Cucurbitane Triterpenes from the Fruiting Bodies and Cultivated Mycelia of *Leucopaxillus gentianeus*

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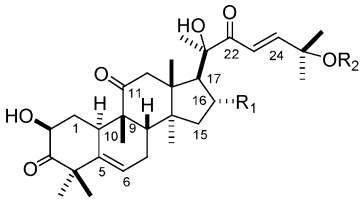
A reinvestigation of the fruiting bodies of the mushroom *Leucopaxillus gentianeus*, allowed the isolation of two minor cucurbitane triterpenes, namely, cucurbitacin D (**5**) and the new metabolite 16-deoxycucurbitacin B (**6**). The latter compound lacks an oxygenated substituent at C-16, an unprecedented structural feature among congeners of cucurbitacin B. The cucurbitanes present in the fruiting bodies were compared with those extracted from mycelia grown on the modified Melin−Norkans (MMN) culture medium. Cucurbitacins B (**1**) and D (**5**), as well as leucopaxillones A (**3**) and B (**4**), were isolated from both sources; in contrast, 16-deoxycucurbitacin B (**6**) and a mixture of fatty acid esters of cucurbitacin B (**2**) were absent in the mycelia. A new triterpene, 18-deoxyleucopaxillone A (**7**), was isolated from the mycelia, but was not detected in the fruiting bodies. The antiproliferative activity of the isolated triterpenes was determined against the NCI-H460 human tumor cell line, in comparison with the antitumor compound topotecan, a well-known topoisomerase I inhibitor.

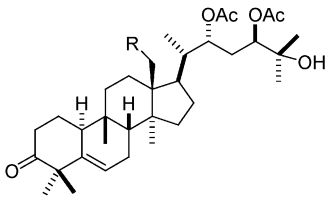
Higher fungi (Basidiomycetes) are a rich source of secondary metabolites, often endowed with unprecedented structural features and remarkable bioactivities.[1−3] In a previous paper, we have reported the structures of the major cucurbitane triterpenes isolated from the fruiting bodies of *Leucopaxillus gentianeus* (Quél.) Kotl. (syn. *L. amarus* (Alb. & Schw.: Fr.) Kühner, family Tricholomataceae).[4] Among these, the highly bitter cucurbitacin B (**1**) is considered a chemical deterrent and is involved in defense mechanisms, to protect the mushroom against parasites and predators. In our ongoing program on bioactive compounds from Basidiomycetes,[5] we decided to reinvestigate a new collection of this same species, in search for minor constituents. At the same time, it was considered interesting to isolate metabolites present in cultures of mycelia obtained from the same fungal material. In fact, very few studies exist on the distribution of secondary metabolites in the various parts of fungi and on the comparison of the chemical contents of the fruiting bodies with those of mycelia.[6]

The antiproliferative activity of cucurbitacins against human tumor cell lines has been known for many years;[7,8] however, the molecular targets involved in their cytotoxicity are still little studied.[9,10] Nowadays, a wide interest is evident in the discovery of new topoisomerase I inhibitors, as potential anticancer lead compounds.[11] For these reasons, we tested the inhibitory activity of the triterpenes isolated from *L. gentianeus* against a human tumor cell line expressing high levels of topoisomerase I.

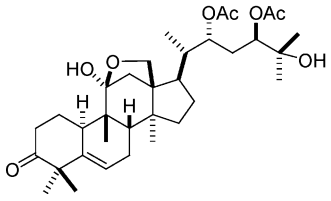
A new crop of *L. gentianeus* was collected in a wood about 250 km from that of our previous investigation,[4] occurring, however, in an identical habitat (coastal wood dominated by pines and evergreen oaks). The fruiting bodies were extracted with EtOAc, and the crude extract was separated by column chromatography according to a previous protocol.[4] In addition to the triterpenes previously isolated from the same fungal species,[4] namely, cucurbitacin B (**1**), cucurbitacin B esters (**2**), and leucopaxillones A (**3**) and B (**4**), two additional cucurbitanes, **5** and **6**, only partially characterized in our former investigation, because of their small amounts, were reisolated and their structures were firmly established.



