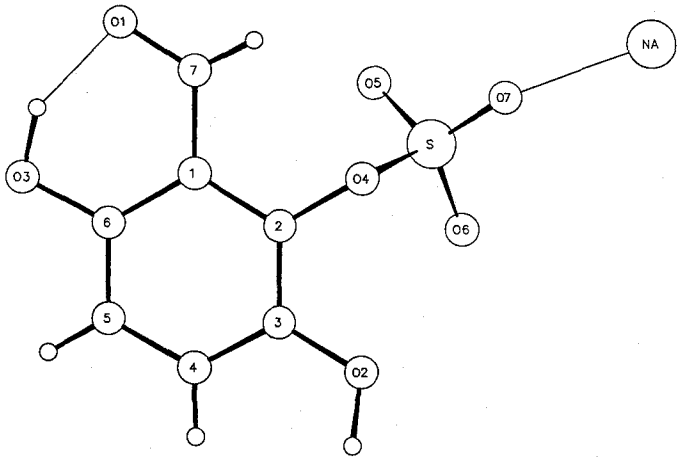

An X-ray perspective drawing for polyclinal (**1**).

the sulfated metabolite siphonodictyal D isolated from the burrowing sponge *Siphonodictyon coralliphagum*, possess an analogous substitution pattern of phenolic hydroxyl groups as polyclinal (**1**).

Subsequent collections of *P. planum* were used to investigate the distribution of polyclinal within three distinct regions of the ascidian colonies. The colonies were dissected into the stalk, the pulpy inner mesenchyme and the zooid-rich surface layer of the colonies. The concentrations of polyclinal in these different colony parts, based on wet weights, were determined to be 5.8×10^{-5} g/g, 7.8×10^{-4} g/g and 2.5×10^{-3} g/g in the stolons, the cortex and the zooid rich outer layers of the colonies, respectively. The higher concentration of polyclinal in the zooid-rich surface layer of the colonies suggests that polyclinal may function as a chemical defense against predators which would be consistent with previous observations on the distribution of predator deterrent gorgonian secondary metabolites in the outer more accessible portions of the colonies[14]. Due to the instability of polyclinal in the agar-squid preparations used in our feeding preference assays, we were unable to perform ecologically relevant bioassays to investigate this metabolite's potential ichthyodeterrent properties.

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7 Polyclinal (**1**) ^{1}H NMR (CD_3 OD, 360 MHz) 10.26 (1H, s, H-1'), 7.27 (1H, d, $J = 6.8$ Hz, H-4), 6.68 (1H, d. $J = 6.8$ Hz, H-5). ^{13}C NMR (CD_3 OD, 50 MHz) 196.8 (C-1'), 157.0 (C-6), 143.5 (C-3), 142.1 (C-2), 128.6 (C-4), 116.4 (C-1) 115.6 (C-5) IR (nujol) 3550-3350, 1670, 1640, 1630, 1585, 1300, 1220, 1180, 1075, 1038, 950 cm^{-1}. Negative LR-FABMS obsd. *m/z* 767, 533, 511, 489, 255 and 233.
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9 Derivative **2**. ^{1}H NMR (CD_3 OD, 360 MHz) 7.98 (1H, s, H-1'), 7.31 (1H, d, $J = 6.8$ Hz, H-4), 7.05 (1H, d, $J = 6.8$ Hz, H-5), 2.37 (6H, s, -OAc), 2.26 (3H, s, -OAc), 2.03 (6H, s, -OAc). LRDEIMS obsd. *m/z* 323, 280, 238, 196, and 154.
10 Polyclinal crystalized in the common monoclinic space group $P2_1/c$ with $a = 8.588$ (2), $b = 10.405$ (1), $c = 10.304$ (2) A, and $\beta = 101.30$ (1)°. The structure was solved routinely, and the conventional crystallographic residual for the 1128 (93%) observed ($|F_0| > 3\sigma(F_0)$) reflections is 0.047. Archival X-ray crystallographic data have been deposited with and can be ordered from the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, U.K. Please give a complete literature citation when ordering.
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New tambjamine class alkaloids from the marine ascidian *Atapozoa* sp. and its nudibranch predators. Origin of the tambjamines in *Atapozoa*

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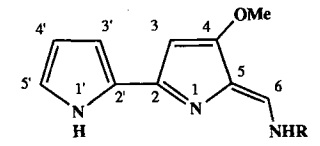
Summary. Two new tambjamine class alkaloids, possessing ichthyodeterrent properties, have been isolated from the organic extracts of the marine ascidian *Atapozoa* sp. and its nudibranch predators. The structure of the new metabolites were elucidated through interpretation of their physical and spectral data and by comparison with spectral data for related compounds. Microscopic examination of *Atapozoa* considering the yellow color of the tambjamines suggested that *Atapozoa* is capable of the de novo biosynthesis of these metabolites.
Key words. Atapozoa; ascidian; chemical defense; tambjamine class alkaloids; origin of secondary metabolites.

