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Constituents of the stem bark of *Discopodium penninervium* and their LTB_4 and COX-1 and -2 inhibitory activities

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Abstract

The stem bark of *Discopodium penninervium* afforded a withanolide, 6α,7α-epoxy-1-oxo-5α,12α,17α-trihydroxywitha-2,24-dienolide (**1**) and a coloratane sesquiterpene, 7α,11α-dihydroxy-4(13),8-coloratadien-12,11-olide (**4**) along with five known compounds, withanone (**2**), 5α,17β-dihydroxy-6α,7α-epoxy-1-oxowitha-2,24-dienolide (**3**), 7α,11α-dihydroxy-8-drimen-12,11-olide (**5**), withasomnine (**6**), and (*E*,*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**7**). The identity of the compounds was established on the basis of spectroscopic data analysis. All compounds were assessed for inhibition of leukotriene metabolism in an in vitro bioassay using activated human neutrophile granulocytes, and for in vitro cycloxygenase-1 and -2 inhibition from sheep cotyledons and seminal vesicles, respectively. In the leukotriene biosynthesis assay all compounds tested at a concentration of 50 μM exhibited activity with percentage inhibitions ranging from 11.5 to 36.6. The withanolide, **1**, displayed a 46.4% inhibition of COX-2 and a 22.9% inhibition of LTB_4 formation at 50 μM concentration. Compounds **4** and **6** inhibited LTB_4 biosynthesis but showed minor inhibition of COX-1 and COX-2. The remaining compounds, on the other hand, were found to be inactive on COX enzymes.

Keywords: *Discopodium penninervium*; Solanaceae; LTB_4; Cyclooxygenase; Withanolide; Coloratane sesquiterpene; 6α,7α-Epoxy-1-oxo-5α,12α,17α-trihydroxywitha-2,24-dienolide; 7α,11α-Dihydroxy-4(13),8-coloratadien-12,11-olide

1. Introduction

Discopodium penninervium Hochst (Solanaceae), a shrub or small tree, is the only species in the genus and endemic to Ethiopia. The leaves and barks are used for the treatment of schistosomiasis, leprosy and stomach-ache in Ethiopian folk medicine (Geyid et al., 2005).

Previous phytochemical investigation of *D. penninervium* revealed the presence of 5α,17β-dihydroxy-6α,7α-epoxy-1-oxowitha-2,24-dienolide, withanone and withanolide A in the roots (Habtemariam and Gray, 1998), and 5,6-epoxy-16-oxygenated withanolides, jaborosalactone-L, and 17-epiacnistin-A in the leaves (Habtemariam et al., 1993, 2000). The latter *Discopodium* withanolides have been shown to exhibit cytotoxic activities to both human and murine cancer cell lines and immunosuppressive activity in rat spleen cells in vitro (Habtemariam et al., 2000).

In the continuation of a search for biologically active constituents from Ethiopian medicinal plants, we report herein the isolation and structural elucidation of a new withanolide, **1**, and a new coloratane sesquiterpene, **4**, together with five known compounds from the stem bark of *D. penninervium* (see Fig. 1). The anti-inflammatory activity of the compounds was assessed in vitro using leukotriene formation, and cycloxygenase-1 and -2 inhibitory assays.

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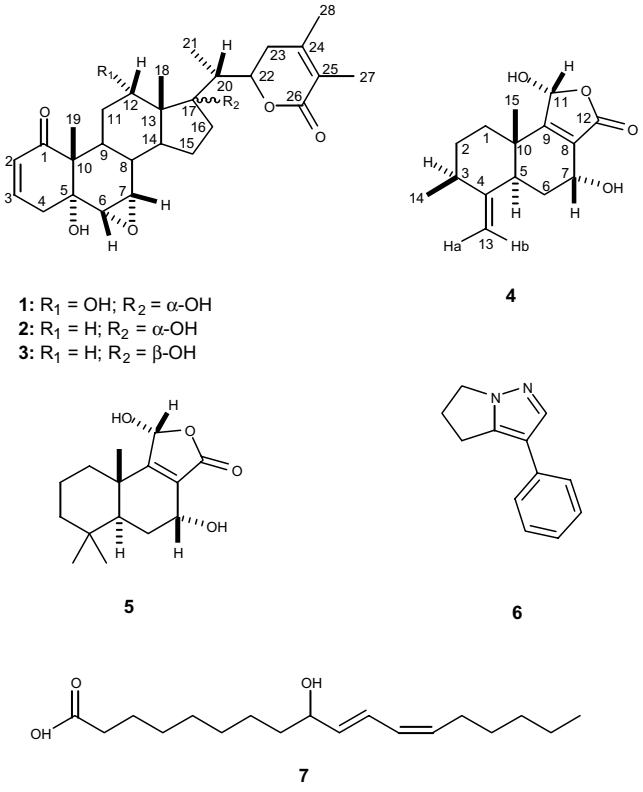

