Nitrone protecting groups for enantiopure *N*-hydroxyamino acids: synthesis of N-terminal peptide hydroxylamines for chemoselective ligations†

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Received 19th March 2010, Accepted 13th May 2010
First published as an Advance Article on the web 22nd June 2010
DOI: 10.1039/c004490c

The synthesis of enantiopure *N*-benzylidene nitrones of *N*-hydroxy-α-amino acids and their incorporation using standard Fmoc-based peptide chemistry into solid-supported peptide chains is described. Deprotection and resin cleavage affords *N*-terminal peptide hydroxylamines, which are the key substrates for chemoselective ligations with C-terminal peptide α-ketoacids. This general route is applicable to a variety of different N-terminal residues and provides a general approach to the solid phase synthesis of peptide hydroxylamines.

Introduction

We have recently reported a novel chemoselective amide-forming ligation of α-ketoacids and *N*-alkyl hydroxylamines (Scheme 1).[1] This reaction does not require any reagents, proceeds under mild conditions, tolerates unprotected functional groups, and produces only carbon dioxide and water as byproducts. In preliminary studies, we have demonstrated that this amide-formation has the potential to serve as a general peptide ligation for the joining of unprotected α-peptide fragments.[2] Our efforts, and those of other groups,[3] suggest that this process will be useful for the preparation of medium to large peptides by ligation of shorter fragments at numerous different ligation sites. This uniquely selective reaction therefore has the potential to compliment the powerful native chemical ligation[4,5] by providing a general amide-forming ligation reaction that is not limited to peptides or substrates possessing an N-terminal cysteine residue or sulfur-containing derivatives.[6]

The greatest obstacle to the widespread application of this reaction to the synthesis of larger peptides has been the lack of synthetic approaches for the preparation of the requisite C-terminal α-ketoacids and N-terminal hydroxylamines. In each case, general methods that not only preserve adjacent stereochemistry and tolerate unprotected functional groups but also interface with standard, established technologies for peptide synthesis must be identified. For the synthesis of the C-terminal α-ketoacids, we have reported a stereoretentive approach based on cyanosulfur ylides that is compatible with Fmoc-based solid phase synthesis.[7] We have also recently disclosed a novel reagent for the preparation of solid-supported N-terminal hydroxylamines.[8] This is a convenient method for the rapid preparation of peptide hydroxylamines, but suffers from modest yields and is best suited for exploratory, rather than preparative, applications. The preparation of N-terminal glycine hydroxylamines is possible *via* nucleophilic displacement on N-terminal bromoacetates, a protocol we have recently employed in our synthesis of the human GLP-1 (7-36) peptide *via* the ketoacid–hydroxylamine ligation of two fully unprotected 15-mer peptide fragments.[9] In this case, the choice of an N-terminal glycine as the ligation site was governed by the synthetic methods available for the preparation of the hydroxylamine rather than any particular advantage of the sterics of the glycine hydroxylamine. On the contrary, our experience to date suggests that N-terminal

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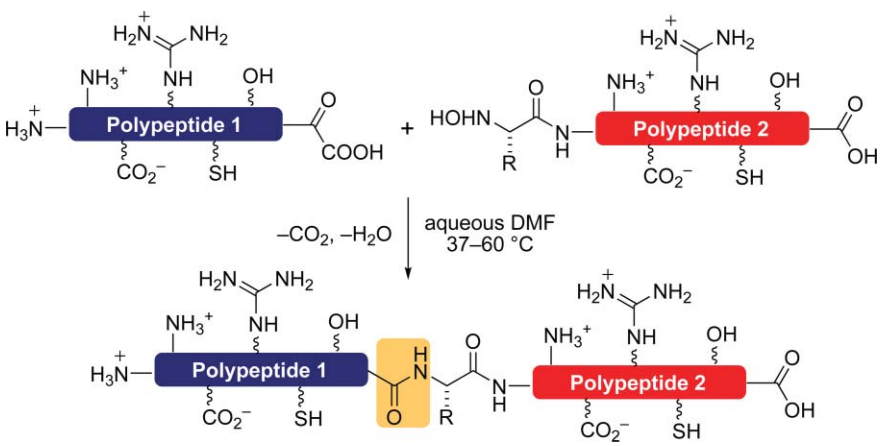
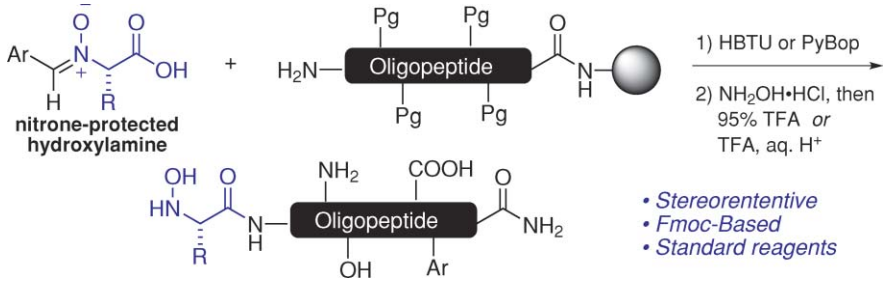
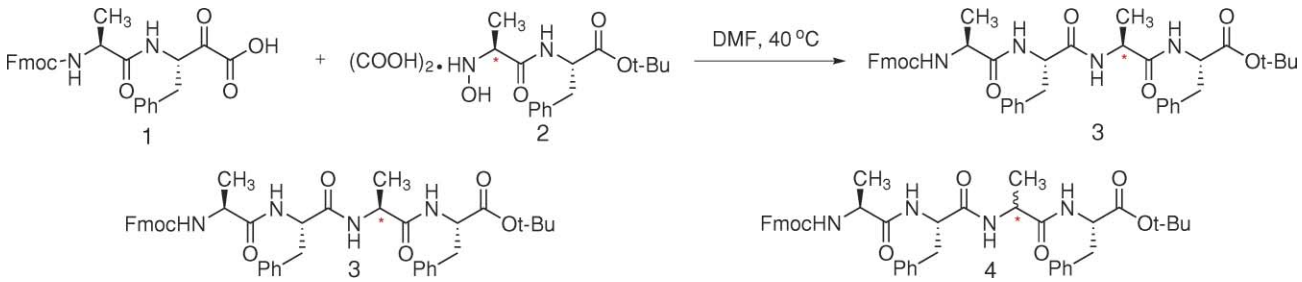
† Electronic Supplementary Information (ESI) available: Experimental procedures, spectra and characterization data. See DOI: 10.1039/c004490c



Scheme 1 Ketoacid–hydroxylamine amide ligations of unprotected peptide fragments.



Scheme 2 Synthesis of N-terminal peptide hydroxylamines by monomer coupling.



Scheme 3 Confirmation of stereochemical integrity of chiral N-hydroxylamines during ligations with α-ketoacids.

glycine hydroxylamines are more prone to decomposition than substituted residues.

The most straightforward approach to introducing enantiopure N-terminal peptide hydroxyamino acid residues into a synthetic peptide would be the coupling, under standard conditions, of a suitably protected *N*-hydroxyamino acid to the peptide N-terminal amine. The successful implementation of this strategy requires four discrete, but interconnected successes: 1) the preparation of enantiomerically pure peptide hydroxylamines; 2) the identification of a chemically and configurationally stable protecting group for the peptide hydroxylamines; 3) the coupling of the *N*-hydroxyamino acid monomers without epimerization; and 4) deprotection of the hydroxylamine and isolation of the side-chain deprotected N-terminal peptide hydroxylamines. A further complication is that N-terminal peptide hydroxylamines are somewhat unstable towards oxidation or elimination, constraining the methodologies that can be employed for their synthesis, manipulation, and purification. Once prepared and isolated in pure form, they can be stabilized as their TFA or oxalate salts, but the relative lability of intermediates necessary for the synthesis of the appropriate amino acid monomer as well as the final peptides demands careful consideration and innovative solutions.

We now report a general, comprehensive approach to the synthesis of N-terminal peptide hydroxylamines by the coupling of enantiomerically pure, nitrone-protected N-hydroxyamino acids to solid-supported peptides (Scheme 2). This chemistry is compatible with common reagents, resins, and protection strategies used in Fmoc-based solid phase peptide synthesis. We have rigorously established the preservation of stereochemistry in the synthesis of the amino acid monomers as well as during their coupling using typical protocols. Several approaches to the unique challenges of deprotecting, isolating, and purifying the N-terminal peptide hydroxylamines are described. In preliminary work, these studies have allowed us to demonstrate that chemoselective peptide ligations can occur at N-terminal residues containing unprotected side chains.

