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A novel diterpene hydroperoxide, glutinosin C, from *Isodon glutinosa*

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Abstract—A novel diterpene hydroperoxide, glutinosin C, has been isolated from the leaves of *Isodon glutinosa*. Its structure and relative stereochemistry were established on the basis of the spectral features and confirmed by single crystal X-ray analysis.

Plants belonging to the genus *Isodon*, are known to be a rich source of *ent*-kaurane diterpenoids,[1,2] most of which have been shown to have antitumor and anti-inflammatory activities.[3]

In our previous studies on the leaves of *Isodon glutinosa*, four *ent*-kauranoids, and one abietanoid were isolated.[4–6] Among them, the abietane diterpenoid, pisiferic acid, was reported to be active against the growth of Gram-positive bacteria.[7] Our recent investigation on the bioactive diterpenoids from the ethyl acetate extracts of the leaves of this plant, led to the discovery of an interesting *ent*-abietane diterpenoid with a hydroperoxyl group, glutinosin C **1**. The structure was established by extensive 2D NMR spectroscopy and X-ray analysis. It is the first time that the structure and relative stereochemistry of a naturally occurring diterpenoid with a hydroperoxyl substituent have been determined by single crystal X-ray crystallography.

The 70% Me_2CO extracts of the air-dried and powdered leaves of *I. glutinosa* (3 kg) were partitioned with EtOAc to afford the EtOAc extracts (200 g), which was subjected to silica gel column chromatography using $CHCl_3$, $CHCl_3$/Me_2CO (9:1, 8:2, 7:3, 6:4) and Me_2CO as eluents. Compound **1** (62 mg) was purified from the $CHCl_3$/Me_2CO (8:2) fraction after repeated column chromatographic separations followed by recrystallization from Me_2CO.

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Compound **1**, giving a molecular ion peak at m/z 334 in the EI MS spectrum, was shown to have a molecular formula of $C_{20}H_{30}O_4$ by HREI MS (found 334.2133, calcd 334.2144). Unlike common diterpenoids isolated from this species, the EI MS spectrum of **1** exhibited two intense fragment ions at m/z 318 and 302, which were attributable to the loss of one oxygen atom and two oxygen atoms from the molecular ion, respectively. However, compounds such as diterpenoids usually show $[M-H_2O]^+$ ion peaks in EI MS spectra. The rare occurrence of the quite intense $[M-O]^+$ ion peak (13%) and $[M-O_2]^+$ ion peak (60%) is characteristic of peroxides.[8] An absorption at 242 nm in the UV spectrum was indicative of an α,β-unsaturated ketone moiety with two substituents.[9] The observation of absorptions at 1663, 3233 and 3416 cm^{-1} in the IR spectrum, revealed the presence of a carbonyl group and at least one hydroxyl group. The carbonyl stretching frequency of 1663 cm^{-1} accompanied by two vibrations at 1622 and 1605 cm^{-1}, suggested an unsymmetrical system.[10]

The ^1H NMR spectrum of **1** displayed two olefinic signals [δ_H 6.84 (1H, s) and 6.39 (1H, s)], two multiplets for two protons attached to oxygenated carbons [δ_H 4.09 (1H, m), 3.91 (1H, m)], three methyl singlets [δ_H 1.60 (3H, s), 0.83 (3H, s), 0.79 (3H, s)], and a methyl doublet [δ_H 1.27 (3H, d, $J=7.0$ Hz)]. Observed in the ^{13}C NMR (including DEPT) spectra of **1** were one conjugated carbonyl carbon, five quaternary carbons including an oxygenated one and two olefinic carbons, four methines including the two olefinic carbons, six methylenes including an oxygen-bearing one, and four methyls, which clearly indicated a diterpenoid skeleton.

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The typical methyl doublet at δ_H 1.27 (3H, d, $J=7.0$ Hz) in the ^1H NMR spectrum, along with an oxygenated methylene carbon signal at δ_C 66.4 (t) in the ^{13}C NMR spectrum, indicated that **1** was a 16-oxygenated abietane-type diterpenoid. From the downfield shift of the two vinyl carbons [δ_C 166.7 (s) and 146.6 (d)] and the upfield shift of the carbonyl carbon [δ_C 188.0 (s)], the presence of a ketone conjugated with two double bonds was inferred, and which was supported by the above IR evidence. Following precedent in diterpenoid ring systems, the conjugated system should be in ring C. The carbonyl carbon was assigned as C-12, with two double bonds at $\Delta^{9,11}$ and $\Delta^{13,14}$, respectively. The results were confirmed by the simultaneous ^1H–^{13}C long-range correlations of H-14 (δ_H 6.84) with the carbonyl carbon [δ_C 188.0 (C-12)], two vinyl quaternary carbons [δ_C 166.7 (s, C-9) and 141.5 (s, C-13)], a methine carbon [δ_C 35.2 (C-15)], a methylene carbon [δ_C 37.3 (C-7)], and an oxygenated quaternary carbon [δ_C 79.5 (C-8)] in the HMBC spectrum (Fig. 1).

