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Diastereomeric and enantiomeric selective accumulation of cypermethrin in the freshwater mussel *Unio gibbus* and its effects on biochemical parameters

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ABSTRACT

Synthetic pyrethroids are a family of chiral pesticides with a large number of stereoisomers. Cypermethrin (CYP) is used in a variety of agricultural crops, but also has public health and veterinary uses. In this work, the freshwater mussel (*Unio gibbus*) was chosen to evaluate the stereoselectivity of CYP through the use of gas chromatography with mass-spectrometry. The effects of CYP on mussels were examined by measuring neurotoxicity and oxidative stress biomarkers during its uptake. The investigation was performed under laboratory conditions using nominal CYP concentrations $C_1 = 100$ μg/L and $C_2 = 150$ μg/L over 96 h. Preferential bioaccumulation of *cis*-CYP isomers was observed. Furthermore, enantiomeric characterization revealed enantioselective accumulation, most probably related to mussel metabolism. Antioxidant enzyme activities (superoxide dismutase (SOD), and catalase (CAT)), and levels of reduced glutathione (GSH) and malondialdehyde (MDA) were determined in digestive gland after 4 days of exposure. CYP significantly inhibited acetylcholine esterase activity, by 51% and 57%, respectively, in mussels treated with 100 and 150 μg/L doses. The highest and lowest CYP concentrations elicited an increase of 67 and 63%, respectively, in SOD activity compared to the controls, while CAT activity was increased by 65 and 73%. A statistically significant decrease in GSH levels (40%) was observed only with the highest CYP concentration tested (150 μg/L). In addition, lipid peroxidation was significantly higher (67%) than in controls. These results provided information on CYP-enantioselective uptake and potential biomarkers that could be effectively applied for the biomonitoring of freshwater ecosystem.

1. Introduction

Synthetic pyrethroids are good examples of chiral compounds [1–3]. These pesticides are anthropogenic analogs of naturally-occurring pyrethrins extracted from the flowers of *Chrysanthemum cineraraefolum* [4]. Cypermethrin [(RS)-α-cyano-3-phenoxybenzyl(1RS)-*cis*-*trans*-3-(2,2-dichlorovinyl)-2,2 dimethyl cyclopropane carboxylate] is a pyrethroid used to kill insects, fungi and other organisms affecting crops, thus improving agricultural production. It has three chiral carbon atoms located at 1C and 3C in the cyclopropane carboxylic acid moiety and C in the alcohol component. It therefore consists of eight optical isomers: two *cis* diastereomers of 1R-*cis*-αR + 1S-*cis*-αS and 1S-*cis*-αR + 1R-*cis*-αS and two *trans* diastereomers of 1R-*trans*-αR + 1S-*trans*-αS and 1S-*trans*-αR + 1R-*trans*-αS [5,6]. In Tunisia, CYP is commonly used in agricultural activities around freshwater reservoirs. Several studies have suggested that aquatic invertebrates and fish are extremely sensitive to CYP [7,8]. There is growing concern and awareness of the potential impact of pesticides on the environment, food safety and human health. Monitoring and quantification of these organic pollutants are therefore important for environmental protection.

Bivalves can filter large volumes of water, process microalgae, bacteria, sediments and particulates, and, potentially, bioaccumulate different chemicals in their tissues. Therefore, these organisms have long been recognized as valuable indicators of pollution, and extensive background information is now available on their biological responses to a wide range of both inorganic and organic chemicals [9,10]. Freshwater bivalves, such as Unionidae, can accumulate various environmental chemicals and have been used as sentinels for study of environmental pollution [11]. However, the exposure impact of CYP on freshwater mussels has, so far, not been extensively investigated. The mussel, *Unio gibbus*, which is abundant in freshwater environments in Tunisia [23], may be a suitable model organism for characterizing potential impact of CYP. The characterization of the quality of freshwater systems requires specially designed biological methods for assessing

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the health status of biota. In this sense, biomarkers are useful tools for assessing environmental health [12].

