International Journal of Chemical Science
Online ISSN: 2523-2843, Print ISSN: 2523-6075; Impact Factor: RJIF 5.22
Received: 25-01-2021; Accepted: 12-02-2021; Published: 15-03-2021
www.chemicaljournals.com
Volume 5; Issue 2; 2021; Page No. 01-05

Leptadeniamide, a new ceramide from *Leptadenia hastata* Pers. (Decne) (Asclepiadeceae) and antimicrobial activity

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Abstract
Leptadeniamide, new ceramide (1) together with six known compounds (2—7) have been isolated from the leaves extract of *Leptadenia hastata*. Their structures were established using IR, NMR 1D (^{1}H and ^{13}C) and 2D (COSY and HSQC) spectroscopy, mass spectrometry and by comparison with related data reported in the literature. The antimicrobial activity of methanol, hexane and ethyl acetate extracts were also screened against *Staphylococcus aureus* ATCC43300, *Klebsiella pneumoniae* NR41916, *Shigella flexineri NR*518, *Klebsiella pneumoniae* ATCC13883, *Pseudomonas aeruginosa* PM 601, *Salmonelle enterica* NR13555, *Staphylococcus aureus* NR46003, *Salmonelle enterica* NR4311, *Staphylococcus aureus* NR46374 and three yeasts *Candida krusei* ATCC 6258, *Candida albicans* ATCC L 26 and *Candida Prapsilosis* ATCC 22019. The methanol extract exhibited moderate activity against *Klebsiella pneumoniae* ATCC13883 and *Staphylococcus aureus* NR46374 with MIC = 500µg/mL and ethyl acetate extract against *Staphylococcus aureus* NR46374 with MIC = 500µg/mL. All compounds showed no antimicrobial activity.

Keywords: leptadeniamide, ceramide, *leptadenia hastata*, antimicrobial activity

Introduction
Infectious diseases remain a major public health problem throughout the world [1]. Pharmacological industries have produced many new antibiotics in the last three decades. However, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to develop resistance to drugs [2]. Medicinal plants represent a rich source of phytochemical agents some of which are antimicrobial [3]. *Leptadenia hastata* Pers. (Decne) is a perennial plant of the Asclepiadaceae family. It's one of the important medicinal herbs used by the traditional healers for the treatment of various diseases such as diabetes mellitus, stomach upset, scabies, hypertension, catarrh, skin diseases, sexual potency, trypanosomosis, prostate inflammation and rheumatism [4—5]. Previous pharmacological studies on *L. hastata* revealed its antibacterial, cytotoxic, anti-androgenic and antioxidant activities [6]. The phytochemical studies on *L. hastata* reported the isolation of steroids, tritrepenes and saponins [7—8]. To the best of our knowledge, no pharmacological and phytochemical studies have been done on *L. hastata* collected in Cameroon. This prompted us to undertake more investigation on this species. We here report the isolation and structure elucidation of a new ceramide, (leptadeniamide) (1) and six known compounds ursan-12-en-3-O-*β*-D-glucopyranoside (2), n-octacosanol (3), a mixture of stigmasterol and *β*-sitosterol (4), hyl-10-*epi*-pheophorbide A (5), di-(2'-ethylhexyl) ester phthalic acid (6) and a mixture of *β*-amyrin and lupeol (7) from *L. hastata* and their antimicrobial activities.

Materials and Methods
Instrumentation
Column chromatography (CC) was performed on silica gel 60 (70-230 mesh, Merck). The melting point of the new compound was recorded in an open capillary using Stuart melting point apparatus (SMP-3) and in uncorrected. ESI-MS spectra (ionization voltage 3kV) were registered on a Q-TOF Ultima spectrometer (Waters). A spectrometer Bruker Avance AV-500 (125 and 500 MHz) was used for NMR spectra. IR spectra were recorded with a Nicolet Avatar IR spectrophotometer (Thermo scientific, USA) TMS as internal standard.

