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Chiral separation of a diketopiperazine pheromone from marine diatoms using supercritical fluid chromatography

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ABSTRACT

The proline derived diketopiperazine has been identified in plants, insects and fungi with unknown function and was recently also reported as the first pheromone from a diatom. Nevertheless the stereochemistry and enantiomeric excess of this natural product remained inaccessible using direct analytical methods. Here we introduce a chiral separation of this metabolite using supercritical fluid chromatography/mass spectrometry. Several chromatographic methods for chiral analysis of the diketopiperazine from the diatom *Seminavis robusta* and synthetic enantiomers have been evaluated but neither gas chromatography nor high performance liquid chromatography on different chiral cyclodextrin phases were successful in separating the enantiomers. In contrast, supercritical fluid chromatography achieved baseline separation within four minutes of run time using amylose tris(3,5-dimethylphenylcarbamate) as stationary phase and 2-propanol/CO_2 as mobile phase. This very rapid chromatographic method in combination with ESI mass spectrometry allowed the direct analysis of the cyclic dipeptide out of the complex sea water matrix after SPE enrichment. The method could be used to determine the enantiomeric excess of freshly released pheromone and to follow the rapid degradation observed in diatom cultures. Initially only trace amounts of c(D-Pro–D-Pro) were found besides the dominant c(L-Pro–L-Pro) in the medium. However the enantiomeric excess decreased upon pheromone degradation within few hours indicating that a preferential conversion and thus inactivation of the L-proline derived natural product takes place.

1. Introduction

Diketopiperazines (DKPz) are natural products formed by the cyclization of two amino acids. They have a broad range of biological activities due to their chiral, rigid functionalized structure, which allows them to bind to different kinds of receptors [1]. This compound class is also of pharmacologic interest due to antibacterial, antifungal, antitumor and several other functions [1]. These smallest possible dipeptides are widespread in nature. In bacteria, several proline containing DKPz seem to play a role in cell to cell signaling [2]. In mammals, c(His–Pro) is endogenous to the brain [3] and in insects DKPz possessing antibacterial properties have been reported [4]. DKPz occur also in fungi and plants with hitherto unknown function [3]. In the diatom *Seminavis robusta*, c(Pro–Pro) (Scheme 1) acts as a sex pheromone. In a mating culture of these diatoms, sex is induced by light and follows a strictly daily pattern of pheromone production and degradation. Pheromone production and reception by the mating partners occurs between 6 to 10 h after the onset of illumination [5]. Interestingly, both synthetic enantiomers c(D-Pro–D-Pro) and c(L-Pro–L-Pro) showed biological activity but circular-dichroism (CD)-spectroscopy suggested that predominantly c(L-Pro–L-Pro) is excreted by sexually active cells. Usually, pheromone receptors only perceive specifically one isomer which opened the question how such an ambiguous activity of two enantiomers can be rationalized. Possible models include that e.g. the reception involves a racemization of the pheromone before or during receptor interactions. Alternatively an unspecific perception by a sloppy receptor might be envisaged [5]. In depth mechanistic investigation of this process was however prevented by the lack of an accurate chiral analysis of the natural product. So far the stereochemistry of c(Pro–Pro) is only accessible through CD-spectroscopy [5,6], making it impossible to quantitatively determine the enantiomeric excess of this pheromone in complex biological matrices. A chiral separation procedure for c(Pro–Pro) would facilitate further biological investigations and could also provide new analytical possibilities in the analysis of DKPz in general.

One specific problem working with DKPz is that elevated temperatures used in some chiral derivatization techniques and

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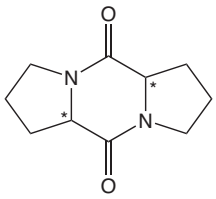

Scheme 1. Structure of c(Pro–Pro).

analytical methods as gas chromatography (GC) can lead to racemization and side reactions [7,8]. High-performance liquid chromatography (HPLC) on a teicoplanin chiral stationary phases [9] offers derivatization-free analytical methods of native amino acids and peptides. Other common stationary phases are α-, β- and γ-cyclodextrines [10]. They can be permethylated (–PM) or possess free hydroxyl groups (–OH), enabling the separation of a broad range of compounds. Diastereomers of several DKPz [11] and cyclic dipeptide enantiomers containing aromatic groups [12] can be separated using a β-cyclodextrin column but to the best of our knowledge no method has been reported for the analytical problem at hand.

Supercritical fluid chromatography (SFC) has been introduced 1962 as powerful chromatographic tool [13]. Lower operating temperatures compared to GC decrease the risk of racemization of the analyte [14] making SFC a method with high potential for the enantioselective detection of thermolabile analytes [15,16]. SFC shows complementary recognition to HPLC and GC thereby enabling the separation of analytes not or only poorly accessible with those techniques. Advantages over common chiral HPLC are often the reduction of analysis times and the wastage of solvent [17]. Matthijs et al. [18] compared the separation of several acidic, basic and neutral pharmacological drugs using polar organic solvent chromatography, normal phase liquid chromatography and SFC. The latter method generally let to the best chiral separations, showing its potential for a wider use. Several other studies support the broad application range of this method, covering enantiomers of amino acid derivatives [19], halogen and methoxy substituted phenylglycidols [20], 1-phenyl-1-propanol [21] as well as several drugs of different compound classes [22]. The rising number of methods for the enantiomeric separation and preparative purification of drugs by SFC illustrates increasing importance for separation sciences and industrial purification [23,24]. SFC can be problematic for very polar substances which are only soluble in water [25], however, the direct injection of aqueous formulations in the mobile phase is possible [26]. In this study we investigate the chiral separation of c(Pro–Pro) on different cyclodextrine- and polysaccharide-derivate based stationary phases in GC, HPLC and SFC. After method-evaluation, the enantiomeric excess of the diatom sex pheromone out of complex biological samples is determined and processes during pheromone degradation are monitored.

