Betulinic Acid: Isolation from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*, Antimalarial Activity, and Crystal Structure of the Benzyl Ester*

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Abstract: The known lupane-type triterpene betulinic acid (**3**) was isolated for the first time from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*. It was found to exhibit moderate to good *in vitro* antimalarial activity against asexual erythrocytic stages of the human malaria parasite *Plasmodium falciparum*. A first X-ray structure analysis succeeded after conversion into its benzyl ester **4**.

Key words: *Triphyophyllum peltatum*, *Ancistrocladus heyneanus*, Dioncophyllaceae, Ancistrocladaceae, triterpenes, betulinic acid, antimalarial activity, *Plasmodium falciparum*, crystal structure.

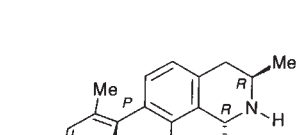
Introduction

The Dioncophyllaceae (2) and the Ancistrocladaceae (3) are small, but chemically most productive families of tropical vines, indigenous to African and South-East Asian rain forests. The plants are typical not only for their morphological properties, but, in particular, also for their chemical constituents, the naphthylisoquinoline alkaloids (4), among them dioncophylline A (**1**) and ancistrocladine (**2**). These naturally occurring biaryls apparently constitute the first tetrahydroisoquinoline alkaloids that are derived from acetic acid units, and not, as usually, from aromatic acids. In the search for further, related or structurally different chemical constituents of these interesting plants, we have also examined less polar fractions.

Materials and Methods

Plant material

Triphyophyllum peltatum (Dioncophyllaceae) was collected in the Parc de Taï, West Ivory Coast, in February 1993 and identified by one of us (L. A. A.). A specimen has been deposited at the Centre National de Floristique, Université d'Abidjan, Ivory Coast. Plants of the Indian species *Ancistrocladus heyneanus* (Ancistrocladaceae) were cultivated in the Botanical Garden, University of Würzburg, from fully matured seeds collected 1992 in India. Cultivation details were described earlier (5, 6).



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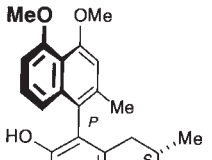

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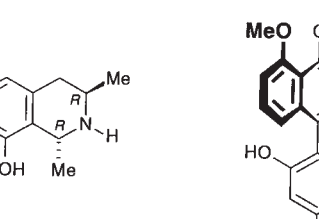

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Instrumentation and materials

Melting points: Reichert-Jung thermovar hot-plate (uncorrected); ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz): Bruker DMX 600; Optical rotation: Perkin-Elmer 241 MC; El-MS: Finnigan MAT 8200; Elemental analysis: LECO CHNS-932; IR: Perkin-Elmer 1420. CC: silica gel 63–200 mesh (Merck); TLC: silica gel F 254 (Merck).

Extraction and isolation (exemplarily from T. peltatum*)*

The air-dried powdered root bark of *T. peltatum* (1400 g) was extracted with petroleum ether (30–75 °C) in a Soxhlet apparatus. The crude extract obtained by evaporation of the solvent in vacuo (30 g), was chromatographed on silica gel with $CHCl_3$/MeOH (97 : 3) as the eluent. Recrystallization from CH_2Cl_2/methanol (95 : 5) gave **3** (2.0 g). Colorless crystals, m. p. 315 °C (Lit. (7) 316–318 °C), $[\alpha]_D^{20}$: +6.7° (*c* 0.4, pyridine) [Lit. (8) +6.8° (*c* 0.4, pyridine]. IR, mass, ^{1}H-NMR, and ^{13}C-NMR spectroscopic data are fully identical to those of an authentic sample. Under the same conditions, isolation of **3** (26 mg) succeeded also from *Ancistrocladus heyneanus* (100 g).

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Plasmodium falciparum in vitro *assays of betulinic acid* (**3**)

P. falciparum (NF 54, clone A1A9) asexual erythrocytic stages were continuously maintained *in vitro*, according to the method of Trager and Jensen (9). Briefly, the suspensions were kept in RPMI medium, at 37 °C, and under an atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2. The parasites were subinoculated every 3–4 days, with initial culture conditions of 1 % parasitaemia and 1 % haematocrit.

Antiplasmodial activities were tested *in vitro*, based upon methods described earlier (10–12). Betulinic acid (**3**) was first dissolved in DMSO (Merck) at a concentration of 1 mg/20 μl. This solution was further diluted with physiological saline to obtain a stock solution of 500 μg/ml, and applied in a series of seven four-fold dilutions, with final concentrations ranging from 50 to 0.012 μg/ml. The compound was tested in replicates of six.

