Scientific paper

A New Ceramide Isolated from *Ficus lutea* Vahl (Moraceae)

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Abstract

In addition to benjaminamide (**2**), β-amyrin, β-amyrin acetate, lupeol, betulinic acid, β-sitosterol glucoside, a new ceramide glycoside was isolated from the woods of *Ficus lutea* Vahl (Moraceae). Using mass fragmentation pattern, 1 and 2D NMR spectra and by comparison with published data, the new compound was characterized as 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,5*E*,12*E*)-2*N*-[(2'*R*)-hydroxyhexadecanoyl]-octadecasphinga-5,12-dienine (**1a**) for which the trivial name lutaoside was proposed. Some isolated compounds were evaluated for their antimicrobial activities. Compounds **1a** and **2** showed some antimicrobial activity.

Keywords: *Ficus lutea*, moraceae, ceramide, lutaoside, antimicrobial activity.

1. Introduction

The Moraceae family consists of about 50 genera and nearly 1400 species including important groups such as *Artocarpus*, *Morus* and *Ficus.*[1] The genus *Ficus* consists of trees and shrubs that possess latex-like material within their vasculatures, affording protection and self-healing for physical assaults.[2] A number of *Ficus* species are used as food and for medicinal properties in traditional Chinese medicine especially amongst people where these species grow.[3] *Ficus benjamina* is used as an ornamental plant in University of Yaoundé I, Cameroon.[4] Previous phytochemical studies of *Ficus* resulted in the isolation of flavonoids, coumarins, alkaloids, steroids, triterpenes, ceramides and salicylic acids.[4–12] The strong antioxidant and antibacterial activities exhibited by this genus,[13] in addition to the search for the chemical constituents of Cameroonian medicinal plants,[14] justified further attempts to isolate and identify active compounds. To the best of our knowledge, little phytochemical research has been reported on *Ficus lutea* Vahl.[15]

2. Results and Discussion

The woods of *Ficus lutea* were extracted with CH_2Cl_2/MeOH (1:1) during two days. The extract was submitted to repeated column chromatography and monitored by TLC to afford benjaminamide (**2**), β-amyrin, β-amyrin acetate, lupeol, betulinic acid, β-sitosterol glucoside and a new ceramide glycoside (Fig. 1). The ^{1}H and ^{13}C NMR, and MS of the known compounds were consistent with those reported in the literature.

Lutaoside (**1a**) was obtained as a colourless crystalline solid in fraction F. The molecular formula $C_{40}H_{75}NO_9Na$ was determined by HRFABMS at *m/z* 736.5334 $[M+Na]^+$ (Calcd. 736.5339). In the positive FABMS, fragment ions at *m/z* 714 $[M+H]^+$, 696

