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Constituents of Regenerated Plants of *Ophiorrhiza pumila*; Formation of a New Glycocamptothecin and Predominant Formation of (3*R*)-Deoxypumiloside over (3*S*)-Congener

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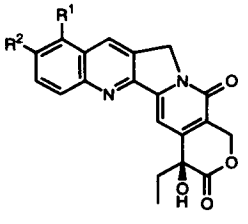
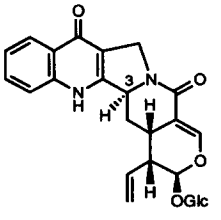
Abstract: The regeneration of plantlets was successful from *Ophiorrhiza pumila* callus cultures, from which a new glucosyloxy camptothecin, 9-β-glucosyloxycamptothecin, together with 15 metabolites including six camptothecin-related alkaloids was isolated. (3*S*)-Deoxypumiloside, one of the plausible biogenetic precursors of camptothecin, could not be found, whereas the (3*R*) epimer was isolated from the regenerated plants. © 1997 Elsevier Science Ltd.

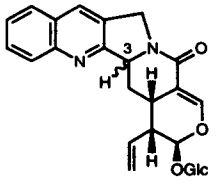
Camptothecin (**1**),[1] first isolated from *Camptotheca acuminata*, is one of the most important alkaloids having inhibitory activity against tumor cells[2] and activity against HIV-I.[3] Recently, camptothecin derivatives such as topotecan®[4] and irinotecan®[5] began to be used as clinical antitumor agents. During our chemical investigations of camptothecin (**1**), we found that *Ophiorrhiza pumila* (Rubiaceae) is a source of camptothecin and related alkaloids. From this plant, the first natural glycosyloxy camptothecin, chaboside (**3**),[6] and a group of plausible biogenetic intermediates, pumiloside (**4**)[7] and deoxypumilosides (**5**, **6**),[7] were isolated. We have recently established the cell and tissue cultures of *O. pumila*. An extensive study on the constituents proved that the production of alkaloids does not take place in the well-growing callus and cell suspension cultures; only anthraquinones and other metabolites were found.[8] In this paper, we describe the successful plant regeneration from *O. pumila* callus cultures and the isolation of the constituents from the regenerated plants.



R^1=R^2=H : Camptothecin (**1**)
R^1=OMe, R^2=H : 9-Methoxycamptothecin (**2**)
R^1=OMe, R^2=OGlc : Chaboside (**3**)



Pumiloside (**4**)

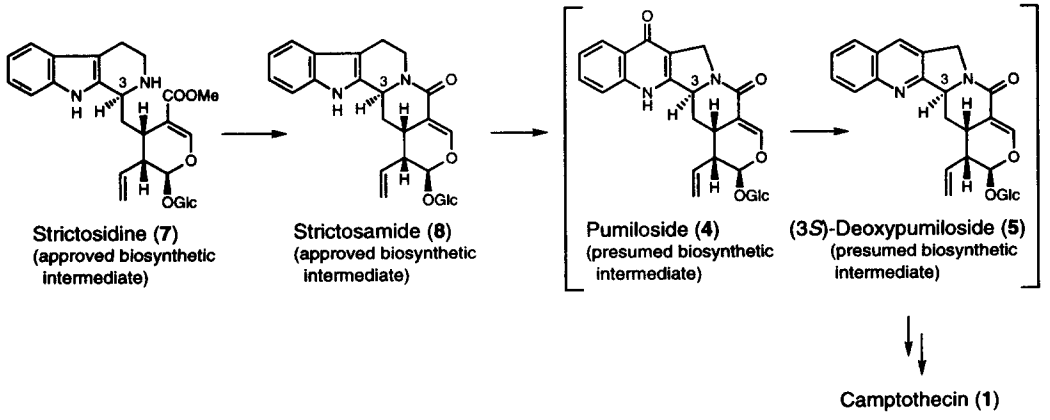


3H-α : (3*S*)-Deoxypumiloside (**5**)
3H-β : (3*R*)-Deoxypumiloside (**6**)

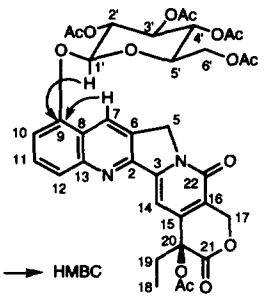
Plant regeneration of *O. pumila* from callus cultures was carried out as follows. Callus cultures originally derived from the leaf segment were maintained on the callus medium[9] in the light (750 lux) at 25°C for 4 weeks and then on the regeneration medium.[9] After the first 4 weeks, the color of the calli changed from light yellow to brown. Ten weeks later the clear differentiation of callus into green leaf buds was observed. Regenerated plants were excised and maintained on A1 medium[9] in the light at 25°C by frequent subculturing on fresh A1 medium at intervals of 6-8 weeks for 4 months.

