GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Isolation of two cassane-type diterpenoids from the bark of *Erythrophleum suaveolens* (Guill. & Perr.) Brenan (Fabaceae)

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GSC Biological and Pharmaceutical Sciences, 2025, 32(03), 314-321

Publication history: Received on 21 August 2025; revised on 27 September 2025; accepted on 30 September 2025

Article DOI: https://doi.org/10.30574/gscbps.2025.32.3.0376

Abstract

Erythrophleum suaveolens is a plant species used to treat Buruli ulcer in Côte d'Ivoire. This plant is rich in cassane-type diterpenoids, compounds known for their multiple biological activities. This study focuses on the isolation, purification, and structural identification of two cassane-type diterpenoids from the ethyl acetate extract of the bark of *Erythrophleum suaveolens*. Two molecules were isolated using conventional purification and isolation methods, namely column chromatography and preparative HPLC. The structural analysis of these compounds was carried out using one-dimensional (1D) and two-dimensional (2D) ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR), COSY, HMBC, and NOESY. The molecules were identified as 3β-acetoxyerythrosuamine and 3β-hydroxy-3-methylbutanoyloxy-6α-hydroxy-norcassamine. These compounds have already been isolated from the root bark of this plant, but this is the first time they have been isolated from the trunk bark. Both compounds belong to the cassane genus, and their discovery enriches the chemistry of *E. suaveolens.*

Keywords: Fabaceae; *Erythrophleum suaveolens;* Cassane diterpenoid; NMR

1. Introduction

Cassane-type diterpenoids are an important class of secondary metabolites in several species of the genus *Erythrophleum* as well as in other Fabaceae. In the genus *Erythrophleum,* despite their high toxicity to humans and livestock, many species are widely used for therapeutic purposes in various local communities [1]. In addition, in Africa and Asia, traditional medicine practitioners empirically used decoctions of leaves or powdered bark from species of the genus *Erythrphleum* to treat various conditions, including cardiovascular disease, dysentery, and diarrhea (decoction of *E. africanum* leaves) [2, 3], blood cancer and mental illness (aqueous extract of leaves and trunk bark of *E. africanum*, aqueous extract of leaves of *E. fordii*) [4, 5], lung diseases in livestock (powder from the bark of *E. chlorostachys*) [6], smallpox, convulsive disorders, pain, swelling (bark of *E. ivorense*) [7, 8], fevers (powder from the bark of *E. chlorostachys*) [6]. The powdered bark of *E. couminga* is used as a purgative and laxative. It was previously used as a poison for fishing and hunting. Pharmaceutical companies used the bark of *E. couminga* as a non-steroidal cardioactive drug, but the project was abandoned due to a shortage of material [9].

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Species of the genus also have various biological activities: cytotoxicity, antimalarial, antioxidant, and anti-inflammatory [10, 11, 12, 13].

Erythrophleum suaveolens, a tree widespread in West Africa, is used in traditional medicine [14]. The objective of this study is to isolate cassane-type diterpenoids from the bark of *Erythrophleum suaveolens.* These molecules will enrich the knowledge base on cassane diterpenoids in this species and may serve as a basis for further pharmacological investigations.

2. Material and methods

2.1. Material

2.1.1. Plant material

The bark of the trunk of *Erythrophleum suaveolens* (Guill. & Perr.) Brenan (Fabaceae) was collected in December in Toumodi (Pacobo: 6°09'56.8"N 4°56'26.1"W), a town located in central Côte d'Ivoire. It was identified and authenticated at the National Floristic Center (CNF) of Félix HOUPHOUËT-BOIGNY University (Abidjan, Ivory Coast). A specimen numbered 10 DIBI ES-2014 was deposited in the herbarium of this center. The bark was scraped from the trunk using a machete, dried in the laboratory away from sunlight, and ground using an electric grinder. The powder obtained was used for the extractions.

2.1.2. Chemical material

The chemical materials consist of distilled methanol, ammonium hydroxide, dichloromethane, sulfuric acid, distilled ethyl acetate, formic acid, acetonitrile, Sephadex® LH 20, and silica gel 60.

