Research Article

ISOLATION AND IDENTIFICATION OF ALKALOIDS FROM *CROTON LOBATUS*

Barthelemy K. Attioua*, Ramiarantsoa Harisolo, Jean Brice Boti, Vincent A. Adiko, Félix Z. Tonzibo, Léon A. Djakoure
Laboratoire de chimie Organique Biologique, UFR Sciences des Structures de la Matière et Technologie, Université de Cocody-Abidjan, Côte d'Ivoire.
*Corresponding author's E-mail: attioua@yahoo.fr

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ABSTRACT

Croton lobatus L. (Euphorbiaceae) is an Ivoirian traditional medicinal plant. Its aerial parts are commonly used as antimalarial and against other diseases. During our past study on this species, several compounds were isolated among which one alkaloid. The aim of this study was to isolate the other alkaloids from its leaves and stems (aerial parts). These were collected together, air-dried and powdered. Three extracts (Cyclohexane, EtOAc - H_2SO_4 and EtOAc - NH_4OH) were realized. Only the third extract was fractionated by aluminum oxide neutral gel column chromatography. Structures of the isolated compounds were established thanks their NMR, HRESIMS and IR spectroscopic data. These fractionations and analyzes have led to *Palmitamide* (1), *Onosmin B* (2), *N-(2-hydroxy-1-phenylpropyl) benzamide* (3), *Onosmin B* (4) and *Aurentiamide acetate* (5). All these compounds were isolated for the first time from *C. lobatus*, except the compound (3), which was isolated from this plant during our past study. These findings confirmed the presence of alkaloid in Croton genus.

Keywords: Croton, Lobatus, Alkaloid, Isolation, Euphorbiaceae, Spectroscopy.

INTRODUCTION

Croton lobatus L. (Euphorbiaceae), a tropical wild plant is commonly used in African traditional medicine to prepare remedies for many diseases (malaria, pregnancy troubles, dysentery)[1]. Interestingly, WENIGER *et al.*[2] have reported the *in vitro* antiplasmodial activity of its aerial parts and roots crude extracts. The *Croton* genus encompasses more than 700 species. Numerous studies have dealt with the constituents of *Croton* and these studies have revealed the presence of sesquiterpenes[3,4], diterpenes[5,6], triterpenes[7], steroids, flavonoids[8] and alkaloids[9]. From the leaves and the stem bark of *C. lobatus,* several compounds were isolated among which sterols, diterpenes, triterpenes, polyphenol and one nitrogen compound[10,11]. The antimalarial property of *C. lobatus* was confirmed by the antiplasmodium falciparum K1 activity of *Geralnylgeraniol* (IC_{50}=1.07 µg/ml) and *Betulinic acid* (IC_{50}=1.45 µg/ml) isolated from its aerial parts by ATTIOUA[12]. During our past study on *C. lobatus*, one alkaloid was clearly identified[10,11]. In this paper, we describe for the first time, the extraction, isolation and the structure of the other alkaloids from *C. lobatus* aerial parts. These include *Palmitanoide* (**1**), Onosmin B (**2**), *N-(2-hydroxy-1-phenylpropyl) benzamide* (**3**), *Onosmin A* (**4**) and *Aurentiamide acetate* (**5**).

MATERIALS AND METHODS

General procedure

Melting points were determined by using a Büchi melting point B-545 apparatus. UV spectra were obtained on a Hewlett Packard 8452A diode array spectrometer. IR spectra were performed using a FT-IR Bruker IFS25 spectrometer. ^1H and ^{13}C NMR spectra and ^1H-^1H COSY, DEPT, 2D-HSQC and 2D-HMBC spectra were recorded from Bruker Avance DMX 400 MHz spectrometers. Chemical shifts are in ppm with reference to TMS. Coupling constants are in Hz. HRESIMS were obtained on an Autospec Micromass (70 eV) instrument (Hewlett Packard). Column chromatography was performed with aluminum oxide neutral gel (100-300 mesh) and TLC with silica gel F_{254}. Alkaloid detection was performed using Dragendorff's reagent, 3% $Ce(NH_4)_2SO_4$ in 85% H_3PO_4, or Ehrlich's reagent.

