Receptor Specificity and Threshold Concentration in Chemotaxis of the Phaeophyte *Cutleria multifida*

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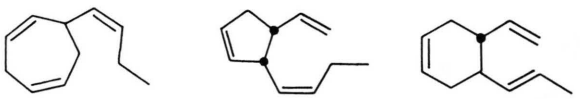
Chemotaxis, *Cutleria multifida* (Phaeophyceae), Sensitivity Threshold, Receptor Specifity, Signal-Receptor Interaction Model

Sexual reproduction has a decisive function during the life cycle of all organisms. In the marine brown alga *Cutleria multifida*, multifidene (**1**), a low molecular weight olefinic hydrocarbon, has been recognized as a chemical messenger for gamete union. Comparative activity tests with 32 specially designed synthetic analogous of the natural attractant demonstrate the high specifity and low threshold concentration (6.5×10^{-12} mol/L) of the gamete's receptor system toward its natural signal (**1**). The findings establish the double bonds of multifidene (**1**) as the molecular coordination points in the messenger-receptor interaction. A tentative model is presented, which takes into account the evidence, that unpolar, but highly polarizable functional groups in the correct spatial arrangement are necessary for maximal mutual interaction.

Introduction

Substances, which act as chemical messengers between individuals of the same species, are usually called pheromones in accordance to a proposal of Karlson and Lüscher [1]. Such chemical signal systems are widespread in nature as a means of interaction and communication. The best known examples are those of the highly developed insects; but even the reproductive cells of primitive plants such as mosses, ferns or marine brown algae use chemotaxis to lure the mature sexual partners for securing their reproduction. Although this type of chemotaxis has been known for over a century by plant physiologists (Thuret, 1854 [2]; Kuckuck, 1899 [3] or Oltmanns 1899 [4]), all attempts to identify these substances failed because of inadequate analytical methods. It was not before 1968 that Machlis [5] reported the first successful isolation and identification of a water soluble sesquiterpenoid sperm attractant from the water mold Allomyces. Very soon another class of compounds with completely hydrophobic character, serving as attractants for the androgametes of the marine brown algae *Cutleria multifida*, *Ectocarpus siliculosus* and *Fucus serratus* respectively, were isolated and identified by Müller and Jaenicke [6–10]. These substances were shown to be C_8- and C_{11}-hydrocarbons with predominant cyclic structures like ectocarpene (**31**) S(+)-6-(*cis*-1-butenyl)-1.4-cycloheptadiene from *Ectocarpus siliculosus* [9], or multifidene (**1**) *cis*-3-(*cis*-1-butenyl)-4-vinyl-cyclopentene and aucantene (**32**) *trans*-4-(trans-1-propenyl)-5-vinyl-cyclohexene from *Cutleria multifida* [10].



ectocarpene (**31**) multifidene (**1**) aucantene (**32**)

Further C_{11}-hydrocarbons – some alicyclic, others linear – were found to be constituents of some Hawaiian *Dictyopteris* species [11, 12] and are obviously biogenetically related to the above mentioned sex attractants. More recent findings indicate that ectocarpene (**31**) may be a general constituent of the essential oils of the sub-class Phaeophycidae, since it has been shown [13], that mature eggs of *Laminaria digitata* produce ectocarpene (**31**) too, along with the actual but in its structure still unknown sex attractant. This, as evidenced by high resolution mass spectrometry, is again a C_{11}-hydrocarbon. It possesses, however, in addition, an oxygen function, which is tentatively assigned to an enol-ether [14] thus preserving the unpolar character of this class of attractants.

* To Prof. Dr. Walther Jaenicke, Erlangen, on the occasion of his 60th birthday.

Reprint requests to Prof. Dr. L. Jaenicke.

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Comparing the known structures of the lures **1**, **31**, or **32** the problem arises, how specific a recognition system has to be to discriminate between C_{11}-hydrocarbons of similar ring size and stereochemistry. Furthermore, the fact, that all these substances belong to the same class of olefinic hydrocarbons implies similar physical properties such as solubility, dipole moments or electron polarizability, which again demand highly specific receptor ligand interaction. To answer the question of specificity, we chose the attracting system of *Cutleria multifida* for two major reasons:

1. Mature female gametes of *Cutleria* secrete three cyclic olefines **1**, **31**, and **32** into the surrounding medium, but only one of them (**1**) is the true and active lure; from this fact we have to expect a highly specific interaction with the trapping system.

