Available online at www.sciencedirect.com

ScienceDirect

Mendeleev Commun., 2018, **28**, 475–478

Mendeleev Communications

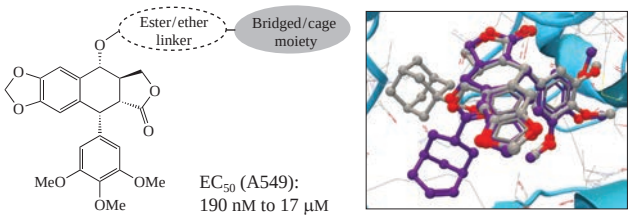
Novel bridged and caged C^4-podophyllotoxin derivatives as microtubule disruptors: synthesis, cytotoxic evaluation and structure–activity relationship

Nikolay A. Zefirov,[a,b] Ann Kruth,[c] Birgit Wobith,[c] Evgenia V. Nurieva,[a] Syed Riyaz,[d] Ch. Venkata Ramana Reddy,[d] Sergei A. Kuznetsov[c] and Olga N. Zefirova*[a,b]

[a] *Department of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation. E-mail: olgaz@org.chem.msu.ru*
[b] *Institute of Physiologically Active Compounds, Russian Academy of Sciences, 142432 Chernogolovka, Moscow Region, Russian Federation*
[c] *Institute of Biological Sciences, University of Rostock, D-18059 Rostock, Germany. E-mail: sergei.kuznetsov@uni-rostock.de*
[d] *Department of Chemistry, Jawaharlal Nehru Technological University Hyderabad, Kukatpally, 500 085 Hyderabad, India. E-mail: riyazsd@gmail.com*

DOI: 10.1016/j.mencom.2018.09.007

New podophyllotoxin C^4-derivatives with bridged and cage moieties were synthesized by the Steglich esterification of podophyllotoxin with polycyclic carboxylic acids or by etherication with (adamantan-1-yl)methanol in the presence of BF_3·Et_2O with the following separation of diastereomers. Most of the target compounds inhibited the growth of human lung carcinoma A549 cells, induced apoptosis, stimulated shortening of microtubules or induced their unusual curling and involution. The activity depends on the slightest differences in the structures of alicyclic moieties and the type of the bond between podophyllotoxin and alicyclic group.



Podophyllotoxin **1** isolated from extracts of *Podophyllum peltatum* possesses anticancer activity due to its ability to interact with colchicine binding domain of dimeric α,β-tubulin and to inhibit its polymerization to microtubules.[1] A great number of analogues of parent molecule are synthesized nowadays, majority of them being obtained by modification at C^4 position of initial molecule.[1–3] Many of these compounds (especially with β-configuration of hydroxyl and/or other substituent at C^4 as in clinically used etoposide) exhibit antitumor properties due to interaction with molecular targets different from tubulin, mainly with enzyme DNA topoisomerase II.[1]

Interestingly, among several hundreds of known tubulin-targeted C^4-podophyllotoxin analogues, only a small number of compounds demonstrate cytotoxicity to cancer cells in low nanomolar range, which is equalvalent to parent molecule. This can be ascribed to the limited cavity volume in the protein. Nevertheless, some C^4-podophyllotoxin derivatives with bulky bridged groups are highly cytotoxic to cancer cells.[4,5] Thus, each of diastereomeric esters **2a** and **2b** (Figure 1) reveals cytotoxicity to human lung carcinoma cell line A549 with EC_{50} = 4 nM.[4]

Relying on these data, in the present work we synthesized and tested a series of podophyllotoxin ester and ether conjugates with different bridged and cage moieties with a specific purpose to evaluate the effect of slightest structural differences in the inserted fragments (general bulkiness, conformational rigidity, the size and relative position of the cycles, *etc.*) on bioactivity. A series of podophyllotoxin-based cage esters **3a–c** and bridged esters **4–7** (see Figure 1) was studied. Both α- and β-isomers of

