



Isolation, characterization and in vitro cytotoxicity of new sesquiterpenoids from *Achillea clavennae*

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Manuscripts

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5 Dear Prof. Merfort,
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13 Please find attached the revised manuscript PLAMED-2013-05-0502-OP.R1 entitled
14 “Isolation, characterization and *in vitro* cytotoxicity of new sesquiterpenoids from *Achillea*
15 *clavennae*” by Snežana Trifunović, Anđelka M. Isaković, Aleksandra Isaković, Ivan Vučković,
16 Boris Mandić, Miroslav Novaković, Vlatka Vajs, Slobodan Milosavljević and Vladimir
17 Trajković.
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23 We have accepted all suggestions and made appropriate corrections designated in yellow color in
24 the revised manuscript. We hope that our answers will satisfy your criteria and criteria of
25 reviewer(s). The manuscript has been corrected by a native English speaker.
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41 Sincerely yours,

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Responses to Reviewers

Abstract:

- Please use *Achillea clavennae* in the abstract and not only the abbreviation *A. clavennae*. Please add also partly in this way "...inducing partly apoptotic death..." (see comment above).

Both corrections have been made.

Introduction:

- Correct page 8, line 9: flavonoids aglycons

The correction has been made.

- Please delete structures of compounds 7 – 10 from Fig 1, as they have already been isolated from the same plant. Compound 4 can remain as this compound was included in the cytotoxicity assay.

The structures of compounds 7 – 10 from Fig. 1 have been deleted. Consequently, oleanolic acid has been assigned a number 7 (before 11). Also, sentences which refer to Fig. 1 (or deleted compounds) have been rewritten:

Abstract: page 2, lines 5 – 7 (revised manuscript)

Introduction: page 4, lines 10 and 11

Results and discussion: page 4, lines 20 – 23, and page 5, line 1

Materials and methods (*Extraction and isolation*): page 14, lines 15, 17, 23, 24, and page 15, lines 1 and 2

Figure legends: Fig. 1 "The structures of compounds 1–11. " have been replaced with "The structures of compounds 1–7. "

Results and discussion:

- Nomenclature should always be the same:

1
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3 Page 10, lane 7: correct: ...and C-10(14) double bonds

4
5 Correct: page 11, lane 7:4,10(14)-

6
7 Correct: page 11, lane 22: ...10(14)

8
9 It is not necessary to write 4(5), it is sufficient 4

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11 The authors have correctly considered this by the chemical name.

12 **We agree with the referee. All corrections have been made.**

13
14 **Page 5, line 12 (revised manuscript): C-4(5) and C-10 has been replaced with C-4 and C-**
15
16 **10(14)**

17 **Page 6, line 12: $\Delta^{4(5),10}$ -germacranolides has been replaced with $\Delta^{4,10(14)}$ -germacranolides**

18
19 **Page 7, line 4: $\Delta^{4(5)}$ and Δ^{10} has been replaced with Δ^4 and $\Delta^{10(14)}$**

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21
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23 - Page 13, lane 2: please simplify:indicated C-5 to be quarternary

24
25 **The correction has been made (page 8, line 7)**

26
27
28 - Please give the exact IC₅₀ values of compounds 3, 4 and cisplatin in U251 and C6 cells
29 including standard deviation. Moreover, the authors write in the figure legend that “the results are
30 presented as mean + SD values of triplicates from a representative of three independent
31 experiments”. The authors must give the results from the three independent experiments!! And
32 not only from one experiment with three technical replicates.
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38 **In accordance with the referee’s request, we have presented the mean IC₅₀ values (with SD)**
39
40 **for each compound (Table 2) and briefly discussed their efficiency in the revised**
41 **manuscript (page 10, lines 12 – 17). Also, we have presented results in Fig. 4 as mean ± SD**
42 **values from three independent experiments, as stated in the revised manuscript (Fig. 4**
43 **legend).**
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48 - Looking at Fig. 5A no concentration dependent activation in U251 cells can be observed, but
49 slightly in C6 cells. Please correct this in the manuscript.

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52 **To comply with the referee’s suggestion, we have deleted the words “dose-dependent”.**

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54 Figure legend 5: please indicate more clearly where a concentration of 25 micromolar and where
55 12.5 and 25 micromolar concentrations were used.
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3 **We have corrected the Fig. 5 legend to indicate more clearly where a concentration of 25**
4 **µM and where 12.5 and 25 µM concentrations were used.**

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6
7 There is a discrepancy between Fig 5C and the manuscript: 32.8% annexin+ cells???

8
9 **The percentage of U251 annexin⁺ cells in Fig. 5C is 22.7% annexin⁺PI⁻ + 10.1% annexin⁺PI⁺**
10 **= 32.8%.**

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15 - The authors have shown that their sesquiterpene lactones induce ROS production, however they
16 did not prove the correlation to apoptosis! This can only be done if they have added an
17 antioxidants together with their compounds and studied cell death. From their results it is also
18 clear that cells not only die from apoptosis. This has to be mentioned.

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22 **The referee is correct in observing that our data do not directly confirm the involvement of**
23 **oxidative stress in the observed cytotoxicity, so the statements to this effect have been**
24 **included in the revised manuscript (page 12, lines 6 – 8 and page 12, lines 23 – 24). Also, the**
25 **possibility that compound 3 actually induced mixed apoptotic/necrotic death has been**
26 **briefly discussed (page 11, lines 16 – 19).**

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33 - It would be favourable that the manuscript is corrected by a native English speaker.

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35 **The manuscript has been corrected by a native English speaker.**
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4 1 **Isolation, characterization and *in vitro* cytotoxicity of new sesquiterpenoids**
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6 2 **from *Achillea clavennae***
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11 4 Snežana Trifunović¹, Anđelka M. Isaković², Aleksandra Isaković², Ivan Vučković¹, Boris
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3 **Abstract**
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8 Further phytochemical investigation into the aerial parts of *Achillea clavennae* has resulted in the
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10 isolation of three new sesquiterpene lactones: two highly oxygenated germacranolides (**1**, **2**) and
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12 the *iso-seco*-guaianolide, 9(*R*)-acetoxy-3-*O*-methyl-*iso-seco*-tanapartholide (**3**). Eight known
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14 compounds were also found in this plant species, of which 9 α -acetoxyanin (**5**), sintenin (**6**) and
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16 oleanolic acid (**7**) were detected for the first time. The structures of the isolated compounds were
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18 elucidated by combined spectroscopic methods (1D and 2D NMR, HRESIMS, CIMS, FTIR).
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20 While the predominant metabolite germacranolide sintenin (**6**) was not cytotoxic, the new *iso*-
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22 *seco*-guaianolide (**3**) displayed cytotoxicity comparable to that of cisplatin and the lactone
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24 apressin (**4**), inducing partly apoptotic death in human U251 and rat C6 glioma cell lines.
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36 **Key words:** *Achillea clavennae* L. (Asteraceae), sesquiterpene lactones, *iso-seco*-guaianolide,
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1 Introduction

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8 The genus *Achillea* L. contains over 100 species, mainly distributed in Europe and the temperate
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10 areas of Asia [1]. Hitherto, about sixty *Achillea* species have been phytochemically investigated,
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12 but far fewer have been biologically tested [2-3]. The composition and antibacterial activities of
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14 the essential oils have been examined the most, even many times for same species, as in the case
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16 of *A. millefolium*, the most popular and wide-spread species of the genus. Alkamides from the
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18 roots were studied in detail, as compounds initially considered relevant for chemotaxonomy.
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20 Nowadays, the leaf exudates (mainly containing flavonoid aglycons) are most frequently
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22 analyzed for this purpose. The main constituents of the investigated plant extracts are terpenoids
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24 (largely sesquiterpene lactones) and flavonoids, classes of compounds with documented
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26 biological activity [2-3]. This fact, as well as the use of many *Achillea* species in traditional
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28 medicine worldwide (for their anti-inflammatory, antispasmodic, stomachic, digestive,
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30 cholagogue, antipyretic, emmenagogue, hemostatic, hypotensive, diuretic and urinary antiseptic
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32 activities), makes this genus interesting for extensive examination.
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39 *Achillea clavennae*, which is the subject of the present study, grows on the elevated carbonate
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41 rocks of the eastern and southern Alps, as well as on mountains in the western part of the Balkan
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43 Peninsula (at altitudes between 1500 and 2500 m). According to the morphological characters
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45 and flavonoid profiles, *A. clavennae* belongs to the group of alpine species (the subgroup *A.*
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47 *moschata*) of the section *Ptarmica* (Mill.) W.D.J. [4]. This species was discovered and described
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49 as a healing plant (cholagogue, stomachic and anthelmintic) at the beginning of the 17th century
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51 [5]. At present, it is used in Croatian folk medicine to treat abdominal pain, the common cold,
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53 influenza and respiratory disorders [6]. Examination of the essential oil of *A. clavennae* from this
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55 locality verified its high antibacterial activity against some respiratory tract pathogens [6-7]. The
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1 extract of a plant collection from Macedonia (containing alkanes, fatty acids, monoterpenes,
2 guaiane sesquiterpenes and flavonoids) also exhibited a wide range of antimicrobial activities [8].
3 Our previous study of the aerial parts of *A. clavennae* from Montenegro (the Komovi Mountain)
4 revealed that guaianolides, bisabolenes and flavonoids were its main constituents. Subsequent
5 work, which was the first assessment of the biological activity of *A. clavennae* constituents,
6 revealed a remarkably high cytotoxic activity of the lactones 9 α -acetoxyartecanin and apressin
7 [9].

