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**Studies on Peptides. CLX.^{1,2)} Synthesis of a 33-Residue Peptide
Corresponding to the Entire Amino Acid Sequence of Human
Cholecystokinin (hCCK-33)**

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An unsulfated form of human cholecystokinin (hCCK-33) was synthesized by successive azide condensations of seven peptide fragments, followed by deprotection with 1 M trimethylsilyl trifluoromethanesulfonate/trifluoroacetic acid. The phenolic group of Tyr (position 27) was selectively sulfated with pyridine-SO₃ complex, after reversible masking of other functional groups with hard base (F⁻)-labile protecting groups, *i.e.*, the amino functions with 9-fluorenylmethoxycarbonyl group and the hydroxyl functions of 4 Ser residues with *tert*-butyldiphenylsilyl groups. In terms of pancreatic protein output and capillary blood flow in dogs, the relative potency of synthetic hCCK-33 with respect to that of synthetic CCK-8 (taken as 1 on a molar basis) was 0.9. In terms of gastric acid and pepsin output in rat *in vivo* assays, synthetic hCCK-33 was about 2- to 3-fold more potent than CCK-8 on a molar basis.

Keywords—unsulfated human CCK-33 synthesis; sulfated hCCK-33 synthesis; hard acid deprotection; trimethylsilyl trifluoromethanesulfonate; *tert*-butyldiphenylsilyl protection; 9-fluorenylmethoxycarbonyl protection; tetrabutylammonium fluoride deprotection; pancreatic protein output; pancreatic capillary blood flow; gastric acid output

As an initial approach for the synthesis of human cholecystokinin (hCCK-33),³⁾ we reported, in the preceding paper,¹⁾ the synthesis of protected hCCK-33, in which unmasked Tyr (position 27) was employed. In order to obtain a quantity of the starting material for the next sulfation step, we decided to synthesize alternatively protected hCCK-33 by using protected Tyr, Tyr(Cl₂-Bzl),⁴⁾ since in the former approach, we observed over-acylation at this free Tyr residue in the fragment condensation reactions. In this paper, we wish to report the total synthesis of hCCK-33, involving in principle, three steps of reactions (Fig. 1); *i.e.*, the alternative synthesis of protected hCCK-33, deprotection to obtain unsulfated hCCK-33, and selective sulfation at the Tyr residue. Prior to the final sulfation reaction, several model experiments were conducted to find suitable conditions.

First, by using Tyr(Cl₂-Bzl), the C-terminal fragment [1'] was alternatively synthesized as shown in Fig. 2. Z(OMe)-Tyr(Cl₂-Bzl)-OH was condensed with a TFA-treated sample of Z(OMe)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂¹⁾ by the Su ester procedure.⁵⁾ This active ester procedure was further employed to condense the rest of the amino acid residues in a stepwise manner.

Starting from the alternatively prepared C-terminal fragment [1'], the available six

