Chemokine Receptor CCR-5 Inhibitors Produced by *Chaetomium globosum*

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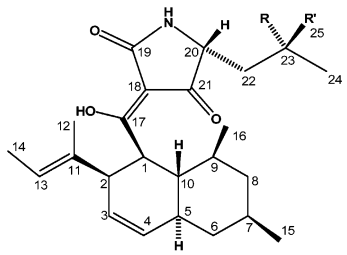
Two novel chemokine receptor CCR-5 inhibitors, Sch 210971 (**1**) and Sch 210972 (**2**), were isolated from the fungal fermentation broth of *Chaetomium globosum* by normal- and reversed-phase HPLC purifications. The structure determination of **1** and **2** was accomplished on the basis of UV, MS, and NMR spectral data analyses including COSY, NOESY, HMQC, and HMBC experiments. The structure and relative configuration of **2** were determined unequivocally by X-ray crystallographic analysis. The major component **2** demonstrated a potent inhibitory activity of $IC_{50} = 79$ nM in the CCR-5 receptor in vitro binding assay.

Human immunodeficiency virus type 1 (HIV-1) infection causes a fatal disease, acquired immunodeficiency syndrome (AIDS), which has become one of the major health crises worldwide since over 38 million infected individuals and 3 million deaths were reported by the end of 2004.[1] Two major classes of approved antiretroviral drugs, the HIV protease and reverse transcriptase inhibitors, are currently used to suppress HIV infection and reduce morbidity and mortality in the combination of antiviral therapy called highly active antiretroviral therapy, or HAART. However, long-term toxicity and adverse drug−drug interactions of existing anti-HIV drugs are major concerns in medical treatments.[2] Furthermore, HIV strains resistant to the current regimen of drugs have been reported due to the increasing use of antiretroviral agents.[3] Hence, there is an urgent demand for developing effective anti HIV-1 infection drugs with novel mechanisms of action. Previous investigations indicated that HIV-1 infection of macrophages, monocytes, and T-cells is mediated by interaction with a cell surface receptor, CCR-5, for the β-chemokines MIP-1α, MIP-1β, and RANTES as a co-receptor with the CD_4 molecule.[4] The CCR-5 receptor, which belongs to the superfamily of seven-transmembrane G-protein coupled receptors (GPCRs), has been identified to play an important role in viral attachment to and entry into the human host cell in the initial stage of HIV-1 infection.[5] Inhibition of the chemokine receptor CCR-5 is an attractive therapeutic target for novel antiviral agents against HIV-1 infection by blocking viral entry into cells. Tremendous research efforts have been made by several drug discovery groups after identification of the CCR-5 receptor as an HIV target to discover potent and selective CCR-5 antagonists as a new class of anti-HIV-1 agents.[6] As part of an antiviral research program searching for novel CCR-5 inhibitors as potential leads for development, a large number of extracts from microbial sources have been screened in CCR-5 membrane binding assays. The screening of these extracts derived from fungi, actinomycetes, or bacteria has led to the discovery of two novel secondary metabolites, Sch 210971 (**1**) and Sch 210972 (**2**), as CCR-5 inhibitors produced by a fungal culture designated as *Chaetomium globosum* (Mer-0229). Described herein are the results of the isolation and structure elucidation, as well as the biological activity of **1** and **2**.



Sch 210971 (**1**): R=COOH R'=OH
Sch 210972 (**2**): R=OH R'=COOH

Results and Discussion

The purification of **1** and **2** was accomplished by normal-phase HPLC (PVA-Sil), followed by reversed-phase HPLC (C-18) through bioassay-guided fractionation to obtain pure **1** (6 mg) and **2** (10 mg) as white solids. Both compounds were soluble in acetone, EtOAc, acetonitrile, MeOH, and CH_2Cl_2, but were insoluble in hexane and H_2O. These compounds were negative in the ninhydrin test for amine groups and positive in the Rydon test for amide functionalities. Since **2** was isolated as a major component with more material available, extensive 2D-NMR studies, as well as X-ray crystallographic analysis, were focused on **2**.

