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Structure-specific Inhibition of Cholesteryl Ester Transfer Protein by Azaphilones

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The effect of thirteen different fungal azaphilones, which have a common 6-isochromane-like ring, was tested on cholesteryl ester transfer protein (CETP) activity *in vitro*. Chaetoviridin B showed the most potent inhibitory activity with an IC_{50} value of $<6.2\,\mu M$, followed by sclerotiorin with an IC_{50} value of $19.4\,\mu M$. Rotiorin, chaetoviridin A and rubrorotiorin had moderate inhibitory activity (IC_{50} ; $30\sim40\,\mu M$), but others showed very weak or no inhibitory activity. The relationship between the structures and their inhibitory activity indicated that the presence of an electrophilic ketone(s) and/or enone(s) at both C-6 and C-8 positions in the isochromane-like ring is essential for eliciting CETP inhibitory activity. The transfer activity of both CE and TG was inhibited by sclerotiorin to approximately the same extent (IC_{50}: 14.4 and $10.3\,\mu M$, respectively). A model of the reaction suggested that sclerotiorin reacts with a primary amine of amino acids such as lysine in the protein to form a covalent bond.

The cholesteryl ester transfer protein (CETP) promotes exchange and transfer of neutral lipids such as cholesteryl ester (CE) and triacylglycerol (TG) between plasma lipoproteins[1~3]. CETP is a very hydrophobic and heat stable glycoprotein with an apparent molecular weight of 74 kDa by SDS-PAGE analysis[4,5]. The cDNA from human liver has been cloned and sequenced[6]. It encodes for a 476-amino acid protein (53 kDa), suggesting that the apparent higher molecular weight is due to addition of carbohydrate residues by post-translational modification.

Evidence has been accumulating for the importance of CETP in atherosclerosis; 1) CETP decreased cholesterol concentration in high density lipoprotein (HDL) *in vitro*[7] and *in vivo*[8], 2) rats and mice deficient in CETP activity have high plasma HDL and are resistant to atherosclerosis, 3) human subjects with a genetic deficiency of CETP have very high HDL and low density lipoprotein (LDL) cholesterol levels and are resistant to atherosclerosis[9,10], 4) human CETP gene-introduced transgenic mice have a redistribution of cholesterol from HDL to LDL[8,11,12], and exhibit a marked increase in susceptibility to diet-induced atherosclerosis[12], and 5) antisense oligonucleotides against CETP inhibited the development of atherosclerosis in cholesterol-fed rabbits[13]. Therefore, CETP is proposed as a novel target of inhibition for anti-atherosclerotic drugs. Interestingly, the mechanism of CETP-mediated lipid transfer remains to be investigated. Recently, many compounds of synthetic[14~16] or natural origin[17~21] have been reported to inhibit CETP activity, such as PD 140195[14], cholesterol derivatives of U-617 and U-95594[15], an isoflavan[16], wiedendiols[17,18] and suberitenones[19] isolated from marine sponges, and U-106305 from a *Streptomyces* sp.[20]. A peptide from hog plasma was also reported to inhibit the activity[22]. On the other hand, fungal stachybotramide was reported to stimulate CETP activity[23]. Thus, small molecules modulating the activity have been searched for extensively from therapeutic and biochemical points of

view.

Recently we found that sclerotiorin, a member of the azaphilone, originally isolated as a fungal yellow pigment[24], showed CETP inhibitory activity. Fungal azaphilones with the common isochromane-like ring in their structures have been reported to show several biological activities such as monoamine oxidase inhibition[25], phospholipase A2 inhibition[26], tumor promotion inhibition[27], gp120-CD4 binding inhibition[28] and acyl-CoA: cholesterol acyltransferase (ACAT) inhibition[29]. However, the relationships between structure of azaphilones and their variety of activities and their mechanism of action are unclear. In the present paper, we describe the effect of azaphilones on CETP activity and show the structure-specific CETP inhibition. In addition, a model of the reaction between an amino acid and sclerotiorin suggests the mechanism of CETP inhibition by sclerotiorin.

Materials and Methods

Chemicals

Sclerotiorin, rubrorotiorin, ochrephilone sclerotioramine, isochromophilones I to VI were isolated from cultures of *Penicillium* spp. as described previously[28,29]. Chaetoviridins A and B were a generous gift from Prof. K. KOYAMA, Meiji College of Pharmacy, and rotiorin was from Prof. M. NATSUME, Faculty of Agriculture, Nagoya University. Deacetylsclerotiorin was prepared as follows. Sclerotiorin (39.3 mg, 0.1 mmol) was treated with 12 equivalents of $NaOCH_3$ (64.6 mg) in methanol (1.97 ml) at room temperature for 44 hours. The reaction mixture was extracted with $CHCl_3$ at pH 7, and was purified by silica gel TLC (Merck) to yield pure deacetylsclerotiorin (34.6 mg, 99% yield). Cholesterol, cholesteryl oleate, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), bovine serum albumin (BSA), phosphatidylcholine (PC), dextran sulfate and EDTA commercially available are of analytical grade. Cholesteryl [1-^{14}C]oleate (1.85 GBq/mmol) and [9,10-^{3}H(N)]triolein (370 GBq/mmol) were obtained from New England Nuclear/DuPont. Phenyl-Sepharose CL-4B, DEAE-Sephadex A-50 and Sephadex G200 superfine were purchased from Pharmacia. Bio Gel A-0.5m (200~400 mesh), nitrocellulose membrane and alkaline phosphate-conjugated goat anti-mouse IgG were were purchased from Bio Rad.

