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ORIGINAL PAPER

New Nortriterpenoid and Ceramides From Stems and Leaves of Cultivated *Triumfetta cordifolia* A Rich (Tiliaceae)

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Abstract To highlight the role of plants in traditional healing, the leaves and the stems of cultivated *Triumfetta cordifolia* were phytochemically studied yielding a new norursane type (**1**), a new ceramide (**2**) and a new piperidinic ceramide derivative (**3**) named, respectively, 2α,19α-dihydroxy-3-oxo-23-nor-urs-12-en-28-oic acid, (2*R*)-2-hydroxy-*N*-[(2*S*,3*S*,4*R*,26*E*)-1,3,4-trihydroxy-26-triaconten-2-yl] tetradecanamide and (2*R*,8*Z*)-2-hydroxy-{(2*S*,3*R*,5*R,6S*)-3,5-dihydroxy-6-[(1*E*,5*Z*)-hexadeca-1,5-dienyl]-2-(β-D-glucopyranosyloxy)methyl piperidine-1-yl} tetracos-8-enamide (**3**). These were obtained together with lupeol (**4**), stigmasterol (**5**), 3-*O*-β-D-glucopyranoside of β-sitosterol (**6**), tormentic acid (**7**) from stems and heptadecanoic acid (**8**), β-carotene (**9**), oleanolic acid (**10**), and 24-hydroxy-tormentic acid (**11**) from leaves. The structures were determined on the basis of NMR data (^{1}H-, ^{13}C-, 2D-NMR analyses), mass spectrometry and confirmed by chemical transformations as well as comparison of spectral data with those reported in the literature. The FRAP method was used to evaluate the antioxidant activity of fractions collected from flash chromatography and isolated compounds. Among the fractions, four reduced Fe^{III}-TPTZ to Fe^{II}-TPTZ while isolated pure compounds showed no activity.

Keywords Cultivated *Triumfetta cordifolia* (Tiliaceae) · Isolation · Structure elucidation · Antioxidant activity

Abbreviations

APT	Attached proton test
CC	Column chromatography
COSY	Correlation spectroscopy
FRAP	Ferric reducing ability of plasma
FT-IR	Fourier transformed infra-red
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
HR-ESI-MS	Higher resolution electrospray mass spectrometry
LCB	Long chain base
MALDI-TOF-MS	Matrix assisted laser desorption ionisation-time of flight-mass spectrometry
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy

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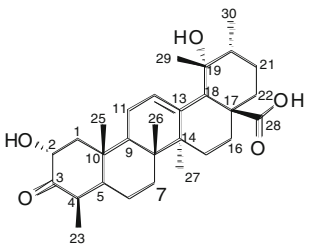
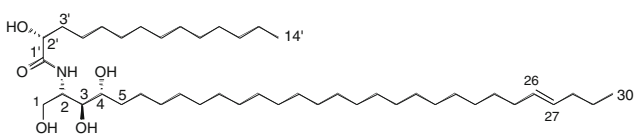
Introduction

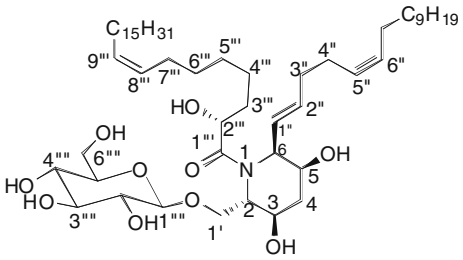
Cultivated *Triumfetta cordifolia* A Rich (Tiliaceae) grows as shrubs; there are similarities to the leaves, flowers and height of the wild species. However, the stems of cultivated *T. cordifolia* are bigger than those of wild species. The people from West and North-west of Cameroon cultivate and use it as foodstuff. The aqueous extract of freshly stems is mixed with other ingredients and accompanied by corn couscous. This meal is essentially eaten by women during the first week after they gave given birth and by convalescing patients. In previous studies, we reported new compounds and other phytoconstituents from twigs and leaves of the wild species. In the continuation of the investigation of cultivated *T. cordifolia,* seven compounds were isolated from the stems and among them, three compounds identified as 2α-19α-dihydroxy-3-oxo-23-nor-urs-12-en-28-oic acid (**1**), (2*R*)-2-hydroxy-*N*-[(2*S*,3*S*,4*R*, 26*E*)-1,3,4-trihydroxy-26-triaconten-2-yl] tetradecanamide (**2**) and (2*R*,8*Z*)-2-hydroxy-{(2*S*,3*R*,5*R*,6*S*)-3,5-dihydroxy-6-[(1*E*,5*Z*)-hexadeca-1,5-dienyl]-2-(β-D-glucopyranosyloxy) methylpiperidine-1-yl}tetracos-8-enamide (**3**) are unreported and four known compounds identified as lupeol (**4**) [1], stigmasterol (**5**) [2], 3-*O*-β-D-glucopyranoside sitosterol (**6**) [3], tormentic acid (**7**) [4]. In addition, four known secondary metabolites were also obtained from the leaves and identified as heptadecanoic acid (**8**) [3], β-carotene (**9**) [5], oleanolic acid (**10**) [1] and 24-hydroxytormentic acid (**11**) [6]. Some of them are endowed with anti-inflammatory, antimicrobial, analgesic and cytotoxic activities [7, 8]. These properties could identify these species as being good health promoters.



Compound **1**



Compound **2**



Compound **3**

This paper deals with the isolation and structural elucidation of new compounds based on spectroscopic methods and some chemical transformations. The antioxidant activity of some fractions obtained from flash chromatography will also be reported.

Experimental Section

Plant Material

T. cordifolia A. Rich. was collected in December 2006 from the Nkongsamba Littoral region of Cameroon and a specimen (N° 12830SRF Cam) has been deposited in the National Herbarium of Yaoundé, Cameroon.

General

IR spectra were recorded on a Perkin-Elmer (FT-IR system spectrum BX spectrometer) using KBr disks. 1D- and 2D-NMR spectra were carried out on a Bruker DRX-400 MHz. Optical rotation was measured with a Perkin Elmer polarimeter model 341 at 589 nm. Melting points were measured in a Stuart Scientific Melting Point apparatus SMP_3 and are uncorrected. The silica gel 60H (particle size <45 μm), GF254 and 60A (size 70–200 μm) were used to perform, respectively, flash chromatography, thin layer chromatography and column chromatography. Iodine-silica gel was employed as developer to visualize the spots on the TLC plates. ESI-FT-ICR-MS and ESI-FT-ICR-MS^n measurements were conducted in the positive ion detection mode on an Explorer High Resolution FT-ICR-MS (Varian-IonSpec Corporation, Palo Alto, CA, USA) fitted with a 9.4-T shielded superconducting magnet and a Micromass Z-spray electrospray source. The used high voltage (HV) was in the −4,000–3,600 V range. Nitrogen was used to assist solvent evaporation. The temperatures of the source and of the probe temperature were 90 and 100 °C, respectively. Ions were trapped in the FT-ICR-MS cell with