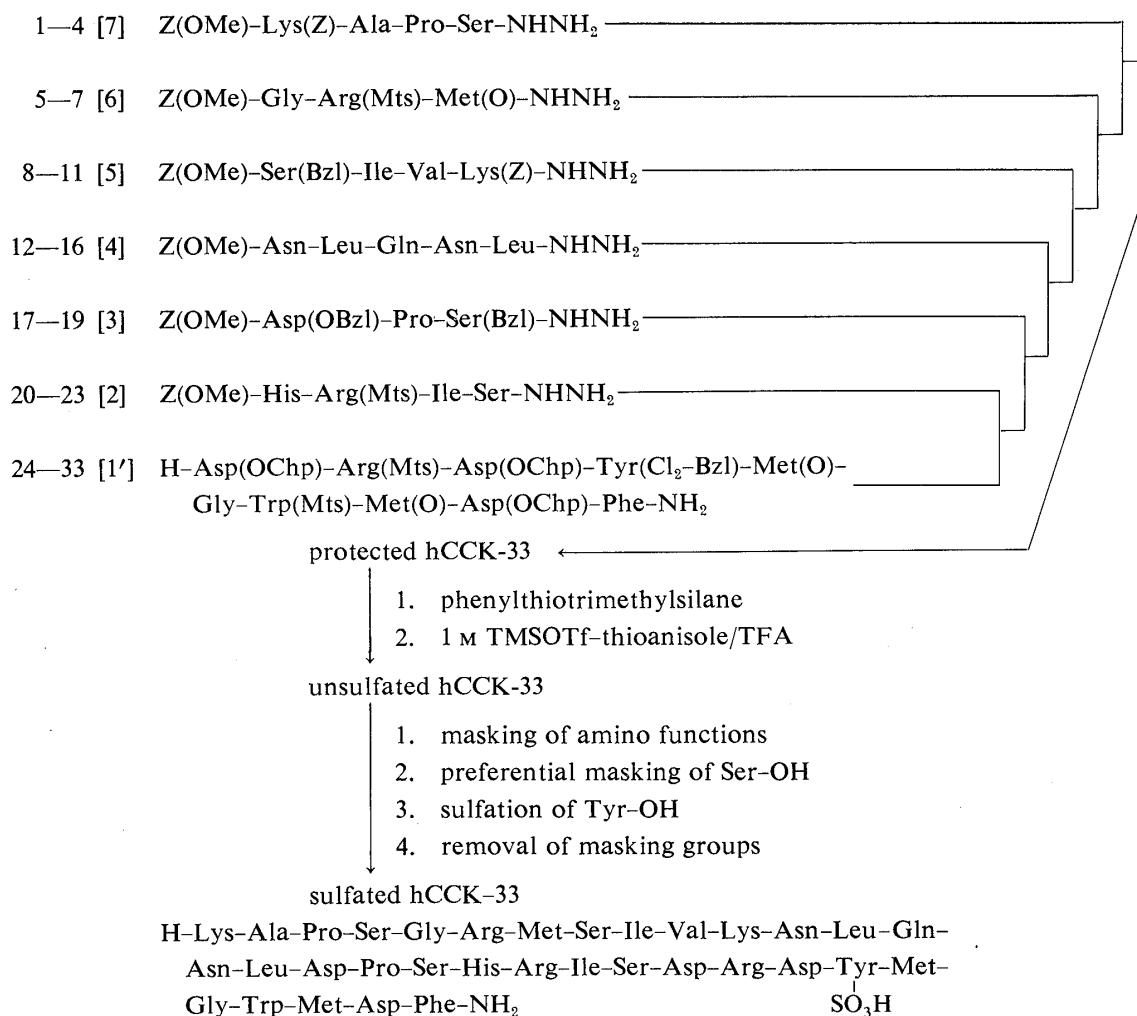


Fig. 1. Synthetic Route to hCCK-33

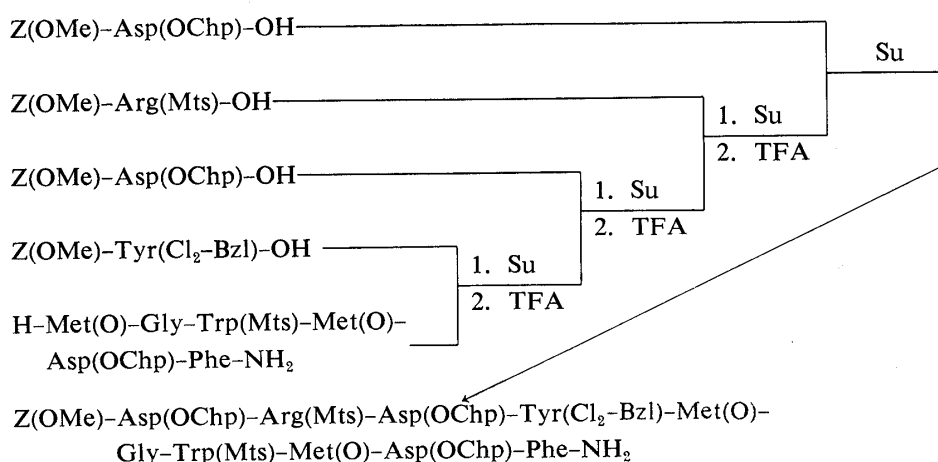


Fig. 2. Synthetic Scheme for the C-Terminal Decapeptide Amide [1'] (Positions 24-33)

fragments were successively condensed by the azide procedure.⁶⁾ DMF was employed as a solvent, except for preparation of the azide of fragment [4]. DMF-DMSO-HMPA (1:1:1) was employed to dissolve fragment [4] as was done in the previous synthesis of protected CCK. The amount of the acyl component was increased from 1.5 to 5 eq as chain elongation

progressed. Compared with the previous synthesis, purification was easier, since every reaction proceeded smoothly without over-acylation. Only three products, protected 22-residue and 26-residue peptides, and protected hCCK-33, had to be purified by gel-filtration on Sephadex LH-60 to remove the acyl components used in excess and the others were easily purified by precipitation from appropriate solvents. As was done in the previous synthesis, recovery of Phe after acid hydrolysis was compared with those of newly added amino acids after each condensation to ascertain the purity of each product, including protected hCCK-33, as shown in Table I. A rational route for preparation of protected hCCK-33 has thus been established.

Next, all protecting groups were removed from protected hCCK-33 to obtain unsulfated hCCK-33. Prior to deprotection, the Met(O) residue was reduced back to Met by brief treatment with phenylthiotrimethylsilane.⁷⁾ The reduced peptide was next treated with 1 M TMSOTf–thioanisole/TFA⁸⁾ in an ice bath for 120 min to remove all protecting groups attached, then the deprotected peptide was purified by gel-filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-Trisacryl M using ammonium bicarbonate buffer. At this stage, a fairly pure material was obtained. The product was further purified by high performance liquid chromatography (HPLC) on a Synchropak RP-P column using a gradient of MeCN in 0.1% aqueous TFA. The homogeneity of synthetic unsulfated hCCK-33 was ascertained by analytical HPLC and amino acid analysis, after 6 N HCl hydrolysis.

Next, in order to sulfate selectively the Tyr residue, several model experiments were conducted by using several amino acids concerned, Tyr, Ser, Trp, Met, His and Lys. As described in the preceding paper,¹⁾ no selective chemical sulfation reagent for the Tyr residue in the presence of other hydroxyamino acid residues (Ser or Thr) is available at the present time. Sulfation with any reagent proceeds predominantly at Ser–OH or Thr–OH, rather than at Tyr–OH. We found that Ser–OH was silylated at a much faster rate than Tyr–OH and thus

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of hCCK-33, Unsulfated hCCK-33 and Protected Intermediates

	Protected peptides							Unsulfated hCCK-33	Sulfated hCCK-33
	24–33	20–33	17–33	12–33	8–33	5–33	1–33		
Asp	2.81	3.09	4.04	5.71	5.82	5.74	6.53	5.72	5.77 (6)
Ser		0.87	1.87	1.57	2.52	2.30	3.69	3.59	3.62 (4)
Glu				1.03	1.07	0.92	1.24	1.11	1.05 (1)
Pro			0.90	0.89	0.92	0.91	2.32	2.02	1.71 (2)
Gly	1.11	1.28	1.11	1.29	1.28	2.28	2.57	2.18	2.09 (2)
Ala							1.20	1.11	1.00 (1)
Val					0.78	0.70	0.81	0.73	0.71 (1)
Met ^{a)}	1.71	1.66	1.82	1.78	1.67	2.33	2.67	2.53	2.86 (3)
Ile		1.08	0.97	0.91	1.69	1.69	1.76	1.49	1.67 (2)
Leu				1.89	1.99	1.69	2.21	2.10	2.05 (2)
Tyr	0.92	1.05	1.04	1.02	1.05	1.03	1.05	0.91	0.94 (1)
Phe	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1)
Lys					0.81	0.78	2.10	1.75	2.11 (2)
His		0.97	0.94	0.80	0.75	0.68	0.73	0.75	0.87 (1)
Trp ^{b)}	0.96	0.86	0.86	0.85	0.72	0.87	0.85	N.D.	N.D.
Arg	0.96	2.17	1.96	1.98	2.06	2.96	3.20	2.69	3.11 (3)
Recov. (%)	80	89	79	77	82	95	84	84	79

