Organic Process Research & Development **2003**, *7*, 28–37

Rapid, Continuous Solution-Phase Peptide Synthesis: Application to Peptides of Pharmaceutical Interest†

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Abstract:

The Fmoc/TAEA and Bsmoc/TAEA methods for the rapid, continuous solution synthesis of peptide segments are shown to be applicable to the gram-scale synthesis of short peptides as well as, for the first time, to the synthesis of a relatively long (22-mer) segment, (hPTH 13−34). In the latter case the crude product was of significantly greater purity than a sample obtained via a solid-phase protocol. The Bsmoc methodology was optimized by a new technique involving filtration of the growing partially deprotected peptide at each coupling−deprotection cycle through a short column of silica gel.

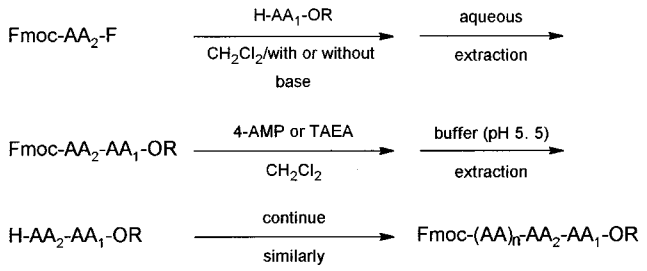
Introduction

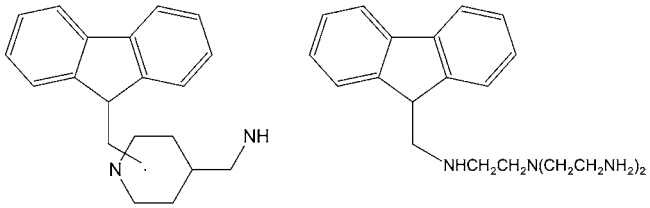
The large-scale synthesis of peptides can be carried out by either classical solution methods or by continuous solid-phase syntheses. Each method suffers from certain deficiencies, for example the need to isolate and purify intermediates in the former case and the problems of handling large amounts of expensive resins or large excesses of reagents in the latter. Upon considering how best to scale up the synthesis of biologically active peptides for clinical testing and eventual commercial production, it seemed desirable to combine the best aspects of both methods, thus leading to our examination of continuous solution techniques.

Results and Discussion

Previously, these continuous solution methods have been used for the small-scale synthesis by Fmoc chemistry of short peptides of six to eight amino acid units.[1] Now we show this technique to be easily adaptable for larger-scale syntheses and, in one case, to the synthesis of a peptide built from 22 amino acid units. The key step of the process (Scheme 1) involves deblocking via 4-(aminomethyl)piperidine (4-AMP) or tris(2-aminoethyl)amine (TAEA) which leads to the formation of adducts **1**[2] or **2**, respectively.

Scheme 1. **Continuous synthesis of short peptides**





1 **2**

These adducts can be removed by extraction with an aqueous buffer of pH 5.5 without disturbing the growing peptide. In addition, in contrast to the case for the solid-phase approach, at any stage the growing peptide can be directly sampled and analyzed.[3] The MALDI mass spectrometric technique represents a convenient method of following the reaction, provided that sampling follows the deblocking process.[4]

Potentially, continuous solution methods should be easily adaptable both for large-scale syntheses and for the synthesis

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† Abbreviations not defined in the text: ACN = acetonitrile; 4-AMP = 4-(aminomethyl)piperidine; Boc = *tert*-butyloxycarbonyl; Bsmoc = 1,1-dioxobenzo[*b*]thiophene-2-ylmethyloxycarbonyl; DBF = dibenzofulvene; DCM = dichloromethane; Dcpm = dicyclopropylmethyl; DEA = diethylamine; DIEA = diisopropylethylamine; Dmcp = dimethyl(cyclopropyl)methyl; DMF = dimethylformamide; Fmoc = 9-fluroenemethyloxycarbonyl; N-HAPyU = 1-(1-pyrrolidinyl-1 H-1,2,3-triazolo[4,5-b]-pyridinylmethylene)pyrrolidinium hexafluorophosphate 3-oxide; N-HATU = 1-[bis(dimethylamino)methylene]-1 H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide; N-HBTU = 1-[bis(dimethylamino)methylene]-1 H-benzotriazolium hexafluorophosphate 3-oxide; hGRF = human growth hormone-releasing factor; HOAt = 7-aza-1-hydoxybenzotriazole; HOBt = 1-hydroxybenzotriazole; hPTH = human parathyroid hormone; MALDI = matrix-assisted laser desorption ionization; Nin = ninhydrin; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PFPhe = pentafluorophenylalanine; reagent R = TFA/thioanisole/1,2-ethanedithiol/anisole(90/5/3/2); TAEA = tris(2-aminoethyl)amine; TFA = trifluoroacetic acid; TFFH = tetramethylfluoroformamidinium hexafluorophosphate; Trt = trityl = triphenylmethyl.

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(2) Structure **1** represents a mixture of the two adducts obtained by addition of 4-AMP to DBF through either the secondary or primary amino functions. See footnote 9 of ref 1b.

(3) For general methods of on-resin monitoring of solid-phase syntheses see: Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC Press: Boca Raton, FL, 1997; p 67.

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Published on Web 12/10/2002

10.1021/op0202179 CCC: $25.00

of longer segments. In the present work, examples of both types are presented. A large-scale route to **3**

Ac−Ser-Asp-Lys-Pro-OH
3

was developed which in one batch led to the preparation of up to 119 g of the acetylated tetrapeptide.[5] In this case 4-AMP was used as the deblocking agent. In many of the syntheses described here protected amino acid fluorides have been used as coupling reagents, but in the large-scale route to **3** the inexpensive reagent diisopropylcarbodiimide (DIC) was also shown to be suitable.

A second short sequence, the octapeptide **4**,[6]

H-D-PFPhe-Gln-Trp-Ala-Val-D-Ala-His-Leu-OMe
4

for which the presence of the methyl ester unit and the hydrophobic N-terminal residue had previously caused difficulties in the attempted solid-phase synthesis,[7] was also obtained via the Fmoc rapid solution-phase approach. The pure protected octapeptide was obtained in 69.2% yield with the last amino acid being introduced as the BOC derivative so that the final deblocking step would proceed to completion via treatment with TFA. The longer sequence **5**,

H-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-
Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-NH_2
5

human parathyroid hormone (hPTH, 13−34),[8] represents a sequence for which the Fmoc-based solid-phase approach presented some deficiencies.[7] In this case also the rapid solution method was applied and led to the formation of a crude product of purity significantly better than that of a corresponding solid-phase synthesis.[9]

As acylating agents, the isolated Fmoc amino acid fluorides[10] were used to effect rapid reaction in the presence of DIEA. The two amino acids which cannot routinely be handled as preformed acid fluorides were introduced via N-HATU (His) or N-HATU/ HOAt (Arg).[11] To maintain solubility of the growing peptide the C-terminal amide residue was protected by the dimethylcyclopropylmethyl (Dmcp) residue.[12] The standard procedure involves use of dichloromethane for all steps: coupling, deblocking, and washing. A disadvantage of this procedure is that during buffer extraction, the desired lower DCM layer must continuously be drawn off at the bottom of the separatory apparatus and repositioned for the next aqueous wash. If a solvent of density lower than that of the aqueous medium could be used, all aqueous washes could simply be drawn off to a waste bottle and discarded.

As a lighter solvent, ethyl acetate was examined, but the results were not always satisfactory, probably because the DBF-scavenging reaction leading to adduct **2** is significantly retarded in ethyl acetate relative to DCM. A hybrid approach was therefore adopted with coupling and deblocking being carried out in DCM; however, prior to the washing steps, two volumes of ethyl acetate were added, and the resulting lighter-than-water mixed solvent was used for the extraction process. After evaporation of the mixed solvent and replacement by fresh DCM, the next coupling reaction was then carried out. In addition to simplifying phase separation, this procedure made for more efficient removal of adduct **2** which is less soluble in EtOAc−DCM (2/1) than in pure DCM.

