Cytotoxic Hydroazulene Diterpenes from the Brown Alga *Cystoseira myrica*

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Cytotoxicity-guided fractionation of the alcohol extract of the brown alga, *Cystoseira myrica*, afforded four new cytotoxic hydroazulene diterpenes, dictyone acetate (**2**), dictyol F monoacetate (**4**), isodictytriol monoacetate (**6**), and cystoseirol monoacetate (**8**), together with two known cytotoxic hydroazulene diterpenes, pachydictyol A (**1**) and dictyone (**3**). The constitution of each isolated compound has been determined on the basis of spectroscopic and chemical evidence.

Key words: Cystoseira, Cytotoxic, Hydroazulenes

Introduction

Marine plants have yielded a variety of secondary metabolites that possess novel chemical structures and interesting pharmacological activities (Stonik and Elyalov, 1986). Recently, researchers have described a wide range of biological activities for algal compounds including anti-HIV, anticoagulant, anticonvulsant, anti-inflammatory, antineoplastic, and cytotoxic activities (Lincolon *et al.*, 1991). A number of diterpenes and sterols have been isolated from the brown algae belonging to the genus *Cystoseira* (Banaigs *et al.*, 1983, Francisco *et al.*, 1977, Combaut *et al.*, 1980).

In a search for bioactive substances from marine brown algae, we collected *Cystoseira myrica* at El-Zafrana, Gulf of Suez. Cytotoxicity-guided fractionation of the alcohol extract afforded four new cytotoxic hydroazulene diterpenes as well as two known ones. The chemotaxonomic implication of these findings is also discussed.

Results and Discussion

An ethanolic extract of the brown alga *Cystoseira myrica* was fractionated on silica gel using a gradient of hexane-ether as gradient solvent. The fractions were monitored by cytotoxicity bioassays using three proliferating mouse cell lines: NIH3T3, SSVNIH3T3, and KA3IT to afford, in order of elution, six compounds (**1**–**4**, **6**, and **8**, Fig. 1). The structures of known compounds **1** (Hirschfeld *et al.*, 1973) and **3** (Enoki *et al.*, 1982) were established by comparing their physical and spectral data with those in the literature. The new compounds **2**, **4**, and **6** are acetate derivatives of known alcohols **3** (Enoki *et al.*, 1982), **5** (Enoki *et al.*, 1983), and **7** (Kusumi *et al.*, 1986). Cystoseirol monoacetate **8** represents a new oxidation pattern for this family of hydroazulene diterpenes.

At the outset we recognized a common feature in the mass spectroscopic behavior in five (**1**–**4** and **6**) of the six natural compounds we isolated. Namely, they each gave rise to a major ion (often the base peak) of *m/z* 159, corresponding to $C_{12}H_{15}$. This can be accounted for by the events shown in Scheme 1. This analysis suggested that there existed a common structural feature within the hydroazulene skeleton for these five compounds, which localized their structural differences to within the substituents attached to C (6) and/or C (11).

Compound **2** was found to have the formula $C_{22}H_{34}O_3$ by mass spectrometry. All twenty-two carbons could be identified in the ^{13}C NMR spectrum. Of the six degrees of unsaturation, implied by the molecular formula of **2**, two were

