture was cooled to room temperature and concentrated in vacuo. The crude product was triturated with diethyl ether, filtered to yield compound **26** (2.9 g, 90%) as a white solid. **<sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):** δ in ppm = 7.36-7.28 (m, 5H, Ph), 5.14-5.04 (m, 2H, CH<sub>2</sub>-Ph), 4.55 (bs, 1H, CH-OH), 3.58 (bs, 1H, CH-N). **<sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>):** δ in ppm = 171.9 (-COOH), 168.4 (-CO<sub>2</sub>Bn), 136.1 (Ph), 128.9 (Ph), 128.5 (Ph), 128.4 (Ph), 69.7 (-C-OH), 66.7 (-CH<sub>2</sub>Ph), 56.1 (-CH-NH). **HRMS (ESI):** *m/z*: calcd for C<sub>11</sub>H<sub>14</sub>NO<sub>5</sub>: 240.0872; [*M*]<sup>+</sup> found: 240.0861.

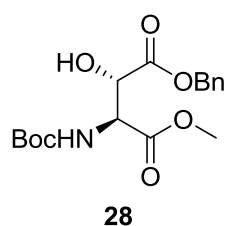
**(2S,3S)-4-(benzyloxy)-2-(tert-butoxycarbonylamino)-3-hydroxy-4-oxobutanoic acid (27)**

The benzyl protected compound **26** (1.0 g, 4.18 mmol) was dissolved in 1:1 dioxane, water (10 mL: 10 mL) and added NaHCO<sub>3</sub> (0.7 g, 8.36 mmol) at 0 °C. A

solution of di-*t*-butyl dicarbonate (1.44 mL, 6.27 mmol) was added dropwise to the reaction mixture and the resulting solution was stirred for 12 h at room temperature. The reaction mixture was then concentrated, dissolved with ethyl acetate (150 mL) and washed three times with 1N HCl solution (3 × 100 mL). The separated organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated in vacuo. Flash column chromatography (10-25% MeOH-CHCl<sub>3</sub>) provided **27** (1.35 g, 95%) as a colorless oil.

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):** δ in ppm = 7.38-7.32 (m, 5H, Ph), 5.50 (d, *J* = 9.2 Hz, 1H, NH), 5.26-5.13 (m, 2H, CH<sub>2</sub>Ph), 4.86 (d, *J* = 9.04 Hz, 1H, CHOH), 4.80 (s, 1H, CH-N), 1.41 (s, 9H, <sup>t</sup>Bu). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):** δ in ppm = 173.2 (COOH), 171.7 (CO<sub>2</sub>Bn), 155.6 (CO-<sup>t</sup>Bu), 134.7 (Ph), 128.6 (Ph), 80.6 (CMe<sub>3</sub>), 71.1 (CH-OH), 68.3 (CH-NH), 56.2 (CH<sub>2</sub>Bn), 28.2 (<sup>t</sup>Bu). **HRMS (ESI):** *m/z*: calcd for C<sub>16</sub>H<sub>21</sub>NO<sub>7</sub>Na: 362.1216; [*M*+Na]<sup>+</sup> found: 362.1213.

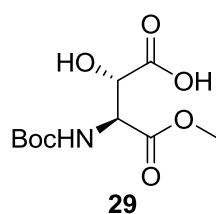
**(2*S*,3*S*)-1-benzyl 4-methyl 3-(tert-butoxycarbonylamino)-2-hydroxysuccinate (28):**



To a solution of **27** (300 mg, 0.88 mmol) in anhydrous DMF (4.5 mL, 0.2 M) were added activated KHCO<sub>3</sub> (176 mg, 1.76 mmol) and methyl iodide (0.19 mL, 1.32 mmol). The reaction mixture was stirred at room temperature for 24 h. The residue was diluted with water and extracted three times with ethyl acetate. The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated. The product was purified by flash column chromatography to afford compound **28** (234 mg, 75%) as a colorless

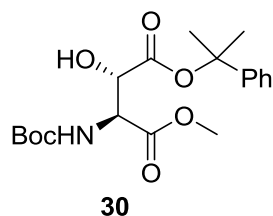
oil.  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 7.41-7.33 (m, 5H, Ph), 5.36 (d,  $J = 9.3$  Hz, 1H,  $\text{NH}$ ), 5.27-5.13 (m, 2H,  $\text{CH}_2\text{Ph}$ ), 4.84 (d,  $J = 9.3$  Hz, 1H,  $\text{CHOH}$ ), 4.73 (dd,  $J = 9.3$  Hz & 5.3 Hz, 1H,  $\text{CH-N}$ ), 3.77 (s, 3H,  $\text{CH}_3$ ), 3.42 (d,  $J = 5.0$  Hz, 1H,  $\text{OH}$ ), 1.43 (s, 9H,  $^t\text{Bu}$ ).  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 171.8 (COOH), 169.9 ( $\text{CO}_2\text{Bn}$ ), 155.3 ( $\text{CO-}^t\text{Bu}$ ), 134.7 (Ph), 128.6 (Ph), 80.3 ( $\text{CMe}_3$ ), 71.1 (CH-OH), 68.3 (CH-NH), 56.2 ( $\text{CH}_2\text{Bn}$ ), 52.8 (OMe), 28.2 ( $^t\text{Bu}$ ). **HRMS (ESI):**  $m/z$ : calcd for  $\text{C}_{17}\text{H}_{24}\text{NO}_7$ : 354.1553;  $[M]^+$  found: 354.1547.

**(2S,3S)-3-(tert-butoxycarbonylamino)-2-hydroxy-4-methoxy-4-oxobutanoic acid**  
**(29)**



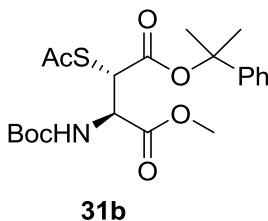
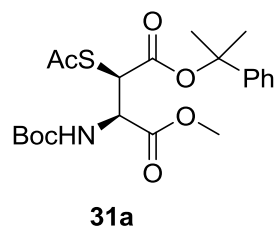
To a solution of **28** (200 mg, 0.56 mmol) in ethyl acetate (4 mL) was added 10% Pd/C (60 mg) at room temperature. The reaction mixture was stirred at room temperature for 12 h under  $\text{H}_2$  atmosphere then filtered through a short pad of celite and washed with chloroform-methanol (1:1). The solvent removed under reduced pressure and the crude product **29** (143 mg, 96%) was direct taken for the next step without further purification.

**(2S,3S)-1-methyl 4-(2-phenylpropan-2-yl) 2-(tert-butoxycarbonylamino)-3-hydroxysuccinate (30)**



To a solution of **29** (330 mg, 1.25 mmol) in anhydrous dichloromethane (10 mL) was added above 1M phenylpropan-2-yl 2,2,2-trichloroacetimidate solution (1.88 mL, 1.88 mmol, 1M solution in hexane) at room temperature. The reaction mixture was stirred for 12 h and the solvent was removed under reduced pressure. The product was purified by flash column chromatography to afford **30** (454 mg, 95%) as a colorless oil.  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 7.37-7.24 (m, 5H, Ph), 5.19 (d,  $J = 10.1$  Hz, 1H,  $\text{NH}$ ), 4.92 (d,  $J = 10.3$  Hz, 1H,  $\text{CHOH}$ ), 4.66 (t,  $J = 1.9$  Hz, 1H,  $\text{CH-N}$ ), 3.79 (s, 3H,  $\text{CH}_3$ ), 3.11 (d,  $J = 4.0$  Hz, 1H,  $\text{OH}$ ), 1.83 (s, 3H,  $\text{CH}_3$ ), 1.77 (s, 3H,  $\text{CH}_3$ ), 1.45 (s, 9H,  $^t\text{Bu}$ ).  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 170.5 (COOH), 170.3 (CO<sub>2</sub>Bn), 155.5 (CO- $^t\text{Bu}$ ), 144.4 (Ph), 128.4 (Ph), 127.5 (Ph), 124.4 (Ph), 85.1 (CMe<sub>2</sub>), 80.3 (CMe<sub>3</sub>), 71.2 (CH-OH), 55.8 (CH-NH), 52.8 (OMe), 29.1 (CH<sub>3</sub>), 28.2 (CH<sub>3</sub>), 26.9 ( $^t\text{Bu}$ ). **HRMS (ESI):**  $m/z$ : calcd for C<sub>19</sub>H<sub>27</sub>NO<sub>7</sub>Na: 404.1685;  $[M+\text{Na}]^+$  found: 404.1697.

**(R)-4-methyl 1-(2-phenylpropan-2-yl) 2-(acetylthio)-3-(tert-butoxycarbonylamino)succinate (31a and 31b):**

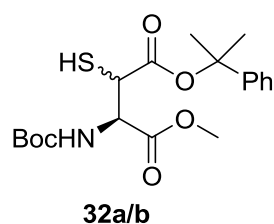


To a stirred solution of **10** (400 mg, 1.05 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL, 0.1 M),  $\text{Et}_3\text{N}$  (0.23 mL, 1.58 mmol) and  $\text{MsCl}$  (0.1 mL, 1.26 mmol) were added slowly at 0 °C. The reaction mixture was stirred at room temperature for 3 h and quenched with saturated  $\text{NH}_4\text{Cl}$  solution. The organic layer was washed with water, brine, dried over  $\text{Na}_2\text{SO}_4$  and concentrated. The mesylated product was purified by flash column chromatography.

Above compound was then dissolved in DMF (4 mL) at room temperature. A solution of DBU salt in AcSH was prepared by adding thioacetic acid (0.18 mL, 2.35 mmol) to DBU (0.18 mL, 1.65 mmol) in DMF (2 mL). The DBU solution was added slowly to the reaction mixture at room temperature and stirred for 1 h. The reaction mixture was then heated to 40 °C for 24 h. The residue was dissolved with ethyl acetate and washed with water, and brine, dried over  $\text{Na}_2\text{SO}_4$  and concentrated. The product was purified by flash column chromatography to yield diastereomeric mixture (**11a**, **11b**) (300 mg, 65%) as a colorless oil. **Compound 11a**:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 7.32-7.22 (m, 5H, Ph), 5.48 (d,  $J = 9.7$  Hz, 1H,  $\text{NH}$ ), 4.95 (d,  $J = 3.7$  Hz, 1H,  $\text{CHOH}$ ), 4.70 (dd,  $J = 10.0$  Hz & 3.9 Hz, 1H,  $\text{CH-N}$ ), 3.67 (s, 3H,  $\text{CH}_3$ ), 2.39 (s, 3H,  $\text{SCOCH}_3$ ), 1.74 (s, 3H,  $\text{CH}_3$ ), 1.72 (s, 3H,  $\text{CH}_3$ ), 1.42 (s, 9H,  $^t\text{Bu}$ ).  $^{13}\text{C}$

**NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 192.4 (SCOMe), 170.0 (COOMe), 168.4 (C=O), 155.8 (CO-<sup>t</sup>Bu), 144.7 (Ph), 128.3 (Ph), 127.3 (Ph), 124.2 (Ph), 84.2 (CMe<sub>2</sub>), 80.2 (CMe<sub>3</sub>), 54.6 (CH-SAc), 52.7 (CH-NH), 48.1 (OMe), 30.1 (COMe), 28.4 (CH<sub>3</sub>), 28.2 (CH<sub>3</sub>), 28.1 (<sup>t</sup>Bu). **HRMS (ESI):**  $m/z$ : calcd for C<sub>21</sub>H<sub>29</sub>NO<sub>7</sub>NaS: 462.1562; [M+Na]<sup>+</sup> found: 462.1559. **Compound 11b: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 7.33-7.23 (m, 5H, Ph), 5.25 (d,  $J$  = 8.3 Hz, 1H, NH), 4.93 (d,  $J$  = 10.2 Hz, 1H, CHOH), 4.72 (d,  $J$  = 4.3 Hz, 1H, CH-N), 3.69 (s, 3H, CH<sub>3</sub>), 2.39 (s, 3H, SCOCH<sub>3</sub>), 1.77 (s, 3H, CH<sub>3</sub>), 1.75 (s, 3H, CH<sub>3</sub>), 1.42 (s, 9H, <sup>t</sup>Bu). **<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):**  $\delta$  in ppm = 192.7 (SCOMe), 170.3 (COOMe), 167.1 (C=O), 155.8 (CO-<sup>t</sup>Bu), 144.9 (Ph), 128.3 (Ph), 127.2 (Ph), 124.3 (Ph), 124.2 (Ph), 84.4 (CMe<sub>2</sub>), 80.4 (CMe<sub>3</sub>), 54.9 (CH-SAc), 52.7 (CH-NH), 49.1 (OMe), 30.1 (COMe), 28.4 (CH<sub>3</sub>), 28.2 (CH<sub>3</sub>), 27.8 (<sup>t</sup>Bu). **HRMS (ESI):**  $m/z$ : calcd for C<sub>21</sub>H<sub>29</sub>NO<sub>7</sub>NaS: 462.1562; [M+Na]<sup>+</sup> found: 462.1559.

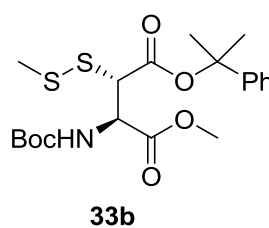
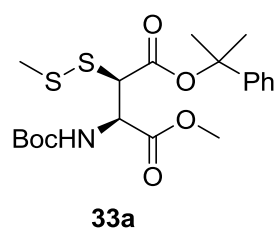
**(R)-1-methyl 4-(2-phenylpropan-2-yl) 2-(tert-butoxycarbonylamino)-3-mercaptosuccinate (32a /b)**



To a solution of above compound (220 mg, 0.5 mmol) in methanol (5 mL, 0.1 M) was treated with 1N NaOH solution (0.5 mL, 0.5 mmol) at 0 °C. The mixture was stirred at 0 °C for 20 min and then carefully neutralized by the addition of 1N HCl at 0 °C. The reaction was diluted with ethyl acetate and washed with water, brine. The

organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The crude product obtained (180 mg, 90%) was directly used for the next step without further purifications.

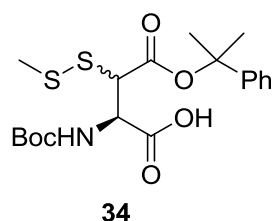
**(R)-1-methyl 4-(2-phenylpropan-2-yl) 2-(tert-butoxycarbonylamino)-3-(methylsulfanyl)succinate (33a and 33b)**



A solution of above crude product (400 mg, 1 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (15 mL) was added dropwise to a solution of *S*-methyl methanethiosulfonate (0.14 mL, 1.2 mmol) and Et<sub>3</sub>N (0.22 mL, 1.5 mmol) at 0 °C. The mixture was stirred at room temperature for 3 h and then concentrated under reduced pressure. The product was purified by flash column chromatography to afford title compound (410 mg, 92%) as colorless oil. **Compound 13a:** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ in ppm = 7.38-7.22 (m, 5H, Ph), 5.62 (d, *J* = 9.9 Hz, 1H, NH), 4.82 (dd, *J* = 10.0 Hz & 4.4 Hz, 1H, CH-N), 4.13 (d, *J* = 4.3 Hz, 1H, CH-SSMe), 3.67 (s, 3H, CH<sub>3</sub>), 2.46 (s, 3H, SSCH<sub>3</sub>), 1.77 (s, 3H, CH<sub>3</sub>), 1.77 (s, 3H, CH<sub>3</sub>), 1.41 (s, 9H, <sup>t</sup>Bu). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ in ppm = 170.5 (COOMe), 168.8 (C=O), 155.8 (CO-<sup>t</sup>Bu), 144.7 (Ph), 128.3 (Ph), 127.3 (Ph), 124.3 (Ph), 84.2 (CMe<sub>2</sub>), 80.2 (CMe<sub>3</sub>), 56.2 (CH-SSMe), 54.6 (CH-NH), 52.7 (CO<sub>2</sub>CH<sub>3</sub>), 31.6 (SSCH<sub>3</sub>), 28.5 (CH<sub>3</sub>), 28.2 (CH<sub>3</sub>), 23.7 (<sup>t</sup>Bu). **HRMS (ESI):** *m/z*: calcd for C<sub>20</sub>H<sub>29</sub>NO<sub>6</sub>NaS<sub>2</sub>: 466.1334; [*M*+Na]<sup>+</sup> found: 466.1346. **Compound 13b:** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ in ppm = 7.41-7.20 (m, 5H, Ph), 5.30 (bs, 1H, NH), 4.74 (t, *J* = 7.1 Hz, 1H, CH-N), 4.00 (od, *J* = 6.1 Hz, 1H, CHSSMe), 3.7 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>),

2.33 (s, 3H, SSCH<sub>3</sub>), 1.79 (s, 3H, CH<sub>3</sub>), 1.77 (s, 3H, CH<sub>3</sub>), 1.43 (s, 9H, <sup>t</sup>Bu). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ in ppm = 170.4 (COOMe), 168.2 (C=O), 155.0 (CO-<sup>t</sup>Bu), 144.9 (Ph), 128.2 (Ph), 127.2 (Ph), 124.5 (Ph), 83.7 (CMe<sub>2</sub>), 80.5 (CMe<sub>3</sub>), 55.8 (CH-SSMe), 54.3 (CH-NH), 52.6 (CO<sub>2</sub>CH<sub>3</sub>), 28.6 (CH<sub>3</sub>), 28.2 (SSCH<sub>3</sub>), 27.9 (CH<sub>3</sub>), 23.1 (<sup>t</sup>Bu). HRMS (ESI): *m/z*: calcd for C<sub>20</sub>H<sub>29</sub>NO<sub>6</sub>NaS<sub>2</sub>: 466.1334; [*M*+Na]<sup>+</sup> found: 466.1346.

**(*R*)-2-(tert-butoxycarbonylamino)-3-(methylsulfanyl)-4-oxo-4-(2-phenylpropan-2-yloxy)butanoic acid (34)**



A solution of above compound (20 mg, 0.05 mmol) in THF: H<sub>2</sub>O (0.9 mL: 0.1 mL) was added 1N LiOH (45 μL, 0.45 mmol) at 0 °C. The mixture was stirred at room temperature for 12 h and then carefully neutralized by the addition of 1N HCl at 0 °C. The reaction was diluted with ethyl acetate and washed with water, brine. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The product was purified by flash column chromatography to afford title compound (9 mg, 50%) as colorless oil.

**Compound 14:** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ in ppm = 7.32-7.22 (m, 5H, Ph), 5.48 (d, *J* = 9.7 Hz, 1H, NH), 4.95 (d, *J* = 3.8 Hz, 1H, CHOH), 4.70 (d, *J* = 3.9 Hz, 1H, CH-N), 2.39 (s, 3H, SCOCH<sub>3</sub>), 1.74 (s, 3H, CH<sub>3</sub>), 1.72 (s, 3H, CH<sub>3</sub>), 1.42 (s, 9H, <sup>t</sup>Bu). HRMS (ESI): *m/z*: calcd for C<sub>19</sub>H<sub>27</sub>NO<sub>6</sub>NaS<sub>2</sub>: 452.1178; [*M*+Na]<sup>+</sup> found: 452.1161.

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# CHAPTER 4

## Additional studies:

### Synthesis of bioactive compounds for medicinal chemistry applications

1. Microwave-assisted copper catalyzed one-pot four-component synthesis of multi-functionalized dihydropyridines, *J. Comb. Chem.* **2010**, *12*, 577-581.

Pasunooti, K. K.; Jensen, C. N.; Chai, H.; Leow, M. L.; Zhang, D.-W.; Liu, X.-W.\*

2. A microwave-assisted, copper-catalyzed three-component synthesis of dihydropyrimidinones under mild conditions, *Tetrahedron Lett.* **2011**, *52*, 80-84.

Pasunooti, K. K.; Chai, C.; Jensen, C. N.; Gorityala, B. K.; Wang, S.; Liu, X.-W.\*

3. A general and mild copper-catalyzed three-component synthesis of protected homoallyl amines, *Tetrahedron Lett.* **2009**, *50*, 2979-2981.

Pasunooti, K. K.; Leow, M. L.; Vedachalam, S.; Gorityala, B. K.; Liu, X.-W.\*

# **CHAPTER 4.1**

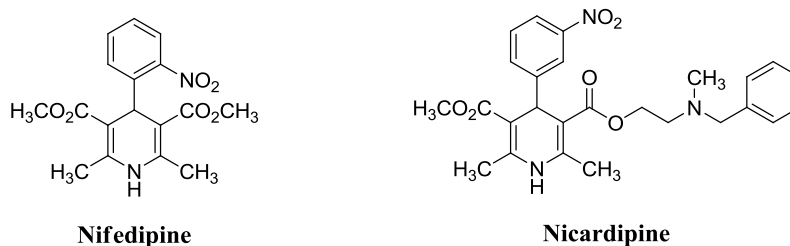
**Microwave-assisted copper catalyzed one-pot four-component synthesis of multi-functionalized dihydropyridines**

## 1. INTRODUCTION

Synthesis of bioactive molecules should preferably be facile, fast and efficient with minimal work-up.<sup>1</sup> In order to suite such requirements, multi-component reactions that directly yield target molecules have attracted considerable attention in both organic and medicinal chemistry.<sup>2</sup> Many chemical processes require a large amount of energy from heating which can have considerable adverse effects on the environment. Therefore, microwave technology is becoming increasingly attractive as an alternative energy source to assist reactions under milder conditions.<sup>3</sup> Microwave irradiation is an extremely powerful tool for reducing reaction time and increasing desired product yields, thus producing many efficient organic reactions.<sup>4,5</sup> Over the past few years our group has extensively focused on the development of new facile and efficient methodologies to synthesize bioactive compounds for medicinal chemistry applications.<sup>6-8</sup>

Multi-substituted 1,4-dihydropyridines (1,4-DHPs) are analogues of NADH co-enzymes and have a wide range of biological activity with such derivatives proving to act as vasodilator, bronchodilator, antiatherosclerotic, antitumor, geroprotective, hepatoprotective and anti-diabetic agents.<sup>9-14</sup> The major biological significance of 1,4-DHPs is that they act as  $\text{Ca}^{2+}$  channel blockers enabling them to be important drugs in the treatment of cardiovascular disease including hypertension, as exemplified by Nifedipine and Nicardipine (Figure 1).<sup>15,16</sup> Recent studies have shown that these 1,4-DHP pharmacophores possess neuroprotectant and platelet anti-aggregatory activities.<sup>17,18</sup> In addition, it has also been indicated that DHPs can be used in the treatment of Alzheimer's disease owing to their antiischemic activity and also as chemosensitizers in tumor therapy.<sup>19</sup> Hence, the development of new methods that lead to multi-

substituted DHPs via an efficient and convenient procedure are of great interest for medicinal chemists.



**Figure 1** Biologically active dihydropyridines.

Multi-component reactions have proven to be a valuable asset in medicinal chemistry, drug design and drug discovery owing to their simplicity, efficiency and high selectivity. Such protocols can reduce the number of steps and present advantages such as, low energy consumption and produce little to no waste leading to desired environmentally friendly processes. The multi-component symmetrical 1,4-dihydropyridine yielding Hantzsch reaction was first established by Hantzsch in 1881 and has attracted considerable attention over the years due to its efficiency to yield bioactive dihydropyridines.<sup>20</sup> However, the classic reaction using aldehyde, ethylacetoacetate and ammonia under acetic condition suffers from disadvantages such as high temperatures, long reaction times, harsh reaction conditions and incomplete conversion of reactants. Thus, over recent years significant effort has been made to find efficient procedures that maintain the simplicity of the Hantzsch reaction but produce better yields. A large number of optimized procedures have been reported by many research groups, the majority of them employed catalytic methods in order to synthesize DHPs. These protocols utilize ionic liquids,<sup>21</sup> triphenyl phosphine,<sup>22</sup> iodine,<sup>23</sup> Baker's yeast,<sup>24</sup> silica supported acids,<sup>25</sup> ceric ammonium nitrate (CAN),<sup>26</sup> organo-catalysts,<sup>27-29</sup> polymers<sup>30,31</sup> and metal-triflates.<sup>32-34</sup> However, many of the

methods still suffer from drawbacks such as high reaction temperatures, incomplete conversion of reactants, expensive metal precursors, stoichiometric amounts of catalyst, environmentally toxic catalysts or prolonged reaction time. In contrast, copper catalysts have become increasingly appealing due to their affordability, high activity and low toxicity. Copper can be used with considerable advantages including mild reaction conditions, high catalytic efficiency, shorter reaction times and no formation of by-products. Recently, our group has developed a copper-catalyzed one pot three-component synthesis of homoallylamines with great success.<sup>35</sup> In this study we focus on the development of new, fast and efficient methodology utilizing microwave technology with a copper catalyst for the preparation of biologically active 1,4-dihydropyridine derivatives *via* a one step multi-component reaction.

