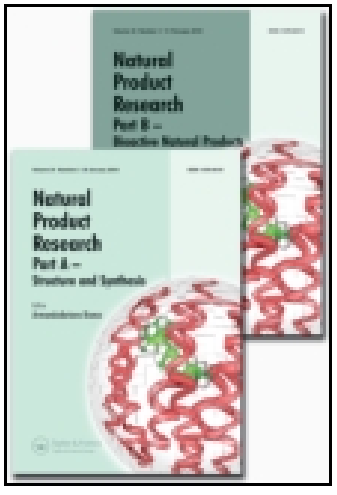


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# New natural compounds from *Rhododendron lepidotum*

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Phytochemical investigation of the aerial parts of *Rhododendron lepidotum* yielded 8-[2′,6′-dimethoxy-4′-(1″,2″,3″-trihydroxy-propyl)-phenyl]-7-hydroxy benzopyranone (**1**), 3-*O*-β-D-glycopyranosyl betulinic amide (**2**) and 8-hydroxy-7,7′-oxydicoumarin (**4**) and five known compounds. Among the known molecules, betulinic amide (**3**) was earlier reported as a semi-synthetic product. The structures of new molecules **1**, **2** and **4** were elucidated on the basis of extensive spectroscopic investigations (1D NMR, 2D NMR and mass spectrometry).

**Keywords:** *Rhododendron lepidotum*; betulinic amide; 8-hydroxy-7,7′-oxydicoumarin; pinitol



## 1. Introduction

The genus *Rhododendron* includes widely distributed flowering plants (over 1000 species) found throughout the world except Africa and South America. *Rhododendrons* are high-altitude plants mainly inhabiting a vast section of Southeastern Asia stretching from Northwestern Himalayas through Nepal, western and central China. More than 90% *Rhododendron* species are found in this region. *Rhododendron lepidotum* is found in India (Kashmir to Arunachal Pradesh), Nepal, Bhutan, China and Burma (Chung et al. 2007).

In the earlier studies on the phytochemical investigation of *R. lepidotum*, we have isolated a number of coumarins and coumarin glycosides (Khan et al. 2008; Ahmad et al. 2010; Shakeel-u-Rehman et al. 2010). Encouraged by the earlier results, the isolation of new natural products from *R. lepidotum* was taken up on large scale. So the plant material (aerial) was collected from the earlier site (Sonamarg, Kashmir) in September 2009 and subjected to methanol extraction for isolation of natural products. Here we report the isolation of three new (**1**, **2** and **4**) and five known molecules. Among the known molecules, betulinic amide (**3**), bergapten (**5**), xanthotoxin (**6**), pinitol (**7**) and coumarin (**8**) are reported for the first time from *R. lepidotum* (Figure 1).

## 2. Results and discussion

Aerial parts of *R. lepidotum* were collected from Sonamarg, Kashmir, India, in September 2009. The air-dried powdered material was defatted and extracted with methanol. The concentrated methanol extract was subjected to column chromatography over silica gel. Repeated column chromatography of methanol extract afforded compounds **1**–**8**. Compound **1** was isolated as a white amorphous powder (m.p.: 208°C). The HR-EI-MS showed a molecular ion peak at *m/z*

