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Re.: Expediente No. **05.059.203**

Solicitud de Patente PCT Entrada en Fase Nacional, para: **AVERMECTINA B1 Y DERIVADOS DE MONOSACARIDOS DE AVERMECTINA B1 QUE TIENEN UN SUSTITUYENTE ALCOXIMETILO EN LA POSICION 4" O 4'**

Solicitante: **MERIAL LIMITED**

RESPUESTA AL EXAMEN DE FONDO

MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pié de mi firma, atentamente me permito presentar respuesta al oficio No. 08494 notificado por fijación en lista el 24 de julio de 2009 correspondiente al examen de fondo según el artículo 46 de la Decisión 486, estando dentro del término legal en los siguientes términos:

Presento copia de los siguientes artículos requeridos por su Despacho según el artículo 46 de la Decisión 486:

1. **TSUKAMOTO, YOSHIHISA ET AL:** "Avermectin chemistry and action: ester and ether-type candidate photoaffinity probes" BIOORGANIC & MEDICINAL CHEMISTRY (2000), 8 (1), 19-26, 2000, XP002280644.
2. **SHOOP, W. L. ET AL.** "Efficacy in sheep and pharmacokinetics in cattle that led to the selection of eprinomectin as a topical endectocide for cattle" INTERNATIONAL JOURNAL FOR PARASITOLOGY (1996), 26 (11), 1227-1235, 1996, XP002280645.
3. **JONES, TODD K. ET AL:** "Synthesis and biological activity of 4a,4-disubstituted avermectins" JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY (1994), 42 (8), 1786-90, 1994, XP002280646

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Cumplido así con lo dispuesto, atentamente solicito a Ud. se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

De la Señora Jefe,


Margarita Castellanos Abondano
C.C. No. **39.779.654** de Usaquén
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Avermectin Chemistry and Action: Ester- and Ether-type Candidate Photoaffinity Probes

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Abstract—Avermectin B_{1a} (**1**) is a potent anthelmintic, insecticide, miticide and chloride channel activator on interaction with a specific nerve membrane site analyzed by binding assays with [³H]**1**. Candidate photoaffinity probes were prepared replacing the dioleandrosyl substituent with potential isosteric esters and ethers approximating the original overall atom length and terminating in a phenyl moiety substituted with azido, iodo or hydroxy. Several of the candidates met the goal of high potency on mouse, housefly and fruit fly brain chloride channels with IC₅₀ values of 7–57 nM in competing for the [³H]**1** binding site. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

The avermectins are potent anthelmintics, insecticides and miticides used to control pests of humans, veterinary animals and crops.^{1,2} The prototype avermectin B_{1a} (**1**) consists of a macrocyclic lactone with a 13 α -dioleandrosyl substituent (R) (Fig. 1). Compound **1** and many analogues bind to vertebrate and invertebrate γ -aminobutyric acid (GABA)-gated and invertebrate glutamate-gated chloride channels to increase ion conductance, thereby disrupting inhibitory or excitatory action. These mechanisms are established by physiological, biochemical and molecular biological investigations^{3–8} with an important contribution from radioligand binding studies with [5 α -³H]**1** and its 22,23-ditritio derivative [³H]ivermectin.^{9–13} Photoaffinity labeling has been successfully applied by Merck scientists to the action of the avermectins, using radioligand **2** which is 4''-amino-4''-deoxy-**1** with 4-azido-3-¹²⁵iodosalicylic acid attached to the amino group through a β -alanyl- ϵ -aminocaproyl spacer (Fig. 1).^{14,15} Photoaffinity probe **2** labels a 47-kDa polypeptide from head membranes of the fruit fly *Drosophila melanogaster* and

53-, 47- and 8-kDa polypeptides from the free-living nematode *Caenorhabditis elegans* but does not bind to rat brain membranes and, therefore, is selective for the insect and nematode membranes.^{16–18} Although target site selectivity is a distinct advantage for safe use of a pharmaceutical or pesticide, it does not enable a single probe to be employed for comparing vertebrate and invertebrate targets and defining differences in their binding sites within the chloride channel, as has been successfully applied to chloride channel blockers.¹⁹

The goal of this investigation is to design a candidate photoaffinity probe effective for both mammals and insects. The failure of **2** to act in mammalian brain¹⁶ is conceivably due to the far greater length of its R substituent than that of **1**. An alternative is that **2** has amide bonds versus only ether linkages in the R substituent of **1**. The candidate photoaffinity probes **3–6** prepared here, therefore, include ester and ether moieties more nearly isosteric with the dioleandrosyl substituent (Fig. 1). Potency is evaluated using the [³H]**1** binding assay with membranes from mouse brain and housefly (*Musca domestica*) and fruit fly heads.

Keywords: antiparasitics; labelling; neurologically-active compounds; receptors; substituent effects.

Additional or alternative key words: Chloride channel; insecticides; isosteres.

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Structural modifications

Esters 3a–f of 1-aglycone (Figs 1 and 2). Key intermediate **9** was synthesized by a two-step reaction from 5-*O*-*tert*-butyldimethylsilylavermectin B_{1a} aglycone (**7**).²⁰ Treatment of **7** with the anhydride prepared in situ from *N*-(2,2,2-trichloroethoxycarbonyl)- β -alanine and

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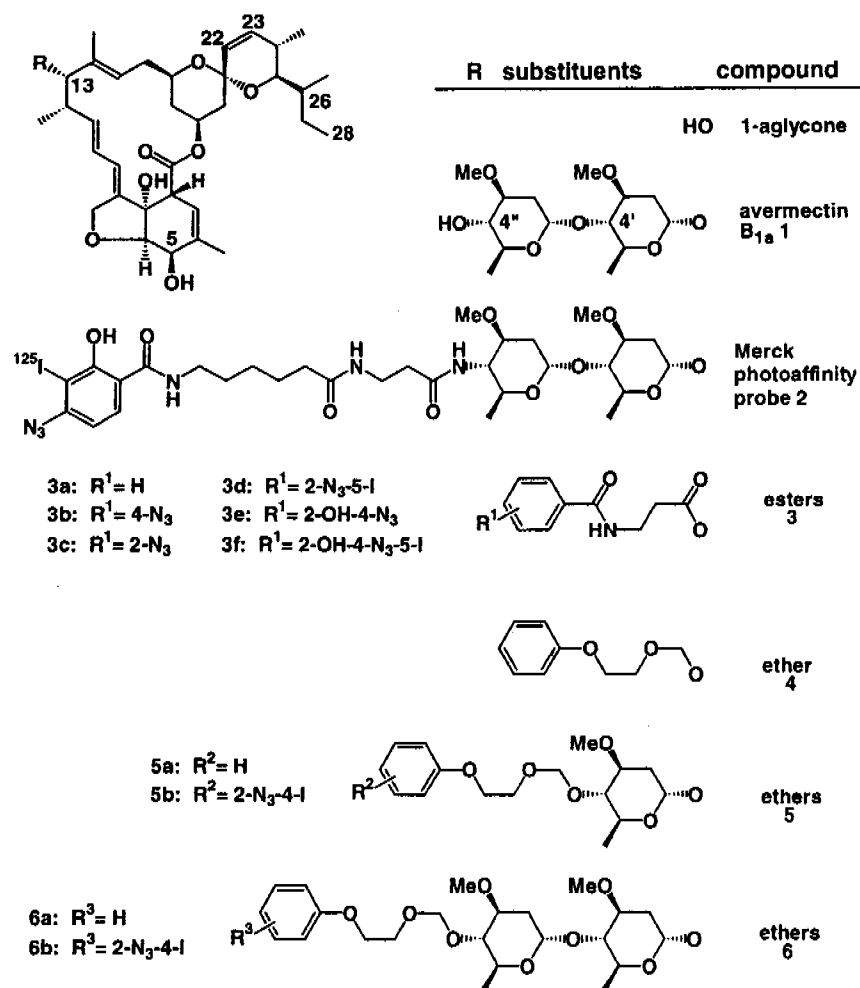


Figure 1. Structures of avermectin B_{1a} (1) and the corresponding aglycone (1-aglycone) and of an amide (2), esters (3) and ethers (4–6) derived therefrom.

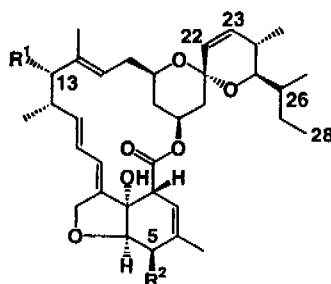
pivaloyl chloride in the presence of triethylamine gave ester 8 in high yield, and reductive cleavage of the 2,2,2-trichloroethoxycarbonyl group of this ester afforded 9. Acylation of the deprotected amino group of 9 was carried out with benzoyl chloride for 3a or with the previously known azidobenzoic acid derivatives in the presence of 1,3-dicyclohexylcarbodiimide (DCC) for 3b–d or with the *N*-hydroxysuccinimidyl ester of 4-azido-2-hydroxybenzoic acid and 4-hydroxy-2-iodobenzoic acid for 3e and 3f, respectively. Finally, desilylation of the 5-hydroxy group gave the esters 3a–f.^{14,20,21}

Ethers 4 of 1-aglycone, 5a and 5b of 1-monosaccharide and 6a and 6b of 1 (Figs 1 and 3). The ether-type derivatives (4–6) were obtained from 7, 5-*O*-*tert*-butyldimethylsilylavermectin B_{1a} monosaccharide (12), and 5-*O*-*tert*-butyldimethylsilylavermectin B_{1a} (14)²² by coupling with phenoxyethoxymethyl chloride or 2-(2-azido-4-iodophenoxy)ethoxymethyl chloride followed by deprotection of the 5-hydroxy group.

Structure–activity relationships

General (Fig. 1 and Table 1). Compound 1 differs only 3-fold in potency for the mouse, housefly and fruit fly brain [³H]1 binding site and 1-aglycone is 18- to 68-fold less active. Photoaffinity probe 2 is similar to ivermectin in its affinity for the *C. elegans* membrane binding site despite the long spacer group between the macrocyclic lactone and the terminal photoreactive 4-azido-3,125-iodosalicylate moiety.¹⁶ However, 2 does not bind to the rat brain [³H]ivermectin binding site¹⁶ suggesting that an R substituent more isosteric with that of 1 may be more appropriate. Several of the candidates in the present study met the goal of high potency on mouse, housefly and fruit fly brain chloride channels with IC₅₀ values of 7–57 nM in competing for the [³H]1 binding site.

Esters 3a–f (Fig. 1 and Table 1). The goal was to replace the dioleandrosyl unit with an ester of approximately



compound	R ¹	R ²
7	HO	OSiMe ₂ Bu- <i>t</i>
8		OSiMe ₂ Bu- <i>t</i>
9		OSiMe ₂ Bu- <i>t</i>
10a: R ³ = H 10d: R ³ = 2-N ₃ -5-I 10b: R ³ = 4-N ₃ 10e: R ³ = 2-OH-4-N ₃ 10c: R ³ = 2-N ₃ 10f: R ³ = 2-OH-4-N ₃ -5-I		OSiMe ₂ Bu- <i>t</i>

reaction conditions

7 → 8: Cl₃CH₂OCONHCH₂CH₂CO₂H, Et₃N, *t*-BuCOCl, CH₂Cl₂, then 7, *i*-Pr₂NEt, 4-DMAP (99%). 8 → 9: Zn dust, AcOH, H₂O, THF (57%). 9 → 10a: PhCOCl, Et₃N, CH₂Cl₂ (87%). 9 → 10b: 4-N₃-PhCO₂H, DCC, CH₂Cl₂ (65%). 9 → 10c: 2-N₃-PhCO₂H, DCC, CH₂Cl₂ (77%). 9 → 10d: 2-N₃-5-I-PhCO₂H, DCC, CH₂Cl₂ (72%). 9 → 10e: 2-HO-4-N₃-PhCO₂-Su, CH₂Cl₂ (66%). 9 → 10f: 2-HO-4-N₃-5-I-PhCO₂-Su, CH₂Cl₂ (66%). 10 → 3: HF-Py, MeCN; 3a (86%), 3b (100%), 3c (99%), 3d (79%), 3e (69%), 3f (91%).

Figure 2. 5-*O*-(*tert*-Butyldimethylsilyl)-13-*O*-(2-aminopropionyl) avermectin B_{1a} aglycone (9) and precursors (7 and 8) and derivatives (10) thereof. Su refers to *N*-hydroxysuccinimidyl ester of the acid.

10-atom equivalent length including an aryl moiety that would potentially bear azido, iodo and possibly hydroxy substituents. The β -alanil group was used as a candidate isosteric spacer unit. All of the esters are potent in the housefly receptor assays (IC₅₀ values 7–31 nM) while 3c and 3e are best for the mouse preparation (IC₅₀ values 57 and 105 nM) and 3d–f for the fruit fly membranes (IC₅₀ values 26–56 nM). On this basis iodo-containing compounds 3d and 3f are candidates for radiolabeling, especially for the insect receptor. While a portion of the activity of the esters may be due to hydrolysis to 1-aglycone, this is probably not a major factor since the IC₅₀ values of 3e for mouse brain and housefly membrane preparations were not changed by treating with a potent esterase inhibitor (phenyl saligenin cyclic phosphonate or phenylmethanesulfonyl fluoride, 10 μ M, 30 min preincubation, data not detailed here) and the ester linkage can be substituted with a non-hydrolyzable ether moiety with enhanced potency relative to the aglycone (see below).

