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Tumor promoting diterpenes from *Euphorbia leuconeura* L.

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Abstract

Diterpene esters of the phorbol and ingenol types are known to be highly active tumor promoting agents that typically occur in members of the *Euphorbiaceae*. In the present work, *Euphorbia leuconeura*, a rare indoor plant, is analyzed for its tumor promoting potential. Latex as well as total leaf extracts exhibited Epstein–Barr-virus (EBV) inducing activity comparable to 12-*O*-tetradecanoyl-phorbol-13-*O*-acetate, a well known tumor promoter. The activity of individual fractions correlated with their ingenol ester content. Three ingenol esters with EBV inducing activity could be isolated and identified. They belong to the milliamine type of diterpene esters that contain aromatic peptidyl groups. Two of them (milliamines L and M) are already known from *E. milii*. The third compound is identified as an isomer of milliamine F with a novel 3,20-diester arrangement. The data show a close relationship between *E. leuconeura* and the more popular indoor plant *E. milii* whose latex is also used as a powerful molluscicide.

Keywords: *Euphorbia leuconeura*; Indoor plant; *Euphorbiaceae*; Ingenol esters; Tumor promotion; Epstein–Barr-virus induction

1. Introduction

In two-stage chemical carcinogenesis, tumor promotors such as phorbol and ingenol diterpenes strongly enhance tumor formation by carcinogenic chemicals (Evans & Soper, 1978; Hecker, 1968). A preliminary screening of several available *Euphorbiaceae* (spurge) indoor plant species had shown that *Euphorbia leuconeura* contained high EBV-inducing activity for human lymphocytes. This method is generally accepted to test unknown substances and extracts for their tumor promoting potential (Kloz, Hergenhahn, Fellhauer, & Hecker, 1989; Polack et al., 1992). The assay is based on the combination of the luciferase reporter gene with an EBV early promoter (Polack et al., 1992).

E. leuconeura is a succulent plant with attractive leaves. It is easy to cultivate and is occasionally used as an indoor plant. Its origin is in Madagascar and it belongs to the *Euphorbia lophogona* group (section *Goniostema* Baill). The tumor promoting compounds are now chemically characterized. *E. leuconeura* is related to *Euphorbia milii* which is also known among gardeners as *E. splendens* or 'crown of thorns'. The latex of *E. milii* contains a unique group of ingenol esters that were first described by Uemura and Hirata (1971) and are termed milliamines. The latter are peptidyl esters containing substituted di- or tri-anthraniloyl groups. At least 14 different milliamines have been identified (Marston & Hecker, 1984a, 1984b; Zani, Marston, Hamburger, & Hostettmann, 1993). Some of the milliamines possess high molluscicidal activity, and the latex of *E. milii* finds practical application

Abbreviations: EBV, Epstein–Barr-virus; LC–MS, liquid chromatography/mass spectroscopy; NMR, nuclear magnetic resonance; TPA, 12-*O*-tetradecanoyl-phorbol-13-*O*-acetate.

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as a molluscicide against schistosomiasis-transmitting aquatic snails of the genus *Biomphalaria* (De Vasconcellos & Schall, 1986). Several tested milliamines had no tumor promoting activity in animal experiments, although the activation of protein kinase C was comparable to that of TPA (Marston & Hecker, 1984a, 1984b; Zani et al., 1993).

We now describe the identification of three bioactive ingenol esters from *E. leuconeura* as milliamines. One of the compounds has a new structure. The characterization of the present milliamines as EBV-inducers makes a tumor promoting potential more likely than apparent from the somewhat contradictory previous data (Marston & Hecker, 1984a, 1984b; Zani et al., 1993).

2. Results and discussion

As measured by HPLC after alkaline hydrolysis of the diterpene ester fraction, the latex of *E. leuconeura* contained 0.66 ± 0.17 μg ingenol/mg fresh weight. The factor between fresh and dry weight of latex was 4.6. Total leaf extracts contained 16.2 μg ingenol derivatives/g fresh weight. The conversion factor of fresh to dry leaf weight was 8.9.

