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Rommel S. Talan

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A Dissertation

entitled

Chemical Ligation of Glycopeptides

by

Rommel S. Talan

Submitted as partial fulfillment of the requirements for

the Doctor of Philosophy in Chemistry

________________________________________
Dr. Steven J. Sucheck, Committee Chair

________________________________________
Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

August 2010
Advances in the chemical synthesis of homogeneous glycoproteins and glycopeptides have facilitated the structural studies of glycopeptides and glycoproteins, understanding the roles of glycans on protein function and development of glycopeptide-based therapeutics. Decarboxylative condensation and the reaction between thioacids and 2,4-dinitrobenzenesulfonamides are highly promising protocols for the chemoselective ligation of glycopeptide fragments that do not depend on thiol-capture methods and are relatively tolerant of steric crowding at the reaction centers. We have extended the synthetic scope of these ligation techniques to the synthesis of O-linked glycopeptides, as well as, the generation of N-linkage.
Investigations into decarboxylative condensation, as presented in Chapter 2, involved the synthesis of a series of protected and unprotected glycosyl dipeptides, which contained the α-keto acid moiety at the C-terminus, followed by their ligation to a series of O-tert-butyl protected N-hydroxylamino acids to afford O-tert-butyl-protected glycosyl tripeptides. The reactions were carried out under both anhydrous and aqueous conditions at neutral pH to produce glycopeptide products in yields ranging from 15% to 86% depending on the amino acids present at the ligation junction. The best yields were obtained when both the α-keto acid and the N-hydroxylamino acid contained medium-sized chains. In addition to the expected tripeptide product, 2,5-substituted oxazoles were isolated when O-tert-butyl protected N-hydroxylamines of glycine were employed in the reaction. The formation of the oxazole is believed to result from an intramolecular cyclization of the O-tert-butyl ester on a nitrilium ion intermediate followed by aromatization. A decarboxylative condensation between O^{18}-labeled phenylpyruvic acid and N-hydroxyphenethylamine oxalate salt resulted in amide products lacking the O^{18} label, providing further support for the nitrilium ion in the reaction pathway.

Integral to these efforts was the initial explorations of the solid-phase synthesis of the α-keto acid and the N-hydroxylamine which was laid out in Chapter 3. A select number of amino acids was loaded onto the Ellman “safety catch” 4-sulfamylbutyryl linker with loading efficiencies ranging from 26 to 80%. Activation of the amino acyl resin with iodoacetonitrile and subsequent displacement with alanine cyanoketophosphorane afforded the crude dipeptide cyanoketophosphorane product in very low yields. The requisite N,O-bis-Fmoc protected amino acids were obtained from
the corresponding free hydroxylamines in excellent yields. The coupling of the bis-Fmoc alanine to the alanine-preloaded Wang-resin was incomplete due to severe steric hindrance which could not be resolved by using higher equivalents of the hydroxylamine, longer reaction times, and two coupling cycles. Cleavage of the dipeptide hydroxylamine from the resin was attended by premature loss of the Fmoc protecting groups.

The application of the amide bond-forming reaction between sulfonamides and thioacids to the synthesis of glycosyl amides and glycopeptides was demonstrated in Chapter 4. β-N-glucosyl asparagine was synthesized from the corresponding N-glucosylsulfonamide and β-thioaspartic acid in good yields at mild conditions and short reaction times. A series of N-peptidyl and N-glycopeptidyl-2,4-dinitrobenzenesulfonamides consisting of short MUC 1 sequences was successfully assembled on the solid phase following Fmoc/tBu strategy with yields from 40% to 85%. Likewise, a series of Boc- and Fmoc-protected amino trityl thioesters were prepared from the corresponding amino acids in good to excellent yields. The analogous preparation of peptide thioesters suffered from low yields and epimerization. The reaction of the side-chain unprotected thioacids derived from the trityl thioesters with alanine sulfonamide proceeded chemoselectively and efficiently, especially at hindered junctions. Similarly, the ligation of histidine thioacid with N-glycopentapeptidylsulfonamide bearing unprotected peptide functionalities and protected glycan afforded the desired product in good yield (71%).
Acknowledgment

I have come this far!

That I have made it this far is due in no small measure to the generosity, support and understanding of many individuals. The successful completion of this dissertation would have been nearly impossible without the constant, meticulous mentoring, and unrelenting support of my esteemed advisor, Prof. Steven J. Sucheck. I am greatly indebted to him for taking me in, coming from the natural product isolation background, to the synthesis world. Although the transition was rough, his willingness to share his time and knowhow, both practical and conceptual, and encouragement had been instrumental in moving my research forward. Not to be forgotten are the numerous suggestions and corrections on scientific writing, presentation and poster-making. That the mentoring extended into the area of how to get acculturated, sometimes delivered in a humorous way, is greatly appreciated.

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August 2010
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List of Abbreviations

AcOH = acetic acid
Bn = Benzyl
Boc = \textit{tert}-butoxycarbonyl
2-Cl-Z = 2-chlorobenzyloxy carbonyl
Cbz = carbobenzyloxy
DCC = 1,3-dicyclohexylcarbodiimide
DIBAL-H = diisobutylaluminum hydride
DIPEA = \(N,N\)-diisopropylethylamine
DMAP = 4-(\(N,N\)-dimethylamino)pyridine
DMDO = dimethyldioxirane
DME = dimethoxyethane = dimethylglycol
DMF = \(N,N\)-dimethylformamide
DMSO = dimethylsulfoxide
dNBS = 2,4-dinitrobenzenesulfonyl chloride
EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Fmoc = 9-fluorenylethylcarbonyl
HATU = \(N\)-[(dimethylamino)-\(1H\)-1,2,3-triazolo[4,5-b]-pyridin-1-ylmethylene]-\(N\)-methylmethanaminium hexafluorophosphate \(N\)-oxide
HBTU = \(N\)-[(\(1H\)-benzotriazole-1-yl)(dimethylamino)methylene]-\(N\)-methylmethanaminium hexafluorophosphate \(N\)-oxide
HOBt = 1-hydroxybenzotriazole
HOOBt = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HONp = 4-nitrophenol
HOSu = N-hydroxysuccinimide
iPr = isopropyl
ivDde = 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl)
MBHA = p-methylbenzhydrylamine resin
mCPBA = m-chloroperoxybenzoic acid
Me = methyl
MeOH = methanol
MesNa = 2-mercaptopethane sulfonic acid, sodium salt
NMP = N-methylpyrrolidone
NBS = N-bromosuccinimide
OcHex = cyclohexyl ester
OSu = O-succinimidyl
PBS = phosphate buffered saline
PEG = polyethylene glycol
Ph = phenyl
py = pyridine
PyBOP = (1H-benzotriazol-1-yl)oxy)tris(pyrrolidino)phosphonium hexafluorophosphate
tBu = tert-butyl
TCEP = tris-carboxyethylphosphine
TES = triethylsilane

xx
TFA = trifluoroacetic acid
TFE = 2,2,2-trifluoroethanol
Tf₂O = triflic anhydride
THF = tetrahydrofuran
TIPS = triisopropylsilane
TLC = thin layer chromatography
TMP = 2,4,6-trimethylpyridine
TMSOTf = trimethylsilyl trifluoromethanesulfonate = trimethylsilyl triflate
Tn = 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl
Trt = trityl
TrtSH = trityl mercaptan
UV = ultraviolet
Z = benzyloxy carbonyl
CHAPTER 1

CHEMICAL LIGATION STRATEGIES FOR GLYCOPEPTIDE SYNTHESIS

1.1 Introduction

Glycopeptides and glycoproteins are found in nature as a heterogeneous mixture of several, and possibly tens, of structures having the same peptide chain but of a variety of carbohydrate architectures. The carbohydrate structures can range from simple to complex for a given glycosylation site while the number of glycosylated sites can also vary.\(^1\) \(^4\) This ensemble of differential carbohydrate structures, collectively termed glycoforms, is brought about by the inherent way in which the biological glycosylation machinery operates. Whereas the polypeptide chain is under the direct control of the cellular transcriptional and translational machinery, the glycosylation event of the peptide is influenced by the expression levels and subcellular distribution of glycosyltransferases and glycosidases.\(^5\)

There are two main sites in the peptide chain where glycosylation occurs which help to classify the resulting glycopeptide. The reducing end of the carbohydrate may be linked to the amide nitrogen of the asparagine residue, in which case the carbohydrate is \(N\)-linked. Alternatively, the reducing end may be appended to the hydroxyl oxygen of
serine or threonine residues, in which case the glycan is O-linked. The carbohydrate modification of the peptide chain (glycosylation) is carried out by glycosyltransferases and glycosidases on the nascent (co-translational) or fully synthesized (post-translational) polypeptide. N-linked glycosylation begins in the lumen of the endoplasmic reticulum. The asparagine in the consensus sequence Asn-X-Ser/Thr of the nascent peptide accepts en bloc a 14-residue oligosaccharide from dolichol oligosaccharide precursor (Scheme 1A). In general, three glucose units and one mannose residue are removed to give the high mannose subtype which is then exported to the Golgi complex. The glycan undergoes further processing in the Golgi complex, including removal of three more mannose units and attachment of various glycosyl units, to give the hybrid and complex subtypes (Scheme 1B). O-linked glycosylation occurs exclusively in the Golgi complex and starts with the addition of GalNAc to serine or threonine residues of a fully synthesized polypeptide (Scheme 2). In contrast to N-linked glycans, there is no known consensus sequence for the addition of GalNAc, although it appears to be common at proline-rich sites that promote β turns. As the glycopeptide transits through the Golgi complex it may acquire more glycosyl units. Sialylation of GalNAc terminates further glycosylation and results in a structure commonly encountered in cancer cells (Scheme 2). Addition of Gal and GlcNAc generate the Core 1 and Core 3 subtypes, respectively, which can then be further elaborated (Scheme 2). Both processes of glycan (N- and O-linked) diversification give rise to the observed heterogeneity of this class of biomolecules.
Scheme 1. (A) Vertebrate N-Glycosylation of the Peptide Chain in the Lumen of the ER. The asparagine residue in the consensus sequence, Asn-X-Thr/Ser, of the nascent peptide accepts the oligosaccharide from dolichol pyrophosphate in the presence of OST. This event is followed by removal of three glucose and one mannose units. (B) Vertebrate Diversification of N-Glycans in the Golgi Complex. This process generates three N-glycan subtypes, namely, high mannose, hybrid and complex (Ref: Varki et al., ed. Essentials of Glycobiology. Cold Spring Harbor, New York, 1999, 85-100).
Scheme 2. \(O\)-Linked Glycosylation of Serine and Threonine Residues and Associated Diversifications. Diversification of \(O\)-linked glycans results in several core structures, the more common ones are shown above. An early and alternate modification of the GalNac residue with sialic acid generates the tumor-associated sialy Tn antigen which terminates further glycan modification (Ref: Varki \textit{et al.}, ed. \textit{Essentials of Glycobiology}. Cold Spring Harbor, New York, 1999, 101-113).

As an illustration of the heterogeneity of glycoproteins, bovine pancreatic RNase is a mixture of unglycosylated peptide designated RNase A with a molecular weight of 13,682 kDa and glycosylated variants referred to as RNase B.\(^2\) The natural population of bovine pancreatic RNase B is composed of five glycoforms of the oligomannose series Man-5 to Man-9 associated with the single N-glycosylation site at Asn-34 (Figure 1).\(^2\) Kamoda and co-workers\(^4\) confirmed the presence of the five glycoforms in a study that involved the enzymatic cleavage of the oligosaccharides of bovine pancreatic RNase B with \(N^4\)-(acetyl-\(\beta\)-D-glucosaminyl)asparagine amidase (PNGase F; EC 3.2.2.18, recombinant) followed by Fmoc labeling of the liberated glycosylamine. In addition, the
positional isomers of Man₇GlcNac₂ were resolved into three peaks in the normal phase HPLC using an amide-silica column.

Figure 1. Capillary Electrophoresis of Bovine Pancreatic RNase B. The natural population of RNaseB consists of five glycoforms of the oligomannose series, Man-5 to Man-9, labeled peaks 5 to 9 in the chromatogram (Ref: Rudd et al. Biochemistry. 1994, 33, 17-22).

Other types of protein glycosylation exist in nature. This includes Glc-β-Asn⁸ and GalNAc-Asn,⁹ first observed in cell surface glycoprotein of Halobacteria. Glc-α-Asn linkage was identified in nephritogenoside, a glycopeptide isolated from rat glomerular basement membranes.¹⁰ Serine and threonine residues in murine estrogen receptors were found to be O-glycosylated with GlcNAc.¹¹,¹² This diversity is further expanded by alternative sites for glycosylation including tyrosine residue first identified in retinal glycogen¹³ and the hydroxylated side-chain amine of lysine in endogeneous adinopectin.¹⁴ Those types of glycosidic linkages and alternative sites of glycosylation will not be included in this review.
The natural heterogeneity of glycoproteins complicates efforts to delineate the role of glycosylation on the over-all properties, including biological function, of the resulting glycopeptide because of the inherent difficulty of isolating pure compounds in sufficient quantities.\textsuperscript{1,2} Despite these challenges, great strides have been made in understanding the roles glycopeptides and glycoproteins play in the development, growth, and survival of the organism, aided in no small part by chemical synthesis. This includes protective and barrier functions such as in the case of mucins;\textsuperscript{15} cell-cell and cell-matrix interactions that are critical in the organization and maintenance of normal tissue architecture;\textsuperscript{16-18} assistance in proper protein folding, protein trafficking, glycoproteins’ half-life, immune system regulation;\textsuperscript{5,19,20} fertilization of ovum and embryogenesis;\textsuperscript{15,18} as hormones and surface receptors for host-pathogen interactions;\textsuperscript{5,19} and bioluminescence.\textsuperscript{21} Direct evidence for many of these roles has been provided by isolation of the target glycopeptides\textsuperscript{16-18} and total synthesis of some proteins.\textsuperscript{22}

Chemical synthesis of glycopeptides offers the opportunity of precise control on the structure of the carbohydrate domain and site of glycosylation and therefore provides vital information on the effects of glycosylation on the properties of the glycopeptide.\textsuperscript{1,2} Aside from providing an efficient alternative approach to the tedious isolation of pure glycopeptides from natural sources, such synthetic skill and understanding can be deployed for practical ends, i.e., in the preparation of glycopeptide-based vaccines,\textsuperscript{23-26} therapeutics and diagnostics.\textsuperscript{27-29} An example of a glycopeptide-based vaccine is shown below (Figure 2A) which is composed of two important regions: (1) A sialy-Tn antigen on a short sequence of MUC1 (B-cell epitope) and linked to the (2) Ovalbumin T\textsubscript{H}-cell
epitope through a triethylene glycol spacer acid. This vaccine construct elicited a strong and highly specific humoral response of the IgG type in mice. The antibody generated exclusively recognizes the glycopeptide derived from MUC1; neither the nonglycosylated MUC1 segment, nor the identical sialyl-Tn attached to a different peptide chain, MUC4, was recognized. Because the immune response was effective only for a third of the immunized population, an improved version was synthesized. In this case, sialyl-Tn was anchored on a 20-mer peptide sequence of MUC1 and conjugated to tetanus toxoid protein carrier (Scheme 2B). This multivalent vaccine, with at least 20 molecules of the glycopeptide loaded per molecule of carrier, elicited a strong immune response in all vaccinated mice. Neutralization experiments strongly indicated that the antibodies produced were specific to the glycopeptide unit of the vaccine. The unglycosylated MUC1-peptide, MUC-1 peptides displaying different glycan structures and the same sialyl-Tn on a different peptide sequence were weakly or not recognized by the antibodies produced in response to the vaccine.
Figure 2. Glycopeptide-Based Vaccines: (A) Vaccine constructed from the sialyl-Tn presenting MUC1 and linked to a short sequence of ovalbumin. (B) An improved version composed of sialyl-Tn MUC1 glycopeptide conjugated to tetanus toxoid.

The chemical synthesis of glycopeptides entails the preparation of two distinct but equally important domains: polypeptide and carbohydrate. With the advent of solid phase peptide synthesis, together with the plethora of peptide protecting groups and resins, peptide preparation of moderate size, approximately 40 residues, has become routine.\textsuperscript{30,31} The preparation of carbohydrates from its monomers in a way that could rival the solid phase technique for peptides is far from settled but tremendous inroads into their synthesis have been gained through the years.\textsuperscript{32-35} For the synthesis of small glycopeptides, an excellent review was provided by Davis.\textsuperscript{36} These fragments, appropriately functionalized at the termini, may be further linked together to arrive at larger, more complex glycopeptides, including one that approaches the biologics level. At
this molecular size, chemical ligation is the preferred approach and one that serves to highlight the convergence of these two powerful groups of synthetic methodologies.

In its simplest sense, what chemical ligation does is to generate a new amide bond or an analog structure exclusively between the respective termini of two unprotected peptide and/or glycopeptide fragments (Scheme 3).\textsuperscript{37} To enable this ligation event, the termini are functionalized with groups that are mutually reactive with one another. This process may be reiterated to achieve the desired peptide size using the same or unrelated chemistries.\textsuperscript{38,39} Since the fragments are prepared separately, each segment may display unrelated carbohydrates that ultimately redounds to glycan diversity in the glycopeptide product. This modular approach to fragment preparation allows two or more groups to work simultaneously, especially for challenging targets. Because the linking occurs at the peptide unit, chemistries that are used for peptide ligation are applicable to glycopeptide synthesis as well. Whatever the nature of the chemistry involved, it is paramount that the glycosidic bond to the peptide chain, and all glycosidic bonds for that matter, survive the ligation step and any post-ligation manipulations. In addition, suppression of racemization of the C-terminal side of the acyl donor is important to preserve the biological activity of the glycopeptide product. The use of fully unprotected peptidic side-chains, as well as the carbohydrate domains, allows the ligation to be carried out in aqueous conditions. Moreover it helps reduce the number of post-ligation steps such as the removal of protecting groups that may promote unforeseen side-reactions to occur.
Scheme 3. Principle of Chemical Ligation.

The total synthesis of proteins and glycoproteins, however, remain a daunting task, employing the concerted efforts of several individuals and groups to come up with the most efficient routes to the targets.\textsuperscript{40} This drives the continued refinement and development of chemical ligation protocols. Chemical ligation protocols can be divided into two general classes based on the structural elements utilized to bring the reacting fragments together and will serve as the organizing principle for this review. One is based on the ability of the mercapto group to readily undergo exchange with thioesters or related derivative as the basis for linking the reacting fragments. The other relies on a group of chemistries that does not involve thioester exchange to bring forth the ligation of the peptide segments. Specific examples that embody each class of ligation protocols will be given. The focus will be on chemistries that have been shown to be useful for glycopeptide synthesis and emerging ligation strategies that show promise in this field. Additionally, only those reactions that provide a native amide linkage will be discussed. Ligations that produce a non-peptide bond at the junction have been reviewed.\textsuperscript{41}
1.2 Cysteine and Thiol-Based Ligation Protocols

Cysteine-enabled ligation chemistry as epitomized by native chemical ligation (NCL) and its antecedents are the first to make headway into the preparation of peptides and glycopeptides with a native amide backbone. This chemistry takes advantage of the nucleophilicity of the sulfur atom and has its beginnings in the work of Wieland and associates. This group obtained the dipeptide valylcysteine starting from the aqueous mixture of valine phenyl thioester and cysteine, the first demonstration of amide-bond formation through a S to N acyl shift. It would be years before the value of this transformation was fully exploited in the form of NCL.

1.2.1 Native Chemical Ligation

Unarguably, NCL has positioned itself as a ligation method of choice for targets bearing cysteine residues located strategically in the sequence. NCL has been applied as well to peptide targets where cysteine replaced nonessential residues to allow the ligation reaction to take place. An example is the cysteine-deficient small microbial ribonuclease, barnase, in which Lys was substituted with Cys to create the requisite ligation site. Its synthetic utility has been convincingly demonstrated in the synthesis of hundreds of proteins and equally useful for the synthesis of glycopeptide, both secretory and membrane bound. The largest polypeptide synthesized by NCL to date is the “covalent dimer” of HIV-protease with 203 amino acid residues. The reaction involves the nucleophilic attack of the thiolate of the N-terminal cysteine in a peptide to a C-terminus thioester of another peptide (Scheme 4). This process links the two peptide fragments together and results in two reactive sites that are in close proximity to one
another. This favorable geometric arrangement allows a facile intramolecular $S$ to $N$ acyl shift to give the amide product.$^{43}$

![Scheme 4. Mechanism of Native Chemical Ligation.](image)

**1.2.2 Removable Auxillaries-Assisted NCL and Related Approaches**

The dependence of NCL for the cysteine residue to capture its peptide partner has been often cited as its major drawback. Driven by this limitation, various innovations were pursued to extend its usefulness to targets without cysteine or have cysteine residues but at locations not useful for the ligation scheme. An alternative is to use cysteine itself$^{50-52}$ or a sulhydryl-functionalized amino acid side-chain, such as β-mercaptophenylalanine,$^{53}$ to effect the ligation step. The ligation product is then subjected to selective desulfurization to give the desired residue, alanine or phenylalanine at the ligation site. Desulfurization of cysteine residue is possible in the presence of its Acm-protected analog.$^{51}$ Where such modification is not possible or does not have said amino acid residues at the desired point of ligation, anchoring of a removable auxillary to the N-
terminus offer similar outcomes, and in fact is widely practiced (Scheme 5A). This later approach has been fruitful in the preparation of complex glycopeptides.

Thiol-based auxillaries tethered to the amino of the N-terminal residue included the $N^\alpha$-ethanethiol, $N^\alpha$-oxyethanethiol,\textsuperscript{54} 2-mercaptobenzyl,\textsuperscript{55} 1-phenyl-2-mercaptoethyl\textsuperscript{56} (Scheme 5B). $N^\alpha$-ethanethiol and $N^\alpha$-oxyethanethiol are the first of these auxillaries to have appeared,\textsuperscript{54} introduced by the same group who published the landmark NCL two years earlier.\textsuperscript{43} These auxiliaries are stable under the ligation conditions employed and allow thioester exchange. However, attachment of the auxiliary to the N-terminus generates a secondary amine and as a consequence of the increased steric bulk, the S to N acyl shift is slower compared to the primary amine nucleophile of NCL.\textsuperscript{54,55,57,58} In some cases, the reaction stops at the initial thioester ligated product especially if nonglycine residues are located on either side of the nascent peptide bond.\textsuperscript{54} Slow rearrangement to the amide product encourages the competing side reaction to hydrolyze the initially ligated thioester.\textsuperscript{59} In addition to slower reaction rates, a number of these auxiliaries are not easily removed at the end of the synthesis when a native amide bond is so desired. 1-Phenyl-2-mercaptoethyl, employed by Low and co-workers for the synthesis of cytochrome b562, requires HF for its removal\textsuperscript{56} whereas $N^\alpha$-oxyethanethiol is detached under milder Zn reduction, although histidine-rich sequences have a tendency to bind the Zn metal.\textsuperscript{54} In the case of the 2-mercaptobenzyl auxiliary featured in the work of Offer and Dawson, there was no demonstration of the cleavage of the auxiliary.\textsuperscript{55} Viewed from the context of glycopeptide synthesis, notwithstanding the slower reaction rate, the survival of the glycosidic link becomes an issue for these auxiliaries. The application of
this approach to glycopeptide synthesis has to await the development of TFA-cleavable auxiliaries that leave the glycosidic bond untouched.

![Diagram of Thio-Auxiliary-Assisted NCL of C-Terminal Thioester and Peptide with Noncysteine Residue at the N-terminus.](image)

Scheme 5. (A) Thio-Auxiliary-Assisted NCL of C-Terminal Thioester and Peptide with Noncysteine Residue at the N-terminus. (B) Structures of Auxiliaries Appended at the N-Terminus.

Increasing the electron density on the benzyl moiety through ring substitution with methoxy groups enhanced the ligation efficiency and TFA-lability of the auxiliaries. A particular embodiment of such innovation was the 4,5,6-trimethoxy-2-mercaptopbenzyl (Tmb) and 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliaries (Figure 5B). Tmb was
used for the auxiliary-assisted ligation of the 62-mer peptide of α-spectrin and auxiliary removal was achieved using TFA (Scheme 6A).\textsuperscript{57} 1-(2,4-Dimethoxyphenyl)-2-mercaptoethyl auxiliaries incorporated to a 9-mer glycopeptide fragment having a protected monosaccharide generated the desired glycopeptide product with its 4-mer thioester partner, provided that the ligation junction consists only of glycine residues (Scheme 6B).\textsuperscript{58}
Scheme 6. Ligation of Peptide C-Terminal Thioesters with Peptides Bearing Thiol-Auxiliaries Appended to the N-Terminus. (A) The Tmb auxiliary was employed for the ligation. (B) 1-(2,4-Dimethoxyphenyl)-2-mercaptoethyl facilitated the ligation reaction of the glycan-bearing fragment.

In an elegant piece of work by the Danishefsky group, Tmb was used in tandem with the in-situ generated thioester that they have pioneered. This group has established the use of the S-phenolic esters from its O-phenolic ester disulfide precursor as a variation of the thioester moiety used in NCL (Scheme 7). Under reducing conditions
the disulfide generates a free thiol group that subsequently rearranges to the aromatic thioester. While certainly an interesting reactive intermediate to consider by analogy to NCL, it has been pointed out that it is strictly not necessary for the ligation to occur.\textsuperscript{60} The S-phenolic ester reacts with peptides having N-terminal cysteine following a mechanistic manifold typical of NCL. The S-phenolic ester and its cysteine-bearing peptide partner lend themselves to the convergent synthesis of glycopeptide fragments, thus allowing diversity of carbohydrate structures in the final product with both N- and O-linkage present (Scheme 7, right side). For N-terminal cysteine-deprived peptides, anchoring the Tmb group essentially led to the same outcome (Scheme 7, left side). The Tmb and O-phenolic ester modified peptides, under reducing conditions, allowed access to glycopeptides having one or more structurally complex carbohydrate domains.\textsuperscript{59} Like its predecessor, these carbohydrates can be distinct from one another and both O- and N-linkage can exist on the same peptide domain. This culminated in the synthesis of a multifunctional glycopeptide displaying three carbohydrate domains from three glycopeptide constructs in a reiterative fashion (Scheme 8).\textsuperscript{59} The middle segment, Fragment 2, appropriately functionalized with a protected N-terminal cysteine, as well as C-terminal O-phenolic ester, was ligated at its C-terminus to the Tmb-auxiliary modified Fragment 3. Upon deprotection of the N-terminal cysteine in the conjoined segment using 10% morpholine/DMF and hydroxylamine, the first fragment was incorporated to the final product through the NCL phenolic ester variation.\textsuperscript{59} An optional two-step sequence for the detachment of the auxiliary was devised to avoid the problem of N to S acyl shift at the ligation center.\textsuperscript{59} This involved the formation of disulfide bond on the mercapto group of the auxiliary and finally treatment with TFA.
Scheme 7. Ligation of Glycopeptide Fragments Using Phenolic Thioester Acyl Donors and Auxillary-Based Acceptors.
Scheme 8. Reiterative Synthesis of N-linked Glycopeptide with Three Carbohydrate Domains. Peptide chains were unprotected unless otherwise noted.

1.2.3 Sugar Assisted Ligation

The sugar assisted ligation (SAL) strategy was introduced by Wong and co-workers for ligations at cysteine-free junctions (Scheme 9). Instead of a removable thiol auxiliary anchored at the N terminus of a peptide, the capture element resides in the sugar unit as a thioacetamido group at C-2. The thioacetamido group fulfills the functions of the N-terminal cysteine of NCL. The peptide thioester is sequestered by this thiol handle at the transthioesterification step. Subsequent S to N acyl transfer provides the ligation product that was found to be rate-limiting. Once completed the thioacetamido group is
reduced using catalytic hydrogenation (Pd/Al₂O₃, H₂, 6M Gn·HCl, TCEP, phosphate buffer, pH = 5.8) to provide the unmodified glycopeptide.


The chemistry is capable of ligation of the three common forms of glycopeptides, namely, α-O and β-O-linked and N-linked glycopeptides. One example is the synthesis of the diptericin ε, an α-O-linked glycoprotein of bacterial origin having 82-residues, none of which is cysteine. This was previously prepared by the Bertozzi group that involved mutating the glycine-25 residue to cysteine to enable the synthesis using NCL. In the present work, the glycoprotein was broken down into three segments and coupled from C- to N-terminus (Scheme 10A). SAL conjoined the middle segment peptide thioester (residues 37-52) and the glycan-bearing C-terminal segment (residues 53-82) at the Gly-Val junction. The resulting fragment presented a cysteine residue at the N-terminus that enabled it to be linked to the N-terminal segment (residues 1-36) by NCL. The synthesis was completed by desulfurization of the cysteine residue. SAL
proved to be useful too for the synthesis of a portion of the cancer-associated α-O-linked glycoprotein MUC1. Ligation was executed at the His-Gly junction between a 5-mer peptide thioacid and 15-mer fragment bearing two glycan moieties, one of which had the modified thiol handle (Scheme 10B). ⁶⁴
Scheme 10. (A) Synthesis of Diptericin ε by SAL and NCL. (B) Synthesis of MUC1 Diglycopeptide by SAL.
Extensive research into the scope and limitations of the chemistry unearthed several factors that affect reaction rates and yields. SAL requires an internal location of the residue bearing the thiol-modified glycan proximal to the N-terminus, irrespective of the glycopeptide class. For optimal results, the glycosylated amino acid preferably is the second residue from the N-terminus.\textsuperscript{61,62,64} We refer to this protocol as first-generation SAL, also called traditional SAL (Figure 3). Extended versions of SAL (exvG1-SAL) have the thiol-modified glycan located farther away from the N-terminus by addition of more residues N-terminal to the glycosylated amino acid (Figure 3). In these cases, yields were viable for ligation reactions where the glycosylated amino acid is as far as the seventh residue from the N-terminus. Nonetheless, there was a gradual reduction in yields as the thiol-modified glycan was moved farther from the N-terminus.\textsuperscript{64} Molecular dynamics simulations revealed a favorable proximal distance of the reactive sites of the traditional SAL which proceeds through a 14-membered transition state for $O$-linked glycopeptides. The extended versions of SAL, in contrast, exhibited longer distances between the reactive sites. These extended versions proceed through a 17-29-membered transition states for a glycan position on the third to seventh residue, respectively.\textsuperscript{64}

![Figure 3. Comparison of the Position of the Glycosyl Amino Acid in First-Generation SAL (G1-SAL) and Extended Versions of First-Generation SAL (exvG1-SAL). The exvG1-SAL is obtained by attachment of more amino acids at the N-terminus.](image-url)

The steric bulk of the amino acids at the ligation junction play a significant role in SAL containing a monosaccharide unit regardless of the type of glycosidic bond and how far the glycosylated residue is from the N-terminus. In most cases, there was a proportional reduction in yields or prolonged reaction times as the ligation site becomes crowded.\textsuperscript{61,62,64} The ligation is influenced by the steric demands of the thioester-bearing residue more than the N-terminal residue of the glycosylated fragment.\textsuperscript{62,64} An example is the Val-Gly linkage. The reaction between the thioester peptide bearing C-terminal Gly and glycopeptide with Val at the N-terminus led to the product.\textsuperscript{62} In contrast, a Val situated at the C-terminus of the peptide thioester did not provide the product when reacted with a glycopeptide possessing a N-terminal Gly.\textsuperscript{62} The reaction was permissive for ligation sites occupied by less to moderately bulky amino acids, such as Gly and Ala, as well as, residues that behave as a general base during the reaction, such as Asp and His, despite being bulky.\textsuperscript{62,64} Ligations involving difficult junctions, for example Val, typically exhibited slow reaction rates.\textsuperscript{61,62} Illustrative of this difficulty is the less than 5% conversion after 48 h for the Val-Ala and Val-Gly linkage; the major product was the unrearranged ligation thioester intermediate.\textsuperscript{62} The steric influence of the C-terminal residue of the thioester, however, is modulated in the presence of more complex glycans.\textsuperscript{66}

Elaboration of the C-2 thiol-bearing monosaccharide unit can have profound influence on the extent of the ligation reaction.\textsuperscript{66} Ligation reactions are possible when additional saccharide units were installed on the C-4 and C-6 positions of the peptide bound sugar unit, but proved to be deleterious when executed at C-3 (Figure 4).
reaction was sensitive to steric bulk of the N-terminal amine and glycan moiety when elaboration was performed on C-4. Synthetically useful yields can be obtained from ligations involving modification at the C-6 but were generally lower than the C-4 substituted thiol-substituted glycan.\textsuperscript{66}

Figure 4. Elaboration of the Monosaccharide Unit of the First-Generation SAL. Sites on the peptide-bound sugar modified with additional glycan units are highlighted with positional numbers. (A) For easy comparison, the structure of G1-SAL containing a monosaccharide unit is provided. (B) Modification at C-4 of the peptide bound carbohydrate with one glycan unit. (C) Modification at C-4 with a disaccharide. (D) Elaboration of the C-6 position. (E) Additional sugar units appended on the C-4 and C-6 positions.
SAL employs desulfurization as the final step to remove the thiol handle on the sugar. The reaction proceeds efficiently with a conversion of up to 90% isolated yield.\(^6^6\) Despite these favorable outcomes, not all peptidic side chains can survive this modification, notably that of cysteine. Initial efforts involved the protection of the Cys residue with acetamidomethyl that rendered the side chain relatively inert to the reductive conditions.\(^6^3\) Side-chain protection of the cysteine residue was not necessary in the second-generation of SAL (G2-SAL).\(^6^5\) The capture element, mercaptoacetic acid, was esterified to the C-3 position of the sugar (Figure 5). Removal of this capture element can be effected by simple hydrolysis using 5% hydrazine in aqueous dithiothreitol. Hence Acm protection of the cysteine residue was not required.\(^6^5\) But this repositioning of the capture element was accompanied with prolonged reaction times that were not satisfactorily resolved even by situating the glycan farther from the N-terminus.

Figure 5. Comparison of the Thiol-Capture Element of the First-Generation SAL (G1-SAL), Second-Generation SAL (G2-SAL) and Extended Versions of Second-Generation SAL (exvG2-SAL). Note the repositioning of the capture element in the G2-SAL to the C-3 of the carbohydrate. The linkage of the capture element in G2-SAL is ester in contrast to G1-SAL which is an amide.
The ligation reaction was carried out in a mixture of NMP and guanidine/HEPES buffer (pH = 8.5) to minimize the hydrolysis of the thiol auxillary and thioester which was significant in the standard ligation buffer consisting of guanidine/NaH$_2$PO$_4$/thiophenol (pH = 8.5, 37 °C). The O-linked glycopeptide fragment used in the G2-SAL had the glycosylated amino acid and cysteine residues situated second and third, respectively, from the N-terminus. The G2-SAL tolerated the presence of unprotected cysteine residue but it was accompanied by protracted reaction times of 2.5 to 4 days and yields that ranged from 22% (His-His linkage) to 84 % (Gly-Gly linkage) as compared to the first-generation SAL (G1-SAL) for the same ligation sites. To improve the ligation outcomes, extended versions of second-generation SAL (exvG2-SAL) was implemented whereby the glycosyl-bearing serine, and cysteine residues were located third and fourth, respectively, from the N-terminus. When compared to G2-SAL with the same residues at the ligation sites, a little more than half showed improvement in yields. The remaining ligation sites mostly showed slight decrease in yields. exvG2-SAL of noncysteine containing glycopeptides provided yields (58-68%) that were either comparable to or less than their cysteine containing counterparts for the same ligation sites.

Further developments involved locating the glycan farther from the N-terminus by chain extension on the N-terminal side of the glycosylated residue (at least fourth position). These further developments gave comparable or lower yields compared to the G2-SAL and shorter extensions of G2-SAL for the Gly-Gly linkage. A notable exception was that for the His-Gly linkage with a marked improvement of 31-35 % in
yields as compared to G2-SAL and shorter extensions of G2-SAL. Despite these admirable developments, in almost all cases, the long reaction times were not resolved satisfactorily.

The sugar can serve the role of a removable auxiliary for challenging ligation reactions provided that a threonine, serine or aspartic acid is close to the desired ligation site. The sugar can be cleaved enzymatically or chemically once its purpose has been served. A preliminary work on a glycosyl undecapeptide involving an amidase, PNGase A, lend support to the feasibility of such proposal.\textsuperscript{62} The glycopeptide was deglycosylated but the reaction was low yielding and incomplete.\textsuperscript{62} Furthermore this has the drawback of using a complex and expensive structure only to be degraded later in the synthesis. In this case, it may be more attractive to use a small, less structurally complex, and less expensive auxiliary on the aforementioned amino acids to assist in the ligation. Side-chain assisted ligation (SCAL) attempts to address this issue by using a simple cyclohexane-based derivative as capture element.\textsuperscript{67,68}

1.2.4 Side-Chain Assisted Chemical Ligation

Similar to SAL, SCAL employs a thiol auxiliary anchored to the hydroxyl and carboxyl side-chains of amino acids via an ester bond.\textsuperscript{67} The auxiliary is a mercaptoacetic acid linked to a cyclohexane or cyclopentane-based template (Scheme X). The auxiliary engages the peptide thioester to allow the S to N acyl shift to occur efficiently (Scheme 11). When the glycosyl amino acid was located penultimate to the N-terminus, the ligation reaction showed preference for less hindered amino acids at the ligation junction.
and those with side chains that act as general base. The relative configuration of the substituents on the ring and ring size, at most six-membered, did not affect the reaction rates as long as the auxiliary was connected to aspartic acid. The synthetic utility of SCAL was demonstrated for the synthesis of a C-terminal 59-mer section of the HIV-1 Tat protein.\textsuperscript{68} It should be noted that the efficient removal of the auxiliary has yet to be addressed.

1.2.5 Direct Oxo-Ester Ligation

In this ingenious variation of NCL, the Daneshsky group\textsuperscript{69} replaced the now de rigueur thioester partner of the cysteine-presenting peptide with an oxo-ester such as the \textit{p}-nitrophenyl ester (Scheme 12). Competition experiments strongly indicated the thiol capture of the oxo-ester by cysteine. The unprotected oxo-ester peptide is more suited for ligations involving a hindered amino acid residue at the C-terminus which include threonine, valine, isoleucine, \textit{d}-allo-isoleucine, and proline. The reaction medium incorporated \textit{p}-nitrophenol to retard the hydrolysis of the oxo-ester in addition to the standard buffer components of NCL (Gn\textcdot\text{HCl}, NaH\textsubscript{2}PO\textsubscript{4}, TECP). The oxo-mediated ligation provided better yields and shorter reaction times compared to the traditional thioester-mediated NCL based on a single comparison experiment. Ligation reaction times ranged from 2 h for the octapeptide \textit{p}-nitrophenyl ester having a threonine C-terminal residue to 15 h for the proline C-terminal variant. The threonine-oxo ester bearing peptide had the highest yield (79\%) of ligation product, whereas the proline variant had the lowest (50\%).

1.3 Thiol-Auxillary and Cysteine-Free Ligation Protocols

More general ligation protocols that do not depend on thiol-based auxillaries and capture agents are also under development. Moreover, the expedient preparation of their respective fragments on the solid phase is still under investigation. The potential of these emerging ligation platforms in glycopeptide synthesis will be of great interest especially considering that some of these methods are less sensitive to steric factors found at the ligation junctions and therefore offer the opportunity to select a ligation site irrespective of the identity of the amino acids at the junction. While the metal\textsuperscript{70} and metal-free ligations\textsuperscript{71} have been around for quite some time and have been convincingly demonstrated for their usefulness in glycopeptide synthesis, decarboxylative condensation,\textsuperscript{72} thioacid-sulfonamide ligation\textsuperscript{73} and related reactions using electrophilic arenes,\textsuperscript{74} direct aminolysis of peptide thioester\textsuperscript{75}, and Staudinger ligation\textsuperscript{76} are emerging technologies in the ligation area. These recent developments broaden the available tools to access glycopeptides that allows investigators to tailor the synthetic approach to the unique requirements of a particular target and in conjunction with the expediency of the preparation of the building blocks.