## 2. RESULTS AND DISCUSSION

First, the establishment of the reaction conditions was undertaken by investigating the effect of different solvents, temperatures, reaction times and amounts of catalyst using benzaldehyde, ethylacetoacetate, dimedone, and ammonium acetate as standard components (Table 1).<sup>36</sup> Initially, a mixture of benzaldehyde, ethylacetoacetate, dimedone and ammonium acetate in dichloromethane was stirred in the presence of  $\text{Cu}(\text{OTf})_2$  (10 mol%) at room temperature for 24 h and the dihydropyridine product was produced in a low yield (38%) due to incomplete conversion of the reactants, leading to the assumption that more thermal energy may be needed. To focus on the effect of temperature and time, the reaction was carried out at 50 °C for 12 h which allowed the heterocyclic product to form with a slightly higher yet unsatisfactory yield (52%).

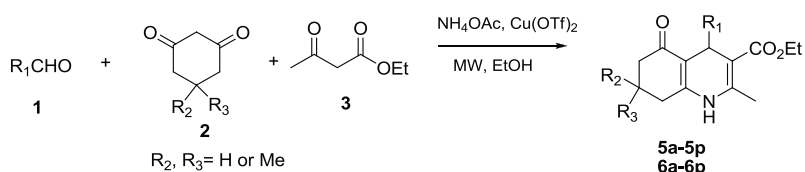
Our attention then moved on to investigate the effect of solvents on the product yields and dichloromethane was replaced by acetonitrile and ethanol. The reactions were stirred for 24 h at room temperature and both solvents resulted in incomplete conversion of reactants, ethanol however gave higher yields. The temperature was then raised to 70 °C for both acetonitrile and ethanol and the mixture was stirred for 12 h. In the case of acetonitrile as the solvent the reaction led to decomposition of the product, therefore producing low yields (55%), however, ethanol produced the desired DHPs in a significantly higher yield (82%). We then focused on lowering the reaction time from 12 h, in which microwave irradiation was utilized due to its ability to lower reaction times and increase yields. A range of temperatures, times, and the amounts of catalysts were screened in order to optimize the one-pot multi-component reaction. For both acetonitrile and ethanol the reaction was then carried out using microwave irradiation at 80 °C for 1 h at 200 W. The reaction utilizing acetonitrile as a solvent suffered from incomplete

conversion of reactants, as indicated by TLC monitoring and thus the reaction in ethanol produced the desired product in much higher yield (95%) than acetonitrile did (65%), proving ethanol to be the most effective solvent. At the same time, decreasing the amount of copper catalyst from 10 mol% to 5 mol% and 2 mol% did not show any significant impact on the product yield. The investigation at this point indicated that ethanol at 80 °C in the presence of 2 mol% Cu(OTf)<sub>2</sub> with a reaction time of 1 h under microwave irradiation were the appropriate conditions for the one-pot synthesis.

Next, we proceeded to lower the reaction time further in order to speed up the reaction and produce desirable yields therefore producing a more efficient reaction. The reaction mixture of benzaldehyde, ethylacetoacetate, dimedone, ammonium acetate and Cu(OTf)<sub>2</sub> (2 mol%) in ethanol was stirred under microwave irradiation at 80 °C for 30 minutes and the DHP product was produced with a similar yield (95%). The temperature was then raised to 100 °C and the reaction mixture was stirred for 15 minutes. Such changes produced the product in similar yields as comparing to that running at 80 °C (95%). When the reaction time was then lowered to 5 minutes at a temperature of 120 °C the resulting DHP product was obtained in a lower yield (65%) suggesting that the temperature was too high which led the components to decompose. From the above investigations, ethanol at 100 °C in the presence of 2 mol% Cu(OTf)<sub>2</sub> with a reaction time of 15 minutes under microwave irradiation at a power of 200 W was established as the optimized reaction conditions for the four-component one-step synthesis.

Under the optimized conditions the copper-catalyzed reactions of a variety of substituted aldehydes were undertaken and a small library of DHPs was created (Table 2). The reactions of different aldehydes possessing a range of substituents from electron-withdrawing (**6c**) to electron-donating (**6d**) produced the heterocyclic products in excellent yields, ranging from 82% to quantitative yields. Un-substituted (**6a**), hetero aromatic (**6k**) and bulky aldehydes such as naphthal (**6p**) were also investigated and led to the production of the corresponding DHPs in excellent yields (81-96%). The use of aldehydes with electron-withdrawing substituents showed no significant effect on the yields of the corresponding DHPs when compared to reactions utilizing aldehydes which possessed electron-donating substituents (compare compounds **6c** and **6d**) and also when compared to those that were un-substituted and those possessing bulky substituents (compare compounds **6c** and **6a**, **6c** and **6p**). We then proceeded to replace the dimedone with 1,3-cyclohexanedione and repeated the reaction with the same substituted aldehydes. Compared with the initial reaction utilizing dimedone, the reactions involving 1,3-cyclohexanedione produced the corresponding DHPs in equally high yields (82% to quantitative yields) (comparing compounds **5b** and **6b**) and similarly the nature of the aldehyde substituents showed no significant effect on the yields (compare compounds **5c** and **5d**, **5c** and **5a**, **5c** and **5p**).

**Table 2.** Cu(OTf)<sub>2</sub> Catalyzed Hantzsch Synthesis of Dihydropyridine Derivatives from Substituted Aromatic Aldehydes



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Entry	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	Product	Yield (%)
1	C <sub>6</sub> H <sub>5</sub>	H	H	<b>5a</b>	95
2	4-MeC <sub>6</sub> H <sub>4</sub>	H	H	<b>5b</b>	quantitative
3	4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	H	H	<b>5c</b>	91
4	4-OMeC <sub>6</sub> H <sub>4</sub>	H	H	<b>5d</b>	98
5	4-CO <sub>2</sub> MeC <sub>6</sub> H <sub>4</sub>	H	H	<b>5e</b>	92
6	4-ClC <sub>6</sub> H <sub>4</sub>	H	H	<b>5f</b>	90
7	4-SMeC <sub>6</sub> H <sub>4</sub>	H	H	<b>5g</b>	quantitative
8	3,4-Me <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	H	H	<b>5h</b>	88
9	3-OHC <sub>6</sub> H <sub>4</sub>	H	H	<b>5i</b>	98
10	2-CF <sub>3</sub> C <sub>6</sub> H <sub>4</sub>	H	H	<b>5j</b>	95
11	2-Furyl	H	H	<b>5k</b>	88
12	3-Thiophenyl	H	H	<b>5l</b>	92
13	n-Pentyl	H	H	<b>5m</b>	82
14	Cyclohexyl	H	H	<b>5n</b>	90
15	2-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	H	H	<b>5o</b>	82
16	1-Naphthyl	H	H	<b>5p</b>	92
17	C <sub>6</sub> H <sub>5</sub>	Me	Me	<b>6a</b>	96
18	4-MeC <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6b</b>	quantitative
19	4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6c</b>	92

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20	4-OMeC <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6d</b>	quantitative
21	4-ClC <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6e</b>	98
22	4-SMeC <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6f</b>	quantitative
23	3,4-Me <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	Me	Me	<b>6g</b>	92
24	3-OHC <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6h</b>	90
25	2-CF <sub>3</sub> C <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6i</b>	87
26	C <sub>5</sub> H <sub>4</sub> N	Me	Me	<b>6j</b>	85
27	2-Furyl	Me	Me	<b>6k</b>	93
28	3-Thiophenyl	Me	Me	<b>6l</b>	86
29	n-Pentyl	Me	Me	<b>6m</b>	94
30	Cyclohexyl	Me	Me	<b>6n</b>	88
31	2-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	Me	Me	<b>6o</b>	81
32	1-Naphthyl	Me	Me	<b>6p</b>	91

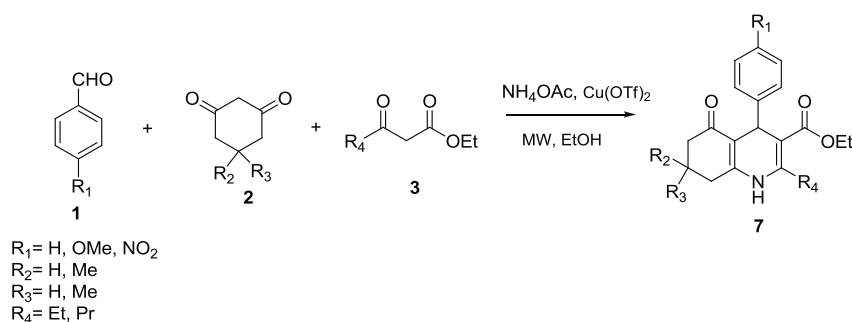
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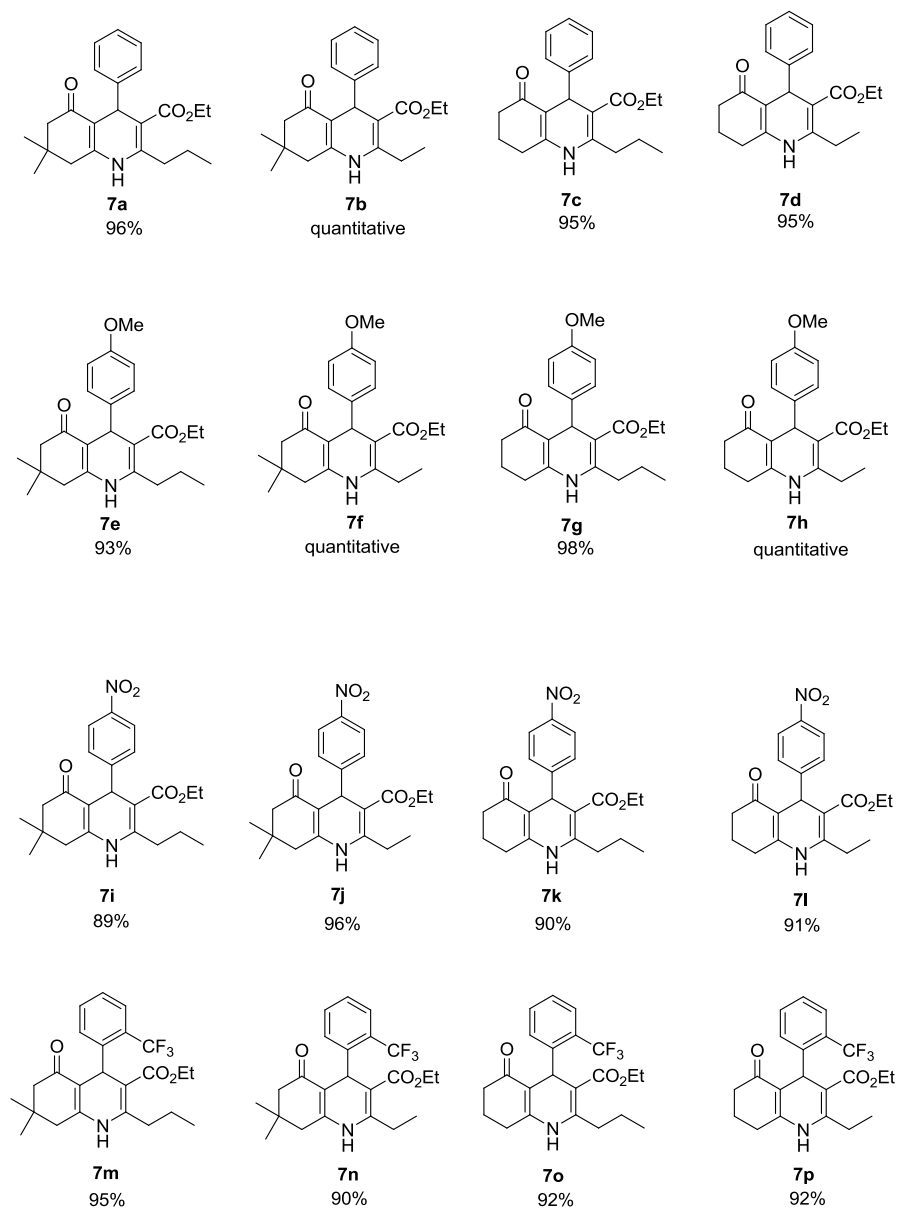
Our attention then focused on replacing ethylacetoacetate in the one-step synthesis (Scheme 1). In the reaction of benzaldehyde, ethyl acetoacetate, dimedone and ammonium acetate the  $\beta$ -ketoester was replaced by ethyl propionylacetate and the corresponding DHP (**7b**) was produced in quantitative yields. At the same time, ethyl butyrylacetate was employed to replace ethyl acetoacetate and the corresponding DHP, **7a**, was formed in similarly high yields (95%). This methodology was also tested with electron-donating (OMe) and electron-withdrawing (NO<sub>2</sub>) substituted benzaldehydes which yielded the desired products, **7e**, **7f**, **7i**, **7j**, in noticeably high quantities (89% to quantitative). The electron withdrawing NO<sub>2</sub> group seemed to have little

effect on the DHP yield when compared to the electron donating OMe product and also the un-substituted product (compare compounds **7f** and **7j**, **7f** and **7b**).

The dimedone component was then replaced with 1,3-cyclohexanedione and the reactions with the ethyl acetoacetate substitution were repeated. The resulting DHPs (**7c**, **7d**, **7g**, **7h**, **7k**, **7l**) were synthesized in equally high yields (90% to quantitative yields) (compare compounds **7a** and **7c**) and the electron withdrawing nitro (NO<sub>2</sub>) group exhibited little effect on the yield when compared to the electron donating methoxy (OMe) product and the un-substituted product (compare compounds **7l** and **7d**, **7l** and **7h**). In medicinal chemistry, fluorine containing components are utilized due to their useful biological functions. Therefore, we carried out the reactions with 2-trifluoromethyl benzaldehyde and the resulting 1,4-dihydropyridine compounds **7m-7p** were produced with similar yields (90–95%).

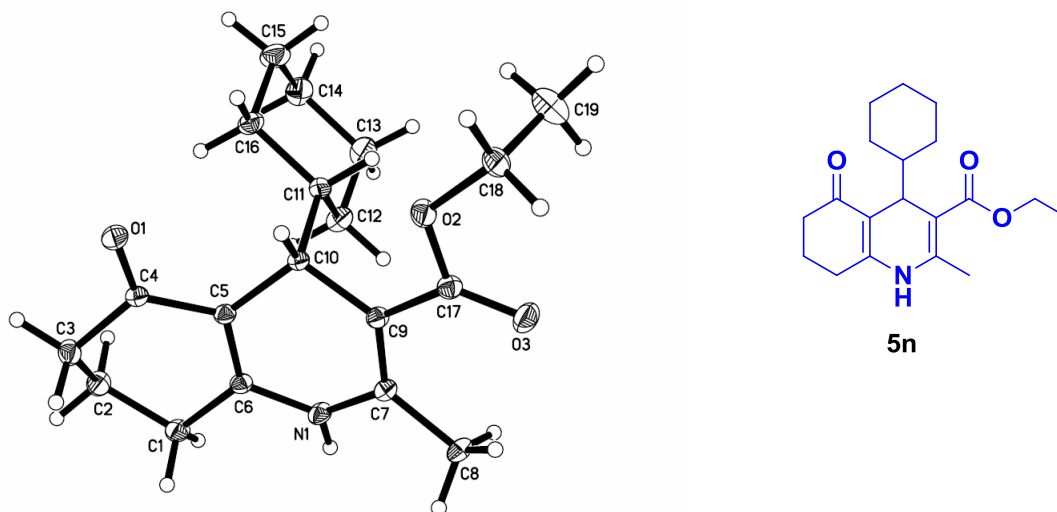
**Scheme 1** Cu(OTf)<sub>2</sub> Catalyzed Hantzsch Synthesis of Dihydropyridine Derivatives.





The products were characterized by  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, HR-MS, and IR spectroscopy and indicated the copper catalyzed reaction yielded pure compounds. The structure of the ethyl 4-cyclohexyl-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate motif was further confirmed by X-ray crystallography (Figure 2, **5n**).

**Figure 2** X-ray crystal structure of ethyl 4-cyclohexyl-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**5n**).



### 3. CONCLUSION

In summary, we have developed a simple and efficient method to generate a range of DHP analogues in excellent yields via a microwave-assisted one-pot four-component reaction. The protocol utilizes  $\text{Cu}(\text{OTf})_2$  in small quantities and mild reaction conditions avoiding work-up and column purification. Reaction times were considerably reduced and product yields increased up to quantitative yields under microwave irradiation. This methodology can be exploited to construct new multi-substituted DHPs, which are of great interest due to their extensive pharmaceutical and biological applications. Currently, biological tests of the synthesized 1,4-DHP analogues are in progress in our laboratory.

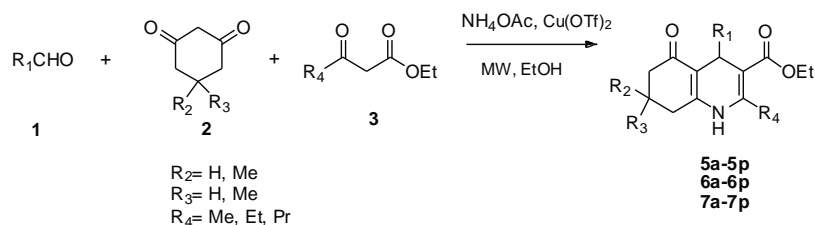
## 4. EXPERIMENTAL

### General Methods

Unless otherwise noted, all reactions were carried out in oven dried glassware under an atmosphere of nitrogen and distilled solvents were transferred by syringes. Solvents and reagents were purified according to the standard procedure prior to use. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 nm). Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on 300 MHz Bruker ACF 300 and 400 MHz Bruker DPX 400 NMR spectrometers. The residual solvent signals were taken as the reference (2.50 ppm for  $^1\text{H}$  NMR spectra and 39.5 ppm for  $^{13}\text{C}$  NMR spectra in  $\text{DMSO-}d_6$ , 7.26 ppm for  $^1\text{H}$  NMR spectra and 77.0 ppm for  $^{13}\text{C}$  NMR spectra in  $\text{CDCl}_3$ ). Chemical shift ( $\delta$ ) is reported in ppm, coupling constants ( $J$ ) are given in Hz. The following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal. Infrared spectra were recorded on a Bio-RAD FTS 165 FT-IR Spectrometer and reported in  $\text{cm}^{-1}$ . Samples were prepared in thin film technique. HR-MS (ESI) spectra were recorded on a Waters Q-T of premier<sup>TM</sup> mass spectrometer. Microwave experiments were conducted in a CEM Discover<sup>TM</sup> system.

## General Experimental Procedures

**For dihydropyridine derivatives from substituted aromatic aldehydes: (5a-5p, 6a-6p, 7a-7p)**



Substituted aromatic aldehyde **1** (1.0 mmol), dimedone or 1,3- cyclohexanedione **2** (1.0 mmol),  $\beta$ -ketoester **3** (1.0 mmol), ammonium acetate (1.0 mmol),  $\text{Cu}(\text{OTf})_2$  (0.02 mmol) and EtOH (2 mL) were added to a 10 mL microwave vessel equipped with a magnetic stirrer. The mixture was heated under microwave irradiation (200 W) at 100 °C for 15 minutes. The progress of reaction was monitored by TLC. After completion, the reaction mixture was cooled to room temperature to allow solidification. Buchner filtration with hexane washing of the precipitates gave pure products (**7a-7p**) without further purification.

## Spectral Details of Dihydropyridine Derivatives

### Ethyl 2-methyl-5-oxo-4-phenyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5a)<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (95% yield); **m.p.** 261-262 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.12 (s, 1H), 7.20-7.04 (m, 5H), 4.90 (s, 1H), 3.98 (q, *J* = 7.0Hz, 2H), 2.48 (m, 2H), 2.28 (s, 3H), 2.22-2.17 (m, 2H), 1.93-1.74 (m, 2H), 1.12 (t, *J* = 7.0Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.1, 167.4, 151.9, 148.3, 145.4, 128.3, 127.9, 126.1, 111.6, 104.1, 59.5, 37.2, 36.1, 26.6, 21.3, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3284, 3073, 2974, 1689, 1607, 1477, 1379, 1284, 721, 694 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>19</sub>H<sub>24</sub>NO<sub>3</sub>: 312.1600, found: 312.1600; Elemental Analysis: Anal. Calcd for C<sub>19</sub>H<sub>21</sub>NO<sub>3</sub>: C, 73.29; H, 6.80; N, 4.50. Found: C, 72.96; H, 7.13; N, 4.29%.

### Ethyl 2-methyl-5-oxo-4-p-tolyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5b)<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (quantitative yield); **m.p.** 244-245 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.12 (s, 1H), 7.07-6.99 (m, 4H), 4.89 (s, 1H), 4.00 (q, *J* = 7.0Hz, 2H), 2.52-2.50 (m, 2H), 2.31 (s, 3H), 2.28-2.22 (m, 2H), 2.23 (s, 3H), 1.94-1.76 (m, 2H), 1.16 (t, *J* = 7.0Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.1, 167.4, 151.7, 145.4, 145.2, 135.0, 128.8, 127.8, 111.7, 104.1, 59.4, 37.2, 35.6, 26.6, 21.3, 21.0, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3277, 3074, 2955, 1695, 1612, 1481, 1379, 1284, 823 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>20</sub>H<sub>24</sub>NO<sub>3</sub>: 326.1756, found: 326.1750.

### Ethyl 2-methyl-4-(4-nitrophenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5c)<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (90% yield); **m.p.** 201-202 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.32 (s, 1H), 8.10 (d, *J* = 8.6Hz, 2H), 7.44 (d, *J* = 8.6Hz, 2H), 5.03 (s, 1H), 3.99 (q, *J* = 7.0Hz, 2H), 2.52-2.49 (m, 2H), 2.34 (s, 3H), 2.25-2.20 (m, 2H), 1.94-1.76 (m, 2H), 1.12 (t, *J* = 7.0Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.1, 166.9, 155.6, 152.5, 146.5, 146.1, 129.2, 123.7, 110.6, 102.8, 59.7, 37.0, 36.9, 26.6, 21.2, 18.8, 14.5; **FT-IR**(KBr):  $\nu_{\max}$  3296, 3082, 2949, 1703, 1604, 1516, 1472, 1381, 1284, 825 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>19</sub>H<sub>21</sub>N<sub>2</sub>O<sub>5</sub>: 357.1450, found: 357.1446.

**Ethyl 4-(4-methoxyphenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5d)**<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (98% yield); **m.p.** 213-214 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.08 (s, 1H), 7.06 (d, *J* = 8.6Hz, 2H), 6.75 (d, *J* = 8.6Hz, 2H), 4.85 (s, 1H), 3.99 (q, *J* = 7.1Hz, 2H), 3.67 (s, 3H), 2.51-2.47 (m, 2H), 2.28 (s, 3H), 2.18-2.13 (m, 2H), 1.92-1.75 (m, 2H), 1.14 (t, *J* = 7.1Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.1, 167.4, 157.8, 151.6, 145.0, 140.6, 128.8, 113.6, 111.8, 104.3, 59.4, 55.3, 37.2, 35.1, 26.6, 21.3, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3267, 3070, 2945, 2902, 1686, 1618, 1489, 1386, 1288, 1117, 827 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>20</sub>H<sub>24</sub>NO<sub>4</sub>: 342.1705, found: 342.1697.

**Ethyl 4-(4-(methoxycarbonyl)phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5e)**

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (92% yield); **m.p.** 190-191 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.20 (s, 1H),

7.79 (d,  $J = 8.3\text{Hz}$ , 2H), 7.28 (d,  $J = 8.3\text{Hz}$ , 2H), 4.95 (s, 1H), 3.96 (q,  $J = 7.1\text{Hz}$ , 2H), 3.80 (s, 3H), 2.50-2.48 (m, 2H), 2.29 (s, 3H), 2.25-2.12 (m, 2H), 1.93-1.69 (m, 2H), 1.09 (t,  $J = 7.1\text{Hz}$ , 3H);  $^{13}\text{C NMR}$  (75MHz, DMSO- $d_6$ ):  $\delta$  196.2, 167.4, 167.0, 153.4, 153.0, 146.0, 129.4, 128.2, 127.6, 110.8, 103.5, 59.9, 52.4, 36.9, 36.6, 26.5, 21.0, 18.5, 14.4; **FT-IR**(KBr):  $\nu_{\text{max}}$  3290, 3074, 2953, 2902, 1726, 1697, 1607, 1485, 1381, 1286, 827  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{21}\text{H}_{40}\text{NO}_5$ : 370.1654, found: 370.1656.

**Ethyl 4-(4-chlorophenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate(5f)**<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (90% yield); **m.p.** 247-248 °C;  $^1\text{H NMR}$  (300MHz, DMSO- $d_6$ ):  $\delta$  9.18 (s, 1H), 7.24 (d,  $J = 8.5\text{Hz}$ , 2H), 7.15 (d,  $J = 8.5\text{Hz}$ , 2H), 4.88 (s, 1H), 3.98 (q,  $J = 7.1\text{Hz}$ , 2H), 2.50-2.46 (m, 2H), 2.29 (s, 3H), 2.24-2.13 (m, 2H), 1.93-1.73 (m, 2H), 1.11 (t,  $J = 7.1\text{Hz}$ , 3H);  $^{13}\text{C NMR}$  (75MHz, DMSO- $d_6$ ):  $\delta$  195.1, 167.2, 152.0, 147.2, 145.8, 130.7, 129.7, 128.2, 111.3, 103.5, 59.6, 37.1, 35.8, 26.6, 21.2, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\text{max}}$  3280, 3076, 2953, 1693, 1647, 1599, 1485, 1386, 1283, 827  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{19}\text{H}_{21}\text{ClNO}_3$ : 346.1210, found: 346.1215.

