Hapten Synthesis and Development of Immunoassays for Methoprene

Joanne V. Mei,[†] Chih-Ming Yin,[*,†] Louis A. Carpino,[‡] and Bruce S. Ferguson[§]

Departments of Entomology and Chemistry, University of Massachusetts, Amherst, Massachusetts 01003, and ImmunoSystems, Inc., 4 Washington Avenue, Scarborough, Maine 04074

Two immunoassay formats were developed for the detection of low levels of the insect growth regulator methoprene. The generation of methoprene-specific antibodies needed for such assays relied on the preparation of a methoprene-carrier immunogen. 11-Methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoic acid was covalently bound to a protein carrier via a spacer group. Two activated ester methods were used to prepare the immunogen, one of which forms a water-soluble, activated ester of methoprene. Polyclonal antibodies raised against the methoprene immunogene were highly specific for methoprene. An indirect enzyme-linked immunosorbent assay (iELISA) and a competitive inhibition enzyme immunoassay (CIEIA) were developed using the polyclonal antisera. The range of the methoprene iELISA was from 5 to 300 ng/mL (ppb), with an I_{50} of 50 ng/mL, while the CIEIA had a range from 1.0 to 10 ppb, with an I_{50} of 3.5 ppb.

INTRODUCTION

The insect growth regulator (IGR) methoprene [isopropyl 11-methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoate (1)] is a chemical analogue of the insect juvenile hormones (JHs) (Figure 1, 5–7). Methoprene was prepared to be more effective than the JHs, both in terms of potency and in terms environmental stability (Henrick et al., 1973, 1976, 1978). Two other juvenile hormone like IGRs, hydroprene (3) and kinoprene (4), have also found commercial uses. Methoprene-based products have been developed for the control of ants, aphids, fleas, flies, mosquitoes, and stored-product pests. The tobacco industry regularly uses methoprene for the control of the cigarette beetle and tobacco moth. Because of the high cost of methoprene, an immunoassay would be useful to prevent overapplication of the IGR. This would constitute a unique use for pesticide immunoassays.

Methoprene residues are monitored to ensure proper application rates and efficacy of control (Heckman and Conner, 1989). Detection of methoprene from various matrices has been accomplished by gas chromatography (Wright and Jones, 1976; Miller et al., 1975; Kortvelyessy et al., 1984), gas chromatography–mass spectrometry (Dunham and Liebrand, 1974), high-pressure liquid chromatography (Dunham et al., 1975; Bergot et al., 1978; Chamberlain, 1985; Allen and Dickinson, 1990), and infrared spectroscopy (Giang and Jaffe, 1980). These methods rely on extensive sample preparation and cleanup procedures. Despite improvements in HPLC, the method still remains time-consuming (Heckman and Conner, 1989).

Here we describe the development of two immunoassays for methoprene which should simplify the sample cleanup procedures and increase the number of samples monitored per day. Immunochemical methods for residue analysis offer many advantages for large-scale screening of environmental samples over conventional methods (Hammock and Mumma, 1980; Hammock et al., 1987; Mumma and Brady, 1987; Jung et al., 1989). As regulatory agencies begin to accept these methods as viable analytical tools (Hammock et al., 1990; O'Rangers, 1990), their applications will become more widespread.

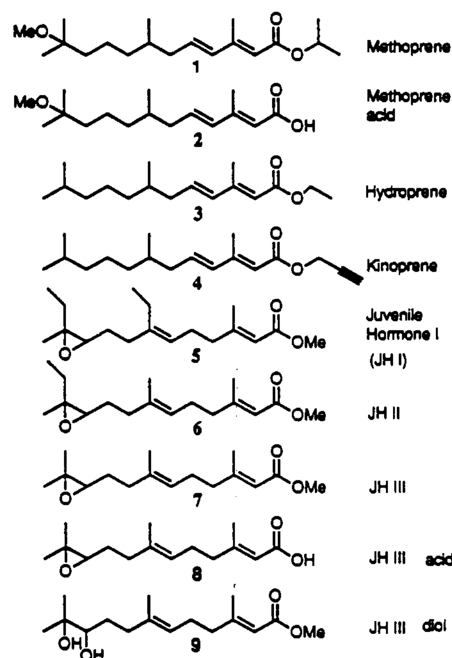

Figure 1. Structure of methoprene and related compounds including hydroprene, kinoprene, and the JHs.

Methoprene, like most pesticides, is a small molecule and does not by itself elicit an immune response. The sensitivity and specificity of an immunoassay are dependent on the ability to raise specific antisera against the targeted molecule. For methoprene to become antigenic, it must be derivatized and coupled to a large carrier molecule. This paper describes the use of a spacer group incorporated between methoprene and the carrier molecule to form an immunogen, the production of polyclonal antisera against the resulting conjugate, and the development of an indirect enzyme-linked immunosorbent assay (iELISA) and a competitive inhibition enzyme immunoassay (CIEIA) for methoprene determination. The methoprene CIEIA was used to determine residues on spiked tobacco.

* To whom correspondence should be addressed.
† Department of Entomology.
‡ Department of Chemistry.
§ ImmunoSystems, Inc.

0021-8561/91/1439-2083$02.50/0 © 1991 American Chemical Society

MATERIALS AND METHODS

Reagents and Equipment. *S*-Methoprene, isopropyl 11-methoxy-3,7(*S*),11-trimethyl-2(*E*),4(*E*)-dodecadienaote (>90%), 5-(^{14}C)methoprene, hydroprene, and kinoprene were gifts from the Sandoz Crop Protection Corp. (Palo Alto, CA). Racemic methoprene (mixture of *R* and *S* isomers) was also obtained from Sandoz. Insect juvenile hormones JH I and JH III, Tween 20 [polyoxyethylene (20) sorbitan monopalmitate], tris(hydroxymethyl)aminoethane hydrochloride, and thimerosal (ethylmercurithiosalicylic acid, sodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA, fraction V) and Freund's complete and incomplete adjuvants were obtained from Gibco (Grand Island, NY). Human serum albumin (HSA, fraction V, fatty acid free), 3,3′,5,5′-tetramethylbenzidine (TMB), and urea hydrogen peroxide were purchased from Calbiochem (La Jolla, CA). Goat anti-rabbit horseradish peroxidase conjugated antibody (IgG-HRP) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). 1,3-Dicyclohexylcarbodiimide (DCC), 4-(*N*,*N*-dimethylamino)pyridine (DMAP), 2-(trimethylsilyl)ethanol, tetraethylammonium fluoride (Et_4NF), and N-hydroxysuccinimide (NHS) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Tobacco, shredded and whole leaf, was provided by R. J. Reynolds Tobacco Co. (Winston-Salem, NC).

Liquid scintillation counting was done on a Packard Tri-carb spectrometer. Ultraviolet and visible spectroscopic analyses were measured in 1-cm cuvettes in an LKB Ultraspec 4050. iELISAs were performed in flat-bottom, polystyrene 96-well microtiter plates (Easy-Wash, Corning), and absorptions were determined using either a MR 600 microtiter plate instrument from Dynatech Instruments (Torrence, CA) or a UV_{max} microplate reader (Molecular Devices, Menlo Park, CA) at 450 nm; data were recorded as optical density and reported as percent inhibition. Methoprene immunoassay test kits for the CIEIA (Envirogard Methoprene) are now available through Millipore Corp. (Bedford, MA). Optical activity was determined with a Rudolf Research (Fairfield, NJ) polarimeter. Infrared spectra were recorded on a Perkin-Elmer 1420 ratio recording spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) was performed on a Perkin-Elmer R12A spectrometer (60 MHz). Chemical shifts (δ) are reported downfield from tetramethylsilane (TMS) as an internal standard in $CDCl_3$ (Aldrich). TLC was performed on precoated silica gel 60 glass plates with fluorescent indicator (254 nm) (Aldrich). Flash chromatography was carried out according to the method of Still et al. (1978).

EXPERIMENTAL PROCEDURES

Hapten Synthesis. Methoprene hapten synthesis (Mei, 1988) was carried out by first converting methoprene to its carboxylic acid by base hydrolysis in ethanol. The free acid (2) was coupled to a spacer group in three steps outlined in Figure 2. The resulting methoprene–spacer acid derivative (12) was coupled to a carrier protein by two activated ester methods (Figure 3). Intermediates were characterized by TLC, IR, ^{1}H NMR, and elemental analysis.