An ether extract from total latex (Evans & Soper, 1978) was tested in an in vitro EBV-assay for inducing potential. 12-*O*-tetradecanoyl phorbol 13-*O*-acetate (TPA), a well known tumor promoting standard, served as a positive control. The induction caused by

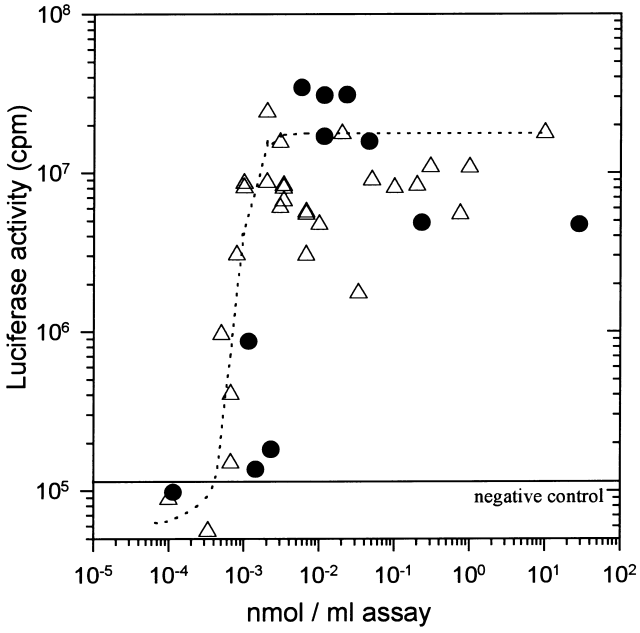

Fig. 1. Induction of luciferase activity by extracts of *Euphorbia leuconeura* (•) and by TPA (△). The ingenol content was determined after hydrolytic cleavage of the esters. The luciferase gene was under the tight control of an EBV early promoter (see Section 4).

the *E. leuconeura* extract was comparable to the TPA standard when related to ingenol content (Fig. 1). For more detailed information about the inducing compounds, the latex extract was fractionated by HPLC and peak materials detected at a wavelength of 220 nm were collected. The resulting 16 different fractions (Fig. 2) were employed for determination of ingenol

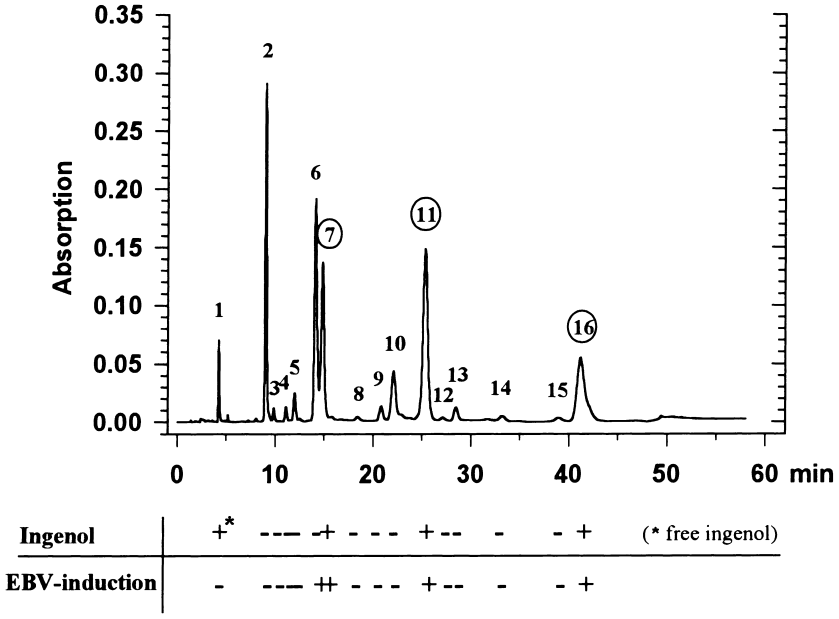

Fig. 2. Fractionation of latex extract of *E. leuconeura* by HPLC. Elution was detected at 220 nm. Each peak was analyzed for ingenol ester content (by hydrolytic cleavage) and for EBV induction. Peaks 7 (compound **3**), 11 (compound **2**) and 16 (compound **1**) were isolated and identified. The presence of ingenol and the response strength in the EBV induction assay are indicated.

after hydrolytic cleavage and of EBV inducing potential. Both parameters were closely correlated. With the exception of fraction 6, only the ingenol containing fractions exhibited an EBV inducing potential. A contamination of fraction 6 by the closely adjacent fraction could, however, not be excluded.

