Synthesis of the 3-Repeat Region of Human Tau-2 by the Solid Phase Assembly of Backbone Amide-Protected Segments

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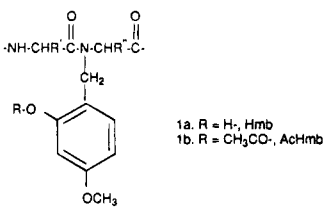
Abstract: The synthesis, purification, and use of *N*-(2-acetoxy-4-methoxybenzyl)(AcHmb) backbone amide protected segments in the preparation of Asp[158]-Leu[251] from the 3-repeat region of human tau-2 has been investigated. Fmoc/*tert*-butyl based[1,2] fully protected segments of 9−21 residues, containing appropriately placed AcHmb protection, were prepared on Polyhipe SU500 resin through a 2-chlorotrityl linker. Protected segments, cleaved with 0.75% trifluoroacetic acid in dichloromethane, were easily purified in 100s of milligram quantities by either silica gel chromatography or standard aqueous acetonitrile based reverse phase preparative HPLC. Purified protected segments, typically soluble at >500 mg mL^{-1} in dimethylformamide, coupled efficiently and economically to a growing resin-bound assembly, to give the 94-mer human tau-2 with an overall purified yield of 2.50 μmol (16.7%).

Introduction

As part of on-going structural studies into proteins associated with Alzheimer's disease,[3] we have prepared the 94-mer Asp[158]-Leu[251] from the 3-repeat region of human tau-2 (**2**),[4] by solid phase segment assembly.[5] Continuing our studies into the uses of the *N*-(2-hydroxy-4-methoxybenzyl)(Hmb) (**1a**) backbone amide protection system in solid phase peptide synthesis,[6] we wish to report that AcHmb (**1b**) backbone substitution[7] confers substantially improved solubility properties to fully protected peptide segments. Backbone amide protection effectively eliminates the often encountered problem of low protected



1a. R = H-, Hmb
1b. R = CH_3CO-, AcHmb

segment solubility,[5a,d,e,8] simplifies segment purification,[5a,8b,9] and enhances segment coupling kinetics.[8b,9] We have readily purified protected segments (up to 21 residues in length) corresponding to regions of the desired target peptide, in 100s of milligram quantities. The AcHmb substituted fully protected segments were extremely soluble in dimethylformamide (>500 mg mL^{-1}), allowing concentrated and therefore efficient resin-bound sequential assembly of the target 94-mer.

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[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1995.

(1) For a general review of Fmoc-polyamide chemistry, see: Atherton, E.; Sheppard, R. C. Solid Phase Peptide Synthesis: A Practical Approach; Oxford University Press: Oxford, 1989; 1−203.

(2) Abbreviations for amino acids and nomenclature of peptide structures follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem,* **1971**, *247*, 997). All chiral amino acids were of the L-configuration. Other abbreviations are as follows: AAA = amino acid analysis; Acm = acetomidomethyl; Ac_2O = acetic anhydride; HMPAA = 4-hydroxymethylphenoxyacetic acid; Boc = *tert*-butoxycarbonyl; BOP = benzotriazolyloxytris(dimethylamino)phosphonium hexafluoro phosphate; Bu^t = *tert*-butyl ether; OBu^t = *tert*-butyl ester; DCC = dicyclohexylcarbodiimide; DMF = *N,N*-dimethylformamide; DCM = dichloromethane; DIC = diisopropylcarbodiimide; DIEA = diisopropylethylamine; DMAP = 4-dimethylaminopyridine; EDT = 1,2-ethanedithiol; FABMS = fast atom bombardment mass spectrometry; Fmoc = 9-fluorenylmethoxy carbonyl; HOBt = 1-hydroxybenzotriazole; HPLC = high pressure liquid chromatography; MALDITOF-MS = matrix assisted laser desorption time of flight mass spectrometry; NMM = *N*-methylmorpholine; Pfp = pentafluorophenyl; Pfp-OH = pentafluorophenol; Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TBTU = *O*-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate;Trt = trityl; TFA = trifluoroacetic acid; TFE = trifluoroethanol; TES = triethylsilane.

(3) A number of proteins associated with Alzheimers disease have been identified, although their exact function during disease are as yet unknown. E.g.: (a) Smith, M. A.; Siedlak, S. L.; Richey, P. L.; Mulvihill, P.; Ghiso, J.; Frangione, B.; Tagliavini, F.; Giaccone, G.; Bugiani, O.; Praprotnik, D.; Kalaria, R. N.; Perry, G. *Nature Medicine* **1995**, *1(4)*, 365−369.

(4) Tau constitutes the principle protein element of intracellular neurofibrillary tangles, pathological legions characteristic of Alzheimers disease, e.g., see: Grundke-Iqbal, I.; Iqbal, K.; Tung, Y.-C.; Quinlan, M.; Wisniewski, H. M.; Binder, L. I. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4913−4917.

(5) For reviews, see: (a) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, *49(48)*, 11065−11133. (b) Benz, H. *Synthesis* **1994**, 337−358. (c) Riniker, B.; Florsheimer, A.; Fretz, H.; Sieber, P.; Kamber, B. *Tetrahedron* **1993**, *49(41)*, 9307−9320. (d) Atherton, E.; Brown, E.; Priestly, G.; Sheppard, R. C.; Williams, B. J. In *Peptides: Synthesis, Structure, Function. Proceedings of the 7th American Peptide Symposium;* Rich, D. H., Gross, E, Eds.; Pierce Chemical Company, Rockford, IL, 1981; pp 163−175. Other relevent papers of interest: (e) Barlos, K.; Gatos, D.; Schafer, W. *Angew. Chem. Int. Ed. Engl.* **1991**, *30(5)*, 590−593. (f) Barlos, K.; Gatos, D.; Frischmann, P.; Schafer, W. In *Peptides 1994, Proceedings of the 23rd European Peptide Symposium;* Maia, H. S. L, Ed.; ESCOM Science Publishers B. V. Leiden, Netherlands, 1995; pp 26−27. (g) Yajima, H.; Kiso, Y. *Chem. Pharm. Bull*, **1974**, *22*, 1087−1094 (assembly of 58-mer trypsin inhibitor). (h) Protein Synthesis Group, Shanghai Institute of Biochemistry. *Sci. Sin.* **1975**, *18*, 745−768 (assembly of 27-mer glucogen).

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(7) Quibell, M.; Turnell, W. G.; Johnson, T. *Tetrahedron Lett.* **1994**, *35(14)*, 2237−2238.

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H-Asp[158]-Leu-Lys-Asn-Val-Lys-Ser-Lys-Ile-Gly-Ser-Thr-Glu-Asn-Leu-Lys-His-Gln-Pro-Gly-Gly-Gly-Lys-Val-Gln-Ile-Val-Tyr-Lys-Pro-Val-Asp-Leu-Ser-Lys-Val-Thr-Ser-Lys-Cys-Gly-Ser-Leu-Gly-Asn-Ile-His-His-Lys-Pro-Gly-Gly-Gly-Gln-Val-Glu-Val-Lys-Ser-Glu-Lys-Leu-Asp-Phe-Lys-Asp-Arg-Val-Gln-Ser-Lys-Ile-Gly-Ser-Leu-Asp-Asn-Ile-Thr-His-Val-Pro-Gly-Gly-Gly-Asn-Lys-Lys-Ile-Glu-Thr-His-Lys-Leu[251]-OH

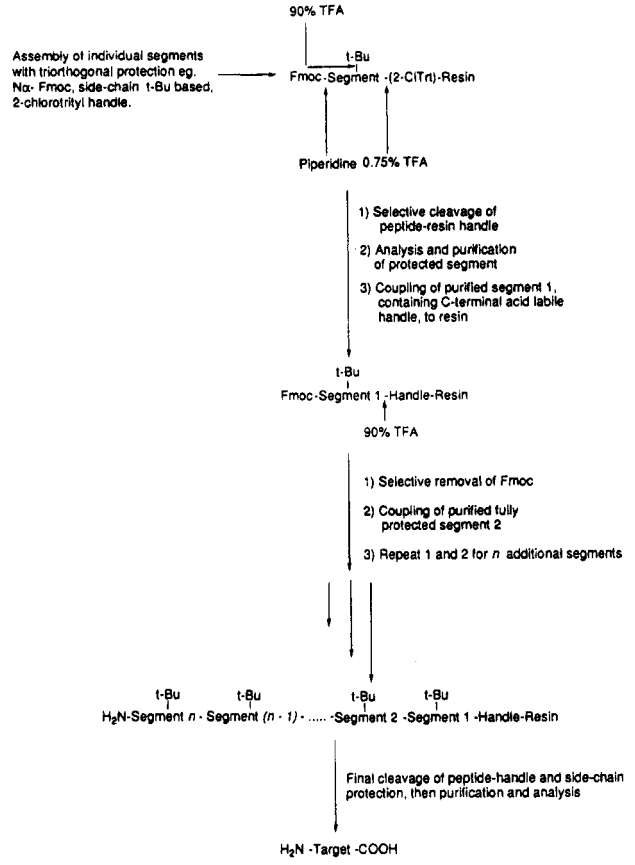
(2)

Results and Discussion

The solid phase segment assembly approach toward the synthesis of large peptides has long been recognized to have potential advantages over the conventional stepwise methodology. It offers the possibility of isolating, purifying and characterizing individual peptide segments before assembly on the solid phase. In principle, an improved quality of crude final cleaved product will be obtained, facilitating purification, which otherwise, using the stepwise method, may become virtually impossible to achieve. This potentially attractive strategy (generally depicted in Scheme 1) can be divided into distinct stages, each of which requires an appropriate solution for the overall strategy to be successful: (1) The assembly of the individual protected segments must be achieved in a smooth and efficient manner. (2) Protected segments must be easily and cleanly removed from the solid support while retaining the α-amino and side chain protecting groups. (3) Purification of the crude protected segments should be readily accomplished. (4) The protected segments should be soluble in a suitable coupling medium at a concentration providing good reaction kinetics. (5) Activation of the protected peptide segments must be unambiguous and coupling to the resin bound peptide should be near quantitative and epimerization free.[10]

The use of segment assembly strategies for protein synthesis, both on the solid-phase and in solution, has been hindered by unsatisfactory general solutions to the latter three stages. The major contributing factor leading to this shortfall is the often encountered low solubility of fully protected segments. The general problem of solubility is not only restricted to solution but also manifests itself during the actual solid phase assembly of protected peptides in the so-called "difficult sequences". Here, the growing, freely solvated peptide-resin matrix, suddenly and largely unpredictably, undergoes a dramatic change during the synthesis, to a poorly solvated state. The major contributing factor leading to this poor solvation is the formation of protein-like extended β-sheet type structures through amide backbone hydrogen bonding.[11] We have recently described a solution to the difficult sequence problem[6a,b] by removing the potential for backbone hydrogen bond formation, through the substitution of selected amides during a synthesis with the Fmoc/Bu[t] compatible *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) protecting group.[6] Protected peptide solubility in solution is presumably governed by largely the same factors, with a decrease in solubility being paralleled by a transition from an unordered soluble species to an ordered inter- and intramolecularly hydrogen-bonded species exhibiting a regular β-structure. We were hopeful that the incorporation of backbone protection into fully protected peptides would enhance their solubility in solution and describe here that a dramatic and positive effect is indeed achieved. This article demonstrates the utilization of backbone protected segments in a flexible and efficient solid phase segment assembly strategy. High protected segment solubility provides simple purification and a straightforward solution to the third and fourth stages outlined above. Improved solubility should additionally benefit the fifth stage, through the preparation of more concentrated coupling media and hence better coupling kinetics.

Scheme 1. Basic Principles of the Solid Phase Segment Assembly Approach toward the Synthesis of Small Proteins



(i) Choice of Segments. The initial stage of a solid phase segment assembly strategy is the division of the target sequence into a set of smaller segments. The 3-repeat region of human tau-2 from Asp[158]-Leu[251] (**2**) offers an almost ideal target to

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(12) Quibell, M.; Johnson, T. in *Peptides 1994, Proceedings of the 23rd European Peptide Symposium;* Maia, H. S. L., Ed.; ESCOM Science Publishers B. V: Leiden, Netherlands, 1995, pp 173−174.

