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A Minor Diterpenoid with a New 6/5/7/3 Fused-Ring Skeleton from *Euphorbia micractina*

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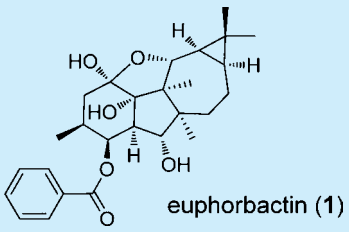
Supporting Information

ABSTRACT: A novel diterpenoid with an unprecedented 6/5/7/3 fused-ring skeleton, euphorbactin (**1**), was isolated from an ethanol extract of the roots of *Euphorbia micractina*. The structure was determined by extensive spectroscopic studies, especially by 2D NMR and CD data analysis. A proposed biosynthetic pathway and preliminary investigations of the biological activity of compound **1** are also discussed.



Species of the genus *Euphorbia* (Euphorbiaceae) are sources of various secondary metabolites with interesting chemical structures and significant bioactivities. In particular, diterpenoids, which display a variety of parent skeletons, exert a range of biological effects, including anti-inflammatory, antimicrobial, antiproliferation, cytotoxic, and modulation of multidrug resistance activities.[1] Notably, ingenol mebutate from the sap of *E. peplus* has recently been approved in the United States, countries of the European Union, Australia, and Brazil for the treatment of actinic keratosis, a precursor to a form of squamous-cell carcinoma.[2] In addition, a nontumor-promoting 12-deoxytigliane diterpenoid from *E. fischeriana* and other species of Euphorbiaceae, prostratin, exhibits potent in vitro activity in inducing HIV expression in latently infected cell lines and primary cells. It has been advanced into preclinical trials, although the mechanism of action has not yet been completely elucidated.[3] These observations have promoted increased interest in the research of diterpenoids in species of this genus.[4] The challenge of semi- or total synthesis of these diterpenoids has also drawn great interest in recent years.[5]

Euphorbia micractina Boiss. is widely distributed at high altitudes (2700−5000 m) in western mainland China, and its roots are used in Chinese folk medicine for the treatment of tumors and warts.[6] As part of a program to access the chemical diversity of Chinese traditional medicines and study their biological effects, especially focusing on minor constituents, we investigated *E. micractina* roots. In a previous study, nine new triterpenoids including several uncommon triterpene hydroperoxides, 17 new lathyrane diterpenoids with a 5/11/3 fused-ring system, and 12 new minor diterpenoids possessing rare 5/6/8, 5/6/7/3, and 5/6/6/4 fused-ring skeletons, together with 45 known compounds, were characterized in several fractions obtained from an EtOH extract from the roots. Some of these compounds showed antiviral activity against HIV-1 replication, selective cytotoxic activity against the A2780 ovarian cancer cell line, and vascular-relaxing activity against phenylephrine-induced vasoconstriction.[7] Our investigation of the remaining fraction with activity against HIV-1 replication from the same extract led to the isolation of a bioactive minor diterpenoid (**1**), which possesses an unprecedented carbon skeleton (Figure 1).[8] Herein, we report details of the isolation, structure elucidation, postulated biogenetic pathway, and biological activity of this compound.

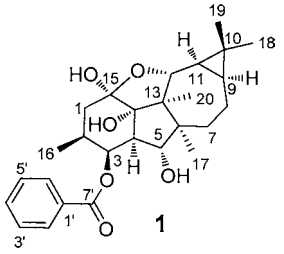


Figure 1. Structure of euphorbactin (**1**).