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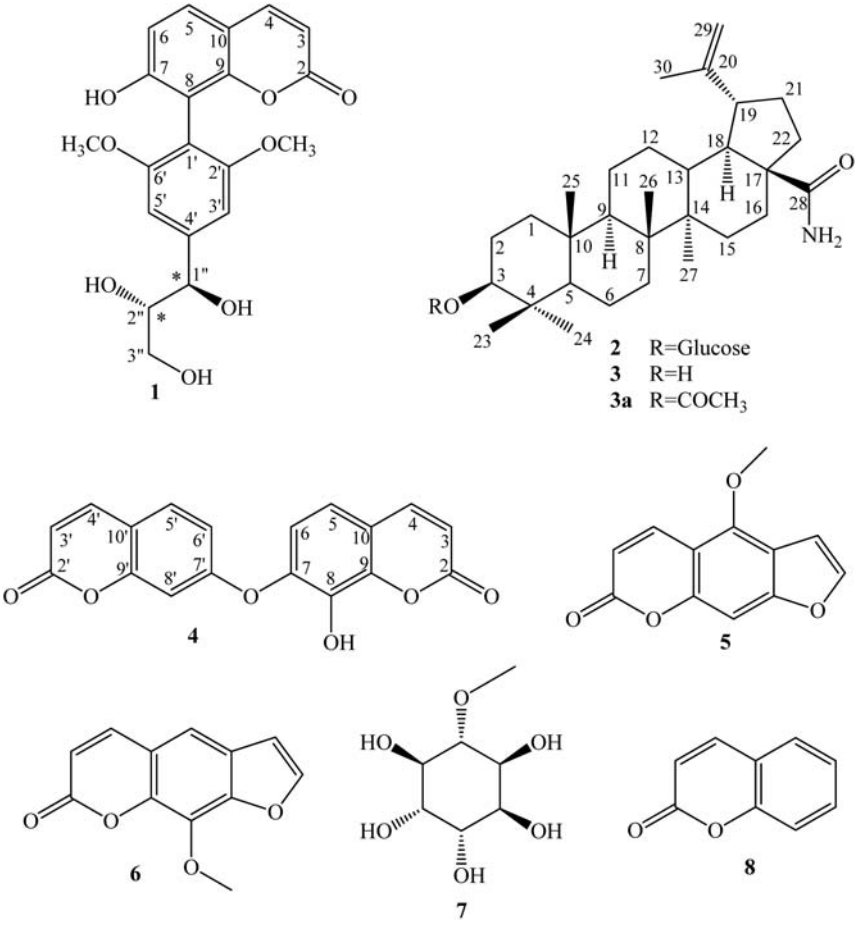


Figure 1. Molecular structures of the isolated compounds.

388.1147. From elemental analysis, HR-EI-MS and other spectral data ($^1$H NMR and $^{13}$C NMR), compound **1** was assigned the molecular formula $C_{20}H_{20}O_8$. The UV spectrum exhibits absorption bands at 219, 261 and 309 nm characteristics of a coumarin nucleus. The infrared (IR) spectrum shows absorption bands for hydroxyl (3419 $cm^{-1}$), carbonyl (1725 $cm^{-1}$) and aromatic ring (1615, 1522, 1500 and 1459 $cm^{-1}$). The $^1$H NMR spectrum of compound **1** shows four doublets at $\delta$ 6.26 and 7.92 with $J = 9.5$ Hz and at $\delta$ 7.17 and 6.92 with $J = 8.5$, which is more or less similar to that of 7,8-dihydroxy coumarin (Zhang et al. 2007). Upon close examination of $^1$H NMR spectrum, it can be inferred that compound **1** consists of two moieties: a disubstituted coumarin (giving four characteristic doublets) and a tetrasubstituted aryl (exhibiting two phenyl protons and two methoxyls). The six proton singlet at $\delta$ 3.86 represents two $-OCH_3$ groups in an identical magnetic environment. A 2H singlet in the aromatic region at $\delta$ 6.70 represents another set of two aromatic protons in an identical magnetic environment. The decoupled $^{13}$C NMR and DEPT show 19 signals, out of which nine carbon signals ($\delta$ 160.1, 112.1, 145.0, 118.2, 112.8, 147.0, 130.2, 144.7 and 113.5) can be safely attributed to coumarin nucleus. Two signals at $\delta$ 76.1 and 77.4 represent two oxygenated carbons, a methylenic resonance at $\delta$ 59.3 represents $CH_2OH$ carbon, an intense signal at $\delta$ 55.5 can be attributed to two magnetically equivalent methoxyls and the rest five signals (a strong signal of two magnetically equivalent carbons at $\delta$ 103.4 and four singlets at $\delta$ 125.9, 136.2, 148.2 and 148.4) are attributed

