Further Halotyrosine Derivatives from the Marine Sponge *Suberea* aff. *praetensa*[§]

Anake Kijjoa[a,b], Rawiwan Watanadilok[c], Pichai Sonchaeng[c], Pichan Sawangwong[d], Madalena Pedro[b], Maria São José Nascimento[b], Artur M. S. Silva[e], Graham Eaton[f] and Werner Herz[g*]

[a] Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, 4099-003 Porto, Portugal
[b] Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia de Universidade do Porto, Rua Anibal Cunha, 4050-017 Porto Portugal
[c] Bangsaen Institute of Marine Science (BIMS), Burapha University, Bangsaen, Chonburi 20131, Thailand
[d] Department of Aquatic Science, Faculty of Sciences, Burapha University, Bangsaen, Chonburi 20131, Thailand
[e] Departamento de Química, Universidade de Aveiro, 3810 Aveiro, Portugal
[f] Department of Chemistry, University of Leicester, University Road, Leicester LE 7RH, UK
[g] Department of Chemistry and Biochemistry, The Florida State University, Tallahassee, FL 32306-4390, USA. FAX: 1-850-64-8281. E-mail: jdulin@chem.fsu.edu

[*] Author for correspondence and reprint requests

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Suberea aff. *praetensa*, Halotyrosine Derivatives, Anticancer Activities

Reexamination of the marine sponge *Suberea* aff. *praetensa*, (Row) from the Gulf of Thailand furnished in addition to bromotyrosine derivatives found previously 5-bromo- and 5-chlorocavernicolin, cavernicolins 1 and 2, two other brominated tyrosine metabolites, a known bisoxazolidone and a new unusual rearranged tyrosine metabolite subereatensin. Several of the metabolites exhibited significant inhibitory effects against five human cancer cell lines.

Introduction

In an earlier article (Kijjoa *et al.*, 2001) we described isolation of the bromotyrosine derivatives fistularin-3, agelorins A and B and the new 11,17-dideoxyagelorins A and B as well as clionasterol from a Gulf of Thailand collection of the marine sponge *Suberea* aff. *praetensa* (Row). The only other previous report on a *Suberea* species dealt with *Suberea creba* from the Coral Sea (Debitus, *et al.* 1998). In order to obtain more material for biological tests we have now carried out two additional collections from the same locality. A collection of November 1999 furnished again clionasterol and fistularin-3 as well as 5-chloro- and 5-bromocavernicolin (**1a** and **2**) previously isolated from *Aplysia* (*Verongia*) *cavernicola* (D'Ambrosio *et al.*, 1984, Guerriero *et al.*, 1984). A second collection of February 2001 furnished clionasterol, fistularin-3, agelorins A and B, the amides **3a** previously reported from *Aplysina fistularis* (Tymiak and Rinehart, 1981), and **4a** (previously reported by Sharma and Burkholder, 1967, from *Verongia cauliformis* and by Tymiak and Rinehart, 1985, from *Aplysina fistularis*), the epimeric dibromolactams cavernicolin 1 (**5**) and cavernicolin 2 (**6**) earlier isolated from *Aplysina cavernicola* (D'Ambrosio *et al.*, 1982) and the bis-oxazolidone **7a** (see Chart 1), previously reported from *Verongia lacunosa* (Borders *et al.*, 1974) and *Aplysina fulva* (Gopichand and Schmitz, 1979). An unusual new constituent was the rearranged tyrosine derivative **8** which we have named subereatensin. Acetate **1b**, **4a** and its acetate **4b** exhibited significant inhibitory effects against five human cancer cell lines.

Materials and Methods

^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker AMC instrument operating at 300.13 and 75.47 MHz, respectively.

[§] Dedicated to Professor Dr. Nuno R. Grande, founder of Instituto de Ciências Biomédicas de Abel Salazar, on the occasion of his 70th birthday.