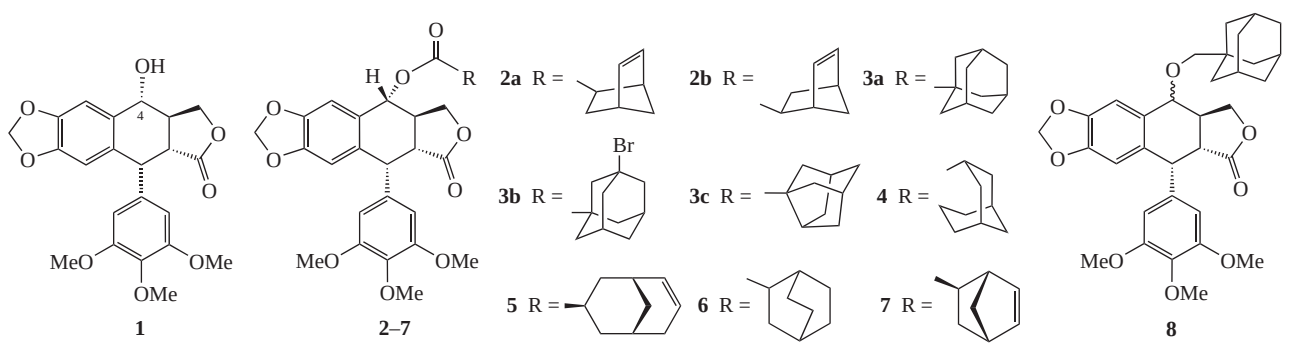


Figure 1 Podophyllotoxin **1** and its derivatives with bridged and cage moieties synthesized earlier[4] (**2a**,**b**) and in this work (**3a–c**, **4–8**); compounds **5–7** were obtained as diastereomeric mixtures (relative configuration is indicated for **5** and **7**).

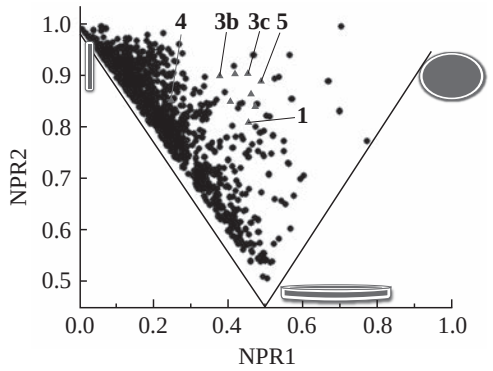


Figure 2 Normalized principal moment of inertia (NPR) plot (vertices relate to the molecular shapes of sphere, rod and disk[6]). The NPR values of compounds **3**–**8** are indicated as triangles (NPR values of 1100 FDA-approved drugs randomly taken from GOSTAR proprietary database[7] are shown as black points for comparison).

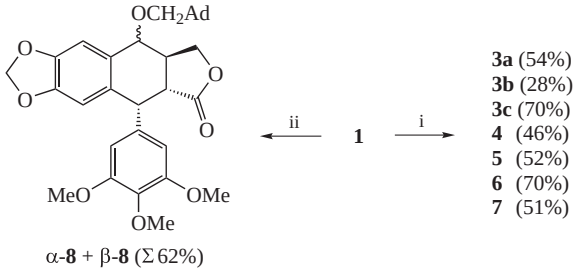
adamantane ethers **8** were obtained to investigate the impact of linker type on bioactivity (α-**8** with the same configuration of substituent at C^4 as in podophyllotoxin is forming a pair to **3a**).

Quantitative evaluation of the 3D shapes of compounds **3**–**8** using normalized principal moment of inertia (NPR) analysis derived by Sauer and Schwartz[6] indicates that the proposed alicyclic moieties provide an essential structural diversity to the set of podophyllotoxin derivatives (Figure 2).

The calculated NPR values of the molecules under study occupy rather broad region in the isosceles triangle of the NPR plot whose vertices relate to the molecular shapes of sphere, rod and disk. This region extends from the left edge of the NPR plot (occupied by NPR values of the most of FDA approved drugs) to the middle of triangle (close to podophyllotoxin point) signifying the diversity of overall shape of proposed set of molecules.