In this study, CYP enantiomers were determined in exposed mussel tissues, through the use of gas chromatography coupled to tandem mass-spectrometry (GC-MS/MS). Moreover, effects of CYP on *U. gibbus* were evaluated in digestive gland (the main organ of CYP accumulation), to assess molecular-level responses. These included levels of reduced glutathione (GSH) and malondialdehyde (MDA), activities of superoxide dismutase (SOD), catalase (CAT) and acetylcholine esterase (AChE).

2. Materials and methods

Adult *U. gibbus* (7.5–8.3 cm in length) were collected from a permanent stream (called Sejenane), a tributary of the Ichkeul lake (northern Tunisia: 37°11, 603′N; 009°34, 764′E). The animals were divided into groups of 10 and acclimated for 4 days in 3-L glass beakers. Water temperature was between 18 and 19 °C, dark/light rhythm was maintained constant (16 h/8 h). Glass beakers were filled with natural fresh water changed every 48 h. The following experimental treatment was established in triplicate glass beakers for 96 h. The animals were divided into control and treated groups. The treated groups were exposed to two nominal CYP concentrations C1 (100 μg/L) and C2 (150 μg/L). One control group was handled in parallel but left untreated (Control). After 4 days of exposure, mussels were removed and immediately frozen at −80 °C prior to chemical and biochemical assays. Five randomly selected mussels from each of the two CYP treated groups were lyophilized, the CYP content of the soft tissue determined. The other five animals were used for biochemical response determination.

2.1. Chemical and biochemical analysis

2.1.1. Chemical analysis

2.1.1.1. Standards and reagents. Analytical standard was purchased from Dr. Ehrenstorfer (Augsburg, Germany). As surrogate standard d_6-*trans*-CYP was chosen and purchased from the same commercial firm. Organic solvents were obtained from J.T. Baker "for use in HPLC" quality (Deventer, The Netherlands). Standard solutions were prepared in ethyl acetate ("for gas chromatography" quality from Merck, Darmstadt, Germany). Calibration curves were prepared at different concentrations ranging between 0.4 and 150 ng mL^{-1}. Solid phase extraction (SPE) cartridges were obtained from Isolute Biotage (Uppsala, Sweden) (C18, 2 g/15 mL) and from Interchim (Montluçon, France) (Basic alumina, 5 g/25 mL).

2.1.1.2. Extraction and cleanup procedures for mussels. The procedure used for sample extraction was previously described [13]. Briefly, 0.1 g of freeze-dried sample was spiked with 10 μL of a 10 ng/L d_6-*trans*-CYP solution. Extraction was performed with 20 mL of hexane:dichloromethane 2:1 and assisted by ultrasound (ultrasonic bath, UCI-200, Raypa, Terassa, Barcelona, Spain) for 15 min. This extraction was repeated twice and all solvent dried by a N_2 stream. A following tandem SPE (basic alumina and C18 cartridges, 30 mL acetonitrile as eluent) was used to clean up the sample. The eluent was evaporated under N_2 and the sample reconstituted in 100 μL of ethyl acetate into an autosampler vial for GC–MS/MS injection.

2.1.1.3. Extraction and cleanup procedures for water. A water sample (20 mL) was collected from each beaker at the end of the exposure. After fortifying with d_6-*trans*-CYP (10 ng) as surrogate standard, water samples were extracted with 1 mL of chloroform with the assistance of sonication (ultrasonic bath, UCI-200, Raypa, Terassa, Barcelona, Spain) for 5 min at 35 °C. The emulsion was centrifuged at 3500 rpm for 5 min. The organic phase was transferred to a vial and completely evaporated under nitrogen stream. Then, the sample was re-dissolved in 100 μL ethyl acetate into an autosampler vial for GC–MS/MS injection.

