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Benzhydrylamine linker grafting: a strategy for the improved synthesis of *C*-terminal peptide amides‡

Dianne Alewood,[a§] Gene Hopping,[a] Andreas Brust,[a,b] Robert C. Reid[a] and Paul F. Alewood[a]*

The standard *p*-MBHA resin used during Boc-chemistry synthesis of peptides carrying *C*-terminal carboxamides is compromised by batch-to-batch variations in its performance. This can cause artificially 'difficult' couplings during peptide chain assembly, which may ultimately lead to failed syntheses given the inability to achieve acceptable coupling yields. To overcome these problems, we have developed a new approach by grafting a functionalized benzhydrylamine linker onto well-characterized and well-performing PAM resins. We combine optimized Boc-chemistry, high-performing PAM resins and new benzhydrylamine-based linkers to achieve improved syntheses of peptide amides. Here we present the synthesis of two new benzhydrylamine linkers and their attachment to selected PAM resins. This novel solid support was evaluated through the synthesis of selected 'difficult' conotoxins and monitoring the coupling efficiency using quantitative ninhydrin assay. The results show a superior performance of the novel linker solid support compared to the standard *p*-MBHA resins routinely used. In summary, we describe an alternative linker-resin system that allows improved access to *C*-terminal amide peptides employing Boc/Bzl chemistry.

Keywords: benzhydrylamine linker; *C*-terminal peptide carboxamide; peptide synthesis; difficult sequences; conotoxin

Introduction

Conotoxins are a rich source of bioactive molecules making them outstanding starting points for drug discovery research [1–6]. Many native conotoxins are produced by the venom glands in the form of *C*-terminal carboxamides [1–6]. Such peptide amides are usually synthesized by solid phase methodologies by coupling protected amino acids to an appropriately amino functionalized polymer support. The peptides are then obtained after chain assembly by employing suitable cleavage conditions [7,8].

The SPPS repertoire for peptide amides employing the Fmoc/t-Bu strategy contains a range of suitable amino functionalized handles, for example, mild acid-sensitive linker systems such as Rink amide linker [9], xanthenyl linker [10], PAL handle [11] and dibenzoylhepta-1,4-diphenylamine linker [12]. In contrast, the Boc/Bzl-strategy is limited to only a few solid supports with the 4-methybenzhydryl amine resin (*p*-MBHA), the most widely used for the synthesis of *C*-terminal peptide carboxamides [13,14]. Solid phase synthesis success is largely dependent on the properties and quality of the solid support used [15,16], and a vast array of polystyrene-based resins are available from a large number of vendors. These popular resins are typically functionalized polystyrene-divinylbenzene-copolymers with inherent quality control issues and little analytical data provided. Although the quality of resins has improved generally over the years, the batch-to-batch variation of *p*-MBHA (Scheme 1) resins for the synthesis of peptide amides employing Boc-chemistry remains a persistent and a difficult issue for peptide chemists. By contrast, the Boc-chemistry synthesis of *C*-terminal acids is well served by the availability of the well-studied and characterized PAM (4-hydroxymethyl-phenylacetamidomethyl) resin (Scheme 1) [17,18].

Both PAM and *p*-MBHA resins (Scheme 1) are obtained from divinylbenzene cross-linked polystyrene through chemical modifications that introduce functionality. In particular, the chemical processes involved to introduce the *p*-MBHA moiety have inherent problems such as unwanted cross-linking, formation of secondary amine groups or side reactions giving rise to other functionalities [19]. These process-related and unwanted side reactions, most likely, lead to the high variability in the commercially available *p*-MBHA resins.

With the aim of developing a more robust resin for the synthesis of peptides carrying *C*-terminal carboxamides, we developed a dual linker approach as follows. Using a well-performing

* *Correspondence to: Paul F. Alewood, Institute for Molecular Bioscience, The University of Queensland, St Lucia 4072, Australia. E-mail: p.alewood@imb.uq.edu.au*

a *Institute for Molecular Bioscience, The University of Queensland, St Lucia 4072, Australia*

b *Xenome Ltd, 120 Meiers Road, Indooroopilly 4068, Australia*

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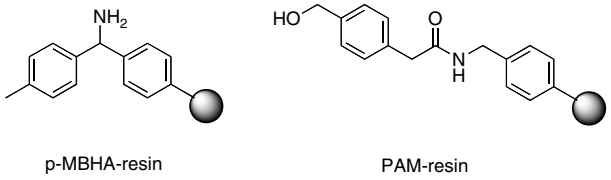
§ *Deceased September 9, 2009.*

Abbreviations used: *HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; HF, hydrogen fluoride; LC–MS, liquid chromatography mass spectrometry; O, in an aminoacid sequence stands for 4-trans-hydroxy-proline; PAM, 4-hydroxymethyl-phenylacetamidomethyl; p-MBHA, 4-methylbenzylhydrylamine; RT, room temperature; SV, substitution value of resin (mmol/g).*



Scheme 1. Handles used for solid phase peptide synthesis employing Boc-chemistry.

Boc-Phe-PAM-resin as the starting resin, we designed and grafted an appropriately functionalized benzhydrylamine linker with chemical properties (TFA stable, HF labile) suitable for Boc/Bzl chemistry.

