MUREIDOMYCINS A~D, NOVEL PEPTIDYLNUCLEOSIDE ANTIBIOTICS WITH SPHEROPLAST FORMING ACTIVITY

II. STRUCTURAL ELUCIDATION

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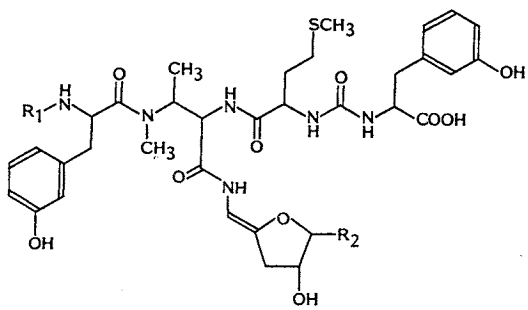
Structures of new antibiotics, mureidomycins (MRD's) A~D, were deduced from spectroscopic analyses and degradation studies. Two residues of *m*-tyrosine, one residue of 2-amino-3-*N*-methylaminobutyric acid (AMBA) and methionine are present in all components of the complex. Uracil is contained in MRD's A and C, while dihydrouracil in MRD's B and D. Methionine and *m*-tyrosine are connected through an ureido bond, and uracil or dihydrouracil is linked to AMBA *via* enamine sugar moiety. In addition, MRD's C and D contain a glycine residue at the *N*-terminal.

Mureidomycins (MRD's) A~D (Fig. 1) which were specifically active against *Pseudomonas aeruginosa* have been isolated from the culture filtrate of *Streptomyces flavidovirens* SANK 60486[1,2]. In this paper we report the structural elucidation of these antibiotics based on spectral analyses and chemical degradation studies.

Fig. 1. Structures, elemental formula and molecular weights of MRD's.



MRD's	R_1	R_2	Formula	MW[a]
A (1)	H	Uracil	$C_{38}H_{48}N_8O_{12}S$	840
B (2)	H	Dihydrouracil	$C_{38}H_{50}N_8O_{12}S$	842
C (3)	Glycine	Uracil	$C_{40}H_{51}N_9O_{13}S$	897
D (4)	Glycine	Dihydrouracil	$C_{40}H_{53}N_9O_{13}S$	899

[a] Lowest isotope composition.

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Table 1. ^{1}H NMR assignment for MRD A.

Shift	Coupling (J, Hz)	Assignment
7.08	1H, t (7.8)	*m*-Tyr
7.00	1H, t (7.8)	*m*-Tyr
6.88	1H, d (7.8)	Uracil
6.64	1H, d	*m*-Tyr
6.62	1H, d	*m*-Tyr
6.57	1H, d	*m*-Tyr
6.56	2H, m	*m*-Tyr
6.53	1H, d	*m*-Tyr
5.82	1H, d (1.5)	Sugar
5.77	1H, br s	Sugar
5.31	1H, d (8.3)	Uracil
4.76	1H, dq (9, 7)	AMBA
4.44	1H, d (8.8)	AMBA
4.29	1H, dd (9.7, 4.4)	*m*-Tyr
4.25	1H, dt (5.8, 2)	Sugar
4.07	1H, m	Methionine
4.06	1H, m	*m*-Tyr
2.88	1H, dd (13.6, 4)	*m*-Tyr
2.87	3H, s	AMBA
2.79	1H, br dd (14, 5)	*m*-Tyr
2.70	1H, ddd (17.1, 6, 2)	Sugar
2.63	1H, dd (13.7, 7.3)	*m*-Tyr
2.52	1H, dd (14, 10)	*m*-Tyr
2.47	1H, br dd (17, 3)	Sugar
2.27	2H, m	Methionine
1.86	3H, s	Methionine
1.75	2H, m	Methionine
1.04	3H, d (6.8)	AMBA

m-Tyr: *m*-Tyrosine.

Table 2. ^{13}C NMR assignment for MRD A.

