A New P-Glycoprotein Inhibitor from the Caper Spurge (*Euphorbia lathyris*)

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The macrocyclic lathyrane polyester *Euphorbia* factor L_{10} (**1a**) has been obtained from the seeds of the caper spurge (*E. lathyris*). The interaction of L_{10} (**1a**) and its acetyl derivative (**1b**) with P-glycoprotein, a multidrug transporter overexpressed in cancer cells and responsible for resistance to chemotherapy, was investigated. The results established lathyrane diterpenoids as a novel chemotype for P-glycoprotein inhibitors.

Over the past few decades, there has been an intense interest in spurges (*Euphorbia* spp. vv.) as renewable sources of industrial raw material.[1] Studies in this area can be traced back to Calvin's seminal concept of plants as "photosynthetic factories" to produce fuels,[2] but the capacity of spurges to accumulate specific fatty acids in their seeds was later recognized and pursued.[3] As a result, mass cultivation of *E. lathyris* L. (caper spurge) and *E. lagascae* Spreng. was established for the production of oleic acid and vernolic acid, respectively.[3,4] Apart from its status as an "arable crop", the caper spurge is also a medicinal plant and a prolific producer of fine chemicals of biomedical relevance. Thus, its seeds are a convenient source of ingenol[5] and contain relatively large amounts of nonirritant macrocyclic diterpenoids of the lathyrane-type[6] as well as dimeric coumarins.[7] Related macrocyclic diterpenoids were also isolated from the roots[8,9] and callus culture of the plant.[9]

The lathyrane constituents of the caper spurge are known as L-factors, and are endowed with a remarkable potential to generate complex polycyclic structures by transannular cyclization.[10] As part of an investigation on the construction of libraries of natural products-like compounds to screen against useful biological targets,[10] large amounts of the macrocyclic L-factors were needed. We therefore embarked on a large-scale isolation of these compounds from commercial seeds of the caper spurge. In the course of these studies, a new macrocyclic diterpenoid ester was obtained. This compound, named L_{10}, showed powerful inhibition of the transport activity of P-glycoprotein, a multidrug transporter overexpressed in cancer cell plasma membranes as an efflux pump conferring cellular resistance to anticancer chemotherapy. The structure elucidation of L_{10} and the mechanism of its interaction with P-glycoprotein are detailed here.

The macrocyclic L-factors L_1, L_2, L_3, and L_8 were obtained from an acetone extract of the seeds by gravity column chromatography and were further purified by crystallization, as described in the Experimental Section. ^{1}H NMR inspection evidenced significant amounts of an additional product only in the mother liquors from the crystallization of L_8. Further purification by chromatography on Sephadex and next on neutral alumina eventually afforded a new noncrystalline lathyrane diester, which was named *Euphorbia* factor L_{10} (**1a**). The isolation yield of **1a** was ca. 0.004%, almost 1 order of magnitude lower than that of the *Euphorbia* factor L_8. The HRMS of **1a** showed the molecular formula $C_{28}H_{42}O_6$, corresponding to an unsaturation index of eight, while the IR spectrum displayed hydroxyl (3460 cm^{-1}) and ester carbonyl (1736 cm^{-1}) absorptions. Since ^{13}C NMR data indicated the presence of two olefin double bonds and three carbonyls, L_{10} was tricyclic. The ^{1}H NMR spectrum in $CDCl_3$ showed signals diagnostic of two olefin methines (δ 6.56, d, J = 11.3 Hz, and δ 5.52, d, J = 10.7 Hz)), one oxymethylene (AB system centered at δ 3.81), one oxymethine (δ 5.26, dd, J = 3.0, 4.0 Hz), and two shielded cyclopropane methines (δ 1.38, m, and 1.11, m). The bicyclic structure and the presence of a cyclopropane ring suggested a lathyrane skeleton for L_{10}, while the ester moieties could be identified as an acetate and a caproate. Acetylation of the natural product afforded a monoacetate (**1b**) and caused a downfield shift of the oxymethylene protons ($\Delta\delta$ +0.41), while the HMBC spectrum correlated the caproate carbonyl (δ 173.2) with the oxymethine proton at δ 5.26. These observations located the hydroxyl group and the caproate at an oxymethine and an oxymethylene carbon of the natural product, while the remaining acetate had to be tertiary and connected to the deshielded carbon at δ 94.6. Further structural details were obtained by 2D NMR measurements (COSY, HMBC). The observed correlations were analyzed starting from the methyls and the oxymethylene appendices to the bicyclic system, and this analysis yielded four fragments expressed in correlations centered on the C-16 methyl (C-1, C-2, C-3), the C-18/C-19 methyls (C-9, C-10, C-11), the C-20 methyl (C-12, C-13, C-14), and the 17-methylene (C-5, C-6, C-7). These fragments were next combined via scalar proton−proton correlations (COSY H-3/H-4/H-5 and H-7a,b/H-8a,b/H9/H-11/H-12) and long-range proton-carbon correlations (HMBC H-1a,b/C-14; H-4/C-14; H-4/C-15) into a lathyrane framework. The relative configuration at the protonated stereogenic carbons and at the endocyclic double bond was established by dipolar proton−proton correlations (NOESY H-12/H-5; H-8β/H-19; H-4/H-2; H-4/H-3; H-4/H-17a; H-5/H-7β; H-5/H-8β; H-17b/H-7α). Assuming the β-configura-

