Complanine, an inflammation-inducing substance isolated from the marine fireworm *Eurythoe complanata*†

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The marine fireworm, *Eurythoe complanata*, is known as a dangerous animal for humans because it induces skin inflammation through its small setae. Here, the inflammation-inducing substance was successfully isolated from the whole body using a methanolic extraction, and the inflammatory activity was determined using a bioassay. The structure was spectroscopically revealed to be a trimethylammonium with an unsaturated carbon chain and was named complanine. Complanine enhanced PKC activity in combination with TPA *in vitro*. This may explain the molecular mechanism behind its inflammation-inducing activity.

The amphinomid polychaetes, including *Eurythoe complanata*, are known as the "fireworms" of marine animals. They are frequently found at sandy, muddy beaches or in shallow water in temperate and subtropical zones. The animals respond to predators by bristling their parapodial setae. They are known as dangerous animals for humans because, if they are handled, serious skin inflammation results.[1] The reaction is considered to be an allergic dermatitis, and the symptoms usually persist for several days. The inflammation-causing substance is likely injected when penetration into skin breaks the hollow setae.[2] The toxin-producing glands of the fireworms were not found to be communicating with the parapodial setae according to analysis by electron microscopy.[3] However, a bioactive substance related to inflammation was reported in pharmacological studies.[4]

The identification of the inflammatory substance from polychaetes has been attempted in previous studies, but the active substances have not been identified. Substances that induce contracting or relaxing of the ileum in rat or guinea pig have been identified from the methanolic extract of *E. complanata*.[5] The ileum assay is useful for searching for substances related to histamine or adenosine mediated inflammation, but the assay is limited in ability to explain the total physiological reaction. In the present study, we provide the first report of the isolation and structural elucidation of the active substance, complanine, showing its inflammatory activity by direct induction of inflammation in a mouse footpad.

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Specimens of *Eurythoe complanata* (225 g) were collected from a tidal wetland on Awase beach of Okinawa island and extracted with 80% aqueous ethanol. The concentrated extract was partitioned between ethyl acetate and water. The aqueous layer was chromatographed on a TSK-G3000S (Tosoh, Japan) polystyrene gel, then eluted with aqueous ethanol. Further purification was carried out by repeating the chromatography on a silica gel with elution by $CHCl_3$–MeOH–H_2O–AcOH. The identification of the active substance was guided by a bioassay.

Bioassay of inflammation-inducing activity was carried out as follows: the sample was dissolved in an adequate concentration (2–5%) of saline and 0.05 ml of solution was injected (s.c.) into the left footpads (right for a negative control) of male 4-week-old ddY mice. The time course of footpad swelling was examined over 4 h. The inflammatory activities were evaluated by measuring the thickness of the footpad over time compared to a negative control. The activity (% swelling) was defined by [(thickness of tested footpad − intact footpad)/(intact footpad)] × 100 (%).

The inflammation-inducing substance of *E. complanata* was obtained from the aqueous layer of a methanolic extract of the whole body. Fractionation using silica-gel column chromatography, guided by a bioactivity assay, lead to the successful purification of the bioactive amphiphilic substance (*ca.* 10 mg).

The inflammatory compound exhibited a positive Dragendorff reaction, thus it was assumed to contain a nitrogen atom. The compound provided a molecular ion peak of 311.2704 by positive HRESIMS, and the molecular formula was deduced to be $C_{18}H_{35}N_2O_2^+$ (M^+), $\Delta = 0.9$ mmu. The formula was assigned as cationic. Thus the molecule was isolated as an acetate salt due to the final chromatographic condition. The counter anion was exchanged into a chloride ion using ion-exchange chromatography. In the analysis of ^{1}H and ^{13}C NMR, and also IR spectra, two discontinuous *Z*-double bonds (^{13}C NMR: δ 128.3, 129.9, 130.3 and 133.7 ppm), one trimethylammonium moiety [^{1}H NMR: δ 3.02 (9H, s)], and one amide bond (^{13}C NMR: δ 182.0 ppm, IR 1575 cm^{-1}) were observed. The connectivities were assigned by the COSY (from H1′ to H3′, from H1 to H4 and from H5 to H11, respectively) and HMBC correlations (NMe–C1′; H2′,3′–C4′; H1–C4′,C2,C3; H5–C4; H8–C7; H10–C9,C11 and H11–C9,C10, respectively, selected). A detailed analysis of the 1D and 2D NMR data (Table 1) disclosed the planar structure of the compound (isolated as an acetate salt) as shown in Fig. 1, and we named it complanine. The novel trimethylammonium cationic structure could be characterized by an unsaturated carbon chain and an amino alcohol *N*-acylated with a GABA derivative. A survey of natural product literature revealed that related compounds possessing a *vic*-amino alcohol with an unsaturated carbon chain

Table 1 NMR assignments for complanine. D_2O was used for measuring spectra. Only reliable HMBC assignments are presented. †* Assignments interchangeable, respectively

