MANUMYCINS E, F AND G, NEW MEMBERS OF MANUMYCIN CLASS ANTIBIOTICS, FROM *Streptomyces* sp.

YUE-ZHONG SHU,* STELLA HUANG, RICHARD R. WANG, KIN SING LAM, STEVEN E. KLOHR, KEVIN J. VOLK, DOLORES M. PIRNIK, J. SCOTT WELLS, PRABHAVATHI B. FERNANDES† and PRAMATHESH S. PATEL†

Bristol-Myers Squibb Pharmaceutical Research Institute,
5 Research Parkway, Wallingford, CT 06492, U.S.A.
†Bristol-Myers Squibb Phamaceutical Research Institute,
P. O. Box 4000, Princeton,
NJ 08543, U.S.A.

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Three new manumycin class antibiotics, namely manumycins E, F and G, were isolated from the culture broth of *Streptomyces* sp. strain WB-8376. Their structures were established by spectroscopic methods, and the *S* configuration of C-4 in the epoxycyclohexenone moiety was determined by CD exciton chirality method for each of the three compounds. Manumycins E, F and G are active against Gram-positive bacteria, and have moderate inhibitory effects on the farnesylation of p21 ras protein. They demonstrated weak cytotoxic activity against human colon tumor cell HCT-116.

The manumycin class of antibiotics, in addition to manumycin itself,[1~3] includes asukamycin,[4] U-56407,[5] U-62162,[6] colabomycin,[7] alisamycin,[8] and manumycins B, C and D.[9] They have shown antibacterial activity mainly against Gram-positive bacteria. Very recently, it has been reported that manumycin and its analogs inhibit ras farnesyltransferase and the growth of *Ki*-ras-activated murine fibrosarcoma in mice, raising its potential as an antitumor agent.[10]

As a part of our ongoing studies on the search for new bioactive secondary metabolites from microorganisms, we found that a *Streptomyces* strain, WB-8376, produced three new manumycin class antibiotics, designated as manumycins E, F and G. In this paper, we report the fermentative production, isolation, structure elucidation and biological activity of these three new antibiotics.

Materials and Methods

Taxonomy

The manumycin-producing organism, strain WB-8376, was isolated from a soil sample collected in Princeton, New Jersey. Taxonomy of the strain WB-8376 was studied using International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB,[11] and those by WAKSMAN.[12]

Fermentation

Frozen vegetative preparations of *Streptomyces* strain WB-8376 were maintained in 10% glycerol - 5% sucrose solution stored at −80°C for use as working stock. To prepare an inoculum for the production phase in shake flask culture, 4 ml of the vegetative stock were transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium rB consisting of soluble starch 0.5%, glucose 0.5%, fishmeat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, sodium chloride 0.2% and calcium carbonate 0.1%. This seed culture was incubated at 28°C for 3 days on a rotary shaker set at 250 rpm. Four ml of seed culture were transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium K-18 consisting of Pharmamedia

2%, glucose 5% and calcium carbonate 0.7%. The production cultures were incubated at 28°C and 250 rpm on the same shaker for 5~6 days. For production in laboratory fermentors (50-liter nominal volume), 20 ml of the seed culture were transferred to a 2-liter Erlenmeyer flask containing 400 ml of seed medium rB. This second seed culture was incubated at 28°C and 250 rpm for 3 days. Four seed cultures were combined and transferred to the fermentor containing 30 liters of production medium K-18. The incubation temperature was 28°C, agitation rate was 300 rpm, and the air flow was 0.7 volume per minute. The back pressure of the fermentor was set at 0.35 kg/cm^2.

Isolation and Purification of Manumycins

The isolation and purification of manumycins from a 5-liter shake flask culture are summarized in Fig. 1.

Determination of Biological Activities

Cytotoxicity was assessed by the XTT-assay[13] using HCT-116 human colon carcinoma cell. Cells were plated at 4,000 cells/well in 96-well microtiter plates, and 24 hours later drugs were added and serially diluted. The cells were incubated at 37°C for 72 hours at which time the tetrazolium dye, XTT, was added. The results are expressed as an IC_{50}, which is the drug concentration required to inhibit cell proliferation (absorbance at 450 nm) to 50% of that of untreated control cells.

