Notes

Isolation and Structure of Antagonists of Chemokine Receptor (CCR5)

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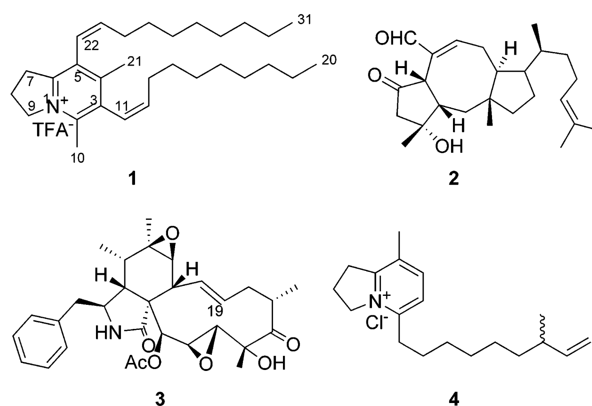
Received January 8, 2004

Human CCR5 is a G-coupled receptor that binds to the envelope protein gp120 and CD4 and mediates the HIV-1 viral entry into the cells. The blockade of this binding by a small molecule receptor antagonist could lead to a new mode of action agent for HIV-1 and AIDS. Screening of natural product extracts led to the identification of anibamine (**1**), a novel pyridine quaternary alkaloid as a TFA salt, from *Aniba* sp.; ophiobolin C from fermentation extracts of fungi *Mollisia* sp.; and 19,20-epoxycytochalasin Q from *Xylaria* sp. Formation of the TFA salt of anibamine is plausibly an artifact of the isolation. The identity of the natural counterion is unknown. Anibamine·TFA competed for the binding of ^{125}I-gp120 to human CCR5 with an IC_{50} of 1 μM. Ophiobolin C and 19,20-epoxycytochalasin Q exhibited binding IC_{50} values of 40 and 60 μM, respectively.

CCR5 is a cell surface G protein-coupled seven-transmembrane receptor specific for the chemokines MIP-1α, MIP-1β, and RANTES. It acts as a co-receptor for M-tropic HIV-1 via its interactions with the viral envelope gp120 protein of HIV-1 at or near the V3 loop after the loop has gone through a conformational change as a result of initial interaction with CD4.[1−4] This helps the non-syncytium-inducing (NSI) strains of HIV-1 to gain entry into their target cells. Viral envelope protein gp120 is a high-affinity ligand for the CCR5 receptor. β-Chemokines block gp120/CCR5 binding and the viral infection. Thus, blockade of gp120 binding to CCR5 by a small molecule antagonist has potential to contribute significantly toward anti-HIV therapy.[5] The potential to find a new drug with a novel mechanism of action has created a lot of enthusiasm by a large number of groups leading to the discovery of a series of potent small molecule synthetic antagonists,[6] and a selected few have entered preclinical and clinical development (e.g., SCH351125, TAK220, and AK602).[6,7]

Screening of natural product extracts derived from microbial fermentations and plants using human CCR5 receptor, ^{125}I-gp120, and soluble CD4 in a filter binding assay format led to the identification of three active extracts: two microbial and one plant. Bioassay-guided fractionations of the CH_3OH extract of *Aniba* sp. (Lauraceae) led to the isolation of a new compound trivially named anibamine (**1**). Fractionation of the methyl ethyl ketone extracts of *Mollisia* sp. and *Xylaria* sp. afforded ophiobolin C (**2**)[8−10] and 19,20-epoxycytochalasin Q (**3**),[11] respectively. The isolation, structure elucidation, and biological activities of these compounds are herein described.



Stems of *Aniba* sp. (collected in Guyana) were extracted with CH_3OH and partitioned with hexane and CH_2Cl_2. Reversed-phase HPLC using aqueous CH_3CN containing TFA followed by silica gel chromatography of the latter extract yielded compound **1** as a colorless solid. HRESIMS analysis of the TFA salt of compound **1** produced a molecular ion at *m/z* 424 and analyzed for a molecular formula of $C_{30}H_{50}N$, which was supported by the ^{13}C NMR spectrum (Table 1). The UV spectrum of compound **1** exhibited absorption maxima at λ_{max} 222 and 284 nm. The IR spectrum showed absorption bands for an aromatic ring and TFA. The ^{1}H and ^{13}C NMR and DEPT spectra in CD_3CN revealed the presence of four methyl groups including two aromatic methyls, 17 methylenes (one attached to a nitrogen atom), four olefinic protons, and five olefinic quaternary carbons (Table 1). The COSY spectrum of **1** indicated two independent spin systems, one for the three methylenes C7−C9 and the second for aliphatic chains terminating with an olefin on one side and a ω-methyl group on the other side. The one-bond ^{1}H and ^{13}C connectivity was established by an HMQC experiment. The

