Pyripyropenes, Novel ACAT Inhibitors Produced by *Aspergillus fumigatus*

III. Structure Elucidation of Pyripyropenes E to L

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Eight new pyripyropenes, E to L, were isolated from the culture broth of *Aspergillus fumigatus* FO-1289-2501 selected as a higher producer by NTG mutation. Structural elucidation indicated that all the pyripyropenes have the same pyridino-α-pyrone sesquiterpene core as pyripyropenes A to D. Among them, pyripyropene L showed the most potent inhibition against acyl-CoA: cholesterol acyltransferase (ACAT) activity with an IC_{50} value of 0.27 μM in rat liver microsomes.

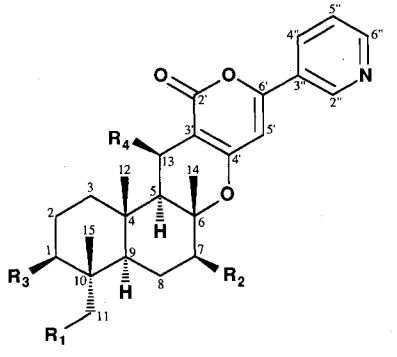
We reported the isolation, biological properties and planar structures of pyripyropenes A to D, novel polyoxygenated metabolites of *Aspergillus fumigatus* FO-1289[1~3], which are inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT). They showed very potent ACAT inhibition in rat liver microsomes with nanomolar levels of IC_{50} values. Recently, we demonstrated the relative and absolute stereochemistry of pyripyropene A[4]. To obtain a large amount of pyripyropene A, the parent strain FO-1289 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) to yield a mutant FO-1289-2501 as a high producer of pyripyropenes. Further isolation study from the jar fermentation broth of the mutant strain led to the discovery of eight new pyripyropenes E to L (Fig. 1). In this paper, the isolation, structure elucidation and biological properties of these pyripyropenes are described.

Materials and Methods

General Experimental Procedures

To obtain high pyripyropene producing strains, the parent *Aspergillus fumigatus* FO-1289 was treated with NTG according to the established method. *Aspergillus fumigatus* FO-1289-2501 selected showed about 10-fold higher production of pyripyropene A than the parent strain. The strain was grown on slants containing soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, and agar 2.0% in distilled water (pH 6.0). The slants were incubated at 27°C and stored in tubes sealed with screw caps at room temperature.

Fig. 1. Structures of pyripyropenes A to L.



	R_1	R_2	R_3	R_4
Pyripyropene A	$-OCOCH_3$	$-OCOCH_3$	$-OCOCH_3$	-OH
Pyripyropene B	$-OCOCH_2CH_3$	$-OCOCH_3$	$-OCOCH_3$	-OH
Pyripyropene C	$-OCOCH_3$	$-OCOCH_2CH_3$	$-OCOCH_3$	-OH
Pyripyropene D	$-OCOCH_3$	$-OCOCH_3$	$-OCOCH_2CH_3$	-OH
Pyripyropene E	-H	-H	$-OCOCH_3$	-H
Pyripyropene F	-H	-H	$-OCOCH_2CH_3$	-H
Pyripyropene G	-H	-H	$-OCOCH_3$	-OH
Pyripyropene H	-H	-H	$-OCOCH_2CH_3$	-OH
Pyripyropene I	$-OCOCH_2CH_3$	$-OCOCH_2CH_3$	$-OCOCH_2CH_3$	-OH
Pyripyropene J	$-OCOCH_3$	$-OCOCH_2CH_3$	$-OCOCH_2CH_3$	-OH
Pyripyropene K	$-OCOCH_2CH_3$	$-OCOCH_3$	$-OCOCH_2CH_3$	-OH
Pyripyropene L	$-OCOCH_2CH_3$	$-OCOCH_2CH_3$	$-OCOCH_3$	-OH

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Kieselgel 60 (E. Merck), SS 1020T (Senshu Sci. Co.,) and Sephadex LH-20 (Pharmacia) were used for column chromatography. HPLC was carried out using JASCO (TRI ROTAR V) and Waters 600E systems.

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.

Single Crystal X-Ray Analysis

A colorless plate crystal having approximate dimensions of $0.4 \times 0.4 \times 0.3$ mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC-5R diffractometer with graphite monochromated CuKα radiation. The data were collected at a temperature of 23 ± 1°C using the ω-2θ scan technique to a maximum 2θ value of 140.0°. Pertinent crystal, data collection, and refinement parameters are summarized in Table 3. Neutral atom scattering factors were taken from CROMER and WABER[5]. Anomalous dispersion effects were included in Fcalc[6]; the values for $\Delta f'$ and $\Delta f''$ were those of CROMER[5]. All calculations were performed using the TEXSAN[7] crystallographic software package of Molecular Structure Corporation. The structure was solved by direct methods[8,9]

ACAT Activity

ACAT activity was carried out in an enzyme assay using rat liver microsomes as reported previously[10].

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (i.d. 6 mm, ADVANTEC). Bacteria were grown on Mueller-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

Fermentation

Different fermentation conditions including media were tested for convenient, stable and high production of pyripyropenes. *A. fumigatus* FO-1289-2501 was precultivated in a 500-ml Erlenmeyer flask containing 100 ml of a medium consisting of glucose 0.5%, yeast extract 0.5%, and mannitol 0.5% in distilled water, adjusted to pH 6.0 before sterilization. The culture was shaken reciprocally at 300 rpm at 27°C for 72 hours. Two hundred ml of the seed culture broth was inoculated into a 30-liter jar fermentor containing 20 liters of the same medium. After a 48-hour incubation, the second seed culture was inoculated at 1% into a 400-liter jar fermentor charged to 200 liters with a production medium consisting of 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%, $CaCO_3$ 0.2% and nicotinic acid (Merck) 0.002% in distilled water, adjusted to pH 6.5 before sterilization. The fermentation was carried out for 120 hours with an agitation rate of 200 rpm and an aeration rate of 0.5 v/v/minute at 27°C. The production of pyripyropenes was determined by HPLC as reported previously[2]. The production of pyripyropene A was observed at 96 hours and increased at least up to 120 hours.

Isolation

The 120-hour cultured whole broth (200 liters) was

Fig. 2. Isolation procedures of pyripyropenes E to L.

