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Chemical Structure and Pharmacological (Curarizing) Properties of Various Indole Alkaloids Extracted from an African Strychnos

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Abstract—The chemical separation and identification of ten alkaloids extracted from the roots of *Strychnos usambarensis* are described. The elucidation of their structure by means of elementary analysis, I. R., U.V. and mass spectrometry allows their classification into bistertiary amines, hybrid and bisquaternary ammonium derivatives.

This classification is reflected in the pharmacological properties. One of the tertiary amine alkaloids (usambarensine) presents atropine-like and spasmolytic activities while the bisquaternary ammonium compounds (dihydrotoxiferine, calebassine, C-curarine and afrocurarine) are competitive neuromuscular blocking agents.

Introduction

The curarizing properties of various alkaloids contained in the bark of *Strychnos usambarensis* roots are empirically used for a long time as an arrow poison by the Banyambo hunters in Rwanda, Africa.

The extraction, separation and identification of these alkaloids have been systematically pursued in the laboratory of Pharmacognosy during several years (1, 2). More recently, their pharmacological properties have been described (3).

In this paper, we present the last results of our work, insisting on the chemical aspects.

The ten indole alkaloids so far identified may be separated into three groups following the characteristics of their amine functions. The first one contains three tertiary alkaloids; the second is also represented by three substances containing simultaneously a quaternary ammonium and a tertiary amine function (hybrid alkaloids), the last one includes 4 quaternary ammonium compounds.

It will be seen that this chemical subdivision has pharmacological implications, the tertiary alkaloids working mainly on the smooth muscles, the quaternary agents on the motor end-plate.

Therefore, they will be examined in this order as well for the chemical as for the pharmacological aspects.

Methods and Results

Part I. Extraction, separation and identification of the alkaloids.

After methanolic extraction, fractionated precipitation and freeze-drying in order to avoid the destruction of the unstable compounds, the alkaloids are redissolved in water or acetone and separated by thin layer and column chromatography. This procedure yields fractions containing at least 10 tertiary, hybrid and quaternary alkaloids identified by their ultraviolet (U.V.), infrared (I.R.) mass and nuclear magnetic resonance (NMR) spectra (4,5). They are separated into three main groups.

1. *The tertiary alkaloids.* Besides harman, found for the first time in *Loganiaceae*, we have isolated two new bis-indole alkaloids, usambarensine and

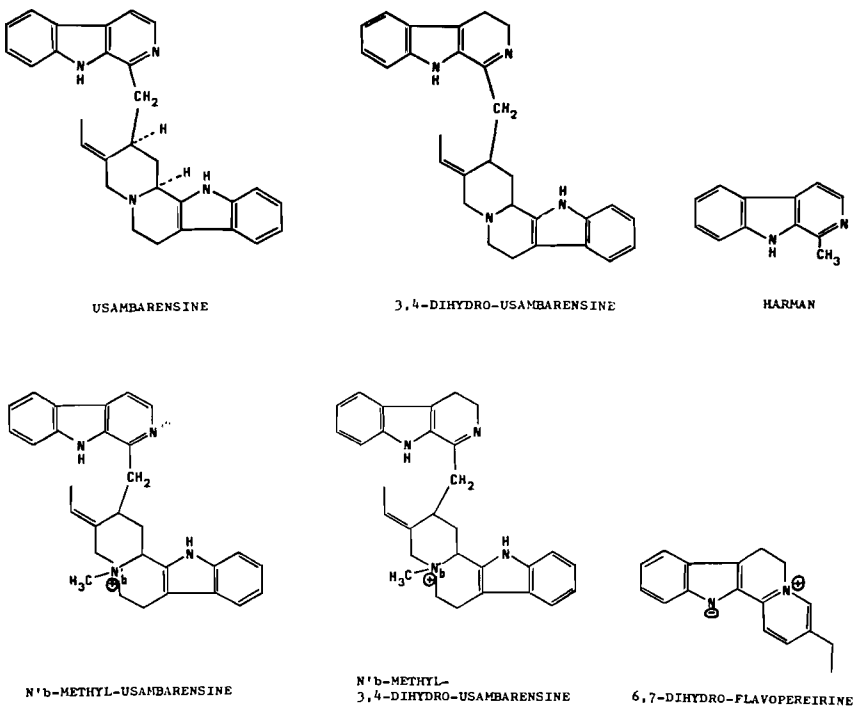


FIG. 1

A. — Chemical structures of usambarensine, 3,4-dihydro-usambarensine and harman.

B. — Chemical structures of N'*b*-methyl-usambarensine, N'*b*-methyl-3,4-dihydro-usambarensine and 6,7-dihydroflavopereirine.

dihydro-usambarensine (6, 7, 8). Their chemical structure is represented at figure 1, A.

