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In vitro Serotonergic Activity of Black Cohosh and Identification of N_ω-Methylserotonin as a Potential Active Constituent

SHARLA L. POWELL[†], **TANJA GÖDECKE**[†], **DEJAN NIKOLIC**[†], **SHAO-NONG CHEN**[†], **SOYOUN AHN**[†], **BIRGIT DIETZ**[†], **NORMAN R. FARNSWORTH**[†], **RICHARD B. VAN BREEMEN**[†], **DAVID LANKIN**[†], **GUIDO F. PAULI**[†], and **JUDY L. BOLTON**[†,*]

[†]Department of Medicinal Chemistry and Pharmacognosy and UIC/NIH Center for Botanical Dietary Supplements Research, College of Pharmacy, 833 S. Wood Street, M/C 781, University of Illinois at Chicago, Chicago, Illinois, 60612-7231

Abstract

Cimicifuga racemosa(L.) Nutt. (syn. *Actaea racemosa* L., black cohosh) is used to relieve menopausal hot flashes, although clinical studies have provided conflicting data, and the active constituent(s) and mechanism(s) of action remain unknown. Since serotonergic receptors and transporters are involved with thermoregulation, black cohosh and its phytoconstituents were evaluated for serotonergic activity using 5-HT_7 receptor binding, cAMP induction, and serotonin selective reuptake inhibitor (SSRI) assays. Crude extracts displayed 5-HT_7 receptor binding activity and induced cAMP production. Fractionation of the methanol extract lead to isolation of phenolic acids and identification of N_ω-methylserotonin by LC/MS-MS. *Cimicifuga* triterpenoids and phenolic acids bound weakly to the 5-HT_7 receptor with no cAMP or SSRI activity. In contrast, N_ω-methylserotonin showed 5-HT_7 receptor binding (IC_{50} 23 pM), induced cAMP (EC_{50} 22 nM), and blocked serotonin reuptake (IC_{50} 490 nM). These data suggest N_ω-methylserotonin may be responsible for the serotonergic activity of black cohosh.

Keywords

black cohosh; *N*-methylserotonin; *Cimicifuga*; menopause; serotonin

INTRODUCTION

Women can expect to spend one third of their lives in the post-menstrual phase, with an estimated 55–75% of women experiencing symptoms such as hot flashes, depression, mood swings, sleep deprivation, and loss of sexual drive (1). Among those women suffering these symptoms, approximately 20% will find the symptoms intolerable, and up to 30% of these women will seek out treatment through their healthcare providers (1, 2). Traditionally, the standard treatment recommended to perimenopausal and post-menopausal women to relieve some of these symptoms was hormone replacement therapy (HRT). However, the high HRT usage was prior to the release of the Women's Health Initiative findings (3, 4), which showed that women on HRT had an increased risk of developing various hormone-dependent cancers (1, 3). Alternatives to traditional HRT are needed to alleviate menopausal symptoms without adverse side effects, leading millions of women to turn to botanical dietary supplements (5). *Cimicifuga racemosa* (L.) Nutt. (syn. *Actaea racemosa* L. (Ranunculaceae), black cohosh) is a popular and well documented medicinal plant, native to eastern North America. The roots and rhizomes have many traditional ethnobotanical uses,

[*]Corresponding author (Tel: (312) 996-5280; Fax: (312) 996-7107; Judy.Bolton@uic.edu).

including relief from rheumatism, as a diuretic, a tonic, an astringent, and for gynecological disorders (1, 6). In addition, black cohosh extracts have become a popular treatment for vasomotor menopausal symptoms, potentially providing an alternative to HRT (1, 6). However, some clinical trials have found no effect of black cohosh extracts on reducing the vasomotor symptoms of menopause (4, 7), although other studies have shown efficacy in alleviating symptoms such as hot flashes, sweating, mood swings, irritability, and sleeplessness (4, 7–9). Conflicting clinical trial results emphasize that the effects of black cohosh on menopausal symptoms and the mechanism(s) of action remain unclear.

Originally, black cohosh was thought to work through estrogenic pathways similar to traditional HRTs (1, 4, 10, 11). An estrogen-like activity was reported initially (4); however, these effects could not be confirmed in later studies which showed no classical estrogenic activity (1, 7, 12, 13). For example, a previous study demonstrated that black cohosh exhibited no estrogenic or antiestrogenic effects in an ovariectomized rat model (14). Interestingly, selective serotonin reuptake inhibitors (SSRIs), which operate through the serotonin transporter (SERT), have been shown to alleviate hot flashes (2). Besides the SERT, the 5-HT_7 and 5-HT_{1A} serotonin receptors are also involved in thermoregulation, which suggests that agonists for these receptors might be beneficial for the alleviation of hot flashes (15). Accordingly, the serotonergic system was examined as an alternative pathway through which black cohosh may reduce menopausal hot flashes. Black cohosh extracts had an affinity for various serotonin receptors, particularly the 5-HT_{1A}, 5-HT_{1D}, and 5-HT_7 (14). The black cohosh extracts also produced receptor-mediated functional activity as demonstrated by induction of cAMP in the human embryonic kidney (HEK) cells stably over-expressing the 5-HT_7 receptor. Data reporting agonistic activity for the 5-HT_7 receptor (14) support the need for *in vitro* evaluation of black cohosh extracts in order to determine the active constituents responsible for serotonergic activity.

The *in vivo* efficacy of a black cohosh extract for relief of menopausal hot flashes is currently being evaluated in a four-arm randomized double-blind, placebo-controlled Phase II clinical trial (16). The same clinical extract was tested for *in vitro* serotonergic activity by measuring 5-HT_7 receptor competitive binding, the induction of cAMP in 5-HT_7-transfected HEK293 cells, and the ability to block the reuptake of serotonin into hSERT-transfected HEK293 cells in the SSRI assay. In addition to the black cohosh clinical extract, several pure compounds isolated from black cohosh, such as triterpenes and polyphenolics (Figure 1) were tested, as well as other fractions obtained through bioassay-guided fractionation (Figure 2).

MATERIALS AND METHODS

Materials and Reagents

All chemicals and reagents were from Fisher (Hanover Park, IL) or Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. All cell culture media were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was acquired from Biowest (Miami, FL), and dialyzed FBS was acquired from Gemini Bioproducts (West Sacramento, CA). The purity of all reference compounds, including radiochemicals, was determined based on certificates of analysis (CoA) provided by the respective suppliers, and included with the compounds. [^{3}H] Lysergic acid diethylamide (LSD, CoA purity ≥ 99%) was obtained from PerkinElmer Life Sciences (Boston, MA). [^{3}H] 5-Hydroxytryptamine trifluoroacetate ([^{3}H] 5-HT, CoA purity ≥ 99%) was purchased from GE Healthcare UK (Piscataway, NJ). Poly-D-lysine hydrobromide was obtained from BD Biosciences and had a CoA purity > 98% (Bedford, MA). The quantitative ^{1}H NMR (qHNMR) analysis method (17) was used to verify compound purity. 3-(2-Aminoethyl)indol-5-ol (serotonin) as a hydrochloride salt was from Sigma-Aldrich (purity: CoA ≥ 98%; determined by qHNMR > 99% with 0.2% of

structurally unrelated impurities). 3-(2-Methylaminoethyl)indol-5-ol (N_{ω}-methylserotonin) was obtained as oxalate salt from Sigma-Aldrich (purity: CoA > 99%; determined by qHNMR > 97.5%, with 2.5% of structurally unrelated impurities) and was used in the stated bioassays.

