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Avermectins and Flea Control: Structure–Activity Relationships and the Selection of Selamectin for Development as an Endectocide for Companion Animals

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Abstract—Evaluation of a wide range of avermectin derivatives for flea activity in an in vitro feeding screen using the cat flea, *Ctenocephalides felis,* revealed a narrow structure–activity relationship (SAR) with activity surprisingly associated with monosaccharides and especially their C-5-oximes. We discovered commercially exploitable flea activity in a single compound, selamectin **33**, which also possessed the necessary antiparasitic spectrum and margin of safety for development as a broad-spectrum companion animal endectocide.

Introduction

Commercial products containing avermectins (e.g. Dectomax® and Ivomec®) or milbemycins (e.g. Cydectin®) are well established for the treatment of a wide range of both *endo*- and ecto-parasite infections of livestock and hence are referred to as endectocides. Although safe for use as an endectocide in livestock, ivermectin **2** produces idiosyncratic toxicity in collie dogs and crossbred collies at doses required to give effective control of gastrointestinal nematodes. Its use in dogs (as Heartgard™), at the very low dose required for safety in all dog breeds, provides only prophylactic control of heartworm. Milbemycin oxime **20** has also been commercialised (as Interceptor®). The compound's greater safety permits doses that provide additional control of gastrointestinal nematodes but it is ineffective against fleas, the major ectoparasite of commercial importance of cats and dogs. We set ourselves the objective of finding a safe, broad-spectrum endectocide for companion animals from this class of macrolides. Most importantly we wished to identify a compound which had systemic in vivo activity against fleas. Prior to the inception of our work, significant flea activity was not associated with the structural class.

Avermectins and milbemycins have been the subjects of extensive research and are known to bind to chloride channels in a wide range of invertebrates and vertebrates.[1] However, their antiparasitic potencies are also known to be dramatically altered by (sometimes minor) structural modification.[2] We therefore screened a wide range of compounds in an in vitro feeding screen using the cat flea, *Ctenocephalides felis*. Close analogues of the flea active structures discovered were then prepared and the optimum compound, selamectin **33**, was identified. Extensive evaluation demonstrated that the compound satisfied all our antiparasitic spectrum and safety criteria (including safety in collies) and it was developed as a safe, broad-spectrum endectocide for cats and dogs.[3]

Results

The majority of the compounds evaluated have been previously reported and are referenced in the Tables. Syntheses of novel avermectin derivatives, which generally follow reported strategies,[4] are shown in Schemes 1 and 2. C-5-oximes and related compounds were obtained by selective oxidation employing manganese dioxide followed by treatment of the crude ketone so obtained with the appropriate nitrogen nucleophile (Scheme 1).

C-4′-deoxygenation of the sugar was achieved via selective protection of the C-5-alcohol as its *tert*-butyldimethylsilyl ether and thiocarbonylation of the C-4′-

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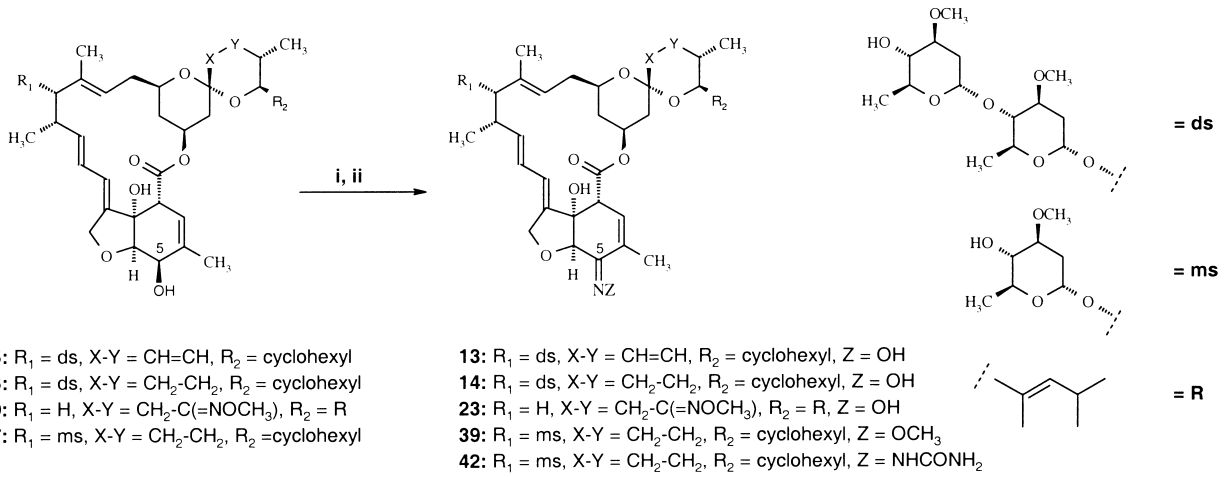
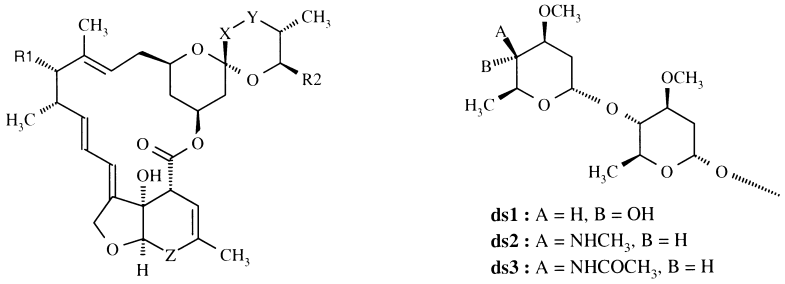


