Isolation, Characterization and Biological Activities of Novel Triprenyl Phenols as Pancreatic Cholesterol Esterase Inhibitors Produced by *Stachybotrys* sp. F-1839

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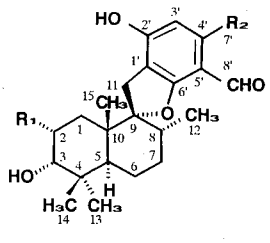
Ten triprenyl phenol metabolites were isolated as inhibitors of pancreatic cholesterol esterase from cultures of *Stachybotrys* sp. F-1839 by solvent extraction and column chromatographies. Combination of spectroscopic analyses revealed that two of these compounds are K-76 (**1**) and stachybotrydial (**2**), and that the remaining eight are new congeners (designated F1839-A (**3**), -B (**4**), -C (**5**), -D (**6**), -E (**7**), -F (**8**), -I (**9**) and -J (**10**)). These compounds inhibited pancreatic cholesterol esterase by 50% at 6×10^{-5} to 1.1×10^{-1} M. Inhibition of the enzyme by compound **2**, the most potent one among these compounds, was time-dependent and irreversible. When administered to normal rats, **2**, at a single oral dose of 100 mg/kg, reduced [^{14}C]cholesterol absorption by 50~60%. In cholesterol-fed mice, dietary supplementation of **2** (0.1%) for 14 days resulted in a 20% reduction in serum total cholesterol level without causing significant change in the high density lipoprotein cholesterol level.

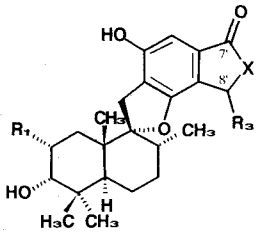
The absorption of cholesterol from the intestinal lumen has been shown to play a role in controlling the steady state concentration of cholesterol in plasma[1,2]. Cholesterol esterase in pancreatic juice binds to the intestinal mucosa and plays a key role in the intestinal absorption of dietary cholesterol[3~6]. GALLO *et al.* have shown that removal of cholesterol esterase from the pancreatic juice results in a marked reduction in cholesterol absorption in rats[7]. Therefore, inhibition of pancreatic cholesterol esterase is a target for the treatment and/or prevention of hypercholesterolemia and atherosclerosis[8,9].

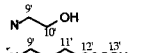
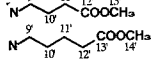
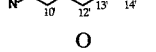
During the course of the screening for cholesterol esterase inhibitors of microbial origin, we found that a fungal strain, *Stachybotrys* sp. F-1839, produces a series of active metabolites. Two of these compounds have been identified to be K-76 (**1**) and stachybotrydial (**2**), which have previously been isolated as an inhibitor of complement activation from *Sta. complementi*[10~12] and as a metabolite of *Sta. cylindrospora*[13], respectively. The remaining eight metabolites are novel triprenyl phenols (Fig. 1). In this report, we describe the isolation, structure elucidation and biological activities of these

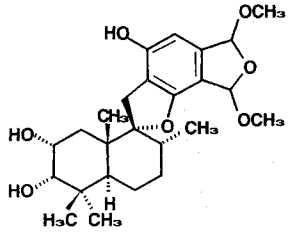
Fig. 1. Structures of compounds **1**~**12**.



Compound	R_1	R_2
1	OH	CHO
2	H	CHO
9	H	CH_3



Compound	R_1	R_3	X
3	OH	H	NH
4	OH	OCH_3	NH
5	OH	OCH_3	NH
7	OH	H	
8	OH	H	
10	H	H	
12	H	H	O



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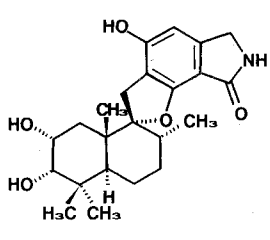

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compounds as inhibitors of pancreatic cholesterol esterase.

Experimental

Microorganism and Taxonomic Study

Strain F-1839 was isolated from a soil sample collected in Shizuoka Prefecture, Japan. The pure culture was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba-shi, Japan, under the name of *Stachybotrys* sp. F-1839 with an accession number of FERM-P 12818. The strain was subcultured on potato glucose agar slants. Taxonomic studies were performed according to the method of AINSWORTH[14,15]. For morphological observations, the strain was grown at 27°C for up to 3 weeks. Detailed observation of mycelium and spore morphologies was performed under a light microscope. The color names used were based on the Color Standard of Nippon Shikisai Co., Japan[16].

Fermentation

A medium consisting of 3% glucose, 1% soybean meal, 0.3% polypeptone, 0.3% meat extract, 0.3% yeast extract, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% antifoam, pH 7.8 (adjusted prior to autoclaving) was used for both seed and production cultures. A loopful of a slant culture was inoculated into a 500-ml Erlenmeyer flask containing 160 ml of medium, which was then incubated at 29°C for 3 days on a rotary shaker at 220 rpm. A 0.5-ml portion of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 160 ml of medium and the flask was incubated as above for 3~7 days. Alternatively, a 0.5-ml portion of the seed culture was transferred to a 500-ml Sakaguchi flask containing 80 ml of medium, which was then incubated at 28°C for 7 days on a reciprocal shaker at 100 rpm.

Isolation of **1**, **2** and **9**

The combined broth (2.62 liters) from 3-day cultures in Erlenmeyer flasks was extracted twice with 3 liters of methyl ethyl ketone, and the organic extract was concentrated *in vacuo* to give an oily residue (5.0 g). The residue was subjected to a chromatography on a silica gel column (400 ml, FL-60D, Fuji-Davison Chemical). After washing with *n*-hexane (1 liter), the column was successively developed with *n*-hexane - EtOAc (4 : 1, 3 : 2 and then 2 : 3, 1 liter each). Compounds **2** and **9** appeared in the 3 : 2 fractions and **1** in the 2 : 3 fractions. Fractions containing **1** and **2** were concentrated independently to dryness and washed with 10 ml of methyl ethyl ketone, giving yellow powders of **1** (1,077 mg) and **2** (603 mg), respectively. After concentration, fractions containing **9** were dissolved in a small volume of MeOH, and then an equal volume of *n*-hexane was added to precipitate **9** (20 mg).