**Ethyl 2-methyl-4-(4-(methylthio)phenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5g)**

The title compound was prepared according to the general procedure. The product was obtained as a green solid; (quantitative yield); **m.p.** 204-205 °C;  $^1\text{H NMR}$  (300MHz, DMSO- $d_6$ ):  $\delta$  9.15 (s, 1H), 7.11 (m, 4H), 4.87 (s, 1H), 4.00 (q,  $J = 7.1\text{Hz}$ , 2H), 2.53-2.48 (m, 2H), 2.43 (s, 3H), 2.31 (s, 3H), 2.24-2.20 (m, 2H), 1.94-1.76 (m, 2H), 1.15 (t,  $J = 7.1\text{Hz}$ , 3H);  $^{13}\text{C NMR}$  (75MHz,

DMSO- $d_6$ ):  $\delta$  195.1, 167.3, 151.8, 145.4, 145.2, 135.3, 128.5, 126.3, 111.5, 103.9, 59.5, 37.2, 35.6, 26.6, 21.3, 18.7, 15.4, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3279, 3073, 2953, 1694, 1607, 1477, 1381, 1285, 827  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{20}\text{H}_{24}\text{NO}_3\text{S}$ : 358.1477, found: 358.1472.

**Ethyl 4-(3,4-dimethylphenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5h)**

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (88% yield); **m.p.** 245-246  $^{\circ}\text{C}$ ;  **$^1\text{H}$  NMR** (300MHz, DMSO- $d_6$ ):  $\delta$  9.07 (s, 1H), 6.95-6.84 (m, 3H), 4.85 (s, 1H), 4.00 (q,  $J = 7.1\text{Hz}$ , 2H), 2.53-2.49 (m, 2H), 2.29 (s, 3H), 2.23-2.17 (m, 2H), 2.14 (s, 3H), 2.12 (s, 3H), 1.93-1.73 (m, 2H), 1.16 (t,  $J = 7.1\text{Hz}$ , 3H);  **$^{13}\text{C}$  NMR** (75MHz, DMSO- $d_6$ ):  $\delta$  195.1, 167.4, 151.6, 145.8, 145.0, 135.4, 133.7, 129.5, 129.1, 125.2, 111.7, 104.2, 59.4, 37.2, 35.6, 26.6, 21.3, 20.1, 19.4, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3306, 3073, 2970, 2941, 1693, 1622, 1600, 1479, 1381, 1284, 800  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{21}\text{H}_{26}\text{NO}_3$ : 340.1913, found: 340.1920; Elemental Analysis: Anal. Calcd for  $\text{C}_{21}\text{H}_{25}\text{NO}_3$ : C, 74.31; H, 7.42; N, 4.13. Found: C, 74.26; H, 7.19; N, 4.32%.

**Ethyl 4-(3-hydroxyphenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5i)**

The title compound was prepared according to the general procedure. The product was obtained as a green solid; (98% yield); **m.p.** 235-236  $^{\circ}\text{C}$ ;  **$^1\text{H}$  NMR** (300MHz, DMSO- $d_6$ ):  $\delta$  9.10 (s, 1H), 9.08 (s, 1H), 6.98-6.93 (m, 1H), 6.59-6.58 (m, 2H), 6.48-6.45 (m, 1H), 4.84 (s, 1H), 4.00 (q,  $J = 7.1\text{Hz}$ , 2H), 2.51-2.46 (m, 2H), 2.28 (s, 3H), 2.20-2.18 (m, 2H), 1.93-1.74 (m, 2H), 1.15 (t,  $J = 7.1\text{Hz}$ , 3H);  **$^{13}\text{C}$  NMR** (75MHz, DMSO- $d_6$ ):  $\delta$  195.1, 167.5, 157.4, 149.5, 145.1, 129.1, 118.6, 114.9, 113.1, 111.5, 104.0, 59.5, 37.2, 35.8, 26.6, 21.3, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3404,

3285, 3071, 2970, 1674, 1611, 1481, 1375, 1294, 1182, 780  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{19}\text{H}_{22}\text{NO}_4$ : 328.1549, found: 328.1543.

**Ethyl 2-methyl-5-oxo-4-(2-(trifluoromethyl)phenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5j)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (95% yield); **m.p.** 206-207 °C;  **$^1\text{H}$  NMR** (300MHz,  $\text{DMSO}-d_6$ ):  $\delta$  9.11 (s, 1H), 7.51-7.05 (m, 4H), 5.40 (s, 1H), 4.01-3.83 (m, 2H), 2.48-2.46 (m, 2H), 2.24 (s, 3H), 2.19-2.05 (m, 2H), 1.91-1.72 (m, 2H), 1.03 (t,  $J = 7.1\text{Hz}$ , 3H);  **$^{13}\text{C}$  NMR** (75MHz,  $\text{DMSO}-d_6$ ):  $\delta$  194.6, 167.3, 151.8, 147.9, 144.6, 132.6, 131.3, 126.7, 126.4, 112.4, 105.2, 59.4, 37.2, 33.3, 26.7, 21.2, 18.6, 14.3; **FT-IR**(KBr):  $\nu_{\text{max}}$  3289, 3076, 2945, 1703, 1608, 1479, 1379, 1294, 1186, 1142, 766  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{20}\text{H}_{21}\text{F}_3\text{NO}_3$ : 380.1474, found: 380.1467.

**Ethyl 4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5k)**<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (88% yield); **m.p.** 214-215 °C;  **$^1\text{H}$  NMR** (300MHz,  $\text{DMSO}-d_6$ ):  $\delta$  9.21 (s, 1H), 7.36 (s, 1H), 6.23 (s, 1H), 5.83 (s, 1H), 5.07 (s, 1H), 4.07 (t,  $J = 6.9\text{Hz}$ , 2H), 2.49-2.46 (m, 2H), 2.26 (s, 3H), 2.25-2.08 (m, 2H), 1.91-1.73 (m, 2H), 1.18 (t,  $J = 6.9\text{Hz}$ , 3H);  **$^{13}\text{C}$  NMR** (75MHz,  $\text{DMSO}-d_6$ ):  $\delta$  194.9, 167.2, 158.9, 152.8, 146.2, 141.4, 110.6, 108.2, 104.5, 101.1, 59.6, 37.1, 30.0, 26.6, 21.3, 18.6, 14.7; **FT-IR**(KBr):  $\nu_{\text{max}}$  3285, 3078, 2945, 1691, 1609, 1481, 1375, 1287, 1217, 1180, 716  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{17}\text{H}_{20}\text{NO}_4$ : 302.1392, found: 302.1398.

**Ethyl 2-methyl-5-oxo-4-(thiophen-3-yl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5l)**<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (92% yield); **m.p.** 240-241 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.15 (s, 1H), 7.30-7.27 (m, 1H), 6.88-6.86 (m, 2H), 5.01 (s, 1H), 4.09-4.02 (m, 2H), 2.50-2.47 (m, 2H), 2.27 (s, 3H), 2.24-2.21 (m, 2H), 1.96-1.78 (m, 2H), 1.17 (t, *J* = 7.1Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.2, 167.4, 152.2, 148.6, 145.7, 127.9, 125.7, 120.3, 110.9, 103.5, 59.5, 37.2, 31.1, 26.6, 21.3, 18.7, 14.7; **FT-IR**(KBr):  $\nu_{\max}$  3277, 3073, 2949, 1690, 1622, 1481, 1385, 1283, 1180, 752 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>17</sub>H<sub>20</sub>NO<sub>3</sub>S: 318.1164, found: 318.1172.

#### **Ethyl 2-methyl-5-oxo-4-pentyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5m)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (82% yield); **m.p.** 139-140 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 8.88 (s, 1H), 4.12-3.99 (m, 2H), 3.80 (s, 1H), 2.39-2.37 (m, 2H), 2.20 (s, 3H), 2.27-2.12 (m, 2H), 1.95-1.78 (m, 2H), 1.34-1.08 (m, 11H), 0.78 (t, *J* = 6.9Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.4, 167.7, 152.6, 145.8, 110.8, 103.5, 59.3, 37.2, 36.7, 32.0, 29.5, 26.6, 24.3, 22.5, 21.5, 18.6, 14.7, 14.3; **FT-IR**(KBr):  $\nu_{\max}$  3300, 3084, 2953, 2868, 1697, 1647, 1607, 1481, 1390, 1285, 1180 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>18</sub>H<sub>24</sub>NO<sub>3</sub>: 306.2069, found: 306.2063.

#### **Ethyl 4-cyclohexyl-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5n)**

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (90% yield); **m.p.** 229-230 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 8.93 (s, 1H), 4.12-3.99 (m, 2H), 3.79 (d, *J* = 5.2Hz, 1H), 2.50-2.29 (m, 2H), 2.24-2.09 (m, 5H), 1.93-1.78 (m, 2H), 1.58-1.50 (m, 2H), 1.43-1.38 (m, 3H), 1.19 (t, *J* = 7.1Hz, 3H), 1.05-0.96 (m, 4H), 0.81-0.74 (m, 2H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.6, 168.3, 152.9, 145.5, 109.3, 102.2, 59.3, 37.4, 34.5, 29.0, 28.9, 26.8, 26.7, 26.6, 21.3, 18.5, 14.7; **FT-IR**(KBr):  $\nu_{\max}$  3277, 3076, 2928, 2905,

2838, 1694, 1614, 1605, 1485, 1381, 1285, 731  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{19}\text{H}_{28}\text{NO}_3$  : 318.2069, found: 318.2067; Elemental Analysis: Anal. Calcd for  $\text{C}_{19}\text{H}_{27}\text{NO}_3$ : C, 71.89; H, 8.57; N, 4.41. Found: C, 72.07; H, 8.83; N, 4.56%.

**Ethyl 2-methyl-4-(2-nitrophenyl)5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5o)**<sup>26</sup>

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (82% yield); **m.p.** 139-140 °C;  **$^1\text{H}$  NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  9.18 (s, 1H), 7.69-7.66 (m, 1H), 7.56-7.52 (m, 1H), 7.42-7.39 (m, 1H), 7.31-7.26 (m, 1H), 5.63 (s, 1H), 3.98-3.80 (m, 2H), 2.49-2.42 (m, 2H), 2.28 (s, 3H), 2.24-2.03 (m, 2H), 1.88-1.67 (m, 2H), 0.98 (t,  $J = 7.1\text{Hz}$ , 3H);  **$^{13}\text{C}$  NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  194.7, 167.1, 152.1, 148.4, 146.1, 142.4, 133.3, 131.3, 127.3, 123.9, 111.4, 103.5, 59.5, 37.0, 32.4, 26.7, 21.1, 18.7, 14.3; **FT-IR**(KBr):  $\nu_{\text{max}}$  3302, 3088, 2976, 1690, 1618, 1501, 1381, 1342, 1292, 856, 791  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{19}\text{H}_{21}\text{N}_2\text{O}_5$  : 357.1450, found: 357.1447.

**Ethyl 2-methyl-4-(naphthalen-1-yl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5p)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (85% yield); **m.p.** 144-145 °C;  **$^1\text{H}$  NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  9.18 (s, 1H), 8.72-8.69 (m, 1H), 7.77-7.74 (m, 1H), 7.64-7.33 (m, 5H), 5.67 (s, 1H), 3.82-3.66 (m, 2H), 2.50-2.42 (m, 2H), 2.31 (s, 3H), 2.20-2.01 (m, 2H), 1.82-1.66 (m, 2H), 1.05 (t,  $J = 6.9\text{Hz}$ , 3H), 0.81 (t,  $J = 7.0\text{Hz}$ , 3H);  **$^{13}\text{C}$  NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  195.2, 167.5, 151.5, 147.3, 144.6, 133.2, 130.9, 128.0, 126.8, 126.7, 126.3, 125.5, 125.3, 113.2, 106.1, 59.4, 37.2, 31.7, 26.8, 21.2, 18.6, 14.3; **FT-IR**(KBr):  $\nu_{\text{max}}$  3198, 3063, 2949, 1694, 1605, 1495, 1381, 1296, 773  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{23}\text{H}_{24}\text{NO}_3$  : 362.1756, found: 362.1747.

**Ethyl 2,7,7-trimethyl-5-oxo-4-phenyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6a)** <sup>27</sup>

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (96% yield); **m.p.** 212-213 °C; **<sup>1</sup>H NMR** (300MHz, CDCl<sub>3</sub>): δ 7.32-7.09 (m, 5H), 6.25 (s, 1H), 5.06 (s, 1H), 4.06 (q, *J* = 7.1Hz, 2H), 2.36 (s, 3H), 2.29-2.05 (m, 4H), 1.20 (t, *J* = 7.1Hz, 3H), 1.07 (s, 3H), 0.93 (s, 3H); **<sup>13</sup>C NMR** (75MHz, CDCl<sub>3</sub>): δ 195.5, 167.5, 148.2, 147.0, 128.0, 127.9, 126.0, 106.1, 59.8, 50.7, 41.1, 36.6, 32.7, 29.4, 27.2, 19.4, 14.2; **FT-IR**(KBr):  $\nu_{\max}$  3273, 3075, 2959, 1697, 1605, 1489, 1381, 1282, 696 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>21</sub>H<sub>26</sub>NO<sub>3</sub>: 340.1913, found: 340.1912.

**Ethyl 2,7,7-trimethyl-5-oxo-4-p-tolyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6b)** <sup>27</sup>

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (Quantitative yield); **m.p.** 262-263 °C; **<sup>1</sup>H NMR** (300MHz, CDCl<sub>3</sub>): δ 7.18 (d, *J* = 7.9 Hz, 2H), 6.99 (d, *J* = 7.8Hz, 2H), 6.67 (s, 1H), 5.01 (s, 1H), 4.06 (q, *J* = 7.1Hz, 2H), 2.32 (s, 3H), 1.21 (t, *J* = 7.1Hz, 3H), 1.05 (s, 3H), 0.94 (s, 3H); **<sup>13</sup>C NMR** (75MHz, CDCl<sub>3</sub>): δ 194.7, 167.4, 149.8, 145.3, 145.2, 135.0, 128.7, 127.8, 110.6, 104.3, 59.4, 50.7, 35.9, 32.6, 29.6, 26.9, 21.0, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3271, 3076, 2959, 1701, 1605, 1493, 1379, 1280 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>22</sub>H<sub>28</sub>NO<sub>3</sub>: 354.2069, found: 354.2061.

**Ethyl 2,7,7-trimethyl-4-(4-nitrophenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6c)**

The title compound was prepared according to the general procedure. The product was obtained as a green solid; (92% yield); **m.p.** 180-181 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.22 (s, 1H), 8.09 (d, *J* = 8.7 Hz, 2H), 7.41 (d, *J* = 8.7Hz, 2H), 4.97 (s, 1H), 3.95 (q, *J* = 7.8Hz, 2H), 2.50-2.41

(m, 2H), 2.31 (s, 3H), 2.27-1.94 (m, 2H), 1.10 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.81 (s, 3H);  $^{13}\text{C}$  NMR (75MHz, DMSO- $d_6$ ):  $\delta$  194.7, 166.9, 155.5, 150.5, 146.6, 146.1, 129.2, 123.6, 109.5, 102.8, 59.7, 50.5, 40.9, 37.1, 32.6, 29.5, 26.9, 18.8, 14.5; FT-IR(KBr):  $\nu_{\text{max}}$  3286, 3076, 2959, 1674, 1603, 1489, 1379, 1225, 833  $\text{cm}^{-1}$ ; HRMS(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{21}\text{H}_{25}\text{N}_2\text{O}_5$ : 385.1763, found: 385.1758.

**Ethyl 4-(4-methoxyphenyl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6d)**<sup>34</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (Quantitative yield); **m.p.** 256-257 °C;  $^1\text{H}$  NMR (400MHz, DMSO- $d_6$ ):  $\delta$  9.01 (s, 1H), 7.05 (d,  $J = 8.5$  Hz, 2H), 6.74 (d,  $J = 8.5$  Hz, 2H), 4.79 (s, 1H), 3.97 (q,  $J = 7.1\text{Hz}$ , 2H), 3.67 (s, 3H), 2.43-2.25 (m, 2H), 2.17 (s, 3H), 2.14-1.95 (m, 2H), 1.13 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.85 (s, 3H);  $^{13}\text{C}$  NMR (100MHz, DMSO- $d_6$ ):  $\delta$  194.7, 167.4, 157.7, 150.0, 145.1, 140.5, 128.9, 113.5, 110.7, 104.4, 59.5, 55.3, 50.7, 35.4, 32.6, 29.6, 27.0, 18.7, 14.6; FT-IR(KBr):  $\nu_{\text{max}}$  3269, 3078, 2957, 1699, 1599, 1510, 1375, 1279, 849  $\text{cm}^{-1}$ ; HRMS(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{22}\text{H}_{28}\text{NO}_4$ : 370.2018, found: 370.2013; Elemental Analysis: Anal. Calcd for  $\text{C}_{22}\text{H}_{27}\text{NO}_4$ : C, 71.52; H, 7.37; N, 3.79. Found: C, 71.98; H, 7.3; N, 4.35%.

**Ethyl 4-(4-chlorophenyl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6e)**<sup>27</sup>

The title compound was prepared according to the general procedure. The product was obtained as a green solid; (98% yield); **m.p.** 237-238 °C;  $^1\text{H}$  NMR (300MHz, DMSO- $d_6$ ):  $\delta$  9.10 (s, 1H), 7.24 (d,  $J = 8.5$  Hz, 2H), 7.15 (d,  $J = 8.5\text{Hz}$ , 2H), 4.84 (s, 1H), 3.96 (q,  $J = 7.1\text{Hz}$ , 2H), 2.50-2.38 (m, 2H), 2.29 (s, 3H), 2.19-1.94 (m, 2H), 1.11 (t,  $J = 7.1\text{Hz}$ , 3H), 0.99 (s, 3H), 0.82 (s, 3H);  $^{13}\text{C}$

**NMR** (75MHz, DMSO- $d_6$ ):  $\delta$  194.7, 167.1, 150.1, 147.0, 145.9, 130.7, 129.8, 128.1, 110.1, 103.6, 59.6, 50.6, 36.1, 32.6, 29.5, 26.9, 18.8, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3275, 3071, 2934, 1703, 1602, 1494, 1381, 1280, 833  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{21}\text{H}_{25}\text{ClNO}_3$ : 374.1523, found: 374.1515.

**Ethyl 2,7,7-trimethyl-4-(4-(methylthio)phenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6f)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (Quantitative yield); **m.p.** 242-243  $^{\circ}\text{C}$ ;  **$^1\text{H}$  NMR** (300MHz, DMSO- $d_6$ ):  $\delta$  9.05 (s, 1H), 7.09 (s, 4H), 4.81 (s, 1H), 3.97 (q,  $J = 7.1\text{Hz}$ , 2H), 2.49-2.25 (m, 2H), 2.40 (s, 3H), 2.28 (s, 3H), 2.19-1.94 (m, 2H), 1.13 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.85 (s, 3H);  **$^{13}\text{C}$  NMR** (75MHz, DMSO- $d_6$ ):  $\delta$  194.7, 167.3, 149.9, 145.4, 145.1, 135.3, 128.6, 128.1, 110.3, 104.0, 59.5, 50.7, 35.9, 32.6, 29.6, 27.0, 18.8, 15.3, 14.6; **FT-IR**(KBr):  $\nu_{\max}$  3273, 3076, 2957, 1701, 1601, 1495, 1381, 1279, 843  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{22}\text{H}_{28}\text{NO}_3\text{S}$ : 386.1790, found: 386.1792.

**Ethyl 4-(3,4-dimethylphenyl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6g)**

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (92% yield); **m.p.** 262-263  $^{\circ}\text{C}$ ;  **$^1\text{H}$  NMR** (400MHz, DMSO- $d_6$ ):  $\delta$  8.98 (s, 1H), 6.93-6.83 (m, 3H), 4.77 (s, 1H), 3.97 (q,  $J = 7.1\text{Hz}$ , 2H), 2.43-2.31 (m, 2H), 2.26 (s, 3H), 2.11 (s, 3H), 2.10 (s, 3H), 2.17-1.94 (m, 2H), 1.15 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.87 (s, 3H);  **$^{13}\text{C}$  NMR** (100MHz, DMSO- $d_6$ ):  $\delta$  194.7, 167.4, 149.8, 145.7, 145.0, 135.3, 133.7, 129.4, 129.3, 125.3, 110.5, 104.4, 59.4, 50.7, 35.8, 32.6, 29.7, 26.9, 20.0, 19.4, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\max}$

3275, 3078, 2960, 1697, 1604, 1499, 1381, 1281, 843  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{23}\text{H}_{30}\text{NO}_3$ : 368.2226, found: 368.2221; Elemental Analysis: Anal. Calcd for  $\text{C}_{23}\text{H}_{29}\text{NO}_3$ : C, 75.17; H, 7.95; N, 3.81. Found: C, 75.56; H, 8.09; N, 4.11%.

**Ethyl 4-(3-hydroxyphenyl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6h)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (90% yield); **m.p.** 148-150  $^{\circ}\text{C}$ ;  **$^1\text{H}$  NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  9.07 (s, 1H), 9.01 (s, 1H), 6.97-6.92 (m, 1H), 6.60-6.57 (m, 2H), 6.47-6.45 (m, 1H), 4.80 (s, 1H), 3.99 (q,  $J = 7.1\text{Hz}$ , 2H), 2.44-2.25 (m, 2H), 2.28 (s, 3H), 2.19-1.96 (m, 2H), 1.15 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.87 (s, 3H);  **$^{13}\text{C}$  NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  194.7, 167.4, 157.3, 149.9, 149.4, 145.2, 129.0, 118.6, 115.0, 113.1, 110.4, 104.1, 59.5, 50.8, 36.1, 32.6, 29.6, 27.0, 18.7, 14.6; **FT-IR**(KBr):  $\nu_{\text{max}}$  3526, 3456, 3192, 3080, 2957, 1701, 1681, 1587, 1491, 1373, 1273, 770  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{21}\text{H}_{26}\text{NO}_4$ : 356.1862, found: 356.1855; Elemental Analysis: Anal. Calcd for  $\text{C}_{21}\text{H}_{25}\text{NO}_4$ : C, 70.96; H, 7.09; N, 3.94. Found: C, 71.06; H, 7.24; N, 4.29%.

**Ethyl 2,7,7-trimethyl-5-oxo-4-(2-(trifluoromethyl)phenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6i)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (87% yield); **m.p.** 177-178  $^{\circ}\text{C}$ ;  **$^1\text{H}$  NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  9.05 (s, 1H), 7.51-7.23 (m, 4H), 5.39 (s, 1H), 4.04-3.93 (m, 2H), 2.49-2.25 (m, 2H), 2.24 (s, 3H), 2.16-1.86 (m, 2H), 1.03 (t,  $J = 7.1\text{Hz}$ , 3H), 0.99 (s, 3H), 0.80 (s, 3H);  **$^{13}\text{C}$  NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  194.2, 167.3, 150.0, 147.6, 144.8, 132.4, 131.1, 126.8, 126.7, 126.5, 111.1, 105.2, 59.4, 50.6,

33.2, 32.5, 29.5, 26.8, 18.7, 14.3; **FT-IR**(KBr):  $\nu_{\max}$  3277, 3070, 2965, 1736, 1697, 1604, 1493, 1381, 1273, 1155, 768  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{22}\text{H}_{25}\text{F}_3\text{NO}_3$ : 408.1787, found: 408.1777; Elemental Analysis: Anal. Calcd for  $\text{C}_{22}\text{H}_{24}\text{F}_3\text{NO}_3$ : C, 64.85; H, 5.94; N, 3.44. Found: C, 64.75; H, 6.17; N, 3.03%.

**Ethyl 2,7,7-trimethyl-5-oxo-4-(pyridin-4-yl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate**  
**(6j)**<sup>34</sup>

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (85% yield); **m.p.** 194-195 °C; **<sup>1</sup>H NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  9.20 (s, 1H), 8.38 (d,  $J = 4.9\text{Hz}$ , 2H), 7.13 (d,  $J = 4.9\text{Hz}$ , 2H), 4.87 (s, 1H), 3.97 (q,  $J = 7.0\text{Hz}$ , 2H), 2.45-2.26 (m, 2H), 2.32 (s, 3H), 2.20-1.96 (m, 2H), 1.10 (t,  $J = 7.0\text{Hz}$ , 3H), 0.99 (s, 3H), 0.81 (s, 3H); **<sup>13</sup>C NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  194.7, 166.9, 155.9, 150.7, 149.7, 146.7, 123.3, 109.2, 102.4, 59.7, 50.6, 36.3, 32.6, 29.5, 26.8, 18.8, 14.5; **FT-IR**(KBr):  $\nu_{\max}$  3292, 3070, 2959, 1674, 1645, 1630, 1601, 1503, 1381, 1283, 787  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[M+H]^+$ : calcd. for  $\text{C}_{20}\text{H}_{25}\text{N}_2\text{O}_3$ : 341.1865, found: 341.1869.

