In Vitro Metabolism of the *N*-Alkyl-*N*-(5-isothiazolyl)- and *N*-(Alkylisothiazolin-5-ylidene)phenylacetamides. Evidence of Proinsecticidal Activity

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In vitro studies have demonstrated that *N*-(4-chloro-3-methyl-5-isothiazolyl)-*N*-methyl-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide (**2a**) undergoes NADPH-dependent metabolism, which is catalyzed by monooxygenase enzymes, in rat liver microsomes. The primary metabolite in rat was found to arise from ring-methyl hydroxylation, while *N*-demethylation to give *N*-(4-chloro-3-methyl-5-isothiazolyl)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide (**1**) was also observed to occur, but at a slower rate. In microsomal proteins prepared from tobacco budworm midgut tissues, the reverse was observed, as **1** is the predominant metabolite, while ring-methyl hydroxylation occurs at a slower rate. The overall rate of metabolism in trout liver microsomes was found to be ≈50-fold slower than in rat and afforded **1** as the predominant metabolite. Metabolism studies conducted on the *N*-alkyl-*N*-(5-isothiazolyl)- and *N*-(alkyl-isothiazolin-5-ylidene)phenylacetamides (**2** and **3**) have shown that the ring-alkylated isomers **3** were converted to **1** more rapidly than isomers **2** in all three species. In general, the rate of conversion to **1**, or bioactivation, increased with increasing radical or carbocation stability of the alkyl group in rat and trout liver. In tobacco budworm, however, bioactivation was highest in the ethyl and *n*-propyl analogues. The ratio of bioactivation in tobacco budworm to that in trout, used as a predictor of selectivity, was observed to be highest with the methyl group.

Keywords: *Isothiazolylphenylacetamide; isothiazolinylidenephenylacetamide; metabolism; proinsecticide*

INTRODUCTION

The *N*-(isothiazol-5-yl)phenylacetamides are members of a new class of compounds possessing excellent broad spectrum activity against a wide range of agricultural insect pests (Hackler et al., 1995). Their mode of action is through inhibition of mitochondrial electron transport activity at complex I (Thoreen et al., 1996; Johnson et al., 1996). Developing commercial agrochemicals having this ubiquitous mode of action and possessing favorable environmental attributes has been challenging. In a previous paper (Samaritoni et al., 1997), we reported that *N*-alkylation of **1** affords isomers **2** and **3** (Figure 1), both of which were found to be significantly less toxic to fish while retaining efficacy toward targeted insect pests. Experimental evidence was presented which suggests that **2** and **3** function as "proinsecticides" requiring in vivo dealkylation to give the highly active **1**. In the present paper we further substantiate the role of metabolic activation of **2** and **3** in tobacco budworm (TBW) larvae midgut, trout liver, and rat liver microsomal preparations through measurements of in vitro metabolism and intrinisic activity at the target site. The preparation and characterization of analogues **2a**−**g** and **3a**−**g** have been reported elsewhere (Samaritoni et al., 1997).

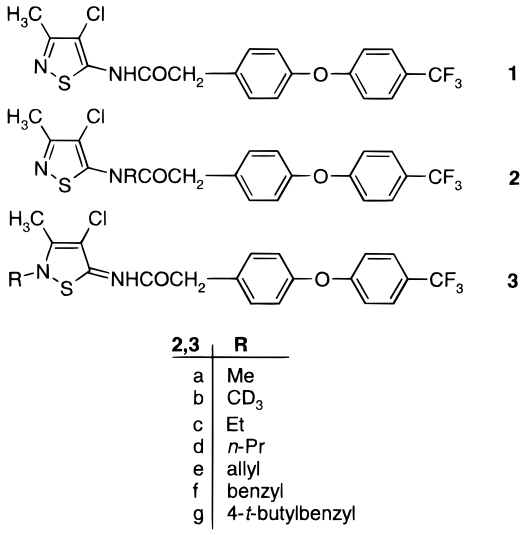

Figure 1. Isothiazolyl- and isothiazolinylidenephenylacetamides.

EXPERIMENTAL PROCEDURES

Reagents. All analogues were dissolved in ethanol at 10 mM and stored at −20 °C before use. NADH (nicotinamide adenine dinucleotide), NADPH (nicotinamide adenine dinucleotide phosphate), isocitrate, isocitrate dehydrogenase, and bovine serum albumin (BSA) were obtained from the Sigma Chemical Co., St. Louis, MO. Piperonyl butoxide (PBO) was obtained from Chem Services, West Chester, PA. An insect artificial diet was obtained from Southland Products, Stoneville, MS. All other chemicals were of reagent grade. Solvents were all of HPLC grade. Fresh bovine hearts were obtained from a local slaughterhouse (Shoup Processing, Inc., Frankfort, IN). Live rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish market.

