Defensive Secretion of Ecdysteroids in *Pycnogonum litorale* (Arthropoda, Pantopoda)

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In response to disturbance, the pycnogonid *Pycnogonum litorale* discharges a mixture of eight ecdysteroids (ES). Repeated intensive molestation causes 99% secretion of the endogenous ES present. The concentration of the total ES in the defensive effluent is 1.0×10^{-3} mol·l^{-1}. 20-Hydroxyecdysone 22-acetate, the predominant ES, reaches 0.8×10^{-3} mol·l^{-1}. This is sufficient to deter significantly feeding by the common shore crab *Carcinus maenas*, a generalist predator in the habitat of the pycnogonid. There is evidence that the secreted ES of *P. litorale* contribute to its unpalatability. The present paper describes for the first time defensive secretion in marine arthropods.

Introduction

Defensive secretion of feeding deterrents is widespread in terrestrial arthropods (see review by Pasteels *et al.*, 1983) and freshwater arthropods (see review by Scrimshaw and Kerfoot, 1987). In marine arthropods, the existence of substances poisonous to humans and other terrestrial vertebrates is known (Hashimoto and Konosu, 1978). However, only recently has evidence for chemical defense in a marine predator-prey relationship been shown in the primitive arthropod *Pycnogonum litorale*. This pycnogonid is unpalatable to a generalist predator in its habitat, the common shore crab *Carcinus maenas* (Tomaschko, 1994). In juvenile male pycnogonids, the unpalatability could be partly assigned to high levels of 20-hydroxyecdysone 22-acetate (20E22Ac), the predominant ecdysteroid in *P. litorale*. However, in adult pycnogonids the endogenous 20E22Ac levels are two to four times below the critical level for significant feeding deterrence in *C. maenas* when assuming homogenous ecdysteroid distribution within their bodies. Nevertheless, the adults are spared by *C. maenas* as well.

In order to investigate the significance of ecdysteroids (ES) as defensive chemicals in adults, the present work examines whether *P. litorale* secretes 20E22Ac or other ES in response to disturbance. Secretion of ES could produce locally high ES levels around the pycnogonids and consequently increase their deterrent potency.

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Materials and Methods

Animals

P. litorale was reared as described previously (Bückmann and Tomaschko, 1992).

Determination of ES-levels in the defensive effluent

The body surface of 4 adult male pycnogonids was dried by cautiously swabbing them with small pieces of paper tissue. Thereafter, each animal was pressed against the inner wall of an Eppendorf vial (2.2 ml) with a glass rod (5 mm in diameter). After removal of the pycnogonids, small droplets remained in the vials. The mass of the discharged fluid was determined by weighing the vials before and after the experiment. The effluent of each animal was mixed with 90 µl 18% (v/v) acetonitrile in water and directly submitted to RP-HPLC. The results presented represent mean values from the four animals.

Determination of the secreted amounts of ES in response to disturbance

Data were obtained from repeated tests with groups of animals, each consisting of eight adult males. Experimental and control animals were kept individually in glass vials in 1 ml sea water. Three different provocation experiments were performed. Animals of the first group were locally disturbed by compressing two randomly chosen legs one after another with forceps. Second group

animals were provoked extensively by pressing them for 30 sec at different parts of the body and the legs against the bottom of the glass vial with the blunt end of a glass rod (4 mm diameter). Animals of the third group received the same extensive treatment on five successive days. Subsequent to each provocation, the water was renewed. The water samples of all animals within one group were pooled. Control animals were kept undisturbed for 5 days, and the water was renewed and pooled every 24 h.

Extraction of ES from pycnogonids

Control animals as well as experimental animals were pooled groupwise, frozen in liquid nitrogen, lyophilized, pulverized in a mortar, and homogenized in 20 ml 70% (v/v) methanol in water. The samples were centrifuged for 2 min at 6000 × *g* and the pellet re-extracted twice with 100% methanol. The combined supernatants were then concentrated to a very small volume, filled up to 10 ml with water (final methanol concentration < 5%), and applied onto a pre-washed (10 ml methanol and 10 ml water) C 18 Sep-Pak cartridge (Waters). The ES were eluted with 7 ml 60% (v/v) methanol in water after the cartridge wash with 5 ml water and 5 ml 25% (v/v) methanol in water. The 60% fraction was evaporated to dryness under reduced pressure for HPLC analysis.

Isolation of secreted ES from the sea water

The pooled water samples of each group were applied onto pre-washed Sep-Pak cartridges. The 60% methanol fractions (see above) were evaporated to dryness for HPLC analysis.