Numbers in parentheses are theoretical. a) Met+Met(O). b) 4 N MeSO₃H hydrolysis

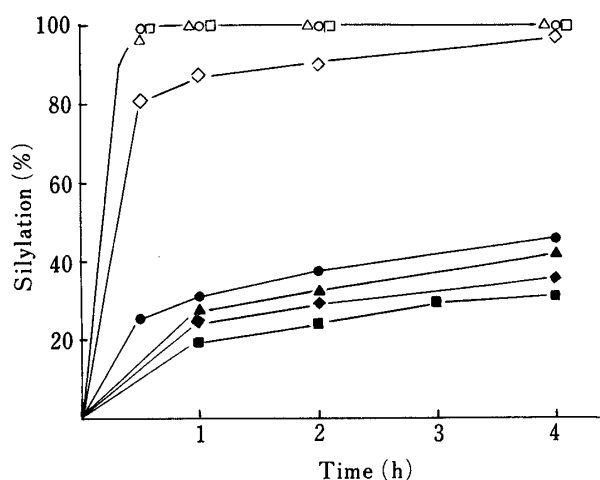


Fig. 3. *tert*-Butyldiphenylsilylation of Ser and Tyr

imidazole (20 eq) + adduct (20 eq)	BuPh ₂ Si Ser	BuPh ₂ Si Tyr
none	—○—	—●—
phenol	—□—	—■—
<i>m</i> -cresol	—△—	—▲—
methylthiophenol	—◇—	—◆—

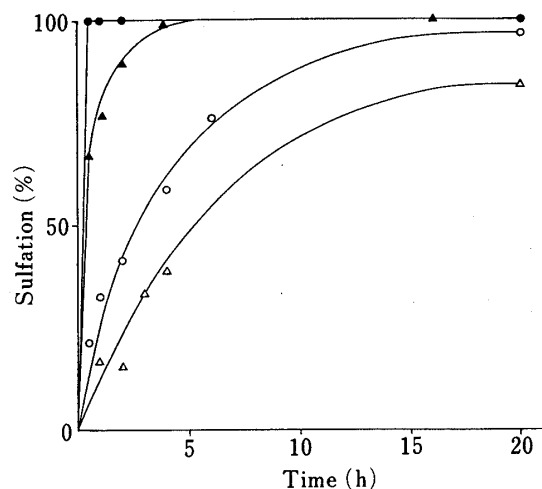


Fig. 4. Sulfation of Z(OMe)-Tyr-OMe and Z(OMe)-Ser-OMe in DMF-Pyridine with Pyridine-SO₃ Complex or Pyridinium Acetylsulfate

Sulfation of Z(OMe)-Ser-OMe by Pyr-SO₃
 —●—, PAS —▲—; Z(OMe)-Tyr-OMe by Pyr-SO₃
 —○—, PAS —△—.

selected *tert*-butyldiphenylsilyl (tBuPh₂Si) chloride⁹) as a silylating reagent. Under sulfation conditions with pyridine-SO₃ complex, the Ser(tBuPh₂Si)-derivative remained intact even after 24 h, while two other derivatives so far tested, trimethylsilyl and *tert*-butyldimethylsilyl derivatives, decomposed. Z(OMe)-Ser-OMe was silylated quantitatively with tBuPh₂Si-Cl in the presence of imidazole within 30 min. Partial silylation of Tyr-OH was found to be effectively suppressed by addition of a phenol compound under ice-cooling. Of three phenol compounds so far tested, phenol gave a somewhat better result than the others. Addition of phenol (20 eq) suppressed silylation of Tyr-OH from 46% to 31% (after 4 h) as shown in Fig. 3. The tBuPh₂Si group is known to be cleaved by brief treatment (0 °C, 60 min) with 1 M tetrabutylammonium fluoride (Bu₄NF)¹⁰) in DMF, while Tyr(SO₃H) was found to be stable under this hard base treatment. When examined by thin layer chromatography (TLC), His, Met and Trp remained unchanged under silylation and desilylation conditions. From these model experiments, we reached the conclusion that preferential sulfation of Tyr-OH in the presence of Ser-OH can be conducted by reversible masking of the latter function with the tBuPh₂Si group. Thr is absent in hCCK-33.

The Fmoc group was introduced by Carpino and Han¹¹) in 1970 as a base-labile protecting group for amino functions. This group was found to be cleaved by treatment with 1 M Bu₄NF¹²) in DMF, together with the tBuPh₂Si group. Thus, we decided to mask the α- and ε-amino functions of two Lys residues (positions 1 and 11) with Fmoc groups during sulfation. When examined by TLC, partial acylation of Tyr with Fmoc-OSu¹³) was found to be effectively suppressed by addition of phenol.

Pyridinium acetylsulfate (PAS reagent) was recently introduced by Penke *et al.*¹⁴) as a sulfation reagent. This reagent was used in DMF-pyridine by Penke *et al.*¹⁵) or in TFA by Kurano *et al.*¹⁶) for preparation of porcine CCK-33, after masking of Ser-OH with an acetyl or phenoxyacetyl group, respectively. Considering the acid-instability of Tyr(SO₃H) and the presence of an unmasked Trp residue in our unsulfated hCCK-33, we decided to sulfate the

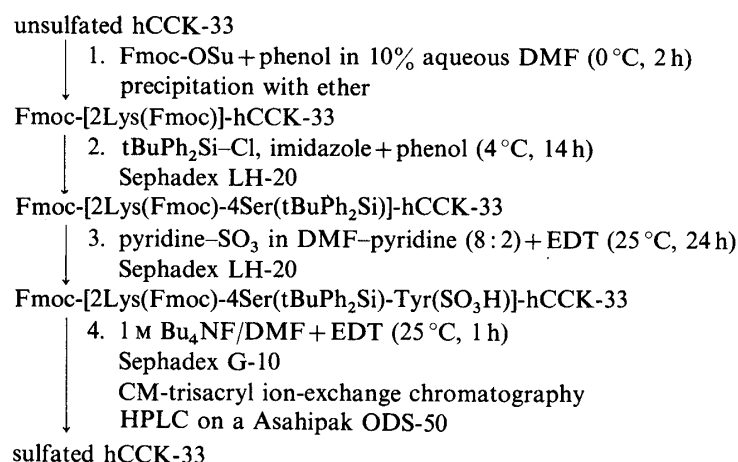


Fig. 5. Procedure for Conversion of Unsulfated hCCK-33 to Sulfated hCCK-33

Tyr residue under basic conditions. In the presence of pyridine in DMF, pyridine- SO_3 complex sulfated Z(OMe)-Tyr-OMe more readily than the PAS reagent (Fig. 4). A similar tendency was also observed in the sulfation reaction of Z(OMe)-Ser-OMe . When examined by TLC, His was found to be partially sulfated with pyridine- SO_3 complex (32%, after 4 h), but addition of water regenerated His quantitatively within 60 min. The use of EDT was effective to suppress partial oxidation of Met and modification of Trp during sulfation.