Freeze-dried regenerated plants (52.6 g) were extracted with hot MeOH to give the extract (19.5 g). The MeOH extract was dissolved with H_2O. It was extracted with $CHCl_3$ and then with *n*-BuOH to give the $CHCl_3$ extract (2.9 g) and the *n*-BuOH extract (5.9 g), respectively. Camptothecin (**1**), 9-methoxycamptothecin (**2**) (from the $CHCl_3$ extract), chaboside (**3**), pumiloside (**4**), (3*R*)-deoxypumiloside (**6**) and strictosamide (**8**) (from the *n*-BuOH extract) were isolated together with six other compounds.[10]

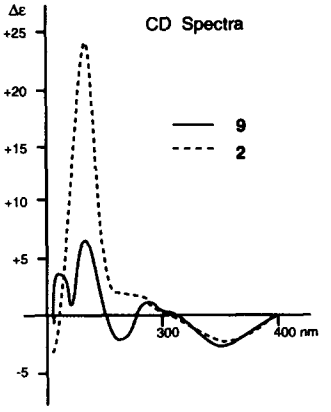
In 1989 we reported the isolation of "deoxypumiloside" and gave the structure of (3*S*)-deoxypumiloside to it.[7a] We recently found that *O. pumila* produces both the C-3 epimeric pair of (3*S*)- and (3*R*)-deoxypumiloside (**5** and **6**), and that the "deoxypumiloside" we reported as a possible biogenetic intermediate possessing the *S* configuration at C-3 was the major isomer and had the (3*R*) instead of (3*S*) configuration.[7b] In order to examine the possible existence of (3*S*)-deoxypumiloside (**5**), the crude *n*-BuOH fraction (20 mg) containing (3*R*)-deoxypumiloside (**6**) was acetylated. From this fraction, only (3*R*)-deoxypumiloside tetraacetate was isolated together with loganin pentaacetate, sweroside tetraacetate and one new alkaloid (**9**, 2.6 mg). This observation is of great interest from the view point of camptothecin biosynthesis. Feeding experiments by Hutchinson demonstrated that strictosidine (**7**) and strictosamide (**8**), both possessing (3*S*)-configuration, are true biosynthetic intermediates.[1a, b] It looked quite natural that pumiloside (**4**) and (3*S*)-deoxypumiloside (**5**) were the biosynthetic intermediates leading to camptothecin (**1**). Concerning with deoxypumiloside, however, we now isolated only the (3*R*)-epimer (**6**) from the regenerated plants; in the wild plant as well the (3*R*)-epimer (**6**) was predominant over the presumed biosynthetic intermediate (**5**). Recently, Kobayashi *et al.* reported that in the marine sponge (+)-keramaphidine B is contained as the dominant component over the enantiomer that is believed to be the true biosynthetic intermediate of manzamine.[11] Our result is quite similar to the reported observation about keramaphidine B.



