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OXIDATION PRODUCTS OF HYPERFORIN FROM *HYPERICUM PERFORATUM*

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Key Word Index—*Hypericum perforatum*; Hypericaceae; supercritical CO_2 fractionation; oxidized hyperforins.

Abstract—The isolation of two oxidation products of hyperforin from the aerial parts of *Hypericum perforatum* and their structure determination by means of 2D NMR methods is reported. The products had the same 1-(2-methyl-1-oxopropyl)-2,12-dioxo-3,10β-bis(3-methyl-2-butenyl)-11β-methyl-11α-(4-methyl-3-pentenyl)-5-oxatricyclo[6.3.1.$0^{4,8}$]-3-dodecene skeleton. In addition, one of them, with the same number of carbons as hyperforin ($C_{35}H_{52}O_5$), contained a 1-methyl-1-hydroxyethyl group in the 6β-position, whereas the other compound (a hemiacetal, $C_{32}H_{46}O_5$), presumably a degradation product of hyperforin, exhibited a 6-hydroxy function. The latter was an inseparable mixture of 6α- and 6β-hydroxy epimers undergoing (according to phase sensitive NOESY) mutual interconversion.

INTRODUCTION

As a part of our examination of wild-growing Yugoslavian plant species exhibiting medicinal properties, we have examined the aerial parts of the very well known medicinal plant *Hypericum perforatum* L. (Ref. [1] and references therein). Our main objective was to test the applicability of a procedure for the isolation of active principles (mainly those exhibiting antibiotic properties), involving ethanol extraction of the air-dried aerial parts of *H. perforatum*, followed by fractionation of the alcoholic extract with supercritical CO_2. This procedure was previously successfully applied to the isolation of rosmanol-9-ethyl ether, a diterpene with antioxidant properties, from sage [2]. The antibiotic activity of the extract of *H. perforatum* was mainly attributed to the acylphloroglucide hyperforin (**3**), a rather unstable compound which is easily oxidised (during storage) to more polar derivatives [3].

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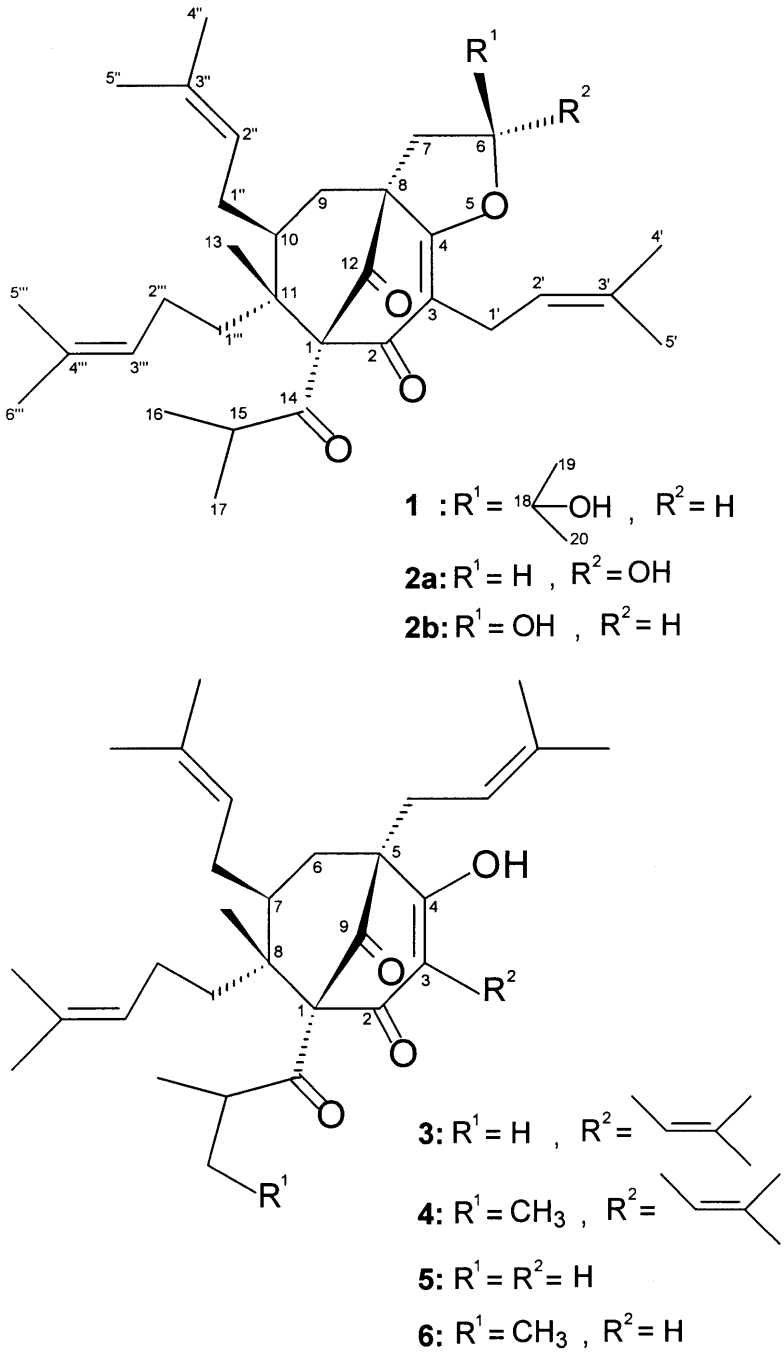
RESULTS AND DISCUSSION

