Affinity Probes for the Avermectin Binding Proteins†

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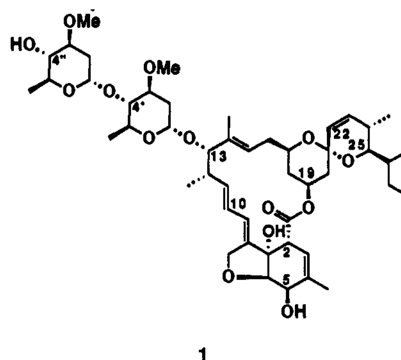
The design and synthesis of a series of avermectin affinity probes used in the identification and purification of the avermectin binding proteins is described. These modified avermectins fall into two design classes: ligands to covalently modify specific avermectin binding proteins [an ^{125}I-labeled aryl azide photoprobe (15) and a tritiated aziridine analog (6)] and ligands for affinity chromatography applications [three biotinylated compounds (10, 12, and 13) and one resin-bound derivative (9)]. The binding affinities of these compounds for the *Caenorhabditis elegans* avermectin binding protein is presented as well as their biological activities against *C. elegans* and *Artemia salina*.

Avermectin B_{1a}[1] (1, AVM), the primary fermentation product of *Streptomyces avermitilis*, is a structurally complex natural product with pronounced pharmacological activities. For instance, ivermectin (IVM), its 22,23-dihydro derivative, has found widespread use as a potent, broad spectrum anthelmintic agent.[2] The pronounced biological activity exhibited by this class of macrolides has elicited substantial synthetic interest directed toward the preparation of analogs with enhanced and/or altered biological activity profiles.[3] Considerable research also has been directed toward the identification and cloning of avermectin receptors from *Caenorhabditis elegans*.[4–6] Elucidation of the structure and molecular properties of these AVM binding proteins is an important goal in understanding the mechanism of AVM-modulated chloride ion transport.

The bioactivity of avermectin is believed to be mediated by stimulation of a specific chloride ion transport system; however, its exact mechanism of action remains unclear.[7]



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For instance, in invertebrates, electrophysiological experiments showed that avermectins enhance γ-aminobutyric acid (GABA) mediated increases in membrane permeability to chloride ions,[8,9] yet also open GABA-insensitive chloride channels.[8] In addition, avermectins have been demonstrated to increase chloride ion permeability in systems that do not possess GABA receptors[10] and modulate specific glutamate-gated anion channels.[11]

Given that avermectins initiate their physiological and biochemical effects by interacting with specific binding proteins located on the plasma membranes of their target cells, the development of high affinity ligands that bind specifically with these proteins has been a significant goal of our research. Their availability will provide powerful tools for biochemical investigations, facilitate receptor isolation, help clarify the mechanism of action of this important class of compounds, and ultimately lead to the synthesis of more efficacious, rationally designed avermectin derivatives.

The design and synthesis of several distinct types of affinity probes for the AVM binding proteins and their

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† This paper is dedicated to Professor Ralph Hirschmann on the occasion of his 70th birthday.

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(1) 2a*E*,4*E*,5′*S*,6*S*,6′*R*,7*S*,8*E*,11*R*,15*S*,17a*R*,20*R*,20a*R*,20b*S*)-6′-[(*R*)-*sec*-butyl]-7-[[2,6-dideoxy-[4-*O*-(2,6-dideoxy-3-*O*-methyl)-α-L-*arabino*-hexopyranosyl]-3-*O*-methyl-α-L-*arabino*-hexopyranosyl]oxy]-5′,6,6′,7,-10,11,14,15,17a,20,20a,20b-dodecahydro-20,20b-dihydroxy-5′,6,8,19-tetramethylspiro[11,15-methano-2*H*,13*H*,17*H*-furo[4,3,2-*pq*][2,6]-benzodioxacyclooctadecin-13,2′-[2*H*]pyran]-17-one.

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biochemical characteristics are described in this report. These probes are intended for application in diverse species, consequently, the need for several different types of affinity ligands was envisaged. These novel AVM derivatives can be placed in two general design classes: structurally modified avermectins for use as either affinity labeling reagents or affinity chromatography probes.

Reactive avermectin affinity labeling reagents were designed with the intention of covalently modifying the ivermectin binding protein with high selectivity, thus enabling identification of receptor sites: an aziridine (**6**) and an aryl azide (**15**) were the two cross-linking agents selected for this purpose. Use of photoactive ligands such as **15** permits superior control over experimental conditions, since the reactive function is released via photolysis. These strategies are complementary because cross-linking efficiencies for photoprobes are quite poor whereas electrophilic agents, like aziridine **6**, are more reactive and consequently less selective.[24]

Affinity chromatography represents another viable approach to purify the IVM receptor. Avermectin was modified for affinity chromatography applications wherein one terminus was tethered directly to CH-Sepharose 4B (**9**). Alternatively, double affinity chromatography probes in which one end binds with receptor proteins and the other end incorporates the biotin function (which binds to streptavidin) also could permit selective purification of AVM binding proteins. Three bifunctional, biotinylated AVM analogs (**10**, **12**, and **13**) therefore were synthesized. Biotinylated derivative **13** bears a cleavable disulfide linkage and presents an alternative mode of freeing purified receptor proteins (other than biotin displacement) from the polymer support.