Compound **1** was obtained as a colorless gum with $[\alpha]^{20}_{D}$ +99 (*c* 0.20, MeOH). Its IR spectrum showed absorption bands assignable to hydroxy (3461 cm^{-1}), carbonyl (1705 cm^{-1}), and aromatic ring (1602 and 1494 cm^{-1}) functionalities. The positive and negative mode ESIMS of **1** exhibited quasimolecular ion peaks at *m*/*z* 479 $[M + Na]^+$ and 455 $[M-H]^-$, respectively. The molecular formula of $C_{27}H_{36}O_6$, with 10 degrees of unsaturation, was deduced from HRESIMS at *m*/*z* 479.2408 $[M + Na]^+$ (calcd for $C_{27}H_{36}O_6Na$, 479.2404), which was supported by the NMR data (Table 1). The ^1H NMR spectrum of **1** in acetone-d_6 showed resonances attributable to the following: (a) a benzoyl group at δ_H 8.04 (2H, d, *J* = 7.5 Hz, H-2′ and H-6′), 7.65 (1H, t, *J* = 7.5 Hz, H-4′), and 7.51 (2H, t, *J* = 7.5 Hz, H-3′ and H-5′); (b) three oxymethines at δ_H 5.68 (d, *J* = 8.5 Hz, H-3), 4.13 (dd, *J* = 9.0 and 5.5 Hz, H-5), and

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Table 1. NMR Spectroscopic Data for Euphorbactin (1)[a]

position	δ_H	δ_C
1a	2.12 dd (13.5, 10.0)	41.5
1b	1.87 dd (13.5, 6.5)	
2	2.15 m	32.1
3	5.68 d (8.5)	72.4
4	2.72 dd (9.0, 8.5)	57.2
5	4.13 dd (9.0, 5.5)	72.3
6		46.1
7a	1.48 dd (13.0, 5.5)	33.5
7b	1.28 dd (13.0, 13.0)	
8a	1.50 m	20.8
8b	0.96 m	
9	0.64 m	25.8
10		20.7
11	0.62 dd (9.0, 7.0)	26.5
12	3.28 d (9.0)	75.0
13		57.9
14		84.0
15		103.1
16	0.91 d (6.0)	18.1
17	0.94 s	18.6
18	1.00 s	28.6
19	0.97 s	15.6
20	1.04 s	10.3
1′		131.2
2′	8.04 d (7.5)	130.3
3′	7.51 t (7.5)	129.4
4′	7.65 t (7.5)	134.0
5′	7.51 t (7.5)	129.4
6′	8.04 d (7.5)	130.3
7′		166.8
O*H*-5	3.54 d (5.5)	
O*H*-14	4.02 s	
O*H*-15	5.47 s	

[a]NMR data (δ) were measured at 500 MHz for ^{1}H and at 125 MHz for ^{13}C in acetone-d_6. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on DEPT, ^{1}H−^{1}H gCOSY, gHSQC, and gHMBC experiments.

3.28 (d, J = 9.0 Hz, H-12); (c) four tertiary methyl groups at δ_H 1.04 (H_3-20), 1.00 (H_3-18), 0.97 (H_3-19), and 0.94 (H_3-17), and (d) a secondary methyl group at δ_H 0.91 (d, J = 6.0 Hz, H_3-16). In addition, the spectrum showed resonances due to three exchangeable hydroxy protons at δ_H 5.47 (s, O*H*-15), 4.02 (s, O*H*-14), and 3.54 (d, J = 5.5 Hz, O*H*-5), as well as partially overlapping signals with complex coupling patterns between δ_H 0.62 and 2.72 that could be attributed to several aliphatic methylene and/or methine units. Besides the resonances of the benzoyl moiety, the ^{13}C NMR and DEPT spectra of **1** showed 20 carbon resonances corresponding to the above-described functional units, as well as to five quaternary carbons (one oxygen-bearing at δ_C 83.9 and one dioxygen-bearing at δ_C 103.1), four aliphatic methine units, and three aliphatic methylene units. Together, these spectroscopic data indicate that **1** is an unusual tetracyclic diterpene alcohol benzoate possessing three free hydroxy groups and an epoxy unit, for which the structure was further elucidated by 2D NMR spectroscopic analysis.

