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Attraction Pheromone of The Benthic Diatom *Seminavis robusta*: Studies on Structure-Activity Relationships

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Abstract

Recently the first pheromone of a marine diatom was identified to be the diketopiperazine (*S*,*S*)-diproline. This compound facilitates attraction between mating partners in the benthic diatom *Seminavis robusta*. Interestingly, sexualized *S. robusta* cells are attracted to both the natural pheromone (*S*,*S*)-diproline as well as to its enantiomer (*R*,*R*)-diproline. Usually stereospecificity is a prerequisite for successful substrate-receptor interactions, and especially pheromone perception is often highly enantioselective. Here we introduce a structure-activity relationship study, to learn more about the principles of pheromone reception in diatoms. We analyzed the activity of nine different diketopiperazines in attraction and interference assays. The pheromone diproline itself, as well as a pipecolic acid derived diketopiperazine with two expanded aliphatic ring systems, showed the highest attractivity. Hydroxylatoin of the aliphatic rings abolished any bioactivity. Diketopiperazines derived from acyclic amino acids were not attrative as well. All stereoisomers of both the diproline and the pipecolic acid derived diketopiperazine were purified by enantioselective high-performance liquid chromatography, and application in bioactivity tests confirmed that attraction pheromone perception in this diatom is indeed not stereospecific. However, the lack of activity of diketopiperazines derived from acyclic amino acids suggests a specificity that prevents misguidance to sources of other naturally occurring diketopiperazines.

Keywords Structure-activity relationship · Pheromone · Enantiomers · Diatoms · Diketopiperazine

Introduction

Diatoms are unicellular algae and are considered major primary producers in many aquatic environments (Field et al. 1998). A characteristic of diatoms is their biomineralized cell wall formed out of silicate. During vegetative cell division, a new cell wall is synthesized within the constraints of the parent cell. In many diatoms this leads to a decrease of the average cell size within a population during vegetative growth (Chepurnov et al. 2004). When a certain sexual size threshold is reached, cells can engage in sexual reproduction to restore their initial cell size and avoid death by extreme miniaturization (Chepurnov et al. 2004; Frenkel et al. 2014a). While centric diatoms release gametes for mating under the influence of environmental cues (Chepurnov et al. 2004), pheromones are involved in mate finding in different pennate species (Basu et al. 2017; Frenkel et al. 2014a; Sato et al. 2011). Recently we reported the first diatom pheromones from the model species *Seminavis robusta* (Gillard et al. 2013; Moeys et al. 2016). In this diatom, sex-inducing pheromones (SIPs) produced by both mating types (MT^+ and MT^-) induce a cell cycle arrest in the opposite sex. In addition, SIPs induce the production and the perception capabilities of the attraction pheromone, the L-proline derived diketopiperazine (*S,S*)-diproline **1**, (Fig. 1). This pheromone is produced by MT^- cells in response to SIP produced by MT^+ cells (SIP^+) and mediates chemoattraction of the latter in a simultaneous chemotactic and chemokinetic movement (Bondoc et al. 2016; Moeys

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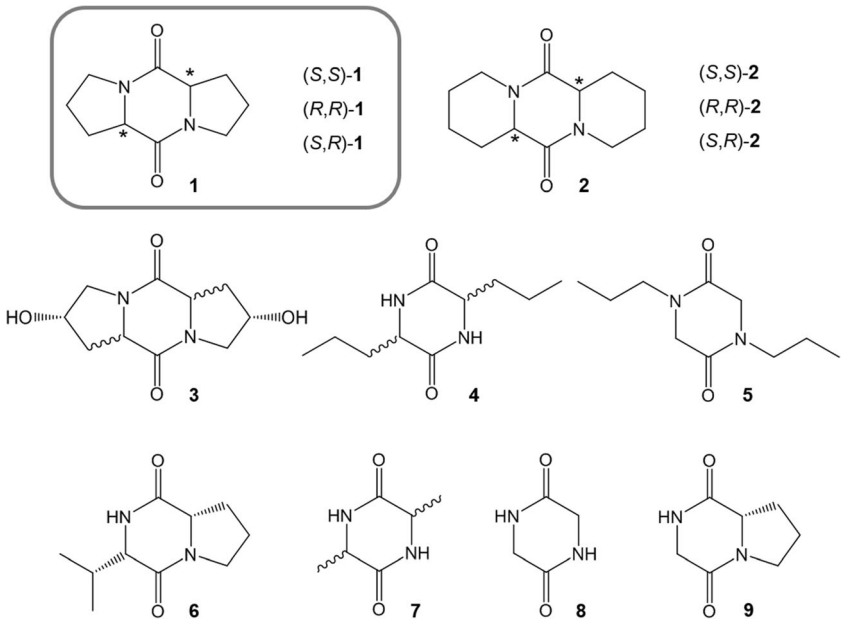

Fig. 1 Compounds tested in this study. The natural pheromone (*S*,*S*)-**1** (gray box) was synthesized stereospecifically, as well as with undefined stereochemical composition (**1**). Stereoisomers of **1** and **2** were separated by chiral HPLC

et al. 2016). Upon pairing, cells produce gametes, which fuse to zygotes, and subsequently auxosporulation restores the initial cell size (Chepurnov et al. 2008, 2002).

