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Pyridine Alkaloids from *Senna multijuga* As Acetylcholinesterase Inhibitors

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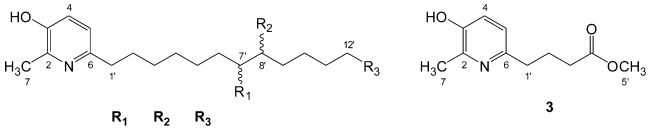
Supporting Information

ABSTRACT: As part of an ongoing research project on *Senna* and *Cassia* species, five new pyridine alkaloids, namely, 12′-hydroxy-7′-multijuguinol (**1**), 12′-hydroxy-8′-multijuguinol (**2**), methyl multijuguinate (**3**), 7′-multijuguinol (**4**), and 8′-multijuguinol (**5**), were isolated from the leaves of *Senna multijuga* (syn. *Cassia multijuga*). Their structures were elucidated on the basis of spectroscopic data analysis. Mass spectrometry was used for confirmation of the positions of the hydroxy groups in the side-chains of **1**, **2**, **4**, and **5**. All compounds exhibited weak in vitro acetylcholinesterase inhibitory activity as compared with the standard compound physostigmine.

Species belonging to the genus *Senna* are well-known for their various applications in traditional medicine and for being sources of alkaloids, including those belonging to the rare piperidine and pyridine classes, which display a variety of biological activities.[1] *Senna multijuga* (Rich.) Irwin et Barneby (syn. *Cassia multijuga* Rich.) (Fabaceae, Caesalpinioideae), popularly known as "aleluia", "canafístula", "caquera", "pau-cigarra", and "topeiuia", is a common tree in the Brazilian Atlantic Forest and other biomes in Africa.[2] It is widely used for ornamental decoration because of its attractive yellow flowers and evergreen leaves.[3] In Brazil, medicinal applications have not been reported frequently for this species, although aqueous preparations from its seeds have been employed in the treatment of ophthalmic and skin infections, which provides evidence of potential antimicrobial properties.[4] Regarding its ethnobotanical utilization, *S. multijuga* leaves have been used as a sedative by indigenous tribes during rituals.[3] Previous phytochemical studies have demonstrated the presence of flavonoid glycosides and pyridine alkaloids in this species, with these substances shown to exhibit moderate acetylcholinesterase (AChE) inhibitory activity.[5] A further investigation on the leaves of *S. multijuga* was carried out, in the hope of finding new unusual pyridine alkaloids in an EtOH extract of these leaves and to evaluate their action as AChE inhibitors. To this end, this extract was subjected to successive liquid−liquid partitioning and acid−base extraction, which yielded a bioactive alkaloidal fraction. This fraction was chromatographed using preparative TLC followed by HPLC separations and afforded five new pyridine alkaloids (**1**−**5**). These compounds were then assessed for their AChE inhibitory activity by means of bioautography and microplate screening assays.



	R_1	R_2	R_3
1	OH	H	OH
2	H	OH	OH
4	OH	H	H
5	H	OH	H

3

RESULTS AND DISCUSSION

Compound **1** was isolated as a white solid, with mp 66.3−67.5 °C. Its molecular weight was measured by HRMS, and the molecular formula was established as $C_{18}H_{31}NO_3$, which implied four degrees of unsaturation. The observed protonated molecular ion at m/z 310.2378 $[M + H]^+$ was close to the value calculated for $C_{18}H_{32}NO_3$, 310.2377. The UV spectrum, with absorptions at 222 and 287 nm, suggested the presence of an aromatic moiety as a chromophore. The IR absorption band at 3178 cm^{-1} was assigned to the hydroxy groups, and the bands at 1585, 1487, and 1464 cm^{-1} were attributed to pyridine ring. The 1D and 2D NMR data were related to those of 12′-hydroxy-7′-multijuguinone,[5] except for an absent carbonyl group at C-7′ on the straight side-chain. However, the presence of a hydroxy group in compound **1** was supported by the NMR spectra (Tables 1 and 2) and EIMS (Figure S1, Supporting Information). Examination of the ^1H, ^{13}C, and DEPT NMR spectra recorded in methanol-d_4 (Tables 1 and 2) showed the presence of a 2,3,6-trisubstituted pyridine ring [δ 7.06 (1H, d, J = 8.0 Hz, H-4), 6.92 (1H, d, J = 8.0 Hz, H-5), δ 146.5 (C-2),