Ethers 4, 5a, 5b, 6a and 6b (Fig. 1 and Table 1). Ether linkages were examined as one way to rule out the possibility of esterases or amidases limiting the potency of

candidate photoaffinity probes. The 13-*O*-(2-phenoxyethoxymethyl) derivatives as prepared from 1-aglycone (4), 1-monosaccharide (5a) and 1 (6a) retain a high level of potency in the housefly receptor assay (IC₅₀ values 19–97 nM). However, the 2-azido-4-iodophenoxy analogues (5b and 6b) are ~100-fold less active than their phenoxy counterparts with mouse and 2–5-fold less active with housefly. Further optimization is required in the phenoxy substituents for a general photoaffinity probe.

Experimental

Chemistry

General. ¹H NMR spectra were recorded for CDCl₃ solutions with a Bruker AM-300 spectrometer. Fast atom bombardment (FABMS) (both low- and high-resolution, LR and HR) was conducted with the Fisons ZAB2-EQ spectrometer. Analytical thin-layer chromatography (TLC) was performed on silica gel with fluorescent indicator using precoated plastic sheets. Preparative TLC involved 20×20 cm plates coated with

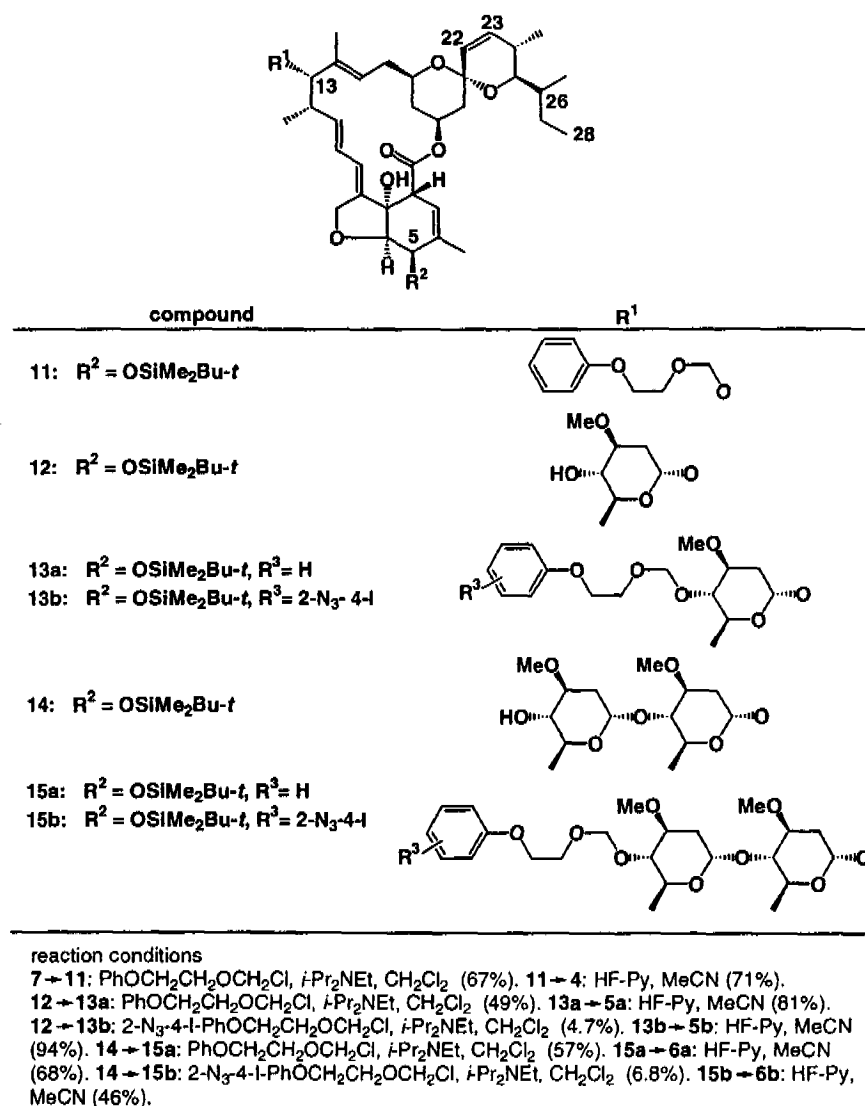


Figure 3. 5-*O*-(*tert*-Butyldimethylsilyl)-13-*O*-(2-phenoxyethoxymethyl)avermectin B_{1a} aglycone (11) and related compounds (12–15).

0.25 or 0.5 mm silica gel containing PF₂₅₀ indicator. Compounds were visualized with short wavelength UV light. Column flash chromatography was performed using silica gel 60. All final products were purified to homogeneity as determined by TLC and ¹H NMR and had appropriate ¹H NMR spectra (Table 2) and molecular mass by FABMS.

5-*O*-(*tert*-Butyldimethylsilyl)-13-*O*-[2-(2,2,2-trichloroethoxycarbonylamino)propionyl]-avermectin B_{1a} aglycone (8). To a solution of *N*-(2,2,2-trichloroethoxycarbonyl)-β-alanine²³ (530 mg) in CH₂Cl₂ (7 mL) in an ice bath were added triethylamine (280 μL) and pivaloyl chloride (250 μL). The mixture was stirred for 30 min at 0 °C and a solution of 7²⁰ (141 mg) in CH₂Cl₂ (3 mL), diisopropylethylamine (174 μL), and 4-(*N,N*-dimethylamino)-pyridine (DMAP) (123 mg) was added at 0 °C. The

mixture was stirred at room temperature for 18 h and poured into ice-cold water (40 mL). The resulting mixture was extracted with ethyl acetate (40 mL, and then 3 × 20 mL) and the combined organic layer was washed with water (20 mL) and brine (20 mL), then dried over Na₂SO₄ and evaporated in vacuo. The residue was purified by silica gel column chromatography (hexane-ethyl acetate gradient) to give 188 mg (99%) of 8.

5-*O*-(*tert*-Butyldimethylsilyl)-13-*O*-(2-aminopropionyl)-avermectin B_{1a} aglycone (9). A mixture of 8 (184 mg), water (10 μL), acetic acid (100 μL) and zinc dust (747 mg) in tetrahydrofuran (THF) (8 mL) was stirred for 4.5 h at room temperature. The reaction mixture was filtered through Celite and the solid was washed with ethyl acetate (80 mL). The filtrate and the ethyl acetate were combined and washed with water (20 mL) and

Table 1. Structure–activity relationships of avermectin derivatives as inhibitors of [³H]avermectin binding in brain membranes of mouse, housefly and fruitfly

Compound	IC ₅₀ , nM ± SD (n)		
	Mouse	Housefly	Fruit fly
<i>Avermectin series</i>			
1	2.9 ± 0.9 (10)	1.5 ± 0.6 (9)	4.2 ± 1.3 (5)
1-aglycone	196 ± 46 (3)	64 ± 20 (4)	77 ± 18 (3)
<i>Esters</i>			
3a	653, 424 ^a	13 ± 5 (3)	73 ± 28 (4)
3b	170 ± 98 (4)	15 ± 10 (3)	243 ± 86 (3)
3c	57 ± 34 (3)	15 ± 9 (3)	477, 304 ^a
3d	185 ± 72 (3)	31 ± 12 (5)	44 ± 38 (3)
3e	105 ± 28 (3)	6.7 ± 3.7 (3)	54 ± 24 (3)
3f	130 ± 2 (3)	21 ± 15 (3)	56, 26 ^a
<i>Ethers</i>			
4	29 ± 18 (5)	19 ± 8 (4)	—
5a	38 ± 7 (30)	33 ± 21 (3)	—
5b	3400, 506 ^a	174, 154 ^a	—
6a	33 ± 13 (3)	97, 62 ^a	—
6b	3684, 1210 ^a	201, 153 ^a	—

^aValues from separate experiments.

brine (20 mL), then dried over Na₂SO₄ and evaporated in vacuo. The residue was purified by preparative TLC (CH₂Cl₂:MeOH, 10:1) to give 85 mg (57%) of **9**.

5-O-(tert-Butyldimethylsilyl)-13-O-(2-benzoylamino-propionyl)-avermectin B_{1a} aglycone (10a). To a solution of **9** (17.5 mg) in CH₂Cl₂ (1 mL) were added triethylamine (8 μL) and benzoyl chloride (3 μL) at 0°C. The reaction mixture was stirred at room temperature for 24 h and purified by preparative TLC (hexane:ethyl acetate, 2:1) to give 17.2 mg (87%) of **10a**.

5-O-(tert-Butyldimethylsilyl)-13-O-[2-(4-azidobenzoyl)-aminopropionyl]avermectin B_{1a} aglycone (10b). A solution of **9** (16.9 mg), 4-azidobenzoic acid²⁴ (6.1 mg), and DCC (10 mg) in CH₂Cl₂ (1 mL) was stirred in the dark at room temperature for 15 h. The reaction mixture was purified by preparative TLC (hexane:ethyl acetate, 2:1) to give 13.1 mg (65%) of **10b**.

5-O-(tert-Butyldimethylsilyl)-13-O-[2-(2-azidobenzoyl)-aminopropionyl]avermectin B_{1a} aglycone (10c). The above procedure with **9** (14.3 mg), 2-azidobenzoic acid²⁵ (6.0 mg) and DCC (10 mg) in the dark gave 13.1 mg (77%) of **10c**.

5-O-(tert-Butyldimethylsilyl)-13-O-[2-(2-azido-5-iodobenzoyl)aminopropionyl]-avermectin B_{1a} aglycone (10d). The above procedure with **9** (17.0 mg), 2-azido-5-iodobenzoic acid²⁶ (11 mg) and DCC (10 mg) in the dark gave 16.7 mg (72%) of **10d**.

5-O-(tert-Butyldimethylsilyl)-13-O-[2-(4-azido-2-hydroxybenzoyl)aminopropionyl]-avermectin B_{1a} aglycone (10e). A solution of **9** (17.8 mg) and *N*-hydroxysuccinimidyl-4-azido-2-hydroxybenzoate²⁶ (6.9 mg) in CH₂Cl₂ (1 mL) was stirred in the dark at 0°C for 30 min and then at room temperature for 25 h. The reaction mixture was purified by preparative TLC (hexane:ethyl acetate, 2:1) to give 14.2 mg (66%) of **10e**.

5-O-(tert-Butyldimethylsilyl)-13-O-[2-(4-azido-2-hydroxy-5-iodobenzoyl)-aminopropionyl]avermectin B_{1a} aglycone (10f). The above procedure with **9** (17.8 mg) and *N*-hydroxysuccinimidyl-4-azido-2-hydroxy-5-iodobenzoate²⁷ (14.5 mg) in the dark gave 16.0 mg (66%) of **10f**.

5-O-(tert-Butyldimethylsilyl)-13-O-(2-phenoxyethoxymethyl)avermectin B_{1a} aglycone (11). To a solution of **7** (20 mg) and 2-phenoxyethoxymethyl chloride (106 mg) in CH₂Cl₂ (150 μL) was added diisopropylethylamine (120 μL). The reaction mixture was stirred for 3 days, poured into ice-cold saturated aqueous NaHCO₃ (20 mL), extracted with CH₂Cl₂ (4×10 mL) and the combined organic layer was washed with NaHCO₃ solution (2×10 mL) then dried over Na₂SO₄ and evaporated in vacuo. The residue was purified by preparative TLC (hexane:ethyl acetate, 6:1) to give 16.3 mg (67%) of **11**.

5-O-(tert-Butyldimethylsilyl)avermectin B_{1a} monosaccharide (12). A solution of avermectin B_{1a} monosaccharide²⁸ (326 mg), imidazole (183 mg), and *tert*-butyldimethylsilyl chloride (202 mg) in DMF (4 mL) was stirred for 40 min. Then additional imidazole (183 mg) and *tert*-butyldimethylsilyl chloride (202 mg) were added followed by stirring for 75 min. The reaction mixture was poured into ice-cold water (50 mL) and extracted with hexane:ethyl acetate (1/1) (4×25 mL). The combined organic layer was washed with water (2×25 mL) and brine (25 mL) then dried over Na₂SO₄ and evaporated in vacuo. The residue was purified by silica gel column chromatography (hexane–ethyl acetate gradient) to give 190 mg (50%) of **12**.

5-O-(tert-Butyldimethylsilyl)-4'-O-(2-phenoxyethoxymethyl)avermectin B_{1a} monosaccharide (13a). To a solution of **12** (21.9 mg) and 2-phenoxyethoxymethyl chloride (from treatment of 2-phenoxyethanol and paraformaldehyde in toluene with HCl gas) (260 mg) in CH₂Cl₂ (150 μL) was added diisopropylethylamine (243 μL). The reaction mixture was stirred for 1 day and poured into ice-cold saturated aqueous NaHCO₃ (10 mL), extracted with ethyl acetate (4×10 mL) and the combined organic layer was washed with aqueous NaHCO₃ (2×10 mL) then dried over Na₂SO₄ and evaporated in vacuo. The residue was purified by preparative TLC (CH₂Cl₂:MeOH, 30:1) to give 12.7 mg (49%) of **13a**.

5-O-(tert-Butyldimethylsilyl)-4'-O-[2-(2-azido-4-iodophenoxy)ethoxymethyl]avermectin B_{1a} monosaccharide (13b). To a solution of **12** (40 mg) and 2-(2-azido-4-iodophenoxy)ethoxymethyl chloride (from 2-(2-azido-4-iodophenoxy)ethanol and paraformaldehyde with HCl gas as above) (101 mg) in CH₂Cl₂ (100 μL) was added diisopropylethylamine (243 μL). The reaction mixture was stirred for 3 days with work up as for **13a** to give 2.6 mg (4.7%) of **13b**.

