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Isolation, Structure and HIV-1 Integrase Inhibitory Activity of Exophillic Acid, a Novel Fungal Metabolite from *Exophiala pisciphila*

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HIV-1 integrase is one of the three enzymes that are critical for replication and spread of HIV and its inhibition is one of the most promising new drug targets for anti-retroviral therapy with potential advantage over existing therapies. This paper describes the isolation and structure elucidation of exophillic acid, a novel dimeric 2,4-dihydroxy alkyl benzoic acid, derived from *Exophiala pisciphila*, a fungus isolated from a soil sample collected in Georgia, USA. Exophillic acid (**1**) and aquastatin A (**2**), a related compound, inhibited the strand transfer reaction of HIV-1 integrase with IC_{50} values of 68 and 50 μM, respectively.

HIV-1 integrase is one of the three enzymes (protease and reverse transcriptase are the other two) that are critical for replication and spread of virus. It catalyzes three essential steps that include assembly, endonucleolytic cleavage (3′-end processing) of the viral DNA and strand transfer of the viral DNA into the host cell DNA.[1~4] Inhibitors of reverse transcriptase and protease have led to a number of clinical agents that continue to have enormous impact on the control of spread of HIV-1 infection and prolongation of lives. Unfortunately, emergence of multi-drug resistant virus strains particularly in drug naïve patients has become a serious problem and anti-HIV-1 therapy with a new mode of action is needed. HIV-1 integrase is absent in the host cells and its essentiality for viral replication presents it to be an exceptional target for the development of a non-toxic anti-retroviral therapeutic agent. Recently much progress has been made in identification of inhibitors of this enzyme.[5~9]

Screening of natural product extracts has discovered inhibitors and antagonists of many therapeutically relevant biological targets. Natural products have been particularly good sources of novel anti-infective agents. Screening using recombinant HIV-1 integrase (50~220 AA) led to the discovery of a number of natural product inhibitors exemplified by integric acid,[10,11] and cytosporic acid[12]. Continued screening with the strand transfer format of the assay[13] led to the discovery of a novel dimeric 2,4-dihydroxy alkyl benzoic acid trivially named exophillic acid (**1**), isolated from *E. pisciphila*. The isolation, structure elucidation and HIV-1 inhibitory activity of **1** and a related compound aquastatin A (**2**)[14] are described herein.

Producing Organism and Fermentation

The fungus (MF6720) was isolated from a soil sample collected in Georgia, USA and was identified as *E. pisciphila* by micromorphological features as described by DE HOOG[15] and a microscopic picture is depicted in Figure 1. For the production of exophillic acid, the culture was grown on a YMEJ seed medium followed by vermiculite based solid production media as described in the experimental section.

Isolation of Exophillic Acid (**1**)

