New Chemical Constituents of Euphorbia quinquecostata and Absolute Configuration Assignment by a Convenient Mosher Ester Procedure Carried Out in NMR Tubes

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Two new compounds, an ent-isopimarane-type diterpene, 3(R),12(R)-dihydroxy-ent-8(14),15-isopimaredien-18-al (1), and a dihydrobenzo(b)furane neolignan, (−)-trans-9-acetyl-4,9′-di-O-methyl-3′-de-O-methyldehydrodiconiferyl alcohol (2), along with five known compounds, 7,7′-dihydroxy-6,8′-bicoumarin (bicoumol) (3), 3,4-dimethoxycinnamaldehyde (4), 6-hydroxy-7-methoxycoumarin (isoscopoletin), N-butylaniline, and vanillin, have been isolated from an ethyl acetate-soluble extract of the stem wood of Euphorbia quinquecostata. The structures of compounds 1 and 2 were elucidated on the basis of spectroscopic data interpretation, and single-crystal X-ray diffraction analysis was used to confirm the structure and relative stereochemistry of 1. The absolute configuration of 1 was established by a convenient Mosher ester procedure in which the sample was treated with MTPA chlorides in deuterated pyridine directly in NMR tubes. All isolates were evaluated for the induction of quinone reductase in Hepa1c1c7 hepatoma cells and for the inhibition of the transformation of murine epidermal JB6 cells.

Several phorbol dibutyrate receptor-binding (PDBu) inhibitory diterpenoids of the ent-atisane and ingenane classes have been isolated from Euphorbia quinquecostata Volk. (Euphorbiaceae) in our previous study.1 As part of a project directed toward the search for novel, plant-derived cancer chemopreventive agents,2–4 we have reinvestigated the chemical constituents of an EtOAc-soluble extract of a re-collection of E. quinquecostata, with the intention of obtaining a larger quantity of the compound 17-hydroxy-ingenol 20-hexadecanoate for additional biological testing. During this study, two new compounds, 3(R),12(R)-dihydroxy-ent-8(14),15-isopimaredien-18-al (1) and (−)-trans-9-acetyl-4,9′-di-O-methyl-3′-de-O-methyldehydrodiconiferyl alcohol (2), along with five known compounds, were isolated and characterized. All isolates were evaluated for their potential cancer chemopreventive properties utilizing in vitro assays to determine quinone reductase induction in murine Hepa1c1c7 hepatoma cells5 and the inhibition of the transformation of murine epidermal JB6 cells.6 This article reports the isolation and structure elucidation of compounds 1 and 2, as well as the biological evaluation of all isolates obtained in these two assays. In addition, we wish to present a convenient procedure for determining the absolute configuration of natural products possessing secondary hydroxyl groups, as performed on 1 using the Mosher ester method carried out directly in NMR tubes. It is anticipated that this procedure will have wide applicability to other natural product laboratories, since the reaction conditions have been simplified and very small sample amounts are needed.

Results and Discussion

Compound 1 was isolated as colorless needles (CHCl3–MeOH, 1:1), mp 149–150 °C, [α]D23 +7.0° (c 0.50, CHCl3).