Surprisingly, during structure elucidation of the pheromone it became evident that also (*R*,*R*)-diproline, the enantiomer of the natural product was bioactive in concentrations similar to the stereoisomeric natural pheromone (Gillard et al. 2013). Such unspecificity in pheromone perception is only reported in rare cases since chirality is usually crucial for pheromone-receptor interactions. In insects, where pheromone chemistry is intensively studied, the activity of only one pheromone enantiomer is by far most prevalent (Mori 2007). Only in few cases enantiomers show activity without inhibitory effects, but mostly the enantiomer of the natural product is less active (Mori 2007; Pierce et al. 1991). In the case of algal pheromones, in general little is known about their structure - activity relations and about reception processes (Frenkel et al. 2014a; Pohnert and Boland 2002). No information at all is available about diatom pheromone reception and receptors. We report here a first study on structure-activity relationships to analyze fundamental aspects of the specificity of attraction pheromone perception in the benthic diatom *S. robusta*. We approach this task with two objectives: firstly to elucidate the role of the stereocenters in the active metabolites and secondly, to test if other, potentially naturally occurring diketopiperazines might interfere with pheromone perception. Therefore, we systematically evaluated the stereospecificity of pheromone reception after the development of a stereoselective purification method by HPLC and tested thirteen diketopiperazines, including stereoisomers, in attraction and interference assays. Our findings demonstrate the unspecificity of pheromone perception for stereoisomers of the natural pheromone and indicate the requirement of a piperazine-2,5-dione nucleus with two adjacent unsubstituted rings for activity.

Methods and Materials

Chemical Syntheses

Thirteen compounds, including the natural pheromone, (*S*,*S*)-diproline ((*S*,*S*)-**1**), were tested in this study (Fig. 1). Compounds **1-5** were synthesized by us, while compounds **6-9** were obtained from Bachem AG (Bubendorf, Switzerland, **6** & **9**), Sigma Aldrich Chemie AG (Steinheim, Germany, **7** as a mixture of all stereoisomers), and Acros Organics (Geel, Belgium, **8**). Structure confirmation was performed by ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopy on a 400 MHz NMR spectrometer (AVANCE III, Bruker) and by high-resolution mass spectrometry on a UHPLC system (Dionex UltiMate® 3000, Thermo Fisher Scientific, Dreieich, Germany) coupled to ESI-Orbitrap MS (Q-Exactive Plus, Thermo Fisher Scientific, Dreieich, Germany). NMR Signals were calibrated relative to the solvent residual signal ($CDCl_3$ or DMSO).

Synthesis of octahydrodipyrrolo[1,2-a:1′,2′-d]pyrazine-5,10-dione (diproline)(1) and (S,S)-1 Diproline of unknown stereochemical composition (**1**) or (*S*,*S*)-diproline ((*S*,*S*)-**1**) were synthesized from 4 g racemic proline (Alpha Aesar, Karlsruhe, Germany) or 10 g (*S*)-proline methyl ester hydrochloride (Apollo Scientific, Manchester, United Kingdom) respectively, according to Nakamura et al. (2006). NMR spectroscopy confirmed the chemical identity as compared to the

literature (Nakamura et al. 2006; Jainta et al. 2008). Detailed NMR data for (*S*,*S*)-1 and (*S*,*R*)-1 can be found in Table S1. HR-MS: calculated exact mass for $C_{10}H_{14}N_2O_2$ $(M+H)^+$: 195.11280, found: 195.11278 (**1**), 195.11253 ((*S*,*S*)-**1**).

Synthesis of decahydrodipyrido[1,2-a:1′,2′-d]pyrazine-6,12-dione (2) and 3,6-dipropylpiperazine-2,5-dione (4) Synthesis of both analogs was modified from Rappath (2005). For the synthesis of **2** racemic pipecolic acid (1 g, abcr GmbH, Karlsruhe, Germany) was dissolved in ethylene glycol under an atmosphere of argon and heated under reflux at 170 °C for 6 h. After cooling to 130 °C, water was added, and the reaction was stirred at RT overnight. The product was extracted with dichloromethane. After evaporation, the resulting crude product precipitated within 2 days. It was filtered and recrystallized in ethanol to give **2** as a white solid with predominatedly (*S*,*R*)-2 (Fig. 4e). Structure confirmation by NMR spectroscopy in $CDCl_3$ was performed. Detailed NMR data for all stereoisomers of **2** are compiled in Table S2, and NMR spectra are shown in Fig. S1-S3. HRMS: calculated exact mass for $C_{12}H_{18}N_2O_2$ $(M+H)^+$: 223.14410, measured: 223.14386.

For the synthesis of **4,** (*S*)-norvaline (500 mg, Alpha Aesar, Karlsruhe, Germany) was used as educt and treated as described above. **4** was obtained as white powder after filtration and recrystallization in ethanol. The structure was confirmed by NMR spectroscopy (Fig. S4): ^{1}H-NMR (DMSO, 400 MHz): δ [ppm] 8.08 (2H, s, NH), 3.79 (2H, s, CH), 1.57–1.71 (4H, m, CH_2), 1.24–1.36 (4H, m, CH_2), 0.84–0.88 (6H, t, $^3J_{H\text{-}H}$ = 7.6 Hz, CH_3); ^{13}C-NMR (DMSO, 100 MHz): δ [ppm] 168.0, 53.6, 34.7, 17.2, 13.8; HRMS: calculated exact mass for $C_{10}H_{18}N_2O_2$ $(M+H)^+$: 199.14410, found: 199.14403.

Synthesis of 2,7-dihydroxyoctahydro-5H,10H-dipyrrolo[1,2-a:1′,2′-d]pyrazine-5,10-dione (3) The synthesis was performed as described by Nonappa et al. (2011) in a microwave (Biotage Initiator Classic, Biotage, Uppsala, Sweden) reaction with (2*S*,4*R*)-4-hydroxypyrrolidine-2-carboxylic acid (560 mg, Sigma Aldrich, Steinheim, Germany) as educt. The structure was confirmed by ^{1}H NMR spectroscopy as compared to the literature (Nonappa et al. 2011). HRMS: calculated exact mass for $C_{10}H_{14}N_2O_4$ $(M+H)^+$: 227.10263, measured: 227.10248.

