685 $cm^{-1}$ band, characteristic of tetrahedral boron, is not seen in spectra of the crystalline enzyme-inhibitor complex (Fig. 1c). The indole displacement experiments, however, have shown that 2-phenylethaneboronic acid saturates the active site of the crystalline enzyme at *p*H 5.0. We conclude, therefore, that the intense $\alpha$-chymotrypsin band at 767 $cm^{-1}$ obscured the less intense band at 775 $cm^{-1}$ because of trigonal boron in the chymotrypsin-inhibitor complex at *p*H 5.5 (Fig. 1c).

At *p*H 7.0, as is seen in Fig. 1c, the band at 684 $cm^{-1}$ is present in the spectra of the crystalline enzyme-inhibitor complex. The position of the band at 684 $cm^{-1}$ indicates that at this *p*H chymotrypsin binds 2-phenylethaneboronic acid in the tetrahedral configuration. X-ray studies indicate that in an analogous system, the crystalline complex of subtilisin BPN′ with 2-phenylethaneboronic acid, the inhibitor is bound to the active site of the enzyme in tetrahedral configuration (*14*).

These data and other results (*4–6, 15*) lead to the following interpretation of the inhibition mechanism. First an enzyme-inhibitor complex is formed in which the boron remains in trigonal form (*15*). The *p*H dependence of the inhibition, which has been reported to be similar to that observed in the catalytic reaction (*4, 6, 16*), occurs in a subsequent step which leads to a tetrahedral adduct between enzyme and inhibitor.

The tendency of boron to form weak complexes with a variety of functional groups is well known (*17–19*). Our data indicate that strong complex formation with a boronic acid derivative that can interact with the specific binding site of the enzyme and a functional group in the active site involves a tetrahedral adduct as suggested by Koehler and Lienhard (*4*).

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19 August 1974; revised 25 February 1975

## Anthopleurine: A Sea Anemone Alarm Pheromone

Abstract. *The sea anemone* Anthopleura elegantissima *responds with characteristic contraction to a pheromone released by wounded conspecifics. The alarm pheromone was isolated by ion-exchange chromatography and identified by chemical and spectroscopic methods as the quaternary ammonium ion (3-carboxy-2,3-dihydroxy-*N,N,N*-trimethyl)-1-propanaminium. Median effective concentration of the crystalline pheromone is 3.5* $\times$ $10^{-10}$ *mole per liter of seawater.*

Evidence has been reported for the existence of pheromones in several marine invertebrates (*1*). With one exception the marine pheromones examined to date are large molecules not readily amenable to detailed chemical characterization (*2*). Unlike airborne pheromones that must be relatively small molecules to be volatile, waterborne pheromones need not be small to be soluble in water (*2*). The only marine pheromone whose structure is known is crustecdysone, a polar steroid that appears to be a sex pheromone in certain crabs (*3*).

We now report the discovery, isolation, and chemical structure of an alarm pheromone in the sea anemone *Anthopleura elegantissima* (Cnidaria, Anthozoa).

The aggregating form of *A. elegantissima* was collected from exposed intertidal rocks in Monterey, California. Animals from the single clone (*4*) used for bioassays were transferred in groups of six animals to 24 glass bowls (250 ml) and maintained in the laboratory with running seawater for 2 months before being used for experiments. An average illumination of 2300 lux

