Isolation, Structure, and HIV-1 Integrase Inhibitory Activity of Cytosporic Acid, a Fungal Metabolite Produced by a *Cytospora* sp.

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Received November 15, 2002

HIV-1 integrase is a critical enzyme for replication of HIV, and its inhibition has the potential to lead to an anti-retroviral therapy that has advantages over existing therapies. Cytosporic acid (**1**) is a polyketide-derived novel natural product that was isolated from a fermentation broth of the filamentous fungus *Cytospora* sp. collected from Puerto Rico. It inhibited strand transfer reaction of HIV-1 integrase with an IC_{50} of 20 μM. The isolation, structure elucidation, relative stereochemistry, and activity of **1** are described.

HIV-1 integrase is one of the three enzymes that are critical for viral replication. It catalyzes three essential steps that include assembly, endonucleolytic cleavage (processing) of the viral DNA, and strand transfer of the viral DNA into the host cell DNA.[1] The other two key enzymes are reverse transcriptase and protease. While inhibitors of the latter two enzymes have led to successful clinical agents that are effective in managing the disease, the emergence of multidrug-resistant virus even in drug naïve patients has become a serious cause for concern. This clearly demonstrates a critical need for new therapies to manage the disease. HIV-1 integrase is a target of choice because of its absence in the host cells and its critical requirement for viral replication. Therefore, inhibitors of integrase have potential to afford novel nontoxic anti-HIV clinical agents. This approach has been recently validated by the discovery of a potent diketo acid lead[2] and subsequent modifications leading to a clinical candidate.[3]

Natural products have been very good sources of novel inhibitors for many biological targets and have been particularly good sources of anti-infective agents. Screening of natural product extracts against recombinant HIV-1 integrase led to the discovery of several classes of natural product inhibitors including equisetin[4] and integramides.[5] Continued screening of fungal extracts led to the discovery of a novel polyketide-derived natural product named herein cytosporic acid (**1**) produced by a *Cytospora* sp.

The producing fungus was isolated from leaf litter of *Manilkara bidentata* collected in Puerto Rico and identified by its micromorphology as *Cytospora* sp. (MF 6608). The culture was grown in a vermiculite-based solid medium or a liquid medium, as described in the Experimental Section. The culture was extracted with methyl ethyl ketone and was chromatographed on Sephadex LH-20. The HIV-1 integrase inhibitory fractions were further chromatographed on reversed-phase HPLC and crystallized from nitromethane to afford **1** (270 mg/L) as colorless needles.

High-resolution ESI-MS analysis of **1** revealed a molecular formula $C_{22}H_{34}O_6$, indicating six degrees of unsaturation. The ^{13}C NMR spectrum of **1** in C_5D_5N (see Experimental Section) corroborated the assigned molecular formula. The IR spectrum of **1** showed absorption bands assigned for various types of keto (ν_{max} 1699, 1666, 1639 cm^{-1}) and hydroxy groups (3404 cm^{-1}). The UV spectrum showed absorption bands at λ_{max} 275 nm due to an α-hydroxy-α,β-unsaturated keto group present in a ring. The DEPT spectrum of **1** indicated the presence of four methyls, seven methylenes, and five sp^3 methines. The quaternary carbons in the ^{13}C NMR spectrum were assigned to three carbonyls [an alicyclic ketone (δ 211.3), conjugated cyclohexenone (δ 194.9), and an acid (δ 177.5)], two sp^2 carbons (δ 146.4 and 137.9), and a sp^3 carbon (δ 58.6). The four methyl groups include two secondary, one tertiary, and an angular methyl as revealed by the ^1H NMR spectrum. The methyl groups and other proton-bearing carbons were correlated to the respective attached protons by a 2D HMQC experiment. The $^1H-^1H$ spin systems were deduced by a 2D COSY experiment, which revealed three fragments consisting of C18−C19, C5−C10, and C11(C16)−C15. These fragments were assembled together by a 2D HMBC experiment. The methyl group H_3-20 exhibited four strong HMBC correlations to C-3 (δ 137.9), C-4 (δ 58.6), C-5 (δ 42.8), and C-17 (δ 211.3), typical of angular methyl groups. The latter carbonyl group (δ 211.3) showed correlations to H-5, H_2-18, and H_2-19, thus unequivocally connecting C-5 to C-18 via the C-17 keto group. The sp^2 carbon C-3 exhibited strong HMBC correlations to both the angular (H_3-20) and the secondary methyl group H_3-16 (δ 1.37) and the H-12 methine, therefore establishing the C-4 to C-12 via C-3 and thus the entire right-hand side of the structure of cytosporic acid. The HMBC correlation of H-10 (δ 2.43) to the conjugated carbonyl (δ 194.9) connected the remainder of the left-hand side of the molecule and completed the structure **1** for cytosporic acid. The COSY fragments were verified by the respective HMBC correlations (Table 1, Supporting Information).

