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The Chemistry of the Polymyxin Antibiotics

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1. *Introduction*

Despite the fact that most of the antibiotics belonging to the polymyxin group have been known since the period between 1947 and 1950 and that some of them have been in clinical use for many years, their structures were practically unknown until very recently. They have been considered mostly as 'minor antibiotics', although they are actually the most active inhibitors of the growth of gram-negative bacteria. Mainly their side effects have limited their wide clinical application. New methods in analysis and synthesis of polypeptides have allowed the structures and chemical relationships in this group of antibacterial substances to be revealed in recent years. It is now possible to give a better classification and to help in this way to clarify some of the confusion existing generally in the biological and medical literature[1].

Several reviews[2–5] are available dealing mainly with biological and clinical aspects. This article is therefore confined to the chemistry of these antibiotics.

2. *Discovery, isolation and nomenclature*

In 1947, the discovery of antibiotic substances from *B. polymyxa* and *B. aerosporus* was announced almost simultaneously by three independent groups. STANSLY et al.[6] described the production, isolation and purification of an active principle from *B. polymyxa* under the name of 'polymyxin', while AINSWORTH et al.[7] reported on the antibiotic 'aerosporin' obtained from *B. aerosporus* Greer. BENEDICT and LANGLYKKE[8] described a medium for obtaining culture filtrates active against *Brucella bronchiseptica*. All three antibiotics had an almost selective bacteriostatic and bactericidal activity against gram-negative bacteria. It soon became apparent that the three groups were dealing with a family of chemically and biologically closely related polypeptides[9]. Comparative chemical and biological studies were undertaken in collaboration between the groups and the results discussed at a symposium organized by the New York Academy of Sciences in 1948[10]. 'Polymyxin' was accepted as generic name for these antibiotics and alphabetical suffixes were adopted for the different types which differed in their amino acid composition. Aerosporin thus became polymyxin A and STANSLY's antibiotic polymyxin D. Other active principles from different strains of *B. polymyxa* were designated as polymyxin B, C and E. Polymyxin B and polymyxin E were later fractionated by counter-current distribution into two components, which were named B_1, B_2[11] and E_1, E_2[12,13] respectively. Only very recently SUZUKI et al. fractionated crude polymyxin D by the same method and named the main biologically active entities polymyxin D_1 and D_2[14].

In 1948, MURRAY and TETRAULT[15] isolated an antibiotic from a non-hemolytic strain of *B. circulans* under the name of 'circulin'. It resembled the polymyxin-type antibiotics chemically and biologically and was later fractionated into two components called circulin A and B, besides possibly some other components[16,17].

In the same year MCLEOD[18] described an antibiotic principle from a culture of *B. krzemieniewsky*, a mucoid

[1] The abbreviations are in accordance with the suggestions of the Committe on Nomenclature at the Fifth European Peptide Symposium (Pergamon Press, Oxford 1963). Z = benzyloxycarbonyl, Tos = tosyl, BOC = *t*-butyloxycarbonyl, PHT = phthaloyl, DNP = dinitrophenyl, OBu^t = tertiary butylester; MOA = (+)-6-methyloctanoic acid, IOA = 6-methylheptanoic acid.

[2] P. G. STANSLY, Am. J. Med. *7*, 807 (1949).

[3] E. JAWETZ, *Antibiotic Monographs*, No. 5 (Medical Encyclopedia, Inc., New York 1956), p. 11.

[4] B. SCHWARTZ, *Experimental Chemotherapy* (Academic Press, New York, London 1964), Vol. III, p. 217.

[5] R. O. STUDER, *Progress in Medicinal Chemistry* (Ed. ELLIS and WEST, Butterworths, London), Vol. 5, in press.

[6] P. G. STANSLY, R. G. SHEPARD, and H. J. WHITE, Bull. Johns Hopkins Hosp. *81*, 43 (1947).

[7] G. C. AINSWORTH, A. M. BROWN, and G. BROWNLEE, Nature *160*, 263 (1947).

[8] R. G. BENEDICT and A. F. LANGLYKKE, J. Bact. *54*, 24 (1947).

[9] T. S. G. JONES, Biochem. J. *43*, 26 (1948).

[10] *Symposium 1948*, Ann. N.Y. Acad. Sci. *51*, 855 (1949).

[11] W. HAUSMANN and L. C. CRAIG, J. Am. chem. Soc. *76*, 4892 (1954).

[12] S. WILKINSON and L. A. LOWE, J. chem. Soc. *1964*, 4107.

[13] T. SUZUKI, K. HAYASHI, K. FUJIKAWA, and K. TSUKAMOTO, J. Biochem., Tokyo *57*, 226 (1965).

[14] T. SUZUKI, K. HAYASHI, Y. SUKETAN, and K. TSUKAMOTO, Experientia *22*, 354 (1966).

[15] F. J. MURRAY and P. A. TETRAULT, Proc. Soc. Am. Bact. *1*, 20 (1948).

[16] D. H. PETERSON and L. N. REINECKE, J. biol. Chem. *181*, 95 (1949).

[17] J. H. DOWLING, H. KOFFLER, H. C. REITZ, D. H. PETERSON, and P. A. TETRAULT, Science *116*, 147 (1952).

[18] C. MCLEOD, J. Bact. *56*, 749 (1948).

variant of *B. circulans*, and called it 'circulin'. Because it soon became obvious that this substance differed from the circulin described by MURRAY and TETRAULT[15], it was renamed polypeptin by mutual agreement between the groups involved[19].

Colistin was isolated in 1950 by KOYAMA et al.[20,21] from cultures of *Aerobacillus colistinus*, an organism taxonomically related to *B. polymyxa*. Later it became known also under the name of colomycin, colimycine or colo-mycin, but it should not be confused with the Russian antibiotic colimycin[22], which is related to neomycin. Commercial colistin was later separated into the three components A, B and C[23,24]. Very recently it was shown that colistin A and polymyxin E_1 are identical, as are colistin B and polymyxin E_2[12,13].

The youngest member of the family, polymyxin M, was isolated in 1958 from a strain of *B. polymyxa* obtained from soil near Moscow[25,26].

