# An Expeditious Procedure for the Isolation of Ingenol from the Seeds of *Euphorbia lathyris*

Giovanni Appendino,*,† Gian Cesare Tron,† Giancarlo Cravotto,† Giovanni Palmisano,† and Jasmin Jakupovic*,‡

*Dipartimento di Scienza e Tecnologia del Farmaco, via Giuria 9, 10125 Torino, Italy, and Institut für Organische Chemie, Technische Universität Berlin, Strasse des 17. Juni 135, 10623 Berlin, Germany*

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A short and practical process for the isolation of ingenol (**1a**) from an agricultural commodity (the seeds of *Euphorbia lathyris*) is described. Macrocyclic diterpene esters are obtained as byproducts, and the esterification pattern of the *Euphorbia* factors $L_2$ (**3**), $L_3$ (**4a**), and $L_8$ (**4b**) was established by 2D NMR measurements. Full spectroscopic data for these compounds are reported.

Over the past few years there has been considerable interest as biological tools in a series of biogenetically related diterpenoids referred to as "phorboids", and exemplified by phorbol, ingenol (**1a**), and resiniferatoxin (RTX).[1] These compounds are typical of plants from the family Euphorbiaceae and have been the subject of intense multidisciplinary research, culminating in the discovery of their cellular targets [the enzyme protein kinase C (PKC) for 12,-13-phorbol-diesters and ingenol-3-monoesters;[2] the R-type vanilloid receptor for RTX ][3]. Certain derivatives of phorbol, ingenol, and resiniferonol also show potential as chemotherapeutic agents, but the mechanism underlying their potent anticancer[4] and antiviral[5] activity is still unknown.

The complex molecular architecture of these compounds has spurred chemical research aimed at the characterization of their pharmacophore and their total synthesis.[6] In this context, ingenol (**1a**) lags behind the two other archetypal phorboids, because, despite efforts spanning more than a decade, it has not yet yielded to synthesis.[7] Moreover, the key elements of its pharmacophore and its affinity profile for the various PKC isoforms are still poorly defined.[8] This shortage of biochemical information on ingenol is surprising, inasmuch as its derivatives are the more common irritants of spurges (*Euphorbia* spp.)[9] and occur in plants of horticultural relevance and agronomic potential as nonfood crops.[10] A better understanding of the structure−activity relationships of compounds within the ingenol esters series and information on their biosynthesis will assist and rationalize the selection of chemotypes lacking (or with a reduced) skin irritancy, and thus more suitable for mass cultivation.[11] The outstanding anticancer[4a] and anti-HIV[5b] activity of certain esters of ingenol and the ongoing synthetic activity in this area[7] provide a further rationale for improving the accessibility to ingenol itself, at present a very expensive research chemical.[12]

Ingenol (**1a**) is widely distributed within spurges,[9] but the seeds of the caper spurge (*E. lathyris* L., mole plant) are the only commercial source of this compound.[13] This plant has received worldwide attention as a renewable source of industrial raw materials (hydrocarbons and oleic acid),[14] and mass cultivation has been attempted in western Europe and the United States. In 1991, Hecker published a detailed procedure for the isolation of ingenol from the seeds of *E. lathyris*.[13] This procedure is labor-intensive and relies on a series of solvent partitions and selective adsorption on Si gel to separate a fraction containing ingenol esters. This is then subjected to hydrolysis, and the parent alcohol is eventually purified by column chromatography. To streamline this isolation procedure, we have developed an alternative and shorter isolation protocol that involves only limited handling of the skin-irritant oil from the caper spurge and also yields its macrocyclic diterpenoid constituents.

The seeds of the caper spurge are a complex matrix, containing, in addition to fats (40−47%) and proteins (15%),[15] a series of diterpenoid esters known as Euphorbia Factors $L_1$−$L_9$.[16] The major diterpene constituents are $L_1$ (**2**) and $L_3$ (**4a**), which are based on the macrocyclic polyols epoxylathyrol and lathyrol, respectively. Small but relatively constant amounts of the ingenol monoesters $L_4$ (**1b**), $L_5$ (**1c**), and $L_6$ (**1d**) are also present, and powerful tumor-promoting activity in a mouse-skin model has been reported for $L_5$ and $L_6$.[16] We reasoned that the isolation of ingenol (**1a**) from the seeds of *E. lathyris* involves three basic steps, namely, the separation of a crude diterpenoid fraction from the oil, the hydrolysis of the L-factors, and the separation of ingenol from the polyols resulting from the hydrolysis of the macrocyclic diterpenoid esters (lathyrol, epoxylathyrol, 7-hydroxylathyrol, isolathyrol, jolkinol).

