Preparation and Purification of β-Amyloid (1–43) via Soluble, Amide Backbone Protected Intermediates

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We report a new synthetic strategy for the preparation of the amyloidogenic protein fragment βA4, through an easily purified (and characterized) intermediate of high aqueous solubility. This novel intermediate consists of βA4(1–43) with backbone amide protection, provided by the *N*-(2-hydroxy-4-methoxybenzyl) group, on residues Phe[20] and Gly[25,29,33,38]. The new strategy enabled derivatized βA4(1–43) to be isolated and purified by standard reverse-phase HPLC procedures and characterized by electrospray mass spectrometry (ESMS).

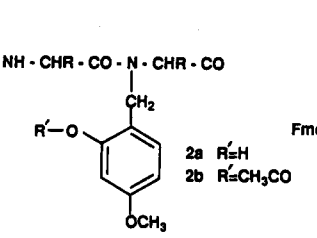
Introduction

βA4[1,2] is a sparingly soluble proteolytic fragment[3] of 42 or 43 amino acid residues present as the main constituent of extracellular proteinaceous deposits known as amyloid plaques. These, along with neurofibrillary lesions represent the major neuropathological characteristics of Alzheimer's disease.[4] As part of ongoing structural studies, βA4(1–43) of high purity was required.

H_2N-D[1]AEFRHDSGYEVHHQKLVFF[20]-
AEDVGSNKGAIIGLMVGGVVIAT[43]-COOH
(1)

The chemical synthesis of this important molecule has been investigated by a number of groups[5–7] with varying degrees of success. Two major factors make this sequence a difficult target for stepwise solid-phase peptide synthesis. Firstly, the hydrophobic composition of the C-terminal region of βA4 results in an association between resin-bound peptide chains[8] (aggregation), a so-called "difficult sequence".[9,10] Aggregation leads to gross steric hindrance and a significant decrease in the rates of deprotection and coupling.[11] Secondly, failure to achieve near-quantitative reactions leads to an accumulation of amino acid deletion sequences which may be extremely difficult to separate from the target peptide. Factors inducing main chain association on a solid support also exist in solution. The result is that βA4 is extremely insoluble, leading to major difficulties in the purification and analysis of synthetic products.

The association of peptide chains is mediated through ionic and hydrophobic interactions along with interchain hydrogen bonding (primarily via the secondary amide bonds along the peptide backbone). The latter effect constitutes the major interaction and can be prevented by replacement of secondary by tertiary amide bonds along the peptide chain.[12] This disaggregation phenomenon arises from steric hindrance effects and leads to a rotation of the tertiary amide bond plane. The result is a separation and disturbance of the β-sheet structure of the aggregate.[13] Previous studies have shown that a number of model hydrophobic peptides, synthesized in solution, with appropriate backbone amide bond substitution, exhibit a dramatic increase in solubility.[14–16] We have recently devised a secondary amide bond protecting group,[17] the *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) system (**2a**), suitable for use in solid-phase peptide synthesis, which prevented interchain association during the synthesis of a number of "difficult sequences", prepared on Kieselguhr supported polydimethylacrylamide (Pepsyn KA) resin at an initial loading of 0.1 mmol g^{-1}. The resulting crude products were shown, by analytical HPLC, to contain fewer



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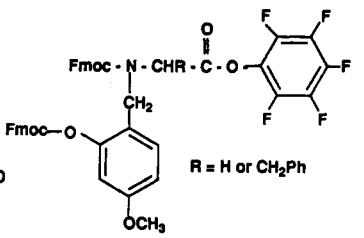


