Two Novel Plumeran Indole Alkaloids Isolated from *Aspidosperma cylindrocarpon* (Apocynaceae)

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Two novel indole alkaloids with plumeran skeleton, *N*-benzoyl-12-demethoxycylindrocarine and *N*-cinnamoyl-12-demethoxycylindrocarine (**1** and **2**, resp.), were isolated from the MeOH extract of the stem bark of *Aspidosperma cylindrocarpon* MÜLL.ARG. These compounds were obtained by high-speed counter-current chromatography, and their structures were elucidated on the basis of their NMR (1D and 2D) data. They were tested *in vitro* against chloroquine-resistant strains of *Plasmodium falciparum*, and only **2** showed a weak activity (IC_{50} 127.97 ng/ml with respect to the standard drug). Several other known compounds, comprising steroids, flavonoids, the rarely found atraric acid, as well as the previously reported alkaloid **3**, were also isolated by conventional chromatographic techniques.

Introduction. – The *Aspidosperma* genus (Apocynaceae) comprises *ca.* 57 species, grouped in seven series according to the chemical characteristics of its alkaloid content [1]. Its occurrence is restricted to the Central and South Americas, between Mexico and Argentina [2]. Several species of *Aspidosperma* present biological activities, being used in folk medicine to treat malaria and leishmaniosis [3], *Diabetes mellitus,* and hypercholesterolemia [4], and against erectile dysfunction [5], amongst others [6].

Aspidosperma cylindrocarpon MÜLL.ARG. from the *Polyneura* series, is popularly known as '*peroba-osso*' or '*peroba-rosa*', and has a high content of indole alkaloids (IAs). A total of 17 IAs have been isolated and identified from this species up to date [2]. Many other related IAs were obtained through organic synthesis [7a–7d].

In the present article, the isolation and characterization of two novel plumeran indole alkaloids, N^1-benzoyl-12-demethoxycylindrocarine (= methyl (2β,12β,19α)-1-benzoylaspidospermidin-21-oate; **1**) and N^1-cinnamoyl-12-demethoxycylindrocarine (= methyl (2β,12β,19α)-1-[(2*E*)-3-phenylprop-2-enoyl]aspidospermidin-21-oate; **2**) described (*Fig. 1*). Also, several other compounds were isolated (*Fig. 1*), including

