Isochromophilones III ~ VI, Inhibitors of Acyl-CoA : Cholesterol Acyltransferase Produced by *Penicillium multicolor* FO-3216

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New azaphilones named isochromophilones III ~ VI were isolated from the culture broth of *Penicillium multicolor* FO-3216 as inhibitors of acyl-CoA : cholesterol acyltransferase (ACAT). Their structures were elucidated by NMR and other spectroscopic analyses. The IC_{50} values of isochromophilones III, IV, V and VI for ACAT activity in an enzyme assay using rat liver microsomes were calculated to be 110, 50, 50 and 120 μM, respectively.

Acyl-CoA : cholesterol acyltransferase (ACAT) is believed to be one of the promising inhibition sites for the treatment of atherosclerosis and hypercholesterolemia.[1~3] In the course of the screening for ACAT inhibitors of microbial origin,[4~7] we found new ACAT inhibitors isochromophilones III ~ VI (**3**~**6**, Fig. 1) produced by a fungal strain FO-3216. They are classified in an azaphilone group like isochromophilones I (**1**), II (**2**)[8] and sclerotiorin (**7**).[9~11] In this paper, taxonomy of the producing strain and fermentation, isolation, structure elucidation and biological properties of **3**~**6** are described.

Results

Taxonomy of Producing Organism

Strain FO-3216 was originally isolated from a soil sample collected at Shirokane, Minato-ku, Tokyo, Japan. For the identification of the fungus, Czapek yeast extract agar (CYA), malt extract agar, 25% glycerol nitrate agar and potato-dextrose agar were used. This strain grew rapidly to form mistletoe gray to dark covert gray colonies with diameter of 50 ~ 70 mm after incubation for 14 days at 25°C. Reverse of the colonies was dark orange to grayish red brown. Yellowish orange soluble pigment was produced on CYA colonies.

Morphological observations was done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (Hitachi S-430). When the strain FO-3216

Fig. 1. The structure of isochromophilones.

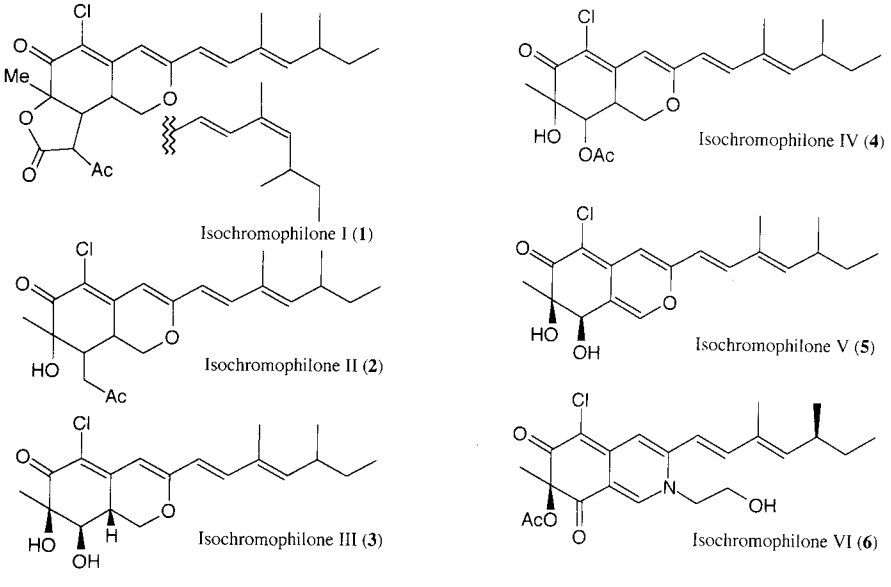


Table 1. Physico-chemical properities of **3**~**6**.

