Journal of Chromatography, 173 (1979) 313–320

CHROM. 11,732

SEPARATION OF POLYMYXINS AND OCTAPEPTINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SHIGERU TERABE*, RYUSEI KONAKA and JUN'ICHI SHOJI
Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553 (Japan)
(Received January 9th, 1979)

SUMMARY

Seventeen decapeptide antibiotics of the polymyxin group and nine octapeptide antibiotics of the octapeptin group have been successfully separated on a commercial reversed-phase material with tartrate buffer–acetonitrile containing sodium 1-butanesulphonate and sodium sulphate as the mobile phase. All of the components of EM49 (a complex of octapeptins A and B) were preparatively separated by use of a large-diameter column, and the structures of two new components, named octapeptins A_4 and B_4, were deduced from the results of the fatty acid and amino acid analyses.

INTRODUCTION

Polymyxins (Table I), a group of polypeptide antibiotics produced by strains of *Bacillus polymyxa* and related species, have a general structure composed of a cyclic heptapeptide moiety and a side-chain consisting of a tripeptide with a fatty acyl residue. A large number of compounds, which are heterogeneous in acyl and/or amino acid residues, belonging to the polymyxin family have been reviewed by Vogler and Studer[1] and Shoji[2]. All of the polymyxins reported to date can be separated into single components by a counter-current distribution method[3] or thin-layer chromatography[4].

Octapeptins (Table II) have structures similar to those of polymyxins but the side-chains consist of only one amino acid with a fatty acyl residue. EM49[5] (later named octapeptin[6]) has been separated into four major components, EM49α, EM49β, EM49γ and EM49δ, on a CM-cellulose column, but both EM49α and EM49δ are still complexes[7,8].

It is considered that the complete separation of these peptides by high-performance liquid chromatography (HPLC) is very useful for identifying these antibiotics, for determining the relative contents of the components and for examining the purity. Recently a few papers have appeared on the separation of peptide anti-

* Present address: Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

TABLE I

STRUCTURES OF POLYMYXINS

FA → L-Dab → L-Thr → W → L-Dab → L-Dab → X → Y
↑ ↓
Z ← L-Dab ← L-Dab ←

Dab = 2,4-diaminobutyric acid.

Polymyxin	*W*	*X*	*Y*	*Z*	*FA**
M_1	L-Dab	D-Leu	L-Thr	L-Thr	a-C_9
M_2	L-Dab	D-Leu	L-Thr	L-Thr	i-C_8
D_1	D-Ser	D-Leu	L-Thr	L-Thr	a-C_9
D_2	D-Ser	D-Leu	L-Thr	L-Thr	i-C_8
C_1	Dab	Phe	Thr	Thr	a-C_9
C_2	Dab	Phe	Thr	Thr	i-C_8
S_1	D-Ser	D-Phe	L-Thr	L-Thr	a-C_9
E_1 (colistin A)	L-Dab	D-Leu	L-Leu	L-Thr	a-C_9
E_2 (colistin B)	L-Dab	D-Leu	L-Leu	L-Thr	i-C_8
B_1	L-Dab	D-Phe	L-Leu	L-Thr	a-C_9
B_2	L-Dab	D-Phe	L-Leu	L-Thr	i-C_8
B_3	L-Dab	D-Phe	L-Leu	L-Thr	n-C_8
F_1	(Dab (5), Thr (1), Leu (2), Ser (1), Ile (1))				a-C_9
F_2					i-C_8
F_3					n-C_8
T_1	L-Dab	D-Phe	L-Leu	L-Leu	a-C_9
T_2	L-Dab	D-Phe	L-Leu	L-Leu	i-C_8

* a-C_9 = 6-methyloctanoyl; i-C_8 = 6-methylheptanoyl; n-C_8 = octanoyl.

biotics by HPLC[9,10], and Tsji and Robertson[11] have reported the separations of polymyxin B_1 and B_2 and of E_1 and E_2 by reversed-phase chromatography with linear-gradient elution.