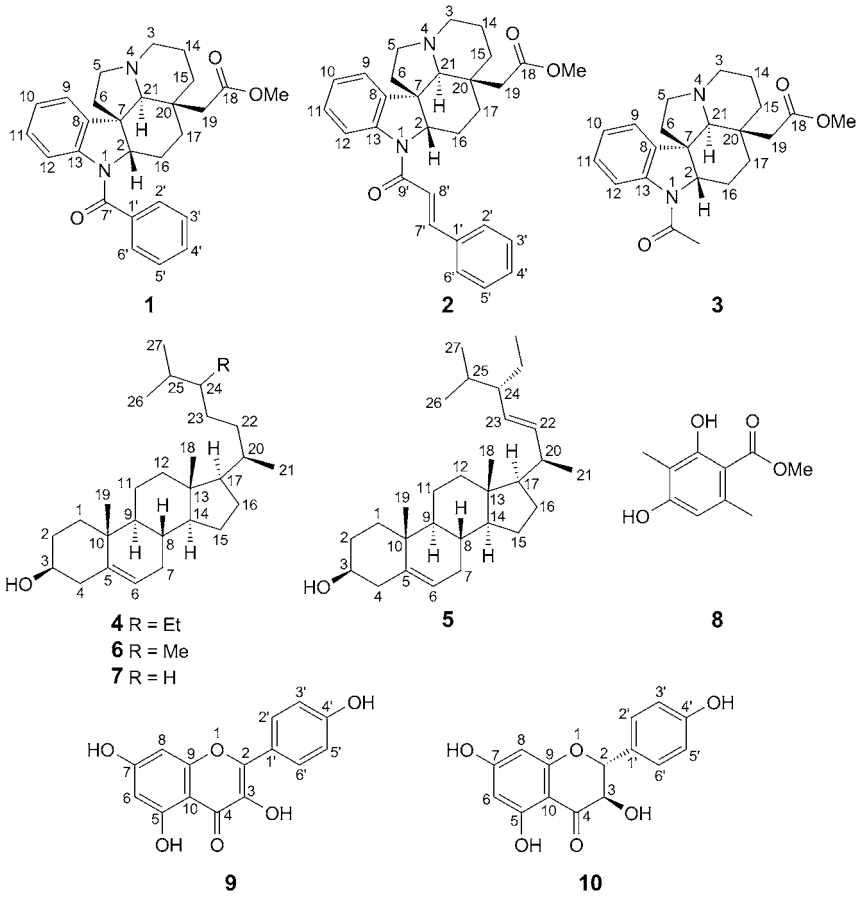

Fig. 1. *Structures of the compounds isolated from* A. cylindrocarpon

another known alkaloid, N^1-acetyl-12-demethoxycylindrocarine (= methyl (2β,12β,-19α)-1-acetylaspidospermidin-21-oate; **3**) [1][7c]; four known steroids, stigmast-5-en-3-ol (**4**), stigmasta-5,22-dien-3-ol (**5**), ergost-5-en-3-ol (**6**), and cholest-5-en-3-ol (**7**) [8]; methyl 2,4-dihydroxy-3,6-dimethylbenzoate (atraric acid; **8**), a rarely found metabolite of higher plants according to the 'Dictionary of Natural Products' [9][10], and two flavonoids, kaempferol (**9**) and aromadendrin (**10**) [11]. All the compounds were isolated trough conventional column chromatography, except for the two novel alkaloids **1** and **2**, which were obtained in pure form through high-performance counter-current chromatography (HP-CCC). The structures were established by spectrometric techniques, mainly 1D- and 2D ^{1}H- and ^{13}C-NMR, as well as HR-ESI-MS.

Results and Discussion. – By fractionation of the stem-bark MeOH extract of *A. cylindrocarpon* and through classical chromatographic methods, eight compounds, **3**–**10**, were isolated (*Fig. 1*). The known compounds **4**–**10** were identified on the basis of

their ^{1}H- and ^{13}C-NMR data, complemented by ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-NOESY, HSQC, and HMBC experiments [12]. These experiments also provided complete and unambiguous assignments of the ^{1}H- and ^{13}C-NMR chemical shifts of **1** and **2** (*Fig. 1*).

Compound **1** was obtained as a yellow oil. The IR spectrum showed bands at 2943–2789 (C–H stretching), and 1732 cm^{-1} (stretching of the γ-lactone C=O), in addition to further bands at 1643, 1477, 1392 (C=C stretching of the benzene ring), and 750 cm^{-1} (C–H bending of a monosubstituted benzene ring) [13].

Comparative analysis of the ^{1}H- and ^{13}C-DEPTQ-NMR spectra of compound **1** in (D_5)pyridine at 60° revealed signals corresponding to 27 C-atoms (*Table 1*). In addition, the typical structural features of an indole alkaloid endowed with a plumeran skeleton were detected: the presence of at least seven quaternary C-atoms (five sp^2 and two sp^3), eleven CH (nine sp^2 aromatics and two sp^3), eight CH_2 groups (all sp^3), and one Me group, as established in the literature [14][15].

The ^{1}H- and ^{13}C-DEPTQ-NMR spectra of **1** allowed us to deduce the presence of 27 C-atoms, comprising seven nonhydrogenated (two sp^3 (δ(C) 52.8 and 36.1) and five sp^2 quaternary C-atoms (three of them attributed to the aromatic ring, at δ(C) 134.8, 141.1, and 129.0, besides a CO group at δ(C) 170.3 and a COOMe group δ(C) 171.3)), eleven CH groups (two sp^3 (δ(C) 67.8 and 68.8) and nine sp^2 (δ(C) 118.0–130.1, attributed to a monosubstituted aromatic ring)), eight sp^3-CH_2 groups (δ(C) 21.6–52.9, including one linked to COOMe at δ(C) 42.9)), and one ester MeO group (δ(C)50.7). This led to the expanded molecular formula $(C)_7(CH)_{11}(CH_2)_8(MeO)$, suggesting an additional aromatic ring, most likely a benzoyl group (δ(H) of H–C(2′/6′) 7.74–7.70), which has been reported before for other alkaloids isolated from *A. cylindrocarpon* [1][2].

