

Chemical Synthesis of a Heat-stable Enterotoxin Produced by Enterotoxigenic *Escherichia coli* Strain 18D

Shoko YOSHIMURA, Makoto MIKI, Haruo IKEMURA, Saburo AIMOTO, Yasutsugu SHIMONISHI,*
Tae TAKEDA,[†] Yoshifumi TAKEDA,[†] and Toshio MIWATANI[†]

Institute for Protein Research, Osaka University, Suita, Osaka 565

[†]Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565

(Received July 26, 1983)

Two peptides with the two primary structures of 18 amino acid residues proposed for a heat-stable enterotoxin from enterotoxigenic *Escherichia coli* strain 18D were synthesized by solution methods and their physicochemical and biological properties were compared with those of native toxin. One of these peptides (Asn-Thr-Phe-Tyr-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr) showed the same heat-stability and ¹H-NMR spectrum as those of the native toxin and evoked fluid secretion in suckling mice at a dose of 1.5–2.0 ng, which is similar to the effective dose of native toxin. Moreover, its toxicity was neutralized by antisera against the native toxin. The other peptide (Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Tyr-Pro-Ala-Cys-Ala-Gly-Cys-Asn) showed different physicochemical and biological behaviors from those of the native toxin and exhibited toxic activity at a dose of 50–80 ng, which is about thirty times the effective dose of the native toxin. Moreover, its toxicity was not neutralized by antisera against the native toxin. These observations indicate that the former peptide is identical to native toxin but that the latter peptide differs in properties from the native toxin.

Enterotoxigenic *Escherichia coli* (ETEC) produces two types of enterotoxin, singly or together, which cause acute diarrhea in man and various domestic animals: a high-molecular-weight, heat-labile enterotoxin (LT) and a low-molecular-weight, heat-stable enterotoxin (ST).¹⁾ There are several reports^{2,3)} that LT shows similar immunological properties to those of cholera toxin and exerts its effect by stimulating adenylate cyclase in the epithelial cells of the small intestine. However, little is known about ST, except that it may affect the guanylate cyclase-cyclic GMP system.⁴⁻⁷⁾ This absence of information is mainly because the large quantities of pure ST necessary for biological studies cannot be obtained from a natural source.

In 1981, Chan and Giannella⁸⁾ reported that the amino acid sequence of an ST isolated from the culture supernatant of ETEC strain 18D was as illustrated in Fig. 1. Slightly before their report, So and McCarthy⁹⁾ determined the nucleotide sequence of DNA encoding an ST produced by a bovine strain of ETEC and hence deduced the amino acid sequence of the ST. The amino acid sequence reported by Chan and Giannella⁸⁾ was very similar to that of the C-terminal 18 residues in the amino acid sequence deduced from the nucleotide sequence by So and McCarthy,⁹⁾ but differed in two residues at positions 11 and 18 from the N-terminus. To examine this difference in the two sequences, we¹⁰⁾ reinvestigated the amino acid sequence of an ST (named ST_p in this paper) produced by the strain of ETEC used by Chan and Giannella.⁸⁾ We found that the sequence of ST_p was different from that proposed by Chan and Giannella,⁸⁾ but identical to that deduced from the nucleotide sequence of DNA by So and McCarthy,⁹⁾ as illustrated in Fig. 1. Recently, Lazure *et al.*¹¹⁾ purified an ST produced by a porcine strain F11 (P155) of ETEC and reported the amino acid sequence (Fig. 1). Their sequence was identical to that for ST_p determined by us. Thus, the amino acid sequence of ST_p produced by ETEC strain 18D was established. However, it seemed interesting to confirm the structure of ST_p by chemical synthesis and also to study the effect of amino acid substitution in the sequence of ST_p on its biological

properties. Since it is difficult to obtain much toxin by cultivation of bacteria, chemical synthesis of ST_p also seemed useful in providing a way to obtain the large amounts of toxin necessary for biological studies.

In this paper, we describe the chemical syntheses of two peptides with the amino acid sequences proposed by Chan and Giannella⁸⁾ (designated as ST_p[Tyr,¹¹ Asn¹⁸]) and by us¹⁰⁾ (designated as ST_p) for an ST isolated from strain 18D of ETEC. We also report some physicochemical and biological properties of the synthetic peptides.

Experimental

All chemicals used for preparative experiments were of reagent grade. Chemicals used for analysis were of special grade and solvents were redistilled before use. All amino acids used were of the L-configuration except glycine. DEAE-Sephadex ion-exchanger was purchased from Pharmacia Japan. LiChrosorb RP-8 was obtained from Merck Japan. Melting points were measured by the capillary method and are given as uncorrected values. Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter. Peptide samples were hydrolyzed in 6 M HCl^{††} in evacuated sealed tubes at 105 °C for 24 or 48 h, and amino acids in the hydrolysates were analyzed in a Hitachi KLA-5 analyzer. Proton NMR spectra were recorded with a JEOL GX-500 spectrometer. Samples were dissolved in 0.3 ml of dimethyl-*d*₆ sulfoxide containing 15% acetonitrile-*d*₃ at a concentration of 1–3 mM and measurements were made at 10 °C after addition of 33 μl of D₂O. Chemical shifts were measured from tetramethylsilane added as an internal standard. A large peak caused by remaining H₂O was saturated by the ¹H-homogated decoupling method. Fast atom bombardment (FAB) mass spectra were recorded with a JEOL double-focusing mass spectrometer JMS-HX100 equipped with an FAB ion source, as described previously.¹⁰⁾ Native ST_p was isolated from the culture supernatant of ETEC strain 18D and purified by high-performance liquid chromatography, as described previously.¹⁰⁾ The abbreviations used in this paper are those recommended by the IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations: MBzl, *p*-methylbenzyl; TFA, trifluoroacetic acid; TEA, triethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl

^{††} 1 M = 1 mol dm⁻³.

sulfoxide.

Boc-Cys(MBzl)-Asn-OBzl (Ia-1). Boc-Asn-OBzl¹²⁾ (32.2 g, 0.1 mol) was dissolved in TFA (150 ml) and stirred at room temperature for 30 min. The solution was concentrated under reduced pressure to a syrup, which was mixed with dioxane containing 1 equiv of HCl. The mixture was treated with ether and the resulting precipitate was redissolved with TEA (14.0 ml, 0.1 mol) in DMF (200 ml) and mixed with Boc-Cys(MBzl)-ONSu (46.5 g, 0.11 mol) in DMF (100 ml). The solution was stirred at room temperature for 1 d 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 NaHCO₃ and water. The washed solution was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to a solid, which was crystallized from a mixture of AcOEt, ether and hexane; wt 50.8 g (96.0%), mp 124–125°C, $[\alpha]_D^{23}$ –28.0° (c 1.0, DMF).

Found: C, 60.99; H, 6.74; N, 7.82; S, 5.98%. Calcd for C₂₇H₃₅O₆N₃S: C, 61.23; H, 6.66; N, 7.93; S, 6.04%.

Boc-Gly-Cys(MBzl)-Asn-OBzl (Ia-2). Compound Ia-1 (42.4 g, 80 mmol) was treated with TFA (150 ml) at room temperature for 30 min. The solution was concentrated to dryness under reduced pressure and the residue was mixed with dioxane containing 1 equiv of HCl. The mixture was concentrated under reduced pressure to a syrup, which was solidified in ether. The solid was dissolved in DMF (200 ml) and mixed with TEA (11.2 ml) and Boc-Gly-ONSu (24.0 g, 88 mmol). After 1 d at room temperature, the solution was concentrated to a syrup under reduced pressure. The syrup was dissolved in AcOEt, washed successively with 0.1 M HCl, 5% aq NaHCO₃ and water, and dried over anhydrous Na₂SO₄. The dried solution was concentrated under reduced pressure to a solid, which was recrystallized from a mixture of AcOEt, ether and hexane; wt 40.5 g (86.4%), mp 119.5–120.5°C, $[\alpha]_D^{23}$ –26.8° (c 1.0, DMF). Found: C, 59.17; H, 6.48; N, 9.38; S, 5.38%. Calcd for C₂₉H₃₈O₇N₄S: C, 59.37; H, 6.53; N, 9.55; S, 5.45%.