to a tetrasubstituted aromatic ring. The connectivities between different protons and their respective carbons were established on the basis of HSQC spectrum. Thus, the protons resonating at $\delta$ 5.12, $\delta$ 4.25 and $\delta$ 3.58 displayed one-bond correlations with C-1″ ($\delta$ 76.1), C-2″ ($\delta$ 77.4) and C-3″ ($\delta$ 59.3), respectively. Similarly, a two-proton singlet peak at $\delta$ 6.70 showed strong correlation with carbons resonating at $\delta$ 103.4 (C-3′ and C-5′). The well-established heteronuclear one-bond connectivities between the protons at $\delta$ 6.26, 7.92, 7.17 and 6.92 and respective carbons at $\delta$ 112.1 (C-3), $\delta$ 145.0 (C-4), $\delta$ 118.2 (C-5) and $\delta$ 112.8 (C-6) were also obvious in the HSQC spectrum. In the HMBC spectrum, H-1″ exhibited interaction with C-3′ and C-5′ at $\delta$ 103.4 and also with C-3″ resonating at $\delta$ 59.3. The long-range interaction between the protons at $\delta$ 6.70 [H-C (3′) and H-C (5′)] with the carbons resonating at $\delta$ 136.2 (C-1′) and $\delta$ 76.1 (C-1″) confirms their position at C-3′ and C-5′. The methoxyl protons resonating at $\delta$ 3.86 are linked with C-2′ and C-6′ at $\delta$ 148.2 and 148.4. Out of the two possibilities for the attachment of hydroxyl group at C-7 and C-8, the 1–3 HMBC correlations were helpful. There is strong correlation between H-5 ($\delta$ 7.17) with C-7 ($\delta$ 147.0) and H-6 resonating at $\delta$ 6.92 with C-8 ($\delta$ 130.2). Thus, hydroxyl group can be safely placed at C-7 with $\delta$ 147.0. Had hydroxyl been at C-8 position, then both chemical shift values ($\delta$ 147.0 and 130.2) for C-7 and C-8 carbons would have been below $\delta$ 140 because such a carbon resonates at $\delta$ 135 (Pukalskas et al. 2002). The attachment of aryl moiety at C-8 is further supported by the absence of correlation between C-1′ carbon and C-6 proton. From the above correlations, the structure of **1** can be elucidated as 8-[2′,6′-dimethoxy-4′-(1″,2″,3″-trihydroxy-propyl)-phenyl]-7-hydroxy benzopyranone.

From the coupling constant value ($J = 8.1$ Hz) between C-1″H and C-2″H, it can be inferred that the two protons have relative anti-configuration (Kihumbu et al. 2002). From the above spectroscopic data, compound **1** can be unambiguously characterised as 8-[2′,6′-dimethoxy-4′-(1″,2″,3″-trihydroxy-propyl)-phenyl]-7-hydroxy benzopyranone.

Compound **3** was isolated as a white solid showing molecular ion peak at *m/z* 455.3759 [$M^+$] which corresponds to the molecular formula $C_{30}H_{49}NO_2$. The structure of compound **3** was characterised as betulinic amide by comparison of its $^1$H and $^{13}$C NMR spectral data with that reported in the literature (Ziegler et al. 2004). This is the first report for the isolation of betulinic amide (**3**) from any natural source. Compound **3** was derivatised to 3-*O*-acetyl betulinic amide (**3a**) (Dorr et al. 2011) which further confirmed the structure of **3**.

Compound **2** was isolated as a white amorphous powder. It showed a molecular ion peak at *m/z* 617.4284 in the mass spectrum that corresponds to molecular formula $C_{36}H_{59}NO_7$. The $^1$H and $^{13}$C NMR were found to be identical with betulinic amide except for the presence of additional peaks of sugar moiety. The $^1$H NMR spectrum displayed the signal of anomeric proton at $\delta$ 4.96 (1H, d, $J = 7.2$ Hz). $^{13}$C NMR of **2** showed a group of six sugar signals at $\delta$ 105.3 (C-1′), 78.3 (C-3′), 76.7 (C-5′), 74.2 (C-2′), 70.9 (C-4′) and 61.8 (C-6′). The $^1$H, $^{13}$C NMR and MS spectral analysis of **2** indicated it to be a glycoside of **3**. As seen from $^{13}$C NMR, there are six signals of glycosidic carbons for a hexose sugar. The hexose residue was considered to be $\beta$-D-glucopyranose because the chemical shifts of sugar carbons were in good agreement with those reported in the literature (Gauthier et al. 2006). Furthermore, hydrolysis of **2** in 1.0 N HCl gave betulinic amide and a sugar moiety. The nature of the sugar part was determined by comparison of its $^1$H NMR values with that of glucose providing further proof for the structure of **2**.

