Indole Glucoalkaloids from *Chimarrhis turbinata* and Their Evaluation as Antioxidant Agents and Acetylcholinesterase Inhibitors

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As part of our study on bioactive agents from Brazilian rainforest plants, two new glucoalkaloids, 3,4-dehydro-strictosidine (**1**) and 3,4-dehydro-strictosidinic acid (**2**), were isolated from *Chimarrhis turbinata,* along with seven known glucoalkaloids, cordifoline (**3**), strictosidinic acid (**4**), strictosidine (**5**), 5α-carboxystrictosidine (**6**), turbinatine (**7**), desoxycordifoline (**8**), and harman-3-carboxylic acid (**9**). The structures of the new alkaloids were established on the basis of comprehensive spectral analysis, mainly 1D and 2D NMR experiments, as well as high-resolution HRESIMS. Alkaloid **3** showed strong free-radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) as well as pronounced antioxidant activity evidenced by redox properties measured by ElCD-HPLC. Additionally, alkaloids **1**−**9** were submitted to TLC screening for acetylcholinesterase inhibitors. Both **7** and **8** were shown to be moderate acetylcholinesterase inhibitors at a concentration of 0.1 and 1.0 μM, respectively. In an in vitro rat brain assay, **7** showed moderate activity (IC_{50} 1.86 μM), compared to the standard compound, galanthamine (IC_{50} 0.92 μM).

Species of the Rubiaceae have been used for various medicinal purposes throughout the world,[1] and our systematic studies on Brazilian plants of this family have revealed marked diversity of the secondary metabolites present and their biological activities.[2] In a previous contribution, we reported the isolation, structure elucidation, and cytotoxic activity of turbinatine (**7**), an indole alkaloid isolated from leaves of *Chimarrhis turbinata* DC. (Rubiaceae).[3] As part of our search for bioactive substances from endemic species of the Amazon, *C. turbinata* was studied further after detection of antioxidant activity in its aqueous and ethanol extracts, indicated by strong free-radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), as well as significant antioxidant activity determined by redox properties measured by electrochemical detector coupled to an HPLC (ElCD-HPLC). Additionally, a TLC screening procedure for acetylcholinesterase (AchE) inhibitors was used as a preliminary test to detect potential bioactive compounds to treat Alzheimer's disease (AD).[4] The great interest in the search for potential natural AchE inhibitors is due to the recent discovery of galanthamine, an alkaloid, with long-acting, selective, reversible, and competitive activity as an AchE inhibitor, used as a drug for the treatment of AD. In this paper the details of the isolation and structure elucidation of two new analogues of strictosidine (**1** and **2**), as well as the isolation of six known indole glucoalkaloids (**3**−**8**), and the β-carboline alkaloid **9**, together with their antioxidant and acetylcholinesterase inhibitory activity data, are presented. Antioxidant indole glucoalkaloids as acetylcholinesterase inhibitors are being reported for the first time. Air-dried bark and leaves from *C. turbinata* were separately powdered and extracted with ethanol. Both ethanolic extracts were dissolved in MeOH−H_2O 80% and partitioned with EtOAc and *n*-BuOH. The aqueous phase from the bark extract and the *n*-BuOH phase from the leaf extract were purified using preparative HPLC, affording alkaloids **1**−**9**.

