Antiarol Cinnamate and Africanoside, a Cinnamoyl Triterpene and a Hydroperoxy-cardenolide from the Stem Bark of *Antiaris africana*

Authors Bertin Vouffo[1,2], Etienne Dongo[2], Petrea Facey[1], Andrea Thorn[3], George Sheldrick[3], Armin Maier[4], Heinz Herbert Fiebig[4], Hartmut Laatsch[1]

Affiliations
[1] Department of Organic and Biomolecular Chemistry, University of Göttingen, Göttingen, Germany
[2] Department of Organic Chemistry, Faculty of Science, University of Yaounde I, Yaounde, Cameroon
[3] Department of Inorganic Chemistry, University of Göttingen, Göttingen, Germany
[4] Oncotest GmbH, Freiburg, Germany

Key words
- *Antiaris africana*
- Moraceae
- antiarol cinnamate
- africanoside
- sterol hydroperoxide
- periplogenin

Abstract

From the methanol extract of the stem bark of the African tree *Antiaris africana* Engler, two new bioactive metabolites were isolated, namely, the α-amyrin derivative 1*β*,11α-dihydroxy-3*β*-cinnamoyl-α-amyrin (antiarol cinnamate, **1**) and a cardiac glycoside, 3*β*-*O*-(α-L-rhamnopyranosyl)-14*β*-hydroperoxy-5*β*-hydroxy-19-oxo-17*β*-card-20(22)-enolide (africanoside, **2a**), together with the known compounds *β*-amyrin and its acetate, *β*-sitosterol and its 3-*O*-*β*-D-glucopyranoside, friedelin, ursolic and oleanolic acid, 19-norperiplogenin, strophanthidol, strophanthidinic acid, periplogenin (**3a**), 3-epiperiplogenin, strophanthidin (**3b**) and 3,3′-dimethoxy-4′-*O*-*β*-D-xylopyronosyl-ellagic acid. Their structures were established on the basis of their spectroscopic data and by chemical methods, while **3a** was additionally confirmed by X-ray crystal structure analysis. The aglycone moiety possessing a hydroperoxy group was found for the first time in cardenolides. Compounds **1** and **2a** showed no activity against bacteria, fungi, and microalgae; however, the crude extract exhibited a high toxicity against *Artemia salina* and a selective antitumor activity against human tumor cell lines. Africanoside (**2a**) effected a concentration-dependent inhibition of tumor cell growth with a mean IC_{50} value of 5.3 nM.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Antiaris africana Engler is a tree belonging to the Moraceae family. It is usually about 15–20 meters high, but can grow sometimes up to 40 meters, has a white latex and alternate dissymmetric leaves [1,2]. The wood is used in some regions of Africa as timber [2,3], and the bark extract is used in traditional medicine for the treatment of chest pains [3]. Leaf decoctions are applied in the treatment of syphilis [2], and the latex is a purgative agent. It is also used to treat sore throat and leprosy [2]. According to previous phytochemical studies, the plant contains sterols and terpenoids such as butyrospermol, α-amyrin and lupenyl acetates, α-amyrin and lupenyl cinnamates as well as betaines, tryptophane, *β*-phenylalanine and cardiac glycosides [3,4].

In our research program to study species of the genus *Antiaris*, we isolated two new compounds, antiarol cinnamate (1*β*,11α-dihydroxy-3*β*-cinnamoyl-α-amyrin, **1**) and africanoside [3*β*-*O*-(α-L-rhamnopyranosyl)-14*β*-hydroperoxy-5*β*-hydroxy-19-oxo-17*β*-card-20(22)-enolide, **2a**] from the methanol extract of the stem bark of *Antiaris africana*, together with 14 known compounds: *β*-amyrin and its acetate, *β*-sitosterol and its 3-*O*-*β*-D-glucopyranoside, friedelin, ursolic and oleanolic acid, strophanthidol, strophanthidinic acid, strophanthidin, periplogenin, 3-epiperiplogenin, 19-*nor*cardenolide, and 3,3′-dimethoxy-4′-*O*-*β*-D-xylopyronosyl-ellagic acid. Their structures were established using 1D, 2D NMR spectra, EI and ESI mass spectra and by chemical derivatization.

Materials and Methods

General

The optical rotations were measured on a Perkin-Elmer polarimeter (model 241). IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer with KBr pellets. NMR spectra were measured on Varian Inova 600 (599.740 MHz), Varian Inova 500 (499.879 MHz) and Varian Unity 300 (300.145 MHz) spectrometers. ESI mass spec-

received October 28, 2009
revised April 9, 2010
accepted April 20, 2010

Bibliography
DOI http://dx.doi.org/10.1055/s-0030-1249958
Published online June 8, 2010
Planta Med 2010; 76: 1717–1723 © Georg Thieme Verlag KG Stuttgart · New York ·
ISSN 0032-0943