A doublet at $\delta$ 4.96 ($J_{H1'-H2'} = 7.15$ Hz) with anomeric carbon resonating at $\delta$ 105.3 indicated that the sugar moiety is attached to the aglycone part via β-linkage. The attachment of hexose sugar with the aglycone part was determined from the HMBC experiment. The HMBC experiment established the correlation between anomeric proton resonating at $\delta$ 4.96 and $\delta_{C\text{-}3}$ 78.1.

Dicoumarinyl ether (**4**) was isolated as a white crystalline solid and analysed for $C_{18}H_{10}O_6$ from the molecular ion peak at *m/z* 322.0467 in HR-EI-MS and other spectral data. The $^1$H NMR

showed two pairs of AB-type doublets [$\delta$ 6.17, 7.82 ($J = 9.5$ Hz, H-3, H-4); 6.19, 7.85 ($J = 9.4$ Hz, H-3′, H-4′)], two doublets [$\delta$ 7.0 ($J = 8.4$ Hz, H-5) and 7.45 ($J = 8.5$ Hz, H-5′)] and a multiplet of three protons [$\delta$ 6.7–6.8 (H-6, H-6′, H-8′)]. The IR band at 3505 $cm^{-1}$ revealed the presence of hydroxyl group. Upon comparison of the spectral data ($^1$H and $^{13}$C NMR) of **4** with that of 8-methoxy-7,7′-oxydicoumarin (Reisch et al. 1988; Reisch et al. 1989), it is found that these two compounds possess more or less a similar structure except the lack of a methoxyl signal at $\delta$ 4.0 in compound **4**. Thus, the structure of **4** can be safely characterised as 8-hydroxy-7,7′-oxydicoumarin.

The structures of betulinic amide (**3**) (Ziegler et al. 2004; Dorr et al. 2011), 3-*O*-acetyl betulinic amide (**3a**) (Dorr et al. 2011), bergapten (**5**) (Liu et al. 2004; Chunyan et al. 2009), xanthotoxin (**6**) (Elgamal et al. 1979), pinitol (**7**) (Murray et al. 1982) and coumarin (**8**) (Jain et al. 2007) were established by comparison of their spectral data with that reported in the literature.



## 3. Experimental

### 3.1. *General experimental procedures*

$^1$H NMR spectra were recorded as $\delta$ values on 200 and 500 MHz nuclear magnetic resonance (NMR) and $^{13}$C NMR on 50 MHz using deuterated acetone/DMSO as a solvent and TMS as the internal standard. IR spectra were recorded as KBr pellets in $cm^{-1}$ on a Hitachi 270-30 spectrophotometer (Japan). Melting points were determined on a Buchi melting point apparatus. UV spectra were scanned in methanol on specord S100. HR-EI-MS were recorded on an Agilent Technologies G6540-UHD LC/MS Q-TOF (Agilent Technologies, Santa Clara, CA, USA). Column was run using silica gel (60–120 mesh). Thin layer chromatography plates were visualised under UV light and after exposure to iodine vapour in iodine chamber.

### 3.2. *Plant material*

The aerial parts of *R. lepidotum* were collected from Sonamarg, Kashmir, in September 2009 from the same place where it was previously collected in 2007 (Ahmad et al. 2010). Moreover, the plant was confirmed by comparison of the collected sample with the Herbarium specimen collected in 2007 under the specimen number 1455/93.