The parasites were incubated for 24 h in microtitre plates (Falcon, MicroTest III). After that time, ^3H-hypoxanthine (Amersham) was added to the suspensions, at a concentration of 0.5 μCi/well, and the parasites were incubated under the same conditions for further 18 h. The incorporated radioactivity was measured by a liquid scintillation counter (Philips, PW 4700).

Synthesis of betulinic acid benzyl ester (**4**)

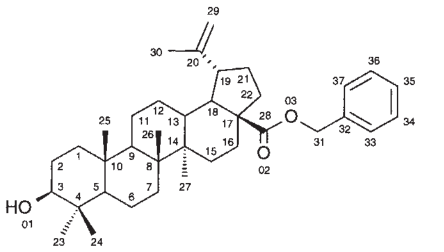
A mixture of betulinic acid (**3**) (50 mg, 109.6 μmol), benzyl bromide (0.10 ml, 0.84 μmol), Cs_2CO_3 (30 mg, 92.07 μmol), and K_2CO_3 (30 mg, 217.0 μmol) in dry acetone (10 ml) was heated at reflux for 10 h. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (99 : 1) as the eluent, to give **4** (58 mg, 98 %) as colorless crystals directly suited for the X-ray structure analysis (see below): m.p. 202 °C, $[\alpha]_D^{20}$: −2.0° (*c* 1.0, EtOH); IR (KBr): ν_{max} = 3510, 2910, 1680, 1445, 1155, 1130, 1035, 880, 750 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ = 0.67–3.04 (43H), 3.19 (1H, dd, *J* = 11.5 Hz, *J* = 4.7 Hz, H-3), 4.61 (1 H, br. s, H-29), 4.74 (1 H, br. s, H-29), 5.11 (1 H, d, *J* = 12.3 Hz, OC*H*HPh), 5.16 (1 H, d, *J* = 12.3 Hz, OCH*H*Ph), 7.32–7.39 (5 H, m); ^{13}C-NMR ($CDCl_3$): δ = 15.32, 15.82, 15.96, 18.27, 20.86, 25.53, 27.39, 27.39, 27.92, 28.00, 29.53, 29.58, 30.57, 32.10, 34.30, 36.91, 37.16, 38.18, 38.20, 38.71, 38.83, 40.64, 42.37, 46.91, 49.42, 49.48, 50.55, 55.34, 56.53, 65.70, 78.94, 109.54, 128.02, 128.21, 128.45, 136.47, 150.53, 175.77; EI-MS: *m/z* = 546 (M$^+$), 528 (M$^+$ − H_2O), 455 (M$^+$ − C_7H_7), 437 (528 − $C_7H_7^+$), 91 ($C_7H_7^+$); Anal. Calcd. for $C_{37}H_{54}O_3$ (546.76): C, 81.28 H, 9.94; Found: C, 80.99 H, 9.88:

X-ray analysis of **4**

Crystal data of betulinic acid benzyl ester (**4**) (from petroleum ether/ethyl acetate, see above) are: size of the crystal: 0.2 × 0.35 × 2.5 mm, orthorhombic, space group $P\ 2_12_12_1$, a = 8.464(2) Å, b = 14.163(3) Å, c = 25.726(4) Å, V = 3084(1) Å^3, z = 4, d = 1.178 g/cm^3, F(000) = 1200 and μ(MoKα) = 0.07 mm^{-1}, graphite monochromator, $2\theta_{max}$ = 55°, ω scan, 7053 unique reflections of which 5978 with ($F > 3\sigma(F)$), F_o/parameter ratio = 16.51, empirical absorption correction, maximum transmission = 0.254, minimum transmission = 0.214. The structure was solved by direct methods on a Siemens P4 and refined using the SHELXTL-Plus program system. Atoms different to hydrogen were refined anisotropically. Hydrogen atoms were refined isotropically. The final discrepancy index was R = 0.072, R_w = 0.074. Atom coordinates for this structure have been submitted to the Cambridge Crystallogrpahic Data Centre.

Results and Discussion

From the petroleum ether extracts of the root bark of *Triphyophyllum peltatum* (Dioncophyllaceae) and *Ancistrocladus heyneanus* (Ancistrocladaceae), we could isolate a hydroxycarboxylic acid, $C_{30}H_{48}O_3$, which turned out to be the known triterpenoid betulinic acid. This compound is most widespread in nature and has been isolated from numerous species of higher plants (13–17), although not yet from Dioncophyllaceae or Ancistrocladaceae.