The inhibition of ras farnesyltransferase was assessed by using p21 ras protein according to the procedures of MANNE and co-workers.[14]

Instrumental Analyses

Specific rotations ($[\alpha]_D$) were measured on a Perkin Elmer 241 polarimeter. UV spectra were recorded at a Shimadzu UV 2100 spectrophotometer. IR spectra were recorded at a Perkin Elmer FT-IR 1800 spectrometer. NMR spectra, including COSY, long range COSY, NOESY (mixing time, 0.8 seconds), HETCOR and HMBC (increment delay, 0.07 seconds), were taken on a Bruker AM-500 spectrometer in acetone-d_6. High resolution mass spectra (HR-MS) were obtained at a Kratos MS50 spectrometer with FAB ionization mode at the acceleration voltage of 8.0 kV. Tandem mass spectra were obtained on a Sciex API III triple quadruple mass spectrometer equipped with an ionspray interface, and MS/MS daughter spectra were generated by using a collision energy of 60 eV. Circular dichroism (CD) spectra were measured at a Jasco J500A spectropolarimeter.

Results and Discussion

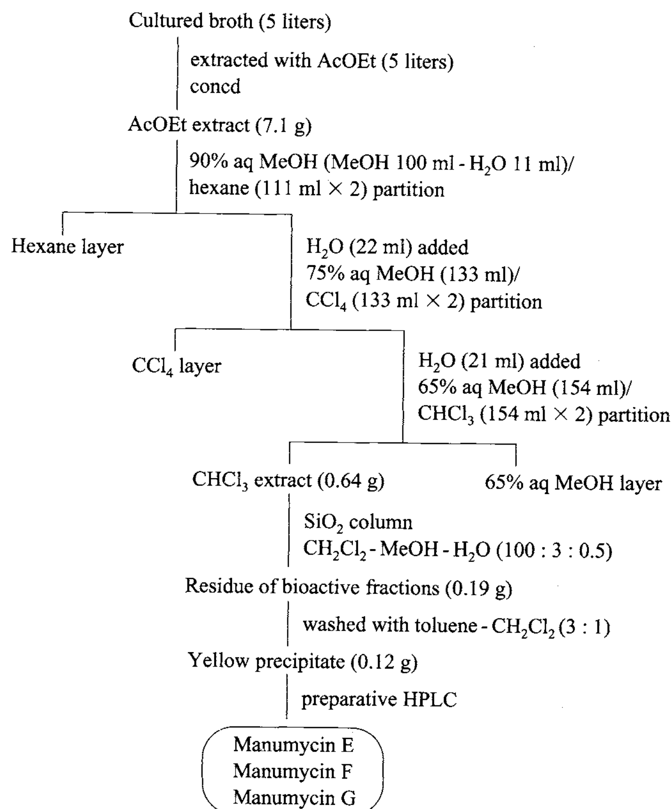
Taxonomy

Microscopic studies on strain WB-8376 grown on ISP morphological agar media (ISP-2, ISP-3 and ISP-4) showed that aerial mycelium is gray and developed smooth spores born in spiral chains. Strain WB8376 has a Type I cell wall (L-DAP and no diagnostic sugars) based on analysis of whole cell hydrolysates, placing it in the Genus Streptomyces.[15] The colony reverse shows a dark yellow-brown pigment on all morphology media with no diffusible pigments. Melanin was not produced on Peptone Iron Agar (ISP-6) or Tyrosine Agar (ISP-7). The modified Arai & Mikami test[16] for melanin was also negative. Strain WB-8376 utilized glucose, xylose, rhamnose, fructose (weak), raffinose, galactose and salicin (weak), but did not utilize mannitol, inositol, sorbitol, arabinose and sucrose for growth when the above carbon sources were incorporated into medium ISP-9 as the sole carbon source. Strain WB-8376 was deposited with the American Type Culture Collection with the accession number of ATCC 55484.

