THE STRUCTURE OF POLYMYXIN S_1
(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XXI[1])

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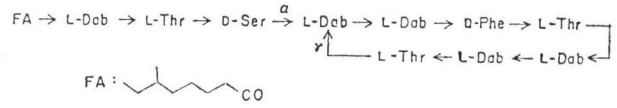
(Received for publication October 3, 1977)

Amino acid analysis on the acid hydrolyzate of polymyxin S_1 revealed its amino acid composition. Isolation of the constituent amino acids and measurement of their optical activities clarified their chiralities: Dab(5L), Thr(3L), Ser(1D) and Phe(1D). The constituent fatty acid was identified with anteisononanoic acid by gas chromatography and mass spectrometry. By the action of polymyxin acylase, deacyl polymyxin S was obtained. Successive EDMAN degradation reaction on deacyl polymyxin S revealed the amino acid sequence. Further evidence for the structure of polymyxin S_1 was obtained by partial acid hydrolysis on tetra-(DNP)-polymyxin S_1.

The isolation of polymyxin S_1 from the culture broth of *Bacillus polymyxa* Rs-6, which is active against Gram-negative bacteria, has been reported in the preceding paper[1]. Structural studies described here led to the structure of polymyxin S_1 as shown in Fig. 1.

Fig. 1. Structure of polymyxin S_1



When polymyxin S_1 was hydrolyzed with hydrochloric acid and the hydrolyzate was analyzed with an automatic amino acid analyzer, 2,4-diaminobutyric acid (abbreviated as Dab hereafter), Ser, Thr and Phe were found in the ratio listed in Table 1. These amino acids were isolated from the hydrolyzate by preparative paper chromatography. Their molecular rotations and ORD curves were measured. From comparison of their molecular rotations with those of the pure isomers (Table 2) and their COTTON effects in the ORD curves, Dab and Thr were deduced to be L-form, and Ser and Phe to be D-form. Thus, the constituent amino acids of polymyxin S_1 were concluded to be Dab(5L), Thr(3L), Ser(1D) and Phe(1D).

Table 1. Amino acid analysis of polymyxin S_1 tetrahydrochloride

	Thr	Ser	Phe	Dab*
Found (μmoles/mg)	2.10	0.68	0.70	3.54
(moles/mole)	(3)	(1)	(1)	(5)

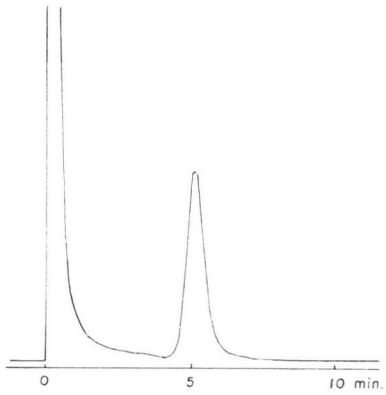
* 2,4-Diaminobutyric acid.

The acid hydrolyzate of polymyxin S_1 was extracted with ethyl ether. When the ethereal extract was methylated and then analyzed with gas chromatography, a main peak was observed as illustrated in Fig. 2. The retention time of the peak was recognized to be identical with that of methyl anteisononanoate which was prepared from polymyxin E. The methylated product was then subjected to gas chromatography-mass spectrometry, which gave supporting evidence for the presence of anteisononanoic acid. This fatty acid commonly occurs in polymyxin group of antibiotics, together with isooctanoic acid. We also have reported the presence of this fatty acid in brevistin[8].

Table 2. Comparison of molecular rotations

Amino acid	$[M]_D$ (in 5 N HCl)
Isolated Dab	$+38.3\pm1.9°$
L-Dab	$+39.0\pm1.1°$
Isolated Thr	$-17.5\pm2.9°$
L-Thr	$-17.9°$
Isolated Ser	$-12.7\pm2.4°$
L-Ser	$+15.9°$
Isolated Phe	$+8.1\pm3.6°$
L-Phe	$-7.4°$

