NOVEL MACROCYCLIC ANTIBIOTICS: MEGOVALICINS A, B, C, D, G AND H

II. ISOLATION AND CHEMICAL STRUCTURES OF MEGOVALICINS

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Myxococcus flavescens AJ12298 was found to produce the complex of macrocyclic antibiotics named megovalicins.

The physico-chemical studies revealed that megovalicins C and B were identical to myxovirescin A_1 and antibiotic M-230B, respectively, and that megovalicins A, D, G and H were closely related new antibiotics.

During our screening program for new antibiotics, we found that *Myxococcus flavescens* AJ12298, a newly isolated myxobacterium, produced a mixture of homologous antibiotics. From the antibiotics complex, we have isolated six biologically active compounds and named megovalicins A (**1**), B (**2**), C (**3**), D (**4**), G (**5**) and H (**6**).

In the preceding paper[1], taxonomy of megovalicin-producing organisms, and production and some biological activities of the antibiotics have been described. We here report the isolation procedure and the structural elucidation of megovalicins.

Materials and Methods

Instruments

Mass spectra were measured on a Jeol DX-300 spectrometer. UV spectra were recorded using a Hitachi 200-20 spectrophotometer. NMR spectra were obtained on a Jeol GX-400 spectrometer with ^1H NMR at 400 MHz and ^{13}C NMR at 100 MHz. Chemical shifts were given in ppm using TMS as an internal standard.

Preparative and analytical HPLC were performed with a Shimadzu LC-4A liquid chromatograph system.

Isolation of Megovalicins

M. flavescens AJ12298 was cultured in a fermentation tank with working volume of 10 kiloliters at 27°C for 70 hours. Aeration rate was 1/20 vvm, and agitation speed was 175 rpm. The production medium employed was as follows; soluble starch 0.5%, sucrose 0.3%, Polypeptone 0.25%, yeast extract (Difco) 0.1%, $MgSO_4$ 0.05%, KH_2PO_4 0.025% and TMA-812 (Toshiba) 0.015%, pH 7.3.

Antibiotic activity was determined by the paper-disk method using *Escherichia coli* 508[1] as an indicator.

The grown cells (10.5 kg, wet) were collected by centrifugation and extracted twice with 18 liters of MeOH. The resulting MeOH extracts were combined and diluted with a half volume of water (resulted in *ca.* 67% MeOH soln) and passed through a Diaion HP-20 column (Mitsubishi Chemical Industries Limited, 15 liters). The column was washed with 67% MeOH (15 liters) and 75% MeOH (30 liters) and the active principle was eluted with 100% MeOH.

The active fraction eluted with MeOH was divided into two portions and each of them was subjected to the following procedure.

After the evaporation of MeOH the residue was dissolved in 220 ml of $CHCl_3$ - hexane (10 : 1) and was applied to a Silica gel column (Merck, 1 liter). The column was washed with $CHCl_3$ (2 liters) and $CHCl_3$ - EtOAc (1 : 4, 3 liters). The active principle was eluted subsequently with $CHCl_3$ - MeOH (10 : 1). The active fraction was evaporated *in vacuo* and subjected to silica gel column chromatography using EtOAc - $CHCl_3$ - MeOH (10 : 9 : 1). The active fractions were collected and evaporated to give the mixture of megovalicins.

Each component of megovalicins could be separated on an ODS column (LRP-1, Whatmann, 1 liter) with a stepwise MeOH gradient (75% MeOH for the first step, 85% for the second step yielding **1**, **2** and **3**, 90% for the third step yielding **4**, 95% for the forth step yielding **5** and **6**).

Finally, **3** and **5** were recrystallized from EtOH and water to give colorless needles. The component **6** was recrystallized from $CHCl_3$ and hexane to give colorless needles. The other components **1**, **2** and **4** were finally purified by repeated runs of reversed phase HPLC (μBondasphere, 19×150 mm, Millipore), eluting with a linear gradient of 50 to 100% MeOH in water.