Isolation

The isolation of manumycins E, F, and G was monitored by antibacterial assay, and the procedure is illustrated in Fig. 1. Briefly, the fermented broth was extracted with ethyl acetate, the extract was dissolved in aqueous methanol, and partitioned against pre-equilibrated hexane, carbon tetrachloride and chloroform

Fig. 1. Isolation and purification procedures for manumycins E, F and G.



sequentially. The chloroform extract was purified by silica gel flash column. The bioactive fractions were collected, concentrated and then washed with nonpolar organic solvent mixture to give a yellow precipitate (0.12 g). A part of the precipitate (63 mg) was chromatographed on a reversed phase HPLC column (Dynamax C_{18}, 25 cm × 10 mm, 8 μm, Rainin Co.) with UV detection at 250 nm. A gradient elution was carried out by beginning with 40% 0.01 M potassium dihydrogen phosphate and 60% acetonitrile - methanol (3 : 1, v/v) and increasing the acetonitrile - methanol mixture (3 : 1, v/v) to 100% over a time course of 50 minutes. The flow rate was 4 ml/minute. Three bioactive compounds were eluted at 17.2 minutes, 24.8 minutes and 26.8 minutes to yield manumycin G (7.5 mg), manumycin E (9.0 mg) and manumycin F (6.5 mg), respectively.

Physico-chemical Properties

Manumycins E, F and G were each obtained as yellow amorphous powders. They were soluble in dimethyl sulfoxide, acetone, acetonitrile and methanol - chloroform mixture (20 ~ 80% methanol), sparingly soluble in ethyl acetate, but were insoluble in hexane and water. Listed in Table 1 are additional physico-chemical data of manumycins E, F and G.

The IR, ^1H NMR and ^{13}C NMR spectra of manumycin E are reproduced in Figs. 2, 3 and 4.

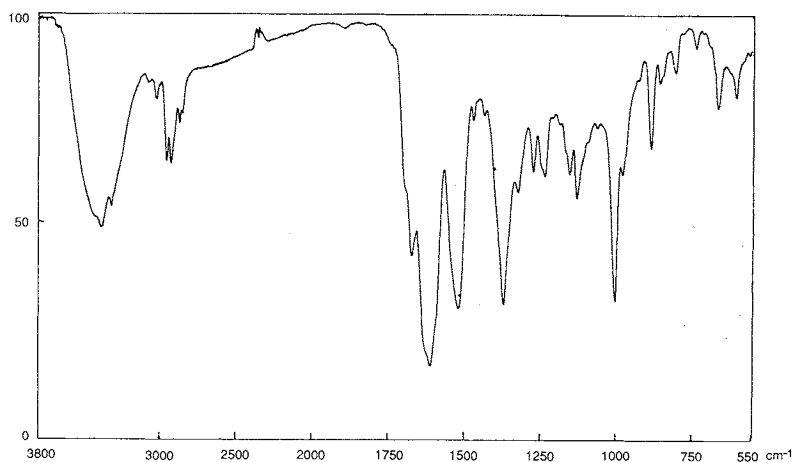
Structure Studies

Manumycin E showed a chemical composition of $C_{30}H_{34}N_2O_7$ by high resolution mass spectrometry,

Table 1. Physico-chemical properties of manumycins E, F and G.

	Manumycin E	Manumycin F	Manumycin G
Appearance	Yellow amorphous powder	Yellow amorphous powder	Yellow amorphous powder
MP	>250°C (dec)	>250°C (dec)	>250°C (dec)
Molecular formula	$C_{30}H_{34}N_2O_7$	$C_{31}H_{34}N_2O_7$	$C_{28}H_{30}N_2O_7$
High resolution MS			
Found:	534.2347	546.2360	506.2039
Calcd:	534.2366	546.2366	506.2053
$[\alpha]_D$	+128° (c 0.01, CH_3COCH_3)	+500° (c 0.03, CH_3CN)	+380° (c 0.02, CH_3CN)
UV λ_{max} (CH_3CN) nm (log ε)	316 (4.3906)	316 (4.4755)	312 (4.5016)
IR (KBr) cm^{-1}	3400, 2930, 1700, 1640, 1545, 1380, 1000	3400, 2930, 1675, 1620, 1520, 1465, 1370, 1000	3425, 2930, 1670, 1610, 1520, 1380, 1310, 1000
CD λ_{max} (CH_3CN) nm	$[\theta]_{348}$ 1.532×10^5, $[\theta]_{307}$ -1.798×10^5	$[\theta]_{348}$ 1.165×10^5, $[\theta]_{307}$ -1.476×10^5	$[\theta]_{348}$ 1.814×10^5, $[\theta]_{307}$ -2.165×10^5

