# Cyclotides: Natural, Circular Plant Peptides that Possess Significant Activity against Gastrointestinal Nematode Parasites of Sheep[†]

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ABSTRACT: The cyclotides are a novel family of backbone-cyclized cystine-knot containing peptides from plants that have been shown to possess insecticidal activity against *Helicoverpa* larvae, an important pest of corn and cotton. In the current study, we investigated the in vitro effects of the cyclotides on the viability of egg, larval, and adult life stages of two species of economically important gastrointestinal nematode parasites of livestock, *Hemonchus contortus* and *Trichostrongylus colubriformis*. The cyclotides showed significant activity in inhibiting development of nematode larvae and motility of adult worms. Activities were comparable to some currently used anthelmintic compounds in these in vitro assay systems. A series of alanine mutants of the prototypic cyclotide kalata B1 were assayed against larvae to determine regions of the peptide responsible for activity. It was observed that anthelmintic activity was dramatically reduced as a consequence of the mutation of a large number of residues that are found clustered on one surface. Activities toward larvae were equivalent in the naturally occurring L-isomer of kalata B1 and a synthetic all-D-isomer, indicating that there is no chiral requirement for anthelmintic activity. The clustering of important residues and the lack of chiral selectivity further support the proposed mode of action of the cyclotides, which involves a membrane-based interaction rather than an interaction at a specific receptor. The cyclotide-induced leakage of a fluorescent dye from vesicles used as a model membrane mimetic further confirms the membrane lytic ability of cyclotides. The relative potency of kalata B1 and kalata B2 in causing membrane leakage is consistent with the order of their anthelmintic activity. These results demonstrate that the cyclotides show potential for use in the control of gastrointestinal nematode parasites.

The cyclotides are a fascinating family of circular, disulfide-rich peptides discovered in plants from the Violaceae and Rubiaceae families (*1*). They incorporate a cystine knot motif (*2*) that interlocks three conserved disulfide bonds. Together with the head-to-tail cyclized backbone, this structural motif imparts exceptional stability to the cyclotides. Figure 1 shows a sequence alignment of representative members of the cyclotide family, highlighting the conserved Cys residues. Six backbone loops, formed by the segments between adjacent cysteines, may be thought of as the functional epitopes protruding from the cystine knot core.

The cyclotides exhibit a diverse range of biological activities, including anti-HIV (*3*), antimicrobial (*4*), neurotensin antagonist (*5*), cytotoxic (*6, 7*), antifouling (*8*), and hemolytic (*9*) properties, although their natural role is postulated to be in plant defense (*10–13*). In a study of their insecticidal activity, the prototypic cyclotide, kalata B1[1] was incorporated into an artificial diet for *Helicoverpa punctigera* larvae and had a dramatic effect on growth and development of the larvae (*14*): none developed beyond the first instar

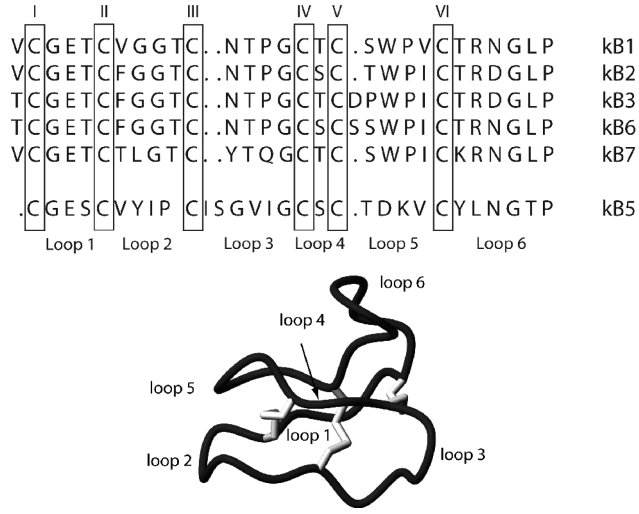


FIGURE 1: Sequence alignment of selected peptides from the cyclotide family. The loops numbered 1−6 represent the backbone segments between adjacent cysteines (labeled with Roman numerals I−VI). For convenience, the cyclotide sequences appear as a linear representation but are head-to-tail cyclized in nature. Schematic representation of kalata B1 showing the cyclic backbone (dark ribbon) and three disulfide bonds (light ribbons).

