New Bioactive Diterpenoid from *Euphorbia decipiens*

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One new diterpene ester with a tricyclic lathyrane-or myrsinol-type skeleton has been isolated from *Euphorbia decipiens* Boiss. & Buhse. In addition to one new, the known constituents β-amyrin, β-amyrin acetate, methyl (2,4-dihydroxy-3-formyl-6-methoxy) phenyl ketone (2), and 1,1-bis(2,6-dihydroxy-3-acetyl-4-methoxyphenyl)methane (3) have been isolated from the same source. The structure elucidation of the isolated compounds was based primarily on 1D and 2D-NMR analysis, including COSY, HMQC, HMBC and NOESY correlations. Compound 1 showed inhibitory activity against prolyl endopeptidase, and also analgesic activity.

Introduction

Plants of the genus *Euphorbia* have been the source of a large number of biologically active compounds. The macrocyclic and polycyclic diterpenes isolated from different species of *Euphorbia* plants with ingenane, tigliane and daphnane skeletons have skin-irritant, tumour-promoting and anti-tumour activities [1–3]. Some esters of myrsinol isolated from *E. myrsinites* showed anti-HIV-1 reverse transcriptase (RT) inhibition [4]. As a part of our program to characterize the chemical constituents of the Iranian *Euphorbia* plants, our group has investigated *Euphorbia decipiens* Boiss. & Buhse, an endemic plant from the mountain Kandovan in the north of Karaj, Tehran, Iran. From this plant we have already reported the isolation of eight novel diterpene esters [5–7]. In order to obtain the minor compounds, the plant was again collected and extracted with acetone [8] and another eight new diterpene esters were isolated from it [9]. We describe here the isolation of, characterization and structure elucidation of one new tricyclic diterpenoid ester from the remaining soluble fraction. Compound 1 showed inhibitory activity against prolyl endopeptidase. Prolyl endopeptidase (PEP, EC 3.4.21.26) is the only serine protease known to cleave a peptide substrate in the C-terminal side of a proline residue [10]. Prolyl endopeptidase plays an important role in the metabolism of peptide hormones and neuropeptides and was recognized to be involved in learning and memory [11,12]. Moreover, alterations of PEP enzyme level and activity seems to be associated with several health disorders such as Alzheimer’s disease, depression, mania, thrombosis, AIDS and cancer [13]. Specific inhibitors of PEP are expected to have anti-amnesic effects. Many PEP inhibitors have been synthesized as candidates for the treatment of neuropathological disorders [14]. Compound 1 also showed analgesic activity.

Results and Discussion

Compound 1 was assigned the molecular formula C_{32}H_{44}O_{12} on the basis of HREIMS. Its IR spectrum showed peaks characteristic for the presence of hydroxyl (3460 cm\(^{-1}\)), carbonyl groups (1740, 1725 cm\(^{-1}\)) and for unsaturation (1640, 1590 cm\(^{-1}\)).

The \(^1\)H NMR spectrum of 1 showed five downfield signals due to the protons geminal to oxygen-bearing groups; three due to oxymethine groups [\(\delta\) 5.24 (t, \(J = 3.4\) Hz, H-3), 6.13 (d, \(J = 11.7\) Hz, H-5) and 4.79 (d, \(J = 6.3\) Hz, H-7)] and two due to oxymethylene groups [\(\delta\) 3.89 (d, \(J = 12.0\) Hz, H-17), 4.19 (d, \(J = 12.0\) Hz, H-11), with a relatively large coupling constant (\(J = 12.0\) Hz), suggesting the presence of free alkoxy group of the decipinone-type skeleton [5]. \(^1\)H NMR also showed four singlets for acetate methyl groups at
δ 2.08, 2.03, 1.99 and 1.92. The upfield shift of the last peak may be due to an anisotropic effect, which has been observed earlier [5]. The spectrum also showed three methyl signals in the molecule which comprise of a secondary methyl at δ 0.91 (d, J = 6.4 Hz, 3x H-16), one olefinic methyl at δ 1.75 (s, 3x H-19) and one tertiary methyl at δ 1.62 (s, 3x H-20) which seems to be geminal to an oxygen bearing group. 1H NMR also showed signals for two double bonds: an exocyclic methylene at δ 4.85 (br s, H-18’) and 4.84 (br s, H-18), and disubstituted double bond at δ 6.03 (dd, J = 1.9, 6.2, 9.3 Hz, H-8) and 5.73 (dd, J = 4.6, 9.4 Hz, H-9). Further analysis revealed the presence of one butanoyl, and four acetyl groups as ester moieties that were supported by fragment ions at m/z 71 [C8H12CO]+, and 43 [CH3CO]+.

