New Isochromophilones VII and VIII Produced by *Penicillium* sp. FO-4164

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New isochromophilones VII and VIII were isolated from the culture broth of *Penicillium* sp. FO-4164. The structures were elucidated as 6*H*-2-benzopyran-6,8(7*H*)-dione, 5-chloro-3-(3′,5′-dimethyl-1′,3′-heptadienyl)-1,7,8a-trihydro-7,8a-dihydroxy-7-methyl-7-acetate for isochromophilone VII and 6*H*-2-benzopyran-6-one,5-chloro-3-(3′,5′-dimethyl-1′,3′-heptadienyl)-1,7,8,8a-tetrahydro-7,8-dihydroxy-7-methyl-8-acetate for isochromophilone VIII. Isochromophilones VII and VIII inhibited diacylglycerol acyltransferase activity with IC_{50} values of 20.0 and 127 μM and acyl-CoA : cholesterol acyltransferase activity with IC_{50} values of 24.5 and 47.0 μM, respectively.

During our screening programs for microbial enzyme inhibitors affecting lipid metabolism[1~3], a fungal strain FO-4164 was found to produce diacylglycerol acyltransferase (DGAT) and acyl-CoA : cholesterol acyltransferase (ACAT) inhibitors. Six components of azaphilone family were isolated from the culture broth of the strain. Two components were novel members named isochromophilones VII and VIII (Fig. 1), but the others were identified as isochromophilone IV[4], sclerotiorin[5,6], rubrorotiorin[7,8] and ochrephilone[9]. Recently, we have reported different components of the azaphilone family, isochromophilones I and II[10~12], as gp120-CD4 binding inhibitors, and isochromophilones III to VI[4] as ACAT inhibitors. In this paper, the taxonomy of the producing strain, its fermentation, and the structure determination and biological activity of isochromophilones VII and VIII are described.

Materials and Methods

General Experimental Procedures

Fungal strain FO-4164 isolated from a soil sample was used for production of isochromophilones. Kieselgel 60 (E. Merck) was used for column chromatography. HPLC was carried out using a JASCO system (TRI ROTAR V).

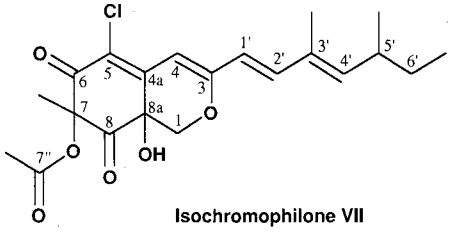
Spectroscopic Studies

UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a Horiba FT-210 infrared spectrometer. Melting points were measured with a Yanaco micro melting point apparatus. Optical rotations were obtained with a JASCO DIP-370 digital polarimeter. EI-MS spectra were recorded on a JEOL JMS-D 100 mass spectrometer at 20 eV. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer. The various NMR spectra were obtained on a Varian XL-400 spectrometer.

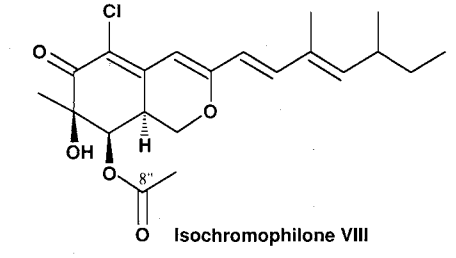
Taxonomic Studies

For the identification of the fungus, Czapek yeast extract agar, malt extract agar, CZAPEK's agar, potato dextrose agar (Difco) and YpSs agar (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$

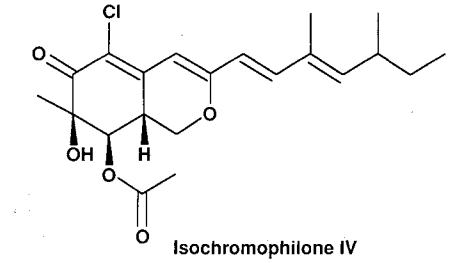
Fig. 1. Structures of isochromophilones VII, VIII and IV.



