JOC
The Journal of Organic Chemistry

VOLUME 66, NUMBER 1 JANUARY 12, 2001

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Articles

Facile Solid-Phase Synthesis of Sulfated Tyrosine-Containing Peptides: Total Synthesis of Human Big Gastrin-II and Cholecystokinin (CCK)-39[1,2]

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Received June 12, 2000

Chemical synthesis of tyrosine *O*-sulfated peptides is still a laborious task for peptide chemists because of the intrinsic acid-lability of the sulfate moiety. An efficient cleavage/deprotection procedure without loss of the sulfate is the critical difficulty remaining to be solved for fluoren-9-ylmethoxycarbonyl (Fmoc)-based solid-phase synthesis of sulfated peptides. To overcome the difficulty, TFA-mediated solvolysis rates of a tyrosine *O*-sulfate [Tyr(SO_3H)] residue and two protecting groups, ^{t}Bu for the hydroxyl group of Ser and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for the guanidino group of Arg, were examined in detail. The desulfation obeyed first-order kinetics with a large entropy (59.6 $J \cdot K^{-1} \cdot mol^{-1}$) and enthalpy (110.5 $kJ \cdot mol^{-1}$) of activation. These values substantiated that the desulfation rate of the rigidly solvated Tyr(SO_3H) residue was strongly temperature-dependent. By contrast, the S_N1-type deprotections were less temperature-dependent and proceeded smoothly in TFA of a high ionizing power. Based on the large rate difference between the desulfation and the S_N1-type deprotections in cold TFA, an efficient deprotection protocol for the sulfated peptides was developed. Our synthetic strategy for Tyr(SO_3H)-containing peptides with this effective deprotection protocol is as follows: (i) a sulfated peptide chain is directly constructed on 2-chlorotrityl resin with Fmoc-based solid-phase chemistry using Fmoc-Tyr(SO_3Na)-OH as a building block; (ii) the protected peptide-resin is treated with 90% aqueous TFA at 0 °C for an appropriate period of time for the cleavage and deprotection. Human cholecystokinin (CCK)-12, mini gastrin-II (14 residues), and little gastrin-II (17 residues) were synthesized with this method in 26−38% yields without any difficulties. This method was further applied to the stepwise synthesis of human big gastrin-II (34 residues), CCK-33 and -39. Despite the prolonged acid treatment (15−18 h at 0 °C), the ratios of the desulfated peptides were less than 15%, and the pure sulfated peptides were obtained in around 10% yields.

Introduction

Sulfation of a tyrosine residue is one of the ubiquitous posttranslational modifications, and a vast number of tyrosine *O*-sulfated proteins have been identified from a wide variety of natural sources.[3] This modification is catalyzed by tyrosylprotein sulfotransferase, a membrane-bound enzyme within the *trans*-golgi compartment. The

10.1021/jo000895y CCC: $20.00 © 2001 American Chemical Society
Published on Web 12/13/2000

tyrosine sulfation is suggested to be involved in many biological functions of secretory proteins. Tyrosine *O*-sulfate [Tyr(SO_3H)] residues are also present in a few biologically active peptides such as gastrin-II,[4] cholecystokinin (CCK),[5] caerulein,[6] leucosulfakinins,[7] leech-derived anticoagulant hirudin,[8] and a higher plant-derived growth factor phytosulfokines.[9] The negative charge of a Tyr(SO_3H) residue would be important not only for peptide folding but also for specific ligand−receptor interaction.[3f]

A prerequisite for investigating the biological functions of peptides and proteins is that efficient methods for obtaining a large amount of them are available; however, chemical synthesis of a Tyr(SO_3H)-containing peptide is still a great challenge. The major difficulty lies in the intrinsic acid-lability of a Tyr(SO_3H) residue. Although many synthetic studies on sulfated peptides have been reported,[10] a general and facile synthetic strategy with wide applicability has not been established yet. If a peptide chain was constructed using a nonsulfated Tyr derivative as a building block, it must be sulfated later without affecting the other susceptible residues (*post-assembly sulfation*). This approach requires special manipulations to achieve the selective sulfation of a Tyr residue. The amino groups of the N-terminus and Lys residues, the alcoholic hydroxyl groups of Ser and Thr residues, and the phenolic hydroxyl groups of Tyr residues that do not need to be sulfated often require protection depending on the sulfating reagent used.[11] The applied protecting groups, in addition, should be removed without deteriorating the sulfated Tyr residue. For example, Sakakibara and co-workers[10c] employed an acid-stable and base-labile phenoxyacetyl group for protection of the Ser residue in nonsulfated porcine CCK-33 and selectively sulfated the Tyr residue using pyridinium acetyl sulfate[11d] in TFA. During this reaction, the amino groups did not need to be protected. In a synthesis of human CCK-33 reported by Yajima and co-workers,[10d] a sequential protection and deprotection procedure was employed: (i) complete deprotection of nonsulfated human CCK-33 constructed by a solution-phase method, (ii) reprotection of the free α- and ϵ-amino groups with Fmoc group, (iii) preferential masking of the alcoholic hydroxyl groups with *tert*-butyldiphenylsilyl (TBDPS) group, (iv) sulfation of the Tyr residue using a pyridine−SO_3 complex,[11f] and (v) simultaneous deprotection of the Fmoc and the TBDPS protecting groups[12] using tetra-*n*-butylammonium fluoride. We have also reported an orthogonal procedure for the selective sulfation of a Tyr residue.[10g,i] The alcoholic hydroxyl groups and amino groups on a peptide chain were masked with safety-catch-type protecting groups, and the Tyr residue was selectively sulfated using a DMF−SO_3 complex.[13] Several sulfated peptides were prepared with the postassembly sulfation method; however, this approach is generally time-consuming and reduction of reactivity is anticipated in the sulfation of a large peptide.

On the other hand, recent advances in the Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS)[14] enabled us

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(1) Abbreviations used are as follows: All amino acids are of the L-configuration. AcOH, acetic acid; $AcONH_4$, ammonium acetate; Boc, *tert*-butoxycarbonyl; ^{t}Bu, *tert*-butyl; CCK, cholecystokinin; Clt resin, 2-chlorotrityl chloride resin; DIPCDI, *N,N*-diisopropylcarbodiimide; EDT, 1,2-ethanedithiol; Fmoc, fluoren-9-ylmethoxycarbonyl; FT-IR, Fourier transform infrared spectrometry; HFIP, hexafluoro-2-propanol; HOBt, 1-hydroxybenzotriazole; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; LSIMS, liquid secondary-ion mass spectrometry; MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NMM, *N*-methylmorpholine; PAL, 5-(4′-aminomethyl-3′,5′-dimethoxyphenyl)valeryl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Pfp, pentafluorophenyl; PyBOP, benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate; TFE, 2,2,2-trifluoroethanol.

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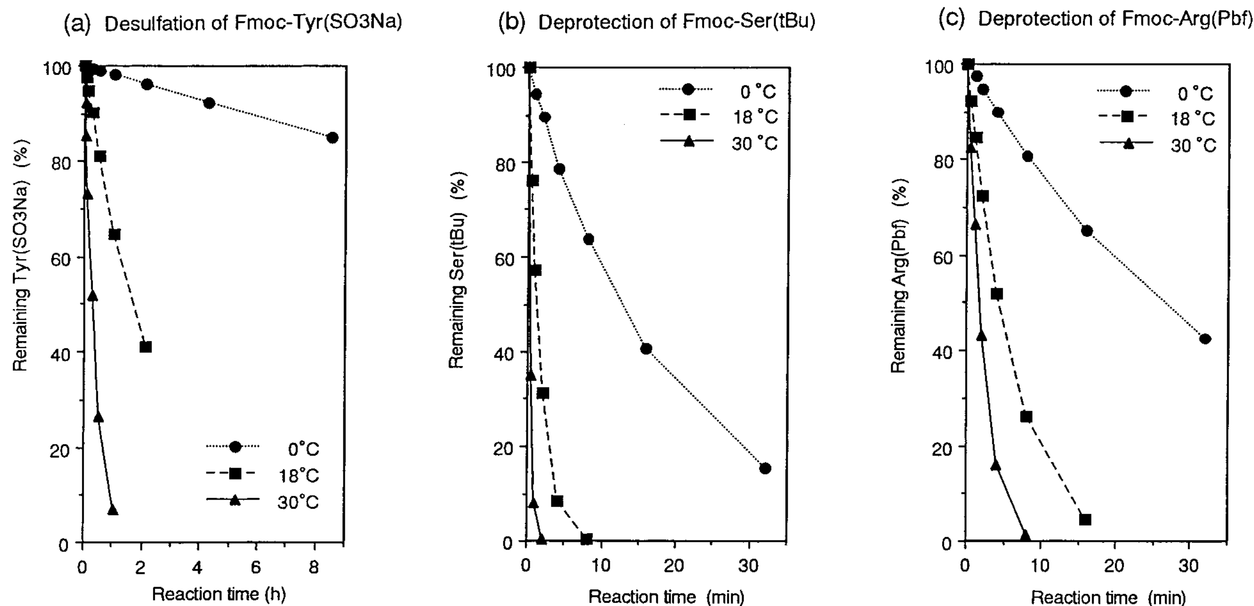