Boc-Ala-Gly-Cys(MBzl)-Asn-OBzl (Ia-3). Compound Ia-2 (38.1 g, 65 mmol) was treated with TFA (150 ml) at room temperature for 25 min and then concentrated to dryness under reduced pressure. The residue was dissolved with dioxane containing 1 equiv of HCl and precipitated by adding ether. The precipitate was redissolved in DMF (150 ml), mixed with TEA (9.1 ml) and Boc-Ala-ONSu (20.0 g, 70 mmol) and stirred at room temperature for 1 d. The solution was concentrated to dryness under reduced pressure, redissolved in AcOEt, and then washed with 0.1 M HCl, 5% aq NaHCO₃ and water. The washed solution was dried over anhydrous Na₂SO₄ and then concentrated under reduced pressure to a solid, which was recrystallized from AcOEt, ether and hexane; wt 41.5 g (97.0%), mp 148–149°C, $[\alpha]_D^{23}$ –33.8° (c 1.0, DMF).

Found: C, 57.84; H, 6.59; N, 10.65; S, 4.75%. Calcd for C₃₂H₄₃O₈N₅S·0.5H₂O: C, 57.65; H, 6.65; N, 10.51; S, 4.80%.

Boc-Ala-Gly-Cys(MBzl)-Tyr-OBzl (Ib-3). Boc-Gly-Cys(MBzl)-Tyr-OBzl¹³⁾ (41.3 g, 65 mmol) was dissolved in TFA (160 ml) and stirred at room temperature for 35 min. The solution was concentrated under reduced pressure to a syrup, which was treated with 1 equiv of HCl in dioxane and then with ether. The precipitate formed was dissolved in DMF (100 ml) and mixed with TEA (9.1 ml) and Boc-Ala-ONSu (21.0 g, 72 mmol). The solution was stirred at room temperature for 2 h and then concentrated to a syrup under reduced pressure. The syrup was redissolved in AcOEt and washed successively with 0.2 M HCl, 5% aq NaHCO₃ and water. The washed solution was dried over anhydrous Na₂SO₄ and then concentrated under reduced pressure to a semisolid material, which was recrystallized from AcOEt and ether; wt 34.5 g (75.2%), mp 126–128°C, $[\alpha]_D^{18}$ –31.3° (c 1.0, DMF).

Found: C, 62.04; H, 6.53; N, 7.91; S, 4.39%. Calcd for C₃₇H₄₆O₈N₄S·0.5H₂O: C, 62.08; H, 6.62; N, 7.83; S, 4.47%.

Boc-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Asn-OBzl (Ia). Compound Ia-3 (30.0 g, 45 mmol) was dissolved in TFA (150 ml), stirred at room temperature for 20 min, and concentrated to a syrup under reduced pressure. The syrup was treated with dioxane containing 1 equiv of HCl and material was precipitated with ether. The precipitate was dissolved in DMF (150 ml) and mixed with TEA (6.3 ml) and Boc-Cys(MBzl)-ONSu (21.2 g, 50 mmol). The solution was stirred at room temperature for 1 d and then concentrated to dryness under reduced pressure. The residue was dissolved in AcOEt, washed with 0.1 M HCl, 5% aq NaHCO₃ and water, and then dried over anhydrous Na₂SO₄. The dried solution was concentrated under reduced pressure to a gelatinous solid, which was recrystallized from a mixture of MeOH and ether; wt 32.5 g (83.6%), mp 187–188°C, $[\alpha]_D^{23}$ –24.6° (c 1.0, DMF).

Found: C, 59.32; H, 6.56; N, 9.42; S, 7.29%. Calcd for C₄₃H₅₆O₉N₆S₂·0.5H₂O: C, 59.09; H, 6.57; N, 9.62; S, 7.32%.

Boc-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl (Ib). Compound Ib-3 (31.8 g, 45 mmol) was dissolved in TFA (100 ml) and stirred at room temperature for 40 min. The solution was concentrated under reduced pressure to a syrup, which was solidified in ether. The solid was dissolved with TEA (6.3 ml) and Boc-Cys(MBzl)-ONSu (21.1 g, 50 mmol) in DMF (200 ml) and stirred at room temperature for 1 d. The solution was concentrated under reduced pressure to a syrup, which was redissolved in AcOEt and washed with 0.3 M HCl, 5% aq NaHCO₃ and water. The washed solution was dried over anhydrous Na₂SO₄ and concentrated to a syrup under reduced pressure. The material was crystallized from CHCl₃ and ether; wt 39.0 g (94.9%), mp 89–91°C (decomp), $[\alpha]_D^{14}$ –19.6° (c 1.0, DMF).

Found: C, 61.72; H, 6.71; N, 7.60; S, 6.67%. Calcd for C₄₈H₅₉O₉N₅S₂·H₂O: C, 61.85; H, 6.60; N, 7.52; S, 6.87%.

Boc-Tyr-Pro-Ala-OMe (IIa-1). Z-Pro-Ala-OMe¹⁴⁾ (16.7 g, 50 mmol) was dissolved in MeOH (300 ml) containing 1 equiv of HCl and hydrogenated over 5% palladium-charcoal catalyst under atmospheric pressure. The catalyst was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in DMF (20 ml). Meanwhile, Boc-Tyr-N₂H₃ (16.2 g, 55 mmol) was dissolved in DMF (100 ml), cooled below –20°C and mixed with 4.39 M HCl in dioxane (29.5 ml). The solution was stirred with isopentyl nitrite (7.8 ml, 60 mmol) at the same temperature for 20 min and mixed with the above DMF solution and TEA (25.2 ml). The solution was stirred at 0°C for 4 d in a refrigerator. The precipitate formed was removed by filtration and the filtrate was concentrated under reduced pressure to an oil, which was redissolved in AcOEt. The solution was washed successively with 0.5 M HCl, 5% aq NaHCO₃ and water. Then it was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to a syrup, which was crystallized from ether and hexane; wt 21.2 g (91.4%), mp 74–76°C, $[\alpha]_D^{23}$ –33.5° (c 1.0, DMF).

Found: C, 59.89; H, 7.66; N, 8.76%. Calcd for C₂₃H₃₃O₇N₃: C, 59.60; H, 7.18; N, 9.07%.

Boc-Cys(MBzl)-Cys(MBzl)-Tyr-Pro-Ala-OMe (IIa-2).

Compound IIa-1 (18.5 g, 40 mmol) was treated with TFA (80 ml) at room temperature for 25 min and concentrated to dryness under reduced pressure. The residue was dissolved in DMF (80 ml). Meanwhile, Boc-Cys(MBzl)-Cys(MBzl)-N₂H₃¹³⁾ (24.1 g, 44 mmol) was dissolved in DMF (80 ml) and cooled below –20°C. The solution was mixed with 4.39 M HCl in dioxane (23.6 ml) and isopentyl nitrite (6.5 ml, 50 mmol) and stirred at –20°C for 35 min. The solution was mixed with the above solution and TEA (20.1 ml) and then stirred at 0°C for 3 d in a refrigerator. The precipitate formed

was removed by filtration and the filtrate was concentrated to an oil under reduced pressure. The oil was dissolved in AcOEt and washed successively with 0.1M HCl, 5% aq NaHCO₃ and water. The washed solution was dried over anhydrous Na₂SO₄ and then concentrated to an oil under reduced pressure. The oil was purified by chromatography on a column of silica gel using a mixture of AcOEt:benzene (v/v, 1/1) as eluent. The purified material was crystallized from AcOEt and hexane; wt 28.1 g (80.0%) mp 102–103°C, $[\alpha]_D^{25}$ –52.4° (c 0.5, DMF).