Plant material

Stems and leaves from *C. lobatus* were collected in the surroundings of Abidjan, Ivory Coast in October 2003. A voucher specimen (No 8950) was deposited in the Herbarium of the Botanical Garden of Cocody University, Abidjan, Ivory Coast. Fresh stems and leaves were dried in the open air without exposure to direct sunlight. The dried sample was powdered and weighed. A total amount of 1200 g was used in this reported study.

Extraction and isolation of alkaloids

The dried and powdered leaves and stems of *C. lobatus* were extracted by maceration at room temperature in EtOH (3×5 L) for 12 h. The combined extracts were concentrated under reduced pressure until a volume of 500 mL, which was washed with cyclohexane (4×500 mL). The previous residue of the EtOH extract (350 mL) was then washed with a mixture of EtOAc – H_2SO_4 10% (49:1 v/v) (5×350 mL). Finally the residue of the EtOAc – H_2SO_4 extract was washed with a mixture of EtOAc – NH_4OH (19:1 v/v) (4×300 mL). Only the EtOAc – NH_4OH extracts were evaporated under reduced pressure to dryness, 3.53 g of crud extract were obtained. The screening of this extract using Dragendorff has revealed the presence of alkaloid. This was later confirmed by Ehrlich's reagents. Then, this extract was subjected to a series of column chromatography, using aluminum oxide neutral gel as

stationary phase. The elution was performed with a mixture of CH_2Cl_2-EtOAc then EtOAc – MeOH, following a gradient of polarity. For the first fractionation, we used a chromatography column with the sizes: 3.5 cm of diameter (d) and 17.5 cm of height (h). Elution with a mixture of CH_2Cl_2 - EtOAc (90: 10 v/v) gave fraction I, that with a mixture of CH_2Cl_2-EtOAc (50:50 v/v) to EtOAc - MeOH (99:01 v/v) gave the fraction II and the fraction III was obtained with a mixture of EtOAc - MeOH (95:5 to 85:15 v/v). The fraction I (200 mg) was purified by recristallization in MeOH to give 63.2 mg of compound (**1**) (R_f 0.5 in CH_2Cl_2- EtOAc 85:15 v/v). The fraction II (1325 mg) was purified by column chromatography (d: 1.51 cm, h: 10 cm) on aluminum oxide neutral gel. The elution with a mixture of CH_2Cl_2-EtOAc (55:45 v/v) gave sub-fraction II_1, then a mixture of CH_2Cl_2-EtOAc (40:60 v/v) the sub-fraction II_2 and with CH_2Cl_2-EtOAc (25:75 v/v) the sub-fraction II_3. These sub-fractions were purified by recristallization in cyclohexane. The sub-fraction II_1 gave compound (**2**) (25 mg, R_f 0.7 in CH_2Cl_2- CH_3OH 95:5 v/v), the sub-fraction II_2 gave compound (**3**) (18 mg, R_f 0.55 in CH_2Cl_2- CH_3OH 95:5 v/v) and the sub-fraction II_3, the compound (**4**) (21 mg, R_f 0.5 in CH_2Cl_2- CH_3OH 95:5 v/v). The latest fraction (III) 500 mg, was purified by column chromatography (d: 1.51 cm, h: 10 cm) on aluminum oxide gel. The elution with CH_2Cl_2 - EtOAc (10:90 v/v) gave the sub-fraction III_1. This sub-fraction was then purified by recristallization in cyclohexane to afford compound (**5**) (15 mg, R_f 0.45 in CH_2Cl_2- CH_3OH 95:5 v/v). All these compounds were soluble in chloroform.