Shift	m	Assignment
178.0	s	Carbonyl
174.1	s	Carbonyl
169.9	s	Carbonyl
166.9	s	Carbonyl
165.1	s	Uracil 4
157.7	s	Ureido
155.5	s	*m*-Tyr 3′
154.7	s	*m*-Tyr 3′
150.5	s	Uracil 2
143.9	s	Sugar
139.6	d	Uracil 6
139.2	s	*m*-Tyr 1′
135.2	s	*m*-Tyr 1′
130.2	d	
129.2	d	
121.1	d	
121.0	d	*m*-Tyr
120.7	d	(2′, 4′, 5′, 6′) × 2
115.7	d	
114.6	d	
113.1	d	
101.9	d	Uracil 5
96.5	d	Sugar
93.0	d	Sugar
72.1	d	Sugar
56.4	d	*m*-Tyr 2
55.1	d	*m*-Tyr 2
53.0	d	Methionine 2
52.1	d	AMBA 4
50.7	d	AMBA 3
38.2	t	*m*-Tyr 3
36.2	t	*m*-Tyr 3
33.0	t	Sugar
30.7	t	Methionine 3
29.8	q	AMBA NCH_3
29.3	t	Methionine 4
14.2	q	Methionine SCH_3
13.1	q	AMBA 4

m: Multiplicity, *m*-Tyr: *m*-tyrosine.

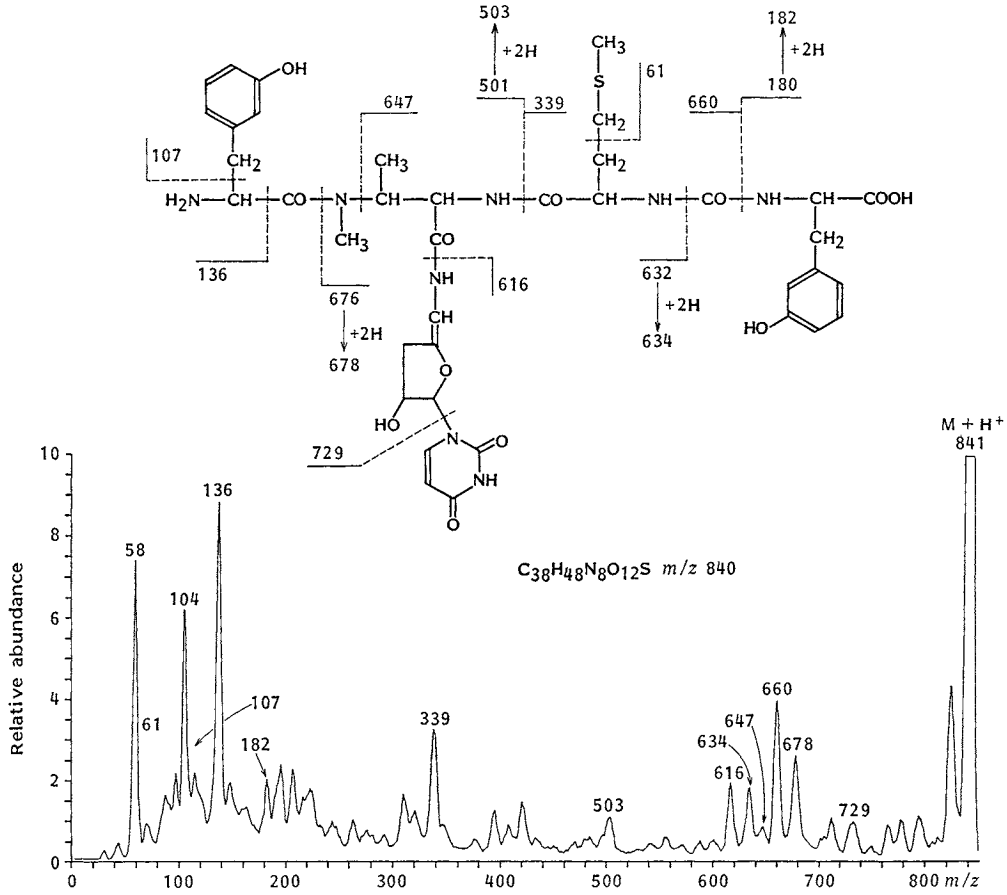
Structure of MRD A

The molecular formula of MRD A (**1**) was determined to be $C_{38}H_{48}N_8O_{12}S$ by high-resolution fast atom bombardment mass spectrometry (HRFAB-MS)[1], and was consistent with the results of the elemental analysis and ^{13}C NMR study. The assignments of ^{1}H and ^{13}C NMR signals are listed in Tables 1 and 2, respectively, and the FAB-MS-MS spectrum of the parent ion peak (m/z 841) are shown in Fig. 2. The structure of **1** was deduced from these data as well as analyses of the degradation products.

