10´-Hydroxyusambarensine, a New Antimalarial Bisindole Alkaloid from the Roots of *Strychnos usambarensis*.

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Reinvestigation of *Strychnos usambarensis* Gilg resulted in the isolation of a tertiary phenolic bisindole alkaloid, 10´-hydroxyusambarensine (1), which was identified by detailed spectroscopic methods. Compound 1 was moderately active against two strains of *Plasmodium falciparum in vitro*.

Strychnos usambarensis Gilg (Loganiaceae) is a tree used traditionally by people of the Banyambo tribe who live along the Akagera river on the border between Rwanda and Tanzania. The leaves and roots are used as the main ingredient of a curarizing arrow poison[1]. This curarizing activity is mainly due to minor quaternary (dimeric) alkaloids such as curarine, calebassine, dihydrotoxiferine[2]. Tertiary bases such as usambarensine and akagerine are also found in the root bark[3]. A number of these alkaloids , which form the usambarane group, showed antiplasmodial, antigiardial, antiamoebic and antimitotic activities[4,5]. In a continuation of our search for potential antiplasmodial compounds from the roots of *Strychnos usambarensis* and other *Strychnos* species[4], we have isolated a new antiplasmodial usambarensine derivative, 10′-hydroxyusambarensine (**1**).

UV spectra of 10′-hydroxyusambarensine (**1**) , displayed maxima at 227, 250 , 291, 298, 362 nm in neutral solution and at 228, 270, 298, 311, 379 nm in acidic solution (bathochromic shift) similar to that of usambarensine[3]. A bathochromic shift was also observed in alkaline solution, suggesting a phenolic substituent (or an anhydronium base).

The molecular weight, 449.2333, established by high resolution FABMS and by ESIMS, corresponds to $C_{29}H_{29}N_4O$. The IR spectrum indicated NH and/or OH, orthodisubsituted benzene and trisubstituted benzene absorptions respectively at 3400, 744, 820 cm^{-1}, but no detectable CO absorption.

The structure of compound **1** was deduced mainly from analysis of ^1H - ^1H COSY, HMQC, HMBC and NOESY spectral data (Table 1). The ^1H (400 MHz) and ^{13}C NMR spectra (100 MHz) are similar to those of usambarensine[3,6]: They are comparable in the upfield region, but only seven aromatic protons are noted in the downfield region. Two coupled deshielded protons at δ 8.1 and 7.83 due to H-5′ and H-6′ were observed, and C-5′ and C-6′ were established by HMQC at δ 137.2 and 114.4, respectively. We noted also the presence of one ethylidene side chain and seven other aromatic protons. The COSY spectrum showed the seven aromatic protons to be in two groups (three and four protons) represented by one four spin system and one ABX pattern. The ABX pattern of the three remaining aromatic protons was explained by a substitution on C-10 or C-11 in one indole moiety. The protons, resonating at δ 7.45, 7.01, 7.05 and 7.25 belong to a corynanium skeleton, and those at δ 7.47, 7.10 and 7.41 to a harman ring. The protonated aromatic carbons were assigned by correlations observed in the HMQC and HMBC spectra. The HMBC spectrum also allowed assignments of the quaternary carbons. H-11′ is correlated to C-9′ and C-13′, H-12′ to C-8′ and C-10′ and H-9′ to C-11′, C-13′, C-10′ and C-7′. H-6′(pyridinic proton firmly attributed) is correlated to C-13′ and C-8′, and H-5′ to C-6′, C-7′ and C-3′. These correlations and the ^{13}C shifts of C-10′, C-9′, C-11′ and C-12′, compared with those of usambarensine[6], suggested that the -OH substituent is located on C-10′ (harman ring). All the ^{13}C shifts from C-8′ to C-13′ in **1** are in agreement with the values calculated for a 10′-hydroxy substituted usambarensine[7] and these ^{1}H and ^{13}C shifts are similar to those of 10′-hydroxy-tetrahydrousambarensine[8]. In the corynane ring, similar HMBC correlations were observed (Table 1) and all the chemical shifts of the protons and carbons (Table 1) were in agreement with structure **1** as compared with the values described for alkaloids possessing similar sub-structures, when available.[3,6,8,9,10.]