8 In the present study, the aerial parts of this alpine species from the Prokletije Mountains
9 (Montenegro) were analyzed. Three new sesquiterpene lactones (**1-3**) and eight known
10 compounds (apressin (**4**), 9 α -acetoxyanin (**5**), sintenin (**6**), oleanolic acid (**7**), chrysetunone, 11-
11 dehydroxy,11-hydroperoxyindicumenone, sesamin, centaureidin) were isolated and structurally
12 determined. In addition, the *in vitro* cytotoxic activity of the predominant lactones **3**, **4** and **6** was
13 examined.

15 Results and Discussion

17 Fractionation of the crude extract (petrol-Et₂O-MeOH, 1:1:1) of the air-dried aerial parts of the
18 title plant, by repeated silica gel column chromatography and preparative TLC, gave six
19 sesquiterpene lactones (new lactones **1-3**, apressin (**4**) [10], 9 α -acetoxyanin (**5**) [11] and sintenin
20 (**6**) [12]), two bisabolenes (chrysetunone [13] and 11-dehydroxy,11-hydroperoxyindicumenone
21 [9]), lignan sesamin [14], flavonol centaureidin [15], and oleanolic acid (**7**) [16] (**Fig. 1**).
22 Apressin (**4**), chrysetunone, 11-dehydroxy,11-hydroperoxyindicumenone, sesamin and
23 centaureidin had already been identified in the aerial parts of *A. clavennae* [9], but compounds **5**,

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3 1 **6, and 7** were found for the first time in this plant species. Oleanolic acid, atypical for the genus
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6 2 *Achillea*, had previously been reported only in *A. teretifolia* [17]. Bisabolene derivatives also
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8 3 rarely occur in this genus (*A. odorata* [18], *A. cretica* [19] and *A. clavennae* [9]), especially
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10 4 hydroperoxy-bisabolenes, previously found only in *A. clavennae*.

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15 6 **Fig. 1**

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20 8 The molecular formula C₁₉H₂₄O₈, for the lactone **1**, was deduced from the positive-ion mode
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22 9 HRESIMS (*m/z* 381.15398 [M+H]⁺; calc. 381.15439). The CI mass spectrum showed the
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25 10 pseudomolecular ion, [MH-O₂]⁺ (*m/z* 349), [MH-O₂-HOAc]⁺ (*m/z* 289) and [MH-2HOAc]⁺ (*m/z*
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27 11 261) ions, indicating the presence of the hydroperoxy and two acetoxy groups. The
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30 12 germacranolide core of **1**, with C-4 and C-10(14) double bonds, α-methylene-γ-lactone group and
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32 13 the substitution at C-1, C-3 and C-9, was recognized in ¹H NMR spectrum (**Table 1**). Singlets at
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34 14 δ 2.03 and δ 2.08, and broad singlet at δ 8.75 originated from two OAc and HOO groups,
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37 15 respectively. Low-field signals at δ 5.33 *dd* (*J*₁ = 5.0 Hz, *J*₂ = 11.5 Hz), δ 5.14 *brd* (*J* = 10.5 Hz)
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40 16 and δ 4.24 *brd* (*J* = 10.0 Hz) belonged to their geminal protons: H-3, H-9 and H-1. H-3 was
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42 17 distinguished on the basis of HMBC correlations H-3(δ 5.33)/C-5 and H₃-15/C-3(δ 75.6).
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45 18 Carbons at δ 76.8 and δ 86.1, connected in the HSQC spectrum with protons at δ 5.14 and δ 4.24,
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47 19 respectively, showed HMBC correlations with H₂-14, which only proves C-9 and C-1 positions
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50 20 of remaining substituents. Other significant HMBC correlations regarding the arrangement of this
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52 21 part of molecule were missing. Taking into account the presence of the lactone sintonin in this
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55 22 plant sample (biogenetically related to **1**), and COSY and NOESY correlations (**Fig. 2**) of the
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57 23 proton geminal to OAc (δ 5.14), as well as of H geminal to OOH (δ 4.24), it was deduced that the
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1 former proton is H-9 and the latter is H-1. Moreover, NOEs H-9/ H-7, H-8 α , H-1 and H-1/H-2 α ,
2 H-3, H-5 revealed α -position of these protons, hence β -position of all substituents. One more
3 high-frequency shifted *brd* ($J = 10.5$ Hz) at δ 5.45 was assigned to H-5 on the basis of HMBC
4 correlations H₃-15,H-3/C-5(122.2). Due to the lack of the important HMBC correlations,
5 coupling patterns in COSY spectrum and NOESY cross-peaks (**Fig. 2**) were crucial for
6 distinguishing CH₂-2 and CH₂-8 groups, as well as identifying α and β protons. NOEs H-6/H₃-15
7 and H₃-15/H-14' disclosed *syn*-orientation of Me-15 and CH₂-14 on the β -face of the trans-4-
8 cyclodecene. Such geometry affects spatial closeness of α -pseudo-axial protons H-1, H-3, H-5
9 and H-9, as well as of β -pseudo-axial H-2 with H-14' and Me-15, and also H-8 β with H-14 and
10 H-6, which was evidenced by corresponding NOE correlations (**Fig. 2**). Finally, **1** was identified
11 as 3 β ,9 β -diacetoxy-1 β -hydroperoxy-6 β ,7 α H-germacra-4,10(14),11(13)-trien-12,6 α -olide. Three
12 substituted $\Delta^{4,10(14)}$ -germacranolides have not previously been found in the genus *Achillea*.

14 Fig. 2

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16 For lactone **2**, closely related to **1**, the structure was determined from the positive-ion mode
17 HRESIMS, CIMS, and ¹H NMR spectra. Prior to recording 2D NMR spectra, this compound was
18 decomposed in the NMR tube, therefore the stereochemical data were obscured. According to
19 [M+H]⁺ ion at m/z 367.17513 (calc. 367.17485) in HRESIMS the molecular formula C₁₉H₂₆O₇
20 was established. CI mass spectrum contained [MH-HOAc]⁺ (m/z 307), [MH-HOAc-H₂O]⁺ (m/z
21 289) and [MH-2HOAc]⁺ (m/z 247) ions, implying the existence of a hydroxy group (instead of
22 hydroperoxy in comparison with **1**) and two acetoxy groups in the molecule. The similarity of the
23 ¹H NMR data of **2** (Table 1) to that of **1** indicated the same basic structure. ¹H NMR spectrum of

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3 1 **2** exhibited signals of two acetoxy groups (δ 2.03 *s* and δ 2.08 *s*, respectively) and their geminal
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6 2 protons at δ 5.29 *dd* ($J_1 = 5.0$ Hz, $J_2 = 11.4$ Hz, H-3) and δ 5.10 *brdd* ($J_1 = 3.2$ Hz, $J_2 = 7.6$ Hz, H-
7
8 3 9). Signals of H-5 (δ 5.39 *brd*, $J = 10.4$ Hz), Me-15 (δ 1.58 *d*, $J = 1.6$ Hz), H-14 (δ 5.56 *brs*) and
9
10 4 H-14' (δ 5.48 *brs*) documented double bonds Δ^4 and $\Delta^{10(14)}$, respectively. Broad doublet at δ 4.16
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12 5 ($J = 9.8$ Hz) originated from H-1, which is geminal to the hydroxy group. Missing the
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14 6 characteristic low-field H-13 and H-13' doublets of the exomethylene group, as well as *brs* of the
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16 7 hydroperoxy group, and the appearance of quintet of H-11 (δ 2.74, $J = 7.6$ Hz) and doublet of
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18 8 Me-13 (δ 1.19, $J = 7.6$ Hz) were the main distinctions of ^1H NMR spectrum of **2**, in comparison
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20 9 with **1**. It is very possible that the lactone **2** (3,9-diacetoxy-1-hydroxy-6 β ,7 α ,11H-germacra-
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22 10 4,10(14)-dien-12,6 α -olide) was formed from sintenin, via corresponding 1,10-epoxy derivative.
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24 11 The hydroperoxide **1** and sintenin might have the same precursor, most probably costunolide or
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26 12 corresponding mono- or di-acetoxy derivative.
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35 **Table 1**