Fig. 2. IR spectrum of manumycin E.



indicating an unsaturation number of fourteen. The compound exhibited a UV maximum at 316 nm due to extended unsaturation, and strong IR absorption bands due to hydroxyl and amide (3400 cm^{-1}), carbonyl (1700 cm^{-1}) and polyene (1640 cm^{-1}) groups. The NMR data (Tables 2 and 3) obtained from ^1H, ^{13}C, DEPT COSY NOESY, HETCOR and HMBC experiments showed overall similarity to those reported for the manumycin class compounds. The presence of a cyclohexene epoxide moiety (C_1-C_6) and two all *trans*-triene moieties (C_7-C_{13} and $C_{1'}$-$C_{7'}$, respectively) was clear from the characteristic ^1H-^1H coupling spin systems (^3J of all involved vicinal protons and ^4J between 3-H and 4-H) (Fig. 5, Table 2). The NOEs of 12-H (δ 6.60) *versus* NH-14 (δ 8.77), 2'-H (δ 6.36) *versus* NH-0' (δ 8.32) (Fig. 5), and the ^{13}C-^1H long range couplings between C_{13} and 11-H, and between $C_{1'}$ and 3'-H (Table 3) further demonstrated the two trienes extended to two triene acylamino moieties, *i.e.*, the lower side chain (C_7-C_{13}-N_{14}) and the upper side chain ($N_{0'}$-$C_{1'}$-$C_{7'}$), respectively. Connectivities of the three partial structure segments (C_1-C_6, the lower side chain and the upper side chain) were established by the HMBC experiment; long range correlations of C_2 (δ 129.2), C_1 (δ 189.9) and C_3 (δ 128.9) *versus* NH-0' (δ 8.32) were observed, thus the upper side chain is located on C_2. On the other hand, the long range correlations

Fig. 3. ^{1}H NMR spectrum of manumycin E (500 MHz, acetone-d_6).

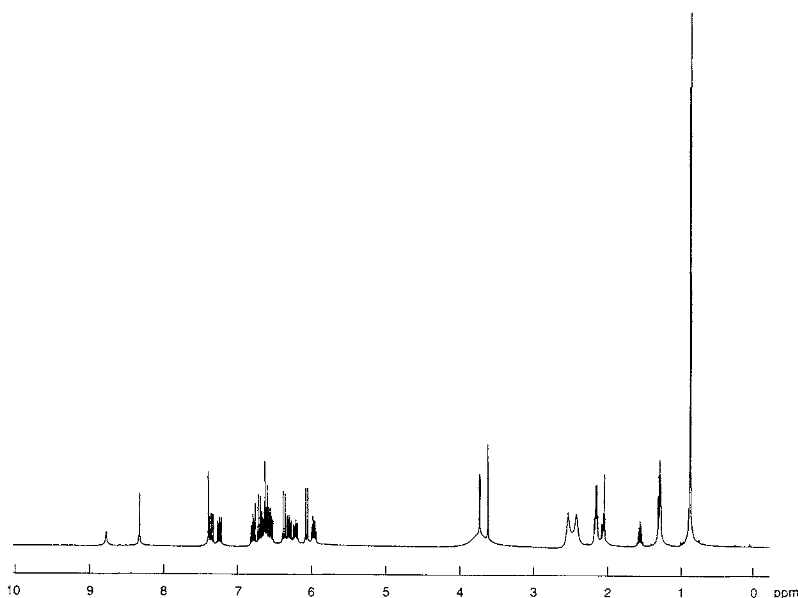
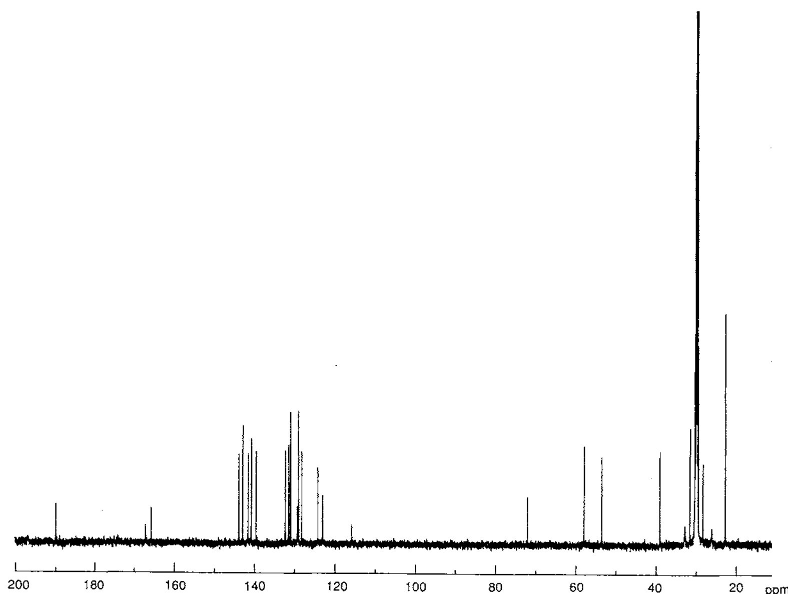