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A molecular formula of \( \text{C}_{20}\text{H}_{30}\text{O}_{3} \) was determined for 1 from its HREIMS at \( m/z \) 318.2183 (calcld for \( \text{C}_{20}\text{H}_{30}\text{O}_{3}, 318.2195 \)). The \( ^1\text{H} \) NMR spectrum of compound 1 displayed characteristic signals for an aldehyde proton at \( \delta_{1\text{H}} 9.41 \) (1H, s, H-18), four olefinic protons at \( \delta_{1\text{H}} 5.76 \) (1H, dd, \( J = 17.5, 10.7 \) Hz, H-15) and 5.09–5.17 (3H, overlapped, H-16a, H-16b, and H-14), two oxygenated methine protons at \( \delta_{1\text{H}} 3.81 \) (1H, dd, \( J = 11.6, 4.3 \) Hz, H-3) and 3.57 (1H, dd, \( J = 12.2, 4.3 \) Hz, H-15), and three tertiary methyl singlets at \( \delta_{1\text{H}} 1.11 \) (3H, s, CH3-19), 1.06 (3H, s, CH3-17), and 0.87 (3H, s, CH3-20). The \(^{13}\text{C} \) and DEPT NMR spectral data indicated that compound 1 contains 20 carbons, including three methyls, six methylenes, seven methines, and four quarternary carbons. An olefinic proton \( (\delta_{1\text{H}} 5.76, \text{H-15}) \) of 1 appeared as a double doublet with coupling constants of 17.6 and 10.5 Hz. These coupling constants and the chemical shift \( (\delta_{1\text{C}} 145.9, \text{d, assigned by HMQC correlation}) \) of C-15 suggested the presence of a vinyl group in the molecule of 1.\(^{6,7} \) On the basis of the molecular formula determined by HREIMS and from the three unsaturated units (an aldehyde group and two double bonds) inferred from the NMR data, it was apparent that three rings were present in the molecule of 1. All of this evidence was suggestive that compound 1 is a pimarane-type diterpene.\(^{6,8,9} \) The positions of the two hydroxy groups and the aldehyde group, as well as the double bond between C-8 and C-14 in 1, were determined on the basis of the observed HMBC correlations from H-3 to C-18, C-19, C-5, and C-4, from H-12 to C-15, C-17, and C-14, from H-15 to C-12, C-13, C-14, and from CH3-19 to C-18, C-3, C-4, and C-5. In the NOESY spectrum of 1, H-18 correlated to H-3 and H-5, CH3-19 correlated to CH3-20, and H-12 correlated to H-9 and H-15. These observations indicated that both OH-3 and OH-12 adopt an \( \alpha \)-orientation, while the aldehyde and vinyl groups are \( \beta \)-oriented. A suitable crystal was obtained from \( \text{CHCl}_3-\text{MeOH} \) (\( \sim 1:1 \)), and the X-ray diffraction structure of compound 1 (Figure 1) confirmed its structure and relative configuration.

The Mosher ester procedure\(^{10,11} \) and modified Mosher method\(^{12,13} \) are based on the diastereomeric effect of an introduced phenyl ring to assign the absolute stereochemistry of organic compounds by measuring and comparing the NMR data of the resultant diastereomeric \( \alpha \)-methoxy-\( \alpha \)-(trifluoromethyl)phenylacetic acid (MTPA) esters. The MTPA esters of compounds may become depleted after measurement of the NMR spectrum, the sample quantities available for chemical transformation and absolute configuration determination for these compounds may become detected after measurement of the necessary physical data and evaluation of biological activity. In the present study, we have determined the absolute configuration of 1 using a very convenient Mosher ester procedure carried out in NMR tubes. Two portions (each 2.0 mg) of 1 were treated with \((S)-(\pm)-\alpha \)- and \((R)-(\pm)-\alpha \)-methoxy-\( \alpha \)-(trifluoromethyl)phenylacetic acid (6.0 \mu L) in deuterated pyridine (0.5 mL) directly in separate NMR tubes (Experimental Section) at room temperature, which afforded the \((R)\)- and \((S)\)-MTPA ester derivatives \((1r)\) and \((1s)\), respectively, of 1. The \(^1\text{H} \) NMR spectra of \((1r)\) and \((1s)\) were obtained by measuring the reaction NMR tubes directly. [Partial \(^1\text{H} \) NMR spectra in the diagnostic regions are shown in Figure 2, while the entire \(^1\text{H} \) NMR spectra of the \((R)\)- and \((S)\)-MTPA esters of 1 are shown in the Supporting Information.] Although strong proton signals of the excess MTPA chlorides and MTPA acids (hydrolysis products from MTPA chlorides, due to the trace amount of \( \text{H}_2\text{O} \) in deuterated pyridine and the moisture of the experimental environment) were present in the \(^1\text{H} \) NMR spectra of \((1r)\) and \((1s)\) (Supporting Information), the undis- turbed signals (H-15, H-14, H-16, CH3-17, CH3-19, and CH3-20) of the diastereomeric MTPA esters \((1r)\) and \((1s)\) were clearly different (Figure 2) and the observed chemical shift differences (\( \Delta \delta_{1r-S} \), Figure 3) unambiguously indicated the absolute configurations of C-3 and C-12 of 1 to be R.
and S, respectively. Thus, the structure of compound 1 was assigned as 3α,12β-di-hydroxy-ent-8(14),15-isopimaradien-18-ol. This assignment was consistent with the relative configurations of C-3 and C-12 determined from the X-ray analysis of 1.