2. The attractant, multifidene (**1**), is a *cis*-disubstituted cyclopentene of given shape and space-filling. Minor variation of geometry in the side chains or shift of the double bond in the ring should not seriously affect shape and conformation of the signal molecule. In other words: Large differences in the attractive potential are not caused by dramatic sterical or physical alterations, but should be related nearly exclusively to the interaction of the messenger molecule with the receptor system.

A related and important question is that of threshold concentrations, which only can be answered with a reproducible biological activity test [15]. In addition, knowledge of the partition coefficients of the attractants and their derivatives between the source (organic solvent droplet as an artificial gynogamete) and the sea water medium is essential. Both parameters, the "apparent biological threshold concentrations" and the partition coefficient for a given hydrocarbon, can be determined with high accuracy as will be shown in the Methods section. From these data the effective threshold concentration of the ligand is easily calculated.

Materials and Methods

Attractants and homologues

All the synthetic compounds tested were racemic mixtures, prepared according to the references given in Table I. With very few exceptions all compounds were purified to stereochemical homogeneity using preparative G. L. C. Only the carbonyl compounds **23** to **26** contained minor amounts $- \sim 5\% -$ of trans-disubstituted isomers, and the hydrocarbon **4** had a stereochemical purity of only 80%.

Cultures

Microgametes of *Cutleria multifida* (Smith) Grev. were obtained from the uniseriate variant CMV-M1b. Culture conditions and concentrations of microgametes by positive phototaxis were as described previously [15].

Biological activity test [15]

Stock solutions of most hydrocarbons were made at 10^{-3} molar concentrations in FC 78 (fluorocarbon of high density $- d = 1.7$ g/ml $-$, 3 M-Company Düsseldorf, West Germany). In cases with poor solubility saturated solutions had to be prepared, the concentration determined by gas chromatography and adjusted to the next lower step of the dilution series. The dilution series consisted of half-logarithmic steps, and 0.1 µl droplets of these dilutions were placed on the bottom of a small polystyrene wet chamber filled with the culture medium [16]. Four minutes after addition of male gametes of *Cutleria multifida* in the dark a microphotograph was taken and the population of microgametes per field over the attractant droplets was compared to that of a solvent blank. The result of each experiment could be expressed by calculating the following "luring-quotient":

$$Q = \frac{\text{gametes per standard-area of sample droplet}}{\text{gametes per standard-area of pure solvent (FC 78)}}$$

Several parallel runs were made for statistical treatment of data. According to the definition of Q the attractivity threshold of a given hydrocarbon is that concentration, at which the ratio of cells per standard-area of attractant droplet over the number of cells per standard-area of the blank approaches 1.00.

Determination of partition coefficients

With respect to the very low solubility of hydrocarbons in water their partition coefficients between the organic solvent FC 78 and the culture medium [16] had to be determined. The following procedure was found to be most effective even at very low concentrations:

200 µl of a 0.1 to 1.0% solution of a given hydrocarbon in FC 78 were stirred magnetically in a centrifuge tube for one hour with 6.0 ml of culture medium saturated with FC 78 (a time of at least 40 min proved to be necessary for saturation of the aqueous phase with the hydrocarbon). The finely dispersed droplets of FC 78 were centrifuged down. 5.0 ml of the aqueous phase were withdrawn and reextracted by stirring with 200 µl of pure FC 78. After one hour of equilibration (magnetic stirring) the two phases were separated by centrifugation. The hydrocarbon content of the organic layer was determined by G. L. C. and from these data the partition coefficients were calculated. Because of the low solubility of hydrocarbons in water the second partition step with fresh FC 78 removed all of the dissolved attractant from the aqueous phase, thus allowing correct comparison of hydrocarbon contents in both, sea water and the original organic solution. Several repetitive injections from the same solution were made for statistical treatment of the integrated peak areas. Furthermore, the whole partition process was repeated several times at different concentrations to exclude oversaturation artifacts. In general the reproducibility proved to be better than ± 4% in all cases. All partition coefficients below 100 were simply determined by stirring 0.1% hydrocarbon solutions in FC 78 with equal volumes of culture medium followed by direct G. L. C.-analysis of both phases without further treatment. All analytical procedures were performed with a Hewlett-Packard 5750 G Research Gaschromatograph equipped with a 3370 B automatic integrator. The stationary phase and support used were 10% Apiezon L on Chromosorb W (60–80 mesh, AW, DMCS treated) in a 1.5 m × 4 mm glass column.