2.1.1.4. GC–MS/MS determination. Analyses were performed on an Agilent Technologies 7890A coupled to a 7000A GC/MS Triple Quad. As regards the chromatographic conditions, we selected a BGB-172 (20% tert-butyldimethylsilyl- β-cyclodextrin in 15% phenyl-, 85%-methylpolysiloxane) of 30 m × 0.25 mm and 0.25 μm of film thickness column (BGB Analytik, Switzerland) [14]. Previously optimized mass spectrometric parameters for the same instrument were used [13]. The selected ionization mode was negative chemical ionization (NCI). The ion source temperature was 250 °C and ammonia was the reagent gas at 2.04×10^{-4} Torr. *Cis* and *trans* CYP isomers could be clearly differentiated under these conditions (Fig. 1). In addition, our methodology enabled discrimination between enantiomers of both *cis* diastereoisomers showing a distinct peak for each enantiomer. However, the enantiomers of *trans* diastereoisomers could not be successfully differentiated under the conditions used. Because of this, each *trans* diastereoisomer peak corresponds to a pair of CYP enantiomers.

2.1.1.5. Enantiomeric factor and diastereoisomeric ratio. The enantiomeric distribution resulting from this analysis was described by the enantiomeric factor (EF) values. The EF for each enantiomer pair was determined from their respective peak areas (see CYP chromatogram in Fig. 1) by the following formula:

$$EF = Ai/A_T \quad (1)$$

where Ai is the area of the first eluting enantiomer and A_T is the sum of areas of both enantiomers ($EF = Area_1 / (Area_1 + Area_2)$). One EF was defined for each enantiomeric pair: EF*cis*1, EF*cis*2. However, EF*trans*1 and EF*trans*2 could not be calculated because it was not possible to separate enantiomers of *trans*-CYP in this study. With this formula, a racemic mixture of an enantiomeric pair, which means an equal proportion of both enantiomers, is always represented by an EF value of 0.5. In addition, the ratio of diastereoisomers was calculated to assess how CYP diastereoisomers are distributed in the mussel. First of all, R*cis*/*trans* was defined as the ratio between *cis* and *trans* isomers. Besides, R*cis*1/*cis*2 was the proportion of the isomer *cis*1 with respect to the isomer *cis*2.

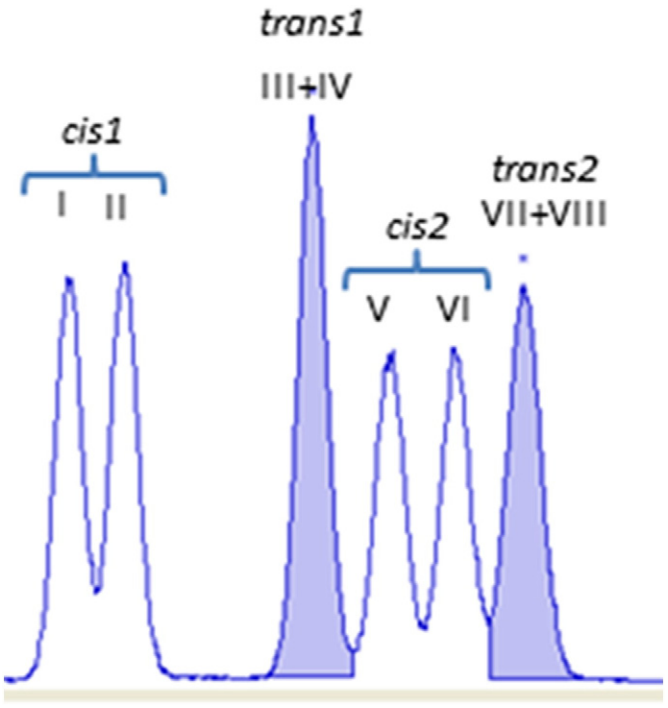

Fig. 1. Chromatogram of cypermethrin analytical standard. Peak assignation (*trans*-isomers are shaded): I, 1R-3R-αR; II, 1S-3S-αS; III, 1S-3R-αS; IV, 1R-3S-αR; V, 1R-3R-αS; VI, 1S-3S-αR; VII, 1R-3S-αS; VIII, 1S-3R-αR. i, 1R-3R; ii, 1S-3S; iii, 1S-3R; iv, 1R-3S.