Electrospray ionization (ESI) mass spectral data from LC/MS experiments showed a protonated molecular ion $(M + H)^+$ at *m*/*z* 446 indicating the molecular mass of 445 Da for **2**. The molecular formula of $C_{25}H_{35}NO_6$ was deduced by elemental analysis. UV absorption at 220 and 295 nm revealed the presence of a conjugation system suggestive of tetramic acid in **2**. Absorption bands in the IR spectrum at 3436, 1710, and 1645 cm^{-1} suggested the presence of hydroxyl, carboxyl, conjugated carbonyl, and amide carbonyl groups, respectively. The ^{13}C NMR spectrum contained 25 carbon resonances representing two carbonyl, two amide/acid carbonyl, two olefinic quaternary, three vinyl methine, one oxygenated quaternary, one nitrogenated methine, six methine, three methylene, and five methyl carbons. The ^1H NMR spectrum was consistent with ^{13}C NMR data showing three vinyl proton signals at δ 5.66 (H-3), 5.66 (H-4), and 5.19 (H-13), one nitrogenated methine signal at δ 3.80 (H-20), two allylic methine resonances at δ 3.94 (H-1) and 3.00 (H-2), two sets of a geminal methylene doublet of doublets (AB spin system) at δ 2.50 (H-22) and 1.75 (H-22), one allylic methyl singlet at δ 1.57 (CH_3-12), one allylic methyl doublet at δ

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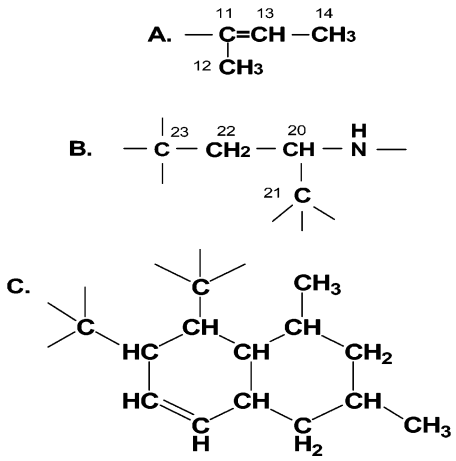

Figure 1. Partical structure assignments of **2** based on COSY and HMQC data.

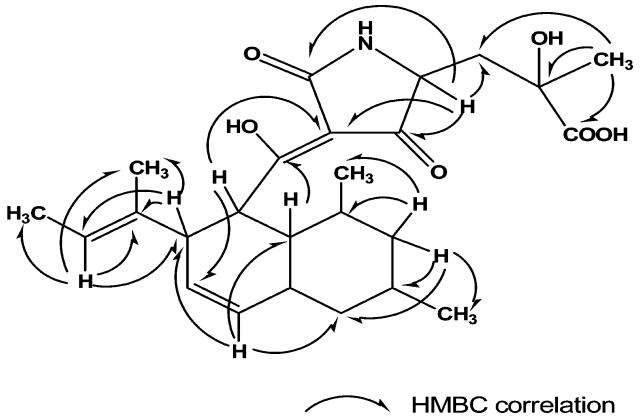

Figure 2. Some important $^{1}H-^{13}C$ long-range coupling HMBC data of **2**.

1.50 (CH_3-14), one methyl singlet at δ 1.49 (CH_3-24), and two methyl doublets at δ 0.91 (CH_3-15) and 0.85 (CH_3-16). Further 2D-NMR analysis with $^{1}H-^{1}H$ correlation data permitted the establishment of three partial structures, A, B, and C, on the basis of COSY and HMQC experiments, as shown in Figure 1. The assignments of these three fragments were confirmed by long-range $^{1}H-^{13}C$ correlation data generated from HMBC experiments. As shown in Figure 2, the correlations of H-13 to C-2, as well as H-2 to C-11, C-12, and C-13, indicated attachment of fragment A to fragment C at position 2. The correlation of H-20 to C-18, C-19, and C-21 suggested the formation of a 2,4-pyrrolidinedione ring from fragment B as a core unit of tetramic acid. The carbon chain extension on the methylene group (CH_2-22) attached to a hydroxyl-carboxylic disubstituted two-carbon moiety was assigned on the basis of correlations of H-24 to C-22, C-23, and C-25. The final connectivity of the decalin moiety and tetramic acid through the enol carbon C-17 was established by HMBC correlations of H-1 to C-18 and H-10 to C-17. Thus, the structural assignments of all protons and carbons for **2** were completed.