Buffers

Experiments described below were performed with Na/K phosphate buffer (ionic strength 0.16, pH 7.4) containing 30.4 mM Na_2HPO_4 and 8.4 mM KH_2PO_4 unless otherwise stated. The concentration of EDTA is 0.025% and the pH of all solutions, including Na/K phosphate buffer of different concentrations, is adjusted to 7.4 except when stated.

Preparation of HDL and LDL

Human plasma (850 ml) was brought to a saline density (d(g/ml) = 1.019) by the addition of saturated KBr solution. Centrifugation was carried out for 24 hours at 150,000 × *g* at 16°C. The upper layer (chylomicron and VLDL) was removed. The lower layer (LDL and HDL) was brought to d = 1.050. Centrifugation was repeated for 24 hours at 150,000 × *g* at 16°C, and the upper (LDL) and lower layers (HDL) were separated. The LDL fraction was recentrifuged using the same conditions. The HDL fraction was brought to d = 1.21 and recentrifuged for 48 hours at 150,000 × *g* at 16°C to obtain the upper layer (HDL). Each resulting solution was dialyzed against Na/K phosphate buffer containing EDTA. Finally, HDL (15 ml, 48 mg protein/ml) and LDL (20 ml, 22 mg protein/ml) fractions were obtained. Protein concentration was determined by the method of LOWRY *et al.*[30] using BSA as a standard.

Preparation of Apo A-I

HDL solution (4 mg protein/ml, 25 ml) was extracted first with an equal volume of ether-ethanol (3 : 2, v/v). The water yellowish phase was then extracted repeatedly (six times) with aliquots of ether-ethanol (3 : 1, v/v) until the water phase became white. At this point, organic solvents (ether and some ethanol) were removed from the protein solution by blowing N_2 over it. Then, to the protein solution was added 6 M urea in Na/K phosphate buffer (5 ml), and the solution was dialyzed at room temperature against 10 mM Tris-HCl buffer (pH 8.6) containing 6 M urea. The dialyzed protein solution (15.4 mg protein/ml, 5 ml) was applied to the Sephadex G200 superfine column (2.5 × 100 cm) using 10 mM Tris-HCl buffer (pH 8.6) containing 6 M urea.

Reconstitution of [^{14}C]CE- and [^{14}C]CE/[^{3}H]TG-HDL

An ethanol solution (1.0 ml), containing 22.5 μmol of phosphatidylcholine, 5.63 μmol of cholesterol and 0.64 μmol of cholesteryl [1-^{14}C]oleate (3.7×10^2 KBq), was rapidly injected into 22 ml of Na/K phosphate buffer containing EDTA through a glass syringe with a 25 gauge needle. After mixing for 5 minutes under a stream of N_2, 1.9 ml of 200 mM sodium cholate and 15.4 mg of apo A-I in Na/K phosphate buffer containing EDTA were added

to the lipid mixture while stirring. The solution was dialyzed exhaustively at 4°C against Na/K phosphate buffer containing EDTA in order to remove ethanol and sodium cholate. The solution of reconstituted [^{14}C]CE-HDL thus obtained was diluted to 25 ml with Na/K phosphate buffer containing EDTA.

An ethanol solution (1.0 ml), containing 22.5 μmol of phosphatidylcholine, 5.63 μmol of cholesterol, 0.64 μmol of cholesteryl [1-^{14}C]oleate (3.7×10^2 KBq) and 0.563 μmol of [^{3}H]triolein (3.7×10^2 KBq), was treated in a similar manner as described above to prepare [^{14}C]CE/[^{3}H]TG-HDL.

Partial Purification of CETP

Human plasma was obtained by centrifugation of blood at $16,000 \times g$ for 20 minutes. Two liters of glass distilled water and 100 ml of 10% dextran sulfate were added to 1 liter of the stirred human plasma. This was followed by the addition of 4 M $CaCl_2$ to the final concentration of 0.1 M. The mixture was stirred gently for 15 minutes and then centrifuged at $16,000 \times g$ for 20 minutes in order to remove the insoluble dextran sulfate-lipoprotein complex.

Solid NaCl was added to the stirred dextran sulfate supernatant fraction in order to increase the ionic strength to 0.5. The mixture was applied to a Phenyl-Sepharose CL-4B column (2.5×15 cm), which was previously equilibrated with 0.5 M NaCl containing EDTA. The column was washed with 1,300 ml of the NaCl solution and with 800 ml of Na/K phosphate buffer containing EDTA. The CETP fraction was eluted with 600 ml of 2.9 mM Na/K phosphate buffer (ionic strength 0.0075) containing EDTA.