The new compound (**9**)[12] obtained from the *n*-BuOH fraction has a light blue fluorescence under the UV lamp at the wavelength of 365 nm. The UV spectrum of **9** showed absorptions maxima at 372, 357, 317, 302, 288, 261, and 216 nm, similar to those of 9-methoxycamptotecin (**2**). High resolution FAB-MS displayed a protonated molecular ion at *m/z* 737.2169 corresponding to the molecular formula $C_{36}H_{37}N_2O_5$. In the ^{1}H-NMR spectrum, a set of three aromatic protons due to the 1, 2, 3-trisubstituted benzene ring system in A ring, two singlet aromatic protons due to H-7 (δ 8.68) and H-14 (δ 7.25), protons due to one ethyl group, one sugar unit and five acetyl groups and two methylene protons were observed. In the ^{13}C-NMR spectrum, the amide carbonyl (δ 157.3) and the lactone carbonyl (δ 167.5) carbons were detected. The sugar part was determined as the β-linked glucose by both of the coupling constants in the ^{1}H-NMR and chemical shifts in the ^{13}C-NMR. In the HMBC spectrum, cross peaks between the anomeric proton (δ 5.34) and C-9 (δ 152.2) and between 7-H (δ 8.68) in the ring B and C-9 (δ 152.2) were observed. From these observations, this new compound was deduced to be 9-β-glucosyloxycamptothecin pentaacetate (**9**) that is the second example of gluco-camptothecin. The absolute configuration at the C-20 position was presumed to be the *S* configuration as the other camptothecins on the basis of CD comparisons. The absolute configuration of the sugar in **9** was assumed to be D as chaboside (**3**). Determination of the structure of **9** including the absolute configuration by total synthesis will be described elsewhere.



9-β-Glucosyloxycamptothecin pentaacetate (**9**)



In conclusion, we succeeded in the plant regeneration of *O. pumila* from cultured calli and it was found that regenerated plants of *O. pumila* maintain an alkaloid productive ability and produce camptothecin-related alkaloids.

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References and Notes

1. a) Hutchinson, C. R. *Tetrahedron* **1981**, *37*, 1047-1065. b) Cai, J.-C.; Hutchinson, C. R. in *The Alkaloids*, Brossi, A., ed., Academic Press Inc., New York, **1983**, Vol 21, pp. 101-137. c) Wall, M. E.; Wani, M. C.

in *The Monoterpenoid Indole Alkaloids*, Chapter 13, (Saxton, J. E., ed.), John Wiley & Sons Ltd., London. **1994**, pp. 689-713.