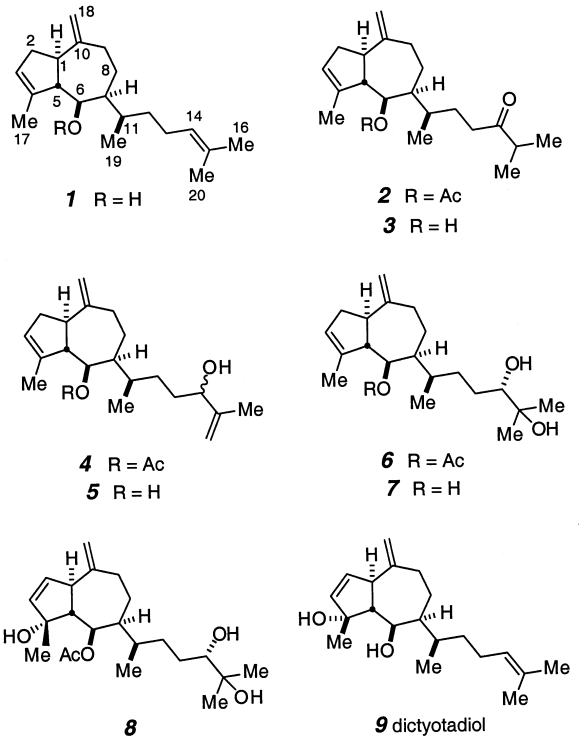
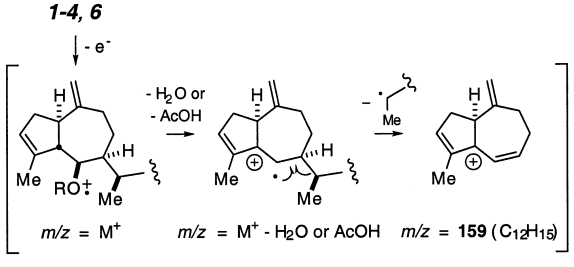


Fig. 1. Structures of new (**2**, **4**, **6**, and **8**) and known (**1** and **3**) compounds isolated from *Cystoseira myrica* as well as related known hydroazulenes (**5**, **7**, and **9**).

accounted for as carbon-carbon double bonds (δ = 107.82, 125.22, 140.27, and 152.13) and two more by the presence of acetate (δ = 171.01) and ketone (δ = 215.34) carbonyl groups in the molecule, compound **2** was thus bicyclic. The IR spectrum contained ketone (1710 cm^{-1}) and acetate (1733 cm^{-1}) carbonyl absorption bands. Mass fragments at *m/z* 286 [$M^+ - AcOH$] and 159 (90) [$C_{12}H_{15}^+$] implied that it was an acetoxy ketone **2**



Scheme 1. Common mass spectral fragmentation events to *m/z* 159 ion.

closely related to **1** (cf. Fig. 1). Both ^1H and ^{13}C NMR resonance (Table I) characteristic of the isobutenyl group in **1** were lacking in **2**. When the ^1H- and ^{13}C NMR data for **2** were compared with those we obtained for known compound **3** (Enoki *et al.*, 1982), it was apparent that the only significant difference between the two molecules could be rationalized in terms of **2** being the 6-acetyl derivative of **3**. Specifically, H (6) was deshielded from δ 4.06 ppm in **3** to 5.32 ppm in **2** and of C (6), from 73.81 ppm in **3** to 78.69 ppm in **2**. This along with the presence of ^1H- and ^{13}C NMR resonance associated with an acetoxy group [δ_H = 2.04, δ_C = 21.98 and 171.01] led to the assignment of structure **2**, dictyone acetate (Fig. 1). This was confirmed by chemical transformation of **3** into **2** with acetic anhydride in pyridine. **2** was concurrently identified by two of the authors in another brown alga, *Dictyota dichotoma* collected from the Red Sea (Gedara *et al.*, 2002).

Compound **4** was found by mass spectrometry and ^{13}C NMR spectroscopy to have the molecular formula $C_{22}H_{34}O_3$. The IR spectrum of **4** contained hydroxyl (3437 cm^{-1}) and carbonyl (1736 cm^{-1}) absorption bands. A three-proton singlet at δ 2.04 in the ^1H NMR spectrum, resonance at δ = 171.14 and 21.98 in the ^{13}C NMR spectrum, and a significant ion at *m/z* 286 [$M^+ - CH_3CO_2H$] in the mass spectrum, indicated the presence of an acetate ester in **4**. Other spectral similarities between **4** and the known diterpene dictyol F **5** (Enoki *et al.*, 1983) suggested that **4** were a monoacetate of **5**. Similar to the differences seen between **2** and **3**, the ^1H NMR resonance for H (6) was deshielded from δ 3.98 in **5** (Enoki *et al.*, 1983) to δ 5.34 in **4**, and the ^{13}C NMR resonance for C (6) from 74.4 in **5** (Enoki *et al.*, 1983) to 79.01 in **4**. Thus, **4** were deduced to be dictyol F monoacetate (Fig. 1).

