Communications to the editor

THE STRUCTURES OF TWO NEW POLYMYXIN GROUP ANTIBIOTICS

Sir:

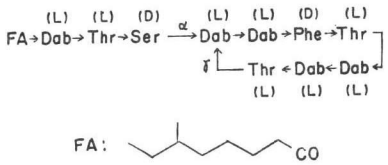
Two new members of the polymyxin group of antibiotics, named polymyxins S_1 and T_1, have been isolated in our laboratory from culture broths of strains identified as *Bacillus polymyxa* Rs-6 and *Bacillus polymyxa* E-12, respectively. These antibiotics are strong basic substances soluble in water, and are primarily active against Gram-negative bacteria *in vitro* and *in vivo*, though polymyxin T_1 exhibits somewhat higher activities against Gram-positive bacteria than other polymyxin group antibiotics do.

The hydrochloric acid salts of polymyxins S_1 and T_1 were obtained as colorless amorphous powders, whose molecular formulae were indicated by elemental analysis to be $C_{53}H_{91}N_{15}O_{15}\cdot 4HCl\cdot H_2O$ and $C_{58}H_{102}N_{16}O_{12}\cdot 5HCl\cdot 2H_2O$ respectively. The infrared absorption spectra of both the antibiotics indicated the presence of peptide bond, but the absence of lactone and carboxyl function.

Automatic amino acid analyses[1] carried out with Hitachi KLA-5 on the acid hydrolyzates of both the antibiotics revealed the amino acid composition of polymyxin S_1 to be Dab* (5), Thr (3), Ser (1) and Phe (1), and that of polymyxin T_1 to be Dab (6), Thr (1), Leu (2) and Phe (1). These amino acids were isolated from the hydrolyzates by preparative paper chromatography and the use of a porous polymer Amberlite XAD-2 column. From their $[M]_D$ values and ORD curves measured, it was concluded that only Ser and Phe in polymyxin S_1 and Phe in polymyxin T_1 were present in D-configurations, and that all other amino acids were present in L-configurations. The fatty acids liberated in the acid hydrolyzates of polymyxins S_1 and T_1 were extracted with ether, methylated and analyzed with gas chromatography.[1] A main peak of identical retention time with methyl anteisononanoate was observed with both the specimens, and the identification was confirmed with gas chromatography-mass spectrometry.[1] By the action of Polymyxin Acylase,[1,2] deacyl polymyxin S_1 and deacyl polymyxin T_1 were readily prepared.

A successive EDMAN degradation reaction was applied to deacyl polymyxin S_1 (6.0 μmoles) with some modifications. 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 in order to diminish the loss of the remaining peptide. The PTC-peptide in the 4th step of the reaction was heated at 40°C for 180 minutes in TFA and then at 80°C for 10 minutes in a mixture of acetonitrile - 2 N HCl (1 : 1), because the formation of PTH-peptide (linear) from PTC-peptide (ring) followed opening the peptide ring was anticipated at this step. The PTH-peptide thus formed was used for the next step of the reaction. This successive reaction proceeded up to the 10th step, revealing the presumable amino acid sequence of polymyxin S_1 as in Fig. 1.

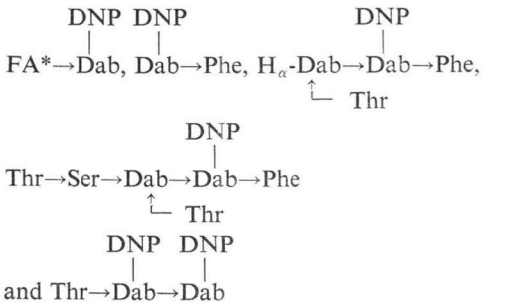
Fig. 1. Structure of polymyxin S_1.