HPLC analyses

Qualitative and quantitative HPLC analyses of the ES were performed on a Shimadzu system with an LC-9A pump and an SPD-6AV UV monitor set at 254 nm, controlled by an IBM PC computer. Reversed-phase chromatograms were obtained with an RP-18 Supersphere column (Merck 250 × 4 mm) using a linear gradient of 18–32% (v/v) acetonitrile (Roth) in water, containing 0.1% TFA (Roth) for 40 min at 1 ml/min flow rate. The collected ecdysteroid fractions were further purified on a normal phase system [(Merck Lichrosorb Si 60, 125 × 4 mm) using dichlormethane/propanol-2/water (125:30:2 (v/v)] at 2 ml/min. ES were identified on the basis of co-migration on both reversed phase (RP) and normal phase (NP) HPLC with reference substances that were extracted from adult pycnogonids (Bückmann *et al.*, 1986). The amounts of ES were determined by peak area calculation using 2 nmol of 20-hydroxyecdysone (20 E) as an external standard.

Chemicals

Ecdysone (E) and 20 E were from Sigma, other ecdysteroid standards were extracted from pycnogonids as described by Bückmann *et al.* (1986). All other chemicals were of reagent grade and from various suppliers (Merck, Fluka, Carlo Erba).

Results

ES in undisturbed animals

The ES in undisturbed males were qualitatively and quantitatively in accordance with the pattern of ES that had been identified by Bückmann *et al.* (1986) from a mixture of adult males and females (Fig. 1). Hence it follows that there is no major sex-specific difference in the content of free ES in adults. The eight ES in detail are: 20-hydroxyecdysone, 20-hydroxyecdysone 22-glycolate, the 25*R* and 25*S* isomers of 20,26-dihydroxyecdysone 22-acetate, 22-deoxy-20,26-dihydroxyecdysone, 20-hydroxyecdysone 22-acetate (20 E 22 Ac),

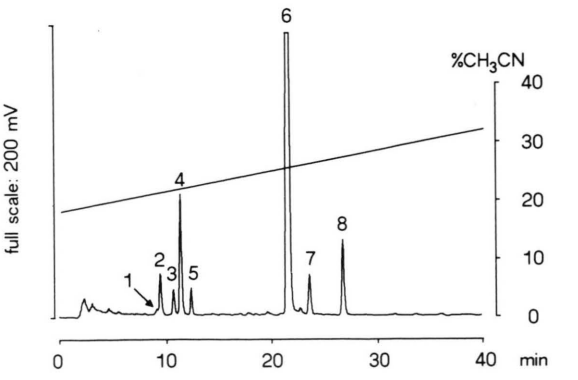

Fig. 1. HPLC analysis of ecdysteroids in *P. litorale* on an RP-18 Supersphere column. Linear gradient of 18–32% (v/v) acetonitrile in water, containing 0.1% trifluoroacetic acid for 40 min at 1 ml/min flow rate, detection at 254 nm. For numbers of ES see legend to Fig. 2.