2-(Trimethylsilyl)ethyl 4-(Benzyloxy)butanoate (11). DMAP (0.734 g, 6.00 mmol) and DCC (5.92 g, 28.7 mmol) were added to a solution of 4-(benzyloxy)butanoic acid (4.44 g, 22.9 mmol), prepared according to the method of Sudo et al. (1967), in dichloromethane (CH_2Cl_2, 50 mL) with stirring at 0 °C. 2-(Trimethylsilyl)ethanol (4.07 g, 34.4 mmol) in CH_2Cl_2 (50 mL) was added dropwise (Sieber et al., 1977) over a 1-h period. The mixture was allowed to stand at −20 °C for 48 h. *N*,*N*′-Dicyclohexylurea (DCU) was filtered from the mixture and the filtrate was washed with 10% aqueous $NaHCO_3$ (3 × 50 mL), water (3 × 50 mL), 10% aqueous acetic acid (3 × 50 mL), water (3 × 50 mL), and saturated NaCl (3 × 50 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure, leaving a yellow liquid which was purified by flash chromatography (ethyl acetate/hexane 10:90) to give 6.26 g of 2-trimethylsilyl ester (11) as a pale yellow liquid (93%): ^{1}H NMR ($CDCl_3$, 60 MHz) δ 0.04 (s, 9 H, $SiMe_3$), 0.98 (t, J = 7 Hz, 2 H, O-CH_2**CH_2**$SiMe_3$), 1.8–2.0 (m, 2 H, C3-CH_2), 2.43 (t, J = 6 Hz, 2 H, C2-CH_2), 3.62 (t, J = 6 Hz, 2 H, C4-CH_2), 4.01 (t, J = 7 Hz, 2H, O-**CH_2**CH_2SiMe_3), 4.32 (s, 2 H, benzylic CH_2), 7.16 (s, 5 H, Ph); IR (neat) 3020 (w, Ph), 1730 (s, COOR), 1250 (m, Si-C), 700, 750 cm^{-1} (m, Ph).

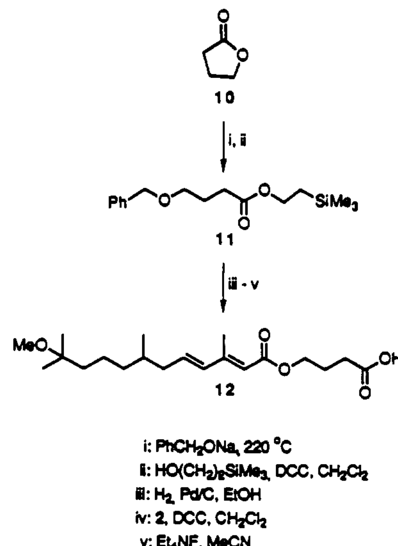

Figure 2. Synthesis of the protected four-carbon spacer group (11) and preparation of methoprene–spacer–acid (12).

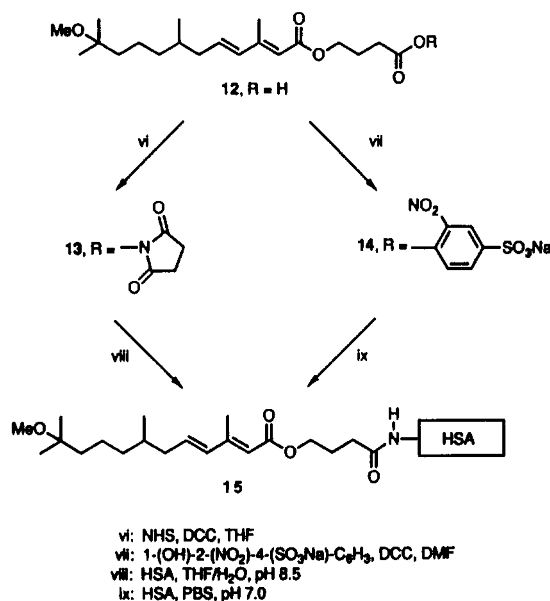

Figure 3. Preparation of the methoprene immunogen (15).

2-(Trimethylsilyl)ethyl 4-Hydroxybutanoate. Palladium on activated carbon (5%, 300 mg, Aldrich) was added to a cold solution of the 2-(trimethylsilyl)ethyl ester (11) (3.10 g, 10.5 mmol) in absolute ethanol (100 mL) in a 500-mL hydrogenation flask. The mixture was shaken under 2 atm of hydrogen for 1.5 h. The catalyst was removed by filtration, and the solvent was evaporated under reduced pressure to give the crude hydroxy ester as a clear liquid (2.11 g, 98%): ^{1}H NMR ($CDCl_3$, 60 MHz) δ 0.04 (s, 9 H, $SiMe_3$), 0.98 (t, J = 7 Hz, 2 H, O-$CH_2CH_2SiMe_3$), 1.8–2.0 (m, 2 H, C3-CH_2), 2.42 (t, J = 6 Hz, 2 H, C2-CH_2), 3.60 (t, J = 6 Hz, 2 H, C4-CH_2), 4.01 (t, J = 7 Hz, 2 H, O-$CH_2CH_2SiMe_3$); IR (neat) 3400 (br, m, OH), 1730 (s, COOR), 1250 cm^{-1} (m, C-Si).

11-Methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoic Acid (2). A mixture of methoprene (1) ($[\alpha]_D$ = +5.2°) and 5-(^{14}C)-methoprene [1.09 g, 3.51 mmol, 1.5 × 10^4 counts per min (cpm)] was added to a 2.5 M KOH ethanol/water (70:30) solution. The reaction mixture was stirred at room temperature for 18 h, and the solution was concentrated under reduced pressure. Water (10 mL) was added, and the aqueous solution was extracted with diethyl ether (3 × 25 mL). The aqueous solution was acidified with 50% H_2SO_4 and extracted with ether (3 × 25 mL), and the combined ether extracts were dried ($MgSO_4$). The solvent was removed, leaving an orange residue which was purified by flash chromatography (ethyl acetate/hexane 20:80) to give the acid (2) as a yellow oil (1.04 g, 95%): ^{1}H NMR ($CDCl_3$, 60 MHz) δ

0.93 (d, J = 6 Hz, 3 H, C7-Me), 1.11 (s, 6 H, C11-Me), 1.2–1.5 (m, 7 H, allylic H), 2.0–2.20 (m, 2 H, C6-CH_2), 2.29 (s, 3 H, C3-Me), 3.13 (s, 3 H, OMe), 5.6–5.7 (m, 1 H, vinylic 2-H), 6.2–6.3 (m, 2 H, vinylic 4,5-H), 11.2 (br s, 1 H, acidic H); IR (neat) 3500–2500 (s br, COOH), 1690 (s, C=O), 1610 (s, C=C), 1260 cm^{-1} (s, C—O).

2-(Trimethylsilyl)ethyl 4-[11-Methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoyloxy]butanoate. DMAP (13.4 mg, 110 μmol) and DCC (142 mg, 688 μmol) were added to cold (0 °C) solution of methoprene acid (2) (150 mg, 559 μmol) in CH_2Cl_2 (10 mL). A solution of 2-(trimethylsilyl)ethyl 4-hydroxybutanoate (130 mg, 698 μmol) in CH_2Cl_2 (5 mL) was added with stirring over 15 min. The mixture was stirred at 0 °C for 5 min and allowed to stand at −20 °C for 24 h. DCU was filtered from the solution, which was then washed with 10% aqueous $NaHCO_3$ (3 × 10 mL), water (3 × 10 mL), 10% aqueous acetic acid (3 × 10 mL), water (3 × 10 mL), and saturated NaCl (2 × 25 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure, leaving a yellow residue which was purified by flash chromatography (ethyl acetate/hexane 10:90) to give the 2-(trimethylsilyl)ethyl ester as a pale yellow oil (95.5 mg, 66%): ^1H NMR ($CDCl_3$, 60 MHz) δ 0.11 (s, 9 H, $SiMe_3$), 0.7–1.0 (m, 5 H, C7-Me, O–$CH_2CH_2SiMe_3$), 1.2–1.5 (m, 9 H allylic H), 1.7–2.6 (m, 7 H, C3-Me, allylic H), 3.15 (s, 3 H, OMe), 3.9–4.4 (m, 4 H, O–CH_2), 5.6–5.7 (m, 2 H, vinylic H), 6.0–6.2 (m, 1 H, vinylic H); IR (neat) 1730 (s, C=O), 1710 (s, C=O), 1610 (m, C=C), 1250 cm^{-1} (m, Si—C). Anal. Calcd for $C_{25}H_{46}O_5Si$: C, 66.04; H, 10.20. Found: C, 65.98; H, 9.97.

