Abietane Diterpenoids from *Salvia pachyphylla* and *S. clevelandii* with Cytotoxic Activity against Human Cancer Cell Lines

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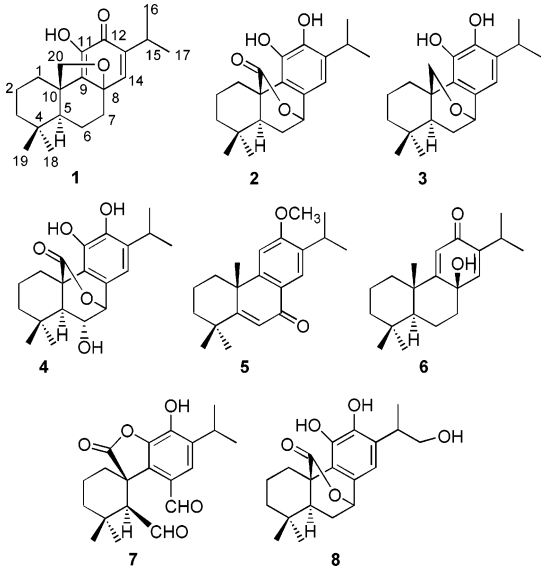
A phytochemical study has been carried out on the aerial parts of *Salvia pachyphylla* and *S. clevelandii*. From *S. pachyphylla*, the known diterpenes carnosol (**2**), rosmanol, 20-deoxocarnosol (**3**), carnosic acid, isorosmanol (**4**), 7-methoxyrosmanol, 5,6-didehydro-*O*-methylsugiol (**5**), 8β-hydroxy-9(11),13-abietadien-12-one (**6**), 11,12-dioxoabieta-8,13-diene, and 11,12-dihydroxy-20-norabieta-5(10),8,11,13-tetraen-1-one were isolated, together with the new diterpene pachyphyllone (**1**). From *S. clevelandii*, the known diterpenes rosmadial (**7**), 16-hydroxycarnosol (**8**), abieta-8,11,13-triene, and taxodone were obtained, together with carnosol (**2**), rosmanol, and carnosic acid. The structure of the new compound (**1**) was identified on the basis of spectroscopic data analysis. Several of these compounds (**1**−**8**) were evaluated against a small panel of human cancer cell lines.

Over five hundred species of the genus *Salvia* (Lamiaceae) are found worldwide, and they have been used in traditional medicinal systems for generations.[1,2] In the course of a study of the chemical composition of the flora used in Latin American popular medicine, we have performed phytochemical studies of extracts of the aerial parts from two *Salvia* species, *S. pachyphylla* Munz and *S. clevelandii* (A. Gray) E. Greene. Both are endemic plants to Baja California Peninsula (Mexico) and California (USA), and *S. pachyphylla* is used by indigenous communities for its medicinal properties in the treatment of flu symptom.[3] There do not appear to be any previous studies on the phytochemistry of *S. pachyphylla*. Tucker et al.[4] studied the volatile terpenes in *S. clevelandii* and found that the major components were camphor (32%) and 1,8-cineole (20%). In this paper, the major secondary metabolites isolated from these two species are described, and the cytotoxic effects against five human cancer cells are reported for eight of the compounds obtained (**1**−**8**).

A cold acetone extract of the aerial parts of *S. pachyphylla* was chromatographed on silica gel repeatedly to give the known secondary metabolites carnosol[5,6] (**2**), rosmanol,[7] 20-deoxocarnosol[8] (**3**), carnosic acid,[6] isorosmanol[9] (**4**), 7-methoxyrosmanol,[10] 5,6-didehydro-*O*-methylsugiol[11] (**5**), 8β-hydroxy-9(11),13-abietadien-12-one[12] (**6**), 11,12-dioxoabieta-8,13-diene,[13] and 11,12-dihydroxy-20-norabieta-5(10),8,11,13-tetraen-1-one[14] and the new diterpene **1**.

