Synthesis of a Partial Sequence of Proinsulin Using the A-Chain of Natural Insulin. II.[1] Synthesis of a Peptide Corresponding to Positions 53—81 of Bovine Proinsulin

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For the synthesis of bovine proinsulin, the *S*-sulfonate of the A-chain of bovine insulin was elongated by two single-step reactions using Boc–Arg(Tos)–ONSu and Boc–Leu–Glu(OBu^t)–Gly–Pro–Pro–Gln–Lys[Z(2-Cl)]–N_3. The *S*-sulfonate of the Leu–Glu–Gly–Pro–Pro–Gln–Lys[Z(2-Cl)]–Arg–A-chain, corresponding to positions 53—81 of bovine proinsulin, was obtained after removal of the protecting groups. The *S*-sulfonates of the elongated derivatives of the A-chain were shown to be very soluble not only in water, but also in organic solvents such as DMF and DMSO in the presence of water. Therefore, these *S*-sulfonates could react with protected peptides in homogeneous solution. Furthermore, the *S*-sulfonates could easily be purified by ion-exchange chromatography. The *S*-sulfonates of the A-chain and its elongated derivatives are thus useful intermediates for the synthesis of proinsulin.

Weinert *et al.*[2] proposed that the *S*-sulfonate of the des-Gly[1]–A-chain, prepared by the Edman degradation of the A-chain of natural insulin, can be used as starting material for the synthesis of proinsulin. The procedure has the advantage that the des-Gly[1]–A-chain can be coupled by various methods with protected peptides which have a glycine residue at the carboxyl end. However, the procedure is complicated, since the des-Gly[1]–A-chain must be prepared from the A-chain by a laborious degradation process. One of the authors (Y.S.)[1] reported the synthesis of a partial sequence of proinsulin using the *S*-sulfonate of the A-chain[3,4] prepared by sulfitolysis of natural insulin. The procedure merits special attention as a method for direct elongation of the A-chain.

We are working on further elongation of the peptide chain of the A-chain of bovine insulin using our procedure for the synthesis of proinsulin. This paper describes the synthesis of a partial sequence corresponding to positions 53—81 of bovine proinsulin[5] (Fig. 1). First, Boc–Leu–Glu(OBu^t)–Gly–Pro–Pro–Gln–Lys–[Z(2-Cl)]–N_2H_3[6] (X) was synthesized (Fig. 2), two fragments, Boc–Leu–Glu(OBu^t)–Gly–OH (IV) and Boc–Pro–Pro–Gln–Lys[Z(2-Cl)]–OMe (VIII), being synthesized separately and coupled together to give the heptapeptide methyl ester (IX), which was then converted to the corresponding hydrazide (X). The tripeptide (IV) was synthesized by two routes. (a) Z–Glu(OBu^t)–ONSu[7] was coupled with H–Gly–OEt to give Z–Glu(OBu^t)–Gly–OEt (I). The dipeptide ethyl ester (I) was then catalytically hydrogenated. The resulting dipeptide ester was not isolated, but coupled directly with Boc–Leu–ONSu[8] to give Boc–Leu–Glu(OBu^t)–Gly–OEt (II). The resulting tripeptide ester was converted to the corresponding acid (IV) by saponification. (b) Z–Glu(OBu^t)–ONSu[7] was condensed with H–Gly–OBzl to give Z–Glu(OBu^t)–Gly–OBzl (III). The dipeptide benzyl ester (III) was catalytically hydrogenated and the resulting dipeptide was condensed with Boc–Leu–ONSu[8] to give IV.

The synthesis of the tetrapeptide, Boc–Pro–Pro–Gln–Lys[Z(2-Cl)]–OMe (VIII), was started by condensing Boc–Gln–ONp[9] with H–Lys[Z(2-Cl)]–OMe (V) to give Boc–Gln–Lys[Z(2-Cl)]–OMe (VI). VI was then treated with TFA and the resulting dipeptide ester was coupled with Boc–Pro–Pro–ONSu (VII) prepared by condensing Boc–Pro–Pro–OH[10] with HONSu using DCC, to give Boc–Pro–Pro–Gln–Lys[Z(2-Cl)]–OMe (VIII). The tetrapeptide (VIII) thus synthesized was treated with TFA and the resulting tetrapeptide ester was condensed with the tripeptide (IV) using DCC in the presence of HOBt.[11] The isolated Boc–Leu–Glu(OBu^t)–Gly–Pro–Pro–Gln–Lys[Z(2-Cl)]–OMe (IX) was converted to the corresponding hydrazide (X) by the usual method of hydrazinolysis.