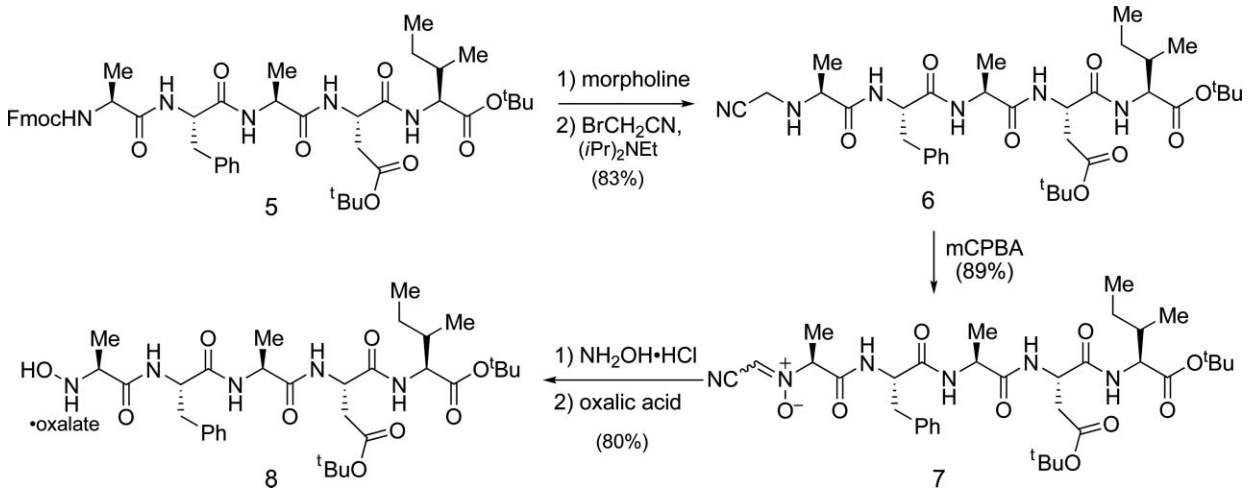
Results and discussion

Stereoretention of peptide *N*-hydroxylamines during ligations with *α*-ketoacids

In our preliminary studies on the α-ketoacid–hydroxylamine ligation, we had occasionally detected peptide epimers at the former hydroxylamine stereocenter, but we were unsure if epimerization had occurred during the preparation of the peptide hydroxylamine or during the ligation itself. In order to determine at which step epimerization occurred, enantiomerically enriched (>99% ee as determined by chiral HPLC) dipeptide **2** was carefully prepared and subjected to ligation with α-ketoacid dipeptide **1** to afford tetrapeptide **3** (Scheme 3). Comparison of the α-ketoacid–hydroxylamine ligation product with diastereomeric standard **4** by SFC chiral column revealed preservation of stereochemistry. This result, and related observations in our laboratory, suggested that epimerization occurred during the preparation of the peptide hydroxylamine. Indeed, epimerization at some stage of the synthesis of certain hydroxylamines was confirmed by their reduction with Zn or RANEY®® Ni and analysis of the resulting amines.[10]

Synthesis of enantiopure *N*-hydroxyamino acids

Our first task therefore became the identification of a reliable, general method for the preparation of enantiopure *N*-hydroxyamino acids. Our previous forays into the synthesis of enantiopure N-terminal peptide hydroxylamines relied on the late-stage oxidation of the N-terminal amine of fully assembled peptides using a 3-step protocol reported by Fukuyama.[11] For example, monoalkylation of N-terminal



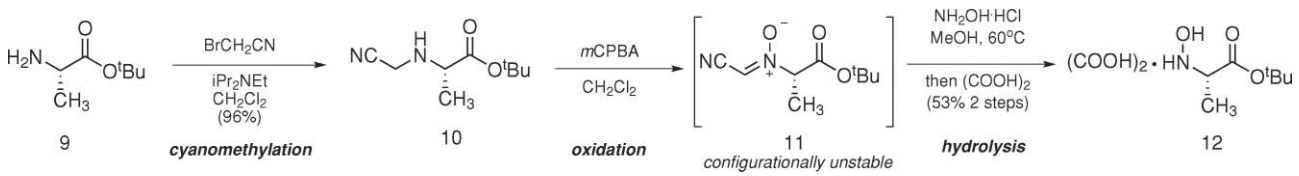
Scheme 4 Synthesis of an N-terminal peptide hydroxylamine by late-stage application of the Fukuyama protocol.

amine **5** with bromoacetonitrile followed by regioselective oxidation of the resulting secondary amine with mCPBA afforded "cyano-nitrone" **7** that was hydrolyzed to the corresponding hydroxylamine with excess hydroxylamine hydrochloride in MeOH (Scheme 4). The *N*-hydroxyamino peptide **8** was isolated by precipitation as its oxalate salt. This process, which is best executed in solution phase, works remarkably well for the preparation of short peptide hydroxylamines but suffers from the inconvenience of extensive manipulations of often sparingly soluble side-chain protected peptides and attendant poor yields.

In shifting our goal to developing a suitable protection and coupling strategy for enantiopure *N*-hydroxyamino acids, we re-evaluated the available methods for the preparation and coupling of the requisite monomers. Poloński, Miller and others have reported the synthesis of non-racemic *N*-benzylidene nitrones derived from α-amino acids including alanine, phenylalanine and leucine by oxidations of the corresponding benzylidene imines.[12] This method offers the most direct route to our targeted monomers, but its execution is often capricious and not readily extended to amino acids containing diverse, protected side-chain functionalities. Other methods for the synthesis of non-racemic peptide hydroxylamines include S_N2 displacements of activated, chiral α-hydroxyacids, which require the preparation of corresponding enantiopure starting materials[13] and the direct oxidation of amino esters with dimethyldioxirane at cryogenic temperatures.[14] We therefore elected to focus our attention on refinements of the Fukuyama procedure to provide a reliable, general method for the preparation of enantiopure *N*-hydroxyamino acids. This approach would also allow us to readily test a variety of protecting group strategies for the peptide hydroxylamines.

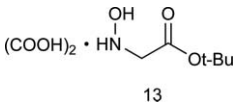
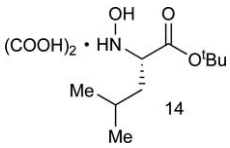
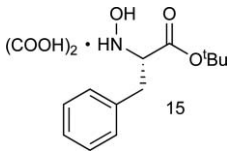
The Fukuyama protocol has proven to be a remarkably robust approach to the synthesis of primary hydroxylamines. It is readily executed on a variety of reaction scales and substrates. In applying this approach to the synthesis of peptide hydroxylamines, we did occasionally detect epimerized products, the origin of which we eventually traced to the "cyano-nitrone" intermediate. If not immediately hydrolyzed to the hydroxylamine, these "cyano-nitrones" undergo epimerization, presumably due to the highly electron-deficient nature of this moiety. Fortunately, as long as this intermediate is used promptly the stereochemical integrity is maintained. In general, the alkylation and oxidation steps occur without difficulty. In order to avoid epimerization at the cyano-nitrone stage, we usually conducted the oxidation, hydrolysis and isolation of the hydroxylamine oxalates without purification of intermediates.[15] In some batches, we have occasionally found the precipitation of the salts to be difficult or low yielding. For this reason, we recommend purification of the cyanomethylated amine prior to the oxidation step, despite the fact that this reaction usually proceeds cleanly and in excellent yield. The resulting hydroxylamine salts were proven to be enantiomerically pure by conversion to the N-benzylidene nitrone derivatives followed by HPLC-analysis on chiral columns (vide infra).

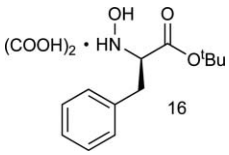
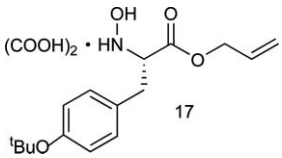
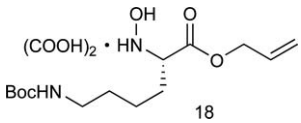
With this information, we developed a general route to the synthesis of nitrone protected hydroxylamines (Scheme 5 and Table 1). For the preparation of *N*-hydroxyamino acids lacking side-chain functionality, we began with *O*-*tert*-butyl protected α-amino acids. For amino acids with side-chain functionality, such as L-tyrosine and L-lysine, we employed *O*-allyl protected amino acids. In either case, *N*-alkylation with bromoacetonitrile proceeded smoothly and the resulting secondary amine (*e.g.* **10**) was isolated by column chromatography. Oxidation with *m*CPBA provided the stereochemically labile cyano-nitrone (*e.g.* **11**), which was immediately hydrolyzed to the free hydroxylamine and isolated as its mono oxalate salt. The oxalate salts are chemically and



Scheme 5 Preparation of enantiopure L-alanine-derived α-*N*-hydroxyamino acid.

Table 1 Enantiopure hydroxylamines prepared by the Fukuyama protocol[a]

	 13	 14	 15
cyanomethylation	[b]	99	97
oxidation and hydrolysis	60	97	80

	 16	 17	 18
cyanomethylation	90	86	86
oxidation and hydrolysis	56	98	90

[a] See Scheme 5 reaction conditions. [b] This hydroxylamine amine was prepared from *tert*-butylbromoacetate and *O*-(2-methoxyisopropyl)hydroxylamine, followed by treatment of this product with oxalic acid (See Supporting Information for details†).

configurationally stable and can be stored without difficulty for several months or longer.

Protecting groups for α-*N*-hydroxyamino acids

In selecting a protecting group for the *N*-hydroxyamino acid monomers, we wished to identify an approach that would be chemically orthogonal to side-chain protection strategies employed in Fmoc-based solid phase peptide synthesis.[16] We were further constrained by potential reactions of the hydroxylamines, which are prone to N–O bond cleavage under reductive conditions or by elimination reactions, particularly when dealing with α-peptide-derived hydroxylamines.[17] Although we briefly explored either *N*-monoprotection or *N*,*O*-bis-protection with carbamate protecting groups,[18] we found some of these derivatives to be unstable towards deprotection and migration of the carbamates was occasionally observed.[19] In certain cases, *O*-protection with silyl groups,[20] trityl derivatives[21] or as acetals[22] was viable, but in our hands we could not develop this into a general strategy for the protection and coupling of *N*-hydroxyamino acid monomers.

