Total Synthesis of Ferrichrome

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Two different sequence hexapeptides for the synthesis of ferrichrome were prepared using N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithine by means of *N*-hydroxysuccinimide plus *N,N*-dicyclohexylcarbodiimide or Woodward reagent K method. The linear hexapeptides (XII·HCl and XIX·HCl) were cyclized by excess *N,N*-dicyclohexylcarbodiimide to give cyclo-triglycyltri-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithyl (XX). Detosylation, acetylation and successive reductive debenzylation of XX afforded deferri-ferrichrome (XXIV). Addition of ferric iron to XXIV gave ferrichrome, which had the same crystalline form, superimposable infrared and well-agreeing visible spectra and the same value in optical rotation compared with the data in the literature of natural product.

Ferrichrome (I), shown in Fig. 1, is one of a family of iron binding molecules[1] in nature and a representative of siderochrome.[2] It is an extremely powerful and relatively selective chelating agent for ferric iron[3] and it has been assumed this substance acts as a specific iron transport agent, a shuttle necessary for the permeation of the metal through the cellular membrane, or a coenzyme in heme biosynthesis.[4]

Neilands first actually isolated ferrichrome as a crystalline organo-iron pigment from the smut fungus, *Ustilago sphaerogena*, in 1952.[5] Subsequent study on the structure[6] of I showed that it binds a ferric iron mainly through ionic forces and that it is a cyclic hexapeptide built up from three residues of N^{δ}-acetyl-N^{δ}-hydroxy-L-ornithine together with three residues of glycine. The amino acid sequence within the peptide moieties of this naturally occurring ferric trihydroxamate was established by Neilands and collaborators in 1963 as cyclo-triglycyltri-N^{δ}-acetyl-N^{δ}-hydroxy-L-ornithyl.[7] Conformation in solution of I was recently studied by Llinas *et al.*[8]

The chemical synthesis of ferrichrome has been accomplished by Keller-Schierlein and Maurer[9] in 1969 using L-δ-nitronorvaline and glycine. The linear hexapeptide, *t*-butyloxycarbonylglycyltri-L-δ-nitronorvalylglycylglycine methyl ester, was prepared by means of mixed anhydride method using *t*-butyloxycarbonyl group as amino protecting group. The cyclization of the linear hexapeptide was carried out by the *p*-nitrophenyl ester method in pyridine. The reduction of the nitro group to hydroxyamino was accomplished with zinc in aqueous ammonium chloride.

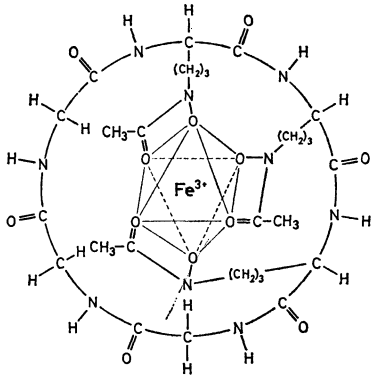
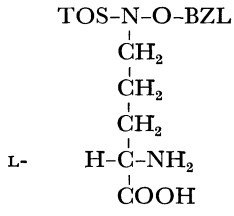


Fig. 1. Structure of ferrichrome (I).

Without isolation of the product, acetylation was carried out with acetic anhydride in pyridine and *O*-acetyl group the removed by treatment of the product with methanolic ammonia. After addition of the ferric chloride, the product, ferrichrome, was purified by countercurrent distribution and crystallized from methanol.

In this paper we also describe the total synthesis of ferrichrome using N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithine (II), shown in Fig. 2, which has been reported in the paper from our laboratory.[10]



(II)

Fig. 2. N^{δ}-Tosyl-N^{δ}-benzyloxy-L-ornithine: TOS, *p*-toluenesulfonyl; BZL, benzyl.

II has a protected hydroxyamino group at δ-position of L-ornithine and therefore fulfils prerequisites for incorporation into peptides of more complex structures. The linear hexapeptides (XII·HCl and XIX·HCl) were prepared *via* two different routes, shown in Fig. 3 and Fig. 4, in order to ascertain whether or not the protected cyclic peptides obtained from the linear hexapeptides possess the same optical rotatory power. Figure 3 indicates the synthesis of glycyltri-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine hydrochride (XII·HCl), in which glycines are the *N*- and *C*-terminal amino acid residues to remove possible racemization at the step of the cyclization reaction. For a study of the relationship between the optical purity and the coupling method at the preparation of XII·HCl, the linear hexapeptide (XII·HCl) was prepared by a step-by-step elongation using *N*-hydroxysuccinimide plus *N,N*-dicyclohexylcarbodiimide[11] or Woodward reagent K[12] as a coupling reagent. The final product (XII·HCl), obtained by the different coupling methods, possessed the same specific rotation, $-8.1°$ and $-8.2°$ in chloroform each other, and its homogeneity was demonstrated by thin-layer chromatography.