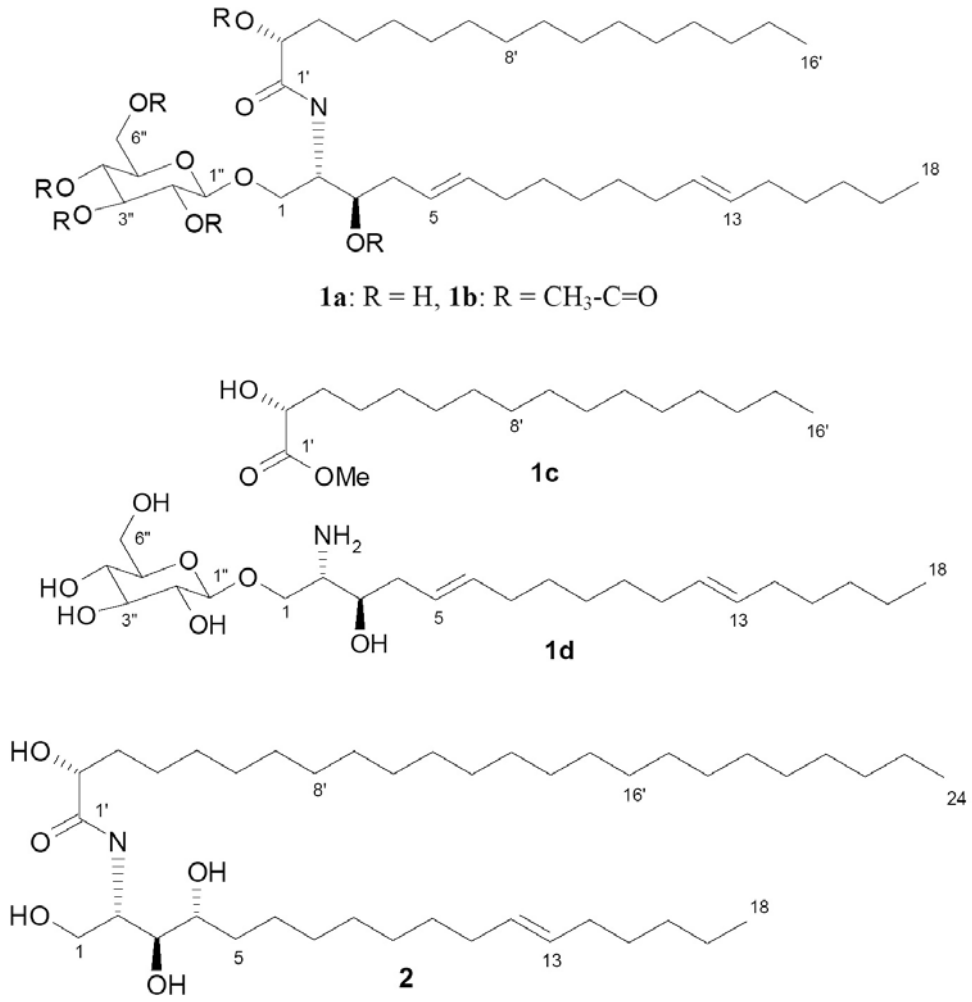

Fig. 1. Structure of compounds **1a, 1b, 1c, 1d** and **2**.

$[M+H–H_2O]^+$, 678 $[M+H–2H_2O]^+$ and 552 $[M+H–C_6H_{11}O_5]^+$ were observed. The IR spectrum of **1a** indicated absorption bands at ν 3445 cm^{-1} (OH), and strong absorption bands for a secondary amide at ν 1647 and 1586 cm^{-1}. These data were supported by the signals at δ 53.0 and 175.0 in ^{13}C NMR spectrum which confirm the presence of C–N and C=O, respectively. Compound **1a** gave a positive reaction in the Molisch test, suggesting the presence of a sugar moiety. The ^1H and ^{13}C NMR spectral data (Table 1) of **1a** indicated the presence of an amide linkage, two long chain aliphatic moieties, suggesting the sphingolipid (glycolipid) nature of the molecule. The ^1H NMR spectral data indicated the presence of a broad singlet centered at δ 1.28 (methylene protons) and two terminal methyl protons at δ 0.90 (t, J = 6.4 Hz, 6H), which confirm the presence of two long aliphatic chain. The methylene protons close to the double bond appeared at δ 1.99–2.25 (m, 8H). A downfield doublet at δ 8.50 (d, J = 8.2 Hz), exchangeable with D_2O, was assigned to the amide proton. The olefinic protons appeared at δ 5.50 (dt, $J_{5,6}$ = 15.4 Hz, $J_{4,5}$ = 5.3 Hz), 5.00 (dd, $J_{5,6}$ = 15.4 Hz, $J_{6,7}$ = 4.7 Hz), 5.54 (dd, $J_{12,13}$ = 16.2 Hz, $J_{11,12}$ = 4.8 Hz) and 5.40 (dd, $J_{13,12}$ = 16.2 Hz, $J_{13,14}$ = 4.6 Hz) were assigned to the C-5/C-6 and C-12/C-13 protons, respectively. Two carbinol protons were observed at δ 4.58 (dd, J = 10.7, 4.9 Hz) and δ 4.40 (dd, J = 10.7, 4.3 Hz). The anomeric proton appeared at δ 4.49 (d, J = 7.8 Hz) suggesting a β-configuration of sugar moiety.

