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The Chemistry and Biology of Ratjadone

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Ratjadone (**1**), a remarkably cytotoxic secondary metabolite, was isolated in 1994 by Höfle et al. from *Sorangium cellulosum* collected as a soil sample at Cala Ratjada (Mallorca, Spain).[1] It belongs to a family of so-called orphan ligands which include polyketides like leptomycin,[2] callystatin A,[3] and other related compounds.[4] In initial biological evaluations, it was found that ratjadone exhibits high cytotoxicity in cultured mouse cell lines (L929) with an IC_{50} value of 50 pg mL^{-1}. Additionally, it was found that this compound inhibits the growth of the HeLa cell line (KB3.1) at remarkably low concentrations (40 pg mL^{-1}).[5] We initiated the total synthesis of ratjadone in order to provide molecular tools that can be used to investigate the biological effects of individual substructures and to contribute to a better understanding of its mode of action. Our total synthesis of ratjadone was therefore set up to allow the rapid assembly of various ratjadone diastereomers and derivatives from three fragments (Scheme 1).[6a–d] During our manuscript preparation, Williams et al. reported the synthesis of (−)-ratjadone.[6e]

The pivotal steps in the synthesis are a Wittig reaction for the junction of the fragments **B** and **C** followed by a Heck reaction for the attachment to the **A** fragment. For the synthesis of diastereomers, the enantiomeric fragments **A′**, **B′**, and **C′** were synthesized according to our original strategy (Scheme 1). From these different fragments, diastereomeric ratjadone frameworks could be assembled in just two steps, with only three further transformations remaining to obtain ratjadone or any of its diastereomers. By using this strategy, we were able to generate the diastereomeric compounds (**2** – **5**) and analogues (**6** – **9**) shown in Scheme 2.

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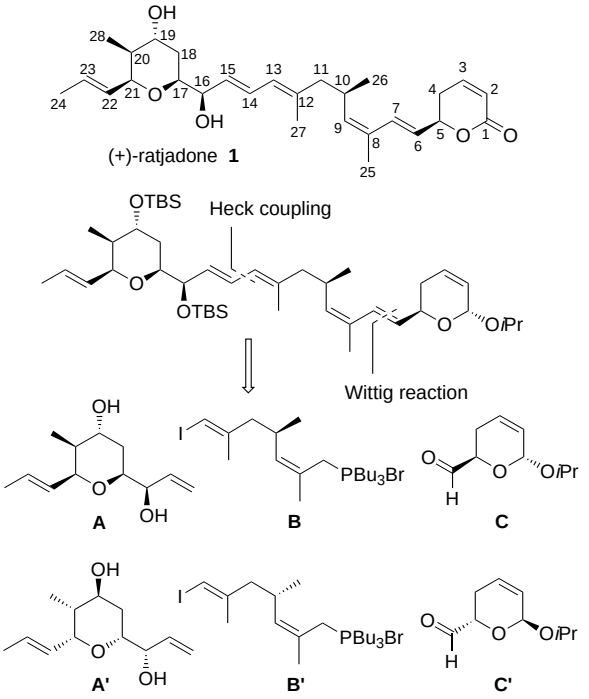
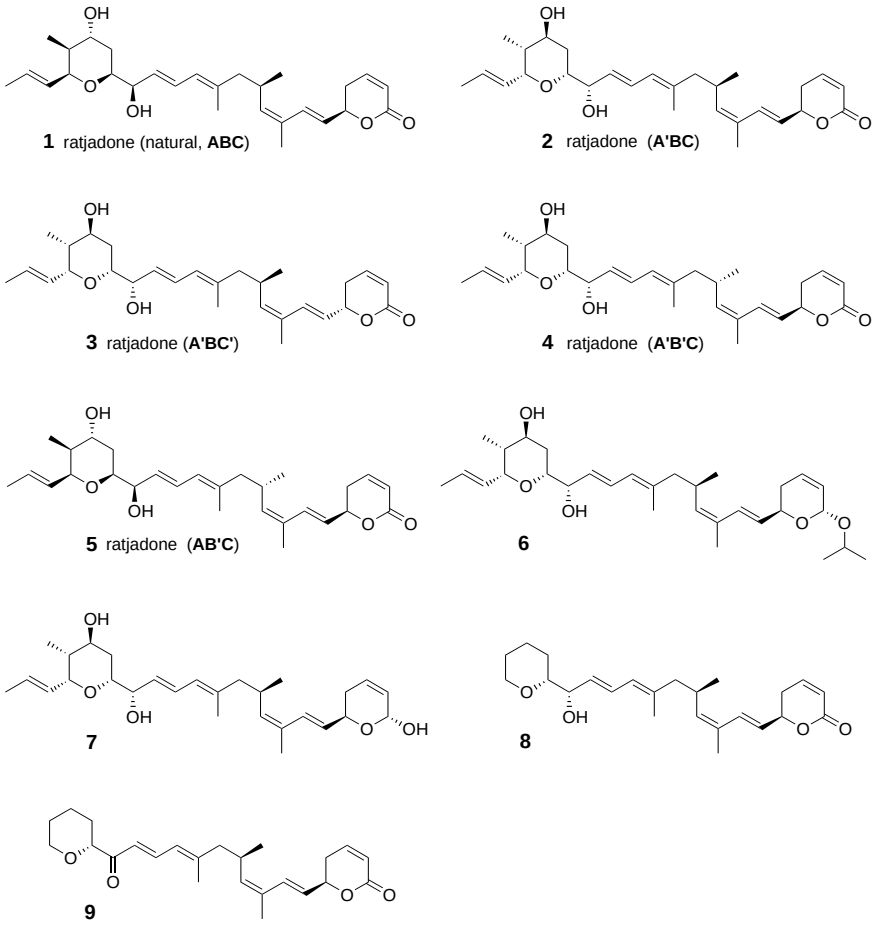