The characteristic feature of polymyxins and octapeptins in chromatography is their strong basicity because they have five or six 2,4-diaminobutyric acid (Dab)

TABLE II

STRUCTURES OF OCTAPEPTINS

FA → D-Dab → L-Dab → L-Dab → X → Y
↑ ↓
L-Leu ← L-Dab ← L-Dab ←

Dab = 2,4-diaminobutyric acid.

Octapeptin	*Synonym*	*X*	*Y*	*FA**
A_1	EM49β	D-Leu	L-Leu	a-$C_{11}h^3$
A_2	EM49α	D-Leu	L-Leu	i-$C_{10}h^3$
A_3		D-Leu	L-Leu	n-$C_{10}h^3$
A_4	—	D-Leu	L-Leu	i-$C_{11}h^3$
B_1	EM49δ	D-Leu	L-Phe	a-$C_{11}h^3$
B_2	EM49γ	D-Leu	L-Phe	i-$C_{10}h^3$
B_3		D-Leu	L-Phe	n-$C_{10}h^3$
B_4	—	D-Leu	L-Phe	i-C_9h^3
C_1	333-25	D-Phe	L-Leu	a-C_9h^3

* a-$C_{11}h^3$ = 3-hydroxy-8-methyldecanoyl; i-$C_{10}h^3$ = 3-hydroxy-8-methylnonanoyl; n-$C_{10}h^3$ = 3-hydroxydecanoyl; i-$C_{11}h^3$ = 3-hydroxy-9-methyldecanoyl; a-C_9h^3 = 3-hydroxy-6-methyloctanoyl.

residues. In this study ion-pair reversed-phase chromatographic conditions were employed with success, and the relationship between the order of elution and structure is discussed. Another purpose of this study was to resolve completely all of the components of EM49 and to deduce their structures.

EXPERIMENTAL

Reagents

Polymyxin M[12] was kindly donated by Dr. G. S. Katrukha of the A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry (Moscow, U.S.S.R.), and polymyxin F[13] and EM49[5] by Dr. E. Meyers of the Squibb Institute for Medical Research (Princeton, N.J., U.S.A.). Polymyxin E and B were purchased from Kayaku Antibiotics Research Laboratory (Tokyo, Japan) and Chas. Pfizer (Groton, Conn., U.S.A.), respectively. Other polymyxins[14,15] and octapeptin C_1[16,17] were prepared in the manner reported by Shoji *et al.*[14–17]. All of the polymyxins designated by the same capital letter but with different arabic numeral subscripts were used as mixtures, except for polymyxin T_1.

HPLC-quality acetonitrile (Wako, Osaka, Japan) and reagent-grade sodium 1-butanesulphonate (Eastman-Kodak, Rochester, N.Y., U.S.A.) were used. Water was purified by using an ionic-exchange column, reverse osmosis and finally single distillation.

Apparatus

The liquid chromatograph consisted of a Waters Model 6000A pump, a Rheodyne Model 7120 injector and a Japan Spectrooptics UVIDEC-100 variable-wavelength UV detector. The columns (20 cm × 4 mm I.D. for analytical purposes and 25 cm × 10 mm I.D. for preparative purposes) were packed with Nucleosil $5C_{18}$ (4-mm I.D. column) or $10C_{18}$ (10-mm I.D. column) (Machery, Nagel & Co., Düren, G.F.R.) by the technique recommended by Machery, Nagel & Co. with slurry solvent B (Machery, Nagel & Co.).

Procedure

The peptide antibiotics were dissolved in distilled water to a concentration of about 1 mg/ml for analytical work and 10 mg/ml for preparative work. The solutions were kept at about 4° when not in use. The amounts of sample injected were 5–50 and 500–1000 μg for analytical and preparative work, respectively. All experiments were run at room temperature. The flow-rates were 1.0 ml/min for the 4-mm I.D. column and 4.0 ml/min for the 10-mm I.D. column and the inlet pressures were 1800–2500 p.s.i. for the former and about 1000 p.s.i. for the latter. The detector was operated at 220 nm.