4-[11-Methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoyloxy]butanoic Acid (12). 2-(Trimethylsilyl)ethyl 4-[11-methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoyloxy]butanoate (164 mg, 361 μmol) was added to a solution of Et_4NF (5.38 g, 3.61 mmol) in dry CH_3CN (distilled from calcium hydride, 10 mL) and heated to 45 °C for 6 h. The reaction mixture was concentrated under reduced pressure, and water (25 mL) was added. The aqueous solution was washed with hexane (2 × 25 mL) and acidified (50% aqueous H_2SO_4). The solution was extracted with diethyl ether (3 × 25 mL) and dried over $MgSO_4$, and the solvent was removed, leaving an orange residue. The residue was purified by flash chromatography (ethyl acetate/hexane/acetic acid 50:50:0.1) to give the acid (12) as a yellow oil (79.4 mg, 68%): ^1H NMR ($CDCl_3$, 60 MHz) δ 0.81 (d, J = 6 Hz, 3 H, C7-Me), 1.12 (s, 6 H, C11-Me), 1.3–1.5 (m, 9 H, allylic H), 1.9–2.1 (m, 4 H, C6′, C3-CH_2), 2.2–2.5 (m, 5 H, C3′-Me, C1-CH_2), 3.15 (s, 3 H, OMe), 4.15 (t, J = 6 Hz, 2 H, C4-CH_2), 5.6–5.7 (m, 1 H, vinylic H), 6.0–6.1 (m, 2 H, vinylic H); IR (neat) 3500–2500 (m br, COOH) 1710 (s, C=O), 1610 cm^{-1} (m, C=C). Anal. Calcd for $C_{20}H_{34}O_5$: C, 67.76; H, 9.67. Found: C, 67.60; H, 9.65.

Preparation of Activated Esters. Two activated esters of compound 12, methoprene–spacer–acid, were prepared for conjugation to the carrier protein (Figure 3).

N-Succinimidyl 4-[11-Methoxy-3,7,11-trimethyl-2(E),4(E)-dodecadienoyloxy]butanoate (13). DCC (156 mg, 756 μmol) was added to a cold (0 °C) solution of methoprene–spacer–acid (12) (135 mg, 381 μmol) and *N*-hydroxysuccinimide (NHS) (54.7 mg, 475 μmol) in tetrahydrofuran (THF) (5 mL) (Lauer et al., 1974; Baehr et al., 1976). The mixture was stirred for 5 min and allowed to stand at −20 °C for 24 h. DCU was filtered from the solution, and CH_2Cl_2 (15 mL) was added. The organic solution was washed with 10% acetic acid (3 × 10 mL), water (3 × 10 mL), and saturated NaCl (3 × NaCl) and dried over $MgSO_4$. The solvent was removed under reduced pressure, leaving the *N*-hydroxysuccinimidyl ester (13) as a yellow oily residue (134 mg, 78%), which was used without further purification: ^1H NMR ($CDCl_3$, 60 MHz) δ 0.8 (d, J = 6 Hz, 3 H, C7-Me), 1.2 (s, 6 H, C11-Me), 2.8 (s, 4 H succinimidyl CH_2), 3.15 (s, 3 H, OMe), 4.15 (t, J = 6 Hz, 2 H, C4-CH_2), 5.6–5.7 (m, 1 H, vinylic H), 6.0–6.1 (m, 2 H, vinylic H); IR (neat 3320 (m, NH, DCU), 1820, 1790, 1740, 1710 (m, m, s, s, C=O), 1610 (m, C=C), 1250, 1210, 1160 cm^{-1} (s, C—O).

Sodium 1-[4-(11-Methoxy-3,7,11-trimethyl-2(E),4(E)-dodecadienoyloxy)butanoyloxy]-2-nitro-4-benzenesulfonate (14). Methoprene–spacer–acid (12) (60.1 mg, 169 μmol) was added to a solution of sodium 1-hydroxy-2-nitro-4-benzenesulfonic acid (HNSA) (41 mg, 170 μmol) (King, 1921; Aldwin and Nitecki, 1987; Mei, 1988) in dimethylformamide (DMF, 2 mL). DCC (43.6 mg, 211 μmol) was added, and the solution was stirred at room temperature for 24 h. DCU was filtered from the solution, and the filtrate was washed with diethyl ether (5 mL). The solvent was removed under reduced pressure with gentle heating. Diethyl ether was added to the orange residue until it solidified (trituration). The ether was removed, and the oily solid was taken up in water (3 mL) and lyophilized, leaving the ester (14) as a fluffy yellow solid (76.1 mg, 77%). The crude material was analyzed for ester content as described below.

Analysis of Esters of 1-Hydroxy-2-nitro-4-benzenesulfonic Acid (HNSA). The above reaction results in a mixture of the ester (14) and free HNSA. The percent ester in the mixture was determined by base hydrolysis of a mixture of the 1-hydroxy-2-nitro-4-benzenesulfonate ester (14) and free HNSA. Reaction of HNSA with either base or nucleophiles generates a bright yellow dianion (16) with λ_{max} = 406 nm (Aldwin and Nitecki, 1987) (Figure 4). This allowed for the quantitative determination of HNSA esters from crude mixtures. The methoprene HNSA ester mixture was dissolved in phosphate buffer (0.01 M, pH 7.0, 1 mL), to give a yellow solution. The amount of free dianion was measured by reading the absorption of the solution at 406 nm (A_{406}). The methoprene HNSA ester was completely hydrolyzed by the addition of 5 N NaOH (50 μL), and a second absorption was taken of the brightly colored yellow solution [A_{406}-(NaOH)]. The difference between the two absorptions, divided by the second absorption, represented the total amount of methoprene HNSA ester in the preparation (Aldwin and Nitecki, 1987; Mei et al., 1990). UV spectra before and after hydrolysis of a ester mixture are shown in Figure 4. The mixture contained approximately 60% ester.

Conjugation of Activated Esters to Carrier Protein. (*1*) *NHS Ester Reaction.* Succinimidyl ester (13) (4.17 mg, 9.53 μmol) in 0.5 mL of THF was added to a solution of HSA (13.15 mg, 196 nmol) in THF/H_2O (56:44, pH 8.5, adjusted with 0.1 M NaOH, 0.5 mL) and stirred for 48 h at room temperature (Figure 3) (Baehr et al., 1976). The protein did not fully dissolve under these reaction conditions. The solution was transferred to a dialysis tube (12 000 MW cutoff, Spectrapor) and dialyzed against phosphate-buffered saline (PBS, 0.05 M, 0.05 M NaCl, pH 7.4, 1.5 L) and against water (1.5 L). Dialysis was ended when the radioactivity of aliquots (1 mL) of washings was equal to background, as measured by scintillation counting. The contents of the dialysis tube were lyophilized, giving the methoprene–protein conjugate (15) as a fluffy white solid (12.87 mg).

(*2*) *HNSA Ester Reaction.* HNSA ester (14) (10.52 mg, 21.62 μmol) in PBS (0.1 M, 0.1 M NaCl, pH 7.0, 0.5 mL) was added to a solution of HSA (4.97 mg, 72.6 nmol) in PBS (1 mL) (Aldwin and Nitecki, 1987). The reaction was stirred for a minimum of 15 min (Figure 3) (Mei, 1988; Mei et al., 1990). Longer reaction times did not significantly increase the number of haptens conjugated to the protein (Table I). An aliquot of the reaction mixture (10 mL) was added to phosphate buffer (0.01 M, pH 7.0, 1 mL). The absorption was measured at 406 nm to assay the amount of dianion (16) liberated by nucleophilic reaction with the protein. The reaction mixture was dialyzed and lyophilized, as described above, to give the methoprene–protein conjugated (15) as a fluffy white solid (8.43 mg).