The specific peaks for butanoyl in the 1H and 13C-NMR spectra further confirmed the presence of butanoyl group in this molecule. A triplet at δ 0.89 (t, J = 7.0 Hz, 3x H-4’) and signals for H-3’ and H-2’ which were overlapped with peaks at about δ 1.5 and δ 2.1 in the 1H NMR spectra of 1. Their corresponding peaks in 13C NMR (DEPT) at δ 13.6 q, 17.8 t and 37.0 t according to HMQC data together with the cross peaks between H-5 and H-2 with the butanoyl carbonyl carbon at δ 171.8 in HMBC suggested a butanoate ester at position 5.

A combination of 13C and DEPT NMR spectra showed 42 carbon atoms, including eight CH3, five CH2, nine CH, and ten quaternary carbons, of which eight are oxygenated (one tertiary alcohol, one tertiary ester, one ketonic and five ester carbonyls).

The 1H-1H and 13C-13C connectivities were supported by the 1H-1H-COSY and HMQC spectra. The locations of ester groups were established by an HMBC spectrum. HMBC correlations of δC 171.8 to δH 6.13 (H-5) indicated that butanoyl group was at C-5 (Table 2). HMBC correlations of ca. δC 170 to δH 5.24 (H-3), 4.79 (H-7) and 4.19 (H-17) indicated that acetate groups were at C-3, C-7, and C-17.

The stereochemistry of 1 was determined by comparison of the 1H NMR coupling constants of 1 with those recorded for myrsinol esters (5,6,7,9,15) with similar structure as well as by NOESY spectra. The coupling constant of H-3 (t, J = 3.4 Hz) and H-4 (dd, J = 3.4, 11.6 Hz) indicated that H-2 to H-4 must lie on one face of the molecule with the same dihedral angle between H-2/H-3 and H-3/H-4. The J (11.0 Hz) between H-4 and H-5 showed the trans relationship between them. The coupling constant of H-12 (d, J = 8.0 Hz) indicated the trans relationship between H-12 and H-11.

The NOESY cross peaks between δ 5.24 (H-3)/δ 2.28 (H-4), δ 6.13 (H-5)/δ 3.87 (H-12), established that H-5, H-12 must be located on one face of the molecule. In NOESY spectra cross peaks between δ 4.79 (H-7, H-18) with δ 3.87 (H-12) and δ 3.30 (H-11) were also detected, through which we concluded that H-7 and H-11 must be on one face of the molecule and H-12 and H-18 in another.

The 1H and 13C-NMR data are very similar to those recorded for decipide [5]. The up field shift for C(13) and the down field shift for C(15) (δ 80.9 (s) and 87.9 (s), resp. are the main difference to the 13C NMR data of decipide. The above data confirm the position of OH and AcO at C(13) and C(15), respectively [9].

In addition to the new compound 1, some known constituents such as methyl (2,4-dihydroxy-3-formyl-6-methoxy) phenyl ketone [16], 1,1-bis (2,6-dihydroxy-3-acetyl-4-methoxyphenyl) methane [16], β-amyrin [17], and β-amyrin acetate [18] have been isolated for the first time from our investigated source. Their structures were established by comparing their spectral data and physical constants.

