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NOTES

Inhibition of Cholesteryl Ester Transfer Protein by Fungal Metabolites, L681,512

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The cholesteryl ester transfer protein (CETP) promotes exchange and transfer of neutral lipids such as cholesteryl ester (CE) and triacylglycerol (TG) between plasma lipoproteins. Evidence is accumulating for involvement of CETP in atherosclerosis[1,2]. Therefore, CETP is expected as a novel target of inhibition for anti-atherosclerotic agents.

In the course of our screening for CETP inhibitors of microbial origin[3~7], four active compounds were isolated from the culture broth of a fungal *Fusarium* sp. FO-6651. Based on its physico-chemical properties and NMR data, they were identified with L681,512 compounds[8,9], which were previously reported as elastase inhibitors. In this paper, the fermentation, isolation and *in vitro* CETP inhibitory activity of L681,512 compounds are described. Furthermore, we show that L681,512-1 proves effective in the *ex vivo* assay using human CETP and apo A-I transgenic mice.

Characteristics of the Producing Strain

The fungal strain FO-6651 was isolated from a soil sample collected at Nikko, Tochigi, Japan. This strain grew well on potato dextrose agar, corn meal agar, malt extract agar, Czapek yeast extract agar and Miura's medium for 4 days at 25°C to form colonies with diameters of 40~60 mm. The colony surface was floccose to felty with a color of white. The reverse color of the colonies was white to yellowish brown. The growth was nil for 14 days at 5°C and 37°C. No teleomorph was observed. Macroconidia having 1 to 3 transverse septa were hyaline in color, slightly curved in shape and 13~40×3.5~5.5 μm in size. From these morphological characteristics, the strain FO-6651 was considered to belong to the genus *Fusarium*.

Fermentation

A slant culture of the strain FO-6651 grown on YpSs agar was used to inoculate a 50-ml test tube containing 10 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Nippon Seiyaku) 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The tube was shaken on a reciprocal shaker for 4 days at 27°C. Two milliliters of the seed culture was transferred into 200 ml of the production medium (sucrose 2.0%, glucose 1.0%, corn steep liquor 1.0%, meat extract 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, trace elements containing in g/liter: $FeSO_4 \cdot 7H_2O$ 1.0, $MnCl_2 \cdot 4H_2O$ 1.0, $ZnSO_4 \cdot 7H_2O$ 1.0, $CuSO_4 \cdot 5H_2O$ 1.0 and $CoCl_2 \cdot 2H_2O$ 1.0 (2 ml), $CaCO_3$ 0.3% and agar 0.1%, pH 6.0) in a 1000-ml Roux-type flask. The fermentation was carried out at 27°C under the static condition. CETP inhibitory activity was observed at day 11 after inoculation and reached a maximun at day 14.

Isolation and Identification

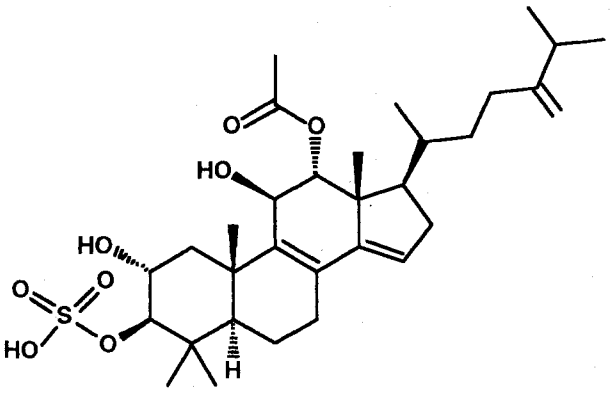
The 14-day old whole broth of *Fusarium* sp. FO-6651 (800 ml) was treated with acetone (800 ml). After centrifugation of the mixture, the supernatant was concentrated and extracted with ethyl acetate (300 ml, twice). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a red powder (428 mg, IC_{50} 14.6 μg/ml). The powder suspended in 30% CH_3CN was subjected to an ODS column (Senshu SSC-ODS-7515-12, 21 ml). The materials were eluted by a linear gradient from 30% CH_3CN (100 ml) to 100 % CH_3CN (120 ml) and each 4.8 ml of the elution was collected. The fractions (11th to 23rd) were concentrated and extracted with ethyl acetate to give a brown powder (287 mg, IC_{50} 12.7 μg/ml). The powder was purified by HPLC (YMC-pack D-ODS-5, 20×250 mm; a linear gradient from 60% CH_3CN in 50 mM NaCl to 75 % CH_3CN in 50 mM NaCl for 60 minutes; UV at 220 nm; 6.0 ml/minute). Four active fractions 1 to 4, eluted as peaks with retention times of 48.7, 65.8, 43.5 and 45.5 minutes were concentrated and

Table 1. Isolation of L681,512-1, 2, 3 and 4 from ethyl acetate extracts of the culture broth of *Fusarium* sp. FO-6651.