1.3.1 Metal and Metal-Free Mediated Coupling

Silver ions convert thioesters and thioacids to active oxoesters which then undergo condensation with the N-terminal amino group of the peptide fragment partner (Scheme 13A). An early demonstration of this procedure in segment coupling was provided by Blake and Li,\textsuperscript{70} who in 1981 reported the preparation of human [Gly17]-β-endorphin in aqueous phase (Scheme 13B). The required peptide segments, a C-terminal
peptide thioester and N-terminal amino peptide, were readily prepared on the solid phase. Protection of the carboxyl side-chain was unnecessary in the coupling reaction because silver ions can selectively activate the C-terminal thioacid residue. Beyond the citraconyl protecting group on lysine residues, other side-chain functionalities were not protected during the reaction with the amine component. The reaction was initiated by addition of silver nitrate and N-hydroxysuccinimide to a solution of the peptide thioacid and N-terminal amino peptide.
Silver-mediated peptide coupling is not limited to thioacids as described in a review by Aimoto (Scheme 13A).\textsuperscript{77} Peptides with S-alkyl thioester modification at the C-terminus undergo condensation with N-terminal amine peptides. This approach, he pointed out, allows the use of Boc-protection for amine side-chains rather than the unstable citraconyl group, improves solubility, and circumvents the oxidation and hydrolysis associated with the thioacids. Application of this method to glycopeptide synthesis was realized in the chemoenzymatic synthesis of eel calcitonin (Scheme 13C).\textsuperscript{78} The synthesis of the thioester bearing the N-linked N-acetylglucosamine was achieved on the solid phase. A Boc-strategy for the assembly of the peptide was employed because the C-terminal thioester was introduced at the start of the synthesis and is incompatible with the deblocking conditions of the Fmoc strategy. The glycosidic N-linkage survived the cleavage step involving HF to give the partially protected glycopeptide thioester (10 residues) in 12% yield.\textsuperscript{78} The other fragment, 22 residues long, was obtained by routine Boc-solid phase method.\textsuperscript{78} Segment condensation was carried out in DMSO using AgNO\textsubscript{3}, HOOBt and DIPEA in an overnight reaction at room temperature to give the partially protected glycopeptide in 55% yield. After removal of protecting groups on lysine, cysteine and the N-terminal residue, enzymatic elongation of the monosaccharide unit on the glycopeptide was performed using endo-β-N-acetylglucosaminidase.\textsuperscript{78}

A purely synthetic approach for the silver-mediated ligation of glycopeptides having complex carbohydrate structures was developed in the laboratory of Danishefsky\textsuperscript{71} who used the O-phenolic esters equipped with a disulfide moiety as the acyl donor. Previous work has shown that it rearranges to the S-phenolic form upon
reduction of the disulfide which is suited to undergo an NCL-type ligation.\textsuperscript{60} Non-cysteine terminal amines, however, failed to capture this type of thioester\textsuperscript{71} (Scheme 14A, insert). In a reaction analogous to the Aimoto method, AgCl was found to enhance the reactivity of the \( S \)-phenolic ester and interestingly, primes the disulfide bond for cleavage, although mechanistic details are still lacking\textsuperscript{71} (Scheme 14A) This outcome eliminated the use of the disulfide reducing agent TCEP-HCl (tris(2-carboxyethyl)phosphine hydrochloride) necessary for the conversion of the ester to the thioester. This reaction, termed AgCl-assisted phenolic ester directed amide coupling (PEDAC-AgCl), gave a high yield of the 23-mer \( N \)-linked glycopeptide containing unprotected pentameric glycan (Scheme 14B).\textsuperscript{71}
Scheme 14. (A) Ligation of O-phenolic Ester Peptide with Non-cysteine Terminal Peptide in the Presence of Silver Ions. (B) Synthesis of N-linked and (C) O-linked Glycopeptides.
Control experiments on the above-mentioned reaction indicated that silver ion can be omitted from the mixture, a fortuitous finding that makes possible the ligation without protecting the cysteine residue; lysine still requires protection.\textsuperscript{71} As a replacement for the silver ions, TCEP was brought back to the reaction mixture to initiate the thioester formation. This TCEP-assisted phenolic ester directed amide coupling (PEDAC-TCEP) was enlisted for the synthesis of the 65-mer \textit{O}-linked glycopeptide bearing the fully protected tetrasaccharide glycophorin domain\textsuperscript{71} (Scheme 14C).

PEDAC-TCEP it turned out is orthogonally compatible with the C-terminal alkyl thioester which is not the case for the PEDAC-AgCl. This important finding opened the way for the coupling of an \textit{N}-linked glycopeptide fragment to a peptide acyl acceptor that has a resident C-terminal alkyl thioester using PEDAC-TCEP\textsuperscript{71} (Scheme 15). The resulting glycopeptide is subsequently activated using PEDAC-AgCl for coupling to the third fragment.\textsuperscript{71} PEDAC-TCEP proved to be a powerful ligation protocol for the coupling of two fragments bearing substantial non-identical \textit{N}-linked sugar domains; one has 12 monosaccharide units, the other 11 units\textsuperscript{71} (Scheme 16). The resulting bidomainal 31-mer glycopeptide, obtained in excellent yield, represents a target that does not lend easily to the direct glycosylation of the peptide chain.
Scheme 15. Tandem PEDAC-TECP and PEDAC-AgCl Approach for the Synthesis of N-Linked Glycopeptide.
Scheme 16. Ligation of Glycopeptide Fragments Bearing Extensive Carbohydrate Regions Using PEDAC-TCEP.
1.3.2 Decarboxylative Condensation

Decarboxylative condensation represents a novel ligation strategy that is more akin to the imine ligation methods in terms of the capture step. In imine ligation methods, the C-terminal-ester glycoaldehyde forms an imine with the N-terminal amine of the nucleophilic segment. As such decarboxylative condensation does not depend on thiol-based capture elements to bring the fragments together. However, it differs from the imine ligation methods in three important respects: (1) There is no requirement for N-terminal amino acid residues having nucleophilic side-chains to be present on the nucleophilic segment, (2) it does not involve an acyl transfer step and (3) it generates an acyclic secondary amide bond instead of the cyclic tertiary amide structure at the ligation juncture. The ligation is based on the condensation of α-keto acid peptide with hydroxylamine peptide (Scheme 17). Addition of the hydroxylamine to the ketone moiety provides the unstable hemiaminal intermediate, that after losing water and carbon dioxide gives the imidic acid, the tautomer precursor of the amide.

Scheme 17. Proposed Reaction Mechanism of Decarboxylative Condensation.
Model studies on decarboxylative condensation involved the ligation of several simple and complex peptide fragments. Results indicated that the reaction has good tolerance for bulky amino acids at the ligation juncture and can deliver the desired product starting from unprotected peptide fragments. The reaction conditions are relatively mild and proceed in aqueous media.\textsuperscript{72}

1.3.3 Nucleophilic Aromatic Substitution-Mediated Ligations

Tomkinson and co-workers reported in 1998 the amide bond-forming reaction between 2,4-dinitrobenzenesulfonamides and thioacids.\textsuperscript{73} The reaction proceeds with the \textit{ipso} attack of the thioacid to the sulfonamide to give a Meisenheimer type complex (Scheme 18A). The sulfonated amine traps the nearby thioacid upon decomposition of the Meisenheimer type complex. Crich and co-workers seized this chemistry to prepare peptides, neoglycoconjugates\textsuperscript{80} and N-glycosyl amides.\textsuperscript{81} Amino acid/peptide-derived sulfonamides react with thioacids to give peptides (Scheme 18B).\textsuperscript{80} A similar reaction involving carbohydrate-linker-sulfonamides provides neoglycoconjugates (Scheme 18C).\textsuperscript{80} When carried out using cyclic thioanhydride, such as \textit{N}-Cbz-L-aspartic anhydride, a regioselective ring-opening in the presence of glycosylamine and subsequent trapping of the thioacid with a sulfonamide gives the \textit{N}-glycosyl amide\textsuperscript{81} (Scheme 18D).
Scheme 18. (A) The Proposed Mechanism of Thioacid-Sulfonamide Chemistry and Its Applications in the Synthesis of (B) Peptides, (C) Neoglycoconjugates and (D) N-Glycosyl Amides.

A closely related reaction involves the Sanger reagent, 2,4-dinitroiodobenzene (2,4-dNIB), and Mukaiyama reagent to couple peptide fragments at ambient temperature (Scheme 19A). These electron-deficient arenes and heteroarenes serve to activate the carboxyl group of the thioacid towards nucleophilic attack. Reactions that consist of amino/peptide thioacids, amino/peptide esters, and Sanger reagent as condensing agent provided several dipeptides and a tetrapeptide in moderate to high yields (Scheme 19B). It was noted that the fluoride ion released from the nucleophilic substitution step promotes the partial cleavage of the Fmoc protecting group. This observation led to the use of 2,4-dinitroiodobenzene (Scheme 19C) and Mukaiyama reagent (Scheme 19D). Comparison of ligation yields among these reagents revealed that Mukaiyama reagent consistently performed better in a range of substrates over the 2,4-dinitroiodobenzene and
Sanger reagent. Application of the Mukaiyama reagent to the coupling of protected tetrapeptide fragments afforded the octapeptide product with minimal epimerization and comparable yields to dehydrative coupling conditions.

Scheme 19. (A) Structures of Electrophilic Condensing Agents. (B) Coupling of C-Terminal Peptide Thioacid with Amino Peptide Using the Electron-Deficient Arenes and Heteroarenes. Proposed Reaction Mechanism of the Condensation Reaction Mediated by (C) Sanger Reagent and 2,4-dNIB, and (D) Mukaiyama Reagent.

1.3.4 Direct Aminolysis of Peptide Thioester

As disclosed in the work of Payne and co-workers, the coupling of a peptide thioester directly to a peptide lacking an N-terminal residue can be executed successfully using a carefully concocted reaction solvent (Scheme 20). This solvent consisted of 4:1 v/v NMP:6M Gn-HCl, 1M HEPES, pH = 8.5 that retards the competing hydrolysis reaction of the thioester and promotes formation of the ligation product. Ligation
reactions involving peptide substrates with a range of N-terminal residues and C-terminal residues provided yields that were dependent on steric bulk at the ligation site. The residue at the N-terminus of the peptide exerted more influence on the efficiency of the ligation than the thioester bearing residue. In general, the highest yields were obtained when glycine, aspartic acid and glutamic acid were the N-terminal residue of the peptide. The reaction was tolerant of unprotected cysteine but not lysine which requires protection with ivDde group.

Scheme 20. Direct Aminolysis of Thioester with Peptide Containing Noncysteine N-Terminal Residue

Direct aminolysis of the thioester can be performed in the presence of unprotected glycans. A common thioester fragment consisting of 20-mer repeat unit of MUC1 whose two threonine residues bear a protected α-GalNac and the N-terminus protected with trifluoroacetamide was used to arrive at the trimeric MUC1 repeat hexaglycopeptide (Scheme 21). The thioester fragment was treated with 10 mM NaOH and 5% hydrazine to remove all protecting groups accompanied by hydrolysis of the thioester. With the N-terminal amine group exposed, the resulting glycopeptide was coupled to the protected (common) version of the thioester to give the dimeric glycopeptide product. Treatment
with 5% hydrazine removes the trifluoroacetamide for final coupling with the third protected thioester unit followed by removal of the trifluoroacetamide.

Scheme 21. Synthesis of the Trimeric MUC1 Repeat Hexaglycopeptide from a Common Thioester Fragment by Direct Aminolysis.

1.3.5 Staudinger Ligation

The Staudinger ligation employs phosphinothiols, e.g. o-phosphinobenzenethiol or diphenylmethanethiol, as a temporary link between the C-terminus thioester peptide and N-terminus azido peptide (Scheme 22A). The steps leading to the iminophosphorane intermediate can be gleaned from the classical Staudinger reaction. Electrophilic addition of the peptide azide to the tervalent phosphorus forms phosphazide, which upon
elimination of nitrogen gives the iminophosphorane intermediate (Scheme 22B). Nucleophilic displacement of the sulfur from the thioester by the iminophosphorane nitrogen produces an amide bond in the resultant amidophosphonium salt. Hydrolysis of the salt liberates the desired peptide. This procedure could have been widely applicable were it not for the limited water solubility of phosphinothiols and a high yield that can be obtained only if glycine is at the ligation juncture. Attempts to address the solubility issue and effect of steric encumberance on yields appear to be promising.

Scheme 22. (A) Structures of Phosphinothiols. (B) Mechanism of Staudinger Ligation.

1.4 Summary

For some years now, there was a steady development of methods that are capable of ligation of glycopeptide fragments carrying complex carbohydrate domains. In addition, emerging ligation chemistries are less sensitive to sterics at the ligation centers and less dependent on thiol-capture methods. Such capability allows ligation at any desired site and significantly expands the synthetic targets available to chemical ligation. Combined with the increasing sophistication in the assembly of the glycopeptide building blocks, advances in ligation chemistry places the synthesis of glycopeptides on the
biologics level to be now within the realm of possibility. The exquisite ability to append multiple glycans of known structure in the same peptide chain will undoubtedly deepen our understanding of glycosylation on the functions of the resultant glycoprotein. From this understanding, new glycopeptide-based therapeutics can be developed.
References


CHAPTER 2
SYNTHESIS OF GLYCOPEPTIDES BY DECARBOXYLATIVE CONDENSATION AND INSIGHT INTO THE REACTION MECHANISM

2.1 Introduction

α-Keto acids and hydroxylamines undergo decarboxylative condensation to selectively generate the amide bond. Because the required reactivity has been built into the fragments, the reaction proceeds without the need for external reagents, produces carbon dioxide and water as by-products, and is “traceless.” First reported in 2006 by Bode and co-workers,¹ initial explorations of the chemistry focused on its usefulness in coupling peptide fragments. Peptide segments consisting of three amino acid residues whose side-chains were unprotected provided a high yield of the product¹ and served to underscore its utility in the field of chemical ligation, that currently is dominated by thiol-capture methods such as the native chemical ligation²,³ and auxiliary-mediated NCL⁴,⁵. The reaction is effective in joining fragments having bulky amino acids located at the ligation site and can be carried out in polar, protic solvents, aqueous and buffered media.¹
The reaction occurs just as effectively if $O$-substituted and cyclic hydroxyalamines (isoxazolidine acetals) are used as the partner of the keto acids. The cyclic hydroxyalamines were noteworthy in providing excellent yields of the product in a short period of time as compared to the linear analogues and can proceed in nonpolar, as well as, aqueous solvents.\textsuperscript{1,6} This exceptional reactivity of isoxazolidine acetals was exploited for the synthesis of $\beta^3$-oligopeptides, an important type of peptidomimetics with interesting biological properties.\textsuperscript{6,7} The isoxazolidine acetal of $\beta^3$-amino acids were coupled with keto acids in tert-BuOH-$H_2O$ to afford the methyl $\alpha$-keto esters, which were saponified to the $\alpha$-keto acids for the next cycle of chain elongation. Using this two-step iterative process, several $\beta$-dipeptide and tripeptides were prepared with over-all isolated yields ranging from 23\% to 70\%. The coupling reactions proceed cleanly, chemoselectively, and at lower concentrations with the exception of substrates involving $\beta h$-valine residues that have been noted to be low yielding due to solubility issues.\textsuperscript{6,7}

The peptide $\alpha$-keto acids employed in decarboxylative condensation were derived from the oxidation of the corresponding amino acid phosphorous ylides.\textsuperscript{1} Typical reaction conditions involved exhaustive ozonolysis at low temperatures or treatment with DMDO. Although successful for protected short chain peptides, the method is incompatible for longer, side-chain unprotected peptides, especially those with easily oxidizable functionality, and epimerization was an issue.\textsuperscript{8} Sulfur ylides were found to be a better alternative to phosphorous ylides. C-Terminal peptide sulfur ylides were readily transformed to the corresponding $\alpha$-keto acids by the mild and easy to handle Oxone\textsuperscript{TM} in aqueous media. The Oxone\textsuperscript{TM}-mediated oxidation was tolerant of unprotected tryptophan,
tyrosine, lysine, arginine, histidine, and glutamic acid but not sulfur-bearing residues such as methionine.⁸

An attractive area for the application of decarboxylative condensation is in glycopeptide synthesis (Figure 1). It is envisioned that this unique condensation chemistry would prove ideal for ligating water-soluble, unprotected, and pH-sensitive glycopeptides. Emerging chemistry that is capable of linking two glycopeptide fragments should in principle (a) avoid amino-acid-specific limitations at the ligation junction, (b) be water-tolerant, (c) be devoid of restricted access to peptide thioesters,⁹ and (d) possess high chemoselectivity. Decarboxylative condensation appears to possess these sought-after qualities. Noteworthy of mention is that the chemistry appears to give higher yields when sterically demanding amino acids are located at the ligation junction,¹ a trend that is opposite that of the NCL and Staudinger ligation. This feature makes it well-suited for targets composed mostly of bulky residues where small side chains may not be always available at the desired ligation site such as the tumor-associated glycoprotein MUC1.¹⁰-¹¹

![Figure 1. General Concept for Decarboxylative Condensation.](image-url)
In this investigation, a model system consisting of glycosyl dipeptides with C-terminal α-keto acid moieties (Glycopeptide I) and a series of amino acid containing N-hydroxylamino moieties (Peptide I) were prepared to demonstrate the chemistry (Figure 2). The two components were used to study the amino acid requirements at the ligation junction and evaluate the compatibility of decarboxylative condensation in the presence of glycans. Glycopeptide I is derived from the glycosyl amino acid 8 and amino acid cyanophosphorane. Closely associated with this study is the coupling of isotope-labeled α-keto acid 10 with N-hydroxylamine to further elucidate the mechanism of decarboxylative condensation.

![Figure 2. Important Intermediates for Decarboxylative Condensation.](image)

### 2.2 Synthesis of N-Hydroxylamines

The desired glycine, alanine, valine and phenethylamine hydroxylamines were prepared by a three-step sequence (Scheme 1) that included the cyanomethylation of the amino moiety present on a series of amino acid esters as the first step, followed by oxidation to the nitrone, and finally hydroxyaminolysis to release the hydroxylamines.\(^1,^{13}\) Several reaction conditions were investigated for the moncyanomethylation of glycine (Table 1). This involved varying the number of equivalents of the alkylating agent and
the base (DIPEA), and the identity of the alkylating agent. Monocyanomethylation of glycine was best achieved by using the less reactive alkylating agent, chloroacetonitrile (2 eq.), DIPEA (5 eq.) and stirring the mixture at 70 °C. In contrast, pronounced overalkylation of the amino acid was observed in bromoacetonitrile. Monocyanomethylation of alanine required the more reactive bromoacetonitrile (1 eq.) due to the crowding around the nucleophilic amino group brought about by the α-methyl group. This is consistent with the use of bromoacetonitrile as the alkylating agent for sterically hindered amines as reported by Tokuyama and co-workers. The reaction mixture was stirred at room temperature for 31 h to avoid racemization of the amino acid. Heating the reaction mixture at 70 °C contributes to racemization, as was the case for the alanine benzyl ester (see Section 2.5). Cyanomethylation of valine required three days to complete using bromoacetonitrile due to steric reasons. The same chemical conversion was completed for phenethylamine in one day in the presence of bromoacetonitrile with a high yield of 99%.

Scheme 1. Synthesis of N-Hydroxylamines.
Table 1. Optimization of Conditions for the Preparation of \( N \)-Cyanomethylglycine

![Chemical Structures](attachment:chemical_structures.png)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Haloacetonitrile</th>
<th>DIPEA</th>
<th>Time (h)</th>
<th>Isolated Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ClCH\textsubscript{2}CN, 1.0 equiv</td>
<td>6 equiv</td>
<td>24</td>
<td>3.0%</td>
</tr>
<tr>
<td>2</td>
<td>ClCH\textsubscript{2}CN, 1.5 equiv</td>
<td>5 equiv</td>
<td>24</td>
<td>19.0%</td>
</tr>
<tr>
<td>3</td>
<td>ClCH\textsubscript{2}CN, 2.0 equiv</td>
<td>5 equiv</td>
<td>24</td>
<td>39.0%</td>
</tr>
<tr>
<td>4</td>
<td>BrCH\textsubscript{2}CN, 1.0 equiv</td>
<td>5 equiv</td>
<td>6</td>
<td>4.0%</td>
</tr>
<tr>
<td>5</td>
<td>BrCH\textsubscript{2}CN, 1.0 equiv\textsuperscript{a}</td>
<td>5 equiv</td>
<td>15</td>
<td>29%</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The reaction was catalyzed by TBAI (1/15 equiv).

The conversion of the monocyanomethylated amino acids to the corresponding nitrone was effected smoothly by \( m \)-chloroperoxybenzoic acid (MCPBA) in good to excellent yields. However, the crude glycine nitrone had to be purified twice by flash column chromatography on silica gel. Hydroxyaminolysis of the nitrones in the presence of hydroxylamine-HCl afforded the corresponding hydroxylamines in moderate to excellent yields. Hydroxyaminolysis of glycine nitrone \textsuperscript{21} and alanine nitrone \textsuperscript{24} was performed at 50 °C. At this juncture, it became clear that alanine hydroxylamine \textsuperscript{7} was a racemic mixture and a milder reaction temperature for this step, especially for chiral amines, had to be used. Bearing this in mind, the hydroxyaminolysis of the succeeding nitrones was carried out at 35-40 °C to afford the desired products.
2.3 Synthesis of Amino Acid Cyanoketophosphoranes

The cyanoketophosphoranes were constructed from the corresponding N-protected amino acids (Scheme 2) based on reported procedures. The requisite (cyanomethylene)triphenylphosphorane, ylide 30, for the first step was prepared from the reaction between cyanomethyltriphenylphosphonium chloride and triethyl amine. Coupling between the amino acids and 30 requires anhydrous conditions because water can effectively compete for the coupling agent, EDCI. Since Fmoc-alanine is commercially available as the monohydrate, attempts were made to remove the hydrate. Co-evaporation in toluene was not effective as indicated by the low yield of the product 33 (33%), even though a large excess of EDCI (2.6 eq.) was used to scavenge any residual water. Another attempt involved stirring of the amino acid in CH₂Cl₂-toluene in the presence of Na₂SO₄ and the use of MS 4Å during the reaction that gave a yield of 52%. The coupling of the hydrate-free Boc-alanine, Boc-valine and Fmoc-valine with ylide 30 went smoothly to afford cyano ketophosphoranes 31, 32, 34, respectively, in good yields.

Scheme 2. Synthesis of Cyanoketophosphoranes.
Removal of the Boc-protecting group on cyanoketophosphoranes 31 and 32 was attempted using HCl or TFA. $^1$HNMR analysis of the crude material obtained from the treatment of 31 with HCl-diethyl ether showed the characteristic signals of the expected product. But a singlet at $\delta$ 1.44 with an integration of nine was observed in addition to those signals. It was surmised that this could be the tert-butyl cation generated during the deprotection step that reacted with the triphenylphosphorane moiety. Indeed, examination of the ESI-MS revealed the presence of the alkylated product 35a (ESI-MS: $m/z$ calcd for C$_{27}$H$_{30}$N$_2$OP 473.1994, found: 473.2 (relative intensity: 4%) [M+H]$^+$). In addition, two more peaks were observed, one corresponding to the incompletely deprotected product 35b (ESI-MS: $m/z$ calcd for C$_{24}$H$_{22}$N$_2$O$_3$P 417.1368, found: 417.3 (relative intensity: 100%) [M+H]$^+$) and has the highest intensity, the other corresponding to the completely deprotected, unalkylated product 35c (ESI-MS: $m/z$ calcd for C$_{23}$H$_{22}$N$_2$OP 373.1470, found: 373.4 (relative intensity: 10%) [M+H]$^+$). Running the same reaction at room temperature gave the same $^1$H NMR signals for the product. Deprotection using TFA as the cleavage reagent in the presence of the carbocation scavenger, dimethyl sulfide, showed the same $^1$H NMR pattern. In the case of Boc 32, Boc-deprotection using TFA appears to afford the desired product but was not used further in the next step. Seeking a more general route for the preparation of amino acid cyanoketophosphoranes the N-Boc protected precursors were abandoned in favor of the Fmoc-protected counterparts.

Treatment of 33 and 34 with neat piperidine gave the expected free amino cyano ketophosphoranes 37 and 38. These compounds were used in the next step without
further purification. It is essential that as much as the residual piperidine be removed by co-evaporation with a mixture of CH₂Cl₂-triethyl amine. Likewise, removal of the dibenzofulvene/dibenzofulvene-piperidine adduct was performed by trituration in diethyl ether-hexanes. The residual piperidine can react with the glycosyl amino acid in the next steps resulting in lower yields and separation issues.

2.4 Synthesis of Glycosyl Dipeptide α-Keto Acid

The glycosyl amino acid 8 was prepared from D-galactose via an eight-step process (Scheme 3) following literature procedures.19,20 D-Galactose was peracetylated in the presence of acetic anhydride to give compound 40. This step was followed by selective displacement of the anomeric acetyl group with bromine and Zn-mediated 1,2-elimination to arrive at the galactal 42.19 Azido-nitration of 42 gave the azido nitrate 43.19 The azido group was attached on carbon 2 which serves as the precursor for the acetamido group. Using lithium bromide, the nitrate at the anomeric carbon of 43 was replaced with bromine to afford the galactosyl donor 44.20 Since nonparticipating groups, such as azido, are expected to give α-selectivity, the glycosylation reaction between 44 and the Fmoc-protected threonine benzyl ester 45 gave the target α-glycoside 46.20 The glycosyl acceptor was prepared by protecting the amino group of threonine benzyl ester with Fmoc using 9-fluoromethyl succinimidyl carbonate.21 Afterwards the azido, having served its purpose, was reduced to amino group using zinc/copper couple and acetylated in situ by acetic anhydride to afford compound 47. Hydrogenolysis of the benzyl group of 47 exposed the free carboxyl group to give compound 8.20
The glycosyl amino acid fragment 8 was coupled to alanine cyano ketophosphorane 37 following standard peptide coupling conditions\(^{14}\) to afford the glycosyl dipeptide cyano ketophosphorane 48 in good yields. The piperidine adduct of compound 8, however, was not isolated in this case. The coupling reaction was carried out using HBTU as the condensing agent in the presence of HOBT and the base TMP. Oxidation of the glycosyl dipeptide cyanophosphorane 48 with freshly prepared dimethyldioxirane (DMDO) gave the α-keto acid 2.\(^{22}\) DMDO was prepared from the treatment of acetone with Oxone.\(^{23}\)

Scheme 3. Synthesis of Fmoc-Ac\(_3\)-Tn-α-Thr-Ala Keto Acid 2.
2.5 Decarboxylative Condensation

2.5.1 Decarboxylative Condensation of Fmoc-Ala Keto Acid 49 and Alanine Hydroxylamine Benzyl Ester 7

A model reaction between a simple keto acid and hydroxylamine was carried out in order to obtain a handle on the ligation chemistry, as well as to determine the chiral integrity of the alanine hydroxylamine benzyl ester. Alanine cyanoketophosphorane 33 was oxidized to the α-keto acid 49 with freshly prepared DMDO.\(^{22}\) The ligation reaction was performed at 35-40 °C with DMF as the solvent and afforded the product in moderate yield (Scheme 4). \(^1\)H NMR analysis of the purified product showed two sets of NH signals corresponding to a ratio of 1:2 based on the integration of the NH signals at \(\delta\) 6.71 and 6.60. To eliminate the possibility that these signals are due to rotamers, a variable \(^1\)H NMR analysis was performed at room temperature, 40 °C and 60 °C. The NH signals essentially remained distinct and did not merge at higher temperatures as expected for rotameric compounds.\(^{24}\) Based from these observations, it was concluded that the hydroxylamine, which has been synthesized using harsher conditions, was a racemic mixture, and was not used further in the study. This also prompted the synthesis of the other chiral hydroxylamines at milder temperatures.

Scheme 4. Decarboxylative Condensation of α-Keto Acid 50 and N-Hydroxylamine 7.
2.5.2 Decarboxylative Condensation of Fmoc-Ac₃-Tn-α-Thr-Ala Keto Acid 2 and Hydroxylamines tert-Butyl Ester

α-Keto acid 2 was combined with glycine hydroxylamine 4 under different reaction conditions to give the desired glycosyl tripeptide 51, together with the previously unknown oxazole byproduct 52 (Table 2). The reaction conducted in anhydrous DMF (Table 2, entry 1) proceeded faster than the reported ligation between Fmoc-Ac₃-Tn-α-Thr-Gly keto acid 1 and glycine hydroxylamine²⁵ 4 which was performed under similar conditions (anhydrous DMF, 35-40 °C) for 24 h. The yields for the product and side product are similar to those reported by Sanki and co-workers (glycopeptide product = 41% and byproduct = 22%).²⁵ Since the reaction was completed in 6 h, another ligation reaction was performed, this time at room temperature, entry 2. The reaction proceeded for 24 h and the yield of the product was slightly lower compared to the ligation reaction performed at higher temperature. The ligation reaction conducted in aqueous DMF (Table 2, entry 3) offered a small improvement in yield.

The condensation of α-keto acid 2 with a slightly bulkier hydroxylamine, alanine hydroxylamine 5 in anhydrous DMF afforded the desired glycotripeptide 53 in a high yield of 86% (Table 2, entry 4). Performing the same reaction in DMF-water (5:1) gave a moderate yield of the desired product (Table 2, entry 5). This outcome is consistent with those known in the literature.¹ Surprisingly, in both conditions, the oxazole by-product was not formed. A similar condensation reaction involving Fmoc-Ac₃-Tn-α-Thr-Gly keto acid 1 and alanine hydroxylamine 5 did not give the oxazole by-product and the yields were comparable to the ligation reaction that generates the Gly-Gly linkage.²⁵
Table 2. Decarboxylative Condensation of \( \alpha \)-Keto Acid 2 and Hydroxylamines

<table>
<thead>
<tr>
<th>Entry</th>
<th>( N )-Hydroxylamines</th>
<th>Conditions</th>
<th>Product Yield (%)</th>
<th>By-product Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( R^1 = H )</td>
<td>Anhyd. DMF, 40-42 °C, 6 h</td>
<td>51 (43)</td>
<td>52 (23)</td>
</tr>
<tr>
<td>2</td>
<td>( R^1 = H )</td>
<td>Anhyd. DMF, 20-25 °C, 24 h</td>
<td>51 (39)</td>
<td>52 (29)</td>
</tr>
<tr>
<td>3</td>
<td>( R^1 = H )</td>
<td>DMF-H( _2 )O (5:1), 20-25 °C, 48 h</td>
<td>51 (46)</td>
<td>52 (23)</td>
</tr>
<tr>
<td>4</td>
<td>( R^1 = \text{CH}_3 )</td>
<td>Anhyd. DMF, 35-40 °C, 38 h</td>
<td>53 (86)</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>( R^1 = \text{CH}_3 )</td>
<td>DMF-H( _2 )O (5:1), 35-40 °C, 48 h</td>
<td>53 (54)</td>
<td>—</td>
</tr>
</tbody>
</table>

Considering the above results and those ligation reactions reported in the literature,\textsuperscript{25} the following trends could be summarized (Scheme 5): (1) Low steric bulk of the amino acids at the ligation site tend to afford moderate yields, whereas, the extreme opposite, high steric crowding gives low to zero yields; moderate steric crowding, as exemplified by the Ala-Ala linkage, gives the highest yields; (2) The trend is basically similar in both anhydrous and aqueous conditions; and (3) The oxazole by-product readily forms when the ligation partner is the least sterically demanding glycine hydroxylamine.
Scheme 5. Decarboxylative Condensation Involving Protected Glycosyl Dipeptide α-Keto Acids and N-Hydroxylamino Acids. Yields in parenthesis are from aqueous conditions.

2.6 Mechanistic Studies on Decarboxylative Condensation

The reported mechanism leading to the amide product was re-examined to account for the formation of the oxazole product (Scheme 6). Two pathways (A and B) have been previously proposed that diverge from a common hemiaminal intermediate. In Pathway A, the hemiaminal loses a molecule of CO₂ and water to an imidic acid which upon tautomerization affords the desired glycosyl tripeptides. A mechanism that could give rise to the oxazoles is not readily apparent from the intermediates of this pathway.
Another plausible mechanism for amide formation is illustrated in Pathway B. In this pathway, the hemiaminal loses a molecule of water to afford an \((E/Z)\) mixture of nitrones. This is followed by the loss of a molecule of \(\text{CO}_2\) and water from the nitrone that would give rise to the putative reactive nitrilium ion. There are two possible scenarios for the nitrilium ion. One, it can cyclize via the attack of the tert-butyl ester carbonyl, followed by the aromatization of the ring to provide an oxazole byproduct. Two, the nitrilium ion could undergo addition of a water molecule to generate the imidic acid of Pathway A. Pathway A was proposed as the operative mechanism because previous studies failed to trap the nitrilium ion by addition of nucleophiles such as methanol, thiophenol, cysteine and glycine).\(^1\) The isolation of the oxazole byproduct indicates that the nitrilium ion might be the key intermediate in decarboxylative condensation. As it appears, oxazole byproduct readily formed in the case of the Gly-Gly\(^{25}\) or Ala-Gly junctions but not for the Gly-Ala, Gly-Val,\(^{25}\) and Ala-Ala, suggesting a role for steric bulk of the hydroxylamine partner. It is apparent that the formation of the oxazole byproduct is favored via a nitrilium ion intermediate when the hydroxylamine residue at the ligation site is Gly.
Scheme 6. Proposed Mechanism Leading to the Amide Product and Oxazole Byproduct.

In order to further distinguish between path A and B, a model study was performed involving decarboxylative condensation of unlabeled and O\textsuperscript{18}-labeled phenyl pyruvic acids 54 and 10, respectively with \textit{N}-hydroxyphenethylamine oxalate salt 34 (Scheme 7). In doing so, the \textit{N}-hydroxyphenethylamine oxalate salt 9 was synthesized under the reaction conditions analogous to the synthesis of amino acid hydroxylamines. Commercially available phenylpyruvic acid 54 was used as an $\alpha$-ketoacid. Compound 54 was first labeled with O\textsuperscript{18}-oxygen in the presence of H\textsubscript{2}O\textsuperscript{18} and 0.1N HCl in anhydrous THF to obtain 10 in 84% yield.\textsuperscript{26} Both 54 and 10 showed mass fragments of $m/z = 143.1$ [M+Na-CO\textsubscript{2}]$^+$ and $m/z = 145.1$ [M+Na-CO\textsubscript{2}]$^+$, respectively with a loss of one molecule of CO\textsubscript{2} (Figure 3). Both compounds 10 and 54 exist as the enol tautomer in solution as indicated by $^{13}$C NMR. The carboxylic acid carbonyl carbon and enolate carbon of compound 10 showed an upfield shift of $\delta$ 0.075 and $\delta$ 0.066, respectively, in the $^{13}$C
NMR as compared to 54 (Figure 4). Compound 54 was subjected to decarboxylative condensation in the presence of 9 in MeOH to afford amide 55 ($m/z = 240.3 \ [M+H]^+$) in 41% yield (Scheme 7). Next, O$^{18}$-labeled 10 was reacted with hydroxylamine 9 under the same reaction conditions to provide amide 55 ($m/z = 240.2 \ [M+H]^+$) with complete loss of label in 51% yield.

Initially it was thought that the labeled amide could not be detected by the mass spectrometer or undergoes exchange with water under the conditions employed in mass spectrometry. To rule out this ambiguity, the labeled amide was prepared by an alternative route (Scheme 7). Commercially available benzyl cyanide 56 was hydrolyzed in a mixture of H$_2$O$^{18}$ and anhydrous THF in the presence of HCl gas (bubbled) following literature procedures. The resultant O$^{18}$-labeled carboxylic acid 57 ($m/z = 163.1 \ [M+Na]^+$) was condensed with phenethylamine in the presence of the coupling agent dicyclohexylcarbodiimide. Analysis of the crude product by ESI-MS showed a mass fragment at $m/z = 242.4 \ [M+H]^+$ corresponding to the O$^{18}$-labeled amide 58. The result suggests that the O$^{18}$-labeled amide is fairly stable once formed and does not exchange with O$^{16}$ in the mass spectrometry employed and amide 55 loses its labeling during amidation reaction. Isotope exchange studies conducted on O$^{16}$-amide in the presence of H$_2$O$^{18}$ (100 eq) were consistent with the observation that the amide oxygen is nonexchangeable. Loss of labeling in 55 supports pathway B as the operative mechanism for the decarboxylative condensation of N-hydroxylamines and $\alpha$-keto acids.
Scheme 7. Isotope Labeling Experiment with O^{18}-Labeled Phenylpyruvic Acid.
Figure 3. Mass Spectrum (ESI-MS) of (A) α-Keto Acid 54, (B) Amide 55 from 54, (C) α-Keto Acid 10, (D) Amide 55 from 10, (E) O\textsuperscript{18}-Labeled Phenylacetic Acid 57, and (F) Amide 58 from 57. For clarity the mass fragment is provided alongside the peak of interest.
Figure 4. Selected Regions of the $^{13}$C NMR Spectrum of Phenylpyruvic acid 54 and O$^{18}$-Labeled Analogue 10. Phenylpyruvic acid exists as the enol tautomer in solution. For clarity the $^{13}$C resonances were provided alongside the peaks.

The complete loss of the label in the amide product raises the interesting question of what is the source of the amide oxygen. The proposed mechanism assumes that water attacks the nitrilium ion on the way to the amide product. However the reaction mixture consists of equimolar amounts of H$_2$O$^{16}$ and H$_2$O$^{18}$ upon reaching the nitrilium ion stage. Both isotopes can attack the nitrilium ion and conceivably could give an O$^{18}$-labeled amide product. That this is not the picture that emerged places the hydroxylamine oxygen
as a likely source of the amide oxygen. The hydroxylamine oxygen can undergo a 1,2-shift through a concerted step or ion-pair as illustrated below (Scheme 8). To test this hypothesis would require the synthesis of O\textsuperscript{18}-labeled hydroxylamine.

![Scheme 8. Proposed 1,2-Shift of the Hydroxylamine Oxygen.](image)

The synthesis of the O\textsuperscript{18}-labeled hydroxylamine is constrained by available synthetic transformations involving oxidizing reagents that can be replaced by commercially supplied O\textsuperscript{18}-labeled counterparts. Hydrogen peroxide oxidation of amines in the presence of selenium oxide catalyst has been reported to provide 34-91\% of the nitrone.\textsuperscript{29} However, this reaction employs 30\% H\textsubscript{2}O\textsubscript{2} to effect oxidation but only a 2\% H\textsubscript{2}O\textsubscript{18} is commercially produced. A model reaction consisting of 2\% H\textsubscript{2}O\textsubscript{2} with 5\% SeO\textsubscript{2} failed to furnish the nitrone from N-cyanomethyl phenethylamine 20 after stirring the mixture for 2 days (Scheme 9). A similar reaction performed on phenethylamine did not provide the expected hydroxylamine product.
2.7 Conclusion

Decarboxylative condensation of α-keto carboxylic acids with N-alkylhydroxylamines offers a chemoselective route for joining two peptide fragments under mild and aqueous conditions. This thiol-capture independent ligation chemistry promises to be free from the limitations imposed by the choice of amino acid residues at the ligation site. The application of this chemistry to the ligation of glycopeptide fragments entailed the synthesis of the glycosyl dipeptide α-keto acids and amino acid-based N-hydroxylamines. The key steps for the synthesis of the glycosyl dipeptide α-keto acids were galactosylation of threonine, coupling of galactosyl threonine with the amino acid cyanoketophosphorane and oxidation of the cyanoketophosphorane. Synthesis of the N-hydroxylamines was accomplished following a three-step sequence that included alkylation, oxidation and hydroxyaminolysis.

The condensation reaction of the α-keto acids and N-hydroxylamines proceeded in anhydrous and aqueous media to give yields of the desired glycosyltripeptide that were dependent on the steric bulk of the amino acids located at the ligation junction. The best yields were obtained when the moderately sized alanine-alanine comprised the ligation
junction. Ligation junctions occupied by less bulky amino acid pairs such as glycine-glycine and glycine-alanine provided moderate yields of the desired product. Junctions that included the bulky valine suffered from low yields. This trend is consistent both in anhydrous and aqueous conditions. However, a slightly better yield was observed for the ligation reactions conducted in aqueous medium involving the $N$-hydroxylamine of glycine.