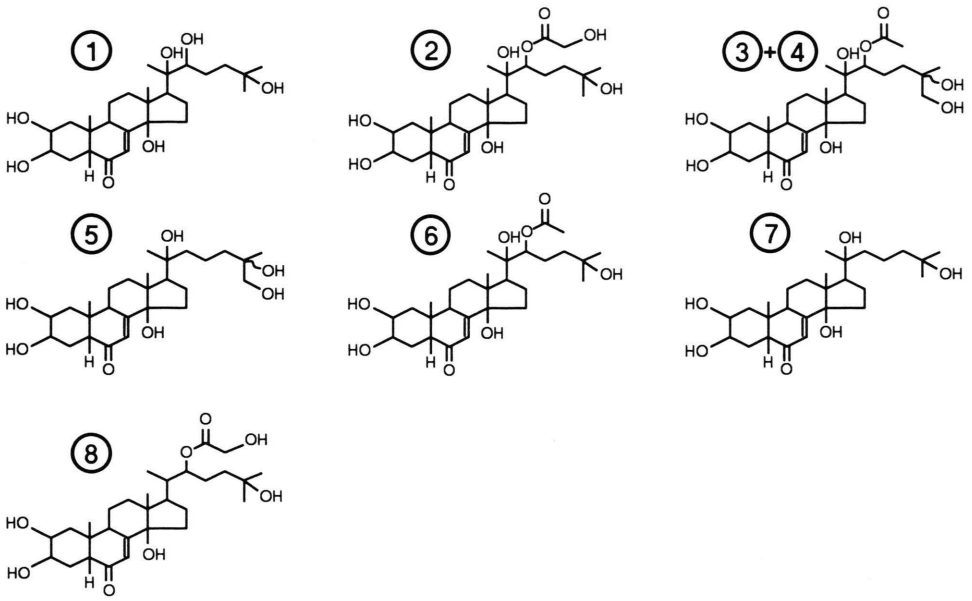

Fig. 2. Ecdysteroids in *P. litorale*. 1: 20-hydroxyecdysone, 2: 20-hydroxyecdysone 22-glycolate, 3 + 4: 25*R* and 25*S* isomers of 20,26-dihydroxyecdysone 22-acetate, 5: 22-deoxy-20,26-dihydroxyecdysone, 6: 20-hydroxyecdysone 22-acetate, 7: 22-deoxy-20-hydroxyecdysone, 8: ecdysone 22-glycolate.

22-deoxy-20-hydroxyecdysone, and ecdysone 22-glycolate (Fig. 2). The predominant compounds is 20E22Ac (Fig. 1, 3a), which makes up about 80% of the total ES.

The discharge of ES in undisturbed animals is low. Only 20E22Ac could be detected in minor amounts in the sea water (Fig. 3a). During five days, the secreted 20E22Ac totalled 1.2 nmol/g fresh weight, corresponding to 1.2% of the 20E22Ac within the pycnogonids.

Secretion of ES in response to disturbance

In all disturbance experiments, the entire spectrum of ES was secreted, quantitatively reflecting the proportions of the endogenous ES pattern.

Compressing two legs (group 1, see Materials and Methods) yielded a total ES secretion of 6.3 nmol/g fresh weight, corresponding to 6.9% of the total ES (Fig. 3b). Extensive molestation for 30 sec (group 2) produced the secretion of 77.0 nmol ES/g, *i.e.* 70% of the total ES (Fig. 3c). The same extensive treatment on five successive days (group 3) caused almost complete secretion of all ES. After 5 days, only 1.2 nmol ES/g (1.4% of total ES) had remained within the animals, whereas 86.1 nmol ES/g (98.6% of total ES) had been secreted (Fig. 3d).

Ecdysteroid-levels in the defensive effluent

The mass of the defensive effluent that was obtained from 4 adult males averaged 450 µg ± 150 µg, calculated as a volume of 450 nl. In accordance with expectations, the secretion contained all eight ES, matching the ES pattern that occurred in the sea water in response to disturbance (Fig. 4). However, the ecdysteroid-levels in the effluent were more than ten times higher than if calculated as homogenous concentrations within the animals. The 20E22Ac and the total ecdysteroid-levels reached 0.8×10^{-3} mol·l^{-1} and 1.0×10^{-3} mol·l^{-1}, respectively.

Discussion

The ecdysteroid secretion of *P. litorale* in response to disturbance is substantial. 20E22Ac, the predominant compound, reaches 0.8×10^{-3} molar concentrations in the defensive secretion. When contained in homogenous concentrations in food

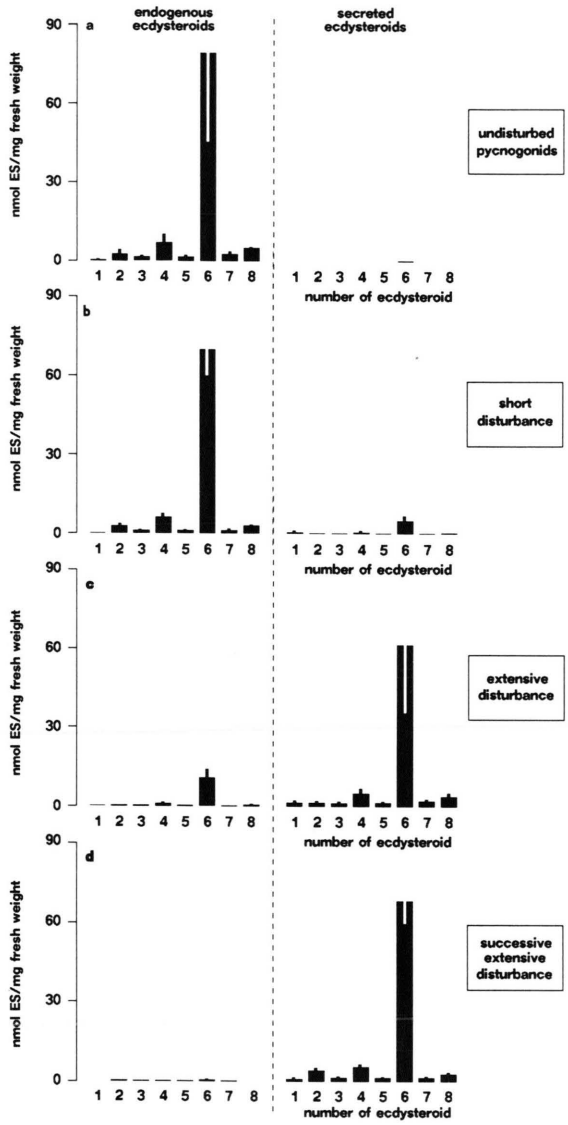

Fig. 3. ES-levels in intact adult males and secretion of ES into sea water in response to different disturbance intensities. Mean values + deviation. For numbers of ES see legend to Fig. 2.

