NOTES

Cochlioquinones and *Epi*-Cochlioquinones: Antagonists of the Human Chemokine Receptor CCR5 from *Bipolaris brizae* and *Stachybotrys chartarum*

K. YOGANATHAN,* LAY-KIEN YANG, CHRISTINE ROSSANT,†
YICUN HUANG, SIEWBEE NG, MARK S. BUTLER
and ANTONY D. BUSS

MerLion Pharmaceuticals,
59A Science Park Drive, The Fleming,
Singapore Science Park, Singapore, 118240

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Chemokines are small proteins (7～16 kDa) that act at G protein-coupled receptors to regulate a variety of physiological and pathophysiological processes[1,2]. The human immunodeficiency virus Type 1 (HIV-1) principally uses CCR5 and CXCR4 chemokine receptors as co-receptors with CD4 to gain entry into target cells[3,4]. Therefore, a molecule that binds to the CCR5 receptor could potentially prevent HIV-1 entry into cells, which makes CCR5 an important target for anti-HIV-1 therapy[5].

In the course of screening our extract library for inhibitors of CCR5[6,7], extracts from *Bipolaris brizae* (Nisikado) Shoem and *Stachybotrys chartarum* were found to displace macrophage inflammatory protein (MIP)-1α from the human chemokine receptor CCR5. Bioassay directed fractionation led to the isolation of three active cochlioquinones from *Bipolaris brizae* (Nisikado) Shoem, namely, 17-methoxycochlioquinone A (**1**), cochlioquinone A (**2**)[8～10] and isocochlioquinone A (**3**)[8～10]. Two active *epi*-cochlioquinones compounds, 11-*O*-methyl-*epi*-cochlioquinone A (**4**) and *epi*-cochlioquinone A (**5**)[11], were isolated from *Stachybotrys chartarum*. Herein, we report the isolation, structure elucidation and biological activity of **1**, **2**, **3**, **4** and **5**.

Materials and Methods

CCR5 Assay

CCR5 receptor binding activity was determined in a 96-well scintillation proximity assay[12] (SPA) using a [^{125}I]-human MIP-1α and membranes prepared from CHO cells over expressing the human CCR5 receptor. The samples were dissolved in 12.5% aqueous DMSO and incubated with 12 μg membranes, 0.17 nM [^{125}I]-MIP-1α, 0.25 mg Wheat Germ Agglutinin-SPA beads in assay buffer (50 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA and a protease inhibitor cocktail), shaking for 5 hours at room temperature. Radioactivity (total binding) was measured after a 2 hours bead settling period. Non-specific binding was defined in the presence of 1 μM recombinant human MIP-1α.

Results and Discussion

Fermentation

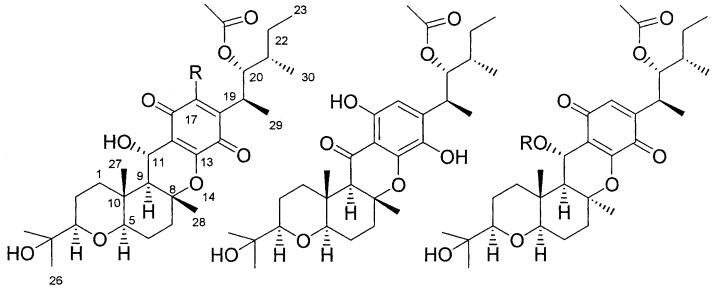
The producing microorganism was identified as *Bipolaris brizae* (Nisikado) Shoem based on morphological characteristics. The strain has been deposited in the MerLion Pharmaceuticals culture collection as F32659. The strain was sub-cultured on malt extract agar (CM057B, Oxoid) for 7 days at 24°C. It was used to inoculate 250 ml Erlenmeyer flasks each containing 50 ml of seed medium composed of 0.4% glucose, 1% malt extract and 0.4% yeast extract. The pH of the medium was adjusted to 5.5 prior to sterilization. The seed flasks were incubated for 5 days at 24°C on a rotary shaker at 200 rpm. A volume of 5 ml of seed culture was used to inoculate 50 ml of liquid medium in a 250 ml flask. The liquid medium is composed of 0.4% yeast extract, 2% glucose and 2% oatmeal. The pH was adjusted to 7.5 and autoclaved at 121°C for 30 minutes. The fermentation was carried out for 9 days at 24°C at 200 rpm.

