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Combined Solid-phase and Solution Approach for the Synthesis of Large Peptides or Proteins

YUJI NISHIUCHI*, HIDEKI NISHIO, TATSUYA INUI, JÓZSEF BÓDI and TERUTOSHI KIMURA

Peptide Institute, Inc., Protein Research Foundation, Minoh-shi, Osaka 562-8686, Japan

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Abstract: In the synthesis of large peptides or proteins, highly homogeneous segments are indispensable for a convergent strategy either on a solid-phase resin or in solution. Employing Boc/Bzl chemistry to prepare fully protected segments with a free α-carboxyl group from the solid support, base-labile linkers are profitable for practical peptide synthesis since they require no special equipment. For this purpose, an *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid (HMFS) linker was adopted. Consequently, there must be high compatibility between the protecting groups of the segment and the anchoring group which is cleavable by treatment with morpholine or piperidine in DMF. Instead of using the 2-bromobenzyloxycarbonyl (BrZ) group for the Tyr residue and the formyl (For) group for the Trp residue, both of which are the most susceptible protecting groups under these base-catalysed conditions, the base-resistant 3-pentyl (Pen) and cyclohexyloxycarbonyl (Hoc) groups were introduced to the respective side-chain functional groups. By applying the present strategy, the authors were able to rapidly synthesize homogeneous protected segments for use in the subsequent segment coupling in solution. In the present paper, the utility of the combined solid-phase and solution approach is demonstrated by synthesizing muscarinic toxin 1 (MTX1) which binds to the muscarinic acetylcholine receptors. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: combined solid-phase and solution approach; *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid (HMFS); 3-pentyl (Pen); cyclohexyloxycarbonyl (Hoc); muscarinic toxin 1 (MTX1)

INTRODUCTION

Today, the chemical synthesis of proteins is of great interest to the peptide chemist, and several techniques using solid phase and solution methods are available. Among them, convergent synthesis involving the preparation and coupling of protected peptide segments is one of the most advantageous approaches for synthesizing large peptides or proteins. However, the problem of insolubility of the protected peptide intermediates in organic solvents commonly used in the peptide synthesis is always

Abbreviations: Acm, acetamidomethyl; BrZ, 2-bromobenzyloxycarbonyl; Bzl, benzyl; CHL, chloroform; cHx, cyclohexyl; ClZ, 2-chlorobenzyloxycarbonyl; CZE, capillary zone electrophoresis; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ESI MS, electrospray ionization mass spectrometry; For, formyl; HATU, *N*-[(dimethylamino)-1H-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HFIP, hexafluoroisopropanol; HMFS, *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid; HOBt, 1-hydroxybenzotriazole; Hoc, cyclohexyloxycarbonyl; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; IEX-HPLC, ion exchange high-performance liquid chromatography; NMP, *N*-methylpyrrolidone; mAChRs, muscarinic acetylcholine receptors; MALDI-TOF MS, matrix assisted laser desorption ionization mass spectrometry; MTX1, muscarinic toxin 1; Pac, phenacyl; Pen, 3-pentyl; RP-HPLC, reversed phase high-performance liquid chromatography; TFE, 2,2,2-trifluoroethanol; Tos, *p*-toluensulfonyl; Trt, triphenylmethyl; TFA, trifluoroacetic acid; WSCI, water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; Xan, 9H-xanthen-9-yl.

* Correspondence to: Peptide Institute, Inc., Protein Research Foundation, Minoh-shi, Osaka 562-8686, Japan. E-mail: yuji@prf.or.jp

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encountered during the elongation and condensation of segments either by the solution or solid-phase strategy.

The authors recently demonstrated the usefulness of a powerful solvent system, a mixture of chloroform (CHL) and TFE [1], for the solution synthesis of complex peptides by synthesizing human midkine, a 121-residue protein [2], and human pleiotrophin, a 136-residue protein [3]. The procedure is based on performing the segment condensation method in solution employing a maximum protection strategy with Boc/Bzl chemistry. To further refine the strategy in solution synthesis, the authors introduced a standard solid phase method on an *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid (HMFS) linker developed by Rabanal *et al.* [4] for synthesizing protected peptide segments (based on the Boc/Bzl or cHx type), which is known as the time-determining step. The HMFS linker belongs to the 9-hydroxymethyfluorene type protecting groups that are designed to be cleaved by nucleophiles, such as piperidine and morpholine, through a β-elimination reaction.

However, several problems needed to be solved in order to synthesize homogeneous segments for use in the subsequent segment coupling reaction in solution. One of the serious side reactions is the dehydration of the side chain amide of the Asn residue to the corresponding nitrile during stepwise elongation of the protected peptide segments on a solid support. This side reaction can be avoided by temporary protection using the 9*H*-xanthen-9-yl (Xan) group on the Asn residues, which is quantitatively removable by a single treatment with TFA [5]. The partial or complete cleavage of the side-chain protecting groups, such as the formyl (For) group on the Trp residue and the 2-bromobenzyloxycarbonyl (BrZ) group on the Tyr residue during detachment of the segments from the HMFS resin by treatment with 20% morpholine in DMF, could also be avoided by using the cyclohexyloxycarbonyl (Hoc) group [6] and the 3-pentyl (Pen) group [7]. Under these conditions, it was possible to rapidly synthesize homogeneous protected peptide segments for use in the subsequent segment coupling reaction in solution.

In order to demonstrate the utility of the combined solid-phase and solution approach, the present strategy was applied to the synthesis of muscarinic toxin 1 (MTX1) [8]. Muscarinic toxins isolated from the venom of snakes belonging to the *Dendroaspis* genus are the only proteins known to bind to the muscarinic acetylcholine receptors (mAChRs) [9]. Among them, MTX1 isolated from the venom of the eastern green mamba, *D. angusticeps*, is of great interest in designing drugs that selectively stimulate M_1 subtype of mAChRs, which might be effective in the treatment of diseases associated with dysfunction of the central or peripheral cholinergic system [10]. It consists of 66 amino acid residues having four intramolecular disulfide bonds, whose pairing has been suggested to be the same as those of short snake toxins [11]. MTX1 was synthesized according to the reported structure by the present procedure in solution and this was compared with the natural product based on HPLC and CZE.

