Improved preparation of β-amyloid(1–43): structural insight leading to optimised positioning of *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide protection

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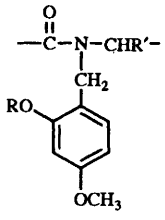
We report an improved synthetic strategy for the preparation of the amyloidogenic protein fragment β-amyloid(1–43). The scheme is based upon the generation of highly soluble *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide-protected intermediates. Optimal positioning of backbone protection at glycines[25,29,33,37] together with phenylalanine[20], was determined from a β-hairpin model derived for the C-terminal region of the resin-bound peptide. This improved scheme provides the final product in 28% overall yield.

Introduction

We have recently described[1] the synthesis and purification of the sparingly soluble peptide β-amyloid(1–43), a major constituent of the extracellular proteinaceous deposits known as amyloid plaques[2,3] that are associated with Alzheimer's disease.[4] Our overall strategy[1] was based upon the straightforward purification of intermediates, rendered soluble by backbone amide protection with the *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) group **1**.[5] This successful strategy



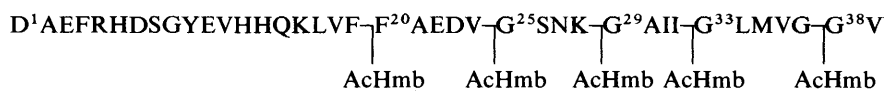
1 R = H, Hmb
R = Ac, AcHmb
R′ = Amino acid side chain

proceeded through the crude intermediate β-amyloid[1–43, (AcHmb)Phe[20], (AcHmb) Gly[25,29,33,38]] **2**, which exhibited a solubility >150 mg cm^{-3} in a 1:1 mixture of 0.1% aq. trifluoroacetic acid (TFA)–acetonitrile, allowing purification by standard reversed-phase high-performance liquid chromatography (RP-HPLC) techniques. Final backbone deprotection of purified compound **2** yielded β-amyloid(1–43) in 7.5% overall yield.

We originally developed the fluoren-9-ylmethoxycarbonyl (Fmoc)/*tert*-butyl-compatible[6] Hmb backbone amide-protection system as a method[7,8] to overcome the 'difficult sequence' phenomenon,[9–11] a problem which has plagued solid-phase peptide synthesis for many years. Difficult sequences suffer from a severe solubility problem during synthesis. The growing peptide chains undergo intermolecular association, leading to an aggregated state in which the nearly quantitative reaction yields required for a successful solid-phase synthesis are precluded.[9b] The formation of interchain hydrogen bonds, through the secondary amide bonds of the peptide backbone (*i.e.*, β-sheet formation), has been accepted as the major contributing factor leading to aggregation. The Hmb protection system was designed to circumvent this problem by removing the potential for backbone hydrogen bond formation. Main-chain Hmb substitution of numerous difficult sequences has inhibited aggregation, leading to superior crude products compared with standard syntheses.[8] From previous experience we had concluded[7,8,12] that Hmb protection need only be introduced at a maximum of every sixth residue in order to inhibit completely chain aggregation, and this 'rule-of-thumb' has held true for all (~20) previously aggregating sequences that we have resynthesized using the main-chain amide-protection approach.

A standard synthesis of β-amyloid(1–43)[1] exhibits all the characteristics of a difficult sequence, with slow and incomplete piperidine-mediated Fmoc removal commencing upon deprotection of the eighth residue, valine[36]. In an attempt to overcome this problem, we originally[1] introduced the (Hmb)glycine[38] residue thereby hoping to prevent the onset of aggregation for at least the next six subsequent residues.[8,12] However, we observed a moderately broadened Fmoc-deprotection profile at methionine[35], only three residues from (Hmb)glycine[38]. To our knowledge, this is the first example in which Hmb incorporation has not provided complete inhibition of aggregation. We surmised that this unexpected aggregation could not be due to the formation of the simple intermolecular β-sheet structures composed of fully extended peptide chains that have usually been attributed to the source of the 'difficult sequence' phenomenon.

