Facile Solid-Phase Synthesis of Sulfated Tyrosine-Containing Peptides: Part II. Total Synthesis of Human Big Gastrin-II and Its C-Terminal Glycine-Extended Peptide (G34-Gly Sulfate) by the Solid-Phase Segment Condensation Approach[1,2)]

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Application of the fluoren-9-ylmethoxycarbonyl (Fmoc)-based solid-phase segment condensation approach to the preparation of sulfated peptides was investigated through the synthesis of human big gastrin-II, a 34-residue sulfated tyrosine [Tyr(SO_3H)]-containing peptide. Highly acid-sensitive 2-chlorotrityl resin (Clt resin) was exclusively employed as an anchor-resin for the preparation of the three peptide segments having the C-terminal Pro residue as well as of the Tyr(SO_3H)-containing resin-bound segment. By using the PyBOP-mediated coupling protocol [PyBOP=benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate], we successively condensed each segment and constructed the 34-residue peptide-resin without any difficulty. The final acid treatment of the fully protected peptide-resin at low temperature (90% aqueous TFA, 0 °C for 8 h), which can detach a Tyr(SO_3H)-containing peptide from the resin and remove the protecting groups concurrently with minimum deterioration of the sulfate, afforded a crude sulfated peptide. After one-step HPLC purification, a highly homogeneous human big gastrin-II was easily obtained in 14% yield from the protected peptide-resin. The sulfate form of the C-terminal glycine-extended gastrin (G34-Gly sulfate), a posttranslational processing intermediate of gastrin-II, was also successfully prepared with the segment condensation approach (11% yield). These results demonstrated the usefulness of the segment condensation protocol for preparing large Tyr(SO_3H)-containing peptides.

Key words segment condensation; sulfated tyrosine-containing peptide; big gastrin-II; 2-chlorotrityl resin; glycine-extended gastrin

Many synthetic achievements of Tyr(SO_3H)-containing peptides have been reported including the solid-phase approach[3)]; however, a versatile synthetic method for them has not been established yet. The major difficulty in the preparation of Tyr(SO_3H)-containing peptides lies in the intrinsic acid-lability of the Tyr(SO_3H) residue. We previously reported a facile solid-phase method for the synthesis of Tyr(SO_3H)-containing peptides.[2,4)] This method is based upon the Fmoc-based stepwise protocol for elongation of the peptide chain[5)] and involves two key features to complete the synthesis in an efficient manner: (i) utilization of highly acid-sensitive 2-chlorotrityl (Clt) resin[6)] to quantitatively detach a sulfated peptide from the solid-support, and (ii) trifluoroacetic acid (TFA)-based deprotection (90% aqueous TFA, 0 °C)[2,4b)] to minimize the deterioration of the sulfate. Sulfated peptides consisting of over 30 amino acid residues, such as big gastrin-II (34 residues), cholecystokinin (CCK)-33 and CCK-39, were prepared using this method without notable difficulties. However, we assume that the larger the sulfated peptides the harder the homogeneous products are to obtain by the stepwise elongation approach. Defective peptides derived from the incomplete coupling often have properties similar to those of the objective peptide, and they would make the final purification time-consuming and difficult. Segment condensation on a solid support (convergent approach) is an attractive alternative to the stepwise elongation approach in the solid-phase peptide synthesis (SPPS). Its advantages have been discussed in many examples.[7,8)] The most promising aspect of the segment condensation approach would be the fact that the purification of peptides synthesized by this approach is much easier than that of peptides synthesized by the stepwise elongation approach. The sizes of the side products derived from incomplete couplings are quite different from that of the desired peptide. Therefore, they are effectively removed by simple chromatographic work-ups.

As a part of our program for establishment of a versatile synthetic method aiming at the biological studies of Tyr(SO_3H)-containing peptides, we investigated the usefulness of the solid-phase segment condensation approach through the synthesis of human big gastrin-II. The C-terminal glycine-extended gastrin sulfate (G34-Gly sulfate), a posttranslational processing intermediate of human big gastrin-II, was also synthesized with the segment condensation approach.

Results and Discussion

The convergent synthesis of human big gastrin-II is outlined in Chart 1. In order to circumvent the racemization problem accompanying the segment condensation, the three Pro residues (Pro^8, Pro^{14} and Pro^{20}) were selected as coupling positions. Thus the sequence of big gastrin-II was divided into four peptide segments: the resin-bound C-terminal segment [**1a**] and three peptide segments with the Pro residues at the C-termini [**2**]—[**4**]. Clt resin was exclusively employed for preparation of these segments. We have already reported the efficient use of the Clt resin for preparation of Tyr(SO_3H)-containing peptides. Detachment of a sulfated peptide from this resin was completed quantitatively under extremely mild conditions without affecting the acid-labile Tyr(SO_3H) residue.[2,4)] In addition, the Clt resin is superior in several aspects to the conventional *p*-benzyloxybenzylalcohol-type resin (Wang resin)[9)] in the preparation of side-chain

