Epiquinamide: A Poison That Wasn't from a Frog That Was[1]

Richard W. Fitch,*[,†] Gordon D. Sturgeon,[†,||] Shaun R. Patel,[‡,||] Thomas F. Spande,[§] H. Martin Garraffo,[§] John W. Daly,[§,#] and Richard H. Blaauw[⊥]

Department of Chemistry, Indiana State University, 600 Chestnut Street, Science S35E, Terre Haute, Indiana 47809, Department of Chemistry, Wabash College, P.O. Box 352, Crawfordsville, Indiana 47933, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland 20892, and Chiralix B.V., P.O. Box 31070, 6503 CB Nijmegen, The Netherlands

Received August 28, 2008

In 2003, we reported the isolation, structure elucidation, and pharmacology of epiquinamide (**1**), a novel alkaloid isolated from an Ecuadoran poison frog, *Epipedobates tricolor*. Since then, several groups, including ours, have undertaken synthetic efforts to produce this compound, which appeared initially to be a novel, β2-selective nicotinic acetylcholine receptor agonist. Based on prior chiral GC analysis of synthetic and natural samples, the absolute structure of this alkaloid was established as (1*S*,9a*S*)-1-acetamidoquinolizidine. We have synthesized the (1*R**,9a*S**)-isomer (*epi*-epiquinamide) using an iminium ion nitroaldol reaction as the key step. We have also synthesized *ent*-**1** semisynthetically from (−)-lupinine. Synthetic epiquinamide is inactive at nicotinic receptors, in accord with recently published reports. We have determined that the activity initially reported is due to cross-contamination from co-occurring epibatidine in the isolated material.

Nicotinic acetylcholine receptors (nAChRs) are a class of ligand-gated ion channels characterized by activation by the alkaloid nicotine and are important targets for the development of therapeutics.[2−4] These receptors are involved in cellular signaling in both the peripheral (PNS) and central (CNS) nervous systems. In the PNS, nicotinic receptors mediate muscle contraction by initiating action potentials at the muscle end-plate, as well as coupling to mechanosensitive hair cells in the inner ear.[5] In the central nervous system, nAChRs mediate much of the fast synaptic transmission in several areas of the brain. Activation of different nAChR subtypes is considered to be responsible for the myriad pharmacological effects of nicotine.[2] Ganglionic-type receptors mediate many of the toxic effects, while the central neuronal subtypes, especially $\alpha4\beta2$, are considered to be responsible for the cognitive enhancing as well as the addictive properties of nicotine.[4] The $\alpha7$ homopentamer has been implicated in inflammation and schizophrenia, and it has been observed that a high percentage (>80%) of schizophrenics are heavy smokers, with the behavior possibly serving as a form of self-medication.[2,3]

Our interest in nAChRs stems from the ability of a number of amphibian alkaloids[6,7] to selectively modulate these receptors (Figure 1). Epibatidine is a well-known highly potent nicotinic agonist.[8] Histrionicotoxin,[9] gephyrotoxin,[10] and several of the pumiliotoxins[11] are blockers of nicotinic receptors.

In 2003, we reported a novel alkaloid, epiquinamide (**1**), from an Ecuadoran poison frog, *Epipedobates tricolor*,[12] along with a screening method based on functional fluorescence assays.[12,13] Since then, several groups, including ours, have undertaken synthetic efforts to produce this compound, which appeared initially to be a novel β2-selective nicotinic acetylcholine receptor agonist.[13−18]

We have synthesized the (1*R*,9a*R*)-enantiomer of epiquinamide (*ent*-**1**) as well as the racemic (1*R**,9a*S**)-diastereomer (**2**), which is described herein. We have compared them to the natural product and the (1*S*,9a*S*)-(+)-enantiomer synthesized previously.[13a] The absolute configuration of natural epiquinamide has been established as (1*S*,9a*S*) by chiral gas chromatograpic analysis and comparison with *ent*-**1** and the natural product.[13b] Synthetic and biological studies by two separate groups have demonstrated that the synthetic enantiomers lack biological activity in several assays.[16,17] Our group has observed this as well with our synthetic isomers of epiquinamide. However, re-evaluation of the natural extract clearly indicated activity in binding and functional fluorescence assays (see below). Thus it is clear that epiquinamide itself lacks activity by itself, and some other component is likely to be responsible for activity in the purified compound. We have considered several possibilities for this, and they are discussed herein.