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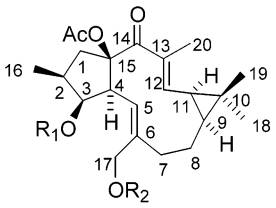
tion typical of all lathyranes for the tertiary C-15 oxygen function,[6,11] the isolathyrol structure **1a** could be eventually deduced for the *Euphorbia* factor L_{10}. Isolathyrol derivatives are very rare, and only one of them, tentatively formulated as **1c**, had been previously described.[12] The detection of NOESY correlation between the two olefinic protons, between the enone proton (H-12) and H-19 and H-8β, and between the oxymethylene protons and H-4 and H-7α showed that **1a** assumes a conformation with both the 20-methyl and the 6-oxymethylene *syn*-oriented on the α-face of the macrocycle. The presence of a free hydroxyl in **1a** is remarkable, since all the macrocyclic diterpenoids isolated previously from the seeds of the caper spurge are peracylated.[5,6,11]



	R_1	R_2
1a	$C_5H_{11}CO$	H
1b	$C_5H_{11}CO$	Ac
1c	C_6H_5CO	Ac

The recent observation that certain macrocyclic diterpenoids of the jatrophane type show a strong inhibition of drug efflux from multidrug-resistant cells[13] prompted us to investigate the interaction of L_{10} (**1a**) and its acetyl derivative (**1b**) with P-glycoprotein. Two different approaches were pursued. The first one is the direct binding to a purified recombinant cytosolic domain of the transporter, an interaction that can be monitored by quenching of the intrinsic protein fluorescence.[14] The second one is the inhibition of P-glycoprotein-mediated cellular drug efflux, a process that is amenable to monitoring by flow cytometry.[15] Figure 1A shows that L_{10} could bind to the cytosolic domain of P-glycoprotein with high affinity. Binding produced a remarkable maximal quenching of intrinsic fluorescence (78.5 $\pm$ 7.1%) and was characterized by a dissociation constant of 16.9 $\pm$ 4.9 μM, as determined with the Grafit program.[16] Acetylation of the 17-hydroxyl was detrimental to binding, leading to a much decreased maximal quenching (13.8 $\pm$ 1.5%). Figure 1B shows that L_{10} (**1a**) could also strongly inhibit P-glycoprotein-mediated daunomycin transport. Thus, a 95% increase in intracellular drug accumulation was measured at a 5 μM concentration of **1a**, a level of activity higher than that of the specific and potent P-glycoprotein inhibitor cyclosporin A (Figure 1B). Interestingly, acetylation of the primary hydroxyl of **1a** had only a modest effect on the inhibitory activity of L_{10}, since **1b** could still afford an 80% increase in daunomycin accumulation (Figure 1B). Since **1b** was only marginally active in binding experiments, this suggests that, in addition to a cytosolic site, **1a** can also bind to a second, higher-affinity, site, probably located within the transmembrane domain of the transporter and at least partly overlapping with the drug-binding site. Binding of L_{10} to this high-affinity site is likely to be responsible for the inhibition of P-glycoprotein-mediated drug transport activity. Dihydro β-agarofuran sesquiterpenes were reported to inhibit the drug transport by a P-glycoprotein-like multidrug transporter upon binding to a similar site.[17]

Taken together, these observations establish lathyrane diterpenoids as a novel chemotype of P-glycoprotein inhibitors and further validate the caper spurge as a remarkable source of bioactive compounds.

