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New Compounds from the Tree Fern *Metaxya rostrata* C. Presl

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Abstract: Four new natural compounds, namely 2-deprenyl-7-hydroxy-rheediaxanthone B, (2E)-2-(hydroxyl hexyliden)-cyclopropyl-1→3-diglucoside and (6E)-6[2-(β-glucopyranosyloxy)-cyclopropyliden] hexanoic acid methylester as well as the first glucocerebroside from a fern, (4E)-1-O-(β-glucopyranosyl)-*N*-(2'-hydroxytetracosanoyl)-4,8-sphingadienine (d18:2/h24:0-GlcCer), were isolated for the first time from the rootlets of the tree fern *Metaxya rostrata* C. Presl. Two of these compounds contain a very rare cyclopropyliden structure which until now is only known from two other compounds from this plant. In addition, this is the first report of the tannin proanthocyanidin A2 and the triterpenes squalene and fern-9(11)-ene in this tree fern, of which the two latter were isolated from the leaves. The structures of all compounds were elucidated on the basis of detailed spectroscopic and mass spectrometric analyses, including 1D-, 2D-NMR and HR Q-TOF-MS.

Keywords: *Metaxya rostrata*; cyclopropyliden derivatives; glucocerebroside; xanthone; spectroscopic analyses.

1. Introduction

The tree fern *Metaxya rostrata* C. Presl (Metaxyaceae) has a wide distribution in lowland rain forests of Central and South America [1]. Based on the ethnomedicinal use in gastrointestinal disorders, the chemical composition of this plant was investigated. In our previous studies two very unusual new compounds, (2E)-2-(6-hydroxyhexyliden)cyclopropyl-β-glucopyranoside and (6E)-6[2-(β-glucopyranosyloxy)cyclopropylidene] hexanoic acid [2], two cytotoxic proanthocyanidins (cinnamtannin B-1 and aesculitannin B), common sterols and sugars were isolated from an aqueous extract of the rhizomes [3]. The fractionation of a dichloromethane extract of the rootlets resulted in the isolation of 2-deprenylrheediaxanthone B, which showed a very interesting cytotoxic activity causing cell death by a mechanism similar to mitotic catastrophe [4], and of other compounds such as betulinic acid, 4-O-β-D-glucopyranosyl-caffeic acid, 4-O-β-D-glucopyranosyl-p-trans-coumaric acid and methyl-α-fructofuranosid [2]. Due to the complex chemical pattern, the study was continued to elucidate further secondary metabolites which might support a chemotaxonomic approach for the correct classification of *Metaxya rostrata* in the group of tree ferns.

