Sexual Pheromones and Related Egg Secretions in Laminariales (Phaeophyta)

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Volatile egg secretions of 15 species belonging to 5 families of the brown algal order Laminariales have been analyzed by high resolution glass capillary gas chromatography. 16 components have been identified and partially determined quantitatively. The composition of the pheromone bouquets and pheromone specificity for induction of spermatozoid-release and -chemotaxis are discussed in respect to ecophysiological functions and significance as chemotaxonomic markers.

Introduction

Sexual reproduction in many brown algae involves communication between gametes by means of specific signal substances, sexual pheromones, preceding fertilization. Generally these are volatile, hydrophobic C_8–C_{11}-hydrocarbons [4]. In species of the brown algal order Laminariales the pheromone secreted by freshly released eggs causes release of spermatozoids from antheridia and, subsequently, chemotaxis. Lamoxirene (**1**), the active compound common to species of the families Laminariaceae, Alariaceae, and Lessoniaceae, has been identified previously [1] and its activity has been confirmed with synthetic samples [2]. In addition to lamoxirene, eggs secrete several chemically related by-products some of which are known as sexual pheromones in other brown algae. In the present study, the composition of egg secretions in selected species of Laminariales is analyzed in detail.

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Materials and Methods

Algal material

In this study, 15 species of widespread geographic origin (Table I) were examined. Clonal gametophyte cultures were established by isolation of germlings from single meiospores or few-celled gametophyte fragments. The cultures were not axenic. Except for *Chorda tomentosa* and *Saccorhiza dermatodea*, all gametophytes are dioecious.

Culture methods

Stock cultures of vegetative gametophytes were kept at 5–8 °C in red fluorescent light (Philips TL 40 W/15, 2–4 μmol $m^{-2}s^{-1}$ photon flux density, 12 h light period). They were propagated by fragmentation with a Teflon piston in a glass homogenizer. Enriched ([3], PES) North Sea-water (salinity 28‰) was used as culture medium with change at 4-week intervals. Formation of gametangia was induced by transfer into fresh culture medium and appropriate temperature conditions (see *Pheromone extraction*) and white fluorescent light (Osram L 40 W/19, 30–60 μmol $m^{-2}s^{-1}$ photon flux density, light period 12 h, or, in extraction No. 14–18, 10 h).

Pheromone extraction

Volatile hydrophobic substances secreted by eggs were extracted using a closed-loop system and ad-

sorbed to a bed of 1.5 mg activated carbon [4, 5]. After desorption with 30 µl dichloromethane (Merck, residue analysis grade), eluates were subjected to glass-capillary gas chromatography. In *Saccorhiza dermatodea, Laminaria hyperborea, Pleurophycus gardneri, Alaria esculenta, Eisenia arborea, Undaria pinnatifida, Macrocystis pyrifera* and *Nereocystis luetkeana* gametogenesis was induced directly by transfer of vegetative gametophytes into the 2 l extraction vessel. In other species, mature gametophytes were transferred one day before beginning of egg release. It was confirmed in blank pre-run experiments that the entire extraction system was free of volatile contaminations. Usually the sampling interval was 7 days, repeated until all eggs were released.

Gas chromatography

It is necessary to analyze brown algal egg secretions under least destructive conditions by avoiding thermal stress and long analysis times. The best way to do gas chromatographic work with algal pheromones is to combine low temperature gas chromatography with hydrogen as carrier gas and the use of on-column injectors. The most appropriate gas chromatographic equipment for this purpose was a Carlo Erba gas chromatograph (Fractovap 2900) equipped with two Grob type on-column injectors and FIDs. The main advantage of this device is that ambient and sub-ambient GC oven temperatures can be achieved with peripheral cryostates. Ultra-pure hydrogen was delivered by hydrogen generators (Dosapro Milton Roy). Recording and quantification of detector signals were performed with Spectra-Physics integrators (SP 4100).

Shortly after extraction, each sample was injected onto several fused silica columns (50 m length, 0.32 mm internal diameter, 0.25 µm film thickness) coated with liquid phase of four different polarities (CP-Sil 5 or SE 30, CP-Sil 9 or SE 54, CP-Sil 19 or OV 1701, and CB-Wax or Carbowax 20M). Sample identification was established by co-injection of authentic references and, in case of the main egg products, additionally by GC-MS analysis.

