Biosynthetic Pathways

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Unprecedented Quassinoids with Promising Biological Activity from *Harrisonia perforata***

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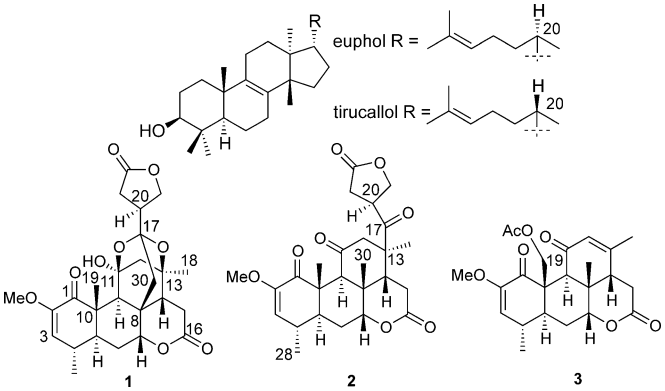
Abstract: *Perforalactone A (**1**), a new 20S quassinoid with a unique cagelike 2,4-dioxaadamantane ring system and a migrated side chain, was isolated from the plant Harrisonia perforata together with two biosynthetically related new quassinoids. The structures of these natural products were elucidated by NMR spectroscopy, X-ray diffraction analysis, computational modeling, and the CD excitation chirality method. The compounds exhibited notable biological properties, including insecticidal activity against Aphis medicaginis Koch and antagonist activity at the nicotinic acetylcholine receptor of Drosophila melanogaster. The structural features of these compounds may be related to their promising biological characteristics. Their biosynthesis and an alternative origin of quassinoid-type natural products are also discussed.*

Natural products are a reliable source of drug and pesticide discovery.[1] In the course of our investigations of bioactive structurally novel natural products derived from plants,[2] we were attracted to quassinoids, which are a class of degraded terpenoids derived from (20*R*)-euphol or (20*S*)-tirucallol from the Simaroubaceae family of plants. Quassinoids exhibit a wide range of biological activities,[3] and their total synthesis has fascinated synthetic chemists for a long time.[4] From the twigs and stem of *Harrisonia perforata* (Blanco) Merr. (Simaroubaceae), we isolated two novel quassinoids: perforalactone A (**1**; Scheme 1), which possesses an unprecedented C_{25} backbone, and its postulated biosynthetic precursor perforalactone B (**2**). Importantly, these compounds are the first reported natural 20*S* quassinoids and they provide clear evidence of the previously overlooked (20*R*)-euphol origin of quassinoids. In the subsequent biosynthetic study, perforalactone C (**3**), a new C_{20} quassinoid with a unique C19 oxygenated functionality was discovered. This compound is presumably derived from **2**. Strikingly, the compounds **1** and **2** exhibited notable biological activities related to their structural features. Herein, we describe the isolation, structural elucidation, possible biosynthesis, and biological activity of these compounds.



Scheme 1. Chemical structures of (20*R*)-euphol, (20*S*)-tirucallol, and perforalactones A–C (**1**–**3**).

Perforalactone A (**1**) was obtained as colorless crystals. Its molecular formula, $C_{26}H_{32}O_9$, was determined by HRESIMS (*m*/*z* 511.1928 $[M+Na]^+$; calcd: 511.1944). The ^1H and ^{13}C NMR spectroscopic data of **1** indicated the presence of a methoxy group and 25 carbon atoms, including two tertiary methyl groups and one secondary methyl group (Table 1). Two carbonyl functional groups giving rise to NMR signals at $\delta_C = 168.5$ and 176.7 ppm indicated the presence of δ-lactone and γ-lactone rings, which was further supported by the characteristic IR absorption bands at 1725 and 1768 cm^{-1}, respectively.[5] Furthermore, the presence of a 2-en-1-one system bearing a methoxy group was indicated by a doublet at $\delta_H = 5.56$ ppm ($J = 2.5$ Hz) in the ^1H NMR spectrum and by the resonances at $\delta_C = 204.8$, 147.5, and 121.0 ppm in the ^{13}C NMR spectrum. This structural assignment was confirmed by HMBC correlation of the olefinic hydrogen atom with the carbonyl moiety and by the characteristic IR absorption band at 1657 cm^{-1}.