RESULTS AND DISCUSSION

Preparation of Protected Peptide Segments on HMFS Resin

The HMFS linker was prepared as reported previously [4]. Loading of the resin with the first Boc-amino acid was performed either by coupling of the preformed linker-amino acid building block onto the resin (method A, Figure 1) or by coupling of the Boc-amino acid onto the linker-functionalized resin (method B). While method B has the advantage of great flexibility regarding the substrate coupled onto the resin, method A allows exact determination of the degree of amino acid substitution. Furthermore, by using method A, the risk of epimerization with the first amino acid resulting from the use of *N*,*N*-dimethyl-4-aminopyridine (DMAP) during the loading reaction could be effectively excluded by recrystallization of the linker-amino acid building blocks (data not shown). When HBTU or HATU is

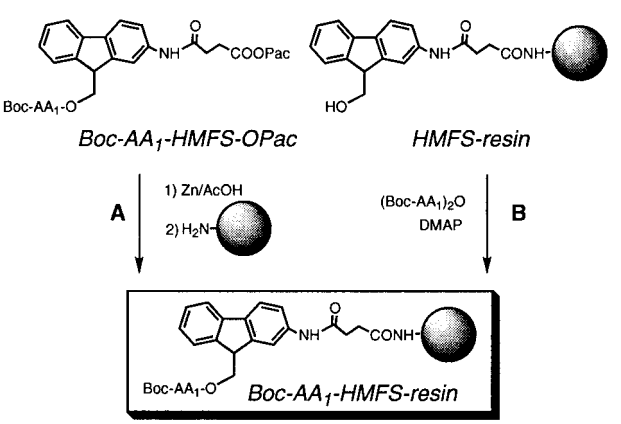

Figure 1 Loading of the resin with the first amino acid.

Table 1 Dehydration of the Asn Residue during the Coupling Reaction with X-Phe-Leu Gly-HMFS resin (X = Gly or Pro)

X	Formation of Boc-Ala(CN)-peptide (%)[a]			
	HATU	HBTU/HOBt	DCC/HOBt	DCC/HOOBt[b]
Gly	5	4	0.7	<0.1
Pro	65	58	11	2

[a] Determined by HPLC after detachment of Boc-peptide from the HMFS resin by treatment with 20% morpholine/DMF.
[b] Boc-Asn/DCC/HOBt or HOOBt (4/4/4 equivalents), Boc-Asn/HATU/DIEA (4/4/6 equivalents) or Boc-Asn/HBTU/HOBt/DIEA (4/4/4/6 equivalents).

used as a coupling reagent to achieve a high degree of coupling efficiency, the major side reaction during the step-wise elongation of the protected peptide segments on a solid support is dehydration of the side chain amide of the Asn residue [12]. The dehydration occurred even in the case of the DCC-mediated coupling reaction, involving the use of HOBt active esters generated *in situ* by the inclusion of HOBt in the activation reaction (Table 1). Thus far, not much attention had been paid to this side reaction in Boc chemistry because the Asn residue can be regenerated from the corresponding nitrile in the final HF treatment of the peptide resin [13]. However, in this strategy, highly homogeneous segments prepared by using HATU or HBTU are indispensable for the subsequent segment condensation in solution. Therefore, the triphenylmethyl (Trt) or Xan group on the Asn residue was employed as a temporary protecting group to suppress this side reaction. While the resistance of the Trt group to the TFA treatment resulted in part of the Trt group remaining even after several coupling cycles, the Xan group could be quantitatively removed by a single TFA treatment.

Cleavage of Protected Peptide Segments from the HMFS Resin

The protected peptide segments from the HMFS resin were detached by treatment with 10% piperidine or 20% morpholine in DMF for 20–30 min. Under these conditions, the formation of aspartimide peptides was examined by evaluating their purities on HPLC using the model tetrapeptide, Boc-Ala-Asp(OcHx)-X-Ala (X = Gly, Phe or Asn); X represents the most susceptible amino acids to base-catalysed conditions (e.g. piperidine and morpholine) through dehydration, transpeptidation and epimerization. In all cases, the extent of the formation of aspartimide peptides was more than 20% when treated with 10% piperidine in DMF for 1 h, but was less than 0.2% when treated with 20% morpholine in DMF for 1 h. However, the aspartimide peptide formation was found to be negligible if 20% morpholine in DMF was used within 30 min.

To detach protected segments from solid supports with the protecting groups intact, there must be a highly compatibility between the protecting groups of the segment and the anchoring group. Thus, base-resistant protecting groups are desirable for our procedure involving the preparation of protected segments on HMFS resin. Among commonly used side-chain protecting groups in Boc chemistry, the For group for the Trp residue and the BrZ group for the Tyr residue are the most susceptible protecting groups under base-catalysed conditions. In fact, partial or complete cleavage of the For group and the BrZ group was observed during detachment of the segments even by treatment with 20% morpholine in DMF for 30 min, which are the optimized conditions for avoiding the formation of aspartimide peptides as described above. These side reactions greatly influence the homogeneity of the protected segments for the subsequent coupling reaction in solution. It was decided, therefore, to employ the Hoc group and the Pen group for the side-chain functional groups of the Trp residue and the Tyr residue, respectively. Both protecting groups developed in the laboratory proved to be suitable for the present strategy in terms of their stability during elongation and detachment of the segments and their removability in the final HF reaction. The Hoc group can be removed by the standard HF procedure without resorting to the use of thiols [6]. The removal of the Pen group in the presence of cation scavengers, such as anisole or *p*-cresol, was accompanied by the production of a slight amount of alkyltyrosine side product, which was comparable to that obtained with the BrZ group [7].