Mass spectra were recorded on a Finnigan 4510 GC-MS system equipped with a fused silica capillary coated with OV 101 (40 m × 0.31 mm). Elution was programmed routinely from 50 °C (5 min isothermal) to 200 °C at 10 °C/min. The ionization potential was 70 eV and the scan range 35–600 Da/s.

Results

Generally, egg secretions contained a complex mixture of various, gamete specific components. In addition to a few major fractions, a large number of trace compounds were detected at least in preparations with high yields of egg products (Fig. 1). The most prominent components of the pheromone bouquets and some by-products were identified and determined quantitatively (Table III). Vegetative gametophytes and those with mature oogonia did not produce any interfering compounds in the examined molecular range before egg release.

From the main secretions the following three compounds have been shown previously to be common to species of the families Laminariaceae, Alariaceae, and Lessoniaceae (extraction-no. 1–11): 6-(1′,2′-*cis*-epoxi-but-3′-enyl)-cyclohepta-1,4-diene (lamoxirene, (**1**)), 6-but-1′-enyl-cyclohepta-1,4-diene (ectocarpene, (**4**)), and *n*-pentadecane (**15**) [1, 2, 6]. Lamoxirene (**1**) acts as a highly potent sexual pheromone in triggering spermatozoid release and chemotaxis in all species of the three families so far examined [2, 7]. Ectocarpene (**4**) has been found in egg secretions of all species of Laminariales. The relative content of (**4**) in pheromone extracts varies, even in preparations from the same gametophyte clone. This is also true for other components in the

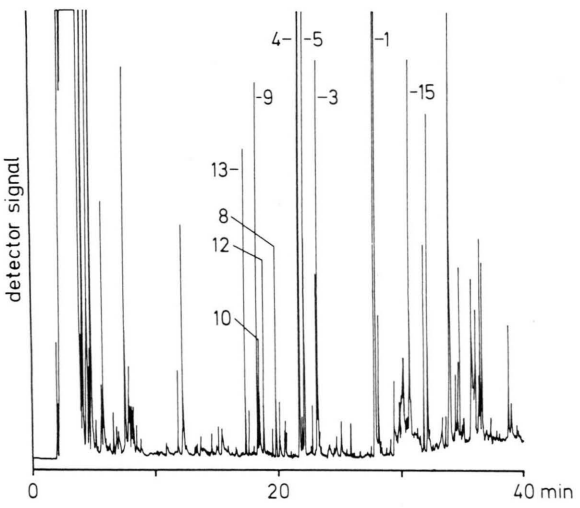
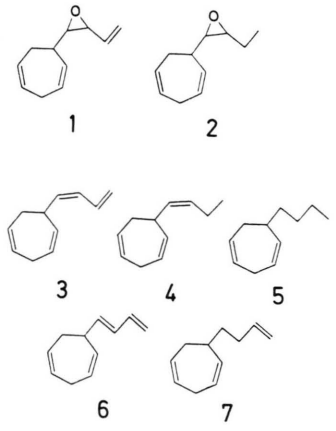


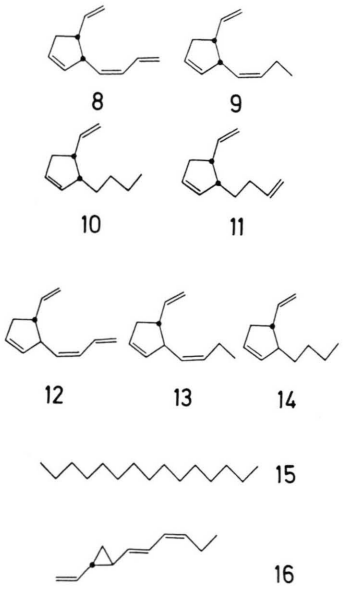
Fig. 1. Gas-chromatographic separation of a pheromone extract from *Undaria pinnatifida* eggs. On column-injection, glass capillary column 50 m × 0.32 mm, CP-Sil 19. Carrier gas: 0.6 ml min^{-1} H_2. Temperature program: 5 min at 25 °C, 5 °C min^{-1}, final temperature 220 °C.

