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Quaternary indole alkaloids from the stem bark of *Strychnos guianensis*

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Abstract

Two new quaternary alkaloids, 9-methoxy-N_b-methylgeissoschizol and guiachrysine together with the known compounds C-alkaloid O, fluorocurine, mavacurine, macusine B and C-profluorocurine, were isolated from *Strychnos guianensis* stembark. The structures of the compounds were elucidated on the basis of spectroscopic studies

Keywords: *Strychnos guianensis*; Loganiaceae; Quaternary alkaloids; 9-Methoxy-N_b-methylgeissoschizol; Guiachrysine; C-alkaloid O; Fluorocurine; Mavacurine; Macusine B; C-profluorocurine; 2D-NMR

1. Introduction

Strychnos guianensis (Aubl.) Mart. (Loganiaceae) is a moderately sized liana well distributed in the basin of middle and upper Rio Orinoco and throughout the entire Amazon basin. It was the first plant source of curare to be collected and identified botanically, and the use of this species in the preparation of curare is very widespread from Colombia to Surinam and in Ecuador and Brazil (Bisset, 1992a, 1992b; Quetin-Leclercq, Angenot & Bisset, 1990). *S. guianensis* was studied pharmacologically by King (1949), West (1937) and by Marini-Bettolo, Casinovi and Hernandez Alvarado (1961) and Marini-Bettolo and Iorio (1956), who also carried out chemical studies. They showed that crude extracts from the root and stem bark displayed muscle-relaxant activity. Several alkaloids were characterized by their chromatographic properties on paper, their coloration with ceric sulfate reagent and sometimes by their UV spectra (Marini-Bettolo & Iorio, 1956). Previously, we have reported the structures of guianensine (Quetin-Leclercq, Llabrès, Warin, Belem-Pinheiro, Mavar-Manga & Angenot, 1995) and 9-methoxygeissoschizol (Mavar-Manga, Quetin-Leclercq, Llabrès, Belem-Pinheiro, Imbiriba da Rocha & Angenot, 1996) from the tertiary alkaloidal fractions, and, recently, the structure of guiaflavine (**8**) (Penelle, Tits, Christen, Brandt, Frédérich & Angenot, 1999). This paper describes the purification, identification, and structural determination of two new quaternary indole alkaloids 9-methoxy-N_b-methylgeissoschizol (**1**) and guiachrysine (**2**), and the occurrence of C-alkaloid O (**3**), fluorocurine (**4**), C-profluorocurine (**5**), mavacurine (**6**), and macusine B (**7**). Preliminary results of a biological approach were also mentioned for **1**, **2**, and **3**.

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2. Results and discussion

After elimination of the tertiary alkaloidal fractions, the crude quaternary extract, precipitated with Mayer's reagent and converted to the chloride form, was separated by MPLC on a Lichrospher RP select B column using a gradient of MeCN in an aqueous solution of heptanesulfonic acid (sodium salt) adjusted to pH 2 with phosphoric acid. The initial fractions contained fluorocurine (**4**), C-profluorocurine (**5**), mavacurine (**6**) and macusine B (**7**); the next fractions yielded 9-methoxy-N_b-methylgeissoschizol (**1**) and C-alkaloid O (**3**), while the latter afforded guiachrysine (**2**) and guiaflavine (**8**). Sometimes, these fractions were submitted to column chromatography on cellulose and eluted with the system **C** of Karrer and Schmid (methyl ethyl ketone saturated with water and 1–3% MeOH) (Schmid, Kebrle & Karrer, 1952).

