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**ARTICLE INFO**

**ABSTRACT**

Seven novel steroid glycosides, acanthifoliosides A-F (1-6) and the methyl ester of 6 (7), were isolated from the marine sponge *Pandaros acanthifolium* as minor components. Acanthifoliosides are characterized by a rare C-15 and C-16 oxidized D ring which was previously found in saponins produced by starfishes. Very uncommon is the presence of additional sugar residues at C-15 or C-16. Their structures were determined on the basis of extensive spectroscopic analyses, including two-dimensional NMR and HRESIMS data. The absolute configurations of the aglycones were assigned by comparison between experimental and TDDFT calculated CD spectra of 1, whereas the absolute configurations of the monosaccharide units were determined by chiral GC analyses of the acid methanolyisates. Some of the acanthifoliosides exhibit moderate antiprotozoal activity but to a lesser extent than the most potent pandarosides.

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Keywords: Marine Sponge Steroidal Saponin Pandaros NMR Circular Dichroism
1. Introduction

Marine biodiversity has been recognized as a prolific source of original secondary metabolites with potential applications in the pharmaceutical industry. Even if most of the terrestrial families of natural products have been found in the marine environment, steroidal saponins are much more scarcely found in marine organisms than in their terrestrial counterparts. These compounds are mainly recognized as chemotaxonomic markers of echinoderms. In marine sponges, triterpenoid saponins were mostly found in the Asteropuss, Melophlus and Erylus genera all belonging to the Astrophyitida order.

During our first investigation of the little studied Caribbean marine sponge Pandaros acanthifolium (Poecilosclerida, Microcionidae), a large family of 19 steroidal saponins named panda rosides was discovered as major constituents of this sponge. So far, the only reported compound from this sponge was the polyether acanthifolicin. Pandarosides are steroidal saponins, characterized by a rare 2-hydroxycyclopentenone D-ring and a glucuronic acid at C-3, some of them bearing additional unsaturations in the A and B cycles. Most of these compounds exhibited interesting antiprotozoal activity, the most active being pandaroside G and its methyl ester, which potently inhibited the growth of Trypanosoma brucei rhodesiense and Leishmania donovani. Because some minor compounds with distinct UV and MS spectra were detected in the HPLC-DAD-MS profiles of some fractions, we decided to go further into the characterization of the secondary metabolome of this sponge, with the hope to isolate additional saponin derivatives.

Herein, we report the isolation and structural elucidation of a novel family of seven steroidal glycosides named acanthifoliosides A–F (1–6) and the methyl ester of 6 (7) as minor constituents of the sponge Pandaros acanthifolium (Fig. 1). Their structures were elucidated by spectroscopic studies, including 1D- and 2D-NMR experiments as well as HRESIMS analyses. All of them lack the cyclopentenone D-ring characteristic of the previous pandarosides. In vitro inhibitory activities of the new metabolites were evaluated against a small panel of parasitic protozoa, i.e. Trypanosoma brucei rhodesiense, Trypanosoma cruzi, Leishmania donovani, and Plasmodium falciparum.

2. Results and discussion

The CH2Cl2/MeOH (1:1) extract of the Caribbean marine sponge Pandaros acanthifolium was fractionated by RP-C18 flash chromatography and two complex polar fractions were further purified by successive semi-preparative RP-C18 HPLC and analytical C8-phenyl HPLC to yield the seven new compounds 1-7 obtained as white amorphous solids.

The molecular formula of acanthifolioside A (1) was determined as C33H52O7 by a pseudomolecular ion at m/z 583.3610 [M+Na]+ in HRESIMS. The bands at 3490, 1688 and 1644 cm⁻¹ in the IR spectrum suggested the presence of hydroxyl, carbonyl and olefinic groups, respectively. The 13C NMR spectrum of 1 confirmed the presence of 33 carbon signals, 28 of them corresponding to a steroid skeleton and five corresponding to a sugar portion according to the COSY and HSQC NMR spectra. The 1H NMR spectrum of 1 evidenced characteristic steroid signals: two methyl groups placed on quaternary carbons at δH 1.06 (s, H 3-19) and 1.02 (s, H 3-18) and four methyl groups placed on tertiary carbons at δH 0.85 (d, J = 6.8 Hz, H3-26), 0.86 (d, J = 6.8 Hz, H3-27), 0.93 (d, J = 6.8 Hz, H3-241), and 1.13 (d, J = 6.9 Hz, H3-21) (Table 1).