5-O-(tert-Butyldimethylsilyl)-4''-O-(2-phenoxyethoxymethyl)avermectin B_{1a} (15a). The procedure for **11** but with **14**²² and a reaction time of 1 day gave 14.7 mg (57%) of **15a**.

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244Table 2. Characteristic 300-MHz ¹H NMR chemical shifts (CDCl₃) of avermectin B_{1a} derivatives

Compound (s)	Chemical shift, δ (multiplicity, coupling constants)				
	C ₅ H	C ₁₃ H	C ₄ H	C ₄ *H	13-Substituent other than saccharide
<i>Avermectin series</i>					
1	4.28 (brs)	3.93 (s)	3.24 (t, 9)	3.16 (t, 9)	
14	4.43–4.45 (m)	3.94 (s)	3.25 (t, 9)	3.17 (t, 9)	
1-monosaccharide	4.29 (brs)	3.96 (s)	3.17 (t, 9)	—	
12	4.43–4.45 (brs)	3.95 (s)	3.17 (t, 9)	—	
1-aglycone	4.29 (brs)	4.00 (s)	—	—	
7	4.41–4.44 (m)	4.01 (s)	—	—	
<i>Intermediates</i>					
8	4.41–4.44 (m)	5.19 (s)	—	—	2.62–2.73 (2H, m, N-C-CH ₂ CO), 3.55 (2H, q, J = 6 Hz, NCH ₂ -C-CO), 4.70 (1H, s, Cl ₃ CCHOCO-N), 4.71 (1H, s, Cl ₃ CCHOCO-N)
9	4.42–4.43 (m)	5.20 (s)	—	—	2.61–2.72 (2H, m, N-C-CH ₂ CO), 2.84 (2H, brs, NH ₂), 3.08 (2H, t, J = 6 Hz, NCH ₂ -C-CO)
<i>Esters</i>					
3a	4.29 (brs)	5.20 (s)	—	—	2.63–2.81 (2H, m, N-C-CH ₂ CO), 3.70–3.86 (2H, m, NCH ₂ -C-CO), 6.85 (1H, t, J = 6 Hz, NH), 7.38–7.49 (3H, m, PhCO), 7.72–7.75 (2H, m, PhCO)
10a	4.41–4.43 (m)	5.20 (s)	—	—	2.64–2.81 (2H, m, N-C-CH ₂ CO), 3.70–3.86 (2H, m, NCH ₂ -C-CO), 6.86 (1H, t, J = 6 Hz, NH), 7.39–7.51 (3H, m, PhCO), 7.72–7.75 (2H, m, PhCO)
3b	4.28–4.32 (m)	5.21 (s)	—	—	2.64–2.81 (2H, m, N-C-CH ₂ CO), 3.71–3.86 (2H, m, NCH ₂ -C-CO), 6.84 (1H, t, J = 6 Hz, NH), 7.06 (2H, dd, J = 7 and 2 Hz, PhCO), 7.75 (2H, dd, J = 7 and 2 Hz, PhCO)
10b	4.41–4.44 (m)	5.21 (s)	—	—	2.64–2.81 (2H, m, N-C-CH ₂ CO), 3.71–3.85 (2H, m, NCH ₂ -C-CO), 6.84 (1H, t, J = 6 Hz, NH), 7.06 (2H, dd, J = 7 and 2 Hz, PhCO), 7.75 (2H, dd, J = 7 and 2 Hz, PhCO)
3c	4.29 (brs)	5.23 (s)	—	—	2.65–2.82 (2H, m, N-C-CH ₂ CO), 3.76–3.86 (2H, m, NCH ₂ -C-CO), 7.17 (1H, dd, J = 8 and 1 Hz, PhCO), 7.22 (1H, td, J = 8 and 1 Hz, PhCO), 7.48 (1H, td, J = 8 and 2 Hz, PhCO), 7.92 (1H, t, J = 5 Hz, NH), 8.11 (1H, dd, J = 8 and 2 Hz, PhCO)
10c	4.42–4.44 (m)	5.23 (s)	—	—	2.65–2.82 (2H, m, N-C-CH ₂ CO), 3.76–3.85 (2H, m, NCH ₂ -C-CO), 7.18 (1H, d, J = 9 Hz, PhCO), 7.22 (1H, td, J = 8 and 1 Hz, PhCO), 7.48 (1H, td, J = 8 and 2 Hz, PhCO), 7.93 (1H, t, J = 5 Hz, NH), 8.11 (1H, dd, J = 8 and 2 Hz, PhCO)
3d	4.27–4.31 (m)	5.23 (s)	—	—	2.63–2.80 (2H, m, N-C-CH ₂ CO), 3.73–3.86 (2H, m, NCH ₂ -C-CO), 6.92 (1H, d, J = 8 Hz, PhCO), 7.76 (1H, dd, J = 8 and 2 Hz, PhCO), 7.90 (1H, t, J = 6 Hz, NH), 8.41 (1H, d, J = 2 Hz, PhCO)
10d	4.41–4.31 (m)	5.23 (s)	—	—	2.65–2.80 (2H, m, N-C-CH ₂ CO), 3.75–3.86 (2H, m, NCH ₂ -C-CO), 6.92 (1H, d, J = 9 Hz, PhCO), 7.76 (1H, dd, J = 9 and 2 Hz, PhCO), 7.90 (1H, t, J = 6 Hz, NH), 8.41 (1H, d, J = 2 Hz, PhCO)
3e	4.27–4.31 (m)	5.21 (s)	—	—	2.67–2.80 (2H, m, N-C-CH ₂ CO), 3.75–3.86 (2H, m, NCH ₂ -C-CO), 6.47 (1H, dd, J = 9 and 2 Hz, PhCO), 6.62 (1H, d, J = 2 Hz, PhCO), 7.02–7.06 (1H, m, NH), 7.28 (1H, d, J = 9 Hz, PhCO), 12.30 (1H, s, HOPhCO)
10e	4.41–4.44 (m)	5.21 (s)	—	—	2.64–2.85 (2H, m, N-C-CH ₂ CO), 3.75–3.85 (2H, m, NCH ₂ -C-CO), 6.47 (1H, dd, J = 9 and 2 Hz, PhCO), 6.62 (1H, d, J = 2 Hz, PhCO), 7.03 (1H, t, J = 6 Hz, NH), 7.28 (1H, d, J = 9 Hz, PhCO), 12.30 (1H, s, HOPhCO)
3f	4.27–4.31 (m)	5.23 (s)	—	—	2.67–2.82 (2H, m, N-C-CH ₂ CO), 3.74–3.83 (2H, m, NCH ₂ -C-CO), 6.75 (1H, s, PhCO), 7.00 (1H, t, J = 6 Hz, NH), 7.67 (1H, s, PhCO), 12.32 (1H, s, HOPhCO)
10f	4.42–4.44 (m)	5.23 (s)	—	—	2.66–2.85 (2H, m, N-C-CH ₂ CO), 3.72–3.83 (2H, m, NCH ₂ -C-CO), 6.75 (1H, s, PhCO), 7.00 (1H, t, J = 6 Hz, NH), 7.67 (1H, s, PhCO), 12.32 (1H, s, HOPhCO)
<i>Ethers</i>					
4	4.27–4.31 (m)	3.96 (s)	—	—	3.80–4.18 (4H, m, OCH ₂ CH ₂ O), 4.65–4.68 (1H, obsc, OCH ₂ O), 4.71 (1H, d, J = 7 Hz, OCH ₂ O), 6.90–6.96 (2H, m, PhO), 7.25–7.31 (3H, m, PhO)
11	4.41–4.44 (m)	3.96 (s)	—	—	3.82–4.16 (4H, m, OCH ₂ CH ₂ O), 4.71 (1H, d, J = 7 Hz, OCH ₂ O), 4.77 (1H, d, J = 7 Hz, OCH ₂ O), 6.90–6.97 (2H, m, PhO), 7.26–7.30 (3H, m, PhO)
5a	4.30 (brs)	3.94 (s)	3.23 (t, 9)	—	3.83–4.20 (4H, m, OCH ₂ CH ₂ O), 4.89 (1H, d, J = 7 Hz, OCH ₂ O), 5.08 (1H, d, J = 7 Hz, OCH ₂ O), 6.91–6.97 (2H, m, PhO), 7.25–7.31 (3H, m, PhO)
13a	4.43–4.45 (m)	3.94 (s)	3.23 (t, 9)	—	3.82–4.16 (4H, m, OCH ₂ CH ₂ O), 4.89 (1H, d, J = 7 Hz, OCH ₂ O), 5.08 (1H, d, J = 7 Hz, OCH ₂ O), 6.91–6.97 (2H, m, PhO), 7.24–7.31 (3H, m, PhO)
5b	4.28–4.32 (m)	3.94 (s)	3.20 (t, 9)	—	3.92–4.02 (2H, m, OCH ₂ CH ₂ O), 4.17 (2H, t, J = 5 Hz, OCH ₂ CH ₂ O), 4.87 (1H, d, J = 7 Hz, OCH ₂ O), 5.06 (1H, d, J = 7 Hz, OCH ₂ O), 6.67 (1H, d, J = 9 Hz, PhO), 7.25 (1H, d, J = 2 Hz, PhO), 7.35 (1H, dd, J = 9 and 2 Hz, PhO)

(continued)

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Table 2 (continued)

13b	4.43–4.45 (m)	3.94 (s)	3.20 (t, 9)	—	3.92–4.02 (2H, m, OCH ₂ CH ₂ O), 4.17 (2H, t, <i>J</i> = 5 Hz, OCH ₂ CH ₂ O), 4.87 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 5.06 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 6.67 (1H, d, <i>J</i> = 9 Hz, PhO), 7.25 (1H, d, <i>J</i> = 2 Hz, PhO), 7.35 (1H, dd, <i>J</i> = 9 and 2 Hz, PhO)
6a	4.30 (brs)	3.94 (s)	3.24 (t, 9)	3.22 (t, 9)	3.92–4.00 (2H, m, OCH ₂ CH ₂ O), 4.15 (2H, d, <i>J</i> = 5 Hz, OCH ₂ CH ₂ O), 4.88 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 5.06 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 6.91–6.97 (3H, m, PhO), 7.25–7.30 (2H, m, PhO)
15a	4.43–4.45 (m)	3.93 (s)	3.24 (t, 9)	3.22 (t, 9)	3.80–4.00 (2H, m, OCH ₂ CH ₂ O), 4.15 (2H, t, <i>J</i> = 5 Hz, OCH ₂ CH ₂ O), 4.88 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 5.06 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 6.91–6.97 (3H, m, PhO), 7.25–7.30 (2H, m, PhO)
6b	4.31 (brs)	3.94 (s)	3.23 (t, 9)	3.20 (t, 9)	3.90–4.05 (2H, m, OCH ₂ CH ₂ O), 4.16 (2H, t, <i>J</i> = 5 Hz, OCH ₂ CH ₂ O), 4.87 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 5.05 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 6.67 (1H, d, <i>J</i> = 9 Hz, PhO), 7.25 (1H, d, <i>J</i> = 2 Hz, PhO), 7.36 (1H, dd, <i>J</i> = 9 and 2 Hz, PhO)
15b	4.42–4.44 (m)	3.93 (s)	3.23 (t, 9)	3.20 (t, 9)	3.80–3.98 (2H, m, OCH ₂ CH ₂ O), 4.16 (2H, t, <i>J</i> = 5 Hz, OCH ₂ CH ₂ O), 4.85 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 5.03 (1H, d, <i>J</i> = 7 Hz, OCH ₂ O), 6.66 (1H, d, <i>J</i> = 9 Hz, PhO), 7.24 (1H, d, <i>J</i> = 2 Hz, PhO), 7.34 (1H, dd, <i>J</i> = 9 and 2 Hz, PhO)

5-*O*-(*tert*-Butyldimethylsilyl)-4''-*O*-[2-(2-azido-4-iodophenoxy)ethoxymethyl]avermectin B_{1a} (15b). The procedure of 13b but with 14 (40 mg) in the dark gave 3.6 mg (6.8%) of 15b.

13-*O*-(2-Benzoylamino-*propionyl*)avermectin B_{1a} aglycone (3a). To a solution of 10a (17.2 mg) in acetonitrile (1 mL) was added HF-pyridine (HF = 68%, 0.1 mL) and the mixture was stirred at room temperature. After 3 h, additional HF-pyridine (0.1 mL) was added and the mixture was stirred for 1 h. The reaction mixture was poured into ice-cold saturated aqueous NaHCO₃ (15 mL) and extracted with ethyl acetate (4 × 5 mL). The combined organic layer was washed with water (5 mL) and brine (5 mL) then dried over Na₂SO₄ and evaporated in vacuo. The residue was purified by preparative TLC (hexane:ethyl acetate, 2:3) to give 12.9 mg (86%) of 3a. MS-LR: C₄₄O₅₇O₁₀N, (MH⁺) 760.

13-*O*-[2-(4-Azido-*benzoyl*)aminopropionyl]avermectin B_{1a} aglycone (3b). The above procedure with 10b (13.1 mg) in the dark gave 11.5 mg (100%) of 3b. MS-LR: C₄₄H₅₆O₁₀N₄, (MH⁺) 801.

13-*O*-[2-(2-Azido-*benzoyl*)aminopropionyl]avermectin B_{1a} aglycone (3c). The above procedure with 10c (13.1 mg) in the dark gave 11.4 mg (99%) of 3c. MS-LR: C₄₄H₅₆O₁₀N₄, (MH⁺) 801.

13-*O*-[2-(2-Azido-5-iodo-*benzoyl*)aminopropionyl]avermectin B_{1a} aglycone (3d). The above procedure with 10d (16.7 mg) in the dark gave 11.7 mg (79%) of 3d. MS-LR: C₄₄H₅₅O₁₀N₄I, (MH⁺) 927; HR (MH⁺) calcd 927.3041, found 927.3020.