Correspondence
Prof. Dr. Hartmut Laatsch
Institute for Organic and Biomolecular Chemistry
Georg-August-University Göttingen
Tammannstrasse 2
37077 Göttingen
Germany
Phone: + 49 551 39 32 11
Fax: + 49 551 39 96 60
hlaatsc@gwdg.de

tra were recorded on a Quattro Triple Quadrupole mass spectrometer, with a Finnigan TSQ 7000 with nano-ESI API ion source. EI-MS was performed on a Finnigan MAT95 (70 eV). High-resolution mass spectra (HR-MS) were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics). Silica gel for flash chromatography: 30–60 µm; J.T. Baker; column chromatography was carried out on MN silica gel 60: 0.05–0.2 mm (230–400 mesh; Macherey-Nagel & Co). Thin-layer chromatography (TLC) was performed on silica gel plates G60, type GF254 (Merck) and DC cards Polygram SIL G/UV_{254} (Macherey-Nagel & Co.) with visualization under UV (254 and 365 nm) and with anisaldehyde/sulphuric acid as spray reagent. Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex; Amersham Biosciences, Ltd. purchased from Sigma-Aldrich Chemie).

Plant material

The plant *Antiaris africana* Engler (Moraceae) was collected from Mont Eloundem (Yaounde) in the Central Province of the Republic of Cameroon, in May 2006. Mr. Victor Nana of the National Herbarium of Cameroon identified the plant. A voucher specimen (N°5925 SRFCam) is deposited at the National Herbarium, Yaounde, Cameroon.

Extraction and isolation

The air-dried and powdered stem bark (8 kg) of *Antiaris africana* Engler was macerated with MeOH at room temperature for 72 h (48 h + 24 h; 2 × 15 L each). Removal of the solvent from the extracts under reduced pressure yielded a dark brown residue (100 g). A part (80 g) of the crude methanolic extract was adsorbed on 100 g of silica gel 60 (0.05–0.2 mm, 230–400 mesh) with 0.1 L methanol as solvent and dried under reduced pressure. The brown powder obtained was submitted to gradient flash chromatography on 600 g of silica gel (column 15 × 15 cm, eluents: *n*-hexane/EtOAc, stepwise gradient, 0, 5, 10, 15, 25, 50, and 100% EtOAc), and 75 fractions each of 500 mL were collected and pooled into 8 major fractions by combination on the basis of TLC. Further separation of these fractions was done by repeated column chromatography using silica gel, Sephadex LH-20 or C-18 reversed-phase columns. Fraction 1 (eluted with *n*-hexane) was a mixture of fatty acids and hydrocarbons and was discarded. Fraction 2 (4.1 g) was eluted with *n*-hexane-EtOAc 9 : 1 and was chromatographed on a 3 × 35 cm silica gel column with *n*-hexane-EtOAc 9 : 1 yielding *β*-amyrin acetate (31 mg), and friedelin (15 mg). Elution with *n*-hexane-EtOAc 85 : 15 gave *β*–sitosterol (4 mg) and *β*-amyrin (8 mg).

The *n*-hexane-EtOAc (8 : 2) eluted fraction 3 (5.1 g) was further separated by silica gel column chromatography (30 × 350 mm, *n*-hexane-ethyl acetate gradient with 15 to 35% EtOAc) to furnish **1** (6 mg). A further subfraction was further purified on Sephadex LH-20 (20 × 750 mm) eluting with CH_2Cl_2-MeOH 3 : 2 to give ursolic acid (8 mg), and oleanolic acid (4 mg). Fractions 4 and 5 were discarded.

From fraction 6 (5.0 g, eluted with *n*-hexane-EtOAc 1 : 1), a precipitate was obtained, which was recrystallized from MeOH to give *β*-sitosterol 3-*O*-*β*-D-glucopyranoside (16 mg). We isolated additionally 19-norperiplogenin (7 mg), periplogenin (**3a**, 35 mg), and strophanthidin (**3b**, 6 mg) when the filtrate was chromatographed on silica gel (25 × 500 mm, *n*-hexane-EtOAc gradient 1 : 1 increasing to 25 : 75). Strophanthidin was further purified on Sephadex LH-20 (20 × 750 mm) eluting with CH_2Cl_2-MeOH 1 : 1.

Further purification of fraction 7 (eluted with *n*-hexane-EtOAc 4 : 6) on silica gel (25 × 300 mm, *n*-hexane-EtOAc gradient 1 : 1 increasing to 2 : 8) gave strophantidol (13 mg), and 3-*epi*-periplogenin (7 mg); the latter was further purified on Sephadex LH-20 (20 × 750 mm, CH_2Cl_2-MeOH 1 : 1).

The last fraction (eluted with *n*-hexane-EtOAc 2.5 : 7.5) gave a white precipitate of **2a** (75 mg). Chromatography of the filtrate on a 15 × 250 mm silica gel column with *n*-hexane-EtOAc 25 : 75 gave strophanthidinic acid (9 mg), and 3,3′-dimethoxy-4′-*O*-*β*-D-xylopyranosyl-ellagic acid (3 mg), which were purified on Sephadex LH-20 (20 × 750 mm) eluting with MeOH.

According to their NMR data, all compounds had a purity of better than 95%, and no further purification steps were performed.

Reduction of africanoside

Africanoside (**2a**, 5 mg) was dissolved in 2 mL MeOH and 2 mg of $NaBH_4$ were added. After stirring for 30 minutes at room temperature, 5 mL of water were added and the reduction product was extracted with CH_2Cl_2 (3 × 10 mL). Evaporation under vacuum gave convallatoxin (**2b**) as a white powder (2.4 mg). After passing through Sephadex LH-20, it was chromatographically pure in three different solvent systems.