In addition to the desired product, oxazole by-products were observed whenever the least sterically hindered $N$-hydroxylamine of glycine was the ligation partner. The formation of the oxazole is believed to result from an intramolecular cyclization of the $O$-tert-butyl ester on the nitrilium ion intermediate followed by aromatization. A decarboxylative condensation between $O^{18}$-labeled phenylpyruvic acid and $N$-hydroxyphenethylamine provided the amide products completely lacking the $O^{18}$-label. Oxygen exchange during mass spectrometric measurements of the amide was ruled out upon detection of the $O^{18}$-labeled amide that was prepared by alternative synthesis. The oxazole and the isotope labeling experiment provide reasonable pieces of evidence for the nitrilium ion as the key intermediate in the condensation.
Experimental Section

General Methods. Amino acids and other fine chemicals were purchased from commercial suppliers and were used without further purification. All solvents used for reactions were dried following the standard procedures. Triethylamine was dried using MS 4Å. Thin-layer chromatography (TLC, silica gel 60, f<sub>254</sub>) were performed in distilled solvents as specified and visualized under UV light or by charring in the presence of 5% H<sub>2</sub>SO<sub>4</sub>/MeOH or cerium ammonium nitrate. Flash column chromatography was performed on silica gel (230-400 mesh) column using solvents as received. <sup>1</sup>H NMR were recorded either on Varian VXR-400 (400 MHz) or INOVA-600 (600 MHz) spectrometer in CDCl<sub>3</sub>, CD<sub>3</sub>OD or DMSO-<i>d</i><sub>6</sub> using residual CHCl<sub>3</sub>, CH<sub>3</sub>OH or DMSO as internal references, respectively. <sup>13</sup>C NMR were recorded either on Varian VXR-400 (100 MHz) or INOVA-600 (150 MHz) spectrometer in CDCl<sub>3</sub>, CD<sub>3</sub>OD or DMSO-<i>d</i><sub>6</sub> using the triplet centered at <i>δ</i>77.273 for CDCl<sub>3</sub>, septet centered at <i>δ</i>49.0 for CD<sub>3</sub>OD, or septet centered at <i>δ</i>39.5 for DMSO-<i>d</i><sub>6</sub> as internal reference, respectively. <sup>31</sup>P NMR was recorded on Varian VXR-400 spectrometer using either CDCl<sub>3</sub> or CD<sub>3</sub>OD as solvent. <sup>1</sup>H-<sup>1</sup>H gCOSY and <sup>1</sup>H-<sup>13</sup>C gHMQC were performed on a INOVA-600 spectrometer. The <sup>1</sup>H NMR data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz, integration, and assignments. Low resolution mass spectra were taken on Esquire-LC electrospray ionization (ESI) mass spectrometer operated in the positive ion mode. High resolution mass spectrometry (HRMS) were performed on a mass spectrometer located at the Mass Spectrometry and Proteomics Facility, The Ohio State University.
Melting points of all crystalline solids were determined using capillary tube and uncorrected.

**General Procedure A. Cyanomethylation of N-Terminal Amino Acids.** To a well-stirred suspension of amino acid tert-butyl ester hydrochloride in anhydrous acetonitrile was added DIPEA, and the resulting solution was stirred under N\(_2\) atmosphere for 5 min. Chloroacetonitrile or bromoacetonitrile was added, and the resulting solution was stirred at 60-70 °C. The completion of the reaction was monitored by TLC. Excess solvent was evaporated to dryness under reduced pressure to get a crude material which was dissolved in CH\(_2\)Cl\(_2\) (50 mL) and washed with saturated NaHCO\(_3\) (50 mL). The aqueous layer was back-extracted with CH\(_2\)Cl\(_2\) (2 x 50 mL). Combined organic layers were washed with brine (1 x 50 mL), dried (anhydrous Na\(_2\)SO\(_4\)) and filtered. The filtrate was concentrated under reduced pressure to get the crude residue.

**General Procedure B. Cyanomethylation of N-Terminal Amino Acids.** To a well-stirred suspension of amino acid tert-butyl ester hydrochloride in anhydrous acetonitrile (10 mL) was added DIPEA dropwise over a period of 15 min, and the resulting solution was stirred under N\(_2\) atmosphere for 5 min. Bromoacetonitrile was added dropwise over a period of 15 min, and stirring was continued at room temperature. The completion of the reaction was monitored by TLC. Excess solvent was evaporated to dryness under reduced pressure to get a crude material which was dissolved in CH\(_2\)Cl\(_2\) (50 mL) and washed with saturated NaHCO\(_3\) (50 mL). The aqueous layer was back-extracted with CH\(_2\)Cl\(_2\) (2 x 50 mL). Combined organic layers were washed with brine (1 x 50 mL), dried (anhydrous Na\(_2\)SO\(_4\)) and filtered. The filtrate was concentrated under reduced pressure to get the crude residue.
Na$_2$SO$_4$) and filtered. The filtrate was concentrated under reduced pressure to get the crude residue.

\[ \text{H}_2\text{N} - \text{O} \text{tBu} \text{N} \text{C} \text{H}_3 \]

**N-Cyanomethylglycine tert-Butyl Ester (16).** Glycine tert-butyl ester hydrochloride (0.20 g, 1.19 mmol) was reacted with DIPEA (96.8 μL, 5.97 mmol) and chloroacetonitrile (75 μL, 1.19 mmol) to furnish the crude material after 24 h following General Procedure A. The crude product was purified by flash column chromatography on SiO$_2$ (8 x 1.8 cm) using ethyl acetate-hexanes (3:7) to afford a clear, colorless oil. Yield: 0.080 g (39%); silica gel TLC $R_f = 0.41$ (ethyl acetate-hexanes = 3:7); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.64 (s, 2H), 3.39 (s, 2H), 1.90 (br.s, 1H), 1.45 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 170.36, 117.60, 82.31, 50.18, 36.96, 28.27; ESI-MS: $m/z$ calcd for C$_8$H$_{14}$NaN$_2$O$_2$ 193.1, found: 193.3 [M+Na]$^+$.

\[ \text{H}_2\text{N} - \text{O} \text{tBu} \text{N} \text{C} \text{H}_3 \]

**N-Cyanomethylalanine tert-Butyl Ester (17).** Alanine tert-butyl ester hydrochloride (0.50 g, 2.78 mmol) was reacted with DIPEA (1.01 mL, 6.11 mmol) and bromoacetonitrile (0.19 mL, 2.78 mmol) to furnish the crude material after 31 h following General Procedure B. The crude product was purified by flash column chromatography on SiO$_2$ (8 x 3.5 cm) using ethyl acetate-hexanes (3:7) to afford a colorless oil. Yield: 0.429 g (84%); silica gel TLC $R_f = 0.56$ (ethyl acetate-hexanes = 0.23); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 3.60 (d, $J = 2.4$ Hz, 2H), 3.38 (q, $J = 7.2$ Hz, 1H), 1.47 (s, 9H), 1.30 (d, $J = 6.6$ Hz, 3H); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 173.36, 117.71,
81.94, 55.85, 35.58, 28.07, 18.62; ESI-MS: m/z calcd for C₉H₁₆NaN₂O₂ 207.1, found: 207.1 [M+Na]+. This compound was reported in the literature.¹

\[
\text{N-Cyanomethylvaline tert-Butyl Ester (18). Valine tert-butyl ester hydrochloride (0.50 g, 2.38 mmol) was reacted with DIPEA (0.87 mL, 5.25 mmol) and bromoacetonitrile (0.17 mL, 2.38 mmol) to furnish the crude material after 3 days following General Procedure B. Purification of the crude residue using flash column chromatography on SiO₂ (8 x 3.5 cm) with ethyl acetate-hexanes (1:9) generated 18 as a colorless amorphous solid. Yield: 0.325 g (64%); silica gel TLC } R_f = 0.37 \text{ (ethyl acetate-hexanes = 1:4); m.p. 25.5-26 °C; } ^1\text{H NMR (600 MHz, CDCl₃): } \delta \text{ 3.50 (dd, } J = 17.4, 33.0 \text{ Hz, 2H, CH₂CN), 2.97(d, } J = 4.2 \text{ Hz, 1H, Val-α-CH), 1.93 (m, 1H, Val-CH), 1.85 (br.s, 1H, NH), 1.45 (s, 9H, } \text{tBu), 0.93 (d, } J = 6.6 \text{ Hz, Val-CH₃, 3H), 0.85 (d, } J = 7.2 \text{ Hz, 3H, Val-CH₃); } ^13\text{C NMR (150 MHz, CDCl₃): } \delta \text{ 173.02 (C=O), 117.95 (CN), 82.11, 82.10, 66.61, 36.97, 31.78, 28.26 (3 x CH₃ of } \text{tBu), 19.36, 17.88; HRMS: } m/z \text{ calcd for } \text{C}_{11}\text{H}_{20}\text{NaN}_{2}\text{O}_{2} \text{ 235.1423, found: 235.1423 [M+Na]+.} \]

\[
\text{N-Cyanomethylalanine Benzyl Ester (19). Alanine benzyl ester hydrochloride was pretreated with dichloromethane and toluene, stirred, and then concentrated to dryness under reduced pressure. The resulting solid was used for the next step. Alanine benzyl ester hydrochloride (4.00 g, 18.5 mmol) was reacted with DIPEA (12.20 mL, 74.1 mmol) and bromoacetonitrile (2.60 mL, 37.1 mmol) in acetonitrile (100 mL) to furnish the crude material after 16 h following General Procedure A. The crude product was purified by}
\]
flash column chromatography on SiO$_2$ (14 x 2.5 cm) using step-wise elution with ethyl acetate-hexanes (2:8 and 3:7) to afford a yellow liquid. Yield: 2.116 g (52%); silica gel TLC $R_f$ = 0.24 (ethyl acetate-hexanes = 3:7); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.38-7.30 (m, 5H), 5.16 (d, $J = 6.0$ Hz, 2H), 3.58 (d, $J = 3.6$ Hz, 2H), 3.53 (q, $J = 7.2$ Hz, 1H), 1.98 (br.s 1H), 1.34 (d, $J = 6.6$ Hz, 3H); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 174.08, 135.59, 128.91, 128.74, 128.53, 117.74, 67.23, 55.58, 35.69, 18.64; ESI-MS: $m/z$ calcd for C$_9$H$_{16}$NaN$_2$O$_2$ 241.1, found: 241.2 [M+Na]$^+$. 

N-Cyanomethyl Phenethylamine (20). Phenethylamine (0.96 g, 7.94 mmol) was reacted with DIPEA (1.57 mL, 9.53 mmol) and bromoacetonitrile (0.570 mL, 7.94 mmol) in acetonitrile (20 mL) to furnish the crude material after 24 h following General Procedure B. The crude product was purified by flash column chromatography on SiO$_2$ (8.5 x 4.5 cm) using step-wise gradient elution in ethyl acetate-hexanes (4:6 and 1:1) to afford a light yellow liquid. Yield: 1.256 g (99%); silica gel TLC $R_f$ = 0.24 (ethyl acetate-hexanes = 1:1); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.32-7.30 (m, 2H), 7.25-7.21 (m, 3H), 3.55 (s, 2H), 2.99 (t, $J = 6.6$ Hz, 2H), 2.82 (t, $J = 7.2$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 139.08, 128.79, 128.74, 126.63, 117.84, 49.90, 37.41, 35.87; ESI-MS: $m/z$ calcd for C$_{10}$H$_{12}$NaN$_2$ 183.0, found: 183.3 [M+Na]$^+$. This compound was reported in the literature.$^{31}$

General Procedure for N-Cyanomethyl N-Oxide Amino Acid Esters. To a well-stirred solution of N-cyanomethyl amine in CH$_2$Cl$_2$ at 0 °C was added $m$CPBA (70-75%), in six portions at 5 min. intervals. The resulting solution was allowed to stir at ambient
temperature. Completion of the reaction was detected by TLC conditions. Sodium thiosulfate \( \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O} \) dissolved in water was added followed by addition of saturated \( \text{NaHCO}_3 \) at 0 °C, and the resulting solution was stirred for 1 h. The reaction mixture was diluted with \( \text{CH}_2\text{Cl}_2 \) and washed with saturated \( \text{NaHCO}_3 \). The aqueous layer was back-extracted with \( \text{CH}_2\text{Cl}_2 \) (3 x 30-400 mL), and the organic layers were washed with brine, dried (anhydrous \( \text{Na}_2\text{SO}_4 \)), and filtered. The filtrate was concentrated to dryness under reduced pressure.

\[ \text{O} \]
\[ \text{N} \]
\[ \text{O} \]
\[ \text{CN} \]
\[ \text{OtBu} \]

**N-Cyanomethyl N-Oxide Glycine tert-Butyl Ester (21).** \( \text{N-Cyanomethyl glycine tert-butyl ester (0.159 g, 0.93 mmol)} \) in \( \text{CH}_2\text{Cl}_2 \) (10 mL) was reacted with \( \text{mCPBA (70-75\%, 0.563 g, 2.45 mmol)} \) following the General Procedure given above. The reaction appeared complete within 5 h. Sodium thiosulfate \( \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O} \) (0.262 g, 1.06 mmol) dissolved in water (2.0 mL) and saturated \( \text{NaHCO}_3 \) (4.0 mL) were used to quench the reaction. Subsequent steps used 30 mL each of the required solvents. The crude nitrone thus obtained was partially purified by flash column chromatography on \( \text{SiO}_2 \) (9 x 4.6 cm) using step-wise elution with acetone-hexanes (1:9 and 2:8). Yield: 0.133 g (78%). The partially purified nitrone was subjected to a second flash column chromatography on \( \text{SiO}_2 \) using step-wise elution with ethyl acetate-hexanes (1:9, 2:8, 1:1) to afford a yellowish brown oil. Yield: 0.070 g (40%); silica gel TLC \( R_f = 0.20 \) (ethyl acetate-hexanes = 2:8); Characteristic peaks in the \(^1\text{H} \text{NMR (600 MHz, CDCl}_3\)): \( \delta 6.76 \) (s, 1H), 4.59 (s, 2H), 1.50 (s, 9H).
**N-Cyanomethyl N-Oxide Alanine tert-Butyl Ester (22).** N-Cyanomethyl alanine tert-butyl ester (0.305 g, 1.66 mmol) in CH₂Cl₂ (15 mL) was reacted with mCPBA (70-75%, 0.787 g, 3.42 mmol) following the General Procedure given above. The reaction appeared complete within 30 min. Sodium thiosulfate Na₂S₂O₃·5H₂O (0.821 g, 3.32 mmol) dissolved in water (3.6 mL) and saturated NaHCO₃ (7.2 mL) were used to quench the reaction. Subsequent steps used 100 mL each of the required solvents except for saturated NaHCO₃ (60 mL). The crude material thus obtained was purified by flash column chromatography on SiO₂ (9 x 4.6 cm) with ethyl acetate-hexanes (3:7) to yield a yellow oil. Yield: 0.286 g (87%); silica gel TLC Rf = 0.65 (MeOH-CH₂Cl₂ = 5:95); ¹H NMR (600 MHz, CDCl₃): δ 6.87 (s, 1H), 4.71 (q, J = 7.2 Hz, 1H), 1.61 (d, J = 7.2 Hz, 3H), 1.43 (s, 9H); ¹³C NMR (100 MHz, CDCl₃): δ 165.12, 112.17, 107.91, 84.48, 74.53, 27.83, 15.65. ESI-MS: m/z calcd for C₉H₁₄NaN₂O₃ 221.1, found: 221.0 [M+Na]⁺. This compound was reported in the literature.¹

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**N-Cyanomethyl N-Oxide Valine tert-Butyl Ester (23).** N-Cyanomethyl valine tert-butyl ester (0.284 g, 1.34 mmol) in CH₂Cl₂ (15 mL) was reacted with mCPBA (70-75%, 0.627 g, 2.72 mmol) following the General Procedure. The reaction appeared complete within 30 min. Sodium thiosulfate Na₂S₂O₃·5H₂O (0.664 g, 2.68 mmol) dissolved in water (2.9 mL) and saturated NaHCO₃ (7.2 mL) were used to quench the reaction. Subsequent steps used 100 mL each of the required solvents except for saturated
NaHCO₃ (60 mL). The crude material thus obtained was purified by flash column chromatography on SiO₂ (9 x 4.6 cm) with ethyl acetate-hexanes (3:7) to yield a colorless amorphous solid. Yield: 0.285 g (94%); silica gel TLC Rf = 0.28 (ethyl acetate-hexanes = 1:4); m.p. 60-61 °C; ¹H NMR (600 MHz, CDCl₃): δ 6.97 (s, 1H, CHCN), 4.17 (d, J = 10.2 Hz, 1H, Val-α-CH), 2.44 (m, 1H, Val-CH), 1.48 (s, 9H, tBu), 1.03 (d, J = 7.2 Hz, 3H, Val-CH₃), 1.01 (d, J = 6.6 Hz, 3H, Val-CH₃); ¹³C NMR (100 MHz, CDCl₃): δ 164.91 (C=O), 112.20 (CN), 107.53 (CHCN), 86.04, 84.62, 31.16, 27.91 (3 x CH₃ of tBu), 18.98, 18.72; HRMS: m/z calcd for C₁₁H₁₈NaN₂O₃ 249.1215, found: 249.1216 [M+Na]⁺.

**N-Cyanomethyl N-Oxide Alanine Benzyl Ester (24).** N-Cyanomethyl alanine benzyl ester (0.998 g, 4.57 mmol) in CH₂Cl₂ (50 mL) was reacted with mCPBA (70-75%, 2.115 g, 9.19 mmol) following the General Procedure given above. The reaction appeared complete within 2.5 h. Sodium thiosulfate Na₂S₂O₃.5H₂O (2.303 g, 9.28 mmol) dissolved in water (10 mL) and saturated NaHCO₃ (20 mL) were used to quench the reaction. Subsequent steps used 100 mL each of the required solvents except for saturated NaHCO₃ (60 mL). The crude nitrone thus obtained was partially purified by flash column chromatography on SiO₂ (8.5 x 5.7 cm) using ethyl acetate-hexanes (25:75) to yield a yellow oil (0.79 g, 74%). Characteristic peaks in the ¹H NMR (600 Hz, CDCl₃): δ 7.42-7.30 (m, 5H), 6.84 (s, 1H), 5.20 (dd, J = 12.0, 27.6 Hz, 2H), 4.83 (q, J = 7.2 Hz, 1H), 1.70 (d, J = 6.6 Hz, 3H).
**N-Cyanomethyl N-Oxide Phenethylamine (25).** *N*-Cyanomethyl phenethylamine (1.099 g, 6.86 mmol) in CH₂Cl₂ (70 mL) was reacted with mCPBA (70-75%, 3.183 g, 13.83 mmol) following the General Procedure given above. The reaction appeared complete within 30 min. Sodium thiosulfate Na₂S₂O₅.5H₂O (3.401 g, 13.72 mmol) dissolved in water (15 mL) and saturated NaHCO₃ (30 mL) were used to quench the reaction. Subsequent steps used 400 mL each of the required solvents except for saturated NaHCO₃ (210 mL). The crude material thus obtained was purified by flash column chromatography on SiO₂ (14 x 7.0 cm) with ethyl acetate-hexanes (35:65) to yield a colorless amorphous solid. Yield: 0.809 g (68%); silica gel TLC R_f = 0.48 (ethyl acetate-hexanes = 1:1); ¹H NMR (400 MHz, CDCl₃): δ 7.37-7.27 (m, 3H), 7.20-7.18 (m, 2H), 6.42 (s, 1H), 4.14 (t, J = 7.2 Hz, 2H), 3.22 (t, J = 6.8 Hz, 2H); ¹³C NMR (150 MHz, CDCl₃): δ 136.11, 129.24, 128.78, 127.72, 112.03, 107.66, 68.77, 33.88; ESI-MS: m/z calcld for C₁₀H₁₀NaN₂O 197.1, found: 197.1 [M+Na]⁺. This compound was reported in the literature.³¹

**General Procedure for the Synthesis of N-Hydroxylamines.** To a well-stirred solution of *N*-cyanomethyl *N*-oxide amine in MeOH was added hydroxylamine hydrochloride and the resulting solution was stirred at a specified temperature. The completion of the reaction was monitored by TLC. The reaction mixture was allowed to attain room temperature, diluted with CH₂Cl₂ (1 part volume with respect to MeOH) and stirred for 5 min. Saturated NaHCO₃ (2-3 parts volume with respect to MeOH) was added and organic
layer was separated. Aqueous layer was extracted with CH$_2$Cl$_2$ (3 x (3-4 parts volume with respect to MeOH)). Combined organic phases were washed with brine (3-4 parts volume with respect to MeOH), dried (anhydrous Na$_2$SO$_4$) and filtered. The filtrate was concentrated to dryness under reduced pressure to provide the crude material. To the above crude material was added oxalic acid in MeOH and the resulting solution was triturated with ether or hexanes. The recovered solids were washed with cold ether or hexanes three times.

![Chemical Structure of N-Hydroxyglycine tert-Butyl Ester (4)](image)

**N-Hydroxyglycine tert-Butyl Ester (4).** Compound 21 (0.070 g, 0.38 mmol) in MeOH (10 mL) was reacted with hydroxylamine hydrochloride (0.138 g, 2.00 mmol) at 50 °C for 7 h following the General Procedure for the Synthesis of N-Hydroxylamines. To the crude material was added oxalic acid (0.100 g, 0.80 mmol) in MeOH (1.0 mL) and the resulting solution was triturated with ether. The liquid containing solids was filtered and washed with cold ether to render 4 as a colorless shiny crystals. Yield: 0.048 g (54%); mp 124-125 °C; silica gel TLC $R_f = 0.30$ (CH$_2$Cl$_2$-MeOH = 6:4); $^1$H NMR (600 MHz, DMSO-d$_6$): $\delta$ 3.51 (s, 2H), 1.40 (s, 9H); $^{13}$C NMR (150 MHz, DMSO-d$_6$): $\delta$ 169.45, 164.05, 81.58, 55.38, 28.39. This compound was reported in the literature.$^{32}$

![Chemical Structure of N-Hydroxyalanine tert-Butyl Ester (5)](image)

**N-Hydroxyalanine tert-Butyl Ester (5).** Compound 22 (0.697 g, 3.52 mmol) in MeOH (88 mL) was reacted with hydroxylamine hydrochloride (1.244 g, 17.90 mmol) at 35-40 °C for 5 h following the General Procedure for the Synthesis of N-Hydroxylamines. To the crude material was added oxalic acid (0.634 g, 3.52 mmol) in MeOH (6.0 mL) and
the resulting solution was triturated with ether. The liquid containing solids was centrifuged to render 6 as a colorless powder. Yield: 0.726 g (100%); silica gel TLC $R_f = 0.23$ (ethyl acetate-hexanes = 1:1); $^1$H NMR (600 MHz, DMSO-$d_6$): δ 3.59 (d, $J = 7.2$ Hz, 1H), 1.42 (s, 9H), 1.14 (d, $J = 7.2$ Hz, 3H); $^{13}$C NMR (150 MHz, DMSO-$d_6$): δ 170.96, 163.03, 81.29, 59.86, 27.67, 13.61. This compound was reported in the literature.$^{1,32}$

![N-Hydroxyvaline tert-Butyl Ester (6)](image)

**N-Hydroxyvaline tert-Butyl Ester (6).** Compound 23 (0.266 g, 1.18 mmol) in MeOH (30 mL) was reacted with hydroxylamine hydrochloride (0.409 g, 5.88 mmol) at 35-40 °C for 36 h following the General Procedure for the Synthesis of $N$-Hydroxylamines. To the crude material was added oxalic acid (0.212 g, 2.36 mmol) in MeOH (3.5 mL) and the resulting solution was triturated with hexanes. The liquid containing solids was centrifuged to render 6 as a colorless powder. Yield: 0.276 g (100%); silica gel TLC $R_f = 0.28$ (ethyl acetate-hexanes = 3:7); m.p. 66-67 °C; $^1$H NMR (600 MHz, DMSO-$d_6$): δ 3.28 (d, $J = 6.6$ Hz, 1H, Val-$\alpha$-CH), 1.87 (m, 1H, Val-CH), 1.43 (s, 9H, tBu), 0.94 (d, $J = 6.6$ Hz, 3H, Val-CH$_3$), 0.87 (d, $J = 6.6$ Hz, 3H, Val-CH$_3$); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 170.6 (C=O); 161.8 (C=O$_{oxalate}$), 81.2 ($\alpha$-CH), 70.9, 27.9, 27.8, 19.6, 18.5; HRMS $m/z$ calcd for C$_9$H$_{19}$NaNO$_3$ 212.1261, found: 212.1263 [M+Na]$^+$.

![N-Hydroxyalanine Benzyl Ester (7)](image)

**N-Hydroxyalanine Benzyl Ester (7).** Compound 24 (0.790 g, 3.40 mmol) in MeOH (60 mL) was reacted with hydroxylamine hydrochloride (1.182 g, 17.00 mmol) at 50 °C for 8
h following the General Procedure for the Synthesis of N-Hydroxylamines. To the crude material was added oxalic acid (0.603 g, 6.80 mmol) in MeOH (10 mL) and the resulting solution was triturated with hexanes. The liquid containing solids was filtered and washed with cold hexanes to render 7 as a colorless amorphous solid. Yield: 0.334 g (41%); mp 121-122; silica gel TLC $R_f = 0.33$ (ethyl acetate-hexanes = 1:1); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.40-7.30 (m, 5H), 5.15 (s, 2H), 3.74 (q, $J = 6.8$ Hz, 1H), 1.17 (d, $J = 7.2$ Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 173.03, 162.83, 136.61, 129.12, 128.73, 128.47, 66.54, 60.25, 14.51; ESI-MS $m/z$ calcd for C$_{10}$H$_{14}$NO$_3$ 196.1, found: 196.2 [M+H]$^+$. This compound was reported in the literature as the HCl salt.$^{32}$

\[ \text{HO} - \text{N} - \text{H} \cdot \frac{1}{2}(\text{COOH})_2 \]

**N-Hydroxy Phenethylamine (9).** Compound 25 (0.789 g, 4.53 mmol) in MeOH (60 mL) was reacted with hydroxylamine hydrochloride (1.574 g, 22.65 mmol) at 35-40 °C for 12 h following the General Procedure for the Synthesis of N-Hydroxylamines. To the crude material was added oxalic acid (0.412 g, 4.53 mmol) in MeOH (4.0 mL) and the resulting solution was triturated with ether. The liquid containing solids was filtered and washed with ether to render 9 as a colorless amorphous flaky solids. Yield: 0.825 g (100%); silica gel TLC $R_f = 0.12$ (ethyl acetate-hexanes = 4:6); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 10.88 (br.s, 3H), 7.32-7.20 (m, 5H), 3.26 (t, $J = 8.4$ Hz, 2H), 2.90 (t, $J = 7.6$ Hz, 2H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 165.52, 137.76, 128.72, 128.59, 126.57, 52.02, 30.15. ESI-MS: $m/z$ calcd for C$_8$H$_{12}$NO 138.1, found: 138.0 [M+H]$^+$. This compound was reported in the literature.$^{31}$
(Cyanomethylene)Triphenylphosphorane (30). To a suspension of (cyanomethyl)-
triphenylphosphonium chloride (10.012 g, 29.64 mmol) in dry CH$_2$Cl$_2$ (144 mL) was
added MS 4Å-dried triethylamine (10.00 mL, 72.14 mmol) dropwise over a period of 15
min. After stirring the mixture for additional 22 h at room temperature under N$_2$
atmosphere, the mixture was washed with chilled water (2 x 40 mL). The organic layer
was dried (MgSO$_4$) for 1 h, filtered, and concentrated under reduced pressure to a brown
yellow solid. The crude material was purified by recrystallization in benzene to furnish
the product as yellowish solids. Yield: 4.673 g (52 %); mp = 191-192 °C; silica gel TLC
$R_f = 0.83$ (CH$_2$Cl$_2$-acetone); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.65-7.62 (m, 6H), 7.51-7.49
(m, 3H), 7.43-7.40 (m, 6H); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 133.10, 132.41, 132.32,
132.26, 132.16, 132.14, 128.75, 128.67; ESI-MS: $m/z$ calcd for C$_{20}$H$_{17}$NP 302.1, found:
302.2 [M+H]$^+$.$^{17}$

General Procedure for Cyanoketophosphoranes

To a well stirred solution of N-protected amino acid (1.0 eq), DMAP (1/15 eq), and EDCI
(1.3-2.0 eq) in CH$_2$Cl$_2$ (5 mL) was added (cyanomethylene)triphenylphosphorane (1.00-
1.05 eq) at ambient temperature under N$_2$ atmosphere. The resulting mixture was stirred
further for a given time (12-19 h) at ambient temperature and the reaction was monitored
using TLC. The reaction mixture was diluted with CH$_2$Cl$_2$ (5 mL) and washed
successively with water (2 x 3 mL), saturated NaHCO$_3$ (1 x 3 mL) and saturated NaCl (1
x 3 mL). The organic phase was dried (MgSO$_4$), filtered and the filtrate was concentrated
to dryness under reduced pressure.$^{16}$
Boc-Protected Alanine Cyanoketophosphorane (31). Boc-protected alanine 26 (0.174 g, 0.92 mmol) was reacted with (cyanomethylene)triphenylphosphorane (0.291 g, 0.97 mmol) in the presence of EDCI (0.229 g, 1.20 mmol) and DMAP (7.0 mg, 0.06 mmol) to furnish the crude material after 12 h following General Procedure for Cyanoketophosphoranes. The crude product was purified by flash column chromatography on SiO₂ (11 x 3.5 cm) using ethyl acetate-hexanes (6:4) to afford a colorless foam. Yield: 0.242 g (56%); silica gel TLC \( R_f = 0.36 \) (ethyl acetate-hexanes = 6:4); \(^1\)H NMR (400 MHz, CDCl₃): \( \delta 7.63-7.47 \) (m, 15H), 5.32 (d, \( J = 7.6 \) Hz, 1H, NH), 4.84 (t, \( J = 6.8 \) Hz, 1H, α-H), 1.45 (d, \( J = 6.8 \) Hz, 3H, CH₃), 1.40 (s, 9H); \(^{13}\)C NMR (100 MHz, CDCl₃): \( \delta 195.66, 155.42, 133.81, 133.71, 133.51, 129.52, 129.31, 123.47, 122.54, 79.10, 60.61, 47.40, 46.14, 28.60, 14.43, 19.54; \(^{31}\)P NMR (80.95 MHz, CDCl₃) 21.32 (s, PPh₃); ESI-MS: \( m/z \) calcd for C₂₈H₂₉NaN₂O₃P 495.2; found: 495.2 [M+Na]⁺. This compound was reported in the patent literature.³³

Boc-Protected Valine Cyanoketophosphorane (32). Boc-protected valine 27 (0.200 g, 0.92 mmol) was reacted with (cyanomethylene)triphenylphosphorane (0.291 g, 0.97 mmol) in the presence of EDCI (0.229 g, 1.20 mmol) and DMAP (7.0 mg, 0.06 mmol) to furnish the crude material after 12 h following General Procedure for Cyanoketophosphoranes. The crude product was purified by flash column chromatography on SiO₂ (12 x 3.5 cm) using ethyl acetate-hexanes (1:1) to afford a colorless foam. Yield: 0.202 g (52%); silica gel TLC \( R_f = 0.33 \) (ethyl acetate-hexanes = 1:1).
1:1); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.63-7.47 (m, 15H), 5.15 (d, $J = 8.8$ Hz, 1H, NH), 4.76 (dd, $J = 4.0$, 8.8 Hz, 1H, $\alpha$-H), 2.27 (m, 1H, $\beta$-H) 1.42 (s, 9H, tBu), 1.03 (d, $J = 6.8$ Hz, 3H, CH$_3$), 0.78 (d, $J = 6.4$ Hz, 3H, CH$_3$); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 194.50, 156.15, 133.74, 133.67, 133.30, 129.29, 129.21, 123.37, 122.75, 78.92, 60.78, 31.67, 28.47, 20.19, 16.92, 14.31; $^{31}$P NMR (80.95 MHz, CDCl$_3$) 21.28 (s, PPh$_3$); ESI-MS: $m/z$ calcd for C$_{30}$H$_{33}$NaN$_2$O$_3$P 523.2; found: 523.3 [M+Na]$^+$. 

**Fmoc-Protected Alanine Cyanoketophosphorane (33).** Toluene was added to Fmoc-Ala-OH·H$_2$O, stirred and concentrated to dryness *in vacuo*. This step was repeated thrice. The dried Fmoc-Ala-OH (1.94 g, 5.89 mmol) was combined with (cyanomethylene)triphenylphosphorane (1.86 g, 6.19 mmol), EDCI (2.26 g, 11.78 mmol) and DMAP (45 mg, 0.37 mmol) in CH$_2$Cl$_2$ (50 mL) to furnish the crude material after 19 h following the General Procedure for Cyanoketophosphoranes. The crude material was purified by flash column chromatography on SiO$_2$ (11 x 5.7 cm) using ethyl acetate-hexane (6:4) to afford a colorless foam. Yield: 1.16 g (33%); silica gel TLC $R_f = 0.35$ (ethyl acetate-hexane = 6:4); The $^1$H NMR was identical to the reported compound: $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.73 (d, $J = 7.7$ Hz, 2H), 7.64-7.50 (m, 17H), 7.36 (t, $J = 7.7$ Hz, 2H), 7.26 (t, $J = 7.7$ Hz, 2H), 5.77 (d, $J = 7.2$ Hz, 1H), 4.96 (quint, $J = 6.9$ Hz, 1H), 4.32 (quint, $J = 8.3$, 2H), 4.18 (t, $J = 8.3$ Hz, 1H), 1.55 (d, $J = 7.2$, 3H); ESI-MS: $m/z$ calcd for C$_{38}$H$_{32}$N$_2$O$_3$P 595.2, found: 595.3 [M + H]$^+$. 
**Fmoc-Protected Valine Cyanoketophosphorane (34).** Fmoc-protected valine 29 (0.312 g, 0.92 mmol) was reacted with (cyanomethylene)triphenylphosphorane (0.291 g, 0.97 mmol) in the presence of EDCI (0.229 g, 1.20 mmol) and DMAP (7.0 mg, 0.06 mmol) to furnish the crude material after 14 h following General Procedure for Cyanoketophosphoranes. The crude material was purified by flash column chromatography on SiO$_2$ (12 x 3.5 cm) using ethyl acetate-hexanes (1:1) to provide a colorless glassy solid. Yield: 0.296 g (52%), silica gel TLC $R_f = 0.36$ (ethyl acetate-hexanes = 1:1); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.74 (d, $J = 7.8$ Hz, 2H, Aromatic), 7.52-7.48 (m, 17H, Aromatic), 7.36 (d, $J = 7.8$ Hz, 2H, Aromatic), 7.26 (t, $J = 6.6$ Hz, 2H, Aromatic), 5.60 (d, $J = 8.4$ Hz, 1H, NH), 4.90 (dd, $J = 3.6$, 8.4 Hz, 1H, $\alpha$-H), 4.33 (m, 2H, Fmoc CH$_2$), 4.22 (t, $J = 7.2$ Hz, 1H, Fmoc CH), 2.44-2.38 (m, 1H, $\beta$-H), 1.07 (d, $J = 6.6$ Hz, 3H, CH$_3$), 0.83 (d, $J = 6.6$ Hz, 3H, CH$_3$); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 194.05, 156.45, 144.30, 144.16, 141.40, 141.38, 133.77, 133.67, 133.47, 133.45, 129.42, 129.29, 127.71, 127.17, 125.43, 123.36, 122.43, 120.03, 66.91, 61.13, 61.04, 47.40, 32.26, 20.24, 16.90; $^{31}$P NMR (80.95 MHz, CDCl$_3$): 21.3 (s, PPh$_3$); ESI-MS: m/z calcd for C$_{48}$H$_{36}$N$_2$O$_3$P 623.6, found: 623.2 [M+H]$^+$.  

Alanine Cyanoketophosphorane (35). To a solution of Boc-protected alanine cyanophosphorane 31 (0.311 g, 0.66 mmol) was added 1.0 M HCl in diethyl ether (9.0 mL, 9.0 mmol) in CH$_2$Cl$_2$ (3 mL) and the mixture was stirred at 0 °C. The reaction was

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monitored by TLC and appeared to stop within 2 h. The mixture was concentrated to dryness under reduced pressure, reconstituted with minimum volume of MeOH, and diethyl ether was added until persistent cloudiness was observed. Filtration of the mixture afforded the crude material as a colorless foam (0.317 g). Silica gel TLC $R_f = 0.45$ (MeOH-CHCl$_3 = 2:8$); $^1$H NMR (400 MHz, MeOH-$d_4$): $\delta$ 7.72-7.53 (m, 17 H), 4.17 (q, $J = 6.4$ Hz, 1H, $\alpha$-H), 1.44 (s, 9H, tBu), 1.39 (d, $J = 2.4$ Hz, 3H, CH3); The crude material was a mixture of three products: alkylated product 35a ESI-MS: $m/z$ calcd for C$_{27}$H$_{30}$N$_2$OP 473.2, found: 473.2 [M+H]$^+$; incompletely deprotected product 35b ESI-MS: $m/z$ calcd for C$_{24}$H$_{22}$N$_2$O$_3$P 417.1, found: 417.3 [M+H]$^+$; desired product 35c ESI-MS: $m/z$ calcd for C$_{23}$H$_{22}$N$_2$OP 373.1, found: 373.4 [M+H]$^+$.

Valine Cyanoketophosphorane (36). A solution of Boc-protected valine cyanophosphorane 32 (0.106 g, 0.21 mmol) in CH$_2$Cl$_2$ (4.8 mL) and TFA (1.20 mL, 16.16 mmol) was stirred at 0 °C for 1.5 h. The mixture was concentrated to dryness under reduced pressure, dissolved in diethyl ether, and hexanes was added until persistent cloudiness was observed. After the mixture was left in the refrigerator overnight, the mother liquor was removed, the solids were washed twice with cold diethyl ether (approximately 10 mL) and the residual solvent was removed in vacuum to afford the crude material as colorless solids. Yield: 0.077 g (92%). Silica gel TLC $R_f = 0.53$ (MeOH-CHCl$_3 = 2:8$); $^1$H NMR (400 MHz, MeOH-$d_4$): $\delta$ 7.77-7.53 (m, 17H), 4.25 (d, $J = 4.0$ Hz, 1H, $\alpha$-H), 2.44-2.40 (m, 1H, $\beta$-H), 1.06 (d, $J = 7.2$ Hz, 3H), 0.93 (d, $J = 7.2$ Hz, 3H); ESI-MS: $m/z$ calcd for C$_{25}$H$_{26}$N$_2$OP 401.2, found: 401.5 [M+H]$^+$. 
Alanine Cyanoketophosphorane (37). Fmoc-protected alanine cyanophosphorane (0.166 g, 0.28 mmol) was taken in anhydrous piperidine (2.79 mL, 28.21 mmol), and the resulting solution was stirred at ambient temperature. The reaction was monitored by TLC and appeared to stop within 15 min. Excess piperidine was removed under reduced pressure, and the crude material thus obtained was coevaporated four times in dry triethylamine (0.16 mL) and dry CH₂Cl₂ (2.80 mL). The resulting solid was triturated in diethyl ether-hexanes, centrifuged, and the residue washed twice with hexane. The residual solvent was evaporated off under reduced pressure to afford the piperidine and N-fluorenylmethylpiperidine-free product as a colorless powder and used in the next. Yield: 0.103 g (99%); The ¹H NMR was identical to the reported compound:¹⁴ ¹H NMR (600 MHz, CDCl₃) δ 7.64-7.60 (m, 3H), 7.60-7.54 (m, 6H), 7.52-7.48 (m, 6H), 4.13 (q, J = 6.6 Hz, 1H), 1.37 (d, J = 7.2 Hz, 3H); ³¹P NMR (80.95 MHz, CDCl₃) δ 20.84 (s); ESI-MS: m/z calcd for C₂₃H₂₂N₂OP 373.1, found: 373.3 [M + H]^⁺.

Valine Cyanoketophosphorane (38). Fmoc-protected valine cyanophosphorane (0.102 g, 0.16 mmol) was treated with neat piperidine (1.70 mL, 17.21 mmol) in the same way as Fmoc-protected alanine cyanophosphorane. Co-evaporation twice in dry triethylamine (0.5 mL) and dry CH₂Cl₂ (1.5 mL), followed by trituration in diethyl ether-hexanes afforded the product as a colorless powder. Yield: 0.064 g (98%); silica gel TLC Rf = 0.48 (MeOH-CHCl₃ = 1:9); Characteristic peaks in the ¹H NMR (600 MHz, CDCl₃) δ
3.85 (d, J = 2.4 Hz, 1H), 3.35 (s, 2H), 2.25-2.10 (m, 1H, β-H), 1.07 (d, J = 6.6 Hz, 3H, CH₃), 0.91 (d, J = 6.6 Hz, 3H, CH₃); ESI-MS: m/z calcd for C₂₅H₂₆N₂O₄ 401.2, found: 401.6 [M + H]⁺.