The low-resolution mass spectrum of **1** showed a molecular ion $[M]^+$ at *m*/*z* 316 ($C_{20}H_{28}O_3$ by HREIMS). The IR spectrum exhibited characteristic bands for hydroxyl (3399 cm^{-1}), α,β-unsaturated ketone (1718 cm^{-1}), and ether (1013 cm^{-1}) groups. In the ^{1}H NMR spectrum, signals for a proton heptuplet (δ 2.95), together with two methyl groups as a doublet at δ 1.09, showed



the presence of a vinylic isopropyl group, with two angular methyl group signals (δ 1.01 and 0.96) also being observed. In the low-field region of the spectrum, a singlet at δ 6.37 (interchangeable with D_2O) was assigned to a vinylic hydroxyl group, two doublets at δ 4.54 and 3.69 with J = 8.0 Hz to an oxymethylene system, and a one-proton singlet at δ 6.78 to H-14. The COSY ^{1}H−^{1}H NMR experiment confirmed the presence of the vinylic isopropyl group and the methylene group linked an oxygen atom. The large difference of the chemical shifts of these protons suggested that they belong to an ether bridge. This was confirmed by a HMBC NMR experiment in which the quaternary carbons at δ 79.0 (C-8) and 136.7 (C-9) showed long-range couplings with the AB system doublets at δ 3.69 and 4.54 (C-20, δ 78.3). In the same spectrum, the carbon signal at δ 182.5 (C-12) showed long-range coupling with a vinylic proton at δ 6.78 (C-14, δ 142.8) and H-15 (δ 2.95) of the isopropyl group on C-13, which together with the long-range coupling between C-14 (δ 142.8) and H-15 was used to establish the partial structure of an isopropyl enone system. The connectivity of this system and the ether bridge was fixed from the long-range

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Table 1. ^{1}H NMR, ^{13}C NMR, and HMBC Data of Compound **1** in $CDCl_3$

position	δ_H[a]	δ_C[b]	HMBC[c]
1	α 1.85 m β 2.56 td (3.8, 13.9)	31.3 (t)	2*, 9, 10, 20
2	1.61 (overlapping) 1.83 (overlapping)	19.9 (t)	
3	1.34 m	41.8 (t)	
4		33.9 (s)	
5	1.65 m	50.3 (d)	6*, 7, 9, 10*, 18, 19, 20
6	α 1.61 overlapping signal β 1.83 overlapping signal	20.1 (t)	7*, 8, 10
7	1.47 m 2.18 dd (2.0, 6.0)	41.9 (t)	5, 8*, 9, 14
8		79.0 (s)	6, 7*, 20
9		136.7 (s)	1, 5, 7, 14, 20, Ar-O*H*
10		48.8 (s)	1*, 5, 6, 20*
11	6.37 s (O*H*)	142.8 (s)	9, 11*, 12
12		182.5 (s)	14, 15, Ar-O*H*
13		142.2 (s)	15*, 16, 17
14	6.78 s	142.8 (d)	7, 9, 12, 15
15	2.95 hept. (6.8)	26.9 (d)	12, 13*, 14, 16*, 17*
16	1.09 d (6.8)	21.8 (q)	13, 15*, 17
17	1.09 d (6.8)	21.2 (q)	13, 15*, 16
18	0.96 s	32.9 (q)	3, 5, 19
19	1.01 s	22.0 (q)	3, 5, 18
20	3.69 d (8.0) 4.54 d (8.0)	78.3 (t)	1, 5, 8, 9, 10*

[a] δ in ppm, J values (in parentheses) in Hz. [b] δ in ppm, data based on DEPT and HSQC experiments. [c] Two-bond coupling enhancements designated by asterisks.

coupling of the enolic proton resonance (δ 6.37) and those of carbons C-9 (δ 136.7) and C-12 (δ 182.5), together with correlations between H-20 (δ 3.69 and 4.54) and C-9 (δ 136.7). The ^{13}C NMR spectrum showed signals for 20 carbon atoms, including a signal resonating at δ 182.5 (s) assigned to the α,β-unsaturated carbonyl group (C-12) and signals at δ 78.3 (t), 79.0 (s), and 142.8 (s) assignable to three carbons bearing oxygens. A NOE effect observed in a ROESY experiment between one proton on C-20 with H-6β and the three-proton singlet corresponding to the β-methyl group at C-4 (Me-19) confirmed the relative configuration of C-8 and C-10 with the ether bridge in the β-position. Analysis of all of the above data, together with the results from ^{13}C NMR, COSY, HSQC, and HMBC experiments (see Table 1), led to the structure for **1** as shown, which has been named pachyphyllone.

From the aerial parts of *S. clevelandii* the known diterpenes carnosol[5,6] (**2**), rosmanol,[7] and carnosic acid,[6] as isolated from *S. pachyphylla*, together with the known diterpenes rosmadial[15] (**7**), 16-hydroxycarnosol[16] (**8**), abieta-8,11,13-triene,[17] and taxodone,[18,19] were obtained and identified by comparison with spectoscopic data published in the literature.