Results and Discussion

Isolation

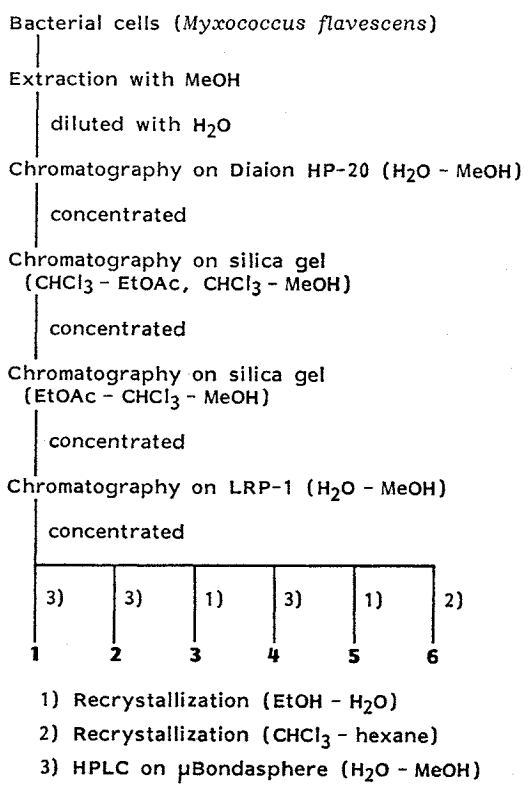
The isolation procedures for megovalicins were outlined in Fig. 1.

Each component of megovalicins could be separated at the 5th step (LRP-1 column chromatography) of the procedure.

Megovalicins C (**3**) and G (**5**) were obtained as colorless needles after recrystallization from a mixture of ethanol and water. Megovalicin H (**6**) was recrystallized from chloroform - hexane. Megovalicins A (**1**), B (**2**) and D (**4**) were obtained as colorless powder by reversed phase HPLC.

Yields from 10.5 kg of wet cells were as follows: **1** (40 mg), **2** (68 mg), **3** (255 mg), **4** (5 mg), **5** (39 mg), **6** (161 mg).

Fig. 1. Isolation procedures for megovalicins A (**1**), B (**2**), C (**3**), D (**4**), G (**5**) and H (**6**).



Chemical Structures

Megovalicins are readily soluble in methanol, ethanol, dichloromethane and chloroform, moderately soluble in ethyl acetate and acetonitrile and insoluble in hexane and water.

The physico-chemical data of megovalicins were summerized in Table 1. ^1H and ^{13}C NMR data were summerized in Tables 2 and 3, respectively.

These data suggested that megovalicins belong to the antibiotics of myxovirescin group, a family of macrocyclic lactam-lactone antibiotics[2]. In this group, only the structures of myxovirescins A_1, A_2[2,3] and antibiotic M-230B[4] have been

Table 1. Physico-chemical properties of megovalicins A (**1**), B (**2**), C (**3**), D (**4**), G (**5**) and H (**6**).

	1	**2**	**3**	**4**	**5**	**6**
Rf[a]	0.56	0.54	0.52	0.44	0.18	0.16
Rt (minutes)[b]	11.7	12.5	13.3	15.0	23.4	24.0
UV λ_{max}^{MeOH} nm (ε)	239	232 (31,000)	239 (29,000)	240	232 (31,000)	239 (29,000)
FAB-MS (*m/z*) (glycerol)	664 $(M+K)^+$, 648 $(M+Na)^+$, 626 $(M+H)^+$, 594 (M^+-OCH_3)	660 $(M+K)^+$, 644 $(M+Na)^+$, 622 $(M+H)^+$, 590 (M^+-OCH_3)	624 $(M+H)^+$, 592 (M^+-OCH_3)	594 $(M+H)^+$	608 $(M+H)^+$, 576 (M^+-OCH_3)	610 $(M+H)^+$, 578 (M^+-OCH_3)
MW	625	621	623	593	607	609
Molecular formula	$C_{35}H_{63}NO_8$	$C_{35}H_{59}NO_8$	$C_{35}H_{61}NO_8$	$C_{34}H_{59}NO_7$	$C_{35}H_{61}NO_7$	$C_{35}H_{63}NO_7$

[a] Rf values on ODS TLC (K18F, Whatmann, CH_3CN - H_2O, 4 : 1).

