Isolation and Structure of Pedilstatin from a Republic of Maldives Pedilanthus sp. 1

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Isolation and Structure of Pedilstatin from a Republic of Maldives *Pedilanthus* sp.[1]

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A new cancer cell growth inhibitor designated pedilstatin (**1**) was isolated from a Republic of Maldives *Pedilanthus* sp. The structure was determined to be 13-*O*-acetyl-12-*O*-[2′*Z*,4′*E*-octadienoyl]-4α-deoxyphorbol on the basis of high-resolution mass spectral and 2D NMR assignments. Pedilstatin was found to significantly inhibit growth of the P388 lymphocytic leukemia cell line with an ED_{50} of 0.28 μg/mL, to afford, at concentrations of 2−5 μM, protection (to 80%) of human-derived lymphoblastoid CEM-SS cells from infection and cell-killing by HIV-1, and to show inhibition of protein kinase C with a K_i of 620 ± 20 nM.

Terrestrial plants are well-established sources of very useful anticancer drugs that range structurally from simple stilbenes[2] and lignans to complex indole alkaloids.[3a−c] Recent advances in the discovery of new plant cancer cell growth inhibitors include a camptothecin glycoside,[4a] taxanes,[4b,c] lignans,[4d,e] acetogenins,[4f] xanthones and benzophenones,[4g] phenols,[4h] sesquiterpenes,[4i] steroids,[4j] and triterpene glycosides[4k] as well as berberine alkaloids.[4l] The higher plants are proving also to be increasingly useful sources of microorganisms producing antineoplastic substances such as paclitaxel and recently sequoiatones A and B from an endophytic fungus of the California coast redwood (*Sequoia sempervirens*).[5] Plants of the family Euphorbiaceae have a long history of use in traditional medical practices.[6] We have been investigating a number of such species from which, for example, we earlier isolated and determined the structures of the phyllanthostatins.[7a−c] Phyllanthostatin 1/phyllanthoside has completed an initial phase 1 human cancer clinical trial.[7d] Because of our continued interest in the Euphorbiaceae, in 1988 we first collected samples of the aerial portion of a Republic of Maldives *Pedilanthus* sp. and completed a scale-up recollection in 1989.

Results and Discussion

Initial separation of a methanol extract gave a dichloromethane (DCM) fraction with significant activity (ED_{50} 0.57 μg/mL) against the murine P388 lymphocytic leukemia cell line. The DCM extract was subjected to a series of chromatographic separations on Sephadex LH-20, and subsequent purification by HPLC afforded a new phorbol-type terpene as a yellow oil designated pedilstatin (**1**) and providing a P388 ED_{50} of 0.28 μg/mL. The molecular formula of pedilstatin (**1**) was deduced as $C_{30}H_{40}O_7$ from HRFAB (*m*/*z* 535.2657 $[M + Na]^+$, Δ 1.5 mmu), implying 11 degrees of unsaturation. The ^{13}C NMR spectrum clearly exhibited 30 carbon resonances, and the APT spectrum of terpene **1** contained signals that were attributed to three carbonyls (δ_C 213.2, 173.5, and 166.0 ppm), two oxygenated quaternary carbons (δ_C 78.0 and 65.2 ppm), two olefinic quaternary carbons (δ_C 143.3 and 137.0 ppm), one aliphatic quaternary carbon (δ_C 25.1 ppm), one oxygenated methine carbon (δ_C 74.7 ppm), six olefinic methine carbons (δ_C 156.1, 146.3, 146.2, 127.0, 126.4, and 114.8 ppm), five aliphatic methine carbons (δ_C 49.6, 47.4, 43.3, 40.6, and 36.9 ppm), one oxygenated methylene carbon (δ_C 69.2 ppm), three aliphatic methylene carbons (δ_C 35.0, 25.1, and 22.0 ppm), and six methyl groups (δ_C 24.1, 21.1, 16.3, 13.7, 11.8, and 10.4 ppm) (Table 1).

The presence of two ester groups was deduced by mass spectroscopy (FAB), which showed the presence of ion fragments at *m*/*z* at 373.2 ($[M + H]^+ - 140$) and 313.2 ($[M + H]^+ - 140 - 60$). The ^1H NMR, COSY, and HMBC spectra revealed those units to be 2*Z*,4*E*-octadienoate and acetate groups. An α,β-unsaturated ketone was ascertained from initial analysis of 2D NMR (COSY, TOCSY, and HMBC), leaving four more unsaturated units unsolved. More detailed interpretation of the 2D spectra located a CH_3-C-CH_3 group (δ_C 24.1/δ_H 1.11, δ_C 25.1, and δ_C 16.3/δ_H 1.14 ppm) fused with a cyclohexane at C-13/C-14 and the ester side chains attached at C-12 and C-13. The final two unsaturated units were secured by observation of a cycloheptene moiety between the cyclohexane and the cyclopentenone when further 2D NMR signals were elucidated. The last two methyls (δ_C 10.4/δ_H 1.71 and δ_C 11.8/δ_H 1.03 ppm) and one oxygenated methylene group (δ_C 69.2/δ_H 3.94 and 3.82 ppm) were assigned at C-2, C-11, and C-6, respectively. These data suggested that **1** was a 4-deoxyphorbol susceptible to epimerization at the C-4 ring juncture.

