Bioassay-Guided Isolation of Epiquinamide, a Novel Quinolizidine Alkaloid and Nicotinic Agonist from an Ecuadoran Poison Frog, *Epipedobates tricolor*

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Analytical HPLC fractionation, combined with an off-line 96-well fluorescent bioassay screen, has been developed and used for the separation and screening of a natural product extract. This method was used to guide the isolation of a novel quinolizidine alkaloid from the methanolic skin extracts of an Ecuadoran frog, *Epipedobates tricolor*. The structure was determined on the basis of MS, IR, and NMR analysis as (1*R**,10*R**)-1-acetamidoquinolizidine (alkaloid **196**). We have named this compound epiquinamide, reflecting its origin and structure. The activity of the isolated compound was determined in five cell lines expressing various nicotinic acetylcholine receptor subtypes. The bioactivity of epiquinamide was evaluated on the basis of membrane potential fluorescence and was found to be β2 selective. This compound represents a new structural class of nicotinic agonists and a potential lead compound for the development of new therapeutics and pharmacological probes for nicotinic receptors. The off-line screening technique was found to be very sensitive for the detection of compounds active at nicotinic receptors.

The screening of crude natural product extracts and combinatorial libraries for activities has been a major source of biologically active compounds for drug development efforts.[1,2] However, whole extract screens can be problematic when multiple, potentially interfering activities or nonspecifically active compounds, such as tannins or leptins, are present.[3] The ability to separate and assess individually such activities without macroscopic isolation represents an extremely useful method for drug discovery.[4−6] The amount of material required for modern microwell biological assays is consistent with that present in analytical HPLC eluents. Moreover, the use of functional assays provides more physiologically meaningful data than do binding assays,[5] which do not distinguish between agonists and antagonists. The functional assay method has been applied to the alkaloid fraction from skin extracts of *Epipedobates tricolor* (Boulenger, 1899),[7] an Ecuadoran poison frog, which previously afforded epibatidine (Figure 1),[8] a potent nonopioid analgetic acting through neuronal nicotinic receptors.[9] Such receptors are attractive targets for drug discovery.[10,11] Preliminary analytical separation with off-line biological evaluation, using fluorescent membrane potential[12−14] and calcium dynamics[12,15−18] assays, revealed the presence of multiple compounds with nicotinic agonist activity. While epibatidine represented the major agonist activity, at least two other agonists were also present. The isolation and characterization of the first of these agonists is described herein. For consistency with the over 500 alkaloids isolated in our laboratory,[19] epiquinamide has been given the code designation **196**, corresponding to its molecular weight.

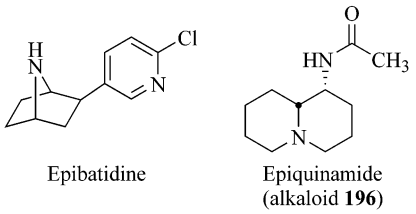

Figure 1.

Results and Discussion

The alkaloid fraction of the methanolic skin extract from 187 frogs of the species *Epipedobates tricolor* collected in 1987,[20] containing ~1 mg/mL total alkaloids, was analyzed by HPLC-MS, while monitoring UV at 260 nm (Figure 2). A large number of compounds were detected in the alkaloid fraction. These included epibatidine, several pumiliotoxins, indolizidines, quinolizidines, and other alkaloids, some of which had been previously characterized,[19] while others were new and of undetermined structure.

For biological evaluation a second LC injection was made using UV monitoring only (Figure 3, upper trace), while the eluent was collected in 96-well microplates. Fractions of 0.125 mL were taken at 0.25 min intervals. These fractions were acidified to convert amines to their corresponding hydrochloride salts (thus reducing volatility), evaporated under N_2, reconstituted in buffer, and analyzed for activity using fluorescent assays in KXα3β4R2 cells,[21−23] a HEK-293 cell line transfected with rat α3β4 ganglionic nicotinic acetylcholine receptors.[24] The cells were assayed for nicotinic activity using Fluo-4,[25] a fluorescent dye sensitive to intracellular calcium. The response for each fraction was plotted as a function of time and is shown in Figure 3 (lower trace) for stimulated elevation of calcium. The activity trace revealed the presence of several active components in the early fractions.