Table I. List of species examined, taxonomic position and geographic origin.

No.		Origin
	Family: Laminariaceae	
1	*Laminaria digitata* (Huds.) Lamour.	Helgoland, North Sea
2	*L. hyperborea* (Gunn.) Fosl.	Helgoland, North Sea
3	*L. groenlandica* Rosenv.	Bamfield, B.C., Canada
4	*L. japonica* Aresch.	Wakkanai, Hokkaido
5	*Pleurophycus gardneri* Setch. et Saund.	Bamfield, B.C., Canada
	Family: Alariaceae	
6	*Alaria esculenta* (L.) Grev.	Newfoundland
7	*Undaria pinnatifida* (Harv.) Sur.	Yoichi, Hokkaido
8	*Eisenia arborea* Aresch.	Bamfield, B.C., Canada
	Family: Lessoniaceae	
9	*Macrocystis pyrifera* (L.) C. Ag.	Santa Barbara, California
10	*M. pyrifera* (L.) C. Ag.	Kaikoura, New Zealand
11	*Nereocystis luetkeana* (Mert.) P. et R.	Santa Barbara, California
	Family: Chordaceae	
12	*Chorda tomentosa* Lyngb.	Tromsö, Norway
13	...	
14	*C. filum* (L.) Lamour.	Aran Isl., Ireland
	Family: Phyllariaceae	
15	*Saccorhiza dermatodea* (de la Pyl.) J. Ag.	Newfoundland
16	...	
17	*S. polyschides* (Lightf.) Batt.	Aran Isl., Ireland
18	...	

pheromone bouquet. Often (**4**) represents the main fraction although it does not represent the sexual pheromone in the corresponding species. Ectocarpene (**4**) is identical with dictyopterene D′ in the essential oil from *Dictyopteris thalli* [8, 9]. *n*-Pentadecane is the most frequent paraffin in sporophytes of *Laminaria digitata, Macrocystis pyrifera* and other brown algae [10, 11].





In addition to lamoxirene (**1**), the more hydrogenated derivative (6-(1′,2′-*cis*-epoxi-butyl)-cyclohepta-1,4-diene (**2**)) has been identified as a minor fraction (about 120 ng in extraction No. 1) in the pheromone bouquet of *Laminaria digitata* (**1**) and (**2**) are the only oxygen containing compounds presently

Table II. Experimental conditions in pheromone extractions.

No.	Species	Fresh weight [g]	Temperature (°C ± 0.5 °C)
1	*Laminaria digitata*	4.0	9
2	*L. hyperborea*	0.7	8
3	*L. groenlandica*	1.5	5
4	*L. japonica*	1.2	12
5	*Pleurophycus gardneri*	0.5	8
6	*Alaria esculenta*	0.7	10
7	*Eisenia arborea*	0.6	12
8	*Undaria pinnatifida*	0.5	12
9	*Macrocystis pyrifera*	0.5	15
10	...	0.7	10
11	*Nereocystis luetkeana*	2.5	12
12	*Chorda tomentosa*	n.d.	2
13	...	3.2	2
14	*C. filum*	1.3	5
15	*Saccorhiza dermatodea*	n.d.	5
16	...	2.5	5
17	*S. polyschides*	1.2	12
18	...	n.d.	8

n.d.: Not determined.

known as gamete secretions in brown algae. Besides ectocarpene (**4**), 6-(1′*Z*,2′-butadienyl)-cyclohepta-1,4-diene (desmarestene (**3**)) and 6-(butyl)-cyclohepta-1,4-diene (**5**) are the quantitatively most important by-products in egg secretions of members of the families Laminariaceae, Alariaceae and Lessoniaceae. As additional cycloheptadiene-derivatives 6-(1′*E*,2′-butadienyl)-cyclohepta-1,4-diene (**6**) and 6-(but-3′-enyl)-cyclo-hepta-1,4-diene (**7**) have been found in trace quantities in *Laminaria digitata* and *Macrocystis pyrifera*, respectively.

