Isolation, Structure Elucidation, and Biological Evaluation of 15-Amido-3-demethoxy-2α,3α-methylenedioxyerythroculine, a New Alkaloid from *Hyperbaena valida*

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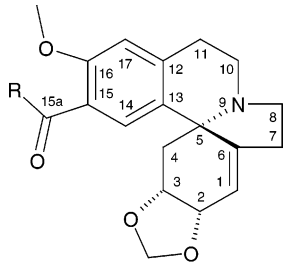
A phytochemical investigation of the leaves of *Hyperbaena valida* resulted in the isolation and characterization of two erythrina-type alkaloids, **1** and **2**, which were found to be antagonists at nicotinic receptors. Compound **1** was assigned as the new 15-amido-3-demethoxy-2α,3α-methylenedioxyerythroculine and compound **2** as the known 3-demethoxy-2α,3α-methylenedioxyerythroculine. Antagonism of a 100 μM nicotine response was observed for alkaloid **1** (IC_{50} value of 94 ± 8 μM) and alkaloid **2** (IC_{50} value of 77 ± 19 μM).

Menispermaceae A. L. Jussieu, the moonseed family, encompasses many medicinal and toxic plants and is widely distributed throughout the tropical world. Native Indians used some of the South American Menispermaceae for their curare-like activity.[1] Indeed, *Chondrodendron tomentosum* Ruiz & Pavon, the plant from which curare is derived, is a member of this family. The extracts of some species have shown significant activity in KB-nasopharynx cell systems as well as antimicrobial activity. *Hyperbaena valida* Miers, a member of the Menispermaceae family, was originally identified in 1867.[2] Plant material from our 1976 Jamaican collection of *H. valida* did not show activity in NCI screening (M. P. Cava, University of Alabama, private communication). Only one species of this genus, *Hyperbaena columbica* (Eichl.) Miers, has been investigated to date, and it was found to contain three alkaloids, two of which were of the erythrina type.[3] As a result of our interest in selective ligands for nicotinic receptors in recent years, a phytochemical study focusing on the leaves of *H. valida* was undertaken to search for related alkaloids in order to assess their biological activities.



1 R = NH_2
2 R = OCH_3

Compound **1** (0.198 g) was isolated from 4.0 kg of the leaves of *H. valida* after chromatography of the total bases with CH_2Cl_2/2% CH_3OH, yielding colorless cubes by recrystallization from CH_2Cl_2/CH_3OH, $[\alpha]_D$ +228 (*c* 0.10, $CHCl_3$). The low-resolution mass spectrum of **1** exhibited a $[M + H]^+$ ion at *m*/*z* 343 with two exchangeable hydrogen atoms, and the high-resolution mass spectrum of **1** indicated the molecular formula to be $C_{19}H_{22}N_2O_4$. The FT-IR spectrum revealed the presence of a conjugated carbonyl group of an amide at 1666 cm^{-1} as well as an aromatic ring at 1585 and 1490 cm^{-1}. The ^1H NMR spectrum of alkaloid **1** in $CDCl_3$ revealed the presence of two aromatic singlets at δ 7.72 and 6.75, an olefinic multiplet at δ 5.96, a pair of methylenedioxy singlets at δ 5.16 and 4.97, two broad, exchangeable protons at δ 7.67 and 5.85, a methoxyl singlet at δ 3.97, and 12 aliphatic multiplets ranging from δ 4.74 to 1.61. COSY NMR data indicated that the nonaromatic protons in **1** belong to three distinct spin systems: two $-CH_2-CH_2-$ systems and one $-CH_2-CH-CH-CH=$ system.