Antibiogram tests of the crude EtOH-extract, using a standard diffusion double layer agar procedure [4] with a range of gram-positive bacteria as test organisms, revealed a moderate activity against *Staphylococcus aureus* and slightly lower activities against *Micrococcus luteus* and *Bacillus subtilis*. There was no activity against gram-negative bacteria such as *Escherichia coli*. Fractionation of the crude extract with supercritical CO_2 at different pressures, according to a previously published procedure [2], yielded five fractions (see Section 3). Whereas the less polar fractions I and II, extracted at lower pressures of CO_2, exhibited antibiotic activity against gram-positive bacteria comparable to that of the crude extract, the activity of the remaining more polar fractions (III–V) was lower. Neither fraction showed activity against gram-negative bacteria (*E. coli*).

Silica gel column chromatography of the main fraction (II) afforded fractions A and B (eluted with toluene, containing 0.4–0.5 and 1.1% EtOAc, respectively), exhibiting similar spectral data to those of the antibiotic hyperforin (**3**) [5–7]. The more polar fraction B contained only a single component



(**1**) with a molecular formula $C_{35}H_{52}O_5$ (as determined by means of EIMS) corresponding to a monooxygenated hyperforin. Fraction A [consisting of two epimeric compounds **2a** and **2b** in the ratio of *ca.* 2.2:1, respectively (as calculated from the ^{1}H NMR integral)] was assigned the molecular formula $C_{32}H_{46}O_5$ from the EIMS data. Whereas compound **1** was inactive on *S. aureus*, showing only low activity against *M. luteus*, the mixture **2a** + **2b**, in comparison to the crude extract, was more active on *M. luteus* and slightly less active on *S. aureus*.

The application of 2D NMR techniques, such as double quantum filtered (DQF) COSY, phase sensitive (PS) NOESY, TOCSY, HMQC and HMBC, together with comparison of the ^{1}H and ^{13}C NMR data to those of **3** [5–7], adhyperforin (**4**) [8] and hyperevolutin A and B (**5** and **6**) [9], enabled assignment of the majority of the NMR signals of the isolated compounds (Tables 1 and 2). The main difference between the ^{1}H NMR and ^{13}C NMR spectra of **1** and **3**, were those concerning the side-chain attached to C-5 in **3** (corresponding to C-8 in

Table 1. ^{1}H NMR data* of compound **1** and the mixture **2a** + **2b**† (300 MHz, $CDCl_3$)