2. *The hybrid alkaloids.* This second group contains the 6, 7 dihydroflavopereirine, a new anhydronium base (9), and the N'*b*-methyl derivatives of usambarensine and dihydro-usambarensine (5, 6) (Fig. 1, B).

3. *The quaternary alkaloids.* The last group contains four identified bis-indole diquaternary alkaloids. Three of these potent products were previously isolated from Calabash curare and some South-American *Strychnos*. They are found for the first time in a *Strychnos* species growing outside the American continent.

The method used for the separation of these 4 substances has, so far, not been described and is therefore given here with some details.

After precipitation and extraction of the tertiary and hybrid alkaloids, the alkaline aqueous phase is brought to *p*H 5 with HCl and a saturated aqueous solution of picric acid is added. An abundant precipitate forms, which is collected and washed with water. The picrate is dissolved in a minimum amount of acetone. The solution is passed through an Amberlite IRA 400 column (Cl^- form) equilibrated with acetone-water (9 : 1). The same mixture is used for the elution. The eluate is then concentrated *in vacuo* to dryness to give crude quaternary alkaloids in the form of chlorides. Further purification over an alumina column (W200-acid form) is required, using methanol as eluent. After evaporation of the eluate, chlorides of the alkaloids are separated by chromatography on cellulose (Whatman CC 31) column using the Karrer's and Schmid's system C (methylethylcetone saturated with water and containing 1–3 % methanol). Two hundred and fifty fractions of 100 ml each are collected in the order of appearance from the column, that is in the order of increasing polarity. After a rapid check on paper chromatograms, the 250 fractions were combined in 22 Fractions (F1 to 22). Each fraction (F2 to F22) contains various alkaloids, which may be separated by a further column chromatography using another system of elution, ethyle-acetate-pyridine-water 75 : 23 : 16,5 (Karrer's and Schmid's system D). Sereval curarizing crude fractions are thus separated over a second column; 20 ml fractions are collected, tested by chromatography and combined following this analysis. Evaporation of the eluate gives substantial amounts of 4 pure alkaloids P1 to P4 which crystallise in methanol-ether.

a. *C-dihydrotoxiferine* (fraction 9 or chloride of alkaloid P1).

The chloride of alkaloid P1 does not melt under 350° C; its picrate decomposes at 182–184° C: $[\alpha]_D = -600°$. These physical data as well as the U.V., I.R. and NMR spectra are superimposable to those of C-dihydrotoxiferine (8) (Fig. 2, A).

C-dihydrotoxiferine, $C_{40}H_{46}N_4$ is a bis-quaternary alkaloid first isolated by Wieland from Calabash curare and later from South-American *Strychnos* species. Its structure was established in 1961 and confirmed by synthesis.

A mass spectrum of P1 further confirmed this identification = *m/e* (relative abundance): 582 (M^+) (3), 566 (31) (M^+-CH_3-H), 552 (base peak) ($M^+-2\ CH_3$ = nor base), 276 (base peak^{++}) (30), 144 (30), 143 (14), 130 (20), 122 (37), 121 (38), 50 (CH_3Cl^{35}), 52 (CH_3Cl^{37}).