Further detailed 2D NMR spectroscopic analysis enabled the construction of the structure of compound **1** (Figure 1a; see also Page S9 and Table S1 in the Supporting Information). However, the existence of ether bridges between C17/C11 and C17/C13 was only evidenced by X-ray crystallographic diffraction with $Cu_{K\alpha}$ radiation (Figure 1c).[6,7] The relative

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Table 1: ^{1}H (500 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for perforalactone A (**1**) in $CDCl_3$.

Position	δ_H [ppm] (J [Hz])	δ_C	Position	δ_H [ppm] (J [Hz])	δ_C [ppm]
1	–	204.8	15α	2.31 (dd, 19.0, 13.0)	28.4
2	–	147.5	15β	2.64 (dd, 19.0, 7.0)	
3	5.56 (d, 2.5)	121.0	16	–	168.5
4	2.51[a]	31.1	17	–	97.7
5	1.94[a]	42.8	18	1.10 (s)	24.2
6α	2.04 (td, 14.5, 3.5)	24.1	19	1.56 (s)	13.8
6β	1.77 (m)		20	2.74 (m)	43.4
7	4.17 (s)	81.2	21α	4.45 (dd 9.0, 6.0)	68.1
8	–	35.5	21β	4.35 (t, 8.0)	
9	2.15 (s)	39.0	22α	2.55 (dd, 17.0,.6.0)	28.8
10	–	48.4	22β	2.50 (dd, 17.0, 9.5)	
11	–	96.2	23	–	176.7
12a	1.90 (d, 13.5)	44.6	28	1.13 (d, 6.5)	19.3
12b	1.73[a]		30a	1.95[a]	36.4
13	–	75.2	30b	–	
14	1.93[a]	46.2	OMe	1.20 (d, 12.0)	55.4
OH	6.65 (s)				

[a] Overlapped signals; the multiplicity could not be observed.

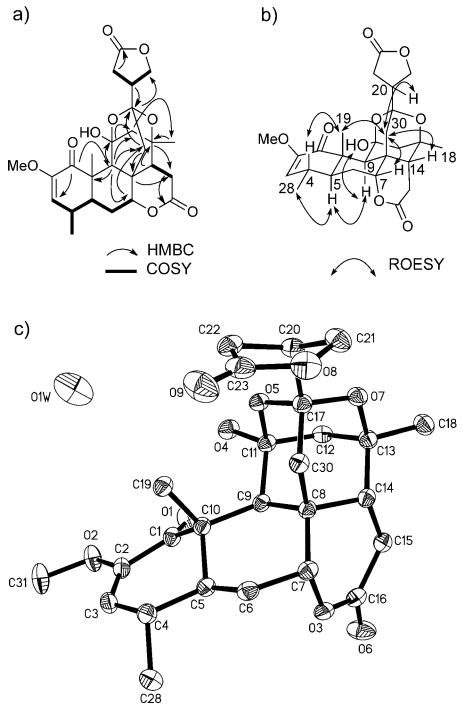


Figure 1. a) Key ^{1}H–^{1}H COSY and HMBC correlations, b) key ROESY correlations, and c) X-ray single-crystal structure of **1**.

and absolute configurations of **1** were also determined by ROESY and X-ray crystallographic experiments (Figure 1b). Thus, the full structure of this novel quassinoid named perforalactone A (**1**) with an unprecedented skeleton was elucidated. Notably, **1** contains a unique cagelike 2,4-dioxa-adamantane ring system not usually encountered in the natural products of the plant kingdom. Previously, only two polyketides[8,9] and two marine natural products,[10,11] one of which is tetrodotoxin,[10] were known to feature this structural moiety. Furthermore, compound **1** is the first naturally occurring 20*S* quassinoid, thus suggesting an alternative origin of quassinoids.[12]

