Letters

A Novel Ergot Alkaloid as a 5-HT$_{1A}$ Inhibitor Produced by *Dicyma* sp.

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Abstract: In the course of a search for small-molecule inhibitors of 5-hydroxytryptamine receptors we have identified a novel ergoline derivative (**1**) from the fungal culture of *Dicyma sp*. This compound has a pK_i of 10.2 versus the 5-hydroxytryptamine$_{1A}$ receptor subtype. The structure was elucidated by extensive NMR spectroscopy and mass spectrometry.

Introduction. Serotonin (5-HT) is a ubiquitous neurotransmitter which is found in both the central and peripheral mammalian nervous systems. The transduction of serotonergic signals across cellular membranes is mediated by a variety of serotonin receptor subtypes. In betweeen them the 5-HT$_{1A}$ receptor subfamily has attracted considerable attention as a target in the development of novel therapeutics for the treatment of depression and anxiety disorders.[1−3] Many compounds of different chemical classes, for example indoles, aminotetracyclines, benzodioxanes, and arylpiperazines, are know to have a high affinity for the 5-HT$_{1A}$ receptors and act as agonist, antagonist, or partial agonist at this receptor site.[4,5]

Ergot alkaloids, having a wide spectrum of central and peripheral pharmacological activity, are used in the treatment of a variety of pathophysiological disturbances.[6,7] All ergot compounds possess the tetracyclic ergoline skeleton as a common structural element that contains a structural relationship to indoleethylamines and catecholamines. It is therefore not surprising that ergot derivatives interact nonselectively with the monoaminergic (adrenergic, dopaminergic, and serotonergic) recognition sites.[8] As a consequence of these interactions, a major challenge in the development of therapeutic agents from this class is the identification of compounds that are sufficiently selective for a single neurotransmitter. Of particular importance to us was the identification of compounds possessing selectivity for the serotonergic system, which has been shown to be involved in psychiatric disorders such as anxiety and depression as well as in physiological processes such as the regulation of sleep pattern and sexual behavior.[9−11]

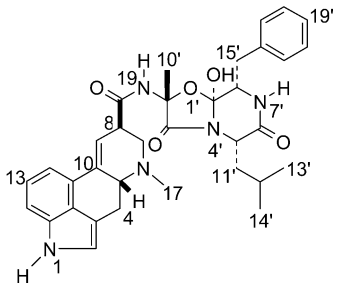

Figure 1. Structure of **1** isolated from *Dicyma* sp.

In an effort to find naturally ocurring 5-HT inhibitors, we extended our search to our culture extract collection and found that extracts of *Dicyma* sp. inhibited 5-HT. Subsequent bioactivity-directed fractionation resulted in the isolation of a novel ergot alkaloid (**1**, Figure 1). In this report, we describe the isolation, structure elucidation and biological activities of **1**.

Results and Discussion. Compound **1** was isolated as a pale yellow solid by biossay-guided fractionation of the organic extracts from liquid cultures of *Dicyma sp*. The identification of **1** was achieved by mass spectrometry and NMR spectroscopy. The ESI MS of **1** showed the molecular ion peak $[M + H]^+$ at *m/z* 598 which was confirmed by positive-ion and negative-ion APCI. Other significant fragments at *m/z* 580 $[(M + H) - H_2O]^+$, *m/z* 433 $[C_{23}H_{23}N_5O_4]^+$, *m/z* 320 $[C_{19}H_{18}N_3O_2]^+$, *m/z* 268 $[C_{16}H_{18}N_3O]^+$, and *m/z* 223 $[C_{15}H_{14}N_2]^+$ were also present. The ion at *m/z* 268 corresponds to free lysergic acid amide, and it is frequently observed in this type of alkaloid.

The ^{1}H NMR spectrum (Table 1) showed characteristic signals for an indole moiety: δ 7.93 ppm (1H, br. s, H-1), 7.15 ppm (1H, dd, $J_1 = 7.5$ Hz and $J_2 = 7.0$ Hz, H-13), 7.19 (1H, d, $J_1 = 7.5$ Hz, H-12), 7.22 (1H, d, $J_1 = 7.0$ Hz, H-14), and 6.94 (1H, br. s, H-2) and the characteristic signal for an *N*-Me group (2.65 ppm), suggesting that compound **1** was a member of the ergot family.[12,13] All other signals of the lysergic acid moiety were further elucidated on the basis of two-dimensional NMR data. Comparison of the chemical shift of H-8 with data of ergotamine and its C-8 epimer suggested the isolated alkaloid to be a derivative of isolysergic acid.[14]