Isochromophilone VII



Isochromophilone VIII



Isochromophilone IV

0.05% and agar 2.0%, pH 6.0) were used. Morphological observations were done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (Hitachi S-430).

DGAT and ACAT Assays

DGAT activity was assayed in an enzyme assay using rat liver microsomes as reported previously[3]. Triacylglycerol formation was measured in an intact cell assay using Raji cells as reported previously[13]. ACAT activity was determined in an enzyme assay using rat liver microsomes as reported previously[14].

Antimicrobial Activity

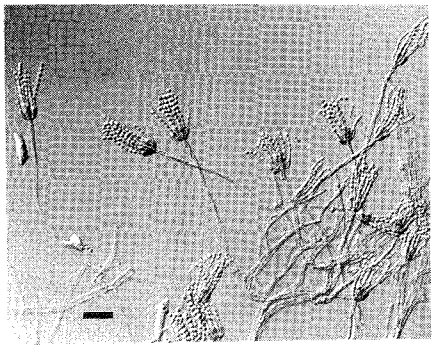
Antimicrobial activity was tested using paper disks (diameter 6 mm, ADVANTEC). Bacteria were grown on Müeller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after 24-hour incubation at 37°C for bacteria and after 48-hour incubation at 27°C for fungi and yeasts.

Results

Taxonomy of the Producing Organism

The fungal strain FO-4164 was originally isolated from a soil sample collected at Saitama, Japan. This strain grew rapidly to form light olive gray to olive gray colonies with a diameter of 45~70 mm after incubation for 14 days at 25°C. At 37°C, growth is nil. Reverse of the colonies was rust brown to dull red. The colony surface was velvety to floccose. Conidia formation was moderate when observed on potato dextrose agar, CZAPEK's yeast extract agar and YpSs agar media. In contrast conidia were poorly produced on CZAPEK's agar and malt extract agar media. When the strain FO-4164 was grown on potato dextrose agar at 25°C for 7 days, the conidiophores were borne from substrate hyphae, and the penicillia were monoverticillate as shown in Fig. 2. The phialides were 10~12.5×2.5 μm. The conidia were globose to subglobose and 2.5~3.0 μm in diameter, and their surface was smooth. From the above characteristics, the strain FO-4164 was identified as a member of the genus *Penicillium*[15,16].

Fig. 2. Photomicrograph of penicillia of strain FO-4164.
Bar represents 10 μm.



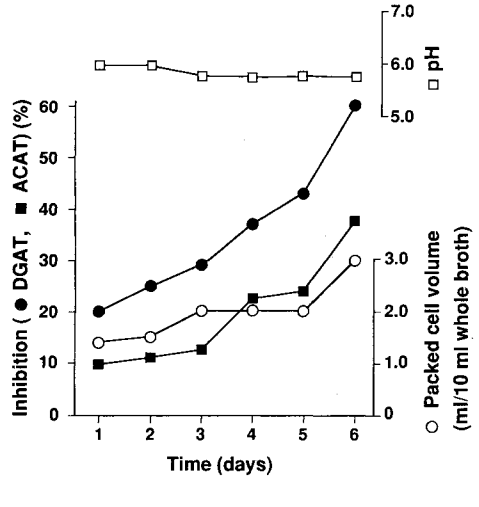
Fermentation

A slant culture of the strain FO-4164 grown on YpSs agar was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flask was shaken on a rotary shaker for 2 days at 27°C. One ml of the seed culture was transferred into 100 ml of a production medium (soluble starch 3.0%, glycerol 1.0%, soybean meal 2.0%, dry yeast 0.3%, KCl 0.3%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, and $CaCO_3$ 0.2%, pH 6.5) in 500-ml Erlenmeyer flasks. The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 3. When 5 μl of ethanol-treated whole broth (1 : 1, v/v) was used for DGAT and ACAT assays, inhibition of DGAT and ACAT activities was observed after 1 day and increased at least up to 6 days.