**Ethyl 4-(furan-2-yl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate**  
**(6k)**<sup>27</sup>

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (93% yield); **m.p.** 246-247 °C; **<sup>1</sup>H NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  9.14 (s, 1H), 7.35 (s, 1H), 6.23 (s, 1H), 5.84 (s, 1H), 5.03 (s, 1H), 4.06 (t,  $J = 6.4\text{Hz}$ , 2H), 2.44-2.22 (m, 2H), 2.26 (s, 3H), 2.21-2.02 (m, 2H), 1.18 (t,  $J = 6.9\text{Hz}$ , 3H), 1.02 (s, 3H), 0.93 (s, 3H); **<sup>13</sup>C NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  194.6, 167.2, 158.9, 150.9, 146.3, 141.2, 110.6, 107.1, 104.4, 101.1, 59.6, 50.7, 32.6, 30.0, 29.6, 26.8, 18.7, 14.7; **FT-IR**(KBr):  $\nu_{\max}$  3279, 3080, 2963, 1676, 1603,

1476, 1396, 731  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{19}\text{H}_{24}\text{NO}_4$ : 330.1705, found: 330.1716.

**Ethyl 2,7,7-trimethyl-5-oxo-4-(thiophen-3-yl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6l)**<sup>27</sup>

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (86% yield); **m.p.** 242-243 °C; **<sup>1</sup>H NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  9.09 (s, 1H), 7.29-7.27 (m, 1H), 6.89-6.84 (m, 2H), 4.98 (s, 1H), 4.03 (q,  $J = 7.1\text{Hz}$ , 2H), 2.42-2.26 (m, 2H), 2.28 (s, 3H), 2.21-2.01 (m, 2H), 1.15 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.87 (s, 3H); **<sup>13</sup>C NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  194.8, 167.3, 150.2, 148.5, 145.9, 127.8, 125.6, 120.2, 109.9, 103.4, 59.5, 50.7, 32.6, 31.2, 29.5, 27.0, 18.8, 14.7; **FT-IR**(KBr):  $\nu_{\text{max}}$  3287, 3084, 2961, 1678, 1667, 1597, 1493, 1391, 1228, 775  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{19}\text{H}_{24}\text{NO}_3\text{S}$ : 346.1477, found: 346.1471.

**Ethyl 2,7,7-trimethyl-5-oxo-4-pentyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6m)**

(6m)

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (94% yield); **m.p.** 147-148 °C; **<sup>1</sup>H NMR** (300MHz,  $\text{DMSO-}d_6$ ):  $\delta$  8.80 (s, 1H), 4.12-4.40 (m, 2H), 3.79 (s, 1H), 2.50-2.01 (m, 4H), 2.20 (s, 3H), 1.21-1.16 (m, 11H), 1.00 (s, 3H), 0.98 (s, 3H), 0.78 (t,  $J = 6.6\text{Hz}$ , 3H); **<sup>13</sup>C NMR** (75MHz,  $\text{DMSO-}d_6$ ):  $\delta$  195.0, 167.6, 150.9, 146.0, 109.6, 103.3, 59.3, 50.9, 36.5, 32.4, 32.0, 29.8, 29.6, 26.8, 24.5, 22.6, 18.6, 14.7, 14.3; **FT-IR**(KBr):  $\nu_{\text{max}}$  3273, 3078, 2936, 1703, 1637, 1595, 1495, 1391, 775  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{20}\text{H}_{32}\text{NO}_3$ : 334.2382, found: 334.2375.

**Ethyl 4-cyclohexyl-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6n)**

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (88% yield); **m.p.** 210-211 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 8.84 (s, 1H), 4.08-4.02 (m, 2H), 3.77 (d, *J* = 4.6Hz, 1H), 2.49-2.10 (m, 4H), 2.17 (s, 3H), 1.61-1.42 (m, 5H), 1.19 (t, *J* = 7.1Hz, 3H), 1.10-0.95 (m, 4H), 1.02 (s, 3H), 1.01 (s, 3H), 0.81-0.75 (m, 2H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 195.2, 168.3, 151.4, 145.8, 108.3, 101.9, 59.3, 51.0, 46.1, 32.3, 30.0, 29.1, 27.1, 26.8, 26.7, 18.6, 14.8; **FT-IR**(KBr):  $\nu_{\max}$  3291, 3074, 2932, 2847, 1697, 1639, 1601, 1493, 1387, 1278, 754 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>21</sub>H<sub>32</sub>NO<sub>3</sub>: 346.2382, found: 346.2381; Elemental Analysis: Anal. Calcd for C<sub>21</sub>H<sub>31</sub>NO<sub>3</sub>: C, 73.01; H, 9.04; N, 4.44. Found: C, 72.9; H, 9.27; N, 4.36%.

**Ethyl 2,7,7-trimethyl-4-(2-nitrophenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6o)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (81% yield); **m.p.** 197-198 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.13 (s, 1H), 7.72-7.27 (m, 4H), 5.64 (s, 1H), 3.96-3.82 (m, 2H), 2.43-2.21 (m, 2H), 2.29 (s, 3H), 2.14-1.85 (m, 2H), 1.01-0.96 (m, 6H), 0.75 (s, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 194.4, 167.0, 150.2, 148.1, 146.3, 142.4, 133.3, 131.1, 127.3, 124.0, 110.3, 103.5, 59.5, 50.5, 32.5, 32.3, 29.3, 26.8, 18.8, 14.3; **FT-IR**(KBr):  $\nu_{\max}$  3292, 3084, 2964, 1697, 1616, 1528, 1487, 1381, 1341, 1283, 755 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>21</sub>H<sub>25</sub>N<sub>2</sub>O<sub>5</sub>: 385.1763, found: 385.1759.

**Ethyl 2,7,7-trimethyl-4-(naphthalen-1-yl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (6p)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (91% yield); **m.p.** 197-198 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.13 (s, 1H), 8.68-8.65 (m, 1H), 7.80-7.36 (m, 6H), 5.64 (s, 1H), 3.83-3.67 (m, 2H), 2.50-2.31 (m, 2H), 2.31 (s, 3H), 2.16-1.84 (m, 2H), 1.00 (s, 3H), 0.84 (t, *J* = 7.1Hz, 3H), 0.80 (s, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 194.8, 167.4, 149.5, 147.1, 144.6, 133.2, 130.9, 128.1, 126.8, 126.7, 126.3, 126.1, 125.5, 125.3, 111.9, 106.0, 59.4, 50.7, 32.5, 31.8, 29.6, 26.8, 18.7, 14.3; **FT-IR**(KBr):  $\nu_{\max}$  3285, 3069, 2953, 1699, 1603, 1485, 1377, 1278, 779 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>25</sub>H<sub>28</sub>NO<sub>3</sub>: 390.2069, found: 390.2071.

**Ethyl 7,7-dimethyl-5-oxo-4-phenyl-2-propyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate**  
**(7a)**

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (96% yield); **m.p.** 195-196 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 9.02 (s, 1H), 7.17-7.03 (m, 5H), 4.87 (s, 1H), 3.97 (q, *J* = 7.1Hz, 2H), 2.70-2.64 (m, 2H), 2.45-2.26 (m, 2H), 2.20-1.94 (m, 2H), 1.60-1.53 (m, 2H), 1.13 (t, *J* = 7.1Hz, 3H), 1.00 (s, 3H), 0.92 (t, *J* = 7.3Hz, 3H), 0.83 (s, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO- *d*<sub>6</sub>): δ 194.6, 167.0, 150.2, 149.6, 148.1, 128.2, 127.9, 126.1, 110.3, 103.9, 59.5, 50.7, 36.3, 33.3, 32.6, 29.6, 26.9, 22.4, 14.5, 14.2; **FT-IR**(KBr):  $\nu_{\max}$  3283, 3082, 2957, 1676, 1609, 1489, 1381, 1227, 700 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>23</sub>H<sub>30</sub>NO<sub>3</sub> : 368.2226, found: 368.2227; Elemental Analysis: Anal. Calcd for C<sub>23</sub>H<sub>29</sub>NO<sub>3</sub>: C, 75.17; H, 7.95; N, 3.81. Found: C, 75.6; H, 8.41; N, 3.92%.

**Ethyl 2-ethyl-7,7-dimethyl-5-oxo-4-phenyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate**  
**(7b)**

The title compound was prepared according to the general procedure. The product was obtained as a white solid; (quantitative yield); **m.p.** 159-160 °C; **<sup>1</sup>H NMR** (300MHz, DMSO- *d*<sub>6</sub>): δ 9.04 (s, 1H), 7.20-7.03 (m, 5H), 4.86 (s, 1H), 3.97 (q, *J* = 7.1Hz, 2H), 2.73-2.67 (m, 2H), 2.46-2.26 (m, 2H), 2.20-1.93 (m, 2H), 1.12 (t, *J* = 7.1Hz, 6H), 1.00 (s, 3H), 0.82 (s, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO- *d*<sub>6</sub>): δ 194.7, 167.0, 151.2, 150.3, 148.2, 128.2, 127.9, 126.2, 110.4, 103.3, 59.6, 50.8, 36.3, 32.6, 29.7, 26.8, 25.0, 14.6, 13.8; **FT-IR**(KBr):  $\nu_{\max}$  3300, 3084, 2963, 1696, 1622, 1477, 1383, 1298, 698 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>22</sub>H<sub>28</sub>NO<sub>3</sub> : 354.2069, found: 354.2068; Elemental Analysis: Anal. Calcd for C<sub>22</sub>H<sub>27</sub>NO<sub>3</sub>: C, 74.76; H, 7.70; N, 3.96. Found: C, 75.12; H, 7.44; N, 4.38%.

#### **Ethyl 5-oxo-4-phenyl-2-propyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7c)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (95% yield); **m.p.** 139-140°C; **<sup>1</sup>H NMR** (300MHz, DMSO- *d*<sub>6</sub>): δ 9.09 (s, 1H), 7.16-7.05 (m, 5H), 4.93 (s, 1H), 3.98 (q, *J* = 7.0Hz, 2H), 2.69-2.63 (m, 2H), 2.49 (m, 2H), 2.18 (m, 2H), 1.87-1.73 (m, 2H), 1.57 (q, *J* = 7.3Hz, 2H), 1.12 (t, *J* = 7.0Hz, 3H), 0.93 (t, *J* = 7.3Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO- *d*<sub>6</sub>): δ 195.0, 167.1, 152.0, 149.5, 148.3, 128.2, 127.8, 126.1, 111.4, 103.8, 59.5, 37.2, 36.1, 33.4, 26.6, 22.4, 21.3, 14.5, 14.2; **FT-IR**(KBr):  $\nu_{\max}$  3283, 3073, 2957, 1678, 1612, 1489, 1385, 1286, 698 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>21</sub>H<sub>26</sub>NO<sub>3</sub> : 340.1913, found: 340.1911.

#### **Ethyl 2-ethyl-5-oxo-4-phenyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7d)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (95% yield); **m.p.** 177-178 °C; **<sup>1</sup>H NMR** (300MHz, DMSO- *d*<sub>6</sub>): δ 9.09 (s, 1H), 7.20-7.05 (m, 5H), 4.90 (s, 1H), 3.98 (q, *J* = 6.9Hz, 2H), 2.72-2.68 (m, 2H), 2.50-2.48 (m, 2H),

2.18 (m, 2H), 1.86-1.72 (m, 2H), 1.13 (t,  $J = 6.9\text{Hz}$ , 3H), 1.06 (t,  $J = 7.0\text{Hz}$ , 3H);  $^{13}\text{C NMR}$  (75MHz, DMSO-  $d_6$ ):  $\delta$  195.0, 167.0, 152.0, 151.0, 148.3, 128.2, 127.8, 126.1, 111.5, 103.3, 59.5, 37.2, 36.1, 26.6, 25.0, 21.2, 14.5, 13.7; **FT-IR**(KBr):  $\nu_{\text{max}}$  3289, 3073, 2949, 1695, 1608, 1479, 1383, 1296, 694  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{20}\text{H}_{24}\text{NO}_3$  : 326.1756, found: 326.1760.

**Ethyl 4-(4-methoxyphenyl)-7,7-dimethyl-5-oxo-2-propyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7e)**

The title compound was prepared according to the general procedure. The product was obtained as a green solid; (93% yield); **m.p.** 183-184 °C;  $^1\text{H NMR}$  (300MHz, DMSO- $d_6$ ):  $\delta$  8.97 (s, 1H), 7.05 (d,  $J = 8.6\text{Hz}$ , 2H), 6.73 (d,  $J = 8.6\text{Hz}$ , 2H), 4.81 (s, 1H), 3.97 (q,  $J = 7.1\text{Hz}$ , 2H), 3.66 (s, 3H), 2.72-2.60 (m, 2H), 2.44-2.25 (m, 2H), 2.19-1.93 (m, 2H), 1.59-1.52 (m, 2H), 1.14 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.92 (t,  $J = 7.3\text{Hz}$ , 3H), 0.84 (s, 3H);  $^{13}\text{C NMR}$  (75MHz, DMSO-  $d_6$ ):  $\delta$  194.6, 167.1, 157.7, 149.9, 149.2, 140.5, 128.8, 113.5, 110.5, 104.2, 59.5, 55.3, 50.7, 35.4, 33.3, 32.6, 29.6, 26.9, 22.4, 14.6, 14.2; **FT-IR**(KBr):  $\nu_{\text{max}}$  3269, 3092, 2955, 1695, 1604, 1487, 1381, 1259, 1219, 853  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{24}\text{H}_{32}\text{NO}_4$  : 398.2331, found: 398.2333; Elemental Analysis: Anal. Calcd for  $\text{C}_{24}\text{H}_{31}\text{NO}_4$ : C, 72.52; H, 7.86; N, 3.52. Found: C, 75.92; H, 8.10; N, 3.94%.

**Ethyl 2-ethyl-4-(4-methoxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7f)**

The title compound was prepared according to the general procedure. The product was obtained as a green solid; (quantitative yield); **m.p.** 187-188 °C;  $^1\text{H NMR}$  (300MHz, DMSO-  $d_6$ ):  $\delta$  8.99 (s, 1H), 7.05 (d,  $J = 8.5\text{Hz}$ , 2H), 6.73 (d,  $J = 8.5\text{Hz}$ , 2H), 4.79 (s, 1H), 3.95 (q,  $J = 7.1\text{Hz}$ , 2H),

3.66 (s, 3H), 2.68 (q,  $J = 7.0\text{Hz}$ , 2H), 2.44-2.24 (m, 2H), 2.19-1.92 (m, 2H), 1.15-1.09 (m, 6H), 0.99 (s, 3H), 0.83 (s, 3H);  $^{13}\text{C NMR}$  (75MHz, DMSO-  $d_6$ ):  $\delta$  194.7, 167.0, 157.7, 150.8, 149.9, 128.8, 113.5, 110.6, 103.6, 59.5, 55.3, 50.7, 35.3, 32.5, 29.7, 26.8, 25.0, 14.6, 13.7; **FT-IR**(KBr):  $\nu_{\text{max}}$  3273, 3076, 2959, 1697, 1601, 1489, 1383, 1296, 1250, 849  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{23}\text{H}_{30}\text{NO}_4$  : 384.2175, found: 384.2184; Elemental Analysis: Anal. Calcd for  $\text{C}_{23}\text{H}_{29}\text{NO}_4$ : C, 72.04; H, 7.62; N, 3.65. Found: C, 72.12; H, 7.94; N, 3.54%.

### **Ethyl 4-(4-methoxyphenyl)-5-oxo-2-propyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7g)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (98% yield); **m.p.** 145-147 °C;  $^1\text{H NMR}$  (300MHz, DMSO- $d_6$ ):  $\delta$  9.04 (s, 1H), 7.04 (d,  $J = 8.7\text{Hz}$ , 2H), 6.73 (d,  $J = 8.7\text{Hz}$ , 2H), 4.84 (s, 1H), 3.97 (q,  $J = 7.1\text{Hz}$ , 2H), 3.66 (s, 3H), 2.72-2.56 (m, 2H), 2.50-2.45 (m, 2H), 2.21-1.16 (m, 2H), 1.98-1.72 (m, 2H), 1.13 (t,  $J = 7.1\text{Hz}$ , 3H), 0.92 (t,  $J = 7.4\text{Hz}$ , 3H);  $^{13}\text{C NMR}$  (75MHz, DMSO-  $d_6$ ):  $\delta$  195.0, 167.2, 157.7, 149.1, 140.7, 128.7, 113.6, 111.7, 104.1, 59.5, 55.3, 37.2, 35.2, 33.4, 26.6, 22.4, 14.6, 14.2; **FT-IR**(KBr):  $\nu_{\text{max}}$  3292, 3078, 2965, 1695, 1605, 1483, 1383, 1234, 833  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{22}\text{H}_{28}\text{NO}_4$  : 370.2018, found: 370.2018.

### **Ethyl 2-ethyl-4-(4-methoxyphenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7h)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (quantitative yield); **m.p.** 176-178 °C;  $^1\text{H NMR}$  (300MHz, DMSO-  $d_6$ ):  $\delta$  9.06 (s, 1H), 7.04 (d,  $J = 8.6\text{Hz}$ , 2H), 6.73 (d,  $J = 8.6\text{Hz}$ , 2H), 4.83 (s, 1H), 3.98 (q,  $J = 7.1\text{Hz}$ , 2H), 3.66 (s, 3H), 2.72-2.65 (m, 2H), 2.51-2.47 (m, 2H), 2.21-2.11 (m, 2H), 1.92-1.72 (m, 2H), 1.12 (t,

$J = 7.1\text{Hz}$ , 6H);  $^{13}\text{C NMR}$  (75MHz, DMSO-  $d_6$ ):  $\delta$  195.0, 167.1, 157.7, 151.7, 150.6, 128.8, 113.6, 111.8, 103.5, 59.5, 55.3, 37.2, 35.2, 26.5, 24.9, 21.3, 14.6, 13.7; **FT-IR**(KBr):  $\nu_{\text{max}}$  3339, 3078, 2978, 1694, 1605, 1483, 1383, 1298, 1260, 849  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{21}\text{H}_{26}\text{NO}_4$  : 356.1862, found: 356.1862.

**Ethyl 7,7-dimethyl-4-(4-nitrophenyl)-5-oxo-2-propyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7i)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (89% yield); **m.p.** 190-191  $^{\circ}\text{C}$ ;  $^1\text{H NMR}$  (300MHz, DMSO- $d_6$ ):  $\delta$  9.19 (s, 1H), 8.09 (d,  $J = 8.5\text{Hz}$ , 2H), 7.41 (d,  $J = 8.5\text{Hz}$ , 2H), 4.99 (s, 1H), 3.96 (q,  $J = 7.1\text{Hz}$ , 2H), 2.72-2.67 (m, 2H), 2.47-2.27 (m, 2H), 2.22-1.94 (m, 2H), 1.58-1.56 (m, 2H), 1.11 (t,  $J = 7.1\text{Hz}$ , 3H), 1.00 (s, 3H), 0.92 (t,  $J = 7.2\text{Hz}$ , 3H), 0.80 (s, 3H);  $^{13}\text{C NMR}$  (75MHz, DMSO-  $d_6$ ):  $\delta$  194.6, 166.7, 155.5, 150.8, 146.1, 129.1, 123.6, 109.3, 102.7, 59.7, 50.5, 37.1, 33.4, 32.6, 29.5, 26.8, 22.4, 14.5, 14.2; **FT-IR**(KBr):  $\nu_{\text{max}}$  3273, 3080, 2967, 1711, 1605, 1520, 1493, 1377, 1344, 831  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{23}\text{H}_{29}\text{N}_2\text{O}_5$  : 413.2076, found: 413.2070; Elemental Analysis: Anal. Calcd for  $\text{C}_{23}\text{H}_{28}\text{N}_2\text{O}_5$ : C, 66.97; H, 6.84; N, 6.79. Found: C, 67.18; H, 6.74; N, 6.65%.

**Ethyl 2-ethyl-7,7-dimethyl-4-(4-nitrophenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7j)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (96% yield); **m.p.** 155-156  $^{\circ}\text{C}$ ;  $^1\text{H NMR}$  (300MHz, DMSO-  $d_6$ ):  $\delta$  9.23 (s, 1H), 8.10 (d,  $J = 8.7\text{Hz}$ , 2H), 7.43 (d,  $J = 8.7\text{Hz}$ , 2H), 4.99 (s, 1H), 3.97 (q,  $J = 7.1\text{Hz}$ , 2H), 2.80-2.67 (m, 2H), 2.44-2.28 (m, 2H), 2.23-1.95 (m, 2H), 1.15-1.09 (m, 6H), 1.00 (s, 3H), 0.81 (s,

3H);  $^{13}\text{C}$  NMR (75MHz, DMSO-  $d_6$ ):  $\delta$  194.6, 166.4, 155.5, 152.2, 150.8, 146.1, 129.1, 123.6, 109.4, 102.1, 59.7, 50.5, 37.0, 32.5, 29.6, 26.7, 25.1, 14.5, 13.7; **FT-IR**(KBr):  $\nu_{\text{max}}$  3279, 3084, 2961, 1713, 1607, 1520, 1487, 1383, 1346, 1298, 831  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{22}\text{H}_{27}\text{N}_2\text{O}_5$  : 399.1920, found: 399.1920.

#### **Ethyl 4-(4-nitrophenyl)-5-oxo-2-propyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7k)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (90% yield); **m.p.** 155-156  $^{\circ}\text{C}$ ;  $^1\text{H}$  NMR (300MHz, DMSO- $d_6$ ):  $\delta$  9.25 (s, 1H), 8.08 (d,  $J = 8.8\text{Hz}$ , 2H), 7.40 (d,  $J = 8.8\text{Hz}$ , 2H), 5.74 (s, 1H), 3.96 (q,  $J = 7.1\text{Hz}$ , 2H), 2.73-2.63 (m, 2H), 2.49-2.48 (m, 2H), 2.27-2.19 (m, 2H), 1.97-1.71 (m, 2H), 1.63-1.51 (m, 2H), 1.10 (t,  $J = 7.1\text{Hz}$ , 3H), 0.93 (t,  $J = 7.3\text{Hz}$ , 3H);  $^{13}\text{C}$  NMR (75MHz, DMSO- $d_6$ ):  $\delta$  195.0, 166.6, 155.7, 152.6, 146.1, 129.1, 123.7, 110.5, 102.6, 59.7, 37.0, 36.9, 33.4, 26.5, 22.4, 21.2, 14.5, 14.2; **FT-IR**(KBr):  $\nu_{\text{max}}$  3300, 3080, 2963, 1695, 1605, 1514, 1487, 1344, 1227, 829  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{21}\text{H}_{25}\text{N}_2\text{O}_5$  : 385.1763, found: 385.1768.

#### **Ethyl 2-ethyl-4-(4-nitrophenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7l)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (90% yield); **m.p.** 171-172  $^{\circ}\text{C}$ ;  $^1\text{H}$  NMR (300MHz, DMSO-  $d_6$ ):  $\delta$  9.28 (s, 1H), 8.08 (d,  $J = 8.7\text{Hz}$ , 2H), 7.40 (d,  $J = 8.7\text{Hz}$ , 2H), 5.00 (s, 1H), 3.97 (q,  $J = 7.1\text{Hz}$ , 2H), 2.79-2.65 (m, 2H), 2.50 (m, 2H), 2.27-2.12 (m, 2H), 1.92-1.72 (m, 2H), 1.16-1.05 (m, 6H);  $^{13}\text{C}$  NMR (75MHz, DMSO-  $d_6$ ):  $\delta$  195.0, 166.5, 155.7, 152.7, 152.1, 146.1, 129.1, 123.7, 110.5, 102.0, 59.7, 37.0, 36.8, 26.5, 25.0, 21.2, 14.5, 13.7; **FT-IR**(KBr):  $\nu_{\text{max}}$  3285, 3084, 2963, 1701, 1605, 1520, 1489, 1383, 1350, 1296, 831  $\text{cm}^{-1}$ ; **HRMS**(ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd. for  $\text{C}_{20}\text{H}_{23}\text{N}_2\text{O}_5$  : 371.1607, found: 371.1601.

**Ethyl 7,7-dimethyl-5-oxo-2-propyl-4-(2-(trifluoromethyl)phenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7m)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (95% yield); **m.p.** 152-153 °C; **<sup>1</sup>H NMR** (300MHz, DMSO-*d*<sub>6</sub>): δ 8.96 (s, 1H), 7.43-7.18 (m, 4H), 5.34 (s, 1H), 3.97-3.79 (m, 2H), 2.61-2.48 (m, 2H), 2.43-2.21 (m, 2H), 2.12-1.81 (m, 2H), 1.55-1.48 (m, 2H), 1.00 (t, *J* = 7.1Hz, 3H), 0.95 (s, 3H), 0.87 (t, *J* = 7.3Hz, 3H), 0.76 (s, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO- *d*<sub>6</sub>): δ 194.2, 167.1, 150.3, 148.3, 147.6, 132.4, 131.0, 126.8, 126.5, 110.8, 105.3, 59.5, 50.6, 33.2, 32.5, 29.6, 26.7, 22.2, 14.3, 14.1; **FT-IR**(KBr):  $\nu_{\max}$  3287, 3080, 2959, 1697, 1607, 1493, 1383, 1153, 1109, 764  $\text{cm}^{-1}$ ; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>24</sub>H<sub>29</sub>F<sub>3</sub>NO<sub>3</sub> : 436.2100, found: 436.2103; Elemental Analysis: Anal. Calcd for C<sub>24</sub>H<sub>28</sub>F<sub>3</sub>NO<sub>3</sub>: C, 66.19; H, 6.48; N, 3.22. Found: C, 66.02; H, 6.46; N, 3.20%.