Based on the large number of synthetically modified avermectin analogs reported to date, it was inferred that the 4″ and 13 positions represented sites that could be readily functionalized without adversely affecting biological activity and binding affinity profiles.[2,4] Variable length spacer arms were incorporated into these affinity probes for optimal binding sensitivity,[12] ranging from aziridine **6** that essentially was tethered directly to the aglycon to the other extreme **13**, where long peptide chains were appended to the terminal oleandrosyl unit. Judicious selection of spacer arms is particularly significant for affinity chromatography applications, because although streptavidin binds biotin with extremely high affinity ($K_a = 10^{15}\ M^{-1}$), its binding site is buried approximately 9 Å below the surface of the protein.[13]

Unlike probes for affinity chromatography applications, ligands used to covalently modify receptor proteins must incorporate a radiolabel. Consequently, aziridine **6** was tritiated in the 5-α position[14] and the photoaffinity probe **15** was radiolabeled with iodine-125.[15,16] Greater sensitivity may be attained using the iodinated photoprobe **15** in lieu of the tritiated aziridyl analog **6** due to the inherent difference in specific activities of ^{125}I and ^{3}H. This difference could be particularly significant since AVM binding proteins are not present in abundant quantities in target tissue.[25]

Table I. Binding Affinity and Bioactivity of AVM Affinity Reagents

compd	*A. salina* immobilization:[a] IC_{100} (ng/mL)	*C. elegans* binding affinity: IC_{50} (nM)	*C. elegans* motility: ED_{50} (ng/mL)
IVM	430	0.1	7
5	3470	5.0	48
9		(0.2)[b]	
10	430	0.22	46
12	1730	0.33	66
13	2600	0.45	75
14	650	0.1	57

[a] Average of two assays. [b] Determined for the non-resin-bound form (**8**).

Biochemistry

Binding affinities and physiological activities were employed to evaluate the new AVM affinity reagents. These results are presented in Table I. Binding affinities were obtained using *C. elegans* membrane homogenates.[4] The ligand employed in the determination of the *C. elegans* IC_{50} values was 22,23-ditritioivermectin ([^{3}H]IVM).[4] Brine shrimp (*Artemia salina*) immobilization[17] and *C. elegans* motility assays[4] provided an assessment of the biological activities of these affinity ligands. The observed binding affinities were quite high, ranging from 0.1 nM for aryl azide **14** to 5 nM for aziridine **5**. The IC_{50} value measured for **14** was identical to that obtained for ivermectin. These probes also exhibited comparable activity in the brine shrimp and *C. elegans* motility assays to that determined for the parent avermectin. The close correlation that exists between the measured binding affinities and the biological effects of these ligands and AVM (or IVM) indicated that specific, high affinity binding indeed occurred with the desired proteins. Schaeffer and Haines have demonstrated a direct correlation between the in vivo potency of AVM derivatives and the affinity binding in *C. elegans* membrane preparations.[4]

Tritiated aziridine **6** exhibited saturable binding with a high affinity binding site from *C. elegans* membrane homogenates. However, due to the low level of AVM binding proteins present in the membrane preparations, cross-linking experiments using this probe proved inconclusive. The B_{max} for the AVM binding protein was determined to be 0.38 pmol/mg,[23] which would require an approximate 40 000-fold purification to achieve homogeneity. Affinity labeling probe **6**, which has a specific activity of only 14 Ci/mmol, would have to exhibit a cross-linking efficiency in excess of 50% to visualize the tagged proteins by autoradiography. Consequently, an affinity reagent, [^{125}I]azido-AVM **15**, bearing an inherently higher specific activity tag was developed.

Radiolabeled photoprobe **15** exhibited, as did [^{3}H]-aziridine **6**, saturable binding with a high affinity binding protein from *C. elegans* worms.[23] This ligand also was shown to be a competitive inhibitor of ivermectin.[23]

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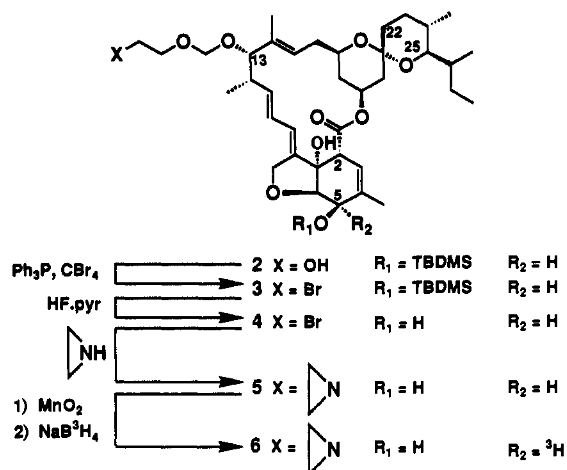
Scheme I



However, unlike 6, the specific activity of 15 was determined to be 1700 Ci/mmol, which permitted significantly greater sensitivity in cross-linking experiments. The successful application of photoprobe 15 in cross-linking experiments is reported elsewhere.[23]

Competition binding assays were performed using the four reagents designed for affinity chromatography applications. Amine 8 (the non-resin-bound form of 9) and biotinylated analogs 10, 12, and 13 exhibited high affinity binding with *C. elegans* membrane homogenates. All binding/affinity chromatography experiments were performed using detergent solubilized protein.

Preliminary results from affinity chromatography experiments have demonstrated that the Sepharose-bound analog 9 removed greater than 95% of the avermectin binding proteins from solution. Biotinylated derivative 10, for instance, also extracted comparable quantities of the binding proteins from solution. Use of 10 allows the experiments to be performed in either of two ways with equal success. For example, the AVM binding sites could be saturated with affinity probe 10 prior to eluting the solution through a monomeric or tetrameric streptavidin column. Alternatively, the streptavidin column could be preloaded with 10 before passing the solution containing the AVM binding proteins through the column. Similar applications of the related chain homologated analogs 12 and 13 may be envisaged.

Chemistry

The synthesis of tritiated aziridine 6 is shown in Scheme I. The starting material for this sequence (2) was 5-O-protected ivermectin aglycon[2] which had been 13-O-alkylated with acetoxyethoxymethyl bromide followed by ammonical methanolysis.[18] Alcohol 2 was converted to bromide 3 and at this juncture, the 5-OTBDMS group was removed using HF·pyr.[19] Treatment of 4 with excess freshly distilled ethyleneamine[20] generated the desired aziridine 5 in excellent yield (67%) after two days at ambient temperature. Only minor amounts (5–10%) of the 2-epi analog were observed and these were readily removed via flash chromatography. Tritium was selectively introduced at the 5-α position by manganese dioxide-mediated allylic oxidation followed by reduction with sodium borotrituride[14] yielding 6 with a specific activity of 14 Ci/mmol.