Plant Material Collection and Authentication
The leaves of *L. hastata* was collected at Mora in the Far North Region of Cameroon in November 2014 and identified by Mr Nana Victor (botanist). A voucher specimen (N° 7798/ SRF/Cam) has been deposited at the Cameroon National Herbarium, Yaounde.

Antimicrobial Assays
The antimicrobial assay was determined by a broth dilution method as previously describe [9] and [10]. 50 µL of extract/compound concentrated at 1 mg/mL were added to wells of the first line. A serial two fold dilution was made by transferring 50µL of the mixture of the first wells to the next one up to the last, final concentrations varying from 500 to 31.25µg/mL. Then, 50 µL of inoculums of 1×10^{5} cells/mL for yeast and 1×10^{6} cells/mL for bacteria were introduced in all the wells except those of the sterility control. Each plate also contained a positive control (Fluconazole or Amoxicillin), a negative control and a blank. Plates were incubated during 24 and 48 hours for bacterial and fungal respectively. The lowest concentration of extract that inhibited the visible growth of a microorganism was defined as minimum inhibitory concentration (MIC). Cut-off points for

significant activity of extracts were as follow: very good (MIC < 62.5 µg/mL), good (62.5 < MIC ≤ 125µg/mL), moderate (250 < MIC ≤ 500 µg/mL) or weak (MIC >500µg/mL).

Isolation and Purification of Compounds

Dry powder (1Kg) of the leaves of *L. hastata* was extracted by maceration using methanol (15 L) for 48 hours. The extract was concentrated under vacuum yielding a brown residue (175g) which was then partitioned with hexane and ethyl acetate to give 25g of hexane extract, 50 g of ethyl acetate extract and 15 g of methanol residual extract. Hexane extract (25g) was subjected to column chromatography on silica gel 60 (200 g) and eluted with (Hexane/EtOAc/MeOH in order of increasing polarity) yielding a novel ceramide (leptadeniamide) (1) (3mg, 3) at EtOAc/MeOH 90:10 in addition to six known compounds identified as ursan-12-en-3-O-*β*-D-glucopyranosyl (3mg, 2), n-octacosanol (4 mg, 3), mixture of stigmasterol and β-sitosterol (3mg, 4), methyl-10-*epi*-pheophorbide A (3 mg, 5), di-(2'-ethylhexyl) ester phthalic acid (5 mg, 6) and the mixture of *β*-amyrin and lupeol (7).

Results and Discussion

Dried and powdered leaves of *L. hastata* were extracted with methanol at room temperature. The residue obtained after evaporation of the solvent was fractionated using n-hexane and ethyle acetate. The phytochemical investigation of the n-hexane leaves extract of *L. hastata* was resulted in the isolation of seven constituents, including one new ceramide (1) and six known compounds ursan-12-en-3-O-*β*-D-glucopyranoside (2) [11] octacosanol (3) [12], a mixture of stigmasterol and *β*-sitosterol (4) [13], methyl-10-*epi*-pheophorbide A (5) [14], di-(2'-ethylhexyl) ester phthalic acid (6) [15] and a mixture of *β*-amyrin and lupeol (7) [16] (Fig. 1).

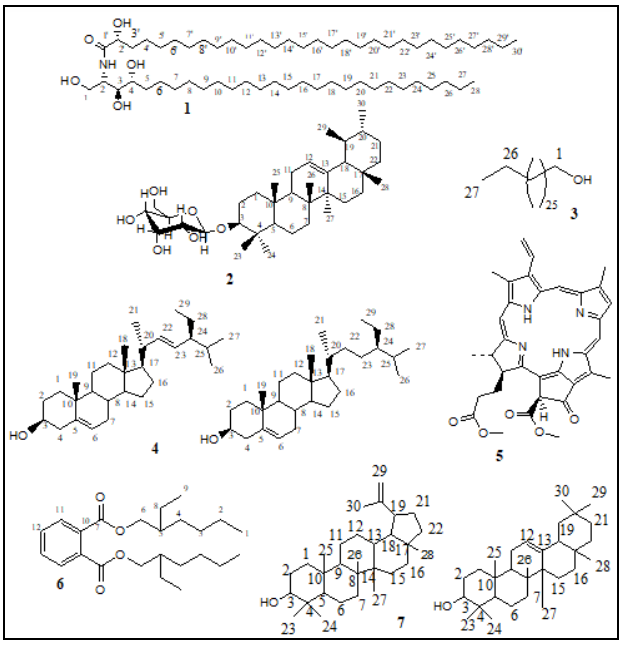

Fig 1: Structures of isolated compounds.