3. Structural elucidation

Fractions 7 (compound **3**), 11 (compound **2**) and 16 (compound **1**) were employed for structural elucidation. A detailed ^{1}H and ^{13}C NMR spectral analysis was carried out with these ingenol derivatives. The three samples gave ^{1}H NMR and ^{13}C NMR spectral analyses which were attributed to the ingenol moiety. The spectral data are summarized in Table 1. Further evidence came from electrospray ionization (ESI) MS fragmentation, which in each case showed characteristic fragments at m/z 313 and at m/z 295. The three compounds differed only in their substituents and sites of esterification. All spectra were interpreted by comparison with those of a nonsubstituted ingenol standard. Chemical shift values (ppm) gave in comparison to free ingenol an indication for the localization of ester-linkages Table 1. In the ^{1}H NMR spectra of the three ingenol derivatives, typical signals for the anthraniloyl moieties of milliamines (δ 6.67–8.81) and of an acetyl group (δ 2.02–2.05) were observed (Table 2). The existence of the acetyl group was also shown by a characteristic MS fragmention pattern (neutral loss of 60 amu).

Compound **1** had a parent molecular mass of 748 Da as established by LC–MS/MS ($[M + Na]^{+}$, m/z 771). The ^{1}H NMR spectral signals attributable to the anthraniloyl moieties exhibited resonances due to a total of twelve aromatic protons. The signals were indicative of the tri-anthraniloyl peptide previously described in *E. milii* (Marston & Hecker, 1983). The MS fragment at m/z 376 of this ester group indicated that anthranilic acid, 3-hydroxy anthranilic acid and benzoic acid were present as shown in Fig. 3.

The chemical shift values of H-3 (δ 5.99) and H-20 (δ 4.45; 4.74) indicated, in comparison with nonesterified ingenol, a 3,20-diester arrangement. A heteronuclear multiple bond correlation (HMBC) ($^{n}J_{(CH)}$ 7.5 Hz) experiment was performed in order to further analyze the attachment of the ester groups. Heteronuclear correlation was achieved between one of the methylene protons at C-20 (δ 4.45) and the carboxyl-C of the acetyl group (δ 172.7). On the other hand, the correlation between H-3 (δ 5.99) and an aromatic carboxyl-C (δ 169.4) proved the esterification of the anthraniloyl moiety at at the C-3 position Fig. 3. In this way, it

Table 1
^{1}H and ^{13}C NMR data for the diterpene moieties of compounds **1**, **2** and **3** (δ in ppm and J (parentheses) in Hz (CD_3OD, 500 MHz, 273 K). Signals were assigned by means of 1-D and 2-D NMR experiments. The numbering of atoms is as shown in Fig. 3.)

Atom No.	**1**		**2**		**3**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C[b]	δ ^{1}H	δ ^{13}C
1	6.10 s	133.7	6.11 s	123.5	5.90 br s	128.1
2	–	139.0	–	[a]	–	141.0
3	5.99 s	84.3	6.07 s	[b]	3.76 s	78.8
4	–	87.1	–	[a]	–	[a]
5	3.83 br s	74.8	3.84 s	74.2	5.56 br s	75.5
7	6.05 s	128.5	6.03 br s	128.2	6.31 br s	127.5
8	4.35	44.8	4.37 br d (10.5)	44.0	4.50 m	44.5
9	–	209.2	–	[a]	–	195.0
10	–	73.1	–	[a]	–	[a]
11	2.65 m	40.4	2.70 m	39.8	2.60 m	39.0
12a	1.78 m	31.8	1.83 m	31.0	1.82 m	30.8
12b	2.38 m	31.8	2.42 m	31.0	2.40 m	30.8
13	0.71 m	24.2	0.73 m	23.8	0.75 m	23.3
14	0.84 dd (8.2, 1.8)	24.0	0.86 m	23.5	0.91 m	23.0
15	–	25.4	–	[a]	–	[a]
16	1.03 s	28.8	1.05	14.9	1.08 s	27.8
17	1.04 s	15.9	1.05	14.9	1.19 s	15.0
18	1.02 d (nd)	17.5	1.05	16.5	0.99 d (7.0)	16.8
19	1.80 s	15.8	1.86 s	14.3	1.79 br s	14.9
20a	4.45 d (12.4)	67.9	4.45 d (12.4)	[b]	4.32 d (12.1)	67.3
20b	4.74 d (12.4)	67.9	4.76 d (12.4)	[b]	4.63 d (12.1)	67.3