13-*O*-[2-(4-Azido-2-hydroxy-*benzoyl*)aminopropionyl]avermectin B_{1a} aglycone (3e). The above procedure with 10e (14.2 mg) in the dark gave 8.6 mg (69%) of 3e. MS-LR: C₄₄H₅₆O₁₁N₄, (MH⁺) 817.

13-*O*-[2-(4-Azido-2-hydroxy-5-iodo-*benzoyl*)aminopropionyl]avermectin B_{1a} aglycone (3f). The above procedure with 10f (16.0 mg) in the dark gave 13.0 mg (91%) of 3f. MS-LR: C₄₄H₅₅O₁₁N₄I, (MH⁺) 943; HR (MH⁺) calcd 943.2990, found 943.3015.

13-*O*-(2-Phenoxyethoxymethyl)avermectin B_{1a} aglycone (4). The procedure for 3a but with 11 (16.3 mg) gave 10.0 mg (71%) of 4. MS-LR: C₄₃H₅₈O₁₀, (MH⁺) 735.

4'-*O*-(2-Phenoxyethoxymethyl)avermectin B_{1a} monosaccharide (5a). The procedure for 3a but with 13a (12.7 mg) gave 9.1 mg (81%) of 5a. MS-LR: C₅₀H₇₀O₁₃, (MH⁺) 879.

4'-*O*-[2-(2-Azido-4-iodophenoxy)ethoxymethyl]avermectin B_{1a} monosaccharide (5b). The procedure for 3a but with 13b (2.6 mg) in the dark gave 2.2 mg (94%) of 5b. MS-LR: C₅₀H₆₈O₁₃IN₃, (MLi⁺) 1052.

4''-*O*-(2-Phenoxyethoxymethyl)avermectin B_{1a} (6a). The procedure for 3a but with 15a (14.7 mg) gave 9.0 mg (68%) of 6a. MS-LR: C₅₇H₈₂O₁₆, (MH⁺) 1023.

4''-*O*-[2-(2-Azido-4-iodophenoxy)ethoxymethyl]avermectin B_{1a} (6b). The procedure for 3a but with 15b (3.3 mg) in the dark gave 1.4 mg (46%) of 6b. MS-LR: C₅₇H₈₀O₁₆IN₃, (MLi⁺) 1196.

Mouse and insect brain [³H]1 binding assays

Brain and head preparations. The studies used whole brain from male mice (25–30 g) and frozen (−70 °C) heads from male and female houseflies (susceptible strain cultured in this laboratory, 4–7 days after emergence as adults) and male and female adult fruit flies (provided by Gerald Rubin of the Department of Molecular and Cell Biology on this campus). The frozen insects at dry ice temperature were shaken in a flask to break them into their body parts and the heads recovered by passing through a 2-mm sieve and recovery on a 1-mm sieve for houseflies or a 1-mm and 0.5-mm sieve, respectively, for fruit flies. Dry ice temperature was maintained for glassware and sieves and the frozen heads were held at −80 °C until used for membrane preparation. Subsequent steps were carried out at 4 °C.

Membrane preparations. Mouse brain homogenate at 10% (w/v) in 0.32 M sucrose was centrifuged at 1000 × g for 10 min and the supernatant at 10,000 × g for 20 min. The pelleted membranes were suspended in 1 mM

EDTA and dialyzed versus distilled water for 5–6 h at 4 °C. Centrifugation (25,000×g, 30 min) gave the final brain membrane fraction. Insect heads were homogenized in 0.25 M sucrose–10 mM Tris–HCl buffer, pH 7.5, and the homogenate was centrifuged at 1000×g for 10 min and the supernatant thereof at 130,000×g for 60 min. The pelleted mouse brain and insect head membranes were suspended in assay buffer (see below) and stored at –80 °C until used.

[³H]1 binding assays. Procedures for the [³H]1 binding assays were based on earlier reports for mammalian brain^{9–12} and houseflies.¹³ The assay buffer was 10 mM phosphate, pH 7.5, containing 200 mM NaCl for mouse and 300 mM NaCl for insect membranes. The incubation mixtures were prepared by adding, in sequence, assay buffer (870 µL), the test compound in dimethyl sulfoxide (DMSO) (10 µL), [³H]1 in DMSO:assay buffer (1:3, 20 µL) and finally the membrane preparation (100 µL) with mixing. The protein level²⁹ was 50, 200 and 400 µg/assay for mouse, housefly and fruit fly, respectively. The final [³H]1 concentration was 2.9 nM (45,000 dpm) for mouse and 1.9 nM (30,000 dpm) for the insects. The mixtures were incubated for 90 min (mouse) or 70 min (insect) at 22 °C and then filtered on Whatman GF/C (mouse) or GF/B (insects) glass-fiber filters followed by three 5 mL rinses with ice-cold NaCl (0.9% w/v) solution containing ethanol (2% v/v). Specific binding was considered to be the difference between total ³H bound with 1.9 or 2.9 nM [³H]1 and nonspecific ³H bound on addition of 5 µM unlabeled 1. The specifically bound [³H]1 with mouse and housefly membranes was directly proportional to protein level. Typical values for percentage and dpm specific binding were 67% and 7700 dpm for mouse, 67% and 7500 dpm for housefly and 86% and 12,000 dpm for fruit fly.

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Efficacy in Sheep and Pharmacokinetics in Cattle that Led to the Selection of Eprinomectin as a Topical Endectocide for Cattle

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Abstract—Shoop W. L., DeMontigny P., Fink D. W., Williams J. B., Egerton J. R., Mrozik H., Fisher M. H., Skelly B. J. & Turner M. J. 1996. Efficacy in sheep and pharmacokinetics in cattle that led to the selection of eprinomectin as a topical endectocide for cattle. *International Journal for Parasitology* 26: 1227-1235. Eprinomectin (MK-397 or 4"-epi-acetylamino-4"-deoxy-ivermectin B₁) is a novel avermectin selected for development as a topical endectocide for all cattle, including lactating cows. The initial efficacy assessments were made in sheep to identify subclasses of the avermectin/milbemycins that possessed inherent activity against a spectrum of nematode parasites. This included examination of several hundred analogs each given orally to a single sheep experimentally infected with a range of parasitic nematodes. Representatives of several subclasses, most notably the 4"-epi-amino avermectin B₁ subclass, were identified as possessing potent, broad-spectrum activity against the endoparasites, whereas subclasses such as those with a variety of synthetic substitutions at C-4a or oximes at C-5 were among the least potent. Eprinomectin, a member of the 4"-epi-amino subclass, possessed potent activity against the range of nematodes when tested at 0.025 mg kg⁻¹ *per os*. Milk and plasma concentration profiles were also made for these and other selected avermectin/milbemycins following topical administration to lactating dairy cattle. The molecular structure of each compound had a significant effect on the milk to plasma ratio, but the ratio of each was constant over time, implying an equilibrium between the 2 compartments. Compounds that were saturated at the C-22,23 bond had milk to plasma ratios ≥ 1.0 , whereas those unsaturated at this bond were generally ≤ 1.0 . The milk to plasma ratio of eprinomectin was ≤ 0.2 . Therefore, not only is eprinomectin the most potent broad-spectrum avermectin/milbemycin identified to date, but it also possesses one of the lowest milk partitioning coefficients in this class of antiparasitics.

Key words: Avermectin; efficacy; eprinomectin; pharmacokinetics.

INTRODUCTION

The structural class of 16-membered macrocyclic lactones known as the avermectin/milbemycins possesses a distinct mode of action and demonstrates unparalleled potency and spectrum of activity against endo- and ectoparasites (Egerton *et al.*, 1979, 1980). Subsequent to the marketing of ivermectin in the

early 1980s, members of this chemical and mode of action class have become some of the most widely used compounds in animal health. Despite attempts at commercial differentiation, none of the recently introduced avermectin/milbemycins provide improved broad-spectrum potency or control a greater range of parasites than ivermectin (Shoop, Mrozik & Fisher, 1995). Further, although various claims are made for these compounds, none can be used in all classes of cattle. Particularly noteworthy are the

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restrictions on the use of current avermectin/milbemycins in lactating dairy cattle. In some countries there are disclaimers against use of these products in any dairy cows of breeding age. In other countries it is possible to treat with an avermectin/milbemycin during a dairy cow's typical 2-month dry period, but many producers are reluctant to risk potential milk residues that could lead either to milk discard or other financial penalties near or during the lactating period.

In an effort to identify a member of the avermectin/milbemycin class that could be used in all classes of cattle, we synthesized and examined a wide variety of analogs to find those that are efficacious against endo- and ectoparasites, are safe and exhibit minimal residue in milk. Because it is not efficient to examine these multidimensional criteria for hundreds of analogs simultaneously, we prioritized our search by examining analogs in sheep to identify subclasses that showed potent endoparasite activity. Sheep provide an excellent test model because their smaller size allows conservation of costly experimental compounds and because many of the nematodes in sheep are congeneric, and in some cases conspecific, to those found in cattle. Select members of the avermectin/milbemycin subclasses that were both potent and broad spectrum in sheep were titrated in cattle against a wide array of endo- and ectoparasites and examined in dairy cattle to determine their milk residue profile. Through an iterative process we identified a compound that would offer utility as the first endectocidal compound for use in all cattle, including lactating dairy cows.

Herein, we (1) present representative probe data from sheep illustrating several subclasses of the avermectin/milbemycins that were examined, (2) show titration data on the best analogs from the probe search in sheep, and (3) present milk-residue data on selected analogs from dairy cattle. From those tests, 4"-epi-acetylamino-4"-deoxy-avermectin B₁ was selected for development as the first topical endectocide for all cattle, including lactating dairy cows. It was subsequently designated MK-397 and then received the generic name, eprinomectin. Full details of its efficacy and safety in cattle will be presented elsewhere.

MATERIALS AND METHODS

Avermectin/milbemycin analogs. All avermectin/milbemycin analogs used in these studies were synthesized at Merck Research Laboratories (Mrozik *et al.*, 1995). Those given to sheep were administered orally in a 60% propylene glycol-40% glycerol formal vehicle at the constant volume rate of 0.1 ml kg⁻¹. Those given to cattle

were administered topically in a probe vehicle formulation comprising 79.5% isopropanol-20% crodamol-0.5% triethanolamine at the constant volume rate of 0.1 ml kg⁻¹.

Sheep probe studies. All sheep used in the structure-activity probe searches were reared helminth free in the Merck Research Laboratories and given experimentally induced helminth infections shortly after weaning. The sheep were mixed breed and sex, 30-50 kg in weight and 4-10 months in age. In all of the probe studies, the sheep were experimentally infected with infective larvae of *Oesophagostomum columbianum* (350 larvae per sheep, day -42), *Ostertagia circumcincta* (7500 larvae per sheep, day -28), *Trichostrongylus colubriformis* (7500 larvae per sheep, day -28), *Haemonchus contortus* (3000 larvae per sheep, day -26), *Cooperia curticei* (7500 larvae per sheep, day -26), *T. axei* (7500 larvae per sheep, day -23), and *C. oncophora* (4000 larvae per sheep, day -23). When the infections reached patency (day 0), the sheep were weighed individually, each was assigned to a treatment or control group randomly, and then the sheep was given the appropriate avermectin/milbemycin analog orally via a calibrated syringe. Each analog was administered to a single sheep. Because of the expense of these experimental compounds and our desire to minimize animal usage, a tactical decision was made not to replicate testing of individual analogs. Instead, we used resources that would have gone into individual replication toward testing multiple compounds in each subclass. This, in effect, provided quasi replication for each structural type but, more importantly, provided additional biological information on subtle structural differences between closely related analogs that would otherwise have been impossible to achieve. Dosages chosen for each analog were selected so as to administer the minimum amount necessary to achieve complete spectrum against the experimentally induced infections based on previous *in vitro* or *in vivo* small laboratory animal test experience. In cases where the analogs showed poor efficacy at a high dosage level, additional testing would only have been academic and thus no attempt was made again to actually achieve the minimum level for maximum control. Those analogs showing maximum efficacy at a low dosage level became candidates for titration studies using larger numbers of sheep. Necropsies were performed on day 7. Abomasa, small intestines and large intestines were removed and processed individually. The abomasa and small intestines were slit longitudinally, washed over 200-mesh sieves (pore size 75 µm), and the contents stored in 10% formalin until microscopical examination. The washed abomasa were also incubated individually overnight in a saline solution with 0.3% Combiotic[®] (Pfizer, New York) (penicillin and dihydrostreptomycin) at 39°C. The next day they were washed again, the sievings fixed in the same manner as described previously, and the counts added to those from the first day. The large intestines were slit longitudinally over 40-mesh screens (pore size 380 µm) and the contents were stored separately in 10% formalin until examination. Worm counts were based on 10% aliquots of the abomasal and small intestine sievings and all of the large intestine sievings. Efficacy for the sheep probe was coded on a scale from 0 to

3 for each helminth species, based on the following: 0=0–50% reduction relative to the control; 1=51–75% reduction relative to the control; 2=76–95% reduction relative to the control; and 3=>95% reduction relative to the control.