a 2V trapping potential. In these conditions, the average mass measurement accuracy was typically better than 2 ppm, and the mass resolution close to 150,000 (m/Δm). After calibration, the identification of the ions was ensured by exact mass measurement and comparison with theoretical isotopic pattern calculated by the "Omega 8 Exact Mass Calculator" software (Varian-IonSpec Corporation, Palo Alto, CA, USA). MS/MS experiments were conducted in SORI–CID (sustained-off resonance irradiation collision induced dissociation) mode. After isolation of parent ion by ejection of other ions, sustained off-resonance irradiation was performed during 60 ms with amplitude of 24 V bp. Nitrogen was used as collision gas. The matrix used for the MALDI-TOF-MS was a solution of 2,5-Dihydroxybenzoic acid (2,5-DHB) 1 M in 50% acetonitrile 50% ultrapure water, 0.1% trifluoroacetic acid (TFA); its measurements were possible using a Bruker Reflex IV time-of-flight mass spectrometer (TOF-MS) (Bruker-Daltonic, Bremen, Germany) equipped with the SCOUT 384 probe ion source, using a nitrogen pulsed laser (337 nm, model VSD-337ND, Laser Science Inc., Boston, MA) with energy output of 400 μJ/pulse. LC-MS analyses were performed on an HPLC system (LC pump P4000 and autosampler AS3000 from Thermo Separation Products) coupled to a LCQ Duo Ion Trap detector (Thermo Electron, Zellik, Belgium) equipped with an ESI interface run in the positive ion mode. The separation of sample components was achieved on an X-Terra MS C18 (5 μm particle size, 3.9 × 150 mm) (Waters, Overijse, Belgium), equipped with an X-Terra MS C18 pre-column (5 μm particle size, 3.9 × 10 mm) and operated at 37 °C. Injection volume was 15 μL. The mobile phase consisted of a mixture of 5 mM ammonium formate buffer at pH 3.8 (A) and acetonitrile (B). Separation conditions for all compounds were as follows: 0.0–0.50 min, A/B hold at 50/50, v/v; 0.50–9.0 min, eluant B increase to 97%; 9.0–12.0 min, eluant B hold at 97%, 12.0–12.5 min, eluant decrease to 50%, 12.5–13.0 min, A/B hold at 50/50, Before each run the column was equilibrated for 6 min at A/B 50/50. The flow rate for column equilibration and analytical runs was 0.4 ml/min. Ionization of the analytes was carried out as follows: sheath gas flow rate (nitrogen), 47 arbitrary units; auxiliary gas flow rate (helium), 18 arbitrary units; spray voltage, 5.0 kV; capillary temperature, 200 °C; capillary voltage, 36 V; Data acquisition was performed in a time segment between 0.2 and 11.5 min after injection. The full MS-MS spectrum of $[M+H]^+$ ions was monitored for all compounds, isolation width was 2.5 *m*/*z*, normalized collision energy was 28.0%. HR-ESI-MS and HR-ESI-MS/MS were carried out using MicroTOF-Q 98 (Bruker-Daltonics, Germany). The method used was tune low MS with source type ESI and the acquisition parameters were: positive ion polarity, set collision cell RF 150 Vpp, set capillary 6,000 V set dry heater 190 °C, set nebulizer 0.4 bar, set dry gas 4.0 L/min.

Extraction and Isolation

Dried stems and leaves of *T. cordifolia* A. Rich. were powdered to give, respectively, 5 and 1.8 kg of the powder. Both powders were separately extracted by maceration at room temperature with 5 (twigs) and 4 L (leaves) of methylene:methanol (1:1). These were extracted three times and each extraction lasted 48 h. The organic extracts were concentrated and yielded 248 and 184 g (for the stems and leaves, respectively) of crude extract which were fractioned by flash chromatography using fine silica gel 60H (particle size <45 μm). The elution was performed with cyclohexane, cyclohexane/EtOAc in order of increasing polarity (3:1 to 1:1 to 1:3), ethyl acetate, acetone and methanol. The fractions collected were indexed A, B, C, D, E, F and G for the twigs and A′, B′, C′, D′, E′, F′ and G′ for the leaves.

Chromatography of Stems

The fraction A (56 g) obtained from cyclohexane:ethyl acetate (3:1, v/v) was purified by CC on silica gel 60A (size 70–200 μm) with different mixtures of cyclohexane:ethyl acetate yielding 150 fractions: 25 mg of lupeol was obtained from the fraction 30–45 eluted with cyclohexane:ethyl acetate (95:5, v/v), 654 mg of sterol mixture (stigmasterol and *β*-sitosterol) was isolated from the fractions 48–52 eluted with cyclohexane:ethyl acetate (82.5:7.5, v/v). The comparative thin layer chromatography of D and E turned out to be identical and led us to mix them. The purification of this mixture (36 mg) was done with methylene chloride and the mixture methylene chloride:methanol in the order of increasing polarity yielding 150 fractions. This gave 6 mg of compound **2** in the fractions 60–63 in the ratio 95:5 (v/v) of methylene chloride:methanol. In the same proportion of solvents, 475 mg of 3-*O*-*β*-D-glucopyranoside of *β*-sitosterol were obtained from the fractions 73–89. The fractions 54–57 collected from the mixture methylene chloride:methanol 95:5 (v/v) were purified with methylene chloride:ethyl acetate in the order of increasing polarity (methylene chloride:ethyl acetate 99:1, 98:2, 96:4, v/v) yielding 126 fractions. An 8-mg amount of compound **1** was isolated from those numbered from 78 to 105 eluted in the ratio 49:1. The part E (25 g) was purified by CC on silica gel 60 A (70–200 μm) with methylene chloride:methanol in order of increasing polarity yielding 83 fractions. Compound **3** (637 mg) was obtained from the fractions 65–87 eluted with methylene chloride:methanol 7:1. Tormentic acid (57 mg) was isolated from G in the mixture methylene chloride:methanol 19:1 (v/v) from the fraction 1–5.

Chromatography of Leaves

The cyclohexane fraction (4 mg) was eluted with petroleum ether:methylene chloride in the order of increasing polarity yielding 98 fractions. Heptadecanoic acid (50 mg) was isolated from the fraction 8–17 in the proportion of mixture of solvents 98:2. Fractions 28–43 yielded 15 mg of β-carotene in the ratio 97:3 (v/v). C′ (20 mg) was chromatographed with cyclohexane:ethyl acetate yielding 80 fractions. The ones eluted with cyclohexane:ethyl acetate 7:3 (v/v) gave 53 mg of oleanolic acid from the fractions 56–70. G′ was purified with methylene chloride:methanol in the order of increasing polarity to give 147 fractions. Among them, 122–128 eluted with methylene chloride:methanol (82.5:7.5, v/v) yielded 5 mg of 24-hydroxytormentic acid.