The *S*-sulfonate of the Arg–A-chain[2] was synthesized by coupling the *S*-sulfonate of the A-chain with Boc–Arg(Tos)–ONSu and removing the protecting group of the arginine residue of the *S*-sulfonate of the Boc–Arg(Tos)–A-chain with anhydrous liquid hydrogen fluoride.[12] In the preceding work,[1] the *S*-sulfonate of the protected Arg–A-chain, Boc–Arg(NO_2)–A-chain, was used directly without removal of the guanidino protecting group of the arginine residue. However, when Boc–Arg(Tos)–ONSu was used for acylation of the *S*-sulfonate of the A-chain, no quantitative coupling of the A-chain could be attained. Thus, in this work the protecting group of the arginine residue was removed from the *S*-sulfonate of the crude Boc–Arg(Tos)–A-chain, the *S*-sulfonate of Arg–A-chain[2] being purified by chromatography on DEAE-Sephadex A-25[13] (Fig. 3).

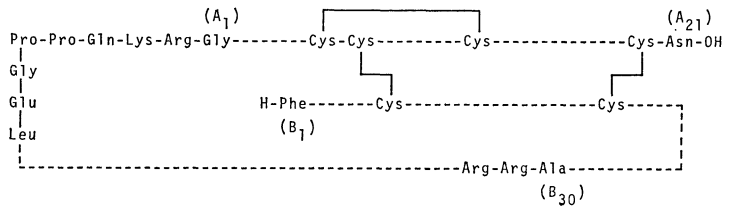

Fig. 1. Structural model of bovine proinsulin.[5]

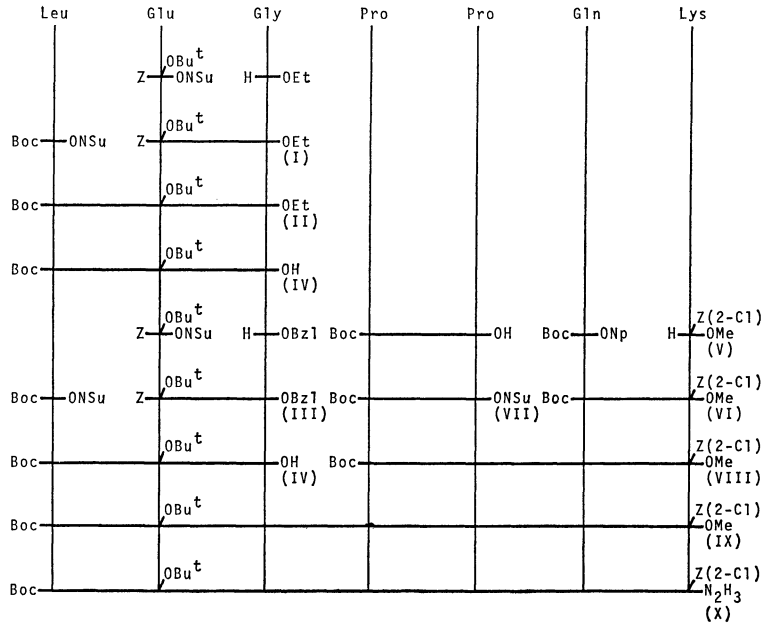

Fig. 2. Scheme for synthesis of protected heptapeptide (sequence corresponding to positions C_{21} to CA_1 in bovine proinsulin).

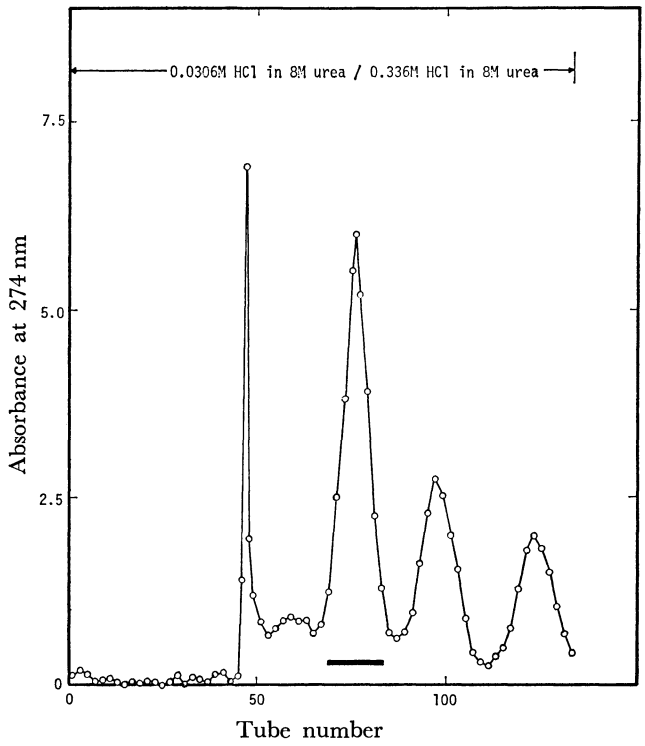

Fig. 3. Chromatogram of the *S*-sulfonate of Arg-A-chain on DEAE-Sephadex A-25 under the conditions described in the text.

