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2,7-DIHYDROXYAPOGEISSOSCHIZINE FROM ROOT BARK OF *STRYCHNOS GOSSWEILERI*

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Key Word Index—*Strychnos gossweileri*; Strychnaceae; root bark; indole alkaloid; 2,7-dihydroxyapogeissoschizine; 2D NMR; cytotoxic activity.

Abstract—In this paper, we describe the isolation and structural determination of 2,7-dihydroxyapogeissoschizine, a new alkaloid from the root bark of *Strychnos gossweileri*. Elucidation of its structure is based mainly on 1D and 2D NMR studies; its conformation was optimized by energy minimization. According to a preliminary test, this substance shows low toxicity to B16 melanoma cells, but not for non-cancer 3T3 fibroblasts cultured *in vitro*.

INTRODUCTION

The root bark of *Strychnos gossweileri* Exell. contains numerous tertiary and quaternary alkaloids [1–8]. In this paper, we report on the isolation and structural determination of a new one, which we have named 2,7-dihydroxyapogeissoschizine, from a cytotoxic fraction of this plant. We also analysed the effects of this alkaloid on cancer and non-cancer cell lines.

RESULTS AND DISCUSSION

2,7-Dihydroxyapogeissoschizine is a non-fluorescent alkaloid giving a pale violet colouration with 1% ceric sulphate in 10% sulphuric acid, becoming yellow after heating. Its UV spectrum, showing maxima at 211, 288 and 323 nm, resembles that of an indolinic alkaloid of the akuammicine-type [9] shifted by *ca* 20 nm for the first band. This spectrum is slightly modified under basic or acidic conditions (see Experimental). The IR spectrum exhibited a band at 1692 cm^{-1}, assignable to an α-β unsaturated ester, and an indoline band (1595 cm^{-1}) [10]. Besides the peak at *m/z* 369 $[M+1]^+$, in accordance with $C_{21}H_{24}N_2O_4$, which is also the base peak, the important features in the FAB mass spectrum are the loss of H_2O and COOMe as evidenced by peaks at *m/z* 351 $[M-H_2O+1]^+$ and 309 $[M-COOMe]^+$, the peak at *m/z* 185 and the 'indole' peaks at *m/z* 144, 143 and 130 [11].

Examination of the ^1H NMR spectrum (Table 1) shows, in addition to the expected quartet of resonances from the aromatic nucleus, the presence of a methoxy group (singlet of three protons at 3.76 ppm) and an ethylidene side chain (quartet of one proton at 5.37 ppm and doublet of three protons at 1.8 ppm). Furthermore, we cannot detect any NH proton, but a deshielded singlet of one proton at 8.05 ppm.

The ^{13}C NMR spectrum (Table 1) in the aromatic region indicates the presence of one carbonyl signal at 169.6 ppm, six protonated and four non-protonated carbon atoms. This does not agree with an akuammicine-type skeleton. The aliphatic region contains two methines, four methylenes, two methyl (OMe and Me) and two deshielded non-protonated carbons (79.3 and 90.7 ppm). This last chemical shift is characteristic of an indoline C-2 with an oxygen substituent [10, 12], while the other could correspond to an indoline C-7 bearing an oxygen atom.

A better understanding of the structure of the molecule was gained by examination of the 2D-COSY spectrum of 2,7-dihydroxyapogeissoschizine (Table 2) where we can assemble three mutually non-interacting spin systems. The first one is a $-CH_2-CH_2-$ unit related to the four protons at 1.9, 2.02, 2.7 and 3.35 ppm. The chemical shifts of these two last protons show that this methylene must be bonded to a nitrogen atom. This fragment probably arises from the tryptamine moiety of the molecule and should correspond to $N-C_5H_2-C_6H_2-$. The second fragment is related to the ethylidene side chain. The vicinal and homallylic connectivities provide the means of assembling the substructure $-CH_2-C=CH-Me$. The chemical shifts of the two protons of the methylene (3.68 and 2.7 ppm) are in accordance with a linkage to a nitrogen atom; this fragment must thus correspond to $N-CH_2-C=CH-Me$. Comparison of the chemical shifts

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Table 1. ^1H and ^{13}C NMR data of **2** in $CDCl_3$

H	δ	Correlations* (carbons)	C	δ	Correlations* (protons)
3	3.44	3, 14	2	90.7	6A, 6B, 14B, 17
5A	2.7	5, 21	3	53.9	3, 5B, 21B
5B	3.35	3, 5, 21	5	48.0	5A, 5B, 21B
6A	1.9	2, 6, 7	6	31.7	6A, 6B
6B	2.02	2, 6, 7	7	79.3	5B, 6A, 6B
9	7.37	9, 13	8	135.0	—
10	7.06	10	9	123.5	9
11	7.27	11	10	123.3	10
12	7.05	12	11	129.3	11
14A	2.04	14, 20	12	109.7	12
14B	2.43	2, 14, 16	13	142.3	9
15	4.28	15, 16, 17	14	27.8	3, 14A, 14B
17	8.05	2, 15, 17, 22	15	31.4	15, 17
18	1.8	18	16	109.2	14B, 15
19	5.37	19	17	135.4	15, 22
21A	2.7	21	18	13.1	18
21B	3.68	3, 5, 21	19	120.4	19
OMe	3.76	22, OMe	20	137.2	14A
			21	52.1	5B, 21A, 21B
			22	169.6	17, OMe
			OMe	52.0	OMe

*Correlations observed by means of X-H CORR and COLOC experiments.

