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Absolute Configuration and *in vitro* Pharmacology of the Poison Frog Alkaloid Phantasmidine

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Abstract

Phantasmidine, a rigid congener of the well-known nicotinic acetylcholine receptor agonist epibatidine, is found in the same species of poison frog (*Epipedobates anthonyi*). Natural phantasmidine was found to be a 4:1 scalemic mixture, enriched in the (2*aR*,4*aS*,9*aS*) enantiomer by chiral-phase LC-MS comparison to the synthetic enantiomers whose absolute configurations were previously established by Mosher's amide analysis. The major enantiomer has the opposite *S* configuration at the benzylic carbon to natural epibatidine, whose benzylic carbon is *R*. Pharmacological characterization of the synthetic racemate and separated enantiomers established that phantasmidine is ~10-fold less potent than epibatidine, but ~100-fold more potent than nicotine in most receptors tested. Unlike epibatidine, phantasmidine is sharply enantioselective in its activity and the major natural enantiomer whose benzylic carbon has the 4*aS* configuration is more active. The stereoselective pharmacology of phantasmidine is ascribed to its rigid and asymmetric shape as compared to the nearly symmetric conformations previously suggested for epibatidine enantiomers. While phantasmidine itself is too toxic for direct therapeutic use, we

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Conflict of Interest Disclosure

Indiana State University and NIH jointly hold a patent on phantasmidine derivatives (Fitch, R. W.; Spande, T. F.; Garraffo, H. H.; Yeh, H. J. C.; Daly, J. W. Nicotinic Receptor Agonists, US 20130281482 A1 and subsequent issuances). Brandeis University holds a patent on the synthesis of phantasmidine derivatives (Snider, B. B.; Zhou, Q. Preparation of Phantasmidine and Analogues Thereof, WO 2012078608 A1).

Supporting Information

Chromatographic and mass spectrometric data for **1** and **2a**; crystallographic data and CIF file for (1*R*,2*R*,4*S*)-epibatidine L-tartrate. The Supporting Information is available free of charge on the ACS Publications website.

CCDC 1562414 contains the supplementary crystallographic data for this paper ((1*R*,2*R*,4*S*) Epibatidine L-Tartrate). These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

believe it is a useful platform for the development of potent and selective nicotinic agonists which may have value as pharmacological tools.

Graphical Abstract

The skin secretions of brightly colored poison frogs have been the source of over 800 alkaloids, many of which are neuroactive.¹ In 2010 we described the isolation and structure elucidation of phantasmidine (1),² a tetracyclic chloropyridine alkaloid closely related to the well-known epibatidine (2a)³ from the Ecuadoran poison frog, *Epipedobates anthonyi* (formerly *E. tricolor*). Epibatidine is a highly potent and selective nicotinic acetylcholine receptor (nAChR) agonist^{4,5} that has been used extensively for pharmacological characterization of nAChR since its isolation in 1992.

Nicotinic acetylcholine receptors (nAChR) are a subset of the cysteine-loop class of ligand-gated ion channels, which include GABA and 5-HT₃ receptors and are important

neurotransmitter receptors in the central and peripheral nervous system.⁶⁻¹⁰ At present there are at least 15 described nAChR subtypes derived from 17 genes encoding subunits thereof (10 α , 4 β and one each of γ , δ or ϵ).⁸ These receptors are involved in a variety of neuromuscular, ganglionic and central neuronal responses⁸ and nAChR dysfunction has been linked to anxiety, addiction, depression, neurodegeneration and certain epilepsies.⁸⁻¹⁰ In order to dissect the functional roles of nAChR, chemical probes are needed with selective activity at individual nAChR subtypes. While numerous synthetic compounds have been developed for subtypes of these receptors, there remains a significant need for highly selective ligands (particularly agonists).^{8,11} Nature is well-suited for this purpose, being a major source of active compounds, often with unforeseen structures and/or mechanisms of action.¹²⁻¹⁴

Based on its structural similarity to epibatidine (**2a**) and preliminary pharmacological evidence obtained during isolation, phantasmidine (**1**) is a promising nAChR ligand. However, it was isolated in quantities far too small (20 μ g) for detailed pharmacological characterization or assignment of absolute configuration.² A synthesis of racemic **1** and resolution by chiral-phase HPLC provided milligram quantities of **1** and *ent*-**1** whose absolute configurations were established by Mosher's amide analysis.^{15,16} With these materials in hand, we determined the absolute configuration of the natural product and investigated nicotinic receptor binding and function which we report here.

