

Isolation and Structures of Novel Fungal Metabolites as Chemokine Receptor (CCR2) Antagonists

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Abstract The chemokine receptor, CCR2, is predominantly expressed on monocytes/macrophages, and on a subset of memory T cells. It binds to several CC type chemokines of the monocyte chemoattractant protein (MCP) family of which MCP-1 exhibits the highest affinity. CCR2/MCP-1 expression/association in monocyte/macrophage/T cells has been associated with inflammatory processes such as rheumatoid arthritis, multiple sclerosis and atherosclerosis. Neutralization of CCR2 with either a peptide or receptor antagonist results in the prevention of joint swelling in rodent models of arthritis. In this paper, bioassay-guided discovery of CCR2 receptor antagonists derived from natural product extracts are reported. These antagonists belong to two main classes exemplified by bis-thiodiketopiperazines and cytochalasins. Six compounds, including emestrin, two new emestrin analogs, and chaetomin represent the first group of compounds. These compounds inhibited the binding of MCP-1 to CCR2 (CHO membrane) with IC_{50} values of 0.8 to $9\ \mu\text{M}$ and exhibited good activity in a whole cell assay using MCP-1 and human monocytes with IC_{50} s ranging from $4\sim 9\ \mu\text{M}$. Cytochalasins A and B represented the second group and inhibited the binding activity with IC_{50} values of 5 and $188\ \mu\text{M}$, respectively. This is the first report of natural product antagonists of the CCR2 receptor.

Keywords CCR2 antagonists, natural products, bis-thiodiketopiperazines, microbial products

Introduction

Chemokines regulate leukocyte development and chemotaxis, and play an important role in their trafficking through the vascular and lymphatic systems. Monocyte chemoattractant protein-1 (MCP-1, CCL2), one of the first chemokines discovered, binds with high affinity and selectivity to the CCR2 receptor and this interaction appears to regulate inflammation in the lung and several other organs. The MCP-1-CCR2 complex is also associated with a number of other disease targets such as autoimmune disorders including rheumatoid arthritis, atherosclerosis, and infectious diseases. The biological significance of MCP-1/CCR2 association in these systems has been recently reviewed [1~5]. Neutralization of CCR2 with either a peptide or small molecule receptor antagonist results in the suppression or prevention of joint swelling in rodent models of rheumatoid arthritis [6, 7]. Our expectation is that a small molecule non-peptide antagonist of the MCP-1 binding to CCR2 may have a beneficial therapeutic effect against many of these disease targets.

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We screened a library of natural product extracts derived from microbial fermentations and plants using a filter-binding assay utilizing ^{125}I -MCP-1 and CHO membranes leading to four hits, all of microbial origin. Bioassay guided fractionation of three of the extracts led to the discovery of a number of compounds dominated by bis-thiodiketopiperazines. These include compounds of the emestrin family including emestrin (**1**), emestrin C (MPC1001, **2**), emestrin D (MPC1001D, **3**), two new members named herein as emestrin E (**4**), secoemestrin C₁ (**5**), and chaetomin (**6**) (Figure 1). Similar fractionation of the fourth extract led to the isolation of cytochalasins A (**7**) and B (**8**), the only compounds isolated in this program that did not contain the bis-thiodiketopiperazine unit. The isolation, structure elucidation and biological activities of these compounds are described.

Results and Discussion

Emestrin (**1**)

Emestrin (**1**) was produced by *Verticimonosporium ellipticus* isolated from leaf litter collected in Puerto Rico and grown on a solid fermentation medium. The mycelia were extracted with methyl ethyl ketone and the extract triturated with CH_2Cl_2 and MeOH. Sephadex LH 20 chromatography of the CH_2Cl_2 soluble material afforded 7 mg/liter of emestrin (**1**). It was characterized by comparison of ^1H , ^{13}C NMR and optical rotation to that of emestrin reported from *Emericella striata* [8, 9].

Emestrins C~E (**2~4**) and Secoemestrin C₁ (**5**)

