Notes

NEW METABOLITES, TETRAHYDROFURAN LIGNANS, PRODUCED BY *Streptomyces* sp. IT-44

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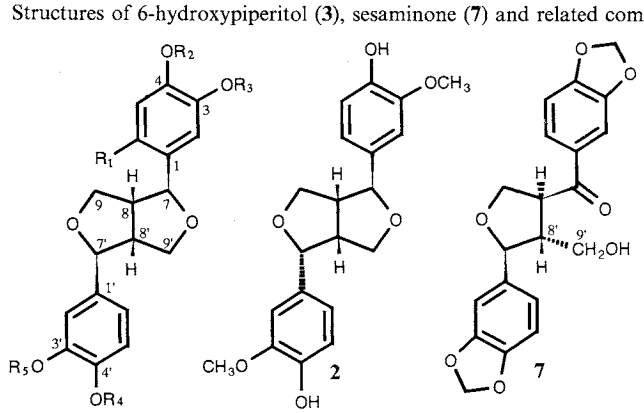
During the course of our screening for new antibiotics, we utilized sesame meal, a waste material from the industrial production of sasame oil, as a nitrogen source for growth of strains of Actinomycetes. An isolate of *Streptomyces* sp. IT-44 showed antibacterial and antitumor activities. Bioassay-guided fractionation of the broth filtrate of *Streptomyces* sp. IT-44 led to the isolation of three furofuran lignan-type antibiotics, determined as pinoresinol (**1**), epipinoresinol (**2**) and a new compound **3**. Four related compounds, piperitol (**4**), sesaminol (**5**), sesamin (**6**) and a new piperonyloyl tetrahydrofuran derivative (**7**) were also isolated by chemical screening. Those compounds were identified as relative compounds by their UV and ^{1}H NMR spectra. In this paper, we report the production, isolation and structure elucidation of the new lignans, compounds **3** and **7**, named 6-hydroxypiperitol and sesaminone, respectively, shown in Fig. 1.

The producing organism was isolated from a soil sample collected at Dunhuang in Gansu Province, China. The strain exhibited the following properties on the various media. The aerial mass color was gray or light brownish-gray to light pinkish-gray and color of vegetative growth was colorless or brownish; melanoid and soluble pigments were not produced. Microscopic observation showed that the mature sporophores formed spirales and had more than 20 spores per chain. The spore surface was smooth or sometimes warty. Sclerotic granules and sporangia were not observed. The cell wall of strain IT-44 in submerged-cultures contained LL-diaminopimelic acid. According to these taxonomic studies, strain IT-44 belongs to the genus *Streptomyces*[1], but the species was not determined.

Compound **3**, obtained as a colorless syrup, was found to have the following physico-chemical properties: It was soluble in chloroform, benzene, ethyl acetate and methanol, and insoluble in water. It gave a positive color reaction with $FeCl_3$, and

Fig. 1. Structures of 6-hydroxypiperitol (**3**), sesaminone (**7**) and related compounds.



1 $R_1=R_2=R_4=H$, $R_3=R_5=CH_3$
3 $R_1=OH$, $R_2-R_3=-CH_2-$, $R_4=H$, $R_5=CH_3$
4 $R_1=H$, $R_2-R_3=-CH_2-$, $R_4=H$, $R_5=CH_3$
5 $R_1=OH$, $R_2-R_3=R_4-R_5=-CH_2-$
6 $R_1=H$, $R_2-R_3=R_4-R_5=-CH_2-$

had UV λ_{max} (MeOH) nm (ε): 203 (83,000), 232 (10,600), 288 (4,500), 302 (4,100) and IR ν_{max} (KBr) cm^{-1}: 3450 (m), 2950 (m), 1640 (s), 1520 (s), 1450 (s), 1290 (s), 1250 (s), 1160 (m), 1040 (s). The UV spectrum was similar to that of other furofuran lignans[2,3]. The molecular formula was determined as $C_{20}H_{20}O_7$ by high-resolution EI mass spectrometry (Calcd: m/z 372.1209, Found: m/z 372.1182 (M^+)). The ^1H NMR spectrum showed close similarities to that of **5**[4] except for the signals at δ_H 5.58 (s, D_2O exchangeable) and δ_H 3.91 (s, 3H). Those signals suggested that one methylenedioxyl group in **5** was replaced by a hydroxyl and a methoxyl. The NOE was observed between the methoxyl signal and a doublet signal at δ_H 6.87 (J=2 Hz), and the latter signal corresponded to position-2 in the 1,3,4-trisubstituted benzene ring. Therefore, the methoxyl and hydroxyl groups were located at C-3′ and C-4′, respectively. Thus, the planar structure of **3** was determined as shown in Fig. 1. The stereochemistry was determined as follows: A doublet at δ_H 4.79 (J=4 Hz) for two protons was assigned to be 7-H and 7′-H. The coupling constant was identical with that of **5**[5],†, and the relative stereochemistry of **3** was estimated to be the same as that of **5** according to KARPLUS' principle[6]. This configuration was also proved by the strong enhancement of 9-Hα and 9′-Hα when 7-H and 7′-H were irradiated (Fig. 2). The ^1H and ^{13}C NMR assignments of **3** and **4** are compared in Tables 1 and 2, respectively. Those results also suggested the structure of **3**. This β, β form of aromatic substitutes on furofuran ring was similar to piperitol (**4**)[7], so compound **3** was designated as 6-hydroxypiperitol.