1,2,3,4,6-Penta-O-acetyl-D-galactopyranoside (40). To a vacuum-dried galactopyranose (20.093 g, 0.11 mol) was added pyridine (100 mL), acetic anhydride (100 mL, 1.06 mol), and the dirty white mixture was stirred at room temperature for 22 h under N₂ atmosphere. The reaction mixture was poured into the Erlenmeyer flask containing crushed ice, and the aqueous solution was extracted with CHCl₃ (3 x 200 mL). The pooled organic phases were neutralized with saturated NaHCO₃ at 0 °C, and the layers separated. The organic layer was dried with Na₂SO₄, filtered, and after azeotropic distillation with toluene to remove the residual pyridine afforded the crude product as a yellow gum (45.267 g) which was used in the next step without further purification. Silica gel TLC Rf = 0.49 (ethyl acetate-hexanes = 1:1).¹⁹

2,3,4,6-Tetra-O-acetyl-D-galactopyranosyl Bromide(41). To a solution of 40 (45.267 g, 0.12 mol) in glacial acetic acid (110 mL) was added chilled 33% hydrobromic acid in acetic acid (150 mL, 2.63 mol) dropwise using addition funnel. The mixture was allowed to stir overnight during which time the mixture was allowed to warm to room temperature. The mixture was poured into ice-water mixture and the aqueous solution was extracted with ice-cold CHCl₃ (3 x 300 mL). The pooled organic extracts were
neutralized with chilled saturated NaHCO₃ and the layers separated. The organic layer was dried (Na₂SO₄), filtered, and concentrated to dryness under reduced pressure to furnish the crude material (39.61 g) as a yellow syrup which was used in the next step without further purification. Silica gel TLC $R_f = 0.72$ (ethyl acetate-hexanes = 2:1).³⁹

3,4,6-Tri-O-acetyl-β-galactal (42). A suspension of activated Zn dust (42.911 g, 0.66 mol) in N-methylimidazole (9.0 mL, 0.11 mol) and ethyl acetate (200 mL) was refluxed for 30 min. A solution of the galactopyranosyl bromide 41 in ethyl acetate (100 mL) was added to the Zn suspension dropwise and the reflux was continued for 3 h. The mixture was filtered over Celite® after allowing it to cool to room temperature. The filtrate was washed with 10% HCl (1 x 200 mL) followed by saturated NaHCO₃ (1 x 200 mL). The organic layer was dried (Na₂SO₄), filtered, and concentrated to dryness under reduced pressure. The crude material was purified by flash column chromatography on SiO₂ in two batches (4.75 x 2.79 in) using ethyl acetate-hexanes (2:8) to furnish a yellow syrup. Yield: 7.096 g (24% over 3 steps); silica gel TLC $R_f = 0.71$ (ethyl acetate-hexanes = 2:1);

¹H NMR (600 MHz, CDCl₃): δ 6.46 (dd, $J = 1.8$, 6.6 Hz, 1H), 5.56-5.55 (m, 1H), 5.43 (dt, $J = 1.2$, 4.8 Hz, 1H), 4.73 (ddd, $J = 1.2$, 2.4, 6.0 Hz, 1H), 4.32 (t, $J = 6.0$ Hz, 1H), 4.26 (dd, $J = 7.2$, 11.4 Hz, 1H), 4.21 (dd, $J = 4.8$, 11.4 Hz, 1H), 2.13 (s, 3H), 2.09 (s, 3H), 2.03 (s, 3H); ESI-MS $m/z$ calcd for C₁₂H₁₆NaO₇ 295.1, found: 295.3 [M+Na]⁺. The ¹H NMR was identical to the reported compound.³⁹
3,4,6-Tri-O-acetyl-2-azido-2-deoxy-α/β-D-galactopyranosyl Nitrate (43). A solution of 42 (3.737 g, 13.83 mmol) in dry acetonitrile (80 mL) was added to a mixture of sodium azide (1.421 g, 21.86 mmol) and ceric(IV) ammonium nitrate (22.926 g, 41.82 mmol) maintained at -15 to -20 °C over a period of 1 h. The reaction mixture was stirred further for 6 h at the same temperature. Ice-cold diethyl ether was added to the reaction mixture, followed by ice water, and the layers were separated. The organic layer was washed several times with ice water until it was colorless, dried (Na₂SO₄), filtered, and concentrated under reduced pressure. The crude material was purified by dissolution in ethyl acetate and precipitation with hexanes to afford colorless crystals. Yield: 2.486 g (48%); Characteristic peaks in the ¹H NMR (600 MHz, CDCl₃): δ 6.34 (d, J = 4.2 Hz, 1H), 5.49 (d, J = 3.0 Hz, 1H), 5.24 (dd, J = 3.6, 10.8 Hz, 1H), 4.36 (t, J = 6.6 Hz, 1H), 2.16 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H). The ¹H NMR was in concordance with the reported compound.²⁰

3,4,6-Tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl Bromide (44). To a solution of 43 (1.00 g, 2.70 mmol) in dry acetonitrile (20 mL) was added lithium bromide (1.17 g, 13.50 mmol). After stirring the mixture for 7 h at room temperature, it was diluted with CH₂Cl₂ (30 mL). The organic layer was washed with cold water (4 x 10 mL), and the pooled aqueous layers were back-extracted with CH₂Cl₂ (3 x 10 mL). The combined organic extracts were dried (Na₂SO₄), filtered and concentrated to dryness under reduced
pressure to afford a yellow syrup (1.299 g). $^1$H NMR analysis showed that the crude product was sufficiently pure to be used in the next step without further purification. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 6.45 (d, $J = 3.6$ Hz, 1H, H-1), 5.49 (d, $J = 3.6$ Hz, 1H, H-4), 5.32 (dd, $J = 3.6$, 10.8 Hz, 1H, H-3), 4.46 (t, $J = 6.6$ Hz, 1H, H-5), 4.15 (dd, $J = 6.6$, 11.4 Hz, 1H, H-6'), 4.08 (dd, $J = 6.6$, 11.4 Hz, 1H, H-6), 3.97 (dd, $J = 3.6$, 10.8 Hz, 1H, H-2), 2.14 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H); ESI-MS $m/z$ calcd for C$_{12}$H$_{16}$BrNaN$_3$O$_7$ 417.2, found: 417.9 [M+Na]$^+$. The $^1$H NMR was in concordance with the reported compound.\(^{20}\)

\[
\begin{array}{c}
\text{HO} \\
\text{CH} \equiv \\
\text{C} \text{O} \\
\text{OBn}
\end{array}
\]

\[\text{FmocCHN} \text{CH}_3\]

\text{N-(9-Fluorenylethoxycarbonyl)-threonine Benzyl Ester (45). The procedure used was analogous to that of Matsumori et al.}^{21}\text{ To a stirred solution of threonine benzyl ester oxalate (4.987 g, 16.66 mmol) in MeOH (100 mL) was added 9-fluoromethyl succinimidyl carbonate (5.659 g, 16.66 mmol), followed by pyridine (3.6 mL, 44.69 mmol). Stirring was continued for 22 h at room temperature. The mixture was diluted with water (400 mL) and extracted with ethyl acetate (3 x 200 mL). The organic extracts were dried (MgSO$_4$), filtered, and concentrated to dryness under reduced pressure. The crude material was purified by flash column chromatography on SiO$_2$ using ethyl acetate-hexanes-CH$_2$Cl$_2$ (1:3:1) to furnish a colorless fluffy solids. Yield: 3.84 g (53%); silica gel TLC $R_f$ = 0.14 (ethyl acetate-hexanes-CH$_2$Cl$_2$ = 1:3:1); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.74 (d, $J = 7.8$ Hz, 2H), 7.59 (d, $J = 7.2$ Hz, 2H), 7.37 (t, $J = 7.2$ Hz, 2H), 7.32-7.26 (m, 7H), 5.76 (d, $J = 8.4$ Hz, 1H), 5.19 (q, $J = 8.4$ Hz, 2H), 4.42-4.36 (m, 4H), 4.20 (t, $J = 7.2$ Hz, 1H), 2.30 (s, 1H), 1.22 (d, $J = 6.6$ Hz, 3H); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 171.24, 156.98, 144.04, 143.83, 141.46, 141.44, 135.36, 128.80, 128.65, 128.36, 127.89, 127.26, 127.25, 125.30, 125.28, 120.15, 120.14, 68.18, 67.56, 67.42, 59.43, 47.28, 20.08; ESI-
MS: \( m/z \) calcd for \( C_{26}H_{25}NaNO_5 \) 454.2, found: 453.5 \([M+Na]^+\). This compound was reported in the literature.\(^{34}\)

\[ \text{N-}(\text{Floren-9-ylmethoxycarbonyl})-O-(3,4,6\text{-tri-O-acetyl-2-azido-2-deoxy-\alpha-D-galactopyranosyl})-L\text{-threonine Benzyl Ester (46).} \]

To a mixture of oven-dried MS 4Å dust (0.79 g), \( \text{Ag}_2\text{CO}_3 \) (1.463 g, 5.31 mmol) under \( \text{N}_2 \) atmosphere and with the exclusion of light was added a solution of compound 45 (1.423 g, 3.30 mol) dissolved in toluene-\( \text{CH}_2\text{Cl}_2 \) (1:1) (30 mL) and stirred for 45 min. \( \text{AgClO}_4 \) (1.231 g, 5.94 mmol) was added to the mixture and stirred for additional 20 min. The azido bromide 44 (1.300 g, 3.30 mmol) dissolved in toluene-\( \text{CH}_2\text{Cl}_2 \) (1:1) (10 mL) was added to the reaction mixture using a syringe over a period of 30 min. The reaction was monitored with TLC (acetone-\( \text{CHCl}_3 \)-hexanes = 1:1:3) and appeared complete after 29 h as judged by the disappearance of the azido bromide. The mixture was filtered over Celite® and rinsed with \( \text{CH}_2\text{Cl}_2 \) (100 mL). The filtrate was extracted with saturated \( \text{NaHCO}_3 \) (3 x 50 mL) and water (2 x 50 mL). The pooled aqueous layers were back-extracted with \( \text{CH}_2\text{Cl}_2 \) (2 x 100 mL). The organic extracts were combined, dried (\( \text{Na}_2\text{SO}_4 \)), filtered, and concentrated to dryness under reduced pressure to afford a white solid (2.600 g). The crude product was used for the next step without further purification. Characteristic peaks in the \( ^1\text{H} \) NMR (600 MHz, \( \text{CDCl}_3 \)): \( \delta \) 7.75 (d, \( J = 7.8 \), 2H, Ar Fmoc), 7.60 (d, \( J = 7.8 \) Hz, 2H, Ar Fmoc), 7.39-7.27 (m, 9H), 5.70 (d, \( J = 9.6 \) Hz, 1H, Thr-NH), 5.42 (d, \( J = 2.4 \) Hz, 1H, H-4), 5.28-5.17 (m, 3H), 4.88 (d, \( J = 3.6 \) Hz, 1H, H-1), 3.57 (dd, \( J = 3.6 \), 11.4 Hz, 1H, H-2), 4.48-4.39 (m, 3H), 4.32 (dd, \( J = 7.8 \), 10.2 Hz, 1H), 4.25-4.19 (m, 2H), 4.05 (d, \( J = 6.6 \) Hz, 2H), 2.13 (s,
3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.32 (d, J = 6.6 Hz, 3H, Thr-CH₃); ESI-MS: m/z calcd for C₃₈H₄₀Na₄N₄O₁₂ 767.3, found: 767.6 [M+Na]⁺. The ¹H NMR was in accordance with the literature.²⁰

**Nα-(Floren-9-ylmethoxycarbonyl)-O-(2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-galactopyranosyl)-L-threonine Benzyl Ester (47).** To a solution of crude 46 (1.0 g) in THF-Ac₂O-AcOH (3:2:1) (44 mL) was added Zn dust (1.160 g, 17.74 mmol) followed by saturated CuSO₄ (2.2 mL). After stirring for 20 min at room temperature, the mixture was filtered through a Celite® bed and rinsed generously with CH₂Cl₂. The filtrate was concentrated to dryness under reduced pressure by co-evaporation with toluene. The crude material thus obtained was purified by flash column chromatography on SiO₂ (10 x 4.8 cm) using acetone-chloroform-hexanes (2:2:6) to furnish the product as a white glassy solid. Yield: 0.303 g (29% over 3 steps); silica gel TLC Rf = 0.19 (acetone-chloroform-hexanes = 2:2:6); Characteristic peaks in the ¹H NMR (600 MHz, CDCl₃): δ 7.74 (d, J = 7.2, 2H, Ar Fmoc), 7.61 (d, J = 7.2 Hz, 2H, Ar Fmoc), 7.39-7.27 (m, 9H), 5.99 (d, J = 9.0 Hz, 1H, AcNH), 5.90 (d, J = 7.8 Hz, 1H, Thr-NH), 5.35 (s, 1H, H-4), 5.15 (d, J = 12.0 Hz, 1H, H-3), 5.08-5.03 (m, 2H, OCH₂Ph), 4.77 (d, J = 3.0 Hz, 1H, H-1), 4.50-4.43 (m, 3H), 4.40-4.37 (m, 1H), 4.24-4.22 (m, 1H), 4.17 (t, J = 6.6 Hz, 1H), 4.09-4.01 (m, 3H), 2.13 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H), 1.95 (s, 3H), 1.27 (d, J = 6.0 Hz, 3H, Thr-CH₃); ESI-MS: m/z calcd for C₄₀H₄₄Na₄N₂O₁₃ 783.3, found: 784.0 [M+Na]⁺. The ¹H NMR was identical to the reported compound.²⁰
$\alpha$-(Floren-9-ylmethoxycarbonyl)-$O$-(2-Acetamido-2-deoxy-3,4,6-tri-$O$-acetyl-$\alpha$-D-galactopyranosyl)-$L$-threonine (8). To the solution of galactosyl threonine benzyl ester 47 (0.477 g, 0.71 mmol) in MeOH (18 mL) was added 10% Pd/C (50% wet) (26% by weight). H$_2$ was introduced to the reaction mixture and stirred for 40 min at room temperature. After the reaction, the mixture was filtered over a celite® bed, rinsed generously with MeOH and concentrated to dryness under reduced pressure. The crude material was purified by flash column chromatography on SiO$_2$ (11 x 4.8 cm) using CHCl$_3$-MeOH (10:1) to give the product as a colorless solid. Yield: 0.330 g (78%); $R_f = 0.32$ (CHCl$_3$-MeOH = 6:1); $^1$H NMR (400 MHz, MeOH-$d_4$) $\delta$ 7.81 (d, $J = 7.2$ Hz, 2H), 7.69 (t, $J = 7.2$ Hz, 2H), 7.42-7.28 (m, 4H), 5.40 (d, $J = 2.8$ Hz, 1H), 5.08 (dd, $J = 11.2$, 2.8 Hz, 1H), 4.61-4.55 (m, 1H), 4.48-4.35 (m, 3H), 4.27 (quint, $J = 8.0$ Hz, 3H), 4.15-4.05 (m, 2H), 2.13 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H), 1.24 (d, $J = 6.4$ Hz, 3H); $^{13}$C NMR (100 MHz, MeOH-$d_4$) $\delta$ 175.38, 173.81, 172.28, 172.25, 172.11, 159.26, 145.52, 145.28, 142.82 128.94, 128.33, 126.30, 126.18, 121.14,121.10, 100.88, 77.89, 69.90, 68.95, 68.32, 67.82, 63.45, 60.12, 23.03, 20.91, 20.79, 20.73, 20.66, 19.3; ESI-MS: $m/z$ calcd for C$_{33}$H$_{38}$NaN$_2$O$_{13}$ 693.2, found: 693.7 [M+Na]$^+$ . The $^1$H NMR was similar to the reported compound. The following peaks matched those reported in the literature: $\delta$ 7.81, 7.69, 7.42-7.28, 5.40, 5.08, 4.61-4.55, 4.48-4.35, 4.27, 4.15-4.05, 2.11, 2.02, 1.99, 1.95, 1.24.
**Fmoc-Ac$_3$-Tn-α-Thr-Ala-Cyanophosphorane Analog 48.** Glycosylamino acid 8 (0.154 g, 0.22 mmol), alanine cyanophosphorane derivative 37 (0.127 g, 0.34 mmol), HBTU (0.178 g, 0.47 mmol), and HOBt (0.071 g, 0.46 mmol) were taken together and dried for 0.5 h in high vacuum before the addition of 3:7 anhydrous DMF-CH$_2$Cl$_2$ (9 mL), and the resulting mixture was stirred for 15 min at ambient temperature. TMP (33 µL, 0.25 mmol) was added under N$_2$ atmosphere and stirring was continued at ambient temperature. The reaction was monitored by TLC (hexanes-CHCl$_3$-acetone-MeOH = 6:2:1:1) and appeared complete in 3.5 h. The reaction mixture was diluted with CH$_2$Cl$_2$ (12 mL) and successively washed with cold 1% aqueous citric acid (1 x 8 mL), and cold saturated NaHCO$_3$ (1 x 8 mL). The organic phase was dried (MgSO$_4$), filtered, and the filtrate was concentrated to dryness under reduced pressure. The crude material was purified by flash column chromatography on SiO$_2$ (7 x 4.5 cm) using hexanes-CHCl$_3$-acetone-MeOH (0.5:0.5:1:8 and then 1:1:2:6) to afford a colorless amorphous solid. Yield 0.14 g (61%); silica gel TLC $R_f$ = 0.31 (hexanes-CHCl$_3$-acetone-MeOH = 1:1:2:6); $^1$H NMR (600 MHz, CDCl$_3$): δ 7.73 (d, $J = 7.8$ Hz, 2H, aromatic), 7.64 (t, $J = 6.6$ Hz, 3H, aromatic), 7.55 (m, 14H, aromatic), 7.36 (t, $J = 7.2$ Hz, 2H, aromatic), 7.27 (m, 2H, aromatic), 7.10 (d, $J = 6.6$ Hz, 1H, Ala-NH), 6.81 (d, $J = 9.6$ Hz, 1H, NHAc), 5.83 (d, $J =$ 7.2 Hz, 1H, Fmoc NH), 5.28 (d, $J = 2.4$ Hz, 1H, H-4), 5.13 (m, 1H, Ala-α-CH), 4.86 (d, $J =$ 3.6 Hz, 1H, H-1), 4.84 (dd, $J = 3.0$, 11.4 Hz, 1H, H-3), 4.52 (m, 1H, H-2), 4.36 (m, 2H, Fmoc CH$_2$), 4.13-4.22 (m, 4H, Thr-α-CH, β-CH, H-5 & Fmoc CH), 4.03 (d, $J = 5.4$ Hz, 2H, H-6 & H-6'), 2.13 (s, 3H, CH$_3$CO), 1.99 (s, 3H, CH$_3$CO), 1.85 (s, 3H, CH$_3$CO), 1.81
(s, 3H, CH$_3$CO), 1.50 (d, $J = 7.2$ Hz, 3H, Ala-CH$_3$), 1.16 (d, $J = 6.6$ Hz, 3H, Thr-CH$_3$);

$^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 193.93 (d, $J_{CP} = 3.6$ Hz, C=O), 170.74 (C=O), 170.43 (C=O), 170.30 (C=O), 170.09 (C=O), 167.83 (C=O), 156.16 (C=O), 143.79, 143.62, 141.20, 141.19, 133.48 (d, $J_{CP} = 10.4$ Hz), 133.48 (d, $J_{CP} = 2.6$ Hz), 129.26 (d, $J_{CP} = 12.9$ Hz), 127.66, 127.07, 127.06, 125.13, 122.03 (d, $J_{CP} = 93.2$ Hz), 119.91, 119.89, 98.54 (C-1), 75.46, 68.66, 67.25, 67.19, 66.91, 62.11, 57.26, 51.06 (d, $J_{CP} = 9.3$ Hz), 47.02 (d, $J_{CP} = 2.9$ Hz), 22.79, 20.72, 20.64, 20.61, 19.80, 16.53; $^{31}$P NMR (161.9 MHz, CDCl$_3$) $\delta$ 21.1 (s, PPh$_3$); HRMS: $m/z$ calcd for C$_{56}$H$_{57}$NaN$_4$O$_{13}$P 1047.3557, found 1047.3565 [M+Na]$^+$. 

**General Procedure for DMDO Oxidation and Decarboxylative Ligation.** To a well-stirred solution of appropriate cyanophosphorane (1 equiv.) in acetone and water was added freshly prepared DMDO (approximately 2 equivalents) and the resulting solution was stirred at ambient temperature. Reaction was monitored by TLC and appeared to stop after 5 min. Solvent was removed under reduced pressure and the crude material thus obtained was left in high vacuum. The product $\alpha$-keto acid was detected by mass spectrum. To the crude $\alpha$-keto acid was added appropriate $N$-hydroxyamino acid tert-butyl ester oxalate salt (1.5 to 2 equiv.) and the mixture was dried in high vacuum for at least 15 min. Anhydrous DMF or DMF-water (5:1) was added into the reaction under N$_2$ atmosphere and the resulting solution was allowed to stir at 40 °C. Progress of the reaction was monitored by TLC. The reaction mixture was diluted with CH$_2$Cl$_2$ (3 mL), washed with water (1 x 2 mL), and the aqueous layer was back-extracted with CH$_2$Cl$_2$ (1 x 2 mL). The pooled organic phases were dried (Na$_2$SO$_4$), filtered, and concentrated to dryness under reduced pressure.
\(N^\alpha\)-(Floren-9-ylmethoxycarbonyl)-Alanylalanine Benzyl Ester (50).\) Alanine cyanophosphorane 33 (0.068 g, 0.11 mmol) was first converted to \(\alpha\)-keto acid 49 in quantitative yield with DMDO (4 mL, approximately 2 equiv) in acetone (3 mL) and water (0.55 mL). Crude material 49 was dried in high vacuum for 24 h and reacted with \(N\)-hydroxylalanine benzyl ester oxalate salt 7 (0.039 g, 0.14 mmol) in anhydrous DMF (2.5 mL) at room temperature to produce dipeptide 50 after 63 h, following the General Procedure for Decarboxylative Ligation. The crude material was purified by flash column chromatography on SiO\(_2\) (6.5 \(\times\) 2.5 cm) using ethyl acetate-hexanes (4:6) to furnish the diastereomeric product as a colorless amorphous solid. Yield: 0.027 g (50%); silica gel TLC \(R_f = 0.72\) (ethyl acetate-hexanes = 8:2); \(^1\)H NMR (600 MHz, CDCl\(_3\)): \(\delta\) 7.74 (d, \(J = 7.2\) Hz, 2H), 7.57 (d, \(J = 5.4\) Hz, H), 7.38 (t, \(J = 7.2\) Hz, 2H), 7.34-7.29 (m, 7H), 6.71 (s, 0.27H), 6.60 (s, 0.50 H), 5.47 (s, 0.50H), 5.41 (s, 0.3H), 5.18-5.10 (m, 2H), 4.60 (quint, \(J = 7.2\) Hz, 1H), 4.36 (d, \(J = 6.6\) Hz, 2H), 4.30-4.24 (m, 1H), 4.19 (q, \(J = 6.6\) Hz, 1H), 1.39 (d, \(J = 7.2\) Hz, 3H), 1.37 (d, \(J = 6.6\) Hz, 3H); \(^1^3\)C NMR (150 MHz, CDCl\(_3\)): \(\delta\) 172.75, 172.68, 172.01, 156.10, 143.99, 143.90, 141.49, 141.47, 135.39, 128.83, 128.69, 128.38, 128.35, 127.93, 127.27, 125.27, 120.19, 67.49, 67.44, 67.42, 67.38, 67.26, 50.55, 48.40, 47.26, 29.90, 19.01, 18.86, 18.42, 18.41; ESI-MS: \(m/z\) calcd for C\(_{28}\)H\(_{29}\)N\(_2\)O\(_5\) 473.2, found 473.0 [M+H]\(^+\).
Glycotripeptide tert-Butyl Ester (51) and Glycopeptide-Derived Oxazole Byproduct (52). Cyanophosphorane 48 (0.053 g, 0.0517 mmol) was first converted to α-keto-acid 2 in quantitative yield with DMDO (4 mL, approximately 2 equiv.) in acetone (2 mL) and water (250 µL). Crude material 2 was dried in high vacuum for 0.5 h and reacted with N-hydroxy glycine tert-butyl ester oxalate salt 4 (0.015 g, 0.0620 mmol) in anhydrous DMF (1.5 mL) at 40-42 °C to afford glycotripeptide 51 and 52 after 6 h, following the General Procedure for Decarboxylative Ligation. Crude material was purified by flash column chromatography on SiO₂ (10 x 3 cm). Elution with MeOH-CH₂Cl₂ (1.5:98.5) yielded desired product 51 as colorless amorphous solid. Yield 0.019 g (43%); silica gel TLC R_f = 0.22 (MeOH-CH₂Cl₂ = 2.5:97.5, run twice); ¹H NMR (600 MHz, CDCl₃): δ 7.74 (d, J = 7.2 Hz, 2H, aromatic), 7.58 (d, J = 7.2 Hz, 2H, aromatic), 7.38 (t, J = 7.2 Hz, 2H, aromatic), 7.29 (t, J = 7.2 Hz, 2H, aromatic), 7.17 (d, J = 8.4 Hz, 1H, NHAc), 7.13 (d, J = 6.6 Hz, 1H, Ala-NH), 6.54 (t, J = 4.2 Hz, 1H, Gly-NH), 5.93 (d, J = 7.2 Hz, 1H, Fmoc NH), 5.36 (d, J = 2.4 Hz, 1H, H-4), 5.16 (d, J = 3.0 Hz, 1H, H-1), 5.07 (dd, J = 3.0, 12.0 Hz, 1H, H-3), 4.56 (m, 2H, H-2 & Ala-CH), 4.38 (d, J = 7.2 Hz, 2H, Fmoc CH₂), 4.28 (dd, J = 3.6, 7.2 Hz, 1H, Thr-α-CH), 4.20 (t, J = 6.6 Hz, 2H, H-5 & Fmoc CH), 4.17 (dd, J = 3.6, 6.6 Hz, 1H, Thr-β-CH), 4.05 (d, J = 6.6 Hz, 2H, H-6 & H-6′), 3.91 (t, J = 4.8 Hz, 2H, Gly-CH₂), 2.13 (s, 3H, CH₃CO), 1.997 (s, 3H, CH₃CO), 1.99 (s, 3H, CH₃CO), 1.98 (s, 3H, CH₃CO), 1.44 (s, 9H, tBu), 1.41 (d, J = 7.2 Hz, 3H, Ala-CH₃), 1.15 (d, J = 6.6 Hz, 3H, Thr-CH₃); ¹³C NMR (150 MHz, CDCl₃): δ 172.34 (C=O), 171.03 (C=O), 170.83
(C=O), 170.66 (2 x C=O), 168.77 (C=O), 168.48 (C=O), 156.20 (C=O), 143.98, 143.86, 141.49, 127.97, 127.30, 125.34, 125.27, 120.24, 120.21, 98.80 (C-1), 83.06, 75.08, 68.22, 67.45, 67.43, 67.19, 62.23, 57.35, 49.18, 47.84, 47.32, 42.37, 29.91, 28.20 (3 x CH₃ of tBu), 23.17, 20.99, 20.98, 20.88, 20.01, 16.50; HRMS: m/z calcd for C₄₂H₅₄NaN₄O₁₅ 877.3483, found 877.3501 [M+Na]⁺, and 52 as colorless amorphous solid. Yield 0.010 g (23%); Rᵣ = 0.24 (MeOH-CH₂Cl₂ = 2.5:97.5, run twice); ¹H NMR (600 MHz, CDCl₃): δ 7.74 (d, J = 7.2 Hz, 2H, aromatic), 7.59 (t, J = 7.8 Hz, 3H, NHAc & aromatic), 7.38 (t, J = 7.2 Hz, 2H, aromatic), 7.30 (m, 2H, aromatic), 7.26 (br.s, 1H, Ala-NH), 6.13 (s, 1H, Oxazole H-4), 5.84 (d, J = 6.6 Hz, 1H, Fmoc NH), 5.35 (d, J = 2.4 Hz, 1H, H-4), 5.21 (d, J = 3.0 Hz, 1H, H-1), 5.18 (dd, J = 3.0, 12.0 Hz, 1H, H-3), 5.04 (m, 1H, Ala-CH), 4.63 (m, 1H, H-2), 4.39 (dd, J = 3.0, 6.6 Hz, 2H, Fmoc CH₂), 4.28 (dd, J = 3.0, 6.6 Hz, 1H, Thr-α-CH), 4.22 (m, 3H, H-5, Thr-β-CH & Fmoc CH), 4.06 (d, J = 6.6 Hz, 2H, H-6 & H-6'), 2.14 (s, 3H, CH₃CO), 2.00 (s, 3H, CH₃CO), 1.99 (s, 3H, CH₃CO), 1.87 (s, 3H, CH₃CO), 1.49 (d, J = 6.6 Hz, 3H, Ala-CH₃), 1.37 (s, 9H, tBu), 1.15 (d, J = 6.0 Hz, 3H, Thr-CH₃); ¹³C NMR (100 MHz, CDCl₃): δ 170.92 (C=O), 170.75 (C=O), 170.72 (C=O), 170.67 (C=O), 168.58 (C=O), 157.05, 156.70 (C=O), 156.12, 144.00, 143.87, 141.53, 127.99, 127.32, 125.34, 125.30, 120.26, 120.22, 107.42 (Oxazole C-4), 98.20 (C-1), 84.96, 74.38, 68.35, 67.47, 67.35, 67.15, 62.18, 57.01, 47.81, 47.34, 44.79, 29.92, 28.26 (3 x CH₃ of tBu), 22.97, 21.03, 20.99, 20.88, 20.69, 16.22; HRMS: m/z calcd for C₄₂H₅₂NaN₄O₁₄ 859.3378, found 859.3375 [M+Na]⁺.
Glycotripeptide tert-Butyl Ester (53). Cyanophosphorane 48 (0.056 g, 0.0546 mmol) was first converted to α-keto-acid 2 in quantitative yield with DMDO (4 mL in acetone, approximately 2 equiv.) in acetone (2 mL) and water (250 μL). Crude material 2 was dried in high vacuum for 0.5 h and reacted with N-hydroxyalanine tert-butyl ester oxalate salt 5 (0.016 g, 0.0656 mmol) in anhydrous DMF (1.5 mL) at 35-40 °C to produce glycotripeptide 53 after 38 h as the sole product, following the General Procedure for Decarboxylative Condensation. Crude material was purified by flash column chromatography on SiO₂ (6 x 3.5 cm). Elution with MeOH-acetone-CHCl₃-hexanes (0.5:0.5:1:8) yielded desired product 53 as a colorless amorphous solid. Yield 0.041 g (86%); silica gel TLC Rf = 0.21 (MeOH-acetone-CHCl₃-hexanes = 1:1:1:7); ¹H NMR (600 MHz, CDCl₃): δ 7.76 (d, J = 7.2 Hz, 2H, aromatic), 7.60 (d, J = 7.2 Hz, 2H, aromatic), 7.40 (t, J = 7.2 Hz, 2H, aromatic), 7.30 (m, 3H, NHAc & aromatic), 7.25 (d, J = 6.6 Hz, 1H, Ala-NH), 6.63 (d, J = 6.6 Hz, 1H, Ala-NH), 5.99 (d, J = 6.6 Hz, 1H, Fmoc NH), 5.40 (br.s, 1H, H-4), 5.17 (d, J = 3.0 Hz, 1H, H-1), 5.11 (dd, J = 3.0, 12.0 Hz, 1H, H-3), 4.60 (m, 1H, H-2), 4.55 (m, 1H, Ala-CH), 4.40 (d, J = 7.2 Hz, 2H, Fmoc CH₂), 4.38 (m, 1H, Ala-CH), 4.32 (dd, J = 3.0, 6.6 Hz, 1H, Thr-α-CH), 4.23 (m, 3H, H-5, Thr-β-CH & Fmoc CH), 4.08 (d, J = 6.6 Hz, 2H, H-6 & H-6'), 2.15 (s, 3H, CH₃CO), 2.02 (s, 6H, CH₃CO), 2.00 (s, 3H, CH₃CO), 1.45 (s, 12H, tBu & Ala-CH₃), 1.40 (d, J = 7.2 Hz, 3H, Ala-CH₃), 1.17 (d, 3H, J = 6.0 Hz, Thr-CH₃); ¹³C NMR (150 MHz, CDCl₃): δ 171.85 (C=O), 171.83 (C=O), 171.02 (C=O), 170.78 (C=O), 170.65 (C=O), 170.62 (C=O), 168.32 (C=O), 156.13 (C=O), 143.98, 143.84, 141.48, 141.47, 127.95, 127.28, 125.33,
125.26, 120.22, 120.19, 98.62 (C-1), 82.49, 74.73, 68.22, 67.46, 67.41, 67.20, 62.21,
57.24, 49.29, 49.15, 47.78, 47.30, 29.89, 28.10 (3 x CH₃ of tBu), 23.13, 20.94, 20.86,
20.09, 18.25 (Ala-CH₃), 16.36; HRMS: m/z calcd for C₄₃H₅₅Na₄O₁₅ 891.3640, found
891.3650 [M+Na]+.

[O¹⁸] 3-Phenyl-2-oxopropanoic Acid (10). To a vacuum-dried phenylpyruvic acid 35
(0.1g, 0.609 mmol) under N₂ atmosphere was added 0.5 mL of 0.1N HCl in anhydrous
THF and H₂O¹⁸ (1.0 g, 49.95 mmol). The mixture was stirred at room temperature and
the O¹⁸-exchange was complete in 15 minutes (monitored by ESI-MS). The mixture was
concentrated under reduced pressure in a N₂-flushed rotary evaporator and the residual
solvent was removed in high vacuum to afford 10 as a light yellow amorphous solid
(0.087 g, 84%). ¹H NMR (400 MHz, DMSO-d₆): δ 13.21 (br.s, 1H, COOH), 9.26 (br.s,
1H, HC=COH), 7.75 (d, J = 7.6 Hz, 2H, aromatic), 7.34 (t, J = 7.6 Hz, 3H, aromatic),
7.24 (t, J = 7.2 Hz, 1H, aromatic), 6.40 (s, 1H, HC=OH); ¹³C NMR (100 MHz,
DMSO-d₆): δ 166.4 (C=O), 141.9, 135.0, 129.3, 128.3, 127.2 (C=OH), 109.5 (C=OH);
ESI-MS: phenylpyruvic acid was decarboxylated at 320 °C to give 2-phenylethanal,
m/z calculated for C₈H₈ONa 145.1, found: 145.1 [M+Na]+.

N-(2-Phenylethyl)phenylacetamide (55). A vacuum-dried mixture of O¹⁸-labeled
phenylpyruvic acid 10 (0.063 g, 0.370 mmol) and phenethyl hydroxyl amine oxalate salt
9 (0.081 g, 0.444 mmol) was dissolved in anhydrous MeOH (8 mL). The resulting
mixture was stirred at 35-40 °C under N₂ atmosphere. After 18 h, an aliquot of the
reaction mixture was analyzed by mass spectroscopy (ESI-MS). Excess solvent was removed under reduced pressure. The crude material was purified by flash column chromatography on SiO\(_2\) (9 x 3.5 cm) using MeOH-CH\(_2\)Cl\(_2\) (1:99) to furnish 55 as a light yellow solid. Yield 0.045 g (51%); \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.35-7.16 (m, 8H), 7.02 (d, \(J = 6.4\) Hz, 2H), 5.45 (s, 1H), 3.54 (s, 2H), 3.46 (q, \(J = 6.4\) Hz, 2H), 2.72 (t, \(J = 6.8\) Hz, 2H); ESI-MS: \(m/z\) calcd for C\(_{16}\)H\(_{18}\)NO 240.1, found: 240.3. Similarly, compound 54 (0.076 g, 0.457 mmol) was converted to amide 55 under the same reaction conditions. Yield: 0.045 g (41%); silica gel TLC \(R_f = 0.70\) (MeOH-CH\(_2\)Cl\(_2\) = 1:9); \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.34-7.16 (m, 8H), 7.02 (d, \(J = 6.8\) Hz, 2H), 5.45 (s, 1H), 3.53 (s, 2H), 3.45 (q, \(J = 6.4\) Hz, 2H), 2.72 (t, \(J = 7.2\) Hz, 2H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 171.15, 138.83, 134.94, 129.62, 129.16, 128.85, 128.74, 127.47, 126.58, 44.00, 40.86, 35.62; ESI-MS: \(m/z\) calcd for C\(_{16}\)H\(_{18}\)NO 240.1, found: 240.3. The \(^1\)H and \(^{13}\)C NMR of 55 from either route were found identical to those reported in the literature.\(^1\)

![structure](image)

\([O^{18}]\) \(N\)-(2-Phenylethyl)phenylacetamide (58). To a stirred solution of benzyl cyanide 56 (55 \(\mu\)L, 0.477 mmol) in anhydrous THF (0.5 mL) was added H\(_2\)O\(^{18}\) (0.478 g, 23.9 mmol) dropwise under N\(_2\) atmosphere. HCl (g) was bubbled into the reaction mixture for 10 minutes. The resulting mixture was refluxed at 50 °C for 12 h under N\(_2\) atmosphere. The desired mixture was refluxed from 50 °C for 12 h under N\(_2\) atmosphere. The desired \(O^{18}\)-labeled phenylacetic acid was detected by ESI-MS (\(m/z = 163.15\) [M+Na]\(^+\)). The mixture was concentrated under reduced pressure in a N\(_2\)flushed rotary evaporator. The residual solvent was removed in high vacuum to afford the crude product 57 (0.070 g) which was used in the next step without further purification. To a well-
stirred solution of 57 (0.070 g) in anhydrous CH₂Cl₂ (2 mL) was added freshly distilled dicyclohexylcarbodiimide (0.095 g, 0.460 mmol), phenethylamine 15 (0.060 mL, 0.476 mmol) and DIPEA (0.16 mL, 0.968 mmol). The resulting mixture was stirred at room temperature for 36 h under N₂ atmosphere. ESI-MS of the reaction mixture revealed labeled amide 58 with $m/z = 242.4$ [M+H]$^+$ ($m/z$ calcd for $C_{16}H_{18}N_{18}O$ 242.1).
References


CHAPTER 3

PROGRESS TOWARDS THE SOLID PHASE SYNTHESIS OF $\alpha$-KETO ACIDS AND $N$-HYDROXYLAMINES

3.1 Introduction

$\alpha$-Keto acids and $N$-hydroxylamines undergo a facile decarboxylative condensation that enables the coupling of unprotected peptide fragments at the newly created amide bond.\cite{1,2} Because the reaction promises to be free from amino-acid specific restriction at the ligation site,\cite{1} it is considered an attractive complement to the NCL\cite{3} and Staudinger ligation.\cite{4} The above-mentioned ligation fragments are obtained by synthetic transformations conducted in solution phase and currently practical for small targets.\cite{1,2,5,6} In contrast, larger peptide segments are expected to be inherently tedious and unworkable to be accomplished in solution phase and tend to be prepared by solid phase methods.\cite{7} Whilst the assembly of the peptide chain on the resin can be readily achieved by routine solid phase peptide synthesis (SPPS), there is limited precedence for the introduction of the desired functionality at the C- and N-termini on solid phase. The work reported by Fukuzumi and Bode,\cite{8} and Ju and Bode\cite{9} are the first to tackle this issue.
Initial attempts to generate the N-terminal hydroxylamine peptide on solid support involved the use of the N-sulfonyloxaziridine reagent as related by Fukuzumi and Bode (Scheme 1). Following assembly of the peptide on the resin and deprotection of the N-terminus, the reagent captures the terminal amine using a pendant aldehyde group and the neighboring oxaziridine moiety oxidizes the resulting imine to the nitrone. Hydroxyaminolysis of the nitrone provides the hydroxylamine moiety which is then reacted to the α-ketoacid and finally cleavage of the peptide from the resin. Several pentapeptides having variable N-terminal residues were assembled on the Rink amide MBHA resin following Fmoc/tBu protocols. After deprotection of the N-terminus, the peptide was oxidized with (S)-N-sulfonyloxaziridine and converted to the hydroxylamine upon hydroxyaminolysis. The efficiency of this key transformation could not be directly assessed because the hydroxylamine was ligated with α-keto acid prior to cleavage. The isolated yields of the ligation products ranged from 17 to 22%.