Leptadeniamide (1) was obtained as white amorphous powder, m. p.132.1—133.1 °C and was assigned the molecular formula $C_{58}H_{117}O_5N$ on the basis of TOF-MS ESI+ analysis which showed a *pseudo*-molecular ion peak at *m/z* = 908.4 $[M+H]^+$ with one degree of instauration. The IR spectrum exhibited absorption bands at 1618 and 1542 cm^{-1} due to the amide group [17]. The ^1H NMR spectrum (C_5D_5N, 500MHz) exhibited five characteristic signals of protons geminal to hydroxyl group, three carbinylics protons resonances at δ = 4.27 (m, 1H, H—2'), 4.33 (overlapped dd, 1H, H—3), 4.61 (br dd,1H, H—4) and two diasterotopics protons at δ = 4.61 (dd, 1H, *J* = 3.8, 12.5 Hz, H—1a) and 4.60 (dd, 1H, *J* = 3.8, 12.5 Hz, H—1b) further supported by the absorption band of hydroxyl group(s) in the IR spectrum 3363.8 cm^{-1}[15]. A sixth signal was observed at δ = 5.10 (m, 1H, 2-H), corresponded to a methine proton vicinal to the nitrogen atom of the amide group and confirmed by an amide carbonyl signal at δ = 175.0 in the ^{13}C NMR spectrum.

Compound 1 also showed characteristics signals of two terminal methyl groups at δ = 0.84 and methylenes at δ = 1.29 (brs) [18]. The ^{13}C—NMR (Table 1) showed resonances at δ_C 175.0 C(1') characteristic of a carbonyl group, a methine linked to the amide N-atom at δ_H 52.7 and three carbinylics resonances at δ_C 76.5 (CHOH), 72.2 (CHOH) and 72.7 (CHOH) was further confirmed the presence of three oxygenated carbons. The ^{13}C—NMR showed a downfield signal of one oxymethylene at δ_C 61.7 (CH_2OH), another methine carbon resonating at δ_C 52.7 (CHNH) was due to the presence of an amidomethine functional, signals for several methylene groups in the range of δ_C 26.2-34.2, and the terminal methyl groups of the aliphatic chains at δ_C 14.0.

The presence of four hydroxyl groups was further confirmed by the presence of four oxygenated carbons at δ_C 76.5, 72.7, 72.2 and 61.7 in the ^{13}C NMR spectrum [19].

In the COSY (^1H-^1H) spectrum, the amidomethine proton at δ_H 5.10 (H—2) showed cross peaks with the diastereotopic oxymethylene protons at δ_H 4.61; 4.60 (2H-1a et 1b), the amidoproton at δ_H 8.57 (NHCO) and the oxymethine proton at δ_H 4.36 (H-3). The oxymethine proton at δ_H 4.36 (H—3) have correlation with the oxymethine proton at δ_H 4.30 (H—4). Additionally, H–1a showed the expected correlations with H—2 and no cross peaks were observed between the signals at δ_H 4.62 assigned to H-2′ to any downfield proton signals the latter only showing correlation to upfield signals. In the HMBC spectrum the signal at δ_H 8.57 showed a strong correlation to C-1′. These results confirmed that the fourth hydroxyl group is present at C-2′ of the fatty acid chain. The length of the fatty acid and sphingosine chain was determined based on the ^1H and ^{13}C—NMR spectra and from different characteristic fragmentation peaks observed on the ESI-MS-TOF spectrum (Figure 3). The length of fatty acid chain was found to be composed of twenty-eight carbons from the ESI-MS-TOF (Figure 3), which showed the ion peak at *m/z* = 451.4 for $[CH_3(CH_2)_{27}CHOHCO]^+$ containing, carboxyl and hydroxyl groups, 515.4 for $[CH_3(CH_2)_{23}CH(CHOH)_3(CO)NHCH_2$ $OH+H]^+$. The length of the sphingosine chain was also obtained from the ESI-MS-TOF spectrum, which showed significant fragmentation peaks at *m/z* = 441.4 for $[CH_3(CH_2)_{24}CH(CHOH)_2OH]^+$, 409.2 for $[CH_3(CH_2)_{23}$ $C(CHOH)_2]^+$ and 398.3 for $[CH_3(CH_2)_{23}$ $(CHOH)_2+H]^+$ (Figure 3). Thus, the long chain

