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Integration of Metabolomics and In Vitro Metabolism Assays for Investigating the Stereoselective Transformation of Triadimefon in Rainbow Trout

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ABSTRACT Triadimefon is a systemic agricultural fungicide of the triazole class whose major metabolite, triadimenol, also a commercial fungicide, provides the majority of the actual fungicidal activity, i.e., inhibition of steroid demethylation. Both chemicals are chiral: triadimefon has one chiral center with two enantiomers while its enzymatic reduction to triadimenol produces a second chiral center and two diastereomers with two enantiomers each. All six stereoisomers of the two fungicides were separated from each other using a chiral BGB-172 column on a GC-MS system so as to follow stereospecificity in metabolism by rainbow trout hepatic microsomes. In these microsomes the *S*-(+) enantiomer of triadimefon was transformed to triadimenol 27% faster than the *R*-(−) enantiomer, forming the four triadimenol stereoisomers at rates different from each other. The most fungi-toxic stereoisomer (1*S*,2*R*) was produced at the slowest rate; it was detectable after 8 h, but below the level of method quantitation. The triadimenol stereoisomer ratio pattern produced by the trout microsomes was very different from that of the commercial triadimenol standard, in which the most rat-toxic pair of enantiomers (known as "Diastereomer A") is about 85% of the total stereoisomer composition. The trout microsomes produced only about 4% of "Diastereomer A". Complementary metabolomic studies with NMR showed that exposure of the separate triadimefon enantiomers and the racemate to rainbow trout for 48 h resulted in different metabolic profiles in the trout liver extracts, i.e., different endogenous metabolite patterns that indicated differences in effects of the two enantiomers. *Chirality 22:183–192, 2010.* © 2009 Wiley-Liss, Inc.

KEY WORDS: triadimefon; triadimenol; triazoles; conazoles; rainbow trout; metabolism; metabolomics; stereoisomers; enantiomers; diastereomers; stereoselectivity; exposure

INTRODUCTION

Triadimefon (Fig. 1) is a 1,2,4-triazole fungicide used for the control of powdery mildews and fungi on fruits, vegetables, turf grasses, and other agricultural crops. It is a systemic fungicide that acts by inhibiting steroid demethylation[1] and is enzymatically reduced in plants, soils, and fungi to the more active metabolite, triadimenol,[1] which is also used as an agricultural fungicide. Triadimefon has a single chiral center and thus exists as two enantiomers. The metabolic transformation of triadimefon to triadimenol involves the reduction of the carbonyl group to an alcohol, resulting in the formation of a second chiral center[2] (Fig. 1). Thus, triadimenol consists of two diastereomers: A [enantiomers A1 (1*R*,2*S*) and A2 (1*S*,2*R*)] and B [enantiomers B1 (1*R*,2*R*) and B2 (1*S*,2*S*)], for a total of four stereoisomers. Diastereomers A and B, and the four stereoisomers that make up these diastereomers, are each produced from triadimefon in different relative amounts by plants and fungi, and the proportions of each stereoisomer may differ depending upon the species.[3,4] A similar metabolic pathway (i.e., formation of triadimenol from triadimefon) is followed in animals[1]; to the best of our knowledge, however, stereoselective formation of triadimenol has not been reported for animals.

Triadimefon and triadimenol are suspected of disrupting steroidogenesis in animals, are neurotoxins, and have been shown to cause tumors in rodents.[5] Since triadimefon and triadimenol are both used as agricultural fungi-

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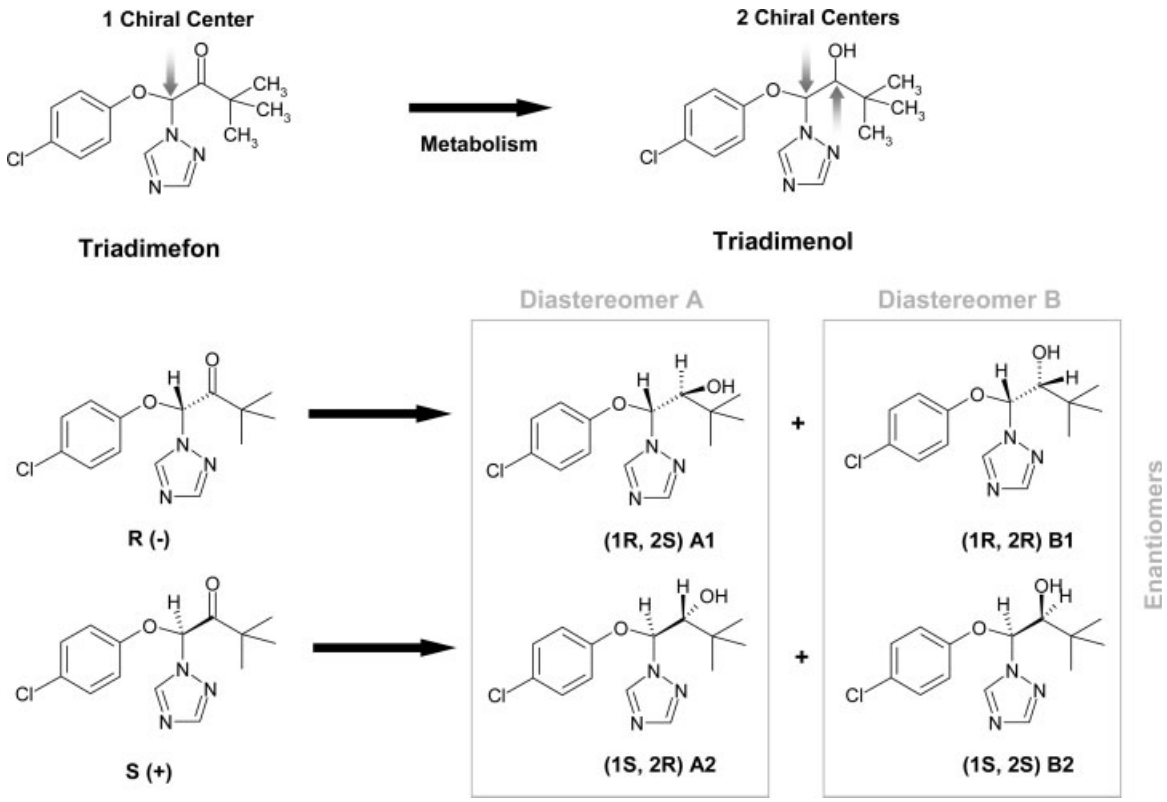