Perforalactone B (**2**) possesses the molecular formula $C_{26}H_{32}O_8$, as determined by HRESIMS (m/z 495.1999 $[M+Na]^+$; calcd: 495.1994). Its IR, ^{1}H NMR, and ^{13}C NMR spectroscopic data (see Table S2) were analogous to those of **1**, thus suggesting that **1** and **2** are structurally similar. The primary difference between **1** and **2** is that **2** has four methyl groups, and its γ-lactone ring is connected to C13 through a ketone group at C17, as suggested by the HMBC correlations of H_3-18/C17, H-12/C17, H-20/C17, and H-21/C17. Detailed 2D NMR spectroscopic analysis (see Figure S7a and Table S1) provided the structure of **2**. However, the ROESY correlations of H_2-21/H_3-18, and H_2-22/H_3-30 only provided a clue to assign the key relative configuration at C20 because of rotation about the C13–C17 and C17–C20 bonds. Thus, computational modeling was used to help determine the orientation of H-20. The potential-energy surface of **2** with the 20-αH configuration was scanned at the AM1 level of theory by rotation about the C13/C17 and C17/C20 bonds, which resulted in 11 minimum-energy points (see Figure S1). All stable conformations were then optimized by DFT at the B3LYP/6-31G* level, and the harmonic vibrational frequencies of each structure were calculated by the same method in GAUSSIAN 03.[13] Seven stable conformations were identified, of which conformers **2a** (accounting for 60% of the conformations in the gas phase) and **2b** (accounting for 38%) are predominantly populated. By the same manner, the two predominant conformers 20-*epi*-**2a** (accounting for 51%) and 20-*epi*-**2b** (accounting for 47%) of the C20 epimer of **2** were found (see Table S3). The distances between the H_2-21 and H_3-18 and the H_2-22 and H_3-30 pairs in conformers **2a** and **2b** are shorter than those in 20-*epi*-**2a** and 20-*epi*-**2b** (Figure 2). Notably, **2** yielded the aforementioned ROESY correlations, whereas 20-*epi*-**2** did not yield analogous ROSEY correlations. Thus, the α orientation of H-20 was unambiguously established. Furthermore, the absolute configuration of **2** was determined to be identical to that of **1**, as evidenced by their nearly congruent circular dichroism curves (see Figures S6b and S11b).

Both perforalactones A (**1**) and B (**2**) have the *S* configuration at C20, which gives us an opportunity to analyze the origin of quassinoids. Previously, quassinoids were suggested to be biogenetically derived from (20*R*)-euphol or (20*S*)-tirucallol. However, only C_{25} quassinoids contain a γ-lactone-ring side chain that includes C20. This side chain is absent in the C_{20}, C_{19}, and C_{18} types.[3a,14] Thus, the true origin of quassinoids must be deduced from the configuration at C20 of C_{25} quassinoids. Because all reported C_{25} quassinoids have the

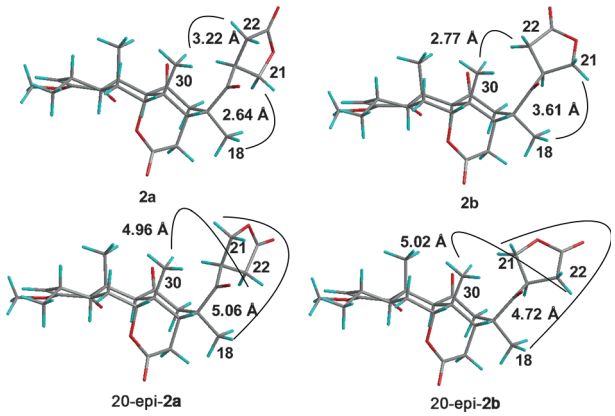
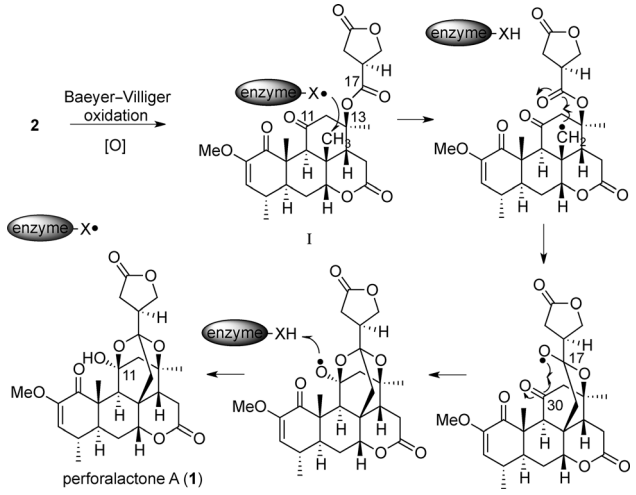