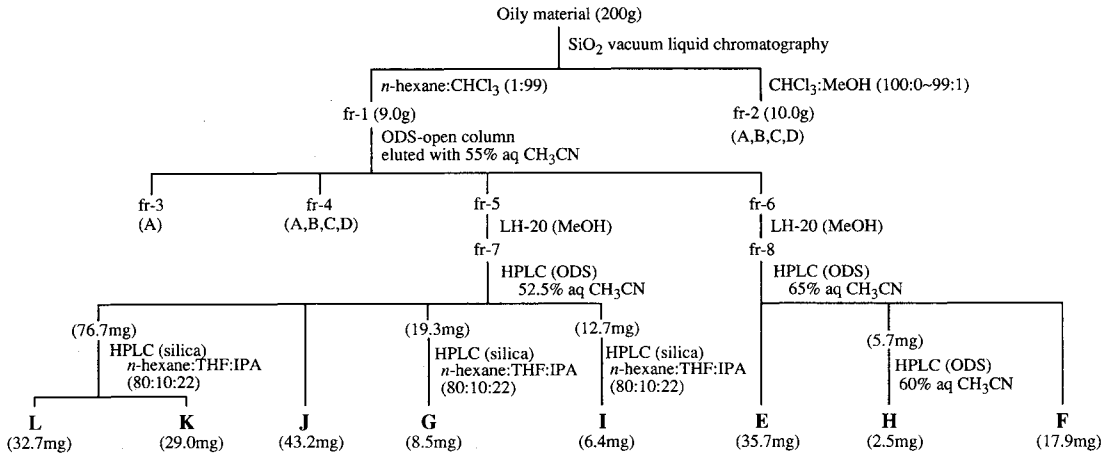
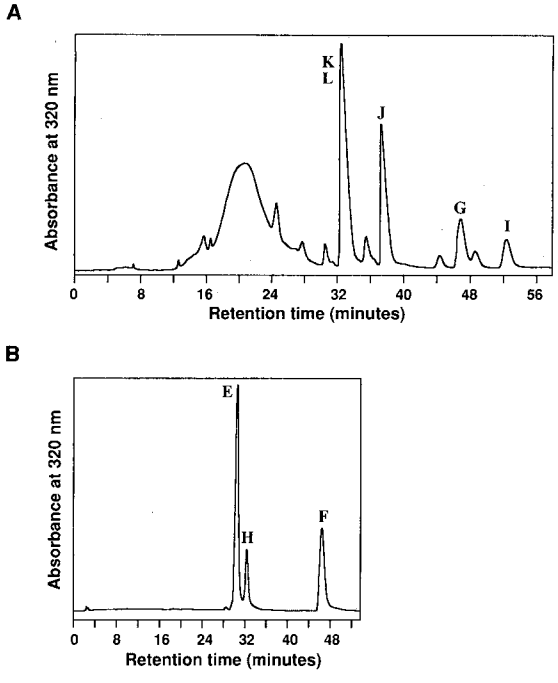


Fig. 3. A chromatographic profile of pyripyropenes E to L separated by preparative HPLC.



A: Column, Senshu pak ODS-H-6251 (30 × 250 mm); solvent, 52.5% aq CH_3CN; UV at 320 nm; 20.0 ml/minute, B: Column, Senshu pak ODS-H-6251 (30 × 250 mm); solvent, 65% aq CH_3CN; UV at 320 nm; 20.0 ml/minute.

extracted with 180 liters of ethyl acetate to yield an ethyl acetate layer and the mycelium. The mycelium was re-extracted with 40 liters of 80% aq acetone. After centrifugation, the acetone extracts were concentrated to an aqueous solution (10 liters), which was extracted with 15 liters of ethyl acetate. Both ethyl acetate extracts (195 liters) were concentrated *in vacuo* to yield a brown oil (410 g). About half of the oil was used for isolation of pyripyropenes E to L as summarized in Fig. 2. The oily material (200 g) was applied on a vacuum liquid chromatography using silica gel (Kieselgel 60, 400 g, 10.5 × 10 cm). The material was eluted stepwise with *n*-hexane - chloroform - methanol solutions (1,200 ml, 20 : 80 : 0, 1 : 99 : 0, 0 : 100 : 0, and 0 : 99 : 1, v/v, respectively), and 200 ml fractions of the eluate were collected. Eluates were pooled into two active fractions; fr-1 (fractions 7 ~ 12) containing all pyripyropenes and fr-2 (fractions 13 ~ 24) containing pyripyropenes A to D. The fr-1 was concentrated *in vacuo* to give a brown material (9.0 g). Then, the residue was subjected to chromatography on an ODS column (Senshu SS 1020T, 200 g). The materials were eluted with 55% aq acetonitrile and 50 ml fractions were collected. The fr-3 (fractions 18 ~ 22) was enriched with pyripyropene A, fr-4 (fractions 23 ~ 32) contained pyripyropenes A to D, fr-5 (fractions 33 ~ 61) contained pyripyropenes G, I, J, K and L, and fr-6 (fractions 62 ~ 67) contained pyripyropenes E, F and H. After concentration, fr-5 was subjected to gel filtration using Sephadex LH-20 (35 × 415 mm; solvent, methanol; 5 ml/fraction). The 39th to 58th fractions (fr-7) containing pyripyropenes G, I, J, K and L were concentrated *in vacuo* to a small volume, and purified by preparative HPLC (Senshu pak ODS-H-6251, 30 × 250 mm; solvent, 52.5% aq CH_3CN; UV at 320 nm; 20.0 ml/minute). As shown in Fig. 3A, pyripyropenes K and L eluted first with the same retention time of 32.5 minutes, followed by pyripyropene J (37.5 minutes), G (47.0 minutes) and I (52.5 minutes). After concentration and ethyl acetate extraction of the four fractions, pure pyripyropene J was obtained as a white powder (43.2 mg), but the other fractions contained impurities. Therefore, pyripyropenes L and K were further purified by preparative HPLC using a silica gel column (Senshu pak silica-5251-N, 20 × 250 mm; mobile phase, *n*-hexane - tetrahydrofuran - isopropyl alcohol (80 : 10 : 22, v/v); UV at 320 nm; 8 ml/minute). Two peaks with retention times of 15.5 and 17.0 minutes were concentrated to give pure pyripyropenes L (32.7 mg) and K (29.0 mg) as white powders, respectively. Under the same HPLC conditions using the silica gel column, pyripyropenes G (retention time of 13.5 minutes) and I (13.0 minutes) were also purified to give pure white powders (8.5 and 6.4 mg, respectively). On the other hand, pyripyropenes E, F and H were purified from the fr-6. After concentration, fr-6 was subjected to Sephadex LH-20 column chromatography under the same conditions as described above for fr-5. The 32th to 40th fractions (fr-8) containing pyripyropenes E, F and H were concentrated to a small volume, which was purified by preparative HPLC (Senshu pak ODS-H-6251, 30 × 250 mm; solvent, 65% aq CH_3CN; UV at 320 nm; 20.0 ml/minute). As shown in Fig. 3B, three peaks with retention times of 30.0, 32.0 and 47.0 minutes were collected and concentrated to give aqueous solutions, which were extracted with ethyl acetate to yield pure pyripyropenes E (35.7 mg) as colorless plate crystals, impure pyripyropene H (5.7 mg) and pure F (17.9 mg) as white powder, respectively. Pyripyropene H was finally purified by preparative HPLC (Senshu pak ODS-H-6251, 30 × 250 mm; solvent, 60% aq CH_3CN; UV at 320 nm; 20.0 ml/minute), eluting with a retention time of 57.0 minutes to give pure pyripyropene H (2.5 mg) as white powder.