Compounds **2~5** were produced by the sterile mycelium of

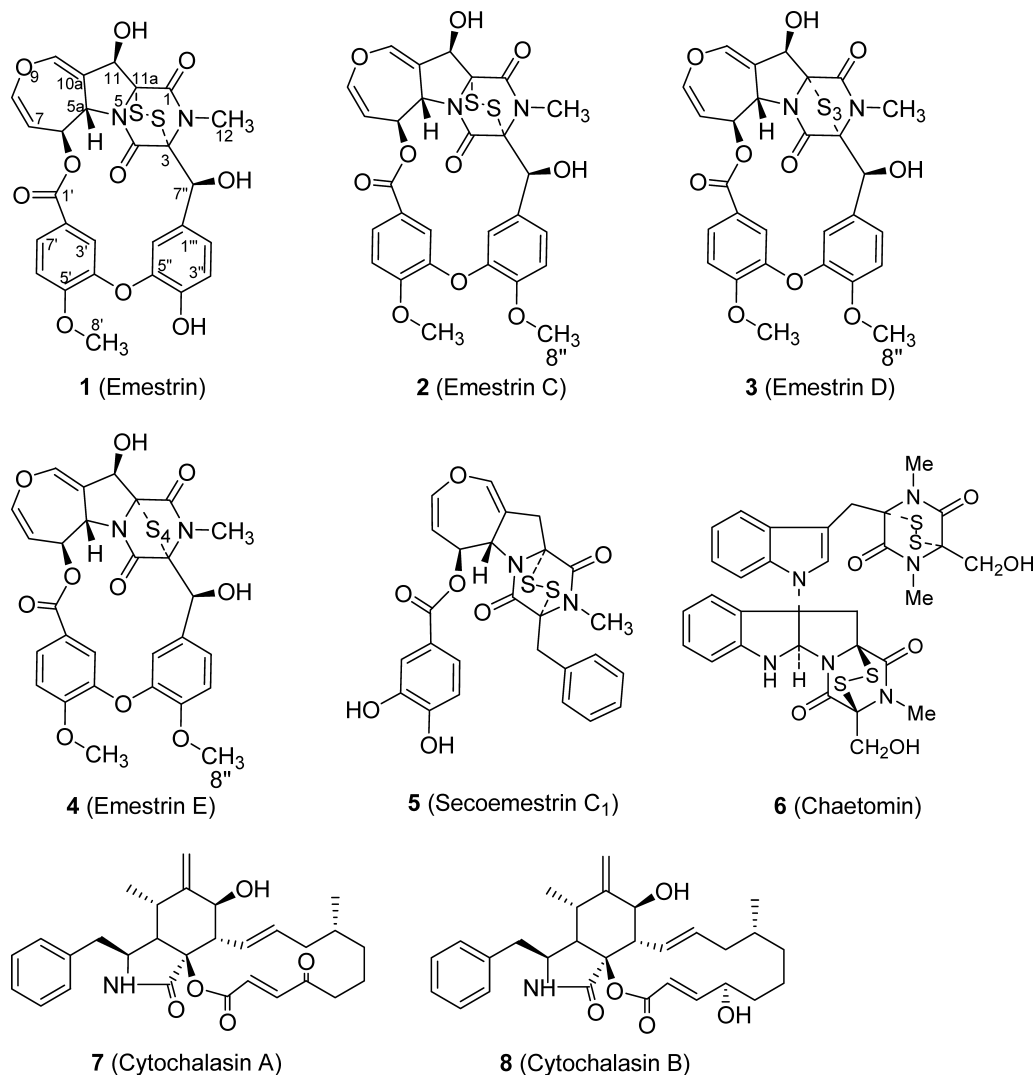


Fig. 1 The structures of compounds **1~8**.

a fungus isolated from Musk Ox dung collected from Alaska and grown in a liquid medium. The fermentation was extracted with methyl ethyl ketone. Gel permeation chromatography of the extract followed by reverse phase and diol HPLC (Figure 2) furnished **2** (0.4 mg/liter), **3** (1.4 mg/liter), **4** (0.2 mg/liter), and **5** (1 mg/liter).

Emestrin C (**2**)

HRESIMS analysis of **2** produced a molecular formula $C_{28}H_{24}N_2O_{10}S_2$ (MW 612) that was equivalent to one methylene unit more than emestrin ($C_{34}H_{16}N_2O_4S_2$, MW 598). The UV spectrum of **2** was identical to that of emestrin. The 1H NMR spectrum (Table 1) of **2** was identical to the spectrum of emestrin except for the presence of an additional aromatic methoxy group appearing at δ_H 3.97 that was assigned to the new methoxy group at C-4". When 1H NMR spectrum of **2** was recorded in a dilute solution of $CDCl_3$ (<1 mM) the signal for OH-7" consistently appeared as a doublet at δ_H 4.77 ($J=12.5$ Hz) and showed a coupling to H-7" (δ_H 5.45, d, $J=12$ Hz) most likely due to six centered hydrogen bonding between OH proton and the keto group. However, at increased concentration (>2 mM) the signal broadened and the coupling to H-7" was not observed and consequently it appeared as a singlet at δ_H 5.45 due to diffusion of hydrogen bonding.

Methylation of emestrin (**1**) with diazomethane overnight at $-10^\circ C$ afforded methyl-emestrin which was shown to be

identical to **2** by direct comparison of HPLC, 1H NMR and specific rotation data. Therefore, emestrin C was elucidated as C-4"-methoxy emestrin (**2**).

Emestrin D (**3**)

HRESIMS of **3** provided a molecular formula $C_{28}H_{24}N_2O_{10}S_3$ (MW 644) and suggested to have an additional sulfur atom in comparison to **2**. The 1H and ^{13}C NMR spectra (Table 1) of **3** was similar to the spectra of **1** and **2**, and were completely assigned from 2D COSY, HMQC and HMBC data (Table 1). The HRESIMS fragmentation of both emestrins C (**2**) and D (**3**) produced a common fragment ion at m/z 531 due to the loss of a molecule of water and sulfur atoms and confirmed the presence of the additional sulfur atom in **3** compared to **2**. Thus, the structure **3** with three sulfur atoms bridging C3-C11a was assigned to emestrin D which is 4"-methoxy emestrin B [10]. While this study was in progress compounds **2** and **3** named as MPC1001 and MPC1001D, respectively, were reported from *Cladorrhinum* sp. as antiproliferative agent with IC_{50} value of 9.3 and 16 nM, respectively, against DU145 human prostate cancer cell line [11].

Emestrin E (**4**)

The molecular formula of **4** ($C_{28}H_{24}N_2O_{10}S_4$) indicated the presence of another sulfur atom when compared to emestrin D, which was likewise assigned to the C3-C11a

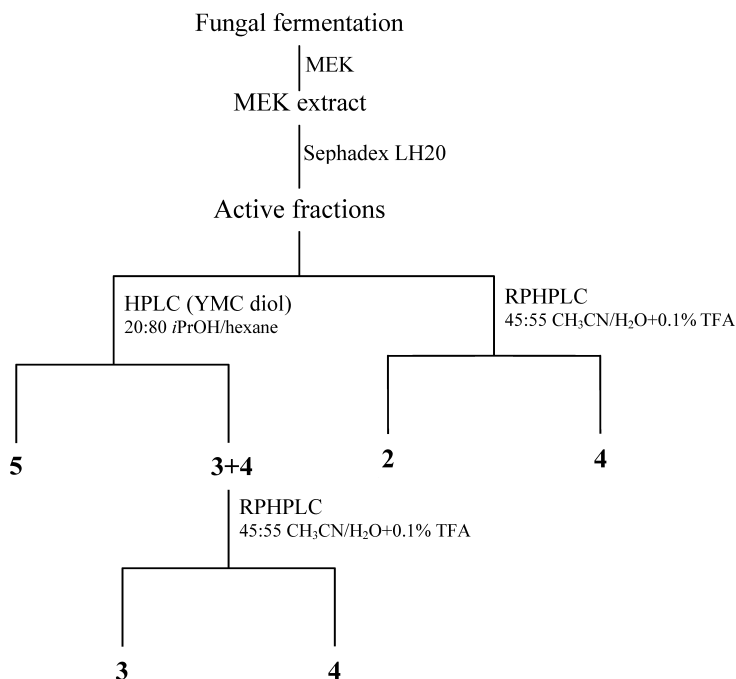


Fig. 2 Isolation of emestrins C~E (**2**~**4**) and secoemestrin C₁ (**5**).