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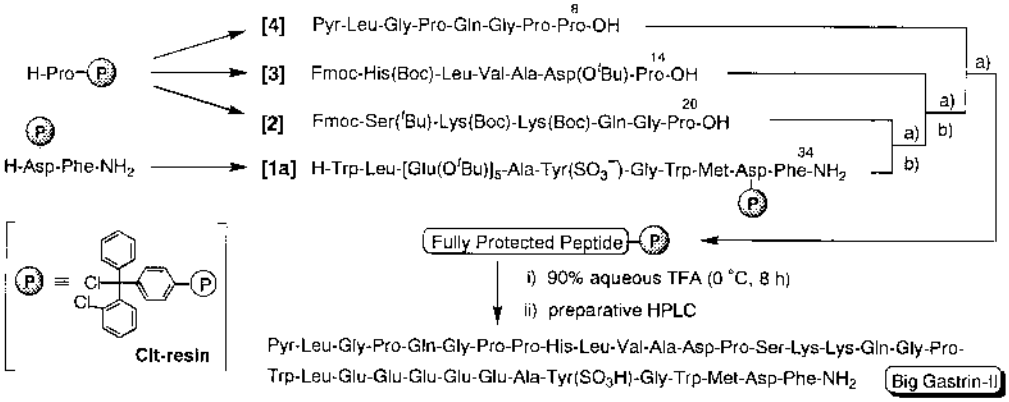

Chart 1. Synthetic Scheme for Human Big Gastrin-II by the Solid-Phase Segment Condensation Approach

a) PyBOP reagent-HOBt-NMM; b) 20% piperidine/DMF.

protected peptide segments with the Fmoc-based SPPS protocols: (i) Clt resin is so sensitive to mild acidic conditions that the acid-sensitive ^{t}Bu-based side-chain protecting groups usually remain intact, and (ii) Clt resin can minimize the premature detachment of a dipeptide from the resin through diketopiperazine (DKP) formation at the dipeptide step.[10] The latter point is especially crucial for the preparation of a peptide having the C-terminal Pro residue[11] by the Fmoc-based SPPS. To suppress this side reaction, the use of sterically hindered trityl-based[10] or *tert*-alcohol-based[12] linkers has been recommended.

First, the C-terminal resin-bound segment [**1a**] containing a Tyr(SO_3H) residue was constructed on Fmoc-Asp(Clt resin)-Phe-NH_2. We utilized the β-carboxyl group of the Asp residue to form an acid-labile ester linkage between the C-terminal dipeptide amide and a Clt resin; the amide-offering linker-resin which allows the quantitative peptide detachment under our acidic cleavage/deprotection conditions (90% aqueous TFA, 0 °C) is not available. The peptide chain was elongated manually according to the general procedures of the Fmoc-based SPPS, and the Tyr(SO_3H) residue was introduced using Fmoc-Tyr(SO_3Na)-OH as a building block. To diagnose the completeness of the elongation reaction, a small portion of the protected 15-mer peptide-resin was subjected to a cleavage reaction with hexafluoro-2-propanol (HFIP)/CH_2Cl_2 (1 : 4 v/v, 25 °C, 30 min).[13] The purity of thus detached protected-peptide on a RP-HPLC chromatogram was promising (Fig. 1a), and its structural correctness was also confirmed by amino acid analysis.[14] We then decided to conduct the segment condensations directly on this resin-bound segment [**1a**].

Two N^{α}-Fmoc protected segments, [**2**] and [**3**], and a peptide segment bearing no protecting groups [**4**] were constructed on H-Pro-Clt resin according to the general procedures of the Fmoc-based SPPS. The suppressed DKP formation and resultant dipeptide (X-Pro) detachment from the resin were substantiated by the fact that the weight of the peptide-resin reasonably increased after incorporation of the third amino acid residue. Quantitative detachment of peptides from the Clt resin can be achieved by treatment with either a mixture of AcOH/trifluoroethanol/CH_2Cl_2 (1 : 1 : 3 v/v)[6] or a mixture of HFIP/CH_2Cl_2 (1 : 4 v/v).[13] However, we routinely use the latter reagent system to eliminate a trace of AcOH in the peptide segments.

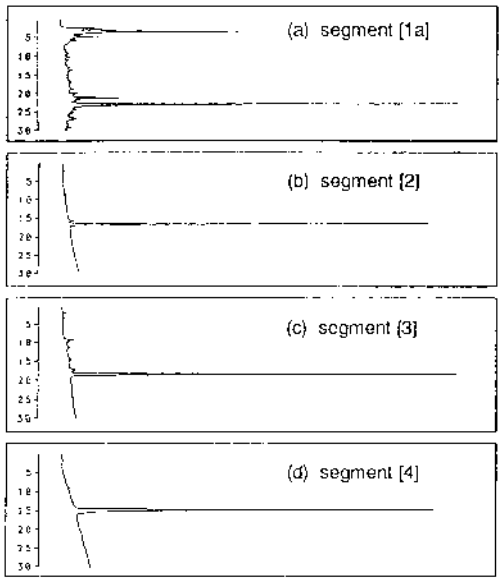

Fig. 1. HPLC Chromatograms of the Segments Used for the Condensations

(a) Elution profile of the protected peptide detached from the resin-bound segment [**1a**]. HPLC conditions for each chromatogram are described in the Experimental section.