Synthesis of Muscarinic Toxin 1 (MTX1)

The protected peptide was assembled in solution from seven segments, which were synthesized with a semi-automatic synthesizer (ACT model 90, Advanced ChemTech, Louisville, KY, USA) using the Boc strategy on HMFS resin except for the C-terminal segment, which was prepared by the solution method. The following side-chain-protected amino acids were employed: Asp(OcHx), Glu(OcHx), Asn(Xan), Gln(Xan), Arg(Tos), Lys(ClZ), His(Bom), Cys(Acm), Ser(Bzl), Thr(Bzl), Tyr(Pen) and Trp(Hoc). Each protected peptide segment was detached from the HMFS resin by treatment with 20% morpholine in DMF for 20–30 min without any side reactions as described above. This detachment procedure could be effectively performed even in the case of sparingly soluble segments (e.g. segment (1–11) in Figure 2) in DMF or NMP, which could be quantitatively extracted with a mixture of CHL and TFE or hexafluoroisopropanol (HFIP) (3/1). The homogeneity of each segment was checked by amino acid analysis, TLC, ESI MS and RP-HPLC. It was found to be more than 95% pure (Figure 3). The segment coupling reaction was carried out according to the scheme shown in Figure 2 using WSCI in the presence of HOBt or HOOBt in DMF or NMP and/or CHL/TFE (3/1).

The protected peptide thus obtained was treated with HF/anisole (9/1) at -5°C for 1 h without addition of thiol to remove all the protecting groups except for Acm groups. The crude product (Figure 4a) was purified by RP-HPLC. The (8 Acm)-peptide (Figure 4b) was treated with $Hg(OAc)_2$ in 50% AcOH at room temperature for 2 h to remove the remaining Acm groups [14]. The Hg ions were removed by adding β-mercaptoethanol followed by gel filtration on Sephadex G-25. The reduced peptide (Figure 4c) was subjected to oxidative folding reaction in 0.1 M $AcONH_4$ buffer (pH 7.8) containing 1 M guanidine hydrochloride at a peptide concentration of

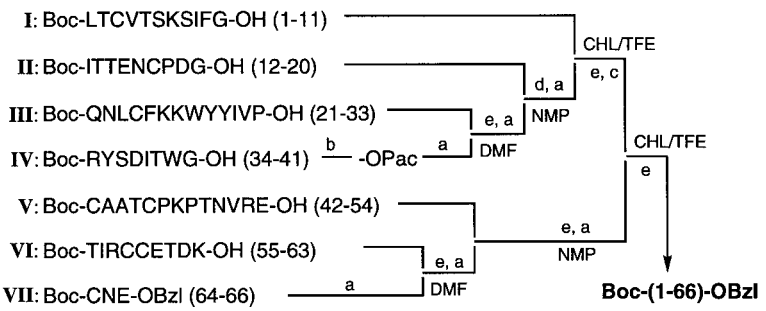

Figure 2 Sequence of segment condensation reactions for the synthesis of MTX1: (a) TFA; (b) Pac-Br/Cs_2CO_3; (c) Zn/AcOH-HFIP; (d) WSCI/HOBt; (e) WSCI/HOOBt.

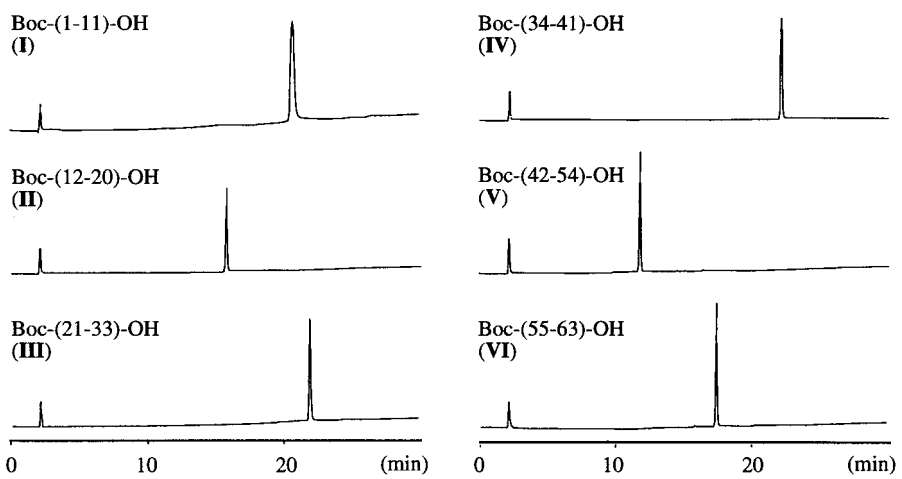

Figure 3 HPLC profiles of protected segments. Column: YMC Pack A-302 ODS (4.6 × 150 mm). Eluent: 45–95% CH_3CN in 0.1% TFA. Running conditions: 25 min gradient, 5 min holding; flow rate: 1 ml/min; temperature: 40°C. Detection: 220 nm.

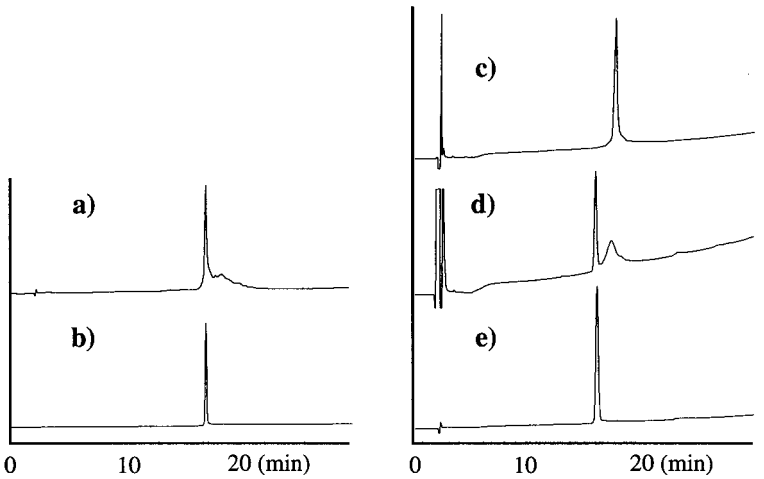

Figure 4 Deprotection and folding of MTX1: (a) crude product obtained after HF treatment; (b) purified (8 Acm)-peptide; (c) (8 SH)-peptide; (d) after folding reaction; (e) purified product. Column: YMC Pack A-302 ODS (4.6 × 150 mm). Eluent: 10–60% CH_3CN in 0.1% TFA. Running conditions: 25 min gradient, 5 min holding; flow rate: 1 ml/min; temperature: 40°C. Detection: 220 nm.

