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Synthesis of a Heat-stable Enterotoxin (STh) Produced by a Human Strain SK-1 of Enterotoxigenic *Escherichia coli*

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A peptide with the amino acid sequence proposed for a heat-stable enterotoxin (STh) produced by a human strain SK-1 of enterotoxigenic *Escherichia coli* was synthesized by a conventional method. Synthetic STh evoked fluid secretion in suckling mice at a dose of 0.8 ng, which is similar to the effective dose of native STh. The fluid secretion was completely inhibited by antisera raised against the native toxin. Furthermore, synthetic STh showed the same ^1H-NMR spectrum and heat-stability as native STh.

Recently, we[1-3] isolated and purified two heat-stable enterotoxins (named STh and STp) that were produced by enterotoxigenic *Escherichia coli* strain SK-1 and strain 18D, respectively, originated from human patients with diarrhea. We determined the sequences of the 19 and 18 amino acid residues of these enterotoxins to be as shown in Fig. 1. Lallier *et al.*[4,5] and Ronnberg *et al.*[6] also isolated ST's from the culture supernatants of enterotoxigenic *E. coli* strain F11(P155) of porcine origin and strain C57/26C2 of human origin and determined their amino acid sequences. These sequences were identical to that of STp determined by us.[3] Our investigations[1-3,7] and those of others[4-6,8] showed that the characteristic features of these heat-stable enterotoxins were as follows: 1) STh and STp have much structural similarity except in a few N-terminal residues. 2) One-third of the amino acid residues are half-cystine residues, which are located in identical positions in the two toxins and are joined intramolecularly by disulfide linkages. 3) The minimum effective doses of the two toxins are similar in suckling mouse assays. 4) The toxicities of STh and STp are both neutralized by both anti-STh and anti-STp antisera. These findings suggested that the common amino acid sequences of STh and STp are very important for expression of their ST toxicity. However, since it is difficult to isolate much toxin from the culture supernatants of bacteria, little is known about the biological and physicochemical properties or the structure-activity relationship of ST, except that it may be involved in the guanylate cyclase-cyclic GMP system.[9-12]

This study was carried out as part of a study on the molecular conformation-activity relationship of ST to confirm the primary structure proposed for STh and to develop a method for synthesizing the large amounts of STh necessary for biological studies. The following paper[13] deals with the synthesis of several shorter analogs of STh and the structure-activity relationship of STh.

Experimental

The general experimental and analytical methods used were described in the preceding paper.[14] YMC-packed column of ODS A-324 (10×300 mm) was obtained from Yamamura Chemical Laboratory Co. Ltd. (Kyoto). LiChrosorb RP-8 (5 μm particle size) was purchased from Merck Japan and packed into a column (8×300 mm) in our laboratory. Aminopeptidase M was obtained from Protein Research Foundation (Minoh, Osaka). Synthetic peptides were hydrolyzed in 4M (1 M=1 mol dm^{-3}) methanesulfonic acid or a mixture of 6 M HCl and propionic acid (v/v, 1/1) in evacuated sealed tubes at 110 °C for 24 h, and amino acids in the hydrolysates were analyzed in a Hitachi type-835 amino acid analyzer. Native STh was isolated from the culture supernatant of enterotoxigenic *E. coli* strain SK-1 and finally purified by high-performance liquid chromatography, as described previously.[2] The abbreviations used in this paper are those recommended by the IUPAC-IUB [*J. Biol. Chem.*, **247**, 977 (1972)]. Additional abbreviations are: MBzl, *p*-methylbenzyl; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; TEA, triethylamine; DMSO, dimethyl sulfoxide.

Boc-Cys(MBzl)-Tyr-OBzl (Ia). Boc-Cys(MBzl)-OH·

	1 5 10 15	
Strain SK-1	Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr	Aimoto et al.[2]
Strain 18D	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr	Takao et al.[3]
Strain 18D	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Tyr-Pro-Ala-Cys-Ala-Gly-Cys-Asn	Chan and Giannella[8]
Strain F11(P155)	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr	Lazure et al.[4-5]
Strain C57/26C2	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr	Ronnberg et al.[6]

Fig. 1. Amino acid sequences of heat-stable enterotoxins (ST) isolated from various strains of enterotoxigenic *E. coli*.

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DCHA (48.1 g, 95.0 mmol) was dissolved with H-Tyr-OBzl·TosOH (54.7 g, 124 mmol) and TEA (4.00 ml) in $CHCl_3$ (1 l) and cooled below −10 °C. The solution was mixed with DCC (21.6 g, 104.5 mmol) and stirred at the same temperature for 1 h and then at room temperature overnight. The precipitate formed was filtered off and the filtrate was concentrated to a syrup under reduced pressure, dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aq $NaHCO_3$, and water. The washed solution was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to a syrup, which was crystallized from AcOEt and hexane; wt 39.4 g (71.7%), mp 136—138 °C, $[\alpha]_D^{22}$ −28.1° (*c* 1.0, DMF).

Found: C, 66.83; H, 6.69; N, 4.89; S, 5.47%. Calcd for $C_{32}H_{38}O_6N_2S$: C, 66.41; H, 6.62; N, 4.84; S, 5.54%.

*Boc-Gly-Cys(MBzl)-Tyr-OBzl (**Ib**).* Compound **Ia** (39.4 g, 68.0 mmol) was dissolved in a mixture of CH_2Cl_2 (60 ml) and TFA (60 ml). The solution was stirred at room temperature for 40 min and concentrated to dryness under reduced pressure. The residue was repeatedly flushed with CH_2Cl_2 under reduced pressure to give a crystalline material, which was dissolved in DMF (500 ml). The solution was stirred with Boc-Gly-ONSu (21.3 g, 78.2 mmol) and *N*-methylmorpholine (15.0 ml) at 2—3 °C overnight. Then it was concentrated under reduced pressure to a syrup, which was dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aq $NaHCO_3$ and water. The washed solution was dried over anhydrous Na_2SO_4 and then concentrated to a syrup under reduced pressure. The syrup was twice crystallized from AcOEt, ether and hexane; wt 37.4 g (86.5%), mp 79.5—81 °C, $[\alpha]_D^{22}$ −21.2° (*c* 1.0, DMF).

