A new bioactive ursane-type triterpenoid from *Croton bonplandianum* Bail

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Abstract. A new triterpenoid 3 α -hydroxy-urs-12,15-dien (**1**) of ursane skeleton along with three others, viz. oleanolic acid (**2**) and ursolic acid (**3**) and β -sitosterol (**4**) were isolated from the root of *Croton bonplandianum* Bail. Their chemical structures were established on the basis of spectroscopic analysis (IR, 1D NMR, 2D NMR, Mass, etc.) and by chemical means. A probable biosynthetic pathway of compound **1** was also proposed. Preliminary investigation with the new compound showed potent activity against a series of fungal pathogens.

Keywords. *Croton bonplandianum*; 3 α -hydroxy-urs-12,15-dien; antifungal.

1. Introduction

Croton bonplandianum Bail belongs to the family of Euphorbiaceae, is native to southern Bolivia, Paraguay, southwestern Brazil and northern Argentina and was reported from India during late 1890 by Kaul.¹ It now occurs widely along roadsides, railway abandoned field in wide open ravines, and paddy or sugarcane fields and on sandy or sandy clay soils. Due to the resemblance of the leaves and flower cymes to that of Tulsi, this plant is often called Ban Tulsi locally. *C. bonplandianum* was reported to have many medicinal uses including the repellent property against the insects,^{2–5} mosquito, *A. aegypti*.⁶ Local people in the remote area of West Bengal, India are using its root against snake bite and the leaf extract against high fever. In addition, the plant is used both as a fuel and as detergent. The methanol extract of *C. bonplandianum* has been found to exhibit antitumour properties using *Agrobacterium tumefaciens* and has larvicidal activities.

Phytochemically croton is rich in secondary metabolites including alkaloids and terpenoids.^{7,8} Diterpene resins found in many species of croton have been used experimentally in the studies of tumour initiation and conceivably prove to be useful in cancer therapy.⁹ Apart from the above limited reports, no systematic study has yet been initiated for the total phytochemical investigation of *C. bonplandianum*.

2. Experimental

2.1 General experimental procedure

Melting points were determined by open capillary method and were uncorrected. IR spectra were measured on Shimadzu 8300 FT-IR spectrophotometer. NMR spectra were recorded on Bruker-Avance 300 and 400 MHz FT-NMR spectrometer. ESIMS was obtained on Applied Biosystem API 2000 and TOFMS were obtained on 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer.

2.2 Plant material

Plants of *C. bonplandianum* used in this experiment were collected from North Bengal, India in May, 2008.

2.3 Extraction and isolation

The air dried roots of *C. bonplandianum* (2 Kg) was chopped into small pieces and extracted with MeOH (2 L) in a soxhlet extractor for 7 days and MeOH was recovered *in vacuo*. The extracted mass (350 g) was suspended in distilled water (1 L) and was partitioned successively between hexane (300 $\times$ 3), EtOAc (300 $\times$ 3) and *n*-BuOH (300 $\times$ 3) to obtain four fractions (hexane phase, ethyl acetate phase, *n*-butanol phase and water phase). The ethyl acetate phase (1.5 g) was purified by repeated column chromatography (silica gel) using petroleum ether (PE) and PE:ethyl acetate of varying concentrations as eluent.

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2.4 Preparation of acetyl derivative of compound 1

Compound **1** (100 mg, 0.23 mmol) in a 50 mL round bottom flask was warmed over a water bath with 10 mL of pyridine and 2 mL of acetic anhydride for 6 h. The reaction mixture was then poured into 50 mL of ice cold water, worked up with ether, dried over anhydrous sodium sulphate. The crude product was then purified over a column of silica gel.

Purification of the gummy material, gave white crystalline monoacetate of mp 223–224 °C. IR spectrum showed peaks at 2850, 1461, 1376, 1248, 1053, 959, 970, 838 cm⁻¹. In the mass spectrum it showed a molecular ion peak at *m/z* 467. The ¹H NMR spectrum of the acylated derivative showed characteristic signals due to five tertiary methyl groups at δ_H 0.68 (3H, s, C-23), 0.69 (3H, s, C-24), 0.76 (3H, s, C-25), 0.84 (3H, s, C-26), 1.01 (3H, s, C-27), 1.25 (3H, s, C-28), and two secondary methyl signals as doublet centred at 0.79 (3H, d, *J* = 6.3 Hz) and 0.92 (3H, d, *J* = 6.3 Hz). The acetate methyl appeared as a singlet centred at 2.33 (3H, s).