Both separations entail the purification of a minor constituent from a complex mixture of neutral compounds having similar polarity. To overcome these obstacles, we explored the possibility of separating the diterpenoid esters from the lipids using a solvent partition, and to selectively hydrolyze the ingenol-based L-factors in the presence of the macrocyclic esters. A successful solvent partition might achieve a considerable mass reduction, because the diterpenoid esters make up only about 3−5% of the oil, whereas the selective hydrolysis of the ingenol-based L-factors (less than 5% of the diterpenoid mixture) would lead to a substantial difference of polarity between the unreacted macrocyclic esters and ingenol, greatly simplifying their chromatographic separation. We describe here how this strategy can be reduced to practice.

## Results and Discussion

Partition of an $Me_2CO$ extract from the seeds between various $H_2O$−MeOH or $Me_2CO$−$H_2O$ mixtures and hydro-

* To whom correspondence should be addressed. Tel. (GA): +39 11 670 7684; (JJ): + 49 30 314 22564. Fax (GA): + 39 11 670 7687; (JJ): + 49 30 314 21105. E-mail (GA): appendin@pharm.unito.it;(JJ): jaku@wap0107.chem-tu-berlin.de.
† Dipartimento di Scienza e Tecnologia del Farmaco.
‡ Institut für Organische Chemie.

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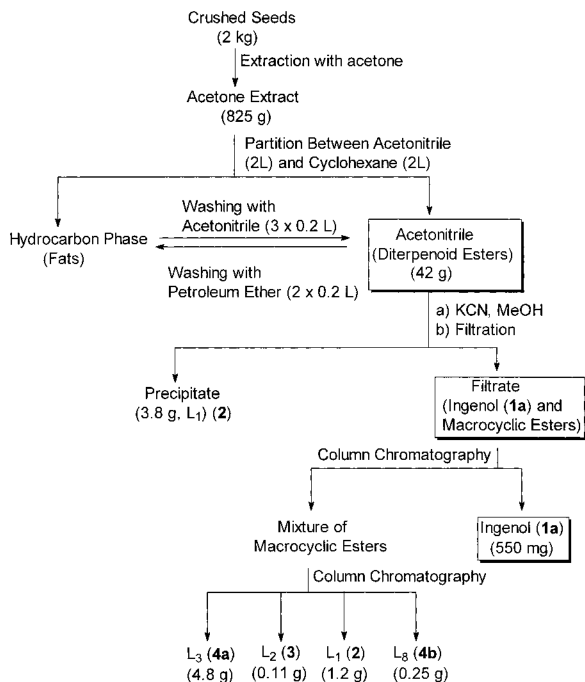
**Scheme 1.** Isolation scheme for ingenol (**1a**) and the *Euphorbia*-Factors $L_1$ (**2**), $L_2$ (**3**), $L_3$ (**4a**), and $L_8$ (**4b**) from the seeds of *E. lathyris*