Fig. 1. The structures of compounds **1**–**7**.

2. Results and discussion

Compound **1** was obtained as amorphous powder and its molecular formula was deduced as $C_{28}H_{38}O_7$ by the LC-ESI-MS data at m/z 487 $[M+H]^+$. The HRMS showed an ion $[M-H]^-$ peak at m/z 485.2553 corresponding to the molecular formula, $C_{28}H_{38}O_7$. An absorption maximum at 223 nm in the UV spectrum was indicative of an α,β-unsaturated δ-lactone structural feature, which was further confirmed by absorption band at 1694 cm^{-1} observed in the IR spectrum. The broad absorption band at 3493 cm^{-1} in the IR spectrum was attributed to hydroxyl stretching. The ^1H NMR spectrum of **1** showed resonances for five methyl groups, with four tertiary methyls at δ 0.90, 1.18, 1.79, 1.91 and one secondary methyl at δ 1.14 (*d*, $J = 6.7$ Hz). The first two methyls resonances were assigned to CH_3-18 and CH_3-19, respectively, whereas the latter two downfield resonances were assigned in turn to CH_3-27 and CH_3-28, and were attached to olefinic carbons. The methyl signal resonated as a doublet was assigned to CH_3-21. Among the ten methine protons that appeared in the ^1H NMR spectrum, the deshielded doublet at δ 5.67 and the double of doublets at δ 6.62, which were coupled to one another, were assigned to H-2 and H-3 respectively. This further confirmed the presence of α,β-unsaturated ketone moiety. A 1H doublet at δ 3.07 and a multiplet at δ 3.33 were assigned to H-6 and H-7, respectively, and the small coupling (3.8 Hz) observed for H-6/H-7 indicated that these hydrogens are axial and equatorial, respectively. This is in agreement with a previous report by Habtemariam and Gray (1998).The presence of an epoxide between C-6 and C-7 was inferred from the low field resonances (56.9 and 57.3 ppm) observed for these two oxygenated carbons in the ^{13}C NMR spectrum. A broad triplet at δ 4.36 was assigned to a methine proton attached to C-12, as it showed a correlation with this carbon at δ 76.3 in the HMQC spectrum, although a methine carbon resonance was absent in the ^{13}C NMR spectrum. This assignment was further substantiated by HMBC cross peaks observed between C-12 and the methyl protons at position 18 and the ^1H–^1H COSY correlation between H-11 and H-12. The remaining methine proton resonances were placed by analysis of HMQC, HMBC and COSY spectra. The upfield carbon resonance at δ 28.3 was assigned to C-9 on the basis of HMBC cross peaks observed for H-12/C-9 and H-19/C-9. All the resonances for methylene protons were assigned unambiguously by HMQC, HMBC and COSY NMR experiments. The relative configuration of the hydroxyl group at position 12 was established on the basis of the small coupling constant of H-12 and by a NOESY NMR experiment (Fig. 2), showing

