

# Bioaccumulation Potential of 4''-*epi*-(Methylamino)-4''-deoxyavermectin B1a Benzoate (Emamectin Benzoate) in Bluegill Sunfish

Amechi C. Chukwudebe,<sup>\*,†</sup> Nick Andrew,<sup>‡</sup> Kurt Drott,<sup>§</sup> James Swigert,<sup>§</sup> and Peter G. Wislocki<sup>†,‡</sup>

Drug Metabolism II Department, Merck Research Laboratories, P.O. Box 450, Three Bridges, New Jersey 08887, Drug Metabolism II Department, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, and Wildlife International Ltd., 8598 Commerce Drive, Easton, Maryland 21601

Rapid uptake of [5-<sup>3</sup>H]MAB1a by bluegill was observed under flow-through aqueous conditions, attaining steady-state concentration by about 21 and 10 days, respectively, in whole fish and viscera. Uptake by fillet was, by comparison, much lower, and steady state was not attained by 28 days. Residue levels on the final exposure day (i.e., day 28) were 90, 40, and 128 µg/kg, respectively, in whole fish, fillet, and viscera. Following 14 days of depuration, these residue levels all declined by about 90%. The steady-state bioconcentration factors for whole fish, fillet, and viscera were 80, 30, and 116, respectively, indicating that emamectin benzoate will neither bioconcentrate in individual aquatic organisms nor biomagnify in the food chain. Analysis of 28-day-exposed fish by HPLC showed that the only significant metabolite was the N-demethylated derivative, comprising about 14% and 9%, respectively, of total residues in fillet and viscera, while parent emamectin benzoate was about 63% and 49%, respectively.

**Keywords:** *Avermectin; bioaccumulation; bioconcentration; HPLC; biomagnification; bluegill sunfish; emamectin benzoate; metabolism*

## INTRODUCTION

Avermectins are a class of macrocyclic lactones produced by the soil actinomycete *Streptomyces avermitilis*. Abamectin, a natural avermectin product, is currently registered in the United States and worldwide as a miticide. Emamectin benzoate or MK-244 [4''-*epi*-(methylamino)-4''-deoxyavermectin B1 benzoate], a derivative of abamectin, is a mixture of two avermectin homologues, each with a molecular mass of ~900 Da. By specification it contains at least 90% of 4''-*epi*-(methylamino)-4''-deoxyavermectin B1a (MAB1a) benzoate and not more than 10% of 4''-*epi*-(methylamino)-4''-deoxyavermectin B1b (MAB1b) benzoate. These components differ by only a methylene group on the isobutyl side chain of the B1a component (Figure 1). This minor structural difference between MAB1a and MAB1b coupled with the preponderance of MAB1a in emamectin benzoate and the nearly identical biological activities of the two homologues indicates that [<sup>3</sup>H]-MAB1a can be used as a test substance for emamectin benzoate. Emamectin benzoate is currently under development by Merck Research Laboratories as a pesticide for the control of lepidopteran insects on a number of crops including celery, lettuce, cole crops, and tomatoes. To assess the potential impact of emamectin benzoate on the environment, the bioaccumulation potential of [<sup>3</sup>H]MAB1a, its major component, in bluegill sunfish was examined. By understanding the nature, levels, distribution, and accumulation of emamectin benzoate residues following fish exposure in treated

water, the safety of these residues with respect to environmental and human exposures can be better assessed.

## MATERIALS AND METHODS

**Chemicals.** Radiolabeled MAB1a [[5-<sup>3</sup>H]4''-*epi*-(methylamino)-4''-deoxyavermectin B1a or [<sup>3</sup>H]MAB1a] was prepared by the Labeled Compound Synthesis Group at Merck Research Laboratories in Rahway, NJ, with specific activity of about 11 mCi/mg and radiochemical purity >98%. In rat and goat metabolism studies (M. Mushtaq, unpublished data), residue results based on either [<sup>14</sup>C]MAB1a or [<sup>3</sup>H]MAB1a were similar, indicating that the tritium label in [<sup>3</sup>H]MAB1a is metabolically stable. Unlabeled MK-244 and the related avermectin derivative, 4''-*epi*-amino-4''-deoxyavermectin B1a (AB1a), were also synthesized at Merck Research Laboratories in Rahway, NJ. The syntheses of these compounds have been described earlier (Mrozik et al., 1989), and their structures are shown in Figure 1. All solvents and other chemicals used in this study were of analytical grade.

**Test Organism.** Juvenile bluegill sunfish (*Lepomis macrochirus*) from the same year class, with estimated age of 6–12 months, were obtained from Osage Catfisheries, Inc., in Osage Beach, MO, held for 14 days at 22 ± 1 °C in medium-hard well water under static conditions, and then further acclimated to flow-through conditions for at least 48 h before exposure to [<sup>3</sup>H]MAB1a. The bluegill, with lengths and weights averaging 46.0 ± 5.0 mm and 1.5 ± 0.6 g, respectively, were fed flaked fish food (Zeigler Brothers, Inc., Gardners, PA), and the excess food was siphoned daily from test chambers after feeding. With the exception of day 0 samples, all fish feeding and sampling schedules were coordinated such that the fish digestive tracts were allowed to clear for approximately 24 h before each sampling.