The polymer-supported synthesis of keto acid or its precursor, peptide cyanoketophosphorane, takes advantage of the ylide chemistry that has been previously developed in solution phase by Wasserman.\textsuperscript{10} Weik and Rademan\textsuperscript{11} generated the obligatory ylide functionality on-resin by treatment of the polystyrene-bound triphenyphosphane with bromoacetonitrile under microwave irradiation, and then triethylamine (Scheme 2A). In the work reported by Ju and Bode,\textsuperscript{9} a tetrahydrothiophene attached to a four linker spacer that terminates in the carboxyl group served as the precursor of the sulfur ylide (Scheme 2B). This thiophene-derived linker was anchored on the Rink amide MBHA resin and then alkylated.\textsuperscript{9} The preferred loading of amino acids on polystyrene-bound phosphorus ylide was with EDCI/DMAP and requires exclusion of moisture for efficient reaction.\textsuperscript{11} In contrast, the solid-bound sulfur ylide offers a wider choice of coupling agents for loading the first residue.\textsuperscript{9} After loading of the C-terminal residue, chain elongation was executed following Fmoc/tBu strategy.\textsuperscript{9,11}

Subsequent oxidation of the ylide provides the reactive α,β-diketonitriles that are easily converted to keto acids under aqueous conditions. Unlike the polystyrene-bound amino peptide ylide,\textsuperscript{11} the presence of spacer atoms between the sulfur ylide and the polymer matrix allows the recovery of intact peptide sulfur ylide that can be stored, and then oxidized to the keto acid as needed.\textsuperscript{9} The isolated yields of the tetrapeptides assembled on the sulfur-based ylide ranged from 33\% to 52\%.\textsuperscript{9} These side-chain unprotected tetrapeptide sulfur ylides were rapidly converted to the keto acids using Oxone and after ligation with hydroxylamine peptide provided the penta- and hexapeptide products in yields of 27\% to 45\%.\textsuperscript{9}
Scheme 2. (A) On-resin Synthesis of Peptide Cyanoketophosphorane and (B) Peptide Keto Acids via the Sulfur Ylide.

The use of (S)-N-sulfonyloxaziridine reagent and phosphorus and sulfur ylides demonstrates the feasibility of solid phase synthesis of the ligation fragments.\textsuperscript{8,9,11} These approaches however require synthesis of new advanced intermediates or specially designed resin linkers.\textsuperscript{9,11} The available solid-phase peptide synthesis platforms are varied enough that it is conceivable that the modified amino acid residues used in the solution phase studies, that is the \(N\)-hydroxylamine amino acid and amino acid cyanoketophosphoranes, can be incorporated into a suitable solid phase technique with minor changes. From this perspective, the synthesis of the required ligation fragments,
therefore, entails the coupling of the modified amino acids at the start or at the end of the synthesis to endow the C- and N-terminus of the peptide with the desired functionality. Depending on the manipulations carried out on the peptidyl resin, the desired precursors may be obtained as shown in Scheme 3. Displacement of the peptide from the resin with the amino acid cyanoketophosphorane will generate the peptide cyanoketophosphoranes (Scheme 3A). On the other hand, capping the N-terminus with amino acid N-hydroxylamine and cleavage will provide the peptide hydroxylamine (Scheme 3B). This approach greatly simplifies the preparation of the required fragments on-resin. This study evaluates the synthesis of the ligation fragments on solid support by incorporation of the modified amino residues on existing solid phase technologies.

Scheme 3. Solid Phase Synthesis of Ligation Fragments: (A) Peptide Cyanoketophosphorane and (B) Protected Peptide Hydroxylamine.
3.2 Solid Phase Synthesis of Peptide Cyanoketophosphoranes

The Ellman “safety catch” 4-sulfamylbutyryl linker allows modification of the C-terminus with various nucleophiles at the cleavage step.\textsuperscript{12} This linker, introduced in 1996, is stable to the conditions commonly encountered in solid phase peptide synthesis\textsuperscript{13-16} and with much improved reactivity than its aryl sulfonamide predecessor.\textsuperscript{17} Upon alkylation of the sulfonamide nitrogen using iodoacetanitride or diazomethane, the linker becomes extremely susceptible towards nucleophilic attack resulting in the incorporation of the nucleophile at the C-terminus and serves to release the peptide from the resin. Nucleophiles such as amines, amino acid esters, mercaptans and α-amino acid coumarin derivatives were successfully employed to displace the peptide from the resin.\textsuperscript{12,13,15} Amino acid cyanoketophosphoranes were anticipated to behave similarly, thus providing access to the precursors of α-keto acids, peptide cyanoketophosphoranes. The proposed strategy is illustrated in Scheme 4. The peptide is assembled on the resin, followed by activation of the resin, and finally displacement with the desired amino acid cyanoketophosphorane.

Fmoc-protected glycine, alanine and proline were selected for coupling to the 4-sulfamylbutyryl PS resin.\textsuperscript{12} The amino acids were coupled to the resin using \textit{in situ} activation with PyBOP (3-4 eq with respect to the available amine on resin) in the presence of DIPEA (5-8 eq) at -20 °C. The coupling of the first residue was initially attended by low loading efficiency. While some investigators have encountered similar results when employing such conditions for the loading of β-branched amino acids,\textsuperscript{14} others have been very successful in this regard especially for less hindered residues.\textsuperscript{13} Amino acyl fluorides were used to circumvent the poor loading\textsuperscript{14} and in some instances, they have been preferred for the convenience they afford\textsuperscript{15,16} as the reaction can be performed at ambient temperature in an SPPS vessel. But rather than synthesize the individual amino acyl fluoride,\textsuperscript{18} efforts were focused on the optimization of the coupling conditions. Better coupling yields were obtained when higher equivalents of the amino acids (4 eq), PyBOP (4 eq), and DIEA (8 eq) were used, the volume of solvents was kept to a minimum,\textsuperscript{19} and the solvents and DIEA were dried using MS 4Å. It was also important to pre-swell the resin\textsuperscript{16} to solvate the resin matrix for better reagent permeation.\textsuperscript{7} Equally important was the use of solvents and reagents free from amines that could cleave off the Fmoc-group\textsuperscript{20} or promote racemization.\textsuperscript{21} Double couplings were performed to ensure high loading efficiency. Shown in Table 1 are the optimized conditions for loading of the amino acids on the resin with the exception of proline.
Table 1. Loading of the Fmoc-Protected Amino Acids on the Resin

<table>
<thead>
<tr>
<th>Fmoc-Amino Acids</th>
<th>Coupling Conditions</th>
<th>Loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Glycine (4 eq)</td>
<td>PyBOP (4 eq), DIPEA (8 eq), DMF, -20 °C, 8 h, and r.t. 16 h</td>
<td>4 63-75%</td>
</tr>
<tr>
<td>2 Alanine (4 eq)</td>
<td>PyBOP (4 eq), DIPEA (8 eq), CHCl₃, -20 °C, 8 h</td>
<td>5 80%</td>
</tr>
<tr>
<td>3 Proline (3 eq)</td>
<td>PyBOP (3 eq), DIPEA (5 eq), CHCl₃, -20 °C, 8 h</td>
<td>6 26%</td>
</tr>
</tbody>
</table>

The activation of the resin was carried out in the presence of iodoacetonitrile and DIPEA for extended periods in darkness as reported in the literature (Scheme 5). However, there is no analytical method available to monitor the progress of this reaction although studies indicate alkylation of the sulfonamide tend to proceed to completion. Hence after activation, the resin was treated immediately with alanine cyanoketophosphorane 7. Displacement of proline 6 from the resin using one equivalent of nucleophile 7 in dry THF afforded a crude material whose amount was less than the theoretical yield. MS analysis of the crude material indicated that the putative product 10 was formed (m/z calc’d for C₄₃H₃₉N₃O₄P 692.3, found: 692.1 [M+H]+). Attempted purification of the material by passing through a plug of silica gel did not result in the isolation of the desired compound. The displacement of glycine 4 from the resin tells a strikingly similar story as that for proline. Using one equivalent of the nucleophile in dry THF and a reaction time of 5 h, the crude material obtained was less than the theoretical yield. MS analysis showed that the putative product 8 was present in the crude material (m/z calc’d for C₄₀H₃₅N₃O₄P 652.2, found: 652.3 [M+H]+). Purification of the material by
preparative TLC did not furnish the desired peptide. Performing the same reaction using three equivalents of the nucleophile at 35-40 °C for 24 h in DMF or displacement in the presence of HOBT and DIPEA gave similar results. Displacement of alanine 5 from the resin was no different from the first two examples. The putative product 9 can be detected by MS analysis (m/z calc’d for C_{41}H_{37}N_{3}O_{4}P 666.2, found: 666.3 [M+H]^+) and the mass of the recovered material was less than the theoretical yield. No attempt at this point was made to purify the crude material.

Scheme 5. Activation and Displacement of the Amino Acyl Resin with Amino Acid Cyanoketophosphorane.

An authentic dipeptide, Fmoc protected-Gly-Ala-cyanoketophosphorane 11 was prepared (Scheme 6) to aid in the localization of the putative product on TLC plates. The standard compound was prepared from Fmoc-glycine 1 and alanine cyanoketophosphorane 7 following standard coupling procedures (Scheme 6). The desired dipeptide 11 was obtained in 40% yield together with the piperidine adduct 12 in 35% yield. Co-chromatography of the authentic peptide with the crude material revealed a faint spot corresponding to the Fmoc-Gly-Ala-cyanoketophosphorane 11. Based from these results, it appears that the displacement of glycine from the resin does not proceed to a significant extent because of acute steric encumberance at the reaction center.
Presumably the same is true for the more hindered alanine and proline. Considering that the solution phase preparation of the authentic product also provided the piperidine adduct to such significant degree, it is conceivable that the same side reaction can occur in the solid phase. In conclusion, this approach was not pursued further.


### 3.3 Solid Phase Synthesis of N-Hydroxylamines

An alternative and simpler approach to accessing N-terminal peptide hydroxylamine would involve replacing the amino acid in the last coupling step with a suitably protected hydroxylamine, such as the $N,O$-bis-Fmoc amino acid hydroxylamine (Scheme 7). This compound, in turn, can be derived from the free amino acid hydroxylamine. Despite the established compatibility of the Fmoc group in solid phase peptide synthesis, the behavior of the bis-Fmoc modified hydroxylamine as a whole under those conditions remains unclear.
Conversion of the hydroxylamine 13 and 14 to the bis-Fmoc form 15 and 16 was achieved by treatment of the hydroxylamine with Fmoc-chloroformate (Fmoc-Cl) under Schotten-Bauman conditions in high yield of 83% and 80%, respectively (Scheme 6). The tert-butyl group was removed using TFA in CH$_2$Cl$_2$ to afford the N,O-bis-Fmoc hydroxylamines 17 and 18 which were used in the next step without further purification.

A solid phase model study was carried out on alanine hydroxylamine (Scheme 9). Alanine hydroxylamine 17 (2 eq.) was pre-activated for 2 minutes using PyBOP (2 eq.), HOBt (2 eq.) and DIEA (4 eq.) in a minimum volume of DMF before being loaded on the NH$_2$-alanine-Wang resin (0.150 g, loading = 0.6 mmol/g). The reaction mixture was
agitated by bubbling $N_2$ gas for a given time. The reaction was monitored using Kaiser test, a sensitive color test based on the reaction of the ninhydrin reagent with free amino groups on the resin.\textsuperscript{27} Kaiser test\textsuperscript{19} performed on the resin indicated that the coupling reaction was still incomplete after 4 hours, at which point the reaction mixture was washed away with DMF. A second coupling using 4 eq. of the hydroxylamine was carried out for 4 hours. Disappointingly, the coupling was still unfinished as judged from the Kaiser test. Because of the limited supply of the hydroxylamine it was decided to terminate the reaction and investigate if any hydroxylamine was coupled to the amino acid-resin. Spectrophotometric determination of the Fmoc-derived chromophore released from piperidine treatment of the peptidyl resin\textsuperscript{19} showed that 57\% of the available amino acid pre-loaded on the resin reacted with the hydroxylamine.

\begin{center}
\textbf{Scheme 9. Solid Phase Synthesis of $N,O$-bis-Fmoc Hydroxylamine Peptide.}
\end{center}

Notwithstanding the incomplete coupling reaction, a sample of the resin (20 mg) was cleaved with 0.2 mL of either 95:5 TFA/H$_2$O or 95:2.5:2.5 TFA/TES/H$_2$O under N$_2$ atmosphere. In the case of TFA/H$_2$O-mediated cleavage, only one strongly UV active spot was identified in TLC using 1:1 hexane/ethyl acetate. That same UV active spot was also identified in TFA/TES/H$_2$O- mediated cleavage. Spectral analysis ($^1$H NMR and ESI-MS) of the unpurified peptide did not indicate the presence of the putative product.
Undeterred by this setback, a model reaction between 18 and 21 was carried out to shed light on the failed attempt to obtain the target compound (Scheme 10). Reaction of 18 and 21 was mediated by the coupling agent PyBOP or HBTU in the presence of HOBt and collidine and carried out in anhydrous DMF/CH₂Cl₂. The reaction afforded the desired product 22 in 31% yield. Although steric strain appears to be a primary contributing factor to the low yield, other factors, such as competing side reactions, could not be excluded just yet. Polymer-bound FmocO-succinimidyl, which is structurally similar to bis-Fmoc amino acid hydroxylamine, was reported to be an effective Fmoc-donating agent to amino acids on the solid phase. To determine if the N,O-bis-Fmoc alanine hydroxylamine behaves in a similar way, equimolar amounts of 18 and 21 were mixed under the literature reported conditions of acetone/water/potassium carbonate or the model reaction conditions consisting of DMF/CH₂Cl₂/collidine but without the coupling agent and HOBt additive (Scheme 8). In both conditions, the starting materials remained unconsumed and no new UV active spot was observed in TLC even after stirring for 24 hours. As far as these observations show, the bis-Fmoc amino acid hydroxylamine is stable and the Fmoc groups do not exchange to give 23 and 24, at least under the conditions used for the coupling reactions.
To help resolve the identity of the UV absorbing compound obtained from the cleavage of the incompletely capped amino acid-resin, the crude material, about 11 mg, was purified by flash column chromatography on silica gel to give 2 mg of white solid, equivalent to a yield of 33% based on the amount of hydroxylamine coupled to the amino acid. Spectral analysis of this compound showed the characteristic signals of the Fmoc group. The exact identity of the compound could not be ascertained at this point because mass spectral analysis was inconclusive. The melting point of the compound (204-205 °C) did not correspond to either FmocNHOH (mp 164.5-167.5 °C)\textsuperscript{30} or FmocNHOFmoc (mp 129 °C\textsuperscript{23} or 159.5-161 °C\textsuperscript{30}). What is clear is that the Fmoc group becomes separated from the N-terminal residue during the cleavage step.
3.4 Conclusion

The recently reported methods for the preparation of $N$-hydroxylamines and $\alpha$-keto acids or cyanoketophosphoranes on solid support require the multi-step synthesis of reagents to effect the desired functionality on-resin. These were the $N$-sulfonyolxaziridine to oxidize the terminal amine on resin and the thiophene-based linker for the sulfur-based ylides. These methods, despite offering generality, still require further developments. Alternatively, the ligation fragments may be obtained by incorporation of the amino acid residues bearing the necessary modification on the peptidyl resin. This approach can be interfaced with existing solid phase techniques and simplifies the synthesis scheme.

Unfortunately, the preparation of the ligation fragments for decarboxylative condensation on solid support using the proposed alternative methods proved to be more challenging than anticipated. Despite the rigorous precautions observed on the reaction conditions for the loading of the amino acid to the safety catch linker, the loading efficiency obtained was 80% at best. The displacement of the activated aminoacyl from the activated safety catch linker with the amino acid cyanoketophosphorane as the nucleophile proceeded only as a background reaction presumably due to high steric hindrance. A variety of displacement conditions examined failed to overcome this limitation. Employing the least sterically hindered amino acid, glycine, did not improve the yields.

The solid phase synthesis of the $N$-hydroxylamines encountered a similar fate as that for the cyanoketophosphoranes. The synthesis of the required bis-Fmoc
hydroxylamines were readily achieved from the corresponding free hydroxylamine in a high yielding two-step process. The coupling of the bis-Fmoc hydroxylamine to the amino acyl Wang resin was accomplished with moderate coupling efficiency despite longer reaction times, higher equivalents of the N,O-bis-Fmoc hydroxylamine, and two coupling cycles. Cleavage of the dipeptidyl-resin bearing the N-terminal hydroxylamine was plagued by the loss of the Fmoc group. Although it is possible to introduce the protected hydroxylamine on a polymer-support, it requires an alternative protecting group strategy and further examination of coupling conditions for the efficient capping of the N-terminus.
Experimental Section

General Information

Materials. Fmoc Ala-preloaded Wang resin (substitution: 0.6 mmol/g) and 4-sulfamylbutyryl aminomethyl resin (substitution: 0.9 mmol/g) was procured from Novabiochem. Amino acids and HOBT were purchased from Chem-Impex International and PyBOP was acquired from Acros Organics. All other fine chemicals were sourced from the following commercial suppliers: Acros Organics, Alfa Aesar, Fisher Scientific and Sigma-Aldrich. CHCl₃ and ICH₂CN were passed through a basic alumina plug purchased from Sigma-Aldrich (product #: 265497, pH = 9.4-10.1) and DMF (Acros organics) and DIPEA (Fisher Scientific) are peptide synthesis quality. All solvents used for peptide synthesis were dried and stored over MS 4Å prior to use. Anhydrous CH₂Cl₂ was freshly distilled over CaH₂. Extra dry MeOH was used as received.

Solid Phase Peptide Synthesis. Peptides were manually assembled on Fmoc Ala-preloaded Wang resin or the 4-sulfamylbutyryl aminomethyl resin. The reactions were performed either in a 20-mL plastic syringe reactor cartridge or 15-mL scintillation vial. Agitation for reactions carried out in the reactor cartridge was provided by a stream of N₂ whereas gentle stirring using a magnetic bar was performed for reactions in vials to avoid resin attrition. The following amino acids were used: Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Gly-OH. Double or triple deprotection and couplings were performed as indicated by the Kaiser test. Washing steps were carried out manually by shaking the reactor cartridge anchored on the vacuum manifold for 5 min/ wash under N₂ atmosphere.
Spectral Analyses. Spectrophotometric determination of the amino acid loading was performed on Thermo Electron UV-Vis spectrometer and the data processed using VISIONpro SOFTWARE V4.00. Yields are based on the resin loading provided by the manufacturer. Proton and carbon nuclear magnetic resonance spectra (\(^1\)H NMR and \(^{13}\)C NMR) were recorded on either INOVA-600 (\(^1\)H NMR 600 MHz; \(^{13}\)C NMR 150 MHz) or Varian VXR-400 (\(^1\)H NMR 400 MHz; \(^{13}\)C NMR 100 MHz) spectrometers with solvent resonance as internal standard (MeOH-\(d_4\): \(^1\)H NMR at \(\delta\) 3.31, \(^{13}\)C NMR at \(\delta\) 49.15; CD\(_3\)CN: \(^1\)H NMR at \(\delta\) 1.94). The \(^1\)H NMR data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz, integration, and assignments. Low resolution mass spectra were taken on Esquire-LC electrospray ionization (ESI) mass spectrometer operated in the positive ion mode.

Loading of the First Amino Acid to the Sulfonamide Resin.
To a 15-mL scintillation vial was added the sulfonamide resin (0.200 g) and pre-swollen in dry CHCl\(_3\) (3 mL) for 1 h under N\(_2\) atmosphere. The solvent was removed up to the resin bed and the resin washed twice with CHCl\(_3\) (1.0 mL). To this swollen resin was added Fmoc-amino acid and DIPEA in a minimum volume of CHCl\(_3\) (approximately 1 mL), and stirred for 10 min at room temperature and then 20 min at -20 °C. A solution of PyBOP in CHCl\(_3\) (approximately 1 mL) was added and stirring was continued for 8 h at –20 °C. For coupling with Fmoc-glycine, DMF was employed for pre-swelling and during reaction, and the mixture was stirred further for 16 h at ambient temperature following the 8 h coupling period at -20 °C. The reaction mixture was transferred to a 20-mL
reactor cartridge and washed with CHCl₃ (3 x 5 mL) and the liquid drained with the aid of vacuum. The resin was subjected to a second coupling cycle. The resin was air-dried for a given time and the cake was collected and dried in vacuum.

**Determination of the Loading of the First Residue**

Two 15-mL scintillation vials were prepared containing the dried Fmoc-amino acid resin in the amount corresponding to 1 μmol with respect to Fmoc (approximately 1 mg). Freshly prepared 20% piperidine in DMF (3 mL) was added to each vial and the resin suspension was agitated from time to time for 2 h. The resin suspension was transferred to two 10 mm matched quartz cuvettes and the resin was allowed to settle before the absorbance was taken at 290 nm. Blank reading was performed on 20% piperidine in DMF. The resin loading was calculated according to equation 1 that is based on extinction coefficient = 5253 M⁻¹ cm⁻¹.¹⁹

\[
\text{Loading (mmol/g)} = \frac{\text{Absorbance of sample}}{\text{mg sample} \times 1.75}
\]  

**Representative Activation and Displacement of the Amino Acyl Sulfonamide Resin.**

The Fmoc-alanyl sulfonamide resin (0.288 g, loading = 0.534 mmol/g) was pre-swollen in NMP (4 mL) in reactor cartridge under N₂ atmosphere and washed twice with NMP (2 x 4 mL), taking care to drain NMP to the top of the resin bed with the aid of suction. To the swollen resin was added NMP (1 mL), DIPEA (0.26 mL, 1.54 mmol) and ICH₂CN (0.28 mL, 3.85 mmol). The mixture was agitated for 24 h with the exclusion of light. The
resin was washed successively with NMP (4 x 3 mL), CH₂Cl₂ (3 x 3 mL) and THF (3 x 3 mL). After drying the resin for a period of time under N₂ stream, the resin was transferred to a 15-mL scintillation vial. The resin was pre-swollen in THF (3 mL) for 1 h under N₂ atmosphere. The liquid was drained off to the top of the resin bed and replaced with fresh THF (1 mL). To the pre-swollen resin was added freshly prepared alanine cyanoketophosphorane 7 (0.067 g, 0.18 mmol) and the mixture was stirred for 24 h. The mixture was filtered through a reactor cartridge and washed with CH₂Cl₂ (20 mL). The filtrate was extracted with 1% aqueous citric acid (1 x 10 mL), saturated NaCl (1 x 10 mL) and the aqueous layers were back-extracted with CH₂Cl₂ (1 x 10 mL). The pooled organic layers were dried (Na₂SO₄), filtered, and concentrated to dryness under reduced pressure to afford a light yellow film (0.041 g). ESI-MS: m/z calc’d for C₄₁H₆₇N₉O₄P 666.3, found: 666.3 [M+H]⁺.

Fmoc-protected Glycylalanine Cyanoketophosphorane (11). Fmoc-protected glycine, (0.075 g, 0.25 mmol), alanine cyanophosphorane derivative 7 (0.141 g, 0.38 mmol), HBTU (0.191 g, 0.51 mmol), and HOBt (0.068 g, 0.51 mmol) were taken together and dried for 15 min in high vacuum before the addition of 1:1 anhydrous DMF-CH₂Cl₂ (5 mL), and the resulting mixture was stirred for 15 min at ambient temperature. TMP (0.037 mL, 0.28 mmol) was added under N₂ atmosphere and stirring was continued at ambient temperature. The reaction was monitored by TLC and appeared complete in 24 h. The reaction mixture was diluted with CH₂Cl₂ (5 mL) and successively washed with 1% aqueous citric acid (1 x 3 mL), saturated NaHCO₃ (1 x 3 mL) and saturated NaCl
solution (1 x 3 mL). The organic phase was dried (MgSO₄), filtered, and the filtrate was concentrated to dryness under reduced pressure. The crude material was purified by flash column chromatography on SiO₂ (8 x 3.5 cm) using step-wise gradient elution with ethyl acetate-hexanes (4:6 and then 9:1) to afford the desired product as a colorless film. Yield: 0.066 g (40%); silica gel TLC Rf = 0.20 (ethyl acetate-hexanes = 9:1); ¹H NMR (400 MHz, MeOH-d₄): δ 7.74 (d, J = 7.6 Hz, 2H), 7.65-7.51 (m, 17H), 7.32 (t, J = 7.2 Hz, 2H), 7.23 (t, J = 6.8 Hz, 2H), 4.92 (d, J = 7.2 Hz, 1H), 4.29-4.40 (m, 2H), 4.13 (t, J = 6.8 Hz, 1H), 3.74 (s, 2H, Gly α-CH₂), 1.42 (d, J = 6.8 Hz, 3H, Ala-CH₃); ¹³C NMR (100 MHz, MeOH-d₄): δ 196.80, 171.58, 158.97, 145.40, 145.26, 142.63, 142.60, 134.92, 134.82, 134.79, 134.76, 130.64, 130.51, 128.90, 128.87, 128.29, 126.43, 126.36, 124.11, 123.17, 121.03, 68.29, 52.89, 48.39, 44.83, 39.01, 18.38; ESI-MS: m/z calc’d for C₄₀H₃₄N₃O₄P 674.2, found: 674.3 [M+Na]⁺; and Fmoc-protected Glycine Piperidine Amide (12). Colorless solid; Yield: 0.058 g (35%); silica gel TLC Rf = 0.13 (ethyl acetate-hexanes = 4:6); mp = 124-125 °C; ¹H NMR (400 MHz, CDCl₃): δ 7.77 (d, J = 7.6 Hz, 2H), 7.63 (d, J = 7.6 Hz, 2H), 7.41 (t, J = 7.6 Hz, 2H), 7.32 (t, J=7.2 Hz, 2H), 5.98 (s, 1H), 4.38 (t, J = 7.6 Hz, 2H), 4.25 (t, J = 7.2 Hz, 1H), 4.04 (d, J = 4.0 Hz, 2H), 3.60 (d, J = 5.2 Hz, 2H), 3.33 (t, J = 5.6 Hz, 2H), 1.66 (d, J = 4.8 Hz, 2H), 1.62-1.54 (m, 4H); ¹³C NMR (100 MHz, CDCl₃): δ 166.04, 156.34, 144.05, 141.39, 127.81, 127.20, 125.34, 120.09, 67.24, 47.26, 45.55, 43.32, 42.75, 26.31, 25.56, 24.49; ESI-MS: m/z calc’d for C₂₂H₂₄NaN₂O₃ 387.2, found: 387.3 [M+Na]⁺.

\[ \text{Fmoc} - \text{O} \quad \text{N} \quad \text{O} \quad \text{OfBu} \]

\[ \text{N,O-bis-Fmoc Glycine Hydroxylamine tert-Butyl Ester (15).} \] To a well-stirred mixture of glycine hydroxylamine oxalate salt (0.037g, 0.19 mmol) and Na₂CO₃ (0.103 g, 97
mmol) in THF (1.5 mL) and water (1.5 mL) at 0 °C was added dropwise a solution of Fmoc-chloroformate (0.100 g, 0.39 mmol) in THF (1.0 mL). The mixture was stirred further for 20 min before being concentrated down to a small volume in rotary evaporator. The concentrate was diluted with ethyl acetate (3 mL) and washed with saturated NaHCO$_3$ (1 x 2 mL). The aqueous layer was back-extracted with ethyl acetate (1 x 3 mL) and the pooled organic layer was dried (anhydrous Na$_2$SO$_4$), filtered and concentrated to dryness. Purification of the crude material by flash column chromatography on SiO$_2$ (9 x 2.5 cm) using ethyl acetate-hexanes (9:1) afforded the desired product as a colorless solid. Yield: 0.096 g (83%); silica gel TLC $R_f$ = 0.35 (ethyl acetate-hexanes = 2:8); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.79 (dd, $J = 3.0$, 7.2 Hz, 4H), 7.66 (d, $J = 7.2$ Hz, 4H), 7.42 (q, $J = 6.6$ Hz, 4H), 7.32-7.30 (m, 4H), 4.57 (d, $J = 7.2$ Hz, 2H), 4.53 (d, $J = 7.2$ Hz, 2H), 4.38 (s, 2H, Gly $\alpha$-CH$_2$), 4.35 (t, $J = 7.8$ Hz, 1H), 4.32 (t, $J = 7.2$ Hz, 1H), 1.54 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.35, 156.29, 154.20, 143.48, 142.94, 144.45, 141.40, 128.21, 128.01, 127.41, 127.34, 125.35, 125.33, 120.30, 120.16, 83.13 (OC(CH$_3$)), 71.74, 69.46, 53.19 (Gly $\alpha$-CH$_2$), 46.94, 46.69, 28.22 (tBu); ESI-MS: $m/z$ calc’d for C$_{36}$H$_{33}$NaNO$_7$ 614.2, found: 614.3 [M+Na]$^+$. 

![Fmoc O N OtBu](image)

$N,O$-bis-Fmoc Alanine Hydroxylamine tert-Butyl Ester (16). To a well-stirred mixture of alanine hydroxylamine oxalate salt (0.040 g, 0.19 mmol) and Na$_2$CO$_3$ (0.103 g, 97 mmol) in THF (1.5 mL) and water (1.5 mL) at 0 °C was added dropwise a solution of Fmoc-chloroformate (0.100 g, 0.39 mmol) in THF (1.0 mL). The mixture was stirred further for 20 min before being concentrated down to a small volume in rotary evaporator. The concentrate was diluted with ethyl acetate (3 mL) and washed with
saturated NaHCO₃ (1 x 2 mL). The aqueous layer was back-extracted with ethyl acetate
(1 x 3 mL) and the pooled organic layer was dried (anhydrous Na₂SO₄), filtered and
concentrated to dryness. Purification of the crude material by flash column
chromatography on SiO₂ (10 x 2.5 cm) with ethyl acetate-hexanes (1:9) afforded the
desired product as a colorless foam. Yield 0.094 g (80%); silica gel TLC Rᶠ = 0.43 (ethyl
acetate-hexanes = 2:8); ¹H NMR (600 MHz, CDCl₃):  δ 7.77 (d, J =7.8 Hz, 4H), 7.62-7.65
(m, 4H), 7.39-7.42 (m, 4H), 7.29 (t, J = 7.2 Hz, 4H), 4.87 (apparently s, 1H), 4.46-4.60
(m, 4H), 4.33 (t, J = 7.8 Hz, 1H), 4.28 (t, J = 7.2 Hz, 1H), 1.50 (d, J = 7.2 Hz, 3H), 1.48
(s, 9H); ¹³C NMR (100 MHz, CDCl₃): δ 168.94, 156.71, 154.68, 143.56, 143.52, 143.13,
142.90, 141.48, 141.43, 141.42, 128.21, 128.19, 128.03, 127.42, 127.40, 127.35, 125.38,
125.34, 125.32, 120.30, 120.18, 82.79, 71.58, 69.38, 59.08, 46.98, 46.77, 13.91; ESI-MS:
m/z calc’d for C₃₇H₃₅NaNO₇ 628.2, found: 628.3 [M+Na]^⁺.

N,O-bis-Fmoc Glycine Hydroxylamine (17). To a stirred solution of N,O-bis-Fmoc
hydroxylamine 15 (0.053 g, 89.58 μmol) in CH₂Cl₂ at 0 °C was added TFA (1.79 mL,
24.10 mmol) dropwise. The reaction was allowed to come to room temperature and
appeared complete within 1.5 h. The mixture was concentrated to dryness. The crude
product was taken up in CH₂Cl₂ (5 mL) and concentrated to dryness and this process
repeated thrice to afford the desired product as a light green foam. Yield: 0.044 g (92%);
silica gel TLC Rᶠ = 0.44 (MeOH-CHCl₃ = 9:1 + 3 drops acetic acid/10 mL); ¹H NMR
(400 MHz, CDCl₃): δ 8.22 (br.s, 1H), 7.72 (dd, J = 4.0, 7.6 Hz, 4H), 7.58 (t, J =7.6 Hz,
4H), 7.36 (td, J = 2.8, 7.2 Hz, 4H), 7.27-7.22 (m, 4H), 4.52 (d, J = 7.2 Hz, 4H), 4.40 (s,
2H, Gly α-CH₂), 4.29-4.22 (m, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 172.70, 156.07,
154.16, 143.29, 142.82, 141.47, 141.45, 128.28, 128.09, 127.46, 127.40, 125.29, 125.17, 120.35, 120.21, 69.56, 52.12 (Gly α-CH2), 46.86, 46.67; ESI-MS: m/z calc’d for C_{32}H_{25}NaNO_7 558.2, found: 558.2 [M+Na]^+.

**N,O-bis-Fmoc Alanine Hydroxylamine (18).** To a stirred solution of N,O-bis-Fmoc hydroxylamine 16 (0.053 g, 0.088 mmol) in CH_2Cl_2 at 0 °C was added TFA (1.75 mL, 23.6 mmol) dropwise. The reaction was allowed to come to room temperature and appeared complete within 1.5 h. The mixture was concentrated to dryness. The crude product was taken up in CH_2Cl_2 (5 mL) and concentrated to dryness and this process repeated thrice to afford the desired product as a light yellow foam. Yield: 0.046 g (100%); silica gel TLC R_f = 0.69 (ethyl acetate-hexanes = 1:1); ^1H NMR (600 MHz, CDCl_3): δ 7.73-7.76 (m, 4H), 7.58-7.62 (m, 4H), 7.75 (t, J = 8.4 Hz, 4H), 7.28 (t, J = 6.0 Hz, 4H), 4.90 (s, 1H), 4.54-4.62 (m, 4H), 4.48 (s, 1H), 4.30 (t, J = 6.6 Hz, 1H), 4.25 (t, J = 7.2 Hz, 1H), 1.51 (d, J = 6.6 Hz, 3H); ^13C NMR (100 MHz, CDCl_3): δ 175.77, 156.42, 154.67, 143.45, 143.28, 143.00, 142.75, 141.50, 141.48, 141.45, 141.43, 128.25, 128.23, 128.06, 128.03, 127.44, 127.40, 127.36,125.26, 125.21, 125.16, 120.33, 120.32, 120.18, 71.77, 69.40, 58.07, 46.88, 46.77, 13.62; ESI-MS: m/z calc’d for C_{33}H_{27}NaNO_7 572.2, found: 572.2 [M+Na]^+.

**Representative Solid Phase Coupling of N,O-bis-Fmoc Alanine Hydroxylamine.**

Fmoc-Ala-Wang resin was placed in a plastic syringe reactor cartridge. The resin was treated with 20% piperidine in DMF (1.5 mL) for 5 min with agitation provided by a
stream of N₂ and repeated with fresh piperidine solution (1.5 mL) for 5 min. The reagent was drained off and the resin washed with DMF (3 x 3 mL). This procedure was repeated if the Kaiser test was negative. N,O-bis-Fmoc alanine hydroxylamine 18 (0.099 g, 0.18 mmol) was preactivated with PyBOP (0.094 g, 0.18 mmol), DIPEA (0.059 mL, 0.36 mmol) and HOBt (0.024 g, 0.18 mmol) in DMF (0.6 mL) for 2 minutes before being added to the deprotected pre-loaded resin. The reaction was allowed to continue for 1 h. The resin was washed with DMF (3 x 4 mL), CH₂Cl₂ (3 x 4 mL), and finally MeOH (3 x 4 mL). The resin was air-dried for sometime and the cake was dried in vacuum to afford dipeptidyl resin 20. The amount of hydroxylamine that was successfully coupled to the amino acid-resin was estimated by dividing the absorbance reading at 290 nm by 2 before the value was plugged in to the equation: loading (mmol/g) = (absorbance)/(mg sample x 1.75). This is to take into account that there are twice as many Fmoc-derived chromophore released from the peptidyl resin.

**Solution Phase Synthesis of N,O-bis-Fmoc Alanylalanine Hydroxylamine (22).** N,O-bis-Fmoc alanine hydroxylamine 18 (0.050 g, 0.091 mmol), alanine tert-butyl ester hydrochloride salt 21 (0.025 g, 0.14 mmol), HBTU (0.038 g, 0.10 mmol), and HOBt (0.014 g, 0.10 mmol) were taken together and dried for 15 min in high vacuum before the addition of 25:75 anhydrous DMF-CH₂Cl₂ (2 mL), and the resulting mixture was stirred for 15 min at ambient temperature. TMP (0.027 mL, 0.20 mmol) was added under N₂ atmosphere and stirring was continued at ambient temperature. The reaction was monitored by TLC and appeared complete in 4 h. The reaction mixture was diluted with CH₂Cl₂ (5 mL) and successively washed with cold 1% aqueous citric acid (1 x 3 mL),
cold saturated NaHCO$_3$ (1 x 3 mL) and cold saturated NaCl solution (1 x 3 mL). The organic phase was dried (MgSO$_4$), filtered, and the filtrate was concentrated to dryness under reduced pressure. The crude material was purified by flash column chromatography on SiO$_2$ (9 x 2.5 cm) using ethyl acetate-hexanes (1.5:8.5) to afford the desired product as a white gum. Yield: 0.019 g (31%); silica gel TLC $R_f = 0.14$ (ethyl acetate-hexanes = 2:8); Characteristic signals in the $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.77 (d, $J = 7.2$ Hz, 4H), 7.60 (d, $J = 6.4$ Hz, 4H), 7.41 (t, $J = 7.2$ Hz, 4H), 7.32-7.28 (m, 4H), 4.61-4.54 (m, 3H), 4.49-4.38 (m, 2H), 4.31 (t, $J = 7.6$ Hz, 1H), 4.26 (t, $J = 6.8$ Hz, 1H), 1.50 (d, $J = 7.2$ Hz, 3H), 1.45 (s, 9H); ESI-MS: $m/z$ calc’d for C$_{40}$H$_{40}$NaN$_2$O$_8$ 699.3, found: 699.3 [M+Na]$^+$. 

**Fmoc-Decomposition Product.** Dipeptidyl resin 20 (0.020 g) was treated with TFA-TES-H$_2$O (95:2.5:2.5 v/v/v) or TFA-H$_2$O (95:5 v/v) (0.20 mL) with occasional agitation for 3.5 h under N$_2$ atmosphere. The resin was washed with the cleavage mixture (1.0 mL) and with liberal amount of CH$_2$Cl$_2$. The filtrate was concentrated to dryness under reduced pressure. The crude material was purified by flash column chromatography on SiO$_2$ using ethyl acetate-hexanes (25:75) to afford 2.0 mg of white amorphous solid. This mass is equivalent to a yield of 33% based on the amount of hydroxylamine coupled to the amino acid (average coupling efficiency of the *bis*-Fmoc hydroxylamine to the preloaded resin = 0.339 mmol/g). Silica gel TLC $R_f = 0.42$ (ethyl acetate-hexane = 1:1); mp = 204-205 °C; $^1$H NMR (400 MHz, MeOH-$d_4$): $\delta$ 7.80 (d, $J = 7.2$ Hz, 4H), 7.66 (d, $J = 7.2$ Hz, 4H), 7.39 (t, $J = 7.6$ Hz, 4H), 7.31 (t, $J = 7.6$ Hz, 4H), 4.31 (d, $J = 6.8$ Hz, 4H),
4.21 (t, $J = 7.2$ Hz, 2H); $^{13}$C NMR (150 MHz, MeOH-$d_4$): $\delta$ 158.6, 144.2, 141.4, 127.6, 126.9, 125.0, 119.7, 66.5, 47.2.
References

CHAPTER 4

THIOACID-SULFONAMIDE CHEMICAL LIGATION OF
GLYCOPEPTIDES

4.1 Introduction

The arylsulfonyl group, which includes \(\text{o-nitrobenzenesulfonyl (oNBS)}, \text{p-nitrobenzenesulfonyl (pNBS)}\) and \(\text{2,4-dinitrobenzenesulfonyl (dNBS)}\) (Figure 1), can be harnessed as a temporary protecting group for amines\(^1\) or a coupling agent for amide bond formation\(^4\) with thioacids depending on the reaction conditions employed. The three arylsulfonyl groups, originally introduced by Fukuyama and co-workers\(^1\), as \(\text{N-arylsulfonamides}\) have figured prominently in the selective alkylation of primary amines to secondary types bearing a variety of pendant groups. The protecting group is removed using either mercaptoacetic acid or thiophenol to furnish the secondary amine, which may be further reacted with an aldehyde to arrive at the tertiary amine.

\[\text{Figure 1. Structure of the Arylsulfonamide Groups.}\]
The dNBS has been extensively used for the conversion of \( N \)-arylsulfonamides, in the presence of cesium carbonate and a suitable reaction partner, to amides\(^4\), thioamides, ureas, and thioureas.\(^5\) Likewise, dNBS has been exploited in the chemical ligation of amino acids, peptide fragments, neoglycoconjugates\(^6\) and in three-component coupling reactions involving cyclic thioanhydride.\(^7\)

Scheme 1. Current Synthetic Scope of the dNBS-Mediated Coupling Reaction.
The dNBS-mediated formation of amide bond between sulfonamides and thioacids has considerable potential in glycopeptide synthesis. This particular ligation chemistry offers several advantages: (1) the amide bond formation remains efficient even when bulky amino acids are located at the ligation juncture, (2) good chemoselectivity in the presence of protic solvents, carboxyl and imidazole groups, (3) proceeds under mild conditions and short reaction times, and (4) the dNBS is extremely easy to introduce in solution phase. The chemistry can be employed to (1) conjugate a sulfonamide-modified glycan to amino acids and peptides bearing side-chain thioacid functionality (Scheme 2A) and (2) ligate glycopeptide fragments (Scheme 2B).