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40 16 Molecular formula $\text{C}_{18}\text{H}_{22}\text{O}_7$ for lactone **3** was concluded from HRESI mass spectrum, recorded
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42 17 in the positive-ion mode, using ammonium formate for the ionisation ($[\text{M}+\text{NH}_4]^+$ at m/z
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44 18 368.17165, calc. 368.17038; $[\text{M}+\text{Na}]^+$ at m/z 373.12752, calc. 373.12577). In EI mass spectrum,
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46 19 ions $[\text{M}]^+$ (m/z 350), $[\text{MH}-\text{HOAc}]^+$ (m/z 290) and $[\text{MeC}=\text{O}]^+$ (m/z 43) were discerned, which
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48 20 implied the presence of an acetoxy group and the methyl ketone group. The IR spectrum revealed
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50 21 four carbonyls with absorption bands at 1765 cm^{-1} (γ -lactone), 1749 cm^{-1} (OAc), 1733 cm^{-1}
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52 22 ($\text{C}=\text{O}$) and 1708 cm^{-1} ($\text{C}=\text{O}$). In the ^1H NMR spectrum (**Table 1**) characteristic doublets for α -
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54 23 methylene- γ -lactone group (H-13: δ 6.40, $J = 2.6$ Hz; H-13': δ 5.67, $J = 2.0$ Hz) and a multiplet
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1 of H-7 (δ 3.19) were present. These protons, coupled with each other, were a part of one of the
2 two spin systems recognized in TOCSY spectrum (**Fig. 3**). Besides H₂-13 and H-7, this system
3 included H-6 (δ 5.06 *d*, J = 4.6 Hz), H₂-8 (δ 2.09 *t*, J = 7.0 Hz) and H-9 (δ 5.13 *t*, J = 6.8 Hz), a
4 low-field proton geminal to an acetoxy group. A singlet of OAc was partly overlapped (δ 2.15-
5 2.17) with singlets of two methyl groups bound to sp²-carbons. One of these signals was assigned
6 to Me-14 at carbonyl C-10 and the other to Me-15 at olefinic C-4 (double bound to C-5). The
7 multiplicity of H-6 indicated C-5 to be quaternary. These data, together with the evidence about
8 the existence of separated ABX spin system of five-membered ring, indicated *iso-seco*-
9 guaianolide structure of **3**. ABX system was constituted of diastereotopic CH₂-2 group (A: δ 2.70
10 *dd* (J_1 = 6.2 Hz, J_2 = 18.4 Hz), B: δ 2.34 *dd* (J_1 = 2.2 Hz, J_2 = 18.4 Hz)) and a proton H-3 (X: δ
11 4.31 *brd* (J = 4.8 Hz)) geminal to OMe group (δ 3.42 *s*). Therefore, **3** is 9-acetoxy-3-*O*-methyl-
12 *iso-seco*-tanaparholide. In NOESY spectrum, more or less expected correlations which confirm
13 the former structure appeared: H₂-8/H-13', H-9, H-6, OAc, H-7; H-2/H-2', H-3, OMe; H-2'/H-2,
14 H-3; H-7/H-13', H-6. Because of the remote nature of the C-3 stereocenter and the flexibility of
15 the (C-8)-(C10) fragment, there was not an evidence about stereochemistry at C-3 and C-9. The
16 configuration at C-9 was deduced on the basis of the previous findings that *seco*-guaianolides,
17 *endo*-peroxyguaianolides and bis-epoxy-guaianolides were biosynthetically related: all derived from
18 common cyclopentadiene precursors; *seco*- and *iso-seco*-guaianolides formed by a cleavage of
19 (C-1)-(C-10) bond of corresponding epoxy-guaianolides [20]. Accordingly, 9(*R*)-configuration of
20 **3** is proposed, as compatible with 9 α -position of acetoxy group of presented endoperoxide **4** and
21 bis-epoxide **5**.

22 *Seco*-guaianolides and *iso-seco*-guaianolides mainly occurred in the genera *Artemisia*, *Tanacetum*
23 and *Achillea*. *Iso-seco*-tanaparholide, firstly isolated from *Artemisia rutifolia* in 1986 [21], has

1 recently been identified in *Tanacetum parthenium*, together with its 3-epimer (3 α -OH), and
2 afterwards both were synthesized. A little difference in NMR data of two epimers and unequal
3 optical rotations were exploited for the comprehensive stereochemical revision of previously
4 isolated 'iso-seco-tanaparthalides' from different plants [22]. *Epi-iso-seco-tanaparthalide* was
5 recognized as a major product in some *Achillea* species.

6 Reviewing literature for 3-epimers of 3-O-methyl-iso-seco-tanaparthalides we could not find
7 strong evidence for its distinction [11, 23, 24]. Therefore, we gave up resolving C-3 configuration
8 on the basis of comparing our data with existing spectroscopic data. As well as in the case of iso-
9 seco-tanaparthalides, synthesis of these epimeric compounds is essential for their complete
10 characterization and also required for a revision of the preceding literature data.

12 **Fig. 3**

14 The guianolide **5** was easily determined as 9 α -acetoxyartecanin due to the similarity of its spectral
15 data with those of the previously isolated diastereomer 9 α -acetoxyartecanin from the same plant
16 [9]. 9 α -Acetoxyartecanin was also found in *A. depressa* [11]. In the previous study of 1D and 2D
17 NMR data of eleven 1,2-epoxy- and 1,2:3,4-diepoxy-guianolides, which we had isolated from
18 different *Achillea* species, we noted a relation between the chemical shifts of C(H)-5 fragment
19 and the configuration of the 1,2-epoxy ring [25]. Briefly, a signal of H-5 (vicinal to the epoxide)
20 of 1 α ,2 α -epoxyguaianolides is shielded, while C-5 is deshielded in comparison with
21 corresponding data of appropriate β -epoxy-diastereomers. Here, signals of H-5 and C-5 of **5**
22 appeared at δ 2.65 and δ 50.6, respectively, while the signals of its *bis*- β -epoxy-diastereomer 9 α -

1 acetoxyartecanin were noticed at δ_H 2.87 and δ_C 43.0 [9], which is one more confirmation of the
2 mentioned, previously recognized spectral rule for this type of compounds.

3
4 For testing the *in vitro* cytotoxicity of *A. clavennae* sesquiterpenoids, we have selected the
5 predominant metabolite germacranolide sintenin (**6**), lactone apressin (**4**) and the new *iso-seco*-
6 guaianolide (**3**). The glioma cell lines C6 and U251 were chosen as targets, having in mind that
7 gliomas, the most common primary malignancy of the central nervous system, are extremely
8 aggressive and mostly incurable tumors [26]. The treatment with compound **6** for 24 h had no
9 significant influence on either mitochondrial respiration or numbers of glioma cells (Fig. 4),
10 which is consistent with previous results obtained in various cancer cell lines [27, 28]. Compound
11 **4** was toxic to all tested cells, including primary astrocytes (Fig. 4), which is in accordance with
12 the earlier reports [9]. The new *iso-seco*-guaianolide (**3**) also displayed a significant dose-
13 dependent cytotoxicity towards glioma cells, as determined both by MTT assay for mitochondrial
14 respiration and crystal violet test for cell numbers (Fig. 4). The analysis of the IC_{50} values
15 presented in **Table 2** indicates that compound **3** was significantly more active against glioma
16 cells than compound **4**, but somewhat less efficient than the prototypical anticancer drug
17 **cisplatin**. Importantly, unlike compound **4** and **cisplatin**, compound **3** displayed only a limited
18 toxicity towards rat primary astrocytes (Fig. 4). While this could be due to a lower proliferation
19 rate of primary astrocytes, it nevertheless indicates that transformed glial cells might be more
20 selective to compound **3** than their primary counterparts.

21
22 **Fig. 4**

23 **Table 2**

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5 2 After confirming the *in vitro* antiglioma action of compound **3**, we next assessed its ability to
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7 3 induce programmed cell death known as apoptosis. We first examined activation of caspases, the
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9 4 main apoptosis-executing enzymes [29], using fluorescent-labeled pan-caspase inhibitor ApoStat
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11 5 (Fig. 5A). Flow cytometric analysis revealed that compound **3** induced a dose- dependent caspase
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13 6 activation in both C6 and U251 glioma cells. Furthermore, the cell cycle analysis after PI staining
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15 7 of cellular DNA demonstrated that the exposure to compound **3** increased the numbers of
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17 8 hypodiploid glioma cells with fragmented DNA (sub-G0/G1 compartment) (Fig. 5B), which is a
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19 9 hallmark of apoptosis [30]. Annexin-detectable externalization of phosphatidylserine is another
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21 10 apoptotic marker [31], and the treatment with compound **3** increased the numbers of annexin⁺
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23 11 glioma cells displaying phosphatidylserine in the outer leaflet of cell membrane (Fig. 5C). The
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25 12 effects of compound **3** (25 μ M) on DNA fragmentation (21.5% hypodiploid cells) and
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27 13 phosphatidylserine exposure (32.8% annexin⁺ cells) in U251 cells were only slightly less
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29 14 prominent than those of cisplatin at the same concentration (32.4% and 37.8%, respectively; data
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31 15 not shown). These results clearly demonstrate that compound **3** induces apoptotic death of glioma
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33 16 cells. However, it should be noted that compound **3** also increased the proportion of PI⁺ cells with
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35 17 the cell membrane damage (Fig. 5C), which could be late apoptotic and/or necrotic cells.
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37 18 Therefore, the possibility that compound **3** could actually induce mixed apoptotic/necrotic death
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39 19 requires further investigation.
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Fig. 5

23 Oxidative-stress mediated mitochondrial dysfunction can lead to activation of caspases and
24 subsequent apoptotic cell demise [31]. Thus, we next examined the effects of compound **3** on

1 ROS production and mitochondrial membrane potential in glioma cells. The flow cytometric
2 analysis of cells stained with the redox-sensitive dye DHR demonstrated that compound **3** was
3 able to induce a dose- dependent ROS generation in glioma cell lines (Fig. 6A). The induction of
4 oxidative stress in compound **3**-treated C6 glioma cells was associated with the mitochondrial
5 membrane depolarization, as confirmed by the increase in green-to-red fluorescence ratio
6 (FL1/FL2) of the mitochondria-binding dye DePsipher (Fig. 6B). While these data indicate that
7 oxidative stress and subsequent mitochondrial depolarization are involved in the cytotoxicity of
8 compound **3**, this assumption remains to be directly confirmed.

10 Fig. 6

11
12 The previously isolated structurally related *iso-seco*-guaianolides were found to reduce viability
13 of immortalized human keratinocytes [32] and inhibit nitric oxide synthesis in
14 lipopolysaccharide-activated RAW 264.7 cells [33], probably through interaction with NF- κ B
15 signaling pathway [22]. The high biological activities of these compounds, including new *iso-*
16 *seco*-guaianolide **3**, are most likely connected with their α,β -unsaturated carbonyl electrophilic
17 groups: an α -methylene- γ -lactone and an α,β -unsaturated cyclopentenone. These groups are
18 known to mediate a Michael-type addition reaction with thiol-containing enzymes and proteins.
19 The importance of these structural features is primarily supported by the fact that lactone **6**
20 (without mentioned groups) was inactive in the present study. Accordingly, lactone **4** with only
21 one α,β -unsaturated carbonyl group was less active than **3**.
22 Taken together, our data for the first time demonstrate that compound **3**, a novel *iso-seco-*
23 *tanaparholide* from *A. clavennae*, triggers apoptotic death of glioma cells associated with the
24 induction of oxidative stress and mitochondrial depolarization.