Figure 2. Optimized geometries of the predominant conformers **2a** and **2b** of compound **2**, and 20-*epi*-**2a** and 20-*epi*-**2b** of the C20 epimer of **2** at the B3LYP/6-31G* level of theory in the gas phase. The calculated distances of the key hydrogen-atom pairs are indicated.

R configuration at C20, (20*S*)-tirucallol has been hypothesized to be the only precursor of quassinoids.[12,15] Our finding of naturally occurring 20*S* C_{25} quassinoids thus provides the first evidence in support of the (20*R*)-euphol origin of quassinoids (Scheme 1).

Structure comparisons suggested an unusual 1,4-transannular migration of the γ-lactone ring as a possible route for the biosynthesis of **1** from **2**, in which the activation of the inert C30 methyl group is the key step. Herein, we propose a putative radical biosynthetic pathway to explain the migration (Scheme 2). To initiate the process, a Baeyer–Villiger oxidation breaks the C13/C17 bond to provide an intermediate **I**, which has been proposed as a common intermediate to bridge C_{25} and C_{20} quassinoids.[14] An enzymatic radical then abstracts a hydrogen atom at C30, and the resulting C30 radical attacks at the C17 carbonyl moiety to form the C17/C30 bond. Some dioxygenases and monooxygenases have been reported to be involved in hydrogen abstraction; for example, deacetoxycephalosporin C synthase abstracts a hydrogen atom from a methyl group in its substrate penicillin N to produce an ethylene sulfide.[16] Subsequently, the produced oxygen radical attacks the C11 carbonyl moiety to generate the C17/C11 ether bridge. Finally, the newly generated oxygen radical reabstracts the hydrogen atom from the active-site residue of the enzyme to produce **1** and thereby return the enzyme to its activated state for another reaction cycle.



Scheme 2. Putative biosynthetic pathway from **2** to **1**; X represents an enzyme active-site residue (Cys, Gly, or Tyr) that could form a radical to activate the inert C30 methyl group.

To further investigate this possible biogenetic pathway, we used LC–MS to identify the proposed intermediates in fresh plant extracts. However, after several attempts, no ion peaks of the possible intermediates were detected. Instead, a compound with an ion peak at *m*/*z* 417.2 was observed to co-occur with compound **1** as a minor constituent; its UV spectrum was similar to that of **1**. This compound was isolated, and its ^{1}H and ^{13}C NMR spectroscopic data were observed to be very similar to those of compound **2** (see Table S2), except for the absence of the side chain and the presence of an oxygenated methylene and an acetyl group, thus suggesting that it is a C_{20} quassinoid related to **2** that bears a acetyl group. Its HRESIMS spectrum (*m*/*z* 439.1737 $[M+Na]^+$; calcd: 439.1732) gave the molecular formula $C_{26}H_{32}O_8$, according to its 1D NMR spectroscopic data. Detailed 2D NMR spectroscopic analysis (see Figure S12a and Table S1) gave structure **3** in Scheme 1. The absolute configuration of the compound was determined by applying the CD excitation chirality method.[17,2c] The CD spectrum exhibited a positive split (231 nm, $\Delta\varepsilon = -0.48$; 250 nm, $\Delta\varepsilon = 6.56$) due to the excitation coupling between the two chromophores of the 2-en-1-one and 12-en-11-one systems;[17] this result indicated a clockwise orientation of the transition dipole moments of the two chromophores (see Figure S16b). Thus, the absolute configuration of this compound, perforalactone C (**3**), a C_{20} quassinoid with a 19-OAc group, was determined and is identical to that of **1** and **2**.