Found: C, 61.38; H, 6.95; N, 7.73; S, 7.15%. Calcd for C₄₅H₅₉O₉N₅S₂: C, 61.56; H, 6.77; N, 7.98; S, 7.29%.

Boc-Cys(MBzl)-Cys(MBzl)-Tyr-Pro-Ala-N₂H₃ (**IIa**). Compound **IIa-2** (26.3 g, 30 mmol) was dissolved in MeOH (300 ml), mixed with 100% hydrazine hydrate (60 ml), and then stirred at room temperature for 1 d. The solution was concentrated to dryness under reduced pressure and mixed with water. The precipitate formed was collected and crystallized from MeOH and ether; wt 21.3 g (81.0%), mp 122–123°C, $[\alpha]_D^{25}$ –42.8° (c 0.5, DMF).

Found: C, 59.06; H, 6.46; N, 10.80; S, 7.05%. Calcd for C₄₄H₅₉O₈N₇S₂·H₂O: C, 58.98; H, 6.86; N, 10.94; S, 7.14%.

Boc-Cys(MBzl)-Cys(MBzl)-Tyr-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Asn-OBzl (**IIIa**). Compound **IIIa** (8.65 g, 10.0 mmol) was dissolved in TFA (100 ml) in an ice-bath. The solution was stirred at room temperature for 20 min and concentrated under reduced pressure to a syrup, which was solidified in ether. The solid was dissolved in DMF (30 ml). Meanwhile, compound **IIa-3** (10.52 g, 12.0 mmol) was dissolved in DMF (50 ml), cooled below –20°C and mixed with 4.39M HCl in dioxane (14.7 ml) and isopentyl nitrite (1.82 ml, 14.0 mmol). The solution was stirred below –20°C for 30 min, and then mixed with TEA (10.4 ml) and the solution described above. The mixture was stirred at 0°C for 2 d in a refrigerator. The precipitate formed was removed by filtration and the filtrate was concentrated to a syrup under reduced pressure. The syrup was dissolved in hot AcOEt and kept at room temperature. The precipitate formed was collected and recrystallized from MeOH, DMF and ether; wt 15.1 g (93.8%), mp 173–175°C, $[\alpha]_D^{19}$ –57.6° (c 0.5, DMSO).

Found: C, 60.00; H, 6.06; N, 9.24; S, 7.86%. Calcd for C₈₂H₁₀₃O₁₅N₁₁S₄·2H₂O: C, 59.79; H, 6.55; N, 9.36; S, 7.79%.

Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl (**IIIb**). *Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-N₂H₃*¹³ (14.1 g, 17 mmol) was dissolved in DMF (50 ml), cooled below –20°C and mixed with 4.39M HCl in dioxane (14.7 ml) and then with isopentyl nitrite (2.5 ml, 19 mmol). The solution was stirred at –20°C for 30 min. Meanwhile, compound **IIb** (13.7 g, 15 mmol) was dissolved in TFA (100 ml), stirred at room temperature for 40 min and then concentrated under reduced pressure to a syrup, which was solidified in ether. The solid and TEA (11.2 ml) were added to the azide solution described above and stirred at 0°C for 1 d in a refrigerator. The precipitate was removed by filtration and the filtrate was concentrated to an oil under reduced pressure. The oil was solidified in a mixture of AcOEt and ether and recrystallized repeatedly from EtOH; wt 19.0 g (78.5%), mp 213–214°C, $[\alpha]_D^{14}$ –41.6° (c 0.5, DMSO).

Found: C, 61.18; H, 6.66; N, 9.78; S, 8.09%. Calcd for C₈₂H₁₀₃O₁₅N₁₁S₄: C, 61.14; H, 6.45; N, 9.57; S, 7.95%.

Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-Cys(MBzl)-Cys(MBzl)-Tyr-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Asn-OBzl (**IVa**). Compound **IIIa** (6.44 g, 4.00 mmol) was dissolved with anisole (2.5 ml) in TFA (75 ml) and stirred at room temperature for 60 min. The solution was concentrated under reduced pressure to a syrup, which was solidified in ether. The solid was dissolved in DMF (50 ml). Meanwhile, *Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-N₂H₃*¹³ (4.06 g, 4.80 mmol) was dissolved in DMF (50 ml), cooled below

–20°C, and mixed with 4.39M HCl in dioxane (5.5 ml) and isopentyl nitrite (0.62 g, 5.28 mmol). The solution was stirred at –20––30°C for 30 min and mixed with TEA (4.0 ml) and the solution described above. The mixture was stirred at 2–3°C for 3 d in a refrigerator. The precipitate formed was collected with MeOH, boiled repeatedly in hot EtOH and collected; wt 7.60 g (81.7%), mp 225–227°C, $[\alpha]_D^{19}$ –39.2° (c 0.5, DMF).

Found: C, 60.69; H, 6.82; N, 8.69; S, 8.26%. Calcd for C₁₁₉H₁₅₅O₂₁N₁₅S₆·3H₂O: C, 60.61; H, 6.88; N, 8.91; S, 8.16%.

Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl (**IVb**). Compound **IIIb** (8.05 g, 5.0 mmol) was dissolved in TFA (100 ml). The solution was stirred at room temperature for 30 min and concentrated under reduced pressure to a syrup, which was solidified in ether. The solid was dissolved in DMSO (40 ml). Meanwhile, *Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-N₂H₃*¹³ (5.07 g, 6 mmol) was dissolved in DMF (30 ml), cooled below –20°C, and mixed with 4.39M HCl in dioxane (8.85 ml) and isopentyl nitrite (0.91 ml, 7 mmol). The solution was stirred at –20––30°C for 35 min and mixed with the solution described above and TEA (6.2 ml). The mixture was stirred at 0°C for 2 d in a refrigerator. The precipitate formed was removed by filtration and the filtrate was concentrated to a syrup. The syrup was mixed with 0.2M HCl and the precipitate formed was collected and recrystallized from EtOH; wt 11.0 g (94.8%), mp 236°C (decomp), $[\alpha]_D^{18}$ –51.3° (c 0.32, DMSO).

Found: C, 61.01; H, 6.89; N, 8.90; S, 8.28%. Calcd for C₁₁₉H₁₅₅O₂₁N₁₅S₆·H₂O: C, 61.04; H, 6.76; N, 8.97; S, 8.20%.

Z(OMe)-Phe-Tyr-OEt (**V-1**). *H-Tyr-OEt*·HCl (8.1 g, 33 mmol) was dissolved with TEA (4.6 ml) in DMF (70 ml) and mixed with *Z(OMe)-Phe-ONp* (13.5 g, 30 mmol) in DMF (30 ml). The solution was stirred at room temperature for 6 d and concentrated to dryness under reduced pressure. The residue was dissolved in AcOEt and washed successively with 5% aq NaHCO₃, 0.5M HCl and water. The washed solution was dried over anhydrous Na₂SO₄ and then concentrated under reduced pressure to a syrup, which was crystallized from AcOEt, EtOH and hexane; wt 15.5 g (99.4%), mp 139.5–140.5°C, $[\alpha]_D^{19}$ –9.0° (c 1.0, DMF).

Found: C, 66.36; H, 6.24; N, 5.32%. Calcd for C₂₉H₃₂O₇N₂: C, 66.91; H, 6.20; N, 5.38%.

Boc-Asn-Thr-OEt (**V-2**). *H-Thr-OEt*·HCl (7.34 g, 40 mmol) was dissolved with TEA (5.6 ml) and *Boc-Asn-ONp* (12.4 g, 35 mmol) in DMF (30 ml). The solution was stirred at room temperature for 1 d and concentrated to dryness under reduced pressure. The residue was dissolved in 1-butanol and washed with 5% aq NaHCO₃, 0.1M HCl and water. The washed solution was dried over anhydrous Na₂SO₄ and concentrated to a syrup under reduced pressure. The syrup was crystallized from AcOEt, hexane and ether; wt 8.70 g (69.0%), mp 150–151°C, $[\alpha]_D^{25}$ –15.6° (c 1.0, DMF).

