Hyperatomarin, an Antibacterial Prenylated Phloroglucinol from *Hypericum atomarium* ssp. *degenii*

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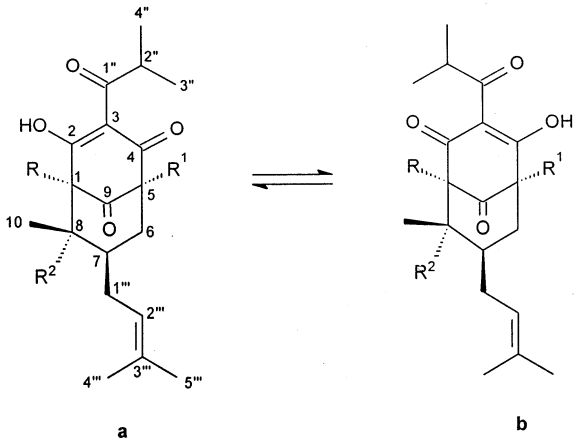
As shown by quantitative ^{1}H NMR measurements, a lipophilic extract of the aerial parts of *Hypericum atomarium* ssp. *degenii* contained a high percentage (3.1% per weight of dried plant material) of a prenylated phloroglucinol (**1**). Compound **1**, named hyperatomarin, occurring in two tautomeric forms (**1a** ⇆ **1b**), was isolated by bioactivity-guided preparative TLC and was identified on the basis of spectral data interpretation. This isolated phloroglucinol exhibited activity against Gram-positive (*Staphyloccocus aureus* and *Microccocus luteus*) and Gram-positive spore-forming bacteria (*Bacillus subtilis* and *B.* IP 5832).

The genus *Hypericum* L. (family Guttiferae) comprises about 400 species that occur commonly in temperate regions throughout the world. In Serbia, 19 species are known.[1] As a result of their numerous biological effects, secondary metabolites of the members of the genus have received considerable attention thus far. Among their active principles, prenylated acylphloroglucinols occupy an important role by showing various activities.[2] The best known example so far is the prenylated phloroglucinol, hyperforin, a constituent of *H. perforatum* (St. John's Wort), which exhibits antibiotic (against Gram-positive bacteria)[3] and antidepressant properties.[4]

Continuing our chemical examination of the flora from Serbia and Montenegro and the search for new compounds of pharmacological interest, we now report the examination of a lipophilic extract (petroleum ether−Et_2O, 2:1) of the aerial parts of *H. atomarium* Boiss. ssp. *degenii* (Bornm.) Hayek,[1,5] also known as *H. annulatum* Moris,[5] an species endemic to the southern parts of the Balkan Peninsula (Albania, Bulgaria, Greece, and southeast Serbia). A previous examination of the methanolic extract of the herb of *H. annulatum* from Bulgaria showed benzophenone *O*-glycosides and xanthones as the main constituents.[6]

The overall appearance of the ^{1}H NMR spectrum of the extract of *H. atomarium* ssp. *degenii* was typical for the overwhelming presence of a mixture of two structurally closely related prenylated phloroglucinols (**1a** and **1b**) in the ratio of 1:0.75, respectively. A quantitative ^{1}H NMR measurement performed on an aliquot of the crude extract revealed 3.1% of **1** (calculated per weight of the dried plant material), based on the integral of low-field OH singlets (δ 19.01 and 18.89) and the aromatic two-proton singlet of 2,6-di-*tert*-butyl-*p*-cresol (BHT) (δ 6.98), as internal standard. According to a preliminary antibacterial test, the crude extract exhibited activity against Gram-positive bacteria (*Staphyloccocus aureus* and *Microccocus luteus*) and Gram-positive spore-forming bacteria (*Bacillus subtilis* and *B.* IP 5832).



Preparative TLC afforded **1** as an inseparable binary mixture containing the same ratio of components **1a** and **1b** as in the crude extract. The occurrence of a pair of low-field singlets (mentioned above), typical for the hydrogen-bonded enolic form of a β-diketone, together with positive (EXSY) cross-peaks between the corresponding ^{1}H NMR signals of **1a** and **1b** observed in the PS NOESY spectrum, clearly indicated a mutual slow chemical exchange between two tautomeric forms (**1a** ⇆ **1b**). In the remainder of this paper, chemical shifts of the same signals of the tautomers when they were separated are presented as δ(**1a**)/δ(**1b**). In case of overlapping, they are denoted as a single chemical shift.