Isolation of **3**, **4**, **5**, **6**, **7** and **10**

The combined supernatant (13.5 liters) obtained from 7-day cultures in Erlenmeyer flasks was applied to a Diaion HP-20 column. After washing the column with water and then 50% aqueous MeOH, active compounds were eluted with MeOH (3 liters). The active fractions were concentrated to yield a brown residue (31.3 g), which was then extracted with EtOAc - MeOH (100 : 15, 300 ml). The extract (11.3 g) was applied to a silica gel column (500 ml), which was successively developed with 1.1 liters each of *n*-hexane, *n*-hexane - EtOAc (3 : 7), EtOAc, EtOAc - MeOH (100 : 5 and 100 : 10). Compound **9** was eluted with EtOAc. Compound **3** and a mixture of **4** and **5** were found in the EtOAc - MeOH (100 : 5) fractions and a mixture of **6** and **7** in the EtOAc - MeOH (100 : 10) fractions. Fractions containing **3** were applied to a column of Chromatorex ODS (20 ml, Fuji-Davison Chemical), washed with 40% aqueous MeOH (200 ml) and then developed with 50% aqueous MeOH (200 ml) to yield purified **3** (962 mg) as a white powder. Compounds **4**, **5**, **6**, **7** and **10**, were purified from respective fractions by preparative HPLC on a YMC-Pack SH-343 S-10 ODS column (20 × 250 mm, YMC Co.) using a mobile phase of 75% aqueous MeOH at a flow rate of 10 ml/minute, yielding 118, 117, 58, 117 and 132 mg of white powders, respectively.

Isolation of **8**

The combined supernatant (2.0 liters) obtained from 7-day cultures in Sakaguchi flasks was applied to a column (400 ml) of Diaion HP-20, washed with 400 ml of water and 500 ml of 50% aqueous MeOH and then developed with 1 liter of MeOH. The active fraction was concentrated to remove organic solvent and the resulting aqueous solution was extracted with 300 ml of *n*-butanol at pH 3. After concentration, the organic extract (4.58 g) was chromatographed on an Amberlite IRA-35 column (300 ml, Roam and Haas) with 2 liters of a linear gradient of 0.01 to 1 M of pyridine-acetic acid buffer, pH 6. Active fractions were combined and extracted with 200 ml of *n*-butanol at pH 3 and the organic extract was washed twice with 200 ml of diluted HCl (pH 3). After evaporation, the resultant residue (1.69 g) was further purified by two successive HPLCs on an Inertsil PREP-ODS column (30 × 250 mm, GL Science) developed with 80% aqueous MeOH at a rate of 25 ml/minute. Active fractions were combined and subjected to a silica gel column (5.4 × 20 mm) developed with EtOAc - acetic acid (98 : 2). Final purification was achieved by HPLC on an Inertsil SIL column (4 × 250 mm, GL Science) developed with chloroform - MeOH - acetic acid (96.5 : 3 : 0.5) at a rate of 1.0 ml/minute, yielding 5.1 mg of **8**.

Assay for Pancreatic Cholesterol Esterase

Pancreatic cholesterol esterase (EC 3.1.1.13) was assayed by determining the formation of cholesteryl oleate as described by VAHOUNY and TREADWELL[17~19]. Briefly, 0.5 ml of 3 mg/ml (0.12 U/ml) porcine pancreatic cholesterol esterase (Sigma) was incubated in 0.154 M potassium phosphate, pH 5.2, at 37°C for 10 minutes in

the absence and presence of test compounds. Subsequently, 0.5 ml of a mixture containing 15.6 mM cholesterol, 93 mM oleate, 40 mM sodium taurocholate, 132 mM NH_4Cl, 8 mg/ml of bovine serum albumin and 0.154 mM potassium phosphate (pH 5.2) was added to the enzyme solution. The mixture was incubated in a shaker bath at 37°C for 30 minutes and the reaction was stopped by heating the mixture in a boiling bath for 2 minutes. The mixture was lyophilized and extracted three times with 3 ml of *n*-hexane containing 0.25 mg of cholesteryl benzoate as an internal standard. After concentration, a portion of the solvent extract was subjected to gas-liquid chromatography (Shimadzu GC-9A) to determine cholesteryl oleate on a 1% Dexsil 300GC/Uniport HP 80/100 column (3 mm O.D. × 0.6 m, GL Science) at 260~320°C.

Spectral Studies

^1H and ^{13}C NMR spectra, including COSY, long range COSY, NOESY, HETCOR and HMBC, were taken on a JEOL GSX-270 NMR spectrometer at 270 MHz and 67.5 MHz, respectively. Chemical shifts were referenced to the solvent peak (pyridine-d_5). UV spectra were recorded on a Shimadzu UV-2100 spectrometer. IR spectra were recorded on a Perkin-Elmer model 1640 FTIR spectrometer. EI-MS and SI-MS were measured on a Hitachi M-2500 mass spectrometer.

Results

Taxonomy

Strain F-1839 showed moderate to good growth on potato glucose, corn meal and Sabouraud agars but grew poorly on Czapek-Dox agar. Optimum growth was observed at pH 6~9 and between 25 and 30°C. Colonies on potato glucose agar were thin and flat and reached 25~30 mm in diameter. The margin of the colony, which lacks phialospores, was distinct and fimbriate, while other regions of the colony turned into pale olive and were rich in phialospores. The reverse side of the colony was brown with an olive margin. Light yellowish-orange to reddish-orange diffusible pigments were produced but exudate was not formed. Colonies on corn meal agar grew to 55~60 mm in diameter and were thin and flat: the surface was white to pale olive and the reverse side was white to pale olive and turned brown in the center. Some dull yellow-diffusible pigments were produced. Colonies on Sabouraud agar reached 30~34 mm in diameter with radiating wrinkles: the surface was dull orange with a pale brown center and the reverse was dull orange with a brown center. No diffusible pigment was produced.

