Five New Bioactive Sesquiterpenes from the Fungus *Radulomyces confluens* (Fr.) Christ.

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Dedicated to Professor Wolfgang Steglich on the occasion of his 65th birthday

Basidiomycetes, Sesquiterpene, Protoilludane, Illudalane, Illudane

The three protoilludanes radulone A (**1**), radulone B (**2**) and radudiol (**3**), the illudalane radulactone (**4**) and the illudane radulol (**5**) were isolated from the extracts of the culture fluids of the basidiomycete *Radulomyces confluens*. The structures of the five new compounds were determined by spectroscopic techniques. Radulone A (**1**) is a potent inhibitor of human and bovine platelet aggregation stimulated by different agonists, inhibiting preferentially the aggregation of human platelets induced by ADP with an IC_{50} value of 2 μM. In addition **1** exhibits cytotoxic and antimicrobial activities. The other four compounds exhibited weak antimicrobial and cytotoxic activity.

Introduction

Platelets play an important role in the development of vascular disorders and arterial thrombosis. The receptors for different agonists and the pathways for the signal transduction offer several interesting targets for pharmacological intervention. In the course of a search for fungal metabolites inhibiting the collagen induced aggregation of bovine platelets, extracts of submerged cultures of several hundred basidiomycetes were tested. Among them, the extracts of *Radulomyces confluens* 95135 showed potent inhibitory activity, and its contents were investigated by bioassay-guided fractionation. In the following we describe the fermentation, isolation, structure elucidation and biological activities of radulone A (**1**) and B (**2**), radudiol (**3**), radulactone (**4**) and radulol (**5**), five new sesquiterpenes produced by *Radulomyces confluens*.

Materials and Methods

General

IR and UV spectra were measured with a Bruker IFS 48 and a Perkin-Elmer Lambda 16 UV/VIS spectrometer, respectively. For analytical HPLC a Hewlett Packard 1090 series II instrument was used. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (*J*) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$=145 Hz and $^nJ_{CH}$=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer, melting points (uncorrected) were determined with a Reichert microscope, and the optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22 °C. All flash chromatography was performed on 60 Å 35–70 μm Matrex silica gel (Grace Amicon) TLC analyses were made on Silica Gel 60 F_{254} (Merck) plates and visualised with anisaldehyde/sulfuric acid and heating.

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Producing organism

Fruiting bodies of *Radulomyces confluens* (Syn.: *Cerocorticium confluens* (Fr.: Fr.) Jül. & Stalpers) were collected growing on wood. The specimen showed all characteristics of the genus and species (Eriksson, 1981). Mycelial cultures were derived from the spore print of a fruiting body. The strain 95135 is deposited in the culture collection of the LB Biotechnolgie, University of Kaiserslautern.

Fermentation and isolation

For maintenance on agar slants and submerged cultivation, *Radulomyces confluens* was grown in YMG medium composed of : yeast extract 0.4%, malt extract 1%, glucose 0.4%, pH 5.5 and agar 1.5% for solid media. For the production of **1** and **4** fermentations were carried out in a Biolafitte C6 fermenter containing 20 l of YMG medium at 22 °C with aeration (2 l air/min) and agitation (120 rpm). 250 ml of a well grown culture in YMG medium were used as inoculum. For the production of **2**, **3** and **5** a fermentation was carried out in a Biostat U fermenter (Braun) containing 100 l of YMG medium. The fermenter was incubated at 22 °C with an aeration rate of 20 liters air/minute and agitation (120 rpm). Ten liters of a well grown seed culture in the same medium served as inoculum.