Each coupling and deblocking cycle took on average 1.5 h. In the case of **5** the first 12 amino acids were added in 1 day, and the remainder, during the next day for an assembly time of 34 h. If the assembly is interrupted, it is preferable to store the material at the washed, Fmoc-protected stage, since reaction with TAEA invariably effects quick solution along with deblocking. Resolubilization of a growing *free amino* peptide was not always achieved.

When assembly of **5** was initiated with 156 mg of phenylalanine Dmcp-amide (90.24 mmol) and 0.36 mmol (0.5 molar excess) of each Fmoc amino acid fluoride or a comparable amount of the free amino acid and N-HATU, the yield of fully protected amide was 325 mg (25.9%). Deblocking of a portion, first via TAEA and then via reagent R,[13] gave free peptide in 70.6% yield which showed by HPLC analysis a purity of 88%. Of the minor impurities present, ES-MS data suggested contamination via material bearing a residual Boc function and an oxidized methionine residue. The crude product was of greater purity than the corresponding material obtained by a manual solid-phase synthesis carried out in DMF on a Rapp Tenta Gel resin[14] (loading 0.14 meq/g) using a 5-fold excess of acid fluoride and 30-min double couplings. For those steps which required the use of N-HATU, 60-min triple couplings were used for the solid-phase synthesis that gave a 22% yield of the fully deblocked peptide. The crude products for the solution- and solid-phase syntheses are compared in Figures 1 and 2.

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(9) No attempts were made to optimize the solid-phase synthesis. The conditions chosen were based on those used for other related syntheses carried out in our laboratory. Any number of other resins or solvent/coupling reagent combinations may well give better results than those described here. It is not our intention to compare the purities of the solution and solid-phase products, but merely to note that the solution method provides a product of acceptable purity.
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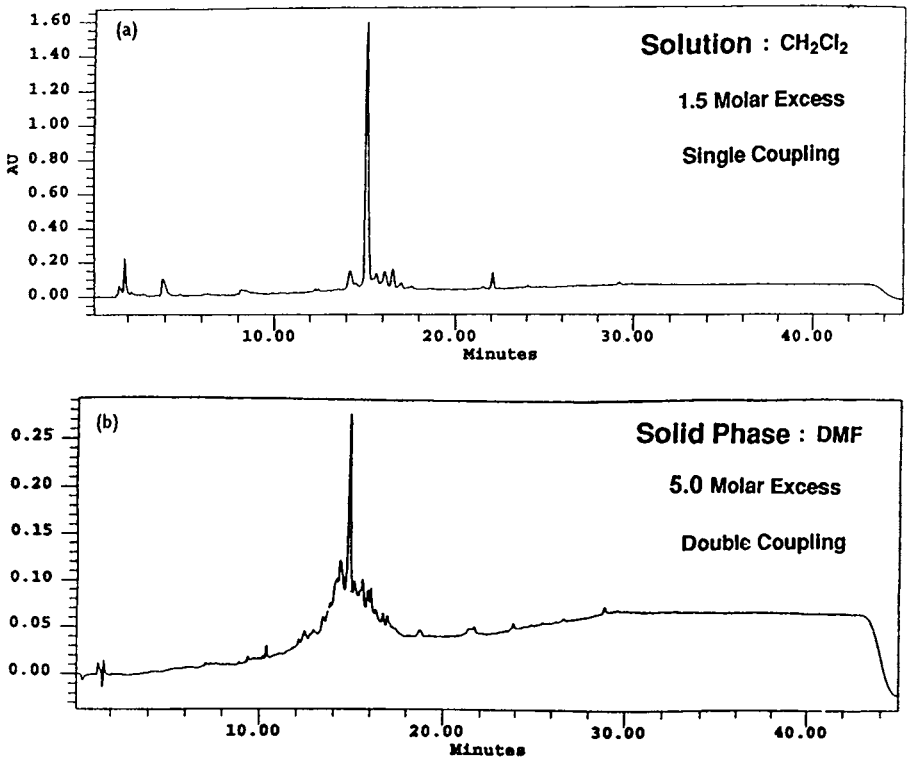


Figure 1. **HPLC: Synthesis of hPTH (13−34) (a) solution phase, (b) solid phase.**

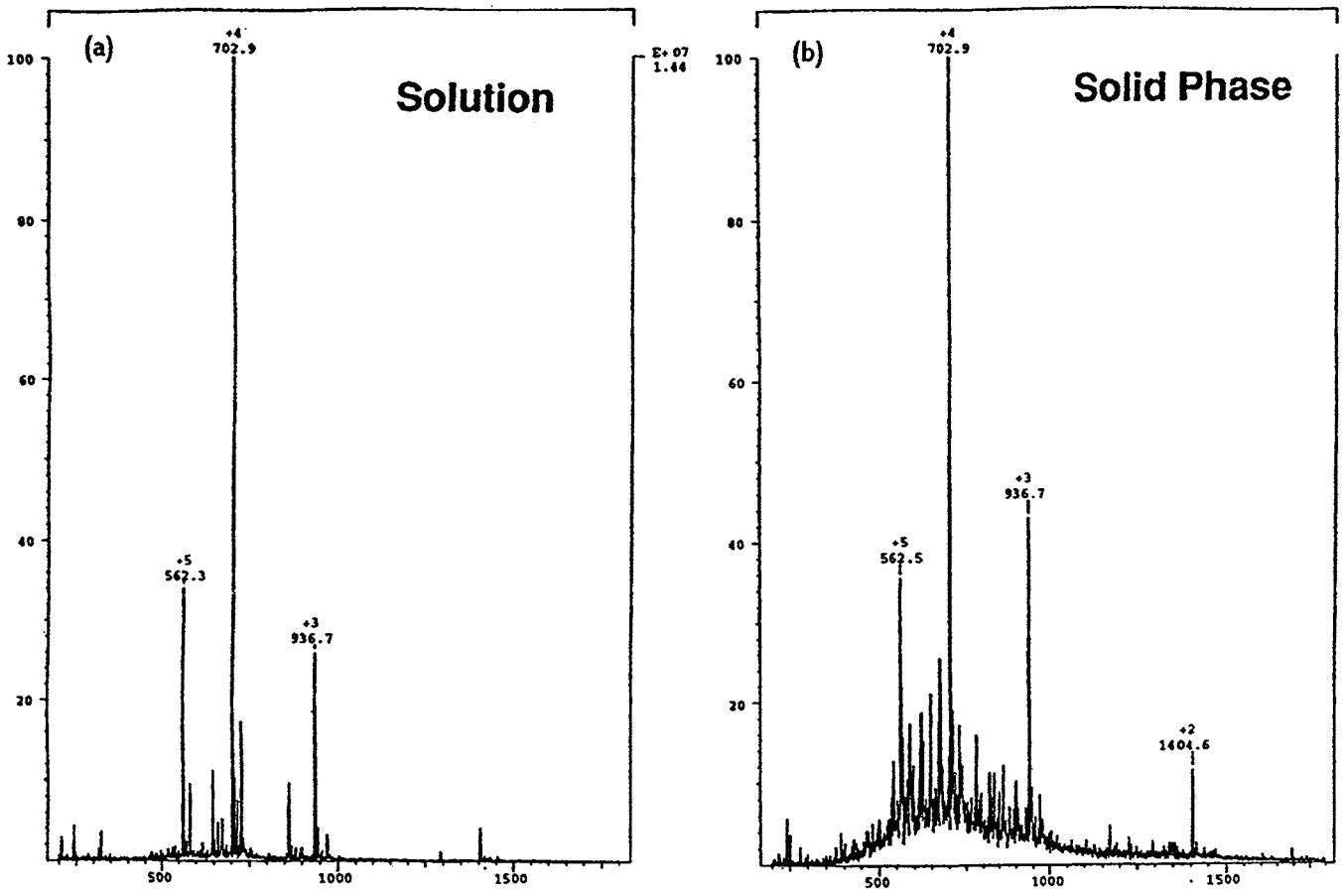


Figure 2. **ES/MS: Synthesis of hPTH (13−34) (a) solution phase, (b) solid phase.**

