THE TOTAL STRUCTURE OF THE ANTIBIOTIC LONGICATENAMYCIN*

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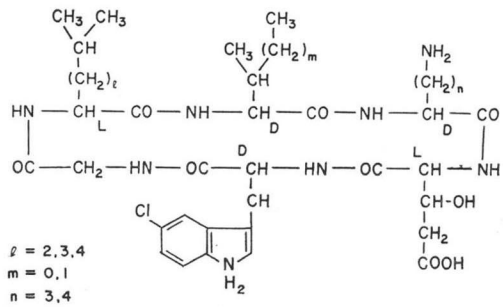
The peptide antibiotic longicatenamycin produced by a *Streptomyces* strain S-520 is a complex mixture of several congeners. The determination of the total structure of longicatenamycin was performed on the mixture using the procedure of N-bromosuccinimide oxidation followed by EDMAN degradation. The structure of the most abundant congener of longicatenamycin can be represented as cyclo (glycyl-L-2-amino-6-methylheptanoyl-D-valyl-D-ornithyl-*threo*-β-hydroxy-L-glutamyl-5-chloro-D-tryptophyl) (Fig. 1: l=4, m=0, n=3). There are three amino acid positions in which variations are found. The antibiotic complex contains compounds in which L-2-amino-6-methylheptanoic acid is replaced with either L-2-amino-5-methylhexanoic acid or L-2-amino-7-methyloctanoic acid; further D-valine with D-isoleucine as well as D-ornithine with D-lysine.

A new antibiotic was isolated from the culture filtrate of *Streptomyces diastaticus* strain S-520 by SHOJI *et al.* in 1970.[1] With their consent, we termed this antibiotic longicatenamycin, the name of which was derived from the unique and characteristic presence of amino acids with unusually long aliphatic side chain. Longicatenamycin possesses antibacterial activity against Gram-positive bacteria.[1]

In our previous papers, all constituent amino acids of this antibiotic were fully clarified.[2,3] It contains one mole of glycine, D-valine (or D-isoleucine), D-ornithine (or D-lysine), *threo*-β-hydroxy-L-glutamic acid, L-2-amino-6-methylheptanoic acid (**2**) (or L-2-amino-5-methylhexanoic acid (**1**), or L-2-amino-7-methyloctanoic acid (**3**)) and 5-chloro-D-tryptophan. Among them, β-hydroxyglutamic acid and the amino acids **1**, **2**, **3** are new naturally occurring amino acids, the structures of which were proposed by SHOJI *et al.*[4,5] and confirmed by us synthetically.[2,3] 5-Chlorotryptophan was first found in the hydrolyzate of longicatenamycin in our recent study.[2,3] The amino acids mentioned in the parentheses above are the alternate residues found in more minor components of the antibiotic complex. For instance, in the hydrolyzate of longicatenamycin the molar ratio of the sum of D-valine and D-isoleucine to glycine corresponds within the experimental error to one. Similarly, D-ornithine is found exchanged with D-lysine as the homologous amino acid and **2** with **1** or **3** in the peptide molecule. Consequently, longicatenamycin could be a complex mixture composed of $2\times2\times3=12$

Fig. 1. Total structure of longicatenamycin.



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congeners maximally. Such occurrence is rather common in peptide antibiotics produced by microorganisms.

Determination of the primary structure of peptide antibiotics usually has been carried out by sequential analysis on a pure congener. In this case, however, separation of the many closely related peptides was extremely difficult, since an efficient separation method like counter current distribution was not readily applicable due to the very poor solubility of the complex in most organic solvents except lower alcohol and dimethyl sulfoxide, and the presence of the acid labile 5-chlorotryptophan. Therefore, in this study, we attempted to carry out the sequential analysis of amino acids using a mixture of the congeners without separation.

Longicatenamycin, as a mixture, was 2,4-dinitrophenylated to give a 2,4-dinitrophenyl (DNP) derivative which showed principally a single spot on a thin-layer chromatogram. In the acid hydrolyzate of DNP-longicatenamycin, only ornithine (or lysine) disappeared in amino acid analysis and ninhydrin-positive DNP-amino acids were detected at the same time. These DNP amino acids were neutral on electrophoresis and insoluble in ether. These were compared with synthetic α-DNP-ornithine (or α-DNP-lysine) and δ-DNP-ornithine (or ε-DNP-lysine) by thin-layer chromatography. Although, the corresponding derivatives of both amino acids were not separated sufficiently, α- and ω-isomers were distinguished satisfactorily. The DNP-amino acids in the hydrolyzate were identical to δ-DNP-ornithine (or ε-DNP-lysine). This fact suggested that longicatenamycin is a cyclic hexapeptide in which a single free amino group is ascribed to the ω-amino function in the ornithine (or lysine) residue.