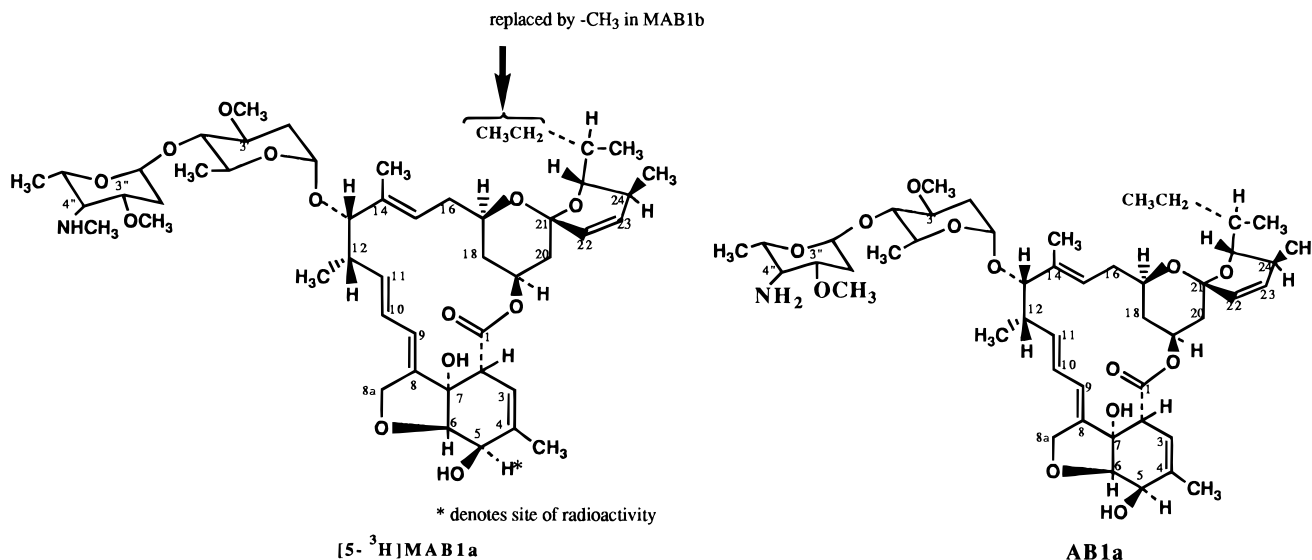
**Test System and Sampling Procedures.** The bioaccumulation of [<sup>3</sup>H]MAB1a in bluegill was studied in Teflon-lined 56-L aquaria containing about 30 L of water. The aquaria (two control and two treated) were positioned inside a temperature-controlled water bath held at ~22 ± 1 °C.

\* Author to whom correspondence should be addressed.

† Merck Research Laboratories, Three Bridges.

‡ Merck Research Laboratories, Rahway.

§ Wildlife International Ltd.



**Figure 1.** Structure of [<sup>3</sup>H]MAB1a and its major metabolite, AB1a.

A primary stock solution of [<sup>3</sup>H]MAB1a in methanol with a specific activity of  $2.25 \times 10^7$  dpm/ $\mu$ g was prepared. Dispensing stock solutions of [<sup>3</sup>H]MAB1a were obtained by 100-fold dilution of the primary stock solution with unlabeled MK-244, resulting in specific activity of  $\sim 2.25 \times 10^5$  dpm/ $\mu$ g. A peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL) delivered [<sup>3</sup>H]MAB1a dispensing stock solution to mixing chambers assigned to the treatment groups. Water flow into the mixing chambers was controlled by rotameters. Dilution of the dispensing stock solution with well water took place in the mixing chamber and the resulting water was split into duplicate treatment group aquaria using a continuous-flow diluter system (Aquatic Technology, Inc., Martinez, CA). For the test system, the diluter delivered a nominal aqueous concentration of 1.6  $\mu$ g/L [<sup>3</sup>H]MAB1a ( $\sim 3.6 \times 10^5$  dpm/L) to the treatment aquaria. For the two control aquaria the diluter supplied analytical grade methanol at the same constant rate that was used for the test system. Well water at pH 8.1 with a dissolved O<sub>2</sub> concentration >5.2 ppm (60% of saturation at 22 °C) was continuously delivered to all aquaria at a rate sufficient for about eight volume changes in 24 h.