Target compounds **3a**–**c**, **4**–**7** were obtained by the Steglich (DCC, DMAP) esterification of podophyllotoxin with the corresponding acids (Scheme 1).† Initial acids were commercially available or obtained as reported.[9–12] Esters **3a**–**c**, **4**–**7** were obtained in 28–70% yields. Compounds **5**–**7** could not be resolved to individual isomers by column chromatography on silicagel and were tested as diastereomeric mixtures. In ^{1}H NMR spectra of the synthesized esters, the resonance of C^4-proton in podophyllotoxin fragment is observed at 5.84–5.91 ppm. In ^{13}C NMR spectra atom C^4 resonates at 72.89–73.41 ppm (for the synthetic details and characteristics of new compounds, see Online Supplementary Materials).

Ether **8** was synthesized by the reaction of podophyllotoxin with (adamantan-1-yl)methanol in the presence of boron trifluoride etherate[13] (see Scheme 1). The mixture of C^4-isomers **8** (α and β) was separated by column chromatography, but due to very close values of retardation factor individual α and β isomers were



Scheme 1 *Reagents and conditions:* i, RCOOH, DCC, DMAP, CH_2Cl_2, ~20 °C, 12 h; ii, (adamantan-1-yl)methanol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, 0 → 20 °C, 6 h, then column chromatography to give α-**8** (23%), β-**8** (11%) and (α+β)-**8** (28%).

† Compound **3a** is mentioned in the patent.[8]

isolated in low yields. In ^{1}H NMR spectra of compound **8**, the resonances of diastereotopic protons OCH_2Ad are observed at 3.04 and 3.05 ppm for α-isomer and at 3.03 and 3.24 ppm for β-isomer. In ^{13}C NMR spectra of ether **8,** the resonance of atom C^4 is revealed at 81.12 ppm for α-**8** and at 74.36 ppm for β-**8** (shifted upfield with respect to the corresponding peaks of C^4 in podophyllotoxin and COH in initial alcohol).

All synthesized podophyllotoxin derivatives were evaluated *in vitro* in a standard calorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay[14] for determination of their cytotoxicity to the human epithelial lung carcinoma cell line A549 (for the specific procedures, see refs. 15–17). The ability of compounds to inhibit the cell growth was also studied using microscopy for direct cell counting over 24 and 48 h of culturing.[18] For each podophyllotoxin derivative its effect on microtubule dynamics and its ability to induce apoptosis were investigated using immunofluorescence microscopy as described.[18–20]

The results of biotests (Table 1) demonstrate that cytotoxicity of the synthesized esters varied from noticeable for **5** and **7** (with EC_{50} in nanomolar range) to moderate for **3a**,**c**, **4** and **6** (with EC_{50} in micromolar range), while compounds **3b** and **8** (both α and β isomers) were not active in MTT test. Total inhibition of cell growth in 48 h was observed at 10 μM for esters **3b**, **4**–**7** and at 100 μM – for esters **3a** and **3c**; at 100 μM ether β-**8** inhibited cell growth for 74%. So, most of the compounds strongly inhibited cell proliferation, and the extent of inhibition correlated with MTT data except bromoadamantane derivative **3b**. At 10 μM all studied podophyllotoxin derivatives, both α and β ethers **8** excepted, induced apoptosis.

At 10 μM esters **3b**, **5**–**7** caused complete (or almost complete) depolymerization of the microtubules typical of podophyllotoxin. An interesting observation was made for compounds **3a**,**c** and **4**, which altered the dynamics of microtubule cytoskeleton in unusual manner stimulating their shortening and week or pronounced 'curling' (Figure 3). This effect is different from the earlier studied tubulin clustering caused by podophyllotoxin–adamantane conjugates with long linker chain[21] and reminds the effect of

Table 1 Results of biotests for compounds **3a**–**c** and **4**–**8**.