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5 2 **Materials and methods**
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10 4 *General experimental procedures*
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12 5 Optical rotations were determined on a Rudolph Research Analytical automatic polarimeter,
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14 6 Autopol IV. IR spectra were measured in the form of transparent films on a Perkin-Elmer FT-IR
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16 7 spectrometer 1725X. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 (at 200
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18 8 and 50 MHz, respectively) and an Bruker Avance III spectrometer (at 500 and 125 MHz,
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20 9 respectively) in CDCl_3 , using TMS as internal standard. Mass spectra were obtained on a
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22 10 Finnigan MAT 8230, BE DCI (150 eV, iso-butane). High-resolution LC/ESI TOF mass spectra
23
24 11 were measured on a HPLC instrument (Agilent 1200 Series) with a Zorbax Eclipse Plus C18
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26 12 column (150 x 4.6 mm i.d.; 1.8 μm) and a diode-array detector (DAD) coupled with a 6210 time-
27
28 13 of-flight LC/MS system (Agilent Technologies). Elemental analysis was performed on a Vario
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30 14 EL III C,H,N,S/O Elemental Analyzer (Elementar). CC was carried out on silica gel 60 (0.063-
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32 15 0.200 mm, Merck). Preparative TLC was performed on silica gel G and F_{254} (0.75 mm layer, 20 x
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34 16 20 cm). Silica gel 60 F_{254} precoated aluminum sheets (0.25 mm, Merck) were used for TLC
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36 17 control. Spots were detected under UV254 and by spraying with 50% H_2SO_4 .
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46 19 *Plant material*

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48 20 The aerial parts of *Achillea clavennae* L. (Asteraceae) were collected at the flowering stage in
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50 21 July 2002 in Montenegro at the mountain Prokletije (Karanfili, altitude ca. 2000 m). The plant
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52 22 sample was identified by Prof. Pedja Janačković (Faculty of Biology, University of Belgrade,
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1 Belgrade, Serbia), and a voucher specimen (BEOU16677) was deposited in the BEOU-
2 Herbarium, Institute of Botany, Faculty of Biology (Belgrade, Serbia).

3 4 *Extraction and isolation*

5 The air-dried aerial parts of *A. clavennae* (270 g) were extracted at a room temperature with a
6 mixture of petrol-Et₂O-MeOH (1:1:1, 2.1 L, 24 h) using the standard procedure [34]. The initial
7 extract (9.0 g) was treated with MeOH (3 × 50 mL) to yield 7.2 g of MeOH soluble portion.
8 Further fractionation was carried out on silica gel dry-column flash chromatography (250 g, 10 ×
9 14 cm), starting the elution with petrol (fraction **F1**, 400 mL) and increasing polarity by adding
10 Et₂O (20% to 100%, **F2-F10**, 350 mL each) and MeOH (15%, **F11**, 350 mL). Fractions were
11 evaporated, and **F7** (petrol-Et₂O 3:7, 423 mg) and **F8** (petrol-Et₂O 2:8, 441 mg) were combined
12 after inspection of analytical TLC (CH₂Cl₂-MeOH 97:3) and ¹H NMR spectra of all fractions.
13 **F6** (petrol-Et₂O 4:6, 558 mg) was chromatographed on silica gel CC (300 g, 45 × 3.5 cm;
14 CH₂Cl₂-MeOH 97:3) to yield 48 subfractions (~20 mL each). Fractions 7-12 (F6a, 62.4 mg), 22
15 (F6b, 24.1 mg), and 25-29 (F6c, 51.0 mg) were further purified by preparative TLC. **Sesamin (3.2**
16 **mg**, F6a/CH₂Cl₂-MeOH 97.5:2.5), **apressin (4, 3.5 mg**, F6b/CH₂Cl₂-MeOH 97:3) and **oleanolic**
17 **acid (7, 2.1 mg**, F6c/CH₂Cl₂-MeOH 97.5:2.5, repeated prep.TLC) were obtained.
18 **F7+8** (864 mg) was divided into 75 subfractions (~30 mL each) by silica gel CC (430 g, 70 × 3.5
19 cm) gradually increasing polarity of eluent (CH₂Cl₂-MeOH, 97.4:2.6 - 96:4). Subfr. F7a (23-26,
20 36.6 mg), F7b (27-33, 74.2 mg), F7c (34-49, 102.1 mg), F7d (54-57, 32.3 mg), and F7e (71-75,
21 56.2 mg) were purified by preparative TLC (always at several plates). Lactone **2** (1.1 mg) was
22 isolated from F7a and lactone **apresin (4, 10.9 mg)** from F7b with the same solvent system
23 (CH₂Cl₂-MeOH 96.5:3.5). Lactone **3** (5.4 mg) was obtained from F7c (CH₂Cl₂-MeOH 97:3). **11-**
24 **Dehydroxy,11-hydroperoxyindicumenone** (1.4 mg) was obtained from F7d together with

1 additional 1.1 mg of **3** (CH₂Cl₂-MeOH 96.5:2.5, repeated TLC). Chrysetunone (**0.5** mg) and
2 centaureidin (**1.4** mg) were isolated from F7e (CH₂Cl₂-MeOH 97:3).

3 Silica gel CC (300 g, 45 × 3.5 cm; CH₂Cl₂-MeOH 97:3) of **F9** (petrol-Et₂O 1:9, 495 mg) yielded
4 40 subfractions (~30 mL each), which were combined after analytical TLC. F9a (4-5, 37.5 mg)
5 yielded lactones **4** (8.5 mg), **1** (1.4 mg), and **6** (1.0 mg) after repeated preparative TLC (solvent
6 system CH₂Cl₂-MeOH 97:3). F9b (6-7, 28.2 mg) was washed out with Et₂O and an insoluble
7 portion was crystallized from CH₂Cl₂ giving 11.4 mg of sintenin (**6**). 9 α -Acetoxycanin (**5**, 0.9
8 mg) was isolated from F9c (13-16, 33.1 mg) after repeated preparative TLC with solvent systems
9 toluen-Et₂O-MeOH (7:2:1) and CH₂Cl₂-MeOH (97:3).

10 Lactone sintenin (10.0 mg) was also isolated from **F10** (Et₂O, 450 mg), which was firstly divided
11 into 20 subfractions (~30 mL each) by silica gel CC (280 g, 40 × 3.5 cm) using solvent system
12 CH₂Cl₂-MeOH (97:3). From combined fraction 4-8 (65.3 mg), this compound was separated as
13 insoluble in Et₂O.

14
15 *3 β ,9 β -diacetoxy-1 β -hydroperoxy-6 β ,7 α H-germacra-4,10(14),11(13)-trien-12,6 α -olide* (**1**):

16 Yellowish gum; [α]_D²² +30.2 (CH₂Cl₂, *c* 0.119); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel.
17 int.): 383 [MH]⁺ (~21), 351 [MH-O₂]⁺ (~34), 291 [351-HOAc]⁺ (~5), 263 [MH-2HOAc]⁺ (~63),
18 245 [263-H₂O]⁺ (~68); HRESIMS: *m/z* 381.15398 [M+H]⁺ (calculated for C₁₉H₂₄O₈+H
19 381.15439); ¹H and ¹³C NMR data, see **Table 1**; COSY and NOESY data, see **Fig. 2**; HMBC: H₃-
20 15/C-3, C-4, C-5; H₂-14/C-9; H-14/C-10; H-13/C-7, C-11; H-3/C-5.

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22 *3,9-diacetoxy-1-hydroxy-6 β ,7 α ,11H-germacra-4,10(14)-dien-12,6 α -olide* (**2**): Yellow gum;

23 [α]_D²² +24.0 (CH₂Cl₂, *c* 0.101); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel. int.): 367 [MH]⁺

1 (~58), 307 [MH-HOAc]⁺ (~82), 289 [307- H₂O]⁺ (~54), 247 [307-HOAc]⁺ (~33); HRESIMS: *m/z*
2 367.17513 [M+H]⁺ (calculated for C₁₉H₂₆O₇+H 367.17485); ¹H NMR data, see **Table 1**.
3
4 *9(R)-acetoxy-3-O-methyl-iso-seco-tanaparholide (3)*: White crystals; [α]_D²² -10.7 (CH₂Cl₂, *c*
5 0.168); IR ν_{\max}^{film} (cm⁻¹): 1765 (γ-lactone), 1749 (OAc), 1733 (C=O), 1708 (C=O); ¹H and ¹³C
6 NMR data, see **Table 1**; HMBC: H₂-8/C-9, C-11, OCOCH₃; OMe/C-3; H-13/C-11; TOCSY data,
7 see **Fig. 3**; NOESY: H₂-8/H-13', H-9, H-6, OAc, H-7; H-2/H-2', H-3, OMe; H-2'/H-2, H-3, OMe;
8 H-7/H-13', H-6; EIMS (70 eV), *m/z*: 350 [M]⁺, 290 [M-HOAc]⁺, 247 [290-COCH₃]⁺, 215 [247-
9 HOCH₃]⁺; HRESIMS: *m/z* 368.17165 [M+NH₄]⁺ (calculated for C₁₈H₂₂O₇+NH₄ 368.17038) and
10 *m/z* 373.12752 [M+Na]⁺ (calculated for C₁₈H₂₂O₇+Na 373.12577); Elemental analysis: found C -
11 61.60, H - 6.42; requires: C - 61.71, H - 6.33%.