carbons gave poor results, but the system of MeCN−cyclohexane led to an excellent separation of the lipids and the diterpenoid fraction (Scheme 1). The basis for this success is unclear, but a similar partition system (MeCN−hexane) was recommended to separate lipophilic tin derivatives and apolar organic compounds in the workup of $Bu_3SnH$ reductions.[17] Partition between equivalent volumes of the two solvents removed about 85% of fats, and a further reduction was achieved washing the acetonitrile phase with petroleum ether, giving an overall mass reduction of >90%. The crude diterpene fraction was then subjected to hydrolysis under several conditions ($NH_2-NH_2$; $H_2O_2-NaHCO_3$; $Cs_2CO_3$; $K_2CO_3$; $NaHCO_3$). No reaction or hydrolysis of the whole diterpenoid mixture was observed. Excellent results were eventually achieved with KCN in MeOH. The rationale for using this system is that the presence of free hydroxyl(s) catalyzes the deacylation of a neighboring ester by the cyanide anion.[18] The macrocyclic esters lack free hydroxyl groups, while all the ingenol-based L-factors have a free hydroxyl on the carbon $\alpha$ or $\beta$ to that bearing the ester functions. Ingenol (**1a**) could then be separated from the unreacted macrocyclic L-factors by chromatography on a short Si gel column. The overall isolation yield was about 0.25−0.30 g/kg of seeds. An unexpected asset of the KCN method is that precipitation of $L_1$ (**2**) takes place during the transesterification of the ingenol-based L-factors, allowing the recovery of the major L-factor by simple filtration (Scheme 1).[19] A certain selectivity in the hydrolysis step could also be achieved with Zemplén methanolysis under carefully controlled conditions (see Experimental Section). This method does not employ toxic chemicals and is thus attractive when large amounts (>10 kg) of seeds are processed. However, yields consistently lower (0.15−0.20 g/kg of seeds) than those obtained with the KCN-protocol were obtained, presumably because of the instability of ingenol (**1a**) in basic medium and the higher load of absorbent necessary for the chromatographic separation of ingenol from lathyrol and epoxylathyrol, the hydrolysis products of $L_3$ and $L_1$, respectively.

**Table 1.** $^1H$ NMR Data for Compounds **1a**−**3**, **4a**, and **4b**[a,b]

| position | 1a | 2 | 3 | 4a | 4b |
|---|---|---|---|---|---|
| 1 | 5.86 q | 3.31 dd | 3.40 dd | 3.52 dd | 3.54 dd |
| 1′ | | 1.35 dd | 1.78 dd | 1.66 dd | 1.65 dd |
| 2 | | 2.07 m | 2.36 m | 2.37 m | 2.39 m |
| 3 | 4.35 br s | 5.48 dd | 5.77 dd | 5.82 dd | 5.83 dd |
| 4 | | 1.86 dd | 2.92 dd | 2.89 dd | 2.90 dd |
| 5 | 3.80 br s | 6.23 d | 6.37 d | 6.20 d | 6.17 d |
| 7 | 6.02 br d | 2.10 m | 5.53 dd | 2.18 br dd | 2.15 br dd |
| 7′ | | 0.92 m | | 2.04 br dddd | 2.05 br ddd |
| 8 | 4.13 br dd | 2.10 m | 2.33 m | 1.95 dddd | 1.94 dddd |
| 8′ | | 1.72 m | 2.21 ddd | 1.74 m | 1.69 m |
| 9 | | 1.08 ddd | 1.34 ddd | 1.14 ddd | 1.14 ddd |
| 11 | 2.37 m | 1.47 dd | 1.50 dd | 1.39 dd | 1.39 dd |
| 12 | 2.25 ddd | 6.59 dq | 6.51 dq | 6.54 dq | 6.52 dq |
| 12′ | 1.74 ddd | | | | |
| 13 | 0.68 ddd | | | | |
| 14 | 0.90 dd | | | | |
| 16 | 1.05 s | 0.65 d | 0.84 d | 0.94 d | 0.93 d |
| 17 | 1.11 s | 2.48 d | 5.50 br s | 5.00 d | 5.01 d |
| 17′ | | 2.30 dd | 5.22 br s | 4.77 br s | 4.75 br s |
| 18 | 0.95 d | 1.20 s | 1.19 s | 1.16 s | 1.16 s |
| 19 | 1.82 d | 1.21 s | 1.26 s | 1.16 s | 1.15 s |
| 20 | 4.16 br d | 1.84 d | 1.81 d | 1.72 d | 1.71 d |
| 20′ | 4.05 br d | | | | |