Plant Materials

Due to the large scale of the overall study, which included a phase II clinical trial, authentic *C. racemosa* (L.) Nutt. (syn. *Actaea racemosa* L., black cohosh) rhizomes/roots (BC #163) were acquired through our industrial partner, Naturex, formerly PureWorld Botanicals (South Hackensack, NJ) (18) and were botanically verified by the UIC/NIH Center for Botanical Dietary Supplements Research and characterized by PCR, vouchers, and microscopy (19). Further details describing the botanical and phytochemical authentication of *C. racemosa* have been communicated (18). Voucher specimens of the plant materials are deposited at Naturex (South Hackensack, NJ) as follows: 75% EtOH extract (BC #163); MeOH extract (BC #066). Milled roots/rhizomes of black cohosh were extracted with 75% ethanol by large-scale percolation, vacuum dried at 45 °C and 29–30 inches of vacuum pressure, and milled through 60-mesh screen to yield a powdered extract. The chemical characterization was performed by HPLC (20), and the extract was standardized to 5.6% of four triterpene glycosides (18). The triterpene glycoside reference materials were isolated and characterized as previously described (18, 21, 22).

Bioassay-guided fractionation

For the bioassay-guided fractionation of black cohosh, which is outlined in Figure 2, dried and milled rhizomes/roots (1 kg) of *C. racemosa* were exhaustively extracted using percolation with 11 L of fresh MeOH (22 × 500 mL) at room temperature, with TLC monitoring. The extract was concentrated *in vacuo* (< 40 °C) to yield 173 g of syrupy residue. The residue was reconstituted in de-ionized water (250 mL) and partitioned with ethyl acetate (20 × 400 mL; 42 g). The water partition was subjected to column chromatography consisting of Amberlite XAD-2, yielding 2 fractions (H_2O and MeOH). A portion of the MeOH-soluble fraction (2.8 g) was further subjected to pH zone refined fast centrifugal partition chromatography (FCPC) using water-butanol-ethylacetate (5:4:1) as the solvent-system. The lower phase was used as mobile phase, adding 2 mg ammonium carbonate per mL phase-volume. 0.2% TFA was added to the stationary phase prior to loading the column. The sample was dissolved in stationary phase and injected, immediately afterwards mobile phase was pumped through the column at a flow-rate of 4 mL/min in a head-to-tail mode. The eluate was combined into seven fractions (1: 0 – 16 mL, 2: 16 – 32 mL, 3: 32 – 40, 4: 40 – 112 mL, 5: 112 –136 mL, 6: 136 – 388 mL, 7: 388 – 508 mL). Fraction 4 contained the cimicifugic acids, which are unique compounds in *Cimicifuga* species. To obtain pure cimicifugic acids, fraction 4 was subjected to a second step of FCPC in a HemWat +5 system [hexane-ethylacetate-methanol-water (3:7:3:7)] adding 0.2% TFA to both phases (23). The column was equilibrated before injecting the sample, which was dissolved in a mixture of both phases and run in a tail to head mode. The flow-rate was set to 4 mL/min (fraction size 4 mL). Solvents were switched to extrusion mode at K = 3 (24), and yielded the cimicifugic acids A, B, E, F and fukinolic acid at K values of K = 3.4 – 3.7, K = 4.8 – 6.0, K = 1.3 – 1.4, K = 2.2 – 2.5, and K = 9.6 – 23.0, respectively, where K represents the distribution constant of the analyte (25). The extraction progression and collected fractions were evaluated using analytical thin layer chromatography (TLC) on 20 × 20 cm pre-coated silica gel 60 F254 glass plates, (Merck, Darmstadt, Germany). Plates were developed at room temperature in the solvent system EtOAc/HCOOH/H_2O (18:1:1) and monitored under UV light (254 and 365 nm) both before and after spraying with Naturstoff reagent (1% 2-aminoethyl-phenylborinate in MeOH). Additional details regarding the extraction procedure have been published separately (26).

Identification of N_ω-methylserotonin

N_ω-methylserotonin was identified by mass spectrometry by means of accurate mass measurements in combination with a CrossFire Commander (Mimas, The University of Manchester, Manchester, UK) database search and comparison with an authentic sample of the compound. Briefly, the XAD-MeOH fraction 7 (Figure 2), active in the 5-HT_7 binding assay, was separated on a Tosoh TSK-Gel Amide-80 column using a gradient from 90–65% acetonitrile/0.1% formic acid over 60 min at a flow rate of 0.2 mL/min. The eluent from the column was introduced into a Micromass (Manchester, UK) Q-TOF-2 hybrid quadrupole/time-of-flight mass spectrometer operated in positive ion electrospray mode. The resolving power was 7000 FWHM. The mass accuracy obtained was better than 10 ppm unless noted otherwise. The ion source parameters were as follows: capillary 3.3 kV, cone voltage 23 V, source block temperature 100 °C, and drying gas temperature 300 °C. Tandem mass spectra were acquired at a quadrupole setting of 13, which corresponds to slightly less than unit resolution of the quadrupole. Tandem mass spectra were acquired at a collision energy of 23 eV using argon as the collision gas at a pressure of 2.0×10^{-5} mBar. The XAD-MeOH fraction 7 and N_ω-methylserotonin were also analyzed using an isocratic HPLC method and LC-MS based on a 50 mm × 4.6 mm i.d., 1.8 μm, Zorbax Eclipse C_{18} (Agilent) column, as well as the gradient HPLC method with LC-MS-MS based on a YMC ODS column to further support the method of unambiguous compound identification.

Cell Culture Conditions

The human 5-HT_7-transfected Chinese hamster ovary (CHO) cell line was generously provided by Dr. David Sibley (National Institutes of Health, Bethesda, MD) and cultured with Ham's F-12 medium containing 10% FBS, 1% sodium pyruvate, 1% geneticin (50 mg/mL), and 1% penicillin/streptomycin. The 5-HT_7-transfected-HEK293 cell line was provided by Dr. Mark Hamblin (University of Washington, Seattle, WA). The 5-HT_7-transfected-HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with puromycin (10 μg/mL), 1% antibiotic-antimycotic, and 10% FBS. Four days before the cAMP assays were performed, the culture medium was replaced with Cellgro Complete serum-free medium from Mediatech (Herndon, VA) supplemented with puromycin (10 μg/mL) and 1% antibiotic-antimycotic. The HEK293 cell line stably expressing human serotonin transporter (hSERT) was a generous gift from Dr. Randy D. Blakely (Vanderbilt University, Nashville, TN). hSERT-transfected-HEK293 cells were maintained in DMEM supplemented with 10% dialyzed FBS, 1% penicillin-streptomycin, 1% glutamax, and 1.2% geneticin (50 mg/mL). Cells were grown in a 37 °C humidified environment with 5% CO_2.