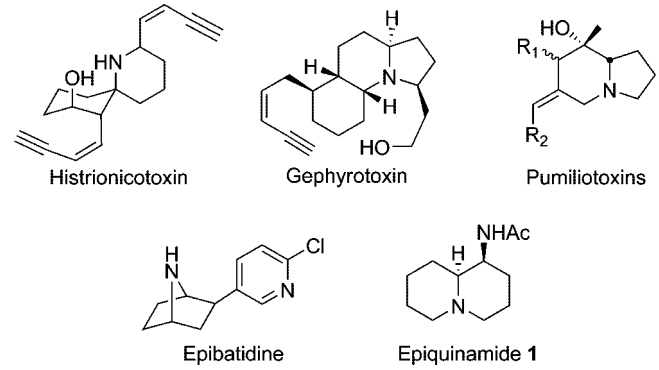


Figure 1. Nicotinically active alkaloids from frogs.

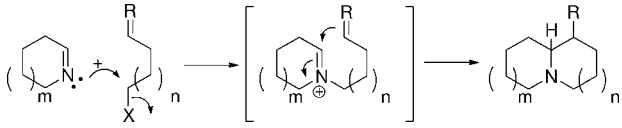
Results and Discussion

Chemistry. Our initial approach to epiquinamide was based on methodology developed for the synthesis of lupine alkaloids used by van Tammelen and Foltz.[19] Construction of the quinolizidine was envisioned as arising from alkylation and subsequent intramolecular iminium ion aldol reaction of a cyclic imine with a dipolar synthon capable of acting sequentially as an electrophile and nucleophile. We see this approach as a general scheme amenable to the core construction of any number of "izidine" azabicycles by variation of the ring size of the imine, chain length of the dipolar synthon, and substitution patterns thereof (Scheme 1). For the

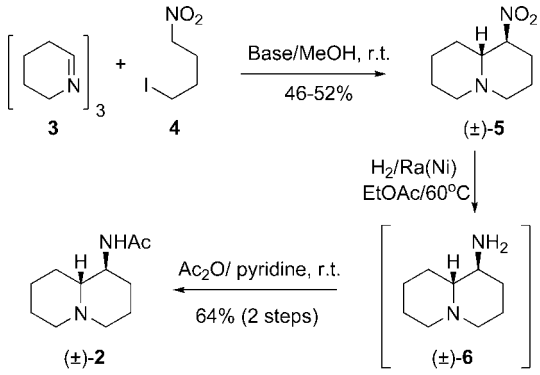
* To whom correspondence should be addressed. Tel: (812) 237-2244. Fax: (812) 237-2232. E-mail: rfitch@indstate.edu.
† Indiana State University.
‡ Wabash College.
§ National Institute of Diabetes and Digestive and Kidney Diseases, NIH.
⊥ Chiralix B.V.
|| Undergraduate student.
Deceased March 5, 2008.

10.1021/np8005452 CCC: $40.75 © 2009 American Chemical Society and American Society of Pharmacognosy
Published on Web 02/06/2009

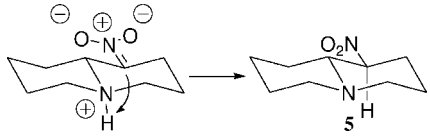
Scheme 1. Alkylation−Iminium Ion Aldol Annulation



Scheme 2. Synthesis of *epi*-Epiquinamide



Scheme 3. Stereoselection in Formation of Nitroquinolizidine **5**

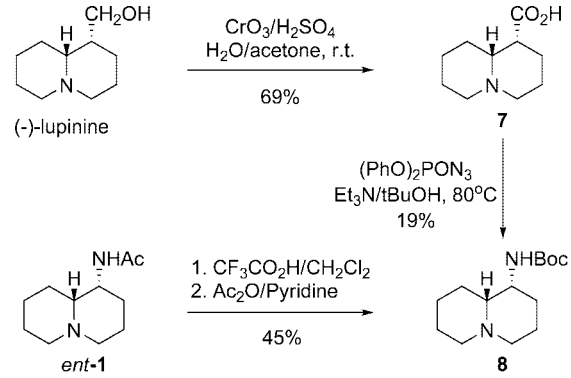


present target, the most obvious sequence involves the reaction of $\Delta^{1,2}$-piperideine (tetrahydropyridine, **3**) with 1-iodo-4-nitrobutane (**4**).

