# Selectively Cytotoxic Diterpenes from *Euphorbia poisonii*

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Bioactivity-guided fractionation of the latex of *Euphorbia poisonii* Pax. (Euphorbiaceae) led to the isolation and characterization of a new tigliane diterpene, 12-deoxyphorbol 13-(9,10-methylene)undecanoate (**3**), together with five known diterpenes (**1**, **2**, **4**−**6**). When evaluated for cytotoxicity in a panel of six human solid tumor cell lines, the diterpene esters, **1**−**3**, **5**, and **6**, were selectively cytotoxic for the human kidney carcinoma (A-498) cell line with potencies for **2** and **3** exceeding that of adriamycin by ten thousand times. Details of the isolations, structural analyses, and cytotoxic activities are described.

There is a pervasive myth in northern Nigeria regarding the toxic nature of *Euphorbia poisonii* Pax. (Euphorbiaceae), trivially called "Tinya" in Hausa. Homicide deaths in rural communities are often attributed to "Tinya" poisoning, the plant is not grazed by livestock, and preparations made from its latex are used as pesticides on millet farms. The latex of *E. poisonii* is extremely irritant to human skin, and it causes blindness when in contact with the eyes. The chemistry[1−7] and bioactivity profiles[8−12] of compounds previously isolated from *E. poisonii* have been studied extensively. Certain phorbol esters, ingenol esters, and daphnane derivatives isolated from Euphorbiaceous plants have been demonstrated to have antileukemic activities,[13] while lathyrane macrocyclics have been reported as cytotoxic against tumor cells in culture.[14,15] Some phorbol esters have also been reported lethal to brine shrimp, but phorbol itself showed no activity at 1000 $\mu$g/mL.[16] Our interest in *E. poisonii* derives from several sources, among which are its uses as garden pesticides in northern Nigeria and the paradoxical activities of its latex components as both procarcinogenic and antitumor agents. Fractionation of the latex of *E. poisonii*, directed by the brine shrimp lethality test (BST),[17,18] led to compounds **1**−**6** which were evaluated for cytotoxicity in a panel of six human solid tumor cell lines,[19−23] with adriamycin as a positive control. All of these, except tigliane (**4**), showed strong cytotoxic selectivity, with potencies approching ten thousand times that of adriamycin, for the human kidney carcinoma (A-498). In a previous paper, we described three bioactive diterpenes that were similarly isolated from the latex of *E. poisonii* but were less selectively cytotoxic.[24] Compound **3** is a novel tigliane diterpene bearing an unusual 9,10-methyleneundecanoate ester at position 13.

## Results and Discussion

Previous studies[1−7] have reported the phytochemical isolation of 12-deoxyphorbol esters and daphnane ortho esters from the latex of *E. poisonii*. This and a previous investigation[24] utilized the BST in an activity-directed isolation project. The 10% aqueous MeOH soluble fraction, of the latex extracts of this plant, exhibited a modest activity against the brine shrimp larvae ($LC_{50}$ 114 $\mu$g/mL). It also showed a significant selectivity for certain cells when tested for cytotoxicity in a panel of human solid tumor cell lines in culture. Bioactivity-directed chromatographic fractionation of the bioactive fraction gave 12-deoxyphorbol 20-acetate 13-angelate (**1**),[1] 12-deoxyphorbol 20-acetate 13-phenylacetate (**2**),[4] 12-deoxyphorbol 13-(9,10-methylene)undecanoate (**3**), 20-hydroxy-12-deoxyphorbol angelate (**4**),[1] resiniferol 20-(4-hydroxy-3-methoxyphenylacetate) 9,13,14-orthophenylacetate (**5**),[3,4,8] and 20-hydroxyresiniferol 9,13,-14-orthophenylacetate (**6**).[4]

