Triterpenes from Pholiota populnea as Cytotoxic Agents and Chemosensitizers to Overcome Multidrug Resistance of Cancer Cells

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ABSTRACT: The detailed mycochemical analysis of the n-hexane extract of Pholiota populnea led to the isolation of four new lanostane diesters, named pholiols A–D (1–4), together with an acyclic triterpene, (3S,6E,10E,14E,18E,22S)-2,3,22,23-tetrahydroxy-6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene (5), ergosterol (6), and 3/β-hydroxyergosta-7,22-diene (7). The isolation was carried out by multistep flash chromatography, and the structures were elucidated using extensive spectroscopic analyses, including 1D and 2D NMR and MS measurements. The isolated metabolites (1–6) were investigated for cytotoxic activity against Colo205 and Colo320 colon adenocarcinoma and nontumoral MRC-5 cell lines. Among the tested compounds, ergosterol (6) showed substantial cytotoxic activity against all cell lines with IC_{50} values of 4.9 μM (Colo 205), 6.5 μM (Colo 320), and 0.50 μM (MRC) with no tumor cell selectivity. A P-glycoprotein efflux pump modulatory test on resistant Colo320 cells revealed that pholiols A (1) and B (2) and linear triterpene polyol 5 have the capacity to inhibit the efflux-pump overexpressed in the cells. Moreover, the drug interactions of triterpenes with doxorubicin were studied by the checkerboard method on Colo 320 cells. Pholiols B (2) and D (4) interacted in synergistic and acyclic triterpene 5 in a very strong synergistic manner; the combination index (CI) values at 50% of the growth inhibition dose (ED_{50}) were found to be 0.348, 0.660, and 0.082, respectively. Our results indicate that P. populnea is a promising source for finding new triterpenes with significant chemosensitizing activity on cancer cells.

Cancer is among the leading causes of morbidity and mortality worldwide. According to Global Cancer Statistics, an estimated 19.3 million new cancer cases, among them 1.148 million new colon cancer instances (representing 6.0% of all cases), were registered worldwide in 2020. A major problem in clinical cancer therapy is the multidrug resistance (MDR) toward cytotoxic drugs. MDR is associated with cellular pharmacokinetic alterations, such as decreased drug accumulation, increased efflux and detoxification capacity, and subcellular redistribution. One of the most widespread mechanisms of resistance involves the efflux of drug molecules out of the cells. In particular, ATP-binding cassette (ABC) transporters have received great interest; one such transporter that has been investigated in detail is P-glycoprotein (P-gp). Triterpenoids were reported to be able to reverse cell resistance to chemotherapy due to P-gp inhibition. Ginsenoside Rh2, isolated from red ginseng, exerts such activity in doxorubicin-resistant human breast cancer MCF-7 cells. Oleanolic acid was reported to be an inhibitor of certain efflux transporters, including P-gp, and thus was found to increase the intracellular concentration of paclitaxel. Maslinic acid, a natural triterpene from Olea europaea L., has attracted increasing interest in recent years because of its promising anticancer activity and dose-dependent enhancing potency of docetaxel (DOC) sensitivity and cellular drug accumulation in MDA-MB-231/DOC cells in a combination treatment. The tetracyclic triterpene alcohol euphol was reported to have cytotoxicity (IC_{50} < 10 μM) against 27 cell lines when screened against a panel of 71 human cancer cells from 15 tumor types. This compound also exhibited antitumoral and antiangiogenic activity in vivo, with synergistic temozolomide interactions in most cell lines. The pentacyclic terpenoid boswellic acid was studied in combination with doxorubicin for the antitumor effects against solid tumors of Ehrlich’s ascites carcinoma grown in mice. The results showed that boswellic acid synergized the antitumor activity of doxorubicin. The fungal metabolite ergosta-7,22-diene-3-one was found to be effective against sensitive (Colo 205) and resistant (Colo 320) human colon adenocarcinoma cells with IC_{50} values 11.6 ± 1.7 μM and 8.4 ± 1.1 μM, respectively, and in a combination assay...
synergism was detected between ergosta-7,22-diene-3-one and doxorubicin with CI index 0.521 ± 0.15 at the 50% growth inhibition dose (ED$_{50}$).8