Synthesis of 1,4-dipropylpiperazine-2,5-dione (5) The synthesis of **5** was carried out according to Dubey et al. (2009) with 1-propyl iodide (Acros Organics, Geel, Belgium) as starting material. The reaction product was purified by column chromatography on silica-gel (Geduran Si 60, Merck, Darmstadt, Germany), using CH_2Cl_2:acetone 1:1 (v:v) (Rf = 0.58). NMR spectroscopy of the product dissolved in $CDCl_3$ confirmed the structural identity as compared to the literature (Emery et al. 2016; Zhou et al. 2014). HRMS: calculated exact mass for $C_{10}H_{18}N_2O_2$ $(M+H)^+$: 199.14410, measured: 199.14407.

Stereoselective Purification of 1 and 2

All stereoisomers of **1** and **2** were purified by enantioselective column chromatography for further bioactivity tests. Method development was carried out on a UHPLC system with a DAD detector (Dionex UltiMate® 3000, Thermo Fisher Scientific, Dreieich, Germany) coupled to ESI-Orbitrap MS (Q-Exactive Plus, Thermo Fisher Scientific, Dreieich, Germany). 1 µl of the mixture of all stereoisomers of **1** (1 mg ml^{-1}) was injected and separated on a LUX 5u Amylose-1 column (100 mm × 4.6 mm, Phenomenex, Torrance, CA, USA) under isocratic conditions using an eluent containing 70% MeOH, 30% deionized water, and 0.1% formic acid. The flow was increased from 0.1 ml min^{-1} to 1 ml min^{-1} within 10 min in a steady gradient and held at 1 ml min^{-1} for additional 5 min. Synthetic (*S*,*S*)-**1** and (*R*,*R*)-**1** were used to verify the retention times of the stereoisomers. The same method was applied to separate a mixture of all stereoisomers of **2** (1 mg ml^{-1}) with 100% MeOH containing 0.1% formic acid as optimal eluent. Purification of all isomers of both compounds was performed on an HPLC system with an MWD detector (Agilent 1100 Series, Agilent, Waldbronn, Germany) and a fraction collector (Agilent 1260 Infinity, Agilent, Waldbronn, Germany) using the developed methods and the same column.

Strains, Culture Conditions & Microscopy

Monoclonal cultures of *S. robusta* MT^+ strains (85A, PONTON36) and MT^- strains (84A, VM3–15), which are maintained at the Belgian Coordinated Collection of Micro-Organisms at Ghent University (http://bccm.belspo.be/about-us/bccm-dcg), were used and experiments were carried out in Ghent (Belgium) and Jena (Germany). Cultures were grown in either natural seawater from the North Sea (Ghent) or 33 g l^{-1} Instant Ocean® Sea Salt (Aquarium Systems, Sarrebourg, France) dissolved in ultra-pure water (Jena), both supplemented with f/2 nutrients according to Guillard (1975), in a 12:12 hr light:dark regime with cool-white fluorescent lamps at approximately 35 µmol photons m^{-2} s^{-1} at 18 °C. Stock cultures were grown in tissue culture flasks or 6-well plates (Sarstedt, Nümbrecht, Germany) for 5 to 7 days until they reached early stationary phase. For bioactivity tests 500 µl or 50 µl of MT^+ cell suspensions from these cultures were inoculated into 24- or 96-well plates respectively (Sarstedt, Nümbrecht, Germany), and 1 ml or 100 µl of fresh medium were added. Cells were either counted or pulse-amplitude modulated minimal fluorescence (F_0) was used as a proxy for biomass to obtain comparable cell numbers for each experiment (Honeywill et al. 2002).

For assessment of culture growth and during bioactivity tests, microscopy was performed using an inverted Leica DM IL LED microscope (Leica, Heerbrugg, Switzerland) mounted with a Nikon DS-Fi2 CCD camera (Nikon, Tokyo, Japan).

Bead Preparation and LC-MS Analysis of Loading Efficacy

Preparation of compound loaded beads was modified after Gillard et al. (2013). All compounds were either dissolved in water (compounds **1**-**3**, **5**-**9**) or MeOH (compound **4**) in stock solutions of 1 mg ml^{-1}. For SPE bead preparation aliquots for all analog concentrations were prepared and diluted in water in a total volume of 1 ml each. For LC-MS analysis 50 µl aliquots of these loading solutions were stored at −20 °C until measurement. The wells of the Oasis® HLB 96-well µ-elution plate (Waters, Eschborn, Germany, 2 mg sorbent per well) were washed and equilibrated with 200 µl MeOH and 200 µl water before loading solutions were applied. Afterwards the sorbent was washed with 50 µl water per well. The flow through of loading and washing was collected separately and stored at −20 °C until LC-MS analysis. The wells were opened with a needle, and SPE beads were flushed out of the wells with 400 µl water, resulting in a bead suspension of 5 mg/ml. Beads suspensions were stored at 4 °C until further usage.