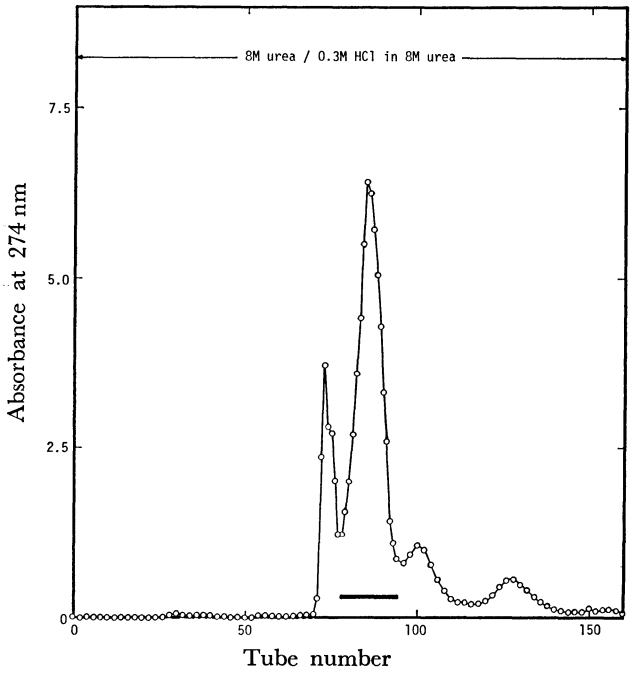

Fig. 4. Chromatogram of the *S*-sulfonate of Leu-Glu-Gly-Pro-Pro-Gln-Lys[Z(2-Cl)]-Arg-A-chain on DEAE-Sephadex A-25 under the conditions described in the text.

The *S*-sulfonate of Arg-A-chain was acylated by protected peptide azides with the sequence, Leu-Glu-Gly-Pro-Pro-Gln-Lys. The *S*-sulfonate of the Arg-A-chain was coupled with Boc-Leu-Glu(OBu^t)-Gly-Pro-Pro-Gln-Lys(Z)-N_3, prepared from the corresponding hydrazide. Considerable cleavage of the N$^\varepsilon$-Z group from the lysine residue occurred when the isolated *S*-sulfonate of the Boc-Leu-Glu(OBu^t)-Gly-Pro-Pro-Gln-Lys(Z)-Arg-A-chain was treated with TFA to remove the Boc group at the amino terminus. The *S*-sulfonate of the Arg-A-chain was then condensed with Boc-Leu-Glu(OBu^t)-Gly-Pro-Pro-Gln-Lys-[Z(2-Cl)]-N_3 protected at the lysine residue with the Z(2-Cl) group,[14] which is more stable than the Z group. The condensation reaction was examined using ratios of 5, 7.5 and 10 equivalents of the protected peptide azide to the *S*-sulfonate of the Arg-A-chain under the conditions described below. The resulting

TABLE 1. AMINO ACID CONTENTS OF DERIVATIVES OF THE A-CHAIN[a]

Amino acid	I[b]	II[b]	III[b]
Lys			1.00 (1)
Arg		1.01 (1)	1.00 (1)
Asp	1.99 (2)	1.96 (2)	1.94 (2)
Ser	1.82 (2)	1.58 (2)	1.79 (2)
Glu	4.35 (4)	4.23 (4)	6.73 (6)
Pro			2.09 (2)
Gly	1.02 (1)	0.99 (1)	1.95 (2)
Ala	1.00 (1)	1.00 (1)	1.00 (1)
Cys	nd[c]	nd[c]	nd[c]
Val	1.59 (2)	1.80 (2)	1.59 (2)
Ile	0.60 (1)	0.84 (1)	0.63 (1)
Leu	2.03 (2)	2.00 (2)	2.96 (3)
Tyr	2.12 (2)	1.94 (2)	1.94 (2)

a) Molar ratios of individual amino acid are shown relative to that of alanine. Numbers in parentheses are theoretical values. b) Compounds I, II, and III are the *S*-sulfonates of the A-chain, Arg-A-chain and Leu-Glu-Gly-Pro-Pro-Gln-Lys[Z(2-Cl)]-Arg-A-chain, respectively. c) Cystine was not determined.