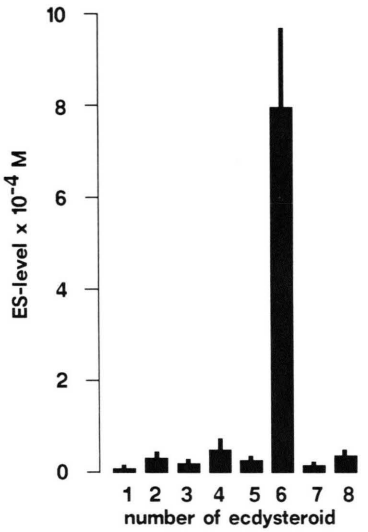

Fig. 4. Levels of ES in the defensive effluent of adult males. Mean values + deviation. For numbers of ES see legend to Fig. 2.

pellets, 20E22Ac reduces food acceptance by *C. maenas* even at a 0.5×10^{-3} molar level (Tomaschko, 1994). Thus, the secreted 20E22Ac in adult pycnogonids significantly contributes to their unpalatability, provided that the levels are still high enough when being perceived by the crabs. This seems to be the case, as recent experiments brought evidence that the exogenous ecdysteroid receptor responsible for feeding inhibition in *C. maenas* is located in the anterior oesophagus region and consequently comes in direct contact with the food (K.-H. Tomaschko, unpublished data). Exogenous ES detection has also been demonstrated by electrophysiological recordings in the lateral antennule of the spiny lobster *Panulirus interruptus* (Spencer and Case, 1984). However, the behavioral function of this low threshold detection is still unknown.

Besides 20E22Ac and 20E, the other six ES in *P. litorale* are also secreted in concentrations equimolar to those within the animals. This may suggest that all eight ES are involved in chemical defense. The almost complete secretion of all ES in response to successive disturbance may even mean that chemical defense is the essential ES function in adults. In molting stages of *P. litorale,* the 20E has at least one additional function. It promotes molting (Bückmann and Tomaschko, 1992), as it does in all other arthropods. The function of ES in arthropods as both hormones and allelochemicals has as yet been unknown. ES occur in all arthropods, where they control molting, growth, and gametogenesis (Koolman, 1990). Just for that very reason, their use as a protective allelochemical against arthropod predators may afford unusually

high levels. As yet, the high ES levels in *P. litorale* are unique in the animal kingdom. The concentrations found in whole animals are even increased by being accumulated in the defensive effluent. The storage of ES in *P. litorale* against a drastic concentration gradient in cells or compartments where they cannot interfere with the basic metabolism is an interesting problem for further studies.

Many insects contain non-ecdysteroidal steroids in their defensive secretions. The defensive effluent of leaf beetles contains polyoxygenated steroid glycosides and cardenolides, the latter occurring as a complex mixture of sometimes more than 10 different cardenolides in a single secretion (Pasteels, 1993). Aquatic hemipterans and dytiscid beetles secrete a mixture of pregnanes, containing almost 1×10^{-1} molar levels of the vertebrate steroid desoxycorticosterone (Scrimshaw and Kerfoot, 1987; Lokensgard *et al.*, 1993), which acts as a feeding deterrent against fish (Gerhart *et al.*, 1991). However, in these cases chemical defense may be the exclusive function of the steroids, as hormonal functions of non-ecdysteroidal steroids in insects are still unknown. On the other hand, an exclusive defensive function of ES may be true for animals other than arthropods, *e.g.* for the zoanthid *Gerardia savaglia,* which contains large amounts of ES (Sturaro *et al.*, 1982; Guerriero and Pietra, 1985).

The present results clearly emphasize the defensive role of ES in *P. litorale*. By concentrating the ES in high levels in the defensive effluent, the critical level for significant feeding deterrence of *C. maenas* is exceeded. This explains why most pycnogonids that were offered to *C. maenas* were seized but released unharmed (Tomaschko, 1994).

Although the present investigations are restricted to adult males, the identical ES patterns in embryos, larvae, and juveniles of both sexes (Tomaschko and Bückmann, 1993) suggest that all developmental stages of *P. litorale* are defended by ES.

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