Before study initiation, the aqueous [<sup>3</sup>H]MAB1a test solution from the diluter system was passed through test aquaria for a 24-h equilibration period. During this period, the [<sup>3</sup>H]MAB1a levels in water were determined, in duplicate, by direct liquid scintillation counting (LSC). The uptake portion was then initiated by transferring the bluegill sunfish (totaling  $\sim 180$ ) to the duplicate control and treated aquaria, in roughly equal amounts. The fish were observed initially and every 24 h thereafter for mortality or adverse behavior.

At each sampling interval during exposure (i.e., days 0, 1, 3, 7, 10, 14, 21, and 28), equal numbers of fish, ranging from one to five, together with duplicate 5-mL aliquots of water were impartially collected from each aquaria and pooled together by treatment. The pooled fish samples were processed by dissection into fillet (body, muscle, skin, skeleton) and viscera (fins, head, internal organs), followed by homogenization with dry ice in a grinder. Thawed duplicate aliquots ( $\sim 0.1$  g) were combusted on a Harvey Model OX400 biological oxidizer, and the resulting <sup>3</sup>H<sub>2</sub>O was trapped in about 15 mL of Monophasic S and quantified by LSC [radiocombustion analysis (RCA)]. Total [<sup>3</sup>H]MAB1a residues in exposure water at the sampling intervals were determined by direct LSC.

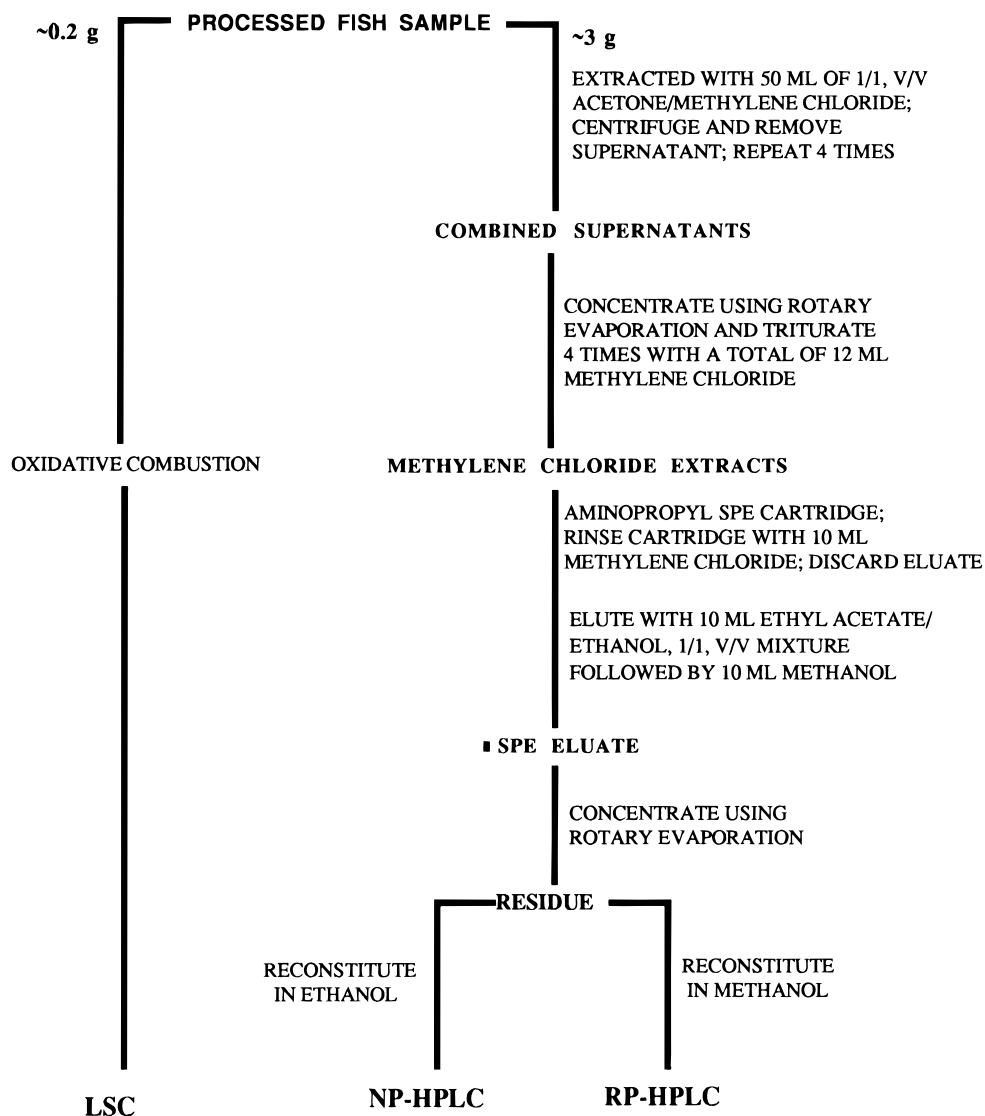
Immediately after the 28-day water and fish sampling, the flow of [<sup>3</sup>H]MAB1a to test aquaria was terminated and replaced with equal volumes of untreated water. The fish were then exposed to this clean water for 14 days. During this depuration period, water (duplicate 5 mL) and fish (one to five) were sampled from each aquaria at intervals of 1, 3, 7, 10, and 14 days and the samples pooled together by treatment. Total [<sup>3</sup>H]MAB1a residue levels in duplicate samples were quantified by either RCA (fish tissues) or direct LSC (water). These [<sup>3</sup>H]MAB1a residue levels in fish and water were

subsequently used to determine daily and steady-state bioconcentration factors as well as uptake and depuration rate constants.