S-sulfonate of Boc–Leu–Glu(OBu^t)–Gly–Pro–Pro–Gln–Lys[Z(2-Cl)]–Arg–A-chain was treated with TFA and the *S*-sulfonate obtained was subjected to chromatography on DEAE-Sephadex A-25[13] (Fig. 4). The best result was obtained with use of 7.5 equivalents of the protected peptide azide.

The N-terminal amino acid residues of the purified *S*-sulfonates of the Arg- and Leu–Glu–Gly–Pro–Pro–Gln–Lys[Z(2-Cl)]–Arg–A-chains were detected as arginine and leucine, respectively, by dinitrophenylation.[15] Amino acid analyses of acid hydrolysates of the purified *S*-sulfonates gave satisfactory results (Table 1).

Thus, the *S*-sulfonate of the A-chain of bovine insulin was elongated by reactions with acylamino acid active ester and acylpeptide azides. During the procedure it became clear that the *S*-sulfonates of the elongated derivatives of the A-chain thus synthesized are very soluble in buffers and organic solvents such as DMF and DMSO containing water, and can react with acylpeptide in homogeneous solution and be purified by chromatography on ion-exchange resin. These *S*-sulfonates seem to be useful intermediates for synthesis of proinsulin. Results on the further elongation of the derivatives obtained will be described in subsequent papers.[16,17]

Experimental

Crystalline bovine insulin was obtained from the Chemical Research Laboratories, Takeda Chemical Industries, Ltd. (Osaka), by courtesy of Dr. M. Fujino. Sephadex was purchased from Pharmacia Co. (Uppsala). All chemicals were of reagent grade and used without further purification. Thin layer chromatography was performed on silica gel G (Merck) using the following solvent systems (volume ratios); $CHCl_3$: MeOH : AcOH (95 : 5 : 3), *n*-BuOH : AcOH : H_2O (4 : 1 : 1), $CHCl_3$: MeOH : AcOH : H_2O (10 : 10 : 1 : 10, lower phase). Peptide samples were hydrolyzed in 6 M HCl in sealed tubes at 105 °C for 48 h, amino acids in the hydrolysates being analyzed in a Hitachi KLA-5 analyzer by the method of Moore *et al.*[18] Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter. Melting points were measured by the capillary method and are given as uncorrected values. 1 M=1 mol dm^{-3}.

Z–Glu(OBu^t)–Gly–OEt (I). H–Gly–OEt·HCl (7.50 g, 53.6 mmol) and TEA (7.6 ml) were dissolved in a mixture of DMF (100 ml) and $CHCl_3$ (100 ml), and then Z–Glu (OBu^t)–ONSu[7] (19.5 g, 44.9 mmol) was added to the solution. The solution was stirred at room temperature for a day and then concentrated to a syrup *in vacuo*. The syrup was dissolved in AcOEt, and the solution was washed successively with 0.1 M HCl and 5% aqueous $NaHCO_3$ and then dried over Na_2SO_4. The dried solution was concentrated to a syrup *in vacuo*; crystals formed on storage in a refrigerator. The crude crystals were collected by filtration with hexane; wt 18.2 g (95.8%). The crude material was recrystallized from AcOEt and hexane; wt 15.6 g (82.1%), mp 76.5—78 °C, $[\alpha]_D^{20}$ −7.3° (*c* 2.0, DMF).

Found: C, 59.63; H, 7.15; N, 6.78%. Calcd for $C_{21}H_{30}O_7N_2$: C, 59.70; H, 7.16; N, 6.63%.

Boc–Leu–Glu(OBu^t)–Gly–OEt (II). Compound I (8.45 g, 20.0 mmol) was dissolved in EtOH (100 ml) and hydrogenated over 5% palladium-charcoal catalyst under atmospheric pressure for 2.5 h. The catalyst was filtered off and washed with EtOH. The filtrate and washings were combined and concentrated to a solid under reduced pressure. The solid was dissolved with Boc–Leu–ONSu[8] (7.20 g, 22.0 mmol) in a mixture of DMF (50 ml) and CH_2Cl_2 (50 ml). The solution was stirred at room temperature for 1 h and then concentrated to a semi-solid residue. The residue was dissolved in $CHCl_3$ and the solution was washed successively with 0.1 M HCl, 5% aqueous $NaHCO_3$ and water. The washed solution was dried and then concentrated to a solid. The solid was collected by filtration with hexane; wt 9.00 g. The crude material was recrystallized from AcOEt and hexane; wt 6.70 g (67.0%), mp 128—130 °C, $[\alpha]_D^{20}$ −22.6° (*c* 1.0, DMF).