Compound **7** was also isolated as a colorless syrup and its physico-chemical properties are as follows: It was soluble in chloroform, benzene, ethyl acetate, acetone, methanol and ethyl ether, and insoluble

Fig. 2. NOE observed for 6-hydroxypiperitol (**3**).

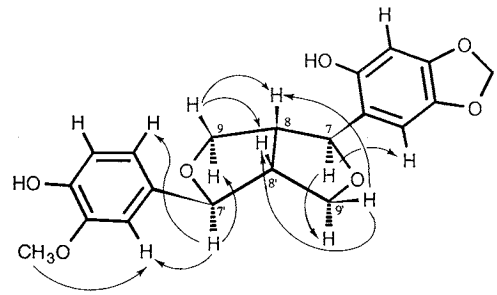


Table 1. ^1H NMR chemical shifts of **3**, **4** and **7** (400 MHz, in $CDCl_3$).

Position	Compound		
	3	**4**	**7**
2	6.51 (s)	6.85 (d, 2)	7.47 (d, 2)
5	6.45 (s)	6.78 (d, 8)	6.88 (d, 8)
6		6.81 (dd, 8, 2)	7.58 (dd, 8, 2)
7	4.79 (d, 4)	4.73 (d, 4)	
8	3.19 (m)	3.07 (m)	4.11 (m)
9 Hα	3.88 (dd, 9, 3)	3.87 (m)	4.14 (m)
Hβ	4.15 (dd, 9, 6)	4.24 (m)	4.28 (m)
$-OCH_2O-$	5.89 (d, 1.5), 5.90 (d, 1.5)	5.95 (s)	6.06 (s)
6-OH	7.77 (s)		
2′	6.87 (d, 2)	6.89 (d, 2)	6.97 (d, 2)
5′	6.89 (d, 8)	6.89 (d, 8)	6.77 (d, 8)
6′	6.80 (dd, 8, 2)	6.82 (dd, 8, 2)	6.85 (dd, 8, 2)
7′	4.79 (d, 4)	4.73 (d, 4)	4.67 (d, 9)
8′	3.19 (m)	3.07 (m)	2.87 (m)
9′ Hα	3.86 (dd, 9, 5)	3.87 (m)	3.66 (dd, 11, 6)[a]
Hβ	4.35 (dd, 9, 7)	4.24 (m)	3.77 (dd, 11, 5)[a]
9′-OH			Obscure
3′-OCH_3-	3.91 (s)	3.91 (s)	
$-OCH_2O-$			5.95 (s)
4′-OH	5.58 (s)	5.57 (s)	

Parentheses represent multiplicity and coupling constant in Hz.
[a] Exchangeable.

† NMR spectral data obtained from sesaminol by JEOL JNM-GX400 spectrometry in this study.

Table 2. ^{13}C NMR chemical shifts of **3**, **4** and **7** (in $CDCl_3$).

Carbon No.	Compound		
	3[a]	**4**[b]	**7**[a]
1	115.0	135.0	131.5
2	106.1	106.5	108.3
3	148.1	147.1	148.5
4	150.8	147.9	152.2
5	99.5	108.2	108.06
6	140.9	119.4	124.9
7	72.5	71.7	197.3
8	53.2*	54.3	50.0
9	85.5**	85.9	70.9
$-OCH_2O-$	101.2	101.1	102.0
1′	132.4	132.9	134.5
2′	108.6	108.6	107.2
3′	146.8	146.7	148.0
4′	145.5	145.2	147.5
5′	114.3	114.3	108.10
6′	119.0	119.0	120.4
7′	72.3	71.7	83.7
8′	52.9*	54.2	52.3
9′	85.3**	85.8	61.4
3′-OCH_3-	56.0	55.9	
$-OCH_2O-$			101.1