We prepared $\Delta^{1,2}$-piperideine (**3**, as the trimer)[20] and 1-iodo-4-nitrobutane (**4**)[21] essentially according to the literature. Nitroiodobutane may be prepared either by direct Kornblum reaction of 1,4-diiodobutane with silver nitrite or by a three-step sequence from 1,4-dichlorobutane involving sequential Finkelstein monoiodination, Kornblum substitution of the iodide by nitrite, and a second Finkelstein substitution of the remaining chlorine. While the latter process is longer, the starting dichloride is only about one tenth the cost of the diiodide and separation of the intermediates provides a cleaner product (see Experimental Section). With both synthons in hand, we set about the cyclization (Scheme 2), which proceeded using either an excess of piperideine trimer or added triethylamine as base to afford racemic 1-nitroquinolizidine **5** in moderate yield as essentially a single diastereomer. While several solvents were evaluated for this reaction, only alcoholic solvents afforded reasonable yields, presumably because of enhanced solubility of the charged intermediates involved in breakdown of the tripiperideine and formation of the iminium salt.[22]

However, the relative configuration of the major diastereomer of **5** was found to be epimeric to that of the natural product with a diastereomeric ratio of ~95:5 by GC-MS. The (1*R*,*9a*S**)-*cis*-stereochemistry was clear from the splitting pattern of the proton adjacent to the nitro group, which displayed large (J = 9.6 and 12.1 Hz) couplings indicating an axial orientation, whereas epiquinamide displays small couplings (largest J = 7.6 Hz, NH coupling, CH couplings <5 Hz) and typically appears as a broadened singlet where the NH is exchanged, consistent with an equatorial disposition. Attempts to epimerize the nitro group using LDA in THF or boiling acetic acid failed to effect any significant change. On examination of the system, the reason for this became clear (Scheme 3). Assuming a chair−chair conformation of the quinolizidine ring, the nitro group would normally be expected to prefer an equatorial orientation. This was borne out by calculation (MM2, Chem3D), which indicated a preference (0.9 kcal/mol) for the equatorial configuration in the (1*R*,*9a*S**)-*cis*-isomer. Surprisingly, when the same calculation was carried out on the acetamide,

Scheme 4. Synthesis of *ent*-Epiquinamide



the axial configuration of the (1*S*,*9a*S**)-*trans*-isomer was found to be preferred, though only by half the energy difference (0.5 kcal/mol). While significant, this energy difference is insufficient to explain the overwhelming preference for the (1*R*,*9a*S**)-*cis*-diastereomer. However, anchimeric assistance by the quinolizidine nitrogen further promotes the observed stereochemistry. Upon formation of the nitronate, reprotonation of the carbon is postulated to occur almost exclusively via suprafacial [1,3] proton transfer from the nitrogen lone-pair to the *aci*-nitro carbon to give **5**. This essentially precludes formation of the desired (1*S*,*9a*S**)-*trans*-diastereomer. While this is not useful for the synthesis of epiquinamide, we envision the excellent degree of stereocontrol to be useful for a number of "izidine" alkaloids (Scheme 1), and further studies are underway in this area.

We transformed nitroquinolizidine **5** to the acetamide by reduction over Raney nickel to afford diamine **6** (Scheme 2). Subsequent acetylation of the crude amine afforded racemic amide **2** in 64% yield over two steps (4% from dichlorobutane over five steps). The NMR spectra were again consistent with the (1*R*,*9a*S**)-*cis*-diastereomer (*epi*-epiquinamide).[17] In order to correlate with the original isolation data that were obtained in acetone-d_6 and CD_3OD, we obtained spectra in each solvent. The acetamide is soluble in $CDCl_3$ as well as the other two solvents and gave quite different proton spectra in each (see Supporting Information), which we ascribe to differences in conformational preference and/or association (especially in $CDCl_3$), perhaps as an acetamide dimer similar to the behavior of carboxylic acids. In order to assess the activity of each enantiomer of **2** independently, we made several attempts to resolve diamine **6** using either D- or L-tartaric acid. This approach has been used previously with excellent results with the similar 1,2-cyclohexanediamine.[23] While we obtained nearly quantitative yields of crystals using 0.5 equiv of the acid in aqueous methanol/acetic acid, we observed only racemic **2** by chiral gas chromatography on a cyclodextrin column after conversion to the free base and acetylation.