6.6×10^{-6} M in the presence of reduced and oxidized glutathione (GSH/GSSG) at room temperature for 3 days (Figure 4d). The molar ratio of peptide/GSH/GSSG was 1/100/10. The product was isolated by RP-HPLC using aqueous CH_3CN containing 0.1% TFA, and then purified by RP-HPLC using aqueous CH_3CN containing 0.1 M phosphate buffer (pH 7.0) followed by 0.1% TFA in 38% yield calculated from the reduced peptide (Figure 4e). Amino acid analysis of the final product gave values which agreed well with the theoretical values. The molecular weight of the product (7510.74: $[M+H]^+$), measured by MALDI-TOF MS agreed well with the theoretical value (7510.56: ($[M+H]^+$) for the peptide which is intramolecularly linked. The synthetic peptide was then compared with the natural peptide on RP-HPLC, IEX-HPLC and CZE systems, and was found to be identical with the natural peptide, as shown in Figure 5.

CONCLUSION

The present study clearly demonstrates that the combination of solid-phase and solution approaches can be successfully applied to the rapid synthesis of large peptides of sufficiently good quality. In particular, the Hoc and Pen protecting groups for the Trp and Tyr residues, respectively, proved to be indispensable for preparing protected peptide segments on base-labile linkers using the Boc/Bzl strategy for the convergent synthesis.

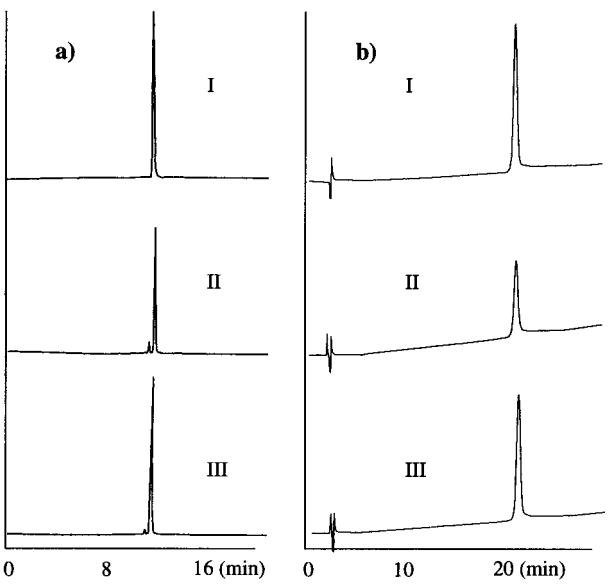

Figure 5 Comparison of the synthetic peptide with natural MTX1: **I**, synthetic peptide; **II**, natural product; **III**, coinjection of the synthetic product and natural MTX1. (a) CZE: column, uncoated fused silica capillary (50 μm × 72 cm); buffer, 20 mM sodium citrate (pH 2.5); voltage, 25 kV; detection, 200 nm; temperature, 30°C. (b) RP-HPLC: column, YMC Pack A-302 ODS (4.6 × 150 mm); eluent, 20–40% CH_3CN in 0.1% TFA; running conditions, 25 min gradient, 5 min holding; flow rate: 1 ml/min; temperature: 40°C; detection: 220 nm.

MATERIALS AND METHODS

Boc-amino acids and other reagents for peptide synthesis were obtained from Peptide Institute Inc. (Osaka, Japan). Boc-Trp(Hoc) and Boc-Tyr(Pen) were synthesized as described [6,7]. Natural MTX1 was purchased from Wako Pure Chemical Industries (Osaka, Japan). TLC was performed on Merck (Darmstadt, Germany) Kieselgel 60F-254 precoated plates. The compounds were visualized with UV light (254 nm) and/or a ninhydrin reagent using the following solvent systems: (1) CHL/MeOH/AcOH, 85/10/5; (2) CHL/MeOH/AcOH, 85/15/5; (3) CHL/80% AcOH/TFE, 6/1/1. HPLC analysis was performed on Shimadzu liquid chromatography model LC-10A or LC-8A (Kyoto, Japan). Capillary zone electrophoresis (CZE) was performed on model 270A apparatus (Applied Biosystems, Foster City, CA, USA). Amino acid analysis was carried out on a JEOL amino acid analyser model JLC-300 (Tokyo, Japan), after hydrolysis of the peptide with 6 M HCl in the presence or absence of 4% thioglycolic acid at 110°C for 22 h. Molecular weights were measured with an ESI MS (HP 1100 LC/MSD, Palo Alto, CA, USA) and a MALDI-TOF MS (JMEX Elite-DE, Tokyo, Japan).

Phenacyl Ester of *N*-(9-(hydroxymethyl)-2-fluorenyl)-succinamic Acid (HMFS-OPac)

TEA (9.9 ml, 70 mmol) was added to a stirred solution of HMFS (20 g, 64.2 mmol) and phenacyl bromide (14.0 g, 70 mmol) in DMF (100 ml) at room temperature. After 3 h, the reaction mixture was partitioned between EtOAc (300 ml) and water (2 l), and the organic layer was washed successively with 1 N HCl (100 ml) and water (3 × 100 ml), and then with brine (100 ml) and dried over anhydrous $MgSO_4$. The dried solution was concentrated to a residue, which was purified by recrystallization from EtOAc and *n*-hexane to yield 26.5 g (96%). Anal. Calc. for $C_{26}H_{23}NO_5$: C 72.71, H 5.40, N 3.26. Found: C 72.55, H 5.52, N 3.23.