Results and Discussion

Chiral-Phase LC-MS and Configurational Analysis.

The absolute configuration of natural phantasmidine (**1** or *ent*-**1**) was not previously determined owing to the tiny amount isolated. However, it was assumed that it would mimic that of epibatidine (**2a**).² The subsequent synthesis, resolution¹⁵ and configurational assignment¹⁶ of the enantiomers of **1** provided material that could be used to establish the absolute configuration of natural **1** by chiral-phase chromatographic comparison. Chiral reverse-phase UHPLC separation of the enantiomers of **1** gave retention times of 4.2 min for (2a*R*,4a*S*,9a*S*)-**1** and 8.5 min for (2a*S*,4a*R*,9a*R*)-**1** (Supporting Information Figures S3-S4).

For assignment of the natural product configuration, a sample of the original extract from which **1** was isolated was used. (Figure 1).^{2,17} This mixture contained epibatidine (**2a**) and *N*-methylepibatidine (**2b**) in addition to small amounts of **1**. Furthermore, **1** and **2b** have the same nominal mass with $[M+H]^+$ at m/z 223/225 for the ³⁵Cl and ³⁷Cl isotopomers, respectively. However, high resolution ESI+ of **1** calculates to m/z 223.0633 for $[^{12}\text{C}_{11}\text{H}_{12}^{35}\text{ClN}_2\text{O}]^+$ and **2b** calculates to m/z 223.0997 for $[^{12}\text{C}_{11}\text{H}_{16}^{35}\text{ClN}_2]^+$, which can be clearly distinguished. Experimental spectra bore this out and the assignments were further supported by mass measurement of all four ³⁵Cl/³⁷Cl and ¹²C/¹³C isotopic peaks for each compound (Supporting Information Table S1 and Figures S2-S6).

Examination of the mass chromatograms and spectra showed unambiguously that the major enantiomer of phantasmidine is the earlier eluting (2a*R*,4a*S*,9a*S*)-isomer **1**. To our surprise, natural phantasmidine is not a single enantiomer, but an approximately 4:1 scalemic mixture of **1** and *ent*-**1** (62.5% ee, Figures S6-S8) eluting at 4.2 and 8.5 min, respectively. On the

other hand, natural epibatidine (**2a**) was previously reported to be a single enantiomer having the (1*R*,2*R*,4*S*)-configuration (see Supporting Information).¹⁸⁻²⁰ Unfortunately, racemic epibatidine could not be completely resolved on this column under any of the conditions tried. However, X-ray analysis of resolved **2a** (as the (2*R*,3*R*)-L-(+)-tartrate salt) and chiral-phase gas chromatography of the N-acetyl derivative **2c**³ on a permethylated cyclodextrin column showed the natural enantiomer to have the (1*R*,2*R*,4*S*)-configuration, consistent with the literature assignment (Supporting Information).¹⁸⁻²¹

The biosynthetic pathways to **1** and **2a** are unknown. The originating organism is presumed to be arthropod prey as frogs raised in captivity contain no alkaloids.^{1,11,22} Natural phantasmidine is a 4:1 mixture of enantiomers that cannot interconvert by epimerization of the three individual stereocenters. Thus, phantasmidine biosynthesis may proceed through an achiral intermediate that could be transformed in a chiral environment to the observed scalemic mixture. However, as the source of these alkaloids is unknown, this remains an open question.

***In Vitro* Pharmacological Evaluation.**

Having the racemate and both enantiomers of **1** in hand, we set about profiling their nAChR pharmacology.

Affinity at nAChR.