1 R_1 = OH; R_2 = Ac
2 R_1 = Oleoyloxy, linoleoyloxy, palmitoyloxy; R_2 = Ac
5 R_1 = OH; R_2 = H
6 R_1 = H; R_2 = Ac



3 R = OH
7 R = H



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Compound **5** was identified by the interpretation of its NMR spectra as cucurbitacin D,[12] a triterpene already isolated from several

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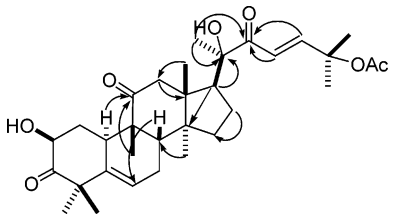

Figure 1. Selected HMBC correlations for 16-deoxycucurbitacin B (**6**).

plants but never found before in a fungal species.[7,13] Cucurbitacin D (**5**) has been reported to be bitter and toxic similar to cucurbitacin B (**1**), from which it differs in having a free hydroxyl group at C-25. Compound **5** was present in the extract of *L. gentianeus* in low amounts (<2 mg/g crude extract), and in contrast to the esters of cucurbitacin B (**2**), the corresponding fatty acid esters of **5** were not found.

The second triterpenoid characterized in the present research, viz., compound **6**, showed spectroscopic data typical of a cucurbitacin structure, but not corresponding to any known cucurbitane derivative.[7,13] Comparison of the ^{13}C NMR spectrum of **6** with those of cucurbitacins B (**1**) and D (**5**) indicated the absence of one sp^3 oxygenated carbon and the presence of an additional methylene carbon in the upfield region. In particular, a DEPT-90 experiment indicated the lack of one of the two CH−O methines of cucurbitacin B (**1**), which 2D NMR experiments (HSQC and HMBC spectra, see Figure 1) proved to be the C-16 oxygenated carbon. This assignment was also corroborated by the chemical shift of the C-17 carbon of compound **6**, which, when compared to the ^{13}C NMR spectrum of **1**, moved upfield due to the absence of the β-hydroxyl (δ 48.9 in **6** vs δ 58.2 in **1**).[14] Compound **6** was thus assigned the structure 16-deoxycucurbitacin B, for which the molecular formula $C_{32}O_7H_{46}$ (MW 542), calculated from proton and carbon counting from the NMR spectra, was in agreement with the observed pseudomolecular ion peaks at *m*/*z* 543 [M + H]$^+$ and *m*/*z* 565 [M + Na]$^+$ in the positive-ion ESIMS (and was confirmed by HRESIMS at *m*/*z* 565.3140, calcd for $C_{32}H_{46}O_7Na$, 565.3136). To the best of our knowledge, compound **6** is the first example of a cucurbitacin B congener lacking the C-16 hydroxyl group.[7,13]

The triterpenoids present in the mycelia of *L. gentianeus* were then examined. An intact fruiting body of the mushroom was sliced, and a piece was used to isolate the mycelium, which was then grown either on the AMG (agar, malt extract, glucose)[15] or on the MMN (modified Melin−Norkrans)[16] culture media. The latter induced a much faster mycelium growth (see the Experimental Section for details) and was, therefore, the medium of choice. After 60 days, the mycelium was frozen at −20 °C and extracted with EtOAc to study the content of secondary metabolites. After liquid chromatographic separations on normal and reversed phases, cucurbitacins B (**1**) and D (**5**), as well as leucopaxillones A (**3**) and B (**4**), were isolated. However, cucurbitacin B esters (**2**) and 16-deoxycucurbitacin B (**6**) were not found in the mycelial extract. On the contrary, a new cucurbitane triterpene was isolated from the mycelia, which was absent in the fruiting bodies. The structure of this compound was determined as **7** on the basis of its NMR and MS spectra. The ^{1}H and ^{13}C NMR spectra indicated a marked similarity with leucopaxillone A (**3**),[4] from which the structure of **7** differs in the absence of a primary hydroxyl group at C-18. This assignment was firmly established by the presence of an additional methyl at δ 15.7 in the ^{1}H NMR spectrum of **7** and the absence of the characteristic signals of the hydroxymethyl group of leucopaxillone A (**3**) (ABq at about δ 3.8 in the ^{1}H NMR spectrum; triplet at about δ 61.6 in the corresponding DEPT ^{13}C NMR spectrum).[4] The positive-ion ESIMS of **7** exhibited a peak at *m*/*z* 581, corresponding to [M + Na]$^+$; this, together with the HRESIMS at *m*/*z* 581.3819 (calcd for $C_{34}H_{54}O_6Na$, 581.3813), indicated a molecular composition of $C_{34}H_{54}O_6$ (MW 558), in full agreement with the NMR data. Compound **7** was thus assigned the structure 18-deoxyleucopaxillone A.