These data were consistent with an ergostane skeleton. Analysis of the 13C NMR spectrum of the aglycone part showed the occurrence of one olefinic quaternary carbon (δC 142.0), three olefinic methines (δC 135.3, 134.3 and 122.5), two secondary oxygenated carbons (δC 74.7 and 72.4), and one carbonyl (δC 218.9) (Table 2). The assignments of 1H and 13C signals of the tetracyclic ring system was established by a combination of COSY, HSQC and HMBC data interpretation, starting from the HMBC correlations of the protons
of the angular methyls Me-18 and Me-19. Long-range correlations from the proton signal at $\delta_H$ 1.06 (H-19) to the carbon resonances at $\delta_C$ 38.4 (C-1), 142.0 (C-5), 51.7 (C-9) and 37.9 (C-10), and the H-4/C-5 and C-6 HMBC correlations placed a first trisubstituted double bond at C-5 (Fig. 2). The key H-20, H-21/C-22 and H-24$^1$, H-25/C-23 HMBC correlations placed the disubstituted double bond at \( \Delta^{22,23} \) of the side chain whereas the HMBC correlations of the protons at $\delta_H$ 2.02 (H-17) and 2.53 (H-20) with the carbon at $\delta_C$ 218.9 placed the ketone at C-16. The resulting two oxygenated methines were placed at C-3 and C-15 due to $^1$H-$^1$H COSY correlations (Fig. 2).

On the other hand, a unique sugar residue was evidenced in the HSQC spectrum of 1 by the characteristic signals of an anomeric proton at $\delta_H$ 4.77 (d, $J = 7.8$ Hz) and $\delta_C$ 103.5. MS-MS fragmentation evidenced the loss of \( m/z \) 132 corresponding to a cyclic pentose. Analysis of the COSY spectrum allowed the assignment of the C-1'/C-2'/C-3'/C-4'/C-5' spin system. The deshielded AMX spin system in the $^1$H NMR spectrum at $\delta_H$ 3.18 (t, $J = 10.9$ Hz), 3.85 (dd, $J = 11.5$, 5.5 Hz) and 3.48 (m) confirmed the presence of a pentopyranose, which was further identified as a xylose by interpretation of the coupling constant values of H-2' (dd, $J = 8.9$, 7.8 Hz) and H-3' (t, $J = 9.0$ Hz). This sugar residue was linked to the aglycone at C-15 because of the key H-1'/C-15 and H-15/C-1' HMBC correlations. The large coupling constant value for the doublet assigned to H-1' ($J = 7.8$ Hz) implied that the xylose was connected to the aglycone through a $\beta$-glycosidic linkage. The D absolute configuration of xylose was obtained by chiral GC analysis of the acetylated sugar residue after acid methanolysis of 1.$^{14}$

Table 1
$^1$H NMR data for acanthifoliosides A-F (1-6) at 500 MHz in CD$_3$OD. $\delta_H$ in ppm (mult., $J$ in Hz).
Figure 2. Key HMBC (H→C) and COSY (bold line) correlations for 1.
The relative configuration of the aglycone part of 1 was assigned on the basis of NOESY NMR data interpretation as well as coupling constant values. First, the hydrogen at H-3 was placed on the α face of the skeleton due to the vicinal coupling constant values of J = 10.9 and 4.5 Hz (δH 3.41, tt) (Fig. 3). The key H-7α/H-9/H-14 nOe correlations strongly suggested that all these protons were placed on the same α side of the molecule. Configuration at C-15 was deduced from the absence of a H-18/H-15 nOe correlation, present in synthetic compounds with the same D-ring and a H-15 on the β face of the steroid.15 In addition, the constant value of J = 7.3 Hz between H-15 and H-14 was consistent with an α orientation of H-15, whereas values of 11.5 or 13.5 Hz were observed in the case of a β orientation for this hydrogen.15,16,17 Because no strong H-17/H-18 nOe correlation was distinguishable we assumed the natural α orientation for H-17 which was also supported by a small H-14/H-17 nOe correlation.18,19 The unusual coupling constant value of 5.5 Hz between H-17 and H-20 was nevertheless questioning. However, the α position for H-17 was further supported by its 13C chemical shift at δC 69.0 ppm and comparison with literature data. During the oxidation of some steroids hydroxylated at C-16, an epimerization at C-17 was observed with a C-16 keto steroid. For the H-17β configuration the authors reported δC 60.7 ppm whereas a strong deshielding at δC 70.9 ppm was observed for the H-17α epimer, very close to our value.20 The coupling constant between H-22 and H-23 (J = 15.4 Hz) was consistent with an E configuration for the disubstituted double bond. Comparison of the chemical shifts of the hydrogen side chains with published data allowed the assignment of the configurations of C-20 (R), C-24 (R) as a usual ergostane.21,22