(13) Bedford, J.; Hyde, C.; Johnson, T.; Jun, W.; Owen, D.; Quibell, M.; Sheppard, R. C. *Int. J. Pept. Protein. Res.* **1992**, *40*, 300−307.

tackle the segment solubility aspect alone, due to the regular spacing of glycine and proline residues. This provides ample opportunity to choose C-terminal segments giving little or no epimerization upon segment coupling.[10] Another important consideration when deciding where to divide the target is that the growing resin-bound assembly needs to be in a freely solvated state[11] during all coupling reactions. This has recently been demonstrated with a study on the segment assembly of β-amyloid(26−43).[12] It was clearly shown that the efficiency of segment coupling to a poorly solvated peptide-resin showed little correlation with the solubility of the incoming segment. The most efficient coupling was shown to occur between an Hmb-protected peptide-resin (i.e., fully solvated) and a soluble (Hmb-protected) segment. Thus, the placement of backbone protection within each segment requires careful consideration and was derived using three main criteria.

(1) Backbone protection need only occur at a maximum of every sixth residue,[6a,b,13] and, as a synthesis progresses, this becomes more flexible. Thus, for segments of approximately 10 residues, placement of backbone protection around the segment center should ensure an even resin-bound distribution as segments are sequentially coupled.

(2) The amide bond being protected needs to be compatible with Hmb chemistry.[14] The only real limitation to this is that an amide bond where the N-terminal residue is β-branched (Val, Ile, Thr) cannot be protected, unless the C-terminal residue is glycine. All other bonds may be readily protected.

(3) A number of recent publications[15] have highlighted the sometimes susceptible nature of the aspartyl amide bond during Fmoc based syntheses, to the repetitive base treatment used for Fmoc removal. We have recently reported[6e] that backbone protection of the aspartyl bond completely inhibits these base catalyzed transformations even in highly susceptible cases. Thus, particularly prone aspartyl bonds,[15a] such as Asp^{233}-Asn^{234} from human tau-2, definitely require protection. Since aspartyl transformations are sequence dependent,[15] it may be prudent to protect aspartyl bonds where possible.

Using the above criteria, the 94-residue target sequence (**2**) was split into eight segments, six of which were C-terminal glycine, one being C-terminal proline and the C-terminal segment itself, shown below. (The residues chosen for backbone protection are bold.)

Segment 1:

Gly^{242}-Asn-Lys-**Lys**-Ile-Glu-Thr-His-Lys-Leu^{251}

Segment 2:

Ser^{231}-Leu-Asp-**Asn**-Ile-Thr-His-Val-Pro-Gly-Gly^{241}

Segment 3:

Gly^{210}-Gln-Val-Glu-Val-Lys-**Ser**-Glu-Lys-Leu-Asp-**Phe**-Lys-Asp-**Arg**-Val-Gln-Ser-Lys-Ile-Gly^{230}

Segment 4:

Ser^{199}Leu-**Gly**-Asn-Ile-His-His-Lys-Pro-Gly-Gly^{209}

Segment 5:

Val^{188}-Asp-Leu-Ser-**Lys**-Val-Thr-Ser-Lys-Cys-Gly^{198}

Segment 6: Gly^{179}-Lys-**Val**-Gln-Ile-Val-Tyr-Lys-Pro^{187}

Segment 7:

Ser^{168}-Thr-Glu-**Asn**-Leu-Lys-His-Gln-Pro-Gly-Gly^{178}

Segment 8:

Asp^{158}-Leu-Lys-Asn-Val-Lys-**Ser**-Lys-Ile-Gly^{167}

After promising initial coupling results (see later), segments 5 and 6 were combined giving segment 9, and 7 and 8 were combined giving segment 10.

(ii) Preparation and Initial Loading of Solid Support. Having targeted the segments required, a protecting group strategy and efficient preparation of the individual protected segments was required. We employed the fluoren-9-ylmethoxycarbonyl[1] group for Nα protection, being removed by base treatment, typically piperidine. Side-chain protection was based on the *tert*-butyl strategy, stable to base, and cleaved by typically 90% TFA with scavengers. The Hmb backbone protection system is compatible with this scheme,[14] being retained during the repetitive base treatment, and cleaved along with the side-chain protection by 90% TFA/scavenger treatment at the end of the synthesis. A number of handles are available that are both compatible with the Fmoc/Bu^t system and selectively cleavable. We chose the 2-chlorotrityl handle described by Barlos et al.[16] which incorporates a number of attractive features. Firstly, peptides are cleaved from this handle under relatively weak acid conditions, typically 0.75% TFA or 20% acetic acid/20% TFE in DCM. The peptide-handle bond is however stable to the weakly acid conditions generated during HOBt activation of Pfp esters (the procedure used in the current work).[17] Secondly, segment 6 contains a C-terminal proline residue which is prone to diketopiperizine formation[18] (basically a cyclization at the dipeptide stage causing premature detachment of the peptide from the handle-resin assembly). This side-reaction is virtually eliminated by the attachment of proline through the 2-chlorotrityl handle.[19]

The solid-phase synthesis described here was performed on polyamide-based resins,[20] requiring an initial investigation into the preparation and loading of the 2-chlorotrityl handle onto polyamide-based solid supports. A summary of the basic chemistry used to prepare the required loaded resins is shown in Scheme 2. The standard method for the loading of resins containing trityl-based handles involves an initial derivitization of the resin (containing appropriate linkers, internal reference amino acids etc.) with a substituted triphenylmethanol, followed by an on-resin conversion of the trityl hydroxyl to halogen (chloro or bromo). The halogen is then displaced by the carboxyl anion of an Fmoc-amino acid generated in the presence of DIEA. This simple and attractive method is well documented for polystyrene based resins,[21] but as far as we are aware has not been described for polyamide based supports. Our attempts

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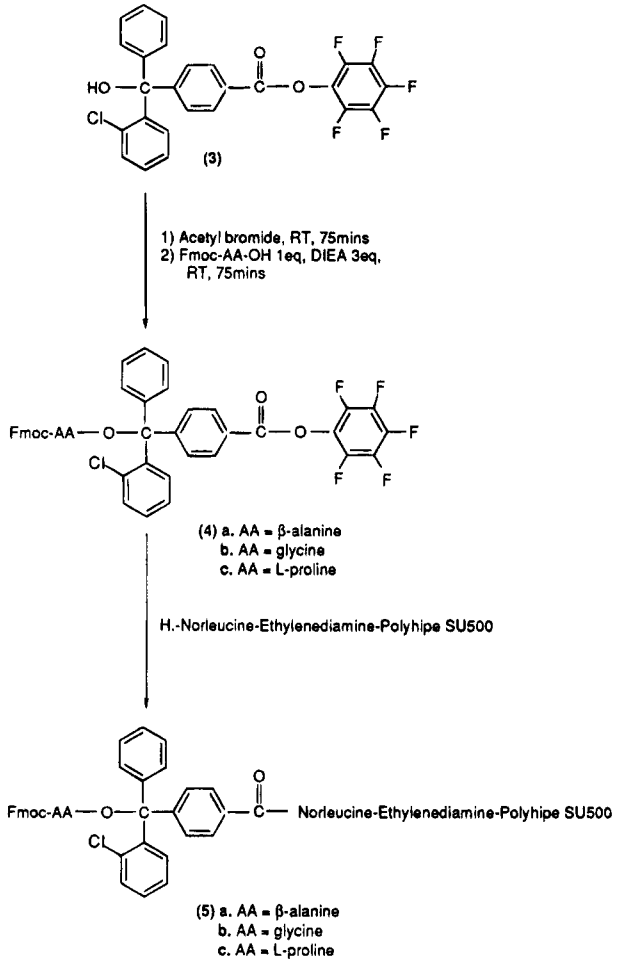
Scheme 2. Loading of *N*α-Fmoc Amino Acids through a Hyperacid Labile 2-Chlorotrityl Handle onto Polyamide Resins, using *N*α-Fluoren-9-ylmethoxycarbonyl Amino Acid (2-Chloro-4′-(carboxypentafluorophenoxy)triphenyl) Methyl Esters (**4a**−**c**)



to follow these methods using polyamide-based resin met with little success, with at best poor loading of around 0.02 mmol peptide/g peptide-resin. Treatment of H.Nle-EDA-Polyhipe SU 500[22] with **3** (see Scheme 2) and then acetylbromide following literature procedures gave a handle-resin matrix that titrated against sodium hydroxide to contain approximately 2.5 mmol/g active bromide, five times the maximum theoretical loading of 0.5 mmol/g. It is likely that the polyamide backbone of the SU 500 resin had protonated during the acetylbromide treatment. Since the following halogen displacement reaction with the Fmoc-aminoacid carboxyl anion is an equilibrium reaction,[21] the presence of increasing amounts of DIEA (to neutralize the protonated resin) severely affected the loading of the Fmoc-amino acid.

Peptide-resins **5a**−**c** were prepared with good loading (0.25−0.275 mmol amino-acid/g peptide-resin) by reaction of *N*α-fluoren-9-ylmethoxycarbonyl-amino acid-(2-chloro-4′-(carboxy-pentafluorophenoxy)triphenyl) methyl esters (**4a**−**c**) (Scheme 2) (prepared in solution), with polyamide resin. Fully characterized key intermediate (**3**) was prepared from the corresponding acid using DCC/Pfp-OH and isolated in 80.3% yield. The acid, 2-chloro-4′-carboxytriphenyl methanol, was prepared by reaction of 4-bromobenzoic acid (1 equiv), butyllithium (2.1 equiv), and 2-chlorobenzophenone (5 equiv) in tetrahydrofuran at −78 °C, under nitrogen, and isolated in 47.6% yield. Compound **3** was converted to the bromotrityl derivative with acetylbromide in dry DCM, followed by reaction with an Fmoc-amino acid (1 equiv) pre-equilibrated with DIEA (3 equiv) in dry DCM, giving derivatives **4a**−**c** in virtually quantitative yields. Peptide-resins **5b,c** were used directly for the preparation of segments 2−10 (see later).

(iii) Preparation, Purification, and Loading of Segment 1. Assembly (8) providing the base peptide-resin used for the sequential addition of purified segments was prepared as outlined in Scheme 3. Peptide-resin **5a** was substituted with the HMPAA linker[23] and then loaded with Fmoc-leucine using standard techniques[1] giving peptide-resin (**6**) (loading of 0.22 mmol leucine/g). (See Scheme 3).

Assembly of the C-terminal protected segment, peptide-resin **7**, proceeded smoothly using standard Fmoc amino acid pentafluorophenyl ester chemistry.[1] Backbone substituted (Hmb)-Lys[245] was incorporated via the *N,O*-bisFmoc-*N*-(2-hydroxy-4-methoxybenzyl)lysine pentafluorophenyl ester derivative[14] as previously detailed. Crude fully protected segment **1** was cleaved (84% by quantitative UV) from peptide-resin **7** using 0.75% TFA in DCM for 45 min. The crude peptide solution was neutralized with NMM and washed twice with a large volume of saturated salt solution, removing the TFA·NMM salt. Evaporation of the dried organic layer in vacuo gave crude segment **1** (500 mg, 185 μmol), which was of a good quality (see Table 1), containing no identifiable amino acid deletion sequences upon analytical HPLC analysis (see Table 2). Simple silica gel purification, achieved on a commercially available "Lobar" column with a chloroform/methanol gradient gave good purification of the crude segment. Fractions were cut and combined for maximum purity, rather than yield, giving highly purified segment 1 (>98% by HPLC, 202 mg, 74.8 μmol, 34.8% overall yield), and a further 174 mg (<95% by HPLC) of slightly inferior quality material, from a single column purification. Coupling of purified segment **1** to H.Nle-NovaSyn KD[24] using BOP[25] mediated activation, gave peptide-resin **8** (loading of 0.048 mmol peptide/g peptide-resin).

(iv) Preparation and Purification of Segments 2−10. Segments 2−10 were prepared (and cleaved) from peptide-resins **5b** and **5c** (0.2−0.3 mmol) as generally detailed for segment 1 and a summary of the analysis and purification method used for each protected segment is given in Table 1 (amino-acid analysis in Table 4). The crude (and final) yields are calculated from the starting quantities of resins **5a**−**c** from which the segment was prepared, making no allowances for resin loss during transfers or the removal of resin fines.

One of the greatest problems encountered in solid phase segment assembly is the purification of the individual fully protected segments.[5,9] To date, no universally-applicable technique exists for segment purification with each segment usually requiring a study in itself, often not reaching a satisfactory outcome. Here, we see probably the single most dramatic effect afforded by backbone substitution of fully protected segments. Purification was easily achieved, the method chosen depending upon the length and crude purity of the segment in question. This was determined by removing all protection from a sample of each segment and analyzing each

(22) A Kieselguhr supported polystyrene polymer impregnated with a polyamide gel, containing approximately 0.5 mmol of reactive methylsarcosine ester/g: Small, P.; Sherrington, D. C. *J. Chem. Soc., Chem. Commun.* **1989**, *21*, 1589−1591.