Isolates

*Antiarol cinnamate [3-O-(E)-cinnamoyl-1β-11α-dihydroxy-α-amyrin, **1**]:* White amorphous powder, R_f: 0.45 (*n*-hexane/EtOAc 75 : 25); $[\alpha]_D^{20}$: + 24.0 (*c* 0.1, $CHCl_3$); IR (KBr): ν_{max} = 3309, 3168, 2917, 1713, 1639, 1171, 980 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) and ^{13}C-NMR ($CDCl_3$, 150 MHz): see **Table 1**; EI-MS (70 eV): *m/z* (%) = 588 (8), 570 (100), 422 (15), 389 (8), 255 (8), 217 (18), 131 (10); HR-ESI-MS (pos. ion mode): *m/z* = 611.40691 $[M + Na]^+$ (calcd. for $C_{39}H_{56}O_4Na$: 611.40707).

*Africanoside [3β-O-(α-L-rhamnopyranosyl)-14β-hydroperoxy-5β-hydroxy-19-oxo-17β-card-20(22)-enolide, **2a**]:* White amorphous powder, R_f: 0.62 (EtOAc/MeOH 95 : 5); $[\alpha]_D^{20}$: + 1.0 (*c* 0.1, $CHCl_3$); IR (KBr): ν_{max} = 3422, 2936, 1738, 1707, 1622, 1034, 980 cm^{-1}; ^{1}H-NMR (600 MHz, acetone-d_6) and ^{13}C-NMR (125 MHz, acetone-d_6): see **Table 2**; ESI-MS (pos. ion mode): *m/z* = 589 $[M + Na]^+$, 567 $[M + H]^+$; ESI MS (neg. ion mode): *m/z* = 565 $[M - H]^-$; HR-ESI-MS (pos. ion mode): *m/z* = 567.27985 $[M + H]^+$ (calcd. for $C_{29}H_{43}O_{11}$: 567.27998).

*Convallatoxin [3β-O-(α-L-rhamnopyranosyl)-5β,14β-dihydroxy-19-oxo-17β-card-20(22)-enolide, **2b**]:* White amorphous powder, R_f: 0.58 (EtOAc/MeOH 95 : 5); ^{1}H-NMR (300 MHz, pyridine-d_5, acetone-d_6) and ^{13}C-NMR (125 MHz, pyridine-d_5, acetone-d_6) see **Table 2**; ESI-MS (pos. ion mode): *m/z* = 573 $[M + Na]^+$; ESI-MS (neg. ion mode): *m/z* = 549 $[M - H]^-$, 595 $[M + HCOO]^-$; HR-ESI-MS (pos. ion mode): *m/z* = 551.28503 $[M + H]^+$ (calcd. for $C_{29}H_{43}O_{10}$: 551.28507).

Antimicrobial assay

Agar diffusion tests were performed using the usual protocol [5] with *Bacillus subtilis* (ATCC 6051), *Escherichia coli, Staphylococcus aureus, Streptomyces viridochromogenes* (Tü 57), *Candida albicans,* and *Mucor miehei* (Tü 284) as well as three microalgae: *Chlorella vulgaris, Chlorella sorokiniana* and *Scenedesmus subspicatus*.

The crude extract was dissolved in $CHCl_3$/10% MeOH (concentration 50 mg/mL), in which the paper disks (8 mm diameter) were dipped, dried under sterile conditions (flow box) and placed on agar plates inoculated with the test strains. The plates were incubated at 37 °C for bacteria (12 hours), 27 °C for fungi (24 hours),

Position	δ_C	δ_H	Position	δ_C	δ_H
1	76.6	3.62 dd (4.5, 11.5)	20	39.3	0.91[#c]
2	32.2	1.82 m, 1.94 m	21	31.0	1.24 m, 1.39 m
3	77.6	4.66 dd (4.6, 12.1)	22	41.2	1.29 m, 1.42 m
4	38.1		23	16.3	0.93 s
5	52.3	0.79 m	24	28.0	0.89 s
6	17.7	1.56 m, 1.64 m	25	13.3	1.12 s
7	33.0	1.29 m, 1.49 m	26	17.9	1.04 s
8	41.9		27	23.1	1.16 s
9	56.5	1.67 d (8.1)	28	28.6	0.79 s
10	44.2		29	17.7	0.85 d (6.3)
11	67.5	4.28 dd (3.5, 8.1)	30	21.3	0.91 brs
12	126.6	5.22 d (3.7)	1′	166.6	
13	144.5		2′	118.6	
14	43.7		3′	144.5	
15	26.9	0.99 m, 1.74 m	4′	134.5	
16	27.8	0.92 m, 2.02 m	5′	128.8	
17	33.5		6′	128.1	
18	57.8	1.38[#]	7′	130.2	
19	39.3	1.31 m[c]			

Table 1 ^{13}C-NMR data (150 MHz) and ^{1}H-NMR data (600 MHz) for antiarol cinnamate (**1**) in $CDCl_3$.

