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A New Scaffold for Amide Ligation

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Abstract—Highly chemoselective amide forming ligation reactions have facilitated the synthetic access to proteins and other amide-linked bioconjugates. In order to generalize this approach, a N^{α}-2-phenyl ethanethiol scaffold has been developed to promote *S* to *N* acyl transfer in a manner analogous to native chemical ligation with N-terminal cysteine residues. Analysis of scaffold-mediated ligation reactions in aqueous solution indicate that the ligation rate at Xaa-Gly junctions is sufficient for the synthesis of large polypeptides. In addition, it was found that the ligation rate is independent of the stereocenter in the scaffold and *S*- to *N*-acyl transfer is rate limiting. These studies indicate that the N^{α}-2-phenyl ethanethiol scaffold is a good candidate for the development of a ligation chemistry for the formation of Xaa-Gly peptides and other unhindered amides.

Introduction

The chemical ligation of unprotectected peptides has proven to be a valuable method for protein synthesis. The ligation of an unprotected peptide thioester to another unprotected peptide possessing an N-terminal Cys to give an amide bond in aqueous solution has been termed native chemical ligation.[1] Extension of this approach to even a single non-Cys site would give greater flexibility to the synthesis of proteins and expand the scope of native chemical ligation to embrace most protein targets.[2] In addition, a general approach for chemoselective amide bond formation would facilitate the synthesis of many unhindered natural products such as *N*-linked glycopeptides, as well as RNA: peptide or other bioconjugates.[3]

One approach to overcome the requirement of the Cys residue is the use of a detachable modification that mimics the ligation characteristics of Cys. The only general site available for introduction of a reversible modification is the primary amine group, converting the amine into a comparatively sterically hindered secondary amine (Fig. 1). Ligation at an N-terminal Cys residue combines rapid thioester exchange with efficient *S*- to *N*-acyl migration. Previously, a removable Cys mimic has been described using an aminoxy anchored ethanethiol (Fig. 1C).[2] However, the hydroxylamine derivative failed to exhibit any of the supernucleophilic properties of the parent hydroxylamine which in combination with a six-membered acyl migration to a secondary amine gave extremely poor acyl rearrangement properties.[4] In the same study, an irreversible N^{α}-ethanethiol modification of the N-terminus (Fig. 1B) showed more promising ligation properties in unhindered model systems.[2,5]

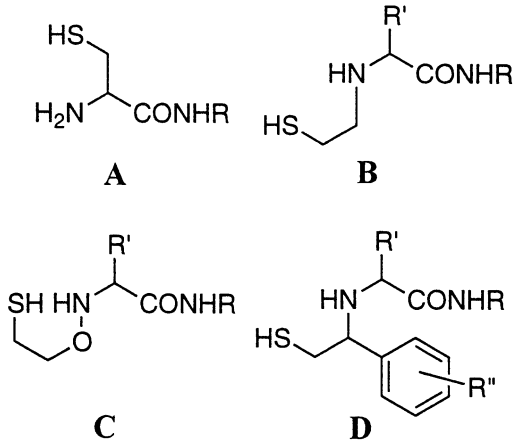

Figure 1. Scaffolds for peptide ligation.