The major agonist activity was that of epibatidine (Figure 1) at 10.2 min. As this compound was previously known,[8] its isolation was not pursued. However, the first peak, at 6.63 min, corresponded to a previously uncharacterized compound (alkaloid **196**), giving a protonated molecular ion by APCI-MS at *m*/*z* 197 and affording a fragment at *m*/*z* 138 (M + H − 59), consistent with a loss of acetamide by McLafferty rearrangement.[26] The 196 molecular weight suggested a molecule with an even number of nitrogens (likely two), one of which appeared to be part of an acetamide. Using otherwise identical chromatographic conditions, but substituting D_2O for water in the mobile phase, afforded *m*/*z* 199 for the deuteronated

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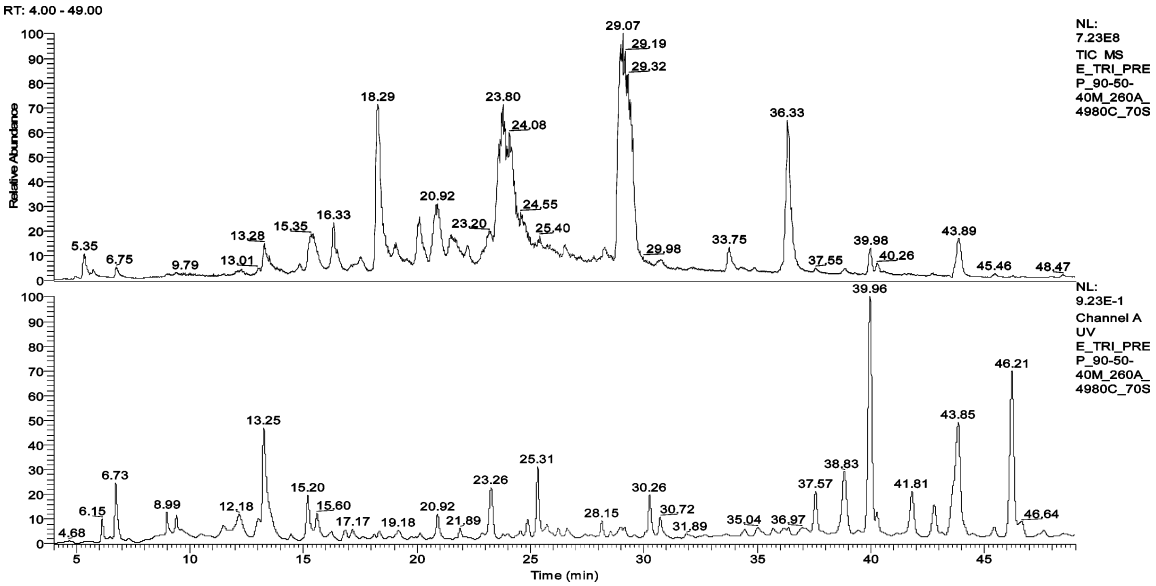

Figure 2. Reversed-phase HPLC-APCI-MS and UV profile of the alkaloid fraction from skin extracts of *Epipedobates tricolor*. The upper trace corresponds to the total APCI ion current, whereas the lower trace corresponds to the UV absorbance at 260 nm. Approximately 80 compounds were present in the crude alkaloid fraction. The peak at 13.25 min corresponds to epibatidine, while the peak at 6.73 min corresponds to epiquinamide.