[a] Due to limited sample (μg range), a 13C NMR spectrum was acquired only from compound **1**; 13C data of **2** and **3** were obtained from HMQC and HMBC spectra, respectively. A low signal to noise ratio precludes detection of some correlations to quaternary carbon atoms.
[b] Compound **2** decomposed during HMQC measurement showing mainly ingenane and ligand resonances.

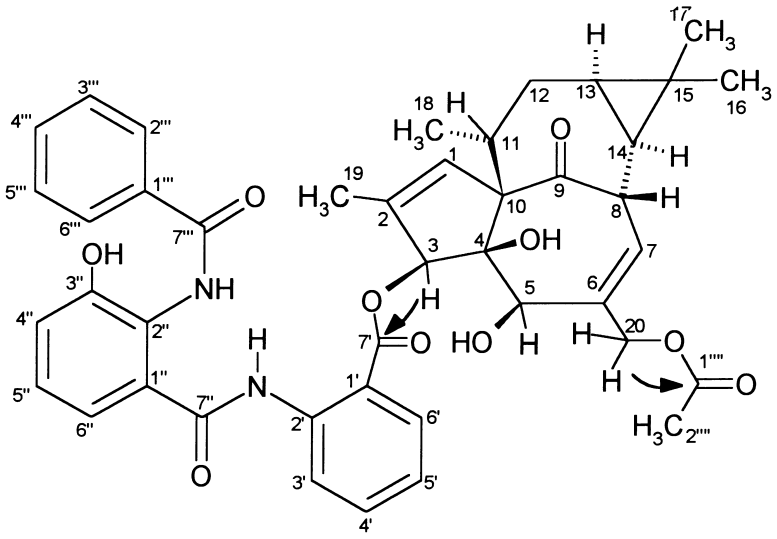

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Fig. 3. Chemical structure of compound **1**, showing the numbering of atoms also used in Tables 1 and 2. Compounds **2** and **3** and their numbering have been previously reported (Zani et al., 1993). Heteronuclear multiple bond correlation (H,C-HMBC) experiments were performed to determine the sites of esterification. Correlations between the protons of the ingenol moiety and the carbonyl-C of the side chains, giving evidence of the localization of the corresponding ester bond are indicated by the arrows.

became obvious that compound **1** is an isomeric form of milliamine F. The latter is a 5,20-diester as shown by Marston and Hecker (1983).

The mass spectrometric data for compounds **2** and **3** had similar fragmentation patterns and molecular ions, $[M + H]^+$ at m/z 629. Addition of ammonium acetate

Table 2
^1H and ^{13}C NMR data for the ester moieties of compounds **1**, **2** and **3** (δ in ppm and J (parentheses) in Hz (CD_3OD, 500 MHz, 273 K). Signals have been assigned by means of 1-D and 2-D NMR experiments. The numbering of atoms is as shown in Fig. 3.)