The mobile phase was prepared by mixing the following two solutions in an appropriate ratio to obtain the desired content of acetonitrile: solution A, tartrate buffer (0.005 *M*), pH 3.0, containing sodium 1-butanesulphonate (0.005 *M*) and sodium sulphate (0.05 *M*); solution B, a mixture of equal volumes of tartrate buffer (0.005 *M*), pH 3.0, and acetonitrile, containing sodium 1-butanesulphonate and sodium sulphate in the same concentration as in solution A. The mobile phase was filtered through a membrane filter (1 or 0.5 μm) and degassed prior to use. The con-

tent of acetonitrile in the mobile phase was adjusted in order to control the retention times of the samples. The contents of acetonitrile in the eluent agent are given with the chromatograms.

For the analyses of constituent fatty acids and amino acids of each component of octapeptin A and B, a total amount of about 3.5 mg of EM49 was injected several times and was fractionated into eight components. The pH of each fraction, concentrated to about 5 ml, was adjusted to 9.0 with sodium hydroxide solution and the solution thus obtained was extracted three times with *n*-butanol. The combined *n*-butanol extract was washed with water and evaporated to dryness. The residue was hydrolysed with constant-boiling hydrochloric acid at 110° for 1 h. The hydrolysate was extracted with three 1-ml portions of diethyl ether. The ethereal extract was dried over anhydrous sodium sulphate, evaporated to about 0.2 ml with a slow stream of nitrogen at 25° and treated with two drops of diazomethane solution in diethyl ether.

The methyl esters produced were analysed with a Shimazu GC-7AG gas chromatograph equipped with a hydrogen flame-ionization detector and a glass column (1.6 m × 3 mm I.D.) packed with 15% diethylene glycol succinate polyester on Chromosorb W (80–100 mesh) at 156°. The flow-rate of the carrier gas (nitrogen) was 45 ml/min. The aqueous layer left after the ether extraction was evaporated to dryness and hydrolysed with constant-boiling hydrochloric acid at 110° for 20 h. After evaporation of the hydrochloric acid, the hydrolysate was analysed with a Hitachi KLA-5 automatic amino acid analyser.

RESULTS AND DISCUSSION

The polymyxin group

As polymyxins have five or four unmasked Dab residues, they are strongly basic. Every attempt to separate polymyxins by reversed-phase liquid chromatography with a mixture of acetonitrile and 0.005 *M* phosphate buffer (pH 7.0) or with a mixture of acetonitrile and 0.01 *M* ammonium sulphate as the mobile phase failed to give good resolution because of peak tailing. In contrast, ion-pair reversed-phase liquid chromatography with a mixture of 0.005 *M* tartrate buffer (pH 3.0) and acetonitrile, containing 0.005 *M* sodium 1-butanesulphonate and 0.05 *M* sodium sulphate as the mobile phase, gave very good separations, as shown in Figs. 1 and 2. When sodium sulphate was not added to the mobile phase the resolutions were poorer and the peaks were broader.

Whereas polymyxin S_1[14] and T_1[15] were available as singly isolated compounds, the other polymyxins listed in Table I were obtained as mixtures having different fatty acyl residues. Three typical chromatograms of polymyxins are shown in Fig. 1. There is no doubt that the main peak in Fig. 1A should be assigned to polymyxin S_1. Mainly two peaks were observed in the chromatogram of colistin (polymyxin E) (Fig. 1B), as expected. The assignments of these two peaks were based on the relative peak areas and the relative retention times. Commercial colistin has been reported[18] to contain generally a larger amount of colistin A than colistin B. Colistin A (polymyxin E_1) has a 6-methyloctanoyl residue (a-C_9) and colistin B (polymyxin E_2) has a 6-methylheptanoyl residue (i-C_8), as shown in Table I. As there is no difference in the polypeptide moieties between the two, colistin B is expected to be eluted faster than colistin A. This relative elution order agrees well with that of polymyxin T_1 and T_2.