A detailed investigation of a carboxy-terminal segment of β-amyloid residues 34–42, together with various Hmb-protected analogues, was undertaken.[13] Using our results, we were able to determine the structure of β-amyloid(34–42) whilst bound to the Kieselguhr-polyamide resin.[13] The resin-bound structure was derived from a combination of Fmoc-deprotection profiles obtained during the initial chain assembly, and cleaved peptide solubilities, light microscopy[14,15] and X-ray analysis of insoluble pellets in solution. We demonstrated that β-amyloid(34–42) formed a β-hairpin structure [Fig. 1(*a*)] on the resin during synthesis. This consists of a reverse turn of valine[36]–glycine[37]–glycine[38]–valine[39], connecting two β-strands (leucine[34] to valine[36] and valine[39] to isoleucine[41]) so as to form an antiparallel, intramolecular β-ribbon. These stable β-hairpin structures could themselves associate to form all-β polymers of anti-parallel molecules, by lateral, intermolecular pairing of self-similar β-strands [Fig. 1(*b*)]. The resulting aggregate thus has a local, pseudo cross-β structure, of anti-parallel β-hairpins rather than of fully extended β-strands. The amide bond of glycine[38], being part of a type-I,-II or -III reverse turn, is directed at approximately 90° with respect to the lateral intra- and inter-molecular hydrogen bonding network. Assuming that residues 34–42 adopt this structure during the synthesis of β-amyloid(1–43), Hmb substitution of glycine[38]



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would not prevent β-hairpin aggregation,† and the consequent lack of protection was indeed observed in our original synthesis.[1] This was probably the main cause of the heterogeneity of the crude product [Fig. 2(a)] and of the low overall final yield.

The β-hairpin model (Fig. 1) for residues 34–42 does, however, predict that a simple shift in the first point of Hmb protection from glycine[38] to glycine[37] should inhibit lateral aggregation early on in the synthesis of β-amyloid(1–43), by disrupting the relatively weak intermolecular self-association of β-strand ii in Fig. 1(*b*). Having found this to be the case for β-amyloid(34–42), we report here a substantial improvement in the quality of the β-amyloid(1–43) crude product, and a four-fold increase in overall yield. Thus a dramatic improvement has been achieved by implementation of a simple modification that was elucidated from a structural understanding of the carboxy-terminal segment of the peptide.[13]

Results and discussion

Our previous synthesis of β-amyloid(1–43)[1] was greatly aided by the use of amide-backbone protection, allowing the isolation and purification of this difficult target molecule through highly soluble, partially protected intermediates. The main impurities contaminating the crude product [Fig. 2(*a*)] were isolated, and identified as Ile[31]- and Ile[32]-deletion peptides. Coupling of these β-branched residues was extremely difficult within the unexpected aggregated environment of the growing peptidyl-resin.[1] The structural insight gained from examination of the various carboxy-terminal segments described earlier, leading to the β-hairpin model,[13] was implemented in a new synthesis of β-amyloid(1–43). The synthesis was performed exactly as described earlier[1] except that the first backbone-protecting group was now incorporated on the nitrogen of Gly[37] rather than Gly[38]. The revised synthesis proceeded smoothly, with no sign of aggregation (standard Fmoc deprotection profiles[6] were observed for every residue), to give the full 43-residue peptide-resin. Upon cleavage, the crude backbone-protected peptide, β-amyloid[1–43, (AcHmb)Phe[20], (AcHmb)Gly[25,29,33,37]] **3** was of a substantially improved quality compared with that obtained in the earlier synthesis [Figs. 2(*a*) and 2(*b*)].

The major component **4** was isolated by C8 RP-preparative HPLC to give penta(acetyl-Hmb)βA4(1–43) (39.2 mg, 16.9%). The product gave a main peak upon HPLC analysis with t_R = 19.90 min (>97%), good amino acid ratios and was confirmed by electrospray mass spectrometry (ESMS) giving the expected molecular ion with m/z = 5506.0. Lability towards TFA was restored to the backbone protection system by de-O-acetylation of the AcHmb groups by using 5% hydrazine hydrate in dimethylformamide (DMF) to give a new main peak (>96%) by analytical HPLC with t_R = 18.46 min. It was noted that the solubility of the product was much less than before the de-O-acetylation reaction, and the HPLC sample required addition of 1,1,1,3,3,3-hexafluoropropan-2-ol for complete dissolution. An aliquot of the de-O-acetylated material was analysed by ESMS and gave two main components, at m/z = 5296.5 (100%) and 5312.3 (20%) [M + 16]. The species with the extra 16 mass units could potentially be the correct peptide but containing oxidised methionine[35]. However, analytical HPLC of a sample treated with ammonium iodide (20 mol equiv.), dimethyl sulfide (20 mol equiv.) and TFA at 0 °C for 1 h, a procedure which should reduce any Met[35](O),[16] gave no change. It appears that any oxidation must have arisen during the electrospray process and the species with m/z 5312.3 (see above) was not a constituent of the actual peptidic material. Recently, Morand *et al.*[17] have described the oxidation of methionyl-, tryptophanyl- and tyrosyl-containing peptides during electrospray ionisation. Also, no mass combinations corresponding to acetyl (+42) were observed above m/z 5296.5, confirming that complete de-O-acetylation had been achieved.

