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New Polyene Macrolide Antibiotics from *Streptomyces* sp. M90025

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Abstract Three polyene macrolide antibiotics including two new compounds were isolated from the culture mycelia of a *Streptomyces* species. The structures of these metabolites were determined as elizabethin, a previously reported 28-membered macrolide and two analogs, using combined spectroscopic methods. These compounds exhibited antifungal activity and cytotoxicity against a human leukemia cell.

Key words: Polyene antibiotics, 28-membered macrolides, *Streptomyces*, elizabethin

Polyene macrolide antibiotics are widely recognized as one of the representative groups of actinomycete metabolites that possess potent antifungal and antiprotozoal activities [2]. Although a significant toxicity and other side effects, as well as the difficulty of stereochemical assignment, hinder the development of these compounds severely for clinical purposes, polyene macrolide antibiotics, including the well-known nystatin and amphotericin B, still remain as a group of the most commonly available drugs for the fungal infections in humans [2, 4, 7].

As a part of the search for novel antifungal metabolites from actinomycetes, the authors encountered a strain of *Streptomyces* from the marine sediment collected off the shore of Geomun Island, Korea, which exhibited potent antifungal activity against *Candida albicans*. Incubation of the strain, followed by extraction and activity-guided separation of the secondary metabolites, yielded several polyene antibiotics possessing 28-membered lactone rings. The structures of the three compounds were determined to be elizabethin and two new analogs, using a combined spectroscopic analysis and comparison of the spectral data with metabolites from the same structural class (Fig. 1) [1, 6, 7]. This report outlines the isolation, structure elucidation, and bioactivity of these compounds.

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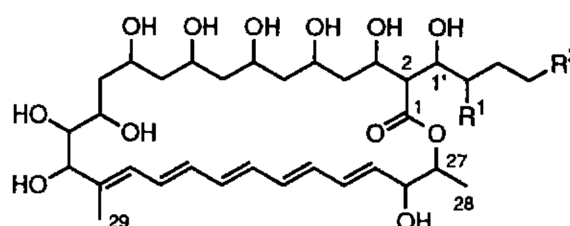

elizabethin: R^1 = H, R^2 = CH_2CH_3
M90025A: R^1 = H, R^2 = CH_3
M90025B: R^1 = CH_3, R^2 = H

Fig. 1. Chemical structures of elizabethin, M90025A, and M90025B.

MATERIALS AND METHODS

General Experimental Methods

The melting points were measured on a Fisher-Johns apparatus and are reported uncorrected. The HRFAB-MS spectra were obtained using a Jeol JMS-HX 110 mass spectrometer provided by the Korea Basic Science Institute, Taejon, Korea. The UV spectra were obtained in methanol using a Milton-Roy spectrophotometer. The IR spectra were recorded on a Mattson GALAXY spectrophotometer. The CD measurements were made in methanol on a JASCO J-710 polarimeter using a 1 cm cell. The optical rotations were measured on a JASCO digital polarimeter using a 5 cm cell. The NMR spectra were recorded in CD_3OD solutions on a Varian Unity 500 spectrometer. The proton and carbon NMR spectra were measured at 500 and 125 MHz, respectively. All chemical shifts were recorded according to an internal Me_4Si. All solvents used were of spectral grade or distilled from glass prior to use.

Taxonomic Studies of Strain M90025

For the identification of the strain, its biochemical properties were examined according to "Bergy's Manual of Systematic

Bacteriology" and its physiological characteristics including the utilization of carbon sources were examined by the method developed by Pridham and Gottlieb [3, 11].

For the fatty acid composition, the mycelium of M90025 (dry weight 0.1 g) was extracted with a Folch solution ($CHCl_3$:MeOH=2:1, 3×2 ml) and saponified. Fatty acid methyl esters were prepared with 5% HCl in MeOH at 80°C for 1 h, extracted with *n*-hexane, concentrated, and then analyzed using a gas chromatograph (HP5890-II plus) equipped with an Omegawax-320 capillary column (0.32 mm×30 m, Supelco) and FID as a detector. The temperatures of injector, oven, and detector were 300, 180, and 300°C, respectively. The retention time and peak area were analyzed by a comparison with those of the authentic samples.

