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# Effect of urochordamines on larval metamorphosis of ascidians

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*Abstract.* Two pteridine-containing bromophysostigmine alkaloids, urochordamine A and B, which were isolated from ascidians as larval metamorphosis promoters, were converted to more polar compounds, urochordamine A′ and B′, respectively, when left standing in protic solvents. These four compounds promoted larval metamorphosis of the ascidian *Halocynthia roretzi* in the order A′ > A > B > B′, and induced metamorphosis of the pediveliger larvae of the mussel *Mytilus edulis galloprovincialis.*
*Key words.* Metamorphosis promoter; ascidian larva; urochordamine; *Ciona savignyi*; *Botrylloides* sp.; *Halocynthia roretzi*; *Mytilus edulis galloprovincialis.*

Larvae of marine organisms swim for a while before embarking on a search for a place to settle and grow. The selection of the place is an important event for marine sessile organisms. In a wide range of marine organisms, chemical substances released from the adults, from prey organisms, or from bacteria are believed to function as chemical cues to initiate settlement and subsequent metamorphosis[1]. However, few chemical cues have been identified to date[2–7].

In the course of our studies on mechanisms of larval settlement and metamorphosis of ascidians, we found that extracts from some marine organisms (including ascidians, sponges, and algae) promoted larval metamorphosis of ascidians. A tadpole larva swims for a species-specific period of time and attaches itself to a surface by its head (settlement). After this its tail is gradually absorbed, and the transition from the larval stage to the adult stage occurs (metamorphosis). We have isolated from two ascidians *Ciona savignyi* (the tunic part) and *Botrylloides* sp. (the whole body) two pteridine-containing physostigmine alkaloids, urochordamine A (**1**) and B (**2**) (fig. 1), which promote settlement/metamorphosis in ascidians[8]. During structure determination of urochordamines, we observed that **1** and **2** were converted to more polar derivatives, urochordamine A′ (**3**) and B′ (**4**), respectively, when left standing in protic solvents. Here, we describe the structures of urochordamines A′ (**3**) and B′ (**4**), and the effect of urochordamines on ascidian larval settlement/metamorphosis.

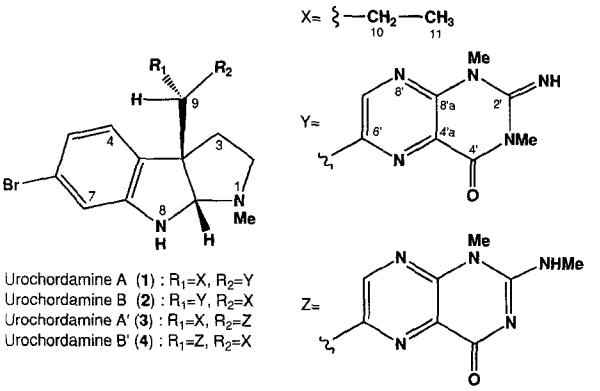


Figure 1. Structures of urochordamines A (**1**), B (**2**), A′ (**3**), and B′ (**4**).

*Rearrangement of urochordamine A (1) and B (2) to urochordamine A′ (3) and B′ (4)*

Urochordamine A (**1**, 50 μg) or B (**2**, 50 μg) was kept at 18 °C in either methanol (100 μL), distilled water (100 μl) containing 2% DMSO, or filtered seawater (100 μl) containing 2% DMSO. A portion (3 μl) of each solution was analyzed daily by HPLC. In all solutions tested, urochordamine A (**1**) and B (**2**) underwent rearrangement into the more polar compounds, urochordamine A′ (**3**) and B′ (**4**), respectively. Compound **1** was rearranged into **3** faster in methanol ($k = 0.75793\ day^{-1}$, $R = 0.996$) than in distilled water ($k = 0.13617\ day^{-1}$, $R = 0.99808$) as shown in figure 2. The rearrangment occurred in seawater as fast as in distilled water (data not shown). Similarly, urochordamine B (**2**) was transformed into urochordamine B′ (**4**).