Found: C, 57.37; H, 8.94; N, 8.33%. Calcd for $C_{24}H_{43}O_8N_3$: C, 57.46; H, 8.64; N, 8.38%.

Z–Glu(OBu^t)–Gly–OBzl (III). H–Gly–OBzl·TosOH (12.1 g, 35.9 mmol) was suspended in THF (200 ml) and mixed with TEA (5.0 ml). The clear solution thus obtained was mixed with Z-Glu(OBu^t)-ONSu[7] (13.0 g, 30.0 mmol). The mixture was stirred at room temperature for 4 d and then concentrated to a syrupy residue, and the residue was dissolved in AcOEt. The solution was washed successively with 0.1 M HCl, 5% aqueous $NaHCO_3$ and water. The washed solution was dried and then concentrated to an oil. The oil was triturated in AcOEt and hexane; wt 13.5 g. The crude product was recrystallized from AcOEt and hexane; wt 12.0 g (82.2%), mp 59.5—61.5 °C, $[\alpha]_D^{19}$ −6.0° (*c* 2.0, DMF). (lit,[19] mp 45—47 °C, $[\alpha]_D^{25}$ −15.9° (*c* 1.01, MeOH)).

Found: C, 64.46; H, 6.65; N, 5.82%. Calcd for $C_{26}H_{32}O_7N_2$: C, 64.45; H, 6.66; N, 5.78%.

Boc–Leu–Glu(OBu^t)–Gly–OH·DCHA (IV). a) Compound II (6.52 g, 13.0 mmol) was dissolved in dioxane (100 ml). To the solution was added 1 M NaOH (15.6 ml) dropwise under cooling with ice-water. The solution was stirred at room temperature for 2 h and then neutralized with 1 M HCl and the dioxane was evaporated off. The residue was mixed with 5% aqueous $NaHCO_3$ and AcOEt. The aqueous layer was separated from the organic layer and acidified with 1 M HCl. The product was extracted several times with AcOEt. The extracts were combined,

dried and concentrated to a syrup; wt *ca.* 5.5 g.

b) Compound III (12.1 g, 24.9 mmol) was dissolved in MeOH (300 ml) and hydrogenated over 5% palladium-charcoal catalyst at 35 °C for 2.5 h. The catalyst was filtered off and the filtrate was concentrated to a solid. The solid was mixed with Boc–Leu–ONSu[8] (9.00 g, 27.4 mmol) and TEA (3.5 ml) in $CHCl_3$ (200 ml), and then stirred at room temperature for 3 d. The reaction mixture was concentrated to a residue, which was dissolved in AcOEt. The solution was washed with 0.1 M HCl and water, dried and concentrated to a syrupy residue. The residue was dissolved with DCHA (5.0 g) in AcOEt. Hexane was added to the soultion and the precipitate formed was collected by filtration; wt 16.0 g. The crude product was crystallized from AcOEt and hexane; wt 13.5 g (82.8%), mp 137—140 °C, $[\alpha]_D^{16.5}$ −13.5° (*c* 1.0, DMF).

Found: C, 62.04; H, 9.78; N, 8.42%. Calcd for $C_{34}H_{62}O_8N_4$: C, 62.35; H, 9.54; N, 8.56%.

H–Lys[Z(2-Cl)]–OMe·HCl (V). Thionyl chloride (8.7 g) was added dropwise in MeOH below −10 °C. To the solution was added H–Lys[Z(2-Cl)]–OH[14] (22.0 g, 69.8 mmol). The mixture was stirred at room temperature for 3 d and concentrated to a syrup, which was crystallized from MeOH and ether; wt 22.5 g. The crude material was recrystallized from MeOH and ether; wt 18.0 g (70.6%), mp 119—121 °C, $[\alpha]_D^{19}$ +12.0° (*c* 2.0, DMF).

Found: C, 49.19; H, 6.04; N, 7.90; Cl, 19.64%. Calcd for $C_{15}H_{22}O_4N_2Cl_2$: C, 49.32; H, 6.07; N, 7.67; Cl, 19.41%.