The stereochemistry still must be considered. The carbon shifts of the indoloquinolizine moiety of **1** were very similar to those observed for usambarensine. In the ^{13}C NMR spectra, the signals of C-3 (δ 55.5), C-6 (δ 19.5), C-5 (δ 52.0) and C-21 (δ 54.6) revealed a 3α cis (*S*) conformation of the C/D quinolizidine ring system[11,12]. The presence of a deshielded aliphatic signal at δ 4.38 in the ^{1}H NMR spectrum (H-3) was also in accordance with this conformation[3,13,14,15]. This hypothesis is corroborated by the lack of Bohlmann bands in the IR spectrum[14]. The 15α-hydrogen (*R*) configuration agrees with the biogenetic hypothesis[15]. Moreover, the chemical shift of C-15 at δ 35.9 confirms this stereochemistry[11]. In the NOESY spectrum, couplings were observed between H-19 and H-21a, H21b and betwenn H-18 and H-15, whereas no couplings were observed between the methyl protons (H-18) and H-21a, H-21b. An *E* orientation of the ethylidene side chain is then firmly established. In corroboration with this stereochemistry, the circular dichroism spectrum of **1** is superimposable on that of usambarensine bromide, whose absolute configuration has been established by single crystal X-ray diffraction[16.]. This permits the assignment of the same (3 *S,* 4 *S,* 15 *R*, 19 *E*) stereochemistry[14].

Results of *in vitro* tests on two strains of *P. falciparum* are shown in table 2. 10′-Hydroxyusambarensine was slightly more active than usambarensine against the two strains ($IC_{50} < 0.5$ µg/mL). The two compounds were more active against the resistant clone than the susceptible one and the activity of **1** against the W2 clone was comparable to these of quinine and chloroquine.

Experimental Section:

General Experimental Procedures. UV and visible spectra were recorded on a Kontron Uvikon spectrophotometer, the IR spectrum was recorded as a KBr pellet on a Perkin-Elmer 1750 FTIR spectrometer. ESIMS and FABMS were obtained, respectively, with a VG Plateform Micromass single quadrupole (VG Analytical, Manchester) (30 eV) and with a VG Autospec - Q (VG Analytical, Manchester) (Liquid sims, Cs^+, 20 keV, resolution > 5000) apparatus. The CD spectrum was measured with a Jobin Yvon CD6 dichrograph.

NMR analyses were carried out with a Bruker DRX 400 Avance spectrometer at 400.13 MHz (^1H) and 100.62 MHz (^{13}C), at 25° C. Chemical shifts are recorded in δ (ppm) based either on δ TMS = 0 and the coupling constants (*J*) are in Hertz. ^{13}C-NMR assignments have been established partly through comparison of chemical shifts with published data for similar compounds, and partly through HMBC and HMQC spectra. All the programs used in performing the 2D NMR experiments come from the Brucker library.

Analytical TLC was performed on precoated Si gel F_{254}, Art. 1.05735 (E. Merck) plates. After development, the dried plates were examined under short-wave (254 nm) or long-wave (366 nm) UV light and sprayed with one of the following reagents: (a) Dragendorff's reagent, (b) Fast Blue Salt B reagent: Spray solution 1: 0.1 g Fast Blue Salt B (Merck 3191) in water; Spray solution 2: NaOH solution (Merck, 0.1 mol/L). LiChroprep Si 60 15 - 25 µm (Merck 9336) was used for column chromatography. Si gel 60 PF 254 (art.1.07747, Merck) was used for purification of alkaloids in preparative TLC (1.25 mm thick, 20 x 40 cm Si gel plates). All solvents used were analytical grade (Merck).

Plant Material: The roots of *Strychnos usambarensis* were collected in March 1970 by one of the authors (LA) in Akagera National Park, Rwanda. They were immediately air-dried in Rwanda before being stored in Belgium at a temperature of 15°C. Voucher specimens of the plant were deposited in the herbarium of the National Botanical Garden of Belgium at Meise and in the herbarium of the Pharmaceutical Institute, at Liège.

Extraction and Isolation: The roots of *S. usambarensis* Gilg (300 g) were macerated with 200 mL of NH_4OH and extracted by 10 L of EtOAc in a Soxhlet apparatus. The extract was concentrated under reduced pressure below 60° C, and extracted with 4% HOAc. The resulting acidic solution was basified to pH 11 with NH_4OH and repeatedly extracted with $CHCl_3$. The $CHCl_3$ was dried over Na_2SO_4 and concentrated to yield a crude alkaloid extract (4.35g). The extract was fractionated first by liquid column chromatography on Merck LichroPrep Si 60 15-25 µm with $CHCl_2$ / MeOH. (Gradient 0 to 3% MeOH) to give 21 fractions. 10´-Hydroxyusambarensine was present in the same fractions as dihydrousambarensine (fractions 18 and 19). Preparative TLC was carried out to separate the two compounds with the system: EtOAc - Isopropanol - NH_4OH (90:8:2) on silica gel plates. On each plate, the main top zone, blue fluorescent under 366 nm, was outlined, scraped off, and eluted with MeOH to yield 15 mg of white amorphous powder (**1**). By HPLC analysis[17] of the EtOAc extract, we observed a concentration of 0.01% of 10′-hydroxyusambarensine in the roots. Usambarensine, obtained in fractions 7-10 was purified as previously described[3] and identified by TLC and spectroscopic comparisons.