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[1] Abbreviations: kB1−7, kalata B1 to kalata B7; L1−L3, first to third instar larvae; EHA, egg hatch assay; LDA, larval development assay.

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and all were approximately 3% of the weight of the control larvae (fifth instar) fed the same diet but without added cyclotide. Increased mortality was also observed, but it was not clear whether this was a direct result of the ingestion of the peptide or whether mortality was associated with starvation, resulting from a possible antifeedant effect of the cyclotide. In a subsequent study, kalata B1 and kalata B2 were fed to *H. armigera* and were shown to retard growth and cause mortality in this related insect species also (*15*). A recent microscopic examination of *H. armigera* midguts showed that cyclotides produce marked disruption of the microvilli, blebbing, swelling, and ultimately rupture of the cells of the gut epithelium (*12*). Given the importance of these pest species and the potential for similar activities to apply to other pests, it was of interest to investigate if the cyclotides might also be active against other agricultural pests.

Gastrointestinal worms are a major health concern in livestock production systems worldwide. The trichostrongylids are the most harmful nematode parasites of small ruminants. Within the superfamily Trichostrongyloidea, several species contribute to parasitism in sheep; the three species of major concern are *Hemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis*. *H. contortus*, or as they are commonly called "barber pole worm", are a major cause of ill health and economic loss in sheep production. This nematode is a bloodsucking parasite that pierces the lining of the abomasum, causing blood plasma and protein loss to the host through ingestion as well as hemorrhage into the abomasum. In heavy infections, populations of *H. contortus* may remove up to 10% of the host's blood per day, leading to rapid death. *H. contortus* infection commonly causes anemia and the loss of serum albumin depletes the host's protein supply and results in edema (bottle jaw). In addition to losses through mortality, major economic losses are attributed to reduced feed efficiency, slow rate of weight gain, poor reproductive efficiency, lowered production of wool and meat, and costs of labor and drugs associated with control.

*T. circumcincta* and *T. colubriformis* are not blood feeders, but rather feed within the mucosa of the abomasum or within the epithelium of the small intestine, respectively. These two species often live in the same sheep. Diseased sheep are underweight, produce less meat and wool as nutrients are diverted from production to fight infection. *T. circumcincta* also reduces the available nutrients to the sheep by depressing food intake. They damage the abomasal wall and cause plasma proteins to leak from the blood into the gut and in some cases will cause diarrhea (scouring). *T. colubriformis* damage the lining of the small intestine and sheep with heavy infections of this parasite rapidly lose condition, develop scours, dehydrate, and may die, while animals with fewer worms are unthrifty and daggy. The associated scours soil the fleece and encourage females of the sheep blowfly (*Lucilia cuprina*) to lay eggs leading to infestations of this damaging ectoparasite.

Currently, the control of nematode parasites is almost entirely based on the use of anthelmintics directed at the adult stages of the parasite. However, the prevalence of anthelmintic resistance has dramatically increased worldwide (*16, 17*). There is now widespread resistance in *H. contortus* to the most recently introduced group of drugs, the macrocyclic lactones (*18, 19*), while *T. colubriformis* and *T. circumcincta* are also showing resistance to this chemical group (*20*). This resistance is expected to undermine the viability of the sheep industry worldwide unless alternative control agents can be developed.

The aim of the current study was to determine whether the cyclotides are toxic toward the gastrointestinal parasites of sheep and, hence, to assess their potential for nematode control. In addition, through the use of an extensive panel of alanine point mutations, the mechanism of action of the cyclotides was investigated. Because many of the other known activities of the cyclotides appear to be associated with membrane disruption, the findings of the current study are of broader importance to understanding the mechanism of action of the cyclotides in general.