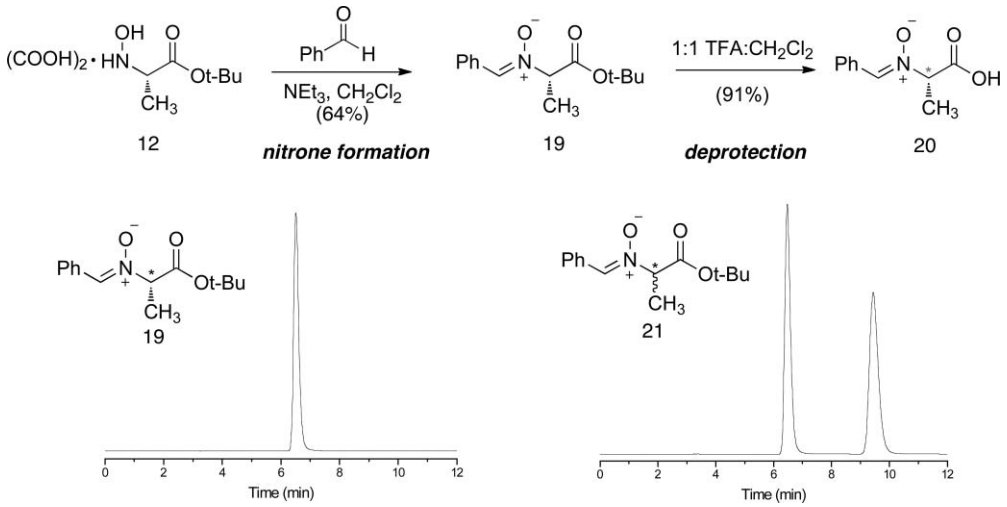
During the course of these studies, our attention turned to a single report of the protection and coupling of *N*-hydroxyamino acid monomers to amines by Bentley and Brooks.[23] This survey of a number of protection and coupling strategies revealed that the *N*-hydroxyamino acids were best protected as their corresponding nitrones. These amino acid monomers could be coupled to a primary amine by the formation of a mixed anhydride with isobutyl chloroformate and deprotected using aqueous acid.

Lacking in this study was the use of enantiomerically pure *N*-hydroxyamino acids. We were initially hesitant to adopt the nitrone protecting group due to fears that the use would lead to epimerization, particularly as we had already established that the cyano-nitrone intermediates employed in the Fukuyama hydroxylamine synthesis were configurationally labile. Furthermore, even if the nitrone-protected *N*-hydroxyamino acid monomers could be prepared in enantiomerically pure form, we were concerned that they would undergo epimerization during the peptide coupling. Ominously, Bentley and Brooks indicated that they experienced difficulty in the peptide coupling when using carbodiimide reagents. The advantage of this approach, however, was the simplicity of the protection step and the ability to modulate the properties of the protected hydroxylamines by variation of the aldehyde used for nitrone formation.

Protection of the hydroxylamines as nitrones was achieved simply by mixing the oxalate salts with benzaldehyde and NEt_3 in DMF or CH_2Cl_2 (Scheme 6). The stereochemical integrity of the *N*-benzylidene nitrones was confirmed by HPLC-analysis on chiral columns by comparison to a racemic standard. We were pleased to find that the *O*-*tert*-butyl, *N*-benzylidene nitrones were chemically and configurationally stable and, like the hydroxylamine salts, could be stored without difficulty or epimerization. Importantly, deprotection of the *tert*-butyl esters with TFA occurred smoothly without affecting the nitrone, which is labile towards aqueous acid. The resulting *N*-benzylidene nitrone α-amino acid (*e.g.* **20**) was also stable and amenable to storage. The same procedures were applied to the synthesis of *N*-benzylidene protected, *N*-hydroxyamino acids from glycine (**22**), L-leucine (**23**), L-phenylalanine (**24**), L-tyrosine (**25**), and L-lysine (**26**) (Table 2).

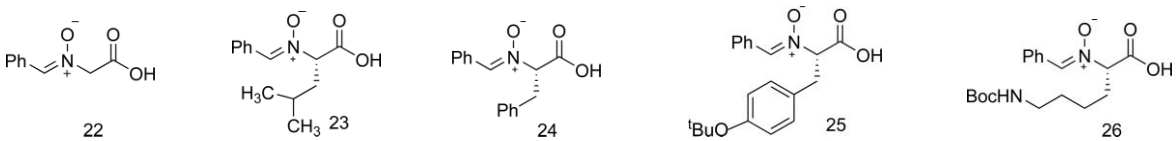
Peptide coupling of nitrone-protected *N*-hydroxyamino acids

The goal of this research project was the identification of a method for the introduction of a suitably protected, enantiomerically pure *N*-hydroxyamino acid to the N-terminus of a solid-supported synthetic peptide. The work of Bentley and Brooks had demonstrated that amide-formation with the *N*-benzylidene nitrone protected hydroxylamines was feasible,[23] but did not address the potential for epimerization during this reaction. Even under the best conditions, epimerization of protected amino acids during peptide coupling is an issue; the use of the electron-deficient nitrones and the potential for the Lewis basic oxygen atom to react with the intermediate activated esters amplified this concern.



Scheme 6 Protection of L-alanine-derived hydroxylamine and confirmation of stereochemical integrity.

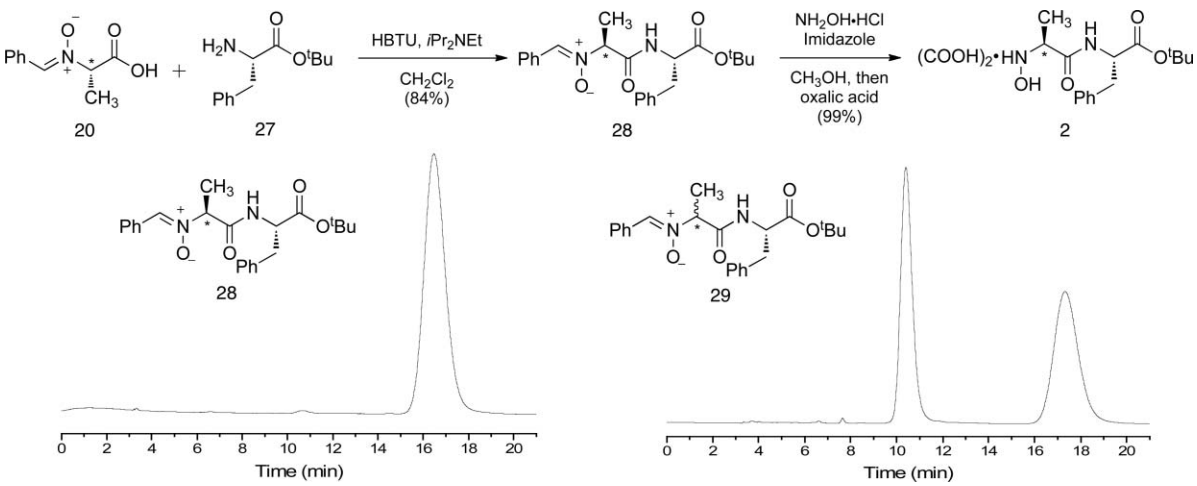
Table 2 Protection α-*N*-hydroxyamino acid monomers as benzylidene nitrones[a]



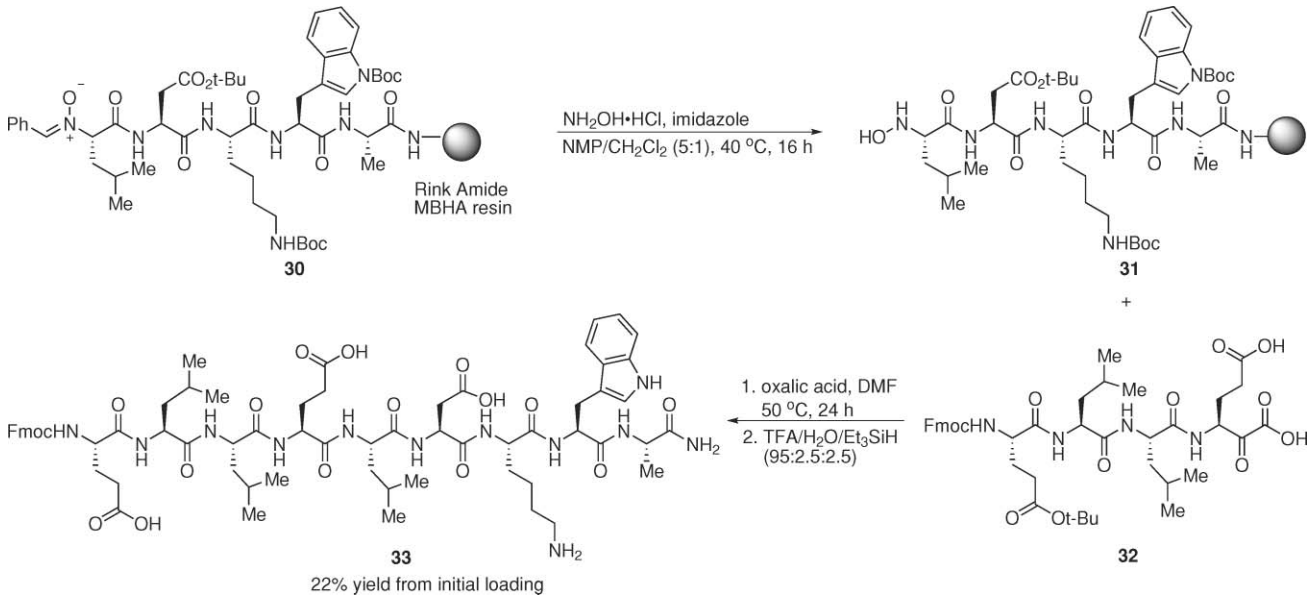
Nitrone formation	99	86	96	75	80
deprotection	93	84	99	59[b]	50[b]

[a] See Scheme 6 for reagents and conditions. [b] Deprotection conditions: $PdCl_2(PPh_3)$ [10 mol %], PPh_3 [30 mol %], morpholine (excess).