Table 1 NMR assignments of emestriins C~E (**2**~**4**) and secoemestrin C₁ (**5**) in CDCl₃

Position	3			2		4		5	
	δ_C	δ_H , mult. <i>J</i> in Hz	HMBC (H→C)	δ_H , mult. <i>J</i> in Hz	δ_H , mult. <i>J</i> in Hz	δ_H , mult. <i>J</i> in Hz	δ_C	δ_H , mult. <i>J</i> in Hz	
1	168.0	—	H-11	—	—	—	166.1	—	
3	77.9	—	H-11	—	—	—	78.3	—	
4	165.2	—	—	—	—	—	162.2	—	
5a	58.9	5.41, dd, 8.0, 2.0	H-6, 7, 10, 11	5.93, dd, 7.5, 2.0	5.69, dd, 8.0, 2.0	5.69, dd, 8.0, 2.0	63.0	5.32, br d, 8.0	
6	74.8	5.29, dt, 8.0, 2.0	H-5a, 7, 8	4.82, dt, 7.5, 2.0	5.23, dt, 8.0, 2.0	5.23, dt, 8.0, 2.0	70.4	5.83, dt, 8.4, 2.0	
7	109.9	5.05, dd, 8.0, 2.0	H-8	4.98, dd, 8.5, 2.0	5.00, dd, 8.0, 2.0	5.00, dd, 8.0, 2.0	105.8	4.68, dd, 8.0, 2.0	
8	138.5	6.37, dd, 8.0, 2.0	H-7, 10	6.34, dd, 8.5, 2.0	6.35, dd, 8.0, 2.0	6.35, dd, 8.0, 2.0	141.1	6.31, dd, 8.0, 2.0	
10	142.9	6.85, d, 2.0	H-5a, 8, 11	6.93, d, 2.0	6.86, d, 2.0	6.86, d, 2.0	139.4	6.67, br d, 2.0	
10a	107.8	—	H-5a, 10	—	—	—	113.4	—	
11	79.7	4.81, s	H-10	4.95, br s	4.91, s	4.91, s	35.3	4.14, br d, 18 3.16, br d, 18	
11a	83.0	—	H-5a, 11	—	—	—	73.4	—	
12	28.8	3.58, s	—	3.43, s	3.27, s	3.27, s	29.4	2.95, s	
1'	166.3	—	H-3', 7'	—	—	—	165.4	—	
2'	122.2	—	H-3', 6'	—	—	—	121.4	—	
3'	119.4	8.05, d, 2.0	H-7'	7.66, d, 2.0	8.25, d, 2.0	8.25, d, 2.0	116.6	7.60, d, 2.0	
4'	146.2	—	H-3', 6'	—	—	—	143.0	—	
5'	154.6	—	H-3', 6', 7', 8'	—	—	—	149.3	—	
6'	112.1	7.04, d, 8.5	—	7.06, d, 8.5	7.09, d, 8.5	7.09, d, 8.5	114.6	6.93 d, 8.8	
7'	125.8	7.75, dd, 8.5, 2.0	H-3', 6'	7.78, dd, 8.5, 2.0	7.84, dd, 8.5, 2.0	7.84, dd, 8.5, 2.0	124.3	7.61, dd, 8.8, 2.0	
8'	56.3	4.06, s	—	4.02, s	4.06, s	4.06, s	—	—	
1''	126.9	—	—	—	—	—	133.7	—	
2''	128.6	7.05, dd, 8.5, 2.0	H-6''	7.13, dd, 8.0, 2.0	7.21, dd, 8.5, 2.0	7.21, dd, 8.5, 2.0	128.5	7.15~7.20	
3''	110.6	6.86, d, 8.5	—	6.95, d, 8.0	6.96, d, 8.5	6.96, d, 8.5	129.2	7.15~7.20	
4''	152.1	—	H-2'', 3'', 6'', 8''	—	—	—	127.4	7.15~7.20	
5''	145.5	—	H-3'', 6''	—	—	—	129.2	7.15~7.20	
6''	125.5	8.30, d, 2.0	H-2''	7.68, d, 2.5	8.43, d, 2.5	8.43, d, 2.5	128.5	7.15~7.20	
7''	76.4	5.31, br s	H-2'', 6''	5.45, d, 12.0	5.37, br s	5.37, br s	36.2	3.68, d, 16 3.63, d, 16	
8''	56.0	3.87, s	—	3.97, s	3.97, s	3.97, s	—	—	
7''-OH	—	—	—	4.77, d, 12.5	—	—	—	—	
11-OH	—	—	—	5.23, s	—	—	—	—	

bridge, and was confirmed by the presence of the fragment ion at m/z 531. The structure of **4** was corroborated by ¹H and ¹³C NMR spectral data (Table 1) comparison with the data of **2** and **3**.