Figure 1. Effect of reaction temperature on the desulfation and the deprotections in neat TFA: (a) desulfation of Tyr(SO_3Na), (b) deprotection of Ser(^{t}Bu), and (c) deprotection of Arg(Pbf).

to directly construct a sulfated peptide chain using Fmoc-Tyr(SO_3Na)-OH[10e,h] as a building block (*preassembly sulfation*). Here the base-labile Fmoc group is employed for N^{α}-protection, and thus repeated acid treatment incompatible with a Tyr(SO_3H) residue is avoidable during the peptide chain construction. This approach seems attractive because of the simplicity and applicability to an automated peptide synthesizer. However, there is a crucial difficulty remaining to be solved in the synthesis with this approach. The effective final cleavage/deprotection conditions applied to the constructed peptide-resin are generally destructive to a Tyr(SO_3H) residue. This becomes a serious problem especially in the case of peptides having a C-terminal amide structure, such as gastrin and CCK, because of the lack of a highly acid-sensitive amide-linker. Therefore, application of the preassembly sulfation has been limited to the synthesis of relatively small and less-complicated Tyr(SO_3H)-containing peptides.

In the course of our efforts to establish a general synthetic method for sulfated peptides, we noticed that TFA, a routinely used reagent for the final cleavage/deprotection step in the Fmoc-SPPS, was not a destructive acid to a Tyr(SO_3H) residue as long as the reaction mixture was kept cold.[10h] Provided that there is a certain TFA-mediated acidolysis system that is sufficient for the final cleavage/deprotection but not destructive to a Tyr(SO_3H) residue, sulfated peptides are expected to be easily prepared with the Fmoc-SPPS. In this article, we describe such an efficient acidolysis system for Tyr(SO_3H)-containing peptides. By combining this deprotection system with highly acid-sensitive 2-chlorotrityl resin as a solid support, we established a general synthetic strategy for sulfated peptides and accomplished the total synthesis of human big gastrin-II and CCK-39.

Results and Discussion

Desulfation vs Deprotection: Kinetic Studies. We previously examined the compatibility of several cleavage/deprotection systems with a Tyr(SO_3H)-containing peptide and found that the desulfation in TFA was significantly temperature-dependent.[10h] To further evaluate the usefulness of this acid to the cleavage/deprotection, TFA-mediated acidolysis rates of Tyr(SO_3Na) and two protecting groups, ^{t}Bu for the hydroxyl group of Ser and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf)[15] for the guanidino group of Arg, were examined. These protecting groups were selected because of their noticeably slow deprotection rates in TFA. The Fmoc-derivatives, Fmoc-Tyr(SO_3Na)-OH, Fmoc-Ser(^{t}Bu)-OH, and Fmoc-Arg(Pbf)-OH, were stirred in neat TFA at a specified temperature (0, 18, or 30 °C), and part of each solution was periodically withdrawn to determine the degree of the desulfation or the deprotections by RP-HPLC. Disappearance of every starting material obeyed good first-order kinetics (Figure 1). From these results, the Arrhenius plot (Figure 2) and activation parameters (Table 1) of each reaction were obtained. The striking feature of the activation parameters determined is that the desulfation was accompanied by a positive entropy term ($\Delta S^{\ddagger} = +59.6$ J·K^{-1}·mol^{-1}) and the largest enthalpy term ($\Delta H^{\ddagger} = +110.5$ kJ·mol^{-1}). These values were well reflected by the strong temperature-dependency of the desulfation rate compared to the rates of the deprotections. The deprotections of Arg(Pbf) and Ser(^{t}Bu) were

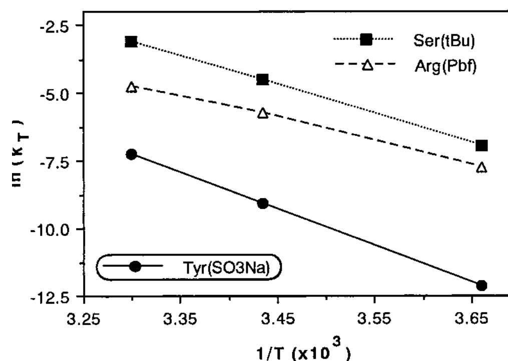

Figure 2. Arrhenius plots for the desulfation rate of Tyr(SO_3-Na) and the deprotection rates of Ser(^{t}Bu) and Arg(Pbf). All the reactions were carried out in neat TFA.

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Table 1. Rates and Activation Parameters for the Desulfation and the Deprotections in Neat TFA at 0 °C

rates and activation parameters[a]	Tyr(SO_3Na) → Tyr	Arg(Pbf) → Arg	Ser(^{t}Bu) → Ser
reaction rate (k_0, s^{-1})	5.30×10^{-6}	4.43×10^{-4}	9.67×10^{-4}
activation energy (E_a, kJ·mol^{-1})	112.8	69.1	89.0
enthalpy of activation ($\Delta H^{\ddagger}$, kJ·mol^{-1})	110.5	66.8	86.7
free energy of activation($\Delta G^{\ddagger}$, kJ·mol^{-1})	94.3	84.2	82.5
entropy of activation($\Delta S^{\ddagger}$, J·K^{-1}·mol^{-1})	59.6	−63.7	15.5
entropy of activation($\Delta S^{\ddagger}$, eu)	14.2	−15.2	3.7

[a] Calculated from the equations $\Delta H^{\ddagger} = E_a - RT$; $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$; $\Delta G^{\ddagger} = -RT\ln(k_0 h/\kappa T)$ at 0 °C. $R = 8.314$ J·K^{-1}·mol^{-1}; $T = 273.15$ K; $h = 6.626 \times 10^{-34}$ J·s; $\kappa = 1.380 \times 10^{-23}$ J·K^{-1}; 1 eu = 4.184 J·K^{-1}·mol^{-1}.

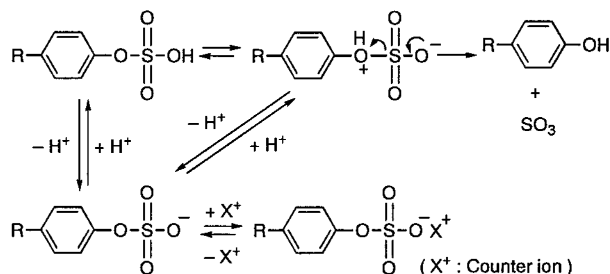

Figure 3. Plausible mechanism for proton-catalyzed desulfation of arylmonosulfates in acidic solution.

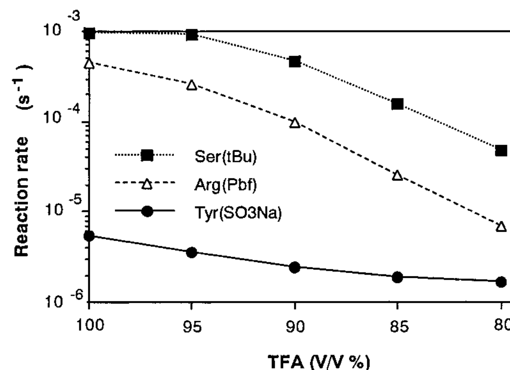

Figure 4. Desulfation and deprotection rates in aqueous TFA at 0 °C.

12 and 56 times faster than the desulfation at 30 °C, but these differences were enhanced to 84 and 182 times at 0 °C, respectively (Figure 2).

