ANTHELMINTIC ACTIVITY OF DIOXAPYRROLOMYCIN

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Dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, and piericidin C_2 produced by UC 11065 were evaluated as anthelmintics. Assays used to examine these compounds included effects on the free-living nematode *Caenorhabditis elegans*, ability to clear target nematodes (*Haemonchus contortus* and *Trichostrongylus colubriformis*) from jirds, and clearance of *Haemonchus contortus* from lambs. A crude extract of UC 11065 containing dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, and piericidin C_2 was active against *C. elegans* and against *H. contortus* in the jird. Purified and/or synthetic samples of dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, and piericidin C_2 were tested in the jird model; only dioxapyrrolomycin exhibited appreciable activity against *H. contortus* ($\geqq$90.9% clearance at 0.33 mg/jird), while none of the compounds showed appreciable activity against *T. colubriformis*. Dioxapyrrolomycin cleared 99.9% of *H. contortus* from lambs at 12.5 mg/kg. An *in vitro* migration study using susceptible and closantel-resistant *H. contortus* showed there is cross-resistance between dioxapyrrolomycin and closantel. Dioxapyrrolomycin appears to be a narrow-spectrum anthelmintic which works through a closantel-like mode-of-action.

In the course of screening for novel metabolites active against brine shrimp, *Artemia salina*, a soil actinomycete (#90413, subsequently UC 11065) was found which produces a mixture of dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, and piericidin C_2. The principal components produced during fermentation were evaluated in a battery of anthelmintic assays, and one of these components, dioxapyrrolomycin, was found to have appreciable anthelmintic activity. Results of those studies are reported herein.

Materials and Methods

Producing Organism

The dioxapyrrolomycin-producing, actinomycete culture was isolated from soil obtained in Michigan, U.S.A. It was given accession number UC 11065 in The Upjohn Culture Collection. The culture was stored as 4 mm diameter agar plugs of vegetative growth (medium ISP-2, Difco) in a liquid nitrogen vapor phase freezer.

Fermentation Conditions

All fermentations were carried out in 500-ml wide-mouth Erlenmeyer flasks containing 100 ml media on a rotary shaker (250 rpm, 3.8 cm throw) at 28°C. The source of inoculum consisted of four 4 mm diameter agar plugs (medium ISP-2, Difco) containing well-sporulated mycelial growth. This was placed in a seed medium (25 g/liter Cerelose, 25 g/liter Pharmamedia, pH 7.2 using ammonium hydroxide), and the seed culture was incubated for 72 hours. The seed culture served as inoculum (5% v/v rate) for shake

flasks containing a production medium (20 g/liter D-galactose, 20 g/liter dextrin, 10 g/liter Soytone (Difco), 2.5 g/liter Solulys (Roquette), 2 g/liter $(NH_4)_2SO_4$, 2 g/liter $CaCO_3$, pH 7.2 using potassium hydroxide). Tap water was used in preparation of seed and production media. Production cultures were incubated for 96 hours prior to harvesting.

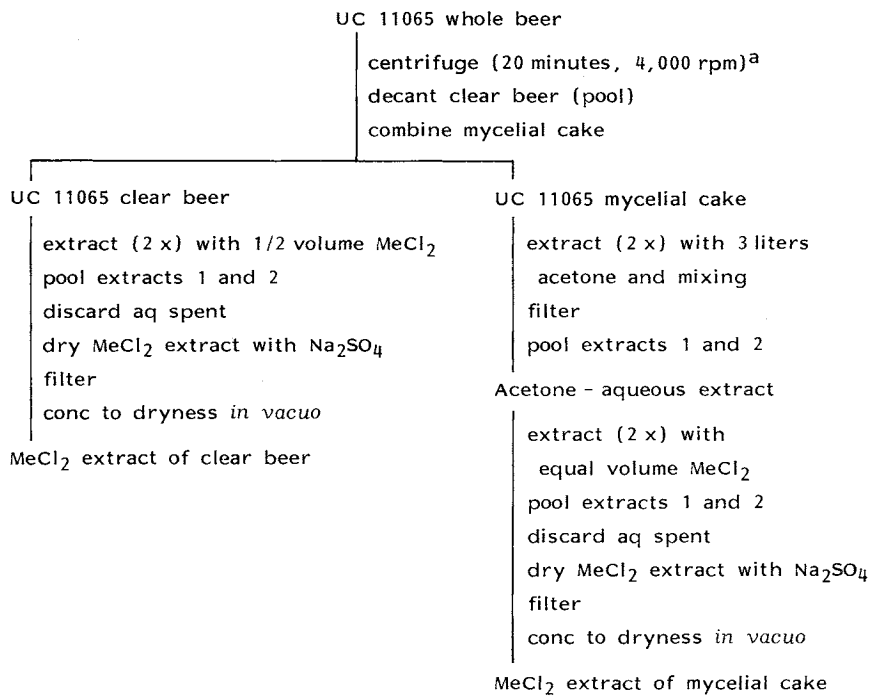
Isolation and Purification (Scheme 1)