3. *Amino and fatty acid composition*

All the members of the polymyxin group are closely related in their chemical, physicochemical and biological properties. They are all basic cyclopeptides with a molecular weight in the region of 1200 containing as characteristic constituents α,γ-diaminobutyric acid, L-threonine and a fatty acid. But it is possible to distinguish between them by the presence of the additional amino acids D-leucine, L-leucine, L-isoleucine, D-phenylalanine, D-valine and D-serine as well as by the nature of the fatty acid. The latter can be either (+)-6-methyloctanoic acid or 6-methylheptanoic acid (Table I).

4. *Structural analysis*

(1) *Polymyxin B_1*. Although several structural features of polymyxin had been determined soon after its isolation[10], the exact chemical studies began in 1954, when HAUSMANN and CRAIG[11] succeeded in separating commercial polymyxin by counter-current distribution into two homogenous fractions called polymyxin B_1 and B_2. The B_1 fraction was subsequently used first in the form of its pentahydrochloride by HAUSMANN for structural analysis. By the method of partial substitution introduced by BATTERSBY and CRAIG[27], the molecular weight of the polypeptide base was determined as 1150 ± 10%. $6M$ of α,γ-diaminobutyric acid, predominantly in the L-form, $2M$ of L-threonine and $1M$ each of L-leucine and D-phenylalanine were found after total hydrolysis together with an optically active fatty acid, which had already been identified in 1949 by WILKINSON[28] as (+)-6-methyloctanoic acid. A careful search for either a free α-carboxyl or a free α-amino group in the intact molecule gave negative results and therefore a cyclic structure[29,30], already envisaged by P. H. BELL et al.[31] in 1949, was proposed.

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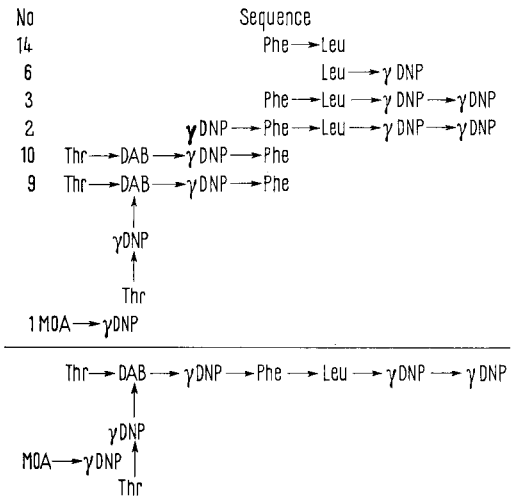
[26] A. S. KHOKHLOV and CH'IH CH'ANG-CH'ING, Biokhimiya *26*, 296 (1961).
[27] A. R. BATTERSBY and L. C. CRAIG, J. Am. chem. Soc. *73*, 1887 (1951).
[28] S. WILKINSON, Nature *164*, 622 (1949).
[29] Considering the recently elucidated structures of the gramicidins A and B[30], which are protected at the *N*-terminus by a formyl group and at the carboxyl end by ethanolamine, this reasoning is no longer correct.
[30] R. SARGES and B. WITKOP, J. Am. chem. Soc. *87*, 2011, 2020, 2027 (1965).
[31] P. H. BELL, J. F. BONE, J. P. ENGLISH, C. E. FELLOWS, K. S. HOWARD, M. M. ROGERS, R. G. SHEPHERD, and R. WINTERBOTTOM, Ann. N.Y. Acad. Sci. *51*, 897 (1949).

Table I. Comparative amino acid and fatty acid composition of the polymyxins

Name	Dab	L-Thr	L-Leu	D-Leu	L-Ile	D-Phe	D-Ser	D-Val	Fatty acid	Structure (year)	Synthesis (year)
Polymyxin A	+	+	–	+	–	–	–	–	MOA		
Polymyxin B_1	6	2	1	–	–	1	–	–	MOA	1963/64	1964
Polymyxin B_2	6	2	1	–	–	1	–	–	IOA	1964	
Polymyxin C	+	+	–	–	–	+	–	–	MOA		
Polymyxin D_1	5	3	–	1	–	–	1	–	MOA	1966	
Polymyxin D_2	5	3	–	1	–	–	1	–	IOA	1966	
Polymyxin M	6	3	–	1	–	–	–	–	MOA		
Colistin A (Polymyxin E_1)	6	2	1	1	–	–	–	–	MOA	1964/65	1965
Colistin B (Polymyxin E_2)	6	2	1	1	–	–	–	–	IOA	1964/65	
Circulin A	6	2	–	1	1	–	–	–	MOA	1965	1965
Circulin B	6	2	–	1	1	–	–	–	MOA		
Polypeptin	3	1	2	–	1	1	–	1	unknown		

The figures give the number of amino acid residues per molecule. The + and – signs state the presence or absence of the amino acids. MOA = (+)-6-methyloctanoic acid; IOA = isooctanoic acid (6-methylheptanoic acid). The table has been compiled from the literature mentioned in the text.

Since at that time no enzyme available was found to attack the molecule, partial hydrolysis with mineral acids remained the only method for structural analysis. Dinitrophenylation of the intact molecule and subsequent partial hydrolysis with HCl led to a number of fragments which could be purified by counter-current distribution and determined in their sequences. With 7 out of 14 identified fragments HAUSMANN[32] was able to propose four possible structures. If the six fragments 14–9 (Figure 1) are logically combined, the branched nonapeptide at the bottom results. The proposed ring structure can now be formed by a peptide bond between the carboxyl group and the amino group of one of the two *N*-terminal threonine residues. This results in a ring structure formed of either 8 or 7 amino acids. The fragment MOA-γ-DNP has then finally to be connected with one of the two resulting side-chains. Furthermore, because the side-chain can be attached either to the α- or γ-amino group of the branching α,γ-diaminobutyric acid residue, two additional possibilities arise in each case, making a total of four possible structures. These were characterized by the following abbreviations[33]: 8α = ring of 8 amino acids and α-connected side-chain; 8γ = ring of 8 amino acids and γ-connected side-chain. In the same way, the 2 seven-membered ring compounds were designated 7α and 7γ (Figure 2).



DAB = α,γ-diaminobutyric acid; γ-DNP = γ-dinitrophenyl-α,γ-diaminobutyric acid.

