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Topoisomerase I Inhibitors from the *Streptomyces* sp. Strain KM86-9B Isolated from a Marine Sponge

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The crude extract of *Streptomyces* sp. strain KM86-9B, isolated from a marine sponge, displayed significant inhibition on topoisomerase I activity. Investigation of the causative components by bioactivity-directed fractionation resulted in the isolation of a series of iso- and anteiso-fatty acids.

Key words : Topoisomerase I inhibitor, Marine actinomycete, Isofatty acids, Anteiso-fatty acids, Marine sponge

INTRODUCTION

Topoisomerases play an important role in the changes of DNA topology which occur during several cellular processes such as DNA replication, transcription, and recombination (Wang, 1987; Slichenmyer *et al.*, 1993). It is observed that topoisomerases are correlated with cell cycle progression and proliferation state of tumor cells (Giovanella *et al.*, 1989; Andoh *et al.*, 1991). Topoisomerase I has been recently recognized as an attractive pharmacological target for the development of novel chemotherapeutics (D'arpa and Liu, 1989; Schneider *et al.*, 1990; Potmesil, 1994; Chen *et al.*, 1994; Wang *et al.*, 1996). Since camptothecin was found to inhibit topoisomerse I, numerous synthetic derivatives of camptothecin have been developed (Dunn, 1994; Potmesil and Pinedo, 1995). Topoisomerase I inhibitors discovered from natural sources include saintopin (Yamashita *et al.*, 1991), corilagin (Berry *et al.*, 1992), chebulagic acid (Berry *et al.*, 1992), sanguiin H-6 (Bastow *et al.*, 1993), xestoquinone (Bae *et al.*, 1993), halenaquinone (Bae *et al.*, 1993), β-lapachone (Li *et al.*, 1993), fagaronine (Larsen *et al.*, 1993), nitidine (Wang *et al.*, 1993), makaluvamine G (Carney *et al.*, 1993), manoalide 23-acetals (Kobayashi *et al.*, 1994), certain flavones (Zahir *et al.*, 1996; Boege *et al.*, 1996), curcumin (Roth *et al.*, 1998), and cryptotanshinone (Lee and Hong, 1998).

In our search for bioactive components from marine bacteria, significant cytotoxicity has been detected in the crude extract of an actinomycete isolated from a marine sponge. Investigation of the causative constituents directed by topoisomerase I inhibition assay led to the isolation of a series of iso- and anteiso-fatty acids. We describe the isolation, characterization, and bioactivities of these fatty acids.

MATERIALS AND METHODS

Microorganisms and culture conditions

Marine sponges were collected under the seashore of Keomun Island, Korea by scuba diver. Squeezed spongial fluid was inoculated on ISP4 medium (Difco) and incubated at 30°C for 2~4 weeks. The pure isolates were maintained on modified ZoBell medium (glucose 20 g, bactopeptone 5 g, yeast extract 1 g, ferric phosphate 0.1 g, agar 15 g, aged sea water 750 ml, distilled water 250 ml, pH 7.4). Difco products of bactopeptone and yeast extract were used for preparation of media. The isolate was cultured at 30°C for 7 days with 50 ml of modified ZoBell broth in 1 L baffled flasks shaken on a rotatory shaker at 150 rpm.

Chemotaxonomical and morphological characteristics

The cultural and morphological characteristics were determined by using media recommended by the International Streptomyces Project (ISP) (Shiring and Gottlieb, 1966). The chemotaxonomical characteristics were analyzed according to Williams *et al.* (1983). After the isolate was incubated on ISP 2 medium at 30°C for 7 days, spore chain morphology was examined using scanning electron microscopy (Phillips model 515).

Topoisomerase I inhibition assay

The assay was carried out using a modification of

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DNA relaxation assay (Yoshikazu *et al.*, 1990). Topoisomerse I purified from HeLa S_3 cell line and pBR322 DNA were used for the assay. The reaction mixture contained in a total volume of 20 μl: 0.2 μg supercoiled pBR322 DNA, 100 mM Tris-HCl (pH 7.9), 0.06 mg/ml BSA, 200 mM KCl, 20 mM $MgCl_2$, 1 mM Dithiothreitol, 1 mM EDTA and 1 unit of topoisomerse I. The mixture was incubated at 37°C for 30 min. One unit of the enzyme is defined as the minimum amount of enzyme giving complete relaxation of 0.2 μg of supercoiled pBR322 DNA under conditions employed. Camptothecin (Sigma) was used as a positive control.