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Table 1. ^{1}H NMR Spectroscopic Data of Alkaloids 1−5 (methanol-d_4, δ in ppm)[a]

	1	2	3	4	5
position	δ_H[b] mult. (J in Hz)	δ_H[b] mult. (J in Hz)	δ_H[b] mult. (J in Hz)	δ_H[b] mult. (J in Hz)	δ_H[b] mult. (J in Hz)
4	7.06, d (8.0)	7.07, d (8.5)	7.08, d (8.0)	7.06, d (8.0)	7.06, d (8.0)
5	6.92, d (8.0)	6.93, d (8.5)	6.94, d (8.0)	6.92, d (8.0)	6.92, d (8.0)
7	2.38, s	2.39, s	2.39, s	2.38, s	2.38, s
1′	2.64, t (7.5)	2.65, t (7.5)	2.68, t (7.5)	2.64, t (8.0)	2.64, t (7.5)
2′	1.63, qt (7.5)	1.64, qt (7.5)	1.94, qt (7.5)	1.63, qt (8.0)	1.63, qt (7.5)
3′	1.34, m	1.35, m	2.34, t (7.5)	1.34, m	1.35, m
4′	1.34, m	1.35, m		1.34, m	1.32, m
5′	1.46, m	1.30, m	3.65, s	1.44, m	1.32, m
6′	1.43, m	1.43, m		1.43, m	1.43, m
7′	3.50, m	1.44, m		3.49, m	1.42, m
8′	1.38, m	3.52, m		1.35, m	3.49, m
9′	1.33, m	1.40, m		1.31, m	1.38, m
10′	1.34, m	1.37, m		1.30, m	1.30, m
11′	1.54, qt (7.0)	1.54, qt (6.5)		1.32, m	1.32, m
12′	3.54, t (7.0)	3.56, t (6.5)		0.90, t (7.0)	0.91, t (7.5)

[a]Recorded at 500 MHz. [b]Multiplicity of signals is given as follows: s, singlet; d, doublet; t, triplet; qt, quintuplet; m, multiplet.

151.5 (C-3), 124.0 (C-4), 122.3 (C-5), and 152.6 (C-6)], a methyl group at δ 2.38 (s, H-7, δ_C 18.1), nine methylenes at δ 2.64 (t, H-1′, J = 7.5 Hz, δ_C 37.5), 1.63 (qt, H-2′, J = 7.5 Hz, δ_C 31.6), 1.34 (m, H-3′, H-4′, and H-10′, δ_C 30.6, 30.3, and 27.0), 1.46 (m, H-5′, δ_C 26.7), 1.43 (m, H-6′, δ_C 38.4), 1.38 (m, H-8′, δ_C 38.3), 1.33 (m, H-9′, δ_C 26.6), and 1.54 (qt, H-11′, J = 7.0 Hz, δ_C 33.6), a hydroxy methylene at δ 3.54 (t, H-12′, J = 7.0 Hz, δ_C 63.0), and a hydroxy methine at δ 3.50 (m, H-7′, δ_C 72.3), which suggested the presence of a long linear side-chain in the structure of **1**, as published previously.[5] Additionally, the side-chain (C-1′−C-12′) attached at C-6 of the pyridine nucleus was also confirmed by an HMBC NMR experiment. Three-bond correlations were observed from H-5 to C-1′, H-1′ to C-5, and H-2′ to C-6 (Figure 1), supporting a 2-methyl-3-hydroxy-6-*n*-alkyl pyridine substitution pattern. The 2,3,6-trisubstituted pyridine ring was confirmed by the HMBC correlations between H-5 and C-3 (^3J) and C-1′ (^3J), which, together with those between H-7 and C-2 (^2J) and C-3 (^3J), indicated the hydroxy group to be located at C-3. This is consistent with a previous study on bioactive pyridine and piperidine alkaloids from *Senna* (*Cassia*) species with similar features to **1**.[1,5] The