Secoemestrin C₁ (**5**)

HRESIMS analysis of **5** afforded a molecular formula C₂₆H₂₂N₂O₇S₂ (MW 538) indicating the presence of two sulfur atoms in the molecule like in emestrin C (**2**). Comparison of the ¹H NMR spectra (Table 1) of **5** with **2** showed the absence of both methoxy groups and H-11 and H-7'' oxymethines, and the presence of a monosubstituted phenyl group indicating the opening of the macrocycle. The

¹H and ¹³C NMR spectra were assigned from COSY, HMQC and HMBC data and are listed in Table 1.

ESIMS fragmentation (Figure 3) of **5** produced a major fragment ion at m/z 385 (80%), that was attributed to the facile loss of the dihydroxybenzoic acid unit which, after elimination of HS₂, produced a stable fragment ion at m/z 320. The ESIMS fragmentation pattern of secoemestrin C₁ differed significantly from the macrocycle-containing compounds **1**~**4** in which only a loss of water are observed. Based on these data structure **5** was assigned to secoemestrin C₁.

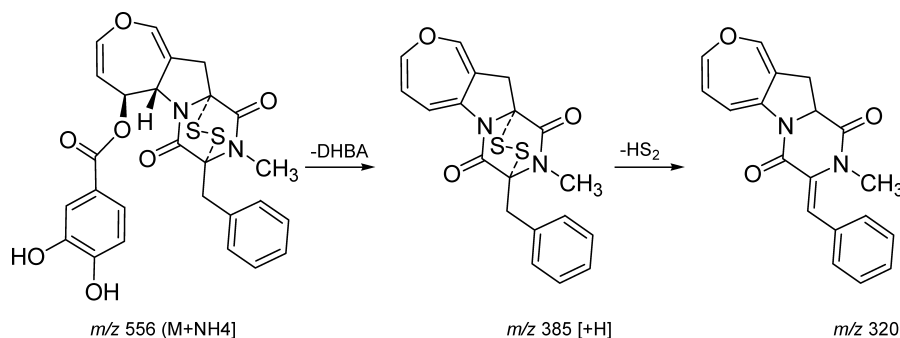


Fig. 3 ESIMS fragmentation of secoemestrin C₁ (**5**).

Chaetomin (**6**)

Sterile mycelia was isolated from a soil sample collected in Argentina and grown on a liquid medium which was extracted by MEK and chromatographed on reverse phase HPLC affording 77 mg/liter of chaetomin (**6**). The structure was confirmed by comparison of ¹H, ¹³C NMR spectra and specific rotation of chaetomin that is reported to be a toxic bacteriostatic agent [12].

Cytochalasins A (**7**) and B (**8**)

These were produced by sterile mycelia isolated from decaying leaves collected in Costa Rica. Gel permeation chromatography of the MEK extract over Sephadex LH 20 followed by reversed phase HPLC led to the isolation of cytochalasins A (**7**, 40 mg/liter) and B (**8**, 120 mg/liter) whose structures were elucidated by comparison of spectral data (UV, ¹H, and ¹³C NMR) [12], with authentic samples.

Biological Activity

All compounds were first evaluated for their activity in the CCR2 membrane binding assay using CCR2 receptor CHO cell membrane and ¹²⁵I-MCP. The results of this assay are presented in Table 2. Emestrins (**1**~**3**) each with two to three sulfur atoms, and with or without a free phenolic group, exhibited (IC₅₀ ~0.8 μM) equal activities in this assay. However, compound **4** with four-sulfur atoms was slightly less active (IC₅₀=1.1 μM). Secoemestrin C₁ (**5**) exhibited IC₅₀=9.3 μM and was ten times less active than **1** indicating the importance of the macrocycle for binding activity. Chaetomin (**6**) showed intermediate activity between **1** and **5** and exhibited an IC₅₀ value of 5.07 μM. The binding activities of cytochalasins A (**7**) and B (**8**) were dramatically different, IC₅₀ values being 5.24 and 188 μM, respectively, showing the importance of the enone moiety of **7** for the activity when compared to enol moiety of **8**. In a similar MIP-1α membrane binding CCR5 assay, chaetomin showed IC₅₀ of 28 μM and exhibited approximately 6 fold selectivity for CCR2 receptor.

Table 2 Inhibition of ¹²⁵I-MCP-1 binding to CCR2 receptor by fungal metabolites (**1**~**8**)

Compound	Membrane	Whole cell
	140LCCR2/CHO IC ₅₀ (μM)	Human monocyte CCR2 IC ₅₀ (μM)
1	0.84±0.3	5.4±0.7
2	0.82±0.4	4.1±0.7
3	0.78±0.4	4.2±0.4
4	1.10±0.5	NA at 10 μM
5	9.30±1.2	7.9±1.0
6	5.07±1.2	9.5±2.9
7	5.24±1.3	NA at 10 μM
8	188.00±15	NA at 10 μM

NA: not active.

These compounds were also evaluated in a whole cell assay using human monocyte cells harvested from Leukopacks. Emestrins **1**~**3** inhibited the ¹²⁵I-hMCP-1 binding to CCR2 receptor in these cells with IC₅₀ values of 5.4, 4.1 and 4.2 μM, respectively, and were amongst the better actives of the series of molecules tested. The activity exhibited by these compounds in this assay was 5~8 fold lower than the results obtained in the membrane binding assay. Emestrin E (**4**) with four sulfur atoms was significantly less active in the cell based assay (IC₅₀ >>> 10 μM) compared to the membrane binding assay. Secoemestrin C₁ exhibited an IC₅₀ of 7.9 μM in the cell based assay which was similar to the membrane binding activity. The whole cell activity of secoemestrin C₁ was about two fold lower than macrocyclic emestrins **1**~**3**. The whole cell activity exhibited by chaetomin was two fold lower than the membrane binding activity (IC₅₀ 9.05 versus 5.07 μM). Neither of the cytochalasins exhibited any whole cell inhibitory activity at 10 μM and were not evaluated further.