A DEAE-Sephadex A-50 column (2.5×15 cm) was equilibrated with Na/K phosphate buffer (ionic strength 0.05) containing EDTA. Concentrated Na/K phosphate buffer (ionic strength 2.0) was added to the CETP fraction to increase the ionic strength to 0.05, and the fraction was applied to the column, which was washed with 300 ml of Na/K phosphate buffer (ionic strength 0.05) containing EDTA, and with 250 ml of Na/K phosphate buffer (ionic strength 0.1) containing 0.05% EDTA. The CETP was eluted with 800 ml of Na/K phosphate buffer (ionic strength 0.1) containing 0.06 M NaCl and EDTA. To concentrate and desalt the large volume of DEAE-Sephadex fraction, the ionic strength was adjusted to 1.0 by the addition of 4 M NaCl, and the fraction was applied to a small Phenyl-Sepharose column (1.5×7.0 cm). CETP was eluted with 0.01% EDTA. The recovery of CETP activity in this concentration process was more than 95%, and the final protein concentration was 2.0 mg/ml.

In Vitro Assay for CETP Activity

The assay for CETP activity was carried out essentially according to the method described by KATO *et al.*[31]. The assay mixtures consisted of 25 μl of reconstituted [^{14}C]CE-HDL (22.5 nmol of phosphatidylcholine, 5.63 nmol of cholesterol, 0.64 nmol of cholesteryl [1-^{14}C]oleate (0.37 KBq) and 15.4 μg of apo A-I) as the donor for cholesteryl ester, 10 μl (32.5 μg as protein) of LDL as the acceptor, 0.21 μmol of DTNB, 5 μl of partially purified CETP (10 μg) and 5 μl of a sample (DMSO solution) in a final volume of 150 μl in Na/K phosphate buffer containing 60 mM NaCl and EDTA in Eppendorf tubes (1.5 ml). The assay incubation was carried out at 37°C.

After a 30-minute incubation, the assay tubes were immediately placed in an ice bath to which was added 30 μl of an LDL-precipitation solution (5 μl of 60 mM $MgCl_2$, 5 μl of 0.1% dextran sulfate and 20 μl of phosphate buffer containing 60 mM NaCl and EDTA). After standing for 20 minutes in an ice bath, the assay mixtures were centrifuged at $13,000 \times g$ for 15 minutes at 4°C. The 140 μl of supernatant solution containing [^{14}C]CE-HDL was carefully transferred into scintillation vials to analyze for radioactivity. On the other hand, after removing without leaving any visible supernatant at the tube bottom, the [^{14}C]CE-LDL precipitate was dissolved in 180 μl of 0.1 M NaOH, and the 140 μl was also analyzed for radioactivity. The CETP mediated cholesteryl ester transfer activity was computed by subtracting the blank values, which include the spontaneous transfer, from the total cholesteryl ester transfer obtained in the presence of CETP. Usually the blank and control transfer values were about 6% and 34% of [^{14}C]CE-HDL added under the assay conditions.

To measure the CE/TG transfer of CETP, reconstituted [^{14}C]CE-HDL was replaced by reconstituted [^{14}C]CE/[^{3}H]TG-HDL as described above in the assay mixture.

Ex Vivo Assay for CETP Activity

Transgenic mice expressing human CETP and human apo A-I[32] were obtained from Jackson Labs, USA. Sclerotiorin dissolved in Cremophor EL solution (4 μl, final 10 mg/kg) was administered to the male mice (n = 4, fasted overnight). Blood was taken at 4 and 24 hours after dosing, and was centrifuged immediately to obtain plasma. The plasma (25 μml) was used as a CETP source to determine the CETP activity as described above.

Transfer of Cholesteryl Ester from Vesicles to CETP

The assay for transfer of CE from [^{14}C]CE/PC vesicles to CETP was carried out according to the methods reported by SWENSON *et al.*[33]. Partially purified CETP (4 mg in 800 μl) was preincubated in the presence or absence of sclerotiorin (final 64 μM) for 15 minutes at 37°C. To the pretreated CETP solution were added unilamellar [^{14}C]CE/PC vesicles (150 nmol PC and 55.5 KBq [^{14}C]CE) in 200 μl of Na/K phosphate buffer (ionic strength 0.16, pH 7.4), and the mixture was incubated for another 15 minutes at 37°C. Then, vesicles and CETP were eluted on a Bio Gel A-0.5m column (1.5 × 40 cm) equilibrated with Na/K phosphate buffer at 4°C. Fractions (2 ml each) were analyzed for CETP activity and radioactivity. CETP in each fraction (10 μl) was also electrophoresed in an SDS-polyacrylamide gel (10%) by the method of LAEMMLI[34], and then transferred to a nitrocellulose membrane. The membrane was blocked with 1.5% gelatin for 1 hour and then incubated with an monoclonal anti-CETP IgG (LT-A4, 1.0 μg/ml)[35] solution for 2 hours. The membrane was washed and then incubated for 2 hours in a solution of alkaline phosphatase-conjugated goat anti-mouse IgG to visualize CETP.