2.2. Methods

2.2.1. Extraction

1 kg of *E. suaveolens* bark powder was subjected to solid-liquid extraction at room temperature in 10 L of distilled methanol (MeOH) for 24 hours. This operation was repeated twice more with the residual marc under the same conditions as before. After filtration, the various solutions were evaporated using a rotary evaporator (Ratavapor, Sartorius Stedim Biotech) and then dried to yield 48.3 g of crude extract. Next, 15 g of this methanolic extract was dissolved in 50 mL of MeOH, then alkalized with a few drops of 25% ammonium hydroxide (NH_4OH), and then supplemented with 100 mL of dichloromethane (CH_2C1_2). To the solution obtained, 100 mL of 1% sulfuric acid was added. The solution obtained was counter-extracted by liquid-liquid extraction with distilled CH_2C1_2 (100 mL x 4). The supernatant phase separated from the dichloromethane phase is first alkalized with NH_4OH to pH = 10, then counter-extracted with distilled ethyl acetate (100 mL x 4) to give, after evaporation using a rotary evaporator, the ethyl acetate extract coded ESA.

2.2.2. Preliminary test: phytochemical screening

The ESA extract underwent phytochemical screening with Dragendorff's reagent. The test proved positive, so the ESA extract was used for fractionation and purification.

2.2.3. Chromatographic methods

Thin-layer chromatography (TLC)

Thin-layer chromatography analyses were performed on aluminum plates (Silicagel 60 F_{254}, Merck, Germany). After development in glass tanks, the plates were observed under ultraviolet light (254 nm and 366 nm). The UV lamp used was manufactured by Vilber Lourmat (230 V, 50 Hz, France).

Flash chromatography

We used a device with an isocratic and gradient pumping system, with a double piston pump. Cartridges weighing 330 g, 120 g, and 24 g from the Grace Resolv® Silica Flash Silica series were used. These cartridges have the advantage of effectively replacing glass columns.

Silica gel column chromatography (CC)

Chromatographic column separations were performed taking into account the mass of the sample (fraction or extract) to be fractionated and its chromatographic profile (CCM). The diameter and height of the column are determined based on the mass of the sample. For effective separation, the mass of silica in the column is equal to 10 times the mass of the sample to be purified **[15].** In this study, the stationary phase used in the various CCM separations was silica gel 60 (40-63 µm, Chromagel, Merck).

Fractionation and purification of ESA extract constituents

The ESA extract is initially purified using flash chromatography. For 2 g of ESA extract, a cartridge containing 10 g of silica gel 60 was used. The elution system consists of a MeOH/ CH_2Cl_2 mixture following an elution gradient of 1:99 (v/v) to 50:50 (v/v). The fractions collected are grouped according to their chromatographic profiles (CCM). A total of 6 fractions (F1 to F6) were collected, and each fraction was then purified on a chromatographic column using a 1.5 cm diameter column and Sephadex® LH 20 as the stationary phase.

Fractions F1 to F5 were purified under the same conditions, using a CH_2Cl_2/MeOH (2:1, v/v) mixture as eluent, following isocratic elution, to give a single subfraction. This fraction was then purified by preparative high-performance liquid chromatography (HPLC) using the H_2O+0.1% methanoic acid (HCOOH)/acetonitrile (ACN) system (85:15 to 50:50, v/v) to provide compound 1.

Fraction F6 is purified under the same conditions as F1-F5, using the CH_2Cl_2/MeOH (1:1, v/v) mixture as eluent, following isocratic elution, to give a single subfraction which is then purified by preparative HPLC (reverse phase) with the H_2O+0.1% HCOOH/ ACN (85:15 to 50:50, v/v) to give compound 2.

2.2.4. Structural identification

The isolated compounds were subjected to the following spectroscopic techniques:

- ^1H and ^{13}C NMR to assign proton and carbon signals.
- Two-dimensional experiments:
    - COSY (Correlation Spectroscopy) to identify couplings between adjacent protons.
    - HMBC (Heteronuclear Multiple Bond Correlation) for long-range proton-carbon correlations (2-3 bonds) to locate substituents and side chains.
    - NOESY to establish spatial proximities (interactions across space), which helps define relative stereochemistry (α/β faces, 3β, 6α positions, etc.).
- Other spectral data (exact mass, IR) as needed to confirm functional groups (hydroxyl, ester, acetoxy) and molecular formula.