**Ethyl 2-ethyl-7,7-dimethyl-5-oxo-4-(2-(trifluoromethyl)phenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7n)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (90% yield); **m.p.** 163-164 °C; **<sup>1</sup>H NMR** (300MHz, DMSO- *d*<sub>6</sub>): δ 8.99 (s, 1H), 7.47-7.19 (m, 4H), 5.34 (s, 1H), 4.02-3.90 (m, 2H), 2.58 (q, *J* = 7.2Hz, 2H), 2.45-2.21 (m, 2H), 2.13-1.81 (m, 2H), 1.07 (t, *J* = 7.4Hz, 3H), 0.99 (t, *J* = 7.1Hz, 3H), 0.95 (s, 3H), 0.75 (s, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO-*d*<sub>6</sub>): δ 194.2, 167.0, 150.3, 150.0, 147.6, 132.4, 131.0, 126.7, 126.5, 110.9, 104.7, 59.5, 50.6, 33.1, 32.5, 29.6, 26.7, 24.9, 14.5, 13.6; **FT-IR**(KBr):  $\nu_{\max}$  3281, 3079, 2957, 1703, 1605, 1489, 1385, 1153, 1107, 766  $\text{cm}^{-1}$ ; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>23</sub>H<sub>27</sub>F<sub>3</sub>NO<sub>3</sub> : 422.1943, found: 422.1943.

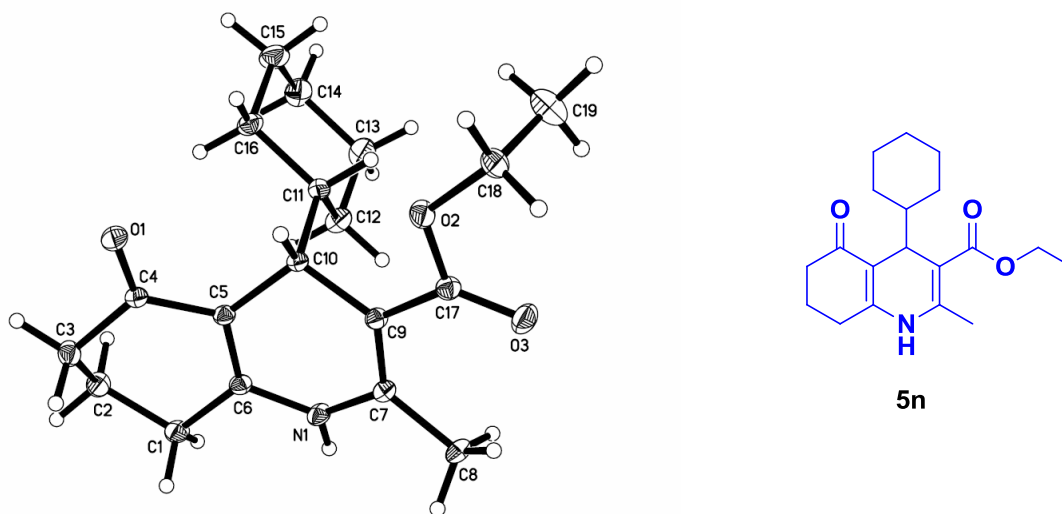
**Ethyl 5-oxo-2-propyl-4-(2-(trifluoromethyl)phenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7o)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (92% yield); **m.p.** 185-187 °C; **<sup>1</sup>H NMR** (300MHz, DMSO- *d*<sub>6</sub>): δ 9.06 (s, 1H), 7.50-7.04 (m, 4H), 5.40 (s, 1H), 3.98-3.83 (m, 2H), 2.66-2.50 (m, 2H), 2.49 (s, 3H), 2.22-2.02 (m, 2H), 1.90-1.71 (m, 2H), 1.59-1.49 (m, 2H), 1.04 (t, *J* = 7.0Hz, 3H), 1.10-0.85 (m, 2H), 0.91 (t, *J* = 7.3Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO- *d*<sub>6</sub>): δ 194.5, 167.1, 152.1, 148.1, 147.9, 132.6, 131.2, 126.7, 126.3, 112.1, 105.4, 59.5, 37.2, 33.3, 33.2, 26.7, 22.2, 21.2, 14.3, 14.1; **FT-IR**(KBr):  $\nu_{\max}$  3275, 3065, 2961, 1699, 1601, 1501, 1385, 1138, 1109, 764 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>22</sub>H<sub>25</sub>F<sub>3</sub>NO<sub>3</sub> : 408.1787, found: 408.1783.

**Ethyl 2-ethyl-5-oxo-4-(2-(trifluoromethyl)phenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (7p)**

The title compound was prepared according to the general procedure. The product was obtained as a brown solid; (92% yield); **m.p.** 205-206 °C; **<sup>1</sup>H NMR** (300MHz, DMSO- *d*<sub>6</sub>): δ 9.08 (s, 1H), 7.50-7.04 (m, 4H), 5.38 (s, 1H), 3.98-3.82 (m, 2H), 2.60 (q, *J* = 7.3Hz, 2H), 2.50-2.40 (m, 2H), 2.20-2.02 (m, 4H), 1.90-1.69 (m, 2H), 1.11 (t, *J* = 7.3Hz, 3H), 1.03 (t, *J* = 7.1Hz, 3H); **<sup>13</sup>C NMR** (75MHz, DMSO- *d*<sub>6</sub>): δ 194.5, 167.0, 152.1, 149.8, 147.9, 132.6, 131.2, 126.7, 126.4, 112.1, 104.7, 59.5, 37.1, 33.3, 26.7, 24.9, 21.2, 14.3, 13.6; **FT-IR**(KBr):  $\nu_{\max}$  3267, 3080, 2978, 1695, 1609, 1493, 1387, 1138, 1117, 770 cm<sup>-1</sup>; **HRMS**(ESI) *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>21</sub>H<sub>23</sub>F<sub>3</sub>NO<sub>3</sub> : 394.1630, found: 394.1629.

## X-ray crystal Structure of Ethyl 4-cyclohexyl-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5n)



**Table 1** Crystal data and structure refinement for ethyl 4-cyclohexyl-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5n).

Identification code	liu35s	
Empirical formula	C <sub>19</sub> H <sub>27</sub> N O <sub>3</sub>	
Formula weight	317.42	
Temperature	103(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P-1	
Unit cell dimensions	a = 7.15420(10) Å	α = 67.9610(10)°.
	b = 9.9445(2) Å	β = 82.8820(10)°.
	c = 12.8353(2) Å	γ = 85.0370(10)°.
Volume	839.12(2) Å <sup>3</sup>	
Z	2	
Density (calculated)	1.256 Mg/m <sup>3</sup>	

Absorption coefficient	0.084 mm <sup>-1</sup>
F(000)	344
Crystal size	0.34 x 0.30 x 0.28 mm <sup>3</sup>
Theta range for data collection	1.72 to 35.00°.
Index ranges	-11<=h<=11, -16<=k<=16, -20<=l<=20
Reflections collected	20658
Independent reflections	7220 [R(int) = 0.0292]
Completeness to theta = 35.00°	97.7 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9769 and 0.9720
Refinement method	Full-matrix least-squares on F <sup>2</sup>
Data / restraints / parameters	7220 / 0 / 210
Goodness-of-fit on F <sup>2</sup>	1.093
Final R indices [I>2sigma(I)]	R1 = 0.0446, wR2 = 0.1295
R indices (all data)	R1 = 0.0628, wR2 = 0.1507
Largest diff. peak and hole	0.571 and -0.533 e.Å <sup>-3</sup>

**Table 2** Atomic coordinates ( x 10<sup>4</sup>) and equivalent isotropic displacement parameters (Å<sup>2</sup>x 10<sup>3</sup>) for ethyl 4-cyclohexyl-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5n). U(eq) is defined as one third of the trace of the orthogonalized U<sup>ij</sup> tensor.

	x	y	z	U(eq)
C(1)	9838(1)	9498(1)	8430(1)	16(1)
C(2)	8070(1)	9471(1)	9229(1)	17(1)
C(3)	6396(1)	10072(1)	8540(1)	17(1)
C(4)	6130(1)	9224(1)	7812(1)	13(1)
C(5)	7786(1)	8547(1)	7411(1)	12(1)
C(6)	9522(1)	8829(1)	7600(1)	13(1)

C(7)	10998(1)	8304(1)	5993(1)	13(1)
C(8)	12834(1)	8498(1)	5264(1)	17(1)
C(9)	9328(1)	7949(1)	5787(1)	12(1)
C(10)	7635(1)	7692(1)	6670(1)	12(1)
C(11)	7421(1)	6041(1)	7347(1)	14(1)
C(12)	9035(1)	5315(1)	8073(1)	18(1)
C(13)	8800(2)	3673(1)	8644(1)	23(1)
C(14)	6928(2)	3356(1)	9380(1)	24(1)
C(15)	5281(1)	4098(1)	8698(1)	23(1)
C(16)	5540(1)	5728(1)	8087(1)	18(1)
C(17)	9137(1)	7720(1)	4738(1)	14(1)
C(18)	6933(1)	7109(1)	3750(1)	19(1)
C(19)	7372(2)	5510(1)	4050(1)	30(1)
N(1)	11096(1)	8553(1)	6982(1)	14(1)
O(1)	4540(1)	9190(1)	7539(1)	18(1)
O(2)	7310(1)	7551(1)	4653(1)	17(1)
O(3)	10388(1)	7653(1)	4020(1)	22(1)

**Table 3** Bond lengths [Å] and angles [°] for ethyl 4-cyclohexyl-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (5n).

C(1)-C(6)	1.4984(11)
C(1)-C(2)	1.5226(12)
C(1)-H(1A)	0.9900
C(1)-H(1B)	0.9900
C(2)-C(3)	1.5219(12)
C(2)-H(2A)	0.9900
C(2)-H(2B)	0.9900
C(3)-C(4)	1.5116(11)
C(3)-H(3A)	0.9900
C(3)-H(3B)	0.9900
C(4)-O(1)	1.2377(10)
C(4)-C(5)	1.4464(11)

C(5)-C(6)	1.3656(11)
C(5)-C(10)	1.5122(11)
C(6)-N(1)	1.3649(10)
C(7)-C(9)	1.3577(11)
C(7)-N(1)	1.3918(11)
C(7)-C(8)	1.4974(11)
C(8)-H(8A)	0.9800
C(8)-H(8B)	0.9800
C(8)-H(8C)	0.9800
C(9)-C(17)	1.4712(11)
C(9)-C(10)	1.5211(11)
C(10)-C(11)	1.5540(11)
C(10)-H(10)	1.0000
C(11)-C(12)	1.5297(13)
C(11)-C(16)	1.5311(12)
C(11)-H(11)	1.0000
C(12)-C(13)	1.5331(13)
C(12)-H(12A)	0.9900
C(12)-H(12B)	0.9900
C(13)-C(14)	1.5236(14)
C(13)-H(13A)	0.9900
C(13)-H(13B)	0.9900
C(14)-C(15)	1.5223(15)
C(14)-H(14A)	0.9900
C(14)-H(14B)	0.9900
C(15)-C(16)	1.5293(13)
C(15)-H(15A)	0.9900
C(15)-H(15B)	0.9900
C(16)-H(16A)	0.9900
C(16)-H(16B)	0.9900
C(17)-O(3)	1.2185(10)
C(17)-O(2)	1.3546(10)
C(18)-O(2)	1.4454(10)
C(18)-C(19)	1.5036(15)
C(18)-H(18A)	0.9900

C(18)-H(18B)	0.9900
C(19)-H(19A)	0.9800
C(19)-H(19B)	0.9800
C(19)-H(19C)	0.9800
N(1)-H(1)	0.8800
C(6)-C(1)-C(2)	111.08(7)
C(6)-C(1)-H(1A)	109.4
C(2)-C(1)-H(1A)	109.4
C(6)-C(1)-H(1B)	109.4
C(2)-C(1)-H(1B)	109.4
H(1A)-C(1)-H(1B)	108.0
C(3)-C(2)-C(1)	109.00(7)
C(3)-C(2)-H(2A)	109.9
C(1)-C(2)-H(2A)	109.9
C(3)-C(2)-H(2B)	109.9
C(1)-C(2)-H(2B)	109.9
H(2A)-C(2)-H(2B)	108.3
C(4)-C(3)-C(2)	111.87(7)
C(4)-C(3)-H(3A)	109.2
C(2)-C(3)-H(3A)	109.2
C(4)-C(3)-H(3B)	109.2
C(2)-C(3)-H(3B)	109.2
H(3A)-C(3)-H(3B)	107.9
O(1)-C(4)-C(5)	122.68(7)
O(1)-C(4)-C(3)	119.38(7)
C(5)-C(4)-C(3)	117.85(7)
C(6)-C(5)-C(4)	118.92(7)
C(6)-C(5)-C(10)	119.73(7)
C(4)-C(5)-C(10)	120.91(7)
N(1)-C(6)-C(5)	119.92(7)
N(1)-C(6)-C(1)	115.95(7)
C(5)-C(6)-C(1)	124.08(7)
C(9)-C(7)-N(1)	119.07(7)
C(9)-C(7)-C(8)	127.91(8)

N(1)-C(7)-C(8)	113.01(7)
C(7)-C(8)-H(8A)	109.5
C(7)-C(8)-H(8B)	109.5
H(8A)-C(8)-H(8B)	109.5
C(7)-C(8)-H(8C)	109.5
H(8A)-C(8)-H(8C)	109.5
H(8B)-C(8)-H(8C)	109.5
C(7)-C(9)-C(17)	121.14(7)
C(7)-C(9)-C(10)	120.46(7)
C(17)-C(9)-C(10)	118.31(7)
C(5)-C(10)-C(9)	109.89(6)
C(5)-C(10)-C(11)	113.24(6)
C(9)-C(10)-C(11)	110.58(6)
C(5)-C(10)-H(10)	107.6
C(9)-C(10)-H(10)	107.6
C(11)-C(10)-H(10)	107.6
C(12)-C(11)-C(16)	109.37(7)
C(12)-C(11)-C(10)	113.87(7)
C(16)-C(11)-C(10)	111.92(7)
C(12)-C(11)-H(11)	107.1
C(16)-C(11)-H(11)	107.1
C(10)-C(11)-H(11)	107.1
C(11)-C(12)-C(13)	110.75(8)
C(11)-C(12)-H(12A)	109.5
C(13)-C(12)-H(12A)	109.5
C(11)-C(12)-H(12B)	109.5
C(13)-C(12)-H(12B)	109.5
H(12A)-C(12)-H(12B)	108.1
C(14)-C(13)-C(12)	110.52(8)
C(14)-C(13)-H(13A)	109.5
C(12)-C(13)-H(13A)	109.5
C(14)-C(13)-H(13B)	109.5
C(12)-C(13)-H(13B)	109.5
H(13A)-C(13)-H(13B)	108.1
C(15)-C(14)-C(13)	111.17(8)

C(15)-C(14)-H(14A)	109.4
C(13)-C(14)-H(14A)	109.4
C(15)-C(14)-H(14B)	109.4
C(13)-C(14)-H(14B)	109.4
H(14A)-C(14)-H(14B)	108.0
C(14)-C(15)-C(16)	111.55(8)
C(14)-C(15)-H(15A)	109.3
C(16)-C(15)-H(15A)	109.3
C(14)-C(15)-H(15B)	109.3
C(16)-C(15)-H(15B)	109.3
H(15A)-C(15)-H(15B)	108.0
C(15)-C(16)-C(11)	111.82(7)
C(15)-C(16)-H(16A)	109.3
C(11)-C(16)-H(16A)	109.3
C(15)-C(16)-H(16B)	109.3
C(11)-C(16)-H(16B)	109.3
H(16A)-C(16)-H(16B)	107.9
O(3)-C(17)-O(2)	121.65(8)
O(3)-C(17)-C(9)	127.61(8)
O(2)-C(17)-C(9)	110.73(7)
O(2)-C(18)-C(19)	110.56(8)
O(2)-C(18)-H(18A)	109.5
C(19)-C(18)-H(18A)	109.5
O(2)-C(18)-H(18B)	109.5
C(19)-C(18)-H(18B)	109.5
H(18A)-C(18)-H(18B)	108.1
C(18)-C(19)-H(19A)	109.5
C(18)-C(19)-H(19B)	109.5
H(19A)-C(19)-H(19B)	109.5
C(18)-C(19)-H(19C)	109.5
H(19A)-C(19)-H(19C)	109.5
H(19B)-C(19)-H(19C)	109.5
C(6)-N(1)-C(7)	122.05(7)
C(6)-N(1)-H(1)	119.0
C(7)-N(1)-H(1)	119.0

C(17)-O(2)-C(18)      116.98(7)

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Symmetry transformations used to generate equivalent atoms:

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36. One reviewer suggested that our methodology corresponds to four- or three-component one-pot reaction. In our methodology, we generally used ammonium acetate as a standard reagent to synthesize dihydropyridines. Although, we never used other substituted amine sources to synthesize dihydropyridine derivatives. So, more precisely our methodology might be called three-component (multi-component) one-pot reaction rather than four-component.

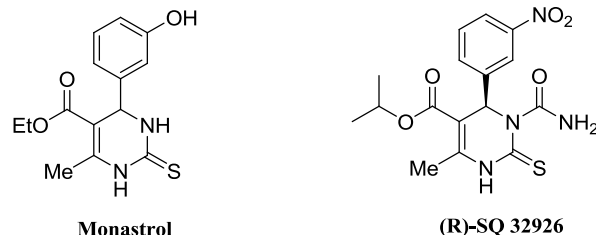
# **CHAPTER 4.2**

**A microwave-assisted, copper-catalyzed three-component synthesis of dihydropyrimidinones under mild conditions**

## 1. INTRODUCTION

The "greening" of global chemical manufacturing by minimizing energy consumption and waste production has become a major concern for the chemical industry. A robust, efficient, and cost-effective chemical process is normally considered as good process chemistry in pharmaceutical synthesis.<sup>1,2</sup> Currently, microwave assisted organic synthesis and one-pot multi-component synthesis are extensively practiced due to their prowess to minimize reaction time, the number of steps, energy consumption, waste production and to maximize synthetic efficiency and environmental benignity.<sup>3,4</sup> Over recent years, great efforts have been made to develop new and facile methodologies for the efficient synthesis of bioactive molecules in our laboratory.<sup>5-7</sup>

Dihydropyrimidinones (DHPMs) have a wide range of biological activities, acting as calcium channel antagonists, anti-hypersensitive, anti-bacterial and anti-inflammatory agents, whilst also possessing cytotoxic activity.<sup>8-12</sup> For example, anti-cancer Monastrol (Figure 1) has been shown to specifically affect mitosis *via* a new mechanism consisting of the specific and reversible inhibition of the motility of the motor protein, mitotic kinesin Eg5.<sup>13,14</sup> At the same time, (R)-SQ 32926 (Figure 1), has been found to have potent anti-hypersensitive activity per oral.<sup>15</sup> It has also been indicated that isolated alkaloids from marine sources containing dihydropyrimidine target demonstrate interesting biological activity.<sup>16</sup> For example, batzelladine alkaloids exhibit potent HIV gp-120-CD4 inhibition.<sup>17,18</sup> Hence, development of new methods that lead to substituted DHPMs *via* an efficient and convenient procedure is of great interest.<sup>19</sup>



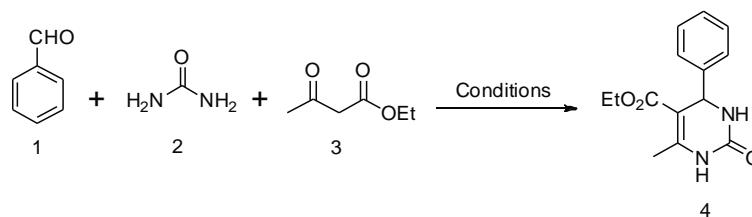
**Figure 1** Biologically active dihydropyrimidinones.

The multi-component DHPM yielding Biginelli reaction was first established in 1893 and was ignored for many years until recently in which the one-pot methodology has gained much interest.<sup>20</sup> A major disadvantage of the acid catalyzed Biginelli reaction is usually obtain lower yields (20%-50%).<sup>21</sup> Over the past few years significant efforts have been made to find new procedures which can maintain the Biginelli reaction simplicity but produce better yields. A large number of optimized procedures have been reported where the most of the protocols employ various catalytic methods in order to synthesize DHPMs.<sup>22</sup> The protocols utilize Lewis acids or metal based catalysts such as  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , *p*-TsOH,  $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ ,  $\text{BF}_3 \cdot \text{OEt}_2$ ,  $\text{InBr}_3$ ,  $\text{LiClO}_4$ ,  $\text{FeCl}_3$ ,  $\text{InCl}_3$  and metal-triflates.<sup>23-25</sup> However, at the practical level, these often require relatively harsh reaction conditions such as high reaction temperatures, expensive or highly acidic catalysts and prolonged reaction times. Further, stoichiometric amounts of catalyst needed if high yields of product are to be achieved. In addition, most of the reactions follow tedious work-up procedures and column purification, which ultimately results in diminished yields.  $\text{Cu}(\text{OTf})_2$  is an excellent triflate surrogate to other metal triflates because it is known for its affordability, high activity and low toxicity and are therefore attracting a considerable amount of attention within organic and medicinal chemistry. Moreover,  $\text{Cu}(\text{OTf})_2$  can be used with considerable advantages such as mild reaction conditions, lower catalyst loading and usually

reaction is devoid of by-products thus making the purification processes more convenient. Recently, we reported the use of  $\text{Cu}(\text{OTf})_2$  in a multi component reaction to synthesize biologically active dihydropyridines and homoallylic amines.<sup>26</sup> Maintaining the quest for synthesizing heterocycles in an efficient manner, herein we report copper catalyzed multi-component synthesis of bio-active dihydropyrimidinones.

## 2. RESULTS AND DISCUSSION

Initially benzaldehyde, ethylacetoacetate and urea were chosen as model substrates to optimize the reaction conditions. In the presence of  $\text{Cu}(\text{OTf})_2$  (10 mol%) and HCl (50 mol%), stirring the above model components at room temperature for 24 h afforded the desired dihydropyrimidone in a satisfactory yield (70%). In order to fine tune the reaction conditions we first examined the effect of temperature, time and the reaction was carried out at 80 °C for 12 h which allowed the heterocyclic product to form in a higher yield (85%). Interestingly, it was observed that the  $\text{Cu}(\text{OTf})_2$  would catalyze the reaction without the presence of HCl thus permitting milder reaction conditions. As shown in Table 1, initial reactions without HCl at 80 °C for 24 h and 12 h yielded the desired product in excellent yields (90%). At the same time, decreasing the catalyst loading from 10 to 5 mol% did not show any significant impact on the product yield. Selection of solvent was critical and solvent has significant impact on product yields. Under the same reaction conditions, when ethanol was replaced by other solvents such as acetonitrile and dichloromethane, it was observed that there was a significant increase in the yield (90%). Notably, reaction in ethanol at 80 °C in the presence of 5 mol%  $\text{Cu}(\text{OTf})_2$  with a reaction time of 12 h was found to be the appropriate condition for this multi-component synthesis.

**Table 1** Optimization of the Biginelli reaction<sup>a</sup>

Entry	Solvent	Temperature [°C]	Catalyst Cu(OTf) <sub>2</sub> [mol%]	Time [h]	Yield (%)
1	EtOH	rt	10	24	70 <sup>c</sup>
2	EtOH	80	10	12	85 <sup>c</sup>
3	EtOH	80	10	24	90
4	EtOH	80	10	12	90
5	EtOH	80	5	12	85 <sup>c</sup>
6	EtOH	80	5	12	90
7	DCM	rt	10	24	70
8	ACN	80	10	24	70
9 <sup>b</sup>	EtOH	150	5	1	40
10 <sup>d</sup>	EtOH	80	5	3	90
11 <sup>d</sup>	EtOH	100	5	2	95
12 <sup>d</sup>	EtOH	120	5	2	95
13 <sup>d</sup>	EtOH	100	2	2	95
<b>14<sup>d</sup></b>	<b>EtOH</b>	<b>100</b>	<b>2</b>	<b>1</b>	<b>95</b>

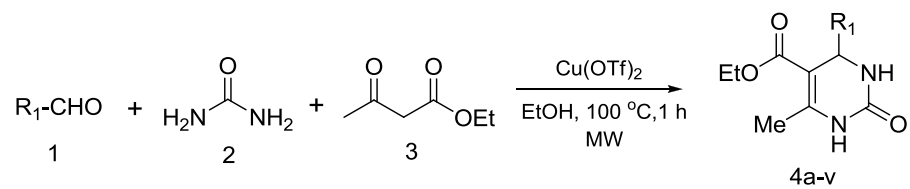
<sup>a</sup>Reaction conditions using benzaldehyde (1.0 mmol), ethylacetoacetate (1.0 mmol), urea (1.5 mmol), Solvent (2 mL) and Cu(OTf)<sub>2</sub> (0.1, 0.05 and 0.02 mmol). <sup>b</sup>Reactions under microwave irradiation, 200 W. <sup>c</sup>Addition of HCl (50 mol%).