Several conotoxin sequences, some of which had proven to be 'difficult' when using conventional *p*-MBHA resins, were chosen to evaluate the grafted linker approach. These included αA-conotoxin EIVA (1–14), ω-conotoxin CVID and α-conotoxins [N11S]-PnIA and [Tyr(SO_3H)15Tyr]-EpI (Table 1). Conotoxin αA-conotoxin EIVA (1–14) [20] was derived from the sequence of the 30 amino acid αA-conotoxin EIVA which blocks α/γ and α/δ nicotinic acetylcholine receptors [20,21]. ω-Conotoxin CVID was discovered in our laboratory [22] and displayed 'difficult' regions when assembled on *p*-MeBHA resins. Its synthesis was always problematic with the 'difficult' regions requiring either extended coupling times (often overnight), or re-coupling with other coupling reagents, or blocking of the unreactive *N*-terminus through acetylation. Therefore, CVID was chosen as a model 'difficult' sequence to examine the effect of the new linker grafting approach. [N11S]-PnIA was derived from a component of the venom from *Conus pennaceus*. [23] EpI, which contains a sulfated tyrosine, was also isolated in our laboratory from the venom of *Conus episcopatus* [24].

Results and Discussion

Linker and solid support synthesis

The chosen solid support for our experiments was the well-performing PAM resin. The PAM linker affords a TFA stabile and HF cleavable ester bond with the first attached amino acid. In our approach, we started either with a Boc-Phe-PAM-resin or a Boc-Leu-PAM resin, both commercially available from multiple vendors. These provided an amino functionality to couple the benzhydrylamine linkers. Our linker design was based on the familiar benzhydrylamine linker principle with appropriate substitution to offer stability to repeated TFA treatment, though readily cleavable by hydrogen fluoride (HF). Furthermore incorporation of carboxylic acid functionality in the linker was necessary for easy attachment to the amino acid loaded PAM resin.

The designed linkers (**4a**/**4b**) were readily synthesized, starting from ethyl phenoxyacetate, in four steps with an overall yield of 20% (**4a**) and 27% (**4b**) (Scheme 2). Friedel–Crafts acylation of ethyl phenoxy acetate with benzoyl chloride (R = H) or p-toluoyl chloride (R = CH_3) and $AlCl_3$ in nitrobenzene gave exclusively the 4-substituted benzophenone isomer **1a/1b,** which was conveniently separated from the nitrobenzene solvent by hydrolysis of the ester and extraction of the carboxylate salt into the aqueous phase. Oxime **2** (**a** or **b**) was formed in near quantitative yield by reaction of the benzophenone derivative **1** (**a** or **b**) with hydroxylamine. The *syn* and *anti* isomers formed in approximately equal amounts and their separation was not attempted. The reduction of oxime 2 **(a** or **b)** to amino acid **3** (**a** or **b**; both stereoisomers obtained) was achieved with best results by the Bouveault–Blanc procedure with sodium metal in absolute ethanol. The amino functionality of the racemic linker **3** (**a** or **b**) was protected with the Boc group by acylation with di-*tert*-butyl dicarbonate under Schotten–Baumann conditions, and **4a** and **4b** were obtained as stable crystalline solids by recrystallization from ethanol. The coupling of linker **4a/4b** to the Boc-Phe-PAM or alternative Boc-Leu-PAM resin was near quantitative using symmetric anhydride activation with a coupling yield of 99.9% after 60 min, as confirmed by quantitative ninhydrin assay [25]. The structure of the obtained dual linker construct (**4a**/**4b**-Phe-PAM) used for further peptide synthesis is also shown in Scheme 2.

Peptide synthesis: assembly, cleavage, peptide resin stability in TFA

The grafted resins (Boc-**4a/b**-Phe-PAM-resin) were used for the chain assembly of the 'difficult' conotoxins (Table 1) using standard HBTU activation, *in situ* neutralization protocols [26–28]. The coupling yields were monitored by the quantitative ninhydrin assay [25] and indicated for all synthesized peptides the average coupling yields of >99.5%. αA-conotoxin EIVA (1–14) was assembled using both linkers (**4a** and **4b**) with no observable difference in coupling yields. HF cleavage was performed using *p*-cresol (10 vol%) or alternatively *p*-cresol/*p*-thiocresol (5 vol% each) as scavenger, to evaluate the effect of different cleavage times and cleavage temperatures. Optimum cleavage times were found to be approximately 2 h at 0–2 °C. Figure 1 depicts the effect of additional *p*-thiocresol as scavenger, resulting in cleaner crude peptide product (cf Figure 1(B) with Figure 1(A)). It appears that the cleavage of the peptide–linker bond is promoted by *p*-thiocresol as experiments using only *p*-cresol as scavenger showed additional product with a mass of 1812 Da (Figure 1(A), peaks 3 and 4). This mass corresponds to the peptide with the linker (**4a**)-Phe-moiety still attached. LC–MS analysis reveals two products with identical mass due to the use of linker **4a** as a racemic mixture, which gives rise to two diastereomeric peptidyl-linker (**4a**)-Phe adducts. Quantitative cleavage can be achieved by the addition of *p*-thiocresol (Figure 1(B)).