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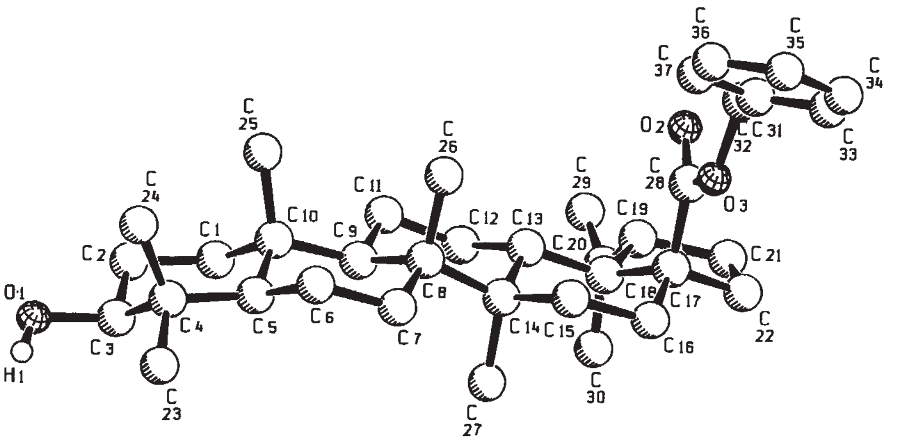

Fig. 1 Structure of **4** in the crystal; hydrogens omitted for reasons of clarity.

Betulinic acid has been found to exhibit a wide range of biological activities, e.g. antitumor activity against human melanoma (15) and against mouse skin two-stage carcinogenesis (17), leishmanicidal activity against amastigotes of *Leishmania amazonensis* (18), spasmogenic activity on isolated rat fundus (13), anti-inflammatory activity in mouse paw and ear edema tests (16), and activity as a human immunodeficiency virus (HIV) inhibitor (14). Given the high antiplasmodial activity of extracts of *T. peltatum* (11) and the use of related plants against malaria in folk medicine (19), we have now for the first time tested the antimalarial activity of betulinic acid (**3**). Using a test system described earlier (11, 12), **3** exhibits a moderate antiplasmodial *in vitro* activity (IC_{50} = 10.46 μg/ml) against asexual erythrocytic stages of *P. falciparum*, the causative agent of the most dangerous and lethal type of malaria.

For reasons of comparison, the activities of a series of reference drugs were determined in the same system. They yielded the following IC_{50} values: chloroquine (0.005 μg/ml), quinine (0.063 μg/ml), mefloquine (0.026 μg/ml), halofantrine (0.015 μg/ml), and artemisinin (0.039 μg/ml). Betulinic acid is virtually nontoxic ($IC_{50} > 20$ μg/ml) to a wide range of cultured human cell lines. It only shows a selective cytotoxic activity towards human melanoma cells, with IC_{50} values of 1–5 μg/ml (15).

Chemically, it is interesting to note that despite the rigidity of the molecular framework of betulinic acid, which is based on the lupane skeleton, no crystal structure analysis of this important natural product has previously been described, except for the closely related natural product anemosapogenin (20). For this reason, in order to obtain crystals suited for an X-ray diffraction analysis, we have prepared different derivatives of **3**. Of these, the benzyl ester **4**, as prepared by treatment of **3** with $PhCH_2Br/K_2CO_3/Cs_2CO_3$ in acetone, gave nice colorless plates from petroleum ether/ethyl acetate.

The crystal structure analysis (Fig. **1**) shows the expected extended ring system, the four annulated chair-type cyclohexane rings and the terminal envelope-type cyclopentane ring. The angular methyl groups C-25 (at C-10), C-26 (at C-8), and C-27 (at C-14), the axial β-methyl group C-24 (at C-4), as well as the likewise angular carboxylic acid ester functionality C-28/O-02/O-03 (at C-17) stick orthogonally out of the polycyclic ring system. This ring array is not entirely flat, but slightly bent to a convex shape (seen from above), thus minimizing steric interaction of the mentioned angular groups. Consequently, the axial exocyclic C,C bonds to these substitutents are not truly parallel to each other, but rather radially divergent. Only the 3-hydroxy function, the α-methyl group (C-23) at C-4, and the 2-propenyl substitutent at C-19 occupy equatorial positions. Characteristic is the near-coplanarity of the entire ester functionality -CO-O-CH_2 (C-28, O-02, O-03, C-31) including the adjacent C-atoms (C-17 and C-32) on both sides.

The X-ray structure analysis of **4** fully confirms the anticipated structure **3** of betulinic acid. Furthermore, the first isolation of betulinic acid from Dioncophyllaceae and Ancistrocladaceae species clearly shows these plants to be capable of not only preparing very special and unique polyketide-derived naphthylisoquinoline alkaloids, but also "conventional", widespread natural products. The isolation and structure elucidation of further compounds from these interesting plants is underway.

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