2.5 Bioassay

Suitable fungal strains were procured from the microbiology laboratory of our institute. DMSO (dimethyl sulphoxide) was used as solvent to prepare different concentrations of the triterpenoid. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in petridishes and were incubated at 37 °C for 48 h. Culture media for fungal pathogens were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber.

3. Results and discussion

During our search towards bioactive natural products in tropical plants,¹⁰ we have investigated the chemical constituents of the MeOH extract of roots of *C. bonplandianum*, which showed potent antifungal activity against a series of fungal and bacterial pathogens. The investigation yielded the isolation of a new triterpene 3 α -hydroxy-urs-12,15-dien (**1**), along with two known triterpenoid oleanolic acid (**2**), ursolic acid (**3**) and most abundant β -sitosterol (**4**). All the compounds (**1**, **2** and **3**) have been screened for their antimicrobial activity.

The MeOH extract of the roots of *C. bonplandianum* was suspended in water, partitioned successively between hexane, EtOAc and *n*-BuOH to get four

fractions (hexane phase, ethylacetate phase, *n*-butanol phase and water phase). Since results of preliminary screening with the EtOAc soluble fraction was found to be most active against the fungal strain used, it was purified (through repeated column chromatography over silica gel) to isolate the active ingredients viz. 3 α -hydroxy-urs-12,15-dien (**1**), along with two known triterpenoids, oleanolic acid (**2**) and ursolic acid (**3**) and most abundant β -sitosterol (**4**). The structures of all the compounds were elucidated on the basis of chemical and spectroscopic analysis and by comparison (for compounds **2**, **3** and **4**) with the spectral data of already reported compounds.

The elucidation of the structure of the new compound was accomplished by extensive analysis of its spectral data. Compound **1** was obtained as a white powder of melting point (mp) 244–246 °C, $[\alpha]_D +4.0$, UV inactive, showed strong absorption band at 3422 (–OH), 2850, 1461, 1376 (gem dimethyl), 1053, 970, 959, 838 cm⁻¹ in the IR spectrum indicative of the presence of hydroxyl, olefinic and gem dimethyl groups. ¹³C NMR spectrum at ambient temperature displayed signals for thirty carbons which were distinguished as eight methyls, seven methylenes, nine methines (five aliphatic, one oxygenated and three olefinic) and six quaternary with the aid of DEPT experiment. The ¹H NMR spectra of compound **1** (table 1) showed characteristic proton signals due to six tertiary methyl groups at δ_H (proton chemical shift) 0.68 (3H, s, C₂₃-Me), 0.69 (3H, s, C₂₄-Me), 0.76 (3H, s, C₂₅-Me), 0.84 (3H, s, C₂₆-Me), 1.01 (3H, s, C₂₇-Me), 1.25 (3H, s, C₂₈-Me), and two secondary methyl signals as doublet centred at 0.79 (3H, d, *J* = 6.3 Hz, C₂₉-Me) and 0.92 (3H, d, *J* = 6.3 Hz, C₃₀-Me). The assignment of the signals of the methyl groups and the remaining ¹H and ¹³C signals were performed through analysis of the HSQC, HMBC and COSY experiments and the results were found consistent with pentacyclic triterpene skeleton and its molecular formula was suggested to be C₃₀H₄₈O on the basis of ESIMS and TOFMS data [*m/z* = 424 (M⁺)]. On acetylation it gave crystalline acetate of mp 223–224 °C. The molecular formula of this acetate was suggested to be C₃₂H₅₀O₂ [ESIMS data *m/z* = 467 (M+1)]. The difference in mass spectral data of **1** and its acetate indicated the formation of a monoacetate and hence indicated the presence of only one hydroxyl group in compound **1**. IR spectrum of the monoacetate showed peaks at 2850, 1461, 1376 (gem dimethyl), 1248 (–OCOMe), 1053, 959, 970, 838 cm⁻¹. The appearance of a sharp peak at 1376 cm⁻¹ in the IR spectra of compound **1** and its monoacetate revealed the presence of a gem dimethyl group in ring A of the pentacyclic triterpenoid skeleton and the concordance of δ_H

Table 1. ^1H , ^{13}C chemical shift of compound **1** and ^{13}C chemical shifts of **1**^a, **2** and **3**.