After these model experiments, we converted the above unsulfated form of hCCK-33 to sulfated hCCK-33 by the following sequence of reactions (Figs. 1 and 5). 1) Treatment with Fmoc-OSu in the presence of TEA to protect all the amino functions (0°C, 2 h). Phenol was added to protect the Tyr residue. 2) Treatment with $\text{tBuPh}_2\text{Si-Cl}$ in the presence of imidazole to protect preferentially four Ser-OH functions (4°C for 14 h, a longer time than in the model experiment). Phenol was added to minimize silylation at the Tyr residue.³⁾ Treatment with pyridine- SO_3 complex in 20% pyridine in DMF to sulfate Tyr-OH (25°C, 24 h). EDT was added to protect Met and Trp. 4) Treatment with 1 M Bu_4NF in DMF to cleave the tBuPh_2Si and Fmoc protecting groups (4°C, 1 h, then 25°C, 1 h). EDT was added to quench dibenzofulvene derived from the Fmoc group.

The crude sample of hCCK-33 thus sulfated was purified by ion-exchange chromatography on CM-Trisacryl M using gradient elution with 0.2 M NH_4HCO_3 buffer, followed by HPLC on an Asahipak ODS-50 column with isocratic elution [MeCN (31%) in 0.1 M AcONH_4]. The former purification was effective to remove over-sulfated and unsulfated hCCKs and the latter to remove the Met(O)-derivatives. This HPLC column gave a better separation of the desired product than the YMC-ODS 302 column. The overall yield was 15% from unsulfated hCCK-33. The yield seems to depend entirely on the silylation conditions employed. Though we have not yet established the optimal silylation conditions, the yield was 13%, when silylation was conducted at 25°C for 3 h.

The purity of synthetic hCCK-33 thus obtained was ascertained by analytical HPLC and amino acid analysis after acid hydrolysis. The presence of $\text{Tyr(SO}_3^-)$ was confirmed by leucine-aminopeptidase (LAP) digestion.

Synthetic hCCK-33 was submitted to parallel bioassay with synthetic CCK-8 to determine the activity. Bioassay was carried out on pancreatic capillary blood flow and pancreatic protein output in pentobarbital-anesthetized mongrel dogs ($n=4$). Pancreatic capillary blood flow was measured by the use of a laser Doppler perfusion monitor¹⁷⁾ and protein concentration in pancreatic juice was measured by the method of Lowry *et al.*¹⁸⁾

Bolus injection of synthetic hCCK-33 and CCK-8 (1.0, 3.125, 6.25, 12.5, 25, 50, 100, 200 pmol/kg of body weight) were given *via* a femoral vein catheter at 60 min intervals. Pancreatic

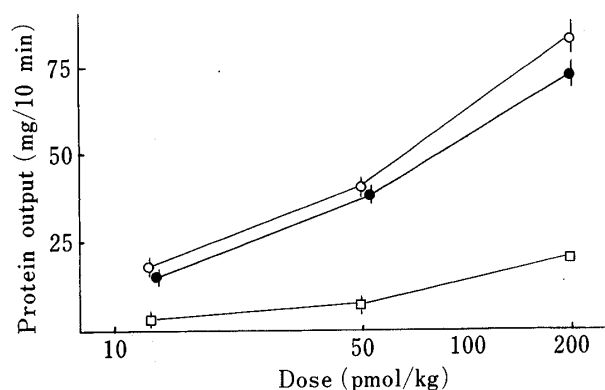


Fig. 6. Increased Pancreatic Protein Output (mg/10 min) in Anesthetized Dogs ($n=4$) in Response to Three CCK-Peptides

—○—, CCK-8; —●—, hCCK-33; —□—, unsulfated hCCK-33.

Relative potencies (mol. basis), CCK-8 1.0, hCCK-33 0.92, unsulfated-33 0.074.

blood flow was increased by administration of synthetic hCCK-33 in a dose-dependent manner. Pancreatic protein output was also increased by administration of synthetic hCCK-33 in a dose-dependent manner. An increase was observed at the minimum dose of 3.125 pmol/kg. The maximal effect was observed at a dose of 200 pmol/kg. As to the effects on pancreatic capillary blood flow and pancreatic protein output, the activity of synthetic hCCK-33 was 92% of that of synthetic CCK-8 on a molar basis (Fig. 6). In terms of gastric acid, pepsin output and pancreatic secretion in rat *in vivo* preparation, synthetic hCCK-33 was about 2 to 3-fold more potent than CCK-8 on a molar basis. In the stimulation of pepsinogen secretion from isolated guinea pig gastric glands, synthetic hCCK-33 was as effective as CCK-8 on a molar basis. It has been reported that CCK-8 is 2.5 times more potent on a molar basis than the entire porcine CCK-33 molecule.¹⁹⁾ Thus, our synthetic hCCK-33 can be judged to have activity comparable to or higher than that of natural porcine CCK-33. In the above dog assay systems, the relative potency of unsulfated hCCK-33 with respect to CCK-8 (taken as 1 on a molar basis) was 0.074. Thus, the important role of the sulfate moiety for biological activity of CCK was confirmed.

We wish to conclude that we have obtained a highly active preparation of hCCK-33 by the new methods described herein, without exposure of the peptide to strong base, as had been done by Penke *et al.*¹⁵⁾ and Kurano *et al.*¹⁶⁾ in their syntheses of porcine CCK-33.

Experimental

General experimental procedures described herein are essentially the same as described in the preceding paper.¹⁾ R_f values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: R_{f1} , CHCl_3 -MeOH- H_2O (8:3:1); R_{f2} , n -BuOH-AcOH-pyridine- H_2O (4:1:1:2); R_{f3} , n -BuOH-AcOH-AcOEt- H_2O (1:1:1:1). The ninhydrin color intensity was measured with a Shimadzu dual wavelength TLC scanner, model CS-900. Fast atom bombardment mass spectra (FAB-MS) were obtained on a JEOL JM HX-100 double-focusing mass spectrometer equipped with a FAB ion source and a data processor (JEOL JMA-3100). LAP was purchased from Sigma (Lot. No. L-6007). CCK-8 was purchased from Protein Institute Inc., Osaka, Japan.

HPLC was conducted with a Waters 204 compact model. A laser Doppler perfusion monitor (model LD5000, Medpacific Corp., Seattle, Washington, U.S.A.) was used to measure pancreatic capillary blood flow.

Z(OMe)-Tyr(Cl_2 -Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ [1'] (Positions 27-33)—A TFA-treated sample of Z(OMe)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ (4.57 g, 3.63 mmol) was dissolved in DMF (25 ml) containing TEA (0.51 ml, 1 eq), then Z(OMe)-Tyr(Cl_2 -Bzl)-OSu (2.62 g, 1.2 eq) and NMM (0.40 ml, 1 eq) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 5.26 g (92%), R_{f1} 0.64. Physical constants and analytical data are listed in Table II, together with those for protected intermediates.