13 *Cells and reagents*

14 All reagents were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Human glioma
15 cell line U251 and rat glioma cell line C6 were kindly donated by Dr Pedro Tranque (Universidad
16 de Castilla-La Mancha, Albacete, Spain). The primary astrocytes were isolated from brains of
17 newborn Albino Oxford rats as previously described [35], in accordance with the ethical
18 guidelines stated in the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised
19 in 1985). Tumor cell lines and primary rat astrocytes were grown in HEPES (20 mM)-buffered
20 RPMI 1640 cell culture medium, supplemented with 5% fetal bovine serum, 2 mM L-glutamine,
21 10 mM sodium pyruvate and 1% of antibiotic/antimycotic mixture at 37 °C in a humidified
22 atmosphere with 5% CO₂. The cells were prepared for experiments using trypsin/EDTA and
23 incubated in 96-well flat-bottom cell culture plates (2 × 10⁴ cells/well) for the cell viability

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3 1 assessment, or 6-well plates (5×10^5 cells/well) for the flow cytometric analysis (plates were
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6 2 from Sarstedt, Nümbrecht, Germany). Cells were rested for 24 h and then treated with
7
8 3 compounds **3**, **4** and **6**. Purity of **3**, **4** and **6** was confirmed by elemental analysis ($> 99\%$). Control
9
10 4 cultures were exposed to the corresponding amount of vehicle (DMSO). We observed no
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12 5 influence of DMSO on any of the parameters tested (data not shown). Cisplatin ($\geq 99.9\%$,
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14 6 Sigma-Aldrich), a well known anticancer drug was used in some experiments as a positive
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16 7 control. Incubation times and concentrations of compounds are stated in figure legends.
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24 9 *Cell viability*

25 10 Mitochondrial dehydrogenase activity, as an indicator of cell viability, was assessed by
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27 11 mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
28
29 12 bromide (MTT) to formazan, and the number of viable cells was determined using crystal violet
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31 13 assay, exactly as previously described [36]. The MTT and crystal violet absorbance was
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33 14 measured in an automated plate reader (Sunrise, Tecan, Dorstet, UK) at 570 nm. The IC_{50} values
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35 15 were calculated using GraphPad Prism software.
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43 17 *Caspase activation*

44 18 Activation of caspases was measured using cell-permeable FITC-conjugated pan-caspase
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46 19 inhibitor ApoStat (R&D Systems, Minneapolis, MN), according to manufacturer's instructions.
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48 20 The increase in green fluorescence (FL1) as a measure of caspase activity was detected using
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50 21 FASCalibur flow cytometer (BD, Heidelberg, Germany). The results are presented as mean
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52 22 intensity of ApoStat fluorescence.
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59 24 *Apoptosis analysis*

1 DNA fragmentation as a marker of apoptotic cell death was analyzed by measuring the DNA
2 content of ethanol-fixed cells stained with DNA-binding dye propidium iodide (PI), exactly as
3 previously described [36]. Red (FL2) fluorescence of PI-stained cells was measured, and
4 percentage of hypodiploid, apoptotic cells (sub-G compartment of the cell cycle) was determined.
5 Alternatively, apoptotic cell death was assessed after double staining with annexinV-FITC and
6 PI, according to manufacturer's instructions (BD, Heidelberg, Germany). Annexin binds the
7 phosphatidylserine exposed on the membrane of apoptotic cells, while PI labels the DNA in cells
8 with membrane damage. The green (FL1) and red (FL2) fluorescence of annexinV-FITC/PI
9 labeled cells was analyzed, and annexin⁺/PI⁻ and annexin⁺/PI⁺ cells were considered as early and
10 late apoptotic cells, respectively. Flow cytometry analyses were conducted on FACSCalibur flow
11 cytometer using CellQuest Pro software (BD, Heidelberg, Germany).

12 13 *Determination of oxidative stress and mitochondrial membrane potential*

14 Intracellular production of reactive oxygen species (ROS) was determined using redox-sensitive
15 fluorescent dye dihydrorhodamine 123 (DHR; Invitrogen, Paisley, UK). DHR (5 μ M) was added
16 to cell cultures at the beginning of the treatment. The mean intensity of green (FL1) fluorescence
17 corresponding to oxidative stress was determined using FACSCalibur flow cytometer.
18 Mitochondrial membrane potential was determined using DePsipher (R&D Systems), a lipophilic
19 cation that has the property of aggregating upon membrane polarization forming an orange-red
20 fluorescent compound. If the potential is disturbed the dye remains or reverts to its green
21 monomeric form. The cells were stained with DePsipher as described by the manufacturer and
22 fluorescence was detected by flow cytometry. The results are presented as green/red fluorescence
23 ratio (mean FL1/FL2), the increase of which reflects mitochondrial depolarization.

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5 **Conflict of Interest**

6 There are no conflicts of interest.

For Peer Review

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3 **1 Figure legends**
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8 **Fig. 1** The structures of compounds 1–7.
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12 **Fig. 2** COSY (a: ) and NOESY (b: ) correlations of 1.
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17 **Fig. 3** TOCSY correlations () of 3.
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22 **Fig. 4.** Antiglioma effects of compounds 3, 4 and 6. C6 cells, U251 cells and rat primary
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24 astrocytes were incubated with different concentrations of compounds 3, 4, 6, or cisplatin, and
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26 mitochondrial respiration (MTT) and cell numbers (crystal violet) were assessed after 24 h (the
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28 dashed lines represents the viability of untreated cells, which was arbitrarily set to 100%). **The**
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30 **results are presented as mean ± SD values from three independent experiments.**
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36 **Fig. 5** Compound 3 induces apoptosis in glioma cells. (A-C) **U251 cells (left) and C6 cells (right)**
37
38 **were incubated with 12.5 μM (A) or 25 μM (A-C) of compound 3 for 24 h.** Cells were stained
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40 with pan-caspase inhibitor ApoStat (A), PI (B) or annexinV-FITC/PI (C), and caspase activity
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42 (A), DNA fragmentation (B) and phosphatidyl-serine exposure (C) were assessed using flow
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44 cytometry. The histograms (A, B) and dot-plots (C) from a representative of three independent
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46 experiments are presented.
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52 **Fig. 6** Compound 3 induces oxidative stress and mitochondrial depolarization in glioma cells. (A)
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54 U251 and C6 cells were incubated with compound 3 (12.5 μM or 25 μM) for 24 h and ROS
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56 production in DHR-stained cells was investigated using flow cytometry. The histograms from a
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3 1 representative of three independent experiments are shown. (B) C6 cells were incubated with
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5 2 compound **3** (25 μ M) for 24 h and mitochondrial membrane potential in DePsipher-stained cells
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8 3 was assessed using flow cytometry. The histograms from a representative of three independent
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10 4 experiments are presented (the values of FL1/FL2 are mean fluorescence ratios reflecting
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12 5 mitochondrial depolarization).
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Table 1 NMR data of **1**, **3** (500 MHz for ^1H , CDCl_3) and **2** (200 MHz for ^1H)

H/C	1		2		3	
	δ_{H}	$\delta_{\text{C}}^{\text{a}}$	δ_{H}	$\delta_{\text{C}}^{\text{a}}$	δ_{H}	$\delta_{\text{C}}^{\text{a}}$
1	4.24 <i>brd</i> (10.0)	86.1	4.16 <i>brd</i> (9.8)			c
2 α	2.50 <i>dddd</i> (1.5, 5.0, 13.0)	34.8	2.55 <i>ddd</i> (1.5, 5.0, 12.8)		2.70 <i>dd</i> (6.2, 18.4), 2.34 <i>dd</i> (2.2, 18.4)	40.7
2 β	2.11 <i>m</i> ^b		2.05 <i>m</i> ^b			
3	5.33 <i>dd</i> (5.0, 11.5)	75.6	5.29 <i>dd</i> (5.0, 11.4)		4.31 <i>brd</i> (4.8)	79.8
4		144.2				173.1
5	5.45 <i>brd</i> (10.5)	122.2	5.39 <i>brd</i> (10.4)			c
6	4.31 <i>dd</i> (9.5, 10.0)	79.0	4.33 <i>t</i> (10.0)		5.06 <i>brd</i> (4.6)	77.0
7	2.84 <i>ddd</i> (2.0, 10.0, 10.0)	46.8	2.05 <i>m</i> ^b		3.19 <i>m</i>	40.5
8 α	2.29 <i>brd</i> (14.5)	33.2	2.45 <i>m</i>		2.09 <i>t</i> (7.0)	34.2
8 β	1.93 <i>brdd</i> (14.5, 10.0)		2.05 <i>m</i> ^b			
9	5.14 <i>brd</i> (10.5)	76.8	5.10 <i>brdd</i> (3.2, 7.6)		5.13 <i>t</i> (6.8)	75.5
10		145.3				c
11		138.0	2.74 <i>quint</i> (7.6)			138.0
12		168.8				c
13	6.28 <i>d</i> (3.5)	119.8	1.19 <i>d</i> (7.6)		6.40 <i>d</i> (2.6)	124.0
13'	5.52 <i>d</i> (3.0)				5.67 <i>d</i> (2.0)	
14	5.56 <i>brs</i>	117.6	5.56 <i>brs</i>		2.16 <i>s</i> ^b	29.6
14'	5.46 <i>brs</i>		5.48 <i>brs</i>			
15	1.58 <i>brs</i>	12.6	1.58 <i>d</i> (1.6)		2.16 <i>s</i> ^b	14.3
OOH	8.75 <i>brs</i>					
OAc	2.08 <i>s</i> ^b	170.2, 20.7	2.08 <i>s</i>		2.17 <i>s</i>	171.2, 20.6
OAc	2.06 <i>s</i> ^b	169.4, 21.0	2.03 <i>s</i>		/	/
OMe	/	/	/		3.42 <i>s</i>	57.6

^a Detected via HSQC and HMBC spectra. ^b Overlapped signals. ^c Not observed.