10′-Hydroxyusambarensine (1): White amorphous powder. On TLC, gives a yellow turning to violet-black coloration with fast blue salt B reagent; UV(MeOH) λ_{max} (log ε) 227 (4.18), 253 (3.69), 291 (3.67), 298 (3.75), 362 (3.03) (MeONa) 228 (4.41), 284 (3.73), 305 (3.61), 390 (2.93),(MeOH + HAc) 228 (4.47), 270 (3.71), 298 (3.52), 311 (3.47), 379 (2.80), 410 (2.82); IR (KBr) ν_{max} : 3433, 2924, 2853, 1635, 1563, 1452, 1413, 1384, 1203, 1107, 928, 822, 744 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; CD_{MEOH} ($\Delta\varepsilon_{nm}$) $\Delta\varepsilon_{225}$ +0.068; $\Delta\varepsilon_{235}$ -3.65; $\Delta\varepsilon_{287}$ +0.31; $\Delta\varepsilon_{296}$ +0.410; ESIMS *m/z* 449 [M^+] (40), 434 (10), 300 (5), 249 (10), 233 (10), 224 (100), 217 (50), 185 (5), 169 (10), 144 (20), 115 (18), 64 (70); HRFABMS *m/z* 449.2333 (calcd for $C_{29}H_{29}N_4O$, 449.234137). **Usambarensine** CD_{MEOH} ($\Delta\varepsilon_{nm}$) $\Delta\varepsilon_{225}$ +1.30; $\Delta\varepsilon_{236}$ -4.34; $\Delta\varepsilon_{287}$ +0.30; $\Delta\varepsilon_{297}$ +0.89. UV, IR, MS, ^1H and ^{13}C NMR data were in agreement with literature values[3,6].

***In vitro* Antiplasmodial Testing:** Continuous *in vitro* cultures of asexual erythrocytic stages of two *P. falciparum* strains, (FCA 20 (chloroquine-sensitive) from Ghana and W-2 (chloroquine-resistant) from Indochina), were maintained following the procedure described by Trager and Jensen[18] at 37°C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640

(ICN, 1060120) supplemented with 32 mM $NaHCO_3$ (Bio-Whittaker), 25 mM HEPES (N-(2-hydroxyethyl)piperazine N'-2-ethanesulfonic acid, BDH 44147 4J.), 1.67 gL^{-1} glucose (Merck), 30 mgL^{-1} Gentamycin (Sigma) and 10% human pooled serum. Parasites were subinoculated every 3-4 days with initial conditions of 1% parasitaemia and 1% haematocrit.

10′-Hydroxyusambarensine or usambarensine bases (1 mg) were dissolved in a minimal amount of DMSO and diluted with culture medium to give stock solutions of 200 µg/mL. Chloroquine diphosphate (Sigma C6628) and Quinine base (Aldrich 14590-4) were used as antimalarial references. Each test sample was applied in a series of 8 fourfold dilutions (final concentrations ranging from 20 µg/mL to 0.0012 µg/mL) and was tested in duplicate. *In vitro* testing of samples was performed as described by Desjardins et al.[19] and modified by Mirovsky et al[20]. A suspension of 200 µL of *P. falciparum*-infected red blood cells with final parasitemia of 0.5% and hematocrit of 1.5% was incubated for 36 h in 96-well microtiter plates in the presence of 25 µL of test sample and of 25µL of ^3H hypoxanthine (0.5 µCi/well). Two series of controls were performed, one with infected red blood cells in the absence of sample and the other with uninfected red blood cells, again with no sample. Next, the contents of each microtiter plate were harvested in a Packard-Canberra Unifilter 96 GF/C. The filter papers were dried for 24 h at 22°C. The radioactivity was measured with a Packard-Canberra liquid scintillation counter. The results were expressed as percentage of growth inhibition. The sigmoid dose-response curve was used to derive IC_{50} values as the mean of two experiments. Controls without test samples defined 100% incorporation.