After 6 days of fermentation in the 20 l scale the culture fluid was separated from the mycelia by filtration. Compounds **1** and **4** were removed from the culture fluid (13 l) by adsorption to HP21 resin (30 x 300 mm, Mitsubishi) and eluted with acetone. The crude extract (1097 mg) was applied onto a column (25 x 250 mm) containing silica gel (0.063–0.2 mesh, Merck 60) and eluted with 700 ml cyclohexane : ethylacetate 50:50 v/v. 438 mg of a crude product were obtained and applied onto another silica gel column (15 x 145 mm, 0.063–0.2 mesh, Merck 60). Elution with cyclohexane : ethylacetate 50:50 v/v yielded 315 mg of an enriched product. 142 mg of pure **1** and 4.1 mg of pure **4** were obtained by preparative HPLC (LiChrosorb CN, 7 µm, Merck, column 250 x 25 mm), elution with cyclohexane : *tert.* butylmethylether 60 : 40 v/v (Rt 30 minutes) and 10 : 90 (Rt 45 minutes), respectively. The 100 l fermentation was extracted in the same way, and the crude extract (6.6 g) was fractionated by silica gel chromatography with toluene : ethanol 20:1 as eluent. Four fractions containing **1**, **2**, **3** and **5** as the main products were obtained. The impure fraction containing **5** was purified by repeated silica gel chromatography using the following systems; toluene : ethanol 40:1, heptane : ethylacetate 4:1, toluene : ether 20:1 and toluene : acetonitrile 10:1, yielding 21 mg of pure **5**. 40 mg of **3** were isolated after silica gel chromatography with toluene : ethanol 20:1 and toluene : ether 1:1, while the fraction containing **2** was further purified by repeated silica gel chromatography in the systems; heptane : ethylacetate 4:1, toluene : ethanol 40:1, heptane : ethylacetate 8:1 and dichloromethane : acetone 20:1. Final yield: 95 mg of pure **2.**

Radulone A (**1**) was obtained as a colourless oil. $[\alpha]_D$ – 182° (*c* 2.7 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 283 nm (21,000). IR (KBr): 3430, 2925, 1670, 1630, 1595, 1455, 1380, 1345, 1310, 1145, 1115, 1030, 950 and 935 cm^{-1}. See Tables I and II for NMR data. EIMS (70 eV), *m/z* (rel. int.): 246.1249 (100%, M^+, $C_{15}H_{18}O_3$ requires 246.1256), 231 (15%), 217 (63%), 203 (39%), 201 (23%), 175 (21%), 173 (24%), 159 (17%), 145 (14%), 122 (30%).

Radulone B (**2**) was obtained as a colourless oil. $[\alpha]_D$ –96° (*c* 2.0 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 255 (19,200) and 282 nm (19,700). IR (KBr): 3439, 2956, 2867, 1647, 1619, 1460, 1416, 1372, 1331,1253, 1219, 1166, 1144, 1127, 1083, 1026, 996, 754, 632 and 581 cm^{-1}. See Tables I and II for NMR data. EIMS (70 eV), *m/z* (rel. int.): 232.1463 (73%, M^+, $C_{15}H_{20}O_2$ requires 232.1463), 217 (M-CH_3, 100%), 203 (33%), 176 (53%), 137 (27%), 95 (38%), 83 (15%).

Radudiol (**3**) was obtained as white crystals (EtOH), m.p. 47–49 °C. $[\alpha]_D$ + 27° (*c* 1.0 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 258 nm (7,500). IR (KBr): 3345, 2952, 2928, 2867, 1645, 1463, 1377, 1317, 1225, 1134, 1103, 1080, 1055, 1034, 1016, 1000, 898, 872 and 612 cm^{-1}. See Tables I and II for NMR data. EIMS (70 eV), *m/z* (rel. int.): 221.1908 (<4%, M^+-OH, $C_{15}H_{25}O$ requires 221.1905), 238 (<1%), 194 (100%), 176 (86%), 161 (68%), 136 (38%), 81 (37%). FABMS (positive ions), *m/z*: 261 (M^++Na). CIMS (CH_4), *m/z* (rel. int.): 221 (M-H_2O+H^+, 30%), 203 (100%), 177 (85%), 176 (34%), 161 (19%), 109 (11%), 95 (10%).

Radulactone (**4**) was obtained as a colourless oil. $[\alpha]_D$ + 24° (*c* 0.5 in $CHCl_3$). UV (MeOH), λ_{max}

(ε): 252 nm (10,300). IR (KBr): 3430, 2955, 2925, 1715, 1610, 1465, 1450, 1400, 1385, 1335, 1290, 1265, 1170, 1110, 1085 and 1045 cm^{-1}. See Tables I and II for NMR data. EIMS (70 eV), *m/z* (rel. int.): 246.1245 (100%, M^+, $C_{15}H_{18}O_3$ requires 246.1256), 231 (52%), 229 (23%), 228 (27%), 213 (22%), 203 (40%), 169 (32%), 155 (10%), 128 (14%), 115 (17%).

Radulol (**5**) was obtained as a colourless oil. $[\alpha]_D$ + 164° (*c* 0.6 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 248 nm (21,200). IR (KBr): 3433, 3016, 2953, 2862, 1642, 1454, 1377, 1360, 1286, 1257, 1158, 1130, 1085, 1037, 976, 957, 879, 859, 572 and 490 cm^{-1}. See Tables I and II for NMR data. EIMS (70 eV), *m/z* (rel. int.): 218.1657 (69%, M^+, $C_{15}H_{22}O$ requires 218.1671), 203 (M-CH_3, 100%), 173 (84%), 145 (32%), 107 (23%), 91 (27%), 77 (19%).