EI mass spectra were measured on a Hitachi Perkin-Elmer RMV-6M instrument. For HRMS samples were run using +FAB ionization with Xe gas at GKV on a KRATOS CONCEPT III, 2 sector mass spectrometer. The accelerating voltage was 8, KV Silica gel for column chromatography was Si Gel 60 (0.2–0.5 mm Merck), for analytical and preparative TLS Si gel G-60 GF 254 Merck.

Animal material

Suberea aff. *praetensa* (Row) was collected from a trawl net on the sea shore of Ban Phae village at the gulf of Thailand, Rayong Province, Thailand, in November 1999 (first extraction) and February 2001 (second extraction). Identification of the sponge by Professor Rob van Soest, Department of Coelenterates and Porifera Zoological Museum, University of Amsterdam and voucher (BIMS-1954) on deposit in the reference collection of the Museum of the Institute of Bangsaen Institute of Science, Burapha University, Bangsaen, Chonburi 20131, Thailand, was mentioned previously (Kijjoa *et al.*, 2001). The collections were frozen immediately at –20 °C for one night prior to extraction.

Extraction, isolation and characterization of the constituents

A. First extract. The sample (490 g fresh weight) was thawed, homogenized with EtOH (1.5 l), allowed to stand overnight in a dark chamber and filtered. The residue on the filter paper was reextracted twice with EtOH (1.5 l). The aqueous alcoholic extracts were combined,evaporated at reduced pressure to ca. 180 ml, and partitioned with EtOAc (3 × 300 ml). The EtOAc solutions were combined and concentrated at reduced pressure to give a residue (5.8 g) which was chromatographed over Si gel (54 g) and eluted with petrol-$CHCl_3$, $CHCl_3$-Me_2O and $CHCl_3$-MeOH, 200 ml frs being collected as follows: Frs 1–4 (petrol-$CHCl_3$, 7:3 v/v), 5–19 (petrol-$CHCl_3$, 1:1), 20–31 (petrol-$CHCl_3$, 3:7), 32–42 (petrol-$CHCl_3$, 1:9), 43–65 ($CHCl_3$-Me_2O, 9:1), 66–82 ($CHCl_3$-Me_2O, 7:3), 83–108 ($CHCl_3$-Me_2O, 1:1), 109–130 ($CHCl_3$-Me_2O, 1:9). Recrystallization of frs 23–28 (52 mg) from petrol and $CHCl_3$ gave clionasterol (23 mg) identified by MS and ^{1}H NMR spectrometry. Frs 49–57 (140 mg) on purification by PTLC (Si gel, $CHCl_3$-Me_2O-HCO_2H, 70:30:0.1) gave fistularin-3 (32 mg) identified by comparison with material isolated previously (Kijjoa *et al.*, 2001), 5-bromocavernicolin (**2**, 18 mg) and 5-chlorocavernicolin (**1a**, 22 mg) identified by ^{1}H and ^{13}C NMR spectrometry, COSY, NOESY, HMBC, and by comparison with the literature (for **1a**, D'Ambrosio *et al.*, 1985, for **2** Guerriero *et al.*, 1984) (all structures in Fig. 1).