The proton and hydrogen-bearing carbon resonances in the NMR spectra of **1** were assigned unambiguously by ^{1}H−^{1}H gCOSY and HSQC spectroscopic data interpretation. In the ^{1}H−^{1}H gCOSY spectrum of **1**, the homonuclear coupling correlations of H_2-1/H-2/H_3-16, H-3/H-4/H-5/O*H*-5, H_2-7/H_2-8/H-9/H-11/H-12, and H-2′(H-6′)/H-3′(H-5′)/H-4′ revealed the presence of fragments with vicinal couplings (Figure 2, thick lines). The HMBC spectrum of **1** showed two- and

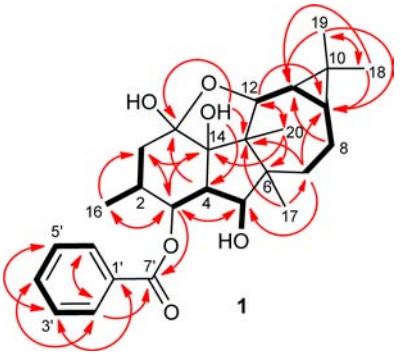

Figure 2. Main ^{1}H−^{1}H COSY (black thick lines) and three-bond HMBC correlations (red arrows, from ^{1}H to ^{13}C) of euphorbactin (**1**).

three-bond correlations from H_2-1 to C-2, C-3, C-14, and C-15; from H-3 to C-1, C-7′, C-14, and C-16; from H-4 to C-3, C-14, and C-15; from H_3-16 to C-1, C-2, and C-3; from O*H*-14 to C-4, C-14, and C-15; and from H-2′(H-6′) to C-7′ (Figures 2 and S15−S24, Supporting Information). These correlations, in combination with the chemical shifts of the proton and carbon resonances, indicated the presence of a six-membered ring moiety consisting of C-1−C-4, C-14, and C-15 in **1** with a secondary methyl (CH_3-16), a benzoyloxy functionality, a hydroxy group, and two oxygen atoms at C-2, C-3, C-14, and C-15, respectively. HMBC correlations from H-4 to C-5; from H-5 to C-3 and C-17; from H_3-17 to C-5, C-6, and C-13; from H_3-20 to C-6, C-13, and C-14; and from O*H*-14 to C-13, together with the corresponding shifts, demonstrated the presence of a five-membered ring fused to positions C-4 and C-14 on the six-membered ring, which was substituted with a hydroxy group at C-5 and two tertiary methyl groups (CH_3-17 and CH_3-20) at C-6 and C-13. HMBC correlations from H_2-7 to C-5, C-6, C-8, C-9, and C-13; from H-12 to C-9, C-11, and C-20; from H_3-17 to C-7; and from H_3-20 to C-12 revealed the presence of a seven-membered ring that was fused to the five-membered ring at C-6 and C-13, which was substituted at C-12 by an oxygen atom. In addition, HMBC correlations from H_3-18 and H_3-19 to C-9, C-10, and C-11; from H_3-18 to C-19; and from H_3-19 to C-18, together with their shifts, indicated the presence of a cyclopropane ring fused to the seven-membered ring at C-9 and C-11, which was substituted with two tertiary methyl groups (CH_3-18 and CH_3-19) at C-10. The presence of an ether linkage between C-12 and C-15 and a hydroxy group at C-15 to form a hemiketal was deduced from the molecular composition and the chemical shifts of H-12, C-12, and C-15 (Table 1).Therefore, the gross structure of **1** was determined as shown in Figure 2.

The relative configuration of **1** was deduced from NOE difference experiments. In the NOE difference spectrum of **1**, irradiation of H-4 enhanced the intensity of H-2, H-3, H_3-17, O*H*-14, and O*H*-5; irradiation of O*H*-14 enhanced O*H*-5, O*H*-15, and H_3-20; and irradiation of H_3-20 gave enhancements of H-9, H-11, H_3-17, and O*H*-14, indicating these protons to be in a cofacial position. In addition, H-5 and H_3-19 were enhanced upon irradiation of H-12, revealing that these protons are

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cofacial as well (Figures 3 and S15−S22 in Supporting Information). In addition, the ^{1}H NMR and ^{1}H−^{1}H gCOSY