Boc–Gln–Lys[Z(2-Cl)]–OMe (VI). Compound V (1.80 g, 4.93 mmol) was dissolved in DMF (30 ml) and mixed with TEA (0.7 ml). The mixture was stirred with Boc–Gln–ONp[9] (2.00 g, 5.45 mmol) at room temperature for 1 d and then concentrated to dryness. The residue was dissolved in AcOEt and washed successively with 0.1 M HCl, 1 M Na_2CO_3 and water. The washed solution was dried and then concentrated to a syrup, which was crystallized from AcOEt and hexane; wt 2.33 g. The crude material was recrystallized from AcOEt and hexane; wt 2.30 g (83.6%), mp 95.5—97.5 °C, $[\alpha]_D^{19}$ −7.6° (*c* 2.0, DMF).

Found: C, 53.91; H, 6.93; N, 9.87; Cl, 6.40%. Calcd for $C_{25}H_{37}O_8N_4Cl$: C, 53.90; H, 6.70; N, 10.06; Cl, 6.37%.

Boc–Pro–Pro–ONSu (VII). A solution of Boc–Pro–Pro–OH[10] (25.0 g, 80.1 mmol) and HONSu (10.0 g, 87.0 mmol) in THF (400 ml) was cooled to −10—−20 °C, DCC (16.5 g, 80.1 mmol) then being added. The solution was stirred at the same temperature for 1 h and at room temperature overnight and the resulting precipitate was filtered off. The filtrate was concentrated to a syrup, which was dissolved in AcOEt. The solution was washed with 5% aqueous $NaHCO_3$ and water saturated with NaCl, dried, and concentrated to a syrup, which was crystallized from AcOEt and hexane; wt 24.6 g. The crude material was recrystallized from 2-propanol; wt 23.5 g (71.6%), mp 147.5—149.5 °C, $[\alpha]_D^{19}$ −90.0° (*c* 2.0, DMF).

Found: C, 55.72; H, 6.63; N, 10.33%. Calcd for $C_{19}H_{27}O_7N_3$: C, 55.73; H, 6.65; N, 10.26%.

Boc–Pro–Pro–Gln–Lys[Z(2-Cl)]–OMe (VIII). Compound VI (11.1 g, 19.9 mmol) was dissolved in TFA (30 ml). The solution was stirred at room temperature for 40 min and concentrated to a syrup, which was washed with ether and dried over NaOH. The dried material was dissolved in THF (100 ml), mixed successively with TEA (3.4 ml) and compound VII (9.0 g, 22.0 mmol), and stirred at room temperature for 1 d. The mixture was stirred with TEA (1.2 ml) and compound VII (4.0 g, 9.8 mmol) at room temperature for 2 d and then concentrated to an oil. The oil was dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aqueous $NaHCO_3$ and water. The washed solution was dried and concentrated to a syrup, The syrup was solidified in a mixture of AcOEt and hexane, and reprecipitated from AcOEt and hexane; wt 12.5 g (83.9%), mp 70.5—72.5 °C, $[\alpha]_D^{16.5}$ −55.2° (*c* 1.0, DMF).

Found: C, 55,65; H, 6.96; N, 10.88; Cl, 4.63%. Calcd for $C_{35}H_{51}O_{10}N_6Cl$: C, 55.95; H, 6.84; N, 11.19; Cl, 4.72%.

Boc–Leu–Glu(OBu^t)–Gly–Pro–Pro–Gln–Lys[Z(2-Cl)]–OMe (IX). Compound VIII (2.25 g, 3.00 mmol) was dissolved in TFA (7 ml). The solution was stirred at room temperature for 60 min and then concentrated to a syrup. The syrup was solidified in ether and dried over NaOH. The dried solid was dissolved in THF (80 ml) with Boc–Leu–Glu(OBu^t)–Gly–OH, which was prepared from compound IV (2.00 g, 3.05 mmol). The solution was mixed with *N*-methylmorpholine (0.64 ml) and cooled to 0 °C. To the solution were added HOBt (0.60 g, 4.44 mmol) and DCC (0.68 g, 3.30 mmol). The mixture was stirred at 0 °C for 1 h and at room temperature for 1 d and then HOBt (0.2 g) and DCC (0.3 g) were added. The precipitate formed was filtered off after 3 d, and the filtrate was concentrated to dryness. The residue was dissolved in AcOEt, washed successively with 0.1 M HCl, 5% aqueous $NaHCO_3$ and water, and concentrated to a residue, which was crystallized from AcOEt and hexane; wt 2.81 g. The crude product was recrystallized from AcOEt and hexane; wt 2.57 g (77.4%), mp 96—98 °C, $[\alpha]_D^{16.5}$ −53.6° (*c* 1.0, DMF). Amino acid ratio in the acid hydrolysate: Glu, 2.22 (2); Gly, 1.00 (1); Pro, 2.21 (2); Leu, 0.99 (1); Lys, 1.10 (1).