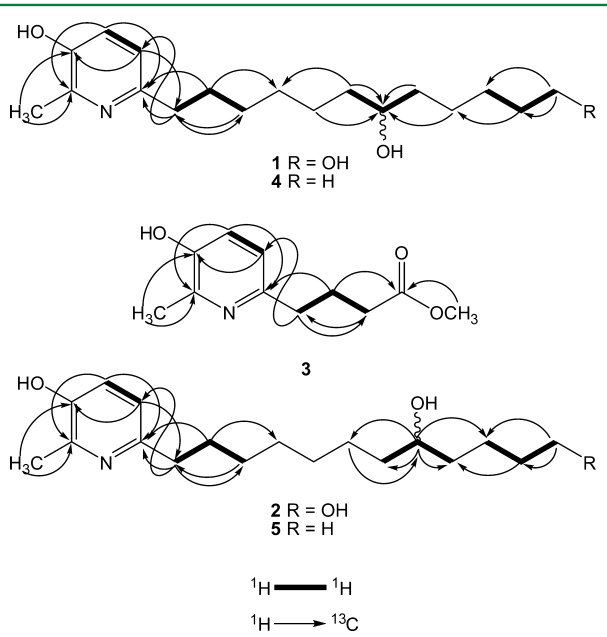

Figure 1. Key COSY (bold) and HMBC (arrow) correlations for compounds **1**−**5**.

Table 2. ^{13}C NMR Spectroscopic Data of Alkaloids 1−5 (methanol-d_4, δ in ppm)[a]

	1	2	3	4	5
position	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.
2	146.5, C	146.5, C	146.9, C	146.5, C	146.5, C
3	151.5, C	151.5, C	151.6, C	151.4, C	151.5, C
4	124.0, CH	124.0, CH	123.8, CH	124.0, CH	124.0, CH
5	122.3, CH	122.3, CH	122.5, CH	122.3, CH	122.3, CH
6	152.6, C	152.6, C	151.4, C	152.6, C	152.6, C
7	18.1, CH_3	18.1, CH_3	18.3, CH_3	18.1, CH_3	18.1, CH_3
1′	37.5, CH_2	37.5, CH_2	36.7, CH_2	37.5, CH_2	37.6, CH_2
2′	31.6, CH_2	31.6, CH_2	26.7, CH_2	31.6, CH_2	31.6, CH_2
3′	30.6, CH_2	30.7, CH_2	34.2, CH_2	30.6, CH_2	30.7, CH_2
4′	30.3, CH_2	30.3, CH_2	175.6, C	30.3, CH_2	30.3, CH_2
5′	26.7, CH_2	30.5, CH_2	52.0, CH_3	26.7, CH_2	30.5, CH_2
6′	38.4, CH_2	26.7, CH_2		38.4, CH_2	26.7, CH_2
7′	72.3, CH	38.4, CH_2		72.4, CH	38.4, CH_2
8′	38.3, CH_2	72.4, CH		38.3, CH_2	72.4, CH
9′	26.6, CH_2	38.2, CH_2		26.5, CH_2	38.1, CH_2
10′	27.0, CH_2	23.1, CH_2		33.1, CH_2	29.0, CH_2
11′	33.6, CH_2	33.7, CH_2		23.7, CH_2	23.8, CH_2
12′	63.0, CH_2	63.0, CH_2		14.4, CH_3	14.4, CH_3

[a]Recorded at 125 MHz.

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hydroxy group at the end of the straight side-chain (C-12′) was assigned by DEPT data analysis, and the hydroxy group attached at C-7′ was defined by HMBC data analysis (see also the Supporting Information). The key correlations are represented in Figure 1. All this evidence was in agreement with the assumption made on the substitution pattern of the pyridine ring and the hydroxy groups located at C-7′ and C-12′ on the straight side-chain of alkaloid **1**, which also was clearly supported by the EIMS (Figure S1, Supporting Information). The overall data analysis led us to propose the structure of the new pyridine alkaloid **1** as 2-methyl-3-hydroxy-6-*n*-(7′,12′-dihydroxydodecyl)pyridine (12′-hydroxy-7′-multijuguinol).