	3	**4**	**5**	**6**
Appearance	Yellow powder	Yellow powder	Yellow powder	Red powder
FAB-MS *m/z* (M+H)	353	395	351	434
HR FAB-MS *m/z* (found)	375.1333 $(M+Na)^+$	395.1611 $(M+H)^+$	351.1384 $(M+H)^+$	434.1744 $(M+H)^+$
m/z (calcd)	375.1350	395.1646	351.1363	434.1734
Molecular formula	$C_{19}H_{25}O_4Cl$	$C_{21}H_{27}O_5Cl$	$C_{19}H_{23}O_4Cl$	$C_{23}H_{28}NO_5Cl$
Optical rotation $[\alpha]_D$ (MeOH)	+150° (*c* 1.07)	−341° (*c* 0.20)	−108° (*c* 0.19)	+2170° (*c* 0.10)
Melting point	84~85°C	83~85°C	58~61°C	122~125°C
UV λ_{max}^{MeOH} nm (ε)	205 (8500), 215 (7050,sh), 264 (4800), 386 (21500)	205 (11800), 267 (7900), 390 (39400)	248 (7000), 314 (4000,sh), 322 (4300,sh), 340 (4000,sh), 355 (7700), 390 (9800,sh), 407 (10200), 430 (7500,sh), 464 (2700,sh)	235 (15600), 345 (22500,sh), 370 (23400), 485 (3900)
IR ν_{max} (KBr) cm^{-1}	3250, 2960, 2920, 2870, 1670, 1610, 1560, 1460, 1265.	3430, 2960, 2930, 1870, 1750, 1670, 1610, 1560, 1270	3420, 2960, 2925, 2850, 1760, 1630, 1560, 1530, 1460, 1210.	2960, 2870, 1730, 1700, 1640, 1595, 1535, 1500, 1370, 1340.

Fig. 3. UV spectra of **3**~**6**.

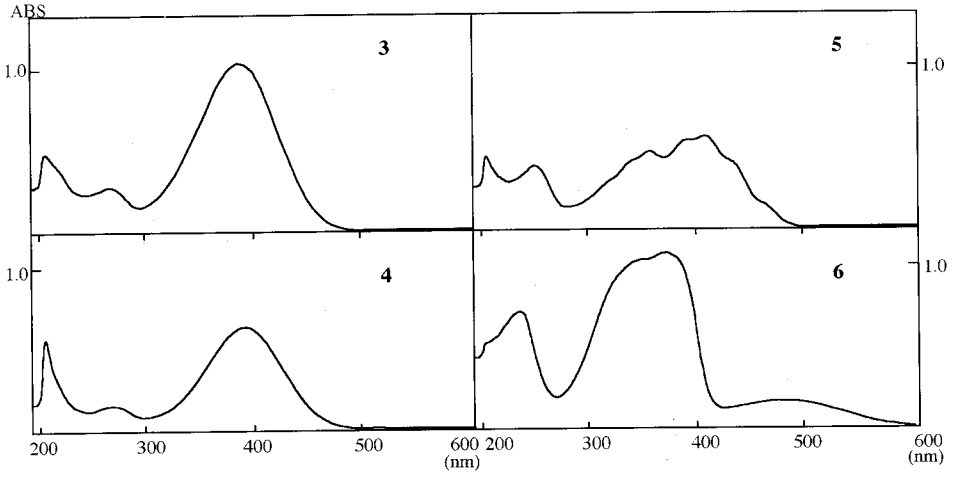
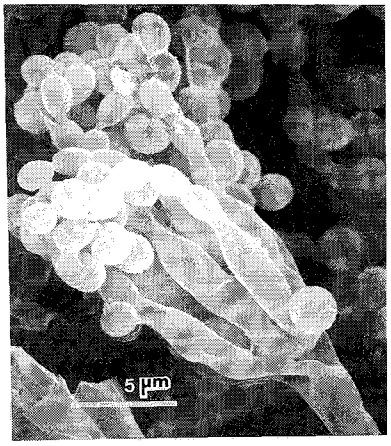


Table 2. ^1H NMR spectral data of **3**~**6**.