The HR-ESI-MS of **1** exhibited a base peak corresponding to $[\mathbf{1}+H]^+$ at *m*/*z* 431.2314 ($C_{27}H_{31}N_2O_3^+$; calc. 431.2329, $\Delta(m/z) = -3.47$ ppm). These data, in combination with the ^{13}C-NMR spectrum, suggested the molecular formula $C_{27}H_{30}N_2O_3$.

Compound **2** was obtained as a white oil. The IR spectrum showed bands at 3059–2727 (C–H stretching) and 1737 cm^{-1} (stretching of the γ-lactone C=O), besides further bands at 1658, 1614, 1402 (C=C stretching of the benzene ring) and 976 and 860 (C–H bending of monosubstituted benzene ring), and 765, 702 cm^{-1} (C=C stretching in alkenes) [13].

The ^{1}H- and ^{13}C-DEPTQ-NMR spectra of **2** revealed the presence of 29 C-atoms (*Table 1*). These spectra displayed the same characteristic signals as **1**, plus those for two additional quaternary C-atoms (one of them corresponding to an aromatic C-atom (δ(C) 141.9), and the other to a CO group (δ(C) 164.3), besides the COOMe group at δ(C) 171.9)), seven additional sp^2-CH groups (five of them attributed to aromatic C-atoms (δ(C) 128.0–129.9) and two to olefinic C-atoms (δ(C) 143.6 and 118.9)), eight sp^3-CH_2 signals (δ(C) 21.5–55.6, including the one linked to COOMe at δ(C) 42.3), and the MeO signal (δ(C) 51.2). These data provided the expanded molecular formula $(C)_7(CH)_{13}(CH_2)_8(MeO)$, indicating the presence of an additional aromatic ring, possibly due to a cinnamoyl group, whose occurrence as a structural moiety of related alkaloids from *A. cylindrocarpon* was previously reported [1][2].

The HR-ESI-MS of **2** displayed a base peak corresponding to $[\mathbf{2}+H]^+$ at *m*/*z* 457.2498 ($C_{29}H_{33}N_2O_3^+$; calc. 457.2486, $\Delta(m/z)$ 2.62 ppm), These data, in combination with the ^{13}C-NMR spectrum, provided the molecular formula $C_{29}H_{32}N_2O_3$. Peaks

Table 1. *^1H- and ^{13}C-NMR Data* (at 500 and 125 MHz, resp.) *of the Two Novel Indole Alkaloids,* **1** *and* **2**, *in (D_5)Pyridine and $CDCl_3$ as Solvent, Respectively, and with TMS as internal reference.* δ in ppm and J in Hz)[a])