Fig. 1. The metabolic transformation of triadimefon to triadimenol. The reduction of a carbonyl group to an alcohol yields a second chiral center and four stereoisomers.

cides, there is concern regarding potential human exposure, as well as concern about their residues in the environment. Triadimenol, for example, has been detected in water samples from ditches and streams at concentrations of up to a few micrograms per liter.[6] Thus, it is important to understand the fate of these compounds in the environment as well as the impact of their exposure to humans and various wildlife species. In addition, the enantioselective loss of triadimefon to form triadimenol during metabolic transformation is of concern. Established data show that a wide variety of chiral pesticides occur nonracemically in various environmental compartments, are transformed enantioselectively in environmental microcosms, or produce enantioselective toxic effects on various organisms.[7] For similar reasons, since it is known that triadimenol diastereomer A is 10 times more acutely toxic to rats (oral LD_{50}) than is diastereomer B,[8] the possible stereoselective formation of triadimenol is an important issue for both human health and ecological risk assessment.

In vivo and in vitro metabolism studies are useful for understanding the fate of a xenobiotic in an organism. In vitro experiments using microsomal material from various organs (e.g., liver) can shed light on such metabolism events with a minimum of expense and provide valuable information on toxicant exposure.[2,9] In certain cases the chemical that enters the organism is metabolized to a more toxic form inside the organism. For example, the organophosphorus insecticide parathion is biotransformed to paraoxon, which acts as a more potent cholinesterase inhibitor.[10]

While metabolism studies provide information on xenobiotic metabolites, metabolomics, a relatively recent addition to the array of molecular techniques used to assess chemical exposures, uses various types of analytical instrumentation to detect and identify changes in endogenous metabolites in selected tissues and/or biofluids of various organisms after exposure.[11] Changes in these endogenous metabolites can be the direct result of exposure to a xenobiotic, and can be used to assess xenobiotic exposure even after the chemical stressor is no longer present. In the environmental arena, metabolomics involves a comparison of endogenous metabolite patterns in tissues or fluids from organisms exposed to some environmental stressor, such as a toxic chemical, with those from unexposed controls, using advanced data analysis techniques. This approach allows graphical depiction of any significant differences in the patterns, which often provides information about toxicity mechanisms, pathways, and possible biomarkers of exposure. Among those analytical techniques most commonly used for metabolomics studies, nuclear magnetic resonance (NMR) spectroscopy provides a powerful approach for obtaining information-rich spectra for a wide variety of tissues and biofluids.[12]

Rainbow trout (*Onchorhynchus mykiss*) have historically been among one of the most frequently tested freshwater fish with respect to toxicology and physiology for the

purpose of assessing ecological risk from exposure to specific xenobiotics.[13] Here, we report results of investigations of the in vitro stereoselective metabolism of triadimefon to triadimenol in rainbow trout liver microsomes, as well as results of complementary NMR metabolomic experiments involving exposure of the separate triadimefon enantiomers and the racemate to rainbow trout in vivo. To our knowledge, this is the first time that xenobiotic metabolism has been used to invoke the influence of chirality on endogenous metabolite distributions as measured through metabolomics.

EXPERIMENTAL

Reagents and Chemicals

Triadimefon and triadimenol were obtained in 99.4 and 96.4% purity, respectively, from the U.S. Environmental Protection Agency National Pesticide Standard Repository (Ft. Meade, MD). Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, phosphate, and trizma buffers were purchased from Sigma Chemical (St. Louis, MO). Methanol and acetonitrile were from Fisher Chemicals (Fair Lawn, NJ) and were of analytical grade. Perchloric acid (60–62%) was obtained from J.T. Baker (Phillipsburg, NJ). All reagents used for metabolomic analyses were purchased from Sigma-Aldrich (St. Louis, MO) and were of ACS reagent grade. Reagent water for all experiments was produced by a Barnstead Nanopure Infinity water purification system (Thermo Scientific, USA). Solvents for the various experiments were pesticide grade.

GC-MS Analysis of Triadimefon and Triadimenol Enantiomers

GC-MS analysis (e.g., of the fungicide standards and the microsome metabolism extracts) was by use of a Hewlett-Packard 5973 mass spectrometer linked to a 6890 gas chromatograph equipped with a BGB 172 (BGB Analytik AG, Switzerland) chiral column. Column description: 30 m × 0.25 mm ID × 0.25 μm film thickness; chiral stationary phase, 20% *tert*-butyldimethylsilylated-B-cyclodextrin. GC conditions were as follows: injection, splitless at temp. 275°C; column temp. program, 150–220°C at 4°/min, followed by temp. hold for 60 min; helium gas flow, 1.5 ml/min; MS inlet temp., 275°; MS source temp., 230°; and fragmentation voltage, 70 eV. Sample injection volume was 1 μl. Detection was by selected ion monitoring (SIM); SIM ions were *m/z* 181, 208, and 210 for triadimefon and *m/z* 128 and 168 for triadimenol. Quantification was by comparison of enantiomer peak areas to standards of similar concentration analyzed the same day; quality control included analysis of at least one standard per day as well as analysis of a standard before and after each 10 samples. These standards were referenced to a standard curve of triadimefon or triadimenol enantiomer peak area versus concentration.

