Cucurbitane Triterpenoids from *Leucopaxillus gentianeus*

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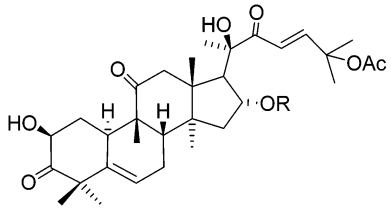
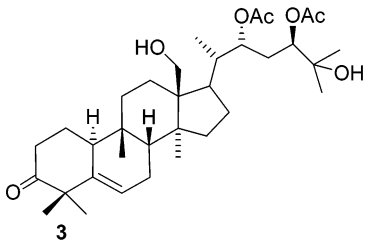
In addition to the known bioactive triterpene cucurbitacin B (**1**), two new cucurbitane triterpenoids, namely, leucopaxillones A (**3**) and B (**4**), exhibiting a new oxygenation pattern among cucurbitacins, have been isolated from the mushroom *Leucopaxillus gentianeus* (syn. *L. amarus*). Cucurbitacin B (**1**) imparts a bitter taste to the flesh of the fungus; however, it occurs in the fruiting bodies mainly esterified as tasteless fatty acid esters **2a**−**c**. In vitro growth inhibitory effects of compounds **1**−**4** on proliferation of four different human tumor cell lines (A549, CAKI-1, HepG2, MCF-7) were evaluated by using a 1-day MTT assay. Only cucurbitacin B was highly active on all lines. Free cucurbitacin B is presumed to be formed in vivo by an enzyme-mediated scission of esters **2a**−**c**, thus constituting a chemical weapon that protects the mushrooms against parasites and predators. Compounds **1**−**4** are structurally different from the other few cucurbitacins isolated from Basidiomycetes, being, instead, more similar to those occurring in plants. In particular, cucurbitacin B (**1**) seems to represent an interesting example of secondary metabolite convergence between distant taxa such as fungi and vascular plants, where they likely exert a similar role of protection. The structures of the compounds were established by means of spectroscopic methods and X-ray diffraction on a single crystal. The absolute configuration of leucopaxillone A has been assigned on the basis of CD chirality rules.

In our ongoing program to search for novel bioactive compounds from higher mushrooms (Basidiomycetes),[1] an EtOAc extract of the fruiting bodies of *Leucopaxillus gentianeus* (Quél.) Kotl. (synonym: *L. amarus* (Alb. & Schw.: Fr.) Kühner, family: Tricholomataceae) showed promising activity in the *Artemia salina* lethality assay.[2] *L. gentianeus* is a medium-sized, robust mushroom with a dull, reddish-brown to cinnamon-brown, coarsely striate cap; it is widely distributed in the Mediterranean areas, forming rings and arcs under conifers and mixed forests, and fruiting generally in late fall. The mushroom is distinguished by a strongly bitter taste and for this reason is not consumed, although it is not considered toxic. The bitter constituents of *L. gentianeus* have not yet been subjected to any phytochemical investigation. Therefore, we report herein the isolation, structure elucidation, and human tumor cells growth inhibition properties of six cucurbitane triterpenoids from *L. gentianeus*, namely, the known cucurbitacin B (**1**), the corresponding new 16-oleyl (**2a**), 16-linoleyl (**2b**), and 16-palmityl (**2c**) esters, and two cucurbitanes with a new oxidation pattern, which have been given the trivial names leucopaxillones A (**3**) and B (**4**).



1 R = H
2a R = Oleyl
2b R = Linoleyl
2c R = Palmityl



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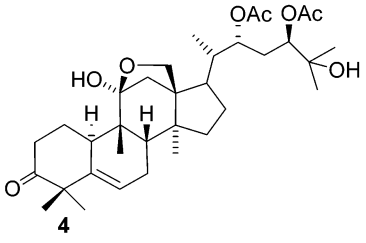

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Results and Discussion

Fungal material was collected along the Tyrrhenian coast of Central Italy and extracted with EtOAc. A preliminary separation of polar compounds from the more lipophilic fraction was obtained by partitioning the crude residue between hexane and 95% aqueous MeOH. Both subextracts showed good activity against *A. salina*[2] and were therefore submitted to the fractionation process. Using a series of chromatographic separations on both flash Si gel and RP columns, and performing final purifications on semipreparative RP-HPLC, compounds **3**, **4**, and a small amount of **1** were isolated from the MeOH subextract, while **2a**−**c** and the majority of **1** were isolated from the hexane layer. On TLC plates, the compounds gave a dramatically different response to UV light and spray reagents. In fact, leucopaxillones A (**3**) and B (**4**) gave a couple of faintly absorptive spots under 254 nm UV light, which became light-blue and purple-bluish, respectively, when sprayed

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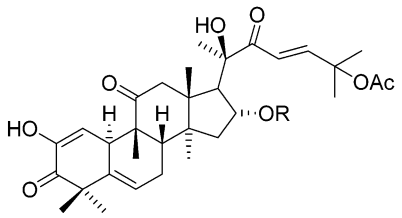
with the sulfovanillin reagent (see the Experimental Section). In contrast, the spots of compounds **1** and **2a**−**c** showed an intense absorption at 254 nm, but were faintly colored upon exposure to the vanillin solution. Preparative separation of individual compounds **2a**−**c** was not feasible under different chromatographic conditions (CC and HPLC); therefore, their IR, NMR, and MS data were recorded as a mixture.