Compound **1** was isolated as a yellowish amorphous solid with strong fluorescence detected at 357 nm, which is compatible with an aromatic 5,6-dihydro-β-carboline chromophore, as corroborated by an indole N-H IR band (3370 cm^{-1}). The molecular formula of this alkaloid was determined as $C_{27}H_{32}N_2O_9$ (13 degrees of unsaturation) by HRESIMS ($[M + H]^+$ *m/z* 529.2166). Interpretation of the ion peak at *m/z* 367.1596 $[M - Glc + H]^+$ suggested the presence of a glucose moiety in **1** and indicated a similarity with the strictosidine pattern of fragmentation.[5] A detailed understanding of the molecular structure of this alkaloid was obtained from NMR spectral analysis (Tables 1 and 2). The unsubstituted indole moiety was deduced from the presence of four aromatic hydrogens at δ 7.38 (d, J = 7.5 Hz), 6.90 (dd, J = 7.5; J = 7.5 Hz), 7.02 (dd, J = 7.5; J = 7.5 Hz), and 7.20 (d, J = 7.5 Hz). A singlet at δ 7.72 corresponding to an enolic hydrogen (H-17) and a doublet at δ 5.81 (J = 9.0 Hz) attributed to a hemiacetalic hydrogen (H-21), in addition to signals for the methylene and methine hydrogens, H-14 and H-15, respectively, and signals for olefin hydrogens, H-18 and H-19, suggested the presence of a *seco*-iridoid moiety, as in strictosidine-type alkaloids. The ^{13}C NMR data (Table 2) confirmed this assumption. Comparison of the NMR data of **1** with those reported for strictosidine (**5**)[6] indicated in this alkaloid the absence of the signal corresponding to C-3 (δ_H = 4.42, δ_C = 51.2), compatible with the structure, 3,4-dehydro-strictosidine. An additional resonance at δ 138.5 in the ^{13}C NMR spectrum was assigned to C-3 and gave additional support to the proposed structure of **1**, which was corroborated by COSY, HMQC, HMBC, and NOE experiments

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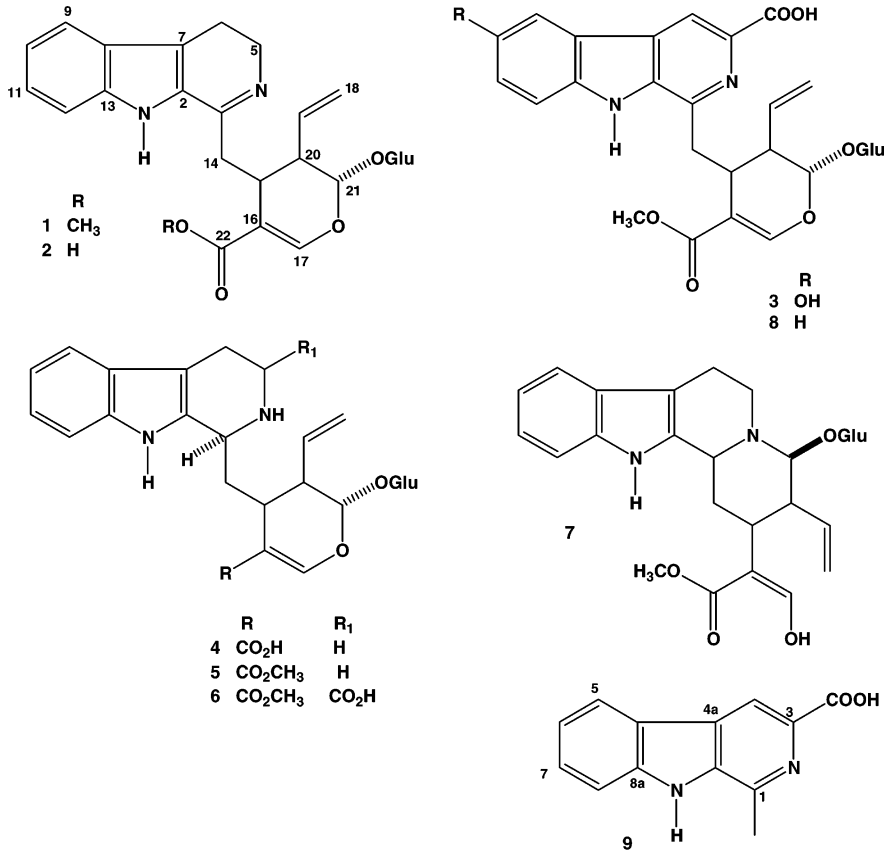
Chart 1



(Figure 1). COSY and NOESY experiments were fundamental to assign the relative configuration of C-15, C-20, and C-21 of this alkaloid, which was inferred to have the same stereochemistry as strictosidine. The structure of **1** was thus defined as 3,4-dehydro-strictosidine.