Fig. 4. ^{13}C NMR spectrum of manumycin E (125 MHz, acetone-d_6).



of C_4 (δ 71.9) and C_5 (δ 57.8) *versus* 7-H (δ 6.07), and C_4 *versus* 8-H (δ 6.68) indicated that the lower side chain is connected to C_4 (Fig. 5, Table 3). The presence of an amino-hydroxy-cyclopentenone unit was confirmed by measuring the ^{13}C NMR spectrum of mamumycin E in acetone-d_6-$CDCl_3$ solvent mixture, which avoided the line broadening of $C_{1''}$ and $C_{3''}$, as similarly observed by ŌMURA and co-workers for the same unit in asukamycin[4]. The NMR observation of a hydroxy proton (δ 13.5, C_3''-OH) in hydrogen-bonding suggested that the cyclopentenone unit is connected to the lower side chain through

Table 2. ^{1}H NMR data of manumycin E, manumycin F and manumycin G (acetone-d_6).

Proton position	Manumycin E δ ^{1}H ppm (mult, Hz)	Manumycin F δ ^{1}H ppm (mult, Hz)	Manumycin G δ ^{1}H ppm (mult, Hz)
3-H	7.38 (d, 2.8)	7.38 (d, 2.8)	7.38 (d, 2.6)
5-H	3.72 (dd, 2.8, 4.2)	3.72 (dd, 2.8, 4.1)	3.72 (dd, 2.6, 4.1)
6-H	3.61 (d, 4.2)	3.61 (d, 4.1)	3.60 (d, 4.2)
7-H	6.07 (d, 14.9)	6.07 (d, 15.0)	6.06 (d, 14.8)
8-H	6.68 (dd, 14.9, 11.0)	6.70 (dd, 15.0, 11.0)	6.70 (dd, 14.8, 11.0)
9-H	6.78 (dd, 14.5, 11.0)	6.78 (dd, 14.5, 11.0)	6.79 (dd, 14.3, 11.0)
10-H	6.55 (dd, 14.5, 11.4)	6.55 (dd, 14.5, 11.4)	6.53 (dd, 14.3, 11.6)
11-H	7.36 (dd, 14.8, 11.4)	7.36 (dd, 14.8, 11.4)	7.36 (dd, 15.1, 11.6)
12-H	6.60 (d, 14.8)	6.63 (d, 14.8)	6.62 (d, 15.1)
2′-H	6.36 (d, 14.9)	6.36 (d, 14.9)	6.37 (d, 14.8)
3′-H	7.24 (dd, 14.8, 11.4)	7.24 (dd, 14.8, 11.4)	7.24 (dd, 14.8, 11.4)
4′-H	6.31 (dd, 14.9, 11.4)	6.31 (dd, 14.9, 11.4)	6.32 (dd, 14.8, 11.4)
5′-H	6.62 (dd, 14.9, 10.7)	6.62 (dd, 14.9, 10.7)	6.66 (dd, 14.9, 10.7)
6′-H	6.21 (dd, 15.1, 10.7)	6.17 (dd, 15.3, 10.7)	6.17 (dd, 15.2, 10.7)
7′-H	5.92 (dt, 15.1, 7.1)	5.92 (dd, 15.3, 7.0)	5.92 (dd, 15.2, 6.8)
8′-H	2.15 (dd, 14.6, 7.1)	2.07 (m)	2.39 (septet, 6.8)
9′-H	1.30 (m)	1.73 (m), 1.12 (m)	1.00 (d, 6.7)
10′-H	1.56 (septet, 6.7)	1.72 (m), 1.31 (m)	1.00 (d, 6.7)
11′-H	0.88 (d, 6.7)	1.60 (m), 1.12 (m)	
12′-H	0.88 (d, 6.7)	1.72 (m), 1.31 (m)	
13′-H		1.73 (m), 1.12 (m)	
4″-H	2.53 (br s)	2.53 (br s)	2.44 (br s)
5″-H	2.42 (br s)	2.41 (br s)	2.44 (br s)
NH-14	8.77 (s)	8.76 (s)	8.78 (s)
NH-0′	8.32 (s)	8.31 (s)	8.33 (s)