To evaluate the binding efficacy of the compounds on the SPE material, LC-MS analysis of the loading solution, loading flow through, and wash flow through for each analog in a theoretical concentration of 20 nmol per mg beads was performed. Therefore, 2 µl of each solution was injected into a UHPLC system (Dionex UltiMate® 3000, Thermo Fisher Scientific, Dreieich, Germany) coupled to ESI-Orbitrap MS (Q-Exactive Plus, Thermo Fisher Scientific, Dreieich, Germany). Separation was performed on a Kinetex® C18 column (2.1 × 50 mm, 1.7 µm, Phenomenex, Aschaffenburg, Germany). The solvent composition was held at 100% A (0.1% formic acid, 2% acetonitrile in water) for 0.2 min, changing in a linear gradient within 7.8 min to 100% B (0.1% formic acid in acetonitrile), held at 100% B for 1 min before returning to 100% A in 0.1 min, and held at 100% A for 0.9 min. A constant flow rate of 0.4 ml min^{-1} was used. For ionization, a spray voltage of 3.3 kV and a capillary temperature of 360 °C were applied. Gas flows were set at 60 arbitrary units for sheath gas, 20 arbitrary units for auxiliary gas, and 5 arbitrary units for sweep gas. The measurement was done in positive ionization mode in a mass range between *m/z* 50 and 750 at 35000 resolution (FWHM). To assess the binding efficacy, peaks were integrated from the chromatographic profiles and compared between samples.

Bioactivity Tests

Bioactivity tests were modified from Gillard et al. (2013). Therefore, freshly inoculated MT^+ cultures in 24- or 96-well plates were dark-synchronized for 36 hrs (Gillard et al. 2008). MT^+ cells were induced by addition of one-third of sample volume of sterile-filtered MT^- medium just prior to illumination (approx. 35 µmol photons m^{-2} s^{-1}). Attraction and interference assays were carried out between 6 and 8 hr after illumination.

Attraction Assays Compound-loaded beads with a binding efficacy of more than 62%, which is simlar to the binding efficacy of the natural pheromone, were added to each well (5 µg per well in 24-well plates, 1 µg per well in 96-well plates). Three pictures of each well were taken 12 min after bead addition, and the number of cells at the bead surface was counted. The attraction was monitored in three or four wells per compound concentration per experiment.

Interference Assays Bioactivity of all compounds that were inactive in the attraction assay or could not be applied due to improper loading on the beads, was tested in an interference assay in 96-well plates. Therefore, dilutions of all compounds in deionized water were prepared from stock solutions (1 mg ml^{-1} as described above), 10 µl of each dilution were applied to each three wells, resulting in concentrations of 0.01 µM, 0.1 µM, 1 µM, 10 µM, and 100 µM concentrations of the tested compounds per well. After 12 min 1 µg of beads, loaded with the natural pheromone (*S*,*S*)-**1** (20 nmol per mg beads), were applied, and after additional 12 min the attraction towards the beads was determined as in the attraction assay.

Statistical Analysis and Modeling

All statistical analysis was carried out using SigmaPlot (Sysstat Software GmbH, Erkrath, Germany) and data are given as mean ± standard error of the mean (SEM). The cell counts at beads for both assay types were summarized per well and three or four wells were tested in each experiment per compound concentration. Data were analyzed by One-Way ANOVA within each concentration level and a subsequent Holm-Sidak post-hoc test was applied for multiple comparisons against the control treatments. For structural comparisons of stereoisomers of **1** and **2** 3D models were prepared in Chem3D (ChemOffice Professional, Perkin Elmer).

Results

Bioactivity of Diproline Analogs

In a first approach, we analyzed the specificity of pheromone reception using a series of synthetic diketopiperazines in attraction assays (Fig. 2). The accumulation of induced MT^+ cells around compound-loaded SPE beads was recorded 12 min after adding the beads (Fig. 2b). Since not all compounds could be applied due to insufficient binding to the Oasis® HLB beads (Table 1), only test compounds with a binding efficacy similar or higher to that of the natural