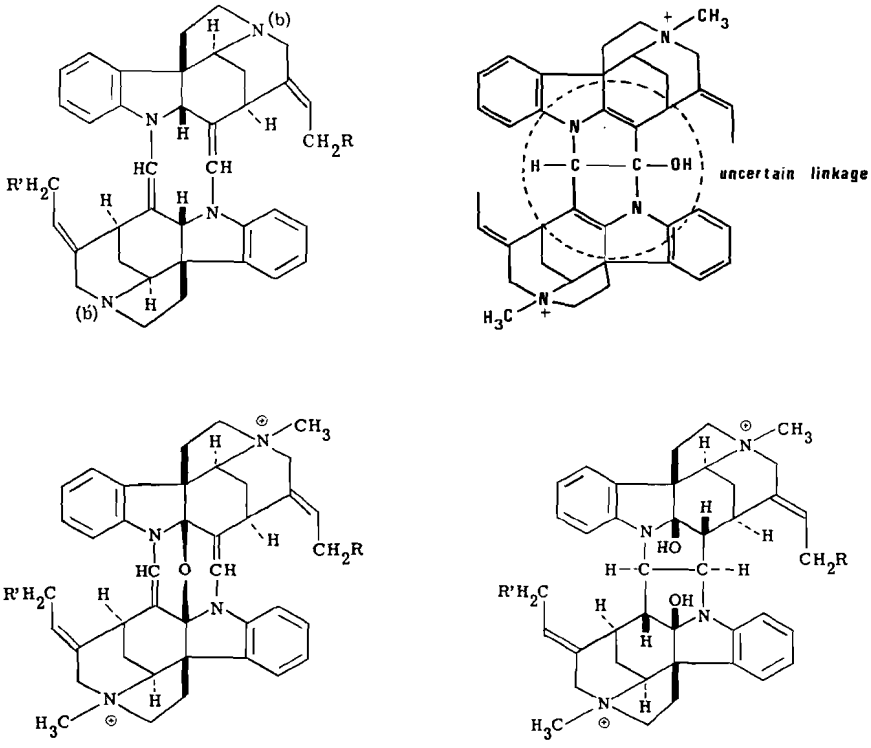


FIG. 2

A. Chemical structure of C-dihydrotoxiferine (F9 or P1), C-toxiferine and alloferine.
R = R′ = H C-dihydrotoxiferine $N_{(b)} = N_{(b')} = CH_3$
R = R′ = OH C-toxiferine $N_{(b)} = N_{(b')} = CH_3$
R = R′ = OH alloferine $N_{(b)} = N_{(b')} = C_3H_5$

B. Afrocurarine $C_{40}H_{44}N_4O^{++}$ 2 Cl^- (hypothetical structure).

C. Chemical structure of C-curarine. R = R′ = H

D. Chemical structure of C-calebassine. R = R′ = H

The mass spectra were obtained by D. Carter with an AEI MS 9 high resolution spectrometer (School of Pharmacy, University of London). The peak intensities are given as % of the largest peak above 120 m.u.which is taken as the base peak.

Furthermore, a comparison has been made with the MS of alloferine (1):

(1) We warmly thank Dr. J. Werli for a generous gift of Alloferine Roche.

m/e (relative abundance): 666 (M^+) (1), 624 ($M^+-C_3H_5-H$) (12), 584 (100) (nor base), 292 (base peak^{++}) (33), 292,5 (10), 293 (11), 144 (60), 143 (20), 138 (34), 137 (20), 130 (25), 76 ($C_3H_5Cl^{35}$), 78 ($C_3H_5Cl^{37}$).

b. Afrocurarine (fraction 11 or chloride of alkaloid P2).

The chloride of alkaloid P2 does not melt under 350° C; its picrate decomposes at 169–171° C. The hypothetical structure in Fig. 2, B is proposed for this new alkaloid ($C_{40}H_{44}N_4O$ - M.W.: 596) on the basis of the following data:

1) the elementary analysis: N = 8,48 %; Cl = 10,69 %

2) The U.V. spectrum (in methanol) shows the following λ max. (log. ϵ): 203 (4,52), 253 (4,07), 272 (4,10), 312 (4,18) and 418 (4,14). There is no significant shift by acidification or basification. The orange-yellow colour of the alkaloid is explained by this spectrum.

3) The I.R. spectrum shows bands at $\sqrt{}$ 3400 (OH), 1630, 1595 (C=C or C=N), 1575, 1480, 1455, 1380, 1320, 1285, 1245 and 750 (unsubstituted indole) cm^{-1}. This spectrum is very similar to that of dihydrotoxiferine.

4) The M.S. shows the following fragmentation data: (cfr Table I), the base peak at m/e 548 ($C_{38}H_{36}N_4$, found: 548, 2910; calculated: 548, 2940) corresponds to the elimination of H_2O from the nor base peak at m/e 566 (40) ($C_{38}H_{38}N_4O$, found: 566, 3056; calc.: 566, 3046). There are other peaks at m/e 596 (M^+) (1), 594 (1), 580 (5) (M^+-CH_3-H), 426 (21) ($C_{30}H_{24}N_3$, found: 426, 1969; calc.: 426, 1970; loss of $C_8H_{12}N$ from the base peak), 283 (3), 274 (15) (base peak^{++}), 144 (8), 143 (7), 130 (7), 122 (33), 121 (10), 50 (CH_3Cl^{35}), 52 (CH_3Cl^{37}).

As this new alkaloid, found in an African curare, gave the save colour reaction (blue) with cerium sulphate and has the same molecular formule than C-curarine, we have called it: afrocurarine.