(23) Atherton, E.; Logan, C. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1981**, 538−546.

(24) A Kieselguhr supported polydimethylacrylamide polymer containing an ethylenediamine spacer, with approximately 0.05−0.1 mmol/g Nle internal reference amino acid.

(25) Castro, B.; Dormay, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, *16*, 1219.

Table 1. Summary of the Crude Yield, HPLC Analysis, Method of Purification, and Final Isolated Yields of Fully Protected Segments Spanning the 3-Repeat Sequence of Human Tau-2 (Asp^{158}-Leu^{251})

segment	structure	MW	crude yield, HPLC R_t (min)	purification method	purified yield
1	Fmoc- Gly^{242}-Asn(Trt)-Lys(Boc)-(AcHmb)Lys(Boc)-Ile-Glu(OBu^t)-Thr(Bu^t)-His(Trt)-Lys(Boc)-Leu^{251}-Handle-βAla-OH	2699.3	500 mg (84%), 31.83 (81.3%)[a]	Lobar, 1	202 mg (34.8%) 74.8 μmol
2	Fmoc-Ser(But)^{231}-Leu-Asp(OBut)-(AcHmb)Asn(Trt)-Ile-Thr(Bu^t)-His(Trt)-Val-Pro-Gly-Gly^{241}-OH	2161.2	445 mg (82%), 31.88 (70.3%)[a]	Lobar, 1	258 mg (47.8%) 119.4 μmol
3	Fmoc-Gly^{210}-Gln(Trt)-Val-Glu(OBut)-Val-Lys(Boc)-(AcHmb)Ser(Bu^t)-Glu(OBu^t)-Lys(Boc)-Leu-Asp(OBut)-(AcHmb)Phe-Lys(Boc)-Asp-(OBu^t)-(AcHmb)Arg(Pmc)-Val-Gln(Trt)-Ser(Bu^+)-Lys(Boc)-Ile-Gly^{230}-OH	4637.5	670 mg (72%), 30.69 (84.2%)[b]	diphenyl, 3	449 mg (48.4%) 96.8 μmol
4	Fmoc-Ser(Bu^t)^{199}Leu-(AcHmb)Gly-Asn(Trt)-Ile-His(Trt)-His(Trt)-Lys(Boc)-Pro-Gly-Gly^{209}-OH	2398.8	470 mg (65%), 29.85 (61.3%)[c]	C_4 butyl, 2	119 mg (16.5%) 49.6 μmol
9 (5 + 6)	Fmoc-Gly^{179}-Lys(Boc)-(AcHmb)Val-Gln(Trt)-Ile-Val-Tyr(But)-Lys(Boc)-Pro-Val-Asp(OBu^t)-Leu-Ser(But)-(AcHmb)Lys(Boc)-Val-Thr(But)-Ser(Bu^t)-Lys(Boc)-Cys(Acm)-Gly^{198}-OH	3721.5	600 mg (81%), 15.56 (43.2%)[d]	diphenyl, 3	115 mg (20.6%) 30.9 μmol
10 (7 + 8)	Fmoc-Asp(OBu^t)^{158}-Leu-Lys(Boc)-Asn(Trt)-Val-Lys(Boc)-(AcHmb)Ser(But)-Lys(Boc)-Ile-Gly-Ser(Bu^t)-Thr(But)-Glu(OBut)-(AcHmb)Asn(Trt)-Leu-Lys(Boc)-His(Trt)-Gln(Trt)-Pro-Gly-Gly^{178}-OH	4477.2	470 mg (53%), 18.67 (49.4%)[e]	diphenyl, 3	207 mg (23.1%) 46.2 μmol

[a] HPLC conditions: Vydac C_8 (250 × 4.6 mm) column, 10−90% B in A gradient over 27 min and then 15 min at 90% B (1.5 mL/min flow, 215 nm UV detection), where buffer A = 0.1% aqueous TFA and buffer B = 90% acetonitrile/10% buffer A. [b] Vydac phenyl (250 × 4.6 mm) column, gradient as in *a*. [c] Vydac C_4 (250 × 4.6 mm) column, gradient as in *a*. [d] Vydac phenyl (250 × 4.6 mm) column, 65−95% B in A gradient over 27 min. [e] Vydac phenyl (250 × 4.6 mm) column, 80−95% B in A gradient over 27 min.

Table 2. Analysis of Crude Base Peptides from Each Fully Protected Segment Prior to Purification[a]

segment	base peptide	HPLC R_t (min) (main peak %)	MALDITOF-MS obsd (expected)
1	*H*-Gly^{242}-Leu^{251}-*OH*	9.46 (97.8%)	1166Da (1167Da)
2	*H*-Ser^{231}-Gly^{241}-*OH*	11.07 (96.3%)	1108Da (1108Da)
3	*H*-Gly^{210}-Gly^{230}-*OH*	11.27 (89.5%)	2391Da (2391Da)
4	*H*-Ser^{199}-Gly^{209}-*OH*	8.67 (69.7%)	1117Da (1116Da)
5	*H*-Val^{188}-Gly^{198}-*OH*, $AcmCys^{197}$	10.04 (82.2%)	1207Da (1207Da)
6	*H*-Gly^{179}-Pro^{187}-*OH*, $AcmCys^{197}$	10.83 (86.3%)	1031Da (1031Da)
7	*H*-Ser^{168}-Gly^{178}-*OH*, $AcmCys^{197}$	3.73 (85.4%)	1167Da (1166Da)
8	*H*-Asp^{158}-Gly^{167}-*OH*, $AcmCys^{197}$	9.95 (89.1%)	1101Da (1101Da)
9 (from 5 + 6)	*H*-Gly^{179}-Gly^{198}-*OH*, $AcmCys^{197}$	12.17 (46.2%)	2219Da (2221Da)
10 (from 7 + 8)	*H*-Asp^{158}-Gly^{178}-*OH*, $AcmCys^{197}$	10.48 (51.3%)	2249Da (2249Da)

[a] Conditions: Vydac C_8 (250 × 4.6 mm) column, 10−90% B in A gradient over 27 min (1.5 mL/min flow, 215 nm UV detection), where buffer A = 0.1% aqueous TFA and buffer B = 90% acetonitrile/10% buffer A. HPLC integrations refer to the percent major peak, confirmed by MALDITOF-MS. Mass analysis of impurities identified the presence of deletion sequences in segments 3−10, defining the purification method required for the corresponding fully protected segment.

Table 3. Data for the Analysis of Intermediates during the Sequential Assembly of Purified Fully Protected Segments 2−10[d]

coupling reaction: protected segment + resin-bound assembly	coupling efficiency individual (cumulative)	intermediate crude peptide for analysis	HPLC R_t (min) (main peak %)	MALDITOF-MS obsd (expected)
2 + Seg1-R	98% (98%)	Fmoc-Ser^{231}-Leu^{251}-OH, $AcHmb^{234,245}$	17.70 (98.1%)[a]	2837Da (2836Da)
3 + Seg2−1-R	101% (98%)	H-Gly^{210}-Leu^{251}-OH	12.66 (84.7%)[a]	4632Da (4631Da)
4 + Seg3−2−1-R	94.5% (92.5%)	Fmoc-Ser^{199}-Leu^{251}-OH, $AcHmb^{210,216,221,224,234,245}$	16.69 (88.7%)[a]	7020Da (7020Da)
9 + Seg4−3−2−1-R	86.5% (80%)	H-Gly^{179}-Leu^{251}-OH, $AcmCys^{197}$	21.68 (77.3%)[b]	7932Da (7932Da)
10 + Seg9−4−3−2−1-R	97% (77.5%)	H-Asp^{158}-Leu^{251}-OH, $AcmCys^{197}$	11.12 (64.7%)[c]	10164Da (10164Da)

[a] HPLC conditions: Vydac C_8 (250 × 4.6 mm) column, with 10−90% B in A gradient over 27 min (1.5 mL/min flow, 215 nm UV detection), where buffer A = 0.1% aqueous TFA and buffer B = 90% acetonitrile/10% buffer A. [b] 10−40% B in A gradient over 27 min. [c] 25−35% B in A gradient over 27 min. N-Terminal serine intermediates were cleaved Fmoc protected due to the possibility of Nα-trifluoro-acetylation[26] complicating HPLC analysis. [d] Coupling efficiencies were calculated by UV quantification of Fmoc release after coupling. After each coupling peptide intermediates were cleaved and characterized.

crude base peptide (see Table 2). The purification of AcHmb backbone substituted fully protected segments was then achieved by one (or a combination) of three simple purification protocols (see Experimental Section for details). If the impurities in the crude segment were derived from the loss of a side-chain protecting group, the only one showing any observable loss being the N^{τ}-trityl group on histidine, Lobar silica gel purification was sufficient. If the base peptide derived from the protected segment exhibited significant amino acid deletions (identified by mass analysis), then the protected segment required preparative HPLC purification. Vydac C_4 purification was suitable for short segments (around 10 residues), with longer segments (up to 21 residues) requiring the less polar Vydac diphenyl medium. Both preparative procedures were performed using aqueous acetonitrile eluents containing 0.1% TFA. These simple and convenient purification techniques, using standard HPLC conditions, provided protected segments (all base peptides >96% by HPLC analysis) rapidly in 100s of mg (16.5−48.4% overall isolated yields). This offers a substantial improvement compared to the sometimes complex methods used for the isolation of nonbackbone protected segments effectively removing the constraints previously encountered with segment purification.

(v) Assembly of Segments. (i) Initial Studies Defining Optimized Procedures. Our early investigations into the preparation and coupling of backbone protected segments[12] focused upon the use of Hmb rather than AcHmb protected segments. However, here a thorough investigation involving

Table 4. Amino Acid Analysis Data (Hydrolysis in Vacuo at 110 °C for 24 h in 6 N HCl) for All Crude Resin-Bound Assemblies

peptide-resin	Asx	Thr	Ser	Glx	Pro	Gly	Cys	Val	Ile	Leu	Nle	Tyr	Phe	β-Ala	His	Lys	Arg
H-Seg1-Resin1[a]	1.04	0.87		1.02		1.01			0.90	1.00	1.17			1.07	0.95	2.91	
	(1)	(1)		(1)		(1)			(1)	(1)	(1)			(1)	(1)	(3)	
H-Seg2-esin1	1.89	0.90	0.76		1.08	1.94		0.98	0.92	1.00	1.23				0.92		
	(2)	(1)	(1)		(1)	(2)		(1)	(1)	(1)	(1)				(1)		
H-Seg3-Resin1	2.04		1.64	4.16		1.90		2.78	0.94	1.00	1.22		0.95			3.78	1.04
	(2)		(2)	(4)		(2)		(3)	(1)	(1)	(1)		(1)			(4)	(1)
H-Seg4-Resin1	0.98		0.83		0.95	3.14			0.82	0.98	1.15				1.47	1.00	
	(1)		(1)		(1)	(3)			(1)	(1)	(1)				(2)	(1)	
H-Seg5-Resin1	0.93	0.90	1.69			1.03	n.d	1.75		0.91	1.16					2.00	
	(1)	(1)	(2)			(1)		(2)		(1)	(1)					(2)	
H-Seg6-Resin1				1.05	1.06	1.00		1.59	0.66		1.15	1.00				1.87	
				(1)	(1)	(1)		(2)	(1)		(1)	(1)				(2)	
H-Seg7-Resin1	1.20	0.98	0.95	2.16	1.07	2.13				1.00	1.14				0.91	1.00	
	(1)	(1)	(1)	(2)	(1)	(2)				(1)	(1)				(1)	(1)	
H-Seg8-Resin1	1.88		0.87			1.00		0.88	0.95	0.94	1.02					2.77	
	(2)		(1)			(1)		(1)	(1)	(1)	(1)					(3)	
H-Seg9-Resin1	1.00	0.97	1.78	0.74	2.71	1.81	n.d	3.14	0.51	1.00	1.23	0.71				3.40	
	(1)	(1)	(2)	(1)	(1)[c]	(2)		(4)	(1)	(1)	(1)	(1)				(4)	
H-Seg10-Resin1	2.25	0.97	1.48	2.40	1.16	3.00		0.60	0.64	1.50	1.83				0.53	3.16	
	(3)	(1)	(2)	(2)	(1)	(3)		(1)	(1)	(2)	(1)				(1)	(4)	
H-Seg1-Resin2[b]	1.02	0.95		1.09		1.01			0.95	1.01	1.66			1.08	1.00	2.88	
	(1)	(1)		(1)		(1)			(1)	(1)	(1)			(1)	(1)	(3)	
H-Seg2-1-Resin2	2.76	1.72	0.72	1.05	0.96	2.75		0.85	1.81	1.96	1.74			1.06	1.84	3.00	
	(3)	(2)	(1)	(1)	(1)	(3)		(1)	(2)	(2)	(1)			(1)	(2)	(3)	
H-Seg3-2-1-Resin2	5.63	2.13	2.58	5.76	0.98	6.06		4.03	3.22	3.51	2.14		1.00	1.16	2.24	8.00	1.08
	(5)	(2)	(3)	(5)	(1)	(5)		(4)	(3)	(3)	(1)		(1)	(1)	(2)	(7)	(1)
H-Seg4-3-2-1-Resin2	6.73	2.25	2.96	6.05	1.88	8.88		4.08	4.33	4.73	2.24		1.00	1.19	4.42	9.26	1.00
	(6)	(2)	(4)	(5)	(2)	(8)		(4)	(4)	(4)	(1)		(1)	(1)	(4)	(8)	(1)
H-Seg9-4-3-2-1-Resin2	8.58	3.45	5.17	7.92	4.88	11.69	n.d	7.96	5.57	6.07	2.15	0.86	1.00	1.09	4.39	13.21	1.13
	(7)	(3)	(6)	(6)	(3)[c]	(10)		(8)	(5)	(5)	(1)	(1)	(1)	(1)	(4)	(12)	(1)
H-Seg10-9-4-3-2-1-Resin2	10.20	3.99	6.53	8.85	5.77	12.67	n.d	7.71	5.57	7.00	2.51	0.82	0.99	1.10	5.06	15.23	1.05
	(10)	(4)	(8)	(8)	(4)[c]	(13)		(9)	(6)	(7)	(1)	(1)	(1)	(1)	(5)	(16)	(1)