Atom No.	**1**		**2**		**3**	
	δ ^1H	δ ^{13}C	δ ^1H	δ ^{13}C[b]	δ ^1H	δ ^{13}C
1′	–	117.6	–	[a]	–	[a]
2′	–	142.3	–	[a]	–	[a]
3′	8.72 d (8.3)	121.8	7.70 d (8.0)	127.5	7.64 d (8.1)	127.3
4′	7.61 dd (7.5; 8.3)	135.6	6.67 dd (8.0; 7.1)	116.3	6.68 dd (8.1; 7.1)	116.0
5′	7.20 dd/7.5; 8.0)	124.4	7.25 dd (8.2; 7.1)	133.0	7.25 ddd (8.3; 7.1)	133.1
6′	8.02 d (8.0)	132.1	6.81 d (8.2)	117.5	6.79 d (8.3)	117.5
7′	–	169.4	–	168.0	–	168.0
1″	–	121.2	–	[a]	–	[a]
2″	–	124.8	–	[a]	–	[a]
3″	–	153.4	8.05 d (8.0)	131.0	8.29 dd (8.0)	131.7
4″	7.15 d (8.0)	121.2	7.18 dd (7.2; 8.0)	122.8	7.19 dd (8.0; 7.0)	122.8
5″	7.27 dd (8.0; 7.5)	128.5	7.65 dd (7.2; 8.4)	134.5	7.67 ddd (8.3; 7.0)	135.2
6″	7.33 d (7.5)	120.2	8.77 d (8.4)	120.5	8.81 d (8.3)	120.5
7″	–	168.8	–	172.0	–	[a]
1‴	–	137.8	–	–	–	–
2‴/6‴	7.94 d (7.3)	128.9	–	–	–	–
3‴/5‴	7.50 dd (7.3; 7.4)	129.8	–	–	–	–
4‴	7.58 t (7.4)	133.3	–	–	–	–
7‴	–	169.4	–	–	–	–
1⁗	–	172.7	–	171.0	–	171.0
2⁗	2.02 s	20.9	2.05 s	20.0	2.05 s	20.0

[a] Due to limited sample (μg range), a 13C NMR spectrum was acquired only from compound **1**; 13C data of **2** and **3** were obtained from HMQC and HMBC spectra, respectively. A low signal to noise ratio precludes detection of some correlations to quaternary carbon atoms.
[b] Compound **2** decomposed during HMQC measurement showing mainly ingenane and ligand resonances.

(NH_4OAc) to compound **3** led to the ammonia adduct (M + 18 amu). Compound **2** gave a strong Na-adduct (M + 23 amu). To achieve a uniform distribution between $[M + H]^+$ and $[M + NH_4]^+$ we finally diluted all samples 1:1 with 200 mM NH_4OAc prior to analysis. The MS data obtained indicated the presence of isomeric compounds.

The ^1H NMR spectra were also characteristic for milliamines, showing signals due to the ingenol moiety as well as the aromatic pseudopeptide moiety. The integration of the aromatic region accounted in both cases for 8 protons. A prominent MS fragment at m/z 256 was attributed to a di-anthraniloyl moiety. Compounds **2** and **3** both gave the ^1H NMR signals (δ 2.02 s) of an acetyl group. The sites of esterification differed in the two milliamines. Compound **3** showed strong shifts of H-5 (δ 5.56) and H-20 (δ 4.33; 4.65) compared to free ingenol. Compound **2** exhibited shifts of H-3 (δ 6.07) and H-20 (δ 4.45; 476). In HMBC experiments of compound **2** heteronuclear correlations between the protons of C-20 (δ 4.45) and the carbonyl-C of the acetyl group (δ 171), and between the proton of C-3 (δ 6.07) and the aromatic carbonyl group (δ 168) were observed. Compound **3** gave a clear correlation of one of the protons of C-20 (δ 4.33) with the carbonyl group (δ 171) of the acetyl residue. No signal of H-5 and the aromatic carbonyl group was obtained. Still, it could be concluded the acetyl groups were esterified at C-20 in both compounds. The differences in the spectra of these compounds could be explained by different positions of the peptidyl groups. Therefore, compound **3** was a 5,20-diester which is identical to milliamine M. Compound **2** was a 3,20-diester identical to milliamine L (Zani et al., 1993).

It should be noted that an apparent discrepancy exists with regard to the tumor promoting potential of the milliamines. Marston and Hecker (1983); 1984a; 1984b) concluded from their experiments with an in vivo mouse skin assay that milliamines A and C were no tumor promoters but skin irritants. On the other hand, milliamine C acted as an inhibitor of specific binding of [^{3}H]-phorbol-12,13-dipropionate to an in vitro epidermal fraction of mouse skin (Schmidt et al., 1983; Marston & Hecker, 1984a, 1984b). In our experiments, each of the three milliamines proved to be a strong EBV inducer. The inducing activity was as efficient as that of the well known tumor promoter TPA. A large scale exposure to milliamines could result from the proposed use of latex fractions as molluscicide against the schistosomiasis-transmitting snails of the genus *Biomphalaria* (De Vasconcellos & Schall, 1986).