Compound **2** was obtained as a yellow oil, and the positive HRMS exhibited the protonated molecular ion at *m/z* 310.2376 $[M + H]^+$ (calcd for $C_{18}H_{32}NO_3$, 310.2327), appropriate for being an isomer of **1** ($C_{18}H_{31}NO_3$), which suggested four degrees of unsaturation. Analysis of the ^1H and ^{13}C NMR spectra of compound **2** revealed the presence of a 2,3,6-trisubstituted pyridine ring similar to that of **1** (Tables 1 and 2), as evidenced by the signals at δ 7.07 (1H, d, J = 8.5 Hz, H-4) and 6.93 (1H, d, J = 8.5 Hz, H-5). Additionally, the methyl protons of CH_3-7 at δ 2.39, analyzed together with ^{13}C and HMBC NMR data, confirmed that the pyridine moiety is similar to **1**. However, a shielding effect was detected at C-10′ (δ_C 23.1), for which the chemical shift was attributed to the γ-effect generated by two hydroxy groups. This led to the assumption that C-12′ (δ_C 63.0) and C-8′ (δ_C 72.4) are connected to the hydroxy groups in **2**, instead of C-12′ and C-7′ as in alkaloid **1**.[6,7] These data, supported by the ^{13}C NMR, DEPT, COSY, HMQC, and HMBC spectra, agreed with a pyridine system bearing a hydroxy group, a methyl, and a long-chain substituent. According to these data, the structure of **2** was determined as 2-methyl-3-hydroxy-6-*n*-(8′,12′-dihydroxydodecyl)pyridine (12′-hydroxy-8′-multijuguinol). The structures of the new alkaloids **1** and **2** suggest that the biogenetic origin of the side-chain in these alkaloids is derived from the acetate pathway (Figure S2, Supporting Information).

Compound **3** was isolated as a yellow oil, with the positive HRMS exhibiting a protonated ion at *m/z* 210.1127 $[M + H]^+$ (calcd for $C_{11}H_{16}NO_3$, 210.1125), consistent with a molecular formula of $C_{11}H_{15}NO_3$, containing five degrees of unsaturation. The IR spectrum showed absorption bands similar to those of **1** and **2**, except for a band at 1732 cm^{-1}, which corresponds to an ester carbonyl. The ^1H and ^{13}C NMR spectra of compound **3** exhibited a substitution pattern in the pyridine moiety similar to those of **1** and **2** (Tables 1 and 2). However, the side-chain linked at C-6 was different from the long-chain observed for alkaloids **1** and **2**. Analysis of the ^1H and ^{13}C NMR spectra of compound **3** revealed the presence of a short side-chain with three methylene signals at δ 2.68 (t, H-1′, J = 7.5 Hz, δ_C 36.7), 1.94 (qt, H-2′, J = 7.5 Hz, δ_C 26.7), and 2.34 (t, H-3′, J = 7.5 Hz, δ_C 34.2), an ester carbonyl at δ_C 175.6 (C-4′), and a methoxy group at δ 3.65 (H-5′, δ_C 52.0). Furthermore, the side-chain from C-1′ to C-5′ was deduced by using the COSY spectrum and linked at C-6 of the pyridine ring by the sequence of three-bond correlations observed between H-5 and C-1′, H-1′ and C-5, and H-2′ and C-6 (Figure 1), which supports the structure of **3** (methyl multijuguinate) as 2-methyl-3-hydroxy-6-(methyl-4-butanoate)pyridine.