Table 1. Conotoxin carboxamides synthesized to evaluate grafted linker strategy

ID	Sequence	Conus sp.	Pharmacology	Reference
EIVA (1–14)	GCCGPYONAACHOC-NH_2	*ermineus*	alpha α	[20,21]
CVID	CKSKGAKCSKLMYDCCSGSCSGTVGRC-NH_2	*catus*	omega ω	[22]
[N11S]-PnIA	GCCSLPPCAASNPDYC-NH_2	*pennaceus*	alpha α	[23]
[Tyr(SO_3H)15Tyr]-EpI	GCCSDPRCNMNNPDYC-NH_2	*episcopates*	alpha α	[24]

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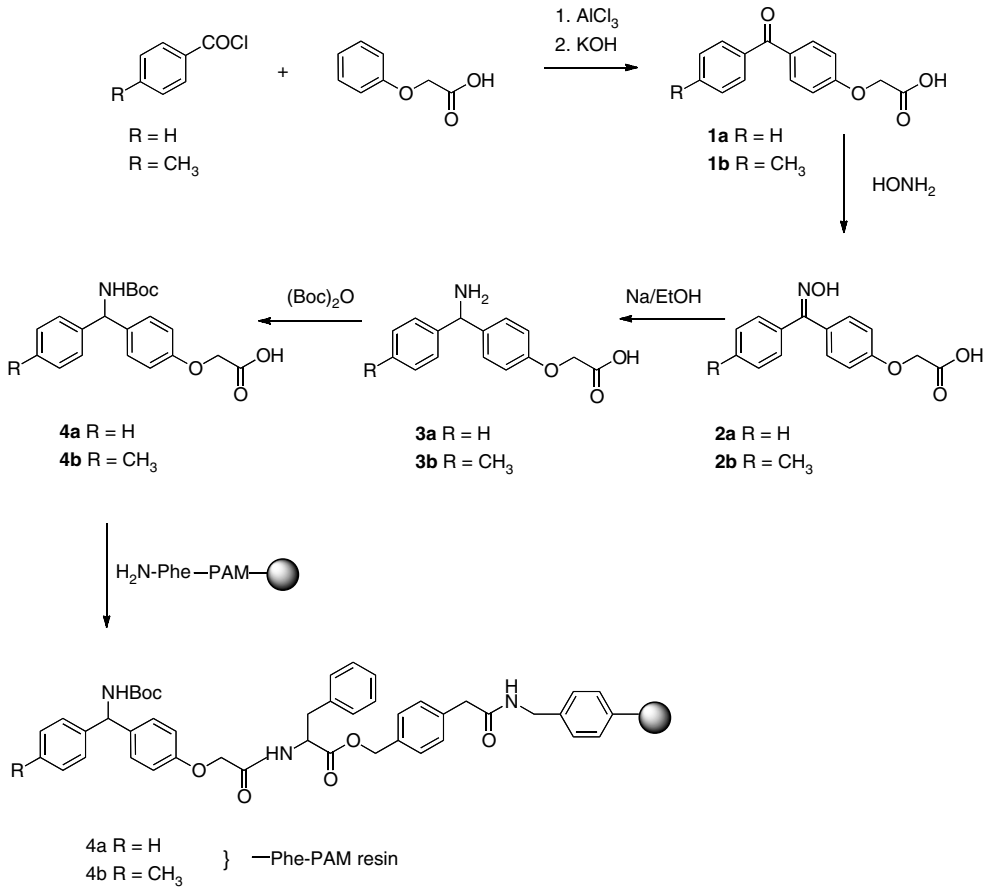

Scheme 2. Synthesis sequence of benzhydrylamine linker **4a** and **4b** and dual linker construct, **4a/4b**-Phe-PAM resin.

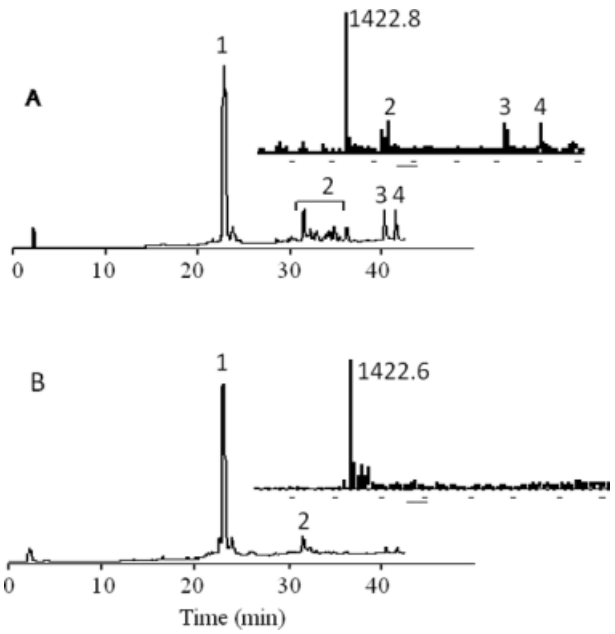

Figure 1. αA-EIVA (Peak 1: $MH^+ = 1422.6$) crude peptide obtained from assembly on **4a**-Phe-PAM resin and HF cleavage for 2 h at 0–2 °C. (A) use of *p*-cresol as scavenger; (B) use of *p*-cresol/*p*-thiocresol as scavenger. Peak 2 relates to some persistent HF cleavage typical adducts, with peaks 3 and 4 relating to peptide having still linker-**4a**-Phe attached (M + 389), which is due to insufficient peptide-linker cleavage.

To evaluate the stability of the peptide–linker bond for long syntheses, the test peptide αA-EIVA (1–14) while attached to either **4a- or 4b-**Phe-PAM resin was stirred at room temperature in neat TFA for 20 h, which equates to approximately 600 deprotection cycles. On filtration, the resulting TFA solution was collected, then diluted with water and lyophilized. Any residual solids were collected, weighed and analyzed by LC–MS and the percentage of cleaved peptide product was estimated using initial peptide resin loading. Linker **4a** was very stable under these conditions, resulting in less than 5% premature cleavage. Linker **4b** was less stabile and showed a cleavage of peptide αA-EIVA from the resin in amounts of about 30% (data not shown). The increased acid sensitivity of linker **4b** is attributed to the electron donating property of the additional methyl group to stabilize the intermediate carbocation formed during cleavage. Thus linker **4a** is the linker of choice where longer peptide sequences are desired.