Hemipholiota populnea (Pers.) Kuyper & Tjall.-Beuk. (syn. Pholiota destruens (Brond.) Quel., Hemipholiota populnea) is member of the Strophariaceae family, distributed worldwide wherever cottonwood occurs. This mushroom species is usually saprophytic, but sometimes parasitic, and grows on broad-leaved woods, mainly on various poplars, but also on willow and birch, playing an important role in decomposing the deadwood of cottonwoods. The chemistry and pharmacology of P. populnea were poorly studied previously, with only pholiotic acid, 3,5-dichloro-4-methoxybenzaldehyde, and 3,5-dichloro-4-methoxybenzyl alcohol, with weak antifungal and pholicotic activity of this species was reported.10

In the current study, the detailed mycochemical analysis of a methanolic extract of H. populnea, isolation, structure determination, and pharmacological evaluation of its chemical compounds are highlighted. The present paper reports four new lanostane diesters (1–4), one acyclic triterpene tetraol (5), and the known compounds ergosterol (6) and 3β-hydroxyergosta-7,22-diene (7). The isolated compounds 1–6 were investigated for cytotoxic activity against human colon adenocarcinoma cells (Colo205, Colo320) and the MRC-5 cell line. The combination with doxorubicin and efflux pump inhibitory activity of the compounds on drug-resistant Colo 320 cells were also assayed. In addition, antimicrobial activity against different bacterial strains was evaluated by the microdilution method.

**RESULTS AND DISCUSSION**

Four lanostane triterpenes (1–4) and an acyclic triterpene tetraol (5) were isolated from the n-hexane-soluble phase of the MeOH extract prepared from the mushroom P. populnea by a combination of multiple flash chromatography steps. The structure elucidation was carried out by extensive spectroscopic analysis, including 1D and 2D NMR ($^1$H−$^1$H COSY, HSQC, HMBC, and ROESY) and HREIMS experiments.

Based on the HREIMS data, the molecular formulas of compounds 1 and 2 were C$_{38}$H$_{58}$O$_{10}$ and C$_{39}$H$_{60}$O$_{10}$, respectively, differing only in a methylene group. The $^1$H and $^{13}$C NMR spectra of 1 and 2 (Tables 1 and 2) were similar except for a singlet signal with three-proton intensity at δ$_C$ 3.71 appearing in the $^1$H NMR spectrum and the extra resonance at δ$_C$ 51.7 in the $^{13}$C NMR spectrum of 2. These findings suggested that 2 was the O-methyl derivative of 1. In accordance with the elemental compositions, the $^{13}$C NMR spectra presented 38 and 39 resonances in compounds 1 and 2, respectively. Based on the HSQC spectra, besides the additional methoxy group in 2, 10 methyl, 10 methylene, and 5 methine groups, and 13 nonprotonated carbons were commonly present in the compounds. Considering the elemental compositions and the chemical shift values of the nonprotonated carbons, two carbonyl groups (δ$_C$ 197.9/197.9 and δ$_C$ 173.7/172.1, 170.8/171.2, and 171.4/170.6), an unsaturation (δ$_C$ 139.2/139.1 and δ$_C$ 163.1/163.0), and two sp$^3$ carbons attached to oxygens (δ$_C$ 76.2/76.2 and 69.6/69.5) were present in 1 and 2. The remaining four signals (δ$_C$ 39.3/39.2, 40.5/40.5, 44.9/44.9, and 47.8/47.8 for 1/2) could be ascribed to sp$^3$ nonprotonated carbons. Based on the characteristic HMBC correlations of the angular methyl singlets [H-18 with C-12, C-13, C-14, and C-17; H-19 with C-1, C-5, C-9, and C-10; H-21 with C-17, C-20, and C-22; H-29 and H-30 with C-3, C-4, and C-5], it was concluded that 1 and 2 are based on a lanostane skeleton. The HMBC correlations of the O-methine doublets at δ$_H$ 4.85/4.83 with C-1, C-4, C-29, and C-30 confirmed their assignment to H-3 in 1/2, while HMBC cross-peaks of the O-methine groups at δ$_C$ 73.0/69.9 (C-2) with protons at δ$_H$ 4.85/4.83 (H-3) suggested that the lanosterol skeleton bears oxygen-containing substituents at both C-2 and C-3. The HMBC correlations of the acetyl CO signal with H-3 and the acetyl methyl signal suggested the presence of an acetate group at C-3, while, based on the HMBC correlations of H-2/C-1′, H-2′/C-3′, H-2′/C-4′, H-2′/C-5′, H-4′/C-5′, and H-2′/C-6′, a 3-hydroxy-3-methylglutarate moiety was attached to C-2 in compounds 1 and 2. The additional H-7′/C-6′ HMBC correlation in compound 2 suggested that 2 was the methyl ester of compound 1. Further to these, the HMBC correlations of the doublet of doublets at δ$_H$ 1.88/1.85 (assigned to H-5) with C-4, C-10, C-19, C-29, and C-30 confirmed this assignment, while long-range correlations of H-5 with the methylene at δ$_C$ 36.2/36.2 and with the carbonyl at δ$_C$ 197.8/197.8 enabled the assignment of C-6 and suggested the presence of a keto group in position 7. In parallel, the HMBC correlations between the diastereotopic protons (H-22) and the methylene group at δ$_C$ 32.6/32.5 (C-23) and carbonyl group at δ$_C$ 214.9/214.8 (C-24) and between the methyl singlets of H-26 and H-27 and C-24 and nonprotonated carbon at δ$_C$ 76.2/76.2 (C-25) led to the conclusion that a 24-keto-25-hydroxy side chain was present at C-17. Based on the complete $^1$H and $^{13}$C NMR assignments.
Table 1. $^1$H NMR Data (800 MHz) of Compounds 1–4 in CDCl₃