[a] Resin1 refers to 2-ClTrt-Nle-EDA-SU 500 (Scheme 2). [b] Resin2 refers to Nle-Pepsyn KD. [c] The AAA values for proline are high due to the presence of Cys(Acm) which hydrolyses to thioproline and co-elutes with proline upon analysis.

small scale couplings of segments 1 and 2[27] indicated that slightly improved crude products were obtained whilst coupling with AcHmb protected segments.

Segment 1 coupled with BOP mediated activation to H.Nle-NovaSyn KD, gave the base peptide H-Gly^{242}-Leu^{251}-OH (ex AcHmb), 97.8% by analytical HPLC with MALDITOF-MS at 1166Da (theoretical mass = 1167Da). A repeat of this reaction using a modified segment 1 containing Hmb backbone protection coupled with virtually identical incorporation compared to above. However, the base peptide from this assembly H-Gly^{242}-Leu^{251}-OH (ex Hmb) was only 80.5% by analytical HPLC. The crude peptide contained two main later eluting impurities, which gave a characteristic UV profile for "Hmb" containing species upon diode array detection, with masses of (M + 209) and (M + 220), respectively. These species were not identified but appear to contain a modified and nonTFA labile Hmb group.

Since protection of the Hmb 2-hydroxyl functionality of the incoming segment by acetylation gave a superior product, we required a method able to selectively remove Fmoc from a coupled segment, whilst retaining on-resin Hmb 2-hydroxyl acetylation. Our standard method for Fmoc removal, 20% piperidine in DMF, also gives quantitative de-O-acetylation of the *N*-(2-acetoxy-4-methoxybenzyl) group[7] and thus cannot be applied for selective Fmoc removal. The de-O-acetylation reaction is presumably a nucleophilic cleavage, thus the use of a non-nucleophilic base, still able to perform Fmoc cleavage, should allow for selective reaction. Treatment of assembly 8 with 1% 1,8-diazabicyclo[5.4.0]undec-7- ene(DBU)/DMF[28] gave upon TFA cleavage H-Gly^{242}-Leu^{251}(AcHmb(Lys^{245}))-OH, 94.4% by analytical HPLC with MALDITOF-MS at 1344Da (theoretical mass = 1343Da). The fully deprotected peptide H-Gly^{242}-Leu^{251}-OH was present at 2.1% indicating that >95% of the de-Fmoc chains still contained acetylated Hmb 2-hydroxyl. This is an approximate figure based upon HPLC integration, not allowing for the increased chromophore present in H-Gly^{242}-Leu^{251}(AcHmb(Lys^{245}))-OH.

Trial coupling reactions involving protected segment 2 and assembly 8 (Scheme 3) initially treated with DBU, gave a significant amount of Nα-acetylated segment 1 (MALDITOF-MS at 1209Da) upon cleavage and analysis. Clearly, the *O*-acetyl group from AcHmb was able to migrate to the Nα-terminus, effectively capping segment 1 during the coupling reaction and eliminating the use of on-resin AcHmb protection during coupling as a viable method.

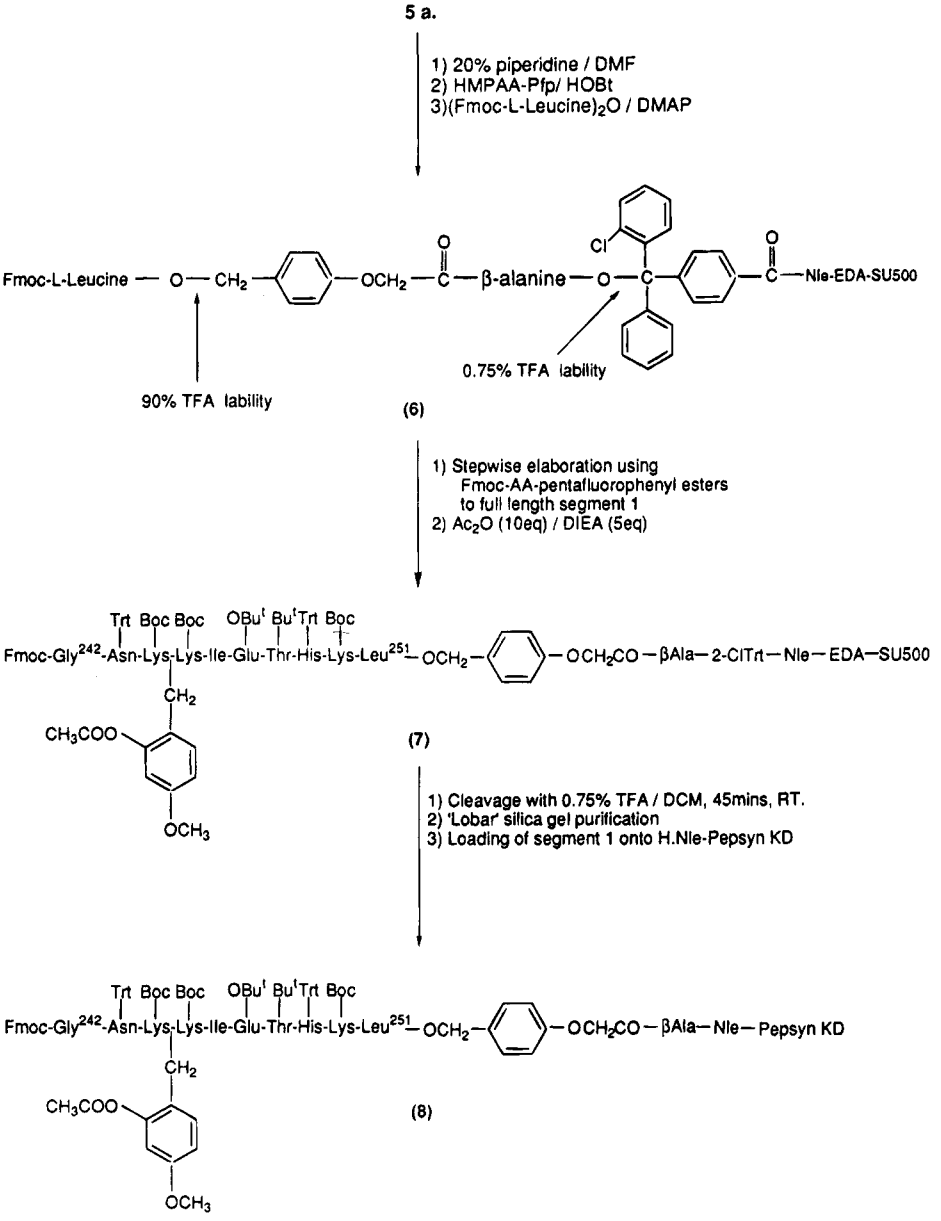
(ii) Optimized General Coupling of AcHmb Backbone Protected Segments. Purified fully protected segments 2−10 were sequentially assembled from peptide-resin 8 (initially treated with 20% piperidine/DMF), using the appropriate AcHmb fully protected segment (2 equiv), BOP (2 equiv) and DIEA (2 equiv) in a minimum volume of DMF. Six hours reaction, gave good incorporation of each incoming segment, and the coupled peptide-resin was capped (with simultaneous Hmb 2-hydroxyl acetylation) with Ac_2O/DIEA after each coupling cycle.

Samples of the crude assembled peptides were cleaved and analyzed after each segment addition and are summarized in Table 3.

Commencing from assembly 8, the individual coupling of segments 2 (11-mer), 3 (21-mer), 4 (11-mer), 9 (20-mer), and 10 (21-mer) were remarkably efficient ranging from 86.5% (segment 9) to mainly >95% incorporation. The addition of fully protected segment 3 using the standard 6 h/2 equiv reaction

(26) Hubener, G.; Gohring, W.; Musiol, H.-J.; Moroder, L. *Pept. Res.* **1992**, *5(5)*, 287−292.
(27) Unpublished results.
(28) Wade, J. D.; Bedford, J.; Sheppard, R. C.; Tregear, G. W. *Pept. Res.* **1991**, *4*, 194−195.

Scheme 3. Basic Peptide-Resin Structure from Which Segment 1 Was Initially Prepared, Incorporating the Double Handle Strategy[a]



[a] Prior cleavage of a C-terminal elongated segment 1, through the hyperacid labile 2-chlorotrityl handle, purification, and reattachment through achiral β-alanine gives 8, the base assembly for the addition of the remaining segments. The chiral integrity of the C-terminal Leu^{251} is protected through this scheme.

was shown to be virtually quantitative by UV estimation. A sample of the fully deprotected crude peptide, H-Gly^{210}-Leu^{251}-OH, was of an excellent quality by HPLC analysis (Figure 1), with a major species (84.7%) giving MALDI-TOF-MS at 4632Da (theoretical mass = 4631Da). The on-resin amino acid analysis indicated a less efficient coupling (at approximately 85%), having a Phe:Leu ratio of 1:3.51, instead of the expected ratio of 1:3.00 (see Table 4). These ratios seem somewhat misleading, since the crude peptide (Figure 1) was obviously of a better quality than suggested by amino acid analysis, appearing to more closely reflect the quantitative UV estimation of coupling efficiency. The method of quantitative UV measurement of Fmoc release from a coupled peptide-resin, taking into account the gain in peptide-resin weight during coupling, probably gives a better reflection of the course of a segment coupling. This excellent result for the coupling of a 21 residue segment is no doubt derived from a combination of high segment solubility (and hence concentration) and superior solvation of the growing backbone protected peptide-resin matrix.[6b,12] The solid-phase addition of such large segments has to our knowledge only been described by one group,[29] whose optimized procedures gave 45% addition of a 17-mer to a 14-mer-resin with 72 h reaction. It appears that our new procedures have overcome the restrictive hurdle concerning the use of short (commonly up to 10 residue) segments during solid phase segment condensations.[5b,8b]

Since the addition of the 21-mer segment 3 had occurred so readily, we decided that it would be prudent to initially perform the expected slow coupling of Pro^{187} (C-terminal of 6) onto Val^{188} (N-terminal of 5) giving segment 9, followed by purification and subsequent incorporation of the 20-mer product. Unfortunately, the initial preparation of 9 was performed by coupling 1 equiv of crude segment 6 with resin-bound segment 5 that had undergone Fmoc removal by DBU treatment. The

(29) Ball, H. L.; Mascagni, P. In *Proceedings of thse Second Japanese Symposium on Peptide Chemistry;* Yanaihara, N., Ed.; ESCOM: Leiden, In press. In contrast, large segments have been assembled in solution, e.g., see: ref 8d.