An additional uptake phase with the same nominal [<sup>3</sup>H]MAB1a aqueous concentration (1.6  $\mu$ g/L, or  $3.6 \times 10^5$  dpm/L) was also conducted to generate sufficient treated fish and water samples for residue characterization. Two treated and two control aquaria were also used for this supplemental exposure. About 90 fish were introduced into the treated aquaria and a similar number into the control aquaria. On day 28 of this supplemental exposure, about 1 L of water was collected from each aquaria for the characterization of [<sup>3</sup>H]MAB1a residues in water. The treated and control fish, totaling about 180, were also collected and used for the characterization of incurred tissue residues.

**Extraction and Characterization of Residues.** The flow sheet for the extraction and characterization of [<sup>3</sup>H]MAB1a residues in the supplemental 28-day fish samples is given in Figure 2. The processed fillet or viscera ( $\sim 3$  g), in duplicate, were mixed with anhydrous sodium sulfate (approximately 6 times the weight of tissue) and extracted four times with a total of about 200 mL of acetone/methylene chloride (1/1 v/v) mixture, using a mechanical homogenizer. Extracts were centrifuged and the resulting supernatants concentrated to near dryness by rotary evaporation and reconstituted in methylene chloride; an aliquot was applied to an aminopropyl SPE cartridge preequilibrated with methylene chloride. The initial eluate was discarded and the SPE cartridge washed with methylene chloride. This latter methylene chloride eluate was also discarded. Sorbed [<sup>3</sup>H]MAB1a residues were then eluted with about 10 mL of ethyl acetate/ethanol (1/1 v/v) mixture followed with 10 mL of methanol. The eluted residues were concentrated to dryness by rotary evaporation and then dissolved in either methanol or ethanol for reversed phase (RP) and normal phase (NP) HPLC, respectively. The 28-day supplemental exposure water was concentrated via aminopropyl SPE cartridge, and the sorbed residues were eluted with methanol. Total radioactivity and [<sup>3</sup>H]MAB1a content of the methanol SPE eluate were determined by direct LSC and RP-HPLC, respectively.

RP-HPLC characterization of the isolated residues was conducted with a Shimadzu gradient HPLC system and Pharmacia Frac-100 fraction collector connected in series with a Zorbax, 5- $\mu$ m particle size, 4.5 mm i.d.  $\times$  250 mm SBC<sub>18</sub> column. A flow rate of 1 mL/min was used with a mobile phase consisting of water (containing 5 mM ammonium acetate) and methanol/acetonitrile 3/2 (v/v) mixture (containing 5 mM ammonium acetate) at the following gradient compositions: 0–50 min, 80–82% methanol/acetonitrile; 50–52 min, 82–100% methanol/acetonitrile; 52–60 min, held isocratically at 100% methanol/acetonitrile. NP-HPLC was conducted with the same Shimadzu HPLC system using a Zorbax, 5- $\mu$ m particle size, 4.5 mm i.d.  $\times$  250 mm RXSIL column and a



**Figure 2.** Flow sheet for the extraction and characterization of [ $^3\text{H}$ ]MAB1a residues in processed fish samples.

mobile phase of isooctane (containing 5 mM triethylamine) and ethanol (containing 5 mM triethylamine) at the following gradient compositions: 0–20 min, 30–60% ethanol; 20–22 min, 60–100% ethanol; 22–27 min, held isocratically at 100% ethanol. The flow rate was 1 mL/min. For both HPLC systems, column eluates from sample extracts and reference standards (MAB1a, AB1a) were monitored at 245 nm with a variable wavelength detector. The HPLC column eluates from sample extracts were also collected in 1-mL fractions and counted by direct LSC for the determination of radiochromatographic profiles.