The relative stereochemistry of **1** was elucidated by the application of the scalar couplings, NOESY correlations, ChemDraw 3D modeling, and examination of Dreiding models. The large couplings (>11 Hz) between H-5, H-10 and H-5, H-6 indicated their respective 1,2-diaxial relationships in a chair−chair conformation. H-10 showed NOESY correlations (Figure 1, Supporting Information) to H-6 and H_3-20, thus indicating 1,3-diaxial orientations of these protons. Similar NOESY correlations of H-6 and H-8 proved their diaxial orientations. These stereochemical assignments were complemented by the 1,3-diaxial NOESY correlations of H-5 to H-7β (δ 1.99) and H-9β (δ 2.56). In

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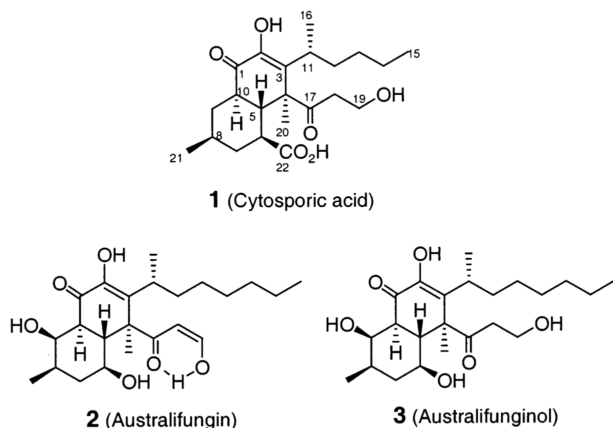
10.1021/np020533g CCC: $25.00 © 2003 American Chemical Society and American Society of Pharmacognosy
Published on Web 03/21/2003

addition, H-5 showed strong NOESY correlation to H-18β (δ 3.86) due to its buttressing effect from the top face. The presence of a strong NOESY correlation of H-18α (δ 3.23) to H-11 (δ 1.86) and H-20 to H-12 (δ 1.86), indicating their spatial proximity, and the absence of the NOESY correlations of either of the two protons at C-18 to the methyl protons at C-16 provided evidence for the α-orientation of the C-16 methyl group, which is consistent with the stereochemistry proposed for the related compound australifungin (**2**), which was isolated from *Sporormiella australis*.[6] The ChemDraw 3D model (Figure 1) and Dreiding model supported the NOESY correlations and scalar couplings and the assigned stereochemistry of cytosporic acid (**1**) that is similar to the reported stereochemistry of australifunginol (**3**) and australifungin (**2**).[6]



1 (Cytosporic acid)

2 (Australifungin) **3** (Australifunginol)

The NMR spectral data of **1** are consistent with the reported data of **2** and **3**. However, unlike **3**,[6] the NMR spectrum of **1** did not exhibit exchange broadening. Cytosporic acid (**1**) consists of a branched chain containing six carbons at C-3 and has a free carboxyl group at C-6 compared to an eight-carbon branched chain present at C-3 and a hydroxy group at C-6 in **3**. The free carboxyl group probably stabilizes **1** into a stable conformational and tautomeric state.

Cytosporic acid (**1**) inhibited strand transfer reaction of HIV-1 integrase in an in vitro assay[4b] with an IC_{50} of 20 μM. Because of the structural similarity, **2**, and **3** were also evaluated in the strand transfer assay. Australifungin (**2**) exhibited an IC_{50} value of 20 μM, and the activity was indistinguishable from **1**. However, **3** did not show any inhibition at 200 μM. The lack of activity of the latter compound indicates that the β-keto aldehyde of **2** and the carboxyl group of **1** are critical for the activity and may be responsible for the binding at the active site by interacting with the required bivalent cation. Due to poor activity of these compounds, they were not evaluated in any other HIV-1 integrase assays including cell-based assays.

Experimental Section

General Experimental Procedures. For general experimental procedures see ref 7.