*The structure of urochordamine A′ (3) and B′ (4)*

Urochordamine A′ (**3**)[9] has a molecular formula of $C_{22}H_{26}BrN_7O$ as determined by the HRFAB mass spectrum (*m/z* 486.1428, $\Delta$ − 1.2 mmu), which was the same as **1**[10]. The $^1H$ and $^{13}C$ NMR spectra of **3** in DMSO-$d_6$ (table) were also superimposable on those of **1**, except for proton signals at $\delta$ 2.58 (d, $J = 3.9$ Hz) and $\delta$ 7.61 (q, $J = 3.9$ Hz), which appeared as singlets at $\delta$ 3.38 and 8.31 in **1** (table). In addition, a large downfield shift was observed for the C4′ carbonyl carbon ($\delta$ 158.8 for **1**;

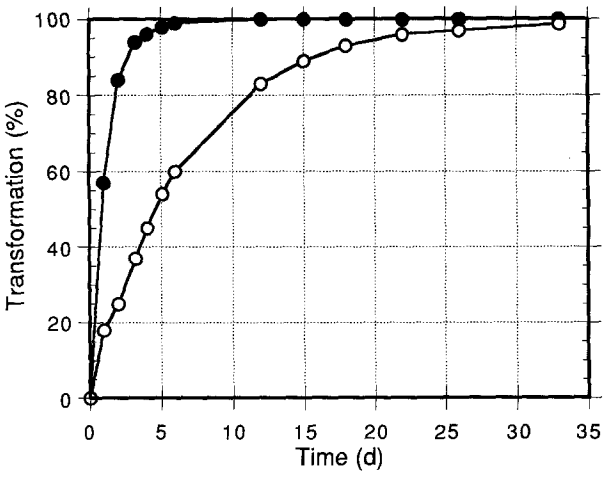


Figure 2. Rearrangement of urochordamine A (**1**) to urochordamine A′ (**3**) either in methanol or distilled water. Fifty microgram of **1** was incubated at 18 °C in methanol (100 µl) or distilled water (100 µl) containing 2% of dimethylsulfoxide (DMSO). Percentages of rearranged products were determined by HPLC. Conditions: column, Asahipak ODP-50, 5 µm, 4.6 × 250 mm; mobile phase, 40% MeOH-water containing 0.01% of trifluoroacetic acid; flow rate, 0.4 ml/min; detector, UV 254 nm. Retention time: **1**, 10.2 min; **2**, 7.6 min; **3**, 42.4 min; **4**, 19.2 min. ● = in methanol; ○ = distilled water.

δ 165.8 for **3**). The HMBC spectrum of **3** revealed no correlation between the 2′-NMe protons and C4′ carbonyl carbon, observed for **1**. This evidence, together with spectral data, led to the assignment of structure **3** (fig. 1). The coupling constant between NMe and NH was compatible with that reported for an aplysinopsin-type compound[11]. Accordingly, urochordamine B′ (**4**)[12] had the structure shown in figure 1. The rearrangement mechanism can be depicted as in figure 3. Attack by an alkoxide ($RO^-$) or a hydroxide group ($HO^-$) on the C4′ carbonyl carbon (ii) results in ring opening (iii → iv). Then, ring closure (v → vi) from the sterically less hindered conformer (v) leads to a stable structure (viii).