amino base and fatty acid of **1** are assigned as 2-amino-docosan-1, 3, 4-triol and 2-hydroxyoctadecanoic acid, respectively.

Table 1: ^{1}H and ^{13}C NMR data and HMBC correlations of **1**[a] (δ in ppm, J in Hz)

		1	
Positions	**δ_H (Mult., J (Hz))**	**δ_C**	**HMBC**
1a	4.61 (dd, *J*=12.5, 3.8)	61.7 CH_2	
1b	4.60 (dd, *J* = 12.5, 3.8)	61.7 CH_2	
2	5.03-5.10 (*m*)	52.7 CHN	
3	4.30—4.33 (*m*)	76.5 CH	
4	4.27 (d, *J* = 10Hz)	72.2 CH	
5	2.00—24 (*m*)	33.6 CH_2	
6	1.51—1.62 (*m*)	26.3 CH_2	
7-25	1.15—1.29 (brs)	29.9-30.8 (CH_2)	7, 25
26	1.40—1.42 (m)	32.5	
27	1.30—1.33	26.3	
28	0.84 (t, J = 9.0Hz)	14.0 CH_3	27, 28
NH	8.57 (d, *J* = 9.0Hz)	/	
1'	/	175.0	
2'	4.61(dd, *J* = 4.0, 7.6 Hz)	72.7	
3'	2.00-.24 (m)	33.6	
4'	1.51-1.62 (m)	26.3	
5'-27'	1.15-1.29 (br.s)	29.9-30.8 (CH_2)	5', 27'
28'	1.40-1.42 (m)	32.5 CH_2	
29'	1.30-1.33 (m)	26.3 CH_2	
30'	0.84 (t, *J* = 9.0Hz)	14.0 CH_3	30', 29'

[a] Mesured in pyridine-d_6

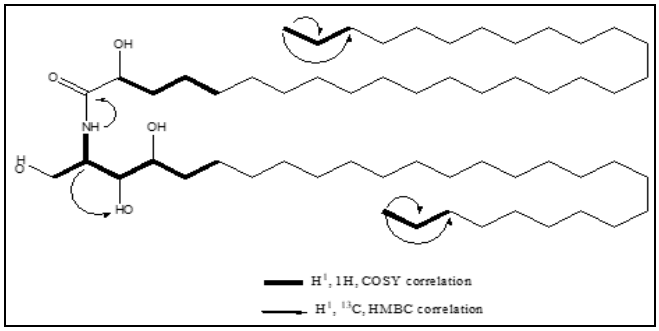

Fig 2: Select HMBC and COSY correlations of compound 1

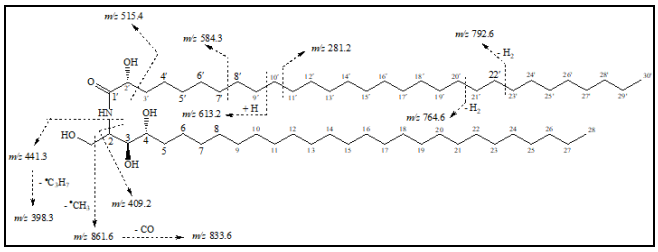

Fig 3: Mass fragmentation pattern of compound 1

In addition ^{1}H-NMR spectrum corresponded to that of the synthetic ceramide (2*S*, 2′*R*,3*S*,4*R*)-2-(2-hydroxy tetracosano ylamino)hexadecane-1,3,4-triol, with respect to the signals due to H–1a, H–1b, H–2, H–3, H–4, and (H–2′) (Table 2) [19] .