Compound **1** was identified as the known cucurbitacin B[3−8] by comparison with an authentic commercial sample (LC-MS and 2D NMR spectra). Furthermore, the ^{1}H and ^{13}C NMR data were superimposable with those reported.[5,8]

Comparison of 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, HSQC) NMR spectra, and MS data of **1** and **2a**−**c**, along with the data of a degradation experiment (vide infra), revealed that the latter compounds were diesters containing both an acetyl group and a fatty acid ester moiety. One of the acyl groups was placed at C-25, the typical substitution position for the cucurbitacins, as inferred by the characteristic low-field resonance (δ 1.58) of the C-25 methyl group signals in the ^{1}H NMR spectrum.[9] The resonances attributable to the triterpene ring system carbons of **1** and **2a**−**c** were identical within ±0.7 ppm, with the notable exception of the signals of the cyclopentane ring, which showed small, but significant chemical shift differences in the range of ±2−4 ppm. The latter could be explained by the occurrence in compounds **2a**−**c** of another ester group at C-16, as confirmed by the downfield shift of H-16 compared to the corresponding signal of **1** (δ_H 5.19 in **2a**−**c** vs 4.36 in **1**), whose coupling constant (7.7 Hz) is characteristic of a β-oriented proton in a 16-*O*-acyl cucurbitacin B derivative.[10,11] Therefore, compounds **2a**−**c** exhibited the same basic structure and oxidation pattern as compound **1**, the only ambiguity to be solved being the relative position of the acetoxy and the fatty ester moieties at C-16 and at C-25. Eventually, formulas **2a**−**c** were established by analysis of HMBC correlation cross-peaks of the ester carboxylic carbons. In fact, a cross-peak between the carbon at δ_C 169.5 and the acetate CH_3 protons at δ_H 2.03 and a cross-peak between the resonance of the carbon at δ_C 173.1 and the 2′-H_2 signal (δ_H 2.2) allowed assignment of the ^{13}C NMR signal of each ester carboxylic group. Consequently, the three-bond connectivity between the carbon at δ_C 173.1 and the H-16 signal proved the position of the fatty acid ester at C-16 and then of the acetoxy moiety at C-25. Alkaline hydrolysis of **2a**−**c** was attempted in order to confirm the structure and to ascertain the nature of the fatty acids. On exposure to NaOH or LiOH in THF/H_2O (2h, 65 °C), no ester hydrolysis was observed, while oxidation of the ketol group occurred to give diosphenols **5a**−**c** (H-1 vinyl proton δ 5.95 d, $^3J_{H,H}$ 2.3 Hz; enol 2-OH, δ 5.90 s), in accordance with the known behavior of α-hydroxyketones in alkaline solution in the presence of oxygen.[12] The structures of compounds **5a**−**c** as 16-O-esters of cucurbitacin E were confirmed by comparison of their NMR data with those reported for cucurbitacin E.[4,7,13] Sodium methoxide in MeOH (45 min, 35 °C), on the other hand, gave, in addition to a mixture of fatty acid methyl esters, a mixture of cucurbitacin derivatives that could not be separated and structurally characterized. However, NMR spectra of this fraction revealed that oxidation of the 1,2-ketol group again occurred, with concomitant Michael addition of a MeOH molecule or of the free C-16 hydroxyl group to the C-24 carbon of the highly electrophilic conjugated enone system in the side chain.

GC−MS analysis of the fatty acid methyl ester mixture from methanolysis of **2a**−**c** revealed the presence of methyl



5a R = Oleyl
5b R = Linoleyl
5c R = Palmityl

oleate as the major component (about 85%), along with methyl linoleate (about 15%) and methyl palmitate (<1%). The identity of each ester was established by comparison with the retention times and mass spectra of standard fatty acid methyl esters and reference mass spectra.[14]

Leucopaxillone A (**3**) was isolated as colorless crystals, $[\alpha]_D$ +24°. EIMS analysis yielded an $[M]^+$ ion at *m/z* 574 corresponding to the molecular formula $C_{34}H_{54}O_7$ on the basis of HREIMS and proton and carbon counting from the NMR spectra. These showed the presence of commonly encountered cucurbitacin functional groups such as a ketone carbonyl at C-3 (δ_C 215.1), a trisubstituted Δ^5 olefin (δ_H 5.67), and a C-25 oxidized carbon (δ_C 72.5); in addition, there was a hydroxymethyl group (ABq at δ 3.8, $^2J_{AB}$ 13.6 Hz) linked to a quaternary carbon, and two acetate ester groups (two methyl singlets at δ 2.03 and 2.09 and two coincident singlet carbonyl signals at δ 171.1) that accounted for the extra four carbons with respect to the C_{30} cucurbitacin triterpene formula. HMBC analysis placed one acetate ester at C-22 on the basis of correlations between the oxymethine proton at δ 4.89 and the signals of an acetate carbonyl and the C-21 methyl at δ 14.0. The C-24 acetate was similarly located by correlation of the oxymethine proton at δ 4.86 and the signals of the other acetate carbonyl and the C-26 and C-27 methyls (δ 1.21 and 1.22). Structural assignments and the relative configuration of **3** were conclusively established by single-crystal X-ray crystallography. Leucopaxillone A (**3**) recrystallized from MeCN/*i*PrOH to yield colorless plates. The subsequent X-ray structure solution confirmed the cucurbitane skeleton and the oxidation pattern of leucopaxillone A (**3**), indicating an *anti* arrangement of the two acetoxy groups in the side chain and the presence of a β-oriented hydroxymethyl group attached to C-13 (Figure 1). The latter structural features were unprecedented among cucurbitacins.