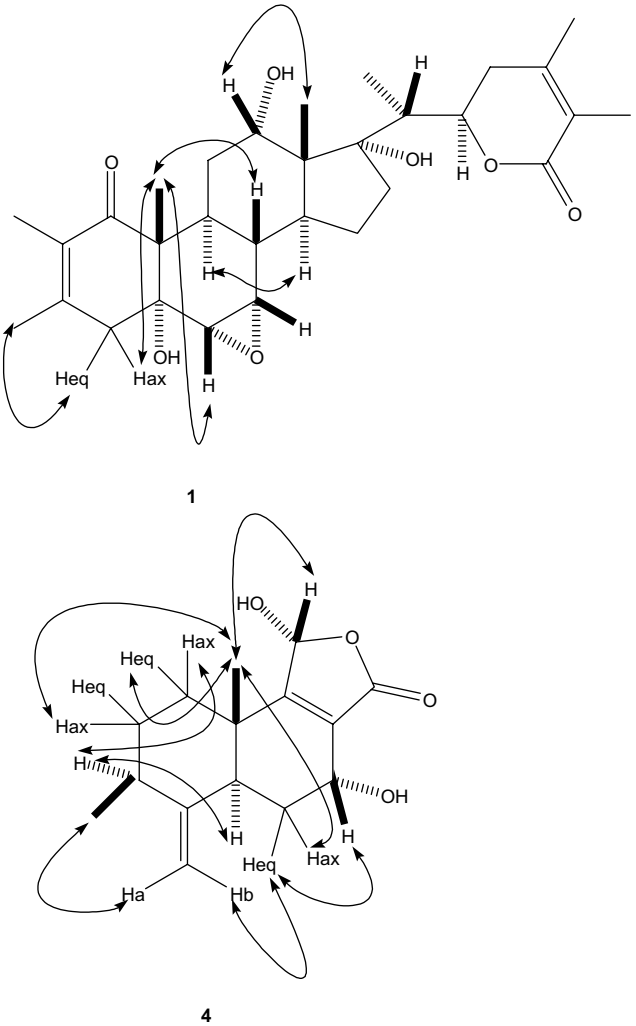

Fig. 2. Major NOE correlations in compounds **1** and **4**.

NOE enhancement between H-12 and CH_3-18. The relative stereochemistry of C-17, C-20 and C-22, on the other hand, are the same as in compound **2**. Comparison of the above data with those in the literature indicated that the structure of **1** is very closely related to that of withanone (**2**) (Habtemariam, 1997), except for the presence of an additional hydroxyl group at position 12 in **1**. Thus, the structure of **1** was established as 6α,7α-epoxy-1-oxo-5α,12α,17α-trihydroxywitha-2,24-dienolide.

Compound **4** was obtained as white needles in *n*-hexane/EtOAC. Its molecular formula $C_{15}H_{20}O_4$ was determined by HRMS (*m/z*; measured 263.1291 $[M-H]^-$; calc. 263.1289). The proton and ^{13}C NMR data were suggestive of a coloratane skeleton for **4** (Wube et al., 2005). The IR spectrum showed a strong absorption band at 1762 and 1740 cm^{-1}, indicating the presence of an α,β-unsaturated lactone (Sakio et al., 2001). This structural feature was further supported by an absorption maximum at 210 nm in the UV spectrum and double bond carbon resonances at δ 129.5 (C-8) and 170.0 (C-9), and a signal for a lactone carbonyl carbon at δ 170.7 (C-12) in the ^{13}C NMR spectrum. In addition a hydroxyl stretch band at 3436 cm^{-1} was observed in the IR spectrum. The ^{13}C and DEPT NMR spectra also revealed the presence of two methyls, four methylenes, four methines, and five quaternary carbons.