[a] *J* (Hz). For **1a**: 1,19 = 1.5; 7,8 = 4.5; 8,14 = 12.5; 11,12 = 3; 11,12′ = 8.5; 12,12′ =15; 12,13 = 6; 12′,13 = 8.5; 13,14 = 8.5. For **2**: 1,1′ = 14; 1,2 = 8; 1′,2 = 12.5; 2,3 = 3.5; 2,16 = 6.5; 3,4 = 3.5; 4,5 = 9.5; 8,9 = 3.5; 8′,9 = 12; 9,11 = 8; 11,12 = 11.5; 12,20 = 1.5; 17,17′ = 3.5; 17′, 7 = 2. For **3**: 1,1′ = 14; 1,2 = 8; 1′,2 = 12; 2,3 = 3.5; 2,16 = 6.5; 3,4 = 3.5; 4,5 = 8; 7,8 = 9; 7,8′ = 3; 8,8′ = 15; 8,9 = 3.5; 8′,9 = 12; 9,11 = 8.5; 11,12 = 11.5; 12,20 = 1.5. For **4a** and **4b**: 1,1′ = 15; 1,2 = 8.5; 1′,2 = 12; 2,3 = 3.5; 2,16 = 6.5; 3,4 = 3.5; 4,5 = 10; 7,7′ = 14; 7,8 = 6; 7′,8′ = 13; 7′,8 = 2; 8,8′ = 15.5; 8,9 = 4; 8′,9 = 12; 9,11 = 8; 11,12 = 11.5; 12,20 = 1.5. [b] Other signals: For **2**: 2.03 (s, OAc-5), 2.13 (s, OAc-15), 3.59 (d, *J* = 15 Hz, OPhAc-3), 3.55 (d, *J* = 15 Hz, OPhAc-3), 7.32−7.22 (m, OPhAc-3). For **3**: 1.29 (s, OAc-5), 2.21 (s, OAc-15), 8.06 (AA′, OBz-3), 7.45 (BB′, OBz-3), 7.58 (C, OBz-3), 7.93 (AA′, OBz-7), 7.35 (BB′, OBz-7), 7.50 (C, OBz-7). For **4a**: 1.82 (s, OAc-5), 2.21 (s, OAc-15), 8.02 (AA′, OBz-3), 7.44 (BB′, OBz-3), 7.57 (C, OBz-3). For **4b**: 1.83 (s, OAc-5), 2.22 (s, OAc-15), 9.22 (d, *J* = 2 Hz, ONic-3), 8.27 (ddd, *J* = 8, 2, 2 Hz, ONic-3), 7.41 (br dd, *J* = 8, 5 Hz, ONic-3), 8.79 (dd, *J* = 5, 2 Hz, ONic-3).

Column chromatography of the unesterified diterpenoid fraction afforded the *Euphorbia* Factors $L_1$−$L_3$ and $L_7$−$L_9$. Despite a fairly similar structure, all these compounds except $L_{7a}$ and $L_{7b}$[20] could be easily separated by gravity column chromatography. Compounds $L_2$ (**3**), $L_3$ (**4a**), and $L_8$ (**4b**) have not yet been completely characterized, because their esterification pattern was unknown.[21,22] To settle this matter, 2D NMR experiments (HMBC spectra) were carried out, locating the aromatic ester groups at C-3 in $L_2$ and $L_3$, and at C-3 and C-7 in $L_8$. The unusual chemical shift of the 5-acetyl group in **3** ($\delta$ 1.29, Table 1) can be rationalized on the basis of strong anisotropic shielding from the benzoyl groups at C-3 and C-7. Full $^1H$ and/or $^{13}C$ NMR data for these compounds and for ingenol (**1a**) and $L_1$ (**2**) were not available and are reported in Tables 1 and 2.

In conclusion, we have developed a short and simple procedure (Scheme 1) for the isolation of ingenol from an agricultural commodity. The availability of ingenol will be useful to the many ongoing programs aimed at the total synthesis of this compound and should spur studies on the characterization of its pharmacophore and the exploitation of the anticancer and anti-HIV potential of its derivatives. The method also affords large amounts of the nonirritant macrocyclic diterpenes $L_1$ and $L_3$ (ca. 2.5 g/kg of seeds, each) which might be interesting starting material for biomimetic cyclizations and the construction of libraries of derivatives.