As just noted, 4-deoxyphorbol esters are the least stable of the tigliane diterpenes. They occur as either A/B ring *trans* or *cis*. These compounds readily epimerize to their A/B *cis* isomer to form the 4α-deoxyphorbol esters, when

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Table 1. Pedilstatin (**1**) ^{1}H and ^{13}C NMR, APT, and HMBC Spectral Summary (δ in ppm)

position	APT	δ_C	δ_H	HMBC
1	CH	156.1	6.97 (1H, br, s)	H-10, H-19, 9-OH
2	C	143.3		H-19
3	CO	213.2		H-1, H-19
4	CH	49.6	2.72 (1H, m)	H-1, H-5β
5	CH_2	25.1	3.37 (1H, dd, 5, 15) 2.42 (1H, dd, 5, 15)	H-4, H-7, H-20β
6	C	137.0		H-5β, H-20
7	CH	126.4	5.05 (1H, br, s)	H-5β, H-14, H-20
8	CH	40.6	1.89 (1H, br, s)	H-10
9	C	78.0		H-7, 9-OH, H-10, H-18
10	CH	47.4	3.44 (1H, m)	H-1, H-5β, 9-OH
11	CH	43.3	1.64 (1H, dq, 6, 10)	H-12, H-18
12	CH	74.7	5.46 (1H, d, 10)	H-11, H-14, H-18
13	C	65.2		H-12, H-14, H-16, H-17
14	CH	36.9	0.73 (1H, d, 5)	H-7, H-16, H-17
15	C	25.1		H-12, H-14, H-16, H-17
16	CH_3	24.1	1.11 (3H, s)	H-17
17	CH_3	16.3	1.14 (3H, s)	H-16
18	CH_3	11.8	1.03 (3H, d, 6)	H-11, H-12
19	CH_3	10.4	1.71 (3H, s)	
20	CH_2	69.2	3.82 (1H, d, 12) 3.94 (1H, d, 12)	H-5β, H-7
1′	CO	166.0		H-12, H-2′, H-3′
2′	CH	114.8	5.52 (1H, d, 11)	
3′	CH	146.3	6.55 (1H, t, 11)	H-5′
4′	CH	127.0	7.32 (1H, dd, 11, 15)	H-2′, H-6′
5′	CH	146.2	6.05 (1H, ddd, 7, 7, 15)	H-3′, H-6′, H-7′
6′	CH_2	35.0	2.14 (2H, dd, 7, 15)	H-4′, H-5′, H-7′, H-8′
7′	CH_2	22.0	1.42 (2H, qt, 7)	H-5′, H-6′, H-8′
8′	CH_3	13.7	0.87 (3H, t, 7)	H-6′, H-7′
9′	CO	173.5		H-10′
10′	CH_3	21.1	2.02 (3H, s)	
9-OH			5.13 (1H, br, s)	

Table 2. Comparison ^{1}H NMR Data for Compounds **1**, **2**, and **3** (δ_H in ppm)[9]

proton position	**1**	**2**	**3**
H-1	6.97	7.57	7.10
H-5α/β	3.37/2.42	2.50	3.45/2.50
H-7	5.05	5.59	5.15
H-10	3.44	3.28	3.54

Table 3. ^{13}C NMR Spectral Comparison of Compounds **1**, **4**, and **5** (δ_C in ppm)[10]

carbon position	**1**	**4**	**5**
C-1	156.1	159.8	156.2
C-2	143.3	136.4	143.3
C-3	213.2	209.7	213.3
C-4	49.6	44.2	49.6
C-5	25.1	29.6	25.1
C-10	47.4	54.2	47.4
C-18	11.8	15.1	11.9

exposed to acidic or alkaline conditions.[8] Because of distinct differences in the chemical shift values of certain protons in the ^{1}H NMR spectra, the A/B ring epimers of 4-deoxyphorbol esters have been distinguished. The most significant shifts were those reported for H-1, H-5, H-7, and H-10, which indicated that the H-4 proton of pedilstatin (**1**) is in the α-orientation, yielding a *cis* A/B ring juncture (Table 2).[9] The A/B *cis* ring assignment was confirmed by comparison of the ^{13}C NMR data of **1** with the data reported for the two known isomers **4** and **5** (Table 3).[10] Thus, pedilstatin (**1**) was assigned the structure 13-*O*-acetyl-12-*O*-(2′*Z*,4′*E*-octadienoyl)-4α-deoxyphorbol, and this was reminiscent of our experience with the diterpenes of *Pimelia prostrata* of the family Euphorbiaceae.[11]