We were therefore pleasantly surprised to find that *N*-benzylidene protected, *N*-hydroxyamino acids can be easily and cleanly coupled to an N-terminal peptide amine in excellent yield and without epimerization (Scheme 7). A number of standard coupling reagents including HBTU, TBTU, PyBop, and DIC may be used, with similar results. In most cases, no special precautions or unusual conditions were needed to effect the coupling. This success allowed us to extend these methods to the incorporation of a variety of *N*-benzylidene protected *N*-hydroxyamino acids onto solid-supported peptide chains. Our further studies, as reported below, confirm that the nitrone protecting groups are compatible with standard methods, resins,



Scheme 7 Peptide coupling of nitrone protected L-alanine-derived hydroxylamine and confirmation of stereochemical integrity.



Scheme 8 On-resin nitrone deprotection and α-ketoacid–hydroxylamine peptide ligation.

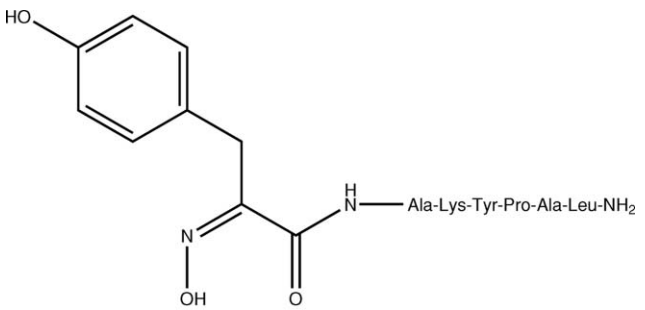
reagents and protecting groups employed in Fmoc-based peptide synthesis.

Coupling and deprotection of nitrone-protected N-terminal peptide hydroxylamines onto solid-supported peptides

The remaining task in this research plan was the identification of reaction conditions for the late stage deprotection of the hydroxylamine. Strategically, this could be effected either in tandem with resin cleavage and side-chain deprotection to give the free N-terminal hydroxylamine that, following suitable purification, could be used in the ligation reaction. Alternatively, we envisioned strategies whereby the nitrone protection would be preserved while the side chain functionalities were deprotected. This would be an important step in the eventual development of a stable, orthogonally reactive hydroxylamine protecting group that can be used for segment condensation approaches to the preparation of longer peptides. Finally, there are cases in which it may be desirable to liberate the hydroxylamine while the peptide is still bound to the resin, a method that we have demonstrated in our prior work on hydroxylamine synthesis.[24] Although not the focus of this study, we were pleased to find that many resin-bound peptide nitrones could be deprotected using these conditions to afford the resin-bound hydroxylamines suitable for on-bead peptide ligations (Scheme 8).

We have placed greater emphasis on methods that deliver the fully unprotected peptide hydroxylamines by nitrone hydrolysis under acidic conditions. A variety of acidic conditions, such as aqueous HCl,[25] promote nitrone hydrolysis, but are complicated by the extremely fast recombination of the hydroxylamine and the aldehyde upon workup.[26] Thus, deprotection reactions that appear to be successful when monitored during the reaction course often deliver significant amounts of the starting nitrone upon isolation! This finding, along with our observations of the properties of the nitrone-protected peptides, led us to adopt a somewhat unorthodox approach to the liberation of the peptide hydroxylamines: following resin cleavage, the peptide nitrones were precipitated from Et_2O and treated with 10% aqueous TFA for 5–10 min before being repeatedly passed through a short column of C18 silica gel and a resin-bond hydroxylamine (5 times). A final elution with CH_3CN afforded the crude peptide hydroxylamines, which were purified by preparative HPLC (Scheme 9).

Resin cleavage/side-chain deprotection of *N*-benzylidene nitrone peptides were conducted without the use of silane scavengers, when such scavengers were used during resin cleavage we observed the *N*-benzyl hydroxylamine peptide. Unexpectedly, nitrone hydrolysis under these conditions is easier for the larger amino acid residues, such as leucine, phenylalanine and tyrosine. Glycine and alanine-derived nitrones, in particular, are considerably more resistant to hydrolysis. Although the peptide nitrones appear to be indefinitely stable, the free hydroxylamines are prone to oxidation to the corresponding oximes during reaction workup and subsequent purification. Indeed, the major side product from the isolated peptides of the ligation reactions in general is the corresponding peptide oximes (*e.g.* **38**).



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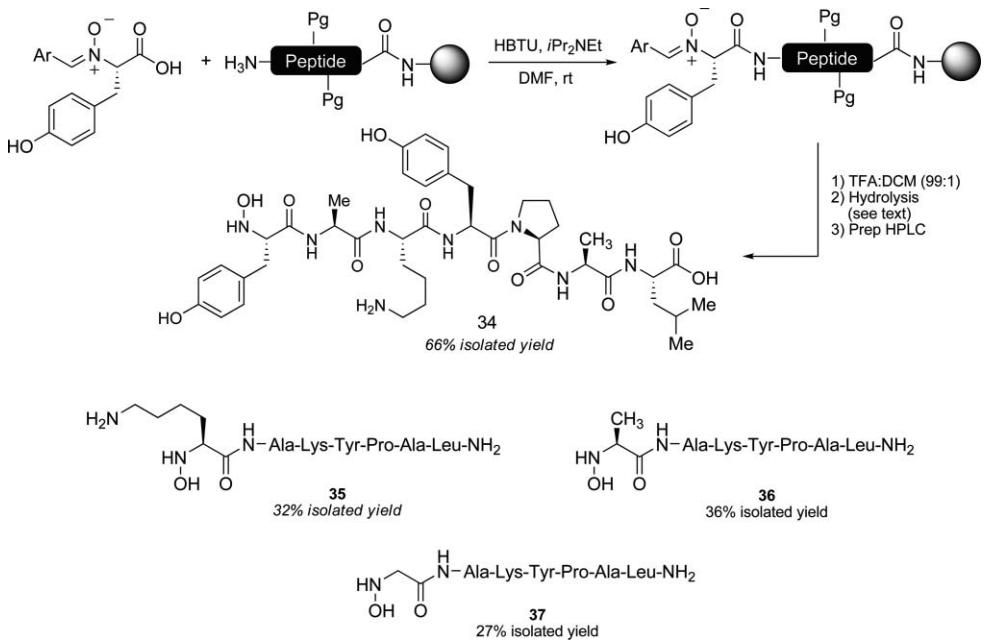
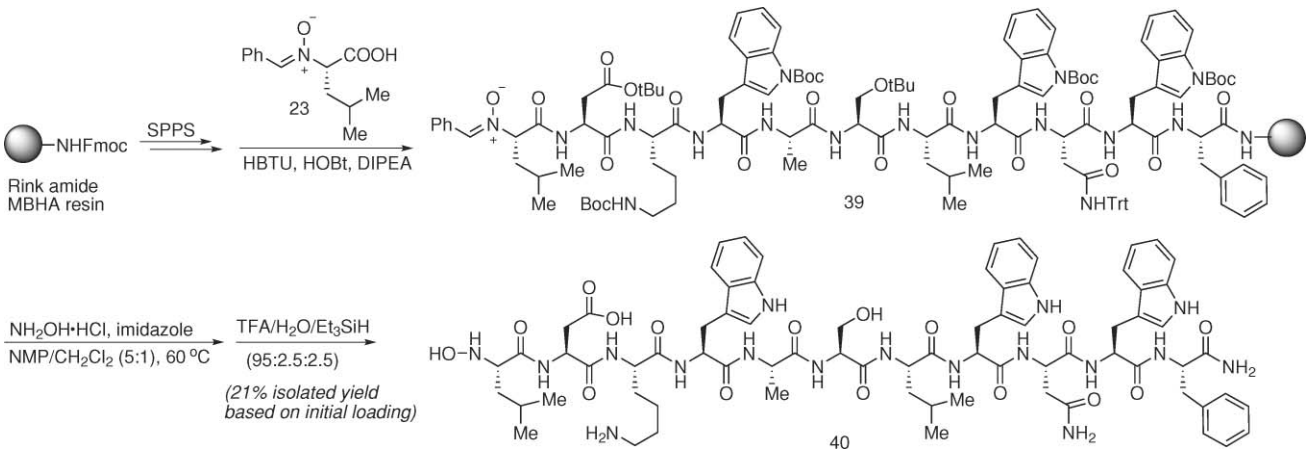
This oxidative side reaction can be mitigated by taking care to shield the peptides from oxygen, but some amount of oxime is usually detected. It can be removed during purification of the final, fully deprotected peptide hydroxylamines by reverse phase preparative HPLC but detracts from the overall yield of the desired product.



Scheme 9 Synthesis of N-terminal peptide hydroxylamines by amide coupling and deprotection.



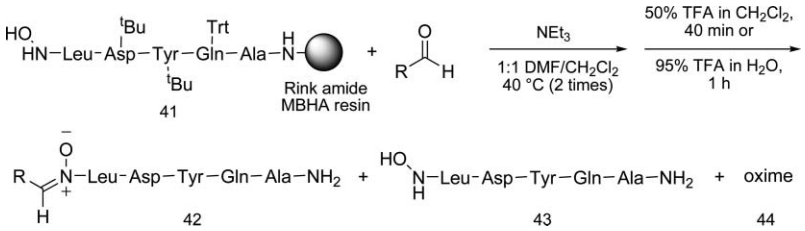
Scheme 10 Solid phase peptide synthesis of an 11-mer peptide hydroxylamine.