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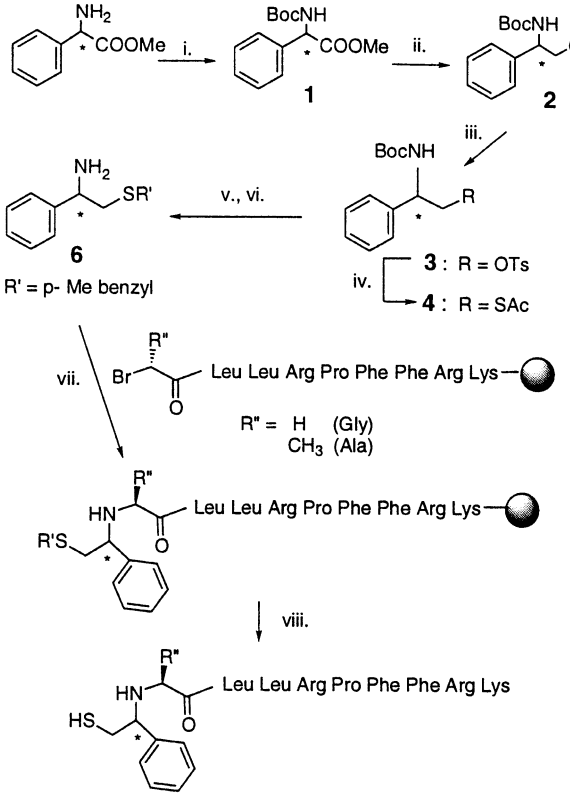
The addition of a phenyl ring to the N^{α}-ethanethiol modification to introduce post ligation lability (Fig. 1D) has some attractive features.[6] Detailed studies on amide bond protection in peptide synthesis have demonstrated that a methoxybenzyl group is acid labile when substituted at an amide bond but acid stable as a benzylamine on the peptide terminus.[7,8] The introduction of the N^{α}-terminal scaffolds should therefore be compatible with standard methods of peptide synthesis, side-chain deprotection and peptide-resin cleavage. Following ligation, the suitably substituted scaffold could be cleaved from the backbone amide with acid. Furthermore, any ring substituent will have a minimal effect on the nucleophilic properties of the thiol side chain. Therefore, photolabile scaffolds (such as those based on α-methyl-6-nitroveratrylamine linkers)[9] can be considered for ligation with all their corresponding advantages for chemoselective bioconjugation of chemically fragile moieties. In contrast, when the nitro and thiol functionalities are both located on the aromatic ring, the nucleophilicity of the thiophenol is too depressed to support the initial thioester exchange step of the ligation.[10]

The use of a five-membered *S*- to *N*-acyl rearrangement and the replacement of the N-terminal aminoxy with an alkylamine should favor the *S*- to *N*-acyl shift. However, the introduction of the phenyl group results in a stereocenter that could strongly effect the rearrangement between L-amino acids. Indeed, such a difference has been observed previously in related work.[11] Consequently, an unambiguous synthetic route to the two enantiomers was required for comparison. The resulting N^{α}-2-phenylethanethiol scaffold is ideal for determining the suitability for this scaffold for ligation, although the resulting benzylamide cannot be removed under acidic conditions. The N^{α}-2-phenylethanethiol scaffold was incorporated into N-terminal Glycyl and L-Alanyl model peptides in order to test their ligation properties in neutral aqueous solution.

Results and Discussion

Both enantiomers of the N^{α}-2-phenyl ethanethiol scaffold were synthesized from commercially available D- and L-phenylglycine (Scheme 1) by a modified route to these compounds, previously reported as amino peptidase inhibitors.[12] The scaffold was then incorporated onto the N-terminus of a polypeptide on the solid support by using the sub-monomer approach.[2,13] The N-terminal residue is introduced in the sequence via the symmetric anhydride of the corresponding α-bromo acid (readily available from D-amino acids) coupled to the peptide resin as its symmetric anhydride.[14] Subsequent reaction with an excess of the amine in the presence of base allows the displacement of the bromine and introduction of the scaffold (Scheme 1). The substitution occurs with inversion of configuration as previously described.[15] For example, (*R*)-α-bromo propionic acid forms the *N*-alkyl-L-Ala-peptide-resin.



Scheme 1. Synthesis of the scaffold and introduction onto peptide: (i) $(t\text{-Bu})_2C_2O_3$, dioxane/water 1:1, quant; (ii) $NaBH_4$, EtOH, 70%; (iii) TsCl, py, 55%; (iv) $CH_3COS^-Cs^+$, DMF, 80%; v. NaOMe, *p*-methyl benzylbromide, 90%; vi. TFA, quant; (vii) DIEA, DMF; (viii) HF, *p*-cresol 5% v/v.

