Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron 60 (2004) 907–918
Tetrahedron

Total chemical synthesis of large CCK isoforms using a thioester segment condensation approach

Kouki Kitagawa,[a,*] Hiroshi Adachi,[a] Yumi Sekigawa,[a] Takeshi Yagami,[b] Shiroh Futaki,[c] Yuan Jun Gu[d] and Kazutomo Inoue[d]

[a]*Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Kamishin'ei-cho 5-13-2, Niigata 950-2081, Japan*
[b]*National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan*
[c]*Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*
[d]*Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan*

Received 8 October 2003; accepted 13 November 2003

Abstract—Silver-ion mediated thioester segment condensation was applied to the chemical synthesis of high molecular weight isoforms of cholecystokinin (CCK). Three building blocks, a C-terminal Tyr(SO_3H)-containing segment and two partially protected thioester segments having a C-terminal Pro residue, were prepared using Fmoc-based chemistry and 2-chlorotrityl chloride (Clt) resin as a solid support. The entire peptide chain was successfully synthesized by two consecutive silver-ion mediated condensation reactions using these building blocks. A brief TFA treatment of the final condensation product gave highly homogeneous CCK-58 in a satisfactory yield. This peptide exhibited glucose-dependent insulinotropic activity at levels comparable to CCK-33. These results demonstrate the usefulness of the silver-ion mediated segment condensation approach in the preparation of large sulfated peptides.

1. Introduction

Cholecystokinin (CCK) is a peptide hormone that is distributed in both the brain and the gastrointestinal tract, and is reported to be involved in central and pancreatic regulations.[1] This peptide is also known to have several isoforms, such as CCK-8, -22, -33,[1a,b] -39,[1e] and -58[1f] (Fig. 1). The different forms of CCK are generated from the post-translational processing of a common precursor protein, pro-CCK.[2] Although CCK-58 has been shown to be the major circulating isoform in various animal species including humans, the significance of the molecular diversity of CCK remains unclear. Every form has a tyrosine *O*-sulfated residue [Tyr(SO_3H)] at the seventh

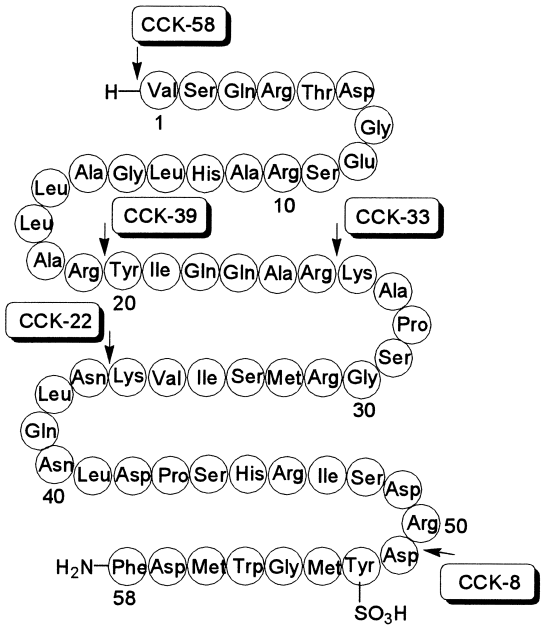


Figure 1. Sequence of CCK peptides.

Keywords: Sulfated peptide; Cholecystokinin (CCK); Peptide thioester; Silver-ion mediated thioester segment condensation; 2-Chlorotrityl chloride resin; Fmoc-based SPPS.

Abbreviations: All, amino acids are of the L-configuration; AcOH, acetic acid; $AcONH_4$, ammonium acetate; Boc, *tert*-butoxycarbonyl; ^{t}Bu, *tert*-butyl; Bum, *tert*-butoxymethyl; CCK, cholecystokinin; Clt, resin, 2-chlorotrityl chloride resin; DIEA, diisopropylethylamine; DIPCDI, *N,N*-diisopropylcarbodiimide; Fmoc, fluoren-9-ylmethoxycarbonyl; 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; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Pfp, pentafluorophenyl; PyBOP, benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate; Su, *N*-hydroxysuccinimidyl; TFA, trifluoroacetic acid; Trt, trityl; WSCDI, water-soluble carbodiimide.

* Corresponding author. Tel.: +81-25-268-1241; fax: +81-25-268-1230; e-mail address: kouki@niigata-pharm.ac.jp

0040–4020/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2003.11.037

position from the C-terminus that is crucial for its biological activity. Even though the shortest CCK-peptide, CCK-8, possesses the characteristic pharmacological properties of CCK peptides, it is important to understand the physiological roles of the larger CCK isoforms in order to clarify the purpose of the molecular diversity.

A prerequisite for investigating the biological properties of the larger CCK isoforms is the availability of an efficient method for obtaining sufficient quantities. However, chemical synthesis of CCK peptides has focused on CCK-8 due to the synthetic difficulties caused by the inherent acid lability of Tyr(SO_3H) residues.[3] These difficulties are exemplified by the challenging syntheses of porcine CCK-33 by Sakakibara et al.[4] and human CCK-33 by Yajima et al.[5] Both syntheses were achieved by solution methods adopting a post-assembly sulfation approach. On the other hand, Fmoc-based solid-phase synthesis of porcine CCK-33 using Fmoc-Tyr(SO_3Na)-OH as a building block (a pre-assembly sulfation approach) was reported by Penke et al. in 1991.[6] In their synthesis, a newly developed acid-labile linker-resin was used. Nevertheless, the total yield of the objective peptide was unsatisfactory. Thereafter, two reports on chemical synthesis of larger isoforms of CCK-related peptides were published.[7] In these reports, the acid labile Tyr(SO_3H) residue was replaced by a hydrolysis-resistant analogue Phe(p-CH_2SO_3Na),[8] while the Met and the Trp residues were replaced by norleucine and 2-naphthylalanine residues, respectively. Analogues of CCK-33, -39, and -58 having these unnatural amino acid residues, were reported to exhibit considerably less potent biological activity than CCK-8.[7]

We recently established a facile solid-phase method for the synthesis of Tyr(SO_3H)-containing peptides.[9] This method was successfully applied to the direct solid-phase syntheses of human CCK-33 and -39.[9b] However, a general method for the synthesis of large sulfated peptides having over 50 amino acid residues, such as CCK-58, remains necessary. Therefore, we examined the applicability of a silver-ion mediated thioester segment condensation approach[10] to the synthesis of large Tyr(SO_3H)-containing peptides. Numerous large peptides have been prepared using this approach and it has been successfully applied to the preparation of post-translationally modified peptides, such as glycosylated peptides[11] and phosphorylated peptides.[12] In this paper, we report the synthesis of the larger isoforms of the sulfated peptides CCK-39 and -58, using the silver-ion mediated thioester segment condensation approach.

2. Results and discussion

2.1. Synthetic plan

The amino acid sequence of human CCK peptides is shown in Figure 1. Considering the acid-lability of the Tyr(SO_3H) residue, it is desirable to keep the number of protecting groups to a minimum, as these groups are removed by a final acid treatment. The silver-ion mediated thioester segment condensation approach[10] meets this requirement, because protection of the side-chain functional groups is unnecessary, except for free amino groups. By adopting this approach to construct the entire peptide chain in combination with a brief TFA treatment to remove the acid-labile amino protecting groups, we can expect efficient preparation of a large Tyr(SO_3H)-containing peptide (Fig. 2). For synthesis of the objective CCK-58, the two Pro residues (Pro^{28} and Pro^{43} in Figure 1) were selected as coupling positions in order to circumvent the racemization problem accompanying thioester segment condensation.[10] As shown in Figure 2, we divided the 58-residue peptide into three segments: the C-terminal Tyr(SO_3H)-containing segment, CCK(44–58), and two thioester segments having C-terminal Pro residues, CCK(29–43) and CCK(1–28).

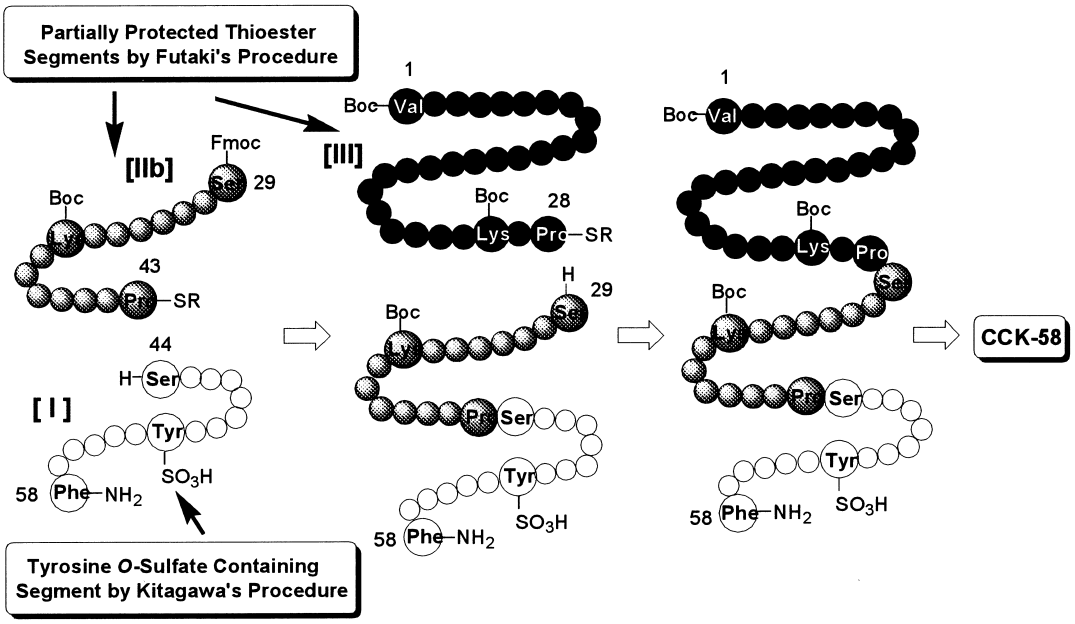


Figure 2. Synthetic plan for CCK-58.