There appears to be no difficulty in applying these methods to the synthesis of longer peptide hydroxylamines. In ongoing work, we have routinely applied these methods to the synthesis of unprotected peptide hydroxylamines in the range of 10–20 amino acids. For example, the 11-mer peptide hydroxylamine **40** was prepared by Fmoc-based solid phase peptide synthesis without complications (Scheme 10).

Modulation of the nitrone protecting group: preliminary results

When *N*-benzylidene nitrones are employed, the stability of the nitrone and its ease of deprotection appears to be correlated to the steric properties of the amino acid residue. Thus, smaller amino acids, such as glycine and alanine, form stable nitrones that can be difficult to deprotect. Depending on the particular application of the α-ketoacid–hydroxylamine ligation, peptide nitrones of either increased or decreased hydrolytic stability are sometimes desired. As is always the case in peptide chemistry, the properties and chemical reactivity of a given peptide fragment are highly dependent on its sequence and solubility. Our chemistry is no exception and the choice of nitrone and its ease of hydrolysis are often sequence and application specific and we do not currently have a predictive tool as to whether or not the *N*-benzylidene nitrones will be easy or difficult to hydrolyze. Our only generalization to date is that the nitrones of glycine and alanine are usually more resistant.

In a preliminary attempt to identify other nitrone protecting groups that have either greater or diminished stability, depending on the application, we have synthesized and screened a number of different peptide nitrones and explored their behavior under typical conditions for side-chain deprotection and purification (Scheme 11). Our observations from this study are summarized in Table 3. In general, more electron-deficient nitrones demonstrated greater stability, while the electron-rich benzylidenes were more prone to nitrone cleavage upon resin release or HPLC purification. We attribute these counterintuitive findings to the very high affinity



Scheme 11 Studies on the stability of various nitrones as hydroxylamine protecting groups.

Table 3 Preliminary studies of nitrone stability to resin cleavage and purification.[a]

entry	R =	50% TFA in CH_2Cl_2	95% TFA in H_2O	Prep-HPLC
1	*p*-CN-C_6H_4	stable	stable	stable
2	trans-cinnamyl	stable	stable	stable
3	2,6-di-Cl-C_6H_3	stable	stable	stable
4	2,4,6-tri-Me-C_6H_2	stable	unstable	unstable
5	2,5-di-(MeO)-C_6H_3	stable	unstable	unstable
6	2,6-di-(MeO)-C_6H_3	unstable	unstable	unstable

[a] See Scheme 11 for peptide structures. These studies are specific to this peptide sequence and may vary depending on the N-terminal hydroxylamine and the peptide sequence.

of the electron-deficient aldehydes to the hydroxylamines. In contrast, the electron-rich arylaldehydes are slower to recombine should cleavage occur.

In the majority of cases, the use of the *N*-benzylidene nitrones (*i.e.* those derived from benzaldehyde) are ideal, but in more sophisticated applications of the α-ketoacid-hydroxylamine ligation we have occasionally needed to modulate the properties of the nitrone to either enhance or suppress its hydrolysis. The ability to change the aldehyde component offers a convenient and powerful means of achieving this. As a caveat, it should be noted that regardless of the nitrone protecting group employed, they are not stable towards the ligation conditions in the presence of another hydroxylamine. In all case studies to date, nitrone exchange occurs, even on the most hindered of substrates. Nitrone protection is therefore not a suitable solution to the challenge of iterative fragment condensations using the α-ketoacid–hydroxylamine ligation, thus other protecting group strategies for the hydroxylamine will be needed for this application.[27]

Peptide-ligations at various N-terminal hydroxylamine residues

The potential impact of the α-ketoacid–hydroxylamine amide-formation on the chemical synthesis of peptides and proteins lies in its ability to effect peptide ligations at a wide variety of ligation sites. In our studies on the synthesis of peptide α-ketoacids, we have noted that certain unprotected α-ketoacids are not good ligation partners due to interactions of the side chains with the α-ketoacid functionality. These studies, and ongoing work in our laboratory, have established that C-terminal glycine, alanine, leucine, phenylalanine, proline, isoleucine, valine and glutamic acid are all viable. Due to the prior difficulties of their synthesis, we have performed fewer studies on the residue-specificity of unprotected N-terminal hydroxylamines in the ligation of α-peptide fragments. Using the materials prepared during the synthesis of various nitrone protected peptide hydroxylamines, we briefly explored their ligations with C-terminal peptide α-ketoacids (Scheme 12).

All N-terminal hydroxylamines tested to date give at least some of the desired ligation product. The major differences appear to be in the stability of the hydroxylamines towards oxidation. For example, the N-terminal lysine hydroxylamine employed for the synthesis of **48** was particularly prone to oxime formation, thereby diminishing the yield of the ligated product. It must be noted that these ligations were unoptimized and performed a single time under a single set of conditions simply to test the viability of each of the N-terminal hydroxylamine ligation sites. In our work on the application of the α-ketoacid–hydroxylamine ligation to longer peptides, we have found that optimization of the reaction conditions as a function of solubility of the peptide fragments and products is usually required. We therefore leave such specific optimizations to synthetic applications of the ligation reaction.

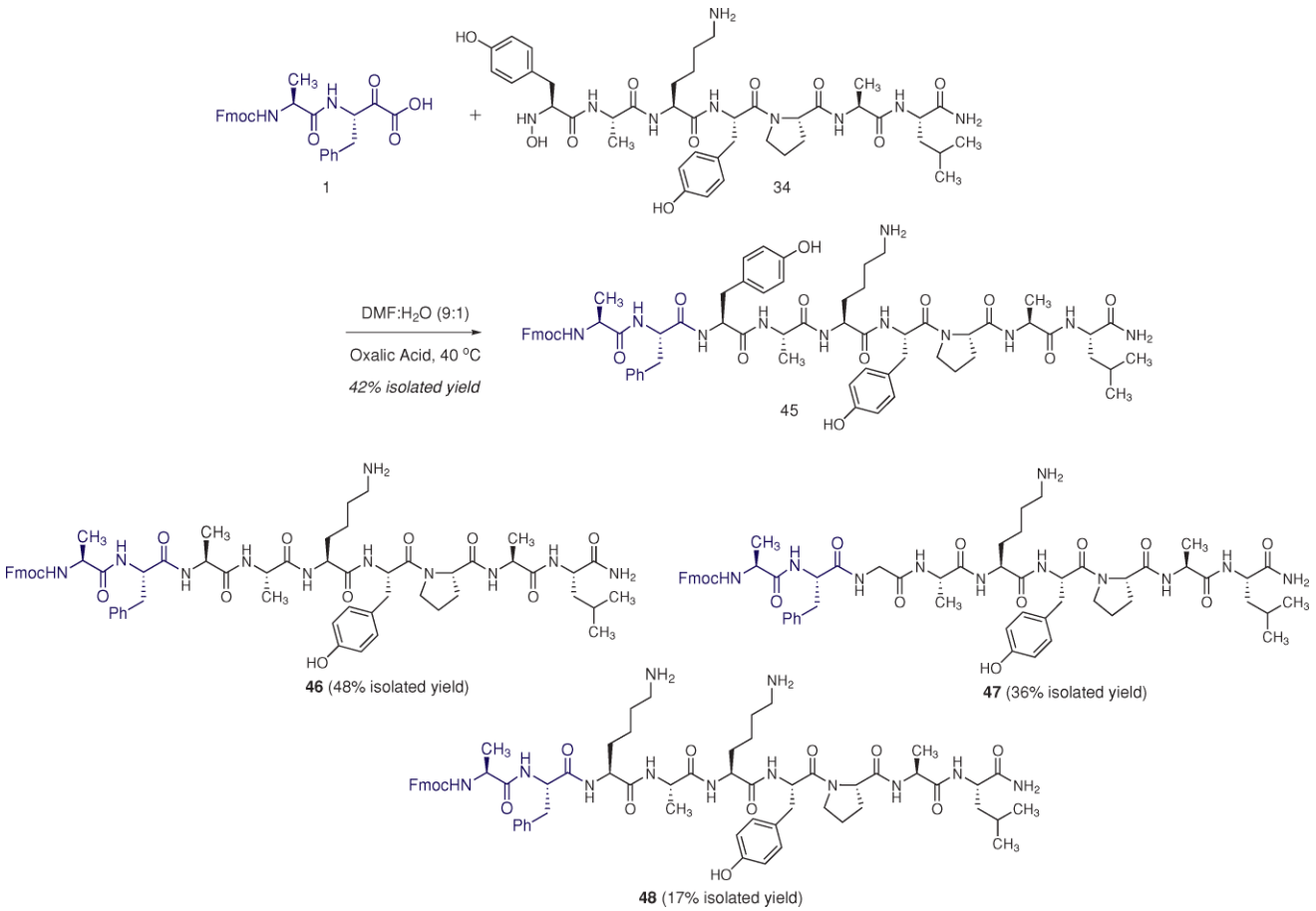
Conclusions

Enantiomerically pure *N*-hydroxyamino acids can be reliably prepared by Fukuyama's method and protected as their *N*-benzylidene nitrones. Unlike other approaches, this method is both general and delivers stable, protected *N*-hydroxyamino acids suitable for introduction into peptides with standard coupling reagents and without epimerization. The solid-supported nitrone-protected hydroxylamines can be cleaved from either side-chain protected or side-chain unprotected peptides without interference from common side-chain functional groups. Importantly, these findings greatly expand the range of N-terminal peptide hydroxylamines that can be prepared and isolated, an important and necessary step for developing the α-ketoacid–hydroxylamine amide-ligation as a general method for peptide synthesis.