Compound **2** was obtained as a pale yellow amorphous powder. HRESIMS analysis gave a protonated molecular ion $[M + H]^+$ peak at *m/z* 515.2056, corresponding to the molecular formula $C_{26}H_{30}N_2O_9$. The IR spectrum showed absorptions at ν_{max} 3440 and 3368 cm^{-1}, indicating the presence of −OH and −NH functions, respectively. Inspection of the NMR data (^1H, ^{13}C, DEPT, and 2D experiments) showed a structure similar to **1**, including also a glucose moiety, confirmed by the ESIMS fragment ion at *m/z* 353.1596 $[M - Glc + H]^+$ and NMR data. The only difference in the ^1H and ^{13}C NMR spectra (Tables 1 and 2) of alkaloid **2** was the absence of signals corresponding to a methoxyl group ($\delta_H = 3.69$, $\delta_C = 53.0$), which is consistent with the presence of a carboxylic acid function, corroborated by the signal at δ 175.0 (C=O) and the IR absorption at 3440 cm^{-1} assigned to the carboxylic hydroxy group. HMBC cross-peaks (Figure 1) confirmed the ^1H and ^{13}C NMR assignments, and the proposed structure for compound **2** as the new 3,4-dehydro-strictosidinic acid.

Compounds **3**−**6** were identified as cordifoline,[7] strictosidinic acid,[8] strictosidine,[8] and 5α-carboxystrictosidine,[8] respectively, after comparison of their NMR spectral data with reported values. Compound **7** was previously isolated from this plant.[3] The β-carboline alkaloids **8** and **9** were identified as desoxycordifoline[7] and harman-3-carboxylic acid,[9] respectively. After careful analysis of the literature, ^{13}C NMR data for compounds **3** and **9** were found to be unpublished and thus these data are included in Table 2.

Compounds **1**−**9** were tested toward the DPPH radical (Table 3).[10] Alkaloid **3** showed strong scavenging activity against the DPPH radical (IC_{50} 18.3 μg/mL) using the antioxidant standard rutin (IC_{50} 12.3 μg/mL) as reference,

Table 1. ^1H NMR Spectral Data for Compounds **1** and **2** in CD_3OD[a] (500 MHz)

position	**1**	**2**
2		
3		
5	3.20 m	3.20 m
		3.68 m
6	3.40 m	3.45 m
7		
8		
9	7.38 d (7.5)	7.48 d (8.0)
10	6.90 dd (7.5; 7.5)	7.06 dd (8.0, 8.0)
11	7.02 dd (7.5; 7.5)	7.15 dd (8.0, 8.0)
12	7.20 d (7.5)	7.34 d (8.0)
13		
14	2.20 m	
	2.40 m	
15	2.98 m	2.92 m
16		
17	7.72 s	7.59 s
18 *cis*	5.16 d (10.5)	5.25 d (11.0)
18 *trans*		
	5.28 d (17.5)	5.35 d (17.5)
19	5.75 ddd (8.0; 10.5; 17.5)	5.92 ddd (8.0, 10.5, 17.5)
20	2.38 m	2.35 m
21	5.81 d (9.0)	5.89 br s
22		
OCH_3	3.69 s	
1′	4.77 d (8.0)	4.80 d (7.0)
2′	3.14 m	3.43 m
3′	3.30 m	3.42 m
4′	3.16 m	3.28 m
5′	3.32 br dd (5.7; 6.3)	3.26 m
6′	3.91 br dd (5.7; 11.0)	4.00 br dd (5.5; 12.0)
	3.57 dd (6.3; 11.0)	3.68 dd (6.5; 12.0)

[a] Chemical shifts (δ) downfield from TMS, *J* couplings (in parentheses) in Hz. Assigments were aided by $^1H-^1H$ COSY and HMQC data.