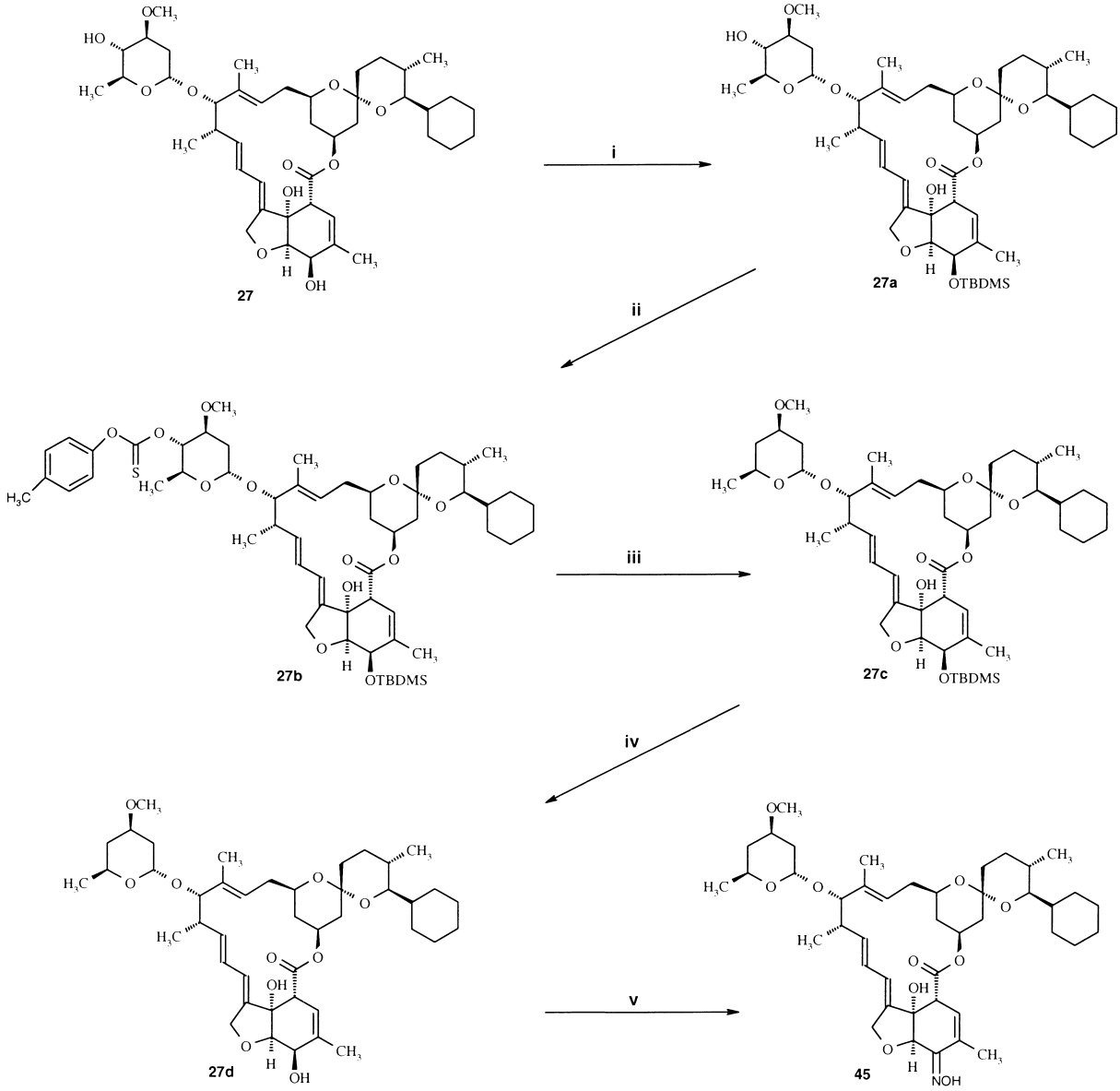
Scheme 1. Synthesis of oximes **13**, **14**, **23** and **39** and semicarbazone **42**. Reagents and conditions: i. manganese dioxide, diethyl ether, 25 °C, 3–20 h; ii. 12.2–27.6 equiv of hydroxylamine hydrochloride, 30 equiv of methoxylamine hydrochloride or 5.43 equiv of semicarbazide hydrochloride, methanol, dioxan, water, 25–50 °C, 2–48 h, 9–66% for two steps.

Table 1. Avermectins, milbemycins and C-5-oxime derivatives



Compound number	R_1	R_2	X–Y	Z
1,[5,a]	ds1	*sec*-Butyl	CH = CH	CH(◄OH)
2,[6,b]	ds1	*sec*-Butyl	CH_2-CH_2	CH(◄OH)
3,[7,c]	ds2	*sec*-Butyl	CH = CH	CH(◄OH)
4,[7,d]	ds3	*sec*-Butyl	CH = CH	CH(◄OH)
5[8,e]	ds1	Cyclohexyl	CH = CH	CH(◄OH)
6[9]	ds1	Cyclohexyl	CH_2-CH_2	CH(◄OH)
7[8]	ds1	Cyclohexyl	$CH_2CH($⋯OH)	CH(◄OH)
8[10]	ds1	Cyclohexyl	$CH_2CH($⋯OCH_3)	CH(◄OH)
9[8]	ds1	Cyclohexyl	CH = CH	CH(◄OCH_3)
10[8]	ds1	Cyclohexyl	$CH_2CH($⋯OH)	CH(◄OCH_3)
11[11]	ds1	*sec*-Butyl	CH = CH	C = NOH
12[12]	ds1	*sec*-Butyl	CH_2-CH_2	C = NOH
13	ds1	Cyclohexyl	CH = CH	C = NOH
14	ds1	Cyclohexyl	CH_2-CH_2	C = NOH
15[11]	ds1	Cyclohexyl	$CH_2CH($⋯OH)	C = NOH
16[10]	ds1	Cyclohexyl	$CH_2CH($⋯OCH_3)	C = NOH
17[13]	H	Isopropyl	CH_2-CH_2	CH(◄OH)
18[14]	H		$CH_2CH($⋯OH)	CH(◄OH)
19[15,f]	H		$CH_2C(=NOCH_3)$	CH(◄OH)
20[16,g]	H	Methyl/ethyl	CH_2-CH_2	C = NOH
21[16]	H	Isopropyl	CH_2-CH_2	C = NOH
22[17]	H		$CH_2CH($⋯OH)	C = NOH
23	H		$CH_2C(=NOCH_3)$	C = NOH

[a]Abamectin.
[b]Ivermectin.
[c]Emamectin.
[d]Eprinomectin.
[e]Doramectin.
[f]Moxidectin.
[g]Milbemycin oxime.



Scheme 2. Synthesis of oxime **45**. Reagents and conditions: i. 1.43 equiv of *tert*-butyldimethylsilyl chloride, 2.2 equiv of imidazole, dimethylformamide, 25 °C, 3.5 h, 60%; ii. 2 equiv of *para*-tolyl chlorothionoformate, pyridine, dichloromethane, 25 °C, 3 h; iii. 89.2 equiv of tributyltin hydride, 0.01 equiv of 2,2′-azobis(isobutyronitrile), toluene, 110 °C, 1 h; iv. 1.31 equiv of *para*-toluenesulphonic acid, methanol, 25 °C, 35% over 3 steps; v. (a). manganese dioxide, diethyl ether, 25 °C, 22 h; (b). 11.7 equiv of hydroxylamine hydrochloride, methanol, dioxan, water, 25–40 °C, 4 h, 76% over 2 steps.

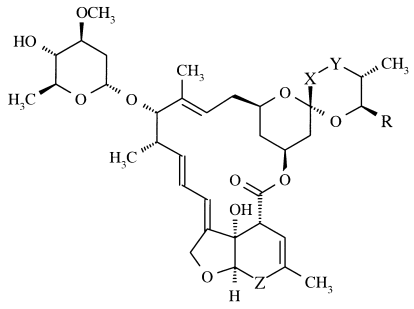
alcohol. Tributyltin hydride reduction of the thiocarbonate, desilylation with *para*-toluenesulphonic acid in methanol, followed by oxidation and oxime formation completed the sequence (Scheme 2).

The insecticidal activity of avermectin derivatives against fleas was determined using an in vitro feeding assay employing the cat flea *Ctenocephalides felis*. The selection of this model, in which fleas feed through an artificial membrane upon blood containing the test compound, is consistent with our objective of identifying systemically active molecules. Potency of a test compound is most conveniently expressed as its minimum detectable dose (MDD), which is defined as the concentration which caused at least 30% mortality at 24 h post-feeding.

