Further Halotyrosine Derivatives from the Marine Sponge Suberea aff. praetensa

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Z. Naturforsch. \textbf{57c}, 732–738 (2002); received April 9/May 21, 2002

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 \textit{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, \textit{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 \textit{et al.}, 1984, Guerrero, \textit{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 \textit{et al.}, 1982) and the bis-oxazolidone 7a (see Chart 1), previously reported from Verongia lacunosa (Borders \textit{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

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

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\textsuperscript{8} Dedicated to Professor Dr. Nuno R. Grande, founder of Instituto de Ciências Biomédicas de Abel Salazar, on the occasion of his 70\textsuperscript{th} 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 200 ml frs being collected as follows: Frs 1–4 (petrol-CHCl3, 7:3 v/v), 5–19 (petrol-CHCl3, 1:1), 20–31 (petrol-CHCl3, 3:7), 32–42 (petrol-CHCl3, 1:9), 43–65 (CHCl3-Me2O, 9:1), 66–82 (CHCl3-Me2O, 7:3), 83–108 (CHCl3-Me2O, 1:1), 109–130 (CHCl3-Me2O, 1:9). Recrystallization of frs 23–28 (52 mg) from petrol and CHCl3 gave clionasterol (23 mg) identified by MS and 1H NMR spectrometry. Frs 49–57 (140 mg) on purification by PTLC (Si gel, CHCl3-Me2O-HCO2H, 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 1H and 13C 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-CHCl3, 3:2), 14–24 (petrol-CHCl3, 2:3), 25–35 (petrol-CHCl3, 1:4 v/v), 36–46 (CHCl3, 47–61 (CHCl3, Me2O, 4:1), 62–73 (CHCl3-Me2O, 3:2), 74–82 (CHCl3-Me2O, 2:3), 83–99 (CHCl3-Me2O, 1:4). Recrystallization of frs 7–8 (252 mg) from petrol-CHCl3 furnished clionasterol (187 mg). Purification of frs 14–19 (184 mg) by PTLC (Si gel, CHCl3-Me2O-HCO2H, 95:5:0.1) gave 36 mg of unknown viscous material. PTLC (Si gel, CHCl3-Me2O-HCO2H, 85:15:0.1) of frs 36–39 (194 mg) gave subereatasin (8, 36 mg). Recrystallization of frs 40–46 (324 mg) from CHCl3-petrol gave 3a (153 mg) (Tymiak and Rinehart, 1985) further characterized as the acetate 3b. Recrystallization of frs 47–48 (222 mg) from CHCl3-Me2O 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, CHCl3-Me2O-HCO2H) gave 51 mg of a mixture of cavernicolin 1 (5, major) and cavernicolin 2 (6, minor), identified by 1H and 13C 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 CHCl3-Me2O afforded the bis-2-oxazolidone 7a (Fig. 2) (420 mg) previously reported from Verongia lacunosa (Borders et al., 1974) and Aplysina
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 C₈H₉NO₃₃⁷Cl: 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); ¹³C 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); ¹³C 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).