The earlier eluting minor fraction **5** in crude peptide **3** contained a species corresponding to the required material but missing a single AcHmb group, and had m/z = 5327.8 (M − 178), and a second species with m/z 5522.3 (M + 16). These two components co-eluted by analytical HPLC, even on shallow gradients. The extra 16 mass units in component **5** could be due to the oxidation of the sole methionine of β-amyloid-(1–43) during the electrospray process (as above) or to the actual presence of oxidised Met[35] in the peptide. We resolved this by treating compound **5** with ammonium iodide (20 mol equiv.), dimethyl sulfide (20 mol equiv.) and TFA at 0 °C for 1 h. An aliquot of reduced fraction **5** was isolated by gel filtration in quantitative yield and re-analysed by analytical HPLC, and gave an unchanged main peak (85%), and a new, slower eluting component (15%). Reduced fraction **5** was also analysed by ESMS, which showed two main components, with m/z = 5328.5 (M − 178) and 5506.8. The mass peak at m/z = 5522.3 was now completely absent, with the reduction procedure providing a new species at m/z = 5506.8, the correct mass for penta(acetyl-Hmb)βA4(1–43). These data unequivocally show the second component fraction **5**, present at around 15%, to be the target material but with oxidised methionine[35].

The major component in fraction **5** was a species missing a single AcHmb group. Since we have shown[18] the AcHmb group to be completely stable to treatment with TFA for at least 16 h, it is unlikely that this species has arisen due to cleavage of one of the five backbone-protecting groups. Two further possibilities, however, readily present themselves. First, the Hmb 2-hydroxy functions may not have been fully acetylated with the overnight acetic anhydride–diisopropylethylamine (DIPEA) treatment. This potential problem was not observed in our previous synthesis of β-amyloid(1–43)[1] prepared by an almost identical scheme, nor was it encountered during the initial development of the acetylation reaction. In addition, fraction **5** was sequenced from the amino terminus, and the target sequence was observed up to valine[18]. After this residue no signal data were obtained, strongly suggesting that the amide of Phe[20] was completely protected, and that full acetylation had been achieved. From considerations of steric hindrance, as (Hmb)phenylalanine[20] had been fully acetylated, one could assume that all available (Hmb)glycines were also fully acetylated. Secondly, if the initial treatment of the peptidyl-resin with di-*tert*-butyl dicarbonate was performed in DMF containing trace amines, partial substitution of the Hmb 2-hydroxy function with the *tert*-butoxycarbonyl (Boc) group may have occurred (the hydroxy site only substitutes under base-catalysed conditions), leaving these sites unavailable for acetylation. The *N*-(2-*tert*-butoxycarbonyl-4-methoxy) group is TFA-labile.[19] It seems likely that the latter problem has

† Similarly, a substitution of proline[38] for glycine[38] in the synthesis of β-amyloid(34–42, proline[38]) also shows the onset of aggregation at methionine[35].