Experimental

General experimental

All reactions utilizing air- or moisture-sensitive reagents were performed in dried glassware under an atmosphere of nitrogen.



Scheme 12 Ligation of unprotected N-terminal hydroxylamine peptides.

CH_2Cl_2 was distilled over CaH_2. THF was distilled from Na/benzophenone. CH_3OH and DMF were dried by passage over molecular sieves under Ar atmosphere. *N*,*N*-Diisopropylethylamine (DIPEA) was distilled from CaH_2. Other reagents were used without further purification. Oxone® was purchased from Alfa Aesar. Thin layer chromatography (TLC) was performed on Merck precoated plates (silica gel 60 F_{254}, Art 5715, 0.25 mm) and was visualized by fluorescence quenching under UV light or by staining with potassium permanganate or ninhydrin. Preparative thin-layer chromatography (PTLC) was performed using plates prepared from silica gel EMD 60 PF254 (Art 7749). Column chromatography was performed on E. Merck Silica Gel 60 (230–400 Mesh) using a forced flow of 0.5–1.0 bar. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were measured on a Bruker Avance II 500 spectrometer. Chemical shifts are expressed in parts per million (PPM) downfield from residual solvent peaks and coupling constants are reported as hertz (Hz). Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. Infrared (IR) spectra were recorded on a JASCO FT/IR-430 spectrophotometer and are reported as wavenumber (cm^{-1}). Optical rotations $[\alpha]_D^T$ were measured at temperature T on a Jasco P–2000 polarimeter operating at the sodium D line with a 100 mm path length cell, and are reported as follows: (concentration (g/ml × 100), solvent). Reverse phase HPLC was performed using the following columns: YMC R-ODS-10A C-18 (250 × 4.6 mm for analytical and 250 × 20 mm for preparative), or Zorbax Eclipse XDB-C8 4.6 × 150 mm. All separations utilized a gradient of iPrOH or CH_3CN and millipore H_2O, each containing 0.1% TFA. Peptides were prepared by standard Fmoc manual solid-phase synthesis protocols using Rink amide MBHA resin with a loading of 0.70 mmol/g.

General procedure for the preparation of *N*-hydroxyamino acid ester oxalates

(*S*)-*tert*-Butyl 2-(hydroxyamino)propanoate oxalate (12). The following procedure is representative. A solution (0.1 M) of (*S*)-1-(*tert*-butoxy)-1-oxopropan-2-aminium chloride (0.516 g, 2.85 mmol, 1.00 equiv) in CH_3CN (27.0 mL) was treated with $BrCH_2CN$ (0.330 mL, 3.13 mmol, 1.10 equiv) and DIPEA (1.00 mL 5.74 mmol, 2.00 equiv), and the reaction was stirred at rt overnight. The mixture was transferred to a separatory funnel and saturated aq $NaHCO_3$ (100 mL) was added. The solution was extracted with CH_2Cl_2 (2 × 100 mL), the organic phases were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified by flash chromatography on silica (20% EtOAc in hexanes) to provide compound **10** as a colorless oil (0.51 g, 97% yield). (*S*)-*tert*-butyl 2-(cyanomethylamino) propanoate (**10**) (1.09 g, 5.92 mmol, 1.00 equiv) was dissolved in CH_2Cl_2 (20 mL), stirred, and cooled to 0 °C. To the cooled reaction mixture, mCPBA (2.55 g, 14.8 mmol, 2.00 equiv) was added in several in portions over 30 min. The

solution was allowed to warm to rt and stirred for 45 min. The reaction was quenched by addition of saturated aq $Na_2S_2O_3$ (15 mL) and saturated aq $NaHCO_3$ (25 mL) and the resulting biphasic mixture stirred for an additional 30 min. The mixture was then transferred to a separatory funnel and the organic phase removed. The aqueous phase was extracted with CH_2Cl_2 (2 × 30 mL), the organic phases were combined, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to provide the cyano-nitrone as a yellow oil. The yellow oil was immediately dissolved in MeOH (57.7 mL), hydroxylamine hydrochloride (2.05 g, 29.6 mmol, 5.00 equiv) was added to the solution, and stirred overnight at 60 °C. The reaction was allowed to cool to rt and CH_2Cl_2 (20 mL) was added to provide a white precipitate which was removed by vacuum filtration. Concentration of the filtrate *in vacuo* afforded a yellow oil. The oil was dissolved in CH_2Cl_2 (20 mL), transferred to a separatory funnel and extracted with saturated aq $NaHCO_3$ (30 mL). The aqueous phase was washed with CH_2Cl_2 (2 × 20 mL), the organic phases were combined, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to a reduced volume. Oxalic acid (1.45 g, 1.18 mmol, 2.00 equiv) in MeOH (2 mL) was added to the solution, followed by cold Et_2O (50 mL), producing a white precipitate which was collected by vacuum filtration and rinsed several times with cold Et_2O to afford compound **12** as a white solid (0.768 g, 53% yield). mp 138–140 °C; $[\alpha]_D^{20}$ −19.3 (*c* 0.15, MeOH); ^{1}H NMR (DMSO-d_6) δ 9.70 (br s, 3H, OH), 3.71–3.68 (m, 1H, NHCH), 1.42 (s, 9H, $OCMe_3$), 1.18 (d, 3H, *J* = 6.5 Hz, Me); ^{13}C NMR (DMSO-d_6) δ 170.8, 163.2, 81.4, 59.8, 27.7, 13.5; IR (KBr) ν 3424.4, 2296.3, 2937.0, 1747.1, 1736.1, 1631.9, 1217,8, 1157.5; ESI calcd for $C_7H_{16}NO_3$ $[M+H]^+$ *m*/*z*: 162.1, found 162.5.

(*S*)-*tert*-Butyl 2-(hydroxyamino)-4-methylpentanoate oxalate (14). (*S*)-*tert*-Butyl 2-(cyanomethylamino)-4-methylpentanoate (0.550 g, 2.28 mmol, 1.00 equiv) was converted to the corresponding *N*-hydroxylamine oxalate according to the general procedure and isolated as a white solid (0.650 g, 97% yield). mp 97–100 °C; $[\alpha]_D^{20}$ +1.7 (c 0.34, MeOH); ^{1}H NMR (D_3COD) δ 3.94 (t, 1H, *J* = 7.0 Hz, NHCH), 1.80–1.77 (br m, 1H, $CHMe_2$), 1.70–1.68 (br m, 2H, i$PrCH_2$), 1.53 (s, 9H, $OCMe_3$), 0.99 (t, 6H, *J* = 5.5 Hz, 2 × Me); ^{13}C NMR (D_3COD) δ 169.2, 165.0, 85.0, 64.3, 37.5, 28.2, 26.1, 23.2, 22.0; IR (KBr) ν 3426.4, 2963.5, 1743.8, 1596.2, 1253.9, 1158.0 cm^{-1}; ESI calcd for $C_{10}H_{22}NO_3$ $[M+H]^+$ *m*/*z*: 204.2, found 204.5.

(*S*)-*tert*-Butyl 2-(hydroxyamino)-3-phenylpropanoate oxalate (15). (*S*)-*tert*-Butyl 2-(cyanomethylamino)-3-phenylpropanoate (2.72 g, 10.5 mmol, 1.0 equiv) was converted to the corresponding *N*-hydroxylamine oxalate according to the general procedure and isolated as a white solid (3.21 g, 93% yield). mp 89–92 °C; $[\alpha]_D^{20}$ +16.7 (*c* 0.12, MeOH); ^{1}H NMR (D_3COD) δ 7.34–7.26 (m, 5H, Ph), 4.20–4.17 (br m, 1H, NHCH), 3.31–3.26 (m, 1H, PhCH_2), 3.03 (dd, 1H, *J* = 13.5, 9.5 Hz, PhCH_2), 1.33 (s, 9H, $OCMe_3$); ^{13}C NMR (D_3COD) δ 168.6, 164.4, 135.8, 130.6, 129.7, 128.5, 84.8, 66.7, 34.7, 28.0; IR (thin film) ν 2980.4, 2561.0, 2492.5, 1740.9, 1642.5, 1253.9, 1155.1, 1253.9, 1155.1 cm^{-1}; HRMS (ESI) calcd for $C_{13}H_{19}NO_3Na$ $[M+Na]^+$ *m*/*z* 260.1263 found 260.1241.

(*S*)-Allyl 3-(4-*tert*-butoxyphenyl)-2-(hydroxyamino) propanoate oxalate (17). (*S*)-Allyl 3-(4-*tert*-butoxyphenyl)-2-(cyanomethylamino)propanoate (1.02 g, 3.10 mmol, 1.00 equiv) was converted to the corresponding *N*-hydroxylamine oxalate according to the general procedure and isolated as a white solid (1.17 g, 98% yield). mp 105.0–106.5 °C; $[\alpha]_D^{20}$ −3.2 (*c* 0.19, MeOH); ^{1}H NMR (CD_3OD) δ 7.13 (d, 2H, *J* = 8.0 Hz, Ar), 6.93 (d, 2H *J* = 8.5 Hz, Ar), 5.86–5.78 (m, 1H, OCH_2CHCH_2), 5.27–5.17 (m, 2H, $CHCH_2$), 4.59–4.58 (m, 2H, OCH_2), 4.04 (t, 1H, *J* = 7.0 Hz, NHCH), 3.05 (dd, 1H, *J* = 14.0, 6.5 Hz, $ArCH_2$), 3.00–2.94 (m, 1H, $ArCH_2$), 1.32 (s, 9H, $OCMe_3$); ^{13}C NMR (CD_3OD) δ 169.1, 163.1, 154.7, 131.4, 129.8, 124.2, 118.1, 115.3, 78.4, 66.1, 65.6, 33.0, 27.9; IR (thin film) ν 3424.9, 3258.6, 2977.0, 1739.9, 1507.1, 1365.8, 1236.6, 1162.8 cm^{-1}; HRMS (ESI) calcd for $C_{16}H_{23}NO_4$ $[M+H]^+$ *m*/*z*: 294.1705, found 294.1707.