For purity control, each purified compound was checked by thin-layer chromatography (TLC) for homogeneity, and by additional spectra to ensure that there was no mixture.

3. Results

3.1. Structural elucidation of compound 1

Compound 1, soluble in methanol, was obtained as an amorphous solid. Its IR spectrum shows certain characteristic bands, such as those at υ_{max} 1735, 1721, and 1594 cm^{-1}, which indicate the presence of three ester functions, and the band at υmax 1669 cm^{-1}, which suggests the presence of a ketone function. The hydroxyl and amine groups give broad absorption bands centered at υ_{max} 3342 and 3323 cm^{-1}. The mass of this compound was determined using the HRESI-QTOF-SM^+ method. The HRESI-QTOF-SM^+ mass spectrum shows the peak of the pseudomolecular ion $[M+H]^+$ at *m/z* 494.2713, corresponding to a molecular mass of 493.2635 g/mol. This value is consistent with the molecular formula $C_{26}H_{39}NO_8$ (calculated mass 493.2676). The fragmentation pattern of this molecule is typical of diterpenoid cassanes and resembles those of 3β-hydroxy-3-methyl-butanoyloxy)-nor-erythrosuamine and 3β-hydroxy-3-methyl-butanoyloxy-6α-hydroxy-nor-cassamine **[11].** The difference with these compounds is the loss of the acetate fragment (CH_3-COO^-; m/z 59 g/mol) in the form of acetic acid (CH_3-COOH) observed at *m/z* 60 g/mol. The ^1H and ^{13}C NMR spectra of compound 1, performed in deuterated methanol, confirm the link between this compound and those of 3β-hydroxy-3-methyl-butanoyloxy)-nor-erythrosuamine and 3β-hydroxy-3-methyl-butanoyloxy-6α-hydroxy-nor-cassamine **[11].** Indeed, there is a very strong resemblance between the spectra of these three molecules. However, on the proton spectrum of compound 1, a singlet is observed at δ_H 2.00 ppm, whereas on those of 3β-hydroxy-3-methyl-butanoyloxy)-

nor-erythrosuamine and 3β-hydroxy-3-methyl-butanoyloxy-6α-hydroxy-nor-cassamine, this singlet resonates at δ_H 2.49 (*s*, 2H) and 2.45 (*s,* 2H) ppm, respectively. The HSQC spectrum indicates that the proton at δ_H 2.00 ppm (*s*, 3H) is carried by the carbon at δ_C 20.8 ppm. Also, the HMBC spectrum data allowed the acetoxy group (CH_3COO) to be fixed in the C-3 position on the carbon chain. Indeed, the carbon at δ_C 172.1 ppm (C=O) correlates in $2J_{CH}$ with the proton at δ_H 2.00 ppm, and in $3J_{CH}$ with the proton at δ_H 4.72 ppm (HC-OH; C-3). We also observe correlations in $3J_{CH}$ between the carbon at δ_C 77.0 ppm (HC-OH) and the protons at δ_H 1.96 ppm (H-9) and 2.81 ppm (H-14). We can therefore deduce that the secondary alcohol at δ_C 77.0 ppm is in position C-7. The carbon of the ketone function observed at δ_C 209.6 ppm (C=O, ketone) correlates in $2J_{CH}$ with the protons at δ_H 2.75 ppm (H-5) and δ_H 4.05 ppm (H-7). We can also deduce that the ketone function is in position C-6. According to the NOESY spectrum, the proton in position (H-7) sees protons H-3, H-5, H-9, and H-18, which are behind the plane. Comparing our spectra with those of this compound allowed us to identify compound 1 as 3β-acetoxyerythrosuamine (Figure 1).

Table I provides information on ^1H and ^{13}C NMR chemical shifts, COSY, NOESY, and HMBC correlations.