Two 3H signals, a singlet and a doublet, were observed in the ^1H NMR spectrum of **4.** The singlet at δ 0.98, which showed HMBC cross peaks with C-1 at δ 35.1 and C-9 at δ 170.0, was assigned to CH_3-15. The methyl doublet gave HMBC correlations with C-2 at δ 32.6, C-3 at δ 39.1 and C-4 at δ 153.5 was assigned to CH_3-14. The signals for the H-13a and H-13b protons were clearly assigned based on NOE correlations observed between CH_3-14 and H-13a and between H-6α and H-13b in the NOESY spectrum. Four 1H signals at δ 2.13, 2.53, 4.48, and 6.22 were assigned to H-3, H-5, H-7, and H-11, respectively. The chemical shifts for the remaining methylene protons were assigned by a detailed analysis of HMQC and HMBC spectra. Exocyclic double bond carbon resonances at δ 153.5 (C-4) and 105.1 (C-13), observed in the ^{13}C NMR spectrum, were characteristic of coloratane sesquiterpenes. The carbon resonances at δ 97.8 and 59.7 were assigned to a hemiketal C-11 and the hydroxyl bearing C-7, respectively. The relative configuration of the five asymmetric centres in the coloratane skeleton was determined by analysis of the coupling constants and NOESY experiments. The NOESY spectrum of **4** showed correlations between the protons of H-3 and H-1α as well as the H-3 and H-5. These correlations require that H-3 and H-5 are *cis* to each other, which would enable the C-3 and C-5 protons to have α-orientation. Similarly, H-11 exhibited a NOE correlation (Fig. 2) with CH_3-15, and this implied that H-11 and CH_3-15 are on the same relative axial face as each other. This would imply that hydroxyl group at C-11 be equatorial and alpha. A small coupling constant value of H-7 (δ 4.48, *d*, $J = 3$ Hz) suggested an α-orientation for the hydroxyl group. On the basis of these observations, compound **4** was established structurally as 7α,11α-dihydroxy-4(13),8-coloratadien-12,11-olide.

The known compounds were identified by analysis of their physical and spectral data and by comparison with published values as withanone (**2**) (Habtemariam and Gray, 1998), 5α,17β-dihydroxy-6α,7α-epoxy-1oxowitha-2,24-dienolide (**3**) (Nittala and Lavie, 1981), 7α,11α-dihydroxy-8-drimen-12,11-olide (**5**) (Sakio et al., 2001), withasomnine (**6**) (Houghton et al., 1994), and (*E*,*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**7**) (Murakami et al., 1992).

The drimane sesquiterpene, 7α,11α-dihydroxy-8-drimen-12,11-olide (**5**), was previously isolated from the animal species, *Dendrodoris carbunculosa* (Sakio et al., 2001) and this is the first report of its occurrence in the plant kingdom. Pyrazole alkaloids appear to be rare in plants of the Solanaceae, although they have been found to occur, for example in *Newbouldia laevis* (Bignoniaceae) (Aladesanmi et al., 1998), *Elytraria acaulis* (Acanthaceae) (Ravikanth et al., 2001) and *Withania somnifera* (Solanaceae) (Schroeter et al., 1966). The occurrence of withasomnine (**6**) in *D. penninervium* is interesting from a chemotaxonomic point of view because it was also isolated from the Indian medicinal plant *W. somnifera*. Moreover, this is the first report of the occurrence of drimane and coloratane sesquiterpenes in the family Solanaceae.

All compounds were evaluated for inhibition of leukotriene metabolism as well as COX-1 and -2 enzymes in vitro. The results presented in Table 3 show that all compounds tested in the leukotriene biosynthesis assay showed some inhibitory effects on LTB_4 formation. The sesquiterpene **4** displayed the highest activity against LTB_4. On the other hand, compound **5**, the biogenetic precursor of compound **4** was found to be three times less active than compound **4**. Thus, the presence of an exocyclic methylene group at position 4 might enhance the LTB_4 inhibitory activity of compound **4**. This is the first report on LTB_4 inhibitory activity of withanolides. Recently, we have reported the leukotriene biosynthesis inhibitory effect of drimane and coloratane sesquiterpenes obtained from the stem bark of *Warburgia ugandensis* (Wube et al., 2006) and results of our study revealed that compounds having a dialdehyde structural feature displayed superior inhibitory effects on the stable metabolite of 5-LOX, LTB_4. The in vitro COX-1 and -2 test results revealed that at a concentration of 50 μM, compounds **4** and **6** showed dual COX-1 and -2 inhibition, whereas compounds **2**, **3**, **5**, and **7** produced no inhibitory effects. The new withanolide, compound **1**, displayed a selective inhibition of the COX-2 enzyme with an IC_{50} value close to 50 μM. Unfortunately, due to lack of compound higher amounts could not be tested. This is the second report on the cycloxygenase inhibitory activity of withanolides and the selective COX-2 inhibitory potency of compound **1** is much higher than the withanolide obtained from *W. somnifera* (Jayaprakasam and Nair, 2003). As compared to compounds **2** and

3, the COX-2 inhibitory potency of compound **1** might be enhanced by the hydroxyl group at position 12.