*Effect of urochordamines on metamorphosis of ascidians*

In order to obtain some structure-activity relationships of urochordamines, we tested the promoting activity of four urochordamines on ascidian *Halocynthia roretzi* larvae. Urochordamines dissolved in DMSO were added to each well of a twelve-well plate; each well contained fifteen larvae in 4 ml of artificial seawater[13]. The plate was incubated at 13.2 °C in the dark. In the presence of promoters, the larval tail began to be absorbed two hours after addition of **1** (seven hours after hatching) (fig. 4b); metamorphosed larvae settled on the bottom of the plate very loosely with stolon in one day (fig. 4d). In the absence of urochordamines, however, larvae continued swimming until they died. It should be noted that larvae underwent metamorphosis even when they were cultured at a high density. Effects of urochordamines A, B, A′, and B′ (**1**–**4**) on larval metamorphosis (i.e., morphological change as shown in figure 4b) are shown in figure 5. The percentages of metamorphosed larvae at a concentration of 25 µg/ml

$^1H$ and $^{13}C$ NMR Data of Urochordamines A (**1**) and A′ (**3**) in DMSO-$d_6$.

| Urochordamine A (**1**) | | | | | Urochordamine A′ (**3**) | | | |
|---|---|---|---|---|---|---|---|---|
| Atom | $^{13}C$ mult | $^1H$ mult | *J* (Hz) | HMBC correlations | $^{13}C$ mult | $^1H$ mult | *J* (Hz) | HMBC correlations |
| 2 | 51.7 t | α 2.19 m | | | 51.7 t | α 2.14 m | | |
| | | β 2.31 m | | C8a | | β 2.29 m | | |
| 3 | 36.82 t | α 1.82 dt | 12, 6.0 | C3a, C3b, C8a, C9 | 37.1 t | α 1.81 m | | |
| | | β 2.06 dt | 12, 6.5 | C3a, C3b, C9 | | β 2.04 m | | C2, C3a, C3b |
| 3a | 60.2 s | | | | 60.2 s | | | |
| 3b | 133.1 s | | | | 133.1 s | | | |
| 4 | 125.1 d | 6.80 d | 7.8 | C3a, C6, C7a | 125.0 d | 6.81 d | 7.3 | C3a, C7a |
| 5 | 118.9 d | 6.66 dd | 7.8, 1.4 | C3b, C6, C7 | 119.0 d | 6.66 d | 7.3 | C3b |
| 6 | 120.5 s | | | | 120.6 s | | | |
| 7 | 109.8 d | 6.51 d | 1.4 | C3b, C5, C6, C7a | 109.8 d | 6.50 s | | |
| 7a | 153.1 s | | | | 153.0 s | | | |
| 8 | | 6.43 br.s | | | | 6.45 br.s | | |
| 8a | 84.0 d | 4.66 br.s | | | 84.0 d | 4.72 br.s | | |
| 9 | 52.5 d | 3.06 dd | 13, 2.0 | C3a, C3b, C8a, C10, C11, C6′, C7′ | 52.8 d | 3.12 d | 10 | |
| 10 | 22.5 t | α 1.57 m | | | 22.6 t | α 1.56 m | | |
| | | β 1.86 dt | 13, 7.3 | C11 | | β 1.88 m | | |
| 11 | 12.3 q | 0.57 t | 7.3 | C9, C10 | 12.4 q | 0.55 t | 7.3 | C9, C10 |
| 2′ | 148.2 s | | | | 154.7 s | | | |
| 4′ | 158.8 s | | | | 165.8 s | | | |
| 4′a | 125.9 s | | | | 129.1 s | | | |
| 6′ | 149.7 s | | | | 152.1 s | | | |
| 7′ | 147.1 d | 8.19 s | | C6′, C8′a | 145.7 d | 8.25 s | | C6′, C8′a |
| 8′a | 147.4 s | | | | 146.9 s | | | |
| 1-Me | 36.76 q | 2.20 s | | C2, C8a | 36.8 s | 2.20 br.s | | |
| 1′-Me | 29.7 q | 3.59 s | | C2′, C8′a | 29.0 s | 3.58 s | | C2′, C8′a |
| 3′-Me | 29.0 q | 3.38 s | | C2′, C4′ | | | | |
| 2′-NHMe | | | | | 28.7 s | 2.88 d | 3.9 | C2′ |
| 2′-NH | | 8.31 s | | | | 7.61 q | 3.9 | |