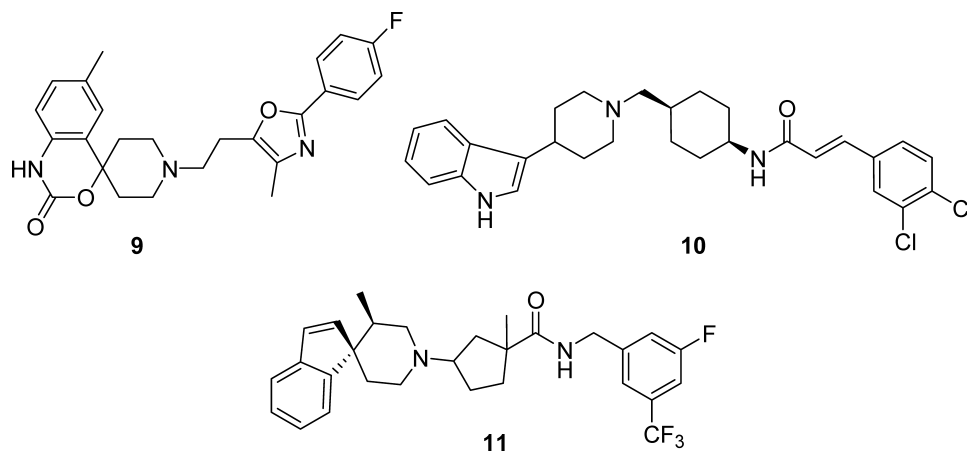


Fig. 4 Structures of compounds **9**~**11**.

Emestrin has been reported to induce DNA fragmentation [13], and causes acute poisoning in mice (LD_{50} 13 mg/kg) [14]. Cytochalasins are known for a variety of biological activities. For example, they effect phagocytosis [15], inhibit glucose transport [16~18], inhibit HIV-1 protease [19], and antagonize the monocyte-CCR5 receptor [20].

In summary, we have reported here a number of known and new *bis*-thiodiketopiperazines and cytochalasins as antagonists of the CCR2 receptor. Although a number of synthetic (exemplified by compounds **9** [5], **10** [21] and **11** [22], Figure 4) potent (nM) antagonists of the CCR2 receptor have been reported in patent literature, these compounds constitute the first report of natural product antagonists.

Experimental

General Procedure

All biological material was collected in strict accordance with the Rio Convention on Biodiversity. All reagents were purchased from Sigma-Aldrich (cat # listed) or otherwise listed. NMR spectra were recorded on Varian Inova 500 MHz instruments operating at 500 MHz for ^1H and 125 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.

The materials used for assays such as Bovine serum

albumin Bovine (A-7030), polyethylenimine (P-3143, in 0.10% water), protease inhibitors cocktail (P-8340, stored at -20°C), bacitracin (B-0125), hMCP-1 (Peprotech, Rocky Hill, NJ), ^{125}I -hMCP-1 (New England Nuclear, NEX-332), BSA (30% solution, Calbiochem #126625), and microscint 0 (Packard) were obtained from the listed sources and used as received. Radioactivity was measured using Topcount NXT manufactured by Packard. Fermentation trace elements (g/liter) are as follows: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.025), CaCl_2 (0.1), H_3BO_3 (0.056), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 7\text{H}_2\text{O}$ (0.019), and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), and were prepared in 0.6 N HCl.

Fermentation and Isolation of Emestrin (1)

Verticimonosporium ellipticus (MF6808, isolated from leaf litter collected in Puerto Rico), was inoculated into seed flasks by transferring an 1 ml aliquot of a frozen culture into a 250 ml unbaffled Erlenmeyer flask containing 50 ml of NAS seed medium (in g/liter, corn steep powder 2.5; tomato paste 40, oat flour 10, glucose 10, trace elements 10 ml, pH 6.8). The seed culture was incubated at 25°C on a gyratory shaker (220 rpm) for 3 days before transferring to NPF2 production medium (in g/liter of water, dextrose 150; urea 4; NZ amine type A 4.0; K_2HPO_4 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25; KCl 0.25; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.90; CaCO_3 16.5). A 220 ml of a liquid nutrient aliquot was added to 675 ml of vermiculite in 2-liter roller bottles. 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.

A 6 liters fermentation was extracted with 7.2 liters of methyl ethyl ketone. The organic extract was concentrated under reduced pressure to 100 ml of mostly aqueous suspension which was extracted with 100 ml CH_2Cl_2 . The

CH_2Cl_2 extract that contained most of the emestrin was concentrated to give 3 g of oil that was chromatographed on a 2-liter Sephadex LH 20 column and eluted with MeOH. Emestrin was highly retained and eluted after two column volumes of eluent. Concentration of the fractions under reduced pressure afforded 42 mg (7 mg/liter) of emestrin (**1**) as a colorless amorphous powder homogenous by HPLC [Rt=5.5 minutes, Zorbax XDB (4.6×150 mm), 40~60, $\text{CH}_3\text{CN} - \text{H}_2\text{O} + 0.1\%$ TFA, 1 ml/minute] and ^1H and ^{13}C NMR spectra.

Methylation of Emestrin

To a cooled (-10°C) solution of emestrin (7 mg) in a 1 : 1 mixture of CH_2Cl_2 and THF (1 ml) was added an excess of an ethereal solution of freshly prepared diazomethane. After stirring at -10°C for 4 hours, the yellow solution was stored in a freezer overnight after which the volatile material was removed under a stream of nitrogen. The methylated product was purified by RPHPLC (Zorbax RX C-8, 21×250 mm, 45~55, $\text{CH}_3\text{CN} - \text{H}_2\text{O} + 0.1\%$ TFA, flow rate 12 ml/minute). Compound **2** eluted in fractions eluting in between 38~43 minute and were lyophilized to afford 3.5 mg of **2** as a colorless amorphous powder. $[\alpha]_{\text{D}}^{25} = 87^\circ$ (*c* 0.4, MeOH).