Subsequently, we focused on lowering the reaction time and in order to decrease the reaction time which is 12 h under conventional heating conditions, we sought microwave technology as an important tool to increase the reaction conditions and yields. Microwave assisted organic synthesis (MAOS) proved to be advantageous for a wide range of organic transformations and has a huge impact on synthetic organic chemistry.<sup>27</sup> The reaction condition

was slightly different than conventional heating, when microwaves were applied to the reaction protocol. Here also, we chose ethanol as our choice of reaction medium because of its polarity which is suitable for microwave heating. We employed the established optimized conditions and irradiated the same model substrates, *i.e.*, benzaldehyde, ethylacetoacetate and urea with 5 mol% Cu(OTf)<sub>2</sub> in ethanol with microwaves at 150 °C for 1h. Unfortunately, there was a rapid decrease in the overall yield (40%) and this could be attributed to the high temperature which led to the decomposition of the components. To our delight, when temperature is decreased to 100 °C desired DHPM was yielded in 90%. Finally, it was found that at 100 °C with a catalytic loading of 2 mol% Cu(OTf)<sub>2</sub> when the components were irradiated with microwaves for 1 h quantitative amounts of DHPM was achieved (Entry 14) in superheated ethanol.

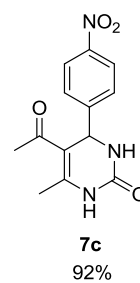
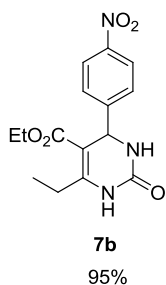
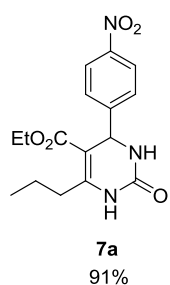
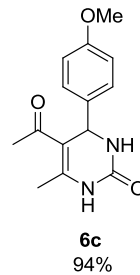
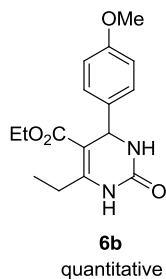
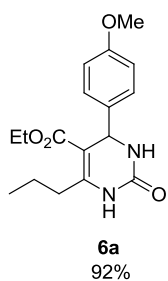
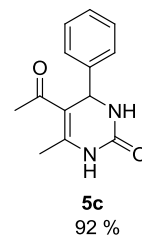
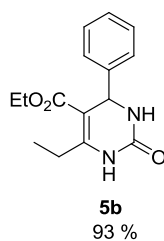
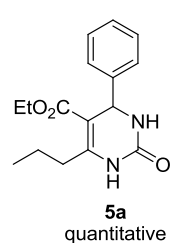
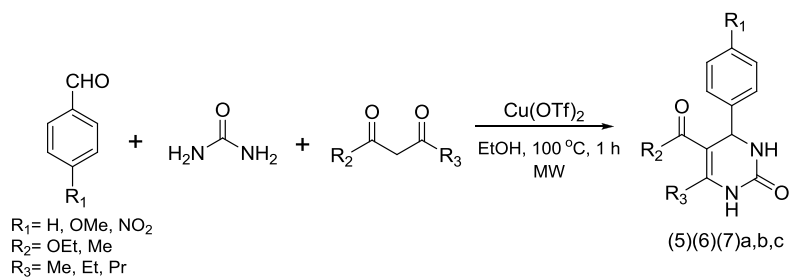
To study the ability of the methodology, a variety of aldehydes with ethylacetoacetate and urea were utilized and a library of substituted DHPMs were synthesized (Table 2). Both electron withdrawing and electron donating substituents on the aldehyde aryl ring were well tolerated. *ortho*-, *meta*- and *para*- Bromo and chloro benzaldehydes (**4b–4g**) produced the desired products in similarly high yields (90%-quantitative) indicating that the electron-withdrawing substituent and its relative position had no significant effect on the yields. In addition, *p*-NO<sub>2</sub> (**4j**), 2-CF<sub>3</sub> (**4n**), *p*-CO<sub>2</sub>Me (**4r**) and 2-I (**4s**) substituted benzaldehydes were employed and the subsequent DHPMs were produced in equally high yields (90%-quantitative). Similar trend was observed with electron donating substituents. Introduction of 3-OH (**4k**), *p*-Me (**4l**), 3,4-Me<sub>2</sub> (**4m**), *p*-SMe (**4q**) and *p*-OMe (**4t**) on benzaldehyde aryl ring produced corresponding DHPMs in excellent quantities (95%-quantitative). At the same time, bulky aromatic naphthyl groups allowed the desired product (**4h–4i**) to form in equally high yields (92%-quantitative). Attempt to extend this protocol to various aliphatic, alicyclic and

heterocyclic aldehydes was successful. 3-Thiophenyl (**4o**), 2-furyl (**4p**), n-pentyl (**4u**) and cyclohexyl (**4v**) aldehyde were utilized and superior yields were obtained (90-95%), irrespective of the nature of aldehyde in shorter reaction time. To study the ability of the methodology, a variety of aldehydes with ethylacetoacetate and urea were utilized and a library of substituted DHPMs were synthesized (Table 2). Both electron withdrawing and electron donating substituents on the aldehyde aryl ring were well tolerated. *ortho*-, *meta*- and *para*- Bromo and chloro benzaldehydes (**4b–4g**) produced the desired products in similarly high yields (90%-quantitative) indicating that the electron-withdrawing substituent and its relative position had no significant effect on the yields. In addition, *p*-NO<sub>2</sub> (**4j**), 2-CF<sub>3</sub> (**4n**), *p*-CO<sub>2</sub>Me (**4r**) and 2-I (**4s**) substituted benzaldehydes were employed and the subsequent DHPMs were produced in equally high yields (90%-quantitative). Similar trend was observed with electron donating substituents. Introduction of 3-OH (**4k**), *p*-Me (**4l**), 3,4-Me<sub>2</sub> (**4m**), *p*-SMe (**4q**) and *p*-OMe (**4t**) on benzaldehyde aryl ring produced corresponding DHPMs in excellent quantities (95%-quantitative). At the same time, bulky aromatic naphthyl groups allowed the desired product (**4h–4i**) to form in equally high yields (92%-quantitative). Attempt to extend this protocol to various aliphatic, alicyclic and heterocyclic aldehydes was successful. 3-Thiophenyl (**4o**), 2-furyl (**4p**), n-pentyl (**4u**) and cyclohexyl (**4v**) aldehyde were utilized and superior yields were obtained (90-95%), irrespective of the nature of aldehyde in shorter reaction time.

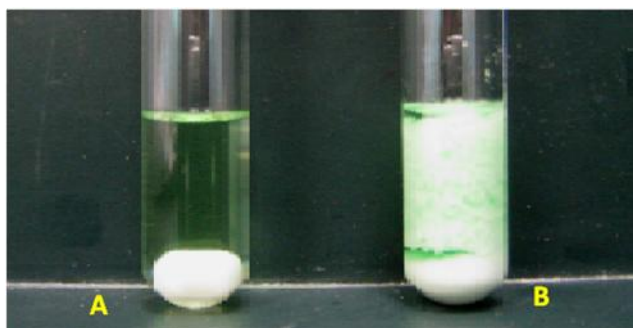
**Table 2** Synthesized dihydropyrimidinone derivatives.

Entry	R <sup>1</sup>	Product	Yield (%)
1	C <sub>6</sub> H <sub>5</sub>	<b>4a</b>	quantitative
2	2-BrC <sub>6</sub> H <sub>4</sub>	<b>4b</b>	quantitative
3	3-BrC <sub>6</sub> H <sub>4</sub>	<b>4c</b>	95
4	4-BrC <sub>6</sub> H <sub>4</sub>	<b>4d</b>	96
5	2-ClC <sub>6</sub> H <sub>4</sub>	<b>4e</b>	90
6	3-ClC <sub>6</sub> H <sub>4</sub>	<b>4f</b>	92
7	4-ClC <sub>6</sub> H <sub>4</sub>	<b>4g</b>	quantitative
8	1-Naphthyl	<b>4h</b>	quantitative
9	2-Naphthyl	<b>4i</b>	92
10	<i>p</i> -NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	<b>4j</b>	90
11	3-OHC <sub>6</sub> H <sub>4</sub>	<b>4k</b>	95
12	<i>p</i> -MeC <sub>6</sub> H <sub>4</sub>	<b>4l</b>	quantitative
13	3,4-Me <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	<b>4m</b>	95
14	2-CF <sub>3</sub> C <sub>6</sub> H <sub>4</sub>	<b>4n</b>	95
15	3-Thiophenyl	<b>4o</b>	92
16	2-Furyl	<b>4p</b>	90
17	<i>p</i> -MeSC <sub>6</sub> H <sub>4</sub>	<b>4q</b>	quantitative
18	<i>p</i> -(CO <sub>2</sub> Me)C <sub>6</sub> H <sub>4</sub>	<b>4r</b>	95
19	2-IC <sub>6</sub> H <sub>4</sub>	<b>4s</b>	90
20	<i>p</i> -MeOC <sub>6</sub> H <sub>4</sub>	<b>4t</b>	quantitative
21	n-Pentyl	<b>4u</b>	95
22	Cyclohexyl	<b>4v</b>	94

We next replaced ethylacetoacetate with 1,3-diketones such as ethyl butyl acetate, ethyl propionyl acetone and acetylacetone and we were pleased to find that our  $\text{Cu}(\text{OTf})_2$  catalyzed multi-component reaction furnished the respective DHPMs in quantitative yield (Scheme 1). This three-component one-step methodology with the 1,3-diketones substitution was tested with neutral, electron-donating (OMe) and electron-withdrawing ( $\text{NO}_2$ ) substituted benzaldehydes in order to establish the effect of the paired substitutions on the product yields. The resulting DHPMs (**5a-5c**, **6a-c**, **7a-c**) were formed in equally high yields (92%-quantitative).

**Scheme 1** Synthesized dihydropyrimidinone derivatives.

Each compound was filtered upon synthesis, avoiding tedious work-up and column purification (Figure 2). The products were characterized by  $^1\text{H}$ ,  $^{13}\text{C}$  NMR, IR, HR-MS spectroscopy, indicating the copper catalyzed reaction yielded pure compounds.



**Figure 2.** Dihydropyrimidinone synthesis: direct solidification avoiding work-up and column purification. Where **A** represents before microwave irradiation and **B** represents after the reaction.

### 3. CONCLUSION

We have successfully developed an efficient, clean and environmentally friendly procedure to generate a library of DHPMs in excellent yields *via* a microwave-assisted multi-component reaction. The protocol utilizes  $\text{Cu}(\text{OTf})_2$  in catalytic amounts and mild reaction conditions and above all it does not require work-up or column purifications hence devoid of formation of the by-products. Reaction times were considerably reduced and product yields increased under microwave irradiation. This green methodology can be employed to construct new substituted DHPM scaffolds, which are of great interest due to their extensive biological applications. Further applications of microwave irradiation and  $\text{Cu}(\text{OTf})_2$  are now being studied in our laboratories for the asymmetric synthesis of dihydropyrimidones under environmentally friendly conditions.

## 4. EXPERIMENTAL

### General experimental procedure for the synthesis of dihydropyrimidinones:

Aldehyde (1.0 mmol), ethylacetoacetate (1.0 mmol), urea (1.5 mmol), Cu(OTf)<sub>2</sub> (0.02 mmol) and EtOH (2 mL) were added to a microwave vial equipped with a magnetic stirrer. The reaction vessel was sealed and irradiated within the microwave reactor at a temperature of 100 °C for 1 h with a maximum power of 200 W. The reaction mixture was cooled to room temperature overnight and the resulting precipitate was filtered and washed with water and hexane. The identity and purity of the products was confirmed by TLC, high-resolution mass spectrometry, IR and <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy (see the Supporting Information for full details).

### Material and Instrumentation

Unless otherwise noted, all reactions were carried out in oven dried glassware under an atmosphere of nitrogen and distilled solvents were transferred by syringes. Solvents and reagents were purified according to the standard procedure prior to use. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 nm). Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on 300 MHz Bruker ACF 300 and 400 MHz Bruker DPX 400 NMR spectrometers. The residual solvent signals were taken as the reference (2.50 ppm for <sup>1</sup>H NMR spectra and 39.5 ppm for <sup>13</sup>C NMR spectra in DMSO-*d*<sub>6</sub>, 7.26 ppm for <sup>1</sup>H NMR spectra and 77.0 ppm for <sup>13</sup>C NMR spectra in CDCl<sub>3</sub>). Chemical shift ( $\delta$ ) is referred in terms of ppm, coupling constants (*J*) are given in Hz. Following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br =

broad signal. Infrared spectra were recorded on a Bio-RAD FTS 165 FT-IR Spectrometer and reported in  $\text{cm}^{-1}$ . Samples were prepared in thin film technique. HRMS (ESI) spectra were recorded on a Finnigan/MAT LCQ quadrupole ion trap mass spectrometer, coupled with the TSP4000 HPLC system and the Crystal 310 CE system. Microwave experiments were conducted in a CEM Discover<sup>TM</sup> system.

### General experimental procedure for the synthesis of dihydropyrimidinones

Aldehyde (1.0 mmol), ethylacetoacetate or equivalent (1.0 mmol), urea (1.5 mmol),  $\text{Cu}(\text{OTf})_2$  (0.02 mmol) and EtOH (2 mL) were added to a 10 mL microwave vial equipped with a magnetic stirrer. The reaction vessel was heated under microwave irradiation (200 W) at 100 °C for 1h. The progress of reaction was monitored by TLC. After completion, the reaction mixture was cooled to room temperature to allow solidification. The resulting precipitate was filtered and washed with water and hexane. The identity and purity of the products was confirmed by TLC, high-resolution mass spectrometry, IR and  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy. Pure products (**4a-4v**, **5a-5c**, **6a-6c**, and **7a-7c**) were obtained without further purification.

### Ethyl 6-methyl-2-oxo-4-phenyl-1,2,3,4 tetrahydropyrimidine-5-carboxylate (**4a**)<sup>23(a)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 202-204 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  in ppm = 9.19 (s, 1H, NH), 7.73 (s, 1H, NH), 7.35-7.21 (m, 5H, Ar-H), 5.15 (d, 1H,  $J=3.2\text{Hz}$ ,  $\text{CH-Ph}$ ), 3.98 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 2.25 (s, 3H,  $\text{CH}_3$ ), 1.09 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  in ppm = 165.8 ( $\text{EtOC=O}$ ), 152.6 ( $\text{C=O}$ ), 148.8 ( $\text{Me(NH)C=C}$ ), 145.3 (C-Ar), 128.8 (C-Ar), 127.7 (C-Ar), 126.7 (C-Ar), 99.7 ( $\text{C=C(CH)CO}_2\text{Et}$ ), 59.6 ( $\text{CH}_2\text{-CH}_3$ ), 54.4 ( $\text{CH-Ph}$ ), 18.2 ( $\text{CH}_3$ ), 14.5 ( $\text{CH}_2\text{-CH}_3$ ); FT-IR (KBr):  $\nu = 3256, 3121,$

2945, 1730, 1703, 1647, 1464, 1290, 1226, 1090, 756  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_3$ : 261.1239, found: 261.1237.

#### **Ethyl 4-(2-bromophenyl)-6-methyl-2-oxo-1,2,3,4 tetrahydropyrimidine-5-carboxylate (4b)**

The title compound was prepared according to the general procedure. The product was obtained as a pale green powder; (quantitative yield); m.p. 205-207 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.28 (s, 1H, NH), 7.69 (s, 1H, NH), 7.58-7.16 (m, 4H, Ar-H), 5.61 (d, 1H,  $J=2.4\text{Hz}$ ,  $\text{CH-Ar}$ ), 3.89 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 2.30 (s, 3H,  $\text{CH}_3$ ), 0.99 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*6):  $\delta$  in ppm = 165.4 ( $\text{EtOC=O}$ ), 151.7 ( $\text{C=O}$ ), 149.7 ( $\text{Me(NH)C=C}$ ), 143.8 (C-Ar), 133.1 (C-Ar), 129.8 (C-Ar), 129.2 (C-Ar), 128.9 (C-Ar), 122.7 (C-Ar), 98.7 ( $\text{C=C(CH)CO}_2\text{Et}$ ), 59.5 ( $\text{CH}_2\text{-CH}_3$ ), 54.5 ( $\text{CH-Ar}$ ), 18.1 ( $\text{CH}_3$ ), 14.4 ( $\text{CH}_2\text{-CH}_3$ ); FT-IR (KBr):  $\nu$  = 3221, 3099, 2978, 1701, 1643, 1445, 1285, 1225, 1092, 746  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{14}\text{H}_{15}\text{BrN}_2\text{O}_3$ : 339.0344, found: 339.0343.

#### **Ethyl 4-(3-bromophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4c)**

The title compound was prepared according to the general procedure. The product was obtained as a pale green powder; (quantitative yield); m.p. 205-207 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.28 (s, 1H, NH), 7.69 (s, 1H, NH), 7.58-7.16 (m, 4H, Ar-H), 5.61 (d, 1H,  $J=2.4\text{Hz}$ ,  $\text{CH-Ar}$ ), 3.89 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 2.30 (s, 3H,  $\text{CH}_3$ ), 0.99 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*6):  $\delta$  in ppm = 165.4 ( $\text{EtOC=O}$ ), 151.7 ( $\text{C=O}$ ), 149.7 ( $\text{Me(NH)C=C}$ ), 143.8 (C-Ar), 133.1 (C-Ar), 129.8 (C-Ar), 129.2 (C-Ar), 128.9 (C-Ar), 122.7 (C-Ar), 98.7 ( $\text{C=C(CH)CO}_2\text{Et}$ ), 59.5 ( $\text{CH}_2\text{-CH}_3$ ), 54.5 ( $\text{CH-Ar}$ ), 18.1 ( $\text{CH}_3$ ), 14.4 ( $\text{CH}_2\text{-CH}_3$ ); FT-IR (KBr):  $\nu$  = 3221, 3099, 2978, 1701, 1643, 1445, 1285, 1225, 1092, 746  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{14}\text{H}_{15}\text{BrN}_2\text{O}_3$ : 339.0344, found: 339.0343.

**Ethyl 4-(4-bromophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4d)**

22 (o)

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (96% yield); m.p. 225-226 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.24 (s, 1H, NH), 7.76 (s, 1H, NH), 7.52 (d, 2H, *J*=8.4Hz, Ar-H), 7.19 (d, 2H, *J*=8.4Hz, Ar-H), 5.12 (d, 1H, *J*=3.2Hz, CH-Ar), 3.98 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.24 (s, 3H, CH<sub>3</sub>), 1.09 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>);  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 165.8 (EtOC=O), 152.5 (C=O), 149.3 (Me(NH)C=C), 144.8 (C-Ar), 131.9 (C-Ar), 129.1 (C-Ar), 120.9 (C-Ar), 99.3 (C=C(CH)CO<sub>2</sub>Et), 59.8 (CH<sub>2</sub>-CH<sub>3</sub>), 54.1 (CH-Ar), 18.4 (CH<sub>3</sub>), 14.6 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3246, 3111, 2949, 1701, 1649, 1458, 1288, 1221, 1088, 781 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>14</sub>H<sub>15</sub>BrN<sub>2</sub>O<sub>3</sub>: 339.0344, found: 339.0336.

**Ethyl 4-(2-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4e)**<sup>23</sup>

(a)

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (90% yield); m.p. 223-224 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.25 (s, 1H, NH), 7.68 (s, 1H, NH), 7.40-7.24 (m, 4H, Ar-H), 5.61 (d, 1H, *J*=2.7Hz, CH-Ar), 3.87 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.28 (s, 3H, CH<sub>3</sub>), 0.97 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>).  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 165.4 (EtOC=O), 151.8 (C=O), 149.8 (Me(NH)C=C), 142.2 (C-Ar), 132.1 (C-Ar), 129.8 (C-Ar), 129.5 (C-Ar), 129.2 (C-Ar), 128.2 (C-Ar), 98.3 (C=C(CH)CO<sub>2</sub>Et), 59.5 (CH<sub>2</sub>-CH<sub>3</sub>), 51.9 (CH-Ar), 18.1 (CH<sub>3</sub>), 14.4 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3354, 3223, 3107, 2978, 1694, 1639, 1450, 1368, 1230, 1098, 744 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>14</sub>H<sub>15</sub>ClN<sub>2</sub>O<sub>3</sub>: 295.0849, found: 295.0850.

**Ethyl 4-(3-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4f)**<sup>23</sup>

(a)

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (92% yield); m.p. 200-201 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.26 (s, 1H, NH), 7.78 (s, 1H, NH), 7.37-7.18 (m, 4H, Ar-H), 5.14 (s, 1H, CH-Ar), 3.99 (q, 2H, *J*=6.8Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.25 (s, 3H, CH<sub>3</sub>), 1.09 (t, 3H, *J*=6.8Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.6 (EtOCC=O), 152.4 (C=O), 149.4 (Me(NH)C=C), 147.7 (C-Ar), 133.4 (C-Ar), 131.0 (C-Ar), 127.7 (C-Ar), 126.7 (C-Ar), 125.4 (C-Ar), 99.1 (C=C(CH)CO<sub>2</sub>Et), 59.8 (CH<sub>2</sub>-CH<sub>3</sub>), 54.1 (CH-Ar), 18.3 (CH<sub>3</sub>), 14.5 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr):  $\bar{\nu}$  = 3250, 3113, 2940, 1711, 1647, 1475, 1429, 1223, 1090, 768 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>14</sub>H<sub>15</sub>ClN<sub>2</sub>O<sub>3</sub>: 295.0849, found: 295.0846.

**Ethyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4g)**

23 (a)

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 217-218 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.24 (s, 1H, NH), 7.77 (s, 1H, NH), 7.39 (d, 2H, *J*=8.5Hz, Ar-H), 7.25 (d, 2H, *J*=8.5Hz, Ar-H), 5.14 (d, 1H, *J*=3.2Hz, CH-Ar), 3.98 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.25 (s, 3H, CH<sub>3</sub>), 1.09 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.7 (EtOCC=O), 152.4 (C=O), 149.2 (Me(NH)C=C), 144.3 (C-Ar), 132.2 (C-Ar), 128.9 (C-Ar), 128.6 (C-Ar), 99.3 (C=C(CH)CO<sub>2</sub>Et), 59.7 (CH<sub>2</sub>-CH<sub>3</sub>), 53.9 (CH-Ar), 18.3 (CH<sub>3</sub>), 14.5 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr):  $\bar{\nu}$  = 3237, 3117, 2978, 1701, 1647, 1460, 1288, 1221, 1088, 781 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>14</sub>H<sub>15</sub>ClN<sub>2</sub>O<sub>3</sub>: 295.0849, found: 295.0843.

**Ethyl 6-methyl-4-(naphthalen-1-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4h)**<sup>22 (d)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 253-254 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.24 (s, 1H, NH), 8.30 (d, 1H,  $J=8.2$ Hz, NH), 7.95-7.55 (m, 3H, Ar-H), 7.54-7.39 (m, 4H, Ar-H), 6.05 (d, 1H,  $J=3.0$ Hz,  $\underline{\text{CH}}$ -Ar), 3.79 (q, 2H,  $J=7.1$ Hz,  $\underline{\text{CH}_2}$ -CH<sub>3</sub>), 2.49 (s, 3H, CH<sub>3</sub>), 0.80 (t, 3H,  $J=7.1$ Hz, CH<sub>2</sub>- $\underline{\text{CH}_3}$ ); <sup>13</sup>C NMR (100 MHz, DMSO-*d*6):  $\delta$  in ppm = 165.7 (EtOC=O), 152.1 (C=O), 149.2 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 140.9 (C-Ar), 133.9 (C-Ar), 130.5 (C-Ar), 128.9 (C-Ar), 128.3 (C-Ar), 126.5 (C-Ar), 126.2 (C-Ar), 126.1 (C-Ar), 124.7 (C-Ar), 124.1 (C-Ar), 99.6 (C= $\underline{\text{C}}$ (CH)CO<sub>2</sub>Et), 59.5 ( $\underline{\text{CH}_2}$ -CH<sub>3</sub>), 50.2 ( $\underline{\text{CH}}$ -Ar), 18.2 (CH<sub>3</sub>), 14.3 (CH<sub>2</sub>- $\underline{\text{CH}_3}$ ); FT-IR (KBr):  $\nu$  = 3231, 3109, 2986, 2930, 1705, 1645, 1465, 1317, 1221, 1088, 791, 777 cm<sup>-1</sup>; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>18</sub>H<sub>18</sub>N<sub>2</sub>O<sub>3</sub>: 311.1396; found: 311.1389.