Racemic phantasmidine (**1**) and the separated enantiomers were first evaluated for affinity using [³H]-epibatidine binding²³ in membranes from rat forebrain and cell lines expressing various combinations of rat nicotinic receptor subunits.^{24,25} The results are shown in Table 1. Racemic **1** has significantly lower binding affinities than epibatidine (**2a**) at $\alpha 3\beta 4$ and $\alpha 4\beta 2$ receptor subtypes (50-fold and 10-fold respectively) as well as at native receptors in rat forebrain (25-fold). In contrast, the affinity of **1** at the $\alpha 7$ subtype is only 2-fold less than **2a**. Further, the affinity of the enantiomers are markedly different, with the major natural enantiomer (2*aR*,4*aS*,9*aS*)-**1** being the eutomer, with 30-44-fold higher affinity than the distomer *ent*-**1**. It is also important to note that racemic **1** and the two enantiomers have at least 30-fold higher affinities for $\alpha 4\beta 2$ over $\alpha 3\beta 4$ receptors. This was somewhat surprising as natural **1** initially appeared to show $\beta 4$ -functional selectivity.² To evaluate this, these synthetic compounds were tested for functional activity using radioisotopic based ion-flux assays and electrophysiology.

Function – Rubidium Ion Efflux.

Racemic **1** was first tested using ⁸⁶Rb efflux in transfected cells expressing subunits for two of the nAChR subtypes used for binding ($\alpha 3\beta 4$ and $\alpha 4\beta 2$) which represent the major peripheral and central receptor subtypes, respectively.²⁵ The results given in Table 2 indicate that racemic **1** is a very potent partial agonist for $\alpha 4\beta 2$ receptors with an EC₅₀ of 0.20 μ M and 42% efficacy relative to 100 μ M nicotine. In $\alpha 3\beta 4$ receptors the racemate is also quite potent and a near-full agonist with an EC₅₀ of 0.75 μ M and 91% efficacy. The differences in functional potency vs **2a** are less pronounced than those for affinity being 18-fold for $\alpha 3\beta 4$ and 8-fold for $\alpha 4\beta 2$ receptors. Racemic **1** has differing enantioselectivity in the two subtypes, but as with affinity profiles, the eutomer is the (2*aR*,4*aS*,9*aS*)-**1**. In $\alpha 3\beta 4$ receptors

the eudismic ratio essentially mirrors that for binding affinity at 47, while in $\alpha 4\beta 2$ receptors the eudismic ratio is a substantial 280. (2a*R*,4a*S*,9a*S*)-**1** is essentially equally potent and efficacious with the racemate, while (2a*S*,4a*R*,9a*R*)-*ent*-**1** is of much lower potency (56 μ M) but still a full agonist. This may be ascribed to the difference in the functional (resting) state vs the high-affinity desensitized state observed in radioligand binding assays.²⁷

Function – Electrophysiology.

Racemic **1** and the two enantiomers were further evaluated by electrophysiology in *Xenopus* oocytes expressing rat nicotinic $\alpha 7$, $\alpha 3\beta 2$, or $\alpha 4\beta 2$ receptors (Table 3 and Figure 2).²⁸ As epibatidine (**2a**) is also known to have activity at serotonin receptors,²⁹ 5-HT_{3A} receptors were included as well.

For $\alpha 7$ nAChR, racemic **1** is a full agonist vs acetylcholine with an EC₅₀ value of 9.9 μ M, while for the $\alpha 3\beta 2$ and $\alpha 4\beta 2$ nAChR, it is a partial agonist with very high selectivity (3200-fold) for the latter. For 5-HT_{3A} receptors, the racemate is also a partial agonist vs *meta*-chlorophenylbiguanide (mCPBG), but had slightly lower potency than for $\alpha 3\beta 2$ and $\alpha 7$ nAChR. Like the racemate, both enantiomers of **1** are full agonists for the $\alpha 7$ receptor and highly selective partial agonists for $\alpha 4\beta 2$. (2a*R*,4a*S*,9a*S*)-**1** is again the eutomer. The eudismic ratio is 31 for $\alpha 4\beta 2$, but only 5 for $\alpha 7$.

Toxicology.