Reaction of Sclerotiorin with Lysine

To sclerotiorin (15 mg, 38 μmol) and lysine (34 mg, 191 μmol) in methanol (1,275 μl) was added 0.2 N NaOH (375 μl), and the mixture was incubated at room temperature for 1 hour. The reaction products (30 mg) extracted with *n*-BuOH at pH 7.0 were purified by HPLC under the following conditions; YMC Pack ODS 20 × 250 mm, a 30-minute linear gradient from 30% to 40% CH_3CN, 6.0 ml/minute. Two new peaks appeared with retention times of 25.5 and 18.5 minutes. The fractions were pooled and concentrated to give the sclerotiorin-lysine adducts **1** (2.6 mg) and **2** (5.7 mg). The structures of **1** and **2** were elucidated by NMR and MS.

1: ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (s, H-1), 7.03 (s, H-1), 6.99 (dd, *J* 15.0, 1.0 Hz, H-1), 6.44 (d, *J* 15.0 Hz, H-1), 5.72 (d, *J* 10.0 Hz, H-1), 4.74 (dd, *J* 8.5, 6.0 Hz, H-1), 2.88 (dd, *J* 7.5, 7.0 Hz, H-1), 2.52 (m, H-1), 2.28 (m, H-1), 2.03 (m, H-1), 1.90 (d, *J* 1.0 Hz, H-3), 1.67 (dd, *J* 7.5, 7.0 Hz, H-1), 1.50 (s, H-3), 1.48 (m, H-1), 1.45 (m, H-1), 1.42 (m, H-2), 1.33 (m, H-1), 1.02 (d, *J* 7.0 Hz, H-3), and 0.89 (t, *J* 7.0 Hz, H-3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 200.2, 189.2, 174.8, 153.1, 148.8, 148.7, 146.7, 141.1, 134.3, 118.2, 117.3, 113.0, 100.9, 84.8, 69.0, 41.0, 36.7, 33.2, 31.7, 29.5, 29.3, 24.9, 21.1, 13.2, and 12.9; HRMS calcd for $C_{25}H_{34}N_2O_5Cl$ 477.2156, found 477.2159.

2: ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (s, H-1), 7.10 (s, H-1), 7.08 (d, *J* 15.5 Hz, H-1), 6.45 (d, *J* 15.5 Hz, H-1), 5.78 (d, *J* 9.5 Hz, H-1), 4.13 (m, H-2), 3.53 (m, H-1), 2.54 (m, H-1), 1.93 (s, H-3), 1.87 (m, H-2), 1.82 (m, H-2), 1.48 (m, H-2), 1.47 (s, H-3), 1.46 (m, H-1), 1.35 (m, H-1), 1.03 (d, *J* 6.5 Hz, H-3), and 0.90 (t, *J* 7.5 Hz, H-3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.3, 188.6, 174.2, 151.2, 148.9, 148.4, 146.3, 142.7, 133.8, 117.0, 116.7, 112.0, 100.2, 84.2, 55.9, 55.3, 36.2, 32.0, 31.2, 30.7, 28.9, 23.2, 20.6, 12.8, and 12.4; HRMS calcd for $C_{25}H_{34}N_2O_5Cl$ 477.2156, found 477.2157.

Results

Inhibition of CETP Activity by Azaphilones

The effect of thirteen azaphilones on CETP activity was tested. Their structures and IC_{50} values for CETP inhibition are summarized in Table 1. Among them, chaetoviridin B showed the most potent CETP inhibition with an IC_{50} value of <6.2 μM, followed by sclerotiorin (IC_{50}; 19.4 μM). Deacetylsclerotiorin showed the same IC_{50} value (19.4 μM) as sclerotiorin, indicating that the acetyl residue in the sclerotiorin molecule is not essential for CETP inhibition. Rotiorin, chaetoviridin A and rubrorotiorin were moderate inhibitors (31.5, 31.6, and 41.1 μM, respectively), but the others showed very weak or no inhibitory activity against CETP.

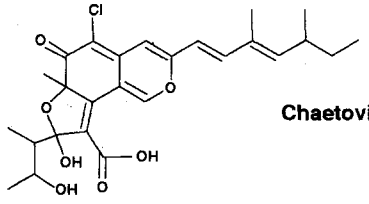
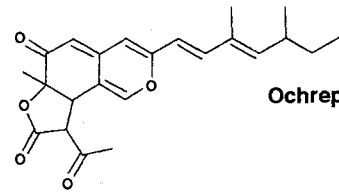
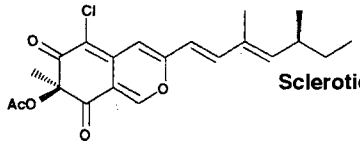
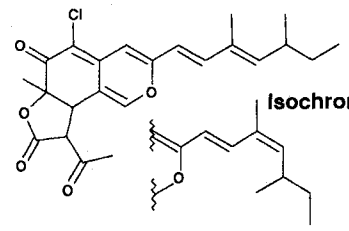
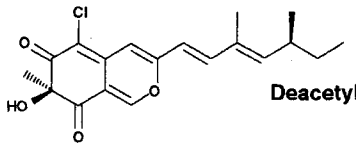
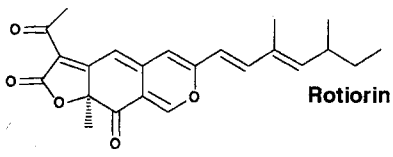
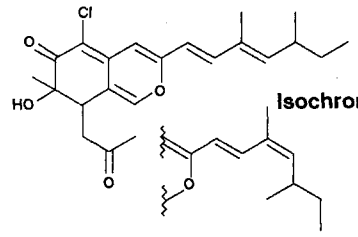
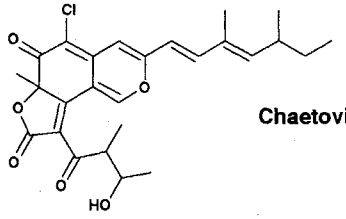
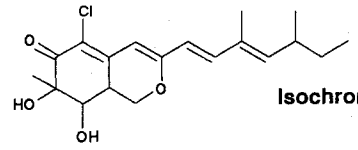
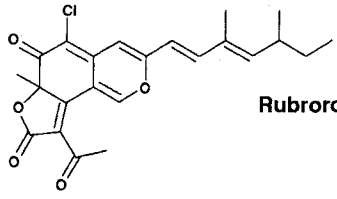
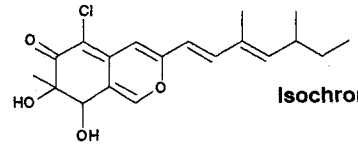
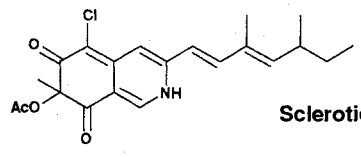
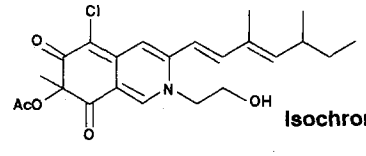
Inhibition of CETP-mediated CE/TG Transfer by Sclerotiorin