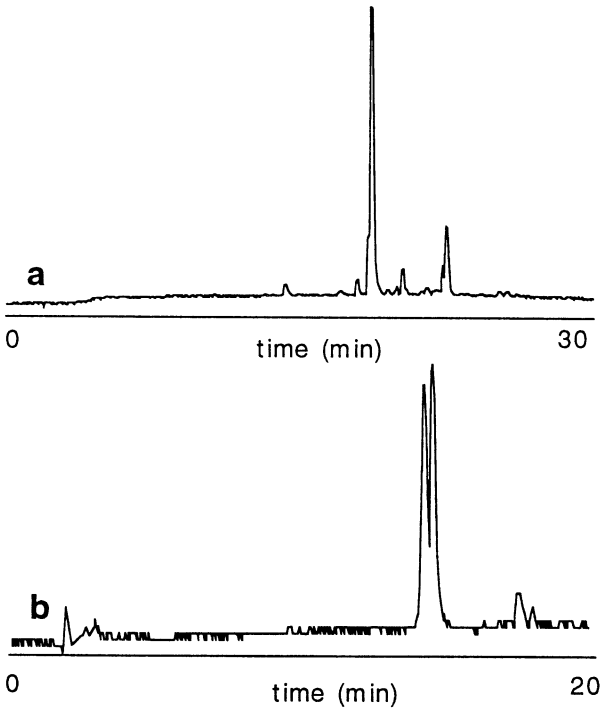


Figure 2. (a) HPLC profile of crude N^{α}-(*R*)-(Pet) **G**LLRPFFRK. Analytical HPLC conditions: Vydac C18 column (5 μm, 0.46×15 cm), 0–67% B in A, linear gradient for 30 min (1 cm^3 min^{-1}, 215 nm UV monitor detection) where buffer A is 0.1% aqueous TFA and buffer B is 10% water, 0.09% TFA in acetonitrile. (b) HPLC coinjection profile of N^{α}-(*R*)- and (*S*)- (Pet) **G**LLRPFFRK. Analytical HPLC: same as above, Vydac C4 column, 15-45% B in A, linear gradient for 20 min.

Subsequent deprotection and cleavage from the resin yields the N^{α}-2-phenyl ethanethiol (Pet) peptide. This procedure proved a robust route to the unambiguous on-resin preparation of the N^{α}-modified peptides, and satisfies one of the principle requirements of a Cys mimic: the necessity of an easy and quantitative route for the derivatization of the peptide (Fig. 2A). It is significant to note that the diasteroemeric peptides generated from the (*R*) and (*S*) scaffolds have different HPLC retention times (Fig. 2B). This suggests that use of a racemic scaffold would complicate the purification of the resulting peptide diastereomers.

Peptide ligations were performed under conditions similar to those used for the assembly of large polypeptides by native chemical ligation.[1,16] The performance of the ligation at an Ala-Gly ligation site was explored (Fig. 3) and in less than 20 h, the peptides were ligated to form the LWAPYRAA(Pet)GLLRPFFRK product (Fig. 3B). The rearrangement was confirmed by the stability of the product to treatment with hydroxylamine (Fig. 3C) which efficiently cleaves thioester intermediates.[17]

The results from ligations in which the residues on either side of the ligation site have been varied are summarized in Table 1. These studies show that the N^{α}-phenyl ethanethiol-Gly peptide undergoes efficient ligation with both Ala and Gly and remarkably, the ligation rate is independent of the stereochemistry of the scaffold. In contrast, neither scaffold was competent for ligation when attached to an N-terminal Ala peptide. The transthioesterification proceeded efficiently but no rearrangement occurred as determined by hydroxylamine treatment. The accumulation of a thioester intermediate suggests that the steric effects of the Ala side chain of the N-terminal residue prevents rearrangement. Previous studies on native ligation rates with different C-terminal amino acids have demonstrated that only the β-branched amino acids and Pro have ligation rates significantly slower than Ala.[18] As a result, the N^{α}-phenyl ethanethiol-scaffold should be compatible