Finally, **1** was evaluated for toxicity in Swiss-Webster mice.³⁰ The racemate and individual enantiomers showed significant toxicity, but were sharply enantiodiscriminant with a eudismic ratio of around 100. The racemate has a highly variable LD₅₀ of 270 $\pm$ 190 μ g/kg. The LD₅₀ of (2a*R*,4a*S*,9a*S*)-**1** is 72 $\pm$ 14 μ g/kg, while *ent*-**1** is much less toxic (LD₅₀ >10 mg/kg). Surprisingly **1** has a very narrow, almost all or nothing response. A dose of 40 μ g/kg of the eutomer results in piloerection, slight elevated respiration and complete recovery in less than 1 min, whereas 50 μ g/kg gives piloerection, elevated respiration, hyperactivity (running) progressing to tonic clonic seizures and death in less than 30 sec. Because of sample limitations a confident LD₅₀ could not be obtained for *ent*-**1**. However, the highest dose (10 mg/kg) of the distomer produces similar effects to the eutomer in affected animals. Racemic **2a** was found to have an LD₅₀ of 1.5 $\pm$ 0.3 μ g/kg. The observed signs of toxicity for epibatidine and phantasmidine were similar, consistent with a common mechanism of action and the LD₅₀/EC₅₀ ratios are similar for the two compounds with (2a*R*,4a*S*,9a*S*)-**1** being 48-fold less toxic than racemic **2a**. For reference, the LD₅₀ values in mouse are reported to be 300 μ g/kg for nicotine and 1730 μ g/kg for cytosine, with similar time courses of action.³¹

Structure-Activity Relationships Between Phantasmidine and Known nAChR Ligands.

At nAChR, epibatidine (**2a**) lacks stereoselectivity with eudismic ratios between 1 and 5.^{4,5,32,33} Observing natural **2a** near one of its two lowest energy conformations (Figure 3), it is apparent that a near bilateral symmetry exists with few steric differences on either side of the plane as was noted when the activity of the enantiomers of **2a** was initially reported.^{5,34} This conformation is generally observed in docked models of epibatidine with nicotinic receptors and in an X-ray structure with an acetylcholine binding protein (2BYQ).³⁵⁻³⁷ On

the other hand, **1** is significantly enantioselective with eudismic ratios averaging 30-50 and its structure lacks any significant bilateral symmetry. Steric interactions (2-5 kcal/mol) between the hydrogens on the azabicyclic and pyridine of **2a** disfavor it occupying the conformation represented by **1**, though favorable interactions in a binding pocket could perhaps compensate for the energetic penalty. However, the conformation of **1** is held rigidly in place by the dihydrofuran ring junction, preventing its rotation into the preferred conformational space occupied by **2a**. Thus the two represent complementary spatial probes of nAChR binding sites. While ~10-fold less potent than **2a**, **1** remains highly potent at nAChR (~100-fold greater than nicotine) and is a selective partial agonist for $\alpha 4\beta 2$. This profile is quite similar to varenicline (Chantix, **4**)³⁸ and the naturally occurring *Laburnum* alkaloid cytisine (**5**),³⁹ both of which are used clinically for smoking cessation. This similarity is borne out when the structures and molecular electrostatic potential maps are compared (Figure 3) and are consistent with results previously reported.^{35,40} Of course, the toxicity of **1** makes it more of a lead compound or probe than a viable clinical candidate.

Conclusions

Phantasmidine (**1**), a rigid structural relative of epibatidine (**2a**), is a highly potent nicotinic receptor agonist. Natural phantasmidine is not a single enantiomer, but an approximately 4:1 mixture of **1** and *ent*-**1** and questions remain as to its biosynthetic origin. Structurally and pharmacologically distinct from **2a**, **1** is a potent and selective partial agonist for $\alpha 4\beta 2$ nAChR. While **1** is too toxic to be a viable candidate for the clinic, the rigid scaffold of **1** and its enantioselectivity make it a useful lead compound for the design and synthesis of selective nAChR probes.

Experimental Section

General Experimental Procedures.