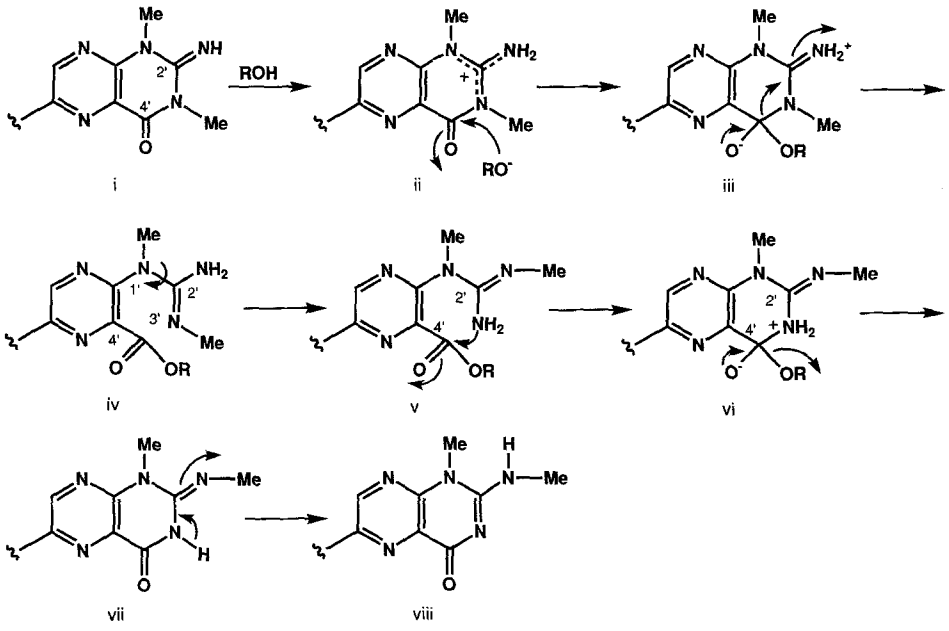


Figure 3. A possible rearrangement mechanism of urochordamine A (**1**) and B (**2**) in protic solvents.

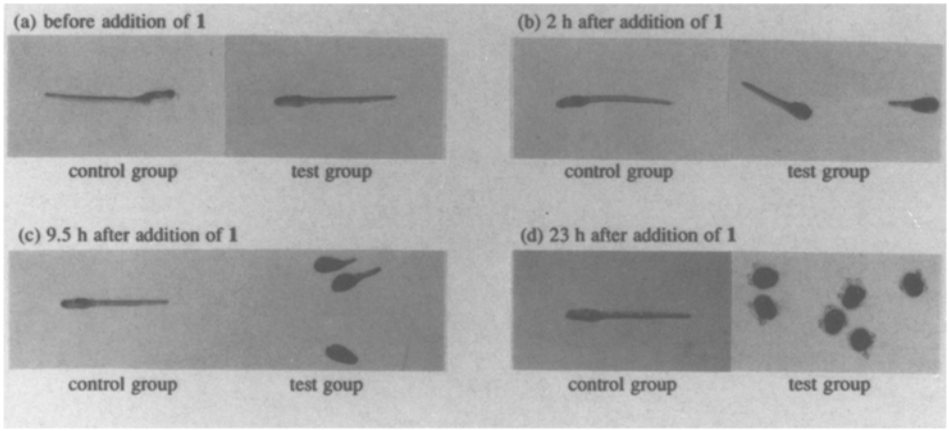


Figure 4. Metamorphosis of *H. roretzi* larvae induced by urochordamine A (**1**). Fifteen 5-h-old larvae were incubated in 4 ml of artificial seawater at 13.2 °C in the dark. To the test group was added 10 µl of DMSO solution of **1** to make up a final concentration of 25 µg/ml. Only 10 µl of DMSO was added to the control group.

were similar whether induced by **1** or **3** (fig. 5). The promoting activity of **2** was lower than that of **1** or **3**. Interestingly, **4** showed no promoting activity at a concentration of 25 µg/ml. Compound **3** induced larval metamorphosis even at a concentration of 0.25 µg/ml, while **1** was ineffective at a concentration of 2.5 µg/ml. Therefore, the order of promoting activity is **3** > **1** > **2** > **4**, indicating that both the structure of the pteridine moiety and the stereochemistry at C9 are important for larval metamorphosis-promoting activity.