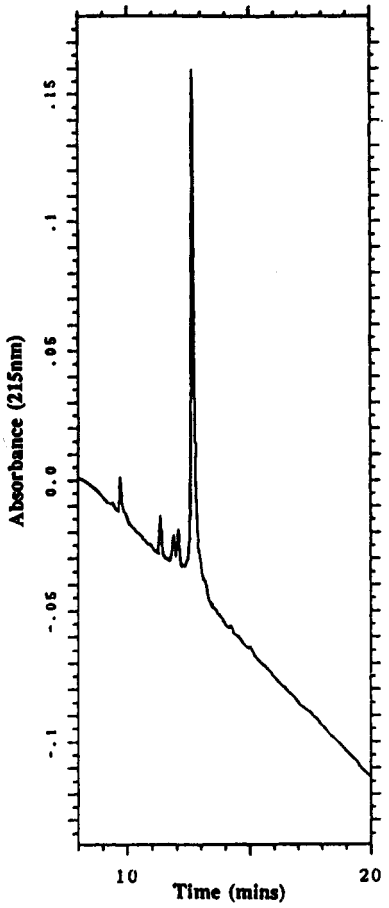


Figure 1. Analytical HPLC of the crude 42-mer H-Gly^{210}-Leu^{251}-OH from the coupling the 21-mer segment 3 onto the resin-bound 21-mer H-Segment-2-1-Resin. Coupling conditions were 2 equiv of AcHmb backbone protected segment 3 with BOP activation, for 6 h in a minimum of DMF. Conditions: Vydac C_8 (250 × 4.6 mm) column, 10−90% B in A gradient over 27 min (1.5 mL/min flow, 215 nm UV detection), where buffer A = 0.1% aqueous TFA and buffer B = 90% acetonitrile/10% buffer A.

reaction was therefore prone to the earlier identified side-reaction of on-resin AcHmb *O*-acetyl to *N*α-acetyl transfer, and a significant proportion of segment 5 was prematurely capped. The coupled and ninhydrin negative peptide-resin gave only 70% incorporation of segment 6; however, crude segment 9 was readily purified and isolated with 20.6% yield. The incorporation of purified segment 9 under standard conditions gave the lowest individual incorporation at 86.5% by quantitative UV. Leaving the reaction longer than 6 h would undoubtedly have improved this coupling yield. However, since there is the possibility of modification of the free Hmb 2-hydroxyls upon longer coupling, the reaction was terminated after 6 h. The on-resin amino-acid analysis (Table 4) proved interesting since segment 9 contains the only tyrosine residue present in the target sequence, and the already incorporated segment 3 contains the only phenylalanine residue. Reassuringly, the Tyr:Phe ratio found was 0.86:1.00, correlating very well with the UV measurement of 86.5% incorporation of segment 9. The addition of the final 21 residues was performed through the coupling of segment 10, which was prepared by a prior coupling, and purification, of segment 8 with resin-bound segment 7.

The full length fully protected peptide-resin (365 mg) was initially treated with 20% piperidine/DMF followed by cleavage (97% by post-cleaved resin AAA) with a TFA/EDT/TES/water cocktail. Crude H-Asp^{158}-Leu^{251}, AcmCys^{197}-OH (127 mg) was readily soluble in 0.1% aqueous TFA, with analytical HPLC analysis (Figure 2a), containing a major species, with an earlier

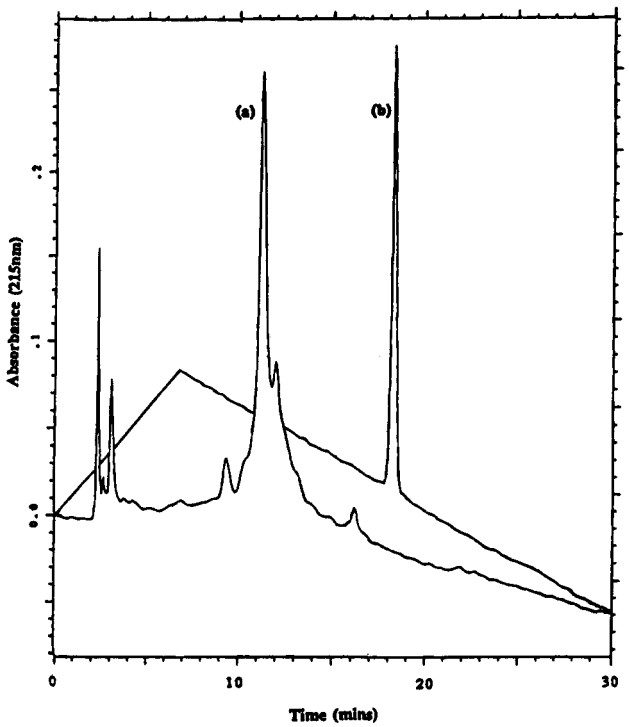


Figure 2. Analytical HPLC of (a) crude and (b) purified H-Asp^{158}-Leu^{251}, AcmCys^{197}-OH. Conditions: Vydac C_8 (250 × 4.6 mm) column, 25−35% B in A gradient over 27 min (1.5 mL/min flow, 215 nm UV detection), where buffer A = 0.1% aqueous TFA and buffer B = 90% acetonitrile/10% buffer A.

and later eluting shoulder. The crude peptide was purified by C_8 preparative HPLC giving H-Asp^{158}-Leu^{251}, AcmCys^{197}-OH.18TFA (30.5 mg, 2.50 μmol, 16.7%). The final yield is based upon an initial 15 μmol assembly, making no allowances for removal of quantities of peptide-resin assembly for analysis after each segment addition, and is thus a conservative value. The purified peptide was analyzed by analytical HPLC giving a single species (>99%) (Figure 2b), with MALDITOF-MS at 10164.4 ± 2.2Da (average of 15 determinations) (theoretical mass = 10164.7Da) (Figure 3). The final product gave good amino acid ratios, consistent with the 94-mer product.

A sample of the full length Fmoc protected peptide-resin was also cleaved, giving crude Fmoc-(Asp^{158}-Leu^{251}, AcmCys^{197}, AcHmb(Ser^{164,} Asn^{171}, Val^{181}, Lys^{192}, Gly^{201}, Ser^{216}, Phe^{221}, Arg^{224}, Asn^{234}, Lys^{245})-OH. This crude partially protected peptide was analyzed by analytical HPLC showing a main species (>75%) (Figure 4a) with MALDITOF-MS at 12172Da (theoretical mass = 12169Da) (Figure 5). The crude HPLC (Figure 4a) shows the later eluting Fmoc-protected product and earlier eluting non-Fmoc species, probably corresponding to the various capped sequences accumulated during the synthesis. These were readily separated, and the crude partially protected 94-mer was purified using C_8 preparative HPLC. Reanalysis by HPLC gave a single species (>99%) (Figure 4b). Backbone protected intermediates are readily soluble in aqueous media, and we have previously described the use of such partially protected peptides[6g,7] in a purification strategy for the isolation of low solubility peptides. The strategy provides the target sequence via an initial purification of highly soluble backbone protected intermediates, before the removal of backbone protection as a final step. This potentially powerful scheme is not required here since the crude deprotected peptide H-Asp^{158}-Leu^{251}, AcmCys^{197}-OH was itself soluble in aqueous media and isolatable by standard preparative HPLC. However, the strategy may well be useful for the isolation of other less soluble targets

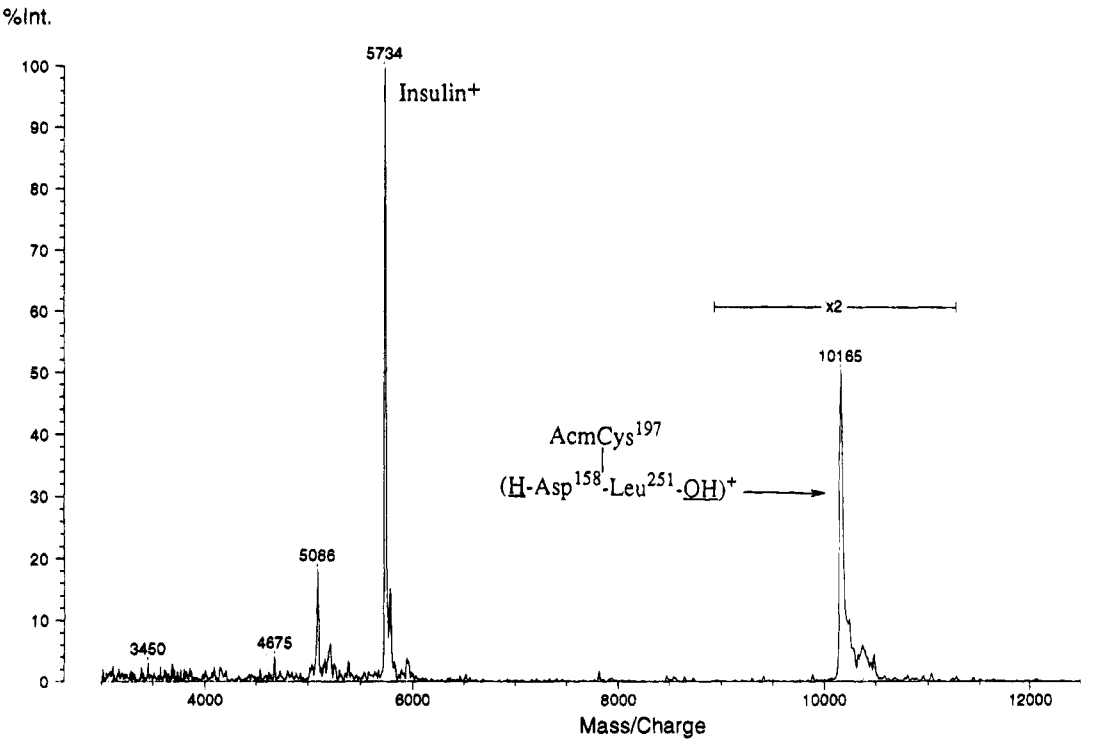


Figure 3. MALDITOF-MS spectrum (with insulin internal calibration) of purified H-Asp[158]-Leu[251], AcmCys[197]-OH (required mass = 10164.7Da).

and is an optional feature inherent in our overall backbone protection scheme.

Summary

We have described here a solid phase assembly strategy in which fairly large (up to 21 residues) fully protected segments are readily purified then efficiently coupled to a growing resin-bound peptide. The coupling efficiency remained high as the length of resin-bound peptide increased and continued to show excellent incorporation for the addition of a 21-mer to a 73-mer-resin, at 97%. A major factor in such efficiencies is the full solvation of the growing peptide chain,[6b,12,13] which is ensured by the incorporation of appropriately placed backbone protection[6b] with the addition of each new segment. Limitations in terms of the size of peptide-resin using this strategy are as yet unknown, and certainly in this case there was no indication that the addition of further segments would be immediately problematic. A solution to the remaining major problem in segment assembly, the C-terminal epimerization of activated segments, is currently being investigated by a number of groups.[30] If this can be resolved, along with our solution to the solubility problem,[5,8] then the true potential of solid phase segment assembly may be realized. Our improved segment assembly methods, in conjunction with the recently described chemical strategies for peptide ligation,[31,32] provide a major tool for the synthetic peptide chemist to actively contribute to the exciting field of protein molecular biology.

Experimental Section

Equipment, Materials, and Methods. Continuous flow Fmoc-polyamide methods reviewed by Atherton and Sheppard[1] were used exclusively. Fmoc amino acid pentafluorophenyl activated esters (Novabiochem, Beeston, Nottingham, UK, NG9 2JR) were used exclusively except for Ser(OBu[t]) and Thr(OBu[t]) which were coupled as the dihydrooxobenzotriazine esters (Novabiochem, UK). Also, Fmoc-His(Trt)-OH was coupled using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation in the presence of HOBt and DIEA. Fmoc *N*-carboxyanhydrides (Propeptide, Vert Le Petit, France) were commercially available. *N,O*-bisFmoc-*N*-(2-hydroxy-4-methoxybenzyl) amino acid pentafluorophenyl esters[14] were prepared as previously detailed. Polyhipe SU 500 resin was purchased from Phase Separations Ltd, Deeside, UK, CH2 2NU. NovaSyn KD resin was purchased from Novabiochem, UK. All solvents were purchased and purified as previously detailed.[1]

Solid phase peptide synthesis was performed on an LKB "Biolynx" 4170 automated synthesizer programmed to perform acylation reactions (in DMF) for 45 min unless otherwise stated and Fmoc deprotection reactions (in 20% piperidine/DMF v/v) for 10 min. Amino acid side-chain protection was as follows: lysine (N[ε]-Boc), serine and threonine (*tert*-butyl ether, Bu[t]), aspartic and glutamic acid (*tert*-butyl ester, OBu[t]), glutamine and asparagine (Trt), histidine (N[τ]-Trt), tyrosine(*tert*-butyl ether, Bu[t]), arginine (Pmc), and cysteine (Acm). 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 obtained using ion-exchange resin with manufacturers buffer solutions and post-column detection by ninhydrin. Analytical HPLC (600E Programmable Solvent Delivery System, 994 diode array detector, Waters Ltd, Watford, Herts, England), monitored at 215nm, was performed using (a)Vydac 208TP54, C8 column (250 × 4.6 mm), (b)Vydac 214TP54, C4 (butyl) (250 × 4.6 mm) or (c) Vydac 219TP54, phenyl (250 × 4.6 mm). A 10−90% B in A gradient over 25 min at (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 using (a) Vydac 214TP1022, protein C4 column (22 × 250 mm) or (b)Vydac 219TP1022, diphenyl column at 10 mL/min flow rate and 215 nm UV detection, solvents A