Assignment	^{1}H NMR/ppm	^{13}C NMR/ppm	HMBC correlations
NMe	3.02 (9H, s)	53.6	C1′
1′	3.22 (2H, m)	66.3	
2′	1.98 (2H, quint., $J = \sim$7.6 Hz)	20.9†	C1′,C4′
3′	2.28 (2H, t, $J = 7.2$ Hz)	32.4	C1′,C2′,C4′
4′		182.0	—
1	3.09 (1H, m), 3.22 (1H, m)	46.3	C4′,C2,C3
2	3.64 (1H, br. m)	70.3	
3	2.08 (2H, m)	23.5	
4	1.41 (2H, m)	34.4	
5	5.31–5.37 (1H, m)	128.3*	C4
6	5.31–5.37 (1H, m)	129.9*	
7	2.43 (2H, t, J= 6.5 Hz)	25.8	
8	5.31–5.37 (1H, m)	130.3*	C7
9	5.31–5.37 (1H, m)	133.7*	
10	1.98 (2H, m)	19.5†	C9,C11
11	0.84 (3H, t, J= 7.6 Hz)	14.4	C9,C10

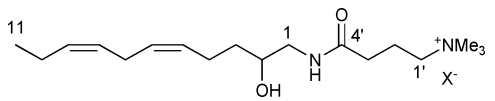

Fig. 1 Structure of complanine. Isolated as an acetate salt. The counter anion can be exchanged by ion-exchange chromatography.

were known. Among them, obscuraminols have been isolated from another marine source, the ascidian *Pseudodistoma obscurum* from Tarifa Island, Spain.[6] However, these compounds were simple amino alcohols without any other chemical functionalities.

The inflammation-inducing activity of complanine is shown in Fig. 2. The activity was characterized as an acute swelling effect in the mouse footpad. Swelling of the footpad was rapidly induced after injection of complanine and disappeared after several hours. This acute inflammatory reaction induced by complanine was statistically significant and reproducible. Complanine did not show any acute toxicity up to concentrations of 100 mg kg^{-1} by i.p. injection. Since the activity of complanine induced irritation in the animals, we concluded that complanine is one of the inflammation-inducing substances produced by *E. complanata*.

The molecular mechanism of biological activity of complanine was examined by a PKC activation assay,[7] because signal transduction by classical PKC plays an important role in the inflammatory process.[8,9] Fig. 2 shows the result of PKC (mixture of α, β, and γ isoforms) activation with complanine in the absence or presence of TPA. Complanine alone showed weak activation of PKC at concentrations higher than 0.5 mM in the presence of Ca^{2+}. Interestingly, complanine significantly and dose-dependently enhanced PKC activity in the presence of 1.2 μM TPA. This synergistic activation of PKC by complanine and TPA was comparable to that of L-phosphatidylserine and TPA. Since the combination of complanine and L-phosphatidylserine did not show any synergistic activation of PKC, we hypothesize that complanine binds to the phospholipid binding site of classical PKC, but not to the diacylglycerol/phorbol ester binding site. Since various novel PKC isozymes have been identified, the effect of complanine on multiple PKC isozymes is an important subject for future study. The activation of PKC by complanine was hypothesized to be one of the molecular mechanisms by which *E. complanata* induced inflammation.

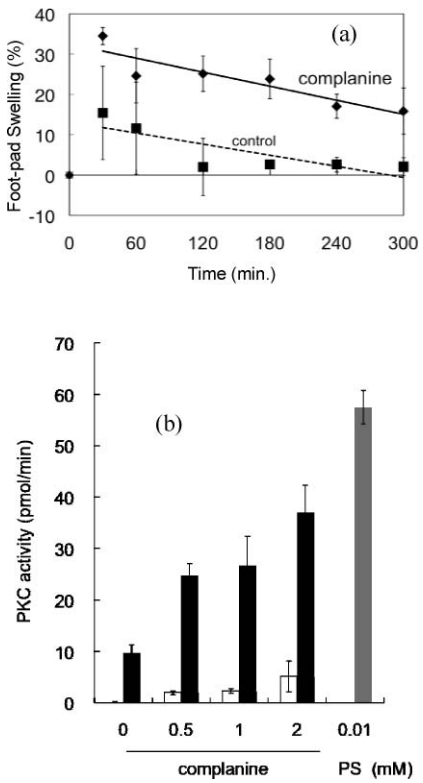

Fig. 2 Biological activities of complanine. (a) Typical inflammation profile induced by complanine. The inflammation-inducing activity (% swelling) was defined by [(thickness of tested footpad − intact footpad)/(intact footpad)] × 100 (%). Values are the mean ± SD of triplicate experiments. 1% Solution of carrageenan was used as a positive control to confirm reproducibility. (b) Activation of PKC by complanine in combination with TPA. PKC activity was determined after incubation with the indicated concentrations of complanine in the presence (solid columns) or absence (open columns) of 1.2 μM TPA. Values are the mean ± SD of triplicate experiments.

The ecological role of complanine remains to be determined. The organ producing this compound is not known and we have no evidence how this molecule may be used as a defensive substance. However, because of its structure and inflammation-inducing

biological property, complanine could play an important role in the defense mechanisms of multiple animals.

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Notes and references

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