Several runs were also made which involved stopping at the stage of the 12-mer to examine the effect of base. With DIEA as base 850 mg of phenylalanine Dmcp-amide gave 3.17 g (54.4%) of the fully protected 12-mer. If no base is used in the coupling step, a comparable yield of 53.2% was obtained. For sequences which begin with a small hydrophilic

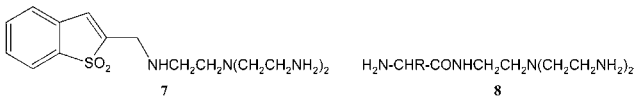
amino acid such as glycine, as in segment hPTH(1−12), **6**

H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-OH
6

the first step was modified by substitution of DEA/CH_3CN[15] for TAEA/DCM in the deblocking step in order to avoid loss of the glycine unit in the aqueous washings. In this case, following evaporation of excess DEA and CH_3CN, the residue is dissolved in DCM and treated with Fmoc-Leu-F. The DBF present from the first deblocking step does not interfere in the coupling process and following addition of TAEA is removed along with that generated in the second step. The same technique can be used in any step in which emulsion formation accompanies workup following TAEA treatment. As an example DEA/CH_3CN deblocking applied only at the octa- and undecapeptide stages in the case of **6** leads to a 30% increase in the yield of the fully protected segment.

As a further improvement of the rapid, continuous process for large-scale work we considered switching from Fmoc to the more recently developed Bsmoc chemistry.[16]

In this case, also previously examined for small-scale syntheses, a simplification of the process is brought about by the ease of removal of byproducts **7** and **8** formed following the deblocking step by avoidance of the use of an acidic buffer.



7 **8**

Both **7** and **8**, the former derived from the protecting group and the latter from excess amino acid fluoride, are soluble in water or saturated sodium chloride and removed by simple extraction. There is less chance for emulsion formation than with buffer extraction of **1** or **2**. In addition, less TAEA is required since the deblocking process is significantly faster in the Bsmoc case. Acceleration of Bsmoc- over Fmoc-deblocking is seen wherever simple primary or secondary amines are used for the deblocking step.

The Bsmoc process was first examined in the case of the 9-amino acid segment **9**

H-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH_2
9

of human growth hormone releasing factor, hGRF(21−29).[17] A preliminary synthesis showed that, following the deblocking (TAEA) and aqueous extraction steps, a small spot always remained at the baseline upon sampling of the reaction mixture by TLC analysis. If not removed at each step the material responsible for this spot contributed to the slow but steady build-up of an impurity which was visible as a diffuse background absorption by HPLC analysis. No attempt was made to identify this material. To avoid this problem in each cycle, a step was added which involved filtration through a short bed of silica gel of the partially deblocked peptide dissolved in a mixture of ethyl acetate and methanol.

A second problem made evident during the preliminary synthesis involved the incomplete washing out of the residue derived from the excess of Bsmoc-Gln(Trt)-F used in the introduction of glutamine.[18] Switching to Dmcp protection[12,18] in place of the trityl group avoided this difficulty. When these modifications were introduced into the protocol for the assembly of **9**, a 3 mmol run gave 3.6 g (69.3%) of the completely deblocked 9-mer. LC/MS analysis showed several unidentified impurities along with about 5% of the peptide retaining a BOC residue (Figure 3). Presumably this could be eliminated by a more complete final deblocking procedure. Detailed conditions and HPLC curves for each stage in the

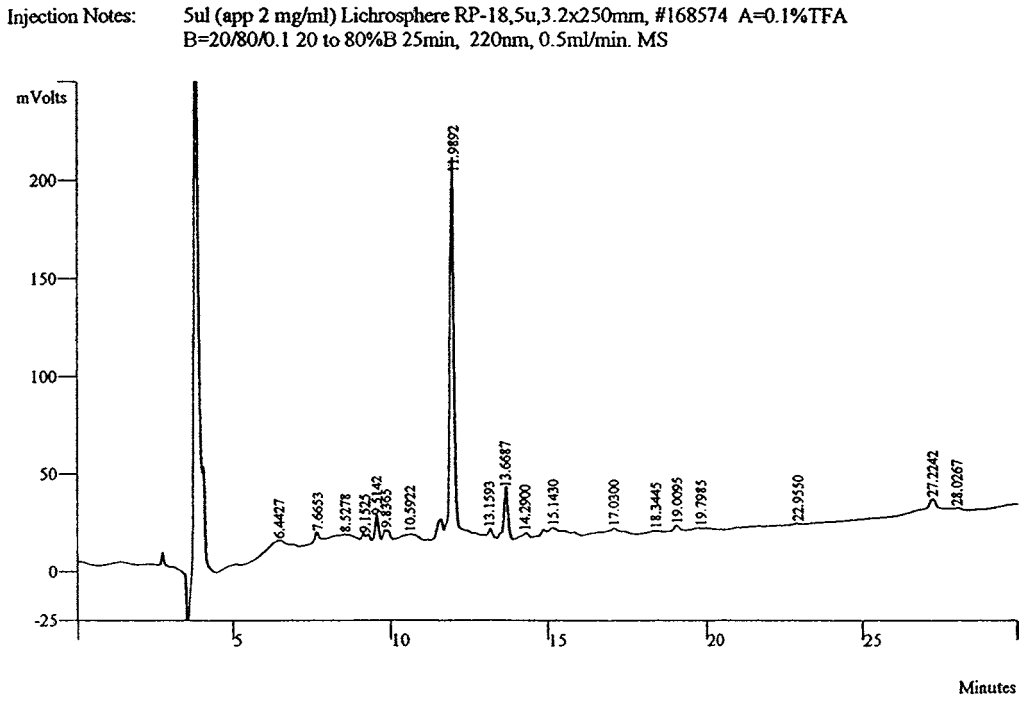


Figure 3. **Fully deprotected GRF (21−29) obtained by the improved standard procedure.**

synthesis are shown in Table 1 and Figure 3a−i (Supporting Information). With these modifications in the standard procedure for the rapid continuous Bsmoc protocol it is believed that this approach has advantages relative to the Fmoc approach, especially for larger-scale syntheses. For shorter peptides the step involving filtration through a bed of silica gel may be omitted.

Summary

It is shown that peptides of 4−22 amino acids in length can be synthesized by rapid, continuous solution methods using either Fmoc or Bsmoc chemistry. Deblocking byproducts formed in the former case are removed with a phosphate buffer, while in the latter simple aqueous extractions are possible. The latter method is thus experimentally simpler to execute. Earlier, small milligram-scale syntheses of such peptides have been described, whereas in the present work these techniques have been scaled up to quantities of 3−100 g. For the first time it was shown that the method can be used for a longer sequence, namely a 22-mer. In the Bsmoc case the process was refined to improve the quality of the crude product by adding in each cycle filtration through a short bed of silica gel to remove a persistent polar impurity present in small amount.

Experimental Section

General. All peptides were identified by mass spectral analysis and in some cases amino acid analysis and, where possible, co-injection onto an HPLC column in the presence of an authentic sample. All new compounds showed consistent IR and NMR spectral data and elemental analyses of C, H, and N that agreed with the theoretical values (±0.3%). The phosphate buffer of pH 5.5 was prepared by dissolving 90 g of $NaHPO_4 \cdot H_2O$ in 500 mL of water. After complete dissolution 32.7 g of dibasic phosphate (Na_2HPO_4, 99.5%) was added and the mixture stirred until totally dissolved. The buffer solution was filtered if necessary and checked with a pH meter to verify the pH. If necessary, dilute NaOH or H_3PO_4 was added to adjust the pH. HPLC analyses were carried out with the system A = H_2O (0.1% TFA). B = ACN (0.1% TFA) according to the various gradients given.