Discussion

The avermectins, milbemycins and C-5-oxime derivatives shown in Table 1 were devoid of activity in the flea feeding assay at the initial screening dose of 1 μg/mL. Their MDDs were not determined as a value of 0.25 μg/mL in this assay provides an activity threshold; only compounds of at least this potency were subsequently effective against fleas on cats and dogs at doses up to the limit imposed for viability as a commercial product. Inspection of Table 2 shows that this threshold level of in vitro activity is only found amongst monosaccharide C-5-oximes. The most potent in vitro flea active (MDD 0.1 μg/mL) was 25-cyclohexyl-25-de(1-methylpropyl)-5-deoxy-22,23-dihydro-5-(hydroxyamino)-avermectin B1a monosaccharide (selamectin) **33**. Compounds with alternative C-5 substituents, prepared

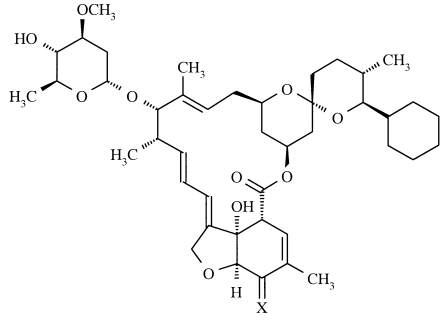
Table 2. Avermectin monosaccharides and C-5-oxime derivatives



Compound number	R	X–Y	Z	Flea MDD μg/mL
24[18]	*sec*-Butyl	CH = CH	CH(◀OH)	*[b]
25[18]	*sec*-Butyl	CH_2-CH_2	CH(◀OH)	*
26[11]	Cyclohexyl	CH = CH	CH(◀OH)	*
27[11]	Cyclohexyl	CH_2-CH_2	CH(◀OH)	1
28[11]	Cyclohexyl	$CH_2CH($····OH)	CH(◀OH)	*
29[10]	Cyclohexyl	$CH_2CH($····OCH_3)	CH(◀OH)	1
30[11]	*sec*-Butyl	CH = CH	C = NOH	1
31[11]	*sec*-Butyl	CH_2-CH_2	C = NOH	0.25
32[11]	Cyclohexyl	CH = CH	C = NOH	0.25
33[11,a]	Cyclohexyl	CH_2-CH_2	C = NOH	0.1
34[11]	Cyclohexyl	$CH_2CH($····OH)	C = NOH	0.25
35[10]	Cyclohexyl	$CH_2CH($····OCH_3)	C = NOH	1
36[11]	Cyclohexyl	$CH_2C(=NOCH_3)$	C = NOH	1

[a]Selamectin.
[b]*Inactive at 1 μg/mL, MDD not determined.

Table 3. C-5-derivatives of 25-cyclohexyl-25-de(1-methylpropyl)-5-deoxy-22,23-dihydro-avermectin B_{1a} monosaccharide.



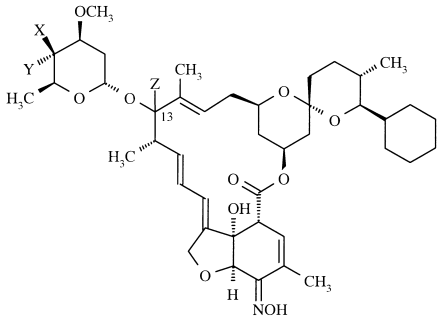
Compound number	X	Flea MDD μg/mL
37[19]	O	*[a]
38[19]	CH2	0.25
39	NOMe	1
40[20]	NNH2	*
41[21]	NNMe2	*
42	NNHCONH2	*

[a]*Inactive at 1 μg/mL, MDD not determined.

in a follow-up series, all possessed inferior activity (Table 3).

To probe the structure–activity relationship (SAR) of the sugar moiety, a series of C-5-hydroxyimino-monosaccharides, each with a single modification at C-4′ was prepared. Inspection of Table 4 again reveals inferior activity for these analogues, with only the epimeric alcohol **43** retaining moderate potency. This is in contrast to the antiparasitic SAR reported in the literature[23] and generated in our laboratories for the corresponding position of disaccharides which is tolerant to substitution, with, for example, 4″-*O*-acetyl and 4″-*epi*-acetylamino-4″-deoxy derivatives retaining full potency. The report that disaccharides epimeric at C-13 possess full anthelmintic potency coupled with an improved margin of safety [24] prompted the preparation of the final compound **49**, the C-13 epimer of **33**. Whilst flea activity was retained with this modification, potency was substantially reduced.

Table 4. 25-Cyclohexyl-25-de(1-methylpropyl)-5-deoxy-22,23-dihydro-5-(hydroxyimino)-avermectin B_{1a} monosaccharide derivatives.



Compound number	X	Y	Z	Flea MDD μg/mL
43[11]	OH	H	◀H	0.25
44[11]	H	OAc	◀H	*[a]
45	H	H	◀H	*
46[11]	CH_3	OH	◀H	*
47[11]	H	NHAc	◀H	*
48[11]	NHAc	H	◀H	*
49[22]	H	OH	····H	1

[a]*Inactive at 1 μg/mL, MDD not determined.

From this work it was concluded that **33** was uniquely potent and the compound was selected for development.

Conclusion

Systematic evaluation of avermectin derivatives for flea activity has revealed a narrow SAR with activity surprisingly associated with monosaccharides and especially their C-5-oximes. We have discovered commercially useful levels of activity in one compound, selamectin **33**, which also has the necessary antiparasitic spectrum and margin of safety for development as a broad-spectrum companion animal endectocide.[3]

Experimental

General

All common reagents and solvents were purchased at highest commercial quality and used without further purification. All solutions used in work up procedures were saturated. All reactions were monitored by thin-layer chromatography carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as visualising agent. E. Merck silica gel (kieselgel 60, 230-400 mesh) was used for column chromatography. Yields, which are unoptimised, refer to chromatographically and spectroscopically (^{1}H NMR) homogenous materials.

^{1}H NMR spectra were acquired on a Varian Inova 500 spectrometer in $CDCl_3$ at 30 °C using 3 mm tubes and calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. High resolution mass spectra (HRMS) were recorded on a Micromass Autospec Q under electrospray conditions.