Table 1. Physico-chemical properties of pyripyropenes E to L.

	Pyripyropene E	Pyripyropene F	Pyripyropene G	Pyripyropene H
Appearence	Colorless crystal	White powder	White powder	White powder
Molecular weight	451	465	467	481
Molecular formula	$C_{27}H_{33}NO_5$	$C_{28}H_{35}NO_5$	$C_{27}H_{33}NO_6$	$C_{28}H_{35}NO_6$
FAB-MS (*m/z*)				
Positive	452 $[M+H]^+$	466 $[M+H]^+$	468 $[M+H]^+$	482 $[M+H]^+$
	474 $[M+Na]^+$	488 $[M+Na]^+$	490 $[M+Na]^+$	504 $[M+Na]^+$
EI-MS (*m/z*)	451 $[M]^+$	465 $[M]^+$	467 $[M]^+$	481 $[M]^+$
	391 $[M-AcOH]^+$	391 $[M-PrOH]^+$	449 $[M-H_2O]^+$	463 $[M-H_2O]^+$
	376 $[M-AcOH-CH_3]^+$	376 $[M-PrOH-CH_3]^+$	434 $[M-CH_3-H_2O]^+$	451 $[M-2CH_3]^+$
			389 $[M-AcOH-H_2O]^+$	448 $[M-CH_3-H_2O]^+$
			374 $[M-AcOH-CH_3-H_2O]^+$	389 $[M-PrOH-H_2O]^+$
				374 $[M-PrOH-CH_3-H_2O]^+$
HREI-MS (*m/z*)				
Found	451.2338	465.2495	467.2296	481.2455
HRFAB-MS (*m/z*)				
Found				
Calcd	451.2350 (for $C_{27}H_{33}NO_5$)	465.2506 (for $C_{28}H_{35}NO_5$)	467.2299 (for $C_{27}H_{33}NO_6$)	481.2455 (for $C_{28}H_{35}NO_6$)
$[\alpha]_D^{28}$ (*c* 1.0, CH_3OH)	+113°	+122°	+102°	+104°
UV $\lambda_{max}^{CH_3OH}$ (nm)	231, 320	231, 320	231, 320	231, 320
IR ν_{max}^{KBr} (cm^{-1})	3440, 2940, 1736, 1641, 1579, 1385, 1219, 1022	3429, 2943, 1714, 1645, 1579, 1404, 1190, 1124, 1019	3448, 2947, 1707, 1643, 1578, 1435, 1245, 1126	3430, 2943, 1716, 1643, 1579, 1435, 1124, 1020
Melting point	174~176°C	NT	248~250°C (dec.)	NT

	Pyripyropene I	Pyripyropene J	Pyripyropene K	Pyripyropene L
Appearence	Colorless crystal	Colorless powder	Colorless crystal	Colorless crystal
Molecular weight	625	611	611	611
Molecular formula	$C_{34}H_{43}NO_{10}$	$C_{33}H_{41}NO_{10}$	$C_{33}H_{41}NO_{10}$	$C_{33}H_{41}NO_{10}$
FAB-MS (*m/z*)				
Positive	626 $[M+H]^+$	612 $[M+H]^+$	612 $[M+H]^+$	612 $[M+H]^+$
	648 $[M+Na]^+$	634 $[M+Na]^+$	634 $[M+Na]^+$	634 $[M+Na]^+$
EI-MS (*m/z*)	625 $[M]^+$	611 $[M]^+$	611 $[M]^+$	611 $[M]^+$
	607 $[M-H_2O]^+$	593 $[M-H_2O]^+$	593 $[M-H_2O]^+$	593 $[M-H_2O]^+$
	551 $[M-PrOH]^+$	537 $[M-PrOH]^+$	551 $[M-AcOH]^+$	537 $[M-PrOH]^+$
	533 $[M-PrOH-H_2O]^+$	519 $[M-PrOH-H_2O]^+$	533 $[M-AcOH-H_2O]^+$	519 $[M-PrOH-H_2O]^+$
	477 $[M-2PrOH]^+$	463 $[M-2PrOH]^+$	477 $[M-AcOH-PrOH]^+$	477 $[M-AcOH-PrOH]^+$
	459 $[M-2PrOH-H_2O]^+$	445 $[M-2PrOH-H_2O]^+$	459 $[M-AcOH-PrOH-H_2O]^+$	459 $[M-AcOH-PrOH-H_2O]^+$
	403 $[M-3PrOH]^+$	403 $[M-AcOH-2PrOH]^+$	403 $[M-AcOH-2PrOH]^+$	403 $[M-AcOH-2PrOH]^+$
	385 $[M-3PrOH-H_2O]^+$	385 $[M-AcOH-2PrOH-H_2O]^+$	385 $[M-AcOH-2PrOH-H_2O]^+$	385 $[M-AcOH-2PrOH-H_2O]^+$
HREI-MS (*m/z*)				
Found		611.2719		
HRFAB-MS (*m/z*)				
Found	626.2955		612.2806	612.2796
Calcd	626.2953 (for $C_{34}H_{44}NO_{10}$)	611.2719 (for $C_{33}H_{41}NO_{10}$)	612.2797 (for $C_{33}H_{42}NO_{10}$)	612.2797 (for $C_{33}H_{42}NO_{10}$)
$[\alpha]_D^{28}$ (*c* 1.0, CH_3OH)	+69°	+65°	+58°	+58°
UV $\lambda_{max}^{CH_3OH}$ (nm)	231, 320	231, 320	231, 320	231, 320
IR ν_{max}^{KBr} (cm^{-1})	3442, 2940, 1738, 1643, 1581, 1435, 1186, 1088	3442, 2944, 1736, 1643, 1581, 1435, 1236, 1184, 1041	3440, 2945, 1738, 1634, 1581, 1435, 1240, 1222, 1088	3440, 2944, 1738, 1643, 1581, 1435, 1244, 1188
Melting point	NT	248~250°C (dec.)	NT	148~150°C