(*S*)-Allyl 6-(*tert*-butoxycarbonylamino)-2-(hydroxyamino) hexanoate oxalate (18). (*S*)-Allyl 6-(*tert*-butoxycarbonylamino)-2-(cyanomethylamino)hexanoate (3.22 g, 9.90 mmol, 1.00 equiv) was converted to the corresponding *N*-hydroxylamine oxalate according to the general procedure, and isolated as a white solid (3.49 g, in 90% yield). mp 105–106.5 °C; $[\alpha]_D^{20}$ −1.1 (*c* 0.54, MeOH); ^{1}H NMR (CD_3OD) δ 6.01–5.93 (m, 1H, OCH_2CHCH_2), 5.37 (dd, 1H, *J* = 15.5, 1.5 Hz, $CHCH_2$), 5.26 (d, 1H, *J* = 10.5 Hz, $CHCH_2$), 4.70 (d, 2H, *J* = 5.5 Hz, OCH_2), 3.84 (br s, 1H, NHCH), 3.03 (t, 2H, *J* = 6.7 Hz, $NHCH_2$), 1.76 (br d, 2H, CH_2), 1.43–1.36 (m, 13H, 2 × CH_2 and $OCMe_3$); ^{13}C NMR (CD_3OD) δ 171.0, 164.4, 157.3, 131.8, 118.0, 78.6, 65.8, 64.5, 39.6, 29.4, 27.8, 27.5, 22.6; IR (thin film) ν 3390.2, 2936.5, 2975.6, 1746.2, 1692.7, 1522.0, 1263.2, 1175.4 cm^{-1}; HRMS (ESI) calcd for $C_{14}H_{26}N_2O_5Na$ $[M+Na]^+$ *m*/*z*: 325.1739, found 325.1734.

General procedure for the preparation of *N*-benzylidene nitrones

(*S*)-*N*-Benzylidene-1-carboxyethanamine oxide (20). The following procedure is representative. (*S*)-*tert*-butyl 2-(hydroxyamino)propanoate oxalate (**12**) (0.25 g, 0.99 mmol, 1.0 equiv) was dissolved in CH_2Cl_2, (9 mL). To this solution, benzaldehyde (0.15 mL, 1.5 mmol, 1.5 equiv) and triethylamine (0.41 mL, 3.0 mmol, 3.0 equiv) were added and the mixture stirred overnight. The reaction was transferred to a separatory funnel, and H_2O (40 mL) was added. The organic phase was removed; the aqueous solution was extracted with CH_2Cl_2 (2 × 40 mL), and the organic phases were combined and washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The product was purified by flash column chromatography on silica (30% EtOAc in hexanes), to afford (*S*)-*N*-benzylidene-1-*tert*-butoxy-1-oxopropan-2-amine oxide (**19**) as white foam (0.20 g, 81% yield). (*S*)-*N*-Benzylidene-1-*tert*-butoxy-1-oxopropan-2-amine oxide (**19**) (0.491 g, 1.98 mmol, 1.00 equiv) was dissolved in a 1 : 1 solution of CH_2Cl_2/TFA (20 mL) and stirred for 2 h at rt. The solvent was reduced to minimum volume, and cold Et_2O (15 mL) was added to the solution to provide a white precipitate. The precipitate was collected by vacuum filtration, and washed several times with Et_2O to afford **20** (0.30 g, 78% yield). mp 141–142 °C; $[\alpha]_D^{20}$ −23.39 (*c* 1.03, MeOH); ^{1}H NMR (CD_3OD) δ 8.28 (dd, 2H, *J* = 7.5, 1.5 Hz, Ph), 7.96 (s, 1H, Ph*CH*NO), 7.50–7.46 (m, 3H, Ph), 5.03 (q, 1H, *J* = 7.0 Hz, CH), 1.71 (d, 3H, *J* = 7.0 Hz, Me); ^{13}C NMR (CD_3OD) δ 171.0, 140.0, 132.6, 131.4, 130.7, 129.6, 73.5, 15.6; IR (KBr) ν 3436.0, 29.0, 1752.0, 1633.4, 1148.4, 1082.8; HRMS (ESI) calcd for $C_{10}H_{12}NO_3$ $[M+H]^+$ *m*/*z*: 194.0817, found 194.0819.

(*S*)-*N*-Benzylidene-1-carboxy-3-methylbutan-1-amine oxide (23). (*S*)-*tert*-Butyl 2-(hydroxyamino)-4-methylpentanoate

oxalate (**14**) (0.96 g, 3.30 mmol, 1.00 equiv) was converted to the corresponding *N*-benzylidene nitrone ester according to the general procedure, and purified by flash column chromatography on silica (15% EtOAc in hexanes) to provide (*S*)-*N*-benzylidene-1-*tert*-butoxy-4-methyl-1-oxopentan-2-amine oxide as a white solid (0.83 g, 86% yield). (*S*)-*N*-benzylidene-1-*tert*-butoxy-4-methyl-1-oxopentan-2-amine oxide (0.340 g, 1.17 mmol, 1.00 equiv) was dissolved in 1 : 1 CH_2Cl_2/TFA (12 mL) and stirred for 2 h at rt. The solution was concentrated *in vacuo* and compound **23** was isolated without further purification as a colorless oil (0.230 g, 84% yield). $[\alpha]_D^{20}$ −86.9 (*c* 0.16, MeOH); ^{1}H NMR (CD_3OD) δ 8.29 (d, 2H, *J* = 6.5 Hz, Ph), 8.00 (s, 1H, Ph*CH*NO), 7.51–7.47 (m, 3H, Ph), 4.96 (dd, 1H, *J* = 10.5, 4.5 Hz, CH), 2.32–2.26 (m, 1H, iPrCH$_2$), 1.86–1.80 (m, 1H, iPrCH$_2$), 1.62–1.57 (m, 1H, *CH*Me$_2$), 1.02–1.00 (m, 6H, Me × 2); ^{13}C NMR (CD_3OD) δ 169.8, 139.5, 131.3, 130.1, 129.4, 128.4, 75.4, 37.4, 24.7, 22.2, 20.5; IR (thin film) ν 2959.2, 1672.4, 1458.4, 1199.9; HRMS (ESI) calcd for $C_{13}H_{16}NO_3$ $[M-H]^-$ *m*/*z*: 234.1, found 234.6.

(*S*)-*N*-Benzylidene-1-carboxy-2-phenylethanamine oxide (24). (*S*)-*tert*-Butyl 2-(hydroxyamino)-3-phenylpropanoate oxalate (**15**) (0.16 g, 0.48 mmol, 1.0 equiv) was converted to the corresponding *N*-benzylidene nitrone according to the general procedure, and was purified by flash column chromatography on silica (15% EtOAc in hexanes) to provide (*S*)-*N*-benzylidene-1-*tert*-butoxy-1-oxo-3-phenylpropan-2-amine oxide as a white solid (0.150 g, 96% yield). (*S*)-*N*-Benzylidene-1-*tert*-butoxy-1-oxo-3-phenylpropan-2-amine oxide (2.57 g, 7.90 mmol, 1.00 equiv) was dissolved in 1 : 1 CH_2Cl_2/TFA (40 mL) and stirred for 2 h at rt. The solution was concentrated *in vacuo* and compound **24** was isolated as a white solid without further purification (2.11 g, 99% yield). mp 155–157 °C; $[\alpha]_D^{20}$ −84.4 (*c* 0.32, MeOH); ^{1}H NMR (CD_3OD) δ 8.10 (d, 2H, *J* = 7.5 Hz, Ph), 7.45–7.37 (m, 4H, Ph and Ph*CH*NO), 7.26–7.16 (m, 5H, Ph), 4.88 (dd, 1H, *J* = 10.5, 4.0 Hz, CH), 3.54 (m, 1H, Ph*CH*$_2$), 3.37 (dd, 1H, *J* = 14.3, 4.7 Hz, Ph*CH*$_2$); ^{13}C NMR (CD_3OD) δ 169.5, 140.2, 137.4, 132.1, 130.4, 129.7, 129.3, 129.2, 127.8, 79.3, 35.9; IR (KBr) ν 3438.9, 3029.1, 2863.2, 1725.9, 1456.4, 1229.8, 1130.5; HRMS (ESI) calcd for $C_{16}H_{16}NO_3$ $[M+H]^+$ *m*/*z*: 270.1130, found 270.1117.