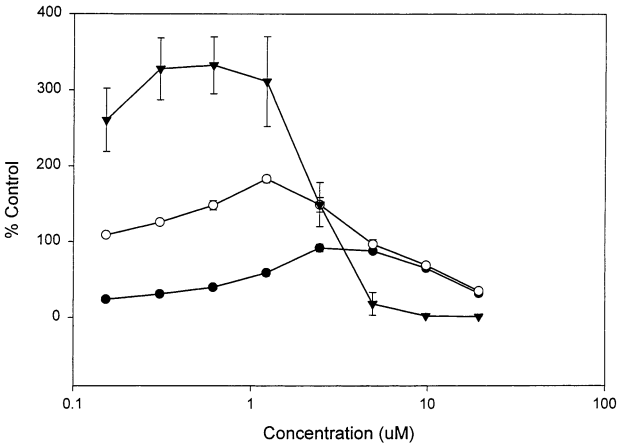
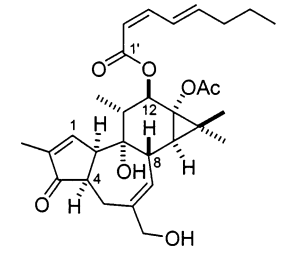
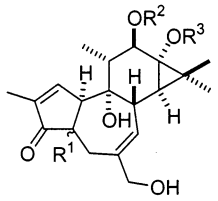


Figure 1. Effect of pedilstatin on HIV-1_{RF} infection of CEM-SS cells. Protection (●) and toxicity (○) are expressed as a percent of cell control values, while reverse transcriptase counts (▼) are normalized to values for the virus control.



1



2, R^1 = β-H, R^2 = 2-methylaminobenzoyl, R^3 = Ac
3, R^1 = α-H, R^2 = 2-methylaminobenzoyl, R^3 = Ac
4, R^1 = β-H, R^2 = R^3 = isobutyrate
5, R^1 = α-H, R^2 = R^3 = isobutyrate

The tigliane-type diterpenes known as phorbol esters are widely distributed in plant species of the family Euphorbiaceae.[12] These compounds were the first tumor-promoting agents to be isolated from natural sources[13] and are now known to be powerful activators of protein kinase C.[14] However, more recently Boyd and co-workers[6] have found that some of these diterpenes are not tumor promoters and instead are very effective antiviral (HIV-1) agents. Pedilstatin (**1**) was examined preliminarily for anti-HIV-1 properties. Initially, pedilstatin appeared to afford protection of human-derived lymphoblastoid CEM-SS cells from infection and cell-killing by HIV-1. (Figure 1). Maximal protection (approximately 80% of control) was observed at pedilstatin levels between 2 and 5 μM. The EC_{50} value for the XTT data from two independent assays is 1.03 μM ± 0.256 (n = 6). However, as is seen by noting reverse transcriptase activity, substantial virus quantities were present in the supernatants of these cells at this concentration. In fact at lower levels of the compound, a spike in

HIV-1 reverse transcriptase activity occurred with levels at least three times those seen in nontreated virus controls. Similar findings have been described previously for other phorbols, such as PMA and prostratin.[6,15] Additionally, after an initial increase in XTT-measured metabolic activity, a further increase in the concentration of pedilstatin caused marked loss of metabolic activity in the CEM-SS cells with an IC_{50} of 13.3 μM ± 1.5 (n = 6). Presently, it is unknown whether the initial higher metabolic activity observed is actually due to increased cell number. However, upon visual inspection of the cells growing in 96-well plates, the cell pellets appeared larger but more diffuse. The biological properties of pedilstatin (**1**) may merit further investigation, for example to ascertain whether this agent behaves more similarly to classical tumor-promoting phorbols such as PMA or to non-tumor-promoting ones such as prostratin.[6]