General Procedure for Preparation of *N*-(9-((*N*$^{\alpha}$Boc-amino)acyloxymethyl)-2-fluorenyl)-succinamic Acid (Boc-AA-HMFS Linker Derivatives)

A solution of Boc amino acid (48 mmol) and dicyclohexylcarbodiimide (DCC) (4.95 g, 24 mmol) in DCM (50 ml) was stirred for 30 min at room temperature. To a solution of HMFS-OPac (8.59 g, 20 mmol) in DMF (30 ml) at 0°C was added the anhydride solution through a filter to remove DC urea, followed by DMAP (0.24 g, 2.0 mmol). The resulting mixture was stirred at 4°C for 15 min and then at room temperature for an additional 2 h, after which it was concentrated *in vacuo*. The resulting residue was partitioned between EtOAc (300 ml) and 1 N HCl (150 ml). The organic layer was washed successively with water (2 × 100 ml), saturated $NaHCO_3$ (3 × 100 ml) and water (2 × 100 ml), and then with brine (100 ml) and dried over anhydrous $MgSO_4$. After removal of the solvent, the solid was crystallized from EtOAc and *n*-hexane to give the product. Part of the product (15 mmol) was dissolved in AcOH (100 ml) with stirring after addition of zinc dust (19.6 g, 20 equivalents) at 40°C for 30 min. After removal of the zinc dust by filtration, the filtrate was evaporated to a residue, which was triturated with 1 N HCl, washed successively with water, and dried. Crystallization from EtOAc and *n*-hexane yielded the desired product.

***N*-(9-((*N*$^{\alpha}$Boc-glycyl)oxymethyl)-2-fluorenyl)succinamic Acid (Boc-Gly-HMFS Linker)**. The title compound was obtained in 93% yield (6.54 g). Anal. Calc. for $C_{25}H_{28}N_2O_7$: C 64.09, H 6.02, N 5.98. Found: C 64.07, H 6.01, N 5.85.

***N*-(9-((*N*$^{\alpha}$Boc-alanyl)oxymethyl)-2-fluorenyl)succinamic Acid (Boc-Ala-HMFS Linker)**. The title compound was obtained in 90% yield (6.51 g). Anal. Calc. for $C_{26}H_{30}N_2O_7$: C 64.71, H 6.27, N 5.81. Found: C 64.62, H 6.36, N 5.62.

***N*-(9-((*N*$^{\alpha}$Boc-prolyl)oxymethyl)-2-fluorenyl)succinamic Acid (Boc-Pro-HMFS Linker)**. The title compound was obtained in 94% yield (7.17 g). Anal. Calc. for $C_{28}H_{32}N_2O_7$: C 66.12, H 6.34, N 5.51. Found: C 65.94, H 6.60, N 5.39.

General Procedure for Attachment of the Preformed Boc-AA-HMFS Linker Derivatives onto Amino Functionalized Supports

A 1% cross-linked (aminomethyl)polystyrene resin (0.83 mmol amino function/g, 2.41 g) was washed with NMP, 10% DIEA/NMP and NMP. To the resin was added 1.5 equivalents of the Boc-amino acid-HMFS linker derivative in 20 ml of NMP containing HBTU/HOBt/DIEA (1.5/1.5/2.2 equivalents). The resulting mixture was agitated for 1 h, drained and washed with NMP. The qualitative ninhydrin test was used to monitor the loading of the preformed Boc-amino acid-HMFS linker derivative. If the test was positive, the procedure was repeated.

General Procedure for the Solid-phase Assembly of Protected Segments

Each segment was synthesized using a semi-automatic peptide synthesizer (Model ACT-90, Advanced ChemTech, Louisville, KY, USA) on the respective Boc-amino acid-HMFS resin (2 mmol). The functional groups of the side chains were protected using the following groups: Bzl for Ser and Thr, Hex for Asp and Glu, Xan for Asn and Gln, Tos for Arg, Bom for His, ClZ for Lys, Acm for Cys, Hoc for Trp and Pen for Tyr. Solvents were used in the ratio of 15–20 ml/g resin. One cycle of the synthesis consisted of the following: (a) DCM (6 × 1 min); (b) 50% TFA/DCM (2 + 16 min); (c) DCM (2 × 1 min); (d) NMP (1.5 min); (e) 10% DIEA/NMP (1 min); (f) NMP (2 × 1 min); (g) addition of Boc-amino acid/HBTU/HOBt/DIEA (4/4/4/6 equivalents) in NMP (30 min); (h) NMP (6 × 1 min). In the case of removal of the Xan group, 1% triisopropylsilane (TIS) was added to the reaction mixture to quench the cation generated during TFA treatment. Coupling reactions were monitored after every cycle by the Kaiser ninhydrin test to determine the extent of completion. Steps (g) to (h) were repeated for coupling reactions that were determined to be incomplete.

General Procedure for the Detachment of Protected Segments from the Resin

The peptide resin derivatives (2.0 mmol) in a 200-ml cylindrical vessel with a sintered glass bottom were treated with 80 ml of 20% morpholine in DMF for 30 min. In the case of the segments which were insoluble or sparingly soluble in DMF, they were extracted with a mixture of hexafluoroisopropanol (HFIP) and DCM (1/4, 80 ml) after the morpholine solution was drained. After filtration, DMF solution or HFIP/DCM solution was evaporated to dryness with the aid of a vacuum pump at room temperature. The resultant residue was triturated with 1 N HCl, washed successively with water, and dried. Each segment was purified by recrystallization, reprecipitation or silica-gel chromatography using appropriate solvents. The homogeneity of each segment was confirmed by amino acid analysis, TLC, ESI MS or RP-HPLC. The Rf values on TLC, retention time (R_t) on RP-HPLC, mass spectral data on ESI MS and amino acid composition as well as yields are given in Table 2.