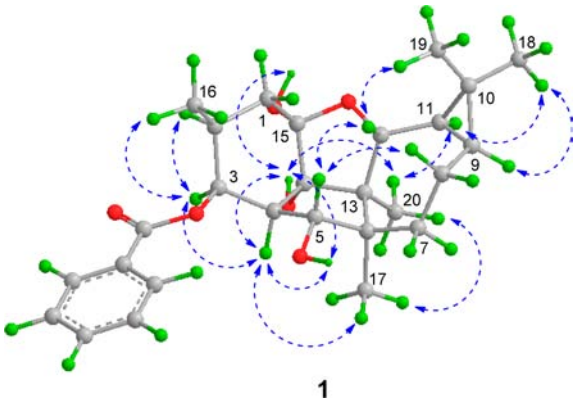


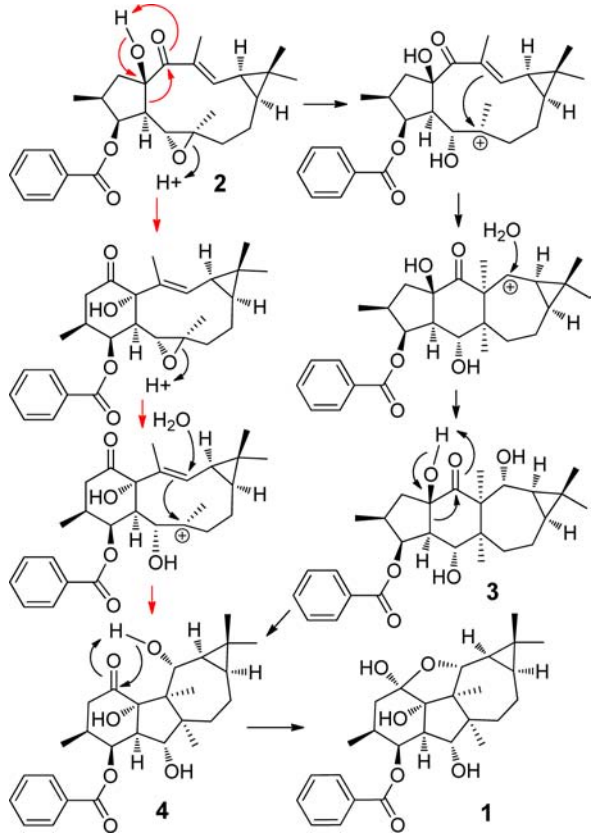
Figure 3. Main NOE enhancements (blue dash lines with double arrows) of euphorbactin (**1**).

spectra of **1** indicated no coupling between H-2 and H-3, suggesting that in the solution the six-membered ring adopts a twist conformation in which the dihedral angle of the two vicinal protons is perpendicular. This was supported by the NOE enhancements of H-2 and H_3-16 upon irradiation of H-3. Accordingly, compound **1** was assigned with the relative configuration as depicted in Figure 3, for which the 6/5/7/3 *cis*-fused-ring system, together with the tetrhydrofuran ring, constructs a cage-like framework.

The absolute configuration of **1** was preliminarily assigned by using the benzoate sector rule for circular dichroism (CD) data, wherein the Cotton effect of benzoate due to the $\pi\rightarrow\pi^*$ intramolecular charge-transfer transition at ca. 225 nm was demonstrated to be useful for determining the absolute configuration of a variety of cyclic secondary alcohols, including those in which the carbinyl carbon is flanked by two methylene groups and those in which one of the carbons adjacent to the carbinyl carbon is bulkier and also more polar.[9] According to the benzoate sector rule, a negative Cotton effect at 225 nm in the CD spectrum of **1** indicated that α,β and β,γ bonds mainly fall in the positive benzoate sectors (Figure S28, Supporting Information), predicting the 3*S* configuration. The absolute configuration was supported by comparison of the experimental CD spectrum with the ECD spectrum predicted from quantum mechanical time dependent density functional theory (TDDFT) calculations.[10] The theoretically calculated ECD spectrum of **1** was in good agreement with the experimental CD spectrum (Figure S2, Supporting Information). Therefore, the structure of compound **1** was determined, and the name euphorbactin was assigned.