Large-Scale Synthesis of Acetyl-Ser-Asp-Lys-Pro-OH. For this synthesis 4-aminomethylpiperidine was used in place of TAEA.[1] The solvent volumes given for this preparation could undoubtedly be reduced, but the synthesis was not optimized. Below are the two TLC systems which were used in monitoring the coupling and deblocking reactions:

TLC System One: methylene chloride:acetone 9:2, UV/ninhydrin

TLC System Two: *n*-butanol:acetic acid: water 4:1:1, UV/ninhydrin

A 4-L Erlenmeyer flask equipped with a magnetic stir bar was charged with 45.9 g (0.221 mol) of L-proline-*tert*-butyl ester hyrochloride, 113.9 g (0.243 mol) of Fmoc-Lys-(BOC)-OH, and 1.2 L of methylene chloride. The suspension was stirred, and 30.7 g (0.243 mol) of diisopropylcarbodiimide and 22.4 g (0.221 mol) of triethylamine were added. The mixture was stirred for 2 h. TLC (System One) indicated that the reaction was complete. With the suspension being stirred, 441 mL (3.661 mol) of 4-aminomethylpiperidine was added, and the mixture was stirred for 1 h. The suspension was filtered, the precipitate was washed with portions of methylene chloride, and the filtrate was diluted to a volume of 16 L with methylene chloride. The solution was then washed with 3 × 8.0 L (total of 24 L) of 10% (w/v) phosphate buffer, pH 5.5. The methylene chloride solution was dried over 300 g of sodium sulfate, and TLC (System Two) indicated completion of the reaction. The solution was vacuum-filtered and concentrated to a volume of 0.9 L. The mixture was vacuum-filtered, and the solid was washed with 3 × 0.1 L of methylene chloride. The volume of this solution was 1.2 L.

The solution was stirred, and100.0 g (0.243 mol) of Fmoc-Asp(O-*t*-Bu)-OH and 30.7 g (0.243 mol) of diisopropylcarbodiimide were added. The mixture was stirred for 1 h. TLC (System One) indicated that the reaction was complete. With the suspension being stirred, 441 mL (3.661 mol) of 4-aminomethylpiperidine was added and the mixture stirred for 1 h. The suspension was vacuum-filtered, the precipitate was washed with portions of methylene chloride, and the filtrate was diluted to a volume of 16 L with methylene chloride. The solution was washed with 3 × 8.0 L (total of 24 L) of 10% (w/v) phosphate buffer, pH 5.5. The methylene chloride solution was dried over 300 g of sodium sulfate, and TLC (System Two) indicated completion of the reaction. The solution was vacuum-filtered and concentrated to a volume of 0.9 L. The mixture was vacuum-filtered, and the solid was washed with 3 × 0.1 L of methylene chloride. The volume of this solution was 1.2 L. The solution was stirred, and 93.2 g (0.243 mol) of Fmoc-Ser-(*t*-Bu)-OH and 30.7 g (0.243 mol) of diisopropylcarbodiimide were added. The mixture was stirred for 1 h. TLC (System One) indicated that the reaction was complete. With the suspension being stirred, 441 mL (3.661 mol) of 4-aminomethylpiperidine was added and the mixture stirred for 1 h. The suspension was vacuum-filtered, the precipitate was washed with portions of methylene chloride, and the filtrate was diluted to a volume of 16 L with methylene chloride. The solution was washed with 3 × 8.0 L (total of 24 L) of 10% (w/v) phosphate buffer, pH 5.5. The methylene chloride solution was dried over 300 g of sodium sulfate, and TLC (System Two) indicated completion of the reaction. The solution was vacuum-filtered and concentrated to dryness. The residue was dissolved in 0.3 L of methylene chloride and vacuum-filtered, and the solid was washed with 2 × 0.17 L of methylene

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(18) Any amino acid which carries a hydrophobic group as part of the R-group of species **8** should be subject to this difficulty. The Asn analogue can be handled similarly.

chloride. The filtrate was stirred, and 0.16 L (1.695 mol) of acetic anhydride and 22.4 g (0.221 mol) of triethylamine were added. The solution was stirred for 1 h. TLC (System Two) indicated the reaction was complete. The solution was concentrated to an oily residue. The residue was triturated with 3 × 0.5 L of diisopropyl ether. The solid was vacuum-filtered, and the white solid was dried to yield 119.3 g (71%) of the fully protected tetrapeptide as a white solid. ESI MS calcd [M + 1] 756, found [M + 1] 756.6; [M + Na] 778.7. See Figures 4 and 5 (Supporting Information).

An Erlenmeyer flask equipped with a magnetic stirrer was charged with 17.9 g (0.023 mol) of the white solid, and 0.179 L of a 95% (v/v) solution of trifluoroacetic acid in methylene chloride was added. The solution was stirred for 2 h, and HPLC (95/5 0.1% trifluoroacetic acid/acetonitrile isocratic at 220 nm, 1.0 mL/min, 5 μm, C_{18} column) indicated that the reaction was complete. The solution was concentrated, and the residue was triturated with 2 × 0.1 L of diisopropyl ether. The solid was filtered to give the crude peptide which was purified by preparative HPLC on a Prochrome C_8 amicon column (80 mm × 40 cm). The crude peptide was lyophilized to reduce TFA and any residual solvents, dissolved in deionized water and eluted with unbuffered water. Analytical HPLC was used to follow the separation. Desired fractions were combined, filtered with a 0.45 μm filter to remove any particulates, and lyophilized to a powder. The yield was 10.46 g (65.0%), based on the weight of tetrapeptide determined relative to a known standard. According to the HPLC trace UV detection (220 nm) gave a final purity of 99.7%. The analytical chromatogram of the purified product (with its corresponding peak denoted by an asterisk) is shown in Figure 6 (Supporting Information). ESI MS calcd [M + 1] 488, found [M + 1] 488.4; [M + Na] 510.3. See Figure 7 (Supporting Information).

Synthesis of the Protected form of Octapeptide 4. In carrying out this synthesis the assembly was initially stopped at the tripeptide stage to verify by ^{1}H NMR examination that the methyl ester group survived treatment with the deblocking agent TAEA. Once this had been established, the remainder of the synthesis was carried out without stopping at any other stage.

(a) Synthesis of Fmoc-His(Trt)-Leu-OMe. To a stirred solution of H-Leu-OMe·HCl (293 mg, 1.6 mmol) and Fmoc-L-His(Trt)-OH (1.9 g, 1.76 mmol) in 20 mL of CH_2Cl_2 was added DIEA (856 μl, 5.1 mmol) and then N-HAPyU (691 mg, 1.6 mmol). The reaction mixture was stirred for 30 min, the reaction being followed by TLC ($HCCl_3$, MeOH, HOAc; 94/5/1). The reaction was stopped by washing with H_2O (5 mL), saturated $KHSO_4$ (5 mL), H_2O (5 mL) and saturated NaCl (5 mL). After drying over $MgSO_4$, the solvent was evaporated in vacuo to give a white solid which was recrystallized from hexane to give 1.0 g of the pure dipeptide as white crystals (83.3%); ^{1}H NMR ($CDCl_3$) δ 0.87−0.96 (dd, 6, Leu), 1,25−1.27 (m,1, Me_2CH), 1.6−1.7 (d, 2, CH_2-CH), 2.9−3.00 (d, 2, His), 3.67 (s, 3, OCH_3), 4.3−4.4 (m, 3, Fmoc), 4.5 (m, 2, His-Leu), 6.65.(m, 2, His), 7.0−7.7.-(m, 23, Fmoc + Trt + aryl).