Sheep titration study. Numerous titrations of the most promising compounds from the sheep probe studies were made and we present a study of the 3 best analogs from the 4"-epi-amino subclass. For this study, 30 sheep of mixed breed and sex were reared helminth free in Merck Research Laboratories and were given experimentally induced helminth infections shortly after weaning. The species composition and timing of the experimental infections were the same as in the sheep probe studies. One sheep died of viral pneumonia prior to treatment and was excluded from further analysis. When the infections became patent (day 0) the remaining 29 sheep were allocated randomly to 10 groups and the 10 groups were allocated randomly to 1 of the following treatments: vehicle only (60% propylene glycol–40% glycerol formal) or 4"-epi-acetylaminio-4"-deoxy-avermectin-B₁ at 0.1, 0.05, or 0.025 mg kg⁻¹ or 4"-epi-methoxyacetylaminio-4"-deoxy-avermectin-B₁ at 0.1, 0.05, or 0.025 mg kg⁻¹ or 4"-epi-formylaminio-4"-deoxy-avermectin-B₁ at 0.1, 0.05, or 0.025 mg kg⁻¹. All dosages were administered orally. Necropsies were performed on day 7 and the sheep were processed and worm counts made according to that described in the section on the sheep probe. The data for worm burdens +1 were transformed to common logarithms and analysed by analysis of variance and Duncan's multiple range test.

Cattle milk residue studies. Promising avermectin/milbemycins from the sheep probe test were each tested in at least 2 lactating Holstein cows following a topical dose at 0.5 mg kg⁻¹. The cows were at various stages of lactation and were kept indoors and tethered to keep them from grooming. The drug solutions were administered topically along the midline of the back of each cow from the withers to the tailhead using a calibrated pipette. All milk and plasma samples were collected during the morning of days 0, 1, 2, 3, 4, 5, 6, 7, 10 and 14 after treatment. The ratio of the drug concentration in milk to that in the plasma was calculated individually for all compounds evaluated in dairy cows and was used to discriminate between desirable and undesirable profiles. The calculations were based on all time points from the dairy cows and no data were eliminated as outliers.

Analysis of milk and plasma samples. The B-series avermectin/milbemycins can be determined in biological samples at parts-per-billion (ng ml⁻¹) concentrations following dehydrative aromatization in an acetic acid–pyridine reagent mixture to a fluorescent derivative (Tolan *et al.*, 1980). This basic method has been modified for increased speed and sensitivity by others through the use of the more powerful nucleophilic 1-methylimidazole as an acetylation catalyst (Tway, Wood & Downing, 1981) and substitution of the better leaving group trifluoroacetic anhydride for acetic anhydride as the acetylation reagent (DeMontigny, Shim & Pivnichny, 1990). Accordingly, the concentration of each of the avermectin/milbemycins in this study was deter-

mined using this modified fluorogenic derivatization procedure to generate the aromatic fluorophore. Fluorescence detection of each derivative was at a 475-nm detection wavelength under 375-nm excitation. Chromatographic conditions were regulated for each compound, and quantitation was achieved relative to a calibration line of fluorescence peak height as a function of authentic analytical reference standards of known concentration for each compound. The typical analytical interday precision measured on fortified milk samples of known concentration over the concentration range 1–50 ng ml⁻¹ analyte was <7% relative standard deviation. The accuracy exceeded 90% (i.e. <10% mean relative error) with the use of an internal standard for each compound. The limit of quantitation and limit of detection were 1.25 and 0.1 ng ml⁻¹, respectively. Similar results were obtained with plasma samples. The anhydride reagent solution was prepared by mixing 3.0 ml of trifluoroacetic anhydride with 7.0 ml of acetonitrile. This solution was prepared on a daily basis. Separately, *N*-methylimidazole, also in acetonitrile (1:1 v/v) was prepared on a weekly basis. The analytical reference standard stock solutions of each analyte were prepared by dissolving each avermectin/milbemycin (of known purity) in acetonitrile (0.1 mg ml⁻¹). Working solutions were prepared by diluting the stock reference solutions to a series of known concentrations in acetonitrile. The calibration curve milk samples were prepared by adding measured volumes of the acetonitrile working standard solutions to fresh, drug-free milk. Typically, 150 µl of *N*-methylimidazole solution in acetonitrile (1:1 v/v) was added to the dry residue obtained following extraction. This solution was then transferred to a 1.1-ml sample vial. A Shimadzu LC-9A autosampler was programmed to add 200 µl of the trifluoroacetic anhydride solution in acetonitrile (30% v/v) automatically. After mixing, 50 µl of the analyte solution was injected directly into the liquid chromatograph.

RESULTS

Sheep probe studies

Six subclasses consisting of 60 avermectin/milbemycins were selected to illustrate some of the structure–activity relationships we observed (Table 1). The subclasses chosen represent substitutions in some of the synthetically more accessible regions of the molecule, such as C-4a, C-5, C-13, C-4', C-4" and C-22,23 and include both milbemycin and aglycone, monosaccharide and disaccharide avermectins. Avermectin B₁ is shown to illustrate these regions (Fig. 1).

It is evident from the data in Table 1 that C-4"-epi-amino analogs (Compounds 41–50) possess the most consistent broad-spectrum activity at the lowest dosages of any subclass that was examined. These members consistently show high-order activity against all 7 nematode species at 0.05 mg kg⁻¹ *per os* in this test. The biological activities of members in

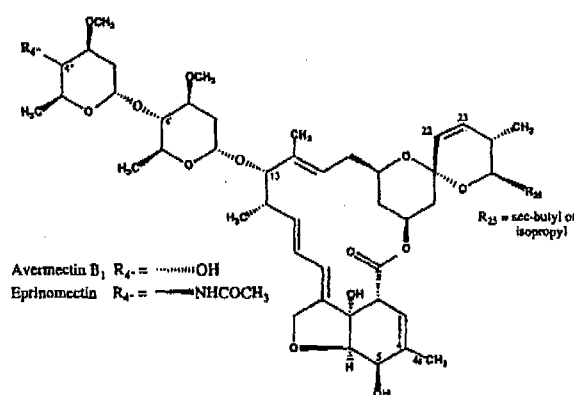


Fig. 1. Molecular structure of avermectin B₁ and eprinomectin.

this subclass can be compared directly to ivermectin (Compound 51). Whereas ivermectin shows a slight weakness against *Cooperia oncophora* at the 0.1 mg kg⁻¹ dosage, most of the C-4"-epi-amino analogs have no such weakness at half the dosage. In further comparison, certain subclasses such as the C-4a (Compounds 1-10) and C-5 analogs (Compounds 11-20) do not, in general, show consistent high-order activity against the helminths at dosages near 0.05 mg kg⁻¹. Likewise, even though some members of the C-13 aglycones (Compounds 21-30) do achieve high-order activity against the spectrum of helminths, they do so only at dosages ≥ 0.2 mg kg⁻¹.

This database also demonstrates how a slight modification to a very potent, broad-spectrum molecule can render it virtually devoid of useful biological activity. For example, Compound 43 is one of the most potent, broad-spectrum agents in these tests and yet when an acetoxy substituent was placed at its C-4a position such as in Compound 4, or a methoxyethoxymethyl-5-oxime was placed at its C-5 position such as in Compound 18, then virtually all useful anthelmintic activity was lost, even if the dosage was doubled.

In general, the relative order of susceptibility of the species in our database to the macrocyclic lactones can be calculated to show that *Oes. columbianum* was the most susceptible, followed in order of decreasing susceptibility by *H. contortus*, *T. axei*, *O. circumcincta*, *T. colubriformis*, *C. oncophora* and *C. curticei*. When susceptibility to the macrocyclic lactones is ranked from the perspective of anatomical site, the large intestine harbored the most susceptible species, followed by those from the abomasum and lastly the group of 3 species from the small intestine was the least sensitive.

Sheep titration studies

Among the several hundred avermectins/milbemycins examined initially in the sheep probe studies, Compounds 41, 42 and 43 demonstrated sufficient potency and spectrum in sheep to justify examination by titration (Table 2). All 3 compounds produced near maximal efficacy against all nematode species at the 0.1 mg kg⁻¹ dosage, but only Compound 43 produced $\geq 97\%$ efficacy against all 7 nematode species at both the 0.1 and 0.05 mg kg⁻¹ dosages. Activity for this compound diminished slightly at the 0.025 mg kg⁻¹ dosage, but even then it had fallen only to 93%, 91% and 93% against *O. circumcincta*, *C. curticei* and *C. oncophora*, respectively. Given that the average number of worms in each control sheep was 17720, the 0.025 mg kg⁻¹ dosage still removed 96% of all worms. No adverse reaction was observed in any sheep.

Plasma and milk concentrations in dairy cattle

The plasma distribution profiles obtained from selected avermectin/milbemycins were similar following topical administration to dairy cattle in that each attained a maximum concentration (C_{max}) in plasma within the first 2 days of drug application and the plasma depletion typically obeyed first-order exponential kinetic decay. Profiles of plasma concentration as a function of time for 3 avermectin/milbemycins are shown in Fig. 2.

Figure 2 illustrates 2 important observations critical to the selection of a compound for use in dairy animals. First, milk and plasma concentrations could differ dramatically between individual avermectin/milbemycins, suggesting a link between molecular structure and differential partitioning. Some compounds were observed to partition predominately in the milk relative to the plasma, and others observed to do the reverse. The 3 compounds in Fig. 2 were chosen to illustrate the range of milk/plasma concentrations observed in the dairy cattle. The compound in the top box of Fig. 2 possesses a highly desirable milk/plasma profile, whereas the compound in the lower box shows an undesirable proclivity to partition in the milk compartment. The compound in the middle box shows an intermediate state.

Second, despite different C_{max} values for the milk and plasma, all milk disposition profiles for the avermectin/milbemycins that we examined were consistently parallel to the corresponding plasma profiles, i.e. both fluids reached C_{max} at the same time and depleted at a similar rate throughout the 14-day profile. The ratio of the concentration in milk (M) to that in the plasma (P) for each compound was a

Table 1— Select sheep probe data for 60 avermectin/milbemycin analogs from 6 structural subclasses each given orally to a single sheep which had been experimentally infected with 7 species of nematodes

Compound no.	Chemical name	Dosage (mg/kg)	Nematodes HOTTCCO ^a
<i>C-4a analogs</i>			
1	4a-Hydroxy-avermectin-B ₁	0.1	3233033 ^b
2	4a-Hydroxy-22,23-dihydroavermectin-B ₁	0.2	3331203
3	4a-Hydroxy-4"-epi-acetylamino-avermectin-B ₁	0.1	0000000
4	4a-Acetoxy-4"-epi-acetylamino-4"-deoxy-avermectin-B ₁	0.1	0000000
5	4a-Benzoyl-4"-epi-acetylamino-4"-deoxy-avermectin-B ₁	0.1	1000000
6	4a-O-Tetrahydropyranyl-4"-epi-acetylamino-4"-deoxy-avermectin-B ₁	0.1	2000133
7	4a-O-Methoxyethoxymethyl-22,23-dihydro-avermectin-B ₁ monosaccharide	0.1	3332001
8	4a-O-Methoxyethoxymethoxy-22,23-dihydro-avermectin-B ₁	0.1	2032233
9	4a-O-Methoxyethoxymethoxy-avermectin-B ₁	0.1	3332023
10	4a-O-Oleandroxyloxy-avermectin-B ₁	0.1	3010022
<i>C-5 analogs</i>			
11	Avermectin-B ₁ -5-oxime	0.1	3321013
12	4"-epi-Acetylamino-4"-deoxy-avermectin-B ₁ -5-oxime	0.1	1000111
13	4"-epi-Methylamino-4"-deoxy-avermectin-B ₁ -5-oxime	0.3	3301333
14	4"-epi-Acetylamino-4"-deoxy-22,23-dihydro-avermectin-B ₁ -5-oxime	0.2	2001132
15	4"-epi-Methylsulfone-4"-deoxy-avermectin-B ₁ -5-oxime	0.1	1011333
16	4"-epi-Acetylamino-4"-deoxy-4a-hydroxy-avermectin-B ₁ -5-oxime	0.2	2000320
17	4"-C-Methyl-avermectin-B ₁ -5-oxime	0.2	3222333
18	4"-epi-Acetylamino-4"-deoxy-avermectin-B ₁ -5-O-methoxyethoxy-methyl-oxime	0.2	0000000
19	4"-epi-Acetylamino-4"-deoxy-avermectin-B ₁ -5-O-acetyl-oxime	0.2	2000323
20	Milbemycin α1-5-oxime	0.2	2233000
<i>C-13 aglycone analogs</i>			
21	13-α-Fluoro-13-deoxy-22-hydro-24-dehydro-avermectin-B ₁ aglycone	0.4	3333102
22	13-α-Fluoro-13-deoxy-22,23-dihydro-avermectin-B ₁ aglycone	0.35	3333332
23	13-β-Fluoro-13-deoxy-22,23-dihydro-avermectin-B ₁ aglycone	0.2	3223011
24	13-β-Fluoro-13-deoxy-avermectin-B ₁ aglycone	0.2	3333333
25	13-O-Methoxyethoxymethyl-avermectin-B ₁ aglycone	0.2	3333333
26	13-O-Methoxyethoxymethyl-22,23-dihydro-avermectin-B ₁ aglycone	0.1	1233233
27	13-O-Methoxyethoxymethyl-avermectin-B ₁ aglycone	0.2	0333133
28	13-O-Methylthiomethyl-avermectin-B ₁ aglycone	0.1	0331003
29	13-O-Methyl-22,23-dihydro-avermectin-B ₁ aglycone	0.1	2332302
30	13-Methoxime-13-deoxy-avermectin-B ₁ aglycone	0.2	3333022
<i>C-4' monosaccharide analogs</i>			
31	Avermectin-B ₁ monosaccharide	0.15	2233330
32	4'-O-Methoxyethoxymethyl-avermectin-B ₁ monosaccharide	0.1	3333223
33	4'-O-Methoxyethoxymethyl-22,23-dihydro-avermectin-B ₁ monosaccharide	0.1	3210003
34	4'-O-Methylthiomethyl-avermectin-B ₁ monosaccharide	0.1	3333333
35	13-epi-22,23-dihydro-avermectin-B ₁ monosaccharide	0.1	2233303
36	4'-O-Tetrahydropyranyl-avermectin-B ₁ monosaccharide	0.1	3332333
37	4'-epi-Methylsulfonyl-4"-deoxy-13-epi-avermectin-B ₁ monosaccharide	0.1	1332020
38	4'-epi-Acetylaminoethylthio-4"-deoxy-avermectin-B ₁ monosaccharide	0.1	3333033
39	4'-O-(2"(5"-Acetylamino-4"-methoxy-7"-methyl oxapimyl))-avermectin-B ₁ monosaccharide	0.05	3332333
40	4'-O-(2"(5"-Hydroxy-4"-methoxy-7"-methyl-oxapimyl))-avermectin-B ₁ monosaccharide	0.1	3333133
<i>C-4"-epi-amino analogs</i>			
41	4"-epi-Methoxyacetylamino-4"-deoxy-avermectin-B ₁	0.05	3333333
42	4"-epi-Formylamino-4"-deoxy-avermectin-B ₁	0.05	3333333
43	4"-epi-Acetylamino-4"-deoxy-avermectin-B ₁	0.05	3333333
44	4"-epi-Methanesulfonylamino-4"-deoxy-avermectin-B ₁	0.05	3333333
45	4"-epi-Propionylamino-4"-deoxy-avermectin-B ₁	0.05	3333333
46	4"-epi-Methoxycarbonylamino-4"-deoxy-avermectin-B ₁	0.1	3333233
47	4"-epi-Acetylamino-3"-des(methoxy)-4"-deoxy-avermectin-B ₁	0.05	3333333
48	4"-epi-N-Methoxyacetyl-N-methyl-amino-4"-deoxy-avermectin-B ₁	0.05	3333333
49	4"-epi-N-Acetyl-N-methyl-amino-4"-deoxy-avermectin-B ₁	0.05	3333333
50	4"-epi-N-Methylthioacetyl-amino-4"-deoxy-avermectin-B ₁	0.05	3332333
<i>C-22,23 single bond analogs</i>			
51	22,23-Dihydro-avermectin-B ₁	0.1	3333323
52	Avermectin-B ₂	0.1	3333233
53	10,11,22,23-Tetrahydro-avermectin-B ₁	0.2	3000003
54	23-Methoxime-22,23-dihydro-avermectin-B ₁	0.05	3333233
55	4"-epi-Methylamino-4"-deoxy-22,23-dihydro-avermectin-B ₁	0.15	3301233
56	4"-epi-Acetylamino-4"-deoxy-22,23-dihydro-avermectin-B ₁	0.15	3333333
57	4"-epi-Formylamino-4"-deoxy-22,23-dihydro-avermectin-B ₁	0.1	3333103
58	4"-epi-Acetylamino-4"-deoxy-avermectin-B ₂	0.1	0333233
59	13-epi-22,23-Dihydro-avermectin-B ₁	0.1	3333003
60	13-epi-Avermectin-B ₂	0.1	3333333