[#] Signals not interpretable; [c] Interchangeable; *J* in Hz, shifts as *δ* values

and 24–26 °C under daylight for microalgae (96 hours). The diameter of the inhibition zones was then measured. Actinomycin D for bacteria (from our own sample collection), amphotericin B for fungi (Sigma Aldrich), and staurosporine for algae (from our own sample collection) were used as positive controls at 40 µg/platelet.

Brine shrimp microwell cytotoxicity assay

Cytotoxicity of the crude extracts was determined with brine shrimps (*Artemia salina*) using a microwell cytotoxicity assay [6]. The mortality rate was determined according to Duracková et al. [7]. Actinomycin D as a positive control showed a mortality rate of 100% at 10 µg/mL.

Human tumor cell line cytotoxicity assay

A modified propidium iodide assay was used to examine the cytotoxic activity of the compounds against human tumor cell lines. The test procedure was described elsewhere [8]. Cell lines tested were derived from patient tumors, engrafted as a subcutaneously growing tumor in NMRI nu/nu mice, or obtained from American Type Culture Collection, National Cancer Institute, Bethesda, MD, USA, or Deutsche Sammlung von Mikroorganismen und Zellkulturen. Doxorubicin hydrochloride (Medac) was used as a positive control, and was tested in concentrations up to 5.2 µM.

X-ray structure determination of periplogenin (3a)

Colorless crystals of **3a** were obtained from a solution of hexane and ethyl acetate (1 : 1). A suitable crystal (0.20 × 0.07 × 0.05 mm^3) was mounted at 100 K on a Bruker Smart 6000 CCD diffractometer equipped with a rotating anode generator and Incoatec Helios optics using Cu-K_α radiation (λ = 1.54178 Å). A total of 50 430 reflections were measured, of which 3250 were independent.

The asymmetric unit consisted of 1.9 periplogenin molecules with an impurity of strophanthidin (**3b**), which replaced one of the two periplogenin molecules in 10% of the unit cells. It also contained one molecule of crystal water. The crystal investigated belonged to the monoclinic space group $P2_1$.

The structure was determined by direct methods. All atoms except hydrogens were refined anisotropically by full-matrix least-squares methods on F^2 using SHELXL97 [9] to give a final R-factor of 0.0281. All nonhydrogen atoms were visible in the difference map and refined using a riding model, except for H_2O. Those hydrogens could be seen in the electron density and were refined accordingly. Several disordered groups were modelled and their occupancy refined and fixed in later stages of the refinement. The Flack x parameter [10] of 0.06(11) confirmed the absolute configuration of periplogenin (**3a**).

Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre: CCDC 749301 contains the supplementary crystallographic data. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax + 44-(0)1223–336 033 or email: deposit@ccdc.cam.ac.uk).

Supporting information

^{1}H-NMR and ^{13}C-NMR spectra of compounds **1, 2a, 3a** and 3′-epi-periplogenin, crystal data and structure refinement for **3a** and bond lengths [Å] and angles [°] in **3a** are available as Supporting Information.

Results and Discussion

The methanol extract of the stem bark of *Antiaris africana* Engler was subjected to silica gel and Sephadex LH-20 column chromatography to afford 16 compounds. Antiarol cinnamate (**1**, **Fig. 1**) was obtained as a white amorphous powder that gave a positive response to the Liebermann-Buchard reaction. Its (+)-HR-ESI mass spectrum exhibited an $[M + Na]^+$ *quasi*-molecular ion peak at *m/z* = 611.40691, ascribable to a molecular formula $C_{39}H_{56}O_4Na$ (calculated: 611.40707), calculating for 12 double bond equivalents in the molecule. Its IR spectrum showed a strong absorption band at 1713 cm^{-1}, indicating an *α,β*-unsaturated ester. Bands at 3309 and 1171 cm^{-1} pointed to the presence of a secondary alcohol in the molecule.

The ^{13}C-NMR spectrum showed 39 signals, which were resolved by an APT experiment into 8 methyls, 16 methines, 7 methylenes and 8 quaternary carbons. Nine of these signals were assigned to

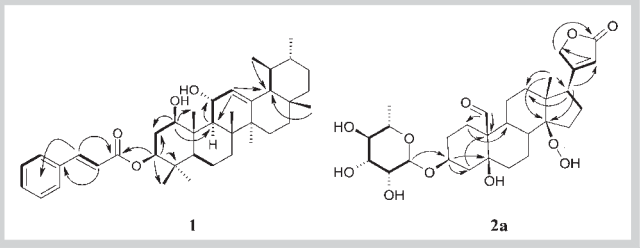

Fig. 1 Chemical structures of antiarol cinnamate (**1**); africanoside (**2a**) and convallatoxin (**2b**); periplogenin (**3a**) and strophanthidin (**3b**).

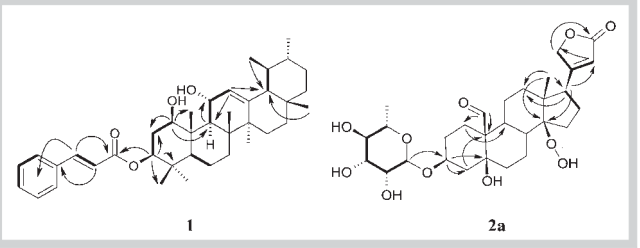

Fig. 2 Selected COSY (–) and HMBC (→) correlations of antiarol cinnamate (**1**) and africanoside (**2a**).

