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NOTE

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Studies on Terpenoids Produced by Actinomycetes

Isolation and Structural Elucidation of Antioxidative Agents, Naphterpins B and C

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Dedicated to the late Prof. Katsumi Kakinuma

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Abstract As a result of screening for terpenoids produced by Actinomycetes, naphterpins B and C, two new congeners of naphterpin were isolated from *Streptomyces* sp. CL190 and their structures were determined by NMR spectral analysis.

Keywords naphterpin, terpenoid, antioxidative agent, *Streptomyces* sp. CL190

It has recently been revealed that all *Streptomyces* organisms utilize the MEP pathway for production of the starter units, isopentenyl diphosphate (IPP) and dimethylally diphosphate (DMAPP), for the biosynthesis of terpenoids [1]. Some of the members of this genus, however, use the mevalonate pathway as well for production of terpenoids as secondary metabolites. Such examples include naphterpin [2], terpentecin [3], napyradiomycin [4], BE-40644 [5] and furaquinocin [6]. We were interested in this phenomenon and initiated a screen for terpenoids of actinomycete origin. As a first step we examined in detail the minor metabolites of a naphterpin producer, *Streptomyces* sp. CL190 [7], with the aim of isolating new terpenoids from this organism.

Since most terpenoids are hydrophobic in nature, we examined lipophilic fraction of the fermentation broth of *Streptomyces* sp. CL190 by TLC, and fractions positive to vanillin-H_2SO_4 color reaction were analyzed by ^{1}H-NMR. Fractions showing sharp methyl singlet signals were judged to contain terpenoids and subjected to further purification. As a result, we succeeded in isolating two terpenoids, naphterpins B and C from *Streptomyces* sp. CL-190. We report herein their structures and biological activities.

Streptomyces sp. CL-190 was cultivated in 15 ml test tubes each containing 5 ml of PC-1 medium (starch 1%, polypepton 1%, meat extract 1% and molasses 1%, pH 7.2 before sterilization) 28°C for two days. Each one ml of the broth was transferred to 500 ml baffled Erlenmeyer flasks containing 100 ml of K-medium (starch 2.5%, soybean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4%, pH 6.2 before sterilization) and cultivated on a rotary shaker at 28°C for 6 days. The fermentation broth was filtered and the filtrate was extracted with AcOEt. The mycelium was extracted with acetone and after removal of the acetone, the aqueous residue was extracted with AcOEt. Both solvent extracts were combined and concentrated *in vacuo* to give an oily residue which was subjected to flash column chromatography (silica gel column, 25×480 mm, equilibrated with hexane : AcOEt=4 : 1). After washing the column with the same solvent, the column was eluted with 200 ml of $CHCl_3$: MeOH=20 : 1. This fraction was concentrated under reduced pressure and the residue was purified by preparative TLC (Merck silica gel 60 F_{254}, 20×20 cm) developed with a solvent ($CHCl_3$: MeOH=20 : 1). Two yellow bands (Rf=0.31 and 0.28) were separately scraped off and eluted with AcOEt to give crude samples that were named naphterpin B and naphterpin C. Each

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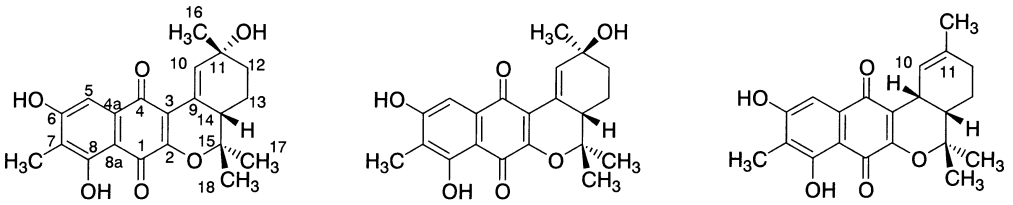

Fig. 1 Structures of naphterpin B, naphterpin C and naphterpin.