H‡	1	2a	2b
6	4.53 *dd* (5.6, 10.8)	5.98 *dd* (3.6, 6.0)	6.11 *ddd* (5.0, 7.0, 7.6)
7α	1.74 *dd* (5.5, 13.0)	1.84 *d* (13.8)	2.14 *dd* (5.0, 13.9)
7β	2.63 *dd* (10.9, 13.0)	2.86 *ddd* (1.7, 6.1, 13.8)	2.57 *dd* (7.6, 13.9)
9α	~2.0§	2.48 *dd* (2.8, 12.4)	~1.95§
9β	1.49 *t* (12.6)	~1.6§	~1.6§
10	~1.60§	~1.65§	~1.65§
13	1.01 *s*	1.03 *s*	1.01 *s*
15	~1.95§	~2.0§	~2.0§
16	0.97 *d* (6.5)	0.96 *d* (6.5)	0.98 *d* (6.5)
17	1.06 *d* (6.5)	1.04 *d* (6.5)	1.06 *d* (6.4)
19	1.35 *s*	–	–
20	1.18 *s*	–	–
1′A	2.99 *dd* (7.6, 14.3)	2.97 *f dd* (7.0, 14.2)	2.97 *f dd* (7.0, 14.2)
1′B	3.11 *dd* (6.9, 14.3)	3.09 *f dd* (7.4, 14.2)	3.09 *f dd* (7.4, 14.2)
2′	5.04 *m*	~5.05 *m*	~5.05 *m*
4′	1.62 *br s*	1.62 *br s*	1.62 *br s*
5′	1.67 *br s*	1.67 *br s*	1.67 *br s*
1″A	1.7§	~1.75§	~1.75§
1″B	~2.1§	~2.15§	~2.15§
2″	4.92 *br t* (~6.5)	4.93 *m*	4.93 *m*
4″	1.67 *br s*	1.67 *br s*	1.67 *br s*
5″	1.54 *br s*	1.54 *br s*	1.54 *br s*
1‴A	~1.3 *m*	~1.3§	~1.3§
1‴B	~2.05§	~2.05§	~2.05§
2‴A	~1.9§	~1.95§	~1.95§
2‴B	~2.1§	~2.15§	~2.15§
3‴	5.04 *m*	~5.05 *m*	~5.05 *m*
5‴	1.57 *br s*	1.57 *br s*	1.57 *br s*
6‴	1.62 *br s*	1.62 *br s*	1.62 *br s*
OH	not observed	3.66 *dd* (1.7, 3.7)	3.72 *d* (7.0)

*Assigned by means of DQF COSY, PS NOESY, TOCSY and HMQC.
†The signals of the epimers were distinguished according to the relative intensities.
‡For the notation of protons see formulae **1**, **2a** and **2b**.
§Overlapped with other signals; detected via 2D correlations.

our compounds). Whereas **3** contained ^{1}H NMR signals of four gem-dimethyl substituted double bonds, i.e. vinyl protons (4H, δ 4.8–5.3) and allylic methyls (8×3H, δ 1.6–1.8), in compound **1** the following signals associated with three trisubstituted double bonds were observed: δ 5.04, (2H, *m*, H-3‴ and H-2′), 4.92 (*br t*, $J = ca.$ 6.5 Hz, 1H, H-2″), 1.67, (6H, *br s*, H-4″ and H-5′), 1.62 (6H, *br s*, H-4′ and H-6‴), 1.57 (3H, *br s*, H-5‴) and 1.54 (3H, *br s*, H-5″). The presence of these double bonds in **1** was also corroborated by the occurrence of six olefinic ^{13}C NMR signals, three (δ 121.2, 122.3, 124.8) exhibiting direct (HMQC) correlations to the vinyl protons and the remaining three sp^2-carbons (δ 131.0, 132.5, 133.5) being non-protonated. A signal of the proton from the missing vinyl group was replaced by a *dd* (H-6, δ 4.53, $J_{6,7\alpha} = 5.6$ and $J_{6,7\beta} = 10.8$ Hz), typical for a proton α-positioned to an oxygen and coupled to an adjacent methylene (H-7), with a HMQC correlation to the carbon resonating at δ 90.1. The remaining non-protonated vinyl carbon, bearing two methyl groups, of this double bond was replaced in **1** by a $Me_2C(18)$–O moiety as revealed from the following ^{13}C and ^{1}H NMR data: δ_C 70.9 (*s*, C-18), 26.8 (*q*, C-19), 24.1 (*q*, C-20) and, δ_H 1.19 (3H, *s*, H-20) and 1.35 (3H, *s*, H-19), together with HMBC correlations between the protons of these methyls and carbons C-6 and C-18 (Fig. 1). Another difference between **1** and (ad)hyperforin (and also hyperevolutins) was the absence of a low-field signal (δ *ca.* 7) of a 4-enolic hydroxyl proton. Compound **1** contained a tertiary hydroxyl group (ν_{max} 3475 cm^{-1}) which could not be acetylated using a standard procedure (Ac_2O/pyridine, room temp.). It was converted into a trichloroacetylcarbamoyl (TAC) derivative by reaction (in the NMR tube) with trichloroacetyl isocyanate (TAI) reagent [10], effecting a downfield shift of H-6, H-19 and H-20 ($\Delta\delta$ *ca.* 0.3–0.4 ppm) corresponding to a β-acylation shift, thus revealing the attachment of the hydroxyl group to C-18. The HMBC cross peaks (Fig. 1) clearly demonstrated the link between this side chain and the rest of the molecule. This, together with the evidence quoted so far, accorded with a tetrahydrofuran substructure, obtained by an heterocyclization involving oxidative addition of two oxygens (one of them being the 4-enolic hydroxyl) to the double bond of the 5-isopentenyl side chain of hyperforin. The HMBC correlations also confirmed the same structure of the remaining part of the molecule as that of hyperforin.