B. Second extract. The sample (1.3 kg fresh weight) on homogenization with EtOH (2 l), filtration, two reextractions of the residue on the filter paper with EtOH, concentration of the combined filtrates to ca. 300 ml, partitioning with EtOAc (3 × 500 ml) and concentration of the EtOAc layers at reduced pressure gave 32 g of crude extract which was chromatographed on Si gel (120 g) and eluted as before, 200 ml frs being collected as follows: Frs 1–13 (petrol-$CHCl_3$, 3:2), 14–24 (petrol-$CHCl_3$, 2:3), 25–35 (petrol-$CHCl_3$, 1:4 v/v), 36–46 ($CHCl_3$), 47–61 ($CHCl_3$, Me_2O, 4:1), 62–73 ($CHCl_3$-Me_2O, 3:2), 74–82 ($CHCl_3$-Me_2O, 2:3), 83–99 ($CHCl_3$-Me_2O, 1:4). Recrystallization of frs 7–8 (252 mg) from petrol-$CHCl_3$ furnished clionasterol (187 mg). Purification of frs 14–19 (184 mg) by PTLC (Si gel, $CHCl_3$-Me_2O-HCO_2H, 95:5:0.1) gave 36 mg of unknown viscous material. PTLC (Si gel, $CHCl_3$-Me_2O-HCO_2H, 85:15:0.1) of frs 36–39 (194 mg) gave subereatensin (**8**, 36 mg). Recrystallization of frs 40–46 (324 mg) from $CHCl_3$-petrol gave **3a** (153 mg) (Tymiak and Rinehart, 1985) further characterized as the acetate **3b**. Recrystallization of frs 47–48 (222 mg) from $CHCl_3$-Me_2O gave **4a** (57 mg) also characterized as the acetate (Sharma and Burkholder, 1970, Tymiak and Rinehart, 1985); the mother liquor on purification by PTLC (Si gel, $CHCl_3$-Me_2O-HCO_2H) gave 51 mg of a mixture of cavernicolin 1 (**5**, major) and cavernicolin 2 (**6**, minor), identified by ^{1}H and ^{13}C NMR spectrometry, COSY, NOESY, HMBC and comparison with data in the literature (D'Ambrosio *et al.*, 1982), and 112 mg at a more polar fraction containing fistularin-3, agelorin A and agelorin B which were identified by comparison with the compounds obtained from *Suberea* aff. *praetensa* previously (Kijjoa *et al.*, 2001). Recrystallization of frs 51–54 (632 mg from $CHCl_3$-Me_2O afforded the bis-2-oxazolidone **7a** (Fig. 2) (420 mg) previously reported from *Verongia lacunosa* (Borders *et al.*, 1974) and *Aplysina*

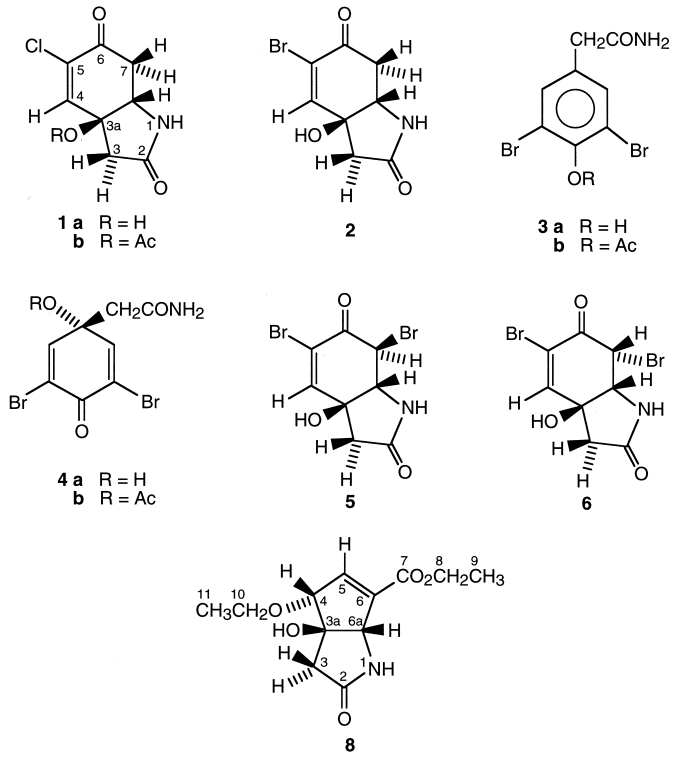

Fig. 1. Structures of compounds from *Suberea* aff. *praetensa.*

fistularis forma fulva (Gopichand and Schmitz, 1979) which was characterized by the new mono- and diacetates **7b** and **7c** (Fig. 2, *vide infra*).

