2,5,6-Tribromo-1-methylgramine, an Antifouling Substance from the Marine Bryozoan *Zoobotryon pellucidum*

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In our screening for antifouling substances using laboratory-reared larvae of the barnacle *Balanus amphitrite*, the crude extract of the marine bryozoan *Zoobotryon pellucidum* exhibited promising activity. Bioassay-guided isolation led to the isolation of 2,5,6-tribromo-1-methylgramine as an effective inhibitor. Its inhibitory activity was 6 times as strong as that of bis-(*n*-butyltin)-oxide (TBTO), while its toxicity to cyprid larvae was one-tenth that of TBTO. This substance also inhibited settlement of the blue mussel *Mytilus edulis*.

Key words: antifouling substance, barnacle, mussel, bryozoan, *Zoobotryon pellucidum*, 2,5,6-tribromo-1-methylgramine

Fouling organisms such as barnacles and blue mussels cause serious problems on ship hulls, in aquaculture systems, and in cooling systems of power plants. Organotin compounds such as TBTO [bis-(*n*-butyltin)-oxide] have been used as effective chemicals against these organisms. However, there is serious concern over the effect of heavy-metal compounds on the marine environment, and less toxic substitutes for these organometallic chemicals are urgently required.

It is well known that many marine invertebrates such as sponges and corals remain remarkably free from settlement by fouling organisms, suggesting that they have biologically active compounds preventing the larvae of other marine organisms from settlement and growth on their body.^{1–4)} So far, several compounds with such activities have been found among marine invertebrates.^{5–10)} These compounds are considered to play an important role in the antifouling mechanism of marine organisms.

This report describes the isolation of 2,5,6-tribromo-1-methylgramine from a bryozoan *Zoobotryon pellucidum*, which showed antifouling activities against the barnacle *Balanus amphitrite* and the blue mussel *Mytilus edulis*.

Materials and Methods

Test Cyprids of the Barnacle

Nauplii of *B. amphitrite* were obtained from reared adult barnacles which had been fed with *Artemia salina* in the laboratory at 23°C. Cyprids were reared from the first stage of nauplius larvae according to the method of Kado.¹¹⁾ The day of metamorphosis to cyprid was defined as day 0. Cyprids were stored in the dark at 5°C until the day of use.¹²⁾

Biological Assay with Cyprids

Each test sample was dissolved in 0.1 ml of methanol and dried in a polystyrene Petri dish (35 mm in diameter) at 23°C, and 5 ml of filtered seawater (through a 0.45 µm mesh; salinity, 32) was added. Ten cyprids

(day 5–7) of *B. amphitrite* were inoculated into each Petri dish. Triplicate dishes were prepared in each set of experiments. After 24 h incubation at 23°C in the dark, the number of settled individuals on the inner surface of the Petri dishes was counted.

Collection of *Z. pellucidum* and Preliminary Assay

Z. pellucidum was collected from the intertidal region of Shimizu harbor, Shizuoka Prefecture, in July, 1991. The specimens (50 g) were soaked in acetone (50 ml) for 24 h at room temperature. The acetone extract (1 ml) was diluted with methanol (19 ml), and 0.1 ml of the diluted solution was dried in a Petri dish. In the biological assay with cyprids (*vide supra*), no settled individual was observed in any of the dishes, suggesting that *Z. pellucidum* contained inhibitor(s) to the larval settlement of *B. amphitrite*.

Isolation of Active Substance

The active substance was isolated from *Z. pellucidum* using the bioassay method described above. Fresh *Z. pellucidum* (2.0 kg) were soaked in acetone (3.0 l) for 24 h at room temperature, and the extract was evaporated and partitioned between ethyl acetate (EtOAc) and H₂O. The inhibitory activity was recovered in the EtOAc layer (4.9 g). The organic layer was chromatographed twice on silica gel with CHCl₃/MeOH then benzene/acetone. Further purification of the active fraction (308 mg) was performed by HPLC on ODS with a mixture of MeOH–2.5% aq. NH₄OH (15:1) to isolate the active material, which gave white needles (mp. 105–107°C) after recrystallization from hexane. This active substance (121.1 mg) gave a 0.006% yield from the fresh *Z. pellucidum* (2.0 kg).

Structural Elucidation of the Active Substance

The structure of the active substance was elucidated mainly by spectroscopic analysis as follows. The UV absorption spectrum (UV) in MeOH was recorded on a Shimadzu UV-2100S spectrophotometer. ¹H-NMR and ¹³C-NMR spectra were recorded on a Varian Unity 500 spectrometer using CDCl₃ as solvent. Electron-impact mass spectra (EIMS) were obtained with a JEOL JMS-SX102 mass spectrometer.

Biological Assay with Blue Mussels

Antifouling activity was also evaluated according to the procedure of Ina *et al.*¹³⁾ using blue mussels *M. edulis* collected from Shimizu harbor. The activities were expressed as ++, +, ±, and – in order of descending activity, and the assay was repeated twice.

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Results

Activities against Barnacle Cyprids

Inhibitory activity on larval settlement was examined in the presence of 1.00, 0.50, 0.25, 0.13, 0.06, 0.03, and 0.015 ppm of the isolated substance. As shown in Fig. 1, the settlement rate of cyprid larvae in the control (0 ppm) was found to be $63 \pm 12\%$ (mean $\pm$ S.D.). At higher concentrations of the substance (0.03 ppm and above), all cyprids failed to settle. Further, all cyprids survived at concentrations from 0.03 to 0.13 ppm. At higher concentrations, some cyprids sank and remained immobile; they were judged to be dead. At concentrations of 0.25, 0.50, and 1.00 ppm, the mortality rates were 7 ± 6 , 23 ± 12 , and $53 \pm 15\%$, respectively.