| atom# | $\delta_H$ (Hz) | $J_{HH}$ (Hz) |
|-------|-----------------|---------------|
| 1β    | 2.25, dd (12.2, 4.1) | 2.22, dd (12.3, 4.3) |
| 1α    | 1.57, t (12.2) | 1.54, t (12.3) |
| 2     | 5.22, td (10.8, 4.1) | 5.20, td (10.8, 4.3) |
| 3     | 4.85, d (10.8) | 4.83, d (10.8) |
| 5     | 1.88, dd (13.3, 4.0) | 1.85, dd (13.3, 4.0) |
| 6β    | 2.46, m (2H) | 2.44, m (2H) |
| 6α    | 1.70, m | 1.72, m |
| 7     | 2.06, m | 2.06, m |
| 11β   | 2.31, m (2H) | 2.30, m (2H) |
| 11α   | 1.81, m | 1.79, m |
| 15β   | 1.75, m | 1.74, m |
| 15α   | 2.10, m | 2.07, m |
| 16β   | 1.41, m | 1.40, m |
| 16α   | 2.01, m | 2.01, m |
| 17    | 1.47, m | 1.44, m |
| 18    | 0.67, s | 0.65, s |
| 19    | 1.32, s | 1.30, m |
| 20    | 1.43, m | 1.40, m |
| 21    | 0.93, d (6.6) | 0.92, d (6.6) |
| 22    | 1.85, m | 1.83, m |
| 23    | 1.33, m | 1.31, m |
| 24    | 2.59, m | 2.57, ddd (17.1, 9.8, 5.1) |
| 25    | 2.51, m | 2.49, ddd (17.1, 9.3, 6.1) |
| 26    | 1.41, s | 1.38, s |
| 27    | 1.41, s | 1.39, s |
| 28    | 0.94, s | 0.92, m |
| 29    | 1.03, s | 1.00, s |
| 30    | 0.93, s | 0.91, m |
| 2′    | 2.57, m | 2.54, AB (15.4) |
| 2′    | 2.70, m | 2.68, AB (15.4) |
| 4′    | 1.40, s | 1.35, s |
| 5′a   | 2.55, br m | 2.62, AB (15.4) |
| 5′b   | 2.72, br m | 2.71, AB (15.4) |
| 7′    | 3.71, s | 3.73, s |
| 3′OAc | 2.09, s | 2.07, s |
| 25′-OH | 3.82, s | 3.83, s |
| 3′-OH | 3.91, s | 3.96, s |