Next, we examined the effect of exposure time on metamorphosis. About 20% of 6-h-old larvae metamorphosed when exposed to 25 µg/ml of **3** for 15 min, which was a much lower proportion than at 30 and 60 min-exposures. However, exposure to 25 µg/ml of **1** for 60 min was necessary to induce metamorphosis, while 30-min exposure was ineffective. These results may indicate that urochordamines promote larval metamorphosis in ascidians without a chemoreception pathway. Finally, it should be noted that urochordamines induced metamorphosis of pediveliger larvae of the mussel *Mytilus edulis galloprovincialis*: 87, 73, 26, and 50% of larvae metamorphosed in 6 days after treatment with 2.5 µg/ml of **1**, **2**, **3**, and **4**, respectively. Urochordamines are the first compounds to be identified which

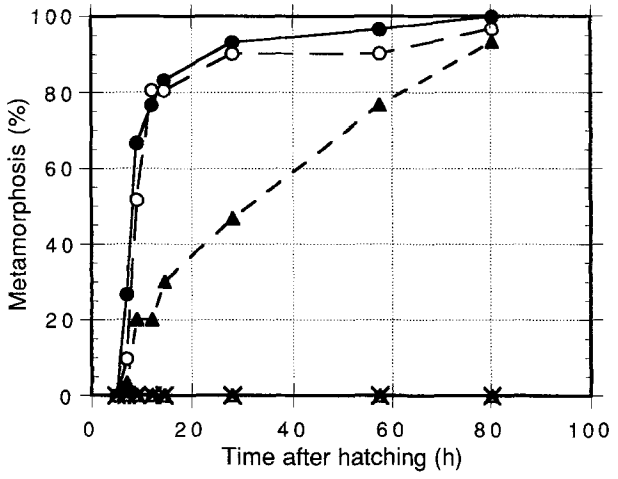


Figure 5. Effects of urochordamine A (**1**), B (**2**), A′ (**3**), or B′ (**4**) on metamorphosis of *H. roretzi* larvae. Assay conditions were the same as described in figure 4. Four compounds at a concentration of 25 μg/ml were tested on 6-h-old larvae. The number of metamorphosed larvae (morphological changes as shown in figure 4b) was counted under a microscope. ● = 1; ▲ = 2; ○ = 3; △ = 4, x = control.

induce metamorphosis of pediveliger larvae. The results again imply that urochordamines do not act via chemoreception, but via an internal mechanism.