For preparation of the Tyr(SO_3H)-containing segment, the facile solid phase approach for the Tyr(SO_3H)-containing peptides developed by our group[9] is potentially useful. In this protocol, two key features are involved in completing the synthesis in an efficient manner: (i) utilization of a highly acid-sensitive 2-chlorotrityl (Clt) resin[13] to quantitatively detach the sulfated peptide from the solid support, and (ii) TFA deprotection at low temperature to minimize the deterioration of the sulfate. With regard to the peptide thioester segments, preparation of these had been limited to Boc-based chemistry until recently (Boc-strategy in Figure 3). This is due to the nucleophilicity of piperidine, a standard deprotection reagent for the N^{α}-Fmoc group in Fmoc-based SPPS. Resin-bound thioester is generally unstable to repeated exposure to piperidine. However, several groups have reported new methods for preparing peptide thioester using Fmoc-based chemistry,[14] either with a thioester-compatible deprotection reagent for the N^{α}-Fmoc group[14b] or with a special linker, such as an alkanesulfonamide-type 'safety-catch' linker[14c–e] and a backbone amide linker.[14f] In this study, we decided to employ a novel protocol developed by Futaki et al.[14a] in which thioesterification is carried out on a fully protected peptide after Fmoc-based peptide-chain assembly (Fmoc-strategy in Figure 3). Application of Clt resin[13] is critical to prepare such side-chain protected peptides with Fmoc-based SPPS. In addition, the sterically hindered Clt resin is able to minimize the premature detachment of dipeptides having a C-terminal Pro residue (X-Pro) from the resin through diketopiperazine formation. We previously demonstrated the usefulness of Clt resin for Fmoc-based preparation of protected peptide segments having C-terminal Pro residues and the obtained peptides were successfully employed in solid-phase segment condensation aiming to synthesize a 34-residue sulfated peptide, human big gastrin-II.[15] In this way, the preparation of both the Tyr(SO_3H)-containing segment and the thioester segments having a C-terminal Pro residue rely upon the unique characteristics of a Clt resin.

2.2. Segment synthesis

2.2.1. Synthesis of Tyr(SO_3H)-containing segment. The C-terminal sulfated peptide, CCK(44–58): [**I**], was prepared as shown in Figure 4. 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,[9] was linked with Clt resin via the β-carboxyl group of Asp, and each Fmoc-amino acid including Fmoc-Tyr(SO_3Na)-OH[6] was added to the peptide-resin in a stepwise manner. After completing the peptide chain assembly, cleavage of the peptide from the resin and deprotection were concurrently conducted with 90% aqueous TFA (0 °C, 12 h). Despite this long TFA treatment, desulfated peptide was estimated to account for ca. 10% of total peptide by HPLC analysis (Fig. 4(a)). The crude sulfated peptide was purified by HPLC to give an analytically pure segment [**I**] in 18% yield (Fig. 4(b)). The structural correctness of this peptide was ascertained by amino acid analysis and liquid secondary-ion mass spectrometry (LSIMS), and the fidelity of the sulfate was confirmed by LSIMS in the positive- and negative-ion modes.[16]

2.2.2. Synthesis of thioester segments. The method for peptide thioester synthesis developed by Futaki et al.[14a] consists of the following steps; (i) construction of a peptide chain on Clt resin by Fmoc-based SPPS, (ii) cleavage of the fully protected peptide from the Clt-resin under extremely weak acidic conditions, (iii) thioesterification of the protected peptide by reaction with HS-$(CH_2)_2$COOEt or HS-$(CH_2)_2CONH_2$, and (iv) deprotection of the peptide with TFA. We require thioester segments bearing a TFA-labile amino protecting group in order to construct a large Tyr(SO_3H)-containing peptide sequence, and therefore re-introduction of a Boc protecting group to the functional amino groups (N^{α}- and N^{ε}-amino groups of a peptide) was also conducted as step (v) in this sequential scheme.

We describe here, as an example, the detailed preparation of

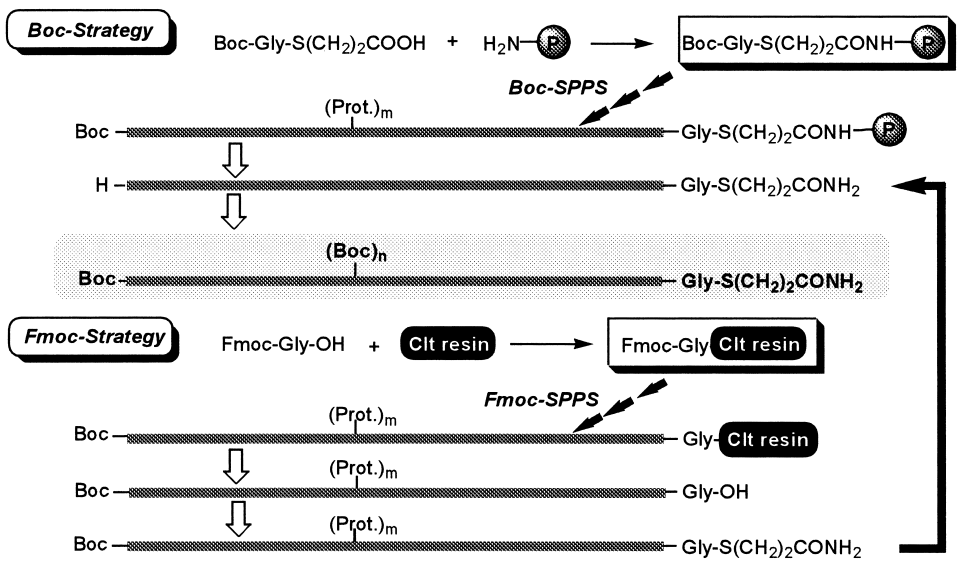


Figure 3. Preparation of peptide thioesters via Boc- or Fmoc-strategy.

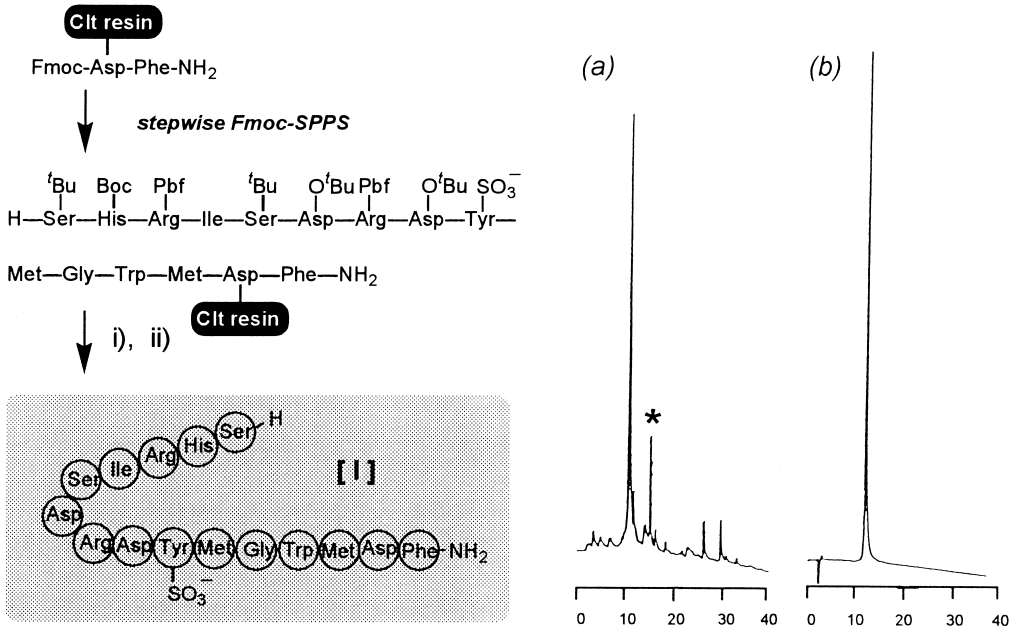


Figure 4. Synthesis of C-terminal Tyr(SO_3H)-containing segment [**I**]. *Reagents*: (i) 90% aqueous TFA, 0 °C, 12 h; (ii) preparative HPLC. *Inlet*: HPLC chromatograms of (a) crude [**I**] after cleavage/deprotection (step i) and (b) HPLC-purified [**I**]. An asterisk in (a) shows the desulfated peptide produced during step (i). [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6×150 mm); elution, a linear gradient of B in A (20 to 40% in 40 min); flow rate, 0.8 ml/min].

thioester segment, Boc-[Lys(Boc)^{26,36}]-CCK(20–43)-S$(CH_2)_2$COOEt: [**IIa**], which was used for the synthesis of CCK-39 (Fig. 5, scheme). This 24-mer peptide was constructed on an Fmoc-Pro-Clt resin according to the general procedure of Fmoc-based SPPS. The N-terminal residue was introduced as a Boc-protected derivative, Boc-Tyr(^{t}Bu)-OH. The suppressed diketopiperazine formation and the subsequent dipeptide (Asp-Pro in this case) detachment from the resin were substantiated by the fact that the weight of the peptide-resin increased reasonably