Found: C, 49.85; H, 7.68; N, 11.57%. Calcd for C₁₅H₂₇O₇N₃: C, 49.85; H, 7.53; N, 11.63%.

Boc-Asn-Thr-N₂H₃ (**V-3**). Compound **V-2** (8.67 g, 24.0 mmol) was dissolved in MeOH (100 ml) and mixed with 100% hydrazine hydrate (30 ml). The solution was stirred at room temperature for 1 d and then concentrated to an oil under reduced pressure. The oil was crystallized from MeOH and ether; wt 7.90 g (94.8%), mp 186–188°C (decomp), $[\alpha]_D^{25}$ –25.2° (c 1.0, DMF).

Found: C, 44.10; H, 7.22; N, 20.33%. Calcd for C₁₃H₂₅O₆N₅·0.5H₂O: C, 44.18; H, 7.30; N, 19.82%.

Boc-Asn-Thr-Phe-Tyr-OEt (**V-4**). Compound **V-1** (10.4 g, 20.0 mmol) was dissolved with anisole (5 ml) in TFA (30 ml), stirred at room temperature for 30 min and then

concentrated to a syrup under reduced pressure. The syrup was dissolved in DMF (60 ml). Meanwhile, compound V-3 (7.63 g, 22.0 mmol) was dissolved in DMF (50 ml) and cooled below -20°C . The solution was mixed with 4.39 M HCl in dioxane (14.8 ml) and isopentyl nitrite (3.25 ml, 25 mmol). The solution was stirred below -20°C for 30 min and mixed with the solution described above and TEA (11.9 ml). The mixture was stirred at 0°C for 6 d in a refrigerator. The precipitate formed was removed by filtration and the filtrate was concentrated to a syrup under reduced pressure. The syrup was dissolved in AcOEt and washed successively with 0.5 M HCl, 5% aq NaHCO_3 and water. The washed solution was dried over anhydrous Na_2SO_4 and concentrated to a gelatinous solid under reduced pressure. The solid was crystallized from EtOH and ether; wt 11.3 g (84.3%), mp $139.5\text{--}140.5^{\circ}\text{C}$, $[\alpha]_D^{25} -27.5^{\circ}$ (c 1.0, DMF).

Found: C, 58.03; H, 6.94; N, 10.19%. Calcd for $\text{C}_{33}\text{H}_{45}\text{O}_{10}\text{N}_5 \cdot 0.5\text{H}_2\text{O}$: C, 58.22; H, 6.81; N, 10.29%.

Boc-Asn-Thr-Phe-Tyr-N₂H₃ (V). Compound V-4 (10.1 g, 15.0 mmol) was dissolved in MeOH (150 ml) and mixed with 100% hydrazine hydrate (20 ml). The solution was stirred at room temperature for 1 d and then concentrated to a crystalline solid under reduced pressure. The solid was collected with water and recrystallized from DMF and ether; wt 9.0 g (91.8%), mp $204\text{--}205^{\circ}\text{C}$, $[\alpha]_D^{25} -42.3^{\circ}$ (c 1.0, DMF).

Found: C, 54.80; H, 6.74; N, 14.25%. Calcd for $\text{C}_{31}\text{H}_{43}\text{O}_9\text{N}_7 \cdot \text{H}_2\text{O}$: C, 55.10; H, 6.71; N, 14.51%.

Boc-Asn-Thr-Phe-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-Cys(MBzl)-Cys(MBzl)-Tyr-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Asn-OBzl (VIa). Compound IVa (2.3 g, 1.0 mmol) was dissolved in TFA (20 ml) and stirred at room temperature for 60 min. The solution was concentrated to dryness under reduced pressure and the residue was dissolved in DMF (10 ml). Meanwhile, compound V (0.79 g, 1.2 mmol) was dissolved in DMF (5 ml) and cooled below -20°C . The solution was mixed with 4.39 M HCl in dioxane (1.5 ml) and isopentyl nitrite (0.18 ml, 1.4 mmol) and stirred below -20°C for 30 min. The solution was mixed with the solution described above and TEA (1.1 ml) and stirred at 0°C for 5 d in a refrigerator. The precipitate formed was removed by filtration and the filtrate was concentrated to an oil under reduced pressure. The oil was mixed with 0.1 M HCl and the resulting precipitate was collected with water. The precipitate was crystallized from DMF and ether; wt 2.50 g (89.6%), mp 225°C (decomp), $[\alpha]_D^{25} -34.7^{\circ}$ (c 0.34, DMSO). Amino acid ratio in the acid hydrolysate: Asp, 1.96 (2); Thr, 0.84 (1); Glu, 0.91 (1); Pro, 1.03 (1); Gly, 1.00 (1); Ala, 2.03 (2); Leu, 0.92 (1); Tyr, 1.75 (2); Phe, 0.93 (1).

Found: C, 60.09; H, 6.62; N, 9.77; S, 6.60%. Calcd for $\text{C}_{141}\text{H}_{178}\text{O}_{28}\text{N}_{20}\text{S}_6 \cdot \text{H}_2\text{O}$: C, 60.24; H, 6.45; N, 9.97; S, 6.83%.

Boc-Asn-Thr-Phe-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl (VIb). Compound IVb (4.65 g, 2 mmol) was dissolved in TFA (100 ml) and stirred at room temperature for 45 min. The solution was concentrated under reduced pressure to a syrup, which was solidified in ether. Meanwhile, compound V (1.58 g, 2.4 mmol) was dissolved in DMF (20 ml) and cooled below -20°C . The solution was mixed with 6.72 M HCl in dioxane (1.75 ml) and isopentyl nitrite (0.37 ml) and stirred below -20°C for 35 min. Then, the mixture was stirred at 0°C for 4 d in a refrigerator with TEA (2.0 ml) and the solid prepared as described above and dissolved in DMSO (5 ml). The precipitate was removed by filtration and the filtrate was concentrated to a syrup under reduced pressure. The syrup was precipitated from EtOH and ether; wt 5.0 g (89.6%), mp 243°C (decomp), $[\alpha]_D^{25} -39.4^{\circ}$ (c 0.35, DMF). Amino acid ratio in the acid hydrolysate: Asp, 1.95 (2); Thr, 0.89 (1); Glu, 0.99 (1); Pro, 1.02 (1); Gly, 1.00 (1); Ala, 1.95 (2); Leu, 0.95 (1); Tyr, 1.85 (2); Phe, 0.97 (1).

Found: C, 60.14; H, 6.74; N, 9.77; S, 6.99%. Calcd for $\text{C}_{141}\text{H}_{178}\text{O}_{28}\text{N}_{20}\text{S}_6 \cdot \text{H}_2\text{O}$: C, 60.24; H, 6.45; N, 9.97; S, 6.83%.

HF-treatment and Air-oxidation: Compound VIa or VIb (69.8 mg, 25 μmol) was treated in the presence of anisole (0.1 ml) with anhydrous liquid hydrogen fluoride (3 ml) at 0°C for 60 min. The HF-reagent was evaporated under reduced pressure. The residue was dissolved in 99% formic acid (1 ml) and washed three times with hexane. The solution was diluted to 5×10^{-5} M with distilled water and rapidly adjusted to pH 8.0 with aqueous ammonia. The solution was kept at room temperature for 4 d with occasional stirring. Free thiol became undetectable during this period. The solution was freeze-dried to a powder. The powder was dissolved in a small amount of water, applied to a column (2×90 cm) of Sephadex G-25, and eluted with 0.01 M NH_4HCO_3 . The toxic fractions were collected and lyophilized. The lyophilized material was dissolved in water, applied to a column (2×33 cm or 1.6×25 cm) of DEAE-Sephadex A-25 (acetate form), and washed sufficiently with water. The adsorbed material was eluted with a linear-gradient of 0 to 0.5 M acetic acid. The toxic fractions were collected, lyophilized and purified on a reversed-phase column of HPLC, as described below.