Serotonin Receptor Competitive Binding Assay

Cells (human 5-HT_7 CHO) were plated into dishes (150 mm × 10 mm) and cultured to confluence in order to collect membranes as previously described (27). Briefly, a hypotonic buffer (15 mM Tris, 1.25 mM $MgCl_2$, and 1 mM EDTA, pH 7.4) was added to the dishes and incubated at 4 °C for 10–15 min. The cells were scraped from the dishes on ice and the lysate was centrifuged. The hypotonic buffer was removed and the membrane pellet was suspended in TEM buffer (75 mM Tris, 12.5 mM $MgCl_2$, 1 mM EDTA, pH 7.4). The cell membranes were homogenized and centrifuged twice at 12,000 g for 20 min. The pellets were dissolved in TEM buffer and stored at −80 °C. Protein concentrations were determined according to the protein assay method using bovine serum albumin (BSA) as the standard from Bio-Rad Laboratories (Hercules, CA).

The 5-HT_7 receptor binding assay was performed as described previously (27) with minor modifications using human recombinant CHO cell membrane and [^{3}H] LSD (5.67 nM) in an incubation buffer (50 mM Tris, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4). The plant extracts,

tested at 50 μg/mL, were considered active if binding was inhibited by 50% or more. After a 1 h incubation at 37 °C, the mixture was filtered over a 934-AH Whatman filter that had been presoaked in 0.5% polyethylenimine (PEI) and washed three times in ice-cold wash buffer (50 mM Tris buffer, pH 7.4) and simultaneously washed and aspirated five times using a 96-well Tomtec-Harvester (Orange, CT). Each filter was dried, suspended in Wallac microbeta plate scintillation fluid (PerkinElmer Life Sciences, Boston, MA), and counted with a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA). Serotonin (1 μM) was used to define nonspecific binding, which accounted for < 10% of total binding. The percent inhibition of [^{3}H] ligand bound to each 5-HT$_7$ receptor was determined using Equation 1.

$$\%\,\text{sample binding} = [1 - (\text{cpm}_{\text{sample}} - \text{cpm}_{\text{blank}})/(\text{cpm}_{\text{DMSO}} - \text{cpm}_{\text{blank}})] \times 100 \quad (1)$$

Serotonergic evaluation of N_{ω}-methylserotonin against the other 5-HT receptors was conducted by MDS Pharma (Bothell, WA) and performed in accordance with published assay protocols (28).

Intracellular cAMP Assay

The active samples in the 5-HT$_7$ binding assay were further tested at a concentration of 40 μg/mL of plant extract for modulation of intracellular levels of cyclic adenosine monophosphate (cAMP) to determine if the active components in the extracts acted as agonists or antagonists of the 5-HT$_7$ receptor. Extracts that induced at least 5 pmol/mL of cAMP at the tested concentration were considered to be agonists. The elevation of intracellular cAMP was assayed as previously described (29) with minor modifications. Briefly, human 5-HT$_7$-transfected HEK293 cells were grown for four days in serum-free media and then plated in poly-D-lysine-coated 12-well plates (100×10^4 cells/well) and left for 24 h. The following day, the cells were washed twice with 2 mL of incubation buffer (150 nM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, 500 μM isobutylmethylxanthine, 1 μM ascorbic acid, 10 μM pargyline, pH 7.4), and then incubated for 20 min at 37 °C. The compounds and extracts were added and the plates were incubated at 37 °C for 10 min. Forskolin (20 nM) and serotonin (10 nM) were used as positive controls. If stimulation could be blocked by co-incubation of serotonin with the 5-HT$_7$ receptor antagonist SB-269970, the treatment was considered receptor mediated. The reactions were terminated by aspirating the buffer and adding 0.1 M HCl with 0.1% Triton-X detergent to lyse the cells. The cells were collected in microcentrifuge tubes and centrifuged for 5 min at 1000 rpm at room temperature. The cell supernatant was obtained and stored at −80 °C until the treated samples were analyzed. An aliquot of the cell supernatant (100 μL) was transferred to the Trevigen HT Direct cyclic AMP EIA competitive immunoassay kit for the quantitative determination of cyclic AMP in treated cells as per the manufacturer's protocol (Trevigen Inc., Gaithersburg, MD). The plate was read immediately in a Powerwave 200 microplate spectrophotometer (BioTek Instruments, Winooski, VT) at 405 nm. The concentration of cAMP was determined by measuring the optical density (OD) minus the non-specific binding (NSB). The intensity of the yellow color was inversely proportional to the concentration of cAMP. The percentage of cAMP bound was determined using equation 2, based on the maximum available binding (B_{max}). The amount of cAMP was calculated using a standard curve, and the data were expressed as pmol/mL induction and compared to the basal cellular cAMP level observed.

$$\%\,\text{bound} = (\text{Net OD}/\text{Net } B_{\text{max}}\,\text{OD}) \times 100 \quad (2)$$

Selective Serotonin Reuptake Inhibition (SSRI) Assay

The SSRI assay was performed as previously described (30) with minor modifications. Briefly, 200 μL of poly-D-lysine solution (20 μg/mL) was added to each well of 24-well plates. After shaking the plates for 5 min, the solution was aspirated, and the plates were dried for 1 h. hSERT-HEK293 cells (15×10^4 cells/well) were plated in the pre-coated 24-well culture plates. After 24 h, the plates were washed with KRH buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM Hepes, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, pH 7.4), incubated with the extracts at 37 °C in KRH buffer containing D-glucose (1.8 g/L), L-ascorbic acid (100 μM), and pargyline (100 μM) for 10 min, and then treated with [^{3}H] 5-HT (20 nM). Fluoxetine (100 μM) was used as a positive control to validate the method. Cells were incubated for an additional 10 min. Uptake of [^{3}H] 5-HT was terminated by washing the plates three times with KRH buffer (30). After adding 200 μL of 1% SDS to each well, the plates were incubated at room temperature for 30 min while gently shaking. The cell solution was transferred to scintillation vials with 4 mL Cytoscint. The tubes were counted using a Beckman LS 5801 (Schaumburg, IL) liquid scintillation counter. The percent inhibition was determined using Equation 1 as previously described. All *C. racemosa* extracts were subjected to SSRI assays, at 40 μg/mL, and were considered active if inhibition was ≥ 50%.

Statistics

The results of the serotonin receptor binding, intracellular cAMP, and selective serotonin reuptake inhibition assays were all determined as the average ± standard deviation of at least two independent experiments in triplicate determinations. Data were analyzed using the GraphPad Prism Version 4.01 to determine IC_{50} and EC_{50} values. Significant difference for cAMP column statistics compared to DMSO control was determined using the one way ANOVA and one sample t-test. IC_{50} values provided by MDS Pharma were determined by a non-linear, least squares regression analysis using MathIQ (ID Business Solutions Ltd., UK).