(b) Synthesis of Fmoc-D-Ala-His(Trt)-Leu-OMe (Two Phase). Without further purification the dipeptide described above (300 mg, 1.39 mmol) was dissolved in 5 mL of CH_2-Cl_2, and the solution was treated with 2 mL of TAEA with stirring for 10 min. The reaction mixture, initially containing a white precipitate, was extracted with saturated NaCl solution (2 × 4 mL), followed by a phosphate buffer of pH 5.5 (3 × 4 mL). To the clear organic layer (10 mL) was added 4 mL of 5% Na_2CO_3, followed by 147 mg (0.47 mmol) of Fmoc-D-Ala-F. The mixture was stirred for 30 min, and the organic layer containing Fmoc-D-Ala-His(Trt)-Leu-OMe [Nin(−)] was collected and washed with 5% $NaHCO_3$, H_2O, and NaCl. After drying over $MgSO_4$, the solvent was evaporated in vacuo to give a white solid, which after recrystallization from CH_2Cl_2/hexane gave 300 mg (90%) of the tripeptide ester as white crystals, ^{1}H NMR ($CDCl_3$) δ 8.84−0.96 (dd, 6, ($C\underline{H}_3$)$_2$CH, 1.3 (m, 1, ($C\underline{H}_3$)$_2C\underline{H}$, 1.4 (d, 3 $C\underline{H}_3$CH), 1.6−1.7 (m, 2, $Me_2CHC\underline{H}_2$), 2.9 (d, 20 Im$C\underline{H}_2$), 3.7 (s, 3, OCH_3, 4.25−4.4 (m, 3, $\underline{C}HCH_2$) 4.5−4.8 (m, 3, Leu-His-Ala), 6.67 (s, 2, His), 7.0−7.8 (m, aromatic).

(c) Synthesis of Boc-D-PFPhe-Gln(Trt)-Trp-Ala-Val-D-Ala-His(Trt)-Leu-OMe. Without further purification the tripeptide described above (120 mg, 1.14 mmol) was dissolved in 5 mL of CH_2Cl_2, the solution was treated with 2 mL of TAEA, and the mixture was stirred for 10 min. After the mixture was washed with saturated NaCl (2 × 4 mL), followed by phosphate buffer, pH 5.5 (3 × 4 mL), the volume was adjusted to 10 mL with CH_2Cl_2, and 4 mL of 5% Na_2CO_3 was added followed by 62 mg (0.18 mmol) of Fmoc-Val-F. The reaction mixture was stirred for 30 min, and the organic layer was separated and treated with 3 mL of TAEA to start a new cycle. The same procedure was repeated four times using Fmoc-L-Ala-F (56 mg, 0.18 mmol), Fmoc-Trp-F (77 mg, 0.18 mmol), Fmoc-Gln(Trt)-F (110 mg, 0.18 mmol), and Boc-D-PFPhe-F (64 mg, 0.18 mmol). After the last coupling was completed, the organic layer (20 mL) was collected, washed with H_2O (5 mL), 5% $NaHCO_3$ (5 mL), and saturated NaCl (5 mL), and dried over $MgSO_4$; the solvent was evaporated in vacuo to give a white precipitate. Recrystallization from CH_2Cl_2/hexane gave 170 mg (69.2%) of the pure protected octapeptide; TLC using $CHCl_3$/MeOH/HOAc (94/5/1) showed one spot (R_f = 0.68); ^{1}H NMR ($CDCl_3$) δ 0.7 (d, 6, ($C\underline{H}_3$)$_2$CH), 0.9 (d, 6, ($C\underline{H}_3$-CH), 1.35 (m, 2, CH_2Leu), 1.49 (m, 2, CH_2Gln), 3.03 (d, 2 CH_2Gln), 3.26 (d, 2, CH_2His), 3.7 (s, 3, OCH_3), 4.2 (d, 2, CH_2PFPhe), 4.45. (d, 2, CH_2Trp, 6.8 (s, 1, His), 6.9 (s, 1, His), 7.0−7.3 (m, 30, aromatic).

Synthesis of Octapeptide 4. To 90 mg of the protected octapeptide described above was added 2 mL of reagent B [TFA, phenol, water, triisopropylsilane (8.8/0.5/0.5/0.2)]. The reaction mixture was stirred at room temperature for 2 h and poured into a centrifuge tube, which contained 10 mL of anhydrous ether (cooled in a dry ice/ethanol bath). The mixture was centrifuged twice, and the liquid was decanted away. The deblocked peptide was precipitated in the same tube from methanol/ether. After the precipitate had separated, the tube was centrifuged and washed with anhydrous cold ether three times. The solid was dried by passage of dry N_2

gas over the surface for 30 min. The off-white precipitate (39 mg, 77%) was 97% pure based on HPLC analysis for which the main peak was found at 20.88 min for the system B (10−40) 20 min, hold 5 min. Co-injection under these conditions with an authentic sample confirmed the identity of the deblocked octapeptide methyl ester. See Figure 8 (Supporting Information).

Amino Acid Analysis (University of Massachusetts Core Facility)

amino acid	number of amino acids	
	calcd	found
glutamic acid	1	1.06
histidine	1	1.01
alanine	2	2.07
valine	1	1.05
leucine	1	1.04

Note: Trp and PFPhe did not show up in this analysis. Mass spectrometry (University of Nebraska): MS/FAB: calcd M 1074; found [M + 1] = 1075.4. See Figure 9 (Supporting Information).

Synthesis of h-PTH(23−34), H-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-NH_2. (a) Preparation of Fmoc-Phe-NH-Dmcp. To a solution of Fmoc-Phe-F (2 g, 5.14 mmol) and H_2N-Dmcp•*p*-TsOH (2 g, 7.4 mmol) in 35 mL of CH_2Cl_2 was added DIEA (2.4 mL, 14.8 mmol). The reaction mixture was stirred for 15 min, diluted with 10 mL of CH_2Cl_2, and then washed with 20 mL of H_2O, 20 mL of 10% $NaHCO_3$, 20 mL of H_2O, and 20 mL of saturated NaCl solution. After drying over $MgSO_4$ the solution was evaporated in vacuo to give a white solid, which was crystallized from ether/hexane to give 1.9 g (78.98%) of the amide as a white solid, mp 124−125 °C, which was used without further purification in steps (b) and (c) below.