All reagents and solvents were purchased commercially and used as received. All LC and GC solvents were HPLC grade or higher. Phantasmidine (**1**) was synthesized as the racemic free base and resolved as described previously.¹⁵ Enantiomers were separated on Chiralcel OJ-H or Chiralpak AD (Daicel, Japan) columns with the same elution order. The earlier eluting, more biologically active enantiomer **1** was assigned the (2*aR*,4*aS*,9*aS*) configuration by Mosher's amide analysis.¹⁶

Gas chromatography-mass spectrometry was carried out using a Trace GC Ultra interfaced to an iTQ 1100 ion trap mass spectrometer (Thermo Scientific). Achiral GC separations were performed using an RTX-5-Amine column (Restek Corporation) 30 m x 0.25 mm, 0.5 μ m d_f , using constant flow (1 mL/min) with vacuum compensation, surged splitless injection, surge pressure 250 kPa, surge time 0.7 min, splitless time 1 min, injector temp 250 °C, oven program 100 °C for 1 min, ramped to 280 °C at 10 °C/min and held 10 min. Chiral-phase GC separations were carried out using a β -Dex 120 column (Supelco) 30m x 0.25 mm, 0.25 μ m d_f , using constant flow splitless injection (1 mL/min) with vacuum compensation, splitless time 1 min, injector temp 100 °C held for 1 min, then ramped to 230 °C at 1 °C/min and held for 10 min. Electron impact MS parameters were: solvent delay 3 min, electron energy 70 eV, automatic gain control, 3 microscans per full scan.

Liquid chromatography-mass spectrometry was performed using an Acquity H-class UHPLC (Waters) interfaced to a Q-Exactive Focus quadrupole-orbitrap mass spectrometer (Thermo Scientific) equipped with an electrospray ion source operating at 3.5 kV with a capillary temperature of 250 °C in positive ion full MS mode with a resolution of 70,000 at m/z 200. The MS was externally calibrated in ESI-(+) mode using a standard calibration mix of caffeine, MRFA and Ultramark 1621 (Thermo Pierce 88323).

Achiral LC separations were carried out using a Kinetex C18 column (3 mm x 150 mm, dp 1.7 μ m, Phenomenex) using a gradient of CH₃CN and H₂O containing 0.1% CH₃CO₂H starting at 5% CH₃CN and ramping to 50% CH₃CN over 10 min, then to 99% CH₃CN over 2 min and held 1 min at a flow rate of 500 μ L/min and a column temperature of 30 °C (Supporting Information Figure S1).

Chiral-phase LC separations were performed using a Lux Cellulose 2 column (4.6 mm x 100 mm, dp 3 μ m, Phenomenex) with isocratic elution using 5 mM aqueous NH₄HCO₃-CH₃CN (40:60) at a flow rate of 500 μ L/min. For these separations, mass spectra were collected with a narrowed m/z range of 200-235 in order to maximize sensitivity for the analytes in question. For reproducibility of retention times and conditions a blank injection was performed prior to analysis of samples. To reduce risk and assess the potential for carryover, a blank injection was performed between injection synthetic materials and the natural extract. Analysis of this injection showed no detectable carryover. High-resolution-accurate mass measurements were made for the four ³⁵Cl/³⁷Cl and ¹²C/¹³C isotopologues. These conditions gave a clean separation of enantiomers with the (2a*R*,4a*S*,9a*S*) enantiomer eluting at 4.2 min and the (2a*S*,4a*R*,9a*R*) enantiomer at 8.5 min (Figures 2, S2-S7).

X-ray diffraction was performed on a Bruker-Nonius Kappa Apex II CCD diffractometer using graphite-monochromated Mo K α radiation. All diffractometer manipulations, including data collection, integration, scaling, and absorption corrections were carried out using the Bruker Apex II software. Full details are provided in the Supporting Information (Figure S13).

Cell Lines.

Cell lines expressing various combinations of nicotinic receptor subunits were generated as previously described.^{24,25} Cells were maintained in culture with weekly subculture in a 5% CO₂ humidified incubator in modified Eagle medium (MEM) until use. For assays, cells were seeded in multiwell plates and used at or near confluence.

Radioligand Binding.