It is a bis-quaternary dimeric indole alkaloid. Its central diazacyclo-octane ring is different from the other members of the C_{40}-alkaloid families. Afrocurarine can be assigned to the C-dihydrotoxiferine family, by its mass spectrum.

Further experiments will perhaps resolve the nature of the dimeric linkage.

c. *C-curarine* (fraction 13 or chloride of alkaloid P3).

Alkaloid P3 gives the same chromatographic values as reference C-curarine (1) in different systems on Whatman 1 paper. The U.V. spectrum was moreover superimposable to that of C-curarine (8). The mass spectrum of P3 also shows very little fragmentation similar to P1, P2 and P4 (cf. Table I) and confirms the identification.

The peaks at m/e 596 (M^+) (1), 580 (M^+-CH_3-H) (10), 567 (50), 566 (base

(1) Prof. G. B. Marini-Bettolo (Roma) kindly made available a sample of C-curarine isolated from South-American *Strychnos*.

peak = nor base) appear at 14 mass units higher than corresponding peaks in dihydrotoxiferine, indicating an ether bridge of the C-curarine structure (Fig. 2, c); other peaks at m/e 458 (9), 443 (4), 301 (10), 283 (base peak^{++}) (19), 144 (9), 143 (6), 130 (9), 122 (32), 121 (28), 50 (CH_3Cl^{35}), 52 (CH_3Cl^{37}) ([1]).

C-curarine ($C_{40}H_{44}N_4O$, MW 596) is a white alkaloid isolated from Calabash curare and South-American *Strychnos* species (8).

X-ray diffraction studies of the di-iodide of C-curarine have provided the structure for the central octacyclic ring and ether bridge; the quaternary $N^{\oplus}—N^{\oplus}$ distance is 8,50 Å, close to the value (8,8 Å) found for the synthetic ganglioplegic compound, hexamethonium bromide (9).

d. *C-calebassine* (fraction F15 or chloride of alkaloid P4).

Chloride of alkaloid P4 shows physical data as well as U.V., I.R. and NMR spectra superimposable to those of C-calebassine (8).

The mass spectrum is characteristic notably by the appearance of strong peaks at m/e 92,106 (>100), 107 (>100), 121 (base peak) and 122 (33), due to fragments from the piperidine portion of the molecule; other peaks are present at m/e 616 (M^+) (<1), 614 (<1) 578 (2,5), 564 (4), 55 (32) ($M^+—2CH_3—2H_2O$), 443 (15) (m/e 550–107 m.u.), 144 (9), 143 (8), 130 (8).

C-calebassine ($C_{40}H_{48}N_4O_2$) is also a bis-quaternary alkaloid isolated from Calabash curare and South-American *Strychnos* species. Its structure is given in Fig. 2, D.

TABLE I

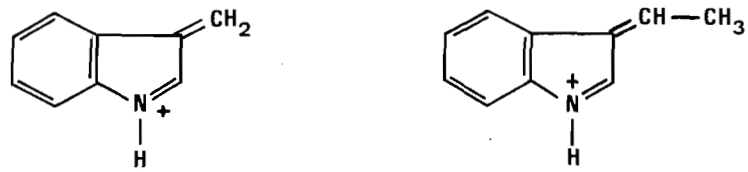
The principal peaks in the mass spectra of bisquaternary alkaloids

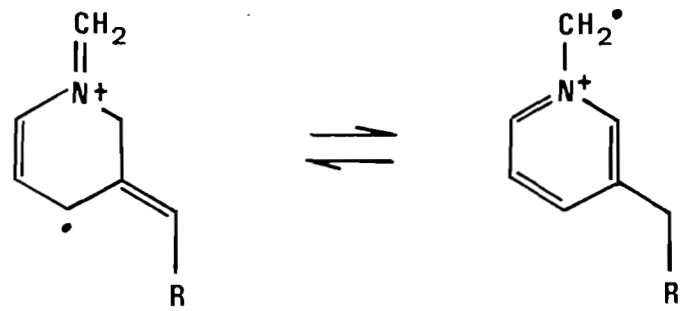
Alkaloids	Indole peaks (Fig. 3, A and B)	Piperidine peaks (Fig. 3, C and D)	Base peak^{++}	Base peak	M^+
Dihydrotoxiferine	130, 143, 144	121, 122	276	552	582
Afrocurarine	130, 143, 144	121, 122	274	548	596
C-curarine	130, 143, 144	121, 122	283	566	596
Calebassine	130, 143, 144	121, 122	275	550	616
Alloferine	130, 143, 144	137, 138	292	584	666