Acid hydrolysis of **1** gave three ninhydrin-positive substances, which were separated by Sephadex G-10 column chromatography. The first amino acid showed the same molecular ion as tyrosine in FAB-MS. Although its ^{1}H NMR spectrum was also similar to that of tyrosine, the coupling pattern of four aromatic protons was identical with that of *meta*-substituted benzene ring. Direct comparison of this amino acid with the authentic sample by HPLC and amino acid analysis revealed its identity with *m*-tyrosine.

The second one was found to be an unusual *N*-methyl diamino acid with the molecular formula

Fig. 2. The MS-MS spectrum of the *quasi* molecular ion of **1**, M+H$^+$ *m/z* 841 by FAB-MS.



of $C_5H_{12}N_2O_2$ determined by HRFAB-MS (calcd for M+H$^+$ 133.0977, observed 133.0981). In the ^{1}H NMR spectrum of this amino acid the couplings between the methyl protons at 1.27 ppm and the methine proton at 3.51 ppm, and the latter and another methine proton at 3.75 ppm were shown. These spectral data suggested 2- or 3-*N*-methyl-2,3-diaminobutyric acid as its structure. The structure was finally determined as 2-amino-3-*N*-methylaminobutyric acid (AMBA) by its identity with the authentic sample synthesized from D-threonine as described in the Experimental section.

The ^{1}H NMR spectrum of the third product revealed that it consisted of one molecule of *m*-tyrosine and one of AMBA. The dipeptide structure is suggested by the parent ion (*m/z* 296) and two daughter ions (*m/z* 133 and 136), corresponding to each amino acid.

The other degradation product was isolated from the acid hydrolysate, and characterized by MS-MS experiment as a novel hydantoin compound containing one residue of *m*-tyrosine and one of methionine (Fig. 3). To confirm the existence of methionine, hydrolysis of **1** with cyanogen bromide was carried out and obtained a homoserine lactone derivative (Fig. 4) in a good yield. It is well known that the peptide containing methionine is specifically cleaved at its carbonyl side to give homoserine lactone[8]. It was therefore clearly indicated that in **1** methionine was linked to *m*-tyrosine through the ureido bond, and not through the amide bond. During acid hydrolysis, the hydantoin

compound seemed to be generated by a condensation reaction. This was consistent with the result of MS-MS experiment (Figs. 2, 5 and see below).

In addition, the presence of uracil in **1** was suggested by the ^{1}H and ^{13}C NMR spectral analyses, in which the chemical shift data were in accordance with literature values[4], and it was recognized in the acid hydrolysate of **1** by HPLC analysis.

The remaining part of **1** was expected to have the formula $C_5H_7NO_2$ with two exchange-

Fig. 3. The structure of the hydantoin compound and assignment of the fragment ion peaks detected by the MS-MS experiment.

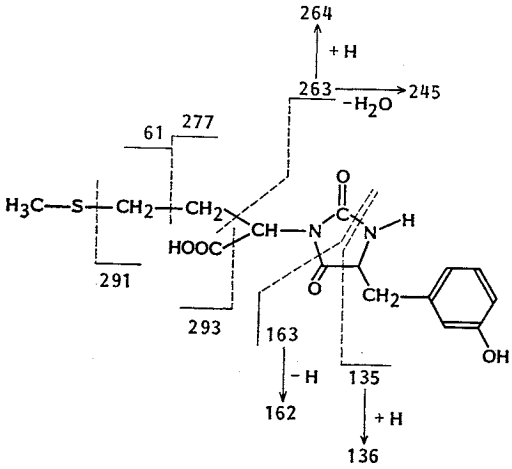


Fig. 4. The structure of the homoserine lactone derivative obtained from the cyanogen bromide degradation and assignment of the fragment ion peaks detected by the MS-MS experiment.