Silica Column Chromatography: Open column silica gel chromatography was performed using 25 g silica gel (mesh 70~230) per g of Preparation A (combined methylene chloride extracts from the clear beer and mycelial cake) to be purified. The silica column was poured and equilibrated with two bed volumes *n*-hexane. Preparation A was absorbed onto two times its weight of silica gel and loaded onto the head of the column. The silica gel column was then eluted with two bed volumes *n*-hexane, followed by four bed volumes of 85% hexane: 15% EtOAc, and finished with two bed volumes EtOAc. Silica column fractions were collected in 20 ml volumes. Twenty μl column fraction aliquots were then tested for bioactivity in the *A. salina* assay.

Artemia salina Assay: Twenty μl of sample was placed in a 250 μl assay well. To this was added 160 μl of saline and 20 μl of an *A. salina* suspension, resulting in a total volume of 200 μl with ~20 to 30 *A. salina* per well. The assay well was then covered and allowed to stand for 6 hours after which each well was evaluated by visualization under a binocular microscope. Scores from 0 (no dead or impaired organisms) to 5 (no live organisms) were recorded. Wells scoring 3 or higher were considered active.

High Performance Liquid Chromatography: Analytical HPLC for sample analysis and peak identification for bioactive fractions of Preparation A was performed on a Hewlett-Packard (HP) 1090A with Diode Array Detector and PC work station. Separation was performed on an HP 2.1 mm × 200 mm ODS RP column preceded by an HP ODS guard column. Elution was achieved with isocratic 65% acetonitrile: 35% NH_4OAc (pH 4.0) for 5 minutes followed by a 20 minute linear gradient to 100% acetonitrile. Column temperature was maintained at 65°C and column eluate was monitored by UV detection at 240 nm. Mobile phase flow rate was maintained at 0.5 ml/minute throughout the entire

Scheme 1.



[a] Whole beer dispensed into 1-liter plastic centrifuge bottles. $MeCl_2$: Methylene chloride.

separation. Sample injections of 1~25 μl were performed automatically by the HP 1090A HPLC.

Preparative HPLC was performed on a Waters Prep LC 3000 with a variable wavelength UV/VIS detector and Waters 745B integrator. Separation was performed on three Waters 25 mm × 100 mm C-18 Radial Pak cartridges in series preceded by a Waters Radial Pak C-18 guard column. Elution was achieved with isocratic 60% acetonitrile: 40% NH_4OAc (pH 4.0) for 40 minutes. The column was maintained at ambient temperature with column eluate monitored by UV detection at 254 nm. Mobile phase flow rate was maintained at 34.2 ml/minute throughout the separation. Column fractions were collected automatically by an Isco Foxy fraction collector using peak detection (Isco 2150 Peak Separator) and 1 minute peak collection time windows. Sample solutions were pumped directly onto the head of the column with maximum injection volumes of 50 ml.

Chemicals and Solvents: All organic solvents used for HPLC and open column liquid chromatography were of HPLC grade or higher quality. Ammonium acetate buffer was prepared from Mallinkrodt AR grade NH_4OAc and pH adjusted with Mallinkrodt AR grade glacial acetic acid. Buffer solutions were passed through 0.2 μm nylon 66 filters prior to use with chromatographic systems.