Isolation

The 6-day old broth (1.9 liters) was extracted with 2 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brown material (6.2 g). The material was dissolved in acetonitrile (40 ml), which was kept at 4°C to give a yellow crystal of sclerotiorin (450 mg). The crystals were removed by filtration, and the supernatant was used for purification by preparative HPLC (Senshu pak ODS-H-6251, 30×250 mm; 70% aq CH_3CN; UV at 345 nm;

Fig. 3. A typical time course of production of DGAT and ACAT inhibitors by *Humicola* sp. FO-4164.



20.0 ml/minute). Under the conditions, ochrephilone, isochromophilones VII, VIII and IV, sclerotiorin and rubrorotiorin were eluted as peaks with retention times of 29.0, 31.5, 38.5, 39.5, 41.0 and 58.0 minutes, respectively. The fractions were concentrated and extracted with ethyl acetate to give pure isochromophilone IV (66.0 mg), ochrephilone (13.0 mg), sclerotiorin (280 mg) and rubrorotiorin (17.2 mg) and impure isochromophilones VII (27.0 mg) and VIII (32.0 mg). Therefore, isochromophilone VII was further purified by preparative HPLC using 60% aq CH_3CN solvent to give a peak with a retention time of 43 minutes, and isochromophilone VIII using 65% aq CH_3CN with a retention time of 53 minutes. As a result, pure isochromophilones VII (11.3 mg) and VIII (16.6 mg) were obtained as yellow powders.

Physico-chemical Properties

Physico-chemical properties of isochromophilones VII and VIII are summarized in Table 1. The IR spectra (KBr) showed the absorption at 1677 cm^{-1} for isochromophilone VII and at 1672 cm^{-1} for isochromophilone VIII, suggesting the presence of $\alpha\beta$-unsaturated ketone in both molecules[1,7]. Isochromophilone VIII showed the same UV spectrum as isochromophilone IV with maxima at 210, 265 and 388 nm (Fig. 4), indicating the presence of the same chromophore in their structures. However, optical rotations were +385° for VIII and −341° for IV[4], suggesting that they are antipodes.

Structure of Isochromophilone VII

The molecular formula of isochromophilone VII was determined to be $C_{21}H_{25}O_6Cl$ by HREI-MS analysis. ^{13}C and ^1H NMR spectra ($CDCl_3$) showed 21 carbon and 25 proton signals, respectively. The DEPT spectra indicated the presence of five $-CH_3$, one $-CH_2-$, one $-O-CH_2-$, one $-CH-$, four $=CH-$ and nine quaternary carbons. To fulfill the molecular formula, the presence of one hydroxyl group was suggested. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum (Table 2). From the ^1H-^1H COSY spectrum (Fig. 5), two proton sequences, $=C^{4'}H-C^{5'}H(CH_3)-C^{6'}H_2-CH_3$ and $-C^{1'}H=C^{2'}H-$, were determined. ^{13}C-^1H long range couplings of ^2J and ^3J observed in the HMBC spectrum (Fig. 5) showed the cross peaks from C-3'-CH_3 (δ 1.82) to C-2' (δ 142.9), C-3' (δ 132.2) and C-4' (δ 148.1), forming the 3,5-dimethyl-1,3-heptadienyl moiety containing the above two proton sequences. The cross peaks from H-2' (δ 7.07), H-1' (δ 6.04) and H-4 (δ 6.18) to C-3 (δ 162.2), from H-4 to C-1' (δ 118.2) and from H-1' to C-4 revealed the presence of an extended conjugation system. Furthermore, the cross peaks from H_2-1 (δ 4.02, 4.74) to C-3, C-4a (δ 142.8) and C-8a (δ 67.9), and from H-4 to C-3 and C-8a, and ^{13}C shifts of C-1 (δ 70.0) and C-3 revealed a pyran ring. The HMBC coupling from

Table 1. Physico-chemical properties of isochromophilones VII and VIII.