Purification of the active compounds

Five liters of culture broth of KM86-9B was centrifuged to separate broth filtrate and mycelia cake. The mycelia was extracted with acetone three times at room temperature. The acetone extract was evaporated to dryness to give 3.5 g of residue. This residue was chromatographed on a C-18 reversed-phase flash column eluting with solvent systems of MeOH:H_2O (2:1), MeOH:H_2O (4:1), followed by MeOH, EtOAc and CH_2Cl_2. A total of 9 fractions were obtained. A portion of fraction #4 (F4, 1600 mg) was subjected to a reversed-phase HPLC with an eluting solvent of acetonitrile:H_2O (30:1) (flow rate, 1.5 ml/min; RI detector) to afford compounds **1** (9.7 mg, t_R: 17.3 min), **2** (4.1 mg, t_R: 18.2 min), **3** (16.9 mg, t_R: 20.5 min), **4** (3.6 mg, t_R: 24.3 min), **5** (13.3 mg, t_R: 27.0 min), **6** (3.8 mg, t_R: 28.9 min), and **7** (7.3 mg, t_R: 33.3 min).

1 (14-methylpentadecenoic acid, iso-16:1): LREIMS m/z 254 $[M]^+$; ^1H nmr (500 MHz, CD_3OD) δ 5.35 (2H, m, olefinic), 2.27 (2H, t, *J*=7.6 Hz, H-2), 2.03 (4H, m, allylic H), 1.60 (2H, m, H-3), 1.52 (1H, septet, *J*=6.5 Hz, H-14), 1.24~1.40 (10H, m, methylene protons), 1.20 (2H, m, H-13), 0.88 (6H, d, *J*=6.7 Hz, H-15, 16). ^{13}C nmr (125 MHz, CD_3OD) δ 177.89 (C-1), 130.91, 130.80, 39.82 (C-13). 35.08 (C-2), 31.04~28.11 (methylene carbons), 29.14 (C-14), 26.15 (C-3), 23.03 (C-15, 16).

2 (hexadecenoic acid, 16:1): LREIMS m/z 254 $[M]^+$; ^1H nmr (500 MHz, CD_3OD) δ 5.34 (2H, m, olefinic), 2.27 (2H, t, *J*=7.6 Hz, H-2), 2.03 (4H, m, allylic H), 1.60 (2H, m, H-3), 1.24~1.40 (16H, m, methylene protons), 0.88 (3H, t, *J*=7.4 Hz, H-16).

3 (12-methyltetradecanoic acid, anteiso-15:0): LREIMS m/z 242 $[M]^+$; ^1H nmr (500 MHz, CD_3OD) 2.26 (2H, t, *J*=7.6 Hz, H-2), 1.59 (2H, m, H-3), 1.55 (1H, m, H-12), 1.25~1.35 (16H, m, methylene protons), 1.18 (2H, m, H-11), 0.88 (3H, d, *J*=6.7 Hz, H-15), 0.87 (3H, t, *J*= 5.6 Hz, H-14). ^{13}C nmr (125 MHz, CD_3OD) δ 179.58 (C-1), 39.10 (C-11), 34.42 (C-2), 31.01~24.07 (methylene carbons), 19.92 (C-15), 11.38 (C-14).

4 (cyclopropane fatty acid): ^1H nmr (500 MHz, CD_3OD) 2.27 (2H, t, *J*=7.6 Hz, H-2), 1.60 (2H, m, H-3), 1.25~1.40 (m, methylene protons), 0.89 (3H, t, *J*= 7.0 Hz, terminal methyl), 0.68 (2H, m), 0.59 (1H, td, *J*=15.6, 8.3 Hz), -0.33 (1H, q, *J*=8.3 Hz).

5 (14-methylpentadecanoic acid, iso-16:0): LREIMS m/z 256 $[M]^+$; ^1H nmr (500 MHz, CD_3OD) 2.27 (2H, t, *J*=7.6 Hz, H-2), 1.59 (2H, quintet, *J*=6.5 Hz, H-3), 1.52 (1H, septet, *J*=6.5 Hz, H-14), 1.26~1.34 (18H, m, methylene protons), 1.17 (2H, m, H-13), 0.88 (6H, d, *J*=6.7 Hz, H-15, 16). ^{13}C nmr (125 MHz, CD_3OD) δ 177.76 (C-1), 40.34 (C-13), 35.06 (C-2), 31.14~30.34 (C-4~11), 29.24 (C-14), 28.63 (C-12), 26.19 (C-3), 23.15 (C-15, 16).

6 (hexadecanoic acid, 16:0): LREIMS m/z 256 $[M]^+$; ^1H nmr (500 MHz, CD_3OD) 2.27 (2H, t, *J*=7.6 Hz, H-2), 1.60 (2H, quintet, *J*=6.5 Hz, H-3), 1.25~1.35 (24H, m, methylene protons), 0.88 (3H, t, *J*=6.7 Hz, H-16).

