Isochromophilones I and II, Novel Inhibitors against gp120-CD4 Binding Produced by *Penicillium multicolor* FO-2338

I. Screening, Taxonomy, Fermentation, Isolation and Biological Activity

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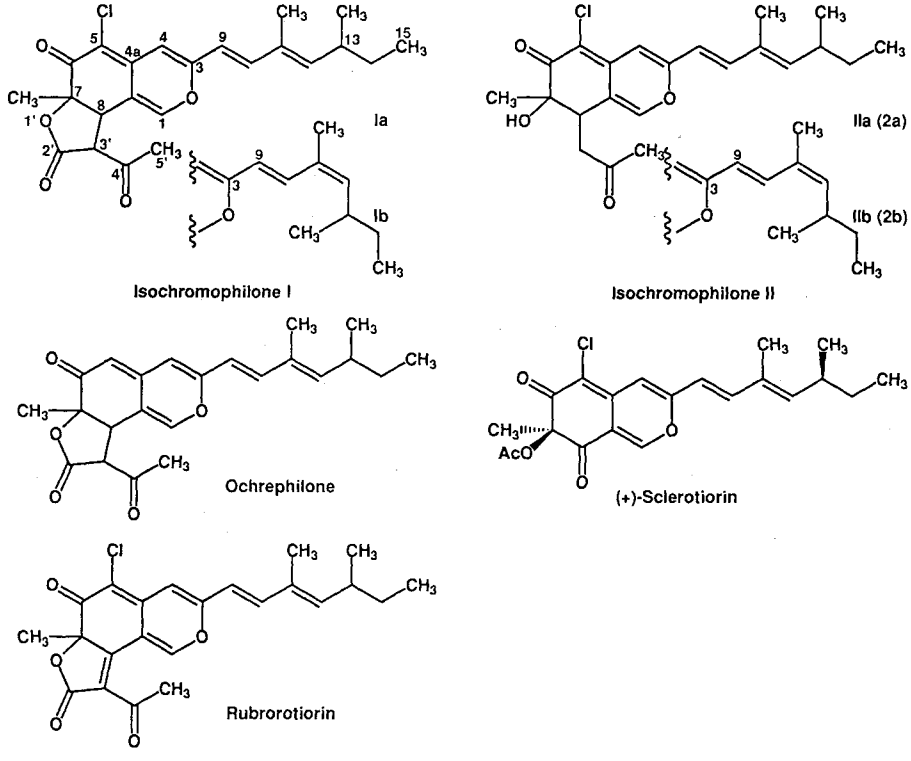
Isochromophilones I and II, the first novel gp120-CD4 binding inhibitors of microbial origin, were isolated from a cultured broth of a soil fungus designated as *Penicillium multicolor* FO-2338. These compounds were obtained as yellow powders from the cultured broth together with the known related compounds sclerotiorin, ochrephilone and rubrorotiorin. Isochromophilones I and II ($C_{23}H_{25}O_5Cl$ and $C_{22}H_{27}O_4Cl$, respectively) have an azaphilone skeleton and a chlorine atom. Isochromophilones strongly inhibited gp120-CD4 binding (IC_{50}: 6.6 and 3.9 μM, respectively), but the other related compounds did not. Isochromophilone II inhibited significantly HIV replication in peripheral human lymphocytes at 25 μM.

Blocking of human immunodeficiency virus (HIV) entry, which begins with highly specific binding of the HIV gp120 envelope protein to a CD4 molecule on the surface of most susceptible cells[1~3], is one of the most important targets for HIV therapies[4]. In our screening program for new inhibitors against gp120-CD4 binding from microorganisms, we discovered novel inhibitors, isochromophilones I and II (Fig. 1), from a cultured broth of the fungal strain FO-2338.