High-performance Liquid Chromatography (HPLC): HPLC was performed on LiChrosorb RP-8 (Merck, 5 μm). Two columns (8×300 mm and 4×250 mm) were packed in our laboratory for semipreparative separation and for analysis, respectively. Both columns were equilibrated with 0.01 M ammonium acetate (pH 5.7) containing 10% acetonitrile and the sample solution was injected onto one of these columns. The column was developed at a flow rate of 1 ml/min with a linear-gradient of 10–30% acetonitrile in 0.01 M ammonium acetate (pH 5.7) with increase in the acetonitrile concentration at a rate of 1%/min.

Biological Assay: ST activity was assayed in suckling mice of 2–4 d old, as described previously.¹⁶⁾ The fluid accumulation ratio of each animal was calculated as the ratio of the weight of the entire intestine to that of the rest of the body. The minimal amount of ST giving a fluid accumulation ratio of over 0.09 was designated as 1 mouse unit, as described previously.¹⁶⁾

Results and Discussion

Isolation of Biologically Active Synthetic Peptides. We recently determined the amino acid sequence of an ST (named ST_h in this paper) isolated from a human strain SK-1 of ETEC.^{16,17)} The sequence was identical with that of ST_p except for the N-terminal three residues and the fourth residue from the C-terminus, as shown in Fig. 1. Interestingly, six half-cystine residues were found at common positions and joined intramolecularly. The disruption of the disulfide bonds in ST_p by reduction destroyed the biological activity of ST.⁸⁾ Furthermore, we found that native ST_p and ST_h have the same potency in a fluid accumulation test using suckling mice^{10,17)} and their toxicity is neutralized by homologous and heterologous anti-ST_p and anti-ST_h antisera.²²⁾ These findings suggested that ST_p and ST_h have the similar secondary structures including disulfide linkages, which play an important part for expression of the toxicity of ST, although the mode of disulfide bond formation has not yet been determined. Moreover, we recently prepared the protected peptide with the amino acid sequence of ST_h and deprotected all the protecting groups from the peptide followed by air-oxidation. We found that the crude air-oxidized product is highly toxic and that the purified

	1	5	10	15	
(a)	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Tyr-Pro-Ala-Cys-Ala-Gly-Cys-Asn				Chan and Giannella ⁸⁾
(b)	---ACC-ACA-TTT-TAC-TGC-TGT-GAA-CTT-TGT-TGT-AAT-CCT-GCC-TGT-GCT-GAA-TGT-TAT				So and McCarthy ⁹⁾
	-Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr				
(c)	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr				Takao <i>et al.</i> ¹⁰⁾
(d)	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr				Lazure <i>et al.</i> ¹¹⁾
(e)	Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr				Aimoto <i>et al.</i> ¹⁷⁾

Fig. 1. Comparison of amino acid sequences proposed for ST: (a) Data reported for an ST isolated from ETEC strain 18D in Ref. 8; (b) deduced from the nucleotide sequence encoding an ST of bovine origin;⁹⁾ (c) data proposed for an ST (=ST_p) isolated from ETEC strain 18D by us;¹⁰⁾ (d) data proposed for an ST isolated from a porcine strain F11(P155) of ETEC in Ref. 11; (e) data proposed for an ST (=ST_h) isolated from a human strain SK-1 of ETEC by us.¹⁷⁾

peptide shows the same biological and physicochemical properties as native ST_h.¹⁸⁾ Hence, we considered that two peptides with the amino acid sequences proposed for ST_p, which have similar amino acid sequences to that of ST_h, can be synthesized by a similar method to that used for synthesis of ST_h. The sequences were first divided into four segments and the protected derivatives of these fragments were prepared separately by conventional solution methods. Then, the segments were coupled sequentially from the C-terminus to the

N-terminus by an azide method¹⁹⁾ to reduce undesirable racemization, as illustrated in Fig. 2. The protected fragments Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-N₂H₃¹³⁾ and V were used for the syntheses of both the two protected peptides VIa and VIb.

Then, all the protecting groups were removed by the HF-method¹⁵⁾ and free peptides were air-oxidized under similar conditions to those described previously.¹⁸⁾ The air-oxidized solutions of peptides were directly applied to a reversed-phase column of HPLC, as illustrated in

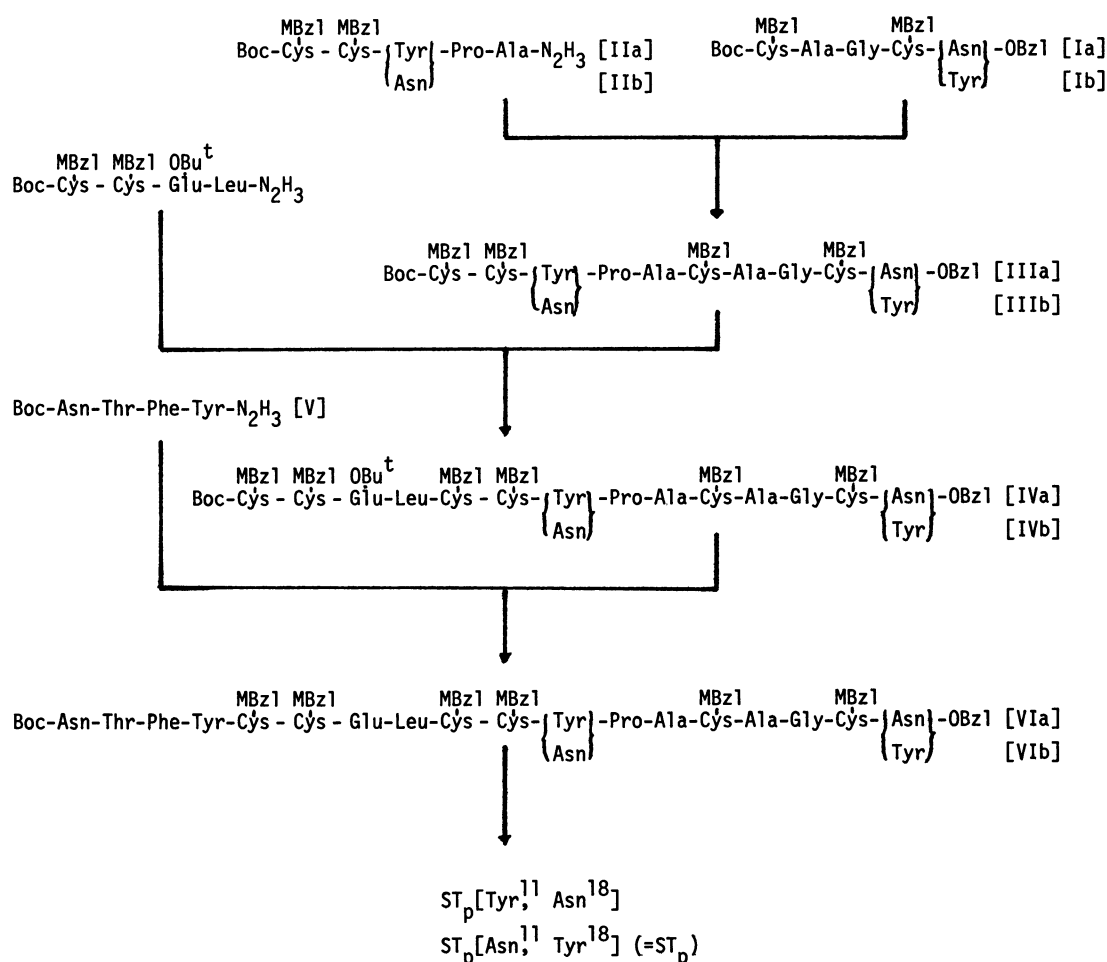


Fig. 2. Scheme for syntheses of ST_p[Tyr¹¹, Asn¹⁸] and ST_p.