Conotoxin CVID has proven in our hands to be a very difficult peptide to synthesize when employing commercially available *p*-MBHA resins. To evaluate a range of commercially available *p*-MBHA resins, we performed the parallel assembly of conotoxin CVID which employed a single 10 min coupling strategy as previously reported [26–28]. Several of these resins failed to deliver CVID in any quantity. Subsequently, we selected the best two performing commercially available *p*-MBHA resins for comparison with our grafted solid support **4a**-Phe-PAM and **4b**-Phe-PAM resins, as well as an amino acid linker variation **4a**-Leu-PAM. We used PAM resins from four different vendors to investigate whether the results were related to the quality of the PAM resins.

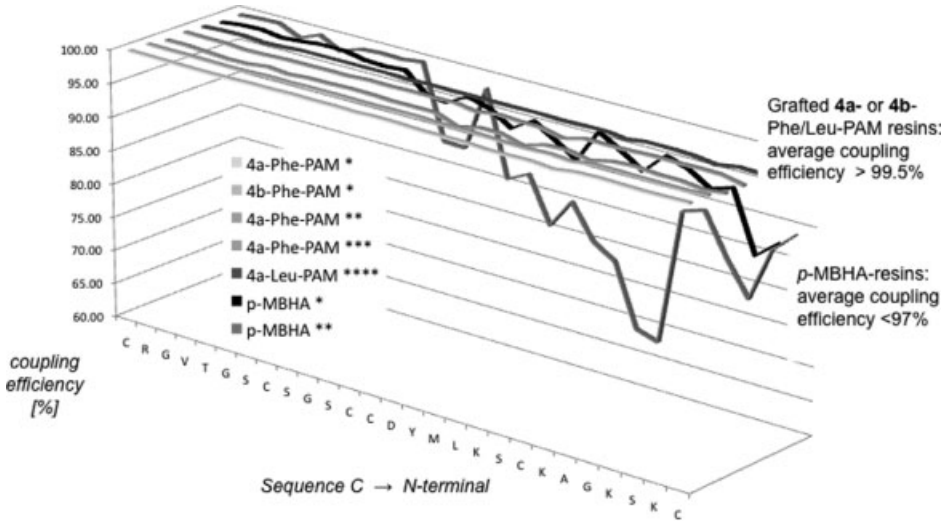

Figure 2. CVID assembly: coupling yields (10 min) obtained by the ninhydrin assay [25]: grafted solid supports outperform investigated *p*-MBHA resins with superior coupling efficiency. The grafted linker (**4a/4b**)-Phe/Leu-PAM resins show excellent coupling efficiency independent from the vendor source (different vendors are indicated by asterisk) while *p*-MBHA resins shows batch-to-batch variation.

The coupling yields of chain assembly were monitored using the quantitative ninhydrin assay [25] after a coupling time of 10 min (Figure 2). We observed that all **4a** or **4b** grafted PAM resins were far superior to the selected *p*-MBHA resins (Figure 2) and delivered consistent average coupling yields of >99.5% (10 min). The *p*-MBHA resins had coupling yields as low as 65% resulting in average coupling yields over the whole synthesis of <97%. It would appear that the chain assembly difficulties on *p*-MBHA resin are related to the chemistry that introduced the *p*-MBHA handle [19] rather then the peptide itself.

Using both linkers (**4a**- and **4b**-Phe-PAM), an optimized synthesis of CVID was performed and was compared to an optimized *p*-MBHA resin synthesis. The linker synthesis resulted in excellent crude peptide quality (Figure 3(A)). Whereas **4a** and **4b**-Phe-PAM coupling yields were satisfactory after 10 min, it was necessary to double couple amino acids on the *p*-MBHA resin starting from amino acid 11 onwards with extended coupling times required. It is obvious that certain regions in the sequence are coupling less efficiently (amino acid 11–18) with deterioration towards the end of the sequence where *p*-MBHA resin is used (Figure 2). Even the extended reaction times and the multiple couplings did not improve average coupling yields to above 99%. Thus it appears that the synthetic difficulties are only partially peptide sequence related and are mainly caused by the poorly performing *p*-MBHA solid support.

The grafted dual linker resin **4a** -Phe-PAM was further employed for the synthesis of conotoxins [N11S]-PnIA and [Tyr(SO_3H)15Tyr]-EpI. In both cases, peptides were obtained with >95% recovery and average coupling yields of >99.6%. Figure 3(B) and (C) display HPLC traces of crude peptides [N11S]-PnIA and [Tyr(SO_3H)15Tyr]-EpI with excellent peptide purity.

All reduced peptides obtained (CVID, [N11S]-PnIA and [Tyr(SO_3H)15Tyr]-EpI) were oxidized into the disulfide folded peptides and delivered material with identical properties (data not shown) to those previously reported by us [22,24].