Found: C, 56.21; H, 7.49; N, 11.19; Cl, 3.23%. Calcd for $C_{52}H_{80}O_{15}N_9Cl$: C, 56.43; H, 7.29; N, 11.39; Cl, 3.20%.

Boc–Leu–Glu(OBu^t)–Gly–Pro–Pro–Gln–Lys[Z(2-Cl)]–N_2H_3 (X). Compound IX (5.53 g, 5.00 mmol) was dissolved in MeOH (50 ml) and mixed with $NH_2NH_2 \cdot H_2O$ (2.5 g). The solution was stirred at room temperature for 3 d and concentrated to a residue, which was collected with ether; wt 5.76 g. The crude material was reprecipitated from $CHCl_3$ and ether; wt 3.82 g (67.0%), mp 114.5—117.5 °C.

Found: C, 53.83; H, 7.36; N, 13.61; Cl, 3.66%. Calcd for $C_{51}H_{80}O_{14}N_{11}Cl \cdot 2H_2O$: C, 53.79; H, 7.44; N, 13.18; Cl, 3.11%.

S-Sulfonate of the A-Chain. The *S*-sulfonate of the A-chain was prepared by the method of Zahn and Drechsel[4] with some modifications. Bovine insulin (1.0 g) was suspended in a solution of 0.05 M NaOAc (36 ml) and dioxane (26 ml). To the suspension was added EDTA·2Na (0.08 g) to give a clear solution, which was adjusted to pH 7.6 with NaOH. Then a solution (10.4 ml) containing Na_2SO_3 (2.3 g) at pH 7.6 and a solution (10.4 ml) containing $Na_2S_4O_6$ (5 g) at pH 7.6 were added at 37—38 °C alternately over a period of 30 min. The *S*-sulfonate of the A-chain was separated from the *S*-sulfonate of the B-chain as follows: The solution was stirred at 37—38 °C for 2 h and at room temperature for 1 d. The resulting precipitate, mainly consisting of the *S*-sulfonate of the B-chain, was removed by centrifugation, and the upper phase, mainly containing the *S*-sulfonate of the A-chain, was desalted on a column of Sephadex G-25 using 0.1 M NH_4HCO_3. The eluate with absorption at 274 nm was lyophilized; wt 493 mg. The lyophilized powder was charged on a column of Dowex 50×2 (1.7×11 cm) equilibrated with 10% HCOOH and 10% AcOH in 8 M urea, and the *S*-sulfonate of the A-chain was eluted with the same buffer. The eluate with absorption at 274 nm was desalted on a column of Sephadex G-25 and lyophilized; wt 361.1 mg. The *S*-sulfonate of the A-chain was also recovered from the precipitate consisting mainly of the *S*-sulfonate of the B-chain during purification of the

latter on a column of carboxymethylcellulose;[20] wt 88.1 mg. For amino acid ratio in the acid hydrolystae, see Table 1.

S-Sulfonate of Arg-A-Chain. *a) Boc-Arg(Tos)-ONSu.* Boc-Arg(Tos)-OH[21] (8.75 g, 20.4 mmol) was dissolved with HONSu (2.80 g, 24.3 mmol) and DCC (4.10 g, 19.9 mmol) in THF (350 ml) below 0 °C. The solution was stirred at the same temperature for 1 h and at room temperature for 3.5 h. The precipitate formed was filtered off and the filtrate was concentrated to a syrup, which was crystallized from AcOEt; wt 6.70 g (62.6%). Although the product contained some impurities, it was used without further purification.