Biological Assays

The platelet aggregation assay was carried out as described previously (Lorenzen *et al.*, 1994). The tests for cytotoxic effects (Zapf *et al.*, 1995), hemolytic activity (Kuschel *et al.*, 1994) and the antimicrobial activity in the serial dilution assay (Anke *et al.*, 1989) have been described previously. COS-7 cells were grown in D-MEM medium supplemented with 10% fetal calf serum incubated in a humified atmosphere containing 5% CO_2. Effects on COS-7 cells were determined as described for HeLa S3 cells (Zapf *et al.*, 1995).

Results and Discussion

Fermentation and isolation

As detected by analytical HPLC, production of radulone A (**1**) and radulactone (**4**) in the culture broth started immediately. Interestingly, only small amounts of compounds **2**, **3** and **5** were formed during fermentations in the 20 l scale. The fermentors were harvested when the activity of the crude extract in the platelet aggregation assay reached a maximum, approximately after 6 days. The extraction of the culture broth and isolation of the metabolites **1** to **5** is described in the experimental section.

Structural elucidation

The structures of radulone A (**1**) and B (**2**), radudiol (**3**), radulactone (**4**) and radulol (**5**) are shown in Figure 1. The determination of their structures is based on data from NMR spectroscopy and mass spectrometry, the 1D NMR data are given in Tables I and II while other spectroscopic data are given in the experimental part.

High resolution EIMS data for radulone A (**1**) indicate that its elemental composition is $C_{15}H_{18}O_3$, suggesting that **1** has an unsaturation

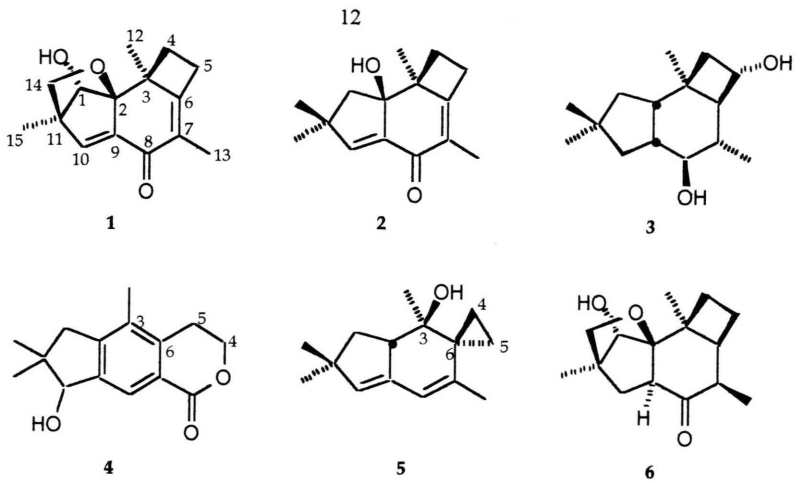

Fig. 1.

Table I. ^1H (500 MHz) NMR data (δ; multiplicity; *J*) for radulones A (**1**) and B (**2**), radudiol (**3**), radulactone (**4**) and radulol (**5**) in $CDCl_3$, with the $CHCl_3$ signal (7.26 ppm) as reference. The coupling constants *J* are given in Hz. (α and β where appropriate).