Bioactivity Test

The antifungal activity was determined with the agar diffusion method using a 6 mm paper disk and the activity was measured according to the diameter of the clear zone. For activity against the mycelial phase, the test organism, *Candida albicans*, was incubated in a CO_2 incubator under a 5% CO_2 atmosphere at 37°C for 2 days. After placing a paper disk with 10 μg of the compounds, the agar plate was incubated for 1 day and the clear zone measured.

The human chronic myelogeneous leukemia cell K562 for the cytoxicity assay was maintained in a RPMI 1640 medium (Life Technologies, U.S.A.) containing 10% FBS and 20 μg/ml kanamycin. The activity was analyzed by a MTT assay and the results were measured at 540 nm with a microplate reader (Bio-Rad Model 3550, U.S.A.).

Elizabethin (1)

HRFAB-MS *m/z* $(M+Na)^+$ 693.3811 (calcd *m/z* 693.3826 for $C_{35}H_{58}O_{12}Na$); mp 217–220°C (decomposed); UV λ_{max} (MeOH) nm (log ε) 357 (4.44), 338 (4.45), 322 (4.29), 308 (4.05), 294 (sh, 3.82), 204 (3.89); $[\alpha]_D^{25}$-52.6° (*c* 0.37, MeOH); CD (MeOH) nm (Δε) 354 (-8.1), 336 (-9.3), 320 (-6.2), 306 (-3.7) nm.

RESULTS AND DISCUSSION

Taxonomy

The producing strain M90025 was isolated from the sandy sediment off the coast of Geomun Island, Korea. and maintained on a yeast extract-malt extract agar containing 70% natural sea water. The colony morphology of the strain grown at 30°C for 3 days was yellow brown, opaque, round, regular, entire and matt type vegetative, having a white aerial mycelium with spiral sporopores (*Spirales*). The strain was Gram positive and immobile. The optimum tempertature of growth was at 30 °C and the strain did not grow at 40°C or 10°C. A scanning electron micrograph of

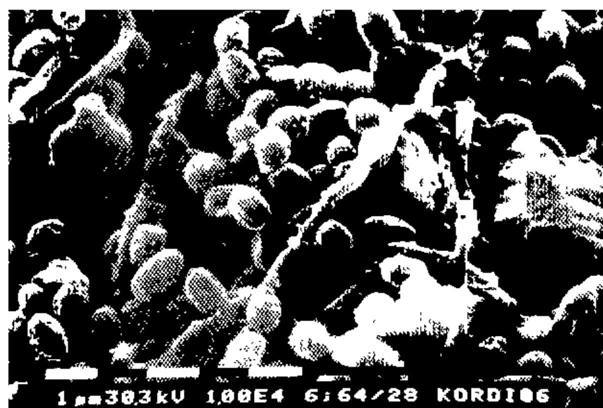

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. M90025 cultured in ISP-4 medium.

the strain showed the characteristic patterns of *Streptomyces*, that is, straight chains tangled with numerous spores which are cylindrical with a smooth surface (Fig. 2).

The basic biochemical and physiological properties of the strain are summarized in Table 1 [3, 11]. From an analysis of cell wall materials, only the L,L-form of diaminopimelic acid was found and a cellular sugar analysis with TLC showed the presence of only arabinose.

Table 1. Basic biochemical and physiological properties of strain M90025.

Temperature range for growth	15–35°C
Optimum temperature for growth	28–32°C
Enzyme activity of	
arginine dihydrolase	-
β-galactosidase	-
lysine decarboxylase	-
ornithine decarboxylase	-
Decomposition of	
chitin	+
gelatin	+
starch	+
Production of indole	-
Production of H_2S	-
Reduction of nitrate	-
Utilization of carbon source	
L-arabinose	+
D-glucose	+
inositol	+
D-mannitol	+
melibiose	+
L-rhamnose	+
sorbitol	+
sucrose	+
Utilization of	
citrate	-
tryptophan	+
VP-test	+

A cellular fatty acid analysis revealed the presence of *iso*-palmitic acid (*i*-C16:0, 24.6%), *iso*-pentadecanoic acid (*i*-C15:0, 20.3%), and *n*-heptadecanoic acid (C17:0, 16.4%) as the major constituents, with a minor presence of palmitic acid (C16:0, 7.1%). Based on these biochemical and physiological characteristics, the strain M90025 was assigned to the genus *Streptomyces*.