Figs. 3A and 4A. Materials in the peaks obtained by HPLC were fractionated and subjected to biological assay. Toxic materials were mainly recovered in the peaks shown by bars in Figs. 3A and 4A, respectively, and the materials in these peaks were compared with native ST_p ,¹⁰⁾ isolated from ETEC strain 18D,⁸⁾ as illustrated in Figs. 3B and 3C, and 4B and 4C, respec-

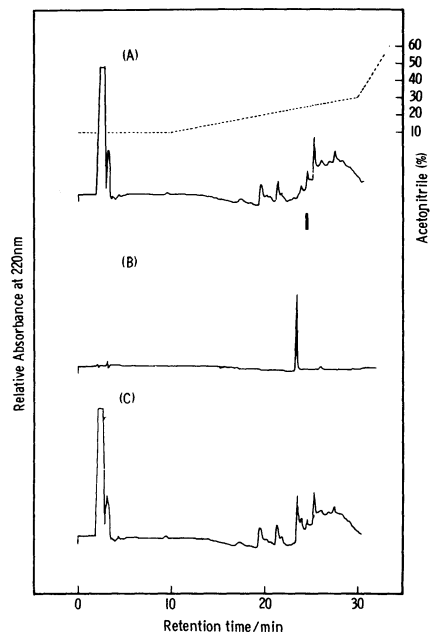


Fig. 3. HPLC profiles on a LiChrosorb RP-8 column ($5\mu\text{m}$, $4\times 250\text{mm}$) of: (A) HF-treated and air-oxidized solution of **VIa**; (B) purified native ST_p ; (C) a mixture of (A) and (B). The toxic activity was found in the peak marked by a bar in (A).

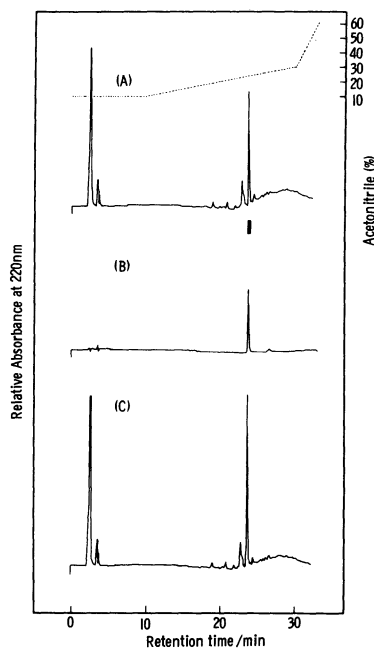


Fig. 4. HPLC profiles on a LiChrosorb RP-8 column ($5\mu\text{m}$, $4\times 250\text{mm}$) of: (A) HF-treated and air-oxidized solution of **VIb**; (B) purified native ST_p ; (C) a mixture of (A) and (B). The main toxic activity was observed in the peak marked by a bar in (A).

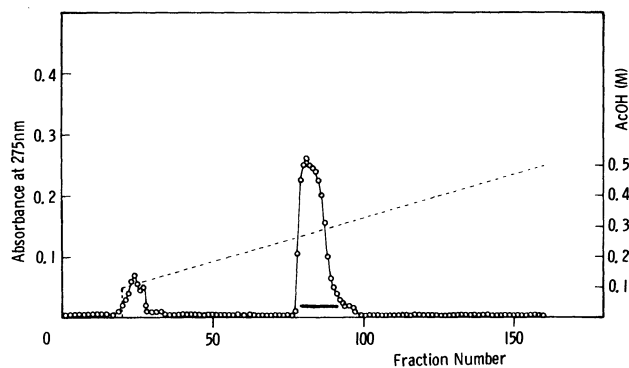


Fig. 5. Chromatography on DEAE-Sephadex A-25 of HF-treated and air-oxidized solution of compound **VIa**. The toxic fraction is shown by a horizontal bar. Column size: $2\times 33\text{cm}$; fractionation size: 10ml/tube ; flow rate: 100ml/h .

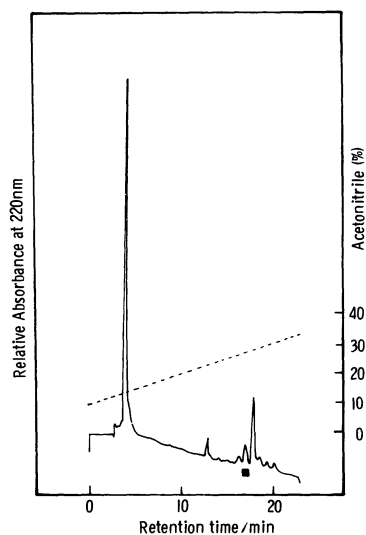


Fig. 6. Semipreparative HPLC of the toxic fraction shown by a horizontal bar in Fig. 5 on a LiChrosorb RP-8 column ($5\mu\text{m}$, $8\times 300\text{mm}$). The toxicity was observed in the peak fraction shown by a horizontal bar.

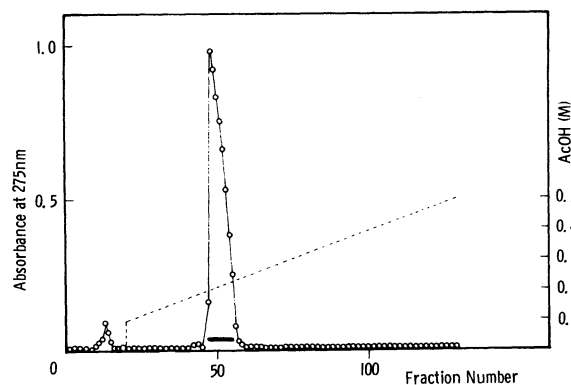


Fig. 7. Chromatography on DEAE-Sephadex A-25 of HF-treated and air-oxidized solution of compound **VIa**. The toxic fraction shown by a horizontal bar was collected. Column size: $1.6\times 25\text{cm}$; fractionation size: 5ml/tube ; flow rate: 35ml/h .

tively. In Fig. 3A, the toxic fraction was eluted later than native ST_p ; the peak fraction eluted at the same retention time as that of native ST_p showed no toxic activity. On the contrary, the toxic peak was eluted with the same retention time as that of native ST_p , in Fig. 4A. Furthermore, the total toxic activity of the air-oxidized solution of compound **VIa** was only one-hundredth of that of compound **VIb**.

The HF-treated and air-oxidized solution of **VIa** was purified on DEAE-Sephadex A-25 and toxic fractions were collected, as shown in Fig. 5. The toxic fractions shown by a horizontal bar were collected and purified further on a semipreparative HPLC column by the reversed-phase mode, as illustrated in Fig. 6. The

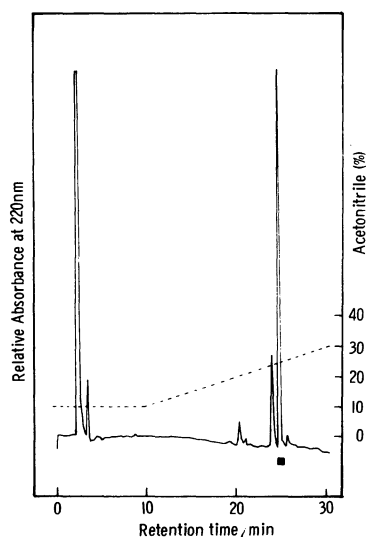


Fig. 8. HPLC profile on a LiChrosorb RP-8 (5 μ m, 8 $\times$ 300 mm) of the fraction shown by a horizontal bar. The toxicity was observed in the peak fraction shown by a horizontal bar.