C	1		1 ^a	2 ^b	3 ^b
	δC	δH	δC	δC	δC
1	40.5	1.03 (m), 1.59 (br. s)	40.5	39.0	39.2
2	29.7	1.65 (m), 1.94 (m)	29.7	28.1	28.2
3	71.8	3.50 (m)	74.0	78.2	78.2
4	33.9		33.9	39.4	39.6
5	56.8	0.86 (br. s)	56.7	55.9	55.9
6	18.7	1.39 (m), 1.48 (m)	18.7	18.8	18.8
7	31.9	1.51 (m), 1.82 (m)	31.8	33.4	33.7
8	39.7		39.7	39.8	40.1
9	50.2	1.55 (m)	50.0	48.2	48.1
10	36.5		36.5	37.4	37.5
11	24.3	1.84 (m), 1.98 (m)	24.3	23.8	23.7
12	121.7	5.33 (s, t-like)	122.6	122.6	125.7
13	140.7		139.6	144.8	139.3
14	42.3		42.3	42.2	42.6
15*	129.2		129.2	28.4	28.8
16*	138.3		138.3	23.8	25.0
17	45.8		45.8	46.7	48.1
18	55.9	2.26 (d, $J = 4.3$ Hz)	55.9	42.1	53.6
19	56.0	2.21 (m)	56.0	46.6	39.5
20	56.7	1.43 (br. s)	56.6	31.0	39.4
21	31.6	1.52 (d $J = 5.6$ Hz), 2.26 (d, $J = 5.6$ Hz)	31.8	34.3	31.1
22	37.2	2.01 (br. s), 2.21 (br. s)	36.9	33.2	37.4
23	21.1	0.68 (s)	21.1	28.8	28.8
24	29.1	0.69 (s)	29.1	16.5	16.5
25	28.2	0.76 (s)	28.2	15.6	15.7
26	19.8	0.84 (s)	19.8	17.5	17.5
27	26.0	1.01 (s)	26.0	26.2	24.0
28	19.4	1.25 (s)	19.3	180.0	179.7
29	19.0	0.79 (d, $J = 6.3$ Hz)	19.0	33.4	17.5
30	23.0	0.92 (d, $J = 6.3$ Hz)	23.0	23.8	21.4

^aDenotes acetyl derivative of compound **1**; ^bmeasured in pyridine d_5 . *Olefinic hydrogens attached to these carbons appeared as an AB q centred at 5.11 ppm with $J = 15.1$ and 8.5 Hz.

values in the ^1H NMR spectrum for the ring A with that of olean or ursane skeleton clearly rejected the possibility of the presence of friedelan skeleton. Mass fragmentation of the compound also followed the same pattern as was observed for typical pentacyclic triterpenoids.¹¹ A possible mass fragmentation pattern is depicted in scheme 1.

The comparison of the chemical shift positions (δH) of the A, B and C rings of **1**, especially the resonances of the methyl groups and two secondary methyl signals on ring E provides the most useful indicator for the presence of ursane-type triterpenoid skeleton.¹² A hydroxy methine signal at δH 3.5 (1H, m, H-3) and a trisubstituted olefinic signal at δH 5.33 (1H, d, $J = 2.7$ Hz, H-12). However, the splitting nature and small J value may be considered by the consideration of long range coupling involving H-12 and H-19, both are

closer in space. The through space distance is obviously more important than the number of intervening bonds, suggesting that **1** is a 3-hydroxy-urs-1,2-en type triterpenoid without possessing angular carboxyl group at C-28.¹⁰ Additionally, **1** showed an AB quartet ($>\text{CH}=\text{CH}<$) centred at δH 5.11 (2H, $J = 15.1$ Hz and 8.5 Hz, H-15 and H-16) signifying the presence of another olefinic double bond having two olefinic protons that must be attributed to the vinylic protons of a disubstituted double bond in a six membered ring.^{13,16}