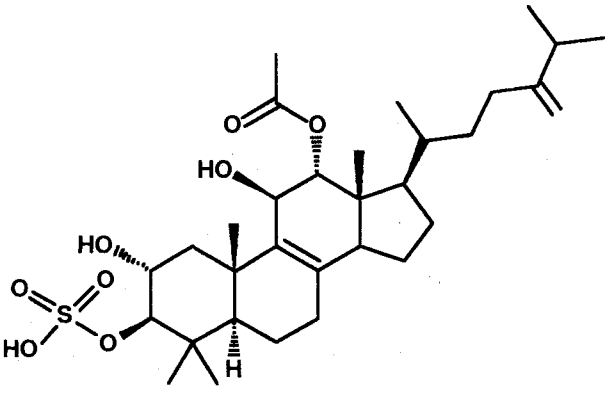
Step	Weight (mg)	Activity (IC_{50}: μg/ml)	Total activity (Weight/Activity)	Yield (%)
EtOAc ectracts	428	14.6	29.3	100
ODS column	287	12.7	22.6	77
HPLC			18.0	61
L681,512-1	133	8.2	16.2	(54)
L681,512-2	14.1	8.7	1.62	(4.5)
L681,512-3	2.75	33	0.08	(0.3)
L681,512-4	4.34	34	0.13	(0.4)

Starting from 800 ml of culture broth.

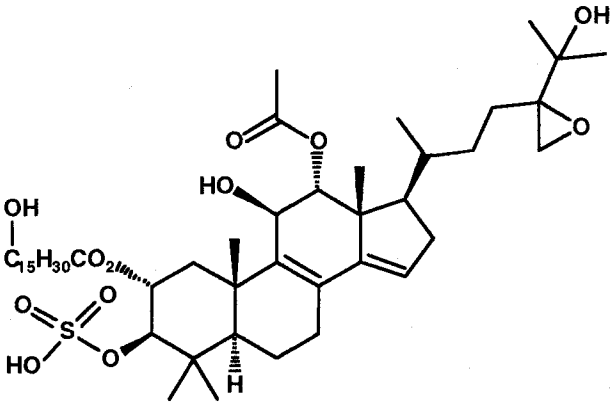
Fig. 1. Structures of L681,512-1 to -4.



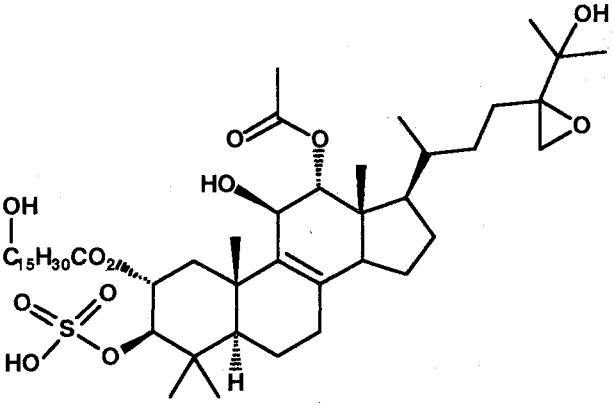
L681,512-1



L681,512-2



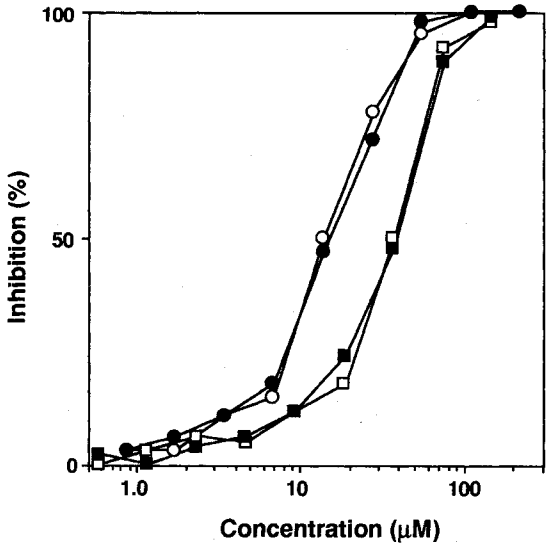
L681,512-3



L681,512-4

Fig. 2. CETP inhibition by L681,512.