The biotinylated (10, 12, and 13) and the Sepharose-bound (9) analogs were generated as shown in Scheme II. Acylation of the readily available amine 7[21] was achieved using Fmoc-β-AlaOH [Fmoc, (9-fluorenyloxy)carbonyl] with dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole (DCC/HOBT) under standard conditions. The silyl protecting group on the 5-hydroxyl was removed with HF·pyridine[19] prior to Fmoc group cleavage (5% piperidine in CH_2Cl_2 at ambient temperature) to yield amine 8 in 44% from 7. The amine thus formed was acylated with a variety of different agents. Reaction of 8 with activated CH-Sepharose 4B[22] produced the polymer-bound avermectin derivative 9. Amine 8 also was acylated with commercially available NHS-biotin (NHS, *N*-hydroxysuccinimide), generating 10. Treatment of 8 with Fmoc-ϵ-caproic acid yielded, after protecting group removal, chain-homologated amine 11. Amine 11 was subjected to acylation with NHS-biotin and NHS-SS-biotin [biotin-$NH(CH_2)_2SS(CH_2)_2COOSu(SO_3Na)$] as described previously to produce 12 and 13, respectively.

Photoaffinity probe 15 was synthesized from amine 11. Reaction of 11 with succinimido 4-azidosalicylate produced 14 in near quantitative yield. The [^{125}I]-radiolabel was introduced using $Na^{125}I$ and chloramine-T.[15] Radiolabeled 15 ultimately was obtained in pure form by reverse-phase HPLC with a specific activity of 1700 Ci/mmol.

Conclusion

In this article, we have demonstrated efficient syntheses of diverse affinity probes to facilitate identification and isolation of the avermectin binding proteins via covalent modification or by affinity chromatographic techniques. These structurally modified AVM affinity reagents exhibited biological profiles comparable to that of avermectin, with high specificity for the key binding proteins. The successful application of these disparate affinity reagents to the identification and isolation of the avermectin binding proteins is reported elsewhere.[23]

Experimental Section

NHS-Biotin, NHS-SS-Biotin, and 4-azidosalylicidamidoOSu were obtained from Pierce Chemicals. $Na^{125}I$ and NaB^3H_4 were obtained from Amersham. ^1H NMR spectra were recorded on Varian XL-300 or XL-400 instruments in $CDCl_3$ with tetramethylsilane as internal reference. Mass spectra were obtained

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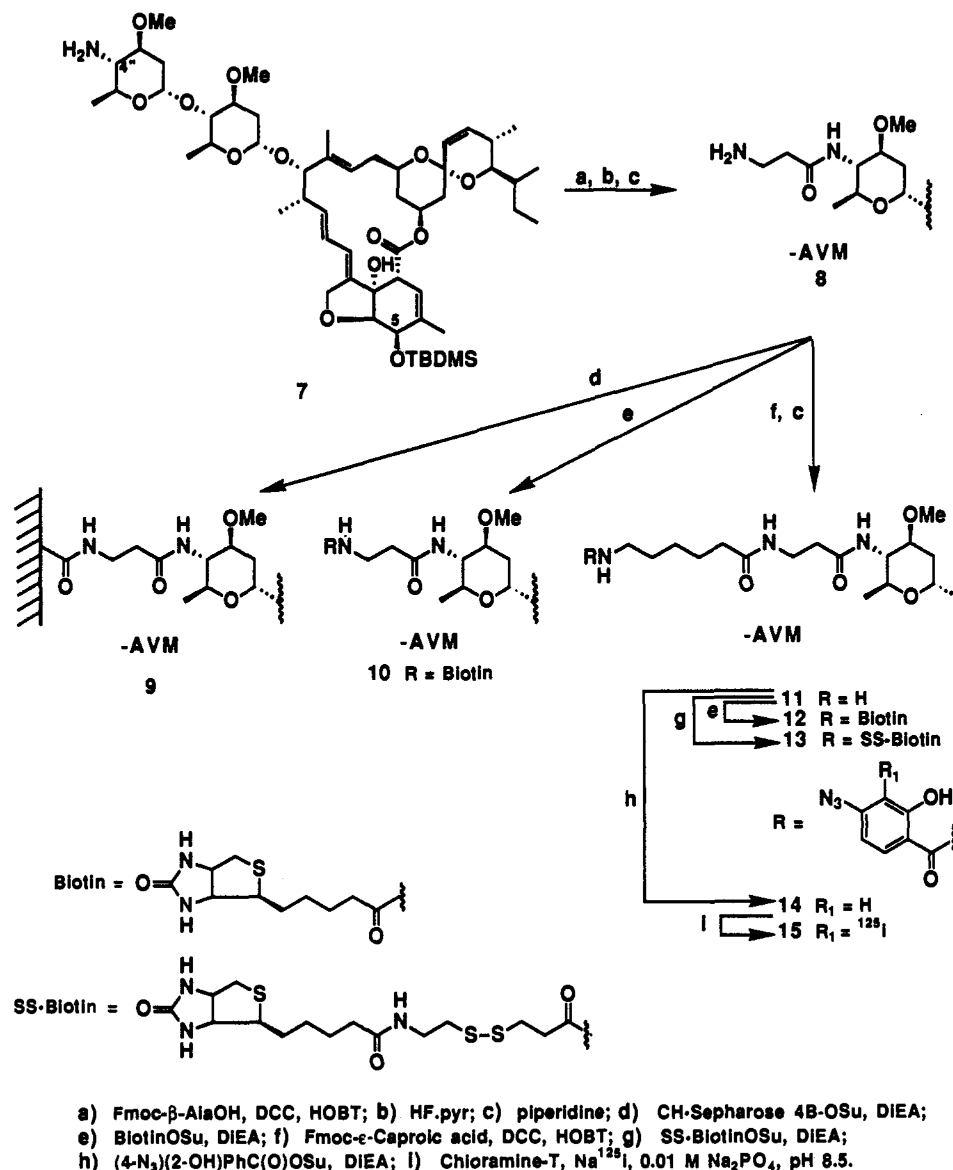
(25) For instance, in *C. elegans* tissue preparations used for avermectin binding assays, the AVM binding proteins are present at levels of 0.34 pmol/mg.[4]