Results and Discussion

Like any other olfactory cell the attractant receptor system is a primary sensory cell responsible for perception and transfer of chemical signals into modified motility or behaviour. In this sense male gametes of *Cutleria multifida* are attracted to an artificial female (FC 78 droplets with known quantities of attractant) and show identical behaviour as during the primary events of natural copulation [17]. This possibility of imitating the initial stage of the mating process with a source of definite attractant concentration enabled us to compare a large number of structurally modified "multifidenes". The results thus obtained are compiled in Table I, which gives a detailed synopsis of threshold concentrations as well as of Q-values and partition coefficients.

The following findings (Table I) prove, that for *Cutleria* androgametes multifidene (**1**) is the specific messenger interacting with a correspondingly specific receptor:

i) As seen from the selected concentration/activity curves of Fig. 1 none of the tested synthetic analogues and structurally related compounds reaches the activity of the natural messenger (**1**). The low threshold concentration of 6.5×10^{-12} mol per liter sea water medium indicates, that the androgamete's receptor system is highly specialized, thus being particularly well suited for the detection of the chemical signal. This threshold exceeds the well studied chemotactic activities of bacteria by three to four powers [28]. However, since the number of binding sites per gamete is unknown, the stoichiometry of interaction between signal and receptor (efficiency) cannot yet be ascertained.

ii.a) Minute geometric alterations of the messenger molecule *e.g. cis* to *trans* within the butenyl side chain (compound **4**), or trans substitution at the ring (compound **3**) are recognized and answered with 32 or 25 fold increased threshold concentrations, compared to that of (**1**).

b) Larger structural modifications such as increase of length in either side chain (compounds **5**, **6**, **7**, and **8**) require considerably (ca. 120 fold) higher threshold concentrations, demonstrating again, that only multifidene (**1**) is the true active messenger (Fig. 2, R = C_2H_5).

c) Any introduction of an additional alkyl substituent (compounds **6**, **9**, **10**, **11**, and **12**) decreases activity and points to an accurate fitting of the ligand to the receptor binding site. These facts indicate such a very close contact of the messenger and receptor area that the distance between the longitudinal axis of the odor molecule and its surrounding receptor pit is less than the radius of a methyl group (< 2 Å) [29]. On the other hand, the contributions of the various methyl groups are not identical. This indicates distinct areas on the binding site, which offer the molecule space to orient the hindering methyl group distal to the area of contact.

iii) Further insight into the mechanism of interaction between ligand and receptor has been obtained by synthesizing the partly hydrogenated "multi-

Table I. Threshold concentrations and partition coefficients.

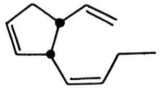
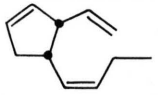
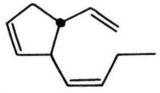
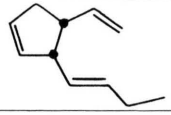
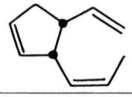
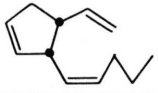
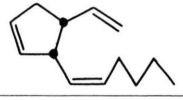
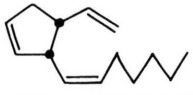
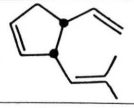
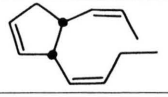
Comp./Refer.	Cmol/l FC78 Q-Values													$^{K}FC78/H_2O$	Threshold-concentrations mol/l sea water
	$10^{-9} \times 3.1$		$10^{-8} \times 3.1$		$10^{-7} \times 3.1$		$10^{-6} \times 3.1$		$10^{-5} \times 3.1$		$10^{-4} \times 3.1$		$10^{-3} \times 3.1$		
1 [18] 	0.98	1.09	***1.15***	***1.49***	***2.33***	***2.97***	***23.2***							4 800	6.5×10^{-12}
2 [18] 				0.96	0.92	0.99	***1.17***	***1.96***	***5.18***	***7.88***				4 800	6.5×10^{-10}
3 [19] 				0.97	1.03	***1.35***	***3.00***							6 200	1.6×10^{-10}
4 [19] 				1.09	0.94	***1.69***	***3.06***	***6.88***						4 800	2.1×10^{-10}
5 [18] 				1.01	1.11	***1.48***	***3.22***							2 200	4.5×10^{-10}
6 [18] 						0.96	1.08	***1.37***	***2.19***	***5.87***				10 000	1.0×10^{-9}
7 [18] 										0.96	0.99			16 500	–
8 [18] 						0.94	1.06	1.01	1.09	***1.53***	***2.41***			17 800	5.6×10^{-9}
9 [18] 				0.99	1.09	1.07	***1.20***	***1.45***	***2.07***					5 200	6.0×10^{-10}
10 [18] 				1.01	1.02	***1.22***	***1.51***	***2.85***						11 000	9.1×10^{-11}