Table 1 ^1H and ^{13}C NMR chemical shift (1D and 2D) of compound 1 (MeOD)

N° atom	^{13}C (δ, ppm)	^1H (δ, ppm ; *m* ; *J*, Hz)	COSY	HMBC	NOESY
1	37,2	1,51 ; 1H ; *m* 1,85 ; 1H ; *m*	H-2α H-2β	C-3 ; C-9 ; C-20 -	- -
2	24,9	1,69; 1H; *m* 2,21 ; 1H ; *m*	H-1α ; H-3α H-1β ; H-3β	- -	- -
3	80,3	4,72 ; 1H ; *dd* (12,4 ;3,03)	H-2α	C-1 ; C-19 ; C25	-
4	43,3	-	-	-	-
5	65,0	2,75 ; 1H ; *s*	-	C-6; C9, C-19; C18 C-20	H-18
6	209,6	-	-	-	-
7	77,0	4,05 ; 1H ; *d* (11,4)	-	C-8; C14, C-6	H-17
8	52,1	1,84 ; 1H ; *m*	H-9	-	H-20
9	47,1	1,96 ; 1H ; *m*	H-8	C-20	-
10	37,2	-	-	-	-
11	27,7	1,12 ; 1H ; *m* 1,93 ; 1H ; *m*	H-12α H-12β	- C-12; C13	- -
12	24,8	2,11 ; 1H ; *m* 3,31 ; 1H ; *m*	H-11α H-11β	C-11; C-15 C-16	- -
13	167,4	-	-	-	-
14	41,9	2,81 ; 1H ; *m*	H-17	C-8; C-15	H-15
15	113,1	5,81 ; 1H ; *s*	-	C-12; C-13; C-16	H-14
16	169,7	-	-	-	-
17	14,9	1,21 ; 3H ; *d* (6,8)	H-14	C-8; C-13	H-7
18	25,7	1,18 ; 3H; *s*	-	C-19; C-5; C-5; C-10	H-5
19	174,8	-	-	-	-
20	14,9	0,96; 3H; *s*	-	C-1; C-5; C-9; C-10	H-8
21	60,2	4,33 ; 2H ; *m*	H-22	C-16	-
22	49,6	3,28 ; 2H ; *m*	H-21	C-23	-
23	33,8	2,71 ; 3H ; *s*	-	C-21	-

24	52,0	3,70 ; 3H ; *s*	-	C-19	-
26	172,1	-	-	-	-
26	20,8	2,00 ; 3H ; *s*	-	C-25	-

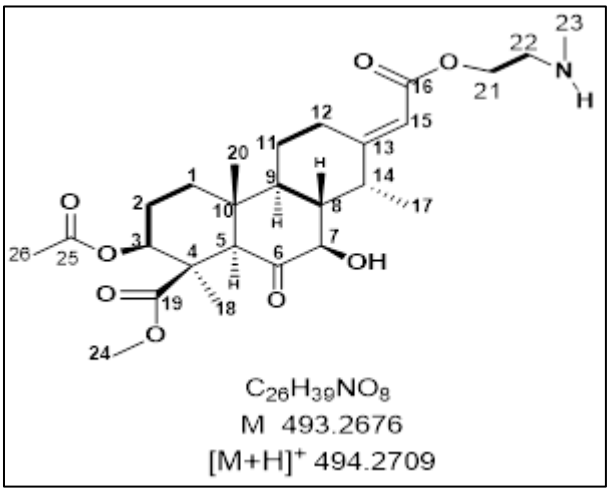

Figure 1 Structure of 3*β*-acétoxyérythrosuamine

3.2. Structural elucidation of compound 2

Compound 2 was obtained as an amorphous solid, soluble in methanol. Its IR spectrum also indicates the presence of carbonyl groups (ester, ketone, and amide) as evidenced by absorption bands observed at υ_{max} 3343, 3321, 1733, 1713, 1706, and 1670 cm^{-1}. The molecular mass of this compound was determined using high-resolution electrospray ionization in positive mode (HRESI-QTOF-SM$^+$). The HRESI-QTOF-SM$^+$ mass spectrum shows the pseudo-molecular ion $[M+H]^+$ peak at *m/z* 552.3177, giving a molecular mass of 551.3099 g/mol. This value corresponds to the molecular formula $C_{29}H_{45}NO_9$ (calculated mass 551.3094).