Protected peptides for the segment condensations should be reasonably pure and free from the deletion sequences. Therefore, purification of the protected segments becomes another critical problem. Because segments [**2**] and [**3**] bear the acid-sensitive ^{t}Bu/Boc protecting groups, special care must also be taken for their purification. Segment [**2**] was prepared by the following work-ups (Chart 2): (i) detachment of the N^{α}-Fmoc deprotected peptide from the resin using HFIP/CH_2Cl_2 (1 : 4, 20 °C for 30 min), (ii) purification by RP-HPLC using an elution system of CH_3CN and 0.1% TFA, and (iii) Fmoc-protection of the free N^{α}-amino group using fluoren-9-ylmethoxycarbonyl succinimide (Fmoc-OSu). Effectiveness of this temporary N^{α}-deprotection/reprotection strategy to obtain a pure protected peptide segment has been reported by Nishiyama and Okada.[15] The detached crude segment [**3**] containing an extremely acid-sensitive His(Boc) residue[16] was contaminated by a small amount of the peptide with the side-chain deprotected His residue (*ca.* 5% on TLC). This exceptionally acid-sensitive peptide was there-

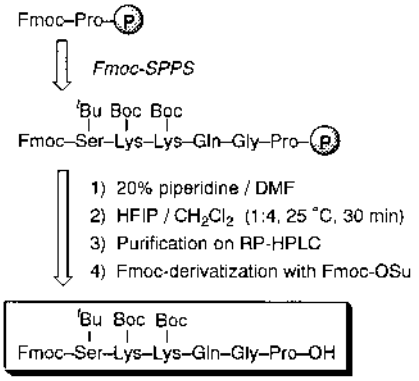

Chart 2. Preparation of Segment [**2**]

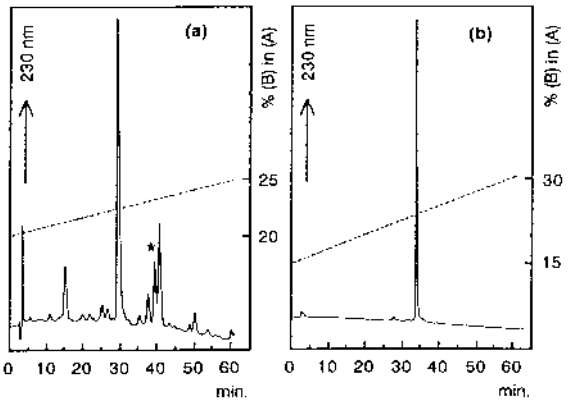

Fig. 2. HPLC Chromatograms of (a) Crude G34-II Obtained after Cleavage/Deprotection and (b) Purified G34-II

Asterisk in (a) shows the desulfated peptide produced during the deprotection. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6×150 mm); elution system, a gradient of CH_3CN (B) in 0.1 M $AcONH_4$ (A) over 60 min at a flow rate of 0.75 ml/min for (a) and (b)].

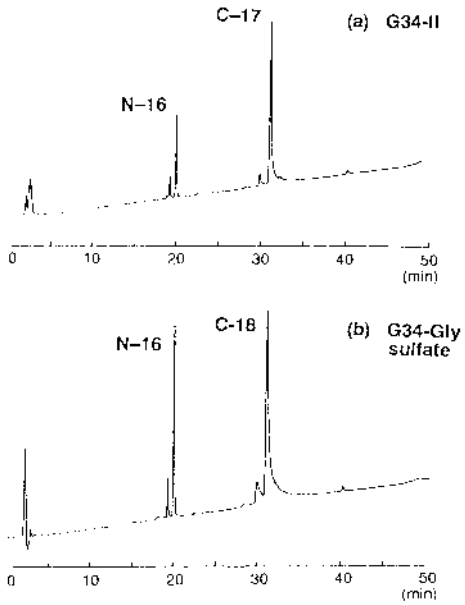

Fig. 3. HPLC Chromatograms of Lysyl Endopeptidase Digest of (a) G34-II and (b) G34-Gly Sulfate

HPLC conditions are described in the Experimental section. N-16 corresponds to the position 1 to 17 fragment and C-17 corresponds to the position 19 to 34 fragment of G34-II. C-18 corresponds to the position 19 to 35 fragment of G34-Gly sulfate.

fore purified by silica gel column chromatography with the N^{α}-Fmoc protected form. The N-terminal segment [**4**] that has no protecting groups was purified by RP-HPLC following the cleavage. After such purification, each segment exhibited high homogeneity on HPLC chromatograms (>97%) as shown in Fig. 1. LSIMS spectra and amino acid ratios in their acid hydrolysates also supported their structural correctness.

