ISOCHROMOPHILONES I AND II, NOVEL INHIBITORS AGAINST gp120-CD4 BINDING FROM *Penicillium* sp.

Sir:

Blocking of human immunodeficiency virus (HIV) entry, which begins with highly specific binding of the HIV gp120 envelope protein with 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 the cultured broth of *Penicillium* sp. FO-2338. In this communication, we describe the fermentation, isolation, structure elucidation and biological activity of them.

The inhibitory activities against gp120-CD4 binding were determined by enzyme-linked immunosorvent assay (ELISA) using recombinant soluble CD4 and recombinant gp120 as described by GILBERT, M. *et al*[5]. The reagents CD4 and gp120, and also anti-CD4 (horse radish peroxidase-conjugated mouse monoclonal anti-CD4) were generaous gift from Genentech Inc. (CA, U.S.A.).

Fermentation of *Penicillium* sp. FO-2338 was carried out 27°C for 68 hours in a 30-liter jar fermentor containing a medium (20 liters) consisting of sucrose 2%, glucose 1%, corn steep liquor 1%, meat extract 0.5%, $CaCO_3$ 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.5%, trace metal solution 1% and agar 0.1% (pH 6.0). The cultured broth (20 liters) was extracted with equal volume of ethyl acetate. The extract was concentrated to dryness under reduced pressure. Crystals of sclerotiorin[6,7] (Fig. 1.) were obtained from a methanol solution of the residue. The mother liquor was chromatographed on a silica gel column using mixtures of *n*-hexane-ethyl acetate (25:75 and 45:55) as developing solvent systems. The active fractions were collected and applied to a Chromatorex ODS column which was developed with THF-water systems (25:75 and 45:55). The active fractions were rechromatographed on a silica gel column with mixtures of *n*-hexane-THF (95:5 and 90:10). Finally, the two active substances, isochromophilones I and II, were purified by HPLC using ODS and silica gel as yellow powders (9.9 mg and 14.4 mg, respectively).

Physico-chemical properties of isochromophilones I and II are summarized in Table 1. 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). The UV and IR spectra of them were

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

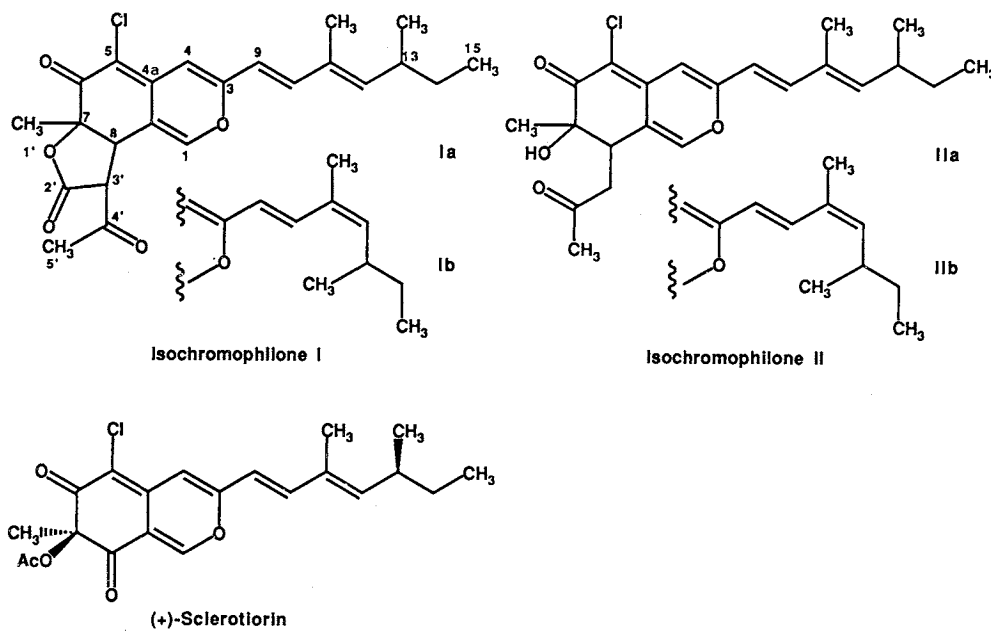

Table 1. Physico-chemical properties of isochromophilones I and II.

	Isochromophilone I	Isochromophilone II
Appearance	Yellow powder	Yellow powder
Molecular formula	$C_{23}H_{25}O_5Cl$	$C_{22}H_{27}O_4Cl$
HREI-MS Found:	416.1396	390.1604
Calcd:	416.1389	390.1596
UV $\lambda_{max}^{CH_3OH}$ nm	252, 357, 393, 410, 464 (sh)	248, 340 (sh), 355, 393, 407, 430 (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

very similar to each other. In the IR spectrum of isochromophilone II, however, the absorption of γ-lactone carbonyl (ν_{max} 1780 cm^{-1}) was not observed.