Palmitamide (**1**): White powder; mp: 105-106°C; UV λ_{max} (MeOH) 215 nm; IR ν_{max} (KBr) in cm^{-1}: 1647 (strong, $H_2NC=O$), 3375-3310 (N-H), 2918 and 2849 (CH_2, CH_3). ^1H and ^{13}C NMR ($CDCl_3$) δ (ppm): 2.24 (t, *J=7.5 Hz*, H-2), 1.60 (m, H-3), 1.25 (br s, H-4 to H-15, CH_2), 0.87 (t, J=6.9Hz, H-16, CH_3), 5.40 (br s, H-17, NH_2); 175.7 (C-1, C=O), 36.0 (C-2), 29.2 -29.6 (C-4 to C-13, CH_2), 14.1 (C-16, CH_3). HRESIMS: $[M+H]^+$ at m/z 256.45918, formula $C_{16}H_{33}NO$. See Table 1 for additional NMR spectral data.

Onosmin B (**2**): white amorphous solid; mp: 139-141°C; UV λ_{max} (MeOH) nm: 242, 293 and 302 nm; IR ν_{max} (KBr) in cm^{-1}: 3345 (sharp, N-H), 3012 (wide, N-H), 728 and 987 (benzyl), 1698 (strong, C=O). ^1H and ^{13}C NMR ($CDCl_3$) δ (ppm): 3.77 (br s, H-8, O-CH_3), 51.6 (C-8, O-CH_3), 6.31 to 7.77 (aromatic protons). HRESIMS: $[M+H]^+$ at m/z 256.13371, formula $C_{16}H_{17}NO_2$. See table 1 for additional NMR spectral data.

N-(2-Hydroxy-1-phenyl-propyl)-benzamide (**3**): White crystals; mp: 157-158°C; UV λ_{max} (MeOH) nm: 293, 310; IR ν_{max} (KBr) cm^{-1}: 3369, 2957 (middle, CH_3), 1638 (strong, C=O). HRESIMS: $[M+H]^+$ at 256.32024, formula $C_{16}H_{17}NO_2$.

Onosmin A (**4**) was white amorphous solid, mp 187-189°C; UV λ_{max} (MeOH) nm: 242, 293 and 302 nm; IR ν_{max} (KBr) in cm^{-1}: 3345 (sharp, N-H), 3012 (wide, N-H), 728 and 987 (benzyl), 1699 (strong, C=O), 3015 (O-H of acid). ^1H and ^{13}C NMR ($CDCl_3$) δ (ppm): 169.8 (COOH), HRESIMS: $[M+H]^+$ at m/z 242.29294, formula $C_{15}H_{15}NO_2$. See Table 2 for additional NMR spectral data.

Aurentiamide acetate (**5**): white amorphous solid, mp =180-182°C, UV λ_{max} (MeOH) nm: 242, 293 and 302 nm; IR ν_{max} (KBr) in cm^{-1}: 1670 (HNC=O) and 1634 (HNC=O), 1741 ($COOCH_3$). ^1H and ^{13}C NMR ($CDCl_3$) δ (ppm): 7.15 to 8.12 (aromatic protons), 170.1 (C-14), 2.01 (br s, H-15, $OCOCH_3$). HRESIMS: $[M+H]^+$ at m/z 445.53782 formula $C_{27}H_{28}N_2O_4$. See table 2 for additional NMR spectral data.

RESULTS AND DISCUSSION

The fractionation by aluminum oxide neutral gel (100-300 mesh) column chromatography of the EtOAc – NH_4OH crude extract from the leaves and stems of *C. lobatus* gave three fractions (I, II and III). Future purification of these fractions by a combination of column chromatography and recristallisation methods has led to the isolation of five alkaloids (figure 1).