Concerning anti-inflammatory drugs, selective COX-2 inhibitors are considered to have advantages over non-selective NSAIDs by a lower risk of gastrointestinal side effects (Smith et al., 2000). Furthermore, it has been evident that COX-2 and 5-LOX pathways are both involved in cell proliferation and angioneogenesis. Therefore the withanolide **1** represents interesting features of a dual inhibitor of COX-2 and leukotriene formation and may serve for the development of anti-inflammatory and cancer chemo preventive agents. However, further structural variations should be evaluated in order to improve the inhibitory potency of **1** in the COX-2 and LTB_4 assays.

3. Experimental

3.1. General experimental procedures

Melting points were determined with Kofler microscope and are uncorrected. Optical rotations were measured on a Perkin–Elmer 241 MC polarimeter. UV spectra were recorded on Shimadzu UV-160A spectrophotometer. Perkin–Elmer 881 infrared spectrophotometer was used in recording the IR spectra. NMR spectra were recorded at 500 MHz for ^1H and 125 MHz for ^{13}C on a Bruker AVANCE 500 spectrometer. All spectra were measured in $CDCl_3$, except for compounds **1** and **4**, which were dissolved in $(CD_3)_2CO$. HRMS were determined with Micromass QTOF Ultima using the internal standard TCA, which had $[M-H]^- = m/z$ 514.2839. Mass spectra were also obtained by LC-ESI-MS analysis on a Thermo-Finnigan LCQ Deca XP Plus mass spectrometer connected to a Surveyor LC-system (Thermo-Finnigan). The absorbance for LTB_4 quantification was conducted using a Tecan RAIN BOW photometric ELISA plate reader.

Chromatographic separation were performed by analytical TLC on Si gel 60 F_{254} (0.2 mm thick), column chromatography on Si gel 60 (70–240 mesh), size exclusion chromatography on Sephadex LH-20, solid phase separation on Isolute C_{18} (10 g) columns, and semipreparative HPLC with LiChrospher® RP-18 (10 μm, 250 times 10 mm i.d.) column.

Türks solution, Na_2EDTA, $CaCl_2 \cdot 2H_2O$ p.a., anhydrous D-glucose, $MgCl_2 \cdot 6H_2O \cdot KCl$, Tris p.a., formic acid and ethanol p.a. were purchased from Merck. Trypan blue solution and eicosatetraenoic acid were obtained from Sigma Chemicals. Ca ionophor A 23187 and epinephrine-hydrogentartrate were bought from Fluka. A LTB_4 EIA kit, purified PGHS-1 and -2, indomethacin, NS-398 and arachidonic acid were obtained from Cayman Chemicals, Ann Arbor, MI, USA. A PGE_2-EIA kit was obtained from R&D systems, Minneapolis, MN, USA. TRIS/HCl buffer (pH 8.0) was bought from Roth. Hematin was obtained from porcine, ICN, Aurora, OH, USA. Zileuton was purchased from Sequoia Research Products Ltd., Oxford, UK.

3.2. Plant material

The stem bark of *D. penninervium* was collected in April 2001 from Dinsho towards Addis Ababa and Dodola in Bale zone, Ethiopia and identified by Mr. Melaku Wondafrash, the National Herbarium, Department of Biology, Addis Ababa University. A voucher specimen (collection number 1487) has been deposited in the National Herbarium, Department of Biology, Addis Ababa University, Addis Ababa, Ethiopia.