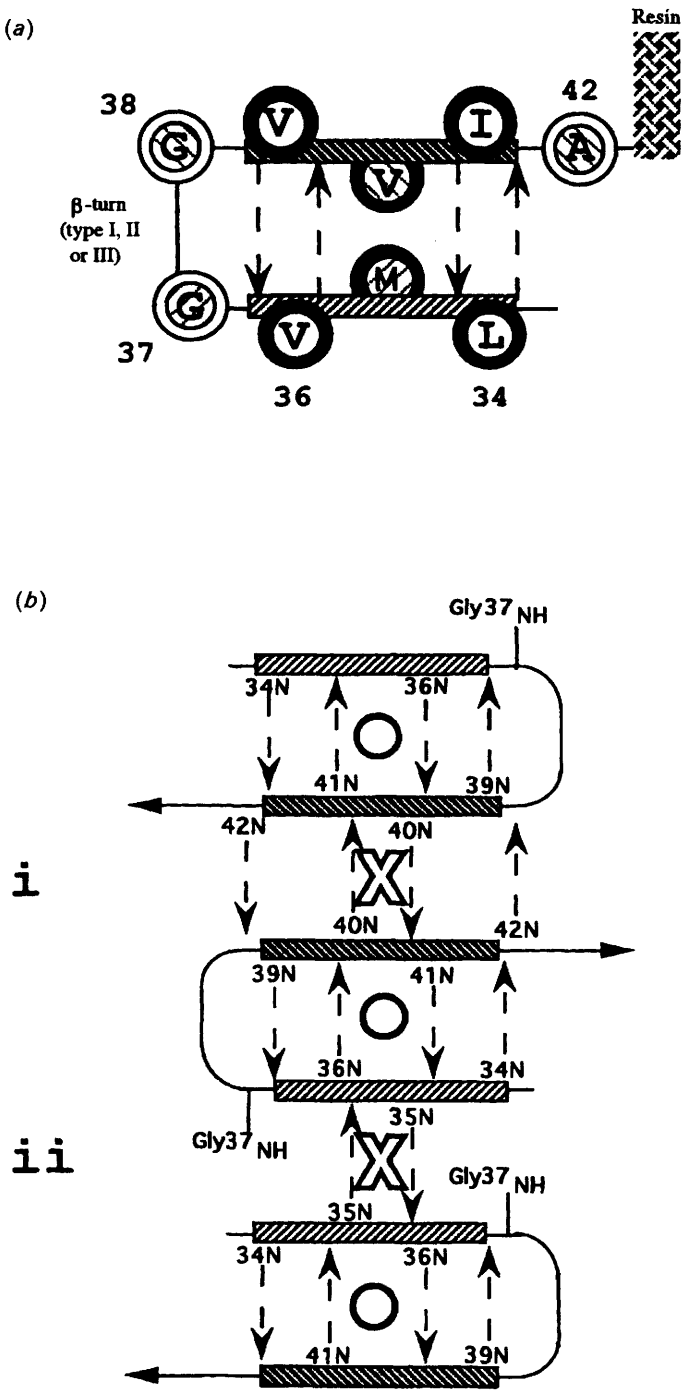

Fig. 1 (*a*) β-Hairpin model for residues (one-letter code) 34–42 of β-amyloid(1–43). Letters denoting side-chains are encircled in black or white according to their high or low propensity, respectively, to stabilise a β-strand within an anti-parallel β-sheet (see ref. 13). The main chain is represented as a thin line, with the two predicted β-strands (Leu^{34} to Val^{36} and Val^{39} to Ile^{41}) as thicker, strippled bars. Intermain-chain hydrogen bonds are dashed arrows, pointing from the amide nitrogen to the carboxyl oxygen. The plane of the resulting β-ribbon lies in the page, and depth is indicated by degrees of shading. (*b*) Laterally associated anti-parallel β-hairpin molecules of β-amyloid(34–42). Owing to the zig-zag of each β-strand, quartets of side-chains form pores of two different types between neighbouring β-strands. Each pore-type is associated with a particular local network of main-chain hydrogen bonds between neighbouring anti-parallel strands, labelled with a cross, ×, or circle, ○, respectively (adapting the notation of Finkelstein and Nakamura [22]). The amide nitrogen atoms involved in inter- and intra-molecular hydrogen bonding are highlighted. For example, 39N indicates the amide nitrogen of valine^{39}, whose hydrogen atom is hydrogen bonded to the carbonyl oxygen of valine^{36}.