Scheme II



on JEOL HX-10A mass spectrometer. HPLC chromatography was performed using Waters Prep LC3000 with a Zorbax RX8 4.6- × 250-mm column. Flash chromatography was performed using E. M. Merck silica gel 60, mesh 230–400. All compounds were purified to homogeneity as determined by TLC and/or reverse-phase HPLC.

***Artemia salina* Immobilization[17] Procedure.** Brine shrimp (*A. salina*) eggs obtained from a local pet store were hatched in a 3% (w/v) aqueous NaCl solution and the live larvae harvested using a pipet. Each test compound (0.025 mL of a solution containing 1 mg/mL of the compound in acetonitrile) was placed separately (in duplicate) in a well of the first column of a 96-well (8 rows X 12 columns) culture plate and diluted with an additional 0.025 mL of acetonitrile. Half of this solution was transferred to the corresponding well of the next column, diluted with 0.025 mL acetonitrile and the 2-fold serial dilution process repeated across the row. The brine shrimp were added to each well in 0.20 mL of brine. The IC_{100} values were determined after 6 h at room temperature using a microscope.

***C. elegans* Binding[4] Assay.** *C. elegans* membrane preparations were obtained by washing *C. elegans* worms with 50 mM HEPES buffer, pH 7.4. The worms were homogenized and centrifuged at 1000*g*. The pellet was discarded and the supernatent centrifuged at 28 000*g*. The resulting pellet was resuspended in HEPES buffer to approximately 12.5 μg protein/mL. The membrane preparations (1.0 mL) were incubated with [^{3}H]-IVM at 22 °C for 45 min in the presence and absence of new AVM analogs. After termination of incubation by rapid filtration over Whatman GF/B filters and rinsing with cold HEPES buffer containing 0.25% Triton X-100, the filters were placed in vials containing Aquasol II and the radioactivity was determined by scintillation counting.

***C. elegans* Motility[4] Assay.** *C. elegans* worms were rinsed with Kreb's bicarbonate buffer (124 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 1.3 mM $MgSO_4$, pH 7.4, at 22 °C), washed twice by centrifugation at 500*g* for 2 min, and then resuspended in Krebs buffer. Aliquots (50 μL) containing approximately 100 worms were placed in test tubes. The compounds were dissolved in dimethyl sulfoxide and added to the test tubes containing the worms in a final volume of 500 μL 1% DMSO. After 16 h at 22 °C, the motility was determined using a microscope. The percentage of immotile worms was then determined at several concentrations for each derivative.

13-*O*-[(2-Bromoethoxy)methyl)]-22,23-dihydro-5-*O*-(*tert*-butyldimethylsilyl)avermectin B_{1a} Aglycon (3). To 200 mg of alcohol 2 (258 μmol) in 2 mL of CH_2Cl_2 at 0 °C was added 91 mg of CBr_4 (275 μmol) followed by 68 mg of Ph_3P (258 μmol). The solution was stirred for 30 min at 0 °C and then at room temperature for 3 h. The crude was purified without workup by flash chromatography on silica gel using 85:15 hexanes/EtOAc as eluant to yield 140 mg 3 (65%) as a colorless oil. TLC: 3:1 hexanes/EtOAc, R_f = 0.76. MS: calcd for $C_{43}H_{69}BrO_9Si$ + Li, 843.4059; found (M + Li), 843.4074.

Partial data ^{1}H NMR (300 MHz, $CDCl_3$, δ): 5.60–5.80 (m, 3 H), 5.30 (s, 1 H), 5.25 (m, 1 H), 5.12 (br d, 1 H), 4.68 (AB, J = 7.1 Hz, 2 H), 4.60 (AB, J = 14.4 Hz, 2 H), 4.41 (br s, 1 H), 4.10

(s, 1 H), 4.03 (m, 1 H), 3.93 (m, 1 H), 3.80 (m, 2 H), 3.64 (m, 1 H), 3.64 (t, J = 6.0 Hz, 2 H), 3.30 (br s, 1 H), 3.16 (br d, J = 4.5 Hz, 1 H), 2.51 (m, 1 H), 2.27 (m, 2 H), 1.96 (dd, J_1 = 3.6 Hz, J_2 = 11.8 Hz, 1 H), 1.77 (s, 3 H), 1.56 (s, 3 H), 1.12 (d, J = 6.9 Hz, 3 H), 0.90 (s, 9 H), 0.83 (d, J = 6.7 Hz, 3 H), 0.76 (d, J = 5.3 Hz, 3 H), 0.11 (s, 6 H).