In order to access the correct diastereomer in enantiopure fashion, we embarked on a semisynthesis from the commercially available (1*R*,9a*R*)-*trans*-(−)-lupinine (Scheme 4). While the starting material is rather expensive (~$300/g), we needed very little material to establish the stereochemistry of the natural product, as the semisynthesis was based on simple functional group manipulation of the side chain.

The amide was prepared in straightforward fashion by oxidation of the primary alcohol to the carboxylic acid (**7**) using the Jones reagent in acetone.[24] While the oxidation worked well by TLC, workup was rather cumbersome owing to difficulties in separating **7** from the chromium salts. Ultimately, addition of silica gel and quenching with ammonia and 2-propanol allowed most of the chromium salts to be adsorbed and the amino acid separated by filtration and purified by flash chromatography. Acid **7** was subjected to a modified Curtius rearrangement using diphenylphosphoryl azide,[25] hydrolyzed, and directly acetylated (Ac_2O/pyridine) to afford the amide (*ent*-**1**, 24% from lupinine). While this furnished

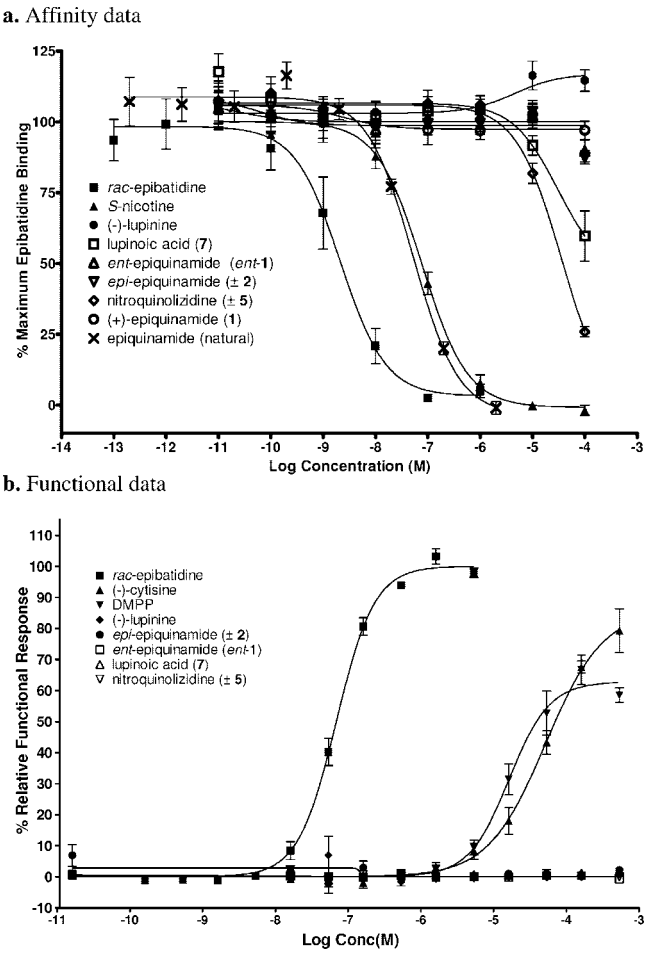


Figure 2. Pharmacologic analysis of epiquinamide and intermediates: (a) affinity data; (b) functional data.

the target compound, the amide was very difficult to separate from the symmetrical urea, formed as a result of reaction of hydrolyzed amine with unhydrolyzed isocyanate during the workup process. We then focused on the *N*-Boc derivative (**8**), which was prepared by replacing toluene with *t*-BuOH as the solvent. While **8** proved difficult to separate from aromatic byproducts, it could be smoothly deprotected with trifluoroacetic acid and acetylated under Schotten−Baumann conditions.[13a] This allowed for the neutral aromatic byproducts to be separated from the acetamide by a simple acid−base extraction, allowing isolation of *ent*-**1** as a clean solid without further chromatography. However, comparison of chiral gas chromatographic retention times with that of the natural product and co-injection clearly showed we did not have the natural enantiomer.[13b] Similar comparison of a sample of (+)-epiquinamide synthesized by Blaauw and co-workers[13a] did show identity and established the absolute configuration of the natural product as (1*S*,9a*S*), making his the first total synthesis of epiquinamide.