Table 2: ^{1}H NMR data (δ and J values) of compound (1) and synthetic ceramide in C_5D_5N

H	Leptadeniamide	Synthetic ceramide[a]
H-1a	4. 61 (dd, *J* = 3.8, 12.5 Hz)	4.52 (dd, *J* = 4.5, 10.7 Hz)
H-1b	4.60 (dd, *J* = 3.8, 12.5 Hz)	4.43 (dd, *J* = 5.0, 10.6 Hz)
H-2	5.10 (m)	5.12 (m)
H-3	4.34 (dd, *J* = 4.5, 6.5 Hz)	4.36 (dd, *J* = 4.6, 6.6 Hz)
H-4	4.27 (m)	4.29 (m)
H-2'	4.61 (dd, *J*= 4.0, 7.6)	4.63 (dd, *J* = 4.0, 7.6 Hz)

Based on the biogenetic considerations, the absolute stereochemistry of C-2, C-3, C-4 and C-2' in **1** was then proposed as 2*S*, 3*S*, 4*R*, 2'*R* [20]. Based on these evidences, compound (**1**) could be assigned the structure of ((2'*R*)-2-hydroxy-N-(2*S*, 3*S*, 4*R*) —1, 3, 4—trihydroxyoctacosan-2-yl) nonacosanamide) to which the trivial name Leptadeniamide was given and described as a new ceramide derivative.

Leptadeniamide (1)

White powder, mp 132.1—133.1 °C — IR ($CHCl_3$ + MeOH): ν_{max} = 3332, 2954, 2949, 1618, 1542 cm^{-1}. — ^{1}H (500 MHz, C_5D_5N): δ = 0.86 (t, *J* = 9.0 Hz, 6H, 28 —H, 30'—H), 1.30—1.33 (m, 2H, H-27), 1.40—1.42 (m, 2H, H-26), 4.27 (d, *J* = 10.0 Hz, 1H, H-4), 1.74 (m, 2H, 7—H), 1.95 – 2.01 (m, 4H, 9-H, 12-H), 2.05 (m, 2H, 5-H), 2.10 (m, 3 -H), 4.27 (d, *J* = 10Hz, 1H, H-4), 4.38 (m, 1H, 3-H), 4.46 (dd, J = 5.0, 10.0 Hz, 1H, 1b-H), 4.54 (dd, J = 5.0, 10.0 Hz, 1H, 1a-H), 4.66 (m, 1H, 2 -H), 5.10 (m, 1H, 2-H), 4.61(dd, *J* = 4.0, 7.6 Hz, H-2'), 2.00-.24 (m, H-3') 1.51-1.62 (m, H-4') 1.15-1.29 (br.s, H-5'-27') 1.40-1.42 (m, H-28') 1.30-1.33 (m, H-29') 7.06 (s, 1H, OH), 7.37 (s, 1H, OH), 7.39 (s, 1H, OH), 7.63 (s, 1H, OH), 8.57 (d, J = 8.0 Hz, 1H, NH). — ^{13}C NMR (125 MHz, C_5D_5N): δ = 14.0 (C-28, C-30'), 26.3 (C-27), 32.5 (C-26), 29.9-30.8 (C-7-25), 26.3 (C-6), 33.6 (C-5), 76.5 (C-3), 52.7 (C-2), 61.7 (C-1), 73.7 (C-2), 72.2 (C-4), 76.5 (C-3), 175.0 (C-1'), 72.7 (C-2'), 33.6 (C-3'), 26.3 (C-4'), 29.9-30.8 (C-5'-27'), 32.5 (C-28'), 26.3 (C-29');TOF-MS-ESI+: 908.4 (Calcd. for $C_{58}H_{117}O_5N$ $[M+H]^+$, 908,6). Important; ^{1}H—^{1}H COSY and HMBC correlations are illustrated in Figure 3