Table 1. Antiproliferative Activity of Compounds **1**−**7** against the NCI-H460 Human Tumor Cell Line

compound	IC_{50} (μg/mL)
topotecan	0.008
cucurbitacin B (**1**)	0.011
cucurbitacin B esters (**2**)	>30
16-deoxycucurbitacin B (**6**)	0.06
cucurbitacin D (**5**)	0.12
leucopaxillone A (**3**)	3.5
leucopaxillone B (**4**)	0.3
18-deoxyleucopaxillone A (**7**)	10.6

A close comparison of the structural features of the seven cucurbitane triterpenes (**1**−**7**) isolated from *L. gentianeus* provides information on possible biogenetic pathways. It is interesting to note that two of the isolated cucurbitanes, viz., the abundant leucopaxillone A (**3**) and the less abundant 18-deoxyleucopaxillone A (**7**), are not oxygenated at C-11. Considering that most naturally occurring cucurbitane triterpenes are oxidized at C-11, mainly as ketones,[7] some authors have postulated that oxidation at this position may occur at a very early stage of the cucurbitacin biogenesis, i.e., before the cucurbitane skeleton is formed by the migration of the C-10 methyl group to C-9.[13,17] Balliano et al., however, have demonstrated that the enzymes in *Cucurbita maxima* induce direct cyclization of squalene 2,3-epoxide to an unfunctionalized cucurbitane derivative, proving that the C-11 position is oxidized at a later stage, after the construction of the tetracyclic cucurbitane skeleton.[18] Therefore, the leucopaxillones are likely formed at an early stage of the biogenetic cascade of *L. gentianeus* cucurbitanes, while the highly oxidized cucurbitacins B (**1**) and D (**5**) are later products. In particular, 18-deoxyleucopaxillone A (**7**), which is the least oxygenated of all the cucurbitanes isolated from *L. gentianeus*, might be the precursor of the other derivatives, which are subsequently formed by oxidation at carbons C-11, C-18, C-20, C-2, and C-16. Interestingly, compound **7** was found only in the fungal mycelia. The occurrence of 16-deoxycucurbitacin B (**6**) is of interest for relating the biogenesis of the C-16 non-hydroxylated leucopaxillones to those of the C-16 hydroxylated cucurbitacins B (**1**) and D (**5**) and indicates that oxidation at C-16 possibly occurs after the introduction of the enone system in the side chain. The latter unit presumably derives from selective oxidation of the OH-22 group of the leucopaxillones, followed by β-elimination of the OAc-24 group.

The fatty acid esters (**2**) of cucurbitacin B are absent in the mycelia, a finding in agreement with the hypothesis that these esters are used as inactive reserve metabolites in a chemical defense system, to protect the fruiting bodies of *L. gentianeus*, where the biologically active cucurbitacin B (**1**) would function as a deterrent. Thus, cucurbitacin B may be biosynthesized in mycelia and accumulated in fruiting bodies as esters (**2**), which are then cleaved by lipases in damaged tissues to repel the external attack of predators and parasites.[4] However, given the limited amounts isolated from fruiting bodies, it is hard to establish whether the free alcohol **1** is naturally occurring in this part of the mushroom or is derived from incomplete esterase inactivation during the extraction process.

In Table 1 the IC_{50} values (μg/mL) of compounds **1**−**7** are summarized, after 72 h exposure to the human lung carcinoma NCI-H460 cell line, in which topoisomerase I is overexpressed. Topotecan, a well-known antitumor compound and topoisomerase I inhibitor, was employed as a positive control in this assay. Cucurbitacin B (**1**) was found to be the most active metabolite in the group, with an IC_{50} value very similar to that of topotecan. The IC_{50} of cucurbitacin D (**5**) was an order of magnitude higher than that of cucurbitacin B (**1**), reflecting the important role of

acetylation at the OH-25 in enhancing the bioactivity of **1**. Leucopaxillones **3**, **4**, and **7** were much less active, with the exception of leucopaxillone B (**4**), which showed a moderate inhibitory activity. 16-Deoxycucurbitacin B (**6**) was only slightly less active than **1** in the present assay; this finding was rather surprising, since the presence of a free OH-16 group has been suggested to be an important structural requirement for cucurbitacin bioactivity.[19] This assumption has apparently been confirmed by the reduced bioactivity of cucurbitacin B esters (**2**), as compared to cucurbitacin B (**1**), which was attributed to acylation of the C-16 hydroxyl group.[4]