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10.1021/np049974l CCC: $27.50 © 2004 American Chemical Society and American Society of Pharmacognosy
Published on Web 05/13/2004

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Assignments of Anibamine·TFA (**1**) in CD_3CN

position	δ_C	δ_H (mult, J in Hz)
2	149.0	
3	135.9	
4	155.6	
5	132.2	
6	155.2	
7	33.0	3.24
8	21.3	2.30, pent, 8.0
9	58.6	4.6, t
10	18.3	2.50, s
11, 22	123.0, 122.1	6.3, brd, 12.4
12, 23	139.5, 139.4	6.0, m
13, 24	29.7, 29.5	1.78, m
14, 25	29.3, 29.2[a]	1.40, m
15, 26	29.95, 29.95[a]	1.21, m
16, 27	30.03, 29.99[a]	1.21, m
17, 28	30.06, 30.06[a]	1.21, m
18, 29	32.6	1.21, m
19, 30	23.3	1.25, m
20, 31	14.4	0.83, t
21	19.1	2.26, s
TFA-CO	164.2	
TFA-CF_3	115.9	

[a] Chemical shifts may be interchanged.

structural fragments were connected to each other by an HMBC ($^{n}J_{XH}$ = 7 Hz) experiment (Table 1). The methylene protons H_2-7, H_2-8, and H_2-9 showed two- and three-bond HMBC correlations to C-6 (δ 155.2) in addition to other carbons and establishing a five-membered ring that is fused with a pyridine ring. The HMBC correlation of H_2-9 to C-2 (δ 149.0) confirmed the fusion through the quaternary nitrogen. The substitutions around the fused pyridine ring were established as follows. The protons of one of the aromatic methyl (H_3-10) groups exhibited strong HMBC correlations to C-2 and C-3 (δ 135.9), indicating that it was substituted at C-2 and was next to the nitrogen atom of the pyridine ring. The second aromatic methyl (H_3-21) displayed strong HMBC correlations to three aromatic carbons, C-3, C-4 (δ 155.6), and C-5 (δ 132.2), thus confirming its substitution at C-4, and allowed its placement *meta* to the first aromatic methyl group. The HMBC correlations from H-11 (H-22) to C-2, C-3, C-4, C-5, and C-6 confirmed the overlap of the two protons and established the substitutions of the chain at C-3 and C-5. As expected, the ^{13}C NMR spectrum showed separations of the shifts of C11−C17 from C22−C28 but not the distal carbons C18−C20 and C29−C31, which were indistinguishable. The H-11 (H-22) appeared as a broad doublet and showed a coupling of J = 12.4 Hz with H-12 (H-23), thus suggesting a *Z*-geometry to olefins and establishing the structure of anibamine (**1**). Formation of the TFA salt of anibamine is plausibly an artifact of the isolation. The identity of the natural counterion is unknown. Louludinium chloride (**4**), a related alkaloid, was reported from blue green alga in 1998 with moderate activity against the KB cell line assay.[12]

Ophiobolin C (**2**) was isolated (180 mg/L) from a *Mollisia* sp. by reversed-phase HPLC of the methyl ethyl ketone (MEK) extract, and its structure was confirmed by comparisons with an authentic sample (LCESIMS, ^{1}H and ^{13}C NMR).[8−10]

The MEK extract of a *Xylaria* sp. fermentation was chromatographed by reversed-phase HPLC, providing 1000 mg/L of 19,20-epoxycytochalasin Q (**3**). The structure of **3** was elucidated by comparison of mass and ^{1}H and ^{13}C NMR spectra with the published data of 19,20-epoxycytochalasin Q isolated from *Xylaria hypoxylon*.[11]

CCR5 Receptor Binding Activity. Anibamine·TFA (**1**) effectively competed with ^{125}I-gp120 for the binding with human CCR5 receptor and exhibited an IC_{50} value of 1 μM. Compounds **2** and **3** were poorer inhibitors of binding and showed IC_{50} values of 40 and 60 μM, respectively. Ophiobolins and cytochalasins are known to exhibit a variety of activities including inhibitions of HIV-1 integrase (ophiobolins)[13] and HIV-1 protease (cytochalasins).[14]

Three tricyclic phenolic lactone (e.g., fuscinarin) antagonists of MIP1α-CCR5 (IC_{50} 20−154 μM) isolated from the fungus *Oidiodendron griseum*[15] and two triterpene glycoside antagonists of ^{125}I-Rantes-CCR5 (K_i 30 and 5 μM) isolated from sea cucumber[16] were recently described.