Found: C, 63.31; H, 6.58; N, 6.32; S, 5.13%. Calcd for $C_{34}H_{41}O_7N_3S \cdot 1/2H_2O$: C, 63.33; H, 6.57; N, 6.52; S, 4.97%.

*Boc-Cys(MBzl)-Thr-N_2H_3 (**Ic**).* Boc-Cys(MBzl)-OH·DCHA (52.8 g, 104 mmol) was dissolved with H-Thr-OEt·HCl (24.8 g, 135 mmol) and TEA (4.3 ml) in $CHCl_3$ (1 l). The solution was cooled below −10 °C, mixed with DCC (23.6 g, 114 mmol) and then stirred at the same temperature for 1 h and at room temperature overnight. The precipitate formed was filtered off and the filtrate was concentrated to a syrup under reduced pressure. The syrup was dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aq $NaHCO_3$, and water. The washed solution was dried over anhydrous Na_2SO_4 and concentrated to a syrup under reduced pressure. The syrup was dissolved in EtOH (500 ml), mixed with 100% hydrazine hydrate (25.2 ml), and stirred at room temperature overnight. The gelatinous solid formed was collected with ether and reprecipitated twice from MeOH and ether; wt 37.5 g (81.9%), mp 125—127 °C, $[\alpha]_D^{22}$ −19.7° (*c* 1.0, DMF).

Found: C, 54.12; H, 7.54; N, 12.70; S, 7.15%. Calcd for $C_{20}H_{32}O_5N_4S$: C, 54.53; H, 7.32; N, 12.72; S, 7.28%.

*Boc-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (**1**).* Compound **Ib** (35.0 g, 55.0 mmol) was dissolved in TFA (100 ml) and stirred at room temperature for 1 h. The solution was concentrated to a solid under reduced pressure. Meanwhile, compound **Ic** (29.1 g, 66.0 mmol) was dissolved in DMF (150 ml) and cooled to −40—−50 °C. The solution was mixed with 4.39 M HCl in dioxane (45.1 ml) and isopentyl nitrite (9.7 ml) and stirred at −20 °C for 30 min. Then it was mixed with *N*-methylmorpholine (21.8 ml) and a solution of the above solid in DMF. The mixture was stirred at 0—2 °C overnight in a refrigerator and then concentrated to dryness under reduced pressure. The residue was dissolved in AcOEt and washed with 0.1 M HCl, 5% aq $NaHCO_3$, and water. The precipitate formed during the washing was collected and dissolved in hot EtOH and insoluble material was filtered off. The solution was concentrated under reduced pressure to a gelatinous solid, which was crystallized from EtOH; wt 38.3 g (73.7%), mp 157.5—159 °C, $[\alpha]_D^{18}$ −29.0° (*c* 1.0, DMF).

Found: C, 62.30; H, 6.72; N, 7.58; S, 6.86%. Calcd for $C_{49}H_{61}O_{10}N_5S_2$: C, 62.33; H, 6.51; N, 7.48; S, 6.79%.

*Z-Asn-Pro-Ala-OMe (**IIa**).* Z-Pro-Ala-OMe[15] (40.8 g, 122 mmol) was hydrogenated over 5% palladium-charcoal in the presence of HCl (1 equiv) in MeOH. The catalyst was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue was dissolved with Z-Asn-ONp (52.0 g, 134 mmol) and TEA (17.1 ml) in DMF (600 ml). The solution was stirred at room temperature overnight and then concentrated to a syrup under reduced pressure. The syrup was dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aq $NaHCO_3$ and water. Crystals formed during the washing procedure were collected and recrystallized from a mixture of AcOEt, EtOH, and ether containing a trace amount of water; wt 39.5 g (72.2%), mp 115.5—118.5 °C, $[\alpha]_D^{22}$ −67.8° (*c* 1.0, DMF).

Found: C, 55.31; H, 6.29; N, 12.11%. Calcd for $C_{21}H_{28}O_7N_4 \cdot 1/2H_2O$: C, 55.13; H, 6.39; N, 12.25%.

*Boc-Cys(MBzl)-Cys(MBzl)-OMe (**IIb**).* Boc-Cys(MBzl)-OH·DCHA (114.0 g, 225 mmol) and H-Cys(MBzl)-OMe·HCl (62.1 g, 225 mmol) were dissolved in $CHCl_3$ (1.5 l) and cooled below −10 °C. The solution was stirred with DCC (51.1 g, 248 mmol) at the same temperature for 1 h and at room temperature overnight. The precipitate formed was filtered off and the filtrate was concentrated to an oil under reduced pressure. The oil was dissolved in AcOEt and insoluble material was filtered off. The filtrate was washed successively with 0.1 M HCl, 5% aq $NaHCO_3$, and water, and then dried over anhydrous Na_2SO_4. The dried solution was concentrated under reduced pressure to a syrup, which was crystallized from AcOEt and ether; wt 115.3 g (97.3%), mp 101—102.5 °C, $[\alpha]_D^{23}$ −52.3° (*c* 1.0, DMF).

Found: C, 61.62; H, 7.12; N, 4.94; S, 11.61%. Calcd for $C_{28}H_{38}O_5N_2S_2$: C, 61.51; H, 7.01; N, 5.12; S, 11.73%.

*Boc-Cys(MBzl)-Cys(MBzl)-N_2H_3 (**IIc**).* Compound **IIb** (115.1 g, 210 mmol) was dissolved in a mixture of DMF (200 ml) and MeOH (500 ml). Then 100% hydrazine hydrate (52.7 g) was added and the mixture was stirred at room temperature for 24 h. The precipitate formed was collected with ether; wt 109.2 g (94.9%), mp 149.5—150.5 °C, $[\alpha]_D^{23}$ −29.8° (*c* 1.0, DMF).