Compounds **4** and **5** were isolated as white solids with mp 93.5−94.0 and 80.9−81.5 °C, respectively. Their positive HRMS exhibited protonated ion peaks at *m/z* 294.2428 and 294.2429 $[M + H]^+$ (calcd for $C_{18}H_{32}NO_2$, 294.2428), consistent with the same molecular formula of $C_{18}H_{31}NO_2$, containing four degrees of unsaturation, and 16 amu less than **1** and **2**. This implied that there was one less hydroxy group in the side-chain of **4** and **5**. Analysis of the ^1H and ^{13}C NMR data revealed that **4** and **5** have structural profiles similar to those of **1** and **2**, except for one more methyl group instead of a hydroxy methylene at the end of the straight side-chain. This observation was confirmed by analysis of the ^{13}C NMR, DEPT, and HMBC data (Tables 1 and 2) and is in agreement with the structures of **4** (7′-multijuguinol) and **5** (8′-multijuguinol) as 2-methyl-3-hydroxy-6-*n*-(7′-hydroxydodecyl)-pyridine and 2-methyl-3-hydroxy-6-*n*-(8′-hydroxydodecyl)-pyridine, respectively.

The positions of the hydroxy groups in the straight side-chain (C-7′ or C-8′) for alkaloids **1**, **2**, **4**, and **5** were established unambiguously by the EIMS. Fragment ions exhibiting relative abundance lower than 5% were not considered in this study (Figure S1, Supporting Information). Analysis of the mass spectra of alkaloids **1** and **4** evidenced a fragment ion at *m/z* 222, which was attributed to a homolytic α-cleavage that gives rise to the oxonium ion typical of alcohols and is in agreement with the hydroxy groups at C-7′.[8,9] This classic fragmentation reaction was also detected in the mass spectra of alkaloids **2** and **5**, with 14 mass units higher (*m/z* 236) than the former, supporting the location of the hydroxy groups at C-8′ (Figure S1, Supporting Information).

The absolute configuration at C-7′ in the side chain of the alkaloid **4** was proposed by the application of Mosher's method.[10] Treatment of **4** with (*S*)-MTPA-Cl using deuterated pyridine-d_5 as solvent yielded a mixture of (*R*)-MTPA-(*R*)-7′-multijuguinol (**4a**) and (*R*)-MTPA-(*S*)-7′-multijuguinol (**4b**) (Figure 2 and Figures S174−S176, Support Information).

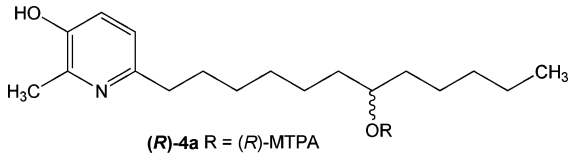

Figure 2. MTPA ester derivatives mixture **4a** and **4b**.

These data were consistent with the optical rotation ($[\alpha]_D^{28}$ 0) observed for compound **4**, which suggested that pyridine alkaloid **4** occurs in *S. multijulga* as a racemic mixture. The optical rotation values ($[\alpha]_D^{28}$ 0) found for alkaloids **2**, **3**, and **5** revealed that all isolated compounds are racemic mixtures, and these results should be consistent with the same absolute configuration occurring for these compounds.

As part of our bioprospecting program aiming at the discovery of novel anti-Alzheimer's agents, pyridine alkaloids **1**−**5** were tested for their AChE inhibitory activity by means of bioautography and microplate assays, and the results are presented in Table 3. The preliminary TLC assay suggested moderate activity for compounds **1**, **4**, and **5**, which were active at the minimum amounts of 1.5, 0.8, and 1.5 μg, respectively, as compared to physostigmine (positive control, 0.1 μg). Compounds **2** and **3** proved to be weakly active, since the minimum amount required for detection of inhibitory activity was over 1.5 μg. In addition, the microplate assay confirmed the preliminary results from the TLC test. Alkaloids **1** and **4** furnished moderate AChE inhibition of 51% and 52% at 350 μM, respectively, as compared to physostigmine (positive

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Table 3. Acetylcholinesterase Inhibitory Activity of Alkaloids 1−5

compound	AChE inhibitory activity	
	bioautography[a]	microplate[b]
1	1.5	51 ± 0.5
2	6.0	28 ± 0.5
3	3.0	40 ± 0.6
4	0.8	52 ± 0.4
5	1.5	19 ± 1.2
physostigmine[c]	0.1	94 ± 0.2

[a]Minimum amount required for AChE inhibitory activity on TLC plates (in μg). [b]Percentage of AChE inhibition at 350 μM. [c]Positive control.

