Further Chemical and Pharmacological Characterization of *Bersama yangambiensis* Toussaint (Melianthaceae)

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Abstract □ A bufadienolide glycoside was isolated and purified from *Bersama yangambiensis*. Partial characterization was accomplished by spectrometric data, and these data suggest that the bufadienolide is a new compound. Preliminary pharmacologic investigations confirmed the cardiotonic properties of this compound and showed an antitumor action *in vitro*.

Keyphrases □ *Bersama yangambiensis* Toussaint (Melianthaceae)—chemical and pharmacological characterization, isolation and identification of a bufadienolide □ Bufadienolides—isolation from *Bersama yangambiensis* Toussaint, pharmacological testing for cardiotonic and antitumor properties □ Cardiotonics, potential—pharmacological testing of a bufadienolide from *Bersama yangambiensis* Toussaint □ Antitumor agents, potential—pharmacological testing of a bufadienolide from *Bersama yangambiensis* Toussaint □ Medicinal plants—chemical and pharmacological characterization of *Bersama yangambiensis* Toussaint

Extracts from various *Bersama* species have been widely used medicinally by Africans, and the pharmacological activity of these preparations has been documented (1–4). In the last 10 years, however, only the species *Bersama abyssinica* Fresen, subspecies *abyssinica*, has been studied (5–8) and its extracts have shown not only cardiotonic activity but also the ability to inhibit the growth of tumor cells (6–8). The active compounds were isolated and identified as bufadienolides, in the genin form, and were shown to differ on the basis of their general properties and molecular weights. These compounds were isolated from the bark by Kupchan *et al.* (6). Lock (5), however, showed that they were present in the leaves but absent in the bark. Thus the distinct possibility exists that the plants utilized in those studies may have been different species or subspecies, especially since the classification of the genus *Bersama* is highly controversial (9, 10).

Bersama yangambiensis is known under three vernacular names (11): Kilulu Kasai, Nde (Ngwaka dialect), and Ianaolo a bowa bo ndomba (Turumbu dialect), a name that is similar to "bowa bo ndomba" which is the vernacular name for *Turreaenthus Africana* (Meliaceae).

Previously, we reported the presence of a bufadienolide in *B. yangambiensis* (12). This report deals with the chemical and main pharmacological characteristics of this bufadienolide.

RESULTS AND DISCUSSION

Compounds A and C show no aldehyde, acetoxy, or methoxy functions in the bufadienolide molecule, as evidenced by a lack of absorption at 2720 and 2750 cm.$^{-1}$ (aldehyde) and at 1215 cm.$^{-1}$ (acetoxy) in the IR spectra and no signals at 10 p.p.m. (aldehyde), 2 p.p.m. (acetoxy), and 3.5 p.p.m. (methoxy) in the NMR spectra (13)[1].

The sequence of peaks at 418, 400, 382, and 364 mass units in the mass spectrum suggests the presence of a 3-hydroxyl or epoxy function on the bufadienolide skeleton. Under electron impact, three molecules of water were removed from peak 418 and this peak corresponds to a fragment of a larger molecule which is composed of this fragment coupled with sugars. The presence of these sugars makes the bufadienolide completely nonvolatile and highly thermolabile in the spectrometer source. Because of this nonvolatility, peaks of heavy mass were very small. There were signals

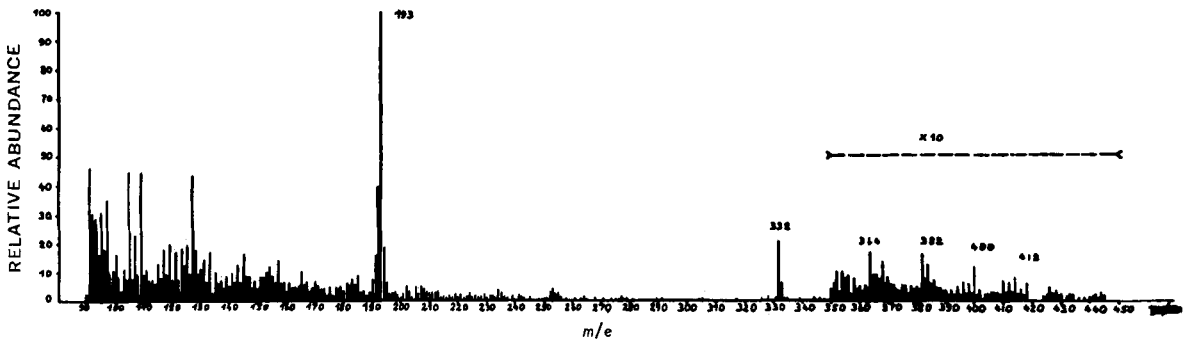
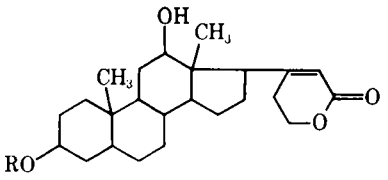