9-Methoxy-N_b-methylgeissoschizol (**1**) is a monomeric quaternary indole alkaloid that gave a grey color with the ceric sulfate–sulfuric acid reagent. Its UV spectrum showed maxima at 207, 220, 259 and 291 nm according to an indole chromophore. There was no bathochromic shift in alkaline solution, indicating the absence of any phenolic group. There was no more hypsochromic shift in acidic solution. No carbonyl band was observed in its IR spectrum. The HRESIMS gave a molecular ion at m/z 341.2223 that was consistent with the elemental composition $C_{21}H_{29}N_2O_2$. The structure of **1** was mainly deduced from ^{1}H-and ^{13}C-NMR spectra. Three aromatic protons (two doublets and one triplet) appeared in the ^{1}H-NMR whose chemical shifts and couplings were indicative of a 9- or 12-substituted indole nucleus. An one proton quartet was observed at 5.99 ppm, coupled in the COSY spectrum with a three-proton doublet at 1.84 ppm suggesting the presence of an ethylidene side-chain. This proton was also weakly coupled (COSY ^{1}H–^{1}H) with one proton of a methylene group at 4.35 ppm, the other proton of this methylene was observed at 3.9 ppm. This group corresponded to C(21)H_2. A three-proton singlet at 3.9 ppm indicated the presence of a methoxyl group. One other three-proton singlet at 3.19 ppm indicated a quaternary N_b–Me group. The 2D-COSY spectrum also allowed to deduce one other spin system: a CH_2–CH_2 unit related to the protons at 3.32, 3.42, 3.77 and 3.82 ppm. The chemical shift of the last two protons was in agreement with a linkage to a quaternary nitrogen atom. This fragment probably arises from the tryptamine moiety and should correspond to N–C(5)H_2–C(6)H_2. The connectivities of other protons in the 2D-COSY spectrum provided the means of assembling the following substructure: CH–CH_2–CH–CH_2–CH_2. The chemical shift of the protons of the last methylene of this fragment (3.51 ppm) was in agreement with linkage to an oxygen atom. Furthermore, the presence of a hydroxyl group was confirmed by acetylation at room temperature. The ^{13}C chemical shifts (total decoupling, DEPT and C–H correlation) fully supported this structure and allowed to localize the substituted position on the aromatic ring. Comparison of the observed chemical shifts of C-8, C-9, C-10, C-11, C-12 and C-13 with those calculated using substituent-induced chemical shifts (Verpoorte, van Beek, Riegman, Hylands & Bisset, 1984) for the corresponding atoms of 9-methoxygeissoschizol indicated the same substitution, as in guianensine (Quetin-Leclercq et al., 1995), C- alkaloid O (Borris, Guggisberg & Hesse, 1983) and guiaflavine (Penelle et al., 1999). The HMBC spectrum confirmed this attribution and also allowed the assignments made for the quaternary carbons. The H-11 signal correlated to C-9, C-12 and C-13, while H-10 correlated to C-8, C-9 and C-12; H-12 was coupled to C-8 and C-10. The ^{13}C-NMR spectrum confirmed the presence of a C-21 compound and clearly showed the presence of an N_b-Me group (δ 48.9 ppm), a methoxyl group (δ 54.6 ppm), C-19 (δ 132.25 ppm), C-20 (δ 129.4 ppm), C-5 (δ 60.08 ppm), C-3 (δ 64.9 ppm), C-21 (δ 63.0 ppm) and eight indolic carbons. The above spectral features confirmed the structure of a quaternary corynantheine-type compound. The stereochemistry still must be considered. The positive Cotton effect observed between 235 and 266 nm and negative between 266 and 285 nm indicates a structure with C/D *cis* rings and a H-3α configuration (Toth, Hetenyi, Clauder & Kajtar, 1978). The proton chemical shift of the *N*-methyl group is also in accord with a C/D *cis* ring junction. Indeed, this stereochemistry is supported by the relatively low field position at δ 3.19 ppm of the quaternary N_b-methyl, suggesting a *cis*-relationship between the N_b-methyl and the H-3 (Arata, Aoki, Hanaoka & Kamei, 1975). A NOESY experiment confirms this relationship. Indeed, a correlation between the N_b-methyl and the H-3 is observed. The H-15α (*S*) configuration agrees with the biogenetic hypothesis (Klyne & Buckingham, 1974). The orientation of the ethylidenic side-chain is proposed to be *E* because of the chemical shifts observed for C-21 and C-15 (Coune, Angenot & Denoël, 1980; Lounasmaa, Jokela, Hanhinen, Miettinen & Salo, 1994; Mavar-Manga et al., 1996). Methylation of authentic 9-methoxygeissoschizol confirmed our hypothesis. After TLC control on Si gel F_{254}, using the solvent system MeOH–25% aqueous MeCOONa solution–Me_2CO (65:35:20) (Paesen, Quintens, Thoithi, Roets, Reybrouck & Hoogmartens, 1994), retention factors of compound **1** and authenthic methylated sample were found to be the same. A confirmation by HPLC analysis was also performed. Alkaloids were detected at 250 nm by a diode-array detector. The mixture of compound **1** and authentic 9-

methoxygeissoschizol after methylation gave only one peak.

C-alkaloid O (**3**) was identified by comparison of its spectral values with an authentic sample. C-alkaloid O has previously been isolated from a sample of calabash curare collected on Rio Içа borders (Colombia/Brazil) (Giesbrecht, Meyer, Bächli, Schmid & Karrer, 1954), and a reinvestigation of this alkaloid led to the determination of its chemical structure (Borris et al., 1983). However, it is the first time that this alkaloid is described in a plant. The interpretation of the NMR spectra led to some little differences with the existent data. The shifts of C-5, C-10, C-12 and C-17 (δ 61.6, 101, 106.2 and 50.4) were deduced from the interpretation of its 2D NMR spectra (Table 3) and were consistent with literature data (Verpoorte et al., 1984). Furthermore, an HPLC comparison of this isolated alkaloid with an authentic sample was carried out. Retention times (R_t) of both the samples were the same, and a mixture of these alkaloids gave only one peak.

The MPLC pooled fractions containing compounds **4** and **5** were submitted to column chromatography on cellulose, eluted with the system C of Karrer and Schmid to yield pure products. Compound **4** (m/z 325 $[M]^+$) was identified as fluorocurine after comparison by TLC, UV spectrum, IR, CD curve and diode-array HPLC with an authentic sample isolated in our laboratory from *S. variabilis*, and comparison with literature data (Tits, Franz, Tavernier & Angenot, 1981). Compound **5** (m/z 343 $[M]^+$ and UV spectrum maxima 210, 250, 295 nm) was identified as C-profluorocurine by comparison with literature data, which described C-profluorocurine isolated from a sample of *Strychnos toxifera* and calabash curare (Asmis, Bächli, Giesbrecht, Kebrle, Schmid & Karrer, 1954; Hesse et al., 1964). Indeed, compound **5** gave a red-violet color with $Ce(SO_4)_2$ becoming olive-green on standing, and a carmin color with conc. HNO_3. Moreover, compound **5** became fluorescent in a mixture with anhydrous methanol and gaseous chlorhydric acid (1:1) (Fritz, Wieland & Besch, 1958).

Compounds **6** and **7** were submitted to column chromatography on cellulose, eluted with the system C of Karrer and Schmid to yield pure products. Compound **6** was identified as mavacurine (m/z 309 $[M]^+$) after comparison by TLC, UV spectrum, IR, MS and diode-array HPLC with an authentic sample isolated in our laboratory from *S. variabilis*, and after comparison with literature data (Tits et al., 1981; Coune, Tits & Angenot, 1982). Compound **7** was identified as macusine **B** (m/z 309 $[M]^+$) by comparison with an authentic sample isolated in our laboratory from *S. usambarensis* (Angenot, 1975) and comparing with literature data (Battersby, Binks, Hodson & Yeowell, 1960; Battersby & Yeowell, 1964; Khan, Hesse & Schmid, 1967).