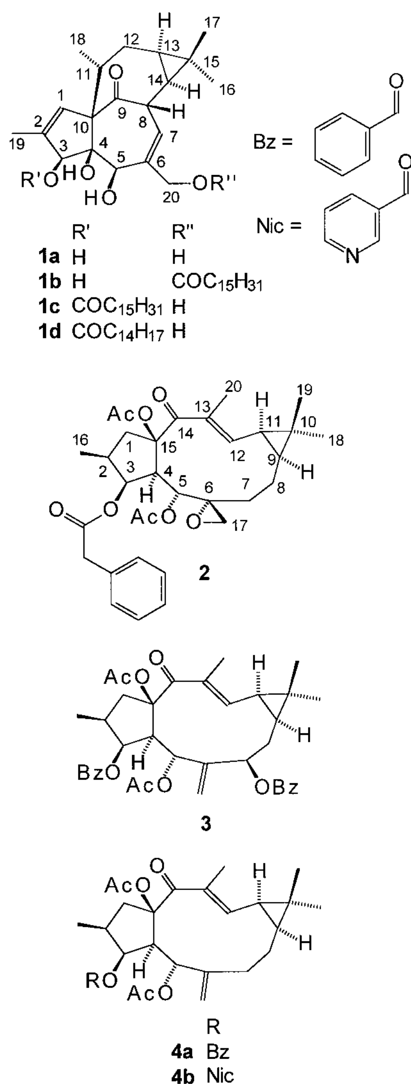


**Table 2.** $^{13}$C NMR Data for Compounds **1a**, **3**, **4a**, and **4b**[a]

| carbon | 1a | 3 | 4a | 4b |
|---|---|---|---|---|
| 1 | 129.2 d | 47.9 t | 48.5 t | 48.6 t |
| 2 | 140.6 s | 37.6 d | 37.9 d | 37.7 d |
| 3 | 80.1 d | 79.6 d | 80.8 d | 81.6 d |
| 4 | 84.3 s | 52.9 d | 52.1 d | 52.2 d |
| 5 | 75.1 d | 64.1 d | 65.4 d | 65.5 d |
| 6 | 139.1 s | 142.0 s | 144.5 s | 144.3 s |
| 7 | 127.3 d | 78.6 d | 34.9 t | 34.9 t |
| 8 | 44.0 d | 28.7 t | 21.6 t | 21.0 t |
| 9 | 207.7 s | 31.5 d | 35.4 d | 35.4 d |
| 10 | 72.6 s | 24.6 s | 25.3 s | 25.3 s |
| 11 | 34.9 d | 27.7 d | 28.5 d | 28.5 d |
| 12 | 31.0 t | 142.7 d | 146.5 d | 146.6 d |
| 13 | 23.3 d | 135.5 s | 134.2 s | 134.2 s |
| 14 | 23.0 d | 197.5 s | 196.7 s | 196.5 s |
| 15 | 23.8 s | 91.9 s | 92.5 s | 92.4 s |
| 16 | 28.5 q | 14.1 q | 14.2 q | 14.2 q |
| 17 | 15.4 q | 119.7 t | 115.4 t | 115.5 t |
| 18 | 17.4 q | 28.7 q | 29.0 q | 28.9 q |
| 19 | 15.5 q | 16.6 q | 16.8 q | 16.8 q |
| 20 | 66.7 t | 12.7 q | 12.4 q | 12.4 q |

[a] Other signals: For **3**: 169.3 (s, OAc-5); 20.9 (q, OAc-5), 169.7 (s, OAc-15); 21.8 (q, OAc-15), 165.9 (s, OBz-3); 130.3 (s, OBz-3), 129.6 (d, OBz-3); 128.3 (d, OBz-3); 133.1 (d, OBz-3), 165.6 (s, OBz-7), 130.1 (s, OBz-7), 129.6 (d, OBz-7), 128.3 (d, OBz-7), 133.1 (d, OBz-7). For **4a**: 170.1 (s, OAc-5); 20.9 (q, OAc-5), 169.7 (s, OAc-15), 21.9 (q, OAc-15), 166.1 (s, OBz-3); 130.1 (s, OBz-3); 129.6 (d, OBz-3), 128.3 (d, OBz-3), 133.1 (d, OBz-3). For **4b**: 170.1 (s, OAc-5), 21.0 (q, OAc-5), 169.6 (s, OAc-15), 22.0 (q, OAc-15), 164.8 (s, ONic-3), 151.0 (d, ONic-3), 126.0 (s, ONic-3), 137.1 (d, ONic-3), 123.3 (d, ONic-3), 153.4 (d, ONic-3).