(*S*)-*N*-Benzylidene-5-(*tert*-butoxycarbonylamino)-1-carboxypentan-1-amine oxide (26). (*S*)-Allyl 6-(*tert*-butoxycarbonylamino)-2-(hydroxyamino)hexanoate oxalate (**18**) (2.06 g, 5.25 mmol, 1.00 equiv) was dissolved in DMF (26 mL) and converted to the corresponding *N*-benzylidene nitrone according to the general procedure, and purified by flash chromatography on silica (45% EtOAc in hexanes) to afford (*S*)-1-(allyloxy)-*N*-benzylidene-6-(*tert*-butoxycarbonyl amino)-1-oxohexan-2-amine oxide as white foam (1.64 g, 80% yield). To a round bottom flask charged with a magnetic stir bar, $Cl_2Pd(PPh_3)_2$ (0.53 g, 0.13 mmol, 10 mol%) PPh_3 (0.11 g, 0.41 mmol, 30 mol%), and THF (7 mL) were stirred under nitrogen for 40 min. (*S*)-1-(Allyloxy)-*N*-benzylidene-6-(*tert*-butoxycarbonylamino)-1-oxohexan-2-amine oxide was placed under an inert atmosphere of nitrogen and dissolved in THF (6 mL). The solution was add to the palladium slurry with a syringe and morpholine (1.2 mL) was added dropwise to the reaction mixture. After stirring 2 h at rt, the solution was concentrated *in vacuo* and the resultant yellow solid dissolved in H_2O (50 mL) and washed with CH_2Cl_2 (3 × 50 mL). The aqueous solution was then acidified with 1 N HCl to a pH ~ 3 and extracted with CH_2Cl_2 (3 × 70 mL). The organic phases were combined, dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was recrystallized from Et_2O to provide compound **26** as a white solid in (0.23 g, 50% yield). mp 138–140 °C; $[\alpha]_D^{20}$ −22.8 (*c* 0.14, MeOH); ^{1}H NMR ($CDCl_3$) δ 8.27 (d, 2H, *J* = 7.5 Hz, Ph), 7.58–7.48 (m, 4H, Ph and Ph*CH*NO), 4.52–4.49 (m, 1H, CH), 3.10 (br s, 2H, NH*CH*$_2$), 2.35–2.31 (m, 1H, CH*CH*$_2$), 2.13 (br d, 1H, CH*CH*$_2$), 1.56–1.53 (m, 2H, CH$_2$), 1.48–1.40 (m, 11H, CH$_2$ and OC*Me*$_3$); ^{13}C NMR ($CDCl_3$) δ 169.4, 156.1, 139.7, 132.5, 130.4, 128.9, 128.7, 79.2, 75.2, 40.1, 31.3, 29.4, 28.4, 23.1; IR (thin film) ν 3340.5, 2932.2, 1687.4, 1520.1, 1165.7 cm^{-1}; HRMS (ESI) calcd for $C_{18}H_{26}N_2O_5Na$ $[M+Na]^+$ *m*/*z*: 373.1739, found 373.1731.

(*S*)-*N*-Benzylidene-2-(4-*tert*-butoxyphenyl)-1-carboxyethanamine oxide (25). (*S*)-Allyl 3-(4-*tert*-butoxyphenyl)-2-(hydroxyamino)propanoate oxalate (**17**) (2.0 g, 5.2 mmol, 1.0 equiv) was dissolved in DMF (13 mL) and converted to the corresponding nitrone according to the general procedure. The product was purified by flash column chromatography on silica (15% EtOAc in hexanes) to afford (*S*)-1-(allyloxy)-*N*-benzylidene-3-(4-*tert*-butoxyphenyl)-1-oxopropan-2-amine oxide as a colorless oil (1.51 g, 75% yield). (*S*)-1-(Allyloxy)-*N*-benzylidene-3-(4-*tert*-butoxy phenyl)-1-oxopropan-2-amine oxide (1.34 g, 3.51 mmol, 1.00 equiv) was converted to the carboxylic acid according to the above procedure to afford compound **25** as a white solid (0.710 g, 59% yield). mp 130–132 °C; $[\alpha]_D^{20}$ −211.2 (*c* 0.16, MeOH); ^{1}H NMR ($CDCl_3$) δ 8.01 (d, 2H, *J* = 7.5 Hz, Ph), 7.50–7.38 (m, 3H, Ph), 7.08 (d, 2H, *J* = 8.0 Hz, Ar), 6.83 (d, 2H, *J* = 8.5 Hz, Ar), 6.77 (s, 1H, Ph*CH*NO), 4.46 (dd, 1H, *J* = 11.5, 3.5 Hz, CH), 3.49–3.44 (m, 1H, Ar*CH*$_2$), 3.36 (dd, 1H, *J* = 10.5, 3.5 Hz, Ar*CH*$_2$), 1.22 (s, 9H, OC*Me*$_3$); ^{13}C NMR ($CDCl_3$) δ 168.9, 155.1, 140.5, 132.8, 130.5, 130.2, 129.6, 128.9, 128.0, 124.8, 78.8, 76.3, 37.8, 28.8; IR (thin film) ν 2976.1, 1734.6, 1506.6, 1450.6, 1237.1, 1160.4; HRMS (ESI) calcd for $C_{20}H_{24}NO_4$ $[M+H]^+$ *m*/*z*: 342.1705, found 342.1715.

General procedure for the synthesis of N-terminal peptide hydroxylamines

α-*N*-Hydroxy-Tyr-Ala-Lys-Pro-Ala-Leu-NH$_2$ (34). The peptide sequence Try-Ala-Lys-Pro-Ala-Leu attached to Rink amide resin (0.110 g) was swelled in CH_2Cl_2 (2.0 mL) for 5 min. (*S*)-*N*-Benzylidene-2-(4-*tert*-butoxyphenyl)-1-carboxyethanamine oxide (**25**) (0.110 g, 0.310 mmol, 4.00 equiv) and HBTU (0.120 g, 0.310 mmol, 3.90 equiv) were dissolved in DMF (1.00 mL), followed by addition of DIPEA (0.110 mL, 0.630 mmol, 8.00 equiv). After agitation for 5 min, the resin was drained and the solution containing compound **25** was added to the resin; the solution was agitated until reaction completion was confirmed by Kaiser test. The resin was consecutively rinsed several times with DMF, CH_2Cl_2, DMF, and MeOH. The resin was dried under vacuum, placed in a glass vial and treated with a solution of 99% TFA in CH_2Cl_2. After agitation for 30 min, the resin was filtered and washed with TFA (1.0 mL). The filtrate was placed under a stream of N_2 and reduced to a volume of 1.0 mL. Cold Et_2O was added to the solution, inducing a white precipitate that was collected by vacuum filtration and rinsed several times with cold Et_2O to provide the *N*-benzylidene nitrone peptide as a white solid (0.050 g). The peptide (0.025 g) was dissolved in a solution of

5% TFA in H_2O. The solution was passed through a column of C-18 silica layered with hydroxylamine Wang resin (0.134 g, 2.00 mmol/g, 10.0 equiv) several times. The resulting solution was then lyophilized and the crude hydroxylamine was purified by preparative HPLC using a gradient of 5–50% iPrOH in H_2O over 30 min with a flow rate of 10.0 mL/min and monitored at 220 nm. Compound **34** was isolated as a white solid (0.015 g, 66% yield). ^{1}H NMR (D_3COD) δ 7.10–7.02 (m, 4H), 6.73–6.69 (m, 4H), 4.59 (br m, 1H), 4.47 (br m, 1H), 4.38–4.21 (m, 4H), 4.13 (q, 1H, J = 7.0 Hz), 3.88–3.74 (m, 2H), 3.57–3.51 (m, 2H), 3.17–3.00 (m, 2H), 2.94–2.80 (m, 6H), 2.15 (br m, 1H), 2.00–1.96 (m, 3H), 1.71–1.56 (m, 8H), 1.40–1.29 (m, 7H), 1.18–1.16 (m, 2H), 0.97–0.91 (m, 6H); HPLC retention time: 13.3 min. at 220 nm, column: YMC R-ODS-10A 250 × 20 mm, flow rate: 1 mL/min, gradient: 5–50% iPrOH in H_2O over 30 min. HRMS (ESI) calcd for $C_{41}H_{62}N_9O_{10}$ $[M+H]^+$ 840.4620, found 840.4611.

General procedure for the chemoselective ligation of peptide fragments

Fmoc-Ala-Phe-Tyr-Ala-Lys-Tyr-Pro-Ala-Leu-NH_2 (45)

Hydroxylamine **34** (11.5 mg, 0.013 mmol, 1.0 equiv), and α-ketoacid **5** (0.010 g, 0.020 mmol, 1.5 equiv) were dissolved in 0.40 mL of 90% DMF in H_2O, and stirred overnight at 40 °C. The reaction was allowed to cool to room temperature and concentrated in vacuo. The ligated product was purified by preparative HPLC with a gradient of 40–50% iPrOH in H_2O over 30 min with a flow of 10 mL/min, and monitoring at 254 nm. Compound **45** was isolated as a white solid (0.007 g, 42% yield). ^{1}H NMR (D_3COD) δ 8.18–8.04 (m, 2H), 7.81–7.77 (m, 2H), 7.68–7.64 (m, 2H), 7.40–7.00 (m, 12H), 6.71–6.68 (m, 4H), 4.50–4.18 (m, 9H), 3.99–3.98 (m, 1H), 3.61 (br m, 1H), 3.49–3.45 (m, 1H), 3.05–2.85 (m, 7H), 19.4 (br m, 2H), 1.84 (br m, 1H), 1.68–1.57 (m, 7H), 1.40–1.15 (m, 12H), 0.95–0.91 (m, 6H); HPLC retention time: 12.4 min at 254 nm, column: YMC R-ODS-10A 250 × 20 mm, flow rate: 1 mL/min, gradient: 40–50% iPrOH in H_2O over 32 min; HRMS (ESI) calcd for $C_{68}H_{86}N_{11}O_{13}$ $[M+H]^+$ m/z: 1264.6407, found 1264.6427.

Acknowledgements

This work was supported by the National Institutes of Health, National Institute of General Medical Science (NIGMS) [NIH R01-GM76320] and the David and Lucille Packard Foundation (Fellowship to J.W.B). Generous support from Bristol Myers Squibb, Roche, Amgen, and Eli Lilly is gratefully acknowledged.

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