RESULTS AND DISCUSSION

Bioassay-guided fractionation (Figure 2)

Since the serotonin receptor 5-HT_7 is involved in thermoregulation, bioassay-guided fractionation was performed using the 5-HT_7 competitive binding assay and the MeOH crude extract. The choice of MeOH as an extraction solvent was based on practical phytochemical considerations, including viscosity, evaporation handling, and limitations in the supply of the clinical extract. In addition, while 75% EtOH and 100% MeOH extracts are of similar polarity and can be mutually substituted from a phytochemical perspective, neat MeOH extracts contain less of the polar interfering substances, such as sugars. The decision to prepare a 75% EtOH extract for clinical studies was based on both biological (serotonergic activity, factors affecting *in vivo* studies) and phytochemical analyses previously detailed (18).

Our initial studies, using the previously mentioned 75% EtOH clinical extract, demonstrated serotonergic activity with an IC_{50} value of 55 μg/mL. The MeOH crude extract had similar serotonergic activity (IC_{50} 87 μg/mL). After separating the triterpenes and sugars from the crude MeOH extract by solvent partitioning, an enriched MeOH fraction (XAD-MeOH) was obtained by chromatography on the XAD column that exhibited more than ten-fold higher serotonergic binding activity compared to the clinical and crude MeOH black cohosh extracts (IC_{50} 5 μg/mL) (Figure 2). Both the clinical extract and XAD-MeOH fraction were also evaluated for functional serotonergic activity. The 5-HT_7 receptor is a G-protein coupled receptor that stimulates adenylate cyclase. Therefore, agonist occupancy of the

receptor dissociates the G_α subunit of the G-protein and activates adenylate cyclase to catalyze the formation of cAMP (15). Forskolin, a compound known to stimulate all types of adenylate cyclase, and serotonin were used as positive controls. Co-incubation of serotonin with the 5-HT_7 receptor antagonist SB-269970 could block this stimulation; however, the activation caused by forskolin could not be completely blocked. Therefore, cAMP production can be used to characterize serotonergic regulators with potential agonistic or antagonistic properties at functional 5-HT_7 receptors. A dose-dependent response was observed for both the clinical extract and the XAD-MeOH fraction in the HEK293 5-HT_7 receptor cell line (Figure 3), although the XAD-MeOH fraction was found to induce significantly more cAMP. The clinical extract and enriched XAD-MeOH fraction were also tested for SSRI activity. The SSRI assay was used to evaluate whether the tested plant extracts or compounds could target the SERT and inhibit serotonin reuptake employing hSERT-transfected-HEK293 cells. Fluoxetine was used as a positive control to validate the method. These black cohosh fractions showed no ability to inhibit the reuptake of serotonin (data not shown).

Pure compounds belonging to two major phytoconstituent classes of black cohosh (Figure 1) were also evaluated in the 5-HT_7 competitive binding, cAMP induction, and SSRI assays to determine whether or not they are serotonergically active and if the serotonergic activity of the black cohosh extract is affected by their presence. Seventeen individual triterpenes, isolated as previously described (18, 21, 22), were tested in the 5-HT_7 competitive binding assay at 50 μM (Table 1). The triterpenes tested displayed no significant activity for binding to the 5-HT_7 receptor. For example, 23-*epi*-26-deoxyactein (also known as 27-deoxyactein) (31), 26-deoxyactein, and actein (*R,S*), which are among the most abundant triterpenes in *C. racemosa* (Figure 1) and commonly used markers for chemical standardization of black cohosh dietary supplements, exhibited IC_{50} values ≥ 100 μM (Figure 2). Cimicifugic acids A, B, E, and F (Figure 1), being characteristic and abundant constituents in *Cimicifuga* species, all had IC_{50} values greater than 500 μM, while fukinolic acid had an IC_{50} value of 100 μM (Figure 2). None of the 17 triterpenes (Table 1) induced cAMP activity (data not shown). The cimicifugic and fukinolic acids also failed to increase the production of cAMP and did not inhibit serotonin reuptake (data not shown). The triterpenes were not tested in the SSRI assay due to limited compound availability. Alkaloids, tannins, and flavonoids are among the other main constituents found in black cohosh (21, 22, 32–35). It was previously shown that antioxidant compounds (methyl caffeate, ferulic acid, caffeic acid, cimicifugic acids A and B, fukinolic acid, and cimiracemate A and B) and cimipronidines were contained in various extracts of black cohosh (32, 36) which may have chemopreventive properties (36); however, these compounds are unlikely to be responsible for the serotonergic activity of black cohosh.

Identification of N_ω-methylserotonin

Since none of the previously isolated pure compounds had serotonergic activity, bioassay-guided fractionation was pursued with the XAD-MeOH fraction (Figure 2), mainly composed of phenolic compounds without triterpenes and sugars. All seven fractions resulting from subsequent FCPC separation were evaluated for 5-HT_7 receptor binding. Fractions 2 and 3 contained cimipronidine alkaloids and had low receptor binding activity. Fraction 4 contained the polyphenolic acids which exhibited weak 5-HT_7 receptor binding activity (Figure 2, Table 1, IC_{50} ~ 100 μg/mL) and no functional activity (data not shown). Fraction 7 showed enhanced 5-HT_7 binding activity (Figure 2, IC_{50} < 2.5 μg/mL). Positive ion electrospray LC-MS analysis of this active fraction revealed the presence of an abundant protonated molecule of *m/z* 191 eluting at 22 min (Figures 4A and 4B). Accurate mass measurements determined an elemental composition of $C_{11}H_{14}N_2O$ (− 5.4 ppm). A database search (CrossFire Commander) revealed only 13 compounds with this formula, one of which

was N_ω-methylserotonin. The positive ion product and tandem mass spectrum of this compound and the corresponding compound in fraction 7 (Figures 4C and 4D) exhibited a prominent loss of methylamine (31 mass units) from the precursor ion, indicating the presence of a secondary amine in the structure. Of all the compounds in the database, only N_ω-methylserotonin was consistent with these analytical data. Confirmation was obtained by establishing that the retention time and fragmentation pattern of this compound in fraction 7 were identical to those of the authentic N_ω-methylserotonin (Figure 4). The XAD-MeOH fraction 7 and N_ω-methylserotonin were also analyzed using an isocratic HPLC method and LC-MS based on a Zorbax Eclipse C_{18} column, as well as the gradient HPLC method with LC-MS-MS based on a YMC ODS column (data not shown) leading to the unambiguous identification of N_ω-methylserotonin as a new *C. racemosa* phytoconstituent.

To our knowledge, this is the first report that N_ω-methylserotonin has been identified in *C. racemosa* roots/rhizomes. Extracts from another species, *Ranunculus sceleratus* L., (cursed buttercup, marsh-crowfoot), belonging to the same family as black cohosh (Ranunculaceae), also contains serotonin and other tryptamine analogues (49, 50). Due to the very low levels of N_ω-methylserotonin in the black cohosh extract, isolation of the target compound was not attempted. A fully characterized sample of N_ω-methylserotonin, analyzed by MS, 1D and 2D NMR, and qHNMR for purity evaluation, was used in the bioassays. In the black cohosh extract, N_ω-methylserotonin is present at very low levels (~30 ppm) that do not allow detection by NMR due to the relatively low sensitivity and dynamic range of the method. Mass spectrometry was used in this instance as a well-established method for the detection and identification of known compounds present in complex mixtures at low levels. In this study we employed an approach that is widely accepted not only in pharmaceutical industry, but also in the chemical industry and forensics and regulatory area. This approach includes determination of molecular composition by accurate mass measurement combined with the comparison of retention times and fragmentation patterns of the test compound with the authentic standard. For the purpose of comparison of retention characteristics, we employed two different chromatographic modes (normal vs. reversed phase and two different reversed phase columns and elution systems), which further strengthens the identification of N_ω-methylserotonin as the active principle.