*2α-19α-Dihydroxy-3-oxo-23-nor-urs-12-en-28-oic acid (**1**)*

Colorless powder; M.p. 187–189 °C; [α] +10.4 (*c* 0:06, DMSO); IR (KBr) ν_{max} 3,577, 3,446, 1,712, 1,687 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); HR-ESI-MS *m/z*: 495.3081 (calc. 495.3081) $[C_{29}H_{44}O_5 + Na]^+$, Retro Diels-Alder 287.1620 $[C_{16}H_{24}O + Na]^+$.

*(2R)-2-Hydroxy-N-[(2S,3S,4R,26E)-1,3,4-trihydroxy-26-triaconten-2-yl]tetradecanamide (**2**)*

Brown solid; M.p. 136–138 °C; [α] −12.86 (*c* 0.14; C_5H_5N); IR (KBr) ν_{max} 3,334, 1,644, 1,624 cm^{-1}; ^{1}H and ^{13}C NMR (Table 2); HR-ESI-MS *m/z*: 732.6447 $[C_{44}H_{87}O_5N + Na]^+$, 718.6283 $[C_{43}H_{85}O_5N + Na]^+$, 704.6163 $[C_{42}H_{83}O_5N + Na]^+$ ESI-MS/MS: 340 $[C_{17}H_{35}O_4N + Na]^+$, 314 $[C_{17}H_{34}O_4N]^+$, 288 $[C_{15}H_{30}O_4N]^+$, MALDI-TOF-MS: 654 $[C_{41}H_{77}O_3N + Na]^+$, 664 $[C_{39}H_{79}O_5N + Na]^+$, 668 $[C_{42}H_{79}O_3N + Na]^+$, 682 $[C_{43}H_{81}O_3N + Na]^+$.

Methanolysis A 1-mg sample of **2** was refluxed (70 °C) for 18 h in 3.5 ml of methanol containing 1.5 ml of 0.9 N HCl under magnetic stirring. The medium was neutralized with sodium carbonate and extracted with chloroform. The organic phase was subjected to MALDI-TOF-MS analysis and LCB was identified at *m/z* 484, $[C_{30}H_{61}O_3N + H]^+$.

*(2R,8Z)-2-Hydroxy-{(2S,3R,5R,6S)-3,5-dihydroxy-6-[(1E,5Z)-hexadeca-1,5-dienyl]-2-(β-D-glucopyranosyloxy)methylpiperidine-1-yl}tetracos-8-enamide (**3**)*

Brown solid; M.p. 201–203 °C; [α] +3.89 (*c* 0.18; MeOH); IR (KBr) ν_{max} 3,336, 3,205, 2,918, 2,849, 1,622, 1,546, 1,469, 1,278, 1,068 cm^{-1}; ^{1}H and ^{13}C NMR (Table 3); HR-ESI-MS: 894.6938 $[C_{52}H_{95}O_{10}N + H]^+$, 880.6717 $[C_{51}H_{93}O_{10}N + H]^+$, 866.6652 $[C_{50}H_{91}O_{10}N + H]^+$, 852.6465 $[C_{49}H_{89}O_{10}N + H]^+$, 838.6347 $[C_{48}H_{87}O_{10}N + H]^+$, 754.5045 $[C_{42}H_{75}O_{10}N + H]^+$, 736.5278 $[C_{42}H_{73}O_9N + H]^+$, 574.4852 $[C_{36}H_{63}O_4N + H]^+$, 534.4463 $[C_{26}H_{47}O_{10}N + H]^+$.

Acetylation and Oxidative Cleavage of the Double Bond A 11-mg sample (10.4 μmol) of compound **3** was dissolved in 4 mL of pyridine and 4 mL of acetic anhydride was added to this solution under magnetic stirring at room temperature. The reaction was stopped after 1 h and the acetylated product was obtained after evaporation under vacuum. This product was dissolved in 4 mL of a mixture of dioxane/water (3:1); two equivalents of pyridine, two drops of 4% wt of an aqueous solution of osmium tetroxide and four equivalents of sodium periodate were added. The

Table 1 The 1H-NMR (400 MHz) and 13C-NMR (100 MHz) spectral data of compound **1** measured in DMSO-d6; δ in ppm, J in Hz

Position	δ_H	δ_C (APT)	Position	δ_H	δ_C (APT)	Position	δ_H	δ_C
1	1.03 (m)	50.9	11	1.45 (m)	24.6	21	0.87 (m)	28.9
	2.20 (dd, 6.4, 12.8)			2.00 (br d, 6.4)			1.21 (m)	
2	4.20 (m, 5.5, 6.5, 10.9)	72.2	12	5.16 (br s)	127.6	22	1.49 (m)	38.1
							1.58 (m)	
3	–	213.5	13	–	139.9	23	0.89 (d, 6.7)	12.1
4	2.45 (m)	43.2	14	–	42.4	24	–	–
5	0.97 (m)	54.9	15	0.97 (m), 1.15 (m)	27.2	25	1.13 (s)	15.0
6	1.40 (m), 1.70 (m)	26.0	16	1.39 (m), 1.49 (m)	26.7	26	1.09 (s)	17.2
7	1.47 (m), 1.62 (m)	37.9	17	–	47.8	27	1.27 (s)	24.7
8	–	40.4	18	2.37 (s)	54.2	28	–	180.1
9	1.65 (m)	45.1	19	–	72.4	29	1.07 (s)	25.0
10	–	37.8	20	2.44 (m)	42.5	30	0.82 (d, 6.7)	17.5
OH-2	4.70 (d, 5.5)	–	OH-19	3.72 (s)	–	–	–	–

Table 2 The ^{1}H NMR (400 MHz) and ^{13}C (APT) NMR (100 MHz, C_5D_5N) spectral data of compound **2**

Position	δ(H) (m, J/Hz)	δ(C) (APT)	COSY	HMBC
1a	4.38 (dd, 4.0, 10.8)	62.8	4.45	52.8, 78.1
1b	4.45 (dd, 8.0, 10.8)	62.8	4.38	52.8, 78.1
2	5.10 (br s)	52.8	4.30, 4.38, 4.45, 8.51	62.8, 78.1, 175.8
3	4.30 (dd, 4.8, 7.2)	78.1	4.22	34.5, 52.8, 62.8, 73.1
4	4.22 (m)	73.1	1.90, 2.20	
5a and 5b	1.90 (m), 2.20 (m)	34.5	1.20	130.4, 130.5
24, 27	1.93 (m), 2.12 (m)	33.0, 33.2	5.40, 5.50	130.4, 130.5
25	5.40 (m, 7.2, 16.0)	130.5	1.93	33.0, 33.2
26	5.50 (m, 7.2, 16.0)	130.6	2.12	33.0, 33.2
6-23, 28, 29 4′-14′ (CH_2)	1.20 (br s)	22.8, 23.1, 24.0, 26.0 30.0, 32.1	0.80	14.0, 22.8, 30.0, 32.1
NH	8.51 (d, 8.0)			52.8, 175.8
1′		175.8		
2′	4.58 (dd, 3.6, 7.8)	72.8	1.97, 2.15	26.0, 36.5, 175.8
3′a and 3′b	1.97 (m), 2.15 (m)	36.5	1.20	
15′, 30 (CH_3)	0.80 (t, 8.0)	14.0	1.20	22.8, 30.0, 32.1

reaction was left under magnetic stirring at room temperature for 3 h. Quenching was done with 10 mL of an aqueous solution of sodium thiosulfate and left under magnetic stirring during 20 min about. The organic layer was extracted three times with 15 mL of methylene chloride and concentrated under vacuum. The analysis by MALDI–TOF of the organic residual phase gave important peaks at *m/z* 241 $[C_{16}H_{33}O + H]^+$, 924 $[M' + Na]^+$ and 940 $[M' + K]^+$.