Table 2. $^{13}$C NMR Data (200 MHz) of Compounds 1–4 in CDCl₃

| atom# | $\delta_C$ (Hz) | type |
|-------|-----------------|------|
| 1     | 40.1, CH₂ | 40.1, CH₁ |
| 2     | 70.3, CH₂ | 69.9, CH₁ |
| 3     | 79.0, CH₂ | 78.9, CH₁ |
| 5     | 49.3, CH₂ | 49.3, CH₁ |
| 6     | 36.2, CH₂ | 36.2, CH₁ |
| 7     | 197.8, C | 197.8, CH₂ |
| 8     | 139.2, C | 139.1, CH₂ |
| 9     | 163.1, C | 163.0, CH₂ |
| 10    | 40.5, C | 40.5, CH₂ |
| 11    | 23.8, CH₂ | 23.8, CH₂ |
| 12    | 29.9, CH₂ | 29.9, CH₂ |
| 13    | 44.9, C | 44.9, C |
| 14    | 47.8, C | 47.8, C |
| 15    | 31.8, CH₂ | 31.8, CH₂ |
| 16    | 28.6, CH₂ | 28.6, CH₂ |
| 17    | 48.9, CH | 48.9, CH |
| 18    | 15.8, CH₃ | 15.7, CH₃ |
| 19    | 19.4, CH₃ | 19.4, CH₃ |
| 20    | 35.9, CH | 35.9, CH |
| 21    | 18.5, CH₃ | 18.5, CH₃ |
| 22    | 30.1, CH₂ | 30.0, CH₂ |
| 23    | 32.6, CH₂ | 32.5, CH₂ |
| 24    | 214.9, C | 214.8, C |
| 25    | 76.2, C | 76.2, C |
| 26    | 26.5, CH₂ | 26.6, CH₁ |
| 27    | 26.5, CH₂ | 26.5, CH₁ |
| 28    | 25.0, CH₂ | 25.0, CH₁ |
| 29    | 17.3, CH₂ | 17.2, CH₂ |
| 30    | 27.6, CH₂ | 27.6, CH₂ |
| 1′    | 170.8, C | 171.2, C |
| 2′    | 44.9, CH₂ | 44.8, CH₁ |
| 3′    | 69.6, C | 69.5, C |
| 4′    | 27.1, CH₂ | 27.3, CH₂ |
| 5′    | 44.8, CH₂ | 44.7, CH₂ |
| 6′    | 173.7, C | 172.1, C |
| 7′    | 51.7, CH₁ | 51.7, CH₁ |
| 3′OAc | 171.4, C | 170.6, C |
| 20′   | 20.9, CH₃ | 20.9, CH₃ |

(Table 1 and 2), compounds 1 and 2 were identified as fasciculic acid A$^{11,12}$ derivatives. According to the observed NOE correlations of H-18 with H-11β, H-15β, H-16β, and H-20, those of H-19 with H-1β, H-2, H-11β, and H-29, and of H-28 with H-15α, H-16α and H-17, the relative stereochemistry as depicted in structural formulas 1 and 2 was proposed for the two compounds. Thus, compound 1, named as pholiol A, was assigned as the 3-O-acetyl-7,24-diketo analogue of fasciculic acid A$^{11,12}$ while pholiol B (2) was assigned as its methyl ester. The relative configuration of the C-3′ chiral center could not be determined on this basis and is only tentatively given as $S$, based on the close chemical shift values with those of similar compounds$^{11,12}$ and on assuming that similar metabolic pathways led to the formation of the structurally similar compounds in the different fungal species.

The molecular formulas of 3 (C₃₈H₆₀O₁₀) and 4 (C₃₉H₆₂O₁₀) derived from HRESIMS measurements differed only in a methylene group, similarly to those of compound pair 1 and 2. As compared to pholios A (1) and B (2), compounds 3 and 4 contained two additional hydrogens, nominally corresponding to the saturation of the double bond in 1/2. The $^1$H and $^{13}$C NMR spectra of 3 and 4 were highly similar to those of compounds 1 and 2. The most striking differences were the presence of a triplet signal with one hydrogen intensity at $\delta_H$ 4.13/4.14 in the $^1$H NMR spectrum and in parallel the absence of the carbonyl resonance belonging to C-7 and the appearance of resonance at $\delta_C$ 72.8/72.8 in the $^{13}$C
NMR spectrum of 3/4. Thus, at first sight, the reduction of the keto group at C-7 to a hydroxy group was envisaged. The 1H and 13C NMR spectra of 3 and 4 were almost identical, except for the presence of a singlet with a three-hydrogen intensity at δH 3.73 in the 1H NMR spectrum and a carbon signal at δC 51.7 in the 13C NMR spectrum of 4. This suggested that 4 was the methyl ester analogue of compound 3. Analysis of the HSQC and HMBD data showed that a hydroxy group was attached to C-12 (δH 4.13/4.14, each 1H, t, J = 7.7 Hz; δC 72.8/72.8 for 3/4; HMBD correlation between H-18 and C-12) and a methylene group was present in position 7 based on the HMBD correlation of H-5 and H-6 with C-7. 2D NMR experiments allowed the complete 1H and 13C NMR assignments of 3 and 4 as listed in Tables 1 and 2. Compound 3, named as pholiol C, was identified as the 24-keto derivative of fusicanic acid B,12 while pholiol D (4) was identified as its methyl ester. The similar chemical shifts and NOE correlations suggested that the relative configurations of the C-2, C-3, C-5, C-10, C-14, C-17, and C-20 (and C-1’) chiral centers of 3 and 4 were identical to those determined for pholiols A (1) and B (2). The NOE correlations of H-12 with H-11α, H-17, and H-28 (Figure S26) suggested that the hydroxy group occupied the β position (thus, the C-12 chiral center had an R configuration) in pholiols C (3) and D (4).