Found: C, 59.26; H, 7.06; N, 10.04; S, 11.60%. Calcd for $C_{27}H_{38}O_4N_4S_2$: C, 59.31; H, 7.01; N, 10.25; S, 11.73%.

*Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-OMe (**IId**).* Compound **IIa** (39.5 g, 88.0 mmol) was hydrogenated over 5% palladium-charcoal in the presence of HCl (1 equiv) in MeOH (400 ml). The catalyst was filtered off and the filtrate was concentrated to a solid under reduced pressure. Meanwhile, compound **IIc** (48.1 g, 88.0 mmol) was dissolved in DMF (500 ml) and cooled below −20 °C. The solution was stirred with 4.39 M HCl in dioxane (60.1 ml) and isopentyl nitrite (13.0 ml) at the same temperature for 30 min. Then, the solution was mixed with the above solid and *N*-methylmorpholine (29.1 ml) and stirred at 4 °C for 3 d. The solution was concentrated under reduced pressure to a syrup, which was solidified in AcOEt. The solid was dissolved in $CHCl_3$, washed with water, and then dried over anhydrous Na_2SO_4. The dried solution was concentrated under reduced pressure to a syrup, which was crystallized from a mixture of $CHCl_3$, EtOH, and ether; wt 64.6 g (88.5%), mp 183 °C, $[\alpha]_D^{23}$ −65.0° (*c* 1.0, DMF).

Found: C, 57.89; H, 6.84; N, 9.98; S, 7.60%. Calcd for $C_{40}H_{56}O_9N_6S_2$: C, 57.95; H, 6.81; N, 10.14; S, 7.74%.

*Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-N_2H_3 (**II**).* Compound **IId** (45.6 g, 55.0 mmol) was dissolved in DMF (500 ml). The solution was mixed with 100% hydrazine hydrate (55 ml) and stirred at room temperature overnight. The solution was concentrated under reduced pressure to a

syrup, which was triturated in ether. The solid formed was reprecipitated from hot EtOH and ether; wt 42.7 g (93.6%), mp 188.5—191 °C, $[\alpha]_D^{23}$ −54.6° (*c* 1.0, DMF).

Found: C, 55.56; H, 6.96; N, 13.23; S, 7.50%. Calcd for $C_{36}H_{56}O_8N_8S_2 \cdot H_2O$: C, 55.30; H, 6.90; N, 13.23; S, 7.59%.

Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (III). Compound **I** (18.9 g, 20.0 mmol) was dissolved in TFA (40 ml) and stirred at room temperature for 40 min. The solution was concentrated to a solid under reduced pressure. Meanwhile, compound **II** (19.9 g, 24.0 mmol) was suspended in DMF (80 ml), cooled below −20 °C, and mixed with 4.39 M HCl in dioxane (16.4 ml) and isopentyl nitrite (3.4 ml). The suspension gradually became clear, and was stirred at −20 °C for 60 min. Then it was mixed with *N*-methylmorpholine (7.92 ml) and a solution of the above solid in DMF (80 ml). The mixture was stirred at 0—2 °C for 4 d and then concentrated to dryness under reduced pressure. The residue was washed with a mixture of AcOEt and water and then reprecipitated from hot EtOH and ether; wt 27.9 g (85.0%), mp 199 °C (dec), $[\alpha]_D^{18}$ −51.2° (*c* 1.0, DMSO).

Found: C, 60.15; H, 6.66; N, 9.33; S, 7.96%. Calcd for $C_{83}H_{105}O_{16}N_{11}S_4 \cdot H_2O$: C, 60.09; H, 6.50; N, 9.29; S, 7.73%.

Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-OEt (IVa). Z-Glu(OBu^t)-OH·DCHA (51.9 g, 100 mmol) was dissolved with H-Leu-OEt·HCl (23.5 g, 120 mmol) and TEA (2.80 ml) in $CHCl_3$ (1 l) and cooled below −10 °C. The solution was mixed with DCC (22.7 g, 110 mmol) and stirred at the same temperature for 1 h and then at room temperature overnight. The precipitate formed was filtered off and the filtrate was concentrated to an oil under reduced pressure. The oil was dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aq $NaHCO_3$, and water. The washed solution was dried over anhydrous Na_2SO_4 and concentrated to a syrup under reduced pressure. The syrup was dissolved in MeOH (500 ml) and hydrogenated over 5% palladium-charcoal in the presence of HCl (1 equiv). The catalyst was filtered off and the filtrate was concentrated to a solid under reduced pressure. Meanwhile, compound **IIc** (49.2 g, 90.0 mmol) was dissolved in DMF (400 ml) and cooled below −20 °C. The solution was stirred with 4.39 M HCl in dioxane (60 ml) and isopentyl nitrite (13.3 ml) at the same temperature for 30 min and then mixed with *N*-methylmorpholine (29.0 ml) and the above solid. The mixture was stirred at 4 °C for 24 h in a refrigerator. The precipitate formed was filtered off and the filtrate was concentrated to a syrup under reduced pressure. The syrup was solidified in water and the solid was twice recrystallized from EtOH and water and from AcOEt and hexane; wt 64.1 g (82.9%), mp 149—152 °C, $[\alpha]_D^{23}$ −26.7° (*c* 1.2, DMF).

Found: C, 61.32; H, 7.87; N, 6.52; S, 7.29%. Calcd for $C_{44}H_{66}O_9N_4S_2$: C, 61.51; H, 7.74; N, 6.52; S, 7.47%.

Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-N_2H_3 (IV). Compound **IVa** (25.7 g, 30.0 mmol) was dissolved in MeOH (400 ml) and mixed with 100% hydrazine hydrate (30 ml). The solution was stirred at room temperature for 2 d and mixed with water. The resulting precipitate was collected by filtration and reprecipitated from EtOH and ether; wt 19.9 g (78.5%), mp 204 °C (dec), $[\alpha]_D^{23}$ −19.8° (*c* 1.0, DMF).