Structural Elucidation of the Settlement Inhibitor

The spectral data of the substance were as follows: UV (MeOH): $\lambda_{\max}$ 232 (ϵ 46,000), 298 (ϵ 9,700), 307 (ϵ 9,000) nm; IR (CHCl₃): 1455, 1415, 1340, 1305 cm⁻¹; HR-EIMS: 427.8550 (calcd for C₁₂H₁₃⁷⁹Br⁸¹Br₃N₂, 427.8567), 425.8559 (calcd for C₁₂H₁₃⁷⁹Br⁸¹Br₂N₂, 425.8588), 423.8606 (calcd for C₁₂H₁₃⁷⁹Br⁸¹Br₂N₂, 423.8601), 421.8623 (calcd for C₁₂H₁₃⁷⁹Br₃N₂, 421.8628); ¹H-NMR (CDCl₃, 500 MHz, ppm): δ 7.94 (1H, s; 4-CH), 7.58 (1H, s; 7-CH), 3.71 (3H, s; 8-CH₃), 3.51 (2H, s; 9-CH₂), 2.25 (6H, s; 10-CH₃ $\times$ 2); ¹³C-NMR (CDCl₃, 125 MHz, ppm): δ 136.5 (s; 7a-C), 128.3 (s; 3a-C), 123.4 (d; 4-C), 117.3 (s; 5-C), 117.2 (s; 2-C), 115.3 (s; 6-C), 114.0 (d; 7-C), 111.7 (s; 3-C), 54.3 (t; 9-C), 45.2 (q; 10-C $\times$ 2), 31.7 (q; 8-C); HMBC cross peaks: 4-H, 5-C;

Fig. 1. Relationship between settlement rate and mortality rate of barnacle cyprids of *B. amphitrite* in the presence of isolated substance from the bryozoan *Z. pellucidum*.

●, settlement rate; ○, mortality rate.

Fig. 2. Structure of inhibitor for larval settlement of *B. amphitrite* isolated from *Z. pellucidum*.

4-H, 7a-C; 7-H, 5-C; 7-H, 3a-C; 9-H₂, 2-C; 9-H₂, 3-C; 9-H₂, 3a-C; 9-H₂, 10-C; 10-H₃, 9-C.

These data coincided well with those of 2,5,6-tribromo-1-methylgramine¹⁴⁾ (TBG) which was first isolated from a different species of bryozoan *Z. verticillatum* as an inhibitor of cleavage of fertilized sea-urchin eggs (Fig. 2).

Activities against Blue Mussels

As shown in Table 1, TBG showed ++ and ++-+ activity at 30 and 8 ng/cm², respectively. A unit was also calculated to express the relative activity of TBG according to the equation of Ina et al.¹³⁾ The activity was 6.8–13.6 times as strong as that of cupric sulfate (CuSO₄), a positive control.

Discussion

MIC (minimum inhibitory concentration) and LC₃₀ (30% lethal concentration) of TBG and the positive control (TBTO) on larval settlement of *B. amphitrite* are shown in Table 2. The MIC of TBG was about one-sixth that of TBTO, whereas the toxicity was only one-tenth that of the antifoulant. These results indicate that the settlement

Table 1. Antifouling activities of TBG and CuSO₄ against the blue mussel *M. edulis*

Substance	Sample amount (ng/cm ²)					Unit*
	80	30	16	8	1.6	
TBG	++	++	++	++~+	++~±	680–1360
CuSO ₄	++	+				100

$$* \text{Unit} = 100 \times \frac{\text{CuSO}_4 \text{ (minimum dose for ++ activity, } \mu\text{mol/cm}^2\text{)}}{\text{Sample (minimum dose for ++ activity, } \mu\text{mol/cm}^2\text{)}}$$

Table 2. MIC for larval settlement and LC₃₀ values of TBG and TBTO (ppm) for reared cyprids

Substance	MIC	LC ₃₀
TBG	0.03	0.60
TBTO	0.20	0.06

MIC: Minimum inhibitory concentration.

LC₃₀: 30% lethal concentration.

Fig. 3. Variations of TBG contents of *Z. pellucidum* from different sampling site and period.

inhibitory activity of TBTO is due to its toxicity, while that of TBG is much less dependent on its toxicity.

Surviving cyprids which had been exposed to 0.25 ppm of TBG for 24 h were rinsed with filtered seawater and transferred into a new Petri dish with fresh seawater. The settlement rate of these cyprids was as high as that of the controls, suggesting that the decrease of the settlement ability of the larvae is attributable to the presence of TBG. In similar experiments using TBTO, its toxicity interfered with the survival of cyprids at antifouling concentrations.

In order to examine monthly and locational variations of TBG content in *Z. pellucidum* in summer seasons, we also collected the bryozoan from Owase harbor, Mie Prefecture, and from Shimizu harbor in July and August, 1992, and analyzed the extracts by HPLC. The results are shown in Fig. 3 and suggest that the content of TBG in *Z. pellucidum* is independent of sampling site and period.

TBG also inhibited the settlement of *M. edulis*, another important fouling organism (Table 1).

These results suggest that TBG in *Z. pellucidum* may play an important role as a defense substance against the settlement of other organisms. TBG thus has potential as an industrial antifoulant in future because of its potent activity and relatively low toxicity.

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