The segment condensation was promoted by a PyBOP-mediated coupling protocol [PyBOP-reagent[17] (3 eq), *N*-hydroxybenzotriazole (HOBt) (3 eq), *N*-methylmorpholine (NMM) (9 eq)], which was the slightly modified protocol used for the convergent synthesis of human little gastrin-I, a non-sulfate form of 17-residue gastrin, by Barany and colleagues.[8a] Four equivalents of each segment were used as an acyl component for its condensation step. Because of the low solubility of segment [**2**] in DMF, a mixture of DMF and DMSO (7 : 3 v/v) was used as a solvent for the segment condensation between [**1a**] and [**2**], while the other two segment condensations were conducted in neat DMF. After each condensation reaction (20 °C, 15 h), the possible unreacted N^{α}-amino groups were acetylated with Ac_2O in the presence of pyridine. Following the completion of the peptide chain assembly, the fully protected peptide-resin was subjected to cleavage/deprotection with 90% aqueous TFA (0 °C for 8 h). The ether-precipitated crude peptide (Fig. 2a) was easily purified by one-step preparative RP-HPLC at neutral pH (pH 7.0),[18] and the pure human big gastrin-II (Fig. 2b) was obtained in 14% yield from the cleavage/deprotection. This yield was comparable to that of human big gastrin-II synthesized by the stepwise elongation protocol (13%).[2] The final product prepared by the segment condensation approach coincided with the authentic material obtained by the stepwise approach in all analytical aspects. Lysyl endopeptidase digestion of the human big gastrin-II generated two peptide fragments. They were separated by RP-HPLC (Fig. 3a) and assigned as the N-terminal 16-peptide (N-16) and the C-terminal 17-peptide (C-17) based upon amino acid analysis of their acid hydrolysates and LSIMS.

As an extension of gastrin-II synthesis, we next prepared the C-terminal Gly-extended form of big gastrin-II (G34-Gly sulfate) with the segment condensation approach. The non-sulfated forms of G-Gly peptides[19] are posttranslational processing intermediates of gastrin-I, and their intrinsic biochemical and physiological properties have been documented recently. For example, G-Gly peptides are reported to have mitogenic effects on human colon cancer[20] and pancreatic cancer cell lines.[21] Based on these findings, G34-Gly sulfate is also attracting much interest from biochemical and physiological standpoints. The C-terminal Gly residue is not amidated in the G34-Gly sulfate, thus the C-terminal resin-bound segment [**1b**] for this peptide was newly prepared starting with Fmoc-Gly-Clt resin. The three segments were successively condensed on [**1b**] as described above. After the segment condensations, the fully protected peptide-resin was subjected to cleavage/deprotection with 90% aqueous TFA (0 °C, 8 h). The ether-precipitated crude peptide was easily purified by RP-HPLC at neutral pH to give a homogeneous G34-Gly sulfate. The total yield of purified peptide was 11%

from the cleavage/deprotection, and the structure of this final product was ascertained by amino acid analysis of its acid hydrolysate and LSIMS. Lysyl endopeptidase digestion of this product generated two peptides (Fig. 3b), and they were assigned as N-16 and the C-terminal 18-peptide (C-18). Biological properties of the synthesized G34-Gly sulfate will be reported elsewhere.

In this report, we demonstrated the applicability of the solid-phase convergent approach to the synthesis of the 34- and 35-residue sulfated gastrin peptides. Very mild acidolytic deprotection with TFA (0 °C) followed by one-step purification by RP-HPLC gave human big gastrin-II in a yield comparable to that obtained by the stepwise elongation approach. Having the advantage of the segment condensation, the crude deprotected peptide was less complicated and therefore easily purified by simple RP-HPLC. However, the yields from the two synthetic experiments were not as good as we expected. Several factors, such as presence of the oxidation-sensitive Met and the alkylation-sensitive Trp residues, and the insufficient deprotection of the protecting groups, would be responsible for these results.

As a result of recent advances in the SPPS, small- to medium-size peptides can be obtained in reasonable yields by the stepwise elongation approach; however, the purity and yields of the large peptides (more than 50 amino acid residues) are generally unsatisfactory. The solid-phase convergent synthesis is an attractive alternative to overcome this problem. In the future, large sulfated peptides will be in demand to elucidate the biological function of sulfated tyrosine-containing proteins and to examine the protein-protein interactions promoted by the tyrosine sulfate residues.[22] The thioester segment condensation approach[23] is another promising method to obtain such large-size sulfated peptides. A study on this approach is underway in our laboratory.[24]

Experimental

General Fmoc-amino acid derivatives, PyBOP reagent, Clt resin (substituted level, 1.47 mmol/g resin), and H-Pro-Clt resin (substituted level, 0.43 mmol/g resin) were purchased from Watanabe Chemical Co., Ltd. (Hiroshima, Japan). Fmoc-His(Boc)-OH (cyclohexylamine salt) was purchased from Novabiochem Japan (Tokyo) and used after purification.[25] Lysyl endopeptidase from *Achromobacter lyticus* M497-1 (EC 3.4.21.50) was purchased from Wako Pure Chemicals Ind., Ltd. (Osaka, Japan). Other chemicals were of analytical grade and were used without further purification. Amino acid ratios in an acid hydrolysate [propionic acid/12 N HCl (1 : 1 v/v, 110 °C, 24 h) for resin-bound peptides and 6 N HCl containing a few drops of phenol (110 °C, 24 h) for peptides] were determined with a Shimadzu LC amino acid analyzer system using *o*-phthalaldehyde protocol. *Rf* values were determined on precoated silica gel plates $60F_{254}$ (Merck) using the following solvent systems (v/v): Rf_1, *n*-BuOH/AcOEt/AcOH/H_2O (1 : 1 : 1 : 1); Rf_2, $CHCl_3$/MeOH/H_2O (8 : 3 : 1, lower layer). For detection, ninhydrin, UV light, and I_2 vapor were used. LSIMS spectra were measured on a VG ZAB-2SE double-focusing mass spectrometer (VG Analytical Ltd., Manchester, England). A mixture of thioglycerol and glycerol (1 : 1) was used as a matrix. The pseudo-molecular ions of peptides are shown as average masses, unless exceptionally noted.