Acetic acid induced abdominal constriction in mice is widely used method for evaluation of peripheral analgesic effect [19]. On this model compound 1 markedly reduced the number of mouse abdominal contractions (Table 1). The various doses of the compound inhibited the acetic acid induced abdominal constriction to a significant extent (P < 0.05, P < 0.01). Maximum inhibition of about 60% was produced at a dose of 20 mg/kg. The analgesic effects of compound 1 produced at 10 and 20 mg/kg were comparable to that shown by ibuprofen and aspirin as standard reference drugs 100 mg/kg. The non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin, ibuprofen and indomethacin can reduce the acid induced writhes by inhibiting the synthesis of prostaglandin which make the nociceptors more sensitive to pain producing agents such as bradykinin [20]. Therefore,
Table 1. Effect of compound 1 and standard compounds aspirin and ibuprofen on acetic acid induced writhing test in mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose [mg/kg] i.p.</th>
<th>Total number of abdominal constriction in 20 min.</th>
<th>Inhibition [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% DMSO</td>
<td>10 ml/kg</td>
<td>75±5</td>
<td>–</td>
</tr>
<tr>
<td>Compound 1</td>
<td>3</td>
<td>50±5</td>
<td>33.3*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29.8±5</td>
<td>60.3**</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>35.2±5</td>
<td>53.1***</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>30.3±6.4</td>
<td>59.6**</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>100</td>
<td>41.8±6.2</td>
<td>44**</td>
</tr>
<tr>
<td>Aspirin</td>
<td>100</td>
<td>38.7±4.3</td>
<td>48.4**</td>
</tr>
</tbody>
</table>

Mice received intraperitoneally different doses of compound 1 and standard compounds 30 min. prior to the administration of 0.9% acetic acid. Data show the number of writhes (mean ± S. E. M.) produced between 5–25 min. after the administration of acetic acid. (n = 4–10).

* p< 0.05 and ** p < 0.01.

The results obtained in the present study suggest NSAIDs like activity of the compound 1. Further investigations are necessary to elucidate the exact mechanism of the analgesic effect of this compound.

For cardiovascular effects various doses of compound 1 were injected intravenously and no detectable changes in blood pressure and heart rate were observed up to the maximum dose of 3 mg/kg. However, higher doses could not be tested due to limited solubility of the compound in required volume of injection (1 ml/kg).

Low molecular weight inhibitors of PEP have been reported in the literature but the majority of these are synthetic. Most of the natural inhibitors have been isolated as PEP inhibitors from microbial origin but PEP inhibitors from plants have been rarely investigated [21]. The compound 1 has shown IC_{50} of 21.8 ± 0.5567 µM (Table 3) with the positive control of PEP (Z-Pro-linal), which is as good in activity as previously reported natural inhibitors [14,22].

**Experimental Section**

**General**

Column chromatography (CC): silica gel, 70–230 mesh. Flash chromatography (FC): silica gel 230–400 mesh. TLC: pre-coated silica gel G-25-UV_{254} plates; detection at 254 nm, and by ceric sulphate reagent. Optical rotations: Jasco-DIP-360 digital polarimeter. UV and IR Spectra: Hitachi-UV-3200 and Jasco-320-A spectrophotometer, respectively. $^1$H- and $^{13}$C NMR, COSY, HMOC and HMBC Spectra. Bruker spectrometers operating at 500 and 400 MHz; chemical shifts $\delta$ in ppm and coupling constants in Hz. Ei-, CI MS: JMS-HX-110 with a data system.