Fermentation and Isolation of Emestrins C~E (**2**~**4**) and Secoemestrin **C**₁ (**5**)

Sterile mycelia (MF6822, isolated from Musk Ox dung collected from Alaska), was inoculated into seed flasks by transferring one ml aliquot of a frozen culture into a 250 ml unbaffled Erlenmeyer flask containing 50 ml of NAS seed medium. The seed culture was incubated at 25°C on a gyratory shaker (220 rpm) for 4 days before transfer to NPGG liquid production medium (in g/liter, glycerol 75, glucose 75; tomato paste 5, NZ amine type A 4.0; K_2HPO_4 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25; KCl 0.25; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.50; CaCO_3 10). A 10 ml aliquot of the seed culture was added to a 250 ml Erlenmeyer flask containing 50 ml aliquot of the production media and shaken at 220 rpm at 22°C for 24 days. Fungal growth in each flask was extracted with 60 ml of methyl ethyl ketone.

A 1 liter fermentation broth of MF6822 was extracted with 1 liter of methyl ethyl ketone (MEK), layers were separated and MEK extract was concentrated under reduced pressure to afford a solid material. Dried MEK extract was dissolved in 50 ml methanol charged on to a 2 liter column of Sephadex LH 20 and eluted with methanol at a flow rate of 10 ml/minute. Active components **2**~**5** eluted in 1.3~1.5 column volumes of eluent. These fractions were pooled to give 62 mg of a residue. The residue was dissolved in 1.5 ml methanol and

chromatographed on a Zorbax RX -8 (21×250 mm) column eluting at a flow rate of 8 ml/minute with a 40 minutes gradient of 20 to 90% aqueous CH_3CN containing 0.1% TFA. Fractions eluting in the 15 to 30 minutes range showed activity. The active fractions were analyzed by HPLC, combined into two pools, concentrated under reduced pressure and lyophilized to yield 8 and 3 mg of colorless amorphous powders, respectively.

A one fourth portion of the 3 mg fraction was further purified by HPLC using a Zorbax RX C-8 (4.6×250 mm) column eluting with 45% aqueous CH_3CN containing 0.1% TFA at a flow rate of 1 ml/minute. The process was repeated four times and fractions eluting at Rt=15.12 and Rt=17.15 minutes were concentrated and lyophilized to give 0.2 and 0.4 mg of emestrin **E** (**4**) and emestrin **C** (**2**), respectively, as colorless amorphous powders.

A tenth portion of the 8 mg fraction was further purified by HPLC using a YMC diol (4.6×250 mm) column eluting with a 20% isopropyl alcohol/hexane at a flow rate of 1 ml/minute and repeated ten times. Fractions eluting at Rt=14.13 and Rt=31~34 minutes were concentrated under vacuum and lyophilized to yield 1 mg of secoemestrin **C**₁ (**5**) as a colorless amorphous powder as well as 3 mg of an enriched fraction containing emestrins **D** (**3**) and **E** (**4**). The latter fraction was further purified by HPLC on a Zorbax RX C-8 (4.6×250 mm) column eluting with a 45% aqueous CH_3CN containing 0.1% TFA at a flow rate of 1 ml/minute. Pooled fractions eluting at Rt=12.85 minutes from multiple runs were concentrated and lyophilized to give 1.4 mg of **3**, and an additional small amount of **4**.

Emestrin **C** (**2**): RPHPLC Rt=17.15 minutes [Zorbax RX C-8 (4.6×250 mm) 45 : 55 $\text{CH}_3\text{CN} - \text{H}_2\text{O} + 0.1\%$ TFA, 1 ml/minute]; $[\alpha]_{\text{D}}^{25} = +75^\circ$ (*c* 0.16, MeOH), UV (MeOH) λ_{max} (ϵ) 207 (26435), 221 (sh), 266 (sh), 283 (sh) nm; IR (ZnSe) ν_{max} 3464 br, 2933, 1685, 1267 cm^{-1} ; ^1H and ^{13}C NMR (see Table 1); LCESIMS (*m/z*) 1247 $[\text{2M} + \text{Na}]^+$, 531 $[\text{M} - \text{H}_2\text{O} - 2\text{S}]$; HRESIFTMS (*m/z*) 635.0770 (calcd for $\text{C}_{28}\text{H}_{24}\text{N}_2\text{O}_{10}\text{S}_2 + \text{Na}$ 635.0765).

Emestrin **D** (**3**): RPHPLC Rt=12.85 minutes [Zorbax RX C-8 (4.6×250 mm), 45 : 55 $\text{CH}_3\text{CN} - \text{H}_2\text{O} + 0.1\%$ TFA, 1 ml/minute]; $[\alpha]_{\text{D}}^{25} = +89.3^\circ$ (*c* 0.56, MeOH); UV (MeOH) λ_{max} (ϵ) 206 (76388), 221 (sh), 255 (34352), 286 (sh) nm; IR (ZnSe) ν_{max} 3443 br, 2933, 1682, 1268 cm^{-1} ; ^1H and ^{13}C NMR (see Table 1); LCESIMS (*m/z*) 1306 $[\text{2M} + \text{NH}_4]^+$; HRESIFTMS (*m/z*): 667.0493 (calcd for $\text{C}_{28}\text{H}_{24}\text{N}_2\text{O}_{10}\text{S}_3 + \text{Na}$, 667.0491), 627.0554 (calcd for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{O}_9\text{S}_3 + \text{H}$, 627.0566), 531.1341 (calcd for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{O}_9 + \text{H}$, 531.1404).