In this paper, we describe the details of the discovery,

Fig. 1. Structures of isochromophilones I and II, ochrephilone and (+)-sclerotiorin.



taxonomy, fermentation, isolation and biological activity; a preliminary account of these compounds has appeared[5]. Structure elucidation including their relative configuration will be reported separately[6].

Results

Screening of Inhibitors against gp120-CD4 Binding and Discovery of Isochromophilones

In the primary screening, the inhibitory activities against gp120-CD4 binding were determined by enzyme-linked immunosorvent assay (ELISA) using recombinant soluble CD4 (sCD4) and recombinant gp120 as described by GILBERT, M. *et al.*[7]. The reagents sCD4 and gp120, and also horse radish peroxidase-conjugated mouse monoclonal anti-CD4 (anti-CD4-HRP) were generous gifts from Genentech Inc. (CA, U.S.A.).

To the 96 well plate coated with gp120 (2.0 μg/ml), sCD4 (1.0 μg/ml) and a sample solution were added, and then incubated for 1 hour at room temperature. After the plate was washed, anti-CD4-HRP was added to the plate. The bound sCD4 was assayed colorimetrically.

In the secondary screening, anti-HIV activity was assayed by HIV-1 p24 capture assay in Diagen GmbH, Germany, as follows; peripheral human lymphocytes were isolated by density gradient centrifugation. After stimulation by mitogen the cells were infected with a standardized preparation of HIV. Subsequently, the infected cells were cultured in the presence of the agent for 4 days. The amount of viral core protein p24 synthesized and released by the infected cells was determined by ELISA technique on days 2, 3 and 4. By comparing with a standard preparation, the amount of protein (p24) produced by the virus-infected cells was calculated.

Using this ELISA method, the cultured broth of bacteria, actinomycetes and fungi isolated mainly from soil samples were subjected to the screening. Table 1 shows the results. Among over eight thousand soil isolates, only 3 strains were picked out as cultures showing both gp120-CD4 binding inhibition and anti-HIV activity. *Streptomyces* sp. OH-5196 was found to produce actinomycins which have an inhibitory activity against gp120-CD4 binding. On the other hand, an actinomycete WK-3419[8] and a fungus FO-2338[5] were found to produce new compounds which inhibit gp120-CD4 binding and anti-HIV activity. The inhibitors produced by FO-2338 were named isochromophilones. The inhibitors (chloropeptins) produced by strain WK-3419 has been reported preliminary[8].

Taxonomy of the Isochromophilone-producing Organism

Strain FO-2338 was originally isolated from a soil sample collected at Shirokane, Minato-ku, Tokyo, Japan. From the characters described bellow, the fungus was identified as *Penicillium multicolor*.

For the identification of the fungus Czapek yeast extract agar (CYA), malt extract agar, 25% glycerol nitrate agar (G25N) and yeast extract-soluble starch agar (YpSs) were used. This strain grew rapidly to form mistletoe gray to ivy colonies with diameter of 40~65 mm after incubation at 25°C for 14 days. The reverse of colonies was light brown to reddish brown. Yellowish orange soluble pigment was produced on CYA, G25N and YpSs colonies.

Morphological observation was done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (Hitachi S-430). When strain FO-2338 was grown on YpSs agar at 25°C for 7 days, the conidiophores born from substrate hyphae and penicillia were mainly monoverticillate as shown in Fig. 2. The phialides were $8\sim10\times2\sim2.5$ μm. The conidia were globe to subglobe and 2~2.5 μm in diameter and its surface was smooth. From the above characteristics, strain FO-2338 was identified as *Penicillium multicolor*[9] and named *Penicillium multicolor* FO-2338. This strain was deposited at the National Institute of Bioscience and

Table 1. Results of screening for gp120-CD4 binding inhibitors from microorganisms.