Z(OMe)-Asp(OChp)-Tyr(Cl_2 -Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ [1'] (Positions 26-33)—A TFA-treated sample of the above 7-residue peptide amide (4.95 g, 3.13 mmol) was dissolved in DMF (30 ml) containing TEA (0.43 ml, 1 eq), then Z(OMe)-Asp(OChp)-OSu [prepared from 2.70 g (1.5 eq) of the DCHA salt] in THF (15 ml) and NMM (0.41 ml, 1.2 eq) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 5.23 g (90%), R_{f1} 0.75.

TABLE II. Physical Constants and Analytical Data for Fragment [1'] and Its Intermediates

Z(OMe)-Protected peptide (positions)	mp (°C)	[α] _D ²⁰ (°) (DMF)	Formula	Analysis (%)		
				Calcd (Found)		
				C	H	N
7-Residue (27—33)	215—218	−20.4	C ₇₇ H ₉₁ Cl ₂ N ₉ O ₁₇ S ₃	58.47 (58.17)	5.80 (5.96)	7.97 (7.98)
8-Residue (26—33)	200—202	−23.9	C ₈₈ H ₁₀₈ Cl ₂ N ₁₀ O ₂₀ S ₃	58.95 (58.66)	6.07 (6.37)	7.81 (7.79)
9-Residue (25—33)	238—243	−21.9	C ₁₀₃ H ₁₃₀ Cl ₂ N ₁₄ O ₂₃ S ₄ · H ₂ O	57.55 (57.52)	6.19 (6.31)	9.12 (9.27)
10-Residue [1'] (24—33)	230—233	−13.5	C ₁₁₄ H ₁₄₇ Cl ₂ N ₁₅ O ₂₆ S ₄	58.45 (58.48)	6.32 (6.42)	8.97 (8.94)

TABLE III. Physical Constants and Analytical Data for Protected hCCK-33 and Its Intermediates

Z(OMe)-Protected peptide (positions)	mp (°C)	[α] _D ²⁰ (°) (DMF)	Formula	Analysis (%)		
				Calcd (Found)		
				C	H	N
14-Residue (20—33)	234—237	−17.6	C ₁₄₄ H ₁₉₂ Cl ₂ N ₂₄ O ₃₃ S ₅ · 4H ₂ O	55.96 (55.63)	6.55 (6.47)	10.88 (11.24)
17-Residue (17—33)	230—232	+8.9	C ₁₇₀ H ₂₂₁ Cl ₂ N ₂₇ O ₃₉ S ₅ · 6H ₂ O	56.62 (56.59)	6.51 (6.33)	10.49 (10.61)
22-Residue (12—33)	245—248	−22.2	C ₁₉₅ H ₂₆₃ Cl ₂ N ₃₅ O ₄₇ S ₅ · 5H ₂ O	56.15 (55.96)	6.60 (6.52)	11.76 (11.87)
26-Residue (8—33)	265—270	−26.0	C ₂₃₀ H ₃₁₂ Cl ₂ N ₄₀ O ₅₄ S ₅ · 8H ₂ O	56.65 (56.53)	6.78 (6.78)	11.49 (11.80)
29-Residue (5—33)	260—262	−8.0	C ₂₅₂ H ₃₄₆ Cl ₂ N ₄₆ O ₆₀ S ₇ · 6H ₂ O	56.22 (56.01)	6.70 (6.35)	11.97 (12.22)
33-Residue (1—33)	260—263	−20.0	C ₂₇₇ H ₃₈₁ Cl ₂ N ₅₁ O ₆₇ S ₇ · 6H ₂ O	56.38 (56.10)	6.71 (6.71)	12.10 (11.93)

Z(OMe)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ [1'] (Positions 25—33)—A TFA-treated sample of the above 8-residue peptide amide (4.75 g, 2.65 mmol) was dissolved in DMF (30 ml) containing TEA (0.37 ml, 1 eq), then Z(OMe)-Arg(Mts)-OSu [prepared from 3.28 g (2 eq) of the CHA salt] in THF (20 ml) and NMM (0.35 ml, 1.2 eq) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.15 g (74%), *R*_f 0.78.

Z(OMe)-Asp(OChp)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ [1'] (Positions 24—33)—A TFA-treated sample of the above 9-residue peptide amide (4.15 g, 1.95 mmol) was dissolved in DMF (40 ml) containing TEA (0.27 ml, 1 eq), then Z(OMe)-Asp(OChp)-OSu [prepared from 1.68 g (1.5 eq) of the DCHA salt] in THF (15 ml) and NMM (0.26 ml, 1.2 eq) were added and the mixture was stirred for 18 h. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 3.57 g (78%), *R*_f 0.70.

Z(OMe)-His-Arg(Mts)-Ile-Ser-Asp(OChp)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ (Positions 20—33)—The azide, prepared from fragment [2] (7.99 g, 2 eq), in DMF (40 ml) and NMM (0.60 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of fragment [1'] (10.73 g, 4.58 mmol) in DMF (30 ml) containing TEA (0.64 ml, 1 eq) and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 11.99 g (87%), *R*_f 0.73. Physical constants and analytical data are listed in Table III, together with those of other protected peptides.

Z(OMe)-Asp(OBzl)-Pro-Ser(Bzl)-His-Arg(Mts)-Ile-Ser-Asp(OChp)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ (Positions 17—33)—The azide, prepared from 3.46 g (1.5 eq) of fragment [3], in DMF (10 ml) and NMM (0.52 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above 14-residue peptide amide (11.89 g, 3.94 mmol) in DMF (30 ml) containing TEA (0.55 ml,

1 eq), and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 8.64 g (63%), R_f 0.71.

Z(OMe)-Asn-Leu-Gln-Asn-Leu-Asp(OBzl)-Pro-Ser(Bzl)-His-Arg(Mts)-Ile-Ser-Asp(OChp)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ (Positions 12–33)—The azide, prepared from 7.57 g (4 eq) of fragment [4], in DMF-DMSO-HMPA (1 : 1 : 1, 90 ml) and TEA (0.41 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above 17-residue peptide amide (8.50 g, 2.43 mmol) in DMF (30 ml) containing TEA (0.34 ml, 1 eq) and the mixture was stirred for 48 h. The product was purified by gel-filtration on Sephadex LH-60, followed by precipitation from DMF with AcOEt; yield 4.84 g (49%), R_f 0.73.