Compound **2** is an asymmetrical bisindole quaternary alkaloid that gave a blue-green color with the ceric sulfate–sulfuric acid reagent. The same coloration is observed by spraying guianensine (Quetin-Leclercq et al., 1995), strychnochrysine (Gadi Biala et al., 1998), afrocurarine (Caprasse, Angenot, Tavernier & Anteunis, 1984), and guiaflavine (Penelle et al., 1999). The orange color of guiachrysine (**2**) is consistent with its UV spectrum showing maxima at 205, 254, 320 and 426 nm. This highly conjugated chromophore is similar to that of afrocurarine, strychnochrysine and guiaflavine because it is not modified in alkali and therefore differing from an anhydronium base (Gribble, 1988). The electrospray mass spectrum gave a molecular peak at m/z 628 consistent with the elemental composition $C_{40}H_{44}N_4O_3$. Furthermore, the molecular weight, 627.3306, established by high resolution ESIMS, corresponds to the elemental composition $C_{40}H_{44}N_4O_3$, $[M - H]^+$. The presence of a corynanium afrocurarine-like moiety as suggested by the UV spectrum is confirmed by mass fragments at m/z 145, 157, 169, 181, 193, 205, 247 and 277 (Caprasse et al., 1984). However, the structure of **2** is mainly deduced from 2D NMR spectra and comparison with analogous compounds data (Caprasse et al., 1984; Frédérich et al., 1998; Gadi Biala et al., 1998; Massiot et al., 1983b; Penelle et al., 1999; Quetin-Leclercq et al., 1995; Wenkert, Cheung & Gottlieb, 1978). In the aromatic region, two deshielded pyridinic protons at δ 8.05 and 8.41, due to H-14′ and H-21′, were observed and therefore, the shifts of C-14′ and C-21′ could be established by HSQC at δ 121.8 and 143.97, respectively. In the ^1H–^1H COSY spectrum of the aromatic region, we detected one set of four benzenic protons from an unsubstituted indole and another set of three protons from 9 or 12 substituted indole nucleus. The protons at δ 7.19 (t), 7.0 (d) and 6.53 (d) can be attributed to the corynanium part. Indeed, a comparison with the spectra of guianensine (Quetin-Leclercq et al., 1995), 9-methoxygeissoschizol (Mavar-Manga et al., 1996), C-alkaloid O (Borris et al., 1983) and guiaflavine (Penelle et al., 1999) indicates that the methoxyl group, corresponding to the three protons singlet assigned at δ 3.91, could be, according to Verpoorte et al. (1984), at the 9-position of the corynanium part. Consequently, the four remaining aromatic protons at δ 7.53 (d), 7.33 (t), 7.21 (d) and 7.03 (t) belong to the strychnan ring. The protonated aromatic carbons were assigned by their direct correlations observed in the HSQC and DEPT spectra. The HMBC spectrum confirms these attributions and allows the assignments of the quaternary carbons. The H-11′ correlated to C-9′, C-10′, and C-13′ (δ 156.9, 101.4, 142.5), while the H-10′ is correlated to C-8′, C-9′ and C-12′ (δ 117.4, 156.9 and

106.3); the H-12′ is coupled to C-8′, C-9′ and C-10′ (δ 117.4, 156.9 and 101.4). Similar correlations were observed for the indoline ring: the H-2, H-9 and H-11 correlated to C-13 (δ 145.9), while the H-10 and H-12 correlated to C-8 (δ 132.4), as shown in Table 1. Examination of NMR spectra reveals for the corynanium part, three methylene groups at δ 56.3, 22.2 and 25.3 ppm, which were assigned to C-5′, C-6′ and C-19′, respectively. In the COSY spectrum, a strong correlation between H-5′ and H-6′, indicating a dihydroflavopereirine skeleton, was observed (Caprasse, Coune & Angenot, 1983). The third methylene unit H_2-19′ was clearly coupled to the methyl protons H_3-18′ (δ 1.385). The HMBC spectrum showed H_3-18′ correlated to C-19′ and to C-20′ (δ 25.3, 138.95). The other half of the structure was also deduced from NMR data. The chemical shifts of the protons and the carbons (Table 1) support the structure of a strychnan skeleton with a N_b-methyl group (δ 49.31) (Wenkert et al., 1978), with four methylene groups (C-5, C-6, C-14 and C-21), and five methine groups (C-2, C-3, C-15, C-16 and C-17). The chemical shifts of C-18, C-19 and C-20 (δ 58.0, 136.1, 129.6) were in agreement with a 18-hydroxy group and a N_b-methyl group when we compare data of **2** with strychnochrysine (Gadi Biala et al., 1998), and guiaflavine (Penelle et al., 1999) with longicaudatine (Massiot et al., 1983b). The assignments of H-17 at δ 4.68 and C-17 at δ 70.1 were consistent with a 17-hydroxy group (Macomber, 1998). The linkage between the two moieties of **2** was deduced from the presence of a lowfield singlet at δ 7.40 (H-17′) (Massiot et al., 1983a), which correlated not only to C-16′ and C-15′ but also to C-2 (δ 70.1) and C-17 (δ 70.1). The deshielding of the isolated H-17′ enamine signal could be explained by the presence of a highly conjugated chromophore (Caprasse et al., 1984). As compared to the spectrum of strychnochrysine, the H-17′ signal was shifted to downfield in **2**, a result of an incremental effect of the methoxyl substituent on the dihydroflavopereirine chromophore. As compared to the spectrum of longicaudatine (Massiot et al., 1983b) or guiaflavine (Penelle et al., 1999), the C-17 signal is upfield, a result of configurational changes due to the opening of the seven-membered ring and similar to that of strychnochrysine and protostrychnine (Baser, 1978). The signal for C-16, which is two bonds away from the OH group, is (as would be expected) shifted downfield. The stereochemistry still must be considered. The proposed relative configurations of C-2, C-7, C-3, and C-15 are those commonly