[b] Retention times (Rt) on reversed phase HPLC (Nova-pack 8NVC18, Millipore, a linear gradient of 50 to 100% CH_3CN for 20 minutes at 1.5 ml/minute).

Table 2. ^1H NMR data for megovalicins A (**1**), B (**2**), C (**3**), D (**4**), G (**5**) and H (**6**)[a].

Proton[b]	Chemical shift (ppm)[c], multiplicity (*J*(Hz))					
	1	**2**	**3**	**4**	**5**	**6**
2-H	2.64, 1H, ddq (6.8, 8.2, 6.8)	—	2.62, 1H, ddq (6.8, 8.2, 6.8)	2.61, 1H, ddq (6.8, 8.2, 6.8)	—	2.60, 1H, ddq (6.8, 8.2, 6.8)
3-H	1.3~1.5[d], m	6.59, 1H, d (11.5)	1.3~1.5[d], m	1.3~1.5[d], m	6.59, 1H, d (11.5)	1.3~1.5[d], m
4-H	1.3~1.5[d], m	2.50, 1H, m	1.3~1.5[d], m	1.3~1.5[d], m	2.52, 1H, m	1.3~1.5[d], m
8-H, 10-H	1.3~1.5[d], m	2.2~2.4, 4H, m	2.2~2.4, 4H, m	2.2~2.4, 4H, m	1.2~1.3[d], m	1.2~1.3[d], m
9-H	3.52, 1H, m	—	—	—	1.2~1.3[d], m	1.2~1.3[d], m
29-H	1.16, 3H, d (6.8)	1.85, 3H, s	1.15, 3H, d (6.8)	1.16, 3H, d (6.8)	1.87, 3H, s	1.16, 3H, d (6.8)
30-H	0.86, 3H, d (6.6)	1.01, 3H, d (7.0)	0.85, 3H, d (6.8)	0.85, 3H, d (6.8)	1.01, 3H, d (6.6)	0.86, 3H, d (6.8)
33-H	4.19, 1H, d (11.0)	4.18, 1H, d (11.0)	4.19, 1H, d (11.0)	1.78, 3H, s	4.17, 1H, d (11.0)	4.16, 1H, d (11.0)
	3.89, 1H, d (11.0)	3.89, 1H, d (11.0)	3.90, 1H, d (11.0)	—	3.95, 1H, d (11.0)	3.95, 1H, d (11.0)
34-H	3.37, 3H, s	3.37, 3H, s	3.37, 3H, s	—	3.35, 3H, s	3.34, 3H, s

[a] Only the characteristic signals compared with those of **3** (myxovirescin A_1) were listed in this table. (The whole spectrum of **3** was in complete agreement with that reported for myxovirescin A_1[2].)

[b] As indicated in Fig. 2.

[c] Chemical shifts in $CDCl_3$ solution from internal TMS.

[d] Overlapping signals.

Table 3. ^{13}C NMR data for megovalicins A (**1**), B (**2**), C (**3**), D (**4**), G (**5**) and H (**6**).