General Procedure of the Fmoc-Based SPPS The Fmoc-based SPPS was carried out manually. Side-chain protecting groups used in the synthesis were as follows: ^{t}Bu for Asp, Glu, and Ser; Boc for Lys and His. The N-terminal Pyr (Pyr=pyroglutamic acid) residue was incorporated into the peptide chain without N^{α}-protection. The N^{α}-Fmoc groups were deprotected by gentle agitation in 20% piperidine/DMF (1+20 min). When Fmoc-Gln or Fmoc-Glu(O^{t}Bu) was the N-terminal residue of peptide-resin, concentration of the piperidine was reduced to 10%. After the N^{α}-Fmoc deprotection, the peptide-resin was washed with DMF (×6), and the next amino acid was then incorporated. The coupling reaction was promoted in DMF by a DIPCDI-HOBt coupling protocol [Fmoc-amino acid (3 eq), DIPCDI (3 eq), and HOBt (3 eq)] or a PyBOP-mediated coupling protocol [Fmoc-amino acid (3 eq), PyBOP reagent (3 eq), and NMM (9 eq)]. For introduction of a Gln residue, an active ester protocol [Fmoc-Gln-OPfp (5 eq), HOOBt[26] (5 eq), and NMM (5 eq)] was employed. After gentle agitation for 1.5 h and washing with DMF (×6), part of the peptide-resin was subjected to the Kaiser test.[27] The completed peptide-resin was finally washed consecutively with DMF (×5), MeOH (×5) and ether (×5), and then dried *in vacuo*.

RP-HPLC for Purification and Purity Assessment RP-HPLC was conducted with an Hitachi L-6200 system. A crude peptide sample was purified by RP-HPLC using a column of Cosmosil $5C_{18}$-AR-300 (20×150 mm) or μBondasphere $5C_{18}$ 100 Å (19×150 mm). A solvent system consisting of solvent A (0.1 M $AcONH_4$) and solvent B (CH_3CN) was used for elution of the Tyr(SO_3H)-containing peptides, and a solvent system consisting of solvent C (0.1% TFA in water) and solvent D (0.1% TFA in CH_3CN) was used for elution of other peptides. An appropriate flow rate was used for elution, and the absorbance was detected at 230 nm or 275 nm depending on the peptide. The solvent was removed by lyophilization and, in the case of the Tyr(SO_3H)-containing peptides, the lyophilized residue was again dissolved in 0.025 M NH_4HCO_3 (20 ml). Lyophilization afforded a fluffy powder as a final purified peptide. Purity of the purified material was assessed by analytical HPLC using a column of Cosmosil $5C_{18}$-AR (4.6×150 mm) unless otherwise noted. A solvent system consisting of solvent A and solvent B or a system consisting of solvent C and solvent D was used for elution.

Preparation of the Resin-Bound C-Terminal Segment [1a] Fmoc-Asp-Phe-NH_2 was prepared by a solution-phase method and attached to Clt resin according to the procedure of Barlos *et al.*[6c] The resin-bound C-terminal segment [**1a**] was constructed on thus obtained Fmoc-Asp(Clt resin)-Phe-NH_2 (225 mg, 0.08 mmol) in a stepwise manner according to the general procedures of Fmoc-based SPPS. After incorporation of the N-terminal Fmoc-Trp-OH, the N^{α}-Fmoc group was removed, and the resultant peptide-resin was dried *in vacuo* (362 mg). In order to evaluate the synthetic efficiency, the protected peptide-resin (30 mg, 6.2 μmol) was treated with HFIP/CH_2Cl_2 (1 : 4, 1 ml) at 25 °C for 30 min, then filtered. The filtrate was condensed using a N_2 stream, and the residue was dissolved in a mixture of CH_3CN/0.1 M $AcONH_4$. A HPLC chromatogram [HPLC conditions: column, Cosmosil $5C_{18}$-AR (6.0×150 mm); elution system, a linear gradient of B in A (30 to 80% in 30 min); flow rate, 1 ml/min; absorbance was detected at 275 nm] of this crude peptide is shown in Fig. 1a. Amino acid ratios in the acid hydrolysate of this crude peptide were as follows (theoretical values are shown in parentheses): Asp 1.09 (1), Glu 5.25 (5), Gly 1.31 (1), Ala 1.14 (1), Met 0.89 (1), Leu 0.89 (1), Tyr 1.00 (1), Phe 1.00 (1), Trp not determined (2).

Preparation of the Segments [2]—[4] Each segment was constructed on H-Pro-Clt resin (331 mg, 0.14 mmol) according to the general procedures of the Fmoc-based SPPS. After incorporation of the N-terminal residue, the peptide-resin with or without the N^{α}-Fmoc group was treated with a cleavage reagent, HFIP/CH_2Cl_2 (1 : 4 v/v), at 25 °C for 30 min. The resin was removed by filtration and the filtrate was condensed using a N_2 stream. The crude peptide in the concentrate was precipitated by addition of dry ether (50 ml). It was then collected by centrifugation, washed with ether, and dried *in vacuo*.