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Table 1. ^{1}H (600) MHz and ^{13}C (150 MHz) NMR Data of Tautomers **1a** and **1b** in C_6D_6 (room temperature), Referenced against TMS as Internal Standard

	1a		**1b**	
position	δ_H, mult. (*J*, Hz)	δ_C, mult.[a]	δ_H, mult. (*J*, Hz)	δ_C, mult.[a]
1	3.19 d (2.4)	65.6 d	3.27 d (2.4)	70.8 d
2		198.1 s		191.2 s
3		112.5 s		112.8 s
4		192.5 s		199.5 s
5	3.37 ddd (2.4, 4.2, 6.6)	61.7 d	3.22 ddd (2.4, 3.0, 6.0)	56.6 d
6	2.02−2.10 m	32.8 t	2.02−2.10 m	32.3 t
7	∼1.34 m	42.0 d	∼1.34 m	41.5 d
8		48.5 s		48.1 s
9		204.5 s		204.5 s
10	0.93 s	20.5 q	1.03 s	20.9 q
1′	1.21 ddd (4.8, 12.0, 15.9)	29.6 t	1.19 ddd (4.8, 12.0, 15.9)	29.7 t
	∼1.42 m		∼1.42 m	
2′	∼2.14 m	∼22.6 t[b]	∼2.14 m	∼22.6 t[b]
	∼1.87 m		∼1.87 m	
3′	∼5.05 m	124.0 d	∼5.05 m	124.2 d
4′		132.0 s		131.9 s
5′	1.59 bs	∼18 q[c]	1.58 bs	∼18 q[c]
6′	1.66 bs	∼25.8 q[b]	1.66 bs	∼25.8 q[b]
1″		209.3 s		209.0 s
2″	4.10 sept (6.6)	35.8 d	4.09 sept (6.6)	35.8 d
3″	0.99 d (6.6)	∼19 q[d]	1.01 d (6.6)	∼19 q[d]
4″	1.12 d (6.6)	∼19 q[d]	1.12 d (6.6)	∼19 q[d]
1‴	1.79 ddd (7.2, 10.8, 14.4)	29.6 t	1.97−1.99 m (2H)	29.7 t
	∼1.96 m			
2‴	4.79 bt (7.2)	123.8 d	4.71 bt (7.2)	123.9 d
3‴		133.0 s		133.0 s
4‴	1.39 bs	∼18 q[c]	1.36 bs	∼18 q[c]
5‴	1.58 bs	∼25.8 q[b]	1.57 bs	∼25.8 q[b]
OH	19.01 s		18.89 s	

[a] Multiplicity derived from H,C *J*-resolved spectrum. [b] Pair of partly overlapped resonances ($\Delta\delta_C$ −0.04 ppm). [c] One of four close resonances (δ_C 18.1, 17.8, 17.7, 17.6). [d] One of three close resonances (δ_C 19.1, 18.8, 18.7).

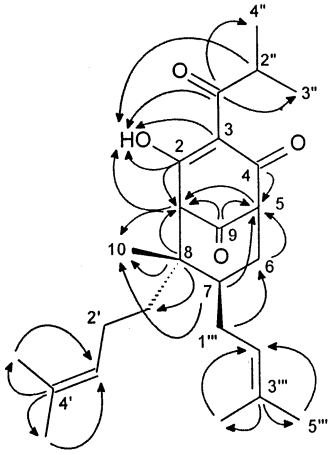

Figure 1. HMBC (C→H) correlations of **1a**.

The DCIMS of **1**, exhibiting a $[M + H]^+$ peak at *m/z* 401, and the ^{13}C NMR spectrum, in which most of the resonances were split into pairs of close signals belonging to the tautomers, indicated a molecular formula of $C_{25}H_{36}O_4$, with eight degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **1** (Table 1), assigned by means of various 2D NMR techniques, indicated a close structural similarity to hyperpapuanone (**2**), a [3.3.1]bicyclic prenylated phloroglucinol that has been previously isolated from *H. papuanum*.[7] By analogy to **2**, compound **1** contained an enolized β-diketone moiety (δ_C 198.1/191.2, C-2; 112.5/112.8, C-3; 192.5/199.5, C-4 and enolic OH, mentioned above) substituted, according to the HMBC data (Figure 1), at C-3 by an isobutyryl unit (δ_H 0.99/1.01 d, J = 6.6 Hz, H_3-3″; 1.12 d, J = 6.6 Hz, H_3-4″; δ_H 4.10/4.09 sept, J = 6.6 Hz, H-2″). The presence of a prenyl side chain at C-7, apparent in the ^{1}H NMR spectrum (Table 1), whose protons (H_2-1‴, H-2‴, H_3-4‴, H_3-5‴), according to the TOCSY and COSY spectra, constituted the same coupling network with the ring protons H-7 (δ_H ∼1.34 m) and H_2-6 (δ_H 2.02−2.10 m), was another common feature of **1** and **2**. Contrary to those of **2**, the ^{1}H NMR and ^{13}C NMR spectra of **1** contained signals of two methines assigned to the ring junctions C-1 (δ_C 65.6/70.8 d) and C-5 (δ_C 61.7/56.6 d). Protons H-1 (δ_H 3.19/3.27 d) and H-5 (δ_H 3.37/3.22 ddd), separated by a carbonyl (δ_C 204.5, C-9), as indicated by the HMBC spectrum (Figure 1), exhibited mutual long-range coupling ($^4J_{1,5}$ = 2.4 Hz) which was due to their *W*-spatial arrangement, typical for the protons from ring junctions. The multiplicity of H-5 (ddd) was in agreement with its additional coupling to an adjacent methylene, which, according to the COSY and TOCSY data, was assigned as H_2-6. At the same time, H-1, exhibiting no further coupling, was attached to a quaternary carbon (δ_C 48.5/48.1 s, C-8) as indicated by the HMBC spectrum (Figure 1). The remaining side chains bonded to C-8, according to the HMBC data (Figure 1), were identified as a methyl (δ_H 0.93/1.03 s, H_3-10) and a homoprenyl group whose protons (H_2-1′, H_2-2′, H-3′, H_3-5′, H_3-6′, Table 1) comprised an independent coupling network (consistent with the COSY and TOCSYspectral data). Finally, the remaining HMBC connectivities (Figure 1), not mentioned in the text, were in accordance with the bicyclic structure **1**. The assignment of the tautomers was based on NOEs of the enolic hydroxyl to H-1 in **1a** and H-5 in **1b**, respectively, as well as comparison of ^{13}C NMR chemical shifts of C(2)−C(3)−C(4) to those in the tautomers of **2**.[7]