Compound	Cytotoxicity[a] EC_{50}/μM	Cell growth inhibition (48 h) (%)[b]	Effect on the microtubules
3a	17.2 ± 0.9	10 μM (27)	'Curling' of microtubules, 100 μM (48 h)
3b	> 50 000	10 μM (100)	Depolymerization of microtubules, 10 μM (48 h)
3c	12.2 ± 0.2	10 μM (23)	Shortening and weak 'curling' of microtubules, 100 μM (48 h)
4	2.59 ± 0.02	1 μM (24)	Shortening and weak 'curling' of microtubules, 10 μM (24 h)
5	0.42 ± 0.09	1 μM (98)	Depolymerization of microtubules, 10 μM (24 h)
6	1.41 ± 0.07	1 μM (40)	Depolymerization of microtubules, 10 μM (24 h)
7	0.19 ± 0.03	10 μM (100)	Depolymerization of microtubules, 10 μM (24 h)
α-**8**	> 50 000	100 μM (0)	No effect on microtubules, 100 μM (48 h)
β-**8**	> 50 000	100 μM (74)	No effect on microtubules, 100 μM (48 h)
2a	0.004 ref. 4		Not determined, ref. 4
2b	0.004 ref. 4		Not determined, ref. 4
1	0.014 ± 0.002	1 μM (100)	Depolymerization of microtubules, 10 μM (24 h)

[a] Results of three to six independent experiments. [b] 100% inhibition of cell growth was observed at 10 μM (48 h) for compounds **3b**, **4**–**7**, and at 100 μM (48 h) for esters **3a** and **3c** (all the compounds except for ethers **8** caused apoptosis).

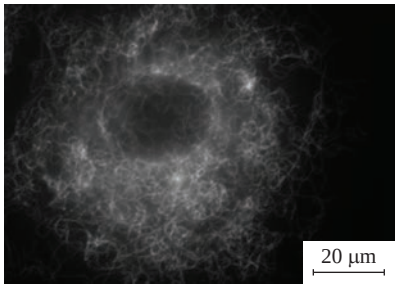


Figure 3 Immunofluorescence microscopy image of the microtubules in carcinoma A549 cells treated with 100 μM of compound **3a**: 'curling' of microtubules.

adamantane derivatives of 2-methoxyestradiol,[22] but is more pronounced for compound **3a**. The rests of microtubules around centrosomes were still observed as star-like structures after partial microtubule depolymerization with noradamantane derivative **3c** (see Online Supplementary Materials). Both isomers of ethers **8** did not alter microtubule dynamics. Hence, it can be deduced that the ability of β-**8** to inhibit cell growth without apoptosis induction and without any effect on microtubules most likely is connected with its action to DNA topoisomerase II (see above).

Several interesting results were obtained during the structure–activity relationship studies. First, the activity tends to diminish with expansion of cycles size in carcass (as in pair **6**/**7** or **3c**/**3a**). However, the general bulkiness of the latter does not always directly correlate with cytotoxicity and the strength of effect on microtubules (compare **5** with **3c**), and other parameters, for example, configuration of the atom bonded to podophyllotoxin, play an essential role. This is demonstrated by noticeable cytotoxicity enhancement of diastereomers **5** with *exo*-bicyclo[3.3.1]nonene group in comparison with their analogue **4** with *endo*-bicyclo[3.3.1]nonane moiety. Most surprising is a drop of cytotoxicity observed for the diastereomeric mixture **7** in comparison with equal in lipophilicity and volume lead-compounds **2a**,**b**,[4] which differ only by position of the bridged group bonding to initial molecule. To explain the difference in activity of these very close podophyllotoxin derivatives, we performed computer molecular modeling of ligand–tubulin interactions using a model of the colchicine-binding site (PDB ID: 1SA1). The structures of the compounds were previously submitted to a conformational MMFF force field optimization and then automated molecular docking was carried out with AutoDock 4.2. The obtained binding modes of lead molecule **2b** and its *exo*-analogue (1*S*,2*R*,4*S*)-**7** are shown in Figure 4(*a*). As it is seen, the compounds occupy various positions in colchicine domain of tubulin, the location of both podophyllotoxin and bridged groups being different. Moreover, the values of scoring functions, though being negative for both compounds, differ by an order of magnitude (most probable to steric reasons) and can explain the contrast in cytotoxicity values.