Z(OMe)-Ser(Bzl)-Ile-Val-Lys(Z)-Asn-Leu-Gln-Asn-Leu-Asp(OBzl)-Pro-Ser(Bzl)-His-Arg(Mts)-Ile-Ser-Asp(OChp)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ (Positions 8–33)—The azide, prepared from fragment [5] (2.10 g, 4 eq), in DMF (20 ml) and TEA (0.10 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above 22-residue peptide amide (2.53 g, 0.62 mmol) in DMF (10 ml) containing TEA (86 μ l, 1 eq) and the mixture was stirred overnight. The product was purified by gel-filtration on Sephadex LH-60, followed by precipitation from DMF with AcOEt; yield 1.95 g (67%), R_f 0.63.

Z(OMe)-Gly-Arg(Mts)-Met(O)-Ser(Bzl)-Ile-Val-Lys(Z)-Asn-Leu-Gln-Asn-Leu-Asp(OBzl)-Pro-Ser(Bzl)-His-Arg(Mts)-Ile-Ser-Asp(OChp)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ (Positions 5–33)—The azide, prepared from fragment [6] (0.98 g, 4 eq), in DMF (5 ml) and NMM (0.15 ml, 4 eq) were added to an ice-chilled solution of a TFA treated sample of the above 26-residue peptide (1.51 g, 0.33 mmol) in DMF (5 ml) containing TEA (46 μ l, 1 eq) and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 1.45 g (86%), R_f 0.77.

Z(OMe)-Lys(Z)-Ala-Pro-Ser-Gly-Arg(Mts)-Met(O)-Ser(Bzl)-Ile-Val-Lys(Z)-Asn-Leu-Gln-Asn-Leu-Asp(OBzl)-Pro-Ser(Bzl)-His-Arg(Mts)-Ile-Ser-Asp(OChp)-Arg(Mts)-Asp(OChp)-Tyr(Cl₂-Bzl)-Met(O)-Gly-Trp(Mts)-Met(O)-Asp(OChp)-Phe-NH₂ (Protected hCCK-33)—The azide, prepared from fragment [7] (0.81 g, 5 eq), in DMF (5 ml) and NMM (38 μ l, 5 eq) were added to an ice-chilled solution of a TFA-treated sample of the above 29-residue peptide amide (1.20 g, 0.23 mmol) in DMF (5 ml) containing TEA (32 μ l, 1 eq) and the mixture was stirred for 24 h. The product was purified by gel-filtration on Sephadex LH-60, followed by precipitation from DMF with AcOEt; yield 0.76 g (58%), R_f 0.77.

H-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH₂ (Free form of hCCK-33)—Protected hCCK-33 (317 mg, 54.7 μ mol) in DMF (3 ml) was treated with phenylthiotrimethylsilane (300 μ l, 30 eq) at room temperature for 60 min, then the solvent was removed by evaporation and AcOEt was added to give a powder; yield 279 mg (89%), R_f 0.72.

The reduced form of protected hCCK-33 (100 mg, 17.4 μ mol) was treated with 1 M TMSOTf-thioanisole/TFA (5 ml) in the presence of *m*-cresol (244 μ l, 130 eq) and EDT (38 μ l, 23 eq) in an ice-bath for 2.5 h, then dry ether was added. The resulting powder was dissolved in ice-chilled MeOH-H₂O (1 ml–2 ml) and 2-mercaptoethanol (200 μ l) and 1 M NH₄F (600 μ l, 36 eq) were added. The pH of the solution was adjusted to 8.0 with TEA, then after 30 min, to 6.0 with 1 N AcOH. After the removal of some insoluble material by centrifugation, the solution was applied to a column of Sephadex G-25 (3.3 \times 105 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front main peak (8.6 ml each, tube Nos. 30–44, monitored by ultraviolet (UV) absorption measurement at 280 nm) were collected and the solvent was removed by lyophilization to give a powder; yield 64.2 mg (95.4%).

The crude product was next purified by ion-exchange chromatography on CM-Trisacryl M (2.0 \times 4.2 cm), which was eluted with a linear gradient formed from pH 8.6, 0.2 M NH₄HCO₃ buffer (250 ml) through a mixing flask containing pH 7.9, 0.01 M NH₄HCO₃ buffer (250 ml). The fractions corresponding to the main peak (8.2 ml each, tube Nos. 24–31, monitored by UV measurement at 280 nm) were collected and the solvent was removed by lyophilization to give a fluffy powder; 20.1 mg (31.3%). Subsequent purification was performed by reverse phase HPLC on a Synchropak RP-P column (0.4 \times 25 cm), which was eluted with a gradient of MeCN (25 to 35% in 30 min)

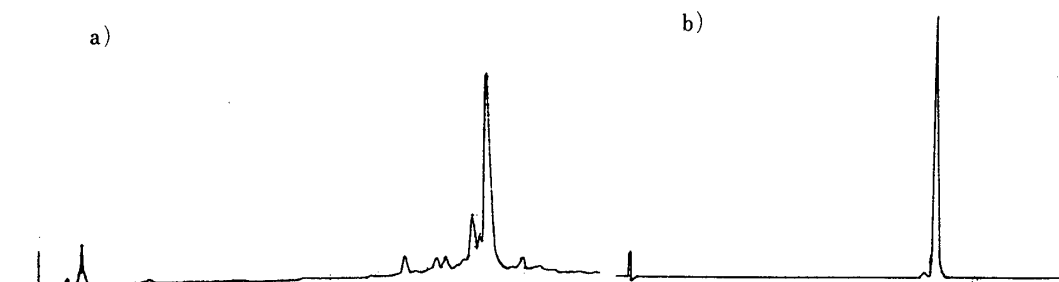


Fig. 7. HPLC Purification of Unsulfated hCCK-33

a) CM-Purified sample on Synchropak RP-P. b) HPLC-Purified sample on YMC AM-3020DS.

in 0.1% aqueous TFA at a flow rate of 1.0 ml/min. The eluate corresponding to the main peak (Fig. 7a, retention time 37 min, detected by UV measurement at 280 nm) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 10.6 mg (53%), $[\alpha]_D^{20} - 65.7^\circ$ ($c = 0.1$, 0.5 N AcOH). The purified peptide exhibited a single peak in HPLC (Fig. 7b, retention time 27 min), when a YMC AM-302-ODS column (4×150 mm) was eluted with a linear gradient of MeCN (40–45% in 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min. FAB-MS m/z : 3864.4 ($M + H$)⁺ (Calcd for $C_{167}H_{264}N_{51}O_{49}S_3$: 3864.9). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical): Asp 3.62 (4), Ser 4.53 (4), Pro 1.66 (2), Gly 2.13 (2), Ala 1.18 (1), Val 1.10 (1), Met 2.70 (3), Ile 2.28 (2), Leu 2.44 (2), Tyr 1.12 (1), Phe 1.00 (1), Lys 2.14 (2), His 1.08 (1), Trp 0.99 (1), Arg 3.24 (3), Asn and Gln were not determined (recovery of Phe, 77%).