*5-Chlorocavernicolin (**1a**).* + FAB (in NBA): *m/z* 202 (MH^+, 20); HRMS (NBA): *m/z* 202.02707; calcd for $C_8H_9NO_3$ ^{35}Cl: 202.02710; ^1H NMR (DMSO) δ 7.96 *brs* (NH), 7.06 *s* (H-4), 6.21 *s* (OH), 3.88 *t* (*J* = 5.1 Hz, H-7a) 2.92 *dd* (*J* = 16.5, 4.6 Hz) and 2.69 *dd* (*J* = 16.5, 6.0 Hz, H-7α and H-7β), 2.54 *d* and 2.45 *d* (*J* = 16.5 Hz, H-3a, H-3b); ^{13}C NMR (DMSO) δ 188.89 (C-6), 172.72 (C-2), 145.84 (C-4), 130.18 (C-5), 73.41 (C-3a), 58.56 (C-7a), 43.78 (C-3), 40.08 (C-7). Acetate **1b** ^1H NMR (DMSO) 8.16 brs (NH), 7.30 *s* (H-4), 4.28 *t* (*J* = 5.3 Hz, H-7a), 3.04 *dd* (*J* = 16.5, 5.1 Hz, H-7a), 2.77 *dd* (*J* = 16.5, 5.4 Hz, H-7b), 2.91 *d* (*J* = 17 Hz, H-3a), 2.77 *d* (*J* = 17, H-3b), 20.4 *s*, (3p, OAc); ^{13}C NMR (DMSO) δ 188.18 (C-6), 171.38 (C-2), 169.86 (Ac), 141.03 (C-4), 132.14 (C-3), 79.38 (C-3a), 56.07 (C-7a), 42.36 (C-3), 21.19 (Ac-Me).

*5-Bromocavernicolin (**2**).* + FAB (in NBA): *m/z* 246 (MH^+, 100); HRMS (NBA): *m/z* 245.97655; calcd for $C_8H_9NO_3$ ^{79}Br: 245.97658, ^1H NMR (DMSO) δ 7.96 *brs* (NH), 7.29 *s* (H-4), 6.23 *s* (OH), 3.88 *t* (*J* = 5.4 Hz, H-7a), 2.93 *dd* (*J* = 16.4, 4.8 Hz) and 2.71 *dd* (*J* = 16.4, 6.0 Hz, H-7a and 7b), 2.61 *d* and 2.45 *d* (*J* = 16.5, H-3a,b); ^{13}C NMR (DMSO) δ 188.91 (C-6), 172.65 (C-2), 150.07 (C-4), 122.45 (C-5), 74.15 (C-3a), 58.60 (C-7a), 43.58 (C-3), 39.78 (C-7). Guerriero *et al.* commented on the unusually low enantiomeric purity (approximately 10%) of their 5-bromocavernicolin which had $[\alpha]_D^{23}$ + 0.036° (0.00084 g ml^{-1}, MeOH). Our own sample failed to exhibit a measurable rotation in MeOH or $CHCl_3$.

*Cavernicolin 1 (**5**) and Cavernicolin 2 (**6**).* +FAB (in NBA) for the 2:1 mixture: *m/z* 326 (MH^+); HRMS (in NBA): *m/z* 325.88509; calcd. for $C_8H_8NO_3$ $^{79}Br_2$. (MH^+) 325.88517; ^1H NMR (DMSO) of major isomer **5**, δ 8.60 *brs* (NH), 7.44 *s* (H-4), 5.39 *d* (*J* = 10.5, H-7), 3.94 *dd* (*J* = 10.4, 4.8, H-7a), 2.85 *d* (*J* = 16.5 Hz, H-3a), 2.22 *d* (*J* = 16.5 Hz, H-3b), ^{13}C NMR of **5** δ 183.65 (C-6), 173.59 (C-2), 150.54 (C-4), 118.96 (C-5), 75.45 (C-