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a Büchi SMP-20 apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer model 237 spectrophotometer. HRMS were obtained on a MAT 95ST Finnigan MAT apparatus (70 eV, EI mode). $^1$H and $^{13}$C NMR spectra were obtained on a Bruker AM 400 spectrometer (400 and 100 MHz, respectively). $^1$H and $^{13}$C NMR chemical shifts refer to C*H*Cl$_3$ at 7.26 ppm, and *C*DCl$_3$ at 77.0 ppm, respectively. Si gel 60 and Si gel 40 (70–230 and 35–70 mesh, respectively, Merck) were used for open-column chromatography.

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

**Isolation of Ingenol (1a).** [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, should be carried out wearing latex gloves and face protection and avoiding contact with the skin.] A sample (2 kg) of seeds was crushed in a Waring blender, and the resulting mash was transferred into a 4-L separatory funnel containing Me$_2$CO (2 L) and a wad of cotton with a layer of sand (ca. 1 cm) at the bottom. Further Me$_2$CO (2 L) was then added, and percolation was started after 24 h. After collection of ca. 6 L of solvent, TLC (hexane–EtOAc 9:1) showed that the extraction of the L-factors was complete. Removal of the Me$_2$CO left an oil (825 g) that was partitioned between cyclohexane (2 L) and MeCN (2 L). The lower cyclohexane phase was removed and further extracted with MeCN (3 × 200 mL). The pooled MeCN phases were washed with petroleum ether (2 × 200 mL) and evaporated, leaving 42 g of residue as a semicrystalline paste. The latter was dissolved in MeOH (70 mL), and KCN (6 g) was added. After a few hours a precipitate started to appear. The course of the reaction was followed by TLC (hexane–EtOAc 3:7; $R_f$ ingenol (**1a**) 0.32; $R_f$ L$_1$ 0.80; $R_f$ lathyrol 0.42; $R_f$ epoxylathyrol 0.38). After 7 days the reaction mixture was filtered to remove the precipitate of L$_1$ (**2**) (3.8 g) and evaporated in a hood. The residue was suspended in ca. 50 mL of EtOAc, and Si gel 40 (ca. 70 g) was added. After removal of the solvent, the slurry was charged on top of a Si gel 60 column (100 g) packed with hexane–EtOAc (5:5). Elution with this solvent (ca. 350 mL) removed the unreacted macrocyclic esters. The solvent was then changed to hexane–EtOAc (2:8) to recover ingenol (**1a**, 550 mg, 0.027%) as a foam: [α]$^{25}_{D}$ +41° (*c* 0.10, MeOH); IR (KBr) 3350, 1713, 1626, 1462, 1392, 1271, 1149, 1017 cm$^{-1}$; $^1$H NMR data, see Table 1; $^{13}$C NMR data, see Table 2; HREIMS *m/z* 348.194 [M]$^+$ (0.5) (calcd for $C_{20}H_{28}O_5$, 348.194).

**Isolation of the Macrocyclic *Euphorbia* Factors.** The fraction eluted with hexane–EtOAc (5:5) was chromatographed on a Si gel column (ca. 150 g) eluted with mixtures of hexane–EtOAc. Elution with hexane–EtOAc (7:3) gave, in order of elution, L$_3$ (**4a**) (4.8 g), L$_2$ (**3**) (110 mg), and L$_1$ (**2**) (1.2 g); elution with hexane–EtOAc 5:5 afforded L$_8$ (**4b**) (255 mg) and L$_7$ (mixture of L$_{7a}$ and L$_{7b}$ (20), 930 mg).