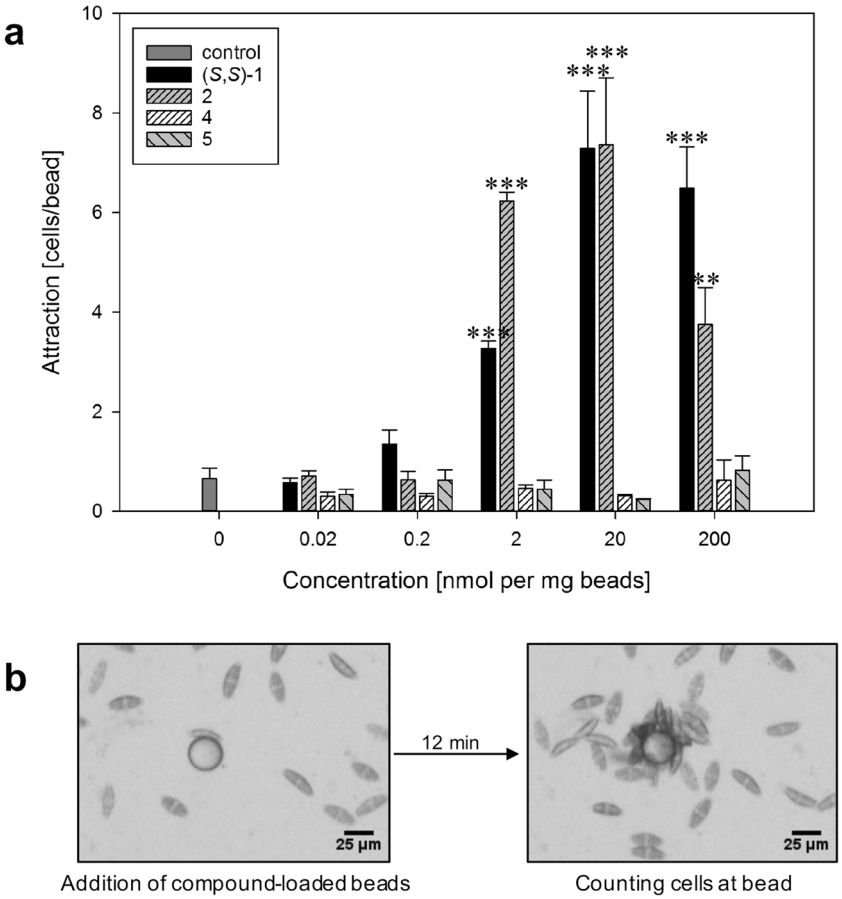

Fig. 2 Attraction of *S. robusta* towards compound-loaded beads. To induced cells beads loaded with the pheromone (*S*,*S*)-**1** or **2**, **4** and **5** were applied and attraction was monitored after 12 min by counting the number of attached cells per bead (**a**). Statistical analysis was performed by One-Way ANOVA of each concentration level followed by Holm-Sidak post-hoc test with multiple comparisons against the control treatment (n = 3, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The principle of the attraction assay is shown in (**b**)

pheromone (*S*,*S*)-**1** were tested in attraction assays (Fig. 2a). Cells were significantly attracted towards beads loaded with compound **2** in a concentration range between 2 and 200 nmol per mg beads ($P < 0.001$ for 2 and 20 nmol per mg beads; $P = 0.008$ for 200 nmol per mg beads) in comparison to unloaded beads (control treatment). This activity was similar to that of the natural pheromone (*S*,*S*)-**1** in the same concentration range ($P < 0.001$ for 2 to 200 nmol per mg beads). Compounds **4** or **5** did not cause an increased accumulation on the beads compared to the control over the entire concentration range. Also for compound **6,** no attraction was observed (Fig. S5).

To assess also the compounds that do not bind to the polymer beads used in the bioassay and to further characterize the inactive compounds, we developed an

Table 1 Binding efficacy of compounds towards SPE beads. All compounds were loaded onto SPE beads in a theoretical concentration of 20 nmol per mg beads. Before and after loading as well as after washing LC-MS of the loading and washing flow through was performed to calculate the amount of bound compound and assess the potential usage in attraction assays

Compound	m/z $[M+H]^+$	% load	% flow through load	% flow through wash	% on bead
(*S*,*S*)-**1**	195.11	100	31.4	1.5	67.1
(*R*,*R*)-**1**	195.11	100	26.8	1.5	71.7
(*S*,*R*)-**1**	195.11	100	35.7	1.5	62.8
(*S*,*S*)-**2**	223.14	100	0	0	100
(*R*,*R*)-**2**	223.14	100	0	0	100
(*S*,*R*)-**2**	223.14	100	1.7	0	98.3
3	227.10	100	105.6	4.7	0
4	199.14	100	6.3	0.2	93.5
5	199.14	100	4.6	0.1	95.3
6	211.14	100	1.3	0	98.7
7	143.08	100	96.1	4.5	0
8	115.05	100	75.4	2.5	22.1
9	155.08	100	86.5	4.8	8.7

interference assay (Fig. 3). By application of dissolved test substrates before addition of beads loaded with 20 nmol per mg beads (*S*,*S*)-**1**, the interference of pheromone perception by these compounds could be examined. In positive controls, high amounts of dissolved (*S*,*S*)-**1,** added before the introduction of pheromone loaded beads, significantly decreased cell attraction at concentrations of 10 μM ($P = 0.012$) and 100 μM ($P = 0.002$). The dissolved pheromone, thus, dominates over the gradient caused by pheromone loaded beads and abolishes an attractive response. In this assay, only compound **4** decreased cell attraction significantly at an elevated 100 μM concentration ($P = 0.012$) without exhibiting toxic effects (verified by microscopic observation). The other compounds did not show any activity and do not interfere with pheromone perception. For further analysis, we focused on the stereochemistry of the bioactive compounds **1** and **2**.

Separation of Stereoisomers

Since both (*S*,*S*)- and (*R*,*R*)-diproline were bioactive in a previous study (Gillard et al. 2013), we aimed at analyzing whether also (*S*,*R*)-diproline as well as the corresponding stereoisomers of **2** could function as attractants. We developed a purification protocol by enantioselective column chromatography on an HPLC-system. We were able to separate the compounds on a LUX Amylose-1 column (Phenomenex, USA) using an isocratic solvent composition (70% MeOH, 30% deionized water and 0.1% formic acid) with a flow gradient. Separation was achieved for all stereoisomers of both compounds **1** and **2** (Fig. 4a, e), and purification was performed so that enantiopure compounds (Fig. 4b-d, f-h) were obtained, which could be used for further testing. Only (*R*,*R*)-diproline still contained ~ 3% (*S*,*S*)-diproline.

Bioactivity of Stereoisomers

The activity of purified stereoisomers of **1** and **2** was evaluated in bead attraction assays (Fig. 5a). All stereoisomers were bioactive and attracted the cells significantly compared to control beads at concentrations of 20 nmol per mg beads ($P = 0.016$ for (*S*,*S*)-**1**, $P = 0.018$ for (*R*,*R*)-**2**, $P < 0.001$ for (*S*,*R*)-**2**) and 200 nmol per mg beads ($P < 0.001$ for (*S*,*S*)-**1**, (*S*,*S*)-**2** and (*R*,*R*)-**2**, $P = 0.026$ for (*R*,*R*)-**1** and (*S*,*R*)-**1**, $P = 0.043$ for (*S*,*R*)-**2**). It has to be noted that the attraction efficacy varies in-between assays (compare Fig. 2), which could be due to the different cell size or nutrient availability during the assays (Chepurnov et al. 2002). The 3D structures highlight the planar character of the diketopiperazine element in all six active compounds, while the flanking ring structures are more flexible (Fig. 5b, c).