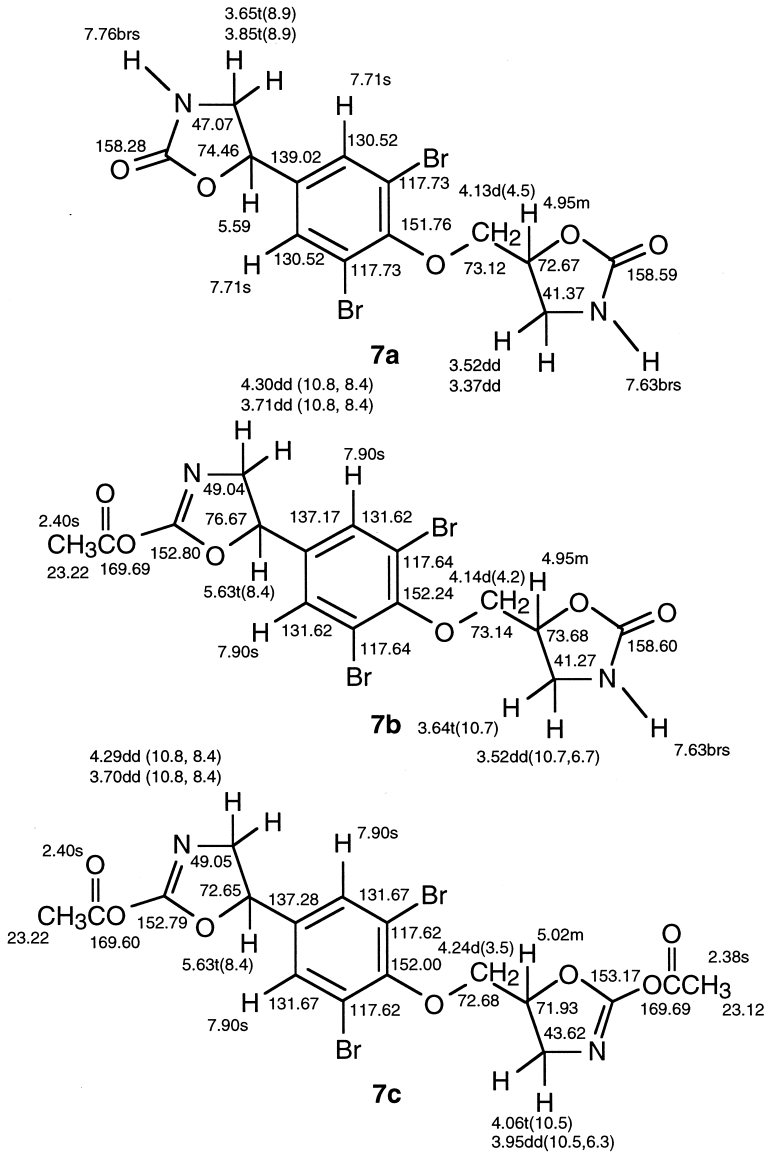

Fig. 2. ^{1}H and ^{13}C NMR Spectra of compounds **7a–7c.**

3a), 68.04 (C-7a) 58.00 (C-7), 42.09 (C-3); ^{1}H NMR of minor isomer **6** δ 8.19 *brs* (NH), 7.33 *s* (H-4), 5.26 *d* (*J* = 4.1 Hz, H-7), 4.22 *d* (*J* = 4.1 Hz, H-7a), 2.62 *d* (*J* = 16.8 Hz, H-3a), 2.42 *d* (*J* = 16.8 Hz, H-3b); ^{13}C NMR (DMSO) of **6** δ 183.73 (C-6), 173.08 (C-2), 149.27 (C-4), 118.74 (C-5), 74.13 (C-3a), 63.35 (C-7a), 52.69 (C-7), 44.10 (C-3).

*Bis-oxazolidone **7a** and its mono- and diacetates **7b** and **7c.*** Compound **7a** has been reported previously. For comparison with the structures assigned to the new mono- and diacetates **7b** and **7c** the ^{1}H and ^{13}C NMR spectra of **7a** are included with those of **7b** and **7c** in Fig. 2. Assignments for **7a–c** were made by COSY, NOESY and HMBC. Acetylation of 55 mg of **7a** by Ac_2O-pyridine in the usual fashion followed by the usual work-up and purification by preparative thin layer chromatography (Si gel, $CHCl_3$-Me_2O-HCO_2H, 80:20:01) gave monoacetate **7b**, + FAB MS (in NBA) *m/z*

Table I. ^{1}H and ^{13}C NMR spectra of compound **8**[a].