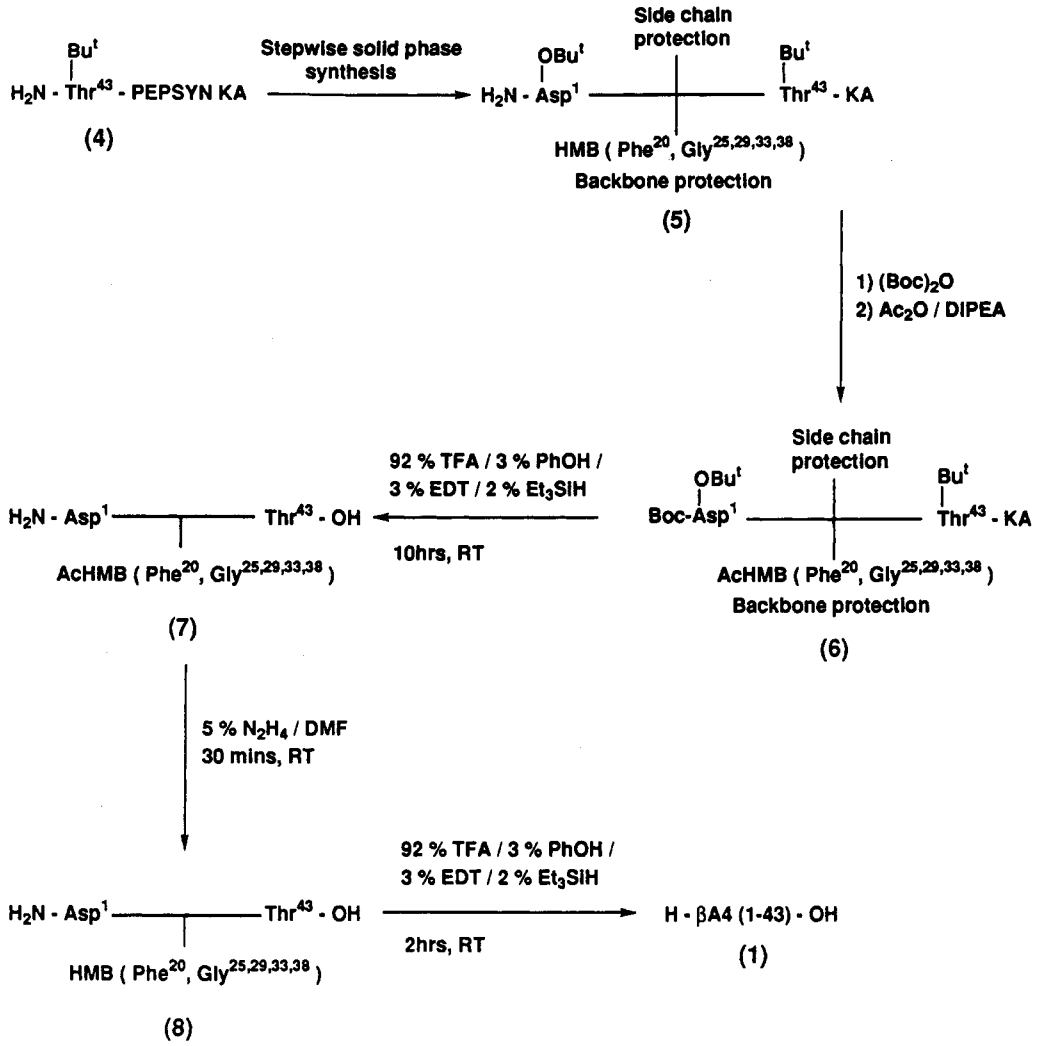
(3)

• Abstract published in *Advance ACS Abstracts,* March 1, 1994.

(1) Glenner, G. G.; Wong, C. W. *Biochem. Biophys. Res. Commun.* **1984,** *120,* 885.
(2) Masters, C. L.; Simms, G.; Weinman, N.; Multhaup, G.: McDonald, B. L.; Beyreuter, K. *Proc. Natl. Acad. Sci. U.S.A.* **1985,** *83,* 4245–4249.
(3) Selkoe, D. J.; Abraham, C. R.; Podlisny, M. B.; Duffy, L. K. *J. Neurochem.* **1986,** *46,* 1820–1834.
(4) Glenner, G. G. *Cell* **1988,** *57,* 307.
(5) Barrow, C. J.; Zagorski, M. G. *Science* **1991,** *253,* 179–182.
(6) Burdick, D.; Soreghan, B.; Kwon, M.; Kosmoski, J.; Knauer, M.; Henschen, A.; Yates, J.; Cotman, C.; Glabe, C. *J. Biol. Chem.* **1992,** *267* (1), 546–554.
(7) Hendrix, J. C.; Halverson, K. J.; Lansbury, P. T., Jr. *J. Am. Chem. Soc.* **1992,** *114,* 7930–7931.
(8) For a recent discussion on interchain association see Kent, S. B. H.; Alewood, D.; Alewood, P.; Baca, M.; Jones, A.; Schnolizer, M. In *Innovations and Perspectives in Solid Phase Synthesis, 2nd International Symposium,* Epton, R., Ed.; Intercept Ltd Publ.: U.K., 1992; pp 1–22.
(9) Kent, S. B. H. In *Peptides, Structure and Function,* Proceedings of the 9th American Peptide Symposium; Deber, C. M., Hruby, V. J., Kopple, K. D, Eds.; Pierce Chemical Co.: Rockford, IL, 1985; pp 407–414.
(10) Atherton, E.; Sheppard, R. C. In *Peptides, Structure and Function,* Proceedings of the 9th American Peptide Symposium; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chemical Co.: Rockford, IL, 1985; pp 415–418.
(11) Nguyen, O.; Sheppard, R. C. In *Peptides 1988,* Proceedings of the 20th European Peptide Symposium, Jung, G., Beyer, E., Eds.; Walter de Gruyter: Berlin, 1988; pp 151–153.
(12) Milton, R. C. de L.; Milton, S. C. F.; Adams, P. A. *J. Am. Chem. Soc.* **1990,** *112,* 6039.
(13) Narita, M.; Ishikawa, K.; Junn-Yann, C.; Kim, Y. *Int. J. Pept. Protein Res.* **1984,** *24,* 580–587.
(14) Narita, M.; Ishikawa, K.; Nakano, H.; Isokawa, S. *Int. J. Pept. Protein Res.* **1984,** *24,* 14–24.
(15) Kemp, D. S.; Grattan, J. A.; Reczek, J. *J. Org. Chem.* **1975,** *40,* 3464.
(16) Weygand, F.; Steglich, W.; Bjarnason, J.; Akhtar, R.; Khan, N. *Tetrahedron Lett.* **1966,** *29,* 3483.
(17) Johnson, T.; Quibell, M.; Owen, D.; Sheppard, R. C. *J. Chem. Soc. Chem. Commun.* **1993,** *4,* 369–372.