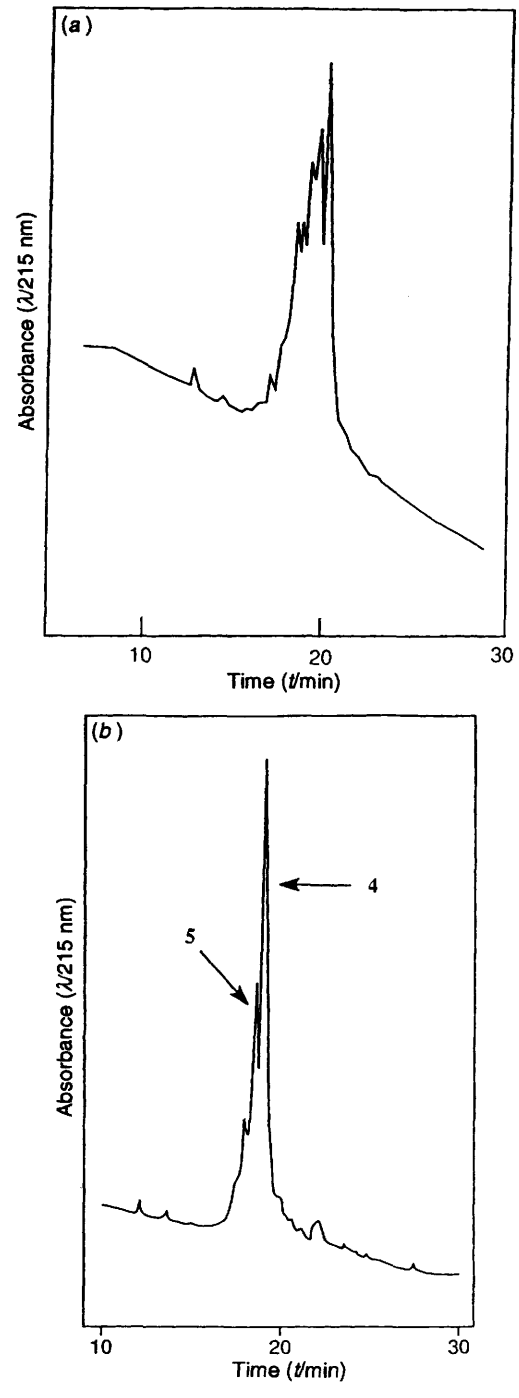

Fig. 2 (*a*) C8 Analytical HPLC of crude β-amyloid [1–43, (AcHmb)Phe^{20}, (AcHmb)Gly^{25,29,33,38}]. (*b*) C8 Analytical HPLC of crude β-amyloid [1–43, (AcHmb)Phe^{20}, (AcHmb)Gly^{25,29,33,37}].

occurred, causing sporadic 2-hydroxy-group *tert*-butoxycarboxylation of the (Hmb)Gly groups but not of (Hmb)Phe^{20}. It may be wise to couple the final amino acid through the N^{α}-Boc-protected derivative where available in future, to eliminate any possibility of the aforementioned problem. However, fraction **5**, missing a single AcHmb group will, after reductive treatment [converting the 15% Met^{35}(O) present into Met^{35}], eventually generate the desired purified β-amyloid(1–43). Thus, the main impurities (~25%) in the new crude product are no longer deletion peptides (as were obtained with protection of glycine^{38}),[1] but species which can be chemically manipulated to give the final, fully deprotected target peptide material. Thus, the combined yield of purified (AcHmb)βA4(1–43) that can be

subsequently backbone deprotected to yield βA4(1–43) was 28.2%, a four-fold increase when compared with the analogous synthesis utilising protection of Gly^{38} (rather than protection of Gly^{37} as here) which gave an isolated yield of 8.0% at this stage.[1]

The final deprotection step, removal of the backbone Hmb groups, was mediated by a TFA–scavenger cocktail for 2 h. The product, βA4(1–43), was isolated by ethereal precipitation with ~ 100% recovery (based on peptide weight). ESMS gave a main species (theory requires m/z 4616.2) with m/z = 4615.5 (100%), and no sign of M + 136 combinations, indicating complete removal of Hmb had been achieved. Again a mass peak at 4631.8 (M + 16) (20%) was observed. Since the peptide from which this product derives was shown earlier to be free of $Met(O)^{35}$, and chemical oxidation would not normally be expected to occur upon treatment of a methionine-containing peptide with TFA, this probably arose during the electrospray process. However, it was not possible to confirm this chromatographically, due to the notorious HPLC difficulties associated with βA 42/43-mers.[20] These chromatographic problems serve to highlight the purification difficulties encountered during the attempted preparation of βA 42/43-mers without the use of backbone protection.

The example described here demonstrates that careful attention must be paid to potential compact structures that may be adopted by the protected peptide during synthesis. A combination of ease of backbone protection incorporation[5] and careful structural analysis of target sequences will indicate the most favourable positions for Hmb substitution. In general, backbone protection provides a means of preventing chain association in systems that involve intermolecularly hydrogen-bonded β-sheet structures.[7,8] Where potential β-turn motifs are identified, with flanking regions prone to formation of β-sheet and consequent intermolecular aggregation, backbone amide protection at the $i + 2$ position[21] within the turn will probably not inhibit aggregation completely. Protection at the i, $i + 1$ or $i + 3$ positions will be effective, either by preventing formation of the turn (chains will then adopt extended structures), or by preventing lateral intermolecular aggregation of the β-hairpin monomeric units.

Conclusions

Compared with an earlier preparation of β-amyloid(1–43), modifying the first position of Hmb protection from $glycine^{38}$ to $glycine^{37}$ leads to a much improved quality of the crude product and a four-fold increase in final yield to 28.2%. This modification was defined from prior structural analysis of the carboxy-terminal portion of the sequence, and the prediction that protection of $glycine^{37}$ would lead to complete inhibition of aggregation was found to be correct. These findings indicate that Hmb backbone amide protection cannot only be introduced as an inhibitor of intermolecular aggregation, but is also useful as a structural probe for defining the relative positions of residues within a β-turn that forms during solid-phase peptide synthesis. This type of information may provide a more rational knowledge-based approach to protecting-group strategies in solid-phase peptide synthesis.