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2. Materials and Methods

2.1. General experimental procedures

The solvents for extraction and fractionation were analytical-grade and obtained from VWR (Vienna, Austria). Accelerated solvent extraction was performed with a Dionex accelerated solvent extractor (ASE) model 200 (Sunnyvale, USA). NMR spectra were recorded on a Bruker Avance DRX 600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) using a 5 mm switchable quadruple probe (QNP, ^{1}H, ^{13}C, ^{19}F, ^{31}P) with z axis gradients and automatic tuning and matching accessory. The resonance frequency for ^{1}H NMR was 600.13 MHz, for ^{13}C NMR 150.92 MHz. All measurements were performed with solutions in deuterated methanol at 298 K. Standard 1D and gradient-enhanced (ge) 2D experiments like double quantum filtered (DQF) COSY, TOCSY, NOESY, HSQC and HMBC were performed as provided by the manufacturer. Chemical shifts were internally referenced to the residual, non-deuterated solvent signal for ^{1}H (δ = 3.31 ppm) and the carbon signal of the solvent for ^{13}C (δ = 49.00 ppm), respectively. The analysis of ^{1}H–^{1}H coupling constants (given in Hertz) was supported by the program Daisy within the processing software TopSpin (Bruker BioSpin, Rheinstetten, Germany). LC/MS analyses were performed on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D ion trap mass spectrometer equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). HPLC separation was carried out on an Acclaim 120 C_{18} column, 2.1×150 mm, 3 µm (Dionex, Germering, Germany) at 25°C and a flow rate of 0.5 mL/min. 1% aqueous formic acid and acetonitrile were used as mobile phase A and B, respectively. The following gradient program was used: 10% B (0 min), 10% B (2 min), and 23% B (15 min). UV data were recorded from 190 to 400 nm. The eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: capillary voltage: ±3.7 kV, nebulizer: 26 psi (N_2), dry gas flow: 9 L/min (N_2), and dry temperature: 340 °C. Positive and negative ion mode multistage mass spectra up to MS^3 were measured in automated data-dependent acquisition (DDA) mode using helium as collision gas, an isolation window of 4 Th, and a fragmentation amplitude of 1.0 V. Offline ESI-MS^n spectra were obtained on the same instrument and HR-ESI-MS spectra were recorded on an ESI-Qq-TOF mass spectrometer (micrOTOF-Q II, Bruker Daltonics) using direct infusion. The CD spectra were measured on a Spectropolarimeter Jasco J-810 (QS 1 mm; Cremella, Italy). For TLC silica gel plates (Merck, Germany, 0.25 mm) and the mobile phases (1) EtOAc/HCOOH/MeOH/H_2O (70 : 8 : 8 : 11); (2) $CHCl_3$/MeOH/H_2O (70 : 22 : 3); (3) EtOAc/MeOH/ H_2O (81 : 11 : 8) were used; detection: (A) anisaldehyde/H_2SO_4-reagent; (B) Naturstoff-reagent A/PEG 400 under UV 366.

2.2. Plant Material

Rhizomes and leaves of *Metaxya rostrata* (*M. r.*) were collected in February 2009 around La Gamba, near Golfito in south-western Costa Rica, and authenticated in the Herbarium of the Museo National in San Jose by Dr. Werner Huber. Voucher specimens (MR0209) are deposited at the Herbarium of the Department of Pharmacognosy, University of Vienna, Austria.

2.3. Extraction and Isolation

Ground, dried rootlets of *Metaxya rostrata* (1 kg) were extracted by sonification with ethylacetate at 40°C yielding 5.1 g ethylacetate-extract. The extract was subjected to column chromatography (CC) on Sephadex LH-20 (CC-1) under elution with EtOAc/MeOH, 7:3 (column diameter: 3 cm; height: 90 cm) to obtain 24 fractions (*SC-1.1* to *SC-1.24*).

Fractions *SC-1.3* (80 mg) and *SC-1.7* (100 mg) were further fractionated by solid phase extraction on SPE-cartridges (C-18, 6 mL, Bond Elut, Varian) under sequential elution with 4 reservoir volumes each of 50, 60, 70, 80, 85, 90, 95 and 100% MeOH. In subfraction 15 of *SC-1.3*, compound **1** (4 mg) was isolated. In subfractions 1 to 5 from *SC-1.7*, compound **2** crystallized (10.2 mg).

Fraction *SC-1.19* (190 mg) was submitted to CC on Sephadex LH-20 under use of MeOH/H_2O mixtures of decreasing polarity (50 to 100 % MeOH) as mobile phase to obtain 21 subfractions. In subfraction 13, proanthocyanidin A2 (5 mg) was isolated.

Another extraction of 300 g pulverized, dried rhizomes by accelerated solvent extraction at 40°C with MeOH yielded 30.6 g methanol extract. For detannification this extract was extracted twice with water by sonfication followed by centrifugation and partition of the aqueous supernatant with butanol. After evaporation of the butanol phase the residue (=7.5 g detannified extract) was submitted to CC on Sephadex LH-20 under elution with 80% MeOH (CC-2) to obtain 13 subfractions *SC-2.1* to *SC-2.13*.