In order to determine the amino acid sequence, *i.e.*, the total primary structure of longicatenamycin, partial hydrolysis in 6 N hydrochloric acid was carried out at 110°C for 1.5 hours in an evacuated tube. The hydrolyzate was separated by paper electrophoresis. β-Hydroxyglutamic acid was detected in an acidic part, and glycine as well as 5-chlorotryptophan in a neutral part, all as free amino acids. From a basic part a tripeptide, composed of the residual amino acids, *i.e.*, **2** (or **1**, **3**), valine (or isoleucine) and ornithine (or lysine), was isolated. From the result of a subtractive EDMAN degradation on this tripeptide, as shown in Table 1, the partial sequence 2(**1**, **3**)-Val(Ile)-Orn(Lys) could be deduced. In this experiment the homologous amino acids belonging to the same pair showed similar behaviors keeping nearly the original molar ratios. This indicates that the simultaneous sequential analysis using the complex mixture of many congeners proceeded successfully in this case.

With a view to obtain other peptide fragments, the following various conditions were applied to effect partial hydrolysis, *e.g.*, i) concentrated hydrochloric acid, 37°C, 24 hours, ii) N hydrochloric acid, room temperature, 7 days, iii) 2 N sodium hydroxide, 70°C, 8 days,

Table 1. EDMAN degradation of the basic tripeptide.

Proposed sequence	(**1**	**2**	**3**)	(Val	Ile)	(Orn, Lys)
Composition	0.42	0.54	0.15	0.92	0.30	1.00
1st Cycle	—	0.11	—	0.80	0.26	1.00
2nd Cycle	—	—	—	0.25	—	1.00
3rd Cycle	—	—	—	—	—	—

1: L-2-Amino-5-methylhexanoic acid; **2**: L-2-Amino-6-methylheptanoic acid; **3**: L-2-Amino-7-methyloctanoic acid.

Table 2. Subtractive EDMAN degradation of the peptide obtained by NBS oxidation.

Proposed sequence	(Gly)	(1	2	3)	(Val	Ile)	(Orn, Lys)	(HyGlu)	(ClTrp)
Composition	1.2	0.35	0.55	0.24	0.64	0.36	1.2	0.46	—
1st Cycle	0.32	0.30	0.45	0.11	0.70	0.30	0.75	0.41	—
2nd Cycle	t	t	—	—	0.72	0.28	0.87	0.56	—
3rd Cycle	—	—	—	—	0.21	t	1.00	0.55	—
4th Cycle	—	—	—	—	—	—	0.20	1.00	—
5th Cycle	—	—	—	—	—	—	—	—	—

1: L-2-Amino-5-methylhexanoic acid; 2: L-2-Amino-6-methylheptanoic acid; 3: L-2-Amino-7-methyloctanoic acid; HyGlu: *threo*-β-Hydroxy-L-glutamic acid; ClTrp: 5-Chloro-D-tryptophan; t: trace.

Table 3. Recovered amino acids from PTH fractions in EDMAN degradation.

Proposed sequence	(Gly)	(1	2	3)	(Val	Ile)	(Orn, Lys)	(HyGlu)	(ClTrp)
1st Cycle	++	—	—	—	—	—	—	—	—
2nd Cycle	—	0.36	0.50	0.15	t	—	t	—	—
3rd Cycle	—	t	—	—	0.84	0.16	t	—	—
4th Cycle	—	—	—	—	—	—	++	t	—
5th Cycle	—	—	—	—	—	—	—	++[a)]	—

1: L-2-Amino-5-methylhexanoic acid; 2: L-2-Amino-6-methylheptanoic acid; 3: L-2-Amino-7-methyloctanoic acid; HyGlu: *threo*-β-Hydroxy-L-glutamic acid; ClTrp: 5-Chloro-D-tryptophan; t: trace; a): detected as PTH amino acid.

iv) N sodium hydroxide, 42°C, 24 hours, v) N sodium hydroxide, 37°C, 6 days, vi) acetic acid-water (1:1), 100°C, 24 hours *etc.*, all in evacuated tubes. However, in every case, no peptide fragment other than the tripeptide described above was obtained. Furthermore, several proteolytic enzymes, including thermolysin which is known to have the specificity for cleavage at amino side of hydrophobic amino acid residues, were inactive against this cyclic peptide.