Discussion

In the benthic diatom *Seminavis robusta* an unspecific perception of the attraction pheromone (*S*,*S*)-diproline as well as for its (*R*,*R*)-enantiomer has been reported (Gillard et al. 2013). However, the putative diproline receptor and its binding capabilities remained poorly characterized. To obtain further insights into diproline sensing we performed a series of structure-activity analyses with nine diketopiperazines (Fig. 1). For the assays,

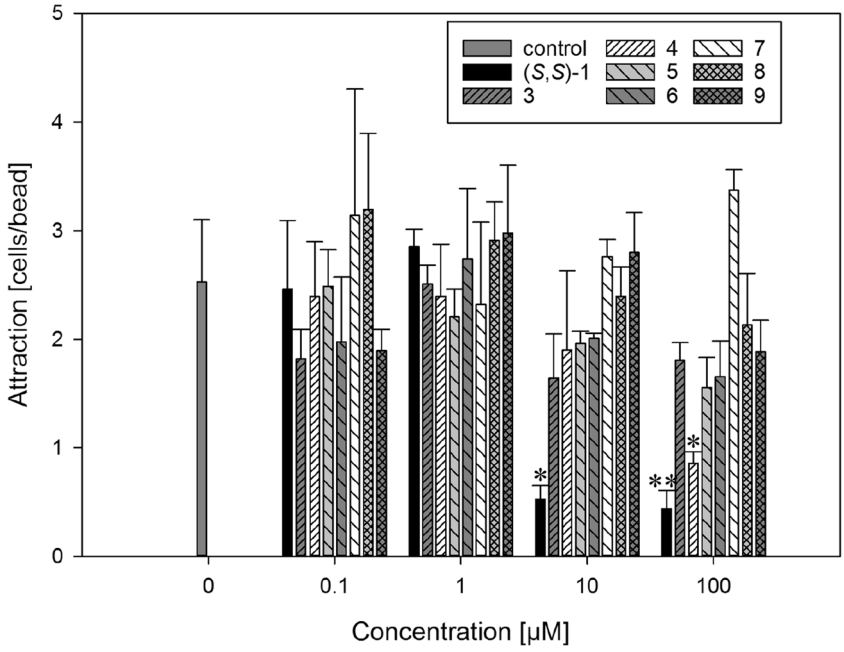

Fig. 3 Interference of pheromone perception. Compounds were added in solution to induced cells 12 min before beads loaded with 20 nmol (*S*,*S*)-**1** per mg beads were applied. Attraction towards the beads was monitored and cells at beads were counted after 12 min ($n = 3$). Statistical analysis was performed by One-Way ANOVA of each concentration followed by Holm-Sidak post-hoc test with multiple comparisons against the control treatment (* $P < 0.05$, ** $P < 0.01$). Only compounds that could not be tested or were inactive in the attraction assay are included