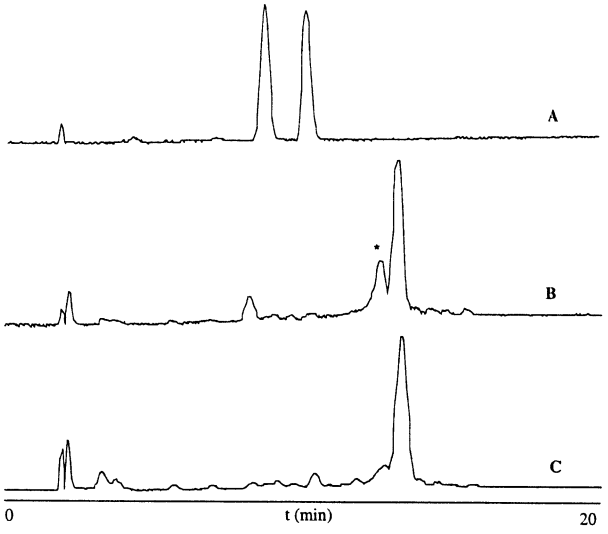


Figure 3. Analytical HPLC traces for the ligation LWAPYRA**A** thioester with N^{α}-(*S*)-(Pet)**G**LLRPFFRK.amide. A: $t=0$; B: $t=20$ h; C: ligation mixture at 20 h after treatment with H_2NOH (pH 6.5). Analytical HPLC conditions: Vydac C4 column (5 μm, 0.46×15 cm), 15–45% B in A, linear gradient for 20 min (1 cm^3 min^{-1}, 215 nm UV monitor detection) where buffer A is 0.1% aqueous TFA and buffer B is 10% water, 0.09% TFA in acetonitrile. (∗ **5** in Figure 4).

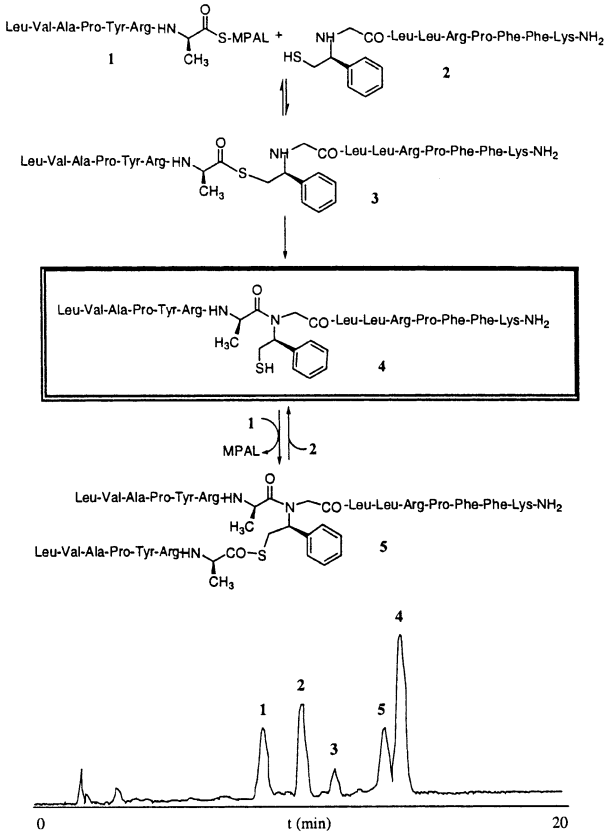


Figure 4. Ligation scheme and HPLC trace at t = 2 h ($t_{1/2}$) for the ligation of thioester LWAPYR**A**-MPAL (**1**) with N^{α}-(*R*)-Pet **G**LLRPFFK.amide (**2**) to give product **4**. Peaks were identified by E/S MS: **1**: 1147.9 (calcd 1148.2); **2**: 1269.7 (calcd 1270.6); **3**: 2198.5 (calcd 2199.7); **4**: 2198.3 (calcd 2199.7); **5**: 3128.5 (calcd 3129.8). HPLC conditions; see Figure 3.