Microorganisms	Number of strains	Strains active against gp120-CD4 binding	Strains active in cell infection assay
Bacteria	307	1	0
Actinomycetes	5796	27	2 (OH-5196, WK-3419)
Fungi	2164	6	1 (FO-2338)
Total	8267	34	3

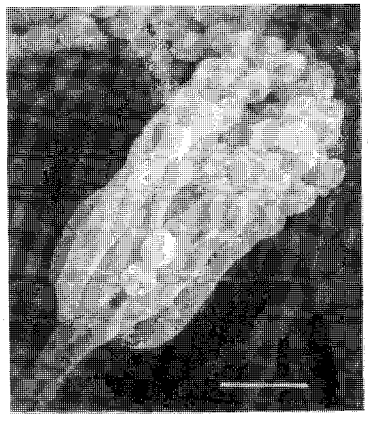
Human Technology, Agency of Industrial Science and Technology of Japan, as FERM P-13405.

Fermentation

The production of isochromophilones I and II were carried out as follows. A roopful of mycelia from a slant culture of *Penicillium multicolor* FO-2338 was transferred into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 2.0%, yeast extract 0.2%, polypeptone 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.1% and agar 0.1% (adjusted to pH 5.8 before sterilization). The flask was incubated at 27°C on a rotary shaker at 200 rpm for 3 days to give a seed culture. The seed culture (400 ml) was transferred to a 30-liter jar fermentor containing a production medium (20 liters) consisting of sucrose 2.0%, glucose 1.0%, corn steep powder 1.0%, meat extract 0.5%, yeast extract 0.5%, KH_2PO_4 0.1%, $CaCO_3$ 0.3%, trace metal solution (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, $CoCl_2 \cdot 2H_2O$ 1.0) 1% and agar 0.3% (pH 6.0). Fermentation was carried out at 27°C for 4 days. Figure 3 shows, a typical time course of isochromophilone production by *Penicillium multicolor* FO-2338. Isochromophilone I production was analyzed with HPLC, but isochromophilone II could not be detected because its peak overlapped those of other metabolites. The production of isochromophilone I began after day-2 and reached a maximum after day-3.

Fig. 2. Scanning electron micrograph of penicillia of strain FO-2338 on YpSs agar.

Scale: 5 μm.



Isolation

The isolation procedures for isochromophilones I and II and related compounds are summarized in Fig. 4. The cultured broth (20 liters) was subjected to organic solvent extraction, column chromatography using silica gel and ODS, and then HPLC using SSC ODS-SH and SSC silica gel to give yellow powders of isochromophilones I (25 mg) and II (10 mg), in addition to the known compounds sclerotiorin[10,11] (*ca.* 100 g), ochrephilone[13] (30 mg) and rubrorotiorin[11,12] (3.0 mg).

Physico-chemical Properties

Physico-chemical properties of isochromophilones I and II are summarized in Table 2. Both of compounds are easily soluble in chloroform, methanol, ethanol, acetone, ethyl acetate and benzene, insoluble in hexane and water. Their EI-MS fragment patterns suggested that they contain a chlorine atom. Their molecular formulae, $C_{23}H_{25}O_5Cl$ and $C_{22}H_{27}O_4Cl$ were determined by HREI-MS (*m/z* 416.1396 (M^+) and 390.1604 (M^+), respectively). In the IR spectrum of isochromophilone I, the absorption of γ-lactone carbonyl (ν_{max} 1780 cm^{-1}) was observed. In that of isochromophilone II, however, its absorption was not observed.

The side chain moiety, 3,5-dimethyl-1,3-dien, of isochromophilones were easily changeable at C-12 position of them to be a complex of two isomers, a and b, respectively like TL-1 and TL-2[14] (Fig. 1). It was confirmed their HPLC and ^1H NMR spectrum.

Isochromophilone I has an azaphilone skeleton,

Fig. 3. Tipical time course of isochromophilone production by *P. multicolor* FO-2338 in a 30-liter jar fermentor.