3.3. Extraction and isolation

The air-dried powdered stem barks of *D. penninervium* (600 g) were extracted successively with petroleum ether and dichloromethane in a Soxhlet apparatus. The dichloromethane extract was concentrated at reduced pressure to give 5 g of a greenish residue. After removal of chlorophyll with Sephadex LH-20 eluting with CH_2Cl_2–MeOH (1:1), the residue (1.2 g) was subjected to solid-phase separation using a H_2O/MeOH (100:0 → 0:100) gradient elution and ten fractions of 100 ml each were collected. Fraction 4, eluted with H_2O-MeOH (7:3), was purified by semi-prep. HPLC using MeCN/H_2O (35:65 → 55:45) gradient elution for 25 min to afford **5** (2 mg). Fr. 8, eluted with H_2O/MeOH (3:7), was subjected to semi-prep. HPLC using MeCN/H_2O (4:6 → 1:1) gradient elution for 40 min to yield **1** (3 mg), **3** (6.5 mg) and **2** (9 mg) at 24, 32 and 34.7 min, respectively. Fr. 9 and 10 were combined and applied to a Sephadex-20 column using CH_2Cl_2 as eluent to give 56 subfractions of 20 ml each. Subfr. 19–27 gave **4** (11 mg) after purification by semi-prep. HPLC using a MeCN/H_2O (35:65 → 65:35) gradient system as eluents. Subfr. 31–46 were further chromatographed on semi-prep. HPLC using a MeCN/H_2O (45:55 → 8:2) gradient elution for 45 min to afford **6** (2 mg) and **7** (14 mg) at 9 and 37 min, respectively.

3.4. In vitro leukotriene metabolism, cycloxygenase-1, and -2 inhibitory assays

The leukotriene metabolism inhibitory assay was conducted as described previously (Adams et al., 2004) using human neutrophile granulocytes with some modification. After isolation of human neutrophile granulocytes from human blood, cell vitality test, cell concentration determination, and incubation with test sample solution the samples were diluted 40-fold and the free LTB_4 concentration was measured using a competitive LTB_4 EIA kit. LTB_4 biosynthesis inhibition was quantified by measuring the absorption at 405 nm after addition of 50 μl aqueous Na_3PO_4 as stop solution. Inhibition was expressed in percent in relation to a control using abs. EtOH. Inhibition values are means of three experiments and each sample was tested in duplicate. Zileuton was used as a positive control.

The cycloxygenase-1 and -2 assays were done with purified PGHS-1 from ram seminal vesicle for COX-1 and purified PGHS-2 from sheep placental cotyledons for COX-2

as reported previously (Fiebich et al., 2005; Rollinger et al., 2005). The percent inhibitions at 50 μM concentration of test compounds were determined for both enzymes. Inhibition values were means of three experiments and each sample was tested in duplicate. Indometacin and NS-398 were used as positive control for COX-1 and -2, respectively.

3.5. 6α,7α-Epoxy-1-oxo-5α,12α,17α-trihydroxywitha-2,24-dienolide (*1*)

Colourless optically inactive amorphous powder, UV λ_{max}^{MeOH} nm (log ε) 223 (3.86), IR ν_{max}^{MeOH} cm^{-1}: 3493, 1694, 1385, 1130, 1094, ^1H NMR (500 MHz $(CD_3)_2CO$) and ^{13}C NMR (125.8 MHz $(CD_3)_2CO$) data, see Table 1, ESI-MS (70 eV) *m/z* (rel. Int.): 487 $[M+H]^+$ (34), 469 $[M-OH]^+$ (100), 452 (62), 171 (14); HRMS found 485.2553; $C_{28}H_{38}O_7$, calc. 485.2545.

3.6. 7α,11α-Dihydroxy-4(13),8-coloratadien-12,11-olide (*4*)

White needles from *n*-hexane-EtOAC mixture, m.p. 143–145 °C, $[\alpha]_D^{28}$ +90.0 (MeOH; *c*0.90), UV λ_{max}^{MeOH} nm (log ε): 210 (3.96), IR ν_{max}^{MeOH} cm^{-1}: 3436, 1762, 1740, 1177, 1036, 940, 904, ^1H NMR (500 MHz $(CD_3)_2CO$) and ^{13}C NMR (125.8 MHz $(CD_3)_2CO$) data, see Table 2, HRMS found 263.1291; $C_{15}H_{20}O_4$, calc. 263.1289.