0022-3263/94/1959-1745$04.50/0

Scheme 1



side-products compared to standard synthetic material.[18] The amide bond protected residue is readily introduced through stable crystalline *N,O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl) amino acid pentafluorophenyl esters (3).[19] We therefore aimed to prepare βA4(1–43) via a soluble backbone-protected form. Here we describe the successful preparation, purification, and analysis of a protected intermediate, leading to purified βA4(1–43).

Results and Discussion

The strategy adopted for the synthesis of βA4(1–43) is illustrated in Scheme 1. The stepwise synthesis was performed on a LKB Biolynx automatic synthesizer using the Fmoc/*tert*-butyl procedure.[20] Prepacked vials (0.5 mmol, Novabiochem, UK) of Fmoc-amino acid pentafluorophenyl esters (appropriate side-chain protection where necessary) with 1-hydroxybenzotriazole catalyst or dihydrooxobenzotriazine esters were used. Reactions were routinely performed under standard acylation[20] (45 min, reaction not monitored for residual free amino group) and deprotection conditions (10 min, 20% piperidine in DMF), see Experimental Section.

A standard synthesis of βA4(1–43) by Fmoc/*tert*-butyl chemistry on Pepsyn KA (Kieselguhr supported polydimethylacrylamide resin) exhibited aggregation upon deprotection of the eighth residue, Val[36]. In continuous-flow Fmoc synthesis, aggregation is readily detected by spectrometric monitoring, in real time, of the release of Fmoc deprotection products into the reagent flow stream.[20] Addition of the following methionine residue remained incomplete even upon repeated coupling with extended acylation reaction times. The peptide was therefore resynthesized using Hmb backbone protection at Gly[38,33,29,25] and Phe[20]. These positions were chosen by the following criteria:

(1) Substitution must be implemented before aggregation occurs. (2) Experience and experimental evidence[18,21] has shown that backbone substitution is effective at preventing aggregation for at least the six following residues. (3) We have observed that coupling to N-terminal Hmb-substituted peptidyl-resin is often slower than standard couplings.[17,18] Thus where possible, the most stereochemically unhindered site for substitution is preferred. (4) Onset of aggregation has not been observed in

(18) Hyde, C.; Johnson, T.; Owen, D.; Quibell, M.; Sheppard, R. C. *Int. J. Pept. Protein Res., in press.*

(19) Full chemical synthesis and spectroscopic details of new protected monomers (3), studies into their coupling and coupling of the following residue, are submitted for publication. These details are also available as supplementary material from this journal.

(20) For an overall review of methods, see Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis: A Practical Approach*; Oxford University Press; Oxford, 1989; pp 1–203.

(21) Bedford, J.; Hyde, C.; Johnson, T.; Jun, W.; Owen, D.; Quibell, M.; Sheppard, R. C. *Int. J. Pept. Protein Res.* **1992**, *40*, 300.

Table 1

step	time
Gly onto (HMB)Gly	15 min
Lys(Boc) onto (HMB)Gly	2 h
Val/Ile onto (HMB)Gly	3 h
Phe onto (HMB)Phe	16 h

peptides longer than 21 residues,[22] i.e. no further substitutions were deemed necessary after Phe[20].

Resynthesis was performed as described using standard protocols for all non-Hmb residues. All Hmb-substituted amino acids were coupled via their pentafluorophenyl esters **3**, to the growing peptide chain for 2 h. As expected, subsequent Fmoc deprotection profiles gave a total absorbance twice that from the previous residue. Coupling to an N-terminal Hmb group was then performed at maximal concentration in a flask, using a 10-fold excess of Fmoc-*N*-carboxy anhydride in dichloromethane. These have been shown[17,19] to be the optimal coupling conditions for this particular type of reaction and typical reaction times are given in Table 1.

On completion of the acylation reaction, the washed resin was returned to the automated peptide synthesiser. Fmoc deprotection areas at this point were typically 1.1–1.3 times that expected. This arose due to acylation of reexposed Hmb hydroxyl sites that have previously undergone O → N acyl transfer.[18] O-Acylated sites are cleaved by piperidine, along with the Fmoc group. This general procedure was used at all Hmb-substituted sites except when coupling Gly[37] onto (Hmb)Gly[38]. Here, a double 2-h coupling with FmocGlyOPfp/HOBT was used; this allowed for unattended machine operation overnight. It should be noted that only resin-bound (Hmb)glycine undergoes complete acylation with incoming, Pfp-activated amino acid residues.[17–19] After addition of the final Hmb residue, stepwise elaboration gave the final assembly **5**. Previous studies[17,18] have shown the Hmb group to be labile to acidolysis conditions typically used during the final step of side-chain deprotection and peptide cleavage from the support. In order to solubilize βA4(1–43) backbone amide protection must be retained. Recent work has shown[23] that a simple base-catalyzed acetylation of the 2-hydroxyl function renders the Hmb group stable to TFA based acidolysis for over 16 h. Acetylation in the absence of base gave no observable 2-hydroxyl substitution.