Table 1. Ligations between peptide thioesters and the N^{α}-phenyl ethanethiol (Pet)-peptides[a]

	$t_{1/2}$ (min)			
	R-(Pet) GLLRPFFRK	*S*-(Pet) GLLRPFFRK	*R*-(Pet) ALLRPFFRK	*S*-(Pet) ALLRPFFRK
LWAPYRAG-thioester	40	30	n.p.[b]	n.p.[b]
LWAPYRAA-thioester	140	120	n.p.[b]	n.p.[b]

[a]Values were determined by inspection of analytical HPLC traces for time courses of each ligation. Differences in half lives for the two stereoisomers are within the experimental error.
[b]No rearranged product was detected after 48 h of reaction.

with Xaa-Gly (where Xaa is any amino acid except for Pro and possibly β-branched) ligation sites.

Although ligation takes place in a single practical step with the addition of two unprotected peptides to a neutral buffered aqueous solution, it is usefully considered as consisting of two distinct steps: a reversible thioester exchange followed by an intramolecular *S*- to *N*- acyl shift. In the case of cysteine mediated native chemical ligation, the initial thioester exchange reaction is rate limiting and migration rapid. The intermediate unrearranged *S*-acyl peptide is not observed. Similar results were obtained with model studies using a 2-mercaptobenzyl scaffold.[10] In contrast, thioester-linked ligation intermediates are observed with the N^{α}-phenyl ethanethiol scaffold. The ligation profile after 2 h (Fig. 4) shows substantial quantities of unrearranged thioester intermediate (**3**), suggesting that intramolecular rearrangement is rate determining in this reaction. This is consistent with the observation that added thiophenol, commonly used to activate the thioester in the native ligation reaction[19] had no effect on the rate of ligation. These results suggest that the use of more activated thioesters such as *S*-nitrobenzoate thioesters[1] would be unlikely to increase the ligation rate. In order to accelerate the rate of ligation in this system, the migration rate must be improved. Previous studies on *O*- to *N*-intramolecular acyl transfer, have demonstrated that the solvent[8,20] or the pH[2] dramatically alter the rate of rearrangement. Despite this limitation, the ability to perform peptide ligations at highly abundant X-Gly junctions[21] would greatly facilitate the synthesis of many protein sequences.

Conclusion

This study demonstrates that the N^{α}-phenyl ethanethiol scaffold is a candidate for chemoselective ligation of unprotected polypeptides at Gly residues. Future studies will focus on derivatives of the benzyl group to introduce acid or photolability to the linker. A cleavable derivative of this scaffold may have applications in a variety of amide bond forming reactions in which a high level of chemoselectivity is necessary[3] such as cyclic peptides, *N*-linked glycosylation and polyamide–peptide conjugates.[22] In addition, this scaffold may be useful as a linker for solid-phase protein synthesis, uniquely positioning the linker at the N-terminus of the first peptide.

Experimental

Reagents

Boc-amino acids for peptide synthesis were from Midwest Biotech (Fishers, IN, USA), 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) from Quantum-Appligene (Carlsbad, CA, USA), di-*iso*-propylethylamine from Applied Biosystem (Foster City, CA, USA), TFA from Halocarbon (River Edge, NJ, USA), DMF from EM-Science (Gibbstown, NJ, USA). (*R*)- and (*S*)-phenyl glycine methyl esters and all reagents for the synthesis of the two enantiomeric scaffolds were from Aldrich (Milwakee, WI, USA), solvents from J.T.Baker (Phillipsburg, NJ, USA).

