NOTES

A Cholesteryl Ester Transfer Protein Inhibitor from an Insect-associated Fungus

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Atherosclerosis is caused by the deposition of lipids, especially cholesterol, on to arterial walls, and continued lipid deposition leads to coronary heart disease.[1,2] Epidemiological studies show the risk of coronary heart disease correlates with increased cholesterol levels associated with low-density lipoprotein (LDL) and inversely correlates with increased cholesterol levels associated with high-density lipoprotein (HDL).[1,2] The transfer of cholesterol in the form of cholesteryl esters from HDL to LDL is mediated by cholesteryl ester transfer protein (CETP), a 66~77 kDa plasma glycoprotein.[3] Studies have shown that CETP activity increases in the presence of a high fat, high cholesterol diet, and that individuals deficient in CETP activity are usually resistant to atherosclerosis.[3] CETP, which has been purified, cloned, and overexpressed in bacteria and Chinese hamster ovary cells, can conveniently be used in an *in vitro* assay system.[4~6] Since CETP inhibitors could be important therapeutic agents, we began a program to search for such inhibitors among the secondary metabolites produced by fungi living in close association with plants and insects. In the course of this program, we detected a compound with CETP inhibitory activity from an insect-associated fungus.

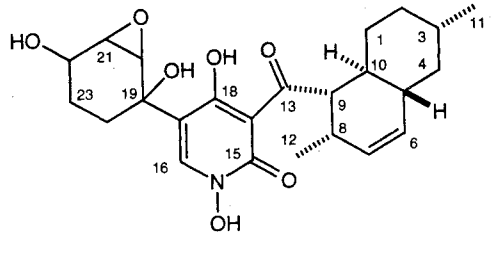
Fermentation and Compound Isolation

The fungus CLR243, tentatively identified as *Cytospora*, was collected in Lake Placid, Florida (U.S.) and isolated from the seed stores of the ant *Pogonomyrmex badius*. CLR243 was cultured in Sabouraud dextrose broth (DIFCO) using 100 ml media in 500 ml Erlenmeyer flasks (1.0 liter total), shaken at 275 rpm at 24°C, and harvested when samples of the culture exhibited maximum CETP inhibition. The fungal cultures were filtered through cheesecloth to remove the mycelia and the clarified broth was extracted twice with ethyl acetate. Evaporation of the solvent *in vacuo* yielded 900 mg of a crude brown extract. The organic extract was first subjected to a 50.0 × 2.0 cm Sephadex LH-20 column (1 : 1 CH_2Cl_2 - MeOH) and 9 ml fractions collected. The active fractions were pooled and applied to a dry-packed C-18 column (10.0 × 1.5 cm column) and eluted with step gradients of 50, 70, and 100% MeOH - H_2O using nitrogen pressure. The fractions were combined according to their TLC profile (SiO_2) and assay results indicated that the majority of the compound was eluted by 70% MeOH - H_2O. The compound appeared to be pure (40 mg) as judged by ^1H NMR analysis.

Results and Discussion

The ^1H and ^{13}C NMR spectra of compound **1** in d_4-methanol showed resonances for 27 non-exchangeable protons and 24 carbons in the molecule. The molecular formula was determined by HRFAB-MS as $C_{24}H_{31}NO_7$, requiring ten degrees of unsaturation and four exchangeable protons. When treated with ninhydrin, the compound did not develop a color and presumably lacked a primary amine. The strong absorptions at 3600~3100 cm^{-1} and 1650 cm^{-1} in the IR spectrum indicated the presence of hydroxyl groups and an unsaturated carbonyl functionality. From a combination of short and long-range COSY, HMQC, and HMBC experiments (Table 1), the structure shown as **1** was deduced.

Construction of the unsaturated decalin ring began with the 9.5 Hz *cis* coupling between the olefinic protons H-6 (5.38 ppm) and H-7 (5.58 ppm). The homonuclear correlation between H-7 and H-8 (2.83 ppm), the four-bond correlation between H-7 and H-9 (4.43 ppm), and the 7.5 Hz coupling between H-8 and the C-12 methyl group formed part of the decalin structure. The coupling of H-10 (1.55 ppm) to H-5 (1.82 ppm) and H-9 (4.4 ppm), and the HMBC correlation of C-5 (43.2 ppm) to H-6, H-7 and H-9 completed the first ring. The remaining connectivity of the decalin fragment was deduced by the observed COSY correlations between H_b-4 (0.78 ppm) and H-5 (1.82 ppm), and HMBC correlations between H_3-11 methyl to C-2, C-3, C-4, that of H-10 (1.55 ppm)



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to C-1 and C-5, and that of H-9 (4.43 ppm) to the carbonyl at 212.1 ppm (C-13).