Compound 5 was identified as (6E,10E,14E,18E)-2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene regarding its 1H and 13C NMR chemical shift values, identical with literature data.13 The 35S,22S configuration of 5 can be suggested based on the opposite optical rotation data than that of the 3R,22R isomer.14 Compound 6 was found to be identical in all of its spectroscopic characteristics with that of ergosterol.15 3β-Hydroxyergosta-7,22-diene was detected in the n-hexane fraction with the use of an authentic standard.

**Cytotoxic Activity.** The isolated compounds (1–6) were tested for their cytotoxic activity on sensitive Colo 205 and resistant Colo 320 cell lines and on the normal MRC-5 embryonal fibroblast cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with doxorubicin as a positive control. Among the studied compounds, ergosterol (6) showed substantial cytotoxic activity against the tumor cell lines with IC50 values of 4.9 μM (Colo 205) and 6.5 μM (Colo 320) (Table 3).

| compound       | Colo205 (IC50 μM) | Colo320 (IC50 μM) | MRC5 (IC50 μM) |
|----------------|------------------|------------------|----------------|
| ergosterol     | 4.9 ± 0.6        | 6.5 ± 0.2        | 0.5 ± 0.1      |
| doxorubicin    | 2.5 ± 0.3        | 7.4 ± 0.2        | >20            |

The compound was more potent against the MRC-5 cell line (IC50 0.50 μM). Pholiols B (2) and D (4) and compound 5 possessed weak inhibitory activities (IC50 > 25 μM) against the tested cell lines without any selectivity (Table S2).

**MDR Efflux Pump Inhibitory Activity.** The inhibitory activities of compounds 1–3, 5, and 6 on efflux function were evaluated by measuring the intracellular accumulation of rhodamine 123, a well-known P-glycoprotein substrate fluorescent dye, within the Colo320 MDR cells. Tarividar, a strong P-gp inhibitor, was used as positive control. All tested compounds were dissolved in DMSO, and the final concentration (2.00%) of the solvent was investigated for any effect on the retention of rhodamine 123. The results revealed inhibition of P-gp MDR efflux pump activity manifested by pholiols A (1) and B (2) and triterpene 5. In general, compounds with fluorescence activity ratio (FAR) values greater than 1 were considered to be active P-gp inhibitors, while compounds with FAR values greater than 10 were considered to be strong MDR modulators. The sterol compounds with methyl ester functionality (2) and the polyhydroxy-squalene derivative (5) exerted the highest anti-MDR effect in this bioassay with FAR values of 6.880 and 6.638, respectively.

| sample          | conc., μM | FAR   |
|-----------------|-----------|-------|
| tariquidar      | 0.2       | 5.533 |
| pholiol A (1)   | 20        | 3.418 |
| pholiol B (2)   | 20        | 6.880 |
| pholiol C (3)   | 20        | 0.967 |
| compound 5      | 20        | 6.638 |
| ergosterol (6)  | 2         | 1.053 |
| DMSO            | 200.00%   | 0.828 |

“Positive control.

**Combination Studies with Doxorubicin.** Compounds 2 and 4–6 were tested for their capacity to reduce the resistance of the MDR Colo 320 cell line to doxorubicin. A checkerboard microplate combination assay was performed, which is a widely used in vitro method for the assessment of drug interactions. Experimental data were analyzed using CompuSyn software, which enabled the determination of the most effective ratios of combined agents and calculation of combination indices (CI). Based on the combination indices, the type of interaction could be defined according to the literature.16 Pholiols B (2) and D (4), with a 3-hydroxy-3-methyl-glutarate methyl ester moiety, and 5 interacted in an synergistic manner, and CI values at 50% of the ED50 were found to be 0.348, 0.660, and 0.082, respectively. The outstanding potency of 5, designated as very strong synergism (CI = 0.082), is promising. In this assay ergosterol (6) was found to have an additive effect in combination with doxorubicin.

