# Bioactive Diterpenoids, a New Jatrophane and Two *ent*-Abietanes, and Other Constituents from *Euphorbia pubescens*

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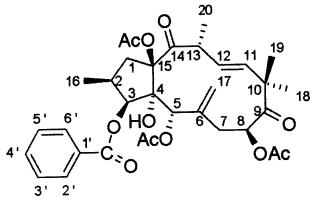
A new jatrophane diterpene, pubescenol (**1**), known *ent*-abietane lactones, helioscopinolide A (**2**) and B (**3**), and taraxerone, 24-methylenecycloartanol, and vanillin have been isolated from *Euphorbia pubescens*. Diterpenes **1**−**3** and previously described pubescene D (**4**) were shown to be moderate inhibitors of the growth of MCF-7, NCI-H460, and SF-268 human tumor cell lines, whereas compounds **2** and **3** also exhibited significant antibacterial activity against *Staphylococcus aureus*.

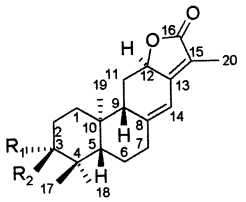
The genus *Euphorbia* has provided a wide range of polyoxygenated macrocyclic diterpenes, as jatrophanes and lathyranes, whose biological properties continue to be explored. Recent investigations revealed that macrocyclic diterpenes are promising modulators of multidrug resistance in tumor cells,[1−4] as well as microtubule-interacting agents.[5]

*Euphorbia pubescens* Vahl (Euphorbiaceae), an herb that grows wild in Portugal near banks of streams and rivers, has afforded several bioactive diterpenes.[3,6] In the search for new anticancer agents we carried out a reinvestigation of its constituents, which led to the isolation and characterization of a new jatrophane diterpene, pubescenol (**1**), two known *ent*-abietanolide derivatives (**2**, **3**), two triterpenes, and 4-hydroxy-3-methoxybenzaldehyde. Here, we also report the in vitro effect of diterpenes **1**−**3** on the growth of the human tumor cell lines MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and SF-268 (CNS cancer), as well as that of diterpene **4**, previously isolated from the same species.[3] The antibacterial activity of compounds **2** and **3** was also evaluated.

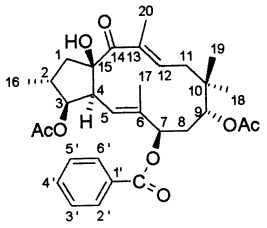
Pubescenol (**1**) was isolated as a white amorphous powder, whose molecular formula ($C_{33}H_{40}O_{11}$) was obtained from HREIMS (*m*/*z* 612.2574). Its IR spectrum exhibited characteristic bands at $\nu_{max}$ 1747 (ester carbonyl) and 1722 $cm^{-1}$ (ketone), an aromatic ring (1601, 1584, and 714 $cm^{-1}$), and a hydroxyl group (3428 $cm^{-1}$). The presence of acetyl groups and one benzoyl group was revealed by the EIMS data, which exhibited fragment peaks at *m*/*z* 552 [M − HOAc]$^+$, *m*/*z* 492 [M − 2 × HOAc]$^+$ and a base peak at *m*/*z* 105 [$C_6H_5CO$]$^+$. These structural features were corroborated by the NMR spectra of **1**, which showed the corresponding $^1$H and $^{13}$C signals for the acetyl and benzoyl groups. A broad singlet at $\delta$ 2.58, without correlation in the HMQC spectrum, confirmed the existence of a hydroxyl group in the molecule. The presence of one exomethylene group ($\delta$ 5.02 and 5.35), a *trans* disubstituted double bond ($\delta$ 5.48, d, $J$ = 15.6 Hz and 5.90, dd, $J$ = 9.6 and 15.6 Hz),



**1**



**2** $R_1$ = OH; $R_2$ = H
**3** $R_1$ = H; $R_2$ = OH



**4**

three oxymethine protons ($\delta$ 4.96, d, $J$ = 3.2 Hz; 5.94, s; 5.38 d, $J$ = 8.8 Hz), as well as two secondary ($\delta$ 1.25, d, $J$ = 6.8 Hz; 1.40, d, $J$ = 7.2 Hz) and two tertiary methyl groups ($\delta$ 1.18 and 1.42) was also revealed by the $^1$H NMR spectrum. $^{13}$C NMR and DEPT spectra of **1** showed signals corresponding to four $CH_3$, three $CH_2$ (one $sp^2$, $\delta_C$ 119.5), seven methines (two olefinic at $\delta_C$ 132.7 and 135.3 and three methines geminal to ester functions at $\delta_C$ 72.4, 75.9, and 86.6), and six quaternary carbons (two keto functions at $\delta_C$ 204.8 and 208.8, a C−OH at $\delta_C$ 88.5, and an olefinic carbon at $\delta_C$ 136.1). The above data suggested a bicyclic diterpenoid skeleton for **1**.[7] Proton−carbon correlations were assigned from the HMQC experiment, whereas the $^1$H−$^1$H COSY ($^3J$ couplings) spectrum revealed the structure of four proton spin-systems A−D (see Supporting