H	**1**	**2**	**3**	**4**	**5**
1α	–	2.10; d; 14.3	1.42; dd; 8.0, 13.4	2.62; d; 16.3	1.55; dd; 9.9, 12.3
1β	3.66; s	1.71; d; 14.3	1.54; dd; 9.0, 13.4	2.79; d; 16.3	1.74; dd; 7.4, 12.3
2		–	2.36; ddd; 8, 9, 10	–	3.03; ddd; 2.4, 7.4, 9.9
4α	1.79; ddd; 2.8, 10, 10	1.69; dd; 3, 10, 10	1.48; dd; 7.1, 11.4	4.48; t; 5.9	1.00; ddd; 4.0, 5.8, 9.5
4β	2.65; dd; 9, 9, 10	2.67; dd; 8, 8, 10	2.14; dd; 7.4, 11.4	–	0.52; ddd; 4.0, 6.5, 9.5
5α	3.09; m; 1, 9, 10, 16	3.02; 1.7, 8.3, 10.0, 16.3	–	2.96; t; 5.9	0.80; ddd; 4.5, 6.5, 9.5
5β	2.72; ddd; 2.9, 8.5, 16.1	2.73; dd; 3.0, 8.5, 16.3	3.98; ddd; 7, 7, 7	–	0.92; ddd; 4.5, 5.8, 9.5
6	–	–	1.70; dd; 7, 8	–	–
7	–	–	1.50; m	–	–
8	–	–	3.16; dd; 9.6, 10.8	7.99; s	5.98; s
9	–	–	2.12; dddd; 7, 10, 11, 11	–	–
10α	6.79; s	6.57; s	1.25; dd; 11, 12	4.69; s	5.22; d; 2.4
10β	–	–	1.77; dd; 6.8, 12.2	–	–
12	1.46; s	1.27; s	1.08; s	2.18; s	1.04; s
13	1.65; d; 1	1.66; d; 1.7	1.11; d; 6.5	–	1.51; s
14α	3.72; d; 16.8	1.23; s	1.01; s	1.05; s	1.04; s
14β	2.77; d; 16.8	–	–	–	–
15	1.31; s	1.15; s	1.12; s	1.17; s	1.13; s

Table II. ^{13}C (125 MHz) NMR data (δ; multiplicity) for radulones A (**1**) and B (**2**), radudiol (**3**), radulactone (**4**) and radulol (**5**) in $CDCl_3$, with the $CDCl_3$ signal (77.0 ppm) as reference.

C	**1**	**2**	**3**	**4**	**5**
1	85.6; d	47.1; t	41.2; t	44.2; t	39.8; t
2	89.2; d	88.1; s	44.6; d	147.4; s	53.1; d
3	46.1; s	51.7; s	31.0; s	131.3; s	71.9; s
4	25.7; t	24.8; t	43.7; t	66.6; t	9.2; t
5	28.5; t	28.1; t	71.3; d	25.2; t	6.2; t
6	167.9; s	168.6; s	60.7; d	138.2; s	32.6; s
7	126.4; s	126.2; s	41.4; d	124.2; s	141.1; s
8	185.3; s	186.2; s	76.3; d	124.2; d	119.9; d
9	141.6; s	141.0; s	48.0; d	143.8; s	139.2; s
10	143.7; d	150.2; d	46.4; t	83.0; d	133.1; d
11	56.5; s	44.6; s	38.4; s	44.1; s	44.5; s
12	23.5; q	23.1; q	28.7; q	14.9; q	20.2; q
13	9.7; q	9.4; q	18.0; q	165.9; s	19.3; q
14	68.3; t	29.8; q	28.6; q	21.5; q	27.8; q
15	11.9; q	28.2; q	29.6; q	26.8; q	29.7; q

index of 7. As the NMR data demonstrate the presence of two double bonds and one carbonyl group, radulone A (**1**) consequently has 4 rings. The HMBC correlations shown in Fig. 2 establish the protoilludane skeleton with C-1, C-2 and C-14 oxygenated, and the HMBC correlation between 14-H_2 and C-2 proved the position of the fourth ring. A Dreiding model of radulone A (**1**) shows that the C-1 hydroxyl group is axial, and the NOESY correlations between 1-H and 12-H_3, 14-Hα and 15-H_3 are in agreement with 1-H being equatorial. In addition, in C_6D_6 (the NMR data in this solvent are not reported) the C-1 hydroxyl proton was clearly visible as a broad singlet, with NOESY correlations to 1-H, 10-H, 12-H_3 and 15-H_3. Radulone A (**1**) shows structural similarities with the antibiotic sesquiterpene coprinolone (**6**), isolated from *Coprinus psychromorbidus* (Starratt *et al.*, 1988).

Radulone B (**2**) is obviously related to radulone A (**1**), it is reasonable to suggest that it is a biosynthetic precursor, lacking the C-1 and C-14 oxygenation. The HMBC correlations (see Fig. 2) demonstrate the structure of **2**, and NOESY correlations show that the relative configuration of C-2 and C-3 are the same as in **1**.