The high-resolution mass spectroscopy (HRFABMS) of, hitherto unreported, **3** suggested a molecular formula of $C_{32}H_{48}O_6$. Compound **3** has a UV maximum at 235 nm, indicating the presence of an $\alpha,\beta$-unsaturated carbonyl group. Significant peaks in the CI-MS of **3** at $m/z$ 511, 313, and 294 identified the parent diterpene skeleton as a 12-deoxyphorbol[9] with one acyl substituent. The $m/z$ peak at 313 arose by the loss of $H_2O$ and a fatty acid, with a mass unit of 198, from the molecular ion. The $^{13}$C-NMR data of **3** (Table 1) displayed a tigliane skeleton, similar to **1**, **2**, and **4** (Chart 1), together with a 12-carbon substituent at C-13. The $^{13}$C-NMR spectrum also showed two trisubstituted double bonds, one ketone and one ester carbon. The molecular formula of **3** suggested the presence of a total of nine unsaturation equivalents. Hence, the remaining five unsaturation equivalents should be due to cyclic forms.

The structure of the subsituent at C-13 of **3** was determined by a careful analysis of the DEPT and $^1$H-NMR signals of the 12-carbon ester moiety. The presence of one methyl group, one ester carbonyl carbon, two methines, and eight methylene carbons, one of which resonated at $\delta$ 13.9, established an unusual cyclopropane undecanoate structure for the 12-carbon substituent. The exomethylene carbon was assigned to C-9′,10′ because the $^1$H-NMR spectrum of **3** showed one tertiary methyl group at $\delta$ 0.89 (d, $J$ = 6.5 Hz) besides that assigned to C-18. The carbon resonaces of the cyclopropane ring ($\delta$ 23.3, 14.2, 13.9) and the tertiary methyl group ($\delta$ 18.1) are comparable to those reported for similar compounds ($\delta$ 27.6, 13.0, 12.4 for the cyclopropane ring and $\delta$ 19.2 for the attached methyl group).[25,26] However, the stereochemistries of the cyclopropane ring could not be unequivocally assigned.

The NMR spectra of **5** and **6** showed the characteristic

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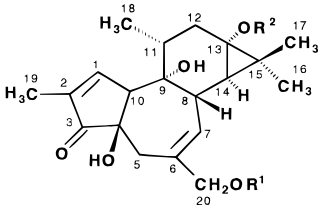
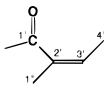
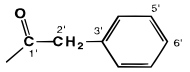
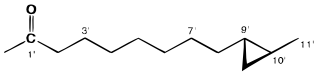
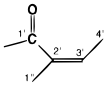
**Table 1.** $^{13}$C-NMR Data of Bioactive Tigliane-Type Diterpenes (**1**−**4**) from the Latex ($\delta$ in $CDCl_3$, 125.7 MHz)

| C | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | 161.46 | 161.36 | 161.36 | 161.34 |
| 2 | 132.79 | 132.83 | 132.74 | 132.74 |
| 3 | 209.06 | 208.97 | 209.27 | 209.44 |
| 4 | 73.61 | 73.56 | 73.77 | 73.75 |
| 5 | 39.00 | 38.99 | 38.61 | 38.46 |
| 6 | 134.77 | 134.82 | 139.77 | 139.97 |
| 7 | 134.07 | 133.15 | 130.55 | 130.41 |
| 8 | 39.54 | 39.39 | 39.15 | 39.13 |
| 9 | 75.98 | 75.87 | 75.98 | 76.19 |
| 10 | 55.73 | 56.68 | 55.75 | 55.66 |
| 11 | 36.43 | 36.30 | 36.29 | 36.31 |
| 12 | 31.88 | 31.65 | 31.98 | 31.91 |
| 13 | 63.09 | 63.91 | 63.24 | 63.22 |
| 14 | 32.72 | 32.29 | 32.55 | 32.77 |
| 15 | 22.92 | 22.99 | 26.62 | 22.86 |
| 16 | 23.64 | 23.00 | 23.13 | 23.62 |
| 17 | 15.37 | 15.30 | 15.38 | 15.37 |
| 18 | 18.56 | 18.48 | 18.56 | 18.56 |
| 19 | 10.12 | 10.08 | 10.11 | 10.07 |
| 20 | 69.78 | 69.69 | 68.34 | 68.22 |
| 1′ | 169.42 | 170.76 | 175.21 | 169.48 |
| 2′ | 127.33 | 41.64 | 31.89 | 127.34 |
| 3′ | 141.06 | 133.82 | 22.66 | 140.70 |
| 4′ | 20.46 | 129.28 | 29.99 | 20.41 |
| 5′/1″ | 16.01[a] | 128.58 | 29.72 | 15.96[a] |
| 6′ | | 127.26 | 29.44 | |
| 7′ | | 128.58 | 29.33 | |
| 8′ | | 129.28 | 26.83 | |
| 9′ | | | 23.29 | |
| 10′ | | | 14.18 | |
| | 20.97 (A) | 20.94 (Ac) | 18.05 (11′) | |
| | 170.80 | 173.48 | 13.88 (exo) | |