Table 1
^{1}H- and ^{13}C-NMR data of compound **1** (recorded in 400/100 MHz in CD_3OD)

Position	^{1}H[a]	Homonuclear correlation (H/H)	^{13}C[b]	HMBC (H/C correlations)[c]
2	–		126 (C)	–
3	4.63 (*br s*)	14A, 14B	64.9 (CH)	–
5A	3.82 (*m*)	5B, 6A, 6B	60.1 (CH_2)	C-7
5B	3.77 (*m*)	5A, 6A, 6B		C-3, C-7, *N*-CH_3
6A	3.42 (*m*)	5A, 5B, 6B	19.35 (CH_2)	C-2, C-7
6B	3.32 (*m*)	5A, 5B, 6A		C-2, C-7
7	–		104.4 (C)	–
8	–		116.3 (C)	–
9	–		155 (C)	–
10	6.54 (*d*, 8)	11	99.7 (CH)	C-8, C-9, C-12
11	7.09 (*t*, 8)	10, 12	123.8 (CH)	C-9, C-12, C-13
12	6.98 (*d*, 8)	11	104.8 (CH)	C-8, C-10
13	–		139 (C)	–
14A	2.65 (*m*)	3, 14B, 15	30.3 (CH_2)	–
14B	2.3 (*m*)	3, 14A, 15		C-3, C-15, C-16, C-20
15	3.23 (*m*)	14A, 14B, 16A, 16B	30.8 (CH)	C-14, C-16, C-17, C-19, C-20
16A	1.62 (*m*)	15, 16B, 17	35.8 (CH_2)	C-14, C-17, C-18, C-20
16B	1.44 (*m*)	15, 16A, 17		C-14, C-17
17A–B	3.51 (*m*)	16A, 16B	59.0 (CH_2)	C-15
18	1.84 (*d*, 5.6)	19, 21A	12.7 (CH_3)	C-19, C-20
19	5.99 (*q*)	18	132.25 (CH)	C-15, C-18, C-21
20	–		129.4 (C)	–
21A	4.35 (*d*, 14)	15, 18, 21B	63.0 (CH_2)	C-19, C-20
21B	3.71 (*d*, 14)	21A		C-3, C-15, C-19, C-20
N-CH_3	3.19 (*s*)		48.9 (CH_3)	C-3, C-5, C-21
OCH_3	3.9 (*s*)		54.6 (CH_3)	C-9

[a] Chemical shift (δ) in ppm from TMS, multiplicities and coupling constants in Hz are in parentheses, overlapped signals *J* unresolved.
[b] Chemical shift (δ) in ppm from TMS.
[c] Correlations from H to the indicated carbons.

accepted from a biogenetic hypothesis: H-2β (2*S*), 7β (7*R*), H-3α (3*S*), H-15α (15*R*) (Klyne & Buckingham, 1974). The establishment of the C-16 configuration of **2** is critical, because the influence of the tetrahydropyridinic linkage ring is difficult to determine. Nevertheless, Massiot et al. (1988) established a ^{13}C-NMR database for the assignments of the spectra of the retuline and isoretuline series of tertiary alkaloids, which differ in the orientation of the hydroxymethyl group at C-16 and in the piperidinic ring conformation (boat in desacetylretuline, chair in desacetylisoretuline). According to the values of chemical shifts of C-2, C-14, and C-16, it was deduced that guiachrysine should belong, as strychnochrysine, to the isoretuline series. In support of this assumption, the coupling constant between H-2 and H-16 (J = 11 Hz) is in good agreement with a H-16α (16*R*) configuration (Tavernier, Anteunis, Tits & Angenot, 1978), which was also encountered in longicaudatine (Massiot et al., 1983b), strychnochrysine and recently in guiaflavine. The configuration of C-17 was finally deduced from the large coupling constant between H-17 and H-16 (10 Hz), which indicated an antiperiplanar position of both protons. This H-17β (17 *R*) configuration is similar to that of guianensine (Quetin-Leclercq et al., 1995), longicaudatine Y (Massiot et al., 1983a), strychnochrysine (Gadi Biala et al., 1998) and 3′,4′,5′,6′-tetradehydrolongicaudatine Y (Frédérich et al., 1998), but opposite to that of guiaflavine (Penelle et al., 1999).

Concurrently with the above structural work, mice and frogs bioassays have been initiated. In mice, the crude quaternary extract administered intraperitoneally possesses an LD_{100} of about 7 mg/kg. The poisonous effects appear fairly rapidly, and death occurs after 5–10 min. Compound **2** possesses an LD_{100} of about 4–6 mg/kg and death occurs fairly rapidly (5–10 min). Compound **1** and compound **3** did not seem toxic after injection of about 12 and 13 mg/kg, respectively. Another minor unknown fraction possesses an LD_{100} of about 1.5 mg/kg. Experiments performed on isolated frog nerve–muscle preparations bathed in a physiological solution containing 1 mM Ca^{2+}, 8 mM Mg^{2+} and neostigmine (3 μM) (Molgo, Lemeignan & Lechat, 1979; Lundh & Thesleff, 1977) revealed that the addition of compound **1** to the medium did not affect the resting membrane potential of the muscle fibers. However, in all experiments performed compound **1** reduced in a dose-dependent manner (2.108–40.653 μM) the amplitude of spontaneous miniature endplate potentials and of endplate potentials evoked by nerve stimulation, as shown in a typical experiment (Fig. 1). These results indicate that compound **1** blocks nicotinic acetylcholine receptors without affecting the membrane potential of skeletal muscle fibers. Interestingly, once the endplate potential amplitude had been reduced to 95% of its control value by compound **1**, the addition of 20 μM 3,4-diaminopyridine to the medium completely reversed the neuromuscular blockade and restored endplate potential amplitude to values close to controls. 3,4-Diaminopyridine is known to increase quantal acetylcholine release from motor nerve terminals and to reverse competitive inhibitors