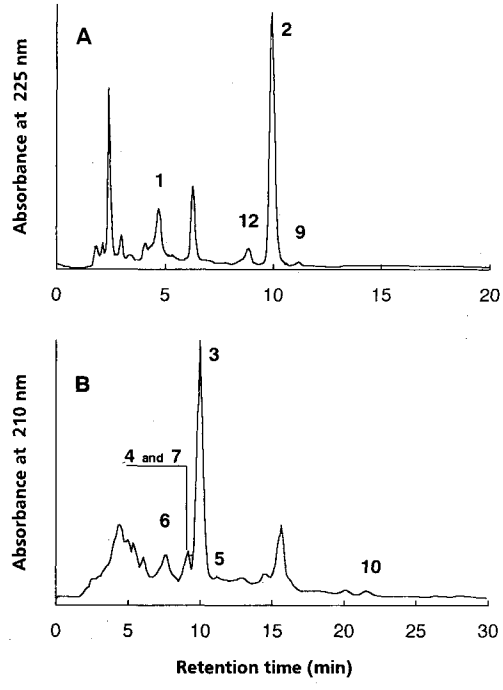
Hyphae were hyaline, septate and 3~6 μm in width and branched intricately. Phialophores arose simply from the hyphae and produced three to four septa from the foot cells. They were 50~70 μm × 2~4.5 μm in size and were erect or slightly flexuous with a slight apical swelling. At the tips of the phialophores, three to six ellipsoidal or tenpin-shaped erect phialides, 6~10 μm × 3~4 μm in size, were formed. They had a smooth surface and were pale yellowish-brown. The phialospores were subglobose or oval, 6~9 μm × 3~5 μm in size, with rough or warty surface and formed slimy masses at apices of the phialide.

These characteristics of the strain F-1839 were closely related to those of *Stachybotrys chartarum*[20], while the strain F-1839 differed from *Sta. chartarum* in that it produces diffusible pigments and lacks branched phialophores.

Fermentation and Isolation

When *Stachybotrys* sp. F-1839 was grown in Erlenmeyer flasks, inhibitory activity against cholesterol esterase appeared on day two and reached a maximum on day three. At this time, **1** and **2** as well as a small amount of **9** accumulated (Fig. 2A). After 7 days of cultivation, **3** accumulated as the major product along with minor metabolites, **4**, **5**, **6**, **7** and **10** (Fig. 2B). From

Fig. 2. The elution profiles of methylethylketone extracts of 3-day (A) and 7-day (B) cultures of *Stachybotrys* sp. F1839.



Cultured broth was extracted with an equal volume of methyl ethyl ketone, and a 10-μl aliquot of the organic extract was subjected to HPLC analyses. Analytical conditions: (A) eluent, acetonitrile-H_2O (75:25) at 1 ml/minute; detection, absorbance at 225 nm; column, Capcell pak UG120 Å (4.5 × 150 mm): (B) eluent, MeOH-H_2O (50:50) at 1 ml/minute; detection, absorbance at 210 nm; column, Inertsil ODS (4.5 × 200 mm).

Table 1. Physico-chemical properties of **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10**.

	3	**4**	**5**	**6**
Appearance	White amorphous powder	White amorphous powder	White amorphous powder	White amorphous powder
$[\alpha]_D^{25}$	−29.6° (*c* 0.1, MeOH)	—	—	—
Molecular formula	$C_{23}H_{31}NO_5$	$C_{24}H_{33}NO_6$	$C_{24}H_{33}NO_6$	$C_{23}H_{31}NO_5$
Molecular weight	401	431	431	401
High resolution MS (*m/z*)	$(M+H)^+$ (FAB)	$(M+H)^+$ (FAB)	$(M+H)^+$ (FAB)	$(M+H)^+$ (FAB)
Calcd:	402.2280	432.2386	432.2386	402.2281
Found:	402.2272	432.2381	432.2384	402.2289
UV λ_{max} nm (ε) (in MeOH)	218 (37,424), 262 (6,553), 301 (3,182)	218 (41,271), 271 (6,230), 308 (2,910)	217 (33,069), 267 (5,293), 302 (2,525)	225 (31,615), 255 (10,385), 298 (4,924)
IR λ_{max} (KBr) cm^{-1}	3405, 2960, 2938, 2875, 1686, 1625, 1466, 1389, 1348, 1332, 1259, 1087, 1045, 960, 945, 878, 772, 671	3384, 2960, 2937, 2875, 1694, 1630, 1464, 1389, 1348, 1331, 1259, 1094, 1044, 960, 945, 882, 857, 776, 681, 595	3404, 2960, 2938, 2875, 1702, 1629, 1459, 1389, 1348, 1330, 1260, 1093, 1044, 960, 944, 881, 775, 684	3356, 2958, 2939, 2867, 1655, 1629, 1458, 1314, 1243, 1093, 1044, 883

	7	**8**	**9**	**10**
Appearance	White amorphous powder	White amorphous powder	White amorphous powder	White amorphous powder
Molecular formula	$C_{25}H_{35}NO_6$	$C_{28}H_{39}NO_7$	$C_{23}H_{32}O_4$	$C_{29}H_{41}NO_6$
Molecular weight	445	501	372	499
High resolution MS (*m/z*)	$(M+H)^+$ (FAB)	M^+ (EI)	M^+ (EI)	M^+ (EI)
Calcd:	446.2542	501.2726	372.2299	499.2931
Found:	446.2250	501.2725	372.2303	499.2955
UV λ_{max} nm (ε) (in MeOH)	217 (31,462), 262 (6,408), 302 (2,314)	217 (33,069), 267 (5,293), 300 (2,525)	229 (11,384), 282 (9,412), 325 (3,683)	218 (32,041), 261 (7,357), 301 (2,323)
IR λ_{max} (KBr) cm^{-1}	3385, 2961, 2937, 2875, 1655, 1628, 1466, 1348, 1331, 1261, 1075, 1045, 945, 758	3410, 2960, 2940, 2880, 1660, 1464, 1350, 1330, 1260, 1180, 1085, 1080, 1042, 940, 775	3447, 3125, 2965, 2936, 2890, 1669, 1611, 1502, 1456, 1433, 1406, 1388, 1320, 1252, 1112, 1079, 1060, 1004, 987, 962, 843, 786, 592, 566, 520	3198, 2937, 2871, 1736, 1670, 1642, 1466, 1388, 1348, 1332, 1258, 1078, 989, 938, 903, 770, 533

2.62 liters of the 3-day cultures, 1,077, 603 and 20 mg of **1**, **2** and **9**, respectively, were isolated. Compounds **3**, **4**, **5**, **6**, **7** and **10** were isolated from 13.5 liters of a 7-day culture with yields of 962, 118, 117, 58, 117, and 132 mg, respectively. Compound **8** (5.1 mg) was isolated from 2.0 liters of a 7-day culture in Sakaguchi flasks.