Position	δH	δC	NOESY	COSY	HMBC
1	8.14 *brs* (NH)		H-6a	H-6a	C-3,3a,6a
2		174.23 *s*			
3′	2.37 *d* (17.4)	43.43 *t*	H-3″,4		C-2,3a,4,6a
3″	2.19 *d* (17.4)		H-3,4,OH		
3a		81.56 *s*			
4	4.1–4.2 *m*	83.16 *d*	H-8″,5,9,10a,b,OH		C-3,5,6,7,9,10a
5	6.75 *d*(2.5)	141.97 *d*		H-4	
6		137.80 *s*			
6a	4.29 *brs*	67.40 *d*	NH,OH		
7		163.40 *s*			
8[c]	4.1–4.2 *m*	60.55 *t*			
9[b]	1.24 *t* (7)	14.02 *q*	H-4	H-4	C-8
10a	3.69 *dq* (9.2,7)	65.93	H-8a,b,10b,11	H-10b,11	C-4
10b	3.55 *dq* (9.2,7)		H-4,4,10a,11	H-10a,11	C-4
11b	1.13 *t* (7)	15.41 *q*	H-10a,b		C-10a
OH	5.36 *brs*		H-3b,6a		C-3,3a,6a

[a] In DMSO at 300 MHz resp. 75 MHz; [b] Intensity 3 protons; [c] Intensity 2 protons.

479 (MH^+), 460; HRMS (in NBA) *m/z* 478.92773; calcd. for $^{12}C_{15}{}^{1}H_{14}{}^{15}N_2{}^{16}O_6{}^{79}Br^{81}Br$ 478.92777 (MH^+), and diacetate **7c**, FAB MS (in NBA) *m/z* 521 (MH^+); HRMS (in NBA) 520.93824; calcd. for $^{12}C_{17}{}^{1}{}_{H17}{}^{14}N_2{}^{16}O_7{}^{79}Br^{81}Br$ (MH^+) 520.93833 (MH^+).

*Subereatensin (**8**).* Gum; MS FAB (in NBA): *m/z* 256 (M + H^+, 100); HRMS FAB (in NBA) *m/z* 256.11858, calcd for $C_{12}H_{18}NO_5$ (M + H^+) *m/z* 256.11850; $[\alpha]_D$ + 25.51 (C = 0.002 g/ml, MeOH); 1H and ^{13}C NMR spectra, NOESY, COSY and HMBC correlations in Table I. The presence of a conjugated carbethoxy group was indicated by the signals of C-5 through C-9, H-5, H-8 and H-9 and the correlations shown in Table I; H-5 was coupled to one of the signals in the two proton multiplet at δ 4.1–4.2 and attached to a carbon at δ 83.16 carrying an ethoxy group; the remaining signals and correlations were characteristic of the five-membered amide ring of the various cavernicolins with a hydroxyl group on carbon-3a. A possible route to the formation of **8** may involve oxidative cleavage of a precursor of type **9** to **10** followed by an aldol condensation (Fig. 3).

Cytotoxicity assays

a) Cell lines. Human tumor cell lines MCF-7 (breast), NCI-H460 (lung), SF-268 (CNS), TK-10 (renal) and UACC-62 (melanoma) were provided by the National Cancer Institute, Bethesda, MD.

b) Cell growth assay. Stock solutions in DMSO were stored at −20 °C providing uniform samples for retests. The frozen concentrates were diluted to the desired concentration with the cell culture medium prior to the assays. Effects on the growth of human cancer cell lines were evaluated by the procedure adopted in the U. S. National Cancer Institute's *in vitro* anticancer drug screening program which uses the sulforhodamine B (SRB) assay to assess growth inhibition (Skehan *et al.*, 1990). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum, 2 mM glu-