The ^{13}C GASPE NMR spectrum indicated that **1** contains 10 downfield carbon signals including a carbonyl resonance at δ 166.5 and a methylenedioxy signal at δ 94.4 as well as nine aliphatic carbon atoms including one quaternary, two methine, five methylene, and one methoxyl signal for a total of 19 carbons. HMQC and HMBC correlation data indicated the presence of a 1,2,4,5-tetrasubstituted aromatic ring, which is part of an isoquinoline alkaloid moiety incorporating one of the CH_2-CH_2- fragments mentioned above. Similar correlations also suggested that the aromatic ring possesses a methoxyl substituent at C-16 and a primary amide substituent at C-15.

Additional NMR correlation data from HMQC and HMBC experiments revealed that the remaining CH_2-CH_2- fragment was attached to the nitrogen atom N-9 and that C-5 is fully substituted. Taking into account the remaining correlations, it was determined that the C-5 spiro carbon atom in the isoquinoline moiety is also part of a cyclohexene ring bearing a methylenedioxy group as well as a pyrrolidine ring encompassing the adjacent nitrogen atom. The resulting structure for compound **1** is as shown, and a summary of the ^1H and ^{13}C NMR chemical shifts in $CDCl_3$ appears in Table 1.

The relative stereochemistry of alkaloid **1** was determined by analysis of coupling constants and NOE enhancements. On the basis of its observed coupling constants with the H-4 methylene protons, $J_{3,4\alpha} = 11.6$ Hz and $J_{3,4\beta} = 6.0$ Hz, H-3 was shown to occupy an axial position. Furthermore, saturation of both the H-2 and H-3 NMR resonances resulted in mutual NOE enhancements as well as enhancements of H-14, establishing the 2α,3α-configuration for the methylenedioxy group. With the fixed relative stereochemistry established, the absolute stereochemistry was then determined by comparison of the optical rotation measured for **1** ($[\alpha]_D$ +228) with literature values for erythrina-type alkaloids of identical relative stereochemistry and established absolute stereochemistries.[3] A

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Table 1. NMR Assignments for Alkaloids **1** and **2** in $CDCl_3$

	1 (^{1}H)	**1** (^{13}C)	**2** (^{13}C)
1	5.96 (m, 1H)	117.6 (d)	117.6 (d)
2	4.74 (m, 1H)	73.0 (d)	72.9 (d)
3	4.42 (ddd, 6.0, 6.5, 11.6 Hz, 1H)	72.0 (d)	72.1 (d)
4	2.30 (dd, 6.0, 11.6 Hz, 1H)	38.8 (t)	38.5 (t)
	1.61 (dd, 11.6, 11.6 Hz, 1H)		
5		63.9 (s)	64.0 (s)
6		147.5 (s)	148.0 (s)
7	2.59 (m, 1H)	27.4 (t)	27.4 (t)
	2.36 (m, 1H)		
8	3.03 (m, 1H)	46.6 (t)	46.7 (t)
	2.75 (m, 1H)		
10	3.55 (m, 1H)	40.6 (t)	40.7 (t)
	3.24 (m, 1H)		
11	3.12 (m, 1H)	22.5 (t)	22.6 (t)
	2.68 (m, 1H)		
12		140.0 (s)	140.2 (s)
13		128.8 (s)	127.5 (s)
14	7.72 (s, 1H)	131.2 (d)	130.4 (d)
15		118.5 (s)	117.6 (s)
15a		166.5 (s)	166.3 (s)
16		156.4 (s)	157.9 (s)
17	6.75 (s, 1H)	112.4 (d)	113.1 (d)
OMe-15a			52.1 (q)
OMe-16	3.97 (s, 3H)	56.0 (q)	56.1 (q)
OCH_2O	5.16 (s, 1H)	94.4 (t)	94.4 (t)
	4.97 (s, 1H)		
NH_2-15a	7.67 (b, 1H)		
	5.85 (b, 1H)		

literature search confirmed that the isoquinoline erythrina-type alkaloid **1** possesses a new structure.