In the same manner, the protons of the oxygenated methylene [δ_H 4.09 (1H, m) and 3.91 (1H, m)] displayed HMBC correlations with a methine carbon [δ_C 35.2 (C-15)], a vinyl quaternary carbon [δ_C 141.5 (C-13)], and a methyl carbon [δ_C 16.1 (q, C-17)], which further confirmed that **1** is an 8,16-dioxygenated 9(11),13(14)-dien-12-one-abietane diterpenoid. As required by its molecular formula, the two oxygenated substituents must be a hydroxyl group and a hydroperoxyl group. However, the assignments of these two oxygenated groups to C-8 and C-16 could not be determined solely using the HMBC technique in our experiment. Considering a plausible biosynthetic origin for **1** and the stability of the structures, it was more reasonable that the hydroperoxyl group was attached to C-8 and the hydroxyl group to C-16.

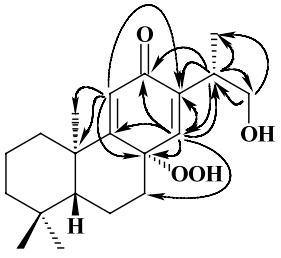


Figure 1. The key correlations of compound **1** from the HMBC spectrum.

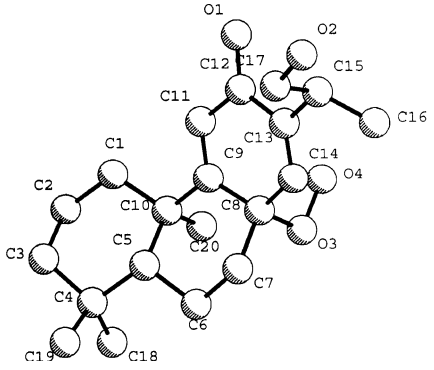
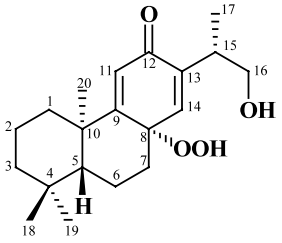
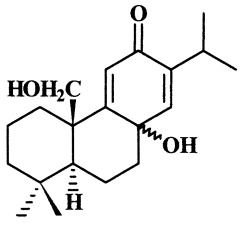


Figure 2. X-Ray crystallographic structure of compound **1**.



1

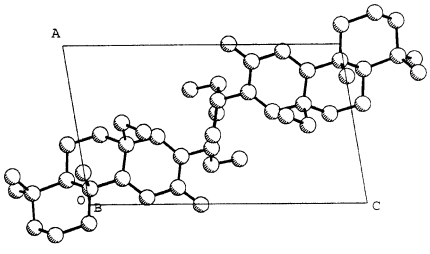


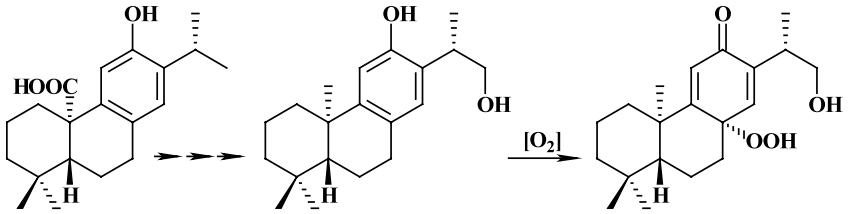
2

Comparison of **1** with a known abietane diterpenoid, 8,20-dihydroxy-abieta-9(11),13-dien-12-one **2** isolated from *Austrocedrus chilensis*,[10] showed that the two structures looked very similar, each possessing the same α,β-unsaturated ketone conjugated system in the C-ring and similar asymmetric centers. However, the optical rotation of **1** was negative while that of **2** was positive,[10] which allowed us to establish **1** as an *ent*-abietane diterpenoid.

As C-8 is a quaternary carbon and the σ-bond between C-13 and C-15 can rotate freely, the stereochemistry of C-8 and C-15 could not be simply deduced from the ROESY spectrum. Fortunately, **1** was obtained as colorless flat crystals after several recrystallizations. The analysis of the single crystal X-ray diffraction[11] (Fig. 2) of **1** not only confirmed the presence of the hydroperoxyl group at C-8, but also established the hydroperoxyl group as being in the α orientation and the stereochemistry of C-15 as *S*.

Thus, the structure of **1** was finally characterized as 15(*S*)-16-hydroxymethyl-8α-hydroperoxy-*ent*-abieta-9(11),13(14)-dien-12-one, and named glutinosin C.





3 4 1

Figure 3. Postulated biogenesis of **1**.