Radioligand binding assays were performed using [³H]-epibatidine in membranes from either rat forebrain or HEK cells transfected with nicotinic receptor subunits as previously described.²³⁻²⁵ Briefly, membranes were incubated with radioligand in the presence of the unlabeled test compound for 4 h at room temperature and collected by rapid filtration and washing. Radioactivity was determined using a liquid scintillation counter. Nonspecific binding was determined in the presence of 300 μ M nicotine. K_i values were calculated from IC₅₀ values according the Cheng-Prusoff equation.²⁶

⁸⁶Rb Efflux.

Rubidium ion efflux was performed as previously described.²⁵ Briefly, plated cells at near confluence were preloaded with [⁸⁶Rb⁺], followed by washing and stimulation with the test compound. Extracellular medium was separated and cells were then lysed. The ratio of counts for extracellular medium and cell lysate as a percentage of total counts were calculated as response relative to 300 μM nicotine.

Electrophysiology.

Oocyte electrophysiology was conducted essentially as previously described.²⁸ Female frogs (*Xenopus laevis*) were purchased from Xenopus Express Inc. Oocytes were dissected from anesthetized frogs and defolliculated prior to injection of RNA. The mRNAs for rat α7, α3, α4, β2 nAChR and 5-HT_{3A} receptor subunits were transcribed *in vitro* from linearized cDNA using the mMessage (Ambion) according to manufacturer instructions. The total volume of RNA injected was approximately 50 nL at 1 μg/μL concentration. For the expression of heteromeric receptors (i.e. α3β2 & α4β2), the mRNAs for each subunit were mixed at a ratio of 1:1 (25 nL of each was injected). Current responses were obtained by two-electrode voltage clamp recording at a holding potential of −60 mV. All electrophysiological experiments were done at room temp (~24 °C). Electrodes contained 3 M KCl and had a resistance of <1 MΩ. The oocytes were continuously perfused with bath solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES). Drugs were applied using a synthetic quartz perfusion tube (0.7-mm i.d.) operated by a computer-controlled valve for 3 to 4 seconds. Drugs were applied at an interval of 4-5 min. For determining EC₅₀, the peak amplitude of currents obtained with different concentrations were normalized to that obtained with 1 mM ACh for each oocyte expressing nAChR, or 10 μM *meta*-chlorophenylbiguanide (mCPBG) for oocytes expressing 5-HT_{3A} receptors. Acetylcholine (ACh) and mCPBG were purchased from Sigma-Aldrich, and the stock solutions were diluted directly in the bath solution. Concentration-response curves were fit using GraphPad Prism Software with standard built-in algorithms. Values for log EC₅₀ and *n*_H were determined by fitting to the Hill equation $I = I_{\max} / (1 + EC_{50} / [A]^{n_H})$, where *I* is the current at a given agonist concentration, *I*_{max} is the maximal current obtained at a saturating agonist (ACh) concentration, EC₅₀ is the agonist concentration that elicits a half maximal current, [A] is the agonist concentration, and *n*_H is the Hill coefficient.⁴¹ Comparison of EC₅₀ and *I*_{max} values used a two-tailed unpaired t-test. Statistical differences were deemed significant at the level of *P* < 0.05. Currents from α3β2 and α4β2 receptors were sensitive to block by dihydro-β-erythroidine (DHβE). A 2 min pre-application of 10 nM DHβE completely blocked currents induced by 10 nM (±)-1 (n=4). Currents from α7 receptors were sensitive to block by methyllycaconitine (MLA). A 2 min pre-application of 30 nM MLA completely blocked currents induced by 10 μM (±)-1 (n=4). The IC₅₀ of MLA vs 10 μM (±)-1 is 33 ± 0.5 nM (n=3). Currents from 5HT_{3A} were sensitive to block by tropisetron. A 2 min pre-application of 30 nM tropisetron completely blocks currents induced by 100 μM (±)-1 (n=4).

Toxicology.