Compound **2** (6.6 mg) was isolated from a fraction eluted with CH_2Cl_2/1% CH_3OH, giving colorless needles after recrystallization from dichloromethane/hexane, mp 90−91 °C. On the basis of mass spectrometric data indicating an $[M + H]^+$ ion at *m*/*z* 358, with no exchangeable hydrogen atoms, as well as a ^{1}H NMR spectrum that was similar to that of **1** except for the presence of an additional methoxyl singlet and the lack of exchangeable amide protons, alkaloid **2** was determined to be the C-15 methyl ester analogue of **1**. The ^{13}C NMR spectrum and correlation data, combined with UV and IR data, confirmed this proposal. The relative stereochemistry of **2** was determined to be identical to that of alkaloid **1** by comparison of their coupling constants and NOE enhancements. The H-3 proton in alkaloid **2** was shown to occupy an axial position on the basis of its coupling constants with adjacent H-4 methylene protons, $J_{3,4\alpha} = 11.6$ Hz and $J_{3,4\beta} = 6.0$ Hz, and NOE interactions between H-2, H-3, and H-14 established the 2α,3α-configuration for the methylenedioxy group in **2**. The absolute stereochemistry of alkaloid **2** was not determined.

Compound **2** is a known alkaloid named 3-demethoxy-2α,3α-methylenedioxyerythroculine, previously isolated from *Hyperbaena columbica*.[3] Since the ^{13}C NMR assignments for **2** were not given in this reference, they are included in Table 1. In order to remain consistent with the literature naming convention used for alkaloid **2**, alkaloid **1** is now named 15-carboxamido-3-demethoxy-2α,3α-methylenedioxyerythroculine. A structurally related erythrina-type alkaloid, erythramide, also containing a carboxamido group at the C-15 position was previously isolated from *Cocculus laurifolius* DC.[4]

Following the protocol for biological testing, no agonist activity was observed for alkaloid **1** or **2**, while antagonism of the 100 μM nicotine response was observed. Alkaloid **1** showed an IC_{50} value of 94 ± 8 μM, while alkaloid **2** showed an IC_{50} value of 77 ± 19 μM. Thus, it appears these compounds are approximately equipotent inhibitors of α3β4 nicotinic receptors. This value is quite similar to that of dihydro-β-erythroidine, a structurally related erythrina alkaloid and blocker of nicotinic channels. DHβE is known to be a weak competitive blocker in these cells, with an IC_{50} based on ^{86}Rb efflux of ~100 μM.[5] While weak at α3β4, DHβE is much more potent at α4β2, the predominant subtype in the brain. Thus, erythrina-type alkaloid **1** or **2** may show promise in the brain as well. Such studies are in progress.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fischer Johns apparatus and are uncorrected. Optical rotations were measured on a Rudolph Research Analytical Autopol II spectrophotometer. IR spectra were recorded on a Nicolet Magna 760 FT-IR spectrophotometer as a KBr pellet. UV spectra were recorded on a Cary 100 spectrophotometer. NMR spectra were recorded on a Bruker Avance 400 spectrometer (^{1}H at 400 MHz and ^{13}C at 100 MHz) in $CDCl_3$ solution at 25 °C, and the residual solvent peak was used as a secondary reference. The accurate mass measurement was performed on a Micromass Q-Tof 2 hybrid quadrupole time-of-flight mass spectrometer, equipped with a Z-spray interface using reserpine as the external mass calibrant.

Plant Material. *Hyperbaena valida* is an arborescent shrub approximately 6 m tall with green fruit. The leaves of the plant were collected from the rocky coastal woodland (altitude 25 ft) of Bulls Bay, Hanover, in Jamaica in July 1976. A voucher specimen of *H. valida* was deposited at the Institute of Jamaica, in Kingston, Jamaica, #63,385.