More generally, a possible health risk from *E. leuconeura* due to milliamines could arise when leaves or the stems are wounded so that exposure to latex can occur. Nursery activities such as cutting or transferring plants into new pots could establish a contamination risk. *E. leuconeura* is occasionally used as an indoor plant due to its attractive leaves. *E. milii* is even more popular. Special care should be taken in handling these species.

4. Experimental

4.1. Cultivation of plants and collection of latex

E. leuconeura plants were kept in a glasshouse under typical indoor conditions (temperature: day/night 22°C/18°C, low light conditions: 1 klx). Plants were potted in commercially available soil (Fruhstorfer Einheitserde type T). Nutrients were supplied weekly by a nutrient solution (Flory 9, Euflor). White latex was drained into tubes after making a scalpel incision into the stem and the leaves of the plant. The tubes were sealed, weighed and extracted at once with 1 ml acetone at 4°C.

4.2. Extraction of diterpene esters

Plant leaf samples and latex were extracted according to Evans and Soper (1978). Dried samples were dissolved in methanol/water (17:3) and partitioned against hexane to remove nonpolar substances. The methanol/water ratio was subsequently changed to 1:2. The diterpene esters were then extracted from this methanol/water phase with diethylether. The recovery of TPA added prior to the extraction procedure was 90%.

4.3. Hydrolysis and quantification of diterpenoids

The diterpene esters were transformed to their parent alcohols by alkaline hydrolysis with 0.5 M KOH in methanol for 20 min at 25°C. The method was adapted from Evans and Kinghorn (1975) and from Girin, Paphassarang, David-Eteve, Chaboud, and Raynaud (1993). Subsequently the samples were fractionated by TLC on silica gel 60 (Merck, Darmstadt, Germany) with *n*-hexane and propan-2-ol (2:1, v/v), as solvent system. Ingenol, phorbol (both from Sigma, Deisenhofen, Germany) and 12-deoxyphorbol (produced by hydrolysis of 12-deoxyphorbol-13-*O*-tetradecanoate (Sigma)) were used as standards for TLC (R_f-values: phorbol = 0.28; ingenol = 0.47; 12-deoxyphorbol = 0.68). The corresponding regions on the silica gel plate were removed, extracted with methanol and analyzed by HPLC on an RP-18 column (250 × 4.6 mm, Spherisorb ODS2, 5 μm, Bischoff, Leonberg) with an acetonitrile/water gradient (flow rate: 1 ml/min; solvent A: 100% H_2O; solvent B: 88% acetonitrile, 12% H_2O; 5 min A, linear gradient to B in 20 min, 5 min B, linear gradient back to A in 2

min; retention time for ingenol: 19.4 min). The compounds were detected by UV-absorption at 220 nm. Identification of ingenol and 12-deoxyphorbol after hydrolysis was carried out by mass spectrometry (Finnigan MAT SSQ 7000) and by comparison of retention times of standards. Detection limit of ingenol was at 0.2 nmol.

4.4. Fractionation, isolation and identification of ingenol esters

The ether extracted diterpene fraction from latex (see above) was fractionated by HPLC on a RP-18 column (250×4.6 mm, Spherisorb ODS2, Type NC, 5 μm, Bischoff). Compounds were detected by UV-absorption at 220 nm in an acetonitrile/water gradient with 62% aqueous acetonitrile for 60 min followed by 88% aqueous acetonitrile. The flow rate was 1 ml/min and peak fractions were collected. The purification scheme of Fig. 2 was employed to quantitate ingenol after hydrolytic cleavage of ester bonds, to obtain the EBV induction data and to isolate defined compounds for characterization by ^{1}H and ^{13}C NMR and LC–MS.

4.5. Cell culture and Luciferase (Luc) assay

Raji Cells, which contain a reporter gene under the control of the EBV-DR promoter in an autoreplicative plasmid, were described in Polack et al. (1992). Instead of the CAT reporter gene we used a firefly luciferase gene to study the induction of the early EBV genes during an abortive cycle. Cells were grown in media RPMI 1640, 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin, 300 μg/ml hygromycin B at 37°C (media and supplements were supplied by Gibco BRL, Life Technologies), in a humidified atmosphere with 5% CO_2. The cells were treated with 1–20 μl plant extract (or known inducer) for 2 days in 24-well plates. The final volume per well was 1.5 ml. Control measurements were carried out with plant extracts from non-*Euphorbiaceae* indoor plants (*Ficus benjamina*, *Ficus elastica*) and with pure methanol instead of plant extract. The procedure for the luciferase assay was according to the Promega Luciferase Assay system. Luciferase activity was determined in a Berthold Autolumat LB 953. The light response was always in the linear range of the reaction. Relative luciferase activity was calculated on a protein basis (Bradford, 1976).