Figure 1—*Mass spectrum of Compound A; base peak = 193. Possible interpretation is as follows: M^+ (genin) 418, 400 ($-H_2O$), 382 ($-H_2O$), and 364 ($-H_2O$). $C_{24}H_{30}O_6$ has a calculated mass of 414.2041; 414.2044 is the measured mass.*

which differed from the main characteristic peaks by one or a few mass units; they were due to formation, by thermolysis, of more or less hydrogenated derivatives of a similar structure (Fig. 1).

The actual mass of peak 414 is particularly easy to measure under the experimental conditions and was determined by the peak-matching technique. It suggests the elemental structure of $C_{24}H_{30}O_6$. Thus, four oxygen atoms are external to the basic bufadienolide structure and can be accounted for in the following manner. Evidence for the presence of a 3β-hydroxyl group, the usual position for sugar linkage, is suggested by the signal at 1.71 p.p.m. in the NMR. The position of the signals of the protons HA, HB, and HC of the α-pyrone, the shift (→0.240 p.p.m.) of the signal given by the

[1] IR spectra were recorded on a Perkin-Elmer model 237 spectrophotometer. NMR spectra were recorded on a Varian model A60. Mass spectra were recorded on an A.E.I. model M.S. 902. GLC was performed on a Varian Aerograph model 1200. UV spectra were recorded on a Perkin-Elmer model 402, and optical rotatory dispersion was recorded on a Perkin-Elmer 141 polarimeter.



A: R = two aldohexose
B: R = one aldohexose
C: R = H
D: R = /, Δ 4–5

2-hydroxyl and/or epoxy groups

C-18 methyl group when changing from $CDCl_3$ to dimethyl sulfoxide in the NMR (13), and the hypsochromic shift (300 → 295.5 nm.) of the classical UV absorption peak of the bufadienolide suggest the presence of a hydroxyl group in the 12β-position. At least one tertiary hydroxyl function is present, because the 3400-cm.$^{-1}$ absorption band of the IR spectrum is not completely removed by acetylation. Although an epoxy bridge between positions 14β and 15β is ruled out by the NMR spectrum, such a bridge could be present at other sites.

The glycosidic nature of the compound is supported by the following evidence: the elemental analysis of the products, higher R_f values of Compounds B and C which resulted from the sequential hydrolysis of Compound A (Compound D is the anhydrogenin), and the mass spectra. The molecular mass of the genin coupled with two completely acetylated sugars would be about 1020. The only mass spectrum of the acetylated derivatives that could be obtained displayed a low ionic intensity in the range of masses around 1000. However, the presence of a peak corresponding to the compound was obviously demonstrated, but its true localization was difficult due to a large preceding zone of very low intensity.

The bufadienolide molecule contains no methylpentoses since the R_f's and retention times of the sugars in the chromatographic systems, as well as their reactions to the detecting reagents, suggest only the presence of two aldohexoses.

On the basis of this discussion, the formulas for Compound A and its hydrolysis products are proposed; the position of the 2-hydroxyl or epoxy groups was not determined.

PHARMACOLOGY OF COMPOUND A

Cardiovascular Activity—The purified compound satisfies the criterion for cardiotonic drugs (14) because it exhibits bitterness, systolic standstill of isolated frog heart under perfusion (Straub preparation), inotropic action on spontaneously beating isolated rabbit auricles, and typical alteration of the ECG of the cat.