Scheme 2. (A) N-Glycosyl Amide from N-Glycosylsulfonamide and (B) a Glycopeptide from N-Glycopeptidylsulfonamide and Peptide Thioacid.
The conjugation of the glycan to peptides is an important step in the synthesis of glycoproteins and various synthetic approaches have been developed for the generation of the N-linkage. These include dehydrative coupling conditions employing various peptide coupling agents (Scheme 3A), the adaptation of the Staudinger Ligation (Scheme 3B), hydroxamate-glycosyl fluoride tandem (Scheme 3C), glycosyl aryltrihaloacetoimidate donors (Scheme 3D), and microwave-assisted amide formation between carboxylic acids and glycosyl isonitriles (Scheme 3E). Equally viable methods have been reviewed by Davis such as the Ritter reaction (Scheme 3F), isothiocynate-derivatized glycans (Scheme 3G), and silylated amide-glycosyl sulfoxide coupling partners (Scheme 3H). All this progress is highly commendable but limitations such as racemization, low to moderate yields, unnatural amide appendages whose removal require additional steps, substrate scope encompassing simple glycan donors and acceptors, use of highly toxic reagents requires the need for further developments. Recently, a reaction based on the sulfonamide and thioacid afforded neoglycoconjugates. A glycosyl donor in the form of N-glycosylsulfonamide, as illustrated in Scheme 1a, has the capacity to provide the highly sought β-configured glycosyl amides that include the N-linked glycopeptides/proteins. This approach may lend itself to the convergent assembly of this type of glycopeptide considering that the reaction is relatively unaffected by the steric bulk of the reacting fragments.
Scheme 3. Chemical Strategies for the Synthesis of N-Glycosyl Amides and Peptides. Reactions provided are for illustration purposes only and may have several variations that have not been included.
Harnessing the innate ligation efficiency of the sulfonamide-thioacid chemistry to access relevant glycopeptide targets would certainly require the preparation of longer peptide fragments. The straightforward synthesis of the requisite ligation fragments is therefore of high priority. The rapid adoption of this technique by other investigators is predicated on this aspect. To this end, a fewer number of steps for the functionalization of the ligation fragments and seamless integration of such manipulations into existing peptide technologies are highly desirable. On-resin synthesis has been the most expedient way of accessing those fragments and the successful synthesis of the N-peptidylsulfonamides and peptide thioacids on solid support will undoubtedly unleash the potential of this particular ligation chemistry to the fullest.

The on-resin sulfonation of peptides has been accomplished before by Miller and co-workers.\textsuperscript{18,19} \textit{o}-NBS and \textit{p}-NBS have been coupled to the N-terminus of a nascent peptide bound to Rink amide MBHA resin.\textsuperscript{18} In a variation of this strategy, a number of \textit{o}-NBS-amino acids were synthesized using Schotten-Baumann conditions and coupled to the resin-bound peptide.\textsuperscript{19} In both instances, the \textit{N}-arylsulfonyl group was used as a protecting group to aid in the controlled alkylation of the N-terminus, and thus was removed afterwards. The solid phase preparation of the \textit{N}-peptidylsulfonamide requires that the \textit{N}-arylsulfonyl group remain intact through the cleavage step in order to effect the desired amide bond formation with its ligation partner off-resin. This is a departure from the procedure developed by Miller and co-workers\textsuperscript{18,19} in which the arylsulfonyl group was used temporarily. However, there is no experimental precedence for the cleavage of the \textit{N}-peptidylsulfonamide from the resin. Apart from this, the preferred \textit{N}-arylsulfonyl
group in the ligation chemistry, the dNBS, has not been used for the N-terminal sulfonation of the peptide on-resin.

A number of methods have been developed for the synthesis of thioacids and thioesters on-resin but only those methods with the thioester functionality already formed at the start of the synthesis and provide the thioacid upon cleavage will be mentioned (Scheme 4). As such, these methods rely on Boc chemistry to assemble the peptide due to the labile nature of the thioester to the basic deprotection schemes of the Fmoc/tBu strategy. Blake and Li\textsuperscript{20} coupled the amino thioacid onto the 4-carboxymethylphenylbenzhydryl chloride to afford the linker 1 and chain extension was performed using Boc-amino acids. After peptide assembly, final deprotection and cleavage from the resin was achieved using the corrosive agent HF. The approach put forth by Crich and co-workers\textsuperscript{21} circumvented the necessity of preparing the individual thioacids for loading onto the resin and cleavage with HF. Instead, the fluorenylmethylthiol which has been previously used to generate the thioacids in solution phase,\textsuperscript{6} was modified into the N-[9-(thiomethyl)-9H-fluoren-2yl]succinamic acid linker and incorporated into the resin to give 2.\textsuperscript{21} As with the Blake methodology, assembly involved coupling of Boc-amino acids. Cleavage from the resin was simply effected with piperidine treatment.

A method that relies on the intrinsic nature of thioesters to undergo thiolysis to a greater extent than hydrolysis was developed recently\textsuperscript{22,23} The hydrothiolysis of the thioesters proceeds in solution readily but suffers significant reduction in yield (based on
HPLC) when the hydrothiolysis reaction was carried out on the resin-bound peptide.\textsuperscript{23} Use of PEG-based Chem Matrix resin 3 appeared to offer a high cleavage yield;\textsuperscript{24} this however, was based on spectrophotometric measurements of the resin before and after cleavage. Control of pH is essential to prevent the release of hydrogen sulfide at low pH.\textsuperscript{23}


The application of the thioacid-sulfonamide chemistry in glycopeptide synthesis and the preparation of the fragments were explored in this investigation. In particular, \textit{N}-glycosylsulfonamide was evaluated for whether or not it can act as a glycosyl donor of thioacids to provide the \textit{β}-configured glycosyl amides. Equally important, the \textit{N}-peptidylsulfonamides and C-terminal peptide thioacids were prepared to evaluate the suitability of the ligation chemistry in the presence of glycans, as well as, unprotected peptide functional groups. Integral to these efforts was the synthesis of the \textit{N}-peptidylsulfonamide on the solid phase to demonstrate the ease with which to access
longer peptide fragments and facilitate the adoption of the chemistry. The trityl thioesters were developed as precursors of the thioacids to lay the groundwork for using the trityl group in the solid phase synthesis of thioacids following the Fmoc/tBu strategy.

4.2 Conjugation of N-glycosylsulfonamide to Thioacids: Facile Synthesis of N-(β-D-Glucopyranosyl)-L-asparaginate

To demonstrate that an N-glycosylsulfonamide can serve as a glycosyl donor of thioacids, a model reaction consisting of N-glucosyl-2,4-dinitrobenzenesulfonamide and β-thioacid asparagine was performed to access the N-(Asn) glycoside. The β-thioacid asparagine 10, however, was not commercially available, and was synthesized in five steps starting from 9-fluorenylmethanol 4 (Scheme 5) following literature procedures.6,25

9-Fluorenylmethanol 4 was converted to tosylate 5 using tosyl chloride in the presence of pyridine as the base. Performing the said transformation using tosyl chloride, CH₂Cl₂, and K₂CO₃ did not afford the desired product. Displacement of the tosylate with potassium thioacetate provided thioester 6 in good yield. This was followed by reduction using DIBAL-H to the corresponding 9-fluorenylmethylthiol 7 in excellent yield. Thiol 7 was coupled to the commercially-available protected-aspartic acid 8 to give thioester 9. Selective Fmoc-deprotection of thioester 9 generated the desired thioacid 10 which was used in the next step as a crude material.
Thioacid 10 was condensed with  \( N-(2\text{-acetamido}-3,4,6\text{-O-acetyl}-2\text{-deoxy-}\beta\text{-D-glucopyranosyl})-2,4\text{-dinitrobenzenesulfonamide} \) 11 in the presence of cesium carbonate and DMF (Scheme 6). The reaction was completed in one hour with an isolated yield of 69% of the desired \( N-(\beta\text{-D-glucosyl})\text{asparagine derivative} \) 12. The observed yield was comparable with other methods such as the Staudinger ligation reaction involving glycosyl azides.\(^{11,12,14}\) The preservation of the \( \beta \)-configuration in the product is a valuable feature of this method in light of the fact that it is a crucial aspect of convergent synthesis.

Scheme 5. Synthesis of \( \beta \)-Thioaspartic Acid 10.

Scheme 6. \( N \)-Glycosylation of \( \beta \)-Thioaspartic Acid.
4.3 Solid Phase Synthesis of Ligatable N-Peptidylsulfonamides

A general strategy for the solid phase synthesis of ligatable N-peptidylsulfonamides is shown in Scheme 7. The peptide is assembled on the resin employing standard coupling conditions for Fmoc-amino acids. After assembly and Fmoc-deprotection of the N-terminus amino acid residue, the terminal amino group is reacted with 2,4-dinitrobenzenesulfonyl chloride (dNBS-Cl) to generate the resin bound N-peptidylsulfonamide. The peptide is cleaved off the resin using standard cleavage protocols that will preserve the dNBS moiety in the peptide product. This approach obviates the requirement for preparing the individual dNBS-amino acids.


4.3.1 Evaluation of Solid Phase Conditions for N-Terminal Sulfonation

A rapid screening of conditions useful for dNBS coupling was performed on NH$_2$-Ala-Wang resin. The coupling reaction was performed for 4 h and Kaiser tests$^{27,28}$ indicated the need for double couplings. The dNBS-alanine was cleaved from the resin following TFA treatment to give the dNBS-alanine and unreacted alanine as the primary products. The combination of dichloromethane and pyridine was found optimal for
introducing dNBS (Table 1, entry 2), the same conditions used in the solution phase as reported earlier, although the solid phase required higher equivalents of the 2,4-dinitrobenzenesulfonylchloride (4 eq) as expected. Other conditions that were explored such as CH$_2$Cl$_2$-DIPEA, DMF-pyridine, DMF-DIPEA, and CH$_2$Cl$_2$-pyridine-DMAP (0.1 eq) proved to be unsatisfactory. The results from the coupling conditions using DIEA are consistent with the observations of Fukuyama and co-workers who have noted that DIPEA promotes decomposition of the sulfonamide via intramolecular Meisenheimer complex.

### Table 1. On-resin 2,4-dNBS Modification of the N-terminus

<table>
<thead>
<tr>
<th>Entry</th>
<th>2,4-dNBS-Cl, equivalents$^d$</th>
<th>Solvent</th>
<th>Base</th>
<th>Ratio of 13:14$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4 (8)</td>
<td>CH$_2$Cl$_2$</td>
<td>py</td>
<td>84:16</td>
</tr>
<tr>
<td>2</td>
<td>4 (4)</td>
<td>CH$_2$Cl$_2$</td>
<td>py</td>
<td>86:14$^c$</td>
</tr>
<tr>
<td>3</td>
<td>4 (4)</td>
<td>CH$_2$Cl$_2$</td>
<td>DIPEA</td>
<td>67:33</td>
</tr>
<tr>
<td>4</td>
<td>4 (4)</td>
<td>DMF</td>
<td>py</td>
<td>30:70</td>
</tr>
<tr>
<td>5</td>
<td>4 (4)</td>
<td>DMF</td>
<td>DIPEA</td>
<td>29:71</td>
</tr>
<tr>
<td>6</td>
<td>4 (4)</td>
<td>CH$_2$Cl$_2$</td>
<td>py + DMAP$^d$</td>
<td>89:11</td>
</tr>
</tbody>
</table>

$^a$Values in parenthesis are the number of equivalents for the second coupling. $^b$The ratio was determined from the $^1$H NMR of the crude product using the integration of the corresponding α-H signal. $^c$Flash column chromatography on silica gel afforded 40% yield of compound 13 based on resin loading. $^d$Used 0.10 equivalents of DMAP.
4.3.2 Solid Phase Synthesis of N-Peptidylsulfonamides

We elected to apply this method toward the synthesis of a short segment of the tandem repeat comprising the ectodomain of MUC1. MUC1 is a heavily glycosylated transmembrane protein that is closely associated with cancers of epithelial origin and is a relevant target of glycopeptide-based anti-cancer therapeutics.29-31 The 20-amino acid-tandem repeat possesses several possible glycosylation sites at the threonine and serine residues found along the sequence NH$_2$-P$_1$DTRP$_7$G$_8$STAPPA$_{15}$G$_{16}$VTSA$_{20}$COOH.29 The tandem repeat is rich in β-branched amino acids providing only two ligation sites involving glycine, namely the P$_7$-G$_8$ and H$_{15}$-G$_{16}$ linkage. Neither an Ala-Ala linkage nor cysteine residues are part of the sequence. This effectively limits the available ligation sites accessible to Staudinger ligation, decarboxylative condensation, and the absence of cysteine would require a modified NCL approach. The sulfonamide-thioacid chemistry is particularly suited to this target as evidenced in the literature for its prodigious reactivity at hindered ligation sites.6

To show that the dNBS group can be coupled to any N-terminal residue on-resin, the solid phase synthesis of the last five amino acid residues at the C-terminus was performed in stages, i.e. starting from the dipeptide, then progressing to pentapeptides. The peptides were manually assembled on the Wang resin using Fmoc chemistry. Coupling of the amino acids was achieved using PyBOP and HOBt in DMF. The dNBS was used in the last coupling step (Scheme 7). The sulfonation of peptide 15 (Table 2, entry 1) was accomplished in two coupling cycles using 4 equivalents of dNBS-Cl and 16 equivalents of pyridine. Longer peptides, however, required higher equivalents of dNBS-
Cl and pyridine but can be performed in one coupling step without any adverse effect on the integrity of the product. An example is tetrapeptide 16 (Table 2, entry 2) which failed to be completely sulfonated even after three coupling cycles following the same procedure for N-peptidylsulfonamide 15. Fortunately this can be easily resolved by using 8 equivalents of dNBS-Cl and 48 equivalents of pyridine. N-terminal sulfonation of pentapeptide 17 and glycosylpentapeptide 18 was achieved using the improved conditions for tetrapeptide 16. The successful on-resin coupling of dNBS at β-branched amino acids such as serine and valine and in the presence of protected glycan provides peptide fragments for ligation at hindered sites. This offers another avenue for accessing glycopeptides that may be troublesome for other ligation techniques.
Table 2. N-Peptidyl-2,4-dinitrobenzenesulfonamides

<table>
<thead>
<tr>
<th>Entry</th>
<th>dNBS Peptides</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="15.png" alt="Image" /></td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td><img src="16.png" alt="Image" /></td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td><img src="17.png" alt="Image" /></td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td><img src="18.png" alt="Image" /></td>
<td>49</td>
</tr>
</tbody>
</table>
Figure 2. (A) Kaiser test: Tube 1 is a positive Kaiser test for the presence of free amine; Tube 2 is a negative Kaiser test indicating the absence of free amine. (B) Manual solid phase peptide synthesis in a reaction cartridge. (C) dNBS-peptidyl resin. (D) Purified and freeze-dried dNBS-peptide.
Cleavage of peptides from the resin is generally effected by TFA in the presence of various nucleophilic additives to capture highly reactive cationic species. The TFA-TIPS-H₂O cleavage mixture was adequate for peptide 18. 1,2-Ethanedithiol (EDT)-containing cleavage cocktails such as Reagent K and R, are sometimes required for peptides having several residues that are highly susceptible to deleterious side reactions. The dNBS group is highly labile in the presence of this thiol. However, this was not the case when cleavage employing reagent K afforded the target compound with a similar yield to the thiol-free cleavage conditions. The acidic nature of the reaction could have suppressed the ability of EDT to displace dNBS.

Figure 3. Pure glycopentapeptidyl sulfonamide (18) cleaved from the resin using (A) reagent K and (B) TFA-TIPS-H₂O. Reverse-phase HPLC eluting with 3-90% MeOH-H₂O (0.1% TFA) gradient, UV detection at 254 nm.
4.4 Synthesis of Peptide Thioacid Precursors

The Fmoc-based chemistry for the assembly of peptides on the solid phase is relatively milder compared to the Boc chemistry. This strategy, however, presents some unique challenges. The thioester bond in the C-terminus is labile under basic conditions. Repeated deprotection steps using piperidine and concomitant exposure of the N-terminal amino group can potentially cleave the peptide prematurely from the resin. Modification of the deprotection mixture to protect the thioester bond was advanced by Aimoto but has not found widespread use. The sulfhydryl group preferably must be easily incorporated into the resin/linker so as to circumvent the need to prepare individual amino thioacids. After assembly, global deprotection and cleavage of the peptide from the resin must be effected smoothly without accompanying side reactions of the C-terminus thioester moiety. The Barlos resin appears to be a likely candidate for the on-resin synthesis of thioacids.

Barlos resin is used for the synthesis of side-chain protected peptides that allow further modification of the C-terminus off-resin. The resin is based on the trityl group that is highly acid-labile. The hypothesis is that the trityl group is bulky enough to make the thioester bond inaccessible to nucleophiles during chain extension and deprotection cycles. To lay the groundwork for using the Barlos resin on which to assemble the peptide thioacids, trityl mercaptan was proposed to functionalize the amino acids via dehydrative coupling conditions. Initially, the bulkiness of the trityl group was a concern in this study. One example of trityl thioester that appeared in the literature, i.e. Z-L-Val-
STrt,\textsuperscript{38} showed that the reaction can be realized. Results obtained from this work will help inform on how to proceed with the solid phase synthesis.

### 4.4.1 Evaluation of Conditions for the Solution Phase Synthesis of Amino Acid Thioesters

The development of conditions for the synthesis of trityl thioesters was performed on histidine, the residue that constitutes the thioacid part of the the H\textsubscript{15}-G\textsubscript{16} linkage. Direct access to the thioacid from the oxyacid function by preactivation of the carboxyl group in PyBOP followed by displacement with NaSH (Table 3, entry 1) was not realized. It is very likely that hydrogen sulfide consumed the condensing agent and precluded any further activation of the carboxyl group and/or deprotected the amino group. Goldstein\textsuperscript{39} and Crich\textsuperscript{6} reported the synthesis of thioacids by thiolysis of \textit{N}-hydroxysuccinimidyl esters with NaSH or H\textsubscript{2}S. This mild method has been used for the preparation of thioacids of acetic acid, Boc-Leu-OH, Boc-Arg(Z)\textsubscript{2}-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Asp(OcHex)-OH, Boc-Ser(Obn)-OH,\textsuperscript{39} Fmoc-HomoCys(Me), Fmoc-Asp-$\alpha$-CO\textsubscript{2}Me, Boc-Asp-$\alpha$-CO\textsubscript{2}Me, Boc-Glu-$\alpha$-CO\textsubscript{2}tBu\textsuperscript{6} but not for Fmoc-His(Trt)-OH. A similar reaction involving trityl mercaptan as the nucleophile failed to deliver the thioester product (Table 3, entry 2). This outcome was traced to the difficulty of generating the succinimide ester rather than the consumption of the added mercaptan with the excess condensing agent.
Further screening of conditions to prepare the trityl thioester involved condensing agents routinely employed in peptide synthesis. Carbodiimide-based reagents gave very low yields of the desired product (Table 3, entries 3 and 4). Reagents that convert the acid to the corresponding OBT esters such as HBTU (Table 3, entry 5) and PyBOP (Table 3, entry 6) gave better yields of 25 and 45%, respectively. HATU afforded the product in high yield of 92%.
Table 3. Synthesis of Thioacid and Trityl Thiesters

<table>
<thead>
<tr>
<th>No.</th>
<th>Conditions</th>
<th>Product</th>
<th>Isolated Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Preactivation of 19 (1 eq) in PyBOP (1.1 eq), HOBt (1.1 eq), DIPEA (1.1 eq), DMF for 5 min before addition of NaSH (3 eq).</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>A mixture of 19 (1 eq), DCC (3 eq), Osu (2 eq) and CH$_2$Cl$_2$ was stirred for 2 h before addition of TrtSH (1.1 eq); further stirring for 17 h.</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>19 (1.0 eq), DCC (2.2 eq), DMAP (0.2 eq), TrtSH (1.5 eq), CH$_2$Cl$_2$, 4 h</td>
<td>21</td>
<td>$^b$N/A</td>
</tr>
<tr>
<td>4</td>
<td>19 (1.0 eq), EDCI (2.2 eq), DMAP (0.20 eq), TrtSH (1.5 eq), CH$_2$Cl$_2$, 4 h</td>
<td>21</td>
<td>3%</td>
</tr>
<tr>
<td>5</td>
<td>19 (1.0 eq), HBTU (2.2 eq), HOBt (2.2 eq), DIPEA (4.4 eq), TrtSH (1.5 eq), CH$_2$Cl$_2$, 4 h</td>
<td>21</td>
<td>25%</td>
</tr>
<tr>
<td>6</td>
<td>19 (1.0 eq), PyBOP (1.1 eq), HOBt (1.1 eq), DIPEA (2.2 eq) TrtSH (1.1 eq), DMF, 15 h</td>
<td>21</td>
<td>45%</td>
</tr>
<tr>
<td>7</td>
<td>19 (1.0 eq), HATU (1.5 eq), DIPEA (4.4 eq), DMF, 2 h</td>
<td>21</td>
<td>92%</td>
</tr>
</tbody>
</table>

$^a$All reactions were carried out at room temperature under N$_2$ atmosphere.

$^b$N/A = Not applicable. Crude material was not purified because of paucity of putative product upon TLC examination.
4.4.2 Solution Phase Synthesis of Amino Acid Thioesters

Although HATU is very effective for the coupling of the hindered nucleophile to histidine, PyBOP was preferred as the condensing agent for economic reasons and proved to be just as effective for less hindered amino acids. Boc-Gly-OH 22, Fmoc-Gly-OH 23, Fmoc-Ser(OtBu)-OH 24, and Fmoc-Pro-OH 26 were converted to the corresponding thioesters 27, 28, 29, and 31, respectively, in high yields (Scheme 8). The preparation of the Fmoc-protected threonine thioester 30 appeared to be the exception with a yield of 66%. Seeking to improve the yield of threonine thioester 30, the coupling reaction was performed in the presence of HATU. Surprisingly HATU gave a lower yield (44%) of the threonine thioester as compared to the PyBOP-mediated reaction. It is important that the coupling reaction be monitored with TLC, especially at the beginning of the reaction and must be worked up once the reaction was almost complete. Reappearance of the starting amino acid on TLC was observed for the preparation of the Fmoc-protected glycine thioester 23 after prolonged reaction time.

Scheme 8. Synthesis of Trityl Thioesters in Solution Phase.
4.4.3 Solid Phase Synthesis of Dipeptides and Off-Resin Thioesterification

The dipeptides were assembled on the acid-labile Barlos resin employing the orthogonally compatible Fmoc/tBu strategy (Scheme 9). Cleavage of the peptides from the resin with AcOH/TFE/CH$_2$Cl$_2$ (2:2:6) provided the side-chain protected peptides which allow further manipulations at the C-terminus. Prior to cleavage, the histidyl-alanine peptide was capped with acetyl group. The peptide 32 was readily purified by trituration with hexanes with a yield of 100%. In the case of the prolyl-alanine 33, the Fmoc-protecting group at the N-terminus was not replaced with acetyl group in order to allow for the visualization of the product under UV. This also had the added convenience of shorter synthetic steps. Moreover, the Fmoc group is stable in the presence of the trityl mercaptan as shown by successful synthesis of Fmoc-protected amino acid thioesters. The Fmoc-protected dipeptide 33, however, exhibited appreciable solubility in the triturating solvent hexanes. This resulted in a respectable yield of 68%. The $^1$H NMR spectrum of 33 indicated some impurities but the material was pure enough to be used in the next step.
Scheme 9. (A) On-Resin Synthesis of Peptides and (B) Off-Resin Thioesterification.

Because HATU was shown to afford good yields of the thioesters from relatively hindered amino acids, HATU was used for the conversion of the dipeptides to the thioesters. Dipeptide 32 was reacted with trityl mercaptan at 35-40 °C for 5 h to afford the product 34 with a low yield of 25% (Scheme 9). \(^1\)H NMR analysis of the purified thioester 34 showed two sets of signals corresponding to two stereoisomers with approximately 1:1 ratio based on the integration of several peaks. To rule out the possibility of rotamers, the thioester was deprotected in the presence of TFA/TIPS/CH\(_2\)Cl\(_2\). The two bulky trityl groups on the peptide could potentially increase the rotational energy barrier such that two species are detected in the \(^1\)H NMR spectrum. \(^1\)H NMR analysis of the detritylated peptide showed the characteristic doubling of signals which indicated that the thioacid was a mixture of diastereomers. Epimerization presumably occurred in the histidine residue as it was the site of carboxyl activation.
Performing the thioesterification reaction at room temperature for prolonged periods improved the yield (42%) but epimerization remains a problem. The thioesterification of Fmoc-protected prolyl-alanine 33 appeared to have delivered the target thioester 35, albeit at a low yield of 36% (Scheme 9).

4.5 Ligation Reaction

4.5.1 Model Ligation of Amino Thioacids with dNBS-Alanine

Conversion of the trityl thioesters to the corresponding thioacids was achieved using TFA-TIPS-CH$_2$Cl$_2$ (45:5:50) in quantitative yields under N$_2$ atmosphere and used in the next step without further purification (Scheme 10). The cleavage cocktail also deprotected the side-chains of the amino thioacids. The identity of the thioacid was ascertained by mass spectral analysis of the crude material. Each of the thioacids was reacted with dNBS-alanine tert-butyl ester 41 in the presence of cesium carbonate and anhydrous DMF (Scheme 10). The ligation of Fmoc-protected glycine thioacid 37 was accomplished with a yield of 66% of didpeptide 43. Fmoc-protected thioacids bearing hydroxyl side-chains such as serine 38 and threonine 39 reacted with the sulfonamide to afford the dipeptide 44 and 45 in yields of 93% and 100%, respectively. The reaction was tolerant of basic aromatic side-chains as illustrated by the reaction of Fmoc-protected histidine thioacid 36 with the sulfonamide to provide the dipeptide 42 in 74%. The reactions were completed in 1 or 2 h, with the relatively hindered thioacids 36, 39, and 40 reacting faster with the sulfonamide compared to the less hindered ones. These results are consistent with those reported in the literature, providing further support to the impressive ability of the thioacid-sulfonamide chemistry to effect ligation at hindered
sites. The thioacids generated from the trityl thioester precursors behaved essentially the same as those that were derived from fluorenlylmethyl mercaptan.\textsuperscript{6} Previous work on this chemistry employed mostly amino acids with nonpolar side-chains, protected sulfhydryl group, one example of heteroaromatic side-chain, \textit{i.e.} tryptophan, Boc-L-Asp-α-OBn as spectator amine, and acetyl-protected carbohydrates.\textsuperscript{6} A closely related reaction of thioacids with amine mediated by 2,4-dinitroiodobenzene, Sanger and Mukaiyama reagents was executed on protected fragments with the notable exception of L-Tyr-OMe, and Z-Arg-OH as spectator amine.\textsuperscript{38} This work demonstrates the wider range of functional group tolerance of the chemistry that includes aromatic and basic side-chains, as well as, hydroxyl groups.

Scheme 10. Dipeptides from Sulfonamide 41 and Trityl Thioester-Derived Thioacids.

**4.5.2 Ligation of the dNBS-Glycosyl Pentapeptide 18 with Histidine Thioacid 36**

The tolerance of the thioacid-sulfonamide chemistry in the presence of protected glycan has been amply demonstrated by the synthesis of neoglyconjugates\textsuperscript{6} and conjugation of glycosyl amine to simple thioacids and β-thioaspartic acid to afford β-configured glycosyl amides.\textsuperscript{26} Nonetheless, the application of the thioacid-sulfonamide
chemistry in the chemical ligation of glycopeptides eagerly awaits experimental validation. As the first step, ligation at the H15-G16 site of MUC1 was initiated. Glycopentapeptidyl sulfonamide 18, which was previously prepared by solid phase, was selected for chemical ligation with Fmoc-protected histidine thioacid 36 (Scheme 11). The simultaneous presence of multiple functional groups in the reacting fragments, such as hydroxyl, imidazole, and carboxyl, as well as the protected glycan O-linked to the peptide will provide valuable insights to the limits of the chemoselectivity and efficiency of the chemistry. The ligation reaction was performed at 0.5 M total concentration of the reactants and the reaction appeared complete in 2 h. The progress of the reaction was monitored by the disappearance of the starting sulfonamide in ESI-MS. TLC was initially used but quickly abandoned in favor of ESI-MS due to the difficulty of localizing the spot of the putative product. HPLC purification of the crude peptide afforded the target side-chain deprotected glycosyl hexapeptide 47 in 71%. This yield is comparable to dipeptide 42 which was obtained from the ligation of histidine thioacid 36 with that of sulfonamide 41. This successful ligation reaction underscores the practicality of the chemistry in the chemical ligation of side-chain deprotected glycopeptides.
Scheme 11. Ligation of dNBS-Glycosyl Pentapeptide 18 with Histidine Thioacid 36.

4.6 Conclusion

Arylsulfonamides have a propensity of forming the amide bond with thioacids. The dNBS group, in particular, has been exploited in the ligation of amino acid and peptide fragments, as well as, the synthesis of neoglycoconjugates. The current study widens the scope of the chemistry to encompass glycopeptide synthesis on two very important fronts. First, the chemistry allows the coupling of the N-glycosylsulfonamide to the aspartic acid side chain to generate the β-N-linkage that is characteristic of N-linked glycopeptides. The glycosylation of the β-thioacid asparagine was achieved in 1 h using N-glucosyl-2,4-dinitrobenzenesulfonamide to provide N-(Asn) glycoside in a yield that was comparable to other methods such as the Staudinger ligation. Second, the reaction permits the joining of peptide fragments bearing an O-linked glycan en route to the O-linked glycopeptides. The ligation reaction is gratifyingly tolerant of unprotected peptide
functionalities such as imidazole, hydroxyl, carboxyl, and the glycosidic linkage in good yields and short reaction times. This is the first piece of evidence indicating that the reaction proceeds in the presence of multiple unprotected side-chain functional groups, as well as, carboxyl and glycan moieties.

Just as important as the chemistry is the demonstration that the required \(N\)-peptidylsulfonamides can be readily synthesized on-resin. The synthesis of \(N\)-peptidyl-2,4-dinitrobenzenesulfonamides on Wang resin has been achieved explicitly for the purpose of using them as building blocks in chemical ligation. A general route was developed for the capping of resin-bound peptides with dNBS to afford the sulfonamides in moderate to good yields. This approach obviates the need for preparing dNBS-amino acids\(^{19}\) for N-terminal functionalization. The dNBS remains attached to the peptide\(^{18,19}\) through cleavage from the resin, which until now has not been shown to survive TFA treatment. The dNBS coupling can be carried out in the presence of a protected glycan. The ease with which this N-terminal functionalization can be executed using a cheap, commercially available sulfonating agent, coupled with the efficient chemistry, it is believed, would facilitate the rapid adoption of this chemistry for glycopeptide ligation.

The synthesis of the peptide thioacid building block was pursued using an alternative strategy that eventually, it is hoped, would allow the synthesis on the solid phase using the Fmoc/tBu strategy. Trityl thioesters were developed as precursors of the thioacids. Thioesterification of the amino acids was predominantly accomplished using PyBOP. HATU was reserved for the more hindered amino acids and for the dipeptides
which were obtained by solid phase synthesis on Barlos resin. Epimerization was observed for the dipeptide but not for the amino acids. The thioacids behaved as expected when reacted with the sulfonamide. The ligation outcome was in consonance with the reported efficiency of the reaction involving bulky amino acids at the ligation site and further revealed the chemoselectivity of the reaction in the presence of unprotected hydroxyl and imidazole side chains.
Experimental Section

General Information

Materials. Fmoc Ala-preloaded Wang resin (substitution: 0.6 mmol/g) was procured from Novabiochem. His-preloaded (substitution: 0.617 mmol/g) and Ala-preloaded (substitution: 0.824 mmol/g) Barlos resins, amino acids and HOBt were purchased from Chem-Impex International and PyBOP was acquired from Acros Organics. Fmoc-Ac$_3$-Tn-α-Thr-OH was obtained from Sussex Research. All other fine chemicals were sourced from the following commercial suppliers: Acros Organics, Alfa Aesar, Fisher Scientific and Sigma-Aldrich. Pyridine, and peptide grade DMF and DIEA were dried over 3Å and 4Å molecular sieves, respectively. Anhydrous CH$_2$Cl$_2$ was freshly distilled over CaH$_2$. Extra dry MeOH was used as received.

General Procedures. RP-HPLC analyses were carried out on a Shimadzu LC-20AT prominence liquid chromatograph equipped with DGU-20A$_3$ prominence degasser. Data was processed with Shimadzu LC solution software. Analytical RP-HPLC was performed on a Premier C8 column (150 x 4.6 mm, 5 μm) with a flow rate of 1.0 mL/min, whereas semi-preparative RP-HPLC was accomplished on a Restek UltraC8 column (150 x 10.0 mm, 5 μm) with a flow rate of 5.0 mL/min. Samples were eluted with a gradient of water (0.1% TFA) and MeOH (0.1% TFA) over a period of 35 min and UV detection at 254 nm. Proton and carbon nuclear magnetic resonance spectra ($^1$H NMR and $^{13}$C NMR) were recorded on either INOVA-600 ($^1$H NMR 600 MHz; $^{13}$C NMR 150 MHz) or Varian VXR-400 ($^1$H NMR 400 MHz; $^{13}$C NMR 100 MHz) spectrometers with solvent resonance as internal standard (MeOH-$d_4$: $^1$H NMR at $\delta$ 3.31, $^{13}$C NMR at $\delta$ 49.15;
CD$_3$CN: $^1$H NMR at $\delta$ 1.94). The $^1$H NMR data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz, integration, and assignments. $^1$H-$^1$H gCOSY was performed on the INOVA-600 spectrometer. Low resolution mass spectra were taken on Esquire-LC electrospray ionization (ESI) mass spectrometer operated in the positive ion mode.

![Chemical Structure](image)

**9-Fluorenymethyl p-toluenesulfonate (5).** To a well-stirred mixture of fluorenylmethanol (2.00 g, 10.19 mmol) in CHCl$_3$ (10 mL) was added dropwise a mixture of p-toluene sulfonyl chloride (1.94 g, 10.18 mmol) in pyridine (1.66 mL, 20.38 mmol). The mixture was stirred for 4 h at room temperature under N$_2$ atm. After completion of the reaction, the reaction mixture was diluted with CHCl$_3$ (60 mL), and washed with saturated aqueous NaHCO$_3$ (3 x 10 mL), followed by brine (1 x 10 mL). The organic phase was dried (anhydrous Na$_2$SO$_4$), filtered, and the filtrate concentrated to dryness under reduced pressure. Recrystallization of the crude material (CHCl$_3$-petroleum ether = 3:1) gave the product as off-white solids. Yield: 2.24 g, (63%); $R_f$ = 0.60 (ethyl acetate-hexane = 3:7); $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.78 (d, $J$ = 7.8 Hz, 2H), 7.74 (d, $J$ = 7.8 Hz, 2H), 7.55 (d, $J$ = 7.2 Hz, 2H), 7.41 (t, $J$ = 7.8 Hz, 2H), 7.32-7.28 (m, 4H), 4.29 (d, $J$ = 1.2 Hz, 1H), 4.28 (s, 1H), 4.24 (t, $J$ = 6.0 Hz, 1H), 2.43 (s, 3H); ESI-MS: $m/z$ calc'd for C$_{21}$H$_{18}$NaO$_3$S 373.1, found: 373.1 [M+Na]$^+$. This compound was reported in the literature.$^{25}$
9-Fluorenlyl methyl thioacetate (6). A solution of fluorenlylmethyl p-toluenesulfonate (1.60 g, 4.57 mmol), potassium thioacetate (0.62 g, 5.43 mmol), and 18-crown-6 (0.12 g, 0.46 mmol) in DMF (15 mL) was stirred at room temperature under N₂ atm for 2 h. After completion of the reaction, the mixture was diluted with ethyl acetate (100 mL), and washed with water (2 x 48 mL), then with brine (2 x 48 mL). The brine wash was re-extracted with ethyl acetate (2 x 100 mL). The organic extracts were pooled together, dried (anhydrous Na₂SO₄), filtered, and concentrated to dryness under reduced pressure. Purification of the residue by flash column chromatography on SiO₂ (10.5 x 7 cm) using ethyl acetate -hexane (3:97) afforded the product as colorless amorphous solid. Yield: 1.00 g (86%); Rᶠ = 0.28 (ethyl acetate -hexane = 5:95); ¹H NMR (400 MHz, CDCl₃) δ 7.78 (d, J = 7.6 Hz, 2H), 7.68 (d, J = 7.6 Hz, 2H), 7.43 (t, J = 7.6 Hz, 2H), 7.35 (t, J = 7.6 Hz, 2H), 4.20 (t, J = 5.6 Hz, 1H), 3.56 (d, J = 6.0 Hz, 2H), 2.30 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 195.56, 145.56, 141.21, 127.86, 127.27, 124.79, 120.04, 46.81, 32.60, 30.84; ESI-MS: m/z calcd for C₁₆H₁₄NaOS 277.1, found: 277.0 [M+Na]⁺. The ¹H and ¹³C NMR were identical to the reported compound.⁶

9-Fluorenlylmethylthiol (7). To a well-stirred solution of 9-fluorenlylmethyl thioacetate (0.15 g, 0.59 mmol) in THF (6 mL) at -78 °C and under N₂ atm was added DIBAL-H (1.30 mL, 1.30 mmol, 1.0 M in hexane) dropwise. The mixture was allowed to come to
room temperature over a period of 30 min. The reaction was quenched by addition of 2N HCl and was stirred vigorously for 10 min. The aqueous layer was extracted with diethyl ether (2 x 20 mL), followed by brine (1 x 20 mL). The brine wash was re-extracted with diethyl ether (1 x 20 mL). The organic extracts were pooled together, dried (anhydrous Na₂SO₄), filtered, and concentrated to dryness under reduced pressure. Purification of the crude material by flash column chromatography on SiO₂ (10 x 2.5 cm) using ethyl acetate-hexane (1:99) afforded the product as a colorless gum. Yield: 0.116 g (93%); Rf = 0.18 (ethyl acetate-hexane = 2:98); ¹H NMR (400 MHz, CDCl₃) δ 7.80 (d, J = 7.6 Hz, 2H), 7.64 (d, J = 7.6 Hz, 2H), 7.44 (t, J = 7.6 Hz, 2H), 7.39-7.35 (m, 2H), 4.20 (t, J = 5.6 Hz, 1H), 3.23 (dd, J = 5.6, 8.4 Hz, 2H), 1.24 (t, J = 8.4 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 145.30, 141.66, 127.84, 127.30, 124.57, 120.17, 49.38, 28.10; ESI-MS: m/z calcd for C₁₄H₁₁⁺ 179.1, found: 179.1 [M-SH]⁺. The ¹H and ¹³C NMR were identical to the reported compound.⁶

![Chemical Structure](image)

α-Benzyl-N-tert-butoxycarbonyl-L-β-aspartyl 9-fluorenymethyl thioester (9). To a well-stirred solution of α-Benzyl-N-tert-butoxycarbonyl-L-β-aspartic acid (0.113 g, 0.35 mmol), 9-fluorenymethylthiol (0.089 g, 0.42 mmol) and DMAP (4.0 mg, 0.035 mmol) in dichloromethane (2.40 mL) at 0 °C was added DCC (0.079 g, 0.38 mmol) in dichloromethane (0.20 mL). The mixture was stirred for 1 h at 0 °C and another 16 h at room temperature. After completion of the reaction, the suspension was filtered, washed...
with generous amounts of dichloromethane and concentrated to dryness under reduced pressure. Purification of the crude material by flash column chromatography on SiO$_2$ (8 x 3.5 cm) using ethyl acetate -hexane (1:9) provided the desired product as a colorless gum. Yield: 0.171 g (95%); $R_f = 0.39$ (ethyl acetate -hexane = 1:4); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.76 (d, $J = 7.6$ Hz, 2H, ArFmoc), 7.63 (dd, $J = 2.0, 7.2$ Hz, 2H, ArFmoc), 7.41 (td, $J = 3.6, 7.2$ Hz, 2H, ArFmoc), 7.35-7.31 (m, 5H, ArFmoc, OCH$_2$Ph), 5.39 (d, $J = 8.4$ Hz, 1H, Asp-NH), 5.15 (s, 2H, OCH$_2$Ph), 4.58-4.54 (m, 1H, Asp-$\alpha$-CH), 4.13 (t, $J = 5.2$ Hz, 1H, Fmoc CH), 3.51 (d, $J = 6.0$ Hz, 2H, Fmoc CH$_2$), 3.21 (dd, $J = 4.8, 16.4$ Hz, 1H, Asp-$\beta$-CH), 3.05 (dd, $J = 4.4, 16.8$ Hz, 1H, Asp-$\beta$-CH), 1.46 (s, 9H, C(CH$_3$)$_3$); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 196.65 (C=O), 170.76 (C=O), 155.39 (C=O), 145.33, 145.27, 141.21, 135.30, 128.67, 128.53, 128.45, 127.94, 127.93, 127.27, 124.81, 124.76, 120.08, 80.30 (C(CH$_3$)$_3$), 67.63 (OCH$_2$Ph), 50.60 (Asp-$\alpha$-CH), 46.70 (Fmoc CH$_2$), 45.52 (Fmoc CH), 32.48 (Asp-$\beta$-CH), 28.45 (C(CH$_3$)$_3$); ESI-MS: $m/z$ calcd for C$_{30}$H$_{31}$NaNO$_5$S 540.2, found: 540.3 [M+Na]$^+$. The $^1$H and $^{13}$C NMR were identical to the reported compound.