### 3.3. *Extraction and isolation*

Air-dried and coarsely powdered plant material (aerial part, 2.80 kg) was defatted with hexane for 48 h. The defatted material was dried and extracted with methanol for 48 h. The methanolic extract thus obtained was concentrated under reduced pressure to give crude extract (364.0 g). The methanolic extract was dissolved in a minimum amount of methanol and adsorbed on silica gel to form slurry. The dried slurry was subjected to silica gel column chromatography. Repeated column chromatography using different percentages of petroleum ether–ethyl acetate and ethyl acetate–methanol afforded three new compounds: 8-[2′,6′-dimethoxy-4′-(1″,2″,3″-trihydroxy-propyl)-phenyl]-7-hydroxy benzopyranone (**1**), 3-*O*-β-D-glucosyl betulinic amide (**2**), a dicoumarinyl ether 8-hydroxy-7,7′-oxydicoumarin (**4**) and five known compounds: betulinic amide (**3**), bergapten (**5**), xanthotoxin (**6**), pinitol (**7**) and coumarin (**8**).

#### 3.3.1. *8-[2′,6′-Dimethoxy-4′-(1″,2″,3″-trihydroxy-propyl)-phenyl]-7-hydroxy benzopyranone (**1**)*

White amorphous powder (27.0 mg). HR-EI-MS: *m/z* 388.1147 (calculated for $C_{20}H_{20}O_8$, 388.1158). m.p.: 206–210°C. UV $\lambda_{max}$ ($CH_3OH$) nm: 219, 261, 309. IR (KBr) $cm^{-1}$: 3419,

1725, 1615, 1522, 1500, 1459. $^1$H NMR (acetone-$d_6$): $\delta$ 6.26 (1H, d, $J = 9.5$ Hz, H-3), 7.92 (1H, d, $J = 9.5$ Hz, H-4), 7.17 (1H, d, $J = 8.5$ Hz, H-5), 6.92 (1H, d, $J = 8.5$ Hz, H-6), 6.70 (2H, s, H-3′, 5′), 3.86 (6H, s, H-2′ OMe, H-6′ OMe), 5.12 (1H, d, $J = 8.1$ Hz, H-1″), 4.25 (1H, m, H-2″), 3.58 (1H, dd, $J = 2.5$, 6.5 Hz, H-3″a), 3.86 (1H, dd, $J = 2.5$ and 6.5 Hz, H-3″b); $^{13}$C NMR (acetone-$d_6$): $\delta$ 160.1 (C-2), 148.4 (C-6′), 148.2 (C-2′), 147.0 (C-7), 145 (C-4), 144.7 (C-9), 136.2 (C-1′), 130.2 (C-8), 125.9 (C-4′), 118.2 (C-5), 113.5 (C-10), 112.8 (C-6), 112.1 (C-3), 103.4 (C-3′, C-5′), 77.4 (C-2″), 76.1 (C-1″), 59.3(C-3″), 55.5 (C-2′ OMe, C-6′ OMe).