Thus, the completed assembly **5** was initially N^{α}-protected with the Boc group by reaction with di-*tert*-butyl dicarbonate (in DMF) in the absence of base (reaction for 2 h at which time the Kaiser ninhydrin test[24] gave a negative response). Reaction with acetic anhydride/diisopropylethylamine in DMF overnight gave quantitative acetylation of the Hmb groups.[23] Cleavage of the protected peptide-resin **6** mediated by TFA yielded the penta(acetyl-Hmb) backbone-protected product **7**. The crude product **7** (137mg) exhibited remarkable solubility with full dissolution in 1 mL of 0.1% aqueous TFA/acetonitrile (1:1 v/v). Analytical HPLC of crude **7** was of a rather poor quality (Figure 1).

During assembly, a broadened Fmoc-deprotection profile was observed at Met[35], only three residues from (Hmb)-Gly[38]. The profile shape was not as broad as usually encountered in a fully associated state,[20] but did indicate the presence of some peptide secondary structure (Figure 2). This was unexpected as backbone substitution usually inhibits chain association for a minimum of six residues after its insertion.[17,21] This is the first time in over 15 syntheses of difficult sequences in which Hmb substitutions have been incorporated that aggregation so close to an Hmb residue has been observed.[17,18] It implies that the associated state encountered here is more complex than the simple intermolecular, extended β-sheet type structures that are generally held to be responsible for aggregation in difficult sequences.[8,22]

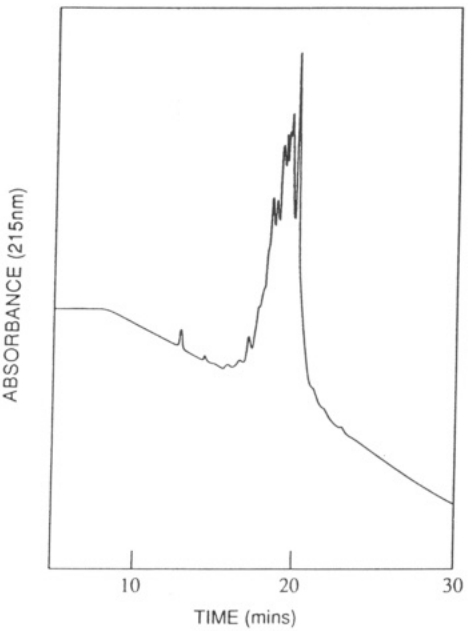


Figure 1. Analytical HPLC of crude penta(acetyl-Hmb)βA4-(1–43) (**7**).

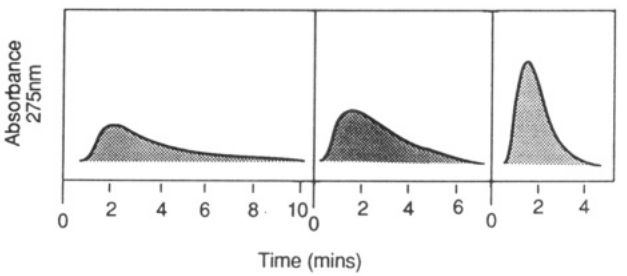


Figure 2. Typical spectrometric peak profiles from the Fmoc-deprotection flow stream (at 275 nm) for peptidyl-resins in an aggregated state (left), a normal unaggregated state (right), and the profile obtained upon deprotection of Met[35] (center).

The solubility of the crude penta(acetyl-Hmb) product **7** enabled its purification by preparative HPLC using C8 reverse-phase packing. Analytical HPLC of the purified product exhibited a single peak (Figure 3) with >98% purity, that gave a major molecular ion species at m/z = 5506.8 (theory required 5505.95) (Figure 4) by electrospray mass spectrometry (ESMS). None of the minor lower mass peaks correlated with possible deletion peptides. In conjunction with amino acid analysis, the fraction was confirmed as the partially protected target peptide **7**.

ESMS analysis of two earlier-eluting fractions (homogeneous on analytical HPLC) isolated during the purification procedure indicated these to potentially be Ile

(22) Meister, S. M.; Kent, S. B. H. In *Peptides: Structure and Function;* Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1984; pp 103–106.

(23) Quibell, M.; Turnell, W. G.; Johnson, T., *Tetrahedron Lett.*, in press.

(24) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.

Figure 3. Analytical HPLC of purified penta(acetyl-Hmb)βA4-(1–43) (7).

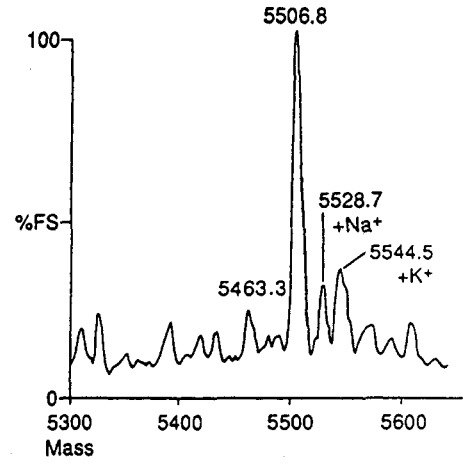


Figure 4. Electrospray mass spectrum of purified penta(acetyl-Hmb)βA4(1–43) (7).

deletion combinations (m/z = 5280.8 and 5393.8, i.e. 226.15 and 113.15 units low). During the initial assembly it was observed that the Fmoc-deprotection area of Ile[31] appeared approximately 25% lower than previous areas.