The mass spectra of the four alkaloids so far identified show characteristic fragmentation processes summarized in table I. The base peak is the nor base, corresponding to the loss of 2 CH_3Cl (P1, P3) or 2 C_3H_5Cl (Alloferine), and to the further elimination of H_2O from tertiary alcoholic function (s) (P2, P4). The appearance of strong peaks in the lower mass range (see m/e 121, 122 or 137, 138) due to fragments from the non-indolic portion of the molecule, is of considerable

([1]) This spectrum is very similar to that of C-curarine, published after the writing of this paper. It is the sole spectrum of a di-quaternary alkaloid described in ref. 10.

diagnostic utility, mainly to distinguish between alkaloids of the toxiferine and dihydrotoxiferine groups with the ethylidene side chain.





$R = CH_3$ m/e 121 ($C_8H_{11}N$)

$R = CH_2OH$ m/e 137 ($C_8H_{11}ON$)

FIG. 3

Chemical characteristics of indole and piperidine moieties obtained by fragmentation of the four bisquaternary alkaloids.

Part II. Pharmacological properties of the alkaloids.

The ten alkaloids separated and identified in the first part of this work have been studied for their possible action on noradrenergic, muscarinic and nicotinic receptors.

From a pharmacological as well as a chemical point of view, they may be subdivided in three classes which will be examined successively. The various technics used have already been described (3).

1. *The tertiary and hybrid alkaloids have been studied on the rat isolated intestinal muscle (muscarinic receptors) and Vas deferens (noradrenergic receptors).*

a) *Isolated intestinal muscle.* Cumulative dose-response curves are obtained by using the method of Ariëns (1964) (11) with carbachol as agonist. The isolated ileon is placed at 37° C in Tyrode solution adequately buffered and oxygenated. The isometric contractions are recorded under an initial force of 1 g. The substances are left during 20 min in the bath before a new curve is recorded.

In these conditions, only usambarensine presents a clearcut antagonistic effect. At a concentration of 3.10^{-6}M a shift of the dose-response curve to the right is observed demonstrating a competitive inhibition between usambarensine and carbachol. At higher concentrations (10^{-5}M and more), there is a progressive lowering of the maximal response suggesting the presence of a non competitive antagonism at these higher concentrations (Fig. 4). The pD'_2 of this phenomenon, calculated according to van Rossum (1963) (12) is 4.76. In our experimental conditions, the pA_2 of atropine is 8.41. The other tertiary alkaloids are devoided of significant effect on the rat isolated intestinal muscle. In the other classes, only fraction 13 (C-curarine) presents a weak inhibiting action.

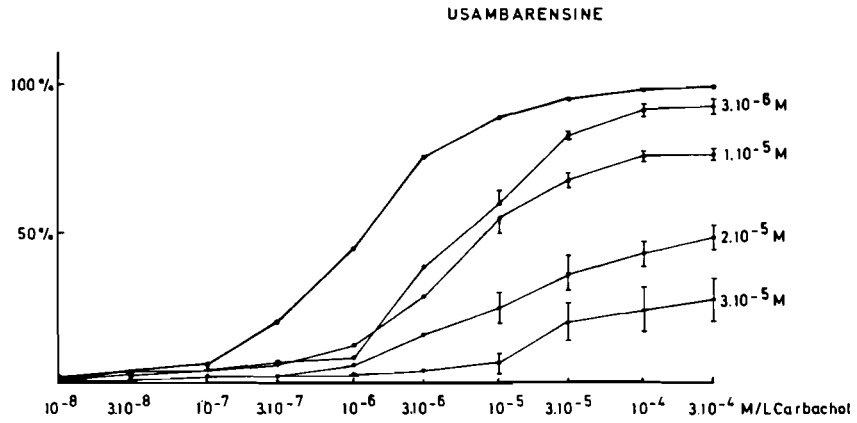


FIG. 4

Inhibiting properties of usambarensine on the carbachol induced cumulative dose-response curves of the rat intestine.

b) *Isolated Vas deferens.* Cumulative dose-response curves for the rat isolated *Vas deferens* with *l*-noradrenaline bitartrate as agonist have been obtained in the same experimental conditions as described for the intestinal muscle. The initial applied force is 200 mg.

No reproducible and statistically significant potentiating or inhibiting effects have been obtained.