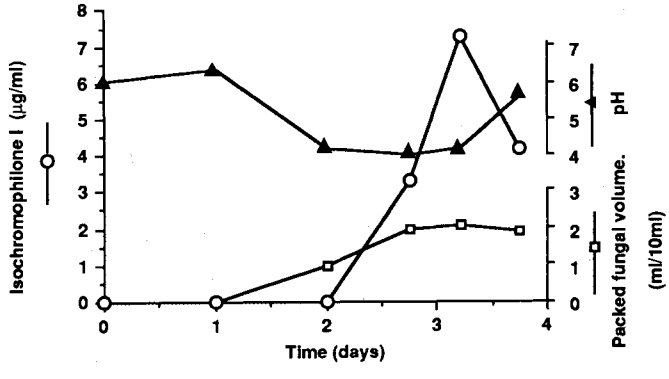


Fig. 4. Isolation procedures for isochromophilones and related compounds.

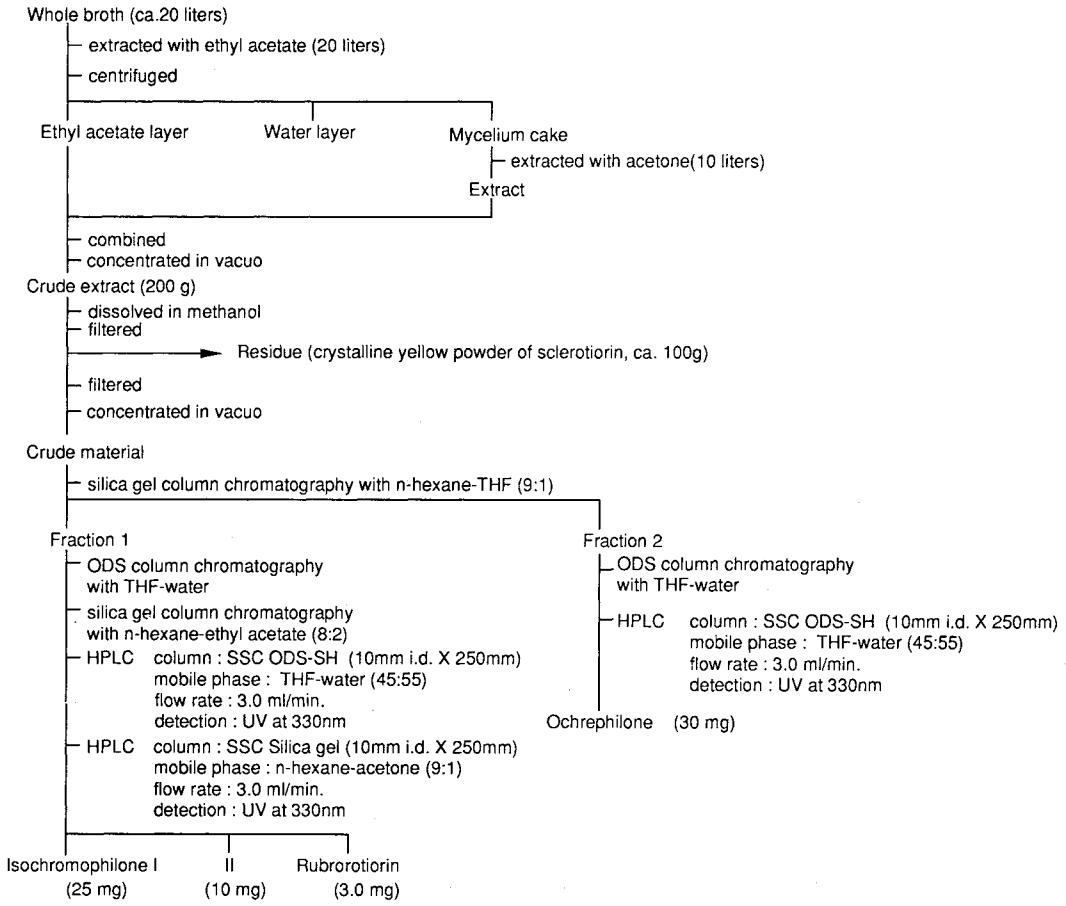


Table 2. Physico-chemical properties of isochromophilones I and II.