Antimicrobial Activity

All the isolates were subjected to the dilution assay for *in vitro* antimicrobial activity against *Staphylococcus ATCC43300, Staphylococcus aureus NR46374, Staphylococcus. aureus NR4300, Klebsiella NR41916, Shigella flexineri NR518, Klebsiella pneumonia ATCC 13883, Pneudmonas enteric NR 13555, Staphylococcus aureus NR 46003, Salmonelle enterica NR4311, Candida krusei ATCCL 6258, Candida albicans ATCC L 26* and *Candida parapsilosis ATCC 22019*. The tests were carried out according to the protocols described in the literature [19] and [20]. Isolates were obtained from the Yaounde Central Hospital, Cameroon and the reference strains from BEI resources and the American Type Culture Collection. Two antimicrobial agents, amoxicillin and fluconazole, were used as positive controls in these tests. The results of the antimicrobial activity presented in table 2 indicated that some extracts were moderately active depending on the microbial strain with MIC values ranging from > 500 to 500 µg/mL. The crude methanol extract was moderately active

against *Staphylococus aureus* ATCC43300, *Staphylococcus* aureus NR46374, *Salmonella* enterica NR13555 and *Pseudomonas aeruginosa* PM601 with MIC values of 500 µg/mL, residual methanol extract was moderately active against *Staphylococcus* aureus NR46374, *Salmonella* enterica NR13555 and *Pseudomonas* aeruginosa PM60 (MIC= 500µg/mL) and the ethyle acetate extract was moderately active against *Salmonella enterica* NR13555 and *Pseudomonas aeruginosa* PM601 (MIC =500µg/mL). All compounds showed no activity against all tested microbial strains.

Table 3: Antimicrobial activity of some *Leptadenia hastata* leaves extract

Extracts Microbial strain	CMELH	MRELLH	EAELLH	HELLH	Amoxicillin	Fluconazole
Staphylococusaureus ATCC43300	500	>500	>500	>500	1	ND
Staphylococcus.aureus NR43003	>500	>500	>500	>500	32	ND
Staphylococcusaureus NR46374	500	500	>500	>500	1	ND
Shigellaflexineria NR518	500	>500	>500	>500	1	ND
Klebsiellapneumonia NR41916	>500	>500	>500	>500	128	ND
Klebsiellapneumonia ATCC13883	>500	>500	>500	>500	1	ND
Salmonella enterica NR13555	500	500	500	>500	128	ND
Salmonellaenterica NR4311	>500	>500	>500	>500	2	ND
Pseudomonasaeruginosa PM601	500	500	500	>500	2	ND
*Candidakrusei*6258	>500	>500	>500	>500	ND	32
Candidaalbicans ATCC L 26	>500	>500	>500	>500	ND	32
Candidaparapsilosis ATCC 22019	>500	>500	>500	>500	ND	16

Conclusion

Leptadeniamide (1) new compound was isolated from hexane leaves extract of *Leptadenia hastata* (Asclepiadeceae). Its chemical structure was elucidated using NMR and ESI-MS-TOF data and by comparison with the literature value. The crude methanol extract exhibited a moderate activity against *Staphylococus aureus* ATCC43300, *Staphylococcus aureus* NR46374, *Salmonella enterica* NR13555 and *Pseudomonas aeruginosa* PM601 with MIC values of 500 µg/mL, residual methanol extract also showed moderate activities against *Staphylococcus aureus* NR46374, *Salmonella enterica* NR13555 and *Pseudomonas aeruginosa* PM60 (MIC= 500µg/mL) and the ethyl acetate extract was moderately active against *Salmonella enterica* NR13555 and *Pseudomonas aeruginosa* PM601 (MIC =500µg/mL). No activity was recorded for all tested compounds.

Acknowledgements

The authors are grateful to the Department of Organic chemistry of the University of Yaounde I for providing some consumables. We also thank the team of the Laboratory of NMR and molecular imaging, University of Mons for undertaking the spectral analysis of compound.

Compliance with Ethical Standards

Conflict of Interest

The authors declare no conflict of interest

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