Compound **6** was analyzed for $C_{22}H_{36}O_4$ by mass spectrometry and ^{13}C NMR spectroscopy. Inspection of ^1H NMR and ^{13}C NMR data of **6** clearly indicated that the substituents in the carbon skeleton of the bicyclic rings was identical to those present in **4**. The major difference was the replacement of resonance associated with the isopropenyl group by a set that could be attributed to the corresponding hydrated tertiary carbinol. Namely, singlets at δ 1.20 and δ 1.15 in the proton spectrum and resonance at δ 23.41 and 26.61 in the

Table I. ^{13}C-NMR data[a] in **$CDCl_3$** for compounds **1**–**4** ,**6**, and **8**.

Carbon No.	Pachydictyol **1**	Dictyone acetate **2**	Dictyone **3**	Dictyol F monoacetate **4**	Isodictytriol monoacetate **6**	Cystoseirol monoacetate **8**
1	46.22 (d)	46.19 (d)	45.84 (d)	46.20 (d)	46.52 (d)	49.56 (d)
2	34.71 (t)	34.16 (t)	33.96 (t)	34.16 (t)	34.29 (t)	133.10 (d)
3	124.20 (d)	125.22 (d)	123.57 (d)	125.15 (d)	125.10 (d)	136.50 (d)
4	141.60 (s)	140.27 (s)	142.53 (s)	140.46 (s)	140.44 (s)	96.60 (s)
5	60.64 (d)	57.50 (d)	59.24 (d)	58.00 (d)	57.50 (d)	54.60 (d)
6	75.30 (d)	78.69 (d)	73.81 (d)	79.01 (d)	78.58 (d)	78.24 (d)
7	47.97 (d)	46.49 (d)	49.20 (d)	46.54 (d)	47.10 (d)	48.26 (d)
8	23.71 (t)	23.08 (t)	23.88 (t)	23.59 (t)	23.66 (t)	24.52 (t)
9	40.84 (t)	39.90 (t)	40.76 (t)	40.20 (t)	40.39 (t)	38.15 (t)
10	152.74 (s)	152.13 (s)	152.79 (s)	152.40 (s)	152.20 (s)	152.65 (s)
11	34.94 (d)	34.65 (d)	34.22 (d)	34.64 (d)	34.30 (d)	33.50 (d)
12	35.21 (t)	38.80 (t)	37.83 (t)	33.40 (t)	33.34 (t)	32.44 (t)
13	25.80 (t)	30.55 (t)	27.12 (t)	32.30 (t)	29.40 (t)	29.06 (t)
14	124.92 (d)	215.34 (s)	216.51 (s)	75.98 (d)	78.26 (d)	78.10 (d)
15	131.73 (s)	41.04 (d)	41.08 (d)	147.70 (s)	73.25 (s)	73.29 (s)
16	25.93 (q)	18.44 (q)	18.50 (q)	17.87 (q)	26.61 (q)	26.69 (q)
17	16.11 (q)	15.52 (q)	16.30 (q)	15.55 (q)	15.59 (q)	17.00 (q)
18	107.32 (t)	107.82 (t)	107.02 (t)	107.79 (t)	107.94 (t)	108.91 (t)
19	17.73 (q)	15.94 (q)	18.22 (q)	16.23 (q)	18.04 (q)	19.51 (q)
20	17.90 (q)	18.44 (q)	18.42 (q)	111.20 (t)	23.41 (q)	23.49 (q)
Oac		21.98 (q) 171.01 (s)		21.98 (q) 171.14 (s)	21.97 (q) 171.46 (s)	21.91 (q) 171.36 (s)

[a] Multiplicity (s, d, t, q) of each carbon is assumed, based upon the observed sign of the DEPT signal (i.e., positive for δ and q, negative for t, and no signal for s).