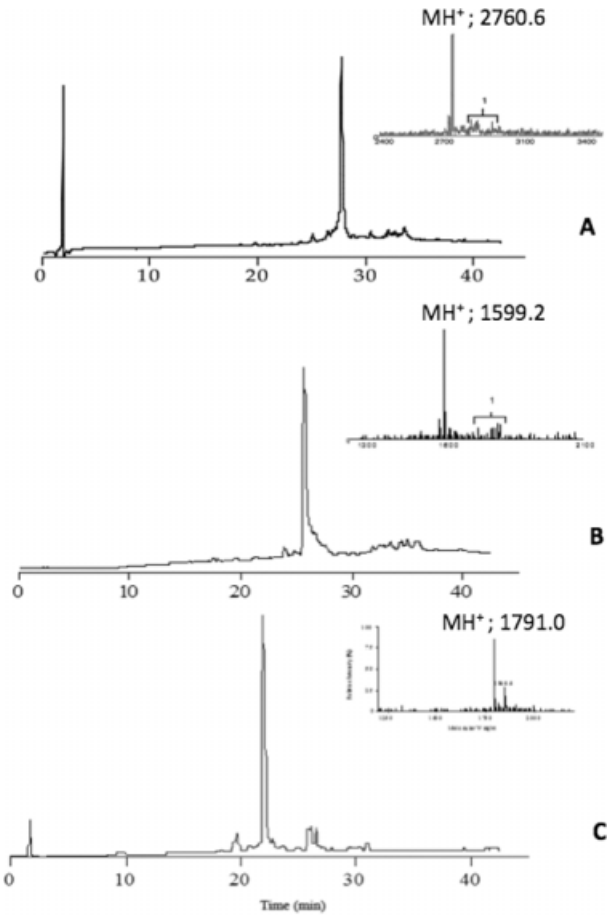

Figure 3. HPLC–MS obtained for crude CVID (A), crude [N11S]-PnIA (B) and crude [Tyr(SO_3H)15Tyr]-EpI (**C**) synthesized on **4a**-Phe-PAM resin.

Conclusions

The synthesis of several conotoxin carboxamides using commercially available *p*-MBHA resins showed strong 'batch-to-batch'

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variations which resulted in artificial difficult peptide assembly. The new benzhydrylamine linkers **4a** and **4b** when grafted on high-performing PAM resins led to the facile synthesis of these conotoxins previously thought to contain difficult sequences. This linker grafting strategy adds to the arsenal of synthetic tools for the successful synthesis of *C*-terminal peptide amides employing the Boc/Bzl-strategy.

Experimental Section

Materials and methods

Protected Boc-amino acid derivatives were from Peptide Institute (Osaka, Japan), Auspep P/L (Melbourne, Australia) or Novabiochem (Sydney, Australia). The following side chain protected Boc-amino acids were used: Cys(4-MeBzl), His(Dnp), Hyp(Bzl), Tyr(BrZ), Thr(Bzl), Ser(Bzl), Asn(Xan), Lys(ClZ), Arg(Tos). All other Boc-amino acids were unprotected. DMF, DCM, DIEA and TFA were all peptide synthesis grade supplied by Auspep P/L (Melbourne, Australia). HBTU was supplied by Richelieu Technologies (Hyacinthe, Quebec, Canada). HPLC-grade acetonitrile (EM Science, Gibbstown, NJ, USA) was supplied by Laboratory Supply (Sydney, Australia). Boc-Phe-PAM-resin was supplied by Applied Biosystems (Foster City, CA, USA, SV = 0.74 mmol/g), Bachem (Bubendorf, Switzerland, SV = 0.7 mmol/g) and Novabiochem (Laufelfingen, Switzerland, SV = 0.71 mmol/g). Boc-Leu-PAM resin was supplied by Peptides International (Louisville, KY, USA, SV = 0.56 mmol/g). *p*-MBHA resin was supplied by Applied Biosystems (SV = 0.4 mmol/g) and Bachem (Switzerland). *p*-Cresol and *p*-thiocresol were of the highest grade available from Fluka (Seelze, Germany). All ethanol used was dry 100% absolute ethanol from CSR (Homebush Bay, Australia).

NMR spectra were recorded on a Varian Gemini 300 spectrometer at room temperature and were referenced to the residual solvent peak for d_6-DMSO solutions, δ_H 2.50, δ_C 39.5 ppm or for D_2O solutions, DSS δ_H 0.00 and 1,4-dioxane δ_C 67.4 ppm.

RP-HPLC data were acquired by elution of a Zorbax C18, 300 Å, 3.5 μm column (Hewlett Packard, Australia) with a linear gradient from 0–60% or 0–72% acetonitrile using buffers A = 0.1% aqueous TFA, B = 90% acetonitrile/0.085% aq. TFA at a flow rate of 1 ml/min with UV detection at 214 nm.

Synthesis of linkers 4a/4b

Ethyl phenoxy acetate

A solution of phenoxy acetic acid (100 g, 0.65 mol) in ethanol (500 ml) and concentrated H_2SO_4 (25 ml) was refluxed for 14 h then evaporated to about one-third of the original volume on a rotary evaporator. The residue was dissolved in ether, washed with water (200 ml), 10% K_2CO_3 (aq), (3 × 50 ml) saturated brine (2 × 200 ml) and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure, then further under high-vacuum pressure for 18 h, to give a colorless oil (108 g, 0.60 mol, 92%).