Radudiol (**3**) has no unsaturations except for the three rings, and differs from the radulones in the oxygenation pattern. The fact that so many carbons are protonated makes the structure reasonably simple to determine, besides the obvious COSY correlations only the HMBC correlations from 12-H_3, 14-H_3 and 15-H_3 are needed to put the protoilludane skeleton together. The relative stereochemistry was determined by the NOESY correlations observed between 2-H and 1-Hβ, 4-Hβ, 5-H and 9-H, between 5-H and 7-H, between 8-H and 6-H, 13-H_3 and 10-Hα, and between 9-H and 7-H. 12-H_3 gave NOESY correlations to 1-Hα and 4-Hα,

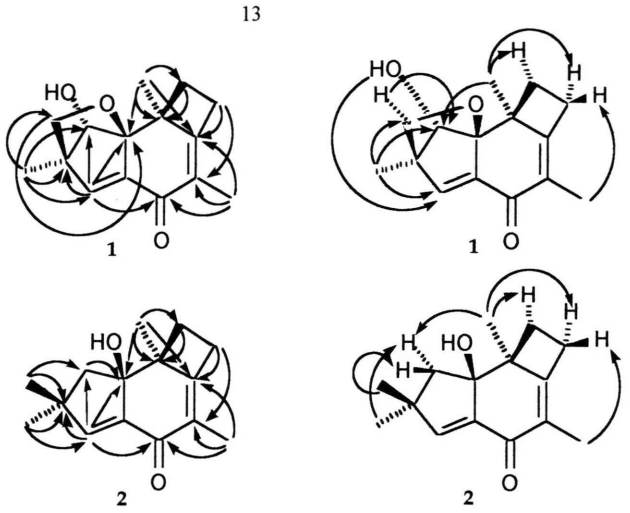

Fig. 2. Pertinent HMBC (left) and NOESY (right) correlations observed for radulones A (**1**) and B (**2**) in $CDCl_3$. The NOESY correlation between 1-OH and 10-H was observed in C_6D_6.

14-H_3 to 1-Hβ, 2-H, 9-H and 10-Hβ, while 15-H_3 gave correlations to 1-Hα and 10-Hα.

Radulactone (**4**) is a dihydroisocoumarin sesquiterpene with an illudalane skeleton. Its structure was determined by the HMBC correlations from 1-H_2 to C-2, C-3 and C-9, 4-H_2 to C-6 and C-13, from 5-H_2 to C-3, C-6 and C-7, from 10-H to C-2, C-8 and C-9, and from 12-H_3 to C-2, C-3 and C-6, besides the usual correlations from 14-H_3 and 15-H_3. The NOESY correlations between 8-H and 10-H, and between 12-H_3 and 1-H_2 as well as 5-H_2 confirmed the structure. Interestingly, the isomeric illudalane sesquiterpene with the hydroxyl group attached to C-1 instead of C-10, calomelanolactone, has been isolated from *Pityrogramma calomelanos* (Bardonille *et al.*, 1978).

The structure of the illudane radulol (**5**) was determined by the HMBC correlations from 12-H_3 to C-2, C-3 and C-6, from 4-H_2 and 5-H_2 to C-3, C-6 and C-7, from 13-H_3 to C-6, C-7 and C-8, and from 14-H_3 and 15-H_3 to C-1, C-10 and C-11, besides to each other. The illudane skeleton is established by the HMBC correlations from 2-H to C-9, and from both 8-H and 10-H to C-2. The ^1H coupling constants of the cyclopropane ring show typical values for illudane sesquiterpenes. The relative stereochemistry was established by the NOESY correlations between 2-H and 1-Hβ as well as 4-H_2, between 12-H_3 and 1-Hα as well as 5-Hα, and between 15-H_3 and 1-Hα.

Biological properties

Radulone A (**1**) is a potent inhibitor of human and bovine platelet aggregation stimulated by different inducers. The effects of **1** on the aggregation of human and bovine platelets stimulated with different inducers are compared in Table III. **1** inhibited preferentially the aggregation of human platelets induced by ADP with an IC_{50} of 2 μM. The IC_{50} values of **1** for the other inducers are significantly higher. A specific interference of **1** with one of the receptors seems unlikely, because **1** inhibited TPA induced platelet aggregation. TPA is an activator of the protein kinase C (PKC) and triggers the PKC dependent pathways of signal transduction. The inhibitory mechanism may be due to

Table III. Effect of radulone A (**1**) on platelet aggregation.