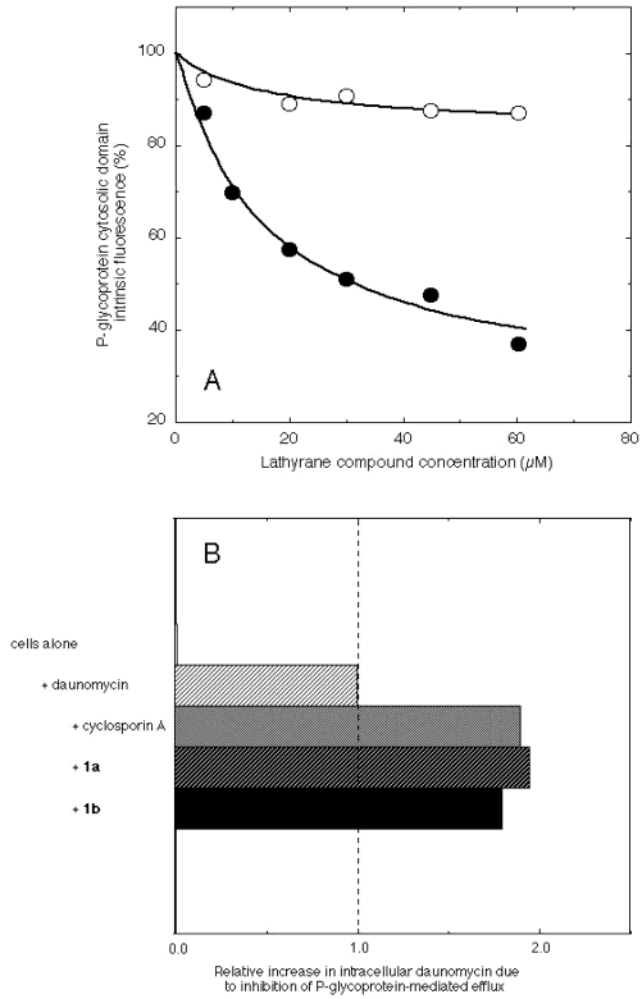


Figure 1. Interaction of lathyranes **1a** and **1b** with P-glycoprotein and inhibition of extracellular daunomycin efflux. (A) Binding of **1a** (closed symbols) and **1b** (open symbols) to the purified cytosolic-domain NBD2 of P-glycoprotein. (B) Daunomycin intracellular accumulation due to inhibition of its transport by **1a**, **1b**, or cyclosporin A in P-glycoprotein-overexpressing leukemic K562/R7 cells.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Shimadzu DR 8001 spectrophotometer. MS (EI, 70 eV) were taken on VG 7070 EQ spectrometers. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer (500 and 125 MHz, respectively) with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. Silica gel 60 (70−230 mesh, Merck) and Sephadex LH-20 were used for open-column chromatography (CC). Known compounds were identified by direct comparison with authentic samples available from previous work.[5]

Plant Material. The seeds of *E. lathyris* were purchased from F. W. Freiherr von Rotenhan, 97500 Ebelsbach, Germany.

Isolation of the Macrocyclic Diterpenoids. [N.B. The oil from *E. lathyris* is highly irritant to skin and mucous membranes and displays tumor-promoting activity. All manipulations, including crushing of the seeds and the chromatographic purifications, should be carried out wearing latex gloves and face protection, avoiding contact with the skin.] A sample (15 kg) of seeds was crushed in a Waring blender, and the resulting mash was transferred into a 15 L extraction vessel and extracted with acetone (1 $\times$ 20 L; 2 $\times$ 12 L).