Noting the significant resemblance of epiquinamide to acetylcholine, we prepared the methiodides of *ent*-**1** and **2**, along with nitroquinolizidine **5** and lupinine, to produce closer bioisosteres. Each was smoothly prepared by treatment of the quinolizidine with methyl iodide in dry acetone, affording crystalline methiodides (see Experimental Section).

Pharmacology. Natural epiquinamide, isolated as described in our original report,[12] and the synthetic isomers described herein and earlier[13] were evaluated for activity in both affinity and functional assays. Affinity was assessed by [^{3}H]-epibatidine binding in rat cerebral cortical membranes, which primarily express the $\alpha4\beta2$ nicotinic receptor subtype (Figure 2a).[26] Functional assays were performed using fluorescence assays based on intracellular calcium in transfected HEK cells expressing $\alpha3$ and $\beta4$ nicotinic receptor subunits (Figure 2b).[27] In all instances, synthetic epiquinamide diastereomers were found to be inactive, having no affinity or functional activity up to 100 μM either as the free bases or as methiodides. Curiously, the isosteric ammonium lupinoate and nitroquinolizidine did show weak inhibition of binding at 100 μM and minor functional inhibition of nicotinic receptor activation at 500 μM. This is too weak to be of great interest for nicotinic work, but studies in other systems is ongoing given their similarity to nipecotic acid, a well-known GABA agonist.[28]

Previous reports described racemic and enantiopure epiquinamide and its diastereomeric analogue to be inactive at nicotinic receptors, in contrast to our initial observations regarding the natural material.[15,16] This prompted us to reexamine the natural material, which retains activity, having approximately the same potency as *S*-nicotine (Figure 2). We were initially puzzled by this and examined the isolated material thoroughly for the presence of contamination. The possibilities are (1) that epiquinamide coelutes with a very potent agonist; (2) that epiquinamide's activity is potentiated by a trace impurity; or (3) that the collected fractions were contaminated by splashover from nearby wells or a stray droplet from the fraction collector. A coeluting agonist is possible, though unlikely. Potentiation of the reference nicotine response in the assay was not observed, ruling out such a coeluting substance. Interwell cross-contamination is the most likely culprit here. The collection of the fractions was done in 96-well plates at 0.25 min intervals in six successive injections. Epiquinamide was collected along one row and epibatidine in the next successive row. Thus it is possible that droplet or splashover contamination occurred.

Examination of isolated epiquinamide by gas chromatography−mass spectrometry showed it to be >99% pure (Figure 3a). We looked for epibatidine as a likely culprit, as it is present in significant quantity in the extract and is an extraordinarily potent agonist. We were unable to locate it by looking for the parent ions at *m*/*z* 208 and 210 (Cl isotopes). However, these ions are of very low abundance in the electron impact mass spectrum of epibatidine. By looking instead for the *m*/*z* 68 and 69 peaks, which are the base peak and ~95% abundance, respectively, we were able to detect, by profiling these ions, a trace amount of epibatidine in the isolated material (Figure 3b). Though sample amounts preclude precise determination, by estimation, there is perhaps 0.1% of epibatidine present in the sample. While tiny, this amount would be consistent with the observed potency of the isolated material from [^{3}H]-epibatidine binding, which is approximately 1000-fold less than that of epibatidine. Although it remains possible that we have some other trace agonist, we believe epibatidine contamination to be the cause of the observed activity. While it was not observed during the original collection (each fraction was examined by GC-MS), the concentration at which it would have been present would have been nearly undetectable in the fractions. As further confirmation, the original extract was rechromatographed and the bioactivity reexamined (see Supporting Information). In the raw extract, weak activity can be observed near the elution time of epiquinamide, but is relatively insignificant and clearly not the robust activity originally observed.