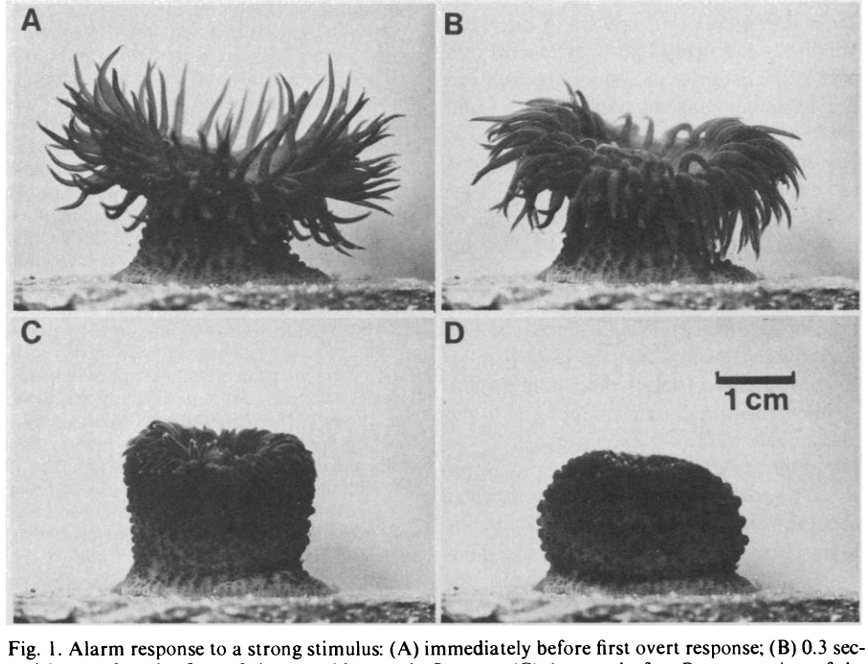


Fig. 1. Alarm response to a strong stimulus: (A) immediately before first overt response; (B) 0.3 second later, after the first of three rapid tentacle flexures; (C) 1 second after B, contraction of the mesenterial retractors; (D) 1.5 seconds after C, constriction of the marginal sphincter.

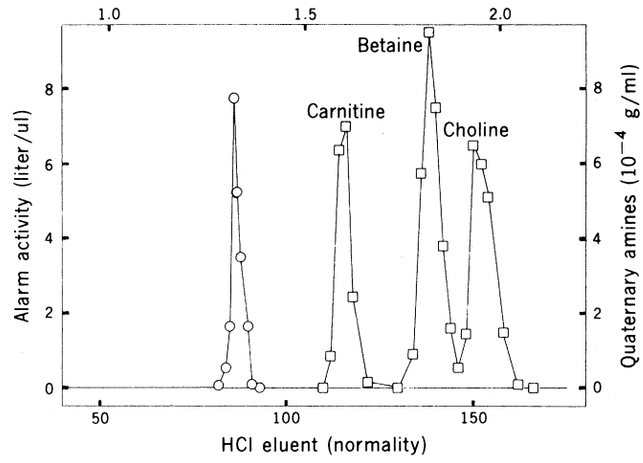


Fig. 2. Ion-exchange chromatography of alarm substance and related compounds. A sample containing crystalline alarm substance (1 mg), choline chloride (5 mg), betaine hydrochloride (5 mg), and carnitine hydrochloride (5 mg) in 1.0 ml of 0.50*N* HCl was applied to a column of Dowex 50 X-8(H+) and eluted with an HCl gradient. Fractions were analyzed biologically for alarm activity (circles) and photometrically (*7*) for quaternary ammonium salts (squares). Alarm substance concentration was insufficient to appear in the photometric assay.

was provided for 12 hours daily. Bowls were arranged in vertical tiers of four, so that water siphoned into the uppermost bowl drained successively into each of the lower three bowls.

When an anemone was mechanically damaged, first the other anemones in that bowl, and subsequently the anemones downstream in that tier, responded with a characteristic contraction, elicited by no other stimuli. This "alarm response" consists of a series of one to four rapid (about 0.3-second duration), convulsive, radially symmetrical flexures of the tentacles toward the base of the column (Fig. 1B). With stronger stimuli these rapid flexures precede an eventual contraction of the mesenterial retractor muscles (Fig. 1C), and constriction of the marginal sphincter (Fig. 1D). A filtered aqueous extract of *A. elegantissima* evokes alarm behavior indistinguishable from that caused by wounded animals.

Progress in the purification of alarm substance was monitored by two methods. For the bioassay of ion-exchange column fractions, we prepared tenfold dilutions in distilled water, then mixed 0.5 ml of each dilution into a bowl of anemones, and recorded the number of animals responding with at least one rapid flexure within 30 seconds. We crudely estimated the median effective concentration ($EC_{50}$) from the proportion of positive responses to the most dilute solution that evoked at least one alarm response. When precision was desired, we began as above with the most dilute effective solution, then mixed 0.5 ml of that solution into each of 12 of the 24 test bowls, recording the number of positive responses as above. This initial solution was then diluted or concentrated as appropriate and administered to the other 12 bowls to measure biological activity as a function of log dose. Additional doses were administered at 2-hour intervals, a period of time sufficient for full recovery of responsiveness (*5*). We computed $EC_{50}$ and associated 95 percent confidence intervals graphically by the method of Litchfield and Wilcoxon (*6*).