Carbon[a]	Chemical shift (ppm)[b], multiplicity[c]					
	1	**2**	**3**	**4**	**5**	**6**
C-1	176.1 s	166.7 s	176.0 s	176.0 s	166.2 s	176.2 s
C-2	37.2 d	126.2 s	37.2 d	37.2 d	125.2 s	37.4 d
C-3	41.3 t	149.1 d	41.0 t	40.8 t	149.1 d	40.8 t
C-4	30.4 d	33.8 d	30.4 d	30.5 d	33.4 d	30.7 d
C-5	36.3 t[d]	37.0 t	36.5 t	36.7 t	36.8 t	37.0 t
C-6	26.6 t	27.5 t	26.5 t	26.5 t	26.8 t[g]	26.6 t[g]
C-7	25.8 t	23.7 t	23.8 t	23.8 t	29.4 t[h]	29.6 t[h]
C-8	37.1 t[d]	42.5 t	42.6 t	42.6 t	28.9 t[h]	29.0 t[h]
C-9	71.2 d	212.3 s	212.3 s	212.3 s	29.2 t[h]	29.3 t[h]
C-10	37.5 t[d]	43.0 t	43.1 t	43.1 t	29.6 t[h]	29.9 t[h]
C-11	23.0 t	22.3 t	22.1 t	21.9 t	27.2 t[g]	27.0 t[g]
C-12	34.5 t	34.7 t	34.7 t	34.7 t	35.0 t	35.1 t
C-13	45.3 d	45.1 d	45.3 d	45.0 d	45.0 d	45.3 d
C-14	140.2 d	139.6 d	139.6 d	137.0 d	139.8 d	140.4 d
C-15	125.9 d	125.9 d	125.9 d	125.7 d	124.8 d	125.4 d
C-16	130.7 d	130.1 d	130.1 d	126.8 d	129.9 d	130.3 d
C-17	134.3 s	134.7 s	134.6 s	135.6 s	133.7 s	134.3 s
C-18	30.4 t[e]	30.3 t	30.2 t	34.6 t	30.6 t[e]	30.9 t[e]
C-19	31.1 t[e]	30.5 t	30.5 t	29.7 t	31.7 t[e]	31.3 t[e]
C-20	73.4 d[f]	73.5 d	73.2 d	73.6 d[f]	73.7 d[f]	73.7 d[f]
C-21	71.5 d	71.6 d	71.7 d	71.7 d	71.5 d	71.6 d
C-22	35.8 t	36.4 t	36.0 t	35.6 t	35.8 t	35.9 t
C-23	68.8 d	69.0 d	68.9 d	69.4 d	68.8 d	68.6 d
C-24	45.4 t	45.4 t	45.4 t	45.3 t	45.3 t	45.4 t
C-26	171.2 s	171.5 s	171.1 s	171.5 s	171.1 s	171.4 s
C-27	73.6 d[f]	73.9 d	73.6 d	74.4 d[f]	73.8 d[f]	74.1 d[f]
C-29	17.8 q	12.7 q	17.5 q	17.3 q	12.7 q	17.2 q
C-30	19.9 q	20.3 q	19.7 q	19.7 q	20.2 q	19.6 q
C-31	28.6 t	28.4 t	28.4 t	28.4 t	28.5 t	28.6 t
C-32	12.0 q	11.9 q	11.9 q	11.9 q	11.9 q	11.9 q
C-33	71.1 t	71.1 t	71.1 t	17.0 q	70.3 t	70.7 t
C-34	58.4 q	58.3 q	58.3 q	—	57.9 q	58.1 q
C-35	34.1 t	34.1 t	34.0 t	34.0 t	33.9 t	34.0 t
C-36	18.2 t	18.3 t	18.2 t	18.2 t	18.2 t	18.3 t
C-37	13.7 q	13.8 q	13.7 q	13.7 q	13.7 q	13.7 q

[a] As indicated in Fig. 2.
[b] Chemical shifts in $CDCl_3$ solution from internal TMS.
[c] Multiplicities in the off-resonance decoupling.
[d~h] Assignments may be interchanged in each case.

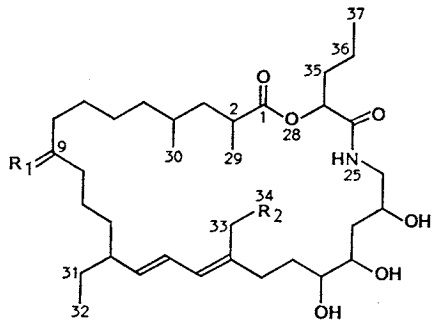
reported (Fig. 2).