Position	2a		2b		
	δ_C	δ_H	δ_C[b]	δ_C[c]	δ_C[d]
1	24.9	1.66 m, 2.00 m	24.9	24.3	25.0
2	25.6	1.67 m, 1.72 m	25.7	26.0	25.7
3	74.2	4.06 m	74.2	73.8	73.83
4	35.9	1.56 m, 2.15 m	36.0	36.3	35.6
5	74.3		74.3	73.7	73.77
6	37.0	1.56 m, 2.05 m	37.0	36.5	37.1
7	18.7	2.16 m	18.7	18.1	18.7
8	42.4	1.92 m	42.4	42.4	42.1
9	39.7	1.72 m	39.8	39.6	39.7
10	55.6		55.6	55.6	55.4
11	22.9	1.45 m	22.9	22.9	22.8
12	40.0	1.44 m	40.0	40.1	39.8
13	51.3		51.3	51.3	50.0
14	85.2		85.2	85.2	84.5
15	32.5	1.12 m, 1.65 m	32.5	32.8	32.4
16	27.4	1.86 m, 2.10 m	27.5	27.6	27.4
17	50.1	2.81 m	50.1	50.3	51.2
18	16.0	0.86 s	16.0	16.3	16.2
19	208.4	10.08 s	208.3	208.1	208.2
20	176.1		176.0	176.1	175.6
21	73.9	4.90 dd, (18.1, 1.7)	73.9	74.0	73.2
22	117.8	5.85 brs	117.8	117.7	117.8
23	174.4		174.3	175.8	174.4
1′	100.4	4.85 brs	100.4	100.4	100.7
2′	72.1	3.77 brs	72.2	72.1	72.9
3′	72.4	3.59 m[a]	72.5	72.4	72.6
4′	73.4	3.39 t (9.3)	73.5	73.7	74.0
5′	70.1	3.63 m[a]	70.1	70.1	70.8
6′	18.1	1.20 d (6.2)	18.1	18.1	18.9

Table 2 ^{13}C-NMR data (125 MHz) and ^{1}H-NMR data (300 MHz) for africanoside (**2a**) in acetone-d_6 and for convallatoxin (**2b**) in acetone-d_6 and pyridine-d_5.

[a] Interchangeable; *J* in Hz, shifts as δ values; [b] Reduction product and [c] commercial **2b** in acetone-d_6; [d] Commercial **2b** sample in pyridine-d_5

a *trans*-cinnamoyl fragment, which was confirmed by comparison of its ^{13}C- (**Table 1**) and ^{1}H-NMR data with those in the literature [11, 12]. The 30 remaining carbon signals were indicative for a triterpenoid which in its ^{13}C-NMR spectrum exhibited resonances of 8 methyls, 3 oxygenated methines (δ = 77.6, 76.6 and 67.5) and 2 olefinic carbons (δ = 144.5 and 126.6); the ^{1}H-NMR spectrum confirmed these assignments. These data (**Table 1**) indicated that **1** is an urs-12-ene-1,3,11-triol derivative [13], which was confirmed by COSY correlations, mainly by cross signals between the protons H-12 and H-11, H-11 and H-9, H-3 and H-1 with H-2 and H-2′ (**Fig. 2**).

The position of the cinnamoyl group at C-3 of the triterpene moiety was determined easily using HMBC spectra, which demonstrated a correlation between H-3 and the cinnamoyl CO group. This spectrum was further helpful to confirm the positions of hydroxy groups at C-1 and C-11, and the skeleton of the molecule (**Fig. 2**). The large axial-axial coupling constant ($J_{9,11}$ = 8.1 Hz) of H-11 indicated the equatorial 11α-position of the hydroxy group at C-11. Related 11α-hydroxy-ursane triterpenes have been reported from taxonomically different plants [14, 15]. The 1D NOE spectrum of **1** showed after irradiation of H-1, an NOE for H-3, H-9 and H-5, suggesting the *β*-orientation of the hydroxy group at C-1; this was further confirmed by irradiation of H-3, which in turn showed an NOE for H-1, H-9 and H-5 (**Fig. 2**). Thus antiarol cinnamate (**1**) was deduced to be 3*β*-*O*-(*E*)-cinnamoyl-1*β*-11α-dihydroxyurs-12-ene.

Africanoside (**2a**, **Fig. 1**) was obtained as a white powder, which showed in its (+)-HR-ESI mass spectrum a *quasi*-molecular ion peak at *m/z* = 567.27985 (calculated: 567.27998 for $[M + H]^+$) corresponding to the molecular formula $C_{29}H_{43}O_{11}$. An IR band at

1738 cm^{-1} was in accordance with an α,β-unsaturated-γ-lactone, and the signal at 1707 cm^{-1} indicated the presence of an aldehyde group. ^{1}H- and ^{13}C-NMR spectra and 2D NMR data showed clearly that compound **2a** is the α-l-rhamnopyranosyl derivative [16] of a cardenolide (α,β-unsaturated steroid γ-lactone) [18]. For the sugar moiety, the anomeric proton signal appeared at $\delta_H = 4.85$ (broad singlet, 1H) and $\delta_C = 100.4$. The methyl signal of a 6-deoxy sugar appeared at $\delta_H = 1.20$ (d; $J = 6.2$ Hz) and $\delta_C = 18.7$. Its broadband decoupled ^{13}C-NMR spectrum showed 29 carbon signals with 2 methyls, 11 methine, 10 methylene and 6 quaternary carbons according to its APT spectrum, which further confirmed that **2a** is a cardiac glycoside.