carbon spectrum corresponding to diastereotopic methyl groups along with a tertiary carbinol resonance at δ 73.25 for C(15) suggested the vicinal diol structure present in **6** (Fig. 1). Once again differences for H(6) and C(6) between **6** and known **7** (Kusumi *et al.*, 1986) were consistent with the former being the 6-acetyl analog of the latter. Thus, deshielding of H (6) from δ 3.94 in **7** [9] to 5.38 in **6** and of C (6), from 74.4 in **7** [9] to 78.58 in **6**, along with resonance associated with the C (6) acetoxy group [δ = 171.46 and 21.97] led to the assignment of structure **6** as isodictytriol monoacetate (Fig. 1).

Compound **8** was analyzed for $C_{22}H_{36}O_5$ by mass spectrometry and ^{13}C NMR spectroscopy. Fragment ion 159 was not present in the mass spectrum of **8** (cf., Scheme 1), suggesting that structural differences resided within the bicyclic moiety. The oxygen-containing functionalities in **8** were identified as one secondary and two tertiary hydroxyl groups [δ_H = 3.30 (dd), δ_C = 78.1 (positive DEPT), 73.29 (no DEPT), and 96.6 (no DEPT)] and a single secondary acetoxy moiety [δ_H = 2.04 and 5.48 (dd), δ_C = 78.24, 171.36, and 21.91] as ascertained from ^{1}H- and ^{13}C NMR spectroscopy. Upon comparing the NMR data for compounds **8** and **6**, it was evident that the side chain comprising C (11) to C (16) was identical in both and that the substitution pattern within the five membered ring were different. From ^{1}H- and ^{13}CNMR data of **8** it was surmised that the C (17) methyl group [δ_H = 1.24 (s), δ_C = 17.00 (positive DEPT)] was attached to a quaternary carbon bearing a hydroxyl function [δ_C = 96.6 (no DEPT)]. The alkene within the cyclopentene ring was now di- rather than trisubstituted [$\delta_{H\ (2)\ and\ H\ (3)}$ = 5.85 (dd, J = 5.7 and 1.8 Hz) and 5.77 (dd, J = 5.7 and 1.7 Hz, $\delta_{C\ (2)\ and\ C\ (3)}$ = 133.1 and 136.5]. The presence of a methylcyclopentenol substructure like that assigned here for cystoseirol monoacetate **8** (Fig. 1) has been observed in other hydroazulene diterpenes. One particularly relevant example is dictyotadiol (**9**), whose relative configuration was deduced by x-ray analysis (Faulkner *et al.*, 1977) and absolute configuration by circular dichroism studies (Arroyo *et al.*, 1991). The same A-ring

Sample	Cell lines NIH3T3	SSVNIH3T3	KA3IT
Alcohol extract of *C. myrica*	5	40	20
Pachydictyol (**1**)	10	20	5
Dictyone acetate (**2**)	15	35	5
Dictyone (**3**)	7.5	20	5
Dictyol F monoacetate (**4**)	10	35	10
Isodictytriol monoacetate (**6**)	7.5	35	5
Cystoseirol monoacetate (**8**)	7.5	35	5

Table II. Cytotoxicity [IC_{50} (μg/ml)] of the alcohol extract and of purified diterpenes from the brown alga *Cystoseira myrica* in *vitro* proliferating mouse cell lines.

cyclopentenol moiety is also observed in a related hydroazulene diterpene (Wright *et al.*, 1993, König *et al.*, 1993).