Table I. Continued

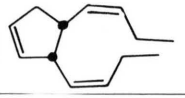
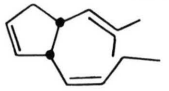
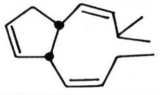
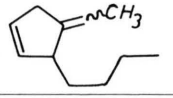
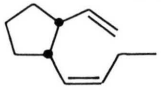
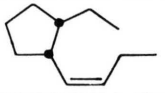
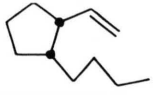
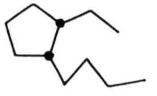
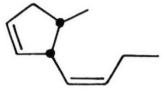
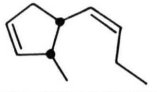
Comp./Refer.	Cmol/l FC78 Q-Values									^{K}FC78/H_2O	Threshold-concentrations mol/l sea water
	$10^{-9} \times 3.1$	$10^{-8} \times 3.1$	$10^{-7} \times 3.1$	$10^{-6} \times 3.1$	$10^{-5} \times 3.1$	$10^{-4} \times 3.1$	$10^{-3} \times 3.1$				
11 [18] 					0.99	0.96	***1.15***	***1.32***		10 200	3.0×10^{-9}
12 [18] 				1.02	1.07	1.04	***1.16***	***1.19***	***1.59***	***1.65***	9 600	3.2×10^{-9}
13 [18] 										0.94	16 100	–
14 [18] 			1.02	1.09	***1.13***	***1.30***	***1.85***				–	–
15 [20] 					1.01	***1.19***	***3.21***	***6.95***	***10.4***	***17.3***	7 400	4.2×10^{-10}
16 [21] 				0.99	0.95	0.96	1.09	***1.55***	***3.23***		12 200	2.6×10^{-9}
17 [21] 									1.06	***1.19***	11 700	2.7×10^{-8}
18 [22] 							1.02	1.07	***1.28***	***1.46***	15 300	2.0×10^{-8}
19 [19] 			1.01	1.06	1.06	***1.30***	***2.76***	***3.01***	***13.9***	***14.7***	5 900	5.3×10^{-10}
20 [20] 					1.02	1.06	1.11	***1.44***	***2.63***	***6.06***	5 900	5.3×10^{-9}