Experimental

Equipment, materials and methods

Continuous-flow Fmoc-polyamide methods reviewed by Atherton and Sheppard[6] were used exclusively. Fmoc amino acid pentafluorophenyl activated esters (Novabiochem, UK) were used exclusively except for Ser(OBu^t) and Arg(Mtr) which were coupled as the dihydrooxobenzotriazine esters (Novabiochem, UK). Fmoc *N*-carboxyanhydrides (Propeptide, Vert Le Petit, France), used for coupling Lys(Boc), Val, Ile and Phe residues to Hmb-protected amino acids and Fmoc-L-Thr(OBu^t)-Pepsyn KA resin (Milligen) were commercially available. *N,O*-BisFmoc-*N*-(2-hydroxy-4-methoxybenzyl) amino acid pentafluorophenyl esters[5] were prepared as previously detailed. All solvents were purified as previously described.[6] NAP-10 analytical gel filtration columns were obtained from Pharmacia Biotech, Uppsala, Sweden.

Solid-phase peptide synthesis was performed on an LKB 'Biolynx' 4170 automated synthesizer programmed to perform acylation reactions (in DMF) for 45 min and Fmoc deprotection reactions (in 20% piperidine in DMF v/v) for 10 min. All chiral amino acids used were of the L-configuration. Amino acid side-chain protection was as follows: lysine (N^{ε}-*tert*-butoxycarbonyl, Boc), serine and threonine (*tert*-butyl ether, Bu^t), aspartic and glutamic acid (*tert*-butyl ester, OBu^t), glutamine (Trt), histidine (N^{im}-trityl, Trt), tyrosine (*tert*-butyl ether, Bu^t), arginine (N^{G}-4-methoxy-2,3,6-trimethylbenzenesulfonyl, Mtr). Peptide hydrolyses were performed at 110 °C for 24 h in 6 mol dm^{-3} HCl containing a trace of phenol, in evacuated, sealed tubes. Analysis of hydrolysis products was performed on a Beckman 7300 analyser. Separation was obtained using ion-exchange resin with manufacturer's buffer solutions and post-column separation detection was by ninhydrin. Amino acid sequencing was performed on an Applied Biosystems 470A protein sequencer, using PTH chemistry. Analytical HPLC was performed on a Brownlee Aquapore RP300 C8 or C4 (butyl) column (250 × 4.6 mm). A 10–95% B in A gradient over a period of 25 min (1.5 cm^3 min^{-1}) was used unless otherwise stated, where A = 0.1% aq. TFA and B = 90% acetonitrile–10% A. Preparative HPLC was performed on a Vydac 208TP1022 C8 column (25 × 2.5 cm) at 10 cm^3 min^{-1} and 215 nm UV detection. ESMS was performed on a VG BioQ spectrometer.

(1) Preparation of βA4[1–43, (Hmb)Phe^{20}, $Gly^{25,29,33,37}$]-Pepsyn KA

Fmoc-L-Thr(OBu^t)-Pepsyn KA (0.097 mmol equiv. g^{-1}; 500 mg) was swollen in DMF for 10 min, any fine particulates removed, and the resin loaded onto the Biolynx synthesizer. The sequence was 'stepwise elaborated' to the 43-residue sequence, giving the following Fmoc deprotection data (measured at 304 nm) (peak height, peak area): Thr^{43} (0.77, 35.5), Ala^{42} (0.67, 38.7), Ile^{41} (0.61, 37.4), Val^{40} (0.60, 35.9), Val^{39} (0.53, 37.1), Gly^{38} (0.62, 36.1), Gly^{37} (2.09, 85.3), Val^{36} (0.60, 38.9), Met^{35} (0.63, 39.6), Leu^{34} (0.44, 36.2), Gly^{33} (1.85, 84.7), Ile^{32} (0.59, 37.9), Ile^{31} (0.61, 36.9), Ala^{30} (0.53, 38.3), Gly^{29} (1.82, 83.0), Lys^{28} (0.83, 54.9), Asn^{27} (0.61, 38.6), Ser^{26} (0.60, 38.0), Gly^{25} (1.91, 84.2), Val^{24} (0.81, 49.1), Asp^{23} (0.57, 36.9), Glu^{22} (0.59 , 38.0), Ala^{21} (0.61, 37.6), Phe^{20} (2.01, 84.8), Phe^{19} (0.70, 42.1) and Val^{18} (0.59, 36.6); the remaining residues had deprotection values very similar to those of Val^{18}. All residues were coupled (0.5 mmol vials) under standard conditions except Hmb residues which were coupled for 3 h.