PrOH: propionic acid, AcOH: acetic acid, NT: not tested.

Physico-chemical Properties of Pyripyropenes E to L

The physico-chemical properties of pyripyropenes E to L are summarized in Table 1. The molecular formulae were determined on the basis of high resolution electron impact mass spectra HREI-MS and FAB-MS. All the pyripyropenes showed the same UV absorption maxima at 231 and 320 nm as pyripyropenes A to D[2], suggesting the presence of a common pyridino-α-pyrone sesquiterpene skeleton. Correlation between the optical rotations and molecular weights indicated two groups of pyripyropenes; 1) pyripyropenes E, F, G and H showed large optical rotations (+102~122°) and low molecular weights (451~481) and 2) pyripyropenes I, J, K and L showed small optical rotations (+58~69°) and high molecular weights (611~625). This suggested structural similarity within each group.

Structure of Pyripyropene L

The molecular formula of pyripyropene L was determined to be $C_{33}H_{41}NO_{10}$ on the basis of HRFAB-MS measurements (*m/z* found 612.2796, calcd 612.2797 for $C_{33}H_{42}NO_{10}$ $[M+1]^+$). The ^1H NMR spectrum ($CDCl_3$) displayed signals due to 41 protons and the ^{13}C NMR spectrum showed 33 resolved peaks (Table 2), supporting the molecular formula. The carbons were classified into six methyl, five methylene, one oxy methylene, two methine, three oxy methine, three quaternary, five sp^2 methine, four sp^2 quarternary and four carbonyl carbons by analysis of the DEPT spectra. The NMR spectra are very similar to those of pyripyropene A except for the presence of two more methylenes for pyripyropene L. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum (Table 2).

Table 2-1. ^{1}H and ^{13}C NMR chemical shifts of pyripyropenes E to L.

Carbon No.	Pyripyropene E		Pyripyropene F	
	^{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	80.1	4.49 (1H, dd, J=11.5, 4.5 Hz)	79.8	4.51 (1H, dd, J=11.5, 5.0 Hz)
C-2	23.5	1.70 (2H, m)	23.5	1.70 (2H, m)
C-3	37.1	1.17 (1H, m)	37.1	1.20 (1H, m)
		1.78 (1H, m)		1.78 (1H, m)
C-4	37.7		37.8	
C-5	51.4	1.50 (1H, dd, J=12.5, 4.5 Hz)	51.4	1.51 (1H, dd, J=13.0, 4.5 Hz)
C-6	80.8		80.8	
C-7	40.2	2.13 (1H, dt, J=12.5, 3.0 Hz)	40.2	2.13 (1H, dt, J=13.0, 3.0 Hz)
		1.68 (1H, m)		1.68 (1H, m)
C-8	19.2	1.80 (1H, m)	19.2	1.80 (1H, m)
		1.44 (1H, m)		1.42 (1H, m)
C-9	55.0	1.08 (1H, m)	55.0	1.09 (1H, m)
C-10	36.7		36.8	
C-11	28.1	0.90 (3H, s)	28.1	0.90 (3H, s)
C-12	15.1	0.88 (3H, s)	15.1	0.93 (3H, s)
C-13	17.3	2.51 (1H, dd, J=17.0, 5.0 Hz)	17.3	2.51 (1H, dd, J=17.0, 4.5 Hz)
		2.23 (1H, dd, J=17.0, 13.0 Hz)		2.23 (1H, dd, J=17.0, 13.0 Hz)
C-13-OH				
C-14	20.7	1.26 (3H, s)	20.7	1.26 (3H, s)
C-15	16.6	0.93 (3H, s)	15.1	0.89 (3H, s)
1-O-CO-(CH_2)-CH_3	21.1	2.05 (3H, s)	9.3	1.14 (3H, t, J=7.5 Hz)
1-O-CO-CH_2-CH_3			28.0	2.33 (2H, dd, J=15.0, 7.5 Hz)
1-O-CO-(CH_2)-CH_3	170.8		174.2	
7-O-CO-(CH_2)-CH_3				
7-O-CO-CH_2-CH_3				
7-O-CO-(CH_2)-CH_3				
11-O-CO-(CH_2)-CH_3				
11-O-CO-CH_2-CH_3				
11-O-CO-(CH_2)-CH_3				
C-2′	163.9		164.0	
C-3′	100.2		100.3	
C-4′	162.8		162.8	
C-5′	99.3	6.41 (1H, s)	99.4	6.42 (1H, s)
C-6′	155.6		155.6	
C-2″	146.6	8.97 (1H, d, J=2.0 Hz)	146.6	8.98 (1H, d, J=2.0 Hz)
C-3″	127.6		127.6	
C-4″	132.7	8.08 (1H, ddd, J=8.0, 2.5, 1.5 Hz)	132.8	8.09 (1H, ddd, J=8.0, 2.0, 1.5 Hz)
C-5″	123.5	7.37 (1H, dd, J=8.0, 4.5 Hz)	123.6	7.38 (1H, dd, J=8.0, 4.5 Hz)
C-6″	151.0	8.63 (1H, dd, J=4.5, 1.5 Hz)	151.0	8.64 (1H, dd, J=4.5, 1.5 Hz)