Scheme 1. *Retrosynthesis of natural (+)-ratjadone (**1**, **ABC**) and diastereoisomers.*

First, natural (+)-ratjadone (**1**, **ABC**) and the one with an enantiomeric **A** fragment (**2**, **A'BC**) were subjected to biological testing. Interestingly, changing the absolute configuration of **A** resulted in a compound that was significantly less cytotoxic than the natural ratjadone. At the same time we analyzed their ability to inhibit the proliferation of tumor cells. With the aid of flow cytometry we were able to distinguish between cell vitality (which corresponds to cytotoxicity) and cell number (which corresponds to tumor growth inhibition) of Jurkat cells at different concentrations of **1** and **2** (Figure 1). Whereas **2** is significantly less cytotoxic than **1**, both compounds inhibit tumor growth to the same extent at different concentrations.

In a different experiment, the effect of ratjadone (**1**) on the growth of glioblastoma cells was investigated. A photograph of these cells before and after treatment with ratjadone is given in Figure 2. After treatment with 50 nM ratjadone the cells lose their typical dendritic shape and separate from the solid support to form globular-shaped cells, which is in this case indicative of the influence of ratjadone.

The growth inhibition and cytotoxicity of ratjadone and its diastereomers were next investigated for different tumor cells (HM02, HEPG2, and MCF7 cell lines, Table 1). To unravel the effects of individual building blocks on the biological activity, the diastereomeric ratjadone **3** derived from fragments **A'**, **B**, and **C'** was next examined and this compound had about the same biological activity as analogue **2**. On the other hand, compound **4** (**A'B'C**), having enantiomeric **A** and **B** fragments but natural **C** fragment, shows a complete loss of tumor growth inhibition. The same result, that is, an almost complete loss of the biological activity, was obtained for compound **5** (**AB'C**) in which only the configuration at C10 was changed (compared to natural ratjadone (**1**)). It is our hypothesis that C10 plays the pivotal role in determining the biological activity by governing the overall conformation of the molecule. In contrast, inversion of the configuration at the other centers only introduces small distortions from the optimal geometry for binding. This results in only small effects on the tumor growth inhibition, but fortuitously decreases the cytotoxicity. Removing the carbonyl group of the unsaturated lactone moiety (as in compounds **6** and **7**) also causes complete loss of tumor growth inhibition. This strongly points toward the α,β-unsaturated lactone acting as a Michael acceptor. Interestingly, the compound with a simplification in the **A** fragment (compound **8**) can still be a potent antitumor agent. On the other hand, the hydroxy group at C16 seems to be important for the biological activity since the compound with the keto



Scheme 2. *(+)-Ratjadone (**1**), diastereomeric ratjadones (**2**–**5**), and analogues (**6**–**9**) synthesized for biological evaluation.*