General methods

Electrospray ionization mass spectrometry (ESI-MS) was performed on an API-III triple quadrupole mass spectrometer (PE-Sciex). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all the observed protonation states of a peptide using MacSpec software (Sciex). Theoretical masses of peptides and proteins were calculated using MacProMass software (Beckman Research Institute, Duarte, CA, USA).

HPLC. Analytical reversed-phase HPLC was performed on a Hewlett Packard HPLC 1050 system using Vydac C-18 columns (5 μm, 0.46×15 cm). Semipreparative reversed–phase HPLC was performed on a Rainin HPLC system using a Vydac C-18 column (10 μm, 1.0×25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute bound peptides. The flow rates used were 1 mL/min (analytical), and 5 mL/min (semipreparative).

Peptide synthesis

Peptides were prepared by manual solid-phase synthesis (SPPS) using the in-situ neutralization/HBTU activation for Boc- chemistry as previously described;[23] couplings were carried out with a 5-fold excess of activated amino acid for a minimum of 15 min and monitored by quantitative ninhydrin test.[24] N-Terminal β-mercaptopropionic acid-leucine (MPAL) thioester peptides were synthesized according to a published procedure on a *t*-Boc-Leu-OCH_2-Pam preloaded resin (Applied Biosystems).[18] C-Terminal peptides were synthesized on a MBHA (*p*-methyl benzhydrylamine) resin; after the Boc- deprotection on the last residue, bromoacetic acid (Gly) or (*R*)-α-bromopropionic acid (Ala) were coupled as the symmetric anhydride.[13]

After synthesis, the peptides were treated with HF (1 h, 0 °C) in the presence of 5% (v/v) p-cresol as scavenger for cleavage from the resin and side-chain deprotection.

Synthesis of the scaffold

All reactions were monitored to completion by TLC using the following systems as eluants: $CHCl_3$/MeOH 12:0.5; toluene/ethylacetate 95:5. ^{1}H NMR spectra were recorded on a Varian Mercury 200.

(*N-tert*-Butyloxycarbonyl) phenylglycine methylester 1. Phenylglycine methylester hydrochloride (3.0 g, 15 mmol) was dissolved in dioxane/water 1:1 (150 mL) containing triethylamine (5.1 mL, 37.5 mmol, 2.5 equiv) and di-*tert*-butylcarbonate (3.9 g, 18 mmol, 1.2 equiv). The mixture was stirred for 2 h at room temperature, the bulk of dioxane was evaporated and ethylacetate (250 mL) was added. The mixture was separated,

washed with aqueous HCl (2 M, 200 mL), water (200 mL), dried (Na_2SO_3) and evaporated yielding **1** (3.9 g; quant) as a white solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.35 (m, 5H), 5.50 (m, 1H), 5.31 (m, 1H), 3.72 (s, 3H), 1.43 (s, 9H).

(*N*-*tert*-Butyloxycarbonyl)-2-amino-2-phenyl-ethanol 2. Boc aminoester **1** (3.9 g, 15 mmol) was dissolved in ice-cold ethanol/water 1:1 (300 mL) and $NaBH_4$ (560 mg, 15 mmol) was carefully added. The mixture was stirred at room temperature o/n; HCl (2 M) was added dropwise to destroy the hydride excess until effervescence stopped; the solution was concentrated to about half of the volume and ethylacetate (200 mL) was added. The two layers were separated, the organic layer washed once with 5% aqueous HCl (200 mL), once with saturated aqueous NaCl (200 mL), dried (Na_2SO_3) and evaporated yielding **2** (2.44 g, 69%) as a white solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.31 (m, 5H), 4.75 (m, 1H), 4.20 (t, J = 6.5 Hz, 1H), 3.85 (d, J = 6.5 Hz, 2H), 1.42 (s, 9H).