13-*O*-[(2-Bromoethoxy)methoxy]-22,23-dihydroavermectin B_{1a} Aglycon (4). To 140 mg of silyl ether 3 (167 μmol) in 14 mL of methanol at room temperature was added 14 mg of *p*-toluenesulfonic acid (80 μmol). The solution was stirred for 1.5 h, then poured into 10 mL of saturated $NaHCO_3$, extracted with EtOAc, washed with brine, and dried ($MgSO_4$). The solution was filtered, concentrated under reduced pressure, and purified by flash chromatography on silica gel to yield 81 mg (67%) 4 as a colorless oil. TLC: 3:2 hexanes/EtOAc, R_f = 0.36. MS: calcd for $C_{37}H_{55}O_9$ + Li, 729.3190; found (M + Li), 729.3226. Partial data ^1H NMR (300 MHz, $CDCl_3$, δ): 5.60–5.82 (m, 3 H), 5.34 (s, 1 H), 5.26 (m, 1 H), 5.10 (br d, J = 6.7 Hz, 1 H), 4.64 (m, 6 H), 4.24 (br t, J = 6.4 Hz, 1 H), 4.06 (m, 2 H), 3.91 (m, 2 H), 3.77 (m, 2 H), 3.63 (m, 1 H), 3.44 (t, J = 5.9 Hz, 2 H), 3.21 (br s, 1 H), 3.15 (br d, J = 7.4 Hz, 1 H), 2.50 (m, 1 H), 2.41 (d, J = 8.3 Hz, 1 H), 1.93 (dd, J_1 = 3.4 Hz, J_2 = 12.1 Hz, 1 H), 1.82 (s, 3 H), 1.47 (s, 3 H), 1.10 (d, J = 7.0 Hz, 3 H), 0.91 (t, J = 7.2 Hz, 3 H), 0.80 (d, J = 6.6 Hz, 3 H), 0.74 (br s, 3 H).

13-*O*-[[2-(Ethyleneamino)ethoxy]methoxy]-22,23-dihydroavermectin B_{1a} Aglycon (5). To 75 mg of bromide 4 (103 μmol) in 2 mL of CH_2Cl_2 at room temperature was added 250 μL of freshly distilled ethyleneamine. The solution was stirred at room temperature for 48 h and then concentrated under reduced pressure at ambient temperature. The crude was purified by flash chromatography on silica gel using gradient elution (1:2:97 to 1:6:93 NH_4OH/MeOH/$CHCl_3$) to yield 47 mg (67%) of 5 as a white powder. TLC: 1:4:95 NH_4OH/MeOH/$CHCl_3$, R_f = 0.25. MS: calcd for $C_{39}H_{59}NO_9$, 685.4190; found (M + H), 686.4289. Partial data ^1H NMR (300 MHz, $CDCl_3$, δ): 5.61–5.83 (m, 3 H), 5.38 (s, 1 H), 5.27 (m, 1 H), 5.15 (br d, 1 H), 4.54–4.72 (m, 3 H), 4.25 (br d, J = 5.5 Hz, 1 H), 3.23 (br s, 1 H), 3.16 (br s, 1 H), 2.40 (t, J = 5.5 Hz, 2 H), 1.94 (dd, J_1 = 3.2 Hz, J_2 = 12.1 Hz, 1 H), 1.88 (s, 3 H), 1.75 (s, 2 H), 1.50 (s, 3 H), 1.17 (br s, 5 H), 0.96 (t, J = 7.3 Hz, 3 H), 0.85 (d, J = 6.6 Hz, 3 H), 0.80 (br s, 3 H).

13-*O*-[[2-(Ethyleneamino)ethoxy]methoxy]-22,23-dihydro-5α-tritioavermectin B_{1a} Aglycon (6). To 40 mg of aziridine 5 (58 μmol) in 2 mL of EtOAc at room temperature was added 400 mg of MnO_2. The solution was stirred for 45 min, filtered through Celite, and concentrated under reduce pressure. The crude product was purified by flash chromatography on silica gel using 1:4:95 NH_4OH/MeOH/$CHCl_3$ as eluant to yield 29 mg (73%) 5-oxo derivative as a white powder. TLC: 1:4:95 NH_4OH/MeOH/$CHCl_3$, R_f = 0.43. MS: calcd for $C_{39}H_{57}NO_9$, 683.4033; found (M + H), 684.4122. To 500 μg of 5-oxo derivative (0.7 μmol) in 100 μL of MeOH at 0 °C was added 10 μL of NaB^3H_4 (1 μmol, 3.4 μg/μL in methanol, 60 Ci/mmol). The ice bath was removed and the solution stirred for 10 min. The crude was placed directly on a preparative TLC plate (250 μm, silica gel) and developed with 1:4:95 NH_4OH/MeOH/$CHCl_3$. Product 6 was identical by TLC to 5 and its specific activity was 14 Ci/mmol. TLC: 1:4:95 NH_4OH/MeOH/$CHCl_3$, R_f = 0.25.

4″α-[[[(9-Fluorenyloxy)carbonyl]-β-alanyl]amino]-4″-deoxy-5-*O*-(*tert*-butyldimethylsilyl)avermectin B_{1a}. To 750 mg of amine 7 (761 μmol) dissolved in 10 mL of CH_2Cl_2 at 0 °C was added sequentially 260 mg of Fmoc-β-AlaOH (837 μmol), 113 mg of HOBT (837 μmol), and 172 mg of DCC (837 μmol). The solution was stirred at 0 °C for 1 h and then at room temperature overnight. The solution was diluted with 20 mL of Et_2O, filtered, and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel using 3:1 EtOAc/hexanes as eluant to yield 575 mg of product (59%) as a pale yellow solid. HPLC: 9:1 MeOH/H_2O, 2 mL/min, t_R = 16.7 min. TLC: 3:1 EtOAc/hexanes, R_f = 0.32. MS: calcd for $C_{72}H_{102}N_2O_{16}Si$ + Li, 1285.7159; found (M + Li), 1285.7176. Partial data ^1H NMR (300 MHz, $CDCl_3$, δ): 7.76 (t, J = 7.4 Hz, 2 H), 7.57 (t, J = 6.4 Hz, 2 H), 7.25–7.41 (m, 4 H), 5.65–5.77 (m, 4 H), 5.53 (dd, J_1 = 2.4 Hz, J_2 = 9.9 Hz, 1 H), 5.47 (m, 1 H), 5.40 (s, 1 H), 5.35 (m, 1 H), 5.31 (s, 1 H), 5.12 (br d, J = 8.8 Hz, 1 H), 4.98 (br s, 1 H), 4.77 (s, 1 H), 4.60 (AB, J_{AB} = 16.1 Hz, 2 H), 4.57 (m, 1 H), 4.40 (m, 2 H), 4.12 (s, 1 H), 3.45 (s, 3 H), 3.38 (s, 1 H), 3.23 (t, J = 8.9 Hz, 1 H), 3.11 (s, 3 H), 1.77 (s, 3 H), 1.48 (s, 3 H), 1.26 (d, J = 6.0 Hz, 3 H), 1.17 (d, J = 5.9 Hz, 3 H), 1.11 (d, J = 7.0 Hz, 3 H), 0.91 (s, 9 H), 0.11 (s, 6 H).