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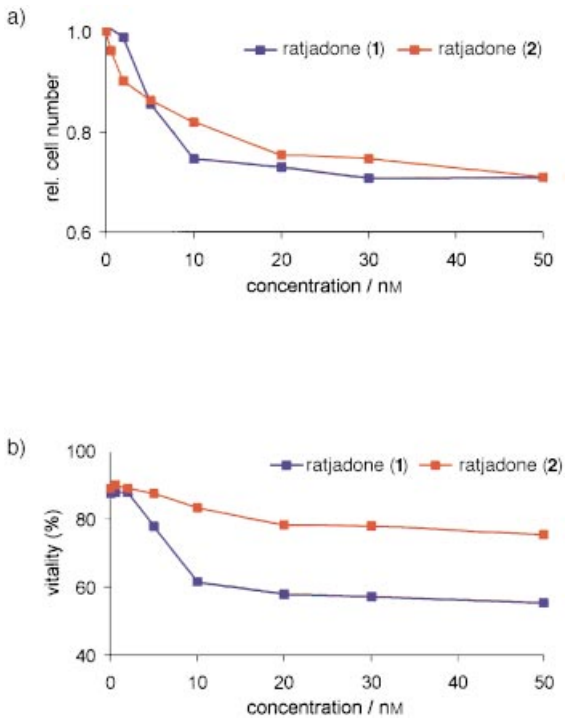

Figure 1. *Flow cytometric determination of cell vitality and relative cell number of Jurkat cells. The diagram shows relative cell number (a) and cell vitality (b) against ratjadones* ***1*** *and* ***2****. The cells were treated with ratjadones in concentrations ranging from 2 to 50 nM and incubated for 48 h. The experiments were run in 12-well microtiter plates. The cell vitality was analyzed with the aid of propidium iodide.*

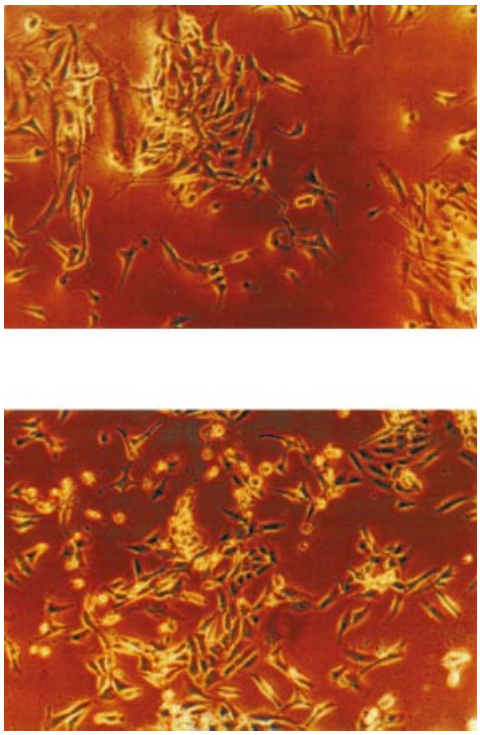

Figure 2. *Glioblastoma cells (U87-MG) in the absence (top) and in the presence of 50 nM ratjadone (1) (bottom). Experiments were performed in 6-well microtiter plates. The cells were incubated for 48 h in DMEM medium with 10% newborn-calf serum at 37 °C and 5% CO_2. Pictures were taken with an Olympus BH-2 microscope (magnification 200 ×). It can be seen that the cells form an aberrant globular shape in the presence of ratjadone.*

group at that position (**9**) showed only little biological activity. Table 1 gives the GI_{50}, TGI, and LC_{50} values for active ratjadone derivatives tested on three different cell lines.

A rationale for the dramatic effects associated with changes at the configuration at C10 can be given by analyzing the conformational preferences. The C8–C9 *Z*-configured double bond introduces an allylic strain which requires the C10-methyl group to be orientated almost perpendicular to the plane of the C8–C9 double bond with both alkenyl chains pointing away from each other (Figure 3).