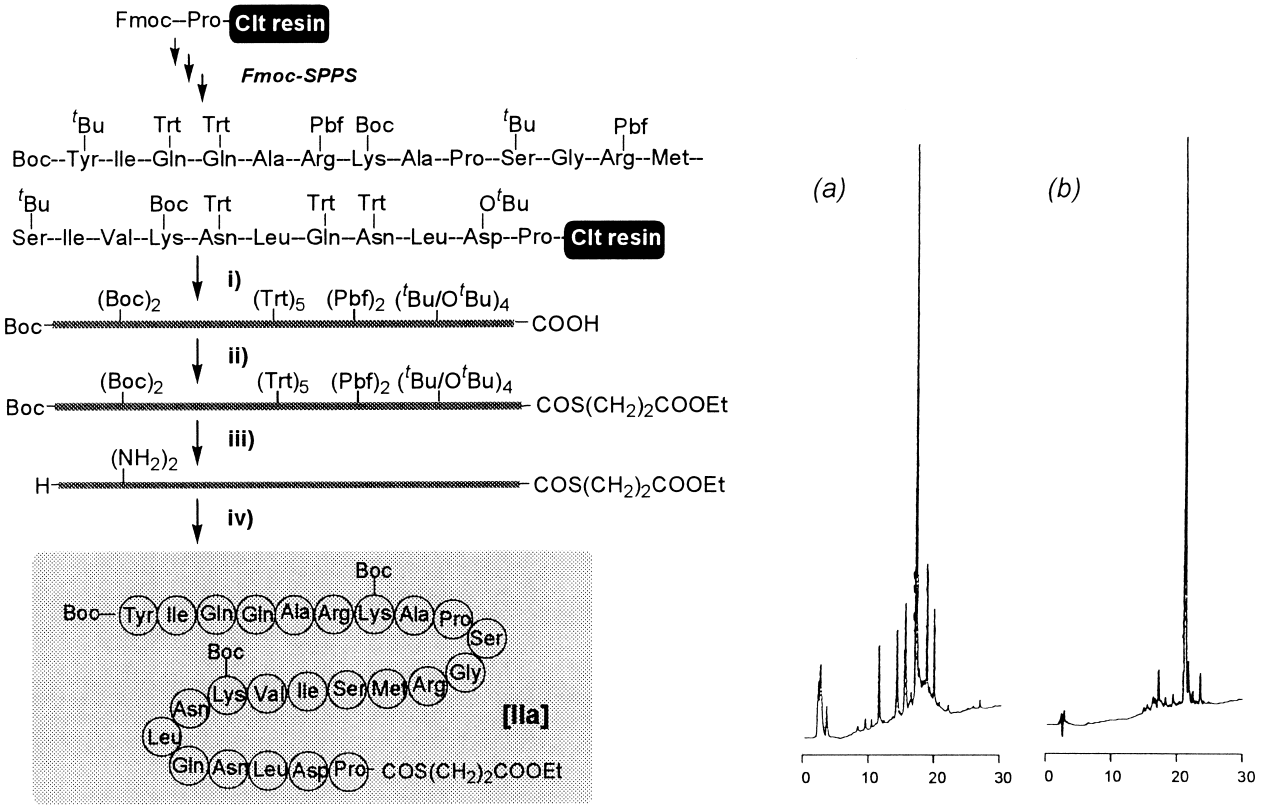


Figure 5. Synthesis of thioester segment [**IIa**]. *Reagents*: (i) HFIP/CH_2Cl_2 (1:4), 25 °C, 30 min; (ii) HS-$(CH_2)_2$COOEt (25 equiv.), WSCDI·HCl (15 equiv.), and HOBt (15 equiv.), 4 °C, 20 h; (iii) 95% aqueous TFA, 25 °C, 3 h; (iv) Boc-OSu (75 equiv.), and NMM (75 equiv.), 25 °C, 20 h. *Inlet*: HPLC chromatograms of (a) crude peptide after step (iii) and (b) crude peptide [**IIa**] after step (iv). [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6×150 mm); elution, a linear gradient of D in C (20 to 45% in 30 min for (a) and 30 to 50% in 30 min for (b)); flow rate, 0.8 ml/min].

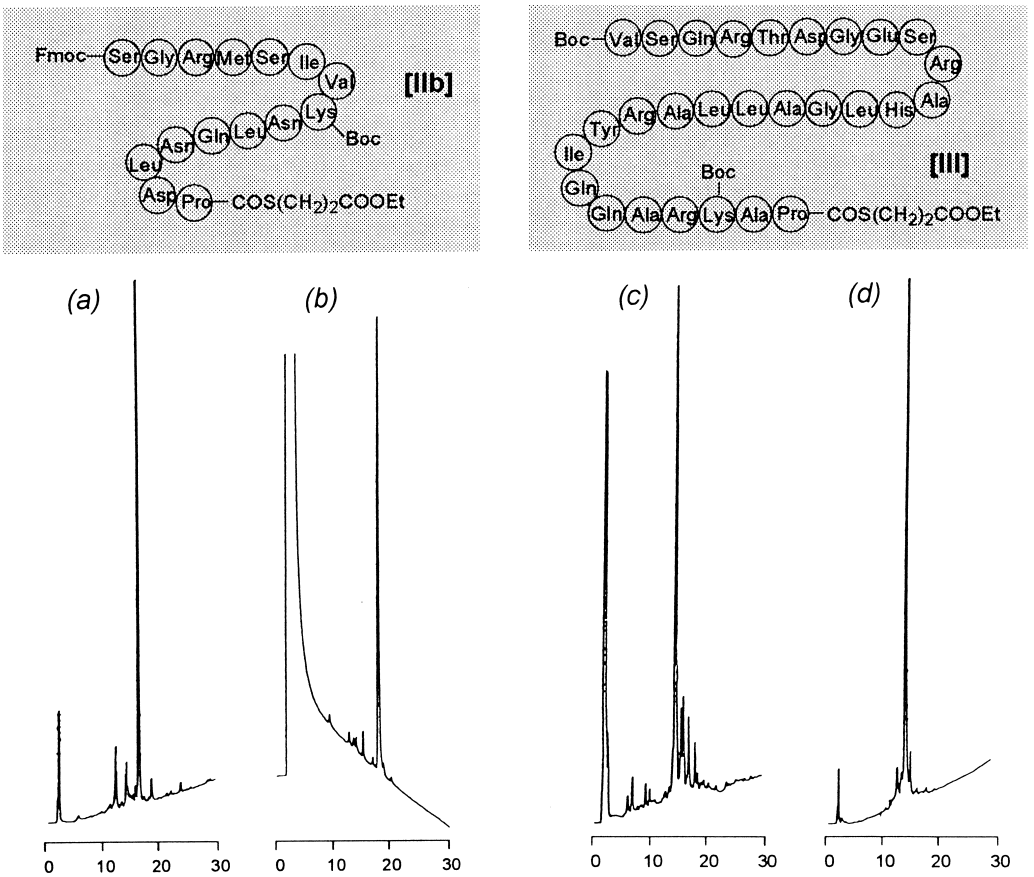


Figure 6. HPLC chromatograms of crude intermediates for preparation of thioester segment [**IIb**] and [**III**]; (a) crude intermediate peptide for [**IIb**] after deprotection (step (iii)), (b) crude intermediate peptide for [**IIb**] after Boc-derivatization (step (iv)), (c) crude intermediate peptide for [**III**] after deprotection (step (iii)), and (d) crude intermediate peptide for [**III**] after Boc-derivatization (step (iv)). Steps (iii) and (iv) correspond to the synthetic steps shown in Figure 5. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6×150 mm); elution, a linear gradient of D in C (30 to 60% in 30 min for (a), 25 to 40% in 30 min for (c), and 30 to 45% in 30 min for (d)), and a linear gradient system of B in A (30 to 65% in 30 min for (b)); flow rate, 1 ml/min for (a), (c), (d) and 0.8 ml/min for (b)].

after incorporation of the third amino acid residue. Detachment of the fully protected peptide from the Clt resin was achieved by treating the peptide-resin with a mixture of hexafluoro-2-propanol (HFIP)/CH_2Cl_2 (1:4, 30 min).[17] We used this cleavage reagent instead of the usual AcOH/trifluoroethanol/CH_2Cl_2[13] cleavage system in order to avoid contamination with trace amounts of AcOH. The fully protected peptide was then directly subjected to thioesterification by reaction with excess ethyl 3-mercaptopropionate (25 equiv.). The reaction was completed with the aid of water-soluble carbodiimide (WSCDI·HCl, 15 equiv.) in the presence of *N*-hydroxybenzotriazole (HOBt, 15 equiv.) in DMF. After overnight stirring at 4 °C, none of the starting material was detected on TLC. The crude product was isolated after washing with ether several times, and then subjected to deprotection with 95% aqueous TFA (25 °C, 3 h). HPLC purification of the crude deprotected peptide (Fig. 5(a)) gave a homogeneous peptide thioester. Finally, Boc groups were introduced to the functional N^{α}- and N^{ε}-amino groups by reacting with excess Boc-OSu (25 equiv. for each amino group) in the presence of *N*-methylmorpholine (NMM) in DMF (25 °C, 3 h). The Boc-protected peptide thioester (Fig. 5(b)) was purified by HPLC to give a homogeneous thioester segment [**IIa**]. The overall yield of thus obtained thioester segment was 11% from the fully protected peptide. Structural correctness of the intermediates at each step and [**IIa**] was confirmed by mass spectrometry.

Two thioester segments used for the synthesis of CCK-58, Fmoc-[Lys(Boc)^{36}]-CCK(29–43)-S$(CH_2)_2$COOEt: [**IIb**] and Boc-[Lys(Boc)^{26}]-CCK(1–28)-S$(CH_2)_2$COOEt: [**III**], were prepared in the essentially same manner. By taking the second segment condensation into account, the N-terminus of segment [**IIb**] was protected with an Fmoc-group. HPLC chromatograms of the crude intermediates obtained after TFA treatment to remove the acid-labile protecting groups (Fig. 6(a) and (c)) were satisfactory. Following preparative HPLC purification, these intermediates were converted to segments [**IIb**] and [**III**] by reacting with Boc-OSu (Fig. 6(b) and (d)). Segment [**III**] was purified by HPLC before segment condensation. On the other hand, segment [**IIb**] was used for the segment condensation without HPLC purification because of the solubility problem. The overall yields of thus obtained thioester segments were 26% for [**IIb**] and 21% for [**III**], respectively, from the fully protected peptides. The structural correctness of the partially protected thioester segments, [**IIb**] and [**III**], was ascertained by amino acid analysis of their acid hydrolysates and LSIMS.

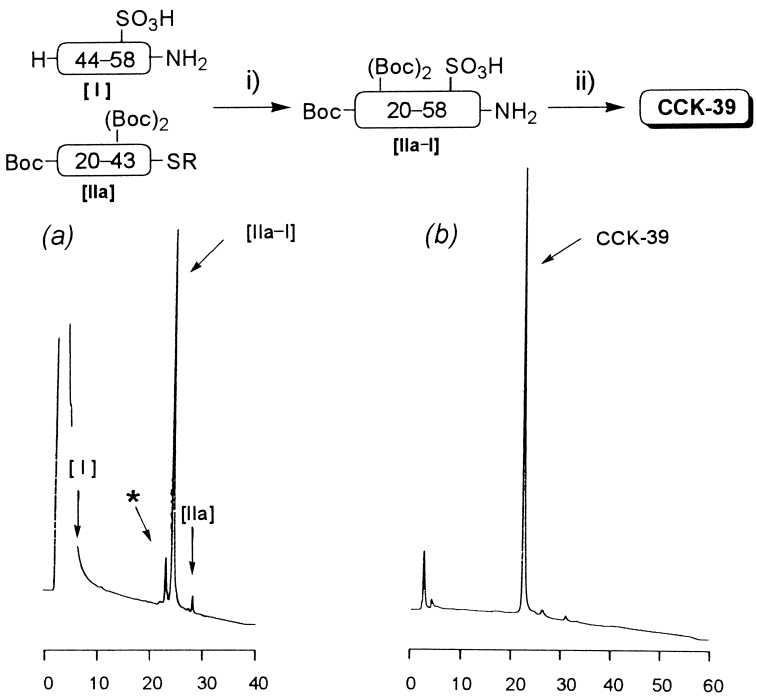


Figure 7. Synthesis of CCK-39 by thioester segment condensation approach. *Reagents*: (i) $AgNO_3$ (3 equiv.), HOOBt (30 equiv.), and DIEA (20 equiv.) in DMSO, 25 °C, 24 h; (ii) 90% aqueous TFA, 0 °C, 2 h. *Inlet*: HPLC chromatograms of (a) a crude condensation product (step (i)) and (b) a crude CCK-39 after the TFA treatment (step (ii)). An asterisk in (a) shows the hydrolyzed product of thioester segment [**IIa**]. The peak of [**I**] is detected as a shoulder after solvent peak in these HPLC conditions. [HPLC conditions: column, Cosmosil $5C_{18}$-AR (4.6×150 mm); elution, a linear gradient of B in A (20 to 50% in 40 min for (a) and 23 to 33% in 60 min for (b)); flow rate, 1 ml/min for (a) and 0.8 ml/min for (b)].

