Flavones and Sesquiterpene Lactones from *Achillea atrata* subsp. *multifida*: Antimicrobial Activity

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Four flavones (**1**−**4**) and nine sesquiterpene lactones (**5**−**13**), one of them (**5**) a new compound, were isolated from the aerial parts of *Achillea atrata* L. subsp. *multifida*. Although the crude extract demonstrated *in vitro* inhibitory activity against *Candida albicans* and *Bacillus subtilis*, all isolated flavones were active against *B. subtilis*. Flavones **1**, **2**, and **3** were also active against *C. albicans*, while **1** and **3** exhibited activity against *E. coli*, as well. None of the tested lactones (**7**, **9**, **12**, and **13**) showed any antimicrobial activity.

The central part of the Balkan peninsula abounds in endemic plant species. One such is a sort of yarrow, identified as *Achillea atrata* L. subsp. *multifida* (DC) Heim (section Ptarmica, family Asteraceae).[1] It is used in traditional medicine as a remedy for bronchial and laryngeal troubles, as well as for pulmonary infections. In the course of our phytochemical studies of the Yugoslavian flora belonging to the Asteraceae and a search for compounds of pharmacological interest, the investigation of the aerial parts of this species was undertaken with an emphasis on the antimicrobial activity of the whole extract and its constituents.

Because the aerial parts of the members of this genus usually contain sesquiterpene lactones,[2−5] the standard extraction procedure[6] for isolation of these compounds has been applied. Si gel column chromatography of the crude extract, followed by rechromatography of selected fractions exhibiting antimicrobial activity and/or containing compounds that could be interesting from a pharmacological as well as a phytochemical point of view (e.g., sesquiterpene lactones), afforded the four flavones, santin (**1**),[7] centaureidin (**2**),[8] apigenin (**3**),[9] and its 7-*O*-*β*-glucoside (**4**).[10] In addition, nine sesquiterpene lactones, all belonging to a 12,6α-olide group (with 11αH configuration), have been isolated: 8α-hydroxy-1α,4α,5α,11*β*H-guaia-10(14)-en-12,6α-olide (**5**, a new guaianolide), the elemanolide temisin (**6**),[11] three germacranolides [balchanolide (**7**),[12] 1*β*-hydroperoxy-8α-hydroxygermacra-4,10(14)-dien-6*β*,7α,11*β*H-12,6α-olide (**8**),[13] shonachalin A (**9**)[13]], and four eudesmanolides [11*β*,13-dihydroreynosin (**10**),[13] 1*β*-hydroxy-6*β*,7α,11*β*H-selin-3-en-6,12-olide (**11**),[14] 8α-hydroxy-11*β*,13-dihydrobalchanin (**12**),[15] and artapshin (**13**)[15]]. The lactones and flavones isolated previously from other plants were identified by comparison of their spectral data with the published ones.

Antimicrobial activity of the crude extract, pure compounds (**1**−**4**, **7**, **9**, and **12**+**13**) and two principal antibiotics used in therapy (nystatin and penicilin G) was detected by the agar well diffusion method.[16] None of the tested lactones exibited any activity, but the crude extract and flavones **1**−**4** were active against *Bacillus subtilis*, with bacteriostatic zones of 6−8 mm. Activity against *Candida albicans* was demonstrated by the crude extract (7 mm), **1** (8 mm), **2** (11 mm), and **3** (10 mm). Only **1** and **3** (6−8 mm) were active against *Escherichia coli*, while no activity was found against Gram-positive bacteria *Staphylococcus aureus* and the fungus *Aspergillus niger*. Minimal inhibitory concentrations (MIC) for *C. albicans* and *E. coli* of flavones **1**−**3** were also determined. The MIC of **3** for *C. albicans* was comparable to that measured for nystatin (3.12 *μ*g/mL) and also to the literature data regarding the activity of this antibiotic.[17] With the increase of superinfections with fungi, particularly with *Candida*, as a consequence of overusing antibacterial antibiotics, or as a complication in chemotherapy of immunocompromised patients, these compounds may offer some potential as therapeutic agents.