Methanolysis A 2-mg sample of (**3**) was refluxed (70 °C) for 18 h in 3.5 mL of methanol containing 1.5 mL of 0.9 N hydrochloride acid under magnetic stirring. The medium was neutralized with sodium carbonate and extracted with chloroform. The organic phase was subjected to the LC-ESI-MS analysis and the peak observed at *m/z* 424 corresponding to retention time 10.96 and some fragment ion led us to identify fatty acid methyl ester as methyl-2-hydroxyhexacos-17-enoate.

FRAP Assay

The FRAP assay is one of the methods used today to evaluate antioxidant power. It was developed by Benzie and Strain [9]. Practically, it is based on a mixture of 1.5 mL of 10 volumes of 300 mmol/L acetate buffer, pH 3.6 + 1 volume of 10 mmol/L 2,4,6-tripyridyl-*S*-triazine (TPTZ) in 40 mmol/L hydrochloric acid + 1 volume of 20 mmol/L ($FeCl_3$) pre-warmed at 37 °C. Then 50 µL of the sample and the standards were added to the mixture and homogenized by vortex. The absorbance at 593 nm was read against a reagent blank at a pre-determined time after sample-reagent mixing. The test was performed at 37 °C and a 0–4 min reaction time window was used.

Results and Discussion

The crude organic extracts of stems and leaves were separately subjected to repeated column chromatography to yield seven and four compounds, respectively. Among those obtained from stems there were a new nor-ursane derivative **1**, a new ceramide **2** and a new piperidinic cerebroside **3.**

Compound **1** was obtained as a white powder from a mixture of methylene chloride/ethyl acetate (49:1, v/v). Its positive mode HR-ESI-MS spectrum showed a peak at *m/z* 495.3081 (calc. 495.3081) corresponding to the molecular formula $[C_{29}H_{44}O_5 + Na]^+$ accounting for eight double bond equivalents. It responded to the Liebermann–Burchard test of triterpenes. The reddish-purple color observed was indicative of a triterpene type of triterpenoid. The IR spectrum showed intense absorption bands at 3,577 (free OH), broad band at 3,446 (OH of carboxylic function), 1,712 (C=O of carbonyl function), 1,687 (C=C stretching) cm^{-1}. Two signals at δ_H/δ_C 5.19 (1H, br *s*)/127.9 and 139.9 corresponding to the trisubstituted double bond of Δ [12] ursene type [1] were exhibited on the ^{1}H- and ^{13}C-NMR (APT) spectra (Table 1) of **1**. These spectra presented also six intense signals of methyl groups in which, there were two at δ_H/δ_C 0,89 (3H, d, $J = 6.7$ Hz)/12.1; 0.82 (3H, d, $J = 6.7$ Hz)/17.1 and four singlets at δ_H/δ_C 1.07/25.0; 1.09/17.2; 1.13/15.0; 1.27/24.2. This meant that, among the eight Me groups of urs-12-enes, two are oxidized or missing but on the APT spectrum, we had two signals of quaternary

Table 3 The ^{1}H-NMR (400 MHz CD_3OD) and ^{13}C-NMR (APT) (100 MHz, CD_3OD) spectral data of compound **3**

Position	δ_H (m, J/Hz)	δ_C (APT)	COSY	HMBC
1′a and 1′b	3.82 (dd, 4.8, 10.2)	69.7	4.08, 4.25, 4.27	51.6, 104.7
	4.08 (dd, 9.2, 10.2)		3.82, 4.25, 4.27	75.5, 104.7
2	4.25 (br d, 9.2)	51.6	3.82, 4.08, 3.61	69.7, 75.5, 177.2
3	3.61 (dd, 9.7, 10.0)	75.5	1.55, 1.70, 4.25	32.9, 51.6, 69.7
4	1.55 (m), 1.70 (m)	32.9	3.61, 4.02	54.8, 75.5
5	4.12 (br d, 4.8, 9.2,)	73.1	3.61, 4.02, 5.49, 5.75	32.9, 54.8, 132.0, 134.4
6	4.02 (m)	54.8	1.55, 4.25, 3.61, 4.12	51.6, 73.1, 177.2
1″	5.49 (dd, 9.2, 14.6)	132.0	2.01, 2.08, 5.75	33.7, 73.1, 134.4.
2″	5.75 (br d, 14.6)	134.4	2.01, 2.08, 5.49	33.7, 132.0
3″a and 3″b	2.01 (br d, 4.8)	33.7	4,12, 5.49, 5.75, 2.01	73.1, 132.0
	2.08 (br d, 9.2)			
4″a and 4″b	1.98 (br s), 2.00 (br s)	33.4	5.38, 5.39, 5.41, 5.75	131.2
			2.05	
1‴	–	177.2	–	–
2‴	4.03 (dd, 4.8, 9.2)	73.0	1.65, 1.75	35.7, 177.2
3‴a and 3‴b	1.65 (m), 1.75 (m)	35.7	1.30, 4.02	26.1, 30.5, 30.7, 73.0
5″, 6″, 8‴, 9‴	5.38 (dd, 4.8, 9.2), 5.39 (dd, 4.8, 9.2), 5.41 (br d, 5), 5.42 (br s)	130.7, 130.8 130.9, 131.2	1.94, 2.00, 2.08	26.1, 28.4, 33.7,
7″, 7‴, 10‴	2.00 (m), 2.10 (m)	28.4	1.30, 5.38, 5.39, 5.41, 5.42	23.7, 30.7, 26.1, 30.5 130.7, 130.8, 130.9, 131.2
8″-15″, 4‴-6‴, 11‴-23‴$(CH_2)_n$	1.30 (br s)	23.7, 26.1, 30.7, 30.8	0.90, 1.94, 2.00	28.4, 35.7, 14.5
16″, 24‴2x (CH_3)	0.90 (t, 6.9)	14.5	1.30	23.7, 26.1, 30.5, 30.7
Glucosyl				
1⁗	4.27 (d, 9.0)	104.7	3.19, 3.37, 4.25	69.7, 75.0
2⁗	3.19 (dd, 9.0, 9.2)	75.0	3.37, 4.27	77.9, 104.7
3⁗	3.37 (dd, 9.0, 9.2)	77.9	3.30	71.6
4⁗	3.30 (br d, 8.5)	71.6	3.29, 3.37	78.0
5⁗	3.29 (ddd, 4.8, 8.5, 9.2)	78.0	3.68, 3.88	62.6,
6⁗a and 6⁗b	3.88 (br d, 12.1)	62.6	3.29	71.6, 78.0
	3.68 (dd, 4.8, 12.1)			

carbons at δ_C 180.1 corresponding to the carboxylic function C-28 [10] and at δ_C 213.5 attributable to the ketone at C-3 [10]. From the above-mentioned data, it was deduced that one of the Me groups is missing and the other is oxidized.