Fig. 1

Due to the low optical rotation of the α,γ-diaminobutyric acid isolated after total hydrolysis, HAUSMANN and CRAIG[11] pointed out the possibility that 1 out of the 6 residues might be of the D-configuration. BISERTE and DAUTREVAUX[34], who confirmed these four possible structures later, found by treatment of the hydrolysate of the fragment MOA-Dab with D-amino acid oxidase that this residue should belong to the D-series.

The analytical methods available at that time did not allow any further distinction between these four possible structures, and we therefore decided in 1957 to settle the question by a synthetic approach (see chapter 5/1). All the four compounds were obtained synthetically and compared carefully with natural polymyxin B_1. The 2 eight-ring structures[33,35], although very similar in their chemical and physicochemical behaviour to polymyxin B_1, could easily be excluded due to their low antibiotic activity. The 2 seven-ring compounds[36,37] proved at first to be nearly indistinguishable from the natural antibiotic, only their optical rotation was some 20% lower. Two experi-

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[34] G. BISERTE and M. DAUTREVAUX, Bull. Soc. Chim. biol. *39*, 795 (1957).
[35] R. O. STUDER, K. VOGLER, and W. LERGIER, Helv. chim. Acta *44*, 131 (1961).
[36] R. O. STUDER, W. LERGIER, and K. VOGLER, Helv. chim. Acta *46*, 612 (1963).
[37] K. VOGLER, R. O. STUDER, P. LANZ, W. LERGIER, E. BÖHNI, and B. FUST, Helv. chim. Acta *46*, 2823 (1963).

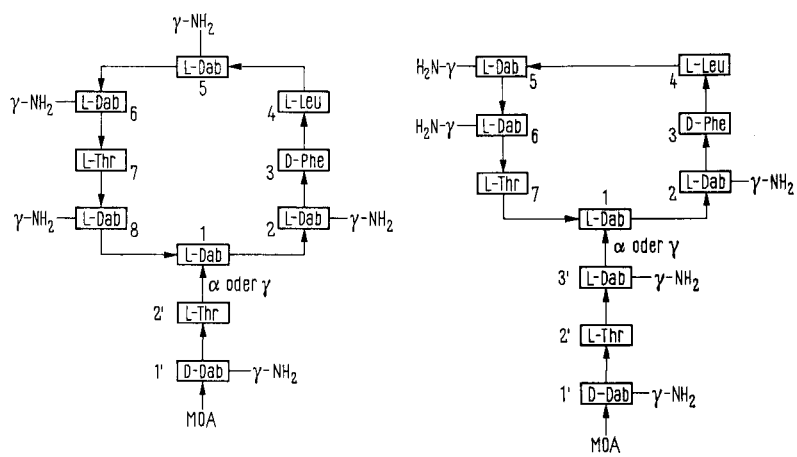

Fig. 2

ments proved that neither was identical with natural polymyxin B_1. First, a special strain of *K. pneumoniae* was found[37], giving a large difference in the in vitro activity between 7α, 7γ and the natural compound (Table II). A second useful criterion was found in the ORD, but only in the form of the *Ni* complexes first prepared by BRINTZINGER[38]. Large differences could be observed in both the site and the amplitude of the corresponding Cotton curves between 7α and 7γ on the one hand and polymyxin B_1 on the other (Figure 3)[37]. Because these differences could not be explained by low purity, partial racemization, or side-reactions during the synthesis, it was concluded that none of the four proposed structures was identical with natural polymyxin B_1[37].

In 1963 we were able to obtain the bacterial enzyme Nagarse (Subtilopeptidase A, EC 3.4.4.16) from Japan[39], an enzyme able to attack polymyxin B_1. The degradation products from the natural and the synthetic peptides showed different chromatographic behaviour. While this work was in progress T. SUZUKI et al.[40,41] found that Nagarse degrades the side-chain step by step down to the ring peptide. Isolation and structural analysis of the fragments led unequivocally to the structure 7α, but, in addition to this result, T. SUZUKI et al. demonstrated that the α,γ-diaminobutyric acid residue adjacent to the fatty acid is not of D- but of L-configuration. Independently WILKINSON and LOWE[42] obtained analogous results. We subsequently synthesized this compound and proved it to be identical with natural polymyxin B_1 (Figure 4) in all respects[43].

(2) *Polymyxin B_2*. Polymyxin B_2 was shown by HAUSMANN and CRAIG[11] to have the same amino acid composition as polymyxin B_1. As the only difference, they found instead of (+)-6-methyloctanoic acid an optically inactive fatty acid, which was later shown by WILKINSON and LOWE[44] to be 6-methylheptanoic acid. Recently WILKINSON and LOWE[45] were able to prove that polymyxin B_2 differs from B_1 only in the replacement of (+)-6-methyloctanoic acid by 6-methylheptanoic acid (Figure 4).

(3) *Polymyxin D_1*. Only recently T. SUZUKI et al.[14] fractionated polymyxin D by counter-current distribution and named the main biologically active components polymyxin D_1 and D_2. After total hydrolysis of polymyxin D_1 5*M* of α,γ-diaminobutyric acid, predominantly in the L-form, 3*M* of L-threonine and 1*M* each of D-serine and D-leucine were found together with (+)-6-methyloctanoic acid. From the sequences of the peptides isolated by partial hydrolysis and enzymatic degradation, they proposed the structure of polymyxin D_1 (Figure 4)[14].