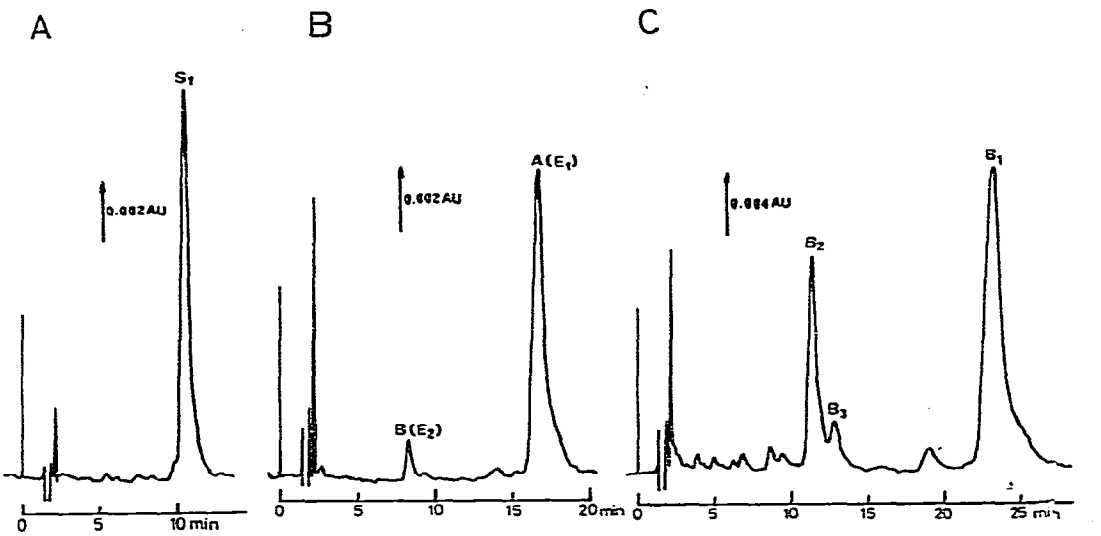
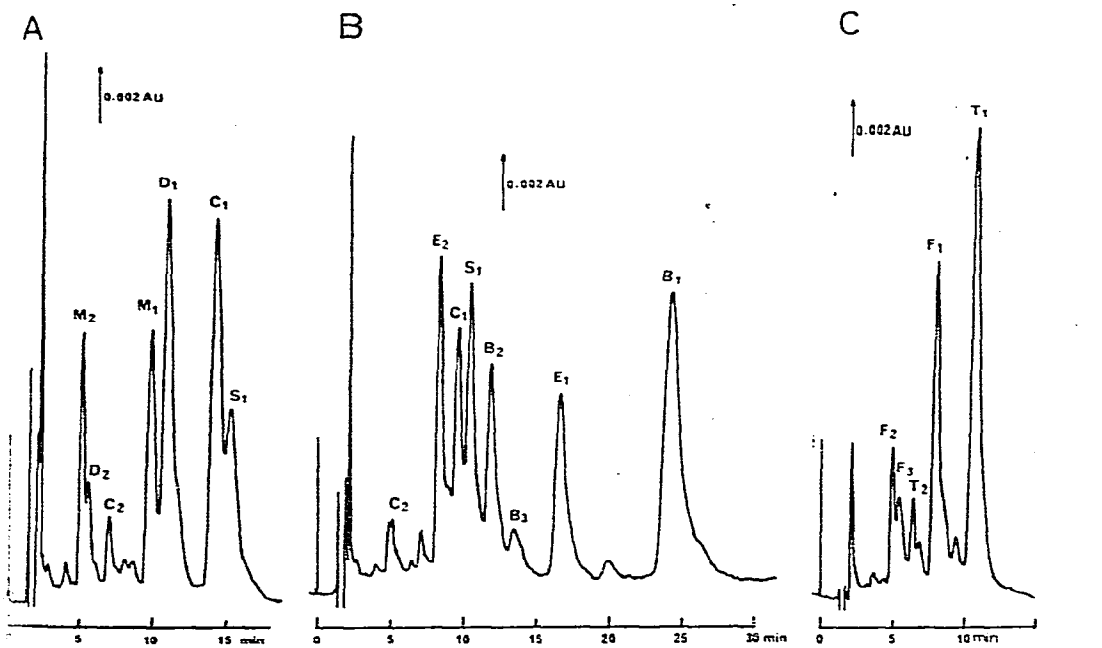