Serotonergic and SSRI activity of N_ω-methylserotonin

N_ω-methylserotonin was evaluated in the 5-HT_7 receptor binding assay, and it revealed a potent binding affinity, IC_{50} 23 pM (Figure 5). To test selectivity, N_ω-methylserotonin was also screened against all the known serotonin receptors (MDS Pharma). N_ω-methylserotonin showed a higher selectivity for the 5-HT_1, 5-HT_6, and 5-HT_7 subfamilies (Table 2), particularly the 5-HT_{1A} and 5-HT_7 receptors, and the least selectivity for the 5-HT_3 and 5-HT_4 receptors which do not appear to be associated with thermoregulation. In general, the addition of the methyl group to the omega nitrogen appears to increase the selectivity for most of the serotonin receptors when compared to serotonin itself. In the cAMP induction assay, N_ω-methylserotonin demonstrated a strong ability to increase intracellular cAMP levels (Figure 6). cAMP formation could be reversed by co-incubation with the 5-HT_7 antagonist, SB 269970 (Figure 6A), which indicated the process was receptor mediated. The EC_{50} was determined to be 22 nM, and the maximum cAMP induction (43 pmol/mL) was observed at 100 nM, the highest tested concentration. Additionally, N_ω-methylserotonin showed strong SSRI activity, IC_{50} 490 nM, in this assay (Figure 7) compared to the SSRI antidepressant, fluoxetine hydrochloride (Prozac, IC_{50} 150 nM). While the fluoxetine and N_ω-methylserotonin SSRI activities appear to be of a comparable level, the black cohosh extracts failed to exhibit any SSRI activity.

Quantitative analysis of the clinical extract also indicated the presence of N_ω-methylserotonin and the amount was determined to be 0.0031% by weight of the excipient-

free extract. Thus N_ω-methylserotonin was independently identified in both a 75% EtOH and a 100% MeOH extract suggesting that methylation of serotonin was not an artifact of the extraction process. Further analysis found 100 μg/mL of the clinical extract would contain approximately 16 nM of N_ω-methylserotonin. At this concentration, cAMP induction is expected to be 17 pmol/mL; however, less than half of that amount was found to be induced in the clinical extract (Figure 3) probably due to the fact that other phytoconstituents in the extract may modulate the activity. In contrast, the XAD-MeOH fraction induced twice the amount of cAMP expected at 100 μg/mL (Figure 3).

Black cohosh has become a very popular alternative therapy for alleviating menopausal symptoms, particularly hot flashes (1, 4, 9). However, many questions about its efficacy as well as mechanism(s) of action or active constituent(s) remain unclear. *In vitro* and *in vivo* experiments designed to determine the mechanism(s) of action found that black cohosh does not seem to work through classical estrogenic pathways (7, 12, 13). For example, an *in vivo* animal study using ovariectomized rats found that black cohosh failed to maintain consistent body weight, did not increase uterine weight, and did not lead to cell differentiation of the vaginal tract, all of which are markers of estrogenic activity (14). Since black cohosh exhibited no estrogenic or antiestrogenic activity *in vivo*, an alternative serotonergic mechanism linked to thermoregulation (14) was examined. In support of this hypothesis, several Phase II and III clinical trials have found that SSRIs were able to reduce hot flashes in breast cancer survivors treated with tamoxifen, raloxifene, aromatase inhibitors, soy products, and/or vitamin E (2, 37). Additional studies have also shown that SSRIs have an affinity to the 5-HT_{1A} receptor, resulting in its downregulation (38, 39). As a result, serotonergic pathways could play a role in black cohosh mediated thermoregulation, although the precise mechanism of action remains unclear. In addition to serotonergic pathways, it is possible that dopaminergic (41–43) and opiate systems (44, 45) play a role in mitigation of hot flashes. Similarities exist between hot flashes experienced as a result of going through menopause or opiate withdrawal (46), which is why opiate-dependent animal models have been used to evaluate hot flashes *in vivo* (47). It has been reported that black cohosh extracts behave as competitive ligands and partial agonists for opiate receptors (44, 48) similar to serotonin receptors (14). A competitive binding assay using a CHO cell line stably transfected with human μ opioid receptors and the same clinical extract used in this study gave an IC_{50} of 170 μg/mL (44) compared to 55 μg/mL for the 5-HT_7 receptor. Although direct comparisons between the opiate experiments and the serotonergic studies are difficult, these data suggest at least two relevant activities are found in black cohosh and both may play a role in thermoregulation of hot flashes.

In summary, the *in vitro* serotonergic activity of black cohosh extracts appears to be mediated by N_ω-methylserotonin. N_ω-methylserotonin was active in both the 5-HT_7 receptor and the SERT pathways, both of which have been shown to affect thermoregulation and can increase serotonin activity. N_ω-methylserotonin displayed 5-HT_7 receptor binding activity at pM levels, cAMP induction activity at nM levels, and SSRI activity in the μM range. Therapeutically, a 120 mg daily dose of the black cohosh clinical extract would contain approximately 3.7 μg of N_ω-methylserotonin. The current *in vitro* studies suggest that N_ω-methylserotonin could be responsible for *in vivo* serotonergic activity, although further studies are in progress to evaluate oral bioavailability. The data have also shown that while triterpenes and *Cimicifuga* polyphenols are suitable markers for chemical standardization, they do not correlate with the serotonergic activity. It is possible that effective black cohosh extracts, beneficial for relieving menopausal symptoms, should be biologically standardized to compounds that show serotonergic activity, such as N_ω-methylserotonin. However, further studies are necessary and ongoing in our laboratory, and will be reported in due course.

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ABBREVIATIONS USED

B_{max}	maximum available binding
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CoA	certificate of analysis
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
FCPC	fast centrifugal partition chromatography
HEK	human embryonic kidney
HRT	hormone replacement therapy
hSERT	human serotonin transporter
KRH	Krebs/Ringer/Hepes
LSD	lysergic acid diethylamide
MeOH	methanol
***N*-methylserotonin**	3-(2-Methylaminoethyl)indol-5-ol
NSB	non-specific binding
OD	optical density
PEI	polyethylenimine
qHNMR	quantitative ^{1}H NMR
SB-269970	(*R*)-3-[2-[2-(4-Methylpiperidin-1-yl)ethyl]pyrrolidine-1-sulfonyl]phenol hydrochloride
SERT	serotonin transporter
SSRI	selective serotonin reuptake inhibitor
TIC	total ion chromatogram
5-HT	5-hydroxytryptamine, serotonin, 3-(2-Aminoethyl)indol-5-ol