No.	**3**	**4**	**5**	**6**
1α	3.82 dd (1H, *J*=11.0,14.0)	3.83 dd (1H, *J*=10.0,14.0)	7.38 s (1H)	7.86 s (1H)
1β	4.85 dd (1H, *J*=5.0,11.0)	4.36 dd (1H, *J*=5.0,10.0)		
4	6.10 s (1H)	6.12 s (1H)	6.53 s (1H)	7.02 s (1H)
8	3.46 d (1H, *J*=10.0)	5.01 d (1H, *J*=10.0)	4.36 s (1H)	
8a	3.12 ddd (1H, *J*=5.0,10.0,14.0)	3.45 ddd (1H, *J*=5.0,10.0,14.0)		
9	5.99 d (1H, *J*=16.0)	5.99 d (1H, *J*=16.0)	6.06 d (1H, *J*=16.0)	6.25 d (1H, *J*=16.0)
10	7.01 d (1H, *J*=16.0)	6.96 d (1H, *J*=16.0)	7.03 d (1H, *J*=16.0)	6.93 d (1H, *J*=16.0)
12	5.62 br.d (1H, *J*=11.0)	5.60 d (1H, *J*=10.0)	5.66 br.d (1H, *J*=10.0)	5.70 br.d (1H, *J*=10.0)
13	2.46 ddd (1H, *J*= 8.0,11.0,15.0)	2.46 m (1H)	2.46 ddd (1H, *J*= 8.0,8.0,15.0)	2.47 m (1H)
14	1.20~1.40 m (2H)	1.20~1.40 m (2H)	1.20~1.40 m (2H)	1.20~1.40 m (2H)
15	0.85 t (3H, *J*=8.0)	0.85 t (3H, *J*=7.0)	0.86 t (3H, *J*=8.0)	0.88 t (3H, *J*=10.0)
16	0.99 d (3H, *J*=8.0)	0.99 d (3H, *J*=7.0)	1.01 d (3H, *J*=7.0)	1.02 d (3H, *J*=7.0)
17	1.81 d (3H, *J*=1.0)	1.81 d (3H, *J*=1.0)	1.83 s (3H)	1.84 d (3H, *J*=1.0)
7-Me	1.55 s (3H)	1.58 s (3H)	1.30 s (3H)	1.55 s (3H)
7-Ac				2.16 s (3H)
8-Ac		1.42 s (3H)		
1'				4.02 dd (2H, *J*=5.0,5.0)
2'				3.92 dd (2H, *J*=5.0,5.0)

Fig. 2. Scanning electron micrograph of penicillia of strain FO-3216 on potato-dextrose agar.

Scale: 5 μm.



was grown on potato-dextrose agar at 25°C for 7 days, the conidiophores born from substrate hyphae, and penicillia were mainly monoverticillate as shown in Fig. 2. The phialides were $7.5\sim10\times2\sim3$ μm. The conidia were globe to subglobe and $2.5\sim3$ μm in diameter and its surface was smooth.

From the above characteristics, strain FO-3216 was identified as the *Penicillium multicolor*,[12] and named *Penicillium multicolor* FO-3216. This strain was deposited in the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan, as FERM P-14152.

Fermentation

A slant culture of strain FO-3216 grown on YpSs agar (soluble starch 1.5%, yeast extract 0.4%, $MgSO_4\cdot7H_2O$ 0.05%, KH_2PO_4 0.1% and agar 2.0%, pH 6.0) was inoculated into two 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4\cdot7H_2O$ 0.05%, polypeptone 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). Two hundred ml of the seed culture was transferred in a 30-liter jar fermenter, containing 20 liters of a production medium (soluble starch 3.0%, glycerol 1.0%, soybean meal 2.0%, dry yeast 0.3%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4\cdot7H_2O$ 0.05% and KH_2PO_4 0.05%). The fermentation was carried out at 27°C. This three-day cultured broth was used for the isolation of **3**~**5**.