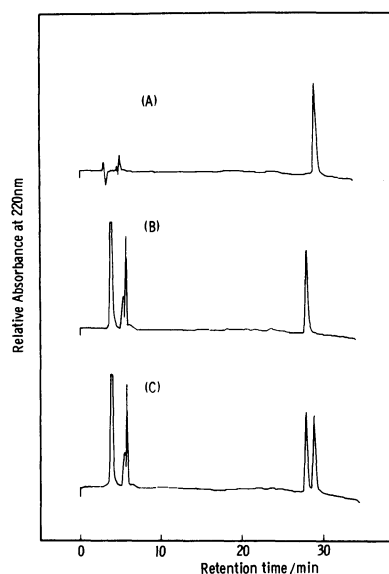


Fig. 9. HPLC profiles of two synthetic preparations on LiChrosorb RP-8 (5 μ m, 8 $\times$ 300 mm); (A) Purified synthetic ST_p [Tyr¹¹, Asn¹⁸]; (B) purified synthetic ST_p ; (C) a mixture of (A) and (B).

purity of the separated fraction shown by a bar was also confirmed by analytical HPLC, as shown in Fig. 9A. The toxic peptide ST_p [Tyr¹¹, Asn¹⁸] was obtained in about 1–2% yield from compound **VIa**.

The HF-treated and air-oxidized solution of compound **VIb** was also purified on DEAE-Sephadex A-25, as illustrated in Fig. 7. The toxic fractions shown by a horizontal bar were combined, lyophilized and purified further on a semipreparative column of reversed-phase HPLC, as shown in Fig. 8. The purity of the toxic fraction was examined by analytical HPLC, as illustrated in Fig. 9B. The yield of purified synthetic peptide ST_p was about 13%, based on the amount of compound **VIb**. As illustrated in Fig. 9C, definite differences were observed between the retention times of the two synthetic peptides ST_p [Tyr¹¹, Asn¹⁸] and ST_p on a reversed-phase column of HPLC. The amino acid compositions of purified synthetic ST_p [Tyr¹¹, Asn¹⁸] and ST_p are summarized in Table 1.

TABLE 1. AMINO ACID COMPOSITIONS OF SYNTHETIC ST_p [Tyr¹¹, Asn¹⁸] AND ST_p [Asn¹¹, Tyr¹⁸]^{a)}

	Synthetic		Native ST_p ^{b)}
	ST_p [Tyr ¹¹ , Asn ¹⁸]	ST_p [Asn ¹¹ , Tyr ¹⁸]	
Asp	2.00	2.04	2.01 (2)
Thr	1.00	0.90	0.99 (1)
Glu	1.11	0.98	1.16 (1)
Pro	1.07	1.01	1.27 (1)
Gly	0.99	1.01	1.16 (1)
Ala	2.00	2.00	2.00 (2)
1/2·Cys	5.25	3.08	4.92 (6)
Leu	0.96	0.96	1.13 (1)
Tyr	1.86	1.87	1.92 (2)
Phe	0.98	0.90	0.99 (1)

a) Values are expressed as molar ratios to alanine.
b) Data cited from Ref. 10.

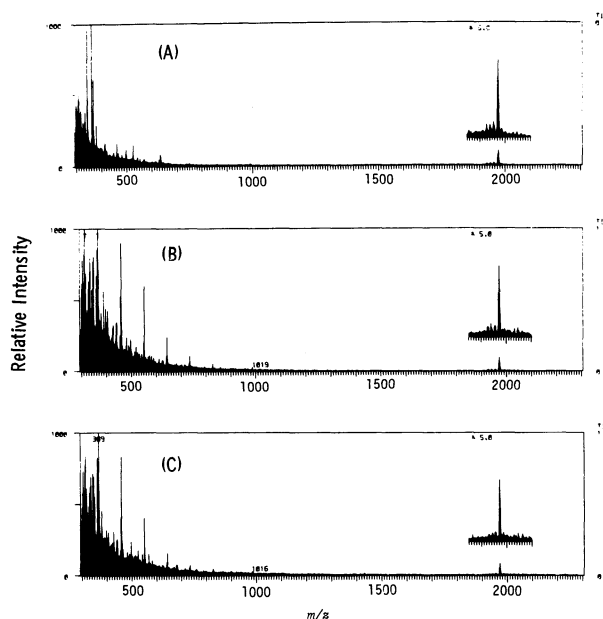


Fig. 10. Positive FAB mass spectra of A) synthetic ST_p [Tyr¹¹, Asn¹⁸]; B) synthetic ST_p ; and C) purified native ST_p .

Physicochemical Properties of Synthetic Peptides $ST_p[Tyr^{11}, Asn^{18}]$ and ST_p . *i) Mass Spectra:* Purified synthetic peptides $ST_p[Tyr^{11}, Asn^{18}]$ and ST_p were subjected to mass measurement by FAB mass spectrometry²⁰⁾ and the mass spectra were compared with that of native toxin as shown in Fig. 10. Like native ST_p , both $ST_p[Tyr^{11}, Asn^{18}]$ and ST_p gave strong signals at $m/z=1971$ (m/z means the ratio of the mass number of an ion m to the number of charges of ion z). The results indicate that the two synthetic $ST_p[Tyr^{11}, Asn^{18}]$ and ST_p both have the same molecular weight (M_r :1970) as native ST_p and are intramolecularly linked by three disulfide bonds, although the positions of the disulfide linkages were not determined.

ii) Proton NMR Spectra: To determine whether either of the synthetic peptides had the same steric features as the native toxin, we compared the 1H -NMR spectra of the two synthetic preparations ($ST_p[Tyr^{11}, Asn^{18}]$ and ST_p) with that of the native toxin. As illustrated in Fig. 11, proton chemical shifts of synthetic ST_p were superimposable on those of the native toxin, but the spectrum of synthetic $ST_p[Tyr^{11}, Asn^{18}]$ was different from that of the native toxin. The results strongly indicate that synthetic ST_p has the same three dimensional structure, including three disulfide linkages, as native toxin.

Comparison of Heat-stabilities by High-performance Liquid Chromatography (HPLC).

Recently we reported a method for testing the heat-stability of ST by HPLC.¹⁸⁾ We found that synthetic ST_h had the same heat-stability as native ST_h and the peak heights on HPLC of synthetic and native ST_h were greatly decreased, although not abolished completely, by heating the compounds at 100°C for 30 min. Using this procedure, we compared the heat-stabilities of synthetic $ST_p[Tyr^{11}, Asn^{18}]$ and ST_p with that of native

ST_p . Figure 12 shows HPLC profiles of synthetic and native peptides heated at 100°C for various periods. Both synthetic peptides showed similar heat-stability to that of native toxin, although synthetic $ST_p[Tyr^{11}, Asn^{18}]$ had lower toxic activity than native ST_p and its toxicity could not be neutralized by antiserum raised against native ST_p , as described below.

Biological Activities of Synthetic Peptides.

Synthetic peptides $ST_p[Tyr^{11}, Asn^{18}]$ and ST_p were examined by the fluid accumulation test in suckling mice.¹⁶⁾ The minimum effective dose of synthetic peptide $ST_p[Tyr^{11}, Asn^{18}]$ was 50–80 ng, which was about 30 times that of native ST_p . On the other hand, the minimum effective dose of synthetic peptide ST_p was 1.5–2 ng, which was almost the same as that of native ST_p .

The immunological properties of synthetic peptides $ST_p[Tyr^{11}, Asn^{18}]$ and ST_p were tested by measuring neutralization of their ST activities with antisera against purified native ST_h ²¹⁾ and ST_p .²²⁾ The toxic activity of synthetic $ST_p[Tyr^{11}, Asn^{18}]$ was not neutralized by either of these antisera, but those of native ST_p and synthetic ST_p were both neutralized by these antisera.