Determination of Hapten Density. Hapten density of protein conjugates was determined directly for both the NHS and HNSA ester conjugation reactions by scintillation counting of conjugates, all of which contained the 5-(^{14}C)methoprene tracer (Table I). The hapten density of protein conjugates prepared from HNSA esters was also determined by visible spectrophotometry, whereby aliquots from reaction mixtures were removed at various times and assayed for the amount of dianion generated before and after base hydrolysis, as described above (analysis of HNSA esters, Figure 4). Values from both the radiolabeled tracer and spectrophotometric determinations are compared for the HNSA method (Table I).

Immunization. Female New Zealand white rabbits were each immunized first with an intravenous injection of the methoprene immunogen (200 μg per animal, 53 molecules of methoprene per molecule of protein) in 0.25 mL of PBS. One month later the animals were boosted subcutaneously with 200 μg of the immunogen and 0.25 mL of Freund's complete adjuvant. A second booster shot of the immunogen (200 μg per animal) in Freund's incomplete adjuvant was given intramuscularly to each animal 2 weeks after the first booster shot. Whole blood was collected

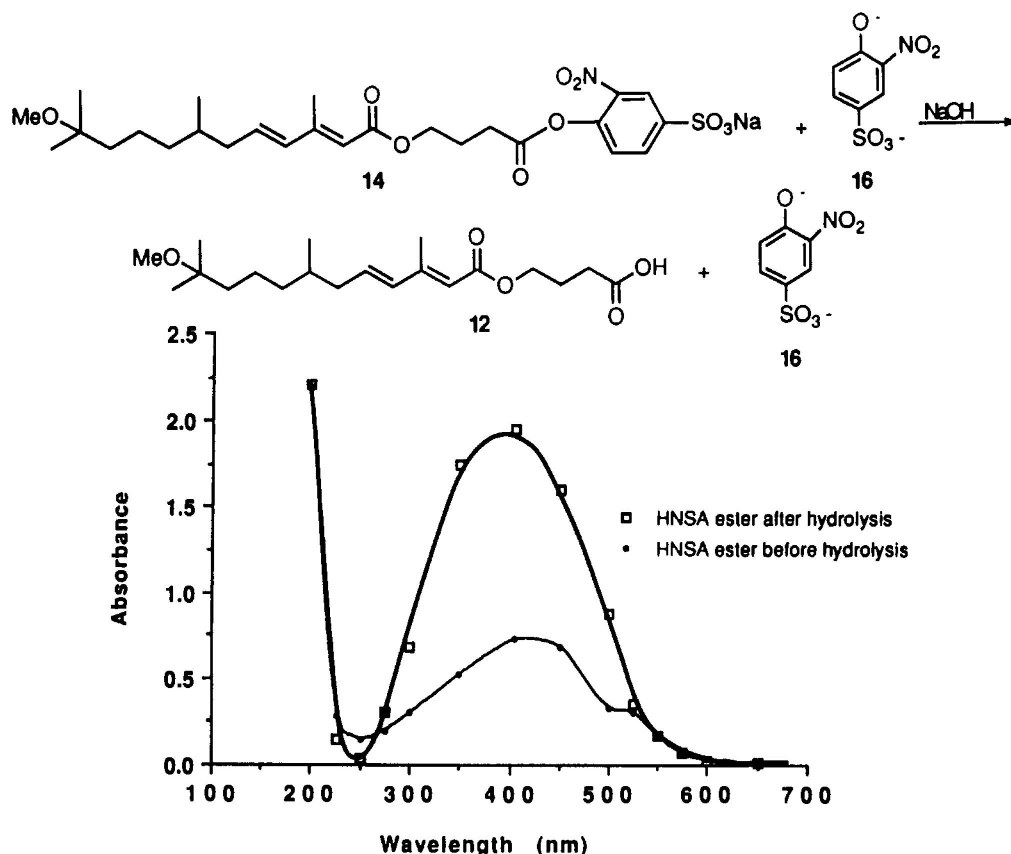

Figure 4. Determination of the amount of HNSA ester (14) in a mixture: absorbance of dianion before (●) (thin line) and after base hydrolysis (□) thick line) of the ester indicates the concentration of active ester.

Table I. Comparison of Hapten Density for Activated Esters

ester	reaction time, h	residues reacted		ester	reaction time, h	residues reacted	
		$^{14}C^a$	$A_{406}{}^b$			$^{14}C^a$	$A_{406}{}^b$
NHS[c]	24	21		HNSA[d]	0.25	31	34
	48	53			0.5	28	32
	48	48			3	28	27

[a] 5-(^{14}C)Methoprene tracer was used to determine hapten density. [b] Absorbance at 406 nm was used to determine hapten density. [c] N-Hydroxysuccinimide. [d] 1-Hydroxy-2-nitro-4-benzenesulfonate.

1 week after the second booster, with further serum collected biweekly until the antibody titer decreased. All blood collected was allowed to coagulate (room temperature, 30 min), centrifuged to remove red blood cells, and incubated in a water bath (60 °C, 20 min), and then thimerosal (0.01%) was added. The serum was stored under refrigeration, frozen for storage (-70 °C), or used withouut further treatment.

Indirect Enzyme-Linked Immunosorbent Assay. To select the best serum and coating conjugate, checkerboard assays (Herman, 1988) in the format of an iELISA (Mei et al., 1990) were conducted with conjugates from different coupling reactions and antisera from different collections and different animals. Coating conjugates were solubilized in carbonate coating buffer.

Corning 96-well microtiter plates were coated with the methoprene-protein conjugate (31 mol of methoprene/mol of carrier) at 0.1 μg/mL in carbonate buffer (0.05 M, pH 9.6, 0.1 mL/well) and incubated for 4-6 h at 37 °C. The plates were washed three times with TBS-Tween 20 buffer (20 mM tris, 500 mM NaCl, 0.1% thimerosal, 0.5% Tween 20, pH 7.5). Methoprene, in methanol (5 μL/well), was added to wells at increasing concentrations, followed by rabbit anti-methoprene antiserum diluted 1/15 000 with antibody buffer (TBS-Tween 20, 1% BSA, 0.1 mL/well). The plates were incubated in a moist atmosphere (room temperature) for at least 4 h and washed three times with TBS-Tween 20. Goat anti-rabbit IgG-HRP (diluted approximately 15 000×) was added to each well at 0.1 mL/well, and plates were incubated for 2 h (room temperature). The plates were washed three times with TBS-Tween 20, and a substrate solution consisting of sodium acetate/citric acid buffer (0.1 M, pH 6.0, 10.0 mL), urea hydrogen peroxide (5 mM, 1.0 mL), and TMB (42 mM in dimethyl sulfoxide, 0.1 mL) (Liem et al., 1979; Bos et al., 1981) was added to each well at 0.1 mL/well. The plates were developed from 20 min to 1 h, with the reaction being stopped by the addition of sulfuric acid (2.0 N, 50 μL). The absorption was determined at 450 nm in the Dynatech reader.

Competitive Inhibition Enzyme Immunoassay. Using the same anti-methoprene antiserum, a competitive inhibition enzyme immunoassay (CIEIA) was also formatted by immobilizing the antibody to the microtiter plate wells (1:40 000, 200 μL/well, Millipore kit). A horseradish peroxidase (HRP, Sigma) conjugate of methoprene was prepared by reacting the NHS-activated ester (13) with the enzyme. Standard curves were prepared by adding standard methoprene solutions (100 μL/well) and the conjugate (diluted 1:35 000 in a diluent buffer, pH 6.8, 100 μL/well) and the conjugate 9diluted 1:35 000 in a diluent buffer, pH 6.8, 100 μL/well) to antibody-coated wells. Standard solutions of methoprene were prepared in 60% acetonitrile/water (v/v) and diluted with distilled water (100 μL) in 2.0 mL). Plates were incubated for 1 h, and wells were washed with cool tap water (five times) and tapped dry. Substrate (urea peroxide, 4.3 mM, 80 μL) was added, followed by the chromogen (TMB, 2.6 mM, 40 μL). The contents of the wells were mixed and incubated for 30 min. The reaction was stopped by the addition of sulfuric acid (2.5 N, 40 μL), and the absorbance was determined at 450 nM in the Molecular Devices reader.