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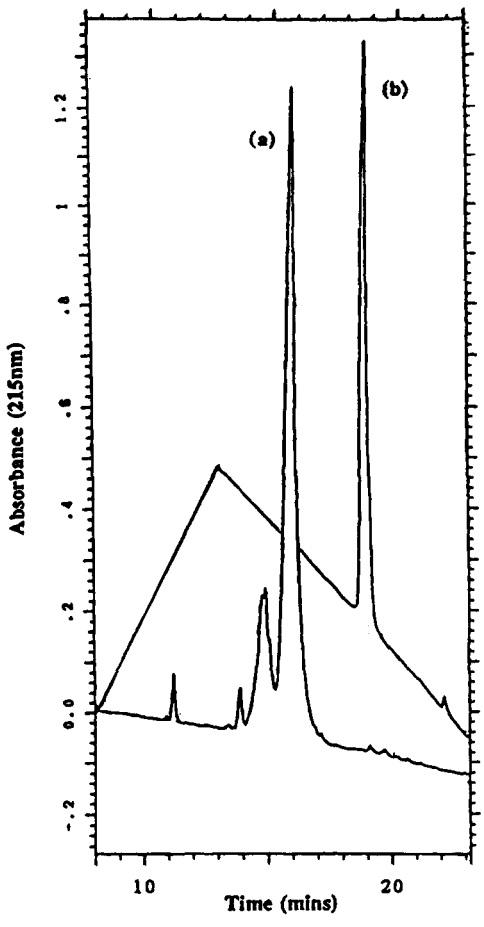

Figure 4. Analytical HPLC of (a) crude and (b) purified Fmoc-Asp[158]-Leu[251], AcmCys[197], AcHmb(Ser[164], Asn[171], Val[181], Lys[192], Gly[210], Ser[216], Phe[221], Arg[224], Asn[234], Lys[245])-OH. Conditions: Vydac C_8 (250 × 4.6 mm) column, 10−90% B in A gradient over 27 min (1.5 mL/min flow, 215 nm UV detection), where buffer A = 0.1% aqueous TFA and buffer B = 90% acetonitrile/10% buffer A.

and B as above. All HPLC columns were purchased from HiChrom Ltd., Reading, Berkshire, England.

MALDITOF-MS were obtained on a Kratos MALDI III bench-top linear/reflectron mass spectrometer (Kratos UK Ltd., Macclesfield, Cheshire, England), using α-cyano-4-hydroxycinnamic acid or sinapinic acid matricies. In general, the sinapinic acid matrix gave superior results for the larger peptides (>6000 Da). Matrix was dissolved in acetone containing 2% (v/v) 0.1% aqueous trifluoroacetic acid, to give a saturated solution. Matrix (0.15−0.2 mL) was applied to a sample slide and allowed to evaporate. The peptide sample was applied to successive matrix spots in a series of dilutions to find the quantity giving the best signal. Calibration by insulin and/or myoglobin was external or internal depending upon the accuracy required. Most analyses were performed with the instrument in the linear mode; when extra accuracy was required, the reflectron option was used. All masses were determined using positive mode ionization; as required, the negative mode was used to confirm the mass observed.

FAB MS were obtained on an MS80 RF double focusing spectrometer, with the FAB gun operating at 6 kV krypton atoms and 20 mA discharge current. Samples were dissolved in dimethyl sulfoxide. Melting points are uncorrected. Thin layer chromatography samples were run on precoated aluminium sheets of silica gel 60 F_{254} (Merck). All organic materials were purchased from Aldrich, Poole, Dorset, England, and used as supplied.

(1) Preparation of Handle Building Blocks (4a−c). (i) 2-Chloro-4′-carboxytriphenylmethanol. *p*-Bromobenzoic acid (10.5 g, 50 mmol) was dissolved in dry tetrahydrofuran (400 mL)/hexane (100 mL), cooled to −78 °C, and flushed with nitrogen. *n*-Butyllithium (10 M solution in hexane, 11 mL, 110 mmol) was added, and the mixture was stirred at −78 °C for 45 min. 2-Chlorobenzophenone (54.1 g, 250 mmol) in dry THF (150 mL) was added over 10 min, and the mixture allowed to warm to room temperature (approximately 1 h). The mixture was poured onto 0.1 M HCl (500 mL) with stirring, and the organic layer was extracted with diethyl ether (4 × 250 mL). The combined organics were extracted with 1 N NaOH (2 × 500 mL), the organic layers were discarded, and the basic aqueous layer was back washed with diethyl ether (2 × 500 mL). The basic aqueous layer was acidified with 5 N HCl to pH 2 under vigorous stirring and then extracted with diethyl ether (4 × 250 mL). The combined etherals were dried (Na_2SO_4) and removed in vacuo, giving a crude white solid (12.7 g).

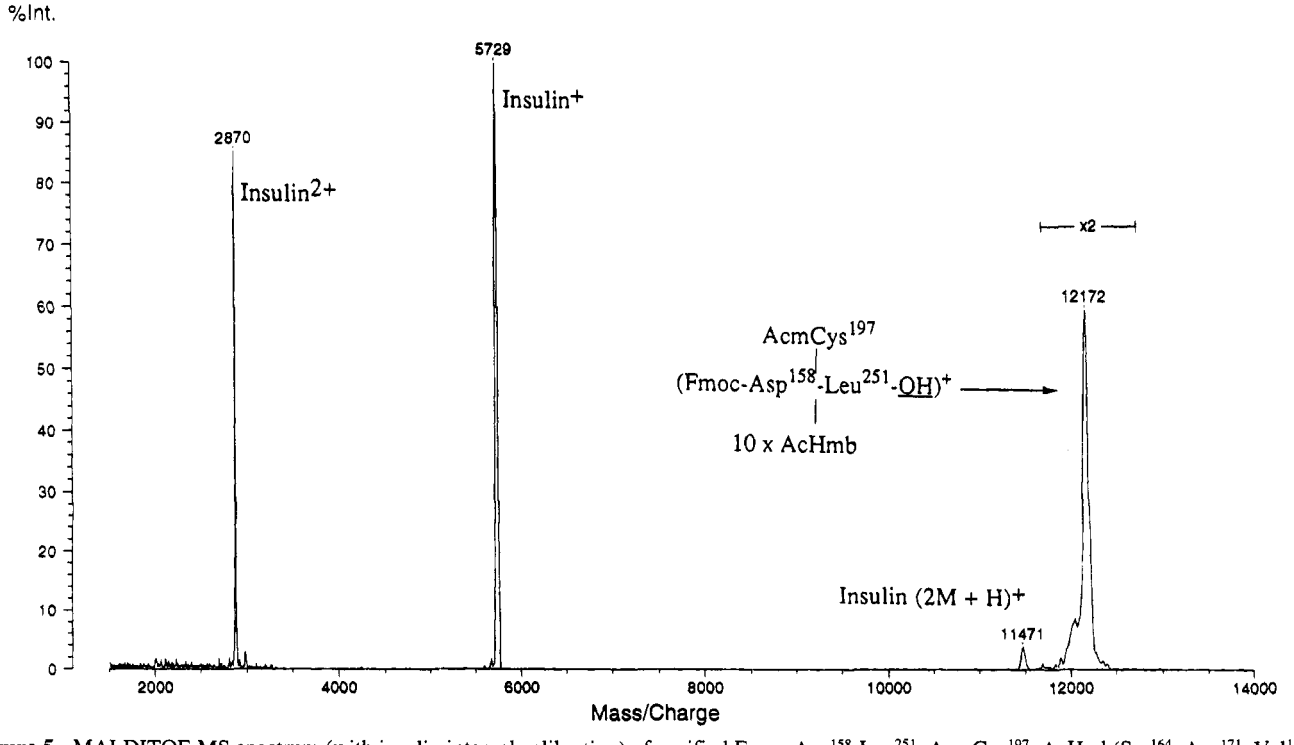

Figure 5. MALDITOF-MS spectrum (with insulin internal calibration) of purified Fmoc-Asp[158]-Leu[251], AcmCys[197], AcHmb(Ser[164], Asn[171], Val[181], Lys[192], Gly[210], Ser[216], Phe[221], Arg[224], Asn[234], Lys[245])-OH (required mass = 12169Da).

Crude solid was ground to a fine powder, extracted with boiling water (5 × 100 mL), and then dried in vacuo giving a fine white solid: yield 8.02 g (47.6%); mp 154−156 °C; $C_{20}H_{15}ClO_3$ = 338.82, req-(fnd) %C 70.89 (70.71), %H 4.46 (4.68), N 10.48 (10.40); FAB MS, obs 339.0 $[M + H]^+$ (45%), 321.0 [M − 18] (100%); analytical HPLC, system (a), R_t = 20.39 min (96.3%).

(ii) 2-Chloro-4′-carboxytriphenylmethanol Pentafluorophenyl Ester (3). 2-Chloro-4′-carboxytriphenylmethanol (7.0 g, 20.75 mmol) was dissolved in dry tetrahydrofuran (50 mL), cooled in an ice-water bath, and stirred. Pentafluorophenol (4.184 g, 22.8 mmol, 1.1 equiv) in dry THF (15 mL) was added followed by dicyclohexylcarbodiimide (4.684 g, 22.8 mmol, 1.1 equiv) in dry THF (10 mL). The mixture was stirred for 3 h and then analyzed by TLC (75% hexane/25% chloroform) showing (254 nm UV detection): R_f 0.2 (new main spot), R_f 0.0 (starting acid virtually absent), R_f 0.75 (faint).

After 3 h reaction the mixture was filtered and washed with THF (3 × 10 mL), and the combined filtrate was removed in vacuo. The residue was flashed twice from hexane giving a viscous precipitous oil (12.0 g). The oil was dissolved in 10% chloroform/90% hexane (20 mL) and loaded onto silica gel (Kiesel gel 60 (Merck), 250 g) prepacked in 10% chloroform/90% hexane. The column was eluted with the above solvent (1.5 L), giving the compound with R_f 0.75, which was discarded. The solvent was gradually changed (over 1 L) to chloroform, and fractions corresponding to the product (R_f = 0.2 in 25% hexane/75% chloroform) were combined and reduced in vacuo. The residue was recrystallized from hexane as a fine white solid: yield 8.4 g (80.3%); mp 52−54 °C (shrinks), 58−61 °C (glasses); $C_{26}H_{14}ClF_5O_3$ = 504.9, req(fnd) %C 61.85 (61.90), %H 2.79 (2.91). FAB MS, obs 505.1 $[M + H]^+$ (15%), 487.0 [M − 18] (100%); analytical HPLC, system (a), R_t 28.02 min (98.4%).

(iii) Nα-Fluoren-9-ylmethoxycarbonyl-β-alanine-(2-chloro-4′-(carboxypentafluorophenoxy)triphenyl) Methyl Ester (4a). 2-Chloro-4′-carboxytriphenylmethanol pentafluorophenyl ester (3) (345 mg, 0.68 mmol) was dissolved in dry DCM (10 mL), neat acetyl bromide (2 mL) was added, and the mixture was left at room temperature for 75 min. The mixture was evaporated in vacuo, and the resultant oil was evaporated six times from hexane giving a white moose. TLC in 75% hexane/25% chloroform gave a major new UV positive spot with R_f = 0.05.

The crude bromide was redissolved in dry DCM (5 mL), and Fmoc-β-alanine (212 mg, 0.68 mmol) was previously pre-equilibrated with diisopropylethylamine (264 mg, 3 equiv, 2.04 mmol) in DCM (5 mL) for 5 min added. The mixture was left at room temperature for 75 min, then diluted with DCM (35 mL), and washed with saturated $NaHCO_3$ (2 × 50 mL). The dried (Na_2SO_4) organic layer was evaporated in vacuo, then evaporated three times from ether and dried in vacuo giving a white moose: yield 550 mg (101%); analytical HPLC, system (a), R_t 30.48 min (91.1%), R_t 17.94 min (2.1%) Fmoc-β-alanine, R_t = 28.05 min (4.5%) (ester (3)). mp 61−64 °C (shrinks), 68−70 °C (glasses); $C_{44}H_{29}ClF_5NO_6$ = 798.2, req(fnd) %C 66.21 (66.39), %H 3.66 (3.86), %N 1.75 (1.71); FAB MS, obs 820.0 $[M + Na]^+$ (30%), 799.1 $[M + H]^+$ (20%).