25-Cyclohexyl-5-demethoxy-25-de(1-methylpropyl)-5-(hydroxyimino)-avermectin A_{1a} (13). A solution of alcohol **5**[8] (500 mg, 0.556 mmol) in diethyl ether (50 mL) containing a suspension of activated manganese dioxide (1 g) was stirred at 25 °C for 15 h. A further portion of manganese dioxide (200 mg) was added and stirring continued for a further 3 h after which the mixture was filtered through Celite® and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of dioxan (20 mL) and methanol (20 mL). A solution of hydroxylamine hydrochloride (538 mg, 7.74 mmol) in water (20 mL) was added and the mixture was stirred at 50 °C for 2 h. The cooled mixture was poured into a mixture of diethyl ether (50 mL) and water (50 mL). The layers were separated and the organic phase was washed with water (50 mL) and brine (50 mL). The aqueous phase was extracted with diethyl ether (50 mL×2). The organic phases were combined, dried (Na_2SO_4) and evaporated under reduced pressure to provide a white solid (552 mg) which was purified by column chromatography on silica gel (50 g, dichloromethane:ethyl acetate, 2:1, v/v) to provide oxime **13** (296 mg, 58%) as an amorphous white solid: R_f = 0.24 (dichloromethane:ethyl acetate, 1:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.11 (broad s, 1H, NO*H*), 5.96 (dt, *J* = 10.4, 2.4 Hz, 1H, C=C*H*CH=CHCH), 5.83–5.82 (m, 1H, C*H*=C(CH_3)C=N), 5.84 (dd, *J* = 9.4, 15.4 Hz, 1H, C=CHCH=C*H*CH), 5.74 (dd, *J* = 9.8, 2.8 Hz, 1H, CH=C*H*CH), 5.70 (dd, *J* = 10.4, 15.4 Hz, 1H, C= CHC*H*=CHCH), 5.55 (dd, *J* = 9.8, 2.5 Hz, 1H, C*H*= CHCH), 5.50–5.44 (m, 1H, C*H*OC=O), 5.40 (d, *J* = 3.4 Hz, 1H, C*H*(O)(O)CH_2), 5.01 (broad d, *J* = 10.1 Hz, 1H, CH_2CH=C(CH_3)), 4.79 (d, *J* = 4.0 Hz, 1H, C*H*(O)(O)CH_2), 4.76 (dd, *J* = 14.6, 2.4 Hz, 1H, OC*H*H), 4.70 (dd, *J* = 14.6, 2.4 Hz, 1H, OCH*H*), 4.68 (s, 1H, C*H*C=NOH), 3.95 (broad s, 1H, CO*H*), 3.90 (broad s, 1H, C*H*(O)), 3.91–3.82 (m, 1H, C*H*(O)(CH_2)(CH_2)), 3.83 (dq, *J* = 9.0, 6.3 Hz, 1H, C*H*(O)(CH_3)CH), 3.77 (dq, *J* = 9.2, 6.3 Hz, 1H, C*H*(O)(CH_3)CH), 3.63 (ddd, *J* = 13.3, 9.0, 4.8 Hz, 1H, C*H*(OCH_3)), 3.49 (ddd, *J* = 13.7, 9.2, 4.8 Hz, 1H, C*H*(OCH_3)), 3.43 (s, 3H, OC*H*$_3$), 3.42 (s, 3H, OC*H*$_3$) 3.42 (quintet, *J* = 2.3 Hz, 1H, C*H*C=O), 3.32 (d, *J* = 10.0 Hz, 1H, C*H*C_6H_{13}), 3.25 (t, *J* = 9.0 Hz, 1H, C*H*(O)(CH)(CH)), 3.17 (t, *J* = 9.2 Hz, 1H, C*H*(OH)(CH)(CH)), 2.80 (broad s, CHO*H*, 1H), 2.57–2.50 (m, 1H, C=CHCH=CHC*H*CH_3), 2.35–2.22 (m, 5H), 2.01 (ddd, *J* = 12.1, 4.8, 2.5 Hz, 1H, CH*H*C), 1.95–1.94 (m, 3H, CH=C(C*H*$_3$)C=N), 1.83–1.77 (m, 3H), 1.70–1.48 (m, 8H), 1.50 (broad s, 3H, CH_2CH=CC*H*$_3$), 1.33–1.19 (m, 4H), 1.28 (d, *J* = 6.3 Hz, 3H, OCHC*H*$_3$), 1.26 (d, *J* = 6.3 Hz, 3H, OCHC*H*$_3$), 1.18 (d, *J* = 7.0 Hz, 3H, C= CHCH=CHCHC*H*$_3$), 0.93 (d, *J* = 7.3 Hz, 3H, CH= CHCHC*H*$_3$), 0.87 (q, *J* = 12.0 Hz, 1H, CH*H*CHOC=O); HRMS, calcd for $C_{50}H_{73}NO_{14}$ (M + Na^+) 934.492877, found 934.489348.

25-Cyclohexyl-5-demethoxy-25-de(1-methylpropyl)-22,23-dihydro-5-(hydroxyimino)-avermectin A_{1a} (14). A solution of alcohol **6**[9] (334 mg, 0.37 mmol) in diethyl ether (20 mL) containing a suspension of activated manganese dioxide (670 mg) was stirred at 25 °C for 15 h. A further portion of manganese dioxide (334 mg) was added and stirring continued for a further 2 h after which the mixture was filtered through Celite® and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of dioxan (10 mL) and methanol (10 mL). A solution of hydroxylamine hydrochloride (314 mg, 4.52 mmol) in water (10 mL) was added and the mixture was stirred at 50 °C for 2 h. The cooled mixture was concentrated under reduced pressure to remove the methanol and dioxan and then extracted with diethyl ether (50 mL×2). The organic phases were combined, washed with water (50 mL) and brine (50 mL), dried (Na_2SO_4) and evaporated under reduced pressure to provide a colourless glass (669 mg). The crude product was purified by column chromatography on silica gel (25 g, dichloromethane:ethyl acetate, 4:1, v/v) and then reverse phase preparative HPLC (250×21.2 mm diameter Zorbax® 8 μ ODS column at ambient temperature and eluted at 9 mL min^{-1}, methanol:water, 90:10, v/v) to provide oxime **13** (141 mg, 42%) as an amorphous white solid: R_f = 0.25 (dichloromethane:ethyl acetate, 1:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (broad s, 1H, NO*H*), 5.95 (dt, *J* = 10.4, 2.4 Hz, 1H, C=C*H*CH= CHCH), 5.83 (m, 1H, C*H*=C(CH_3)C=N), 5.78 (dd, *J* = 9.4, 15.0 Hz, 1H, C=CHCH=C*H*CH), 5.74 (dd, *J* = 10.4, 15.0 Hz, 1H, C=CHC*H*=CHCH), 5.46–5.39 (m, 1H, C*H*OC=O), 5.40 (d, *J* = 3.2 Hz, 1H, C*H*(O)(O) CH_2), 4.99 (broad d, *J* = 10.7 Hz, 1H, CH_2CH=C(CH_3)), 4.79 (d, *J* = 4.2 Hz, 1H, C*H*(O)(O)CH_2), 4.76 (dd, *J* = 14.4, 2.4 Hz, 1H, OC*H*H), 4.70 (dd, *J* = 14.4, 2.4 Hz, 1H, OCH*H*), 4.68 (s, 1H, C*H*C=NOH), 3.98 (broad s, 1H, CO*H*), 3.95 (broad s, 1H, C*H*(O)), 3.86–3.81 (m, 1H, C*H*(O)(CH_2)(CH_2)), 3.84 (dq, *J* = 9.0, 6.3 Hz, 1H, C*H*(O)(CH_3)CH), 3.77 (dq, *J* = 9.2, 6.3 Hz, 1H, C*H*(O) (CH_3)CH), 3.63 (ddd, *J* = 13.3, 9.0, 4.8 Hz, 1H, C*H* (OCH_3)), 3.49 (ddd, *J* = 13.6, 9.2, 4.9 Hz, 1H, C*H*(OCH_3)), 3.42 (s, 6H, 2×OC*H*$_3$) 3.41 (quintet, *J* = 2.3 Hz, 1H, C*H*C=O), 3.25 (t, *J* = 9.0 Hz, 1H, C*H*(O)(CH)(CH)), 3.17 (t, *J* = 9.2 Hz, 1H, C*H*(OH)(CH)(CH)), 3.08 (broad d, *J* = 10.0 Hz, 1H, C*H*C_6H_{13}), 2.60 (broad s, CHO*H*, 1H), 2.56–2.49 (m, 1H, C=CHCH=CHC*H*CH_3), 2.35–2.22 (m, 4H), 1.97 (ddd, *J* = 11.9, 5.2, 1.4 Hz, 1H, CH*H*C), 1.95–1.94 (m, 3H, CH=C(C*H*$_3$)C=N), 1.81–1.78 (m, 3H), 1.66–1.43 (m, 12H), 1.50 (broad s, 3H, CH_2CH= CC*H*$_3$), 1.37 (t, *J* = 11.9 Hz, 1H, C*H*HC), 1.30–1.14 (m, 4H), 1.28 (d, *J* = 6.3 Hz, 3H, OCHC*H*$_3$), 1.26 (d, *J* = 6.3 Hz, 3H, OCHC*H*$_3$), 1.17 (d, *J* = 7.0 Hz, 3H, C= CHCH=CH CHC*H*$_3$), 0.85 (q, *J* = 12.0 Hz, 1H, CH*H*CH OC=O), 0.80 (broad d, *J* = 5.4 Hz, 3H, CHC*H*$_3$); HRMS, calcd for $C_{50}H_{75}NO_{14}$ (M + Na^+) 936.508527, found 936.512494.