Standard Curves and Cross Reactivity. Standard curves were obtained from the iELISA by adding solutions of S-methoprene (to triplicate or quadruplicate wells) in concentrations ranging from 0.5 to 500 ppb prior to the addition of methoprene-specific antiserum (Figure 5). The iELISA format was used to determine cross reactivity of the antiserum. Related materials, such as JH I, JH III, the methoprene intermediates used to prepare the immunogen (Figure 6), and the IGRs hydroprene and kinoprene (Figure 7), were tested. Standard curves in water were also obtained for the CIEIA by adding solutions of S-meth-

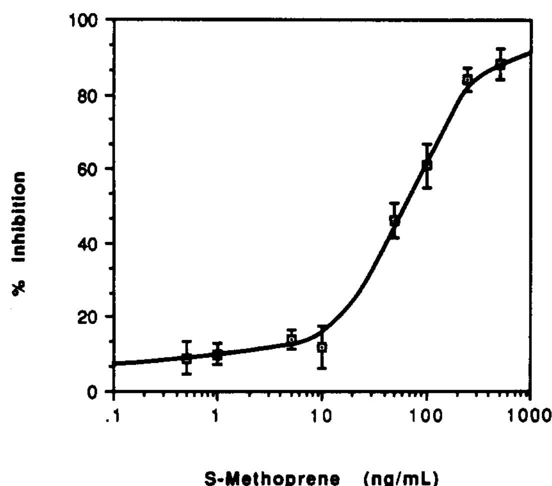

Figure 5. Percent inhibition of the antiserum binding to immobilized methoprene by free methoprene in the indirect ELISA. Fifty percent inhibition (I_{50}) occurred at about 50 ng/mL. Vertical bars indicate standard error ($N = 3$ for each point).

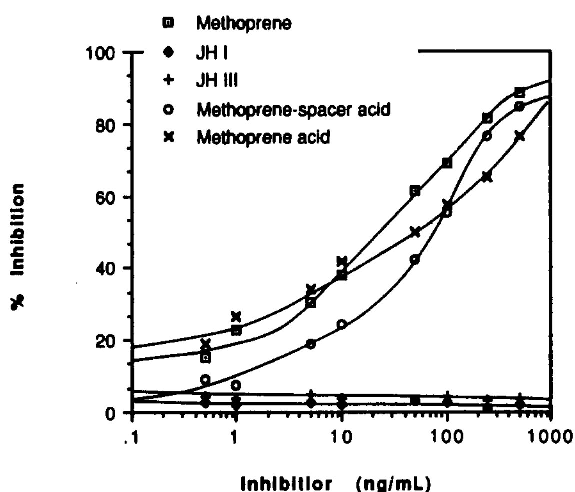

Figure 6. Cross reactivity of the methoprene antiserum with methoprene derivatives and JHs using the iELISA. The JHs showed virtually no cross reaction with the antiserum. Due to the complexity of the figure, standard error bars were omitted ($N = 4$ for each point, SE < 3%).

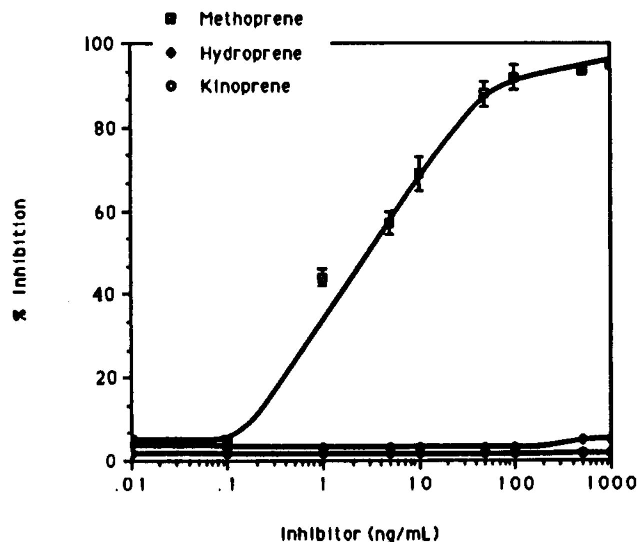

Figure 7. Cross reactivity of the methoprene antiserum with methoprene analogues, hydroprene and kinoprene, using the iELISA. I_{50} is approximately 4 ng/mL for S-methoprene in this assay.

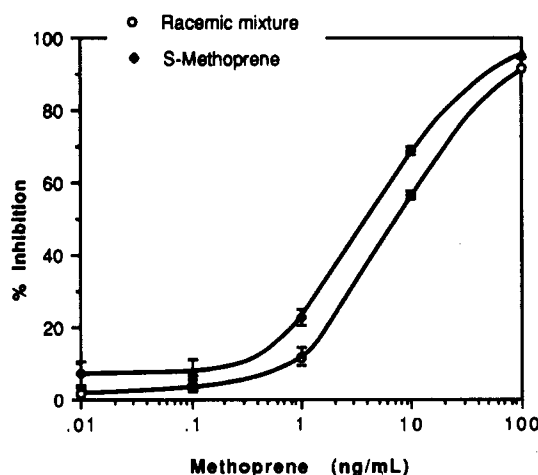

Figure 8. Standard curve for the methoprene competitive inhibition enzyme immunoassay (CIEIA). Standards were prepared in the absence of the matrix. Vertical bars indicate standard error ($N = 3$).

oprene and racemic methoprene in concentrations ranging from 0.1 to 100 ppb, along with enzyme-labeled S-methoprene (Figure 8).

Extraction of Tobacco. Both the iELISA and CIEIA formats were used to determine methoprene residues from spiked tobacco. Because the iELISA required more extensive cleanup procedures (Mei et al., 1990), the CIEIA format was adapted for tobacco use and provided enhanced assay simplicity. Various types of tobacco were spiked in 3-8 ppm concentrations with S-methoprene or with a racemic mixture of R- and S-methoprene isomers (Kabat tobacco formulation) and extracted.

One gram of methoprene-spiked dried, ground, or chopped tobacco was shaken with 24 mL of 60% (v/v) acetonitrile for 30 min (200 rpm on an orbital shaker). The supernatant (2-3 mL) was filtered through a Millex LCR syringe filter (Millipore Corp., Bedford, MA). The filtered supernatant was further diluted with distilled water (100 μL into 2.0 mL), and 100 μL of this solution was added to antibody-coated wells, followed by the methoprene-HRP conjugate as described above. Figure 9 illustrates a spike and recovery curve using the CIEIA on five varieties of tobacco.

RESULTS AND DISCUSSION

Immunogen Design. Methoprene has no reactive moiety for conjugation to the protein carrier. Either end of the methoprene molecule could have been derivatized to incorporate a functional group. Literature on radioimmunoassays (RIA) for the JHs has shown that JH-protein conjugates were prepared by conjugating the acid (8) directly to the carrier as an amide at C-1 (Lauer et al., 1974; Baehr et al., 1976; Goodman et al., 1990) or as the succinimidyl ester of the diol (9) (Strambi, 1981; Strambi et al., 1981) (Figure 1). Conjugation of JH through C-1 removed the ester function of the native hormone, while conjugation through C-11 completely changed the configurations of carbons 11 and 12. Despite the simplicity of JH quantification by RIA, use of the technique has not become widespread due to the need for extensive preparation of samples and the need to pay scrupulous attention to protocols to achieve reproducibility (Granger and Goodman, 1983, 1988; Goodman et al., 1990). Antiserum specificity and cross reactivity for the various JH homologues have also contributed to difficulties in using the JH RIA.