The second fungal strains F32923 (*Stachybotrys chartarum*) has been deposited in the MerLion

* Corresponding author: yoga@merlionpharma.com
† Present address: Upstate Ltd., Cowley Rd., Cambridge CB4 ODS, UK.

Table 1. Physico-chemical properties of 17-methoxycochlioquinone A (**1**) and 11-*O*-methyl-*epi*-cochlioquinone A (**4**).

	1	**4**
appearance	Yellow oil	Yellow solid
Molecular formula	$C_{31}H_{46}O_9$	$C_{31}H_{46}O_8$
ESI-MS $(M+Na)^+$	585.3042(calcd 585.3040)	569.3071 (calcd 569.3090)
$[\alpha]^{25}_D$	+141° (*c* 0. 20, $CHCl_3$)	+38° (*c* 0.12, $CHCl_3$)
UV (MeOH) λ_{max} (log ε) nm	283 (4.04), 386 (3.52)	270 (4.04), 385 (3.52)
IR ν_{max} (NaCl) cm^{-1}	3521, 2968, 2879, 1730, 1660, 1645, 1456	3468, 2971, 2936, 1734, 1678, 1645, 1454



(**1**) R = Me
(**2**) R = H

3

(**4**) R = Me
(**5**) R = H

Pharmaceuticals culture collection. The strain was sub-cultured and fermented using the same conditions described above.

Isolation of Active Constituents of *Bipolaris brizae* (Nisikado) Shoem

The freeze-dried fermentation broth (2 liters) of *Bipolaris brizae* (Nisikado) Shoem was extracted 3 times with CH_2Cl_2-MeOH (1 : 1) and evaporated to dryness under vacuum. The dry extract (14.5 g) was partitioned 3 times between hexane and 90% MeOH in H_2O (1 : 1). The 90% MeOH portion was adjusted to 70% with H_2O, and then partitioned 3 times with $CHCl_3$. The active $CHCl_3$ fraction (1.7 g) was subjected to reverse phase preparative HPLC (isocratic elution for 70 minutes; mobile phase: 0.1% HCOOH in acetonitrile+0.1% HCOOH in H_2O (65 : 35); flow rate: 10 ml/minute) to give compounds **1** (6 mg), cochlioquinone A (**2**) (1 mg) and isocochlioquinone A (**3**) (3 mg). Cochlioquinone A (**2**) and isocochlioquinone A (**3**) were identified by comparison of their spectral properties with those reported in the literature[8~10].

Isolation of Active Constituents of *Stachybotrys chartarum*

The freeze-dried fermentation broth (2 liters) of *Stachybotrys chartarum* was extracted 3 times with CH_2Cl_2-CH_3OH (1 : 1) and evaporated to dryness under vacuum. The crude extract (2 g) was partitioned 3 times between hexane and 90% MeOH in H_2O (1 : 1). The active hexane fraction (0.5 g) was subjected to reverse phase preparative HPLC (gradient elution; mobile phase: 0.1% HCOOH in acetonitrile+0.1% HCOOH in H_2O (50 : 50→100 : 0 over 30 minutes); flow rate: 12 ml/minute) to give (**4**) (3 mg) and *epi*-cochlioquinone A (**5**) (1 mg). *Epi*-cochlioquinone A (**5**) was identified by comparison of its spectral properties with those previously reported[11].

Structure Elucidation

The molecular formula ($C_{31}H_{46}O_9$) of compound **1** was established by analysis of the ^{13}C NMR, multiplicity-edited HSQC spectrum and positive HR-ESIMS. The physico-chemical properties of **1** are given in Table 1. The IR spectrum showed characteristic absorption bands from OH

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for 17-methoxycochlioquinone A (**1**) in $CDCl_3$.