At the same time, the potential homoallylic strain (*syn*-pentane interactions) due to the trisubstituted C12–C13 double bond can be minimized by a conformation in which the two methyl groups on C10 and C12 have an *anti* relationship (Figure 3). To confirm these considerations, an ^{1}H NMR spectroscopic analysis of the conformation of ratjadone in solution (CD_3OD) was performed. The analysis was done by selective irradiation of the protons at C7, C10, and C26 in 1D-ROESY experiments with Gaussian pulses of 40 ms duration and a spin

Table 1. *Growth inhibition and LC_{50} values for ratjadones* ***1*** *–* ***5*** *and* ***8*** *tested against different cell lines. All values are given in ng mL^{-1}.[a]*

Ratjadone	Cell line HM02			Cell line HEPG2			Cell line MCF7		
	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
1	0.36	2.3	90	0.8	> 100[b]	> 100	1.1	> 100[c]	> 100
2	< 5.0	24	250	13	> 500[d]	> 500	16	> 500[e]	> 500
3	17.0	> 100[f]	> 100	85	> 100	> 100	25	> 100	> 100
4	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
5	70	> 100	> 100	> 100	> 100	> 100	75	> 100	> 100
8	5.0	140	> 500	58	> 500[g]	> 500	100	> 500[h]	> 500

[a] The investigations were performed according to the NCI protocol[12] with cultured tumor cell lines (HMO2, HEPG2, and MCF7). Tumor cells were cultured in 96-well microtiter plates, medium: RPMI 1640 with 10% fetal calf serum. After 24 h the compounds were added in concentrations of 0.1, 0.5, 1, 5, 10, 50, and 100 ng mL^{-1} and cultured for additional 48 h. After that time the cell number was determined (protein determination with sulforhodamine). The compounds were dissolved in MeOH or water. The methanol concentration in the test experiments was maximally 0.1%. From the concentration – activity curves the following data were obtained: GI_{50} = concentration at which half of the cells were inhibited in their growth; TGI = concentration at which a complete inhibition of cell growth was observed; LC_{50} = concentration that reduced the cell number after 24 h to 50%. [b] 65% growth inhibition at 100 ng mL^{-1} [c] 64% growth inhibition at 100 ng mL^{-1}. [d] 80% growth inhibition at 500 ng mL^{-1}. [e] 80% growth inhibition at 500 ng mL^{-1}. [f] 90% growth inhibition at 100 ng mL^{-1}. [g] 60% growth inhibition at 500 ng mL^{-1}. [h] 60% growth inhibition at 500 ng mL^{-1}.

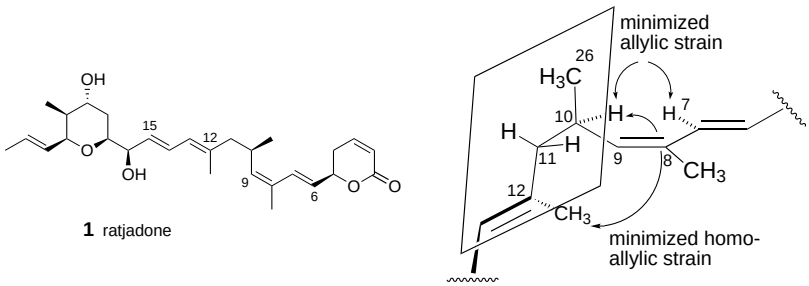

*Figure 3. Minimization of the allylic strains derived from the two trisubstituted double bonds and substituents at C10 are responsible for a kink (ca. 90°) in the alkenyl chain of ratjadone (**1**).*

lock time of 250 ms. Irradiation of the proton H7 gave NOE signals at H5 and H10. On the other hand, irradiation of H10 gave signals for H7 and H26, but none for H9, indicating a coplanar orientation of H7 and H10.

Additionally, the conformation was supported by Monte Carlo simulations.[7] Using the GB/SA solvent model in an MM2* force field Monte Carlo search gave a global minimum of 37.59 kcal mol^{-1} for ratjadone, which was found twice (convergent), with five additional conformations within 1 kcal mol^{-1}. By using the same parameters, compound **3** (**A'BC'**) gave essentially the same overall conformation with a global minimum of 38.68 kcal mol^{-1} (twice, convergent), with 60 additional conformations within 1 kcal mol^{-1}. Figure 4 shows the global minimum for **1** (**ABC**) and the overlay with **A'BC'** in which the carbon atoms C10, C11, and C26 are aligned.