The hydroazulene skeleton is typical of diterpenoid metabolites derived from the brown algae of the genus *Dictyota.* Such structures have also been found in the marine moluscs, *Aplysia depilans* (Minale and Riccio, 1976). However, the work described here apparently represents the first time such diterpenoid skeletons have been observed in *Cystoseiraceae,* suggesting a related chemotaxonomy of the brown algae belonging to *Cystoseiraceae* and *Dictyotaceae.*

Finally, cytotoxicity assays (Shier, 1983, Abbas *et al.*, 1992) of the total alcohol extract of *Cystoseira myrica* as well as of the individual compounds after purification (Table II) were carried out *in vitro* using three proliferating mouse cell lines, a normal fibroblast line NIH3T3 and two virally transformed forms SSVNIH3T3 and KA3IT. All compounds exhibited moderate cytotoxicity on the cancer cell line KA3IT (IC_{50} = ~ 5 μg/ml) and showed reduced cytotoxicity towards the normal NIH3T3 cells. The total alcohol extract showed more cytotoxic activity against the normal cell line than on the other virally transformed forms.

Experimental

Plant material, apparatus, and methods

^{1}H NMR spectra were recorded at 300 or 500 MHz and ^{13}C NMR at 75 MHz. Chemical shifts are given in δ (ppm) relative to TMS as internal standard. Overlapped protons in the region of δ 1.2–2.3 are not listed, only discreet resonance from that region of the spectrum is listed. Infrared spectra were determined on thin films cast from $CHCl_3$, recorded on a Protégé-400 (S. S. P.) spectrophotometer. Electron impact mass spectra were determined at 70 ev on a Kratos MS-25 instrument. Thin layer chromatography was performed on silica gel (kieselgel 60, F254) of 0.25 mm layer thickness. Preparative thin layer chromatography (PTLC) was performed on silica gel plates (20 cm × 20 cm) of 500 μm thicknesses. The alga *Cystoseira myrica* was collected in June 1998, at El-Zaafarana, near the middle of the Gulf of Suez. A voucher sample was identified by Professor Yahia El-Azab, Department of Botany, Faculty of Science, Mansoura University and deposited at the Pharmacognosy Department, Faculty of Pharmacy, Mansoura University.

Extraction and isolation

The alga was air-dried in the shade at room temperature and ground to a powder with a mortar and pestle. This powder (1 kg) was slurred in ethanol (~ 5 l) and allowed to stand at room temperature for several days. Filtration and concentration of the filtrate provided a crude extract that was dissolved in a small amount of methanol, stored at ~ 0 °C overnight, and filtered to remove fats. The filtrate was again evaporated under reduced pressure to afford a dark brown viscous oily residue (~20 g, ~ 2% of the dry weight of the alga). This residue was chromatographed on a silica gel column using a hexane-Et_2O gradient. Fractions of ~ 50 ml were collected. The fractions containing a single compound were combined and further purified by preparative TLC to give compounds in the following order:

Pachydictyol A (**1**)

Fractions 5–9 were combined. PTLC using hexane-EtOAc (19:1, v/v) afforded pachydictyol A (30 mg, 0.003% dry wt.). IR (cm^{-1}): 3514 (OH),

1644 (C = C), EIMS *m/z* (rel. int.): 288 (25) [M^+, $C_{20}H_{32}O$], 270 (25) [$M^+ - H_2O$], 255 (5) [$M^+ - CH_3 - H_2O$], 159 (62) [$C_{12}H_{15}^+$]. ^{1}H NMR ($CDCl_3$) δ 5.33 [1H, br s, H (3)], 5.12 [1H, t, *J* = 7.2, H (14)], 4.74 [2H, br s, H (18), H (18′)], 3.92 [1H, br d, *J* = 7.8, H (6)], 1.80 [3H, s, Me (17)], 1.68 [3H, br s, Me (16)], 1.60 [3H, s, Me (20)], and 0.99 [3H, d, *J* = 6.0, Me (19)]. ^{13}C-NMR (Table I). Spectral properties compare favorably to literature data (Hirschfeld *et al.*, 1973).