Position	**1**				**2**			
	HSQC		HMBC		HSQC		HMBC	
	δ(H)	δ(C)	(^2J(H→C))	(^3J(H→C))	δ(H)	δ(C)	(^2J(H→C))	(^3J(H→C))
2	4.66–4.46 (*m*)	67.8			4.39 (*dd*, *J* = 11.3, 6.4)	66.2		6; 17; 21
3	2.95–2.93 (*m*), 2.04–2.02 (*m*)	52.9			3.11 (br. *d*, *J* = 10.6), 2.07–2.04 (*m*)	55.6		21
5	3.14–3.12 (*m*), 2.35–2.33 (*m*)	51.8			3.21 (*dt*, *J* = 9.1, 3.2), 2.21–2.28 (*m*)	52.3		21
6	2.36–2.35 (*m*), 1.75–1.69 (*m*)	38.8			2.10–2.08 (*m*), 1.61–1.59 (*m*)	39.3	5	2; 21
7	–	52.8		9	–	52.9	2; 6; 21	5; 9; 16
8	–	134.8		10; 12	–	135.3		2; 10; 12; 21
9	7.42 (*d-like*, *J* = 7.4)	124.5			7.28 (*d*, *J* = 8.8)	128.1		
10	7.15 (*t-like*, *J* = 7.4)	127.7			7.10 (*t*, *J* = 7.1)	124.6		
11	7.47–7.44 (*m*)	128.3			7.26 (*t*, *J* = 7.4)	122.2	10	9
12	7.47–7.44 (*m*)	118.0			8.33 (*d*, *J* = 8.0)	118.6	11	10
13	–	141.1			–	137.6	2	2; 9; 11
14	1.74–1.72 (*m*), 1.39–1.33 (*m*)	21.6			1.96–1.94 (*m*), 1.61–1.59 (*m*)	21.5	3; 15	
15	1.75–1.72 (*m*), 1.39–1.37 (*m*)	34.6		19	1.71–1.67 (*m*), 1.48–1.45 (*m*)	34.9		3; 19
16	2.32–2.29 (*m*), 1.70–1.66 (*m*)	25.9			2.11–2.08 (*m*), 1.48–1.46 (*m*)	27.0		
17	1.70–1.66 (*m*), 1.40–1.98 (*m*)	24.3		19	2.12–2.11 (*m*), 1.37 (br. *d*, *J* = 13.6)	24.4	19; 21	19; 21
18	–	171.3	19	MeO	–	171.9	19	MeO
19	2.46 (*d*, *J* = 14.2), 2.12 (*d*, *J* = 14.2)	42.9		19	2.29 (*d*, *J* = 14.3), 2.05 (*d*, *J* = 14.3)	42.3		15b; 17a; 21
20	–	36.1	19		–	35.9	19; 21	
21	2.75 (*s*)	68.8		19	2.60 (*s*)	69.3		3; 5; 6; 15; 17
1′	–	129.0			–	141.9	2′/6′	8′
2′/6′	7.74–7.70 (*m*)	127.3	3′/5′		7.62 (br. *d*, *J* = 6.6)	128.0		
3′/5′	7.47–7.44 (*m*)	128.7			7.45–7.35 (*m*)	128.9		
4′	7.47–7.44 (*m*)	130.1			7.45–7.35 (*m*)	129.9		
7′	–	170.3			7.89 (*d*, *J* = 15.3)	143.6	8′	
8′	–	–			6.81 (*d*, *J* = 15.3)	118.9	7′	
9′					–	164.3	8′	7′
MeO	3.57 (*s*)	50.7			3.58 (*s*)	51.2		

[a]) Number of H-atoms bound to C-atoms deduced by comparative analysis of {^{1}H}- and DEPTQ-^{13}C-NMR spectra. Chemical shifts and coupling constants were obtained of 1D ^{1}H-NMR spectrum. ^{1}H,^{1}H-COSY and ^{1}H,^{1}H-NOESY experiments were also used for these assignments. Superimposed ^{1}H signals were described without multiplicity, and chemical shifts were deduced from HSQC and HMBC spectra.

corresponding to the ions $[2M+H]^+$ at m/z 913.4898 ($C_{58}H_{65}N_4O_6^+$; calc. 913.4899; $\Delta(m/z) = -0.1$ ppm) and $[2M+Na]^+$ at m/z 935.4707 ($C_{58}H_{64}N_4NaO_6^+$; calc. 935.4718; $\Delta(m/z) = -1.17$ ppm) were also observed.

A number of signals in the HSQC spectra of both **1** and **2** were useful to identify their plumeran skeletons: the chemical shift of H–C(2) around 4.0 (δ(H) 4.66 – 4.46 for **1** and 4.39 for **2**) and of H–C(21) (δ(C) 68.8 and 69.3, for **1** and **2**, resp.). Another important feature, in this case, is the behavior of the two diasterotopic H-atoms at C(19). The signals of these H-atoms, in presence of the COOMe substituent, were observed as *doublet*s at δ(H) 2.46 and 2.12, and 2.29 and 2.05, for **1** and **2**, respectively. Their coupling constants (J(19a,19b) of 14.2 and 14.3, resp.) are typical of geminal H-atoms.

