Spermatozoid Chemotaxis in *Laminaria digitata* (Phaeophyceae). III. Pheromone Receptor Specificity and Threshold Concentrations

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The pheromone receptor specificity and threshold concentrations have been determined for spermatozoid chemotaxis in *Laminaria digitata* in structure-activity studies. The results are in agreement with the current concept on the pheromone receptor interaction in brown algae, which is based on mutually-induced dipole-dipole interaction, dispersion forces and hydrophobic forces. Comparison with the receptor specificity in pheromone-induced spermatozoid release in the same species indicates that different pheromone receptors are involved in these two functions.

Introduction

Chemotaxis is an important aspect of the fertilization process in many brown algae (Phaeophyceae) where flagellated male gametes are attracted by sessile, pheromone-releasing female cells (Maier and Müller, 1986; Maier, 1993).

In the life cycle of the kelp *Laminaria* the gametes are formed by free-living microscopic gametophytes which are characterized by dioecism and oogamy. Here, egg secretions have a dual function: in addition to chemotaxis they mediate the release of spermatozoids from unicellular antheridia. Pheromone-triggered spermatozoid release has not only been found in representatives of the brown algal order Laminariales (including *Laminaria*), but also in the Desmarestiales and Sporochnales (Maier and Müller, 1986) which by some authors are considered to be closely related (Clayton, 1984; Maier *et al.*, 1988). Spermatozoid release has been studied most intensively in *Laminaria digitata* (Maier, 1982; Maier *et al.*, 1988).

Eggs of *Laminaria digitata* produce a complex bouquet of low molecular weight hydrocarbons among them substances known as pheromones of other brown algae like ectocarpene (**7, 8**) and desmarestene (**6**) (Maier *et al.*, 1987). One particular compound, 6-(1′,2′-*cis*-epoxy-but-3′-enyl)-cyclohepta-1,4-diene (lamoxirene **1**) was found to induce spermatozoid release and chemotaxis at very low concentrations (Marner *et al.*, 1984; Maier *et al.*, 1988, 1992). Lamoxirene (**1**) is regarded as specific for the order Laminariales (Müller and Maier, 1985; Maier *et al.*, 1987).

Upon perception of the pheromone in spermatozoids of *Laminaria digitata* the apical part of the antheridial wall is dissolved within a few seconds. The cells are released, become motile and then react by directed movement to the same substance. This sequence of different activities must be subject to coordinated, fast and specific regulatory mechanisms in chemoreception and signal transduction.

In a previous study the specificity of the pheromone receptor for spermatozoid release and the pheromone-binding mechanism was explored by analyzing the structure-activity relationship in pheromone structure, using a number of specifically synthesized pheromone analogues (Maier *et al.*, 1988). The concentration-accumulation relationship in chemotaxis with lamoxirene (**1**) has also been investigated (Maier *et al.*, 1992) and it was concluded that probably more than one receptor species are involved in chemoreception. The present article deals with the structure-activity re-

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lationship in pheromone chemoperception related to chemotaxis and a differentiation of pheromone receptors on *Laminaria digitata* spermatozoids based on binding specificity.

Materials and Methods

An unialgal, clonal stock culture of male gametophytes of *Laminaria digitata* (Huds.) Lamour., originating from Heligoland, North Sea, Germany, was maintained at 6 ± 1 °C. Gametogenesis in subcultures was induced at 12 ± 1 °C as described previously (Maier and Müller, 1990). Gametophytes were used after 10–13 days under induction conditions. Spermatozoids were released by mild temperature shock as described by Maier and Müller (1990) immediately before each chemotaxis assay. In this way dense suspensions of cells were obtained which were not in contact with pheromone prior to the chemoaccumulation assay.