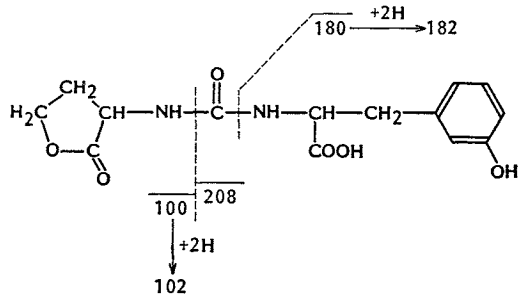
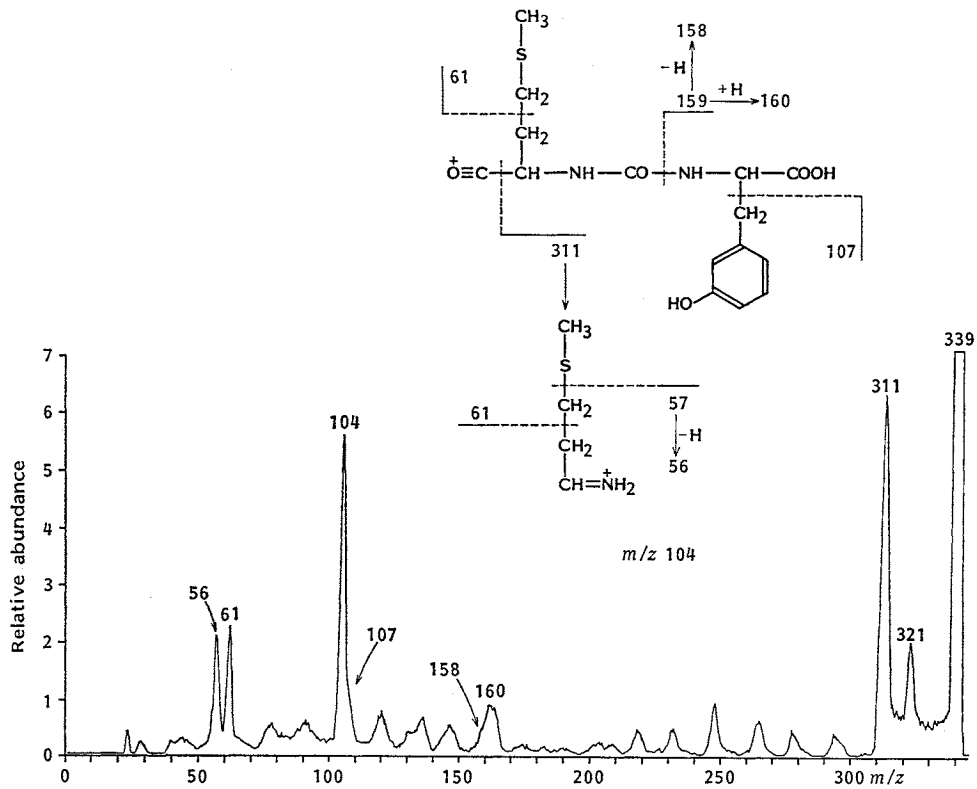
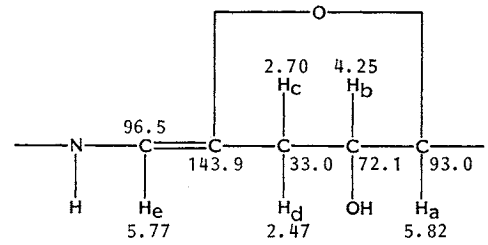


Fig. 5. The MS-MS spectrum of the ion at *m/z* 339 from **1** by FAB-MS.