The absolute configuration of **3** was assigned by analysis of the CD spectrum, which showed a negative band ($\Delta\epsilon$ = −1.0) at 292 nm for the n−π^* transition of the β,γ-unsaturated ketone chromophore. The low intensity of $\Delta\epsilon$ and the absence of a red-shifted absorption (compared to *cisoid* β,γ-unsaturated ketones) were in agreement with the *transoid* geometry of the chromophore, in the definition

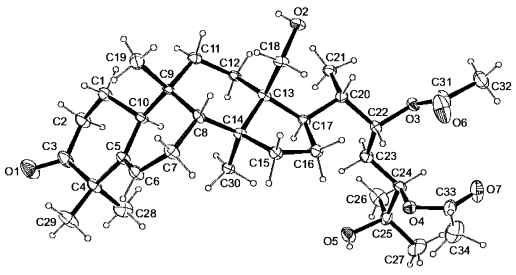


Figure 1. ORTEP-generated perspective (hydrogen atoms excluded) of leucopaxillone A (**3**). Thermal ellipsoids are drawn at the 20% probability level. One molecule of cocrystallized 2-propanol has been omitted for clarity.

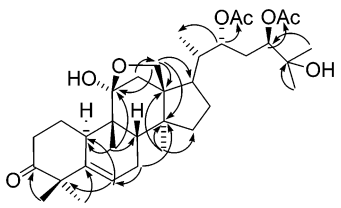


Figure 2. Selected HMBC correlations for leucopaxillone B (**4**).

given by Kirk.[15] Application of the semiempirical sector rule for the n$-\pi^*$ carbonyl transition[15] led to the absolute configuration depicted in formula **3**. In leucopaxillone A, the mechanism of optical activity arising from the interaction between the magnetic dipole moment of the carbonyl group and the electric dipole moment of the olefinic double bond can be considered dominant, as there is no other significant chromophore nearby. The absolute configuration obtained for leucopaxillone A (**3**) is identical to that assigned to the known cucurbitane triterpenes.

Once the structure of compound **3** was established, that of leucopaxillone B, $[M]^+$ ion at *m/z* 588 in the EIMS spectrum, could be inferred as cucurbitane **4** by the NMR spectra, showing, with respect to **3**, an extra five-membered cyclic hemiacetal formed by ring closure of the 13-CH_2OH group onto the C-11 carbonyl group. This structural feature was confirmed by HMBC correlations between the ether H_2-18 protons (ABq at δ 3.90) and the C-11 hemiacetal quaternary carbon at δ 110.9 and between the H_2-12 protons and the ether carbon at δ 69.1 (see Figure 2).

Table 2. In Vitro Cell Growth Inhibitory Activity of Compounds **1**−**4** from *Leucopaxillus gentianeus*

	IC_{50} (μM)			
compound	A549	CAKI 1	HepG2	MCF-7
cucurbitacin B (**1**)	46.6	7.1	0.76	0.78
cucurbitacin B esters (**2a**−**c**)	>300	>300	>300	>300
leucopaxillone A (**3**)	>300	107.8	96.7	7.94
leucopaxillone B (**4**)	154.7	30.3	86.9	86.9

Table 2 shows the IC_{50} (the concentration required to inhibit 50% of cell growth) values of triterpenes **1**−**4** on proliferation of human lung carcinoma (A549), kidney carcinoma (CAKI 1), epatoblastoma (HepG2), and breast adenocarcinoma (MCF-7) cell lines, in a 1-day MTT assay.[16] In general, the four lines showed a different sensitivity toward the tested compounds, the MCF-7 and A549 lines exhibiting the lowest and highest resistance, respectively. Cucurbitacin B (**1**) showed a high activity on all lines, in accordance with previous investigations;[8,13,17−20] in particular, epatoblastoma and breast adenocarcinoma cells appear notably sensitive to growth inhibition. Leucopaxillone A (**3**), though less active, exhibited a specific growth inhibitory activity against the MCF-7 line. The significant diminution in activity observed for compounds **3** and **4** and the inactivity of esters **2a**−**c** confirmed earlier