Compound **1** is characterized by the 6/5/7/3 fused-ring skeleton, which has never been identified in a natural product. Two plausible biosynthetic pathways for **1** are postulated in Scheme 1 (shown as black and red arrows, respectively). The biosynthetic precursor of **1** is proposed to be the co-occurring 3-benzoyloxy-5,6-epoxylathyr-12-en-15-ol-14-one (**2**) or/and euphoractin F (**3**).[7] An enzyme-catalyzed transannular cyclization of **2**, with a concomitant addition of one molecule of water, generates **3**, which is supported by the acid-catalyzed transformation of lathyrane analogues.[7c,11] An α-ketol rearrangement of **3** would give an intermediate (**4**), which would then undergo a sequential and/or simultaneous intramolecular nucleophilic addition to afford **1**. Alternatively, precursor **2**

Scheme 1. Plausible Biosynthetic Pathway of Euphorbactin (1)



would be transformed into intermediate **4** through the α-ketol rearrangement, followed by a similar transannular cyclization and intramolecular nucleophilic addition to produce **1**. As the absolute configurations of the lathyrane analogues and structurally related derivatives from species of the genus *Euphorbia* were unambiguously determined by single-crystal X-ray crystallography,[7] the biosynthetic pathways support the assignment of the absolute configuration for **1**. The α-ketol rearrangement has been proposed to be involved in the biosynthesis of various natural products,[12] and such rearrangements have been conducted using acidic or basic conditions or employing metal catalysis.[13] Indeed, this approach has been used with success in a number of natural product syntheses.[14] In order to exclude the possibility of **1** being fortuitously formed by some type of catalytic effect during the isolation procedure, the putative precursors **2** and **3** were separately refluxed in acetone or methanol with or without silica gel (the main solvents and absorbent used in the isolation procedure) for 48 h. The rearrangement did not occur under the simulated conditions, thus supporting that compound **1** is a true natural product.

In the in vitro bioassays performed in this study, compound **1** showed activity against HIV-1 replication,[7c] with IC_{50} and SI values 28.6 ± 0.8 μM and 86.1, respectively (the positive control zidovudine gave IC_{50} = 0.05 ± 0.03 μM and SI = 682.2). Other assays assessed antiviral activity against the herpes simplex virus 1 (HSV-1) and the influenza virus A/Hanfang/359/95 (H3N2);[15] inhibitory activity against the release of glucuronidase in rat polymorphonuclear leukocytes (PMN) induced by PAF;[16] cytotoxicity against several human cancer cell lines; and inhibitory activity against protein tyrosine

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phosphatase 1B (PTP1B). However, **1** was inactive at a concentration of 10 μM in each assay.

In conclusion, euphorbactin (**1**) was isolated as the minor component with activity against HIV-1 replication from the extract of *E. micractina* roots. The new structure provides a framework for synthesis and biological evaluation. In particular, the plausible biosynthetic pathway associated with the different types of co-occurring diterpenoids provides an important clue for further studies of biomimetic and total synthesis, chemical transformation, structural modification, and structure−activity relationships, as well as biosynthesis of the diverse diterpenoids from the genus *Euphoria*.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, ECD calculations; IR, ESIMS, HR-ESIMS, 1D and 2D NMR, UV, and CD spectra of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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(8) Euphorbactin (**1**): white amorphous powder, $[\alpha]^{20}_{D}$ + 99 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 224 (4.26), 251 (3.63) nm; CD (MeOH) 225 ($\Delta\varepsilon$ −1.80) nm; IR (Nujol) ν_{max} 3461, 2956, 2929, 2873, 1705, 1602, 1584, 1496, 1453, 1379, 1365, 1315, 1273, 1175, 1144, 1118, 1045, 1001, 978, 908, 897, 884, 712 cm^{-1}; ^{1}H NMR (acetone-d_6, 500 MHz) data, see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz) data, see Table 1; (+)-ESIMS *m*/*z* 479 $[M + Na]^+$, (−)-ESIMS *m*/*z* 455 $[M - H]^-$; (+)-HRESIMS *m*/*z* (+)-HR-ESIMS *m*/*z* 479.2408 $[M + Na]^+$ (calcd for $C_{27}H_{36}O_6Na$, 479.2404). For plant material and isolation procedures of compound **1**, see Supporting Information.

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