For **1**, the presence of the additional aromatic ring can be confirmed by the HMBCs ($^3J(H \rightarrow C)$) between C(1′) and H–C(3′)/H–C(5′) and between C(4′) and H–C(2′)/H–C(6′). The presence of the COOMe group in C(19) can be evidenced by the HMBCS ($^2J(H \rightarrow C)$) between C(18) and CH_2(19), and by the HMBC ($^3J(H \rightarrow C)$) between C(18) and the H-atoms of the MeO group. The HMBC ($^2J(H \rightarrow C)$) between C(20) and CH_2(19), established the position of the side chain, characteristic of this type of indole-alkaloid skeleton [14].

For **2**, the location of the C=C bond, *i.e.*, C(7′)=C(8′), was deduced from the HMBCs between the C-atoms resonating at δ(C)143.6 and 118.9, and the olefinic H-atoms with signals at δ(H) 7.89 (H–C(7′)) and 6.81 (H–C(8′)), respectively. The vicinal *trans*-coupling ($^3J = 15.3$ Hz) between these H-atoms was confirmed by the ^{1}H,^{1}H-COSY spectrum. The HMBC spectrum shows the ($^2J(H \rightarrow C)$) and ($^3J(H \rightarrow C)$) correlations between the C(9′), and the H–C(8′) and H–C(7′), and the ($^3J(H \rightarrow C)$) correlation between C(1′) and H–C(8′), in agreement with the presence of an *N*-cinnamoyl group **2**. The additional aromatic ring can be confirmed by the HMBCs ($^2J(H \rightarrow C)$) between C(1′) and H–C(2′)/H–C(6′) and between H–C(2′)/H–C(6′), and H–C(3′)/H–C(5′), as well as the connection of C(1′) to C(7′) by the HMBC ($^3J(H \rightarrow C)$) with H–C(8′).

The relative configuration of **2** was suggested based on the spatial interactions observed in the ^{1}H,^{1}H-NOESY spectrum, as outlined in *Fig. 2*. For **2**, the ^{1}H,^{1}H-NOESY interactions H–C(2) one H-atom of CH_2(19), as well as the interactions H–C(2) one H-atom of the CH_2(6), and H–C(2)/H–C(8′) indicated that these groups are β-oriented.

The alkaloid N^1-acetyl-12-demethoxycylindrocarine (**3**) has been already isolated from other natural sources [1][16], but it is more often cited as a product of organic synthesis [7c][17]. However, the NMR data provided in all these articles were insufficient or incomplete, so they are presented in the *Exper. Part*.

The antimalarial activities of **1** and **2** are compiled in *Table 2*. In despite of several alkaloids isolated from *Aspidosperma* species, which were tested *in vitro* against *P. falciparum* strains and showed remarkable activities [18], these two new alkaloids did not display the same performance. The compound **1** showed a chloroquine-resistant *P. falciparum* strain growth inhibition less than 50% at both tested concentrations. So, the IC_{50} value was not even evaluated, once the sample is considered inactive. The compound **2** showed a growth inhibition rate $>50\%$ (72%) at the concentration of

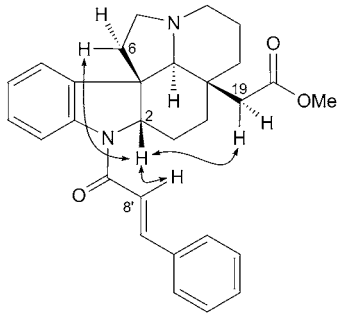

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Fig. 2. The main NOE interactions *of compound* **2**.

Table 2. P. falciparum *Chloroquine-Resistant Strain* (W2) *Growth Decrease* [%] *and* IC_{50} *Values of* **1** *and* **2**

Sample	Concentration [μg/ml]	Growth decrease [%]	IC_{50} [μg/ml]	Classification
1	25	30	> 50	Inactive
	50	43		
2	25	43	25 – 50	Weakly active
	50	72		

50 μg/ml, which led to the determination of the IC_{50} value of 25 – 50 μg/ml, implying a weak activity.

These results can be explained by the lateral chain at C(20). The compounds that are structurally characterized by the presence of a free Et group are more active than those in which the corresponding C-atoms are involved in a tetrahydrofuran ring [18]. So, it can be inferred that the presence of the strongly oxidized MeO-containing lateral chain is responsible for the decrease of the activity.