The fragmentation pattern of compound 2 suggests that it is a cassane-type diterpenoid [16]. Some fragments are also comparable to those of 6α-hydroxy-norcassamine [17]. Specifically, the ion $[(M+H)-75]^+$ observed *at m/z* 478.2111 corresponds to the loss of HO-CH_2-CH_2-$NHCH_3$; the ion [(M+H)-75-118]+ observed at 360.1566 corresponds to the loss of HO-CH_2-CH_2-$NHCH_3$; the ion $[(M+H)-75-118-60]^+$ observed at 300.1415 corresponds to the loss of CH_3O-CO; and the ion $[(M+H)-31]^+$ observed at *m/z* 522.2316 corresponds to the loss of CH_3-NH_2 (Figure 2).

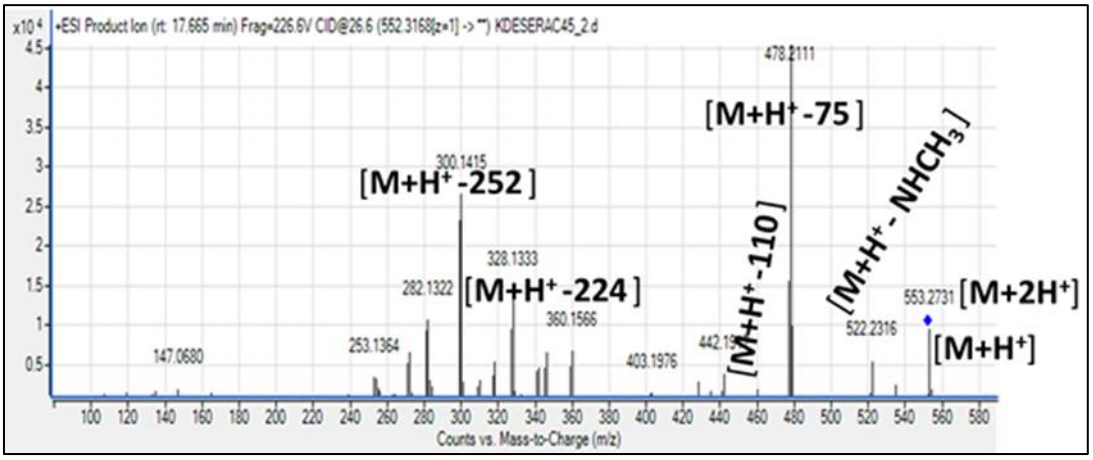

Figure 2 ESI-MS/MS mass spectrum of 3β-hydroxy-3-methyl-butanoyloxy-6α-hydroxy-nor-cassamine

The fragment that differentiates 6α-hydroxy-norcassamine from compound 2 is the 3-hydroxy-3-methylbutanoate group ($HO\text{-}C(CH_3)_2\text{-}CH_2\text{-}COO^-$), which captures a proton to form 3-hydroxy-3-methylbutanoic acid (Figure 3), corresponding to the loss of a mass unit of 118 g/mol.

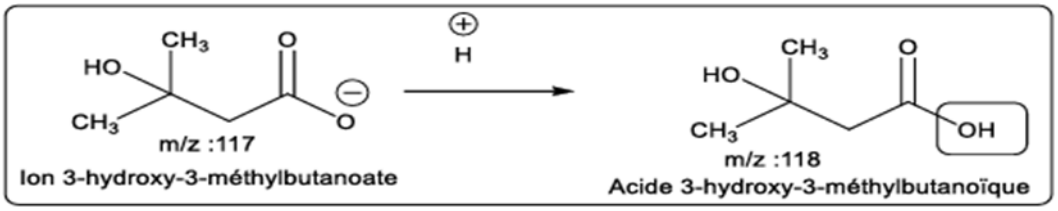

Figure 3 Conversion of the 3-hydroxy-3-methylbutanoate ion to 3-hydroxy-3-methylbutanoic acid