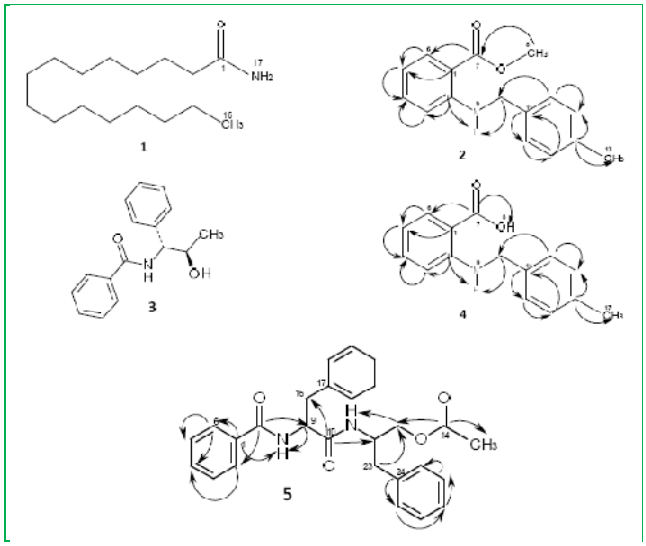

Figure 1: structures of *Palmitanoide* (**1**), Onosmin B (**2**), *N-(2-hydroxy-1-phenylpropyl)benzamide* (**3**), *Onosmin A* (**4**) and *Aurentiamide acetate* (**5**). Black arrow: COSY ($^3J_{H-H}$) and HMB ($^2J_{C-H}$, $^3J_{C-H}$ and $^4J_{C-H}$) correlations.

This is in confirming with the results of alkaloid tests realized with Dragendorff and Ehrlich reagents. The compound (**1**) was *Palmitamide* or *Hexadecanamide,* an aliphatic alkaloid derivative. It was isolated as a white powder with a melting point (mp: 105-106°C), UV absorption band of 215 nm. The IR spectrum on KBr disc showed stretching band of the carbonyl $H_2NC=O$ at 1647 cm^{-1}. For the N-H, absorption was observed between 3375 and 3310 cm^{-1}. The two strong bands at 2918 cm^{-1} and 2849 cm^{-1} could be attributed to the methyl (CH_3) and methylene (CH_2) groups. NMR spectra were recorded in $CDCl_3$ and spectral data are reported in table 1. The ^1H NMR spectrum exhibited one methyl group at δ 0.87 ppm (t; H-16, *J=6,9 Hz*). The singlet at δ 1.25 ppm (br s) indicated an aliphatic chain $(CH_2)_n$. Amide protons ($H_2NC=O$) gave wide signal between δ 5.52 and 5.37 ppm. ^{13}C NMR spectra confirmed the presence of amide group with the peak at δ 175.7 ppm ($H_2NC=O$).

Table 1: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of compounds (**1**) and (**2**) in $CDCl_3$ (δ in ppm, J in Hz).

Position	1		2	
	δ_C	δ_H (mult. Hz)	δ_C	δ_H (mult. Hz)
1	175.7	-	108.3	-
2	36.0	2.24 (t; 7.5)	149.6	-
3	25.5	1.60 (m)	113.7	6.57 (d; 8.4)
4	29.2	1.25 (br s)	132.9	7.31 (dd; 8.4; 8.1)
5	29.3	1.25 (br s)	117.6	6.77 (dd; 8.1; 7.8)
6	29.5	1.25 (br s)	131.0	7.77 (d; 7.8)
7	29.6	1.25 (br s)	167.1	-
8	29.6	1.25 (br s)	51.6	3.77 (br s)
9	29.6	1.25 (br s)	-	4.0 (brs)
10	29.6	1.25 (br s)	47.1	4.27 (brs)
11	29.6	1.25 (br s)	139.9	-
12	29.6	1.25 (br s)	126.1	6.93 (d, 6.6)
13	29.3	1.25 (br s)	129.0	6.91 (d, 6.6)
14	31.9	1.25 (br s)	136.2	-
15	22.7	1.25 (br s)	129.0	66.91 (d, 6.6)
16	14.1	0.87 (t; 6.9)	126.1	6.93 (d, 6.6)
17		5.4 (br)	25.1	2.37 (br s)

Table 2: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of compounds (**4**) and (**5**) in $CDCl_3$ (δ in ppm, J in Hz).

