ISOLATION AND STRUCTURAL ELUCIDATION OF AN ANTIOXIDATIVE AGENT, NAPHTERPIN

Sir:

Active oxygen species cause a variety of diseases such as inflammation, autoimmune disease, diabetes, rheumatism, cardiovascular diseases and cancer-initiation[1,2]. Thus, it could be expected that antioxidative agents may prevent these diseases. We have screened antioxidants of microbial origin by employing the Yagi method after minor modification using rat liver microsome free from vitamin E[3]. As a result, we succeeded in isolating a new antioxidative agent, named naphterpin. In this paper, we report the fermentation, isolation and structure determination of naphterpin.

The producing microorganism *Streptomyces* sp. CL190 was isolated from Ishigaki Island, Okinawa Prefecture, Japan. It was inoculated into test tubes containing 12 ml of the seed medium consisting of glucose 2.5%, soybean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4% (pH 6.2, before sterilization). After incubation at 27°C for 2 days on a reciprocal shaker, an aliquot of the broth (2 ml) was transferred into 500-ml Erlenmeyer flasks each containing 100 ml of the same medium as the seed culture and incubated at 27°C for 2 days on a rotary shaker. The fermentation broth (600 ml) was transferred into a 50-liter jar fermenter containing 30 liters of the same medium, and cultivation was carried out at 27°C for 28 hours with agitation 400 rpm and aeration at 30 liters per minute.

The mycerial cake collected by centrifugation from the whole fermentation broth (60 liters) was stirred with 10 liters of acetone. The solvent extract was concentrated *in vacuo* to a small volume and active materials were extracted twice with each 1 liter of ethyl acetate. The separated organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The oily residue was applied to a silica gel column (5.0 × 30.0 cm) packed with a mixture of *n*-hexane and ethyl acetate (4 : 1). The column was developed with the same solvent system, and the pooled active fraction was concentrated to dryness. The dry residue (626 mg) was rechromatographed on a silica gel column (3.0 × 24.5 cm) packed with a mixture of chloroform, methanol and conc aq ammonia (200 : 20 : 1). The active elute was concentrated *in vacuo* and the residue (269 mg) was dissolved in a small amount of chloroform and methanol (1 : 1) to be further purified by column chromatography on Sephadex LH-20 (2.5 × 87 cm) developed with the same solvent mixture. The active fraction was evaporated to dryness *in vacuo*, and crystallization from a small volume of ethyl acetate gave orange plates of naphterpin.

Its physico-chemical properties are shown in Table 1. The molecular formula of naphterpin was determined as $C_{21}H_{22}O_5$ by HREI-MS (*m/z* 354.1514 (M^+), calcd: 354.1470) and elemental analysis (calcd: C 71.17, H 6.26, O 22.57, found: C 71.13, H. 6.22, O 22.90). The UV absorption of naphterpin suggested the presence of a 1,4-naphthoquinone nucleus[4~6].

The ^{13}C and ^1H spectral data of naphterpin are summarized in Table 2. Detailed spectral analysis of naphterpin aided by ^1H-^1H COSY, spin-decoupling experiments and ^{13}C-^1H COSY revealed the presence of a 3,4-disubstituted 1-methylcyclohexene skeleton as shown in Fig. 1.

The sequence from 10-H (6.01 ppm) to 12-H (1.95 ppm) through 9-H (3.47 ppm), 14-H (1.75 ppm) and 13-H (1.25 and 1.95 ppm) was revealed by ^1H-^1H COSY. Since one of the methylene protons 13-H (1.95 ppm) was overlapped with another methylene proton 12-H (1.95 ppm), correlation between C-12 and C-13 was also supported by a spin decoupling experiment irradiating one of the 13-H methylene protons appearing at a higher field (1.25 ppm). Allylic couplings of the olefinic proton with 16-H (CH_3, 1.64 ppm) and 12-H were

Table 1. Physico-chemical properties of naphterpin.