Tobacco Budworm (*Heleothis virescens*) Larvae Toxicity Bioassay. Ten third-instar larvae (50−70 mg each) were used for each concentration of the compound tested. The larvae were kept in separate Petri dishes with an artificial diet. For topical assays, 1.0 μL of the compound dissolved in

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acetone was applied along the dorsal surface of the larvae. For injection bioassays, 0.5 μL of the compound dissolved in DMSO was injected into the midventral section of the larvae using a 10 μL Hamilton syringe fitted with a 30 gauge needle. Controls received carrier solvent alone. LC_{50} values were determined according to the Spearman−Karber method 48 h after application of the experimental compound (Hamilton et al., 1978). For experiments employing PBO, 1 μL of neat PBO was applied to the dorsal surface of the larvae 2−4 h prior to application of the test compound. Less than 5% mortality was observed for the control groups.

In Vitro Metabolism by Microsomal Proteins Prepared from Tobacco Budworm Midgut, Rat Liver, or Trout Liver. For insects, ≈100 last instar tobacco budworm larvae (400−500 mg each) were starved overnight and dissected. The midguts were removed and washed with buffer (0.2 M sucrose, 0.15 M KCl, 0.05 M potassium phosphate, pH 7.4) to remove any remaining food contents. The cleaned midguts were placed in fresh buffer at 4 °C and homogenized using a glass−Teflon pestle tissue grinder. The homogenate was centrifuged at 9000*g* (4 °C) for 15 min to remove unbroken cells, nuclei, and mitochondria. The supernatant was collected and centrifuged at 100000*g* for 1 h. The resulting pellet was washed by suspending in fresh buffer and centrifuging for 20 min at 200000*g*. The resulting pellet, consisting primarily of proteins and lipids comprising a microsomal fraction, was suspended into ≈1 mL of buffer, and the protein concentration was determined using the method of Lowry et al. (1951). Test compounds were added to a final concentration of 25 μM to a suspension of the microsomal protein (1.0 mg/mL) in incubation buffer consisting of 0.15 M KCl, 0.05 M Tris, 0.01 M $MgCl_2$, and 1% BSA, pH 7.4, plus a NADPH regenerator system consisting of 0.007 M isocitrate and 0.5 unit/mL isocitrate dehydrogenase. After a 1 min incubation at 30 °C, the reaction was initiated by the addition of 0.5 mM NADPH (final concentration). Samples (0.5 mL) were taken after 0, 15, 30, 45, and 60 min of incubation and pipetted into 5 mL of methylene chloride containing [^{14}C]aldrin (50 000 dpm) as an internal standard, quickly vortexed, and placed on ice. The samples were then centrifuged to separate the two phases, and the organic phase was removed. This extraction procedure was repeated twice, and the organic fractions were combined and evaporated to dryness under nitrogen. The extraction procedure was determined to remove >99% of total radioactivity from the aqueous phase. The residue was brought up into 200 μL of ethanol, and 100 μL was injected into the HPLC using an autosampler.

For metabolism studies employing microsomes prepared from trout livers, a similar protocol was employed, except that total protein concentration was 2.0 mg/mL, sample size was 1.0 mL, and the incubation temperature was 22 °C. In some cases, the supernatant fraction obtained after centrifugation of the homogenate at 9000*g* for 15 min (S-9 fraction) was used as the source of protein. No differences were observed in either the rate or profile of product formation when using the S-9 fraction, compared to using a microsomal fraction. For metabolism studies using microsomal protein prepared from male adult Sprague−Dawley rat livers, procedures were similar to those described above, except that the incubation temperature was 37 °C, the protein concentration was 0.84 mg/mL, the substrate concentration employed was either 25 or 100 μM, and no BSA was added to the incubation buffer.

HPLC Conditions. Reversed phase HPLC was performed using a Waters Model 600 multisolvent delivery HPLC coupled to a Waters 990 photodiode array detector and a Flo-One\Beta radioactive flow detector. The solvent-to-scintillant ratio was 4:1, resulting in an overall counting efficiency of >87%. The column employed was a Supelco LC-18-DB (5 μm particle size, 25 cm long × 4.6 mm i.d.). The solvent conditions were initially 30% acetonitrile/water + 10 mM trifluoroacetic acid (TFA), going to 60% acetonitrile + 10 mM TFA, employing a 15 min linear gradient at a flow rate of 1.5 mL/min. The gradient was then increased from 60% to 100% acetonitrile + 10 mM TFA using a linear gradient over 15 min. Chromatography continued isocratically for 7 min at 100% acetonitrile + 10 mM TFA. [^{14}C]Aldrin, the internal standard used to determine recovery of material, eluted at about 20 min and was monitored by the radioactive flow detector. A standard curve was constructed to quantify the peak area corresponding to **1**. Retention times of the substrate and metabolites shown in Figure 2 varied slightly from sample to sample due to chromatography conducted using different batches of C_{18} columns. Identity of peaks were confirmed using appropriate standards.