Found: C, 59.40; H, 7.69; N, 9.74; S, 7.53%. Calcd for $C_{42}H_{64}O_8N_6S_2$: C, 59.69; H, 7.63; N, 9.94; S, 7.59%.

Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (V). Compound **III** (16.4 g, 10 mmol) was dissolved in TFA (35 ml) and stirred at room temperature for 80 min. The solution was concentrated to a solid under reduced pressure. Meanwhile, compound **IVa** (10.1 g, 12.0 mmol) was suspended in DMF (45 ml) and cooled below −20 °C. The suspension was mixed with 4.39 M HCl in dioxane (8.2 ml) and isopentyl nitrite (1.77 ml) and stirred at the same temperature for 60 min, when it gradually became clear. The resulting solution was mixed with *N*-methylmorpholine (4.0 ml) and a solution of the above solid in DMF (40 ml). The mixture was stirred at 0 °C for 4 d in a refrigerator and then concentrated to dryness under reduced pressure. The residue was collected with AcOEt, boiled in hot EtOH, cooled to room temperature, and filtered; wt 20.1 g (85.4%), mp 220 °C (dec), $[\alpha]_D^{18}$ −47.7° (*c* 1.0, DMSO).

Found: C, 60.95; H, 6.64; N, 8.75; S, 8.19%. Calcd for $C_{120}H_{157}O_{22}N_{15}S_6$: C, 61.23; H, 6.72; N, 8.92; S, 8.17%.

Z-Asn-Tyr-OEt (VIa). Z-Asn-OH (20.0 g, 75.0 mmol) was dissolved with H-Tyr-OEt·HCl (20.3 g, 82.5 mmol) and TEA (11.6 ml) in DMF (200 ml). The solution was cooled below −10 °C and mixed with HOBt (11.1 g, 82.5 mmol) and DCC (14.7 g, 71.3 mmol). The mixture was stirred at the same temperature for 1 h and at room temperature overnight. The precipitate formed was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue was washed successively with 0.1 M HCl and water and then reprecipitated from DMF and water; wt 31.6 g (96.9%), mp 170.5—172.5 °C, $[\alpha]_D^{20}$ +12.0° (*c* 1.0, DMSO).

Found: C, 60.45; H, 5.94; N, 9.05%. Calcd for $C_{23}H_{27}O_7N_3$: C, 60.39; H, 5.95; N, 9.19%.

Z-Ser-Asn-Tyr-OEt (VIb). Compound **VIa** (9.15 g, 20.0 mmol) was dissolved in MeOH (150 ml) and hydrogenated over 5% palladium-charcoal at atmospheric pressure in the presence of HCl (1 equiv). The catalyst was filtered off and the filtrate was concentrated to a solid under reduced pressure. Meanwhile, Z-Ser-N_2H_3 (5.60 g, 20.0 mmol) was dissolved in DMF (80 ml) and cooled below −20 °C. The solution was mixed with 4.39 M HCl in dioxane (13.7 ml) and isopentyl nitrite (2.95 ml) and stirred at the same temperature for 30 min. Then, the solution was mixed with *N*-methylmorpholine (6.60 ml) and the above solid, and stirred at 0—2 °C for 2 d. The precipitate formed was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue was crystallized from AcOEt and recrystallized from hot EtOH and AcOEt; wt 8.70 g (80.0%), mp 194—196 °C, $[\alpha]_D^{25}$ +6.2° (*c* 0.9, DMF).

Found: C, 57.22; H, 5.84; N, 10.21%. Calcd for $C_{26}H_{32}O_9N_4$: C, 57.35; H, 5.92; N, 10.29%.

Z-Asn-Ser-OMe (VIc). Z-Asn-OH (13.3 g, 50.0 mmol) was dissolved with H-Ser-OMe·HCl (8.60 g, 55.0 mmol) and *N*-methylmorpholine (6.05 ml) in DMF (150 ml). The solution was cooled below −10 °C and mixed with HOBt (7.4 g, 55 mmol) and DCC (9.8 g, 47.5 mmol). The mixture was stirred at the same temperature for 1 h and at room temperature overnight. The precipitate formed was filtered off and the filtrate was concentrated to crystalline material under reduced pressure. The material was recrystallized from AcOEt; wt 16.1 g (88.0%), mp 197—199 °C, $[\alpha]_D^{25}$ +8.4° (*c* 1.0, DMSO).

Found: C, 52.33; H, 5.68; N, 11.46%. Calcd for $C_{16}H_{21}O_7N_3$: C, 52.31; H, 5.76; N, 11.44%.

Z-Asn-Ser-N_2H_3 (VId). Compound **VIc** (16.0 g, 43.6 mmol) was dissolved in DMF (300 ml), mixed with 100% hydrazine hydrate (22.0 ml), and stirred at room temperature overnight. The crystalline material formed was boiled in hot EtOH and collected after cooling; wt 16.1 g (100%), mp 209 °C (dec), $[\alpha]_D^{23}$ −3.9° (*c* 1.0, DMSO).

Found: C, 47.76; H, 5.88; N, 18.97%. Calcd for $C_{15}H_{21}O_6N_5 \cdot 1/2H_2O$: C, 47.87 H, 5.89; N, 18.61%.