Table 1. ^{13}C NMR and ^1H NMR Data ($CDCl_3$) for Compounds **3** and **4**

	leucopaxillone A (**3**)		leucopaxillone B (**4**)	
position	δ_C (multiplicity)[a]	δ_H multiplicity (*J* in Hz)	δ_C (multiplicity)[a]	δ_H multiplicity (*J* in Hz)
1	25.9 (CH_2)	α = 2.09 br β = 1.48−1.38 m	26.4 (CH_2)	1.93−1.79 and 1.72−1.66 m
2	38.7 (CH_2)	α = 2.61−2.52 m β = 2.43−2.34 m	38.6 (CH_2)	α = 2.60 m (11.6, 6.5) β = 2.35 m
3	215.1 (C)		214.9 (C)	
4	51.1[b] (C)		51.4 (C)	
5	142.9 (C)		143.4 (C)	
6	120.2 (CH)	5.67 br	119.5 (CH)	5.67 br d (4.2)
7	24.7 (CH_2)	α = 2.43−2.34 m β = 1.86−1.74 m	24.2 (CH_2)	α = 1.93−1.79 m β = 2.42 m
8	43.2 (CH)	1.86−1.74 m	44.8 (CH)	1.72−1.66 m
9	51.0[b] (C)		45.2 (C)	
10	38.5 (CH)	2.61−2.52 m	38.2 (CH)	2.66 br d (11.3)
11	32.6 (CH_2)	α = 1.48−1.38 m β = 1.86−1.74 m	110.9 (C)	
12	24.4 (CH_2)	α = 1.57−1.51 m β = 2.03 br	40.3 (CH_2)	α = 2.48d (12.1) β = 1.93−1.79 m
13	35.2 (C)		26.7 (C)	
14	49.2 (C)		49.4 (C)	
15	25.7 (CH_2)	α = 2.01 br β = 1.73−1.62 m	27.6 (CH_2)	2.54 br
16	34.2 (CH_2)	α = 1.00 br β = 1.32 dd (10.6, 12.5)	35.2 (CH_2)	1.40 br
17	47.6 (CH)	1.57−1.51 m	39.3 (CH)	1.93−1.79 m
18	61.6 (CH_2)	4.07 d (13.6), 3.53 d (13.6)	69.1 (CH_2)	3.90 and 3.66 d (8.5)
19	27.3 (CH_3)	0.89 s	17.0 (CH_3)	1.02 s
20	38.9 (CH)	2.11 br	41.4 (CH)	1.77 d (12.2)
21	14.0 (CH_3)	1.03 d (7.5)	13.2 (CH)	1.00 d (7.5)
22	72.4 (CH)	4.89 d (10.0)	71.8 (CH)	4.79 d (9.8)
23	26.4 (CH_2)	1.86−1.62 m	26.7 (CH_2)	1.93−1.79 and 1.56−1.46 m
24	75.8 (CH)	4.86 d (10.0)	75.6 (CH)	4.86 d (9.7)
25	72.5 (C)		72.5 (C)	
26	28.8[c] (CH_3)	1.22[b] s	27.3[e] (CH_3)	1.22[h] s
27	25.2[c] (CH_3)	1.21[b] s	25.5[e] (CH_3)	1.21[h] s
28	19.5 (CH_3)	0.89 s	19.6 (CH_3)	0.86 s
29	27.2 (CH_3)	1.20 s	28.7 (CH_3)	1.26 s
30	22.8 (CH_3)	1.25 s	23.2 (CH_3)	1.24 s
1′	171.1 (C)		171.0[f] (C)	
2′	21.5[d] (CH_3)	2.09[c] s	21.5[g] (CH_3)	2.07[i] s
1″	171.1 (C)		170.9[f] (C)	
2″	21.0[d] (CH_3)	2.03[c] s	21.1[g] (CH_3)	2.04[i] s
OH		1.14 br		1.56−1.46 br

[a] Multiplicities were determined by DEPT and HSQC. [b−i] These values can be interchanged.

studies that have demonstrated the importance for cucurbitacin bioactivity of a highly electrophilic conjugated ketone in the side chain and of a free C-16 hydroxyl group.[10] A likely explanation for these structural requirements is a hydrogen-bonding interaction between the two groups, which could activate the α,β-unsaturated ketone toward nucleophilic attack by a biological macromolecule.

Leucopaxillones are novel cucurbitane triterpenes with the structural novelty residing in the oxidation of C-18 and in the side chain; moreover, compound **4** exhibits an additional tetrahydrofuran hemiacetal ring. Cucurbitacin B (**1**), instead, has been isolated from numerous genera of plants,[4−8,19−23] though its existence in the fungal kingdom has been unreported. On the other hand, cucurbitacin B derivatives **2a**−**c** are the first examples of cucurbitacin long chain fatty acid esters found in nature, the acetate being the most frequently encountered ester at C-16. Cucurbitacins are highly oxidized triterpenes encountered in several plant families, mainly in the Cucurbitaceae; in contrast, they occur quite rarely in higher fungi. In fact, so far they have been isolated only from *Hebeloma vinosophyllum*[24] and *Russula lepida*,[25,26] two unrelated fungal species. Interestingly, the cucurbitane derivatives of the latter two species are structurally quite different from those (**1**−**4**) of *L. gentianeus*.

L. gentianeus is an ectomycorrhizal mushroom, widely distributed over Europe and Northern and Central America. Its strong bitterness is a characteristic permanent feature, independent of the growth place. *L. gentianeus* is associated with Pinaceae (*Pinus*) and Fagaceae (*Quercus*) plants, and it has never been found under different host trees or cucurbitacin-producing plants. This observation points to an endogenous biosynthesis of cucurbitacins **1** and **2** inside the fungal cells.

It is interesting to note that, in living organisms, some secondary metabolites are found only in one species or in a few strictly related taxa: these compounds can be useful chemotaxonomic markers, as they often indicate an evolutionary relationship. On the other hand, some metabolites are scattered in organisms that bear no taxonomic similarity. This evolutionary convergence is often observed for compounds playing some specific activity in nature: in this case the metabolites arise independently in nonrelated species, probably under the pressure of environmental mediated stimuli. Cucurbitacin B should belong to the latter category: it might represent an interesting example of secondary metabolites' convergence between fungi and vascular plants, where it likely exerts a similar ecological role (the intensely bitter cucurbitacins are known to be toxic to a range of herbivores and to protect plant tissues from grazing animals).[21,25] However, nothing is known on the presence of cucurbitacins in different species, belonging either to the same genus (*Leucopaxillus*) or to the same family (Tricholomataceae) or even to the same class (Basidiomycetes) as *L. gentianeus*. Various bitter mushrooms have been investigated, but the molecules responsible for their bitterness are completely different from cucurbitacin B; see for instance the β-carboline alkaloids of *Cortinarius infractus*.[27]