Table I. Continued

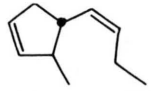
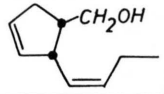
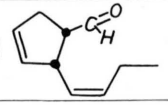
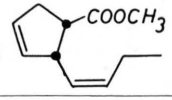
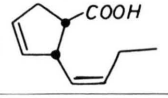
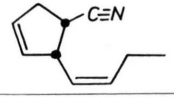
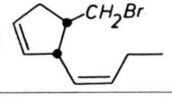
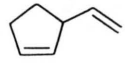
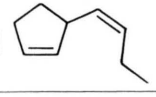
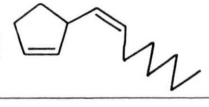
Comp./Refer.	Cmol/l FC78 Q-Values													^{K}FC78/H_2O	Threshold-concentrations mol/l sea water
	$10^{-9} \times 3.1$		$10^{-8} \times 3.1$		$10^{-7} \times 3.1$		$10^{-6} \times 3.1$		$10^{-5} \times 3.1$		$10^{-4} \times 3.1$		$10^{-3} \times 3.1$		
21 [20] 							1.02	1.03	***1.25***	***2.06***	***3.75***	***9.09***		–	–
22 [18] 							***1.09***	***1.45***	***1.62***					1.4	7.2×10^{-7}
23 [18] 	0.99	1.02	1.10	***1.24***	***1.25***	***1.81***	***4.42***	***6.98***	***14.2***					30	1.0×10^{-9}
24 [18] 	1.17	***1.16***	***1.59***	***2.63***	***Positive paralysed***									170	1.8×10^{-11}
25 [23] 												1.06		–	–
26 [18] 	1.07	1.13	1.01	***1.18***	***1.28***	***1.86***	***5.87***	***7.29***	***8.05***	***2.96***	***Paralysed***			13	2.4×10^{-9}
27 [24] 	0.99	1.08	1.06	***1.39***	***2.16***	***4.67***								2 100	1.4×10^{-11}
28 [25] 											1.04	0.98	0.88	–	–
29 [25] 					0.91	0.95	0.97	1.05	***1.83***	***3.57***	***4.72***			2 700	3.7×10^{-9}
30 [25] 											1.02	0.99	1.05	12 000	–

Table I. Continued

Comp./Refer.	Cmol/l FC78 Q-Values								$^{K}FC78/H_2O$	Threshold concentrations mol/l sea water
	$10^{-9} \times 3.1$	$10^{-8} \times 3.1$	$10^{-7} \times 3.1$	$10^{-6} \times 3.1$	$10^{-5} \times 3.1$	$10^{-4} \times 3.1$	$10^{-3} \times 3.1$			
31 [26]				0.99	1.01	***1.21***	***1.67***	***3.79***	***9.87***	***15.8***	3 500	2.9×10^{-9}
32 [27]				1.07	1.14	1.18	1.19	***1.52***	***1.19***	***2.37***	***1.85***	3 100	3.2×10^{-8}
33				1.03	1.07	1.05	1.07	1.08	0.97	***1.45***	***1.79***	–	–

fidenes" (**15**, **16**, **17**, and **18**). Whereas the loss of one double bond increases the threshold 65 fold, the loss of two double bonds increases the threshold 1215 (**16**) or 4000 fold (**17**) respectively. Thus the activity of compounds which lost two double bonds is of the same order as found for the completely hydrogenated compound **18**. This non-linear loss of activity compared to the absence of one, two or all three unsaturations might be due to a cooperative binding of the messenger with all three double bonds to the area of contact. Some other derivatives as **19**, **20**, and **21** support in addition the importance of each double bond in general as well as their spatial arrangement towards each other in particular. Although space filling and physical properties of compounds **19** and **20** have been practically retained, their different thresholds underline the need for an allylic arrangement of the π-electron systems. A similar comparison of **1**, **2**, and **15**, where **1** and **2** have identical configuration, again shows the demand for a very exact mutual interaction of ligand and binding site, since the response of **2** corresponds to that of the dihydro-derivative **15**. This observation is easily explained, if only two of the three double bonds of **2** are involved in the binding process. According to these findings Fig. 3 presents a tentative model of interaction, which accomodates all experimental results mentioned before.

The receptor may possess precisely fixed electrophilic groups corresponding to the positions of double bonds of the ligand thereby inducing opposite polarizations between both. Subsequent or concerted dipolar interaction could effect binding and change of conformation of the ensuing complex. Further proof of this hypothesis comes from the derivatives **24** and particularly **27**. Although in this latter molecule the essential vinyl substituent (compare **1** to **29**) has been replaced by the bromo-methyl-group, requiring about equal space, the receptor response is not considerably decreased. This leads to the more general conclusion, that other substituents of suitable size and polarizability including small permanent dipole moments pointing in the right direction may simulate the binding properties of the original double bond [30].

iv) The group of "oxygenated multifidenes" **22** to **27** offers additional insight into the receptor area and the basic principles of interaction. As can be seen from Table I the receptor response is obviously a distinct function of the polarity of the messenger