	Isochromophilone VII	Isochromophilone VIII
Appearance	Yellow powder	Yellow powder
Molecular formula	$C_{21}H_{25}O_6Cl$	$C_{21}H_{27}O_5Cl$
Molecular wight	408	394
FAB-MS (*m/z*)		
Positive	409 $[M+H]^+$	395 $[M+H]^+$
	431 $[M+Na]^+$	417 $[M+Na]^+$
HREI-MS (*m/z*)		
Calcd:	408.1333	394.1540
Found:	408.1348	394.1519
$[\alpha]_D^{20}$ (*c* 1.0, MeOH)	+559 °	+385 °
UV λ_{max}^{MeOH}(nm) (ε)	215 (6,100)	210 (12,000)
	268 (7,900)	265 (6,100)
	400 (51,000)	388 (48,500)
IR υ_{max}^{KBr} (cm^{-1})	2962, 1740, 1677	3429, 2960, 2925
	1612, 1560, 1273	1745, 1672, 1612
	1244	1560, 1228
Melting point	78~81°C	59~61°C
Solubility		
Soluble	EtOH, CH_3CN, EtOAc	EtOH, CH_3CN, EtOAc
	MeOH, $CHCl_3$	MeOH, $CHCl_3$
Insoluble	H_2O, *n*-Hexane	H_2O, *n*-Hexane

Fig. 4. UV spectra of isochromophilones VII and VIII (in MeOH).

VII: —— VIII: – – – –.

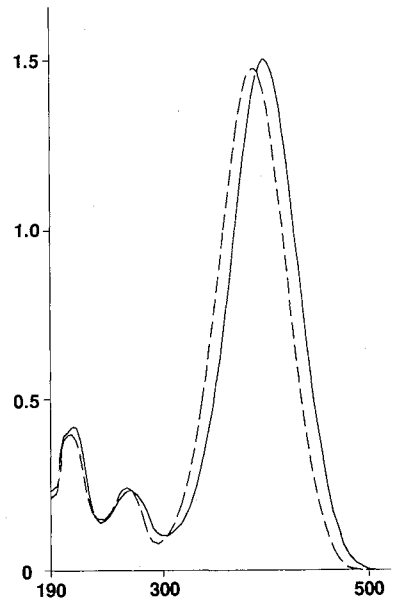


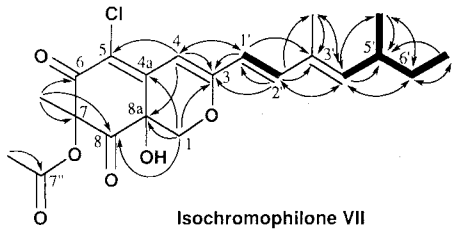
Table 2. ^{1}H and ^{13}C NMR chemical shifts of isochromopilones VII and VIII.