Z-Asn-Ser-Ser-Asn-Tyr-OEt (VIe). Compound **VIb** (8.70 g, 16.0 mmol) was hydrogenated over 5% palladium-charcoal in MeOH (100 ml). The catalyst was filtered off and the filtrate was concentrated to a solid under re-

duced pressure. Meanwhile, compound **VId** (6.50 g, 17.6 mmol) was suspended in DMF (100 ml) and cooled below −20 °C. The suspension was mixed with 4.39M HCl in dioxane (12.0 ml) and isopentyl nitrite (2.60 ml). The resulting solution was stirred at the same temperature for 30 min. The solution was mixed with *N*-methylmorpholine (5.80 ml) and the above solid. The mixture was stirred at 0 °C for 3 d and the precipitate formed was filtered off. The filtrate was concentrated under reduced pressure to a gelatinous solid, which was collected with AcOEt. Half the solid was boiled in hot EtOH and collected after cooling; wt 5.2 g (82.9%), mp 215 °C (dec), $[\alpha]_D^{23}$ +2.1° (*c* 1.0, DMSO).

Found: C, 52.05; H, 5.75; N, 12.81%. Calcd for $C_{33}H_{43}O_{13}N_7 \cdot 1/2H_2O$: C, 52.52; H, 5.88; N, 12.99%.

Z-Asn-Ser-Ser-Asn-Tyr-N_2H_3 (VI). Compound **VIe** (8.7 g, 8.0 mmol) was dissolved with 100% hydrazine hydrate (8.0 ml) in DMF (80 ml) and stirred at room temperature overnight. The resulting precipitate was collected with EtOH. The crystalline material was boiled in hot EtOH and filtered after cooling to room temperature; wt 5.0 g (93.9%), mp 207 °C (dec), $[\alpha]_D^{20}$ −9.2° (*c* 1.1, DMSO).

Found: C, 49.13; H, 5.88; N, 17.09%. Calcd for $C_{31}H_{41}O_{12}N_9 \cdot H_2O$: C, 49.66; H, 5.78; N, 16.81%.

Z-Asn-Ser-Ser-Asn-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys-(MBzl)-Tyr-OBzl (VII). Compound **V** (1.18 g, 0.5 mmol) was dissolved with anisole (0.2 ml) in TFA (10 ml) and stirred at room temperature for 2 h. The solution was concentrated under reduced pressure to a syrup. Meanwhile, compound **VI** (549 mg, 0.75 mmol) was dissolved in a mixture of DMF (5 ml) and DMSO (5 ml) and cooled below −10 °C. The solution was mixed with 4.39 M HCl in dioxane (0.51 ml) and isopentyl nitrite (0.11 ml) and stirred at −10 °C for 60 min. Then, the solution was mixed with *N*-methylmorpholine (1.0 ml) and a solution of the above syrup in DMF (5 ml). The mixture was stirred at 0 °C for 10 d, concentrated under reduced pressure, and then mixed with EtOH. The precipitate formed was collected, boiled in hot EtOH, and collected after cooling to room temperature; wt 1.13 g (78.0%), mp 214 °C (dec), $[\alpha]_D^{18}$ −40.0° (*c* 1.0, DMSO). Amino acid ratio in the acid hydrolysate: Asp, 2.93 (3); Thr, 0.97 (1); Ser, 1.61 (2); Glu, 1.03 (1); Pro, 0.98 (1); Gly, 1.00 (1); Ala, 1.04 (1); 1/2Cys, not determined; Leu, 1.05 (1); Tyr, 1.93 (2).

Found: C, 58.11; H, 6.40; N, 10.41; S, 6.76%. Calcd for $C_{142}H_{178}O_{32}N_{22}S_6 \cdot H_2O$: C, 58.50; H, 6.22; N, 10.57; S, 6.60%.

Deprotection, Air-oxidation and Purification of Toxic Peptides. Compound **VII** (290 mg, 100 μmol) was treated with anhydrous liquid hydrogen fluoride (10 ml) containing anisole (0.87 ml) at 0—2 °C for 60 min. The HF-reagent was removed under reduced pressure and the residue was dissolved in 99% formic acid (3.0 ml). The solution was washed three times with hexane and diluted with water to 5×10^{-5} M concentration of the peptide. The solution was adjusted to pH 8.0 by adding aqueous ammonia and kept at room temperature for 4 d with occasional stirring. Then it was adjusted to pH *ca.* 7.0 by adding glacial acetic acid and divided into two portions, which were each applied to a column (3×25 cm) of DEAE-Sephadex A-25 (acetate form). The adsorbed material was washed with water and eluted with 1500 ml of a linear gradient of 0 to 0.5 M AcOH. Fractions with toxicity were collected and lyophilized. The lyophilized material was purified on a reversed-phase column (Merck, LiChrosorb RP-8 5 μm, 8×300 mm) by HPLC with a linear gradient of 10% to 35% acetonitrile in 0.01 M ammonium acetate (pH 5.7). Fractions of eluate with toxicity were collected and lyophilized; wt 4.90 mg (4.8%).

Aminopeptidase M Digestion of ST_h. Native or synthetic ST_h (*ca.* 100 μg) was dissolved in 0.1 M Tris HCl buffer (100 μl) at pH 7.6 and digested with aminopeptidase M (5 μg) for 30 min at 40 °C. The reaction was stopped by addition of 1 M formic acid (100 μl). The solution was directly applied to a YMC-packed column ODS A-324 (10×300 mm) and the adsorbed material was eluted with a linear-gradient of 10—45% acetonitrile in 0.05% hexafluorobutyric acid (pH 2.51).