Compound **1** readily generated **11** upon treatment with MeOH. Compound **2** spontaneously yielded **12** by the intramolecular Cannizzaro reaction[13].

Physico-chemical Properties and Structure Elucidation

Compounds **1** and **2** were identified as K-76[10,21] and stachybotrydial[13], respectively, by comparing physico-chemical properties of respective compounds with those of the authentic compounds.

The physico-chemical properties of compounds **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10** are summarized in Table 1. They were soluble in pyridine and dimethyl sulfoxide, moderately soluble in lower alcohols and methyl ethyl ketone, slightly soluble in EtOAc but practically insoluble in *n*-hexane and water. These compounds gave positive responses to iodine vapor, sulfuric acid, anthrone and phosphomolybdic acid. Their molecular formulae were assigned as shown in Table 1 based on their high-resolution mass spectra together with the data obtained by NMR spectral analyses.

The NMR data obtained from ^1H, ^{13}C, DEPT, ^1H-^1H COSY, NOE, HETCOR and COLOC experiments (see Tables 2 and 3 for ^1H and ^{13}C NMR data) showed that compounds **3**~**10**, like **1**, **2** and other triprenyl phenols[10,13,21~23], have substituted spiro[4-hydroxy-benzofuran-2(3*H*),1′-6′-hydroxy-2′,5′,5′,8′a-tetramethyl-decahydronaphthalene] structures. Thus, in compound **9**, for example, four methyl carbons at δ_C 15.8 (C-12), 29.1 (C-13), 22.6 (C-14) and 16.1 (C-15), a methylene carbon at δ_C 31.4 (C-11), three quaternary carbons at δ_C 38.1 (C-4), 98.4 (C-9) and 42.7 (C-10), and two partial structures, C-1–C-2–C-3 and C-5–C-6–C-7–C-8–C-12, were pieced together into a drimane skeleton. This assignment was based on COLOC experiments in which the following long-range couplings were observed: from 12-CH_3 to carbons at positions 7, 8 and 9; from both 13-H_3 and 14-CH_3 to carbons at positions 3, 4 and 5; from 15-CH_3 to carbons at positions 1, 5, 9 and 10; from

Table 2. ^{1}H NMR spectral data for **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10**.

Proton	3	4	5	6
1	1.76 (1H, dd, 12.0, 4.0)[1)]	1.76 (1H, dd, 12.2, 4.2)	1.76 (1H, dd, 12.0, 4.2)	1.78 (1H, dd, 12.2, 4.0)
	2.38 (1H, t, 12.0)	2.42 (1H, t, 12.2)	2.38 (1H, t, 12.0)	2.42 (1H, t, 12.2)
2	4.34 (1H, ddd, 12.0, 4.0, 2.4)	4.32 (1H, ddd, 12.2, 4.2, 2.4)	4.30 (1H, ddd, 12.0, 4.2, 2.4)	4.32 (1H, ddd, 12.2, 4.0, 2.8)
3	3.79 (1H, d, 2.4)	3.73 (1H, d, 2.4)	3.70 (1H, d, 2.4)	3.78 (1H, d, 2.8)
5	2.48 (1H, dd, 12.6, 1.8)	2.54 (1H, dd, 12.6, 2.3)	2.51 (1H, dd, 12.6, 2.6)	2.69 (1H, dd, 12.6, 2.2)
6	1.42 (1H, m*)	1.38 (1H, m*)	1.38 (1H, m*)	1.37 (1H, m*)
	1.68 (1H, m*)	1.57 (1H, m*)	1.55 (1H, m*)	1.62 (1H, m*)
7	1.59 (2H, m*)	1.54 (1H, m*)	1.55 (1H, m*)	1.50 (1H, m*)
		1.68 (1H, m*)	1.67 (1H, m*)	1.83 (1H, m*)
8	1.76 (1H, m*)	1.76 (1H, m*)	1.77 (1H, m*)	1.71 (1H, m*)
11	3.13 (1H, d, 16.6)	3.11 (1H, d, 17.2)	3.11 (1H, d, 16.3)	3.02 (1H, d, 16.1)
	3.60 (1H, d, 16.6)	3.59 (1H, d, 17.2)	3.57 (1H, d, 16.3)	3.51 (1H, d, 16.1)
12	0.84 (3H, d, 6.0)	0.89 (3H, d, 6.0)	0.88 (3H, d, 6.1)	0.84 (3H, d, 6.3)
13	1.22 (3H, s)	1.20 (3H, s)	1.20 (3H, s)	1.30 (3H, s)
14	0.91 (3H, s)	0.91 (3H, s)	0.90 (3H, s)	0.91 (3H, s)
15	1.03 (3H, s)	1.03 (3H, s)	1.02 (3H, s)	1.03 (3H, s)
3′	7.54 (1H, s)	7.30 (1H, s)	7.32 (1H, s)	6.73 (1H, s)
7′				4.24 (2H, s)
8′	4.14 (1H, d, 17.0)	6.02 (1H, d, 1.2)	6.24 (1H, d, 1.8)	
	4.24 (1H, d, 17.0)			
8′-OCH_3		3.32 (3H, s)	3.54 (3H, s)	
NH	9.22 (1H, br s)	9.61 (1H, br s)	9.55 (1H, br s)	8.58 (1H, br s)