Dictyone acetate (**2**)

Fractions 10–16 were combined. PTLC using hexane-EtOAc (9:1, v/v) afforded dictyone acetate **2** as an oil (25 mg, 0.0025% dry wt.). IR (cm^{-1}): 1733 (OAc), 1710 (C = O), 1644 (C = C), EIMS *m/z* (rel. int.): 286 (14) [$M^+ - AcOH$, $C_{20}H_{30}O^+$], 159 (90) [$C_{12}H_{15}^+$], 43 (100) [$C_3H_7^+$]. ^{1}H NMR ($CDCl_3$) δ 5.33 [1H, br s, H (3)], 5.32[1H, dd, *J* = 4.1, 3.6 Hz, H (6)], 4.79 [1H, br s, H (18)], 4.76 [1H, br s, H (18′)], 2.04 [3H, s, Me (Ac)], 1.6 [3H, br s, Me (17)], 1.08 [6H, d, *J* = 7.0, Me (16), Me (20)], and 0.87 [3H, d, *J* = 6.8, Me (19)]. ^{13}C-NMR (Table I).

Dictyone (**3**)

Fractions 18–25 were combined. PTLC using mixtures of hexane-EtOAc (17:3, v/v) afforded dictyone **3** as a pale yellow oil (20 mg, 0.002% dry wt). IR (cm^{-1}): 3480 (OH), 1713 (C = O), 1644 (C = C), EIMS *m/z* (rel. int.): 304 (5) [M^+, $C_{20}H_{32}O_2^+$], 286 (12) [$M^+ - H_2O$, $C_{20}H_{30}O^+$], 159 (45) [$C_{12}H_{15}^+$], 145 (12), 107 (30), 71 (40), 43 (100) [$C_3H_7^+$]. ^{1}H NMR ($CDCl_3$) δ 5.33 [1H, br s, H (3)], 4.06 [1H, dd, *J* = 8.1, 3.3 Hz, H (6)], 4.74 [2H, br s, H (18), H (18′)], 1.85 [3H, br s, Me (17)], 1.1 [6H, d, *J* = 6.9, Me (16), Me (20)], and 0.95 [3H, d, *J* = 6.3, Me (19)]. ^{13}C-NMR (Table I). The spectral properties are identical to those reported for **3** (Enoki *et al.*, 1982).

Dictyol F monoacetate (**4**)

Fractions 27–39 were combined. PTLC using hexane-EtOAc (8:2, v/v) afforded dictydiol monoacetate **4** as a colorless oil (12 mg, 0.0012% dry wt). IR (cm^{-1}): 3437 (OH), 1736 (OAc), 1644 (C = C). EIMS *m/z* (rel. int.): 286 (85) [$M^+ - AcOH$, $C_{20}H_{30}O^+$], 268 (22) [$M^+ - AcOH - H_2O$], 200 (32), 159 (100) [$C_{12}H_{15}^+$], 105 (24), 91 (16), 43 (76) [$C_3H_7^+$]. ^{1}H NMR ($CDCl_3$) δ 5.34 [1H, dd, *J* = 8.4, 3.0, H (6)], 5.33 [1H, br s, H (3)], 4.94 [1H, br s, H (20)], 4.83 [1H, br s, H (20′)], 4.79 [1H, br s, H (18)], 4.76 [1H, br s, H (18′)], 4.02 [1H, t, *J* = 6.1, H (14)], 2.04 [3H, s, Me (Ac)], 1.71 [3H, br s, Me (17)], 1.62 [3H, br s, Me (16)], and 0.83 [3H, d, *J* = 6.2, Me (19)]. ^{13}C-NMR (Table I).

Isodictytriol monoacetate (**6**)

Fractions 72–83 were combined. PTLC using mixtures of hexane- EtOAc (3:1, v/v) afforded isodictytriol monoacetate **6** as a colorless oil (13 mg, 0.0013% dry wt). IR (cm^{-1}): 3450 (OH), 1736 (OAc), 1640 (C = C), EIMS *m/z* (rel. int.): 304 (11) [$M^+ - AcOH$], 286 (5) [$M^+ - AcOH - H_2O$], 159 (100) [$C_{12}H_{15}^+$], 105 (24), 91 (17), 43 (82) [$C_3H_7^+$]. ^{1}H NMR ($CDCl_3$) δ 5.38 [1H, dd, *J* = 8.6, 3.7, H (6)], 5.33 [1H, br s, H (3)], 4.79 [1H, br s, H (18)], 4.76 [1H, br s, H (18′)], 3.30 [1H, dd, *J* = 9.5, 2.0, H (14)], 2.04 [3H, s, Me (Ac)], 1.61 [3H, br s, Me (17)], 1.2 and 1.15 [each 3H, s, Me (16), Me (20)], and 0.89 [3H, d, *J* = 6.2, Me (19)]. ^{13}C-NMR (Table I).