The cyclopentene-pheromones multifidene (**9**) (*cis*-3-[1′*Z*-butenyl]4-vinyl-cyclopentene), viridiene (**8**) (*cis*-3-[1′*Z*,3′-butadienyl]4-vinyl-cyclopentene and *cis*-3-butyl-4-vinyl-cyclopentene (**10**) are common by-products in egg secretions of species of the three families mentioned above. Moreover, *trans*-viridiene (**12**) has been found frequently. Additionally, *trans*-multifidene (**13**) is present in extracts of *Laminaria japonica*, whereas 3-*trans*-(butyl)-4-vinyl-cyclopentene (**14**) and 3-*cis*-(but-3′-enyl)-4-vinyl-

Table III. Pheromone extraction yields*.

No.	Species	(**1**)	(**3**)	(**4**)	(**5**)	(**8**)	(**9**)	(**10**)	(**12**)	(**15**)	(**16**)
										n-C_{15}	
1	*Laminaria digitata*	5.6	0.5	9.7	0.9	+	0.1	0.1			
2	*L. hyperborea*	0.3	+	0.1						1.1	
3	*L. groenlandica*	0.2		0.6	0.2					5.2	
4	*L. japonica*	0.5	+	0.7	+	+	+		0.1	0.5	
5	*Pleurophycus gardneri*	1.2		0.1							
6	*Alaria esculenta*	2.8	0.6	1.1	0.2	0.1			0.2	3.7	
7	*Eisenia arborea*	0.6	0.2	0.5	0.1	0.1	+		0.2		
8	*Undaria pinnatifida*	6.1	0.8	19.5	2.6	0.3	0.9	0.8	0.3	0.2	
9	*Macrocystis pyrifera*	2.9	0.6	0.7	0.2	0.1	+	+	0.1	0.2	+
10	...	2.9	0.3	7.3	0.7	0.1	0.5	+	0.1	1.5	
11	*Nereocystis luetkeana*	0.1		0.1							
12	*Chorda tomentosa*			12.2	4.6		90.6	4.1		1.3	1.6
13	...			23.4	5.8		206.5	6.1		2.0	3.8
15	*Saccorhiza dermatodea*			0.6	+					+	+
16	...			3.8							
17	*S. polyschides*			3.7							
18	...			2.7	+						+

(*): In micrograms as *n*-undecane, ± 15%.
(+): Traces below 50 ng.

cyclopentene (**11**) have been identified as traces in *Macrocystis pyrifera*. Hormosirene (**16**) {*trans*-1-(1′*E*,3*Z*-hexadienyl)-2-vinylcyclopropane} has also been found in minor amounts in secretions of *Macrocystis pyrifera*. The compounds **2**, **6**, **7**, **11**, **12**, **13**, and **14** have not been reported before as natural products [4, 23, 24].

In *Chorda tomentosa* (Chordaceae), eggs secrete multifidene (**9**) as the main product. (+)-multifidene (**9**) {(+)-(3*S*,4*S*)-3-(1′*Z*-butenyl)-4-vinyl-cyclopentene [12]} has been identified as the specific sexual pheromone. It triggers spermatozoid release and is probably also involved in chemotaxis [13]. Ectocarpene (**4**), 6-butyl-cyclohepta-1,4-diene (**5**), *cis*-3-(butyl)-4-vinyl-cyclopentene (**10**), hormosirene (**16**) and *n*-pentadecane have been found as by-products (Table III). Oxygen-containing as well as highly unsaturated compounds like desmarestene (**3**) or viridiene (**8**) are lacking. Attempts to identify the substances secreted by eggs of *Chorda filum* have been unsuccessful as yet. In this species, gas chromatograms of egg secretions are very different from those of any other member of Laminariales hitherto investigated, including *C. tomentosa*.