In summary, we have described a new pyridine quaternary alkaloid, anibamine (**1**), from an *Aniba* sp., that is a modest inhibitor of the binding of gp120 and CCR5. In addition, the CCR5 antagonistic activities of ophiobolin C (**2**) and 19,20-epoxycytochalasin Q (**3**) are also described.

Experimental Section

General Experimental Procedures. All biological material was collected in strict accordance with the Rio Convention on Biodiversity. All reagents were purchased from Sigma-Aldrich (cat # listed) or as otherwise stated. The UV and IR spectra were recorded on Perkin-Elmer instruments. All NMR spectra were recorded on Varian Inova 400 MHz instruments operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. An 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. HP1100 was used for analytical HPLC.

^{125}I-gp120/CCR5 Binding Assay. Cell membranes were prepared from CHO cells stably transfected with human CCR5 receptor. These membrane preparations (2 μg/well) were incubated with ^{125}I-YU2-gp120 (20 000 CPM) and CD4 (4 nM final concentration) with or without test compounds in 125 mL/well of binding buffer (50 mM HEPES, pH 7.2 w/0.5% BSA, 5 mM $MgCl_2$, 1 mM $CaCl_2$) containing protease inhibitor cocktail (10 μg/mL of leupeptin, aprotinin, chymostatin, and 0.1 mM phenylmethylsulfonyl fluoride (Sigma P-7626) in a 96-well plate. The test compounds were tested in triplicate with 5-fold dilutions starting with 20 mM for compound **1** and 200 mM for compounds **2** and **3**. After incubation for 1 h at 23 °C, the reaction was harvested through glass fiber filters. The amount of compound required to inhibit 50% of the specific binding of ^{125}I-gp120 from the average of the three experiments provided the IC_{50} values.

Isolation of Anibamine·TFA (1). Stems of an unidentified *Aniba* species (Lauraceae) were collected near Bartica, Guyana, in September 1992. A voucher specimen documenting the collection (Tiwari 931) is preserved in the herbarium of the New York Botanical Garden. The air-dried plant material was coarsely ground and extracted with CH_3OH at ambient temperature. A portion of the CH_3OH extract was concentrated and diluted with H_2O to produce a solution of 9:1 CH_3OH−H_2O. It was sequentially partitioned with one volume each of hexane and CH_2Cl_2. A 100 mg portion of the gum was purified by a reversed-phase HPLC using a Zorbax SB CN column (21 × 250 mm) eluting with a 60 min gradient of 55−60% aqueous CH_3CN containing 0.1% TFA at a flow rate of 8 mL/min. Fractions eluting with 60% CH_3CN at 60−70 min were pooled, concentrated, and lyophilized to give a 4.3 mg fraction, which was chromatographed on a small pipet filled with silica gel. The mixture was charged on to the column in CH_2Cl_2. After washing with CH_2Cl_2, the column was eluted with 5% CH_3OH−CH_2Cl_2 to afford 1.2 mg of **1** as an amorphous solid: UV (CH_3OH) λ_{max} 222 (ϵ 36 270), 284 (17 140); IR (ZnSe) ν_{max} 3405, 2924, 2854, 1687, 1467, 1198, 1123, 799; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS m/z 424.3920 (calcd for $C_{30}H_{50}N$,

424.3938), 338.2850 (calcd for $C_{24}H_{36}N$, 338.2848), 324.2697 (calcd for $C_{23}H_{34}N$, 324.2691), 310.2548 (calcd for $C_{22}H_{32}N$, 310.2535).

Isolation of Ophiobolin C (2). The fungal culture of *Mollisia* sp. (GB5328) was isolated from dead bark of *Tsuga canadensis* collected in Hamburg Mountain, Sussex Co., NJ, and grown on a KF seed and modified AD2 production medium[13] and was extracted with 1.2 volumes of methyl ethyl ketone. A 10 mL portion of the extract was concentrated under reduced pressure to give 28 mg of a gum, which was chromatographed on a reversed-phase Zorbax RX C-8 (21 × 250 mm) column eluted at 8 mL/min with a 30 min gradient of 30−90% aqueous CH_3CN. Fractions eluting at 37−38 min contained all of the CCR5 binding activity and were lyophilized to give 6 mg of **2** as a colorless powder.

Isolation of 19,20-Epoxycytochalasin Q (3). The fungal culture of *Xylaria* sp. (MF6837) was isolated from a fruiting body collected in Puerto Rico. The culture was grown on a KF seed and AD2 growth media[13] and was extracted with 1.2 volumes of MEK. A 30 mL aliquot of the extract was concentrated to give 300 mg of a gum. Fifty percent of this extract in 0.5 mL of CH_3OH was chromatographed on the same Zorbax RX C-8 column and eluted with the same gradient described above. Lyophilization of the fractions eluting at 23−24 min afforded 45 mg of **3** as a colorless solid.

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NP049974L