Preparative Separation of Triadimefon Enantiomers

Triadimefon enantiomers were separated by Chiral Technologies (West Chester, PA) using preparative supercritical fluid chromatography. Racemic triadimefon (2 g) was applied to a Chiralpak®ADH® (Chiral Technologies) preparative column (3.0 cm i.d. × 25 cm long). Elution was by CO_2:MeOH/85%:15% at 25°C, flow rate was 2.0 ml/min, and detection was by UV at 275 nm. Preparative yields were 0.89 and 0.90 g for the 1st and 2nd eluting enantiomer, respectively, with an enantiomeric excess of >99% for each. The optical rotation in methanol of the first eluting enantiomer was (−) and that of the second was (+) as determined by Chiral Technologies using a PDR Chiral Advanced Laser Polarimeter under ambient conditions.

Trout Microsome Preparation

Juvenile rainbow trout of mixed gender (Lake Burton Fish Hatchery, Clarkesville, GA) were held for a 10-day acclimation period in 50 l fiberglass aquaria with carbon-filtered, recirculating, dechlorinated tap water chilled to 12°C. Trout livers were excised upon cervical dislocation, washed, and coarsely minced in an ice-chilled 1.15% KCl solution. The KCl solution was then drained and a wet weight of liver material was recorded. The liver was transferred to a prechilled glass homogenizer where 4 ml volumes of a 0.25 M sucrose solution were added per gram of liver weight. The sample was homogenized on ice and then transferred to an ice-chilled centrifuge tube. The liver homogenate was centrifuged in a high speed centrifuge (Beckman) at 8000*g* for 20 min at 4°C. Next, the supernatant was carefully decanted and ultracentrifuged at 100,000*g* for 60 min at 4°C. The supernatant was then discarded and the microsome pellet was resuspended with a 1:1 volume per gram of original wet weight with a pH 8.0, 0.066 M trizma base buffer containing 0.25 M sucrose and 5.4 mM EDTA. Trout liver microsome total protein content was determined using the Bradford assay and microsomal material was stored at −80°C until use.

Trout Microsome Incubation Procedure

Incubations of microsome samples with triadimefon were conducted in microcentrifuge tubes placed in a cooling block at 11°C. Microsomal suspensions were prepared at a final concentration of 0.125 mg microsome protein/ml in trizma buffer (100 mM) at pH 8.0. Next, triadimefon stock solutions (in acetonitrile and stored in amber vials at 4°C) were added to achieve a final substrate concentration level of 20–40 μM while not exceeding 1% organic solvent. The system was vortexed and allowed to stand for 10 min in the cooling block to ensure temperature equilibration. In a separate vial, a NADPH-regenerating system (NRS) was preincubated at 11°C for 10 min with 100 mM trizma buffer at pH 8.0 and a final reaction concentration containing 0.5 mM NADP, 7 mM glucose-6-phosphate, 1.25 mM $MgCl_2$, and 1.5 U of glucose-6-phosphate dehydrogenase. Incubation was initiated by the addition of 250 μl of the NRS to the microsomal suspension, and the low temperature trout microsome assays were conducted for 0–48 h.

Each assay sample, taken at selected time points, was terminated with a 1:1 addition of MTBE, vortexed, and immediately placed on ice. The samples were centrifuged at 4°C for 10 min at 10,600*g*, and the MTBE was transferred to seal-cap vials containing a polyspring microinsert for subsequent analysis of triadimefon and triadimenol.

Fish Exposures and Liver Tissue Collection for Metabolomic Analysis

Juvenile rainbow trout (n = 160) of mixed gender, obtained from the Lake Burton Fish Hatchery (Clarkesville, GA), were randomly assigned to 50 L fiberglass aquaria (20 fish per tank) containing recirculating, dechlorinated tap water chilled to 12°C, and carbon filtered to remove any contaminant residues in the water. Fish were maintained on a 12 h light, 12 h dark photoperiod and fed trout chow (Aquamax, Carolina Pet Supply) during the acclimation period (7 days) before exposure initiation. Food was withheld over the 48 h course of the exposure.

Fish were exposed to each of the triadimefon enantiomers or the racemate via oral gavage at two dose levels (high dose, 720 mg/kg body weight; low dose, 144 mg/kg body weight). Oral gavage stock solutions (1000 mg/l) for each triadimefon enantiomer or racemate were prepared by dissolving each in methanol and warmed gelatin.[14] Fish were lightly anesthetized in tricaine methanesulfonate, MS-222; 20 mg/l buffered with 200 mg/l $NaHCO_3$; (Finquel, Argent, Redmond, WA), removed and weighed, and the corresponding dose was administered via oral gavage by using a syringe. Fish were allowed to recover in clean water before being returned to their respective treatment aquaria. Controls were treated in an identical manner but without the addition of any triadimefon chemical to the methanol/gelatin solution. Twenty-four hours after the exposure, 10 fish from each treatment (e.g., racemate, 720 mg/kg/day) and the corresponding controls were randomly removed and anesthetized in a buffered solution of MS-222, 100 mg/l. Total body weight was measured and livers were excised, weighed, and immediately transferred to preweighed microcentrifuge tubes, then flash-frozen in liquid nitrogen. After an additional 24 h (the 48-h time point), the remaining fish were removed and identically processed. Liver samples were stored at −80°C before further analysis.

Liver Extraction and Preparation for NMR Analysis

Polar liver extracts were prepared according to the procedure published by Viant et al.[15] Briefly, pulverized tissue samples were extracted using 5 ml/g (wet mass) of ice-cold 6% perchloric acid followed by centrifugation at 12,000*g* for 10 min at 4°C. Supernatants were collected and neutralized to pH 7.4 using 2 M K_2CO_3 followed by centrifugation once again to remove salt ($KClO_4$). Finally, 500 μl of each supernatant was lyophilized and reconstituted in 550 μl of sodium phosphate buffered (100 mM, pH 7.4) deuterium oxide (99.96%) containing 0.25 mM TSP. Following another round of centrifugation, samples were pipetted into 5-mm tubes and analyzed by NMR.