4-(Benzoylphenoxy)acetic acid 1a

Anhydrous $AlCl_3$ (26 g) was added to a stirred solution of ethyl phenoxy acetate (16.5 g, 92 mmol) and benzoyl chloride (16 ml, 138 mmol) in nitrobenzene (25 ml). The temperature increased to about 40 °C. Stirring under a $CaCl_2$ drying tube was continued at room temperature for a further 60 min; then the mixture was poured into ice/water (600 ml) and stirred for a further 15 min. The mixture was extracted with ether and the organic layer was washed with 2 M HCl, brine, 10% K_2CO_3 and brine. The ether was removed *in vacuo* and the remaining nitrobenzene solution was diluted with THF (200 ml), water (200 ml), and KOH (12 g) and refluxed with stirring for 60 min. After cooling, ether and water were added and the aqueous layer was separated, washed with ether and acidified with 10 M HCl. Stirring for a further 10 min was required before the product crystallized fully which was then filtered off, washed with water and air dried. Recrystallization from ethanol (5 ml/g) gave white prisms (17.5 g, 68 mmol, 74%) m.p. 151–153 °C.

^{1}H NMR (300 MHz, d_6-DMSO) δ 7.75–7.60 (m, 5 H, ArH), 7.60–7.49 (m, 2 H, ArH), 7.07 (AA′XX′ system $J_{AX} + J_{AX'} = 8.8$ Hz, 2 H, *ortho* to O), 4.82 (s, 2 H, OCH_2). ^{13}C NMR (d_6-DMSO) δ 194.4, 169.7, 161.4, 137.7, 132.1, 132.1, 129.8, 129.3, 128.5, 114.4, 64.6. λ_{max} (KBr) cm^{-1} 1738, 1714, 1652, 1595, 1509, 1424, 1320, 1305,1247, 1184, 1151, 1080, 938, 915, 860, 788, 745, 692, 674, 615. *m/z* 257 (MH^+), 274 (MNH_4^+).

4-(4-Methylbenzoyl)phenoxyacetic acid 1b

This was prepared from ethyl phenoxy acetate (16.5 g, 92 mmol) using the procedure for **1a** except that benzoyl chloride was replaced with 4-toluoyl chloride (18 ml, 136 mmol) resulting in white prisms (19.2 g, 76 mmol, 82%) m.p. 173–175 °C.

^{1}H NMR (300 MHz, d_6-DMSO) δ 7.72 (AA′XX′ system $J_{AX} + J_{AX'} = 8.8$ Hz, 2 H), 7.61 (AA′XX′ system $J_{AX} + J_{AX'} = 8.0$ Hz, 2 H), 7.35 (AA′XX′ system $J_{AX} + J_{AX'} = 8.0$ Hz, 2 H), 7.06 (AA′XX′ system $J_{AX} + J_{AX'} = 8.8$ Hz, 2 H), 4.82 (s, 2 H, OCH_2), 2.40 (s, 3 H, CH_3). ^{13}C NMR (d_6-DMSO) δ 194.1, 169.7, 161.2, 142.5, 134.9, 131.9, 130.1, 129.6, 129.0, 114.3, 64.5, 21.1. *m/z* 271 (MH^+).

4-(Hydroxyimino-phenymethyl) phenoxyacetic acid 2a

A mixture of 4-(benzoyl)phenoxyacetic acid **1a** (27.0 g, 105 mmol), hydroxylamine hydrochloride (15 g, 217 mmol), ethanol (75 ml) and water (125 ml) was stirred and heated until boiling. Solid KOH (45 g) was added in portions over 5 min then the solution was refluxed for a further 10 min. The solution was cooled to 10 °C and acidified to pH 2 with conc. HCl (about 50 ml). The mixture was extracted with EtOAc and the combined extracts were washed with brine, dried over $MgSO_4$ and evaporated to dryness giving the oximes as a white solid (26.2 g, 103 mmol, 98%). The ratio of *syn : anti* oximes was about 1 : 1 as shown by NMR. The spectral data below refer to the mixture of *syn* and *anti* oximes.

^{1}H NMR (300 MHz, d_6-DMSO) δ 13.05 (br s, 1 H, COOH), 11.29 and 11.12 (br s, 1 H, NOH), 7.51–7.20 (m, 7 H, ArH), 6.98 and 6.90 (AA′XX′ systems $J_{AX} + J_{AX'} = 8.9$ Hz, 2 H, *ortho* to O), 4.73 and 4.69 (s, 2 H, OCH_2). ^{13}C NMR (d_6-DMSO) δ 170.1, 170.0, 158.3, 157.7, 154.8, 154.7, 137.2, 133.7, 130.6, 129.7, 128.8, 128.8, 128.3, 128.3, 128.1, 127.3, 125.9, 114.3, 114.0, 64.4. λ_{max} (KBr) cm^{-1} 3242 (br), 1706, 1602, 1509, 1446, 1330, 1308, 1247, 1186, 1162, 1076, 1006, 945, 930, 837, 775, 700, 662. *m/z* 272 (MH^+), 289 (MNH_4^+).

4-(Hydroxyimino-p-tolylmethyl)phenoxyacetic acid 2b

This was prepared from **1b** (28.4 g, 105 mmol) by the method for **2a** yielding the oxime **2b** as a white solid (27.4 g, 102 mmol, 97%). The spectral data below refer to the mixture of *syn* and *anti* oximes.

^{1}H NMR (300 MHz, d_6-DMSO) δ 7.32–7.13 (m, 6 H), 6.96 and 6.89 (AA′XX′ systems $J_{AX} + J_{AX'} = 8.8$ Hz, 2 H, *ortho* to O), 4.73 and 4.68

(s, 2 H, OCH_2), 2.35 and 2.30 (s, 3 H, CH_3). ^{13}C NMR (d_6-DMSO) δ 170.1, 170.0, 158.3, 157.6, 154.7, 154.6, 138.2, 137.7, 134.4, 130.7, 130.6, 129.9, 128.9, 128.6, 128.3, 127.2, 126.0, 64.4, 20.9, 20.8. *m/z* 286 (MH^+).