2.3. Synthesis of CCK-39

As a preliminary experiment, we examined the applicability of the silver-ion mediated thioester segment condensation approach to the synthesis of large CCK peptides by preparing CCK-39 (Fig. 7). Segment condensation between [**I**] (1 equiv.) and [**IIa**] (1 equiv.) was performed with the aid of $AgNO_3$ (3 equiv.), 3, 4-dihydro-3-hydroxy-4-oxo-1, 2, 3-benzotriazine[18] (HOOBt, 30 equiv.), and NMM (20 equiv.) in DMSO.[10e] As an additive, HOOBt was used to form a reactive active ester intermediate. The coupling reaction proceeded smoothly without notable side reactions (Fig. 7(a)). After 24 h, the starting two segments were almost completely consumed to form a protected condensation product, Boc-[Lys(Boc)^{26,36}]-CCK(20–58): [**IIa–I**]. Mass spectrometry revealed that the small peak detected before the main peak on the HPLC chromatogram (Fig. 7(a)) was a hydrolyzed product of the thioester segment.[19] The condensation product was isolated by HPLC in 60% yield. This HPLC-purified product was then subjected to TFA treatment (0 °C, 2 h) to remove the three Boc-groups, and CCK-39 was obtained in high purity without production of the desulfated peptide (Fig. 7(b)). The synthetic peptide was found to be identical to authentic CCK-39[9b] with regard to analytical HPLC, mass spectrometry, and lysyl endopeptidase digestion. The results of amino acid analysis after acid

Table 1. Amino acid ratios in acid hydrolysates of synthetic peptides

A.A.	CCK-39 peptides			CCK-58 peptides				
	Residues	[**IIa–I**]	CCK-**39**	Residues	[**Iib–I**]	Residues	[**III–IIb–I**]	CCK-**58**
Asp	6	5.59	6.34	6	6.58	7	6.14	6.75
Thr						1	1.00	0.97
Ser	4	3.67	3.44	4	3.26	6	5.00	5.04
Glu	3	3.25	3.36	1	1.10	5	5.42	5.26
Pro	2	1.92	2.03	1	1.00	2	1.85	2.05
Gly	2	2.25	2.12	2	1.78	4	4.00	3.96
Ala	2	1.90	2.19			5	5.32	4.94
Val	1	0.78	0.79			2	1.83	1.75
Met	3	1.93	2.33	3	2.51	3	2.05	2.16
Ile	3	2.36	2.62	2	1.63	3	2.68	2.60
Leu	2	2.00	2.00	2	2.00	5	5.13	4.84
Tyr	2	2.00	1.95	1	1.05	2	2.03	1.87
Phe	1	0.92	0.85	1	0.87	1	0.97	0.88
His	1	1.06	0.97	1	1.09	2	1.92	1.96
Trp	1	N.D.	N.D.	1	N.D.	1	N.D.	N.D.
Lys	2	2.04	2.05	1	1.00	2	1.75	2.09
Arg	4	3.37	3.69	3	2.86	7	6.52	6.95

hydrolysis of the CCK-39 and the partially protected intermediate are listed in Table 1. From these results, we concluded that the silver-ion mediated thioester segment coupling strategy is promising for the synthesis of longer sulfated peptides, such as CCK-58.

2.4. Synthesis of CCK-58

The synthesis of CCK-58 is outlined in Figure 8. To construct the entire peptide chain, two segments were successively condensed by the silver-ion mediated reaction. The first condensation reaction between segment [**I**] (1 equiv.) and segment [**IIb**] (1 equiv.) was mostly completed after 24 h at 25 °C (Fig. 8(a)), and the condensation product, Fmoc-[Lys(Boc)^{36}]-CCK(29–58): [**IIb**–**I**], was obtained in 67% yield, after HPLC purification. Following removal of the N^{α}-Fmoc group by a brief treatment with piperidine, the resultant N^{α}-free segment [**IIb**–**I**] (1 equiv.) was condensed with segment [**III**] (1.5 equiv.). In this condensation step, significant amounts of the hydrolyzed products of the thioester segment were produced.[20] Nevertheless, the condensed peptide was detected as the main peak on the HPLC chromatogram (Fig. 8(b)). After HPLC purification, the condensation product, Boc-[Lys(Boc)^{26,36}]-CCK(1–58): [**III**–**IIb**–**I**], was obtained in 40% yield. The final TFA treatment (0 °C, 2 h) resulted in a fully deprotected 58-mer peptide, without the accompanying desulfated peptide (Fig. 8(c)). HPLC purification of this peptide gave highly homogeneous CCK-58 in 45% yield. The results of amino acid analysis after acid hydrolysis of the CCK-58 and the partially protected intermediates are listed in Table 1.

The amino acid composition of the purified CCK-58 coincided well with the theoretical value (Table 1), and the sulfate was confirmed to be intact by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). It is noteworthy that the $[M+H]^+$ was detected without the accompanying desulfated fragment ion $[M+H-SO_3]^+$ on the positive-ion mode MALDI-TOFMS spectrum (Fig. 9(a)). This result reflect the intramolecular stability of the Tyr(SO_3H) residue in CCK-58.[16b] Lysyl endopeptidase digestion of the synthetic CCK-58 resulted in three peptide fragments on the HPLC chromatogram (Fig. 9(b)). These peptide fragments were identified as a 10-mer (positions 27 to 36 of CCK-58, designated as peak 1 in Figure 9(b)), a 22-mer corresponding to CCK-22 (positions 37 to 58, peak 2), and a 26-mer (positions 1 to 26, peak 3), on the basis of the amino acid compositions of their acid hydrolysates. Peptide mass mapping of the digest also supported the structural correctness of the synthetic CCK-58 (Fig. 9(c)).

The insulinotropic activity of the synthetic CCK-58 was investigated using isolated pancreatic islets (male Wister rats) pretreated with glucose (11.1 mmol) as previously

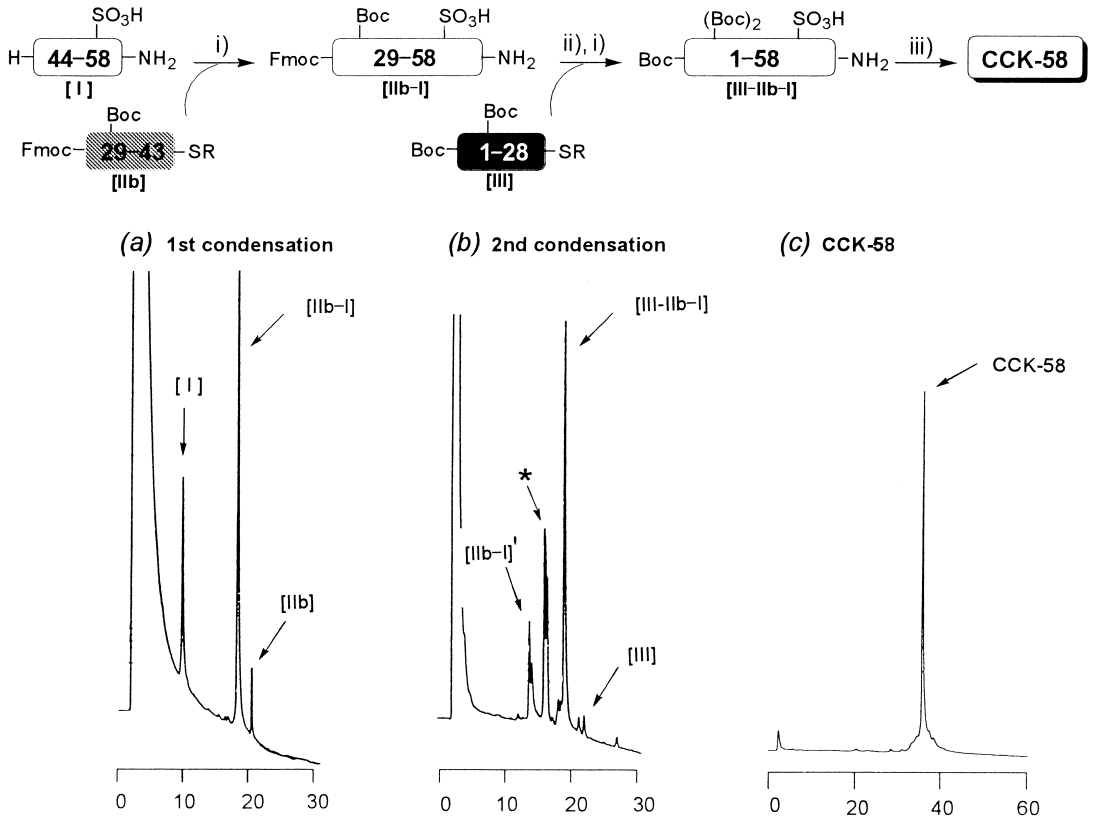
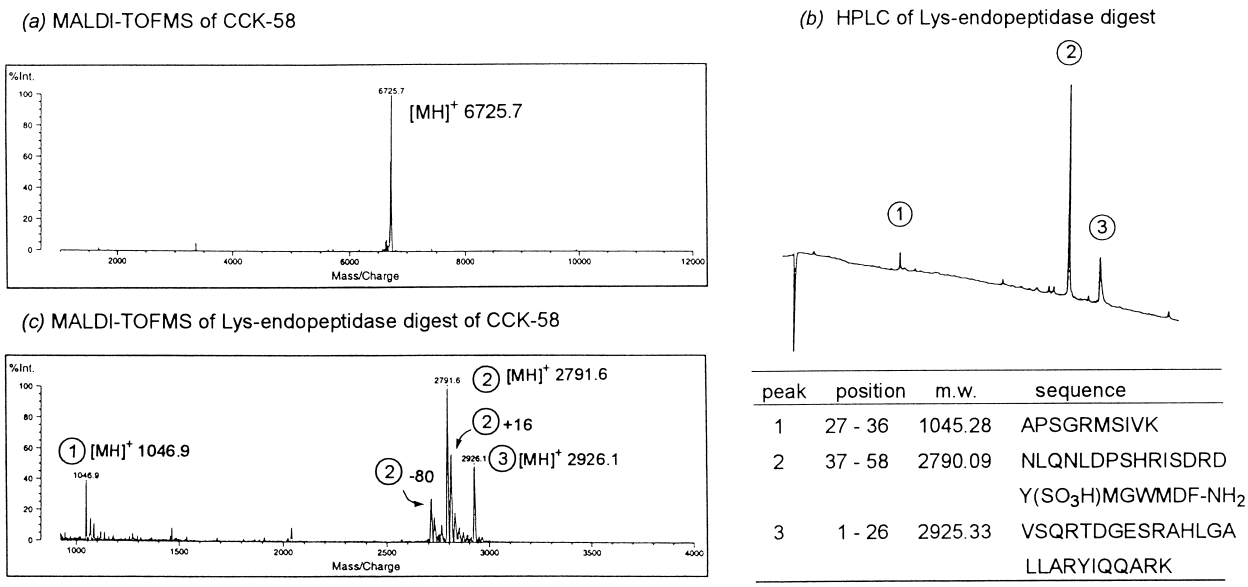