Production and Isolation

A slant culture of *Streptomyces* sp. M90025 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a SYP medium (starch 1%, yeast extract 0.4%, peptone 0.2%) in 50% aged sea water, adjusted to pH 8 by 1 M Tris buffer, and incubated at 27°C for 4 days on a rotary shaker (150 rpm). Fermentation was carried out by a transfer of 5 ml of the seed culture to a 3-l Fernbach flask containing 1 l of the same medium and incubated for 7 days under similar conditions.

The pooled 110 l of culture broth was concentrated using a tangential filtering system (Minikross Lab. System, Micogen, U.S.A.) with a 0.5 μm poresize membrane. The medium contained in a wet mycelial cake was removed by centrifugation for 10 min (8,500 rpm), and the residue (wet weight 2.1 kg) was repeatedly extracted with MeOH (8 l twice). After removing the solvent *in vacuo*, the residual brown gum (97.88 g) was suspended into MeOH (2 l) and filtered. A bioactivity test revealed that the antifungal substances were contained in the filtrate. The solvent was evaporated under reduced pressure and the dark-brown residue (38.94 g) was subjected to adsorption chromatography. After loading the residue onto a Diaion HP20 column (5 cm×60 cm), the column was washed sequentially with distilled water (2 l), MeOH (1 l), and acetone (1 l).

The biologically active MeOH layer (13.51 g) was separated by C_{18} reversed-phase vacuum flash chromatography using sequential mixtures of MeOH and water as eluents (elution order: 50, 30, 20, 10% aqueous MeOH and 100% MeOH). The fractions eluted with 30 and 20% aqueous MeOH were combined (2.80 g) and separated by a reversed-phase HPLC (YMC ODS-A column, 10×250 mm, 42% aqueous MeOH) to yield the polyene macrolides in order of the elution compounds M90025B (**3**), M90025A (**2**), and elizabethin (**1**). The purification of these compounds was established by a reversed-phase HPLC (YMC ODS-A column, 60% aqueous THF) to produce 200.3, 42.4, and 17.2 mg of **1-3**, respectively, as pale-yellow amorphous solids.

Physico-Chemical Properties

The physico-chemical properties of M90025A and M90025B are summarized in Table 2. The molecular formulas of these compounds were established to be $C_{34}H_{56}O_{12}$ and identical to each other by a combined HRFAB-MS and ^{13}C NMR analysis. The UV data showed diagnostic peaks of pentaenes in the region of 360–290 nm [10]. IR absorptions at 3,400 (broad) and 1,725 cm^{-1} revealed the presence of hydroxyl(s) and an ester groups, respectively. The CD data for M90025A and M90025B were highly compatible with those obtained for elizabethin; CD (MeOH) nm (Δε) 354 (-8.1), 336 (-9.3), 320 (-6.2), 306 (-3.7) nm [1, 5].

Structure Determination

The NMR data for **1**, the major metabolite, showed all of the characteristics of a 1,3-polyol containing polyene macrolide. A combination of ^{1}H COSY, TOCSY, gHSQC, and gHMBC experiments determined the gross structure of **1** including the ester linkage between C-1 and C-27. Two-dimensional NMR experiments also confirmed the assignment of all of the signals of the protons and carbons except for those in the middle part of the 1,3-polyol (C-5–

Table 2. Physico-chemical properties of M90025A and M90025B.

	M90025A	M90025B
Appearance	Pale-yellow solid	Pale -yellow solid
MP (°C)	220–225 (dec.)	207–210 (dec.)
Molecular formula	$C_{34}H_{56}O_{12}$	$C_{34}H_{56}O_{12}$
HRFABMS (*m/z*)		
Obsd	679.3669	679.3681
Calcd	679.3669	679.3669
UV λ_{max} (MeOH) nm (log ε)	357 (4.44), 339 (4.47), 322 (4.37), 306 (4.20), 294 (sh, 3.82)	357 (4.38), 338 (4.43), 320 (4.38), 306 (4.25), 294 (sh, 4.05)
CD λ_{max} (MeOH) nm (Δε)	354 (-6.0), 334 (-7.1), 320 (-4.6), 308 (-2.6)	355 (-5.3), 336 (-5.9), 319 (-4.3), 308 (-3.0)
IR vmax (KBr) cm^{-1}	3400, 2935, 1725, 1580, 1415	3400, 2935, 1725, 1575, 1400
$[\alpha]_D^{25}$ (MeOH) ° (*c*)	-54.4 (0.17)	-30.9 (0.16)
Solubility		
Soluble	MeOH, acetone, THF, $CHCl_3$	MeOH, acetone, THF, $CHCl_3$
Insoluble	H_2O, hexane	H_2O, hexane

Table 3. ^{1}H and ^{13}C NMR assignments for M90025A and M90025B.