(*N*-*tert*-Butyloxycarbonyl)-2-amino-2-phenyl-ethanol-*p*-toluenesulphonate 3. *p*-Toluenesulphonylchloride (1.9 g, 9.9 mmol) dissolved in a minimum of pyridine was added to a solution of the Boc-aminoalcohol **2** (2.1 g, 9 mmol) in pyridine (10 mL) at 0 °C and stirred at room temperature o/n. Ethylacetate (100 mL) was added to the mixture followed by aqueous HCl (1 M; 150 mL), separated, washed with aqueous HCl (1 M; 100 mL), water (100 mL), dried (Na_2SO_3), and evaporated. The solid product was triturated in *n*-hexane yielding **3** (2.3 g, 55%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.28–7.65 (m, 9H), 4.9 (bs, 1H), 4.21 (m, 2H), 2.43 (s, 3H), 1.60 (s, 9H).

(*N*-*tert*-Butyloxycarbonyl)-2-amino-2-phenyl-(*S*-acetyl) ethanethiol 4. Cesium thioacetate (5.25 mmol, prepared by mixing 1.75 g of Cs_2CO_3 and 390 μL of thioacetic acid in 10 mL of DMF) was added to a solution of the tosyl derivative **3** (1.5 g, 3.5 mmol) in DMF (1 mL) at 0 °C. The mixture was stirred at room temperature o/n, ethylacetate (100 mL) was added, the mixture separated and the organic layer washed with water (3×100 mL), dried (Na_2SO_3) and evaporated. Chromatography on silica gel (100 g) using toluene/ethylacetate (97:3) as eluant yielded the *S*-acetyl derivative **4** (830 mg, 80%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.4 (m, 5H), 5.1 (m, 1H), 3.25 (m, 2H), 2.37 (s, 3H), 1.40 (s, 9H).

(*N*-*tert*-Butyloxycarbonyl)-2-amino-2-phenyl (*S*-*p*-methyl benzyl) ethylmercaptan 5. The *S*-acetyl derivative **4** (770 mg, 2.6 mmol) was dissolved in dry methanol (60 mL) NaOMe (1 M, 7.8 mL) was added and the mixture stirred for 15 min. *p*-Methyl benzyl bromide (580 mg, 3.2 mmol) was added and the mixture stirred for a further 20 min. H^+ Dowex (ca. 200 mg) was added and the mixture was filtered and evaporated. Chromatography on silica gel (100 g) using toluene/ethylacetate (99:1) as eluant yielded **5** as a white solid (600 mg, 90%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.05–7.40 (m, 9H), 5.15 (bs, 1H), 4.82 (bs, 1H), 3.55 (s, 2H), 2.78 (m, 2H), 2.32 (s, 3H), 1.42 (s, 9H).

2-amino-2-phenyl (*S*-*p*-methyl benzyl) ethanethiol 6. The *N*-Boc protected derivative **5** (170 mg, 0.5 mmol) was dissolved in TFA, left standing for 10 min and evaporated. The crude material was used without further purification.

Coupling to the peptide resin. The crude amine **6** (130 mg, 0.5 mmol) was dissolved in a mixture of DMF (1 mL) and DIEA (0.5 mL, 2.9 mmol). The solution was added to the bromoacetyl peptide-resin (110 mg, 0.05 mmols). For Gly terminal peptide, the reaction was left for 14 h; for the Ala terminal peptide, 24 h. The peptide-resin was washed with DMF, followed by CH_2Cl_2 and dried under vacuum. Peptide sidechain deprotection and cleavage from the resin was carried out by standard HF protcols.[25] Standard workup and HPLC purification yielded peptide, 20 mg (45% on synthesis). ES MS analysis: N^{α}-(Pet)GLLRPFFRK.amide: 1269.7 (calcd 1270.6), N^{α}-(Pet) ALLRPFFRK.amide: 1284.0 (calcd 1284.7).

Ligation reactions. Peptides (1 mg) were dissolved in buffer ($NaHPO_4$, 100 mM; 100 μL) in the presence of 35 mM tris(2-carboxyethyl)phosphineH·Cl, pH 8.5, to give a final peptide concentration of 8 mM. The final pH of the solution after addition of peptide was 7.5.

Acknowledgements

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