The toxicity of the compound was studied in the cat. Animals of 2.5–3 kg. were anesthetized with sodium pentobarbital (35 mg./kg.). Blood pressure was recorded through a carotid canula on a lampblack-darkened drum, and an ECG was registered for 10 sec. every 30 sec. (derivation I). Perfusion was performed through an intracath in the femoral vein which was inserted to about 2 cm. below the diaphragm.

An alcoholic (5%) solution of Compound A was adjusted in order to perfuse the required dosage (0.05, 0.1, or 0.3 mg./kg.) in a total volume of 1.5 ml./0.5 hr. The lethal dose was 0.3 mg./kg.; concentrations of 0.05–0.1 mg./kg. resulted in only wild and reversible ECG alterations and had no effect on blood pressure.

The earliest effects of the compound were observed in the ECG between 13 and 25 min. after starting the perfusion. The PR interval was prolonged, and a decalage of ST occurred. Tachycardia was always observed after 20–25 min. of perfusion; increased automaticity of the infraauricular structure followed, resulting at first in isochrone rhythm. The normal pacemaker always regained control for 2 min.; thereafter, the typical course of "digitalis" intoxication ensued.

There was also a typical transient blood pressure fall about 25 min. after the start of the perfusion of 0.3-mg./kg. concentrations without any premonitory or concomitant alteration in the ECG. Blood pressure became irregular and gradually fell 10 min. later.

After 30 min. of perfusion, a micturition was regularly observed. Death occurred 43–45 min. after starting the perfusion.

Antitumor Activity—Cytotoxic activity (1) was determined *in vitro* on a cell culture obtained from a human epidermoid carcinoma of the pharynx (15). The ED_{50} was 1.55 mg./l.

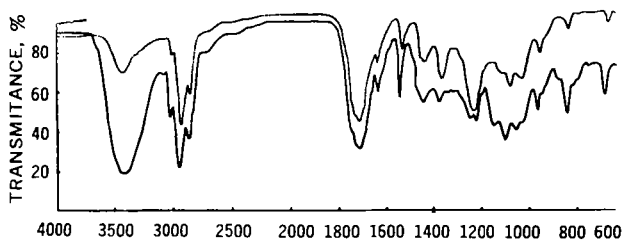


Figure 2—*IR spectra of Compound A and its acetylated derivative (KBr). α-Pyrone bands are at 1720, 1640, and 1538 cm.$^{-1}$; acetyl bands are at 1370 and 1240 cm.$^{-1}$. Key: ——, Compound A; and . . . , acetylated derivative.*

EXPERIMENTAL

Chromatographic Studies—Separation of the sugars isolated from the hydrolysis of the glycoside was accomplished by means of thin-layer, paper, and gas-liquid chromatography. Descending paper chromatography was accomplished on Whatman No. 1 paper utilizing ethyl acetate–pyridine–water (10:4:3) and benzene–1-butanol–pyridine–water (1:5:3:2) as solvent systems. Visualization was obtained by spraying with an alkaline methanolic solution of triphenyl-2,3,5-tetrazolium chloride (1 volume of a 4% solution of triphenyl-2,3,5-tetrazolium chloride + 1 volume of 1 *N* NaOH in methanol) and a 1-butanol–ethanol–water solution of *p*-anisidine phthalate [*p*-anisidine (0.1 *M*) and phthalic acid (0.1 *M*) in 96% ethanol] containing a trace of stannous chloride.

TLC was carried out on silica gel G plates buffered with 0.02 *M* sodium acetate, developed in ethyl acetate–isopropanol (65:35) and chloroform–methanol (80:20), and visualized with anisaldehyde (0.5 ml. anisaldehyde + 0.5 ml. H_2SO_4 + 0.1 ml. glacial acetic acid) in sulfuric acid. GLC was performed on the silylated sugars utilizing silanized chromosorb DMCS, 60–70 mesh impregnated with 10% SE-30 in a 2-m. steel column at a pressure of 1.5 p.s.i. with a programmed temperature from 150 to 300° (6° min. linearly) and detection by means of a flame-ionization detector.