Cucurbitacin B (**1**) is highly bitter, while **2a**−**c** and **3** and **4** are tasteless. We presume, therefore, that a defense mechanism, based on compound **1** as a chemical deterrent, has also developed in the fruiting bodies of *L. gentianeus*, which are thus protected against parasites and predators. In addition, it is likely that compounds **2a**−**c** constitute the "inactive forms" that are rapidly transformed by enzymes into the "active compound" **1** after injury to the mushroom. Indeed, the way by which the bioactive metabolite **1** is "stored" in the tissues as inactive fatty acid ester (**2a**−**c**) is not unprecedented in the fungal kingdom; in particular, inactive fatty acid esters of different terpenoids are characteristic constituents of the chemical defense machinery endowed to most species of the family Russulaceae.[28]

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns hot-stage apparatus and are uncorrected; optical rotations were determined on a Perkin-Elmer 241 polarimeter; UV (in MeOH) and IR (neat or in mini KBr disks) spectra were recorded on a Kontron UVIKON 941 and an FT-IR Perkin-Elmer Paragon 1000 PC spectrometer, respectively; CD spectra were recorded on a Jasco J-710 spectropolarimeter, employing 0.1 cm optical path cuvettes; ^{1}H and ^{13}C NMR spectra ($CDCl_3$) were determined on a Bruker CXP 300 spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C), respectively. ^{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]; the abbreviations s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad are used throughout; coupling constants (*J*) are reported in Hz. EIMS and HREIMS were recorded using a Finnigan MAT 8222 spectrometer, with ionization being induced by electron impact at 70 eV. LC-MS and LC-MS/MS data were obtained using an LCQ DECA ion trap mass spectrometer equipped with an electrospray ionization (ESI) ion source and controlled by Xcalibur software 1.3 (Thermo-Finnigan, San Jose, CA). ESI experiments were carried out in positive ion mode under constant instrumental conditions: source voltage 4.5 kV, capillary voltage 15 V, sheet gas flow 60 (arbitrary units), auxiliary gas flow 15 (arbitrary units), capillary temperature 250 °C, tube lens voltage 15 V.

The system was run in automated LC-MS/MS mode and the HPLC used was a Surveyor LC system (Thermo Finnigan, San Jose, CA). Thin-layer chromatography was performed on silica gel 60 F_{254} Al sheets (Merck) and RP-18 HPTLC F_{254} glass-backed plates (Merck). Compounds were visualized under UV light (254 and 366 nm) and by spraying with a 0.5% vanillin solution in H_2SO_4/EtOH (4:1) followed by heating. Flash column chromatography was performed with Merck Kieselgel 60 (40−63 μm) and Merck LiChroprep RP-18 (25−40 μm). HPLC experiments were performed on a Jasco instrument, equipped with a high-pressure gradient unit, and an UV multiwavelength detector. GC capillary analysis was performed with an HRGC-5160 Mega Series Carlo Erba apparatus (SupelCowax TM10 capillary column, 30 m long, 0.32 mm i.d., 0.25 μm film thickness) in the following conditions: injection "on column", FID detector at 300 °C; H_2 as a carrier gas at 0.3 kg/cm^2 pressure; temperature of the column oven increased from 90 to 180 °C at 40 °C per min, then from 180 to 270 °C at 5 °C per min, finally kept at 270 °C for 20 min. GC−MS analysis was performed with a Finnigan-MAT ITS-40 instrument equipped with a DB-5MS capillary column, 30 m long, 0.25 mm i.d., 0.25 μm film thickness. Chromatographic conditions: injector 250°, detector 270 °C; temperature of the column oven increased from 40 to 300 °C at 8 °C per min, then kept at 300 °C for 20 min; He as a carrier gas. An authentic sample of cucurbitacin B (**1**) was purchased from Apin Chemicals Ltd. (UK). Standard fatty acids were purchased from Sigma and were methylated with diazomethane in Et_2O. Phosphate buffered saline (PBS) solution and MTT salt were supplied by Sigma.

Fungal Material, Extraction, and Isolation. Fruiting bodies of *Leucopaxillus gentianeus* were collected in December 2002 near Cecina (prov. of Livorno). The mushrooms were identified by one of the authors (M.C.). A voucher specimen (accession number MUT 5317) is deposited at the Mycotheca Universitatis Taurinensis (Turin, Italy).

The fruiting bodies (a total of approximately 1.5 kg) were frozen at −20 °C immediately after collection; a few weeks

later, they were minced without defrosting and extracted with EtOAc (3 L), slowly allowing the mixture to warm to 20 °C, under stirring for about 4 h. The resulting material was filtered, and the aqueous phase was separated; the organic layer was dried with Na_2SO_4 and evaporated in vacuo to yield about 9 g of crude extract. The latter was partitioned between 95% aqueous MeOH and hexane to give approximately 4.5 g of each subextract. The hexane subextract was fractionated by means of an MPLC preparative apparatus (Jobin-Yvon), using a silica gel column eluted with hexane/EtOAc mixtures ranging from 9:1 to 1:1. Twelve macrofractions were obtained (A_1−A_{12}). Cucurbitacin B (**1**) (16 mg) was isolated from fraction A_{11}, and the esters **2a**−**c** (35 mg) from fraction A_8, after repetitive separations on RP-18 columns employing different MeCN/H_2O mixtures as solvent systems. The MeOH subextract was similarly separated on a preparative silica gel column eluted with toluene/acetone mixtures, ranging from 5:1 to 1:1. Ten fractions were obtained (B_1−B_{10}). Leucopaxillone A (**3**) (70 mg) was obtained from fraction B_7, and leucopaxillone B (**4**) (29 mg) from fraction B_8, after extensive RP-18 chromatography (MeCN/MeOH/H_2O mixtures in different percentages).