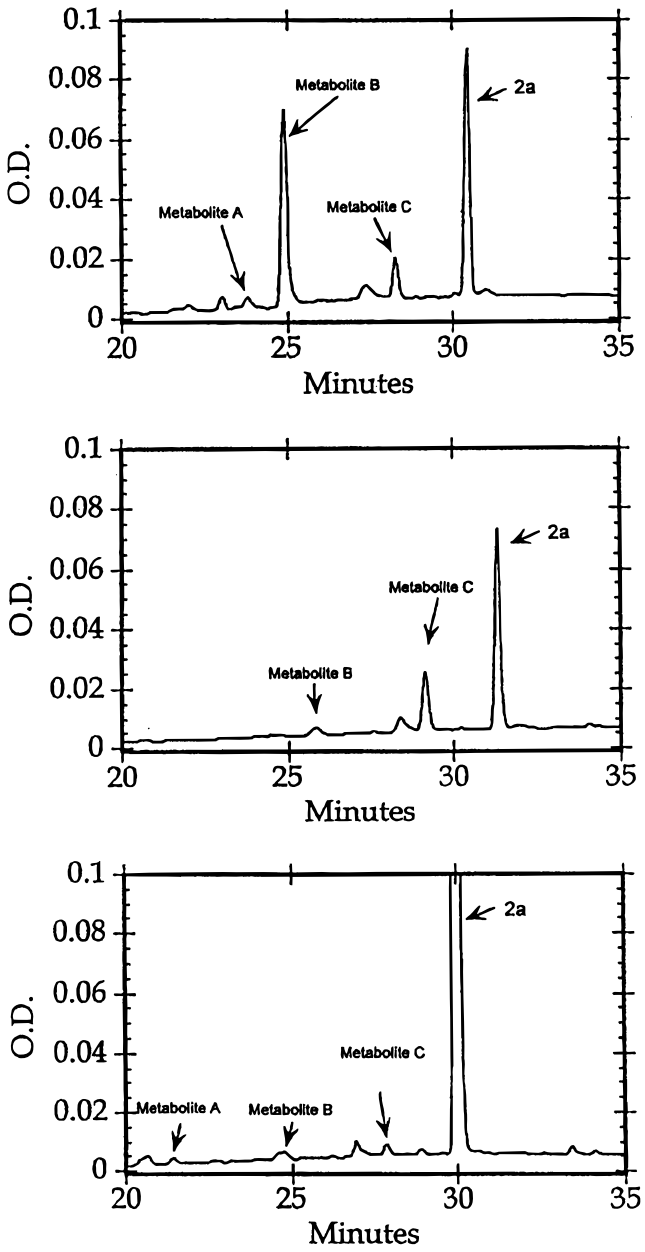

Figure 2. HPLC profile of metabolites of **2a** formed by a 30 min incubation with microsomal proteins prepared from rat liver (top), TBW midguts (middle), and trout livers (bottom), respectively.

Toxicological Bioassays against Trout. Experimental compounds were tested for lethality against rainbow trout (*O. mykiss)* by The Dow Chemical Environmental Toxicology Research Laboratories in Midland, MI. Each compound was tested at 0.1, 1, 10, and 100 ppb against five fish (4−6 months old) in 5 L of water maintained at 12 °C with aeration. Observations for mortality were recorded at 4, 24, 48, 72, and 96 h. For comparative purposes, mortality data are presented as an index value ranging from 0 to 25, which represents the level and rate at which the onset of toxicity is achieved. The index is calculated by summing the number of mortalities at each of the five different time points for a given concentration (i.e., from 0 to 25). The *average* of the mortality sums at all

Table 1. Bioassays of 1, 2a, and 2b against TBW Larvae[a]

compd	MET_{bov} IC_{50} (nM)	LD_{50} TBW_{inj} (μg/larva)	LD_{50} TBW_{inj} + PBO (μg/larva)
1	2.5	0.02	0.005
		0.01−0.02	0.004−0.007
2a	63	0.05	0.12
		0.04−0.07	0.02−0.64
2b	70	0.27	0.56
		0.19−0.38	0.39−0.80

[a] Range of values under LD_{50} indicates 95% confidence intervals. MET_{bov} is the IC_{50} value for 50% inhibition of mitochondria electron transport activity.

four concentrations is then taken as the index. Using this index, compounds having low values represent those that produced a low level of toxicity over a long period of exposure, whereas compounds having a high value were highly toxic in a short period of time.

Diet Egg Bioassays against TBW Larvae. Experimental compounds were serially diluted to 50.0, 12.5, 3.13, 0.78, and 0.20 ppm using 90:10 water/acetone (containing 0.025% Triton X-100). Test units consisted of a 4 cm diameter (1 oz.) plastic cup, containing 7.5 g of insect diet. A 0.25 mL aliquot of compound was added to each cup and allowed to dry. Each test unit was infested with 10 *H. virescens* eggs, capped, and stored at 25 °C and 50% relative humidity. Each dose consisted of five replicates. Observations for mortality were made between 4 and 6 days after treatment. LC_{50} values and 95% confidence intervals were calculated using the Spearman−Karber statistical program.