Position	4		5	
	δ_C	δ_H (mult. Hz)	δ_C	δ_H (mult. Hz)
1	107.3	-	134.8	-
2	150.1	-	127.2	8.12 (d, 8.1)
3	113.7	6.65(d; 8.4)	128.7	7.91 (dd; 8.1; 8.4)
4	133.9	7.31 (dd; 8.4; 8.1)	132.0	7.95 (m)
5	117.6	6.80 (dd; 8.1; 7.8)	128.7	7.91 (dt; 8.4; 8.1)
6	132.6	7.93 (d; 7.8)	127.2	8.1 (d, 8.1)
7	169.8	-	168.5	-
8	-	-	-	7.99 (brs)
9	-	4.0 (brs)	54.6	4.93 (dd; 6.3; 13)
10	47.1	4.27 (brs)	171.8	-
11	139.9	-	-	7.99 (brs)
12	126.1	6.94 (d, 6.7)	51.0	4.55 (m)
13	129.0	6.91 (d, 6.7)	67.6	4.17 (H-α, m), 4.44 (H-β; m)
14	136.2	-	170.1	-
15	129.0	66.91 (d, 6.7)	21.0	2.01 (br s)
16	126.1	6.94 (d, 6.7)	37.5	2.97 (H-α; dd; 6.3; 13) 3.20 (H-β; dd; 6.3; 13)
17	25.1	2.35 (br s)	139.1	-
18			127.0	7.15 (d; 7.5)
19			128.2	7.25 (dd; 7.5; 8.0)
20			125.9	7.02 (m)
21			128.2	7.25 (dd; 7.5; 8.0)
22			127.0	7.15 (d; 7.5)
23			41.0	2.62 (H-α; dd; 6.1; 13) 2.87 (H-β; dd; 6.1; 13)
24			138.8	-
25			128.1	7.15 (d; 7.5)
26			128.9	7.25 (dd; 7.5; 8.0)
27			126.1	7.02 (m)
28			127.0	7.25 (dd; 7.5; 8.0)
29			128.1	7.15 (d; 7.5)

The only methyl gave signal at δ 14.1 ppm and the aliphatic chain, the strong pick at δ 29.6 ppm. The HRESIMS spectrum exhibited the molecular ion peak $[M+H]^+$ at m/z 256.45918 with the molecular formula $C_{16}H_{33}NO_3$ (M= 255.45 g/mol). *Palmitamide* is a natural product already isolated from *Acropora pulchra*[13], in the essential oil of two species of *Alpinia*[14] and from the green alga *Rhizoclonium hieroglyphicum* var[15].