4″α-(β-Alanylamino)-4″-deoxy-5-*O*-(*tert*-butyldimethylsilyl)avermectin B_{1a}. To 500 mg of Fmoc-protected amine (391 μmol) placed in 10 mL of CH_2Cl_2 at 0 °C was added 2 mL of freshly distilled piperidine. The solution was stirred at 0 °C for 15 min and then at room temperature for 2 h. The solution was concentrated at ambient temperature under reduced pressure to a viscous oil and then lyophilized from benzene. The crude was purified by flash chromatography on silica gel using gradient elution (1:4:95 to 1:9:90 NH_4OH/MeOH/$CHCl_3$) to yield 391 mg (91%) free amine as a pale yellow solid. HPLC: 9:1 MeOH/H_2O, 2 mL/min, t_R = 12.4 min. TLC: EtOAc, R_f = 0.18. MS: calcd for $C_{57}H_{92}N_2O_{14}Si$ + Li, 1063.6477; found (M + Li), 1063.6471. Partial data ^1H NMR (300 MHz, $CDCl_3$, δ): 6.97 (br d, J = 6.8 Hz, 1 H), 5.61–5.79 (m, 4 H), 5.50 (dd, J_1 = 3.1 Hz, J_2 = 9.1 Hz, 1 H), 5.39 (s, 1 H), 5.32 (m, 1 H), 5.28 (s, 1 H), 4.95 (br s, 1 H), 4.73 (s, 1 H), 4.59 (AB, J_{AB} = 13.5 Hz, 2 H), 4.39 (s, 1 H), 3.88 (s, 1 H), 3.39 (s, 3 H), 3.30 (s, 3 H), 1.76 (s, 3 H), 1.46 (s, 3 H), 1.21 (d, J = 6.1 Hz, 3 H), 1.17 (d, J = 5.8 Hz, 3 H), 1.11 (d, J = 6.9 Hz, 3 H), 0.88 (s, 9 H), 0.49 (s, 6 H).

4″α-(β-Alanylamino)-4″-deoxyavermectin B_{1a} (8). To 350 mg of silyl ether (317 μmol) in 4 mL of THF at room temperature was added 1 mL of HF·pyr (25 g of HF·pyr, 10 mL of pyridine, 25 mL of THF) and the solution stirred for 12 h. The solution was diluted with 20 mL of Et_2O and poured into 20 mL of water, and the layers were separated. Both layers were neutralized with saturated $NaHCO_3$, the aqueous layer was extracted with Et_2O, and the combined organic layer was washed with brine and dried ($MgSO_4$). The solution was filtered and concentrated under reduced pressure, and the crude purified by flash chromatography on silica gel using 1:9:90 NH_4OH/MeOH/$CHCl_3$ as eluant to yield 256 mg (82%) 8 as a pale yellow solid. TLC: 1:9:90 NH_4OH/MeOH/$CHCl_3$, R_f = 0.13. MS: calcd for $C_{51}H_{78}N_2O_{14}$ + Li, 949.5614; found (M + Li), 949.5632. Partial data ^1H NMR (300 MHz, 2:1 $CDCl_3$/CD_3OD, δ): 5.61–5.86 (m, 4 H), 5.45 (dd, J_1 = 2.7 Hz, J_2 = 9.1 Hz, 1 H), 5.45 (br s, 2 H), 5.10 (m, 1 H), 4.98 (br s, 1 H), 5.72 (s, 1 H), 4.19 (br s, 1 H), 3.89 (s, 1 H), 3.36 (s, 3 H), 3.18 (s, 3 H), 3.15 (s, 1 H), 3.12 (t, J = 8.5 Hz, 1 H), 2.86 (m, 2 H), 2.51 (m, 1 H), 1.74 (s, 3 H), 1.45 (s, 3 H), 1.19 (d, J = 6.1 Hz, 3 H), 1.16 (br s, 6 H).

4″α-(Biotinyl-β-alanylamino)-4″-deoxyavermectin B_{1a} (10). To 4.0 mg of amine 8 (4.2 μmol) in 500 μL of 1:1 THF/CH_2Cl_2 at room temperature was added sequentially 3.5 mg of NHS–biotin (10 μmol) and 3 μL of DIEA (15 μmol). After 1 hr at room temperature, the reaction crude was purified without workup by flash chromatography on silica gel using 1:9:90 NH_4OH/MeOH/$CHCl_3$ as eluant to yield 4.4 mg (88%) of 10 as a white powder. HPLC: 8:2 MeOH/H_2O, 2 mL/min, t_R = 7.5 min. TLC: 1:9:90 NH_4OH/MeOH/$CHCl_3$, R_f = 0.32. MS: calcd for $C_{61}H_{92}N_4O_{16}S$ + Li, 1173.6389; found (M + Li), 1173.6431. Partial data ^1H NMR (400 MHz, $CDCl_3$, δ): 7.04 (t, J = 5.8 Hz, 1 H), 6.41 (s, 1 H), 6.33 (d, J = 8.9 Hz, 1 H), 5.76 (m, 1 H), 5.73 (m, 3 H), 5.52 (dd, J_1 = 2.5 Hz, J_2 = 9.9 Hz, 1 H), 5.41 (br s, 2 H), 5.39 (m, 1 H), 5.28 (s, 1 H), 4.95 (br s, 1 H), 4.74 (s, 1 H), 4.66 (br s, 2 H), 4.49 (br s, 1 H), 4.30 (br s, 2 H), 3.94 (d, J = 6.2 Hz, 1 H), 3.91 (s, 1 H), 3.42 (s, 3 H), 3.30 (s, 3 H), 3.27 (br s, 1 H), 3.20 (t, J = 8.8 Hz, 1 H), 2.88 (dd, J_1 = 4.9 Hz, J_2 = 12.7 Hz, 1 H), 2.71 (d, J = 12.6 Hz, 1 H), 1.85 (s, 3 H), 1.47 (s, 3 H), 1.22 (d, J = 6.0 Hz, 3 H), 1.15 (d, J = 6.1 Hz, 3 H), 1.14 (d, J = 6.8 Hz, 3 H).