In-vivo toxicity assays were performed as previously described.³⁰ The protocol was approved by the Institutional Animal Care and Use Committee of Utah State University (Protocol #2152). Known amounts of the individual alkaloids were dissolved in physiological buffered saline and the solutions stored in sterile injection vials. Fasting (12 h) weanling White Swiss-Webster male mice (15-20 g, Simonsen Labs), were weighed and dosed intravenously, via the tail vein in mice restrained in a plastic mouse block. Immediately after injection the mice were returned to their pen for observation. The LD₅₀ was determined using a modified up-and-down method, calculated using PROC PROBIT procedures in SAS (SAS Institute) on a logistic distribution of the survival data, including 95% confidence intervals,

Molecular Modeling.

Calculated structures (geometry optimization and vibrational frequencies) were generated using Gaussian 09 (Rev. E.01)⁴² using density functional theory at the B3LYP level using the cc-pVDZ basis set. Molecular orbitals and electrostatic potential surfaces were generated through WebMO (v. 17.0.012e)⁴³ using natural charges calculated with NBO 6.0⁴⁴ installed on the Indiana State University chemistry department computing cluster.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- (17). The original NMR sample of purified phantasmidine had been lost during a laboratory closure and the few samples that we had from the original isolation contained so little material as to not be reliably detectable by GC-MS. The unfortunate passing of John Daly and the closure of his laboratory created a problem for the preservation of his group's samples. This is an all-too-common problem in the natural products community. Fortunately, at the suggestion of Daly group members Tom Spande and Martin Garraffo, and after some discussion with NIH administrators, Richard Fitch and Indiana State University were granted custody of the collection. Within the collection, we were able to locate the original extract from which **1** had been isolated.² This sample was used for the chiral-phase UHPLC-MS analysis described herein.
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- (21). In the process of validating the configuration of epibatidine, we identified commercial samples of the enantiomeric tartrates (gifted to the Daly lab in the late 1990s) which were configurationally mis-assigned. This may due to a simple mislabeling, but is instructive in the need to rigorously establish the correct configuration of materials and avoid the sole use of (+) and (–) in descriptions of pharmacologic samples unless absolutely necessary. It is very common for free bases and acid salts of chiral amines to have opposite sign of rotation due to polarization reversal. See Eliel EL; Wilen SH; Mander LN *Stereochemistry of Organic Compounds*; John Wiley and Sons: New York, 1994; Ch. 13, pp. 991–1118 and references cited therein.
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Figure 1. Chiral-phase LC-MS chromatograms showing extracted ions for phantasmidine (**1**), epibatidine (**2a**) and *N*-methylepibatidine (**2b**) in *E. anthonyi* extract. All ions are shown in the top trace. Ions in the mass range indicated on the right are shown in subsequent traces.

Figure 2. Functional profiles of phantasmidine as the racemate (top panel) and individual enantiomers (bottom panels) at rat nicotinic and serotonin receptor-expressing *Xenopus* oocytes.

Figure 3.
Molecular Electrostatic Potential Maps of Epibatidine, Phantasmidine, Varenicline and Cytisine.

Table 1.*In vitro* Binding Affinities of Phantasmidine Enantiomers at Rat nAChR Subtypes ^a

Compounds	K _i (nM) ^b			Forebrain	Selectivity (α4β2/α3β4) ^c
	α3β4	α4β2	α7		
(±)- 1	11 ± 1	0.35 ± 0.03	5.4 ± 0.5	1.1 ± 0.1	31
(2a <i>R</i> ,4a <i>S</i> ,9a <i>S</i>)- 1	9.1 ± 0.4	0.27 ± 0.03	4.4 ± 0.8	0.87 ± 0.09	34
(2a <i>S</i> ,4a <i>R</i> ,9a <i>R</i>)-ent- 1	390 ± 120	12 ± 3	130 ± 20	29 ± 6	33
2 <i>S</i> -(–)-Nicotine	370 ± 22	7.9 ± 0.6	430 ± 130	11 ± 1	47
(±)- 2	0.22 ± 0.01	0.033 ± 0.003	2.7 ± 0.7	0.041 ± 0.001	9
Eudismic Ratio ^d	43	44	30	33	

^a Competition binding assays were carried out in membrane homogenates of stably transfected cells or rat forebrain tissue as described previously. 23-25 The nAChR were labeled with [³H]-epibatidine. The K_d values for [³H]-epibatidine used for calculating K_i values were 0.3 for α3β4, 0.04 for α4β2, 1.8 for α7 and 0.05 for rat forebrain.²⁶

^b Each value shown represents the mean ± SEM of four independent measurements.