Biological Assay. ST activity was assayed in suckling mice of 2—4 d old, as described previously.[1]

Results and Discussion

Synthesis. First the protected linear peptide **VII** with the whole amino acid sequence of ST_h was prepared, as shown in the scheme in Fig. 2. Then the protecting groups of **VII** were removed by treatment with anhydrous liquid hydrogen fluoride.[16] The free linear peptide thus obtained was spontaneously air-

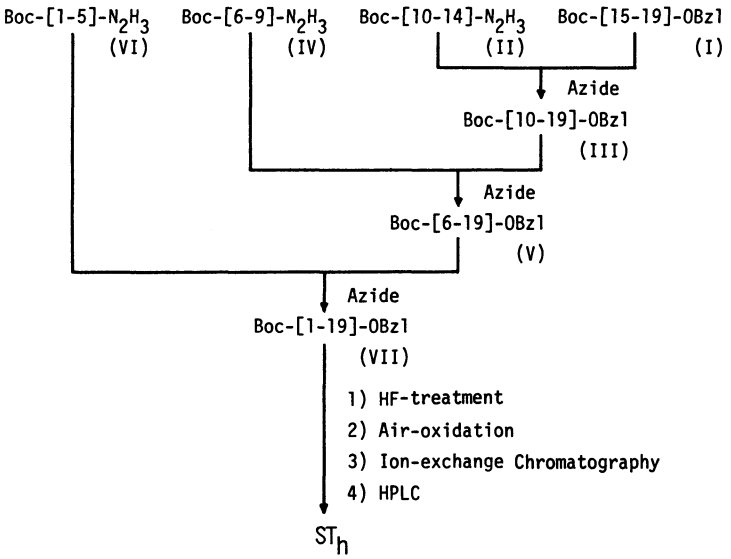

Fig. 2. Scheme for synthesis of ST_h.

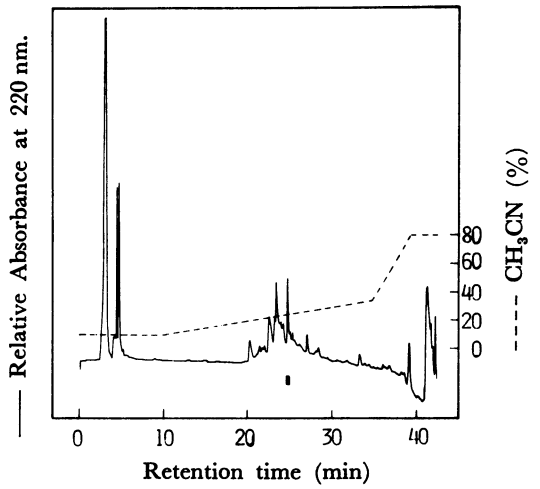

Fig. 3. HPLC profile on a LiChrosorb RP-8 column (5 μm, 4×250 mm) of HF-treated and air-oxidized solution of compound **VII**. The peak marked by a black bar showed toxicity.

oxidized in dilute solution (5×10^{-5}M) at pH 8.0 at room temperature. The air-oxidized solution was examined by high-performance liquid chromatography (HPLC) and results are shown in Fig. 3. Peak fractions were separated and toxicity of each was examined by the fluid accumulation test in suckling mice.[1] High toxicity was observed in the peak fraction shown by a black bar. This fraction was purified by ion-exchange chromatography followed by HPLC. The purified fraction had the same retention time on HPLC as native ST_h under two different conditions, as shown in Fig. 4. The yield of this fraction was 4.8% on the basis of the amount of the protected peptide **VII**. This yield was not surprisingly low, because there are 15 possible modes of formation of ST_h with three disulfide linkages from a linear peptide of ST_h and the environment for formation of disulfide linkages used in this work might be different from that in a biological system. The amino acid composition of synthetic ST_h agreed well with the theoretical one, as shown in Table 1.

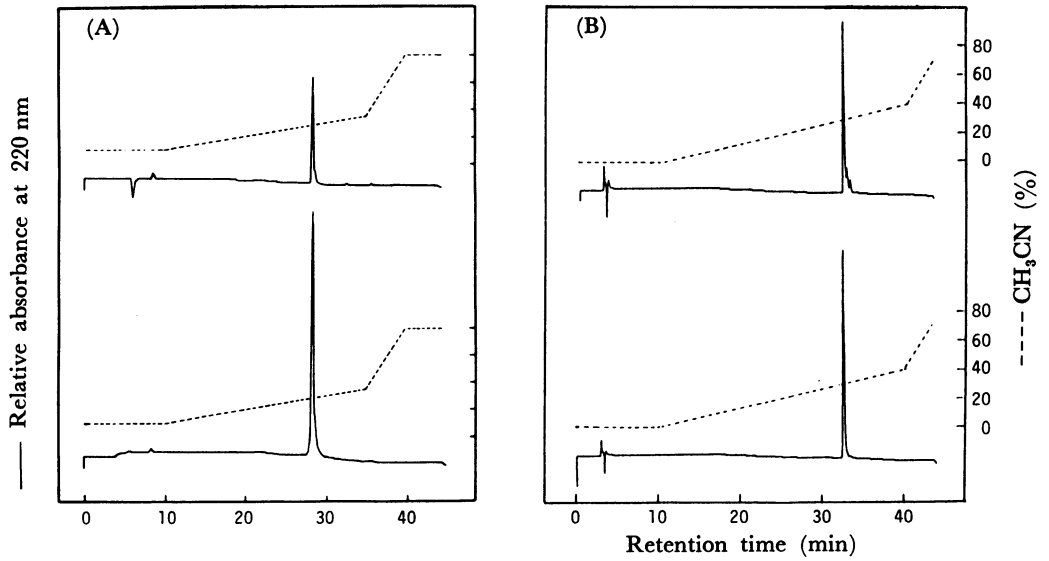

Fig. 4. HPLC profiles of native ST_h (upper) and synthetic ST_h (lower). (A) Column, LiChrosorb RP-8 (5 μm, 8×300 mm); starting solvent, 10% CH_3CN in 0.01 M $AcONH_4$ (pH 5.7). (B) Column, LiChrosorb RP-8 (5 μm, 4×250 mm); starting solvent, 10% CH_3CN in 0.05% TFA (pH 2.35).