5-Demethoxy-23,28-dideoxy-25-(1,3-dimethyl-1-butenyl)-6,28-epoxy-5-(hydroxyimino)-23-(methoxyimino)-milbemycin B (23). A solution of methoxime **19**[15] (250 mg, 0.391 mmol) in diethyl ether (5 mL) containing a suspension of activated manganese dioxide (500 mg) was stirred at 25 °C for 2 h. A further portion of manganese dioxide

(500 mg) was added and stirring continued for a further 0.5 h after which the mixture was filtered through Celite® and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of dioxan (12 mL) and methanol (6 mL). A solution of hydroxylamine hydrochloride (750 mg, 10.79 mmol) in water (6 mL) was added dropwise over 5 min and the mixture was stirred at 25 °C for 48 h. The mixture was concentrated under reduced pressure to remove the methanol and dioxan and then extracted with diethyl ether (50 mL×3). The organic phases were combined, washed with water (50 mL) and brine (50 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (5 g, dichloromethane/diethyl ether 0→5%, v/v) and then reverse phase preparative HPLC (250×21.4 mm diameter Rainin Dynamax® Microsorb™ 5 μ ODS column maintained at 40 °C and eluted at 10 mL min^{-1}, acetonitrile:methanol:water, 50:33:17, v/v/v) to provide oxime **23** (24 mg, 9%) as an amorphous white solid : R_f = 0.54 (dichloromethane:ethyl acetate, 4:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (broad s, 1H, NO*H*), 5.86 (dt, *J* = 11.5, 2.4 Hz, 1H, C = C*H*CH = CHCH), 5.83– 5.82 (m, 1H, C*H* = C(CH_3)C = N), 5.75 (dd, *J* = 11.5, 14.6 Hz, 1H, C = CHC*H* = CHCH), 5.42–5.35 (m, 1H, C*H*OC=O), 5.36 (dd, *J* = 10.3, 14.6 Hz, 1H, C = CHCH = C*H*CH), 5.18 (broad dd, *J* = 9.0, 1.0 Hz, 1H, C*H*($CHCH_3$)$_2$), 4.92 (broad dd, *J* = 9.8, 3.7 Hz, 1H, CH_2C*H* = C(CH_3)), 4.76 (dd, *J* = 14.3, 2.4 Hz, 1H, OCH*H*), 4.71 (dd, *J* = 14.3, 2.4 Hz, 1H, OC*H*H), 4.66 (s, 1H, C*H*C = NOH), 3.84 (s, 3H, C = NOCH_3), 3.77 (broad s, 1H, CO*H*), 3.62 (d, *J* = 10.3 Hz, 1H, OC*H*) 3.50–3.45 (m, 1H, C*H*(O)(CH_2)(CH_2)), 3.40 (quintet, *J* = 2.4 Hz, 1H, C*H*C=O), 3.29 (d, *J* = 14.8 Hz, 1H, C*H*HC = N), 2.65–2.55 (m, 1H, C*H*(CH_3)$_s$), 2.47–2.38 (m, 1H, C = CHCH = CHC*H* CH_3), 2.31 (dq, *J* = 10.3, 6.6 Hz, 1H, C*H*CH_3), 2.26–2.18 (m, 3H), 2.15 (ddd, *J* = 11.9, 5.2, 1.4 Hz, 1H, CH*H*C), 1.94 (m, 3H, CH = C(CH_3)C = N), 1.92 (d, *J* = 14.8 Hz, 1H, CH*H*C = N), 1.87 (t, *J* = 12.6, Hz, 1H, C*H*HC(CH_3) = CH), 1.81 (broad d, *J* = 12.0 Hz, 1H, C*H*HC HOC=O), 1.66 (m, 3H, C(CH_3) = CHCH(CH_3)$_2$), 1.51 (s, 3H, CH_2CH = CCH_3), 1.44 (t, J = 11.9 Hz, 1H, C*H*HC), 1.05 (d, *J* = 6.6 Hz, 3H, CH(CH_3)(CH_3)), 1.00 (d, *J* = 6.6 Hz, 3H, C = CHCH = CHCHCH_3), 0.97 (d, J = 6.7 Hz, 3H, CH(CH_3)(CH_3)), 0.92 (d, *J* = 6.6 Hz, 3H, CHCH_3), 0.88 (q, *J* = 12.0 Hz, 1H, CH*H*CHOC=O); HRMS, calcd for $C_{37}H_{52}N_2O_8$ ($M + Na^+$) 675.362137, found 675.361564.