Table 2. IC₅₀ values for compounds **3**, **4**, **6**, and cisplatin, presented as mean ± SD from three independent experiments (*p < 0.05 compared to **4**, t-test; CV - crystal violet, n.a. - not assessed).

	comp. 6		comp. 4		comp. 3		cisplatin	
	MTT	CV	MTT	CV	MTT	CV	MTT	CV
U251	>100	>100	91.8±7.9	97.7±9.4	36.6±6.8*	50.9±7.2*	20.8±4.3	18.9±3.2
C6	>100	>100	80.1±9.6	90.0±10.5	41.6±8.5*	66.6±9.5*	15.0±5.7	17.6±4.1
astrocytes	n.a.	n.a.	>100	61.8±15.6	>100	>100	42.1±9.1	25.5±5.1

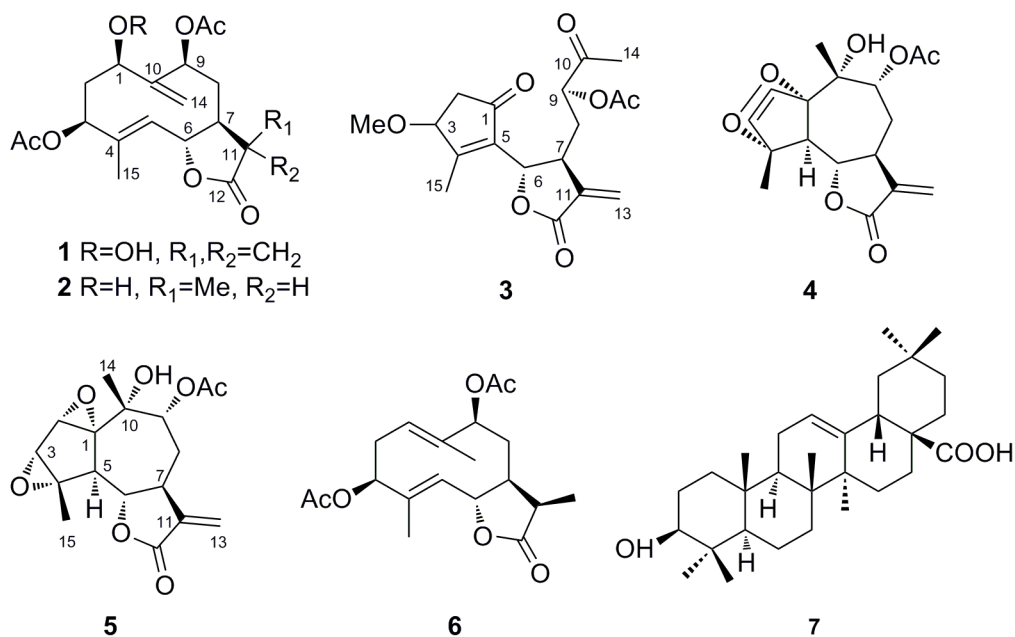
Fig. 1

Fig. 2

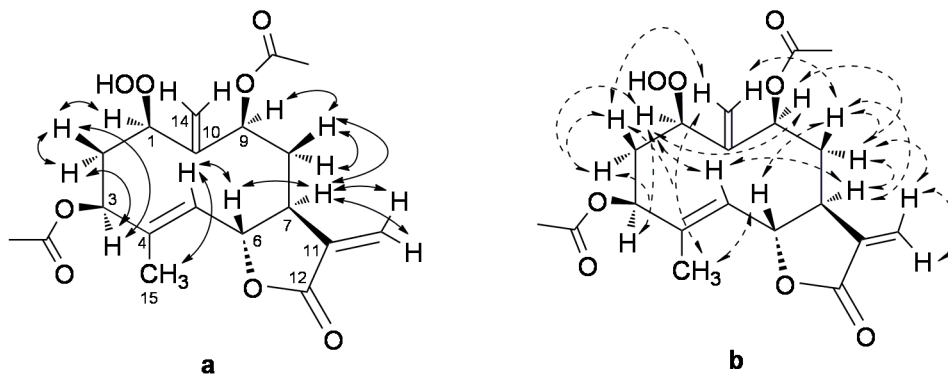


Fig. 3

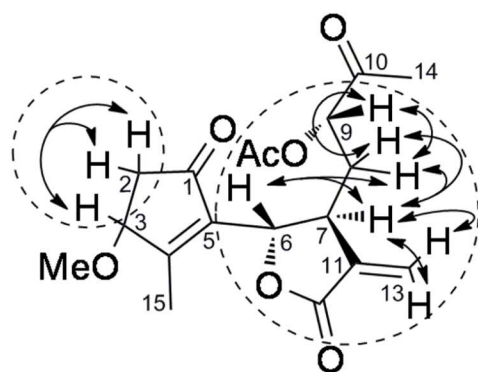


Fig. 4