an amide linkage in which the amidocarbonyl (C_{13}) exists in proximity to $C_{3''}$-OH. The linkage was strongly supported in view of the spectral data for manumycin class compounds having the same structure fragment, despite no notable C-H long range correlations across the amide group were observed for manumycin E. The distinct difference between manumycin E and other known mamumycin class compounds, however, is the terminal group on the upper side chain; the NMR spectra comprised signals clearly assignable to an isopentyl group ($C_{8'}$-$C_{12'}$) attached to $C_{7'}$ (Tables 2 and 3). This has been proven by tandem mass spectrometry; the MS/MS spectrum of the protonated molecular ion (MH^+, m/z 535) exhibited a prominent daughter ion at m/z 177, characteristic of the upper side chain fragment (Fig. 6). The above evidence indicates that manumycin E is a new member of mamumycin class antibiotics (Fig. 7). The configuration of C_4 was determined by the application of exciton chirality method; the CD spectrum (acetonitrile) showed a diagnostic bisignate Cotton effect [λ_{max} 348 nm ([θ] $+1.532\times10^5$); λ_{max} 307 nm ([θ] -1.798×10^5)], indicative of the S configuration for C_4, similar to those of asukamycin[4] and colabomycin[17] but opposite to that of manumycin[3] (Fig. 7). The stereochemistry around the epoxide, however, is not established in this study.

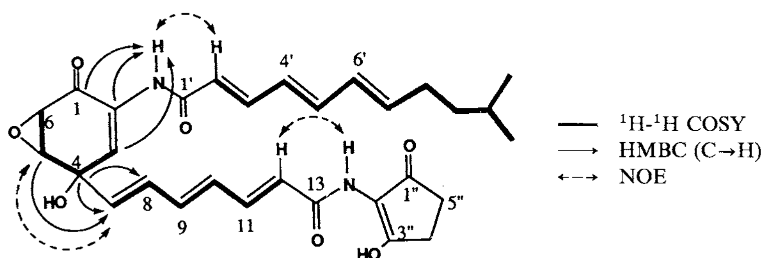
The molecular formula of manumycin F was determined to be $C_{31}H_{34}N_2O_7$ by high resolution mass spectrometry, suggesting that manumycin F may be a configurational isomer of asukamycin. Very recently, the structure of asukamycin first reported in 1979[4] was corrected by ZEECK and co-workers,[9] the revised structure has an all *trans* (7*E*, 9*E* and 11*E*) lower triene side chain instead of that (7*Z*, 9*Z* and 11*E*) originally proposed, and the stereochemistry of epoxide moiety was deduced as 5*R* and 6*S* based on

Table 3. ^{13}C NMR data of manumycin E, manumycin F and manumycin G (acetone-d_6).