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8 Tsukamoto, S., Hirota, H., Kato, H., and Fusetani, N., Tetrahedron Lett. *34* (1993) 4819.
9 Urochordamine A′ (**3**). UV $\lambda_{max}$ 350 (log ε 3.60), 339.5 (3.79), 309 (3.75), and 248.5 nm (4.27). $^1$H and $^{13}$C NMR (DMSO-$d_6$): Table. FABMS (positive, glycerol matrix) *m/z* 486/484 $(M+H)^+$, 232 $(C_{11}H_{14}N_5O)^+$, and 218 $(C_{10}H_{12}N_5O)^+$. HR-FABMS (PEG + NBA matrix) *m/z* 486.1428, calcd for $C_{22}H_{27}{}^{81}BrN_7O$, $\Delta$ − 1.2 mmu).
10 Ultraviolet spectra were recorded on a Hitachi U-2000 spectrophotometer. Infrared spectra were measured on a JASCO IR Report-100 or IR-700 spectrometer. NMR spectra were recorded on a Bruker ARX-500 NMR spectrometer at 27 °C in DMSO-$d_6$. Residual $CHD_2SOCD_3$ (2.49 ppm) and $CD_3SOCD_3$ (39.5 ppm) signals were used as internal standards. FAB mass spectra were measured on a JEOL SX 102 mass spectrometer.
11 Guella, G., Mancini, I., Zibrowius, H., and Pietra, F., Helv. chim. Acta *72* (1989) 1444. In the aplysinopsin-type compound NH*Me* and N*H*Me signals were observed at δ 2.98 (br.d, *J* = 4.8 Hz) and 7.40 (br. q, *J* = 4.8 Hz) in DMSO-$d_6$, respectively.
12 Urochordamine B′ (**4**). UV $\lambda_{max}$ 351.5 (log ε 3.50), 337.5 (3.57), 324.5 (3.57), and 248.0 nm (4.04). $^1$H NMR (DMSO-$d_6$) δ: 0.60 (3 H, *t*, *J* = 7.0 Hz, 11-H$_3$), 1.57 (1 H,m, 3α-H), 1.84 (2 H, m, 10-H$_2$), 2.24 (1 H, m, **3β**-H), 2.25 (3 H, br.s, 1-Me), 2.29 (1 H, m, 2α-H), 2.47 (1 H, m, 2β-H), 2.86 (3 H, br.s, 2′-NMe), 3.16 (1 H, t, *J* = 7 Hz, 9-H), 3.56 (3 H, s, 1′-Me), 4.73 (1 H, s, 8a-H), 6.30 (1 H, s, 8-H), 6.37 (1 H, s, 7-H), 6.53 (1 H, d, *J* = 8.0 Hz, 5-H), 7.15 (1 H, d, *J* = 8.0 Hz, 4-H), 7.65 (1 H, br.s, 2′-NH), and 8.36 (1 H, s, 7′-H). $^{13}$C NMR (DMSO-$d_6$) δ: 12.3 (q, C11), 22.1 (t, C10), 28.6 (s, 2′-NMe), 29.0 (s, 1′-Me), 36.4 (s, 1-Me), 37.7 (t, C3), 51.4 (t, C2), 52.1 (d, C9), 60.1 (s, C3a), 85.0 (d, C8a), 109.4 (d, C7), 118.4 (d, C5), 120.1 (s, C6), 126.5 (d, C4), 129.2 (s, C4′a), 132.7 (s, C3b), 146.42 (d, C7′), 146.48 (s, C8′a), 152.3 (s, C6′), 153.3 (s, C7a), 154.6 (s, C2′), and 165.8 (s, C4′). HMBC correlations: 4-H/C6 and C7a; 5-H/C3b and C7; 7-H/C3b; 8-H/C3b; 8a-H/C2 and C9; 9-H/C3a, C8a, C6′, and C7′; 10-H$_2$/C9 and C6′; 11-H$_3$/C9 and C10; 7′-H/C6′ and C8′a; 1-Me/C2 and C8a; 1′-Me/C2′; 2-NMe/C2′. FABMS (positive, glycerol matrix) *m/z* 486/484 $(M+H)^+$, 232 $(C_{11}H_{14}N_5O)^+$, and 218 $(C_{10}H_{12}N_5O)^+$. HRFABMS *m/z* 486.1443, calcd for $C_{22}H_{27}{}^{81}BrN_7O$, $\Delta$ + 0.3 mmu).
13 Composition of artificial seawater: 460 mM NaCl, 10.1 mM KCl, 9.2 mM $CaCl_2$, 35.9 mM $MgCl_2 \cdot 6H_2O$, 17.5 mM $MgSO_4 \cdot 7H_2O$, 10 mM Tris-HCl, pH 8.2.