Recently, the origins of several biologically active secondary metabolites such as tetrodotoxin, ciguatoxin and okadaic acid, initially isolated from marine macroorganisms, have been traced to their associated microorganisms[2]. In addition, closely related metabolites isolated from phylogenetically diverse marine macroorganisms have been proposed to arise from the biosynthetic capabilities of associated microorganisms[2]. In this paper, we describe the isolation and characterization of the bipyrrole and tetrapyrrole secondary metabolites, including two new tambjamine class alkaloids (**2**–**3**), from the ascidian *Atapozoa* sp. (Polycitoridae, Aplousobranchia)[3] and from several species of nembrothid nudibranchs observed as predators of this ascidian. Tambjamine class bipyrroles and a related tetrapyrrole have been described from bryozoans[4,5], an ascidian[6], and a mutant strain of the bacterium *Serratia marcescens*[7]. Microscopic examination of *Atapozoa*, considering the physical properties of the tambjamine class alkaloids, leads us to conclude that *Atapozoa* is capable of the de novo biosynthesis of these metabolites.

Research with *Atapozoa* sp. and its nudibranch predators has also established the first direct chemical link in predator-prey associations involving ascidians and physically vulnerable molluscs and has demonstrated the in situ ichthyodeterrent properties of the *Atapozoa* secondary metabolites[8].

Materials and methods

Atapozoa sp. is a soft, physically vulnerable, colonial ascidian easily accessible to potential generalist predators. Collections of *Atapozoa* sp. were made in shallow water habitats at several islands in the central Philippines and in diverse areas of the western Pacific including Kwajalein Atoll, Ant Atoll near Ponape, Palau, and Bunaken Island near Manado in Sulawesi[9]. After collection, the ascidians were either frozen or immediately placed in organic solvents. Nudibranchs of the genus *Nembrotha*, including *N. cristata, N. kubaryana* and several unidentified species, were commonly observed grazing on *Atapozoa*. These nudibranchs were also collected and either frozen or stored in organic solvents. Several collections were made of the exuded mucus of *Nembrotha* spp. from the Philippines. The nudibranchs were irritated by rough handling and the blue-green mucus they exuded was collected on filter paper and stored in acetone. After solvent partitioning of the crude extract, the initial fractionations were accomplished by silica gel vacuum flash chromatography with the final purification of secondary metabolites by gel-filtration using Sephadex LH-20 and by C-18 reversed-phase HPLC (8:2 methanol/water with 0.1 M ammonium acetate buffer). A less polar blue pigment was obtained from the nudibranchs by elution from silica gel vacuum flash chromatography using hexane-ether solvent mixtures. This blue pigment was also detected in organic extracts of *Atapozoa* larvae by TLC analysis. The yields of compounds **1**–**3** ranged from

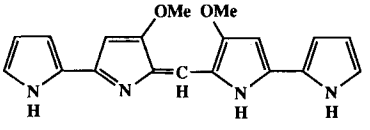


tambjamine C (**1**) R =

tambjamine E (**2**) R =

tambjamine F (**3**) R =

tambjamine A (**4**) R = H



tetrapyrrole (**5**)

1.7% dry mass in the ascidians to as high as 4.1% dry mass in the nudibranchs. Compounds **1**–**3** accounted for as much as 65% of the lipid-extractable mass of the mucus, while the previously reported tetrapyrrole **5** accounted for up to 8.1% of this material.

Structures of tambjamines E and F (**2** and **3**). The major metabolite from the initial collections of *Atapozoa* sp. was readily identified as tambjamine C (**1**) by a comparison of previously reported spectral data for this metabolite[4–10]. Tambjamines A–D were first isolated from the Gulf of California bryozoan *Sessibugula translucens* and its nembrothid nudibranch predators *Tambje abdere* and *T. eliora*.