$\alpha$-Benzyl-N-$\textit{tert}$-butoxycarbonyl-L-$\beta$-aspartyl thioacid (10). A solution of $\alpha$-benzyl-N-$\textit{tert}$-butoxycarbonyl-L-$\beta$-aspartyl 9-fluorenylethyl thioester (0.055 g, 0.11 mmol) in 40% piperidine in DMF (1.06 mL, 4.24 mmol) was stirred for 1.5 h at room temperature. Afterwards, the reaction mixture was concentrated to a small volume and reconstituted with ethyl acetate (5 mL). The organic phase was washed with ice-cold 1M HCl (2 x 2 mL), then chilled water (1 x 2 mL) and finally chilled brine (1 x 2 mL). Each of the washes was back-extracted with ethyl acetate (1 x 1 mL). The pooled organic extracts
were dried (anhydrous Na$_2$SO$_4$), filtered, and concentrated to dryness under reduced pressure to afford a yellowish gum. The crude material was used in the next step. ESI-MS: $m/z$ calcd for C$_{16}$H$_{21}$NaNO$_5$S 362.1, found 362.1 [M+Na]$^+$.6

**Benzyl $N^\alpha$-tert-butoxycarbonyl-N-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-$\beta$-D-glucopyranosyl)-L-asparaginate (12).** To a suspension of cesium carbonate (0.035 g, 0.11 mmol), in anhydrous DMF (0.30 mL) was added $\alpha$-benzyl-$N^\alpha$-tert-butoxycarbonyl-L-$\beta$-aspartyl thioacid (0.043 g, crude, approximately 0.11 mmol). The mixture was stirred for 10 min at room temperature under N$_2$ atm before the addition of $N$-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-$\beta$-D-glucopyranosyl)acetamide (0.031 g, 0.054 mmol). To the resulting red solution was added another portion of anhydrous DMF (0.50 mL). The reaction was judged complete by TLC after stirring for 1 h. The reaction mixture was diluted with ethyl acetate (60 mL), washed with saturated aqueous NH$_4$Cl (2 x 20 mL), followed by brine (1 x 20 mL). The organic phase was dried (anhydrous Na$_2$SO$_4$), filtered, and the filtrate concentrated under reduced pressure. The crude product was purified by flash column chromatography on SiO$_2$ (10 x 2.5 cm) using hexane-CHCl$_3$-MeOH-acetone (60:30:5:5) to furnish the desired product as an amorphous light green solid. Yield: 34.58 mg (69%); $R_f$ = 0.09 (hexane-CHCl$_3$-MeOH-acetone = 60:30:5:5); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.33-7.37 (m, 5H, aromatic), 7.04 (d, 1H, $J = 8.4$ Hz, NHAc$_{C-1}$), 5.96 (d, 1H, $J = 7.8$ Hz, NHAc$_{C-2}$), 5.55 (d, 1H, $J = 8.4$ Hz, Asp-$\alpha$-NH), 5.12-5.20 (m, 3H, H-3, OCH$_2$Ph), 5.01-5.04 (t, 2H, $J = 8.4$ Hz, H-1, H-4), 4.56-4.57 (m, 1H, Asp-$\alpha$-CH), 4.31 (dd, 1H, $J = 4.2$, 12.0 Hz, H-6), 4.08-4.13 (m, 1H, H-2), 4.06 (dd, 1H, $J$
= 1.8, 12.0 Hz, H-6'), 3.72-3.75 (m, 1H, H-5), 2.94 (dd, 1H, J = 4.2, 16.2 Hz, Asp-β-CH), 2.67 (dd, 1H, J = 4.2, 16.2 Hz, Asp-β-CH), 2.08 (s, 3H, CH₃CO), 2.07 (s, 3H, CH₃CO), 2.05 (s, 3H, CH₃CO), 1.95 (s, 3H, CH₃CO), 1.42 (s, 9H, C(CH₃)₃); ¹³C NMR (150 MHz, CDCl₃): δ 172.4 (C=O), 172.2 (C=O), 171.3 (C=O), 170.914 (C=O), 170.910 (C=O), 169.5 (C=O), 155.7 (C=O), 135.5, 128.7, 128.5, 128.4, 80.5 (C-1), 80.3, 73.8, 73.0, 67.7, 67.6, 61.9, 53.6, 50.3 (Asp-α-CH), 38.4 (Asp-β-CH₂), 28.5 (C(CH₃)₃), 23.3 (CH₃CO), 20.96 (CH₃CO), 20.92 (CH₃CO), 20.8 (CH₃CO); ESI-MS: m/z calcd for C₃₀H₄₁N₃NaO₁₃ 674.3, found: 674.3 [M+Na]⁺. The ¹H NMR and ¹³C NMR were identical to the reported compound.¹²

**Solid Phase Peptide Synthesis.** Peptides were manually assembled on Fmoc Ala-preloaded Wang resin (150 mg) using Fmoc/tBu strategy. The reactions were performed in a 20 mL syringe reactor cartridge with agitation provided by a stream of N₂. The following amino acids were used: Fmoc-Ser(OtBu)-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Val-OH, Fmoc-Gly-OH and Fmoc-Ac₃-Tn-α-Thr-OH. The synthesis involved the following steps: (i) Fmoc deprotection with 20 or 40% piperidine in DMF (3 mL) for 25 min; (ii) Kaiser test; (iii) washing with DMF (3 x 3 mL, 5 min/wash); (iv) coupling of Fmoc amino acid (2 eq) for 1-2 h with pre-activation (2 min) in PyBOP (2 eq), HOBt (2 eq), DIPEA (4 eq) and DMF; (vi) Kaiser test; (vii) washing with DMF (3 x 3 mL, 5 min/wash). Double or triple deprotection and couplings were performed as indicated by the Kaiser test.²⁷ Coupling involving Fmoc-Ac₃-Tn-α-Thr-OH was carried out for 7 h using the same amount of reagents as mentioned above. After assembly, the Fmoc group was removed, the resin washed with DMF (3 x 3 mL, 5 minutes/wash), followed by
CH₂Cl₂ (3 x 3 mL, 5 minutes/wash). The resin was transferred to a 20 mL scintillation vial for reaction with 2,4-dinitrobenzenesulfonyl chloride as described below. The peptide was released from the resin following treatment with TFA/H₂O (95:5), unless otherwise noted, for 4 h under N₂ atmosphere. The mixture was filtered, washed with the cleavage cocktail (1-2 mL), then with a liberal amount of dichoromethane, unless otherwise stated. The filtrate was concentrated to dryness in vacuo and the crude peptide was purified. Yields are based on the resin loading provided by the manufacturer.

**General Procedure for N-Terminal Sulfonation (Method A).** To the pre-swollen peptidyl resin was added 2,4-dinitrobenzenesulfonyl chloride (0.096 g, 0.36 mmol), CH₂Cl₂ (1.0 mL) and pyridine (0.12 mL, 1.44 mmol). The mixture was stirred slowly (to prevent resin attrition) at room temperature under N₂ atmosphere. After 4 h, the mixture was washed with CH₂Cl₂ (1 x 6 mL, 5 min/wash), DMF (3 x 3 mL, 5 min/wash) and CH₂Cl₂ (3 x 3 mL, 5 min/wash). The above coupling procedure was performed twice. Kaiser test was used to monitor the completion of the reaction. The resin was given a final MeOH wash (3 x 3 mL, 5 min/wash) prior to drying in high vacuum.

**Improved General Procedure for N-Terminal Sulfonation (Method B).** To the pre-swollen peptidyl resin was added 2,4-dinitrobenzenesulfonyl chloride (0.096 g, 0.36 mmol), CH₂Cl₂ (1.0 mL) and pyridine (0.12 mL, 1.44 mmol). The mixture was stirred slowly (to prevent resin attrition) at room temperature under N₂ atmosphere for 3 h. Afterwards, 2,4-dinitrobenzenesulfonyl chloride (0.096 g, 0.36 mmol) and pyridine (0.24
mL, 2.88 mmol) were added to the mixture and stirring was continued for 3-3.5 h more. The mixture was washed with CH$_2$Cl$_2$ (1 x 6 mL, 5 min/wash), DMF (3 x 3 mL, 5 min/wash), CH$_2$Cl$_2$ (3 x 3 mL, 5 min/wash) and finally MeOH (3 x 3 mL, 5 min/wash) and the resin was dried in high vacuum. Kaiser test was used to monitor the completion of the reaction.

dNBS-Alanine (13). After N-terminal capping following the general procedure (Method A) described earlier, the sulfonamide was cleaved from the resin (140 mg) upon treatment with the cleavage cocktail (1.4 mL). The crude sulfonamide was purified by flash column chromatography on SiO$_2$ (6 x 1.3 cm) using MeOH-CH$_2$Cl$_2$-CH$_3$COOH (5:95:0.6) to give a yellow solid. Yield: 11.6 mg (40%); $^1$H NMR (600 MHz, MeOH-$d_4$): $\delta$ 8.72 (d, $J = 2.4$ Hz, 1H), 8.59 (dd, $J = 2.4$, 8.4 Hz, 1H), 8.34 (d, $J = 8.4$ Hz, 1H), 4.19 (q, $J = 7.2$ Hz, 1H), 1.45 (d, $J = 7.2$ Hz, 3H); $^{13}$C NMR (100 MHz, MeOH-$d_4$): $\delta$ 174.92, 151.42, 149.41, 140.77, 133.51, 127.92, 121.37, 53.58, 19.53; ESI-MS: m/z calcd for C$_9$H$_9$N$_3$NaO$_8$S 342.0, found: 342.0 [M+Na]$^+$, This compound was reported in the literature.$^{41}$

dNBS-Ser-Ala-OH (15). After N-terminal capping following the General Procedure (Method A) described earlier, a portion (18.4 mg) of the recovered resin (147 mg) was treated with the cleavage cocktail (0.2 mL) to afford the crude peptide. The crude peptide was purified by reverse-phase flash column chromatography on a PrepSep C18 plug (2 x
1.5 cm) using 60 mL each of the following solvents in the order indicated: MeOH-H₂O (1:3), MeOH-H₂O (1:1), MeOH-H₂O (3:1) and MeOH to afford a colorless solid. Yield: 3.9 mg (85%); ¹H NMR (600 MHz, MeOH-d₄): δ 8.75 (d, J = 2.4 Hz, 1H), 8.59 (dd, J = 2.4, 9.0 Hz, 1H), 8.35 (d, J = 8.4 Hz, 1H), 4.16 (t, J = 6.0 Hz, IH, Ser-β-CH), 4.13 (q, J = 7.2 Hz, 1H, Ala-α-CH), 3.81-3.75 (m, 2H, Ser-α-CH, and Ser-β-CH), 1.28 (d, J = 7.2 Hz, 3H, Ala-CH₃); ESI-MS: m/z calcd for C₁₂H₁₄N₄NaO₁₀S 429.0, found: 429.2 [M+Na]⁺.

dNBS-Val-Thr-Ser-Ala-OH (16). After N-terminal capping following the improved General Procedure (Method B) described earlier, a portion (20.4 mg) of the recovered resin (158 mg) was treated with the cleavage cocktail (0.2 mL) to give the crude peptide. The crude peptide was purified by semi-preparative RP-HPLC and freeze-dried to afford a colorless fluffy hygroscopic solid. Yield: 5.4 mg (77%); ¹H NMR (600 MHz, MeOH-d₄): δ 8.74 (d, J = 2.4 Hz, 1H), 8.59 (dd, J = 2.4, 9.0 Hz, 1H), 8.35 (d, J = 9.0, 1H), 4.40 (t, J = 5.4 Hz, 1H, Ser-α-CH), 4.38-4.32 (m, 1H, Ala-α-CH), 4.17 (d, J = 4.2 Hz, 1H, Thr-α-CH), 3.97 (d, J = 6.6 Hz, 1H, Val-α-CH), 3.94-3.93 (m, 1H, Thr-β-CH), 3.81 (dd, J = 6.0, 11.4 Hz, 1H, Ser-β-CH), 3.74 (dd, J = 4.8, 11.4 Hz, 1H, Ser-β-CH), 2.11 (sextet, J = 6.6 Hz, 1H, Val-β-CH), 1.39 (d, J = 7.2 Hz, 3H, Ala-CH₃), 1.00 (d, J = 6.0 Hz, 3H, Thr-CH₃), 0.99 (d, J = 6.6 Hz, 3H, Val-CH₃), 0.93 (d, J = 7.2 Hz, 3H, Val-CH₃); ESI-MS: m/z calcd for C₂₁H₃₀N₆NaO₁₃S 629.2, found: 629.3 [M+Na]⁺.
dNBS-Gly-Val-Thr-Ser-Ala-OH (17). After N-terminal capping following the improved General Procedure (Method B) described earlier, a portion (20.3 mg) of the recovered resin (170 mg) was treated with the cleavage cocktail (0.2 mL) to give the crude peptide. The crude peptide was purified by semi-preparative RP-HPLC and freeze-dried to afford a colorless fluffy solid. Yield: 4.4 mg (62%). $^1$H NMR (600 MHz, CD$_3$CN): $\delta$ 8.65 (d, $J = 2.4$ Hz, 1H), 8.51 (dd, $J = 2.4$, 8.4 Hz, 1H), 8.26 (d, $J = 8.4$ Hz, 1H), 7.26 (d, $J = 6.6$ Hz, 1H, NH), 7.21-7.14 (m, 3H, Val-NH, Thr-NH, and Ser-OH), 4.34-4.28 (m, 2H, Ser-α-CH, and Ala-α-CH), 4.26 (dd, $J = 3.0$, 7.2 Hz, 1H, Thr-α-CH), 4.18-4.14 (m, 1H, Thr-β-CH), 4.05 (t, $J = 7.2$ Hz, 1H, Val-α-CH), 3.96 (d, $J = 17.4$ Hz, 1H, Gly-α-CH), 3.92 (d, $J = 17.4$ Hz, 1H, Gly-β-CH), 3.80 (dd, $J = 4.2$, 12.0 Hz, 1H, Ser-β-CH), 3.67 (dd, $J = 4.2$, 11.4 Hz, 1H, Ser-β-CH), 2.09-2.04 (m, 1H, Val-β-CH), 1.34 (d, $J = 7.2$ Hz, 3H, Ala-CH$_3$), 1.09 (d, $J = 6.6$ Hz, 3H, Thr-CH$_3$), 0.88 (d, $J = 3.6$ Hz, 3H, Val-CH$_3$), 0.87 (d, $J = 3.6$ Hz, 3H, Val-CH$_3$); ESI-MS: m/z calcd for C$_{23}$H$_{33}$N$_7$NaO$_{14}$S 686.17, found: 686.3 [M+Na]$^+$. 

dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (18). After N-terminal capping following the improved General Procedure (Method B) described earlier, a portion (20.7 mg) of the recovered resin (205 mg) was treated with TFA/TIPS/H$_2$O (95:2.5:2.5) (0.2 mL) for 3 h
to give the crude glycosylpeptide. The crude material was purified by semi-preparative RP-HPLC and freeze-dried to afford a colorless fluffy solid. Yield: 4.4 mg (49%); $^1$H NMR (600 MHz, CD$_3$CN): $\delta$ 8.66 (d, $J = 2.4$ Hz, 1H), 8.51 (dd, $J = 2.4$, 8.4 Hz, 1H), 8.28 (d, $J = 7.8$ Hz, 1H), 7.25 (d, $J = 7.8$ Hz, 1H, Ser-NH), 7.17-7.15 (m, 3H, Ala-NH, Thr-NH, Val-NH), 6.82 (d, $J = 9.0$ Hz, 1H, NHAc), 5.33 (d, $J = 1.8$ Hz, 1H, H-4), 5.06 (dd, $J = 3.6$, 11.4 Hz, 1H, H-3), 4.99 (d, $J = 3.0$ Hz, 1H, H-1), 4.43 (dd, $J = 2.4$, 8.4 Hz, 1H, Thr-α-CH), 4.39-4.36 (m, 1H, Ser-α-CH), 4.33-4.27 (m, 3H, Ala-α-CH, H-2, and H-5), 4.27-4.22 (m, 2H, Thr-β-CH and Ser-OH), 4.16 (dd, $J = 6.6$, 7.8 Hz, Val-α-CH), 4.06 (s, 1H, H-6'), 4.05 (d, $J = 1.2$ Hz, 1H, H-6), 3.97 (d, $J = 17.4$ Hz, 1H, Gly-α-CH), 3.91 (d, $J = 17.4$ Hz, 1H, Gly-α-CH), 3.74 (dd, $J = 5.4$, 11.4 Hz, Ser-β-CH), 3.69 (dd, $J = 4.8$, 11.4 Hz, Ser-β-CH), 2.09 (s, 3H, CH$_3$CO), 2.08-2.04 (m, 1H, Val-β-CH), 1.97 (s, 3H, CH$_3$CO), 1.90 (s, 3H, CH$_3$CO), 1.87 (s, 3H, CH$_3$CO), 1.36 (d, $J = 7.2$ Hz, 3H, Ala-CH$_3$), 1.18 (d, $J = 6.6$ Hz, 3H, Thr-CH$_3$), 0.90 (d, $J = 6.6$ Hz, 3H, Val-CH$_3$), 0.88 (d, $J = 7.2$ Hz, 3H, Val-CH$_3$); ESI-MS: $m/z$ calcd for C$_{37}$H$_{52}$N$_8$NaO$_{22}$S 1015.28, found: 1015.5. [M+Na]$^+$. 

![dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH](image)

dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (18) from cleavage using reagent K. A portion (18.4 mg) of the resin was treated with reagent K (TFA/thioanisole/H$_2$O/phenol/EDT = 82.5:5:5:5:2.5) (0.2 mL) for 3 h. The resin was washed with neat TFA (2.4 mL), concentrated to dryness under reduced pressure, the
residue washed with cold Et$_2$O four times and dried in high vacuum. Purification of the glycosyl peptide by semi-preparative RP-HPLC and after freeze-drying afforded a white fluffy solid. Yield: 3.8 mg (48%). The $^1$H NMR, ESI-MS and RP-HPLC trace were identical to the compound previously obtained with TFA/TIPS/H$_2$O treatment.

**General Procedure for the Thioesterification of Amino Acids.** A mixture of Boc- or Fmoc-protected amino acid, condensing agent/additive and tritylmercaptan was dried in vacuum for 30 min. To this dried material was added DMF (1.0 mL, unless otherwise noted), followed by DIPEA, and the resulting solution was stirred for a given time under N$_2$ atmosphere at ambient temperature. The completion of the reaction was monitored by TLC. The reaction mixture was diluted with ethyl acetate (40 mL), washed with water (2 x 10 mL), and saturated NaCl (1 x 10 mL). The aqueous layers were back-extracted with ethyl acetate (1 x 20 mL). The pooled organic extracts were dried (Na$_2$SO$_4$), filtered and concentrated to dryness under reduced pressure.

**N-α-Fmoc-N-im-Trityl-Protected L-Histidine Trityl Thioester (21).** Fmoc-protected histidine (0.177 g, 0.29 mmol) was converted to the thioester in the presence of HATU (0.166 g, 0.44 mmol), tritylmercaptan (0.087 g, 0.31 mmol) and DIPEA (0.20 mL, 1.26 mmol) after 2 h following General Procedure given above. The crude material was purified by flash column chromatography on SiO$_2$ (3.5 x 6 cm) using step-wise gradient of acetone-ethyl acetate-hexanes (5:5:90 and 10:10:80) to provide a colorless powder. Yield: 0.231 g (92%); silica gel TLC $R_f = 0.09$ (acetone-ethyl acetate-hexanes =
Boc-Protected Glycine Trityl Thioester (27). Boc-protected glycine (0.050 g, 0.29 mmol) was converted to the thioester in the presence of PyBOP (0.163 g, 0.31 mmol), HOBt (0.042 g, 0.31 mmol), tritylmercaptan (0.087 g, 0.31 mmol) and DIPEA (0.10 mL, 0.63 mmol) after 24 h following General Procedure given above. The crude material was purified by flash column chromatography on SiO₂ (3.5 x 7 cm) using step-wise gradient elution with acetone-hexanes (7.50:92.50) to provide a colorless amorphous solid. Yield: 0.111 g (89%); silica gel TLC \( R_f = 0.25 \) (acetone-hexanes = 1:9, run twice); \(^1\)H NMR (600 MHz, CDCl₃): \( \delta \) 7.28-7.21 (m, 15H, aromatic H), 5.02 (s, 1H, NH), 3.99 (d, \( J = 5.4 \) Hz, 1H, \( \alpha\)-CH₂), 1.43 (s, 9H, tBu); \(^{13}\)C NMR (100 MHz, CDCl₃): \( \delta \) 195.12 (SC=O), 155.60, 143.65, 129.96, 128.00, 127.55, 127.40, 80.37, 70.70, 50.48, 28.46; ESI-MS: \( m/z \) calcd for C₂₆H₂₇NNaO₃S 456.2, found: 455.7 [M+Na]⁺.
**Fmoc-Protected Glycine Trityl Thioester (28).** Boc-protected glycine (0.085 g, 0.29 mmol) was converted to the thioester in the presence of PyBOP (0.163 g, 0.31 mmol), HOBt (0.042 g, 0.31 mmol), tritylmercaptan (0.087 g, 0.31 mmol) and DIPEA (0.10 mL, 0.63 mmol) after 2 h following General Procedure given above. The crude material was purified by flash column chromatography on SiO₂ (3.5 x 7 cm) using acetone-hexanes (15:85) to provide a colorless film. Yield: 0.158 g (100%); silica gel TLC \( R_f = 0.45 \) (acetone-hexanes = 3:7); \(^1\)H NMR (600 MHz, CDCl₃): \( \delta \) 7.72 (d, \( J = 7.6 \) Hz, 2H, aromatic H), 7.54 (d, \( J = 7.6 \) Hz, 2H, aromatic H), 7.36 (t, \( J = 7.2 \) Hz, 2H, aromatic H), 7.28-7.18 (m, 17H, aromatic H), 5.31 (t, \( J = 5.6 \) Hz, NH), 4.36 (d, \( J = 6.8 \) Hz, 2H, Fmoc CH₂), 4.17 (t, \( J = 7.2 \) Hz, 1H, Fmoc CH), 4.05 (d, \( J = 5.6 \), 2H, \( \alpha \)-CH₂); \(^{13}\)C NMR (100 MHz, CDCl₃): \( \delta \) 194.57 (SC=O), 156.11, 143.85, 143.50, 141.39, 129.89, 128.01, 127.85, 127.43, 127.22, 125.22, 120.12, 70.92, 67.38, 50.68, 47.18; ESI-MS: \( m/z \) calcd for \( C_{36}H_{29}NNaO₃S \) 578.2, found: 577.8 [M+Na]⁺.

**N-α-Fmoc-O-tert-Butyl-Protected 1-Serine Trityl Thioester (29).** Fmoc-protected serine (0.109 g, 0.29 mmol) was converted to the thioester in the presence of PyBOP (0.163 g, 0.31 mmol), HOBt (0.042 g, 0.31 mmol), tritylmercaptan (0.087 g, 0.31 mmol) and DIPEA (0.10 mL, 0.63 mmol) after 3 h following General Procedure given above. The crude material was purified by flash column chromatography on SiO₂ (3.5 x 7 cm) using acetone-ethyl acetate-hexanes (3.5:3.5:93) to provide a white foam. Yield: 0.165 g (90%); silica gel TLC \( R_f = 0.20 \) (acetone-ethyl acetate-hexanes = 0.5:0.5:9); \(^1\)H NMR
(600 MHz, CDCl$_3$): $\delta$ 7.77 (t, $J = 7.8$ Hz, 2H, aromatic H), 7.65 (d, $J = 7.8$ Hz, 1H, aromatic H), 7.63 (d, $J = 7.2$ Hz, 1H, aromatic H), 7.40 (t, $J = 7.2$ Hz, 2H, aromatic H), 7.29-7.23 (m, 17H, aromatic H), 5.80 (d, $J = 9.0$, 1H, NH), 4.54 (dd, $J = 6.6$, 10.2 Hz, 1H, Fmoc CH), 4.47 (dt, $J = 3.0$, 12.0, 1H, $\alpha$-H), 4.34-4.27 (m, 2H, Fmoc CH$_2$), 3.85 (dd, $J = 2.4$, 9.0, 1H, $\beta$-CH), 3.47 (dd, $J = 3.0$, 8.4, 1H, $\beta$-CH), 1.20 (s, 9H, tBu); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 197.67 (SC=O), 155.95, 144.05, 143.86, 143.72, 141.46, 130.14, 130.05, 127.91, 127.88, 127.26, 127.22, 125.41, 125.30, 120.16, 73.74, 70.22, 67.46, 62.54, 61.18, 47.31, 27.56 (C(CH$_3$)$_3$); ESI-MS: m/z calcd for C$_{41}$H$_{39}$NNaO$_4$S 664.2, found: 663.7 [M+Na]$^+$. 

$\text{FmocHN STrt}$

N-$\alpha$-Fmoc-$O$-tert-Butyl-Protected L-Threonine Trityl Thioester (30). Fmoc-protected serine (0.113 g, 0.29 mmol) was converted to the thioester in the presence of PyBOP (0.163 g, 0.31 mmol), HOBt (0.042 g, 0.31 mmol), tritylmercaptan (0.087 g, 0.31 mmol) and DIPEA (0.10 mL, 0.63 mmol) after 7 h following General Procedure given above. The crude material was purified by flash column chromatography on SiO$_2$ (3.5 x 8 cm) using acetone-ethyl acetate-hexanes (2.5:2.5:95) to provide a colorless foam. Yield: 0.124 g (66%); silica gel TLC $R_f = 0.25$ (acetone-ethyl acetate-hexanes = 0.5:0.5:9); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.75 (dd, $J = 4.2$, 7.8 Hz, 2H, aromatic H), 7.65 (d, $J = 7.2$ Hz, 1H, aromatic H), 7.63 (d, $J = 7.2$ Hz, 1H, aromatic H), 7.38 (td, $J = 3.6$, 7.8 Hz, 2H, aromatic H), 7.28-7.19 (m, 17H, aromatic H), 5.73 (d, $J = 9.6$ Hz, 1H, NH), 4.56 (dd, $J = 6.6$, 10.2 Hz, 1H, Fmoc CH), 4.34-4.27 (m, 2H, Fmoc CH$_2$), 4.20 (d, $J = 7.8$ Hz, 2H, $\alpha$-CH + $\beta$-CH), 1.09 (s plus overlapped d, 12H, tBu + $\beta$-CH$_3$); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 198.
Fmoc-Protected L-Proline Trityl Thioester (31). Fmoc-protected proline (0.096 g, 0.29 mmol) was converted to the thioester in the presence of PyBOP (0.326 g, 0.63 mmol), HOBt (0.042 g, 0.31 mmol), tritylmercaptan (0.087 g, 0.31 mmol) and DIPEA (0.20 mL, 1.26 mmol) after 3 h following General Procedure given above. The crude material was purified by flash column chromatography on SiO$_2$ (3.5 x 7.5 cm) using step-wise gradient of acetone-ethyl acetate-hexanes (5:5:90) to provide a colorless solid. Yield: 0.138 g (82%); silica gel TLC $R_f = 0.30$ (acetone-ethyl acetate-hexanes = 1:1:8); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.80 (t, $J = 6.8$ Hz, 2H), 7.69-7.63 (m, 2H), 7.43-7.40 (m, 2H), 7.33-7.22 (m, 17H), 4.56-4.39 (m, 3H), 4.33-4.27 (m, 2H), 3.67-3.64 (m, 1H, $\delta$-CH), 3.56-3.48 (m, 1H, $\delta$-CH), 2.12-2.00 (m, 1H, $\beta$-CH), 2.00-1.80 (m, 3H, $\gamma$-CH x 2 & $\beta$-CH); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 198.69 (SC=O), 198.34 (SC=O), 155.11 (C=O), 154.65 (C=O), 144.38, 144.33, 143.97, 143.85, 143.80, 143.68, 141.51, 141.49, 141.46, 141.37, 130.36, 130.07, 129.95, 127.99, 127.93, 127.88, 127.84, 127.44, 127.38, 127.31, 127.27, 127.23, 127.19, 125.61, 125.45, 125.36, 125.31, 120.15, 70.26, 70.16, 68.09, 67.82, 66.62, 66.49, 47.54, 47.36, 46.99, 31.44, 30.21, 24.28, 23.37; ESI-MS: $m/z$ calcd for C$_{39}$H$_{33}$NaNO$_3$S 618.2, found: 617.8 [M+Na]$^+$. The characteristic doubling of signals was consistent with that reported for Boc-protected L-proline 9-fluorenylethyl thioester.$^6$
**Solid Phase Synthesis on Barlos Resin and Off-Resin Thioesterification.** Peptides were manually assembled on Fmoc amino acid preloaded Barlos resin (150 mg) using Fmoc/tBu strategy. Fmoc-Ala-OH and Fmoc-Pro-OH were used for the coupling step. A complete cycle of chain extension includes the following steps: (i) coupling of Fmoc amino acid (2 eq) for 1-4 h with pre-activation (2 min) in PyBOP (2 eq), HOBt (2 eq), DIPEA (4 eq) and DMF; (ii) Kaiser test; (iii) Washing with DMF (3 x 3 mL, 5 min/wash), 2-propanol (2 x 3 mL, 5 min/wash) and DMF (3 x 3 mL, 5 min/wash); (iv) Fmoc deprotection with 20 piperidine in DMF (3 mL) for 25 min; (v) Kaiser test; (vi) Washing with DMF (3 x 3 mL, 5 min/wash), 2-propanol (2 x 3 mL, 5 min/wash) and DMF (3 x 3 mL, 5 min/wash). The reactions were performed in a 20 mL syringe reactor cartridge with agitation provided by a stream of N₂. After chain assembly, the peptidyl-resin was washed with acid-free CH₂Cl₂ (3 x 3 mL, 5 min/wash.) The peptide was cleaved from the resin following treatment with AcOH/TFE/CH₂Cl₂ (2:2:6), for 3-4 h under N₂ atmosphere. The mixture was filtered, washed with the cleavage cocktail (1-2 mL), then with a liberal amount of CH₂Cl₂. The filtrate was concentrated *in vacuo* and the residual acetic acid was co-evaporated with hexane (3 x 60 mL). The residue was triturated with hexanes and centrifuged. The residue was washed twice with hexanes and dried in high vacuum to remove the residual solvent. Yields are based on the resin loading provided by the manufacturer. The peptide was converted to the corresponding peptide thioester following the General Procedure for the amino acid thioester.
**N-Fmoc-L-alanyl-L-histidine (32).** Following assembly on the resin (loading = 0.617 mmol/g) and N-terminal capping, a portion (88.5 mg) of the recovered resin (153.2 mg) was treated with the cleavage cocktail (0.089 mL) to afford the peptide as a white solid. Yield: 26.5 mg (100%); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.73 (s, 1H, imidazole H), 7.35 (s, 9H, aromatic H), 7.14 (s, 1H, His-NH), 7.09 (d, $J$ = 4.2 Hz, 6H, aromatic H), 6.70 (s, 1H, imidazole H), 6.67 (d, $J$ = 6.0 Hz, 1H, Ala-NH), 4.53 (q, $J$ = 6.0 Hz, 1H, His-$\alpha$-CH), 4.23 (pentet, $J$ = 6.0 Hz, 1H, Ala-$\alpha$-CH), 3.22-3.12 (m, 2H, His-$\beta$-CH), 1.90 (s, 3H, CH$_3$CO), 1.28 (d, $J$ = 7.2 Hz, 3H, Ala-CH$_3$); ESI-MS: $m/z$ calcld for C$_{30}$H$_{31}$N$_4$O$_4$ 511.2, found: 511.3 [M$^+$].

**N-Fmoc-L-prolyl-L-alanine (33).** Following assembly on the resin (loading = 0.824 mmol/g) and N-terminal capping, a portion (0.100 g) of the recovered resin (0.173 g) was treated with the cleavage cocktail (1.0 mL) to furnish the peptide as a colorless solid. Yield: 19.7 mg (68%); ESI-MS: $m/z$ calcld for C$_{23}$H$_{24}$N$_2$O$_5$ 408.2, found: 408.9 [M$^+$].

**N-Fmoc-alanylhistidine Trityl Thioester (34).** The peptide (28.6 mg, 0.056 mmol) was reacted with HATU (47.5 mg, 0.12 mmol), tritylmercaptan (34.0 mg, 0.12 mmol), and DIPEA (0.040 mL, 0.0246 mmol) in DMF (0.5 mL) at 35-40 °C to afford the thioester.
after 5 h following the General Procedure given for amino acid thioesters. The crude material was purified by flash column chromatography on SiO$_2$ (1.6 x 13.5 cm) using a step-wise gradient elution with MeOH-CHCl$_3$ (0:100, 1:99, and 2.5:97.5) to afford the thioester as a diastereomeric mixture. Yield: 10.7 mg (25%); silica gel TLC $R_f = 0.47$ (MeOH-CHCl$_3$ = 7.5:92.5); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 8.29 (d, $J = 7.2$ Hz, 1H), 8.23 (d, $J = 7.2$ Hz, 1H), 8.02 (s, 2H), 7.35-7.28 (m, 20H), 7.17-7.14 (m, 29H), 7.09-7.07 (m, 11H), 6.58 (s, 2H), 6.48 (d, $J = 7.2$ Hz, 1H), 6.30 (d, $J = 7.2$ Hz, 1H), 4.73-4.69 (m, 2H), 4.58 (pentet, $J = 7.2$ Hz, 1H), 4.53 (pentet, $J = 7.2$ Hz, 1H), 2.94-2.90 (m, 2H), 2.88-2.85 (m, 2H), 1.97 (s, 3H), 1.96 (s, 3H), 1.41 (d, $J = 6.6$, 3H), 1.38 (d, $J = 7.2$, 3H); ESI-MS: $m/z$ calcd for C$_{49}$H$_{45}$N$_4$O$_3$S 769.3, found: 769.3 [M+H]$^+$. 

\[ \text{\textit{N-Fmoc-L-prolyl-L-alanine Trityl Thioester (35).}} \] The peptide (19.7 mg, 0.048 mmol) was reacted with HATU (62.9 mg, 0.17 mmol), tritylmethanethiol (32.6 mg, 0.12 mmol), DIPEA (0.035 mL, 0.21 mmol) in DMF (0.75 mL) to furnish the thioester after 3 h following the General Procedure given for amino acid thioesters. The crude material was purified by flash column chromatography on SiO$_2$ (1.6 x 14 cm) using a step-wise gradient elution with MeOH-CH$_2$Cl$_2$ (0:100 and 0:599.5) to afford the thioester as a yellowish film. Yield: 11.7 mg (36%); silica gel TLC $R_f = 0.09$ (MeOH-CHCl$_3$ = 1:99); ESI-MS: $m/z$ calcd for C$_{42}$H$_{38}$NaN$_2$O$_4$S 689.2, found: 689.1 [M+Na]$^+$. 

\[ \text{dNBS-Alanine tert-Butyl Ester (41).} \] To a well-stirred mixture of alanine tert-butyl ester hydrochloride salt (0.200 g, 1.10 mmol) and 2,4-dinitrobenzenesulfonyl chloride (0.710
g, 2.66 mmol) in dry CH$_2$Cl$_2$ (1.0 mL) at 0 °C was added pyridine (0.72 mL, 8.80 mmol) dropwise. Cooling was removed and the mixture was stirred further for 16 h. The mixture was diluted with CH$_2$Cl$_2$ (40 mL), washed with 1 N HCl (1 x 10 mL), water (1 x 10 mL) and finally saturated NaCl (1 x 10 mL). Each of the aqueous extracts was back-extracted with CH$_2$Cl$_2$ (1 x 20 mL). The combine organic extracts were dried (Na$_2$SO$_4$), filtered and concentrated to dryness under reduced pressure. Purification of the residue by flash column chromatography on SiO$_2$ (3.5 x 6 cm) using step-wise gradient of ethyl acetate-hexanes (5:95 and 10:90) afforded the product as yellowish amorphous solid. Yield 0.274 g (66%); silica gel TLC $R_f$ = 0.27 (ethyl acetate-hexanes = 2:8); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 8.73 (d, $J = 2.4$ Hz, 1H), 8.52 (dd, $J = 1.8$, 8.4, 1H), 8.29 (d, $J = 8.4$ Hz, 1H), 6.18 (d, $J = 9.0$ Hz, 1H, NH), 4.20-4.15 (m, 1H, $\alpha$-CH), 1.47 (d, $J = 7.2$ Hz, 3H, Ala CH3), 1.29 (s, 9H, tBu); $^{13}$C NMR (150 MHz): $\delta$ 170.75 (C=O), 149.83, 148.06, 140.05, 132.28, 127.27, 121.13, 83.20, 53.56, 27.85 (C(CH$_3$)$_3$), 19.70; ESI-MS: m/z calcd for C$_{13}$H$_{17}$N$_3$O$_8$S 375.1, found: 375.5 [M]$^+$.

**General Procedure for Thioacids and Ligation.** A solution of the amino acid thioester in the cleavage cocktail consisting of TFA-TIPS-CH$_2$Cl$_2$ (50:5:45) was stirred at room temperature under N$_2$ atmosphere for 15 min. Then the mixture was concentrated to dryness under reduced pressure and the crude material thus obtained was left in high vacuum for at least 1 h. The thioacid product was detected by mass spectrometry. To the solution of the crude thioacid (1.2 eq) in dry DMF (0.5 mL) was added CsCO$_3$ under N$_2$ atmosphere. The mixture was stirred for 10 min at room temperature under N$_2$ atm before the addition of dNBS-alanine tert-butyler (1.0 eq). To the resulting red solution
was added another portion of anhydrous DMF (0.50 mL). The reaction was monitored by TLC. The mixture was diluted with ethyl acetate (60 mL), washed with cold saturated NaHCO$_3$ (1 x 20 mL), followed by cold water (1 x 20 mL) and finally, cold brine (1 x 20 mL). The aqueous layers were back-extracted with ethyl acetate (1 x 20 mL). The combined organic phases was dried (anhydrous Na$_2$SO$_4$), filtered, and the filtrate concentrated under reduced pressure.