**Collection and identification**

The plant Euphorbia decipiens Boiss. & Buhse (Euphorbiaceae) was collected at the mountain Kandovan, north of Karaj, Iran, in 1998, and identified by Mr. Bahram Zehzad (plant taxonomist) at the Department of Biological Sciences, Shahid Beheshti University, Eveen, Tehran. A voucher specimen (no. 98,112) has been deposited at the herbarium of the Biology Department of Shahid Beheshti University, Eveen, Tehran.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^{*}$IC_{50} [µM]</th>
<th>Z-Pro-linal (positive control) for PEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.8 ± 0.5567</td>
<td>1.27 ± 0.01 nM</td>
</tr>
</tbody>
</table>

$^*$ IC_{50} values are the mean ± standard mean (SEM) error of three assays.

Table 2. HMBC$^c$ data for 1.

<table>
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<tr>
<th>Protons correlating with carbon resonance.</th>
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</table>

Table 3: In vitro quantitative inhibition of Prolyl endopeptidase by compound 1.
Extraction and purification

The air-dried ground plant (4 kg) was exhaustively extracted with acetone at r.t. The extract was evaporated to yield the residue (62 g). The defatted extract (51 g) was extracted with chloroform. The chloroform extract (44 g) was subjected to CC over a silica gel column (880 g) using hexane with gradient of CHCl₃ upto 100% and followed by methanol. Twenty fractions were collected.

The fraction no. 9 was loaded on preparative plates using system of hexane: EtOAc (55:45) to purify compounds 1 (colorless oil, 24.2 mg). Fraction no. 1 was subjected to CC eluting with Hexane-Chloroform (9.0:1.0) to obtain compounds 2 (6.8 mg) and 3 (9.7 mg). Fraction no. 2 was loaded chromatographed on silica gel column, eluted with a gradient of Me₂CO and petrol. The fraction no. 3 thus obtained was again loaded on silica gel column to give compounds 2 and 3.

Cardiovascular evaluation

The effect of compound 2 on blood pressure was studied in normotensive anaesthetized Wister rats (200–250 g). Animals were anaesthetized with thiopental sodium (60–80 mg/kg, i.p.). The arterial blood pressure was recorded from the carotid artery via the arterial cannula connected to a pressure transducer coupled with a Grass model 79 polygraph. Drugs were injected via the cannula inserted in the jugular vein. Mean blood pressure was calculated as the diastolic blood pressure plus one-third of plus width. Acetyl-choline and noradrenaline (1 µg/kg) were used as a positive control.

Enzyme inhibition assay

Chemicals. Prolyl endopeptidase (Flavobacterium meningosepticum origin) was purchased from Seikagaku Corporation (Tokyo, Japan) and N-benzyloxy carbonyl-Gly-Pro-pNA was procured from BACHEM Fine Chemicals Co. Specific inhibitor of PEP, N-benzyloxy carbonyl-pro-prolinal, was kindly donated by Dr. Hideaki Shimizu, Yachult Central Institute For Microbiological Research, Tokyo, Japan.

PEP inhibition assay

The PEP inhibition activity was assayed by a modification of the method of Yoshimoto et al. [25] 100 M Tris (hydroxymethyl)-aminomethane-HCl buffer containing 1 mM EDTA, pH 7.0, 247 µl, PEP (0.02 unit/300 µl) 15 µl and test sample in 8 µl MeOH, were mixed in 96-well microplate and preincubated for 10 min. at 30 °C. The reaction was initiated by adding 30 µl of 2 mM of N-benzyloxy carbonyl-Gly-Pro-pNA (in 40% 1,4-dioxane) as the substrate. The amount of released \( p \)-nitroaniline was determined spectrophotometrically, as increase in absorption at 410 nm, with 96-wells microplate reader (Molecular Devices, Spectramax 340 USA). The IC₅₀ values were the average of at least three determinations performed in duplicate.