Proton	7	8	9	10
1	1.76 (1H, dd, 12.6, 4.3)	1.26 (1H, m*)	1.17 (1H, tdd, 13.0, 3.7)	1.20 (1H, dt, 12.2, 3.3)
	2.39 (1H, t, 12.6)	1.65 (1H, br t, 11.8)	2.40 (1H, td, 13.0, 3.7)	2.36 (1H, m*)
2	4.33 (1H, ddd, 12.6, 4.3, 2.0)	3.90 (1H, br d, 11.8)	1.88 (1H, m*)	1.77～2.07 (2H, m*)
			2.01 (1H, tdd, 13.0, 3.7, 2.2)	
3	3.74 (1H, d, 2.0)	3.33 (1H, br s)	3.64 (1H, d, 2.2)	3.63 (1H, m*)
5	2.54 (1H, dd, 12.9, 2.4)	1.98 (1H, br d, 12.2)	2.62 (1H, dd, 12.7, 2.2)	2.54 (1H, dd, 12.9, 2.1)
6	1.41 (1H, m*)	1.30 (1H, m*)	1.46 (1H, m*)	1.44 (1H, m*)
	1.67 (1H, m*)	1.43 (1H, m*)	1.69 (1H, m*)	1.71 (1H, m*)
7	1.59 (2H, m*)	1.26 (2H, m*)	1.63 (2H, m*)	1.62 (1H, m*)
				2.00 (1H, m*)
8	1.74 (1H, m*)	1.67 (1H, m*)	1.88 (1H, m*)	2.00 (1H, m*)
11	3.11 (1H, d, 17.0)	2.76 (1H, d, 16.4)	3.02 (1H, d, 12.5)	2.92 (1H, d, 17.1)
	3.60 (1H, d, 17.0)	3.16 (1H, d, 16.4)	3.42 (1H, d, 12.5)	3.58 (1H, d, 17.1)
12	0.83 (3H, d, 6.0)	0.62 (3H, d, 6.2)	0.87 (3H, d, 6.1)	0.92 (3H, d, 5.4)
13	1.25 (3H, s)	0.98 (3H, s)	1.23 (3H, s)	1.28 (3H, s)
14	0.93 (3H, s)	0.78 (3H, s)	0.93 (3H, s)	0.95 (3H, s)
15	1.04 (3H, s)	0.89 (3H, s)	1.00 (3H, s)	1.04 (3H, s)
3′	7.29 (1H, s)	6.82 (1H, s)	6.58 (1H, s)	7.40 (1H, s)
7′			2.69 (3H, s)	
8′	4.15 (1H, d, 16.5)	4.17 (1H, d, 17.0)	10.88 (1H, s)	3.84 (1H, d, 16.6)
	4.39 (1H, d, 16.5)	4.20 (1H, d, 17.0)		4.08 (1H, d, 16.6)
9′	3.69 (1H, m*)	3.53 (2H, m*)		3.34 (1H, m*)
	3.90 (1H, m*)			3.58 (1H, m*)
10′	3.95 (2H, m*)	1.89 (2H, m*)		1.45 (2H, m*)
11′		2.29 (2H, t, 7.4)		1.58 (2H, m*)
12′				2.35 (2H, m*)
13′		3.53 (3H, s)		
14′				3.61 (3H, s)

^{1}H NMR spectra (270 MHz) were measured at 35°C in pyridine-d_5 except for **8** which was measured in $CDCl_3$. [1)] Proton number, multiplicity and coupling constants in Hz are indicated in parentheses. m*, overlapping multiplet.

11-H_2 to carbons at positions 8, 9 and 10. The signals at δ_H 6.58 (3′-H) and at δ_C 168.9 (C-6′), 159.9 (C-2′), 141.8 (C-4′), 111.8 (C-3′), 111.6 (C-1′) and 111.2 (C-5′) were assigned to the penta-substituted aromatic ring. The ^{1}H-^{13}C *meta* couplings were observed between the proton at δ_H 6.58 and the carbons at δ_C 111.6 and 111.2. The signals of C-1′, C-2′ and C-6′ were further long-range coupled to the *geminal*-coupled methylene signals at δ_H 3.02 and 3.42 (11-H_2), which were in turn long-range coupled to C-8, C-9 and C-10.

The NMR results suggested that **9** differs from **2** only in that **9** has, at position 4′, a methyl substituent in place

Table 3. ^{13}C NMR spectral data for **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10**.