25-Cyclohexyl-5-demethoxy-25-de(1-methylpropyl)-22, 23-dihydro-5-(methoxyimino)-avermectin A_{1a} monosaccharide (39). A solution of alcohol **27**[11] (250 mg, 0.33 mmol) in diethyl ether (20 mL) containing a suspension of activated manganese dioxide (250 mg) was stirred at 25 °C for 18 h. A further portion of manganese dioxide (250 mg) was added and stirring continued for a further 2 h after which the mixture was filtered through Celite® and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of dioxan (20 mL) and methanol (20 mL). A solution of methoxylamine hydrochloride (835 mg, 10 mmol) in water (20 mL) was added and the mixture was stirred at 50 °C for 2 h. The mixture was basified with aqueous potassium hydrogen carbonate solution and then extracted with diethyl ether (50 mL×3). The organic phases were combined, washed with water (50 mL) and brine (50 mL), dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified by reverse phase preparative HPLC (250×21.4 mm diameter Rainin Dynamax® Microsorb™ 5 μ ODS column maintained at 40 °C and eluted at 20 mL min^{-1}, methanol:water, 90:10, v/v) to provide oxime **39** (86 mg, 33%) as an amorphous white solid: R_f = 0.39 (dichloromethane:ethyl acetate, 4:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.93 (dt, *J* = 10.4, 2.4 Hz, 1H, C = C*H*CH = CHCH), 5.79–5.78 (m, 1H, C*H* = C(CH_3)C = N), 5.76 (dd, *J* = 9.4, 15.0 Hz, 1H, C = CHCH = C*H*CH), 5.73 (dd, *J* = 10.4, 15.0 Hz, 1H, C = CHC*H* = CHCH), 5.46–5.39 (m, 1H, C*H*OC=O), 4.98 (broad d, *J* = 10.9 Hz, 1H, CH_2C*H* = C(CH_3)), 4.83 (d, *J* = 3.6 Hz, 1H, C*H*(O)(O)CH_2), 4.72 (dd, *J* = 14.4, 2.4 Hz, 1H, OC*H*H), 4.66 (dd, *J* = 14.4, 2.4 Hz, 1H, OCH*H*), 4.56 (s, 1H, C*H*C = NOH), 4.00 (s, 3H, C = NOCH_3), 3.96 (broad s, 1H, CO*H*), 3.91 (broad s, 1H, C*H*(O)), 3.86 (dq, *J* = 9.2, 6.2 Hz, 1H, C*H*(O)(CH_3)CH), 3.65 (tdd, *J* = 11.8, 4.6, 1.9 Hz, 1H, C*H*(OCH_3)), 3.49 (ddd, *J* = 13.6, 9.0, 4.8 Hz, 1H, C*H*(OCH_3)), 3.47 (s, 3H, OCH_3), 3.38 (quintet, *J* = 2.4 Hz, 1H, C*H*C=O), 3.16 (t, *J* = 9.2 Hz, 1H, C*H*(O)(CH)(CH)), 3.07 (broad d, *J* = 7.6 Hz, 1H, C*H*C_6H_{13}), 2.55–2.49 (m, 1H, C = CHCH = CHC*H*CH_3), 2.51 (broad s, CHO*H*, 1H), 2.34–2.22 (m, 3H), 1.97 (ddd, *J* = 12.0, 5.1, 1.5 Hz, 1H, C*H*HC), 1.95–1.94 (m, 3H, CH = C(CH_3)C = N), 1.81–1.77 (m, 3H), 1.66–1.42 (m, 11H), 1.57 (broad s, 3H, CH_2CH = CCH_3), 1.37 (t, *J* = 12.0 Hz, 1H, CH*H*C), 1.30–1.14 (m, 4H), 1.27 (d, *J* = 6.2 Hz, 3H, OCHCH_3), 1.16 (d, *J* = 6.9 Hz, 3H, C = CHCH = CHCHCH_3), 0.82 (q, *J* = 11.8 Hz, 1H, C*H*H CHOC=O), 0.80 (broad d, *J* = 5.5 Hz, 3H, CHCH_3); HRMS, calcd for $C_{44}H_{65}NO_{11}$ ($M + Na^+$) 806.445532, found 806.445465.

25-Cyclohexyl-5-demethoxy-25-de(1-methylpropyl)-22, 23-dihydro-5-(semicarbazono)-avermectin A_{1a} monosaccharide (42). A solution of alcohol **27**[11] (125 mg, 0.33 mmol) in diethyl ether (20 mL) containing a suspension of activated manganese dioxide (125 mg) was stirred at 25 °C for 18 h. A further portion of manganese dioxide (125 mg) was added and stirring continued for a further 2 h after which the mixture was filtered through Celite® and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of dioxan (10 mL) and methanol (10 mL). A solution of semicarbazide hydrochloride (200 mg, 1.79 mmol) in water (5 mL) was added and the mixture was stirred at 25 °C for 48 h. The mixture was concentrated under reduced pressure to remove the methanol and dioxan. Aqueous sodium hydrogen carbonate solution (10 mL) was added and the mixture extracted with diethyl ether (50 mL×3). The organic phases were combined, washed with water (50 mL) and brine (50 mL), dried ($MgSO_4$) and evaporated under reduced pressure to provide semicarbazone **42** (91 mg, 66%) as an amorphous white solid: R_f = 0.14 (ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.50 (broad s, 1H, NN*H*), 5.98 (dt, *J* = 11.0, 2.4 Hz, 1H, C = C*H* CH = CHCH), 5.87–5.86 (m, 1H, C*H* = C(CH_3)C = N), 5.80 (dd, *J* = 9.7, 15.0 Hz, 1H, C = CHCH = C*H*CH), 5.72 (dd, *J* = 11.0, 15.0 Hz, 1H, C = CHC*H* = CHCH), 5.49–

5.43 (m, 1H, C*H*OC=O), 4.99 (broad d, $J=10.8$ Hz, 1H, $CH_2CH=C(CH_3)$), 4.83 (d, $J=3.5$ Hz, 1H, C*H*(O)(O)CH_2), 4.72 (d, $J=2.4$ Hz, 2H, OCH_2), 4.21 (s, 1H, C*H*C=N), 4.07 (broad s, 1H, C*H*(O)), 3.97 (broad s, 1H, CO*H*), 3.86 (dq, $J=9.1$, 6.2, 1H, C*H*(O)(CH_3)CH), 3.66 (tdd, $J=11.8$, 4.3, 1.8 Hz, 1H, C*H*(OCH_3)), 3.56 (ddd, $J=13.5$, 9.1, 4.7 Hz, 1H, C*H*(OCH_3)), 3.47 (s, 3H, OCH_3), 3.32 (quintet, $J=2.4$ Hz, 1H, C*H*C=O), 3.17 (t, $J=9.1$ Hz, 1H, C*H*(O)(CH)(CH)), 3.08 (broad d, $J=7.8$ Hz, 1H, C*H*C_6H_{13}), 2.57–2.51 (m, 2H), 2.34–2.22 (m, 3H), 1.97–1.96 (m, 3H, CH=C(C*H*$_3$)C=N), 1.95 (ddd, $J=12.0$, 4.8, 1.3 Hz, 1H, C*H*HC), 1.81–1.77 (m, 3H), 1.66–1.42 (m, 11H), 1.51 (broad s, 3H, $CH_2CH=CCH_3$), 1.38 (t, $J=12.0$ Hz, 1H, CH*H*C), 1.30–1.14 (m, 4H), 1.28 (d, $J=6.2$ Hz, 3H, OCHC*H*$_3$), 1.17 (d, $J=7.0$ Hz, 3H, C=CHCH=CHCHC*H*$_3$), 0.82 (q, $J=11.8$ Hz, 1H, C*H*HCHOC=O), 0.80 (broad d, $J=5.3$ Hz, 3H, CHC*H*$_3$); HRMS, calcd for $C_{44}H_{65}N_30_{11}$ ($M+Na^+$) 834.451680, found 834.455976.