Synthetic Chemistry

2-(3′,5′-Dichloro-2′-methoxybenzoyl)pyrrole (**1**): Ethyl bromide (16.1 g, 147 mmol) was added to a stirred mixture of Mg turnings (3.5 g, 147 mmol) in diethyl ether (200 ml) and refluxed for 1 hour. A solution of freshly distilled pyrrole (9.0 g, 134 mmol) was added and refluxed for an additional 1 hour. The reaction was cooled in an ice bath and a solution of 3,5-dichloro-2-methoxy benzoyl chloride (134 mmol), triethyl amine (14.8 g, 147 mmol) in tetrahydrofuran (100 ml) was added dropwise. After addition was complete, the ice bath was removed and the reaction was stirred for an additional 2 hours at room temperature. The reaction was poured onto a mixture of ice (100 g) and concentrated hydrochloric acid (10 ml) and stirred for 15 minutes. The aqueous layer was extracted with chloroform (2 × 300 ml), dried over sodium sulfate and filtered. The filtrate was purified *via* Si-60 column chromatography using a mobile phase gradient of 0~15% ethyl acetate in hexane to give **1** (10.8 g, 30.0%) isolated as an off white solid (mp 111~112°C), (literature 114~115°C)[1]. ^1H NMR (300 MHz, $CDCl_3$) δ 10.0 (1H, br s), 7.51 (1H, d, J=2.6 Hz), 7.34 (1H, d, J=2.6 Hz), 7.20~7.18 (1H, m), 6.69~6.68 (1H, m), 6.33~6.30 (1H, m), 3.85 (3H, s).

2,3-Dichloro-5-(3′,5′-dichloro-2′-hydroxybenzoyl)pyrrole, pyrrolomycin C (**3**): Sulfuryl chloride (8.8 ml, 110 mmol) was added dropwise to a solution of **1** (11.8 g, 44 mmol) at 0°C in methylene chloride (100 ml). After addition was complete (*ca.* 1 hour), the reaction was warmed to 20°C and stirred for an additional 1.25 hours. The reaction was concentrated to a semi-solid under reduced pressure, reconstituted in benzene (120 ml), cooled to 0°C, and anhydrous aluminum chloride (14.6 g, 109.7 mmol) was added. The reaction mixture was stirred an additional 2 hours at room temperature and poured onto ice (200 g) and acidified with concentrated hydrochloric acid (10 ml) to pH 3. The aqueous phase was extracted with methylene chloride (3 × 200 ml), dried over anhydrous sodium sulfate and filtered. The residue was purified *via* trituration with ethyl acetate to give **3** (7.3 g, 51%) isolated as a yellow solid (mp 220°C), (literature 220~221°C)[1]. ^1H NMR (300 MHz, DMSO-d_6) δ 13.45 (1H, br s), 10.49 (1H, br s), 7.73 (1H, d, J=2.4 Hz), 7.44 (1H, d, J=2.4 Hz), 6.85 (1H, s), ^{13}C NMR (75 MHz, DMSO-d_6) δ 180.9, 150.6, 131.5, 128.6, 127.7, 127.6, 123.1, 122.9, 121.3, 118.5, 110.1, EI-MS (M^+) m/z 323.

2,3,4-Trichloro-5-(3′,5′-dichloro-2′-hydroxybenzoyl)pyrrole, pyrrolomycin D (**4**). A solution of sulfuryl chloride (1.7 g, 12.3 mmol) in methylene chloride (5 ml) was added to a stirred solution of **1** (1.0 g, 3.7 mmol) at 0°C in methylene chloride (25 ml) at 0°C. The reaction mixture was immediately warmed to room temperature and stirred an additional 5 hours. The reaction mixture was concentrated under reduced pressure and triturated with hexane to give a solid (200 mg, 15% crude yield) consisting of trichlorinated pyrrole and dichlorinated pyrrole isomers in a 4 : 1 ratio. Anhydrous aluminum chloride (150 mg, 1.1 mmol) was added to a suspension of this material in benzene (5 ml) at 0°C. The reaction was stirred an additional 1.5 hours at 20°C and then was poured onto a mixture of ice (50 g) and concentrated hydrochloric acid (4 ml). The aqueous portion was extracted with methylene chloride (2 × 50 ml) and dried over anhydrous sodium sulfate. The filtrate was purified by Si-60 column chromatography using a mobile phase gradient of 0~80% ethyl acetate in hexane to give **4** isolated as a yellow solid (75 mg, 6% yield), mp 192~194°C (literature 195~198°C)[1]. ^1H NMR (300 MHz, CD_3OD) δ 7.56 (1H, d, J=2.6 Hz), 7.33 (1H, d, J=2.6 Hz), EI-MS (M^+) m/z 357.

Anthelmintic Assays

Caenorhabditis elegans Assay: The free-living nematode *C. elegans* was used as a primary anthelmintic assay. This assay has been described by SIMPKIN and COLES[2]. Activity at 50 ppm moves a test material to *in vivo* evaluation.