In two species of the family Phyllariaceae, *Saccorhiza polyschides* and *S. dermatodea*, ectocarpene (**4**) is the only prominent egg product. In addition, traces of 6-butylcyclohepta-1,4-diene (**5**), hormosirene (**16**), and *n*-pentadecane have been found. The function of ectocarpene (**4**) as the specific sexual pheromone in *Saccorhiza* sp. could not be confirmed by bio-assays. In *S. polyschides*, both, (+)- and (−)-ectocarpene (**4**) as well as 6-butyl-cyclo-hepta-1,4-diene (**5**) do not induce spermatozoid release at 1×10^{-7} M, the highest concentration which could be tested. Hormosirene (**16**), however, triggers release at a threshold concentration of 1×10^{-8} M, but this threshold is certainly too high to be attributed to a genuine brown algal pheromone. Lamoxirene (**1**) and multifidene (**9**) are not active.

Re-extraction experiments with known quantities (1×10^{-7} mol in 2 l aqueous solution) of synthetic pheromones introduced into experimental set-ups identical to those used for extraction of algal secretions, gave information on the efficiency for the extraction method. It was surprisingly low. The recovery rates for desmarestene (**3**) and ectocarpene (**4**) were determined to about 5%, those for multifidene (**9**) and *n*-undecane to less than 2% and that for lamoxirene (**1**) to about 1%. These values illustrate the experimental limitations inherent in the extraction method used here, probably mainly due to adsorption of the pheromones to the glass surface of the relatively large extraction vessel. Nevertheless, the closed-loop technique has worked very successful for the past ten years [4]. With smaller volumes, the recovery rates are considerably higher. The relative composition of pheromone extracts depends to a certain extent on differing extraction efficiencies for the various compounds. When corrected for this effect, lamoxirene (**1**) is the main egg product in all species of the families Laminariaceae, Alariaceae, and Lessoniaceae (extraction No. 1−11), followed by ectocarpene (**4**).

In *Laminaria japonica* (No. 4), *Alaria esculenta* (No. 6), *Undaria pinnatifida* (No. 8) and *Macrocystis pyrifera* (No. 9, 10), the number of eggs released within the extraction period have been estimated as 3×10^6, 7×10^6, 1×10^7, 5×10^6 and 5×10^6, respectively. The amount of lamoxirene (**1**), the specific sexual pheromone, secreted by one egg in these species can be calculated to about $2-6 \times 10^{-11}$ g on average. In addition, an equal quantity of by-products may be produced. In *Chorda tomentosa* (No. 12, 13) eggs probably secrete at least 10 times as much multifidene (**9**).

Discussion

The comparative analysis of egg secretions in Laminariales (Table III) reveals some distinct groupings of taxa within this order. Pheromone extracts in species of the families Laminariaceae, Alariaceae, and Lessoniaceae, show practically identical bouquets of lamoxirene (**1**) and by-products. The pheromonal system of members of these three families representing the major group of the order Laminariales appears to be indistinguishable, despite widespread geographic origin (Table I). The presence and specific activity of lamoxirene (**1**) as the spermatozoid-releasing and -attracting pheromone in all these species has been demonstrated previously [2]. Five additional species from Japan show pheromonal cross-reactions [14].

Hence, sexual pheromone specificity and the composition of the pheromone bouquet have remained unchanged since phyletic divergence of these taxa. The only useful fossil record which can be assigned to Laminariales, *Julescraneia grandicornis* [15], dates back to the late miocene or early pleiocene 5−10

million years ago. This extinct species presents good evidence that the family Lessoniaceae was already represented by morphologically highly differentiated plants at that time. This time span also marks the minimum age of the pheromone system common to the order. However, the present distribution of, for example, the genus *Lessonia* in the Southern hemisphere might perhaps date back to the final break-up of Gondwana with separation of Australia and Antarctica at the eocene-oligocene boundary about 40 million years ago. At that time, due to cooling of the earth climate (the species of Laminariales are restricted to cold-temperate waters), transequatorial migration of the Laminariales might also have been possible, accompanied by radiation of the order. The essential role of sexual reproduction in the life history perhaps combined with lacking evolutionary pressure towards changes in a very sensitive and successful signaling mechanism might account for the conservative character of the pheromone system in Laminariales.