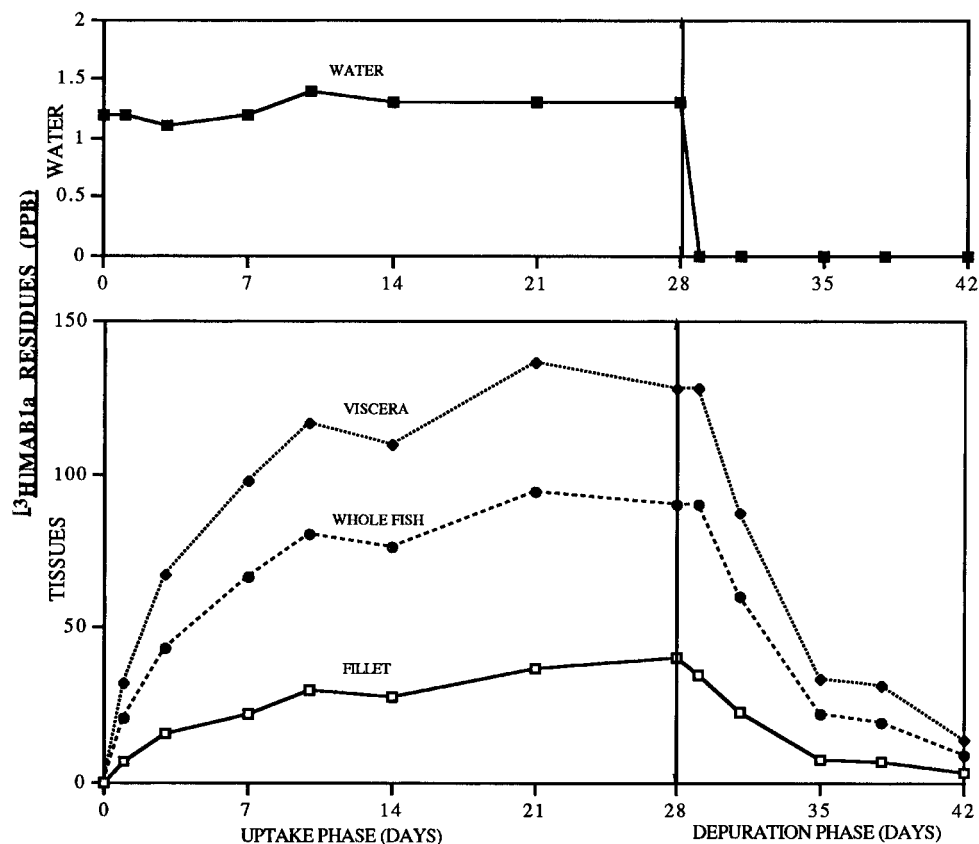
**Calculations.** At each sampling interval during the uptake phase, the daily bioconcentration factor ( $\text{BCF}_0$ ), defined as the ratio of residue concentration in whole fish or fish tissues ( $C$ ) and water ( $C_w$ ), was calculated. Nonlinear regression techniques, from the BIOFAC program (Blau and Agin, 1978), were used to compute uptake and depuration rate constants ( $k_u$  and  $k_d$ , the time required to reach 90% of steady-state level or eliminate 50% of accumulated residues, respectively) as well as the steady-state bioconcentration factor,  $\text{BCF}_B$ . Onset of steady-state levels in tissues were determined by nonlinear regression techniques using a growth-curve model incorporating four parameters: theoretical maximum and minimum concentrations, slope, and intercept. All steady-state concentrations were tested against the predicted theoretical maximum at the  $p < 0.05$  level.

## RESULTS AND DISCUSSION

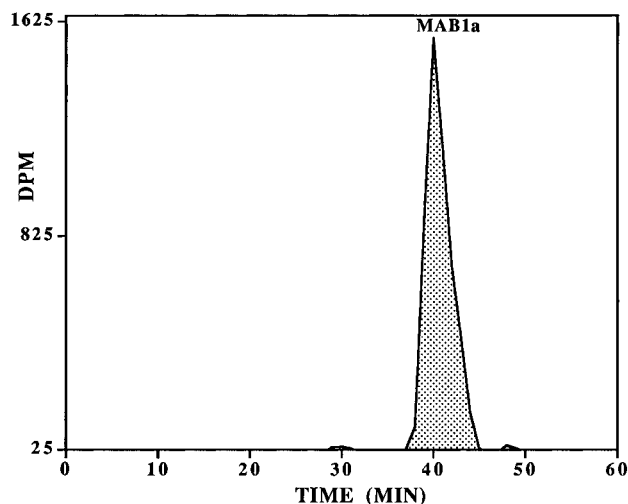
**Bioaccumulation and Depuration Rate Determination.** The bioaccumulation potential of [ $^3\text{H}$ ]-

MAB1a in bluegill under flow-through aqueous conditions was determined in this study. Fish were maintained at a measured water concentration averaging  $\sim 1.2 \mu\text{g/L}$  [ $^3\text{H}$ ]MAB1a over the 28-day exposure period (range: 1.1–1.4  $\mu\text{g/L}$ ) and, using a growth-curve model, these differences in concentration were not statistically significant (Figure 3). Parent [ $^3\text{H}$ ]MAB1a was the only residue found in water, indicating that [ $^3\text{H}$ ]MAB1a was stable in the flow-through water under the ambient conditions of this study (Figure 4). Exposure to [ $^3\text{H}$ ]MAB1a had no adverse effects on the fish. Water quality parameters such as temperature, dissolved  $\text{O}_2$ , and pH did not vary significantly between control and treated aquaria. Fish in control aquaria were similarly unaffected by the well water and methanol.