Haemonchus contortus/*Trichostrongylus colubriformis*/Jird Assay: The jird model was used as an initial *in vivo* anthelmintic assay. This model utilized jirds infected with two important target parasites of ruminants, *H. contortus* and *T. colubriformis* (anthelmintic-sensitive or -resistant worms can be used). Initially, activity was assessed only against *H. contortus* as described by CONDER *et al.*[3], while follow-up studies examined activity against both parasites using the techniques outlined by CONDER *et al.*[4]. A test material was considered highly active if it produced a clearance of $\geqq 90\%$ at $\leqq 1$ mg/jird for either parasite. Cross-resistance with benzimidazoles, ivermectin, and levamisole was examined as described for levamisole by CONDER *et al.*[5].

Haemonchus contortus/Sheep Assay: Lambs monospecifically infected with *H. contortus* was used to evaluate materials. Purpose bred, helminth-free lambs were procured. These lambs were treated with ivermectin (0.2 mg/kg, subcutaneously), vaccinated for sore mouth, and placed in a single, community pen. Three weeks later each lamb was treated with levamisole hydrochloride (8.0 mg/kg, *per os*). Two weeks after treatment with levamisole, all lambs were inoculated *per os* with $\sim$7,500 infective larvae of *H. contortus*. Rectal fecal samples were taken from each lamb 1 to 3 days prior to infection, and these were examined using the double centrifugation technique to verify that the animals were free of trichostrongyles prior to infection. On day 32$\sim$34 postinoculation (PI), a rectal fecal sample from each lamb was examined again using the McMaster counting chamber technique to verify infection; those animals which did not exhibit suitable infection were dropped from the study. Remaining lambs were treated *per os* on day 35 PI; 4$\sim$5 animals received vehicle only. Prior to administration, test materials were prepared in a manner suitable for the substance being examined. All lambs were monitored for toxic signs following treatment. Lambs were killed 7 days after treatment (day 42 PI), and the abomasum was ligated and removed from each animal. Each abomasum was longitudinally sectioned and the contents rinsed into an 80 mesh sieve. Sieve contents were collected in individual containers and fixed in formol-alcohol. Later each sample was transferred to a 1,000-ml graduated cylinder and the volume was brought to 400$\sim$1,000 ml with tap water. The total number of worms in a 10% aliquot was determined. If no worms were found in the 10% aliquot, the entire sample was examined. Total worm number/lamb and percentage clearance for each treatment were calculated. Percentage clearance was determined according to the following formula:

$$\text{Percentage clearance} = [(\text{Mean number of worms recovered from vehicle control lambs} - \text{number of worms recovered from treated lamb})/\text{mean number of worms recovered from vehicle control lambs}] \times 100.$$

A substance was considered highly active if its clearance was $\geqq 90\%$ and moderately active if its clearance was $\geqq 70$ but $< 90\%$.

Haemonchus contortus Migration Assay: Fourth-stage larvae of closantel-resistant (H41) and -susceptible (McM) strains of *H. contortus* were exposed to drugs over 2 or 3 days and viability assessed by counting the number of larvae either passing through or retained by a 50 μm aperture nylon mesh at 37°C. Concentrations required to inhibit migration of 50% of the worms (MIC_{50}) were calculated from a best fit curve to a plot of logit of response to log concentration[6].

Results

The UC 11065 fermentation was processed according to Scheme 1. Final structure elucidation was carried out for each compound using IR, UV, MS, and NMR techniques. Elemental analysis and ORD spectroscopy methods were used for the analysis of dioxapyrrolomycin. In all cases, results were in agreement with the published data of dioxapyrrolomycin[7,8], pyrrolomycin C[1,9], and piericidin C_2[10,11].

Synthesis of pyrrolomycin C was accomplished by using a modification of a procedure by KOYAMA *et al.*[1]. Pyrrole magnesium bromide was reacted with 3,5-dichloro-2-methoxy benzoyl chloride to give **1**.

Chlorination of this intermediate with SO_2Cl_2 at 0°C followed by demethylation with $AlCl_3$ produced pyrrolomycin C in 51% yield. Our results are in contrast to work by KOYAMA *et al.*[1], who report the production of pyrrolomycin C and it's trichlorinated analog pyrrolomycin D in 18 and 16% yields, respectively, when chlorination is performed at 20°C. We have found that yields of pyrrolomycin C can generally be improved with chlorination at 0°C. The trichlorinated analog, pyrrolomycin D, was obtained in low yield (6%) with SO_2Cl_2 when chlorination was performed at 20°C.