b) S-Sulfonate of Arg-A-Chain. The *S*-sulfonate of the A-chain (546 mg) was dissolved in a mixture of DMF (3 ml) and DMSO (3 ml). The solution was mixed with TEA (0.14 ml) and then with a solution of Boc-Arg(Tos)-ONSu (1.10 g) in DMF (4 ml) and DMSO (1 ml). The mixture was stirred at room temperature for 1 d and then mixed with TEA (0.1 ml) and a solution of Boc-Arg(Tos)-ONSu (0.50 g) in DMSO (2 ml). The mixture was stirred at room temperature for 3 d and then mixed with 0.1 M NH_4HCO_3. The precipitate formed was removed by centrifugation and the upper phase was charged on a column of Sephadex G-25 equilibrated with 0.1 M NH_4HCO_3. The column was eluted with 0.1 M NH_4HCO_3 and the eluate with absorption at 274 nm was collected and lyophilized; wt 517.1 mg. Amino acid ratio in the acid hydrolysate: Asp, 1.97 (2); Ser, 1.75 (2); Glu, 4.29 (4); Gly, 1.01 (1); Ala, 1.00 (1); Val, 1.64 (2); Ile, 0.62 (1); Leu, 2.01 (2); Tyr, 2.30 (2); Arg, 0.83 (1). The lyophilized powder and anisole (0.64 ml) were put into the Daiflon-cylinder of an HF-reaction apparatus.[12] Anhydrous hydrogen fluoride (15 ml) was distilled into the cylinder cooled to −78 °C. The mixture was stirred at 0 °C for 90 min and then hydrogen fluoride was evaporated off. The residue was dissolved in 8 M urea (40 ml) with Na_2SO_3 (1.6 g), $Na_2S_4O_6$ (1.6 g) and EDTA·2Na (24 mg) and the solution was adjusted to pH 8 and stirred at room temperature for 3 d. It was then charged on a column (3×77 cm) of Sephadex G-25 equilibrated with 0.1 M NH_4HCO_3, and the column was eluted with 0.1 M NH_4HCO_3. The eluate with absorption at 274 nm was collected and lyophilized; wt 455.1 mg. The lyophilized powder (449.4 mg) was charged on a column (1.6×21 cm) of DEAE-Sephadex A-25 (acetate cycle) previously equilibrated with 8 M urea, and fractionated with a liner gradient of 0.0306 M HCl in 8 M urea (250 ml) to 0.336 M HCl in 8 M urea (250 ml) (Fig. 3). The fraction with absorption at 274 nm (shown by a bar) was collected, desalted on a column (3×57 cm) of Sephadex G-25, and lyophilized; wt 252.7 mg. For amino acid ratio in the acid hydrolysate, see Table 1.

S-Sulfonate of Leu-Glu-Gly-Pro-Pro-Gln-Lys[Z(2-Cl)]-Arg-A-Chain. Compound X (2.01 g, 1.76 mmol) was dissolved in DMF (7.5 ml) and cooled to below −40 °C. The cooled solution was mixed with 4.0 M HCl in dioxane (2.25 ml) and isopentyl nitrite (0.25 g, 2.11 mmol), and then stirred at the same temperature for 75 min. To the mixture were added *N*-methylmorpholine (1.10 ml) and a solution of the *S*-sulfonate of Arg-A-chain (0.68 g) in a mixture of DMSO (10 ml) and water (3.3 ml). The mixture was stirred at 2—3 °C in a refrigerator for 5 d and then 0.1 M NH_4HCO_3 was added. The resulting syrupy material was centrifuged and the supernatant was charged on a column of Sephadex G-25 equilibrated with 0.1 M NH_4HCO_3. The eluate with absorption at 274 nm was lyophilized; wt 848 mg. Amino acid ratio in the acid hydrolysate: Asp, 1.96 (2); Ser, 1.81 (2); Glu, 6.64 (6); Pro, 1.94 (2); Gly, 1.94 (2); Ala, 1.00 (1); Val, 1.63 (2); Ile, 0.62 (1); Leu, 2.89 (3); Tyr, 1.94 (2); Lys, 0.96 (1); Arg, 0.97 (1). The lyophilized powder was dissolved in TFA (20 ml) under cooling in an ice-water bath. The solution was stirred at room temperature for 60 min and then concentrated to a syrup. The syrup was dissolved in 0.1 M ammonium water at pH 8.0, and the solution was lyophilized. The resulting powder was dissolved in 8 M urea (50 ml) with Na_2SO_3 (2.0 g), $Na_2S_4O_6$ (2.0 g) and EDTA·2Na (0.04 g). The solution was stirred at room temperature for 2 d and then desalted using a column of Sephadex G-25 in 0.1 M NH_4HCO_3. The eluate with absorption at 274 nm was lyophilized; wt 770 mg. The lyophilized powder (329 mg) was charged on a column (1.6×15 cm) of DEAE-Sephadex A-25 (acetate cycle) equilibrated with 8 M urea, and the column was eluted with a linear gradient of 8 M urea (250 ml) to 0.3 M HCl in 8 M urea (250 ml) (Fig. 4). The fraction shown by a bar was collected, desalted and lyophilized; wt 213 mg. For amino acid ratio in the acid hydrolysate, see Table 1.

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6) The abbreviations used are those recommended by the IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations: TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; TEA, triethylamine; DMF, *N,N*-dimethylformamide; THF, tetrahydrofuran, DCHA, dicyclohexylamine; DMSO, dimethyl sulfoxide.
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