[38] H. BRINTZINGER, Helv. chim. Acta *44*, 744 (1961).
[39] Japanese Patent No. 5264, 7.5.1963.
[40] T. SUZUKI, K. HAYASHI, K. FUJIKAWA, and K. TSUKAMOTO, J. Biochem. Tokyo *54*, 555 (1963).
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[43] K. VOGLER, R. O. STUDER, P. LANZ, W. LERGIER, and E. BÖHNI, Helv. chim. Acta *48*, 1161 (1965).
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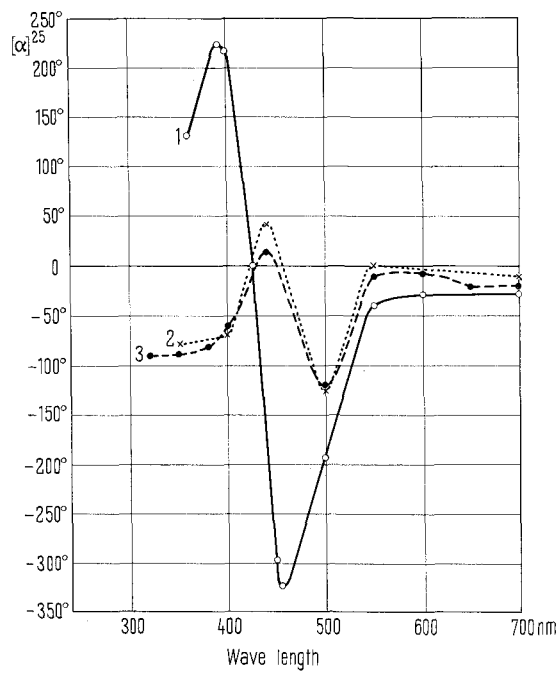

Fig. 3

Table II. Antibiotic activity of the synthetic isomers 8γ, 8α, 7γ, 7α and colistin A in comparison with natural polymyxin B_1, standard deviation ± 20%

	8γ	8α	7γ	7α	Colistin A	B_1
Brucella bronchiseptica ATCC 4617 (in vitro)	900 μ/mg	800 μ/mg	8250 μ/mg	8898 μ/mg	4983 μ/mg	8096 μ/mg
Pseudomonas aeruginosa (in vitro)	322 μ/mg	396 μ/mg	7088 μ/mg	6761 μ/mg	–	10,420 μ/mg
Escherichia coli ATCC 10,536 (in vitro)	241 μ/mg	184 μ/mg	4681 μ/mg	5081 /μmg	5775 μ/mg	5033 μ/mg
Klebsiella pneumoniae ATCC 100,131 (in vitro)	–	–	1297 μ/mg	1514 μ/mg	7381 μ/mg	8421 μ/mg
Escherichia coli 1346 (CD_{50} s.c., mice) (in vivo)	7 mg/kg	7 mg/kg	2.7 mg/kg	2.0 mg/kg	–	0.7 mg/kg

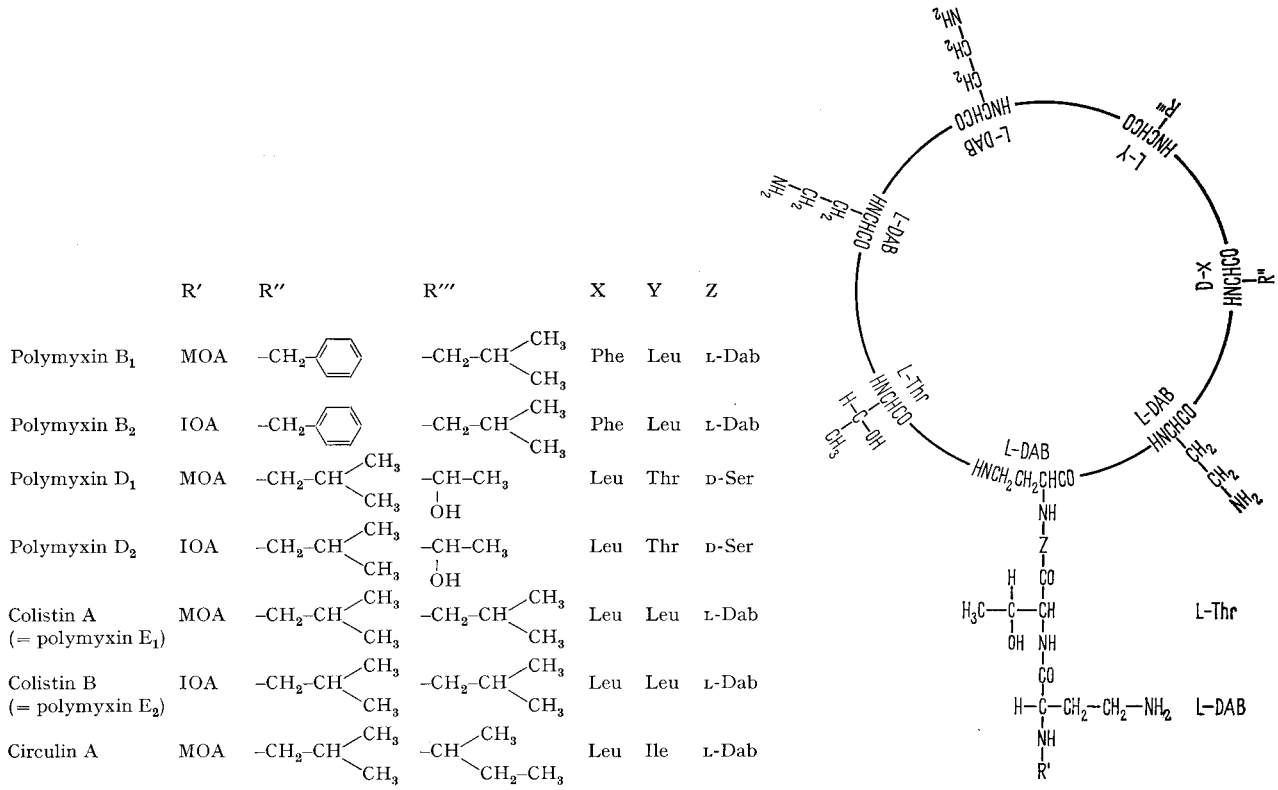

Fig. 4 The structures of the known members of the polymyxin group

(4) *Polymyxin D_2*. Polymyxin D_2 was found by T. SUZUKI et al.[14] to differ from polymyxin D_1 only in the replacement of (+)-6-methyloctanoic acid by 6-methylheptanoic acid (Figure 4).

(5) *Polymyxin E_1 (colistin A)*. Several structures have been proposed for colistin, although already in 1953 ODA[46] demonstrated by paper chromatography that it consists of three different entities. K. SUZUKI[47] formulated it in 1957 as a cyclooctapeptide containing five residues of L-α,γ-diaminobutyric acid and one residue each of L-threonine, L-leucine, D-leucine and (+)-6-methyloctanoic acid. BISERTE and DAUTREVAUX[48] suggested in 1963, after finding an additional L-α,γ-diaminobutyric acid and L-threonine, that colistin is a cyclopentapeptide with a five-membered side-chain.