The TFA stable acetyl-Hmb groups of purified 7 were de-O-acetylated by 5% hydrazine/DMF treatment to give TFA-labile HO-Hmb groups (8). Analytical HPLC analysis (Figure 5) showed a single peak with >98% purity, that gave a major molecular ion species at m/z = 5296.8 (theory required 5295.8) (Figure 6) by ESMS. A peak at m/z = 5312.3 (50% intensity of the main peak), i.e. M + 16 was also observed, possibly arising from Met(O)[35] peptide. This was probably formed during the ESMS process, as analytical HPLC shows a single peak (even on shallower gradients), and no Met(O) was observed on amino acid analysis. In addition, hydrogen peroxide treatment of purified 8 (to generate Met(O)) gave a different retention time on analytical HPLC (elution time 0.5 min earlier compared to that of 8) that could clearly be distinguished from pure 8. No mass combinations

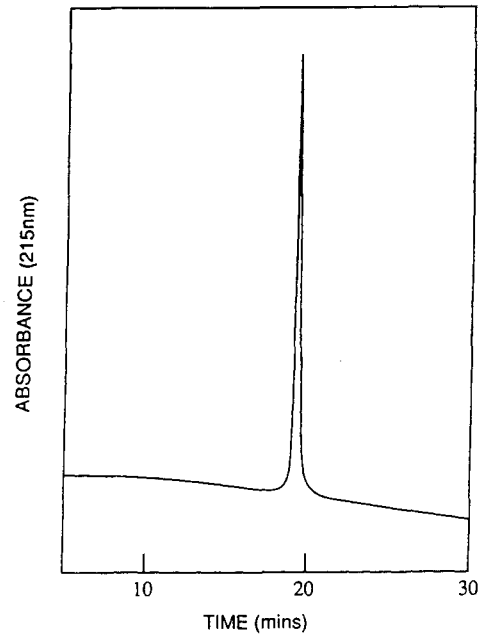


Figure 5. Analytical HPLC of penta(Hmb)βA4(1–43) (8).

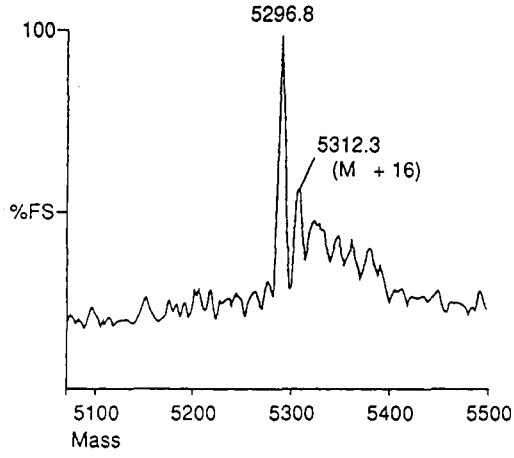


Figure 6. Electrospray mass spectrum of penta(Hmb)βA4(1–43) (8).

corresponding to acetyl were observed above 5296.8, confirming that complete de-O-acetylation had been achieved.

Backbone Hmb groups were removed from Phe[20], Gly[25,29,33,38] using a TFA/scavenger cocktail for 2 h. Precipitation from diethyl ether gave purified βA4(1–43) (1) (yield 8.2 mg, 7.5% overall; based on quantitative amino acid analysis of peptide 8 and the quantitative recovery of 1 by weight). Analytical HPLC (Aquapore C4, butyl column) was determined on a sample dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol. The elution profile obtained (Figure 7) was ill-defined; however, ESMS indicated that only a single species was present, m/z = 4616.5 (100% relative intensity), 4631.8 (20%) [M + 16], and 4655.5 (30%) $[M + K]^+$, 4671.3 [M + 55] (theory required 4615.2) (Figure 8) and no molecular ions indicative of incomplete Hmb removal were observed i.e. combinations of M + 136. As with peptide 8 (shown chemically to contain fully reduced Met) the mass signal at M + 16 probably arose during the ESMS process. Chemical oxidation would not normally be expected to occur during simple TFA treatment of a methionine-containing peptide. However, due to HPLC difficulties it was not possible to

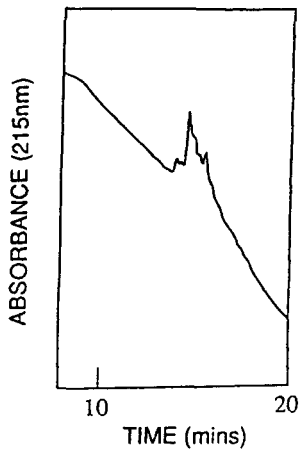


Figure 7. Analytical HPLC (C4, butyl column) of βA4(1–43) (1).