The ^{13}C NMR spectrum of **1** displayed a total of 34 carbon signals, suggesting a molecular formula of $C_{34}H_{39}N_5O_5$, which agreed with the MS data. These carbon resonances were classified as 4 methyl carbons,

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Table 1. ^{13}C and ^{1}H NMR Spectral Data for Compound **1** in $CDCl_3$ at 25 °C

	δ_C, ppm	δ_H, ppm, multiplicity (J_{HH} in Hz)
NH-1		7.93, br. s
2	119.1	6.94, br. s
3	110.4	
4	22.2	3.33, dd (5.0 and 13.9) and 2.83, m
5	59.9	3.83, dd (5.9 and 13.1)
7	49.2	2.98, dd (3.5 and 12.1) and 2.93, dd (2.6 and 12.1)
8	43.7	3.18, m
9	118.5	6.37 dd (1.7 and 5.7)
10	135.5	
11	129.3	
12	112.2	7.19, d (7.5)
13	123.5	7.15 dd (7.5 and 7.0)
14	110.2	7.22, d (7.0)
15	133.8	
16	126.2	
Me-17	41.2	2.65, s
CO-18	175.8	
NH-19		9.37, br. s
2′	85.5	
CO-3′	166.2	
5′	53.3	4.49, dd (5.2 and 8.7)
CO-6′	168.7	
NH-7′		6.93, br. s
8′	62.0	3.62, dd (2.1 and 11.3)
9′	102.6	
Me-10′	24.7	1.576, s
11′	43.5	1.98, m and 1.90, m
12′	24.6	1.98, m
Me-13′	22.9	0.99, d (6.1)
Me-14′	21.8	1.04, d (6.1)
15′	34.8	3.28, dd (2.2 and 13.9) and 2.83, m
16′	135.5	
17′	129.3	7.33, m
18′	127.5	7.27, m
19′	129.3	7.33, m
20′	127.5	7.27, m
21′	129.3	7.33, m
OH-22′		5.44, s

4 methylene carbons, 15 methine carbons, and 11 quaternary carbons. The presence of three amide (or ester) carbonyl resonances in the range of 168−174 ppm and the characteristic signals of a *N*-Me group (2.65 ppm), two amide protons (6.93 and 9.37 ppm), and two methyl doublets (0.99 and 1.04 ppm) confirmed that compound **1** was a member of the ergopeptine alkaloids. There were no signals that could be assigned to the methylene protons of a proline residue or a second alanine, which usually are constituents of ergopeptine alkaloids. Instead, the spectrum showed one pair of methylene protons at δ_H 2.83−3.28 coupled to H-8′. Thus, the proline moiety must be replaced by a phenylalanine residue. These assignments were confirmed by long-range $^{1}H-^{13}C$ correlation of the α-protons with both *β*- and *γ*-carbons and the correlations of the *N*-H with the α-carbon in each residue. Configurations of the remaining asymmetric centers, as shown, are the same as for the regular series of ergopeptines;[12−14] however, absolute configurations at the 2′, 5′, 8′, and 9′ positions of **1** remain to be determined.

Compound **1** was identified as a new ergot alkaloid derivative, on the basis of their spectral data. In two independent experiments, this compound isolated from *Dicyma* sp. was evaluated for affinity for the 5-HT_{1A} receptor. The affinity for 5-HT_{1A} receptor was assayed in terms of the ability to displace the binding of the radioligand [^{3}H]-8-OH-DPAT to the human cloned 5-HT_{1A} receptor stably expressed in HEK 293 cells.[15] The percentage of inhibition for the assay sample was calculated assuming 100% activity for the controls. To assess its selectivity, we measured the potency of this compound at five different 5-hydroxytryptamine receptors and D_2 dopamine receptor. To provide the comparison of the results obtained in the different assays we reported (Table 2) the pK_is.

Table 2. Receptor Binding Profile[a]

	affinity (pK_i)				
receptor	compound **1**	LSD[b]	5-HT[c]	8-OH-DPAT[c]	5-CT[c]
5-HT_{1A}	10.2	8.9	8.4	8.7	9.2
5-HT_{1B}	8.5	nd[d]	8.3	6.5	8.9
5-HT_{2A}	7.8	8.5	6.4	5.0	<5.3
5-HT_{2C}	7.8	8.3	7.3	4.9	5.6
5-HT_6	7.3	8.2	6.9	6.9	6
5-HT_7	8.2	8.2	8.5	8.5	9.4
dopamine D_2	8.5	6.9	nd	nd	nd

[a] All values represent the mean of at least two determinations, with each determination lying within 0.2 log units of the mean. Binding experiments were conducted as follows. Receptors and radioligands used in the binding assay: 5-HT_{1A} (human cloned receptors in HEK 293 cells; [^{3}H]-8-OH-DPAT); 5-HT_{1B} (human cloned receptors in CHO cells; [^{3}H]-5-CT); 5-HT_{2A} (human cloned receptors in HEK 293 cells; [^{3}H]ketanserin); 5-HT_{2C} (human cloned receptors in HEK 293 cells; [^{3}H]mesulergine); 5-HT_6 (human cloned receptors in HeLa cells; [^{3}H]LSD); D_2 (human cloned receptors in CHO cells; [^{125}I]iodosulpride). [b] Data from Nichols et al.[16] [c] Data from Roberts et al.[17] [d] nd means not determined.