Figure 8. Synthesis of CCK-58 by thioester segment condensation approach. *Reagents*: (i) $AgNO_3$ (3 equiv.), HOOBt (30 equiv.), and DIEA (20 equiv.) in DMSO, 25 °C, 24 h; (ii) 25% piperidine in a mixture of DMF and DMSO, 25 °C, 3 h, then gel-filtration on Sephadex LH-20; (iii) 90% aqueous TFA, 0 °C, 2 h. *Inlet*: HPLC chromatograms of (a) crude product after first condensation reaction, (b) crude product after second condensation reaction, and (c) crude CCK-58 after TFA treatment. An asterisk in (b) shows the hydrolyzed product of thioester segment [**III**] and also [**IIb**–**I**]′ in (b) shows the N^{α}-Fmoc deprotected segment of [**IIb**–**I**]. [HPLC conditions: column, Cosmosil 5C_{18}-AR (4.6×150 mm); elution, a linear gradient of B in A (20 to 65% in 30 min for (a), 25 to 55% in 30 min for (b), and 30 to 45% in 60 min for (c)); flow rate, 0.8 ml/min].



peak	position	m.w.	sequence
1	27 - 36	1045.28	APSGRMSIVK
2	37 - 58	2790.09	NLQNLDPSHRISDRD
			$Y(SO_3H)$MGWMDF-NH_2
3	1 - 26	2925.33	VSQRTDGESRAHLGA
			LLARYIQQARK

Figure 9. Characterization of synthetic CCK-58. (a) MALDI-TOFMS spectrum of CCK-58; (b) HPLC chromatogram of lysyl endopeptidase digested CCK-58; and (c) MALDI-TOFMS spectrum of lysyl endopeptidase digested CCK-58. Assignment of the fragmented peptides is summalized in the inserted table. The sulfated fragment (peak 2) was detected as three mass peaks corresponding to the molecular protonated ion, desulfated peptide ion (-80 Da), and oxidized peptide ion (+16 Da), on the peptide mass map.

described.[9b] Synthetic CCK-58 increased the insulin release from the islets to the same degree as CCK-33.

3. Conclusions

We reported herein the usefulness of the silver-ion mediated thioester segment condensation approach in synthesizing large Tyr(SO_3H)-containing peptides. A large isoform of the CCK-peptide, CCK-58, was successfully prepared for the first time, and in good yield, using this approach. Clt-resin was efficiently employed in preparing the sulfated and thioester segments required for peptide chain construction. Notably the thioester segments were prepared using standard Fmoc-based chemistry without using special N^{α}-Fmoc deprotection reagents or special linkers. This method may contribute to the understanding of the functions of sulfated proteins by facilitating the chemical synthesis of large Tyr(SO_3H)-containing peptides.

4. Experimental

4.1. General

Fmoc-amino acid derivatives, diisopropylcarbodiimide (DIPCIDI), WSCDI·HCl, benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)-reagent and Clt resin (substituted level; 1.47 mmol/g, 100–200 mesh) were purchased from Watanabe Chemical Co., Ltd. (Hiroshima, Japan). Fmoc-His(Boc)-OH (cyclohexylamine salt) was obtained from Carbiochem–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 used without further purification. Acid hydrolysis was carried out at 110 °C for 24 h with a mixture of propionic acid and 12 M hydrochloric acid (1:1 v/v) for resin-bound peptides or with 6 M hydrochloric acid containing a few drops of phenol for purified peptides. Amino acid ratios were determined with a Shimadzu LC amino acids analyzer system using the *o*-phatalaldehyde protocol. LSIMS were performed on a VG ZAB-2SE double-focusing mass spectrometer using the Opus operating data system. Glycerol, thioglycerol, and *m*-nitrobenzylalcohol were used as the matrix, either neat or in combination. MALDI-TOFMS was performed on a Kratos Kompact MALDI IV. Sinapinic acid was used as the matrix.

4.2. General procedure for Fmoc-based solid-phase peptide synthesis

Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) was conducted in manually. Side-chain protecting groups used in the synthesis were as follows: ^{t}Bu for Asp, Glu, Ser, Thr, and Tyr; Boc for Lys and His (for segment [**I**]); Trt for Asn and Gln; Pbf for Arg; Bum for His (segment [**III**]). N^{α}-Fmoc protecting groups were cleaved by 1 min treatment with 20% piperidine in DMF followed by a second treatment with the same reagent for 20 min. For deprotection of the N^{α}-Fmoc groups of Gln and Glu(^{t}Bu) residues, the concentration of piperidine was reduced to 10%. After Fmoc cleavage, the peptide-resin was washed with DMF (×6). The next residue was then incorporated using the DIPCDI-HOBt coupling protocol [Fmoc-amino acid (3 equiv.), DIPCDI (3 equiv.), and HOBt (3 equiv.)] or the PyBOP[21]-mediated coupling protocol [Fmoc-amino acid (3 equiv.), PyBOP reagent (3 equiv.), and NMM (9 equiv.)]. After gentle agitation (1.5 h) and washing with DMF (×6), part of the peptide-resin was subjected to the Kaiser test.[22] On completion of the assembly, the peptide-resin was successively washed with DMF (×5), MeOH (×5), and ether (×5), then dried in vacuo.

4.3. General procedure for purification by preparative RP-HPLC and purity assessment by analytical HPLC

Crude peptide was purified by RP-HPLC using a column of Cosmosil $5C_{18}$ AR-300 (20×150 mm) or Cosmosil $5C_{18}$ AR (10×250 mm). Purity of the peptide was assessed using a column of Cosmosil $5C_{18}$ AR (4.6×150 mm). For elution of Tyr(SO_3H)-containing peptides, a solvent system consisting of solvent A (0.1 M $AcONH_4$) and solvent B (CH_3CN) was used. For elution of other peptides, a solvent system consisting of solvent C (0.1% aqueous TFA) and solvent D (CH_3CN containing 0.1% TFA) was used. The absorbance of the eluate was measured at 230 or 275 nm, depending on the peptide.

4.4. Peptide synthesis

4.4.1. CCK(44–58) [I]. Fmoc-Asp-Phe-NH_2 was prepared by the solution method and attached to Clt resin via the β-carboxyl group of Asp, according to the procedure of Barlos et al.[9,13c] The resulting Fmoc-Asp(Clt resin)-Phe-NH_2 (330 mg, 0.15 mmol) was used as the starting dipeptide resin, and each Fmoc-amino acid derivative was added to it, according to the general procedures of Fmoc-based SPPS. Fmoc-Tyr(SO_3Na)-OH was used for incorporation of the Tyr(SO_3H) residue and Fmoc-His(Boc) was incorporated after desalting followed by a brief purification on silica gel column chromatography.[15] After the 15-mer peptide chain was assembled, the N^{α}-Fmoc group was removed. The peptide-resin was then dried in vacuo (620 mg).

The protected peptide-resin, H-Ser(^{t}Bu)-His(Boc)-Arg(Pbf)-Ile-Ser(^{t}Bu)-Asp(O^{t}Bu)-Arg(Pbf)-Asp(O^{t}Bu)-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp(Clt resin)-Phe-NH_2 (100 mg, 17.7 μmol), was treated with pre-cooled 90% aqueous TFA (1.5 ml) at 0 °C for 12 h, then dry ether (50 ml) was added. The formed precipitate was collected by centrifugation and washed twice with ether. The dried product was dissolved in 0.02 M NH_4HCO_3 (10 ml) and applied to a column of Sephadex G-25 (2.5×55 cm). NH_4HCO_3 (0.02 M) was used for elution and the fractions corresponding to the first peak (UV absorbance detected at 280 nm) was pooled. The pooled fractions were lyophilized to give a powder (22.1 mg, 63% on cleavage and deprotection). This sample was purified by preparative HPLC using a linear gradient of B in A (26 to 30% in 40 min) at a flow rate of 2.5 ml/min. After lyophilization, 6.6 mg of pure segment [**I**] was obtained (30% recovery on HPLC purification). This peptide was detected as a sharp single peak at t_R 11.3 min on an analytical HPLC chromatogram [elution, a linear gradient of B in A (20 to 40% in 40 min); flow rate, 0.8 ml/min]. Amino acid ratios in the acid hydrolysate were as follows (theoretical values are given in parentheses): Asp 3.28 (3), Ser 1.91 (2), Gly 1.12 (1), Met 1.61 (2), Ile 1.00 (1), Tyr 1.05 (1), Phe 0.98 (1), Trp not determined (1), His 1.00 (1), Arg 2.01 (2). LSIMS *m/z*: calcd for $C_{83}H_{119}N_{25}O_{27}S_3$ 1995.2 (*M*, average mass); found 1995.9 $[M+H]^+$.