![Figure 3. Key NOESY correlations for the aglycone of 1.](image)

The negative Cotton effects at 325 nm (δε = -0.32) in the CD spectrum of 1 was assigned to the n→π* transition of the ketone at C-16. A conformational analysis was performed on the usual stereoisomer using the Density Functional Theory (DFT) method at B3LYP/6-31+G(d) level, which gave 12 conformers of relative energies below 5 kcal.mol⁻¹. The calculation was run on the 4 lowest energy DFT structures which represent more than 80% of the population. A very good agreement was observed between the experimental CD spectrum of 1 and the TDDFT calculated spectrum of 1 with this stereochemistry (Fig. 4), which allowed us to propose the depicted relative and absolute configurations for the aglycone part of 1 (Fig. 1). Performing the same theoretical CD spectra with all the four stereoisomers at C-15 and C-17, observation was made that the position of the oxygen at C-15 was critical for the sign of the Cotton effect. Placing the oxygen in the α side of the molecule. Configuration at C-15 was deduced from the absence of a H-3-18/H-15 nOe correlation, whereas values of 11.5 or 13.5 Hz were observed in the case of a β orientation for this hydrogen.15,16,17 Because no strong H-17/H-18 nOe correlation was distinguishable we assumed the natural α orientation for H-17 which was also supported by a small H-14/H-17 nOe correlation.18,19 The unusual coupling constant value of 5.5 Hz between H-17 and H-20 was nevertheless questioning. However, the α position for H-17 was further supported by its 13C chemical shift at δC 69.0 ppm and comparison with literature data. During the oxidation of some steroids hydroxylated at C-16, an epimerization at C-17 was observed with a C-16 keto steroid. For the H-17β configuration the authors reported δC 60.7 ppm whereas a strong deshielding at δC 70.9 ppm was observed for the H-17α epimer, very close to our value.20 The coupling constant between H-22 and H-23 (J = 15.4 Hz) was consistent with an E configuration for the disubstituted double bond. Comparison of the chemical shifts of the hydrogen side chains with published data allowed the assignment of the configurations of C-20 (R), C-24 (R) as a usual ergostane.21,22

![Figure 4. Experimental and calculated CD spectra of 1.](image)

The molecular formula of 2 was established as C32H52O7 by HRESIMS (m/z 585.3765 [M+Na]+), which showed the presence of two additional protons in comparison to 1. The 1H and 13C NMR data were similar to those of 1, which indicated the occurrence of a similar steroidal skeleton and sugar residue. Differences were located on the D ring where the lack of the signal at δC 218.9 (C-16) and the appearance of two signals at δC 74.6 / δC 4.26 suggested the replacement of the carbonyl by an hydroxyl group at C-16. The assignment of the C-14/C-15/C-16/C-17/C-20 spin system by the COSY spectrum confirmed this assumption. We assumed that the stereochemistry was the same as for compound 4 where the signals were fully resolved and all four H-14, H-15, H-16 and H-17 hydrogens placed on the α side of the molecule. Indeed, chemical shifts and coupling constants of H-15 and H-16 were found to be very similar for both products. On this basis, the structure of compound 2 was established as (E)-15β-O-(β-D-xylopyranosyl)ergosta-5,22-dien-16-one.

The molecular formula of compound 3 was determined as C32H52O7 by HRESIMS (m/z 585.3613 [M+Na]+), which involved the loss of a methylene unit comparing to 2. Differences in their 1H NMR spectra were easily located on the aglycone side chain, where the lack of the characteristic signal at δH 0.95 (d, J = 6.6, H-24β) suggested that a methyl was absent at C-24. The presence of a new methylene at δH 1.90 and 1.86 and δC 43.4 were also consistent with this assumption. In consequence, the structure of 3 was deduced to be (E)-15β-O-(β-D-xylopyranosyl)cholesta-5,22-diene-3β,16β-diol.