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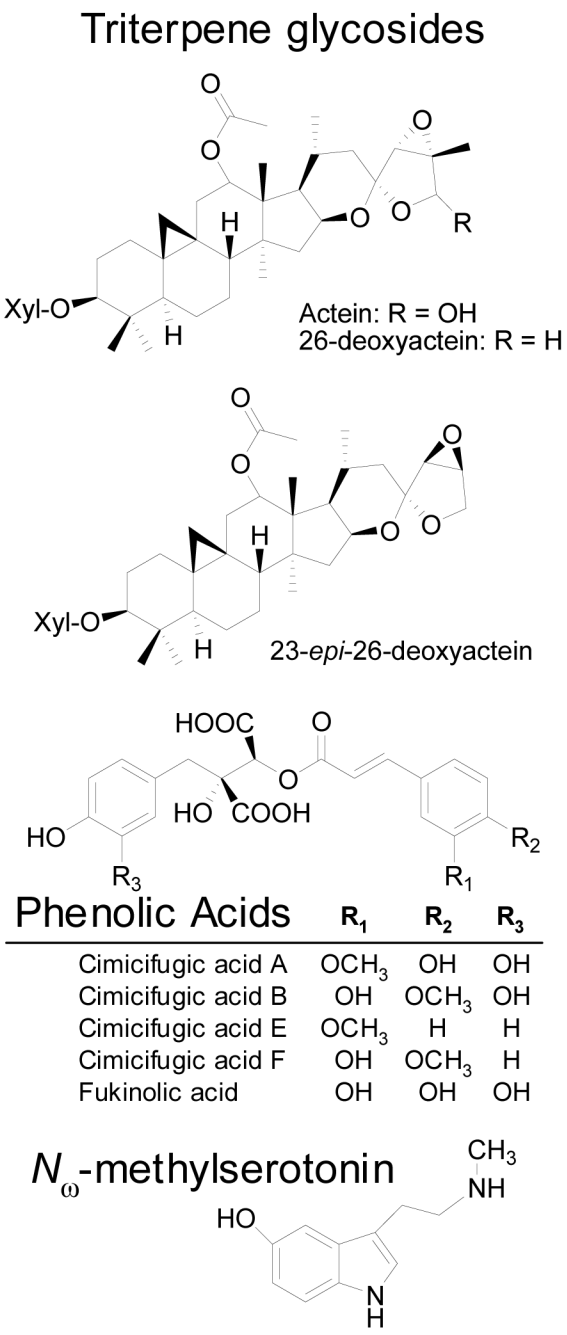

Figure 1.
Key phytoconstituents of black cohosh.

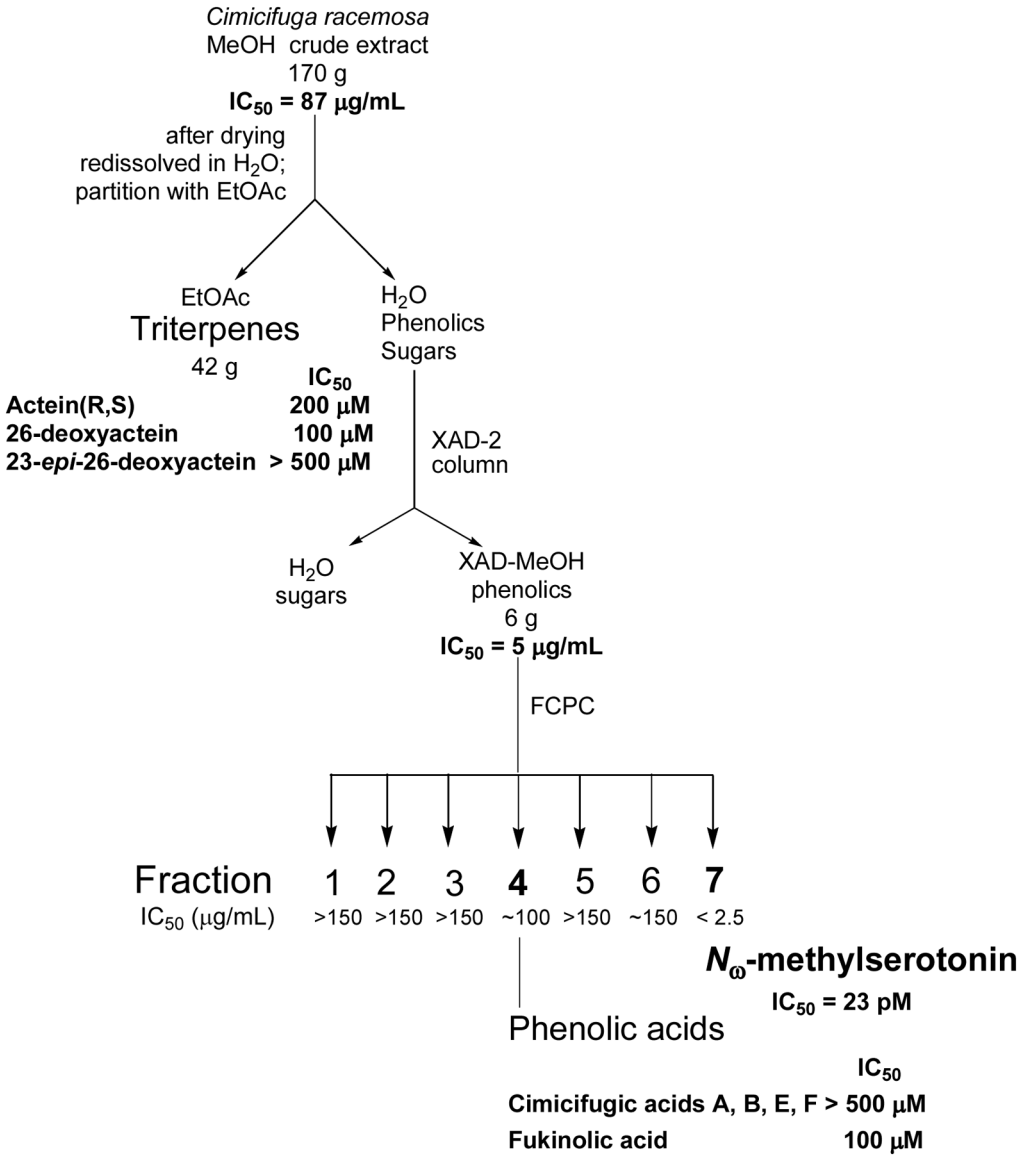

Figure 2. Bioassay guided fractionation scheme

The isolation scheme following the fractionation and subfractionation of the crude MeOH extract. The clinical extract (75% EtOH) had an IC_{50} of 55 μg/mL. The structures of the most relevant triterpenes, phenolic acids, and N_{ω}-methylserotonin are shown in Figure 1.