From ^{13}C NMR and DEPT spectra of compound **1a** it is possible to conclude that it consists of 40 carbon atoms, including two methyl, 25 methylene, 12 methine and one quaternary carbon atoms. Four methine carbons resonating at δ 129.8, 129.1, 128.7 and 128.1 suggested the presence of two double bonds in the molecule which were further confirmed in ^1H NMR spectrum with the presence of four olefinic proton signals at δ 5.54, 5.40, 5.50 and 5.00, respectively.

The glycosphingolipid skeleton was supported by the observation that a proton attached to a nitrogen appea-

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of lutaoside **1a** in pyridine-d_6

Position	δ_C	δ_H
1α	68.8 (t)	4.58 (dd, 10.7; 4.9)
1β	68.8 (t)	4.40 (dd, 10.7; 4.3)
2	53.0 (d)	5.20 (m)
3	75.4 (d)	4.50 (m)
4	33.5 (t)	2.25 (m)
5	128.7 (d)	5.50 (dt, 15.4; 5.3)
6	128.1 (d)	5.00 (dd, 15.4; 4.7),
7	32.8 (t)	2.25 (m)
8–10, 15–17	24.0–26.5[a] (t each)	1.28 (br s)
11	33.0 (t)	2.11 (m)
12	129.8[ε] (d)	5.54[ζ] (dd, 16.2; 4.8)
13	129.1[ε] (d)	5.40[ζ] (dd, 16.2; 4.6)
14	32.1 (t)	1.99 (m)
18	13.0 (q)	0.90 (t, 6.4)
NH	–	8.50 (d, 8.2)
1'	175.0 (s)	–
2'	72.5 (d)	4.20 (t, 7.3)
3'	31.0 (t)	1.80 (m)
4'-14'	28.0–29.1[a] (t each)	1.28 (br s)
15'	21.9 (t)	1.70 (m)
16'	13.0 (q)	0.90 (t, 6.4)
1"	102.9 (d)	4.49 (d, 7.8)
2"	73.5 (d)	4.48 (m)
3"	71.9 (d)	4.00 (m)
4"	70.9 (d)	4.11 (m)
5"	70.0 (d)	4.55 (m)
6"a	61.8 (t)	3.70 (dd, 11.5; 5.5)
6"b	61.8 (t)	3.98 (dd, 11.5; 2.4)

Multiplicities and coupling constants in Hz are given in parentheses. [a] Overlapping signals
Resonances with the same superscripts (ε, ζ) in the same column may be interchanged. br s: broad singlet;
d: doublet; dd: doublet of doublet; m: multiplet; t: triplet; dt: doublet of triplet; s: singlet; t: triplet; q: quartet

red as a doublet at δ 8.50 in the ^{1}H NMR spectrum and that a tertiary carbon at δ 53.0 and a quaternary carbon at δ 175.0 were correlated with this nitrogen proton in the HMBC spectrum (Fig. 2).[16]

The positions of the sugar moiety and the double bonds in the long chain sphingosine part were determined by 2D NMR experiments. The ^{1}H–^{1}H COSY (Fig. 2) displayed spin systems of –HOCH–CH_2–CH_2–CH_2, –CH–HOCH–CH_2–CH=CH–CH_2, –CH_2–CH=CH–CH_2 –CH_2, –OCH_2–CH–, –CH_2–CH_2– and two –CH_2–CH_3. In the HMBC spectrum (Fig. 2) the anomeric proton H-1" showed a correlation with C-1 suggesting the position of the sugar moiety to be at the sphingosine part. Accordingly, the HMBC spectrum of **1a** did not show any correlations of the methyl group to the olefin carbons (δ 129.8, 129.1, 128.7 and 128.1). Further investigation of the EIMS showed some important fragment ions at *m/z* 71 and 97 suggesting one of the double bond to be located at C-12,[17] while the second was clearly located at C-5 due to the correlation of H-2 with C-3, C-4 and one small correlation with C-5, and H-3 with C-4 and C-5/C-6. The geometry of the C-5/C-6 and C-12/C-13 alkenyl bonds were evident to be *trans* based on the vicinal coupling constants ($J_{5,6}$ = 15.4 Hz and $J_{12,13}$ = 16.2 Hz). These *E*-geometries were also deduced from the chemical shifts of the allylic carbons C-4, C-7, C-11 and C-14 (δ 33.5, 32.8, 33.0 and 32.1, respectively).[18] The chemical shifts for the corresponding allylic carbons for the *Z* configuration are usually less than δ 29.0.[19,20]