The feature of the desulfation rate in TFA can be interpreted by a mechanism proposed for the deterioration of arylmonosulfates (ArO−SO_3H) in acidic solution[16] (Figure 3). The inherent acid-lability of Tyr(SO_3H), or the acid-lability of ArO−SO_3H, is attributable to the dominant existence of the unstable proton-donating form [ArO^+(H)−SO_3^-] in acidic solution, which rapidly decomposes to Ar−OH and SO_3. This amphoteric ion is predicted to be highly solvated and stabilized by molecules with a high dipole moment such as TFA and 2,2,2-trifluoroethanol (TFE). The large entropy of activation of the desulfation in TFA substantiates the rigidly solvated amphoteric ion and less-solvated transition state. Therefore, a higher energy barrier should be passed over for the desulfation in polar solvents. Acidolysis rates of arylmonosulfates are actually reported to be affected by the polarity of the solutions as well as the acidity.[11d,16,17] If the polarity is low, arylmonosulfates are expected to rapidly decompose even under weakly acidic conditions. On the contrary, they would be stable even in strongly acidic solutions when the polarity is unusually high. Because of their high polarity, TFA and TFE are supposed to be favorable solvents for the cleavage/deprotection of sulfated peptides.

In an acidolysis system for protected peptides, soft bases are generally dissolved to scavenge the released cations and to promote the deprotections as S_N2-type reactions.[18] However, we previously reported that the addition of a sulfur-containing soft base such as thioanisole and ethanedithiol (EDT) significantly accelerated the desulfation of a Tyr(SO_3H)-containing peptide.[10h] The deprotections, therefore, have to be promoted without the addition of sulfur-containing soft bases. It is considered that the unimolecular deprotection (S_N1-type reaction) is more advantageous to a Tyr(SO_3H)-containing peptide than the bimolecular deprotection (S_N2-type reaction) requiring soft bases. TFA seems again to be a promising deprotection reagent for a Tyr(SO_3H)-containing peptide because the high ionizing power of TFA promotes S_N1-type deprotections. Moreover, the rates of the S_N1-type deprotections were less temperature-dependent (Figure 2). Thus, by using the marked rate difference between the desulfation and the S_N1-type deprotections in cold TFA (Figure 2), a large number of protecting groups can be removed with minimum damage of a Tyr(SO_3H) residue.

In practice, we used aqueous TFA for the deprotection of a Tyr(SO_3H)-containing peptide to diminish side reactions caused by the released cations. Water is known to be a good cation scavenger in TFA.[14] We examined in advance the effects of water addition on the rates of the desulfation and the deprotections. It was found that the deprotections were gradually retarded with increasing water content in the TFA. By contrast, the desulfation rate was not affected significantly (Figure 4). In 90% aqueous TFA at 0 °C, the deprotection of the Pbf group was still 40 times faster than the desulfation. From these results, we concluded that 90−95% aqueous TFA at low temperature could maintain the large rate difference between the desulfation and the S_N1-type deprotections and can be effectively applied to the deprotection of a sulfated peptide.

Establishment of a Facile Solid-Phase Method for the Synthesis of Sulfated Peptides. Previously,[10h] we prepared CCK-12 with the Fmoc-SPPS using PAL-resin as a solid support [PAL = peptide amide linker; 5-(4-(9-fluorenylmethoxycarbonyl)aminomethyl)-3,5-dimethoxyphenoxyvaleric acid linker[19]]. Despite the use of this reportedly acid-sensitive linker-resin, the detachment efficiency of the peptide from the support was no more than 40% under the cleavage/deprotection conditions employed. To improve the detachment efficiency, we

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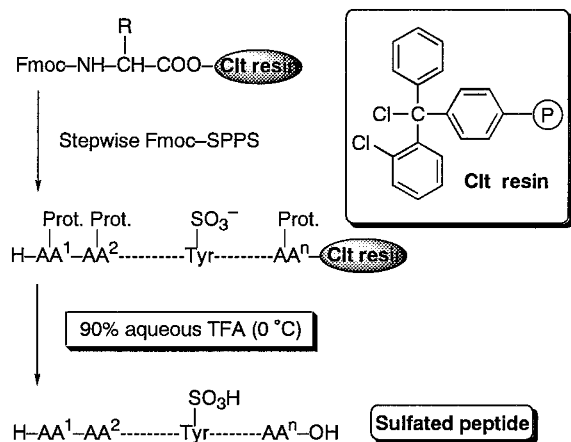

Figure 5. Synthetic scheme of a Tyr(SO_3H)-containing peptide with the Fmoc-based solid-phase approach.

decided to use 2-chlorotrityl (Clt) resin[20] as a solid support. Clt resin was developed to provide peptide segments bearing ^{t}Bu/Boc side-chain protecting groups, and quantitative detachment of a protected peptide from this linker-resin can be achieved under extremely mild acidic conditions.[20,21]

Our strategy for the Fmoc-SPPS of a Tyr(SO_3H)-containing peptide is shown in Figure 5. This approach involves two key features to complete the synthesis in an efficient manner: (1) utilization of Clt resin as a solid support to detach a sulfated peptide quantitatively and (2) deprotection with 90% aqueous TFA at 0 °C to minimize the deterioration of a Tyr(SO_3H) residue. The effectiveness of this approach was evaluated through the synthesis of leucine-enkephalin sulfate[22] [Tyr(SO_3H)-Gly-Gly-Phe-Leu (**1**)], human CCK-12 (**2**), mini gastrin-II (**3**) (14 residues), and little gastrin-II (**4**) (17 residues).[23] The amino acid sequences of the CCK peptides and the gastrin-II peptides are shown in Figure 6.

To overcome the general low recovery of a peptide amide from the amide-offering linker-resin, the C-terminal dipeptide, Fmoc-Asp-Phe-NH_2,[20c] was linked to Clt resin through the β-carboxyl group of the Asp residue. The resulting dipeptide-resin was treated with 20% piperidine/DMF to remove the N^α-Fmoc group; then each building block including Fmoc-Tyr(SO_3Na)-OH was successively introduced into the peptide chain. The PyBOP-mediated coupling protocol [PyBOP = benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate] was used for the peptide chain elongation.[24] For protection of the Arg residue in CCK peptides, we selected Pbf group[15] that was most sensitive to our deprotection conditions (90% aqueous TFA, 0 °C) among the various protecting groups examined. The assembled peptide-resin corresponding to **2** was subjected to the two-step cleavage/deprotection; first with a mixture of HFIP/CH_2Cl_2 (1:4 v/v) for 30 min at 25 °C and then with 90% aqueous TFA for 8 h at 0 °C. Detachment of the protected peptide from the Clt resin was quantitative (>95%), and deterioration of the sulfate during the prolonged deprotection was not significant (ca. 10% of the peptide was desulfated, Figure 7a). Subsequent purification by RP-HPLC afforded highly homogeneous **2** in 26% yield from the protected peptide-resin. Similarly, **3** and **4** were obtained from the corresponding peptide-resin in 34 and 38% yields, respectively (Figure 7b). Deprotection of gastrin-II peptides was continued for 5 h at 0 °C because they contain no Arg(Pbf) residues. The determined amino acid composition of each peptide coincided with the theoretical value, and integrity of the sulfate was ascertained by FT-IR and liquid secondary-ion mass spectrometry (LSIMS). Especially, the negative-ion LSIMS spectra gave direct evidence of the intactness of the sulfate as previously reported.[25,26] The yield of **2** obtained with this method (26%) was significantly improved compared to the previous synthesis[10h] using PAL-resin (7.4% yield from the protected peptide-resin). These results clearly demonstrated the potential of our novel strategy for the synthesis of a Tyr(SO_3H)-containing peptide.