The 6α-H configuration (and also 6β-orientation of the $Me_2C(18)OH$ moiety) was deduced from a NOE between H-6 and a proton of the adjacent C-7 methylene, assigned as H-7α (δ 1.74 *dd*, $J_{6,7\alpha} = 5.5$ and $J_{7\alpha,7\beta} = -13.0$ Hz) on the basis of its upfield shift in comparison to H-7β (δ 2.63 *dd*, $J_{6,7\beta} = 10.9$ and $J_{7\alpha,7\beta} = -13.0$ Hz), the latter being in the plane

Table 2. ^{13}C NMR chemical shifts (δ)* of compound **1** and the mixture **2a** + **2b**† (75 MHz, $CDCl_3$)

C‡	1	2a	2b
1	83.2	83.1	83.3
2	192.7	193.3	193.1
3	116.7	116.9	116.8
4	172.9	173.0	171.5
6	90.1	102.7	104.1
7	30.2	36.1	37.1
8	59.4	58.2	59.1
9	38.1	39.8	38.9
10	43.2	43.7	43.1
11	48.3	47.5	47.8
12	204.4	204.4	204.0
13	13.5	13.4	13.5
14	209.4	210.6	210.5
15	42.0	41.8	41.9
16	21.3	21.2	21.2
17	20.4	20.2	20.2
18	70.9	–	–
19	26.8	–	–
20	24.1	–	–
1′	22.1	22.3	22.2
2′	121.2	120.7	120.8
3′	132.5	132.7	132.7
4′	25.7§	25.5–25.7‖	25.5–25.7‖
5′	17.8¶	17.6–17.8**	17.6–17.8**
1″	27.1	26.9	26.9
2″	122.3	122.1	122.0
3″	133.5	133.1	133.5
4″	25.8	25.5–25.7‖	25.5–25.7‖
5″	17.6¶	17.6–17.8**	17.6–17.8**
1‴	36.4	36.3	36.3
2‴	25.2	25.2	25.1
3‴	124.8	124.7	124.6
4‴	131.0	131.0	131.1
5‴	17.9§	17.6–17.8**	17.6–17.8**
6‴	25.6¶	25.5–25.7‖	25.5–25.7‖

*Assigned by means of DEPT, HMQC and HMBC and the analogy with the ^{13}C NMR data of hyperevolutin A and B [9].
†The signals of epimers **2a** and **2b** were distinguished according to the relative intensity (*ca.* 2:1, respectively).
‡For carbon notation see formulae **1**, **2a** and **2b**.
§The assignments can be interchanged.
‖Partially superimposed methyl signals.
¶The assignments can be interchanged.
**Partially superimposed three methyl signals.

of 12-carbonyl group and thus experiencing its deshielding effect. The NOE between H-6 and H-9α (δ *ca.* 2.0, identified by means of HMQC and TOCSY), observed in the PS NOESY of **1** (Fig. 2), also confirmed the proposed α-geometry of this proton. The remaining NOEs, together with COSY correlations (not shown) were also in agreement with the proposed structure.