Two additional compounds (**2** and **3**) isolated from *Atapozoa* sp. and the nudibranchs also possessed the tambjamine bipyrrole nucleus[11,12] but were derived from different alkylamines to form two new tambjamines, E and F (**2** and **3**). A molecular formula of $C_{12}H_{15}N_3O$ was established for **2** from its M^+ $m/z = 217.1218$ ion in the HREIMS in conjunction with ^1H and ^{13}C NMR data[10]. The close similarity of the downfield bands in both the ^1H and ^{13}C NMR spectra and the UV and IR spectra of **2** with those for **1** established the presence of the identical bipyrrole nucleus. An ethylamine residue was evident from the ^1H NMR bands at δ 3.56 (2H, bq, $J = 7.3$ Hz) and 1.41 (3H, t, $J = 7.3$ Hz) and ^{13}C NMR bands at δ 45.8 (CH_2) and 15.5 (CH_3). These spectral features confidently established the structure of tambjamine E as **2**. Tambjamine F (**3**) was determined to have a molecular formula of $C_{18}H_{19}N_3O$ by interpretation of the M^+ $m/z = 293.1543$ ion in conjunction with ^1H and ^{13}C NMR data[12]. The presence of the tambjamine bipyrrole nucleus was also indicated for **3** by comparison of its NMR, UV and IR spectra with those of **1** and **2**. The ^1H and ^{13}C NMR spectra of **3** also revealed the presence of a phenethylamine residue. Five proton bands were observed between δ 7.20 and 7.35 and ^{13}C NMR resonances at δ 52.5 (C8), 36.7 (C9), 138.7 (C10), 129.3 (C11, 2C),

129.8 (C12, 2C) and 127.3 (C13). The loss of 91 mass units (C_7H_7) from the parent ion in the HREIMS of **3** to yield the base peak at *m/z* 202 results from a cleavage between C8 and C9. This cleavage helped confirm the assignment of phenethylamine as the side chain and establish the structure of tambjamine F as **3**.

Tambjamine A (**4**), isolated in minor quantities from several of the nudibranch predators of *Atapozoa*, was identified by interpretation of the M^+ *m/z* = 189.0899 ion in the HREIMS and a comparison of its ^1H NMR spectra[13] with that previously reported for this compound[4].

The blue-pigmented tetrapyrrole **5**, isolated from *Nembrotha* sp., *N. kubaryana* and their exuded mucus, was identified by interpretation of the M^+ *m/z* = 334.1406 ion that confidently analyzed for $C_{19}H_{18}N_4O_2$ and a comparision of its ^1H NMR spectrum[14] with the previously reported ^1H NMR chemical shifts[5,6] for **5**. The geometry about the C5–C6 double bond was not determined in the previous studies of this blue pigment and was also not addressed in our work.

Tambjamine C (**1**) and a small quantity of **5** were also isolated from the larvae of *Atapozoa* and thus represent the first characterization of secondary metabolites from the larvae of an ascidian. The unpalatability of *Atapozoa* larvae to coral reef fishes (N. Lindquist, pers. obs.) and the presence of deterrent quantities of **1** in these larvae, along with the demonstrated in situ feeding deterrent properties of tambjamine class alkaloids[8], provides convincing evidence for the chemical protection of *Atapozoa* larvae.

Microscopic examination of *Atapozoa* reveals that its intense pigmentation is confined to the granular amebocyte blood cells[3]. Thus the large quantity (0.5%–1.7% dry weight) of the brightly yellow-colored tambjamines should therefore reside within these blood cells. The morula cells, a blood constituent of the ascidian *Ascidia nigra*, have been found to contain virtually all the reducing blood pigments called the tunichromes[15], and more recently, the antimicrobial halocyamines from the ascidian *Halocynthia roretzi* were detected only in its morula cells[16].

The isolation of tambjamines and the tetrapyrrole (**5**) from phylogenetically diverse marine invertebrates[4–6] and the isolation of **5** from a mutant strain of the bacterium *S. marcescens*[7] would suggest that these compounds originate from symbiotic bacteria associated with these animals. However, a transmission electron microscopic investigation of *Atapozoa* larvae, performed expressly for the purpose of identifying bacterial symbionts failed to find any significant quantities of bacteria within the granular amebocytes or in any other part of this ascidian. Thus we conclude that *Atapozoa* is capable of the de novo biosynthesis of the tambjamines observed here.