control, 94% inhibition). These results suggest that the 2-methyl-3-hydroxypyridine moiety is an important site of interaction for the observed activity, but it alone is not responsible for this action. This is because alkaloids **1**−**5** exhibited variations in AChE inhibition depending on the position of the hydroxy group at the alkyl side-chain. Compounds **1** and **4** were more active than their isomers **2** and **5**, suggesting that the hydroxy group at C-7′ established more important interactions than when it is located at C-8′. On the other hand, comparison between the most potent pyridine alkaloids **1** and **4** indicated that oxidation at C-12′ had no influence on the activity. Alkaloid **3**, which also bears a methyl ester, displayed activity similar to that of **1** and **5**. Altogether, the alkyl side-chain clearly contributes to the potency of the alkaloid regarding acetylcholinesterase inhibition.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a 341 LC polarimeter (PerkinElmer) at 28 °C. Melting points were recorded on a digital Microquimica MQAPF-302 apparatus and are uncorrected. UV data were acquired using an Amersham Ultrospec 2100 pro UV/vis spectrophotometer. IR spectra were registered on a Nicolet iS10 FT-IR spectrometer coupled with an ATR accessory (the samples were pressed against a crystal of Ge). The 1D (^{1}H, ^{13}C, and DEPT) and 2D (^{1}H−^{1}H COSY, HMQC, and HMBC) NMR experiments were accomplished on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 125 MHz (^{13}C), at 30 °C, using TMS (δ_{TMS} = 0.00) as internal standard or residual solvent resonances of methanol-d_4 at δ 3.30 and 49.0 as references for ^{1}H and ^{13}C, respectively. High-resolution mass spectra with electrospray ionization were measured on an ultrOTOF$_Q$ (Bruker Daltonics) apparatus, operating in the positive mode. MeOH−H_2O (4:1) was used as solvent system. The EIMS were determined on a gas chromatography Shimadzu QP2010 mass spectrometer at an ionizing potential of 70 eV. The following conditions were employed: carrier gas was He at a constant flow of 1.2 mL/min; column was DB-1MS (30 m × 0.25 mm i.d.) fused-silica capillary with polydimethylsiloxane (0.25 μm film thickness) as the stationary phase; injector temperature was set at 280 °C, with a split ratio of 1:20; oven temperature initially held at 50 °C for 3 min and then increased at a rate of 6 °C/min to 295 °C, which was maintained for 10 min; time run 53 min. The scanned mass range was 40−500 amu.

TLC was performed on silica gel F_{254} plates (0.20 mm, Fluka), and spots were visualized under UV light (254 and 366 nm) and sprayed with Dragendorff's reagent or anisaldehyde−H_2SO_4, followed by charring for 5 min. Preparative TLC was carried out with Analtech 500 μm thick silica gel GF plates (20 × 20 cm, Uniplate) and visualized under UV light at 366 nm. Analytical and preparative HPLC separations were accomplished on a Shimadzu (Kyoto, Japan) CLASS-VP instrument equipped with a binary pump model LC-8A, a UV−vis detector model SPD-10A*vp*, an evaporative light scattering detector model ELSD-LT, a fraction collector model FRC-10A, and an automatic sample injector model SIL-10AF and controlled with the aid of an LC workstation CLASS-VP version 6.14 SP2 software. A Phenomenex Luna C_{18} (250 × 4.60 mm, 5 μm) column and a preparative Phenomenex Synergi Hydro-RP C_{18} 80 Å Axia Packed (100 × 21.20 mm, 4 μm) column protected with the corresponding guard columns were employed. All solvents utilized in the experimental procedures were HPLC-grade or had been previously distilled.

Plant Material. Leaves of *S. multijuga* were collected by M. Pivatto and W. Francisco in Araraquara (São Paulo, Brazil) in July 2010. The plant was identified by Inês Cordeiro from Instituto de Botânica in São Paulo−SP, Brazil. A voucher specimen (SP 384103) has been deposited in the herbarium of this institute.