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**Table 1.** Effect of Compounds **1**−**4** on the Growth of Three Human Tumor Cell Lines[a]

| compound | $GI_{50}$ ($\mu$M) MCF-7 (breast) | NCI-H460 (lung) | SF-268 (CNS) |
|---|---|---|---|
| pubescenol (**1**) | 69.04 ± 4.59 | 55.56 ± 3.95 | 75.16 ± 6.54 |
| helioscopinolide A (**2**) | 67.50 ± 3.04 | 72.78 ± 6.33 | >100 |
| helioscopinolide B (**3**) | 76.99 ± 8.07 | >100 | >100 |
| pubescene D (**4**) | 37.50 ± 3.06 | 37.50 ± 0.65 | 90.12 ± 2.47 |
| doxorubicin[b] | 42.8 ± 8.2 | 94.0 ± 8.7 | 93.0 ± 7.0 |

[a] Results are expressed as $GI_{50}$ (concentrations of compounds that cause 50% inhibition of cell growth). Compounds were tested at a maximum concentration of 100 $\mu$M. Results are the mean ± SEM of 3−6 independent experiments performed in duplicate. [b] Data from the positive control doxorubicin are expressed in nM.

Information). The observed long-range coupling of the exomethylene group with the methylene protons at $\delta$ 1.95 (dd, $J$ = 8.8 and 18.8 Hz, H-7α) and 2.87 (brd, $J$ = 18.8 Hz, H-7$\beta$) and the heteronuclear correlations displayed in the HMBC spectrum established the structural frame of **1** and the position of the functional groups. When compared to similar compounds,[7] the carbon data of **1** showed paramagnetic effects at C-3 (≅10 ppm), C-5 (≅2 ppm), and C-15 (≅3 ppm), which is due to the unusual presence of a hydroxyl group at C-4. A diamagnetic effect at C-1 (≅4 ppm) was also observed.

The relative configuration of **1** was deduced by means of a NOESY spectrum assuming an α orientation for OH-4, as a reference point, by analogy with H-4α found in jatrophane diterpenes.[8−10] NOE effects were observed between OH-4α/H-3, thus providing evidence for the $\beta$ orientation of the benzoyl group, which, in turn, gave a nuclear Overhauser effect at H-5 and at the acetyl methyl group on C-15. NOE cross-peaks between OAc-15/Me-16, Me-16/H-1$\beta$, and H-1$\beta$/H-13 indicated their $\beta$ configuration. Furthermore, on the basis of the NOE correlations between H-13/H-11 (very strong), H-11/Me-19, and H-12/Me-18 it was concluded that H-11 is directed above and H-12 below the plane of the macrocycle and Me-19 is on the $\beta$ and Me-18 on the α face. Similarly, the α orientation of H-8 was deduced from the strong NOE enhancement observed between Me-18/H-8. No NOE effects were observed between OH-4α/OAc-15, supporting the *trans* cyclopentane/macrocyclic ring junction.

The diterpene lactones with an *ent*-abietane skeleton, helioscopinolide A (**2**) and helioscopinolide B (**3**), as well as taraxerone, 24-methylenecycloartanol, and 4-hydroxy-3-methoxybenzaldehyde, were identified by comparison of their spectral data with those reported in the literature.[11−17]

Compounds **1**−**4** were evaluated for their capacity to inhibit the in vitro growth of MCF-7, NCI-H460, and SF-268 cell lines, after continuous exposure for 48 h. Results from Table 1 show that compounds **1** and **4** are moderate growth inhibitors of all the cell lines tested. Compounds **2** and **3** were inactive against the SF-268 cell line even when tested at 100 $\mu$M. An investigation of the antibacterial activity was undertaken using a bioautographic agar overlay method.[18] Helioscopinolides A (**2**) and B (**3**) showed significant activity against *Staphylococcus aureus* 6538P (2.5 $\mu$g/spot) and no effect against *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, even when tested at 40 $\mu$g/spot. Moderate antibacterial activity against *Moraxella catarrhalis* has been previously found for compound **2**.[19]