Fig. 1. Chromatograms of polymyxins. Content of acetonitrile in the mobile phase, 22.5%. Column: Nucleosil $5C_{18}$ (20 cm × 4 mm I.D.). Samples: (A) polymyxin S_1 (4.5 μg), (B) colistin (polymyxin E) (10 μg); (C) polymyxin B (33 μg).

whose elution order was confirmed by comparison of two chromatograms of polymyxin T_1 alone and of a mixture of polymyxin T_1 and T_2. Polymyxin B_1 and B_2[3] were similarly assigned as shown in Fig. 1C. Polymyxin B_3[19] is contained in a smaller



ig. 2. Separation of polymyxins. Content of acetonitrile in the mobile phase: (A) 21%; (B) 22.5%;) 30%. Column: Nucleosil $5C_{18}$ (20 cm × 4 mm I.D.). Sample: (A) a mixture of polymyxin M, D, and S; (B) a mixture of polymyxin C, E, S and B; (C) a mixture of polymyxin F and T.

amount than the other two and has an octanoyl residue (n-C_8), as shown in Table I. The peptide antibiotic having a 3-hydroxydecanoyl residue (n-$C_{10}h^3$) is eluted slower than that having a 3-hydroxy-8-methylnonanoyl residue (i-$C_{10}h^3$), as described later in the separation of octapeptins under similar chromatographic conditions. A similar result has also been observed in the HPLC of cerexins under reversed-phase liquid chromatographic conditions[20]. Taking into account the above, polymyxin B_3 was assigned as shown in Fig. 1C. Other polymyxins designated by the same capital letter but with different arabic numeral subscripts have similarly been assigned as shown in Fig. 2.

The retention times of the polymyxins listed in Table I varied widely when they were eluted under the same conditions. Therefore, the polymyxins investigated were divided into three groups so as to be eluted with moderate retention times. The three groups were chromatographed under different isocratic conditions and nearly complete separations of all polymyxins were obtained, as shown in Fig. 2. If a more complete resolution is required, the content of acetonitrile in the mobile phase can be reduced. The polymyxin E used to obtain Fig. 2 was of a different batch from that used to obtain Fig. 1B.

The retention times of the polymyxins increased in the order of fatty acyl residues i-C_8 < n-C_8 < a-C_9 if their polypeptide moieties were identical. The contribution of an amino acid residue to the order of elution can be evaluated based on a comparison of the retention times of various combinations of polymyxins with an identical fatty acyl group but different amino acid residues, *e.g.*, polymyxin M_1 and D_1, polymyxin E_1 and B_1, and polymyxin M_1 and E_1. It was concluded from the above considerations that the retention times increase in the order Dab < Ser and Thr < Leu < Phe. The latter order seems reasonable in view of the hydrophobicity of each amino acid residue. However, the former seems conflicting, because Dab is expected to be ion paired with 1-butanesulphonate under the chromatographic conditions used, whereas Ser has a hydroxy group. The most probable explanation of this behaviour is the effect of steric hindrance for ion pairing in the position where different amino acid residues are located, *viz.*, the position designated by W in Table I.

The octapeptin group

Octapeptins (Table II) have related structures to polymyxins and good separations were obtained when chromatographic conditions similar to those for polymyxins were employed. Octapeptin C_1, which has been reported to be a single entity[16], was eluted much faster than the other octapeptins when it was injected together with EM49. EM49 has been reported to consist of six components and to be separated into four major components[7]. However, the HPLC separation gave eight peaks, as shown in Fig. 3B, which was obtained under preparative conditions. The resolution of each peak was virtually identical with that obtained with an analytical column, and the amount injected could be increased to 1000 μg without a significant loss of resolution.