Considering some characteristic signals of triterpenes of urs-12-ene type [10] such as the ones observed at δ_H/δ_C 0.97 (H-5, m)/54.9, 1.65 (H-9, m)/45.1 and 2.37 (H-18, s)/54.2, we have observed interesting long range correlations on the COSY and HMBC spectra (Fig. 1). In fact, the proton at δ_H 0.97 showed correlations with those at δ_H 0.89, 1.40, 1.70 and 2.45 as well as their carbons at δ_C 12.1, 26.0 and 43.2, respectively. The multiplicity of signals at δ_H 0.89/12.1 appearing as a doublet and correlating with the proton at δ_H 2.45 confirmed that only one Me group was present at C-4. Additional interactions supporting this structure were noted from both protons at δ_H 0.89 and 2.45 to the carbons at δ_C 54.9 and 213.5. The signal at δ_H 1.65 showed correlation with the proton at δ_H 2.00 and the carbons at δ_C 15.0, 25.0 and 37.8. The protons (δ_H 1.13) of carbon at δ_C 15.0 showed interactions with the carbons at δ_c 37.8 and 50.9. Both protons at δ_H 1.03 and 2.20 linked to the carbons at δ_c 37.8 and 50.9, respectively, interacted with the carbon at δ_C 15.0, 37.8, 54.8, 72.2 and 213.5 showing that the ring A has an acyloin system. The signal at δ_H 2.37 correlated with the carbons at δ_C 25.0, 42.5, 47.8, 72.9, 127.6, 139.9 and 180.1 corresponding to the interactions observed in the derivatives of 19α-hydroxyurs-12-en-28-oic acid [4]. The spatial geometry of hydroxyl and methyl groups in position C-2 and C-4, respectively, were determined using the NOESY spectrum (Fig. 2).

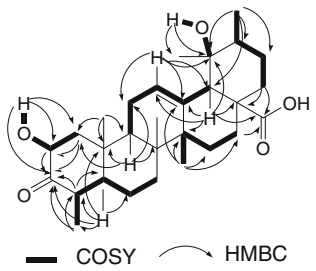


Fig. 1 COSY and HMBC correlations of compound **1**

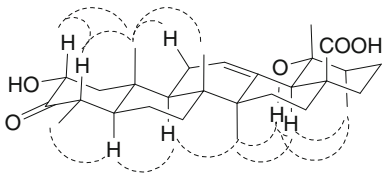
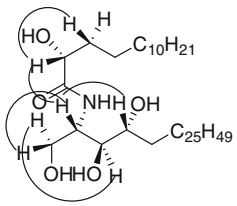


Fig. 2 NOESY interactions of compound **1**

Since, the protons at δ_H 1.13 (H-25) and 1.65 (H-9) have a spatial configuration β and α, respectively [10], it was noted that the one at δ_H 1.13 interacted with the protons at δ_H 2.00 (H-11), 2.45 (H-4) and 4.20 (H-2). This meant that the four protons have the same spatial orientation. The lack of methyl group C-24 and the equatorial position of C-23 were confirmed by the correlations observed between the signal at δ_H 1.65 and the protons at δ_H 0.89, 0.97 and 1.27. The combination of the foregoing data led us to identify **1** as 2α-19α-dihydroxy-3-oxo-23-nor-urs-12-en-28-oic acid.

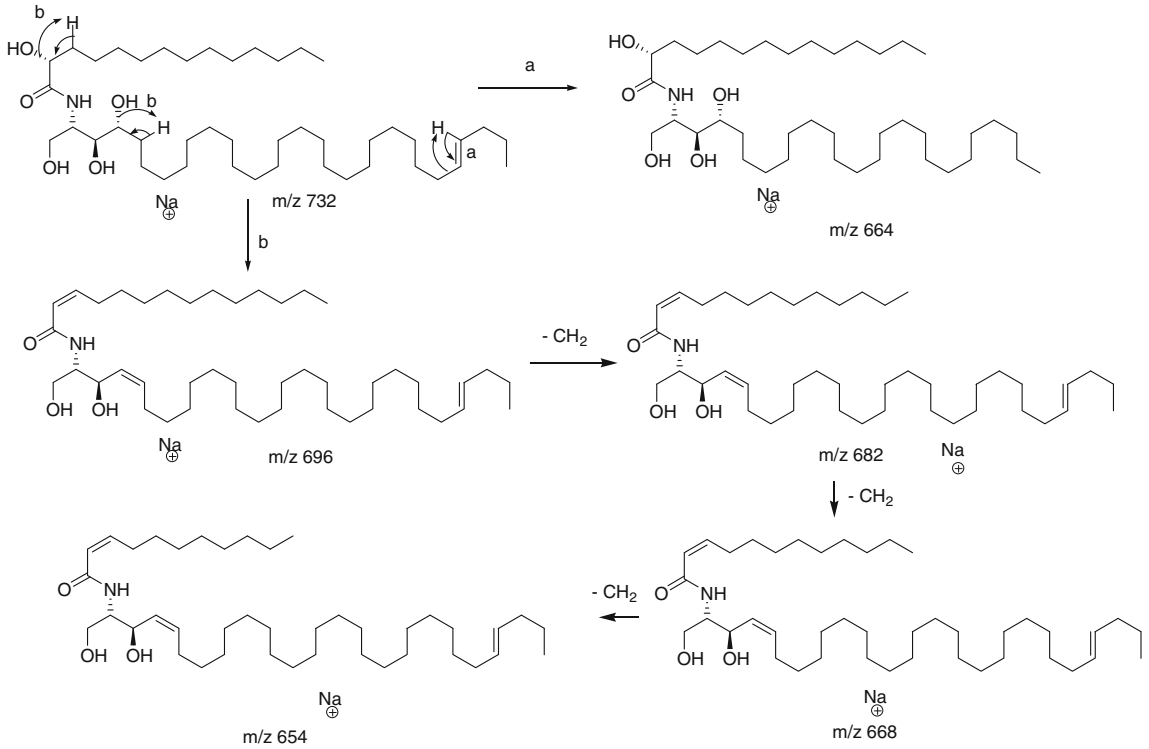
Compound **2** was obtained as a dark solid from a mixture of methylene chloride:methanol (19:1, v/v). Its positive HR-ESI-MS showed a pseudomolecular ion at *m/z* 732.6447 $[M+Na]^+$ (calc. 732.6476), corresponding to the formula $C_{44}H_{87}NO_5$ accounting for two double bond equivalents. The FT-IR spectrum of **2** showed characteristic absorption bands for a free hydroxyl (3,334 cm^{-1}) and an amide carbonyl function (1,624 cm^{-1}) [11]. The ^{1}H- and ^{13}C-NMR spectral data of **2** exhibited a set of signals which were in accordance with those of phytoceramides [11]. In fact, the presence of a N**H** group at δ_H 8.51 (d, $J = 8.0$ Hz) which was exchangeable with deuterated water was observed as well as two multiplets of two olefinic protons at δ_H 5.40 (*m*) and 5.50 (*m*), a broad singlet at δ_H 1.20 (methylene protons), a triplet of 6H at δ_H 0.80 ($J = 8.0$ Hz, two terminal methyl groups) and two carbinol protons at δ_H [4.38 (dd, $J = 4.8$, 10.8 Hz); 4.45 (dd, $J = 8.0$, 10.8 Hz)]. The NMR spectrum of **2** (Table 2) also showed carbon signals at δ_C 175.8, 52.8 and the range 23.9–33.9, further supporting the fatty-acid amine nature of **2.** In addition, we observed the resonances for three oxymethine protons at δ_H/δ_C 4.22 (*m*)/73.1, 4.30 (dd, $J = 4.8$, 7.2 Hz)/78.1 and 4.58 (dd, $J = 3.6$, 7.8 Hz)/72.8