The molecular formula of compound 4 was assigned as C30H50O4 by HRESIMS (m/z 633.4341 [M+Na]+). The 13C and 1H NMR spectra of 4 evidenced that no double bond was present in the structure. Comparing with the spectral data of acanthifoliosides A-C (1-3) significant changes were observed for the aglycone and sugar residue of 4. The 13C NMR spectrum of 4 displayed 35 carbon signals, 29 corresponding to the aglycone part and six to the glycoside. The 1H NMR spectrum exhibited the characteristic steroid signals: two methyl groups on quaternary carbons at δH 0.87 (s, H-19) and 1.05 (s, H-18), three methyl groups on tertiary carbons at δH 0.90 (d, J = 6.8 Hz, H-26), 0.96 (d, J = 6.8 Hz, H-27), and 1.01 (d, J = 6.6 Hz, H-21), and one methyl group on a secondary carbon at δH 0.97 (t, J = 7.1 Hz, H-24β). All these data were consistent with a poriferastane/stigmastane skeleton. The analysis of HSQC correlations clearly showed the occurrence of five secondary oxygenated carbons (δC 63.9, 71.2, 71.9, 70.0 and 86.2), that were placed at C-17, C-15, C-3,
C-23 and C-16 by a combination of COSY and HMBC spectra interpretation. Moreover, the sugar portion of compound 4 showed in the 1H NMR spectrum a new methyl signal at δH 1.30 (d, J = 6.2 Hz, H-6'), which suggested the presence of a deoxysugar. The interpretation of coupling constants of the corresponding 1H NMR signals led us to propose a rhomapyranose unit. Chiral GC analysis allowed us to identify the usual α absolute configuration of this sugar. The H-1' anomic proton exhibited a signal at δH 4.77 (d, J = 3.2 Hz), indicating that the glycoside and the aglycone were connected through an α-glycosidic linkage. Finally, the sugar was linked to the aglycone at C-16 due to the key H-1''/C-16 HMBC correlation. Confirmation was made by the strong deshielding of C-16 from 74 ppm for compounds 2 and 3 to 86 ppm for 4.

The relative stereochemistry of 4 was established by interpretation of coupling constant values and NOESY data, because in this case no overlapping occurred for all the 1H signals of the D ring. NOESY cross-peaks between H-14/H-15/H-16/H-17 led us to assume that all these protons were placed on the same α face. Because such configurations were already observed for saponins isolated from starfishes we decided to confirm this result comparing the coupling constants for H-15 and H-16. Values around 2.0 Hz are consistent with a trans configuration between both hydroxyls on the cyclopentane D ring. Because we measured a constant of 6.2 Hz between these two hydrogens, the relative configuration was cis. After comparison with literature data and modeling with Chem3D (MM2) and MestreJ, using the HLA empirical generalization of the Karplus equation, the only possibility was an all cis relative configuration for the D ring substituents. Determination of the configuration of the side-chain was rendered difficult because the low quantity of material did not allow us to perform the Mosher method. To the best of our knowledge there is no natural steroid with a similar side-chain which prevented any NMR data comparison. There are relatively few examples of such a side-chain in the literature and comparison between chemical shifts was highly uncertain. Nevertheless, two stereochemical studies of closely related 23-deoxybrassinosteroids allowed us to strongly suggest a trans configuration for compounds 4–6. NMR investigations and molecular modeling of a C-23/C-24 syn configuration did not evidence the same noe correlations, while the H-23 signal at δH 3.90 ppm (dd, J = 10.8, 4.8, 1.9 Hz) for 22-deoxy-24-episterasterone characterized by a trans configuration exhibited very similar coupling constant values. Confirmation of both configurations at C-23 and C-24 would nevertheless require deeper stereochemical analyses. Compound 4 was established as 16β-O-(α-l-rhamnopyranosyl)-5α-poriferastane-3β,15β,23S-triol.