[a] C-5′ for **2** and **3**; C-1″ for **1** and **4**.

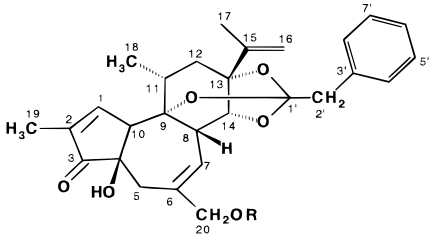
**Chart 1**



| | $R^1$ | $R^2$ |
|---|---|---|
| 1 | Ac |  |
| 2 | Ac |  |
| 3 | H |  |
| 4 | H |  |

features of a daphnane ortho ester diterpene.[8,9] The identity of **5** was confirmed as resiniferol 20-(4-hydroxy-3-methoxyphenylacetate) 9,13,14-orthophenylacetate from the spectral data. The substitution pattern of the benzoate at C-20 was unambiguously resolved by NOE difference experiments. The $^1$H-NMR of **5** revealed the following signals, for the benzoate moiety in the aromatic region, at $\delta$ 6.84 (1H, d, $J$ = 8.0 Hz, H-7″), 6.80 (1 H, d, $J$ = 2 Hz, H-4″), and 6.76 (2H, dd, $J$ = 2, 8.5 Hz, H-8″). Irradiation of the methoxy signal at $\delta$ 3.88 induced a 6.65% NOE enhancement in the signal at $\delta$ 6.80 (1H, d, $J$ = 2 Hz, H-4″). This NOE result was consistent with a 4-hydroxy-3-methoxyphenylacetate substructure for the C-20 substituent. The NMR spectra of **5** and **6** were quite similar except for the absence of the benzoate signals in **5**. At variance with **5**, the C-20 protons of **6** were observed upfield at $\delta$ 4.1, thus confirming C-20 as the attachment site for the benzoate in **5** (Chart 2).

**Chart 2**



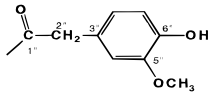
| | R |
|---|---|
| 5 |  |
| 6 | H |

**Table 2.** $^{13}$C-NMR Data of Bioactive Daphnane-Type Diterpenes (**5** and **6**) from the Latex ($\delta$ in $CDCl_3$, 125.7 MHz)