^c Note that binding affinity is inversely proportional to K_i.

^d The eudismic ratio is the ratio of the binding affinity (inversely proportional to K_i) of the more potent enantiomer (eutomer) over the less potent enantiomer (distomer).

Table 2.Agonist activity of phantasmidine at nAChR Subtypes based on ^{86}Rb efflux^a

Compounds	Rat $\alpha 3\beta 4$		Human $\alpha 4\beta 2$		Selectivity ($\alpha 4\beta 2/\alpha 3\beta 4$)
	EC_{50} (μM) ^b	E_{max} (%) ^c	EC_{50} (μM) ^b	E_{max} (%) ^c	
($\pm$)- 1	0.75 $\pm$ 0.007	91 $\pm$ 3	0.20 $\pm$ 0.1	42 $\pm$ 5	3.7
(2a <i>R</i> ,4a <i>S</i> ,9a <i>S</i>)- 1	0.57 $\pm$ 0.04	91 $\pm$ 3	0.20 $\pm$ 0.05	45 $\pm$ 7	2.9
(2a <i>S</i> ,4a <i>R</i> ,9a <i>R</i>)-ent- 1	27 $\pm$ 12	53 $\pm$ 13	56 $\pm$ 54	110 $\pm$ 6	0.49
2 <i>S</i> -($-$)-Nicotine	27 $\pm$ 1	107 $\pm$ 3	2.1 $\pm$ 0.6	103 $\pm$ 3	13
($\pm$)- 2	0.041 $\pm$ 0.005	114 $\pm$ 2	0.025 $\pm$ 0.001	135 $\pm$ 13	1.7
Eudismic Ratio	47		280		

^aFunctional properties of compounds were measured in cells stably expressing rat $\alpha 3\beta 4$ nAChRs and human $\alpha 4\beta 2$ nAChR.²⁵ Assays were performed as described in the Experimental Section.

^bPotency values (EC_{50}) were calculated according to half-maximal response for the individual compounds.

^cEfficacy values (E_{max}) were normalized to activation by 100 μM nicotine. Each value shown represents the mean $\pm$ SEM of 3 to 4 independent experiments.

Table 3

Electrophysiological Properties of Phantasmidine Enantiomers at Rat nAChR Subtypes Expressed in *Xenopus* Oocytes

Compounds	EC ₅₀ (μM) ^a (I _{max}) Hillslope			
	α3β2 ^b	α4β2 ^b	α7 ^c	5HT _{3A} ^d
(±)-1	19 ± 13 (0.25 ± 0.08) 1.57	0.0031 ± 0.0002 (0.32 ± 0.04) 1.6	9.9 ± 0.9 (1.08 ± 0.04) 1.8	29 ± 2 (0.79 ± 0.05) 1.7
(2a <i>R</i> ,4a <i>S</i> ,9a <i>S</i>)-1	ND	0.0015 ± 0.0003 (0.37 ± 0.03) 1.8	4.0 ± 1.0 (1.1 ± 0.1) 1.5	ND
(2a <i>S</i> ,4a <i>R</i> ,9a <i>R</i>)-ent-1	ND	0.045 ± 0.002 (0.43 ± 0.02) 1.7	21 ± 6 (1.02 ± 0.06) 1.3	ND
Acetylcholine	ND	24 ± 6 (1.0 ± 0.1) 1.0	110 ± 29 (1.0 ± 0.1) 1.5	ND
Eudismic Ratio		31	5	

^a I_{max} for α7, α4β2 and α3β2 nAChR are normalized to 1 mM acetylcholine and that of 5HT_{3A} receptor is normalized to 10 μM *meta*-chlorophenylbiguanide. Values are expressed ± SEM for an average of three experiments with four oocytes each.

^b Currents from α3β2 and α4β2 receptors were sensitive to block by dihydro-β-erythroidine.

^c Currents from α7 receptors were sensitive to block by methyllycaconitine.

^d Currents from 5HT_{3A} were sensitive to block by tropisetron.