Appearance	Orange plates	UV λ_{max}^{MeOH} nm (ε)	215 (24,400), 266 (18,800),
MP	224~225°C		315 (12,400), 415 (4,200)
$[\alpha]_D^{23}$ (*c* 0.1, $CHCl_3$)	−648°	$\lambda_{max}^{MeOH+NaOH}$ nm (ε)	208 (27,500), 229 (25,200),
Molecular formula	$C_{21}H_{22}O_5$		291 (20,700), 325 (8,600),
HREI-MS (*m/z*)	Calcd: 354.1470		425 (3,800), 520 (4,200)
	Found: 354.1514 (M^+)	IR ν (KBr) cm^{-1}	3300, 1620, 1600, 1580,
Analysis			1430, 1320, 1275, 1245
Calcd for $C_{21}H_{22}O_5$:	C 71.17, H 6.26, O 22.57	Rf value	0.21 (toluene-acetone, 97 : 3)
Found:	C 71.13, H 6.22, O 22.90	(silica gel 60)	0.22 (hexane-ethyl acetate, 4 : 1)

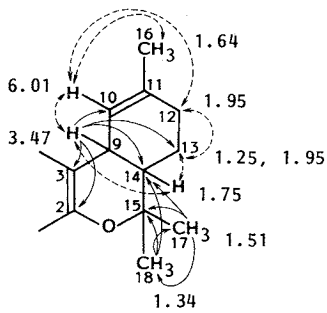
Table 2. ^{13}C and ^{1}H NMR chemical shifts[a] of naphterpin.

No.	^{13}C	^{1}H	No.	^{13}C	^{1}H
1	183.1		11	136.1	
2	153.5		12	29.6	1.95
3	123.3		13	20.4	1.25, 1.95
4	184.8		14	39.7	1.75
4a	131.4		15	80.8	
5	108.4	7.31	16	23.5	1.64
6	161.5		17	25.6	1.51
7	117.2		18	25.1	1.34
8	162.6		7-CH_3	7.8	2.15
8a	107.9		6-OH		8.25
9	31.1	3.47	8-OH		12.20
10	120.0	6.01			

[a] ppm from internal TMS in $CDCl_3$.

Fig. 1. Structural analysis for terpenoid moiety of naphterpin.

HMBC as solid-line arrows and proton spin couplings as dotted-line arrows.



confirmed by long range ^{1}H-^{1}H COSY. The linkage of the cyclohexene ring with a gem dimethyl group will be explained later.

The naphthoquinone moiety was analyzed by utilizing two and/or three bond ^{13}C-^{1}H couplings observed in the heteronuclear multiple-bond correlation (HMBC)[7,8]. The methyl proton (2.15 ppm) at C-7 (117.2 ppm) was coupled to C-6 (161.5 ppm), C-7 and C-8 (162.6 ppm). Based on the ^{13}C chemical shifts, C-6 and C-8 were assigned to phenolic carbons. The phenolic hydroxyl proton 8-OH (12.20 ppm), which was hydrogen-bonded with a quinone carbonyl, was coupled to C-7, C-8 and C-8a (107.9 ppm). Another phenolic hydroxyl proton 6-OH (8.25 ppm) showed couplings to C-5 (108.4 ppm), C-6 and C-7. These aromatic carbons were accommodated in a 1,3-dihydroxy-2-methyl-5,6-disubstituted benzene system by analyzing ^{13}C-^{1}H long range couplings observed with 5-H (7.31 ppm) in the HMBC spectrum. Thus, 5-H was coupled to C-8a, C-4a (131.4 ppm), C-6 and C-7. In addition, 5-H proton was coupled to C-4 (184.8 ppm, quinone carbonyl carbon). By taking into consideration these relations and UV spectral data (*vide supra*), a structure for the naphthoquinone part of naphterpin was determined as shown in Fig. 2.

Fig. 2. HMBC analysis for naphthoquinone moiety of naphterpin.