Fig. 2. Gas chromatogram of fatty acid methyl ester from polymyxin S_1



When polymyxin S_1 was reacted with 2,4-dinitrofluorobenzene, tetra(DNP)-polymyxin S_1 was produced. Acid hydrolysis of the dinitrophenyl derivative of polymyxin S_1 released four moles of γ-DNP-Dab and one mole of free Dab and the unsubstituted residues: three moles of Thr and one mole each of Ser and Phe. This means that only the γ-amino groups of four Dab residues are free and the N-terminal amino group is acylated. Furthermore, a ring structure with a branched chain of a peptide was suggested, in which one Dab residue occurs at the branching point. In the IR spectrum of polymyxin S_1 tetrahydrochloride, the presence of peptide bonds could be observed, but lactone or ester linkages were absent. Paper electrophoresis indicated only basic character. A molecular formula $C_{53}H_{91}N_{15}O_{15}$ anticipated from the above data coincides with the data of elemental analysis already reported[1].

An enzyme preparation, polymyxin acylase from the cells of *Pseudomonas* sp. M-6-3, which is able to deacylate polymyxin E has been reported[4]. By its action, we have previously succeeded in the deacylation of cerexin A[2] and brevistin[3]. Polymyxin S_1 was easily deacylated by the action of this enzyme to afford deacyl polymyxin S.* In an attempt of successive EDMAN degradation reaction on de-

Table 3. EDMAN degradation of deacyl polymyxin S

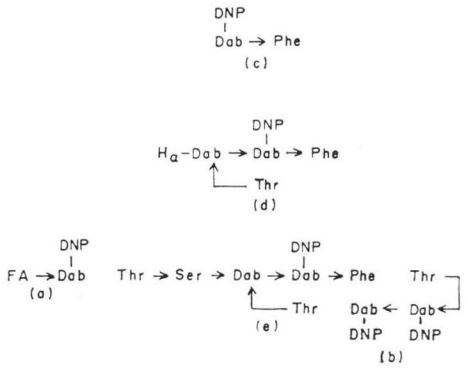
	PTH-Amino acid	Amino acid found (in ratio)			
		Dab	Thr	Ser	Phe
Original peptide	—	5.13	3.00	1.09	1.00
1st step	γ-PTC-Dab*	3.71	3.00	0.97	0.86
2nd step	Thr, ΔThr	3.38	2.00	0.73	0.80
3rd step	Ser**	3.49	2.00	0.31	1.05
4th step	—	2.86	2.00	0.00	1.01
5th step	γ-PTC-Dab	2.08	2.00	0.00	0.83
6th step	Phe	2.10	2.00	0.00	0.47
7th step	Thr, ΔThr	—	—	—	—
8th step	γ-PTC-Dab	—	—	—	—
9th step	γ-PTC-Dab	—	—	—	—
10th step	Thr, ΔThr	—	—	—	—

* Phenylthiohydantoin of γ-phenylthiocarbamyl-2,4-diaminobutyric acid.
** Observed only in a poor amount.

* The subscript number, which implies differentiation of the fatty acyl residue, is not needed for the deacyl derivative.

acyl polymyxin S, some modifications in the procedure were necessarily required. During the reaction, the uncovered Dab residues are converted to γ-phenylthiocarbamyl-2,4-diaminobutyric acid (γ-PTC-Dab) residues, consequently the peptide becomes considerably lipophylic, which causes a considerable loss in the remaining peptide. Therefore the procedure of the EDMAN degradation[5] was modified as follows: The solvents, benzene and ethylenechloride, used for extraction of an excess phenylisothiocyanate (PTC) and phenylthiocarbamyl amino acid (PTC-amino acid) were replaced by cyclohexane and ethyl ether, respectively. Opening of a peptide ring by EDMAN degradation was successfully carried out in the work on octapeptins (EM49) by PARKER and RATHNUM[6]. The 4th step of successive EDMAN degradation reaction on deacyl polymyxin S suggested ring-opening, because the usual procedure in this step resulted in a poor yield of the remaining peptide and only a trace amount of the PTH-amino acid. This was also anticipated from the known structures of polymyxin group antibiotics. Therefore, the PTC-peptide in the 4th step was heated for a prolonged period in trifluoroacetic acid (TFA) to complete the formation of the thiazolinone derivative of the linear heptapeptide, which was then converted to the corresponding phenylthiohydantoin by heating with aqueous acid. The result thus obtained is shown in Table 3. This apparently suggested the amino acid sequence as shown in Fig. 1.