7 (14-methylhexadecanoic acid, anteiso-17:0): LREIMS m/z 270 $[M]^+$; ^1H nmr (500 MHz, CD_3OD) 2.27 (2H, t, *J*=7.6 Hz, H-2), 1.60 (2H, m, H-3), 1.49 (1H, m, H-14), 1.25~1.35 (20H, m, methylene protons), 1.18 (2H, m, H-13), 0.88 (3H, d, *J*=6.7 Hz, H-17), 0.87 (3H, t, 5.6 Hz, H-16).

^1H nmr, ^{13}C nmr, COSY, HMQC experiments were performed at 500 MHz and 125 MHz with a Varian Unity 500 instrument using Varian standard pulse programs. Solutions in CD_3OD were used for all the nmr studies. Chemical shifts were reported relative to the residual solvent peaks (CD_3OD: ^1H δ 3.3, ^{13}C δ 49). YMC-Gel ODS-A 60-500/400 was used for the reversed-phase flash column chromatography. YMC-pack ODS-A (5 μm, 250×10 mm) column was used with an Alltech guard cartridge column for HPLC. HPLC was conducted on a Spectra-Physics isochrom pump equipped with an RI detector (Shodex RI-71). RP-18 F254 S (Merck) was used for TLC. LREIMS was recorded on a Profile HV-3.

RESULTS AND DISCUSSION

The morphological characteristics of the strain KM 86-9B showed grey aerial mycelia and red-brown substrate mycelia on ISP 2 agar medium. The shape of spore-bearing hyphae formed spiral chains and the spores had smooth surfaces. The cell wall type was determined to be Type I containing LL-diaminopimelic acid. Phospholipid pattern was Type PII with phosphatidyl ethanolamine. Consequently, the strain KM86-9B was identified to belong to the genus *Streptomyces* by Bergey's Mannual of Systematic Bacteriology (Lechevalier, 1989).

The acetone extract of mycelia was chromatographed on a C18 reversed-phase vacuum flash column to afford an active fraction #4 (F4, + at a concentration of 1000 μg/ml). F4 was subsequently subjected to C18 reversed-

phase HPLC to afford compounds **1-7** as the causative constituents for topoisomerase I inhibition.

^1H nmr spectrum of **5** revealed that of a typical fatty acid. A triplet at δ 2.27 (2H) was attributable to methylene protons next to a carbonyl function. Doublet methyl protons at δ 0.88 which integrated for six protons suggested the presence of an isopropyl group. This signal was coupled to a septet at δ 1.52 (1H) which is assignable to a methine proton at a branching point. This septet was further coupled to a multiplet at δ 1.17 (2H). In the ^{13}C nmr spectrum of **5**, sixteen carbon signals were observed. They were composed of a carbonyl carbon (δ 177.76), two overlapped methyl carbons (δ 23.15), a methylene carbon (δ 35.06) adjacent to a carbonyl function, a downfield shifted methylene carbon (δ 40.34), and seven methylene carbons (δ 31.4~26.19). These carbons were correlated to the corresponding protons by a HMQC experiment. Based on nmr spectral data, the structure of **5** was deduced to be an iso-fatty acid. EIMS of **5** showed the molecular ion peak at m/z 256. Thus, the structure of **5** was determined to be 14-methylpentanoic acid (iso-16:0). The iso branching of **5** was further confirmed by a weaker signal at m/z 227 and an enhanced peak at m/z 213 in EIMS (Wijekoon *et al.*, 1984).

^1H nmr spectral pattern of **1** was quite similar to that of **5** except for the presence of additional olefinic protons at δ 5.35 (2H) and methylene protons next to the double bond at δ 2.03 (4H), implying the presence of a double bond in the alkyl chain. The geometry of the double bond was deduced to be a Z configuration according to the absence of carbon signals near δ 32~34 in the ^{13}C nmr spectrum of **1** (Jung *et al.* 1996). EIMS of **1** showed a molecular ion at m/z 254. Thus the structure of **1** was deduced to be 14-methylpentadecaenoic acid (iso-16:1). The location of the double bond was not established.

^1H nmr spectrum of **3** was similar to those of **1** and **5**, except for the signals of methyl protons. The methyl signals appeared at δ 0.88 (3H) and δ 0.87 (3H) as a doublet and a triplet, respectively, indicating the presence of an anteiso branching. In the ^{13}C nmr spectrum, the terminal methyl carbon and the branched methyl carbon appeared at δ 11.38 and δ 19.92, respectively, in accordance with the reported data (Omura *et al.*, 1986). EIMS of **3** showed the molecular ion peak at m/z 242. A weaker peak at m/z 199 and enhanced peaks at m/z 213 and m/z 185 supported the presence of an anteiso branching. Thus, the structure of **3** was determined to be 12-methyltetradecanoic acid (anteiso-15:0).