It should be mentioned that there are no reports for the use of *Aspidosperma cylindrocarpon* in traditional medicine. This assay was performed considering potential antiplasmodial activity that is common among the indole alkaloids isolated from *Aspidosperma* species.

Experimental Part

General. The isolation of the novel compounds **1** and **2** was performed by using a *Dynamic Extractions Ltda. Model Spectrum-1000* mini high-speed counter-current chromatograph (Berkshire, United Kingdom), equipped with a self-balancing three-coil centrifuge rotor, containing two prep. multiplayer coils with total capacity of 142 ml, the internal diameter of PTFE tubing was 1.6 mm. Chromatographic purifications were carried out over silica gel (SiO_2; 70 – 230 mesh). Silica gel $60F_{254}$ was used in TLC; sulfuric vanillin and *Dragendorff*'s reagents were used for visualization. FT-IR Spectra: *FTIR-8300 Shimadzu* spectrometer; KBr disk; $\tilde{\nu}$ in cm^{-1}. 1D and 2D ^1H- and ^{13}C-NMR spectra: *Bruker DRX 500* MHz spectrometer, equipped with inverse probes and pulsed-field gradient, operating at 500 (^1H) and 125 (^{13}C) MHz; $CDCl_3$ was used as solvent and TMS as internal standard; chemical shifts, δ, in ppm and coupling constants, J, in Hz. 1D ^1H and ^{13}C spectra were acquired under standard conditions by

using a 5 mm $^{1}H/^{13}C$ dual probe. Standard pulse sequences were used for 2D spectra by using a multinuclear inverse detection 5-mm probe with field gradient. HR-ESI-MS: *UltrOTOF-Q* (*Bruker Daltonics*, Billerica, MA) mass spectrometer; in the positive-ion mode.

Plant Material. The stem bark of *A. cylindrocarpon* MÜLL.ARG. was collected in February 2010 near the Reserva Florestal Vale, Linhares City, Espírito Santo State, Brazil (S 19° 36,211′; O 39° 57,543′). A voucher specimen (CVRD-313) was deposited with the reserve's herbarium.

Extraction and Isolation. The stem bark of *A. cylindrocarpon* MÜLL.ARG. was dried, powdered (3.15 kg), and then exhaustively extracted with MeOH at r.t. for 3 d. After solvent evaporation under reduced pressure, 458.7 g of the crude MeOH extract were obtained. An aliquot (44.56 g) of this extract was then submitted to fractionation by column chromatography (CC, SiO_2; gradient of CH_2Cl_2/MeOH).

Fr. 1 (11.08 g) was successively fractionated by CC (SiO_2; gradient CH_2Cl_2/MeOH) to provide the *Fr. 1.1* containing a mixture of the steroids **4** to **7** (48.4 mg), and of atraric acid (**8**; 6.7 mg), *Frs. 1.8* and *1.9* containing kaempferol (**9**; 307.8 mg) and aromadendrin (**10**; 318.3 mg), and *Fr. 1.15* containing **3** (150.3 mg).

An aliquot (504.9 mg) of *Fr. 1.10* (1.3 g) was submitted to high-performance countercurrent-chromatography (HP-CCC), employing hexane/AcOEt/MeOH/H_2O 1:2:1.75:1, (*v*/*v*/*v*/*v*) (Arizona system). More detailed information on this technique will be published by the authors [19]. This fractionation resulted in 20 fractions, some containing two novel plumeran indole alkaloids **1** (26.6 mg) and **2** (23.2 mg), together with two other simplest compounds, still in elucidation process.

N^1-Benzoylcylindrocarine (= *Methyl (2β,12β,19α)-1-Benzoylaspidospermidin-21-oate*; **1**). Yellow oil. IR (KBr): 2943–2789 (C–H stretching), 1732 (C=O), 1643, 1477, 1392 (benzene ring). ^{1}H- and ^{13}C-NMR (C_5D_5N): see *Table 1*. HR-ESI-MS: 430.2314 ($[M+H]^+$, $C_{27}H_{30}N_2O_3^+$; calc. 430.2329).