Inducer		Human platelets IC_{50}* [μM]	Bovine platelets IC_{50}* [μM]
Collagen	[0.4 mg/ml]	28	12
ADP	[25 μM]	2	12
Thrombin	[0.1 U/ml]	325	51
U 46 619[a]	[4.5 μM]	30	–
Arachidonic acid	[0.02 mg/ml]	53	–
TPA	[50 ng/ml]	38	–

IC_{50} values of **1** for the inhibition of aggregation of human and bovine platelets stimulated by different inducers.
* 50% inhibition of aggregation.
[a] Thromboxane A_2 analogue (UpJohn).
– inducer not applicable.

an unselective reaction of **1** with sulfhydryl and ε-amino functions of cysteine and lysine residues of the receptors and the proteins participating in signal transduction. **1** reacts with lysine and dithiothreitol by forming several biologically inactive adducts. Puri *et al.* reported in 1991, that chemical modification of the cysteine and lysine residues on the platelet surface impair platelet aggregation. They demonstrated, that *o*-phthalaldehyde inhibited ADP induced platelet aggregation by covalent modification of the 100 kDa ADP receptor, called aggregin. One of the sites of modification contains closely spaced cysteine and lysine residues whose integrity is essential to the functions of aggregin (Puri *et al.*, 1991). This may be an explanation for the preferential inhibition of ADP induced platelet aggregation.

Radulone A (**1**) also showed strong cytotoxic activities on HL 60 and L 1210 cells with IC_{50}'s of 2 and 1 μM, respectively, whereas lower cytotoxic effects were observed with HeLa S3 and COS-7 cells (Table IV). In the serial dilution assay **1** exhibited antifungal and antibacterial activities (Table V). No hemolytic effects of **1** on bovine erythrocytes could be observed at concentrations up to 400 μM.

None of the other four sesquiterpenes radulone B (**2**), radudiol (**3**), radulactone (**4**) and radulol (**5**) influenced the aggregation of human or bovine platelets induced with different agonists. The cytotoxic effects of **2**, **3**, **4** and **5** on different cell lines are shown in Table IV. Compared to **1** they are much weaker with radulol (**5**) as the most potent with IC_{50} values of 43 μM for L 1210 and 108 μM for HL 60 cells. Radulactone (**4**) and radulone (**5**) exhibited weak bacterio- and fungistatic effects against *Corynebacterium insiduosum*, *Micrococcus luteus* and *Nematospora coryli* (at 406 μM) and *Bacillus subtilis* and *Penicillium notatum* (at 431 μM), respectively.

Table IV. Cytotoxic activities of the radulones A (**1**) and B (**2**), radudiol (**3**), radulactone (**4**) and radulol (**5**).

Cells	Compound [μM]				
	1 IC_{50}	**2** IC_{50}	**3** IC_{50}	**4** IC_{50}	**5** IC_{50}
HeLa S3	16	–	–	–	431
COS-7	20	n.t.	n.t.	–	n.t.
HL 60	2[a]	431[b]	–	203[a]	108[a]
L 1210	1[a]	431[b]	188[b]	203[a]	43[a]

– IC_{50} not reached by concentrations up to 400 μM.
[a] Lysis of cells.
[b] Inhibition of growth.

Table V. Antimicrobial effects of radulone A (**1**).

Organism	MIC of **1** [μM]
Bacteria	
Escherichia coli K12	203[†]
Salmonella typhimurium TA 98	406[s]
Arthrobacter citreus	203[†]
Bacillus brevis	406[s]
Bacillus subtilis	406[†]
Corynebacterium insiduosum	20[s]
Micrococcus luteus	20[†]
Mycobacterium phlei	–
Streptomyces bikiniensis	41[†]
Fungi	
Nadsonia fulvescens	203[†]
Nematospora coryli	20[†]
Saccharomyces cerevisiae α S 288 c	203[†]
Saccharomyces cerevisiae is 1	41[†]
Fusarium oxysporum	–
Paecilomyces variotii	102[s]
Penicillium notatum	102[s]
Mucor miehei	41[†]
Rhodotorula glutinis	102[†]
Ustilago nuda	20[†]

Minimal inhibitory concentration (MIC) of **1** in the serial dilution assay.
[†] = Bacterio-/fungicidal effects; [s] = Bacterio-/fungistatic effects; – No effects up to 406 μM.

It is interesting to note the differences in biological activities between radulone A (**1**) and B (**2**). Both contain the same conjugated penta-1,4-dien-3-one functionality, which at a first glance would be considered responsible for the potent bioactivity of **1**. Either the oxygen bridge between C-2 and C-14 in radulone A (**1**) is important for the bioactivity *per se*, or it induces a better overlap in the dienone system making it more reactive. The fact that coprinolone (**6**) (Starratt *et al.*, 1988) has antibiotic properties indicates that the former alternative is correct.

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