To evaluate the reliability of the assigned absolute stereochemistry of 1 by this convenient Mosher ester reaction carried out in NMR tubes, both 1R and 1S were individually purified chromatographically. The 1H NMR data of 1R and 1S after purification (summarized in Figure 3) also suggested the absolute configuration of C-3 and C-12 of 1 to be R and S, respectively. The only difference in the present procedure from previously reported Mosher ester methods is that deuterated pyridine and NMR tubes have been used as the reaction solvent and the reaction containers, respectively. In this manner, it is very convenient to monitor reactions by acquiring 1H NMR spectra at intervals, and the absolute stereochemistry can be established from the 1H NMR data of the diastereomeric MTPA esters without further purification. In addition to compound 1, we have shown the applicability of this convenient procedure to the absolute stereochemistry determination of another secondary hydroxyl-containing natural product, philadelphicalactone A, which was isolated from Physalis philadelphica in our recent work, and its absolute configuration has been established by the normal Mosher ester method. The obtained 1H NMR spectral data of (R)- and (S)-MTPA esters of philadelphicalactone A by the present convenient method (Supporting Information) enabled the absolute configuration of C-4 of philadelphicalactone A to be determined as S, which is consistent with the previous result. We believe this convenient procedure will prove to be of wide application in natural products laboratories and will permit the accurate absolute configuration assignment of minor isolates obtained in phytochemical investigations in a time-saving manner.

Compound 2 was isolated as yellowish oil, [α]D 23° = −73.3° (c 0.10, CHCl3). The HREIMS of 2 exhibited a molecular ion peak at m/z 414.1689, consistent with the molecular formula C23H26O7 (calc for C23H26O7, 414.1679). In the 1H NMR spectrum of 2, signals were evident for five aromatic protons (δH 6.88–6.91, 5H), protons of a trans double bond (δH 6.56, 1H, d, J = 15.8 Hz, H-7; 6.16, 1H, dt, J = 15.8, 6.0 Hz, H-8), three methoxy group protons (δH 3.77, 3H, s, Ar-OMe; 3.88, 3H, s, Ar-OMe; 3.40, 3H, s, OMé-9′), and the methyl protons (δH 2.04, 3H, s, OAc) of an acetoxyl group. The 13C NMR spectrum of 2 also displayed the characteristic signals for three methoxy groups (δC 56.0, Ar-OMe × 2, and 50.3, OMe-9′) and an acetoxyl group (δC 170.8, and 20.8). Besides these substituent signals, 18 skeletal carbon resonances appeared in the 13C NMR spectrum of 2, which, in combination with its 1H NMR data, suggested that 2 is a dihydrobenzof[b]furan nedelignan.

In the HMBC spectrum of 2, H-9 (δH 4.45 and 4.09) and the methyl signal (δH 2.04, 3H, s, OMe) correlated to the carbonyl carbon (δC 170.8, s, OAc) of the acetoxyl group, and the methoxy signal at δH 3.40 (3H, s, OMe-9′) correlated to C-9′ at δC 73.2. These observations indicated that the acetoxyl group was attached to C-9 and a methoxy group was located at C-9′. The deuterium exchangeable singlet at δH 5.63 (1H, s, OH-3′) was not correlated with any carbon signals in the HMOC spectrum of 2, while this proton singlet was correlated to C-2′, C-3′, and C-4′ in the HMBC spectrum of 2. This permitted the assignment of the hydroxy group at C-3′. The trans relationship between H-7 and H-8 was determined by comparison of the coupling constant and the chemical shifts of these two protons with those of reported analogues.

Accordingly, compound 2 was assigned as (−)-trans-9-acetyl-4′,9′-di-O-methyl-3′-de-O-methyldehydrodiconiferyl alcohol.