Carbon No.	Isochromopilone VII		Isochromopilone VIII	
	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]
C-1	70.1	4.02 (1H, d, J=12.5 Hz) 4.74 (1H, d, J=12.5 Hz)	67.3	3.85 (1H, dd, J=12.8, 10.8 Hz) 4.48 (1H, dd, J=10.8, 4.9 Hz)
C-3	162.2		162.6	
C-4	100.2	6.18 (1H, s)	101.8	6.09 (1H, s)
C-4a	143.86		144.6	
C-5	121.0		115.9	
C-6	185.5		192.0	
C-7	83.5		76.0	
C-7-CH3	23.4	1.83 (3H, s)	24.6	1.45 (3H, s)
C-7"	169.7			
C-7"-CH3	19.9	2.12 (3H, s)		
C-8	197.1		74.2	5.52 (1H, d, J=3.0 Hz)
C-8"			170.5	
C-8"-CH3			20.6	2.02 (3H, s)
C-8a	68.0		36.8	3.23 (1H, ddd, J=12.8, 4.9, 3.0 Hz)
C-8a-OH		7.35 (1H, brs) c		
C-1'	118.2	6.04 (1H, d, J=15.5 Hz)	118.6	5.98 (1H, d, J=15.2 Hz)
C-2'	142.91	7.07 (1H, d, J=15.5 Hz)	142.2	6.98 (1H, d, J=15.2 Hz)
C-3'	132.2		132.2	
C-3'-CH3	12.4	1.82 (3H, d, J=1.0 Hz)	12.4	1.81 (3H, d, J=1.0 Hz)
C-4'	148.1	5.67 (1H, d, J=10.0 Hz)	147.5	5.62 (1H, d, J=9.2 Hz)
C-5'	35.0	2.46 (1H, m)	35.0	2.45 (1H, m)
C-5'-CH3	20.2	1.00 (3H, d, J=6.5 Hz)	20.2	0.99 (3H, d, J=6.4 Hz)
C-6'	30.1	1.29 (1H, m) 1.42 (1H, m)	30.1	1.30 (1H, m) 1.40 (1H, m)
C-6'-CH3	11.9	0.85 (3H, t, J=7.5 Hz)	11.9	0.84 (3H, t, J=7.2 Hz)

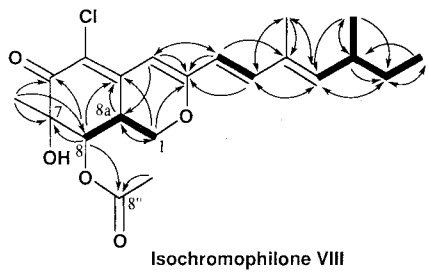
[a] Chemical shifts are shown with reference to $CDCl_3$ as 77.7 ppm.
[b] Chemical shifts are shown with reference to $CDCl_3$ as 7.26 ppm.
[c] This signal was observed in DMSO-d_6.

Fig. 5. ^{1}H-^{1}H COSY and HMBC results for isochromophilones VII and VIII.

^{1}H-^{1}H COSY: ▬, HMBC: H ⟶ C.



Isochromophilone VII



Isochromophilone VIII

C-7"-CH_3 (δ 2.12) to C-7" (δ 169.7) and the fragment ion peak (m/z 349, $[M+H-60]^+$) in EI-MS supported the presence of an acetyl residue. The presence of another 6-membered ring was suggested because of the cross peaks from C-7-CH_3 (δ 1.83) to C-6 (δ 185.4), C-7 (δ 83.4) and C-8 (δ 197.1), from H-1 to C-8, and from H-4 to C-5 (δ 121.0) in the HMBC spectrum and the degree of unsaturation. This structure contained an α,β-unsaturated ketone, which was also suggested by the IR spectrum. Finally the remaining hydroxyl group, acetyl group and Cl should be attached to C-8a, C-7 and C-5, respectively, due to the ^{13}C chemical shifts.

Taken together, the structure of isochromophilone VII was elucidated as shown in Fig. 5.

Structure of Isochromophilone VIII

The ^{13}C NMR spectrum ($CDCl_3$) of isochromophilone VIII (Table 2) was similar to that of isochromophilone VII. Comparison of the spectra data indicated that $-C^{8a}H-$ and $-O-C^{8}H-$ carbons are present in place of the corresponding quaternary carbons for isochromophilone VII. From the ^{1}H-^{1}H COSY spectrum (Fig. 5), an additional proton sequence $-O-C^{8}H-C^{8a}H-C^{1}H_2-O-$ was determined. ^{13}C-^{1}H long range couplings from H-8 (δ 5.52) to C-7 (δ 76.0), C-8" (δ 170.5), and from C-8"-CH_3 (δ 2.02) to C-8" were observed in the HMBC spectrum (Fig. 5). Taken together, the structure of isochromophilone VIII was elucidated as shown in Fig. 5.