Further evidence for the sequence and the branching mode of the peptide ring with a branched chain was obtained as below. Tetra (DNP)-polymyxin S_1 was prepared in the usual way. Amino acid analysis with the acid hydrolyzate indicated it to contain one mole of Dab and four moles of γ-DNP-Dab other than the remaining amino acids. This DNP-derivative (approx. 10 mg) was partially hydrolyzed with a mixture of formic acid and conc.HCl (1 : 1) at room temperature or at 37°C. Several DNP-peptide fragments were isolated by TLC on silica gel with chloroform - ethanol - 14% aqueous ammonia (5 : 7 : 2). A portion of each fragment was hydrolyzed to determine the amino acid composition. Another portion was further dinitrophenylated and then hydrolyzed. Amino acid analysis and detection of DNP-amino acid by TLC were performed with the hydrolyzate. From these results, the following sequences

* Dab: 2,4-Diaminobutyric acid.

were determined for these fragments:



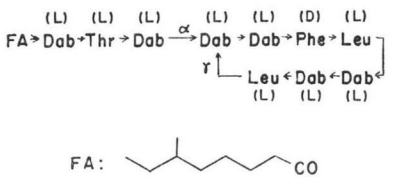
From the above, we concluded the structure of polymyxin S_1 to be as indicated in Fig. 1.

A successive EDMAN degradation reaction on deacyl polymyxin T_1 (approx. 6.0 μmoles) was also carried out as in polymyxin S_1. However, the loss of remaining peptide was larger in this case, because this peptide contained more residues of lipophilic amino acid. Therefore, the result of this reaction did not clarify the sequence beyond the 7th step, but the sequence, Dab→Thr →Dab→Dab→Dab→Phe→Leu, was identified.

Penta (DNP)-polymyxin T_1 was prepared and acid hydrolysis revealed this contained one mole of Dab and five moles of γ-DNP-Dab, similarly. This DNP-derivative was subjected to the cleavage reaction developed for threonyl peptide.[3] Penta (DNP)-polymyxin T_1 (22 mg) was dissolved in dimethylsulfoxide (0.25 ml), trifluoroacetic acid (2.5 μl) and pyridine (5 μl), and dicyclohexylcarbodiimide (DCC) (44 mg) was added. After stirring for a while, the mixture was allowed to stand for 16 hours at room temperature. The reaction mixture was diluted with ethyl acetate. The excess DCC was destroyed by oxalic acid (44 mg), and the resulting precipitate of dicyclohexylurea (DCU) was filtered off. The ethyl acetate solution was washed with saturated $NaHCO_3$ solution and then saturated NaCl solution, and dried (Na_2SO_4). The residue obtained by evaporation of ethyl acetate was triturated with benzene, giving a yellow powder of an intermediate ketone derivative. The yellow powder was then dissolved in acetic acid (1.35 ml), methanol (0.54 ml) and water (0.27 ml). After addition of hydroxylamine hydrochloride (20 mg), the solution was heated at 80°C for 40 minutes. The reaction mixture was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution, water, 1 N HCl and saturated NaCl solution, and dried (Na_2SO_4). After concentration, the reaction product was then purified by TLC on silica gel with chloroform - methanol (1 : 1), being obtained as a yellow powder (4 mg). Acid hydrolysis and amino acid analysis indicated that the product was the DNP-octapeptide formed by cleavage at the Thr residue.

* FA means fatty acyl residues.

Fig. 2. Structure of polymyxin T_1.



EDMAN degradation on this DNP-octapeptide was carried out with the same modification as in our investigation on tetra(DNP)-deacyl octapeptin C_1[4]. This reaction proceeded well, and the result clearly indicated the sequence of this octapeptide to be as indicated in the structure given in Fig. 2. Furthermore, when the remaining peptide of the 1st step of the EDMAN degradation reaction, a cyclic heptapeptide, was dinitrophenylated and hydrolyzed, approximately one mole of α-DNP-Dab was obtained in addition to the other residues. This provided the conclusive evidence for the branching mode in the peptide ring.

Thus, the structure of polymyxin T_1 has been concluded to be as given in Fig. 2.

Throughout these experiments, when quantitative analyses of α-DNP-Dab and γ-DNP-Dab were needed, the procedure for automatic amino acid analysis by the Hitachi KLA-5[1] 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.

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