Compound 1

Colorless oil (24.2 mg): \([α]_D^{23} = -1.96 (c = 0.204, \text{CHCl}_3)\); UV (MeOH) \(λ_{max} = 201.6 \text{ nm}\); IR \(ν_{max}\)
Data of known constituents

**β-Amyrin**

M.p. 193–195°C (CHCl₃ + MeOH). C₃₀H₄₆O₁₀ [α]₂⁰ +68.9° (CHCl₃). – IR \( \nu_{\text{max}} \) (KBr): 1733, 1663 and 1567 cm⁻¹. – ¹H NMR (500 MHz, CDCl₃): \( \delta = 0.87 \) (s, 3 H, 27-H, 26-H, 23-H, 25-H), 3.58 (s, each 3 H, 28-H, 24-H), 0.86 (s, 6 H, 30-H, 29-H), 0.93, 0.96, 0.99, 1.12 (s, each 3 H, 27-H, 26-H, 23-H, 25-H), 3.22 (m, \( W_{1/2} = 12.0 \) Hz, 1 H, 3-H). – EIMS \( m/z \) (rel.int. %): 462 (M⁺, \( \text{C}_{30}\text{H}_{46}\text{O}_{10} \)), 411 (2.1), 408 (0.5), 393 (0.5), 218 (100), 207 (2), 203 (24), 191 (0.5), 189 (5), 175 (5), 161 (4), 141 (5), 135 (8), 133 (4), 123 (16), 121 (19), 119 (9), 109 (7) and 107 (8).

**β-Amyrin acetate**

M.p. 241.0–242.5°C (CHCl₃ + MeOH). C₃₂H₄₄O₁₂ \[\alpha]₂⁰ +68.9° (c = 0.94, CHCl₃). – IR \( \nu_{\text{max}} \) (KBr): 1772, 1635 (C = CH), 1240 (C = O, acetate) and 812 (C = CH) cm⁻¹. – ¹H NMR (500 MHz, CDCl₃) \( \delta = 0.87 \) (s, 3 H, 27-H, 26-H, 23-H, 25-H), 3.58 (s, each 3 H, 28-H, 24-H), 0.86 (s, 6 H, 30-H, 29-H), 0.98 (s, 6 H, 25-H, 26-H), 1.14 (s, 3 H, 27-H), 2.07 (s, 3 H, OCOCH₃), 4.54 (dd, \( J = 6.0, 11.0 \) Hz, 1 H, 3-H), 5.21 (t, \( J = 3.5 \) Hz, 1 H, 12-H). – EIMS \( m/z \) (rel.int. %): 468 (M⁺, \( \text{C}_{32}\text{H}_{52}\text{O}_{2} \)), 453 (2), 408 (M⁺ – CH₃COOH, 4), 218 (100) and 203 (47).

**Methyl (2, 4-dihydroxy-3-formyl-6-methoxy) phenyl ketone (2)**

Plates, m.p. 137–138°C. C₁₉H₁₉O₅. IR \( \nu_{\text{max}} \) (KBr): 3300 (OH), 2710 (CHO), 1710 (C=O), 1595, 1460 (aromatic) cm⁻¹. – ¹H NMR (CDCl₃): \( \delta = 0.87 \) (s, 3 H, 27-H, 26-H, 23-H, 25-H), 3.58 (s, each 3 H, 28-H, 24-H), 0.86 (s, 6 H, 30-H, 29-H), 0.93, 0.96, 0.99, 1.12 (s, each 3 H, 27-H, 26-H, 23-H, 25-H), 3.22 (m, \( W_{1/2} = 12.0 \) Hz, 1 H, 3-H). – EIMS \( m/z \) (rel.int. %): 462 (M⁺, \( \text{C}_{30}\text{H}_{48}\text{O}_{4} \)), 411 (2.1), 408 (0.5), 393 (0.5), 218 (100), 207 (2), 203 (24), 191 (0.5), 189 (5), 175 (5), 161 (4), 141 (5), 135 (8), 133 (4), 123 (16), 121 (19), 119 (9), 109 (7) and 107 (8).

Acknowledgement

We are thankful to Mr. Bahram Zehzad for identification of the plant.