2. *The diquaternary ammonium compounds present interesting neuromuscular blocking properties demonstrated by stimulating the rat sciatic nerve and recording the resulting contraction of the extensor muscles.*

Male rats of approximately 500 g are anesthetized by sodium pentobarbital (30 mg/kg, I.P.) and fixed on a Palmer table. After careful dissection, the extensor muscle is attached to a FTA 10 force transducer and the isometric contractions recorded on a 7702 B Hewlett Packard recorder.

The sciatic nerve is stimulated by single supramaximal pulses (±1,5 V; 0,1 msec; 0,1 Hz). The carotid blood pressure is simultaneously recorded. The alcaloids are intravenously injected in isolated geometrically increasing doses at 30 minutes intervals or in a cumulative manner.

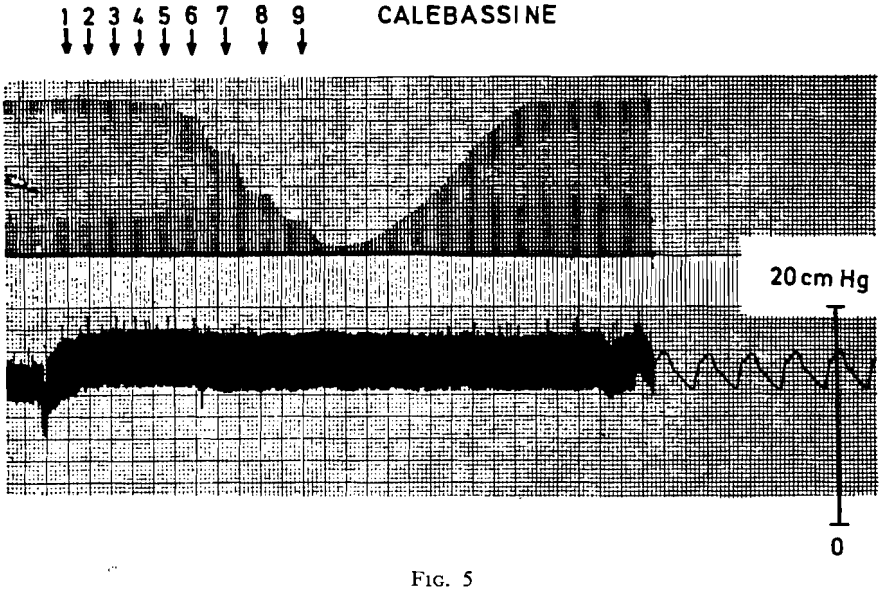


FIG. 5

Neuromuscular blocking action of calebassine, intravenously injected in cumulative doses from 1.66 to 7.2 10^{-7} M/kg (increasing factor 1.2). At the top, contraction of the extensor muscle after electrical stimulation of the sciatic nerve. At the bottom, carotid blood pressure. The paper speed is 5 mm/min. Artificial respiration is applied at the beginning of the injection.

a) In these experimental conditions, dihydrotoxiferine, afrocurarine, C-curarine and calebassine contained in the bark of the *Strychnos usambarensis* roots possess a potent neuromuscular blocking action. A typical experiment is presented for calebassine in Fig. 5.

b) The curarizing properties of the alkaloids are antagonized by inhibiting acetylcholinesterase by means of eserine.

The effect is demonstrated at Fig. 6. An i.v. injection of eserine, 10^{-6}M/kg, partially antagonizes the neuromuscular blocking action of calebassine. It seems, therefore, that the alkaloids are not acting as depolarizing agents. They have curare-like activities by inhibiting the action of acetylcholine on the motor end plate.

c) This assumption is further substantiated by the fact that after the curarizing effect has been fully developed, the muscle remains excitable to a direct current