While these data are somewhat embarrassing to report, they are not entirely unusual in the isolation of natural products. Further, the results are a reminder that the presence of multiple active substances in an extract (in this case compounded by small amounts of sample available) can sometimes lead to erroneous results from cross-contamination that are not always evident *a priori*. This underscores the importance of obtaining synthetic material whenever possible to corroborate both structure and pharmacology. In this case, we were able to produce a correct structure, but were misled by contamination in the pharmacology. Often with more complex structures, the converse occurs. Thus,

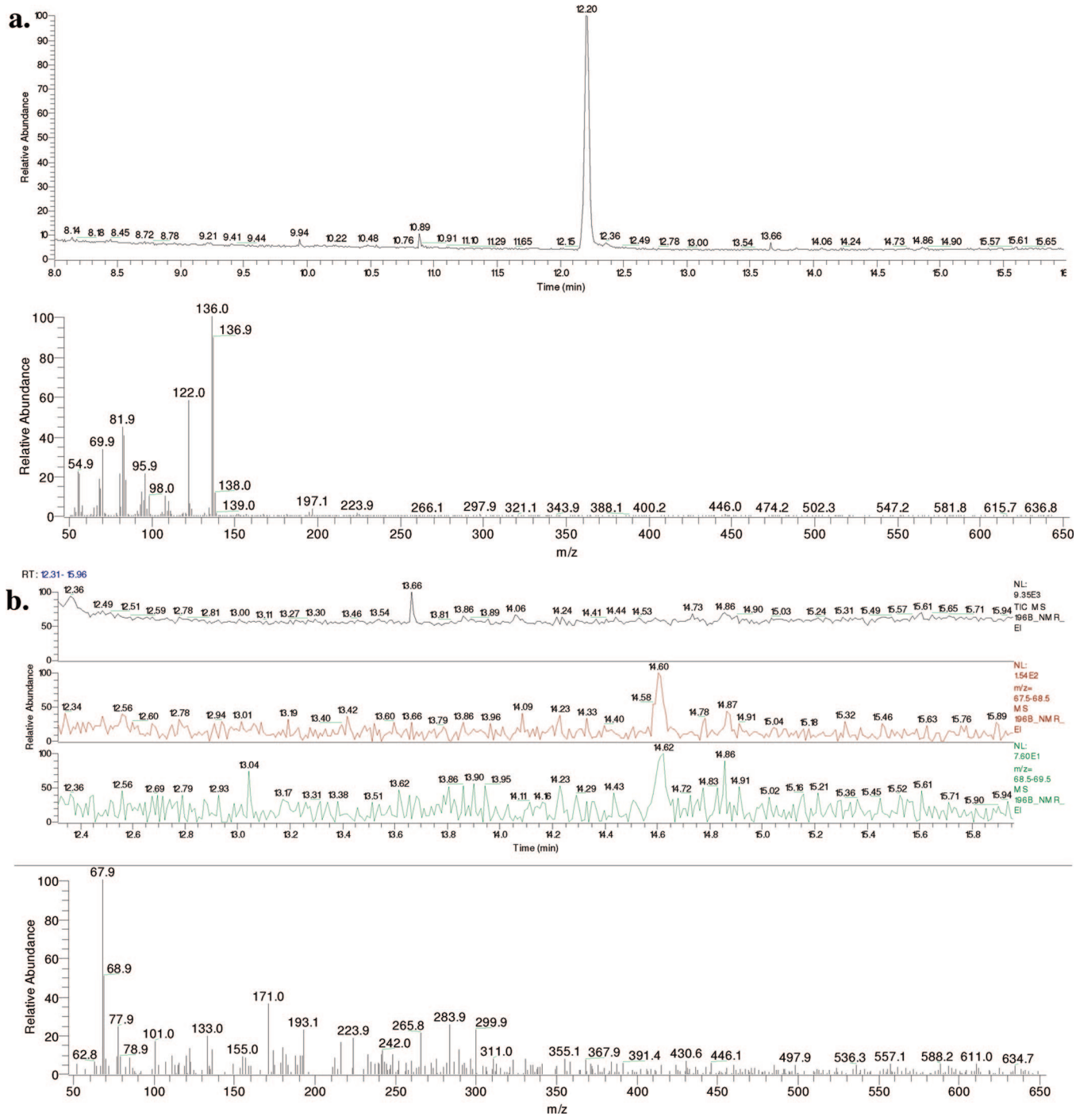


Figure 3. GC-MS data for natural epiquinamide.