Analysis of structure–activity relationship reveals an important role of conformational flexibility of cycles in alicyclic moieties. An enhancement of flexibility in structurally close pairs **3a**/**4**, **3a**/**5**, **3c**/**5** or **3c**/**4** leads to an increase in cytotoxicity and effectiveness of altering microtubule dynamics. According to the data of molecular modeling performed for esters **3a** and (1*S*,3*R*,5*R*)-isomer of **5**, the location of their podophyllotoxin fragments in colchicine domain is rather close, but the positions of cage and bridged moieties have essential differences [Figure 4(*b*)]. Bicyclo[3.3.1]-nonene of (1*S*,3*R*,5*R*)-isomer of **5** is expanded to the interface of α/β tubulin subunits near alkyl part of side chain of Lys352β, while adamantane core of the ester **3a** is located in the pocket formed by hydrophobic side chains of Thr179α and Leu248β residues and hydrophilic side chains of Ser178α and Gln247β

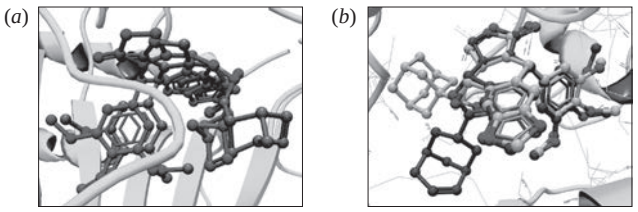


Figure 4 Location of the conjugates (*a*) **2b** (gray) and (1*S*,2*R*,4*S*)-isomer of **7** (black) or (*b*) **3a** (gray) and (1*S*,3*R*,5*R*)-isomer of **5** (black) in α,β-tubulin dimer as predicted by automated docking (AutoDock 4.2; visualized using CLC Drug Discovery Workbench). α-Subunit is presented on the left and β-subunit – on the right (hydrogen atoms are omitted for clarity).

residues. The unfavorable contacts of the latter with lipophilic adamantane core might explain the moderate cytotoxicity of **3a**.

Finally, comparison of the activities of podophyllotoxin–adamantane conjugates **3a** and α-**8** indicates that the ester linker is more cytotoxic than the ether one with equal linker length. Moreover, the latter does not cause any effect on microtubules. Interestingly, the binding mode of **3a** in tubulin [see Figure 4(*b*)] fails to give an explanation for this difference; since the extra-carbonyl oxygen in **3a** does not form any hydrogen bond with the protein in the model. The revealed differences in activity in the pair **3a**/α-**8** as well as in the pairs **2**/**7** or **4**/**5** indicate that total lipophilicity of the inserted alicyclic group does not play a determined role in activity of C^4-podophyllotoxin derivative. This statement is in accordance with the earlier observations.[4]

In conclusion, a series of novel bridged and cage podophyllotoxin derivatives have been synthesized and some of them demonstrated a noticeable cytotoxicity to cancer cells (the lowest EC_{50} is 190 nM for **7**) or unusual microtubule-curling effect. Cytotoxicity and effect on microtubule dynamics were found to be extremely sensitive not only to the slightest changes in the structures of alicyclic moieties, *i.e.* the size of the cycles, their positions and conformational flexibility, but also to the type of the bond between podophyllotoxin and alicyclic group. Computer molecular modeling indicates that bridged or cage moieties are mostly exposed to the interface of α/β tubulin subunits and do not form pronounced favorable hydrophobic contacts with protein. Hence, the activity of cage and bridged C^4-esters of podophyllotoxin seems to be determined by steric hindrance caused by structural elements of alicycles in the protein (and its effect on the position of podophyllotoxin moiety in tubulin) or by unfavorable interactions with hydrophilic side chains of amino acid residues in the protein. These conclusions can be helpful for further design of C^4-podophyllotoxin analogues as putative anticancer agents. The unusual behavior of bromoadamantane ester of podophyllotoxin in A459 cells and the microtubule-curling effect are worth further research and this work is now in progress.

This work was supported by the Russian Foundation for Basic Research (project no. 18-03-00524) and the German organization DAAD (German Academic Exchange Service) under the auspices of a collaborative agreement between M. V. Lomonosov Moscow State University and University of Rostock. S.R. thanks the Council of Scientific Industrial Research New Delhi (CSIR) for the award of CSIR-Senior Associateship [no. 13(8906-A)/2017].

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2018.09.007.

References

1 X. Yu, Z. Che and H. Xu, *Chem. Eur. J.*, 2017, **23**, 4467.
2 Y.-Q. Liu, J. Tian, K. Qian, X.-B. Zhao, S. L. Morris-Natschke, L. Yang, X. Nan, X. Tian and K.-H. Lee, *Med. Res. Rev.*, 2015, **35**, 1.