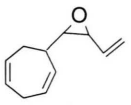
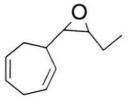
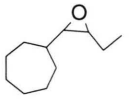
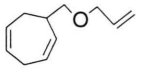
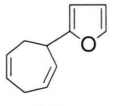
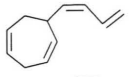
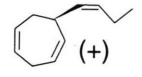
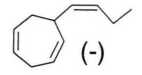
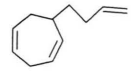
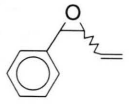
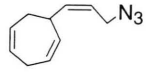
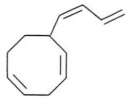
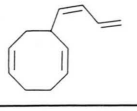
The chemoaccumulation assay described by Müller (1976) was used, employing a microscopic preparation of four 0.1 µl solvent droplets (a, b, c, d) in a square arrangement on a polystyrene petri dish with sea-water. Three droplets (b, c, d) contained a test substance at a defined concentration whereas the fourth droplet (a) was the pure solvent. As a solvent the water-immiscible high density fluorocarbon FC-72 (3 M Company) was used. 4 min after the addition of freshly released spermatozoids to the preparation the distribution of spermatozoids on the surface of the droplets was documented by a flash exposure microphotograph. The number of cells (A, B, C, D) in a standard area on the droplets were counted and the quotients B/A, C/A and D/A (Q-values) calculated. In addition the quotient B/D was determined in order to confirm the absence of unknown external factors influencing the distribution of spermatozoids (mean: 0.99 ± 0.18, n = 416). Assays with $0.70 > B/D > 1.30$ were excluded. Spermatozoid densities in the assays were $1–3\times10^6$ cell ml^{-1}. *Laminaria* spermatozoids are not phototactic and their chemotactic movement (Maier and Müller, 1990) is not influenced by light. Control experiments with four droplets of pure solvent gave a mean Q-value ("blank") of 1.07 ± 0.15 (n = 69). In a series of experiments with 13 different gametophyte subcultures the response of spermatozoids to a standard solution (3×10^{-7} M) of racemic lamoxirene (**1**) gave a mean accumulation factor of S = 2.20 ± 0.74 (n = 114). For each of the extremely slow growing subcultures used in the present study and each day the response of the spermatozoids to this standard solution (F) was compared with S to account for a possible variation in the "fitness" of the cells. Corrected accumulation factors were then calculated as $A_i = (Q/\text{blank} - 1) \times S/F + 1$. For each concentration step, the accumulation factors from 9–75 assays were pooled, giving a mean accumulation factor A. The threshold concentration for a given substance is the lowest concentration at which in comparison to the blank a statistically significant accumulation of spermatozoids was observed with an error probability of $p < 0.5\%$ in the one-tailed Student's t-test.

Stock solutions of test substances (1 mM) and dilution series were made in the solvent FC-72. Racemic mixtures were used except for compound **7** (100% *S*-(+)-ectocarpene), which was purified from the root oil of *Senecio isatideus* (Boland *et al.*, 1982a), and compound **8** (*R*-(−)-ectocarpene, optical purity ≥90% e.e.). References for synthesis and physicochemical data of compounds **1–3:** Marner *et al.* (1984); **4, 9:** Boland and Mertes (1984); **6:** Boland *et al.* (1982b); **11, 12, 13:** Schroer-Sissoko (1988); **5, 8, 10:** unpublished. The substances were of 95% gas chromatographic purity. Compound **10** was a mixture of *cis/trans* isomers (2:3). Synthetic racemic lamoxirene (a mixture of 4 diastereomers) was a gift of F.-J. Marner (Institut für Biochemie, Universität zu Köln). Partition coefficients between the solvent and sea-water at 12 °C ($K_{FC/SW}$) were determined according to Boland *et al.* (1981). The highest concentration for a given substance available to a spermatozoid on the solvent/water interphase in the chemotaxis assay was calculated as the quotient: concentration in the solvent/partition coefficient.

Results

Lamoxirene (**1**), the natural pheromone of *Laminaria* shows the lowest threshold concentration at 1.4×10^{-12} M among the substances tested for their chemoattractive potential (Table I). The concentration response curve spans over 7 orders of magnitude in concentration

Table I. Thresholds and maxima in the concentration response relationships in the chemotaxis of *Laminaria digitata* spermatozoids with synthetic pheromone and structurally related compounds ($K_{FC/SW}$: distribution coefficient for the solvent system FC-72/sea-water.

Compound		Threshold concentration [M]	Maximum accumulation at [M]	$K_{FC/SW}$
1		1.4×10^{-12}	1.4×10^{-6}	7.4
2		1.4×10^{-7}	1.4×10^{-5}	7.8
3		6.3×10^{-8}	$\geq 6.3\times10^{-5}$	33
4		6.0×10^{-7}	6.0×10^{-6}	50
5		1.6×10^{-9}	1.6×10^{-6}	189
6		2.8×10^{-12}	2.8×10^{-8}	779
7		1.2×10^{-8}	$\geq 3.7\times10^{-7}$	2680
8		3.7×10^{-8}	$\geq 3.7\times10^{-7}$	2680
9		4.5×10^{-11}	$\geq 4.5\times10^{-7}$	2217
10		1.4×10^{-9}	4.3×10^{-7}	7
11		7.9×10^{-9}	7.9×10^{-7}	380
12		3.5×10^{-10}	$\geq 3.5\times10^{-7}$	2900
13		3.9×10^{-10}	3.9×10^{-8}	2600

The data for **1** were taken from Maier *et al.* (1992).