4.4.2. Boc-[Lys(Boc)^{26,36}]-CCK(20–43)-S(CH_2)$_2$COOEt [IIa]. Starting with Fmoc-Pro-Clt resin (255 mg, 0.15 mmol), each amino acid derivative was incorporated to the peptide-resin according to the general Fmoc-SPPS protocol. After the N-terminal Boc-Tyr(^{t}Bu)-OH was incorporated, the peptide-resin was dried in vacuo (630 mg). The fully protected peptide-resin (200 mg, 21.0 μmol) was treated with a mixture of HFIP/CH_2Cl_2 (1:4 v/v, 5 ml) at 25 °C for 30 min, then filtered. The filtrate was concentrated using an N_2 stream, and then dry ether (50 ml) was added to precipitate the peptide. After centrifugation, the collected precipitate was dried in vacuo (105 mg, quantitative).

The obtained fully protected peptide, Boc-Tyr(^{t}Bu)-Ile-Gln(Trt)-Gln(Trt)-Ala-Arg(Pbf)-Lys(Boc)-Ala-Pro-Ser(^{t}Bu)-Gly-Arg(Pbf)-Met-Ser(^{t}Bu)-Ile-Val-Lys(Boc)-Asn(Trt)-Leu-Gln(Trt)-Asn(Trt)-Leu-Asp(O^{t}Bu)-Pro-OH (105 mg, 21.0 μmol), was dissolved in ice-cooled DMF (2 ml), then HS-(CH_2)$_2$$COOC_2H_5$ (70.4 μl, 0.53 mmol), WSCDI·HCl (60.3 mg, 0.31 mmol), HOBt (48.2 mg, 0.31 mmol) were added. After the reaction mixture was stirred at 4 °C for 20 h, the solution was concentrated and the resultant residue was triturated with ether (10 ml) three times, giving a powder. The dried powder was treated with 95% aqueous TFA (3 ml) at 25 °C for 3 h, then ether (50 ml) was added. The resultant precipitate was collected by centrifugation and washed twice with ether. This product was dissolved in H_2O (30 ml) and lyophilized to give the crude peptide thioester (51.1 mg, 86% yield from the protected peptide). The crude peptide (51.0 mg) was purified by preparative HPLC [column, Cosmosil $5C_{18}$-AR (10×250 mm); elution, a linear gradient of D in C (20 to 45% in 60 min); flow rate, 1.5 ml/min]. After lyophilization, 15.8 mg of the pure peptide thioester was obtained (31% recovery on HPLC). LSIMS *m/z*: calcd for $C_{123}H_{207}N_{37}O_{36}S_2$ 2844.4 (*M*, average mass); found 2845.1 $[M+H]^+$.

The peptide thioester (15.3 mg, 5.4 μmol) was dissolved in ice-cooled DMF (2 ml), then Boc-OSu (87 mg, 0.4 mmol) and NMM (45 μl, 0.4 mmol) were added. After the reaction mixture was stirred at 25 °C for 20 h, the solution was concentrated and the resultant residue was triturated with ether (10 ml) twice, giving a powder (11.1 mg, 66% yield). The Boc-protected peptide thioester was purified by preparative HPLC [column, Cosmosil $5C_{18}$-AR (10×250 mm); elution, a linear gradient of D in C (35 to 50% in 60 min); flow rate, 2.0 ml/min]. After lyophilization, 7.1 mg of homogeneous segment [**IIa**] was obtained (64% recovery on HPLC). This peptide was detected as a single peak at t_R 20.8 min on an analytical HPLC chromatogram [column, Cosmosil $5C_{18}$-AR (4.6×150 mm); elution, a linear gradient of D in C (30 to 50% in 30 min); flow rate, 0.8 ml/min]. Amino acid ratios in the acid hydrolysate were as follows: Asp 3.51 (3), Ser 1.72 (2), Glu 3.16 (3), Pro 1.85 (2), Gly 1.00 (1), Ala 2.14 (2), Val 0.72 (1), Met 0.77 (1), Ile 1.59 (2), Leu 1.89 (2), Tyr 0.94 (1), Lys 1.82 (2), Arg 1.78 (2). LSIMS *m/z*: calcd for $C_{138}H_{231}N_{37}O_{42}S_2$ 3144.7 (*M*, average mass); found 3145.5 $[M+H]^+$.

4.4.3. Fmoc-[Lys(Boc)^{36}]-CCK(29–43)-S(CH_2)$_2$COOEt [IIb]. The protected 15-mer peptide was constructed on Fmoc-Pro-Clt resin (255 mg, 0.15 mmol). After incorporation of the *N*-terminal Fmoc-Ser(^{t}Bu)-OH, the peptide-resin was washed and dried in vacuo (603 mg). The protected peptide-resin thus obtained (400 mg, 44.6 μmol) was treated with a mixture of HFIP/CH_2Cl_2 (1:4, 10 ml) at 25 °C for 30 min and filtered. The fully protected peptide,

Fmoc-Ser(^{t}Bu)-Gly-Arg(Pbf)-Met-Ser(^{t}Bu)-Ile-Val-Lys(Boc)-Asn(Trt)-Leu-Gln(Trt)-Asn(Trt)-Leu-Asp(O^{t}Bu)-Pro-OH, was isolated from the filtrate using similar methods as described for the preparation of [**IIa**] (115 mg, 92% yield).

The obtained fully protected peptide (109 mg, 40.0 μmol) was dissolved in ice-cooled DMF (3 ml), then HS-$(CH_2)_2$-$COOC_2H_5$ (134.2 μl, 1.0 mmol), WSCDI·HCl (115 mg, 0.60 mmol), and HOBt (69 mg, 0.60 mmol) were added. After the reaction mixture was stirred at 4 °C for 12 h, the solution was concentrated and the resultant residue was triturated with ether (10 ml) three times to give a powder. The dried powder was then treated with 95% aqueous TFA (4 ml) at 25 °C for 3.5 h, and ether (50 ml) was added to the mixture. The precipitate was collected by centrifugation, washed with ether, dissolved in H_2O (30 ml) and lyophilized to give a crude peptide thioester (58.2 mg, 73% yield from the protected peptide). The crude peptide (51.1 mg) was purified by preparative HPLC [column, Cosmosil 5C_{18}-AR (10×250 mm); elution, an isocratic elution of D in C (38% for 10 min) followed by a linear gradient of D in C (38 to 48% in 20 min); flow rate, 2.0 ml/min]. After lyophilization, 29.6 mg of the pure N^{α}-Fmoc protected peptide thioester was obtained (51% recovery on HPLC). LSIMS *m/z*: calcd for $C_{90}H_{140}N_{22}O_{26}S_2$ 2010.4 (*M*, average mass); found 2010.9 $[M+H]^+$.

The N^{α}-Fmoc protected peptide thioester (40.0 mg, 20 μmol) was dissolved in ice-cooled DMF (4 ml), then Boc-OSu (107.5 mg, 0.5 mmol) and NMM (55 μl, 0.5 mmol) were added. After the reaction mixture was stirred at 25 °C for 10 h, the solution was concentrated and the resultant residue was triturated with ether (10 ml) twice, giving a powder (32.5 mg, 76% yield). The obtained [**IIb**] was detected as a sharp peak at t_R 17.8 min with minor impurities on an analytical HPLC chromatogram [column, Cosmosil 5C_{18}-AR (4.6×150 mm); elution, a linear gradient of B in A (30 to 65% in 30 min); flow rate, 0.8 ml/min]. Because of its solubility, [**IIb**] was used for segment condensation without further purification. Amino acid ratios in the acid hydrolysate were as follows; Asp 3.20 (3), Ser 1.67 (2), Glu 1.05 (1), Pro 1.02 (1), Gly 1.00 (1), Val 0.76 (1), Met 0.49 (1), Ile 0.73 (1), Leu 1.98 (2), Lys 0.98 (1), Arg 0.92 (1). LSIMS *m/z*: calcd for $C_{95}H_{148}N_{22}O_{28}S_2$ 2110.5 (*M*, average mass); found 2111.0 $[M+H]^+$.

4.4.4. Boc-[Lys(Boc)^{26}]-CCK(1–28)-S$(CH_2)_2$COOEt [III]. This protected peptide was constructed on Fmoc-Pro-Clt resin (250 mg, 0.15 mmol). After the incorporation of the N-terminal Boc-Val-OH, the peptide-resin was dried in vacuo (680 mg). The protected peptide-resin (200 mg, 23.0 μmol) was then treated with a mixture of HFIP/CH_2Cl_2 (1:4, 5 ml) at 25 °C for 30 min and filtered. The fully protected peptide, Boc-Val-Ser(^{t}Bu)-Gln(Trt)-Arg(Pbf)-Thr(^{t}Bu)-Asp(O^{t}Bu)-Gly-Glu(O^{t}Bu)-Ser(^{t}Bu)-Arg(Pbf)-Ala-His(Bum)-Leu-Gly-Ala-Leu-Leu-Ala-Arg(Pbf)-Tyr(^{t}Bu)-Ile-Gln(Trt)-Gln(Trt)-Ala-Arg(Pbf)-Lys(Boc)-Ala-Pro-OH, was obtained from the filtrate as described for the preparation of [**IIa**] (140 mg, quantitative).

The fully protected peptide (132 mg, 23.0 μmol) was dissolved in ice-cooled DMF (5 ml), then HS-$(CH_2)_2$-$COOC_2H_5$ (81 μl, 0.60 mmol), WSCDI·HCl (70 mg, 0.36 mmol), and HOBt (42 mg, 0.36 mmol) were added. After the reaction mixture was stirred at 4 °C for 20 h, the solution was concentrated and the resultant residue was triturated with ether (10 ml) three times, giving a powder. The dried powder was then treated with 95% aqueous TFA (5 ml) at 25 °C for 3 h, and ether (50 ml) was added to the mixture. The formed precipitate was collected by centrifugation and washed with ether. This precipitate was dissolved in 5% AcOH (3 ml), applied to a column of Sephadex G-10 (4×55 cm), and eluted with 5% AcOH. The fractions corresponding to the first peak (detected with the UV absorbance at 280 nm) were pooled and lyophilized, giving a fluffy powder (72.5 mg, 94% yield from the protected peptide). This peptide thioester (71.5 mg) was purified by preparative HPLC [column, Cosmosil 5C_{18}-AR (20×150 mm); elution, an isocratic elution of D in C (32% for 10 min) followed by a linear gradient of D in C (32 to 40% in 50 min); flow rate, 3.0 ml/min] (30.3 mg, 42% recovery on HPLC). LSIMS *m/z*: calcd for $C_{137}H_{231}N_{47}O_{40}S$ 3208.7 (*M*, average mass); found 3209.5 $[M+H]^+$.