4.6. NMR spectroscopic analysis

^{1}H and ^{13}C NMR spectra were recorded with a Bruker DMX 500 spectrometer (proton frequency: 500.13 MHz) using 2.0 mm capillaries and an inverse geometry TXI 2.5 mm probehead (90°: 9.4 μs ^{1}H; 10.0 μs ^{13}C) in CD_3OD at 273 K ($^{1}H/^{13}C$: 3.30/49.00 ppm). H,C-HMBC spectra were recorded in the absolute value mode, using a coupling constant of 7.5 Hz. Absolute value DQ-COSY and phase sensitive (TPPI) TOCSY (t_{mix}: 35 ms) and H,C-HMQC-spectra were acquired using Bruker standard software (HMQC: aq: 203 ms, sw: 5040 Hz, d1: 1.5 s, $^{1}J(CH)$: 145 Hz, ^{13}C GARP decoupling: 70 μs, number of increments in F1: 320; H,H-TOCSY: aq: 227 ms, sw: 4496 Hz; 512 increments in F1). The ^{13}C NMR-spectrum was recorded with a 2.5 mm dual probehead (90°: 9.0 μs) with broad band decoupling and an acquisition time of 1.9 s (relaxation delay $d1$: 3.5 s). NMR assignments were supplemented by ^{1}H and ^{13}C chemical shift calculation with the ACD HNMR/CNMR Predictor 3.0 (Advanced Chemistry Development, Toronto, Canada), complemented with own data of ingenane in CD_3OD to exclude solvent effects.

4.7. LC–MS

Mass spectra of isolated fractions were obtained with an API 300 LC–MS/MS system (PE Sciex, Thornhill, Canada). Samples were introduced into the mass spectrometer via a syringe pump (flow rate 5 μl/min) (Harvard apparatus, Quebec, Canada). Ionization was achieved in the positive mode with an ion spray interface at an ionization voltage of 4.8 kV. Nitrogen 5.0 (Linde) was used as nebulizer and curtain gas. Nebulizer gas was set to 1.31 l/min, curtain gas to 1.25 l/min. For MS/MS experiments nitrogen was also used as collision gas (pressure in the collision cell $P_{cc} = 0.44$ Pa (3.3×10^{-3} torr) to effect collision-induced dissociation in the MS/MS mode. Lens and quadrupole parameters were optimized to achieve maximum intensity of the signals. LC 2 Tune 1.2 and Multiview 1.2 (PE Sciex, Thornhill, Canada) software were used for data acquisition and evaluation.

Mass spectrometric data of compounds **1**, **2** and **3**:

4.7.1. Compound **1** (fraction 16)

ESI-MS m/z: 771 [M + Na^+], 711 [771-HOAc], 399 [($C_{21}N_2O_5H_{16}$) peptidyl moiety 376 + Na^+], 395 [771–376(peptidyl moiety)], 335 [395-HOAc], 313 [395-NaOAc], 295 [313-H_2O]

4.7.2. Compound **2** (fraction 11)

ESI-MS m/z: 651 [M + Na^+], 591 [651-HOAc], 395 [651–256 ($C_{14}N_2O_3H_{12}$) peptidyl moiety], 335 [395-HOAc], 313 [395-NaOAc], 295 [313-H_2O], 279 [256 peptidyl moiety + Na^+]

4.7.3. Compound **3** (fraction 7)

ESI-MS m/z: 646 [M + NH_4^+], 629 [M + H^+], 611 [M + H^+-H_2O], 569 [629-HOAc], 551 [611-HOAc],

313 [569–256 ($C_{14}N_2O_3H_{12}$) peptidyl moiety], 295 [313-H_2O], 257 [256 peptidyl moiety + H^+]

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