The following physico-chemical studies revealed that megovalicins C (**3**) and B (**2**) were identical to myxovirescin A_1 and antibiotic M-230B, respectively. The structures of the other megovalicins have been elucidated by spectroscopic methods, in particular by direct comparisons of their UV, ^1H NMR, ^{13}C NMR and mass spectra with the corresponding spectra of myxovirescin A_1 and antibiotic M-230B.

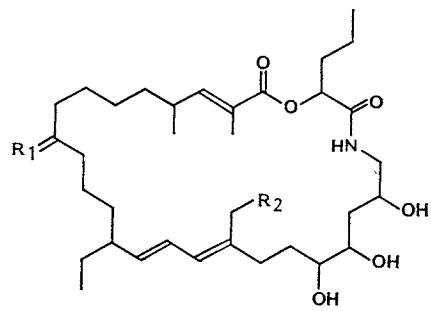
Megovalicin C (**3**)

The physico-chemical data on **3** (Tables 1, 2 and 3), as well as the ^1H-correlation spectroscopy (COSY) spectrum (data not shown) were in complete agreement with those reported for myxovirescin

Fig. 2. Structures of megovalicins A (1), B (2), C (3), D (4), G (5) and H (6).



A (1) R_1 = H,OH R_2 = OCH_3
C (3) R_1 = O R_2 = OCH_3
(myxovirescin A_1)
D (4) R_1 = O R_2 = H
H (6) R_1 = H,H R_2 = OCH_3



B (2) R_1 = O R_2 = OCH_3
(antibiotic M-230B)
G (5) R_1 = H,H R_2 = OCH_3

A_1[2,3]. Therefore we identified **3** as myxovirescin A_1 (Fig. 2).

Megovalicin B (2)

The physico-chemical data on **2** (Tables 1, 2 and 3) were in complete agreement with those reported for antibiotic M-230B[4]. Therefore we identified **2** as antibiotic M-230B (Fig. 2).

Antibiotic M-230B is a 2,3-dehydro form of myxovirescin A. The dehydration of the C2-C3 bond of **2** (antibiotic M-230B) was demonstrated by the UV spectrum (λ_{max} shifted to 232 nm by the resulting α,β-unsaturated ester), by the ^1H NMR spectrum (a new olefinic proton signal (δ 6.59 (1H, d)) and a downfield shift of 29-H methyl signal (from δ 1.15 (3H, d) to δ 1.85 (3H, s)) were observed) and by the ^{13}C NMR spectrum (new olefinic carbon signals (δ 126.2 (s) and δ 149.1 (d)) and upfield shifts of C-1 carbonyl signal (from δ 176.0 (s) to δ 166.7 (s)) and of C-29 methyl signal (from δ 17.5 (q) to δ 12.7 (q)) were observed).

Megovalicin A (1)

The fast atom bombardment mass spectrum (FAB-MS) of **1** showed a quasi molecular ion $(M+H)^+$ at *m/z* 626 which is 2 mass units (2H) higher than that of **3**. In the ^1H NMR spectrum of **1**, the signals of the α-protons (8-H and 10-H) of C-9 carbonyl, present at δ 2.2~2.4 (4H, m) in **3**, were missing, whereas an additional oxygen substituted methine proton (9-H) was found at δ 3.52 (1H, m). In the ^{13}C NMR spectrum of **1**, the signal of the C-9 kotone carbonyl group, found at δ 212.3 (s) in **3**, was missing, whereas an additional signal of an oxy-methine carbon (C-9) was found at δ 71.2 (d). These observations suggest that **1** is a reduced form of **3**, C-9 ketone group being reduced to secondary alcohol group. Due to this change C-8 and C-10 showed upfield shifts and C-7 and C-11 showed small downfield shifts (see ^{13}C NMR spectral data in Table 3). In the other part of the molecule the chemical shifts were nearly identical between **1** and **3**.