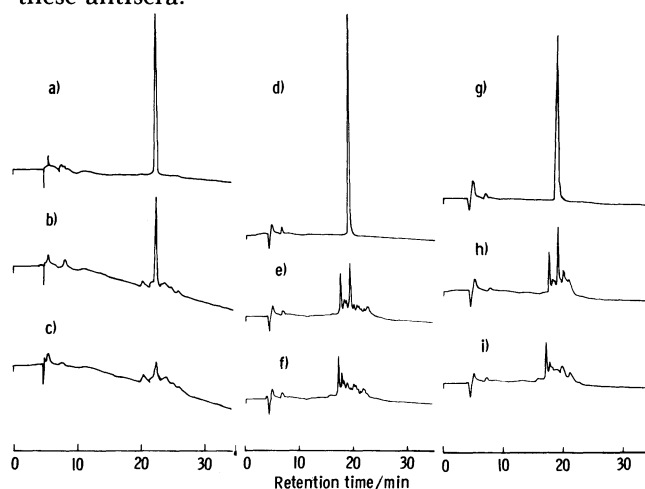


Fig. 12. Comparison of heat-stabilities of synthetic and native ST: (first column) synthetic $ST_p[Tyr^{11}, Asn^{18}]$ a) untreated, b) after 10 min at 100°C, c) after 30 min at 100°C; (second column) synthetic ST_p d) untreated, e) after 10 min at 100°C, f) after 30 min at 100°C; (third column) native ST_p g) untreated, h) after 10 min at 100°C, i) after 30 min at 100°C.

TABLE 2. SUMMARY OF BIOLOGICAL PROPERTIES OF SYNTHETIC PEPTIDES $ST_p[Tyr^{11}, Asn^{18}]$ AND ST_p

	MED ^{a)} ng	Neutralization of ST Activity	
		<i>anti-ST_h</i> Antiserum ^{b)}	<i>anti-ST_p</i> Antiserum ^{c)}
Native ST_p	2.5	+	+
Synthetic $ST_p[Tyr^{11}, Asn^{18}]$	50–80	–	–
Synthetic ST_p	1.5–2.0	+	+

a) Minimum effective dose. b) Antiserum against native ST_h raised in rabbits [21] was used. c) Antiserum against native ST_p raised in rabbits [22] was used.

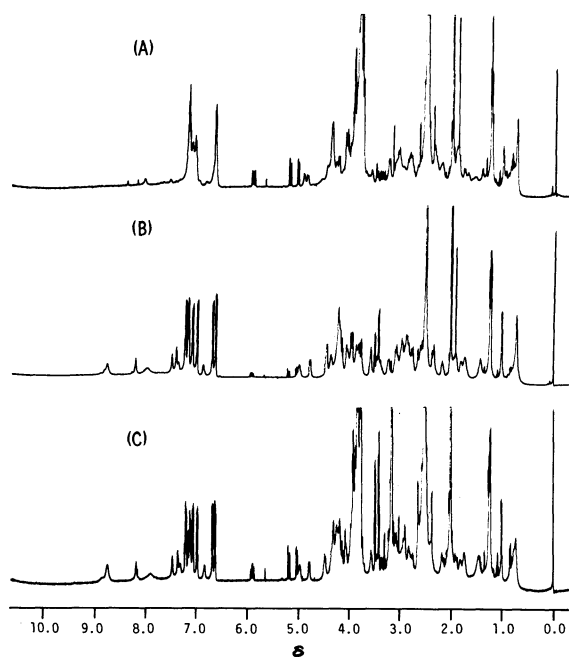


Fig. 11. 500 MHz 1H -NMR spectra of (A) synthetic $ST_p[Tyr^{11}, Asn^{18}]$, (B) synthetic ST_p , (C) native ST_p , measured under the conditions described in the text.

The results, summarized in Table 2, strongly suggest that ST_p[Tyr¹¹, Asn¹⁸] with the amino acid sequence proposed by Chan and Giannella⁸) did not have the same biological and immunological activities as those of native ST_p, whereas ST_p with the sequence proposed by us¹⁰) had similar biological and immunological properties indistinguishable from those of native toxin. The biological and immunological properties of these synthetic peptides will be reported in detail elsewhere.

In this work, two peptides (ST_p[Tyr¹¹, Asn¹⁸] and ST_p) with the amino acid sequences proposed in Ref. 8 and 10 for an ST produced by ETEC strain 18D were synthesized. Analyses of these synthetic peptides gave the following interesting results: 1) Synthetic ST_p was indistinguishable from native ST_p in all physicochemical and biological properties examined, 2) Synthetic ST_p[Tyr¹¹, Asn¹⁸] differed in properties from native ST_p, although Klipstein *et al.*²⁴) recently reported that synthetic ST_p[Tyr¹¹, Asn¹⁸] had similar properties to those of native ST_p, 3) In spite of its difference from native ST_p, synthetic ST_p[Tyr¹¹, Asn¹⁸] had ST activity, although its toxicity was lower than that of native ST_p and could not be neutralized by antiserum against native ST_p. Using the synthetic preparation, it will be possible to examine the structure-activity relationship and the biological and immunological properties of the toxin.

This work was partly supported by a Grant-in-Aid for Scientific Research No. 58122002 from the Ministry of Education, Science and Culture.

References

- 1) H. W. Smith and C. L. Gyles, *J. Med. Microbiol.*, **3**, 387 (1970).
- 2) C. L. Gyles, *J. Infect. Dis.*, **129**, 277 (1974).
- 3) R. L. Guerrant, L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman, *Infect. Immun.*, **10**, 320 (1974).
- 4) M. Field, L. H. Graf, Jr., W. J. Laird, and P. L. Smith, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 2800 (1978).
- 5) J. M. Hughes, F. Murad, B. Chang, and R. L. Guerrant, *Nature*, **271**, 755 (1978).
- 6) P. M. Newsome, M. N. Burgess, and N. A. Mullan, *Infect. Immun.*, **22**, 290 (1978).
- 7) M. C. Rao, S. A. Orellan, M. Field, D. C. Robertson, and R. A. Giannella, *Infect. Immun.*, **33**, 165 (1981).
- 8) S.-K. Chan and R. A. Giannella, *J. Biol. Chem.*, **256**, 7744 (1981).
- 9) M. So and B. J. McCarthy, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 4011 (1980).
- 10) T. Takao, T. Hitouji, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *FEBS Lett.*, **152**, 1 (1983).
- 11) C. Lazure, N. G. Seidah, M. Chretien, R. Lallier, and S. St-Pierre, *Can. J. Biochem. Cell Biol.*, **61**, 287 (1983).
- 12) S.-S. Wang, B. F. Gisin, D. P. Winter, R. Makofske, I. D. Kulesha, C. Tzougraki, and J. Meienhofer, *J. Org. Chem.*, **42**, 1286 (1977).
- 13) H. Ikemura, unpublished.
- 14) E. Schroeder, *Ann. Chem.*, **679**, 207 (1964).
- 15) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
- 16) Y. Takeda, T. Takeda, T. Yano, K. Yamamoto, and T. Miwatani, *Infect. Immun.*, **25**, 978 (1979).
- 17) S. Aimoto, T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Eur. J. Biochem.*, **129**, 257 (1982).
- 18) H. Ikemura, S. Yoshimura, S. Aimoto, Y. Shimonishi, T. Takeda, Y. Takeda, and T. Miwatani, *Chem. Lett.*, **1983**, 101.
- 19) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
- 20) M. Barber, R. S. Bordori, R. D. Sedgwick, and A. N. Tyler, *J. Chem. Soc., Chem. Commun.*, **1981**, 325.
- 21) K. Okamoto, A. Miyama, T. Takeda, Y. Takeda, and T. Miwatani, *FEMS Microbiol. Lett.*, **16**, 85 (1983).
- 22) T. Takeda, Y. Takeda, S. Aimoto, T. Takao, H. Ikemura, Y. Shimonishi, and T. Miwatani, submitted to *FEMS Microbiol. Lett.*, (1983).
- 23) S. Aimoto, H. Watanabe, H. Ikemura, Y. Shimonishi, T. Takeda, Y. Takeda, and T. Miwatani, *Biochem. Biophys. Res. Commun.*, **112**, 320 (1983).
- 24) F. A. Klipstein, R. F. Engert, and R. A. Houghten, *Infect. Immun.*, **39**, 117 (1983).