Fmoc-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Gln-Gly-Pro-OH [2] After incorporation of the N-terminal Fmoc-Ser(^{t}Bu)-OH, the N^{α}-Fmoc group was removed. The dried peptide-resin (417 mg, 0.13 mmol) was then treated with the cleavage reagent (5 ml) to afford crude H-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Gln-Gly-Pro-OH (125 mg, 86% on cleavage step). A portion of this product (35 mg) was purified by HPLC (Cosmosil $5C_{18}$-AR-300 column) using an isocratic elution of 36% D in C at a flow rate of 2 ml/min to give the homogeneous peptide (17 mg, 48% on HPLC purification step). This HPLC-purified product (110 mg, 0.12 mmol) was dissolved in DMF (3 ml), and Fmoc-OSu (63 mg, 0.18 mmol) and Et_3N (34 μl, 0.24 mmol) were then added to this solution. After the mixture was stirred at 20 °C for 5 h, the solution was condensed *in vacuo* and the residue was triturated with ether. The formed precipitate was washed with ether several times, then dried; weight, 95 mg (69% on Fmoc-protection step). On an analytical HPLC chromatogram (Fig. 1b), this product exhibited a single peak at t_R=16.5 min [elution system, a linear gradient of D in C (45 to 75% in 30 min); flow rate, 0.8 ml/min; absorbance was detected at 230 nm]. Rf_1 0.72, Rf_2 0.34. Amino acid ratios in the acid hydrolysate: Ser 0.73 (1), Glu 0.98 (1), Pro 0.85 (1), Gly 1.00 (1), Lys 1.82 (2). LSI-MS: *m/z* Calcd for $C_{56}H_{83}N_9O_{15}$ 1122.3 (M); Found 1123.0 $[M+H]^+$.

Fmoc-His(Boc)-Leu-Val-Ala-Asp(O^{t}Bu)-Pro-OH [3] After incorporation of the N-terminal Fmoc-His(Boc)-OH, the N^{α}-Fmoc protected peptide-resin (450 mg, 0.13 mmol) was treated with the cleavage reagent (5 ml) to

give crude Fmoc-His(Boc)-Leu-Val-Ala-Asp(O^{t}Bu)-Pro-OH (135 mg, 100% on cleavage step). This crude sample (135 mg) was purified by column chromatography on silica gel (1.5×10 cm) using $CHCl_3$–MeOH–H_2O (8 : 3 : 1 v/v, lower layer) as an eluant. The fractions exhibiting a single spot on TLC were combined and concentrated *in vacuo*. By treating the residue with dry ether, the pure segment [**3**] was obtained; weight, 105 mg (78% on purification step). On an analytical HPLC chromatogram (Fig. 1c), this product exhibited a single peak at t_R=18.4 min [elution system, a linear gradient of D in C (40 to 70% in 30 min); flow rate, 0.8 ml/min; absorbance was detected at 230 nm]. Rf_2 0.64. Amino acid ratios in the acid hydrolysate: Asp 1.02 (1), Ala 1.00 (1), Pro 1.06 (1), Val 0.97 (1), Leu 1.00 (1), His 0.80 (1). LSI-MS: *m/z* Calcd for $C_{53}H_{72}N_8O_{13}$ 1029.2 (M); Found 1030.0 $[M+H]^+$.

Pyr-Leu-Gly-Pro-Gln-Gly-Pro-Pro-OH [4] After incorporation of the N-terminal Pyr-OH, the dried peptide-resin (386 mg, 0.13 mmol) was treated with the cleavage reagent (5 ml) to give crude Pyr-Leu-Gly-Pro-Gln-Gly-Pro-Pro-OH (93 mg, 92% on cleavage step). This crude product (93 mg) was purified by HPLC (μBondasphere 5C_{18} 100 Å column) using a linear gradient of D in C (18 to 24% in 60 min) at a flow rate of 2.5 ml/min to afford the pure segment [**4**] (58 mg, 62% on HPLC purification step). On an analytical HPLC chromatogram (Fig. 1d), this product exhibited a single peak at t_R=14.6 min [elution system, a linear gradient of D in C (5 to 50% in 30 min); flow rate, 1 ml/min; absorbance was detected at 230 nm]. Rf_1 0.38. Amino acid ratios in the acid hydrolysate: Glu 1.95 (2), Pro 2.51 (3), Gly 2.00 (2), Leu 1.00 (1). LSI-MS: *m/z* Calcd for $C_{35}H_{53}N_9O_{11}$ 775.9 (M); Found *m/z* 777.0 $[M+H]^+$, 799.0 $[M+Na]^+$.