*,** Exchangeable.
[a] 100 MHz.
[b] 67.5 MHz.

in water. It gave a positive color reaction with 2,4-dinitrophenylhydrazine, and had UV λ_{max} (MeOH) nm (ε): 204 (63,400), 231 (20,700), 278 (7,900), 310 (7,150), IR ν_{max} (KBr) cm^{-1}: 3445 (m), 2885 (m), 1669 (m), 1650 (m), 1505 (s), 1489 (s), 1444 (s), 1251 (s), $[\alpha]_D^{25}$ $-25.0°$ (c 0.140, MeOH) and FAB-MS (positive) m/z 371 $(M+1)^+$. The molecular formula of **7** was determined to be $C_{20}H_{18}O_7$ by HREI-MS (Calcd: m/z 370.1053, Found: m/z 370.0977 $(M)^+$). The ^1H NMR spectrum showed six aromatic protons and they were assigned to two 1,3,4-trisubstituted benzene rings. The presence of each of 2H singlets at δ_H 5.95 and δ_H 6.06 bound to methylene carbons (δ_C 101.1 and 102.0) suggested that the both benzene rings have 3,4-methylenedioxyl groups. The fact that a part of the phenyl group is connected to the carbonyl group was suggested by a carbonyl carbon at δ_C 197.3 and the conjugated property shown by the IR spectrum at 1669 cm^{-1} together with the FAB-MS data, which gave a base peak of the piperonyloyl group at m/z 149[8]. The ^1H-^1H COSY connectivities revealed the skeletal structure as 2,3,4-trisubstituted tetrahydrofuran. The methylene signals corresponding to the 9′ position (δ_H 3.66 and 3.77, δ_C 61.4) were

Fig. 3. Long range ^1H-^{13}C coupling observed in HMBC experiment on sesaminone (**7**).

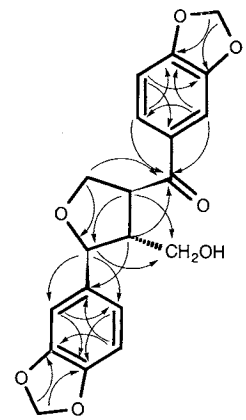
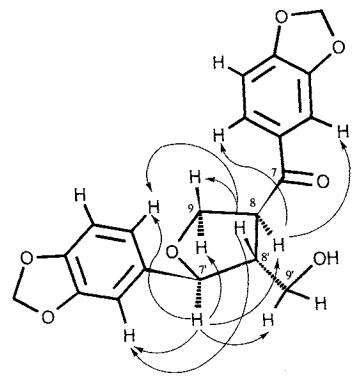


Fig. 4. NOE observed for sesaminone (**7**).



assigned to a hydroxymethyl group adjacent to C-8′. Furthermore, the correlations observed in HMBC spectrum (9-H～C-7, 8′-H～C-7, 8′-H～C-1′ and 7′-H～C-2′, 6′) suggested that the piperonyloyl group and methylenedioxyphenyl group are to C-8 and C-7′, respectively (Fig. 3). Consequently, the planar structure was then determined to be an oxidative ring-opening derivative of sesamin (**6**), so compound **7** was designated as sesaminone. The relative stereostructure was established by a NOE difference experiment. The NOE between 8-H and 7′-H (3%) suggested the relative configuration of those positions to be *cis*. Furthermore, the configuration of 7′-H and 8′-H was deduced to be *trans* based on the following NOE network: 8′-H～2′-H (3%), 8′-H～6′-H (6%) and 7′-H～9′-H (4%). Therefore, an all-*trans* stereochemistry of the three asymmetric centers was established (Fig. 4).

Because furofuran lignans have been reported to be contained in sesame seeds[9], and because these lignans are known to have biological activities such

as antitumor, antimitotic, antimicrobial and antioxidative ones[10], the origins of the two new lignans were examined by quantifying their amounts in sesame meal and fermentation medium with and without an inoculum of strain IT-44. The results showed that the strain produced more than 85 μg of **7** from 1 g of sesame meal after fermentation for 5 days. About 1.5 μg of **7** was obtained from 1 g of sesame meal after extraction, while about 6.5 μg was gained from autoclaved medium containing 1 g of sesame meal without inoculum. On the other hand, about 15 μg of **3** from 1 g of sesame meal was quantified from culture fluid after fermentation, but was not detectable in sesame meal and autoclaved medium without the inoculum. Based on the above results, the new lignans **3** and **7** appear to be biotransformed by *Streptomyces* sp. IT-44.