Fig. 3 NOESY interactions of compound **2**

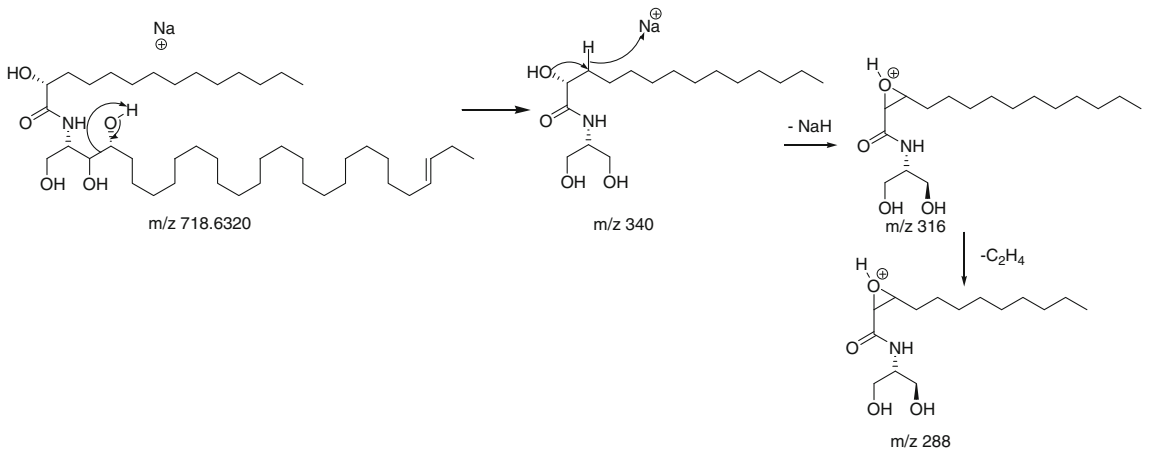


confirming that this ceramide of dihydrosphingolipid type has linked to a fatty acid having acyloin system [11]. The *trans*-configuration of the double bond was evident from the coupling constant between the olefinic protons ($J = 16.0$ Hz) and the chemical shifts of the allylic carbons at δc 33.0 and 33.2, which were expected at 26.5 ppm in case of *cis*-configuration [12]. In the COSY spectrum of **2** (Table 2), the proton signal of the azomethine (δ_H 5.10) was correlated with the hydroxymethylene (δ_H 4.38 and 4.45) and two oxygenated methines (δ_H 4.22 and 4.30) suggesting the location of two hydroxyl groups at C-3 and C-4. The third hydroxyl group was located at C-2′ as result of the correlations observed in the HMBC spectrum (Table 2) from the oxymethine group (δ_H 4.58) and amine group (δ_H 8.50) to the carbonyl ester (δ_C 175.8). The methylene C-3′ (δ_H 1.97 and 2.15) showed correlation with the broad singlet at 30.0. The absolute configuration of asymmetric carbons in phytosphingolipids is 2′*R*, 2*S*, 3*S* and 4*R* [11], and NOESY spectrum showed interactions between the protons geminated to the oxygen, confirming that **2** has the same absolute configurations (Fig. 3). By application of the fragmentation mechanism developed by Fong-Fu et al. [13], the peak observed at *m/z* 664 gave information about the position of the double bond (Scheme 1) by loss of C_5H_8. Its position in the long chain base was confirmed by methanolysis. In fact, this reaction (0.9 N hydrochloric acid:methanol/70 °C/18 h) gave the fatty acid methyl ester and the long chain base which were carefully characterized on the basis of mass spectrometry as methyl-2-hydroxytetradecanoate and 2-amino-25-triacontene-1,3,4-triol (at *m/z* 484, $[C_{30}H_{61}O_3N + H]^+$) which were in accordance with the fragment ion obtained after MS/MS analysis of the peak at *m/z* 718 (Scheme 2). The analyses of the NMR and mass spectroscopy data led to the unambiguous assignment of structure **2** as (2*R*)-2-hydroxy-*N*-[(2*S*,3*S*,4*R*,26*E*)-1,3,4-trihydroxy-26-triaconten-2-yl]tetradecanamide trivially named triumfettamide A.