Protected Boc-(55–66)-OBzl

Segment **VII** (0.71 g, 1.0 mmol) was dissolved in TFA (5 ml) at − 10°C, and then allowed to react for

Table 2 Analytical Data for Segments Synthesized by HMFS Resin

Segment	R_f [a]	R_t [b] (min)	Yield[c] (%)	Amino acid analysis	ESI MS[d] (calculated)
I (1–11)	0.52 (3)	20.8	82	Thr 1.47 (2), Ser 1.75 (2), Gly 0.98 (1), 1/2(Cys)$_2$ 0.72 (1), Val 0.99 (1), Ile 1.01 (1), Leu 0.97 (1), Phe 1.00 (1), Lys 1.12 (1)	1855.1 (1855.6)
II (12–20)	0.42 (2)	15.7	76	Asp 2.00 (2), Thr 1.69 (2), Glu 1.06 (1), Gly 0.94 (1), 1/2(Cys)$_2$ 0.84 (1), Ile 0.99 (1), Pro 0.99 (1)	1465.7 (1464.7)
III (21–33)	0.56 (2)	21.8	64	Asp 0.96 (1), Glu 0.97 (1), 1/2(Cys)$_2$ 0.78 (1), Val 0.87 (1), Ile 0.86 (1), Leu 0.98 (1), Tyr 1.91 (2), Phe 1.00 (1), Lys 1.97 (2), Trp 0.37 (1), Pro 0.88 (1)	2476.4 (2476.8)
IV (34–41)	0.58 (1)	22.1	83	Asp 1.00 (1), Thr 0.91 (1), Ser 0.92 (1), Gly 1.01 (1), Ile 0.92 (1), Tyr 0.97 (1), Trp 0.45 (1), Arg 0.94 (1)	1709.7 (1710.0)
V (42–54)	0.33 (2)	11.6	92	Asp 1.00 (1), Thr 1.50 (2), Glu 1.06 (1), Ala 1.98 (2), 1/2(Cys)$_2$ 1.53 (2), Val 0.98 (1), Lys 1.06 (1), Arg 0.96 (1), Pro 2.05 (2)	2216.4 (2217.0)
VI (55–63)	0.43 (3)	17.3	66	Asp 1.00 (1), Thr 1.90 (2), Glu 1.02 (1), 1/2(Cys)$_2$ 1.40 (2), Ile 0.93 (1), Lys 1.08 (1), Arg 0.99 (1)	1977.2 (1977.8)

[a] TLC: See 'Materials and Methods' for the solvent.
[b] RP-HPLC: Conditions are the same as in Figure 3.
[c] Yield: overall yield from resin.
[d] ESI MS: mass spectra of all peptide segments were observed with a Hewlett Packard 1100 LC/MSD; observed masses were derived from the experimental m/z values for all observed protonation states of a molecular species, using the program ChemStation (HP). The calculated masses were based on the average isotope composition.

40 min at room temperature. After removal of excess TFA *in vacuo*, the residue was triturated with 4.1 N HCl solution in dioxane (0.3 ml, 1.2 mmol) to convert the TFA salt to the HCl salt, the product was precipitated with ether and dried over NaOH *in vacuo*. The dried product and segment **VI** (2.07 g, 1.05 mmol) were dissolved in DMF (20 ml). To the solution, HOOBt (0.18 g, 1.1 mmol) and WSCI (0.20 ml, 1.1 mmol) were added at -10°C, and the whole mixture was allowed to react under stirring for 16 h at room temperature. The product was precipitated by adding an excess of chilled water, and the precipitates were collected by filtration, then washed with water and ether. The product was purified by reprecipitation from DMF and CH_3CN; the yield was 2.42 g (94.5%); amino acid analysis: Asp 1.85 (2), Thr 1.69 (2), Glu 1.97 (2), 1/2(Cys)$_2$ 1.96 (3), Ile 0.92 (1), Lys 1.00 (1), Arg 1.06 (1).

Protected Boc-(42–66)-OBzl

The Boc-(55–66)-OBzl obtained above (2.30 g, 0.90 mmol) was treated with TFA (10 ml) and the product was converted to the HCl salt as described above. The HCl salt and segment **V** (2.09 g, 0.94 mmol) were dissolved in NMP (50 ml). To the solution, HOOBt (0.16 g, 0.99 mmol) and WSCI (0.18 ml, 0.99 mmol) were added at -10°C, and the whole mixture was allowed to react under stirring for 5 h at room temperature. The product was precipitated by adding excess chilled water, and the precipitates were collected by filtration, then washed successively with water and ether. The product was purified by reprecipitation from DMF and CH_3CN to yield 3.96 g (94.3%); amino acid analysis: Asp 2.64 (3), Thr 2.85 (4), Glu 2.84 (3), Ala 1.77 (2), 1/2(Cys)$_2$ 3.41 (5), Val 0.88 (1), Ile 0.87 (1), Lys 2.00 (2), Arg 1.96 (2), Pro 1.84 (2).

Protected Boc-(34–41)-OPac

Cs_2CO_3 (195 mg, 0.6 mmol) was added to the stirred solution of segment **IV** (2.0 g, 1.2 mmol) in DMF (20 ml). To the solution, phenacyl bromide (0.28 g, 1.4 mmol) was added at -10°C, and the mixture was stirred for 3 h at room temperature. The product was precipitated by adding an excess of chilled water, and the precipitates were collected by filtration, then washed with water and ether. The product was purified by triturating with hot MeOH and washed with ether, and dried; 2.0 g (95%); amino acid analysis: Asp 1.00 (1), Thr 0.92 (1), Ser 0.91 (1), Gly 1.02 (1), Ile 0.94 (1), Tyr 0.97 (1), Trp 0.50 (1), Arg 0.95 (1).