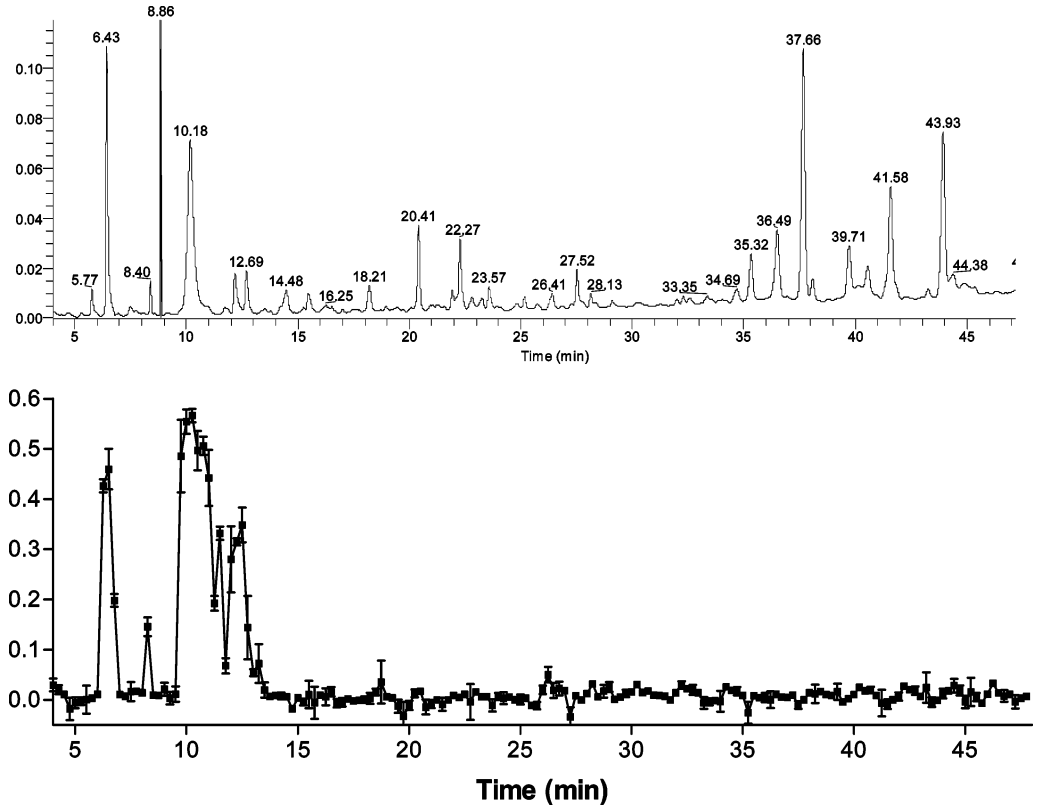

Figure 3. Reversed-phase HPLC bioassay of the alkaloid fraction of skin extracts from *Epipedobates tricolor*. The fraction was separated by HPLC with UV detection at 260 nm (upper trace). Eluate was collected in 96-well plates at 0.25 min intervals, acidified, evaporated to dryness, reconstituted in physiological buffer, and assessed for nicotinic receptor activity at rat $\alpha 3\beta 4$ receptors in KX$\alpha 3\beta 4$R2 cells using a calcium fluorescence assay using Fluo-4 as described in the Experimental Section (lower trace). Peaks at 6.43 and 10.19 min are epiquinamide and epibatidine, respectively. An apparent peak at 8.86 min in the upper trace is due to an electronic noise spike.

molecular ion (M + D), indicating one exchangeable hydrogen. This indicated that the second, basic nitrogen was part of a tertiary amine. The daughter ion remained at *m*/*z* 138, indicating the exchangeable hydrogen was in the putative acetamide moiety.

Analysis of the GC-MS data of the alkaloid fraction taken in the chemical ionization (CI) mode, using NH_3 as the reagent gas, revealed a peak with *m*/*z* 197 (M + H). Subsequent CI-MS/MS experiments afforded fragments progressing from *m*/*z* 197 to 138 to 110, corresponding to consecutive losses of acetamide and ethylene. Surprisingly, CI using ND_3 as the reagent gas showed only ~30% exchange of one hydrogen, and thus it appears that the amide proton does not exchange readily under these conditions,[27] although alcohol and amine protons readily exchange.[8,28] In EI-MS mode the alkaloid showed a parent ion at *m*/*z* 196 and fragments at *m*/*z* 137, 136, and 122, again consistent with loss of acetamide and subsequent loss of

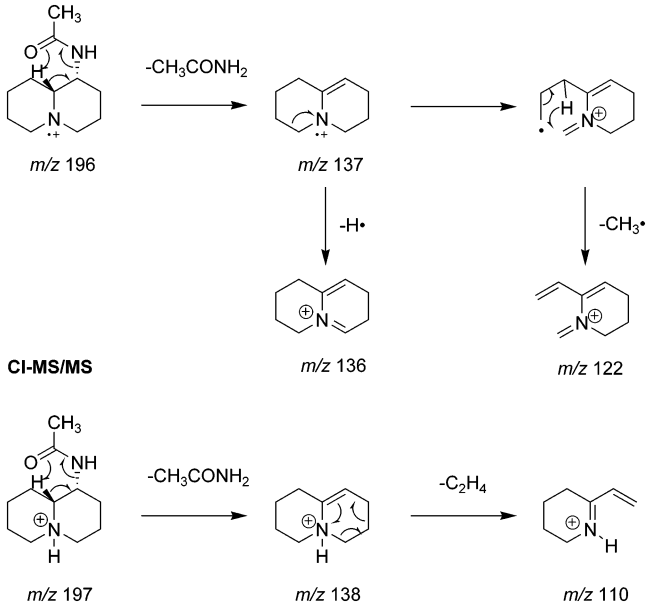

Figure 4. Postulated mass spectral fragmentation pathways of epiquinamide.

hydrogen or methyl from *m*/*z* 137. This progression was confirmed by EI-MS/MS experiments. However, no methyl loss from the parent was observed.[29] An interpretation of the mass spectral fragmentation pathways is shown in Figure 4.

The presence of an amide was also suggested by the GC-FTIR spectrum (Figure 5), which showed a strong carbonyl band at 1706 cm^{-1} and a strong, shouldered band at 1487 cm^{-1}. The lack of CH absorptions above 3000 cm^{-1} indicated the absence of olefinic CH, while Bohlmann bands at 2802 and 2765 cm^{-1} suggested a fused bicyclic "izidine" structure. The closest match available from an EPA vapor-phase infrared database search was an *N*-alkyl acetamidopiperidine, further suggesting the presence of a tertiary amine and an acetamide. A very weak absorption at ~3380 cm^{-1} was seen, consistent with an amide NH; however, noise in this region precluded unequivocal assignment.