An attempt to further purify **4a** by silica gel chromatography using a hexane/chloroform mixture led to degradation of the crude product.

(iv) Nα-Fluoren-9-ylmethoxycarbonylglycine-(2-chloro-4′-(carboxypentafluorophenoxy)triphenyl) Methyl Ester (4b). Prepared as for **4a** using Fmoc-glycine (998 mg, 3.36 mmol), giving a white moose: yield 2.70 g (102%); analytical HPLC, system (a), R_t = 30.08 min (93.1%), R_t = 17.65 min (1.1%) Fmoc-glycine, R_t = 28.01 min (2.6%) (ester (**3**)); mp 82−84 °C (shrinks), 87−89 °C (glasses); $C_{43}H_{27}ClF_5NO_6$ = 784.2, req(fnd) %C 65.86 (65.46), %H 3.47 (3.67), %N 1.79 (1.96); FAB MS, obs 784.0 $[M + H]^+$ (25%).

(v) Nα-Fluoren-9-ylmethoxycarbonylproline-(2-chloro-4′-(carboxypentafluorophenoxy)triphenyl) Methyl Ester (4c). Prepared as for **4a** using Fmoc-proline (270 mg, 0.8 mmol), giving a white moose: yield 680 mg (103.0%); analytical HPLC, system (a), R_t = 31.94 min (92.5%), R_t = 19.16 min (2.1%) Fmoc-proline, R_t = 28.22 min (1.9%) (ester (**3**)); $C_{46}H_{31}ClF_5NO_6$ = 824.2, FAB MS, obs 825.7 $[M + H]^+$ (55%).

(2) Derivitization and Initial Loading of Resins. (i) General Method for Quantitative UV Measurement. Approximately 2−5 mg of the peptide-resin was accurately weighed out and treated with 20% piperidine/DMF (3.5 mL) for 3 min. The solution was then measured for UV absorbance at 290 nm, (a factor for the gain in peptide-resin weight from segment coupling calculated if required) and compared against a standard measurement obtained by an identical treatment of Fmoc-glycine-pepsyn KA (0.10 mmol g^{-1}, 5.0 mg).

(ii) Initial Derivitization of Polyhipe SU 500. Polyhipe SU 500[22] (10 g, approximately 0.5 mmol g^{-1} sarcosine methyl ester) was gently agitated with ethylenediamine (460 mL) overnight.[33] The resin was filtered off, washed thoroughly with DMF and then diethyl ether, and dried in vacuo (the "standard method" used for resin washing). The dried resin was then coupled with Fmoc-Nle (8.83 g, 5 equiv, 25 mmol)/TBTU (7.9 g, 4.9 equiv)/HOBt (3.8 g, 4.95 equiv)/DIEA (6.35 g, 9.9 equiv) in DMF (25 mL). After 1 h, the peptide-resin gave a negative ninhydrin response,[1] was thoroughly washed as usual, and dried in vacuo to give Fmoc-Nle-EDA-SU 500: yield 11.35 g.

(iii) Preparation of Fmoc-β-Alanine-(2-ClTrt)-CO-Norleucine-EDA-SU 500 (5a). Fmoc-L-Nle-EDA-SU 500 (ex 2.ii, 1.0 g, approximately 0.4 mmol) was treated with 20% piperidine in DMF (15 mL) for 3 and 7 min and then thoroughly washed as usual. The free-amino peptide-resin was then coupled with Nα-fluoren-9-ylmethoxycarbonyl-β-alanine-(2-chloro-4′-(carboxypentafluorophenoxy)-triphenyl) methyl ester (**4a**) (545 mg, 0.67 mmol, 1.7 equiv)/HOBt (102.6 mg, 0.67 mmol) in DMF (4 mL) for 2 h. The peptide-resin was thoroughly washed as usual and then treated with Ac_2O (204 mg, 5 equiv)/DIEA (103 mg, 2 equiv) pre-mixed in DMF (4 mL). After 30 min, the peptide-resin was filtered and washed as usual giving **5a**: yield 1250 mg, loading = 0.275 mmol peptide/g peptide-resin.

(iv) Preparation of Fmoc-Leucine-OCH_2-C_6H_4-OCH_2CO-β-alanine-(2-ClTrt)-CO-Norleucine-EDA-SU 500 (6). Fmoc-β-Alanine-(2-ClTrt)-CO-Norleucine-EDA-SU 500 (**5a**) (1250 mg, approximately 0.35 mmol) was treated with 20% piperidine in DMF (15 mL) for 3 and 7 min and then thoroughly washed as usual. The free-amino peptide-resin was then coupled with HMPAA-Pfp (313 mg, 0.9 mmol, mw = 348.2)/HOBt (138 mg, 0.9 mmol) in DMF (8 mL) for 1 h, giving a negative ninhydrin response, then thoroughly washed, and dried in the usual manner.

Fmoc-L-Leucine (1062 mg, 3 mmol) was dissolved in DCM (5 mL)/DMF (1 mL), cooled to 0 °C, and stirred. DIC (187 mg, 1.48 mmol) in DCM (2 mL) was added, and the mixture was stirred at 0 °C for 30 min and then evaporated in vacuo. The resultant symmetric anhydride was dissolved in DMF (6 mL) and added to the above peptide-resin. After 2 min, DMAP (37 mg, 0.3 mmol) in DMF (1 mL) was added with swirling, and the mixture left at room temperature for 2 h. The peptide-resin was then thoroughly washed and dried as usual: yield 1290 mg, loading = 0.22 mmol peptide/g peptide-resin; amino acid ratios: Leu 0.74, β-Ala 0.85, Nle 1.00.

(v) Preparation of Fmoc-Glycine-(2-ClTrt)-CO-Norleucine-EDA-SU 500 (5b). Prepared as detailed for **5a** from Fmoc-L-Nle-EDA-SU 500 (6.0 g, approximately 2.4 mmol) using Nα-fluoren-9-ylmethoxycarbonyl-glycine-(2-chloro-4′-(carboxypentafluorophenoxy)triphenyl) methyl ester (**4b**) (2.65 g, 3.38 mmol, 1.4 equiv)/HOBt (518 mg, 3.38 mmol) in DMF (20 mL) for 2 h: yield 7710 mg, loading = 0.271 mmol peptide/g peptide-resin.

(vi) Preparation of Fmoc-Proline-(2-ClTrt)-CO-Norleucine-EDA-SU 500 (5c). Prepared as detailed for **5a** from Fmoc-L-Nle-EDA-SU 500 (1.0 g, approximately 0.4 mmol) using Nα-fluoren-9-ylmethoxycarbonyl-L-proline-(2-chloro-4′-(carboxypentafluorophenoxy)-triphenyl) methyl ester (**4c**) (552 mg, 0.67 mmol, 1.7 equiv)/HOBt (102.6 mg, 0.67 mmol) in DMF (4 mL) for 2 h: yield 1275 mg, loading = 0.255 mmol peptide/g peptide-resin.

(vii) Preparation of H-Norleucine-NovaSyn KD. NovaSyn KD (1000 mg, approximately 0.1 mmol) was coupled with Fmoc-Nle (106 mg, 3 equiv, 0.3 mmol)/TBTU (96.5 mg, 2.95 equiv)/HOBt (46.5 g, 2.95 equiv)/DIEA (77 mg, 5.95 equiv) in DMF (4 mL). After 1 h, the peptide-resin gave a negative ninhydrin response,[1] was thoroughly washed as usual, and dried in vacuo. Fmoc-Nle-NovaSyn KD (1.02 g, approximately 0.08 mmol g^{-1}) was then treated with 20% piperidine in DMF (15 mL) for 3 and 7 min, thoroughly washed, and dried as usual.

(3) Preparation and Purification of Fully Protected Segments 1−10. (i) General Procedures. (a) Segment Preparation. The appropriate peptide-resin **5b,c** or **6** (800−1250 mg) was sequentially

coupled with 1.25 mmol vials of the appropriately side-chain protected and activated Fmoc-amino acid derivatives (see general experimental introduction). For the incorporation of an Hmb protected residue, the de-Fmoc peptide-resin was initially removed from the Biolynx, washed, and dried. The amino-free resin was then coupled with the appropriately side-chain protected *N,O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl)amino acid pentafluorophenyl ester[14] (3 equiv)/HOBt (3 equiv) in DMF, until ninhydrin negative (from 1−24 h). The washed and dried peptide-resin was then returned to the Biolynx synthesiser, deprotected with 20% piperidine/DMF as usual, then removed, washed, and dried.

The residue immediately following the Hmb-substituted residue was then coupled using the appropriately side-chain protected Fmoc-amino acid-*N*-carboxy anhydride (10 equiv) in DCM (1−24 h).[14] The washed and dried peptide-resin was then returned to the Biolynx synthesizer, and synthesis was continued as usual.

(b) Cleavage of Fully Protected Segments. Fully protected peptide-resins (approximately 1500 mg) were treated with 0.75% TFA/DCM (50 mL) for 45 min at room temperature. The resin was filtered-off and washed with DCM (2 × 25 mL), and the combined organics were treated with NMM (375 μL). The organics were then washed with saturated sodium chloride solution (2 × 750 mL), dried (Na_2SO_4), and removed in vacuo giving the crude fully protected segment. The cleaved peptide-resin was washed and dried in the usual manner, and a sample was analyzed for quantitative UV absorbance at 290 nm, giving an estimation of the peptide-resin cleavage.

(c) Assessment of Crude Segment Quality. A sample of the crude fully protected segment (approximately 1 mg) was treated with 20% piperidine/DMF (approximately 50 μL) for 30 min and then diluted with ice-cold diethyl ether (25 mL). After cooling in dry ice/acetone for 3 min, the mixture was centrifuged for 7 min (3000 rpm). The liquid was decanted, and the etheral wash was repeated. The resultant solid was dried in vacuo and then treated with TFA/EDT/TES/water (90:5:3:2, v/v/v/v, 1 mL) overnight. The solution was sparged with nitrogen to remove volatile solvents and washed with ether (5 × 25 mL) as above. The crude fully deprotected segment was then dissolved in 0.1% aqueous TFA (500 μL), and 50 μL was analyzed by C_8 analytical HPLC. The major peak from HPLC was collected and confirmed as the base peptide from the fully protected segment by MALDITOF-MS, data in Table 2.

Also, a sample of the crude fully protected segment (approximately 1 mg) was dissolved in trifluoroethanol (250 μL) and analyzed by appropriate analytical HPLC, data in Table 1.

(d) Purification Techniques. Three basic purification techniques were utilized.

(a) Method 1. The crude peptide (approximately 500 mg) was dissolved in chloroform (3 mL) and loaded onto a "Lobar LiChroprep Si 60, 37 × 440 mm" silica gel column, pre-equilbrated in chloroform. Using a gradient of 0−40% methanol in chloroform (2 L eluent, gradient over 3 h), and monitoring fractions at 304 nm, desired fractions were identified, combined, and removed in vacuo. The residue was dissolved in TFE (15 mL), transferred to a 50 mL falcon tube, reduced to approximately 2 mL, diluted to 15 mL with distilled water, and lyophilized to give the purified segment.

(b) Method 2. The crude segment (approximately 500 mg) was dissolved in TFE (15 mL)/water (5 mL) and purified by 20 × 1 mL injections on a Vydac protein C_4, 22 × 250 mm preparative column using a high % acetonitrile/0.1% aqueous TFA gradient. Fractions were pooled, diluted with approximately half the pooled volume using water, and neutralized with 0.01 M sodium carbonate. The neutralized solution was freeze-dried, and the lyophilized product was dissolved in chloroform (100 mL), washed with saturated sodium chloride solution (3 × 500 mL), dried (Na_2SO_4), and removed in vacuo. The residue was dissolved in TFE (15 mL), transferred to a 50 mL falcon tube, reduced to approximately 2 mL, diluted to 15 mL with distilled water, and lyopholized to give the purified segment.

(c) Method 3. The crude peptide was purified and worked-up as described in method 2, but using a Vydac diphenyl 22 × 250 mm preparative column.

(ii) Preparation and Purification of Segment 1. Peptide-resin 6 (1000 mg, 0.22 mmol) was elaborated with the following residues.