Fraction *SC-2.2* (634 mg) was further fractionated by CC on silica under elution with water-saturated EtOAc/MeOH (8:2) resulting in the isolation of compound **3** (18 mg) in subfraction 9.

Fraction *SC-2.3* (5.9 g) was partitioned between water and EtOAc and subsequently n-BuOH. The organic phases were evaporated resulting in fraction *P-E* and *P-B*, respectively. Fraction *P-E* (690 mg) was further fractionated by CC on silica (CC-3) and elution with water-saturated EtOAc/MeOH (9:1) resulting in the 14 subfractions *SC-3.1* to *SC-3.14*. Fraction *P-B* (2.75 g) was fractionated in the same manner (CC-4) using water-saturated EtOAc/MeOH (9:1 and 8:2) as mobile phase resulting in the 17 subfractions *SC-4.1* to *SC-4.17*. Subfractions *SC-3.8* from CC-3 and *SC-4.5* and *SC-4.6* from CC-4 were combined (149 mg) and further purified by CC on silica (CC-5) and elution with water-saturated EtOAc/MeOH (9:1) resulting in 7 subfractions *SC-5.1* to *SC-5.7*. Final purification of the combined fractions *SC-5.4, SC-5.5* and *SC-5.6* from CC-5 (79 mg) was achieved by CC on Sephadex LH-20 under elution with MeOH/water (1:1) resulting in the isolation of compound **4** (14 mg).

Accelerated solvent extraction of ground, dried leaves (1 kg) with CH_2Cl_2 resulted in 35.4 g extract. After removal of chlorophylls, the purified extract (2.2 g) was fractionated using VLC (*SC-8*) on silica resulting in 22 fractions. In subfraction 1 (59 mg), the triterpenes squalene and fern-9(11)-en were enriched.

3. Results and Discussion

Compound **1** is a whitish microcrystalline substance. From the NMR data, compound **1** was identified as a glucocerebroside.

The signals of the β-glucopyranose moiety were unambiguously allocated (see Supporting Information). The fatty acid part was saturated and contained a hydroxy group in position 2´´. The shifts in the ^{1}H- und ^{13}C-NMR-experiments correlated with literature [5,6]. In the sphingosin part two double bonds in position 4´ and 8´ were identified, the first in E configuration, the second one undetermined due to the shift degeneracy of the protons 8´ and 9´, respectively.

For further information concerning the side chains of the cerebroside, extensive MS experiments were performed. The HR-ESI-MS data indicated a molecular formula of $C_{48}H_{91}NO_9$. The fragmentation pattern of the lithiated molecule showed good correlation with literature [7]. The most informative fragment ions in the MS^2 and MS^3 spectra of the $[M+Li]^+$ ion were assigned to the loss of the glucosyl moiety (Y_0 ion at *m/z* 670.7), the loss of the hydroxyfatty acid (g_1 ion at *m/z* 466.3), and the combined loss of those two to give the lithiated long-chain base (e_{1b} ion at *m/z* 304.2). These and the other major fragment ions, which could all be assigned to known fragmentation pathways of cerebrosides, allowed the unambiguous determination of the length of the side chains to give the fully saturated hydroxyfatty acid residue with 24 C atoms and the twofold unsaturated sphingosine residue with 18 C atoms.

Based on these results, compound **1** was identified as *(4E)-1-O-(β-glucopyranosyl)-N-(2'-hydroxytetracosanoyl)-4,8-sphingadienine (d18:2/h24:0-GlcCer).* To the best of our knowledge this is a new glucocerebroside and the first representative of this class of compounds isolated from a fern.