For production of **6**, 1 ml of the same seed culture was transferred into each of five 500-ml Erlenmeyer flasks containing 100 ml of the production medium. The flasks were then shaken on a rotary shaker at 27°C. This three-day cultured broth was used as a starting material.

Isolation

The 20 liter broth of the jar fermentation was centrifuged to obtain the mycelium, which was extracted with 10 liters of acetone. After filtration, the acetone extract was concentrated to remove acetone, and the remaining aqueous solution was extracted with 3 liters of ethyl acetate. The extract was concentrated *in vacuo* to dryness to yield a dark red powder (35 g). A portion of the powder (10 g) was dissolved in $CHCl_3$ and applied on a silica gel column (800 g, i.d. 4.0×34 cm). After washing with 1 liter of $CHCl_3$, the active substances were eluted with 1 liter of $CHCl_3$-methanol (100:7). The active fraction was concentrated *in vacuo* to give a pale orange powder (3.7 g). The powder was further chromatographed on a silica gel column (150 g, i.d. 3.0×12 cm). After washing with toluene-acetone (100:5), the fraction containing **4** was eluted with toluene-acetone (100:7), and the mixture of **3** and **5** was eluted with toluene-acetone (100:18). Final purification of isochromophilones were carried out by preparative TLC developed with toluene-acetone (5:1). Active bands was collected and extracted with $CHCl_3$-methanol (9:1). The extracts were concentrated *in vacuo* to yield pure **3** (25 mg), **4** (5 mg) and **5** (10 mg) as yellow powders.

For isolation of **6**, the broth of the flask culture was also centrifuged to obtain the mycelia, which was extracted in the same manner as the mycelia of the jar fermentation. The extract was concentrated *in vacuo* to give a red powder (500 mg) and then it was chromatographed on a silica gel column (60 g, i.d. 2.0×10 cm). After washing with toluene, **6** was eluted with toluene-acetone (100:5). It was further purified by preparative TLC to yield a red powder of **6** (5 mg).

Structure Elucidation of **3**~**6**

The physico-chemical properties of **3**~**6** are summarized in Table 1. The UV spectra of **3**~**6** are shown in Fig. 3. The chemical shifts of ^1H and ^{13}C NMR of **3**~**6** are shown in Tables 2 and 3, respectively.

Isochromophilone V (5)

The molecular formula of **5** was established as $C_{19}H_{23}O_4Cl$ (MW 350) by the analysis of HR FAB-MS; $(M+H)^+$ *m/z* 351.1371 (calcd for $C_{19}H_{24}O_4Cl$ 351.1347). The ^1H-^1H COSY experiment revealed two proton sequences, $-CH=CH-$ and $=CH-CH(CH_3)-CH_2-CH_3$. From HMBC experiments, the two sequences were combined to form a 3,5-dimethyl-1,3-heptadienyl residue and the residue was further connected to 7,8-dihydro-6*H*-2-benzopyran ring as shown in Fig. 4. Bonding of a

Table 3. ^{13}C NMR spectral data of **3**~**6**.

No.	3	4	5	6
1	68.7 t	67.8 t	146.5 d	141.9 d
3	163.9 s	163.3 s	158.0 s	144.8 s
4	101.5 d	101.5 d	105.6 d	111.8 d
4a	146.2 s	145.6 s	140.5 s	100.6 s
5	118.1 s	118.5 s	106.0 s	148.5 s
6	188.8 s	186.9 s	191.1 s	184.4 s
7	74.9 s	75.0 s	76.2 s	84.9 s
8	73.3 d	73.2 d	71.7 d	193.9 s
8a	37.7 d	35.7 d	116.5 s	114.6 s
9	119.0 d	118.9 d	116.3 d	115.0 d
10	141.9 d	141.9 d	142.2 d	145.1 d
11	132.2 s	132.2 s	131.9 s	131.7 s
12	147.2 d	147.2 d	147.9 d	148.0 d
13	34.9 d	35.0 d	35.0 d	35.0 d
14	30.1 t	30.1 t	30.0 t	29.7 t
15	11.9 q	11.9 q	11.9 q	12.0 q
16	20.2 q	20.2 q	20.2 q	20.2 q
17	12.3 q	12.4 q	12.3 q	12.6 q
7-Me	21.2 q	20.7 q	23.8 q	23.2 q
7-Ac C=O				170.3 s
7-Ac Me				20.3 q
8-Ac C=O		170.3 s		
8-Ac Me		20.6 q		
1'				55.4 t
2'				60.9 t

Fig. 4. HMBC and ^1H-^1H COSY experiments for **5**.