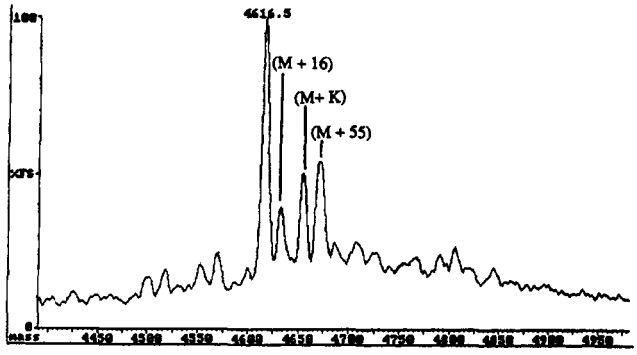


Figure 8. Electrospray mass spectrum of βA4(1–43) (1).

confirm this chromatographically. In addition, sequence analysis confirming the structure of 1 showed no evidence of Met(O). Burdick et al.[6] have previously commented upon the chromatographic behavior of βA4(1–42), suggesting that the ill-defined profile is an intrinsic property of the peptide rather than a reflection of product heterogeneity. The profile may highlight conformational effects induced by the C4 column during the reverse-phase HPLC process, as suggested by Houghten et al.[25,26] for numerous model peptides.

Conclusions

Current methods for the preparation, and especially the purification of highly insoluble peptide sequences appear inadequate. The work described here offers a practical approach to solving these problems, through strategic protection of the peptide backbone amide bonds. Backbone protection using the TFA-stable *N*-(2-acetoxy-4-methoxybenzyl) group at residues Phe[20], Gly[25,29,33], and Gly[38] of βA4(1–43) gave a crude product from stepwise synthesis with a solubility >137 mg/mL in 0.1% aqueous TFA/acetonitrile (fully deprotected βA4(1–43) is virtually insoluble in this solvent). This enabled purification to be achieved by standard reverse-phase chromatography and facilitated acquisition of mass spectral data.

The overall yield of purified βA4(1–43) was low (7.5%), mainly due to an unexpected aggregation state commencing at the deprotection of Met[35]. It is possible that the aggregation observed is not due to simple intermolecular β-sheet type structures of fully extended peptide chains (the positioning of (Hmb)Gly[38] prevents this), and its origin is currently under investigation. The prevention of alternative aggregated conformations should enable the crude purity of backbone protected βA4(1–43) to be substantially improved.

The new strategies outlined here could provide a general solution to the problematic preparation of hydrophobic sequences (e.g. transmembrane-spanning peptides) which are currently very difficult to purify and characterize by available techniques.

Experimental Section

Equipment, Materials, and Methods. Continuous flow Fmoc-polyamide methods reviewed by Atherton and Sheppard[20] were used exclusively. Fmoc amino acid pentafluorophenyl activated esters (Novabiochem, UK) were used exclusively except for Ser(OBut) and Arg(Mtr) which were coupled as the dihydrooxobenzotriazine esters (Novabiochem, UK). Fmoc *N*-carboxy anhydrides (Propeptide, Vert Le Petit, France), used for coupling Lys(Boc), Val, Ile, and Phe residues to Hmb-protected amino acids (see Table 1) and Fmoc-L-Thr(OBu[t])-Pepsyn KA resin (Milligen) were commercially available. *N,O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl) amino acid pentafluorophenyl esters[19] were prepared in house; full experimental details are available from the authors upon request (see also supplementary material). All solvents were purified as previously described.[20]

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/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 N HCl containing a trace of phenol in evacuated, sealed tubes. Analysis of hydrolysis products was performed on a Beckman 7300 analyzer. Separation was obtained using ion-exchange resin with manufacturers buffer solutions and post-column separation detection 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 25 min (1.5 mL/min) was used unless otherwise stated, where A = 0.1% aqueous TFA and B = 90% acetonitrile/10% A. Preparative HPLC was performed on a Vydac 208TP1022 C8 column (25 × 2.5 cm) at 10 mL/min and 215-nm UV detection. Electrospray mass spectra were obtained on a VG BioQ spectrometer.

Preparation of Peptide–Resin Assembly 5. Fmoc-L-Thr-(OBu[t])-Pepsyn KA (0.097 mequivg^{-1}, 250 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 275 nm) (peak height, peak area): Thr[43] (1.24, 71.0), Ala[42] (1.11, 71.6), Ile[41] (1.04, 74.0), Val[40] (1.05, 71.2), Val[39] (0.95, 70.5), Gly[38] (2.56, 146.6), Gly[37] (1.17, 74.4), Val[36] (0.95, 70.9), Met[35] (0.69, 57.0), Leu[34] (0.58, 59.2), Gly[33] (2.14, 117.3), Ile[32] (0.57, 51.0), Ile[31] (0.43, 39.2), Ala[30] (0.50, 40.3), Gly[29] (2.05, 86.6), Lys[28] (0.69, 46.3), Asn[27] (0.83, 46.3), Ser[26] (0.74, 45.4), Gly[25] (2.34, 102.3), Val[24] (1.09, 72.0), Asp[23] (0.77, 41.7), Glu[22] (0.83, 47.8), Ala[21] (0.86, 49.2), Phe[20] (2.39, 105.3), Phe[19] (1.40, 87.6), Val[18] (0.64, 43.6). 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 2 h. Also Gly[37] was double-coupled (2 × 2 h) to (Hmb)Gly[38] using the pentafluorophenyl ester.