	Isochromophilone I	Isochromophilone II
Appearance	yellow powder	yellow powder
EI-MS	416 (M^+)	390 (M^+)
Molecular weight	416	390
HREI-MS	found 416.1396 calcd. 416.1389	found 390.1604 calcd. 390.1596
Molecular formula	$C_{23}H_{25}O_5Cl$	$C_{22}H_{27}O_4Cl$
UV λ_{max}^{EtOH} nm	256(17,500), 273(15,600), 340(sh), 357(12,000), 395(15,200), 412(15,700), 430(sh) and 464(sh)	250(15,000), 318(sh), 340(13,000), 356(14,300), 393(14,300), 410(16,500), 431(12,400) and 464(sh)
IR ν_{max}^{KBr} cm^{-1}	1780, 1720, 1630, 1560, 1520, 1430	1715, 1630, 1560, 1520, 1425
solubility		
soluble in	$CHCl_3$, CH_3OH, EtOH, EtOAc, acetone, benzene	$CHCl_3$, CH_3OH, EtOH,EtOAc, acetone, benzene
insoluble in	hexane, water	hexane, water

γ-lactone moiety and a chlorine atom. Ia and Ib components are easily converted to each other. Isochromophilone II has also an azaphilone skeleton but no γ-lactone. IIa and IIb components are also converted easily to each other. From the above data, the structures of isochromophilones Ia, Ib, IIa and IIb were determined as shown in Fig. 1. The details of structure elucidation will be reported separately[6]. The other compounds, sclerotiorin, ochrephilone and rubrorotiorin were identified from MS and ^1H and ^{13}C NMR data.

Table 3. Inhibition of HIV replication in the viral core protein level.

Sample	Viral core protein p24 systhesized (ng/ml)		
	Day 2	Day 3	Day 4
None	0	97.3	129.6
Isochromophilone II	0	0	13.5

Biological Activities

Isochromophilones I and II inhibited gp120-CD4 binding with IC_{50} values of 6.6 and 3.9 μM, respectively, by ELISA method. Sclerotiorin exhibited no inhibition even at 250 μM. Ochrephilone which lacks a chlorine atom at C-5 of isochromophilone I, weakly inhibited with IC_{50} of 114 μM, but rubrorotiorin exhibited no inhibition even at 300 μM.

Isochromophilone II exhibited anti-HIV activity at 25 μM (Table 3), but exhibited no effect on cell proliferation in lymphocytes at the same concentration although it inhibited somewhat the cell proliferation at 250 μM (data not shown).

Isochromophilones I and II were inactive against bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli* and *Staphylococcus aureus*) and fungi (*Candida albicans*, *Aspergillus niger* and *Piricularia oryzae*) at 1.0 mg/ml on paper disc method.

Discussion

Isochromophilones I and II were isolated as inhibitors against the binding between the HIV gp120 envelope protein and its receptor CD4 molecule, and isochromophilone II was found to have anti-HIV activity, which was assayed the inhibition of HIV replication in the viral core protein (p24) level. It seems that the anti-HIV activity is due to blocking of HIV entry into the cells, which was caused by inhibiting gp120-CD4 binding. To our knowledge, they are the first novel non-peptide compounds and the first microbial metabolites to inhibit gp120-CD4 binding.

The fungus strain, *Penicillium multicolor* FO-2338 produced some other azaphilone compounds, sclerotiorin, ochrephilone and rubrorotiorin than isochromophilones I and II. These compounds exhibited weak or no inhibition against gp120-CD4 binding. It suggests that the existence of a chlorine atom at C-5 and the substitution of a ketone at C-8 on azaphilone skeleton are important in the inhibitory activity against gp120-CD4 binding.

Acknowledgments

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