At about the same time, T. SUZUKI et al.[24] isolated enough pure colistin A by counter-current distribution to perform a structural analysis similar to that of polymyxin B_1. Molecular weight determination either by the method of partial substitution or the spectrophotometric measurements of picrates gave a value of 1360 for the corresponding hydrochloride[24]. The quantitative composition as found by BISERTE and DAUTREVAUX[48] was confirmed by total hydrolysis[24]. Partial acid hydrolysis[49] and enzymatic degradation with Nagarse[50] were both used to break the molecule down into smaller fragments. After isolation and sequential analysis of these peptides, T. SUZUKI et al.[50] proposed a structure for colistin A differing from polymyxin B_1 only in the replacement of D-phenylalanine by D-leucine (Figure 4). Recently, WILKINSON and LOWE[12] succeeded in separating polymyxin E by counter-current distribution into the two components E_1 and E_2. Their structural analysis revealed that polymyxin E_1 and colistin A are identical[51]. A little later, the same conclusion was reached by T. SUZUKI et al.[13].

The final proof for the correctness of this structure came with its total synthesis[52] (chapter 5/2).

(6) *Polymyxin E_2 (colistin B)*. Colistin B, isolated by T. SUZUKI et al.[24] from commercial colistin by counter-current distribution, was found to differ from colistin A only in having 6-methylheptanoic acid in place of the (+)-6-methyloctanoic acid[53] (Figure 4). WILKINSON[51] demonstrated at about the same time that polymyxin

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E_2 is identical with colistin B, a result which was confirmed by T. SUZUKI et al.[13].

(7) *Circulin A*. In 1952 DOWLING et al.[17] reported on the qualitative composition of circulin A, after its isolation from crude circulin by chromatography. Six years later KOFFLER et al.[54,55] found that it consists of L-α,γ-diaminobutyric acid, L-threonine, D-leucine, L-isoleucine and (+)-6-methyloctanoic acid in the molar ratios 6:2:1:1:1. From the sequences of the peptides obtained by partial acid hydrolysis, KOFFLER et al. formulated circulin A tentatively as a cyclodecapeptide with no side-chain[56,57]. Because of the otherwise close chemical and biological similarity to the polymyxin antibiotics this structure aroused the suspicion of T. SUZUKI et al.[58]. Their studies were in analogy to those on the polymyxins based on enzymatic degradation and partial hydrolysis. The deduction of the structure from the peptides thus obtained revealed that circulin A differs from polymyxin E_1 (colistin A) only in the replacement of L-leucine in the latter by L-isoleucine[58] (Figure 4).

(8) *The polymyxins A, C, M, circulin B and polypeptin*. Whereas the polymyxins A[59,60] and C[60] are only known in their qualitative amino acid composition, quantitative amino acid analyses have been performed for polymyxin M[61], circulin B[56,57] and polypeptin[62]. With the exception of the latter, their fatty acid has also been determined, but nothing is yet known about their structure.

5. *Syntheses*

(1) *The syntheses of the analogues 8α, 8γ, 7α and 7γ*. While the amino acids L-threonine, L-leucine and D-phenylalanine are commercially available, the isomers of α,γ-diaminobutyric acid had to be prepared first according to ADAMSON[63] from D- or L-glutamic acid. The (+)-6-methyloctanoic acid had already been prepared by CROMBIE and HARPER[64] starting with optically active amyl alcohol and dihydrofurane. Because the latter substance was not available to us, we used a twofold malonic ester condensation starting with (−)-2-methylbutanol distilled from fusel oil (Figure 5)[65].

The general plan to synthesize cyclopeptides of this kind was to prepare first the corresponding branched, open-chain decapeptides and then to attempt their cyclization.

A possible position for a ring formation seemed to be between the *C*-terminal D-phenylalanine and *N*-terminal L-leucine (Figure 6)[66].

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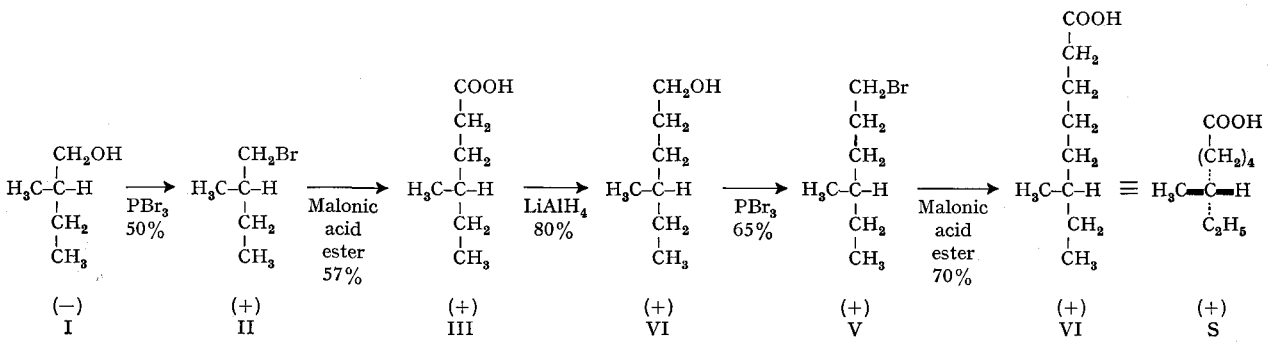

Fig. 5

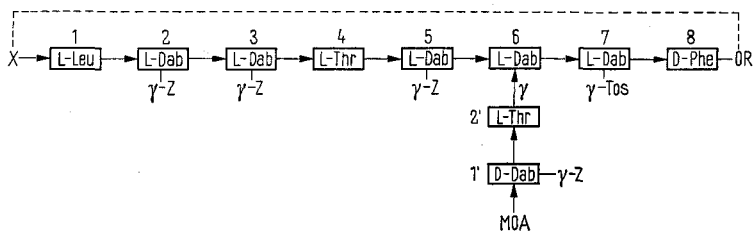

Fig. 6

The open-chain decapeptides 8α[35] and 8γ[66] were obtained by suitably connecting the three main peptides 1–2, 3–5 and 1′–5′ (Figure 7).