Table 2. Observed ^1H–^1H connectivities for **2** in the 2D-COSY spectrum

H-3/H-14A	H-3/H-14B	H-5A/H-5B
H-5A/H-6A	H-5A/H-6B	H-5B/H-6A
H-5B/H-6B	H-6A/H-6B	H-9/H-10
H-9/H-11	H-10/H-11	H-11/H-12
H-12/H-17	H-14A/H-14B	H-14A/H-15
H-14B/H-15	H-15/H-17	H_3-18/H-19
H_3-18/H-21A	H-19/H-21A	H-21A/H-21B

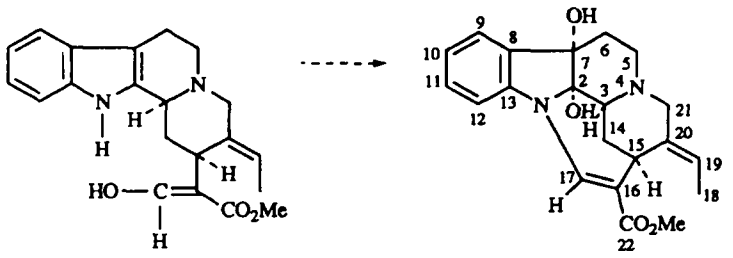
of the protons and carbons of the last spin system shows that they correspond to a CH–CH_2–CH–N unit. The COSY spectrum also indicates that the proton (H-15) at 4.28 ppm is weakly coupled with the proton at δ8.05 ppm. Furthermore, this deshielded proton is also coupled with H-12. The structure was finally refined by the COLOC-8 Hz map (Table 1) indicating coupling between the 8.05 proton and carbons resonating at 31.4, 90.7 and 169.6 ppm. On the basis of these considerations, structure **2** is proposed for 2,7-dihydroxyapogeissoschizine. All the chemical shifts and couplings in the ^1H and ^{13}C, 2D-COSY, X-H CORR and COLOC spectra fully support this structure.

This type of skeleton is biogenetically closely related to geissoschizine (**1**) but with the notable difference that the 1 and 17 positions are joined giving an additional fused ring. Such a seven-membered ring has, to our knowledge, only been observed in apogeissoschizine, obtained after treatment of geissospermine with concentrated hydrochloric acid [13] or by treatment of geissoschizine with trifluoroacetic acid [14]. In mavacurine- and pleiocarpamine-type alkaloids, the additional ring has one carbon less. 2,7-Dihydroxyapogeissoschizine is, therefore, the first natural product possessing this skeleton. The presence of two hydroxyl functions on C-2 and C-7 has already been mentioned in C-alkaloid Y isolated from a calabash curare [15].

The stereochemistry remains to be considered. The chemical shift of C-15 is in accordance with a 15-*S* configuration as compared with alkaloids of the dihydropleiocarpamine-type [10, 16]. This configuration also agrees with the biogenetical hypothesis [17].

In order to analyse the constrained conformational space of this molecule, 17 different arrangements have been built depending on the C-2, C-3, C-7 *S* or *R* configuration, the hybridization character of N4, the *Z,E* conformation of the C-19 chain and the rotation of the acetoxy fragment. For each conformation, all the 147 degrees of freedom describing the geometry have been fully optimized by energy minimization at the quantum chemistry AM1 level [18]. For all these optimized structures, the coupling constants measured in the NMR spectra were compared with the coupling constants calculated from the dihedral angles using the Karplus equations [19]. The best fit was observed for the 2*S*,3*S*,7*S*,*trans*-quinolizidine stereochemistry. The proposed conformation (Fig. 1) was the most energetically favourable with this configuration and, furthermore, had one of the lowest absolute energies of all the configurations tested.

The stereochemistry proposed for C-2 and C-7 is also in agreement with previous observations on mavacurine-type alkaloids. The authors explained that, because this



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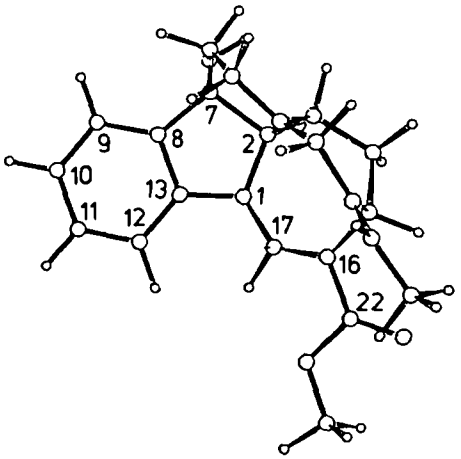

Fig. 1 Proposed conformation for 2,7-dihydroxyapogeissoschizine.

type of molecule has a pronounced hollow-sphere type of geometry, attack must occur on the outside of the sphere, giving derivatives in which the orientation of the substituents is exclusively α [20, 21].