NMR Instrumentation and Measurements

All tissue extract NMR data were acquired at 25°C on a Varian Inova 800 NMR spectrometer (799.7 MHz, ^{1}H) using a cryogenic triple resonance probe. The residual water signal was suppressed by presaturation. Acquisition parameters for the 1D ^{1}H experiments included a 2 sec presaturation, 12 kHz spectral window, 16 k data points and 64 scans. All 1D data were processed with double-size zero-filling and 0.3 Hz line broadening. To aid in metabolite resonance assignments, 2D gradient COSY spectra were collected. Acquisition parameters for these spectra include a 1 sec presaturation, 6 kHz spectral window, 1024 data points, 128 increments, and n-type for the indirect dimension. All ^{1}H chemical shifts were internally referenced to TSP. Peak assignments were made according to previously reported values.[16–18]

Metabolomic Data Reduction and Multivariate Analyses

All NMR spectra were phased and baseline corrected before data reduction. Reduction consisted of segmenting the chemical shift region from 0 to 10 ppm into 500 bins 0.02 ppm in size. All spectral processing and binning was done using the ACD/1D NMR Manager module as part of the ACD/SpecManager software package (Advanced Chemistry Development, Toronto, Canada). The reduced data sets were then imported into Microsoft® Excel. For each binned spectrum, those bins containing water resonances (δ 4.7–5.0) were removed, and the remaining bins were scaled by the total integrated area as a means for normalization. Finally, all variables were mean-centered and unit variance-scaled.[19] To identify changes in metabolite profiles among dosed and control animals, principal components analysis (PCA) was performed using the SIMCA-P+ software package version 10.0.4 (Umetrics, Umea, Sweden).

RESULTS AND DISCUSSION

GC-MS Analysis of Triadimefon and Triadimenol Stereoisomers

Figure 2 shows a typical GC-MS chromatogram (SIM) of a mixture of triadimefon and triadimenol commercial standards at a concentration of 4 μg/ml each obtained using the BGB-172 chiral column. The two triadimefon enantiomers are almost baseline-resolved, while the four triadimenol stereoisomers are separated from each other by approximately one minute or more. The triadimefon peaks are nominally of equal area, indicating the expected racemic form of the compound. The EF of triadimefon is 0.503 ± 0.005 (n = 6), according to eq. 1. It is known that the absolute configurations of the two enantiomers relative to their optical rotation is *R*-(−) and *S*-(+)[20]; the first eluting peak on this BGB-172 column is the (*R*)-(−) enantiomer.

In fungi, plants, and animals, the carbonyl group of triadimefon is reduced to an alcohol. The resulting metabolite, triadimenol, is a considerably more potent fungicide than triadimefon.[21] Since triadimenol has two chiral centers, it exists as four stereoisomers; these exist as two dia-

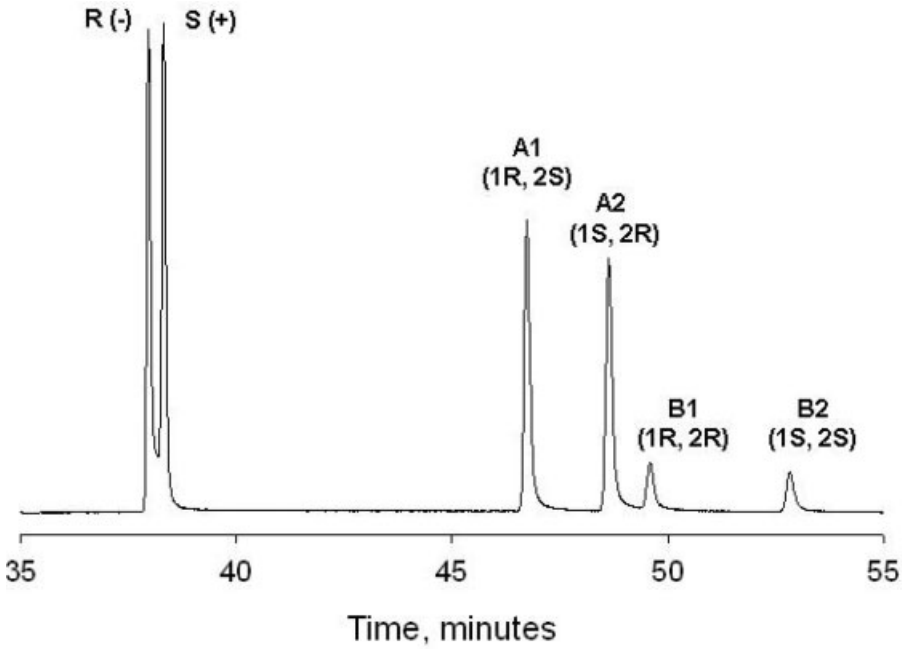

Fig. 2. GC-MS chromatogram (selected ion mode) of a mixed standard of the commercial formulations of triadimefon (first two peaks; ions *m/z* 181, 208, 210) and triadimenol (following four peaks; ions *m/z* 128, 168) showing enantiomer separation on a BGB-172 chiral column.

stereomers, termed A and B, each of which consists of a pair of enantiomers (Figs. 1 and 2). The EF of the diastereomer A enantiomers is 0.497 ± 0.005 (n = 6), and of diastereomer B is 0.483 ± 0.010 (n = 6). (This "diastereomer" terminology is technically incorrect and may be confusing: A and B are commercially called diastereomers, but technically a diastereomer is one isomer of a chemical that has two chiral centers, such as triadimenol, and each of these individual isomers has a counterpart, called an epimer, which is another of the four stereoisomers that differs in configuration at only one of the chiral centers.[22])

Figure 2 shows for a commercial mixture of triadimenol a pattern of chromatographic separation of diastereomers A and B, each of which shows two enantiomer peaks in equal amounts (A1 and A2; B1 and B2); i.e., each diastereomer is a racemic mixture. Diastereomer A is 85% of the total triadimenol, while diastereomer B is about 15%. As shown in Figure 1, A1 and B1 epimers are derived from metabolism of (*R*)-(−)-triadimefon and A2 and B2 are from (*S*)-(+)-triadimefon. Similar to the two triadimefon enantiomers, the *R* stereoisomer elutes before the *S* stereoisomer for each diastereomer (see Fig. 2). Little is known about toxicity of the four individual stereoisomers, except that most of the fungicidal activity of triadimenol can be attributed to the A2 (1*S*,2*R*)-stereoisomer.[23] This is one of the predominant enantiomers in the commercial formulation of triadimenol (see Fig. 2), and contributes to the greater fungal toxicity of diastereomer A over that of B.