A crude extract of UC 11065 containing several components, including dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, and piericidin C_2, was examined for anthelmintic activity against the

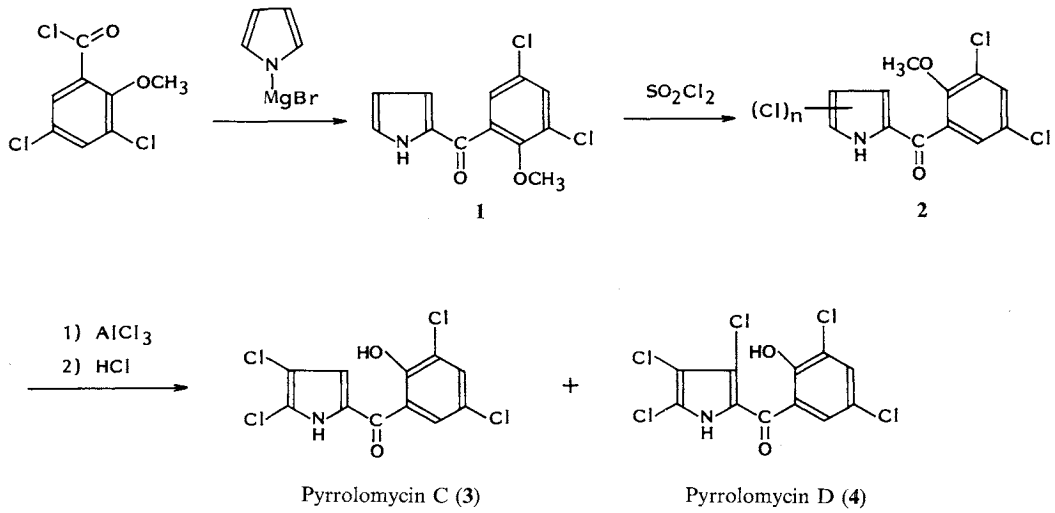


Table 1. Percentage clearance of *Haemonchus contortus* and *Trichostrongylus colubriformis* from jirds inoculated *per os* with ~1,000 exsheathed, infective larvae of each parasite, treated *per os* with dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, or piericidin C_2 on day 10 postinoculation (PI) and necropsied on day 13 PI.

Compound	Purity	Dose (mg/jird)	n (survived to necropsy)	Percentage clearance	
				H. contortus	*T. colubriformis*
Dioxapyrrolomycin	~100%	0.33	3 (1)	90.9	41.5
		0.33	3 (3)	100	41.5
		0.11	3 (3)	100	0
		0.037	3 (3)	96.4	17.2
		0.012	3 (3)	45.8	48.8
Pyrrolomycin C	100%	2.5	3 (0)	—	—
		1.0	3 (3)	48.4	55.4
		1.0	3 (3)	24.1	33.5
		0.92	3 (3)	32.3	56.2
Pyrrolomycin D	100%	2.75	3 (0)	—	—
		1.0	3 (0)	—	—
		0.33	3 (0)	—	—
		0.11	3 (3)	0	0
Piericidin C_2	95%*	2.84	3 (0)	—	—
		0.947	3 (2)	62.1	N.D.**
		1.06	3 (2)	29.5	0

* Pyrrolomycin C makes up the majority of the remainder.
** N.D. = Not done.

free-living nematode *C. elegans* and was found to be highly active at 50 ppm. Based on this response against *C. elegans*, the crude preparation was evaluated against a target parasite, *H. contortus*, in the jird. At 2.5 mg/jird, >99% clearance of parasites was achieved in treated jirds compared to vehicle treated animals and no toxic signs were observed.

A program was initiated to isolate and identify the active component(s) produced by UC 11065, Table 1 shows results obtained against *H. contortus* and a second target parasite, *T. colubriformis* in the jird model for the 4 main components of the crude preparation described above, *i.e.* dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, and piericidin C_2. Although pyrrolomycin C and piericidin C_2 cleared $\leqq$62.1% of *H. contortus* at ~1.0 mg/jird and pyrrolomycin D cleared 0% of *H. contortus* at 0.11 mg/jird (it killed all jirds given a higher dose), dioxapyrrolomycin exhibited strong activity ($\geqq$90.9% clearance at 0.33 mg/jird) against this parasite, which could readily explain results obtained with the crude preparation. It also is worth noting that although neither dioxapyrrolomycin nor pyrrolomycin C are highly active against *T. colubriformis*, both have a hint of activity against this parasite (41.5% clearance at 0.33 mg/jird and 33.5~56.2% clearance at ~1.0 mg/jird, respectively).