**(2*S**,3*S**,4*R**,5*R**,9*S**,11*S**,15*R**)-5,15-Diacetoxy-3-benzoyloxy-14-oxolathyra-6(17),(12*E*)-diene (*Euphorbia* Factor L$_3$) (4a):** white powder; mp 152–154 °C; [α]$^{25}_{D}$ +110° (*c* 0.10, CH$_2$Cl$_2$); UV (EtOH) $\lambda_{max}$ 273, 225 nm; IR (KBr) 1740, 1713, 1649, 1622, 1369, 1277, 1221, 1109, 711 cm$^{-1}$; $^1$H NMR data, see Table 1; $^{13}$C NMR data, see Table 2; HREIMS *m/z* 522.263 [M]$^+$ (1) (calcd for $C_{31}H_{38}O_7$, 522.262).

**(2*S**,3*S**,4*R**,5*R**,7*R**,9*S**,11*S**,15*R**)-5,15-Diacetoxy-3,7-dibenzoyloxy-14-oxolathyra-6(17),(12*E*)-diene (*Euphorbia* Factor L$_2$) (3):** white powder; mp 200–203 °C; [α]$^{25}_{D}$ +120° (*c* 0.15, CH$_2$Cl$_2$); UV (EtOH) $\lambda_{max}$ 270, 230 nm; IR (KBr) 1741, 1718, 1655, 1633, 1425, 1371, 1277, 1109, 715 cm$^{-1}$; $^1$H NMR data, see Table 1; $^{13}$C NMR data, see Table 2; HREIMS *m/z* 642.283 [M]$^+$ (1) (calcd for $C_{38}H_{42}O_9$, 642.283).

**(2*S**,3*S**,4*R**,5*R**,6*R**,11*S**,15*R**)-5,15-Diacetoxy-3-phenylacetoxy-14-oxolathyra-6(17),12*E*-diene-6(17)-epoxide**

**(*Euphorbia* Factor $L_1$) (2):** white powder; mp 195−198 °C; $[\alpha]^{25}_{D}$ +109° (*c* 0.10, $CH_2Cl_2$); UV (EtOH) $\lambda_{max}$ 273, 229 nm; IR (KBr) 1740, 1651, 1622, 1456, 1268, 1126, 900, 725 $cm^{-1}$; $^1$H NMR data, see Table 1; $^{13}$C NMR data, see Hokawa et al.[23] HREIMS *m*/*z* 552.272 [M]$^+$ (1) (calcd for $C_{32}H_{40}O_8$, 552.272).

**(2*S**,3*S**,4*R**,5*R**,9*S**,11*S**,15*R**)-5,15-Diacetoxy-3-nicotinoyloxy-14-oxolathyra-6(17),(12*E*)-diene (*Euphorbia* Factor $L_8$) (4b):** white powder; mp 195−198 °C; $[\alpha]^{25}_{D}$ +130° (*c* 0.14, $CH_2Cl_2$); UV (EtOH) $\lambda_{max}$ 270, 229 nm; IR (KBr) 1747, 1718, 1653, 1626, 1591, 1423, 1288, 1223, 1122, 742 $cm^{-1}$; $^1$H NMR data, see Table 1; $^{13}$C NMR data, see Table 2; HREIMS *m*/*z* 523.257 [M]$^+$ (2) (calcd for $C_{30}H_{37}NO_7$, 522.262).

**Zemplén Methanolysis of the Diterpenoid Fraction.** The diterpenoid fraction obtained after partition of the extract from 2 kg of seeds was dissolved in 430 mL of freshly prepared 0.1 N NaOMe and stirred at 15 °C. The reaction was followed by TLC (see above). After 15 h the reaction mixture was neutralized by the dropwise addition of HOAc (2.6 mL), concentrated to ca. half volume, and then partitioned between $H_2O$ and EtOAc. The organic phase was washed with brine and evaporated, affording a thick oil. The latter was purified by column chromatography (150 g Si gel). Elution with hexane−EtOAc (7:3) afforded 4.6 g of $L_3$ and 5.02 g of $L_1$, and elution with hexane−EtOAc (3:7) gave 928 mg of a mixture of lathyrol and epoxylathyrol. Ingenol (**1a**) (390 mg, 0.019%) was obtained by elution with hexane−EtOAc (2:8).

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