Emestrin **E** (**4**): RPHPLC Rt=15.12 minutes [Zorbax RX C-8 (4.6×250 mm), 45 : 55 $\text{CH}_3\text{CN} - \text{H}_2\text{O} + 0.1\%$ TFA, 1 ml/minute]; UV (MeOH) λ_{max} (ϵ) 205 (22084), 221 (sh),

261 (10563), 290 (sh) nm; IR (ZnSe) ν_{\max} 3360, 2924, 1686, 1267 cm^{-1} ; ^1H and ^{13}C NMR (see Table 1); LCESIMS (m/z) 1370 $[\text{2M}+\text{NH}_4]^+$, 531 $[\text{M}-\text{H}_2\text{O}-4\text{S}]$; HRESIFTMS (m/z) 699.0202 (calcd for $\text{C}_{28}\text{H}_{24}\text{N}_2\text{O}_{10}\text{S}_4+\text{Na}$, 699.0211).

Secoemestrin C_1 (**5**): HPLC $\text{Rt}=14.13$ minutes [YMC diol (4.6 \times 250 mm) 1:4 *i*PrOH-hexane, 1 ml/minute]; RPHPLC $\text{Rt}=12.5$ minutes [Zorbax RX C-8 (4.6 \times 250 mm), 15 minutes 20~90% gradient of aqueous $\text{CH}_3\text{CN}+0.1\%$ TFA, 1 ml/minute), $[\alpha]_{\text{D}}^{25}=-134.1^\circ$ (c 0.4, MeOH), UV (MeOH) λ_{\max} (ϵ) 208 (64901), 221 (sh), 263 (20392), 296 (11030) nm; IR (ZnSe) ν_{\max} 3364 (br), 2925, 1690, 1291 cm^{-1} ; ^1H and ^{13}C NMR (see Table 1); LCESIMS (m/z) 556 $[\text{M}+\text{NH}_4]^+$; HRESIFTMS (m/z): 556.1212 (calcd for $\text{C}_{26}\text{H}_{22}\text{N}_2\text{O}_7\text{S}_2+\text{NH}_4$, 556.1212), 385.0674 (calcd for $\text{C}_{19}\text{H}_{17}\text{N}_2\text{O}_3\text{S}_2$, 385.0681), 320.1150 (calcd for $\text{C}_{19}\text{H}_{16}\text{N}_2\text{O}_3$, 320.1161).

Fermentation and Isolation of Chaetomin (**6**)

The sterile mycelia of MF6838 was isolated from a soil sample collected in Argentina and inoculated into seed flasks by transferring a 1 ml aliquot of a frozen culture into a 250 ml Erlenmeyer flask containing 50 ml of KF seed medium (in g/liter water, corn steep liquor 5; tomato paste 40; oat flour 10; glucose 10; trace elements 10 ml, pH 6.8). The seed culture was incubated at 25°C on a gyratory shaker (220 rpm) for 4 days before transfer to GG1 production medium (in g/liter water, glycerol 75, glucose 10; ardamine pH 5, $(\text{NH}_4)_2\text{SO}_4$ 2, soybean meal 5, tomato paste 5, sodium citrate 2). A 10 ml aliquot of the seed culture was added to a 250 ml Erlenmeyer flask containing production media and shaken at 220 rpm for 20 days at 22°C. Fungal growth in each flask was extracted with 60 ml of methyl ethyl ketone by shaking for 30 minutes.

A 30 ml extract was concentrated under reduced pressure to afford 30 mg semi-solid. This material was dissolved in 1.5 ml methanol and chromatographed on a Zorbax RX C-8 (21 \times 250 mm) column eluting at a flow rate of 8 ml/minute with a 40 minutes gradient of 30 to 70% aqueous CH_3CN containing 0.1% TFA. Fractions eluting between 34 and 36 minutes were pooled, concentrated under reduced pressure and lyophilized to furnish 2.3 mg of chaetomin (**6**) as an amorphous powder. $\text{Rt}=5.79$ minutes (Zorbax RX C-8, 4.6 \times 250 mm, $\text{CH}_3\text{CN}-\text{H}_2\text{O}+0.1\%$ TFA, 1 ml/minute), identified by comparison of UV, ^1H and ^{13}C NMR and mass spectral data with an authentic sample [12].

Fermentation and Isolation of Cytochalasins A (**7**) and B (**8**)

Nonsporulating sterile mycelium GB4200 (isolated from decaying leaves of *Citharexylum macredenium*

(Verbenaceae), Parque Nacional Braulio Carillo, Heredia Province, Costa Rica) were 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 water, 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 NPF2 production medium and conditions as described for production of emestrin.

A 30 ml fermentation was extracted with 36 ml MEK. The extract was concentrated to dryness and chromatographed on a 70 ml Sephadex LH 20 column using MeOH. Concentration of fractions eluting at 0.85 column volume afforded 16 mg of a mixture of cytochalasins. The cytochalasin mixture was chromatographed on RPHPLC (Zorbax RX C-8, 21 \times 250 mm) and eluted with a 35 minutes gradient of 40 to 90% aqueous $\text{CH}_3\text{CN}+0.1\%$ TFA at a flow rate 8 ml/minute. Concentration of fractions eluting at 16~20 and 22~25 minutes under reduced pressure followed by lyophilization afforded 3.6 mg of cytochalasin B (**8**) and 1.2 mg of cytochalasin A (**7**), respectively.

Membrane Binding Assay

Membranes, prepared from CCR2b-transfected CHO cells (2 μg), were incubated with 25 pM ^{125}I -hMCP-1 (2200 Ci/mmol) in buffer containing 50 mM HEPES pH 7.4, 0.5% BSA, 5 mM MgCl_2 , 1 mM CaCl_2 , 0.1 mg/ml bacitracin and 0.01% protease inhibitors cocktail for 1 hour at room temperature. Non-specific binding was measured in the presence of 100 nM unlabeled hMCP-1. Test samples were reconstituted in DMSO and 2 μl were tested in a final volume of 250 μl . The reaction was terminated by filtration onto GF/B filter plates presoaked with 0.33% polyethyleneimine using a Tomtec 9600 Cell Harvester. The filters were washed with 25 mM HEPES pH 7.4 containing 0.5 M NaCl and 0.01% sodium azide. Subsequently, filter plates were dried and counted for radioactivity in a Topcount NXT after addition of 50 μl Microscint 0.