Thus, with the available evidence from MS and IR, a bicyclic structure was proposed, comprising an acetamide as a substituent on a quinolizidine or indolizidine ring. The acetamide was on a six-membered ring, adjacent to the bridgehead, as this was the only position consistent with consecutive losses of acetamide and ethylene via McLafferty and retro-Diels–Alder processes, respectively (Figure 4). The other ring was most likely a six-membered ring, although IR analysis was inconclusive on this point. While the loss of methyl would tend to infer a methylindolizidine, such losses via rearrangement of quinolizidines have been reported.[29]

Having identified this alkaloid as the likely source of biological activity in this region of the analytical HPLC chromatogram, a semipreparative scale HPLC separation was undertaken, which gave roughly the same elution times as Figure 2. The sample was concentrated and chromatographed in six portions (to prevent overloading of the column), and the eluate was collected in 96-well, deep polypropylene plates at 0.25 min intervals. The fractions were acidified with dilute HCl to suppress volatility as before, and 10 μL aliquots were transferred to standard 96-well plates, concentrated, and evaluated for biological activity. Early active fractions were found to contain alkaloid **196** by GC-MS under chemical ionization (CI) conditions. The molecular formula was established as $C_{11}H_{20}N_2O$ by fast-atom-bombardment (FAB) high-resolution mass spectrometric (HRMS) measurements. The most homogeneous of these fractions were pooled and concentrated to afford material for NMR experiments (see Experimental Section). This sample of alkaloid **196** proved sufficient for one-dimensional proton and two-dimensional $^1H-^1H$ COSY spectra in CD_3OD. While these spectra were satisfactory for assignment of most resonances, overlap of signals in the 1–2 ppm range and an obscured resonance under the solvent peak precluded an unambiguous assignment of structure. This situation was improved with acetone-d_6 as solvent (Figure 6). Proton signals in the 1–2 ppm range were far better separated in this solvent. Although a signal at 2.05 ppm was obscured by solvent, its presence and couplings were unambiguously established by comparison with the CD_3OD spectra and by $^1H-^1H$ COSY and TOCSY spectra. An inverse-detected HMQC spectrum was obtained over the course of 3 days. This spectrum, along with the other data, firmly established the structure of alkaloid **196** as (1*R**,10*R**)-1-acetamidoquinolizidine,[30] which we have named epiquinamide, reflecting its structure and origin (Figure 1). A total of 250 μg of epiquinamide, 0.002% based on wet skin weight, was obtained (see Experimental Section). The NMR spectra indicated a *trans*-fused quinolizidine in a chair–chair conformation with the acetamide moiety in an axial orientation.

It is worthwhile noting that this represents one of the rare cases when mass spectrometry and infrared spectroscopy provided a nearly complete structure prior to NMR analysis. Epiquinamide (**196**) represents a new structural class of alkaloids for our laboratory, and while NMR demonstrated the absence of a methyl group (aside from the acetamide) and gave stereochemical information, this is the first case of a new structure, unrelated to our prior alkaloids, to be proposed on the basis of MS and IR spectral data.

Purified epiquinamide (**196**) allowed dose–response profiles to be obtained with several cell lines expressing various nicotinic receptor subtypes[24] (Figure 7, Table 1). Epiquinamide was most potent at β2-containing subtypes, including SH-SY5Y cells[31,32] (human neuroblastoma cells which express a ganglionic-type $\alpha3\beta2$* receptor) and in K-177 cells[33] (HEK-293 cells transfected with human central neuronal $\alpha4\beta2$ receptors). The latter subtype is considered to be responsible for nicotinic agonist-mediated analgesia. Epiquinamide showed somewhat less activity in KX$\alpha3\beta4$R2 cells[23–25] (HEK-293 cells transfected with rat ganglionic-type $\alpha3\beta4$ receptors) and IMR-32 cells[34,35] (human neuroblastoma cells expressing ganglionic type $\alpha3\beta4$* receptors). Only marginal activity was seen in TE-671 cells[31] (human rhabdomyosarcoma cells expressing neuromuscular $\alpha1\beta1\gamma\delta$ receptors). Thus, epiquinamide represents a new structural class of nicotinic receptor agonists, which despite modest potency, displays marked selectivity for nicotinic receptor subtypes. Its successful isolation demonstrates the power of the present bioassay-guided method to identify even modestly active components of natural product extracts.