Removal of the solvent left a yellow-orange oil (7.3 L), which deposited a copious orange precipitate of esculetin (49 g) on standing at room temperature (16 days). The oil was decanted from the precipitate and partitioned between cyclohexane (15 L) and MeCN (15 L). The lower cyclohexane phase was removed and further extracted with MeCN (2 × 2 L). The pooled MeCN phases were washed with petroleum ether (2 × 1 L), concentrated to ca. 1/5 of the original volume, and cooled (4 °C). After 48 h, the voluminous precipitate (21 g) of the *Euphorbia* factor L_1 was removed by filtration. The mother liquors were evaporated to afford a paste (299 g), which was fractionated by gravity column chromatography on silica gel (2 kg) packed with petroleum ether−EtOAc, 95:5. Elution was started with this solvent and next continued with petroleum ether−EtOAc, 9:1, obtaining the *Euphorbia* factors L_3, L_2, and L_1 (16.5, 4.5, and 13.5 g, respectively, after washing the fraction residues with ether). Elution with petroleum ether−EtOAc, 8:2 and next 7:3, afforded 5.7 g of a mixture of L_8 and L_{10} as a yellowish paste. Washing with ether gave pure L_8 (3.3 g), while the mother liquors (3.4 g) were purified by column chromatography on Sephadex LH-20 (60 g, hexane−EtOAc, 8:2, as eluant) and next on neutral alumina (chloroform−acetone, 5:1) to give a further 1.1 g of L_8 and 600 mg of L_{10} (**1a**).

***Euphorbia* Factor L_{10} (=15-acetyl-5-hexanoyl-17-hydroxyisolathyrol, 1a):** colorless viscous oil; $[\alpha]^{25}_D$ +59° (*c* 0.3, CH_2Cl_2); ν_{max} liquid film 3496, 1748, 1732, 1717, 1651, 1645, 1615, 1372, 1271, 1240, 1177, 1007 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.56 (d, J = 11.3 Hz, H-12), 5.52 (d, J = 10.7 Hz, H-5), 5.26 (dd, J = 4.0, 3.0 Hz, H-3), 3.95 (d, J = 12.5 Hz, H-17a), 3.68 (d, J = 12.5 Hz, H-17b), 3.53 (dd, J = 14.0, 8.0 Hz, H-1α), 2.70 (dd, J = 10.7, 3.8 Hz, H-4), 2.40 (t, J = 7.5 Hz, H-2′a,b), 2.37 (m, H-7β), 2.27 (m, H-8α), 2.22 (m, H-7α), 2.18 (m, H-2), 2.02 (s, 15-OAc), 1.83 (s, H-20), 1.70 (quin., J = 7.0 Hz, H-3′a,b), 1.47 (m, H-8β), 1.47 (m, H-1β), 1.38 (m, H-11), 1.37 (m, H-4′a,b), 1.37 (m, H-5′a,b), 1.18 (s, H-18), 1.11 (m, H-9), 1.05 (s, H-19), 0.96 (d, J = 6.7 Hz, H-16), 0.93 (t, J' = 6.9 Hz, H-6′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.6 (s, C-14), 173.2 (s, C-1′), 169.4 (s, 15-OAc), 147.0 (d, C-12), 144.8 (s, C-6), 132.3 (s, C-13), 122.2 (d, C-5), 94.6 (s, C-15), 80.7 (d, C-3), 62.8 (t, C-17), 50.2 (d, C-4), 44.6 (t, C-1), 38.3 (d, C-2), 34.5 (t, C-2′), 34.3 (d, C-9), 31.7 (t, C-7), 31.4(t, C-4′), 29.2 (d, C-11), 29.1 (q, C-18), 28.2 (t, C-8), 24.8 (t, C-3′), 24.8 (s, C-10), 22.4 (t, C-5′), 21.3 (q, 15-OAc), 16.1 (q, C-19), 13.9 (q, C-6′), 13.8 (q, C-16), 12.3 (q, C-20); HREIMS *m/z* 474.2967 $[M]^+$ (2) (calcd for $C_{28}H_{42}O_6$, 474.2981).