Carbon	3	4	5	6	7	8	9	10
1	33.5 (t)	33.6 (t)	33.6 (t)	33.9 (t)	33.9 (t)	33.3 (t)	24.7 (t)	24.8 (t)
2	66.1 (d)	66.2 (d)	66.1 (d)	66.3 (d)	66.3 (d)	67.1 (d)	25.9 (t)	26.1 (t)
3	78.7 (d)	78.8 (d)	78.7 (d)	79.2 (d)	79.1 (d)	79.1 (d)	74.6 (d)	74.9 (d)
4	38.5 (s)	38.6 (s)	38.4 (s)	38.7 (s)	38.7 (s)	38.7 (s)	38.1 (s)	38.2 (s)
5	39.6 (d)	39.7 (d)	39.5 (d)	39.6 (d)	39.9 (d)	39.6 (d)	40.4 (d)	40.5 (d)
6	20.8 (t)	20.9 (t)	20.9 (t)	21.1 (t)	21.2 (t)	21.1 (t)	21.3 (t)	21.3 (t)
7	31.2 (t)	31.3 (t)	31.1 (t)	30.4 (t)	31.6 (t)	31.4 (t)	31.5 (t)	31.7 (t)
8	36.8 (d)	36.9 (d)	36.7 (d)	37.3 (d)	37.1 (d)	37.1 (d)	37.2 (d)	37.4 (d)
9	98.2 (s)	98.7 (s)	98.6 (s)	99.1 (s)	98.5 (s)	98.5 (s)	98.4 (s)	98.8 (s)
10	43.5 (s)	43.7 (s)	43.6 (s)	43.5 (s)	43.9 (s)	43.9 (s)	42.7 (s)	42.8 (s)
11	32.7 (t)	32.4 (t)	32.4 (t)	32.0 (t)	33.0 (t)	32.5 (t)	31.4 (t)	32.8 (t)
12	15.5 (q)	15.6 (q)	15.5 (q)	15.6 (q)	15.8 (q)	15.9 (q)	15.8 (q)	15.9 (q)
13	29.1 (q)	29.1 (q)	29.0 (q)	29.0 (q)	29.4 (q)	29.0 (q)	29.1 (q)	29.1 (q)
14	22.1 (q)	22.2 (q)	22.1 (q)	22.4 (q)	22.4 (q)	22.4 (q)	22.6 (q)	22.7 (q)
15	16.8 (q)	16.9 (q)	16.9 (q)	17.1 (q)	17.2 (q)	17.3 (q)	16.1 (q)	16.2 (q)
1′	117.5 (s)	118.3 (s)	118.3 (s)	113.5 (s)	117.5 (s)	117.9 (s)	111.6 (s)	117.7 (s)
2′	155.1 (s)	156.5 (s)	156.5 (s)	158.3 (s)	155.4 (s)	153.9 (s)	159.9 (s)	155.5 (s)
3′	101.9 (d)	102.4 (d)	102.6 (d)	102.3 (d)	101.9 (d)	102.8 (d)	111.8 (d)	101.9 (d)
4′	135.0 (s)	135.8 (s)	135.5 (s)	147.6 (s)	135.9 (s)	134.2 (s)	141.8 (s)	135.5 (s)
5′	115.1 (s)	114.3 (s)	114.3 (s)	106.7 (s)	113.2 (s)	113.3 (s)	111.2 (s)	112.8 (s)
6′	156.7 (s)	157.7 (s)	157.6 (s)	159.4 (s)	156.7 (s)	156.4 (s)	168.9 (s)	157.0 (s)
7′	172.2 (s)	170.9 (s)	170.7 (s)	45.7 (t)	169.1 (s)	170.0 (s)	21.8 (q)	168.7 (s)
8′	42.9 (t)	83.4 (d)	83.6 (d)	171.2 (s)	48.5 (t)	48.2 (t)	187.8 (d)	47.2 (t)
8′-OCH_3		51.4 (q)	51.9 (q)					
9′					46.0 (t)	42.5 (t)		42.0 (t)
10′					60.5 (t)	24.2 (t)		27.9 (t)
11′						31.8 (t)		22.4 (t)
12′						173.9 (s)		33.5 (t)
13′						52.1 (q)		173.5 (s)
14′								51.2 (q)

^{13}C NMR spectra (67.5 MHz) were measured at 35°C in pyridine-d_5 except for **8** which was measured in $CDCl_3$. Multiplicities are given in parentheses.

of an aldehyde substituent. The methyl and aldehyde substituents at C-4′ and C-5′, respectively, were confirmed by the following long-range couplings: from 7′-CH_3 (δ_H 2.96) to carbons C-3′, C-4′ and C-5′; from 8′-CHO (δ_H 10.88) to carbons C-4′, C-5′ and C-6′.

Compound **10** was characterized by lack of aromatic aldehyde functions that are present in **2**, while it has the following additional signals in the NMR spectra: an *O*-methyl, five methylenes and two carbonyl carbons. In the COLOC spectrum, both the *O*-methyl signal at δ_H 3.61 and the methylene signal (δ_H 2.35) at the terminus of the spin system, –CH_2–CH_2–CH_2–CH_2–, are coupled to the carbonyl carbon at δ_C 173.5 (C-13′). The *geminal*-coupled methylene signals at δ_H 3.34 and 3.58 (9′-H_2) are coupled to carbons at C-7′ and C-8′. Isolated *geminal*-coupled methylene signals at δ_H 3.84 and 4.08 (8′-H_2) are coupled to C-4′, C-5′, C-6′ and C-7′, and H-3′ (δ_H 7.40) is coupled to an amide carbonyl (δ_C 168.7). Thus, it is proposed that **9** has a structure shown in Fig. 1.

The physico-chemical properties of compounds **3**, **4**, **5**, **6** and **7** were very similar to each other. The NMR results showed that these metabolites, like compound **1**, have a hydroxyl substitution at position 2 in the drimane moiety. In the IR spectrum of **3**, the aromatic aldehyde absorption was missing but a band at 1686 cm^{-1}, which is consistent with the presence of α,β-unsaturated γ-lactam, was observed. In addition, a carbonyl signal at δ_C 172.2 and *geminal*-coupled methylene signals (δ_H 4.14 and 4.24; δ_C 42.9), which were coupled to an additional amide proton signal at δ_H 9.22 were observed in the NMR spectra of **3**. The long-range correlations between H-3′ and the carbonyl carbon and between the methylene signals (δ_H 4.14 and 4.24) and carbons C-4′, C-5′ and C-6′ revealed the position of the carbonyl carbon at 7′.

Compound **6**, which has an identical molecular formula with that of **3**, is a regioisomer of **3**. The ^{13}C NMR data for **6** were nearly identical with those of **3** except for slight upfield and downfield shifts of C-4′ and C-5′, respectively. The long-range correlations between the methylene protons at δ_H 4.24 and carbons C-3′ and C-4′ showed that the methylene carbon at δ_C 45.7 of the γ-lactam ring was connected to C-4′.

Compound **4** differs from **3** in that **4** possesses additional methoxy signals (δ_H 3.32 and δ_C 51.4) in the

NMR spectra and has a molecular weight higher than that of **3** by 30 mass units. An apparent down-field resonance of the oxymethine proton (δ_H 6.02) at position 8′ was indicative of a methoxy substitution at this position.

That compound **5** has an identical structural formula with that of **4** was established by a combination of spectroscopic analyses. Since NOE studies revealed that stereochemistry of the drimane moiety in **5** is identical with that in **4** (see below), **5** should be an epimer of **4** at C-8′. ^{1}H NMR results, which showed apparent differences between **4** and **5** in the chemical shifts of the methoxy and the oxymethine signals at position 8′, were consistent with this conclusion.

Compound **7** differs from **3** in that **7** lacks amide proton that is present in **3** but possesses an additional spin system, $-CH_2-CH_2-$, and has a molecular weight that is higher than that of **3** by 44 mass units. One of the two additional methylenes is shifted to δ_H 3.95 and δ_C 60.5, suggesting that it bears oxygen, and the other (δ_H 3.69 and 3.90; δ_C 46.0) is long-range coupled with the methylene at position 8′. Thus, a hydroxyethyl should be attached to the nitrogen atom of the γ-lactam ring in **7**.