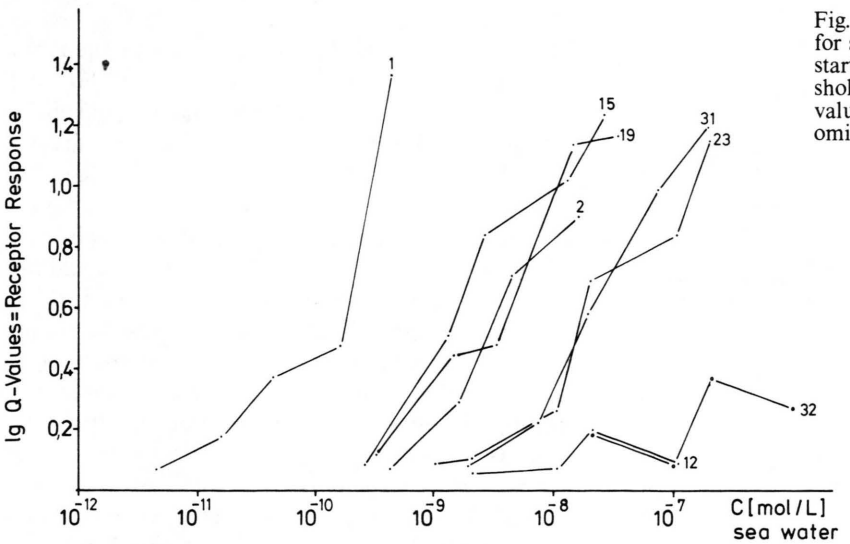


Fig. 1 Concentration/Response curves for selected "multifidenes". The curves start at the calculated effective threshold concentrations. Insignificant *Q*-values at lower concentrations are omitted. Numbers of Tab. I.

molecules. Since the water solubility of these compounds depends on their permanent dipole moment, the partition coefficients between FC 78 and water have been taken as a basis of comparison. By classifying **22** to **27** according to their enhanced water solubility it becomes evident, that the polar forms of the otherwise unchanged molecules do not release meaningful signals (compare **27** and **22**; **24** and **25**). This last finding can be explained best if there is an unpolar area surrounding the binding (trapping) site which has a pronounced affinity for an olefinic hydrocarbon only [31]. Moreover, such a selecting property fulfills the demand on a high signal-to-noise ratio, since the large difference in polarity between the carrier medium water and multifidene **1** secures a safe recognition of the signal molecule.

v) If the response to messenger molecules is followed up to higher concentrations (Fig. 4) a stimulus-response curve is obtained, which corresponds in all respects to the chemosensoric behaviour of bacteria [28]. Along with the sharp decrease of activity at 10^{-6} mol/l the gametes become desoriented and then paralyzed. However, whether this is caused by saturation or by predominantly blocking

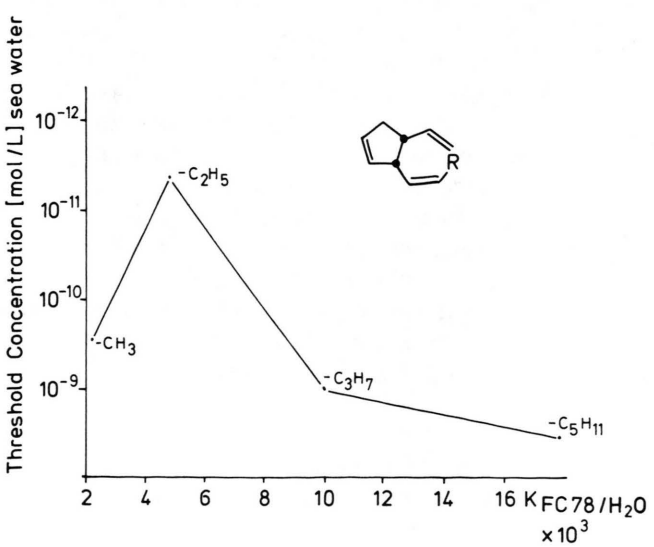


Fig. 2. Structure/Response relationship of homologues "multifidenes". The activity (threshold concentration) of each compound is presented as a function of its partition coefficient (FC 78/H_2O), thus connecting increasing chain length to a physicochemical parameter.