CETP promotes not only CE transfer but TG transfer. To investigate the effect of sclerotiorin on both transfers by CETP, reconstituted HDL doubly labeled with [^{14}C]CE and [^{3}H]TG was used as a donor lipoprotein. The drug inhibited the transfer of both CE and TG with similar IC_{50} values of 14.4 μM and 10.3 μM, respectively (Fig. 1). When reconstituted HDL monolabeled with [^{3}H]TG was used, analogous inhibitory potency of sclerotiorin against TG transfer (IC_{50}: 12.7 μM) was observed (data not shown).

Irreversible Iinhibition of CETP by Sclerotiorin

CETP was preincubated with sclerotiorin (0~25 μM) at 37°C for 20 minutes, and then the preincubated CETP was transferred into the assay solution to start the reaction. By this method, sclerotiorin was diluted about 30 times by the assay solution, the final concentrations of which were expected to show no inhibitory activity

Table 1. Effect of azaphilones on the transfer of [^{14}C]CE from reconstituted [^{14}C]CE-HDL to LDL.

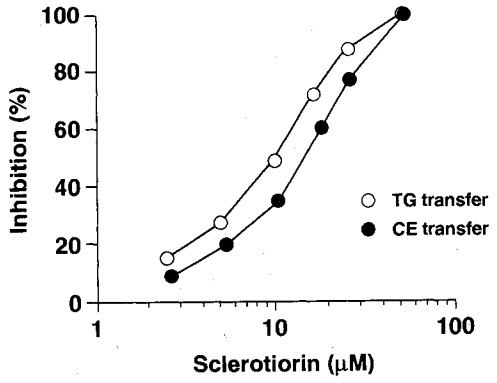
Compound	IC_{50}(μM)	Compound	IC_{50}(μM)
 Chaetoviridin B	<6.2	 Ochrephilone	>262
 Sclerotiorin	19.4	 Isochromophilone I	>219
 Deacetylsclerotiorin	19.4		
 Rotiorin	31.5	 Isochromophilone II	>232
 Chaetoviridin A	31.6	 Isochromophilone III	>265
 Rubrorotiorin	41.1	 Isochromophilone V	>265
 Sclerotioramine	84.8	 Isochromophilone VI	>230

against CETP. However, CETP activity was inhibited in response to the drug concentration at preincubation (Table 2). The IC_{50} at preincubation (14.5 μM) showed a good correlation with those results shown in Table 1 and Fig. 1, suggesting that sclerotiorin inhibits CETP irreversibly.

Ex Vivo Inhibition of CETP by Sclerotiorin

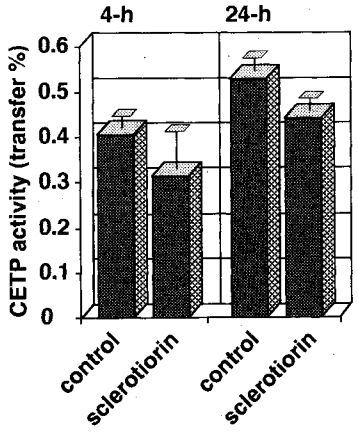
After an oral administration of sclerotiorin (10 mg/kg) to the transgenic mice expressing human CETP and apo A-I, the plasma was assayed for CETP activity. The activity was inhibited 23% at 4 hours after dosing, and inhibition was 16% even at 24 hours (Fig. 2). The *ex*

Fig. 1. Inhibition of CETP-mediated CE and TG transfer by sclerotiorin.