These data were within the experimental error limit identical with those of convallatoxin (**2b**) (**Table 2**) [17] and related glycosides [18], but the additional oxygen atom cannot be connected with carbon and must therefore be part of a hydroperoxide. The presence of intense bands in its IR spectrum at 3422 (OH bond), 1034 (C–O-O bond) and 980 cm^{-1} (O-O bond) confirmed this assumption. The presence of a hydroperoxy group in this compound was unambiguously proven by an exchangeable hydroxy proton signal at $\delta = 4.00$ in the ^{1}H-NMR spectrum, and by reduction using $NaBH_4$. In fact, the (+)-HR-ESI mass spectrum of the reduction product showed a *pseudo*-molecular ion peak at $m/z = 551.2850$ ($[M + H]^+$), indicative for the molecular formula $C_{29}H_{43}O_{10}$. Spectroscopic comparison (**Table 2**) with a commercial convallatoxin (**2b**) sample proved the identity both in ace-

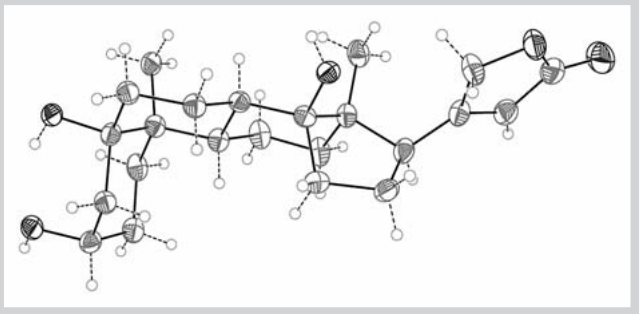

Fig. 3 Crystal structure of periplogenin (**3a**). The structure of **3a** as determined by X-ray crystallographic analysis. Only one of two molecules in the asymmetric unit is shown, with the disordered **3b** removed for clarity. The atoms are shown as ORTEP plots with 50% probability displacement ellipsoids.

Table 3 Cytotoxic activity of africanoside (**2a**) against tumor cell lines in a monolayer cytotoxicity assay.

		IC_{50} (nM)	
Tumor type	**Cell line**	**2a**	**Doxorubicin**
Bladder	BXF 1218 L	5.1	16.4
	BXF 1352 L	6.0	2.6
	BXF T24	5.3	107
Colon	CXF 269 L	6.3	20.8
	CXF HCT116	5.6	24.3
	CXF HT29	6.2	24.3
	CXF RKO	4.6	1.2
Stomach	GXF 251 L	6.5	19.7
Head & neck	HNXF CAL27	8.8	4.5
Lung	LXFA 289 L	1.9	81.2
	LXFA 526 L	6.5	5.2
	LXFA 629 L	1.2	24.9
	LXFL 1121 L	0.7	11.6
	LXFL 529 L	1.1	< 0.5
	LXFL H460	4.9	2.7
Breast	MAXF 401NL	0.9	7.0
	MAXF MCF7	7.6	25.2
Melanoma	MEXF 1341 L	> 1763	9.0
	MEXF 276 L	9.2	2.7
	MEXF 462NL	1.8	4.8
Ovary	OVXF 899 L	6.9	51.7
	OVXF OVCAR3	5.8	5.8
Pancreas	PAXF 1657 L	6.9	2.1
	PAXF 546 L	16.4	51.7
	PAXF PANC1	1.1	6.4
Prostate	PRXF 22RV1	4.8	< 0.5
	PRXF DU145	4.6	1.3
	PRXF LNCAP	6.2	< 0.5
	PRXF PC3M	5.3	9.8
Mesothelioma	PXF 1752 L	7.2	< 0.5
	PXF 698 L	4.9	4.0
Kidney	RXF 1781 L	5.6	59.1
	RXF 393NL	6.2	12.4
	RXF 486 L	4.4	133
Sarcoma	SXF SAOS2	7.8	5.2
	SXF TE671	6.3	< 0.5
Uterus	UXF 1138 L	5.6	1.2
	Mean	5.3	6.9

tone and pyridine [17]. The position of the peroxide group was unambiguously determined due to the fact that the hydroperoxy carbon appears at δ_C = 81–86 [19–22]; therefore the peroxy group could neither be on the sugar moiety nor on the carbon C-5 [19] but must be connected with C-14. It should be stated that the shift of C-14 in **2a** is in the same range as for 14-hydroxycardenolides [18]. This unusual downfield shift of C-14 could explain why the ^{13}C-NMR data of **2a** are identical to those of the reduction product **2b**.