The peptide thioester (28.9 mg, 9 μmol) was dissolved in ice-cooled DMF (3 ml), then Boc-OSu (97.3 mg, 0.45 mmol) and NMM (24.8 μl, 0.23 mmol) were added. After the reaction mixture was stirred at 25 °C for 10 h, the solution was concentrated and the resultant residue was triturated with ether (10 ml) twice (22.5 mg, 73% yield). The Boc-protected peptide thioester was purified again by preparative HPLC [column, Cosmosil 5C_{18}-AR (4.6×150 mm); elution, a linear gradient of D in C (30 to 45% in 30 min); flow rate, 1 ml/min]. After lyophilization, 16.2 mg of pure [**III**] was obtained (74% recovery on HPLC). This peptide was detected as a single peak at t_R 13.4 min on an analytical HPLC chromatogram. Amino acid ratios in the acid hydrolysate were as follows; Asp 1.01 (1), Thr 0.95 (1), Ser 1.74 (2), Glu 4.45 (4), Pro 1.00 (1), Gly 1.91 (2), Ala 5.76 (5), Val 0.96 (1), Ile 0.94 (1), Leu 3.10 (3), Tyr 1.00 (1), His 1.00 (1), Lys 1.00 (1), Arg 3.98 (4). LSIMS *m/z*: calcd for $C_{147}H_{247}N_{47}O_{44}S$ 3408.9 (*M*, average mass); found 3409.3 $[M+H]^+$.

4.4.5. Synthesis of CCK-39. Silver nitrate (1.02 mg, 6.0 μmol), HOOBt (9.8 mg, 60 μmol), and DIEA (5.2 μl, 40 μmol) were dissolved in DMSO (250 μl) and stirred at 25 °C for 1 h, then a DMSO solution (1 ml) containing segment [**I**] (4.0 mg, 2.0 μmol) and segment [**IIa**] (6.3 mg, 2.0 μmol) was added. After the mixture was stirred at 25 °C for 24 h, the formed insoluble material was precipitated by centrifugation. Portions of the supernatant (ca. 100 μl each) were subjected to RP-HPLC for isolation of the condensation product, Boc-[Lys(Boc)^{26,36}]-CCK(20–58): [**IIa–I**], [HPLC conditions: column, Cosmosil 5C_{18}-AR (10×250 mm); elution, a linear gradient of B in A (25 to 50% in 60 min); flow rate, 2.0 ml/min]. The eluate corresponding to the main peak (t_R 38.2 min) was pooled and lyophilized. The resultant residue was again lyophilized from 0.02 M NH_4HCO_3 (30 ml) to give a fluffy powder of [**IIa–I**] (5.96 mg, 60% yield). MALDI-TOFMS *m/z*: calcd for $C_{216}H_{340}N_{62}O_{67}S_4$ 5005.7 (*M*, average mass); found 5006.9 $[M+H]^+$.

HPLC-purified [**IIa**–**I**] (5.0 mg, 1.0 μmol) was then treated with pre-cooled 90% TFA (1 ml) at 0 °C for 2 h, and dry ether (50 ml) was added. The formed precipitate was collected by centrifugation and washed with ether twice. This precipitate was dissolved in 0.02 M NH_4HCO_3 (25 ml) and lyophilized to give crude CCK-39 (4.25 mg, 90% yield). The obtained crude CCK-39 (Fig. 7(b)) was used for final characterization without further purification. MALDI-TOFMS *m/z*: calcd for $C_{201}H_{316}N_{62}O_{61}S_4$ 4705.3 (*M*, average mass); found 4706.4 $[M+H]^+$. Amino acid ratios in an acid hydrolysate of crude CCK-39 and the condensation product [**IIa**–**I**] were listed in Table 1.

4.4.6. Synthesis of CCK-58. Silver nitrate (1.02 mg, 6.0 μmol), HOOBt (9.8 mg, 60 μmol), and DIEA (5.2 μl, 40 μmol) were dissolved in DMSO (250 μl) and stirred for 1 h. A DMSO (1 ml) solution containing segment [**I**] (4.0 mg, 2.0 μmol) and segment [**IIb**] (4.3 mg, 2.0 μmol) was then added. After the mixture was stirred at 25 °C for 24 h, the resultant insoluble material was precipitated by centrifugation. Portions of the supernatant (ca. 100 μl each) were subjected to RP-HPLC for isolation of the condensation product, Fmoc-[Lys(Boc)^{36}]-CCK(29–58): [**IIb**–**I**], [HPLC conditions: column, Cosmosil 5C_{18}-AR (10× 250 mm); elution, an isocratic elution of B in A (35% for 10 min) followed by a linear gradient of B in A (35 to 55% in 50 min); flow rate, 1.75 ml/min]. The eluate corresponding to the main peak (t_R 31.7 min) was pooled and lyophilized. The resultant residue was again lyophilized from 0.02 M NH_4HCO_3 (30 ml) to give a fluffy powder of [**IIb**–**I**] (5.32 mg, 67% yield). MALDI-TOFMS *m/z*: calcd for $C_{173}H_{257}N_{47}O_{53}S_4$ 3971.5 (*M*, average mass); found 3973.7 $[M+H]^+$.

The obtained condensation product [**IIb**–**I**] (16.3 mg, 4.1 μmol) was dissolved in a mixture of DMSO (1 ml) and DMF (2 ml), and piperidine (1 ml) was then added. After the reaction mixture was stirred at 25 °C for 3 h, the solution was applied onto a column of Sephadex LH-20 (2.5×66 cm) and eluted with DMF. The eluate corresponding to the first peak was pooled and concentrated. The resultant residue was dissolved in 0.02 M NH_4HCO_3 (20 ml) and lyophilized to give a fluffy powder (12.0 mg, 78% yield). This peptide was detected as a sharp single peak at t_R 14.7 min on an analytical HPLC chromatogram [elution, a linear gradient of B in A (25 to 45% in 30 min); flow rate, 0.8 ml/min]. MALDI-TOFMS *m/z*: calcd for $C_{158}H_{247}N_{47}O_{51}S_4$ 3749.2 (*M*, average mass); found 3750.0 $[M+H]^+$.

The second segment condensation was conducted in a similar manner. Silver nitrate (0.76 mg, 4.5 μmol), HOOBt (7.5 mg, 46 μmol), and DIEA (4.0 μl, 31 μmol) were dissolved in DMSO (200 μl) and stirred at 25 °C for 1 h, then a DMSO solution (1 ml) containing the Fmoc-deprotected segment [**IIb**–**I**] (3.76 mg, 1.0 μmol) and segment [**III**] (5.15 mg, 1.5 μmol) was added. After the mixture was stirred at 25 °C for 24 h, the resultant insoluble material was precipitated by centrifugation. Portions of the supernatant (ca. 100 μl each) were subjected to RP-HPLC for isolation of the condensation product, Boc-[Lys-(Boc)^{26,36}]-CCK(1–58): [**III**–**IIb**–**I**], [HPLC conditions: column, Cosmosil 5C_{18}-AR (4.6×150 mm); elution, a linear gradient of B in A (25 to 65% in 40 min); flow rate, 0.8 ml/min]. The eluate corresponding to the main peak (t_R 19.4 min) was pooled and lyophilized. The resultant residue was again lyophilized from 0.02 M NH_4HCO_3 (20 ml) to give a fluffy powder of [**III**–**IIb**–**I**] (2.76 mg, 40% yield). This peptide was detected as a sharp single peak at t_R 19.1 min on an analytical HPLC chromatogram. MALDI-TOFMS *m/z*: calcd for $C_{300}H_{483}N_{93}O_{94}S_4$ 7024.2 (*M*, average mass); found 7025.2 $[M+H]^+$.

HPLC-purified [**III**–**IIb**–**I**] (3.8 mg, 0.54 μmol) was then treated with pre-cooled 90% TFA (0.5 ml) at 0 °C for 2 h, and dry ether (50 ml) was added. The formed precipitate was collected by centrifugation and washed with ether twice. This precipitate was dissolved in 0.02 M NH_4HCO_3 (15 ml) and lyophilized to give crude CCK-58. The crude CCK-58 was further purified by HPLC [HPLC conditions: column, Cosmosil 5C_{18}-AR (4.6×150 mm); elution, a linear gradient of B in A (30 to 45% in 60 min) at a flow rate of 0.8 ml/min]. The eluate corresponding to the main peak (t_R 36.4 min) was pooled and lyophilized. The resultant residue was lyophilized again from 0.02 M NH_4HCO_3 (15 ml) to give a fluffy powder, CCK-58 (1.62 mg, 45% yield). This peptide was detected as a sharp single peak at t_R 36.2 min on an analytical HPLC chromatogram under the same conditions used for purification. MALDI-TOFMS *m/z*: calcd for $C_{285}H_{459}N_{93}O_{88}S_4$ 6724.6 (*M*, average mass); found 6725.7 $[M+H]^+$. Amino acid ratios in an acid hydrolysate of the purified CCK-58 and the condensation products, [**IIb**–**I**] and [**III**–**IIb**–**I**], were listed in Table 1.

4.4.7. Lysyl endopeptidase digestion of CCK-39 and CCK-58. Lysyl endopeptidase digestion of synthetic CCK-39 was carried out in a similar manner as described before.[9b] Briefly, the purified CCK-39 (150 μg) was digested in 0.1 M NH_4HCO_3 (pH 8.2, 300 μl) at 37 °C for 4 h. The weight ratio of the enzyme to the substrate was about 1:200. The enzyme digest of the CCK-39 gave three peaks (t_R 14.2, 18.7 and 43.2 min) on an HPLC chromatogram [HPLC conditions: column, Cosmosil 5C_{18}-AR (4.6×150 mm); elution, a linear gradient of D in C (5 to 35% in 60 min); flow rate, 0.8 ml/min; absorbance was detected at 230 nm]. These peaks were identified as a 7-mer: H-YIQQARK-OH (t_R 14.2 min), a 10-mer: H-APSGRMSIVK-OH (t_R 18.7 min), and a 22-mer corresponding to CCK-22 (t_R 43.2 min) on amino acid analysis of the acid hydrolysates (data not shown) and mass spectrometry. LSIMS: 7-mer, *m/z*: calcd for $C_{40}H_{67}N_{13}O_{11}$ 905.5 (*M*, monoisotopic mass); found 906.5 $[M+H]^+$; 10-mer, *m/z*: calcd for $C_{44}H_{80}N_{14}O_{13}S$ 1044.6 (*M*, monoisotopic mass); found 1045.6 $[M+H]^+$; CCK-22, *m/z* calcd for $C_{117}H_{173}N_{35}O_{39}S_3$ 2790.0 (*M*, average mass); found 2791.0 $[M+H]^+$.