The enantiomer composition of the triadimenol formulation used in the metabolism studies reported here corresponds to the relative peak areas depicted in Figure 2, with the EF values reported earlier. This is the formulation supplied by the EPA repository and used in our experiments, and is representative of actual commercial triadimenol. It is noteworthy, however, that different analytical standards of triadimenol may not contain the same ratios of diastereomers A and B as the commercial pesticide formulation. For example, a standard from Sigma (www.sigma.com) contained less than 1% of diastereomer B, as measured by GC-MS, whereas the EPA repository formulation contains about 15%. Since the more abundant diastereomer A is the most toxic, at least to rats[8] and fungi,[23] the Sigma standard would be overall more toxic—to the same or similar species—since the toxicity of the EPA repository product would be diluted by the presence of the less toxic diastereomer B. Thus, it is critical to use the commercial pesticide formulation standard in fate and effect experiments on triadimenol.

Metabolism of Triadimefon in Trout Microsomes

Research results demonstrated that the *S*-(+) enantiomer of triadimefon was metabolized faster than the *R*-(−) enantiomer in trout liver microsomes, as shown in Figure 3 where the enantiomer fraction (EF, eq. 1) of triadimefon is shown to decrease slowly with time, from 0.513 at t_o to 0.495 at t_{48} (2889 min).

$$\mathrm{EF} = \frac{[S\text{-}(+)\ \text{enantiomer}]}{[S\text{-}(+)\ \text{enantiomer}] + [R\text{-}(-)\ \text{enantiomer}]} \qquad (1)$$

Since the decrease in EF was relatively small, additional metabolism studies were conducted with the individual *S*-(+) and *R*-(−) enantiomers. The *S*-(+) enantiomer metabolized 27% faster than *R*-(−) and would theoretically yield a change in EF of 0.07 after 2500 min based upon reaction

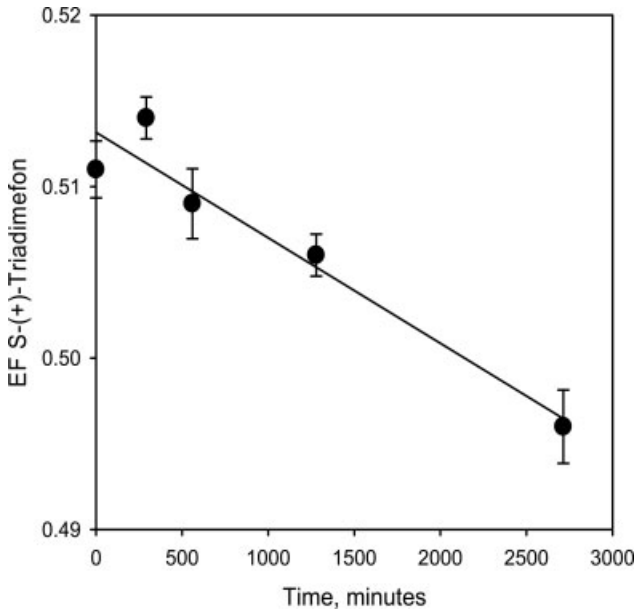

Fig. 3. The enantiomer fraction (EF) of triadimefon decreases with time, indicating that the *S*-(+)-enantiomer metabolizes faster than the *R*-(−).

rates. These results compare favorably with the racemic study, which exhibited a 22% faster rate of *S*-(+) metabolism and an EF change of about 0.02 after 2500 min (see Fig. 3).

Figure 4 is a GC-MS chromatogram (SIM) depicting the distribution of triadimenol stereoisomers formed after in vitro exposure of racemic triadimefon to trout liver microsomes for 480 min. Although the stereoisomer ratio pattern is very different from that of the commercial triadimenol standard (see Fig. 2), it is obvious that the first two eluting peaks correspond to diastereomer A (i.e., 1*R*,2*S* and 1*S*,2*R*) and the second pair to diastereomer B (1*R*,2*R* and 1*S*,2*S*) based upon comparison of retention times with those of the standard. However, it was not possible to ascertain the absolute configuration of each isomer based upon existing information. To address this issue, metabolism studies were conducted using the separate *R*-(−)- and *S*-(+)-triadimefon enantiomers. The *R*-(−) enantiomer produced significant metabolites at 46.5 and 49.6 min while the *S*-(+) yielded metabolites at 48.4 and 53.2 min (see Fig. 4). Assuming that *R* could only produce 1*R*,2*R* and 1*R*,2*S* (see Fig. 1) and knowing that one of the first two eluting peaks had to be either 1*R*,2*S* or 1*S*,2*R* (the composition of diastereomer A), it was concluded that the first