Compound 5 exhibited the molecular formula C47H80O19 as indicated by HRESIMS (m/z 631.4180 [M+Na]+), which involved the presence of an additional unsaturation comparing to 4. Both compounds exhibited very similar NMR spectra and they were proven to share the same side chain and sugar residue. The structural difference lied in the presence of a double bond at C-5/C-6 due to the signals at δH 5.38 (H-6) / δC 122.5 (C-6) and δC 142.1 (C-5) as previously described for acanthifoliosides A-C (1–3). The molecular structure of acanthifolioside F (6) (C49H82O19) was calculated from the HRESIMS spectrum (m/z 971.5198 [M+Na]+). Comparison between the NMR spectra of 6 and those of acanthifoliosides D and E (4 and 5) did not evidence any strong difference between their steroid parts. Changes were mostly observed in the NMR spectra of the sugar part, where for the first time three anomeric protons were evidenced in the 1H NMR spectrum. A α-rhamnopyranose was identified by comparison with previous data of compounds 4 and 5. A β-glucopyranose and a β-glucuronic acid were identified by comparison with previous data obtained for pandarosides. The sugar identification was completed by chiral GC analysis, which allowed us to assign the absolute configuration of the sugar residues as D-GluUA, D-Glu and L-Rha. Finally, interpretation of 13C deshieldings on the glucuronic acid residue in addition to H-1'''/C-4' and H-1''/C-2' HMBC correlations allowed us to identify the sequence of the trisaccharide unit as (β-D-glucopyranosyl-(1→2))-(α-L-rhamnopyranosyl-(1→4))-β-D-glucuronic acid, the GlcUA residue being attached to the aglycon at C-16 due to a H-1''/C-16 HMBC cross-peaks.

The ion at m/z 985.5386 [M+Na]+ in the HRESIMS established the molecular formula of compound 7 as C45H79O19, which indicated the presence of an additional methylene unit in comparison with 6. The 1H and 13C NMR data were very similar to those of 6 except for the new signals at δH 3.76 (s, H-5) and δC 52.8 (O-CH3) which suggested the presence of a methoxyl group. The H2-C=O/C-6' HMBC correlation allowed us to identify the methyl ester of acanthifolioside F (6). This compound could be formed during the extraction process as previously observed for pandarosides.

All acanthifoliosides share a common 15,16 dioxidized D rings with one sugar residue at C-15 or C-16. They consequently differ from the previously isolated pandarosides characterized by a 15,16 diketone enolized at C-16. Mostly found in starfishes, some analogues have been recognized as cytotoxins. Even if the genus Pandaros belongs to the Pocilloclerida order, a recent phylogenetic study by Erpenbeck put apart the genera Pandaros and Ectyoplasia from the other Pocilloclerida sponges, known to produce a large variety of complex alkaloids. They seem to be much more closely related to Asterophorida sponges, a rich source of saponins. Some saponins were already described from Ectyoplasia ferox and our study underlined a high amount of steroid saponins in Pandaros acanthifolium. All these chemotaxonomic considerations are fully consistent with the result of Erpenbeck.

Antiprotozoal bioassays were performed on compounds 1–7. Even if moderate activities were detected on L. donovani and P. falciparum acanthifoliosides appear less potent than pandarosides.
Table 3.
In vitro antiprotozoal and cytotoxic activities of sponge-derived compounds 1-7. IC_{50} values are in µM.

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Stds 0.010 a 2.64 b 0.51 c 0.20 d 0.012 e

Standard compounds: a melarsoprol, b benznidazole, c miltefosine, d chloroquine, e podophyllotoxin. Nt: non tested

Table 2
13C NMR data for acanthifoliosides A-F (1-6) at 125 MHz in CD$_3$OD. $\delta_C$ in ppm.

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3. Experimental section
3.1. General experimental procedures

Optical rotations were measured on Perkin Elmer 343 polarimeter equipped with a 10-cm microcell. CD spectrum of 1 was measured using a JASCO J-810 spectropolarimeter. IR spectra were obtained with a Perkin–Elmer Paragon 1000 FT-IR spectrometer. UV measurements were performed on a Varian Cary 300 Scan UV-Visible spectrometer. Electrospray ionisation (ESI) mass spectra were obtained with a Bruker Esquire 3000 Plus spectrometer in the positive or negative mode. High-resolution mass spectra (HRESIMS) were obtained from a LTQ Orbitrap mass spectrometer (Thermo Finnigan). NMR experiments were performed on a Bruker Avance 500
MHZ spectrometer. Chemical shifts (δ in ppm) are referenced to the carbon (δC, 49.0) and residual proton (δH 3.31) signals of CD3OD, the solvent with multiplicity (s singlet, d doublet, t triplet, m multiplet). Flash Chromatography fractionation was performed on a Armen Spot Flash instrument equipped with a UV detector. Column chromatography was performed using DIOL as the stationary phase (40-63 μm, Merk KGaA 64271 Darmstadt, Germany). HPLC separation and purification were carried out on a Waters 600 system equipped with a Waters 996 Photodiode Array detector coupled with a Sedex 55 ELSD (SEDERE, France), and a Waters 717 plus Autosampler. TLC was performed with Kieselgel 60 F254 (Merck glass support plates) and spots were detected after spraying with 10% H2SO4 in EtOH reagent and heating.