Purified CCK-58 (160 μg) was similarly digested by lysyl endopeptidase, and the digest was analyzed by HPLC under the same conditions used for synthetic CCK-39. Three peaks (peak 1 to peak 3 according to the order of elution, Figure 9(b)) were detected and were identified as H-APSGRMSIVK-OH (peak 1; t_R 18.8 min), CCK-22 (peak 2; t_R 43.3 min), and the N-terminal 26-mer (peak 3; t_R 48.5 min). Amino acid analyses of the acid hydrolysates; peak 1: Ser (2) 1.85, Gly (1) 1.37, Ala (1) 1.00, Pro (1) 0.85, Val (1) 0.75, Ile (1) 0.68, Met (1) 0.57, Lys (1) 0.75, Arg (1)

0.86; peak 2: Asp (6) 6.51, Ser (2) 2.10, Glu (1) 1.39, Gly (1) 1.41, Pro (1) 0.88, Met (2) 1.40, Ile (1) 1.00, Leu (2) 2.00, Tyr (1) 0.97, Phe (1) 1.00, His (1) 1.15, Arg (2) 2.00, Trp (1) N.D.; peak 3: Asp (1) 1.21, Thr (1) 1.00, Ser (2) 2.07, Glu (4) 4.21, Gly (2) 2.14, Ala (4) 3.78, Val (1) 1.00, Ile (1) 0.84, Leu (3) 2.61, Tyr (1) 0.85, His (1) 1.05, Lys (1) 1.13, Arg (4) 3.35. Part of the enzyme digest was then subjected to MALDI-TOFMS and peaks corresponding to the three peptides were detected on the mass spectrum (Fig. 9(c)).

Acknowledgements

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 (no. 10672008 and 13672241) from the Japan Society for the Promotion of Science.

References and notes

1. (a) Mutt, V.; Jorpes, J. E. *Eur. J. Biochem.* **1968**, *6*, 156–162. (b) Mutt, V.; Jorpes, J. E. *Biochem. J.* **1971**, *125*, 57P–58P. (c) Rehfeld, J. F. *J. Biol. Chem.* **1978**, *253*, 4022–4030. (d) Tatemoto, K.; Jörnvall, H.; Siimesmaa, S.; Halldén, G.; Mutt, V. *FEBS Lett.* **1984**, *174*, 289–293. (e) Eysselein, V. E.; Eberlein, G. A.; Hesse, W. H.; Singer, M. V.; Goebell, H.; Reeve, J. R., Jr. *J. Biol. Chem.* **1987**, *262*, 214–217. (f) Eysselein, V. E.; Eberlein, G. A.; Schaeffer, M.; Grandtt, D.; Goebell, H.; Niebel, W.; Rosenquist, G. L.; Meyer, H. E.; Reeve, J. R., Jr. *Am. J. Physiol.* **1990**, G253–G260.
2. Takahashi, Y.; Kato, K.; Hayashizaki, Y.; Wakabayashi, T.; Otsuka, E.; Matsuki, S.; Ikehara, M.; Matsubara, K. *Proc. Natl Acad. Sci. U.S.A.* **1985**, *82*, 1931–1935.
3. General reviews on tyrosine sulfation in proteins: (a) Huttner, W. B. *Nature* **1982**, *299*, 273–276. (b) Huttner, W. B. *Methods Enzymol.* **1984**, *107*, 200–223. (c) Huttner, W. B. *Annu. Rev. Physiol.* **1988**, *50*, 363–376. (d) Huttner, W. B.; Baeuerle, P. A. *Modern cell biology*; Satir, B., Ed.; Alan R. Liss Inc: New York, 1988; Vol. 6, pp 97–140.
4. Kurano, Y.; Kimura, T.; Sakakibara, S. *J. Chem. Soc., Chem. Commun.* **1987**, 323–325.
5. Fujii, N.; Futaki, S.; Funakoshi, S.; Akaji, K.; Morimoto, H.; Doi, R.; Inoue, K.; Kogire, M.; Sumi, S.; Yun, M.; Tobe, T.; Aono, M.; Matsuda, M.; Narusawa, H.; Moriga, M.; Yajima, H. *Chem. Pharm. Bull.* **1988**, *36*, 3281–3291.
6. (a) Penke, B.; Ngyerges, L. *Pept. Res.* **1991**, *4*, 289–295. (b) Yagami, T.; Shiwa, S.; Futaki, S.; Kitagawa, K. *Chem. Pharm. Bull.* **1993**, *41*, 376–380.
7. (a) Miranda, M. T. M.; Liddle, R. A.; Rivier, J. E. *J. Med. Chem.* **1993**, *36*, 1681–1688. (b) Miranda, M. T. M.; Craig, A. G.; Miller, C.; Liddle, R. A.; Rivier, J. E. *J. Protein Chem.* **1993**, *12*, 533–544.
8. Marseigne, I.; Dor, R. A.; Durieux, C.; Pelaprat, D.; Reibaud, M.; Blanchard, J. C.; Roques, B. P. *J. Med. Chem.* **1989**, *32*, 445–449.
9. (a) Kitagawa, K.; Aida, C.; Fujiwara, H.; Yagami, T.; Futaki, S. *Tetrahedron Lett.* **1997**, *38*, 599–602. (b) Kitagawa, K.; Aida, C.; Fujiwara, H.; Yagami, T.; Futaki, S.; Kogire, M.; Ida, J.; Inoue, K. *J. Org. Chem.* **2001**, *66*, 1–10.
10. (a) Blake, J.; Li, C. H. *Proc. Natl Acad. Sci. U.S.A.* **1981**, *78*, 4055–4058. (b) Yamashiro, D.; Li, C. H. *Int. J. Pept. Protein Res.* **1988**, *31*, 322–334. (c) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn* **1991**, *64*, 111–117. (d) Kawakami, T.; Kogure, S.; Aimoto, S. *Bull. Chem. Soc. Jpn* **1996**, *69*, 3331–3338. (e) Aimoto, S. *Biopolymers (Pept. Sci.)* **1999**, *51*, 247–265, and references sited therein.
11. (a) Mizuno, M.; Muramoto, I.; Kawakami, T.; Seike, M.; Aimoto, S.; Haneda, K.; Inazu, T. *Tetrahedron Lett.* **1998**, *39*, 55–58. (b) Haneda, K.; Inazu, T.; Mizuno, M.; Iguchi, R.; Yamamoto, K.; Kumagai, H.; Aimoto, S.; Suzuki, H.; Noda, T. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1303–1306. (c) Mizuno, M.; Haneda, K.; Iguchi, R.; Muramoto, I.; Kawakami, T.; Aimoto, S.; Yamamoto, K.; Inazu, T. *J. Am. Chem. Soc.* **1999**, *121*, 284–290.
12. (a) Sakamoto, H.; Kodama, H.; Higashimoto, Y.; Kondo, M.; Lewis, M. S.; Anderson, C. W.; Appella, E.; Sakaguchi, K. *Int. J. Pept. Protein Res.* **1996**, *48*, 429–442. (b) Kawakami, T.; Hasegawa, K.; Aimoto, S. *Bull. Chem. Soc. Jpn* **2000**, *73*, 197–203. (c) Miyoshi, K.; Otaka, A.; Kaneko, M.; Tamamura, H.; Fujii, N. *Chem. Pharm. Bull.* **2000**, *48*, 1230–1233.
13. (a) Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphoutiu, G.; Sotiriu, P.; Wenqing, Y.; Schafer, W. *Tetrahedron Lett.* **1989**, *30*, 3943–3946. (b) Barlos, K.; Gatos, D.; Kapolos, S.; Papaphoutiu, 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. (d) Barlos, K.; Gatos, D. *Biopolymers (Pept. Sci.)* **1999**, *51*, 266–278.
14. (a) Futaki, S.; Sogawa, K.; Maruyama, J.; Asahara, T.; Niwa, M.; Hojo, H. *Tetrahedron Lett.* **1997**, *38*, 6237–6240. (b) Li, X.; Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **1998**, *39*, 8669–8672. (c) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369–11374. (d) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689. (e) Alsina, J.; Yokum, T. S.; Albericio, F.; Barany, G. *J. Org. Chem.* **1999**, *64*, 8761–8769. (f) Quaderer, R.; Hilvert, D. *Org. Lett.* **2001**, *3*, 3181–3184.
15. Kitagawa, K.; Aida, C.; Fujiwara, H.; Yagami, T.; Futaki, S. *Chem. Pharm. Bull.* **2001**, *49*, 958–963.
16. (a) Yagami, T.; Kitagawa, K.; Futaki, S. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1335–1338. (b) Yagami, T.; Kitagawa, K.; Aida, C.; Fujiwara, H.; Futaki, S. *J. Pept. Res.* **2000**, *56*, 239–249.
17. Bollhagen, R.; Schmiedberger, M.; Barlos, K.; Grell, E. *J. Chem. Soc., Chem. Commun.* **1994**, 2559–2560.
18. König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 2034–2040.
19. MALDI-TOFMS *m/z*: found 3029.1 $[M+H]^+$. This molecular protonated ion corresponds to the Boc-protected peptide carboxylic acid, *m/z* calcd for $C_{133}H_{223}N_{37}O_{41}S$ 3028.5 (*M*).
20. Two peaks in the HPLC chromatogram (t_R 16.40 and 16.50 min in Figure 8(b)) were suspected as hydrolyzed products of thioester segment [**III**] based on the MS determinations (MALDI-TOFMS); *m/z*: found 3293.4 (for peak at t_R 16.40 min) and 3294.9 (for peak at t_R 16.50 min). A calculated *m/z* for Boc-protected peptide carboxylic acid ($C_{142}H_{239}N_{47}O_{43}$) is 3292.7 (*M*). Further characterization for these peaks was not done.
21. Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205–208.
22. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.