Extraction and Isolation. The leaves (4.0 kg) were moistened with 1:1 ammonium hydroxide/water and extracted exhaustively with ethyl acetate/ethanol (9:1). The extract was concentrated and partitioned between dichloromethane (CH_2Cl_2)/2% sulfuric acid (H_2SO_4) to obtain the CH_2Cl_2-soluble neutral fraction (69.7 g). The aqueous layer was basified with NH_4OH and extracted with CH_2Cl_2 to collect the total bases (6.0 g). Total bases (6.0 g) were subjected to silica gel column chromatography using dichloromethane (CH_2Cl_2) and mixtures of dichloromethane/methanol (CH_3OH) to give the erythrina-type alkaloids **1** (198 mg) and **2** (6.6 mg).

15-Carboxamido-3-demethoxy-2α,3α-methylenedioxyerythroculine (1): colorless cubes (recrys. CH_2Cl_2/CH_3OH); mp 219−220 °C (uncorrected); $[\alpha]_D$ +228 (*c* 0.10, $CHCl_3$); IR (KBr) ν_{max} 1666, 1585, 1490 cm^{-1}; UV (EtOH) λ_{max} (log ε) 224 (4.95), 301 (4.61), 330 (4.10) nm; ^{1}H and ^{13}C NMR data, see Table 1; HRMS $[M + H]^+$ *m*/*z* 343.1651 (calcd for $C_{19}H_{23}N_2O_4$, 343.1658).

Nicotinic Receptor Assay. The protocol used for functional analysis of alkaloids **1** and **2** at nicotinic receptors was performed similarly to that previously described[6,7] and is an adaptation of a prior protocol.[8] KXα3β4R2 cells, expressing rat α3 and β4 nicotinic receptor subunits, were provided by Drs. Yingxian Xiao and Kenneth J. Kellar of Georgetown University School of Medicine and grown according to published protocols.[5,9] For assays, cells were seeded into 96-well plates coated with poly-D-lysine and grown to near confluence.[7] The cells were removed from the incubator, allowed to reach room temperature, and then washed twice with 100 μL of HBSS/HEPES (pH 7.4). Subsequently, the medium was replaced with 30 μL of otherwise identical buffer, containing Calcium-3 no-wash dye (Molecular Devices, Sunnyvale, CA, R8091, one bottle diluted to 15 mL with HBSS/HEPES), and the cells were loaded for 1 h in the dark. Stimulated calcium fluorescence was then measured using a Cytofluor II fluorescence plate reader (Applied Biosystems) to which had been attached a holder arm, allowing a 12-channel pipettor to be positioned above the wells, thus facilitating addition of reagents while reading. Excitation and emission wavelengths were set to 485 and 525 nm, respectively. Measurements were made across 12 wells at approximately 6 s intervals, which is as fast as the instrument is capable of reading the full row. Basal fluorescence was measured for 30 s (5 scans), followed by addition of 30 μL of a 2× solution of **1** or **2** and measurement of fluorescence for 90 s (15 scans, to assess agonism). Subsequently, 30 μL of a 3× solution of nicotine di-D-tartrate was added to afford a final concentration of 100 μM, and fluorescence was measured for 30 s (5 scans, to assess antagonism). Finally, 30 μL of a 4× calibrant solution was added to afford a final concentration of 5 μM ionomycin, 100 μM carbamylcholine chloride, and 20 μM carbonyl cyanide-4-(trisfluoromethoxy)phenylhydrazone (FCCP, Sigma), and the resultant fluorescence was measured for 30 s (5 scans, to maximize signal and correct for dye loading and cell count).[8] Addition of compounds was facilitated by interruption of the scan sequence by computer control, followed by addition of reagents and simultaneous restarting of the

scan sequence. Following data collection, responses were calculated as follows:

$$\text{Compound response} = (F_{Max}(\text{Test Compound}) - F_{Basal})/(F_{Max}(\text{Calibrant}) - F_{Basal})$$

$$\text{Nicotine response} = (F_{Max}(\text{Nicotine}) - F_{Basal})/(F_{Max}(\text{Calibrant}) - F_{Basal})$$

While absolute calcium concentrations can be determined by the method of Grinkiewicz et al.,[10] such measurements tend to be unreliable with intensity dyes and are unnecessary for the determination of potency values.

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