Mitochondrial Electron Transport Measurements. Serial dilutions of the compounds to be tested were prepared in ethanol. Mitochondria prepared from bovine hearts (Keeley et al., 1969) were suspended at a protein concentration of 0.05 mg/mL in 25 mL of MET buffer consisting of 0.2 M sucrose, 1 mM EDTA, 20 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris, pH 7.4, and incubated at 37 °C for 2−5 min to ensure temperature equilibration. Three milliliters of the reaction mixture was transferred to each of six cuvettes. A 15 μL aliquot from five serial dilutions of the test compound was added to each of five corresponding cuvettes. Ethanol was used as a control. The enzyme assay was initiated by the addition of 10 μL of 48 mM NADH (prepared in 1% bicarbonate) to give a final concentration of 0.16 mM. Optical density was measured as a function of time (every 30 s for 5 min) using a Shimadzu UV-265 spectrophotometer coupled to a temperature-controlled six cell kinetics unit set at 37 °C, with buffer plus protein in the reference cell. Rates of NADH oxidation were linear over time with r^2 values >0.95 for the rate of reaction in the absence of inhibitor. IC_{50} values were calculated using inhibitor concentrations that gave between 20% and 80% inhibition.

RESULTS AND DISCUSSION

Prior to the metabolism studies, we conducted a series of topical and injection bioassays with **1**, **2a**, and **2b** against TBW larvae in the presence and absence of the synergist PBO. The synergistic activity of PBO on many different compounds is derived from its broad spectrum inhibition of insect cytochrome P450s (Yamamoto, 1973). If metabolic activation is required for these analogues to fully express their insecticidal activity, then coapplication of PBO should reduce the toxicity of these compounds against insects. Compounds not requiring metabolic activation would either be unaffected or made more potent by coapplication of PBO. The results of these bioassay experiments are shown in Table 1.

When injected into TBW larvae without PBO, **1** is observed to be highly active, with an LD_{50} value of 0.02 μg/larva. This is consistent with the high potency of **1** to inhibit mitochondrial electron transport activity. Upon coapplication of PBO, the toxicity of **1** increased 4-fold. This increase in toxicity is significant at the 95% confidence level and is consistent with **1** not requiring metabolic activation but instead possibly being metabolized by monooxygenase enzymes to a less active metabolite.

Compound **2a** is only 2.5-fold less toxic to TBW larvae when injected into the insect in the absence of PBO compared to **1**, even though it is 25-fold less active at inhibiting mitochondrial electron transport activity (see Table 1). When TBW larvae are pretreated with PBO prior to injection, the toxicity of **2a** is observed to *decrease* 2.5-fold, to 0.12 μg/larva. (Although the decrease in toxicity was numerically 2.5-fold, this was not significant at the 0.05 level, due to a slower onset of toxicity in the larvae treated with **2a** and PBO.) This decrease in toxicity would be expected if **2a** required metabolic activation to fully express its toxicity in TBW larvae. When the deuterated analogue **2b** was tested for its toxicity, it was observed to be 5-fold less toxic compared to **2a**, even though both of these molecules have equivalent intrinsic activities to inhibit mitochondrial electron transport activity. Treatment of TBW larvae with PBO prior to exposure to **2b** *decreased* the toxicity of **2b** by a factor of 2, further suggesting that this molecule relies on metabolic activation for at least some of its observed insecticidal activity. This is the same level of protection that PBO afforded to **2a**.

To further understand the metabolic reactions acting on these molecules in vivo, we prepared microsomal protein fractions from various species and measured the rates of in vitro metabolism of **1**, **2a**, and **2b**. In the rat system, we observed a moderate rate of metabolism of **1** and a high rate of metabolism of both **2a** and **2b**. When measuring the rate of metabolism by disappearance of substrate with time, we obtain values of 0.39, 1.52, and 1.60 nmol min^{-1} (mg of protein)$^{-1}$ for **1**, **2a**, and **2b**, respectively. The in vitro metabolism of all three isothiazolylamides is dependent on the presence of NADPH. Addition of clotrimazole (10 μM) to the incubation mixture completely abolished metabolism, and the addition of SKF-525A (100 μM) inhibited metabolism by >90%. These two compounds are general inhibitors of rat liver monooxygenase enzymes (Sheets et al., 1986; Correia, 1995). Inhibition of metabolism of all three isothiazolylamides by these inhibitors suggests that the metabolic reactions measured in vitro are catalyzed primarily by monooxygenase enzymes.

Both **2a** and **2b** were observed to be metabolized at approximately equivalent rates in the rat liver system. The lack of a deuterium isotope effect on the rate of metabolism suggested that *N*-demethylation is not a dominant route of metabolism of these compounds in rats. The profile of metabolites formed from incubating **2a** for 30 min with microsomes prepared from untreated rat livers is shown in Figure 2 (top). The abundance of metabolite A was too low to obtain sufficient material for identification. However, it has a retention time by HPLC identical with that of the aryloxyphenylacetic acid, which would result from hydrolysis of the amide linkage of the ether side chain. Without spectral data, however, this identification is only putative.