25-Cyclohexyl-5-*O*-demethyl-5-*O*-[(1,1-dimethylethyl)dimethylsilyl]-25-de(1-methylpropyl)-22,23-dihydro-avermectin A_{1a} monosaccharide (27a). To a stirred solution of alcohol **27**[11] (3.03 g, 4 mmol) and imidazole (600 mg, 8.8 mmol) in dimethylformamide (10 mL) at 25 °C was added *tert*-butyldimethylsilyl chloride (663 mg, 4.4 mmol). After 3 h *tert*-butyldimethylsilyl chloride (200 mg, 1.33 mmol) was added and stirring continued for 0.5 h. Water (100 mL) was then added and the mixture extracted with dichloromethane (50 mL×2). The organic phases were combined, dried (Na_2SO_4) and evaporated under reduced pressure to provide a yellow oil which was purified by column chromatography on silica gel (100 g, dichloromethane:ethyl acetate, 9:1, v/v) to provide silyl ether **27a** (2.1 g, 60%) as a pale yellow amorphous solid: $R_f=0.57$ (dichloromethane:ethyl acetate, 4:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.84 (dt, $J=10.3$, 2.4 Hz, 1H, C=C*H*CH=CHCH), 5.74 (dd, $J=9.2$, 14.8 Hz, 1H, C=CHCH=C*H*CH), 5.71 (dd, $J=10.3$, 14.8 Hz, 1H, C=CHC*H*=CHCH), 5.39–5.32 (m, 1H, C*H*OC=O), 5.34–5.33 (m, 1H, C*H*=C(CH_3)CHOTBDMS), 4.99 (broad d, $J=10.9$ Hz, 1H, $CH_2CH=C(CH_3)$), 4.83 (d, $J=3.4$ Hz, 1H, C*H*(O)(O)CH_2), 4.67 (dd, $J=14.6$, 2.4 Hz, 1H, OC*H*H), 4.58 (d, $J=14.6$, 2.2 Hz, 1H, OCH*H*), 4.44 (broad d, $J=5.6$ Hz, 1H, C*H*OTBDMS), 4.17 (broad s, 1H, C*H*(O)), 3.95 (broad s, 1H, CO*H*), 3.86 (dq, $J=9.2$, 6.3 Hz, 1H, C*H*(O)(CH_3)CH), 3.82 (d, $J=5.6$ Hz, 1H, C*H*(O)CHOTBDMS, 3.65 (tdd, $J=11.8$, 4.6, 2.0 Hz, 1H, C*H*(OCH_3)), 3.56 (ddd, $J=13.5$, 9.2, 4.6 Hz, 1H, C*H*(OCH_3)), 3.47 (s, 3H, OCH_3), 3.39 (sextet, $J=2.4$ Hz, 1H, C*H*C=O), 3.16 (t, $J=9.2$ Hz, 1H, C*H*(O)(CH)(CH)), 3.07 (broad d, $J=7.2$ Hz, 1H, C*H*C_6H_{13}), 2.59 (broad s, 1H, C*H*OH), 2.54–2.48 (m, 1H, C=CHCH=CHC*H*CH_3), 2.34–2.22 (m, 3H), 1.98 (ddd, $J=11.9$, 5.0, 1.3 Hz, 1H, C*H*HC), 1.79 (broad s, 3H, CH=C(CH_3)C=N), 1.80–1.75 (m, 3H), 1.68–1.40 (m, 11H), 1.51 (broad s, 3H, $CH_2CH=CCH_3$), 1.34 (t, $J=11.9$ Hz, 1H, CH*H*C), 1.30–1.13 (m, 4H), 1.27 (d, $J=6.3$ Hz, 3H, OCHC*H*$_3$), 1.15 (d, $J=6.8$ Hz, 3H, C=CHCH=CHCHC*H*$_3$), 0.93 (s, 9H, t-Bu), 0.82 (q, $J=11.8$ Hz, 1H, C*H*HCHOC=O), 0.79 (broad d, $J=5.2$ Hz, 3H, CHC*H*$_3$), 0.13 (s, 6H, Si(CH_3)$_2$); HRMS, calcd for $C_{49}H_{78}O_{11}Si$ ($M+Na^+$) 893.521112, found 893.522751.

25-Cyclohexyl-5-*O*-demethyl-25-de(1-methylpropyl)-4″-deoxy-22,23-dihydro-avermectin A_{1a} monosaccharide (27d). To a stirred solution of silyl ether **27a** (435.5 mg, 0.5 mmol) in dichloromethane (5 mL) at 25 °C was added pyridine (2 mL) and then *para*-tolyl chlorothionoformate (187 mg, 1 mmol, added dropwise over 1 min). After 2 h *para*-tolyl chlorothionoformate (187 mg, 1 mmol) was added and stirring continued for 1 h. The reaction mixture was then evaporated under reduced pressure and the residue purified by column chromatography on silica gel (20 g, dichloromethane:hexane, 1:1, v/v) to provide thionocarbonate **27b** (420 mg) which was taken up in toluene (80 mL). To this solution was added tributyltin hydride (12 mL, 44.6 mmol) and 2,2′-azobis(isobutyronitrile) (0.8 mg, 0.005 mmol). The mixture was heated at 110 °C for 1 h, then cooled and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (20 g, hexane and then dichloromethane) to provide silyl ether **27c** (271 mg) which was taken up in methanol (50 mL). To this solution was added *para*-toluenesulphonic acid monohydrate (125 mg, 0.657 mmol) and the mixture was stirred at 25 °C for 0.75 h and then poured into aqueous potassium hydrogen carbonate solution (25 mL). Water (25 mL) was added and the mixture extracted with diethyl ether (50 mL×3). The combined organic phases were washed with water (20 mL×3) and brine (20 mL×2), then dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (10 g, dichloromethane:ethyl acetate, 9:1, v/v) and then reverse phase preparative HPLC (250×21.4 mm diameter Rainin Dynamax® Microsorb™ 5 μ ODS column maintained at 40 °C and eluted at 20 mL min^{-1}, methanol:-water, 85:15, v/v/v) to provide alcohol **27d** (131 mg, 35%) as an amorphous white solid: $R_f=0.33$ (dichloromethane:ethyl acetate, 4:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.88 (dt, $J=10.6$, 2.4 Hz, 1H, C=C*H*CH=CHCH), 5.77 (dd, $J=9.3$, 15.0 Hz, 1H, C=CHCH=C*H*CH), 5.71 (dd, $J=10.6$, 15.0 Hz, 1H, C=CHC*H*=CHCH), 5.44 (broad s, 1H, C*H*=C(CH_3)CHOH), 5.42–5.36 (m, 1H, C*H*OC=O), 5.03 (broad d, $J=11.8$ Hz, 1H, $CH_2CH=C(CH_3)$), 4.89 (d, $J=3.4$ Hz, 1H, C*H*(O)(O)CH_2), 4.69 (dd, $J=14.2$, 2.4 Hz, 1H, OC*H*H), 4.66 (dd, $J=14.2$, 2.4 Hz, 1H, OCH*H*), 4.30 (broad t, $J=6.9$ Hz, 1H, C*H*OH), 4.12 (s, 1H, CO*H*), 4.03 (ddq, $J=11.5$, 1.8, 6.2 Hz, 1H, C*H*(O)(CH_3)CH_2), 3.97 (d, $J=6.9$ Hz, 1H, C*H*(O)CHOH), 3.96 (broad s, 1H, C*H*(O)), 3.65 (tt, $J=11.3$, 4.2 Hz, 1H, C*H*(OCH_3)), 3.56 (tdd, $J=11.8$, 4.6, 2.1 Hz, 1H, C*H*(O)CH_2)$_2$), 3.40 (s, 3H, OCH_3), 3.29 (sextet, $J=2.2$ Hz, 1H, C*H*C=O), 3.07 (broad d, $J=7.9$ Hz, 1H, C*H*C_6H_{13}), 2.51 (dsextets, $J=2.4$, 7.0 Hz, 1H, C=CHCH=CHC*H*CH_3), 2.34 (d, $J=6.9$ Hz, 1H, CHO*H*), 2.32 (broad d, $J=13.5$ Hz, 1H, (CH_3)C=CHC*H*H), 2.25 (dt, $J=13.5$, 11.8 Hz, 1H, (CH_3)C=CHC*H*H), 2.14 (broad d, $J=12.4$ Hz, 1H, CH(O)(O)C*H*H), 2.05 (broad d, $J=12.4$ Hz, 1H, C*H*HCH(OCH_3)), 1.97 (ddd, $J=12.0$, 4.9, 1.5 Hz, 1H, C*H*HC), 1.88 (broad s, 3H, CH=C(C*H*$_3$)CHOH), 1.83–1.76 (m, 3H), 1.68–1.40 (m, 11H), 1.55 (broad s, 3H, $CH_2CH=CCH_3$), 1.35 (t, $J=12.0$ Hz, 1H, CH*H*C), 1.30–1.17 (m, 5H), 1.19 (d, $J=6.2$ Hz, 3H, OCHC*H*$_3$), 1.14 (d, $J=7.0$ Hz, 3H, C=CHCH=CHCHC*H*$_3$), 0.80 (q, $J=11.8$ Hz, 1H, C*H*HCHOC=O), 0.79 (broad d,