Table 3. [^{35}S]GTP*γ*S Binding to HEK 293 Cell Membranes Expressing Human 5-HT_{1A} Receptors (pEC_{50}, E_{max}, % Stimulation, Intrinsic Activity Relative to 5-HT) for 5-HT, SB-272183, and Compound **1**[a]

	pEC_{50}	E_{max}	% stimulation	intrinsic activity
5-HT	8.06 ± 0.18	224 ± 20	126 ± 17	1
SB-272183	7.72 ± 0.18	144 ± 2	42 ± 5	0.33 ± 0.03
1	8.70 ± 0.30	229 ± 10	138 ± 13	1.11 ± 0.13

[a] All values are expressed as the mean ± SE mean for four to seven independent experiments.

As can be seen from Table 2, compound **1** was found to display a high degree of selectivity for the 5-HT_{1A} receptor, being over 100-fold selective 5-HT_{2A}, 5-HT_{2C}, 5-HT_6, and 5-HT_7 receptors, and over 50-fold selective for 5-HT_{1B} and dopamine D_2 receptors. Compound **1** was further evaluated using an in vitro functional assay,[18] namely [^{35}S]-GTP*γ*S binding to HEK293 cells stably expressing human 5-HT_{1A} receptors. The standard compound, SB-272183,[19] was also evaluated for comparison showing a partial agonist profile and a pEC_{50} of 7.72 (Table 3). Compound **1** stimulated basal activity with a pEC_{50} of 8.70, indicating that compound **1** is a full agonist at this receptor.

In addition, to better define the binding affinity and selectivity profile, compound **1** was compared with other 5-HT_{1A} agonists (5-HT, 5-CT, 8-OH-DPAT, and LSD) typically used as pharmacological tools for 5-HT research. Reported data for these compounds were included in Table 2. On the basis of these data, compound **1** showed the highest affinity for 5-HT_{1A} receptor, being 10-fold more potent than 5-CT, the most potent of the group. The selectivity pattern was also very encouraging for compound **1**, even comparing it with LSD, the most structurally related one. Both compounds have similar affinity at the 5-HT_7 receptor, but compound **1** has lower affinity than LSD for 5-HT_{2A}, 5-HT_{2C}, and 5-HT_6.

In conclusion, the new ergopeptide derivative **1** is the first example of a high-affinity and selective agonist for the 5-HT_{1A} receptor, discovered from a high-throughput screening from a natural products source.

General Methods. NMR spectra were recorded with a JEOL Alpha-400 NMR spectrometer (399.65 MHz for ^1H and 100.40 MHz for ^{13}C) using $CDCl_3$ as solvent. MS spectra were recorded on an Ion-trap Finigan LCQ. HPLC separations were performed using a Beckman M126 pump equipped with a Beckman M168 UV/Vis diode array detector (190−600 nm).

Microorganisms. The fungal strains were isolated from soil collected in Guatemala. Working stocks were prepared on Potato Dextrose agar (22 g/L Dehydrated Potato, 20 g/L glucose and 17 g/L agar) slants stored at 4 °C. Slants were inoculated from long-term stocks kept at −196 °C or from freeze-dried cultures.

Fermentation. Fermentation was performed in two different steps; 250 mL flasks containing 30 mL of HAGGS1 medium (soybean oil 3%, glycerol 4%, agar (Pronadisa) 1.5%, cotton seed flour 1% and starch 1%) were selected from freshly prepared plates and were fermented during 72 h at 28 °C in orbital shakers (250 rpm). Twenty-five milliliters of these broths was used as inocula for 250 mL fermentations, in HAGGS1 medium, contained in 2 L flasks. The culture was harvested after 6 days.

Detection of 5-HT receptor inhibitory activity: 5-HT receptor inhibitory activity was detected by monitoring the binding competition between a labeled receptor ligand and the compound **1**.