Synthesis of Human Big Gastrin-II by the Segment Condensation Approach. Peptide-Chain Assembly All the reactions (coupling, capping, and N^α-Fmoc deprotection) were carried out in a 1.5-ml plastic tube, and washing work-ups were done in a sintered funnel. The resin-bound C-terminal segment [**1a**] (34 mg, 7.0 μmol) was suspended in a mixture of DMF/DMSO (7 : 3 v/v, 1 ml), and the protected segment [**2**] (31.4 mg, 28.0 μmol), PyBOP reagent (18.2 mg, 35.0 μmol), HOBt (5.4 mg, 35.0 μmol), and NMM (4.0 μl, 28.0 μmol) were then added to this solution. After the reaction mixture was stirred at 20 °C for 15 h, the peptide-resin was filtered and washed with DMF. The resultant peptide-resin was negative to the Kaiser test. The peptide-resin was then re-suspended in DMF (1 ml), and the possible free α-amino groups were capped with Ac_2O (20 μl) in the presence of pyridine (20 μl) for 45 min. After filtration and washing with DMF, the N^α-Fmoc group of the peptide-resin was removed by agitating in 20% piperidine/DMF (1.5 ml) for 30 min. The N^α-deprotected peptide-resin was collected by filtration, washed with DMF, and then subjected to the next segment condensation. The N^α-Fmoc protected segment [**3**] (28.8 mg, 28.0 μmol) and the N-terminal segment [**4**] (21.7 mg, 28.0 μmol) were similarly introduced. Because these segments had sufficient solubility, neat DMF (1 ml) was used as a solvent for the coupling reactions. The Kaiser test after each segment condensation was negative. After completion of the whole peptide-chain assembly, the weight of the dried peptide-resin was 44.2 mg.

Synthesis of Human Big Gastrin-II The dried peptide-resin (44.2 mg, 5.75 μmol) was treated with pre-cooled 90% aqueous TFA (2 ml) at 0 °C for 8 h, then filtered. Dry ether (50 ml) was added to the filtrate, and the formed precipitate was collected by centrifugation, washed with ether, then lyophilized from 0.02 N NH_4HCO_3 (25 ml) to give a fluffy powder; weight, 24.5 mg (100% on cleavage/deprotection step). A portion of this crude peptide (Fig. 2a) (22.5 mg) was purified by HPLC (μBondasphere 5C_{18} 100 Å column) using a linear gradient of B in A (23 to 30% in 60 min) at a flow rate of 3 ml/min. After lyophilization, 3.44 mg of the homogeneous product was obtained (14% from cleavage/deprotection step). On an analytical HPLC chromatogram (Fig. 2b), this product exhibited a single peak at t_R=33.5 min [elution system, a linear gradient of B in A (15 to 30% in 60 min); flow rate, 0.75 ml/min; absorbance was detected at 230 nm]. This peak position coincided with that of the authentic big gastrin-II obtained by the stepwise elongation approach. Amino acid ratios in the acid hydrolysate: Asp 2.00 (2), Ser 1.12 (1), Glu 7.79 (8), Pro 4.72 (5), Gly 4.00 (4), Ala 1.97 (2), Val 0.93 (1), Met 0.62 (1), Leu 2.76 (3), Tyr 0.97 (1), Phe 1.05 (1), Lys 1.88 (2), His 0.94 (1), Trp not determined (2). LSI-MS: *m/z* Calcd for $C_{176}H_{251}N_{43}O_{56}S_2$ 3929.3 (M); Found 3929.8 $[M+H]^+$ and 3849.8 $[M+H-SO_3]^+$ in the positive-ion mode, 3927.8 $[M-H]^-$ in the negative-ion mode.

Synthesis of G34-Gly Sulfate by the Segment Condensation Approach Starting with Fmoc-Gly-Clt resin (165 mg, 0.1 mmol), the resin-bound C-terminal segment [**1b**] was constructed according to the general procedures of the Fmoc-based SPPS. After the N^α-Fmoc group of the N-terminal Trp residue was removed, the peptide-resin was washed and dried *in vacuo* (334 mg).

Each segment was condensed to the peptide-resin in essentially the same manner described in the preparation of big gastrin-II. The protected segment [**2**] (31.4 mg, 28.0 μmol), segment [**3**] (28.8 mg, 28.0 μmol), and the N-terminal segment [**4**] (21.7 mg, 28.0 μmol) were successively introduced onto the resin-bound C-terminal segment [**1b**] (40 mg, 7.0 μmol) by the PyBOP-mediated coupling protocol (20 °C for 15 h). The possible free α-amino groups were capped by Ac_2O in the presence of pyridine (20 μl each) in DMF (1 ml) for 45 min. After completion of the 35-residue peptide chain assembly, the weight of the dried peptide-resin was 50.0 mg.

The dried peptide-resin (50 mg, 4.8 μmol) was treated with pre-cooled 90% aqueous TFA (2 ml) at 0 °C for 8 h, then filtered. By adding dry ether (50 ml) to the filtrate, the crude peptide was precipitated. It was collected by centrifugation, washed with ether, then lyophilized from 0.02 N NH_4HCO_3 (25 ml) to give a fluffy powder; weight, 20.4 mg (100% on cleavage/deprotection step). A portion of this crude peptide (11.8 mg) was purified by HPLC (Cosmosil 5C_{18}-AR column, 4.6×150 mm) using a linear gradient of B in A (15 to 30% in 60 min) at a flow rate of 0.75 ml/min to give the homogeneous product; weight, 1.3 mg (11% from cleavage/deprotection step). This product exhibited a single peak at t_R=28.0 min on a HPLC chromatogram under the conditions described above. Amino acid ratio in the acid hydrolysate: Asp 2.08 (2), Ser 0.98 (1), Glu 7.85 (8), Pro 4.47 (5), Gly 5.00 (5), Ala 2.08 (2), Val 0.95 (1), Met 1.00 (1), Leu 2.88 (3), Tyr 1.01 (1), Phe 1.07 (1), Lys 1.95 (2), His 1.07 (1), Trp not determined (2). LSI-MS: *m/z* Calcd for $C_{178}H_{253}N_{43}O_{58}S_2$ 3987.4 (M); Found 3988.2 $[M+H]^+$ and 3908.3 $[M+H-SO_3]^+$ in the positive-ion mode, 3986.6 $[M-H]^-$ in the negative-ion mode.