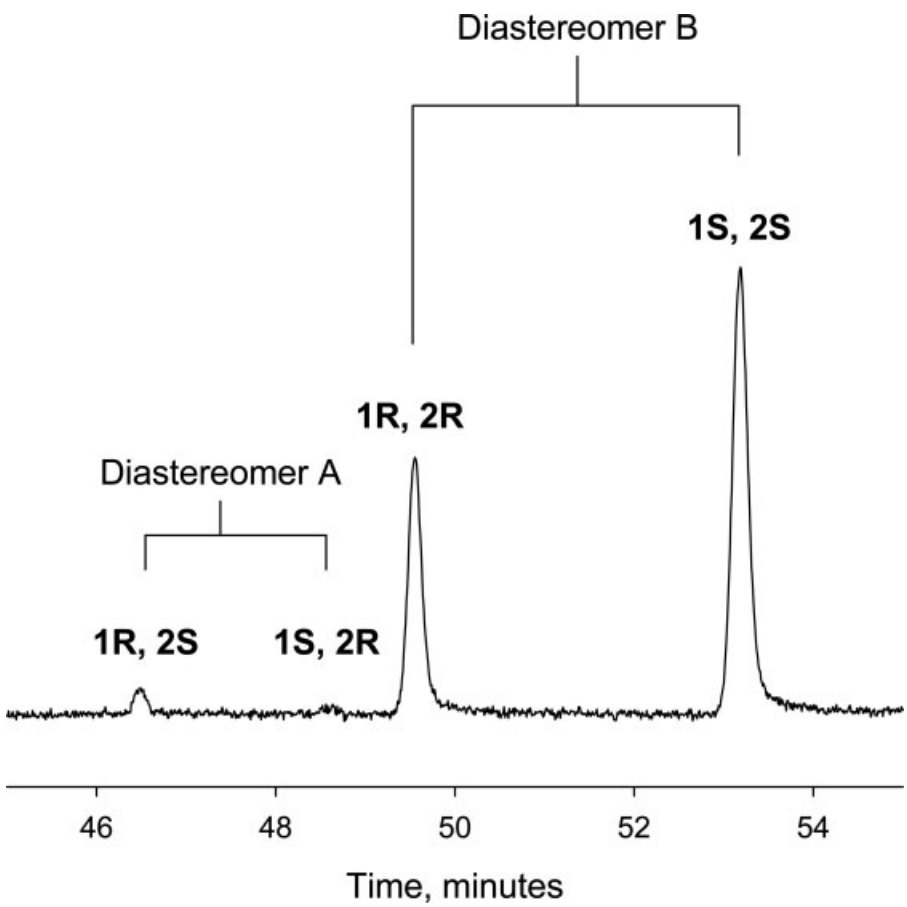

Fig. 4. GC-MS chromatogram (selected ion mode; *m/z* 128, 168) depicting the distribution of tiradimenol enantiomers formed from racemic triadimefon after in vitro exposure to trout liver microsomes for 480 min.

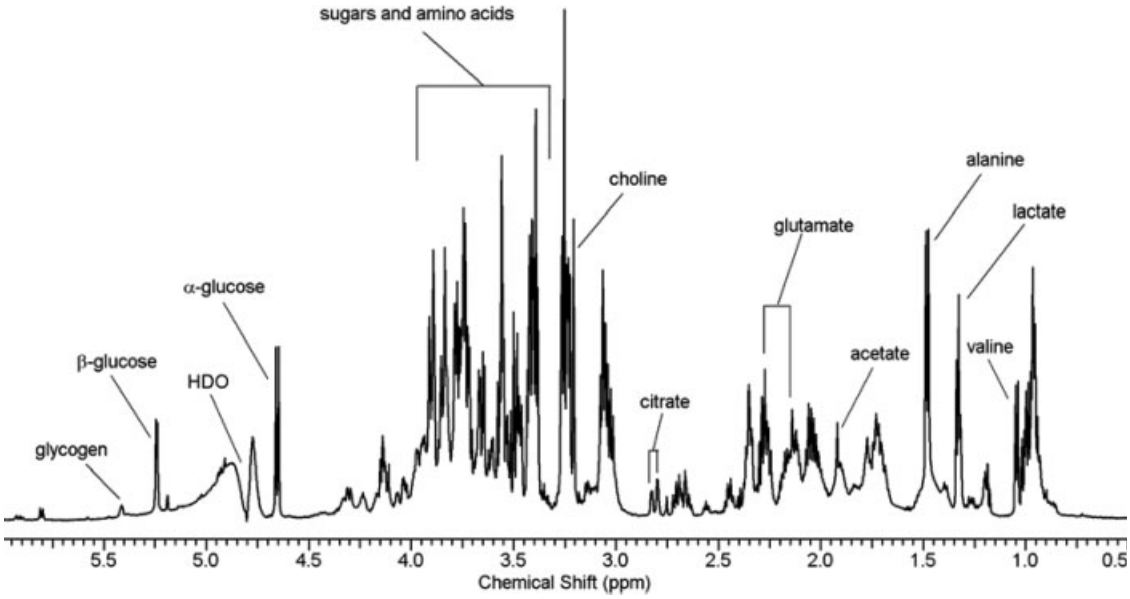

Fig. 5. One-dimensional ^{1}H NMR spectrum (800 MHz) of a typical trout liver extract used in the metabolomics study. "HDO" indicates the resonance produced by residual water in the sample.

peak had to be 1*R*,2*S* (46.5 min) and by default the second peak was 1*S*,2*R* (48.4 min). Following the same logic, the remaining two peaks were determined to be 1*R*,2*R* (49.6 min) and 1*S*,2*S* (53.2 min), respectively.