4″α-[[(ε-Aminocaproyl)-β-alanyl]amino]-4″-deoxyavermectin B_{1a} (11). To 500 mg of 8 (395 μmol) placed in 10 mL of CH_2Cl_2 at room temperature was added 184 mg of [(9-fluorenyloxy)carbonyl]-ε-caproic acid (520 μmol) and 70 mg of HOBT (520 μmol). To this was added 107 mg of DCC (520 μmol). After 2 h, the reaction was filtered through a 1.5-in. plug of silica gel using 96:4 EtOAc/MeOH as eluant and concentrated under reduced pressure. The crude product (460 mg) was dissolved in 10 mL of CH_2Cl_2 at room temperature to which was added 2 mL of freshly distilled piperidine. After 2 h at room temperature, the solution was concentrated under reduced pressure at ambient temperature to a viscous oil which was lyophilized from benzene. The product was purified by flash chromatography on silica gel using 1:9:90 NH_4OH/MeOH/$CHCl_3$ as eluant to yield 310 mg 11 (82%) as a pale yellow powder. TLC: 1:9:90 NH_4OH/MeOH/

$CHCl_3$, R_f = 0.08. MS: calcd for $C_{57}H_{89}N_3O_{15}$ + Li, 1056.6372; found (M + Li), 1056.6393. Partial data ^1H NMR (300 MHz, $CDCl_3$, δ): 6.51 (br s, 1 H), 6.00 (br d, J = 8.8 Hz, 1 H), 5.83 (br s, 1 H) 5.62–5.80 (m, 4 H), 5.52 (dd, J_1 = 2.4 Hz, J_2 = 9.9 Hz, 1 H), 5.40 (br s, 2 H), 5.36 (m, 1 H), 4.98 (br s, 1 H), 4.74 (s, 1 H), 4.66 (s, 2 H), 4.27 (d, J = 5.5 Hz, 1 H), 3.94 (d, J = 6.2 Hz, 1 H), 3.91 (s, 1 H), 3.42 (s, 3 H), 3.31 (s, 3 H), 3.27 (br s, 1 H), 3.20 (t, J = 8.9 Hz, 1 H), 2.89 (br s, 3 H), 2.68 (br s, 2 H), 1.85 (s, 3 H), 1.47 (s, 3 H), 1.22 (d, J = 6.0 Hz, 3 H), 1.16 (d, J = 5.5 Hz, 3 H), 1.14 (d, J = 6.0 Hz, 3 H).

4″α-[[[ε-(Biotinylamino)caproyl]-β-alanyl]amino]-4″-deoxyavermectin B_{1a} (12). To 24 mg of amine 11 (23 μmol) placed in 1 mL of CH_2Cl_2 at room temperature was added 8 mg of BiotinOSu (23 μmol) followed by 6 μL of DIEA (30 μmol). After 10 min at room temperature, the crude was purified without workup by flash chromatography on silica gel using 1:9:90 $NH_4OH/MeOH/CHCl_3$ as eluant to yield 23 mg (79%) 12 as a white powder. HPLC: 8:2 $MeOH/H_2O$, 2 mL/min, t_R = 6.9 min. TLC: 1:9:90 $NH_4OH/MeOH/CHCl_3$, R_f = 0.15. MS: calcd for $C_{67}H_{103}N_5O_{17}S$ + Li, 1288.7230; found (M + Li), 1288.7234. Partial data ^1H NMR (400 MHz, $CDCl_3$, δ): 7.03 (t, J = 5.5 Hz, 1 H), 6.86 (d, J = 8.7 Hz, 1 H), 6.52 (t, J = 5.5 Hz, 1 H), 6.37 (s, 1 H), 5.84 (br s, 1 H), 5.72 (m, 3 H), 5.52 (dd, J_1 = 2.3 Hz, J_2 = 9.7 Hz, 1 H), 5.48 (s, 1 H), 5.40 (s, 2 H), 5.39 (m, 1 H), 4.99 (br s, 1 H), 4.73 (s, 1 H), 4.66 (br s, 2 H), 4.48 (br s, 1 H), 4.31 (m, 2 H), 3.93 (d, J = 6.2 Hz, 1 H), 3.90 (s, 1 H), 3.41 (s, 3 H), 3.29 (s, 3 H), 3.26 (br s, 1 H), 2.88 (dd, J_1 = 4.9 Hz, J_2 = 12.8 Hz, 1 H), 2.72 (d, J = 12.8 Hz, 1 H), 1.84 (s, 3 H), 1.47 (s, 3 H), 1.20 (d, J = 5.0 Hz, 3 H), 1.14 (t, J = 6.2 Hz, 3 H).