(b) Synthesis of Fmoc-Trp(Boc)-Leu-Arg(Pbf)-Lys-(Boc)-Lys(Boc)-Leu-Gln(Trt)-Asp(O-*t*-Bu)-Val-His(Trt)-Asn(Trt)-Phe-NH-$CMe_2C_3H_5$ with Base. Fmoc-Phe-NH−$CMe_2C_3H_5$ (850 mg, 1.8 mmol) was dissolved in 15 mL of CH_2Cl_2 and treated with 7 mL of TAEA; the mixture stirred for 15 min. A white precipitate was formed which was dissolved during subsequent extraction with NaCl. The reaction mixture was extracted with saturated NaCl solution (2 × 7 mL) followed by phosphate buffer of pH 5.5 (2 × 7 mL) and saturated NaCl solution (1 × 7 mL). To the clear organic layer containing H-Phe-NH-$CMe_2C_3H_5$ were added Fmoc-Asn(Trt)-F (1.5 g, 2.5 mmol) and DIEA (436 μl, 2.5 mmol). The reaction mixture was stirred for 20 min after which 7 mL of TAEA was added directly to the same flask. The reaction mixture was stirred for 15 min, and 5 mL of CH_2Cl_2 was added. The resulting mixture was extracted with two 7-mL portions of saturated NaCl solution followed by two 7-mL portions of phosphate buffer (pH 5.5). The clear organic layer containing the Fmoc-deblocked dipeptide amide was dried over $MgSO_4$. The dry solvent mixture containing H-Asn(Trt)-Phe-NH-$CMe_2C_3H_5$ was cooled in an ice bath and there was added Fmoc-His(Trt)-OH (1.6 g, 2.6 mmol), DIEA (872 μL, 2.6 mmol) and N-HATU (950 mg, 2.6 mmol). The reaction mixture was stirred for 30 min. The course of the reaction was controlled by TLC ($CHCl_3$/MeOH/HOAc, 9/1/1). The reaction was stopped by dilution with 40 mL of ethyl acetate followed by washing with H_2O (20 mL, 10% $NaHCO_3$ (20 mL), H_2O (20 mL), and saturated NaCl solution (20 mL). After drying over $MgSO_4$, the solvent was evaporated in vacuo to give an off-white solid, which was dissolved in 20 mL of CH_2Cl_2. The clear organic layer containing Fmoc-His(Trt)-Asn(Trt)-Phe-NHC$Me_2C_3H_5$ was treated with 7 mL of TAEA as described above followed by addition of Fmoc-Val-F (1.1 g, 2.5 mmol). After coupling, the Fmoc group was deblocked using TAEA (7 mL). The next five couplings were made in the same way with 1.1 g (2.5 mmol) of Fmoc-Asp(O-*t*-Bu)-F, 1.6 g (2.5 mmol) of Fmoc-Gln(Trt)-F, 924 mg (2.5 mmol of Fmoc-Leu-F, 1.2 g (2.5 mmol) of Fmoc-Lys(Boc)-F, and 1.2 g (2.5 mmol) of Fmoc-Lys(Boc)-F in 40 mL of CH_2Cl_2. After deblocking the Fmoc group from the nonapeptide using TAEA and following the regular washing procedure described previously, the clear organic layer containing the Fmoc-deblocked nonapeptide was dried over $MgSO_4$ and cooled to 0° C in an ice bath under N_2. Fmoc-Arg(Pbf)-OH (1.6 g, 2.6 mmol), DIEA (872 μl, 2.6 mmol), and a mixture of N-HATU (960 mg, 2.6 mmol) and HOBt (353 mg, 2.6 mmol) were added to the reaction mixture which was stirred for 15 min in an ice bath and for 10 min at room temperature. The reaction was stopped by diluting with 80 mL of ethyl acetate and washing with H_2O (40 mL), 10% $NaHCO_3$ (40 mL), saturated NaCl (40 mL). After the mixture was dried over $MgSO_4$, the solvent was removed to give an off-white solid which was dissolved in 30 mL of CH_2Cl_2. The clear organic layer contained Fmoc-Arg(Pbf)-Lys(Boc)-Lys(Boc)-Leu-Gln(Trt)-Asp(O-*t*-Bu)-Val-His(Trt)-Asn(Trt)-Phe-NH-Dmcp which was treated with 10 mL of TAEA for 15 min. Following the regular washing procedure described above, Fmoc-Leu-F (924 mg. 2.5 mmol) and Fmoc-Trp(Boc)-F (526.44 mg, 2.5 mmol) were added sequentially. The fully protected 12-mer was diluted with 50 mL of CH_2Cl_2 and washed with H_2O, 10% $NaHCO_3$, and saturated NaCl. Solvent was removed, and the remaining solid was dissolved in EtOAc (∼15 mL) and precipitated using hexane (∼20 mL). The white precipitate was collected by suction filtration to give 3.17 g (54.44%) of the fully protected peptide. Of this sample, 200 mg (0.06 mmol) was treated with 2 mL of CH_2Cl_2 and 1 mL of TAEA for 20 min at room temperature. Dilution with 10 mL of CH_2Cl_2 was followed by washing with saturated NaCl solution (2 × 5 mL each), buffer (2 × 5 mL), and saturated NaCl (1 × 5 mL). Solvent was removed, and the residue was treated with reagent R (5 mL) for 2 h (left at room temperature without stirring). Solvent was then evaporated using a rotary evaporator fitted with a dry ice−EtOH trap. Then 10 mL of cold CH_2Cl_2 was added, and the solution was evaporated. This process was repeated three times, and then 10 mL of cold dry ether was added and evaporated. This process was repeated three times, after which 10 mL of cold dry ether was added, and evaporation was repeated again three times with this solvent. A white foam remained which was dissolved in the minimum amount of dry MeOH (1 mL) and transferred to a centrifuge tube. Ten milliliters of cold (Dry Ice−EtOH) dry ether was added, and the test

tube was centrifuged to collect all of the free peptide as the TFA salt. The resulting white solid amounted to 52 mg (62.1%). MS-ES: calcd [M] 1396.01; found [M + 1] 1397.3. See Figures 10 and 11 (Supporting Information).

(c) Synthesis of Fmoc-Trp(Boc)-Leu-Arg(Pbf)-Lys-(Boc)-Lys(Boc)-Leu-Gln(Trt)-Asp(O-*t*-Bu)-Val-His(Trt)-Asn(Trt)-Phe-NHCMe$_2$C$_3$H$_5$ Without Base. The synthesis described under (b) above was repeated except that DIEA was omitted (no-base synthesis). The yield was 53.2%. Upon deblocking, the free peptide exhibited MS data MW (M + 1) found 1397.3. HPLC analysis showed a main peak at 15.85 min in agreement with data for the sample described above, B (5−40) 35 min, hold 5 min.

Rapid Solution Synthesis of hPTH (13−34) 5 in the Presence of DIEA. A solution of Fmoc-Phe-NHCMe$_2$C$_3$H$_5$ (156 mg, 0.24 mmol) in 4 mL of CH_2Cl_2 was treated with 3 mL of TAEA, and the solution was stirred for 10 min after which 10 mL of CH_2Cl_2 was added. The resulting solution was extracted with two 7-mL portions of saturated NaCl solution, two 7-ml portions of phosphate buffer (pH 5.5), and one 7-ml portion of saturated NaCl solution. To the clear organic layer was added Fmoc-Asn(Trt)-F (216 mg, 0.36 mmol) and DIEA (56.5 μl, 0.36 mmol). The reaction mixture was stirred for 20 min, after which 5 mL of TAEA was added and the solution stirred for 15 min. The resulting mixture was extracted up to the 12-mer stage as described above for hPTH(23−34). After deblocking the 12-mer, 40 mL of ethyl acetate was added (since washing in CH_2Cl_2 caused an emulsion), and the resulting mixture was extracted with two 25-mL portions of saturated NaCl solution followed by two 25-mL portions of phosphate buffer. The organic layer was removed, and the residue was dissolved in 10 mL of CH_2Cl_2 and treated in the normal manner with Fmoc-Glu(O-*t*-Bu)-F. Normal workup was followed by coupling with Fmoc-Val-F (148 mg, 0.36 mmol), Fmoc-Arg(Pbf)-OH (233 mg, 0.36 mmol), Fmoc-Glu(O-*t*-Bu)-F (154 mg, 0.36 mmol), Fmoc-Met-F (135 mg, 0.36 mmol), Fmoc-Ser(*t*-Bu)-F (139 mg, 0.36 mmol), Fmoc-Asn(Trt)-F (216 mg, 0.36 mmol), Fmoc-Leu-F (128 mg, 0.36 mmol), Fmoc-His(Trt)-OH (223 mg, 0.36 mmol), and Fmoc-Lys(Boc)-F (170 mg, 0.36 mmol). The coupling for Fmoc-Arg(Pbf)-OH was carried out as described for hPTH(23−34) at 0 °C under N_2 using N-HATU (137 mg, 0.36 mmol) and DIEA (125.5 μl, 0.72 mmol). Each coupling and deblocking on average took about 1.5 h. The first 12 amino acids were coupled in 1 day and the remainder the next day for a total time of 34 h. The fully protected 22-mer was diluted in 40 mL of CH_2Cl_2 and washed with saturated NaCl solution; after solvent was removed, the residue was redissolved in EtOAc and precipitated using hexane. The white precipitate was collected using suction filtration to give 325 mg (25.9%) of the protected peptide. To 100 mg (0.02 mmol) of fully protected peptide there was added 2 mL of CH_2Cl_2 and 1 mL of TAEA; the mixture was kept at room temperature for 20 min, diluted with 10 mL of CH_2Cl_2, and washed with saturated NaCl solution (2 × 5 mL), buffer (2 × 5 mL), and saturated NaCl solution (1 × 5 mL). The solvent was removed, and the residue was treated with 5 mL of reagent R for 2 h (left at room temperature without stirring). Solvent was evaporated, and the free peptide was collected, as described for hPTH-(23−34). The free peptide amounted to 37 mg (70.56%). The HPLC of the crude peptide is shown in Figure 1a (purity 88%). Some of the impurity involved residual undeblocked Boc groups and oxidized methionine. The HPLC solvent system involved B (5−40) 35 min, hold 5 min.