The structure of the new lactone **5** was determined by means of IR, CIMS, and ^{1}H (1D and 2D) NMR spectra. The ^{1}H NMR data of **5**, assigned by means of double quantum filtered (DQF) COSY and phase sensitive (PS) NOESY, are presented in Table 1. Five double-bond equivalents obtained from the molecular formula of $C_{15}H_{22}O_3$ (measured by CIMS) together with the NMR evidence, such as two broad one-proton singlets (δ 5.05 and 5.10) typical for an exocyclic double bond, two methyl doublets (δ 1.43 and 0.93, J ca. 7 Hz), and a double doublet (δ 4.0, J = 9.6 and 10.0 Hz) characteristic for a lactonic proton (H-6), indicated a guaia-10(14)-en-12,6α-olide structure with the *trans*-annelated lactone ring. The methyl doublet at a lower field, according to the characteristic chemical shift and COSY correlation to H-11 (dq, δ 2.60), was readily assigned to H-13. The similarity of $J_{11,13}$ to that in the related 11α-methylguaianolides,[2,18] and the large coupling constant of H-11 to H-7 (J = 10.8 Hz), together with a NOESY H-13,H-7 correlation (Figure 1), indicated 11α-methyl configuration in **5**. The strong NOE between H-15 and H-6 fully accorded with 4*β*-Me geometry. The occurrence of an abundant $[M + H - 18]^+$ fragment (m/z 233) in the CIMS as well as IR evidence (3383 cm^{-1}) were in agreement with a hydroxyl functionality. The OH group was associated

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Table 1. ^{1}H (NMR 300 MHz) Data[a] (assigned by DQF and PS NOESY correlations) of Lactone **5** Measured in $CDCl_3$

H	δ_H
1	2.76, ft, 8.0
2α,2β	2.0−1.7, m
3α,3β[b]	1.75−1.55, m
4	2.39, m
5	2.25, m
6	4.00, dd, 9.6, 10.0
7	1.99, m
8	3.96, m
9β	2.81, dd, 5.2, 12.0
9α	2.08, dd, 10.3, 12.0
11	2.60, dq, 10.8, 7.0
13	1.43, d, 7.0
14	5.10, s
14′	5.04, s
15	0.96, d, 7.0

[a] The following signals are (fully or partially) overlapped: H-1 and H-9β; H-4 and H-5; H-7 and H-9α; H-7 and H-2β; H-2α and H-2β ($\delta_{2\alpha}<\delta_{2\beta}$); H-2α and H-3α; H-3α and H-3β ($\delta_{3\alpha}>\delta_{3\beta}$). [b] Partially obscured by the signal of H_2O (δ ca. 1.6).

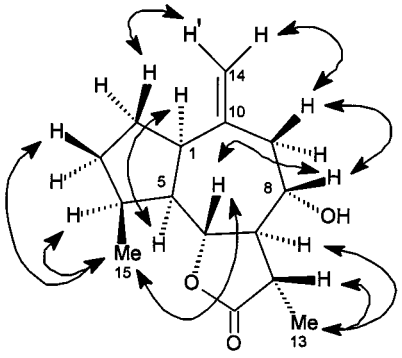

Figure 1. Observed NOESY connectivities for **5**.

with one (carbinol) proton multiplet (δ 3.69) in the ^{1}H NMR assigned by the COSY correlations as H-8. The NOE of this proton to H-6, as well as the magnitudes of scalar coupling to H-9 ($J_{8,9\alpha} = 10.3$ and $J_{8,9\beta} = 5.2$ Hz), were in accordance with 8α-OH geometry. The remaining correlations observed in the DQF COSY in combination with those from the PS NOESY (Figure 1) enabled us to determine the structure and the relative stereochemistry of this molecule.

Experimental Section

General Experimental Procedures. UV spectra were measured as diluted samples in MeOH on a UV−vis Specord M-40 (Carl Zeiss) spectrometer. Melting points were determined using a Boetius PHMK apparatus and are not corrected. IR spectra were recorded on a Perkin−Elmer FT IR 1725 X spectrometer. ^{1}H NMR (200 and 300 MHz) and ^{13}C NMR (50 and 75 MHz) data were registered on a Varian Gemini 2000 and a Bruker AMX 300 NMR spectrometer with TMS as internal standard. The chemical shifts are reported in δ units (ppm). EIMS (70 eV) and CIMS (isobutane, 150 eV) were taken on a Finnigan MAT 8230 double-focusing mass spectrometer. Column chromatography was performed on Si gel 60 Merck, 70−30 mesh. A commercially available Lobar column packed with Si gel 60 Merck (40−60 μm) was used for MPLC. Preparative TLC was performed using 0.2-mm precoated Si gel 60 F_{254} (Merck).

Plant Material. The aerial parts of *Achillea atrata* L. subsp. *multifida* were collected during the flowering season at Piribreg (altitude of ca. 2300 m) on Sara Mountain, Yugoslavia, in July 1992. A voucher specimen (no. 150792AA) has been deposited at the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade.