Compound **3** was isolated as a brown solid from a mixture of methylene chloride:methanol (9:1, v/v). Its positive mode HR-ESI-MS exhibited a pseudomolecular ion at *m/z* 894.6938 $[M+H]^+$, corresponding to a molecular formula $C_{52}H_{95}O_{10}N$ ($C_{52}H_{96}O_{10}N$, calc. 894.7029) indicating six double bond equivalents. The FTIR spectrum of **3** showed broad absorption bands for hydroxyl function (3,336 cm^{-1}), strong absorptions for secondary amides

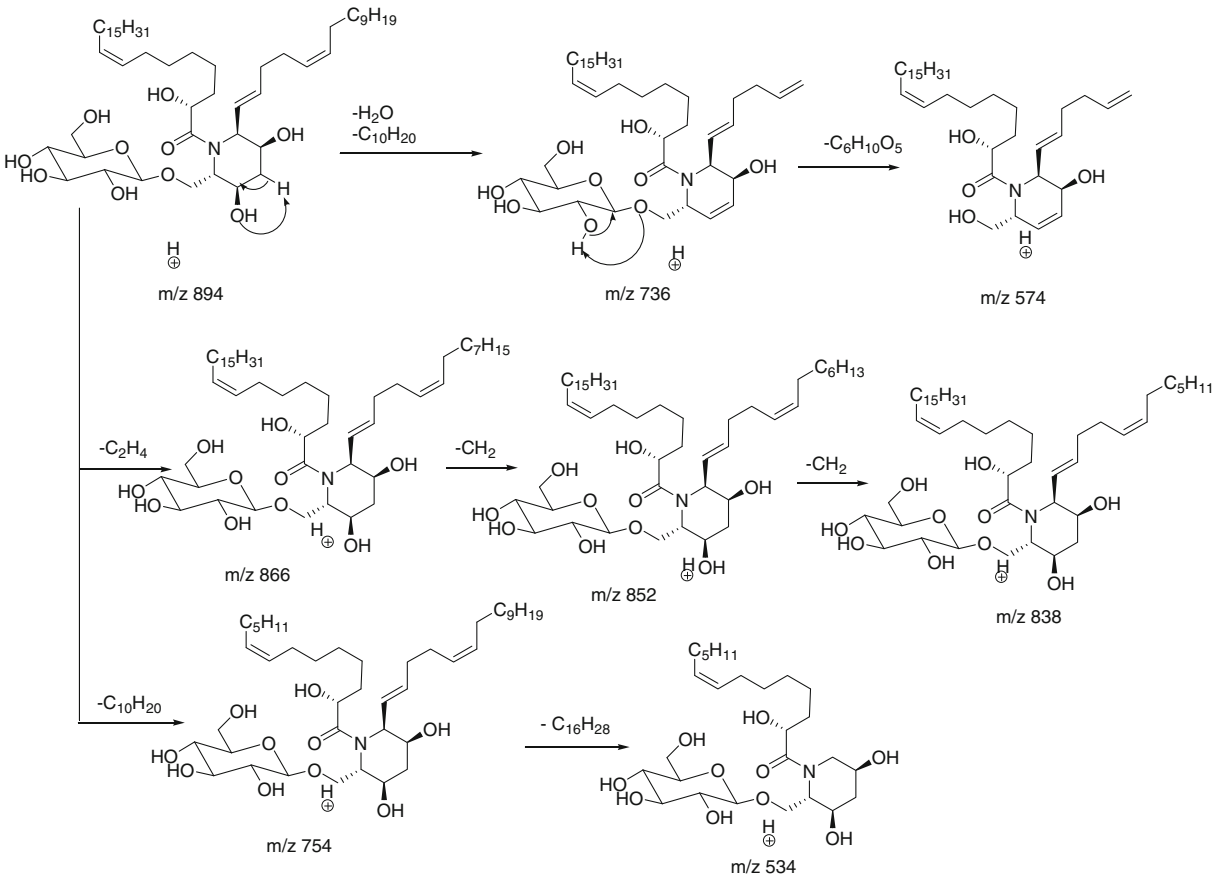


Scheme 1 Fragment ions giving the position of the double bond and other information on **2**



Scheme 2 Fragment ions from MS/MS justifying the identity of the fatty acid side chain and those of the long chain base in the compound **2**

(1,622, 1,546 and 3,205 cm^{-1}) and aliphatic chains (1,469, 2,849 and 2,918 cm^{-1}). The presence of a nitrogen-attached carbon was confirmed by the signal at δ_C 51.6 and a carbonyl signal at δ_C 177.2 in the APT spectrum. **3** gave a positive test result with Molish reagent suggesting the presence of a sugar moiety. Its NMR spectra (Table 3) showed the anomeric proton at δ_H 4.27 (1H, d, $J = 8.7$ Hz; 104.7) and a set of carbon signals at δ_C 75.0, 77.9, 71.6, 78.0, 62.6 consistent with β-D-glucopyranoside. This corroborated with the peak observed on HR-ESI-MS/MS at *m/z* 574.4852 (calc. 574.4830) resulting from $[C_{52}H_{95}O_{10}N\text{-}H_2O\text{-}C_{10}H_{20}\text{-}C_6H_{10}O_5 + H]^+$ (Scheme 3). We observed also, the characteristic signals close to those of phytosphingosine type possessing a 2*R*-hydroxyfatty acid side chain [12]. So, an intense signal of CH_2 sequence characteristic of long aliphatic chains was observed in the NMR spectra between δ_H 1.21 and 1.38 (br *s*) and a triplet of terminal CH_3 groups at δ_H/δ_C (6H, 0.90, t, $J = 6.9$ Hz)/14.5. Furthermore, an oxymethylene at δ_H/δ_C 3.82 (dd, $J = 9.6$, 11.2 Hz) and 4.08 (dd, $J = 4.8$, 11.2 Hz)/69.7 and



Scheme 3 Fragment ions giving indications (sugar and the position of the double bond) of the structure of **3**

three oxymethines at δ_H/δ_C 3.61(dd, $J = 5.8$, 9.7 Hz)/75.5, 4.03 (*m*)/73.0 and 4.12 (*m*)/73.1 were observed. Beside this information, two ethylenic protons in *trans*-geometry [12] at δ_H/δ_C 5.49 (dd, $J = 7.4$, 14.6 Hz)/132.0 and 5.75 (br d, $J = 14.6$ Hz)/134.4 were observed together with four olefinic ones in *cis*-configuration [11] between 5.37 and 5.42 ppm (m, $J = 4.8$, 7.4 Hz)/130.7, 130.8, 130.9, 131.2. From the foregoing data, the structure of **3** was suggested to be a polyunsaturated β-D-glucopyranosyl sphingolipid. Pertinent interactions observed in the HMBC spectrum (Table 3) from the proton of oxymethine at δ_H 4.03 (*m*, 73.0) to C=O and a set of methylene group justifying that the fatty acyl part is α-hydroxylated. Two carbinol protons Ha-1′ (3.82), Hb-1′ (4.08) correlated with the anomeric carbon C-1‴′ led us to attach the sugar moiety at C-1′. Moreover, the long chain base was considered to be of phytosphingosine type because of correlations between the proton H-3 (3.61 (*m*); δ_C 75.5) and the carbons C-1′ (69.7), C-2 (51.6), C-4 (32.9) and C-6 (73.1). An interesting correlation observed from the proton H-6 (4.12) to the carbon at δ_C 54.8 suggested that this ceramide was an azacyclane derivative. The same proton H-6 showed correlation with the olefinic carbons in *trans*-configuration (132.0; 134.4) confirming their position in long chain base side. The correlation between H-6 (4.02)/δ_C 54.8 and the carbons at δ_C 177.2 and 51.6 added to the comparison of spectroscopic data with those presented by Vanderlan et al. [14] and Yamashita et al. [15] ascertained that **3** was a piperidinic derivative.The COSY spectrum (Fig. 4) revealed additional interactions between the proton at δ_H 4.12 and those at δ_H 5.49, 5.75, 4.00; the proton H-5 (4.02) correlated with the ones at δ_H 4.25, 3.61 and 1.55.