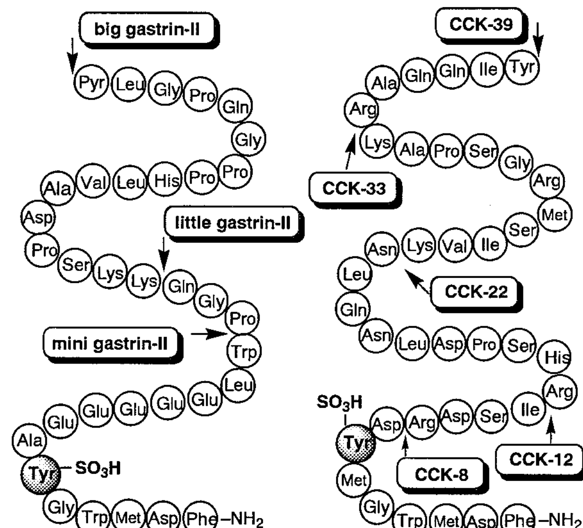

Figure 6. Sequences of human gastrin-II and CCK peptides. The N-terminal Gln residue of little gastrin-II is posttranslationally cyclized to a Pyr (= pyroglutamic acid) residue.

(19) (a) Albericio, F.; Barany, G. *Int. J. Pept. Protein Res.* **1987**, *30*, 206–216. (b) Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730–3743.

(20) (a) Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Wenqing, Y.; Schafer, W. *Tetrahedon Lett.* **1989**, *30*, 3943–3946. (b) Barlos, K.; Gatos, D.; Kapolos, S.; Papaphotiu, G.; Schafer, W. *Tetrahedron Lett.* **1989**, 30, 3947–3950. (c) Barlos, K.; Chatzi, O.; Kapolos, S.; Poulos, C.; Schafer, W.; Wenqing, Y. *Int. J. Pept. Protein Res.* **1991**, *38*, 555–561.

(21) Bollhagen, R.; Schmiedberger, M.; Barlos, K.; Grell, E. *J. Chem. Soc., Chem. Commun.* **1994**, 2559–2560.

(22) Unsworth, C. D.; Hughes, J.; Morley, J. S. *Nature* **1982**, *295*, 519–522.

(23) At an early stage of this investigation, we used a two-step cleavage/deprotection protocol for synthesis of the sulfated peptides. Thus we first examined the extent of desulfation during the cleavage with a mixture of AcOH/TFE/CH_2Cl_2 (1:1:3 v/v) or hexafluoro-2-propanol (HFIP)/CH_2Cl_2 (1:4 v/v) using N^α-Fmoc protected **1** as a model peptide. These experiments showed that about 1.8% of the peptide was desulfated during the cleavage with AcOH/TFE/CH_2Cl_2 (25 °C, 2 h) and that no desulfated peptide was generated during the treatment with HFIP/CH_2Cl_2 (25 °C, 6 h). For the characterization of **1**, the N^α-Fmoc protected peptide-resin was treated with 20% piperidine/DMF then with HFIP/CH_2Cl_2 (0.5 h). Pure **1** was obtained in 58% yield from the peptide-resin after RP-HPLC purification.

(24) Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205–208.

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(26) (a) Arlandini, E.; Gioia, B.; Perseo, G.; Vigevani, A. *Int. J. Pept. Protein Res.* **1984**, *24*, 386–391. (b) Gibson, B. W.; Falick, A. M.; Burlingame, A. L.; Nadasdi, L.; Nguyen, A. C.; Kenyon, G. L. *J. Am. Chem. Soc.* **1987**, *109*, 5343–5348. (c) Gibson, B. W. In *Biological Mass Spectrometry*; Burlingame, A. L., McCloskey, J. A., Eds.; Elsevier Science Publishers B. V.: Amsterdam, 1990; pp 315–336. (d) Carr, S. A.; Hemling, M. E.; Bean, M. F.; Roberts, G. D. *Anal. Chem.* **1991**, *63*, 2802–2824. (e) Gibson, B. W.; Cohem, P. *Method Enzymol.* **1990**, *193*, 480–501. (f) Talbo, G.; Roepstorff, P. *Rapid Commun. Mass Spectrom.* **1992**, *7*, 201–204.

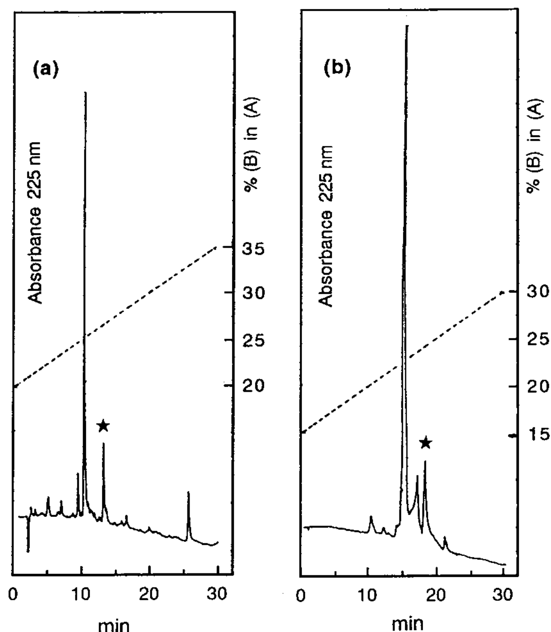

Figure 7. HPLC chromatograms of crude (a) CCK-12 **2** and (b) little gastrin-II **4**. An asterisk in each chromatogram shows the desulfated peptide produced during the deprotection step. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6 × 150 mm); elution system, a linear gradient of CH_3CN (B) in 0.1 M $AcONH_4$ (A); flow rate, 1 mL/min].

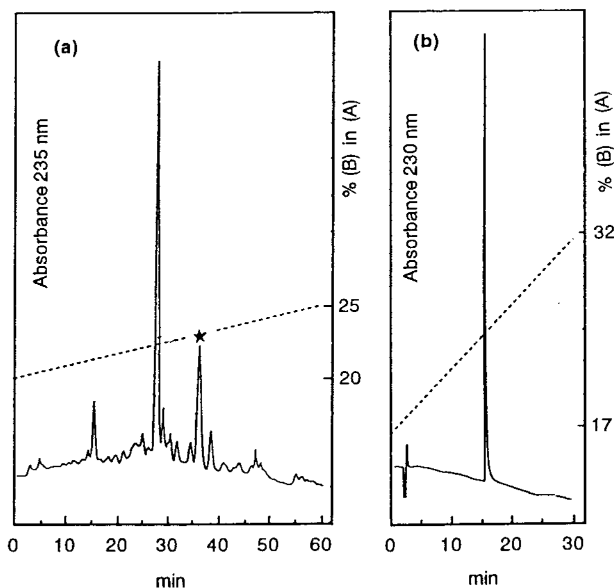

Figure 8. HPLC chromatograms of (a) crude **5** and (b) purified **5**. An asterisk in (a) shows the desulfated peptide (big gastrin-I) produced during the deprotection. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6 × 150 mm); elution system, a linear gradient of CH_3CN (B) in 0.1 M $AcONH_4$ (A); flow rate,1 mL/min].

Synthesis of Human Big Gastrin-II. Gastrins are a family of peptide hormones that stimulate gastric acid secretion.[4] They contain many troublesome residues for chemical synthesis (Figure 6). Several synthetic achievements of gastrin-I peptides, the nonsulfated counterparts of gastrin-II, were reported with solution- or solid-phase methods.[10a,27] It has been recognized that the homogeneous products are difficult to obtain with a conventional solid-phase approach due to several critical side reactions. Sheppard and co-workers pointed out that the Trp residues in gastrin peptides tended to be alkylated by ^{t}Bu cations generated from the protecting groups during the acidolysis.[27c,d] Nucleophilic addition of the Trp residue to the polymer support also reduces the yield of the desired peptide.[28] Various scavenger cocktails, such as TFA−phenol−thioanisole−EDT−H_2O[29a] and TFA−thioanisole−EDT−anisole,[29b] have been effectively used to reduce such side reactions during the cleavage/deprotection. However, these cocktails including sulfur-containing soft bases are not applicable to the deprotection of a sulfated peptide as already discussed.[10h] We attempted the synthesis of human big gastrin-II (**5**, Figure 6) to demonstrate the usefulness of our new strategy.