It is obvious from Figure 4 that there are large differences in triadimenol stereoisomer product distribution after triadimefon exposure to trout liver microsomes relative to that of the standard (see Fig. 2). Using eq. 2, the EF measured for each of the four triadimenol stereoisomers after metabolism for 480 min was: 0.06 (1*R*,2*S*), 0 (1*S*,2*R*), 0.38 (1*R*,2*R*), and 0.56 (1*S*,2*S*).

$$\text{EF } x = \frac{[\text{enantiomer } x]}{[\text{sum of 4 enantiomers}]} \quad (2)$$

These EFs remained relatively unchanged throughout the course of the metabolism study (48 h). The greater abundance of the 1*S* triadimenol stereoisomers (1*S*,2*S* and 1*S*,2*R*; sum EF = 0.56) formation relative to the 1*R* stereoisomers (1*R*,2*S* and 1*R*,2*R*; sum EF = 0.44) formation at 480 min is consistent with the conclusion that the *S*-(+)-triadimefon is metabolized more quickly than the *R*-(−) (see Fig. 3); this assumes that 1*S*,2*S* and 1*S*,2*R* can only arise via the metabolism of *S*-(+)-triadimefon. However, these rates for triadimefon in trout microsomes are not very different, which may relate to the fact that the toxicity of the two enantiomers of triadimefon to fungi are also not very different.[21] The EF of zero of the (1*S*,2*R*) triadimenol stereoisomer indicates no production of this most fungitoxic stereoisomer in trout microsomes, although a very small amount—below the level of quantitation—is indicated in the chromatogram of Figure 4. This may initially appear surprising, but unpublished work by the authors has shown that the relative proportions of the four stereoisomers of triadimenol produced by metabolism of triadimefon can vary greatly from species to species. Since it is known that triadimenol diastereomer A is 10 times more acutely toxic to rats (LD_{50}) than is diastereomer B,[8] the stereoselective formation of triadimenol is an important issue for both human health and ecological risk assessment. Incidentally, the relatively high levels of triadimefon exposure (up to about 40 μM or 1.2 mg/kg) to the microsomes were necessary, as shown by the distribution of triadimenol peak areas in Figure 4; e.g., the peaks of diastereomer A are below quantitation levels. Lower exposure levels would have required unreasonably long exposure times for measureable triadimenol GC peaks to develop.

Based upon the distribution of triadimenol stereoisomers observed in these liver metabolism studies, it can be concluded that the reductive enzyme preferentially delivers the hydride from the *si*-face of the *R*-(−) enantiomer and the *re*-face of the *S*-(+) enantiomer to yield the *R* and *S* alcohol, respectively. Interestingly, this follows both Prelog and anti-Prelog specificity,[24] respectively, and suggests that two separate carbonyl reducing enzymes may be involved in the formation of triadimenol from triadimefon.

Metabolomics: The Effect of Chirality on Responses to Triadimefon Exposure

As described earlier (experimental section), juvenile rainbow trout were exposed (via gavage) to either one of the triadimefon enantiomers or the racemate at two dose levels, 144 and 720 mg/kg/day, and over two time durations, 24 and 48 h. Livers were then excised and extracted using a perchloric acid-based procedure. Subsequent ^{1}H NMR analysis of the extracts revealed a number of metabolites in significant abundance (see Fig. 5), including

numerous amino acids, organic acids, and carbohydrates (i.e., glycogen and glucose). This NMR spectrum is typical of those obtained for the metabolomic analyses conducted in this research.

After spectral processing and binning, the complete dataset for these experiments was assessed using principal components analysis (PCA).[25] PCA is an unsupervised multivariate modeling approach that is useful for determining where the greatest variation lies in the dataset. This variation is captured through the creation of new variables or components that are based on the original variables (or bins in this case). Typically, the variation in the dataset cannot be captured by one component alone, making multicomponent models necessary. In this case, components are derived in such a way that the first component captures the greatest variation in the dataset with the subsequent components capturing progressively less. For metabolomic studies, in practice, the first two or three components are typically most useful.

Plotting the positions of the individual spectra (based on the intensities of the bins) in a coordinate system defined by these components is useful for determining similarities and differences among the spectra (i.e., among the metabolite profiles of the fish livers) relative to the different exposure classes (e.g., control vs. exposed). In such a plot, called a scores plot, a group of fish that all display a particular liver metabolite profile (e.g., the control fish) will cluster together in a particular area of the plot, while another group that displays a different profile (e.g., the exposed fish) will also cluster together, but in a separate area. Thus, one can readily establish whether or not differences exist between the classes to determine if the exposure has produced a detectable response.

Using this approach with the data set generated by this research provided a means to determine whether or not the fish responded differently to the different enantiomers or the racemate of triadimefon, and how these responses changed with the duration of exposure. For example, Figure 6A shows a two-dimensional scores plot of the first two components from a PCA model built using the binned 24 h spectral data acquired from the livers of the fish in the control and high dose classes. Notice that while most of the *S*-(+) enantiomer exposed fish are grouped close to the controls, the fish exposed to the *R*-(−) enantiomer and the racemate are grouped separately. This grouping of the *S*-(+) enantiomer exposed fish with the controls indicates a continued similarity in the metabolite profiles of these two classes after 24 h of exposure. In addition, the separation observed between these classes and the *R*-(−) enantiomer and racemate-exposed fish reflects a change in the liver metabolite profiles of these latter two classes of fish as a result of exposure. After 48 h of exposure, however, this grouping of the *S*-(+) enantiomer-exposed fish with the controls no longer exists (Fig. 6B). At this time, the *S*-(+) enantiomer-exposed fish have become grouped with the *R*-(−) enantiomer and racemate-exposed fish. There was no significant difference in trout responses between controls and the low dose (144 mg/kg/day) classes for either enantiomer or the racemate at either sampling time. This begs the question of what is the appropriate level of in vivo exposure of triadimefon for metabolomics studies. Observation of results in terms of differential metabolite formation depends upon exposure concentration and time. It is possible that enantioselectivity would have been observed at the lower concentration with longer duration of exposure. However, higher concentrations of chemical are often used over shorter durations, and the information gleaned from such exposures is often useful for determining the nature of toxicity. In this case, that approach showed that the two enantiomers of triadimefon produce different metabolic responses as a function of exposure duration.