Position	M90025A			M90025B		
	[13]C		[1]H	[13]C		[1]H
1	172.9	s		173.0	s	
2	60.5	d	2.55 (dd, J = 8.8, 7.3 Hz)	61.2	d	2.53 (dd, J = 8.8, 7.3 Hz)
3	73.3	d	4.18 (ddd, J = 10.8, 7.3, 3.4 Hz)	73.2	d	4.18 (ddd, J = 10.3, 8.8, 3.4 Hz)
4	41.2	t	1.50 (m)	41.1	t	1.51 (m)
5	74.2[a]	d	3.99-4.03 (m)	74.3[a]	d	3.99-4.03 (m)
6	45.2	t	1.45 (m), 1.36 (m)	45.2	t	1.44 (m), 1.36 (m)
7	73.9	d	3.99-4.03 (m)	74.0	d	3.99-4.03 (m)
8	45.3	t	1.65 (m), 1.55 (m)	45.4[b]	t	1.64 (m), 1.55 (m)
9	74.1[a]	d	3.99-4.03 (m)	74.2[a]	d	3.99-4.03 (m)
10	44.3	t	1.50 (m), 1.32 (m)	44.3	t	1.49 (m), 1.32 (m)
11	71.4	d	3.95 (m)	71.5	d	3.93 (m)
12	39.5	t	1.75 (ddd, J = 14.2, 10.7, 3.4 Hz), 1.53 (m)	39.5	t	1.75 (ddd, J = 14.4, 10.7, 3.4 Hz), 1.53 (m)
13	70.3	d	3.26 (br d, J = 10.7 Hz)	70.3	d	3.26 (br d, J = 11.2 Hz)
14	78.3	d	3.71 (dd, J = 8.8, 2.0 Hz)	78.3	d	3.71 (dd, J = 9.3, 2.0 Hz)
15	80.5	d	3.88 (d, J = 8.8 Hz)	80.5	d	3.88 (d, J = 8.9 Hz)
16	138.5	s		138.5		
17	129.9	d	6.05 (br d, J = 11.0 Hz)	129.9	d	6.05 (br d, J = 11.0 Hz)
18	129.0	d	6.47 (dd, J = 15.6, 11.0 Hz)	129.0	d	6.48 (dd, J = 16.1, 11.0 Hz)
19	135.4	d	6.30-6.36 (m)	135.5	d	6.30-6.36 (m)
20	134.1	d	6.30-6.36 (m)	134.1	d	6.30-6.36 (m)
21	134.8	d	6.30-6.36 (m)	135.0	d	6.30-6.36 (m)
22	133.6	d	6.30-6.36 (m)	133.6	d	6.30-6.36 (m)
23	134.2	d	6.30-6.36 (m)	134.1	d	6.30-6.36 (m)
24	131.9	d	6.43 (m)	132.1	d	6.43 (m)
25	134.3	d	6.02 (dd, J = 15.1, 5.6 Hz)	134.4	d	6.02 (dd, J = 15.1, 4.9 Hz)
26	73.2	d	4.10 (dd, J = 5.6, 5.3 Hz)	73.2	d	4.10 (br dd, J = 5.4, 4.9 Hz)
27	75.2	d	4.84 (m)	75.3	d	4.84 (m)
28	17.9	q	1.29 (d, J = 6.3 Hz)	17.9	q	1.28 (d, J = 6.3 Hz)
29	11.7	q	1.78 (d, J = 1.0 Hz)	11.7	q	1.78 (br s)
1'	72.4	d	3.84 (br dd, J = 8.8, 8.3 Hz)	70.3	d	3.99-4.03 (m)
2'	35.9	t	1.53 (m), 1.37 (m)	25.2	d	1.84 (m)
3'	28.6	t	1.53 (m), 1.34 (m)	45.3[b]	t	1.36 (m), 1.20 (m)
4'	23.7	t	1.32 (m)	21.4	q	0.93 (t, J = 6.8 Hz)
5'	14.4	q	0.92 (t, J = 7.3 Hz)			
6'				24.4	q	0.92 (t, J=5.4 Hz)

Assignments for signals in the regions of C-1-C-4, C-11-C-19, and C-24-C-6' were aided by a combination of ^{1}H COSY, TOCSY, gHSQC, and gHMBC experiments Assignments for the remaining parts were made by comparison with those reported for fungichromin [4]. [a,b] Interchangeable signals.