Extraction and Purification—Plants were collected in Yangambi, Democratic Republic of Zaire[2], and were stabilized, 2 kg. of bark at a time, by boiling in 94% ethanol in the presence of calcium carbonate (1 g./10 l.).

Stabilized material corresponding to 8.5 kg. of bark, ground to a fine powder, was extracted by percolation with 94% ethanol until bitterness and characteristic coloration with 85% sulfuric acid could no longer be detected. The extracts (about 60 l.), combined with the ethanolic solution resulting from the stabilization step, were filtered and evaporated under vacuum at room temperature. The resulting residue (1.5 kg.) was dissolved in methanol (2.5 l.) and precipitated by the addition of dichloromethane (7.5 l.). The supernate was absorbed on cellulose (800 g.) and, after removal of the methanol–dichloromethane, extracted with petroleum ether (b.p. 20–50°) in a soxhlet apparatus. The defatted material was transferred to a cellulose column (4 kg.) and eluted with ether containing increasing amounts of dichloromethane followed by dichloromethane with increasing concentrations of methanol.

Fractions eluted with dichloromethane and dichloromethane containing up to 5% methanol were Liebermann positive. These fractions were combined and evaporated; the residue was dissolved in absolute methanol, filtered, and evaporated, yielding 120 g. of residue which was adsorbed onto a 3.5-kg. column of alumina. Fractionation of the column was accomplished by elution with solvents of steadily increasing polarity (benzene, dichloromethane, methanol, and water). The fractions eluted with dichloromethane and dichloromethane containing up to 15% methanol showed a UV absorption maximum at approximately 295 nm. They were combined, filtered, and evaporated, and the residue was dissolved in absolute methanol which was subjected to preparative TLC on silica gel plates (20 × 40 cm.). The plates were developed twice with chloroform–methanol (9:1 v/v), and the samples were removed from the silica gel by prolonged extraction with methanol.

The bitter fractions from the first preparative step also showed a typical coloration and fluorescence with antimony trichloride spray (about 0.22% in $CHCl_3$). These fractions were chromatographed on

[2] Herbarium was identified by M. Vanhaelen in "Jardin Botanique de l'Etat" in Brussels.