The fermentation broth of *E. pisciphila* was extracted

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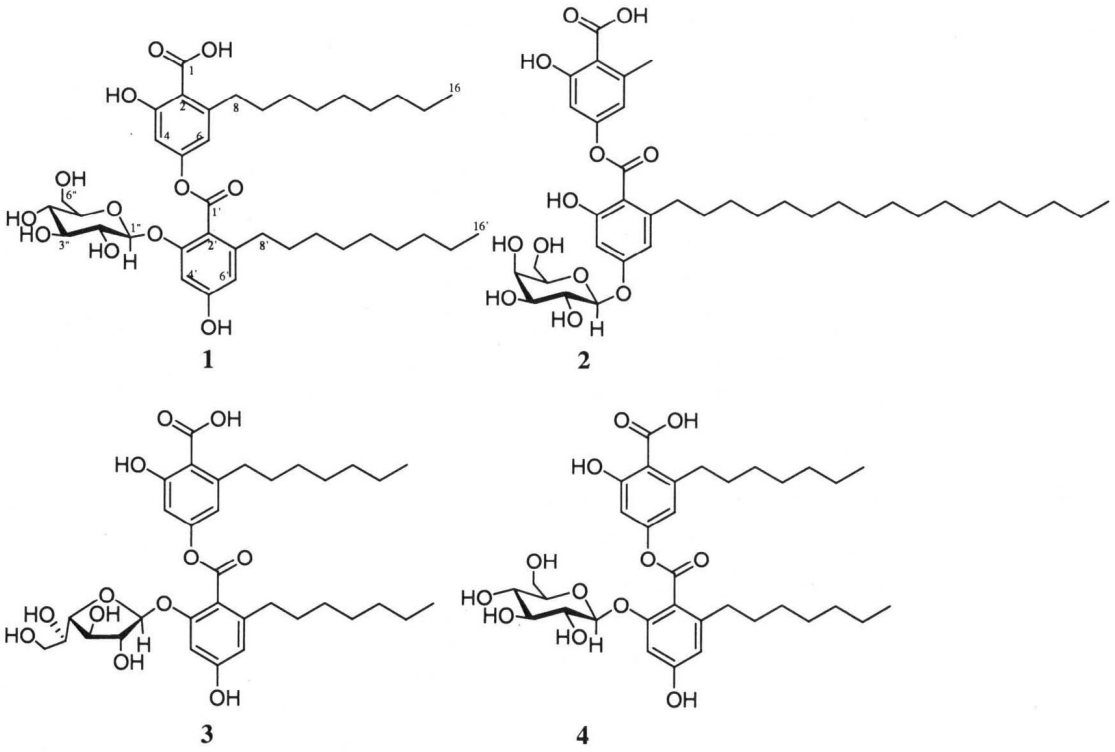

1 2

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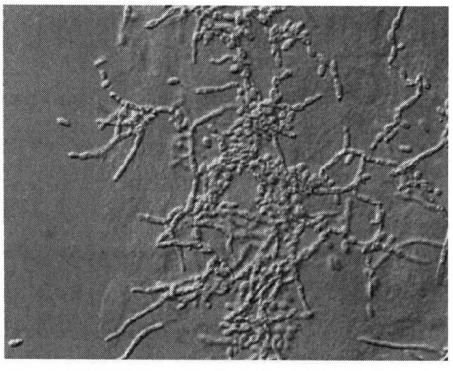
Fig. 1. Photomicrograph of the black yeast *E. pisciphila*.

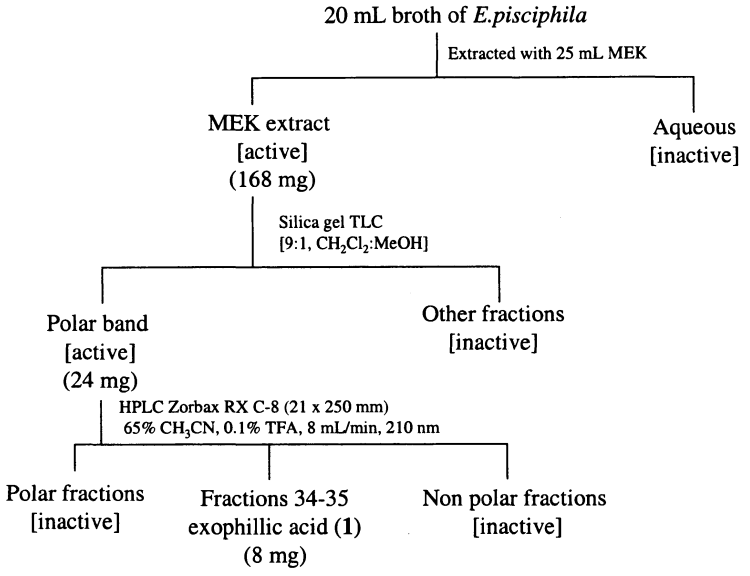


with methyl ethyl ketone and was chromatographed on preparative silica gel plates followed by reversed phase HPLC (Scheme 1) to furnish exophillic acid **1** (400 mg/liter) as a buff colored solid.