From their ^{13}C and ^{1}H NMR spectra, they were found to be a complex of two isomers, respectively. The ratio of the integration of signals of two isomers in ^{1}H NMR was observed to be 2:1 for isochromophilones I and II. ^{13}C and ^{1}H NMR spectral data of the isomers of isochromophilones I and II were summarized in Table 2. These data were assigned from the results of ^{1}H-^{1}H, ^{1}H-^{13}C and ^{1}H-^{13}C long range COSY.

The ^{1}H NMR spectrum of a mixture revealed that both of the isomers, isochromophilones Ia and Ib, have five methyls, one methylene, three methine and five olefinic protons. Because the UV and ^{13}C NMR spectra of the mixture resemble those of sclerotiorin[7], the compounds seem to contain an azaphilone skeleton as sclerotiorin does. ^{1}H-^{1}H COSY and NOE spectra suggested that the isomers (isochromophilones Ia and Ib) have different partial structures as shown in Fig. 2. The ^{13}C NMR data indicated presence of a lactone carbonyl carbon (δ 168.0, C-2'), an acetyl carbonyl carbon (δ 199.6, C-4') adjacent to methyl proton (δ 2.47, H-5'), which was confirmed by ^{1}H-^{13}C long range COSY, and two methine carbons (δ 42.3 and δ 57.2) which were not observed in ^{13}C NMR spectrum of sclerotiorin. The ^{1}H-^{13}C long range COSY experiments revealed that a methyl proton (δ 1.62, 7-CH_3) is coupled to an oxygenated quaternary carbon (δ 83.3, C-7), carbonyl carbon (δ 183.9, C-6) and methine carbon (δ 42.3, C-8). One methine proton (δ 3.90, H-8) connecting a methine carbon (δ 42.3, C-8) and another methine proton (δ 3.80, H-3') coupling to a lactone carbonyl (δ 168.0, C-2') appeared as AB system (J=12.0 Hz). Isochromophilone I has a γ-lactone moiety consisting of an oxygenated quaternary carbon (δ 83.3, C-7), the above methine carbons and lactone carbonyl group. In addition, NOEs were observed between a singlet olefinic proton (δ 7.44, H-1) and a methine proton (δ 3.90, H-8), and between another methine proton (δ 3.80, H-3') and an acetyl methyl proton (δ 2.47, H-5'). Accordingly acetyl moiety was confirmed to be attached to the methine carbon (δ 57.2, C-3') on the γ-lactone ring. Therefore, the structures of isochromophilones Ia and Ib were established as shown in Fig. 1.

From the ^{1}H and ^{13}C NMR spectra, isochromophilone II was found to contain isomers and the same chromophore as that of isochromophilone I and to have a closely related azaphilone skeleton. In the IR spectrum, however, the absorption of γ-lactone carbonyl (ν_{max} 1780 cm^{-1}) observed with isochromophilone I is absent. In addition, in the ^{13}C NMR spectrum of isochromophilone II, carbonyl carbon signal which was observed at δ 168.0 (C-2') in that of isochromophilone I disappeared, on the other hand, a methylene carbon (δ 41.3, C-3') appeared instead of methine carbon (δ 57.2, C-3'). The ^{1}H NMR spectrum also revealed that a methine proton (δ 3.38, H-8) is coupled to two methylene protons (δ 2.35 and δ 3.08, H-3'; J=10.0, 3.0 Hz, respectively). From the above data, the structures of isochromophilones IIa and IIb were determined as shown in Fig. 1.

The inhibitory activities against gp120-CD4 binding were assayed by ELISA method described above. Isochromophilones I and II inhibited gp120-CD4 binding with IC_{50} of 6.6 and 3.9 μM, respectively. Sclerotiorin exhibited no inhibition at 250 μM.

Anti-HIV activity was assayed as follows. Peripheral human lymphocytes were isolated by density gradient centrifugation. After stimulation by

Table 2. ^{13}C and ^{1}H NMR spectral data of isochromophilones Ia, Ib, IIa and IIb δ=ppm in $CDCl_3$.