It is noteworthy that the tetrahydroxy-squalene derivative (5) has the capacity to potentiate the effect of doxorubicin in Colo 320 adenocarcinoma cells by P-gp modulation and strong synergism with doxorubicin, and therefore it represents a promising new class of potential adjuvants of cancer chemotherapy. With regard to the moderate P-gp inhibitory activity and strong synergism of 5 in combination with doxorubicin, the mechanism of its chemosensitizing effect should have another P-gp-independent mechanism.

**Antibacterial Effect.** Compounds 1–6 were inactive against Escherichia coli ATCC 25922, Salmonella enterica serovar Typhimurium 14028s, Staphylococcus aureus ATCC 25923, and S. aureus 27213.8

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** The chemicals used in the experiments were supplied by Sigma-Aldrich Hungary and Molar Chemicals, Hungary. An InsMark IP-Digi1 polarimeter (Shanghai InsMark Instrument Technology Co., Ltd., Shanghai, China) was applied for optical rotation measurements. Flash chromatography...
were acquired on a Bruker Avance III HD 800 MHz spectrometer (12 g of sorbent) using a mixture of acetone (40 g of sorbent), linear gradient from 0:00 to 75:25, t = 50 min). The presence of 3β-hydroxyergosta-7,22-diene (7) was detected in the n-hexane fraction using an authentic standard. Pholiol A ([M] +): amorphous solid; (α,β) = 10 + 0.1 (0.1, CHCl3); HRESIMS m/z 673.39752 [M + H]+ (Δ = 2.7 ppm; C21H18O7); H and 13C NMR data, see Tables 1 and 2; HRESI-MSMS (CID = 35%; rel int %) m/z 611(100), 571(63), 529(44), 469(4).

Pholiol B ([M] +): amorphous solid; (α,β) = 80 + 0.1 (0.1, CHCl3); HRESIMS m/z 689.42289 [M + H]+ (Δ = 4.4 ppm, C23H23O7); H and 13C NMR data, see Tables 1 and 2; HRESI-MSMS (CID = 35%; rel int %) m/z 671(81), 531(100), 513(30), 471(55), 453(56), 435(8), 417(4), 377(1).

Pholiol C ([M] +): amorphous solid; (α,β) = 16 + 0.1 (0.1, CHCl3); HRESIMS m/z 675.41283 [M + H]+ (Δ = 2.2 ppm, C23H25O7); H and 13C NMR data, see Tables 1 and 2; HRESI-MSMS (CID = 35%; rel int %) m/z 613(100), 573(92), 555(6), 531(31).

Pholiol D ([M] +): amorphous solid; (α,β) = 90 + 0.1 (0.1, CHCl3); HRESIMS m/z 1403.85130 [M + Na]+ (Δ = 4.9 ppm, C43H39O12Na); H and 13C NMR data, see Tables 1 and 2; HRESI-MS3 (1408/713; CID = 35%, 45%; rel int %) m/z 653(12), 639(6), 537(100), 477(41), 437(3).

(35S,16E,18E,20S)-3,22,23-Tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetraacostaetaene (5): amorphous solid; (α,β) = 2.3 (0.16, CHCl3) and 15.75 (0.16, MeOH); H and 13C NMR data, Table S1; HRESIMS m/z 479.40900 [M + H]+ (Δ = 1.0 ppm, C34H60O3); HRESI-MSMS (CID = 35%; rel int %) m/z 461(100), 443(31).

Cell Culture. The human colon adenocarcinoma cell lines, the Colo 205 (ATCC-CCL-222) doxorubicin-resistant parent and Colo 320/MDR-LRP (ATCC-CCL-220.1) resistant to anticancer agents expressing ABCB1, were purchased from LGC PROMOchem (Teddington, UK). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM Na-pyruvate, 100 mM Heps, nystatin, and a penicillin–streptomycin mixture in concentrations of 100 U/L and 10 mg/L, respectively. The MRC-5 (ATCC CCL-171) human embryonic lung fibroblast cell line (LG PROMOchem) was cultured in EMEM medium, supplemented with 1% nonessential amino acid mixture, 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM Na-pyruvate, nystatin, and a penicillin–streptomycin mixture in concentrations of 100 U/L and 10 mg/L, respectively. The cell lines were incubated in an humidified atmosphere (5% CO2, 95% air) at 37 °C.