The compound (**2**) was *Onosmin B or Methyl 2-(p-tolylmethylamino)benzoate*, an alkaloid derivative. It was isolated as a white amorphous solid with a melting point (mp) of 139-14°C. Its UV spectrum exhibited absorption band maximum at 242, 293 and 302 nm, corresponding to those of the methyl benzoate and *p*-methylbenzene system. The IR spectrum recorded on KBr disc showed absorption bands of N-H at 3345 cm^{-1} (sharp) and 3012cm^{-1} (wide), those of the benzyl group at 728 and 987cm^{-1}. The carbonyl C=O exhibited stretching band at 1698 cm^{-1}. NMR spectra were recorded in $CDCl_3$. The ^{1}H NMR spectrum displayed singlet signal of H-8 at δ 3.77 ppm due to the methoxy group (O-CH_3). A wide signal ascribable to the secondary amine proton H-9 appeared at δ 4.0 ppm. The benzoate protons are observed at δ 6.57 ppm (H-3), 7.31 ppm (H-4), 6.77 ppm (H-5) and 7.77 ppm (H-6). Protons of the methylbenzene appeared at δ 6.93 ppm (H-12, H-16) and δ 6.91 ppm (H-13, H-15). The ^{13}C NMR spectrum confirmed the presence of methylbenzoate system with signals at δ 108.3 ppm (O=C-OCH_3, C-1), δ 149.6 ppm (C-2) and δ 51.6 ppm (O-CH_3). COSY, HSQC and HMBC spectra gave the best information about the structure of this compound. A combination of COSY, HSQC and HMBC spectral data have led to these sequences: C_8-C_7-C_1; C_6-C_1-C_7-C_2-C_3; C_{17}-C_{15}-C_{13}. For more correlations, see figure 1. The HRESIMS spectrum exhibited molecular ion peak $[M+H]^+$ at m/z 256.13371 with the molecular formula $C_{16}H_{17}NO_2$ (M=255.12). *Onosmin B* was previously isolated from *Onosma hispida* wall[16]. The compound (**3**) was *N-(2-hydroxy-1-phenyl-propyl)-benzamide.* It was already isolated and described from *C. lobatus* during our past work[10,11]. The compound (**4**) was *Onosmin A.* It was isolated as white amorphous solid, mp 187-189°C. Its structure was similar to that of compound (**2**), which was the methyl ester of compound (**4**). Its NMR spectral data (table 2) were similar to those of (**2**). But, a small difference was observed with the carbonyl group of a carboxylic acid (COOH), which showed signal at δ 169.8 ppm (C-7). On the ^{1}H and ^{13}C NMR spectra of the compound (**4**), signals at δ 3.77 ppm (H-8) and δ 51.6 ppm (C-8) were missing; these indicated the absence of a methoxy group (O-CH_3). The HRESIMS spectrum exhibited the molecular ion peak $[M+H]^+$ at m/z 242.29294 with the molecular formula $C_{15}H_{15}NO_2$ (M=241.28 g/mol). *Onosmin A* was also previously isolated from *Onosma hispida* Wall[16]. The compound (**5**) was isolated as a white amorphous solid, mp =180-182°C. It was the *N-benzoyl phenylalaminoyl phenlyalaninolacetate* or the *Aurentiamide acetate.* Its IR spectrum displayed two absorption bands due to the

stretching of the carbonyl C=O (HNC=O) at 1670 and 1634 cm^{-1}, that from the carbonyl of the ester group ($COOCH_3$) appeared at 1741 cm^{-1}. The ^1H NMR spectrum in $CDCl_3$ exhibited the chemical shifts of aromatic protons between δ 8.12 and 7.02 ppm. Protons of amide group gave the wide signal at δ 8.12 ppm (H-8, H-11) and these of the acetate (CH_3-COO) gave a singlet at δ 2.01 ppm (H-15). The ^{13}C NMR spectrum showed carbonyl signals at δ 168.5 ppm (C-7, HNC=O), δ 171.8 ppm (C-10, HNC=O) and δ 170.1 ppm (CH_3O-C=O). The aromatic carbons are observed between δ 125.7 and 139.1 ppm. The structure of this compound was clearly established thanks to COSY, HSQC and HMBC spectral data. The HRESIMS spectrum exhibited the molecular ion peak $[M+H]^+$ at m/z 445.53782 with the molecular formula $C_{27}H_{28}N_2O_4$ (M=444.54 g/mol). *Aurentiamide acetate* was previously isolated from *C. hieronymi* Griseb[17] and *Patrinia villosa* Juss[18].

CONCLUSION

Investigation of the EtOAc-NH_4OH crude extract from *C. lobatus* aerial parts *has* led to the isolation of five alkaloids: *Palmitanoide* (**1**), Onosmin B (**2**), *N-(2-hydroxy-1-phenylpropyl) benzamide* (**3**), *Onosmin A* (**4**) and *Aurentiamide acetate* (**5**). Their structures were determined thanks by NMR, UV, IR and HRESIMS spectroscopic data.

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