3 A. Kamal, S. M. Ali Hussaini, A. Rahim and S. Riyaz, *Expert Opin. Ther. Pat.*, 2015, **25**, 1025.
4 J. L. López-Pérez, E. del Olmo, B. de Pascual-Teresa, A. Abad and A. San Feliciano, *Bioorg. Med. Chem.*, 2004, **14**, 1283.
5 A. Abad, J. L. López-Pérez, E. del Olmo, L. F. García-Fernández, A. Francesch, C. Trigili, I. Barasoain, J. M. Andreu, J. F. Díaz and A. San Feliciano, *J. Med. Chem.*, 2012, **55**, 6724.
6 W. H. Sauer and M. K. Schwartz, *J. Chem. Inf. Comput. Sci.*, 2003, **43**, 987.
7 *GVK Biosciences Pvt. Ltd Proprietary Databases*, https://www.gostardb.com.
8 B. T. Yajie and X. Li, *Patent CN 102875565*, 2013.
9 O. N. Zefirova, E. V. Nurieva, H. Lemcke, A. A. Ivanov, N. V. Zyk, D. G. Weiss, S. A. Kuznetsov and N. S. Zefirov, *Mendeleev Commun.*, 2008, **18**, 183.
10 T. Sasaki, S. Eguchi and T. Toru, *J. Org. Chem.*, 1970, **35**, 4109.
11 L. Tafesse, N. Tsuno and X. Zhou, *Patent ES 2583146*, 2016.
12 J.-H. Liu, G. A. Gauger and P. Kovacic, *J. Org. Chem.*, 1973, **38**, 543.
13 M. K. Zilla, D. Nayak, R. A. Vishwakarma, P. R. Sharma, A. Goswami and A. Ali, Eur. *J. Med. Chem.*, 2014, **77**, 47.
14 T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55.
15 E. V. Nurieva, N. A. Zefirov, A. V. Mamaeva, Yu. K. Grishin, S. A. Kuznetsov and O. N. Zefirova, *Mendeleev Commun.*, 2017, **27**, 240.
16 D. A. Vasilenko, E. B. Averina, N. A. Zefirov, B. Wobith, Yu. K. Grishin, V. B. Rybakov, O. N. Zefirova, T. S. Kuznetsova, S. A. Kuznetsov and N. S. Zefirov, *Mendeleev Commun.*, 2017, **27**, 228.
17 A. A. Beloglazkina, B. Wobith, E. S. Barskaia, N. A. Zefirov, A. G. Majouga, E. K. Beloglazkina, N. V. Zyk, S. A. Kuznetsov and O. N. Zefirova, *Med. Chem. Res.*, 2016, **25**, 1239.
18 O. N. Zefirova, H. Lemcke, M. Lantow, E. V. Nurieva, B. Wobith, G. E. Onishchenko, A. Hoenen, G. Griffiths, N. S. Zefirov and S. A. Kuznetsov, *ChemBioChem*, 2013, **14**, 1444.
19 A. Al-Haddad, M. A. Shonn, B. Redlich, A. Blocker, J. K. Burkhardt, H. Yu, J. A. Hammer III, D. G. Weiss, W. Steffen, G. Griffiths and S. A. Kuznetsov, *Mol. Biol. Cell*, 2001, **12**, 2742.
20 C. S. Potten, *Br. J. Cancer*, 1996, **74**, 1743.
21 O. N. Zefirova, E. V. Nurieva, B. Wobith, V. V. Gogol, N. A. Zefirov, A. V. Ogonkov, D. V. Shishov, N. S. Zefirov and S. A. Kuznetsov, *Mol. Divers.*, 2017, **21**, 547.
22 E. V. Nurieva, N. A. Zefirov, A. V. Mamaeva, B. Wobith, S. A. Kuznetsov and O. N. Zefirova, *Russ. Chem. Bull., Int. Ed.*, 2018, **67**, 688 (*Izv. Akad. Nauk, Ser. Khim.*, 2018, 688).

Received: 15th February 2018; Com. 18/5481