Cucurbitacin B (1) exhibited ^{1}H NMR and ^{13}C NMR spectra identical with those reported[5,8] and of an authentic sample. Moreover, compound **1** and authentic cucurbitacin B were identical by LC-ESI-MS analysis. Analytical column: Alltech Alltima RP-18, 250 × 4.6 mm, 5 μm; eluent: H_2O (A)−MeCN/MeOH, 1:1 (B), from 55% A to 25% A in 35 min; flow rate: 0.9 mL/min; UV detector positioned at 240 nm. Retention time of cucurbitacin B (**1**) under these chromatographic conditions: 28.6 min. ESI-MS molecular ion cluster peaks at *m/z* 581.3 $[M + Na]^+$, 576.1 $[M + NH_4]^+$, 558.8 $[M + H]^+$, 499.2 $[M + H - AcOH]^+$.

Cucurbitacin B esters 2a−c: chromatographically inseparable oily mixture; $[\alpha]_D^{22}$ −1.5° (*c* 0.3, CH_2Cl_2); IR (thin film) ν_{max} 3455, 2926, 2838, 1732, 1694 (sh), 1625, 1368, 1248, 1227 cm^{-1}; ^{1}H NMR ($CDCl_3$) 7.12 (1H, d, *J* = 16.0 Hz, H-24), 6.42 (1H, d, *J* = 16.0 Hz, H-23), 5.78 (1H, m, H-6), 5.45−5.30 (m, olefinic protons of linoleate and oleate moieties), 5.19 (1H, br t, *J* = 7.7 Hz, H-16), 4.42 (1H, dd, *J* = 6.0, 12.6 Hz, H-2), 4.30 (1H, s, OH), 3.62 (1H, br, OH), 3.26 (1H, br d, *J* = 14.5 Hz, H-12a), 2.8−2.6 (m, H-10, H-12b, H-17, and H_2-11′ of linoleate), 2.4−2.2 (3H, m, H-1a and H_2-2′ of fatty acid esters), 2.05 (m, allylic protons of linoleate and oleate moieties), 2.03 (3H, s, acetate C*H*$_3$), 2.02−1.85 (3H, m, H-7a, H-8, and H-15a), 1.58 (6H, s, H_3-26 and H_3-27), 1.60−1.35 (4H, m, H-7b, H-15b, and H_2-3′ of fatty acid esters), 1.42 (3H, s, H_3-21), 1.35−1.20 (m, H-1b and methylene protons of fatty acid ester chains), 1.33 (3H, s, H_3-28 or H_3-29), 1.30 (3H, s, H_3-30), 1.27 (3H, s, H_3-29 or H_3-28), 1.09 (3H, s, H_3-19), 1.03 (3H, H_3-18), 0.87 (3H, br t, *J* = 6.5 Hz, terminal methyl protons of fatty acid esters); ^{13}C NMR ($CDCl_3$) 212.9 (C, C-3), 211.6 (C, C-11), 200.8 (C, C-22), 173.1 (C, fatty ester CO), 169.5 (C, acetate CO), 152.4 (CH, C-24), 140.4 (C, C-5), 130.3 (CH, C-13 linoleate), 130.1 (CH, C-10 oleate), 130.2 (CH, C-9 linoleate), 129.6 (CH, C-9 oleate), 128.1 (CH, C-10 linoleate), 127.8 (CH, C-12 linoleate), 120.3 (CH, C-6), 119.3 (CH, C-23), 79.1 (C, C-25), 77.6 (C, C-20), 73.3 (CH, C-16), 71.6 (CH, C-2), 54.1 (CH, C-17), 50.2 (C, C-4), 49.9 (C, C-13), 48.5 (CH_2, C-12), 48.3 (C, C-9), 47.9 (C, C-14), 43.2 (CH_2, C-15), 42.0 (CH, C-8), 35.9 (CH_2, C-1), 33.8 (CH_2, C-2 fatty ester), 33.7 (CH, C-10), 31.9 (CH_2, C-16 fatty ester), 29.7, 29.5, 29.3, 29.2, 29.1 ($(CH_2)_n$ fatty ester), 29.3 (CH_3, C-28 or C-29), (27.2 (allylic CH_2 fatty ester), 26.4 (CH_3, C-26 or C-27), 26.3 (CH_3, C-27 or 26), 25.6 (CH_2, C-11 linoleate), 24.6 (CH_2, C-3 fatty ester), 23.7 (CH_2, C-7), 23.6 (CH_3, C-21), 22.7 (CH_2, C-17 fatty ester), 21.9 (CH_3, acetate), 21.2 (CH_3, C-29 or C-28), 20.0 (CH_3, C-19), 19.7 (CH_3, C-18), 18.7 (CH_3, C-30), 14.1 (CH_3, fatty ester); EIMS *m/z* 822 $[M]^+$ of **2a** (4), 762 $[M - CH_3COOH]^+$ (54), 667 (35), 652 (22), 481 (100), 480 (79), 464 (45); HREIMS *m/z* 762.5451 $[M - CH_3COOH]^+$ of **2a** (calcd for $C_{48}H_{74}O_7$, 762.5435).