Structure Elucidation of Exophillic Acid (**1**)

High-resolution ESIFTMS analysis of **1** produced a molecular formula of $C_{38}H_{56}O_{12}$ indicating the presence of eleven degrees of unsaturation. The formula was corroborated by the ^{13}C NMR spectral analysis which displayed $2\times CH_3$, $17\times CH_2$, $9\times CH$, $8\times C°$, and $2\times C=O$ (Table 1). The UV spectrum showed absorption maxima at 210, 250, 254 and 295 nm. The IR spectrum showed the presence of hydroxy (3335 cm^{-1}), alkyl (2921 cm^{-1} and 2851 cm^{-1}), ester/acid (1716 cm^{-1}) and aromatic (1658 cm^{-1}) groups. The 600 MHz ^1H NMR spectrum of **1** in C_5D_5N displayed two triplets (δ_H 0.84, J=6.1 Hz; and δ_H 0.88, J=7 Hz) for two terminal methyl groups, two sets of *meta*-coupled aromatic protons, an anomeric proton and four methines corresponding to a hexose residue and 17-methylene group protons. These spin systems were confirmed by the COSY spectrum. One of the 17 methylenes was oxygenated and appeared at δ_C 62.8 while the remaining appeared between δ_C 23.4 and δ_C 36.8 typical of methylene groups present in a long alkyl chain. Two methylene groups were shifted downfield (δ_H 3.29, δ_H 3.25 and δ_{2H} 3.00) and were assigned to two sets of benzylic protons. These showed COSY correlations to methylenes at δ_H 1.83 and δ_H 1.87, respectively, which in turn showed correlations to protons buried in methylene envelop. The anomeric proton (H-1″) of the hexose residue showed a 7.8 Hz coupling with H-2″ which exhibited a

Scheme 1. Isolation of exophillic acid (**1**).



equally large coupling with H-3″ (J=7.8 Hz) suggesting a 1,2,3-triaxial relationship of these three protons. The coupling constant values of H-3″ with H-4″ could not be independently discerned due to the overlap of the two protons in the ^{1}H NMR spectrum. However the configuration of the latter proton was deduced as axial based on the large coupling (J=7.8 Hz) of H-4″ with H-5″ and thus establishing axial configuration of all protons of the hexose residue and confirming it as a β-glucopyranoside. The benzylic methylene protons (δ_H 3.29, δ_H 3.25 and δ_H 3.00) showed HMBC ($^nJ_{XH}$=7 Hz, Table 1) correlations to three aromatic carbons each. The H_2-8 displayed correlations to C-2 (δ_C 114.6), C-6 (δ_C 116.0) and C-7 (δ_C 148.8) and H_2-8′ to C-2′ (δ_C 115.6), C-6′ (δ_C 111.8) and C-7′ (δ_C 144.5) and thus established the location of the two alkyl chains at C-7 and C-7′, respectively. The H-4 showed HMBC correlations to C-2, C-3, C-5 (δ_C 155.5), C-6 and H-6 displayed HMBC correlations to C-2, C-4 (δ_C 109.5), C-5 and C-8 (δ_C 36.8) thus establishing a 3,5-dioxy-7-alkyl-benzoate substitution for the first half of the structure. Similar HMBC correlations of H-4′ and H-6′ established an identical substitution pattern for the second half of the molecule. The HMBC correlation of anomeric proton H-1″ to C-3′ (δ_C 158.5) established the glucoside linkage to C-3′. Finally the connectivity of the benzoate ester linkage was established as follows. Of the four oxygenated aromatic carbons two appeared relatively downfield at δ_C 164.7 and δ_C 162.2 and were assigned to C-3 and C-5′ containing the free phenolic groups, C-3′ (δ_C 158.5) had been already confirmed to possess the ether linkage as glucoside, and thus the remaining oxygen containing carbon C-5 (δ_C 155.5) must be esterified and connected to C-1′. This assignment is consistent with the observed relative upfield chemical shift of C-5 due to benzoate ester substitution compared to phenolic and glucoside ether substituted carbons C-2, C-5′ and C-3′. This was further supported by the benzoate induced relative downfield shifts of two *ortho*-carbons C-4 (δ_C 109.5) and C-6 (δ_C 116.0) compared to phenol induced shifts of the two *ortho*-carbons C-4′ (δ_C 102.2) and C-6′ (δ_C 111.8). Negative ion ESIMS produced strong molecular ion at *m/z* 703 $[M-H]^-$ and a weak ion at *m/z* 279 due to a fragment originating by cleavage of the ester bond with intact carboxy group of top portion of the molecule (Figure 2). The positive ESIMS spectrum produced fragment ions at *m/z* 425 and *m/z* 263 originating from the fragmentation of the lower half of the molecule with or without glucose (Figure 2), respectively, thus confirming that both top and bottom half contained the same alkyl chain. Based on these data structure **1** was assigned to exophillic acid.