Table 1. Amino acid compositions of synthetic and enzymatic-degraded peptides of ST_h[a]

	Synthetic			Native		
	ST_h	ST_h[3-19][b]	ST_h[4-19][b]	ST_h[c]	ST_h[3-19][d]	ST_h[4-19][d]
Asp	2.96(3)	1.98(2)	2.00(2)	2.96(3)	2.00(2)	2.01(2)
Thr	0.93(1)	0.95(1)	0.99(1)	0.96(1)	0.96(1)	1.00(1)
Ser	1.85(2)	0.97(1)	—	1.91(2)	0.97(1)	—
Glu	0.99(1)	1.01(1)	1.01(1)	1.16(1)	1.00(1)	1.04(1)
Pro	1.14(1)	1.14(1)	1.06(1)	1.26(1)	1.07(1)	1.11(1)
Gly	1.03(1)	1.05(1)	1.05(1)	1.21(1)	1.03(1)	1.04(1)
Ala	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
1/2Cys	5.56(6)	5.39(6)	5.50(6)	4.76(6)	5.34(6)	5.36(6)
Leu	1.03(1)	1.04(1)	1.04(1)	1.10(1)	1.05(1)	1.06(1)
Tyr	1.95(2)	2.00(2)	2.00(2)	1.92(2)	2.00(2)	1.99(2)

a) Values were calculated as mol/mol of Ala; numbers in parentheses indicate nearest integer values; b) Preparations from synthetic ST_h; c) Values cited from Ref. 2; d) Preparations from native ST_h.

Biological Activities. The toxicity of synthetic peptide ST_h was examined by the fluid accumulation test in suckling mice. The minimum effective dose of synthetic ST_h was 0.8 ng/100 μl, which was almost the same as that of native ST_h, as shown in Table 2. Furthermore, the toxicity of synthetic ST_h was neutralized not only by homologous anti-native ST_h antiserum but also by heterologous anti-native ST_p antiserum.[7]

Aminopeptidase M Digestion of Native and Synthetic ST_h. To obtain evidence that the synthetic ST_h had the same spacial structure as the native toxin, we treated synthetic and native ST_h with aminopeptidase M and compared the digests by HPLC, as shown in Fig. 5. The profile of the digest of synthetic ST_h was very similar to that of native ST_h. The peak fractions were separated and analyzed as described in the following paper.[13] The two major fractions were identified as ST_h[3-19] and ST_h[4-19], in which two and three N-terminal amino acid residues, respectively, of ST_h were removed. The minor peak fraction between undigested ST_h and ST_h[3-19] was identified as that of peptide ST_h[2-19] lacking the N-terminal Asn residue of ST_h. The same results were obtained on analysis of native ST_h, providing evidence that synthetic ST_h has the same structure as native ST_h.

Proton NMR Spectra. To confirm that synthetic peptide ST_h had the same tertiary structure as native ST_h, including the positions of disulfide linkages, we measured the 500 MHz ^{1}H-NMR spectra of synthetic and native ST_h. As shown in Fig. 6, the proton chemical shifts of synthetic ST_h were superim-

TABLE 2. BIOLOGICAL PROPERTIES OF SYNTHETIC ST_h

	Minimum effective dose (ng/100 μl)*	Neutralization by anti-native ST_h antiserum
Native ST_h	1.0	+
Synthetic ST_h	0.8	+

* Calculated from the recovery of amino acids in acid hydrolysates.

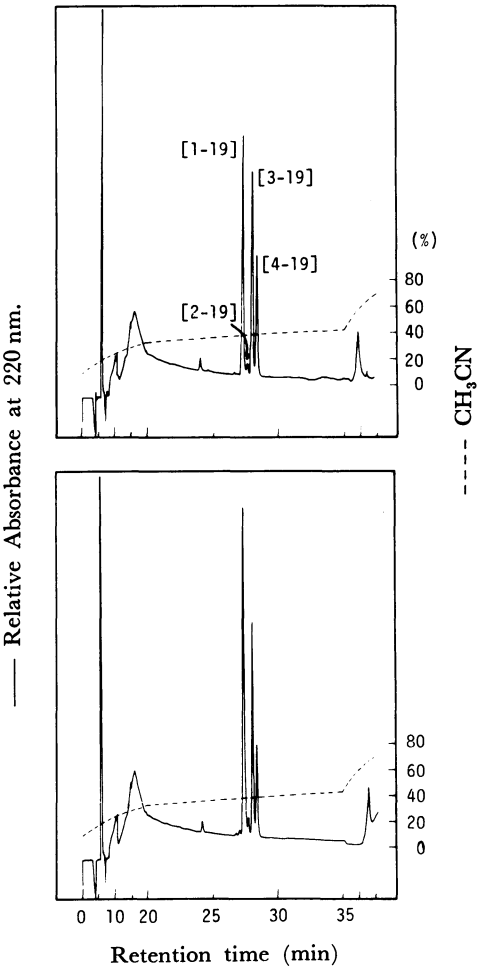

Fig. 5. HPLC profiles on a YMC ODS column (5 μm, 10×300 mm) of aminopeptidase M digests of native ST_h (upper) and synthetic ST_h (lower).

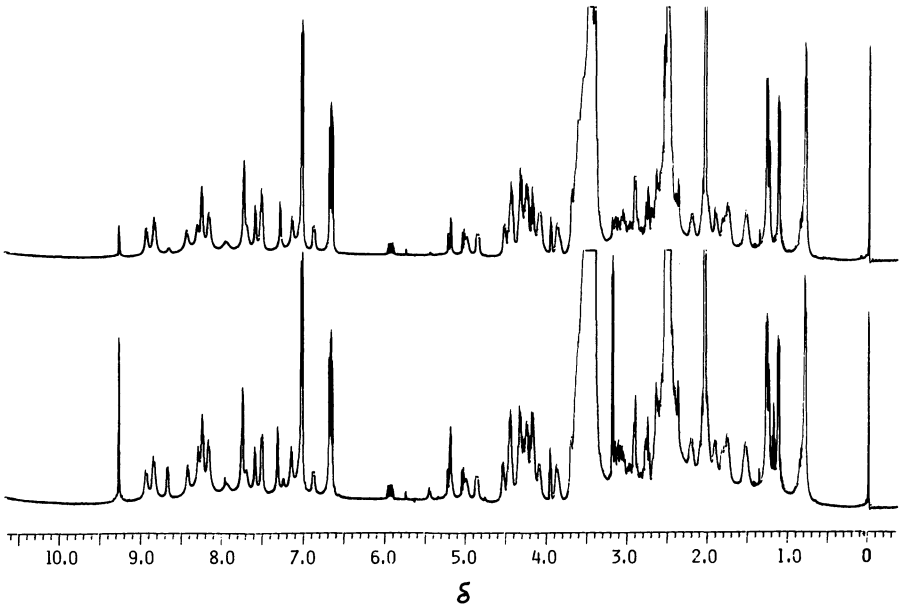

Fig. 6. 500 MHz ^{1}H-NMR spectra of synthetic ST_h (upper) and native ST_h (lower).