it is important that the pharmacognosist work in collaboration with the synthetic/medicinal chemist to produce data that are unequivocal. Indeed in this very extract, we have other compounds, including phantasmidine, which are of significant interest.[5,6] However, because of similar concerns, we are completing the synthesis of phantasmidine prior to releasing its structure and activity, which we hope to report soon.

In conclusion, we have synthesized three and examined the pharmacology of all four stereoisomers of epiquinamide. The *trans* relative configuration has been confirmed and the absolute configuration has been established as (1*S*,9a*S*). All four diastereomers have been found to be inactive at nicotinic acetylcholine receptors, and the misleading activity in the natural material is concluded to be trace contamination by co-occurring epibatidine. This determination would not have been possible without synthetic material, underscoring the importance of chemical synthesis in collaboration with natural products isolation and pharmacology.

Acknowledgment. K. J. Lipscomb and R. J. Tucker are acknowledged for performing exploratory experiments. A generous gift of HEK cells expressing various subunit combinations for nicotinic receptors by Drs. K. J. Kellar and Y. Xiao of Georgetown University Medical School is gratefully acknowledged. This work was performed with funds from the Department of Chemistry and College of Arts and Sciences at Indiana State University (startup funding to R.W.F. and summer stipend to G.D.S.), as well as a Sigma Xi Grant-in-aid (12030168 to K.J.L.), the Lilly Endowment (Wabash College Indiana Internship Initiative summer stipend to S.R.P. and Indiana State University Promising Scholar Award to R.W.F.), and the National Science Foundation (CHE0521075 for a 400 MHz NMR spectrometer at Indiana

State University). Work performed at NIH was supported by the intramural program of NIDDK.