A rapid uptake of radioactivity by whole fish, fillet, and viscera was observed (Figure 3). Residues plateaued by about 21 days (94  $\mu\text{g/kg}$ ) and 10 days (117  $\mu\text{g/kg}$ ), respectively, in whole fish and viscera ( $p = 0.3$  and 0.19, respectively when compared to predicted theoretical maximum; not statistically significant). When these 21- and 10-day observed residue values were compared to the respective theoretical maximum concentrations predicted from the growth-curve model (105 and 130  $\mu\text{g/kg}$ , respectively), the differences were not statistically significant. Concentrations of [ $^3\text{H}$ ]MAB1a residues in fillet were, by comparison, much lower compared to whole fish and viscera, and steady state



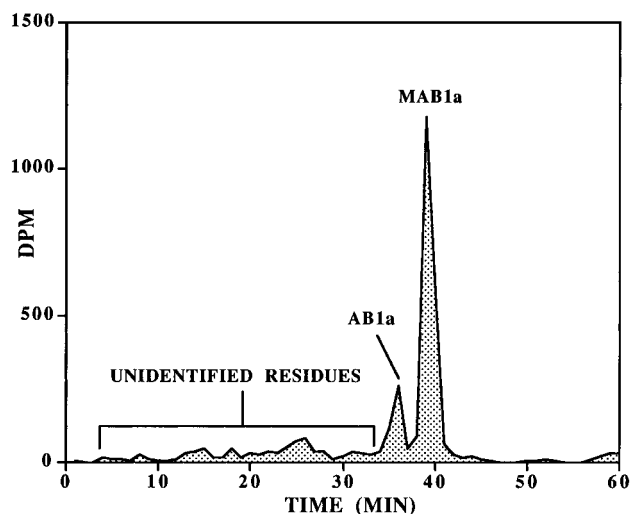
**Figure 3.**  $[^3\text{H}]$ MAB1a residues in water, viscera, whole fish, and fillet during a 42-day exposure and depuration study with bluegill.



**Figure 4.** RP-HPLC of day 28 water residues.

was not attained by 28 days of exposure. Tissue residue levels at the 28-day exposure interval were 90, 40, and  $128 \mu\text{g/kg}$   $[^3\text{H}]$ MAB1a equivalents for whole fish, fillet, and viscera, resulting in  $\text{BCF}_0$  values of 69, 31, and 98, respectively. After 14 days of depuration in untreated water, residue levels in whole fish, fillet, and viscera declined to about 9, 3, and  $14 \mu\text{g/kg}$ , respectively (Figure 3).

$\text{BCF}_B$ ,  $k_u$ , and  $k_d$  values are shown in Table 1. In whole fish, fillet, and viscera, the  $\text{BCF}_B$  values, calculated from the relationship  $k_u/k_d$ , were 80, 30, and 116, respectively. These agree with the 28-day  $\text{BCF}_0$  values for these matrices. Uptake rate constants ( $k_u$ ) in whole fish, fillet, and viscera were 14, 5.5, and  $20 \text{ day}^{-1}$ , respectively. Depuration rate constants ( $k_d$ ) in whole fish, fillet, and viscera varied narrowly between 0.17 and  $0.18 \text{ day}^{-1}$ , and the estimated times to reach 50%



**Figure 5.** RP-HPLC of day 28 fillet residue extract.

residue clearance in these tissues were 3.9, 3.8, and 4.0 days, respectively. The  $\text{BCF}_B$  and the 28-day  $\text{BCF}_0$  values both indicate a low bioaccumulation potential for emamectin benzoate.

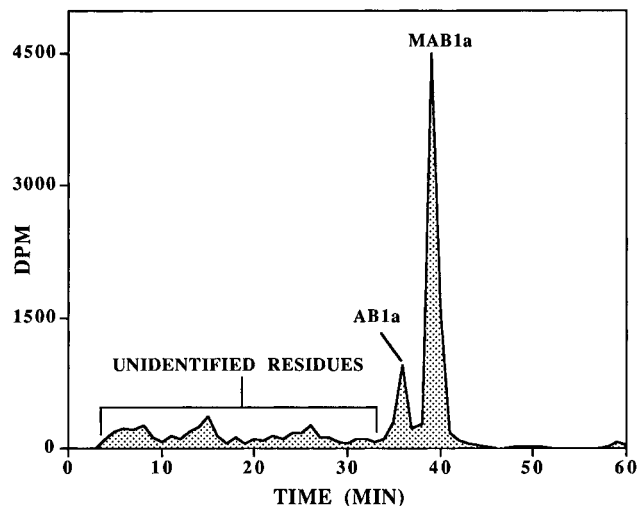
**Nature and Distribution of the Incurred Residues.** The extraction of fillet and viscera from fish collected on day 28 of the supplemental uptake period led to recoveries of 110% and 82%, respectively, of the total radioactivity. These extracts were shown, by HPLC, to consist of parent MAB1a and its N-demethylated product, AB1a (Table 2; Figures 5 and 6).