Stereochemistries of Isochromophilones VIII and IV

Isochromophilones VIII and IV possess the same planar structures. The two compounds have four chiral

Fig. 6. The relative stereochemistries for C-7, 8 and 8a of isochromophilones VII and VIII.

NOE: ⟷.

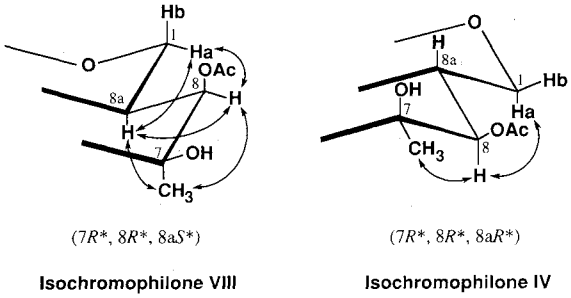


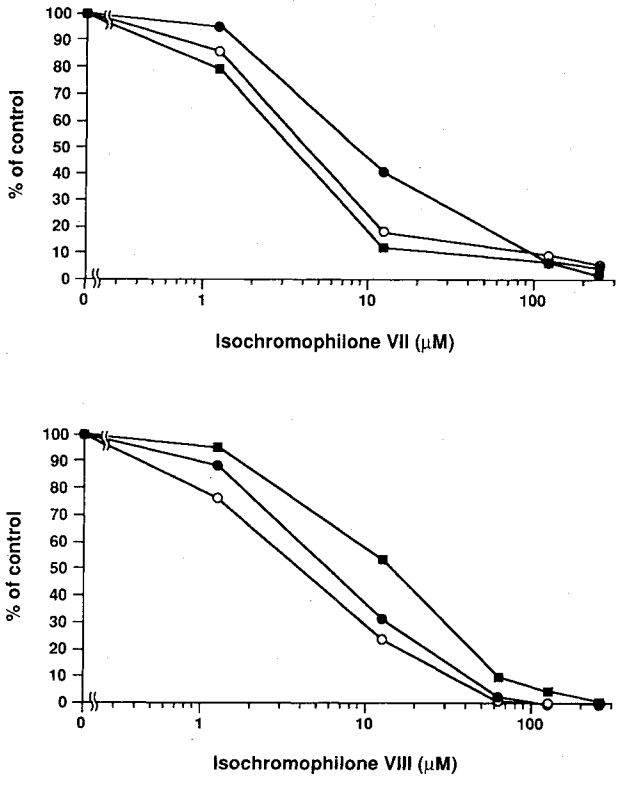
Table 3. Effects of isochromophilones and related compounds on DGAT and ACAT activities in assays using rat liver microsomes.

Compound	IC_{50} (μM)	
	DGAT	ACAT
Isochromophilone VII	20.0	24.5
Isochromophilone VIII	127	47.0
Isochromophilone IV	63.5	50.0
Sclerotiorin	174	>200
Rubrorotiorin	>242	132
Ochrephilone	>262	>200
Amidepsine B	19.2	>200

carbons, and we have studied the relative stereochemistry at the three positions C-7, C-8 and C-8a by NOE experiments (Fig. 6). Molecular modeling (actual models) revealed that the conformation of the angular C-8a proton should be axial in both compounds. In ^1H NMR spectra, the coupling constants of H-8a were assigned to be $J_{8a,1b}=12.8$ Hz, $J_{8a,1a}=4.9$ Hz and $J_{8a,8}=3.0$ Hz for isochromophilone VIII (Table 2) and $J_{8a,1b}=5.0$ Hz, $J_{8a,1a}=14.0$ Hz and $J_{8a,8}=10.0$ Hz for isochromophilone IV[4], indicating that H-8 is equatorial for isochromophilone VIII and is axial for isochromophilone IV. Concerning the conformation of 7-CH_3, NOEs were observed between H-8a and each of Ha-1, H-8 and 7-CH_3 and between H-8 and each of Ha-1 and 7-CH_3 for isochromophilone VIII, and between H-8 and each of 7-CH_3 and Ha-1 for isochromophilone IV, indicating that 7*R**,8*R** configurations are reasonable for the both structures.