No.	Ia		Ib		IIa		IIb	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	146.3	7.44 (1H, s)	145.9	7.54 (1H, s)	145.4	7.42 (1H, s)	145.4	7.44 (1H, s)
3	158.7		158.4		158.1		158.1	
4	105.0	6.54 (1H, s)	105.7	6.56 (1H, s)	104.9	6.47 (1H, s)	104.9	6.47 (1H, s)
4a	113.2		113.2		116.3		116.5	
5	109.2		109.2		105.6		105.6	
6	183.9		183.9		191.4		191.4	
7	83.3		83.3		73.9		73.9	
8	42.3	3.90 (1H, d, J=12.0 Hz)	42.3	3.92 (1H, d, J=12.0 Hz)	40.0	3.38 (1H, dd, J=10.0, 3.0 Hz)	40.0	3.42 (1H, dd, J=10.0, 3.0 Hz)
8a	140.6		140.6		118.7		118.7	
9	116.0	6.05 (1H, d, J=15.0 Hz)	118.4	6.15 (1H, d, J=16.0 Hz)	116.3	6.01 (1H, d, J=15.5 Hz)	119.1	6.12 (1H, d, J=15.5 Hz)
10	142.8	7.04 (1H, d, J=15.0 Hz)	134.3	7.44 (1H, d, J=16.0 Hz)	142.1	7.00 (1H, d, J=15.5 Hz)	133.7	7.41 (1H, d, J=15.5 Hz)
11	131.4		129.8		131.8		129.7	
12	148.4	5.67 (1H, d, J=9.5 Hz)	145.9	5.53 (1H, d, J=10.0 Hz)	147.7	5.56 (1H, d, J=9.5 Hz)	145.3	5.49 (1H, d, J=10.0 Hz)
13	35.0	2.40~2.55 (1H, m)	34.1	2.40~2.55 (1H, m)	34.9	2.20~2.65 (1H, m)	34.0	2.20~2.65 (1H, m)
14	30.0	1.23~1.50 (1H, m)	30.0	1.23~1.50 (1H, m)	30.0	1.20~1.38 (1H, m)	30.2	1.20~1.38 (1H, m)
15	11.9	0.86 (3H, t, J=7.5 Hz)	12.0	0.85 (3H, t, J=7.5 Hz)	11.8	0.82 (3H, t, J=7.5 Hz)	11.8	0.83 (3H, t, J=7.5 Hz)
7-CH_3	23.3	1.62 (3H, s)	22.2	1.65 (3H, s)	26.7	1.28 (3H, s)	26.1	1.31 (3H, s)
11-CH_3	12.3	1.83 (3H, d, J=1.0 Hz)	12.3	1.91 (3H, d, J=1.0 Hz)	12.3	1.79 (3H, d, J=1.0 Hz)	12.3	1.83 (3H, d, J=1.0 Hz)
13-CH_3	20.1	1.05 (3H, d, J=6.5 Hz)	20.0	1.08 (3H, d, J=6.5 Hz)	20.2	0.97 (3H, d, J=6.5 Hz)	20.1	0.99 (3H, d, J=6.5 Hz)
2′	168.0		168.0					
3′	57.2	3.80 (1H, d, J=12.0 Hz)	57.2	3.78 (1H, d, J=12.0 Hz)	41.3	2.35 (1H, dd, J=18.5, 3.0 Hz), 3.08 (1H, dd, J=18.5, 10.0 Hz)	41.2	2.37 (1H, dd, J=18.5, 3.0 Hz), 3.10 (1H, dd, J=18.5, 10.0 Hz)
4′	199.6		199.6		206.4		206.4	
5′	30.1	2.47 (3H, s)	30.2	2.47 (3H, s)	30.5	2.06 (3H, s)	30.5	2.08 (3H, s)

Fig. 2. Partial structures of isomers of isochromophilones Ia and Ib.

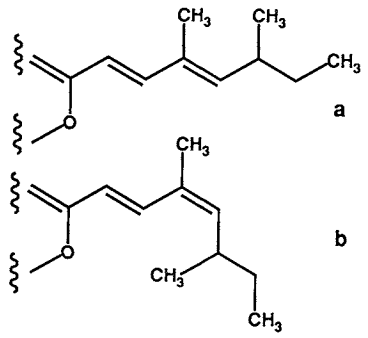

Table 3. Inhibition of HIV replication in the viral core protein level.

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

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 capture-ELISA technique on days 2, 3 and 4. By comparing with a standard preparation, the amount of protein (p24) produced by the rirus infected cells was calculated.

As shown in Table 3, isochromophilone II inhibited significantly HIV replication at 25 μM. It 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).

As mentioned above, isochromophilones I and II were found to inhibit the binding between gp120 and CD4, and also to inhibit strongly HIV proliferation. The inhibition of HIV replication by isochromophilone II is considered to be due to blocking of HIV entry in the cells. They are the first novel non-peptide compounds to inhibit gp120-CD4 binding, and are expected to provide the lead compounds for development of HIV therapies. The details of the structure elucidation and biological activities of isochromophilones I and II will be reported elsewhere.

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