^{13}C NMR spectrum showed all the 30 carbons and thereby confirmed the presence of a triterpene skeleton (table 1). Four downfield signals at δC 121.7 (C-12), 140.7 (C-13), 129.2 (C-15) and 138.3 (C-16) indicated the presence of four sp^2 hybridized carbons. The signals at δC 121.7 and 140.7 are characteristic for a C-12/C-13 double bond in the ursane type structure.¹³ The

signal at δ_C 140.7 is due to the presence of a trisubstituted olefinic carbon at C-13 and peaks at δ_C 138.3 and 129.2 were indicative of the presence of another double bond having two disubstituted olefinic carbon. The ^{13}C values for these two carbons (δ_C 138.3 and 129.2) clearly removed the possibility of any trisubstituted double bond. C-3 carbon appeared at δ_C 71.8 whereas for compounds **2** and **3** it appeared at δ_C 78.2, i.e., a shielding of seven units that signifying a stereochemical change at C-3. Therefore, for compound **1** the hydroxyl group is alpha instead of beta, i.e., it is an 3-epi-urs-1,2-en type triterpenoid. On the other hand, the chemical shift of C-18 (δ_C 55.9) in compound **1** was very close to that in compound **3** (δ_C 53.6), which indicated that these two compounds have the same configuration at C-18. Eight methyl signals appeared at δ_C 21.1

(C-23), 29.1 (C-24), 18.7 (C-25), 19.8 (C-26), 24.3 (C-27), 19.4 (C-28), 19.0 (C-29) and 23.0 (C-30). Assignments of all the carbons were made by comparison with that reported in literature for ursane skeleton¹⁴ and are tabulated in table 1.

The structure of compound **1** was finally established by 2D NMR experiments. The methyl singlet at δ_H 1.25 was correlated with a carbon signal at δ_C 19.4 in the HMQC spectrum were assigned to H₃-28 from HMBC correlation observed from H₃-28 (δ_H 1.25) to C-17 (δ_C 45.8). Another methyl singlet at δ_H 0.84 (3H, s, C₂₆-Me), was correlated with carbon singlet at δ_C 19.8 in the HMQC spectrum, were assigned to H₃-26 from the HMBC correlation observed from H₃-26 (δ_H 0.84) to C-8 (δ_C 39.7). The presence of a trisubstituted olefin between C-12 and C-13 was revealed by the HMBC correlations observed for H₂-11/C-12, H₂-11/C-13, H-18/C-13 and H₃-27/C-13. Finally, 2D NMR techniques were applied to locate the exact position of another double bond. Both the COSY and HMBC spectra gave significant information regarding the position of the double bond between C-15 and C-16. The COSY spectrum revealed two significant correlations of H-18 at δ_H 2.26 (1H, d, J = 4.3 Hz) with a proton at δ_H 5.13 (1H, AB q, J = 15.1 and 8.5 Hz) and a proton at δ_H 5.01 (1H, AB q, J = 15.1 and 8.5 Hz). In the HMBC spectrum (table 2) cross peaks were observed due to H₃-27 at δ_H 1.01 (s, 3H)/ δ_C 129.2 and H₃-28 at δ_H 1.25 (s, 3H)/ δ_C 138.3. Two olefinic protons at δ_H 5.13 (1H, AB q, J = 15.1, 8.5 Hz) and δ_H 5.01 (1H, q, J = 15.1, 8.5 Hz) in effect showed correlations in COSY spectrum. From the above data it was confirmed that compound **1** has a second double bond between C₁₅ and C₁₆. The appearance

Table 2. 1H - ^{13}C long range correlation detected in the HMBC spectra of compound **1** (δ relative to TMS in $CDCl_3$).

Proton	Correlated C
1.01 (H-27)	39.7 (C-8), 140.7 (C-13), 42.3 (C-14), 129.2 (C-15), 50.1 (C-9), 128.3 (C-16)
0.84 (H-26)	39.7 (C-8), 42.3 (C-14)
1.25 (H-28)	45.8 (C-17)
0.68 (H-23)	33.9 (C-4), 56.8 (C-5), 71.8 (C-3)
1.55 (H-9)	36.5 (C-10), 39.7 (C-8), 19.8 (C-26), 28.2 (C-25)
5.33 (H-12)	50.1 (C-9), 42.3 (C-14), 55.9 (C-18)
2.26 (H-18)	121.7 (C-12), 140.7 (C-13), 42.3 (C-14), 128.3 (C-16), 45.8 (C-17), 56.0 (C-19), 56.7 (C-20), 19.4 (C-28)

Scheme 1. Proposed mass fragmentation pattern of compound **1**.

Scheme 2. Probable biosynthetic pathway of compound **1**.

of such an AB quartet for such type of double bond in ring D of pentacyclic triterpenoid, between C₁₅ and C₁₆, was reported in literature.^{15,16} Thus on the basis of the above data the structure of compound **1** was established as 3 α -hydroxy-urs-12,15-diene.