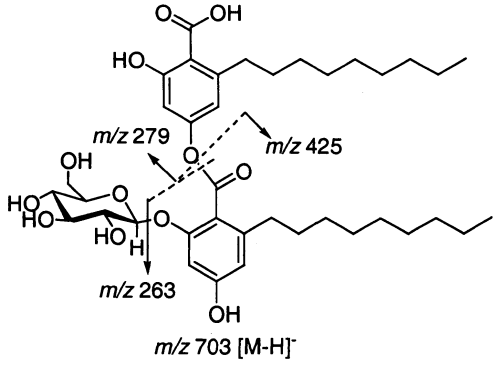
HIV-1 Integrase Activity

Exophillic acid (**1**) inhibited the strand transfer reaction[13] of HIV-1 integrase with an IC_{50} of 68 μM. Aquastatin A (**2**) was almost equally active and showed

Table 1. ^{1}H and ^{13}C NMR assignment of exophillic acid (**1**) in C_5D_5N.

Position	δ_C	type	δ_H	HMBC (H→C)
1	175.1	C°		
2	114.6	C°		
3	164.7	C°		
4	109.5	CH	7.45 (d, 2.0)	2, 3, 5, 6
5	155.5	C°		
6	116.0	CH	7.20 (d, 2.0)	2, 4, 5, 8
7	148.8	C°		
8	36.8	CH_2	3.29 (dt, 13, 7.5)	2, 6, 7, 9, 10
			3.25 (dt, 13, 7.5)	
9	32.8	CH_2	1.83 (t, 7.8)	7, 8, 10, 11
10	30.7	CH_2	1.44 m	8, 9, 11
11	30.4	CH_2	1.32 m	
12	30.4	CH_2	1.23 m	
13	30.4	CH_2	1.23 m	
14	32.8	CH_2	1.23 m	
15	23.4	CH_2	1.23 m	
16	14.7	CH_3	0.84 (t, 6.1)	14, 15
1'	167.4	C°		
2'	115.6	C°		
3'	158.5	C°		
4'	102.2	CH	7.32 (d, 2.0)	2', 3', 5', 6'
5'	162.2	C°		
6'	111.8	CH	6.90 (d, 2.0)	2', 4', 5', 8'
7'	144.5	C°		
8'	34.8	CH_2	3.00 (t, 8.0)	2', 6', 7', 9',10'
9'	32.4	CH_2	1.87 (t,7.8)	7', 8', 10', 11'
10'	30.0	CH_2	1.40 m	8', 11'
11'	30.4	CH_2	1.32 m	
12'	33.4	CH_2	1.23 m	
13'	30.4	CH_2	1.23 m	
14'	32.8	CH_2	1.23 m	
15'	23.4	CH_2	1.23 m	
16'	14.8	CH_3	0.88 (t, 7.0)	14', 15'
1"	103.3	CH	5.68 (d, 7.8)	3', 3"
2"	75.4	CH	4.35 (t, 7.8)	1", 3"
3"	79.2	CH	4.31 (m)	1", 5"
4"	71.5	CH	4.30 (m)	3"
5"	79.4	CH	4.00 (ddd, 2.5, 5.0, 7.8)	3", 4"
6"	62.8	CH_2	4.34 (dd, 5.0., 12.0)	4", 5"
			4.39 (dd, 2.5, 12.0)	4", 5"

Fig. 2. ESIMS fragmentation of exophillic acid (**1**).