Compound **8** differs from **7** in that **8** lacks a hydroxy but possesses an additional methylene signal and a methoxycarbonyl signal in the NMR spectra. Both the methylene proton (δ_H 2.29) at the terminus of a spin system $-CH_2-CH_2-CH_2-$ and the *O*-methyl proton (δ_H 3.53) are coupled to a carbonyl carbon at δ_C 173.9 (C-12′). In the long-range ^{1}H-^{1}H COSY spectrum, a coupling between 8′-H_2 and 9′-H_2 was observed. Accordingly, the structure of **8** was deduced as shown in Fig. 1.

The relative stereochemistry of compounds **3**～**10** was established by differential NOE experiments. Representative results for **9** are shown in Fig. 3. C-15 and 5-H must be *trans-diaxial* in view of a large coupling constant (J=12.7 Hz) of 5-H and the strong NOE from 15-H_3 to 2-H (δ_H 2.01), 6-H (δ_H 1.69), 8-H and 14-H_3. The NOE from 15-H_3 to 11-H (δ_H 3.42) was also critical in establishing the relative stereochemistry of the *spiro* center. The NOE data also allowed stereo-specific proton assignments of *geminal* proton pairs for 1-H_2, 2-H_2, 6-H_2, 7-H_2 and 11-H_2 and the *geminal* methyl groups (13-H_3 and 14-H_3). Similar results were obtained with other congeners. All of the observed nuclear Overhauser enhancements were consistent with the proposed structures for these compounds.

From these findings, the structures of compounds **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10** (designated F1839-A, -B, -C, -D, -E, -F, -I and -J, respectively) were determined as depicted in Fig. 1.

Inhibition of Cholesterol Esterase

The inhibitory activities of **1**～**12** against cholesterol esterase are summarized in Table 4. Compound **2** was the most potent; inhibition was 50% at 60 μM. Compounds **1** and **9** showed moderate activity, giving 50% inhibition at 200 and 270 μM, respectively. Other compounds were far less inhibitory than these compounds, giving 50% inhibition at >1 mM. Compounds **1**～**12** did not affect 3-hydroxy-3-methylglutaryl-CoA reductase and acyl-CoA : cholesterol acyltransferase (ACAT) activities at concentrations up to 1 mM (data not shown).

Inhibition of cholesterol esterase by **2** was time-dependent and irreversible. Thus, when cholesterol esterase that had been preincubated with 150～300 μM of **2** for 20 minutes was assayed before and after ultrafiltration, enzyme activity, which was inhibited by 38～64% before ultrafiltration, did not recover significantly after reducing the concentration of **2** by ultrafiltration (Table 5). As shown in Fig. 4, the kinetics of inactivation of cholesterol esterase by **2** is biphasic, comprising an initial

Fig. 3. NOE data for **9** obtained in pyridine-d_5.

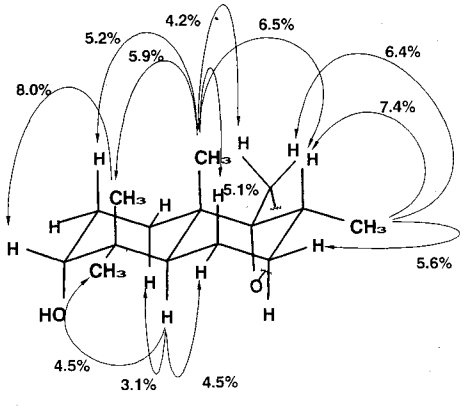


Table 4. Inhibition of pancreatic cholesterol esterase by **2** and related triprenyl phenols.

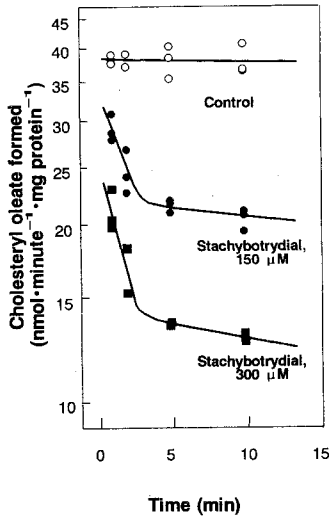
Compound	IC_{50} (mM)
K-76 (**1**)	0.20
Stachybotrydial (**2**)	0.06
F1839-A (**3**)	2.9
F1839-B (**4**)	33
F1839-C (**5**)	110
F1839-D (**6**)	5.4
F1839-E (**7**)	2.9
F1839-F (**8**)	4.9
F1839-I (**9**)	0.27
F1839-J (**10**)	2.6
Dimethyl acetal **11**	1.1
Lactone **12**	1.3

Table 5. Irreversible inhibition of pancreatic cholesterol esterase by **2**.

2 (μM)		Enzyme activity (nmol·minute^{-1}·mg protein^{-1})	Inhibition (%)
at preincubation	at enzyme assay		
Before ultrafiltration			
0	0	48.9	0
150	75	30.3	38
300	150	17.2	64
After ultrafiltration			
0	0	44.9	0
150	16	30.6	32
300	32	20.4	55

Cholesterol esterase (2.4 mg/ml) was preincubated at 37°C for 20 minutes in the presence of the indicated concentrations of **2**. Subsequently, a portion of the mixture was removed and assayed for cholesterol esterase activity. The remaining portion was subjected to ultrafiltration at 4°C using Centricut U-10 (Kurabo, Japan), which traps substances with approximate *M*r of >10,000. Unfiltrated materials were suspended in 0.154 M potassium phosphate, pH 5.2, and assayed for enzyme activity. Each value represents the mean of triplicate determinations.

Fig. 4. Time-dependent inactivation of pancreatic cholesterol esterase by **2**.



Cholesterol esterase (2.4 mg/ml) was preincubated in the absence (○) or presence of 150 μM (●) and 300 μM (■) of **2** at 37°C in 0.154 M potassium phosphate, pH 5.2.