Extraction and Isolation. The dried and powdered leaves (1.9 kg) were extracted with ethanol (27 L × 3) for seven days at room temperature. The solvent was removed under reduced pressure in a rotary evaporator, which furnished a thick syrup (310 g). The crude ethanol extract (310 g) was reconstituted in MeOH−H_2O (4:1), filtered, and successively partitioned with *n*-hexane (200 mL × 6), CH_2Cl_2 (200 mL × 5), and EtOAc (200 mL × 4). All the phases were concentrated under reduced pressure. The CH_2Cl_2 fraction (100 g) was dissolved in 400 mL of 5% HCl aqueous solution (ca. pH 2), filtered, and partitioned with EtOAc (50 mL × 3), to remove nonbasic components. Then, NH_4OH (30%) was added to the aqueous phase until ca. pH 9 and partitioned with CH_2Cl_2 (50 mL × 6). The latter organic layer was washed with H_2O (100 mL × 3) and concentrated, in order to give an alkaloidal fraction (1.42 g), which was purified by preparative TLC with *n*-hexane−$CHCl_3$−EtOAc (1.5:2.0:6.5) as developing solvent (×2). The bands were scraped and eluted with MeOH, to afford a mixture of **1** and **2** (R_f 0.12, 110.0 mg), pure alkaloid **3** (R_f 0.38, 19.0 mg), and a mixture of **4** and **5** (R_f 0.55, 130.0 mg). The mixture containing **1** and **2** was subjected to semipreparative HPLC on RP-C_{18} and eluted with MeOH−H_2O (40:10, flow rate 5.5 mL/min, UV 286 nm), which yielded the pure alkaloids **1** (t_R 82.7 min, 13.4 mg) and **2** (t_R 97.3 min, 31.2 mg). The mixture containing **4** and **5** was submitted to semipreparative HPLC on RP-C_{18} and eluted with MeOH−H_2O (75:25, flow rate 5.5 mL/min, UV 286 nm), which furnished the pure alkaloids **4** (t_R 27.2 min, 23.4 mg) and **5** (t_R 25.8 min, 21.0 mg).

12′-Hydroxy-7′-multijuguinol (1): white solid; $[\alpha]_D^{28}$ 0 (*c* 0.1, MeOH); mp 66.3−67.5 °C; UV (MeOH) λ_{max} (log ε) 222 (3.87), 287 (3.70) nm; IR ν_{max} 3178, 2926, 2848, 1585, 1487, 1464, 1157, 1039, 993, 821, 719, 650 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS *m*/*z* 310.2378 [M + H]$^+$ (calcd for $C_{18}H_{32}NO_3$, 310.2377); TLC R_f 0.12 (1:9 *n*-hexane−EtOAc).

12′-Hydroxy-8′-multijuguinol (2): yellow oil; $[\alpha]_D^{28}$ 0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 222 (3.81), 287 (3.64) nm; IR (film) ν_{max} 3350, 2926, 2854, 1581, 1489, 1462, 1416, 1280, 1126, 831 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS *m*/*z* 310.2376 [M + H]$^+$ (calcd for $C_{18}H_{32}NO_3$, 310.2377); TLC R_f 0.12 (1:9 *n*-hexane−EtOAc).

Methyl multijuguinate (3): yellow oil; UV (MeOH) λ_{max} (log ε) 222 (3.71), 286 (3.48) nm; IR (film) ν_{max} 3342, 2951, 2925, 2850, 1732, 1579, 1489, 1435, 1280, 1161, 1124, 1012, 833 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS *m*/*z* 210.1127 [M + H]$^+$ (calcd for $C_{11}H_{16}NO_3$, 210.1125); TLC R_f 0.52 (1:9 *n*-hexane−EtOAc).

7′-Multijuguinol (4): white solid; $[\alpha]_D^{28}$ 0 (*c* 0.1, MeOH); mp 93.5−94.0 °C; UV (MeOH) λ_{max} (log ε) 222 (3.96), 287 (3.84) nm; IR (film) ν_{max} 3342, 2956, 2920, 2850, 1578, 1461, 1346, 1288, 1267, 1163, 1128, 823, 725, 606 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS *m*/*z* 294.2428 [M + H]$^+$ (calcd for $C_{18}H_{32}NO_2$, 294.2428); TLC R_f 0.65 (1:9 *n*-hexane−EtOAc).