Partially purified CETP was incubated with reconstituted [^{14}C]CE/[^{3}H]TG-HDL and LDL in the presence or absence of sclerotiorin (0~50 μM) at 37°C for 30 minutes. Then, an LDL-precipitation solution was added to the reaction mixture to separate HDL and LDL. The radioactive HDL and -LDL were measured and the inhibition of CE (closed circle) and TG (open circle) transfer by sclerotiorin was calculated as described in Materials and Methods. Each value is the average of duplicate determinations.

Fig. 2. *Ex vivo* inhibition of CETP activity by sclerotiorin.



Sclerotiorin dissolved in Clemophor EL solution (4 μl, final 10 mg/kg) was administered to the transgenic mice (n=4, fasted overnight) expressing human CETP and human apo A-I. A blood was taken at 4 and 24 hours after dosing, and was centrifuged immediately to obtain plasma. The plasma (25 μl) was used as a CETP source to determine the CETP activity as described in Materials and Methods.

Table 2. Irreversible inhibition of CETP by sclerotiorin.

Sclerotiorin (μM)		CETP activity (%)	
Preincubation	Final	(dpm)	
0	0	3896	(100)
2.5	0.08	3599	(92.4)
7.5	0.24	2844	(73.0)
12.5	0.40	2355	(60.4)
25.0	0.80	1704	(43.7)

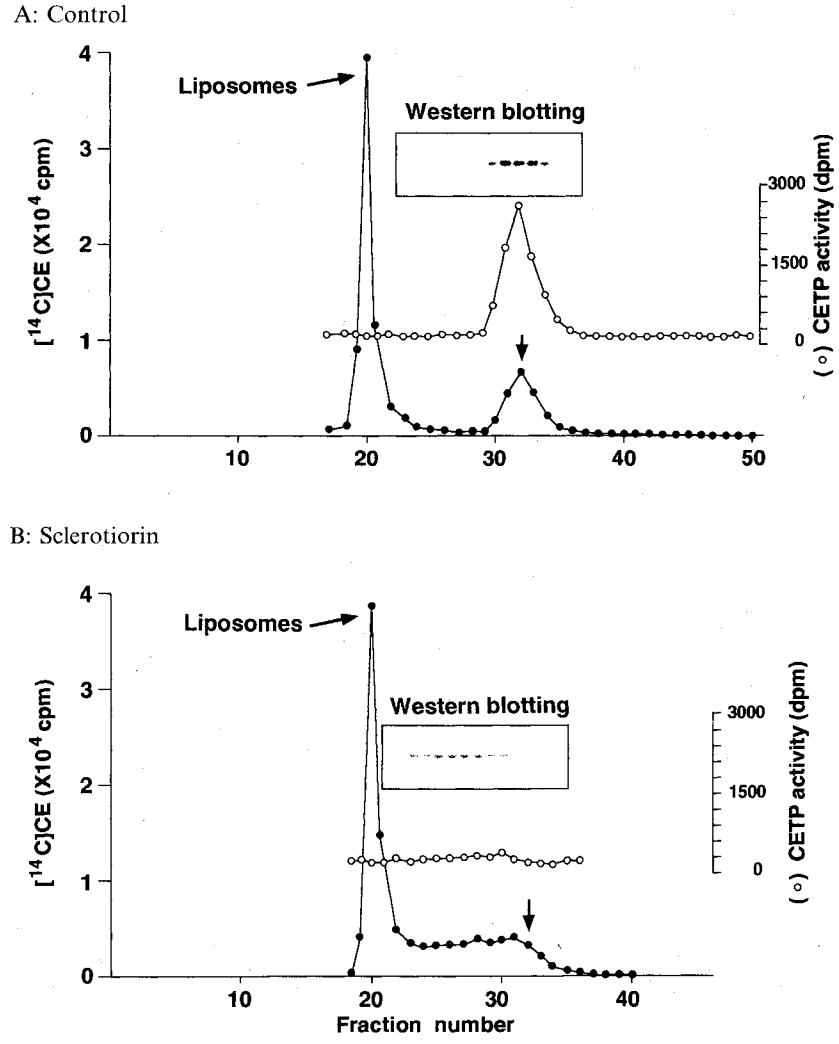
Partially purified CETP (250 μg) and sclerotiorin (0~25 μM) in 50 μl of Na/K phosphate buffer were preincubated at 37°C for 20 minutes. Then, 5 μl of the pretreated CETP was transferred into 145 μl of the assay solution as indicated in "Materials and Methods" to start the reaction.

vivo efficacy under the conditions also suggests the irreversible and bioavailable inhibition of CETP by the drug.

Effect of Sclerotiorin on CE Transfer from Vesicles to CETP

After partially purified CETP was incubated with [^{14}C]CE/PC vesicles at 37°C for 15 minutes, CETP and the vesicles were separated by gel filtration using a Bio Gel column. Through this column, [^{14}C]CE/PC vesicles was eluted as a void volume. From the Western blotting analysis and radioactivity of the fractions, CETP and [^{14}C]CE were eluted together as a peak of the 33rd fraction (Fig. 3A), indicating that [^{14}C]CE was transferred from vesicles to CETP. The ratio of [^{14}C]CE transfer was calculated as 24%. On the other hand, sclerotiorin-preincubated CETP was incubated with [^{14}C]CE/PC vesicles at 37°C for 15 minutes, and Bio Gel column chromatography was carried out as well (Fig. 3B). From the Western blotting analysis, the elution of CETP was shifted to higher molecular weight with a broad peak of the 24th to 29th fractions. Interestingly, about 25% of [^{14}C]CE was associated to the CETP-detectable fractions, suggesting that [^{14}C]CE could be transferred from vesicles to the drug-treated CETP. However, the drug-treated CETP in the fractions lost the overall [^{14}C]CE transfer activity almost completely.