The C-terminal dipeptide was attached to Clt resin, and it was elongated to the 34-residue peptide in a stepwise manner. A His residue was introduced into the peptide chain using Fmoc-His(Boc)-OH[14] as a building block. The assembled peptide-resin was subjected to the cleavage/deprotection with 90% aqueous TFA. Consistent with the results of our kinetic studies, deprotection with 90% aqueous TFA at 0 °C for 8 to 9 h was satisfactory for completing the removal of the protecting groups. Despite this prolonged acid treatment, loss of the sulfate was about 15% (Figure 8a); on the other hand, even brief treatment with 90% aqueous TFA at room-temperature resulted in a drastic production of the desulfated peptide (big gastrin-I, **6**). After purification of the crude peptide by RP-HPLC, highly homogeneous **5** (Figure 8b) was obtained in 13% yield from the protected peptide-resin. The amino acid composition of the acid hydrolysate coincided with the theoretical value. In the positive- and negative-ion LSIMS spectra, this product exhibited the pronounced peaks corresponding to $[M + H]^+$ and $[M - H]^-$, respectively.[30] Moreover, amino acid analyses of two fragments (**N-16** and **C-17** in Figure 9) generated by a lysyl endopeptidase digestion supported the structure of this final product.

Synthesis of Big-Molecular-Form CCK Peptides, CCK-33 and -39. Cholecystokinin (CCK) is a group of peptide hormones that is distributed both in the brain and gastrointestinal tract and participates in the central and pancreatic regulations.[5] This peptide is also known to exist in several molecular forms (Figure 6), such as CCK-8, -22, -33,[5a,b] -39,[5e] and -58,[5f] but the significance of this molecular diversity and the physiological roles of each form remain to be investigated. Every form has a Tyr(SO_3H) residue at the seventh position from the C-terminus that is crucial for the biological activities.

We further demonstrated the effectiveness of our strategy through the synthesis of CCK peptides, CCK-22 (**7**), -33 (**8**), and -39 (**9**). Their synthesis, however, was

(27) (a) Beacharm, J.; Bentley, P. H.; Gregory, R. A.; Kenner, G. W.; MacLeod, J. K.; Sheppard, R. C. *Nature* **1966**, *209*, 585−586. (b) Jaeger, E.; Thamm, P.; Schmidt, I.; Knof, S.; Moroder, L.; Wünsch, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 155−164. (c) Brawn, E.; Sheppard, R. C.; Williams, B. J. *J. Chem. Soc., Perkin Trans. 1* **1983**, 75−82. (d) Brawn, E.; Sheppard, R. C.; Williams, B. J. *J. Chem. Soc., Perkin Trans. 1* **1983**, 1161−1167. (e) Tam, J. P.; Merrifield, R. B. *Int. J. Pept. Protein Res.* **1985**, *26*, 262−273. (f) Kneib-Cordonier, N.; Albericio, F.; Barany, G. *Int. J. Pept. Protein Res.* **1990**, *35*, 527−538.

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(30) Although the desulfated $[M + H - SO_3]^+$ is usually detected as a prominent peak on the positive-ion LSIMS spectra of a Tyr(SO_3H)-containing peptide, the $[M + H]^+$ peak becomes dominant over the $[M + H - SO_3]^+$ peak as the peptide chain is elongated.[31]

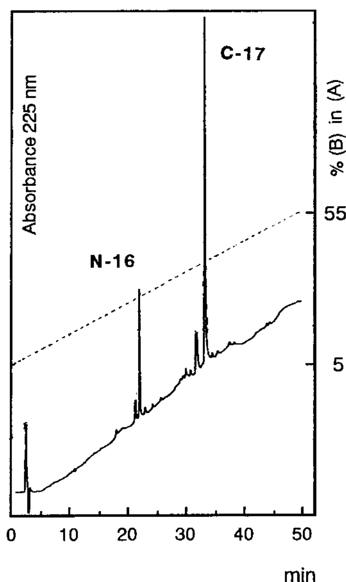

Figure 9. HPLC chromatogram of a lysyl endopeptidase digest of **5**. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6 × 150 mm); elution system, a linear gradient of CH_3CN (B) in 0.1% TFA (A); flow rate, 1 mL/min].

supposed much more difficult than that of big gastrin-II because they contain multiple Arg residues and oxidation-susceptible Met residues in addition to an alkylation-sensitive Trp residue (Figure 6). To minimize the number of protecting groups, Asn and Gln residues were introduced without masking the side-chain. For this purpose, Fmoc-Asn/Gln-OPfp[32] (Pfp = pentafluorophenyl) were used as acylation reagents with the help of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt).[33] Each constructed peptide-resin was subjected to the cleavage/deprotection with 90% aqueous TFA at 0 °C. Due to the presence of multiple Pbf groups in the protected peptides, the deprotection had to be prolonged (10 h for **7** and 15 to 18 h for **8** and **9**). Nevertheless, deterioration of the Tyr(SO_3H) residues was not significant (Figure 10a,c).[34] After one-step HPLC purification, highly homogeneous CCK peptides were obtained (Figure 10b,d). The yields from the protected peptide-resin were not excellent (9.7% for **8** and 7.8% for **9**), but these figures would be satisfactory for a total solid-phase synthesis of a sulfated peptide of 35 to 40 residues in length. The amino acid compositions of their acid hydrolysates coincided with the theoretical values, and intactness of the sulfates was directly evidenced on LSIMS or matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). Lysyl endopeptidase digestion produced two and three fragments from **8** and **9**, respectively (Figure 11). These fragments were identified as YIQQARK (**10**), APSGRMSIVK (**11**), and CCK-22 (**7**). All the analytical data supported the structural correctness of the final products. The insulinotropic activity of the CCK peptides was examined using the isolated pancreatic islets (male Wister rats) pretreated with glucose (11.1 mM). Synthetic human CCK-39 increased the insulin release to the same extent as CCK-8 and -33 at doses of 10^{-8} and 10^{-9} M (Table 2). In this way, CCK peptides were synthesized with our new approach without any difficulties.

Conclusions

A facile and efficient strategy for the Fmoc-SPPS of a Tyr(SO_3H)-containing peptide was established and successfully applied to the synthesis of various sizes of human gastrin-II and CCK peptides. The vital points of our strategy are the use of an extremely acid-sensitive Clt resin as a solid support and the S_N1-type deprotections promoted in TFA having a high ionizing power. To minimize the deterioration of a Tyr(SO_3H) residue, the final deprotection with TFA must be carried out at low temperature without addition of sulfur-containing soft bases. To our knowledge, the synthesis of big gastrin-II and CCK-39 has not been achieved so far. Our novel strategy has general applicability to sulfated peptides regardless of their sizes. The establishment of a facile synthetic protocol would also be helpful for biological studies of sulfated peptides and proteins.

Experimental Section

General Procedures. Fmoc-amino acid derivatives, PyBOP reagent, and Clt resin (substituted level; 1.47 mmol/g resin) were purchased from Watanabe Chemical Co., Ltd. (Hiroshima, Japan). Fmoc-His(Boc)-OH was obtained from Calbiochem-Novabiochem Japan, Ltd. (Tokyo, Japan). Lysyl endopeptidase from *Achromobacter lyticus* M497-1 (EC 3.4.21.50) was purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Other chemicals were of analytical grade and were used without further purification. Acid hydrolysis was carried out at 110 °C for 24 h with a mixture of propionic acid and 12 N HCl (1:1 v/v) for the resin-bound peptides, or with 6 N HCl containing a few drops of phenol for the purified peptides, and the hydrolysate was subjected to an amino acid analysis. For LSIMS analysis, glycerol, thioglycerol, and *m*-nitrobenzyl alcohol were used as a matrix. Sinapinic acid was used as a matrix for MALDI-TOFMS analysis.

Determination of the Rate Constants and Kinetic Parameters. Fmoc-Tyr(SO_3Na)-OH (4.28 mg, 8.47 μmol), Fmoc-Ser(^{t}Bu)-OH (3.25 mg, 8.47 μmol), and Fmoc-Arg(Pbf)-OH (5.50 mg, 8.47 μmol) were separately dissolved in TFA (1.0 mL) and stirred at a specified temperature (0, 18, or 30 °C). At appropriate intervals, 5.0 μL of each solution was withdrawn and diluted with a mixture of CH_3CN and phosphate buffer (0.2 M, pH 7.0) (3:7 v/v, 1 mL). Part of this solution (200 μL) was analyzed by RP-HPLC [column, Wakosil-II $5C_{18}$ (4.6 × 150 mm); elution system, a linear gradient of CH_3CN in 0.1 M $AcONH_4$ (30 to 70% in 20 min); flow rate, 1 mL/min; absorbance was detected at 300 nm]. Under these chromatographic conditions, the t_R of the starting compounds and the reaction products were as follows: 4.8 and 7.8 min for Fmoc-Tyr(SO_3Na)-OH and Fmoc-Tyr-OH; 10.8 and 6.3 min for Fmoc-Ser(^{t}Bu)-OH and Fmoc-Ser-OH; 14.1 and 6.1 min for Fmoc-Arg(Pbf)-OH and Fmoc-Arg-OH, respectively. These experiments were conducted in triplicate and the rates and activation parameters determined from the results are summarized in Table 1.