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Table 1 . ^{1}H (400 MHz) and ^{13}C-NMR (100MHz) Data of 10′-hydroxy-usambarensine (recorded in MeOD).

position	^{1}H^a	COSY H/H correlations	HMBC^b H/C correlations	^{13}C^c
2				133.2
3	4.38 (m -bs)	14a,14b		55.5
5a	3.34 (m)	5b,6a,6b		52.0
5b	3.18 (m)	5a, 6a, 6b	C7 (C19) C20	
6a	3.24 (m)	6b,5a,5b	C21	19.5
6b	2.80 (m)	6a,5a,5b		
7				107.3
8				128.1
9	7.45 (m)	10,11	C11, C13, C7	118.6
10	7.01 (m)	9, 11,12	C8	119.8
11	7.05 (m)	9, 10, 12	C9, C13	122.5
12	7.25 (d)	11 (10)	C10, (C11), C8	112.2
13				138.1
14a	2.51 (m)	14b,15,3		33.3
14b	2.31 (m)	14a,15,3	C2, C16	
15	3.53 (m)	14a,14b,16a,16b		35.9
16a	3.27 (m)	16b,15	C17, C20, C15	38.5
16b	3.00 (dd)	16a,15	C17, C15	
18	0.99 (d, 7.1)	19	C19, C20	13.0
19	5.42 (q, 7.1)	18, 21a	C15, C18, C21	124.5
20				135.2
21a	4.03 (d, 13)	21b, 19		54.6
21b	3.21 (d, 13)	21a	C19, C20	
2′				(137.1)
3′ (17)				145.0
5′	8,11 (d, 5.5)	6′	C6′, C7′, C17	137.2
6′	7,81 (d, 5.5)	5′	(C2′), C13′, C8′	114.4
7′				130.1
8′				122.8
9′	7.47 (d, 2.3)	11′	(C7′),(C10′), C11′, C13′	106.7
10′				152.0
11′	7.1 (dd, 9 and 2.3)	12′ ,9′	C9′, (C10′), C13′	119.3
12′	7.4 (d, 9)	11′	C8′, C10′	113.6
13′				136.9

[a] Chemical shifts (δ) in ppm from TMS. Multiplicities and coupling constants in Hz are in parentheses.

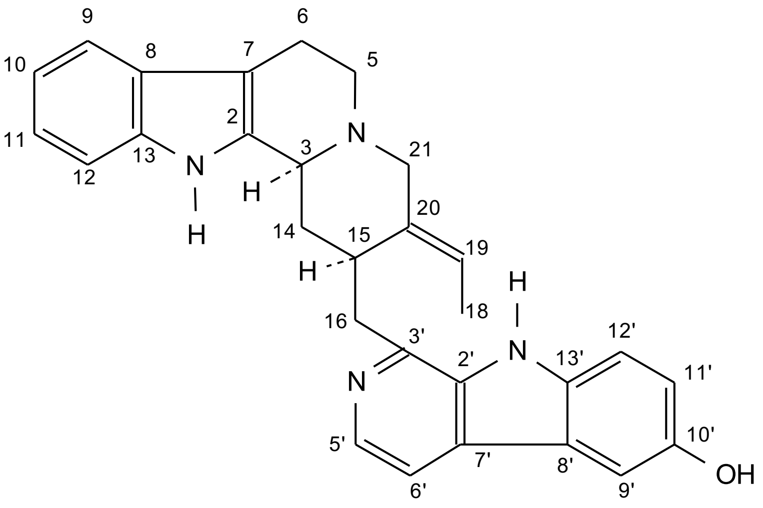
[b] Correlations from H to the indicated carbons.

[c] Chemical shifts (δ) in ppm from TMS.

Table 2. IC_{50} Values of usambarensine, 10′-hydroxyusambarensine and of two reference drugs on two *Plasmodium falciparum* clones.

	FCA 20 GHANA (chloroquine sensitive strain)		**W2 INDOCHINA (chloroquine resistant strain)**	
Compound	**IC_{50} µg/mL ± SD[a]**	**n[b]**	**IC_{50} µg/mL ± SD[a]**	**n[b]**
10’-Hydroxy-usambarensine base	0.480 ± 0.014	3	0.160 ± 0.016	3
Usambarensine base	0.655 ± 0.013	6	0.265 ± 0.023	2
Chloroquine diphosphate	0.01 ± 0.001	9	0.148 ± 0.01	9
Quinine base	0.113 ± 0.003	3	0.122 ± 0.02	2

a. Values are expressed as mean ± standard deviation. All tests were realized in duplicate.
b. “n” = number of experiments.



10'-hydroxyusambarensine