| C | 5 | 6 | C | 5 | 6 |
|---|---|---|---|---|---|
| 1 | 158.22 | 158.32 | 20 | 70.42 | 69.28 |
| 2 | 136.55 | 136.54 | 1′ | 117.78 | 117.76 |
| 3 | 208.39 | 209.00 | 2′ | 41.02 | 41.02 |
| 4 | 73.30 | 73.54 | 3′ | 125.65 | 125.42 |
| 5 | 39.95 | 39.83 | 4′ | 134.07 | 135.02 |
| 6 | 134.99 | 138.91 | 5′ | 127.67 | 127.67 |
| 7 | 130.78 | 130.84 | 6′ | 130.78 | 130.84 |
| 8 | 39.07 | 38.94 | 7′ | 127.67 | 127.67 |
| 9 | 81.09 | 81.19 | 8′ | 126.47 | 126.49 |
| 10 | 55.35 | 55.46 | 1″ | 171.42 | |
| 11 | 33.04 | 33.06 | 2″ | 40.99 | |
| 12 | 35.73 | 35.72 | 3″ | 128.52 | |
| 13 | 84.44 | 84.49 | 4″ | 111.79 | |
| 14 | 80.63 | 80.78 | 5″ | 146.53 | |
| 15 | 146.53 | 146.37 | 6″ | 144.84 | |
| 16 | 110.69 | 110.68 | 7″ | 114.40 | |
| 17 | 18.75 | 18.79 | 8″ | 122.19 | |
| 18 | 19.83 | 19.85 | MeO | 55.97 | |
| 19 | 10.21 | 10.24 | | | |

In the previous literature, the $^{13}$C-NMR of tigliane and daphnane-type diterpenes from *E. poisonii* have not been reported. Thus, the $^{13}$C-NMR assignments for compounds **1**−**6** are shown in Tables 1 and 2, and these were based on DEPT, HETCOR, and COLOC experiments.

Results of the BST and of the cytotoxic activities against six human solid tumor cell lines for **1**−**6** are summarized in Table 3. Compound **3** is more than a thousand times as active as the others in the BST. Compounds **1**−**3**, **5,** and **6** showed a strong cytotoxic selectivity for the human kidney carcinoma (A-498) cell line.

Compound **5** was significantly less active but was still selective for A-498. Of all the tigliane esters isolated, **3**, hitherto unknown, demonstrated the strongest bioactivities and selectivity of over one million times for

**Table 3.** Lethality and Cytotoxicity of Compounds **1**−**6** in the BST Assay and Human Solid Tumor Cell Culture Systems

| compound | $LC_{50}$ ($\mu$g/mL) | $ED_{50}$ ($\mu$g/mL) | | | | | |
|---|---|---|---|---|---|---|---|
| | BST[a] | A-549[b] | MCF-7[c] | HT-29[d] | A-498[e] | PC-3[f] | PACA-2[g] |
| **1** | 191.04 | 18.89 | 41.76 | 30.77 | $2.18 \times 10^{-4}$ | 27.71 | 30.34 |
| **2** | 12.03 | 26.06 | 41.26 | 29.47 | $1.59 \times 10^{-7}$ | 35.10 | 26.90 |
| **3** | $1.10 \times 10^{-2}$ | $2.85 \times 10^{-1}$ | 2.30 | $3.83 \times 10^{-1}$ | $<10^{-7}$ | $2.44 \times 10^{-1}$ | 1.92 |
| **4** | 114.65 | 11.60 | 69.88 | 26.50 | 45.96 | 17.93 | 37.25 |
| **5** | 280.79 | 1.12 | 4.29 | 3.00 | $1.08 \times 10^{-1}$ | 2.90 | 2.44 |
| **6** | NT[i] | $5.41 \times 10^{-2}$ | 30.88 | 7.41 | $1.97 \times 10^{-2}$ | 4.56 | 3.55 |
| adriamycin[h] | NT[i] | $4.16 \times 10^{-3}$ | $3.76 \times 10^{-1}$ | $3.90 \times 10^{-2}$ | $1.87 \times 10^{-3}$ | $6.37 \times 10^{-2}$ | $1.63 \times 10^{-2}$ |

[a] Brine shrimp lethality test.[17,18] [b] Lung carcinoma.[19] [c] Breast carcinoma.[20] [d] Colon adrenocarcinoma.[21] [e] Kidney carcinoma.[19] [f] Prostate adenocarcinoma.[22] [g] Pancreatic carcinoma.[23] [h] Positive control. [i] NT: not tested.