Model Experiments—1. Fmoc-Protection of Lys and Its Deprotection: H-Lys-OH (14.6 mg, 0.1 mmol) in H₂O-DMF (1:9, 2 ml) was treated with Fmoc-OSu (141 mg, 4 eq) in the presence of TEA (59 μ l, 4 eq) in an ice-bath for 60 min; the starting material and a mono-Fmoc-derivative (R_f 0.42) disappeared in TLC and a new ninhydrin-negative spot (R_f 0.66) was detected. The product was isolated by extraction procedure A. The di-Fmoc-derivative thus isolated was dissolved in DMF (1 ml) and the solution was treated with 1 M Bu₄NF/THF (1 ml, 10 eq) in the presence of EDT (39 μ l, 10 eq) at 25 °C for 60 min; the R_f 0.66 compound was fully converted to H-Lys-OH (R_f origin). Z(OMe)-Tyr-OMe (0.1 mmol) in DMF (2 ml) was similarly treated with Fmoc-OSu (4 eq) and TEA (4 eq) in the absence or presence of phenol (30 eq) in an ice-bath for 60 min. As determined by a TLC scanner, formations of Z(OMe)-Tyr(Fmoc)-OMe (R_f 0.98) were 7.8% and 0%, respectively. Thus, addition of phenol was effective to suppress Tyr-modification. When Z(OMe)-His-OMe (0.1 mmol) was similarly treated with Fmoc-OSu and TEA, formation of Z(OMe)-His(Fmoc)-OMe was negligible.

Fmoc-Lys(Fmoc)-OH (0.1 mmol) in DMF-pyridine (8:2, 2 ml) containing pyridine-SO₃ complex (10 eq) was kept at 25 °C for 18 h. No change was observed in the TLC pattern.

2. Preferential tBuPh₂-Silylation of Ser-OH: First, the stability of silylated Z(OMe)-Ser-OMe derivatives under treatment with pyridine-SO₃ complex was examined. Z(OMe)-Ser-OMe (14 mg, 0.05 mmol each) in DMF (1 ml) was treated with R-Cl (R = Me₃Si or tBuMe₂Si or tBuPh₂Si, 10 eq) in the presence of imidazole (20 eq) in an ice-bath for 60 min. The solvent was removed by evaporation and the residue was washed with *n*-hexane. Each product [68 μ mol each, R = Me₃Si (R_f 0.97); R = tBuMe₂Si (R_f 0.99); R = tBuPh₂Si (R_f 0.99)] was dissolved in DMF-pyridine (8:2, 1 ml) containing EDT (20 μ l), and pyridine-SO₃ complex (94 mg, 10 eq) was added. Each solution was kept at 25 °C and periodically examined by means of the TLC scanner. The Me₃Si-compound was desilylated completely within 30 min and the tBuMe₂Si-compound to the extent of ca. 15% within 24 h. However, the tBuPh₂Si-compound remained unchanged even after 24 h.

Next, preferential tBuPh₂-silylation of Ser-OH in the presence of Tyr-OH was examined. A mixture of Z(OMe)-Ser-OMe (0.05 mmol), Z(OMe)-Tyr-OMe (0.05 mmol) and imidazole (20 eq) in DMF (1 ml) was treated with tBuPh₂Si-Cl (20 eq) in the absence or presence of a phenol compound (20 eq each of phenol, *m*-cresol or *p*-methylthiophenol) at 4 °C for 4 h. Each product was quantified by means of the TLC scanner and the results are shown in Fig. 3. When the reaction was carried out at 25 °C for 4 h, Z(OMe)-Tyr-OMe was silylated in 75% yield in the absence of phenol and 44% in presence of phenol.

3. Deprotection of the tBuPh₂Si Group from Z(OMe)-Ser(tBuPh₂Si)-OMe: Z(OMe)-Ser(tBuPh₂Si)-OMe (36 mg, 68 μ mol) in DMF (1 ml) was treated with 1 M Bu₄NF/DMF (1 ml, 15 eq) in the presence of EDT (20 μ l) at 25 °C for 60 min; the starting material (R_f 0.99) disappeared completely and a spot (R_f 0.91) corresponding to Z(OMe)-Ser-OMe was detected.

4. Sulfation of Tyr-OH: A solution of Z(OMe)-Ser-OMe and Z(OMe)-Tyr-OMe (0.05 mmol each) in 20% pyridine in DMF (1 ml) was treated with pyridine-SO₃ complex (5 eq) or PAS (10 eq) at 25 °C, and the solution was examined periodically by means of the TLC scanner. The results are shown in Fig. 4.

Z(OMe)-Trp-OH, Z(OMe)-Met-OH and Z(OMe)-His-OMe (0.05 mmol each) were treated with pyridine-SO₃ complex or PAS for 4 h as stated above. The former two remained unchanged, while Z(OMe)-His-OMe was sulfated in 32% yield by pyridine-SO₃ and 18% by PAS. When H₂O was added (pH 6.0), the sulfated His compound (R_f 0.21) decomposed to regenerate the starting material (R_f 0.68) within 60 min.

Conversion of Unsulfated hCCK-33 to Sulfated hCCK-33—Fmoc-OSu (79 mg, 30 eq) was added to an ice-chilled solution of unsulfated hCCK-33 (30 mg, 7.8 μ mol) and phenol (22 mg, 30 eq) in DMF-H₂O (900–100 μ l) containing TEA (33 μ l, 30 eq) and the mixture was stirred in an ice-bath for 2 h. Dry ether was added. The resulting powder was re-precipitated from DMF with ether. The Fmoc-derivative thus obtained (R_f 0.66) was dissolved in DMF (2 ml), together with imidazole (63 mg, 120 eq) and phenol (88 mg, 120 eq), then tBuPh₂Si-Cl (216 μ l, 120 eq) was added and the solution was stirred at 4 °C for 14 h. Ether was added and the resulting powder was re-precipitated from DMF with ether. The product (R_f 0.77) was purified by gel-filtration on Sephadex LH-20 (4×47 cm) which was eluted with DMF. The desired fractions (9.2 ml each, tube Nos. 21–29, monitored by UV absorption measurement at 280 nm, as was done with other purifications) were combined and the solvent was removed by evaporation.