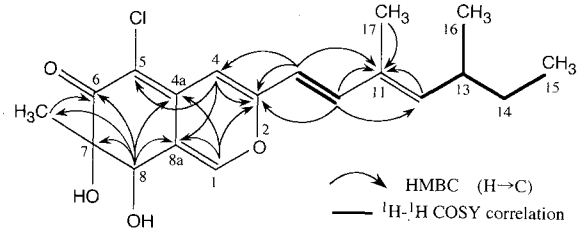


Fig. 5. CD spectra of **9** and **10**.

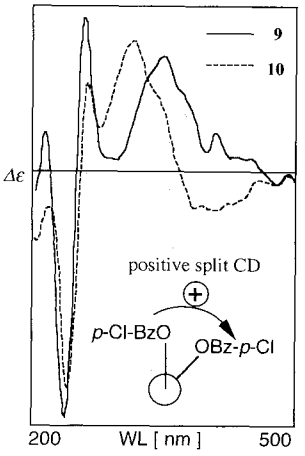
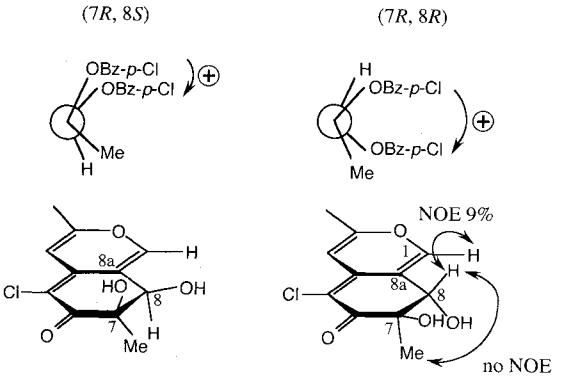


Fig. 6. Elucidation of stereochemistry of **5** by NOEs and the exciton chirality method.



chlorine atom to C-5 was reasonable from its chemical shift (106.0 ppm). Thus the planer structure of **5** was elucidated as shown in Fig. 4, which was the same as that of TL-1 (**8**).[13] However, the ^{13}C chemical shifts of **5** and **8** were different. Therefore, **5** was regarded as a diastereomer of **8**. The stereochemistry of **5** was elucidated by the exciton chirality method.[14] Treatment of **5** with *p*-chlorobenzoylchloride afforded the dibenzoate **9**. The CD spectrum in MeOH of **9** exhibited positive first and negative second Cotton effects (260.0 nm $\Delta\varepsilon$ 16.9, 238.5 nm $\Delta\varepsilon$ −34.3, Fig. 5). Therefore, **9** gave a positive split CD, suggesting that the possible configuration of **5** was 7*R*,8*S* or 7*R*,8*R*. As the NOE was observed between H-1 and H-8 (9%) but not between H-8 and 7-CH_3, the absolute configuration of **5** was deduced to be 7*R*,8*R* (Fig. 6). The configuration of C-13 was not elucidated. Consequently, the structure of **5** was elucidated as (7*R*,8*R*)-7-deacetyl-O^8,8-dihydro-sclerotiorin.