Fermentation of *Cytospora* sp. (MF 6608). The producing fungal isolate is identified as *Cytospora* sp. (Coelomycetes) on the basis of morphological characteristics observed on plate cultures grown on Oatmeal Agar (Difco) for 10 days at 23 °C. These observations include the size, shape, and color of the reproductive structures known as conidiomata, conidiophores, conidiogenous cells, and conida and agree well with the published description for the genus *Cytospora*.[8]

The culture was grown for 3 days at 25 °C in a seed medium, which has been described.[8] Production of **1** was achieved on a solid medium (AD2),[9] prepared by adding 220 mL of liquid to 675 cm^3 large particle vermiculite in a 2-L roller jar, using a 12 mL inoculum from the seed. The roller jars were incubated 22 °C for 18 days. Alternatively, the production of **1** was also achieved on a liquid Medium-4,[10] prepared as 50 mL per 250 mL flask. An aliquot of the growth seed (1−2 mL) was used to inoculate the flasks, which were incubated at 22 °C, 220 rpm, for 18 days.

Isolation of Cytosporic Acid (1). The fermentation broth (30 mL) was extracted with 35 mL of methyl ethyl ketone (MEK) and filtered through Celite. MEK was removed from the extract under reduced pressure, and the solid residue was chromatographed on a 1-L column filled with Sephadex LH-20 and eluted with methanol. This yielded a single active cut, which was further purified by HPLC (Zorbax RX C8, 22.4 × 250 mm, 50% aqueous CH_3CN containing 0.1% TFA at a flow rate of 8 mL/min), affording an active major peak at t_R = 25 min. The residue of the active fraction was recrystallized from nitromethane to yield 8 mg of cytosporic acid (**1**) as colorless fine needles: mp 198−202 °C, $[\alpha]^{25}_D$ +69.5° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (ϵ) 275 nm (24882); IR ν_{max} (ZnSe) 3404, 2950, 2871, 1699, 1666, 1639, 1460, 1383, 1178, 1027 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) 2.86, 1H, brt, J = 11.5 Hz (H5), 2.78, 1H, dt, J = 11.5, 3.5 Hz (H-6), 1.99, 1H, brd, J = 10 Hz (H-7_{ax}), 1.37, 1H, m (H-7_{eq}), 1.38, 1H, m (H-8), 2.56, 1H, brd, J = 13.5 Hz (H-9_{ax}), 1.06, 1H, m (H-9_{eq}), 2.43, 1H, dt, J = 12.5, 4.0 Hz (H-10), 1.86, 1H, m (H-11), 1.83, 1H, m (H-12), 2.50, 1H, m (H-12), 1.17, 1H, m (H-13), 1.26, 1H, m (H-14), 0.82, 3H, t, J = 7.0 Hz (H-15), 1.37, 3H, d, J = 6.5 Hz (H-16), 3.23, 1H, brm (H-18_{α}), 3.86, 1H, brm (H-18_{β}), 4.26, 1H, brm (H-19), 4.40, 1H, brm (H-19), 1.67, 3H, s (H-20), 0.88, 3H, d, J = 6.0 Hz (H-21); ^{13}C NMR (C_5D_5N, 125 MHz) 194.9 (C-1), 146.4 (C-2), 137.9 (C-3), 58.6 (C-4), 42.8 (C-5), 47.6 (C-6), 40.1 (C-7), 30.9 (C-8), 35.0 (C-9), 43.3 (C-10), 36.5 (C-11), 34.7 (C-12), 31.4 (C-13), 23.1 (C-14), 14.3 (C-15), 18.1 (C-16), 211.3 (C-17), 42.1 (C-18), 58.2 (C-19), 14.7 (C-20), 22.2 (C-21), 177.5 (C-22); ESI-MS (*m/z*) 789 $[2M + H]^+$, 787 $[2M - H]^-$, 395 $[M + H]^+$, 393 $[M - H]^-$; HRESI-FTMS (*m/z*) 395.2436 ([M + H], calcd for $C_{22}H_{35}O_6$ 395.2433).

Reisolation of 1 from Liquid Fermentations. A 2-L fermentation broth was extracted with 2 L of MEK and filtered through Celite. MEK was removed from the extract under reduced pressure, and the solid residue was chromatographed on a 2-L column filled with Sephadex LH-20 and eluted with methanol. This yielded a single active cut (3 g), which contained mainly cytosporic acid. A portion (100 mg) of the active cut was further purified as described above, yielding 52 mg of cytosporic acid as a white powder.

Acknowledgment. The authors thank Dr. Guy H. Harris for providing samples of australifungin and australifunginol and Gabe Dezeny for fermentation support.

Supporting Information Available: A copy of ^{1}H and ^{13}C NMR spectra of **1**, Table 1 containing full ^{1}H and ^{13}C NMR assignments including HMBC data, and Figure 1 showing a ChemDraw 3D model and NOESY data. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP020533G