Metabolite B was formed at the highest rate in rat and was purified from a separate experiment employing a larger incubation reaction. Its mass and NMR spectra are shown in Figures 3 and 4, respectively. The mass spectrum of metabolite B shows a molecular ion at 457, 16 Da greater than the substrate molecule, suggesting

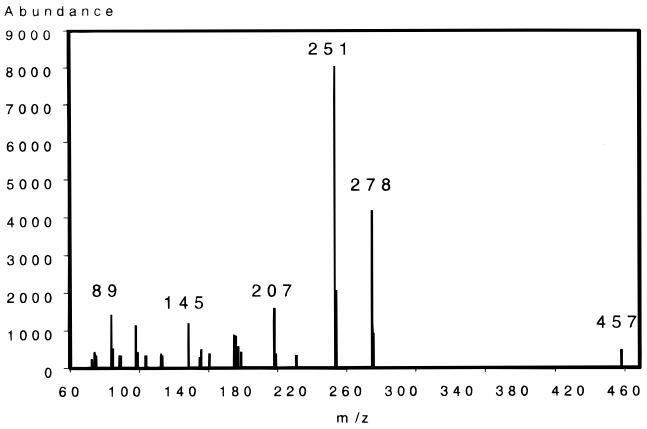

Figure 3. Mass spectrum of metabolite B of **2a**, showing molecular ion at 457 Da.

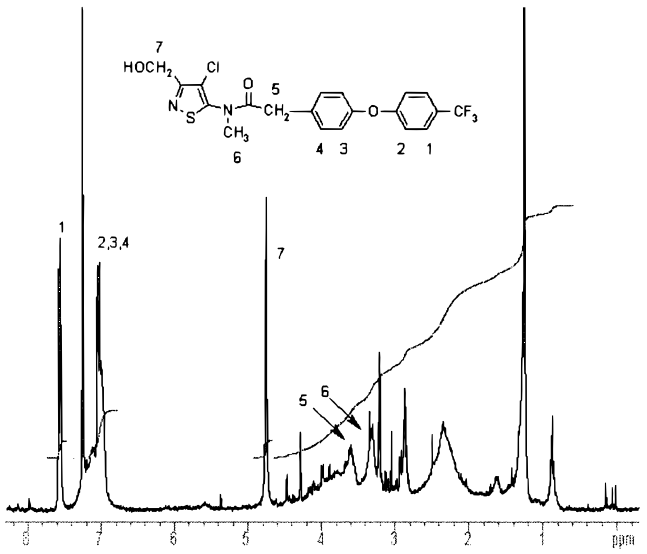

Figure 4. 300 MHz ^{1}H NMR spectrum of metabolite B of **2a** in $CDCl_3$ at room temperature (reference TMS at 0.0 ppm). Numbers represent corresponding protons shown on the structure given.

that the molecule was transformed through a hydroxylation reaction. Its proton NMR spectrum suggests that metabolite B is the result of hydroxylation on the isothiazole methyl substituent. The aromatic region of the spectrum is identical with that of **1**. The methyl resonance at 2.48 ppm, which corresponds to the isothiazole methyl group in the parent molecule **1**, is absent and a new peak appears at 4.75 ppm, corresponding to the two protons of the hydroxymethyl group. Two broad resonances centered at 3.3 and 3.6 ppm represent the NCH_3 and $COCH_2$ groups, respectively. Redissolving the sample in DMSO-d_6 and obtaining the ^{1}H NMR spectrum at 20 °C intervals from room temperature to 80 °C showed progressive sharpening of the peaks of the metabolite due to more rapid rotation about the amide C−N bond. The identity of metabolite B is therefore confirmed as the hydroxymethyl analogue (see Figure 4). Metabolite B was isolated, purified, and tested for its potency to inhibit mitochondrial electron transport activity. The IC_{50} value was determined to be 132 nM, which is 2-fold less active than the parent molecule **2a**. This route of metabolism of **2a** thus represents a detoxification pathway.

Metabolite C has retention times by HPLC and GC identical with those of the demethylated analogue, **1**. Its mass spectrum is also identical with **1**, showing a reduction in molecular weight of the substrate by 15, indicative of loss of a methyl group. We therefore assign the identity of metabolite C as **1**. This molecule is 25-fold more potent at inhibiting mitochondrial electron transport activity compared to **2a** (see Table 1). The formation of metabolite C represents a bioactivation pathway. A general schematic pathway for the metabolism of **2a** is presented in Figure 5.

Only metabolites A and B were formed when **2b** was incubated with rat liver microsomes. Metabolite C was absent from all time points (data not shown), which is consistent with the expected deuterium isotope effect on the rate of the demethylation reaction (Wilkes et al., 1991). When we incubated **2a** with microsomes prepared from the midgut tissues of TBW larvae, we observed substantial differences in both the rate of metabolism and the profile of products formed compared to that which occurs in the rat liver system (see Figure 2, middle). The overall rate of metabolism was slower, and metabolite A was not observed. In the TBW system, the more potent MET inhibitor, metabolite C, is the predominate metabolite, while metabolite B, which is ≈50-fold less active at inhibition of mitochondrial electron transport activity compared to metabolite C, is produced at a much lower level. This differential production of the more intrinsically active metabolite could account for the good activity of **2a** against TBW larvae.

Compound **2a** was metabolized ∼33% more rapidly than **2b** by microsomal protein from TBW midgut [0.20 vs 0.15 nmol min^{-1} (mg of protein)$^{-1}$]. Metabolites B and C are produced using **2b** as the substrate, except that the proportions of these two metabolites are about equal. This is due to both an ≈2-fold decrease in the rate of *N*-demethylation of the deuterated compound and an ≈2.5-fold increase in the rate of ring methyl hydroxylation. Thus, in TBW as well as in rats, *N*-demethylation is a slower reaction in vitro using the deuterated substrate compared to the normal methylated compound.