(R/S)-4-(Amino-phenylmethyl)phenoxyacetic acid 3a

The oxime **2a** (28.4 g, 105 mmol) was dissolved in boiling absolute ethanol (300 ml) and stirred with a very substantial stirring bar (or preferably mechanical stirring). Sodium (30 g) was added through the condenser in portions as rapidly as possible, keeping the reaction refluxing vigorously (about 30 min). Heating was reapplied toward the end of the reaction and stirring was continued until all of the sodium had dissolved. Cold water (350 ml) was added and the mixture was stirred until all solids had dissolved then the solution was acidified to pH 5 with conc. HCl (about 120 ml). The mixture was left to crystallize overnight, then the racemic product was filtered off, washed well with water and dried giving a white powder (15.4 g, 56.7 mmol, 54%) m.p. 233–237 °C.

^1H NMR (300 MHz, D_2O + DCl + DSS) δ 7.55–7.30 (m, 7 H, ArH), 6.96 (AA′XX′ system $J_{AX} + J_{AX'} = 8.7$ Hz, 2 H, *ortho* to O), 5.68 (s, 1 H, CHN), 4.72 (s, 2 H, OCH_2). ^{13}C NMR (D_2O + DCl) δ 173.8, 158.1, 137.3, 130.8, 130.1, 129.8, 127.7, 116.0, 65.7, 58.4. λ_{max} (KBr) cm^{-1} 3200–2400, 1660–1470, 1423, 1338, 1302, 1253, 1200, 1056, 945, 851, 822, 696. *m/z* 258 (MH^+), 241 (M-NH_2).

(R/S)-4-(Amino-p-tolylmethyl)phenoxyacetic acid 3b

This was prepared from **2b** (29.9 g, 105 mmol) using the procedure for **3a** yielding a white powder (17.0 g, 63 mmol, 60%) m.p. 235–238 °C.

^1H NMR (300 MHz, D_2O + DCl + DSS) δ 7.35 (AA′XX′ system $J_{AX} + J_{AX'} = 8.8$ Hz, 2 H), 7.29 (m, 4 H), 7.01 (AA′XX′ system $J_{AX} + J_{AX'} = 8.8$ Hz, 2 H), 5.64 (s, 1 H), 4.77 (s, 2 H), 2.33 (s, 3 H). ^{13}C NMR (D_2O + DCl) δ 173.8, 158.1, 140.1, 134.5, 131.1, 130.6, 129.6, 127.7, 115.9, 65.6, 58.0, 21.0. *m/z* 255 (M-NH_2), 543 (2 MH^+).

(R/S)-4-(tert-Butoxycarbonylamino-phenylmethyl)phenoxyacetic acid 4a

The amino acid **3a** (14.6 g, 56.7 mmol) and K_2CO_3 (15.6 g) were dissolved in water (120 ml) with heating, and then the solution was cooled to room temperature. A solution of di-*tert*-butyl dicarbonate (13.5 g, 62 mmol) in THF (120 ml) was added and the mixture was stirred vigorously for 60 min. Water and ether were added then the aqueous layer was acidified to pH 3 with citric acid and extracted with EtOAc/DCM (2 : 1). The extracts were washed with sat. brine, filtered through Celite (to remove a persistent insoluble by-product), then dried ($MgSO_4$) and evaporated to dryness. The solid residue was recrystallized from ethanol giving white prisms (10.62 g, 30 mmol, 53%) m.p. 154–156 °C.

^1H NMR (300 MHz, d_6-DMSO) δ 7.89 (d, $J = 9.3$ Hz, 1 H, NH), 7.35–7.17 (m, 7 H, ArH), 6.34 (AA′XX′ system $J_{AX} + J_{AX'} = 8.7$ Hz, 2 H, *ortho* to O), 5.76 (d, $J = 9.3$ Hz, 1 H, CHN), 4.63 (s, 2 H, OCH_2), 1.39 (s, 9 H, $(CH_3)_3$. ^{13}C NMR (d_6-DMSO) δ 170.2, 156.6, 155.0, 143.3, 135.6, 128.3, 128.2, 127.0, 126.7, 114.1, 78.1, 64.4, 57.1, 28.3. λ_{max} (KBr) cm^{-1} 3372, 3100–2500, 1744, 1683, 1611, 1506, 1446, 1391, 1365, 1303, 1252, 1223, 1170, 1082, 1046, 1022, 914, 882, 844, 805, 695. *m/z* 358 (MH^+), 302 (M-^{t}Bu), 241 (M-BocNH).

(R/S)-4-(tert-Butoxycarbonylamino-p-tolylmethyl)phenoxyacetic acid 4b

This was prepared from **3b** (16.2 g, 60 mmol) and di-*tert*-butyl dicarbonate (13.5 g, 62 mmol) by the method for **4a** giving white prisms (13 g, 35 mmol, 58%) m.p. 170–171 °C.