The relative configuration of **2** was determined in part by NOESY data. The observation of four 1,3-diaxial couplings between H-1 and H-5, H-5 and H-7, H-7 and H-9, and H-9 and H-1 revealed the α-configuration of these protons. The coupling between H-1 and H-2 further showed the same α-configuration (equatorial) for the H-2 proton. Moreover, observation of NOE coupling between H-10 and CH_3-9 showed that the H-10 proton had the opposite β-orientation, thus forming a *trans* configuration at the decalin ring junction. However, the configurations of two chiral centers at C-20 and C-23 could not be determined due to the lack of references at adjacent carbons.

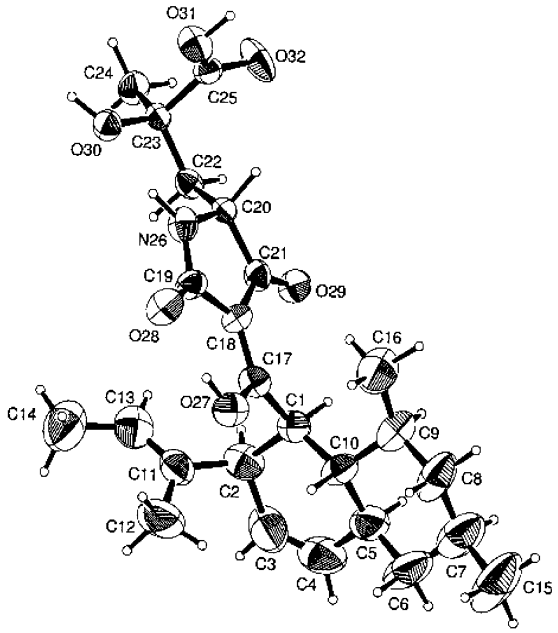

Figure 3. ORTEP (40% probability ellipsoids) showing the crystallographic atom numbering scheme and solid-state conformation of Sch 210972 (**2**).

To complete the configurational determination and confirm the structural assignments by NMR data analysis, compound **2** was crystallized from a methylene chloride/acetone (1:1) solution to obtain weakly diffracting single crystals suitable for X-ray analysis. As shown in Figure 3, in addition to establishing the configurations at C-20 and C-23, the results of the X-ray analysis yielded the complete structure and confirmed the NMR assignments for the remaining chiral centers on the decalin ring.

The molecular mass of **1** was determined to be 445 Da by ESIMS data that showed the protonated molecular ion at *m/z* 446 $(M + H)^+$. The molecular formula was found to be $C_{25}H_{35}NO_6$ by elemental analysis, the same as **2**. Comparison of UV and IR profiles of **1** with **2** clearly indicated that **1** was a simple isomer of **2**. The similar ^{1}H and ^{13}C NMR spectra provided further supportive evidence for the structure of **1**. The significant differences of chemical shifts at C-23 and C-24 (both showing over 2 ppm shift) in comparison to **2** indicated a change of the side chain attached to the tetramic acid ring in **1**. The observation of a large chemical shift difference at H-20 due to the steric effects of hydroxyl and carboxylic acid strongly suggested the opposite stereochemistry arrangements of hydroxyl and carboxylic acid groups at C-23 in **1**.

Both compounds **1** and **2** displayed inhibitory activity in the CCR-5 membrane binding assay. Furthermore, **2** demonstrated highly significant activity, with an IC_{50} value of 79 nM in vitro. Interestingly, as an epimer of **2**, the potency of **1** decreased over 15-fold, with an IC_{50} value of 1.2 μM. These data indicated that the orientation of hydroxyl and carboxylic acid groups on C-23 plays an important role in the receptor binding and provides valuable information for structure−activity relationship (SAR) studies by medicinal chemists. Compounds **1** and **2** were also tested in the CCR-2 binding assay to determine specificity, and there was no inhibitory activity observed at 100 μM concentration.