ES-MS (Biomeasure): Figure 2a; Calcd [M + 3/3] 935.7, found 936.7; Calcd [M + 4/4] 702.0; found 702.9; Calcd [M + 5/5] 561.8, found 562.3.

Solid-Phase Synthesis of hPTH(13−34) 5. Conditions and reagents were as follows:

support quantity	0.5 g
loading	0.14 meq/g
excess amino acid fluoride/acid	5.00×
DMF	1.4 mL
DIEA	147 μL
N-HATU	171 mg (0.45 mmol)

The synthesis was carried out manually on a Rapp Tenta Gel resin (0.5 g) with loading of 0.14 meq/g, and a 5-fold excess of each amino acid. The synthesis started with DMF (5 × 2 mL) and CH_2Cl_2 (3 × 2 mL) washes. The deprotection of the Fmoc group was carried out by 10 mL of 20% piperidine/DMF for 10 min. Washing by DMF (5 × 2 mL) and CH_2Cl_2 (3 × 2 mL) followed. Each coupling step was carried out by adding Fmoc-AA-F (0.45 mmol) for a 30-min period. At the end of the first coupling (Fmoc-Phe-F, 175 mg) an acetylation was carried out to cap unreacted amino groups. The procedure was as follows: (1) CH_2Cl_2 (4 × 1 min), (2) Ac_2O (10 equiv) in pyridine−CH_2Cl_2 (1:19, 1 × 15 min), (3) CH_2Cl_2 (4 × 1 min), (4) MeOH (4 × 1 min), (5) CH_2Cl_2(4 × 1 min). For Fmoc-His(Trt)-OH and Fmoc-Arg(Pbf)-OH, 0.45 mmol, 5 equiv of the acid in DMF (1.2 mL) was used. After 1 min equivalent amounts of N-HATU (171 mg), HOAt, and DIEA (147 μL) in 0.2 mL of DMF were added, and the solution was allowed to stand for 60 min at room temperature with stirring. This process was repeated twice. The following washes were carried out: (1) DMF (4 × 1 min) and (2) CH_2Cl_2 (4 × 1 min). A 50-mg sample of peptide−resin was deblocked using 20% piperidine in DMF and washed as usual with DMF and CH_2Cl_2; the peptide−resin was suspended in TFA−thioanisole−1,2 ethanedithiol−anisole (90:5:3:2) for 2 h. The cleaved peptide was filtered and washed with TFA−CH_2Cl_2 (1:1) (2 mL) and CH_2Cl_2; the combined solutions were evaporated, chased with ether (5 × 40 mL), and centrifuged to give the crude peptide. The HPLC profile is shown in Figure 1b. The yield was 22%. ES-MS (Biomeasure), see Figure 2b: Calcd [M + 2/2] 1403, found 1404.6; Calcd [M + 3/3] 935.7, found 936.7; Calcd [M + 4/4] 702, found 702.9; Calcd [M + 5/5] 561.8, found 562.5.

Rapid Solution Synthesis of hPTH 1−12 (6). To a solution of 3 g (0.01 mol) of Fmoc-Gly-OH and Dcpm-OH (1.25 g, 0.011 mol) in 50 mL of CH_2Cl_2 was added a mixture of DCC (2.06 g, 0.01 mol) and DMAP (0.122 mg, 0.001 mol). The resulting solution was stirred at room temperature for 1 h, after which CH_2Cl_2 was evaporated in vacuo, and 50 mL of ethyl acetate was added. The white precipitate

(DCU) was filtered off, and the ethyl acetate solution was washed with H_2O (20 mL) saturated $NaHCO_3$ solution (20 mL), and saturated NaCl (20 mL) solution and dried over $MgSO_4$. After removal of solvent, the residue was crystallized from EtOAc−hexane to give 2.9 g (76.71%) of white crystals, mp 118−119 °C. The ester (400 mg) was deblocked by treatment with 8 mL of ACN and 6 mL of DEA at room temperature. The resulting solution was stirred at room temperature for 1 h, the solvent was removed, and the residue was dissolved in EtOAc−hexane (1:1) and evaporated. This process was repeated three times to remove all of the DEA. To the residue of H-Gly-OCH(C_3H_5)$_2$ was added, without removing residual dibenzofulvene, 10 mL of CH_2Cl_2 followed by 530 mg of Fmoc-Leu-F. The resulting mixture was stirred for 20 min, after which 7 mL of TAEA was added, and the reaction mixture was stirred for 15 min. Then 5 mL of CH_2Cl_2 was added, and the resulting mixture was extracted with two 10-mL portions of saturated NaCl solution followed by two 10-mL portions of phosphate buffer. To the clear organic layer containing the dipeptide ester was added Fmoc-Asn(Trt)-F (900 mg, 1.5 mmol). The reaction mixture was stirred for 20 min, the course of the reaction being controlled by TLC ($CHCl_3$/MeOH/HOAc, 9/1/2). Five milliliters of CH_2Cl_2 was added, and the resulting mixture was extracted with saturated NaCl solution and buffer solution as described above. The clear organic layer containing the Fmoc-deblocked tripeptide ester was dried over $MgSO_4$. The dried solution was cooled in an ice bath under N_2 and Fmoc-His-(Trt)-OH (929 mg, 1.5 mmol), DIEA (262 μL, 1.5 mmol) and N-HATU (570 mg, 21.5 mmol) were added. The reaction mixture was stirred for 30 min. The course of the reaction was controlled by TLC. The reaction was stopped by diluting with 40 mL of ethyl acetate and washing with H_2O (20 mL), 10% $NaHCO_3$ (20 mL), H_2O (20 mL), and saturated NaCl solution (20 mL). Solvent was removed, and the residue was dissolved in 15 mL of CH_2Cl_2. The clear organic layer containing Fmoc-His(Trt)-Asn(Trt)-Leu-Gly-O−CH(C_3H_5)$_2$ was treated with 8 mL of TAEA as described above, followed by addition of Fmoc-Met-F (560 mg, 1.5 mmol), Fmoc-Leu-F (530 mg, 1.5 mmol), Fmoc-Gln(Trt)-F (919 mg, 1.5 mmol), Fmoc-Ile-F (530 mg, 1.5 mmol), Fmoc-Glu(O-*t*-Bu)-F (640 mg, 1.5 mmol), Fmoc-Ser(*t*-Bu)-F (578 mg, 1.5 mmol), Fmoc-Val-F (509 mg, 1.5 mmol), and Fmoc-Ser(*t*-Bu)-F (578 mg, 1.5 mmol). After the last coupling, the fully protected 12-mer was diluted with 10 mL of CH_2Cl_2 and washed with H_2O, $NaHCO_3$, and saturated NaCl solution, and the solution was dried over $MgSO_4$. After removal of solvent, the residue was dissolved in EtOAc and precipitated using hexane. Chromatography over silica gel using $CHCl_3$/MeOH (9:1) gave the main spot as 120 mg (4.7%) of the correct peptide. The low yield can be explained, since the Fmoc-deblocked 8- and 11-mers led to emulsion formation during the washes with buffer. To improve the yield, instead of using TAEA for deblocking the Fmoc group from the 8- and 11-mers, DEA was used following the directions given below. After coupling of Fmoc-Ile-F all the solvent was removed, and 15 mL of ACN/DEA (7:8) was added; the reaction mixture was stirred at room temperature for 1 h. After removal of solvent, the residue was dissolved in EtOAc and precipitated using hexane to remove most of the dibenzofulvene. The white precipitate was collected by suction filtration and dissolved in CH_2Cl_2 for the next coupling. The same process was used in deblocking the Fmoc group from the 11-mer. The collected 12-mer amounted to 950 mg (37.43%). It is to be noted that the use of DEA in place of TAEA, for these two steps only, improved the yield by 30%. A sample (200 mg, 0.79 mmol) of the fully protected peptide was tested in 2 mL of CH_2Cl_2/1 mL of TAEA. After 20 min of stirring at room temperature, the solution was diluted with 10 mL of CH_2Cl_2 and the whole washed with saturated NaCl solution (2 × 5 mL), buffer (2 × 5 mL), and saturated NaCl solution (5 mL). The solvent was removed, and the residue was treated with reagent R (5 mL) for 2 h at room temperature. The solvent was removed as described for hPTH(23−34), and the free peptide was collected as a white solid, yield 63 mg (60.01%). By HPLC analysis the peptide was 98% pure. ES-MS: MW calcd 1328.9; found [M + 1]: 1327.6 (Figure 12, Supporting Information). The HPLC conditions were as follows: B (5−50) 30 min, hold 5 min (Figure 13, Supporting Information).