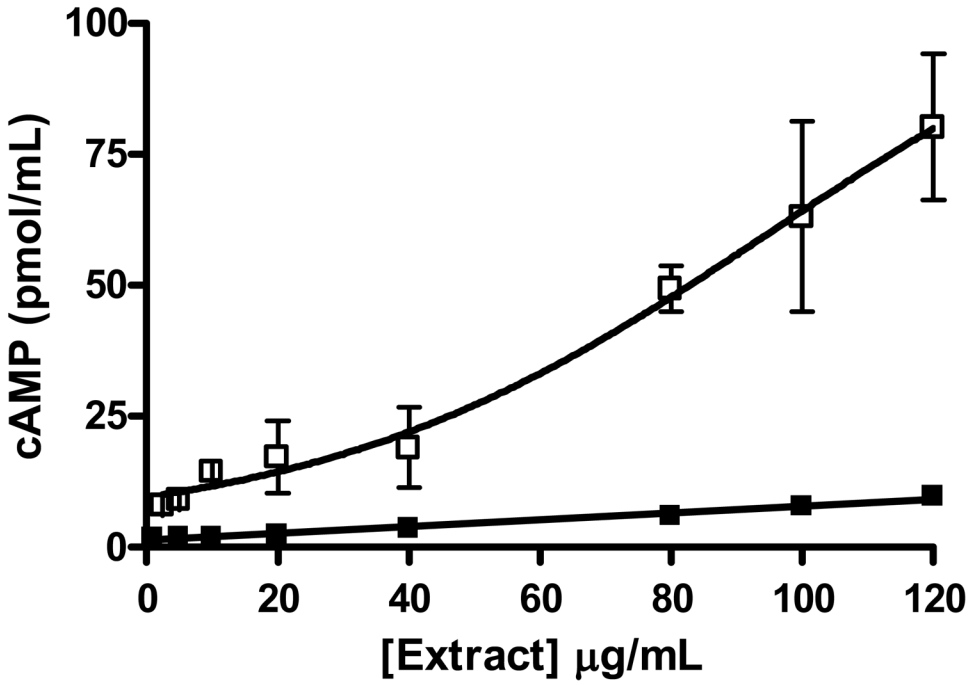

Figure 3. Black cohosh induces intracellular cAMP

Dose-dependent induction of cyclic AMP (pmol/mL) for the clinical black cohosh extract (■) and XAD-MeOH extract, EC_{50} 70 μg/mL (□) mediated through the 5-HT_7 receptor.

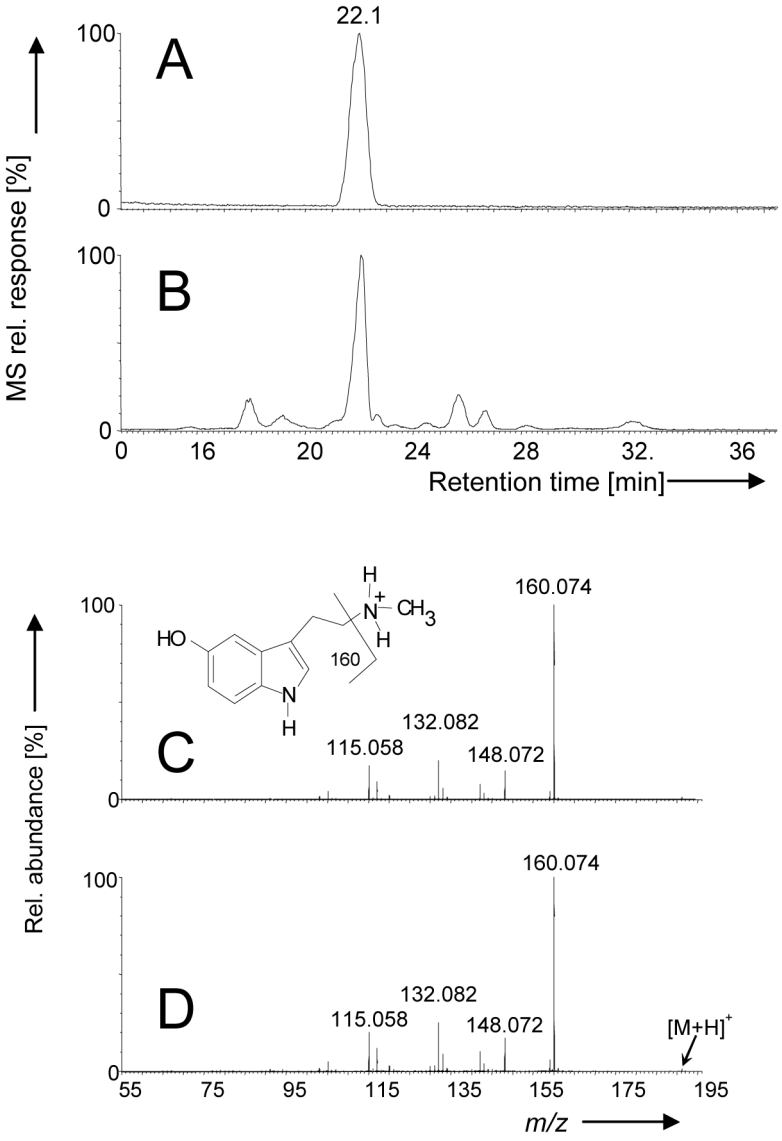

Figure 4. Positive ion electrospray LC-MS analysis and tandem mass spectra of fraction 7 and N_{ω}-methylserotonin

Total ion chromatograms of A) N_{ω}-methylserotonin standard. B) Fraction 7. A comparison of the product ion tandem mass spectra of N_{ω}-methylserotonin and a compound from fraction 7 eluting at 22 min. The major fragment ion corresponds to the loss of methylamine from the side chain. C) N_{ω}-methylserotonin standard. D) Black cohosh fraction 7.

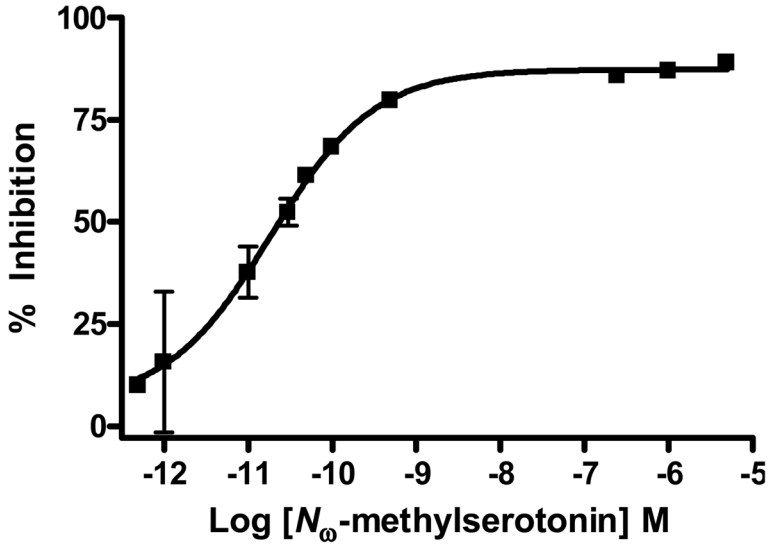

Figure 5. ***N*$_\omega$-methylserotonin can bind to the 5-HT$_7$ receptor**

Percent inhibition of 5-HT$_7$ receptor binding with increasing doses of N_ω-methylserotonin. IC_{50} 23 pM.