Thus, two novel secondary metabolites (**1** and **2**) were isolated from the fungus *C. globosum* and were determined to be chemokine receptor CCR-5 inhibitors. Compound **1** is an epimer of **2** at C-23, and they both belong to the tetramic acid (2,4-pyrrolidinedione) class of antibiotics.[7] Numerous natural occurring tetramic acids have been reported in the scientific literature including coniosetin,[8] vancoresmycin,[9] CJ-17,572,[10] CJ-21,058,[11] ascosalipyrrolidinone A,[12] reutericyclin,[13] crypotocin,[14] xanthobaccin A,[15] LL-49F233,[16] PF1052,[17] lydicamycin,[18] MBP049,[19] equistin,[20] and phomasetin.[21]

Tetramic acid type antibiotics possess antimicrobial activity against various microorganisms including many resistant microbial pathogens.[8] To the best of our knowledge, compounds **1** and **2** are the first natural tetramic acid metabolites possessing CCR-5 antagonistic activity to be reported in the literature.

Experimental Section

General Experimental Procedures. Melting points were recorded on a MEL-TEMP apparatus (Laboratory Devices, Cambridge, MA) and were uncorrected. IR and UV spectra were obtained using a Nicolet FTIR model 10-MX and a Hewlett-Packard "8050 A" ultraviolet−vis spectrophotometer, respectively. Optical rotation was measured on a Perkin-Elmer 243B polarimeter. LC-MS analyses were performed on a triple-stage quadrupole TSQ-7000 mass spectrometer equipped with ESI (electrospray ionization)/APCI (atmospheric pressure chemical ionization) interfaces (Finnigan MAT, San Jose, CA) that linked to a Hewlett-Packard HP-1090 Series X system. HRFABMS data were produced by a JEOL JMS-HX110A mass spectrometer in a glycerol/thioglycerol matrix. Both ^{1}H and ^{13}C NMR spectra were recorded using Varian XL-400 instruments operating at 400 and 100 MHz, respectively.

Microorganism. The producing culture was collected and isolated from sterilized leaves of evergreen plants in Tucson, AZ. It was characterized as a fungus, *Chaetomium globosum*, on the basis of morphological studies and deposited in the American Type Culture Collection with the accession number ATCC 74489. Morphological observations of the producing strain of *Chaetomium globosum Kunze* SCF 1705 were made on the following standardized description of SNA (Synthesischer nährstoffärmer agar): KH_2PO_4 1.0 g, KNO_3 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, glucose 0.2 g, saccharose 0.2 g, agar 2.0 g, and distilled H_2O 100 mL. Pieces of sterile filter paper (about 5 cm round) were placed on the surface of the agar after it had solidified.

Colonies were 50−70 mm in diameter on SNA after 10 days at room temperature, comprised of perithecia scattered over much of the surface, concentrated near the inoculum and around the edge of the filter paper, then in vague concentric rings outward, with little aerial mycelium and the submerged mycelium unpigmented and inconspicuous: perithecia superficial, easily removed from the substrate, single, scattered, or in continuous mass, olivaceous in superficial view, but actually black and covered with a dense layer of straight and coiled grayish hyphae that project 300−400 μm beyond the perithecial wall, resulting in the olivaceous color and hirsute appearance, 700−1250 μm tall and 700−1100 μm wide including the superficial hairs, about 300−600 × 125−350 μm excluding the superficial hyphae; peridium black in surface view, comprised of a textura epidermoidea of brown cells 2.5−4 μm wide, with slightly uneven, slightly thickened walls; hyphae covering peridium of two types, both with scattered septa; the first formed were straight and emerged from the base of the perithecium; the second formed were helically coiled, 2.5−3.5 μm wide, with evenly, slightly thickened walls covered with minute crystals, resulting in a spiny to warty surface appearance; asci not seen, assumed to be deliquescent; ascospores 9.5−10.5 × 8−9 μm, ellipsoidal to slightly lemon shaped, olivaceous, smooth-walled, walls slightly thickened, with a pore about 1 m wide at one end; ascospores holding together in hornlike, black masses called chirrhi, up to 300 μm long and 50−125 μm wide.