## MATERIALS AND METHODS

*Isolation.* Native kalata B1 (kB1) and a range of natural variants (kalata B2, kB2; kalata B3, kB3; kalata B5, kB5; kalata B6, kB6; kalata B7, kB7) were isolated from the above ground parts of *Oldenlandia affinis*. Fresh plant material (500 g) was ground and extracted with 50/50 (v/v) DCM/MeOH, and the crude extract was partially purified by RP flash chromatography, yielding a fraction containing predominantly cyclotides (5 g). This sample was purified further by preparative RP-HPLC to yield pure kalata B1 (125 mg) together with smaller amounts of the other natural variants, as described previously (*1*).

*Synthesis of Mutants.* Alanine mutants and the D-isomer of kalata B1 were synthesized using solid phase methods, as described previously (*21, 22*). Briefly, the peptides were assembled using manual solid-phase peptide synthesis with Boc chemistry. Amino acids were added to the resin using HBTU with in situ neutralization. Cleavage of the peptides from the resin was achieved using hydrogen fluoride with *p*-cresol and *p*-thiocresol as scavengers (9:0.8:0.2 v/v HF/cresol/thiocresol). The crude reduced peptides were purified on a Phenomenex $C_{18}$ column. Gradients of 0.05% aqueous TFA and 90% acetonitrile, 0.045% TFA were employed with a flow rate of 8 mL/min, and the eluent was monitored at 230 nm. These conditions were used in subsequent purification steps. Cyclization and oxidation of the mutants was performed by dissolving the peptides in 50/50 (v/v) 0.1 M ammonium bicarbonate (pH 8.5)/isopropanol (0.5 mg/mL) with reduced glutathione added (final concentration 1 mM). The mixture was stirred at room temperature for 24 h and then purified by RP-HPLC. Correctly folded mutants were identified by their late elution under reverse phase conditions, and $^1$H NMR spectra confirmed the folded states.

*Preparation of Eggs.* The eggs of *H. contortus* and *T. colubriformis* were obtained from the McMaster Laboratory, CSIRO Livestock Industries, Armidale, NSW. The McMaster isolates of *H. contortus* and *T. colubriformis*, both susceptible isolates with no history of exposure to anthelmintics, were utilized in this study. The nematode eggs were obtained from the faeces of infected animals and isolated by passing the faeces through a series of fine sieves (250 $\mu$m and 75 $\mu$m) followed by centrifugation in a stepwise sucrose gradient (10, 25, and 40% sucrose). The eggs were recovered from the interface between the 10 and 25% sucrose layers, washed over a 25 $\mu$m sieve with water to remove residual sucrose

and diluted to obtain 50−60 eggs per 30 μL after the addition of amphotericin B (37.5 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL).

*Egg Hatch Assay (EHA) and Larval Development Assay (LDA).* EHAs and LDAs were conducted in a microtiter plate format, as described previously (*23*). Briefly, 200 μL of 2% agar was deposited into each well of a 96-well microtiter plate. After this had solidified, 30 μL of nematode egg solution was aliquotted into each well. Water (10 μL) was added to the control wells, while 10 μL of cyclotide solution (varying concentrations) was aliquotted into the treatment wells. The final concentration range was 1.0−62.5 μg/mL (0.3−21.5 μM) for LDAs and 104−833 μg/mL (35−288 μM) for EHAs. For each assay, triplicate wells were examined at each concentration. The plates were incubated overnight at 28 °C and the unhatched eggs and L1 larvae present in each well were counted. The percentage inhibition of egg hatching was estimated by comparing the percentage hatch at each cyclotide concentration with the percentage hatch in the control wells.

For the LDA, plates were set up as described above, incubated overnight at 28 °C, and then 20 μL of growth medium was added to each well. The growth medium consisted of Earle's salt solution (10% v/v), yeast extract (1% w/v), sodium bicarbonate (1 mM), and saline solution (0.9% sodium chloride, w/v) (*24*). The nematodes were allowed to feed and develop for four days and then killed using Lugol's iodine solution and scored for the number of fully developed infective stage larvae (L3) present in each well. Each treatment was conducted in at least triplicate and controls for water or 20% ethanol were included in each assay as required.