Most of the ^{13}C resonances of fraction A were split into pairs of close signals (Table 2), thus indicating a mixture of two closely related (epimeric) compounds (**2a** and **2b**). Moreover, the occurrence of positive PS NOESY cross-peaks between some signals of the epimers (see below) clearly indicated mutual slow chemical exchange. The only difference between the spectral data of this mixture and those of **1** (and also of hyperforin) was associated with a part of the molecule originating from a side-chain attached in hyperforin at C-5. The absence of ^1H and ^{13}C NMR signals of the Me_2C of the tertiary carbinol moiety, i.e. C-18, $C(19)H_3$ and $C(20)H_3$ (observed in **1**) and the decrease of molecular mass by 42 amu (in comparison to that of **1**) indicated that **2a** and **2b** were the degradation products of hyperforin. The resonance of the proton corresponding to H-6 in **1** was shifted downfield and divided into two multiplets, one of them (δ 6.11, *ddd*, $J_{6,0H} = 7.0$, $J_{6,7\beta} = 7.6$ and $J_{6,7\alpha} = 5.0$ Hz) belonging to the less abundant **2b** and the other (δ 5.98, *dd*, $J_{6,0H} = 3.6$, $J_{6,7\beta} = 6.0$ Hz) to the major component **2a**. After acetylation of **2a** + **2b**, using the standard procedure (Ac_2O/pyridine, room temp.), the signal of H-6 was shifted downfield to δ 6.68 (*d*, $J_{6,7\beta} = 6.0$ Hz) and 6.80 (*dd*, $J_{6,7\alpha} = 5.8$ and $J_{6,7\beta} = 7.2$ Hz) in **2a** and **2b**, respectively, with concomitant simplification of multiplicity due to the removal of coupling with OH. The ratio of the epimers was also markedly affected by the acetylation. In the 1:4 mixture of acetates the main acetate was that originating from the minor epimer **2b**. All this evidence indicated an epimeric hemiacetal structure for **2a** and **2b**, undergoing mutual chemical exchange, as demonstrated by the occurrence of positive cross-peaks between the signals of H-6 (and also of H-7β) in the PS NOESY. The hemi-acetal structures were also supported by the chemical shifts of C-6 (i.e. δ 104.1 and 102.7, in **2a** and **2b**, respectively) identified by means of HMQC correlations to H-6. The DQF COSY cross-peaks of H-6 enabled identification of the signals of the adjacent methylene (H-7α and H-7β). The assignment of β-orientation in both epimers to a proton of $C(7)H_2$ giving resonance at lower field was based (as in **1**) on the anisotropic effect of the 12-carbonyl group. According to Dreiding models, H-7β in both epimer was in the plane of the 12-carbonyl group, thus explaining its deshielding effect. The occurrence of positive PS NOESY cross-peaks due to the epimerisation at C-6, between H-7β of **2a** and **2b**, corroborated this assignment. In epimer **2b** H-6 coupled to protons resonating at δ 2.14 (*dd*, $J_{7\alpha,6} = 5.0$ and $J_{7\alpha,7\beta} = -13.9$ Hz) and 2.57 (*dd*, $J_{7\beta,6} = 7.6$ Hz, $J_{7\alpha,7\beta} = -13.9$ Hz), assigned as H-7α and H-7β, respectively. In epimer **2a** only H-7β (δ 2.86, *ddd*, $J_{7\beta,OH} = 1.7$, $J_{7\beta,6} = 6.1$ and $J_{7\alpha,7\beta} = -13.8$ Hz) coupled to H-6, while H-7α (δ 1.84, *d*, $J_{7\alpha,7\beta} = -13.8$ Hz) exhibited only geminal coupling. This indicated a *trans* H-6/H-7α relationship with a torsion angle between them of *ca.* 90°. A strong negative PS NOESY cross-peak connecting the signals of H-6 and H-7β in this epimer clearly indicated a *cis*-relationship between these protons, corresponding to 6α-orientation of the hydroxyl group. At the same time, the NOE H-6/H-7α in **2b** was in agreement with a 6β-hydroxy- (and 6α-H) configuration. The observed chemical shifts of H-7 were also in accord with the above proposal. In **2a**, H-7β was deshielded in comparison to the same proton in **2b** which was in accord with the well-