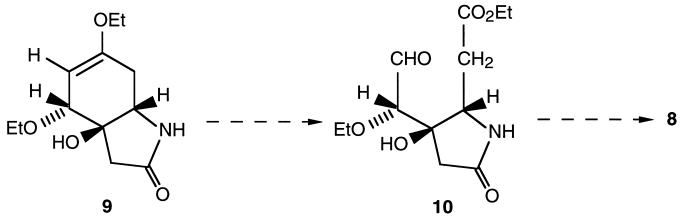

Fig. 3. Suggested origin of compound **8**.

tamine and 50 μg/ml gentamycin at 37 °C in a humidified atmosphere containing 5% CO_2. For the SRB assay each cell line was plated at the density ensuring exponential growth throughout the period of the experiment (according to the growth profiles 7.5×10^4 for NCI-H 460, 1×10^5 cells/ml for UACC-62, 1.5×10^5 for MCF-7, SF-268 and TK-10) in 96-well plates and allowed to attach overnight. Cells were then exposed for 48 hr to serial concentrations of compounds and to the positive control doxorubicin. After the incubation period the adherent cells were fixed *in situ*, washed and stained with SRB. The bound stain was solubilized and the absorbance measured at 492 nm in a microplate reader. The concentration at inhibition of 50% of net cell growth (GI_{50}) was calculated as described elsewhere (Monks *et al.*, 1991). Toxicity was inferred from the SRB assay by comparing the absorbance of the wells containing treated cells after 48 hr with wells containing untreated cells fixed at the time at which compounds were added. Lower absorbances after 48 hr of treatment indicated occurrence of cell death instead of growth arrest.

Results and Discussion

In vitro effects of six compounds from *Subrerea* aff. *praetensa* (Row) and their acetates on the growth of five human cancer cell lines are listed in Table II. Results are given in concentrations causing 50% cell growth inhibition. Inhibition was produced by several compounds. The effect produced by **1b** and **4b** appeared to be associated with real growth inhibition and not to cell death to toxicity, as inferred from the SRB assay. By contrast the effect produced by **4a** seemed to be associated with toxicity because the number of cells remaining after 48 hr exposure was less than before addition of **4a**. While **4b** showed a modest inhibitory effect ($GI_{50} > 20$ μM), **1b** was a potent inhibitor of MCF-7, SF-268 and UACC-62 cancer cell lines ($GI_{50} < 10$ μM).

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Table II. Concentration of compounds from *Suberea* aff. *praetensa* causing 50% cell growth inhibition (GI_{50}) of five human cancer cell lines.[a]

	GI_{50}[μM]				
	MCF-7	NCI-H460	SF268	TK10	UACC-62
1a	92.5 ± 8.5	–	–	> 100	> 100
1b	7.4 ± 0.4	9.5 ± 0.8	–	29.2 ± 1.2	8.2 ± 0.7
2	> 100	> 100	> 100	> 100	> 100
3a	> 100	> 100	> 100	> 100	> 100
4a	4.6 ± 0.3	25.8 ± 0.9	20.9 ± 1.2	10.2 ± 0.9	8.3 ± 0.6
4b	24.3 ± 1.6	79.0 ± 2.9	77.4 ± 2.2	37.3 ± 4.6	33.8 ± 8.4
7a	> 100	> 100	> 100	> 100	> 100
7b	> 100	> 100	> 100	> 100	> 100
7c	> 100	> 100	> 100	> 100	> 100

[a] Doxorubicin was used as a positive control: GI_{50} MCF-7 = 5.5 ± 3.2 × 10^{-2} μM; NCI-H460 = 0.81 ± 0.2 × 10^{-2} μM; SF-268 = 9.3 ± 0.7 × 10^{-2} μM; TK-10 = 57.0 ± 13.2 × 10^{-2} μM; UACC-62 = 9.4 *t* ± 2.3 × 10^{-2} μM. Results are means ± SEM of 3–6 independent observations performed in duplicate.

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