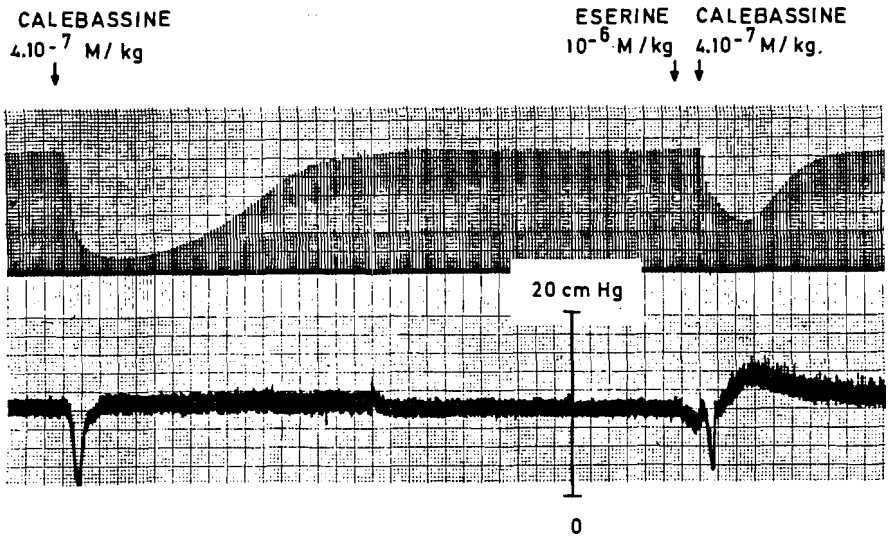


FIG. 6

Partial inhibiting effect of eserine on the neuromuscular blocking action of calebassine.

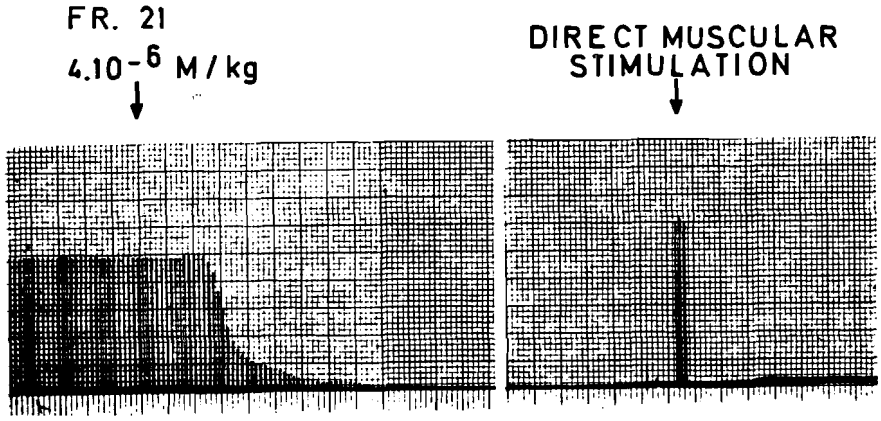


FIG. 7

Effect of direct muscular stimulation after prolonged paralysis produced by fraction 21.

application. A typical example of this phenomenon is shown in Fig. 7 obtained with a yet unidentified potent and long acting polar alkaloid contained in fraction 21. Another unidentified, active alkaloid is present in fraction 19.

d) It must be noted that for some unknown, probably pharmacokinetic reason, a first injection of an alkaloid potentiates the effect of a second administration after complete recovery has been obtained. This potentiating effect is illustrated in Fig. 8 for another substance, C-curarine.

e) From a quantitative point of view, we have compared the curarizing potency of the alkaloids of *Strychnos usambarensis* to that of *d*-tubocurarine.

The inhibitory effects of 9 different concentrations of these drugs (6 determinations for each point) have been plotted on a semi logarithmic paper. The ED 50 are graphically obtained; the standard error deviation (SED) and 95 % confidence limits are calculated. These results are summarized at table II. They