Taken together, isochromophilone VIII has 7*R**,8*R**, 8a*S** configurations and isochromophilone IV has 7*R**, 8*R**,8a*R**.

Fig. 7. Effects of isochromophilones VII and VIII on triacylglycerol (■), phosphatidylethanolamine (○) and phosphatidylcholine (●) formations in the Raji cell assay.



Biological Properties

Effect of Isochromophilones on DGAT and ACAT Activities

The inhibitory activity of isochromophilones against DGAT and ACAT was investigated in *in vitro* assays using rat liver microsomes, and their IC_{50} values are summarized in Table 3. Isochromophilone VII inhibited both activities with similar IC_{50} values of 20.0 μM for DGAT and 24.5 μM for ACAT. Isochromophilone VIII showed weak inhibitory activity against DGAT and ACAT, and is rather specific for ACAT inhibition. Under the same condition, isochromophilone IV inhibited both activities with higher IC_{50} values than isochromophilone VII. However, other related compounds showed only very weak or almost no inhibitory activities against DGAT and ACAT.

To investigate specificity for DGAT inhibition, the effects of isochromophilones VII and VIII were studied on triacylglycerol (TG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) syntheses in intact Raji cells (Fig. 7). TG formation was inhibited dose-dependently with IC_{50} values of 3.2 μM for isochromophilone VII and 15.0 μM for isochromophilone VIII.

However, the drugs inhibited both PC and PE formation to analogous extents, indicating that they are not specific for DGAT inhibition.

Other Biological Activities

Antimicrobial activity was tested at a concentration of 10 μg/paper disk. Isochromophilones IV, VII and VIII showed antimicrobial activity against *Bacillus subtilis* ATCC 6633 (diameter of inhibition zone: 0, 0, 9.0 mm), *Mycobacterium smegmatis* ATCC 607 (12.8, 8.2, 7.8 mm), *Micrococcus luteus* PCI 1001 (0, 0, 8.0 mm) and *Pyricularia oryzae* (0, 8.6, 9.6 mm), respectively. But no antimicrobial activity was observed against the following microorganisms; *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces sake*, *Mucor racemosus* and *Aspergillus niger*.

Discussion

Many azaphilones have been isolated from fungi as phospholipase A2 inhibitors[18], monoamine oxidase inhibitors[19] and tumor promotion inhibitors[20]. We also reported isochromophilones I and II[10~12] as gp120-CD4 binding inhibitors, and isochromophilones III to VI[4] as ACAT inhibitors. In this paper, we have showed that isochromophilones VII, VIII and IV exhibit both ACAT and DGAT inhibitory activities. The inhibitory activities against DGAT and ACAT are summarized in Table 3 for all isochromophilones and other azaphilones we have isolated. Although there was no relationship between gp120-CD4 binding inhibition and ACAT inhibition caused by these azaphilones[4] (data not shown), DGAT and ACAT inhibitions by the drugs seemed parallel (Table 3). It might be that both DGAT and ACAT have a common mechanism for exerting their similar acyltransfer activities, which is possibly blocked by the azaphilones. In this sense, they might be non-specific inhibitors of acyltransferase activity. In an intact Raji cell assay, in fact, TG, PC and PE formations were inhibited to a similar extent by isochromophilones VII and VIII (Fig. 7). In contrast, amidepsine B, as described recently, inhibited DGAT activity specifically (Table 3), which was supported by specific inhibition of TG synthesis in the intact cells[3].

Acknowledgments

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