Some COSY and HMBC correlations of **2a** are shown in ▶ **Fig. 2**. The *O*-glycosidic connection of the sugar moiety with C-3 was determined by HMBC correlations between the anomeric proton H-1′ (δ_H = 4.85) and C-3 (δ_C = 74.2) and the chemical shift of the anomeric carbon (δ_C = 100.4). Further important correlations are observed between H-3 and C-5, between H-18, H-17 and the hydroperoxy carbon C-14, and between H-19 and C-10. Thus africanoside (**2a**) was assigned to be the 14-hydroperoxy derivative of convallatoxin (3-*O*-*α*-L-rhamnopyranosylstrophanthidin, **2b**).

Further compounds were identified as *β*-amyrin and its acetate [23], *β*-sitosterol [24] and its 3-*O*-*β*-D-glucopyranoside [15,24], friedelin [25], ursolic [26] and oleanolic acids [26], strophanthidol, strophanthidinic acid and strophanthidin (**3b**) [18], periplogenin (**3a**) and 3-*epi*-periplogenin [18], 19-norperiplogenin [27] and 3,3′-dimethoxy-4′-*O*-*β*-D-xylopyranosyl-ellagic acid [28].

Periplogenin (**3a**, ▶ **Fig. 1**) is closely related to africanoside (**2a**, ▶ **Fig. 1**). Crystals of **3a** suitable for X-ray structure determination could be obtained. The crystal structure is similar to that of strophantidin (**3b**) [29] and was refined to a final R-factor of 0.0281. The asymmetric unit consisted of 1.9 periplogenin molecules with an impurity of strophanthidin (**3b**), which replaced one of the two **3a** molecules in 10% of the unit cells. This is in accordance with the NMR spectra, which showed signals of a second cardenolide. One of the two steroid molecules in the asymmetric unit is disordered; the other one is not, due to different hydrogen bond formation in the crystal lattice. A similar effect has been described for strophantidin (**3b**) by Hoehne and Seidel [30]. The Flack x parameter [10] of 0.06(11) confirmed the absolute configuration of **3b** as shown in ▶ **Fig. 3**, and also that of the contaminating **3a**.

The crude extract and compounds **1** and **2a** were screened for antibacterial, antimicroalgal and antifungal activities using the agar diffusion method. At a concentration of 500 µg/disk, no significant activity was observed against the organisms tested.

However when subjected to the brine shrimp cytotoxicity assay, the crude extract exhibited 100% mortality against *Artemia salina* at 100 µg/mL, while compounds **1** and **2a** showed 7% and 29% mortality at 10 µg/mL, respectively. The crude extract showed selective antitumor activity against a panel of 6 human tumor cell lines with an IC_{70} value of 0.014 µg/mL, and above-average activity towards 67% of the cell lines as tested.

The cytotoxic effect of africanoside (**2a**) was determined in a monolayer cell proliferation assay using a panel of 37 human tumor cell lines, reflecting 14 different tumor histo types. Africanoside (**2a**) effected concentration-dependent inhibition of tumor cell growth with a mean IC_{50} value of 5.3 nM, which was in the same range as the positive control doxorubicin (mean IC_{50} = 6.9 nM in the same cell line panel) (▶ **Table 3**). Compound **2a** displayed significant *in vitro* tumor cell selectivity towards 7 of the 37 tested tumor cell lines (using an individual IC_{50} value < 1/2 of the mean IC_{50} value as the threshold). Above average activity was pronounced in tumor cell lines of lung cancer (LXFA 289 L, LXFA 629 L, LXFL 1121 L, LXFL 529 L; IC_{50} values ranging from 0.7 nM to 1.9 nM), mammary cancer (MAXF 401NL, IC_{50} = 0.9 nM), melanoma (MEXF 462NL, IC_{50} = 1.8 nM), and pancreatic cancer (PAXF PANC1, IC_{50} = 1.1 nM) (▶ **Table 3**).

Acknowledgements

We thank R. Machinek for the NMR measurements and Dr. H. Frauendorf for the mass spectra. B. Vouffo is thankful for a research fellowship of the German Academic Exchange Service (DAAD).