**Fillet.** Fish fillets from the supplemental 28-day  $[^3\text{H}]$ -MAB1a exposure were used for residue characterization. Total residues in these fillets, by RCA, were about  $23 \mu\text{g/kg}$ . On the basis of RP-HPLC, parent MAB1a and AB1a were found at levels of about 14.5 and  $3.2 \mu\text{g/kg}$ ,

**Table 1. Steady-State Bioconcentration Factors and Uptake and Depuration Rate Constants for Emamectin Benzoate in Bluegill<sup>a</sup>**

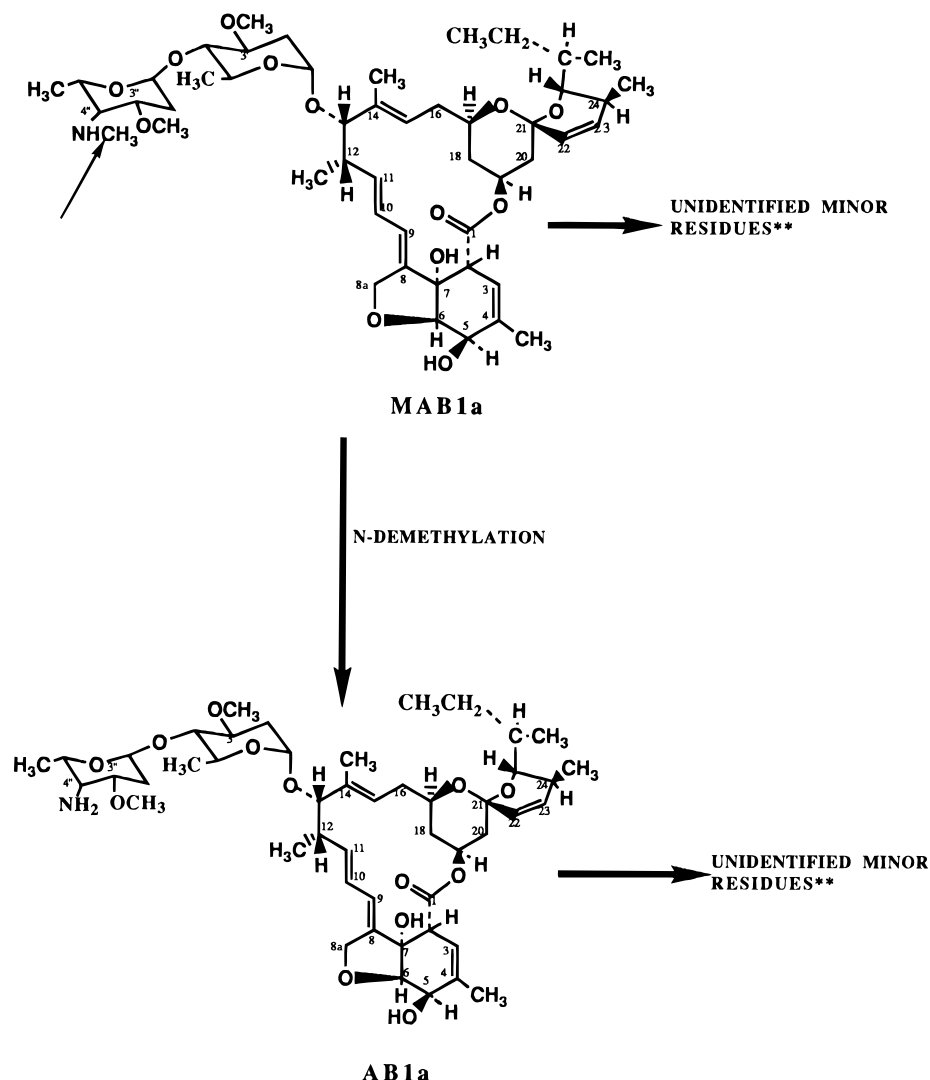
tissue	steady-state BCF <sup>b</sup> (BCF <sub>B</sub> )	uptake rate constant ( $k_u$ ) (day <sup>-1</sup> )	depuration rate constant ( $k_d$ ) (day <sup>-1</sup> )	est time to reach 90% of steady state (days)	est time to reach 50% of clearance (days)
fillet	30	5.5	0.18	13 <sup>c</sup>	3.8
viscera	116	20	0.17	13	4.0
whole fish	80	14	0.18	13	3.9

<sup>a</sup> Determined using the BIOFAC model (Blau and Agin, 1978). Steady-state bioconcentration factor is also referred to as equilibrium bioconcentration factor. <sup>b</sup> BCF<sub>0</sub> values for whole fish, fillet, and viscera on day 28 of exposure were 69, 31, and 98, respectively. <sup>c</sup> Determined by using the highest residue level (on day 28) as the steady state.