Lysyl Endopeptidase Digestion of Big Gastrin-II and G34-Gly Sulfate Purified big gastrin-II (200 μg) was digested by lysyl endopeptidase (2 μg) at 37 °C for 5 h, and the digest was subjected to RP-HPLC analysis [column, Cosmosil 5C_{18}-AR (4.6×150 mm); elution system, a linear gradient of D in C (5 to 55% in 50 min); flow rate, 1 ml/min]. The eluate was monitored by absorbance at 225 nm. Two peak fractions (Fig. 3a) were pooled, and the solvents were removed by lyophilization. Part of each lyophilisate was subjected to LSIMS and amino acid analysis after acid hydrolysis. The first eluting peak (t_R=20.0 min) was determined to be the N-terminal 16-mer (N-16) and the second eluting peak (t_R=31.0 min) was assigned to the C-terminal 17-mer (C-17). N-16: amino acid ratios in the acid hydrolysate, Asp 0.96 (1), Ser 0.91 (1), Glu 2.10 (2), Pro 3.49 (4), Gly 2.00 (2), Ala 1.21 (1), Val 0.95 (1), Leu 2.00 (2), His 1.03 (1), Lys 1.02 (1); LSI-MS, *m/z* Calcd for $C_{73}H_{114}N_{20}O_{22}$ 1622.8 (M, monoisotopic mass); found 1623.8 $[M+H]^+$. C-17: amino acid ratios in the acid hydrolysate, Asp 1.10 (1), Glu 5.91 (6), Pro 1.17 (1), Gly 2.35 (2), Ala 1.07 (1), Met 0.70 (1), Leu 1.00 (1), Tyr 1.15 (1), Phe 1.05 (1), Trp not determined (2); LSI-MS, *m/z* Calcd for $C_{97}H_{127}N_{21}O_{34}S_2$ 2195.3 (M); Found 2218.0 $[M+Na]^+$ and 2115.9 $[M+H-SO_3]^+$ in the positive-ion mode, 2194.3 $[M-H]^-$ in the negative-ion mode.

Purified G34-Gly sulfate (200 μg) was similarly digested by the enzyme, and the produced peptides were separated by HPLC (Fig. 3b), then characterized. The first peak (t_R=20.0 min) coincided with N-16 (LSIMS, *m/z* 1623.9 $[M+H]^+$) and the second peak (t_R=31.1 min) was determined to be the C-terminal 18-mer (C-18). C-18: amino acid ratios in the acid hydrolysate, Asp 1.07 (1), Glu 5.90 (6), Pro 1.00 (1), Gly 3.00 (3), Ala 1.08 (1), Met 0.71 (1), Leu 1.00 (1), Tyr 0.93 (1), Phe 0.93 (1), Trp not determined (2); LSI-MS, *m/z* Calcd for $C_{99}H_{129}N_{21}O_{36}S_2$ 2253.4 (M); Found 2276.0 $[M+Na]^+$ and 2173.9 $[M+H-SO_3]^+$ in the positive-ion mode, 2252.3 $[M-H]^-$ in the negative-ion mode. The small peak appearing just before the N-16 peak in Figs. 3a and 3b corresponds to N-16-Lys, which is derived from insufficient digestion of the Lys–Lys bond. Also, the small peaks appearing before the C-17 and C-18 peaks correspond to big gastrin-II and G34-Gly sulfate, respectively.

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References and Notes

1) Abbreviations used are as follows: All amino acids are of the L-configuration. AcOH, acetic acid; $AcONH_4$, ammonium acetate; Ac_2O, acetic anhydride; Boc, *tert*-butoxycarbonyl; ^{t}Bu, *tert*-butyl; CCK, cholecystokinin; Clt resin, 2-chlorotrityl chloride resin; DIPCDI, *N*,*N*-diisopropylcarbodiimide; DKP, diketopiperazine; Fmoc, fluoren-9-ylmethoxycarbonyl; G34-II, human big gastrin-II; G34-Gly sulfate, C-terminal glycine-extended big gastrin sulfate; HFIP, hexafluoro-2-

propanol; HOBt, *N*-hydroxybenzotriazole; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; LSIMS, liquid secondary-ion mass spectrometry; NMM, *N*-methylmorpholine; Pfp, pentafluorophenyl; PyBOP, benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; Su, *N*-hydroxysuccinimidyl; TFE, 2,2,2-trifluoroethanol; TFA, trifluoroacetic acid.

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