Table I. Inhibitory activities of compounds **1-7** from strain KM86-9B against topoisomerase I

Compound	Inhibition at the concentration of 100 μg/ml
1 (iso-16:1)	++
2 (16:1)	-
3 (anteiso-15:0)	++
4 (cyclopropyl)	++
5 (iso-16:0)	+
6 (16:0)	+
7 (anteiso-17:0)	++
camptothecin (200 μg/ml)	+

^1H nmr spectrum of **7** was quite similar to that of **3**. EIMS of **7** showed the molecular ion at m/z 270 with intense peaks at m/z 213 and m/z 241. Thus the structure of **7** was determined to be 14-methylhexadecanoic acid (anteiso-17:0).

^1H nmr spectrum of **2** was that of a typical straight chain fatty acid with a double bond (δ 5.34, 2H). The geometry of the double bond was assumed to be a Z configuration because the methylene carbons next to the double bond appeared at δ 28.15 and 28.11 in the ^{13}C nmr spectrum of **2**. EIMS of **2** showed the molecular ion at m/z 254. Thus, the structure of **2** was determined to be hexadecaenoic acid (16:1). The location of the double bond was not determined.

^1H nmr spectrum of **6** was similar to that of **2** but the signals of the olefinic protons and the protons adjacent to the double bond were missing. EIMS of **6** showed the molecular ion at m/z 256. Thus the structure of **6** was determined to be hexadecanoic acid (16:0).

^1H nmr spectrum of **4** showed a typical pattern of a cyclopropane moiety (Kobayashi *et al.,* 1993). Signals of the cyclopropane moiety appeared at δ-0.33 (1H, q, *J*=8.3 Hz), 0.59 (1H, td, *J*=15.6, 8.3 Hz) and 0.68 (2H, m). Thus the identity of **4** was suspected to be a fatty acid with a cyclopropane moiety flanked in the alkyl chain. However, the gross structure of **4** could not be determined due to lack of material.

The inhibitory activities of **1-7** against topoisomerase I were detemined and compared with that of camptothecin as shown in Fig. 1. Some of the fatty acids, such as cyclopropane fatty acid and 14-methylhexadecanoic acid (anteiso-17:0) displayed significant inhibition on topoisomerase I, which was comparable to that of camptothecin. The iso- and anteiso-fatty acids exhibited higher potency than straight chain fatty acids. There has been a report that phosphatidylinositol and cardiolipin inhibit topoisomerase I (Umekawa *et al.*, 1988). And it was also found that acidic phospholipids directly interact with topoisomerase I to inhibit its binding to DNA (Tamura *et al.*, 1990). Phosphatidylglycerol and cardiolipin containing unsaturated fatty acid showed potent inhibition on topoisomerase I, while those with saturated fatty acid showed no activity. Thus it was speculated that phospholipids might be involved in DNA replication and transcription via changes of membrane fluidity, which in turn will change

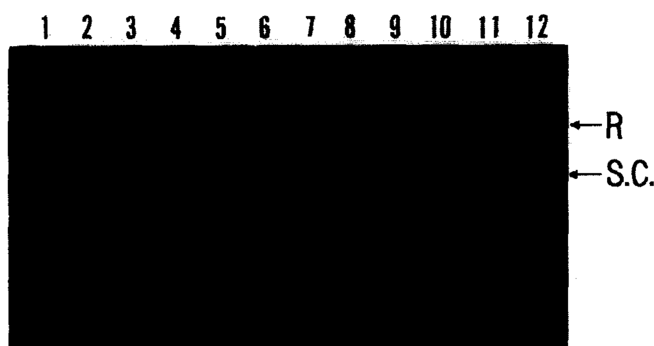

Fig. 1. Effects of fatty acids of the strain KM86-9B on relaxation activity of DNA topoisomerase I.
Each compound was added with a concentration of 0.1 mg/ml. Plasmid DNA (pBR 322, 0.2 g) was treated with 1 unit of topoisomerase I in 20 μl of the reaction mixture: lane 1, pBR322 DNA control; lane 2, no drug; lanes 3 and 4, methanol; lane 5, compound **1**; lane 6, compound **2**; lane 7, compounds **3**; lane 8, compound **4**; lane 9, compound **5**; lane 10, compound **6**; lane 11, compound **7**; lane 12, camptothecin (0.2 mg/ml). "R" and "S.C." denote relaxed and supercoiled DNA, respectively.

the topology of DNA in the cells (Mizushima *et al.*, 1992). Other than phospholipids, there has been a report that unbranched acetylenic fatty acids inhibit topoisomerase I mediated DNA binding (Berry *et al.*, 1991). Thus it appears that the fatty acid moiety might play a major role in the inhibition of topoisomerase I by these phospholipids. In our study, it appears that unsaturation and branching of fatty acid are closely related to their activity on topoisomerase I.

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