No.	^{13}C (δ, m)[a]		^{1}H δ, (m, *J* in Hz)	COSY	HMBC ^{1}H to ^{13}C
1	39.5	t	1.42 m	1b, 2, 2b	3, 10
			2.45 m	1a, 2, 2b	
2	22.5	t	1.44 m	1a, 1b, 2b, 3	1, 3, 24
			1.64 m	1b, 1b, 2, 3	
3	86.0	d	3.23 dd (12, 2)	2a, 2b	5, 24, 25, 26
5	84.2	d	3.15 dd (12, 2)	6a, 6b	3, 6, 10, 27
6	26.2	t	1.54 m	5, 6b, 7a, 7b	5, 10
			1.72 m	5, 6a, 7a, 7b	
7	38.4	t	1.90 m	6a, 6b, 7b	5, 6, 9, 28
			2.08 dt (11.5, 3)	6a, 6b, 7a	
8	84.7	s			
9	52.8	d	1.69 d (10)	11	5, 10, 11, 12, 28
10	37.7	s			
11	64.1	d	4.89 d (10)	9	9, 10, 12, 13, 18
12	118.4	s			
13	152.8	s			
15	183.0	s			
16	132.8	s			
17	157.3	s			
17-OMe	62.8	q	4.01 s		17
18	185.5	s			
19	33.0	d	3.38 m	20, 29	15, 16,17,20,29
20	78.1	d	5.25 br d (10)	19	16, 19, 21, 22, 20-OCOMe, 30
20-OCOMe	171.5	s			
20-OCOMe	22.0	q	1.88 s		OCOMe
21	36.8	d	1.69 m	30	22, 30
22	28.1	t	1.15 m	22b, 23	21, 30
			1.27 m	22a, 23	
23	12.9	q	0.91 t (7.5)	22a, 22b	21, 22
24	72.8	s			
25	27.0	q	1.18 s		3, 24, 26
26	24.7	q	1.16 s		3, 24, 25
27	13.3	q	1.00 s		5, 9, 10
28	21.9	q	1.31 s		7, 8, 9
29	16.2	q	1.19 d (7)	19	16, 19, 20
30	13.1	q	0.93 d (7)	21	20, 21, 22

[a] Assignments based on COSY, multiplicity-edited HSQC and HMBC NMR data.

(broad, 3521 cm^{-1}), ester (1730 cm^{-1}) and carbonyl groups (1660 and 1645 cm^{-1}). The ^{13}C NMR spectrum of **1** (Table 2) showed 31 resolved peaks, which could be classified into seven methyl, one methoxyl, five methylene, three methines, four oxygenated methines and eleven quaternary carbons. The ^{1}H NMR spectrum of **1** (Table 2) displayed signals for four oxymethine groups (δ_H 5.25, br d; 4.89, d; 3.23, dd; 3.15, dd), five methylene groups (δ_H 1.4~2.5, m), one methoxy group (δ_H 4.01, s) and eight methyl groups (δ_H 0.91, t; 0.93, d; 1.00, s; 1.16, s; 1.18, s; 1.19, d; 1.31, s; and 1.88, s). These data were similar to that of cochlioquinone A (**2**)[8~10] except for the presence of a methoxy group and the absence of aromatic methine H-17 (δ_H 6.56) present in cochlioquinone A (**2**)[8~10]. The placement of the methoxy at C-17 was confirmed by the observation of a ^{3}J (OMe to C-17) correlation in HMBC NMR spectrum. This was also in accord with the observed carbon resonance of C-17 at δ_C 157.3 since it is α to both an oxygen and a carbonyl function. These observations supported the assignment of **1** as the 17-methoxy derivative of cochlioquinone A.

Examination of the NMR data of compound **4** (Table 3) suggested that **4** was a cochlioquinone derivative related to *epi*-cochlioquinone A (**5**). The physico-chemical properties of **4** are given in Table 1. A molecular formula of $C_{31}H_{46}O_8$ was determined for compound **4** on the basis of positive HR-ESIMS and ^{13}C NMR data, which was different to **5** by a CH_2 unit. Comparison of ^{1}H and ^{13}C NMR data of **4** with

Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for 11-*O*-methyl-*epi*-cochlioquinone A (**4**) in $CDCl_3$.