Residue (coupling time, deprotection area, deprotection height), Lys^{250}(45 min, 111.3, 1.636), His^{249}(2 h, 112.1, 1.718), Thr^{248}(2 h, 113.04, 1.785), Glu^{247}(90 min, 115.3, 1.621), Ile^{246}(2 h, 113.6, 1.484), (Hmb)Lys^{245}(8 h, 220.6, 2.926), Lys^{244}(5 h, 191.5, 2.408), Asn^{243}(2 h, 126.0, 1.638), Gly^{242}(2 h, Fmoc protected). The peptide-resin was treated with Ac_2O (255 mg)/DIEA (160 mg) for 1 h, and the fully assembled peptide-resin was washed and dried as usual: yield 1652 mg. A sample was analyzed for AAA ratios, see Table 4.

Peptide-resin (1540 mg) was cleaved by the standard method (see above) giving crude fully protected segment 1 (500 mg, 185 μmol). Post-cleaved peptide resin (5.0 mg) gave $Abs_{290} = 0.210$, equating to 84% cleavage. C_8 analytical HPLC (10−90% B in A, 2−27 min, then 90% B in A for 13 min) (fully protected), R_t = 29.31 min (4.2%), 31.83 min (81.3%), 32.13 min (3.8%). C_8 analytical HPLC (10−90% B in A, 2−27 min) (base peptide) R_t = 9.46 min (97.8%), MALDITOF-MS at 1166Da (theoretical mass = 1167Da).

Crude fully protected segment 1 (495 mg) was purified by method 1 giving two fractions. Fraction 1, yield 202 mg, (74.8 μmol, 34.8% overall), C_8 analytical HPLC (10−90% Bin A, 2−27 min, then 90% B in A for 13 min), R_t = 31.92 min (98.1%). Fraction 2 yield 174 mg, C_8 analytical HPLC (10−90% B in A, 2−27 min, then 90% B in A for 13 min), R_t = 29.41 min (7.3%), 31.79 min (92.1%).

(iii) Preparation and Purification of Segment 2. Prepared from peptide-resin **5b** (925 mg, 0.25 mmol) as generally detailed for segment 1: yield 1470 mg. Purified by method 1, see Tables 1 and 2 for analytical data and Table 4 for AAA ratios.

(iv) Preparation and Purification of Segment 3. Prepared from peptide-resin **5b** (800 mg, 0.22 mmol) as generally detailed for segment 1: yield 1215 mg. Purified by method 3 using a 75−95% B in A gradient, 2−27 min, then 95% for 15 min. See Tables 1 and 2 for analytical data and Table 4 for AAA ratios.

(v) Preparation and Purification of Segment 4. Prepared from peptide-resin **5b** (1200 mg, 0.3 mmol) as generally detailed for segment 1: yield 1710 mg. Purified by method 2 using a 65−95% B in A gradient, followed by a second purification by method 1. See Tables 1 and 2 for analytical data and Table 4 for AAA ratios.

(vi) Preparation of Segment 5. Prepared from peptide-resin **5b** (800 mg, 0.22 mmol) as generally detailed for segment 1, but not cleaved: yield 1100 mg. Base peptide data, see Tables 2 and 4 for AAA ratios.

The peptide-resin was treated with 1% DBU/DMF (50 mL) for 3 and 7 min, then washed, and dried in the usual manner.

(vii) Preparation of Segment 6. Prepared from peptide-resin **5c** (1000 mg, 0.25 mmol) as generally detailed for segment 1: yield 1300 mg. Peptide-resin (1275 mg) was cleaved (67% by quantitative UV) by the standard method (see above) giving crude fully protected segment 6 (295 mg, 153 μmol). C_8 analytical HPLC (10−90% B in A, 2−27 min, then 90% B in A for 13 min) (fully protected), R_t = 30.27 min (77.7%). Base peptide data, see Tables 2 and 4 for AAA ratios.

(viii) Preparation and Purification of Segment 9. H.-Segment 5-resin (from vi) (825 mg, 0.15 mmol) was coupled with crude fully protected segment 6 (288 mg, 150 μmol)/BOP (66.3 mg, 150 μmol)/HOBt (23.0 mg, 150 μmol)/DIEA (19.4 mg, 150 μmol) in DMF (6 mL) for 20 h. The peptide-resin was then thoroughly washed and dried: yield 1030 mg. Crude protected segment was cleaved as usual and purified by method 3 using a 65−95% B in A gradient. See Tables 1 and 2 for analytical data and Table 4 for AAA ratios.

(ix) Preparation of Segment 7. Prepared from peptide-resin **5b** (800 mg, 0.22 mmol) as generally detailed for segment 1 but not cleaved: yield 1110 mg. Base peptide data, see Tables 2 and 4 for AAA ratios.

The peptide-resin was treated with 1% DBU/DMF (50 mL) for 3 and 7 min, then washed, and dried in the usual manner. **Preparation of Segment 8.** Prepared from peptide-resin **5b** (800 mg, 0.22 mmol) as generally detailed for segment 1: yield 1250 mg. Peptide-resin (1240 mg) was cleaved (87% by quantitative UV) by the standard method (see above) giving crude fully protected segment 8 (295 mg, 153 μmol). C_8 analytical HPLC (10−90% B in A, 2−27 min, then 90% B in A for 13 min) (fully protected), R_t = 32.22 min (90.3%). Base peptide data, see Tables 2 and 4 for AAA ratios.

(xi) Preparation and Purification of Segment 10. H.-Segment 7-resin (from ix) (1025 mg, 0.2 mmol) was coupled with crude fully

protected segment 8 (465 mg, 215 μmol)/BOP (95.4 mg, 215 μmol)/HOBt (33.0 mg, 215 μmol)/DIEA (27.9 mg, 215 μmol) in DMF (5 mL) for 20 h. The peptide-resin was then thoroughly washed and dried: yield 1225 mg. Purified by method 3 using an 80−95% B in A gradient, 2−27 min. See Tables 1 and 2 for analytical data and Table 4 for AAA ratios.

(4) Assembly of Segments. (i) Initial Loading of Segment 1 onto H-Nle-NovaSyn KD. Assembly 8. To purified segment 1 (ex 3.ii, 177 mg, 66 μmol) was added BOP (29.9 mg, 66 μmol), HOBt (10.1 mg, 66 μmol), and DIEA (8.5 mg, 66 μmol) in DMF (500 mL). After 2 min, H-Nle-NovaSyn KD (ex 2.vii, 750 mg, approximately 0.06 mmol) was added, followed by DMF (1500 mL), and the reaction was left for 3 h. The peptide-resin was washed, dried, then treated with Ac_2O (30.6 mg, 0.3 mmol)/DIEA (15.5 mg, 0.12 mmol), and premixed in DMF (3 mL, 2 min), for 30 min. The peptide resin was then thoroughly washed and dried: yield 860 mg, assembly 8 loading = 0.048 mmol peptide/g peptide-resin.

Peptide-resin (5.0 mg) was treated with 20% piperidine/DMF for 30 min, then washed and dried in vacuo: amino acid ratios see Table 4.

Fmoc deprotected peptide-resin (4 mg) was cleaved (5 h) and worked-up as detailed in 3.i.c.: analytical HPLC (system a), R_t = 9.37 min (97.6%) with MALDITOF-MS at 1166Da (theoretical mass = 1167Da).

(iii) Addition of Segment 2. Purified segment 2 (ex 3.iii, 207 mg, 96 μmol, 2 equiv) was coupled for 6 h to assembly 8 (830 mg) (initially treated with 20% piperidine/DMF) as generally detailed in 4.i: yield 900 mg. Fmoc deprotected peptide-resin (1.0 mg) was analyzed for AAA ratios, see Table 4.

Coupled fully protected peptide-resin (2.0 mg) was cleaved (5 h) and worked-up as detailed in 3.i.c. See Table 3 for analytical data.

(iv) Addition of Segment 3. Purified segment 3 (ex 3.iv, 139 mg, 30 μmol) was coupled to H-Segment2-1-NovaSyn KD (ex 4.iii, deprotected following 4.i.a, 310 mg, approximately 0.015 mmol) following the general method detailed in 4.iii. Yield 367 mg; AAA ratios, see Table 4.

Fmoc deprotected peptide-resin (1.5 mg) was cleaved (5 h) and worked-up as detailed in 3.i.c: analytical HPLC (Figure 1) (system a), R_t = 12.66 min (84.7%), with MALDITOF-MS at 4632Da (H-Gly^{210}-Leu^{251}-OH, theoretical mass = 4631Da).

(v) Addition of Segment 4. Purified segment 4 (ex 3.v, 72 mg, 30 μmol) was coupled to H-Segment3-2-1-NovaSyn KD (ex 4.iv, deprotected following 4.i.a, 350 mg, approximately 0.015 mmol) following the general method detailed in 4.iii: yield 372 mg. 1.0 mg was hydrolyzed (general introduction) and analyzed for AAA ratios, see Table 4.

Coupled fully protected peptide-resin (2.0 mg) was cleaved (5 h) and worked-up as detailed in 3.i.c. See Table 3 for analytical data.

(vi) Addition of Segment 9. Purified segment 9 (ex 3.viii, 111 mg, 30 μmol) was coupled to H-Segment 4-3-2-1-NovaSyn KD (ex 4.v, deprotected following 4.i.a, 351 mg, approximately 0.015 mmol) following the general method detailed in 4.iii: yield 381 mg. 1.0 mg was hydrolyzed (general introduction) and analyzed for AAA ratios, see Table 4.

Fmoc deprotected peptide-resin (1.9 mg) was cleaved (5 h) and worked-up as detailed in 3.i.c. See Table 3 for analytical data.

(vii) Addition of Segment 10. Purified segment 10 (ex 3.xi, 134 mg, 30 μmol) was coupled to H-Segment 9-5-4-3-2-1-NovaSyn KD (ex 4.vi, deprotected following 4.i.a, 360 mg, approximately 0.015 mmol) following the general method detailed in 4.iii: yield 390 mg; AAA ratios, see Table 4.

Coupled fully protected peptide-resin (2.0 mg) was cleaved (5 h) and worked-up as detailed in 3.i.c: analytical HPLC (system a) (Figure 4a), R_t = 15.70 min (75.1%), with MALDITOF-MS at 12172Da (Fmoc-Asp^{158}-Leu^{251}, $AcmCys^{197}$, AcHmb(Ser^{164}, Asn^{171}, Val^{181}, Lys^{192}, Gly^{210}, Ser^{216}, Phe^{221}, Arg^{224}, Asn^{234}, Lys^{245})-OH theoretical mass = 12169Da) (Figure 5).

Purified by C_8 preparative HPLC (single injection) using a 10−90% B in A gradient. Re-analyzed by analytical HPLC (system a) (Figure 4b), R_t = 15.73 min (>98%).

(viii) Large Scale Cleavage and Purification of H-Asp^{158}-Leu^{251}, $AcmCys^{197}$-OH. Peptide-resin (ex 4.vii, 365 mg) was treated with 20% piperidine/DMF (50 mL for 3 and 25 min), then thoroughly washed, and dried. The Fmoc deprotected peptide-resin was then cleaved with TFA/EDT/TES/water (90:5:3:2, v/v/v/v, 10 mL) for 12 h, and a white solid (127 mg) was isolated by the standard nitrogen sparge/etheral precipitation method (see 3.i.c): post-cleaved resin AAA, Phe:Nle ratio of 0.03:2.52, 97% cleavage; analytical HPLC (system a, 25−35% B in A over 25 min) (Figure 2a), R_t = 11.12 min (64.7%).

Crude H-Asp^{158}-Leu^{251}, $AcmCys^{197}$-OH.18TFA was dissolved in 0.1% aqueous TFA (10 mL), and 5 μL aliquots was analyzed by C_8 analytical HPLC. A gradient of 26−32% B in A over 25 min gave three main species with R_t = 4.5 min (17%), 6.4 min (61%) (desired material), 7.9 min (19%). The crude peptide was purified (20 × 500 μL) by C_8 preparative HPLC using a 26−32% B in A gradient. The desired fractions were pooled, freeze-dried, and then redissolved in 0.1% aqueous TFA (10 mL). C_8 analytical HPLC, 25−35% B in A, R_t = 11.19 min (>99%) (Figure 2b) with MALDITOF-MS at 10164.4 ± 2.2Da (n = 15) (H-Asp^{158}-Leu^{251}, $AcmCys^{197}$-OH theoretical mass = 10164.7Da) (Figure 3).

A 50 μL aliquot was hydrolyzed giving the following AAA ratios: Asp/Asn (10) 10.45, Thr (4) 3.84, Ser (8) 7.28, Glu/Gln (8) 8.75, Pro (4) 3.94, Gly (13) 13.37, Val (9) 8.26, Ile (6) 5.36, Leu (7) 7.07, Tyr (1) 0.94, Phe (1) 0.95, His (5) 4.68, Lys (16) 15.59, Arg (1) 0.95: yield 2.50 μmol, 16.7% overall.

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