Activity of pedilstatin (**1**) as a ligand for protein kinase C was measured by inhibition of binding of [20-^{3}H]phorbol 12,13-dibutyrate to protein kinase Cα. The assay was performed as previously described with incubation in the presence of phosphatidylserine (100 μg/mL) for 5 min at 37 °C.[16] The K_i for pedilstatin was 620 ± 20 nM (n = 3 experiments). This value shows good agreement with the measured biological potency of pedilstatin in the P388 assay. The modest potency relative to typical phorbol esters is consistent with the observation that biological activity is dependent on the AB ring system being in a *trans* configuration.[17]

Experimental Section

General Experimental Procedures. All solvents used in the extraction and isolation processes were redistilled prior to use. The fractionation was directed by employing the murine P388 lymphocytic leukemia cell line and followed by thin-layer chromatography using Analtech silica gel GHLF Uniplates (visualized under long- and short-wave UV and developed in an acidic ethanolic solution of *p*-anisaldehyde). Column chromatography was performed with Sephadex LH-20 from Pharmacia. The HPLC purification of pedilstatin was achieved on a Zorbax SB C18 column with aqueous methanol (65%) as mobile phase. The chromatograms were recorded with both a diode array detector (HP1100 series) and an evaporative light scattering detector (SEDEX model 55). The optical rotation value was determined using a Perkin-Elmer 241 polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded with a Varian Inova 500 MHz instrument. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.24 ppm, δ_C 77.0 ppm). High- and low-resolution FAB spectra were obtained from a Kratos MS-50 mass spectrometer (Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln, NE) using a glycerol−triglycerol matrix.

Plant Collection. Approximately 100 kg of the entire plant (*Pedilanthus* sp., possibly *Pedilanthus tithymaloides* (Euphorbiaceae)) was collected in several locations in the Republic of Maldives in March 1989 and was preserved in ethanol for shipment. The plant was identified by Dr. David Lorence at the Pacific Tropical Botanical Garden at Lawai, Kauai, Hawaii. A voucher specimen is maintained at the Arizona State University Cancer Research Institute.

Plant Extraction. The shipping EtOH solution was decanted and concentrated to 4 L and then extracted three times with 4 L of DCM. The plant material was extracted twice with DCM−CH_3OH (1:1), followed by addition of H_2O to effect phase separation. The DCM extracts were evaporated, combined (dry weight 921 g), and dissolved in 12 L of a 1:1 mixture of hexane and CH_3OH−H_2O (9:1). After partitioning three times with hexane, the methanolic phase was adjusted to 3:2 CH_3OH−H_2O and partitioned three times with 12 L of DCM. This DCM extract was evaporated, yielding 570 g of a dark green oil.

Isolation of Pedilstatin (1). The dark green oil (570 g) was chromatographed in batches on a Sephadex LH-20 column using CH_3OH as the eluent. The most active fractions were further fractionated on Sephadex LH-20 columns using the following solvents: methanol−dichloromethane (1:1), hexane−2-propanol−methanol (1:1:1), hexane−toluene−dichloromethane−ethanol (17:1:1:1), hexane−toluene−acetone−methanol (1:4:3:1), toluene−ethyl acetate−methanol (6:1:1), and hexane−dichloromethane−acetone (4:5:1). The resultant most active fraction was purified by HPLC using a reversed-phase C_8 column to afford a yellow oil (40 mg) herein named pedilstatin (**1**), 13-*O*-acetyl-12-*O*-(2′*Z*,4′*E*-octadienoyl)-4α-deoxyphorbol: R_f 0.35 (SiO_2 2% CH_3OH in DCM); $[\alpha]^{24}_D$ −0.85° (*c* 1.18, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$), see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$), see Table 1; FABMS *m/z* 535.3 $[M + Na]^+$ (15), 513.4 $[M + H]^+$ (20), 373.2 (35), 355.2 (80), 313.2 (70), 295.2 (80), 123.1 (100); HRFABMS *m/z* 535.2657 (calcd for $C_{30}H_{40}O_7Na$, 535.2672).

Antiviral Activity. Assays for antiviral effects of pedilstatin were performed as previously described.[6,15] Briefly, CEM-SS cells were maintained in RPMI 1640 medium without phenol red and supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 10 μg/mL gentamicin. Eight test sample dilutions were added in 100 μL quantities to appropriate wells of a 96-well, round-bottomed cell culture plate. A Beckman Biomek 1000 robot was used for drug dilutions and transfers. Exponentially growing cells were added to appropriate wells of the plate at a density of 5000 cells/well in 50 μL. Stock virus ($HIV\text{-}1_{RF}$) was diluted in complete medium to yield 80−90% cell kill in 7 days, and a 50 μL quantity was added to individual wells. Plates were incubated 7 days at 37 °C in a humidified CO_2 atmosphere and were then assayed for cell metabolic activity by XTT-tetrazolium staining and for reverse transcriptase activity.

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