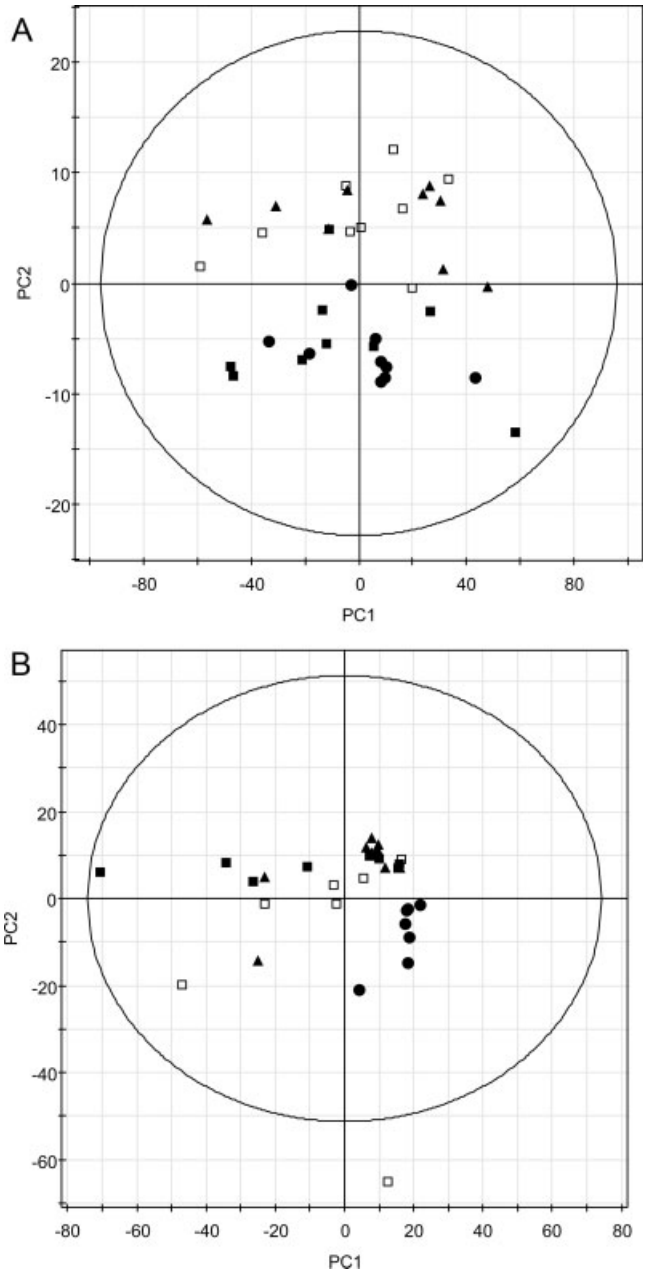

Fig. 6. Two-dimensional PCA scores plots from the (**A**) 24 h and (**B**) 48 h high dose (720 mg/kg) triadimefon exposures. Key: controls (●), racemate (▲), *R*-(−)-enantiomer (□), *S*-(+)-enantiomer (■).

The metabolomic results highlight the significant usefulness of this approach for relating temporal responses in endogenous metabolite patterns to chemical exposures. However, if possible, it is important to anchor such responses to the more classical metabolism measures. For example, the EF data presented in Figure 3 allow for some speculation as to why this effect is seen. Specifically, the delay in the response of the fish to the *S*-(+) enantiomer (Fig. 6A) suggests a difference in the rate of metabolism of this enantiomer compared to that of the *R*-(−) enantiomer and the racemate. As shown in Figure 3, the negative slope of the line, corresponding to a decrease in EF of triadimefon, indicates that the *S*-(+) enantiomer is converted to triadimenol in trout liver microsomes more rapidly than the *R*-(−) enantiomer. As this conversion likely provides a path for reducing the toxicity of triadimefon, the higher rate observed for the *S*-(+) enantiomer might explain why, in the metabolomic analysis, the fish exposed to this enantiomer are still grouped with the controls after 24 h of exposure (Fig. 6A); i.e., the faster metabolism of the *S*-(+) enantiomer reduces its toxicity more than the slower metabolism of the *R*-(−) enantiomer, keeping the *S*-(+) enantiomer-exposed fish more like the controls.

Toxicity reduction is only possible if the products of the *S*-(+) transformation are less toxic than those of the *S*-(+) enantiomer itself. Although adequate data is not available to prove this unequivocally, the fact that diastereomer B is about 10-fold less toxic to rats than diastereomer A, and that the major product of *S*-(+) metabolism, the 1*S*2*S* enantiomer, is one of the components of diastereomer B (see Fig. 4), suggests transformation to less toxic products. In support of this, Figure 4 shows very little production of the other *S*-(+)-triadimefon metabolism product, the 1*S*2*R* enantiomer, which is known to be the most toxic of the four triadimenol enantiomers, at least to fungi.

As the exposure is continued for an additional 24 h, however, the capacity to metabolize the *S*-(+) enantiomer may become overwhelmed, eventually producing an effect similar to the *R*-(−) enantiomer and the racemate. As a result, the *S*-(+) enantiomer is observed to group with the *R*-(−) enantiomer and the racemate in Figure 6B. While these conclusions are based on compelling rationalization, further research will be required to fully test this explanation.

IMPLICATIONS

Preliminary work in the authors' laboratory shows that the stereochemistry of conversion of triadimefon to triadimenol varies with metabolism of the exposed organism; this research is continuing. Since it is known that the stereoisomers of triadimenol differ in their toxicities to fungi[23] and to rats,[8] the biological activity of the triadimenol that is formed probably depends on the relative abundance of its particular stereoisomers. Interspecies comparison of the production of metabolite enantiomers by chiral analysis should be useful in probing the metabolism of pesticide-exposed organisms as well as improving cross-species extrapolation for risk assessment. Finally, in addition to knowledge about the stereochemical exposure parameters, accurate risk assessment would require measurement of acute and chronic toxicity of the separate enantiomers of both triadimefon and triadimenol for each organism of concern. Considerable effort and expense is required to develop such data, but it is a feasible endeavor. Toxicity measurements for the separate enantiomers of chiral pharmaceuticals is the prescribed practice, and such measurements have also been conducted for a few enantiomers of chiral pesticides.[26–28]

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