Extraction and Isolation. The powdered, air-dried aerial parts (718 g) were extracted exhaustively by maceration at room temperature with a mixture of petroleum ether−Et_2O−MeOH (1:1:1) using a standard procedure.[6] After filtration, the extract was concentrated in vacuo to yield 30 g of a residue that was subsequently applied to Si gel column chromatography and eluted with petroleum ether. The polarity of the solvent was gradually increased by adding of Et_2O, and elution was finished with gradual addition of MeOH to Et_2O (up to 50%). The evaporated fractions were combined according to their TLC profiles. Based on their IR spectra and TLC profiles, fractions that contained lactones and flavones (F-1−F-6) were further purified.

From the fraction F-1 [0.7 g, eluted with petroleum ether−Et_2O (4:1)] precipitation from Et_2O afforded 36 mg of yellow crystals that were further purified on a Si gel column [eluent petroleum ether−Et_2O (1:1) and Et_2O] to yield santin (**1**, 32.4 mg).

F-2 [0.62 g, petroleum ether−Et_2O (1:4)], upon crystallization from Et_2O−petroleum ether, gave 55 mg of a white solid that was further chromatographed on Si gel (elution with petroleum ether−Et_2O, gradually increasing the percentage of Et_2O) to afford **7** (18 mg) and the guaianolide **5** (2 mg). From the mother liquor, after evaporation to dryness, repeated crystallization from MeOH gave centaureidin (**2**, 30 mg). Evaporation of the mother liquor and MPLC, eluent, Et_2O−petroleum ether−CH_2Cl_2 (5:3:2), gave several fractions. Only two of them contained lactones, and these were further examined. Preparative TLC of the less polar fraction [eluent, petroleum ether−Me_2CO (5:1), two developments] and repeated preparative TLC [eluent, $CHCl_3$−MeOH (25:1), two developments] gave **2** (3 mg) and **6** (2 mg). The more polar fraction was purified by preparative TLC [eluent, $CHCl_3$−MeOH (25:1), two developments] to obtain **8** (3 mg) and **10** (1.2 mg).

Fraction F-3 [40 mg, petroleum ether−Et_2O (2:8)] upon crystallization from MeOH afforded apigenin (**3**, 10 mg).

Fraction F-4 (1.7 g, Et_2O) was chromatographed on Si gel [eluent, toluene−EtOAc (7:3 and 1:1)] to obtain a mixture (685 mg) of two isomeric lactones. Two portions of this mixture (ca. 10 mg each) were subjected to preparative TLC [eluent, Et_2O−petroleum ether−MeOH (4:2:1)], three developments, to obtain **12** (1.2 mg) and **13** (2.8 mg).

From the fraction F-5 (0.94 g, Et_2O), a portion (470 mg) was rechromatographed on Si gel [eluent, toluene−EtOAc (7:3) and pure EtOAc] to yield lactone **9** (181 mg).

The last fraction F-6 [0.6 g, Et_2O−MeOH (8:2)], upon crystallization from MeOH, gave apigenin-7-glucoside (**13**, 78 mg).

8α-hydroxy-1α,4α,5α,11βH-guaia-10(14)-en-12,6α-olide (5): obtained as a colorless solid (Et_2O−petroleum ether), mp 112−114 °C; IR (film) ν_{max} 3383, 1754, 1664, 1452, 1381 cm^{-1}; ^{1}H NMR (see Table 1); HRCIMS *m/z* $[M + H]^+$ 251.1642 (calcd for $C_{15}H_{23}O_3$, 251.1647); DCIMS *m/z* $[M + H]^+$ 251 (100), 233 (66) $[M + H - 18]^+$.

Bioassays. Antimicrobial activity against Gram-positive bacteria *S. aureus* ATCC 25923, spore-forming *Bacillus* IP 5832, Gram-negative bacteria *E. coli* ATCC 25922, yeast *C. albicans* ATCC 24433, and fungus *A. niger* was determined by the agar well diffusion method.[16] Stationary-phase culture was suspended in saline solution in a concentration of approximately 10^5 cfu/mL, and 200 μL of the suspension was spread over the surface of Mueller−Hinton (for bacteria) and Sabouraud (for yeast and fungi) agar plates. Test samples at a concentration of 50 μg/50 μL were added in the wells, and after incubations of 24 h (bacteria and yeast) or 48 h (fungi), zones of inhibition were measured (as the diameter).

Minimal inhibitory concentrations (MICs) were determined in duplicate, in brain heart infusion broth for *E. coli* and Sabouraud liquid medium for *C. albicans*. Initial concentration (1 mg/mL) of samples was made in DMSO. Serial dilutions (100−0.1 μg/mL) of samples were prepared, and liquid medium was inoculated by cultures in stationary phase, concentration 10^5 cfu/mL. After incubation at 37 °C for 24 h, MICs were determined as the lowest concentration of compound preventing any visible growth.[19]

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