The cyclohexanol epoxide ring was constructed by straightforward analysis of the NMR data. The presence of the epoxide at C-20/C-21 was first suggested by their chemical shifts (60.5 and 57.7 ppm respectively) and confirmed later by peracetylation of **1**. The remaining five sp^2 carbon signals, three oxygens, and one nitrogen atom must make up a pyridone ring. H-16 (8.00 ppm) exhibited correlations to C-15, C-17, and C-18, with a weaker, long-range correlation to C-14. The magnitude of the chemical shifts is consistent with an α-pyridone

Table 1. NMR data for compound **1** (d_4-methanol).

^{13}C (100 MHz)[a]			^{1}H (*J* Hz, 500 MHz)[b,c]	COSY	r-COSY	HMBC
1	30.9	t	1.91 (dq, 12.0, 2.0)	0.88	1.73, 1.55, 1.02	1.55
			0.88	1.91, *1.55		
2	36.6	t	1.73	1.48, 1.02	1.91, 1.48	1.91, 0.92
			1.02 (qd, 12.0, 3.0)	1.73, 1.48	1.91	
3	34.4	d	1.48	1.82, 1.02, 0.92	1.73	1.91, 0.92
4	43.1	t	1.73	0.78	1.91	1.91, 1.55, 0.92
			0.78	*1.82, 1.73		
5	43.2	d	1.82	1.55, *0.78	5.38, 4.43	5.58, 5.38, 4.43
6	131.7	d	5.38 (br d, 9.5)	5.58	2.83, 1.82	1.82
7	132.6	d	5.58 (ddd, 9.5, 4.0, 2.0)	5.38, 2.83	4.43	2.83, 0.80
8	32.4	d	2.83	5.58, 4.43, 0.80	5.38	5.58, 5.38, 4.43, 0.80
9	54.5	d	4.43 (dd, 11.5, 6.0)	2.83, 1.55	5.58, 1.82, 0.80	5.58, 1.55, 0.80
10	37.6	d	1.55 (qd, 11.5, 2.0)	4.43, 1.82, *0.88	1.91	5.38, 4.43
11	22.9	q	0.92 (d, 6.5)	1.48		
12	18.4	q	0.80 (d, 7.5)	2.83	4.43	4.43, 2.83
13	212.1	s				4.43
14	108.5	s				8.00
15	158.7	s				8.00
16	139.0	d	8.00 (s)			
17	114.5	s				8.00, 2.22, 1.67
18	175.8	s				8.00
19	70.5	s				8.00, 3.63, 2.22, 1.82, 1.67, 1.34
20	60.5	d	3.63 (d, 3.5)	3.40		2.22, 1.67
21	57.7	d	3.40 (dd, 3.5, 3.0)	4.10, 3.63		1.82
22	67.5	d	4.10 (td, 7.5, 3.0)	3.40, 1.82, 1.34	2.22, 1.67	2.22, 1.67, 1.34
23	25.8	t	1.82	2.22, 1.34		3.40, 2.22, 1.67
			1.34	1.82, 1.67	2.22	
24	31.8	t	2.22 (ddd, 14.0, 9.0, 2.5)	1.82, 1.67	4.10, 1.34	4.10, 3.62
			1.67	2.22, 1.34	4.10	

[a] Multiplicity was determined from DEPT spectrum. [b] Assignments to carbons were based on HMQC spectrum. [c] Those signals without designated splitting pattern were either multiplets or buried by other signals.

* Data of these proton correlations were observed from DQF-COSY spectrum.

Table 2. Comparative NMR data for **1** and its triacetate.

1 (d_4-methanol)			**1** triacetate ($CDCl_3$)	
^{13}C (100 MHz)		^{1}H (*J* Hz, 500 MHz)	^{13}C (100 MHz)[a]	^{1}H (*J* Hz, 500 MHz)[b]
175.8	s		176.4 s	
158.7	s		155.9 d	
139.0	d	8.00 (s)	139.1 d	7.61 (s)
132.6	d	5.58 (ddd, 9.5, 4.0, 2.0)	131.2 d	5.56 (ddd, 9.5, 4.5, 2.5)
131.7	d	5.38 (br d, 9.5)	130.6 d	5.37 (br d, 9.5)
114.5	s		110.9 d	
108.5	s		107.8 s	
70.5	s		76.8 s	
67.5	d	4.10 (td, 7.5, 3.0)	67.6 d	5.22 (td, 8.5, 3.0)
60.5	d	3.63 (d, 3.5)	56.0 d	4.03 (d, 4.0)
57.7	d	3.40 (dd, 3.5, 3.0)	54.0 d	3.58 (dd, 4.0, 3.0)
54.5	d	4.43 (dd, 11.5, 6.0)	53.0 d	4.26 (dd, 11.0, 6.0)

[a] Multiplicity confirmed by DEPT spectrum. [b] Assignments to carbons based on splitting pattern.

hydroxylated at the C-18 position (175.8 ppm), requiring the final oxygen to be present on the nitrogen as a hydroxamic acid. Connectivity of the *N*-oxo pyridone with the cyclohexyl ring was determined by the correlation of H-16 (8.00 ppm) with C-19 (70.5 ppm), leaving C-14 as the link to the decalin ring *via* C-13 and giving the final structure as shown in **1**.