The tripeptide 3–5 is the key peptide for the synthesis of all four isomers (Figures 7 and 8). It contains in position 3 the branching L-α,γ-diaminobutyric acid residue. For the selective introduction of the dipeptide 1–2 and the pentapeptide 1′–5′ this tripeptide 3–5 must contain three different, selectively removable amino protecting groups. We have used the formyl-[67,68] and the benzyloxycarbonyl group[69] to protect the N^{α}- and the N^{γ}-amino function of the L-α,γ-diaminobutyric acid residue in position 3 and the tosyl group[70] for the N^{γ}-amino group in position 4. The *C*-terminal carboxyl group was blocked by its methylester.

The selection of the tosyl group to protect the N^{γ}-amino function in position 4 offers some problems due to the possibility of pyrolidone ring closure under the usual conditions of peptide bond formation. PODUŠKA and RUDINGER[71,72] have demonstrated that this danger can be avoided by using the CURTIUS azide procedure[73] in the presence of excess glacial acetic acid.

The tripeptide 3–5 was therefore obtained by coupling the azide of N^{α}-*Z*-N^{γ}-Tos-L-α,γ-diaminobutyric acid to D-phenylalanine methylester in the presence of glacial acetic acid, followed by hydrogenation and reaction with N^{α}-For-N^{γ}-*Z*-L-α,γ-diaminobutyric acid by means of dicyclohexylcarbodiimide[74]. This tripeptide may also be obtained in equally good yields by coupling the azide of N^{α}-For-N^{γ}-*Z*-L-α,γ-diaminobutyryl-N^{γ}-Tos-L-α,γ-diaminobutyric acid to D-phenylalanine methylester under these modified conditions. The dipeptide itself was prepared by condensing N^{α}-For N^{γ}-*Z*-L-α,γ-diaminobutyric acid and N^{γ}-Tos-L-α,γ-diaminobutyric acid methylester with dicyclohexylcarbodiimide followed by transformation to the hydrazide.

The side-chain peptide 1–2 was prepared also by the modified azide method using the components N^{α}-*Z*-N^{γ}-Tos-D-α,γ-diaminobutyric acid hydrazide and L-threonine methylester followed by hydrogenation and condensation with (+)-6-methyloctanoic acid either by the acid chloride method or by means of carbonyldiimidazol[75,76]. The ester was finally transformed to the hydrazide in the usual manner.

The synthesis of the pentapeptide 1′–5′ finally was easily achieved by starting with For-L-leucine and linking one residue after the other either by the carbodiimide procedure or by the azide method. Both ways yielded identical pentapeptides.

The branched, open-chain decapeptide 8α was obtained by deformylation of the peptide 3–5 (Figure 7) followed by condensation of the free N^{α}-amino group with the side-chain peptide 1–2 by the azide procedure to the pentapeptide 1–5. Thereafter, the γ-amino group in position 3 was deprotected by hydrogenation and condensed with the pentapeptide 1′–5′ again by the azide method.

The decapeptide of the isomer 8γ was prepared by condensation of the azide of the side-chain peptide 1–2 with the N^{γ}-amino group in position 3 of the hydrogenated peptide 3–5. The resulting pentapeptide was then deformylated by means of HCl/methanol[67] and coupled with the azide of the pentapeptide 1′–5′

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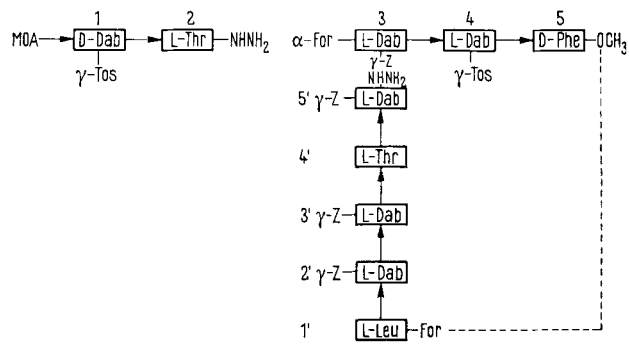

Fig. 7

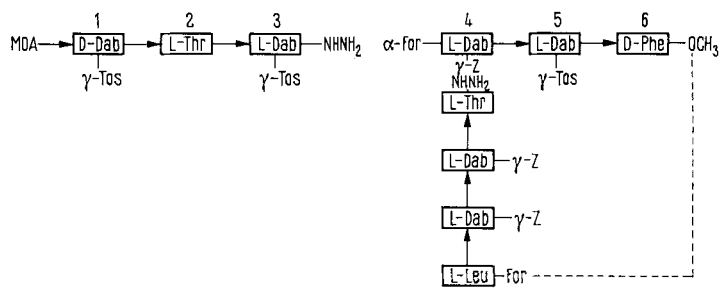

Fig. 8

through the unprotected N^{α}-amino function in position 3 (Figure 7).

In a quite similar way the branched, open-chain decapeptides of the isomers 7α[36] and 7γ[37] could be synthesized (Figure 8).

The tripeptide 4–6 (Figure 8) is identical with the tripeptide 3–5 (Figure 7), already used for the synthesis of the eight-membered ring compounds. The tetrapeptide 1′–4′ is an intermediate product in the synthesis of the pentapeptide 1′–5′ (Figure 7). In order to obtain the side-chain tripeptide 1–3 (Figure 8), the side-chain dipeptide 1–2 originally used (Figure 7) was coupled with N^{γ}-Tos-L-α,γ-diaminobutyric acid methylester by the azide procedure followed by hydrazinolysis of the resulting tripeptide.

From these three peptides the branched, open-chain decapeptides 7α[36] and 7γ[37] were obtained in a way similar to the one described for the synthesis of the decapeptides 8α[35] and 8γ[66].