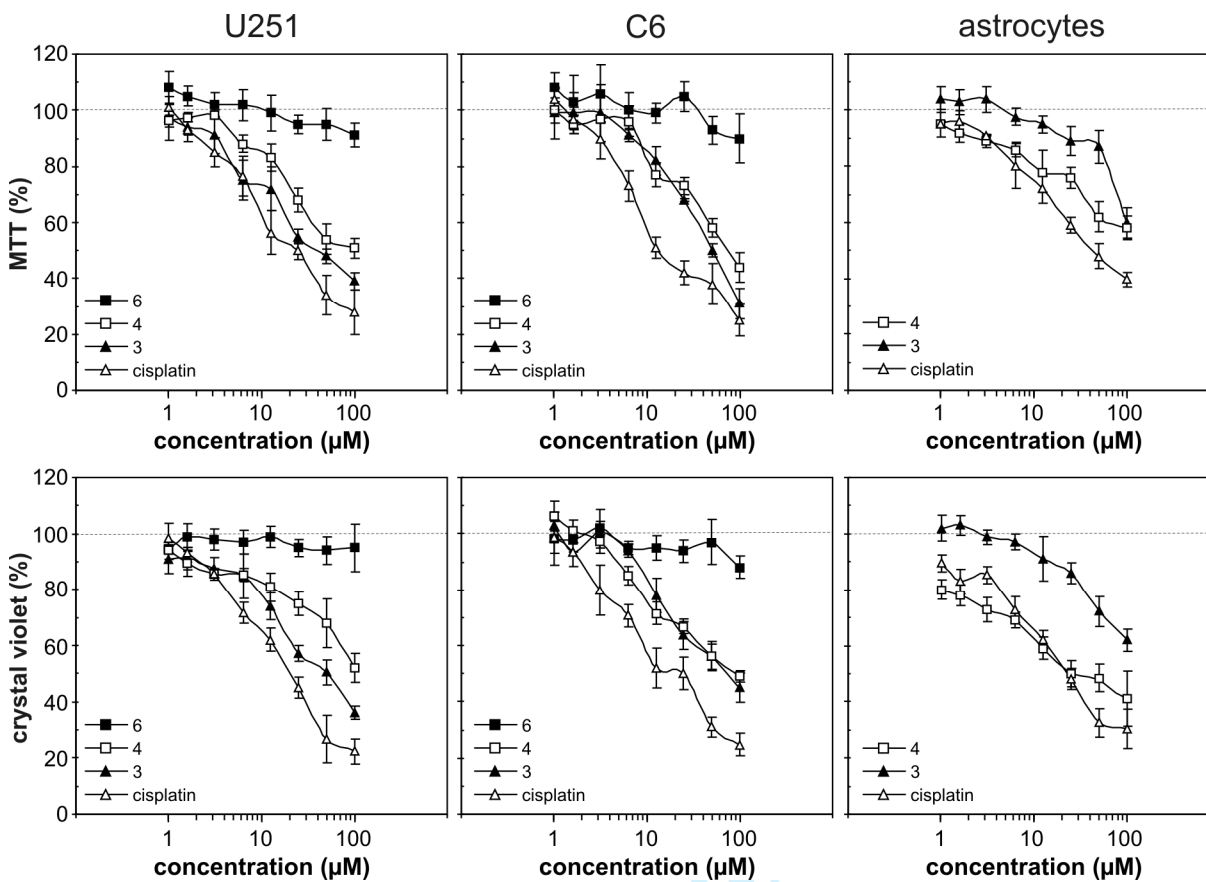


Fig. 5

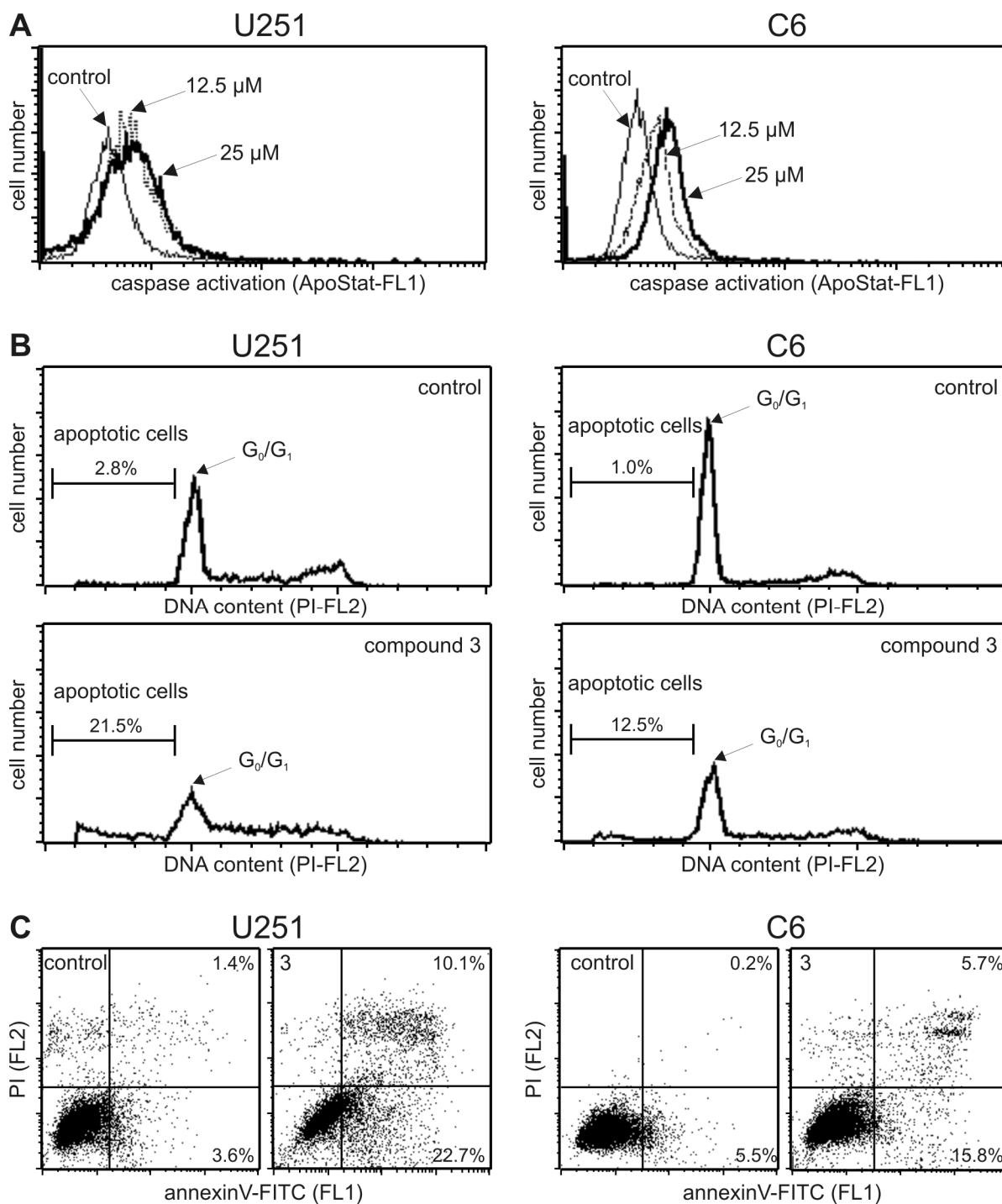
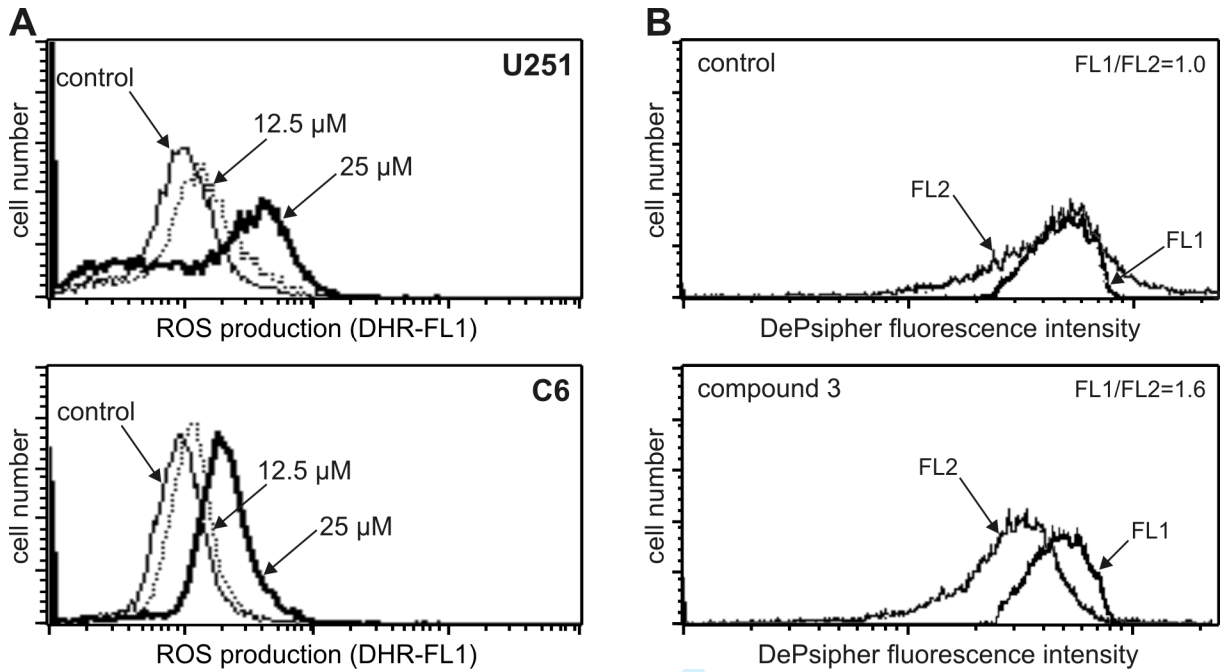
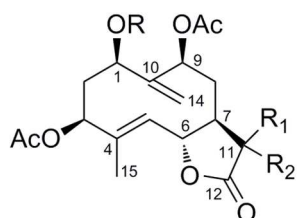


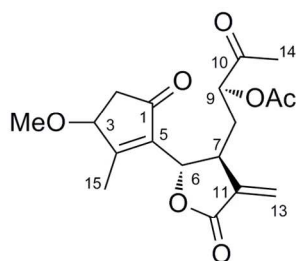
Fig. 6



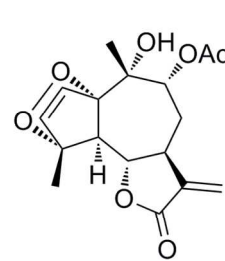


1 R=OH, R₁,R₂=CH₂

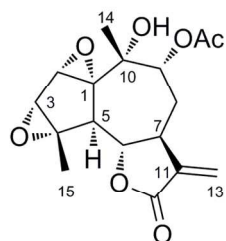
2 R=H, R₁=Me, R₂=H



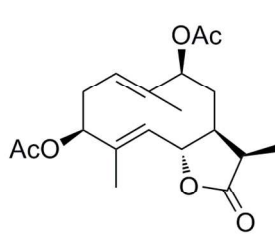
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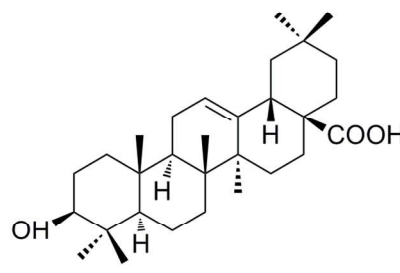
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134x84mm (300 x 300 DPI)

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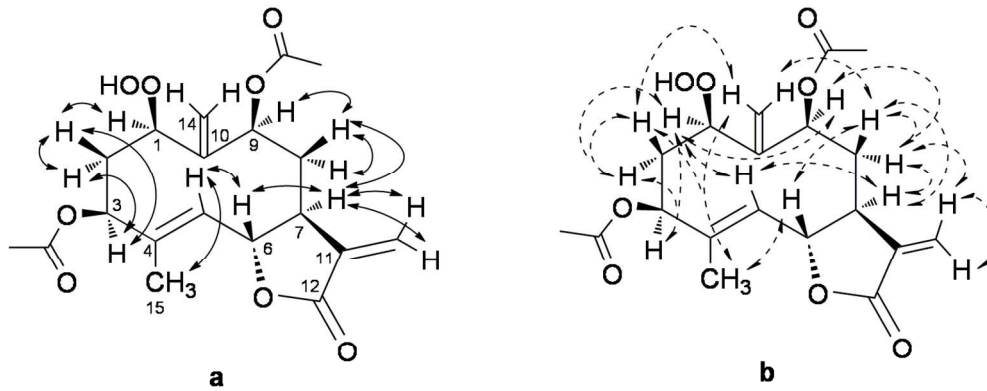