2. Experimental

2.1. Origin of samples

Enantioselective synthesis of both enantiomers of c(Pro–Pro) [27] and extraction of the natural sex pheromone from a mating culture of the diatom *Seminavis robusta* followed published procedures [5]. Briefly, the mating diatoms were cultured in 50 ml seawater medium. After illumination, the cultures were filtered hourly and the cell-free filtrate containing the diatom pheromone was mixed with 15 nmol caffeine used as internal standard. Medium samples were extracted on hydrophilic–lipophilic-balanced solid-phase extraction (HLB–SPE) cartridges, which were eluted with Methanol (MeOH). After determination of the pheromone concentration [5], the extract was evaporated to dryness under a stream of nitrogen, and stored at −20 °C until analysis in this study.

2.2. Chiral GC/MS analysis

One μl of a racemic mixture containing 150 μM c(Pro–Pro) solved in acetonitrile was injected in split 10 mode on a J&W Cyclodex-B^{TM} column (0.252 mm ID × 30 m × 0.25 DF, Agilent, Böblingen, Germany) using an Agilent 6890N gas chromatograph coupled to an orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer (GCT Premier^{TM}, Waters, Manchester, UK). The stationary phase of the column is composed of nonbonded 10.5% β-cyclodextrin in 14% cyanopropyl-phenyl/86% methylpolysiloxane. Helium 5.0 (Linde AG, Pullach, Germany) with a constant flow of 1 ml min^{-1} was used as carrier gas. For method evaluation, the column temperature was kept isothermic at different temperatures (230 °C, 220 °C, 210 °C, 200 °C, and 180 °C), the injector temperature was 250 °C. The scanned mass range was between 50–800 *m/z* with a scan time of 0.19 s^{-1} and an interscan delay of 0.01 s. Data evaluation was done by monitoring the base ion of c(Pro–Pro) at *m/z* = 70.07.

2.3. Chiral HPLC/MS analysis

One μl of a racemic mixture containing 100 μM c(Pro–Pro) solved in water was injected into a ultra-performance liquid chromatography (UPLC) coupled to a time of flight mass spectrometer with electrospray ionization (ESI-MS) (Acquity UPLC®/Q-ToF micro, Waters, Manchester, UK). Analysis was performed with a Nucleodex® screening kit containing α-, β- and γ-Nucleodex (4 mm ID × 30 mm × 5 μm PS, Macherey-Nagel, Dueren, Germany) cartridges. The solvent was kept at 100% water. The scanned mass range was between 40 and 1000 *m/z* with a scan rate of 0.56 scans s^{-1} and an inter-scan delay of 0.04 s in positive mode. The collision energy was 5 V, the sample cone was set to 25 V, the cone gas flow was held at 20 l N_2 h^{-1} and the desolvation gas flow at 700 l N_2 h^{-1}. Data evaluation was done by monitoring the $[M+H]^+$ of c(Pro–Pro) (*m/z* = 195.1).

2.4. Chiral SFC/MS analysis

For method development, 1 μl of c(L-Pro–L-Pro), c(D-Pro–D-Pro) and the racemic mixture (all 260 μM in MeOH/water 1:1) was injected into a ultra-performance convergence chromatography (UPC^2) system coupled to a triple quadrupole mass spectrometer with ESI-MS (Acquity UPC^{2TM}/Xevo TQD, Waters, Eschborn, Germany) using Chiralpak® IA, IB, IC and ID columns (4.6 mm ID × 100 mm × 3 μm PS, Chiral Technologies Europe, Illkirch-Cedex, France). The column temperature was 40 °C. MeOH, acetonitrile and 2-propanol with a flow of 2 ml min^{-1} were used in different proportions as modifiers with CO_2 with a system backpressure of 1500 psi. The gradient was ramped from 5–40% modifier in 4 min and was kept at 40% for 1 min. Detection was performed in single ion monitoring positive mode of *m/z* = 195 with a dwell time of 80 ms and an interchannel delay time of 3 ms. The collision energy was 3 V, the sample cone was set to 20 V, the cone gas flow was held at 20 l N_2 h^{-1} and the desolvation gas flow at 700 l N_2 h^{-1}. For method optimization the pure c(Pro–Pro) enantiomers or their mixtures as well as diatom extracts containing the natural pheromone (all in methanol) were analysed with a gradient ramping from 20–50% 2-propanol/CO_2 in 4 min. Detection was performed in multiple reaction monitoring mode (MRM) (ion pairs *m/z* = 195/97). The collision energy was adjusted to 24 V, the sample cone was set to 40 V.