For the cyclization of these four decapeptides we chose, after some preliminary experiments, the carbodiimide procedure already applied earlier by WIELAND and OHLY[77]. The formyl group at the amino end (Figures 7 and 8) was removed by means of HCl/methanol in dimethylformamide, whereas the saponification of the methylester proceeded smoothly with aqueous alkali in dimethylsulfoxide. The open-chain decapeptides were then submitted to cyclization in high dilution in dimethylformamide/dioxane ($10^{-4}M$/l). Initially we used a tenfold excess of dicyclohexylcarbodiimide, but we obtained a yield of less than 5% of the cyclized product. The application of a 300-fold excess[78] gave an increase in yield up to 15%, estimated for the crude material. This big excess of carbodiimide was easily removed from the reaction mixture by transformation to the corresponding urea compound. Because it was difficult to purify the protected cyclized decapeptides, all the blocking groups were removed by Na in liquid NH_3[79] directly from the crude cyclized product. The materials thus obtained were then purified by counter-current distribution and precipitation of the cyclic bases in concentrated cold ammonia and transformed into the corresponding pentahydrochlorides. By careful analysis it was possible to demonstrate that each of the four compounds corresponded to the anticipated structure, but that none of them was identical with natural polymyxin B_1 (chapter 4/1)[37].

(2) *Synthesis of polymyxin B_1.* For the synthesis of the compound corresponding to the structure proposed by T. SUZUKI et al.[40,41] (7α, all-L-Dab) the scheme was slightly revised[43]. At the chain-ends the easily removable tert. butylester[80] and tert. butyloxycarbonyl residue[81,82] were used to protect the corresponding amino or carboxyl group (Figure 9). The peptides I, II and III were synthesized by the azide and the *p*-nitrophenylester methods[83]. Removal of the tert. butyloxycarbonyl group in I by trifluoroacetic acid[84] and coupling with the azide of II led to the protected octapeptide. This was transformed into the corresponding hydrazide and condensed with III by the azide procedure. After removal of both chain-end protecting groups the cyclization was performed as described for the analogues. The benzyloxycarbonyl groups were then removed by sodium in liquid ammonia. Hydrogenation could not be carried out satisfactorily at room temperature. Again the crude product was purified by counter-current distribution and precipitation of the free base. The end product was finally crystallized as pentaorthophosphate[43].

(3) *The synthesis of polymyxin E_1 (colistin A).* Because the only difference between polymyxin B_1 and polymyxin E_1 is the replacement of D-phenylalanine in the former by a D-leucine in the latter, the same scheme could be used for the synthesis of polymyxin E_1[85] (Figure 9). Only in peptide III had D-phenylalanine to be replaced by D-leucine. As in the case of

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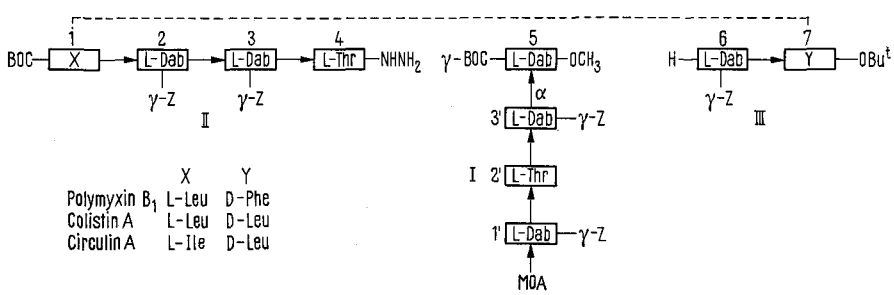

Fig. 9

polymyxin B_1, the final product could be obtained in crystalline form as pentaorthophosphate and it proved to be completely identical with the natural compound[85].

(4) *The synthesis of circulin A.* Efforts have been made to synthesize the compounds with the structures proposed by KOFFLER[56,57] for circulin A and B, which according to the latest information[58] are incorrect. During their intensive studies on the chemistry of α,γ-diaminobutyric acid, RUDINGER et al.[86] synthesized the linear decapeptide corresponding to KOFFLER's circulin A, and cyclized it by means of the azide method. AROLD[87] reported an analogous synthesis of the cyclodecapeptide corresponding to KOFFLER's circulin B. Both groups have used in their studies either the optically inactive (±)-6-methyloctanoic acid[87] or *n*-pelargonic acid[86]. Unfortunately, no details of the comparison with the natural antibiotics are available.

The close relationship between polymyxin E_1 (colistin A) and circulin A[58] allowed us to use the same scheme for the synthesis of the latter (Figure 9)[88]. Only in peptide II had L-leucine to be replaced by L-isoleucine. The cyclopeptide could also be obtained as crystalline pentaorthophosphate and it proved to be identical with the natural product in all respects[88]. Circulin A therefore has the structure proposed by T. SUZUKI et al. (Figure 4)[58].

(5) *Synthetic approaches to other members.* No efforts have yet been made towards the synthesis of the other known members, polymyxin B_2, D_1, D_2 and E_2. Only SILAEV et al.[89–91] have reported on the synthesis of some peptide sequences isolated from partial hydrolysates of polymyxin M.

6. *Concluding remarks*

The chemical data available at present clearly show that the polymyxins, the colistins, the circulins and possibly also polypeptin are chemically so closely related that they may all be comprised under the generic name polymyxin. All the members whose structures are known to date are decapeptides which contain a seven-membered ring with an α-connected side-chain ending with a fatty acid. The differences occur only in so far as D-phenylalanine and D-leucine as well as L-leucine and L-isoleucine in the ring, L-α,γ-diaminobutyric acid and D-serine as well as (+)-6-methyloctanoic acid and 6-methylheptanoic acid in the side-chain are interchangeable. From the amino acid composition of the other members, the close relationship of the producing organisms and their nearly identical biological properties, one may expect analogous chemical structures also for the yet unknown members of the polymyxin group.

It is interesting to note further that only with the α-connection of the side-chain is it possible to deduce a linear decapeptide having α-amino peptide bonds throughout (Figure 10), whereas this is not possible with a γ-connected side-chain[92]. This may suggest that the biosynthesis of this kind of cyclopeptide proceeds through the corresponding linear peptides.

From the fact that polymyxin B, polymyxin D, polymyxin E (colistin) and circulin could each be fractionated into closely related components by extensive counter-current distribution, one might expect that also the polymyxins A and C are not yet single entities.