Fig. 3. Peptide fragments obtained from partial hydrolysis of tetra(DNP)polymyxin S_1.



Further evidence for the structure of polymyxin S_1 was obtained as follows: Tetra(DNP)-polymyxin S_1 was partially hydrolyzed with a mixture of formic acid and conc. hydrochloric acid at 37°C. The hydrolyzate was separated by TLC, affording five DNP-peptide fragments (*a*, *b*, *c*, *d* and *e*) whose sequences were deduced by amino acid compositions and determination of N-terminus as shown in Fig. 3. From these, the structure of polymyxin S_1 was unequivocally determined (Fig. 1). The fragment (*d*) was a key peptide which provided the conclusive evidence for the mode of branching in polymyxin S_1.

Polymyxin S_1 is most closely related to polymyxin D_1 in structure. The only difference is replacement of D-Leu in polymyxin D_1 by D-Phe in polymyxin S_1.

Experimental

ORD curves were recorded with a JASCO Model ORD/UV-5 spectropolarimeter. $[\alpha]_D$ was measured with a Perkin-Elmer Model 141 polarimeter. GC-MS was measured with a Hitachi RMV-6 gas chromatograph-mass spectrometer.

Amino acid analysis was carried out with an amino acid analyzer Hitachi KLA-5 under the normal conditions directed for the instrument except for the following case. When quantitation of γ-DNP-Dab was needed, the procedure was modified as follows: 0.35 M sodium citrate buffer solution, pH 5.28, for the short column was replaced by 0.525 M sodium citrate buffer solution, pH 5.28, because under the normal conditions the peak of γ-DNP-Dab partially overlapped with the peak of ammonia. The preparation of α-DNP-Dab and γ-DNP-Dab used as reference samples have already been reported[8]. The peptides to be analyzed were hydrolyzed with constant boiling hydrochloric acid for 20 hours at 110°C except where stated otherwise.

Amino acid composition of polymyxin S_1

A few mg of polymyxin S_1 tetrahydrochloride was hydrolyzed and the hydrolyzate was analyzed for amino acid composition in the manner described above. The amino acids found are listed in Table 1.

Isolation of constituent amino acids

Polymyxin S_1 tetrahydrochloride, 160 mg, was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzate was extracted with ethyl ether. The ethereal extract was used for analysis of fatty acid as described in the next section. The aqueous residue was concentrated to dryness and applied to three sheets of Toyo Roshi No. 51 (60×60 cm), which were developed with *n*-butanol - acetic acid - water (4: 1: 2) in descending manner. Separated zones of Dab, Ser, Thr and Phe (zones of Ser and Thr were partly overlapped) were extracted with water. The fractions of Ser and Thr were rechromatographed with the same system. Each of the fractions was adsorbed on a small column of Dowex 50×8 (NH_4 form), which was washed with water and eluted with 0.5 N NH_4OH. By evaporation of the eluates Dab (75 mg), Phe (12 mg), Ser (6.5 mg) and Thr (28 mg) were obtained as colorless powders. The sample of Dab was dissolved in a small amount of dil.HCl and evaporated to dryness. The residue was crystallized from water and ethanol to give colorless crystals.

2,4-Diaminobutyric acid monohydrochloride:

Anal. Found: C, 31.13; H, 7.28; N, 18.23; Cl, 22.87.
Calcd. for $C_4H_{10}N_2O_2 \cdot HCl$: C, 31.07; H, 7.17; N, 18.12; Cl, 22.94.

$[\alpha]_D^{25.0}$ +24.7±1.2° (*c* 0.519, 5 N HCl)

ORD: $[\phi]_{209}$ 0, $[\phi]_{223}$ +1610, $[\phi]_{250}$ +520 (*c* 0.2820, 0.5 N HCl)

Phenylalanine:

Anal. Found: C, 65.19; H, 6.46; N, 8.31.
Calcd. for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.31.

$[\alpha]_D^{25.0}$ +4.9±2.2° (*c* 0.203, 5 N HCl)

ORD: $[\phi]_{218.5}$ 0, $[\phi]_{225}$ −5900, $[\phi]_{250}$ −1000 (*c* 0.2483, 0.5 N HCl)

Serine:

Anal Found: C, 34.20; H, 6.70; N, 13.23.
Calcd. for $C_3H_7NO_3$: C, 34.28; H, 6.71; N, 13.33.