Carbon No.	Pyripyropene G		Pyripyropene H	
	^{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	80.3	4.52 (1H, dd, J=11.5, 5.0 Hz)	80.8	4.53 (1H, dd, J=11.5, 5.0 Hz)
C-2	23.3	1.77 (2H, m)	23.4	1.77 (2H, m)
C-3	36.8	1.33 (1H, m)	36.8	1.37 (1H, m)
		2.14 (1H, m)		2.14 (1H, m)
C-4	38.1		38.1	
C-5	56.1	1.47 (1H, m)	56.1	1.48 (1H, m)
C-6	82.2		82.3	
C-7	41.4	2.12 (1H, dt, J=13.0, 3.0 Hz)	41.4	2.14 (1H, dt, J=13.0, 3.0 Hz)
		1.68 (1H, m)		1.55 (1H, m)
C-8	19.5	1.61 ~ 1.78 (2H, m)	19.5	1.77 ~ 1.80 (2H, m)
C-9	55.6	1.02 (1H, dd, J=12.0, 2.5 Hz)	55.6	1.05 (1H, dd, J=12.0, 2.5 Hz)
C-10	37.8		37.8	
C-11	28.3	0.89 (3H, s)	28.3	0.89 (3H, s)
C-12	17.0	1.37 (3H, s)	17.0	1.37 (3H, s)
C-13	60.4	4.96 (1H, br d, J=4.0 Hz)	60.5	4.97 (1H, br d, J=4.0 Hz)
C-13-OH		2.84 (1H, br s)		2.82 (1H, br s)
C-14	22.1	1.65 (3H, s)	22.2	1.65 (3H, s)
C-15	16.6	0.91 (3H, s)	16.6	0.92 (3H, s)
1-O-CO-(CH_2)-CH_3	21.3	2.06 (3H, s)	9.3	1.16 (3H, t, J=7.5 Hz)
1-O-CO-CH_2-CH_3			28.0	2.34 (2H, dd, J=15.0, 7.5 Hz)
1-O-CO-(CH_2)-CH_3	170.9		174.2	
7-O-CO-(CH_2)-CH_3				
7-O-CO-CH_2-CH_3				
7-O-CO-(CH_2)-CH_3				
11-O-CO-(CH_2)-CH_3				
11-O-CO-CH_2-CH_3				
11-O-CO-(CH_2)-CH_3				
C-2′	164.2		164.2	
C-3′	103.3		103.3	
C-4′	162.7		162.7	
C-5′	99.6	6.45 (1H, s)	99.6	6.46 (1H, s)
C-6′	157.1		157.1	
C-2″	146.8	9.00 (1H, d, J=2.0 Hz)	146.8	9.00 (1H, d, J=2.0 Hz)
C-3″	127.3		127.4	
C-4″	132.9	8.10 (1H, ddd, J=8.0, 2.0, 1.5 Hz)	133.0	8.10 (1H, ddd, J=8.0, 2.0, 1.5 Hz)
C-5″	123.6	7.40 (1H, dd, J=8.0, 4.5 Hz)	123.6	7.40 (1H, dd, J=8.0, 4.5 Hz)
C-6″	151.4	8.67 (1H, dd, J=4.5, 1.5 Hz)	151.4	8.68 (1H, dd, J=4.5, 1.5 Hz)

Table 2-2. ^{1}H and ^{13}C NMR chemical shifts of pyripyropenes E to L.

Carbon No.	Pyripyropene I		Pyripyropene J	
	^{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	73.3	4.80 (1H, dd, J=12.0, 5.0 Hz)	73.2	4.78 (1H, dd, J=12.0, 5.0 Hz)
C-2	22.7	1.80 (1H, m)	22.7	1.80 (1H, m)
		1.89 (1H, m)		1.87 (1H, m)
C-3	36.2	1.38 (1H, m)	36.1	1.37 (1H, m)
		2.16 (1H, m)		2.14 (1H, m)
C-4	37.9		37.8	
C-5	54.8	1.53 (1H, m)	54.6	1.53 (1H, m)
C-6	83.4		83.3	
C-7	77.5	5.02 (1H, m)	77.5	5.00 (1H, m)
C-8	25.3	1.62 (1H, m)	25.2	1.58 (1H, m)
		1.79 (1H, m)		1.76 (1H, m)
C-9	45.5	1.62 (1H, m)	45.3	1.60 (1H, m)
C-10	40.5		40.4	
C-11	64.7	3.68 (1H, d, J=12.0 Hz)	64.8	3.66 (1H, d, J=12.0 Hz)
		3.80 (1H, d, J=12.0 Hz)		3.77 (1H, d, J=12.0 Hz)
C-12	17.5	1.44 (3H, s)	17.4	1.42 (3H, s)
C-13	60.3	4.99 (1H, d, J=4.0 Hz)	60.1	4.98 (1H, d, J=4.0 Hz)
C-13-OH		2.90 (1H, br s)		3.10 (1H, br s)
C-14	16.3	1.69 (3H, s)	16.3	1.68 (3H, s)
C-15	13.3	0.90 (3H, s)	13.2	0.87 (3H, s)
1-O-CO-(CH_2)-CH_3	9.18	1.13 (3H, t, J=7.5 Hz)	9.09	1.10 (3H, t, J=7.5 Hz)
1-O-CO-CH_2-CH_3	27.85	2.32 (2H, dq, J=1.5, 7.5 Hz)	27.80	2.29 (2H, dq, J=1.5, 7.5 Hz)
1-O-CO-(CH_2)-CH_3	173.8		173.8	
7-O-CO-(CH_2)-CH_3	9.18	1.21 (3H, t, J=7.5 Hz)	9.11	1.19 (3H, t, J=7.5 Hz)
7-O-CO-CH_2-CH_3	27.87	2.44 (2H, dq, J=4.0, 7.5 Hz)	27.79	2.42 (2H, dq, J=4.0, 7.5 Hz)
7-O-CO-(CH_2)-CH_3	173.3		173.4	
11-O-CO-(CH_2)-CH_3	9.18	1.16 (3H, t, J=7.5 Hz)	20.8	2.07 (3H, s)
11-O-CO-CH_2-CH_3	27.6	2.37 (2H, dq, J=1.5, 7.5 Hz)		
11-O-CO-(CH_2)-CH_3	174.2		170.9	
C-2′	164.0		163.9	
C-3′	102.9		102.9	
C-4′	162.2		162.2	
C-5′	99.4	6.42 (1H, s)	99.4	6.41 (1H, s)
C-6′	157.4		157.2	
C-2″	146.9	9.00 (1H, d, J=2.0 Hz)	146.7	8.98 (1H, d, J=2.0 Hz)
C-3″	127.2		127.1	
C-4″	133.0	8.09 (1H, ddd, J=8.0, 2.0, 1.0 Hz)	133.0	8.07 (1H, ddd, J=8.0, 2.0, 1.0 Hz)
C-5″	123.6	7.40 (1H, dd, J=8.0, 5.0 Hz)	123.6	7.38 (1H, dd, J=8.0, 5.0 Hz)
C-6″	151.6	8.68 (1H, dd, J=5.0, 1.0 Hz)	151.4	8.66 (1H, dd, J=5.0, 1.0 Hz)