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 C₈H₈NO₃⁷⁹Br₂ (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), ¹³C NMR of 5 δ 183.65 (C-6), 173.59 (C-2), 150.54 (C-4), 118.96 (C-5), 75.45 (C-7).
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 $\delta$ 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 $\delta$ 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$_2$O-pyridine in the usual fashion followed by the usual work-up and purification by preparative thin layer chromatography (Si gel, CHCl$_3$-Me$_2$O-HCO$_2$H, 80:20:01) gave monoacetate 7b, + FAB MS (in NBA) $m/z$
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<table>
<thead>
<tr>
<th>Position</th>
<th>δH, ppm</th>
<th>δC, ppm</th>
<th>NOESY</th>
<th>COSY</th>
<th>HMBC</th>
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<tbody>
<tr>
<td>1</td>
<td>8.14 brs (NH)</td>
<td></td>
<td>H-6a</td>
<td>H-6a</td>
<td>C-3a,6a</td>
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<tr>
<td>2</td>
<td>2.37 d (17.4)</td>
<td>174.23 s</td>
<td>H-3',4</td>
<td></td>
<td>C-2a,4,6a</td>
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<tr>
<td>3</td>
<td>2.19 d (17.4)</td>
<td>43.43 t</td>
<td>H-3,4,OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td></td>
<td>81.56 s</td>
<td>H-8,5,9,10a,b,OH</td>
<td>H-4</td>
<td>C-3,5,6,7,9,10a</td>
</tr>
<tr>
<td>4</td>
<td>6.75 d(2.5)</td>
<td>141.97 d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>137.80 s</td>
<td>H-10b,11</td>
<td>C-8</td>
<td></td>
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<tr>
<td>6a</td>
<td>4.29 brs</td>
<td>67.40 d</td>
<td>NH,OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>163.40 s</td>
<td>H-10b,11</td>
<td>C-8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.1–4.2 m</td>
<td>60.55 t</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9b</td>
<td>1.24 t (7)</td>
<td>14.02 q</td>
<td>H-10a,11</td>
<td>C-4</td>
<td></td>
</tr>
<tr>
<td>10b</td>
<td>3.69 dq</td>
<td>65.93</td>
<td>H-8a,b,10b,11</td>
<td>H-10b,11</td>
<td>C-4</td>
</tr>
<tr>
<td>11b</td>
<td>1.13 t (7)</td>
<td>15.41 q</td>
<td>H-10a,b</td>
<td>C-10a</td>
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<tr>
<td>OH</td>
<td>5.36 brs</td>
<td></td>
<td>H-3b,6a</td>
<td>C-3a,6a</td>
<td></td>
</tr>
</tbody>
</table>

Table I. ¹H and ¹³C NMR spectra of compound 8.

479 (MH⁺), 460; HRMS (in NBA) m/z 478.92773; calcd. for $^{12}$C$_{15}$H$_{14}$N$_{2}$O$_{6}$Br$_{8}$ 478.92777 (MH⁺), and diacetate 7c, FAB MS (in NBA) m/z 521 (MH⁺); HRMS (in NBA) 520.93824; calcd. for $^{12}$C$_{17}$H$_{17}$N$_{2}$O$_{7}$Br$_{8}$ (MH⁺) 520.93833 (MH⁺).

*Subereatensin* (8). Gum; MS FAB (in NBA): m/z 256 (M + H⁺, 100); HRMS FAB (in NBA) m/z 256.11850; [α]$_D$ + 25.51 (C = 0.002 g/ml, MeOH); ¹H and ¹³C 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 cavernicolsins 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-
amine and 50 µg/ml gentamycin at 37 °C in a
humidified atmosphere containing 5% CO2. 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 (GI50) was
calculated as described elsewhere (Monks et al.,
1991). Toxicity was inferred from the SRB assay
by comparing the absorbance of the wells contain-
ing treated cells after 48 hr with wells containing
untreated cells fixed at the time at which com-
pounds 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 *Suberea*
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 caus-
ing 50% cell growth inhibition. Inhibition was pro-
duced 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 addi-
tion of 4a. While 4b showed a modest inhibitory
effect (GI50 > 20 µm), 1b was a potent inhibitor of
MCF-7, SF-268 and UACC-62 cancer cell lines
(GI50 < 10 µm).

### Acknowledgements

Work in Portugal was supported by FCT (I & D
222/94) POCTI (QCA III) and FEDER. We thank
the National Cancer Institute, Bethesda, MD, USA
for the generous provision of tumor cell lines.

### Table II. Concentration of compounds from *Suberea* aff. *praetensa* causing 50% cell growth inhibition (GI50) of five human cancer cell lines.

<table>
<thead>
<tr>
<th></th>
<th>GI50[µm]</th>
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<tbody>
<tr>
<td></td>
<td>MCF-7</td>
</tr>
<tr>
<td>1a</td>
<td>92.5 ± 8.5</td>
</tr>
<tr>
<td>1b</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>3a</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>4a</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>4b</td>
<td>24.3 ± 1.6</td>
</tr>
<tr>
<td>7a</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>7b</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>7c</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

* Doxorubicin was used as a positive control: GI50 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^-5 µm; TK-10 = 57.0 ± 13.2 × 10^-2 µm; UACC-62 = 9.4 ± 2.3 × 10^-2 µm. Results are
means ± SEM of 3–6 independent observations performed in duplicate.