No.	^{13}C (δ, m)[a]		^{1}H δ (m, *J* in Hz)	COSY	gHMBC ^{1}H to ^{13}C
1	37.7	t	1.35 m	1b, 2a, 2b	9, 10, 27
			2.05 m	1a, 2a, 2b	2, 3, 10, 27
2	22.7	t	1.48 m	1a, 1b, 2b, 3	10
			1.62m	1a, 1b, 2a, 3	1, 3, 10, 24
3	85.2	d	3.15 dd (12, 2)	2a, 2b	1, 5, 24, 25, 26
5	84.2	d	3.08 dd (12, 2)	6a, 6b	1, 3, 6, 27
6	24.5	t	1.57 m	5, 6b, 7a	5, 10
			1.80 m	5, 6a	5, 7
7	37.5	t	1.72 m	6a, 7b	6, 8
			2.35 m	7a	5, 6, 8, 9
8	80.1	s			
9	53.4	d	1.60 d (9)	11	1, 7, 10, 11, 12, 27
10	35.5	s			
11	68.4	d	4.08 d (9)	9	8, 10, 12, 13, 18, 11-OMe
11-OMe	57.9	q	3.40 s		11
12	119.0	s			
13	153.3	s			
15	182.2	s			
16	150.0	s			
17	135.0	d	6.55 s		12, 15, 19
18	187.0	s			
19	35.5	d	3.20 m	20, 29	16, 20, 29
20	79.5	d	5.02 dd (8, 4)	19, 21	16, 20-OCOMe, 21
20-OCOMe	171.4	s			
20-OCOMe	21.7	q	1.93 s		20-OCOMe
21	37.0	d	1.60 m	30	19, 23
22	27.5	t	1.10 m	22b, 23	30
			1.27 m	22a, 23	
23	12.7	q	0.89 t (7)	22a, 22b	21, 22
24	72.8	s			
25	24.6	q	1.16 s		3, 24, 26
26	27.0	q	1.18 s		3, 24, 25
27	12.7	q	0.60 s		1, 5, 9, 10
28	27.3	q	1.31 s		7, 8, 9
29	17.8	q	1.17 d (7)	19	16, 19, 20
30	14.0	q	0.91 d (7)	21	20, 21, 22

[a] Assignments based on COSY, HSQCED and HMBC.

those of *epi*-cochlioquinone A (**5**)[11] revealed that **4** had a methyl ether group (δ_H 3.40, s; δ_C 57.9) in addition to the *epi*-cochlioquinone A (**5**) skeleton. The observation of an HMBC correlation from the methyl ether protons to δ_C 68.4 allowed placement of the methyl ether group at C-11 and assignment of the structure of **4** as the 11-*O*-methyl derivative of *epi*-cochlioquinone A. The relative stereochemistry of H-11 was deduced to be α from the $J_{9\alpha\text{-}11}$ value of 9 Hz, which required H-11 and H-9α to be in a *cis*-diaxial arrangement.

Biological Activities

17-Methoxycochlioquinone A (**1**), cochlioquinone A (**2**), isocochlioquinone A (**3**), 11-*O*-methyl-*epi*-cochlioquinone A (**4**) and *epi*-cochlioquinone A (**5**) were found to compete effectively with MIP-1α for binding to human CCR5 with IC_{50} values of 100, 11, 50, 7 and 4 μM respectively. Comparison of the activity of **2** and **3** suggested that the quinoid skeleton increased the activity of the cochlioquinones, while substitution of a methoxy group at C-17 in **1** significantly decreased the activity compared to **2**. The IC_{50} values **4** and **5** were similar to that of cochlioquinone A (**2**) (11 μM), which has the same 12,13,15-trisubstituted quinone unit but different stereochemistry at C-8. Cochlioquinones family have been previously reported to inhibit NADH-ubiquinone reductase[10] and acyl-CoA cholesterol acyltransferase[11].

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