the human kidney carcinoma (A-498); the presence of the cyclopropyl ring in the fatty acid ester substituent at C-13 of **3** would seem to contribute to this effect. Compounds that show selective activity at $ED_{50}$ values of 4 $\mu$g/mL and less in the cytotoxicity assay may be considered promising in our search for potential antitumor compounds from plant sources. Potent skin irritancy was noted when handling compound **5**.[9]

## Experimental Section

**General Procedures.** Column chromatography: silica gel (Merck, 60−200 mesh). HPLC: Rainin system equipped with Dynamax software, a Rainin UV-detector (set at 247 nm), normal phase 250 × 21 mm silica gel column. Optical rotations: Perkin-Elmer 241 polarimeter. IR spectra: Perkin-Elmer 1600 FTIR. UV spectra: Beckman DU-7 spectrometer. $^1$H and $^{13}$C-NMR: Varian VXR-500S spectrometer. Mass spectra: Finnigan 4000 spectrometer (low resolution), Kratos 50 spectrometer (high resolution).

**Plant material.** The latex of *E. poisonii* was collected into EtOH from plants found in the Kombosto local government area of Kano state, Nigeria, and preserved in EtOH. The botanical identification of the plant was carried out by Mr. Ali Garko and Dr. Y. Karatella of the Department of Biological Sciences, Bayero University, Kano. A reference sample of the plant is being cultivated in the Botanical Garden of Bayero University, Kano.

**Brine Shrimp Test (BST).** The BST was performed according to standard protocols,[17,18] and $LC_{50}$ values, in $\mu$g/mL, were determined for partitioned fractions, pooled chromatographic fractions, and the isolated compounds.

**Cytotoxic Assays.** Certain fractions and pure compounds were tested for cytotoxicity in a panel of six human solid tumor cell lines at the Purdue Cancer Center, using a 7-day MTT assay and standard protocols for A-549 (lung carcinoma),[19] MCF-7 (breast carcinoma),[20] HT-29 colon (adenocarcinoma),[21] A-498 (kidney carcinoma),[19] PC-3 (prostate adenocarcinoma),[22] and PACA-2 (pancreatic carcinoma)[23] with adriamycin as a positive control.

**Isolation.** The EtOH preserved latex of *E. poisonii* was evaporated under reduced pressure at 35 °C. The concentrated extract (706 g) was added to $H_2O$ and then partitioned with $CH_2Cl_2$. The $CH_2Cl_2$ layer was concentrated (214 g), redissolved in 10% aqueous MeOH, and further partitioned with hexane. The aqueous MeOH layer (33 g) showed significant activity in the BST, giving an $LC_{50}$ value of 114 $\mu$g/mL, and against certain human solid tumor cells in culture (A-549, $ED_{50}$ 4.84 $\mu$g/mL; MCF-7, $ED_{50}$ 26.94 $\mu$g/mL; HT-29, $ED_{50}$ 27.25 $\mu$g/mL; PC-3, $ED_{50}$ 12.23 $\mu$g/mL; and PACA-2, $ED_{50}$ 22.84 $\mu$g/mL); adriamycin gave $ED_{50}$ values of $3.93 \times 10^{-3}$, 1.51 x$10^{-1}$, $3.44 \times 10^{-2}$, $3.22 \times 10^{-2}$, and $2.47 \times 10^{-3}$ $\mu$g/mL in the same run. A portion of the MeOH soluble residue (26 g) was fractionated on an open column of silica gel (250 g) eluted with a hexane−EtOAc gradient. Eluants were analyzed by TLC, pooled into 12 fractions, and tested in the BST. The first fraction (1.44 g, BST $LC_{50}$ 45.8 $\mu$g/mL), eluted with hexane−EtOAc (9:1), the second (275 mg, BST $LC_{50}$ 4.02 $\mu$g/mL) and the third fractions (976 mg, BST $LC_{50}$ 106.49 $\mu$g/mL), eluted with hexane−EtOAc (8.5:1.5), and the eighth fraction (1.17 g, BST $LC_{50}$ 0.50 $\mu$g/mL), eluted with hexane−EtOAc (8:2), were separately refractionated on silica gel columns. On elution with hexane−$CHCl_3$ gradients, the columns for the first, second, and third fractions yielded **1**, **2**, and **5** which were repeatedly purified over normal phase HPLC to give 12-deoxyphorbol 20-acetate 13-angelate (**1**; 88.5 mg, $1.25 \times 10^{-2}$% w/w), 12-deoxyphorbol 20-acetate 13-phenylacetate (**2**; 17.8 mg, $2.52 \times 10^{-3}$% w/w), and resiniferol 20-(4-hydroxy-3-methoxyphenylacetate) 9,13,14-orthophenylacetate (**5**; 81.0 mg, $1.15 \times 10^{-2}$% w/w).