## Experimental Section

**General Experimental Procedures.** Optical rotations were obtained using a Perkin-Elmer 241-MC polarimeter. IR spectra were determined on a Perkin-Elmer 1310 instrument. The NMR spectra were recorded on a Bruker ARX-400 NMR spectrometer ($^1$H 400 MHz; $^{13}$C 100.61 MHz), with TMS as internal standard and $CDCl_3$ as solvent. EI-MS were taken at 70 eV on a Kratos MS25RF spectrometer and the HREIMS on a Finnigan-FT-2001. Column chromatography was carried out on silica gel (Merck 9385). HPLC was carried out on a Merck-Hitachi instrument, with UV detection, using a Merck Lichrospher 100 RP-18 (10 $\mu$m, 250 × 10 mm) column.

**Plant Material.** *E. pubescens* was collected near Porto Alto, Portugal, in June 2000 and identified by Dr. Teresa Vasconcelos (plant taxonomist) of Instituto Superior de Agronomia, University of Lisbon. A voucher specimen (no. 249) has been deposited at the herbarium (LISI) of Instituto Superior de Agronomia.

**Extraction and Isolation.** The air-dried whole plant (5 kg) was powdered and extracted exhaustively with MeOH (6 × 14 L) at room temperature, and the residue (330 g) was suspended in a MeOH/$H_2O$ solution and partitioned sequentially with hexane and $Et_2O$. The ether extract (67 g) was chromatographed on silica gel (1.7 kg), with mixtures of hexane/EtOAc and EtOAc/MeOH (0:1 to 1:0) as eluents, to give fractions A (5 g; *n*-hexane/EtOAc, 1:1 to 2:3); B (3.62 g; *n*-hexane/EtOAc, 2:3 to 3:7), C (0.53 g; *n*-hexane/EtOAc, 1:0 to 11:9), and D (1.46 g; *n*-hexane/EtOAc, 11:9 to 1:1). Fraction A was chromatographed on silica gel (250 g) using *n*-hexane/$CH_2Cl_2$ (1:4 to 0:1) and $CH_2Cl_2$/EtOAc mixtures (1:0 to 0:1). The column chromatographic fractions (100 mL each) were combined into F-$A_1$ (100 mg; $CH_2Cl_2$), F-$A_2$ (1.03 g; $CH_2Cl_2$), and F-$A_3$ (1.13 g; $CH_2Cl_2$/EtOAc, 1:0 to 9:1). F-$A_2$ was repeatedly chromatographed on silica gel columns with *n*-hexane/EtOAc mixtures to yield a mixture (298 mg), which was further purified by HPLC (MeCN/$H_2O$, 3:2, 5 mL/min) to afford 7 mg ($t_R$ 20 min) of **1**. Vanillin (6 mg) and compound **3** (4 mg) were obtained from fractions F-$A_1$ and F-$A_2$. Fraction B furnished **3** (34 mg) and **2** (25 mg), and from fractions C and D were obtained, respectively, taraxerone (13 mg) and 24-methylenecycloartanol (5 mg) (see Supporting Information for details of the isolation and structure elucidation of known compounds).