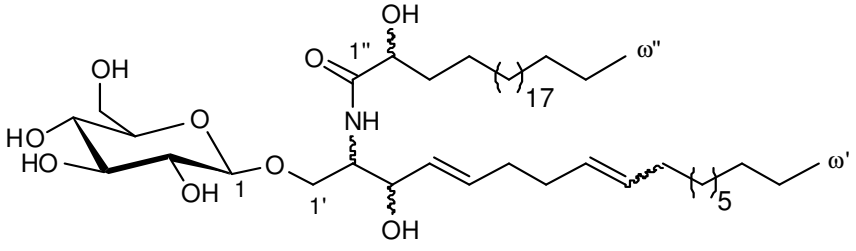
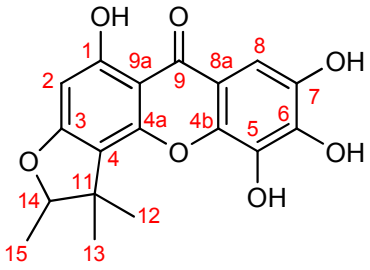


Figure 1. Structure of compound **1** = (4E)-1-O-(β-glucopyranosyl)-N-(2'-hydroxytetracosanoyl)-4,8-sphingadienine (d18:2/h24:0-GlcCer)

Compound **2** was isolated as whitish crystals. The NMR- and mass spectra were highly similar to 2-deprenylrheediaxanthone B, which had been previously isolated from *Metaxya rostrata* [4]. The molecular weight was 344.0932 Da by HR-ESI-MS, fitting to a pentahydroxy-C-prenylxanthone ($C_{18}H_{16}O_7$). Fragmentation of the $[M+H]^+$ ion yielded neutral losses of C_3H_6 (-42 Da) and C_4H_8 (-56 Da), which are typical for C-prenylated aromatic compounds [8,9], and an additional loss of CO (-28 Da) from the fragment ion at *m/z* 288.7 in MS^3. The ^{1}H-NMR spectrum showed two singlets of aromatic protons at δ = 6.12 ppm and 7.10 ppm. Two further singlets at δ= 1.61 ppm and 1.32 ppm as well as a doublet at δ= 1.40 ppm (J = 6.6 Hz) were attributed to methyl groups. The proton of a CH-group appeared at δ = 4.53 ppm as a quartet and the coupling constant of 6.6 Hz proved the substitution with the methyl group. The ^{13}C-NMR shifts of the trimethylfurano part and the furano-anellated, hydroxylated benzene correlated largely with literature [10]. In the pyrano-anellated benzene, signals of C-6 and C-8 showed a high-field shift of 10 and 17.4 ppm, respectively, as compared to 2-deprenyl-rheediaxanthon B [10]. In accordance with the down-field shift of δ = 29.5 ppm for C-7, the position of the additional hydroxyl group at C-7 was verified and the new natural compound identified as 2-deprenyl-7-hydroxy-rheediaxanthone.



Chemical Formula: $C_{18}H_{16}O_7$
Molecular Weight: 344,32

Figure 2. Structure of compound **2** =2-deprenyl-7-hydroxy-rheediaxanthone

According to ^{1}H- and ^{13}C-NMR data, compound **3** comprised a methylen-cyclopropanglycoside which contained the same genin as (1R,2E)-2-(6-hydroxyhexyliden)cyclopropyl-*β*-D-glucopyranoside [2]. The molecular formula of **3** is $C_{21}H_{36}O_{12}$ in accordance with the HR-ESI-MS data. Fragmentation of the $[M+Na]^+$ and the $[M-H]^-$ ion showed a disaccharide consisting of two hexoses to be attached to the genin. They were verified as β-glucopyranoses by NMR experiments and based on the down-field shift of C-3' of 10 ppm together with the corresponding HMBC crosspeaks from H-1´´ to C-3´ and from C-1´´ to H-3´, respectively, the 1→3 linkage of the sugars was proven. Thus, unambiguously, the structure of compound **3** is (2E)-2-(hydroxyhexyliden)cyclopropyl-1→3-diglucoside.