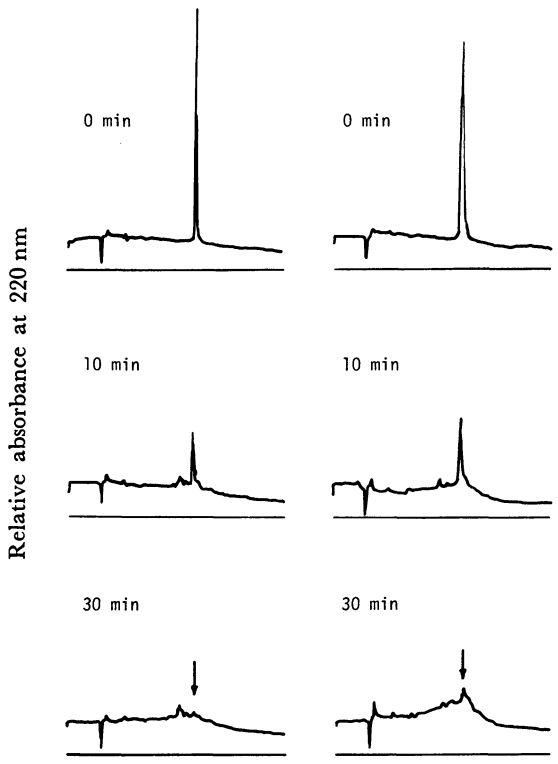

Fig. 7. HPLC profiles on a LiChrosorb RP-8 column (5 μm, 4×250 mm) of heat-treated native ST_h (left column) and synthetic ST_h (right column). The column was developed with a linear-gradient of 10 % to 35 % CH_3CN in 0.01 M $AcONH_4$ (pH 5.7) with increase of 1 %/min of CH_3CN at a flow rate of 0.5 ml/min.

posable on those of native ST_h, indicating that the synthetic peptide had the same three dimensional structure as native toxin.

Heat-stability We examined the heat-stability of synthetic ST_h by HPLC, as described previously.[17] Synthetic ST_h was confirmed to be stable on heating at 100 °C for 30 min like native ST_h, as illustrated in Fig. 7.

Thus, the synthetic ST_h obtained was found to be identical to native ST_h with respect to its physicochemical and biological properties, providing evidence that the amino acid sequence proposed for ST_h, a heat-stable enterotoxin of a human strain SK-1 of enterotoxigenic *Escherichia coli*, is correct. Furthermore, a method was developed for obtaining much toxin. The synthetic ST_h will be used for elucidating the biochemical and biological properties of ST and also for detecting ST-producing bacteria, which are very important epidemiologically.

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References

1) Y. Takeda, T. Takeda, T. Yano, K. Yamamoto, and T. Miwatani, *Infect. Immun.*, **25**, 978 (1979).
2) S. Aimoto, T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Eur. J. Biochem.*, **129**, 257 (1982).
3) T. Takao, T. Hitouji, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *FEBS Lett.*, **152**, 1 (1983).
4) R. Lallier, F. Bernard, M. Gendreau, C. Lazure, N. G. Seidah, M. Chretien, and S. St-Pierre, *Anal. Biochem.*, **127**, 267 (1982).
5) C. Lazure, N. G. Seidah, M. Chretien, R. Lallier, and S. St-Pierre, *Can. J. Biochem. Cell Biol.*, **61**, 287 (1983).
6) B. Ronnberg, T. Wadstrom, and H. Jornvall, *FEBS Lett.*, **155**, 183 (1983).
7) T. Takeda, Y. Takeda, S. Aimoto, T. Takao, H. Ikemura, Y. Shimonishi, and T. Miwatani, *FEMS Microbiol. Lett.*, **20**, 357 (1983).
8) S.-K. Chan and R. A. Giannella, *J. Biol. Chem.*, **256**, 7744 (1981).
9) M. Field, L. H. Graf, Jr., W. J. Laird, and P. L. Smith, *Proc. Natl. Acad. Sci., U.S.A*, **75**, 2800 (1978).
10) J. M. Hughes, F. Murad, B. Chang, and R. L. Guerrant, *Nature*, **271**, 755 (1978).
11) P. M. Newsome, M. N. Burgess, and N. A. Mullan, *Infect. Immun.*, **22**, 290 (1978).
12) R. A. Giannella and K. W. Drake, *Infect. Immun.*, **24**, 19 (1979).
13) H. Ikemura, H. Watanabe, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Bull. Chem. Soc. Jpn.*, **57**, 2550 (1984).
14) S. Yoshimura, M. Miki, H. Ikemura, S. Aimoto, Y. Shimonishi, T. Takeda, Y. Takeda, and T. Miwatani, *Bull. Chem. Soc. Jpn.*, **57**, 125 (1984).
15) E. Schroder, *Ann. Chem.*, **679**, 207 (1964).
16) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
17) S. Aimoto, H. Watanabe, H. Ikemura, Y. Shimonishi, T. Takeda, Y. Takeda, and T. Miwatani, *Biochem. Biophys. Res. Commun.*, **112**, 320 (1983).