![FmocHN N N H O tBu O O N N H - Fmoc - L - histidyl - L - alanine tert-Butyl Ester (42).](image)

**N-Fmoc-L-histidyl-L-alanine tert-Butyl Ester (42).** Treatment of histidine thioester 22 (95.2 mg, 0.10 mmol) with the cleavage cocktail (1.8 mL) furnished the crude thioacid (105.4 mg). ESI-MS: $m/z$ calcd for C$_{21}$H$_{20}$N$_3$O$_3$S 394.1, found: 394.3 [M+H]$^+$. The crude thioacid was reacted with the sulfonamide (33.8 mg, 0.090 mmol) in the presence of CsCO$_3$ (44.0 mg, 0.14 mmol) to produce the dipeptide after 1 h following the General Procedure for Thioacids and Ligation. The crude material was purified by flash column chromatography on SiO$_2$ (2.5 x 13 cm) using MeOH-CHCl$_3$-hexanes (5:45:50) to provide a light yellow solid. Yield: 33.2 mg (74%); silica gel TLC $R_f = 0.21$ (MeOH-CHCl$_3$-hexanes = 10:40:50); $^1$H NMR (600 MHz, DMSO-$d_6$): $\delta$ 7.89 (d, $J = 7.2$ Hz, 2H, aromatic H), 7.67 (t, $J = 9.0$ Hz, 2H, aromatic H), 7.56 (s, 1H, imidazole H), 7.44-7.38 (m, 2H, aromatic H), 7.32 (q, $J = 7.2$ Hz, 2H, aromatic H), 6.81 (s, 1H, imidazole H), 4.27 (dd, $J = 3.6$, 9.6, 1H, His-$\alpha$-CH), 4.23-4.15 (m, 3H, Fmoc CHCH$_2$), 4.11 (q, $J = 7.2$ Hz, 1H, Ala-$\alpha$-CH), 2.94 (d, $J = 12.0$, His-$\beta$-CH), 2.78 (dd, $J = 10.2$, 14.4, 1H, His-$\beta$-CH), 1.38 (s, 9H, tBu), 1.25 (d, $J = 7.2$ Hz, 3H, Ala-CH$_3$); $^{13}$C NMR (150 MHz, DMSO-
Treatment of glycine thioester 28 (79.7 mg, 0.14 mmol) with the cleavage cocktail (2.60 mL) furnished the crude thioacid (76.9 mg) as a colorless solid. ESI-MS: m/z calcd for C_{17}H_{15}NaNO_{3}S 336.1, found: 336.4. The crude thioacid was reacted with the sulfonamide (0.050 g, 0.13 mmol) in the presence of CsCO_{3} (0.065 g, 0.20 mmol) to produce the dipeptide after 2 h following the General Procedure for Thioacids and Ligation. The crude material was purified by flash column chromatography on SiO_{2} (2.5 x 9.5 cm) using MeOH-acetone-CHCl_{3}-hexanes (0.25:0.25:3:6.5). The partially purified material was subjected to another flash column chromatography (2.5 x 6 cm) using ethyl acetate-hexanes (4:6) to afford a colorless amorphous solid. Yield: 0.037 g (66%); silica gel TLC R_{f} = 0.20 (ethyl acetate-hexanes = 4:6); \textsuperscript{1}H NMR (600 MHz, CDCl_{3}): δ 7.77 (d, J = 7.2 Hz, 2H, aromatic H), 7.60 (d, J = 7.2 Hz, 2H, aromatic H), 7.40 (t, J = 7.2 Hz, 2H, aromatic H), 7.31 (t, J = 7.2 Hz, 2H, aromatic H), 6.69 (d, J = 7.2 Hz, 1H, Ala-NH), 5.65 (t, J = 5.4 Hz, 1H, Gly-NH), 4.49 (pentet, J = 7.2 Hz, 1H, Ala-α-CH), 4.41 (d, J = 7.2 Hz, 2H, Fmoc CH_{2}), 4.23 (t, J = 7.2 Hz, 1H, Fmoc CH), 3.96-3.91 (m, 2H, Gly-α-CH), 1.47 (s, 9H, tBu), 1.39 (d, J = 7.2 Hz, 3H, Ala CH_{3}); \textsuperscript{13}C NMR (150 MHz, CDCl_{3}): δ 172.18 (C=O), 168.47 (C=O), 156.73 (C=O), 143.93, 141.44, 127.90, 127.26, 125.27, 120.16, 82.42, 67.41, 48.88, 47.22, 44.53, 28.11 (C(\text{CH}_{3})_{3}), 18.73; ESI-MS: m/z calcd for C_{24}H_{28}NaN_{2}O_{5} 447.2, found: 447.0 [M+Na]\textsuperscript{+}. 

**N-Fmoc-glycyl-L-alanine tert-Butyl Ester (43).**
**N-Fmoc-L-seryl-L-alanine tert-Butyl Ester (44).** Treatment of serine thioester 29 (0.100 g, 0.16 mmol) with the cleavage cocktail (2.50 mL) furnished the crude thioacid (97.7 mg) as a colorless foam. ESI-MS: \( m/z \) calcld for \( C_{18}H_{17}NaNO_4S \) 366.1, found: 366.6 \([\text{M+Na}]^+\). The crude thioacid was reacted with the sulfonamide (0.049 g, 0.13 mmol) in the presence of CsCO\(_3\) (0.064 g, 0.20 mmol) to produce the dipeptide after 2 h following the General Procedure for Thioacids and Ligation. The crude material was purified by flash column chromatography on SiO\(_2\) (2.5 x 9.5 cm) using MeOH-CH\(_2\)Cl\(_2\) (2:98) to provide a yellow amorphous solid. Yield: 55.2 mg (93%); silica gel TLC \( R_f = 0.11 \) (MeOH-CH\(_2\)Cl\(_2\) = 2:98); \(^1\)H NMR (600 MHz, CDCl\(_3\)): \( \delta \) 7.76 (d, \( J = 7.8 \) Hz, 2H, aromatic H), 7.59 (d, \( J = 4.8 \) Hz, 2H, aromatic H), 7.40 (t, \( J = 7.2 \) Hz, 2H, aromatic H), 7.31 (t, \( J = 7.8 \) Hz, 2H, aromatic H), 7.12 (d, \( J = 7.2 \) Hz, 1H, Ala-NH), 5.99 (d, \( J = 7.8 \) Hz, 1H, Ser-NH), 4.46 (pentet, \( J = 7.8 \) Hz, 1H, Ala-\( \alpha \)-CH), 4.39 (d, \( J = 7.2 \) Hz, 2H, Fmoc CH\(_2\)), 4.35 (t, \( J = 6.0 \), 1H, Ser-\( \alpha \)-CH), 4.21 (t, \( J = 7.2 \) Hz, 1H, Fmoc CH), 4.03 (s, 1H, Ser-\( \beta \)-CH), 3.70 (s, 2H, Ser-\( \beta \)-CH & OH); \(^{13}\)C NMR (150 MHz, CDCl\(_3\)): \( \delta \) 172.39 (C=O), 170.61 (C=O), 156.56 (C=O), 143.90, 143.82, 141.43, 127.91, 127.26, 125.27, 120.16, 82.62, 67.48, 63.37, 55.69, 49.23, 47.18, 28.08, 17.99; ESI-MS: \( m/z \) calcld for \( C_{25}H_{30}NaN_2O_6 \) 477.2, found: 477.1 \([\text{M+Na}]^+\).

**N-Fmoc-L-threonyl-L-alanine tert-Butyl Ester (45).** Treatment of threonine thioester 30 (0.066 g, 0.10 mmol) with the cleavage cocktail (2.10 mL) furnished the crude
thioacid (57.0 mg) as a colorless solid. ESI-MS: \( m/z \) calcd for \( \text{C}_{19}\text{H}_{10}\text{NaNO}_{4}\text{S} \) 380.1, found: 380.0 \([\text{M+Na}]^+\). The crude thioacid was reacted with the sulfonamide (0.032 g, 0.084 mmol) in the presence of \( \text{CsCO}_3 \) (0.041 g, 0.13 mmol) to produce the dipeptide after 1 h following the General Procedure for Thioacids and Ligations. The crude material was purified by flash column chromatography on SiO\(_2\) (2.5 x 13 cm) using MeOH-CHCl\(_3\)-hexanes (1:49:50) to provide a yellowish film. Yield: 39.3 mg (100%); silica gel TLC \( R_f = 0.09 \) (MeOH-CHCl\(_3\)-hexanes = 2:48:50); \(^1\)H NMR (600 MHz, CDCl\(_3\)): \( \delta \) 7.77 (d, \( J = 7.8 \) Hz, 2H, aromatic H), 7.60 (d, \( J = 7.2 \) Hz, 2H, aromatic H), 7.41 (t, \( J = 7.2 \) Hz, 2H, aromatic H), 7.32 (t, \( J = 7.8 \) Hz, 2H, aromatic H), 6.98 (d, \( J = 5.4 \) Hz, 1H, Ala-NH), 5.85 (d, \( J = 7.8 \) Hz, 1H, Thr-NH), 4.46-4.43 (m, 2H, Ala-\( \alpha \)-CH & Fmoc CH), 4.41-4.38 (m, 1H, Fmoc CH), 4.34 (dd, \( J = 2.4, 6.0 \) Hz, 1H, Thr-\( \beta \)-CH), 4.24-4.20 (m, 2H, Thr-\( \alpha \)-CH & Fmoc CH), 1.47 (s, 9H, tBu), 1.38 (d, \( J = 7.2 \) Hz, 3H, Ala-CH\(_3\)), 1.19 (d, \( J = 6.6 \) Hz, 3H, Thr-CH\(_3\)); \(^1^3\)C NMR (150 MHz, CDCl\(_3\)): \( \delta \) 172.09, 170.42, 162.79, 156.85, 143.92, 143.82, 141.45, 127.92, 127.25, 125.28, 125.23, 120.18, 120.16, 82.42, 67.40, 67.30, 58.62, 49.06, 47.25, 18.23, 18.12; ESI-MS: \( m/z \) calcd for \( \text{C}_{26}\text{H}_{32}\text{NaN}_{2}\text{O}_{6} \) 491.2, found: 491.6 \([\text{M+Na}]^+\).

**N-Fmoc-L-prolyl-L-alanine tert-Butyl Ester (46).** Treatment of proline thioester 31 (52.8 mg, 0.089 mmol) with the cleavage cocktail (1.4 mL) furnished the crude thioacid (48.7 mg) as a colorless solid. ESI-MS: \( m/z \) calcd for \( \text{C}_{20}\text{H}_{19}\text{NaNO}_{3}\text{S} \) 376.1, found: 376.7 \([\text{M+Na}]^+\). The crude thioacid was reacted with the sulfonamide (27.7 mg, 0.074 mmol) in the presence of \( \text{CsCO}_3 \) (36.1 mg, 0.11 mmol) to produce the dipeptide after 1 h following the General Procedure for Thioacids and Ligation. The crude material was purified by
flash column chromatography on SiO$_2$ (2.5 x 12 cm) using MeOH-CHCl$_3$-hexanes (4:11:85) to provide a colorless amorphous solid. Yield: 34.0 mg (99%); silica gel TLC $R_f = 0.07$ (MeOH-CHCl$_3$-hexanes = 5:10:85); $^1$H NMR (600 MHz, CD$_3$OD): $\delta$ 7.80 (dd, $J$ = 3.6, 7.8 Hz, 4H), 7.66 (d, $J$ = 7.2 Hz, 1H), 7.63 (d, $J$ = 7.2 Hz, 1H), 7.61 (d, $J$ = 7.8 Hz, 1H), 7.59 (d, $J$ = 7.8 Hz, 1H), 7.40 (t, $J$ = 7.8 Hz, 4H), 7.31 (q, $J$ = 7.2 Hz, 4H), 4.43 (dd, $J$ = 6.0, 10.2 Hz, 1H, Fmoc CH), 4.37 (d, $J$ = 6.6 Hz, 2H, Fmoc CH$_2$), 4.31 (dd, $J$ = 3.6, 9.0 Hz, 1H, Pro-α-CH), 4.28-4.21 (m, 5H, Pro-α-CH & Ala-α-CH x 2 & Fmoc CH x 2), 4.20-4.12 (m, 1H, Fmoc CH), 3.62-3.58 (m, 1H, Pro-δ-CH), 3.58-3.54 (m, 1H, Pro-δ-CH), 3.51-3.45 (m, 1H, Pro-δ-CH), 3.45-3.40 (m, 1H, Pro-δ-CH), 2.33-2.25 (m, 1H, Pro-β-CH), 2.25-2.18 (m, 1H, Pro-β-CH), 2.12-2.06 (m, 1H, Pro-β-CH), 2.06-2.00 (m, 1H, Pro-β-CH), 2.00-1.93 (m, 2H, Pro-γ-CH$_2$), 1.93-1.88 (m, 2H, Pro-γ-CH$_2$), 1.45 (s, 3H, tBu), 1.43 (s, 3H, tBu), 1.36 (d, $J$ = 7.2, 3H, Ala-CH$_3$), 1.31 (d, $J$ = 7.2 Hz, 3H, Ala-CH$_3$); $^{13}$C NMR (150 MHz, CD$_3$OD): $\delta$ 175.02, 174.94, 174.85, 174.77, 173.52, 173.43, 173.43, 156.85, 156.69, 145.67, 145.55, 145.27, 145.16, 142.78, 142.71, 142.62, 129.01, 128.96, 128.33, 128.32, 128.29, 126.37, 126.30, 126.18, 121.12, 121.11, 82.73, 82.67, 69.06, 68.83, 61.50, 61.46, 61.19, 61.15, 50.49, 50.04, 48.68, 48.56, 48.53, 48.22, 32.76, 31.57, 28.33 (C(CH$_3$)$_3$), 25.45, 24.52, 17.60, 17.55, 17.48, 17.43; ESI-MS: $m/z$ calcd for C$_{27}$H$_{32}$NaN$_2$O$_5$ 487.2, found: 487.5 [M+Na]$^+$. 

![Image of the compound structure]
**N-Fmoc-His-Gly-Val-(Ac₃-Tn-α-Thr)-Ser-Ala-OH (47).** To the solution of the crude histidine thioacid (1.2 eq) in dry DMF (0.2 mL) was added CsCO₃ (1.1 mg, 3.4 μmol) under N₂ atmosphere. The mixture was stirred for 10 min at room temperature under N₂ atm before the addition of N-peptidylsulfonamide (1.7 mg, 1.7 μmol). To the resulting red solution was added another portion of anhydrous DMF (0.10 mL). The reaction was monitored by ESI-MS and appeared complete in 2 h. The mixture was neutralized with 1N HCl to pH = 5 and concentrated to dryness under reduced pressure. The crude material was purified by semi-preparative RP-HPLC and freeze-dried to afford a colorless fluffy solid. Yield: 1.4 mg (71%); ¹H NMR (600 MHz, CD₃OD): δ 8.71 (s, 1H, imidazole H), 7.81 (d, J = 7.2 Hz, 2H), 7.62 (d, J = 7.8 Hz, 2H), 7.40 (t, J = 7.2 Hz, 2H), 7.33-7.30 (m, 2H), 7.22 (s, 1H, imidazole H), 7.02 (d, J = 9.0 Hz, 1H, NH), 6.65 (d, J = 8.4 Hz, 1H, NH), 5.34 (s, 1H, H-4), 5.14 (dd, J = 3.0, 11.4 Hz, 1H, H-3), 5.11 (d, J = 4.2 Hz, 1H, H-1), 4.63 (s, 1H, Thr-α-CH), 4.50 (t, J = 4.8 Hz, 1H, Ser-α-CH), 4.48-4.40 (m, 3H, His-α-CH & Fmoc CH₂), 4.39-4.34 (m, 2H, H-2, Ala-α-CH), 4.30-4.28 (m, 3H, Thr-β-CH & 2 other protons), 4.20 (t, J = 6.0 Hz, 1H, Fmoc CH), 4.09-4.03 (m, 2H), 3.97 (d, J = 5.4, 2H, Gly-CH₂), 3.82 (dd, J = 4.8, 11.4 Hz, 1H, Ser-β-CH), 3.78 (dd, J = 6.6, 11.4 Hz, 1H, Ser-β-CH), 3.14-3.09 (m, 2H, His-β-CH), 2.19-2.17 (m, 1H, Val-β-CH), 2.14 (s, 3H, CH₃CO), 2.00 (s, 3H, CH₃CO), 1.97 (s, 3H, CH₃CO), 1.93 (s, 3H, CH₃CO), 1.42 (d, J = 7.8 Hz, 3H, Ala-CH₃), 1.28 (d, J = 6.6 Hz, 3H, Thr-CH₃), 1.00 (d, J = 7.2 Hz, 3H, Val-CH₃), 0.99 (d, J = 7.2 Hz, 3H, Val-CH₃); ESI-MS: m/z calcd for C₅₂H₆₈N₉O₁₉ 1122.5, found: 1122.3 [M+H]⁺.
References


SUPPORTING INFORMATION FOR CHAPTER 2

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Page 281: $^1$H NMR of N-(2-Phenylethyl)phenylacetamide 55 Obtained From $[O^{18}]$-Phenylpyruvic Acid

Page 282: Mass Spectrum (ESI-MS) of N-Benzyl-2-phenylethanamide 55 ($m/z = 240.3$ [M+H]$^+$) Obtained From $[O^{18}]$-Phenylpyruvic Acid

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$^1$H NMR of $N$-Cyanomethylalanine tert-Butyl Ester 17
$^{13}$C NMR of $N$-Cyanomethylalanine tert-Butyl Ester 17
$^1$H NMR of N-Cyanomethyl Valine tert-Butyl ester 18
$^{13}$C NMR of N-Cyanomethyl Valine *tert*-Butyl Ester 18
$^1$H NMR of $N$-Cyanomethylalanine Benzyl Ester 19
$^{13}$C NMR of \textit{N}-Cyanomethylalanine Benzyl Ester 19
$^1$H NMR of $N$-Cyanomethyl Phenethylamine 20
$^{13}$C NMR of $N$-Cyanomethyl Phenethylamine 20
$^1$H NMR of $N$-Cyanomethyl $N$-Oxide Glycine tert-Butyl Ester 21
$^1$H NMR of N-Cyanomethyl N-Oxide Alanine tert-Butyl Ester 22
$^{13}$C NMR of N-Cyanomethyl N-Oxide Alanine tert-Butyl Ester 22
$^1$H NMR of $N$-Cyanomethyl $N$-Oxide Valine tert-Butyl Ester 23
$^{13}$C NMR of N-Cyanomethyl N-Oxide Valine tert-Butyl Ester 23
$^1$H NMR of $N$-Cyanomethyl $N$-Oxide Alanine Benzyl Ester 24
$^1$H NMR of $N$-Cyanomethyl $N$-Oxide Phenethylamine 25
$^{13}$C NMR of $N$-Cyanomethyl $N$-Oxide Phenethylamine 25
$^1$H NMR of $N$-Hydroxyglycine \textit{tert}-Butyl Ester 4

\[
\text{HO}^+ \text{N} \text{O} \text{tBu} \cdot \frac{1}{2}(\text{COOH})_2
\]
$^{13}$C NMR of $N$-Hydroxyglycine tert-Butyl Ester 4

![Chemical Structure](image-url)
$^1$H NMR of $N$-Hydroxyalanine tert-Butyl Ester 5
$^{13}$C NMR of $N$-Hydroxyalanine tert-Butyl Ester 5

![Chemical structure of $N$-Hydroxyalanine tert-Butyl Ester 5]
$^1$H NMR of $N$-Hydroxyvaline tert-Butyl Ester 6

![NMR Spectrum](image)

Structure:

\[ \text{HO-}N^+\text{C(O)(O)}_2\text{OtBu} \cdot \frac{1}{2}(\text{COOH})_2 \]
$^{13}$C NMR of N-Hydroxyvaline tert-Butyl Ester 6

$\text{HO}^-\text{NH}^-\text{O(Bu)}\cdot 1/2(\text{COOH})_2$
$^1$H NMR of N-Hydroxyalanine Benzyl Ester 7

$\text{HO}^+\text{N}^\text{\textsuperscript{\texttrademark}}\text{\textsuperscript{\texttrademark}}\text{O}^\text{\textsuperscript{\texttrademark}}\text{\textsuperscript{\texttrademark}}\text{\textsuperscript{\texttrademark}}\text{\textsuperscript{\texttrademark}}\text{\textsuperscript{\texttrademark}}\text{CH}_3 \bullet 1/2(\text{COOH})_2$
$^{13}$C NMR of N-Hydroxyalanine Benzyl Ester 7

\[
\begin{align*}
\text{HO-} & \quad \text{N} \\
& \quad \text{CH}_3 \\
\end{align*}
\]

$\text{O} \quad \text{OfBu} \quad \bullet \quad \frac{1}{2} (\text{COOH})_2$
$^1$H NMR of $N$-Hydroxy Phenethylamine 9

\[ \text{HO}^-\text{N} - \text{C}_8\text{H}_5 \cdot \frac{1}{2}(\text{COOH})_2 \]
$^{13}$C NMR of N-Hydroxy Phenethylamine \textbf{9}
$^1$H NMR of Triphenylcyanophosphorane 30
$^{13}$C NMR of Triphenylcyanophosphorane 30
$^1$H NMR of Boc-Protected Alanine Cyanophosphorane 31
$^{13}$C NMR of Boc-Protected Alanine Cyanophosphorane 31
$^{31}$P NMR of Boc-Protected Alanine Cyanophosphorane 31
$^1$H NMR of Boc-Protected Valine Cyanophosphorane 32
$^{13}$C NMR of Boc-Protected Valine Cyanophosphorane 32
$^{31}$P NMR of Boc-Protected Valine Cyanophosphorane 32
$^1$H NMR of Fmoc-Protected Alanine Cyanophosphorane 33
\(^1\)H NMR of Fmoc-Protected Valine Cyanophosphorane 34

![Chemical Structure](image-url)
$^{13}$C NMR of Fmoc-Protected Valine Cyanophosphorane 34
$^{31}$P NMR of Fmoc-Protected Valine Cyanophosphorane 34
$^1$H NMR of Alanine Cyanophosphorane 35

![NMR spectrum of compounds](image-url)
$^1$H NMR of Valine Cyanophosphorane 36
$^1$H NMR of Alanine Cyanophosphorane 37
$^{31}$P NMR of Alanine Cyanophosphorane 37
\(^1\)H NMR of Valine Cyanophosphorane 38
\(^1\)H NMR of 3,4,6-Tri-O-acetyl-D-galactal 42
$^1$H NMR of 3,4,6-Tri-$O$-acetyl-2-azido-2-deoxy-$\alpha$-$D$-galactopyranosyl Nitrate 43
$^1$H NMR of 3,4,6-Tri-O-acetyl-2-azido-2-deoxy-$\alpha$-D-galactopyranosyl Bromide 44
$^1$H NMR of $N$-(9-Fluorenylmethoxycarbonyl)-threonine Benzyl Ester 45
$^{13}$C NMR of $N$-(9-Fluorenylmethoxycarbonyl)-threonine Benzyl Ester 45
$^1$H NMR of $N^\alpha$-(Floren-9-ylmethoxycarbonyl)-$O$-(3,4,6-tri-$O$-acetyl-2-azido-2-deoxy-$\alpha$-$D$-galactopyranosyl)-$L$-threonine Benzyl Ester

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\(^1\)H NMR of \(N^\alpha-(\text{Floren-9-ylmethoxycarbonyl})-O-(2\text{-acetamido-2-deoxy-3,4,6-tri-O-acetyl-}\alpha\text{-D-galactopyranosyl})\)-L-threonine Benzyl Ester 47
$^1$H NMR of $N^\alpha$-(Fmoc-9-ylmethoxycarbonyl)-O-(2-Aacetamido-2-deoxy-3,4,6-tri-O-acetyl-$\alpha$-D-galactopyranosyl)-L-threonine 8
$^{13}$C NMR of $N^\alpha$-(Floren-9-ylmethoxycarbonyl)-$O$-(2-Acetamido-2-deoxy-3,4,6-tri-$O$-acetyl-$\alpha$-D-galactopyranosyl)-L-threonine 8
$^1$H NMR of Fmoc-Ac$_3$-Tn-α-Thr-Ala-Cyanophosphorane Analog 48
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$^{31}$P NMR of Fmoc-Ac$_2$-Tn-α-Thr-Ala-Cyanophosphorane Analog 48
$^1$H-$^1$H gCOSY NMR of Fmoc-Ac$_3$-Tn-$\alpha$-Thr-Ala-Cyanophosphorane Analog 48
HMBC NMR of Fmoc-Ac$_3$-Tn-α-Thr-Ala-Cyanophosphorane Analog 48
$^1$H NMR of $N^\alpha$-(Floren-9-ylmethoxycarbonyl)-Alanylalanine Benzyl Ester 50
$^{13}$C NMR of $\alpha$-(Fmoc-9-ylmethoxycarbonyl)-Alanylalanine Benzyl Ester 50
Variable Temperature $^1$H NMR of $N^\alpha$-(Fmoc-9-ylmethoxycarbonyl)-Alanylalanine Benzyl Ester 50

R.T.

40 °C

60 °C
\(^1\)H NMR of Glycotripeptide \textit{tert}-Butyl Ester 51
$^{13}$C NMR of Glycotripeptide *tert*-Butyl Ester 51
$^1$H-$^1$H gCOSY NMR of Glycotripeptide tert-Butyl Ester 51
$^1$H NMR of Glycopeptide-Derived Oxazole Byproduct 52
$^{13}$C NMR of Glycopeptide-Derived Oxazole Byproduct 52
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$^1$H NMR of Glycotripeptide tert-Butyl Ester 53
13C NMR of Glycotripeptide tert-Butyl Ester 53
$^{1}H-^{1}H$ gCOSY NMR of Glycotripeptide tert-Butyl Ester 53
$^1$H NMR of [O$^{18}$]-Phenylpyruvic Acid 10
$^{13}$C NMR of [O$^{18}$]-Phenylpyruvic Acid 10
Mass Spectrum (ESI-MS) of \([O^{18}]\)-Phenylpyruvic Acid 10 \([m/z = 145.1 (M+Na-CO_2)^+]\)
$^1$H NMR of $N$-(2-Phenylethyl)phenylacetamide 55 Obtained From [O$^{18}$]-Phenylpyruvic Acid
Mass Spectrum (ESI-MS) of $N$-Benzyl-2-phenylethanamide 55 ($m/z = 240.3$ [M+H]$^+$) Obtained From [O$^{18}$]-Phenylpyruvic Acid
$^1$H NMR of $N$-(2-Phenylethyl)phenylacetamide 55 Obtained from [O$^{16}$]- Phenylpyruvic Acid
$^{13}$C NMR of $N$-(2-Phenylethyl)phenylacetamide 55 Obtained from [O$^{16}$]- Phenylpyruvic Acid
Mass Spectrum (ESI-MS) of N-(2-Phenylethyl)phenylacetamide 55 Obtained from [O^{16}]- Phenylpyruvic Acid
Mass Spectrum (ESI-MS) of O\textsuperscript{18}-Labeled Phenyl Acetic Acid 57 ($m/z = 163.1$ [M+Na]$^+$)
Mass Spectrum (ESI-MS) of O\textsuperscript{18}-Labeled N-(2-Phenylethyl)phenylacetamide \textbf{58} \((m/z = 242.4 \ [M+H]^+)\)
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Page 289: Mass Spectrum (ESI-MS) of Crude Fmoc-Gly-Ala-Cyanoketophosphorane 8 (m/z =652.3 [M+H]+)

Page 290: Mass Spectrum (ESI-MS) of Crude Fmoc-Ala-Ala-Cyanoketophosphorane 9 (m/z =666.3 [M+H]+)

Page 291: Mass Spectrum (ESI-MS) of Crude Fmoc-Pro-Ala-Cyanoketophosphorane 10 (m/z =692.1 [M+H]+)

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Page 295: 13C NMR Fmoc-Protected Glycine Piperidine Amide 12

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Page 297: 13C NMR of N,O-bis-Fmoc Glycine Hydroxylamine tert-Butyl Ester 15

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Page 299: 13C NMR of N,O-bis-Fmoc Alanine Hydroxylamine tert-Butyl Ester 16

Page 300: 1H NMR of N,O-bis-Fmoc Glycine Hydroxylamine 17

Page 301: 13C NMR of N,O-bis-Fmoc Glycine Hydroxylamine 17

Page 302: 1H NMR of N,O-bis-Fmoc Alanine Hydroxylamine 18

Page 303: 13C NMR of N,O-bis-Fmoc Alanine Hydroxylamine 18

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Page 305: 1H NMR of Fmoc-Decomposition Product

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Mass Spectrum (ESI-MS) of Crude Fmoc-Gly-Ala-Cyanoketophosphorane 8 ($m/z = 652.3$ [M+H]$^+$)
Mass Spectrum (ESI-MS) of Crude Fmoc-Ala-Ala-Cyanoketophosphorane 9 (m/z = 666.3 [M+H]^+)<br><br>![Mass Spectrum Diagram]
Mass Spectrum (ESI-MS) of Crude Fmoc-Pro-Ala-Cyanoketophosphorane 10 ($m/z =692.1 \ [M+H]^+$)

![Mass Spectrum of Crude Fmoc-Pro-Ala-Cyanoketophosphorane 10]
$^1$H NMR of Fmoc-Protected Glycylalanine Cyanoketophosphorane 11
$^{13}$C NMR of Fmoc-Protected Glycylalanine Cyanoketophosphorane 11
$^1$H NMR Fmoc-Protected Glycine Piperidine Amide 12
$^{13}$C NMR Fmoc-Protected Glycine Piperidine Amide 12
\(^1\)H NMR of \(N,O\)-bis-Fmoc Glycine Hydroxylamine \(\text{tert-Butyl Ester} \ 15\)
$^{13}$C NMR of $N,O$-bis-Fmoc Glycine Hydroxylamine tert-Butyl Ester 15
$^1$H NMR of $N,O$-bis-Fmoc Alanine Hydroxylamine *tert*-Butyl Ester 16
$^{13}$C NMR of $N,O$-bis-Fmoc Alanine Hydroxylamine \textit{tert}-Butyl Ester 16
$^1$H NMR of $N,O$-bis-Fmoc Glycine Hydroxylamine 17
$^{13}$C NMR of $N,O$-bis-Fmoc Glycine Hydroxylamine 17
$^1$H NMR of $N,O$-bis-Fmoc Alanine Hydroxylamine 18
$^{13}$C NMR of $N,O$-bis-Fmoc Alanine Hydroxylamine 18
\(^1\)H NMR \(N,O\)-bis-Fmoc Alanylalanine Hydroxylamine 22

\[
\text{Fmoc} - \text{N} - \text{O} - \text{OtBu}
\]
$^1$H NMR of Fmoc-Decomposition Product
$^{13}$C NMR of Fmoc-Decomposition Product
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Page 319: $^1$H NMR of Benzy1 $N^\alpha$-$tert$-butoxycarbonyl-$N$-(2-acetamido-3,4,6-tri-$O$-acetyl-2-deoxy-$\beta$-$D$-glucopyranosyl)-$L$-asparaginate 12

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Page 322: $^{13}$C NMR of dNBS-Alanine 13

Page 323: $^1$H NMR of dNBS-Ser-Ala-OH 15

Page 324: $^1$H-$^1$H gCOSY NMR of dNBS-Ser-Ala-OH 15

Page 325: $^1$H NMR of dNBS-Val-Thr-Ser-Ala-OH 16

Page 326: Characteristic region of $^1$H-$^1$H gCOSY NMR of dNBS-Val-Thr-Ser-Ala-OH 16

Page 327: $^1$H NMR of dNBS-Gly-Val-Thr-Ser-Ala-OH 17

Page 328: $^1$H-$^1$H gCOSY NMR of dNBS-Gly-Val-Thr-Ser-Ala-OH 17
Page 329: $^1$H NMR of dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH 18

Page 330: $^1$H-1H gCOSY NMR of dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH 18

Page 331: Analytical RP-HPLC of (A) purified and (B) crude dNBS-Val-Thr-Ser-Ala-OH (16) eluting with 35-90% gradient of H$_2$O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm

Page 332: Analytical RP-HPLC of (A) purified and (B) crude dNBS-Gly-Val-Thr-Ser-Ala-OH (17) eluting with 30-90% gradient of H$_2$O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.

Page 333: Analytical RP-HPLC of (A) purified and (B) crude dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (18) eluting with 30-90% gradient of H$_2$O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.

Page 334: Analytical RP-HPLC of (A) purified and (B) crude dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (18) obtained from reagent K treatment of the resin, eluting with 30-90% gradient of H$_2$O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.

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Page 343: $^{13}$C NMR of N-α-Fmoc-O-tert-Butyl-Protected L-Serine Trityl Thioester 29

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Page 356: Mass Spectrum (ESI-MS) of N-Fmoc-L-prolyl-L-alanine Trityl Thioester 35 ($m/z = 689.1$ [M+Na$^+$])

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Page 358: $^{13}$C NMR of dNBS-Alanine tert-Butyl Ester 41

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Page 363: $^{13}$C NMR of N-Fmoc-glycly-L-alanine tert-Butyl Ester 43

Page 364: $^1$H-$^1$H gCOSY NMR of N-Fmoc-glycly-L-alanine tert-Butyl Ester 43

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Page 370: $^1$H-$^1$H gCOSY NMR of N-Fmoc-L-threonyl-L-alanine tert-Butyl Ester 45

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Page 372: $^{13}$C NMR of N-Fmoc-L-prolyl-L-alanine tert-Butyl Ester 46

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$^1$H NMR of 9-Fluorenymethyl $p$-Toluenesulfonate 5
$^1$H NMR of 9-Fluorenylmethyl Thioacetate 6
$^{13}$C NMR of 9-Fluorenlylmethyl Thioacetate 6
$^1$H NMR of 9-Fluorenymethylthiol 7
$^{13}$C NMR of 9-Fluorenylmethylthiol 7
$^1$H NMR of α-Benzyl-N-tert-butoxycarbonyl-L-β-aspartyl 9-fluorenymethyl thioester 9

![Chemical structure of the compound](image)
$^{13}$C NMR of $\alpha$-Benzyl-$N$-tert-butoxycarbonyl-$\beta$-aspartyl 9-fluorenylmethyl thioester 9
Mass Spectrum (ESI-MS) of $\alpha$-Benzyl-$N$-tert-butoxycarbonyl-L-$\beta$-aspartyl thioacid 10
$^1$H NMR of benzyl $N^\alpha$-tert-butoxycarbonyl-$N$-(2-acetamido-3,4,6-tri-$O$-acetyl-2-deoxy-$\beta$-$D$-glucopyranosyl)-L-asparagine 12
$^{13}$C NMR of benzyl $N^\alpha$-tert-butoxycarbonyl-$N$-(2-acetamido-3,4,6-tri-$O$-acetyl-2-deoxy-$\beta$-$D$-glucopyranosyl)-L-asparagine 12
$^1$H NMR of dNDS-Alanine-OH 13
$^{13}$C NMR of dNBS-Alanine-OH 13
$^1$H NMR of dNBS-Ser-Ala-OH 15
$^{1}\text{H}-^{1}\text{H} \text{gCOSY NMR of dNBS-Ser-Ala-OH 15}$
$^1$H NMR of dNBS-Val-Thr-Ser-Ala-OH 16
Characteristic region of $^1$H-$^1$H gCOSY NMR of dNBS-Val-Thr-Ser-Ala-OH 16
\(^1\text{H NMR of dNBS-Gly-Val-Thr-Ser-Ala-OH 17}\)
$^{1}H-^{1}H$ gCOSY NMR of dNBS-Gly-Val-Thr-Ser-Ala-OH 17
H NMR of dNBS-Gly-Val-(Ac₃-Tn-α-Thr)-Ser-Ala-OH 18
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Figure 1. Analytical RP-HPLC of (A) purified and (B) crude dNBS-Val-Thr-Ser-Ala-OH (16) eluting with 35-90% gradient of H\textsubscript{2}O (0.1\% TFA) and MeOH (0.1\% TFA) over a period of 35 minutes, UV detection at 254 nm.
Figure 2. Analytical RP-HPLC of (A) purified and (B) crude dNBS-Gly-Val-Thr-Ser-Ala-OH (17) eluting with 30-90% gradient of H$_2$O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.
Figure 3. Analytical RP-HPLC of (A) purified and (B) crude dNBS-Gly-Val-(Ac₃-Tn-α-Thr)-Ser-Ala-OH (18) eluting with 30-90% gradient of H₂O (0.1% TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.
Figure 4. Analytical RP-HPLC of (A) purified and (B) crude dNBS-Gly-Val-(Ac₃-Tn-α-Thr)-Ser-Ala-OH (18) obtained from reagent K treatment of the resin, eluting with 30-90% gradient of H₂O (0.1% TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.
H NMR of \( N\text{-}\alpha\text{-Fmoc-}N\text{-im-Trityl-Protected L-Histidine Trityl Thioester} \) \( 21 \)
$^{13}$C NMR of N-$\alpha$-Fmoc-$N$-im-Trityl-Protected L-Histidine Trityl Thioester 21
$^1$H-$^1$H gCOSY NMR of $N$-α-Fmoc-$N$-im-Trityl-Protected L-Histidine Trityl Thioester 21
$^1$H NMR of Boc-Protected Glycine Trityl Thioester 27
$^{13}$C NMR of Boc-Protected Glycine Trityl Thioester 27
$^1$H NMR of Fmoc-Protected Glycine Trityl Thioester 28
$^{13}$C NMR of Fmoc-Protected Glycine Trityl Thioester 28
$^1$H NMR of $N$-$\alpha$-Fmoc-$O$-$\text{ tert}$-Butyl-Protected $\text{L}$-$\text{Serine Trityl Thioester}$ 29
$^{13}$C NMR of N-$\alpha$-Fmoc-$O$-$\text{tert}$-Butyl-Protected L-Serine Trityl Thioester 29
$^{1}H-^{1}H$ gCOSY NMR of $N$-$\alpha$-Fmoc-$O$-$\text{tert}$-Butyl-Protected L-Serine Trityl Thioester 29
$^1$H NMR of $N$-$\alpha$-Fmoc-$O$-tert-Butyl-Protected L-Threonine Trityl Thioester 30
$^{13}$C NMR of $N$-$\alpha$-Fmoc-$O$-$\text{tert}$-Butyl-Protected $L$-Threonine Trityl Thioester 30
$^1$H-$^1$H gCOSY NMR $N$-α-Fmoc-$O$-tert-Butyl-Protected L-Threonine Trityl Thioester 30
$^1$H NMR of Fmoc-Protected L-Proline Trityl Thioester 31
$^{13}$C NMR of Fmoc-Protected L-Proline Trityl Thioester 31
$^1$H-$^1$H gCOSY NMR of Fmoc-Protected L-Proline Trityl Thioester 31
$^1$H NMR of $N$-Fmoc-L-alanyl-L-histidine 32
\(^1\)H-\(^1\)H gCOSY NMR of N-Fmoc-L-alanly-L-histidine 32
Mass Spectrum (ESI-MS) of \(N\text{-Fmoc-L-prolyl-L-alanine}\) \(33\) \((m/z = 408.9 [M]^+)\)
$^1$H NMR of $N$-Fmoc-alanylhistidine Trityl Thioester 34
$^1$H-$^1$H gCOSY NMR of N-Fmoc-alanylhistidine Trityl Thioester
Mass Spectrum (ESI-MS) of N-Fmoc-L-prolyl-L-alanine Trityl Thioester 35 ($m/z = 689.1$ [M+Na]$^+$)
$^1$H NMR of dNBS-Alanine tert-Butyl Ester 41

\[ \text{Structure Image} \]
$^{13}\text{C}$ NMR of dNBS-Alanine \textit{tert}-Butyl Ester 41
$^1$H NMR of N-Fmoc-L-histidyl-L-alanine tert-Butyl Ester 42
$^{13}$C NMR of N-Fmoc-L-histidyl-L-alanine tert-Butyl Ester 42
$^1$H-$^1$H gCOSY NMR of N-Fmoc-L-histidyl-L-alanine tert-Butyl Ester 42
$^1$H NMR of $N$-Fmoc-glycyl-L-alanine tert-Butyl Ester 43
$^{13}$C NMR of N-Fmoc-glycyl-L-alanine *tert*-Butyl Ester 43
$^1$H-$^1$H gCOSY NMR of N-Fmoc-glycyl-L-alanine tert-Butyl Ester 43
$^{1}$H NMR of N-Fmoc-L-seryl-L-alanine tert-Butyl Ester 44
$^{13}$C NMR of N-Fmoc-L-seryl-L-alanine tert-Butyl Ester 44
$^{1}$H-$^{1}$H gCOSY NMR of N-Fmoc-L-seryl-L-alanine tert-Butyl Ester
$^1$H NMR of N-Fmoc-L-threonyl-L-alanine tert-Butyl Ester 45
$^{13}$C NMR of $N$-Fmoc-L-threonyl-L-alanine tert-Butyl Ester 45
$^1$H-$^1$H gCOSY NMR of N-Fmoc-L-threonyl-L-alanine tert-Butyl Ester 45
$^1$H NMR of N-Fmoc-L-prolyl-L-alanine tert-Butyl Ester 46
$^{13}$C NMR of N-Fmoc-L-prolyl-L-alanine tert-Butyl Ester 46
$^1$H-$^1$H gCOSY NMR of N-Fmoc-L-prolyl-L-alanine tert-Butyl Ester 46
$^1$H NMR of $N$-Fmoc-His-Gly-Val-($Ac_3$-Tn-$\alpha$-Thr)-Ser-Ala-OH 47
$^{1}$$^{1}$H gCOSY NMR of N-Fmoc-His-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH 47