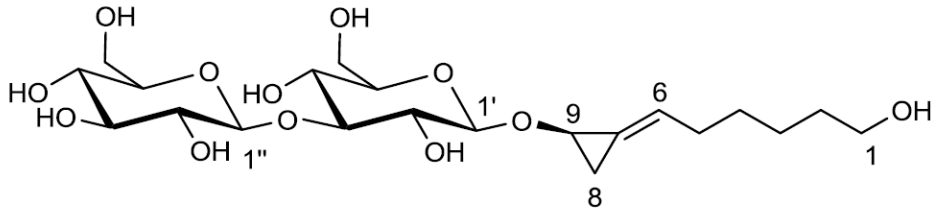


Figure 3. Structure of compound **3** = (2E)-2-(hydroxyhexyliden)cyclopropyl-1→3-diglucoside

The HR-ESI-MS of compound **4** showed an $[M+H]^+$ ion at *m/z* 347.1706, corresponding to a sum formula of $C_{16}H_{26}O_8$. Fragmentation of the $[M+NH_4]^+$ and the $[M+Na]^+$ both showed the characteristic loss of a hexosyl group (-162 Da). In addition, the loss of methanol (-32 Da) from the aglycone was observed in the MS^2 spectra of the $[M+NH_4]^+$ and the $[M+HCOO]^-$ ion. By ^{1}H- and ^{13}C-NMR data, the sugar was identified as glucose. The shifts of the genin in the ^{1}H- und ^{13}C-NMR spectra showed good correlation with (6E)-6[(2R)-2-(*β*-D-glucopyranosyloxy) cyclopropylidene]hexanoic acid

[2]. In ^{1}H-NMR, only the protons at C-2 exhibited a down-field shift of 0.2 ppm and an additional signal of a methyl group occurred at δ = 3.65 ppm. The up-field shifts of 7 ppm, 4.5 ppm and 1.9 ppm, respectively, of C-1, C-2 und C-3 in the ^{13}C-NMR spectrum, together with a HMBC correlation from the methyl protons to the carboxyl carbon, confirmed the structure of a methylester of (6E)-6[(2R)-2-(*β*-D-glucopyranosyloxy)cyclopropylidene] hexanoic acid for compound **4**, which thus was identified as (6E)-6[2-(β-glucopyranosyloxy)-cyclopropyliden] hexanoic acid methylester.

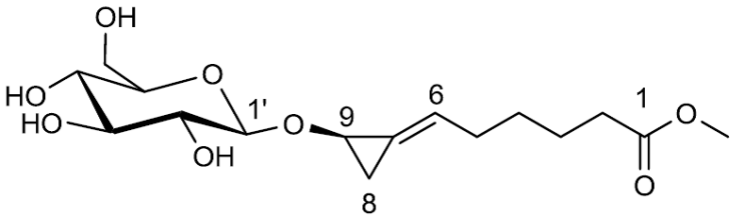


Figure 4. Structure of compound **4** = (6E)-6[2-(β-glucopyranosyloxy)-cyclopropyliden] hexanoic acid methylester

Besides these four new natural compounds this is also the first report of proanthocyanidin A2, squalene and fern-9(11)-ene in *Metaxya rostrata,* the structures of which were elucidated by MS- and NMR experiments showing excellent correlation to published data [11-13].

This investigation of *M. r.* showed that compounds with a cyclopropyliden structure, which have never been found before in nature, might be characteristic for this species. In addition to two earlier described substances [2], a diglucoside and a methylester of this class of compounds were isolated for the first time.

Metaxyaceae are a monotypic family consisting of only two species, namely *Metaxya rostrata* C. Presl and *Metaxya lanosa* A. R. Sm. & H. Toumisto [1]. Phylogenetic studies suggested that the family belongs to the tree ferns [14-18]. Monophyly of the family was strongly supported by DNA-molecular data. Nevertheless, the family was placed at two conflicting phylogenetic positions in chloroplast DNA-based reconstructions [19]. Thus, the outstanding chemical composition of *M. r.* might support chemotaxonomic studies to clarify the true relationship of Metaxyaceae among tree ferns.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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