**Figure 6.** RP-HPLC of day 28 viscera residue extract.

accounting for 63% and 14%, respectively, of total residues (Table 2; Figure 5). Several unidentified minor residues accounted for the remaining radioactivity, with none attaining a level of 5%, or more, of the total residues. The presence of MAB1a and AB1a in fillet was also confirmed by NP-HPLC (not shown).

*Viscera.* Similarly, viscera from the supplemental 28-day [<sup>3</sup>H]MAB1a-exposed fish were used for residue characterization. Total residues in these viscera, by RCA, were about 106  $\mu$ g/kg and, on the basis of RP-HPLC, consisted of parent MAB1a and AB1a at levels of about 52 and 9.5  $\mu$ g/kg, accounting for 49% and 9%, respectively, of total residues (Table 2; Figure 6). Similar to fillet, several minor unidentified residues were also observed. None of these residues individually attained a level of 5%, or more, of the total residues. The presence of MAB1a and AB1a in viscera was further confirmed by NP-HPLC (not shown).

**Figure 7.** Proposed metabolic pathway of MAB1a in bluegill.

**Table 2. Nature and Distribution of [<sup>3</sup>H]MAB1a Residues in Bluegill**

characterization	% of radioactive residues <sup>a</sup>	
	fillet, day 28 exposure	viscera, day 28 exposure
acetone/methylene chloride extraction	100	100
RP-HPLC		
MAB1a	63	49
AB1a	14	9
sum of minor residues <sup>b</sup>	23	42

<sup>a</sup> The total radioactive residues in fillet and viscera were 23 and 106  $\mu\text{g/kg}$ , respectively. Extraction of fillet and viscera led to recoveries of 110 and 82%, respectively, of the total radioactivity. All numbers presented above were normalized to 100% recovery.

<sup>b</sup> Several minor unidentified residues were also observed, each <5% of the total residues. The presence of MAB1a, AB1a, and other minor unidentified residues was also confirmed by NP-HPLC.

**Conclusions.** The equilibrium bioconcentration factors ( $\text{BCF}_B$ ) for emamectin benzoate (measured as [<sup>3</sup>H]-MAB1a) in whole fish, fillet, and viscera were about 80, 30, and 116, respectively. Compounds with  $\text{BCF}_B$  values <100 are considered to be of little environmental concern (Canton and Sloof, 1979) and are not expected to bioconcentrate into tissues (Davies and Dobbs, 1984; Ernst, 1985). These low  $\text{BCF}_B$  values indicate that emamectin benzoate will neither biomagnify in the aquatic food chain or in tissues consumed by humans nor bioaccumulate in whole fish consumed by fish predators.

In whole fish, fillet, and viscera, depuration of emamectin benzoate residues was relatively rapid, with more than 50% clearance occurring after about 4 days in untreated water. This is consistent with results from mammalian metabolism studies in which the compound neither accumulates nor persists in rats or goats (M. Mushtaq, unpublished data). Emamectin benzoate was metabolized in fillet and viscera via N-demethylation to yield AB1a. This metabolite was also found in rat and goat metabolism studies (M. Mushtaq, unpublished data). Thus, the metabolic fate and disposition of emamectin benzoate in representative fish and mammals are similar.

For most organic compounds,  $\text{BCF}_B$  has been directly correlated with simple laboratory measurements such as molecular weight (MW) and the 1-octanol/water partition coefficient ( $\log P$ ) (Davies and Dobbs, 1984; Veith et al., 1979; Zitko and Hutzinger, 1976). Therefore, the low  $\text{BCF}_B$  of emamectin benzoate is probably related to its MW (~900), with the large size acting as a steric barrier and preventing it from being a truly lipophilic molecule. Similarly, at high  $\log P$  values,  $\text{BCF}_B$  does not increase proportionately and may even decrease (Anliker et al., 1981; Sugiura et al., 1978). The relatively high  $\log P$  (~5 at pH 7) of emamectin benzoate (McCauley, 1992) thus indicates that it will not be a truly lipophilic compound *in vivo*. Although the magnitudes of these laboratory-determined physicochemical properties (MW,  $\log P$ ) have been shown to correlate with  $\text{BCF}_B$ , their use as a predictor of  $\text{BCF}_B$  should be

subordinate to data from biological systems, including mammalian disposition studies. This is because, due to involvement of similar processes, *in vivo* data are better predictors of the full range of biological interactions governing the ability of a compound to bioconcentrate in aquatic organisms. In this *in vivo* study, the low uptake of emamectin benzoate and its low persistence in bluegill (a representative aquatic organism) demonstrate that it will neither bioconcentrate in individual organisms nor biomagnify in the food chain—if introduced into a body of water.

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