A general procedure was used to couple to a terminal Hmb residue. The de-Fmoc resin was removed from the synthesizer, washed with DMF and then diethyl ether, and briefly dried in

(25) Houghten, R. A.; DeGraw, S. T. *J. Chromatogr.* **1987**, *386*, 223.
(26) Buttner, K.; Arad, O.; Ostresh, J.; Houghten, R. A. In *Innovations and Perspectives in Solid Phase Synthesis, 1st International Symposium;* Epton, R., Ed.; SPCC: UK, 1990; pp 325–336.

vacuo. The resin was then suspended in solution of the appropriate Fmoc-amino acid-*N*-carboxy anhydride (10 equiv, 0.25 mmol) in dichloromethane (approximately 4 mL) for the times shown in Table 1. The acylated resin was then washed as above, resuspended in DMF, loaded onto the synthesizer, and continued.

The final 43-residue assembly was washed with DMF, *tert*-amyl alcohol, acetic acid, *tert*-amyl alcohol, DMF and diethyl ether, and dried in vacuo over potassium hydroxide pellets. The final peptide-resin weighed 425 mg.

Amino acid analysis of the crude resin-bound peptide gave the following ratios: Asp/Asn 3.65 (4), Thr 0.98 (1), Ser 1.25 (2), Glu/Gln 3.86 (4), Gly 5.83 (6), Ala 3.78 (4), Val 7.86 (6), Met 0.99 (1), Ile 2.28 (3), Leu 1.86 (2), Nle 1.10 (1)[$], Tyr 0.89 (1), Phe 2.91 (3), His 2.68 (3), Lys 1.71 (2), Arg 0.87 (1).

$-Norleucine is an internal reference amino acid attached to the resin during its initial derivatization prior to peptide synthesis. Norleucine is positioned between the acid-labile peptide resin linker and the resin itself and is not part of the target peptide sequence.

High amino acid ratios, especially for valine, are related to the termination of ca. 30% of the peptide at Met[35]. The C-terminal 35–43 sequence, which contains 3 Val residues, remains to contaminate the final product. When amino acid ratios were calculated, these were related to a normalized value from the whole of the peptide, a simple calculation $(3 \times 0.3) + (6 \times 0.7)/0.7 = 7.29$ indicates that the Val figure would be expected to be high.

Preparation of Protected Assembly 6. Assembly **5** was treated with di-*tert*-butyl dicarbonate (10 equiv, 54 mg) in DMF (4 mL) for 2 h (at this point the peptide-resin assembly gave a negative ninhydrin response) and the resin washed with DMF and then diethyl ether. The N-terminal Boc-resin was then treated with acetic anhydride (20 equiv, 51 mg) in DMF (3 mL) with the addition of diisopropylethylamine (10 equiv, 32 mg) in DMF (1 mL) and left overnight. The resin was then washed and dried in the usual manner. Amino acid analysis of the *Nα*-Boc-penta-2-acetoxy-Hmb resin-bound peptide gave the following ratios: Asp/Asn 3.88 (4), Thr 1.11 (1), Ser 1.57 (2), Glu/Gln 4.13 (4), Gly 6.24 (6), Ala 4.14 (4), Val 7.52 (6), Met 0.99 (1), Ile 2.23 (3), Leu 1.96 (2), Nle 1.16 (1), Tyr 0.95 (1), Phe 3.09 (3), His 2.83 (3), Lys 1.83 (2), Arg 0.94 (1).

Peptide-Resin Cleavage. Preparation of Crude Penta-(Acetyl-Hmb)βA4(1–43) (7). Assembly **6** (420 mg) was treated with 92% TFA/3% phenol/3% ethanedithiol/2% triethylsilane (v/w/v/v) (20 mL) for 10 h. Cleaved resin was removed by filtration and washed with 5 × 4 mL of neat TFA, and the combined filtrates N_2-sparged to approximately 3 mL in volume. Ice-cooled diethyl ether (40 mL) was added giving precipitation, the mixture was cooled in acetone/dry ice for 5 min and centrifuged at 3000 rpm for 5 min. The ethereal solution was decanted, and further 5 × 40 mL ether extractions performed. The residue was dried in vacuo to give a white solid (yield 137 mg, 100% cleavage).

Analysis of Crude Peptide 7. Crude peptide **7** (137 mg) was fully dissolved in 0.1% aqueous TFA/acetonitrile (1:1) (1 mL) and diluted to 5 mL with the same solvent mixture. Analytical HPLC (C8 column, main peak, rt = 19.97 min) afforded multiple faster-eluting components (Figure 1). Analytical HPLC (C8 column): gradient 42–62% B in A over 25 min, three main peaks (rt = 13.60, 14.48, 17.42 min). Amino acid analysis (25 μL of solution) gave the following ratios: Asp/Asn 3.77 (4), Thr 0.94 (1), Ser 1.47 (2), Glu/Gln 4.05 (4), Gly 6.00 (6), Ala 3.96 (4), Val 6.80 (6), Met 0.96 (1), Ile 2.35 (3), Leu 2.10 (2), Tyr 0.97 (1), Phe 3.18 (3), His 2.81 (3), Lys 1.83 (2), Arg 0.94 (1).