^aHOTTCCO= *Haemonchus contortus*, *Ostertagia circumcincta*, *Trichostrongylus axei*, *T. colubriformis*, *Cooperia curticei*, *C. oncophora* and *Oesophagostomum columbianum*.

^b0=0-50% reduction relative to the control; 1=51-75% reduction relative to the control; 2=76-95% reduction relative to the control; and 3=>95% reduction relative to the control.

Table 2—Comparative efficacy titration in sheep which had been experimentally infected with 7 nematode species and then given oral treatments of Compound 41 (4"-epi-methoxyacetylaminio-4"-deoxy-avermectin B₁), Compound 42 (4"-epi-formylaminio-4"-deoxy-avermectin B₁), or Compound 43 (4"-epi-acetylaminio-4"-deoxy-avermectin B₁)

Analog	Dosage (mg kg ⁻¹)	No. of sheep	<i>Haemonchus contortus</i>	<i>Ostertagia circumcincta</i>	<i>Trichostrongylus axei</i>	<i>Colubriformis</i>	<i>Cooperia curticei</i>	<i>Oncophora</i>	<i>Oesophagostomum columbianum</i>
Control ^a	—	3	768 (150-2130)	1644 (1380-2050)	4451 (4070-5050)	5440 (5170-5650)	4253 (3530-4790)	1022 (490-1880)	142 (87-207)
Compound 41	0.025	3	99**	67	90	96	88	94	100**
	0.05	3	99**	86	97	99**	93*	95	100**
	0.1	3	99**	99**	99**	99**	99**	99**	100**
Compound 42	0.025	3	99**	97**	98**	89**	27	56	100**
	0.05	3	99**	99**	99**	99**	79	70	100**
	0.1	3	99**	99**	99**	99**	99**	99**	100**
Compound 43	0.025	3	99**	93	99**	98**	91*	93	100**
	0.05	3	99**	99**	99**	99**	97**	99**	100**
	0.1	2	99**	99**	99**	99**	99**	99**	100**

^aGeometric number of worms in the control group, followed by the range in parentheses.
 *, ** Reduction from control level due to treatment at $P < 0.05$, < 0.01 , respectively; Duncan's multiple range t-test.

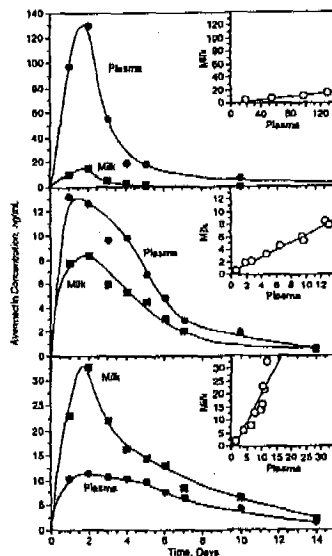


Fig. 2. Representative milk (■) and plasma (●) disposition profiles from 3 avermectin/milbemycins showing $K(M/P)$ values of 0.2 (top box, 3"-desmethoxy-4"-acetylaminio-4"-deoxy-avermectin B₁), 0.6 (middle box, avermectin B₁ monosaccharide) and 1.3 (bottom box, 4"-epi-methylaminio-4"-deoxy-22,23-dihydro-avermectin B₁). Inset in each box shows plot of milk concentration against plasma concentration for each compound and the slope of the line indicates $K(M/P)$.

reproducible constant, K . $K(M/P)$ values calculated for 17 selected compounds are shown in Table 3. The linear plots of milk versus plasma demonstrate the direct relationship between milk and plasma concentration in the dairy cows, and the slope of each line is a measure of the $K(M/P)$ for that avermectin/milbemycin. These data were fit to a linear least-squares regression line for each compound. The mean correlation coefficient (r^2) of these lines was 0.82 ± 0.13 (S.D.).

DISCUSSION

The 4"-epi-amino avermectin subclass contained members demonstrating the most potent and consistent broad-spectrum activity against the 7 nematode species tested in sheep. Within that subclass, 4"-epi-acetylaminio-4"-deoxy-avermectin B₁ was the most potent of all compounds tested and it required dosage levels of $0.025 \text{ mg kg}^{-1} \text{ per os}$ before clear breaks in activity were observed. However, even at that dosage there remained 91% efficacy against the dosage limiting species *C. curticei*, and 96% of all worms were killed. Confirmation of the potency and spectrum for this compound in cattle against both endo- and ectoparasites are presented elsewhere (Shoop et al., 1996).

Table 3—Mean milk to plasma concentration ratios [$K(M/P)$] of 17 avermectin/milbemycins following topical administration of 0.5 mg kg⁻¹ to lactating dairy cattle

Compound No.	Chemical name	Number of cows examined	$K(M/P)$
11	Avermectin-B ₁ -5-oxime	2	0.12
43	4"-epi-Acetylamino-4"-deoxy-avermectin-B ₁	10	0.17
—	3"-epi-4"-Acetylamino-4"-deoxy-avermectin-B ₁	2	0.2
—	3"-Desmethoxy-4"-acetylamino-4"-deoxy-avermectin-B ₁	2	0.2
47	4"-epi-Acetylamino-3"-des(methoxy)-4"-deoxy-avermectin-B ₁	2	0.2
12	4"-epi-Acetylamino-4"-deoxy-avermectin-B ₁ -5-oxime	2	0.2
41	4"-epi-Methoxyacetylamino-4"-deoxy-avermectin-B ₁	2	0.2
42	4"-epi-Formylamino-4"-deoxy-avermectin-B ₁	2	0.2
—	4"-epi-Amino-4"-deoxy-avermectin-B ₁	2	0.3
31	Avermectin-B ₁ monosaccharide	2	0.6
13	4"-epi-Methylamino-4"-deoxy-avermectin-B ₁ -5-oxime	2	0.6
44	4"-epi-Methanesulfonylamino-4"-deoxy-avermectin-B ₁	2	0.7
22	13- α -Fluoro-13-deoxy-22,23-dihydro-avermectin-B ₁ aglycone	2	1.0
55	4"-epi-Methylamino-4"-deoxy-22,23-dihydro-avermectin-B ₁	2	1.3
60	13-epi-avermectin-B ₂	2	1.5
21	13- α -Fluoro-13-deoxy-22-hydro-24-dehydro-avermectin-B ₁ aglycone	2	2.5
26	13-O-Methoxyethoxymethyl-22,23-dihydro-avermectin-B ₁ aglycone	2	2.7

Although we have employed a full range of *in vitro* receptor binding tests, *in vitro* whole organism tests, or small laboratory animal (mostly rodent) *in vivo* tests to guide us in the early phases of identifying useful compounds, they have not been and could not be expected to be accurate predictors of the broad-spectrum requirements demanded from antiparasitics such as the avermectin/milbemycins. We have found the sheep probe test to be an important model for predicting activity in cattle and it has been a useful tool for probing structure-activity relationships among related compounds. Although not inexpensive, the sheep probe model provided direct information on the inherent activity of a compound in a ruminant animal against a full range of endoparasites that are congeneric, and in some cases conspecific, to those found in the bovine. It has also provided invaluable safety and pharmacokinetic information as well.

With regard to residue, milk partitioning relative to plasma is a complex process controlled by many variables, including the physico-chemical properties of the compound and its membrane interactions. In the experiments reported herein, absorption of the avermectin/milbemycins and their detection in the plasma after topical delivery was a rapid process. Following absorption into the plasma, a reversible equilibrium was established between the milk and plasma compartments so that the ratio of the milk and plasma concentrations (M/P) at equilibrium was a constant, $K(M/P)$. The establishment of this equilibrium in the present study is confirmed by the

reproducibility of the M/P concentration ratios for each compound at all sampling times independent of concentrations. The first-order depletion curves in milk and plasma are parallel, indicating that the constant milk to plasma equilibrium was maintained throughout the excretion process.

It is clear from the results listed in Table 3 that the structure of the avermectin/milbemycin molecule has a significant effect on determining the distribution ratio of the drug between milk and plasma, and consequently, on milk residues following topical administration. *In vivo* partitioning of ivermectin into milk has been studied by many workers. The passage of ivermectin from blood to milk in cattle was first demonstrated by Alvinerie *et al.* (1987), who showed that the milk and plasma concentrations for ivermectin were of the same order of magnitude and that the milk concentration decreased according to the terminal half-life observed in the plasma. In sheep, ivermectin was shown to be excreted in the milk at concentrations similar to those in plasma (Bogan & McKellar, 1988). Bogan & McKellar (1988) also showed that the excretion of ivermectin in milk did not diminish the plasma concentration disproportionately, which is consistent with an *in vivo* milk/plasma equilibrium, and the mean $K(M/P)$ calculated for ivermectin from the data reported in that study was 1.1 ($N=15$). Toutain *et al.* (1988) reported the parallel disposition of ivermectin in milk and plasma of cattle with a $K(M/P)$ of 0.77 ± 0.27 . Peak plasma concentration was calculated to occur after 1.45 days and the milk concentration peaked at

a comparable 1.76 days following subcutaneous injection.

Ivermectin's equal partitioning between milk and plasma is an attribute that has not lent it for use in lactating animals. Therefore, we have sought structure-activity relationships that could allow us to exploit different partitioning ratios in the avermectin/milbemycin class. To this end, certain trends can be seen in Table 3 that allow for the categorization of these molecules according to their $K(M/P)$ values. Those avermectin/milbemycins that are saturated at C-22,23 exhibit milk concentrations equal to or greater than plasma concentrations, i.e. $K(M/P) \geq 1.0$, and are found towards the bottom of Table 3. There are also subclasses among the avermectin/milbemycins that exhibit low $K(M/P)$ values of <1.0 . These are the compounds not saturated at the C-22,23 position and some, such as the 4"-epi-acetylamino or 5-oxime substituents, exhibit highly desirable $K(M/P)$ values of ≤ 0.2 . In the avermectin/milbemycin series we have examined, saturation at C-22,23 and subsequent alteration of the stereochemical configuration of the pyran ring is one change that influences partitioning in the milk.

As more is learned about milk partitioning, we may find that some substituent groups can enhance the gains made by unsaturating C-22,23, while others abolish it. Subtle changes to the structures of these complex molecules change their solubilities, membrane interactions and receptor binding. As was observed in the efficacy trials against helminth parasites, structural attributes such as the combination of the 4"-epi-acetylamino substituent to the avermectin B₁ backbone (Compound 43) conferred great potency and spectrum, but it was also found that additional substituents to this combination produced an entire range of outcomes from negligible (Compound 47), to minor (Compound 56), to substantial (Compound 14), to massive (Compound 3 or 4).