3.2. Biological material

The marine sponge was collected off Martinique Island in summer 2003 by SCUBA diving (Canyons de Babodie 14°45.982 N, 61°11.902 W). A voucher specimen (ORMA8362) identified by Dr Jean Vacelet, has been deposited in the Centre d’Océanologie de Marseille (Endoume, France). The sponge was kept frozen from collection until the extraction process.

3.3. Extraction and isolation

The frozen sponge (536 g) was cut into pieces of about 1 cm3 and extracted with MeOH/CH2Cl2 1:1 at room temperature yielding 20.0 g of crude extract after solvent evaporation. The crude extract was fractionated by RP-C18 flash chromatography (elution with a decreasing polarity gradient of H2O/MeOH from 1:0 to 0:1, then MeOH/CH2Cl2 from 1:0 to 0:1). The MeOH 100 % (1.1 g) fraction was then subjected to DIOL column chromatography (elution with an increasing polarity gradient of CH2Cl2/MeOH from 95:5 to 100:0), this separation step yielded seven fractions after the TLC analysis (see details in supporting information). The aliquots corresponding to MeOH (5 to 15 %, 40 mg) were then subjected to RP-C18 semi-preparative HPLC (Phenomenex, Luna C18, 250 × 10 mm, 5 μm) with an isocratic mobile phase of H2O/MeOH/TFA (flow 3.0 mL min-1, 10:90:0.1) to yield pure compounds 5, 6, and 7 (tR: 13.2, 14.8 and 21.1 min; 1.0, 1.7 and 1.2 mg, respectively). On the other hand, the H2O/MeOH 1:3 (220 mg) fraction, which had been obtained from RP-C18 flash chromatography, was then subjected to RP-C18 semi-preparative HPLC (Phenomenex, Luna C18, 250 × 10 mm, 5 μm) with a gradient of H2O/MeOH/TFA (flow 3.0 mL min-1 from 28:72:0.1 to 20:80:0.1) to obtain a complex chemical profile (see supporting information). A subsequent mixture (33.0-34.0 min; 10.0 mg) was finally purified by analytical HPLC (C2-phe nyln) with a gradient of H2O/CH3CN/formic acid (flow 0.5 mL min-1, 50:50:0.1) to afford pure metabolites 5, 4 and 1 (tR: 19.1 and 23.3 min; 3.7 and 3.3 mg, respectively).

3.4. Computational method

Quantum chemical calculations were performed on the four diastereoisomers at C-15 and C-17 of compound 1. The Gaussian03W package75 has been used for the conformational search as well as for circular dichroism calculations. Density functional theory (DFT) with B3LYP functional28 and Pople’s 6.31+G(d) basis set29 was used on the lowest energy conformer. TDDFT was employed to calculate excitation energy (in eV) and rotatory strength R in dipole velocity (Rvel) and dipole length (Rlen) forms. The Boltzmann weighted rotatory strengths were simulated in ECD curve by using a corrected Gaussian function.

\[
\Delta \varepsilon(E) = \frac{1}{2.296 \times 10^{-39} \sqrt{2\pi \Delta}} \sum_a \Delta \varepsilon_{oa} R_{oa} e^{-\frac{(E-E_{oa})^2}{2.296 \times 10^{-39} \sqrt{2\pi \Delta}}}
\]

where Δ is half the width of the band at \( \frac{1}{e} \) peak height expressed in energy units. The parameters ΔEoa and Roa are the excitation energies and the rotatory strengths for transition from 0 to a, respectively, \( \Delta = 0.1 \) eV and Rvel were used.

3.5. Acanthifolioside A (1)

\((E)-3\beta\)-hydroxy-15-β-O-((β-D-xylo pyranosyl) ergosta-5,22-dien-16-one. White amorphous solid; [α]D20 -87.3 (c 0.14, MeOH); CD (MeOH, c 2.5×10-4 M) \( \lambda_{max} (\Delta \alpha) \) 325 (-0.32) nm. IR (thin film): \( \gamma_{max} 3490, 1688 y 1644 \) cm-1; 1H NMR see Table 1; 13C NMR see Table 2; HRESIMS (+): m/z 583.3610 [M+Na]+ (calcd for C33H52NaO7, 583.3605, \( \Delta 0.85 \) ppm).