Leucopaxillone A (3): colorless crystals (CH_3CN/2-propanol, 1:1); mp 203−207 °C; $[\alpha]_D^{22}$ +24° (*c* 0.3, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 267 (3.66) nm; IR (KBr) ν_{max} 3444 (OH), 2931, 1731 (C=O acetate), 1714 (C=O ketone), 1372, 1253, 1027 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\epsilon$) 292 (−1.04) nm; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) data, see Table 1; EIMS *m/z* 574 $[M]^+$ (7), 544 (5), 499 (6), 484 (17), 456 (9), 423 (15), 405 (14), 356 (15), 298 (61), 283 (27), 187 (48), 149 (54), 97 (55), 44 (100); HREIMS *m/z* 574.3865 (calcd for $C_{34}H_{54}O_7$, 574.3870).

Leucopaxillone B (4): colorless oil; $[\alpha]_D^{22}$ +56° (*c* 0.4, CH_2Cl_2); IR (thin film) ν_{max} 3460 (OH), 2973, 1731 (C=O acetate), 1715 (C=O ketone), 1259 cm^{-1}; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) data, see Table 1; EIMS *m/z* 588 $[M]^+$ (3), 570 (4), 528 (3), 510 (4), 450 (4), 356 (13), 265 (8), 147 (48), 32 (100); HREIMS *m/z* 588.3669 (calcd for $C_{34}H_{52}O_8$, 588.3662).

Reaction of Esters 2a−c with NaOH (or LiOH). Cucurbitacin B esters **2a**−**c** (25 mg) were dissolved in THF (1.5 mL) and 0.5 mL of aqueous 4 N NaOH (or LiOH) was added. The solution was kept at 65 °C under stirring for 2 h. After this time, the reaction mixture was quenched with diluted HCl and extracted with Et_2O. The organic solution was dried over Na_2SO_4 and evaporated under vacuo, to yield 10 mg of cucurbitacin E esters **5a**−**c** as a colorless oil: ^{1}H NMR ($CDCl_3$) 7.14 (1H, d, *J* = 15.5 Hz, H-24), 6.41 (1H, d, *J* = 15.5 Hz, H-23), 5.95 (1H, d, *J* = 2.3 Hz, H-1), 5.90 (1H, s, enol OH), 5.76 (1H, m, H-6), 5.40−5.30 (m, olefinic protons of linoleate and oleate moieties), 5.21 (1H, dd, *J* = 7.5, 8.4 Hz, H-16), 4.28 (1H, s, OH), 3.49 (1H, br s, H-10), 3.20 (1H, d, *J* = 14.0 Hz, H-12a), 2.75 (1H, d, *J* = 14.0 Hz, H-12b), 2.68 (1H, d, *J* = 8.4 Hz, H-17), 2.5−2.2 (2H, m, H_2-2′ of fatty acid esters), 2.10−1.80 (m, H-8, H-7a, H-15a, and allylic protons of linoleate and oleate moieties), 2.04 (3H, s, acetate C*H*$_3$), 1.56 (6H, s, H_3-26 and H_3-27), 1.60−1.30 (4H, m, H-7b, H-15b, and H_2-3′ of fatty acid esters), 1.40 (3H, s, H_3-21), 1.35−1.20 (m, methylene protons of fatty acid ester chains), 1.34 (3H, s, H_3-28 or H_3-29), 1.30 (3H, s, H_3-30), 1.27 (3H, s, H_3-29 or H_3-28), 1.05 (6H, s, H_3-18 and H_3-19), 0.90 (3H, br t, *J* = 6.5 Hz, terminal methyl protons of fatty acid esters); EIMS *m/z* 760 $[M - CH_3COOH]^+$ of **5a** (5), 663 (12), 648 (15), 598 (6), 531 (10), 481 (70), 479 (75), 383 (100); HREIMS *m/z* 760.5281 $[M - CH_3COOH]^+$ of **5a** (calcd for $C_{48}H_{72}O_7$, 760.5278).

Methanolysis of Esters 2a−c. A 0.25 mL sample of a NaOMe solution (10% in MeOH, freshly prepared from MeOH and Na) was added to a MeOH solution of **2a**−**c** (20 mg in 1.0 mL of MeOH). The solution was kept at 35 °C under stirring until a TLC analysis showed the disappearance of the starting ester (about 45 min). The reaction was then quenched with diluted HCl and extracted with Et_2O. After solvent evaporation, the resulting mixture was loaded on a silica gel column, eluted with hexane/EtOAc mixtures. This allowed the complete separation of the fatty acid methyl esters from the mixture of cucurbitacin derivatives which could not be further separated. The fatty acid methyl esters fraction (IR band at 1735 cm^{-1}) was analyzed by GC and GC−MS, and individual components (methyl oleate and linoleate in a ratio of 85:15, accompanied by ≤1% methyl palmitate) were identified by peak enrichment and comparison of the mass spectra with those of authentic reference compounds and literature mass spectra.[14] Relative abundances were estimated by integration of the peak areas.