In order to identify the peaks, all of the components were fractionated and each fraction was analysed by a gas chromatograph and an amino acid analyser after hydrolysis (see Experimental). The methyl esters of fatty acids obtained from the acylpeptides were identified based on a comparison of the gas chromatographic retention times between the esters obtained and authentic esters[21]. The results are given in Table III. As the structures of octapeptins have been determined, the assignments of

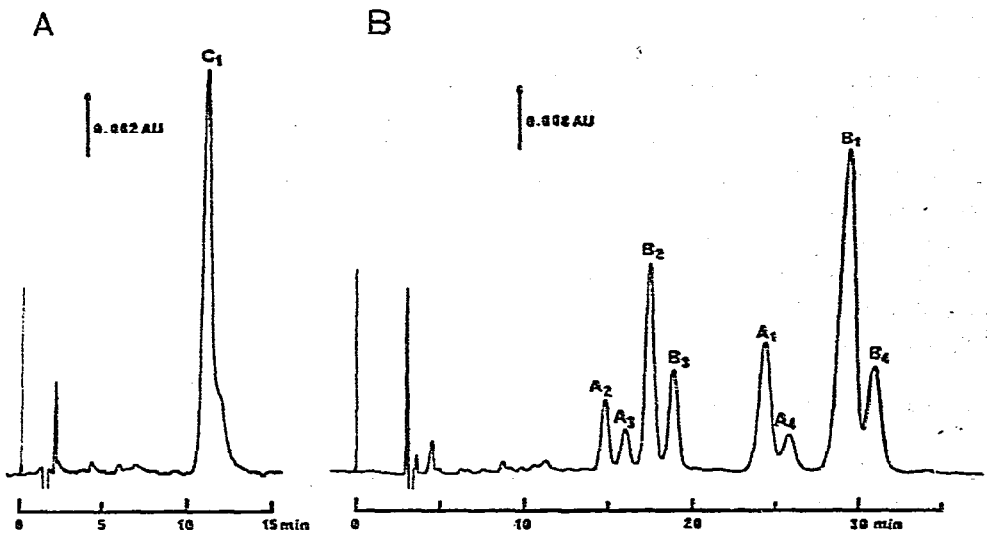


Fig. 3. Chromatograms of octapeptin C_1 and EM49. Content of acetonitrile: (A) 30%; (B) 31%. Column: (A) Nucleosil $5C_{18}$ (20 cm × 4 mm I.D.); (B) Nucleosil $10C_{18}$ (25 cm × 10 mm I.D.). Sample: (A) octapeptin C_1 (5.2 μg); (B) EM49 (a mixture of octapeptin A and B) (250 μg).

octapeptin A_1, A_2, A_3, B_1, B_2 and B_3 are straightforward from Table III. The structures of the remaining two, which were newly found, were deduced by analogy with other octapeptins as shown in Table III. The retention times increased in the order of fatty acyl residues i-$C_{10}h^3$ < n-$C_{10}h^3$ < a-$C_{11}h^3$ < i-$C_{11}h^3$ if the octapeptins had an identical peptide moiety. It can also be concluded that Phe is more hydrophobic than Leu on the basis of the discussion above regarding the polymyxin group. It should be noted that the difference in hydrophobic effects between C_{10} and C_{11} fatty acid residues is much more greater than that between Leu and Phe, *e.g.*, octapeptin B_2 and B_3 are eluted much faster than octapeptin A_1 and A_4.

TABLE III

FATTY ACID AND AMINO ACID ANALYSES ON SEPARATED COMPONENTS OF OCTAPEPTINS (EM49)

Peak *	*Fatty acid*	*Amino acid found (ratio)* **			*Identification*
		Dab	*Leu*	*Phe*	
1	i-$C_{10}h^3$	5.00	3.21 (3)	0.00	A_2
2	n-$C_{10}h^3$	5.00	2.49 (3)	0.00	A_3
3	i-$C_{10}h^3$	5.00	1.91 (2)	1.01 (1)	B_2
4	n-$C_{10}h^3$	5.00	1.76 (2)	0.72 (1)	B_3
5	a-$C_{11}h^3$	5.00	2.77 (3)	0.00	A_1
6	i-$C_{11}h^3$	5.00	2.65 (3)	0.00	A_4 (new)
7	a-$C_{11}h^3$	5.00	1.87 (2)	1.06 (1)	B_1
8	i-$C_{11}h^3$	5.00	1.91 (2)	1.01 (1)	B_4 (new)

* Numbered in order of elution.