4″α-[[[ε-[[β-[[2-(Biotinylamino)ethyl]dithio]propionyl]amino]caproyl]-β-alanyl]amino]-4″-deoxyavermectin B_{1a} (13). To 24 mg of amine 11 (23 μmol) placed in 1 mL of CH_2Cl_2 at room temperature was added 14 mg of NHS-SS-biotin (23 μmol) followed by 6 μL of DIEA (30 μmol). After 40 min at room temperature, the reaction crude was purified without workup by flash chromatography on silica gel using 1:9:90 $NH_4OH/MeOH/CHCl_3$ as eluant to yield 24 mg (73%) of 13 as a white powder. HPLC: 8:2 $MeOH/H_2O$, 2 mL/min, t_R = 16.8 min. TLC: 1:4:95 $NH_4OH/MeOH/CHCl_3$, R_f = 0.09. MS: calcd for $C_{72}H_{112}N_6O_{18}S_3$ + Li, 1451.7356; found (M + Li), 1451.7348. Partial data ^1H NMR (400 MHz, $CDCl_3$, δ): 7.01 (t, J = 5.9 Hz, 1 H), 6.89 (t, J = 5.7 Hz, 1 H), 6.82 (t, J = 5.6 Hz, 1 H), 6.67 (d, J = 9.0 Hz, 1 H), 6.08 (s, 1 H), 5.85 (br s, 1 H), 5.72 (m, 3 H), 5.52 (dd, J_1 = 2.5 Hz, J_2 = 9.8 Hz, 1 H), 5.38 (m, 3 H), 4.98 (br d, J = 6.2 Hz, 1 H), 4.74 (br s, 1 H), 4.66 (br s, 2 H), 4.49 (br t, J = 6.1 Hz, 1 H), 4.31 (m, 2 H), 4.22 (s, 1 H), 3.94 (d, J = 6.1 Hz, 1 H), 3.91 (s, 1 H), 3.41 (s, 3 H), 3.30 (s, 3 H), 2.98 (t, J = 6.8 Hz, 2 H), 2.89 (dd, J_1 = 4.8 Hz, J_2 = 12.7 Hz, 1 H), 2.83 (t, J = 6.3 Hz, 2 H), 2.73 (d, J = 12.6 Hz, 1 H), 2.59 (t, J = 6.8 Hz, 2 H), 2.14 (t, J = 7.2 Hz, 2 H), 1.85 (s, 3 H), 1.47 (s, 3 H), 1.21 (d, J = 6.1 Hz, 3 H), 1.15 (d, J = 5.5 Hz, 3 H), 1.14 (d, J = 6.3 Hz, 3 H).

4″α-[[[ε-[(p-Azidosalicyloyl)amino]caproyl]amino]-β-alanyl]amino]-4″-deoxyavermectin B_{1a} (14). To 24 mg of amine 11 (23 μmol) placed in 1 mL of CH_2Cl_2 at room temperature was added 7 mg of azidosalycidoOSu (23 μmol) followed by 6 μL of DIEA (30 μmol). After 10 min at room temperature, the crude reaction mixture was purified without workup by flash chromatography on silica gel using 1:9:90 $NH_4OH/MeOH/CHCl_3$ as eluant to yield 21 mg (76%) of 14 as a white powder. HPLC: 8:2 $MeOH/H_2O$, 2 mL/min, t_R = 16.8 min. TLC: 1:4:95 $NH_4OH/MeOH/CHCl_3$, R_f = 0.11. MS: calcd for $C_{64}H_{92}N_6O_{17}$ + Li, 1223.6679; found (M + Li), 1223.6694. Partial data ^1H NMR (400 MHz, $CDCl_3$, δ): 7.53 (d, J = 8.6 Hz, 1 H), 7.01 (br t, J = 4.9 Hz, 1 H), 6.59 (d, J = 2.3 Hz, 1 H), 6.46 (m, 2 H), 5.85 (m, 1 H), 5.72 (m, 3 H), 5.57 (d, J = 9.1 Hz, 1 H), 5.52 (dd, J_1 = 2.6 Hz, J_2 = 9.9 Hz, 1 H), 5.40 (br s, 2 H), 5.38 (m, 1 H), 4.96 (br d, J = 6.2 Hz, 1 H), 4.74 (s, 1 H), 4.66 (br s, 2 H), 4.27 (d, J = 5.5 Hz, 1 H), 3.94 (d, J = 6.3 Hz, 1 H), 3.91 (s, 1 H), 3.41 (s, 3 H), 3.31 (s, 3 H), 3.27 (br s, 1 H), 3.20 (t, J = 9.0 Hz, 1 H), 3.06 (m, 2 H), 2.38 (t, J = 5.8 Hz, 2 H), 2.17 (t, J = 6.9 Hz, 2 H), 2.00 (dd, J_1 = 3.3 Hz, J_2 = 12.2 Hz, 1 H), 1.85 (s, 3 H), 1.47 (s, 3 H), 1.21 (d, J = 6.2 Hz, 3 H), 1.16 (d, J = 6.2 Hz, 3 H), 1.13 (d, J = 6.9 Hz, 3 H).

[^{125}I]4″α-[[[ε-[(p-Azido-o-iodosalicyloyl)amino]caproyl]amino]-β-alanyl]amino]-4″-deoxyavermectin B_{1a} (15). To 50 μL of freshly prepared Chloramine-T (3 μg/μL in acetone) at room temperature was added 2 μg of azido AVM 14 in 1 μL of DMSO.[42] Carrier-free ^{125}I (5 mCi in 15 μL of 0.01 M sodium phosphate, pH 8.5) was then added and the reaction maintained at room temperature for 5 min. The acetone was removed under a stream of N_2 and replaced with 50 μL of methanol. The azido AVM 14 and ^{125}I-azido AVM 15 were resolved by reverse-phase HPLC (C18 Vydac column, 4.6-mm × 25-cm, 84:16 methanol/water, isocratic, t_R's of 9.4 and 10.5 min, respectively). The ^{125}I-azido AVM 15 was obtained in 25% yield and was essentially carrier free with a specific activity of 1700 Ci/mmol.

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