Purification of Crude Peptide 7. Crude peptide **7** (137 mg in 5 mL) was purified by preparative HPLC (Vydac C8 column) (9 × 600 μL) injections using a gradient of 42–62% B in A over 25 min. Three main fractions were collected corresponding to analytical retention times 13.5 (F1), 14.5 (F2), and 17.5 min (F3). Each fraction was lyopholized to a white solid, and analytical HPLC (C8 column) gradient 42–62% B in A over 25 min showed each fraction to be homogeneous. Amino acid analysis of fractions F1–3 gave the following ratios:

Fraction 1. Asp/Asn 4.09 (4), Thr 1.01 (1), Ser 1.72 (2), Glu/Gln 4.31 (4), Gly 6.23 (6), Ala 4.12 (4), Val 5.92 (6), Met 0.37*(1), Ile 1.84 (3), Leu 2.18 (2), Tyr 0.89 (1), Phe 3.16 (3), His 3.03 (3), Lys 2.00 (2, std), Arg 0.83 (1).

Fraction 2. Asp/Asn 4.01 (4), Thr 1.01 (1), Ser 1.83 (2), Glu/Gln 4.19 (4), Gly 6.33 (6), Ala 4.18 (4), Val 5.83 (6), Met 0.45*(1), Ile 1.08 (3), Leu 2.13 (2), Tyr 0.87 (1), Phe 3.12 (3), His 2.93 (3), Lys 2.00 (2, std), Arg 0.89 (1).

Fraction 3. Asp/Asn 3.85 (4), Thr 0.97 (1), Ser 1.75 (2), Glu/Gln 3.96 (4), Gly 6.21 (6), Ala 4.06 (4), Val 5.64 (6), Met 0.46*(1), Ile 2.26 (3), Leu 2.27 (2), Tyr 0.82 (1), Phe 2.85 (3), His 2.70 (3), Lys 2.00 (2, std), Arg 0.86 (1).

Methionine ratios (*)were consistantly low in all analyses (numerous nonrelated samples) at the time of analysis.

Each fraction was analyzed by electrospray mass spectrometry, giving penta(acetyl-Hmb)βA4(1–43)-OH (required m/z = 5505.95). Fraction 1: major species at m/z = 5280.6 (M − 226), minor components at m/z = 5328.0, 5408.8, 5522.5. Fraction 2: major species at m/z = 5393.8 (M − 113), minor component at m/z = 5506.5. Fraction 3: major species at m/z = 5506.8, minor components at m/z = 5463.3 (M − 43), 5528.7 (M + Na^+), 5544.5 (M + K^+) (Figure 4).

Analytical HPLC (C8 column) of F3 gave a single peak (>98%, rt = 19.76 min) (Figure 3). Quantitative amino acid analysis gave a yield of 1.94 mmol (10.7 mg, 8.0% overall yield).

De-O-acetylation of Purified Peptide 7. Preparation of Penta(Hmb)βA4(1–43) (8). Purified F3 (10.0 mg) was suspended in 500 μL 5% hydrazine hydrate/DMF and sonicated for 25 min (dissolution occurred quickly upon sonication). The sample was then extracted with 6 × 15 mL of cold diethyl ether (centrifuge between extracts) and dried in vacuo to give a chalky white solid, yield (9.8 mg, 100%).

Amino acid analysis of the de-O-acetylated peptide **8** gave the following ratios: Asp/Asn 3.95 (4), Thr 0.94 (1), Ser 1.69 (2), Glu/Gln 4.10 (4), Gly 6.15 (6), Ala 4.00 (4), Val 5.54 (6), Met 0.88(1), Ile 2.36 (3), Leu 2.07 (2), Tyr 0.86 (1), Phe 2.85 (3), His 2.75 (3), Lys 2.00 (2), Arg 0.88 (1).

Analytical HPLC (C8 column) gave a single peak (>98%, rt =19.09 min), (Figure 5). A sample was analyzed by electrospray mass spectrometry giving (theory requires 5295.8) a main species at m/z = 5296.8 and m/z = 5312.3 (50% intensity) (Figure 6).

Final Cleavage of Hmb-Backbone Protection. Preparation of βA4(1–43) (1). Peptide **8** (9.5 mg) was treated with 92% TFA/3% phenol/3% ethanedithiol/2% triethylsilane (v/w/v/v) (1 mL) for 2 h. The product was isolated by the usual N_2-sparge and ethereal extractions to give a white solid, yield (8.2 mg, 7.5% overall).

A sample was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol and analyzed by analytical HPLC (C4), giving absorbance from 49 to 58% B (Figure 7). A sample was analyzed by electrospray mass spectrometry giving (theory requires 4615.2) a main species at m/z = 4616.5 (100% relative intensity) and minor species at 4631.8 (20%) [M + 16], 4655.5 (30%) [M + K]$^+$, and 4671.3 [M + 55] (Figure 8).

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Supplementary Material Available: Experimental procedures and spectral data for synthetic intermediates (8 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.