** Values in parentheses represent rounded-off ratios.

CONCLUSION

It has been shown that all of the components of polymyxin M, D, C, S, E, B, F and T and octapeptin A, B and C can be separated successfully by use of a chemically bonded C_{18} stationary phase and a mobile phase consisting of a mixture of tartrate buffer and acetonitrile containing sodium 1-butanesulphonate and sodium sulphate. It should be emphasized that even groups of polymyxins and octapeptins that have an identical peptide moiety but isomeric fatty acyl residues can be separated successfully. Two new components, octapeptin A_4 and B_4, have been found in EM49 and their structures have been deduced by the gas chromatographic analysis for fatty acids and by analysis of the amino acids in the hydrolysate.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Dr. Edward Meyers of The Squibb Institute for Medical Research and to Dr. G. S. Katrukha of A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry for providing samples.

REFERENCES

1 K. Vogler and R. O. Studer, *Experientia*, 22 (1966) 345.
2 J. Shoji, *Advan. Appl. Microbiol.*, 24 (1978) 187.
3 W. Hausmann and L. C. Craig, *J. Amer. Chem. Soc.*, 76 (1954) 4892.
4 A. H. Thomas and I. Holloway, *J. Chromatogr.*, 161 (1978) 417.
5 E. Meyers, W. E. Brown, P. A. Principe, M. L. Rathnum and W. L. Parker, *J. Antibiot.*, 26 (1973) 444.
6 E. Meyers, W. L. Parker, W. E. Brown, J. Shoji and Y. Wakisaka, *J. Antibiot.*, 29 (1976) 1241.
7 W. L. Parker and M. L. Rathnum, *J. Antibiot.*, 26 (1973) 449.
8 W. L. Parker and M. L. Rathnum, *J. Antibiot.*, 28 (1975) 379.
9 K. Tsuji, J. H. Robertson and J. A. Bach, *J. Chromatogr.*, 99 (1974) 597.
10 K. S. Axelsen and S. H. Vogelsang, *J. Chromatogr.*, 140 (1977) 174.
11 K. Tsuji and J. H. Robertson, *J. Chromatogr.*, 112 (1975) 663.
12 A. B. Silaev, S. N. Maevskaya, Zh. P. Trifonova, E. P. Yubikova, N. M. Vasil'eva and G. S. Katrukha, *J. Gen. Chem. USSR*, 45 (1975) 2287.
13 W. L. Parker, M. L. Rathnum, L. D. Dean, M. W. Nimeck, .W E. Brown and E. Myers, *J. Antibiot.*, 30 (1977) 767.
14 J. Shoji, T. Kato and H. Hinoo, *J. Antibiot.*, 30 (1977) 1035.
15 J. Shoji, T. Kato and H. Hinoo, *J. Antibiot.*, 30 (1977) 1042.
16 J. Shoji, H. Hinoo, Y. Wakisaka, K. Koizumi, M. Mayama, S. Matsuura and K. Matsumoto, *J. Antibiot.*, 29 (1976) 516.
17 T. Kato and J. Shoji, *J. Antibiot.*, 29 (1976) 1339.
18 T. Suzuki, H. Inouye, K. Fujikawa and Y. Suketa, *J. Biochem. (Tokyo)*, 54 (1963) 25.
19 L. Withander and H. Heding, *J. Antibiot.*, 29 (1976) 774.
20 J. Shoji, T. Kato, S. Terabe and R. Konaka, *J. Antibiot.*, in press.
21 J. Shoji, T. Kato and R. Sakazaki, *J. Antibiot.*, 29 (1976) 1268.