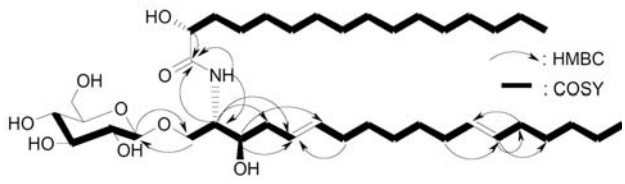

Fig. 2. Important HMBC and ^{1}H–^{1}H COSY correlations for compound **1a**.

The above spectral data revealed compound **1a** to be a glycosphingolipid of the C18-sphinga-5,12-dienine type.[21] The absolute stereochemistry at C-2 and C-3 were proposed as 2*S* and 3*R* (identical to that of D-sphingosine) on the basis of ^{13}C NMR spectral data, since the chemical shifts of C-2 (δ 53.0) and C-3 (δ 75.4) were in agreement with those of the reported natural product hylodendroside-I (δ 52.8 and 75.9).[21]

The length of the long chain base (LCB) and the fatty acid were determined by EIMS, which showed fragment ions at *m/z* 254 and 459 (Fig. 3). On the other hand, the peak at *m/z* 551 in EIMS is due to the elimination of the sugar moiety which is also confirmed in FABMS at *m/z* 552 $[M+H–C_6H_{11}O_5]^+$. Based on the ^{13}C NMR chemical shifts of the chiral center (Table 1) and optical rotation compared with those of the natural and synthetic ceramine,[22] it is clear that compound **1a** has a D-glucopyranosylsphingosine moiety with (2*S*,3*R*, 5*E*,12*E*) geometry. Thus, the structure of lutaoside (**1a**) was assigned as 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,5*E*, 12*E*)-2*N*-[(2'*R*)-hydroxyhexadecanoyl]-octadecasphinga-5,12-dienine, which is described here for the first time.

Acetylation of compound **1a** gave **1b** ($C_{52}H_{87}NO_{15}$). Methanolysis of **1a** gave **1c** and **1d**. The structure of compounds **1b**, **1c** and **1d** (Fig. 1) were confirmed by MS and ^{1}H NMR spectra.

The antifungal and antibacterial activities of compounds **1a** and **2** were determined using the agar diffusion method with 8 mm paper disks loaded with 40 μg of each compound (See Table 2). Compound **1a** exhibited *in vitro* good antimicrobial activity against *Mucor miehei* and *Bacillus subtilis* compared to the nystatin as reference.

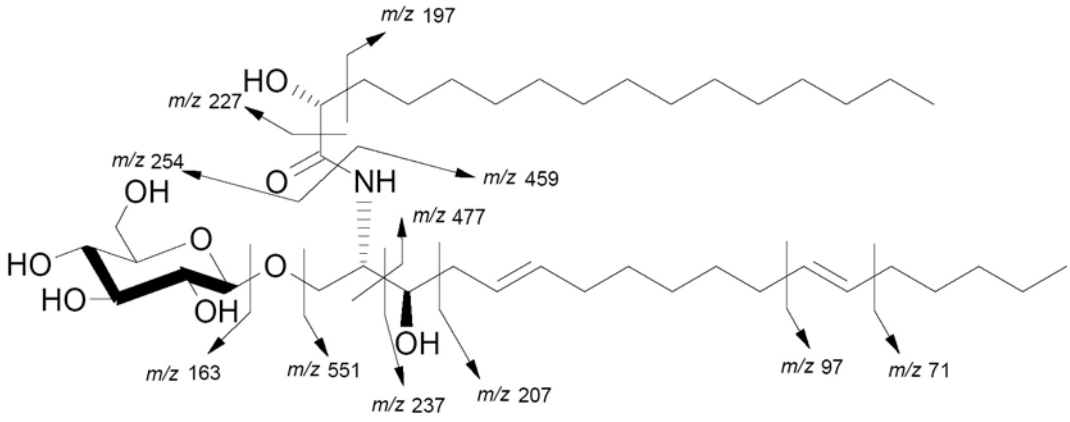

Fig. 3. Important mass fragmentation pattern of compound **1a**.