Cystoseirol monoacetate (**8**)

Fractions 85–94 were combined. PTLC using mixtures of hexane- EtOAc (7:3, v/v) afforded cystoseirol monoacetate **8** as a colorless oil (12 mg, 0.0012% dry wt). IR (cm^{-1}): 3450 (OH), 1732 (OAc), 1640 (C = C), EIMS *m/z* (rel. int.): 320 (5) [$M^+ - AcOH$], 302 (18)[$M^+ - AcOH - H_2O$], 200 (12), 43 (76) [$C_3H_7^+$]. ^{1}H NMR ($CDCl_3$) δ 5.85 [1H, dd, *J* = 5.7, 1.8, H (2)], 5.77 [1H, dd, *J* = 5.7, 1.7, H (3)], 5.48 [1H, dd, *J* = 9.3, 2.1, H (6)], 4.77 [1H, br s, H (18)], 4.72 [1H, br s, H (18′)], 3.30 [1H, dd, *J* = 9.6, 2.0, H (14)], 3.0 [1H, br d, *J* = 9.4, H (1)], 2.04 [3H, s, Me (Ac)], 1.24 [3H, br s, Me (17)], 1.20 and 1.15[each 3H, s, Me (16), Me (20)], and 0.88 [3H, d, *J* = 6.2, Me (19)]. ^{13}C-NMR (Table I).

Acetylation of (**3**)

A solution of dictyone (5 mg) in a mixture of acetic anhydride (500 μl) and a few drops of pyridine was heated for about 4 h in a water bath and then cooled. It was poured into water and extracted with ether. The ether extract was washed

with water and dried over anhydrous sodium sulfate. The solvent free residue was purified by PTLC using hexane: benzene: EtOAc (10:10:3, v/v/v) to afford dictyone acetate **2** as a colorless oil.

Cytotoxicity assays

Cytotoxic assays (Shier, 1983, Abbas *et al.*, 1992) were performed using three proliferating mouse cell lines, a normal fibroblast line NIH3T3 and two virally transformed forms SSVNIH3T3 and KA3IT. Samples of extract or pure compound (5 mg) were dissolved in 62.2 µl of DMSO, and working solutions made by diluting 20 µl of the DMSO solution into 2 ml of sterile medium (Dulbecco's modified Eagle's medium, Sigma Chemical Co. St. Louis, MO, USA). Two-fold or 2.5-fold dilutions of the extracts of pure compounds from 200 µg/ml to 0.5 µg/ml were prepared in triplicate in the wells of 96-well culture trays (Falcon Micro Test III, # 3072, Becton Dickinson Labware, Lincoln Park, NJ, USA) in 200 µl of medium containing 5% (v/v) calf serum (Hyclone Laboratories, Logon, Utah, USA). Inoculums of 2 × 103 cells were added to each well in a 100 µl aliquot of 10% calf serum in medium. The 96-well trays of cells were cultured under standard conditions until uninhibited cultures (control) became confluent. The contents of the wells were decanted, and each cell layer washed with a small amount of the medium. The wells were filled with formal saline (3.7% w/v formaldehyde in 0.15 M NaCl), and allowed to stand at room temperature for at least 30 minutes. The trays was washed with tap water, and attached cells stained by adding two drops of 0.5% (w/v) crystal violet solution in 20% (v/v) aqueous methanol added to each well. The trays was washed with tap water, and the IC50 estimated visually as the approximate concentration that causes 50% reduction in the number of stained cells adhering to the bottom of the wells.

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