A general procedure was used to couple to a terminal Hmb residue. The de-Fmoc resin was removed from the synthesizer, washed successively with DMF and diethyl ether, and briefly dried *in vacuo*. The resin was then suspended in solution of the appropriate Fmoc-amino acid-*N*-carboxyanhydride (10 mmol equiv., 0.5 mmol) in dichloromethane (~ 5 cm^3) for 3 h [residues onto (Hmb)Gly] or 20 h [Phe onto (Hmb)Phe]. The acylated resin was then washed as above, re-suspended in DMF, loaded onto the synthesizer, and synthesis was continued.

The final 43-residue assembly was washed successively with DMF and diethyl ether and dried *in vacuo* over potassium hydroxide pellets. The final peptide-resin weighed 810 mg. Amino acid analysis of the crude resin-bound peptide gave the

following relative proportions: Asp/Asn 3.63 (4), Thr 1.03 (1), Ser 1.44 (2), Glu/Gln 3.57 (4), Gly 6.06 (6), Ala 3.94 (4), Val 5.62 (6), Met 0.99 (1), Ile 3.02 (3), Leu 2.08 (2), Nle 1.24 (1), Tyr 0.84 (1), Phe 2.72 (3), His 2.56 (3), Lys 1.85 (2), Arg 1.05 (1).

(2) Preparation of Boc-βA4[1–43, (AcHmb)Phe^{20}, Gly^{25,29,33,37}]-Pepsyn KA

Resin-bound peptide from preparation (1) was treated with di-*tert* butyl dicarbonate (10 mol equiv., 108 mg) in DMF (6 cm^3) for 2 h, the reaction being monitored by the Kaiser test, then was washed successively with DMF and diethyl ether. The N-terminal Boc resin was then treated with acetic anhydride (20 mol equiv., 102 mg) in DMF (4 cm^3) with the addition of DIPEA (10 mol equiv., 64 mg) in DMF (2 cm^3) and left 2 h. The resin was then washed and dried in the usual manner.

(3) Preparation of crude penta(acetyl-Hmb)βA4(1–43) 3

(i) Peptide-resin cleavage. Peptide-resin assembly from peptide **2** (675 mg) was treated with 92% TFA–3% phenol–3% ethane-1,2-dithiol–2% triethylsilane (v/w/v/v) (20 cm^3) for 5 h. Cleaved resin was removed by filtration, washed with neat TFA (5 × 4 cm^3), and the combined filtrates were sparged with N_2 to ~3 cm^3 in volume. Ice-cooled diethyl ether (40 cm^3) was added to cause precipitation, and the mixture was cooled in acetone–solid CO_2 for 5 min and centrifuged at 3000 rpm for 5 min. The etheral solution was decanted, and further diethyl ether extractions (5 × 40 cm^3) were performed. The residue was dried *in vacuo* to give a solid (209 mg, 90% cleavage by post-cleaved resin amino-acid ratios).

(ii) Analysis of crude penta(acetyl-Hmb)βA4(1–43) 3. Crude peptide **3** (209 mg) was fully dissolved in 0.1% aq. TFA–acetonitrile (1:1) (1 cm^3), and the solution was diluted to 7.5 cm^3 with the same solvent mixture. Analytical HPLC (C8 column) gave a main peak with t_R = 19.91 min (62%), a quicker eluting component with t_R = 19.19 min (22%), along with various minor components [Fig. 2(*b*)]. Analytical HPLC (C8 column), gradient 42–62% B in A over a period of 25 min gave 2 main peaks, with t_R = 14.32 and 18.37 min.

(4) Purification of penta(acetyl-Hmb)βA4(1–43) 3

Crude peptide **3** (209 mg in 7.5 cm^3) was purified by preparative HPLC (Vydac C8 column) (10 × 750 mm^3) injections using a gradient of 42–62% B in A over a period of 25 min. Two main fractions were collected, corresponding to analytical retention times 14.3 (F1, compound **5**) and 18.4 min (F2, compound **4**). Each fraction was lyophilised to afford solid, and analytical HPLC (C8 column) with gradient 42–62% B in A over a period of 25 min showed each fraction to be homogeneous. Amino acid analysis of fractions F1 and F2 gave the following relative proportions: Fraction 1 (compound **5**): Asp/Asn 3.86 (4), Thr 0.98 (1), Ser 1.63 (2), Glu/Gln 4.01 (4), Gly 6.18 (6), Ala 3.96 (4), Val 5.34 (6), Met 0.83 (1), Ile 2.34 (3), Leu 1.92 (2), Tyr 0.90 (1), Phe 2.68 (3), His 2.60 (3), Lys 1.79 (2) and Arg 0.85 (1) (yield 4.76 μmol, 26.2 mg, 11.3% overall).