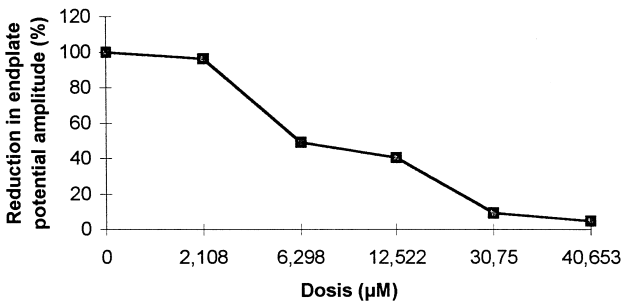
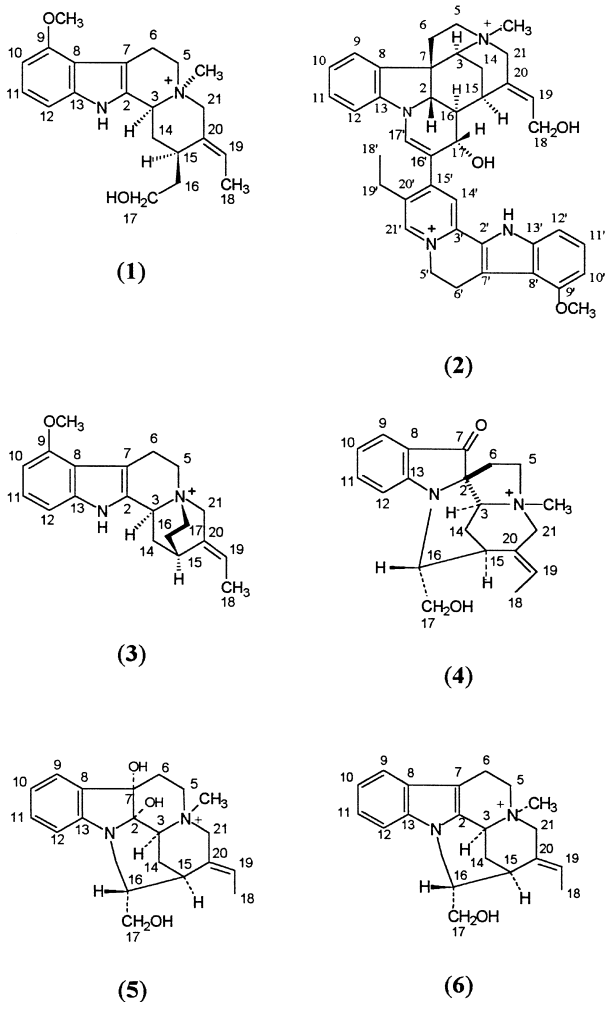
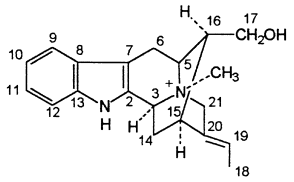
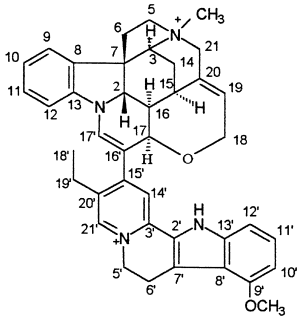


Fig. 1. Influence of **1** on endplate potential amplitude. After addition of 6.298 μM of **1**, the endplate potential amplitude is reduced to 49%. The addition of 40.65 μM of **1** reduced this amplitude to 95%. After each addition of **1**, all the synaptic events were averaged following alignement to the midpoint of their rising phases.





(7)



(8)

of nicotinic acetylcholine receptors (Molgo, 1982). Therefore, the electrophysiological results obtained strongly suggest that compound **1** is a competitive inhibitor of acetylcholine receptors. Further experiments will be needed to determine whether the other compounds guiachrysine (**2**), guiaflavine (**8**) and derivatives also have an inhibitory action on nicotinic acetylcholine receptors.

3. Experimental

3.1. Plant material

Stem bark of *S. guianensis* (Aubl.) Mart. was collected in April, 1988 by L. A. at Manaus, near Rio Taruma in Brazilian Amazonia. Identification of the plant was confirmed by Dr. A. J. M. Leeuwenberg (Wageningen); voucher specimens (INPA Herb. No. 150,295) are deposited at the INPA (Instituto Nacional de Pesquisas da Amazônia) at Manaus, at the Pharmaceutical Institute at Liège (Belgium), and at the Agricultural University at Wageningen (The Netherlands).