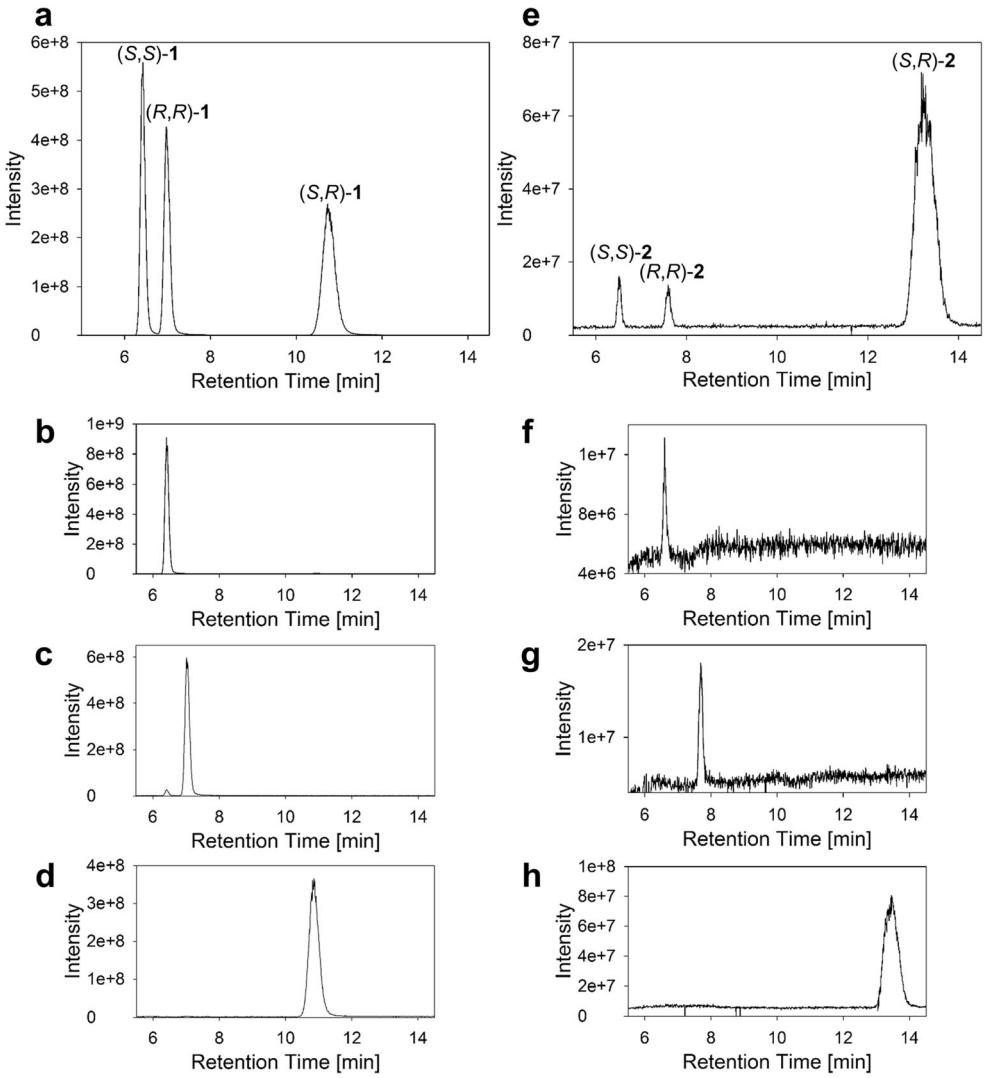

Fig. 4 Stereoselective separation of **1** and **2**. Extracted ion currents of compounds **1** (**a**-**d**) and **2** (**e**-**h**) before (**a**,**e**) and after entioselective column chromatography by HPLC are shown (**b**-**d**, **f**-**h**). Fractions (**b**-**d**, **f**-**h**) were used for subsequent bioassays

we loaded the respective test compounds on HLB beads that were then administered to a culture of *S. robusta* mating type+ , the one that is attracted to diproline. In cases where the pheromone analogs did not bind sufficiently to the polymer beads, we reverted to an interference assay, in which the dissolved test compounds were added before administration of pheromone loaded beads. If the test compounds interact with the receptor in these assays, no or reduced finding capability towards the pheromone gradient is observed. We found the diketopiperazine derived from pipecolic acid (**2**) to be similarly active to the natural pheromone. This implies that hydrophobic recognition of the flanking ring structures on both sides of the diketopiperazine moiety might be necessary for successful pheromone-receptor interactions. Diketopiperazines with only one pyrrolidine ring (**6**, **9**) as well as those without additional ring sub-structures (**4**, **5**, **7**, **8**) were inactive. Also, the introduction of hydroxyl groups as in **3,** rendering the flanking rings more hydrophilic, did not lead to a bioactive pheromone derivative. These results show specificity for diproline recognition with only minor possible structural modifications. Perception of diketopiperazines derived from other proteinogenic amino acids is, therefore, unlikely. This specificity is important in biofilms, since diketopiperazines are known and widely distributed bacterial metabolites that could otherwise interfere with the sexual reproduction of *S. robusta*. (Borthwick 2012; De Rosa et al. 2003). Since we applied compounds **3**, **4** and **7** with unknown stereochemical composition, we can't exclude overlaying inhibitory effects of single stereoisomers within the mixture and, therefore, false negative results. However, in the interference assay (Fig. 3) efficacy in diproline finding was only reduced when the natural pheromone was present and, upon application of compound **4** at higher concentrations. The other metabolites exhibited no inhibitory activity. Thus, we conclude that isomers of **3**, **4**, and **7** do not inhibit pheromone activity in a way sufficient to interfere with the attraction assay.