Carbon No.	Pyripyropene K		Pyripyropene L	
	^{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	73.3	4.80 (1H, dd, J=12.0, 5.0 Hz)	73.5	4.77 (1H, dd, J=12.0, 5.0 Hz)
C-2	22.7	1.82 (1H, m)	22.7	1.81 (1H, m)
		1.89 (1H, m)		1.88 (1H, m)
C-3	36.2	1.38 (1H, m)	36.2	1.36 (1H, m)
		2.15 (1H, m)		2.14 (1H, m)
C-4	37.9		37.8	
C-5	54.8	1.51 (1H, m)	54.7	1.50 (1H, m)
C-6	83.2		83.3	
C-7	77.7	5.00 (1H, m)	77.4	5.00 (1H, m)
C-8	25.2	1.62 (1H, m)	25.2	1.61 (1H, m)
		1.78 (1H, m)		1.78 (1H, m)
C-9	45.5	1.60 (1H, m)	45.4	1.60 (1H, m)
C-10	40.5		40.4	
C-11	64.6	3.73 (1H, d, J=12.0 Hz)	64.7	3.67 (1H, d, J=12.0 Hz)
		3.76 (1H, d, J=12.0 Hz)		3.80 (1H, d, J=12.0 Hz)
C-12	17.4	1.43 (3H, s)	17.4	1.43 (3H, s)
C-13	60.2	4.97 (1H, d, J=4.0 Hz)	60.1	4.99 (1H, d, J=4.0 Hz)
C-13-OH		3.00 (1H, br s)		3.06 (1H, br s)
C-14	16.3	1.68 (3H, s)	16.3	1.68 (3H, s)
C-15	13.3	0.88 (3H, s)	13.2	0.88 (3H, s)
1-O-CO-(CH_2)-CH_3	9.14	1.12 (3H, t, J=7.5 Hz)	21.1	2.02 (3H, s)
1-O-CO-CH_2-CH_3	27.8	2.30 (2H, dq, J=1.5, 7.5 Hz)		
1-O-CO-(CH_2)-CH_3	173.7		170.4	
7-O-CO-(CH_2)-CH_3	21.2	2.14 (3H, s)	9.14	1.20 (3H, t, J=7.5 Hz)
7-O-CO-CH_2-CH_3			27.8	2.42 (2H, dq, J=4.0, 7.5 Hz)
7-O-CO-(CH_2)-CH_3	170.0		173.3	
11-O-CO-(CH_2)-CH_3	9.14	1.14 (3H, t, J=7.5 Hz)	9.11	1.14 (3H, t, J=7.5 Hz)
11-O-CO-CH_2-CH_3	27.5	2.36 (2H, dq, J=1.5, 7.5 Hz)	27.5	2.36 (2H, dq, J=1.5, 7.5 Hz)
11-O-CO-(CH_2)-CH_3	174.2		174.2	
C-2′	163.9		163.9	
C-3′	102.9		102.9	
C-4′	162.1		162.2	
C-5′	99.4	6.41 (1H, s)	99.4	6.42 (1H, s)
C-6′	157.3		157.3	
C-2″	146.8	8.99 (1H, d, J=2.0 Hz)	146.8	8.98 (1H, d, J=2.5 Hz)
C-3″	127.1		127.1	
C-4″	132.9	8.08 (1H, ddd, J=8.0, 2.0, 1.0 Hz)	132.9	8.08 (1H, ddd, J=8.0, 2.5, 1.5 Hz)
C-5″	123.6	7.39 (1H, dd, J=8.0, 5.0 Hz)	123.6	7.39 (1H, dd, J=8.0, 5.0 Hz)
C-6″	151.5	8.67 (1H, dd, J=5.0, 1.0 Hz)	151.5	8.67 (1H, dd, J=5.0, 1.5 Hz)

[a] Each sample was dissolved in $CDCl_3$. 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.

The ^{1}H-^{1}H COSY spectrum revealed the six partial structures I to VI, and ^{13}C-^{1}H long-range couplings of ^2J and ^3J in the HMBC spectrum confirmed the structure of pyripyropene L as shown in Fig. 4. Thus, pyripyropene L has the same core structure of pyridino-α-pyrone sesquiterpene as pyripyropene A[3]. The differences between pyripyropene L and A were found in the acyloxy groups at C-1, C-7, and C-11. Concerning the C-1 position, the cross peaks from H-1 and 1-*O*–CO–C*H*$_3$ (δ 2.02) to 1-*O*–*C*O–CH$_3$ (δ 170.4) indicated the presence of an acetoxy residue in pyripyropene L as well as in pyripyropene A. As for the positions of C-7 and C-11 the cross peaks from H-7, 7-*O*–CO–C*H*$_2$–CH$_3$ (δ 2.42) and 7-*O*–CO–CH$_2$–C*H*$_3$ (δ 1.20) to 7-*O*–*C*O–CH$_2$–CH$_3$ (δ 173.3), and from H$_2$-11, 11-*O*–CO–C*H*$_2$–CH$_3$ (δ 2.36) and 11-*O*–CO–CH$_2$–C*H*$_3$ (δ 1.14) to 11-*O*–*C*O–CH$_2$–CH$_3$ (δ 174.2) indicated that a propionylxy residue was attached at both C-7 and C-11 for pyripyropene L. Fragment ion peaks at *m/z* 537 [M−PrOH]$^+$, 477 [M−PrOH−AcOH]$^+$ and 403 [M−2PrOH−AcOH]$^+$ in EI-MS supported the presence of one acetyl and two propionyl residues. Taken together, the structure of pyripyropene L was elucidated as shown in Fig. 1.