It should be noted that hydroxyamino protecting tosyl and benzyl groups are stable to the reaction conditions employed in the peptide formation and in the deprotection of the α-position. Figure 4 indicates the synthesis of another linear hexapeptide, tri-N^{δ}-tosyl-

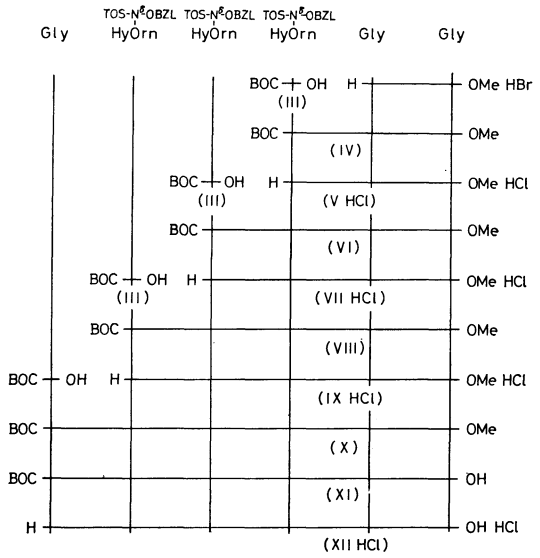


Fig. 3. Synthesis of glycyltri-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine hydrochloride (XII·HCl): HyOrn, N^{δ}-hydroxy-L-ornithine; Gly, glycine; BOC, *t*-butyloxycarbonyl.

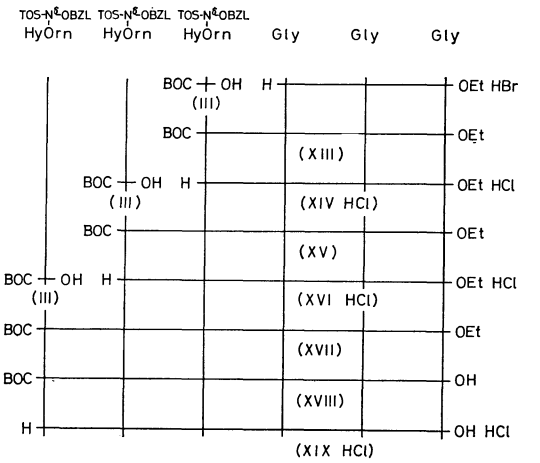


Fig. 4. Synthesis of tri-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithyldiglycylglycine hydrochloride (XIX·HCl).

N^{δ}-benzyloxy-L-ornithyldiglycylglycine hydrochloride (XIX·HCl), in which II is *N*-terminal amino acid and glycine is *C*-terminal amino acid. XIX·HCl, prepared by a step-by-step elongation by means of *N*-hydroxysuccinimide plus *N,N*-dicyclohexylcarbodiimide method, possessed the specific rotation, +3.2° in chloroform.

Various methods for the cyclization of the linear hexapeptides (XII·HCl and XIX·HCl) were undertaken. Namely *N,N*-dicyclohexylcarbodiimide, water soluble carbodiimide (*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide) or *N*-hydroxysuccinimide plus *N,N*-dicyclohexylcarbodiimide method were attempted to cyclize the linear hexapeptides. Since the *N,N*-dicyclohexylcarbodiimide method gave the pure cyclization product in the 41% yield, we describe in detail about the cyclization reaction using *N,N*-dicyclohexylcarbodiimide. The scheme for the cyclization reaction of XII·HCl or XIX·HCl is presented in Fig. 5.

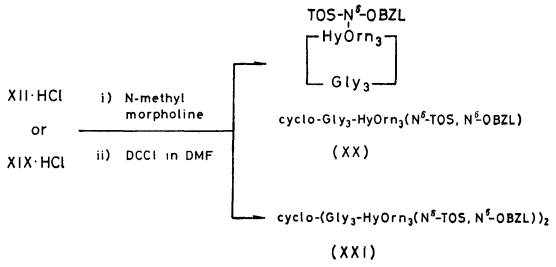


Fig. 5. Cyclization of linear hexapeptide (XII·HCl or XIX·HCl) using *N,N*-dicyclohexylcarbodiimide (DCCI).

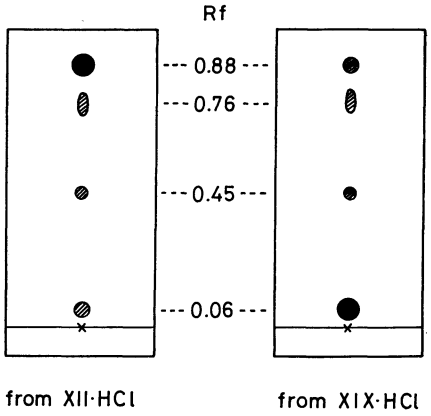


Fig. 6. Thin-layer chromatogram of the crude cyclization products obtained from XII·HCl or XIX·HCl; the R_f value of cyclic hexapeptide is 0.88 and that of dimerization product is 0.06.

The cyclization of XII·HCl using *N,N*-dicyclohexylcarbodiimide in a large amount of dimethylformamide gave a mixture of the desired monomerization product (XX), the dimerization substance (XXI), the unreacted linear hexapeptide (XII) and *N,N*-dicyclohexylurea, which were determined by thin-layer chromatography (Fig. 6).