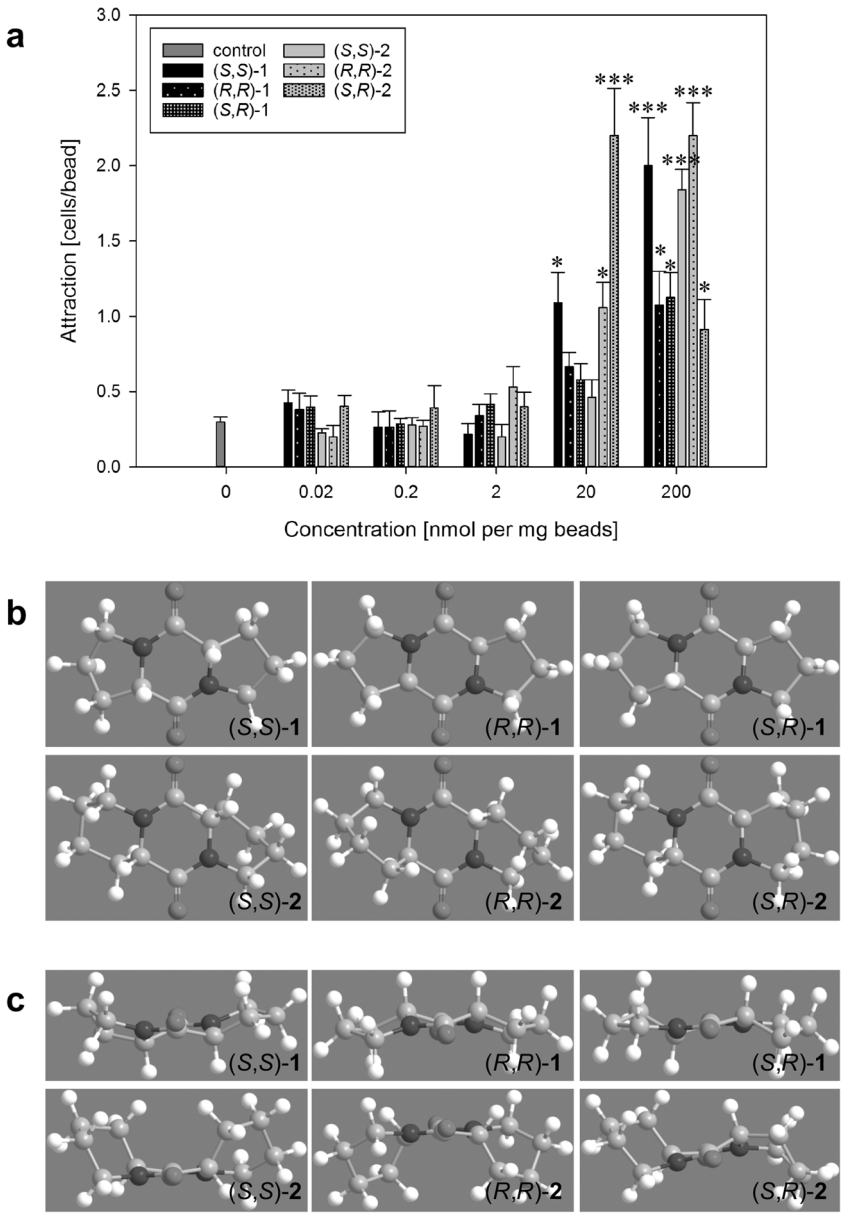

Fig. 5 Bioactivity of the stereoisomers of **1** and **2**. The attraction towards compound loaded beads was monitored as described in Fig. 2 and cells accumulating around the beads were counted ($n = 4$) (**a**). Statistical analysis was performed by One-Way ANOVA of each concentration followed by Holm-Sidak post-hoc test with multiple comparisons against the control treatment (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). 3D structures of the tested compounds were modeled in Chem3D (ChemOffice Professional, Perkin Elmer) and are shown in two distinct views (**b**, **c**)

To further investigate the importance of stereochemistry in pheromone activity, stereoselective purification of diproline was carried out using HPLC. Since in previous experiments diproline stereoisomers could only be separated by supercritical fluid chromatography, there was a need for a semi preparative separation (Frenkel et al. 2014b). Our new protocol achieves separation on an amylose tris(3,5-dimethylphenylcarbamate) column with a simple isocratic elution using MeOH / water / formic acid and can be applied to different diketopiperazines (Fig. 3). Attraction assays using the purified compounds revealed that all stereoisomers of diproline **1** were active underpinning an unspecificity of diproline perception in terms of stereochemistry. These structures are recognized per se by the receptor without prior enzymatic conversion since the pheromone is stable even in extended incubations with *S. robusta* (unpublished data). All stereoisomers of diproline have quasi a flat piperazine-2,5-dione nucleus, and the pyrrolidine moieties are in an envelope conformation (Fig. 5) (Behre et al. 2012). The arrangement of the amide groups in the diketopiperazine ring provides a required but not sufficient motive for pheromone activity since it is also found in compounds **3**-**9** that were inactive. Additional hydrophobic interactions mediated by the pyrrolidine rings apparently facilitate pheromone-receptor interactions. Since the piperidine rings of compound **2** that exhibits activity as well are found in the spatially more demanding chair conformation (Fig. 5) the hydrophobic recognition motive might be more flexible. Pheromone reception of these compounds requires some core elements, including the flat piperazine-2, 5-dione and two adjacent ring systems but is otherwise more

flexible concerning stereochemistry and ring size. Therefore, pheromone recognition might be dependent on correctly positioned polar groups of the diketopiperazine ring in combination with the hydrophobic pyrrolidin / piperidine rings.

The lack of specificity in pheromone perception might pose a risk for a misguidance caused by other diketopiperazines in the biofilm. This is especially true since this is a quite widely distributed compound class (Borthwick 2012). However, the unspecific response towards the diproline stereoisomers in *S. robusta* might be compensated by other components of the multi-step pheromone system of the alga. Production and sensing of diproline are only initiated after cells encounter sex-inducing pheromones (SIPs) from the respective mating partners (Frenkel et al. 2014a; Gillard et al. 2013; Moeys et al. 2016). We suggest that SIPs, which also mediate a cell cycle arrest in the opposite mating type in addition to pheromone production and perception, might, therefore, play a regulatory role in this orchestrated system and control the specificity of the close range interactions in this diatom (Moeys et al. 2016).

Since the only known pheromones in diatoms were those identified in *S. robusta* (Gillard et al. 2013; Moeys et al. 2016), this is the first study on structure-activity relationships in diatom pheromone research. In brown algae that also belong to the Heterokontophytes, a family of fatty acid derived hydrocarbons act as attraction pheromones and their activities have been intensively studied as reviewed in Pohnert and Boland (2002). Structure-activity relationships of lamoxiren, the pheromone of the brown alga *Laminaria* spp. revealed that all stereoisomers exhibited bioactivity, but at different threshold concentrations (Maier et al. 2001, 1994). Brown algal pheromones are often released as enantiomeric mixtures, and in several species their perception relies both on the geometric as well as the electronic structure of the unsaturated hydrocarbons (Pohnert and Boland 2002). These compounds are, thus, not comparable to the rather polar putatively amino acid derived pheromone of *S. robusta*. It will be interesting to see in further studies, whether a diketopiperazine pheromone chemistry can be more universally observed in diatoms and whether similar structural variability as in brown algae is found.

In this study, we found pheromone activity of diketopiperazines with two flanking unsubstituted ring structures. Of the two tested compounds **1** and **2** all stereoisomers proved to be active. This indicates a core-requirement for central structural elements but a missing specificity, when it comes to more subtle structural modifications. It might be concluded that the additional sex inducing pheromones required for successful mating contribute to specificity. Future research on pheromones in other diatom species will show whether similar multi-step systems with non-stereospecific pheromone perception are conserved in these microalgae.

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