From the standpoint of the synthesis of these compounds, approaches other than those used up to now are possible. Experiments to cyclize a linear decapeptide corresponding to the structure 7α proposed by HAUSMANN for polymyxin B_1, having α-amino peptide bonds throughout (Figure 10) have not been successful, possibly due to steric hindrance by the tosyl-protecting groups used to protect the γ-amino functions[92]. Other cyclization methods for the branched open-chain decapeptides, such as the activated ester method, have up to now not resulted in better yields[93]. Experiments to prepare first a cycloheptapeptide and then introduce the side-chain afterwards have not yet been undertaken.

Zusammenfassung. Die Chemie der Polymyxine, einer Klasse von basischen Polypeptid-Antibiotika, begann 1954, als durch Gegenstromverteilung erstmals ein definierter Vertreter, das Polymyxin B_1, in reiner Form isoliert werden konnte (L. C. CRAIG). Partialhydrolyse mit Mineralsäuren führte zum Schluss, dass es sich um Cyclohepta- oder Cyclooctapeptide mit Seitenketten handelt, die α,γ-Diaminobuttersäure (Dab) enthalten (W. HAUSMANN).

Amidartige Verknüpfung der Seitenkette mit einer Fettsäure [(+)-6-Methyloctansäure (MOA) oder 6-

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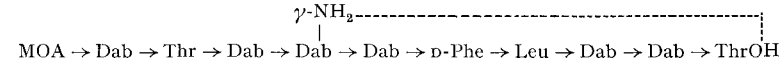

Fig. 10

Methylheptansäure (IOA)] (S. WILKINSON) verleiht diesen Antibiotika den Charakter von Invertseifen. Sie sind besonders gegen gramnegative Erreger wirksam. Schwierigkeiten bereiteten die Aufklärung der Verknüpfungsweise der Seitenkette (α- oder γ-) und die Beantwortung der Frage, ob das Molekül neben D-Phenylalanin in der Ringsequenz noch einen D-α,γ-Diaminobuttersäurerest in Nachbarschaft zur Fettsäure in der Seitenkette enthält. Synthetische Versuche mit D-α,γ-Diaminobuttersäure an dieser Stelle führten zu hochaktiven Produkten, die aber mit natürlichem Polymyxin B_1 nicht identisch waren. Entscheidende Fortschritte wurden mit dem bakteriellen Enzym Nagarse (T. SUZUKI) erzielt, das schrittweise die Seitenkette bis zum Ringpeptid abbaut. Dabei ergab sich, dass den Polymyxinen die allgemeine Struktur eines Cycloheptapeptides mit α-verknüpfter Seitenkette zukommt (Figur 4).

Die Polymyxine B_1, E_1 (Colistin A) sowie Circulin A unterscheiden sich voneinander nur durch eine Variation in der gleichen Dipeptidsequenz des 7-gliedrigen Ringes. Die im Polymyxin B_1 vorhandene Dipeptidsequenz D-Phe-L-Leu ist in Polymyxin E_1 (Colistin A) durch D-Leu-L-Leu und in Circulin A durch D-Leu-L-Ile ersetzt. Im Polymyxin D_1 ist neben dem Ersatz der entsprechenden Sequenz durch L-Leu-L-Thr noch ein α,γ-Diaminobuttersäurerest der Seitenkette durch ein D-Serin ausgetauscht. Die entsprechenden Verbindungen mit dem Index 2 unterscheiden sich von denjenigen mit dem Index 1 durch einen Austausch der (+)-6-Methyloctansäure durch 6-Methylheptansäure.

Die Struktur von Polymyxin B_1, E_1 und Circulin A konnte durch Totalsynthese gesichert werden (K. VOGLER). Weitere Fortschritte in der Erforschung der Natur der noch unbekannten Vertreter sind in Kürze zu erwarten.

SPECIALIA

Chemical Structure of Polymyxin D_1

An antibiotic designated as polymyxin D was first isolated from the culture fluid of a strain of *Bacillus polymyxa* by STANSLY, SHEPHERD, and WHITE in 1947[1]. In 1949 BELL et al.[2] reported that polymyxin D is a basic polypeptide containing L-threonine: D-leucine: D-serine: L-α,γ-diaminobutyric acid in the ratio 3:1:1:5. But no studies on the chemical structure have yet been made.

During the course of our investigation of the culture medium of *Bacillus polymyxa* ATCC 10401, antibiotic substances were isolated from a culture broth in considerable yield. The crude antibiotics were purified mainly by ion exchange chromatography (Amberlite IRC-50, H^+-form) and counter-current distribution using a mixture of *n*-butanol/sec-butanol and 0.1 *N* HCl in the ratio 6:30:40 (v/v) as solvent, and two pure components were isolated. The constituent amino acids of both were found to be threonine: leucine: serine: α,γ-diaminobutyric acid in the molar ratio 3:1:1:5, which agreed with that of polymyxin D reported by BELL et al.[2]. Thus we named the two components polymyxin D_1 and polymyxin D_2, respectively.

Polymyxin D_1, the more active entity biologically, was first hydrolysed with 6 *N* HCl for 20 h at 105 °C and the configurations of the amino acids in the hydrolysate were examined microbiologically[3], and also by means of optical rotatory dispersion. The results agreed with those given by BELL et al.[2], and the amino acid composition of polymyxin D_1 was confirmed as L-threonine: D-leucine: D-serine: L-α,γ-diaminobutyric acid in the molar ratio 3:1:1:5. The methyl ester derivative of the C_9 fatty acid isolated from the acid hydrolysate of polymyxin D_1 was shown to be (+)-6-methyloctanoic acid methylester by gas chromatography.

Polymyxin D_1 was then partially hydrolysed with 6 *N* HCl at 37 °C for 70 h and the resulting peptides were separated by gradient column chromatography. 19 fragments were isolated in apparently pure states as judged by paper chromatography, paper electrophoresis, and thin layer chromatography. With three fragments,

Moa[4] → (α)Dab, Thr → Ser → (α)Dab → (α)Dab → Leu,

(γ)
↑
Thr → (α)Dab → (α)Dab,

Thr

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[4] The following abbreviations are used throughout this paper: Moa = (+)-6-methyloctanoyl residue; Dab = α,γ-diaminobutyric acid residue; → = C to N bond in –CO–NH–; → (α)Dab = → Dab; → (γ)Dab = → Dab.
NH_2 $NH_2(\gamma)$