The R_f value of 0.06 was proved to be of the dimerization product (XXI), which was demonstrated by the molecular weight determination and elemental analysis, while that of 0.88 was of the desired protected cyclic hexapeptide (XX). The product indicating R_f 0.76 was considered to be a linear hexapeptide (XII) and R_f 0.45 was determined to be of dicyclohexylurea. For the removal of XXI, the reaction mixture was treated with hot methanol. After methanol-insoluble material (XXI) had been filtered off, the methanol solution was evaporated to dryness. Furthermore, for the removal of XII and dicyclohexylurea, the residue thus obtained was dissolved in a mixture of dimethylformamide and water (4 : 1) and passed successively through columns of CG-120 and CG-400; the yield of the crystalline XX from XII·HCl was found to be 41% after recrystallization from methanol. The molecular weight determination demonstrated that the molecular size of this compound was corresponded to that of XX. The cyclization reaction of another linear hexapeptide (XIX·HCl) gave the result indicated in Fig. 6. In this case, the main product, obtained in a *ca.* 40% yield, was found to be

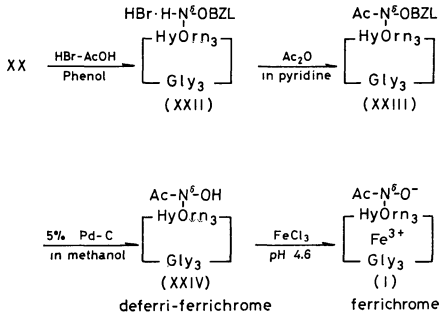


Fig. 7. Transformation of XX to deferri-ferrichrome XXIV and addition of ferric ion to XXIV.

a dimer (XXI). Not more than 7% of the starting material (XIX·HCl) was converted to the cyclic hexapeptide. Since the cyclic hexapeptide, thus obtained, possessed the same specific rotation with that of XX prepared from XII·HCl, the maintenance of optical purity was demonstrated.

The transformation of XX to deferri-ferrichrome (XXIV) and of XXIV to ferrichrome (I) are presented in Fig. 7.

The tosyl group of XX could be removed selectively on treatment with 36% hydrogen bromide in acetic acid in the presence of phenol. However, it was unsuccessful to isolate the detosylated product (XXII·3HBr) in the pure state. The removal of the tosyl group was confirmed by the lacking of the absorptions at 1350 and 1165 cm^{-1} in the infrared spectrum. Experiment in which the crude detosyl-compound was acetylated with acetic anhydride in pyridine, resulted acetyl-derivative (XXIII) in 47% overall conversion (XX to XXIII). On hydrogenation in methanol solution over 5% palladium on charcoal at room temperature under atmospheric pressure for 4 days, XXIII was almost quantitatively converted into XXIV (deferri-ferrichrome), which was ascertained by the comparison with the NMR spectrum obtained from natural product.[13] Figure 8 shows the NMR spectrum of XXIV, in which the characteristic signal of methyl protons in N^{δ}-acetyl group appears at δ 2.61 and the signals of benzyl group are not observable. Figure 9 shows the infrared spectrum of XXIV, in which the removal of the benzyl group is confirmed by the absence of the absorptions at 3025 and 698 cm^{-1}.

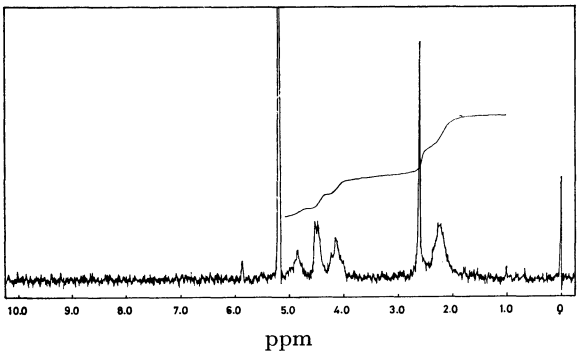


Fig. 8. NMR spectrum of synthetic deferri-ferrichrome (XXIV).

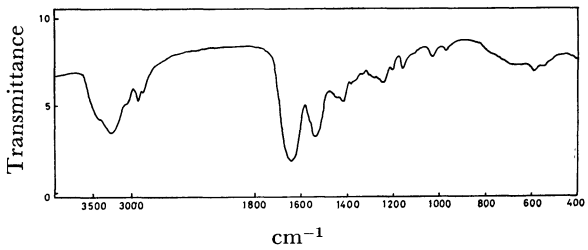


Fig. 9. Infrared spectrum of synthetic deferri-ferrichrome (XXIV).

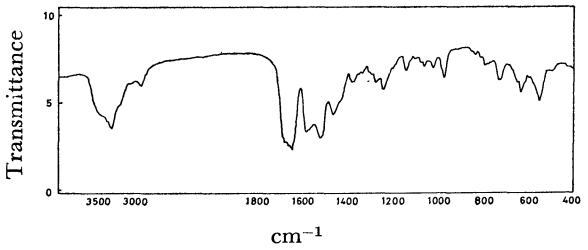


Fig. 10. Infrared spectrum of synthetic ferrichrome (I).