3.6. Acanthifolioside B (2)

\((E)-15\beta\)-O-((β-D-xylopyranosyl)ergosta-5,22-diene-3β,16β-diol. White amorphous solid; [α]D20 -22.5 (c 0.16, MeOH); IR (thin film): \( \gamma_{max} 3436 \) cm-1; 1H NMR see Table 1; 13C NMR see Table 2; HRESIMS (+): m/z 585.3765 [M+Na]+ (calcd for C33H54NaO7, 585.3762, \( \Delta 0.51 \) ppm).

3.7. Acanthifolioside C (3)

\((E)-15\beta\)-O-((β-D-xylopyranosyl)cholesta-5,22-diene-3β,16β-diol. White amorphous solid; [α]D20 -25.0 (c 0.14, MeOH); IR (thin film): \( \gamma_{max} 3422 \) cm-1; 1H NMR see Table 1; 13C NMR see Table 2; HRESIMS (+): m/z 571.3613 [M+Na]+ (calcd for C35H56NaO7, 571.3605, \( \Delta 1.4 \) ppm).

3.8. Acanthifolioside D (4)

16β-O-(α-L-rhamnopyranosyl)-5α-poriferastane-3β,15β,23S-triol. White amorphous solid; [α]D20 -15.6 (c 0.13, MeOH); IR (thin film): \( \gamma_{max} 3420 \) cm-1; 1H NMR see Table 1; 13C NMR see Table 2; HRESIMS (+): m/z 633.4341 [M+Na]+ (calcd for C39H58NaO8, 633.4347, \( \Delta -0.95 \) ppm).

3.9. Acanthifolioside E (5)
16β-O-(α-L-rhamnopyranosyl)poriferast-5-ene-3β,15β,23S-triol. White amorphous solid; [α]D<sub>20</sub> -30.0 (c 0.07 MeOH); IR (thin film): \( \gamma_{\text{max}} \) 3430 cm<sup>-1</sup>; 1H NMR see Table 1; 13C NMR see Table 2; HRESIMS (+): m/z 631.4180 [M+Na]<sup>+</sup> (caled for C<sub>35</sub>H<sub>69</sub>NaO<sub>26</sub>, 631.4171, \( \Delta 1.43 \) ppm).

3.10. Acanthifolioside F (6)

16β-O-[β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosylxoyuronoyric acid]-5α-poriferastane-3β,15β,23S-triol. White amorphous solid; [α]D<sub>20</sub> -20.7 (c 0.15 MeOH); IR (thin film): \( \gamma_{\text{max}} \) 3420 cm<sup>-1</sup>; 1H NMR see Table 1; HRESIMS (+): m/z 971.5198 [M+Na]<sup>+</sup> (caled for C<sub>43</sub>H<sub>69</sub>NaO<sub>30</sub>, 971.5186, \( \Delta 1.24 \) ppm).

3.11. Methyl ester of acanthifolioside F (7)

White amorphous solid; [α]D<sub>20</sub> -19.2 (c 0.13, MeOH); 1H NMR (500 MHz, CD<sub>3</sub>OD) for the uronic residue: δ 4.60 (d, J = 7.6 Hz, H-1), 3.43 (t, J = 7.5 Hz, H-2'), 3.56 (m, H-3' and H-4'), 3.81 (d, J = 9.5 Hz, H-5'), 3.76 (s, CH<sub>3</sub>O-); 13C NMR (125 MHz, CD<sub>3</sub>OD) for the uronic residue: δ 101.8 (C-1'), 112.5 (C-2'), 120.8 (C-3'), 116.5 (C-4'), 171.9 (C-5'), 52.8 (CH<sub>3</sub>O-); HRESIMS (+): m/z 985.5386 [M+Na]<sup>+</sup> (caled for C<sub>43</sub>H<sub>69</sub>NaO<sub>30</sub>, 985.5421, \( \Delta 3.55 \) ppm).

3.12. Methanalysis of acanthifoliosides

0.30 mg of compounds 2, 4 and 6 was dissolved in a HCl (7 N, 1.0 mL)-MeOH solution (Supelco, USA) and heated at 75 °C for 4 h. The reaction mixture was neutralized with NaHCO<sub>3</sub>, evaporated to dryness and then partitioned between CHCl<sub>3</sub> and H<sub>2</sub>O. The H<sub>2</sub>O layer was dried under reduced pressure to afford a mixture of methyl glycosides.