To better understand the toxicity of these compounds in fish, we prepared S-9 and microsomal protein fractions from rainbow trout livers and incubated the three isothiazolylamides (**1**, **2a**, and **2b**) with these preparations. Three metabolites were produced by these in vitro preparations, the same three seen in rat liver preparations according to HPLC. The rates of formation of these metabolites in fish are substantially lower compared to those in rat and TBW. The overall rates of metabolism were 0.007, 0.010, and 0.006 nmol min^{-1} (mg of protein)$^{-1}$ for **1**, **2a**, and **2b**, respectively. The rates of metabolism were similar for both S-9 and microsomal preparations, and the same metabolites were formed in the S-9 and microsomal preparations. On the basis of coretention on HPLC, metabolite C (*N*-demethylated product) is the major metabolite of **2a**, followed by metabolites B and then A (see Figure 1, bottom).

The overall rate of metabolism of these compounds by trout liver microsomes is ≈50-fold slower than that seen in rat liver microsomes. The same metabolites were formed from **2b**, except that the rate of *N*-demethylation was reduced even further by ≈5−10-fold. Thus, very little bioactivation was found to occur with the deuterated compound, which is consistent with the more favorable fish toxicity profile of **2b** compared to **2a** and with the results we obtained using either rat liver or TBW midgut microsomes. The activation reac-

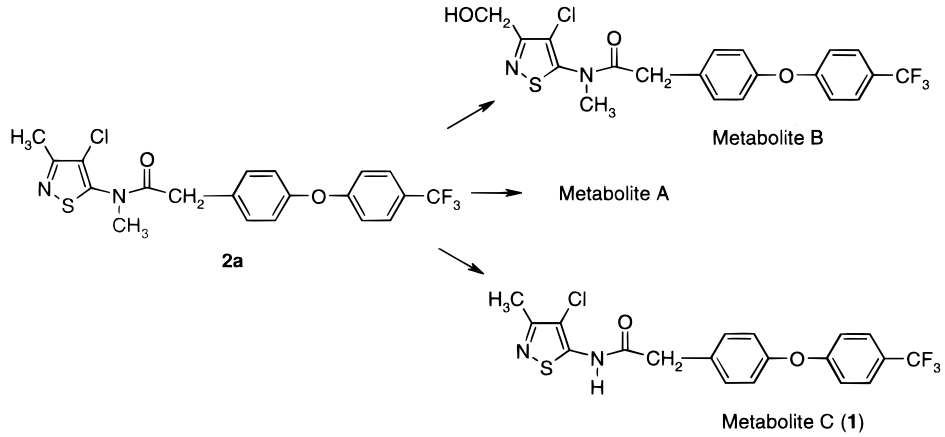

Figure 5. Proposed route of metabolism of **2a** by microsomal proteins prepared from rat liver, TBW midgut, or trout livers. Metabolite A has the same retention time as the aryloxyphenylacetic acid resulting from cleavage of the amide linkage of **2a**, but insufficient spectral data are available to give a definitive structural assignment.

Table 2. Summary of in Vitro and in Vivo Activities for 1−3

R	compd	MET IC_{50} (nM)	TBW LC_{50} (ppm)	fish toxicity index[a]
H	**1**	2.5	0.3	25
amide-N				
methyl	**2a**	63	2.2	12.3
methyl-d_3	**2b**	70	2.8	9.3
ethyl	**2c**	264	1.0	10.3
propyl	**2d**	102	11.2	9.3
allyl	**2e**	430	2.9	14.0
benzyl	**2f**	215	3.0	14.3
tert-butylbenzyl	**2g**	2000	>50	0
ring-N				
methyl	**3a**	705	1.1	11.7
methyl-d_3	**3b**	702	2.8	6.7
ethyl	**3c**	1600	0.6	15.0
propyl	**3d**	1340	1.0	15.3
allyl	**3e**	923	0.4	18.3
benzyl	**3f**	859	2.5	12.7
tert-butylbenzyl	**3g**	2800	14.6	1.0

[a] See Experimental Procedures.

tion (i.e. formation of metabolite C) is most prominent in TBW microsomes, which is fortuitous since this is a major agricultural insect pest that is being targeted with this chemistry. Substitution of deuterium for the protons constituting the *N*-methyl function was found to greatly reduce the rate of formation of metabolite C in all three systems we studied.

To extend this study, we have studied the effect of various alkyl substitutions on **1** at either the amide nitrogen or the isothiazole ring nitrogen atom to affect intrinsic activity, insect pest toxicity, and fish toxicity. The results of these studies are shown in Table 2. The effect of nitrogen atom substitution of the isothiazolylamides on fish toxicity was variable. Clearly the parent molecule, **1**, was most toxic to fish, with a toxicity index of 25. Alkyl substitution at either the amide or ring nitrogen decreased the fish toxicity index to a range of 0−18.3, which represents a safening of the molecule toward fish to >100-fold. Associated with this safening was a concomitant decrease in the efficacy of the molecules against insects, with LC_{50} values increasing from 0.3 ppm for the parent molecule against TBW larvae to a range of 0.4−>50 ppm for the nitrogen-atom-substituted analogues.