The cytotoxic activity of compounds **1**−**8** was evaluated in vitro against A2780 ovarian cancer, SW1573 non-small-cell lung cancer, WiDr colon cancer, T-47D breast cancer, and HBL-100 breast cancer cells. Growth inhibition 50% (GI_{50}) values were determined using the National Cancer Institute (NCI) protocol, with slight modifications,[20] and the results are shown in Table 2. Overall, the most sensitive cell lines against these compounds were A2780 and HBL-100, while the SW1573, WiDr, and T-47D cell lines were more resistant. According to the results obtained, the most active compounds, **2**, **3**, and **8**, showed GI_{50} values in the range 3.6−35 μM for the five cell lines, with the A2780 and HBL-100 cell lines being the most sensitive, with GI_{50} values in the range 3.6−5.4 μM. Compounds **1** and **4**−**7** were less active against all cell lines. The observation that the majority of the compounds assayed showed antiproliferative activity against sensitive and resistant tumor cell lines is a promising result, since colon cancer cells are more resistant than ovarian cancer cells to conventional anticancer drugs.[21] In addition, the results obtained against the breast cancer cells suggest that these compounds may have a mechanism of action independent of the estrogen receptor (ER).[22] T-47D cells are ER positive, while HBL-100 cells lack this receptor and are ER negative.[22] Therefore, some of these abietane compounds may be useful leads for the development of novel antitumor drugs toward hormone-independent breast cancer.

Table 2. Growth Inhibition 50% (GI_{50}) Values against Human Solid Tumor Cells[a]

	cell line				
compound	A2780 (ovarian)	SW1573 (NSCLC)	WiDr (colon)	T-47D (breast)	HBL-100 (breast)
1	14 ± 3.5	23 ± 3.8	36 ± 1.2	32 ± 1.1	22 ± 0.3
2	3.6 ± 1.0	10 ± 8.2	26 ± 1.4	24 ± 2.8	3.9 ± 0.4
3	5.4 ± 2.4	22 ± 6.5	32 ± 3.8	32 ± 2.0	4.6 ± 0.5
4	19 ± 2.0	23 ± 8.6	>100	>100	17 ± 2.2
5	16 ± 3.0	18 ± 5.0	29 ± 2.0	33 ± 7.1	16 ± 1.5
6	19 ± 3.8	29 ± 2.3	83 ± 30	82 ± 31	26 ± 3.3
7	36 ± 2.5	32 ± 2.4	59 ± 36	48 ± 14	26 ± 0.75
8	3.6 ± 0.6	9.0 ± 5.0	28 ± 1.2	35 ± 5.1	3.6 ± 0.3

[a] Values given in μM ± standard deviation and are means of three to five experiments.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter. IR spectra were obtained on a Bruker IFS 28/55 (FTIR) spectrometer. UV spectra were recorded on a JASCO V-560 spectrophotometer. NMR spectra were recorded on Bruker Avance 300 MHz and Bruker Avance 400 MHz spectrometers in $CDCl_3$, unless otherwise noted. Chemical shifts are given in ppm with TMS as the internal standard. Low- and high-resolution mass spectra were run on a VG Micromass ZAB-2F mass spectrometer at 70 eV. Merck silica gel (0.063−0.200 mm) and Sephadex LH-20 were used for column chromatography. Analytical thin-layer chromatography (TLC) and preparative TLC were carried out on precoated Schleicher and Schüll plates.

Plant Material. Stems, leaves, and flowers of *Salvia pachyphylla* were collected from Sierra de San Pedro Mártir, Baja California, Mexico, in November 2000, and identified by one of the authors (J.D.). A voucher plant specimen (BCMEX9783) was deposited at the BCMEX herbarium, Faculty of Sciences, Universidad de Baja California Campus de Ensenada. Stems, leaves, and flowers of *Salvia clevelandii* were collected from North Ensenada, Baja California, Mexico, in January 2001, and also identified by J.D. A voucher specimen (BCMEX8715) was again deposited at the BCMEX herbarium.