3.2. Extraction and isolation

The powdered stem bark (600 g) was macerated for 24 h with MeOH–HOAc (99:1) and percolated with the same mixture. After concentration of the extract under reduced pressure, precipitation by addition of H_2O and filtration, the aqueous solution was then extracted with $CHCl_3$. After separation of $CHCl_3$, the aqueous solution was basified to pH 10–11 and again extracted with $CHCl_3$. During this last operation, a precipitate was obtained. The $CHCl_3$ extracts contained tertiary alkaloids and the terminal aqueous solution quaternary alkaloids. This last solution was acidified with 5 N HCl to pH 5 and precipitated by Mayer's reagent (Evans, 1996). The precipitate was then dissolved in MeOH–Me_2CO–H_2O (6:2:1) and the alkaloids converted to the chlorides by passage through a Amberlite® IRA-420 column and concentrated under reduced pressure to give 6.7 g residue. Two grams of this residue were chromatographed over a Lichrospher® RP-select B Merck column. The mobile phase was a mixture of two solvents; solvent A was an aqueous solution of heptanesulfonic acid (sodium salt) (1 g in 420 ml) adjusted to pH 2 with phosphoric acid, and solvent B was MeCN (Gadi Biala, Tits, Wauters & Angenot, 1996). Because of the complexity of this extract, a linear gradient system of elution was selected: from 15% to 35% B in A. After TLC controls, using the solvent system: MeOH–25% aqueous MeCOONa solution–Me_2CO (65:35:20) (Paesen et al., 1994), the eluates with similar profiles were combined and precipitated by Mayer's reagent. Each precipitate was dissolved in MeOH–Me_2CO–H_2O (6:2:1) and the alkaloids converted to the chlorides by passage through a Amberlite® IRA-420 column. Fractions containing fluorocurine (**4**) and C-profluorocurine (**5**) (detected when the gradient was 15% B in A) were pooled and submitted to column chromatography on cellulose, eluted with system C of Karrer and Schmid (methyl ethyl ketone saturated with water and 1–3% MeOH) (Schmid et al., 1952). This last purification afforded the pure compound **4** (12 mg) and compound **5** (4 mg). Mavacurine (**6**) and macusine B (**7**) were detected in other fractions of the same ratio of the gradient. These fractions were pooled and submitted to column chromatography on cellulose to afford the pure compound **6** (12 mg) and compound **7** (13 mg). 9-Methoxy-N_b-methylgeissoschizol (**1**) (60 mg) was detected in fractions pooled when the gradient was 20% B in A. C-alkaloid O (**3**) (129 mg) was also detected in other fractions pooled when the gradient was 20% B in A. Fractions containing guiachrysine (**2**) (detected when the gradient was 25% B in A) were pooled and submitted to column chromatography on cellulose. This last purification afforded the pure compound **2** (25 mg).

3.3. 9-Methoxy-N_b-methylgeissoschizol (*1*)

TLC: Rf: 0.64 in 65:35:20, grey after pulverisation with $Ce(SO_4)_2/H_2SO_4$. UV λ_{max} nm (MeOH) (log ε): 207 (4.25), 220 (4.32), 259 (3.74), 291 (3.52); (MeONa) λ_{max} (log ε) 205 (4.3), 220 (4.32), 259 (3.75), 291 (3.55); CD_{MeOH} ($\Delta\varepsilon_{nm}$) $\Delta\varepsilon_{235}$ −0.30; $\Delta\varepsilon_{266}$ +0.7567; $\Delta\varepsilon_{288}$ −0.108; $\Delta\varepsilon_{302}$ 0; ESI-MS (50 V) *m/z* (rel. int.): 341 (100) $[M]^+$, 340 (75) $[M - H]^+$, 298, 297, 173, 142. HRESIMS *m/z* 341.2223 (calcd for $C_{21}H_{29}N_2O_2$, 341.2229). FTIR ν_{max} (KBr) (cm^{-1}): 3420, 2926, 1631, 1513, 1462, 1385, 1257, 1107, 739, 570. ^1H- and ^{13}C-NMR recorded on a Bruker DRX 400 MHz spectrometer are presented in Table 1.

Acetylation: To 1 mg of **1** was added 1 ml pyridine and 0.2 ml Ac_2O. After standing 24 h at room temperature, reagents were removed by evaporation to yield the mono-acetylated compound with a $[M]^+$ peak at *m/z* 383 and the diacetylated compound with a $[M]^+$ peak at 425 (ESIMS).

Methylation: To 1 mg of 9-methoxygeissoschizol, 1 ml MeOH and one drop of iodomethane (16 M) were added. After standing 24 h at room temperature, reagents were removed by evaporation to yield the methylated compound.

3.4. Guiachrysine (**2**)

Orange-colored amorphous powder; on TLC gives immediately a blue-green color with $Ce(SO_4)_2/H_2SO_4$; UV (MeOH) λ_{max} nm (log ε) 205 (4.26), 254 (3.96), 320 (3.87), 426 (3.86); ESIMS m/z 628 (15) $[M]^+$ corresponding to $C_{40}H_{44}N_4O_3$, 627 (30) $[M - H]^+$, 609 (70) $[M - H - H_2O]^+$, 314 (100) $[M]^{2+}$; EIMS m/z 594, 277, 272, 247, 235, 205, 181, 168, 157, 145; HRESIMS m/z 627.3306 (calcd for $C_{40}H_{43}N_4O_3$, 627.3335); IR (KBr) ν_{max} 3401, 2924, 2852, 1637, 1600, 1583, 1517, 1483, 1462, 1277, 1256, 1104, 1049, 1017, 925, 900, 784 and 747 cm^{-1}; ^1H- and ^{13}C-NMR in Table 2. The NMR spectra were obtained at 400/100 and 600/150

Table 2
^1H-and ^{13}C-NMR data of compound **2** (recorded in 400/100 and 600/150 MHz in CD_3OD)