In general, it is relatively easy to find an optimized compound against any given single parasite target, but finding one that has the attributes to hit multiple targets, let alone multiple parasite targets, while maintaining low residue profiles in specific host physiological compartments, is much more difficult. We have used our data base to identify such compounds. Elimination of compounds such as those with oxime substituents at C-5 as potential candidates for further consideration is easily done. For example, even though the avermectin-5-oxime compound has an excellent $K(M/P)$ value, both it and its entire subclass of 5-oxime analogs show poor potency and spectrum of activity against the nematode parasites. Likewise, reverse those profiles and the opposite situation is a compound like

ivermectin, which is a superior anthelmintic with excellent potency and spectrum, and yet it does not possess a favorable $K(M/P)$ and is not used in lactating animals. Of course, the worst grouping contains members like the 13-O-methoxy-ethoxymethyl-22,23-dihydro-avermectin B₁ aglycone, which has poor properties as a food animal anthelmintic as well as one of the least desirable $K(M/P)$ values.

In contrast, 4"-epi-acetylamino-4"-deoxy-avermectin B₁ not only was the most potent avermectin that we have ever titrated against adult endoparasites of sheep, but it also had one of the best partitioning profiles of all that were tested in lactating dairy cattle. Therefore, this compound was selected for further tests in cattle and was subsequently given the generic name, eprinomectin.

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Synthesis and Biological Activity of 4a,4''-Disubstituted Avermectins

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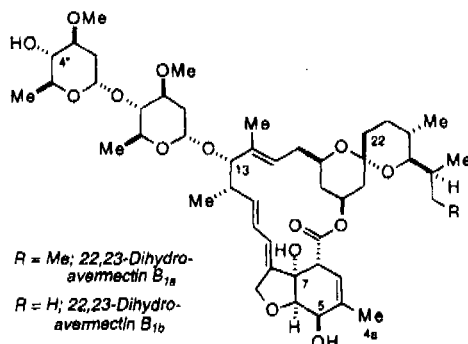
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Allylic oxidation of avermectin B₁ and C₄'-substituted avermectin B₁ analogues at C_{4a} followed by derivatization of the C_{4a} oxygen provides compounds that have an improved safety profile, as judged by mouse LD₅₀ data, and are substantially more potent than avermectin B₁ against flies.

Keywords: Avermectin; anthelmintic; pesticide

INTRODUCTION

The avermectins are naturally occurring macrocyclic lactones with important anthelmintic and pesticidal activity (Fisher and Mrozik, 1984; Davies and Green, 1986; Pulliam et al., 1989; Lankas and Gordon, 1989; Davies and Green, 1991a,b). Avermectin B₁ (abamectin) (Dybas, 1989) and 22,23-dihydroavermectin B₁ (ivermectin) (Chabala et al., 1980) are widely used anthelmintic agents in animal (Benz et al., 1989) and human health (Greene et al., 1989). The structural novelty of avermectins and their economic importance have attracted the attention of a number of synthetic organic chemistry groups concerned with chemical modification and total synthesis (Blizzard et al., 1990). In this vein, several publications and patents have appeared describing modification of avermectin B₁ at C_{4a} (Mrozik, 1984a,b) and C₄' (Mrozik et al., 1989; Mrozik, 1984a,b; Linn and Mrozik, 1991) in an attempt to improve these important antiparasitic drugs.



Avermectin B₁ is isolated as a two-component mixture. The major component (B_{1a}, ≥80%) contains a *sec*-butyl group at C₂₅; the minor component (B_{1b}, ≤20%) contains an isopropyl group at C₂₅. These components are separable by HPLC; however, since they have essentially the same biological activities, they are not separated in practice. All compounds in this paper are mixtures of B_{1a} and B_{1b} components, represented as B₁ and drawn as B_{1a} for clarity.

We report in this paper our observations on the synthesis and biological activity of C_{4a}-substituted aver-

mectin analogues, with emphasis on their activity against flies. We have found that oxidative substitution at C_{4a} increases potency against flies and increases the LD₅₀ in mice of these avermectin analogues.

EXPERIMENTAL PROCEDURES

General. General procedures and instrumentation employed in this work have been reported previously (Jones et al., 1992) except for the following additions. NMR assignments were made with the aid of APT data and literature data (Diez-Martin et al., 1990; Neszmelyi et al., 1989). Standard abbreviations are used for multiplicities with the addition of om for obscured multiplet. Low-resolution FAB mass spectra were acquired on Finnigan MAT TSQ 70B and VG Masslab VG20-250 quadrupole mass spectrometers using as matrix 5:1 dithiothreitol/dithioerythritol doped with lithium acetate or undoped. High-resolution LSIMS data were obtained on a JEOL JMS-HX110 double-focusing (EB) mass spectrometer at 10 kV using Ultramark 1600F as the internal standard and 3-nitrobenzyl alcohol as matrix.

Stomoxys Assay. The antifeedant activity was estimated *in vitro* by evaluating the response of adult *Stomoxys calcitrans* allowed to feed on 2.3 cm diameter Whatman Grade 3 filter paper disks containing bovine blood and test avermectins. Each compound was presented at five or more dosage levels in 0.1 mL of the experimental vehicle (aqueous vehicle containing 0.02% Triton X-100, 5% acetone, and up to 0.2% dimethylformamide) applied to the filter paper disk on top of 7-dram plastic vials. Two hours after application of test compound, 0.4 mL of citrated bovine blood was added to the filter paper disk. Each treatment level consisted of 10 replicate vials, each vial containing four adult flies. The experimental design is such that all groups are completely randomized within the whole, and the test vials are read in numerical order without knowledge of treatment history. Ivermectin is run as a positive control on every assay. The estimated LD₉₀ is calculated from the motility of flies 24 h after exposure to test substances. The LD₉₀ calculation is based on double arcsine transformed data fitted to straight lines with log-dose response curves. Data are reported in Table 1.

Sheep Assay. Anthelmintic efficacy of the compounds was determined in sheep raised helminth free and experimentally infected with the parasites listed in Table 2. When the infections were patent, the sheep were randomly assigned to a treatment or control group. Each compound was tested in one sheep while two sheep served as controls. The compounds were administered in a single oral dosage of 0.1 mg/kg. Control animals were given only the vehicle. Seven days after dosing, the animals were necropsied and the residual worm burdens were determined. The efficacies of the test compounds were recorded as described in footnote a of Table 2.

Estimated Mouse LD₅₀ Assay. Acute toxicity of the compounds was evaluated by calculating the LD₅₀ for male

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Table 1. Avermectin Analogue Activities against Brine Shrimp (*A. salina*) and *S. calcitrans* Adults

compd ^a	X	R	brine shrimp IC ₁₀₀ ^b (ng/mL)	<i>Stomoxys</i> LD ₅₀ (ppm)
1a	OH		356 ^c	40.9
1aD	OH		430 ^c	29.5
1b	epi-AcNH		540 ^c	5.3
2a	OH		760 ^c	33.0
2b	epi-AcNH		870	18.8
4c	epi-AcNH	Bz	870	2.1
4d	epi-AcNH	THP	1500 ^c	2.7
4e	epi-AcNH	Me	650	1.3
4f	epi-AcNH	CH ₂ OBn	870	1.7
4g	epi-AcNH	MEM	650	1.4
4gD	epi-AcNH	MEM	870	0.9
4h	OH	MEM	325	3.6
4hD	OH	MEM	430	2.1
1i	OMEM		1300	75.6
2i	OMEM		1730	8.7
4j	OMEM	MEM	1730	7.1
1k	epi-AcNMe		650	13.1
2k	epi-AcNMe		650	9.8
4l	epi-AcNMe	MEM	870	1.8
1m	OMe		2600	31.7
4mD	OMe	Me	870	22.4

^a All derivatives are of avermectin unless noted as 22,23-dihydro by the addition of a D to the compound number. ^b Brine shrimp data as previously described (Blizzard et al., 1989), average of two assays unless otherwise noted. ^c Multiple assays.

Table 2. Anthelmintic Efficacy of Avermectin Analogues in Sheep^a

compd	H.c. ^b	Os.c.	T.a.	T.c.	C.c.	C.onc.	Oe.c.
1a	3	3	3	3	3	3	3
1aD	3	3	3	3	3	3	3
1b	3	3	3	3	3	3	3
2a	3	2	3	3	0	3	3
2b	0	0	0	0	0	0	0
4c	1	0	0	0	0	0	0
4g	3	3	3	3	3	3	3
1i	3	0	1	0	0	0	3
2i	3	2	3	2	3	1	3
4j	3	0	3	3	3		3

^a Sheep data obtained as described under Experimental Procedures, all compounds tested at 0.1 mg/kg, efficacy as percent reduction from control: 0 = <50%, 1 = 51–75%, 2 = 76–95%, 3 = >95%. ^b H.c., *Haemonchus contortus*; Os.c., *Ostertagia circumcincta*; T.a., *Trichostrongylus axei*; T.c., *Trichostrongylus colubriformis*; C.c., *Cooperia curticei*; C.onc., *Cooperia oncophora*; Oe.c., *Oesophagostomum columbianum*.

CD-1 mice. Each compound was evaluated at several doses in treatment groups of five mice per dose allocated at random from a pool of mice. The compounds were administered orally in a vehicle via a calibrated syringe with a blunt-tipped needle. Seven days after dosing, the number of deaths was determined and the estimated LD₅₀ values were determined by the method of dose-pair responses. This method effectively emulates linear regression while minimizing the number of animals required.

General Procedure A: SeO₂ Oxidation. A 250-mL round-bottom flask fitted with a magnetic stirring bar, septum, and nitrogen inlet was charged with the desired avermectin analogue in CH₂Cl₂ (0.15 M). To the resulting clear solution was added 0.50 equiv of SeO₂ followed by 4 equiv of 90% *t*-BuOOH at room temperature. The resulting solution was stirred at room temperature for ~24 h. The reaction mixture was then concentrated by rotary evaporation and chromatographed with a CH₂Cl₂/MeOH solvent mixture on SiO₂ to provide the 4a-hydroxyavermectin analogue as a foam.

Data for 4a-Hydroxyavermectin B₁ (2a) (Mrozik, 1984a,b): 2.04 g (50%); *R_f* = 0.17 (94:6 CH₂Cl₂/MeOH). MS and combustion data are in the supplementary material.

Data for 4''-epi-(Acetylamino)-4''-deoxy-4a-hydroxyavermectin B₁ (2b): 6.10 g (50%); *R_f* = 0.27 (93:7 CH₂Cl₂/MeOH); ¹H NMR δ 5.86 (m, H₉), 5.78–5.63 (om, H₃, H₁₀, H₁₁, H₂₃, NH), 5.55 (dd, *J* = 9.9, 2.5 Hz, H₂₂), 5.38–5.25 (om, H₁₉, H_{1'}), 4.95

(m, H₁₅), 4.74 (d, *J* = 3.2 Hz, H_{1'}), 4.65 (m, H_{8a}), 4.55 (br d, *J* = 5.0 Hz, H₅), 4.38 (dd, *J* = 10.0, 3.2 Hz, H_{4'}), 4.22 (br s, H_{4a}), 4.03 (m, H_{5'}), 3.97 (d, *J* = 6.3 Hz, H₈), 3.90 (br s, H₁₃), 3.90–3.72 (om, H₁₇, H₈), 3.71–3.50 (om, H_{3'}, H_{3'}), 3.45 (d, *J* = 10 Hz, H₂₅), 3.42 (s, OCH₃), 3.35 (s, OCH₃), 3.32 (m, H₂), 3.18 (t, *J* = 9.0 Hz, H_{4'}), 2.70 (br s, 2 × OH), 2.50 (m, H₁₂), 2.32–2.15 (om, 2 × H₁₆, H₂₄, H_{2eq}), 2.07 (s, CH₃CO), 2.05–1.95 (om, H_{20eq}, H_{2'}eq), 1.75 (m, H_{18eq}), 1.65–1.40 (om, H₂₀, H₂₆, 2 × H₂₇, H_{2'}, H_{2'}), 1.48 (s, 3 × H_{14a}), 1.21 (d, *J* = 6.2 Hz, 3 × H₆), 1.15 (d, *J* = 6.9 Hz, 3 × H_{12a}), 1.10 (d, *J* = 6.6 Hz, 3 × H_{6'}), 0.95–0.85 (om, 3 × H_{24a}, 3 × H_{26a}, 3 × H₂₈, H_{18a}); ¹³C NMR δ 173.2 (C₁), 170.9 [(C=O)NH], 140.2, 139.3 (C₁₄, C₈), 138.1 (C₁₁), 136.3 (C₂₃), 135.1 (C₄), 127.7 (C₂₂), 124.7 (C₁₀), 120.7, 119.9, 118.3 (C₃, C₉, C₁₅), 98.6 (C_{1'}), 95.8 (C₂₁), 94.9 (C_{1'}), 81.9 (C₁₃), 81.0 (C_{4'}), 80.6 (C₇), 79.3 (C_{3'}), 79.2 (C₈), 74.9 (C₂₅), 73.3 (C_{3'}), 68.6, 68.3 (C₁₇, C₁₉), 68.4 (C_{8a}), 67.1, 65.5 (C₅, C_{5'}), 64.4 (C_{4a}), 56.6, 56.1 (2 × OCH₃), 48.5 (C_{4'}), 45.6 (C₂), 40.5 (C₂₀), 39.8 (C₁₂), 36.5 (C₁₈), 35.2 (C₂₆), 34.5 (C_{2'}), 34.2 (C₁₆), 31.8 (C_{2'}), 30.6 (C₂₄), 27.5 (C₂₇), 23.4 (CH₃C=O), 20.2 (C_{12a}), 18.3 (C₆), 17.0 (C_{6'}), 16.4 (C_{24a}), 15.1 (C_{14a}), 13.0 (C_{26a}), 12.0 (C₂₈); MS (FAB) 952 (M + Na, 4), 330 (26), 305 (22), 300 (20), 221 (20), 186 (100), 154 (82), 112 (34). Anal. Calcd for C₅₀H₇₅NO₁₅: C, 64.57; H, 7.95; N, 1.51. Found: C, 64.53; H, 7.95; N, 1.52.