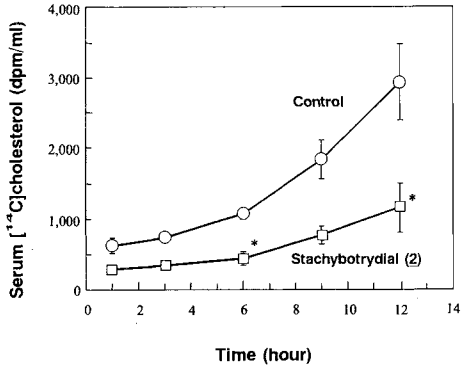
After the intervals indicated, aliquots of the mixture were removed and assayed for enzyme activity.

rapid phase and a following slow one.

Effect of **2** on the [^{14}C]Cholesterol Uptake in Rats

The effect of **2** on cholesterol absorption was examined by determining the incorporation of orally administered [^{14}C]cholesterol into blood in normal-fed rats. When rats were given 100 mg/kg of **2** and [^{14}C]cholesterol simultaneously, the level of [^{14}C]cholesterol in the serum was greatly reduced as compared with that in rats given [^{14}C]cholesterol alone (Fig. 5). The reduction was 50~60% after 1~12 hours.

Fig. 5. Effect of **2** on the [^{14}C]cholesterol absorption in rats.



Male Wistar rats (180~190 g) grown on a normal chow (CE-2 containing 4% fat, Crea Japan) were fasted for 16~18 hours. A dose of 100 mg/kg of **2** (□) was administered orally as a suspension in 0.5% carboxymethylcellulose (CMC). Control animals (○) received an equal volume of CMC. Immediately after the administration, rats were given orally 6 mg of [^{14}C]cholesterol (2 μCi) in 1 ml of a suspension containing 7.8% triolein and 0.38% cholic acid. Blood was collected from the external jugular vein after various intervals to measure radioactivity. Each point is the mean ± SEM from four rats. *$p<0.05$.

Effect of **2** on the Serum Cholesterol Levels in Cholesterol-fed Mice

Dietary supplementation with **2** resulted in a marked reduction of the serum cholesterol level in mice fed a diet containing 1% cholesterol. Thus, when mice were fed with cholesterol for 14 days, the serum total cholesterol level reached to 270~280 mg/dl. Dietary supplemented **2** at 0.025 and 0.1% reduced the level of serum cholesterol by 7 and 20%, respectively (Table 6). The reduction of cholesterol was prominent in fractions other than high density lipoprotein (HDL), and little or no change in HDL cholesterol level was observed. This hypocholesterolemic effect of **2** was comparable with that of dietary cholestyramine at 1.0%. Compound **2** had no effect on the serum triglyceride level under these conditions (data not shown).

Discussion

In the present studies we have isolated ten triprenyl phenol metabolites, **1** and **2** as well as novel related compounds **3**, **4**, **5**, **6**, **7**, **8**, **9**, and **10**, from cultures of *Stachybotrys* sp. F-1839 as inhibitors of cholesterol esterase. Of these compounds, **1**, **2** and **9** showed relatively potent activity, whereas others, which lack

Table 6. Effect of **2** on the serum cholesterol level in cholesterol-fed mice.

Group	Number of animals	Body weight (g)		Serum cholesterol level (mg/dl)		
		0 day	14 day	Total	Non-HDL cholesterol	HDL cholesterol
Exp. 1						
1% Cholesterol diet	8	22.7±0.3	29.9±0.6	272±20 (100)	167±24 (100)	105±5 (100)
1% Cholesterol diet+0.1% **2**	8	22.8±0.3	29.6±1.0	215±15* (79.0)	114±13 (68.2)	101±5 (96.2)
1% Cholesterol diet +1% cholestyramine	8	22.6±0.9	30.0±0.9	201±14* (73.9)	95±17* (56.9)	105±5 (100)
Exp. 2						
1% Cholesterol diet	8	23.0±1.0	30.7±1.4	284±18 (100)	196±22 (100)	89±8 (100)
1% Cholesterol diet+0.1% **2**	8	23.1±1.0	30.2±1.6	226±30 (79.6)	149±35 (76.0)	77±6 (86.5)
1% Cholesterol diet+0.025% **2**	7	23.1±0.9	31.1±1.3	264±19 (93.0)	170±21 (86.7)	93±8 (104.5)

Male ICR mice (21~25 g) were allowed to access freely to water and the diet (AIN diet #76 (Oriental Yeast, Japan) supplemented with 1% cholesterol and 0.5% cholic acid) for 14 days. Where indicated, the diet contained **2** or chlestyramine. Blood was collected by cardiac puncture and total and HDL cholesterol concentrations in serum were determined using Cholesterol Test C Wako and HDL-Cholesterol Test Wako (Wako Chemicals, Japan), respectively. Non-HDL cholesterol concentration was calculated by subtracting the HDL cholesterol value from the total cholesterol value. *$p<0.05$.

aromatic aldehyde groups, were far less inhibitory. In addition, **9**, which lacks one of the aldehyde function of **2** but has a methyl substituent, was 4.5-fold less inhibitory than **2**. These results suggest an important role of the aromatic aldehyde functions of these compounds in the inhibition of cholesterol esterase. Compound **1**, which possesses a hydroxy substitution at position 2 of the drimane moiety of **2**, showed 30% of the activity of **2**, suggesting another structural requirement for potent inhibition. Compound **2** irreversibly inactivated cholesterol esterase. Aldehydes react with primary amines such as ε-amino group of lysine and terminal α-amino group of proteins[24]. It is, however, unclear whether **2** reacts with such residue(s) of cholesterol esterase.

Compound **2** inhibited the [^{14}C]cholesterol absorption in normal-fed rats and reduced the serum cholesterol level in cholesterol-fed mice, suggesting that the hypocholesterolemic effect of **2** is associated with the inhibition of intestinal absorption of cholesterol. It has been suggested that cholesterol esterase and ACAT[25] in the intestinal mucosa are important regulators of cholesterol absorption[26]. Since **2** did not affect ACAT activity, it is suggested that the inhibition of intestinal cholesterol absorption by **2** is due to the inhibition of cholesterol esterase.

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