Carbon position	Manumycin E δ ^{13}C (m)	Manumycin F δ ^{13}C (m)	Manumycin G δ ^{13}C (m)	Key ^{13}C-^{1}H long range coupling (HMBC)
1	189.9 (s)	190.0 (s)	189.9 (s)	3-H, 6-H, NH-0′
2	129.2 (s)	129.3 (s)	129.2 (s)	3-H, NH-0′
3	128.9 (d)	128.2 (d)	128.1 (d)	NH-0′
4	71.9 (s)	72.0 (s)	71.9 (s)	5-H, 7-H, 8-H
5	57.8 (d)	57.9 (d)	57.8 (d)	7-H
6	53.4 (d)	53.3 (d)	53.4 (d)	
7	139.5 (d)	139.6 (d)	139.5 (d)	
8	131.4 (d)	131.5 (d)	131.4 (d)	
9	140.7 (d)	140.8 (d)	140.7 (d)	
10	132.2 (d)	132.4 (d)	132.2 (d)	
11	143.8 (d)	143.9 (d)	143.8 (d)	
12	122.9 (d)	123.0 (d)	122.9 (d)	
13	167.1 (s)	167.2 (s)	167.1 (s)	11-H, 12-H, NH-14
1′	165.6 (s)	165.7 (s)	165.6 (s)	2′-H, 3′-H, NH-0′
2′	124.1 (d)	124.1 (d)	124.1 (d)	
3′	142.8 (d)	143.0 (d)	142.8 (d)	
4′	128.9 (d)	129.3 (d)	129.2 (d)	
5′	141.4 (d)	141.9 (d)	141.6 (d)	
6′	130.9 (d)	128.7 (d)	128.2 (d)	
7′	140.6 (d)	146.0 (d)	147.1 (d)	8′-H
8′	38.8 (t)	41.9 (d)	32.1 (d)	
9′	31.4 (t)	33.4 (t)	22.3 (q)	
10′	28.2 (d)	26.8 (t)	22.3 (q)	
11′	22.6 (q)	26.6 (t)		
12′	22.6 (q)	26.8 (t)		
13′		33.4 (t)		
1″	196.8 (s)*	196.8 (s)*	196.8 (s)*	
2″	115.7 (s)	115.8 (s)	115.8 (s)	
3″	172.9 (s)*	172.9 (s)*	172.9 (s)*	
4″	26.0 (t)	26.1 (t)	26.0 (t)	
5″	32.9 (t)	32.8 (t)	32.9 (t)	

* Signals were observed in a $CDCl_3$ - acetone-d_6 solvent mixture.

Fig. 5. Key connectivities of manumycin E observed in NMR spectra.



aromatic solvent induced shift in ^1H NMR. The NMR (Tables 2 and 3), MS/MS (Fig. 6) and CD (Table 1) spectra of manumycin F closely resembled those of asukamycin,[4,18,19] showing that both compounds have the identical upper and lower side chains and the same 4*S* configuration. Direct spectral comparisons did however, indicate the difference between the two compounds in IR spectrum and in values of specific rotation and [θ] of CD spectrum, implying that manumycin F may be a diastereomer of asukamycin with

Fig. 6. Major fragments in daughter MS/MS spectrum of the *quasi* molecular ion (MH^+) of manumycin E (*m/z* 535), manumycin F (*m/z* 547) or manumycin G (*m/z* 507).

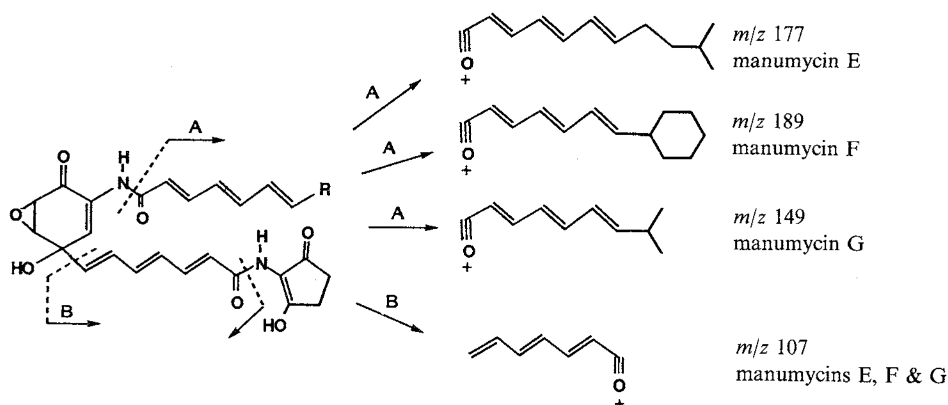


Fig. 7.