IC_{50} value of 50 μM in the strand transfer assay. Both the *ortho*-hydroxy benzoic acid and aliphatic chain may play a significant role for the activity as was observed in the case of integric acid[10,11] and integracins.[16]

Exophillic acid (**1**) is related to aquastatin A (**2**), an unsymmetrical galactopyranoside isolated from *Fusarium aquaeductuum* as an inhibitor of adenosine triphosphatase,[14] and KS-502 (**3**), a symmetrical galactofuranoside isolated from *Sporothrix* sp. KAC-1985 as an inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase.[17] The structure of KS-502 was confirmed subsequently by a total synthesis.[18] TPI 1 (**4**), a glucopyranoside was reported from *Hypomyces* sp. and *Nodulisporium* sp. as inhibitors of phosphatidylinositol-3 kinase and cAMP, endothelin-1 receptor antagonist and antitumor agent.[19]

In summary, we have described isolation and structure of exophillic acid (**1**), a novel dimeric alkyl-benzoate glucoside, which is a modest inhibitor of the strand transfer reaction of HIV-1 integrase.

Experimental

General Procedure

All NMR spectra were recorded on Varian Inova 500 or 600 MHz instruments operating at 500 and 600 MHz for ^{1}H and 125 and 150 MHz for ^{13}C nuclei. An HP1100 was used for analytical HPLC. LC-MS was performed on a Thermo Quest LCQ instrument using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). High Resolution mass spectral analyses were performed on a Thermo Quest FTMS using electrospray ionization. For column chromatography silica gel H (E. Merck 60~200 mesh) was used.

Fermentation and Production of Exophillic Acid (1)

Exophiala pisciphila, MF6720, was inoculated into seed flasks by transferring a 1 ml aliquot of a frozen culture into a 250 ml Erlenmeyer flask containing 50 ml of YMEJ seed medium (in g/liter, yeast extract 4; malt extract 8; dextrose 4; junlon 1.5; pH 7.0). The seed culture was incubated at 25°C on a gyratory shaker (220 rpm) for 3 days before transfer to production medium (in g/liter, dextrose 150; urea 4; NZ amine type A 4.0; K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$

0.25; KCl 0.25; $ZnSO_4 \cdot 7H_2O$ 0.90; $CaCO_3$ 16.5). Fermentations were allowed to grow on a solid production medium in 2-liter roller bottles containing approximately 675 cc of vermiculite to which was added 220 ml of a liquid nutrient. A 10 ml aliquot of the seed culture was added and shaken to coat the vermiculite. The inoculated roller bottles were incubated at 4 rpm on a Wheaton rolling machine at 22°C for 18 days. Fungal growth in each bottle was extracted with 250 ml of methyl ethyl ketone.

Isolation of Exophillic Acid (**1**)

A 25 ml methyl ethyl ketone (MEK) extract of *E. pisciphila* (MF6720) was concentrated under reduced pressure and lyophilized to yield a 168 mg of crude material which was chromatographed on 0.5 mm thick 20×20 cm silica gel plates developed in 9:1 CH_2Cl_2-MeOH. The polar band eluting near the baseline was collected and extracted with 50% CH_2Cl_2-MeOH. The extract was concentrated under reduced pressure to give 24 mg of solid material, which was highly enriched with exophillic acid (**1**). This was further chromatographed by reverse phase preparative HPLC using a Zorbax RX C-8 (21×250 mm) column eluting with 65% aqueous CH_3CN containing 0.1% TFA at a flow rate of 8 ml/minute. Concentration at reduced pressure followed by lyophilization of fractions eluting at 34~35 minutes afforded 8 mg of exophillic acid as a buff powder. $[\alpha]_D^{23} = -13.9°$ (*c* 1.3, CH_3OH), UV (MeOH) λ_{max} 210 (ε 34,054), 250 (10,973), 254 (10,973), 295 (3,243) nm, IR (ZnSe) ν_{max} 3335, 2921, 2852, 1716, 1658, 1587, 1461, 1373, 1238, 1171, 1136, 1043, 828 cm^{-1}, Positive ion ESIMS (*m/z*): 722 $[M+NH_4]^+$, 425, 263; Negative ion ESIMS (*m/z*): 703 $[M-H]^-$, 279; HRESIFTMS: Found: 722.4095 (calcd for $C_{38}H_{56}O_{12}+NH_4$: 722.4115). For ^1H and ^{13}C NMR see Table 1.

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