On the basis of the above findings, we hypothesized that if both the ester and methoxy functions of methoprene were maintained in the immunogen, the resulting antibodies might have a greater specificity for methoprene. Hapten structure and site of conjugation have been shown

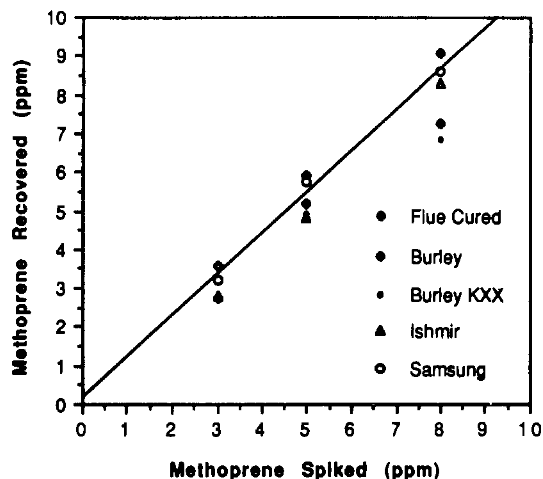

Figure 9. Spike and recovery curve. Tobacco varieties were spiked with 3, 5, and 8 ppm of racemic methoprene. The CIEIA was used to estimate the amount of methoprene recovered after extraction procedures ($N = 2$, $r^2 = 0.992$). Data points for Burley and Burley KXX at 8 ppm appear under data points of the other tobacco varieties.

to profoundly affect the sensitivity and specificity of the resulting immunoassay (Hammock and Mumma, 1980; Vallejo et al., 1982; Mumma and Brady, 1987; Jung et al., 1989; Goodrow et al., 1990). The methoprene immunogen, therefore, was designed with four-carbon spacer group between methoprene and the carrier (Figure 3, structure **15**). The spacer group was covalently bound to methoprene as an ester, which was linked to the carrier as an amide. Spacer groups of the appropriate length have also been shown to increase the immunogenicity of haptens (Kinsky et al., 1983, 1984; Scott et al., 1984). The resulting conjugate (**15**) retains the ester and methoxy functions of methoprene. The spacer group was incorporated through a series of protection/deprotection reactions originally used for peptide synthesis (Figure 2).

Immunogen Synthesis. With these structural requirements in mind, we chose to use the 4-oxybutanoyl moiety as a spacer group (Figure 2). The synthesis of the immunogen (methoprene–spacer–protein, **15**) is outlined in Figures 2 and 3. The preparation of this conjugate required prudent choice of protective groups for each end of the spacer. Cleavage conditions would have to be mild enough so as not to attack either the α,β-unsaturated double bonds or the methoxy function of methoprene.

Central to this approach was the synthesis of 2-(trimethylsilyl)ethyl 4-(benzyloxy)butanoate (**11**). The 2-(trimethylsilyl)ethyl residue was chosen to protect the carboxylic function because it could be selectively removed under mild conditions as the last step in the preparation of methoprene–spacer–acid (**12**). Esters of 2-(trimethylsilyl)ethanol and the carboxylic acid residues of peptides were also shown to be stable under workup conditions and during hydrogenation (Sieber et al., 1977).

The reaction between 4-butyrolactone (**10**) and the sodium salt of benzyl alcohol gave 4-(benzyloxy)butanoic acid (Sudo et al., 1967) (Figure 2). Esterification with 2-(trimethylsilyl)ethanol, followed by catalytic hydrogenation to remove the benzyl protective group (Van Duzee and Adkins, 1935), gave 2-(trimethylsilyl)ethyl 4-hydroxybutanoate (**11**). This alcohol was esterified with 11-methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoic acid (**2**), which was prepared by base hydrolysis of methoprene (**1**). The base hydrolysis of methoprene did not seem to cause isomerization of the double bonds, as determined by 60-MHz NMR. It should be noted, however, that when acids are formed from unsaturated α,β-alcohols or ester, isomerization can occur.

The resulting 2-(trimethylsilyl)ethyl ester was deblocked with tetraethylammonium fluoride as the source of fluoride ion, under mildly basic conditions (Carpino et al., 1978), to afford 4-(11-methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoyloxy)butanoic acid (**12**). This selective removal of the protective groups allowed for the sequential coupling to methoprene acid (**2**) and to the carrier protein to afford the immunogen (**15**).

Conjugation Reactions. Methoprene–spacer–acid (**12**) was conjugated to the carrier protein using two activated ester methods. The NHS ester (**13**) was easily formed by condensation of methoprene–spacer–acid (**12**) with *N*-hydroxysuccinimide (Figure 3). The conjugation reaction was carried out under organic/aqueous reaction conditions, which were unfavorable for the solubilization of the protein. Despite the heterogeneous nature of the reaction, approximately 50 methoprene residues were bound per protein molecule after 48 h (Table I), as determined by the radioactivity of the conjugates.

A water-soluble activated ester was also prepared. Methoprene–spacer–acid (**12**) was esterified with sodium 1-hydroxy-2-nitro-4-benzenesulfonate (HNSA) to give the activated ester (**14**). HNSA was prepared by the nitration of sodium 4-hydroxybenzenesulfonate (King, 1921). This ester was freely soluble in the aqueous solutions used to form the protein conjugate (**15**).

The HNSA ester coupling reaction also has the advantage of using spectroscopy to monitor the progress of the reaction. Both the protein and the active ester were soluble in phosphate-buffered saline at neutral pH. Reaction of the carrier protein was followed by removing aliquots of reaction mixtures and measuring the increase in the 1-hydroxy-2-nitro-4-benzenesulfonate dianion concentration over time [Table I; also see Analysis of Esters of 1-Hydroxy-2-nitro-4-benzenesulfonic Acid (HNSA)]. Generation of free dianion was directly proportional to the amount of acylation of the carrier protein. The ester was stable under neutral reaction conditions, with less than 1% hydrolysis over a 24-h period, in the absence of nucleophiles. This method allowed for a reduction in reaction time, and the incorporation of approximately 30 methoprene residues per protein molecule (Table I). Because both spectral analysis and radioassay indicated similar hapten densities, the HNSA method may be a valuable tool for future immunogen preparations. The method conferred water solubility to an otherwise oily hapten. The formation of such a water-soluble hapten, the properties of which can be determined by spectral analysis, may provide an important contribution to pesticide immunochemical technology.

Immunization. Both mice (Mei, 1988) and rabbits were immunized with the methoprene antigen. All animals produced antibodies which showed specificity for methoprene; however, antisera from one particular rabbit showed negligible affinity for the carrier protein, as assayed by allowing antibodies to bind the unmodified carrier (less than 5% bound with preabsorbing antisera against the carrier). Antiserum from this animal was subsequently used to develop the iELISA and CIEIA.

Indirect ELISA. The iELISA developed was designed to measure the amount of methoprene in a solution by an indirect competition reaction. Such assay formats for pesticides are described by Jung et al. (1989). Methoprene was first immobilized on the solid support by coating the methoprene conjugate to microtiter plate wells. The methoprene coating antigen that gave the best results

contained 31 molecules of methoprene per molecule of protein and was applied at a concentration of 10 ng/well (0.1 mL/well). Under these conditions, whole antisera from one rabbit could be diluted up to 15 000 times. It should be noted that the immunizing antigen contained a higher hapten density than the coating antigen and that the two antigens were prepared from different activated esters under different reaction conditions. With these conditions, the binding of the antiserum from this rabbit to the carrier protein was reduced to less than 1%. We have not witnessed such a lack of response to the carrier protein by antisera from other similarly immunized animals. Methoprene could inhibit the binding of 50% of the antibody at a concentration of 50 ppb in the iELISA (Figure 5).

Competitive Inhibition Enzyme Immunoassay. The CIEIA format greatly reduced the amount of time needed to perform the assay compared to the iELISA (1.5 vs 6–7 h, respectively). The CIEIA also required less tobacco sample preparation, where one need only extract the sample, filter the supernatant, make one dilution, and apply to the assay. The assay measures methoprene on tobacco in a 1–10 ppm range; however, standards provided in the CIEIA kit range from 2 to 20 ppb as a final concentration in the sample plate. This takes into account the dilution factors introduced as a result of extracting tobacco treated with parts per million levels of methoprene (Figure 9). The standard curve prepared using the CIEIA in the absence of the matrix had a range of 1.0–10.0 ppb, with an I_{50} of 3.5 ppb (Figure 8, *S*-methoprene).