able protons and two unsaturation units. The proton correlation spectroscopy (COSY) experiment demonstrated a proton spin system consisted of three methine and two methylene protons as shown in Fig. 6. The methine proton H_a was shown to be coupled to another methine proton H_b, which in turn was coupled to the two methylene protons H_c and H_d. They were weakly coupled to the methine proton H_e. The coupling between H_e and the exchangeable amide proton was also observed in the spectrum measured in DMSO-d_6. In the proton-carbon related spectra, H_a and H_e were shown to be attached to the anomeric and olefinic carbon atoms, respectively. In the ^{1}H NMR spectrum of the pentaacetate of **1** obtained by acetylation with acetic anhydride in pyridine, H_b shifted downfield to 5.31 ppm. This observation indicated that H_b was bound to the carbon bearing a hydroxyl group. From these results the structure of this moiety was determined as an unusual enamine sugar shown in Fig. 6.

Fig. 6. The structure of enamine sugar moiety and NMR assignment.



The total structure of each component was confirmed by the extensive FAB-MS-MS study (Fig. 2). Since the fragment ions at *m/z* 729 and 616 corresponded to the losses of uracil and uracil plus sugar moiety from the parent ion, respectively, it was supposed that uracil was linked to the sugar moiety as in a nucleoside. Two daughter ions at *m/z* 503 and 339 indicated the involvement of *m*-tyrosine, AMBA and nucleoside moiety in the former, and another *m*-tyrosine and methionine in the latter fragment. A series of fragment ions at *m/z* 182, 660 and 634, together with MS-MS spectrum of the fragment ion at *m/z* 339 (Fig. 5) verified location of one *m*-tyrosine at the *C*-terminal, linking to methionine *via* ureido bond. As for the *N*-terminal the loss of another *m*-tyrosine was exhibited by the fragment ions at *m/z* 136 and 678 (Fig. 2), and this was consistent with the result from the Edman degradation. The *m*-tyrosine at the *N*-terminal was estimated to be connected to the methylamino group of AMBA due to the fragment ion at *m/z* 647. The total structure of **1** was thus elucidated as shown in Fig. 1.

Structures of MRD's B~D

MRD B (**2**) had the molecular formula $C_{38}H_{50}N_8O_{12}S$ determined by HRFAB-MS and elemental analysis[1]. The ^{1}H NMR spectrum of **2** was quite similar to that of **1** except that the uracil protons of **1** (6.88 and 5.31 ppm) were replaced by four methylene protons (2.0~2.9 ppm). *m*-Tyrosine, AMBA and the hydantoin compound were found in the acid hydrolysate, but uracil was not. Moreover the main fragment ion detected in the MS-MS experiment of **2** exhibited the same mass number (*m/z* 729) as that of **1**. Since this fragment was generated by the loss of uracil from **1**, it was supposed that **2** contained dihydrouracil instead of uracil, but the other portions of the molecule were identical with **1**. Therefore the structure of **2** was deduced as shown in Fig. 1.

The molecular formula $C_{40}H_{51}N_9O_{13}S$ was determined for MRD C (**3**) by HRFAB-MS and elemental analysis[1]. Acid hydrolysis of **3** gave glycine in addition to *m*-tyrosine, AMBA, the hydantoin compound and uracil. Although the ^{1}H NMR signals of **3** were quite similar to those of **1**, a pair of doublets (3.42 and 3.53 ppm) corresponding to the methylene protons of glycine newly appeared, and methine proton of *m*-tyrosine (4.29 ppm in the spectrum of **1**) shifted down to 4.80 ppm. These facts

indicated that glycine located at *N*-terminal of **3** and linked to *m*-tyrosine *via* amide bond. It was also supported by the fact that Edman degradation gave glycine followed by *m*-tyrosine. From these observations **3** was identified as glycylmureidomycin A.

The molecular formula $C_{40}H_{53}N_9O_{13}S$ was determined for MRD D (**4**) by HRFAB-MS and elemental analysis, and allowed us to speculate that **4** was identical with glycylmureidomycin B. This was corroborated by the analysis of degradation products and spectral studies as performed for **2** and **3**.