Assay for Cytotoxic Effect. The effects of increasing concentrations of the compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The 2-fold serial dilutions of the tested compounds were made starting with 100 μM. Then, 104 human colon adenocarcinoma cells in 100 μL of the medium (RPMI-1640) were added to each well, except for the medium control wells. The adherent human embryonic lung fibroblast cell line (104/well) was seeded in EMEM medium in 96-well flat-bottomed microtiter plates for 4 h before the assay. The serial dilutions of the compounds were made in a separate plate starting with 100 μM and then transferred to the plates containing the adherent corresponding cell line. Culture plates were incubated at 37 °C for 24 h; at the end of the incubation period, 20 μL of MTT (thiazolyl blue tetrazolium bromide) solution (from a 5 mg/mL stock solution) was added to each well. After incubation at 37 °C for 4 h, 100 μL of sodium dodecyl sulfate (SDS) solution (10% SDS in 0.01 M HCl) was added to each well, and the plates were further incubated at 37 °C overnight. Cell growth was determined by measuring the optical density (OD) at 540 nm (ref 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of cell growth was expressed as IC50.
values, defined as the inhibitory dose that reduces the growth of the cells exposed to the tested compounds by 50%. IC₅₀ values and the SD of triplicate experiments were calculated by using GraphPad Prism software version 5.00 for Windows with nonlinear regression curve fit (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Rhodamine 123 Accumulation Assay. The cell numbers of the human colon adenocarcinoma cell lines were adjusted to 2 × 10⁶ cells/mL, resuspended in serum-free RPMI 1640 medium, and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at 2 or 20 μM concentrations, and the samples were incubated for 10 min at room temperature. Tariquidar was applied as positive control at 0.2 μM. DMSO at 2% v/v was used as solvent control. Next, 10 μL (5 μM final concentration) of the fluorochrome and ABCB1 substrate rhodamine 123 (Sigma) were added to the samples, and the cells were incubated for a further 20 min at 37 °C, washed twice, and resuspended in 1 mL of PBS for analysis. The fluorescence of the cell population was measured with a Partec CyFlow flow cytometer (Partec, Münster, Germany). The FAR was calculated as the quotient between FL-1 of the treated/un-treated resistant Colo 320 cell line over the treated/un-treated sensitive Colo 205 cell line according to the following equation:

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\text{FAR} = \frac{\text{Colo320}_{\text{treated}}/\text{Colo320}_{\text{control}}}{\text{Colo205}_{\text{treated}}/\text{Colo205}_{\text{control}}}
\]

Checkerboard Combination Assay. A checkerboard microplate method was applied to study the effect of drug interactions between the compounds (2, 4–6) and the chemotherapeutic drug doxorubicin. The assay was carried out on the Colo 320 colon adenocarcinoma cell line. The final concentration of the compounds and doxorubicin used in the combination experiment was chosen in accordance with their cytotoxicity toward this cell line. The dilutions of doxorubicin were made in a horizontal direction in 100 μL, and the dilutions of the compounds vertically in the microtiter plate in a 50 μL volume. Then, 6 × 10⁴ of Colo 320 cells in 50 μL of medium were added to each well, except for the medium control wells. The plates were incubated for 72 h at 37 °C in a 5% CO₂ atmosphere. The cell growth rate was determined after MTT staining. At the end of the incubation period, 20 μL of MTT solution (from a stock solution of 5 mg/mL) was added to each well. After incubation at 37 °C for 4 h, 100 μL of SDS solution (10% in 0.01 M HCl) was added to each well, and the plates were further incubated at 37 °C overnight. OD was measured at 540 nm (ref 630 nm) with a Multiscan EX ELISA reader. CI values at 50% of the growth inhibition dose were determined using Compusyn software (CompuSyn Inc., Paramus, NJ, USA) to plot four to five data points at each ratio. CI values were calculated by means of the median-effect equation, according to the Chou–Talalay method, where CI < 1, CI = 1, and CI > 1 represent synergism, additive effect (or no interaction), and antagonism, respectively.⁷,¹⁸

Bacterial Strains and Determination of Antibacterial Activity. See ref 8.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c01024.

1D and 2D NMR and HRESIMS spectra of compounds 1–5 (PDF)

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Notes

The authors declare no competing financial interest.

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