To investigate the role of metabolism in bioactivation of the isothiazolylamides and to better understand the relationship between structure and rate of bioactivation, a series of experiments were conducted in which the rate

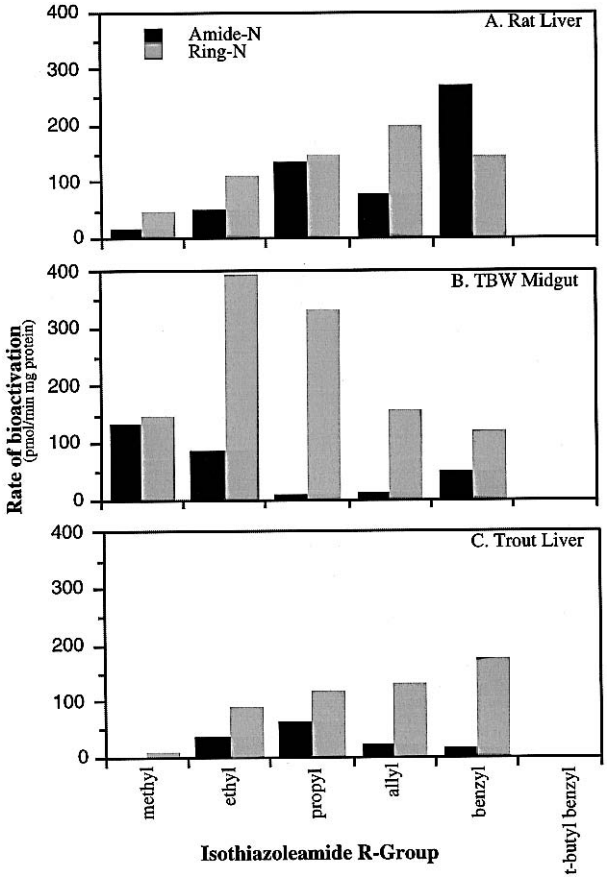

Figure 6. Rate of bioactivation of substrate, reflecting appearance of **1** in (A) rat liver, (B) TBW midgut, and (C) trout liver for amide nitrogen (solid bars) and ring nitrogen (shaded bars) substitutions of **2** and **3**.

of metabolism of each amide- and ring-nitrogen-substituted analogue was measured. These experiments were conducted in vitro with microsomal protein preparations from rat liver, fish liver, and TBW midgut tissues. Rate determinations were made by monitoring the time-dependent change in area of the HPLC peaks of the substrate and parent molecule, as detected by UV absorbance at 260 nm. The time-dependent disappearance of substrate represents overall metabolism, while the appearance of parent **1** represents bioactivation. The results of these experiments are shown in Figure 6.

When comparing the in vitro rate of conversion of the substrate to the active parent molecule **1** (i.e., bioacti-

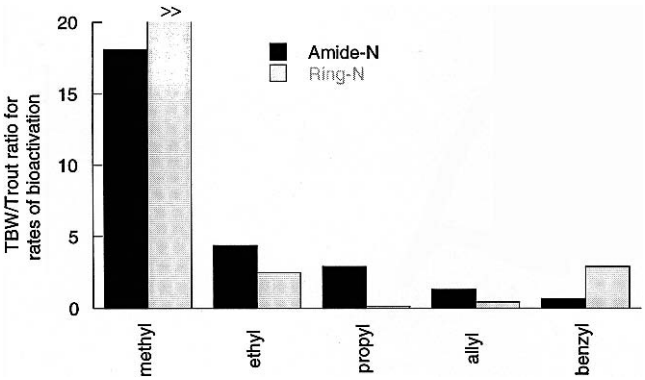

Figure 7. Therapeutic index of target efficacy and nontarget safety represented by the ratio of in vitro rates of bioactivation in TBW vs trout microsomal preparations for amide nitrogen (solid bars) and ring nitrogen (shaded bars) substitutions of **2** and **3**.

vation), we found that ring-substituted isothiazolylamides **3** are more rapidly bioactivated than the corresponding amide-nitrogen-substituted analogues for all three species. The one exception is the amide-benzyl substituent (**2f**) in rat liver microsomes (Figure 6A). There is no measured bioactivation of the *tert*-butylbenzyl substituent when it is positioned in either the amide- or ring-nitrogen positions for any of the species tested. Its low intrinsic activity as an inhibitor of mitochondrial electron transport activity accounts for its low toxicity toward fish. For the ring-nitrogen substituents, the rate of bioactivation generally increases with increasing radical or carbocation stability of the pro-group for rat and trout liver, with the *tert*-butylbenzyl substituents as exceptions. Bioactivation of ring-nitrogen analogues in TBW is highest with the ethyl and propyl analogues. The methyl, allyl, and benzyl analogues are all cleaved at slower rates.