Supporting Information Available: Experimental procedures and selected 1D and 2D NMR spectra for compounds and intermediates are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Presented at the 48th Annual Meeting of the American Society of Pharmacognosy, Portland, ME, July 14−18, 2007; Abstract O4.
(2) Arneric, S. P.; Holladay, M.; Williams, M. *Biochem. Pharmacol.* **2007**, *74*, 1092–1101.
(3) Gundisch, D. *Expert Opin. Ther. Pat.* **2005**, *15*, 1221–1239.
(4) Tapper, A. R.; McKinney, S. L.; Nashmi, R.; Schwarz, J.; Desphande, P.; Labarca, C.; Whiteaker, P.; Marks, M. J.; Collins, A. C.; Lester, H. A. *Science* **2004**, *306*, 1029–1032.
(5) Elgoyhen, A. B.; Vetter, D. E.; Katz, E.; V. Rothlin*, C. V.; Heinemann, S. F.; Boulter, J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3501–3506.
(6) Daly, J. W. *J. Med. Chem.* **2003**, *46*, 447–452.
(7) Daly, J. W.; Spande, T. F.; Garraffo, H. M. *J. Nat. Prod.* **2005**, *68*, 1556–1575.
(8) Spande, T. F.; Garraffo, H. M.; Edwards, M. W.; Yeh, H. J. C.; Pannell, L.; Daly, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 3475–3478.
(9) Daly, J. W.; Karle, I.; Myers, C. W.; Tokuyama, T.; Waters, J. A.; Witkop, B. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1870–1875.
(10) Aronstam, R. S.; Daly, J. W.; Spande, T. F.; Narayanan, T. K.; Albuquerque, E. X. *Neurochem. Res.* **1986**, *11*, 1227–1240.
(11) Daly, J. W.; Tokuyama, T.; Fujiwara, T.; Highet, R. J.; Karle, I. L. *J. Am. Chem. Soc.* **1980**, *102*, 830–836.
(12) Fitch, R. W.; Garraffo, H. M.; Spande, T. F.; Yeh, H. J. C.; Daly, J. W. *J. Nat. Prod.* **2003**, *66*, 1345–1350. The frogs from this collection have since been reclassified as *Epipedobates anthonyi* on the basis of geography: Graham, C. H.; Ron, S. R.; Santos, J. C.; Schneider, C. J.; Moritz, C. *Evolution* **2004**, *58*, 1781–1793. This is an important distinction given the dietary alkaloid source for these frogs, which is presumably geographically dependent: Darst, C. R.; Menendez-Guerrero, P. A.; Coloma, L. A.; Cannatella, D. C. *Am. Nat.* **2005**, *165*, 56–69. For a more comprehensive discussion and revision of the systematics of dendrobatid frogs, see: Grant, T.; Frost, D. R.; Caldwell, J. P.; Gagliardo, R.; Haddad, C. F. B.; Kok, P. J. R.; Means, B. D.; Noonan, B. P.; Schargel, W.; Wheeler, W. C. *Bull. Am. Mus. Nat. Hist.* **2006**, *299*, 1–262.
(13) (a) Wijdeven, M. A.; Botman, P. N. M.; Wijtmans, R.; Schoemaker, H. E.; Rutjes, F. P. J. T.; Blaauw, R. H. *Org. Lett.* **2005**, *7*, 4005–4007. (b) Wijdeven, M.; Wijtmans, R.; Berg, R.; Noorduin, W.; Schoemaker, H.; Sonke, T.; van Delft, F.; Blaauw, R.; Fitch, R.; Rutjes, F. P. J. T.; Spande, T. F. *Org. Lett.* **2008**, *10*, 4001–4003.
(14) Tong, S. T.; Barker, D. *Tetrahedron Lett.* **2006**, *47*, 5017–5020.
(15) Huang, P. Q.; Guo, Z. Q.; Ruan, Y. P. *Org. Lett.* **2006**, *8*, 1435–1438.
(16) Suyama, T. L.; Gerwick, W. H. *Org. Lett.* **2006**, *8*, 4541–4543.
(17) Kanakubo, A.; Gray, D.; Innocent, N.; Wonnacott, S.; Gallagher, T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4648–4651.
(18) Voituriez, A.; Ferreira, F.; Perez-Luna, A.; Chemla, F. *Org. Lett.* **2007**, *9*, 4705–4708.
(19) van Tamelen, E. E.; Foltz, R. L. *J. Am. Chem. Soc.* **1969**, *91*, 7372–7377.
(20) DeKimpe, N.; Stevens, C. *J. Org. Chem.* **1993**, *58*, 2904–2906.
(21) Kurth, M. J.; Ahlberg-Randall, L. A.; Takenouchi, K. *J. Org. Chem.* **1996**, *61*, 8755–8761.
(22) Less polar solvents were observed to produce a precipitate without appreciable further reaction. Though uncharacterized, we suspect it to be the initial quaternary salt from reaction of intact tripiperidine with the iodide. This is consistent with prior observation that protic solvents promote equilibrium between trimer and monomer. See: Schopf, C.; Komnak, A.; Braun, F.; Jacobi, E.; Borgmuth, M. L.; Bullnehimer, M. *Liebigs Ann. Chem.* **1948**, *559*, 1–42. While slower, we found the intermediate chloronitrobutane to be equally competent in the annulation reaction.
(23) Gaslbøl, F.; Steenbøl, P.; Sørensen, B. S. *Acta Chem. Scand.* **1972**, *26*, 3605–3611.
(24) Schopf, C.; Thoma, O.; Schmidt, E.; Braun, W. *Liebigs Ann.* **1928**, *465*, 97–147.
(25) Ninomiya, K.; Shioiri, T.; Yamada, S. *Tetrahedron* **1974**, *30*, 2151–2157.
(26) Houghtling, R. A.; Davila-Garcia, M. I.; Kellar, K. J. *Mol. Pharmacol.* **1995**, *48*, 280–287.
(27) Fitch, R. W.; Xiao, Y.; Kellar, K. J.; Daly, J. W. *Proc. Nat. Acad. Sci. U.S.A.* **2003**, *100*, 4909–4914.
(28) Barrett-Jolley, R. *Br. J. Pharmacol.* **2001**, *133*, 673–678.

NP8005452