Cross Reactivity of Antibodies. Cross recognition to related compounds was examined using the iELISA format. The derivatives used to make the methoprene immunogen, methoprene acid (**2**) and methoprene–spacer–acid (**12**), had I_{50}s of 50 and 70 ng/mL, respectively, indicating that both the analyte portion of the hapten, independent of the spacer arm, and the immunizing hapten inhibited binding (Figure 6). The cross reactivity to methoprene acid can be eliminated from environmental samples by using chromatography to separate methoprene from methoprene acid. Schooley et al. (1975) have shown that methoprene acid is a degradation product formed by microbial metabolism in water samples; however, degradation of methoprene by photodecomposition (Quistad et al., 1975b) or mammalian metabolism (Quistad et al., 1975a) does not generate methoprene acid. Related compounds, JHs I and III, did not cross react with the methoprene antiserum at concentrations up to 800 ng/mL (Figure 6). The methoprene analogues hydroprene (**3**) and kinoprene (**4**) also did not cross react at concentrations up to 1000 ng/mL (Figure 7).

Using the CIEIA, it was found that the antibody was inhibited by less *S*-methoprene compared to the racemic mixture (I_{50} = 3.5 vs ~5 ng/mL, respectively) (Figure 8). The exact composition of *R* and *S* isomers of the racemic mixture was not known, and we did not have access to pure *R*-methoprene. The CIEIA was also very effective at predicting the amount of methoprene (racemic mixture) present on spiked tobacco, indicating that the tobacco matrix had little effect on assay specificity (Figure 9).

Application of the Methoprene iELISA and CIEIA. This paper describes the synthesis of hapten and hapten–carrier conjugates used to raise specific polyclonal antibodies which form the basis for sensitive immunoassays for methoprene. The methoprene immunoassays have the potential for use in the determination of residues from plant and water matrices. The CIEIA format enables the user to process many more samples than other analytical techniques. Collaborative efforts are currently being conducted to determine if the methoprene CIEIA can be adapted to detect residues from grain samples, e.g., whole grain wheat, broken kernels, and flours (Hill et al., 1991). Additional protocols are also being investigated using methanol (80% v/v) as the extracting solvent in place of acetronitrile for plant matrices. Preliminary results indicate that methanol is a suitable substitute for acetonitrile.

ACKNOWLEDGMENT

We thank Sandoz Crop Protection Corp. for the gifts of methoprene, hydroprene, and kinoprene. We also thank Dr. Bai-Xiang Zou for technical assistance with the methoprene indirect ELISA and Dr. Titan Fan and Ms. Karen Larkin at ImmunoSystems for assistance with the CIEIA. This research was funded, in part, by the R. J. Reynolds Tobacco Co., ImmunoSystems, Inc., and the Massachusetts Agricultural Experiment Station. It is published as Contribution No. 2970 from the station.

LITERATURE CITED

Aldwin, L.; Nitecki, D. E. A Water-Soluble, Monitorable Peptide and Protein Crosslinking Agent. *Anal. Biochem.* **1987**, *164*, 494–501.

Allen, C. R.; Dickinson, C. M. Determination of Methoprene in Water Samples by High Performance Liquid Chromatography. *J. Liq. Chromatogr.* **1990**, *13*, 371–381.

Baehr, J. C.; Pradelles, P.; Lebreux, C.; Cassier, P.; Dray, F. A Simple and Sensitive Radioimmunoassay for Insect Juvenile Hormone Using an Iodinated Tracer. *FEBS Lett.* **1976**, *69*, 123–128.

Bergot, B. J.; Anderson, R. J.; Schooley, D. A.; Henrick, C. A. Liquid Chromatographic Analysis of Enantiomeric Purity of Several Terpenoid Acids as Their 1-(1-Naphthyl)Ethylamide Derivatives. *J. Chromatogr.* **1978**, *155*, 97–105.

Bos, E. S.; van der Doelen, A. A.; van Rooy, N.; Schurs, A. H. 3,3′,5,5′-Tetramethylbenzidine as an Ames Test Negative Chromogen for Horse-Radish Peroxidase in Enzyme Immunoassay. *J. Immunol.* **1981**, *2*, 187–204.

Carpino, L. A.; Tsao, J.-H.; Rinsdorf, H.; Fell, E.; Hettrich, G. The β-(Trimethylsilyl)ethoxycarbonyl Amino-protecting Group. *J. Chem. Soc., Chem. Commun.* **1978**, 358–359.

Chamberlain, S. T. Determination of Methoprene in Poultry Manure by High-performance Liquid Chromatography. *Analyst* **1985**, *110*, 879–880.

Dunham, L. L.; Liebrand, R. J. Residue Determination of an Insect Growth Regulator by Mass Fragmentography. *Adv. Spectrom.* **1974**, *6*, 251–257.

Dunham, L. L.; Schooley, D. A.; Siddall, J. B. A Survey of the Chromatographic Analysis of Natural Insect Juvenile Hormones and the Insect Growth Regulator, Altosid. *J. Chromatogr. Sci.* **1975**, *13*, 334–336.

Giang, P. A.; Jaffe, H. Infrared Spectrophotometric Determination of Encapsulated Methoprene in Poly(lactic acid) Formulations. *J. Am. Oil Chem. Soc.* **1980**, *73*, 879–882.

Goodman, W. G.; Coy, D. C.; Baker, F. C.; Xu, L.; Toong, Y. C. Development and Application of a Radioimmunoassay for the Junvenile Hormones. *Insect Biochem.* **1990**, *20*, 357–364.

Goodrow, M. H.; Harrison, R. O.; Hammock, B. D. Hapten Synthesis, Antibody Development, and Competitive Inhibition Immunoassay for *s*-Triazine. *J. Agric. Food Chem.* **1990**, *38*, 990–996.

Granger, N. A.; Goodman, W. G. Juvenile Hormone Radioimmunoassays—Theory and Practice. *Insect Biochem.* **1983**, *13*, 333–340.

Granger, N. A.; Goodman, W. G. Radioimmunoassay: Junvenile Hormones. In *Immunological Techniques in Insect Biology*; Gilbert, L. I., Miller, T. A., Eds.; Springer-Verlag: New York, 1988; pp 215–251.

Hammock, B. D.; Mumma, R. O. Potential of Immunochemical Technology for Pesticide Analysis. In *Pesticide Identification at the Residue Level*; Gould, R. F., Ed.; American Chemical Society: Washington, DC, 1980; pp 321–352.

Hammock, B. D.; Gee, S. J.; Cheung, P. Y. K.; Miyamoto, T.; Goodrow, M. H.; Van Emon, J.; Seiber, J. N. Utility of Immunoassay in Pesticide Analysis. In *Pesticide Science and Biotechnology*; Greenhalgh, R., Roberts, T. R., Eds.; Blackwell Scientific Publications: Boston, MA, 1987; pp 309–316.

Hammock, B. D.; Gee, S. J.; Harrison, R. O.; Jung, F.; Goodrow, M. H.; Li, Q.-X.; Lucas, A. D.; Szekacs, A.; Sundaram, K. M. S. Immunochemical Technology in Environmental Analysis, Addressing Critical Problems. In *Immunochemical Methods for Environmental Analysis*; Van Emmon, J. M., Mumma, R. O., Eds.; American Chemical Society: Washington, DC, 1990; pp 112–139.

Heckman, R. A.; Conner, T. R. Solid-Phase Extraction of Methoprene Residue from Tobacco with On-Line Elution and HPLC Analysis. *LC-GC* **1989**, *7*, 885–856.

Henrick, C. A.; Staal, G. B.; Siddall, J. B. Alkyl 3,7,11-Trimethyl-2,4-dodecadienoates, A New Class of Potent Insect Growth Regulators with Juvenile Hormone Activity. *J. Agric. Food Chem.* **1973**, *21*, 354–359.

Henrick, C. A.; Willy, W. E.; Staal, G. B Insect Juvenile Hormone Activity of Alkyl (2*E*,4*E*)-3,7,11-Trimethyl-2,4-dodecadienoates. Variations in the Ester Function and in the Carbon Chain. *J. Agric. Food Chem.* **1976**, *24*, 207–218.