The eighth fraction (1.17 g) was rechromatographed on silica gel, eluting with a $CH_2Cl_2$−EtOAc gradient, to give a bioactive fraction (BST $LC_{50}$ 0.25 $\mu$g/mL) that was further purified on HPLC, eluting with hexane−MeOH−THF, to give 20-hydroxyresiniferol 9,13,14-orthophenylacetate (**6**; 3.1 mg, $4.39 \times 10^{-4}$% w/w), 20-hydroxy-12-deoxyphorbol 13-(*cis*-9,10-methylene)undecanoate (**3**; 19.5 mg, $2.765 \times 10^{-3}$% w/w), and 20-hydroxy-12-deoxyphorbol 13-angelate (**4**; 35.5 mg, $5.03 \times 10^{-3}$% w/w). The spectral data for the tiglianes (**1**, **2**, **4**) and the daphnane ortho esters (**5** and **6**) isolated here were consistent with reported[1,4,9,27,28] values. Their $^{13}$C-NMR data are shown in Tables 2 and 3. **3** is a new natural compound which appeared as a single peak in HPLC analyses (solvent EtOAc−hexane, 15:85; retention time, 55.8 min; flow rate 10 mL/min; detected at 247 nm, and solvent THF−MeOH−hexane, 0.04:0.36:96; retention time, 72.7 min; flow rate 10 mL/min; detected at 235 nm).

**20-Hydroxy-12-deoxyphorbol 13-(*cis*-9,10-methylene)-undecanoate (3)**: $[\alpha]^{23}_D$ +116 ($c$ = 1.3, $CHCl_3$); UV $\lambda_{max}$ (MeOH) nm 235 (14 400), 201 ($\epsilon$ 27 800); IR $\upsilon$ (film) cm$^{-1}$ 3401, 2924, 2856, 1704, 1645, 1454, 907; CI-MS $m/z$ (rel int) 511 $[MH - H_2O]^+$ (75), 313 (100), 295 (40), 199 (20), 91 (15); HRFABMS $m/z$ 551.3300 ($M^+$) for $C_{32}H_{48}O_6Na$ requires 551.3349; $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 7.59 (bs, H-1), 5.69 (bs, 9-OH), 5.68 (m, H-7), 3.97, 4.04 (AB dq, $J$ = 12.5, 5.0 Hz, $H_2$-20, coupled to OH-20), 3.26 (m, H-10), 2.99 (m, H-8), 2.46, 2.53 (AB q, $J$ = 19.0 Hz, $H_2$-5), 2.33 (bs, OH), 2.06, 1.49 (m, H-12), 1.96 (m, H-11), 1.87 (bs, OH), 1.76 (m, $H_3$-19), 1.65 (m, $H_2$-2′), 1.35−1.25 (m, $H_2$-3′ through H-9′), 1.23 (s, $H_3$-16), 1.15 (m, H-10′), 1.07 (s, $H_3$-17), 1.03, 0.96 (m, exo $CH_2$), 0.85 (superimposed, d, $J$ = 3.5 Hz, H-14), 0.86 (d, $J$ = 6.5 Hz, $H_3$-18), 0.89 (d, $J$ = 6.5 Hz, $H_3$-11′).

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