Effects of H_2O Addition on the Reaction Rates. Acidolysis rates of Fmoc-Tyr(SO_3Na)-OH, Fmoc-Ser(^{t}Bu)-OH, and

(31) (a) Yagami, T.; Kitagawa, K.; Aida, C.; Fujiwara, H.; Futaki, S. In *Peptide Science—Present and Future* (*Proceedings of the 1st International Peptide Symposium)*; Shimonishi, Y., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; pp 370−372. (b) Yagami, T.; Kitagawa, K.; Aida, C.; Fujiwara, H.; Futaki, S. *J. Pept. Res.* **2000**, *56*, 239−249.

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(33) (a) Atherton, E.; Holder, J. L.; Meldal, M.; Sheppard, R. C.; Valerio, R. M. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2887−2894. (b) Peters, S.; Bielfeldt, T.; Meidal, M.; Bock, K.; Paulsen, H. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1163−1171.

(34) We observed a significant difference in acid-sensitivity of Tyr(SO_3H) residues in gastrin-II and CCK peptides; the Tyr(SO_3H) residue in gastrin-II peptides was more sensitive to acidic conditions compared to that in CCK peptides. It seems very likely that this difference is due partly to the ionic interactions between the Tyr(SO_3H) residue and Arg residues in CCK peptides.[31b]

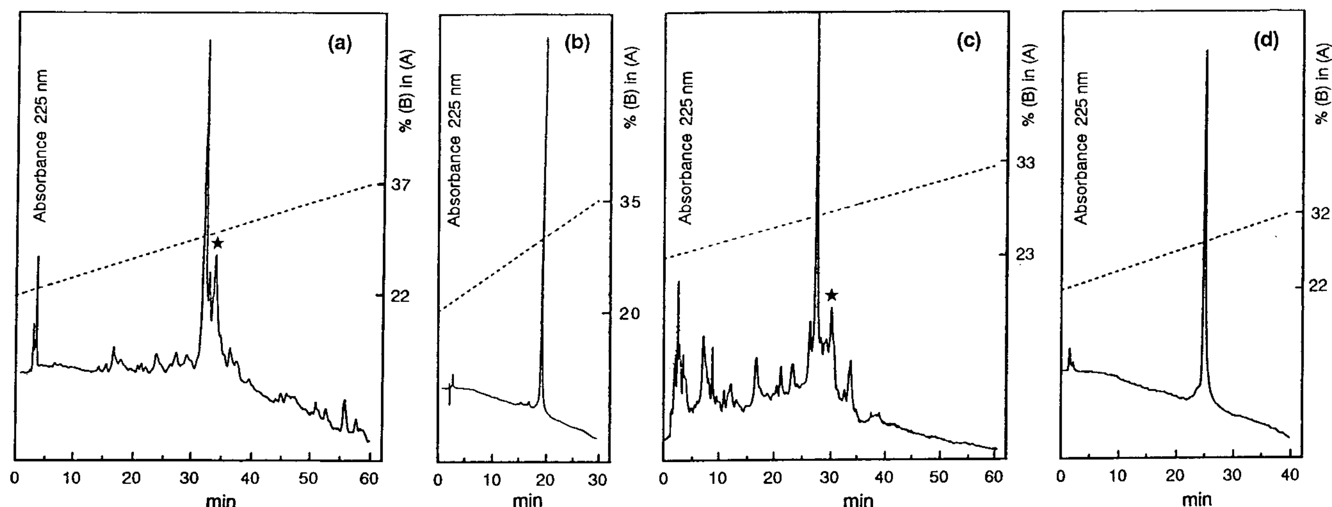

Figure 10. HPLC chromatograms of (a) crude **8**, (b) purified **8**, (c) crude **9**, and (d) purified **9**. Asterisks in (a) and (c) show the desulfated peptides produced during the deprotection. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6 × 150 mm); elution system, a linear gradient of CH_3CN (B) in 0.1 M $AcONH_4$ (A); flow rate, 0.8 mL/min for (a), (c), and (d), 1.0 mL/min for (b)].

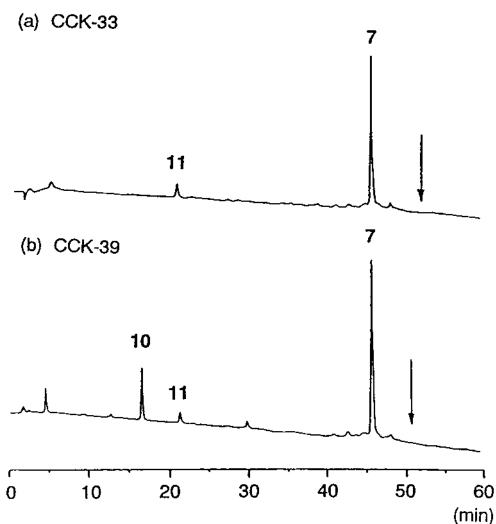

Figure 11. HPLC chromatograms of a lysyl endopeptidase digest of (a) **8** and (b) **9**. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6 × 150 mm); elution system, a linear gradient of CH_3CN in 0.1 M $AcONH_4$, 5 to 65% in 60 min; flow rate, 1 mL/min; absorbance was detected at 225 nm]. Arrows in (a) and (b) show the eluting position of **8** and **9**, respectively.

Table 2. Glucose-Dependent Insulinotropic Effect of the Synthetic CCK Peptides

	n	insulin(μU/h/islet)[a]
glucose (3.3 mM)	3	9.3 ± 2.1
glucose (11.1 mM)	4	14.5 ± 0.9
glucose (11.1 mM) + CCK-8 (10^{-8} M)	7	19.5 ± 4.2
glucose (11.1 mM) + CCK-33 (10^{-9} M)	7	16.7 ± 2.8
glucose (11.1 mM) + CCK-33 (10^{-8} M)	7	19.5 ± 2.6
glucose (11.1 mM) + CCK-39 (10^{-9} M)	7	16.6 ± 2.6
glucose (11.1 mM) + CCK-39 (10^{-8} M)	7	21.6 ± 2.6

[a] Mean ± SD.

Fmoc-Arg(Pbf)-OH at 0 °C in aqueous TFA (95, 90, 85, or 80% TFA) were determined as described above. These experiments were conducted in triplicate, and the results are depicted in Figure 4.

Fmoc-Based Solid-Phase Peptide Synthesis (general procedures). Solid-phase peptide synthesis was conducted manually. Side-chain protecting groups used in the synthesis were as follows: *t*Bu for Asp, Glu, Ser, and Tyr; Boc for Lys and His; Pbf for Arg. Fmoc groups for N^{α}-protection were cleaved by 1 min treatment with 20% piperidine in DMF followed by the second treatment with the same reagent for 20 min. For deprotection of the N^{α}-Fmoc group of Gln and Glu(O*t*Bu) residues, the concentration of piperidine was reduced to 10%. After the Fmoc cleavage, the peptide-resin was washed with DMF (× 6). The next residue was then incorporated with the DIPCDI−HOBt coupling protocol [Fmoc-amino acid (3 equiv), DIPCDI (3 equiv), and HOBt (3 equiv)] or the PyBOP-mediated coupling protocol [Fmoc-amino acid (3 equiv), PyBOP reagent (3 equiv), and *N*-methylmorpholine (NMM) (9 equiv)]. Asn and Gln residues were exceptionally coupled with an active ester protocol [Fmoc-Asn/Gln-OPfp (5 equiv), HOOBt (5 equiv), and NMM (5 equiv)]. After gentle agitation (1.5 h) and washing with DMF (× 6), part of the peptide-resin was subjected to the Kaiser test.[35] On completion of the assembly, the peptide-resin was successively washed with DMF (× 5), MeOH (× 5), and ether (× 5) and then dried in vacuo.