Henrick, C. A.; Anderson, R. J.; Staal, G. B.; Ludvick, G. F. Insect Juvenile Hormone Activity of Optically Active Analogues (2*E*,4*E*)-3,7,11-Trimethyl-2,4-dodecadienoates and of Arylterpenoid Analogues. *J. Agric. Food Chem.* **1978**, *26*, 542–550.

Herman, B. W. Immunoassays of Pesticides. In *Immunological Techniques in Insect Biology*; Gilbert, L. I., Miller, T. A., Ed.; Springer-Verlag: New York, 1988; pp 135–179.

Hill, A. S.; Mei, J. V.; Yin, C.-M.; Ferguson, B. S.; Skerritt, J. H. Determination of the Insect Growth Regulator, Methoprene, in Wheat and Milling Fractions Using an Enzyme Immunoassay. *J. Agric. Food Chem.* **1991**, *39*, 1882–1886.

Jung, F.; Gee, S. J.; Harrison, R. O.; Goodrow, M. H.; Karu, A. E.; Braun, A. L.; Li, Q.-X.; Hammock, B. D. Use of Immunochemical Technologies for the Analysis of Pesticides. *Pestic. Sci.* **1989**, *26*, 303–317.

King, R. Production of Picric Acid from the Sulphonic Acids of Phenol. *J. Chem. Soc.* **1921**, *119*, 2105–2119.

Kinsky, S. C.; Loader, J. E.; Benson, A. L. An Alternative Procedure for the Preparation of Immunologic Liposomal Model Membranes. *J. Immunol. Methods* **1983**, *65*, 295–306.

Kinsky, S. C.; Hashimoto, K.; Loader, J. E.; Benson, A. L. Synthesis of *N*-Hydroxysuccinimide Esters of Phosphatidylethanolamine and Some Properties of Liposomes Containing These Derivatives. *Biochim. Biophys. Acta* **1984**, *769*, 543–550.

Kortvelyessy, G.; Szoradi, S.; Sztruhar, I.; Ladanyi, L. Gas Chromatography of 3,7,11-Trimethyl-11-hydroxy- (or methoxy)-2,4-dodecadienoic Acids and Related Compounds as Their Methyl or Trimethylsilyl Esters. *J. Chromatogr.* **1984**, *303*, 370–374.

Lauer, R. C.; Solomon, P. H.; Nakanishi, K.; Erlanger, B. F. Antibodies to Insect C16-Juvenile Hormone. *Experientia* **1974**, *30*, 560–562.

Liem, H. H.; Cardenas, F.; Tavassoli, M.; Poh-Fitzpatrick, M. B. Quantitative Determination of Hemoglobin and Cytochemical Staining for Peroxidase Using 3,3′,5,5′-Tetramethylbenzidine Dichloride, a Safe Substitute for Benzidine. *Anal. Biochem.* **1979**, *98*, 388–393.

Mei, J. V. The Use of Methoprene in the Preparation of an Immunogen from Non-Immunogenic Insect Growth Regulators with Juvenile Hormone Activity: Development of a Model System. M. S. Thesis, University of Massachussetts, Amherst, MA, 1988.

Mei, J. V.; Yin, C.-M.; Carpino, L. A. An Enzyme-Linked Immunosorbent Assay for the Residue Detection of the Insect Growth Regulator, Methoprene. In *Immunochemical Methods for Environmental Analysis*; Van Emmon, J. M., Mumma, R. O., Eds.; American Chemical Society: Washington, DC, 1990; pp 140–155.

Miller, W. W.; Wilkins, J. S.; Dunham, L. L. Determination of Altosid Insect Growth Regulator in Waters, Soils, Plants, and Animals by Gas-Liquid Chromatography. *J. Am. Oil Chem. Soc.* **1975**, *58*, 10–18.

Mumma, R. O.; Brady, J. F. Immunological Assays for Agrochemicals. In *Pesticide Science* and *Biotechnology*; Greenhalgh, R., Roberts, T. R., Eds.; Blackwell Scientific Publications: Boston, MA, 1987; pp 341–348.

O'Rangers, J. J. Development of Drug Residue Immunoassays, Technical Considerations. In *Immunochemical Methods for Environmental Analysis*; Van Emmon, J. M., Mumma, R. O., Eds.; American Chemical Society: Washington, DC, 1990; pp 27–37.

Quistad, G. B.; Staiger, L. E.; Bergot, B. J.; Schooley, D. A. Environmental Degradation of the Insect Growth Regulator Methoprene. VII. Bovine Metabolism to Cholesterol and Related Natural Products. *J. Agric. Food Chem.* **1975a**, *23*, 743–749.

Quistad, G. B.; Staiger, L. E.; Schooley, D. A. Environmental Degradation of the Insect Growth Regulator Methoprene (Isopropyl(2*E*,4*E*)-11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoate). III. Photodecomposition. *J. Agric. Food Chem.* **1975b**, *23*, 299–303.

Schooley, D. A.; Bergot, B. J.; Dunham, L. L.; Siddall, J. B. Environmental Degradation of the Insect Growth Regulator Methoprene (Isopropyl (2*E*,4*E*)-11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoate). II. Metabolism by Aquatic Microorganisms. *J. Agric. Food Chem.* **1975**, *23*, 293–298.

Scott, D.; Nitecki, D. E.; Kindler, H.; Goodman, J. W. Immunogenicity of Biotinylated Hapten-Avidin Complexes. *Mol. Immunol.* **1984**, *21*, 1055–1060.

Sieber, P.; Andreatta, R. H.; Eisler, K.; Kamber, B.; Riniker, B.; Rink, H. The 2-Trimethylsilylethyl Residue, A Selectively Cleavable Carboxylic Acid Protecting Group. In *Peptides. Proceedings of the 5th American Peptide Symposium*; Goodman, M., Meienhofer, J., Eds.; Wiley and Sons: New York, 1977; pp 543–545.

Still, W. C.; Kahn, M.; Mitra, A. Rapid Chromatographic Technique for Preparative Separation with Moderate Resolution. *J. Org. Chem.* **1978**, *43*, 2923–2925.

Strambi, C. Some Data Obtained by Radioimmunoassay of Juvenile Hormones. In *Juvenile Hormone Biochemistry: Action, Agonism, and Antagonism*; Pratt, G. T., Brook, G. E., Eds.; Elsevier North-Holland Biomedical Press: Amsterdam, 1981; pp 47–57.

Strambi, C.; Strambi, A.; De Reggi, M. L.; Hirn, M. H.; De Laage, M. A. Radioimmunoassay of Insect Juvenile Hormones and Their Diol Derivatives. *Eur. J. Biochem.* **1981**, *118*, 401–406.

Sudo, R.; Kaneda, A.; Itoh, N. Synthesis of Carolic Acid. *J. Org. Chem.* **1967**, *32*, 1844–1846.

Vallejo, R. P.; Bogus, E. R.; Mumma, R. O. Effects of Hapten Structure and Bridging Groups on Antisera Specificity in Parathion Immunoassay Development. *J. Agric. Food Chem.* **1982**, *30*, 572–580.

Van Duzee, E. M.; Adkins, H. Hydrogenation and Hydrogenolysis of Ethers. *J. Am. Chem. Soc.* **1935**, *57*, 147–151.

Wright, J. E.; Jones, R. L. Insect Growth Regulators: Methoprene and Stauffer R-20458 in Pupae of the Stable Fly from Treated Breeding Medium. *Bull. Environ. Contam. Toxicol.* **1976**, *15*, 525–529.

Received for review April 9, 1991. Revised manuscript received July 22, 1991. Accepted August 13, 1991.

Registry No. 2, 40596-67-6; 11, 136586-83-9; 12, 136586-86-2; 13, 136586-87-3; 14, 136586-88-4; methoprene, 40596-69-8; 2-(trimethylsilyl)ethyl 4-hydroxybutanoate, 136586-84-0; 2-(trimethylsilyl)ethyl 4-[11-methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoyloxy]butanoate, 136586-85-1.