Acetylation of **1** with acetic anhydride in pyridine produced a triacetate. The acetylation occurred at C-19, C-22, and the *N*-oxo sites, as evidenced from ^{1}H and ^{13}C NMR spectra in $CDCl_3$ (Table 2). Presumably the hydroxyl on C-18 is relatively unreactive due to hydrogen bonding with the C-13 carbonyl. The hydrogen-bonded proton could be observed both in **1** (17.25 ppm) and its triacetate (16.80 ppm) when their ^{1}H spectra were acquired with $CDCl_3$ solutions.

The relative stereochemistry of compound **1** could be determined only for the decalin fragment. NOe data and coupling constant information indicated that the relative configurations at C-5 and C-10 give a *trans* ring fusion. The quartet of doublets for H-10 (11.5, 2.0 Hz) required three *trans*-diaxial couplings and one axial-equatorial coupling, giving C-9 its assigned configuration. The 6.0 Hz coupling constant for H-8 and H-9 suggested an axial-equatorial relationship, resulting in a *syn* configuration. Finally, the lack of observed nOe between H_3-11 (0.92 ppm) and H-5 suggested that the methyl group is equatorial, which is also supported by the apparent quartet of doublets (12.0, 3.0 Hz) for H-2_{ax} (1.02 ppm). The partial relative stereochemistry is therefore as shown in **1**.

Compound **1** is most closely related to the fungal metabolites apiosporamide[7] and fischerin.[8] Apiosporamide bears the same relative stereochemistry but lacks the *N*-hydroxyl group, while fischerin contains a *cis*-decalin and lacks the C-3 methyl substituent. Compound **1** has weaker activity ($IC_{50}=40\,\mu M$) than other CETP inhibitors (strongest inhibitor at $0.3\,\mu M$).[9] Apiosporamide and fischerin were isolated based on other biological activities, and have not been tested for CETP inhibition.

Experimental

General

^{1}H and 2-D NMR spectra were recorded on Varian Unity 500 spectrometer, while the ^{13}C spectra (100 MHz) were recorded on Varian Unity 400 spectrometer. The ultraviolet spectrum was obtained from Shimadzu UV160U UV-Vis spectrophotometer and the infrared spectrum from Perkin-Elmer 16 PC FT-IR. Mass spectral data was acquired by the University of Illinois, Urbana, Mass Spectometry Facility. Melting point was obtained on Fisher-Johns Melting Point Apparatus and the optical rotation was measured on Perkin-Elmer 241 polarimeter.

CETP Inhibition Assay

Schering-Plough Research Institute performed the assays.[9] The Amersham Scintillation Proximity Assay (SPA) kit was used to test for CETP inhibition. In this CETP-SPA assay, the transfer of [^{3}H]cholesteryl esters from HDL to biotinylated LDL is measured following incubation of donor ([3H]CE-HDL) and acceptor (biotinylated LDL) particles in the presence of human recombinant CETP. Following incubation at 37°C in 96-well plates (Dynatech) for 4 hours, the reaction is terminated by adding 200 ml of streptavidin SPA formulated in an assay termination buffer. Transfer is measured after counting cpm on a TopCount 96-well scintillation counter (Packard). The rate of increase in signal is proportional to the transfer of [3H]cholesteryl esters by CETP.

Physical-chemical Data

Compound **1**. Light yellow solid, mp 148°C (dec.); $[\alpha]_D^{25}=-59.7°$ *c* 0.479, MeOH; HRFAB-MS: *m/z* $[M+H]^+$ 446.2171, observed; calc. for $C_{24}H_{32}NO_7$, *m/z* 446.2178; IR (AgCl) br 3600~3100, 2950, 2905, 1650 cm^{-1}; UV (MeOH) λ_1 231 ($\varepsilon=19000$), λ_2 283 ($\varepsilon=12000$), λ_3 339 ($\varepsilon=12000$).

Compound **1** triacetate. Pale yellow oil; HRFAB-MS: *m/z* $[M+H]^+$ 572.2480, observed; calc. for $C_{30}H_{38}NO_{10}$, *m/z* 572.2495; IR (AgCl) 2950, 2905, 1760, 1740, 1675 cm^{-1}.

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