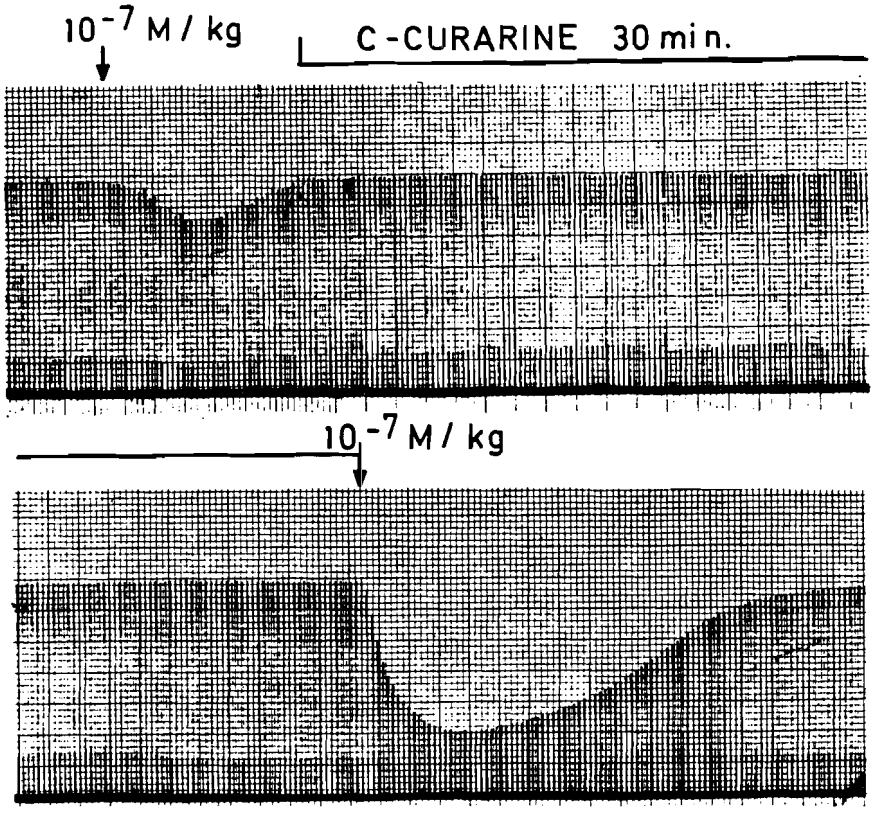


FIG. 8

Potentiating effect of a first injection of C-curarine on a second administration of the same alkaloid after full recovery.

have been submitted to an analysis of variance and the statistical significance between the ED 50 s has been tested by the method of orthogonal contrasts. All the P values are inferior to 0.01. The decreasing order of potency is *d*-tubocurarine, C-curarine, dihydrotoxiferine, calebassine, and afrocurarine. It must be noted that for C-curarine and afrocurarine, the quantities of the purified substances were too small to repeat the dose-response curves more than twice. For this reason, the confidence limits have not been calculated and the ED 50 values are approximative.

TABLE II

Quantitative curarizing activities of the Strychnos alkaloids and d-*tubocurarine*

Substances	ED 50 ± SED (10^{-7} M/kg)	95 % confidence limits	% of activity
d-Tubocurarine	1.44 ± 0.16	(1.03–1.85)	100
C-Curarine	1.53 (1)	—	94
Dihydrotoxiferine	3.82 ± 0.34	(2.95–4.69)	38
C-Calebassine	4.81 ± 0.13	(4.48–5.14)	30
Afrocurarine	30 (1)	—	5

(1) These values are estimated.

Discussion

The ten alkaloids so far separated and identified in the bark of *Strychnos usambarensis* roots may be subdivided in tertiary amines (harman, usambarensine and 3,4-dihydro-usambarensine), hybrid (6,7-dihydroflavopereirine and the N'*b*-methyl derivatives of usambarensine and dihydro-usambarensine) and quaternary ammonium compounds (dihydrotoxiferine, calebassine, afrocurarine and C-curarine). Two other unidentified bisquaternary ammonium compounds are present in fractions 19 and 21.

From a chemical point of view, four of the tertiary and hybrid alkaloids are new substances. That is also the case for afrocurarine (partially identified) in the quaternary ammonium class. Dihydrotoxiferine, calebassine and C-curarine are well known alkaloids of the South American *strychnos* species. Their presence in an African species was not described.

Pharmacologically, the tertiary and hybrid alkaloids are not active in the tests studied, except that usambarensine presents an atropine-like and spasmolytic activity shared by C-curarine.

On the contrary, the quaternary ammonium compounds possess interesting and potent curarimimetic activities. They seem to work as competitive (curare-like) and not as depolarizing (decamethonium-like) agents. Indeed, their inhibitory action may be antagonized by increasing the amount of acetylcholine at the

motor end plate by means of eserine and the muscle remains excitable to a direct application of current after a prolonged inhibition of the neuromuscular preparation.

It is interesting to note that a first injection of these neuromuscular blocking agents potentiates the activity of a second administration during a long period. That can be explained by the presence of a large amount of reserve receptors at the end plate level as postulated by Waser (13) after autoradiographic experimentation.

Finally, we may conclude that the lethal action of the arrow poison prepared by the Banyambo hunters in Rwanda is certainly due to the curarimetic properties of the various bisquaternary ammonium alkaloids described in this paper.

Summary

By means of column and paper chromatography, elementary analysis, I.R., U.V. and mass spectrometry, ten alkaloids have been separated and identified from extracts of the bark of *Strychnos usambarensis* roots. They are bistertiary, hybrid and bisquaternary ammonium derivatives. These last compounds present potent curarimetic activities.

Acknowledgments—We thank Professors A. Denoël and C. L. Lapiere (Liège) and Dr. N. G. Bisset (University of London) for their close interest in this work. We are particularly indebted to Mr. J. Denoël (Director of N.M.R. Centrum, University of Liège) for N.M.R. Spectra recordings.

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