Natural products in the form of decoctions, teas, etc., have been used for millennia for the treatment of disease.[1,2] The isolation of bioactive compounds from natural sources remains a major source of new medicinals and pharmacological probes. Typically, drug discovery efforts in this area have been initiated by the observation of an activity in a crude extract, often based on anecdotal or folklore evidence. Such work would be followed by efforts to isolate the active principles based on wet chemical techniques and reevalu-

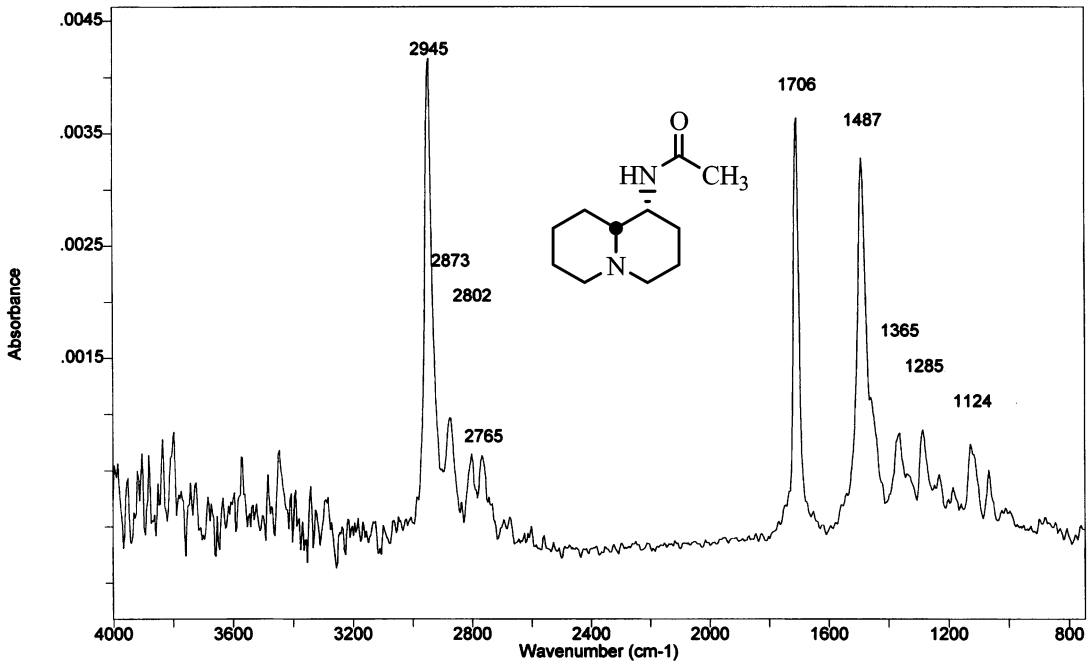

Figure 5. GC-FTIR spectrum of epiquinamide.

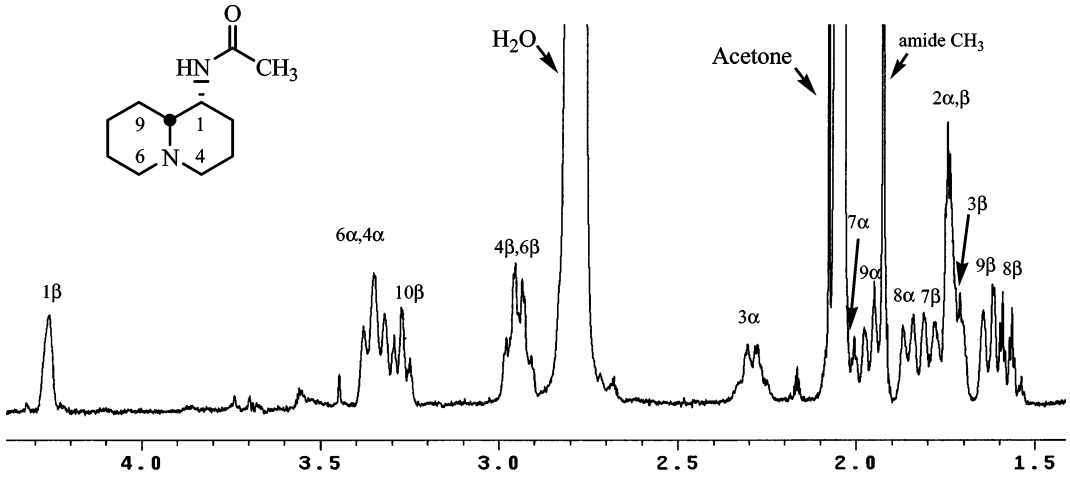

Figure 6. ^{1}H NMR spectrum of epiquinamide in acetone-d_6. Solvent signals are indicated by arrows. Stereochemical α and β designations refer to the *syn*- or *anti*-orientation, respectively, of a given proton relative to the acetamide moiety.