Thus, the structure of **1** can be assigned as shown in Fig. 2.

In the course of structural elucidation of myxovirescin A_1 (**3**), TROWITZSCH *et al.* reduced myxovirescin A_1 with sodium borohydride, and obtained the compound corresponding to **1** (a mixture of C-9 epimer) chemically[2]. But this is the first case that **1** was obtained from the natural products.

Megovalicin H (6)

The FAB-MS of **6** showed a quasi molecular ion $(M+H)^+$ at *m/z* 610 which was 14 mass units lower than that of **3**.

In the ^1H NMR spectrum of **6**, the signals of the α-protons of C-9 carbonyl (8-H and 10-H, δ 2.2~2.4 (4H, m) in **3**) were missing as in the case of **1**, and the great signal attributed to methylene protons was observed at δ 1.2~1.3.

In the ^{13}C NMR spectrum, the carbon signals around C-9 ketone carbonyl group in **3** were missing (as **1**), whereas additional five methylene signals characteristic to the long linear aliphatic chain were observed at δ 26.6~29.9. These observations suggest that **6** is a further reduced form of **3**, C-9 ketone group being reduced to methylene group.

In the other part of the molecule, the chemical shifts were nearly identical to **3**.

Thus, the structure of **6** can be assigned as shown in Fig. 2.

Both myxovirescins C and D discribed without structure in the patent literature[5] have the same molecular formula as **6**, and one of them may be identical to **6**.

Megovalicin G (5)

The FAB-MS of **5** showed a quasi molecular ion $(M+H)^+$ at *m/z* 608 which was 2 mass units (2H) lower than that of **6**.

^1H and ^{13}C NMR spectra of **5** showed close resemblance to those of **6**, except for some signals attributed to the moiety around C2-C3 bond. These signals were changed in the same pattern observed in **2** compared with **3**.

In the UV spectrum, **5** showed the same absorption curve with **2** (λ_{max} 232 nm), which also suggested the presence of C2-C3 double bond, resulting α,β-unsaturated ester.

These observations suggest that **5** is a 2,3-dehydro form of **6**, thus a 2,3-dehydro-9-deoxy form of **3**.

The ^{13}C chemical shift of the allylic methyl (C-29, δ 12.7) suggested the *E* configuration for C2-C3 double bond, which was further confirmed by the nuclear Overhauser effect (NOE) enhancement observed with 4-H on irradiation of 29-H.

Thus, the structure of **5** can be assigned as shown in Fig. 2.

Megovalicin D (4)

The FAB-MS of **4** showed a quasi molecular ion $(M+H)^+$ at *m/z* 594, which was 30 mass units lower than that of **3**. Furthermore, M^+-OCH_3 ion, characteristic for the other megovalicins, could not be detected at all.

In addition, the characteristic signals for CH_2OCH_3 group observed in the ^1H NMR spectrum (33-H: δ 4.19 (1H, d, J=11.0 Hz) and δ 3.90 (1H, d, J=11.0 Hz), 34-H: δ 3.37 (3H, s) in **3**) and in the ^{13}C NMR spectrum (C-33: δ 71.1 (t), C-34: δ 58.3 (q) in **3**) disappeared in **4**. Whereas an additional olefinic methyl signal was observed (^1H NMR: δ 1.78 (3H, s), ^{13}C NMR: δ 17.0 (q)). And the attachment site of this additional methyl group was established as position 17, by the long range coupling detected between this methyl and 16-H olefinic proton.

These observations suggest that **4** is a demethoxy form of **3**, CH_2OCH_3 group at C-17 being substituted by a methyl group.

The ^{13}C chemical shift (δ 17.0) of this additional olefinic methyl (C-33) suggested the *E* configuration for C16-C17 double bond, which was further confirmed by the NOE enhancement observed with

15-H on irradiation of 33-H.

Thus, the structure of **6** can be assigned as shown in Fig. 2.

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