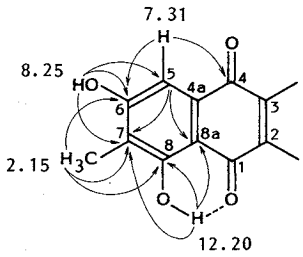
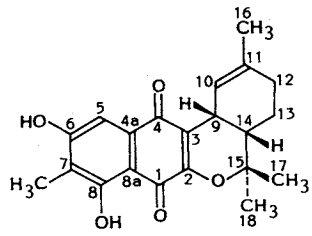
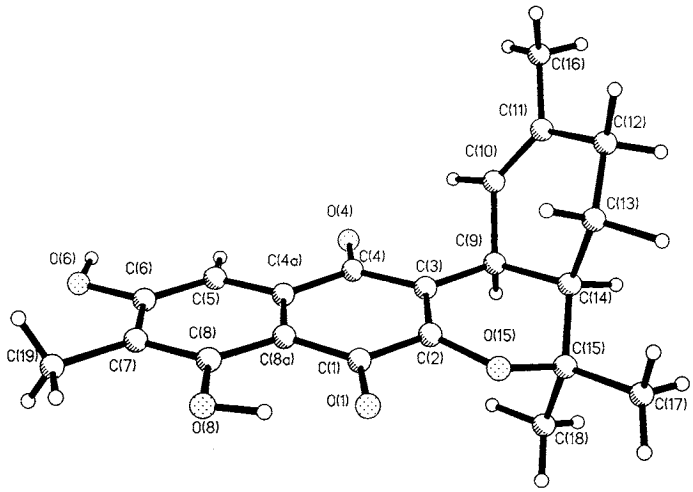


Fig. 3. Structure of naphterpin (relative stereochemistry).



The structure of the cyclohexene ring moiety of naphterpin (Fig. 1) was extended by HMBC spectral analysis; the methyl proton 17-H (1.51 ppm) was coupled to three carbons, *i.e.*, a methine carbon C-14 (39.7 ppm) contained in the cyclohexene ring, a quaternary oxycarbon C-15 (80.8 ppm) and a methyl carbon C-18 (25.1 ppm). Likewise, another tertially methyl proton 18-H (1.34 ppm) was coupled to C-14 and C-15 in addition to C-17. These NMR spectral

Fig. 4. Computer generated perspective drawing of naphterpin.



data proved the relations among C-14, C-15, C-17 (25.6 ppm) and C-18 as shown in Fig. 1.

The methine proton 9-H (3.47 ppm) in the 1-methylcyclohexene ring was coupled to six carbons. Couplings to C-10 (120.0 ppm), C-11 (136.1 ppm), C-13 (20.4 ppm) and C-14 confirmed the cyclohexene ring skeleton. Additional two carbons were C-3 (123.3 ppm) and oxygenated sp^2 carbon C-2 (153.5 ppm). Since four oxygens out of five oxygens in naphterpin had been assigned to the 1,4-naphthoquinone nucleus as described above (Fig. 2), C-2 and C-15 had to be connected by an ether bond as shown in Fig. 1.

Since ^{13}C-^{1}H long range couplings from the methine proton 9-H to the carbonyl carbon C-4 or C-1 could not be observed, the linkage of the two portions of the molecule shown in Figs. 1 and 2 remained unclear. Thus, the complete structure was determined by an X-ray analysis as shown in Fig. 3.

X-Ray photographs displayed orthorhombic symmetry, and accurate lattice constants of $a=8.1261$ (16), $b=14.688$ (3) and $c=15.472$ (3) Å were determined from a least-squares fit to the 2θ-values of reflections measured on a diffractometer. Systematic extinctions, along with the observed optical activity, uniquely required the space group $P2_12_12_1$ with one molecule of composition $C_{21}H_{22}O_5$ forming the asymmetric unit. A total of 1,467 unique reflections with $2\theta \leq 114°$ were collected using Cu Kα radiation and 2θ-θ scans. Of these, 1,371 (92%) were judged observed after correction for Lorentz, polarization and background effects. The structure was solved using direct methods and refined by full-matrix least-squares techniques to a conventional crystallographic residual of 0.041 for the observed data. Crystallographic parameters have been deposited with the Cambridge Crystallographic Data Center. The results of the X-ray analysis are given in Fig. 4, a computer generated perspective drawing of naphterpin.

IC_{50} of naphterpin for the assay system employed in this experiment was 5.3 μg/ml, while vitamin E was 9.4 μg/ml. Naphterpin showed no antimicrobial activities against *Bacillus subtilis* IAM1026, *Escherichia coli* NIHJ, *Candida albicans* IAM4905 and *Pyricularia oryzae* NIAES at the concentration of 1,000 μg/ml.

Addendum in Proof

The absolute configuration of naphterpin has been established as shown in Fig. 3 by X-ray analysis of its monobromoacetyl derivative.

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