Preparation of Homogenates Cell Membranes. Cells transfected with DNA fragments coding for one of the human receptors according to the method described by To et al.[20] or native brain samples were suspended in ice-cold 50 mM Tris buffer (pH 7.7 at 25 °C) and homogenized for about 20 s using an Ultra-Turax blender. Sedimentation of the insoluble particles was achieved using a centrifuge set at 20000 rpm (35000 rcf) for 10 to 20 min. All whole cells and prepared homogenates were stored at −70 °C until use. Assay buffer: 0.1% L-ascorbic acid, 10 μM pargyline, 0.5 mM EDTA, 10 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.4. Procedure: A 70 μL aliquot of tissue preparation suspended in assay buffer (300 μL of suspension per 12 mL assay buffer homogenized with a potter) was added to each well of Picoplates-96 (Packard) followed by 5 μL of **1** in neat DMSO and incubated at room temperature for 15 min. An amount of 20 μL of 10 nM [^3H]-5-CT was then added, and the wells were incubated at 37 °C for 30−45 min. The well contents were then filtered through GF/C plates (Packard no. 6005174) pretreated with 0.3% polyethylenimine and washed four times with 150 μL of ice-cold 50 mM Tris-HCl, pH 7.4. The filters were dried, 30 μL of Microscint O was added, and the scintillations were counted using the Top Count Scintillation Counter. As a control of nonspecific binding, 5 μL of 200 μM 5-hydroxytryptamine (in neat DMSO) was used instead of the test solution. As a control for 50% inhibition, 5 μL of 3 μM ritanserin (in neat DMSO) was used instead of the test solution.

IC_{50} values were estimated from the cpm using a four-parameter logistic curve fit within EXCEL.[21] K_i values were calculated using the method of Cheng and Prusoff.[22] pIC_{50} and pK_i were defined as the negative $\log_{10}$ of the molar IC_{50} and K_i, respectively

[^{35}S]GTPγS Binding Studies. [^{35}S]GTPγS binding studies were carried out essentially as described Thomas et al.[23] with some minor modifications. Briefly, membranes from 10^6 cells were preincubated at 30 °C for 30 min in 20 mM HEPES buffer (pH 7.4) in the presence of $MgCl_2$ (3 mM), NaCl (100 mM), GDP (10 μM), and ascorbate (0.2 mM), with or without compounds. The reaction was started by the addition of 10 μL of [^{35}S]GTPγS (100 pM, assay concentration) followed by a further 30 min incubation at 30 °C. Nonspecific binding was determined using nonradiolabeled GTPγS (20 μL) added prior to the membranes. The reaction was terminated by rapid filtration through Whatman GF/B grade filters followed by 5 × 1 mL washes with ice cold HEPES (20 mM)/$MgCl_2$ (3 mM) buffer. Radioactivity was measured using liquid scintillation spectrometry.

Extraction and Isolation. The fermentations (4 × 250 mL) were harvested. Each fermentation broth was homogenized by adding an equal volume of acetone and shaking on an orbital shaker at 300 rpm for 0.5 h at room temperature. The homogenates were centrifuged at 2000 rpm (4 °C) for 15 min and the aqueous-acetone phases combined to give 2000 mL of solution.

The acetone was eliminated under reduced pressure and the pH adjusted to 7 with 1 N NaOH. The resulting solution was extracted with ethyl methyl ketone (3 × 400 mL). The combined organic phases were dried over Na_2SO_4, and the solvent was evaporated off to give a residue of 1950 mg. Most of this (1900 mg) organic extract was dissolved in 10 mL of MeOH and chromatographed on a Sephadex LH-20 column (90 × 4 cm) developed with MeOH at 10 mL/min. A total of 120 fractions of 10 mL were obtained and monitored by analytical reverse phase HPLC. The most interesting fractions (S27−33) according to their composition and activity were pooled and evaporated to dryness. A total of 850 mg of active material was obtained.

This material was chromatographed on a Flash 40 M: KP−Sil silica cartridge (4 × 15 cm, 32−63 μm, 60 Å, 500−550 m^2/g silica, ref FKO-1107−17044 from Biotage). The Biotage Flash 40 I apparatus was pressurized to 100 psi with compressed air. The cartridge was stabilized with 500 mL of $CHCl_3$/MeOH (40:1). Once stabilized, 800 mg of the active fraction dissolved in 5 mL of $CHCl_3$/MeOH (40:1) were loaded onto the column, and 150 fractions of 8 mL were collected eluting with the same mixture of solvents. Active fractions (F70−90) of this column in the 5-HT receptor inhibition assay contained a major compound visualized by TLC.

Compound **1** (1.6 mg) was finally purified as a pale yellow solid from these fractions using silica gel 60 F_{254} TLC plates (20 × 20 cm, 0.2 mm thickness) on glass support (ref 1.05715 from Merck) and EtOAc/MeOH (9:1) as the eluent. The compound showed a R_f of 0.35 under these conditions.

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