Table 2. ^{13}C NMR Data for Compounds **1**−**3** and **9** in CD_3OD Solutions[a]

carbon	1	2	3	9
1				136.1 s
2	135.2 s	136.2 s	137.2 s	
3	138.5 s	148.0 s	141.9 s	141.7 s
4				115.4 d
4a				127.0 s
4b				121.3 s
5	48.2 t	48.0 t	136.8 s	122.1 d
6	28.0 t	16.9 t	116.3 d	120.1 d
7	107.9 s	105.8 s	138.9 s	128.3 d
8	127.5 s	127.3 s	123.0 s	112.1 d
8a				140.8 s
9	119.1 d	119.1 d	122.5 d	
9a				136.4 s
10	120.5 d	120.6 d	114.3 d	
11	123.7d	123.4 s	153.8 s	
12	112.2 d	112.3 d	106.6 d	
13	138.2 s	138.2 s	132.2 s	
14	35.0 t	35.2 t	33.5 t	
15	32.6 t	35.0 t	35.3 t	
16	108.2 s	112.3 s	109.6 s	
17	157.1 d	153.6 d	155.0 d	
18	119.7 t	119.1 t	120.0 t	
19	135.2 d	136.3 d	135.0 d	
20	45.3 d	45.6 d	45.5 d	
21	97.3 d	96.8 d	97.2 d	
22	172.0 s	175.0 s	169.1 s	
CH_3				20.2 q
COOH			167.0 s	166.7 s
OCH_3	53.0 q		51.8 q	
1′	100.4 d	100.3 d	100.3 d	
2′	74.6 d	78.6 d	78.6 d	
3′	77.9 d	77.9 d	77.9 d	
4′	71.7 d	71.7 d	71.6 d	
5′	78.8 d	74.7 d	74.6 d	
6′	63.0 t	63.0 t	62.9 t	

[a] Chemical shifts (d) downfield from TMS, 125.77 MHz. Assignments and multiplicities were based on DEPT and HMQC experiments.

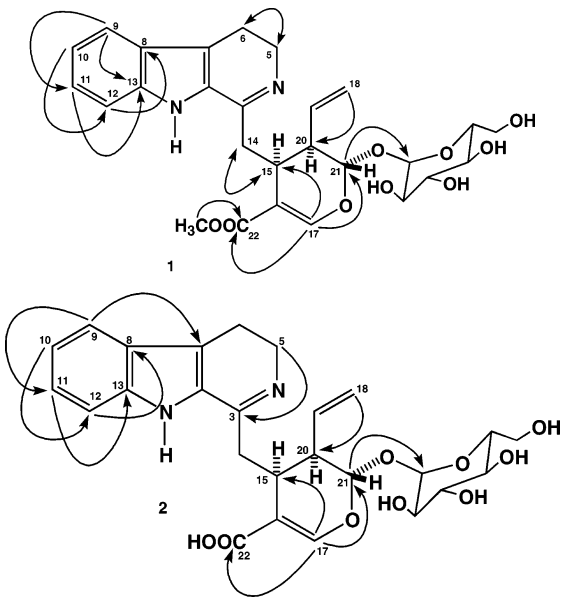


Figure 1. Selected HMBC (H→C) and COSY (↔) correlations of **1** and **2**.

whereas all other isolated alkaloids showed moderate to weak activity (IC > 40 μM). This result indicates that the free-radical scavenging activity of this indole alkaloid is due to its higher hydrogen-donating ability (to DPPH), provided by the hydroxy group at C-10, which results in a more efficient radical-scavenging effect.[11] The electrochemical behavior displayed by these alkaloids showed a good correlation with the radical-scavenging effect measured in the DPPH assay, which clearly indicates that substances with oxidative peaks above +1.2V (Table 3) and a small oxidative capacity have weaker radical-scavenging properties than those that are oxidized at lower potentials.[12] Compounds **1**−**9** were submitted to preliminary TLC screening for selecting potential AchE inhibitors,[13] in which only compounds **7** and **8** inhibited the enzyme at 0.1 and 1.0 μM concentrations. Due to the limited amount of alkaloid **8**, only compound **7** was submitted to further evaluation in an in vitro rat brain assay, to measure its AchE inhibitory potential, and showed a moderate activity (IC_{50} 1.86 μM) when compared with the standard galanthamine (IC_{50} 0.92 μM).