The residue was dissolved in 20% pyridine in DMF (1 ml), then EDT (22 μ l, 30 eq) and pyridine-SO₃ complex (124 mg, 100 eq) were added and the mixture was stirred at 25 °C for 24 h. The solution was applied to a column of Sephadex LH-20 (4×47 cm), which was eluted with DMF as stated above. The desired fractions (tube Nos. 20–24)

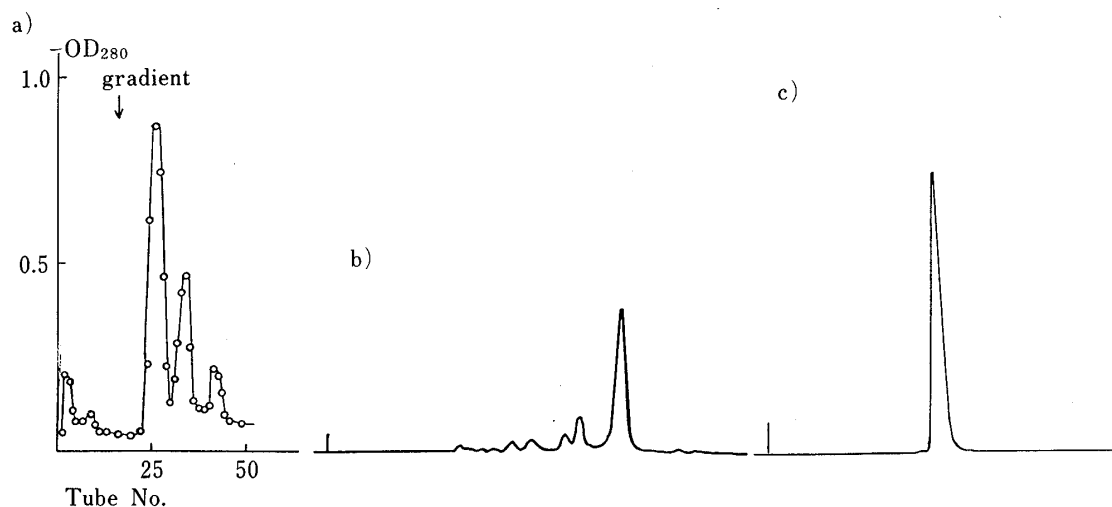


Fig. 8. CM and HPLC Purifications of Sulfated hCCK-33

a) CM-Trisacryl purification. b) HPLC purification of CM-purified sample on Asahipak ODP-50. c) HPLC of purified sample on Asahipak ODP-50.

were combined and the solution was concentrated (to *ca.* 1 ml). This solution was treated with 1 M Bu_4NF in DMF (1.0 ml) in the presence of EDT (22 μl , 30 eq) in an ice-bath for 60 min, then at room temperature for 60 min. Under cooling with ice, 1 M NH_4HCO_3 (4 ml) was added and a small amount of insoluble material was removed by centrifugation. The supernatant was applied to a column of Sephadex G-10 (2.4×49 cm), which was eluted with 0.1 M NH_4HCO_3 buffer (pH 8.2). The fractions corresponding to the front main peak (7.8 ml each, tube Nos. 11–17) were combined and the solvent was removed, together with the salt, by repeated lyophilization to give a white powder; yield 19.2 mg (63.9%).

The crude sample was next purified by ion-exchange chromatography on CM-Trisacryl M (1.6×4.5 cm), which was eluted by gradient formed with 0.2 M NH_4HCO_3 buffer (pH 8.4, 500 ml) through a mixing flask containing 0.01 M NH_4HCO_3 buffer (pH 7.8, 300 ml). The fractions corresponding to the 2nd peak (Fig. 8a) (7.8 ml each, tube Nos. 21–29) were combined and the solvent and the salt were removed by repeated lyophilization to give a powder; yield 7.5 mg (39.1%, overall yield 25.0%). The product obtained here was further purified by HPLC on a Asahipak ODS-50 column (10×250 mm) with isocratic elution [31% MeCN in 0.1 M AcONH_4 (pH 6.5)] at a flow rate of 2 ml per min. The desired eluate (Fig. 8b, t_R , 42 min) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 4.1 mg (61%, overall yield 15% from unsulfated hCCK-33). When silylation was carried out at 25 °C for 3 h, the yield, after similar purifications, was 13%. $[\alpha]_D^{21} - 72.7^\circ$ ($c=0.1$, H_2O). R_f 0.42. Retention time on HPLC was 14 min, when an Asahi Pak ODP-50 (4×150 mm) was eluted with a gradient of MeCN (20–40%, for 30 min) in 0.1 M AcONH_4 (pH 7.8) at a flow rate of 1 ml/min (Fig. 8c). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical): Asp 3.49 (4), Ser 4.22 (4), Pro 1.50 (2), Gly 2.12 (2), Ala 1.13 (1), Val 1.14 (1), Met 2.92 (3), Ile 1.96 (2), Leu 2.07 (2), Tyr(SO_3H) 0.91 (1), Phe 1.00 (1), Lys 2.00 (2), His 0.92 (1), Trp 0.96 (1), Arg 2.87 (3), Asn and Gln were not determined (recovery of Phe, 81%). The Asp–Pro linkage resisted the action of LAP).

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References and Notes

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- 2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Z(OMe)=*p*-methoxybenzyloxycarbonyl, Z=benzyloxycarbonyl, Mts=mesitylenesulfonyl, (O)=sulfoxide, Bzl=benzyl, Cl_2 -Bzl=2,6-dichlorobenzyl, Chp=cycloheptyl, Fmoc=9-fluorenylmethyloxycarbonyl, Su=*N*-hydroxysuccinimidyl, TEA=triethylamine, TFA=trifluoroacetic acid, TMSOTf=trimethylsilyl trifluoromethanesulfonate, DMSO=dimethyl sulfoxide, HMPA=hexamethylphosphorus triamide, EDT=ethanedithiol, DMF=*N,N*-dimethylformamide, NMM=*N*-methylmorpholine, CHA=cyclohexylamine, DCHA=dicyclohexylamine, THF=tetrahydrofuran.
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