To achieve the highest "therapeutic index" of activity against TBW with safety toward fish, it is required that the ratio of bioactivation rates in TBW/trout be high. We therefore plotted the ratio of the rate of bioactivation of the compounds in TBW and trout as a function of the chain length for both the ring- and amide-nitrogen-substituted isothiazolylamides. The results are shown in Figure 7. There is an inverse relationship between the ratio of bioactivation rate and the length/size of the R-group substituent. This relationship holds for both the amide- and ring-substituted analogues, except for the ring-substituted benzyl analogue. Therefore, the shorter alkyl substituents appear, in vitro, to have the highest potential for fish safety. Indeed, the fish toxicity data shown in Table 2 support this conclusion for the ring-nitrogen-substituted analogues, since the methyl analogue is generally safer to fish than the longer substituents. The *tert*-butylbenzyl analogues were not included in this analysis since they were not bioactivated at all. They also had the lowest intrinsic activity to inhibit mitochondrial electron transport activity. For these reasons, the *tert*-butylbenzyl analogues are the safest to fish of all the compounds tested, not causing any mortality at the highest concentration tested. For the same reasons, unfortunately, these compounds are not active in bioassays against TBW larvae.

CONCLUSIONS

N-Alkylated amides **2** and **3** function as proinsecticides and undergo in vitro dealkylation (bioactivation) to form **1**, which is responsible for the toxic effects observed in TBW and trout. However, due to different metabolic profiles and/or rates of metabolism, the amount of **1** present at any particular time is different in the three species and may result in different degrees of safening. In rat, safening may arise from a detoxification pathway involving C-4 methyl hydroxylation, which is the predominant mode of metabolism of **2a**. In trout, the slower rate of conversion to **1** results in considerable safening of this class of insecticide in fish. It is the target pest, TBW, which combines the fastest rate of conversion of **2a** to **1** (activation) with a slower rate of conversion to metabolite B (detoxification), resulting in high levels of activity against this insect pest.

With the incorporation of different alkyl groups on amide or ring nitrogen, the therapeutic index or selectivity is markedly affected, even to the point at which bioactivation can occur at a greater rate in trout than in TBW (propyl, **2c**, and allyl, **2d**, for amide nitrogen substitution).

Although the proinsecticide approach, as applied to the isothiazolylamide class of chemistry, was successful in achieving safety to rainbow trout, there was always a concomitant loss in efficacy toward the target insect pest TBW with the seven analogues. In some instances acceptable (i.e., commercial) levels of TBW activity were achieved, while fish toxicity was reduced by >100-fold. Unfortunately, even this level of safening gave unacceptably high fish toxicity. Therefore, for the *N*-alkylisothiazolylamide series derived from **1**, it appears that the proinsecticide approach is not sufficient by itself to attain the current standards of product safety toward fish while maintaining insecticidal activity against major agricultural pest species.

Taken together, it is apparent that bioactivation of a proinsecticide represents a useful tool to mitigate nontarget toxicity, but not to eliminate it altogether in this case. This is especially true for compounds that act by a mode of action common to both target and nontarget organisms, such as mitochondrial electron transport and uncouplers (Wilkes et al., 1991; Palmer et al., 1990). In these instances, safening a compound by 100−1000-fold may not be adequate and underscores the need to find new target sites with insect pest selectivity.

ABBREVIATIONS USED

1, *N*-(4-chloro-3-methyl-5-isothiazolyl)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **2a**, *N*-(4-chloro-3-methyl-5-isothiazolyl)-*N*-methyl-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **2b**, *N*-(4-chloro-3-methyl-5-isothiazolyl)-*N*-([$^2H_3$]methyl)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **2c**, *N*-(4-chloro-3-methyl-5-isothiazolyl)-*N*-ethyl-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)-oxy]phenyl]acetamide; **2d**, *N*-(4-chloro-3-methyl-5-isothiazolyl)-*N*-propyl-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **2e**, *N*-allyl-*N*-(4-chloro-3-methyl-5-isothiazolyl)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]-acetamide; **2f**, *N*-benzyl-*N*-(4-chloro-3-methyl-5-isothiazolyl)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **2g**, *N*-(*p*-*tert*-butylbenzyl)-*N*-(4-chloro-3-methyl-5-isothiazolyl)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]-phenyl]acetamide; **3a**, *N*-(4-chloro-2,3-dimethyl-3-isothiazolin-5-ylidene)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **3b**, *N*-[4-chloro-3-methyl-2-([$^2H_3$]methyl)-3-isothiazolin-5-ylidene]-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)-oxy]phenyl]acetamide; **3c**, *N*-(4-chloro-2-ethyl-3-methyl-3-isothiazolin-5-ylidene)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)-oxy]phenyl]acetamide; **3d**, *N*-(4-chloro-3-methyl-2-pro-

pyl-3-isothiazolin-5-ylidene)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **3e**, *N*-(2-allyl-4-chloro-3-methyl-3-isothiazolin-5-ylidene)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **3f**, *N*-(2-benzyl-4-chloro-3-methyl-3-isothiazolin-5-ylidene)-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide; **3g**, *N*-[[2-(*p*-*tert*-butylbenzyl)]-4-chloro-3-methyl-3-isothiazolin-5-ylidene]-2-[*p*-[(α,α,α-trifluoro-*p*-tolyl)oxy]phenyl]acetamide.

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