Position	^1H[a]	Homonuclear correlations (H/H)	^{13}C (by HSQC, DEPT and CPD)[b]	HMBC (H/C correlations)[c]
2	4.43 (*d*, 11)	16	70.1 (CH)	C-6, C-7, C-8, C-13, C-17
3	4.505 (*s*)	14A, 14B	73.2 (CH)	C-2, C-8, C-15
5	3.895 (*m*)	6A, 6B	65.5 (CH_2)	C-3, C-6, C-7, C-21
6A	2.85 (*m*)	5, 6B	39.5 (CH_2)	C-2, C-7, C-8
6B	2.3 (*m*)	5, 6A		C-2, C-7
7			51.7 (C)	
8			132.4 (C)	
9	7.53 (*d*)	10	124.7 (CH)	C-7, C-11, C-13
10	7.03 (*t*)	9, 11	123.3 (CH)	C-8, C-12
11	7.33 (*t*)	10, 12	131.2 (CH)	C-9, C-13
12	7.21 (*d*)	11	111.5 (CH)	C-8, C-10
13			145.9 (C)	
14A	2.49 (*d*, 15)	3, 14B, 15	26.1 (CH_2)	
14B	1.91 (*d*, 15)	14A, 3	–	C-20
15	3.33 (*br s*)	14A	27.4 (CH)	–
16	2 (*m*, 10, 11)	2, 17	49.99 (CH)	C-2, C-17, C-20
17	4.68 (*d*, 10)	16	70.1 (CH)	C-15, C-16, C-16′, C-17′
18	4.27 (*d*, 6)	19	58.0 (CH_2)	C-19, C-20
19	6.095 (*t*, 6)	18	136.1 (CH)	C-15, C-21
20			129.6 (C)	
21A	4.475 (*d*, 16)	21B	65.7 (CH_2)	
21B	4.065 (*d*, 16)	21A	–	C-3, C-15, C-19, C-20, N-CH_3
2′			124.9 (C)	
3′			141.6 (C)	
5′	4.71	6′	56.3 (CH_2)	C-3′, C-6′, C-7′, C-21′
6′	3.47	5′	22.2 (CH_2)	C-2′, C-5′, C-7′, C-8′
7′			118.1 (C)	
8′			117.5 (C)	
9′			156.9 (C)	
10′	6.53 (*d*)	11′	101.4 (CH)	C-8′, C-9′, C-12′
11′	7.19 (*t*)	10′, 12′	128.7 (CH)	C-9′, C-10’’, C-13′
12′	7.0 (*d*)	11′	106.3 (CH)	C-8′, C-9′, C-10′
13′			142.5 (C)	
14′	8.05 (*s*)	–	121.8 (CH)	C-2′, C-3′, C-16′, C-20′
15′			157.5 (C)	
16′			122.2 (C)	
17′	7.40 (*s*)	–	133.6 (CH)	C-2, C-16, C-17, C-15′, C16′
18′	1.385 (*t*)	19′A, 19′B	13.9 (CH_3)	C-19′, C-20′
19′A	2.85 (*m*)	18′, 19′B	25.3 (CH_2)	C-15′, C-18′, C-20′, C-21′
19′B	2.93 (*m*)	18′, 19′A		C-15′, C-18′, C-20′, C-21′
20′			138.95 (C)	
21′	8.41 (*s*)	–	143.97 (CH)	C-2′, C-3′, C-5′, C-15′, C-19′, C-20′
OCH_3	3.91 (*s*)	–	55.9 (CH_3)	C-9′
NCH_3	3.36 (*s*)	–	49.31 (CH_3)	C-3, C-5, C-21

[a] Chemical shift (δ) in ppm from TMS, multiplicities and coupling constants in Hz are in parentheses, overlapped signals *J* unresolved.
[b] Chemical shift (δ) in ppm from TMS.
[c] Correlations from H to the indicated carbon.

MHz on Bruker AMX-400 and AMX-II-600 spectrometers.

3.5. C-alkaloid O (**3**)

UV (MeOH) λ_{max} nm (log ε): 227 (4.10), 266 (3.88), 282 (3.77), and 291 (3.73); CD_{MeOH} ($\Delta\varepsilon_{nm}$) $\Delta\varepsilon_{227}$ +0.47; $\Delta\varepsilon_{256}$ −0.12; $\Delta\varepsilon_{291}$ −0.15; $\Delta\varepsilon_{310}$ 0; HRFABMS *m/z* 309.1953 (calculated for $C_{20}H_{25}N_2O$, 309.1966) recorded on a VG Autospec-Q (VG Analytical, Manchester) apparatus; IR (KBr) ν_{max} (cm^{-1}): 3393, 2957, 2926, 2855, 1621, 1598, 1569, 1514, 1461, 1367, 1351, 1257, 1169, 1106, 1071, 1039, 978, 825, 780, 742; ^{1}H- and ^{13}C-NMR recorded on a Bruker DRX 400 MHz spectrometer, are listed in Table 3.

3.6. Fluorocurine (**4**)

UV, MS, IR, and ^{1}H-NMR; see literature (Tits et al., 1981), CD_{MeOH} ($\Delta\varepsilon_{nm}$) $\Delta\varepsilon_{208}$ −0.80; $\Delta\varepsilon_{225}$ −2.88; $\Delta\varepsilon_{260}$ −0.16; $\Delta\varepsilon_{312}$ −3.12; $\Delta\varepsilon_{355}$ 0.72; $\Delta\varepsilon_{415}$ 2.28; $\Delta\varepsilon_{480}$ 0.

3.7. C-profluorocurine (**5**)

For UV, MS, chemical reactions, see literature (Asmis et al., 1954; Hesse et al., 1964; Fritz et al., 1958). IR (KBr) ν_{max} (cm^{-1}): 3413, 2959, 2924, 1632, 1611, 1480, 1462, 1385, 1365, 1313, 1288, 1244, 1209, 1146, 1039, 1011, 960, 936, 879, 823, 758.

3.8. Mavacurine (**6**)

For UV, MS, IR, and ^{1}H-NMR, see literature (Tits et al., 1981).