These considerations about the overall conformation of the molecules and its importance for the biological activity were also supported by CD spectroscopy. The CD spectrum of ratjadone shows the exiton effect (between the dienes of C6–C9 and C12–C15), which is generally maximized at an projection angle of about 70° between chromophores.[8] This conformation is consistent with the release of strain for ratjadone. The CD curves for ratjadone and its diastereoisomers are shown in Figure 5. It

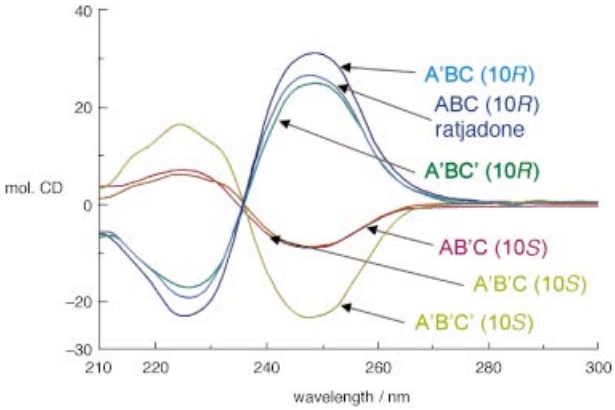

*Figure 5. CD spectra of ratjadone (**1**) and its diastereoisomers. The shape of the CD curves depends on the configuration at C10.*

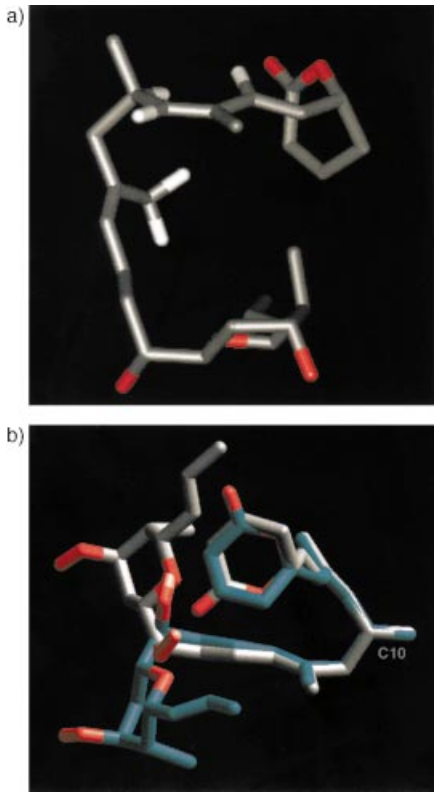

*Figure 4. a) Global energy minimum conformation of ratjadone (**1**, **ABC**) derived from Monte Carlo calculations. b) Overlay of **1** and **3** (**A'BC'**). The carbon atoms C10, C11, and C26 are aligned. The carbon atoms of compound **1** are given in gray, the ones of **3** in green.*

can be seen that the shape of the curves depends on the configuration at C10 and that configurational changes at other positions have only small effects on the overall conformation, which is consistent with the biological data.[9] Taking these data into consideration, we propose that the configuration at C10 induces a helix-like turn that governs the overall conformation of the molecule. Changing this configuration has therefore a more significant effect on the overall conformation than changes at the **A** or **C** fragments and consequently on its biological activity.

A similar structure – activity relationship based on the configuration at these stereocenters was reported for callystatin A by Kobayashi et al.[10] They also reported that inversion of the configuration at both C5 and C10 from *R* to *S* decreases the biological activity. Compared to ratjadone, the biological activity of callystatin seems to decrease more significantly when the configuration at C5 is changed (Figure 6). On the other hand, changing the C10 configuration from *R* to *S* only leads to a 3.5-fold decrease. In contrast to ratjadone, in callystatin the trisubstituted double bond is separated from the C10-methyl group by an *E*-configured disubstituted double bond. Hence, the increased flexibility in callystatin, compared to ratjadone, allows both diastereomeric callystatins (with regard to the configuration at C10) to adopt similar conformations that are required for target binding, thus resulting in similar biological activities.