Fermentation. The CCR-5-active complex was produced when the producing microorganism, *C. globosum*, was grown in an aqueous nutrient medium under submerged aerobic conditions at a temperature of about 24−35 °C and at a pH of about 6.5 to 8.0 with agitation until substantial CCR-5 activity was imparted to the medium. Temperature studies indicated that the organism grew rapidly at about 24 °C. Therefore, the fermentation was conducted employing a single-temperature pattern of about 24 °C for a period of ∼120 h in flasks.

The growth of the organism (packed cell volume) and pH were determined intermittently. During the course of the fermentation, production of the CCR-5-active complex was monitored by the CCR-5 assay. The medium employed for the fermentation contained proteose peptone, yeast extract, cerelose, and soy grits as the major sources of nitrogen and carbon, respectively. Under these conditions, the microorganism produced active components as determined by monitoring the fermentation using the CCR-5 assay.

The fermentation was initiated by addition of the inoculum to the broth. Inoculum volume was between 3.5 and 7.0% of total broth volume. The inoculum was prepared by addition of a sample of 5% inoculum of the frozen whole broth of the producing culture to an appropriate germination medium. A particularly preferred germination medium in grams/liter comprised proteus peptone #3, 5.0; sodium chloride, 5.0; cerelose, 20.0; yeast extract, 3.0; soy grits, 5.0; and sodium potassium phosphate (monobasic), 5.0. The inoculum stage of the fermentation required 24−120 h with 72−96 h being preferred and was conducted at about 24 °C with agitation (250 rpm). A 5% inoculum of this culture was transferred to the same germination medium and grown as described above. Inoculum developed in this manner was transferred to the fermentation medium. A particularly preferred fermentation medium comprised 10 g/L of neopeptone, 40 g/L of cerelose, and 5 g/L of calcium carbonate. The fermentation stage required from 96 to 144 h with 120 h being preferred and was conducted at about 24 °C with agitation (250 rpm). The pH of the solution was adjusted to 7. An antifoam agent such as Antifoam B (Dow Corning) was added to the medium to control foam. An active complex as detected by the CCR-5 assay was produced.

Extraction and Isolation. Solid-phase extraction (SPE) methodology was originally applied on small scale using DZC-18 (Diazem) packing material to yield a crude extract for CCR-5 screening. However, during the extraction process of a large-scale fermentation, a standard ethyl acetate extraction at harvest pH (6.5−7.2) failed to extract Sch 210971 (**1**) and Sch 210972 (**2**) from the fermentation broth due to the polar and acidic nature of the molecules. Further evaluation indicated that **1** and **2** were extractable with ethyl acetate under acidic condition. The fermentation broth (4 L) was adjusted with concentrated HCl (12 N, 37%) to pH 2 and then extracted with 12 L of EtOAc. After removal of solvent under reduced pressure, the crude extract (8 g) was partitioned with a biphasic solvent system (hexane/EtOAc/MeOH/H_2O with 5% acetic acid, 8:2:5:5). The CCR-5-active complex from the upper phase portion (800 mg) was chromatographed by normal-phase HPLC (YMC semipreparative PVA-Sil column 30 × 250 mm with guard 30 × 75 mm, S-5, 2% MeOH in *n*-butyl chloride, 24 mL/min, UV = 295 nm) to obtain pure **1** (6 mg) and the mixture containing **2**. The mixture was further purified by reversed-phase HPLC (YMC semipreparative ODS column, 20 × 250 mm with a guard column 20 × 50 mm, S-5, 70−100% acetonitrile in H_2O with a linear gradient in 15 min, 12 mL/min, UV = 220 nm) to afford pure **2** (10 mg).