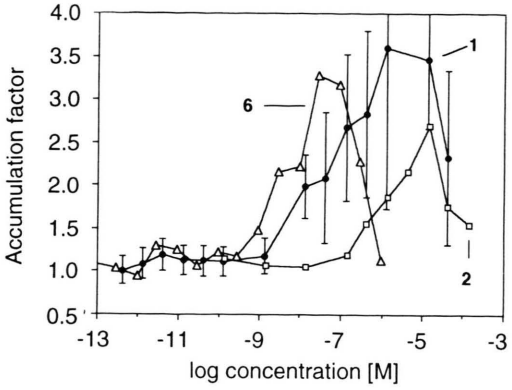


Fig. 1. Concentration response curves for chemoaccumulation of *Laminaria digitata* spermatozoids with synthetic lamoxirene (**1**), desmarestene (**6**) and compound **2.** For clarity, the standard deviations of the data are shown only for **1.**

(Fig. 1) with a maximum in the micromolar range (data taken from Maier *et al.*, 1992). The shape of the curves is similar for all compounds. Hydrogenation of the terminal side chain double bond (**2**) results in a parallel shift of the concentration response curve (Fig. 1) towards higher concentrations by a factor of about 100. The activity in terms of the threshold concentration is drastically reduced by a factor of 10^5 (Table I). Additional saturation of the two ring double bonds (**3**) causes a further, but comparably small reduction in activity while the threshold concentration is essentially the same as for **2.** The ether **4** shows only poor activity, whereas the furyl-cycloheptadiene (**5**, Table I) is a potent chemoattractant inducing spermatozoid accumulations as strong as lamoxirene (**1**) at the same optimum concentration in the micromolar range. The threshold concentration, however, is higher by a factor of 1000. In desmarestene (**6**) the epoxy group of lamoxirene is replaced by a double bond. This compound gives a slightly asymmetric concentration response curve very similar to, but not as broad as that of lamoxirene (**1**) (Fig. 1). The threshold concentrations for desmarestene and lamoxirene are almost the same, but with desmarestene the accumulation maximum is reached at a concentration as low as 2.8×10^{-8} M (Table I) which makes this compound an even more potent attractant at low pheromone concentrations than lamoxirene (**1**). Hydrogenation of the terminal double bond of desmares-

tene (**6**) which results in ectocarpene (**7, 8**) has a deleterious effect on attractive potential: the threshold concentration is increased by a factor of about 10^4. *S*-(+)-ectocarpene (**7**) appears to be slightly more active than *R*-(−)-ectocarpene (**8**) with an error probability p = 5–20% (Student's t-test) for the 3 highest concentrations tested and with a somewhat lower threshold (Table I). Hydrogenation of the double bond in 1′ position of desmarestene resulting in compoung **9** causes only a relatively small reduction in the threshold concentration compared to lamoxirene (**1**) and desmarestene (**6**) (Table I). The accumulation maxima were not reached with the available concentrations of **7–9** due to the high partition coefficients. If the cycloheptadiene ring in lamoxirene (**1**) is replaced by a phenyl ring as in compound **10** the effect on chemoaccumulation in terms of accumulation factors and the position of the maxima appears to be insignificant, though the threshold concentration is increased by a factor of 100 (Table I). 6-(3′-azido-1′ *Z*-propenyl)-1,4-cycloheptadiene (**11**) which was tested as a potential photoaffinity label for the pheromone receptor is also a reasonable effective attractant with high accumulation factors and a maximum at micromolar concentrations (Table I). The threshold concentration at 7.9×10^{-9} M is practically the same as for ectocarpene (**7, 8**). The cyclooctadiene analogues of desmarestene **12** and **13** show only poor activity (**12**) (Table I) compared to desmarestene (**6**).

Developmental studies in which the threshold concentration for pheromone-triggered spermatozoid release with lamoxirene (**1**) was compared with the chemotactic potential of heat-shock released cells of the same age during gamete maturation gave no indication for a differentiation between the pheromone perception systems involved in these two cellular functions, both developed in parallel (data not shown).