^1H NMR (300 MHz, d_6-DMSO) δ 12.95 (broad s, 1 H), 7.83 (d, $J = 9.1$ Hz, 1 H, NH), 7.22–7.07 (m, 6 H), 6.82 (AA′XX′ system $J_{AX} + J_{AX'} = 8.6$ Hz, 2 H), 5.71 (d, $J = 9.2$ Hz, 1 H, CH), 4.62 (s, 2 H, OCH_2), 2.25 (s, 3 H, CH_3), 1.36 (s, 9 H, O^{t}Bu). ^{13}C NMR (d_6-DMSO) δ 170.2, 156.5, 155.1, 140.4, 135.9, 135.7, 128.8, 128.3, 127.0, 114.1, 78.0, 64.5, 56.9, 28.3, 20.6. *m/z* 255 (M-BocNH), 389 (MNH_4^+), 626 (M + 255), 643 (M + 272), 653, 743 (2 MH^+), 760 (2 MNH_4^+).

Attachment of linker 4a/4b to Phe-PAM resin

The Boc-Phe-OCH_2-PAM resin (0.5 mmol) was swollen in DMF for 30 min then drained. The Boc group was removed using neat TFA (2 × 1 min). The resin was flow washed with DMF for 1 min prior to neutralization with 10% DIEA in DMF (2 × 1 min). The resin was then flow washed for 1 min with DMF. Linker **4a** (2 mmol) was dissolved in 3 ml DCM and a minimum amount of DMF, then DIC (157 µl; 1 mmol) was added and the mixture was activated at RT for ca 30 min (the formation of insoluble *N*,*N*′-dicyclohexylurea was not observed). It was necessary to use more DMF to dissolve linker **4b**, but otherwise the procedure was identical. The activated linker was then added to the drained resin and the coupling efficiency measured after 60 min (>99.8% incorporation) using the ninhydrin reaction [25]. The resin was finally washed with DMF, DCM, then dried under nitrogen prior to use in the syntheses.

Chain assembly Boc-chemistry

The chain assembly of the peptides (0.25 mmol resin used) was performed on a manual shaker system using HBTU activation and *in situ* neutralization protocols [26] to couple the Boc-protected amino acid to the resin. The Boc protecting group was removed using 100% TFA, and DMF was used as both the coupling solvent and for flowwashes throughout the cycle. The progress of the assembly was monitored by the quantitative ninhydrin assay [25].

Synthesis of CVID was performed on *p*-MBHA resin supplied by Applied Biosystems and Bachem, as well as on synthesized **4a**-, and **4b**-Phe-PAM (PAM resin supplied by Applied Biosystems, Bachem and Novabiochem) and **4a**-Leu-PAM resin (PAM resins supplied by Peptide International). The coupling efficiencies were monitored and compared at a coupling time of 10 min using the quantitative ninhydrin assay [25]. For results see Figure 2.

αA-EIVA (1–14) was prepared from the linkers **4a** and **4b** linked to a Phe-PAM resin. [Tyr(SO_3H)15Tyr]-EpI was made with linker **4a** attached to Phe-PAM resin. [N11S]-PnIA was made on **4a**-Phe-PAM resin.

Evaluation of stability of peptide-linker bond to TFA

The test peptide αA-EIVA assembled on both **4a-** and **4b**-Phe-PAM resins was stirred at RT in TFA for 20 h. The TFA solution was collected and diluted with water and lyophilized. Any resulting solids were collected, weighed and examined by ESI-MS. The amount of cleaved peptide product was compared to initial peptide loading on the resin used.

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Deprotection and cleavage of peptide resins

Histidine containing peptides require the removal of the Dnp-protecting groups prior to HF cleavage. This deprotection of His(Dnp) is performed by thiolysis (β-mercaptoethanol : DIEA : DMF, 2 : 1 : 7, 2 $\times$ 30 min). After subsequent removal of the *N*-α-Boc group, using TFA and flow-washes with DMF, DCM and drying under nitrogen the peptide resin can be transferred to HF treatment to achieve simultaneous deprotection of side chains and cleavage of the peptide from the resin. This cleavage was carried out in a HF cleavage apparatus supplied by Peptide Institute using a ratio of 9 : 1 HF to scavengers (1 : 1 *p*-cresol:*p*-thiocresol) for 2 h at 0° to 2° (optimized conditions for peptides using linker **4a** and **4b**). After removal of HF, the peptide was precipitated from ether and washed with ether, then dissolved in 50% acetonitrile/0.1% aqueous TFA prior to lyophilization and characterization.

Characterization

The crude, reduced peptides were examined by RP-HPLC and correct molecular weight was confirmed by ESI-MS. Reconstructed data were used to look for the addition of masses (+387 or +402) corresponding to the presence of the linker (**4a**/**4b**)-Phe moiety, indicating insufficient cleavage between peptide and linker-Phe-resin. Peptides α A-EIVA (1–14), [N11S]-PnIA, [Tyr(SO_3H)15Tyr]-EpI were assembled on 0.25 mmol **4a**-Phe-PAM resin (ABI, SV = 0.62) with an average coupling yield [25] of >99.8% (10 min couplings) and the peptides obtained after HF cleavage had >95% peptide recovery and HPLC–MS crude purity >70%, αA-EIVA (1–14) assembled on **4b**-Phe-PAM resin, was obtained in 90% peptide recovery after HF cleavage and 80% purity (HPLC).

CVID when assembled on *p*-MBHA resin (ABI; 0.25 mmol, SV = 0.62 mmol/g) was obtained with 53% peptide recovery and purity by LC–MS of 23%.

CVID when assembled on a 0.25 mmol **4a**-Phe-PAM resin (ABI; SV = 0.63 mmol/g) which resulted in an average coupling yield [25] of 99.7% (10 min couplings) with peptide recovery after HF cleavage of 91% and HPLC crude purity of 73%.

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