Fig. 3. Transfer of [^{14}C]CE from [^{14}C]CE/PC vesicles to CETP.



Partially purified CETP, preincubated in the absence (A) or in the presence (B) of sclerotiorin (64 μM) at 37°C for 15 minutes, was incubated at 37°C with [^{14}C]CE/TG vesicles for another 15 minutes, and then eluted though a Bio Gel A-0.5m column (1.5 × 40 cm). Fractions (2 ml each) were analyzed for radioactivity (closed circle) and CETP activity (open circle). A part of each fraction (10 μl) was electrophoresed in SDS-polyacrylamide gel (10%), and CETP was visualized by Western blotting analysis using monoclonal anti-CETP IgG (LT-A4) and alkaline phosphatase-conjugated goat anti-mouse IgG. Arrows show the elution position of CETP under control conditions. Each value is the average of duplicate determinations.

Reaction of Sclerotiorin with Lysine

Sclerotiorin and lysine were incubated under an alkaline condition, and the sclerotiorin adducts were isolated by preparative HPLC (data not shown). Two adducts were obtained and the structures (Fig. 4) were elucidated by spectroscopic analyses, demonstrating that sclerotiorin reacts with the α- and ε-primary amines of lysine. Thus, this model of the reaction suggested that sclerotiorin, as expected, can react with the protein by covalently modifying primary amines of lysine or *N*-terminal amino acid in the molecule and this suggested to reflect its molecular mode of action.

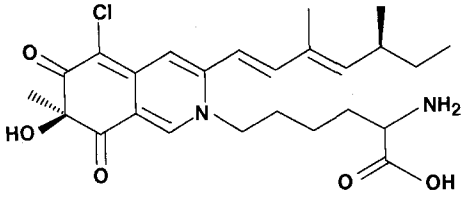
Discussion

From the results summarized in Table 1, structure-specific CETP inhibition by azaphilones was demonstrated. Azaphilones have a common isochromane-like ring with a similar hydrophobic side chain at the C-2 position (Table 1). The chlorine atom at the C-5 position and the acetyl residue at the C-7-OH are not important for the CETP inhibition because rotiorin lacking the chlorine and deacetylsclerotiorin lacking the acetyl

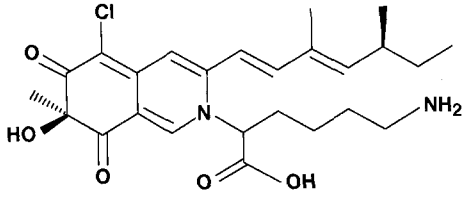
residue at the C-7-OH still showed the inhibitory activity. More importantly, electrophilic ketone(s) and/or enone(s) at the both C-6 and C-8 positions of the isochromane-like ring are necessary for eliciting CETP inhibitory activity (Fig. 5). Furthermore, the oxygen atom of the ring is also essential because in sclerotioramine and isochromophilone VI, where the oxygen atom is replaced by a nitrogen atom, the inhibitory activity is decreased.

The results of dilution experiments of sclerotiorin-pretreated CETP (Table 2) and its *ex vivo* efficacy (Fig. 2) strongly suggested that the drug inhibits CETP irreversibly. It was reported that sclerotiorin reacts with a primary amine such as ammonia and methylamine to yield sclerotioramine and *N*-methylsclerotioramine, respectively[36]. Thus, the pyranyl oxygen changes to a nitrogen of a primary amine in this reaction. In our model reaction, sclerotiorin also reacted with α- and ε-amino residues of lysine to form the sclerotiorin-adducts (Fig. 4). A possible reaction mechanism is shown in Scheme 1. The lone pair of electrons of a primary amine attacks the electrophilic C-10 carbon, where the electron poor state is induced by the C-8 ketone, and the C–O bond is cleaved. Then, nucleophilic attack to the C-2 carbon by the lone pair of electrons of the primary amine occurs again. This model could explain the structure-specific CETP inhibition by azaphilones. Therefore, it is plausible that the drug modifies primary amines such as lysine or *N*-terminal amino acid in the CETP molecule covalently. The amino acid sequence of human CETP revealed 25 lysine residues in the molecule[6], some of which are responsible for CETP activity[37]. Furthermore, the *N*-terminal cysteine of both human and rabbit plasma CETP was modified by *p*-chloromercuriphenyl sulfonate,

Fig. 4. Structure of sclerotiorin adducts **1** and **2**.