Protected Boc-(21–41)-OPac

The Boc-(34–41)-OPac obtained above (2.00 g, 1.09 mmol) was treated with TFA (15 ml) and the product was converted to the HCl salt as described above. The HCl salt and segment **III** (2.71 g, 1.09 mmol) were dissolved in NMP/DMF (80 ml/20 ml). To the solution, HOOBt (0.213 g, 1.30 mmol) and WSCI (0.239 ml, 1.30 mmol) were added at -10°C, and the whole mixture was allowed to react under stirring for 12 h at room temperature. The product was precipitated by adding an excess of chilled water, and the precipitates were collected by filtration, then washed successively with water and ether. The product was purified by triturating with CH_3CN and ether to yield 3.50 g (77.8%); amino acid analysis: Asp 1.88 (2), Thr 0.81 (1), Ser 0.73 (1), Glu 0.97 (1), Gly 0.89 (1), 1/2(Cys)$_2$ 0.65 (1), Vla 0.85 (1), Ile 1.62 (2), Leu 0.99 (1), Tyr 2.84 (3), Phe 1.00 (1), Lys 1.98 (2), Trp 1.03 (2), Arg 0.90 (1), Pro 1.06 (1).

Protected Boc-(12–41)-OPac

Boc-(21–41)-OPac obtained above (3.30 g, 0.788 mmol) was treated with TFA (20 ml) and the product was converted to the HCl salt as described above. The HCl salt and segment **II** (1.27 g, 0.945 mmol) were dissolved in NMP/DMF (40 ml/10 ml). To the solution, HOOBt (0.128 g, 0.945 mmol) and WSCI (0.173 ml, 0.945 mmol) were added at -10°C, and the whole mixture was allowed to react under stirring for 16 h at room temperature. The product was precipitated by adding an excess of chilled water, and the precipitates were collected by filtration, then washed successively with water and ether. The product was purified by triturating with CH_3CN and ether to yield 3.82 g (87.6%); amino acid analysis: Asp 3.61 (4), Thr 2.22 (3), Ser 0.78 (1), Glu 1.87 (2), Gly 1.72 (2), 1/2(Cys)$_2$ 1.55 (2), Val 0.83 (1), Ile 2.44 (3), Leu 0.99 (1), Tyr 2.83 (3), Phe 1.00 (1), Lys 2.00 (2), Trp 0.65 (2), Arg 0.89 (1), Pro 1.90 (2).

Protected Boc-(1–41)-OPac

The Boc-(12–41)-OPac (3.50 g, 0.633 mmol) obtained above was treated with TFA (30 ml) and the product was converted to the HCl salt as described above. The HCl salt and segment **I** (1.28 g, 0.693 mmol) were dissolved in NMP/DMF (80 ml/20 ml). To the solution, HOOBt (0.103 g, 0.756 mmol) and WSCI (0.139 ml, 0.756 mmol) were added at -10°C, and the whole mixture was allowed to react under stirring for 16 h at room temperature. The product was precipitated by adding excess chilled

water, and the precipitates were collected by filtration, then washed successively with CH_3OH and ether. The product was purified by triturating with hot CH_3OH and washed with ether to yield 3.71 g (81.0%); amino acid analysis: Asp 3.36 (4), Thr 3.70 (5), Ser 2.50 (3), Glu 1.89 (2), Gly 2.75 (3), 1/2$(Cys)_2$ 2.53 (3), Val 1.90 (2), Ile 3.47 (4), Leu 2.02 (2), Tyr 2.79 (3), Phe 2.06 (2), Lys 3.15 (3), Trp 0.76 (2), Arg 0.88 (1), Pro 2.00 (2).

Protected Boc-(1–66)-OBzl

Boc-(1–41)-OPac (3.60 g, 0.495 mmol) obtained above was dissolved in HFIP/AcOH (10 ml/40 ml). Zinc dust (1.62 g, 24.7 mmol) was added to the solution and the whole mixture was stirred vigorously under an argon atmosphere at 45°C for 2 h. The zinc dust was removed by filtration, the filtrate was concentrated to a residue, which was triturated with 1 N HCl, washed with water and CH_3CN, and dried. The product was purified by triturating with NMP, washed with CH_3CN and ether, successively, and dried; the yield of Boc-(1–41)-OH was 3.41 g (97.4%). Boc-(42–66)-OBzl (1.85 g, 0.397 mmol) was treated with TFA (20 ml), and the product was converted to the HCl salt as described above. The HCl salt and Boc-(1–41)-OH (3.12 g, 0.437 mmol) were dissolved in DCM/TFE (3/1, 15 ml). HOOBt (71.3 mg, 0.437 mmol) and WSCI (80.0 μL, 0.437 mmol) were added to the solution at -10°C, and the whole mixture was allowed to react under stirring for 14 h at room temperature. The product was precipitated by adding excess CH_3CN, and the precipitates were collected by filtration, then washed with CH_3CN. The product was purified by reprecipitation from DCM/TFE (3/1) and CH_3CN, and dried to yield 4.62 g (98.5%); amino acid analysis: Asp 6.75 (7), Thr 6.66 (9), Ser 2.47 (3), Glu 4.95 (5), Gly 3.05 (3), Ala 1.77 (2), 1/2$(Cys)_2$ 6.39 (8), Val 2.92 (3), Ile 4.62 (5), Leu 2.20 (2), Tyr 3.00 (3), Phe 2.12 (2), Lys 5.37 (5), Trp 0.65 (2), Arg 2.77 (3), Pro 4.00 (4).

Removal of the Protecting Groups from Boc-(1–66)-OBzl

The fully protected peptide, Boc-(1–66)-OBzl (2.0 g, 0.17 mmol) was treated with TFA (10 ml) as described above, and the N^{α}-deprotected product was treated by HF (42.5 ml) in the presence of *p*-cresol (7.5 ml) at -5°C for 1 h. After evaporation of HF *in vacuo*, the residue was triturated with ether and collected by filtration; the yield of the crude product was 1.30 g. The product was purified by preparative RP-HPLC on a YMC-Pak ODS column (30 × 250 mm) using a linear gradient (20–45% CH_3CN in 0.1% TFA/H_2O for 80 min) to obtain 384 mg (28.0%) of purified 8 Cys(Acm)-containing peptide.