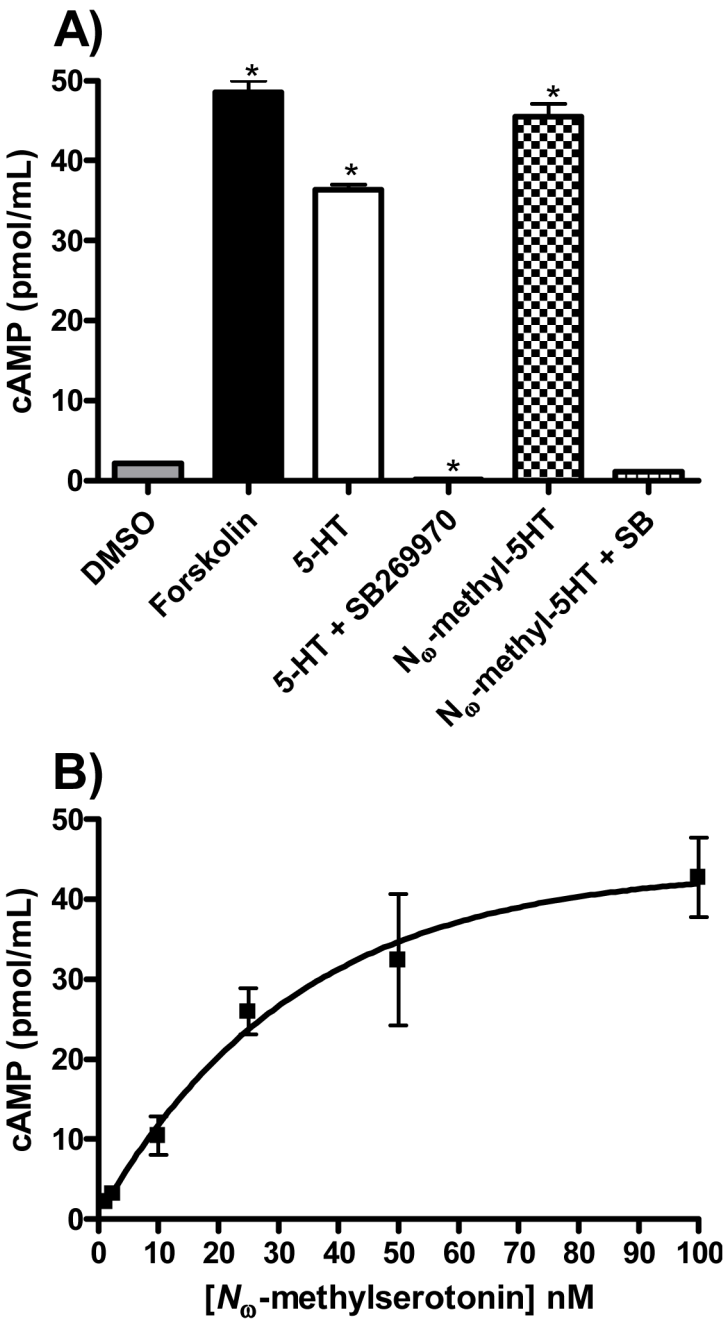

Figure 6. N_{ω}-methylserotonin induces intracellular cAMP

(A) Cyclic AMP intracellular levels of human 5-HT_7 transfected HEK293 cells after treatment with N_{ω}-methylserotonin (100 nM). (B) Intracellular cyclic AMP (pmol/mL) as a response of increasing dosage from N_{ω}-methylserotonin mediated through the 5-HT_7 receptor. *$p < 0.05$.

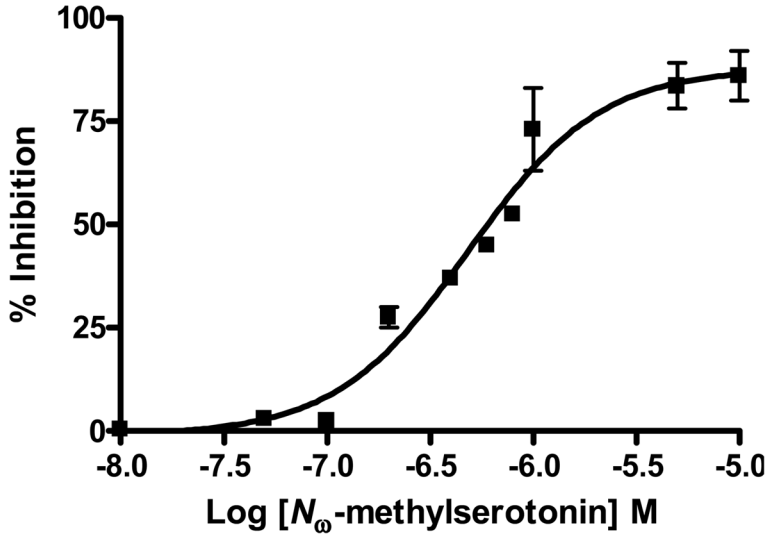

Figure 7. N_{ω}-methylserotonin exhibits SSRI activity

Dose-dependent percent inhibition of selective serotonin reuptake with N_{ω}-methylserotonin. The IC_{50} value was determined to be 490 nM.

Table 1

5-HT_7 Competitive Binding Inhibition of Triterpenes Tested at 50 μM. (NA – not active)

5-HT7 competitive binding	
Compound (50 μM)	**% Inhibition ± SD**
23-*epi*-26-deoxyactein	NA
23-O-acetylshengmanol-3-β-D-arabinoside	NA
25-anhydrocimigenol-3-O- β-xyloside	31 ± 8
26-deoxyactein	38 ± 4
Cimiracemoside P	NA
Cimiracemoside J	NA
Cimiracemoside K	NA
25-O-acetylcimigenol-3-O-β-xyloside	NA
25-O-acetylcimigenol-3-O-α-arabinoside	NA
25-anhydrocimigenol-3-O- β-arabinoside	NA
Actein (R,S)	NA
23-O-acetylshengmanol-3-β-D-xyloside	35 ± 10
Cimiracemoside N	18 ± 4
Cimiracemoside O	19 ± 5
Cimiracemoside L	NA
Cimicifugoside H-2	NA
Cimifugoside H-1	NA

Table 2

Selectivity of N_{ω}-methylserotonin Binding to Serotonin Receptor Subtypes.

	IC50 [nM]		
Receptor	**N_{ω}-methylserotonin[a]**	**Serotonin[c]**	**Positive controls[c]**
5-HT1A	2	1.9	Metergoline: 4
5-HT1B	15	19	Serotonin: 19
5-HT2A	--	410	Ketanserin: 0.65
5-HT2B	97	55	Ketanserin: 290
5-HT2C	101	67	Mesulergine: 0.7
5-HT3	1670	700	MDL-72222: 11
5-HT4	6700	300	R,S-235970-190: 90
5-HT5A	289	100	Methiothepin: 2.9
5-HT6	55	340	Methiothepin: 2.8
5-HT7	0.02[b]	0.86	Methiothepin: 0.4

[a]The results represent primary screening data (three points with duplicate determinations) tested at MDS Pharma, Bothell, WA, except for the binding data with the 5-HT7 receptor.

[b]These represent results performed in triplicate in three independent experiments.

[c]The results represent MDS Pharma results in accordance with published assay protocols.