Spectral analysis (IR, NMR, Mass, etc.) of the prepared acetate derivative provide further evidence in favour of the proposed structure of compound **1** (3 α -hydroxy-urs-12,15-diene). Mass spectrum showed the incorporation of only one acetyl group (at m/z 467) and thus confirming the presence of only one hydroxyl group. IR spectrum of the monoacetate showed peak at 1248 cm⁻¹ in addition to other peaks for the acetate group. In the ¹H NMR spectrum acetate methyl appeared as a singlet centred at δ_H 2.33 (3H, s) and the C₃-H shifted downfield (δ_H 4.61, m, 1H) in comparison to the same (δ_H 3.52, m, 1H) for the hydroxyl methine part in compound **1**. The ¹³C spectrum showed all the carbons for the monoacetate derivative. C-3 appeared at δ_C 74.0 (shifted downfield with respect to that of the original compound, **1**) and the acetate carbonyl signal appeared at δ_C 170.5. The DEPT and COSY spectra of the acetylated derivative were in good agreement to that proposed for the acetylated derivative (**5**) and thus established the structure of compound **1** as 3 α -hydroxy-urs-12,15-dien.

Purification of compound **2** yielded a white powder of mp 306–308 °C and its molecular formula was

suggested to be C₃₀H₅₀O₃ on the basis of FAB MS data [m/z = 458.2 (M)⁺] and its structure was elucidated as oleanolic acid by ¹H and ¹³C NMR data and that already reported in literature.¹⁷

Compound **3** was also obtained as a white powder of mp 280–282 °C. Its molecular formula was suggested to be C₃₀H₅₀O₃ on the basis of FAB MS data [m/z = 458.5 (M)⁺] and was finally identified as ursolic acid by ¹H and ¹³C NMR data and that already reported in literature.¹⁷

Purification of the most polar fraction (8% ethyl acetate in petroleum ether) afforded white crystals of mp 136–137 °C, M⁺ 414, and was identified as β -sitosterol **4** by spectral analysis and by comparison with an authentic sample of β -sitosterol (mixed mp, co-IR, and co-tlc, etc.).

A probable biosynthetic pathway for the formation of the new triterpenoid **1** has depicted in scheme 2 on the basis of the formation of α -amyrin.¹⁸ This is followed by the formation of a transient carbanion intermediate (**S3**) and its subsequent rearrangement to **S5**. Catalytic role of NADP⁺ present in cells/tissue was then suggested for the formation of **1**.

All the compounds (**1**, **2** and **3**) isolated from the ethyl acetate soluble fraction were tested for their anti-fungal activity by the disc diffusion method (table 3) against the microorganisms which are very much native to North Bengal,¹⁹ India; viz. *Calletotricheme camellie*, *Fussarium equisetae*, *Alternaria alternata*, *Curvularia eragrostidies* and *Colletrichum gleosporoides*. These fungal pathogens are responsible to cause wilt disease to tomatoes, pineapple, etc. cultivated traditionally in this region. The MIC values including that of reference sample Bavistin are tabulated in table 3. The MIC values of Bavistin against these fungal pathogens were also determined. A comparison of antifungal activities of compound **1**, **2** and **3** to that of Bavistin showed that although they are less active compare to Bavistin, but

Table 3. MIC of compound **1** against different fungi.

Compound	MIC of 1 in μ g/mL against different fungi				
	CC	CG	AA	FE	CE
1	10	< 15	10	< 10	15
2	<10	20	10	<10	10
3	15	<10	10	<15	<15
Bavistin	2.5	1.25	2.5	2.5	<2.5

all of them exhibited moderately good activity against all the fungal pathogens tested.

4. Conclusion

A new triterpenoid of ursane skeleton has been isolated from the root of *C. bonplandianum* and characterized as 3 α -hydroxy-urs-12,15-dien (**1**) along with two known triterpinic acids, oleanolic acid (**2**) and ursolic acid (**3**). β -sitosterol (**4**) was also obtained as the most polar fraction of the ethylacetate phase. Antifungal potentiality of compound **1** was also detected against five different fungal pathogens. A plausible biosynthetic pathway was also suggested.

Supplementary information

The electronic supporting information can be seen in www.ias.ac.in/chemsci.

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