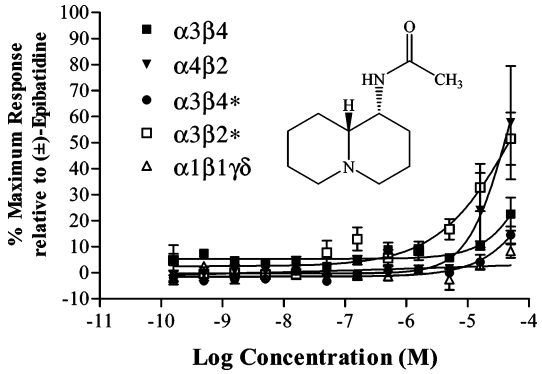

Figure 7. Biological evaluation of epiquinamide (**196**) in cell lines expressing various nicotinic receptor subtypes. Pharmacological parameters were obtained using a cell-based membrane potential assay as described in the Experimental Section.

ation of activity in the isolated fractions. This would be performed in iterative fashion until macroscopic amounts of an apparently active substance were found. Alternatively, natural extracts were subjected to fractionation to afford macroscopic amounts of pure compounds, and these were subsequently evaluated for activity.

While both of these methods can yield active compounds, each has limitations. An approach utilizing iterative wet chemical separation and biological evaluation can often reach a point where activity persists, but little or no material remains. Alternatively, the pure compounds isolated from a given extract may have no activity, or the activity present is due to a trace impurity, which eludes

Table 1. Pharmacological Evaluation of Epiquinamide (**196**) in Cell Lines Expressing Subtypes of Nicotinic Receptors[a]

cell line	subtype	activity at 50 μM
TE-671	human α1β1$\gamma\delta$	8 ± 6
SH-SY5Y	human α3β2*	51 ± 25
IMR-32	human α3β4*	15 ± 8
KXα3β4R2	rat α3β4	23 ± 13
K-177	human α4β2	58 ± 44

[a] Determined using the membrane potential assay as described in the Experimental Section. Activity is expressed as percentage of maximum (±)-epibatidine response ± SD of 2−3 experiments conducted in duplicate.

characterization for lack of material. Although the current method is also subject to this condition, the amounts required for detection of a measurable signal are quite small (ng to μg quantities depending on potency), thus consuming very little material. While plant extracts are generally not sample-limited, for animal sources this can often be the case. The isolation of alkaloids from skins of neotropical poison frogs represents just such a situation. The amounts of alkaloids on a per skin basis are often vanishingly small, and the collection of large numbers of frogs is problematic. This is due to restrictions on collecting as well as declines in frog populations in many areas from environmental causes and habitat destruction.[1,36] Thus, quantities available for structural and biological study can be quite limited. The alkaloid fraction described herein is that of the skins of 183 frogs. While this may sound substantial on a numerical basis, the size of these frogs and the minute quantities of alkaloids present per frog afforded ~6 mg of total alkaloids, of which epiquinamide represents only a very small fraction (~240 μg). Many instrumental methods, especially hyphenated techniques (GC-MS, LC-MS, GC-FTIR, and more recently, LC-NMR), have been developed in recent years[37,38] for the structure elucidation of minute quantities of compounds, and the ability to continuously track and determine biological activities during isolation is essential. In the current study, this is accomplished on the basis of a sensitive assay method amenable to the analysis of very small amounts of material, namely, analytical HPLC eluates in the ng−μg range. Fortunately, in this case, acceptable amounts (~240 μg) of a pure compound were obtained, enabling full spectral (IR, MS, and NMR) and pharmacological characterization. Moreover the analytical HPLC separation/bioassay strategy allowed for the identification of multiple agonist activities in the presence of multiple compounds, including antagonists, which will be reported in due course.

In conclusion, a novel nicotinic ligand, epiquinamide (**196**), has been isolated from skin extracts of an Ecuadoran frog, *E. tricolor*, using a combined approach for microscale separation, bioassay, and structural characterization of biologically active components of complex mixtures. This use of 96-well functional fluorescent bioassays is a rapid and sensitive method for both preliminary biological evaluation and detailed pharmacological analysis. It is suggested that this type of integrated structural and biological evaluation should become more generally applicable to the identification and isolation of bioactive components of natural products and synthetic combinatorial libraries.