Sch 210971 (1): mp 126−128 °C; $[\alpha]^{24}_D$ +127.5 (*c* 0.1, acetone); UV (MeOH) λ_{max} 220, 290 nm; IR (KBr) ν_{max} 3390, 1725, 1650, 1595, 1440 cm^{-1}; ^{1}H NMR (acetone-d_6, 400 MHz) δ 5.67 (2H, br s, H-3, H-4), 5.20 (1H, dq, J = 6.5, 1.0 Hz, H-13), 4.10 (1H, dd, J = 10.0, 2.5 Hz, H-20), 3.95 (1H, dd, J = 8.0, 7.0 Hz, H-1), 3.01 (1H, dt, J = 8.0, 1.0, 1.0 Hz, H-2), 2.22 (1H, dd, J = 14.0, 2.5 Hz, H-22a), 1.90 (1H, dd, J = 14.0, 10.0 Hz, H-22b), 1.85 (1H, m, H-5), 1.62 (1H, m, H-7), 1.58 (3H, br.s, 12-CH_3), 1.51 (3H, br d, J = 6.5 Hz, 14-CH_3), 1.49 (3H, s, 24-CH_3), 1.40 (1H, m, H-10), 1.38 (1H, m, H-9), 1.90 (1H, m, H-6a), 1.64 (1H, m, H-8a), 0.93 (1H, m, H-6b), 0.92 (3H, d, J = 6.5 Hz, 15-CH_3), 0.84 (3H, d, J = 6.5 Hz, 16-CH_3), 0.82 (1H, m, H-8b); ^{13}C NMR (acetone-d_6, 100 MHz) δ 194.8 (C, C-17), 192.8 (C, C-21), 176.9 (C, C-25), 175.6 (C, C-19), 135.3 (C, C-11), 133.5 (CH, C-4), 128.4 (CH, C-3), 121.5 (CH, C-13), 100.6 (C, C-18), 72.9 (C, C-23), 58.9 (CH, C-20), 47.8 (CH, C-2), 46.0 (CH, C-10), 45.8 (CH, C-1), 45.8 (CH_2, C-8), 41.8 (CH_2, C-6), 41.3 (CH_2, C-22), 40.4 (CH, C-5), 39.1 (CH, C-9), 32.4 (CH, C-7), 25.0 (CH_3, C-24), 21.8 (CH_3, C-15), 20.5 (CH_3, C-16), 15.1 (CH_3, C-12), 12.9 (CH_3, C-14); positive ESI-MS (*m*/*z*) 446 (M + H)$^+$; *anal.* C 68.23%, H 7.98%, N 3.11%, calcd for $C_{25}H_{35}NO_6$, C 67.42%, H 7.87%, N 3.15%.

Sch 210972 (2): mp 149−150 °C; $[\alpha]^{24}_D$ +40.0 (*c* 0.1, acetone); UV(MeOH) λ_{max} 220, 295 nm; IR (KBr) ν_{max} 3436, 1710, 1645, 1578, 1447 cm^{-1}; ^{1}H NMR (acetone-d_6, 400 MHz) δ 5.66 (2H, br s, H-3, H-4), 5.19 (1H, dq, J = 6.5, 1.0 Hz, H-13), 3.94 (1H, dd J = 8.0, 7.0 Hz, H-1), 3.80 (1H, dd, J = 10.0, 2.5 Hz, H-20), 3.00 (1H, dt, J = 8.0, 1.0, 1.0 Hz, H-2), 2.50 (1H, dd, J = 14.0, 2.5 Hz, H-22a), 1.91 (1H, m, H-6a), 1.85 (1H, m, H-5), 1.75 (2H, dd J = 14.0, 10.0 Hz, H-22b), 1.66 (1H, m, H-8a), 1.63 (1H, m, H-7), 1.57 (3H, br s, 12-CH_3), 1.50 (3H, br d, J = 6.5 Hz, 14-CH_3), 1.49 (3H, s, 24-CH_3), 1.40 (1H, m, H-10), 1.39 (1H, m, H-9), 0.95 (1H, m, H-6b), 0.91 (3H, d, J = 6.5 Hz, 15-CH_3), 0.85 (3H, d, J = 6.5 Hz, 16-CH_3), 0.83 (1H, m, H-8b); ^{13}C NMR 194.9 (C, C-17), 193.5 (C, C-21), 177.2 (C, C-25), 176.6 (C, C-19), 136.2 (C, C-11), 134.5 (CH, C-4), 129.4 (CH, C-3), 122.3 (CH, C-13), 101.4 (C, C-18), 75.1 (C, C-23), 60.9 (CH, C-20), 48.5 (CH, C-2), 46.9 (CH, C-1), 46.9 (CH, C-10), 46.6 (CH_2, C-8), 42.7 (CH_2, C-22), 42.6 (CH_2, C-6), 41.2 (CH, C-5), 39.9 (CH, C-9), 33.2 (CH, C-7), 27.7 (CH_3, C-24), 22.6 (CH_3, C-15), 21.4 (CH_3, C-16), 16.8