The synthetic ferrichrome (I) was obtained by the reaction of XXIV with three equivalents of ferric chloride in aqueous buffered solution of pH 4.6, at which re-addition of iron to deferri-ferrichrome obtained from naturally occurring ferrichrome was performed by Emery and Neilands.[6b] On recrystallizations from anhydrous methanol, pure synthetic ferrichrome (I) was obtained as long orange needles with the well-agreeing value of elemental analysis. The comparison with the data in the literatures of natural product was made of the melting point, optical rotation and absorptivity in visible absorption; mp 248—251 °C (decomp.) (lit.[5] 240—242 °C (decomp.)), $[\alpha]_D$ +304°± 10° (*c* 0.0425, water) (lit.[9b] +300° (*c* 0.04, water)) and $E_{1cm}^{1\%}$ (428 mμ 39.8 (water) (lit.[5] $E_{1cm}^{1\%}$ (425 mμ) 39.6 (water)). Furthermore, the infrared spectrum of synthetic I, as shown in Fig. 10, corresponded closely to that of natural product described in the literature.[13]

Experimental

All melting points were determined with a Yanagimoto electric micromelting point apparatus unless otherswie indicated and are uncorrected. Optical rotations were measured with a Yanagimoto automatic polarimeter OR-50. The nuclear magnetic resonance spectra were run on a Hitachi Perkin-Elmer R-20 High Resolution NMR spectrometer, using tetramethylsilane as an external or internal standard. Infrared spectra were recorded on a Hitachi G-3 spectrophotometer as KBr disk. Molecular weight was determined by the use of a differential vapor pressure osometer (Hitachi Perkin-Elmer 115 Molecular weight apparatus). The visible spectrum was taken on a Hitachi Recording Spectrophotometer EPS-3. The thin-layer chromatography was carried out on Merck silica gel F_{254} using the solvent system of *n*-butanol–acetic acid–pyridine–water (4 : 1 : 1 : 2 v/v). The paper chromatography was carried out on Toyo Roshi No. 50 using the solvent system of *n*-butanol–acetic acid–water (4 : 1 : 1 v/v).

N$^{\alpha}$-t-*Butyloxycarbonyl*-N$^{\delta}$-*tosyl*-N$^{\delta}$-*benzyloxy*-L-*ornithine (III)*.

This compound was prepared by the procedure described in the previous paper.[15]

N^{α}-t-Butyloxycarbonyl-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine Methyl Ester (IV).[16] *a*): To a stirred solution of *N^{α}-t*-butyloxycarbonyl-*N^{δ}*-tosyl-*N^{δ}*-benzyloxy-L-ornithine (III) (4.92 g, 10 mmol), glycylglycine methyl ester hydrobromide (2.27 g, 10 mmol), *N*-hydroxysuccinimide (2.3 g, 20 mmol) and *N*-methyl morpholine(1.21 ml, 11 mmol) in dimethylformamide (100 ml) was added a solution of dicylcohexylcarbodiimide (2.27 g, 11 mmol) in dimethylformamide (10 ml) at −10 °C. After stirring for 42 hr, the crystalline deposit was removed and the filtrate was evaporated *in vacuo*. The oily residue was dissolved in chloroform (150 ml) and washed successively with water, M sodium bicarbonate, 2 M hydrochloric acid and water. The organic layer was dried over magnesium sulfate and evaporated *in vacuo*. The oily residue was solidified with the aid of petroleum ether; 5.03 g (81%); mp 56—64 °C; $[\alpha]_D$ +6.8° (*c* 1, methanol).

Found: C, 56.35; H, 6.68; N, 8.91%. Calcd for $C_{29}H_{40}N_4O_9S$: C, 56.11; H, 6.50; N, 9.03%.

b): To a stirred suspension of III (9.86 g, 20 mmol) and Woodward reagent K (5.07 g, 20 mmol) in nitromethane (80 ml) was added triethylamine (2.93 ml, 21 mmol) at 5 °C. To the resultant clear solution was added glycylglycine methyl ester hydrobromide (4.5 g, 20 mmol) and triethylamine (2.93 ml, 21 mmol). The mixture was stirred at room temperature for 36 hr and then evaporated *in vacuo*. The oily residue was treated as described above; yield, 8.9 g (72%); mp 61—67 °C; $[\alpha]_D$ +7.0° (*c* 1, methanol).

N^{δ}-Tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine Methyl Ester Hydrochloride (V·HCl). *i*): To the cold solution of IV-a[17] (5.03 g) in ethyl acetate (20 ml) was added 6 M hydrogen chloride in ethyl acetate (30 ml). After stirring for 2 hr at room temperature, the mixture was evaporated *in vacuo*. The oily residue was crystallized from a small volume of ethyl acetate by addition of ether; yield, 4.30 g (95.5%); mp 108—121 °C; $[\alpha]_D$ +22.3° (*c* 1, ethanol).

Found: C, 51.24; H, 5.97; N, 10.06%. Calcd for $C_{24}H_{33}N_4O_7SCl$: C, 51.67; H, 6.09; N, 9.94%.

ii): The peptide hydrochloride (V·HCl) derived from IV-b[17]; mp 109—120 °C; $[\alpha]_D$ +22.1° (*c* 1, ethanol).