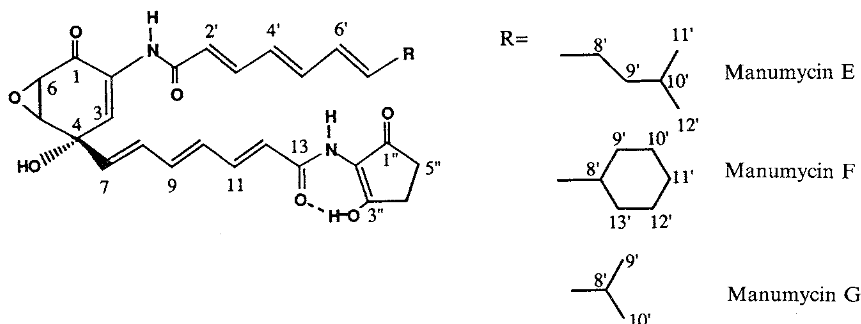


Table 4. Antimicrobial activity of manumycins E, F and G.

	MIC (μg/ml)				MIC (μg/ml)		
	Manumycin E	Manumycin F	Manumycin G		Manumycin E	Manumycin F	Manumycin G
Staphylococcus aureus A9537	2	1	8	*Proteus mirabilis* A9900	128	128	128
S. aureus A9606	4	2	8	*Klebsiella pneumoniae* A9664	128	128	128
S. epidermidis A24548	2	1	8	*Enterobacter cloacae* A9656	128	128	128
Bacillus subtilis A9506	1	0.5	8	*Pseudomonas aeruginosa* A9843	64	128	128
Salmonella typhimurium A27908	2	0.5	32	*Candida albicans* A22803	>50	>50	>50
Micrococcus luteus A9852	8	2	16	*Saccharomyces cerevisae* A25803	>50	>50	>50
M. luteus A21349	4	1	16				
Escherichia coli SGB888	0.32	0.1	2.5				

most likely different stereochemistry at the epoxide moiety. A degradation study[20] is needed to unambiguously establish the epoxide stereochemistry of manumycin F.

Manumycin G, with the molecular formula of $C_{28}H_{30}N_2O_7$, was found to be different from manumycins E and F in the terminal group of upper side chain; a prominent fragment ion at *m/z* 149 was observed in the daughter MS/MS spectrum of the *quasi* molecular ion at *m/z* 507 (Fig. 6). The NMR data of COSY and HMBC experiments further demonstrated the presence of an isopropyl group ($C_{8'}$ to $C_{10'}$) and its connectivity to $C_{7'}$ (Tables 2 and 3). Thus, the structures of manumycins E, F and G were established as shown in Fig. 7.

Biological Activities

Manumycins, E, F and G were tested against a variety of micro-organisms. The results are shown in Table 4. They were active primarily against Gram-positive bacteria and the Gram-negative bacterium *Escherichia coli*, with manumycin F and E being more active. However, they were not active against fungi.

Since manumycin was reported to have significant activity (IC_{50}: 5 μM) in inhibiting ras farnesyltransferase,[10] manumycins E, F and G were also examined in the similar assay by using p21 ras protein[14]. The three compounds exhibited only moderate activity with the IC_{50} value of 100 μM (manumycin E), 92.5 μM (manumycin F) and 120 μM (manumycin G), respectively, implying that the upper side chain of manumycin may play an important role in the enzyme inhibition.

Manumycins E, F and G displayed weak cytotoxic activity on the human colon carcinoma cell line HCT-116 with IC_{50} of 15.6 μg/ml for each of the compounds.

Acknowledgement

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