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10 Tambjamine C (**1**): brown oil; ^1H NMR ($CDCl_3$, 360 MHz). 7.27 (1H, bs, H-6), 7.06 (1H, m, H-3′), 6.70 (1H, m, H-5′), 6.25 (1H, m, H-4′), 5.95 (1H, s, H-3), 3.91 (3H, s, H-7), 3.26 (2H, d, *J* = 6.7 Hz, H-8), 1.99 (2H, dt, *J* = 6.7, 6.7, H-9), 1.01 (6H, d, *J* = 6.7 Hz, H-10,11). ^{13}C NMR ($CDCl_3$, 50 MHz). 164.7 (C-4), 143.4 (C-6), 142.3 (C-2), 123.9 (C-5′), 123.8 (C-2′), 113.2 (C-4′), 111.3 (C-5), 111.2 (C-3′), 92.1 (C-3), 59.0 (C-7), 58.4 (C-8), 30.1 (C-9), 20.0 (2C, C-10,11). UV (MeOH) 405 (ε 30500), 280 (sh), 251 (6600), 204 nm (5300). UV (MeOH + NaOH) 368 (ε 17700), 252 (6900), 203 nm (26600). IR ($CHCl_3$) 3500–2900, 2970, 1670, 1620, 1530, 1410, 1315, 1240, 1165, 1140, 1118, 1035, 1015, 965 cm^{-1}. HREIMS obsd. (M^+) *m/z* 245.1532, $C_{14}H_{19}N_3O$ requires 245.1528, Δ 1.6.
11 Tambjamine E (**2**): yellow crystals ($CDCl_3$); m.p. 68–70 °C. ^1H NMR ($CDCl_3$, 360 MHz) 9.92 (1H, bs, H-1′), 9.50 (1H, bs, NH), 7.36 (1H, bm, H-6), 7.07 (1H, bm, H-3′), 6.76 (1H, bm, H-5′), 6.28 (1H, bm, H-4′), 5.97 (1H, s, H-3), 3.93 (3H, s, H-7), 3.56 (2H, bq, *J* = 7.3 Hz, H-8), 1.41 (3H, t, *J* = 7.3 Hz, H-9). ^{13}C NMR ($CDCl_3$, 50 MHz) 164.6 (C-4), 142.8 (C-6), 142.2 (C-2), 123.8 (C-5′), 123.7 (C-2′), 113.0 (C-4′), 111.4 (C-5), 111.2 (C-3′), 92.3 (C-3), 59.1 (C-7), 45.8 (C-8), 15.5 (C-9). UV (MeOH) 405 (ε 24700), 280 (sh), 257 (5700), 205 nm (4900). UV (MeOH + NaOH) 363 (ε 14400), 251 (6200), 204 nm (24600). IR ($CHCL_3$) 3500–2800, 1664, 1608, 1529, 1342, 1259, 1170, 977 cm^{-1}. HREIMS obsd. (M^+) *m/z* 217.1218, $C_{12}H_{15}N_3O$ requires 217.1215, Δ 1.4.
12 Tambjamine F (**3**): brown oil. ^1H NMR ($CDCl_3$, 360 MHz) 9.88, (1H, bs, H-1′), 7.35–7.20 (6H, H-6,11 (2H), 12 (2H), 13), 7.07 (1H, bm, H-3′), 6.70 (1H, bm, H-5′), 6.27 (1H, bm, H-4′), 5.93 (1H, s, H-3), 3.87 (3H, s, H-7), 3.68 (2H, t, *J* = 7.2 Hz, H-8), 3.03 (2H, t, *J* = 7.2 Hz, H-9). ^{13}C NMR ($CDCl_3$, 50 MHz) 164.7 (C-4), 143.0 (C-6), 142.3 (C-2), 138.7 (C-10), 129.8 (2C, C-12), 129.3 (2C, C-11), 127.3 (C-13), 123.9 (C-5′), 123.6 (C-2′), 113.2 (C-4′), 113.2 (2C, C-3′,5), 92.2 (C-3). 59.0 (C-7), 52.5 (C-8) 36.7 (C-9). UV (MeOH) 407 (ε 32100), 280 (sh), 259 (7000), 205 nm (10200). UV (MeOH + NaOH) 365 (ε 19200), 251 (7600), 203 nm (32600). IR ($CHCl_3$) 3450–2800, 1670, 1620, 1600, 1530, 1420, 1190, 1120, 1035, 1015, 965 cm^{-1}. HREIMS obsd. (M^+) *m/z* 293.1543, $C_{18}H_{19}N_3O$ requires 293.1530, Δ 4.4.
13 Tambjamine A (**4**): yellow oil. ^1H NMR ($CDCl_3$, 360 MHz) 7.49 (1H, bs, H-6), 7.11 (1H, m, H-3′), 6.81 (1H, m, H-5′), 6.32 (1H, m, H-4′), 5.96 (1H, s, H-3), 3.93 (3H, s, H-7). HREIMS obsd. (M^+) *m/z* 189.0899, $C_{10}H_{11}N_3O$ requires 189.0902, Δ 1.6.
14 Tetrapyrrole (**5**): dark blue oil. ^1H NMR ($CDCl_3$, 360 MHz) 11.97 (1H, bs, H-1), 11.77 (2H, bs, H-1′), 7.12 (3H, bs, H-5′ (2H), H-6 (1H)), 6.79 (2H, bd, H-3′), 6.32 (2H, bt, H-4′), 6.07 (2H, s, H-3), 3.94 (6H, s, H-7). HREIMS obsd. (M^+) *m/z* 334. 1406, $C_{19}H_{18}N_4O_2$ requires 334.1398, Δ 2.4.
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