Fig. 2
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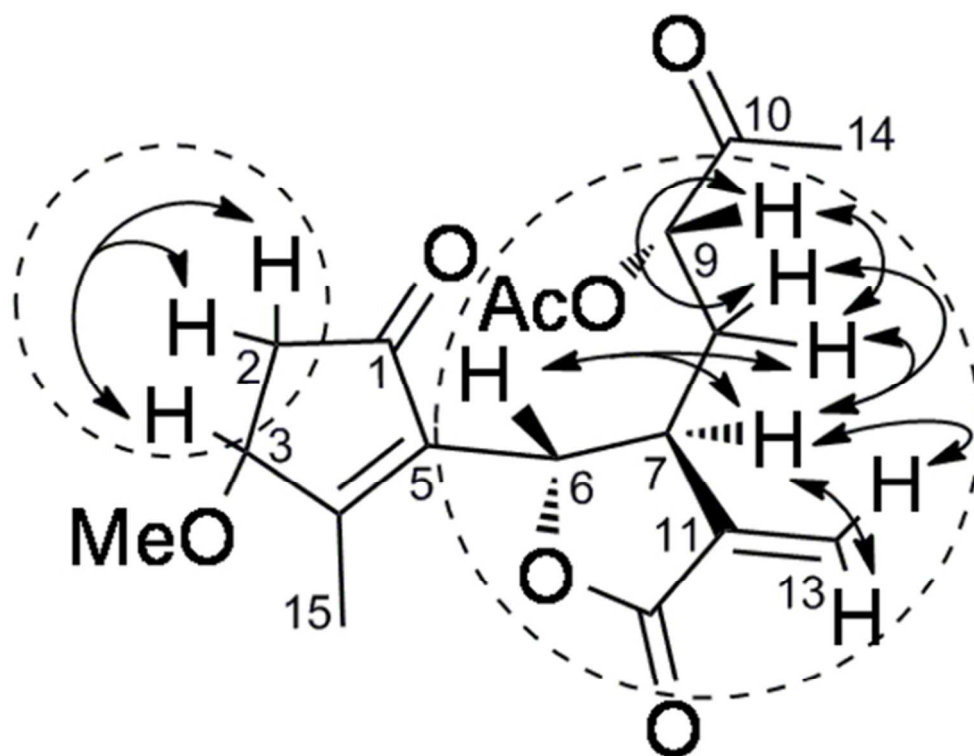
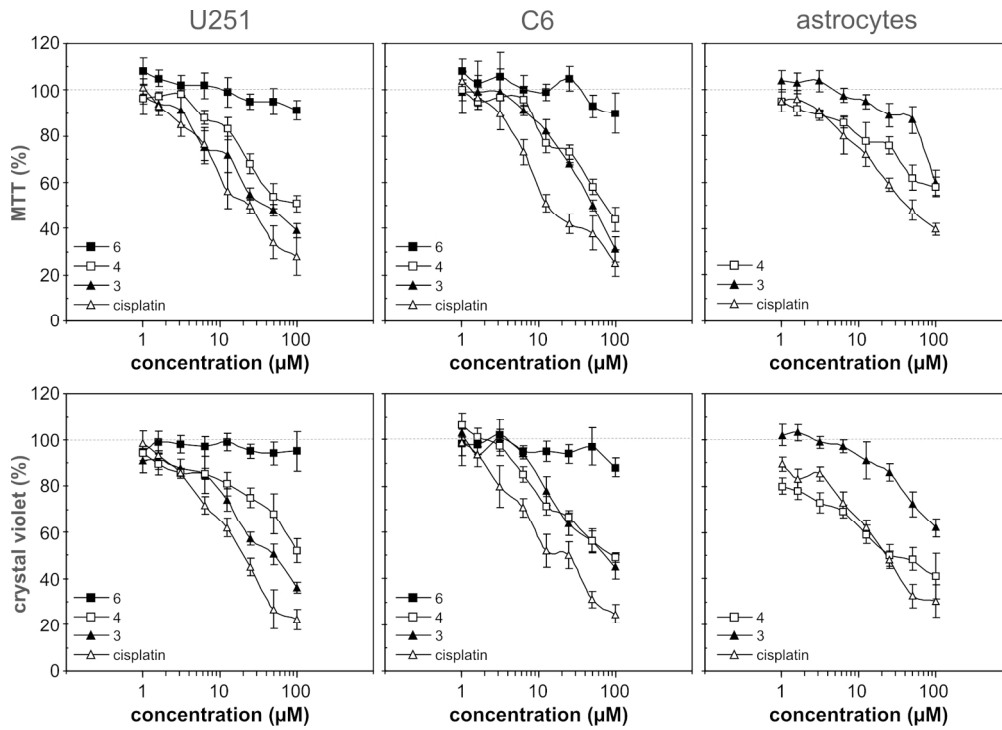
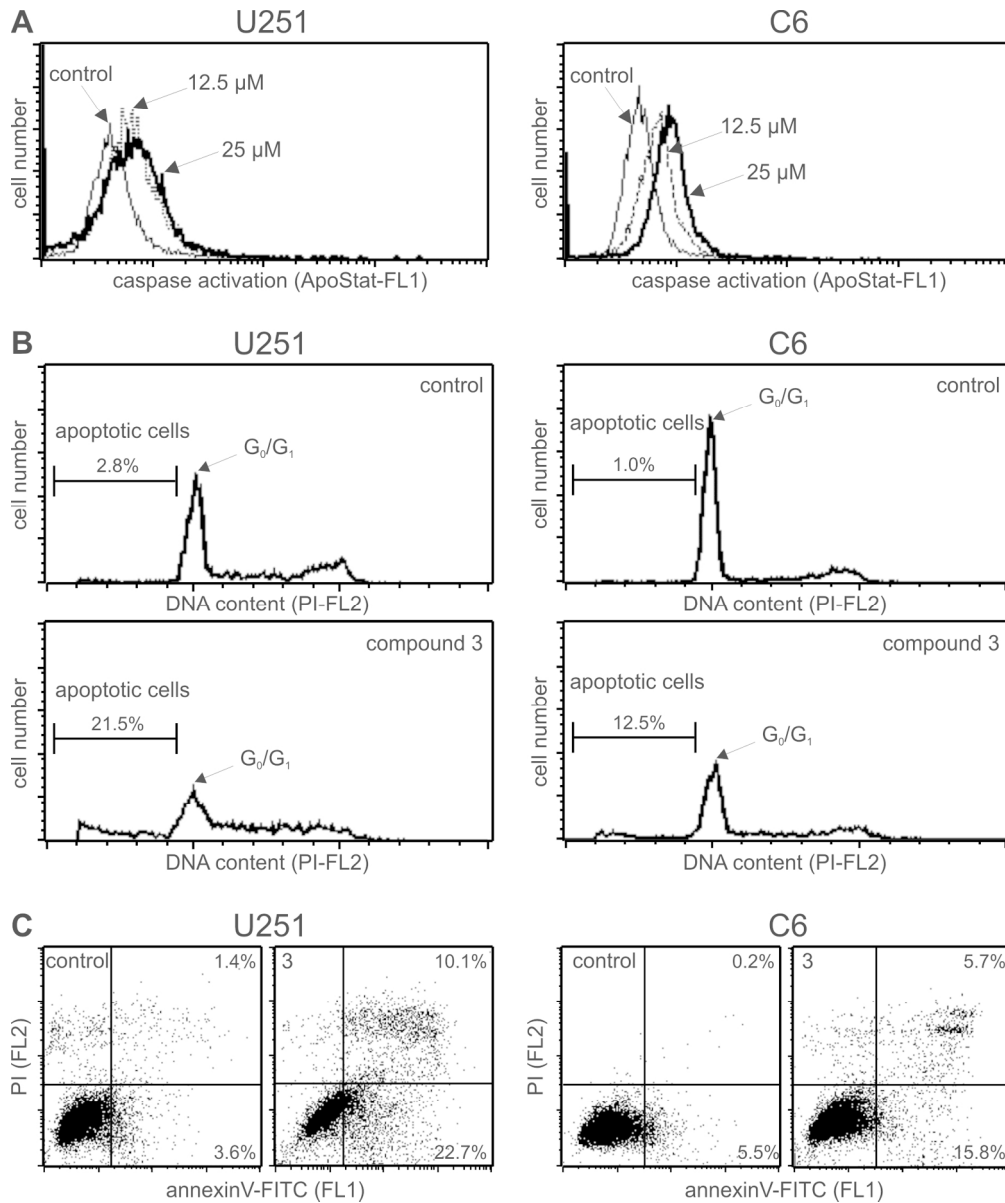


Fig. 3
64x50mm (300 x 300 DPI)

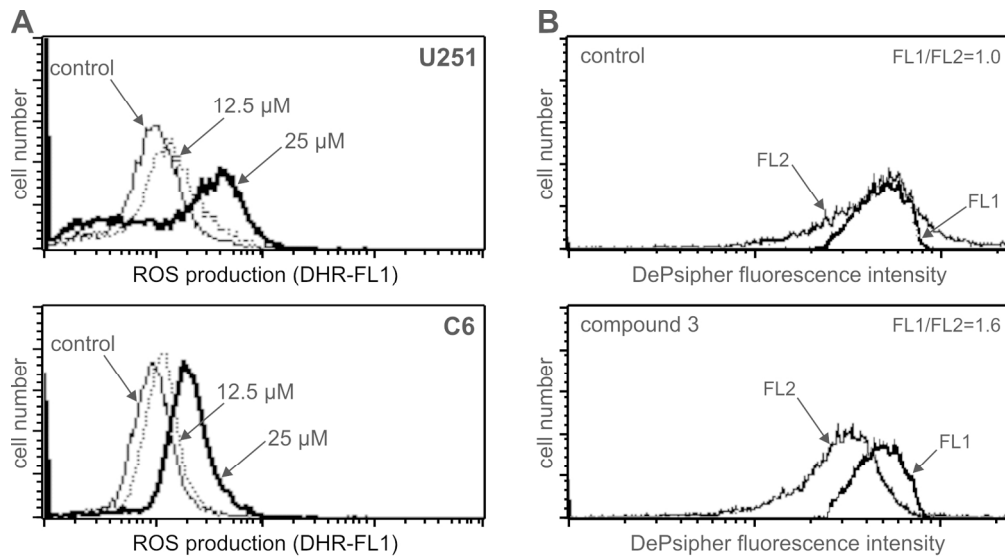


160x114mm (300 x 300 DPI)

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157x187mm (300 x 300 DPI)



160x87mm (300 x 300 DPI)