N^{α}-t-Butyloxycarbonyl-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithyl-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine Methyl Ester (VI). a): To a stirred colorless solution of III (4.17 g, 8.47 mmol), V·HCl (4.72 g, 8.47 mmol), *N*-hydroxysuccinimide (1.96 g, 17.0 mmol) and *N*-methyl morpholine (1.00 ml, 9.00 mmol) in dimethylformamide (50 ml) was added a solution of dicyclohexylcarbodiimide (1.86 g, 9.00 mmol) in dimethylformamide (10 ml) at −15 °C. After stirring for 45 hr at room temperature, the crystalline deposit was removed by suction and the filterate was evaporated *in vacuo*. The oily residue was dissolved in ethyl acetate (200 ml) and washed successively with water, M sodium bicarbonate, water, 2 M hydrochloric acid and water. The organic layer was dried over anhydrous magnesium sulfate and evaporated *in vacuo*. The residual semi-solid was crystallized from ethyl acetate–ligroin (1 : 1) to give an amorphous powder; yield, 6.9 g (82%); mp 112—120 °C; $[\alpha]_D$ −2.8° (*c* 1, methanol). One more recrystallization from the same solvent raised the mp to 120—123 °C.

Found: C, 57.98; H, 6.10; N, 8.25%. Calcd for $C_{48}H_{62}N_6O_{13}S_2$: C, 57.93; H, 6.28; N, 8.45%.

b): The tetrapeptide ester (VI) was obtained by the same procedure employed for the preparation of IV-b; yield, 72.5 %; mp 118—122 °C; $[\alpha]_D$ −2.7° (*c* 1, methanol).

Di-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine Methyl Ester Hydrochloride (VII·HCl). *i*): Removal of the *t*-butyloxycarbonyl group from VI-a using the same reagent employed for the preparation of V·HCl gave VII·HCl; yield, 93.0%; mp 112—126 °C; $[\alpha]_D$ +16.4° (*c* 1, methanol).

Found: C, 55.22; H, 6.17; N, 8.92%. Calcd for $C_{43}H_{55}N_6O_{11}S_2Cl$: C, 55.44; H, 5.95; N, 9.02%.

ii): The tetrapeptide ester hydrochloride derived from VI-b; mp 110—123 °C; $[\alpha]_D$ +16.0° (*c* 1, methanol).

N^{α}-t-Butyloxycarbonyl-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithyldi-N^{δ}-tosyl-N^{δ}-benzoyloxy-L-ornithylglycylglycine Methyl Ester (VIII). *a*): To a stirred colorless solution of III (3.19 g, 6.48 mmol), VII·HCl (6.04 g, 6.48 mmol) and *N*-hydroxysuccinimide 1.50 g, 13.0 mmol) in dimethylformamide (40 ml) was added *N*-methyl morpholine (0.77 ml, 7.0 mmol) at −15 °C. Thereafter dicyclohexlcarbodiimide (1.45 g, 7.0 mmol) in dimethylformamide (10 ml) was added at the same temperature. The mixture was stirred for 48 hr at room temperature. The crystalline deposit was removed and the pale yellow filtrate was evaporated *in vacuo*. The residual semi-solid was triturated several times with water and then the resultant solid was collected by suction. Recrystallization from ethyl acetate–ligroin (3 : 1) gave an amorphous powder; yield, 5.35 g (60.5%); mp 152—154 °C; $[\alpha]_D$ −3.1° (*c* 1, methanol).

Found: C, 58.68; H, 5.92; N, 8.12%. Calcd for $C_{67}H_{84}N_8O_{17}S_3$: C, 58.75; H, 6.18; N, 8.18%.

b) The pentapeptide ester (VIII) was obtained by the same method employed for the preparation of IV-b; yield, 49%; mp 154—156 °C; $[\alpha]_D$ −3.6° (*c* 1, methanol).

Tri-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine Methyl Ester Hydrochloride (IX·HCl). *i*): VIII was converted to the pentapeptide ester hydrochloride (IX·HCl) in the same manner as described above for V·HCl; yield, 91.5%; mp 126—135 °C; $[\alpha]_D$ +5.8° (*c* 1, methanol).

Found: C, 56.52; H, 6.05; N, 8.23%. Calcd for $C_{62}H_{77}N_8O_{15}S_3Cl$: C, 57.02; H, 5.94; N, 8.53%.

ii): IX·HCl from VIII-b by the same procedure employed for the preparation of V·HCl; yield, 96.5%; mp 123—135 °C; $[\alpha]_D$ +4.8° (*c* 1, methanol).

t-Butyloxycarbonylglycyltri-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithylglycylglycine Methyl Ester (X). *a*): To a stirred solution of *t*-butyloxycarbonylglycine (0.43 g, 2.45 mmol), IX·HCl (3.2 g, 2.45 mmol) and *N*-hydroxysuccinimide (0.58 g, 5.0 mmol) in dimethylformamide (20 ml) was added *N*-methyl morpholine (0.30 ml, 2.7 mmol) at −15 °C. After stirring for 30 min at the same temperature, dicyclohexylcarbodiimide (0.56 g, 0.70 mmol) in dimethylformamide (5 ml) was added and the reaction mixture was stirred for 48 hr at room temperature. The crystalline deposit was removed and the yellow filtrate was evaporated *in vacuo*. The crude crystalline hexapeptide ester was collected with the aid of water (50 ml), washed successively with water and dried over phosphorus pentoxide under vacuum. Recrystallization from methanol treated with charcoal gave a colorless powder; yield, 3.0 g (86%); mp 170—172 °C; $[\alpha]_D$ +2.4° (*c* 1, chloroform): IR, 1740 cm^{-1} (–COOMe).