○ L681,512-1; ● L681,512-2;
□ L681,512-3; ■ L681,512-4.



extracted with ethyl acetate to give FO-6651-1 (133 mg, IC_{50} 8.2 μg/ml), -2 (14.1 mg, IC_{50} 8.7 μg/ml), -3 (2.75 mg, IC_{50} 33 μg/ml) and -4 (4.34 mg, IC_{50} 34 μg/ml) as white powders, respectively. The summary of purification from ethyl acetate extracts of the culture broth is shown in Table 1. From various spectroscopic analyses including NMR, FO-6651-1 to -4 were identified with L681,512-1 to 4 (Fig. 1) previously reported as elastase inhibitors[8,9].

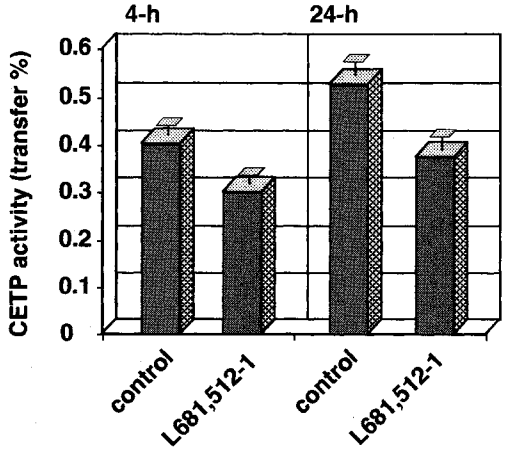
Inhibition of CETP Activity by L681,512

The assay for CETP activity was carried out according to our established method[3]. As shown in Fig. 2, L681,512-1 to -4 inhibited CETP activity in a dose-dependent manner. Furthermore, L681,512-1 (IC_{50}, 13.8 μM) and -2 (14.6 μM) were more potent than L681,512-3 (37.5 μM) and -4 (38.5 μM), indicating that the long acyl side chain in L681,512-3 and -4 is not important for CETP inhibition. When the CETP assay was carried out in the presence of 200 μM BSA, a level of albumin similar to that of human plasma[4], L681,512-1 and -2 gave IC_{50} values of 25.3 and 21.8 μM, indicating that the CETP inhibition by the compounds is partially affected by the presence of BSA in the assay. L681,512-1 to -4 were reported as elastase inhibitors with similar IC_{50} values (2.4, 2.5, 1.9 and 1.6 μM, respectively)[8]. Although these might be no relationship between CETP inhibition and elastase inhibition caused by these compounds, further analyses are necessary to define this point.

Table 2. Effect of preincubation of CETP with L681,512-1 on its activity.

L681,512-1 (μM)		CETP activity	
Preincubation	Final	(dpm)	(inhibiton %)
0	0	3779	(0)
16.8	0.60	3742	(0.3)
33.7	1.12	3667	(3.0)
67.3	2.24	3517	(7.0)
135	4.48	2956	(22)
269	8.98	2095	(45)

Fig. 3. *Ex vivo* efficacy of L681,512-1 in transgenic mice expressing human CETP and apo A-I.



Reversible CETP Inhibition by L681,512

CETP was preincubated with L681,512-1 (0~269 μM) at 37°C for 30 minutes, and then a part of the preincubated CETP was transferred into the assay solution to start the reaction. By this method, the inhibitor was diluted about 30 times by the assay solution. As shown in Table 2, the inhibition (%) of CETP activity at the final drug concentrations, but not at the preincubation concentrations, was almost comparable to the result of Fig. 2, suggesting that

L681,512-1 inhibits CETP reversibly.

Ex Vivo Inhibition of CETP by L681,512

Transgenic mice expressing human CETP and human apo A-I[10] were obtained from Jackson Labs, USA. L681,512-1 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 μl) was used as a CETP source to determine the CETP activity[10]. As shown in Fig 3, the activity was inhibited 26% at 4 hours after dosing, and inhibition was 29% even at 24 hours. The *ex vivo* efficacy under the conditions suggests bioavailable inhibition of CETP by the drug.

Acknowledgments

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