The relative stereochemistry of **1** was assigned from its NOESY data. The occurrence of a NOE in the major tautomer (**1a**) between the tertiary methyl (H_3-10) and the enolic OH (at C-2) indicated the 8β-configuration of the methyl and the orientation of an 8α-homoprenyl side chain.

The 7β-stereochemistry of the prenyl group was established from the weak NOEs between the hydroxyl proton from both tautomers and the methylene protons at C-1‴.

Antimicrobial testing of **1** against Gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*), as well as Gram-positive spore-forming bacteria (*Bacillus subtilis* and *Bacillus* IP 5832), revealed the following MIC values: 1.56 μg/mL (*S. aureus*, *M. luteus*, and *B.* IP 5832) and 3.12 μg/mL (*B. subtilis*). Under the same conditions erythromycin showed MIC values of 0.2 μg/mL (*S. aureus* and *M. luteus*) and 0.39 μg/mL (*B. subtilis* and *B.* IP 5832).

Experimental Section

General Experimental Procedures. The spectra were recorded with the following instruments: optical rotations, Perkin-Elmer 141 MC polarimeter; UV, Cintra 40, GBC UV−vis spectrometer; IR, Perkin-Elmer FT-IR spectrometer 1725 X; ^{1}H and ^{13}C NMR 1D and 2D NMR, Varian Gemini 2000 (200 MHz for ^{1}H) and Bruker DMX 600 (600 MHz for ^{1}H) in C_6D_6 with TMS as internal reference (room temperature); DCIMS (150 eV, isobutane), Finnigan MAT mass spectrometer 8230, double focusing (BE geometry). Silica gel 60 F_{254} precoated aluminum sheets (0.25 mm, Merck) were used for TLC controls and preparative TLC plates (2 mm, Merck) for preparative purification. Elemental analysis was performed using the standard combustion (Pregl) method.

Plant Material. The aerial parts of *Hypericum atomarium* ssp. *degenii* were collected in southeast Serbia at the locality Sićevačka Klisura in June 2002. A voucher specimen (BEOU 14911) has been deposited in the herbarium at the Faculty of Biology, Botanic Garden "Jevremovac", Belgrade.

Extraction and Isolation. Air-dried and powdered aerial parts of *H. atomarium* ssp. *degenii* (88 g) were extracted twice at room temperature with 1:2 ether−petroleum ether (2 × 300 mL) for 24 h, followed by a 30 min extraction on an ultrasonic bath. The combined extracts were concentrated in vacuo to yield an oily light brown residue (8.2 g).

An aliquot of the crude extract (0.5 g), subjected to preparative TLC (approximately 50 mg per plate), using the developing system toluene−ethyl acetate, 95:5, R_f = 0.57, followed by benzene extraction of the purified compound, afforded 155 mg of **1**, corresponding to a yield of 2.9% (per weight of the dried plant material).

Hyperatomarin (1): colorless oil; $[\alpha]^{22}_D$ +19.4° (*c* 0.31, CH_2Cl_2); UV(MeOH) λ_{max} (log ϵ) 279 (3.81) nm; IR (film) ν_{max} 3400, 3048, 2973, 2936, 2858, 1737, 1666, 1551, 1432, 1383 cm^{-1}; ^{1}H and ^{13}C NMR (see Table 1); DCIMS *m*/*z* $[M + H]^+$ 401; *anal.* C 75.16%, H 8.97%, calcd. for $C_{25}H_{36}O_4$, C 74.96%, H 9.06%.

Bioassays. The test microorganisms were *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 4698 (Gram-positive bacteria), and *Bacillus subtilis* ATCC 6633 and *Bacillus* IP 5832 (Gram-positive spore-forming bacteria). Antibacterial activity was determined by the agar dilution method using the Mueller-Hinton medium.[8,9] The antibiotic erythromycin was used as a positive control.

An initial concentration (1 mg/mL) of tested samples was made in MeOH. Serial dilutions (100−0.1 μg/mL) of samples were prepared, and the surface of an agar plate was inoculated by streaking of a bacterial suspension (ca. 10^5 cfu/mL). After incubation at 37 °C for 24 h, minimal inhibitory concentrations (MICs) were determined as the lowest concentrations preventing any visible growth. All experiments were performed in duplicate.

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