Isochromophilone III (3)

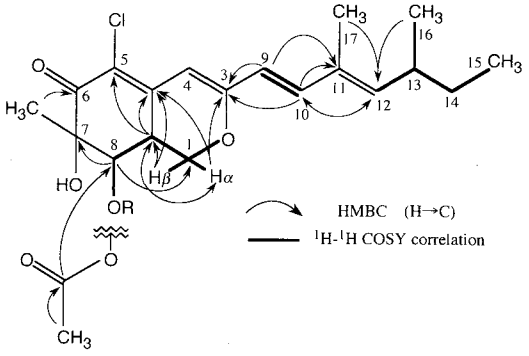
The molecular formula of **3** was established as $C_{19}H_{25}O_4Cl$ (MW 352) by the analysis of HR FAB-MS; $(M+Na)^+$ *m/z* 375.1333 (calcd for $C_{19}H_{25}O_4ClNa$ 375.1350). From the ^1H-^1H COSY, three proton sequences, –O–CH_2–CH–CH–O–, =CH–CH(CH_3)–CH_2–CH_3 and –CH=CH–, were elucidated. The HMBC experiment and the comparison of the chemical shifts of **3** and **5** revealed the planer structure of **3** as shown in Fig. 7. The stereochemistry of **3** was elucidated by the same methods as described for **5**. The CD spectrum of 7,8-bis-*p*-chlorobenzoate of **3** (**10**) in MeOH exhibited positive first and negative second Cotton effects (255 nm $\Delta\varepsilon$ 9.37, 234 nm $\Delta\varepsilon$ −14.6, Fig. 5). Therefore, **10** gave a positive split CD, suggesting that the possible configuration of **3** was 7*R*,8*S* or 7*R*,8*R*. The coupling constants of H-8a ($J_{1\alpha,8a}=14$ Hz, $J_{8a,8}=10$ Hz) indicated that Hα-1, H-8a and H-8 were all axial. The NOE between 7-CH_3 and H-8 (10%) suggested that 7-CH_3 was equatorial (Fig. 8). Thus the absolute configuration of **3** was deduced to be 7*R*,8*R*,8a*R*. Consequently, the

structure of **3** was elucidated as (7*R*,8*R*,8a*R*)-7-deacetyl-1,8a-dihydro-O^8,8-dihydro-sclerotiorin.

Isochromophilone IV (4)

The molecular formula of **4** was established as $C_{21}H_{27}O_5Cl$ (MW 394) by the analysis of HR FAB-MS; $(M+H)^+$ *m/z* 395.1611 (calcd for $C_{21}H_{28}O_5Cl$ 395.1646). The UV spectrum of **4** in MeOH showed the absorption maxima at 205 (ε 11,800), 267 (7,900) and 390 (39,400) nm, which was similar to **3**. Compound **4** showed similar ^1H and ^{13}C NMR spectra except for the presence of one acetyl group in **4** as those of **3**. The same correlations as those of **3** were observed in ^1H-^1H COSY and HMBC of **4**. A cross peak from H-8 proton (δ 3.46) to the acetyl carbonyl carbon (δ 170.3) in HMBC revealed that **4** was 8-*O*-acetyl **3** (Fig. 7).

Fig. 7. HMBC and ^1H-^1H COSY experiments for **3** (R = H) and **4** (R = Ac).



Isochromophilone VI (6)

The molecular formula of **6** was established as $C_{23}H_{28}NO_5Cl$ (MW 433) by the analysis of HR FAB-MS; $(M+H)^+$ *m/z* 434.1744 (calcd for $C_{23}H_{29}NO_5Cl$ 434.1734). The ^1H-^1H COSY and HMBC experiment revealed the structure of **6** as shown in Fig. 9. To

Fig. 8. Elucidation of stereochemistry of **3** by NOEs and the exciton chirality method.

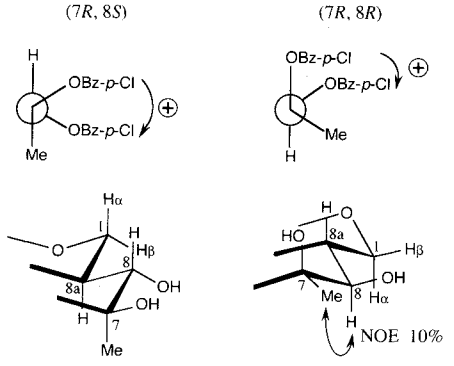


Fig. 9. HMBC and ^1H-^1H COSY experiments for **6**.