$[\alpha]_D^{25.0}$ −12.1±2.3° (*c* 0.223, 5 N HCl)

ORD: $[\phi]_{214}$ 0, $[\phi]_{226}$ −1640, $[\phi]_{250}$ −400 (*c* 0.2820, 0.5 N HCl)

Threonine:

Anal. Found: C, 40.22; H, 7.41; N, 11.47.
Calcd. for $C_4H_9NO_3$: C, 40.33; H, 7.62; N, 11.76.

$[\alpha]_D^{25.0}$ −14.7±2.4° (*c* 0.224, 5 N HCl)

ORD: $[\phi]_{214.5}$ 0, $[\phi]_{226}$ +1710, $[\phi]_{250}$ +310 (*c* 0.3062, 0.5 N HCl)

Identification of the constituent fatty acid

The ethereal extract of the acid hydrolyzate of polymyxin S_1 was methylated with diazomethane. When it was analyzed by GC on a Perkin-Elmer Model 881 equipped with a steel column (6 feet, 1/8-inch diameter) packed with 15% diethylene glycol succinate polymer on Chromosorb W 80~100 mesh at 105°C, a main peak of a retention time of 5.14 minutes was observed. The reference compounds, methyl isooctanoate and anteisononanoate, prepared from polymyxin E, showed retention times of 2.95 and 5.14 minutes under the same condition. When the preparation was subjected to GC-MS the following peaks were observed: *m/e*, 172 (M^+), 157 ($M-CH_3$), 143 ($M-CH_3$-CH_2), 141 ($M-OCH_3$), 115 ($M-CH_3$-CH_2-$CH(CH_3)$), 87 (CH_2–CH_2–$COOCH_3$), 74 (base peak, H_2C=C(OH)–OCH_3) *etc.* In the spectrum intensities of the peaks of 143 and 115 were relatively large, indicating the anteiso-structure of the fatty acid ester.

Deacyl polymyxin S

Polymyxin S_1 tetrahydrochloride (20 mg, 15 μmole) was dissolved on 0.05 M phosphate buffer pH 7.40 (2 ml). Five mg of polymyxin acylase (acetone powder of *Pseudomonas* sp. M-6-3)[4] was added

to the solution, which was then stirred for one day at 37°C. The reaction mixture was centrifuged (3,000 rpm, 10 minutes), and the supernatant was applied to a sheet of Toyo Roshi No. 51, 60×60 cm, which was developed with *n*-butanol - acetic acid - water (4: 1: 2). The zone of deacyl polymyxin S detectable by ninhydrin coloration (Rf *ca.* 0.03) was cut out and extracted with water. Lyophylization gave a colorless powder of deacyl polymyxin S (6.3 μmole).

EDMAN degradation of deacyl polymyxin S

For successive EDMAN degradation reaction on deacyl polymyxin S, the following modifications were made in the published procedure[5] in order to diminish the loss of the remaining peptide. Excess phenylisothiocyanate (PTC) was removed by extraction with cyclohexane twice and then a mixture of cyclohexane and benzene (1: 1) twice, and PTC-amino acid was extracted with ether three times. Furthermore, the PTC-peptide at the 4th step of the reaction was heated at 40°C for 180 minutes in trifluoroacetic acid and then at 80°C for 10 minutes in a mixture of acetonitrile - 2 N HCl (1: 1), because opening of the peptide ring was anticipated at this step. Approximate 6.3 μmole of deacyl polymyxin S was subjected to the above procedure. After the 7th step of this experiment, the analysis of the remaining peptide (subtractive mode) was omitted, because its quantity became extremely small. The extraction of the PTC-amino acid was performed with ethylenechloride after the 8th step. The PTH-amino acids were identified by TLC[5]. The results of the degradation are presented in Table 3.