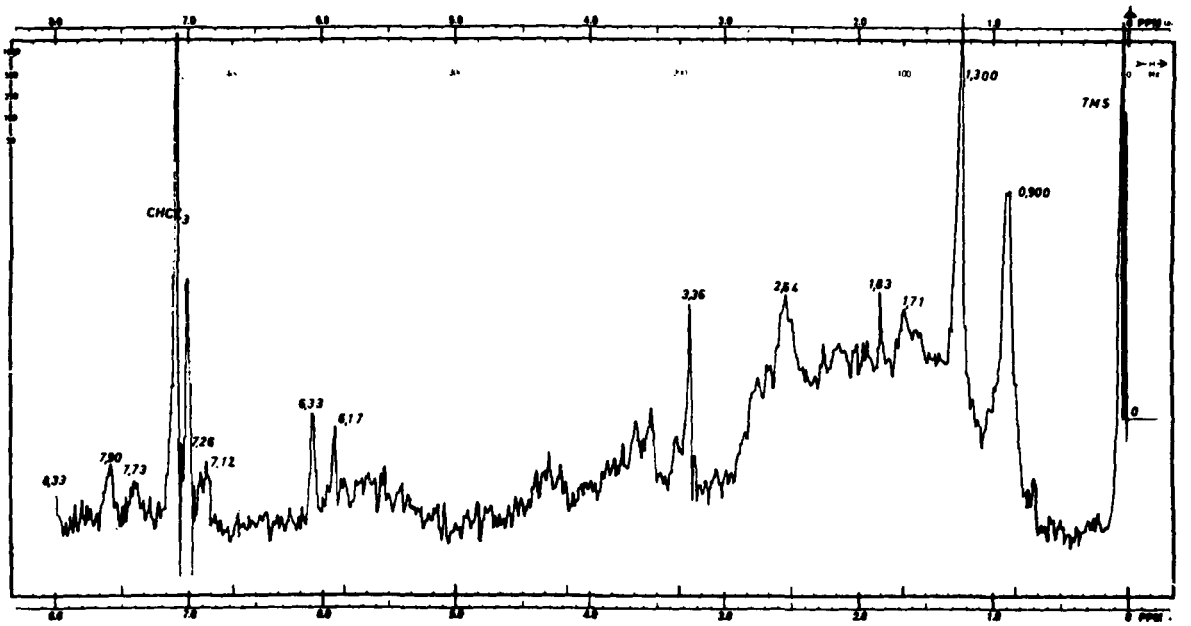


Figure 3—*NMR spectrum of Compound C (5 mg. in $CDCl_3$); accumulated spectrum with CAT (internal standard, tetramethylsilane). $\tau = 9.10$ for C_{18}; $\tau = 8.70$ for C_{19}.*

silica gel, with benzene–methanol–formamide (85:15:1) as the solvent, and were resolved into two spots (A and B). Compound A (lower spot) was dissolved in 3 ml. of absolute methanol, 20 ml. of benzene was added, and the solution was evaporated. This procedure was repeated until a white precipitate was obtained. Compound B (upper spot) was treated in a similar manner, utilizing absolute acetone and cyclohexane, respectively, as the solvents. The purification yield was 0.002%, and Compound A (130 mg.) and Compound B (40 mg.) were obtained in noncrystallizable and chromatographically pure forms.

Hydrolysis of Compounds A and B—Compound A was hydrolyzed to Compound B under mild hydrolysis conditions (5 mg. material; methanol, 0.5 ml.; sulfuric acid, 0.1 *N*, 0.5 ml.; under nitrogen for 72 hr. at 0°). Stronger hydrolytic conditions (5 mg. material; methanol, 0.5 ml.; sulfuric acid, 1 *N*, 0.5 ml.; under nitrogen for 30 min. at 100°) produced the two genins, C and D. These hydrolysates were diluted with an equal volume of water, the methanol was removed *in vacuo*, and the aqueous solution remaining was extracted several times with chloroform to remove the genins and partially hydrolyzed glycoside. This chloroform solution was washed with water, dried over anhydrous sodium sulfate, passed through a small column of silica gel, and purified by precipitation with chloroform and cyclohexane as previously described for Compounds A and B. TLC of these purified products on silica gel G in benzene–methanol–formamide (85:15:1) showed, after spraying with antimony trichloride in chloroform, R_f values of: 0.27, Compound A; 0.34, Compound B; 0.37, Compound C; and 0.41, Compound D.

The aqueous solution remaining after chloroform extraction was diluted with 5 ml. of water and adjusted to a pH of 6.5 with barium hydroxide solution; the barium sulfate was removed by centrifugation. This solution, at pH 6.5, was placed on the top of a small column of cellulose and eluted with water-saturated butanol. Separation of the sugars was achieved by paper, thin-layer, and gas chromatography as previously described.

Characterization of Compounds A and C—Compound A exhibited a UV maximum (in methanol) at 295.5 nm. (log ϵ 3.74) and specific rotation (in methanol) of 35.8 at 589 nm., 37.3 at 578 nm., 43.2 at 546 nm., and 82.2 at 436 nm. Due to the strong absorption of the chromophore in the UV, no optical rotatory dispersion spectrum was recordable below 400 nm. (16). It produced a blue spot which turned greenish blue after 2 hr. with the Liebermann test, no specific coloration or fluorescence with the Rosenheim test, a dark-brown spot which turned reddish brown (after 2 min.) and finally a pale purple with 85% sulfuric acid, and a purplish spot at room temperature which turned blue-gray at elevated temperatures with antimony trichloride. The Legal, Keller-Kiliani, and Raymond tests were negative. Elemental analysis showed: C, 60.00%; H, 7.37% (calc.: C, 59.51%; H, 7.49%). Acetylation produced a yellowish-white noncrystallizable residue. Figure 2 presents a comparison of the IR spectra of Compound A and its acetylated derivative.

Compounds C and A have identical UV spectra and color reactions. Figure 3 presents the NMR spectrum of Compound C.

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