Synthesis of Gastrin-II and CCK Peptides by the Facile Solid-Phase Method. Peptide Chain Assembly. Fmoc-Asp-Phe-NH_2 was prepared by a solution-phase method and attached to Clt resin according to the procedure of Barlos and co-workers.[20c] The resulting Fmoc-Asp(Clt resin)-Phe-NH_2 (307 mg, 100 μmol) was used for the starting dipeptide-resin, and each Fmoc-amino acid derivative was manually incorporated into it. For the synthesis of **4** and **5**, the N-terminal pyroglutamic acid (Pyr) was incorporated into the peptide chains without N^{α}-protection.

Cleavage and Deprotection. The protected peptide-resin was treated with a mixture of HFIP/CH_2Cl_2 (1:4 v/v, approximately 5 mL for 100 mg of the peptide-resin) for 0.5 h at 25 °C and then filtered. The combined filtrate and washing were condensed using a N_2 stream; then dry ether (50 mL) was added to precipitate the peptides. After centrifugation, the collected precipitate was dried in vacuo. The cleavage efficiency was usually about 95% based on the amino acid analysis of the residual resin. The protected peptide thus detached was treated with *precooled* 90% aqueous TFA (approximately 2 mL for 100 mg of the peptide-resin) at 0 °C for an appropriate period of time; then dry ether (50 mL) was added. The formed precipitate was collected by centrifugation and washed twice with ether and then dissolved in 0.025 M NH_4HCO_3 (50 mL) and lyophilized to give a crude peptide sample.

For the synthesis of big gastrin-II (**5**), CCK-22 (**7**), -33 (**8**), and -39 (**9**), the cleavage from the peptide-resin and the deprotection of the protecting groups were concurrently conducted with 90% aqueous TFA at 0 °C for an appropriate period of time.

Purification by Preparative RP-HPLC and Purity Assessment by Analytical HPLC. A crude peptide sample

(35) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595−598.

obtained after deprotection was purified by RP-HPLC using a column of 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) at a flow rate of 3 mL/min was used for elution, and the absorbance was detected at 235 or 275 nm depending on the peptide. The solvent was removed by lyophilization, and the residue was again dissolved in 0.025 M NH_4HCO_3 (10 mL). Lyophilization afforded a fluffy powder as a final purified peptide. The purity of the purified material was assessed by analytical HPLC using a column of Cosmosil 5C_{18}-AR (4.6 × 150 mm) except for **3** and **7**, in which a column of Cosmosil 5C_{18}-AR (6.0 × 150 mm) was used. A solvent system consisting of solvent A (0.1 M $AcONH_4$) and solvent B (CH_3CN) at an appropriate flow rate was used for elution, and the absorbance was detected at 225 or 275 nm.

CCK-12 (2). Starting with the protected peptide-resin (150 mg, 25.5 μmol), 40.6 mg of a crude peptide (Figure 7a) was obtained after cleavage and deprotection (8 h at 0 °C). A portion of this sample (22.5 mg) was purified by preparative HPLC using a linear gradient of B in A (25 to 35% in 60 min). After lyophilization, 5.9 mg of pure **2** was obtained (26% yield from the protected peptide-resin). This sample exhibited a sharp single peak at t_R 11.4 min on an analytical HPLC chromatogram [elution system, a linear gradient of B in A (20 to 35% in 30 min); flow rate, 1 mL/min; absorbance was detected at 225 nm]. Amino acid ratios in the acid hydrolysate were as follows (theoretical values are given in parentheses): Asp 2.76 (3), Ser 0.94 (1), Gly 1.00 (1), Met 1.78 (2), Ile 0.89 (1), Tyr 0.90 (1), Phe 1.05 (1), Trp not determined (1), Arg 0.93 (1). LSIMS *m/z*: calcd for $C_{68}H_{94}N_{17}O_{23}S_3$ 1612.6 ([M − H], monoisotopic mass); found 1612.5 $[M - H]^-$. FT-IR: ν_{max} (KBr) 1049 and 1260 cm^{-1}.

Mini Gastrin-II (3). Starting with the protected peptide-resin (50.0 mg, 10.0 μmol), 19.0 mg of a crude peptide was obtained after cleavage and deprotection (5 h at 0 °C). This sample was purified by preparative HPLC using an elution system; an isocratic elution (22% B in A) for 10 min followed by a linear gradient of B in A (22 to 32% in 60 min). After lyophilization, 6.5 mg of pure **3** was obtained (34% yield from the protected peptide-resin). This sample exhibited a sharp single peak at t_R 15.5 min on an analytical HPLC chromatogram [elution system, a linear gradient of B in A (15 to 45% in 30 min); flow rate, 1 mL/min; absorbance was detected at 275 nm]. Amino acid ratios in the acid hydrolysate: Asp 1.04 (1), Glu 4.74 (5), Gly 1.00 (1), Ala 1.00 (1), Met 0.65 (1), Leu 0.94 (1), Tyr 0.87 (1), Phe 0.95 (1), Trp not determined (2). LSIMS *m/z*: calcd. for $C_{85}H_{108}N_{17}O_{30}S_2$ 1912.0 ([M − H], average mass); found 1911.9 $[M - H]^-$. FT-IR: ν_{max} (KBr) 1050 and 1241 cm^{-1}.

Little Gastrin-II (4). Starting with the protected peptide-resin (102 mg, 20.0 μmol), 43.4 mg of a crude peptide (Figure 7b) was obtained after cleavage and deprotection (5 h at 0 °C). A portion of this sample (20.4 mg) was purified by preparative HPLC using an elution system; an isocratic elution (22% B in A) for 10 min followed by a linear gradient of B in A (22 to 27% in 60 min). After lyophilization, 7.9 mg of pure **4** was obtained (38% yield from the protected peptide-resin). This sample exhibited a sharp single peak at t_R 15.4 min on an analytical HPLC chromatogram [elution system, a linear gradient of B in A (15 to 30% in 30 min); flow rate, 1 mL/min; absorbance was detected at 275 nm]. Amino acid ratios in the acid hydrolysate: Asp 1.02 (1), Glu 5.89 (6), Pro 1.31 (1), Gly 2.00 (2), Ala 1.00 (1), Met 0.31 (1), Leu 1.00 (1), Tyr 0.78 (1), Phe 0.95 (1), Trp not determined (2). LSIMS *m/z*: calcd. for $C_{97}H_{122}N_{20}O_{34}S_2$ 2176.2 ([M − H], average mass); found 2176.3 $[M - H]^-$, 2096.7 $[M - H - SO_3]^-$. FT-IR: ν_{max} (KBr) 1049 and 1239 cm^{-1}.

Synthesis of Big Gastrin-II. Big Gastrin-II (5). Each residue was incorporated stepwise into Fmoc-Asp(Clt resin)-Phe-NH_2 (155 mg, 50.0 μmol) according to the general procedures for the Fmoc-SPPS. The resulting protected peptide-resin (60.0 mg, 10.2 μmol) was treated with 90% aqueous TFA (8 h at 0 °C) for cleavage/deprotection. The crude peptide (37.0 mg) was obtained after lyophilization (Figure 8a). A portion of this sample (13.6 mg) was purified by preparative HPLC using a linear gradient of B in A (23 to 30% in 60 min). After lyophilization, 1.8 mg of pure **5** was obtained (13% yield from the protected peptide-resin). This sample exhibited a sharp single peak at t_R 16.4 min on an analytical HPLC chromatogram (Figure 8b). Amino acid ratios in the acid hydrolysate: Asp 2.09 (2), Ser 1.05 (1), Glu 7.57 (8), Pro 4.97 (5), Gly 3.90 (4), Ala 2.00 (2), Val 0.91 (1), Met 0.72 (1), Leu 2.71 (3), Tyr 0.89 (1), Phe 1.00 (1), Lys 2.12 (2), His 0.94 (1), Trp not determined (2). LSIMS *m/z*: calcd. for $C_{176}H_{251}N_{43}O_{56}S_2$ 3929.3 ([M], average mass); found 3929.8 $[M + H]^+$ in the positive-ion mode, and 3927.8 $[M - H]^-$ in the negative-ion mode.