Table 1 Physicochemical properties of naphterpin B and naphterpin C

	Naphterpin B	Naphterpin C
Appearance	Orange plates	Orange needles
MP	104°C	214~216°C
$[\alpha]_D$	+406° (*c* 0.6, MeOH)	+413° (*c* 0.16 MeOH)
Molecular formula	$C_{21}H_{22}O_6$	$C_{21}H_{22}O_6$
HRFAB-MS (*m/z*)	Calcd: 371.1494 $(M+H)^+$	Calcd: 371.1494 $(M+H)^+$
	Found: 371.1461	Found: 371.1510
UV λ_{max} nm (ε) (MeOH)	284 (15900), 341 (4500)	284 (15000), 340 (4000)
IR (KBr) cm^{-1}	3464, 1629, 1561, 1438, 1328, 1248	3446, 1624, 1559, 1441, 1326, 1247

sample was further purified by HPLC (PEGASIL Silica 60-5, 10×250 mm, $CHCl_3$: MeOH = 40 : 1). Fractions containing naphterpin B and naphterpin C were separately combined and further purified by Sephadex LH-20 column chromatography. Both compounds were crystallized from small amount of AcOEt. Yields of these compounds from 30 liters of fermentation broth were 21.5 mg (naphterpin B) and 11.4 mg (naphterpin C).

Their physicochemical properties are summarized in Table 1. Based on the HRFAB-MS data, the molecular formula of B and C were determined to be identical, *i.e.*, $C_{21}H_{22}O_6$ [B, $(M+H^+)$ 371.1461, calcd. 371.1494; C, $(M+H^+)$ 371.1510, calcd. 371.1494].

Their structures were determined by detailed analysis of ^1H and ^{13}C NMR spectral data summarized in Table 2. The presence of four methyl proton singlets in the ^1H NMR spectra of naphterpin B (1.12, 1.52, 1.56 and 2.12 ppm) and naphterpin C (1.08, 1.36, 1.57 and 2.08 ppm) clearly indicated that these metabolites belonged to a group of terpenoids. In addition, they showed two aromatic protons (7.16 and 7.46 ppm for B and 7.10 and 7.23 ppm for C) and one hydrogen-bonded phenolic proton at *ca.* 12 ppm. These very similar data of B and C suggested that they are structural analogs of naphterpin (Fig. 1).

This conclusion was corroborated by comparison of ^{13}C NMR spectra of B and C to that of naphterpin as shown in Table 2. The ^{13}C NMR spectra of these compounds are very similar; a new quaternary oxygenated carbon signal (68.5 ppm for naphterpin B and 71.6 ppm for naphterpin C), however, was seen in both compounds with concomitant disappearance of a methine signal at 31.1 ppm in naphterpin. No change was observed with the naphthoquinone moiety of these compounds. Further structural information was obtained by analysis of HSQC and HMBC spectra of B (Fig. 2) and C which revealed migration of the double bond at C-10–C-11 in naphterpin to C-9–C-10 in B and C together with the appearance of a quaternary oxygenated carbon in B and C. Other moieties of these molecules remained unchanged suggesting that B and C were stereoisomers to each other at C-11.

In order to determine the stereochemistry at C-11, we planned to utilize NOEs observed with16-CH_3. Obstacle for these experiments, however, was severe overlapping of several protons from C-12 to C-14. Thus detailed analysis of the relevant protons were first started by taking TOCSY spectra with naphterpin C which revealed the proton spin system from 12-H to 14-H (Fig. 3), in particular the presence of a proton (12-Heq) at 2.08 ppm hidden by a strong methyl singlet at the same position. Decoupled difference spectra obtained by irradiation of 14-H