**Pubescenol, (5α,8$\beta$,15$\beta$-triacetoxy-3$\beta$-benzoyloxy-4α-hydroxy-9,14-dioxo-13$\beta$*H*-jatropha-6(17),11E-diene) (1):** white amorphous powder; $[\alpha]_D^{25}$ + 36° (*c* 0.10, $CHCl_3$); IR (film), $\nu_{max}$ 3428, 1747, 1722, 1601, 1584, 1457, 1370, 1278, 1237, 714 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 7.99 (2H, d, $J$ = 7.6 Hz, H-2′, H-6′), 7.59 (1H, t, $J$ = 7.6 Hz, H-4′), 7.44 (2H, t, $J$ = 7.6 Hz, H-3′, H-5′), 5.94 (1H, s, H-5), 5.90 (1H, dd, $J$ = 9.6, 15.6 Hz, H-12), 5.48 (1H, d, $J$ = 15.6 Hz, H-11), 5.38 (1H, d, $J$ = 8.8 Hz, H-8), 5.35 (1H, brs, H-17b), 5.02 (1H, brs, H-17a), 4.96 (1H, d, $J$ = 3.2 Hz, H-3) 4.04 (1H, m, H-13), 3.30 (1H, dd, $J$ = 8.0, 14.0 Hz, H-1α), 2.87 (1H, brd, $J$ = 18.8 Hz, H-7$\beta$), 2.58 (1H, brs, 4-OH), 2.55 (1H, dd, $J$ = 10.8, 14.0 Hz, H-1$\beta$), 2.22 (3H, s, 15-$COCH_3$), 2.20 (1H, m, H-2), 2.04 (3H, s, 8-$COCH_3$), 1.95 (1H, dd, $J$ = 8.8, 18.8 Hz, H-7α), 1.87 (3H, s, 5-$COCH_3$), 1.42 (3H, s, Me-18), 1.40 (3H, d, $J$ = 7.2 Hz, Me-16), 1.25 (3H, d, $J$ = 6.8 Hz, Me-20), 1.18 (3H, s, Me-19); $^{13}$C NMR ($CDCl_3$, 100.61 MHz) $\delta$ 208.8 (C, C-9), 204.8 (C, C-14), 170.2 (C, 8-$COCH_3$), 168.5 (C, 15-$COCH_3$), 167.9 (C, 5-$COCH_3$), 165.4 (C, $COC_6H_5$), 136.1 (C, C-6), 135.3 (CH, C-11), 133.5 (CH, C-4′), 132.7 (CH, C-12), 129.6 (CH, C-2′, C-6′), 129.4 (C, C-1′), 128.6 (CH, C-3′, C-5′), 119.5 ($CH_2$, C-17), 94.6 (C, C-15), 88.5 (C, C-4), 86.6 (CH, C-3), 75.9 (CH, C-5), 72.4 (CH, C-8), 49.9 (C, C-10), 46.7 (CH, C-13), 38.7 ($CH_2$, C-1), 38.7 (CH, C-2), 30.9 ($CH_2$, C-7), 23.9 ($CH_3$, C-18), 23.9 ($CH_3$, C-19), 21.8 ($CH_3$, 15-$COCH_3$), 20.6 ($CH_3$, 5-$COCH_3$), 20.5 ($CH_3$, 8-$COCH_3$), 20.0 ($CH_3$, C-16), 18.9 ($CH_3$, C-20); EIMS *m*/*z* 612 $[M]^+$ (4), 552 $[M - HOAc]^+$ (3), 492 $[M - 2 \times HOAc]^+$ (2), 123 (15), 105 $[C_6H_5CO]^+$ (100), 96 (73), 77 $[C_6H_5]^+$ (9), 43 (26); HREIMS *m*/*z* 612.2574 (calcd for $C_{33}H_{40}O_{11}$, 612.2571).

**Tumor Cell Growth Assay.** Stock solutions of compounds **1**−**4** were prepared in DMSO at 400 times the desired final maximum test concentration and stored at −20 °C. The frozen samples were freshly diluted with culture medium prior the assay. Final concentrations of DMSO did not interfere with

the cell growth. The effects of compounds on the growth of tumor cell lines were evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) for the in vitro anticancer drug screening that uses the protein-binding dye sulforhodamine B (SRB) to assess growth inhibition.[20,21] Three human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and SF-268 (CNS cancer). Cells were exposed for 48 h to five concentrations of compounds starting from a maximum concentration of 100 $\mu$M. Doxorubicin was used as positive control. Growth inhibition of 50% ($GI_{50}$) was determined as described.[20]

**Antibacterial Assay.** Antibacterial assays on pure compounds, fractions, and extracts were performed against *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853, using a bioautographic agar overlay method.[18] Each compound (1.25−40 $\mu$g), dissolved in $CH_2Cl_2$, was applied as a spot (3 mm), with a graduated micropipet, to precoated TLC plates (10 × 10 cm), which were left in a fume cupboard for 24 h to ensure evaporation of the solvents. The overlay medium was mixed with 0.5 mL of a 5% 2,3,5-triphenyltetrazolium chloride (Merck) aqueous solution and inoculated with 250 $\mu$L of the bacterial culture. The plates were incubated at 37 °C for 24 h to reveal active compounds as clear spots against a pink-red background. Ceftazidim (0.8 $\mu$g, Glaxo-Wellcome), dissolved in $H_2O$, was used as positive control.

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**Supporting Information Available:** Details of the NMR experiments performed on compound **1**. Included are Figure 1 showing $^1$H spin systems, selected HMBC correlations of **1**, and details of the isolation and identification of compounds **2** and **3** and taraxerone, 24-methylenecycloartanol, and vanillin. This information is available via the Internet, free of charge, from http://pubs.acs.org.

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