Dioxapyrrolomycin and pyrrolomycin C were examined in sheep monospecifically infected with *H. contortus*. Data from these studies are shown in Table 2. Dioxapyrrolomycin was highly active (percentage clearance of 92.2) at 1.56 mg/kg, while synthetic pyrrolomycin C was essentially inactive at 50 mg/kg.

Jirds infected with ivermectin/benzimidazole- or levamisole/benzimidazole-resistant *H. contortus* were used to examine whether dioxapyrrolomycin has cross-resistance with any of the 3 major classes of broad-spectrum anthelmintics. Data presented in Table 3 show that dioxapyrrolomycin has

Table 2. Percentage clearance of *Haemonchus contortus* from lambs monospecifically inoculated *per os* with ~7,500 infective larvae of the parasite, treated *per os* with dioxapyrrolomycin or pyrrolomycin C on day 35 postinoculation (PI), and necropsied on day 42 PI.

Compound	Purity (%)	Dose (mg/kg)	Percentage clearance
Dioxapyrrolomycin	~100	12.5	100
		6.25	99.9
		3.125	99.7
			~99.9*
		1.56	92.2
		0.78	44.0
Pyrrolomycin C	100	50.0	21.9

* Range indicates multiple studies.

Table 3. Percentage clearance of susceptible, levamisole/benzimidazole-resistant, or ivermectin/benzimidazole-resistant *Haemonchus contortus* from jirds inoculated *per os* with ~1,000 exsheathed, infective larvae of a particular strain of the parasite, treated *per os* with dioxapyrrolomycin, levamisole hydrochloride, albendazole, or ivermectin on day 10 postinoculation (PI), and necropsied on day 13 PI.

Compound	Dose (mg/jird)	Percentage clearance		
		Susceptible	Levamisole/ benzimidazole-resistant	Ivermectin/ benzimidazole-resistant
Dioxapyrrolomycin*	0.11	95.8	98.6	92.7
Levamisole***	0.4	~95.0	51.7	96.4
Albendazole***	0.075	~95.0	36.2	N.D.**
Ivermectin***	0.005	~95.0	98.6	18.7

* 95% pure; pyrrolomycin C makes up the majority of the remainder.

** N.D. = not done.

*** Levamisole (Sigma Chemical Co.), Albendazole (SmithKline Beecham), Ivermectin (Merck & Co.).

approximately equal efficacy against the resistant and susceptible strains studied. The *in vitro* migration assay showed that dioxapyrrolomycin is ~6 times less active against closantel-resistant *H. contortus* than against susceptible worms (Table 4).

Table 4. Minimum inhibitory concentration (MIC_{50}) of dioxapyrrolomycin, closantel, or levamisole for migration *in vitro* of 50% of closantel-susceptible (McM) and -resistant (H41) strains of *Haemonchus contortus*.

Drug	MIC_{50} (μg/ml)	
	McM	H41
Dioxapyrrolomycin	0.219	3.83
Closantel*	8.06	22.1
Levamisole*	0.195	0.195

* Closantel (SmithKline Beecham), Levamisole (Sigma Chemical Co.).

Discussion

Dioxapyrrolomycin has activity of potential utility against the important ruminant parasite, *H. contortus*. Dioxapyrrolomycin and pyrrolomycin C appear to have some, albeit very weak, activity against a second ruminant parasite, *T. colubriformis*, in the jird model, suggesting that manipulation (synthetically or semisynthetically) of the template provided by the "pyrrolomycin" class may provide a novel broad-spectrum anthelmintic. Although lack of cross-resistance with the 3 major classes of broad-spectrum anthelmintics has been demonstrated for dioxapyrrolomycin, migration studies *in vitro* have shown that dioxapyrrolomycin is cross-resistant with closantel (thought to uncouple electron-transport-associated phosphorylation[12]), the primary narrow-spectrum drug used to control *H. contortus* in the field. Based on these data, dioxapyrrolomycin appears to be a narrow-spectrum anthelmintic which works through a closantel-like mode-of-action.

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