Structures of Pyripyropenes I, J and K

The molecular formulae of pyripyropenes J and K are the same as that of pyripyropene L, suggesting that they are positional stereoisomers of the acyloxy groups. In fact, all the spectral data including ^{1}H and ^{13}C NMR, HMBC and EI-MS indicated that pyripyropene J has a propionylxy residue at C-1 and C-7 and an acetoxy residue at C-11 and that pyripyropene K has a propionylxy residue at C-1 and C-11 and an acetoxy residue at C-7. The positions of all the acyloxy residues were confirmed by the HMBC experiments.

The molecular formula of pyripyropene I is a CH$_2$ unit larger than that of pyripyropene L, suggesting a propionylxy residue at all the C-1, C-7 and C-11 positions. All the spectral data supported the structure.

Taken together, the structures of pyripyropenes I, J and K were elucidated as shown in Fig. 1.

Structures of Pyripyropenes E, F, G and H

Comparison of the ^{13}C NMR (Table 2) and the DEPT spectra of pyripyropenes H and I revealed that 1) two propionylxy moieties (CH$_3$–CH$_2$–CO–O–) are lacking in pyripyropene H, and 2) a methylene carbon of C-7 (δ 41.4) and a methyl carbon of C-11 (δ 28.3) were observed in pyripyropene H instead of an oxymethine C-7 (δ 77.5) and an oxymethylene C-11 (δ 64.8) in pyripyropene I. In fact, the following evidence supported the structure of pyripyropene H; 1) observation of cross peaks from the methine proton of H-9 (δ 1.05) to the methylene protons of H$_2$-8 (δ 1.77∼1.80) and H$_2$-7 (δ 2.14, 1.55), in the ^{1}H-^{1}H COSY spectrum, and 2) long-range couplings from H$_2$-7 to C-6 (82.3), from H$_3$-14 (δ 1.65) to C-7, from H$_3$-11 (δ 0.89) to C-1 (δ 80.8) and C-9 (δ 55.6), from H-1 (δ 4.53) to C-11 and C-15 (δ 16.6) and from H$_3$-11 and H$_3$-15 (δ 0.92) to C-1 in HMBC experiments. The results indicated that pyripyropene H is 7,11-didepropionylxy pyripyropene I (Fig. 1).

Comparison of all the spectral data between pyripyro-

Fig. 4. ^{1}H-^{1}H COSY, HMQC and HMBC experiments of pyripyropene L.

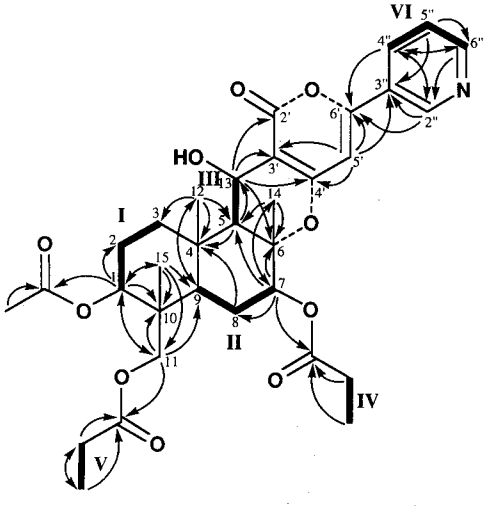


Table 3. Single crystal X-ray crystallographic analysis.

Crystal parameters	
Empirical formula	$C_{27}H_{33}NO_5$
Formula weight	451.56
Crystal dimensions (mm)	0.4×0.4×0.3
Crystal system	Monoclinic
Lattice Parameters:	a=34.905 (5) Å
	b=7.467 (3) Å
	c=9.271 (2) Å
	β=103.02 (3)°
	V=2354 (1) Å^3
Space group	C_2 with Z=4
Density calc (g/cm^3)	1.274
Linear absorption factor (cm^{-1})	6.67
Refinement parameters	
No. of reflections measured	2,304
Nonzero reflections ($I>3.00\sigma$)	1,571
R-indexa Residuals: R[a]	0.083
Residuals: R_w[b]	0.081
Goodness of fit indicator[c]	5.28

[a] $\Sigma \| Fo| - |Fc| / \Sigma |Fo|$.
[b] $[(\Sigma_w(|Fo| - |Fc|)^2 / \Sigma_w Fo)]^{1/2}$.
[c] $[\Sigma_w(|Fo|^2 - |Fc|)^2 / (No - Nv)]^{1/2}$.
No = number of observations.
Nv = number of variables.

penes G and H suggested that pyripyropene G has an acetoxy residue in place of the corresponding propionylxy residue of pyripyropene H. The position of the acetoxy residue in pyripyropene G was confirmed by observing the long-range couplings from H-1 (δ 4.51) and 1-*O*–CO–C*H*$_3$ (δ 2.05) to 1-*O*–*C*O–CH$_3$ (δ 174.2) in HMBC experiments. Thus, pyripyropene G was determined to be 7,11-didepropionylxy pyripyropene L (Fig. 1).

Comparison of all the spectral data between pyripyropenes F and H indicated that pyripyropene F is deficient in the hydroxyl group of pyripyropene H. The structure of pyripyropene F was confirmed by HMBC experiments. Especially, the long range couplings were observed from H-5 (δ 1.51) to C-13 (δ 17.3) and from H$_2$-13 (δ 2.51, 2.23) to C-4 (δ 36.8), C-5 (δ 51.4), C-6 (δ 80.8), C-2′ (δ 164.0), C-3′ (δ 100.3) and C-4′ (δ 162.8). Thus, pyripyropene F was determined to be 13-deoxy pyripyropene H (Fig. 1).

All the spectra data of pyripyropene E indicated that pyripyropene E is 13-deoxy pyripyropene G, which was confirmed by HMBC experiments. The structure of pyripyropene E (Fig. 1) was identical with GERI-BP001[11].