Acetylation of the *Euphorbia* Factor L_{10}. A sample (80 mg) of **1a** was dissolved in dry pyridine (2 mL) and treated with Ac_2O (2 mL). After standing overnight, the reaction was worked up by dilution with water and extraction with EtOAc. The organic phase was washed sequentially with 2 N H_2SO_4 and saturated $NaHCO_3$ and brine and next dried (Na_2SO_4) and evaporated. The residue was purified by gravity CC on silica gel (8 g, hexane−EtOAc, 7:3, as eluant) to afford 64 mg of **1b** as a colorless viscous oil: $[\alpha]^{25}_D$ +59° (*c* 0.3, CH_2Cl_2); ν_{max} liquid film 1745, 1731, 1717, 1651, 1640, 1625, 1372, 1271, 1250, 1240, 1029 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.55 (d, J = 11.3 Hz, H-12), 5.62 (d, J = 10.9 Hz, H-5), 5.30 (dd, J = 4.0, 3.0 Hz, H-3), 4.35 (d, J = 12.5 Hz, H-17a), 4.12 (d, J = 12.5 Hz, H-17b), 3.54 (dd, J = 14.0, 8.0 Hz, H-1α), 2.72 (dd, J = 10.9, 3.7 Hz, H-4), 2.40 (t, J = 7.5 Hz, H-2′a,b), 2.40 (m, H-7β), 2.26 (m, H-8α), 2.04 (m, H-7α), 2.18 (m, H-2), 2.03 (s, 15-OAc), 2.02 (s, 17-OAc), 1.85 (s, H-20), 1.70 (quin., J = 7.0 Hz, H-3′a,b), 1.46 (m, H-8β), 1.43 (m, H-1β), 1.37 (m, H-11), 1.37 (m, H-4′a,b), 1.37 (m, H-5′a,b), 1.18 (s, H-18), 1.10 (m, H-9), 1.05 (s, H-19), 0.96 (d, J = 6.7 Hz, H-16), 0.93 (t, J = 6.9 Hz, H-6′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.4 (s, C-14), 173.0 (s, C-1′), 170.7 (s, 17-Oac), 169.4 (s, 15-OAc), 146.9 (d, C-12), 140.2 (s, C-6), 132.4 (s, C-13), 125.02 (d, C-5), 94.5 (s, C-15), 80.5 (d, C-3), 64.0 (t, C-17), 50.4 (d, C-4), 44.6 (t, C-1), 38.5 (d, C-2), 34.0 (t, C-2′), 34.0 (d, C-9), 32.4 (t, C-7), 31.4 (t, C-4′), 29.3 (d, C-11), 29.1 (q, C-18), 28.4 (t, C-8), 24.8 (t, C-3′), 24.8 (s, C-10), 22.4 (t, C-5′), 21.4 (q, 15-OAc), 20.9 (q, 17-OAc), 16.1 (q, C-19), 13.9 (q, C-6′), 13.8 (q, C-16), 12.3 (q, C-20); HREIMS *m/z* 516.3091 $[M]^+$ (1) (calcd for $C_{30}H_{44}O_7$, 516.3087).

Biological Assays. (A) Binding Assays. The recombinant *C*-terminal cytosolic domain of P-glycoprotein, NBD2, was overexpressed and purified as described previously.[14] Fluorescence experiments were performed at 25 °C by using an SLM-Aminco 8000C spectrofluorimeter. The tryptophan-specific intrinsic fluorescence of 0.5 μM recombinant domain in 1.2−2.0 mL of 20 mM potassium phosphate at pH 6.8, containing 0.5 M NaCl, 20% glycerol, and 0.01% methyl 6-*O*-(*N*-heptylcarbamoyl)-β-D-glucopyranoside, was scanned from 310 to 360 nm upon excitation at 295 nm, integrated, and corrected for buffer contribution. Binding of the lathyrane compounds was monitored by the quenching of intrinsic fluorescence produced by successive additions of aliquots up to 60 μM. The modifications were corrected for the inner-filter effect determined under the same conditions with 0.5 μM *N*-acetyltryptophanamide. Curve fitting of binding related to fluorescence decrease was performed with the Grafit program (Erithacus software) as previously described.[16]

(B) Inhibition of P-Glycoprotein-Mediated Drug Efflux. One million K562/R7 human leukemic cells expressing high levels of P-glycoprotein were incubated for 1 h at 37 °C in 1 mL of RPMI 1640 medium containing a final concentration of 10 μM daunomycin,[15] in the presence or absence of 5 μM lathyrane compound. Cells were washed three times with ice-cold phoshate buffer saline and maintained on ice until analysis by flow cytometry on a FACS-II (Becton-Dickinson Corp., Mountain View, CA). Assays were performed in duplicate, in at least three separate experiments. Cyclosporin A, a potent inhibitor of P-glycoprotein, was used as a positive control at a final concentration of 2 μM. Control experiments showed that cells incubated with lathyrane derivatives in the absence of daunomycin did not exhibit enhanced fluorescence (data not shown).

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