C-10) and polyene (C-20- C-23) chains, which closely overlapped with each other in both ^{1}H and ^{13}C NMR spectra. The assignment of the signals for the protons and carbons at these regions was aided by a comparison of the NMR data with that reported for fungichromin, a structurally similar polyene macrolide from *Streptomyces* spp. [4]. A literature survey revealed that the proposed structure of **1** was identical with that of elizabethin, a polyene macrolide antibiotic produced by *Streptomyces elizabethii* [1, 6]. Although the stereochemistry of the asymmetric centers for **1** and elizabethin were not determined, a comparison of the CD data showed that these two were indeed identical metabolites [1, 5].

The spectral data for M90025A (**2**) were very similar to those obtained for elizabethin, with the loss of signals for an upfield methylene in both ^{1}H and ^{13}C NMR data being the only noticeable difference (Table 3). A combination of the two-dimensional NMR experiments revealed that one of the methylenes in the side chain of elizabethin was missing in **2**. Although the stereochemical assignment for the asymmetric carbon centers was not established, a comparison of the CD data implied that **2** had the same stereochemistry as **1**. Thus the structure of M90025A was defined as an analog of elizabethin possessing a truncated side chain.

The related metabolite M90025B (**3**) was isolated as a pale-yellow amorphous solid, possessing the same molecular

Table 4. Biological activities of polyene macrolides isolated from *Streptomyces* M90025.

	Elizabethin	M90025A	M90025B
Antifungal activity against *C. albicans* (diameter of clear zone, mm)	15.4	12.0	8.9
LC_{50} against K562 (μg/ml)	1.3	3.5	5.5

formula $C_{34}H_{56}O_{12}$ as **1**. The spectral data for this compound were also highly compatible with those obtained for **1**. However, the ^{13}C NMR data showed that the signals of the two methylene carbons of **1** were replaced by those of a methine and a methyl carbon in **3**. A combination of the ^{1}H COSY and gHSQC experiments showed that the *n*-butyl group (C-2'- C-5') of **1** was replaced by a *sec*-butyl group in **3** (Table 3). This interpretation was confirmed by several HMBC correlations between the methyl protons at C-2' and C-6' and the neighboring carbons. Since the CD profile of **3** was very similar to those obtained for elizabethin and **3**, the configurations of the asymmetric carbon centers of these metabolites were also assumed to be identical.

Biological Activity

The antifungal activity was determined using the agar diffusion method against *Candida albicans*. The diameters of the clear zones for **1**, **2**, and **3** were 15.4, 12.0, and 8.9 mm, respectively, at concentrations of 10 μg per disk. The cytotoxicity of the polyene macrolides was determined against the human myelogeneous leukemia cell K562. Compounds **1-3** exhibited a LC_{50} of 1.3, 3.5, and 5.5 μg/ml, respectively (Table 4).

Stereochemical Assignments of 1,3-Polyols

The stereochemical assignment of the 1,3-polyol group, which includes polyene macrolide antibiotics, is widely recognized as a very difficult and laborious task. This is mainly due to the difficulty of obtaining reliable crystals for the X-ray crystallographic analysis and the difficulty of interpreting the complex NMR spectra [9]. Due to the recent development of high-resolution NMR techniques, however, the stereochemistry of these compounds has been consecutively unveiled. Even a general strategy has been proposed that may provide a new tool for the chemical investigation of polyene macrolides and other metabolites containing the 1,3-polyol functionality [8, 9]. The absolute stereochemistry of elizabethin, a polyene macrolide antibiotic of unknown stereochemistry and also the major metabolite of *Streptomyces* M90025, is currently under investigation using combined chemical and spectral methods and the result will be reported in due course.

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