According to present conceptions, brown algal pheromones are synthesized by a common route and biosynthesis starts from more or less unsaturated fatty acids [4, 16]. The aliphatic terminus of the fatty acid precursor is preserved in the C_4-substituent of the cycloheptadiene- or cyclopentene-pheromones. Deviations in the principal ring structures have never been found in brown algal gamete secretions. Each pair of compounds with a corresponding pattern of double-bonds, for example, ectocarpene (**4**)/multifidene (**9**) and desmarestene (**3**)/viridiene (**8**) originates from the same precursor, dodeca-3,6,9-trienoic acid and dodeca-3,6,9,11-tetraenoic acid, respectively. The composition of the pheromone bouquet depends on the specificity of the enzymes involved in biosynthesis and, probably, to a considerable extent on the nature of available fatty acids. For that reason eggs might secrete not only the species-specific pheromone but a complex mixture of compounds. The relative composition of the pheromone bouquet may even vary within extracts from one clone (Table III, extraction No. 9, 10, 15, 16) perhaps reflecting different physiological states of the gametophytes.

However, another explanation could be that pheromone mixtures or single compounds have as yet unknown biological functions which might explain why nonspecificity in biosynthesis has not been eliminated during evolution. Possible functions might include, for example, cooperativity in pheromone perception, or physiological responses in spermatozoids other than release and chemotaxis.

Furthermore, components of pheromone bouquets might, by interaction with pheromone receptors of other species, act as allomones in interspecific competition. Many of them are known as sexual pheromones in brown algae belonging to other orders. Ectocarpene (**4**) is the specific male attractant in *Ectocarpus siliculosus, E. fasciculatus* (Ectocarpales), *Sphacelaria rigidula* (Sphacelariales) and *Adenocystis utricularis* (Dictyosiphonales) and has been also found as a by-product in gamete secretions of *Cutleria multifida* (Cutleriales), *Desmarestia aculeata, D. viridis* (Desmarestiales) and *Cladostephus spongiosus* (Sphacelariales). It can easily be demonstrated that male gametes of *Ectocarpus siliculosus* and *Desmarestia aculeata* are attracted by eggs of *Laminaria digitata*. Desmarestene (**3**) has been identified as signal substance in *Desmarestia aculeata* and *D. viridis*, as well as in *Cladostephus spongiosus*. In *Dictyota dichotoma* (Dictyotales), 1-butyl-2,5-cycloheptadiene (**5**) is the active compound. Multifidene (**9**) has been found as the sexual pheromone in *Cutleria multifida* and viridiene (**8**) in *Syringoderma phinneyi* (Syringodermatales). In *Hormosira banksii, Xiphophora chondrophylla, X. gladiata* (Fucales), *Durvillaea potatorum, D. antarctica, D. willana* (Durvillaeales), *Scytosiphon lomentaria* and *Colpomenia peregrina* (Scytosiphonales), the male attractant has been identified as hormosirene (**16**) [4, 17].

It is significant that eggs of *Chorda tomentosa* (Chordaceae) and *Saccorhiza* spp. (Phyllariaceae) do not secrete the highly unsaturated compounds desmarestene and viridiene or oxygen-containing compounds like lamoxirene. However, it has always to be considered that, due to the experimental limitations in the extraction method used in this study, micro-traces as well as hydrophilic, non-volatile substances in gamete secretions could not be detected. In respect of their egg secretions, *Chorda filum* is very clearly distinct from *C. tomentosa*, substantiating doubts on a phylogenetic relationship of these species. Likewise, the family Phyllariaceae has an isolated position within the order Laminariales [18]. Thus, the composition of the pheromone bouquet may be important as a chemotaxonomic character at the family- or order-level [4, 7]. The pheromone bouquet in *Desmarestia* (Desmarestiales) [19, 20] differs from *Laminaria* essentially in the absence of

lamoxirene. The molecular topologies of lamoxirene (**1**) and desmarestene (**3**) are very similar. The sum formula of the sexual pheromone of *Perithalia caudata* (Sporochnales) is identical with lamoxirene (**1**), $C_{11}H_{14}O$ [21]. Although probably closely related, the chemical structure is different. These findings further strengthen the assumption of a close phylogenetic relationship between the three orders, Laminariales, Desmarestiales, and Sporochnales which is also indicated by striking similarities in other aspects of sexual reproduction [22].

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