In order to have more evidence of the topoisomerase I inhibition potency of cucurbitacin B (**1**), a topoisomerase I-dependent DNA cleavage assay was performed. 7-Ethyl-10-hydroxy-20(*S*)-camptothecin, the active metabolite of irinotecan, an established clinical camptothecin derivative, was used as a reference in the test. Indeed, the cleavage patterns (Figure S1, Supporting Information) showed the ability of cucurbitacin B (**1**) to stimulate the cleavage at the same sites of the reference compound; however, cucurbitacin B was substantially less effective, thus indicating that, in addition to topoisomerase I inhibition, other mechanisms contribute to its cytotoxic activity.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on an FT-IR Perkin-Elmer BX spectrometer. ^{1}H and ^{13}C NMR spectra ($CDCl_3$ and CD_2Cl_2) were determined on a Bruker CXP 300 or on a JEOL Eclipse 400 NMR spectrometer. ^{1}H and ^{13}C chemical shifts (δ, ppm) are relative to residual $CHCl_3$ signals [δ_H 7.26, δ_C (central line of t) 77.1, respectively] or to residual CH_2Cl_2 signals [δ_H 5.0, δ_C 54.20]. ESIMS experiments were carried out using a Finnigan LCQ Advantage MS 1.4 spectrometer, equipped with Xcalibur 1.4 software. HRESIMS were determined on a ICR-FTMS Apex II Bruker Daltonics spectrometer. Thin-layer chromatography was performed on silica gel F_{254} sheets (Polygram) and RP-18 TLC F_{254} sheets. Compounds were visualized under UV light (254 and 366 nm) and by spraying with a sulfanisaldehyde solution followed by heating. Flash column chromatography was performed with Merck Kieselgel 60 (40−63 μm) and Merck LiChroprep RP-18 (25−40 μm).

Fungal Material. Fruiting bodies of *Leucopaxillus gentianeus* were collected in November 2003 in the vicinity of Rome. The mushrooms were identified by one of the authors (M.C.). A voucher specimen (accession number 3515) was deposited at the Mycotheca Universitatis Taurinensis (MUT).

Extraction and Isolation. The fruiting bodies (a total of approximately 1.5 kg) were immediately minced and extracted with ethyl acetate (2 L), under stirring for about 2 h. The mixture was filtered and the solvent was evaporated in vacuo to yield about 4 g of crude extract. The latter was fractionated on a RP-18 column eluted with mixtures ranging from H_2O−MeCN−MeOH (8.5:0.5:1.0) to MeCN−MeOH (6.6:3.3). Thirty-six fractions were obtained (A_{1-36}); cucurbitacin D (**5**) (4 mg) was isolated from fractions A_{17} and A_{18}, and 16-deoxycucurbitacin B (**6**) (5.1 mg) from fractions A_{25} and A_{26}, after repetitive separation on RP-18 columns (employing different mixtures of H_2O−MeCN−MeOH in different percentages) and on silica gel columns, eluted with toluene−EtOAc mixtures.

Mycelial Growth and Extraction. One fruiting body was used to isolate a mycelium sample and was deposited at MUT (accession number 3515). Starting from this sample, the mycelium of *L. gentianeus* was grown on modified Melin−Norkrans (MMN) medium, employing sterile Petri dishes (9 cm diameter), at a constant temperature of 24 °C. After 4−5 weeks, the dishes were completely covered with cotton-like mycelia, and after approximately 2 months, they were frozen at −20 °C. This mycelium material (a total of about 150 g) was minced in a mortar and then extracted with a mixture of EtOAc−MeOH (9.5:0.5) under stirring for 2 h. The mixture was then filtered and the solvent was evaporated in vacuo to yield about 200 mg of crude extract. The latter was fractionated using a RP-18 column eluted with mixtures ranging from H_2O−MeCN−MeOH (7.0:1.0:2.0) to MeCN−MeOH (6.6:3.3). Twenty-five fractions were obtained (B_{1-25}); cucurbitacin D (**5**) (4 mg) was isolated from fraction B_8 and leucopaxillone C (**7**) from fraction B_{17}, after a successive separation on a silica gel column (eluted with mixtures of toluene−EtOAc in different percentages).