Table 2 NMR spectral data of naphterpins

No.	Naphterpin B		Naphterpin C		Naphterpin	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	182.4 (s)		181.2		183.1	
2	154.5 (s)		154.2		153.5	
3	127.0 (s)		125.5		123.3	
4	183.3 (s)		183.9		184.8	
4a	131.4 (s)		131.4		131.4	
5	108.5 (d)	7.16	107.8	7.10	108.4	7.31
6	161.8 (s)		162.3		161.5	
7	117.0 (s)		117.2		117.2	
8	162.2 (s)		162.4		162.6	
8a	107.9 (s)		107.3		107.9	
9	116.1 (s)		115.5		31.1	3.47
10	135.3 (d)	7.46	137	7.23	120	6.01
11	68.5 (s)		71.6		136.1	
12	35.7 (t)	1.61, 1.95	37	2.00, 2.08	29.6	1.95
13	20.1 (t)	1.61, 1.80	22.5	1.24, 2.00	20.4	1.25, 1.95
14	42.4 (d)	2.30	41.8	2.54	39.7	1.75
15	82.4 (s)		82.2		80.8	
16	30.7 (q)	1.52	27.3	1.36	23.5	1.64
17	19.3 (q)	1.12	19.4	1.08	25.6	1.51
18	26.7 (q)	1.56	26.2	1.57	25.1	1.34
7-CH_3	7.7 (q)	2.12	7.8	2.08	7.8	2.15
6-OH		N.D.		N.D.		8.25
8-OH		12.2		12.0		12.2

Spectra were taken in $CDCl_3$. s, d, t and q represent quaternary, methine, methylene and methyl carbons, respectively. N.D.: not detected.

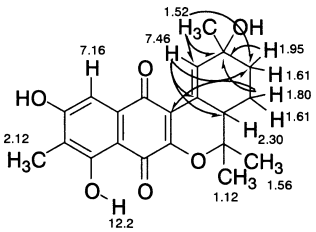

Fig. 2 Important HMBC correlations observed for naphterpin B.

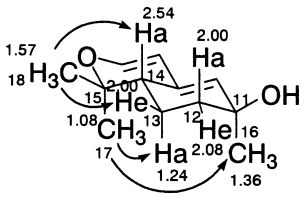

Fig. 3 Stereochemical relationship determined for naphterpin C.

Arrows indicate ROEs.

(2.54 ppm) revealed 13-Hax (1.24 ppm, $J_{13,14}$=10 Hz) and 13-Heq (2.00 ppm, $J_{13,14}$=3 Hz). Similar experiments irradiating 13-Hax identified its coupling partners 12-Hax (2.00 ppm, $J_{12,13}$=10 Hz) and 12-Heq (2.08 ppm, $J_{12,13}$=3 Hz). Thus, the assignments of 12-Hax, 13-Hax and 14-Hax were rigorously established for stereochemical analysis. In the ROESY spectrum of C, ROEs were observed between 17-CH_3 and 16-CH_3 and between 17-CH_3 and 13-Hax. Based on these results, the stereochemistries of 16-CH_3 and 17-CH_3 were determined to be axial as shown in Fig. 3. Very close chemical shifts of 16-CH_3 and 13-Hax that caused insufficient separation of relevant cross peak from diagonal peak hampered to elicit necessary information from the ROESY spectrum.

Similar ROE experiments carried out with B detected ROEs between 17-CH_3 and 13-Hax, and between 18-CH_3 and 14-Hax, but not between 17-CH_3 and 16-CH_3. Thus, the stereostructures of B and C were established as shown

in Fig. 1. B and C are presumably formed by epoxidation of the double bond at C-10 and C-11 in naphterpin followed by ring opening catalyzed by protonation. They are structurally related to methoxydebromomarinone [8] isolated from a marine *Streptomyces* species.

Naphterpins B and C suppressed lipid peroxidation in rat homogenate system [7] (IC_{50} for B 6.5 mcg/ml and for C 6.0 mcg/ml) and their activities were *ca.* 1/10 of that of naphterpin.

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