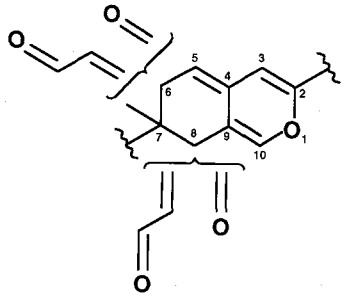


1

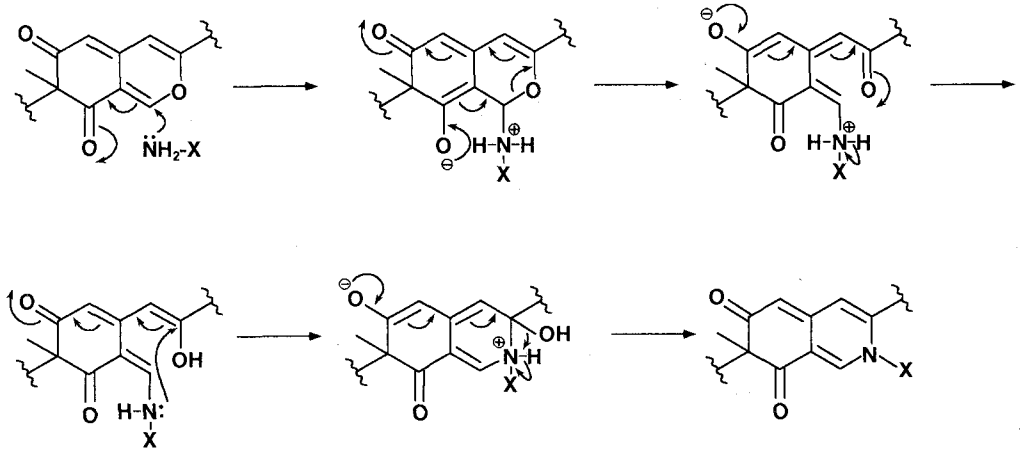


2

Fig. 5. Common substructure of azaphilones essential for eliciting CETP inhibition.



Scheme 1. A possible reaction mechanism for formation of sclerotiorin adducts.



resulting in inhibition of TG transfer activity[38]. Therefore, covalent modification of such residues by sclerotiorin might impair the CETP activity.

CETP promotes not only CE transfer but TG transfer. Inhibitory effects of antibodies or some compounds on the transfers were reported, and three types of inhibition were shown; 1) monoclonal antibodies against CETP inhibited TG transfer selectively[35,39,40], 2) cholesterol analogs were demonstrated to be selective inhibitors of CE transfer[40,41], and 3) certain sulfhydryl alkylating reagents altered both CE and TG transfers[33,42]. Sclerotiorin also inhibited transfer of the both neutral lipids. As a result, it was concluded that CETP has different binding sites for the neutral lipids.

To study the mechanism of CETP, SWENSON *et al.* observed the CE transfer from vesicles to CETP by separating the vesicles and CETP through a gel filtration column[33]. We utilized similar conditions, and the elution profile showed that 24% of [^{14}C]CE was transferred from [^{14}C]CE/PC vesicles to CETP (Fig. 3A), which was consistent with their results[33]. To clarify the inhibitory mechanism of sclerotiorin, its effect on this model was tested (Fig. 3B). Western blotting showed that the elution of sclerotiorin-treated CETP was shifted to higher molecular weight regions, but the same level of [^{14}C]CE (about 25%) was still transferred from vesicles to the drug-treated CETP. However, the [^{14}C]CE bound to the CETP was not further transferred to LDL. These results indicated that the inhibition mechanism of sclerotiorin is different from that of *p*-chloromercuriphenylsulfonate, which also inhibits both CE and TG transfer, since it was found to decrease the transfer of CE and TG from vesicles to CETP[33]. Previous studies showed that the binding of CETP to lipoproteins involves mainly ionic interaction, that is, negatively charged lipoproteins and positively charged CETP[43,44]. JIANG *et al.*[37] demonstrated, in fact, that point mutagenesis of positively charged amino acids including protonated ^{233}Lys within the conserved region of CETP reduced the HDL binding and CETP activities markedly. Therefore, it is plausible that sclerotiorin can modify such important lysine residues especially on surface of the molecule to form a covalent bond as predicted from the model reaction. Unlike the mutagenic CETP, the drug-modified CETP appeared to maintain the ability to bind to vesicles to transfer CE to the CETP molecule although it lost the overall transfer activity completely. Furthermore, the elution shift of the drug-treated CETP to higher molecular weights as shown by the gel filtration column chromatography (Fig. 3B) might be due to the irreversible modification by sclerotiorin.

The biological properties of sclerotiorin have not been studied in detail. We have investigated the effects of the drug on several enzymatic and biochemical reactions such as acyl-CoA: cholesterol acyltransferase, diacylglycerol acyltransferase, phospholipases A2, PAF acetyl hydrolase and gp120-CD4 binding activities (data not shown). Sclerotiorin did not show any inhibitory effects on the reactions at 100 μM except on type II phospholipase A2 (IC_{50} 1.5 μM)[26]. If our model of the reaction mechanism is valid, several lysine residues in the protein molecules might be modified by sclerotiorin. However, most enzymes appear to maintain their activity even after such modification, while CETP and type II phospholipase A2 lost their activity, suggesting differences in functional importance of lysine residues among proteins. Therefore, sclerotiorin is a useful tool to investigate whether lysine residues in proteins play a critical role in their biological functions. The binding site(s) of sclerotiorin on CETP remains to be defined.

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