Cucurbitacin D (5). ^{1}H NMR and ^{13}C NMR spectra were identical with those reported in the literature;[12] positive-ion ESIMS *m*/*z* 539.5 $[M + Na]^+$, 1055.3 $[2M + Na]^+$; negative-ion ESIMS *m*/*z* 515.9 $[M - H]^-$.

16-Deoxycucurbitacin B (6): waxy solid; $[\alpha]_D^{22}$ +20.8 (*c* 0.3, CH_2Cl_2); IR (thin film) ν_{max} 3455 (OH), 2966, 2927, 2881, 2851, 1735 (C═O acetate), 1715 (C═O saturated ketones), 1692 (C═O conjugated ketone), 1628, 1464, 1434, 1387, 1369, 1284, 1248, 1125, 1054, 1022, 987 cm^{-1}; ^{1}H NMR (CD_2Cl_2) δ 7.17 (1H, d, J = 15.6 Hz, H-24), 6.47 (1H, d, J = 15.6 Hz, H-23), 5.82 (1H, m, H-6), 4.45 (1H, dd, $J_{2-1\alpha}$ = 6.0, $J_{2-1\beta}$ =12.9 Hz, H-2), 4.07 (1H, s, HO-20), 3.53 (1H, br, HO-2), 3.21 (1H, d, J = 14.4 Hz, H-12β), 2.76 (1H, brd, $J_{10-1\beta}$ = 13.1 Hz, H-10), 2.70 (1H, d, J = 14.4 Hz, H-12α), 2.44 (1H, m, H-8), 2.36 (1H, m, H-7a), 2.23 (1H, ddd, J_{1a-10} = 3.5, J_{1a-2} = 6.0, J_{1a-1b} = 9.6 Hz, H-1α), 2.09 (1H, m, H-17), 2.03 (3H, s, acetate Me), 2.01 (1H, m, H-7b), 1.61 (1H, m, H-16a), 1.59 (3H, s, H-26 or H-27), 1.56 (3H, s, H-27 or H-26), 1.46 (3H, s, H-21), 1.43−1.34 (3H, m, H-15 and H-16b), 1.35 (3H, s, H-28 or 29), 1.31 (3H, s, H-29 or 28), 1.26 (1H, m, H-1β), 1.14 (3H, s, H-18) 1.08 (3H, s, H-19) 1.00 (3H, s, H-30); ^{13}C NMR (CD_2Cl_2) δ 214.0 (C, C-11), 213.3 (C, C-3), 202.5 (C, C-22), 170.4 (C, acetate CO), 154.1 (CH, C-24) 141.0 (C, C-5) 121.4 (CH, C-6) 119.7 (CH, C-23) 79.7 (C, C-25) 79.5 (C, C-20) 72.4 (CH, C-2), 54.7 (C, C-14), 51.1 (C, C-4), 50.9 (C, C-13), 49.6 (CH_2, C-12), 48.9 (C, C-9), 48.9 (CH, C-8), 43.2 (CH, C-17), 36.8 (CH_2, C-1), 34.60 (CH_2, C-15), 34.5 (CH, C-10), 30.0 (CH_3, C-28 or C-29), 27.1 (CH_3, C-26 or C-27), 26.7 (CH_3, C-27 or C-26), 24.6 (CH_3, C-21), 24.5 (CH_2, C-7), 23.4 (CH_3, acetate), 21.8 (CH_3, C-29 or C-28), 21.6 (CH_2, C-16), 20.4 (CH_3, C-19), 19.6 (CH_3, C-30), 18.7 (CH_3, C-18); EIMS *m*/*z* 482 $[M - CH_3COOH]^+$ (45), 369 (100); positive-ion ESIMS *m*/*z* 565.2 $[M + Na]^+$ 543.7 $[M + H]^+$; HRESIMS *m*/*z* 565.3140 (calcd for $C_{32}H_{46}O_7$-Na, 565.3136).