Table 3. Scavenging Activity for the DPPH Radical and Redox Potentials (V) of the Electrochemically Active Compounds **1**−**9**[a]

compound	IC_{50} (μM)[a]	E_{ox} (+)	E_{red} (−)
1	55.1	1.35	0.45
2	52.3	1.30	0.40
3	18.3	0.70	0.05
4	49.7	1.30	0.40
5	56.0	1.25	0.40
6	47.1	1.30	0.45
7	50.9	1.30	0.45
8	49.9	1.25	0.40
9	45.5	1.35	0.35
rutin	12.3	0.90	0.10

[a] Concentration in μM required to scavenge 50% DPPH free radicals. Rutin was used as standard.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer polarimeter in MeOH at 25 °C, using a sodium lamp operating at 598 nm. UV spectra were recorded on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were run on a Perkin-Elmer FT-IR 600 spectrophotometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Varian DRX-500 spectrometer, using TMS as internal standard. ESIMS were obtained on a VG Platform Fisons instrument, and HRTOF-ESIMS-MS was performed using a Q-TOF (Micromass) (40 eV) instrument. Column chromatography was carried out on silica gel 230−400 mesh (Merck), XAD-4 (Sigma-Aldrich), and Sephadex LH-20 (Pharmacia). TLC was performed using silica gel 60 (Merck, μ = 230 mesh) and precoated silica gel 60 PF_{254} plates. Spots on TLC were visualized under UV light and/or by spraying with anisaldehyde-H_2SO_4 reagent followed by heating at 120 °C and/or Dragendorff reagent. Preparative HPLC was performed on a Prep LC 4000 system (Waters) using C_{18} (250 mm × 21,20 mm, Luna-Phenomenex) columns.

Plant Material. Authenticated *Chimarrhis turbinata* DC. was collected in Reserva do Viro, Belém, PA, Brazil, in February 2000 and identified by Dr. Marina Thereza V. do A. Campos. A voucher specimen was deposited in the Herbarium of the Botanic Garden, São Paulo (Voucher No. Lopes-51).

Extraction and Isolation. Air-dried bark and leaves from *C. turbinata* were separately powdered and extracted with ethanol. Both ethanolic extracts were dissolved in MeOH−H_2O 80% and partitioned with EtOAc and *n*-BuOH. The *n*-BuOH fraction (12.3 g) after solvent evaporation was dissolved into H_2O (5 mL) and submitted to filtration over XAD-4 eluted with H_2O, MeOH−H_2O (20−100% gradient), acetone, MeOH−CH_2Cl_2 (1:1), CH_2Cl_2, and Et_2O. The subfractions obtained (A−K) were compared by TLC and revealed with specific reagents to alkaloids (Dragendorff). Fractions A (5.0 g), B (700.0 mg), and C (253.0 mg) were purified. Fraction A was submitted to VLC (vacuum-liquid chromatography) using octadecylsilane silica and gradient elution of decreasing polarity with H_2O, MeOH, acetone, and CH_2Cl_2 to give seven fractions. Fraction A4 (660.0 mg) was purified by HPLC using MeCN−H_2O−AcOH

(15:84.5:0.5) as mobile phase, UV detection at 237 nm, and a flow rate of 12 mL min^{-1} and afforded 5α-carboxystrictosidine (**6**) (5.0 mg). Fraction B (700.0 mg) was dissolved into MeOH and submitted to gel filtration on Sephadex LH-20 eluted with MeOH, affording 23 fractions (B1−B23). After TLC comparison, fractions B13−B15 were combined (90 mg) and purified by HPLC using MeCN−H_2O−AcOH (15:84.5:0.5) as mobile phase, with UV detection at 237 nm and a flow rate of 12 mL min^{-1}, affording the new derivative **1** (5.2 mg).