Taking into consideration that pisiferic acid was isolated from the same species, and 16-hydroxy-feruginol (enantiomer of **4**) was isolated from *Isodon flavidus*, which has been reported to produce the same type of diterpenoids as *I. glutinosa*,[14] a plausible biosynthetic origin for **1** is from *ent*-pisiferic acid **3**, as shown in Fig. 3. The possible mechanism for the last step of the reaction being similar to that reported in literature.[15]

It is possible that the peroxidation could occur in the course of our long extraction and isolation process. Although our extraction and separation conditions did not involve the use of temperatures above 60°C, or of acid or alkali, the following two experiments were carried out: (1) 5 mg of pisiferic acid was dissolved in 5 ml CH_3OH to which was added 20 mg Si gel H (10–40 μ), and the mixture successively stirred and irradiated at 254 and 365 nm for 12 h; (2) 5 mg of pisiferic acid was dissolved in 5 ml CH_3OH, added to 20 mg Si gel H (10–40 μ), and successively stirred and refluxed at 70°C for 12 h. Starting material was recovered in both cases arguing that compound **1** is not an artifact, and suggesting that the phenolic unit of pisiferic acid is stable under normal extraction and isolation conditions. Of course the true biosynthetic precursor of **1** could readily be the C-20 methyl equivalent of **3**.

Besides the predominance of *ent*-kaurane diterpenoids, *Isodon* genus plants have also been found to produce *ent*-pimarane, *ent*-isopimarane, *ent*-gibberellane diterpenoids, and so on.[2] However, this is the first time that an *ent*-abietane diterpenoid has been discovered in an *Isodon* species. Compound **1** is the first example in which the structure and relative stereochemistry of a naturally occurring hydroperoxyl-containing diterpenoid has been determined by single crystal X-ray crystallography, although a number of peroxy diterpenoids have already been recognized,[16,17] which even include a few abietane diterpenoid hydroperoxides.[18,19]

Glutinosin C (**1**): $C_{20}H_{30}O_4$, colorless crystal, mp: 138–140°C; $[\alpha]_D^{15.2}$: −252.73 (*c* 0.55, MeOH); UV (MeOH) λ_{max} (log ε): 200.5 (4.28), 242 (4.58) nm; IR (KBr) ν_{max}: 3416, 3233, 2982, 2966, 2939, 2928, 2865, 1663, 1622, 1605, 1469, 1442, 1387, 1306, 1264, 1228, 1212, 1159, 1029, 987, 975, 959, 917, 911, 866 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) δ 6.84 (1H, s, H-14), 6.39 (1H, s, H-11), 4.09 (1H, m, H-16a), 3.91 (1H, m, H-16b), 3.52 (1H, m, H-15), 1.60 (3H, s, Me-20), 0.83 and 0.79 (each 3H, s, Me-18 and Me-19), 1.27 (3H, d, J=7.0 Hz, Me-17); ^{13}C NMR (C_5D_5N, 125 MHz) δ 38.1 (t, C-1), 19.1 (t, C-2), 42.1 (t, C-3), 34.3 (s, C-4), 54.6 (d, C-5), 18.2 (t, C-6), 37.3 (t, C-7), 79.5 (s, C-8), 166.7 (s, C-9), 41.3 (s, C-10), 125.0 (d, C-11), 188.0 (s, C-12), 141.5 (s, C-13), 146.6 (d, C-14), 35.2 (d, C-15), 66.4 (t, C-16), 16.1 (q, C-17), 33.5 (q, C-18), 22.0 (q, C-19), 18.9 (q, C-20); EI MS (70 eV) m/z 334 $[M]^+$ (2), 318 (13), 302 (60), 287 (28), 271 (100), 255 (10), 247 (19), 231 (8), 213 (14), 201 (20), 187 (32), 173 (23), 163 (93), 149 (70), 137 (20), 123 (23), 105 (19), 91 (32), 81 (30), 69 (72), 55 (62); HREI MS m/z $[M]^+$ 334.2133 (calcd for $C_{20}H_{30}O_4$ 334.2144).

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11. A crystal of dimensions 0.05×0.20×0.40 mm was used for X-ray measurements on a MAC DIP-2030 diffractometer with a graphite monochromator, with maximum 2θ value of 50.0°. The total number of independent reflections measured was 1609, of which 1600 were considered to be observed ($|F|^2 \geq 8\sigma|F|^2$). Crystal data: $C_{20}H_{30}O_4$, M= 334.46, monoclinic system, space group: $P2_1$, a= 6.9480(3), b=11.1320(3), c=11.8600(6) Å, β=99.246(2)°, V=905.40(6) Å^3, Z=2, d=1.227 g cm^{-3}, Mo Kα radiation, linear absorption coefficient μ=1.0 cm^{-1}. The structure was solved by the direct method SHELX-86[12] and

expanded using difference Fourier techniques, refined by the program and method NOMCSDP[13] and full-matrix least-squares calculations. Hydrogen atoms were fixed at calculated positions. The final indices were $R_f = 0.050$, $R_w = 0.049$ ($w = 1/\sigma|F|^2$).

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