Experimental

General

NMR spectra were measured with Jeol GX-400 spectrometer using TMS as an external standard. The spectra were measured in D_2O unless otherwise indicated. FAB-MS and FAB-MS-MS spectra were obtained on Jeol JMS-HX 100 mass spectrometer. Amino acid analysis was carried out with Hitachi 835 amino acid analyzer. Edman degradation was done with Applied Biosystems 470A protein sequencer[5].

Acid Hydrolysis

A 10-mg aliquot of **1** was hydrolyzed in conc HCl - AcOH (1 : 1, 5 ml) for 18 hours at 105°C in a sealed tube. After evaporation of the solvent under reduced pressure, the residue was analyzed by HPLC (Senshu Pak H-2151) eluted with 5% aqueous CH_3CN containing 0.05% TFA, and chromatographed on Sephadex G-10 column (170 ml) using the upper layer of BuOH - AcOH - H_2O (4 : 1 : 5) as eluent. Each fraction was checked by silica gel TLC. The yields of *m*-tyrosine, AMBA, the dipeptide, and the hydantoin compound were 2.0, 1.2, 0.5 and 0.7 mg, respectively. The ^1H NMR data for AMBA, the dipeptide and the hydantoin compound were as follows: AMBA δ 3.76 (1H, d, J=7.7 Hz), 3.5 (1H, m), 2.63 (3H, s), 1.27 (3H, d, J=6.6 Hz); dipeptide δ 7.11 (1H, t, J=7.7 Hz), 6.68 (1H, br d, J=7.7 Hz), 6.67 (1H, br d, J=7.7 Hz), 6.62 (1H, br s), 4.30 (1H, d, J=4.4 Hz), 4.20 (1H, t, J=7.0 Hz), 3.51 (1H, m), 3.04 (1H, d, J=6.6 Hz), 3.02 (1H, d, J=7.3 Hz), 2.59 (3H, s), 1.00 (3H, d, J=6.9 Hz); hydantoin compound δ 7.04 (1H, t, J=7.7 Hz), 6.62 (1H, br dd, J=2.1 and 7.7 Hz), 6.59 (1H, br d, J=7.7 Hz), 6.52 (1H, br d, J=2.2 Hz), 4.4 (2H, m), 2.94 (2H, d, J=4.0 Hz), 1.96 (1H, m), 1.78 (3H, s), 1.72 (1H, m), 1.60 (1H, m), 1.35 (1H, m). Hydrolysis of the other components were performed as described for **1**.

Synthesis of AMBA

Carbobenzoxy chloride (CBZ-Cl, 25.3 g) was added to D-threonine (16.7 g) solubilized in 2 N NaOH (70 ml) with Na_2CO_3 (8.9 g), and vigorously stirred at room temperature for 1 hour. CBZ-threonine was extracted with EtOAc after acidification and then methylated with CH_2N_2 in ether (100 ml). CBZ-threonine methyl ester thus obtained was combined with PCl_5 (31.2 g) in CH_2Cl_2 (300 ml), and stirred at 20°C for 3 hours. Chlorinated product was purified by silica gel chromatography, and mixed with $CH_3NH_2 \cdot HCl$ (9.7 g) and Et_3N (14.5 g) in MeOH containing K_2CO_3 (3.3 g). The product was purified on silica gel column and was identified with α-CBZ-amino-β-methylaminobutyric acid methyl ester by ^1H NMR. AMBA (0.5 g) was obtained after hydroxylation of this compound with HCl.

Cyanogen Bromide Cleavage

The solution of **1** (20 mg in 0.5 ml of 70% formic acid) was treated with cyanogen bromide (360 mg) for 18 hours at room temperature. After lyophilization, the degradation product was isolated by HPLC system (Senshu Pak H-2151) with 15% aqueous CH_3CN containing 0.05% TFA to yield 2.9 mg. Its ^1H NMR signals were as follows: δ 7.07 (1H, t, J=7.7 Hz), 6.67 (1H, br d, J=7.7 Hz), 6.61 (1H, br d, J=7.7 Hz), 6.60 (1H, br s), 4.1~4.3 (4H, m), 2.97 (1H, dd, J=5.1 and 13.9), 2.75 (1H, dd, J=8.4 and 13.9), 2.33 (1H, m), 1.97 (1H, m).

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