X-Ray Crystallographic Analysis of Pyripyropene E

A single crystal X-ray analysis of pyripyropene E, which was recrystallized from MeOH, was carried out. The analytical data are summarized in Table 3. The non-hydrogen atoms were refined anisotropically. The final cycle of full-matrix least-squares refinement was based on 1571 observed reflections and 297 variable parameters and converged with unweighted and weighted agreement factors of $R=0.083$, $R_w=0.081$. Thus, the relative configuration of pyripyropene E was secured as shown in Fig. 5. The stereochemistry is the same as that of pyripyropene A[4], which was defined by the Mosher NMR method using the two tris-Mosher ester derivatives and application of the Kakisawa-Kashman test. All the pyripyropenes described above are biosynthetically related and are expected to share the same relative and absolute stereochemistry as shown in Fig. 1.

Biological Properties

Effect of Pyripyropenes on ACAT Activity in Microsomes

As shown in Fig. 6, pyripyropenes inhibited ACAT

Fig. 5. Relative molecular structure of pyripyropene E determined by X-ray crystallography.

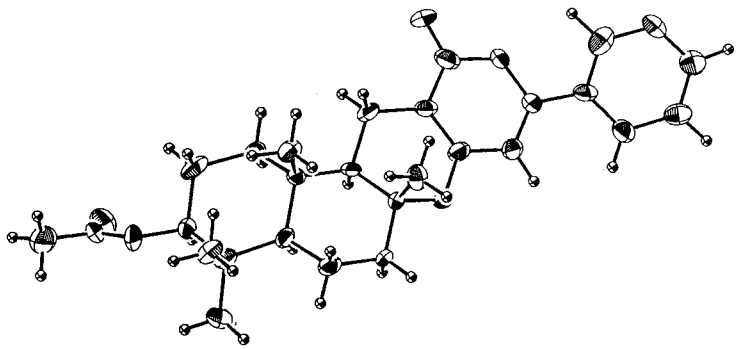
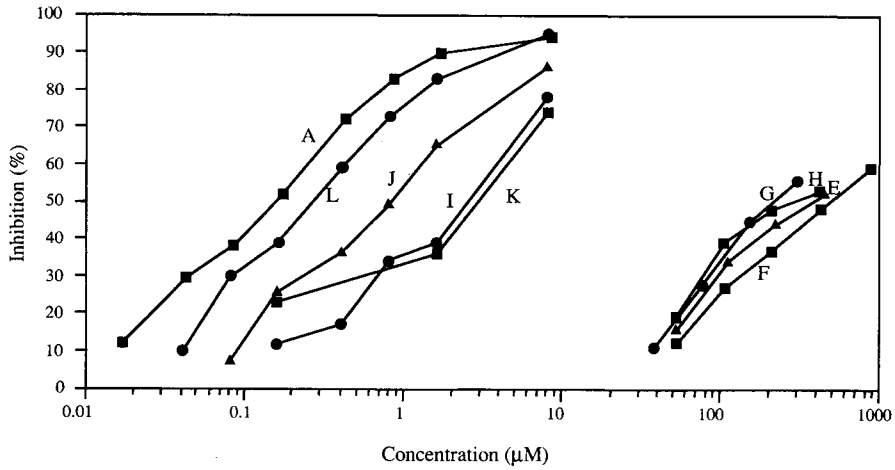


Fig. 6. ACAT inhibition by pyripyropenes A and E to L in the enzyme assay using rat liver microsomes.



activity dose-dependently in the enzyme assay. Among the new pyripyropenes, pyripyropene L showed the most potent inhibitory activity with an IC_{50} value of 0.27 μM, followed by pyripyropenes J (IC_{50} 0.85 μM), I (2.45 μM) and K (2.65 μM). Under the same conditions, the IC_{50} value of pyripyropene A was 0.16 μM, which was almost the same as that reported previously.[1,2] However, pyripyropenes G (IC_{50} 221 μM), H (270 μM), E (399 μM) and F (559 μM) were much less potent ACAT inhibitors than the other pyripyropenes.

Other Biological Activities

No antimicrobial activity was observed at a concentration of 1 mg/ml (10 μg/disk) for pyripyropenes E to L against the following microorganisms; *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces sake*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*.

Discussion

A total of 12 different pyripyropenes A to L have been isolated from *A. fumigatus* FO-1289-2501 selected as a high producer of pyripyropenes when cultured in a production medium supplying 0.002% nicotinic acid. Our recent biosynthetic study demonstrated that intact nicotinic acid was incorporated into the pyripyropene molecule. Eventually, the production of pyripyropene A (*ca.* 30 μg/ml) increased about 10 times over that reported previously.[2] Furthermore, sufficient pyripyropenes E to L were obtained to elucidate the chemical structures. All the pyripyropenes have the common pyridino-α-pyrone sesquiterpene core, and differ in the presence/absence of acyloxy residues at C-7 and C-11, and of the hydroxy residue at C-13. In this paper, the positions of the acyloxy residues at C-1, C-7 or C-11 were confirmed completely by HMBC experiments. The fragment ion peaks in EI-MS also supported the structures of pyripyropenes; the presence of the hydroxy residue at C-13 yielded $[M-H_2O]^+$, then the acyloxy residues fragmented according to the order, C-7 > C-1 > C-11 positions. For example, in case of pyripyropene K having 13-OH, 7-*O*-acetyl, 1-*O*-propionyl and 11-*O*-propionyl residues, fragment ion peaks of *m/z* 593 $[M-H_2O]^+$, 551 $[M-AcOH]^+$, 477 $[M-AcOH-PrOH]^+$ and 403 $[M-AcOH-2PrOH]^+$ were observed.

Pyripyropenes E to H, lacking the acyloxy residues at C-7 and C-11, showed greatly decreased ACAT inhibitory activity (Fig. 6). The result indicates that at least the two acyloxy residues are responsible for exhibiting potent ACAT inhibition. JEONG *et al.* reported that GERI-BP001, which seemed identical with pyripyropene E, showed moderate ACAT inhibitory activity with an IC_{50} value of 50 μM in the similar assay using rat liver microsomes (*cf.* 399 μM in our assay). The discrepancy in ACAT inhibitory activities is unclear. The structure-activity relationships of these pyripyropenes will be reported elsewhere.

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