12-Demethoxy-N^1-cinnamoylcylindrocarine (= *Methyl (2β,12β,19α)-1-[(2E)-3-Phenylprop-2-enoyl]-aspidospermidin-21-oate*; **2**). White oil. IR (KBr): 3059–2727 (C–H stretching), 1737 (C=O), 1658, 1614, 1402 (benzene ring), 976, 860 (benzene ring), 765, 702 (alkenes). ^{1}H and ^{13}C-NMR ($CDCl_3$): see *Table 1*. HR-ESI-MS: 457.2498 ($[M+H]^+$, $C_{29}H_{33}N_2O_3^+$; calc. 457.2491).

12-Demethoxy-N^1-acetylcylindrocarine (= *Methyl (2β,12β,19α)-1-Acetylaspidospermidin-21-oate*; **3**). ^{1}H-NMR ($CDCl_3$): 8.15 (*d*, *J* = 8.0, H–C(12)); 7.23 (*dt*, *J* = 11.4, 7.4, H–C(11)); 7.21 (*d*, *J* = 7.4, H–C(9)); 7.06 (*dt*, *J* = 7.4, 1.0, H–C(10)); 4.10 (*dd*, *J* = 11.3, 6.2, H–C(2)); 3.63 (*s*, MeO–C(18)); 3.19 (*dt*, *J* = 9.2, 3.4, H_a–C(5)); 3.08 (*dt*, *J* = 11.0, 1.9, H_a–C(3)); 2.54 (*s*, H–C(21)); 2.54–2.21 (*m*, H_b–C(5)); 2.36 (*d*, *J* = 13.9, H_a–C(19)); 2.30 (*s*, AcN); 2.26–2.17 (*m*, H_a–C(17)); 2.20–2.00 (*m*, H_a–C(6)); 2.14–1.96 (*m*, H_a–C(16)); 2.10–1.94 (*m*, H_b–C(3)); 2.06 (*d*, *J* = 13.9, H_b–C(19)); 2.00–1.56 (*m*, H_a–C(15)); 1.74–1.56 (*m*, H_b–C(16)); 1.61–1.59 (*m*, H_a–C(14)); 1.60–1.52 (*m*, H_b–C(14)); 1.56–1.38 (*m*, H_b–C(6)); 1.50–1.35 (*m*, H_b–C(17)); 1.49–1.35 (*m*, H_b–C(15)). ^{13}C-NMR: 171.9 (C(18)); 168.5 (CO of AcN); 140.9 (C(13)); 137.4 (C(8)); 127.9 (C(11)); 124.4 (C(10)); 122.3 (C(9)); 118.4 (C(12)); 69.2 (C(21)); 67.6 (C(2)); 53.5 (C(3)); 52.8 (C(7)); 52.3 (C(5)); 51.2 (*Me*O–C(18)); 42.4 (C(19)); 39.2 (C(6)); 35.9 (C(20)); 34.7 (C(15)); 25.9 (C(16)); 24.3 (C(17)); 23.3 (Me of AcN)); 21.5 (C(14)).

In vitro *Antimalarial Test.* The *in vitro* tests of **1** and **2** were carried out with erythrocytes infected with chloroquine-resistant *P. falciparum* (W2 clone), by the lactate dehydrogenase (LDH) assay [20]. The general procedures are described below:

1. Parasite-drug incubation: 20 µl of each dilution of the tested compounds are added to flat-bottomed 96-weel microtiter plates, each containing 180 µl of a suspension of *P. falciparum* infected erythrocytes (1% hematocrit, 2% parasitemia), three times. Controls without drugs, with infected erythrocytes (positive control) or non-infected erythrocytes (negative control), were performed.

2. The microtiter plates are incubated at 37° under a reduced O_2 atmosphere (5% CO_2) for 48 h, with test samples and controls. Then, the plates are frozen (−20° for at least 24 h) to achieve the erythrocytes lysis.

3. Thereafter, 100 µl of *Malstat* reagent and 25 µl of NBT/PES reagent were added to each well of a new, duplicated flat-bottom 96-weels microtiter plates. The cell lysate was then resuspended and transferred to the corresponding wells, thereby initiating the LDH reaction. After 1 h of incubation, the absorbance in each well was registered by a spectrophotometer (540 nm). The values were statistically treated at Origin 8.0, to generate the log dose–response curves.

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