Lysyl Endopeptidase Digestion of 5. Purified **5** (100 μg) was digested by lysyl endopeptidase in 0.1 M NH_4HCO_3 (pH 8.2, 250 μL) at 37 °C for 4 h. The weight ratio of the enzyme to the substrate was 1:200. The enzyme digest of **5** showed two peaks (t_R =21.8 and 32.9 min) on an HPLC chromatogram (Figure 9). These two peaks were identified to be the N-terminal 16-mer (**N-16**, t_R =21.8 min) and the C-terminal 17-mer (**C-17**, t_R =32.9 min) from amino acid analyses of their acid hydrolysates: **N-16**, Asp 0.98 (1), Ser 0.94 (1), Glu 1.91 (2), Pro 4.36 (4), Gly 2.00 (2), Ala 0.98 (1), Val 0.95 (1), Leu 1.90 (2), Lys 0.97 (1), His 0.96 (1); **C-17**, Asp 1.03 (1), Glu 5.80 (6), Pro 0.80 (1), Gly 2.25 (2), Ala 1.07 (1), Met 0.92 (1), Leu 1.00 (1), Tyr 0.95 (1), Phe 1.20 (1), Trp not determined (2). Nonsulfated counterpart, big gastrin-I (**6**), was similarly digested by lysyl endopeptidase. The resulting digest exhibited two peaks (t_R = 21.8 and 34.3 min) on an HPLC chromatogram; the first eluting peak coincided with the t_R of **N-16**, while the last eluting peak was slightly retarded compared with the t_R of **C-17**.

Synthesis of Human CCK-33 and -39. Each residue was incorporated stepwise into Fmoc-Asp(Clt resin)-Phe-NH_2 (307 mg, 100 μmol). There were no doubts about the completion of each coupling step, and repeated coupling was not required. After introduction of the Asn residue (twenty seconds from the C-terminus), the N^{α}-Fmoc group was removed, and the resulting peptide-resin was dried in vacuo. Part of this peptide-resin (50 mg) was used for the preparation of CCK-22, and the rest was further elongated to obtain the longer CCK peptides.

CCK-22 (7). The protected peptide-resin (50 mg, 7.5 μmol) was treated with 90% aqueous TFA (10 h at 0 °C) for cleavage/deprotection. The resulting crude peptide (19.0 mg) was purified by preparative HPLC using a linear gradient of B in A (25 to 32% in 60 min). After lyophilization, 2.8 mg of pure **7** was obtained (13.4% yield from the protected peptide-resin). This sample exhibited a sharp single peak at t_R 16.6 min on an analytical HPLC chromatogram [elution system, a linear gradient of B in A (20 to 45% in 30 min); flow rate, 1 mL/min; absorbance was detected at 230 nm]. Amino acid ratios in the acid hydrolysate: Asp 5.26 (6), Ser 1.84 (2), Glu 1.00 (1), Pro 0.91 (1), Gly 1.00 (1), Met 1.83 (2), Ile 0.76 (1), Leu 1.81 (2), Tyr 0.89 (1), Phe 1.05 (1), His 0.88 (1), Trp not determined (1), Arg 1.78 (2). LSIMS *m/z*: calcd. for $C_{117}H_{172}N_{35}O_{39}S_3$ 2789.0 ([M − H], average mass); found 2788.9 $[M - H]^-$.

CCK-33 (8). The protected peptide-resin (36 mg, 3.8 μmol) was treated with 90% aqueous TFA (15 h at 0 °C) for cleavage/deprotection. The resulting crude peptide (15.0 mg, Figure 10a) was purified by preparative HPLC using an elution system; an isocratic elution (27% B in A) for 10 min followed by a linear gradient of B in A (27 to 30% in 60 min). After lyophilization, 1.5 mg of pure **8** was obtained (9.7% yield from the protected peptide-resin). This sample exhibited a sharp single peak at t_R 18.6 min on an analytical HPLC chromatogram (Figure 10-b). Amino acid ratios in the acid hydrolysate: Asp 5.53 (6), Ser 3.18 (4), Glu 1.03 (1), Pro 2.12 (2), Gly 2.00 (2), Ala 0.89 (1), Val 0.66 (1), Met 2.53 (3), Ile 1.52 (2), Leu 1.80 (2), Tyr 0.93 (1), Phe 1.00 (1), Lys 1.91 (2), His 0.91 (1), Trp not determined (1), Arg 2.72 (3). Recoveries of Val and Ile were slightly low due to steric hindrance of the Ile-Val bond. LSIMS *m/z*: calcd for $C_{167}H_{263}N_{51}O_{52}S_4$ 3945.4 ([M], average mass); found 3946.5 $[M + H]^+$ in the positive-ion mode, and 3944.6 $[M - H]^-$ in the negative-ion mode.

CCK-39 (9). The protected peptide-resin (50 mg, 5.1 μmol) was treated with 90% aqueous TFA (18 h at 0 °C) for cleavage/deprotection. The resulting crude peptide (23.6 mg, Figure 10c)

was purified by preparative HPLC using an elution system; an isocratic elution (27% B in A) for 10 min followed by a linear gradient of B in A (27 to 30% in 60 min). After lyophilization, 1.8 mg of pure **9** was obtained (7.8% yield from the protected peptide-resin). This sample exhibited a sharp single peak at t_R 26.8 min on an analytical HPLC chromatogram (Figure 10-d). Amino acid ratios in the acid hydrolysate: Asp 5.95 (6), Ser 4.07 (4), Glu 3.04 (3), Pro 1.65 (2), Gly 1.85 (2), Ala 1.77 (2), Val 0.67 (1), Met 2.09 (3), Ile 2.29 (3), Leu 2.00 (2), Tyr 1.74 (2), Phe 1.06 (1), Lys 1.79 (2), His 0.97 (1), Trp not determined (1), Arg 3.52 (4). Low recovery of Met was due to the oxidation (Met sulfoxide), and recoveries of Val and Ile were low as in the case of CCK-33. MALDI-TOFMS *m*/*z*: calcd for $C_{201}H_{316}N_{62}O_{61}S_4$ 4705.3 ([M], average mass); found 4706.0 $[M + H]^+$ in the positive-ion mode, and 4704.0 $[M - H]^-$ in the negative-ion mode.

Lysyl Endopeptidase Digestion of 8 and 9. Purified **8** (100 μg) was digested by lysyl endopeptidase in 0.1 M NH_4HCO_3 (pH 8.2, 250 μL) at 37 °C for 4 h. The weight ratio of the enzyme to the substrate was 1:200. The enzyme digest of **8** showed two peaks (t_R = 21.5 and 46.2 min) on an HPLC chromatogram (Figure 11a). The first eluting peak (t_R = 21.5 min) was indistinguishable from a peak of H-APSGRMSIVK-OH (**11**), and the second eluting peak (t_R = 46.2 min) coincided with a peak of CCK-22 (**7**). Purified **9** was similarly digested by lysyl endopeptidase, and the digest was analyzed on HPLC using the same chromatographic conditions (Figure 11b). Three peaks with t_R = 17.5, 21.5, and 46.2 min were detected, and they coincided with peaks of authentic H-YIQQARK-OH (**10**), **11**, and **7**, respectively.

Biological Activity of Synthetic CCK Peptides. Pancreatic islets were isolated from male Wister rats (about 210 to 240 g) by collagenase digestion and dextran gradient separation. Approximately 400 to 600 islets were obtained from each pancreas. Eight to 10 islets were incubated at 30 °C for 60 min in Krebs−Ringer bicarbonate buffer (pH 7.4, 1 mL) containing glucose (11.1 mM) with or without a synthetic CCK peptide. Insulin released into the medium by the islets (Table 2) was assayed with an enzyme immunoassay kit (Sanyo, Kyoto, Japan).

Acknowledgment. This work was supported by a grant from the Promotion and Mutual Aid Corporation for Private Schools of Japan and a Grant-in-Aid for Scientific Research (C) (no. 10672008) from the Japan Society for the Promotion of Science.

Supporting Information Available: Experimental details for the desulfation rates with the cleavage reagents using **1** as a model sulfated peptide and preparation of compound **6**, **10**, and **11**; the positive- and negative-ion mode LSIMS spectra of **5** and **8**; the positive- and negative-ion mode MALDI-TOFMS spectra of **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO000895Y