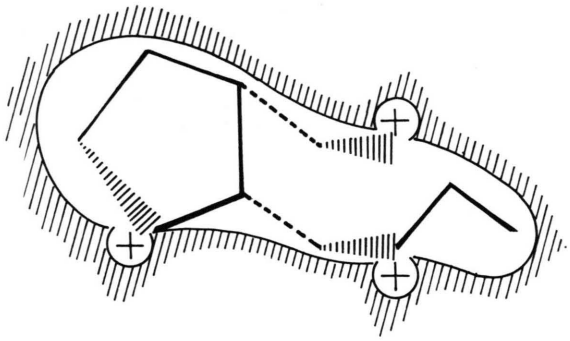


Fig. 3. Proposed binding site of multifidene **1** (3(R), 4(R)-Form as model) showing the geometry of punctual polarizing charges.

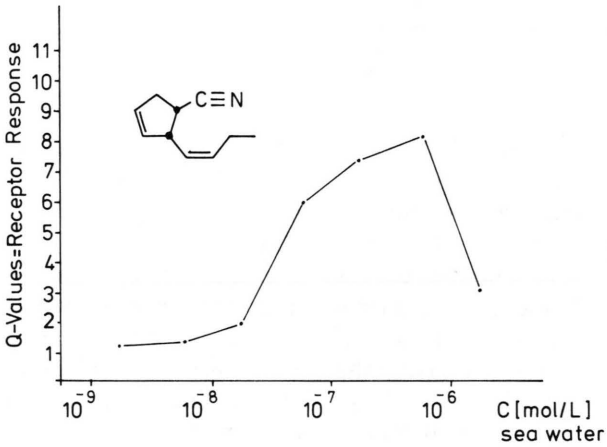


Fig. 4. Concentration/Response Curve; exemplified by the nitrile **26** because of its enhanced water solubility.

of the binding sites cannot be ascertained presently and has to await further investigation.

The slope of the concentration/response curves (Fig. 1 and 4) is almost identical for all compounds with any significant luring activity. From this it may be infered that the signal triggers the same sensory circuit independent of its actual binding strength. Thus the sensomotoric chain seems to consist of at least three links: reception-transmission/modulation-processing as discussed for other biological information.

Conclusions

The data presented make it obvious that the sex attractants of brown algae are recognized by the spermatozoids through specific binding sites, shaped to fit the spatial and steric requirements of the messenger molecules. In this aspect there is no difference to other known chemotactic systems. However, most pheromones isolated so far and molecules acting as chemical attractants or repellents in bacteria, many have at least one functional hetero (N-, O- etc.) group. This is not so in the case of chemical signals that lure algal gametes: They are unsaturated hydrocarbons, most of them alicyclic molecules in which only the overall structure and the position of the double bonds relative to each other may function in molecular interactions. It is improbable that binding is effected by transition metal chelation of the unsaturated molecules. This would require transition metals never found in biomolecules. The evidence given here favours the binding of attractant through polarization of the π-electron systems by charged groups in the receptor site to which the lipophilic compound may be attached by additional entropic forces in the aqueous milieu. It is still too early to hypothesize on the nature of these receptors. However, it has to be kept in mind that the algal gametes are naked cells and their surface is hydrophilic in contrast to the hydrophobic cuticle found in the outer surfaces of the exo-skeletons of insects [33]. Thus binding between lipophilic attractant and androgamete envelope will be slight except at the true binding site of receptors, which not only accomodate the molecules spatially but also will have polarizing groups at proper geometry and distance; consequently these areas will expose towards the surrounding medium more hydrophobic moieties such as aromatic amino acids of proteins and proteoglycans or lipids of glycolipids. The sensitivity of the system is surprisingly high at 1 in 10^{11} and compares very favourably with many odor recognition systems of insects [34]. From this threshold the overall binding energy (consisting of solution enthalpy, entropic terms, true binding energy etc.) calculates to about 16 kcal (67 kJ) mol^{-1}. Since the induced dipole/dipole interactions of the polarized double bonds may not exceed 3 kcal (13 kJ) each, the contributions of all other – in their nature still unknown – terms will be almost half of the total binding energy, thus making the system strongly dependent on changes in the overall milieu.

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