Experimental Section

General Experimental Procedures. Solvents and reagents were obtained from commercial sources and used as received. HPLC separations utilized an Agilent 1100 system consisting of a vacuum degasser, binary pump, autosampler, temperature-controlled column compartment, and variable-wavelength UV detector interfaced to an Isco Foxy Jr. fraction collector equipped with a platform for collection in 96-well plates. Analytical separations were performed on a 4.6 × 250 mm Phenomenex Aqua C_{18} 125 Å, 5 μm reversed-phase column using a CH_3CN-H_2O system, with each component containing 0.05% HOAc to improve peak sharpness. The mobile phase was programmed on a gradient from 90% to 50% H_2O over 40 min, followed by 50% to 0% H_2O over the next 20 min at a flow rate of 0.5 mL/min. Semipreparative separations were performed on a 10 × 250 mm Phenomenex Aqua C_{18} 125 Å, 5 μm reversed-phase column and a 10 × 50 mm guard column, using the same gradient at a flow rate of 2.5 mL/min to afford approximately the same component retention times. LC-MS^n experiments were performed on a Finnigan LCQ ion-trap mass spectrometer equipped with an atmospheric pressure chemical ionization interface using the analytical HPLC system described above. Deuterium-exchange experiments were conducted by replacement of water in the mobile phase with D_2O. GC-MS^n experiments utilized a Finnigan GCQ ion-trap mass spectrometer equipped with a Restek RTX-5MS 0.25 mm × 30 m column operated with a temperature program beginning at 100 °C, holding 1 min and ramping at 10 °C/min to 280 °C and holding for 10 min. CI experiments were performed using NH_3 as the ionization gas (ND_3 for deuterium-exchange experiments). GC-FTIR experiments utilized a Hewlett-Packard 5890 GC interfaced to a Hewlett-Packard 5965B infrared detector (IRD) and a Hewlett-Packard 5971 mass selective detector (MSD). Separations were performed with a Hewlett-Packard HP-5 (0.32 mm × 25 m) column using the temperature program described above. High-resolution mass spectra were obtained using a JEOL SX102 magnetic sector mass spectrometer operating in FAB+ mode. NMR experiments were performed on a Varian Unity INOVA 500 MHz instrument equipped with a 3 mm gradient microprobe. Chemical shifts are expressed as δ ppm relative to TMS, referenced to the appropriate residual proton signal of the deuterated solvent used.

Collection and Extraction. Specimens of *Epipedobates tricolor* were collected at a stream approximately 14 km west of Santa Isabel, Azuay, Ecuador, in September 1987.[20] Skins of 183 frogs totaling 13 g wet weight were macerated three times with 20 mL of CH_3OH each. The combined CH_3OH extracts were diluted with an equal volume of water and extracted with 100 mL of $CHCl_3$ three times. The $CHCl_3$ extract was subsequently partitioned with three 100 mL portions of 0.1 N HCl. The acid solution was then made basic (pH 10) by addition of 2 N NH_4OH. Alkaloids were then extracted with 100 mL of $CHCl_3$ three times. Finally, the extract was dried over anhydrous Na_2SO_4 and carefully evaporated to near dryness. The residue was reconstituted in 6.5 mL of CH_3OH to afford an alkaloid fraction corresponding to ~2 g wet skin equivalent per mL. This alkaloid fraction was used for all subsequent analyses and isolations.

Epiquinamide (alkaloid 196). Six milliters of the above extract was concentrated to a volume of ~600 μL, fractionated by HPLC using six 100 μL injections under the conditions described above, and collected in 96-well deep (2 mL/well) polypropylene plates. A 10 μL aliquot of each fraction was assayed for activity, and the remainder was acidified by the addition of 10 μL of 0.1 N HCl to each well and evaporated to dryness under a stream of N_2. The fractions were reconstituted in 200 μL of CH_3OH and analyzed by GC-MS. The cleanest early bioactive fractions were pooled to afford a sample of epiquinamide. Initial attempts at obtaining NMR data in CD_3OD failed due to a methanol-soluble bleed product of the silica-based column. Evaporation to dryness and dissolution in $CDCl_3$ left the residue behind as a white solid, but failed to give acceptable spectra due to severe line broadening and shift anomalies. Similar results were seen using C_6D_6 as a solvent. Reconstitution of this sample in CD_3OD, however, afforded an acceptable spectrum. Apparently, a paramagnetic impurity was to blame for the unsatisfactory spectra in the poorly coordinating solvents. ^1H NMR analysis in CD_3OD showed this sample to be >90% pure. The sample amount was quantified by addition of 0.1 μL of $CHCl_3$ and comparison of the signal integration with that of the C1 proton of epiquinamide, resulting in an estimate of 104 μg. Similar treatment of the remaining pooled fractions afforded an additional 147 μg of epiquinamide, containing ~10% of an unidentified impurity. A total of ~240 μg, 0.002% based on wet skin weight, was thus obtained: GC-FTIR ν_{max} 3377 vw, 2945 s, 2873 w, 2802 w, 2765 w, 1706 s, 1487 s, 1365 w, 1285 w, 1124 w cm^{-1}; ^1H NMR (500 MHz, CD_3OD, HCl salt) δ 4.14 (1H, br s, H-1β), 3.44 (1H, d, J = 10.9 Hz, H-6α), 3.41 (1H, d, J = 12.9 Hz, H-4α), 3.30 (1H, m, H-10β, under solvent, indicated by COSY), 3.02 (1H, t, J = 14.7 Hz, H-4β), 2.97 (1H, t, J = 13.0 Hz, H-6β), 2.08 (1H, m,