**Ethyl 6-methyl-4-(naphthalen-2-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4i)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow powder; (92% yield); m.p. 210-212 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.24 (s, 1H, NH), 8.30 (d, 1H,  $J=8.2$ Hz, NH), 7.95-7.55 (m, 3H, Ar-H), 7.54-7.39 (m, 4H, Ar-H), 6.05 (d, 1H,  $J=3.0$ Hz,  $\underline{\text{CH}}$ -Ar), 3.79 (q, 2H,  $J=7.1$ Hz,  $\underline{\text{CH}_2}$ -CH<sub>3</sub>), 2.49 (s, 3H, CH<sub>3</sub>), 0.80 (t, 3H,  $J=7.1$ Hz, CH<sub>2</sub>- $\underline{\text{CH}_3}$ ); <sup>13</sup>C NMR (75 MHz, DMSO-*d*6):  $\delta$  in ppm = 165.7 (EtOC=O), 152.1 (C=O), 149.2 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 140.9 (C-Ar), 133.9 (C-Ar), 130.5 (C-Ar), 128.9 (C-Ar), 128.3 (C-Ar), 126.5 (C-Ar), 126.2 (C-Ar), 126.1 (C-Ar), 124.7 (C-Ar), 124.1 (C-Ar), 99.6 (C= $\underline{\text{C}}$ (CH)CO<sub>2</sub>Et), 59.5 ( $\underline{\text{CH}_2}$ -CH<sub>3</sub>), 50.2 ( $\underline{\text{CH}}$ -Ar), 18.2 (CH<sub>3</sub>), 14.3 (CH<sub>2</sub>- $\underline{\text{CH}_3}$ ); FT-IR (KBr):  $\nu$  = 3246, 3121, 2974, 1717, 1651, 1458, 1290, 1225, 1086, 773 cm<sup>-1</sup>; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>18</sub>H<sub>18</sub>N<sub>2</sub>O<sub>3</sub>: 311.1396; found: 311.1389.

**Ethyl 6-methyl-4-(4-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4j)** <sup>23</sup>

(a)

The title compound was prepared according to the general procedure. The product was obtained as a yellow powder; (90% yield); m.p. 201-202 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.35 (s, 1H, NH), 8.22 (d, 2H, *J*=8.7Hz, Ar-H), 7.89 (s, 1H, NH), 7.50 (d, 2H, *J*=8.7Hz, Ar-H), 5.27 (d, 1H, *J*=3.3Hz, CH-Ar), 3.99 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.27 (s, 3H, CH<sub>3</sub>), 1.10 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.5 (EtOC=O), 152.5 (C=O), 152.2 (Me(NH)C=C), 149.9 (C-Ar), 147.2 (C-Ar), 128.1 (C-Ar), 124.3 (C-Ar), 98.6 (C=C(CH)CO<sub>2</sub>Et), 59.9 (CH<sub>2</sub>-CH<sub>3</sub>), 54.1 (CH-Ar), 18.3 (CH<sub>3</sub>), 14.5 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr): ν̄ = 3238, 3123, 2986, 1730, 1705, 1645, 1522, 1348, 1219, 1096, 854, 783 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>14</sub>H<sub>16</sub>N<sub>3</sub>O<sub>5</sub>: 306.1090; found: 306.1087.

**Ethyl 4-(3-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4k)** <sup>23</sup> (a)

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (95% yield); m.p. 190-192 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.35 (s, 1H, NH), 9.15 (s, 1H, OH), 7.68 (s, 1H, NH), 7.09 (t, 1H, *J*=8.0Hz, Ar-H), 6.68-6.61 (m, 3H, Ar-H), 5.07 (d, 1H, *J*=3.0Hz, CH-Ar), 3.99 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.24 (s, 3H, CH<sub>3</sub>), 1.12 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.8 (EtOC=O), 157.8 (C-Ar), 152.7 (C=O), 148.5 (Me(NH)C=C), 146.7 (C-Ar), 129.7 (C-Ar), 117.3 (C-Ar), 114.6 (C-Ar), 113.5 (C-Ar), 99.9 (C=C(CH)CO<sub>2</sub>Et), 59.7 (CH<sub>2</sub>-CH<sub>3</sub>), 54.3 (CH-Ar), 18.2 (CH<sub>3</sub>), 14.6 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr): ν̄ = 3512, 3352, 3244, 3119, 2980, 1718, 1678, 1600, 1460, 1229, 1094, 872, 779 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>14</sub>H<sub>17</sub>N<sub>2</sub>O<sub>4</sub>: 277.1188; found: 277.1184.

**Ethyl 6-methyl-2-oxo-4-p-tolyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4l)**<sup>23(a)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 216-217 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.15 (s, 1H, NH), 7.68 (s, 1H, NH), 7.12 (s, 4H, Ar-H), 5.10 (d, 1H, *J*=3.2Hz, CH-Ar), 3.98 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.26 (s, 3H, CH<sub>3</sub>), 2.24 (s, 3H, CH<sub>3</sub>), 1.10 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.8 (EtOC=O), 152.6 (C=O), 148.6 (Me(NH)C=C), 142.4 (C-Ar), 136.8 (C-Ar), 129.3 (C-Ar), 126.6 (C-Ar), 99.9 (C=C(CH)CO<sub>2</sub>Et), 59.6 (CH<sub>2</sub>-CH<sub>3</sub>), 54.1 (CH-Ar), 21.1 (CH<sub>3</sub>), 18.2 (CH<sub>3</sub>), 14.6 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr): ν̄ = 3231, 3094, 2916, 1701, 1649, 1456, 1285, 1219, 1082, 777 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>15</sub>H<sub>19</sub>N<sub>2</sub>O<sub>3</sub>: 275.1396; found: 275.1400.

**Ethyl 4-(3,4-dimethylphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4m)**

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (95% yield); m.p. 229-230 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.12 (s, 1H, NH), 7.64 (s, 1H, NH), 7.07-6.92 (m, 3H, Ar-H), 5.08 (d, 1H, *J*=3.1Hz, CH-Ar), 3.98 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.23 (s, 3H, CH<sub>3</sub>), 2.18 (s, 3H, CH<sub>3</sub>), 2.17 (s, 3H, CH<sub>3</sub>), 1.11 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.8 (EtOC=O), 152.7 (C=O), 148.5 (Me(NH)C=C), 142.8 (C-Ar), 136.3 (C-Ar), 135.5 (C-Ar), 130.0 (C-Ar), 127.9 (C-Ar), 124.0 (C-Ar), 99.9 (C=C(CH)CO<sub>2</sub>Et), 59.6 (CH<sub>2</sub>-CH<sub>3</sub>), 54.1 (CH-Ar), 20.0 (CH<sub>3</sub>), 19.5 (CH<sub>3</sub>), 18.2 (CH<sub>3</sub>), 14.6 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr): ν̄ = 3339, 3223, 3105, 2978, 1687, 1628, 1462, 1296, 1238, 1096, 822, 798 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>16</sub>H<sub>21</sub>N<sub>2</sub>O<sub>3</sub>: 289.1552; found: 289.1555.

**Ethyl 6-methyl-2-oxo-4-(2-(trifluoromethyl)phenyl)-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4n)**

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (95% yield); m.p. 206-207 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.34 (s, 1H, NH), 7.70-7.65 (m, 2H, Ar-H), 7.51-7.45 (m, 2H, Ar-H), 7.32 (s, 1H, NH), 5.56 (d, 1H, *J*=2.2Hz, CH-Ar), 3.85 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.34 (s, 3H, CH<sub>3</sub>), 0.87 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.2 (EtOC=O), 151.4 (C=O), 150.2 (Me(NH)C=C), 144.6 (C-Ar), 133.8 (C-Ar), 131.3 (C-Ar), 129.0 (C-Ar), 128.5 (C-Ar), 126.4 (CF<sub>3</sub>), 98.6 (C=C(CH)CO<sub>2</sub>Et), 59.4 (CH<sub>2</sub>-CH<sub>3</sub>), 51.0 (CH-Ar), 18.2 (CH<sub>3</sub>), 14.2 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr): ν̄ = 3258, 3105, 2955, 1699, 1643, 1458, 1314, 1234, 1124, 1096, 768 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>15</sub>H<sub>16</sub>N<sub>2</sub>O<sub>3</sub>F<sub>3</sub>: 329.1113; found: 329.1110.

**Ethyl 6-methyl-2-oxo-4-(thiophen-3-yl)-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4o)**

The title compound was prepared according to the general procedure. The product was obtained as a pale brown powder; (92% yield); m.p. 231-232 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 9.19 (s, 1H, NH), 7.75 (s, 1H, NH), 7.47-7.44 (m, 1H, thiofuran-H), 7.15-7.14 (m, 1H, thiofuran-H), 7.00-6.98 (m, 1H, thiofuran-H), 5.22 (d, 1H, *J*=3.2Hz, CH-thiofuran), 4.05 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 2.22 (s, 3H, CH<sub>3</sub>), 1.15 (t, 3H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ in ppm = 165.8 (EtOC=O), 153.1 (C=O), 148.9 (Me(NH)C=C), 146.2 (C-thiofuran), 127.1 (C-thiofuran), 126.6 (C-thiofuran), 121.2 (C-thiofuran), 99.9 (C=C(CH)CO<sub>2</sub>Et), 59.7 (CH<sub>2</sub>-CH<sub>3</sub>), 49.9 (CH-thiofuran), 18.2 (CH<sub>3</sub>), 14.6 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr): ν̄ = 3233, 3102, 2980, 1722, 1701, 1649, 1458, 1288, 1233, 1092, 766 cm<sup>-1</sup>; HRMS (ESI) *m/z* [M+1]<sup>+</sup>: calcd for C<sub>12</sub>H<sub>15</sub>N<sub>2</sub>O<sub>3</sub>S: 267.0803 ; found: 267.0806.

**Ethyl 4-(furan-3-yl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4p)**<sup>22 (p)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a light brown powder; (90% yield); m.p. 199-201 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.24 (s, 1H, NH), 7.75 (s, 1H, NH), 7.55 (s, 1H, furan-H), 6.36-6.35 (m, 1H, furan-H), 6.17-6.14 (m, 1H, furan-H), 5.20 (s, 1H,  $\underline{\text{C}}\text{H}$ -furan), 4.03 (q, 2H,  $J=7.1\text{Hz}$ ,  $\underline{\text{C}}\text{H}_2$ -CH<sub>3</sub>), 2.23 (s, 3H, CH<sub>3</sub>), 1.14 (t, 3H,  $J=7.1\text{Hz}$ , CH<sub>2</sub>- $\underline{\text{C}}\text{H}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 165.5 (EtOC=O), 156.4 (C=O), 152.9 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 149.8 (C-furan), 142.6 (C-furan), 110.8 (C-furan), 105.7 (C-furan), 97.2 (C= $\underline{\text{C}}$ (CH)CO<sub>2</sub>Et), 59.7 ( $\underline{\text{C}}\text{H}_2$ -CH<sub>3</sub>), 48.2 ( $\underline{\text{C}}\text{H}$ -thiofuran), 18.2 (CH<sub>3</sub>), 14.6 (CH<sub>2</sub>- $\underline{\text{C}}\text{H}_3$ ); FT-IR (KBr):  $\nu$  = 3339, 3246, 3111, 2965, 1707, 1647, 1462, 1290, 1230, 1096, 795, 737  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>12</sub>H<sub>15</sub>N<sub>2</sub>O<sub>4</sub>: 251.1032; found: 251.1040.

**Ethyl 6-methyl-4-(4-(methylthio)phenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4q)**

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 201-203 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.18 (s, 1H, NH), 7.71 (s, 1H, NH), 7.15-7.23 (m, 4H, Ar-H), 5.10 (d, 1H,  $J=3.3\text{Hz}$ ,  $\underline{\text{C}}\text{H}$ -Ar), 3.98 (q, 2H,  $J=7.1\text{Hz}$ ,  $\underline{\text{C}}\text{H}_2$ -CH<sub>3</sub>), 2.44 (s, 3H, SCH<sub>3</sub>), 2.24 (s, 3H, CH<sub>3</sub>), 1.11 (t, 3H,  $J=7.1\text{Hz}$ , CH<sub>2</sub>- $\underline{\text{C}}\text{H}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 165.8 (EtOC=O), 152.6 (C=O), 148.8 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 142.0 (C-Ar), 137.4 (C-Ar), 127.3 (C-Ar), 126.5 (C-Ar), 99.6 (C= $\underline{\text{C}}$ (CH)CO<sub>2</sub>Et), 56.7 ( $\underline{\text{C}}\text{H}_2$ -CH<sub>3</sub>), 54.0 ( $\underline{\text{C}}\text{H}$ -Ar), 18.2 (CH<sub>3</sub>), 15.3 (CH<sub>3</sub>), 14.6 (CH<sub>2</sub>- $\underline{\text{C}}\text{H}_3$ ); FT-IR (KBr):  $\nu$  = 3219, 3092, 2978, 2918, 1721, 1705, 1647, 1458, 1285, 1219, 1088, 772  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>15</sub>H<sub>19</sub>N<sub>2</sub>O<sub>3</sub>S: 307.1116; found: 307.1118.

**Ethyl 4-(4-(methoxycarbonyl)phenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4r)**

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (95% yield); m.p. 178-179 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.27 (s, 1H, NH), 7.93 (d, 2H,  $J=8.3\text{Hz}$ , Ar-H), 7.82 (s, 1H, NH), 7.38 (d, 2H,  $J=8.3\text{Hz}$ , Ar-H), 5.21 (d, 1H,  $J=3.3\text{Hz}$ ,  $\underline{\text{C}}\text{H-Ar}$ ), 3.98 (q, 2H,  $J=7.1\text{Hz}$ ,  $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 3.84 (s, 3H,  $\text{CO}_2\underline{\text{C}}\text{H}_3$ ), 2.26 (s, 3H,  $\text{CH}_3$ ), 1.08 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*6):  $\delta$  in ppm = 166.6 (EtOC=O), 165.8 (MeOC=O), 152.6 (C=O), 150.6 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 149.5 (C-Ar), 130.1 (C-Ar), 129.2 (C-Ar), 127.3 (C-Ar), 99.2 (C= $\underline{\text{C}}(\text{CH})\text{CO}_2\text{Et}$ ), 59.9 ( $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 54.5 ( $\text{CO}_2\underline{\text{C}}\text{H}_3$ ), 52.7 ( $\underline{\text{C}}\text{H-Ar}$ ), 18.4 ( $\text{CH}_3$ ), 14.6 ( $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ ); FT-IR (KBr):  $\nu$  = 3244, 3111, 2945, 1730, 1703, 1651, 1458, 1285, 1219, 1096, 788  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{16}\text{H}_{19}\text{N}_2\text{O}_5$ : 319.1294; found: 319.1294.

#### **Ethyl 4-(2-iodophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4s)**

The title compound was prepared according to the general procedure. The product was obtained as a pale green powder; (90% yield); m.p. 195-196 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.26 (s, 1H, NH), 7.82-7.79 (m, 1H, Ar-H), 7.65 (s, 1H, NH), 7.40-7.26 (m, 2H, Ar-H), 7.01-6.96 (m, 1H, Ar-H), 5.44 (s, 1H,  $\underline{\text{C}}\text{H-Ar}$ ), 3.89 (q, 2H,  $J=6.9\text{Hz}$ ,  $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 2.29 (s, 3H,  $\text{CH}_3$ ), 0.99 (t, 3H,  $J=7.0\text{Hz}$ ,  $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*6):  $\delta$  in ppm = 165.4 (EtOC=O), 151.6 (C=O), 149.5 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 146.9 (C-Ar), 139.6 (C-Ar), 129.9 (C-Ar), 129.6 (C-Ar), 128.4 (C-Ar), 99.7 (C= $\underline{\text{C}}(\text{CH})\text{CO}_2\text{Et}$ ), 99.3 ( $\underline{\text{C}}\text{H-Ar}$ ), 59.5 ( $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 18.1 ( $\text{CH}_3$ ), 14.6 ( $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ ); FT-IR (KBr):  $\nu$  = 3341, 3237, 3125, 2976, 1690, 1636, 1449, 1369, 1227, 1096, 781  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_3$ : 387.0206; found: 387.0208.

#### **Ethyl 4-(4-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4t)**<sup>22 (k)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 204-206 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.15 (s, 1H, NH), 7.66 (s, 1H, NH), 7.14 (d, 2H,  $J=8.7\text{Hz}$ , Ar-H), 6.87 (d, 2H,  $J=8.7\text{Hz}$ , Ar-H), 5.09 (d, 1H,  $J=3.1\text{Hz}$ ,  $\underline{\text{C}}\text{H-Ar}$ ), 3.98 (q, 2H,  $J=7.1\text{Hz}$ ,  $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 3.71 (s, 3H, OCH<sub>3</sub>), 2.24 (s, 3H, CH<sub>3</sub>), 1.10 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 165.8 (EtOC=O), 158.9 (C-Ar), 152.6 (C=O), 148.5 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 137.5 (C-Ar), 127.9 (C-Ar), 114.2 (C-Ar), 100.0 (C= $\underline{\text{C}}(\text{CH})\text{CO}_2\text{Et}$ ), 59.6 ( $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 55.5 (OCH<sub>3</sub>), 53.8 ( $\underline{\text{C}}\text{H-Ar}$ ), 18.2 (CH<sub>3</sub>), 14.6 ( $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ ); FT-IR (KBr):  $\nu$  = 3235, 3113, 2955, 1703, 1647, 1514, 1456, 1279, 1221, 1088, 837, 791  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for C<sub>15</sub>H<sub>19</sub>N<sub>2</sub>O<sub>4</sub>: 291.1345; found: 291.1343.

#### **Ethyl 6-methyl-2-oxo-4-pentyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4u)** <sup>22 (o)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a pale green powder; (95% yield); m.p. 227-228 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 8.92 (s, 1H, NH), 7.31 (s, 1H, NH), 4.08-4.03 (m, 3H,  $\underline{\text{C}}\text{H-C}_5\text{H}_{11}$  &  $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 2.15 (s, 3H, CH<sub>3</sub>), 1.36-1.15 (m, 11H, C<sub>5</sub>H<sub>11</sub>), 0.86-0.81 (m, 3H,  $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 165.9 (EtOC=O), 153.3 (C=O), 148.7 (Me(NH) $\underline{\text{C}}=\text{C}$ ), 99.9 (C= $\underline{\text{C}}(\text{CH})\text{CO}_2\text{Et}$ ), 59.5 ( $\underline{\text{C}}\text{H}_2\text{-CH}_3$ ), 50.5 ( $\underline{\text{C}}\text{H-C}_5\text{H}_{11}$ ), 37.1 (pentyl-C), 31.4 (pentyl-C), 23.8 (pentyl-C), 22.5 (pentyl-C), 18.1 (CH<sub>3</sub>), 14.7 ( $\text{CH}_2\text{-}\underline{\text{C}}\text{H}_3$ ), 14.3 (pentyl-C); FT-IR (KBr):  $\nu$  = 3248, 3121, 2957, 2936, 2855, 1724, 1703, 1647, 1383, 1288, 1223, 1086, 779  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for C<sub>13</sub>H<sub>23</sub>N<sub>2</sub>O<sub>3</sub>: 255.1709; found: 255.1712.

#### **Ethyl 4-cyclohexyl-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4v)** <sup>24 (d)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a pale blue powder; (94% yield); m.p. 233-234 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):

$\delta$  in ppm = 8.88 (s, 1H, NH), 7.29 (s, 1H, NH), 4.06-4.02 (m, 2H,  $\text{CH}_2\text{-CH}_3$ ), 3.91 (s, 1H,  $\text{CH-C}_6\text{H}_{11}$ ), 2.15 (s, 3H,  $\text{CH}_3$ ), 1.66-1.57 (m, 4H, cyclohexyl-H), 1.36-1.25 (m, 2H, cyclohexyl-H), 1.19-1.06 (m, 7H, cyclohexyl-H &  $\text{CH}_2\text{-CH}_3$ ), 0.90-0.83 (m, 1H, cyclohexyl-H);  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*6):  $\delta$  in ppm = 166.2 ( $\text{EtOC=O}$ ), 153.7 ( $\text{C=O}$ ), 148.9 ( $\text{Me(NH)C=C}$ ), 98.4 ( $\text{C=C(CH)CO}_2\text{Et}$ ), 59.5 ( $\text{CH}_2\text{-CH}_3$ ), 55.4 ( $\text{CH-C}_6\text{H}_{11}$ ), 45.3 (hexyl-C), 29.0 (hexyl-C), 26.7 (hexyl-C), 26.5 (hexyl-C), 26.4 (hexyl-C), 26.1 (hexyl-C), 18.2 ( $\text{CH}_3$ ), 14.6 ( $\text{CH}_2\text{-CH}_3$ ); FT-IR (KBr):  $\nu = 3235, 3115, 2918, 1722, 1703, 1641, 1450, 1283, 1231, 1096, 789 \text{ cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{14}\text{H}_{23}\text{N}_2\text{O}_3$ : 267.1709 ; found: 267.1715.

### Ethyl 2-oxo-4-phenyl-6-propyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (5a) <sup>22 (k)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 149-150 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.15 (s, 1H, NH), 7.70 (s, 1H, NH), 7.34-7.21 (m, 5H, Ar-H), 5.14 (d, 1H,  $J=3.4\text{Hz}$ ,  $\text{CH-Ph}$ ), 3.98 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 2.65-2.60 (m, 2H,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 1.59-1.52 (m, 2H,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 1.10 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 0.90 (t, 3H,  $J=7.4\text{Hz}$ ,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO-*d*6):  $\delta$  in ppm = 165.5 ( $\text{EtOC=O}$ ), 152.8 ( $\text{C=O}$ ), 145.4 ( $\text{Me(NH)C=C}$ ), 128.8 (C-Ar), 127.7 (C-Ar), 126.7 (C-Ar), 99.5 ( $\text{C=C(CH)CO}_2\text{Et}$ ), 59.7 ( $\text{CH}_2\text{-CH}_3$ ), 54.4 ( $\text{CH-Ph}$ ), 32.8 ( $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 22.1 ( $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 14.5 ( $\text{CH}_2\text{-CH}_3$ ), 14.1 ( $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ); FT-IR (KBr):  $\nu = 3242, 3111, 2965, 1703, 1647, 1458, 1310, 1213, 1096, 787 \text{ cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{16}\text{H}_{21}\text{N}_2\text{O}_3$ : 289.1552, found: 289.1555.

### Ethyl 6-ethyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (5b)

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (93% yield); m.p. 137-139 °C;  $^1\text{H}$  NMR (300 MHz, DMSO-*d*6):  $\delta$  in ppm = 9.20 (s, 1H, NH), 7.73 (s, 1H, NH), 7.34-7.21 (m, 5H, Ar-H), 5.15 (d, 1H,  $J=3.2\text{Hz}$ ,  $\text{CH-}$

Ph), 3.98 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 2.72-2.57 (m, 2H,  $\text{CH}_2\text{-CH}_3$ ), 1.15-1.06 (m, 6H, 2 $\text{CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta$  in ppm = 165.4 (EtOC(=O)), 154.4 (C=O), 152.9 (Me(NH)C=C), 145.3 (C-Ar), 128.9 (C-Ar), 127.7 (C-Ar), 126.7 (C-Ar), 99.9 (C=C(CH)CO<sub>2</sub>Et), 59.7 ( $\text{CH}_2\text{-CH}_3$ ), 54.4 (CH-Ph), 24.6 ( $\text{CH}_2\text{-CH}_3$ ), 14.5 ( $\text{CH}_2\text{-CH}_3$ ), 13.4 ( $\text{CH}_2\text{-CH}_3$ ); FT-IR (KBr):  $\nu$  = 3237, 3111, 2976, 1701, 1643, 1460, 1302, 1217, 1090, 758, 696  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>15</sub>H<sub>19</sub>N<sub>2</sub>O<sub>3</sub>: 275.1396, found: 275.1393.

### 5-Acetyl-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one (5c)<sup>22 (p)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a pink powder; (92% yield); m.p. 238-239 °C;  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta$  in ppm = 9.18 (s, 1H, NH), 7.82 (s, 1H, NH), 7.35-7.24 (m, 5H, Ar-H), 5.26 (d, 1H,  $J=3.4\text{Hz}$ , CH-Ph), 2.29 (s, 3H,  $\text{CH}_3$ ), 2.10 (s, 3H,  $\text{CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta$  in ppm = 194.7 (MeC(=O)), 152.6 (C=O), 148.6 (Me(NH)C=C), 144.7 (C-Ar), 129.0 (C-Ar), 127.8 (C-Ar), 126.9 (C-Ar), 110.0 (C=C(CH)COMe), 54.3 (CH-Ph), 30.8 (CH<sub>3</sub>), 19.4 (CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3283, 3250, 2930, 1699, 1674, 1612, 1595, 1454, 1381, 1265, 1236, 768, 706  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>13</sub>H<sub>15</sub>N<sub>2</sub>O<sub>2</sub>: 231.1134, found: 231.1138.