Found: C, 57.94; H, 6.11; N, 8.74%. Calcd for $C_{69}H_{87}N_9O_{18}S_3$: C, 58.08; H, 6.15; N, 8.84%.

b): The hexapeptide ester was obtained by the same procedure employed for the preparation of IV-b; mp 168—170 °C (from methanol); $[\alpha]_D$ +2.1° (*c* 1, chloroform).

t-Butyloxycarbonylglycyltri-N^{δ}-tosyl-N^{δ}-benzyloxy-L-ornithyl-glycylglycine (XI). *i*): To a stirred suspension of the hexapeptide methyl ester (X) (2.9 g, 2.0 mmol) in acetone (20 ml) was added M sodium hydroxide (4 ml) at room temperature. After 48 hr, resultant pale yellow clear solution was neutralized with citric acid and then evaporated *in vacuo*. Residual solid was collected with the aid of water and recrystallization from methanol gave a colorless powder; yield, 1.6 g (57%);

mp 138—143 °C; $[\alpha]_D$ +1.4° (*c* 1, chloroform); IR absorption of the ester group in X at 1740 cm^{-1} disappeared.

Found: C, 57.56; H, 5.88; N, 8.66%. Calcd for $C_{68}H_{85}N_9O_{18}S_3$: C, 57.81; H, 6.07; N, 8.92%.

ii): The ester-free hexapeptide derived from X-b; yield, 58%; mp 138—143 °C; $[\alpha]_D$ +1.5° (*c* 1, chloroform).

Glycyltri-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithylglycylglycine Hydrochloride (XII·HCl). *i*): To a solution of XI-a (1.6 g, 1.1 mmol) in chloroform (30 ml) was added 6 M hydrogen chloride in ethyl acetate (20 ml) under ice cooling. The solution was then allowed to stand at room temperature for 4 hr and evaporated *in vacuo*. Crystalline deposit was collected with the aid of dry ether; yield, 1.4 g (92%); mp 131—138°C; $[\alpha]_D$ −8.1° (*c* 1, chloroform); R_f 0.75 (tlc).

Found: C, 55.94; H, 6.06; N, 9.10%. Calcd for $C_{63}H_{78}N_9O_{16}S_3Cl$: C, 56.09; H, 5.83; N, 9.35%.

ii) The hexapeptide hydrochloride (XII-HCl) derived from XI-b; mp 132—138 °C; $[\alpha]_D$ −8.2° (*c* 1, chloroform); R_f, 0.75 (tlc).

Glycyltri-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithylglycylglycine (XII). XII was obtained by the treatment of XII·HCl with aqueous ammonia in dimethylformamide. Recrystallization from dimethylformamide-water gave a pure product (XII); mp 212—218 °C; $[\alpha]_D$ −4.2° (*c* 0.5, acetic acid); R_f, 0.76 (tlc).

Found: C, 57.34; H, 5.80; N, 9.30%. Calcd for $C_{63}H_{77}N_9O_{16}S_3$: C, 57.65; H, 5.91; N, 9.61%.

N$^\alpha$-t-Butyloxycarbonyl-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldiglycylglycine Ethyl Ester (XIII). III and diglycylglycine ethyl ester hydrobromide were coupled by the same procedure employed for the preparation of IV-a to give a desired tetrapeptide ester; mp 98—102 °C (from ethyl acetate-ligroin); $[\alpha]_D$ +0.3° (*c* 1, acetic acid).

Found: C, 55.94; H, 6.43; N, 10.19%. Calcd for $C_{32}H_{45}N_5O_{10}S$: C, 55.55; H, 6.56; N, 10.12%.

N$^\delta$-Tosyl-N$^\delta$-benzyloxy-L-ornithyldiglycylglycine Ethyl Ester Hydrochloride (XIV-HCl). Removal of the *t*-butyloxycarbonyl group of XIII was carried out by the same procedure employed for the preparation of V·HCl to give the tetrapeptide ester hydrochloride; mp 115—128 °C; $[\alpha]_D$ +21.7° (*c* 1, methanol).

Found: C, 51.54; H, 5.90; N, 10.89%. Calcd for $C_{27}H_{38}N_5O_8SCl$: C, 51.63; H, 6.10; N, 11.15%.

N$^\alpha$-t-Butyloxycarbonyl-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyl-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldiglycylglycine Ethyl Ester (XV). III and XIV·HCl were coupled by the same procedure employed for the preparation of IV-a to give a desired pentapeptide ester; mp 155—157 °C (from methanol); $[\alpha]_D$ −7.4° (*c* 1, acetic acid).

Found: C, 57.50; H, 6.05; N, 9.08%. Calcd for $C_{51}H_{67}N_7O_{14}S_2$: C, 57.45; H, 6.33; N, 9.20%.

Di-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldiglycylglycine Ethyl Ester Hydrochloride (XVI·HCl). XVI·HCl was obtained by the same procedure employed for the preparation of V·HCl; mp 196—201 °C, $[\alpha]_D$ +17.9° (*c* 1, methanol).

Found: C, 54.78; H, 5.82; N, 9.42%. Calcd for $C_{46}H_{60}N_7O_{12}S_2Cl$: C, 55.10; H, 6.03; N, 9.78%.