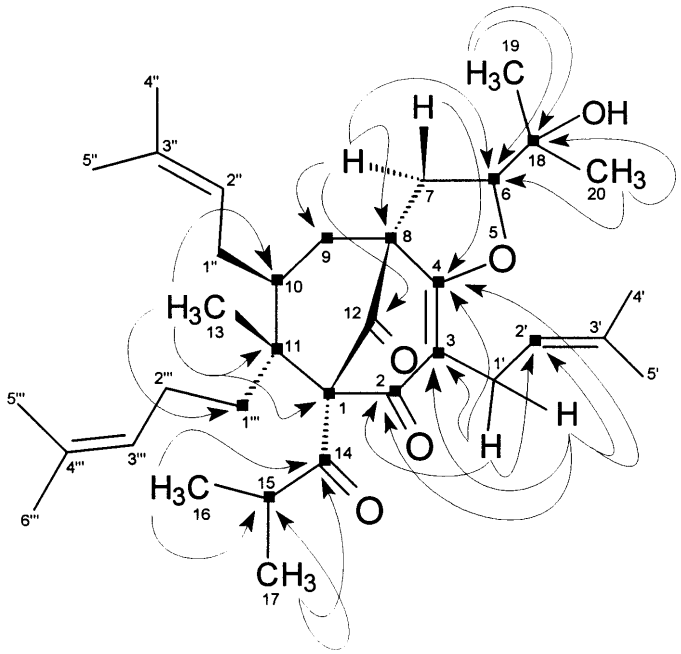


Fig. 1. Significant HMBC correlations of **1**

known anisotropic influence of the vicinal OH group [11].

Introduction of a OH group usually causes a downfield shift of the vicinal *anti*-proton and an upfield shift of the *syn*-proton to the extent of 0.1–0.3 ppm. Due to the same influence, H-7α in **2b** was deshielded ($\Delta\delta$ = 0.40 ppm) in comparison to the same proton in **2a**. The occurrence of a long-range coupling between the hydroxyl proton and H-7β (J = 1.7 Hz) in **2a** indicated a "W"-spatial arrangement in the H–O–C(6)–C(7)–H(β) moiety (probably fixed by intramolecular hydrogen bond to the ethereal oxygen), which is also compatible to the proposed α-orientation in this epimer.

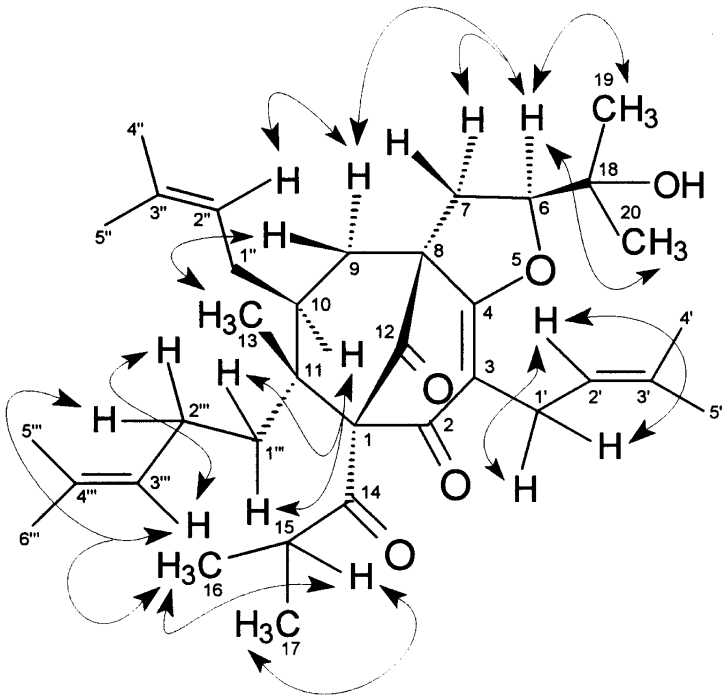


Fig. 2. Significant NOEs in **1**

EXPERIMENTAL

Plant material

Hypericum perforatum (voucher 0794 deposited at the Faculty of Pharmacy, University of Belgrade) was collected at mount Ozren (Southeast Serbia) during the flowering season in July 1994.