Discussion

The results of pheromone structure-activity studies related to chemotaxis in *Laminaria digitata* confirm the significance of precisely positioned polarizable groups or double bonds in the pheromone molecule for biological activity and is thus in agreement with the concept of pheromone binding based on mutually induced dipole/dipole interactions, dispersion forces and hydrophobic forces (Boland *et al.*, 1981, 1982c, 1984; Maier *et al.*, 1988). As a consequence, hydrogenation of double bonds severely reduces activity as is seen in the series **1–3** as well as in **6–9.** Similar results have been obtained in structure-activity studies on the chemoreceptors in pheromone-triggered spermatozoid release in *Laminaria digitata* (Maier *et al.*, 1988) and *Chorda tomentosa* (Maier *et al.*, 1984) and in gamete chemotaxis of other brown algae (Boland *et al.*, 1981, 1982c, 1983, 1984). The effect of individual double bonds is different and depends on their position in the molecule. The terminal side chain double bond is indispensible for effective pheromone-receptor interaction (compare **1, 2, 6, 7, 9**), whereas hydrogenation of double bonds (**3**) or electron delocalization (**10**) in the ring have much less effect.

As in spermatozoid release (Maier *et al.*, 1988) the epoxy group in lamoxirene (**1**) has only minor significance in determining pheromonal activity in spermatozoid chemotaxis since compound **9** and also desmarestene (**6**) possess very high activity in comparison with **1.** The function of the heterocycle may be seen in a conformational constraint in the side chain, similar to a double bond in this position as in **6.** In addition the heterocycle changes the partition coefficient, which makes lamoxirene (**1**) considerably less lipophilic than most other brown algal pheromones. The relationship between pheromone structure and activity in the induction of spermatozoid release have been interpreted in the same way (Maier *et al.*, 1988). High flexibility in the butyl side chain leads to low activity, as in the ether (**4**), although this compound can adopt the conformation of lamoxirene (**1**). Two similar cases are seen in *Desmarestia aculeata,* where compound **9** is significantly less active in spermatozoid attraction than desmarestene (**6**) and in *Syringoderma phinneyi* with viridiene and its corresponding derivative (Boland *et al.*, 1984). Less effective interaction between the partially saturated compounds and the pheromone receptor can be compensated at least at medium concentrations in the water phase by their increased lipophily (higher partition coefficient). Partitioning into the gamete's lipid membranes may lead to a considerably increased concentration in the receptor lipid environment if the pheromone receptor is situated in a membrane. The relatively high activity of compounds **9**

and **6** in *Laminaria* chemotaxis could be due at least partly to this effect. The significance of partition and hydrophobic interaction as the initial steps in pheromone binding has been demonstrated first in studies on *Ectocarpus siliculosus* and *Cutleria multifida* (Boland *et al.*, 1981, 1983).

The absolute configuration of lamoxirene (**1**) is not known at present and pure enantiomers are not available. The differences in activity between *S*-(+)-ectocarpene (**7**) and *R*-(−)-ectocarpene (**8**) are so small that no conclusion can be drawn on the absolute configuration of lamoxirene.

The absolute values of the accumulation factors in *Laminaria* spermatozoid chemotaxis are much smaller than those in *Cutleria* (Boland *et al.*, 1981), for example. This may be partly attributed to the heat-shock method used for spermatozoid release. Premature spermatozoids in such suspensions may be pheromone-insensitive. A background of such cells reduces the accumulation factor and leads to underestimated thresholds.

The receptor profiles in spermatozoid release and in chemotaxis of *Laminaria digitata* spermatozoids are clearly different. Desmarestene (**6**), which is by a factor of 200 less active than lamoxirene (**1**) in spermatozoid release (Maier *et al.*, 1988) acts as a potent attractant in chemotaxis. Furthermore, the aromatic lamoxirene analogue (**10**) as well as the furyl derivative (**5**) were totally inactive in triggering spermatozoid release and **5** acts as a potent, probably competitive inhibitor. In chemotaxis, however, both compounds showed considerable activity. This justifies the conclusion that different pheromone receptor species are involved in spermatozoid release and in chemotaxis. Evidence obtained in a previous study indicated that more than one receptor type is required for chemoaccumulation over the whole response range (Maier *et al.*, 1992) defined as the concentration range between the threshold and the saturating concentration.

Lamoxirene (**1**), desmarestene (**6**) and ectocarpene (**7**) are components in the pheromone bouquet secreted by eggs in many species of the Laminariales (Maier *et al.*, 1987). Compounds **6** and **7** are known as pheromones in other brown algal orders. Desmarestene (**6**) causes spermatozoid release and chemotaxis in *Desmarestia* (Boland *et al.*, 1984). The high activity of desmarestene (**6**) in chemotaxis of *Laminaria* spermatozoids supports the arguments for a close phylogenetic relationship between the Laminariales and the Desmarestiales (Clayton, 1984; Maier *et al.*, 1988).

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