The positioning of the 3-hydroxy-3-methylbutanoate group on the carbon skeleton of compound 2 was determined based on correlations observed in the HMBC spectrum. In this spectrum, the carbonyl at δ^C 172.5 ppm shows a $3J_{CH}$ correlation with the proton at δ_H 4.64 ppm (H-3), allowing it to be assigned to carbon C-3 of the basic cassane-type diterpenoid skeleton. Regarding the stereochemical arrangement of this molecule, the NOESY spectrum shows that the H-3 proton is positioned behind the plane, as it correlates with H-5, H-9, H-17, and H-18, which are also behind the plane. From this, it is deduced that the 3-hydroxy-3-methylbutanoic acid group (Figure 3) is oriented in front of the molecular plane. Based on the analyses above, compound 2 was formally identified as 3β-hydroxy-3-methylbutanoyloxy-6α-hydroxy-nor-cassamine (Figure 4). This molecule has previously been isolated from the root bark of *Erythrophleum suaveolens* [18]; however, this is the first time it has been identified in the trunk bark of this plant.

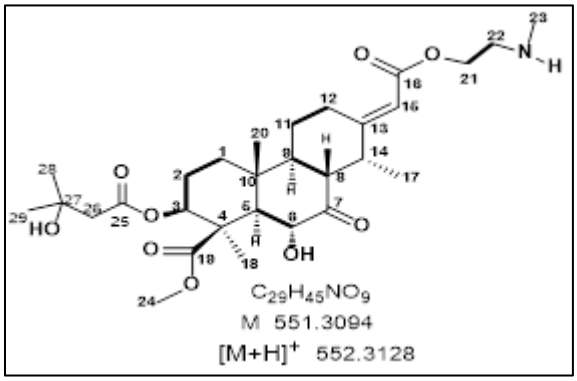

Figure 4 Structure de la 3β-hydroxy-3-méthyl-butanoyloxy-6α-hydroxy-nor-cassamine

We have thus proposed a fragmentation scheme with the major fragments observed at m/z 553.3247, 522.2316, 478.2111, 360.1566, and 300.1415 (Figure 5).