In order to elucidate the complete structure, selective chemical cleavage at carboxyl side of 5-chlorotryptophan by N-bromosuccinimide oxidation was attempted next.[6] Oxidation of longicatenamycin with excess N-bromosuccinimide gave a single ninhydrin-positive product. This result is an additional evidence for the cyclic peptide structure of longicatenamycin. The reaction process could be followed by disappearance of absorption maximum at 280 nm due to indole nucleus and the appearance of a new maximum at 320 nm.

The linear peptide obtained by N-bromosuccinimide oxidation was purified by Sephadex G-10 column and then subjected to EDMAN degradation in the usual manner. Peptides produced on successive degradations were analyzed by amino acid analysis after complete acid hydrolysis. The result is summarized in Table 2. The lower value for β-hydroxyglutamic acid in the original composition of the peptide is due to the partial decomposition of this amino acid during acid hydrolysis.[3] The phenylthiohydantoyl (PTH) amino acid produced on each step of EDMAN degradation was hydrolyzed with hydrochloric acid and the recovered amino acid was analyzed as shown in Table 3. In the case of β-hydroxyglutamic acid, detection of PTH-amino acid by thin-layer chromatography was employed in order to circumvent the problem of the acid catalysed decomposition. Again in the degradation the homologous amino acids in each pair reacted analogously throughout all of the procedures.

From these sequential analyses, the total structure of longicatenamycin was established as

depicted in Fig. 1. In this structure, hydrophobic amino acids like **2** (or **1**, **3**) and valine (or isoleucine) are located in neighboring positions, while hydrophilic amino acids like ornithine (or lysine) and β-hydroxyglutamic acid are linked in another side of the cyclic molecule. This characteristic structural feature may relate to the biological activity and the experimental fact that the tripeptide with hydrophobic amino acids was resistant to the partial acid hydrolysis where glycine, β-hydroxyglutamic acid and 5-chlorotryptophan were liberated as free amino acids.

SHOJI *et al.* observed two spots *i.e.*, LCM-I and LCM-II on thin-layer chromatogram of DNP-longicatenamycin.[7] However after we had found the existence of acid-sensitive 5-chlorotryptophan as a new component,[2,3] SHOJI *et al.* reinvestigated the isolation of the antibiotic without extensive exposure to acidic media. The sample thus obtained gave single spot on tlc with either a DNP or an acetyl derivative. Additionally, in view of the fact that LCM-II was converted to LCM-I with acid and reverse change never occurred even in alkaline solution, LCM-I seems to be an artifact attributed to the acid degradation of 5-chlorotryptophan residue in crude longicatenamycin.

From NMR study of the temperature dependence of NH protons in longicatenamycin containing all congeners, one of six CO-NH protons was found to participate in an intramolecular hydrogen bond. However, an exact assignment of the NMR signals must wait for the complete separation of congeners.

Experimental

All melting points are uncorrected. For amino acid analysis, Hitachi Amino Acid Analyzer KLA-3 was employed. Acidic and neutral amino acids were analyzed on 50-cm column using 0.2 N citric acid buffer at pH 4.25 except 2-amino-7-methyloctanoic acid (**3**) and 5-chlorotryptophan for which 15-cm column and 0.2 N citric acid buffer at pH 5.28 were used. Paper electrophoresis was carried out in a pyridine-acetic acid-water (30:4:966) buffer solution on Toyo filter paper No. 51.

2,4-Dinitrophenyllongicatenamycin. To a mixed solution of longicatenamycin (200 mg) in methanol and aqueous sodium hydrogencarbonate (400 mg), was added a methanolic solution (20 ml) of 1-fluoro-2,4-dinitrobenzene (2 g). The reaction mixture was stirred at room temperature in the dark for 2 hours. A yellow precipitate was filtered and reprecipitated from hot methanol and water. This precipitation was repeated three times; yield 150 mg; mp 256~261°C (decomp.).