Extraction and Isolation. Dried stems, leaves, and flowers of *S. pachyphylla* (460 g) were extracted with acetone at room temperature, and the solvent was removed under reduced pressure at 40 °C, affording an extract (41 g) that was subjected to flash chromatography on silica gel with mixtures of *n*-hexane/ethyl acetate of increasing polarity. Repeated chromatography on silica gel and Sephadex LH-20 elicited the following products: pachyphyllone (**1**) (6 mg), carnosol (**2**) (44 mg), rosmanol (20 mg), 20-deoxocarnosol (**3**) (15 mg), carnosic acid (23 mg), isorosmanol (**4**) (9 mg), 7-methoxyrosmanol (17 mg), 5,6-didehydro-*O*-methylsugiol (**5**) (13 mg), 8β-hydroxy-9,13-abietadien-12-one (**6**) (17 mg), 11,12-dioxoabieta-8,13-diene (8 mg), and 11,12-dihydroxy-20-norabieta-5(10),8,11,13-tetraen-1-one (11 mg).

Dried stems, leaves, and flowers of *S. clevelandii* (427 g) were extracted with distilled acetone at room temperature, and the solvent was removed under reduced pressure at 40 °C, providing an extract (36.2 g), which was subjected to flash chromatography on silica gel with mixtures of *n*-hexane/ethyl acetate of increasing polarity. Repeated chromatography on Sephadex LH-20 and silica gel gave the following products: carnosol (**2**) (17 mg), rosmanol (11 mg), carnosic acid (11 mg), rosmadial (**7**) (2.9 mg), 16-hydroxycarnosol (**8**) (43 mg), abieta-8,11,13-triene (3.7 mg), and taxodone (4.5 mg).

Pachyphyllone (1): amorphous solid; $[\alpha]^{20}_D$ −147.5 (*c* 0.002, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 350 (5.24), 206 (5.01) nm; IR (film) ν_{max} 3399, 2924, 1718, 1644, 1621, 1459, 1367, 1047 cm^{-1}; ^{1}H NMR (400 MHz, see Table 1); ^{13}C NMR (75 MHz, see Table 1); EIMS *m/z*

316 $[M]^+$ (100), 245 (11), 205 (14), 180 (34), 149 (20), 135 (11), 81 (11), 69 (18), 57 (27), 55 (20); HREIMS *m*/*z* 316.2065 (calcd for $C_{20}H_{28}O_3$ 316.2038).

Cytotoxicity Testing. RPMI 1640 medium was purchased from Flow Laboratories (Irvine, UK), fetal calf serum (FCS) from Gibco (Grand Island, NY), trichloroacetic acid (TCA) and L-glutamine from Merck (Darmstadt, Germany), and penicillin G, streptomycin, dimethyl sulfoxide (DMSO), and sulforhodamine B (SRB) from Sigma (St. Louis, MO). The human solid tumor cell lines A2780 (ovarian cancer), SW1573 (non-small-cell lung cancer), WiDr (colon cancer), T-47D (breast cancer), and HBL-100 (breast cancer) were used in this study. These cells lines were a kind gift from Professor Godefridus J. Peters (VU University Medical Center, Amsterdam). Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum and 2 mM L-glutamine in a 37 °C, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and resuspended in antibiotic-containing medium (100 units penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions displaying >97% viability by trypan blue exclusion dye were subsequently counted. After counting, dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated in a volume of 100 μL per well at densities of 6000 (SW1573, HBL-100), 7000 (A2780), 12 000 (WiDr), and 15 000 (T-47D) cells per well, based on their doubling times.

Chemosensitivity tests were performed using the SRB assay of the National Cancer Institute, with slight modifications. Briefly, pure compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicate at different dilutions in the range 1−100 μM. Test compound treatment was started on day 1 after plating at incubation times of 48 h, after which time cells were precipitated with 25 μL of ice-cold 50% (w/v) trichloroacetic acid and fixed for 60 min at 4 °C. The SRB assay was performed by measuring the optical density (OD) of each well at 490 nm, using a ELx800NB absorbance microplate reader (BioTek). Values were corrected for background OD from wells only containing medium. The percentage growth (PG) was calculated with respect to untreated control cells (C) at each of the drug concentration levels based on the difference in OD at the start (T_0) and end of drug exposure (T), according to NCI protocols. Therefore, if T is greater than or equal to T_0, the calculation was $100 \times [(T - T_0)/(C - T_0)]$. If T is less than T_0, denoting cell killing, the calculation was $100 \times [(T - T_0)/(T_0)]$. The effect was defined as the percentage of growth, where 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and 50% cell killing (LC_{50}) represent the concentration at which PG is +50, 0, and −50, respectively. With these calculations a PG value of 0 corresponds to the amount of cells present at the start of drug exposure, while negative PG values denote net cell kill.

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