Tetra(DNP)-polymyxin S_1

Polymyxin S_1 tetrahydrochloride (30 mg) and $NaHCO_3$ (87 mg) were dissolved in water (0.5 ml). A 10% 2,4-dinitrofluorobenzene solution in ethanol (1.0 ml) was added. The mixture was stirred for 16 hours in the dark at room temperature. The reaction mixture was diluted with water and extracted with ethyl acetate. The extract was washed with $NaHCO_3$ solution, water and dil.HCl, and then concentrated to dryness. The residue was dissolved into warm ethyl acetate and the solution was allowed to stand at room temperature to give a yellow precipitate. This was collected by filtration and washed with ether. A yellow powder of tetra(DNP)-polymyxin S_1 (28 mg) was obtained. Acid hydrolysis followed by amino acid analysis revealed the following residues (in ratio): Dab (1.06), γ-DNP-Dab (3.48), Thr (3.00), Ser (0.84) and Phe (1.11).

Partial hydrolysis of tetra(DNP)-polymyxin S_1

Tetra(DNP)-polymyxin S_1 (14 mg) was dissolved in a mixture of formic acid (2.0 ml) and conc. hydrochloric acid (2.0 ml). The mixture was stood for 64 hours at 37°C, and then evaporated to dryness. The residue was applied to three plates of Precoated Silica Gel GF Plate, (20×20 cm, Merck), which were developed with chloroform - ethanol - 14% aqueous ammonia (5: 7: 2). The separated yellow zones were extracted with a mixture of chloroform, methanol and conc. ammoniacal water (2: 2: 1). The extracts were examined by TLC in another solvent system to confirm their homogenities. Thus, five peptide fragments were obtained, with approximate Rf values as follows:

Fragment	Rf value		
	CEN	CM	BAW
a	0.90	0.80	—
b	0.41	0.27	—
c	0.33	—	0.46
d	0.25	—	0.29
e	0.20	—	0.38

Plate: Precoated silica gel GF (Merck)
CEN: Chloroform - ethanol - 14% ammoniacal water (5: 7: 2)
CM: Chloroform - methanol (1: 1) containing 1% formic acid
BAW: *n*-Butanol - acetic acid - water (4: 1: 2)

A portion of each fragment was hydrolyzed to determine the amino acid composition. Another portion was further dinitrophenylated and then hydrolyzed. The acid aqueous solution of the latter hydrolyzate was extracted with ether. The ethereal extract was tested by TLC for DNP-amino acid,

and the aqueous portion was analyzed with an amino acid analyzer. Identification of DNP-amino acid was carried out on the precoated silica gel plate with the same solvents as above. The Rf values of the DNP-amino acids were as follows:

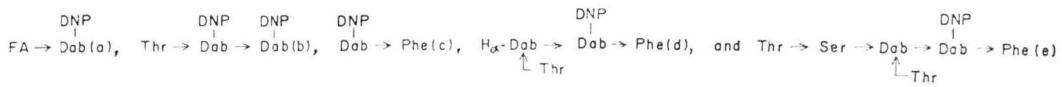
DNP-Amino-acid	Rf value	
	CEN	CM
α,γ-bis(DNP)-Dab	0.71	0.48
DNP-Ser	0.35	0.40
DNP-Thr	0.42	0.44

The amino acids found with these peptide fragments before and after dinitrophenylation and the DNP-amino acids detected by TLC are listed in Table 4.

Table 4. Amino acid found (in ratio)

Fragment	Dab	α-DNP -Dab	γ-DNP -Dab	Thr	Ser	Phe	DNP-Amino acid detected by TLC
a			+				
(After DNP)			+				
b			2.00	1.14			
(After DNP)			2.00	0.11			DNP-Thr
c			0.95			1.00	
(After DNP)			0.0			1.00	bis(DNP)-Dab
d	1.04		0.84	1.04		1.00	
(After DNP)	0.0	0.62	0.63	0.0		1.00	DNP-Thr
e	1.15		0.82	1.95	1.00	1.00	
(After DNP)	1.00		0.60	0.42	0.93	1.00	DNP-Thr

From these results, the following sequences were deduced for these peptide fragments:



Acknowledgement

The authors wish to thank to Prof. YUKIO KIMURA of Mukogawa Women's University for his kind supply of an enzyme preparation of polymyxin acylase. The authors are indebted to Dr. YUZO NAKAGAWA for measurement of GC-MS, and also Dr. KAORU KURIYAMA and TATSUO IWATA for Measurement of ORD and CD, the members of Shionogi Research Laboratory.

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