Sesaminone (**7**) had only a weak activity (MIC value, 200 μg/ml) against *Enterococcus aecium* ATCC 6056 of the 27 kinds of microorganisms tested, but 6-hydroxypiperitol (**3**) had no activity. While pinoresinol (**1**) and epipinoresinol (**2**) showed moderate activity against Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*. Compounds **1**, **2**, and **3** exhibited IC_{50} values of 2.2, 4.0 and 3.1 μg/ml, respectively, against KB cells. No cytotoxic activity of **7** was observed when assessed at the dose up to 10 μg/ml.

Experimental

Taxonomy

The producing strain IT-44 was taxonomically characterized at 27°C by the International Streptomyces Project (ISP) procedures recommended by SHIRLING and GOTTLIEB[11]. The chemical analysis of cell wall diaminopimelic acid isomers was carried out by the method of BECKER *et al.*[12].

Fermentation

The seed medium consisted of glucose 0.5%, soluble starch 2.4%, beef extract 0.3%, yeast extract 0.5%, corn steep liquor 0.4%, $CaCO_3$ 0.4% and $CoCl_2 \cdot 6H_2O$ 0.002% (pH 7.2). After 2 days of shaking culture, one percent of the seed culture was inoculated into each of several 500-ml Erlenmeyer flasks, each containing 100 ml of fermentation medium composed of glucose 0.5%, dextrin 2.5%, sesame meal 2%, corn steep liquor 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03% and $CaCO_3$ 0.3% (pH 7.2). Incubation was carried out on a rotary shaker at 220 rpm at 27°C for 5 days.

Assay of Biological Activity

Cytotoxic activity (IC_{50} value) was determined by the growth inhibition of KB carcinoma cells after exposure to a given compound for 3 days in EAGLE's minimal essential medium supplemented with 10% calf serum. The progress of antibiotic production in cultures was followed by the agar diffusion assay method with *Bacillus subtilis* ATCC 6633. The MIC was measured by the serial dilution assay method on DAVIS's minimal agar medium supplemented with 0.1% meat extract.

Isolation of Aryltetrahydrofurans

The culture filtrate (10 liters) was extracted with ethyl acetate at pH 8.0. After concentration of the extract to dryness *in vacuo*, 921 mg of paste was obtained and partitioned in the mixing solution of methanol, benzene and *n*-hexane (1 : 10 : 5). The evaporated supernatant (*ca.* 700 mg) was subjected to silica gel column chromatography (270 × 16 mm i.d.) using chloroform - methanol as eluant. The eluate at a solvent ratio of 200 : 1 showed the growth inhibition against *Bacillus subtilis* and KB cells. This residue (*ca.* 130 mg) was rechromatographed on a YMC-ODS column (270 × 10 mm i.d.) using a stepwise elution with acetonitrile - water. Compounds **1**, **2** and **3** were obtained in the eluate collected at a ratio of 30 : 70 by the activities against *Bacillus subtilis* and/or KB cells., **4** and **7**, at 35 : 65, and at last **5** and **6** at 45 : 55. The respective fractions were extracted with chloroform, and then the organic layer was concentrated to dryness *in vacuo*. One-half miligram of **3** and about 8.5 mg of **7** were purified through a silica gel flash column (Fuji Davison silica gel BW-300) eluted with a gradient of benzene - ethyl acetate.

Quantitative Analysis

Quantitative analysis of **3** and **7** in test samples was performed by HPLC, monitored at 230 nm on a μBondapak C-18 (Waters, 250 × 5 mm i.d.) using a 30% aqueous acetonitrile solution as eluant, and the calibration curves were determined by use of standard solutions prepared from pure samples of **3** and **7**. Compound **7** could be detected above 0.5 μg/ml but **3** required above 2 μg/ml. The respective retention times of compounds were as follows: **1**, 11.7; **2**, 14.5; **3**, 18.3; **7**, 29.6; **4**, 32.9; **5**, 50.0 and **6**, 58.7 minutes. Samples for testing were prepared by extraction with ethyl acetate at pH 8 after methanol extraction or no extraction, and the concentrate was dissolved in benzene. The insoluble part was redissolved in acetonitrile, and the test

samples were obtained following passage through a silica gel flash column developed with benzene - ethyl acetate.

Acknowledgements

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