N$^\alpha$-t-Butyloxycarbonyl-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldi-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldiglycylglycine Ethyl Ester (XVII). III and XVI·HCl were coupled by the same procedure employed for the preparation of IV-a; mp 192—195 °C (from methanol); $[\alpha]_D$ −5.2° (*c* 1, acetic acid).

Found: C, 58.49; H, 6.09; N, 8.67%. Calcd for $C_{70}H_{89}N_9O_{18}S_3$: C, 58.35; H, 6.23; N, 8.75%.

N$^\alpha$-t-Butyloxycarbonyl-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldi-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldiglycylglycine (XVIII). Hydrolysis of the hexapeptide ester (XVIII) in acetone with M sodium hydroxide was carried out by the same method employed for the hydrolysis of X; mp 122—129 °C (from methanol); $[\alpha]_D$ −2.0° (*c* 1, acetic acid).

Found: C, 57.94; H, 6.09; N, 8.87%. Calcd for $C_{68}H_{85}N_9O_{18}S_3$: C, 57.81; H, 6.07; N, 8.92%.

Tri-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyldiglycylglycine Hydrochloride (XIX·HCl). XIX·HCl was obtained by the same procedure employed for the preparation of XII·HCl; mp 188—198 °C (from ethanol); $[\alpha]_D$ +3.2° (*c* 1, acetic acid).

Found: C, 56.18; H, 5.98; N, 9.13%. Calcd for $C_{63}H_{78}N_9O_{16}S_3Cl$: C, 56.09; H, 5.83; N, 9.34%.

cyclo-Triglycyltri-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyl (XX).

i) From XII·HCl: To a solution of XII·HCl (7.8 g, (5.8 mmol) in dimethylformamide (60 ml), *N*-methyl morpholine (0.66 ml), 6.0 mmol) was added at room temperature. After stirring for 3 hr, the solution was diluted with dimethylformamide to 2 l. The diluted solution of the free base of XII·HCl was added dropwise into a solution of dicyclohexylcarbodiimide (24 g, 115 mmol) in dimethylformamide (4 l) kept at 0—5 °C under stirring over a period of 48 hr. The solution was stirred for another 4 days at room temperature and then evaporated to 200 ml *in vacuo* at 40 °C. To the residual pale yellow solution was added 50% acetic acid (30 ml) under ice cooling to remove an excess of dicyclohexylcarbodiimide as dicyclohexylurea. After 2 hr, crystalline deposit was removed by filtration and the yellow filtrate was evaporated to dryness. Further the residual oil in dimethylformamide (100 ml) was treated wtih 50% acetic acid as described above and this procedure was repeated several times. The oily residue thus obtained was solidified by addition of water. The crude product was collected and dried; yield, 9.15 g. This was dissolved in 1 l of hot methanol and the insoluble substance (550 mg) was removed by filtration. The filtrate was evaporated to dryness and the residue was redissolved in 150 ml of a mixture of dimethylformamide and water (4 : 1). The solution was successively passed through columns (5×20 cm) of CG-120 (H^+ form) and CG-400 (OH^- form). The columns were then washed with the same solvent. The combined effluents were treated with charcoal and evaporated to dryness *in vacuo*. The residue was collected with the aid of water and dried; yield, 5.8 g. Recrystallization of the product from methanol gave the cyclic hexapeptide (XX); yield, 3.1 g (41%); mp 161—167 °C; $[\alpha]_D$ −3.0° (*c* 1, chloroform); R_f 0.88 (tlc); molecular weight, 1308 (calcd 1295) (dimethylformamide as solvent). Ninhydrin reaction was negative. Recrystallization three times from methanol raised the mp to 181—182 °C.

Found: C, 58.43; H, 5.89; N, 9.48%. Calcd for $C_{63}H_{75}N_9O_{15}S_3$: C, 58.45; H, 5.84; N, 9.74%.

Identification of the methanol-insoluble substance as the dimerization reaction product, i.e. cyclo-*(triglycyltri-N$^\delta$-tosyl-N$^\delta$-benzyloxy-L-ornithyl)$_2$.* Recrystallization from acetic acid gave an analytical sample; mp 246—251 °C; R_f 0.06 (tlc); molecular weight, 2603 (calcd 2589) (dimethylformamide as solvent); yield, 550 mg (7.3%).

Found: C, 58.51; H, 5.83; N, 9.98%. Calcd for $(C_{63}H_{75}N_9O_{15}S_3)_2$: C, 58.45; H, 5.84; N, 9.74%.

ii) From XIX·HCl: XIX·HCl was converted to the free base and allowed to cyclize in dimethylformamide using excess dicyclohexylcarbodiimide as a condensing reagent as has been described above; yield, 7%; mp 172—181 °C; $[\alpha]_D$ −2.8° (*c* 1, chloroform). Dimerization product was obtained as main product; yield, 40%; mp 244—250 °C.