Fraction 2 (compound **4**): Asp/Asn 3.76 (4), Thr 0.99 (1), Ser 1.55 (2), Glu/Gln 3.98 (4), Gly 6.25 (6), Ala 4.01 (4), Val 5.66 (6), Met 1.00 (1), Ile 2.82 (3), Leu 2.19 (2), Tyr 1.01 (1), Phe 3.07 (3), His 2.95 (3), Lys 2.06 (2), Arg 0.87 (1) (Yield 7.12 μmol, 39.2 mg, 16.9% overall).

Each fraction was analysed by ESMS which showed: penta-(acetyl-Hmb) βA4(1–43)-OH requires m/z = 5505.95; Fraction 1: major species at m/z = 5327.8 (100%) [M − 178], 5522.3 (65%) [M + 16]. Fraction 2: major species at m/z = 5506.0 (100%), minor components at m/z = 5527.3 (20%) [M + Na^+], 5544.3 (15%) [M + K^+].

(5) Reduction of fraction 1 (compound 5)

Fraction 1 (1.0 mg, 0.2 μmol) was dissolved in TFA (100 mm^3) (*i.e.* ~ 2 mmol dm^{-3} solution). Ammonium iodide (5 mg in 500 mm^3) (20 mol equiv., 50 mm^3) was added, followed by dimethyl sulfide (15 mg in 5000 mm^3 TFA) (20 mol equiv., 80 mm^3), and the mixture was left at 0 °C for 1 h. The mixture was then sparged with nitrogen, and the residue was dissolved in 0.1% aq. TFA–acetonitrile (4:1; 1000 mm^3). A NAP-10 gel filtration column was eluted with 0.1% aq. TFA (5 × 2 cm^3), the sample solution (1000 mm^3) was added, and the first 1000 mm^3 were discarded. The following 1500 mm^3 were collected and freeze-dried to give a solid (0.99 mg). The sample was dissolved in 0.1% aq. TFA–acetonitrile (1:1; 200 mm^3), and an aliquot was analysed by analytical HPLC (C8 column) to give a main peak with t_R = 19.10 min (85%) and a slower eluting component with t_R = 19.71 min (15%). Reduced fraction 1 was analysed by ESMS which showed m/z = 5328.5 (100%) [M − 178] and 5506.8 (60%).

(6) De-*O*-acetylation of purified peptide fraction 2 (compound 4)

Preparation of penta(Hmb)βA4(1–43). Purified F2 (39.2 mg) was suspended in 5% hydrazine hydrate–DMF (1500 mm^3) and the mixture was sonicated for 45 min (dissolution occurred upon sonication). The sample was then extracted with cold diethyl ether (6 × 15 cm^3) (centrifuge between extracts), and dried *in vacuo*. The treatment was then repeated to give a chalky solid (37.5 mg, 100%).

Amino acid analysis of the de-*O*-acetylated peptide gave the following relative proportions: Asp/Asn 3.92 (4), Thr 0.97 (1), Ser 1.64 (2), Glu/Gln 3.96 (4), Gly 6.01 (6), Ala 3.95 (4), Val 5.74 (6), Met 0.98 (1), Ile 2.66 (3), Leu 2.14 (2), Tyr 0.96 (1), Phe 2.88 (3), His 2.81 (3), Lys 2.02 (2) and Arg 0.91 (1).

A sample was dissolved in 1,1,1,3,3,3-hexafluoropropan-2-ol and analysed by analytical HPLC (C8 column); this showed a single peak >96%, with t_R = 18.46 min. Analysis by ESMS gave (theory requires m/z 5295.8) a main species at m/z = 5296.5 (100%), and a peak at m/z = 5312.3 (20%) [M + 16].

(7) Final cleavage of Hmb-backbone protection

Preparation of βA4(1–43). De-*O*-acetylated peptide from preparation (6) (37.5 mg) was treated with 92% TFA–3% phenol–3% ethane-1,2-dithiol–2% triethylsilane (v/w/v/v) (3 cm^3) for 2 h. The product was isolated by the usual N_2 sparge and ethereal extractions, to give a solid (32.4 mg, 16.7% overall).

A sample was analysed by ESMS, which showed (theory requires m/z 4616.2) a main species at m/z = 4616.5 (100%) and minor species at m/z 4631.8 (20%) [M + 16], 4654.8 (30%) [M + K]$^+$ and 4671.3 (35%) [M + 55].

The peptide was further verified for 42 cycles (up to Ala^{42}) by sequence analysis.

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