2. Wang, J. C. *Ann. Rev. Biochem.* **1985**, *54*, 665-697.
3. Priel, E.; Blair, D. G.; Showalter, S. D. U. S. PAT. APPL., 31 pp. avail. NTIS, order No. PAT-APPL-7-520 456.
4. *32nd Annual Meeting of the American Society of Clinical Oncology*, Philadelphia/USA, 18-21, May, 1996; see also *Deutsche Apotheker Zeitung* **1996**, *136*, 2619.
5. a) Sawada, S.; Yokokura, T.; Miyasaka, T. *Ann. N. Y. Acad. Sci.* **1996**, *803*, 13-28. b) Chabot, G. G. *Ann. N. Y. Acad. Sci.* **1996**, *803*, 164-172.
6. a) Aimi, N.; Hoshino, H.; Nishimura, M.; Sakai, S.; Haginiwa, J. *Tetrahedron Lett.* **1990**, *31*, 5169-5172. b) Aimi, N.; Ueno, M.; Hoshino, H.; Sakai, S. *Tetrahedron Lett.* **1992**, *33*, 5403-5404.
7. a) Aimi, N.; Nishimura, M.; Miwa, A.; Hoshino, H.; Sakai, S.; Haginiwa, J. *Tetrahedron Lett.* **1989**, *30*, 4991-4994. b) Kitajima, M.; Masumoto, S.; Takayama, H.; Aimi, N. *Tetrahedron Lett.* **1997**, *38*, 4255-4258.
8. Kitajima, M.; Fischer, U.; Nakamura, M.; Ohsawa, M.; Ueno, M.; Takayama, H.; Unger, M.; Stöckigt, J.; Aimi, N. *Phytochem.* in press.
9. The callus medium contained Murashige and Skoog (MS) salts, 2% sucrose and 0.7% agar supplemented with 1mg/l indole 3-acetic acid. The regeneration medium contained MS salts, 2% sucrose and 0.7% agar supplemented with 1mg/l kinetin. A1 medium contained half-strength MS salts, 1% sucrose and 0.7% agar. Murashige and Skoog salts; Murashige, T.; Skoog, F. *Physiol. Plant.* **1962**, *15*, 437-479.
10. From the $CHCl_3$ extract, **1** (17.5 mg), **2** (12.2 mg), β-sitosterol (65.7 mg), 3-*O*-β-D-glucosyl-β-sitosterol (51.6 mg), phaeophytin a (6.9 mg) and phaeophytin b (2.2 mg) were isolated. From the *n*-BuOH extract, **3** (1.6 mg), **4** (68.9 mg), **6** (23.2 mg), **8** (6.2 mg), 3-*O*-caffeoyl quinic acid methyl ester (6.0 mg) were obtained. 3-*O*-Caffeoyl quinic acid (28.4 mg) was isolated from the H_2O extract (12.4 g).
11. a) Kobayashi, J.; Tsuda. M.; Kawasaki, N.; Matsumoto, K.; Adachi, T. *Tetrahedron Lett.* **1994**, *35*, 4383-4386. b) Kobayashi, J.; Kawasaki, N.; Tsuda. M. *Tetrahedron Lett.* **1996**, *37*, 8203-8204.
12. 9-β-Glucosyloxycamptothecin pentaacetate (**9**) : HR-FABMS (NBA) *m/z*: 737.2169 (Calcd for $C_{36}H_{37}N_2O_{15}$: 737.2194); FAB-MS (NBA) *m/z* (%): 737 (MH^+); ^{1}H-NMR (400 MHz, $CDCl_3$) δ : 8.68 (br-s, 1H, H-7), 7.94 (d, 1H, *J* = 8.5 Hz, H-12), 7.73 (dd, 1H, *J* = 8.5, 7.9 Hz, H-11), 7.25 (s, 1H, H-14), 7.18 (d, 1H, *J* = 7.9 Hz, H-10), 5.68 (d, 1H, *J* = 17.3 Hz, H-17), 5.51 (dd, 1H, *J* = 9.6, 7.8 Hz, H-2'), 5.41 (d, 1H, *J* = 17.3 Hz, H-17), 5.41 (dd, 1H, *J* = 9.6, 9.6 Hz, H-3'), 5.34 (d, 1H, *J* = 7.8 Hz, H-1'), 5.33 (dd, 1H, *J* = 19.2, 1.0 Hz, H-5), 5.26 (dd, 1H, *J* = 19.2, 1.0 Hz, H-5), 5.25 (dd, 1H, *J* = 9.6, 9.6 Hz, H-4'), 4.33 (dd, 1H, *J* = 12.4, 5.3 Hz, H-6'), 4.22 (dd, 1H, *J* = 12.4, 2.5 Hz, H-6'), 3.98 (ddd, 1H, *J* = 9.6, 5.3, 2.5 Hz, H-5'), 2.28 (dd, 1H, *J* = 13.9, 7.6 Hz, H-19), 2.22 (s, 3H, 20-OCO*Me*), 2.14 (dd, 1H, *J* = 13.9, 7.6 Hz, H-19), 2.09, 2.08, 2.07, 2.06 (each s, 3H, 4 x CO*Me*), 0.98 (dd, 3H, *J* = 7.6, 7.6 Hz, H-18). ^{13}C-NMR (125 MHz, CDCl) δ : 153.0 (C-2), 146.0 (C-3), 50.2 (C-5), 128.5 (C-6), 126.0 (C-7), 121.1 (C-8), 152.2 (C-9), 110.2 (C-10), 130.2 (C-11), 124.3 (C-12), 149.4 (C-13), 96.2 (C-14), 145.8 (C-15), 120.6 (C-16), 67.1 (C-17), 7.6 (C-18), 31.8 (C-19), 75.9 (C-20), 167.5 (C-21), 157.3 (C-22), 99.1 (C-1'), 71.0 (C-2'), 72.3 (C-3'), 68.2 (C-4'), 72.3 (C-5'), 61.7 (C-6'), 169.9 (20- O*C*OMe), 169.5 (2'-O*C*OMe), 170.2 (3'- O*C*OMe), 169.4 (4'- O*C*OMe), 170.4 (6'- O*C*OMe), 20.8 (C-20-OCO*Me*), 20.73, 20.67, 20.64, 20.60 (2', 3', 4', 6'-COCO*Me*); CD (c = 0.054 mmol/l, MeOH, 27 °C) Δε (λ nm): 0 (398), -2.51 (352), 0 (311), +1.04 (286), 0 (271), -2.10 (263), 0 (250), +6.47 (231), +1.21 (223), +3.70 (211), 0 (207).

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