Assuming the absolute configurations of phytoceramide to be 2′*R*, and 2*S* [16], the NOESY spectrum showed important interactions between H-6 (4.02) and those at δ_H 3.82, 4.08 and 3.61; H-2 (4.25) interacted with the protons H-5 (4.12) and H-4 (1.70) (Fig. 4). These observations indicated that the correlating protons were close in the space and led us to define the absolute configuration of the other carbons to be 3*R*, 5*R* and 6*S*.

The fragment ion at *m*/*z* 534 obtained from ESI-MS/MS was used to locate the double bonds in the piperidine side chain (Scheme 3). This position was evident after acetylation and oxidative cleavage of double bonds (Scheme 4). The organic phase obtained, was analyzed by MALDI-TOF-MS giving five interesting peaks at 193 $[C_{11}H_{22}O + Na]^+$,

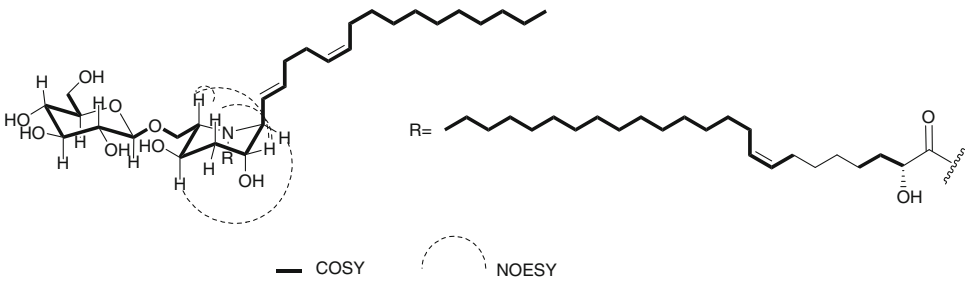
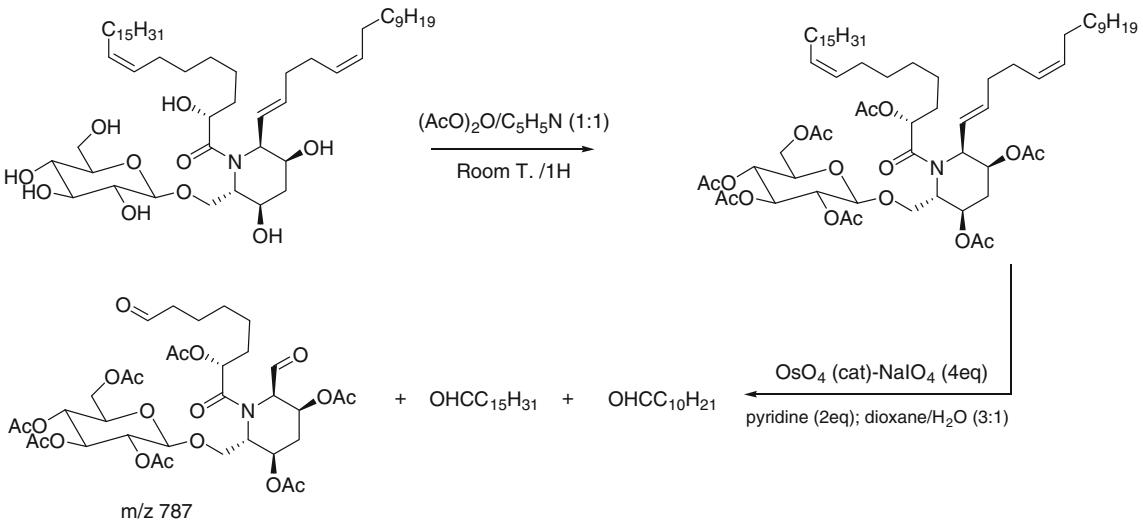


Fig. 4 COSY and NOESY interactions of compound **3**



Scheme 4 Oxidative cleavage of double bonds in compound **3**

209 $[C_{11}H_{22}O + K]^+$, 241 $[C_{16}H_{32}O + Na]^{\cdot+}$, 263 $[C_{16}H_{32}O + K]^+$ and 810 $[C_{35}H_{49}O_{19}N + Na]^+$. The last value justified the belief that **3** had seven free alcohol functions.

Methanolysis solution (aqueous 0.9 N hydrochloric acid:methanol) of **3** was neutralized with sodium carbonate aqueous solution and extracted with chloroform to yield methyl-2-hydroxy-8*Z*-tetracos-8-enoate which was identified after LC-ESI-MS analysis with the peak at *m*/*z* 396 (retention time 10.66 min). From the foregoing data, compound **3** was identified as being (2*R*,8*Z*)-2-hydroxy-{(2*S*,3*R*,5*R*,6*S*)-3,5-dihydroxy-6-[(1*E*,5*Z*)-hexadeca-1,5-dienyl]-2-(*β*-D-glucopyranosyloxy)methylpiperidine-1-yl} tetracos-8-enamide.

The ^{1}H- and ^{13}C-NMR data of the known compounds were similar with those reported in literature.

In the aim to valorise this edible plant, the antioxidant activity of fractions obtained from flash chromatography, evaluated by the FRAP method, was used to carry out this biological activity. Two of them showed a good activity in comparison to those where trolox was used as the standard (Fig. 5). It was noted that, among the four extracts from leaves, one (B′) did not show any activity and the active fraction from stems (the part used as food) was the

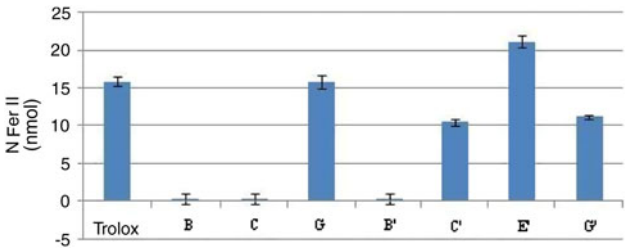


Fig. 5 Result of antioxidant test of flash chromatographic fractions

methanolic one (G). Reduction of Fe^{III}-TPTZ to Fe^{II}-TPTZ was attributable to other secondary metabolites present in trace amounts in the organic extract because not all isolated compounds displayed reducing activity. Although the isolated compounds were not active, some ursolic acid derivatives have been reported as being radical scavenging natural products [17]. In addition, lupeol induces apoptotic death of human pancreatic adenocarcinoma cells [18] and tormentic acid has been reported as being an anti-inflammatory compound [19]. In addition to this wide activity spectrum, ceramide and its derivatives play a significant role in the apoptosis induction of some cancer cells lines [20].

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