References

1 *Berg CC, Hijman MEE, Weerdenburg JCA.* Flore du Cameroun: Moracées. Paris: Muséum National d'Histoire Naturelle, Laboratoire de Phanérogamie; 1985: 104–110
2 *Berhaut J.* Flore illustrée du Sénégal: Dicotylédones, Tome VI: Linacées à Nymphéacées. Dakar: Ministère du Développement Rural et de l'Hydraulique; 1979: 402–405
3 *Okogun IJ, Spiff AI, Ekong DEU.* Triterpenoids and betaines from the latex and bark of *Antiaris africana.* Phytochemistry 1976; 15: 826–827
4 *Esuoso KO, Lutz H, Kutubuddin M.* Unsaponifiable lipid constituents of some underutilized tropical seed oils. J Agric Food Chem 2000; 48: 231–234
5 *Biabani MAF, Baake M, Lovisetto B, Laatsch H, Helmke E, Weyland H.* Anthranilamides: new antimicroalgal active substance from a marine *Streptomyces sp.* J Antibiot 1998; 51: 333–340
6 *Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD.* A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). Planta Med 1993; 59: 250–252
7 *Duracková Z, Betina V, Horniková B, Nemec P.* Toxicity of mycotoxins and other fungal metabolites to *Artemia salina* larvae. Zentralbl Bakteriol Abt II 1977; 132: 294–299
8 *Dengler WA, Schulte J, Berger DP, Mertelsmann R, Fiebig HH.* Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. Anticancer Drugs 1995; 6: 522–532
9 *Sheldrick GM.* A short history of SHELX. Acta Crystallogr 2008; A64: 112–122
10 *Flack HD.* On enantiomorph-polarity estimation. Acta Crystallogr 1983; A39: 876–881
11 *Wood AC, Lee K, Vaisberg JA, Kingston IGD, Neto CC, Hammond BG.* A bioactive spirolactone iridoid and triterpenoids from *Himatanthus sucuuba.* Chem Pharm Bull 2001; 49: 1477–1478
12 *Bhattacharyya J, Barros BC.* Triterpenoids of *Cnidosculus urens.* Phytochemistry 1986; 25: 274–276
13 *Mahato BS, Kundu PA.* ^{13}C NMR spectra of pentacyclic triterpenoids – a compilation and some salient features. Phytochemistry 1994; 37: 1517–1575
14 *Caceras-Castillo D, Mena-Rejon JG, Cedillo-Rivera R, Quijano L.* 21β-Hydroxy-oleanane-type triterpenes from *Hippocartea excelsa.* Phytochemistry 2008; 69: 1057–1064
15 *Topçu G, Ulubelen A.* Triterpenoids from *Salvia kronenburgii.* J Nat Prod 1999; 62: 1605–1608
16 *Agrawal PK.* NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 1992; 31: 3307–3330
17 *Schenk B, Junior P, Wichtl M.* Cannogenol-3-*O*-α-L-rhamnosid und cannogenol-3-*O*-β-D-allomethylosid, zwei neue Cardenolidglykoside aus *Convallaria majalis.* Planta Med 1980; 40: 1–11
18 *Robien W, Kopp B, Schabl D, Schwarz H.* Carbon-13 NMR spectroscopy of cardenolides and bufadienolides. Prog NMR Spectrosc 1987; 19: 131–181
19 *Iida T, Ogawa S, Hosoi K, Makino M, Fujimoto Y, Goto T, Hofmann AF.* Regioselective oxyfunctionalization of unactivated carbons in steroids by a model of cytochrome P-450: osmium porphyrin complex/*tert*-butyl hydroperoxide system. J Org Chem 2007; 72: 823–830
20 *Dang H-S, Davies AG.* Ene reactions of allylically stannylated cholestenes: singlet oxygenation of 7α-triphenylstannylcholest-5-en-3β-ol, and of 7α-triphenylstannyl- and 7α-tributylstannyl-cholest-5-ene-3-one, and the rearrangement of 5α-tributylstannylperoxy-3β-benzoyloxycholest-6-ene and of 7α-tributylstannylperoxy-3β-benzoyloxycholest-5-ene. J Chem Soc [Perkin II] 1992: 1095–1101

21 *Patra A, Chaudhuri SK.* Assignment of carbon-13 nuclear magnetic resonance spectra of some friedelanes. Magn Reson Chem 1987; 25: 95–100
22 *Kuo Y-H, Chiang Y-M.* Six new ursane- and oleanane-type triterpenes from the arial roots of *Ficus microcarpa*. Chem Pharm Bull 2000; 48: 593–596
23 *Knight SA.* Carbon-13 NMR spectra of some tetra- and pentacyclic triterpenoids. Org Magn Reson 1974; 6: 603–611
24 *Kovganko NV, Kashkan ZN, Borisov EV, Batura EV.* ^{13}C NMR spectra of β-sitosterol derivatives with oxidised rings A and B. Chem Nat Comp 1999; 35: 646–649
25 *Ageta H, Arai Y, Suzuki H, Kiyotani T, Kitabayashi M.* NMR spectra of triterpenoids. III. Oleanenes and migrated oleanenes. Chem Pharm Bull 1995; 43: 198–203
26 *Seebacher W, Simic N, Weis R, Saf R, Kunert O.* Complete assignment of ^{1}H and ^{13}C NMR resonances of oleanolic acid, 18-α-oleanolic acid, ursolic acid and their 11-oxo derivatives. Magn Reson Chem 2003; 41: 636–638
27 *Makarevich IF, Dikan LN.* 19-Norcardenolides. Chem Nat Comp 1985; 21: 67–71
28 *Li X-C, Elsohly HN, Hufford CD, Clark AM.* NMR assignments of ellagic acid derivative. Magn Reson Chem 1999; 37: 856–859
29 *Gilardi RD, Flippen JL.* The crystal structure of strophantidin, a cardioactive steroide, $C_{23}H_{23}O_6 \cdot ½H_2O$. Acta Crystallogr 1973; B29: 1842–1848
30 *Hoehne E, Seidel I.* Neubestimmung der Laktonringkonformation in der Kristallstruktur des Strophantidin $C_{23}H_{23}O_6 \cdot ½H_2O$. J Prakt Chem 1978; 320: 151–156