### Ethyl 4-(4-methoxyphenyl)-2-oxo-6-propyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (6a)

The title compound was prepared according to the general procedure. The product was obtained as a yellow solid; (92% yield); m.p. 134-135 °C;  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta$  in ppm = 9.14 (s, 1H, NH), 7.66 (s, 1H, NH), 7.16 (d, 2H,  $J=8.7\text{Hz}$ , Ar-H), 6.87 (d, 2H,  $J=8.7\text{Hz}$ , Ar-H), 5.11 (d, 1H,  $J=3.2\text{Hz}$ , CH-Ar), 3.98 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 3.71 (s, 3H, OCH<sub>3</sub>), 2.66-2.61 (m, 2H,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 1.58-1.55 (m, 2H,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 1.10 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 0.91 (t, 3H,  $J=7.3\text{Hz}$ ,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta$  in ppm = 165.6 (EtOC(=O)), 158.9 (C-Ar), 152.9 (C=O), 152.5 (Me(NH)C=C), 137.5 (C-Ar), 127.8 (C-Ar), 114.1

(C-Ar), 99.8 (C=C(CH)CO<sub>2</sub>Et), 59.6 (CH<sub>2</sub>-CH<sub>3</sub>), 55.5 (OCH<sub>3</sub>), 53.8 (CH-Ar), 32.8 (CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>), 22.1 (CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>), 14.5 (CH<sub>2</sub>-CH<sub>3</sub>), 14.1 (CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3238, 3099, 2965, 1709, 1639, 1464, 1281, 1209, 1082, 800 cm<sup>-1</sup>; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>17</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub>: 319.1658; found: 319.1659.

#### Ethyl 6-ethyl-4-(4-methoxyphenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (6b)

The title compound was prepared according to the general procedure. The product was obtained as a white powder; (quantitative yield); m.p. 139-140 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.16 (s, 1H, NH), 7.65 (s, 1H, NH), 7.15 (d, 2H, *J*=8.1Hz, Ar-H), 6.87 (d, 2H, *J*=8.1Hz, Ar-H), 5.09 (s, 1H, CH-Ar), 3.98 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 3.71 (s, 3H, OCH<sub>3</sub>), 2.65 (q, 2H, *J*=7.1Hz, CH<sub>2</sub>-CH<sub>3</sub>), 1.12-1.09 (m, 6H, 2CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 165.5 (EtOC=O), 158.9 (C-Ar), 154.0 (C=O), 152.9 (Me(NH)C=C), 137.5 (C-Ar), 127.8 (C-Ar), 114.1 (C-Ar), 99.2 (C=C(CH)CO<sub>2</sub>Et), 59.6 (CH<sub>2</sub>-CH<sub>3</sub>), 55.5 (OCH<sub>3</sub>), 53.7 (CH-Ar), 24.5 (CH<sub>2</sub>-CH<sub>3</sub>), 14.5 (CH<sub>2</sub>-CH<sub>3</sub>), 13.4 (CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3235, 3111, 2970, 2932, 1701, 1647, 1462, 1302, 1219, 1094, 787 cm<sup>-1</sup>; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>16</sub>H<sub>21</sub>N<sub>2</sub>O<sub>4</sub>: 305.1501; found: 305.1506.

#### 5-Acetyl-4-(4-methoxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (6c)<sup>22 (p)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a dark brown powder; (94% yield); m.p. 173-175 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.13 (s, 1H, NH), 7.74 (s, 1H, NH), 7.15 (d, 2H, *J*=8.6Hz, Ar-H), 6.88 (d, 2H, *J*=8.6Hz, Ar-H), 5.20 (d, 1H, *J*=3.1Hz, CH-Ar), 3.72 (s, 3H, CH<sub>3</sub>), 2.27 (s, 3H, CH<sub>3</sub>), 2.07 (s, 3H, CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 194.8 (MeC=O), 159.0 (C=O), 152.6 (Me(NH)C=C), 148.2 (C-Ar), 136.8 (C-Ar), 128.1 (C-Ar), 114.3 (C-Ar), 110.1 (C=C(CH)COMe), 55.5 (CH-Ar), 53.8 (OCH<sub>3</sub>), 30.6 (CH<sub>3</sub>), 19.3 (CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3302,

3113, 2947, 2833, 1697, 1612, 1466, 1329, 1234, 1034, 831, 789  $\text{cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_3$ : 261.1239; found: 261.1235.

#### **Ethyl 4-(4-nitrophenyl)-2-oxo-6-propyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (7a)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow powder; (91% yield); m.p. 186-188  $^{\circ}\text{C}$ ;  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  in ppm = 9.32 (s, 1H, NH), 8.22 (d, 2H,  $J=8.6\text{Hz}$ , Ar-H), 7.87 (s, 1H, NH), 7.50 (d, 2H,  $J=8.6\text{Hz}$ , Ar-H), 5.28 (d, 1H,  $J=3.3\text{Hz}$ ,  $\text{CH-Ar}$ ), 3.99 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 2.69-2.58 (m, 2H,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 1.60-1.52 (m, 2H,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 1.10 (t, 3H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 0.91 (t, 3H,  $J=7.3\text{Hz}$ ,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  in ppm = 165.3 ( $\text{EtOC=O}$ ), 153.8 ( $\text{C=O}$ ), 152.5 ( $\text{Me(NH)C=C}$ ), 152.5 (C-Ar), 147.2 (C-Ar), 128.0 (C-Ar), 124.3 (C-Ar), 98.5 ( $\text{C=C(CH)CO}_2\text{Et}$ ), 59.9 ( $\text{CH}_2\text{-CH}_3$ ), 54.1 ( $\text{CH-Ar}$ ), 32.8 ( $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 22.1 ( $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 14.4 ( $\text{CH}_2\text{-CH}_3$ ), 14.1 ( $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ); FT-IR (KBr):  $\nu = 3238, 3111, 2965, 1705, 1636, 1520, 1449, 1350, 1307, 1207, 1086, 804 \text{ cm}^{-1}$ ; HRMS (ESI)  $m/z$   $[\text{M}+1]^+$ : calcd for  $\text{C}_{16}\text{H}_{20}\text{N}_3\text{O}_5$ : 334.1403; found: 334.1409.

#### **Ethyl 6-ethyl-4-(4-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (7b)**

The title compound was prepared according to the general procedure. The product was obtained as a yellow powder; (95% yield); m.p. 215-217  $^{\circ}\text{C}$ ;  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  in ppm = 9.35 (s, 1H, NH), 8.22 (d, 2H,  $J=8.6\text{Hz}$ , Ar-H), 7.87 (s, 1H, NH), 7.50 (d, 2H,  $J=8.6\text{Hz}$ , Ar-H), 5.26 (d, 1H,  $J=3.2\text{Hz}$ ,  $\text{CH-Ar}$ ), 3.99 (q, 2H,  $J=7.1\text{Hz}$ ,  $\text{CH}_2\text{-CH}_3$ ), 2.77-2.55 (m, 2H,  $\text{CH}_2\text{-CH}_2\text{-CH}_3$ ), 1.14-1.05 (m, 6H,  $2\text{CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  in ppm = 165.1 ( $\text{EtOC=O}$ ), 155.3 ( $\text{C=O}$ ), 152.5 ( $\text{Me(NH)C=C}$ ), 152.2 (C-Ar), 147.2 (C-Ar), 128.1 (C-Ar), 124.3 (C-Ar), 97.9 ( $\text{C=C(CH)CO}_2\text{Et}$ ), 59.9 ( $\text{CH}_2\text{-CH}_3$ ), 54.1 ( $\text{CH-Ar}$ ), 24.6 ( $\text{CH}_2\text{-CH}_3$ ), 14.4 ( $\text{CH}_2\text{-CH}_3$ ), 13.4

(CH<sub>2</sub>-CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3231, 3111, 2968, 1697, 1639, 1518, 1454, 1348, 1298, 1209, 1092, 806 cm<sup>-1</sup>; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>15</sub>H<sub>18</sub>N<sub>3</sub>O<sub>5</sub>: 320.1246; found: 320.1240.

**5-Acetyl-6-methyl-4-(4-nitrophenyl)-3,4-dihydropyrimidin-2(1H)-one (7c)** <sup>22 (p)</sup>

The title compound was prepared according to the general procedure. The product was obtained as a pale green powder; (92% yield); m.p. 263-264 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 9.33 (s, 1H, NH), 8.19 (d, 2H,  $J=7.8$ Hz, Ar-H), 7.98 (s, 1H, NH), 7.50 (d, 2H,  $J=7.8$ Hz, Ar-H), 5.38 (s, 1H, CH-Ar), 2.31 (s, 3H, CH<sub>3</sub>), 2.18 (s, 3H, CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  in ppm = 194.4 (MeC=O), 152.5 (C=O), 152.0 (Me(NH)C=C), 149.5 (C-Ar), 147.1 (C-Ar), 128.1 (C-Ar), 124.3 (C-Ar), 109.9 (C=C(CH)COMe), 53.6 (CH-Ar), 31.1 (CH<sub>3</sub>), 19.6 (CH<sub>3</sub>); FT-IR (KBr):  $\nu$  = 3339, 3254, 3138, 1709, 1672, 1607, 1514, 1445, 1348, 1236, 1103, 762 cm<sup>-1</sup>; HRMS (ESI)  $m/z$  [M+1]<sup>+</sup>: calcd for C<sub>13</sub>H<sub>14</sub>N<sub>3</sub>O<sub>4</sub>: 276.0984; found: 276.0988.

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# **CHAPTER 4.3**

**A general and mild copper-catalyzed three-  
component synthesis of protected homoallyl  
amines**

## 1. INTRODUCTION

Lewis acid catalyzed allylations of imines or iminium species with allylating reagents, such as allyl silane or borane reagents are effective methods for the introduction of an amino group into carbon frameworks.<sup>1</sup> In addition, homoallyl amines and derivatives are considered to be useful precursors in natural product synthesis<sup>2</sup>,  $\beta$ -lactam chemistry<sup>3</sup> and are valuable intermediates in drug discovery.<sup>4</sup> The synthesis of homoallyl amines from aldimines and allyltrimethylsilane or allyltributylstannane, is inefficient due to more synthetic transformations.<sup>5,6</sup> A one-pot, three-component reaction was first reported for the preparation of homoallylamines by Panek.<sup>7</sup> Subsequently, Veenstra reported an improved one-pot, three-component reaction for the synthesis of protected homoallyl amines.<sup>8</sup>

The main drawback of this reaction was the use of excess Lewis acid ( $\text{BF}_3 \cdot \text{OEt}_2$ ). Yamamoto,<sup>9</sup> Ollevier,<sup>10</sup> Phukan<sup>11</sup> and Williamson<sup>12</sup> have demonstrated three-component syntheses of homoallyl amines by employing various Lewis acids. All of these methods while offering some advantages, also suffer from disadvantages in terms of the use of expensive catalysts, incomplete reactions, prior silylation of the carbamate and limited substrate scope. Furthermore, their scope for enantioselective synthesis using chiral ligands is extremely limited. Hence, the development of new methods which lead to potentially general and convenient procedures for this transformation is still of interest.

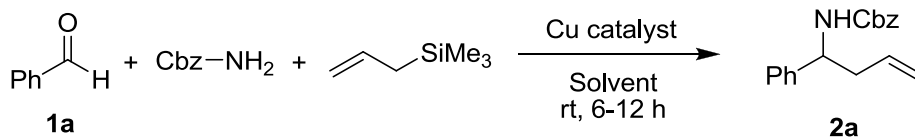
In recent years, copper-catalyzed reactions have received considerable attention as they are more affordable, highly active, less toxic, and inexpensive in contrast to classical Lewis acids. Copper complexes have been studied extensively by Pfaltz,<sup>13</sup> Andrus,<sup>14</sup> and Katsuki<sup>15</sup> using chiral bis and tris oxazoline ligands for asymmetric synthesis. Copper (I or II) triflates have

been widely used as catalysts for various chemical transformations.<sup>16</sup> To the best of our knowledge, the copper-catalyzed three-component reaction for the synthesis of homoallyl amines has not been studied. Herein, we report a mild and efficient synthesis of homoallyl amines using an inexpensive copper catalyst.

## 2. RESULTS AND DISCUSSION

We first optimized the reaction conditions for the copper-catalyzed, one-pot, three-component allylation of in situ generated imines. The results are summarized in Table 1. Initially, 10 mol% of CuOTf was utilized to promote the reaction of benzaldehyde, benzyl carbamate (CbzNH<sub>2</sub>) and allyltrimethylsilane in different solvents. The results were disappointing when dichloromethane and acetonitrile were used as solvents with yields of only 20% and 35% yields being obtained, respectively (entries 1 and 2). When 10 mol% of Cu(OTf)<sub>2</sub> was used in acetonitrile at room temperature the desired homoallyl amine obtained in 80% yield (Table 1, entry 3). Further investigation of the reaction conditions revealed that acetonitrile was the best solvent, and a catalyst loading as low as 5 mol% could be used to afford the highest yield of 92% (entry 4). Changing solvent to dichloromethane reduced the yield significantly to 40% (entry 5). When 20 mol% copper catalyst was used, the yield dropped considerably to 70% (entry 6).

**Table 2** Optimization of the copper-catalyzed one-pot, three-component allylation of in situ-generated imines.

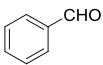
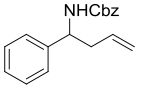
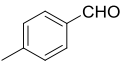
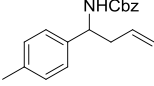
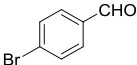
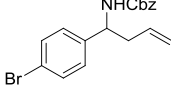
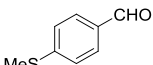
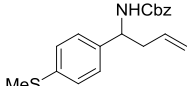
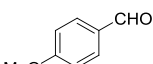
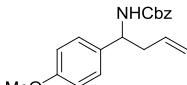
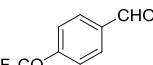
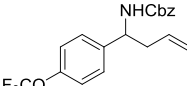
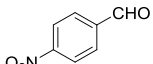
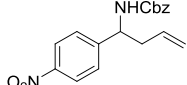


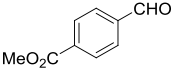
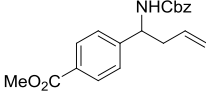
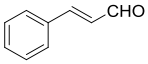
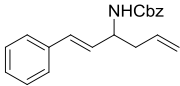

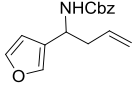
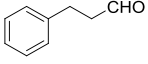
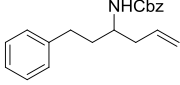
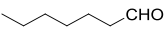
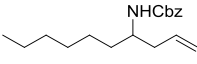
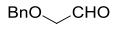
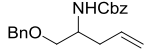
Entry	Catalyst	mol %	Solvent	Time (h)	Yield (%)
1	CuOTf	10	CH <sub>2</sub> Cl <sub>2</sub>	24	20
2	CuOTf	10	CH <sub>3</sub> CN	24	35
3	Cu(OTf) <sub>2</sub>	10	CH <sub>3</sub> CN	12	80
<b>4</b>	<b>Cu(OTf)<sub>2</sub></b>	<b>5</b>	CH <sub>3</sub> CN	<b>10</b>	<b>92</b>
5	Cu(OTf) <sub>2</sub>	5	CH <sub>2</sub> Cl <sub>2</sub>	24	40
6	Cu(OTf) <sub>2</sub>	20	CH <sub>3</sub> CN	12	70

The substrate scope for the three-component reaction of various aldehydes, benzyl carbamate (CbzNH<sub>2</sub>) and allyltrimethylsilane was found to be general (Table 2). In the presence of 5 mol% of copper(II) triflate, a variety of aldehydes were successfully transformed into the corresponding Cbz-protected homoallyl amines at room temperature. Moreover, the reaction conditions were mild and rapid, and no side products were formed. First, we chose 4-substituted

aromatic aldehydes to carry out the allylation reaction. As is evident from Table 2, besides benzaldehyde (entry 1), other substituted aromatic aldehydes also served as good substrates for this reaction. For example, mono-substituted benzaldehydes (methyl, bromo and methylthiol) gave the desired homoallyl amines in excellent yields (>88%) (entries 2-4). Interestingly, aldehydes with electron-donating groups on the phenyl ring (entries 5 and 6) give the expected products in good yields. On the other hand, aromatic aldehydes possessing electron-withdrawing groups on the phenyl ring and  $\alpha,\beta$ -unsaturated aldehydes (entries 7-9) were employed for the synthesis of the corresponding protected homoallyl amines in moderate yields. This methodology could be extended to a variety of functional groups such as a long chain aliphatic, phenyl propionaldehyde. Interestingly, benzyl protected glycolaldehyde gave the desired product in excellent yield, which allows an easy access to aminoalcohols (92%, entry 13, Table 2).

**Table 2** Synthesis of various substituted homoallyl amines<sup>a</sup>

Entry	Aldehyde 1	Time (h)	Homoallyl amine	2 <sup>b</sup>	Yield (%)
1		10		2a	92
2		8		2b	90
3		8		2c	88
4		6		2d	95
5		8		2e	85
6		8		2f	85
7		8		2g	75

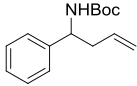
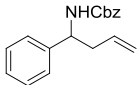
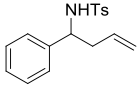
8		12		2h	76
9		6		2i	85
10		12		2j	78
11		6		2k	88
12		8		2l	82
13		8		2m	92

<sup>a</sup> Conditions: Cu(OTf)<sub>2</sub> (5 mol%), Acetonitrile, r.t.

<sup>b</sup> All products (except products 2d, 2f, 2h, 2j and 2m) have been previously reported.<sup>8, 10, 11, 12</sup>

To further study the scope of the reaction, we also examined the reaction of benzaldehyde and allyl trimethylsilane with benzyl carbamate, *tert*-butyl carbamate and *para*-toluene sulfonamide in the presence of 5 mol% Cu(OTf)<sub>2</sub>. The results are summarized in Table 3. The reaction with *tert*-butyl carbamate went to completion to provide the Boc-protected homoallyl amine in 82% yield. Similarly, the benzyl carbamate and *para*-toluene sulfonamide reactions afforded homoallyl amines in excellent yields (92% and 80%, respectively).

**Table 3** Synthesis of protected homoallyl amines from benzaldehyde.

Entry	R <sub>2</sub> -NH <sub>2</sub>	Time (h)	Homoallyl amine	Yield (%)
1	Boc-NH <sub>2</sub>	8		82
2	Cbz-NH <sub>2</sub>	10		92
3	Ts-NH <sub>2</sub>	6		80

Reaction conditions: Cu(OTf)<sub>2</sub> (5 mol%), acetonitrile, r. t.

### 3. CONCLUSION

We have demonstrated a highly efficient synthesis of homoallylamines *via* a copper-catalyzed, one-pot, three-component reaction of aldehydes, carbamates and allyltrimethylsilane. This method offers several advantages including mild reaction conditions, a low quantity of the catalyst (5 mol%), and no formation of by-products. The current method can be applied to a numerous of functionalized substrates. Extension of this work by employing chiral ligands is underway.

## 4. EXPERIMENTAL

Unless otherwise noted, all reactions were carried out in oven dried glassware under an atmosphere of nitrogen and distilled solvents were transferred by syringe. Solvents and reagents were purified according to the standard procedure prior to use. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 nm). Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on a 300 MHz Bruker ACF 300, 400 MHz Bruker DPX 400 and 500 MHz Bruker AMX 500 NMR spectrometers, respectively. The residual solvent signals were taken as the reference (7.26 ppm for  $^1\text{H}$  NMR spectroscopy and 77.0 ppm for  $^{13}\text{C}$  NMR spectroscopy). Chemical shift ( $\delta$ ) is referred in terms of ppm, coupling constants ( $J$ ) are given in Hz. Following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal. Infrared spectra were recorded on a Bio-RAD FTS 165 FT-IR Spectrometer and reported in  $\text{cm}^{-1}$ . Samples were prepared in thin film technique. HRMS (ESI) spectra were recorded on a Finnigan/MAT LCQ quadrupole ion trap mass spectrometer, coupled with the TSP4000 HPLC system and the Crystal 310 CE system.

**General experimental procedure for the synthesis of homoallyl amines:**

To a stirred solution of aldehyde (1 mmol), benzyl carbamate (1.2 mmol) and allyltrimethylsilane (1.5 mmol) in dry acetonitrile (2 mL, 0.5 M) under a nitrogen atmosphere was added  $\text{Cu}(\text{OTf})_2$  (5 mol%) at room temperature. The reaction was stirred until the reaction was complete as indicated by TLC. The reaction mixture was quenched with saturated ammonium chloride solution and diluted with ethyl acetate. The layers were separated, the aqueous layer was extracted with ethyl acetate twice, and the combined organic extract was washed with water and brine. Drying over anhydrous sodium sulfate and subsequent removal of the solvent under vacuum resulted in a colorless oily residue, which was purified by column chromatography on silica gel to give desired product.

**Entry 4, Table 2: Benzyl 1-[4-(methylthio)phenyl] but-3-enylcarbamate (2d):**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 7.34 (br s, 4H), 7.23-7.17 (m, 5H), 5.71-5.61 (m, 1H), 5.13-5.03 (m, 5H), 4.76 (d,  $J = 5.6$  Hz, 1H), 2.51 (s, 2H), 2.47 (s, 3H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm = 155.6, 137.3, 136.3, 133.6, 128.5, 128.1, 126.8, 118.6, 66.8, 54.1, 40.9, 15.9. IR ( $\text{CHCl}_3$ ):  $\nu = 3016, 1716, 1500, 1215 \text{ cm}^{-1}$ . HRMS (ESI):  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{22}\text{NO}_2\text{S}$ : 328.1371  $[\text{M}+\text{H}]^+$ ; found: 328.1377.

**Entry 6, Table 2: Benzyl 1-[4-(trifluoromethoxy) phenyl] but-3-enylcarbamate (2f):**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 7.34-7.32 (m, 7H), 7.17 (d,  $J = 8.2$  Hz, 2H), 5.70-5.60 (m, 1H), 5.14-5.03 (m, 5H), 4.81 (br s, 1H), 2.52 (s, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 155.6, 148.3, 136.2, 133.1, 128.5, 128.2, 127.6, 121.7, 119.1, 119.0, 66.9, 53.8, 40.9. IR ( $\text{CHCl}_3$ ):  $\nu = 3020, 1708, 1508, 1261, 1215 \text{ cm}^{-1}$ . HRMS (ESI):  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{19}\text{NO}_3\text{F}_3$ : 366.1317  $[\text{M}+\text{H}]^+$ ; found: 366.1324.

**Entry 8, Table 2: Methyl 4-[1-(benzyloxycarbonylamino) but-3-enyl] benzoate (2h):**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 8.00 (d,  $J = 8.2$  Hz, 2H), 7.34-7.32 (m, 7H), 5.68-5.58 (m, 1H), 5.23 (br s, 1H), 5.13-5.02 (m, 4H), 4.85 (d,  $J = 5.5$  Hz, 1H), 3.90 (s, 3H), 2.52 (br s, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 166.8, 155.6, 147.2, 136.2, 133.1, 129.9, 129.2, 128.5, 128.2, 126.2, 119.0, 66.9, 54.3, 52.1, 40.8. IR ( $\text{CHCl}_3$ ):  $\nu = 3433, 3020, 1701, 1504, 1280$   $\text{cm}^{-1}$ . HRMS (ESI):  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{22}\text{NO}_4$ : 340.1549  $[\text{M}+\text{H}]^+$ ; found: 340.1552.

**Entry 10, Table 2: Benzyl 1-(furan-3-yl)but-3-enylcarbamate (2j):**  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 7.37-7.35 (m, 5H), 7.33-7.31 (m, 2H), 6.33 (m, 1H), 5.78-5.70 (m, 1H), 5.14-5.09 (m, 4H), 4.83 (br s, 1H), 4.83 (d,  $J = 6.2$  Hz, 1H), 2.52 (d,  $J = 4.9$  Hz, 2H).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 155.7, 143.3, 139.1, 136.4, 133.6, 128.1, 126.4, 118.5, 109.0, 66.8, 46.8, 39.7. IR ( $\text{CHCl}_3$ ):  $\nu = 3016, 1708, 1504, 1334, 1219$   $\text{cm}^{-1}$ . HRMS (ESI):  $m/z$ : calcd for  $\text{C}_{13}\text{H}_{20}\text{NO}_3$ : 238.1443  $[\text{M}+\text{H}]^+$ ; found: 238.1441.

**Entry 13, Table 2: Benzyl 1-(benzyloxy)pent-4-en-2-ylcarbamate (2m):**  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 7.41-7.40 (m, 5H), 7.38-7.33 (m, 5H), 5.86-5.78 (m, 1H), 5.15-5.10 (m, 5H), 4.55 (dd,  $J = 12.0, 24.0$  Hz, 2H), 3.95 (s, 1H), 3.55-3.53 (m, 2H), 2.46-2.39 (m, 2H).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  in ppm 156.0, 138.0, 136.6, 134.3, 128.5, 128.4, 128.1, 127.7, 127.6, 117.9, 73.2, 70.9, 66.6, 50.4, 36.4. IR ( $\text{CHCl}_3$ ):  $\nu = 3437, 3016, 1712, 1508, 1215$   $\text{cm}^{-1}$ . HRMS (ESI):  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{24}\text{NO}_3$ : 326.1756  $[\text{M}+\text{H}]^+$ ; found: 326.1751.

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