8′-Multijuguinol (5): white solid; $[\alpha]_D^{28}$ 0 (*c* 0.1, MeOH); mp 80.9−81.5 °C; UV (MeOH) λ_{max} (log ε) 222 (3.87), 287 (3.76) nm; IR (film) ν_{max} 3421, 3352, 2960, 2926, 2850, 1579, 1462, 1356, 1280, 1167, 1130, 945, 831, 725, 623, 596 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS *m*/*z* 294.2429 [M + H]$^+$ (calcd for $C_{18}H_{32}NO_2$, 294.2428); TLC R_f 0.65 (1:9 *n*-hexane−EtOAc).

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Preparation of the (*R*)-MTPA Ester Derivatives of 4 by the Mosher Ester Procedure. (*R*)-MTPA ester of compound **4** was prepared using the Mosher ester procedure.[10] Compound **4** (1.5 mg) was dried under vacuum, resuspended in pyridine-d_5 (0.75 mL), and transferred into clean NMR tube, respectively. (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*S*)-MTPA-Cl] (10 μL) was immediately added into the NMR tube under an N_2 gas stream, and then the NMR tube were shaken to ensure even mixing. The reaction afforded (*R*)-MTPA ester derivatives (*R*)-MTPA-(*R*)-7′-multijuguinol (**4a**) and (*R*)-MTPA-(*S*)-7′-multijuguinol (**4b**) (Figure 2). The spectra of the mixture of **4a** and **4b** were obtained directly from the reaction NMR tube that was permitted to stand at room temperature and monitored every 1 h by ^{1}H NMR. The reaction was found to be complete after 5 h (Figures S174−S176, Support Information).

Bioautographic Assay. The AChE inhibitory activity of the pure compounds was determined using a TLC bioautographic assay as described previously.[11] Compounds **1**−**5** were spotted in the TLC layers in the 0.1 to 6.0 μg range, which was followed by development with *n*-hexane−EtOAc (1:4, v/v) and subsequent drying. The plates were then sprayed with the enzyme solution (6.66 U/mL), thoroughly dried, and incubated at 37 °C for 20 min (moist atmosphere). Enzyme activity was detected by spraying with a solution consisting of 0.25% 1-naphthyl acetate in EtOH plus 0.25% Fast Blue B salt aqueous solution. Potential acetylcholinesterase inhibitors appeared as clear zones on a purple-colored background. Electric eel AChE type V (product no. C 2888, 1000 U) and the other reagents were purchased from Sigma-Aldrich.

Microplate Assay. The AChE inhibitory activity of compounds **1**−**5** was evaluated using the modified methods of Ellman et al. and Rhee et al. in a 96-well microplate, as described previously.[12] In this method, the enzyme hydrolyzes the substrate acetylthiocholine, which results in production of thiocholine. The latter reacts with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), thereby producing 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, which can be detected at 405 nm. For the experiments, 25 μL of acetylthiocholine iodide aqueous solution (15 mM), 125 μL of DTNB (3 mM) in buffer C, 50 μL of buffer B, and 25 μL of the sample diluted in buffer A were added to the 96-well plates, to give a range of concentrations going from 0.78 to 100 μg/mL. The absorbance was measured at 405 nm every 30 s, three times. Next, 25 μL of a 0.22 U/mL solution of the enzyme was added, and the absorbance was read every 10 min, twice. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the rate of the reaction before the addition of the enzyme from the rate of the enzyme reaction. The percentage of inhibition was calculated in comparison to a blank (10% MeOH in buffer A), and a positive control of physostigmine was used in the same range of concentrations. The following buffers were employed: buffer A: 50 mM Tris-HCl (pH 8); buffer B: 50 mM Tris-HCl (pH 8) containing 0.1% bovine serum albumin V fraction; buffer C: 50 mM Tris-HCl (pH 8) containing 0.10 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$. All of the reagents were purchased from Sigma-Aldrich.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

TLC plates as well as IR, high-resolution mass, ^{1}H and ^{13}C NMR, and selected 2D spectra for compounds **1** to **5** are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

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