18-Deoxyleucopaxillone A (7): colorless, waxy solid; $[\alpha]_D^{22}$ +14.0 (*c* 0.2, CH_2Cl_2); IR (thin film) ν_{max} 3343, 2918, 2850, 1738, 1714, 1468, 1377, 1260, 1089, 1023, 800 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.66 (1H, m, H-6), 4.88 (1H, m, H-22), 4.84 (1H, m, H-24), 2.56 (1H, brm, H-2a), 2.53 (1H, brm, H-10), 2.38 (2H, m, H-2b and H-7a), 2.09 (3H, s, acetate Me), 2.03 (3H, s, acetate Me), 1.99 (1H, br, H-1a), 1.87 (1H, br, H-20), 1.84 (H-7b), 1.82 (1H, br, H-17), 1.79−1.75 (2H, m, H-23), 1.66−1.58 (2H, br, H-11a and H-12a), 1.56 (1H, m, H-8), 1.52 (2H, m, H-15), 1.46 (1H, m, H-1b), 1.30 (2H, br, H-16), 1.29−1.23 (2H, br, H-11b and H-12b), 1.24 (3H, s, H-30), 1.21 (9H, s, H-26, H-27 and H-28), 0.92 (3H, d, H-21), 0.88 (3H, s, H-29), 0.86 (6H, s, H-18 and H-19); ^{13}C NMR ($CDCl_3$) δ 215.3 (C, C-3), 171.0 (C, acetate CO), 170.9 (C, acetate CO), 142.7 (C, C-5), 120.5 (CH, C-6), 75.9 (CH, C-24), 72.5 (C, C-25), 72.4 (CH, C-22), 51.1 (C, C-14), 49.1 (C, C-4), 47.4 (CH, C-8), 46.7 (C, C-9), 43.4 (CH, C-17), 38.8 (CH_2, C-2), 38.7 (2 CH, C-10 and C-20), 35.1 (C, C-13), 34.9 (CH_2, C-16), 32.3 (CH_2, C-11), 30.5 (CH_2, C-12), 28.8 (CH_3, C-26), 27.4 (CH_3, C-29), 27.3 (CH_3, C-28), 26.6 (CH_2, C-23), 26.3 (CH_2, C-15), 25.9 (CH_2, C-1) 25.3 (CH_3, C-27), 24.7 (CH_2, C-7), 22.8 (CH_3, C-30), 21.5 (CH_3, acetate), 21.1 (CH_3, acetate), 18.1 (CH_3, C-19), 15.7 (CH_3, C-18), 13.4 (CH_3, C-21); positive-ion ESIMS molecular ion cluster peaks at *m*/*z* 581.3 $[M + Na]^+$; HRESIMS *m*/*z* 581.3819 (calcd for $C_{34}H_{54}O_6Na$, 581.3813).

Cytotoxicity Assay. The human large-cell lung carcinoma cell line, NCI-H460 (ATCC HTB 177), was used in this study. The cell line was cultured in RPMI-1640 containing 10% fetal calf serum. Antiproliferative activity of the test compounds was assessed by a growth inhibition assay after 72 h exposure. Briefly, cells in the logarithmic phase of growth were harvested and seeded in duplicate into six-well plates. Twenty-four hours after seeding, cells were exposed to test compound, then harvested 72 h later and counted with a Coulter counter. IC_{50} is defined as the inhibitory compound concentration (μg/mL) causing a 50% decrease of cell growth compared to untreated control. All compounds are insoluble in water and were, therefore, dissolved in DMSO prior to dilution in the biological assay. Final concentration of DMSO was at a maximum of 1%.

Topoisomerase I-Dependent DNA Cleavage Assay. A gel purified BamHI-EcoRI fragment of SV40 DNA was used for the cleavage assay. DNA fragments were uniquely 3′-end labeled. Topoisomerase I DNA cleavage reactions (20 000 cpm/sample) were performed with 1, 10, and 50 μM cucurbitacin B (**1**) and with 50 μM 7-ethyl-10-hydroxy-20(*S*)-camptothecin, in 20 μL of 10 mM Tris-HCl (pH 7.6), 150 mM

KCl, 5 mM $MgCl_2$, 15 μg/mL BSA, 0.1 mM dithiotreitol, and the human recombinant enzyme (full length topoisomerase I)[20] for 30 min at 37 °C. Reactions were stopped by adding 1% SDS and 0.3 mg/mL proteinase k and incubating for 45 min at 42 °C. After precipitation, DNA was resuspended in denaturating buffer (80% formamide, 10 mM NaOH, 0.01 M EDTA, and 1 mg/mL dye) before loading on a denaturating 8% polyacrylamide gel in TBE buffer. DNA cleavage levels were visualized with a PhosphoImager 425 model (Molecular Dynamics).

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Supporting Information Available: Figure showing effects of cucurbitacin B (**1**) and 7-ethyl-10-hydroxy-20(*S*)-camptothecin on topoisomerase I-mediated DNA cleavage. This information is available free of charge via the Internet at http://pubs.acs.org.

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