Table 2. Antimicrobial activity of compounds **1a** and **2**

Micro-organisms tested	Sample Compound **1a**	Compound **2**	Nystatin
Chlorella vulgaris	14	–	–
Scenedesmus subspicatus	11	10	–
Chlorella sorokiniana	13	11	–
Mucor miehei	17	13	15
Bacillus subtilis	16	14	14
Candida albicans	12	13	15
Streptomyces viridochromogenes	–	–	14
Escherichia coli	–	–	–
Staphylococcus aureus	–	–	–

Diameter of inhibition zone in mm. Nystatin was used as the reference.

3. Conclusion

This research study reports a new ceramide **1a** isolated in fraction F (EtOAc/10%MeOH). The new compound was fully characterized and it is one of the rare reported phytochemical study on *F. lutea*. Ceramide has already being reported in other *Ficus* species.[4,12] Two isolates (**1a** and **2**) were evaluated for their antimicrobial activities. Compound **1a** exhibited *in vitro* good antimicrobial activity against *Mucor miehei* and *Bacillus subtilis*. However, the chemical constituents as well as the biological activities of *Ficus* species still remain unclear. Therefore, the secondary metabolites and the biological activities reported from *F. Lutea* seem to be worth for further studies.

4. Experimental

4. 1. General

Melting point is uncorrected and was obtained with a micro melting point apparatus (Yanaco, Tokyo-Japan). Optical rotation values were measured with a Horiba SEPA-300 polarimeter, and IR spectra were recorded with JASCO J-20A spectrophotometer. ^{1}H and ^{13}C NMR spectra were acquired with a Jeol EX-400 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Mass spectra were obtained with a Jeol JMS-700 instrument. Column chromatography was conducted on silica gel 60 (Kanto Chemical Co., Inc., Japan), Sephadex LH-20 (Pharmacia, Sweden) and ODS (Fuji Silysia, Japan). TLC analysis was carried out by using precoated silica gel plates (Merck), and the spots were detected by spraying with H_2SO_4/10% vanillin and then heating. Flash chromatography was carried out on silica gel (230–400 mesh). R_f values were measured on Polygram SIL G/UV254 (Macherey-Nagel & Co.).

4. 2. Plant Material

The woods of *Ficus lutea* Vahl were collected in July 2008 at Kribi, South Cameroon. A voucher specimen has been deposited in the National Herbarium, Yaoundé, Cameroon (Ref. N°. 3471/SRFK).

4. 3. Hydrolysis Experiments

Compound **1a** (1.9 mg) was dissolved in a mixture of MeOH (1 mL) and distilled H_2O (1 mL). Then 7% HCl (1 mL) solution was added and the solution was refluxed for 10 h at 50 °C. MeOH was evaporated in vacuum after cooling. The reaction mixture was extracted three times with chloroform (8 mL each). The residue obtained after removal of acid was compared with standard sugar units on silica gel plate using EtOAc:H_2O (9:1) as the solvent system and the sugar was found to be D-glucopyranoside.

4. 4. Extraction and Isolation

The powdered woods of *Ficus lutea* (3.6 kg) were soaked in 15 L of CH_2Cl_2/MeOH (1:1) during two days at room temperature. Solvent was removed under reduced

pressure and 100 g of organic extract was obtained. Part of this dark-green residue (95 g) was subjected to vacuum liquid chromatography (VLC) on silica gel and eluted with pure *n*-hexane (Fraction A), followed by mixture of *n*-hexane/*p*% ethyl acetate in incremental steps (*p*% = 25%, 50%, 75%, 100% for fractions B, C, D, E, respectively) and finally the mixture of ethyl acetate/10% methanol (Fraction F). Six main fractions (A–F) were obtained and, based on analytical TLC, fractions D and E were combined.