Despite the absence of Bohlmann bands in the 2700–2800 cm^{-1} region of the IR spectrum, we propose a *trans*-quinolizidine form for 2,7-dihydroxyapogeissoschizine as in geissoschizine [22] which also lacks these bands. Furthermore, the chemical shift of H-3 (δ 3.89) is in accordance with this configuration [22]. The low field shift of the H-15 (4.28 ppm) can be explained not only in terms of diallylic position, but also by the anisotropy effect from the sp^2 plane of the carbonyl function when the acetoxy fragment has the proposed orientation.

The NOE response between the H_3-18 and H-15 and the absence of effect with H-21 indicate that the ethylidene side chain at C-20 is in the *E* configuration. This is also in accordance with the chemical shifts of H-19 and H-18 as compared with apogeissoschizine and 19,20 isoapogeissoschizine [14].

The effects of 2,7-dihydroxyapogeissoschizine on cancer or non-cancer cell lines *in vitro* were analysed using a quantitative colorimetric test based on the transformation of the tetrazolium salt MTT into dark blue formazan by various dehydrogenase enzymes in active mitochondria, so that the reaction occurs only in living cells [23]. The results showed that 2,7-dihydroxyapogeissoschizine is cytotoxic in B16 melanoma cells above concentrations of 75 $\mu g\,ml^{-1}$, but has no significant activity against non-cancer 3T3 fibroblasts.

Microscopical observation of B16 melanoma cells treated with 75 or 100 $\mu g\,ml^{-1}$ of 2,7-dihydroxyapogeissoschizine showed that most treated cells were arranged in parallel on the plastic culture dishes and appeared elongated. These cells generally failed to pile up and form multilayers as did control cells or cells treated with lower concentrations of 2,7-dihydroxyapogeissoschizine. This could perhaps be explained by the fact that some contact inhibition is restored in these treated cancer cells, thus reducing their growth. But obviously, this type of interpretation has to be confirmed by further tests.

EXPERIMENTAL

Plant material. Root bark of *S. gossweileri* Exell collected in Zaïre, near Matadi by C.D. and identified by Dr Breyne. Reference specimens (HB5690) are deposited at the Botanical Garden of Belgium at Meise.

Extraction and isolation. Extraction followed the procedure recently described [6]. 2,7-Dihydroxyapogeissoschizine was present in the lower organic phase of fr. A. The residue was fractionated by medium-pressure liquid chromatography on silica gel 60 using $CHCl_3$ and then $CHCl_3$–MeOH (99:1). Frs containing 2,7-dihydroxyapogeissoschizine were finally purified by precipitation in hexane.

2,7-*Dihydroxyapogeissoschizine.* UV λ_{max}^{MeOH} nm (logε): 211 (4.1), 288 (4.0), 323 (4.2); λ_{max}^{MeONa} nm (logε): 219 (4.1), 288 (4.0), 326 (4.2); λ_{max}^{+HCl} nm (logε): 211 (4.1), 285 (4.0), 316 (4.2). CD (MeOH; *c* 0.01): $\Delta\varepsilon_{333} = -1.5$, $\Delta\varepsilon_{317} = +0.38$, $\Delta\varepsilon_{305} = -2.3$, $\Delta\varepsilon_{283} = +10$, $\Delta\varepsilon_{240} = -3.8$, $\Delta\varepsilon_{223} = +1.9$, $\Delta\varepsilon_{200} = -3.8$. FTIR ν_{max} cm^{-1}: 3371, 2923, 2855, 1692 (COOMe–C=C), 1595, 1482, 1435, 1383, 1322, 1297, 1259, 1232, 1215, 1154, 1113, 1089, 1028, 1008, 952, 837, 807, 754, 718, 664, 598, 521, 471. FAB MS *m/z* (rel. int.): 369 $[M+1]^+$ (100), 351 (39), 337 (5), 309 (7), 185 (45), 146 (6), 144 (7), 130 (5), 120 (5), 108 (8). 400 MHz ^1H and 100 MHz ^{13}C NMR spectra: Tables 1 and 2.

Calculations. AM1 calculations were performed on a FPS-264 attached processor linked to a VAX-4200 and on a FPS-511EA computer using GAUSSIAN 86-92 programs [24].

Cytotoxicity tests. These tests were made in NUNC 96-well plates as already described [6] in mouse B16 melanoma cells (5000 cells per well) or mouse 3T3 fibroblasts (15000 cells per well). Eight wells were used for each experimental condition.

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