(CH_3, C-12), 13.8 (CH_3, C-14); ESIMS (*m*/*z*) 446 $(M + H)^+$; HRFABMS: *m*/*z* 468 $(M + Na)^+$ (calcd for $C_{25}H_{35}NO_6Na$ 468.2362, found 468.2357); *anal*. C 66.95%, H 7.51%, N 3.58%, calcd for $C_{25}H_{35}NO_6$, C 67.42%, H 7.87%, N 3.15%.

CCR-5 Membrane Binding Assay. A high-throughput screen utilizing a CCR-5 membrane binding assay identifies inhibitors of RANTES binding. This assay utilizes membranes prepared from NIH 3T3 cells expressing the human CCR-5 chemokine receptor, which has the ability to bind to RANTES, a natural ligand for the receptor. In a 96-well plate format, 14 μg (total protein) of membrane preparation and 0.05 nM ^{125}I-RANTES were incubated in the presence (and absence) of compound, fraction, or extract for 1 h. Compounds or extracts were serially diluted over a wide range of 0.001 to 1 μg/mL and tested in triplicate. Reaction cocktails were harvested through glass fiber filters and washed thoroughly. Total counts for replicates were averaged and data reported as the concentration required inhibiting 50% of total ^{125}I-RANTES binding. Compounds with potent activity in the membrane binding assay were further characterized in secondary cell-based HIV-1 entry and replication assays.

The activity of compound **2** was evaluated in the CCR-5 membrane binding assay. Compound **2** was serially diluted over a range of 0.001 to 1.0 μg/mL, and each concentration was tested in replicates of four. Compounds **1** and **2** demonstrated IC_{50} values of 1.2 μM and 79 nM, respectively.

X-ray Crystallographic Analysis of Sch 210972 (2). The compound was crystallized from CH_2Cl_2 and acetone (1:1). Crystal data: $C_{25}H_{35}NO_6$; MW = 445.54, monoclinic, space group $P2_1$, a = 9.038(1) Å, b = 6.396(1) Å, c = 21.569(3) Å, β = 98.78(1)°, V = 1232.2(3) Å^3, Z = 2, D_c = 1.201 g cm^{-3}, μ(Cu Kα radiation) = 0.693 mm^{-1}; crystal dimensions 0.20 × 0.12 × 0.04 mm.

An Enraf-Nonius CAD-4 diffractometer (Cu Kα radiation, graphite monochromator, λ = 1.5418 Å) was used for all measurements. Intensity data ($\pm h$, $+k$, $+l$), θ_{max} = 75°, 2758 nonequivalent reflections, recorded at 296 K by $\omega-2\theta$ scans, yielded only 1006 reflections with $I > 2.0\sigma(I)$ for use in the structure determination and refinement. The crystal structure was solved by direct methods. Parameter refinement by means of full-matrix least-squares iterations converged at $R = 0.051$ ($R_w = 0.063$). Final atomic positional and thermal parameters, bond lengths, bond angles, and torsion angles have been deposited at the Cambridge Crystallographic Data Center as CIF files. Copies of the data can be obtained free of charge, on request, from the Director, Cambridge Crystallographic Data Center, 12 Union Road, Cambridge, CB2 1EZ, UK [fax: +44 (0) 223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

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Supporting Information Available: Tables of crystallographic data for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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