Fraction A (4 g) gave mainly β-amyrin (65.0 mg)[23,24] and β-amyrin acetate (25.1 mg).[25] Fraction B (12 g) was passed through a Sephadex LH-20 column and subjected to silica gel column chromatography and preparative TLC to afford a mixture of sterol (160 mg),[26] lupeol (87.8 mg)[27] and betulinic acid (35.0 mg).[28] Fractions D and E (17 g) were chromatographed on silica gel and eluted with a mixture of *n*-hexane/ethyl acetate of increasing polarity to yield 98 fractions (ca. 100 mL each). Fractions 1–58 (3 g), subjected to column chromatography over silica gel, yielded mainly β-sitosterol glucoside (165.3 mg),[29] while benjaminamide (**2**, 38.9 mg)[4] was obtained in fractions 59–98 (6 g) eluted with $CHCl_3$/MeOH (3:1). Fraction F (13 g) was purified by silica gel column chromatography eluting with $CHCl_3$/MeOH (15:4) to yield lutaoside (**1a**, 16.2 mg).

Lutaoside or 1-*O*-β-D-Glucopyranosyl-(2*S*,3*R*,5*E*,12*E*)-2*N*-[(2'*R*)-hydroxyhexadecanoyl]-octadecasphinga-5,12-dienine (1a)

Colourless crystalline; mp 203–205 °C; R_f = 0.11 ($CHCl_3$/10% MeOH); $[\alpha]_D^{22}$ +8.12 (*c* 0.04, MeOH); IR (film) (ν_{max}/cm^{-1}): 3445 (OH), 3209 (NH), 2924, 2853, 1647, 1586, 1427, 1388, 1217, 1167, 1078, 1047, 1040, 881; ^{1}H NMR (400 MHz, C_5D_5N, 30 °C, TMS) and ^{13}C NMR (100 MHz, C_5D_5N): see Table 1; EIMS: see Fig. 3; FABMS: *m/z* 736 $[M+Na]^+$, 714 $[M+H]^+$, 696 $[M+H-H_2O]^+$, 678 $[M+H-2H_2O]^+$, 552 $[M+H-C_6H_{11}O_5$ (sugar moiety)$]^+$; HRFAB MS: Calcd for $C_{40}H_{75}NO_9Na$ 736.5339 $[M+Na]^+$, found 736.5334.

Acetylation of Lutaoside (1a)

Lutaoside (**1a**, 3.0 mg) was dissolved in pyridine (0.6 mL) and acetanhydride (0.9 mL). The solution was stirred for 7 h at 47 °C. The usual work-up gave **hexaacetoxylutaoside** (**1b**) (2.2 mg, 93%) as an amorphous solid with R_f = 0.91 ($CHCl_3$/10% MeOH); $[\alpha]_D^{22}$ +7.67 (*c* 0.06, pyridine); IR (film) (ν_{max}/cm^{-1}): 3200 (NH), 2915, 2853, 1653, 1580, 1500, 1452, 1419, 1217, 1100, 890; ^{1}H NMR (400 MHz, C_5D_5N, 30 °C, TMS) δ 0.83 (t, *J* = 6.3 Hz, 6H, H-18, H-16'), 1.20-1.30 (br s), 1.50–1.55 (m, 4H, H-3', H-15'), 1.90 (m, 6H, H-6, H-11, H-14), 2.00, 2.05, 2.06, 2.08, 2.12, 2.13 (s, 3H each, CH_3-C=O), 4.09 (dd, *J* = 11.3, 3.7 Hz, 1H, H-1a), 4.12 (m, 2H, H-6"), 4.25 (dd, *J* = 11.3, 5.4 Hz, 1H, H-1b), 4.26 (m, 1H, H-5"), 4.46 (d, *J* = 7.8 Hz, 1H, H-1"), 4.92 (dd, *J* = 10.7, 7.8 Hz, 1H, H-2"), 5.40–5.47 (m, 5H, H-3", H-4", H-6, H-12, H-13), 5.48 (m, 1H, H-2'), 5.50 (m, 1H, H-3), 5.71 (dd, *J* = 15.8, 4.5 Hz, 1H, H-5), 8.53 (d, *J* = 8.2 Hz, 1H, NH); FABMS *m/z* 967 $[M+H]^+$; HRFAB MS Calcd for $C_{52}H_{88}NO_{15}$ 966.6154 $[M+H]^+$, found 966.6138.