Removal of Acm Groups

To a solution of 8 Acm-containing peptide obtained above (380 mg, 0.047 mmol) in 50% AcOH (40 ml), $Hg(OAc)_2$ (132 mg, 0.414 mmol) was added and stirring was continued under argon gas atmosphere for 2 h at room temperature. β-Mercaptoethanol (0.87 ml) was added to the solution and it was stirred for 2 h at room temperature. The solution was then applied to a Sephadex G-25 column (3.2 × 60 cm) and eluted with 1 M AcOH. The principal fraction was collected and lyophilized to obtain 278 mg (71.0%) of 8 SH-peptide.

Oxidative Folding of 8 SH-Peptide

The 8 SH-peptide thus obtained (100 mg, 0.012 mmol) was dissolved in 2 l of 0.1 M $AcONH_4$ buffer (pH 7.8) containing 1 M guanidine hydrochloride, reduced glutathione (817 mg) and oxidized glutathione (163 mg). The ratio of peptide and redox reagent was 1/100/10, and the peptide concentration was 6.6×10^{-6} M. The oxidative folding was carried out under gentle stirring in a stream of argon for 3 days at room temperature. The mixture was acidified to pH 2 by adding TFA, and the folded peptide was purified by RP-HPLC on a YMC-Pak ODS column (4.6 × 150 mm) using a linear gradient (20–40% CH_3CN in 0.1% TFA/H_2O for 80 min). The product was further purified by RP-HPLC on a Capcell-Pak ODS column (4.6 × 150 mm) using a linear gradient [15–35% CH_3CN in 0.1 M phosphate buffer (pH 7.0) for 80 min] followed by RP-HPLC on a YMC-Pak ODS column as described above to obtain 38 mg (38.0%) of the folded peptide; amino acid analysis: Asp 7.17 (7), Thr 7.88 (9), Ser 2.86 (3), Glu 5.18 (5), Pro 4.41 (4), 1/2$(Cys)_2$ 7.11 (8), Gly 3.05 (3), Ala 2.04 (2), Val 2.90 (3), Ile 4.63 (5), Leu 2.09 (2), Tyr 3.02 (3), Phe 2.01 (2), Lys 5.07 (5), Trp 1.63 (2), Arg 3.00 (3). The molecular weight measured by MALDI-TOF MS was 7510.74 ($[M + H]^+$) {theoretical value: 7510.56 ($[M + H]^+$}.

REFERENCES

1. Kuroda H, Chen Y-N, Kimura T, Sakakibara S. Powerful solvent systems useful for the synthesis of sparingly-soluble peptides in solution. *Int. J. Pept. Protein Res.* 1992; **40**: 294–299.

2. Inui T, Bódi J, Kubo S, Nishio H, Kimura T, Kojima S, Matuta H, Muramatsu T, Sakakibara S. Solution synthesis of human midkine, a novel heparin-binding neurotrophic factor consisting of 121 amino acid residues with five disulphide bonds. *J. Pept. Sci.* 1996; **2**: 28–39.
3. Inui T, Nakao M, Nishio H, Nishiuchi Y, Kojima S, Muramatsu T, Kimura T, Sakakibara S. Total synthesis of human pleiotrophin, a 136-residue heparin-binding neurotrophic factor having five disulfide bonds. In *Proceedings of the First International Peptide Symposium*, Shimonishi Y (ed.), Kluwer: Dordrecht, The Netherlands, 1999; 552–554.
4. Rabanal F, Giralt E, Albericio F. Synthesis and applications of a new base-labile fluorene derived linker for solid-phase peptide synthesis. *Tetrahedron* 1995; **51**: 1449–1458.
5. Shimonishi Y, Sakakibara S, Akabori S. Studies on the synthesis of peptides containing glutamine as the C-terminal. 1. Protection of amide-nitrogen with xanthyl group during peptide synthesis. *Bull. Chem. Soc. Jpn* 1962; **35**: 1966–1970.
6. Nishiuchi Y, Nishio H, Inui T, Kimura T, Sakakibara S. N^{in}-Cyclohexyloxycarbonyl group as a new protecting group for tryptophan. *Tetrahedron Lett.* 1996; **37**: 7529–7532.
7. Bódi J, Nishiuchi Y, Nishio H, Inui T, Kimura T. 3-Pentyl (Pen) group as a new base-resistant side chain protecting group for tyrosine. *Tetrahedron Lett.* 1998; **39**: 7117–7120.
8. Karlsson E, Jolkkonen M, Satyapan N, Adem A, Kumlin E, Hellman U, Wernstedt C. Protein toxins that bind to muscarinic acetylcholine receptors. *Ann. N.Y. Acad. Sci.* 1994; **710**: 153–161.
9. Adem A, Karlsson E. Muscarinic receptor subtype selective toxins. *Life Sci.* 1997; **60**: 1069–1076.
10. Jerusalinsky D, Cervenansky C, Walz R, Bianchin M, Izquiredo I. A peptide muscarinic toxin from the green mamba venom shows agonist-like action in an inhibitory avoidance learning task. *Eur. J. Pharmacol.* 1993; **240**: 103–105.
11. Segalas I, Roumestand C, Zinn-Justin S, Gilquin B, Menez R, Menez A, Toma F. Solution structure of a green mamba toxin that activates muscarinic acetylcholinereceptors, as studied by nuclear magnetic resonance and molecular modeling. *Biochemistry* 1995; **34**: 1248–1260.
12. Gausepohl H, Kraft M, Frank RW. Asparagine coupling in Fmoc solid phase peptide synthesis. *Int. J. Pept. Protein Res.* 1989; **34**: 287–294.
13. Mojsov S, Mitchell AR, Merrifield RB. A quantitative evaluation of methods for coupling asparagine. *J. Org. Chem.* 1980; **45**: 555–560.
14. Nishio H, Kimura T, Sakakibara S. Side reaction in peptide synthesis: Modification of tryptophan during treatment with mercury(II) acetate/2-mercaptoethanol in aqueous acetic acid. *Tetrahedron Lett.* 1994; **35**: 1239–1242.