Standard Procedure for Bsmoc/TAEA Large-Scale Synthesis of Peptides in Solution on a Scale of 3 mmol. To the crude Bsmoc-Arg(Pbf)-NH-Dmcp (0.7299 g, 1 mmol) prepared as described below (A) and dissolved in 10 mL of DCM was added 0.75 mL (5 mmol) of TAEA. After stirring for 0.5 h at room temperature the DCM was removed in vacuo, and the residue was stirred with 10% $NaHCO_3$ solution (25 mL) for 10−15 min. The mixture was extracted with EtOAc (3 × 20 mL), and the combined organic extracts were washed with saturated NaCl solution (2 × 25 mL) and water (2 × 25 mL). Without drying, the solvent was removed in vacuo. The residue was dissolved in 10 mL of DCM and the solution treated under stirring with Bmoc-Ser(*t*-Bu)-F (0.5782 g, 1.5 mmol) and 0.35 mL (2 mmol) of DIEA. After 2 h of stirring, TAEA (1.5 mL, 10 mmol) was added, and the resulting mixture was stirred for an additional 0.5 h. DCM was removed in vacuo and the residue treated exactly as given above. Additional coupling and deblocking steps were carried out in the same way. HPLC analysis after both coupling and deblocking could be used to follow the course of the synthesis. Depending on the length of the peptide and the sequence of amino acids, HPLC analysis may not be able to separate the fully protected from the Bsmoc-deprotected segments. For the system described here, this point was reached at the stage of the 15-mer. In addition an unidentified material leading to a diffuse background absorption built up as the chain was extended. This material was avoided by using an improved procedure which is described in detail under (C) below for the 9-mer.

Improved Standard Procedure for the Solution Synthesis of hGRF (21−29) 9 via the Bsmoc/TAEA Method at a 3.0 mmol Scale. (A) Bsmoc-Arg(Pbf)-NH-Dmcp. To a solution of Bsmoc-Arg(Pbf)-OH (6 g, 9.24 mmol), pTsOH·H_2N-Dcmp (3.66 g, 13.5 mmol), and DIEA (6.6 mL, 36 mmol) in 100 mL of DCM at 0 °C under a nitrogen atmosphere was added a mixture of N-HATU (3.42 g, 9

mmol) and HOBt (1.38 g, 9 mmol).[19] The reaction mixture was stirred at 0° for 30 min and 1 h at room temperature. An additional 100 mL of DCM was added, and the mixture was extracted with water (100 mL), 10% $NaHCO_3$ (100 mL), water (100 mL), and saturated NaCl solution (100 mL), dried over $MgSO_4$, and the solvent was removed in vacuo. The residue was dissolved in EtOAc and flash chromatographed using a silica column and EtOAc as eluent. After solvent was removed in vacuo, there remained 6.4 g of the amide as a foam. Yield: 97.4 wt %. The crude product according to ^1H NMR analysis was pure enough to be used as such to initiate the synthesis.

(B) Deprotection of Bsmoc Arg(Pbf)-NH-Dmcp. TAEA (2.25 mL, 15 mmol) was added in one portion to a solution of Bsmoc-Arg(Pbf)-NH-Dmcp (2.1897 g, 3 mmol) in DCM (30 mL) under stirring and at ambient temperature. After 1 h (HPLC indicated the absence of the starting material) the reaction mixture was worked up as mentioned in the "Standard Work-Up Procedure" described below. Yield 1.4468 g (95.1%) of 1-H-GRF29 as a white powder.

(C) Standard Procedure for Working-Up the Reaction Mixture After Deprotection with TAEA. DCM was removed in vacuo, and the residue was stirred with 10% $NaHCO_3$ (50 mL) for 10−15 min. The mixture was extracted with AcOEt (3 × 25 mL), and the combined organic extracts were washed with saturated sodium chloride solution (2 × 25 mL). Without drying, the solvent was removed in vacuo and the residue dissolved in 25 mL of AcOEt/MeOH (1:1) which was flash-filtered though a bed (height = 40 mm, diameter = 30 mm) of silica gel 60 (EM, 230−400 mesh ASTM. The bed of silica gel was washed with an excess of AcOEt/MeOH (1:1) (approximately 75 mL). The combined filtrates were concentrated to dryness in vacuo.

(D) Cycle 1: Coupling of 1-H-GRF29 with Bsmoc-Ser-(*t*-Bu)-F and Deprotection of the Resulting 2-B-GRF29. The residue (1.4468 g) (obtained as described above) was dissolved in DCM (30 mL) and the solution treated under stirring with Bsmoc-Ser(*t*-Bu)-F (1.7346 g, 4.5 mmol) and DIEA (1.05 mL). The reaction mixture was kept stirring at ambient temperature overnight. The next day TAEA (4.5 mL,10 mmol) was added in one portion to the mixture, and after 0.5 h the reaction was complete (by HPLC). The reaction mixture was worked up as mentioned in the "Standard Workup Procedure" described above (C). Yield: 1.7580 g (90.0%).

The remaining cycles were carried out similarly with the full details of the synthesis collected in Table 1 and Figure 3a−i (Supporting Information). Complete deblocking of the protected 9-mer using TFA/H_2O/EDT/thioanisole for 2 h under N_2 gave a sample (3.6 g, 69.3%) for which the major peak showed the correct molecular weight: ESI MS calcd [M + 1] 1102.6; found [M + 1] 1102.4. See Figure 14, Supporting Information.

(19) In model studies it was shown that this "mixed" system afforded less lactam side product during the coupling process. For the model coupling of Fmoc-D-Arg(Pmc)-OH + Dmcp-NH_2 at 0° C in DCM for 30 min the percent of lactam and amide, respectively, were as follows:

coupling system	lactam	amide
N-HATU/HOBt/DIEA	–	97.1
N-HATU/HOBt/TMP	32.9	65.1
N-HBTU/HOBt/DIEA	–	96.2
N-HBTU/HOBt/TMP	54.7	33.1
TFFH/ DIEA	99.0	–
DCC/DMAP	47.0	28.8

Generally when stand-alone coupling reagents derived from HOBt or HOAt are used in the presence of the latter as additive, the choice of additive parallels that built into the coupling reagent. For other cases in which mixed systems are reported, see: (a) Lescrinier, T.; Hendrix, C.; Kerremans, L.; Rozenski, J.; Link, A.; Samyu, B.; Van Aerschot, A.; Lescrinier, E.; Eritja, R.; Van Beeumen, J.; Herdewijn, P. *Chem. Eur. J.* **1998**, *4*, 425. (b) Zhang, Z.; Van Aerschot, A.; Chaltin, P.; Busson, R.; Herdewijn, P. *Collect. Czech. Chem. Commun.* **2001**, *66*, 923. (c) Suter, G.; Stoykova, S. A.; Linden, A.; Heimgartner, H. *Helv. Chim. Acta* **2000**, *83*, 2961. (d) Tselios, T.; Daliani, I.; Probert, L.; Deraos, S.; Matsoukas, E.; Roy, S.; Pires, J.; Moore, G.; Matsoukas, J. *Bioorg. Med. Chem.* **2000**, *8*, 1903.

Acknowledgment

We are indebted to the National Science Foundation (NSF CHE-0078971) the National Institutes of Health (GM-09706), and Biomeasure, Inc, for support of the work carried out in Amherst.

Supporting Information Available

Appropriate HPLC and MS data for all of the peptides described here. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review July 3, 2002.

OP0202179