3.9. Macusine B (**7**)

For UV, MS, see literature (Battersby et al., 1960; Battersby & Yeowell, 1964; Khan et al., 1967; Angenot, 1975). IR (KBr) ν_{max} (cm^{-1}): 3392, 2921, 2877, 1624, 1454, 1383, 1328, 1247, 1190, 1072, 1048, 1035, 1008, 950, 930, 751, 725.

3.10. Guiaflavine (**8**)

For UV, MS, IR, ^{1}H- and ^{13}C-NMR, see literature (Penelle et al., 1999).

Table 3
^{1}H-NMR and ^{13}C-NMR Data of compound **3** (recorded in 400/100 MHz in CD_3OD)

Position	^{1}H[a]	Homonuclear correlation (H/H)	HMBC[b]	^{13}C (by HMQC, DEPT and CPD)[c]	^{13}C[d,e]
2	–	–	–	127.7	127.4
3	4.93 (*t*)	14A, 14B	C-2, C-7, C-14, C-17, C-21	63.2	62.6
5A	3.7 (*m*)	5 B, 6A, 6B	C-3, C-6, C-7, C-17, C-21	61.6	49.9
5B	3.6 (*m*)	5A, 6A, 6B			
6A–B	3.35 (*m*)	5	C-2, C-5, C-7, C-21,	20.4	19.8
7	–			105.7	105.0
8	–			116.1	116.6
9	–			156.1	155.2
10	6.54 (*d*)	11	C-8, C-9, C-12	101.0	106.0
11	7.08 (*t*)	10, 12	C-9, C-13	125	124.6
12	6.98 (*d*)	11	C-8, C-10	106.2	101.1
13	–	–		140.5	139.7
14A	2.65 (*m*)	3, 14B, 15	C-16	32.5	32.0
14B	2.2 (*m*)	3, 14A, 15	C-2, C-3, C-16, C-20		
15	3.29 (*m*)	14A, 14B	C-3, C-17	26.1	25.4
16A–B	2.05 (*m*)	17A, 17B	C-3, C-14, C-17, C-20	25.0	24.5
17A	3.8 (*m*)	16, 17B,	C-5, C-16, C-21		
17B	3.46 (*m*)	16, 17A	C-3, C-5, C-15, C-16	50.4	61.1
18	1.8 (*d*)	19, 21A, 21B	C-19, C-20, C-21	13.1	13.0
19	5.6 (*q*)	18	C-15, C-18, C-21	121.8	121.8
20	–			131.3	130.0
21A	4.41 (*d*)	18, 21B	C-3, C-20	66.2	66.1
21B	4.16 (*d*)	18, 21A	C-5, C-15, C-17, C-19, C-20		
OCH_3	4.86 (*s*)	–	C-9	55.9	56.9

[a] Chemical shift (δ) in ppm from TMS, multiplicities are in parentheses.
[b] Correlations from H to the indicated carbons.
[c] Chemical shift (δ) in ppm from TMS.
[d] Spectra run in CD_3OD + D_2O.
[e] Borris et al., 1983.

3.11. Biological material

3.11.1. Bioassays in mice

Biological experiments were conducted using NMRI male mice (35–39 g). These mice were housed in a standard metal cage and provided food and water ad libitum.

3.11.2. Isolated frog neuromuscular preparations

The cutaneous pectoris muscle and associated nerve were removed from double pithed male frogs (*Rana esculenta*) weighing 20–25 g and pinned to the base of a 2 ml tissue bath superfused with a standard solution containing (mM): NaCl, 115.0; KCl, 2.0; $CaCl_2$, 1.8 and HEPES buffer, 5.0 (pH 7.25). In some experiments, the $CaCl_2$ was reduced and $MgCl_2$ was added at concentrations indicated in the results. In most of the experiments, neostigmine (3 μM) was present in the solution to inhibit acetylcholinesterases. The motor nerve of the isolated neuromuscular preparations was stimulated via a suction microelectrode, adapted to the diameter of the nerve, with current pulses of 0.05–0.1 ms duration and supramaximal voltage (typically 3–8 V). These pulses were generated by a S48K stimulator (Grass Instrument Division, West Warnick, RI) linked to a stimulus isolation unit.

Membrane potentials and synaptic potentials were recorded from endplate regions at room temperature (22–24°C) with intracellular microelectrodes filled with 3 M KCl (8–12 MΩ resistance) using conventional techniques (Fatt & Katz, 1951) and an Axoclamp-2A system (Axon Instruments, USA). Recordings were made continuously from the same endplate before and throughout application of the drug tested. Electrical signals after amplification were displayed on a digital oscilloscope and simultaneously recorded on video tape with the aid of a modified digital audio processor (Sony PCM 701 ES) and a video casette recorder (Sony SLC9F). Data were collected and digitized with the aid of a computer equipped with an analogue and digital I/O interface board (DT2821, Data Translation MA, USA) at a sampling rate of 25 kHz. Computerized data acquisition and analysis was performed with a program kindly provided by Dr. John Dempster (University of Strathclyde, Scotland). Endplate potentials (EPPs) and miniature endplate potentials (MEPPs) were analyzed individually for amplitude and time course. For each experimental condition, synaptic events were averaged following alignement to the midpoint of their rising phases.

3.12. HPLC analysis of *1, 3, 4, 6*

The stationary phase was an ALTIMA C8 column (5 μm, 250 × 4.6) and heptanesulfonic acid (pH 2)/acetonitrile (60/40) at a flow rate of 1 ml/min was used as eluent. Alkaloids were detected at 250 nm by a diode-array detector. Retention time (in min) of these alkaloids were compared with references and appeared at 3.8 min (**4**), 4.9 min (**6**), 5.9 min (**1**) and 9.7 min (**3**).

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