Notably, although nearly all other positions of a quassinoid could be oxygenated, a C19 methyl moiety has never been previously observed to be oxygenated. This functionality, together with the co-occurrence of **3** with **1** in this experiment as a minor isolate and the sole C_{20} quassinoid, strongly suggests that **3** shares a common intermediate **I** and a similar radical mechanism with **1** in its biosynthesis, instead of the involvement of a P450 enzyme. Clearly, the shift of a C30 methyl radical generated by the enzymatic radical to the C19 methyl group and the coupling of a free hydroxyl radical could lead to this unique oxygenation at C19 (see Scheme S1 in the Supporting Information).

The scaffold and structural features of these compounds are unique and differ significantly from those previously reported; thus, these compounds might display unprecedented biological profiles. The two major isolates **1** and **2** were subjected to insecticidal assays because other quassinoids have exhibited insecticidal activity.[3a] Compounds **1** and **2** demonstrated significant activity against the agriculturally relevant sucking pest insect *Aphis medicaginis* Koch, with LC_{50} values of 86.4 and 7.23 μM, respectively; however, they demonstrated no activity against the chewing insects *Spodoptera exigua* (Hübner) and *Mythimna separate* Walker. Moreover, **1** and **2** showed no evident cytotoxicity against

a series of cell lines (see the Supporting Information), thus suggesting the mode of action (MoA) of their insecticidal effect is not attributable to their cytotoxicity, unlike the MoAs of other reported quassinoids.[18]

In an attempt to preliminarily investigate the MoA of these quassinoids, we focused on the insect nicotinic acetylcholine receptor (nAChR) because it is a major and ubiquitous target for insecticide action.[19] Thus, compounds **1** and **2** were tested for their activity at the nAChR on isolated neurons from *Drosophila melanogaster*, which is a model insect commonly used in this assay.[19,20] Both compounds exhibited significant nAChR activity ($IC_{50} = 15.8$ nM for **1** and $IC_{50} = 1.26$ nM for **2**). In particular, **2** showed an IC_{50} value approximating that of the positive reference imidacloprid ($IC_{50} = 0.79$ nM), which is the most commercially important insecticide targeting an insect nAChR. Further biochemical electrophysiological tests suggested that they are all antagonists at the nAChR of the insect.[21] Owing to the paucity of the sample, compound **3** was only evaluated for its cytotoxicity against the HL-60 cell line, and showed moderate activity with an IC_{50} value of 8.7 μM.

Notably, **2** showed higher activities by the same order of magnitude against the *D. melanogaster* nAChR (12.5-fold) and *A. medicaginis* (11.9-fold) as compared with **1**. These data indicate that the insecticidal activity of both compounds might arise from their activity at the nAChR of the insect.[22] However, the binding site and affinity of these compounds may differ from those of neonicotinoid pesticides. Indeed, their structures are quite different; compounds **1** and **2** lack the negatively charged nitro groups that contribute to the activity of neonicotinoid pesticides.[19] These results imply that the isolates could be novel lead compounds as pesticides, especially because neonicotinoid pesticides have been suggested to cause unwanted environmental impact.[23] Although their structures are too complex to be synthesized easily, they could provide valuable inspiration for the development of new pesticides. Furthermore, the remarkable insecticidal and insect-nAChR activity of these quassinoids may be related to the defense system of plants.

Keywords: biosynthetic pathways · insecticidal activity · natural products · nicotinic acetylcholine receptors · structure elucidation

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