We next turned our attention to the mode of action of ratjadone. Figure 7 shows the graphs indicating the number of

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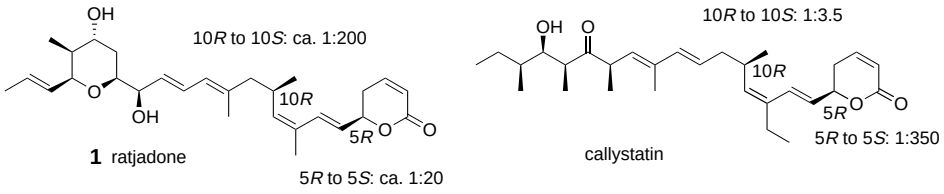

Figure 6. *Comparison of the influence of changes in configuration on the biological effects of callystatin and ratjadone (**1**) (HEPG2 cell line). Changing the configuration at C10 has more severe effects on the biological activity in ratjadone.*

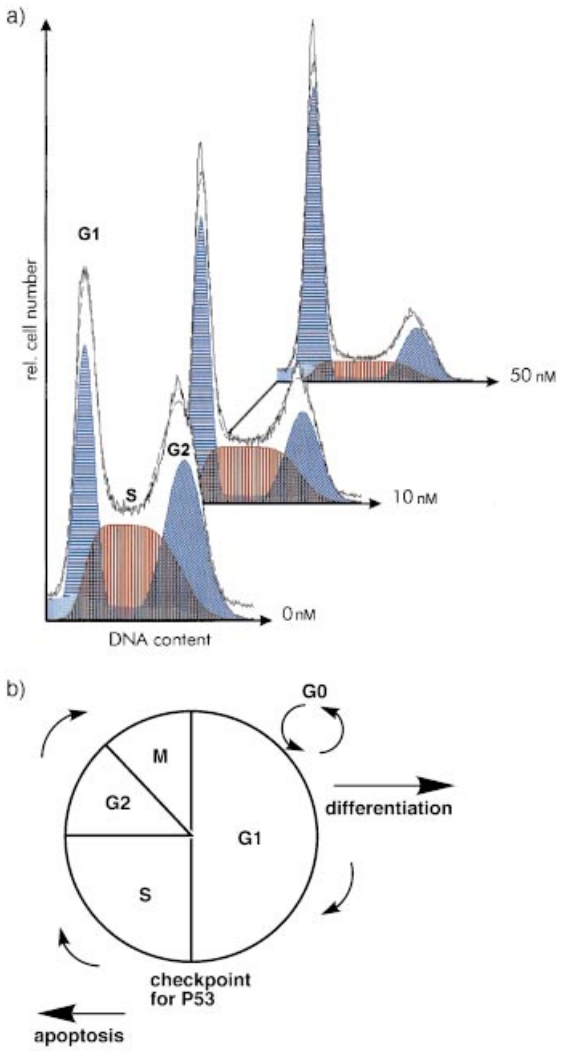

Figure 7. *a) Cell cycle analysis of Jurkat cells. Ratjadone (**1**) induces a G1 arrest. Cell cycle analysis was performed with propidium iodide for measurement of the DNA content. The analysis of the Gaussian curves was done with the program "Wincycle" from Phoenix Flow Systems (San Diego, CA). b) Schematic representation of the cell cycle.*

cells in the individual phases of the cell cycle. It can be seen that the addition of ratjadone decreases the number of cells in the S and G2 phases. At a concentration of 50 nM, the number of cells in the S and G2 phases has gone down from 64% (without ratjadone) to 24%. This clearly demonstrates that both **1** and **2** arrest tumor cells in the G1 phase. Additionally, we could see that the tumor cells undergo apoptosis and that the mode of action depends on P53[11] (by up-regulation of P21), which serves as a checkpoint for chromosome integrity at the interface of the G1 and S phases.

In conclusion, natural ratjadone (**1**) was found to be the most active compound among all its diastereoisomers and analogues, but compounds **2**, **3**, and **8** still showed antitumor activity in a very promising concentration range. Gratifyingly, the significant cytotoxicity of the natural ratjadone could be substantially decreased by changing the configuration of the **A** fragment or at C5. Additionally, the substituents at the **A** ring are obviously not crucial for the biological activity since compound **8**, which was constructed from an **A** ring analogue, still gave a very active ratjadone derivative. This might become of special interest if a synthetic access to this class of compounds is required for drug development. Even though a variety of analogues still need to be tested, we can already say that changing the absolute configuration at the tetrahydropyran ring or just having a simplified analogue instead leads to a significant drop in cytotoxicity while retaining a high antitumor activity.

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