3.13. Derivatization of the Hydrolysate for GC Analysis

The methanalysis products were dissolved in a mixture of dry CH<sub>2</sub>Cl<sub>2</sub>/pyridine (1:1) and an excess of acetic anhydride was added. The reaction was stirred at 25 °C for 8 h. The mixture was then dried and dissolved in AcOEt for GC analysis.

3.14. Chiral GC Analysis

GC analysis was carried out on a Chirasil- L-Val Alltech capillary column (25 m x 0.25 mm, i.d.), using a Hewlett Packard Mass Selective Detector 5972 series. A temperature gradient system was used for the oven, starting at 100 °C for 3 min and increasing up to 200 °C at a rate of 10 °C/min. Peaks of the hydrolysate of pandarosides and sugar standards were detected at 15.3 min (D-glc), 13.2 min (D-xyl), 12.1 min (D-glcUA) and 11.5 min (L-rha). Because some retention times fluctuated, the identity of the enantiomers was confirmed by injection of a mixture of the sample and standards acetylated using the same protocol.

3.15. Antiprotozoal assays

Antimalarial activity against P. falciparum. In vitro parasite growth inhibition was assessed by a modified of [3H]-hypoxanthine incorporation assay using the chloroquine- and pyrimethamine-resistant K1 strain and the standard drug chloroquine. Briefly, compounds were dissolved in 100% DMSO and 2-fold dilution series of the compounds prepared in assay medium (RPMI 1640 supplemented with 5% AlbuMax II, 0.2% w/v glucose, 0.03% L-glutamine) added to each well of microritser plates. Parasite cultures (50 µl) were added to each well reaching a final volume of 100 µl per well (final DMSO concentration \( \leq 0.25% \)). Peaks of the hydrolysate of pandarosides and sugar standards were detected at 15.3 min (D-glc), 13.2 min (D-xyl), 12.1 min (D-glcUA) and 11.5 min (L-rha). Because some retention times fluctuated, the identity of the enantiomers was confirmed by injection of a mixture of the sample and standards acetylated using the same protocol.

Trypanocidal activity against T. brucei rhodesiense was tested using the Trypanosoma brucei STIB 900 strain parasite and the standard drug, melarsoprol, were used for the assay. Minimum Essential Medium (50 µl) supplemented with 25 mM HEPES, 1g/l additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well microtitre plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h the medium was removed and replaced by 100 µl fresh medium with or without a serial drug dilution of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml were prepared. Then 10<sup>6</sup> bloodstream forms of T. b. rhodesiense STIB 900 in 50 µl was added to each well and the plate incubated at 37 °C under a 5% CO<sub>2</sub> atmosphere for 72 h. 10 µl of a resazurin solution (12.5 mg resazurin dissolved in 100 ml double-distilled water) was then added to each well and incubation continued for a further 2-4 h. Then the plates were read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

Trypanocidal activity against Trypanosoma cruzi. Rat skeletal myoblasts (L6 cells) were seeded in 96-well microtitre plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 48 h, the medium was removed and replaced by 100 µl per well containing 5000 trypomastigote forms of T. cruzi Tulahuen strain C24 containing the β-galactosidase (Lac Z) gene.\(^{40}\) After 48 h, the medium was replaced from the wells and replaced by 100 µl fresh medium with or without a serial drug dilution of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml. After 96 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/Nonidet (50 µl) was added to all wells. A color reaction developed within 2-6 h and read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices), which calculated IC<sub>50</sub> values.

Leishmanial activity against Leishmania donovani. Amastigotes of L. donovani strain MHOM/ET/67/L82 were grown in axenic culture at 37 °C in SM medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO<sub>2</sub> in air. One hundred µl of culture medium with 10<sup>6</sup> amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtitre plates. Serial drug dilutions covering a range from 90 to 0.123 µg/ml were prepared. After 72 h of incubation the
plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µl of a resazurin solution was then added to each well and the plates incubated for another 2 h. Then the plates were read in a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro. Decrease of fluorescence (= inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC₅₀ values were calculated.

3.16. Cytotoxicity against L6 cells.

Assays were performed in 96-well microtiter plates, each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4 x 10⁵ L6 cells. Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml were prepared. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Alamar Blue (10 µl, 12.5 mg resazurin dissolved in 100 ml double-distilled water) was then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the microplate reader software Softmax Pro. Podophyllotoxin was the standard drug used.

Acknowledgements

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Supplementary data

Separation and purification details including HPLC profiles, ¹H, ¹³C, 2D NMR spectra for compounds 1-7, experimental and calculated CD spectra of 1.

References and notes