Methanolysis of Lutaoside (1a)

Lutaoside (**1a**, 5.0 mg) was subjected to methanolysis in 0.1 mL of 0.9 M HCl in methanol at 60 °C for 15 h. Methyl ester (**1c**, 2.0 mg, 78 %) was extracted from the methanol solution with hexane. After removal of MeOH, the residue was purified using reverse phase (RP-18) eluting with MeOH/H_2O (3:2) to yield 1-*O*-β-D-glucopyranosylsphingosine (**1d**, 0.9 mg, 85 %) as an amorphous solid.

2*R*-Hydroxyhexadecanoic Methyl Ester (1c)[30]

Amorphous solid; R_f = 0.95 ($CHCl_3$/10% MeOH); $[\alpha]_D^{25}$ –2.3 (*c* 0.08, MeOH); ^{1}H NMR (400 MHz, MeOH, 30 °C, TMS) δ 0.85 (t, *J* = 6.4 Hz, 3H, H-16'), 1.25 (br s), 1.40 (m, H-15'), 1.70 (m, H-3'), 3.88 (s, 3H, OCH_3), 4.25 (m, 1H, H-2'); FABMS *m/z* 287 $[M+H]^+$; HRFAB MS Calcd for $C_{17}H_{35}O_3$ 287.2586 $[M+H]^+$, found 287.2579.

1-*O*-β-D-Glucopyranosylsphingosine (1d)

Amorphous solid; R_f = 0.19 ($CHCl_3$/10% MeOH); $[\alpha]_D^{25}$ +6.8 (*c* 0.1, MeOH); ^{1}H NMR (400 MHz, MeOH, 30 °C, TMS) δ 0.81 (t, *J* = 6.4 Hz, H-18), 120 (br s), 1.99–2.12 (m, 6H, H-6, H-11, H-14), 3.20 (br m, 2H, NH_2), 3.40–3.78 (m, 10H, H-Gluc, H-1, H-2, H-3), 4.22 (d, *J* = 7.9 Hz, 1H, H-1"), 5.17 (m, 4H, H-5, H-6, H-12, H-13); FABMS *m/z* 460 $[M+H]^+$; HRMS (FAB) Calcd for $C_{24}H_{46}NO_7$ 460.3274 $[M+H]^+$, found 460.3268.

5. Antimicrobial Assay

Agar diffusion tests were performed in the usual manner[10] with *Bacillus subtilis* and *Escherichia coli* (on peptone agar), S*taphylococcus aureus* (Bacto nutrient broth), *Streptomyces viridochromogenes* (M Test agar), the fungi *Mucor miehei* and *Candida albicans* (Sabouraud agar), and three microalgae (*Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*).

Compounds were dissolved in an azeotrope chloroform/MeOH (87:13) and 40 µg pro paper disks (Ø 8 mm) were impregnated with each using a 100 µL syringe, dried for 1 h under sterile conditions and placed on the pre-made agar test plates. Bacteria and fungi plates were kept in an incubator at 37 °C for 12 h, micro algae plates for three days at room temperature in a day light incubator. The diameter of inhibition zones was measured.

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Povzetek

Iz lesa *Ficus lutea* Vahl (Moraceae) smo poleg benjaminamida (**2**), β-amirina, β-amirin acetata, lupeola, betulinske kisline in β-sitosterol glukozida, izolirali tudi nov ceramid glikozid. Z uporabo fragmentacije v masnem spektrometru, 1 in 2D NMR spektrov in s primerjavo z že objavljenimi podatki, smo novo spojino karakterizirali kot 1-*O*-β-D-glukopiranozil-(2*S*,3*R*,5*E*,12*E*)-2*N*-[(2'*R*)-hidroksiheksadekanoil]-oktadekasfinga-5,12-dienin (**1a**) ter zanj kot trivialno ime predlagali lutaozid. Nekaterim izoliranim spojinam smo tudi določili antimikrobne aktivnosti. Spojini **1a** in **2** sta pokazali določeno antimikrobno aktivnost.