Whole Cell Binding Assay

Monocytes were harvested from Leukopacks and resuspended to a final density of 2×10^6 cells/ml binding buffer. Cells (200000) were incubated with ^{125}I -hMCP-1 (~25 pM, 2200 Ci/mmol) at room temperature for 45 minutes in buffer containing HEPES (50 mM), MgCl_2 (5 mM), CaCl_2 (1 mM), BSA (0.5%), and protease inhibitor cocktail at pH 7.4. Reactions were terminated by filtration over GF/B filters that had been presoaked in 0.10% polyethyleneimine using a Packard Cell Harvester. The filters were washed with HEPES (25 mM), pH 7.5

containing NaCl (500 mM) and the plates were dried. Plates were counted for ^{125}I radioactivity using Microscint 0 and a Topcount NXT.

References

- Ley K. Arrest, migration, survival, and proliferation of leukocytes and vascular cells: The many faces of chemokine biology. *Microcirculation* 10: 245–246 (2003)
- Daly C, Rollins BJ. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: Therapeutic opportunities and controversies. *Microcirculation* 10: 247–257 (2003)
- Charo IF, Peters W. Chemokine receptor 2 (CCR2) in atherosclerosis, infectious diseases, and regulation of T-cell proliferation. *Microcirculation* 10: 259–264 (2003)
- Rose CE, Jr, Sung SSJ, Fu SM. Significant involvement of CCL2 (MCP1) in inflammatory disorders of the lung. *Microcirculation* 10: 273–288 (2003)
- Cascieri MA, Springer MS. The chemokine/chemokine-receptor family: Potential and progress for therapeutic intervention. *Curr Opin Chem Biol* 4: 420–427 (2000)
- Gong JH, Ratkay LG, Waterfield JD, Clark-Lewis I. An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. *J Exp Med* 186: 131–137 (1997)
- Brodmerkel CM, Collins R, Hall L, Hertel D, Stewart R, Wang A, Xue CB, Diamond S, Friedman S, Vaddi K. In 68th Annual Meeting of American College of Rheumatology, San Antonio, Texas, 2004
- Seya H, Nozawa K, Nakajima S, Kawai K, Udagawa S. Studies on fungal products. Part 8. Isolation of structure of emestrin, a novel antifungal macrocyclic epithiodioxopiperazine from *Emericella striata*. X-ray molecular structure of emestrin. *J Chem Soc Perkin Trans 1* 1986: 109–116 (1986)
- Ooike M, Nozawa K, Kawai KI. An epitetrathiodioxopiperazine related to emestrin from *Emericella foveolata*. *Phytochemistry* 46: 123–126 (1997)
- Nozawa K, Udagawa S, Nakajima S, Kawai K. Studies on fungal products. XIV. Emestrin B, a new epithriothiodioxopiperazine, from *Emericella striata*. *Chem Pharm Bull* 35: 3460–3463 (1987)
- Onodera H, Hasegawa A, Tsumagari N, Nakai R, Ogawa T, Kanda Y. MPC1001 and its analogues: New antitumor agents from fungus *Cladorrhinum* species. *Org Lett* 6: 4101–4104 (2004)
- Cole RJ, Cox RH. *Handbook of Toxic Fungal Metabolites*. Academic Press, New York, 1981.
- Ueno Y, Umemori K, Nimi E, Tanuma S, Nagata S, Sugamata M, Ihara T, Sekijima M, Kawai K, Ueno I. Induction of apoptosis by T-2 toxin and other natural toxins in HL-60 human promyelotic leukemia cells. *Nat Toxins* 3: 129–137 (1995)
- Terao K, Ito E, Kawai K, Nozawa K, Udagawa S. Experimental acute poisoning in mice induced by emestrin, a new mycotoxin isolated from *Emericella* species. *Mycopathologia* 112: 71–79 (1990)
- Ishikawa J, Okano J, Ohki K, Amagai A, Maeda Y, Miyata H. Phagocytosis of *Dictyostelium discoideum* studied by the particle-tracking method. *Exp Cell Res* 288: 268–276 (2003)
- von-Wolff M, Ursel S, Hahn U, Steldinger R, Strowitzki T. Glucose transporter proteins (GLUT) in human endometrium: Expression, regulation, and function throughout the menstrual cycle and in early pregnancy. *J Clin Endocrinol Metabol* 88: 3885–3892 (2003)
- Wndhaber RAJ, Wilkins RJ, Meredith D. Functional characterization of glucose transport in bovine articular chondrocytes. *Pflügers Archiv Eur J Physiol* 446: 572–577 (2003)
- Hagglund CL, Gottschalk I, Lundahl P. Cytochalasin B-binding and transport properties of the Glut1 human red cell glucose transporter. *Recent Res Dev Bioenerget* 1: 117–129 (2000)
- Ondeyka J, Hensens OD, Zink D, Ball R, Lingham RB, Bills G, Dombrowski A, Goetz M. L-696,474, a novel cytochalasin as an inhibitor of HIV-1 protease II. Isolation and structure. *J Antibiot* 45: 679–685 (1992)
- Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, Plachw J, Stangassinger M, Erfle V, Schlondorff D. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: A mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med* 6: 769–775 (2000)
- Gribble AD, Forbes IT, Witherington J. Preparation of 3-substituted indoles or fused pyrroles as antagonists of the chemokine MCP-1 (CCR2B) receptor. GlaxoSmithKline, 2002, WO 2002079151 A1.
- Yang L, Butora G, Parsons WH. Preparation of *N*-cyclopentylpiperidines as modulators of chemokine receptor activity. Merck & Co., Inc., 2002, WO 2002013824 A1.