#### 3.3.2. *3-O-β-D-Glucosyl betulinic amide (**2**)*

White amorphous powder (57.0 mg). HR-EI-MS: *m/z* 617.4284 (calculated for $C_{36}H_{59}NO_7$, 617.4292). m.p.: 210–215°C. IR (KBr) cm$^{-1}$: 3550, 3473, 3419, 3414, 2940, 2868, 1686, 1638, 1617. $^1$H NMR (DMSO): $\delta$ 0.70 (3H, s, $CH_3$-25), 0.81 (3H, s, $CH_3$-24), 0.82 (1H, m, H-5), 0.91 (3H, s, $CH_3$-27), 0.91 (3H, s, $CH_3$-26), 0.97 (3H, s, $CH_3$-23), 1.02 (1H, m, Hα-1), 1.20 (1H, m, Hβ-11), 1.21 (1H, m, Hα-12), 1.24 (1H, m, Hα-15), 1.37 (2H, m, Hβ-6; H-9), 1.38 (1H, m, Hβ-7), 1.41 (1H, m, Hα-11), 1.44 (1H, m, Hα-7), 1.50 (1H, m, Hα-21), 1.57 (3H, m, Hα-16, Hα-6, Hα-22), 1.67 (3H, s, $CH_3$-30), 1.67 (1H, br d, $J = 13$ Hz, Hβ-1), 1.85 (1H, t, H-18), 1.85 (2H, m, H-2), 1.86 (2H, m, Hβ-15, Hβ-12), 2.19 (1H, m, Hβ-21), 2.25 (1H, m, Hβ-22), 2.27 (1H, m, H-13), 2.57 (1H, m, Hβ-16), 3.05 (1H, t, $J = 12$ Hz, H-3), 3.07 (1H, m, H-19), 3.34–3.38 (3H, m, Glc-H), 3.63–3.73 (3H, m, Glc-H), 4.58 (1H, s, Hβ-29), 4.69 (1H, s, Hα-29), $\delta$ 4.96 (1H, d, $J_{H1'-H2'} = 7.15$ Hz, H1′). $^{13}$C NMR (DMSO): $\delta$ 178.3 (C-28), 151.3 (C-20), 109.9 (C-29), 105.3 (C-1′), 78.3 (C-3′), 76.7 (C-5′), 74.2 (C-2′), 70.9 (C-4′), 61.8 (C-6′), 78.3 (C-3), 56.5 (C-17), 56.0 (C-5), 51.1 (C-9), 49.6 (C-18), 47.7 (C-19), 42.9 (C-14), 41.2 (C-8), 39.4 (C-4), 39.2 (C-1), 38.7 (C-13), 37.6 (C-22), 37.3 (C-10), 34.9 (C-7), 32.6 (C-16), 31.0 (C-21), 30.1 (C-15), 28.4 (C-23), 27.7 (C-2), 26.1 (C-12), 21.4 (C-11), 19.2 (C-30), 18.8 (C-6), 16.4 (C-25), 16.3 (C-26), 16.0 (C-24), 14.9 (C-27).

#### 3.3.3. *8-Hydroxy-7,7′-oxydicoumarin (**4**)*

White amorphous powder (39 mg). HR-EI-MS: *m/z* 322.0467 (calculated for $C_{18}H_{10}O_6$, 322.0477). m.p.: 232–236°C. UV $\lambda_{max}$ ($CH_3OH$) nm: 205, 243, 285; IR (KBr) cm$^{-1}$: 3533, 2361, 1681, 1625, 1569, 1510, 1463, 1414, 1323, 1072, 1014, 988, 903, 796, 761. $^1$H NMR (DMSO): $\delta$ 6.17 (1H, d, $J = 9.5$ Hz, H-3), 7.82 (1H, d, $J = 9.5$ Hz, H-4), 7.00, (1H, d, $J = 8.4$ Hz, H-5), 6.18 (1H, d, $J = 9.5$ Hz, H-3′), 7.85 (1H, d, $J = 9.4$ Hz, H-4′), 7.45 (1H, d, $J = 8.5$ Hz, H-5′), 6.75 (3H, m, H-6, H-6′, H-8′); $^{13}$C NMR (DMSO): $\delta$ 163.6 (C-2′), 159.9 (C-2), 156.1 (C-7′), 151.2 (C-8), 146.1 (C-7), 145.5 (C-9′), 145.2 (C-4′), 144.7 (C-4), 144.5 (C-9), 133.0 (C-5), 131.1(C-5′), 119.1 (C-3), 113.6 (C-10′), 112.7 (C-3′), 112.3 (C-10), 111.2 (C-6′), 109.7 (C-6), 101.2 (C-8′).

### 3.4. *Acetylation of 3*

In a typical experiment, compound **3** (0.5 mmol), pyridine (500 μL) and acetic anhydride (500 μL) were stirred at 40°C for 24 h. The reaction mixture was poured into ice-cold water and extracted with EtOAc. The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated *in vacuo* to get the desired acyl derivative (**3a**) in almost quantitative yield.

## 4. Conclusions

Three new compounds: 8-[2′,6′-dimethoxy-4′-(1″,2″,3″-trihydroxy-propyl)-phenyl]-7-hydroxy benzopyranone (**1**), 3-*O*-β-D-glycosyl betulinic amide (**2**) and 8-hydroxy-7,7′-oxydicoumarin (**4**) together with five known compounds were isolated and characterised from *R. lepidotum*.

Among the known molecules, betulinic amide (**3**) is reported for the first time from natural source. The other known isolates are bergapten (**5**), xanthotoxin (**6**), pinitol (**7**) and coumarin (**8**).

## Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1–S5.

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