cyclo-*Triglycyltri-N$^\delta$-benzyloxy-L-ornithyl Hydrobromide (XXII·3HBr).* To a solution of 36% hydrogen bromide in acetic acid (32 ml) and phenol (4.0 g) in a glass-stoppered bottle, XX (3.0 g, 2.3 mmol) was added. After stirring

for 42 hr at room temperature, the solution was evaporated *in vacuo* at 30 °C. The oily residue was solidified by adding ether. The crude product collected by suction with the aid of ether was dissolved in water (200 ml). After water-insoluble materials were filtered off, the solution was washed three times with ether, treated with charcoal and then evaporated to dryness *in vacuo*. To the residual oil, ethanol was added and evaporated *in vacuo* in order to remove a trace of water. After the residual oil was solidified by the addition of anhydrous ether and collected with the aid of ether, the product (XXII·3HBr) was obtained as a hygroscopic colorless powder. The removal of tosyl group was determined by Infrared absorption spectrum; absorptions at 1350 and 1165 cm^{-1} for tosyl group were absent.

cyclo-*Triglycyltri*-N$^{\delta}$-*acetyl*-N$^{\delta}$-*benzyloxy*-L-*ornithyl* *(XXIII)*. XXII·3HBr was dissolved in a mixture of pyridine (20 ml) and acetic anhydride (1.0 ml) at 5 °C. After stirring for 12 hr at room temperature, the reaction mixture was evaporated to a syrup *in vacuo*. The residue was evaporated twice with water in order to decompose an excess of acetic anhydride. The final residue was taken up in *n*-butanol (150 ml) and the solution was repeatedly washed with water. The organic layer was treated overnight with charcoal and evaporated to dryness *in vacuo*. The residue was triturated with ether to give an amorphous product (XXIII); the over-all yield in the conversion of XX into XXIII was 47%. NMR spectrum (CD_3OD, TMS-internal standard) indicates N$^{\delta}$-acetyl protons at δ 2.04. The signal of *O*-acetyl derivative was not detected in NMR spectrum.

cyclo-*Triglycyltri*-N$^{\delta}$-*acetyl*-N$^{\delta}$-*hydroxy*-L-*ornithyl*; *deferriferrichrome (XXIV)*. A solution of XXIII (1.0 g) in methanol (40 ml) was hydrogenated in the presence of 5% palladium charcoal (2.0 g) for 4 days at room temperature. After the catalyst was filtered off, the filtrate was evaporated *in vacuo* at 25 °C. The residue was triturated with ether to give an amorphous XXIV; the yield of the dried product, 620 mg. NMR spectrum (D_2O, TMS-external standard) indicates N$^{\delta}$-acetyl protons at δ 2.61, β,γ-methylene protons of N$^{\delta}$-hydroxyornithine at δ 2.25, δ-methylene protons of that at δ 4.15, α-methyne proton of that at δ 4.86 and methylene protons of glycine at δ 4.49 respectively, as shown in Fig. 8. The signals of δ 5.87 and 4.53 are those of side bands of water.

Ferrichrome (I). To the colorless solution of XXIV (500 mg) in McIlvain buffer (pH 4.6) (25 ml), M ferric chloride (2.2 ml, ca. three equivalents) was added at room temperature under continuous stirring. On addition of the ferric chloride, the reaction mixture formed a reddish-violet solution. After stirring for an additional 2 hr, the solution was completely saturated with ammonium sulfate. This solution was extracted with benzyl alcohol (600 ml × 2) and the reddish-orange benzyl alcohol layer was washed with water (100 ml × 3). Diethyl ether (3.6 l) was added to the washed benzyl alcohol solution and then the solution extracted with water (200 ml × 3). The combined aqueous extract was shaken with diethyl ether (200 ml × 3) and the ether phase discarded. The fine red aqueous solution was then evaporated to dryness *in vacuo* at 28 °C. The residual product was scratched with acetone (50 ml) and acetone-insoluble substance was collected by suction. The reddish-brown powder obtained was briefly refluxed in anhydrous methanol (120 ml), filtered and the filtrate concentrated slightly on the oil-bath. The ferrichrome (I) began to crystallize at once in long orange needles, whoes paper chromatography gave a single spot (R_f 0.28) using the solvent system of *n*-butanol–acetic acid–water (4 : 1 : 1). The yield was 85 mg; mp 248—251 °C (decomp.) determined with Melting Point Apparatus MP-21 (Yamato Scientific Co., Ltd., open capillary in silicone oil) and uncorrected; $[\alpha]_D$ $+304° \pm 10°$ (*c* 0.0425, water); $E^{1\%}_{1cm}$ (428 mμ) 39.8 in water. For analysis, a part of the product was recrystallized three times from anhydrous methanol.

Found: C, 43.26; H, 5.69; N, 16.72%. Calcd for $C_{27}H_{42}N_9O_{12}Fe$: C, 43.79; H, 5.72; N, 17.02%.

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16) This compound was prepared by two different methods described in the text; a) indicates *N*-hydroxysuccinimide plus *N,N*-dicyclohexylcarbodiimide method and b) indicates Woodward reagent K method. This is repeated in the preparations of VI, VIII and X.

17) The symbol, IV-a, indicates the peptide obtained by the *N*-hydroxysuccinimide plus *N,N*-dicyclohexylcarbodiimide method in the preparation of IV and IV-b indicates that obtained by the Woodward reagent K method. This is to be repeated in the following in order to ascertain whether or not the peptides obtained by the different methods possess the same optical rotation.