Table 1
^1H, ^{13}C NMR and HMBC correlations data for compound **1** in acetone-d_6[a]

Position	δ_H (*J* in Hz)	δ_C, multiplicity	HMBC
1		203.6, C	H-19
2	5.67 (*dd*) (10.1, 2.2)	129.3, CH	
3	6.62 (*ddd*) (10.1, 5.2, 2.2)	141.3, CH	
4α	2.56 (*dd*) (14.5, 3.5)	37.8, CH_2	H-2
4β	2.77 (*m*)		
5		74.1, C	H-19
6	3.07 (*d*) (3.8)	56.9, CH	
7	3.33 (*m*)	57.3, CH	
8	1.98 (*m*)	37.2, CH	
9	2.01 (*m*)	28.3, CH	H-12, H-14
10		51.6, C	H-19
11α	2.73 (*m*)	31.4, CH_2	
11β	1.60 (*m*)		
12	4.36 (*t*) (2.2)	76.3, CH	H-18
13		50.2, C	H-18
14	1.84 (*m*)	40.8, CH	H-18
15α	2.07 (*m*)	23.1, CH_2	
15β	1.36 (*m*)		
16α	2.37 (*m*)	37.7, CH_2	
16β	1.45 (*m*)		
17		87.1, C	H-18, H-21
18	0.90 (*s*)	16.4, CH_3	
19	1.18 (*s*)	15.1, CH_3	
20	2.13 (*m*)	43.7, CH	H-21
21	1.14 (*d*) (6.7)	9.2, CH_3	
22	4.55 (*dt*) (12.5, 3.5)	79.7, CH	H-21
23α	2.21 (*m*)	33.4, CH_2	
23β	2.45 (*dd*) (18.5, 9.5)		
24		151.4, C	H-27, H-28
25		121.7, C	H-27, H-28
26		166.9, C	H-27
27	1.79 (*s*)	12.6, CH_3	
28	1.91 (*s*)	20.5, CH_3	

[a] Chemical shifts are in ppm relative to TMS; 500 MHz for ^1H and 125 MHz for ^{13}C.

Table 2
^1H, ^{13}C NMR and HMBC correlations data for compound **4** in acetone-d_6[a]

Position	δ_H (*J* in Hz)	δ_C, multiplicity	HMBC
1α	1.78 (*dt*) (12.6, 4.0)	35.1, CH_2	H-15
1β	1.99 (*m*)		
2α	1.35 (*m*)	32.6, CH_2	H-14
2β	1.80 (*m*)		
3	2.13 (*m*)	39.1, CH	H-13α, H-14
4		153.5, C	H-3, H-6α, H-14
5	2.53 (*d*) (12.0)	44.5, CH	H-6β, H-7, H-13β, H-15
6α	1.85 (*m*)	32.1, CH_2	
6β	1.89 (*m*)		
7	4.48 (*d*) (3.0)	59.7, CH	H-6α
8		129.5, C	H-6 α, H-7, H-11
9		170.0, C	H-15
10		39.4, C	H-6α, H-15
11	6.22 (*s*)	97.8, CH	
12		170.7, C	H-11
13α	4.86 (*bs*)	105.1, CH_2	H-3
13β	4.67 (*bs*)		
14	1.09 (*d*) (6.5)	18.5, CH_3	
15	0.98 (*s*)	17.2, CH_3	

[a] Chemical shifts are in ppm relative to TMS; 500 MHz for ^1H and 125 MHz for ^{13}C.

Table 3
Leukotriene metabolism, COX-1 and -2 inhibitory activities of compounds isolated from *D. penninervium* at 50 μM test concentration and IC_{50} values of the positive controls zileuton, indomethacin and NS-398

Compound	% Inhibition ± SD at 50 μM test concentration		
	LTB_4	COX-1	COX-2
1	22.9 ± 1.98	NA	46.4 ± 2.23
2	28.7 ± 1.54	NA	NA
3	18.9 ± 6.63	NA	NA
4	36.6 ± 1.38	2.1 ± 9.15	11.83 ± 6.86
5	11.5 ± 3.98	NA	NA
6	25.6 ± 3.24	6.8 ± 4.59	6.4 ± 2.31
7	22.5 ± 6.92	NA	NA
Zileuton	10.0 μM (IC_{50})	ND	ND
Indomethacin	ND	1.25 μM (IC_{50})	ND
NS-398	ND	ND	5.0 μM (IC_{50})

NA, not active and ND, not determined.

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