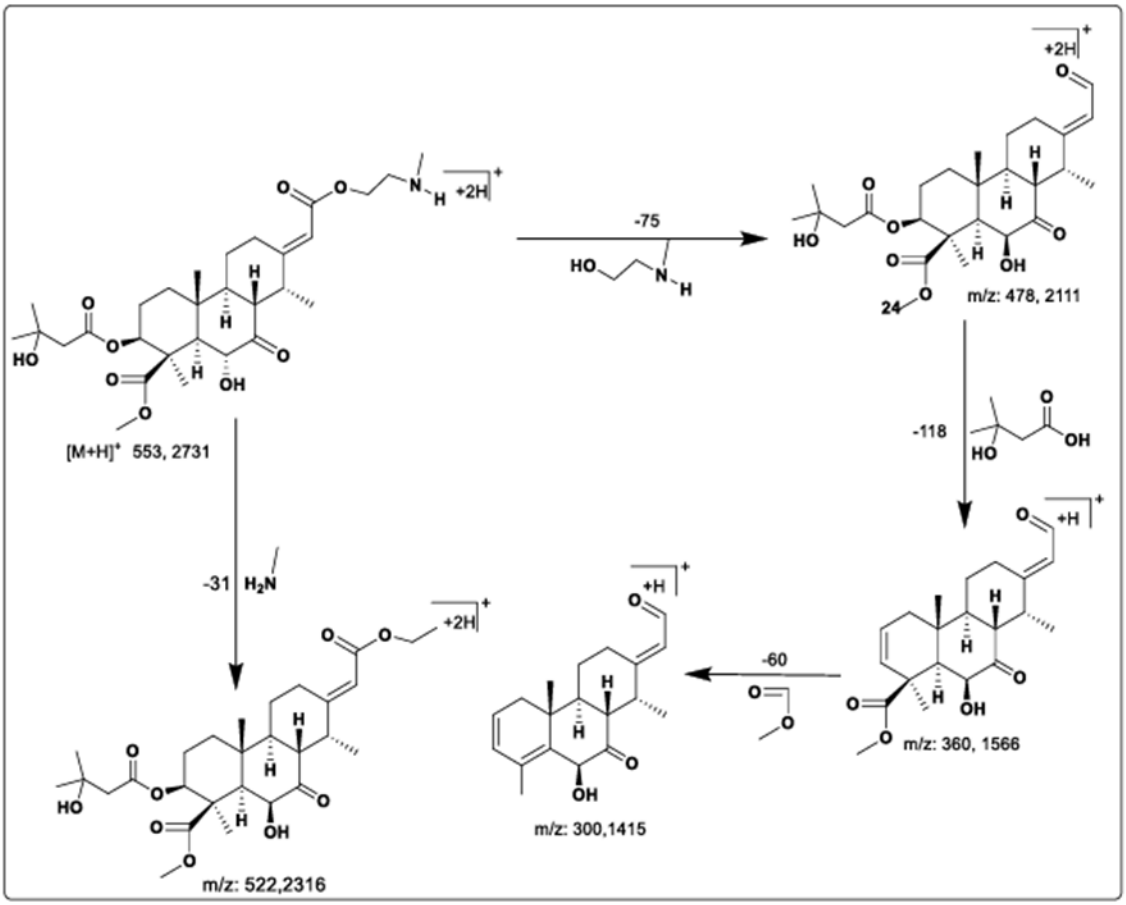

Figure 5 Fragmentation pattern of 3β-hydroxy-3-methyl-butanoyloxy-6α-hydroxy-nor-cassamine

4. Discussion

The structures isolated in this study, namely 3β-acetoxy-erythrosuamine and 3β-hydroxy-3-methylbutanoyloxy-6α-hydroxy-nor-cassamine, are cassane-type diterpenoids with an amine function. Both molecules have previously been reported from the root bark of *E. suaveolens*. Several cassane-type diterpenoids with an amine function, such as 6α-hydroxy-nor-cassamine or 8,9-dehydro-nor-cassamine [1], show similarities with compound 2 isolated in this study (3β-hydroxy-3-methylbutanoyloxy-6α-hydroxy-nor-cassamine), particularly regarding the nor-cassamine skeleton. Likewise, the work of Konan et *al*. [11] reported new cassane-type amine derivatives bearing "3-hydroxy-3-methylbutanoyloxy" groups, which further links compound 2 from our study to these cases.

Although numerous cassane diterpenoids have been described, these two compounds exhibit particular substitutions: an acetoxy group in the first case, and a 3-methylbutanoyloxy group plus a 6α-hydroxy group in the second. These modifications can strongly influence polarity, chemical stability, and potentially biological properties (affinity, activity, toxicity). These compounds could be good candidates for biological activity studies (antioxidant, anti-inflammatory, cytotoxic). Indeed, some cassane-type diterpenoids with amide or hydroxyl functions from *E. suaveolens* reported by Konan et *al.* [11] have shown significant antioxidant capacities. It would be interesting to evaluate the compounds isolated in this study in similar tests, or others, to assess the influence of these specific substitutions (acetoxy vs. hydroxy + bulky ester) on activity.

5. Conclusion

This study allowed the isolation from the trunk bark of *Erythrophleum suaveolens* of two cassane-type diterpenoids newly characterized in the context of trunk bark: 3β-acetoxy-erythrosuamine and 3β-hydroxy-3-methylbutanoyloxy-6α-hydroxy-nor-cassamine. These molecules enrich the chemical repertoire of the species, particularly regarding esterified and hydroxylated substituents. Their discovery paves the way for future biological investigations to explore

their pharmacological potential. For future studies, it is recommended to evaluate their biological activity and, if possible, to synthesize derivatives to establish structure-activity relationships.

Compliance with ethical standards

Disclosure of conflict of interest

Authors declare that there are no conflicts of interest.

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