To isolate alarm substance, whole fresh anemones were blotted, homogenized in a Waring Blendor with an equal volume of 2 percent acetic acid, and centrifuged at 320*g* for 10 minutes. The pellet was reextracted in another volume of 1 percent acetic acid, and the supernatants were combined. The $EC_{50}$ of this extract was 5.9 $\times 10^{-5}$ g of wet anemone per liter of test bowl water (± 15 percent). Ethanol was added to a final concentration of 50 percent by volume, and the mixture was allowed to stand overnight at −4°C, then centrifuged at 12,000*g* for 5 minutes. The clear supernant was passed through a column of Dowex 50 X-8(H+) and the alarm substance eluted with 1*N* $NH_4OH$. Active fractions were pooled, concentrated, and passed through a column of Dowex 1 X-8(OH−), to which the alarm substance did not bind. Dowex 1 effluent and water washes were combined, concentrated, and adsorbed to a column (17 by 290 mm) of Dowex 50 X-8(H+), 100 to 200 mesh, then eluted in 2-ml fractions at 10 ml $hr^{-1}$ with a linear gradient of 0.50 to 2.50*N* HCl.

In Fig. 2 the chromatographic mobility of alarm substance is compared to that of known related compounds on the purification column. Fractions with an $EC_{50}$ less than $4 \times 10^{-3}$ ml/liter were pooled, adjusted to *p*H 6.8 with Dowex 1(OH−), filtered, and concentrated to a viscous syrup that was identified as a homogeneous quaternary amine by paper chromatography (*7, 8*). This syrup was extracted with 20 volumes of hot methanol from which a flocculent white solid precipitated upon the addition of a volume of cold diethyl ether equal to the volume of methanol.

Crystallization of this precipitate from methanol-ether yielded needles [four crystallizations; $EC_{50} = 7.4 \times 10^{-8}$ g/liter (± 14 percent); m.p., 210° to 214°C (with decomposition); $[\alpha]_D^{16} = 48°C$ (*c*, 0.1375 g/100 ml)] which furnished a flocculent reineckate [m.p., 160° to 164°C (decomposition)] in acid medium (*9*). This result in conjunction with the infrared absorption at $\eta_{max} = 1610$ $cm^{-1}$ strongly suggested the presence of a betaine moiety.

The nuclear magnetic resonance (NMR) spectrum of the crystalline material (*10*) revealed a singlet at 2.96 δ [9H, $(CH_3)_3N^+$], a doublet of a doublet at 3.29 δ (2H, $J$ = 7 and 5 hz), a doublet at 4.08 δ (1H, $J$ = 5 hz), and a broad signal at 4.50 δ (1H, *c*). Double irradiation of the signal at 4.50 δ collapsed the doublet at 4.08 δ and doublet of doublet at 3.29 δ to a pair of singlets, thus confirming the following moiety, $-CH_2-CHOH-CHOH-$. The chemical shift of the two-proton signal (3.29 δ) clearly indicated its attachment to the trimethylamino moiety [compare $(CH_3)_2N-CH_2-CH_2OH$, 3.60 δ; choline bitartrate, $CH_2OH$, 4.4 δ; $CH_2-N^+$, 3.70 δ]. On the basis of the preceding spectral data we postulated the following structure for the crystalline compound.

$$(CH_3)_3\overset{+}{N}-CH_2-CHOH-CHOH-COOH\cdot Cl^-$$

**1**

(3-carboxy-2,3-dihydroxy-*N,N,N*-trimethyl)-1-propanaminium chloride

Calculated percentages for elemental analysis for (**1**) $C_7H_{16}ClNO_4$ (312.533), are: C, 39.37; H, 7.49; Cl, 16.60; N, 6.56; combustion analysis yielded C, 39.0; H, 7.54; Cl, 16.5; N, 6.46: these observed percentages are in complete agreement with the postulated structure.

To the best of our knowledge structure **1** has not been previously described as either a naturally occurring or synthetic substance. We therefore propose the trivial name anthopleurine for *A. elegantissima* alarm substance. By virtue of its intra-

specific effects at very low concentrations, anthopleurine clearly meets the criteria for a pheromone (*11*). It is the second pheromone from a marine invertebrate animal to be isolated and fully characterized chemically.

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## Suppression of a Field Population of Houseflies with *Spalangia endius*

Abstract. *Sustained releases of the microhymenopteran pupal parasite* Spalangia endius, *at a commercial poultry installation in north Florida, completely suppressed a population of houseflies within 35 days.*

Control of houseflies, *Musca domestica*, by chemical or physical measures is often inadequate (*1*), and resort to other methods is frequently necessary. The use of predators and parasites of pest insects as an adjunct or replacement measure is a promising alternative (*2*). We found that *Spalangia endius*, a parasitic wasp, originally obtained from housefly pupae collected from an isolated dairy in Alachua County, Florida, could be an effective agent for housefly control (*3*), although it does parasitize other species of muscoid flies (*4*). We report here the successful suppression of a population of houseflies at a commercial poultry installation in north Florida as the result of the release of this parasite during the summer of 1974.