α-2,4-Dinitrophenyl-L-ornithine. δ-Benzyloxycarbonyl-L-ornithine (0.5 g) prepared through the copper complex in the usual manner[8] was suspended in a mixture of water (10 ml) and ethanol (5 ml). An aqueous solution (5 ml) of sodium hydrogencarbonate (0.2 g) and an ethanolic solution (20 ml) of 1-fluoro-2,4-dinitrobenzene (0.3 g) were added to the suspension. The reaction mixture was stirred at room temperature in the dark for 2 hours. After acidification with N hydrochloric acid, it was extracted with ethyl ether. The extract was washed with water, dried with magnesium sulfate and then evaporated *in vacuo*. The residue was dissolved in acetic acid (10 ml) and treated with hydrogen bromide in acetic acid (5 ml) at room temperature for 1.5 hours. To the reaction mixture, water was added and the resulting solution was washed with ethyl ether. The solution was passed through Dowex 50 (H^+ form) column and α-DNP-ornithine was eluted with N ammonia. The eluate was evaporated *in vacuo* to obtain an oily product.[9]

α-2,4-Dinitrophenyl-L-lysine. In a similar manner to that of the ornithine derivative, α-DNP-L-lysine was prepared from ε-benzyloxycarbonyl-L-lysine.

δ-2,4-Dinitrophenyl-L-ornithine. Crystals of δ-DNP-L-ornithine hydrochloride (360 mg) was

obtained from L-ornithine hydrochloride (500 mg) through the copper complex according to the procedure of F. SANGER,[9] mp 215~218°C (decomp.). Found: C, 39.11; H, 4.57; N, 16.45; Cl, 10.37%. Calcd. for $C_{11}H_{14}O_6N_2 \cdot HCl$: C, 39.47; H, 4.52; N, 16.74; Cl, 10.59%.

ε-2,4-Dinitrophenyl-L-lysine. This compound was prepared in a similar manner to that of δ-DNP-L-ornithine.

Hydrolysis of 2,4-Dinitrophenyllongicatenamycin. DNP-longicatenamycin (25 mg) was hydrolyzed with 6 N hydrochloric acid (3 ml) at 110°C for 24 hours. The DNP-amino acid formed showed a positive ninhydrin reaction and was insoluble in ethyl ether. It moved as a neutral amino acid on paper electrophoresis. Rf values on thin-layer chromatogram on developing with methyl ethyl ketone-ethyl ether (3:2) were: DNP-amino acid obtained by the hydrolysis, 0.20; synthetic α-DNP-ornithine (or α-DNP-lysine), 0.10; synthetic δ-DNP-ornithine (or ε-DNP-lysine) 0.20.

Partial Hydrolysis of Longicatenamycin. Longicatenamycin was partially hydrolyzed with 6 N hydrochloric acid in an evacuated tube at 110°C for 1.5 hours. The hydrolyzate was subjected to paper electrophoresis and each band was cut off and extracted with a mixture of water and acetic acid (1:1). β-Hydroxyglutamic acid was detected in an acidic part on tlc, and glycine and 5-chlorotryptophan in a neutral part. From a basic part, a single spot of a peptide was detected on tlc, Rf: 0.42 (*n*-butanol-acetic acid-water, 4:1:2); 0.15 (methyl ethyl ketone-pyridine-water-acetic acid, 35:5:5:1). The subtractive EDMAN degradation of this basic peptide was carried out by the usual procedure.[10] The result is given in Table 1.

N-Bromosuccinimide Oxidation of Longicatenamycin. To a solution of longicatenamycin (100 mg, 0.12 mM) in a mixture of acetic acid (4 ml) and methanol (4 ml), was added N-bromosuccinimide (178 mg, 1 mM) in methanol (4 ml) and the mixture was allowed to stand at room temperature for 50 minutes. After decomposition of excess N-bromosuccinimide with formic acid (2 ml) for 30 minutes, the reaction mixture was concentrated below 35°C. The syrup thus obtained was dissolved in water, and was subjected to chromatography on a Sephadex G-10 column (1.5×50 cm) with water elution. Fractions showing UV absorption at 320 nm were combined and concentrated *in vacuo*. Lyophilization produced a white powder, yield 70 mg.

This linear peptide was subjected to EDMAN degradation. In each step, the phenylthiohydantoyl (PTH) amino acid obtained was hydrolyzed with 6 N hydrochloric acid at 135°C for 12 hours to recover free amino acid which was analyzed by amino acid analyzer. PTH-β-hydroxyglutamic acid obtained in the final degradation step was detected by comparison with authentic sample on tlc, Rf: 0.10 (chloroform-methanol, 9:1); 0.12 (chloroform-methanol-water 45:12:1); 0.65 (chloroform-methanol-acetic acid, 15:5:1); 0.44 (*n*-butanol saturated with water). Furthermore, the residual peptides of each degradation were analyzed after complete hydrolysis with 6 N hydrochloric acid at 110°C for 24 hours.

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