X-ray Crystal Structure Determination. Intensity data were obtained at room temperature on an Enraf-Nonius CAD4 diffractometer, using graphite-monochromatized Mo Kα radiation (λ = 0.71073 Å). Unit-cell parameters were determined by least-squares fitting of 25 centered reflections. Calculations were performed with the WinGX-97 software.[29] The intensities were corrected for *Lp* and absorption.[30] The structure was solved by direct methods (SIR92)[31] and refined on F^2 by full-matrix least-squares using SHELXL-97.[32] All non-H atoms were refined with anisotropic displacement parameters, while hydrogen atoms were placed in calculated positions with isotropic thermal parameters proportional to those of their neighboring atoms. One 2-propanol molecule, cocrystallized with **3**, is present in this crystal structure. Its unusually high displacement parameters indicate partial disorder. Any attempts of refining alternative positions for these atoms were unsuccessful. As there were no heavy atoms, the absolute configuration could not be determined from the Friedel pairs.

The crystal used for this study showed very low diffraction due to its small size. Data collection was then performed over

a restricted range of θ (2−25°). No improvement to the resolution of the model would in fact have been obtained by increasing the θ range because most of the reflections would have been weak. The number of observed reflections/number of parameters ratio is quite low. Anyway no chemically unacceptable bond length or angle was found and the final difference Fourier map was featureless, thus confirming the reliability of the model. The geometry of compound **3** as revealed by the crystallographic study was unexceptional. Both the six-membered saturated rings take a *chair* conformation [apex−apex distances: C3−C10 2.983 Å; C9−C13 3.043 Å]. It is worth noting that ring A shows a slight positional disorder at the most peripheral C3 position. This results also in a disorder of the O1 oxygen atom along the axis normal to the C1−C2−C4−C5 plane, as evidenced by the U11 component of its displacement parameter. Similar positional disorder is also shown by the O6 atom. In both cases any attempt of refining two alternative positions was unsuccessful.

The crystal packing is maintained by van der Waals forces and hydrogen bonds: O2···O6 2.811(13) Å; H2···O6 2.225(9) Å; O2−H2···O6 128.7(5)°; O5···O1 2.993 Å; H5···O1 2.239(13) Å; O5−H5···O1 153.1(8)°.

Atomic scattering factors were taken from *International Tables for X-ray Crystallography*.[33] Diagrams of the molecular structures were produced by the ORTEP program.[34]

Crystal Data:[35] $C_{34}H_{54}O_7 \cdot C_3H_8O$, fw = 634.87; monoclinic, space group $P2_1$, a = 15.189(5) Å, b = 7.530(3) Å, c = 15.593-(5) Å, β = 95.72(3)°, V = 1774.6(12) Å^3, Z = 2, D_c (Z = 2) = 1.188 g cm^{-3}, μ_{Mo} = 0.082 mm^{-1}; specimen 0.35 × 0.32 × 0.07 mm. Data were measured at T = 293(3) K, with Mo Kα (λ = 0.71073 Å) graphite-monochromatized radiation, using $\omega-2\theta$ scan. A total of 5406 reflections were collected in the range 2−25° θ ($-18 < h < 18$, $-8 < k < 8$, $-18 < l < 18$). During data collection three standard reflections were measured every 300 reflections. Convergence was obtained for 416 parameters at R_1 = 6.07%, calculated on 1362 reflections with $I > 2\sigma_I$, R_{all} = 21.80% for 3375 independent reflections, and GOF = 0.956. Extinction coefficient = 0.0065(16), largest peak and hole in the ΔF maps 0.182 and −0.215 e$^-$ Å^{-3}.

Cancer Cell Growth Inhibitory Bioassay. The in vitro inhibitory effects of compounds **1**−**4** on proliferation of human lung carcinoma (A549), epatoblastoma (HepG2), breast adenocarcinoma (MCF-7), and kidney carcinoma (CAKI 1) cell lines were measured with MTT, essentially as described.[16] Human tumor cells were purchased from the American Type Culture Collection (ATCC) and maintained at 37 °C, in a humidified incubator containing 5% CO_2, as adherent cell cultures in the appropriate medium: A549 in F12K medium supplemented with 10% fetal bovine serum (FBS); HepG2 and MCF-7 in MEM medium supplemented with 1 mM sodium pyruvate and 10% FBS; CAKI 1 in Mc Coy's medium supplemented with 10% FBS. Cells were counted, transferred into 96-well microtiter plates, and incubated in the corresponding medium supplemented with 10% FBS for 24 h prior to the addition of test compounds. Test compounds were solubilized in DMSO (100 μL each) and diluted with media, without FBS, to the desired concentration. To microtiter wells emptied of original media, compounds were then added and incubated for 24 h. The final concentration of DMSO (0.25%) showed no interference with the growth of the cell lines. Supernatant was removed from wells, and the cells were washed with PBS solution (pH 7.2, 150 μL/well). A MTT solution (150 μL/well) at 0.5 mg/mL in MEM medium without Phenol Red was added to cells, which were then incubated for 3 h at 37 °C. Excess tetrazolium salt was removed, and precipitated reduced formazan salt was solubilized with DMSO (150 μL/well). Absorbances of the resulting solutions were read at 540 nm on an automated microplate reader (Tecan Rainbow Model). Each experiment was repeated twice in triplicate.

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Supporting Information Available: X-ray crystallographic tables of atomic coordinates, bond lengths and angles, and anisotropic thermal parameters for leucopaxillone A (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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(35) Crystallographic data for the structure of leucopaxillone A (**3**) have been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 235137. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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