Isolation procedure

The air-dried ground aerial parts (6.3 kg) were extracted twice with EtOH (96%, 10 l) at room temp. with occasional stirring for 10 days. After depigmentation of the combined extracts (concentrated to *ca*. 6 l) using 200 g of active charcoal, evaporation of the solvent *in vacuo* yielded the crude extract (702.5 g). For the preparation of suitable material for batch extraction with CO_2, the extract, dissolved in EtOH, was mixed with thermally treated alumino-silicate (500 g), and the resulting suspension was evaporated under reduced pressure. The dry solid residue was passed through a 2-mm sieve and extraction of this (in a pilot plant device), using different pressures of CO_2 and temp yielded five fractions: I (10.5 g, 140 bar, 60°), II (38.9 g, 210 bar, 60°), III (17.1 g, 280 bar, 60°), IV (8.9 g, 350 bar, 70°) and V (7.3 g, 420 bar, 80°). A portion of the main fraction II (20 g) was subjected to CC on silica gel, starting elution with toluene and gradually increasing the polarity by addition of EtOAc. The mixture **2a** + **2b** (206 mg) was isolated from fraction (A) eluted with toluene containing 0.4–0.5% (v/v) EtOAc, after repeated silica gel CC (C_6H_6–EtOAc, 22:3). Fraction B, eluted with toluene containing 1.1% EtOAc (v/v) was rechromatographed on silica gel CC (C_6H_6–EtOAc, 22:3) to afford compound **1** (342 mg) as a waxy solid.

1-(2-*Methyl*-1-*oxopropyl*)-2,12-*dioxo*-3,10*β*-*bis*(3-*methyl*-2-*butenyl*)-6*β*-(1-*methyl*-1-*hydroxyethyl*)-11*β*-*methyl*-11α-(4-*methyl*-3-*pentenyl*)-5-*oxatricyclo*-[6.3.1.$0^{4,8}$]-3-*dodecene* (**1**)

White solid, $[\alpha]_D^{25}$ +68 ($CHCl_3$, *c* 0.20). UV λ_{max}^{EtOH} nm (log ϵ): 273 (4.29); IR ν_{max}^{film} cm^{-1}: 3491, 1730, 1657, 1363, 1377; EIMS (probe) 70 eV, *m*/*z* (rel. int.): 552 [M]+ (74), 537 (4), 348 (34), 347 (32), 305 (17), 293 (21), 204 (50), 161 (16), 135 (99), 109 (22), 95 (35), 93 (29), 69 (100), 57 (28), 43 (67); ^{1}H and ^{13}C NMR: Tables 1 and 2.

1-(2-*Methyl*-1-*oxopropyl*)-2,12-*dioxo*-3,10*β*-*bis*(3-*methyl*-2-*butenyl*)-6-*hydroxy*-11*β*-*methyl*-11α-(4-*methyl*-3-*pentenyl*)-5-*oxatricyclo*[6.3.1.$0^{4,8}$]-3-*dodecene* (**2a** + **2b**)

White solid. UV λ_{max}^{EtOH} nm (log ϵ): 273 (4.06); IR ν_{max}^{film} cm^{-1}: 3431, 1731, 1627; EIMS (probe) 70 eV, *m*/*z* (rel. int.): 510 [M]+ (21), 467 (6), 448 (8), 423 (8), 305 (11), 289 (5), 263 (10), 204 (47), 147 (27), 135 (65), 109 (21), 95 (24), 93 (26), 69 (100), 55 (22), 43 (70); ^{1}H and ^{13}C NMR: Tables 1 and 2.

Bioassay

The anti biogram-tests were carried out in Petri dishes containing two-layers of agar, prepared in two steps according to the standard diffusion procedure [4]. The microorganisms were grown in the Department of Microbiology, Faculty of Chemistry (Belgrade). The measure of activity was based on the diameter of the inhibition zone.

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