The ethanolic fraction (1.5 g) was purified by semipreparative HPLC using MeCN−H_2O−AcOH (15:84.5:0.5) as mobile phase, UV detection at 237 nm, and a flow rate of 12 mL min^{-1}, to afford the new derivative **2** (17.0 mg) and the known compounds **9** (30.0 mg), **3** (24.6 mg), **6** (154.1 mg), and **8** (148.9 mg).

The aqueous fraction from the bark (10 g) was submitted to RP-18 VLC eluted with decreasing polarity eluents as indicated above to give seven fractions. Fraction 1 (9 g) was purified by semipreparative HPLC [MeCN−H_2O−AcOH (15:84.5:0.5); UV detection at 237 nm; flow rate 12 mL min^{-1}], affording compounds **4** (15.0 mg), **5** (20.0 mg), and **6** (15.0 mg) and two unresolved peaks (A, retention time 19.9 min; B, retention time 50.7 min.). Fraction A (28 mg) was further purified by semipreparative HPLC [MeCN−H_2O−AcOH (13: 86.5:0.5); UV detection at 254 nm; flow rate 12 mL min^{-1}], affording **3** (16.4 mg). Fraction B (227.7 mg) was purified by preparative HPLC [MeCN−H_2O−AcOH (20:79.5:0.5); UV detection at 237 nm; flow rate 12 mL min^{-1}] and afforded **7** (14.0 mg) and **8** (90.0 mg).

3,4-Dehydro-strictosidine (1): amorphous yellow powder; UV λ_{max} (MeOH) 358 (3.9), 239 (3.0) nm; IR (KBr) 3496 (OH), 3370 (NH), 1710 (C=O) cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); HRESIMS *m/z* 529.2166 $[M + H]^+$ (calcd for $C_{27}H_{32}N_2O_9$ + H, 529.2186).

3,4-Dehydro-strictosidine acid (2): amorphous yellow powder; UV λ_{max} (MeOH) 356 (3.5), 240 (3.0) nm; IR (KBr) 3440 (OH), 3368 (NH) cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); HRESIMS *m/z* 515.2056 $[M + H]^+$ (calcd for $C_{26}H_{30}N_2O_9$ + H, 515.2030).

Determination of the Radical-Scavenging Activity. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was used as a stable radical in methanol (200 μmol, 2 mL). The reagent was added to 1 mL aliquots of compounds **1**−**9**, previously dissolved in methanol, to yield final concentrations of 100, 80, 40, 20, 10, and 5 μM. Each mixture was shaken and held for 30 min at room temperature, in the dark. Rutin was used as a standard compound. DPPH solution (2 mL) in methanol (1 mL) served as control. The evaluation of the reduced form of DPPH generated in situ was performed measuring the decrease in the current (*I*) on the electrochemical cell of the ElCD.[10] The areas obtained in each chromatogram were normalized and compared with the one obtained for the blank. The radical-scavenging activity of the samples was expressed in terms of IC_{50} (concentration in μM required for a 50% decrease in current (*I*) by the reduced form of DPPH).

Redox Potential. The determination of the optimal potential (E_{ox}) required to apply on each compound was obtained by means of hydrodynamic voltammograms generated for each compound[10] (Table 3).

TLC Screening Assay for Acetylcholinesterase Inhibitors. Samples were dissolved in MeOH or in MeOH−CH_2Cl_2 mixtures to 0.1 and 1.0 mM concentrations. Physostigmine and galathamine were used as reference compounds in two concentrations (0.1 and 0.01 μM). After application of 2 μL of each sample on a silica gel plate, it was sprayed with DTNB/ATCI reagent (1:1 solution of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 1 μM acetylthiocholine iodide in buffer) until saturation of the silica. The plate was allowed to dry for 5 min, and then 3 U/mL of enzyme solution was sprayed. After a while, a yellow background appeared, with white spots showing the inhibitory compounds.[4]

Evaluation in a Rat Brain Assay. The protocol adopted for this in vitro assay is described by Ellman and co-workers.[14]

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