$J = 5.4$ Hz, 3H, CHCH_3); HRMS, calcd for $C_{43}H_{64}0_{10}$ ($M + Na^+$) 763.439719, found 763.441418.

25-Cyclohexyl-5-demethoxy-25-de(1-methylpropyl)-4″-deoxy-22,23-dihydro-5-hydroxyimino-avermectin A_{1a} monosaccharide (45). A solution of alcohol **27d** (91 mg, 0.123 mmol) in diethyl ether (30 mL) containing a suspension of activated manganese dioxide (90 mg) was stirred at 25 °C for 2 h. A further portion of manganese dioxide (90 mg) was added and stirring continued for a further 18 h. A third portion of manganese dioxide (90 mg) and stirring continued for a further 2 h after which the mixture was filtered through Celite® and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of dioxan (10 mL) and methanol (10 mL). A solution of hydroxylamine hydrochloride (100 mg, 1.44 mmol) in water (5 mL) was added and the mixture was stirred at 25 °C for 2 h and then heated at 40 °C for 2 h. The mixture was poured into aqueous potassium hydrogen carbonate solution (25 mL) and water (50 mL) and then extracted with diethyl ether (50 mL×3). The organic phases were combined, washed with water (20 mL×3) and brine (10 mL×2), dried (Na_2SO_4) and evaporated under reduced pressure. The crude product (117 mg) was purified by column chromatography on silica gel (5 g, dichloromethane:ethyl acetate, 9:1, v/v) and then reverse phase preparative HPLC (250×21.4 mm diameter Rainin Dynamax® Microsorb™ 5 μ ODS column maintained at 40 °C and eluted at 20 mL min^{-1}, methanol/water, 85/15, v/v) to provide oxime 45 (70 mg, 76%) as an amorphous white solid : $R_f = 0.43$ (dichloromethane:ethyl acetate, 4:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.04 (broad s, 1H, NO*H*), 5.95 (dt, $J = 10.5$, 2.4 Hz, 1H, C=C*H*CH=CHCH), 5.84–5.83 (m, 1H, C*H*=C(CH_3)CHOH), 5.79 (dd, $J = 9.3$, 15.1 Hz, 1H, C=CHCH=C*H*CH), 5.73 (dd, $J = 10.5$, 15.1 Hz, 1H, C=CHC*H*=CHCH), 5.47–5.40 (m, 1H, C*H*OC=O), 5.03 (broad d, $J = 11.5$ Hz, 1H, CH_2CH=C(CH_3)), 4.89 (d, $J = 3.4$ Hz, 1H, C*H*(O)(O)CH_2), 4.76 (dd, $J = 14.4$, 2.4 Hz, 1H, OC*H*H), 4.70 (dd, $J = 14.4$, 2.4 Hz, 1H, OCH*H*), 4.68 (s, 1H, C*H*C=NOH), 4.04 (ddq, $J = 11.5$, 1.8, 6.3 Hz, 1H, C*H*(O)(CH_3)CH_2), 3.99 (s, 1H, C*H*(O)), 3.97 (broad s, 1H, CO*H*), 3.70 (tt, $J = 12.3$, 4.6 Hz, 1H, C*H*(OCH_3)), 3.66 (tdd, $J = 11.5$, 4.5, 2.0 Hz, 1H, C*H*(O)(CH_2)$_2$), 3.41 (s, 3H, OCH_3), 3.40 (quintet, $J = 2.3$ Hz, 1H, C*H*C=O), 3.07 (broad d, $J = 8.0$ Hz, 1H, C*H*C_6H_{13}), 2.52 (dsextets, $J = 2.5$, 6.9 Hz, 1H, C=CHCH=CHC*H*CH_3), 2.34 (broad d, $J = 13.6$ Hz, 1H, (CH_3)C=CHC*H*H), 2.25 (dt, $J = 13.6$, 11.5 Hz, 1H, (CH_3)C=CHCH*H*), 2.14 (broad d, $J = 12.6$ Hz, CH(O)(O)C*H*H), 2.06 (broad d, $J = 12.3$ Hz, C*H*HCH(OCH_3)), 1.97 (ddd, $J = 12.1$, 4.9, 1.3 Hz, 1H, C*H*HC), 1.94 (broad s, 3H, CH=C(CH_3)C=NOH), 1.83–1.76 (m, 3H), 1.50 (broad s, 3H, CH_2CH=CCH_3), 1.68–1.40 (m, 11H), 1.37 (t, $J = 12.1$ Hz, 1H, CH*H*C), 1.30–1.17 (m, 5H), 1.19 (d, $J = 6.3$ Hz, 3H, OCHCH_3), 1.14 (d, $J = 6.9$ Hz, 3H, C=CHCH=CHCHCH_3), 0.82 (q, $J = 11.5$ Hz, 1H, C*H*HCHOC=O), 0.80 (broad d, $J = 5.5$ Hz, 3H, CHCH_3); HRMS, calcd for $C_{43}H_{63}NO_{10}$ ($M + Na^+$) 776.434968, found 776.435166.

Determination of flea feeding activity. In vitro systemic activity was determined at 38 °C using a membrane feeding apparatus.[25] Culture cats were used to provide regular supplies of an established laboratory strain of fleas (*Ctenocephalides felis*) not more than 48 h post-eclosion. Twenty-four hours prior to testing, up to 30 adult fleas of mixed sex were collected and aspirated into specially designed feeding chambers and held at 25 °C and 75% relative humidity (RH). Immediately prior to testing, the number of live fleas in each feeding chamber was recorded and test solutions (blood plus compound) prepared. Test compounds were made up as stock solutions in dimethylsulfoxide (DMSO) and 50 μl of the stock added to 9.95 mL of blood.

Fleas were fed through a 300 μm mesh for 6 h. Knock-down and mortality were monitored during feeding and for 24 h post feeding. On removal from the feeding chamber, fleas were maintained at 25 °C and 75% RH. Mortality was recorded and efficacy calculated as a percentage of the control fleas. Control fleas were fed on blood containing 0.5% v/v DMSO. The minimum detectable dose (MDD) was defined as the dose producing > 30% mortality of fleas after 24 h post feeding.

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