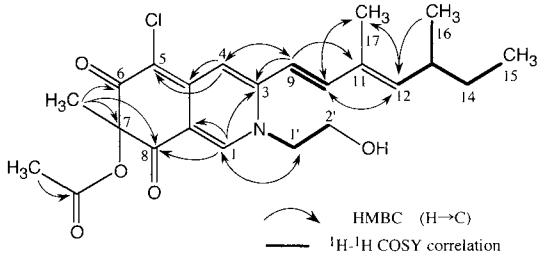
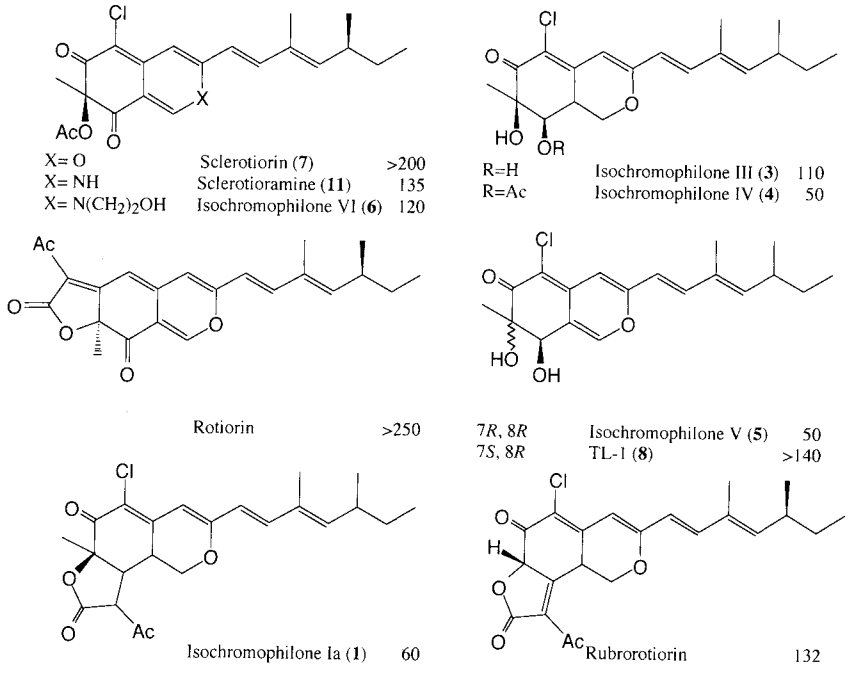


Fig. 10. ACAT inhibitory activities of azaphilones. (IC_{50}: μM)



confirm the absolute configuration of **6**, chemical transformation was done from **7**. Azaphilones are converted with primary amines or ammonia into *N*-containing compounds rapidly.[11] For example, **7** is converted to sclerotioramine (**11**) with ammonia. Treatment of **7** with 2-hydroxyethylamine afforded **6** quantitatively. Thus **6** was shown to have the same configuration as that of **7** (7*R*,13*S*). Consequently, the structure of **6** was elucidated as (7*R*,13*S*)-*N*-2-hydroxyethyl-sclerotioramine.

Biological Properties

Inhibitory Effect of Isochromophilones on ACAT Activity in an Enzyme Assay

The inhibitory activity of isochromophilones and some other azaphilones on ACAT activity was tested in an assay using rat liver microsomes as a source of enzyme and the results are shown in Fig. 10. They showed moderate inhibition against ACAT. Among them, **4** and **6** showed the most potent inhibitory activity with an IC_{50} value of 50 μM.

Other Biological Activities of Isochromophilones

The compound **4** also inhibited the activity of choresteryl ester transfer protein (CETP) with an IC_{50} value of 98 μM. The compounds **3**, **5** and **6** weakly inhibited the activity of CETP in 300 μM. The effect of azaphilones on CETP activity will be reported elsewhere.