Five known compounds, 7,7′-dihydroxy-6,8′-bicoumarin (bicoumal) (3),25 3,4-dimethoxycinnamaldehyde (4),26 6-hydroxy-7-methoxycoumarin (isoscopoletin),27 N-butylaniline,28 and vanillin,29 were isolated along with compounds 1 and 2. The structures of these known compounds were identified by physical and spectroscopic data measurement (1H NMR, 13C NMR, DEPT, 2D NMR, and MS) and by comparing the data obtained with those of published values. Full 1H and 13C NMR data assignments of bicoumal (3) are reported in the Experimental Section for the first time.

The potential of all isolates to induce quinone reductase (QR) in murine Hepatocarcinoma cells and to inhibit the transformation of murine epidermal JB6 cells was evaluated according to previous protocols. The results indicated that only 3,4-dimethoxycinnamaldehyde (4) was significantly active in these assays with a CD (concentration to double induction) value of 9.5 μg/mL (52.8 μM) (QR assay) and an IC50 value of 2.3 μg/mL (12.8 μM) (JB6 assay), respectively.

**Experimental Section**

**General Experimental Procedures.** Melting points were determined on a Kofer hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter. UV spectra were obtained with a Beckman DU-7 spectrophotometer. IR spectra were run on an ATI Mattson Genesis Series FT-IR spectrophotometer. NMR spectral data were recorded at room temperature on Bruker Avance DPX-300 and DRX-500 MHz spectrometers with tetramethylsilane (TMS) as internal standard. EIMS and HREIMS data were obtained on a Finnigan/MAT 90/95 sector-field mass spectrometer, and HRFABMS were obtained on a VG 7070E-HF sector-field mass spectrometer. X-ray crystallographic analysis data collection for compound 1 was carried out on an Enraf-Nonius Kappa CCD area detector with a rotating anode Mo X-ray tube. The direct method DIR was used to locate non-hydrogen atoms, and the WinGX package was used for completing the structure determination, while ORTEP was used to generate Figure 1. Column chromatography was carried out with Si gel G (Merck, 230–400 mesh). Analytical thin-layer chromatography (TLC) was performed on precoated 250 μm thickness Merck Si gel 60 F254 aluminum plates, while preparative thin-layer chromatography was performed on precoated 1000 μm thickness Merck Si gel 60 F254 glass plates.
Plant Material. The stem wood of Euphorbia quinque-esta was collected in Tanzania by Z.H.M. in February 1999. A voucher specimen (PA0177) has been deposited at the University of Illinois Pharmacognosy Field Station, Downers Grove, IL.

Extraction and isolation. The dried and milled stem wood (9.5 kg) was extracted by maceration with MeOH three times (3 × 20 L) at room temperature, one day each time. After filtration and concentration of the methanol extract (1000 mL) at room temperature and monitored every 2 h by 'H NMR. The reaction mixture was transferred from the NMR tube and purified over a small column (10 × 2 cm) of deactivated Florisil (silica gel) and eluted with a gradient of petroleum ether–EtOAc (100:1 to 5:1) to give, in order of polarity, a first fraction (F01), a second fraction (F02), a further fraction (F03), and another fraction (F04), all washed with water.

The EtOAc-soluble extract was subjected to Si gel column chromatography with chloroform as the first solvent system (from 100:1 to 5:1), to give, in order of polarity, a first subfraction (F01), a second subfraction (F02), and a third subfraction (F03). Subfraction F03 was further purified by preparative TLC (Merck 60 Å Si gel, 20 cm) at room temperature, one day each time. After filtration and evaporation of the solvent under reduced pressure, a yield of 9.2 mg of pure compound was obtained as a white amorphous powder.

Preparation of the (R)- and (S)-MTPA Ester Derivatives of 1 by a Convenient Mosher Ester Procedure. Compound 1 (2.0 mg) was transferred into a clean NMR tube and was dried completely under the vacuum of an oil pump. Descriptions of reactions and instrumentation are published in the literature.

The EtOAc-soluble residues. A voucher specimen (PA0177) has been deposited at the Cambridge Crystallographic Data Centre for the structure of this compound has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 182252. Copies of this data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK: [fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].