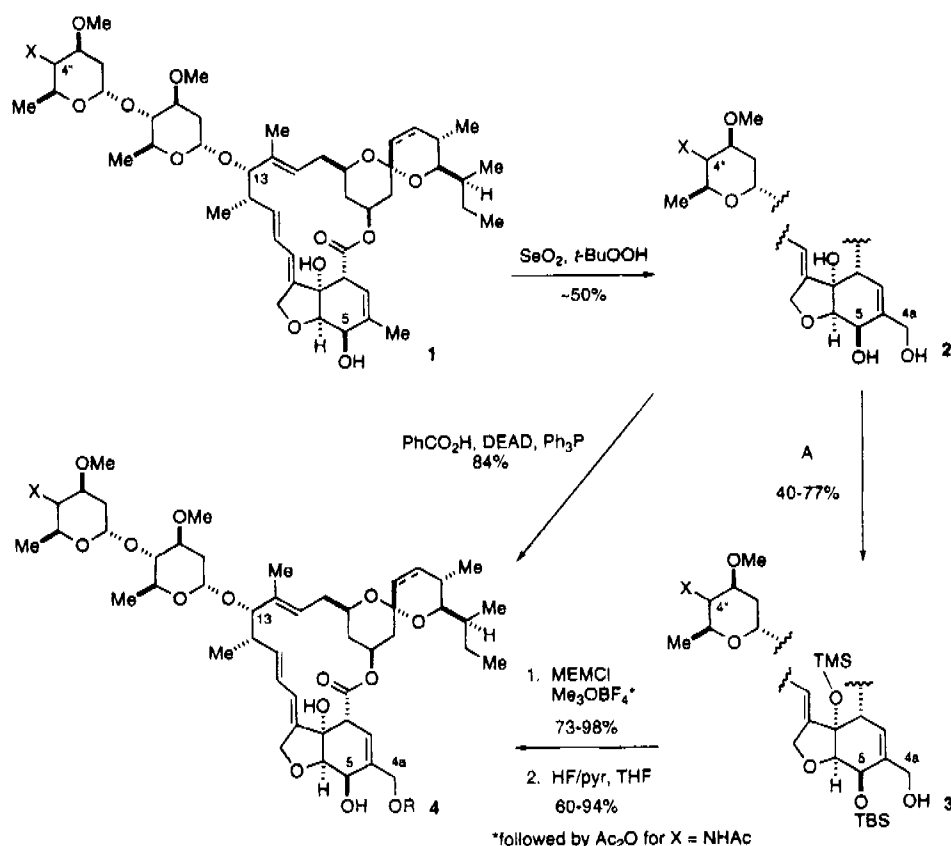
Data for 4''-epi-N-Acetyl-4''-deoxy-N-(methylamino)-4a-hydroxyavermectin B₁ (2k): 1.35 g (46%); *R_f* = 0.19 (94:6 CH₂Cl₂/MeOH); ¹H NMR (selected data) δ 5.86 (m, H₉), 5.78–5.63 (om, H₃, H₁₀, H₁₁, H₂₃), 5.55 (dd, *J* = 9.9, 2.5 Hz, H₂₂), 5.45 (d, *J* = 3.0 Hz, H_{1'}), 5.40–5.30 (m, H₁₉), 4.95 (m, H₁₅), 4.74 (d, *J* = 3.2 Hz, H_{1'}), 4.65 (m, H_{8a}), 4.55 (t, *J* = 5.0 Hz, H₅), 4.22 (br s, H_{4a}), 4.15 (m, H_{5'}), 3.95 (d, *J* = 6.3 Hz, H₈), 3.90 (br s, H₁₃), 3.42 (s, OCH₃), 3.35 (s, OCH₃), 3.32 (m, H₂), 3.18 (s, NCH₃), 2.85 (d, *J* = 5 Hz, OH); MS (FAB) 966 (M + Na, 80), 344 (90), 312 (100), 221 (95). Anal. Calcd for C₅₁H₇₇NO₁₅: C, 64.88; H, 8.22; N, 1.48. Found: C, 64.62; H, 8.33; N, 1.41.

RESULTS AND DISCUSSION

Chemistry. We initially focused on one C_{4'} substituent (*epi*-acetylamino) (Mrozik et al., 1989; Mrozik, 1984a,b; Linn and Mrozik, 1991) and varied the groups at C_{4a}. Once the structure activity relationship was established, we examined the most desirable C_{4a} substituents with other C_{4'} groups. In general, these compounds were prepared by first oxidizing at C_{4a} with SeO₂ as previously reported (Fisher and Mrozik, 1984; Mrozik, 1984a,b; Fraser-Reid et al., 1988), to provide alcohols 2a, 2b, 2i, and 2k. A series of protection and deprotection reactions selectively revealed C_{4a}OH in 3 for derivatization. Finally, complete deprotection provided the desired compounds (4) for testing (Scheme 1).

The oxidation reproducibly provided 4a-hydroxyavermectins in a yield of ~50%; however, only very low yields were obtained with C₅OH protected with either silyl or ester groups. Esters at C_{4a}OH were directly introduced through a Mitsunobu reaction (Mrozik, 1984a,b; Mitsunobu, 1981; Hughes et al., 1988) without the aforementioned protection/deprotection sequence to provide compounds like 4c. Ethers, however, were prepared by silylating with *t*-BuSiMe₂Cl (TBSCl) at C₅-OH and C_{4a}OH, desilylating C_{4a}OTBS, and trimethylsilylating C₇OH and C_{4a}OH with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) followed by hydrolysis of the primary silyl ether with aqueous HOAc to provide 3 from 2. The final silylation with BSTFA was necessary since the tertiary alcohol at C₇ was competitively alkylated by Me₃OBf₄, (2-methoxyethoxy)methyl chloride (MEMCl), and benzyloxymethyl chloride (BOMCl). Alkylation at C_{4a}OH followed by deprotection with pyridine/HF (Trost et al., 1983) provided the desired compounds (4) for testing. The methyl ethers were prepared by alkylation with Me₃OBf₄. This reagent competitively alkylates the C_{4'} acetylamino group, so

Scheme 1



A: a) TBSCl, imidazole, DMF, rt; b) HF/pyr, THF, rt; c) BSTFA, DMF, rt; d) HOAc, H₂O

the reaction was run to alkylate both the amide and alcohol functional groups. Upon complete alkylation, the reaction mixture was quenched with water to provide a C_{4'} amino group that was reacylated with Ac₂O.

Silylation at C_{4'}OH (and C₇OH) was sufficiently slower than silylation at C₅OH and C_{4a}OH (see structure 2), so that good yields of either silylated (allowing the reaction to run for extended periods of time) or free alcohol (stopping the reaction when C₅OH was silylated) at C_{4'} could be obtained. Identical groups could therefore be introduced at C_{4a}OH and at C_{4'}OH by selectively silylating C_{4a}OH and C₅OH and then selectively hydrolyzing the primary silyl group to provide a C₅ monosilyl ether—C_{4a}, C_{4'}, C₇ triol. Pertrimethylsilylation followed by aqueous hydrolysis gave a C_{4a}, C_{4'} diol ready for alkylation. Deprotection once again provided the desired compounds (4) for testing. The analogues with a hydroxyl at C_{4'} were prepared by silylating C_{4a}OH, C_{4'}OH, and C₅OH and then selectively hydrolyzing the primary silyl ether at C_{4a}. As described above, the alcohols at C₇ and C_{4a} were then trimethylsilylated and the primary silyl ether was hydrolyzed with aqueous HOAc. Alkylation followed by hydrolysis with pyridine/HF yielded the desired compounds (4).

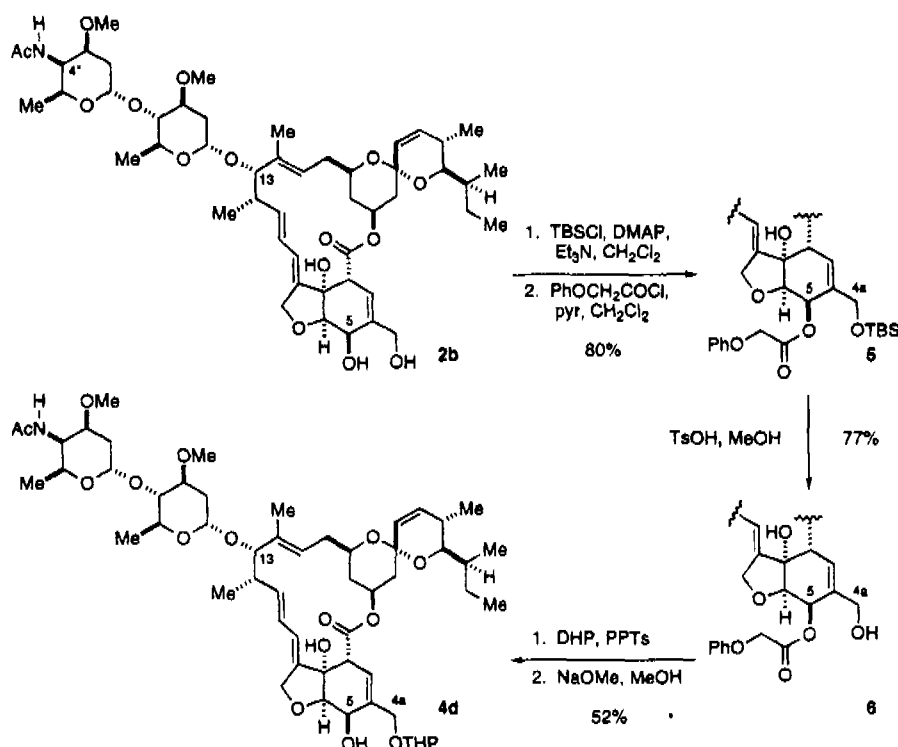
A tetrahydropyran (THP) substituent was attached on C_{4a}OH through a slightly different sequence as shown in Scheme 2. Silylation of C_{4a}OH followed by esterification of C₅OH provided 5. Desilylation at C_{4a} provided the primary alcohol, 6, for derivatization with 3,4-dihydro-2H-pyran (DHP). The ester at C₅ was then removed by methanolysis to provide 4d for testing.

Biological Evaluation. The new avermectin ana-

logues were evaluated for activity in two *in vitro* assays against brine shrimp (Blizzard et al., 1989) (*Artemia salina*) and flies (*Stomoxys calcitrans* adults, Table 1). As shown in Table 1, derivatization at C_{4a} usually increased activity against flies relative to the parent C_{4'} substituted avermectins. Nonbranched ethers (4e–g) were more active than free hydroxyls (2a, 2b, 2i, 2k), an ester (4c), or branched ether (4d). Saturating the C₂₂–C₂₃ double bond of the avermectin analogues produced compounds with increased activity against flies (compare 1aD, 4gD, and 4hD to 1a, 4g, and 4h). The more easily installed MEM group appeared to be as good as or better than a simple methyl substituent for increasing potency against flies (compare 4g, 4mD to 4e, 4j). Both of the acylamino groups at C_{4'} and the groups at C_{4a} served to increase potency against flies relative to 1a. Combined on the same molecule, they provided even more potent compounds (4g and 4l), with the C_{4a} substituent providing a larger impact on efficacy. No strong correlation existed between the brine shrimp and fly data.

Selected compounds were further evaluated for *in vivo* activity against several parasites in sheep (Table 2) (Chabala et al., 1980). As shown in Table 2, a free hydroxyl or ester group at C_{4a} generally reduced anti-parasitic activity in sheep (compare 2a, 2b, and 4c to 1a, 1b, and 1i). However, substitution with the chemically less reactive (and less polar) MEM group restored the activity to levels at or above the parent avermectin's levels (compare 4g and 4j to 1b and 1i). Finally, analysis of these avermectin analogues in a mouse LD₅₀ assay showed that C_{4a} substitution generally provided compounds that were potentially safer in mammals than

Scheme 2

Table 3. Acute Toxicity of Avermectin Analogues in Mice^a

compd	LD ₅₀ (mg/kg)	compd	LD ₅₀ (mg/kg)
1a	19	4c	>560
1aD	27	4e	175
1b	24	4g	>560
2a	205	4gD	>320
		4h	>100
		4hD	310

^a Estimated mouse LD₅₀ as described under Experimental Procedures.

the parent derivatives, with the MEM ether (4g) superior to a methyl ether (4e) (Table 3).

Conclusion. Oxidation of avermectin analogues at C_{4a} followed by derivatization of the added oxygen provides safer compounds, as judged by mouse LD₅₀ data, with substantial improvement in activity against flies. The antiparasitic activity of avermectin can be fully retained (see 4g) by forming a MEM ether with the added C_{4a} oxygen while increasing activity against flies. The improved safety profile and efficacy against flies may potentially expand the use of avermectins.

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Supplementary Material Available: A procedure for 4c, general procedures B–F, selected mass spectral, combustion analytical, and transcribed and assigned NMR data for 2a, 4c, 4e, 4f, 4g, 4gD, 4h, 4hD, 2i, 4j, 4l, and 4mD; procedures and data for 4a-O-TBS-2b, 5, 6, and 4d (14 pages). Ordering information is given on any current masthead page.

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