Antimicrobial and cytotoxic activities of **3**, **5** and **6** were tested. They inhibited the growth of *Staphylococcus aureus* FDA 209P, *Bacteroides fragilis* ATCC 23745 and *Pyricularia oryzae* KF 180 at 50 μg/disk (paper disk method). But they did not inhibit the growth of *Bacillus subtilis* PCI 219, *Micrococcus luteus* PCI 1001, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Escherichia coli* NIHJ JC-2, *Pseudomonas aeruginosa* P3, *Xanthomonas oryzae* KB 88, *Acholeplasma laidlawii* PG 8 KB 174, *Candida albicans* KF 1, *Saccharomyces sake* KF 26, *Aspergillus niger* ATCC 6275 and *Mucor racemosus* IFO 4581 in the same concentration. The IC_{50} values of **3**, **5** and **6** against the growth of B-16 melanoma cells *in vitro* were 33, 36 and 30 μM, respectively.

Experimental

General

NMR spectra were recorded on Varian XL-400 (400 MHz) NMR spectrometer. Mass spectra were obtained on JEOL model JMS-AX505 mass spectrometer. UV-visible spectra were measured on Shimadzu UV-200S spectrometer. IR spectra were recorded on Horiba FT-210 diffraction infrared spectrometer.

Preparation of **9** and **10**

p-Chlorobenzoyl chloride (20 mg, 0.11 mmol), triethylamine (100 μl) and dimethylaminopyridine (1 mg) were added to the solution of **5** (10 mg, 0.028 mmol) in toluene (2 ml). The mixture was stirred at 50°C for 30 hours, then washed with water, dried over Na_2SO_4 and filtered. After evaporation of the filtrate, the resulting material was subjected to TLC and developed with $CHCl_3$ to give **9** (3 mg). Compound **10** (3 mg) was afforded from **3** (10 mg) in the same manner to that described for the preparation of **9**. 7,8-Bis-*p*-chlorobenzoate of **5** (**9**); (MW 626), FAB-MS *m/z* 627 $[M+H]^+$, UV λ_{max}^{MeOH} nm (ε): 200 (52,600), 240 (36,900), 270 (8,100), 280 (3,800), 380 (33,800).

7,8-Bis-*p*-chlorobenzoate of **3** (**10**); (MW 628), FAB-MS *m/z* 629 $[M+H]^+$, UV λ_{max}^{MeOH} nm (ε): 200 (46,500), 240 (36,400), 300 (7,500), 315 (11,300), 335 (18,200), 350 (20,700), 380 (18,200), 400 (16,300), 424 (9,400), 455 (3,140).

ACAT Assay *in Vitro*

ACAT activity was assayed as reported previously.[15]

Discussion

Azaphilones are yellow or orange pigment produced by fungi and their pyranyl oxygens easily change to nitrogens with ammonia. The inhibitory activities against monoamine oxidase,[13] phospholipase A_2[16] and tumor promotion[17] of azaphilones have been reported. Our group has recently reported that **1** and **2** isolated from *Penicillium multicolor* FO-2338 showed inhibitory activity against gp120-CD4 binding.[8] Though the strain FO-3216 also produced **1** and **2** with **3**~**6**, the inhibitory activity of **3**~**6** against gp 120-CD4 binding was weaker than that of **1** and **2** (data not shown). Here we showed that azaphilones had the inhibitory activity against ACAT.

The planar structures of **5** and **8** are same. But the ACAT inhibitory activity of **5** was about three times more potent than that of **8**. Compounds **3** and **4** are also 7*R*, and they showed more potent inhibitory activity than **8**. Therefore, the configuration 7*R* should be important for ACAT inhibitory activity. Sclerotiorin inhibited ACAT less potent than **3**, and rotiorin and rubrotiorin also showed only weak inhibition. It suggested that C-8 should be sp^3 for the inhibitory activity against ACAT.

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