The test area consisted of three open-sided poultry houses containing a total of 6700 caged layers. Our observations indicated that most of the housefly breeding occurred at the larger structure (3 by 114 m), which was separated by a distance of 33 m from the two smaller houses (7 by 25 m). The density of adult flies at the test area and at the check site, a similar poultry installation 6 miles (9.6 km) away where no releases were made, was determined before (test site only) and during the test by using a modification of the Scudder grid (*5*). Three times a week samples of housefly pupae were collected from the breeding area at the test site and at the check site and returned to the laboratory, where they were held for 10 days at 27.8°C and 60 percent relative humidity to allow for emergence of adult flies or pupal parasites. Housefly pupae that did not eclose in that time were examined microscopically for evidence of parasitism or natural mortality. The natural parasitism of pupae collected in the samples at the test site before the test was 24 percent (Fig. 1); at the check site, it averaged 22 percent throughout the 10 weeks of the test.

The *Spalangia endius* released at the test site were reared in the laboratory. The adult wasps were held in plastic cages (61 by 61 by 48 cm) at 26.7°C and 60 percent relative humidity. Also, because the parasites are attracted to the nearest light source, rearing was conducted in total darkness except when adjustments or manipulations were necessary. Three times a week 160,000 2-day-old housefly pupae were introduced into a cage containing 24,000 parasites. After 18 to 24 hours, the pupae were removed, placed in 4-liter paper containers, and held at 60 percent relative humidity and 27.8°C until the next generation of parasites began emerging 18 to 20 days later. Three to 5 days later, the empty fly puparia and emerged adults were removed. The remaining (parasitized) fly pupae were divided into groups of 6000 and held in 2-liter containers.

The test began the week of 23 June. Three times a week for 10 weeks, 14 of the 2-liter containers of parasitized housefly pupae were placed at six release sites adjacent to the larger poultry house.

Four weeks after the initial release (Fig. 1), all housefly pupae collected from the test area were parasitized. Thereafter, for another month, parasitism ranged from a low of 93 percent to a high of 100 percent. However, on 25 August it dropped to 14 percent, a result of the removal of a majority of the manure during the week of 4 August. Most of the existing population of adult *Spalangia* was removed with the manure, as was most of the larval breeding medium. Thus, for several weeks it was difficult to find housefly pupae, and the few that were collected from an isolated group during the week of 25 August were young pupae that were not parasitized at the time they were collected. It is assumed that if the pupae had been collected when they were older, the rate of parasitism would have been higher. Thus, the removal of the adult wasps, combined with the reduced numbers of emerging parasites, which resulted from the removal of many pupal sites, so decimated the parasite population that immigrating houseflies were able to reinitiate a fly population in the larval medium that had a low number of parasites.

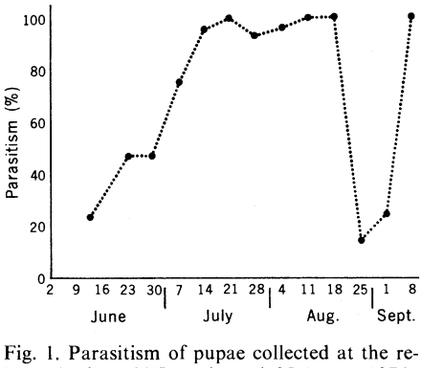


Fig. 1. Parasitism of pupae collected at the release site from 23 June through 25 August 1974.

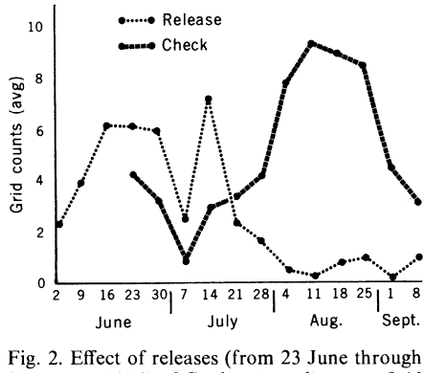


Fig. 2. Effect of releases (from 23 June through 25 August 1974) of *Spalangia endius* on a field population of houseflies.