H-7α), 2.05 (3H, s, amide CH_3), 1.89 (1H, m), 1.86 (2H, m), 1.84 (1H, m), 1.75 (1H, m), 1.70 (1H, m), 1.60 (2H, m); ^{1}H NMR (500 MHz, acetone-d_6, HCl salt) δ 11.81 (1H, br s, ammonium), 8.92 (1H, br s, amide NH), 4.27 (1H, br s, H-1β), 3.36 (1H, d, J = 14.2 Hz, H-6α), 3.32 (1H, d, J = 14 Hz, H-4α), 3.29 (1H, q, J = 10.3 Hz, H-10β), 2.96 (1H, d, J = 12.3 Hz, H-4β), 2.94 (1H, d, J = 12.3 Hz, H-6β), 2.30 (1H, q, J = 10.8 Hz, H-3α), 2.05 (1H, m, 7α, partially obscured by solvent, indicated by COSY), 1.97 (1H, m, J = 14.1 Hz, H-9α), 1.93 (3H, s, amide CH_3), 1.86 (1H, dm, J = 14.5 Hz, H-8α), 1.79 (1H, dm, J = 14.5 Hz, H-7β), 1.74 (1H, pd, J = 1.4, 3.1 Hz, H-2α), 1.73 (1H, dm, J = 3.4 Hz, H-2β), 1.71 (1H, m, H-3β), 1.64 (1H, dm, J = 14 Hz, H-9β), 1.58 (1H, dq, J = 13, 3.5 Hz, H-8β); ^{13}C NMR (125 MHz, acetone-d_6, determined from inverse HMQC) δ 65.6 (C-10), 56.0 (C-6), 55.2 (C-4), 46.8 (C-1), 28.7 (C-2), 27.2 (C-9), 23.3 (C-7), 22.8 (C-8), 22.5 (amide CH_3), 18.6 (C-3). Stereochemical α and β designations refer to a given proton's *syn*- or *anti*-orientation, respectively, relative to the acetamide moiety; EI-MS (*m*/*z*) 197 (2), 196 (<1), 138 (10), 137 (100), 136 (68), 122 (40), 96 (7), 83 (10), 82 (11), 70 (6), 55 (8); EI-MS/MS (on 137, *m*/*z*) 136 (17), 122 (100), 108 (15), 94 (28), 91 (10); CI-MS (NH_3, *m*/*z*) 197, (ND_3, *m*/*z*) 198; CI-MS/MS (NH_3, on 197, *m*/*z*) 138; CI-MS/MS-MS (NH_3, on 197, 138, *m*/*z*) 110; LC-MS (H_2O/CH_3CN, *m*/*z*) 197, (D_2O/CH_3CN, *m*/*z*) 199; HRMS (FAB+, *m*/*z*) found 197.1654, calcd for ($C_{11}H_{20}N_2O$ + H), 197.1649.

Cell Lines. K-177[33] cells were a generous gift of Abbott Laboratories. KXα3β4R2[21−23] cells were a generous gift of Dr. Kenneth J. Kellar of Georgetown University School of Medicine. TE-671[31] rhabdomyosarcoma cells and IMR-32[34,35] and SH-SY5Y[31,32] neuroblastoma cells were obtained from the American Type Culture Collection. Culture conditions were as described previously.[12]

Fluorescent Nicotinic Receptor Bioassay. Assays were performed essentially as previously described.[12,39] Briefly, samples for analysis were prepared in 96-well plates either as timed fractions from HPLC or by serial dilution of stock solutions of pure compounds in Hanks balanced salt solution (HBSS) supplemented with 20 mM 4-hydroxyethylpiperazine-1-sulfonic acid (HEPES) and adjusted to pH 7.4 with sodium hydroxide. Initial studies were conducted using an Applied Biosystems Cytofluor 4000 as previously described.[39] Later studies and detailed pharmacologic analysis were performed on a Flexstation fluorescence plate reader (Molecular Devices, Sunnyvale, CA).[12] Excitation/emission pairs were set to 485/525 nm or 530/565 nm for calcium and membrane potential measurements, respectively, using commercial dye kits supplied by the instrument manufacturer. Cells expressing nicotinic receptors were loaded with dye for 40−60 min in a dark drawer and placed in the plate reader. Three successive programmed additions of compound, nicotine (100 μM final concentration), and calibrant were performed at 16, 120, and 160 s with a total read time of 200 s. The calibrant for calcium measurements consisted of a solution of ionomycin, carbamylcholine chloride, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at final concentrations of 5, 100, and 20 μM, respectively, in HBSS/HEPES.[15] The calibrant for membrane potential was KCl at a final concentration of 40 μM in HBSS/HEPES. Basal fluorescence was subtracted and responses were calculated as a ratio of peak compound or nicotine fluorescence to peak calibrant fluorescence. Responses were subsequently normalized to a maximum response of (±)-epibatidine.

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