*Adult Motility Assays.* The effects of cyclotides on the adult stages of *H. contortus* were assessed by observing the degree of motility shown by worms over a period of exposure to the peptides in vitro. The adult worm recovery and culture methods were as described by Kotze and McClure (2001) (*25*). Briefly, adult worms were recovered from sheep abomasa (approximately 6−10 weeks post infection) by manual picking from the gut contents. They were placed in culture medium (RPMI-1640, HEPES buffer, glucose, bovine serum, antibiotics, and fungicide) for several hours, and then groups of six were placed in separate tubes in 0.5 mL of culture medium. The only significant change from the previously described method (*25*) was the inclusion of 20% newborn bovine serum in the culture medium, and the subsequent maintenance of the worms in an atmosphere of 20% $CO_2$, 5% $O_2$, and 75% $N_2$ (compared to 5% $CO_2$ in air for the earlier study). The cyclotides were added to the tubes at various concentrations and the tubes were kept at 37 °C. Each compound was tested in triplicate. At 24 h intervals, the worms were observed and their degree of motility was scored. The assay tubes were placed onto a warm tray and tubes were held individually near a light for assessment of motility. Each tube was swirled to thoroughly disturb the nematodes and was scored according to the degree of motility shown by the worms using the scoring system described by O'Grady and Kotze (*26*). Briefly, the worms were scored as the following: 3, most individuals showing significant smooth sinusoidal motion, similar to motion at the start of the culture period; 2, significant movement shown by a small number of individuals, at least one individual able to move in a normal sinusoidal fashion; 1, only very limited movement in a small number of individuals, no sinusoidal motion; and 0, no movement.

*Calculations and Statistical Analysis.* The percentage inhibition of egg hatch and percentage inhibition of larval development were calculated using the following equation (*27*):

$$\% \text{ inhibition} = (A - B)/A \times 100$$

where $A$ = the number of newly hatched larvae (EHA) or the number of larvae that had developed into the L3 stage (LDA) in control incubations and $B$ = the number of newly hatched larvae (EHA) or L3 larvae (LDA) in incubations containing different concentrations of cyclotides.

To provide parameters for comparison of the test compounds, we calculated the concentration at which 50% of eggs failed to hatch (in the EHA) or 50% and 99% of larvae failed to achieve full development to L3 larvae (in the LDA) as $IC_{50}$ and/or $IC_{99}$ values. The ovicidal and/or larvicidal $IC_{50}$ and $IC_{99}$ values were calculated using nonlinear regression (sigmoidal dose−response, GraphPad Prism).

*Vesicle Preparation.* The standard buffer was composed of 10 mM HEPES (*N*-(2-hydroxy-ethyl)-piperazine-*N*′-2-ethanesulfonic acid, from Sigma-Aldrich), 107 mM NaCl, 1 mM $Na_2EDTA \cdot 2H_2O$, and approximately 5−6 mM NaOH to adjust the pH to 7.4. The fluorescent dye 5-carboxyfluorescein (CF) was purchased from Sigma-Aldrich. An aqueous stock was prepared containing 50 mM CF in 10 mM HEPES, 107 mM NaCl, and 1 mM $Na_2EDTA \cdot 2H_2O$ and was adjusted using NaOH to pH 7.4. The concentration of CF was determined using an extinction coefficient of 72000 $M^{-1}$ $cm^{-1}$ at 492 nm. The dye solution was stored at 4 °C in the dark. The lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids. A total of 20 mg of lipid was dissolved in 2 mL of chloroform (Sigma-Aldrich) separately and then dried under a stream of nitrogen gas and then vacuum desiccated overnight. Lipids were stored under nitrogen at −20 °C to prevent oxidation. Lipid concentrations were determined by the Stewart assay (*28*).

Dry lipid was dispersed in the standard buffer (in the presence of dye) by vortex mixing, followed by sonication at 37 °C under nitrogen for 45 min. The liposome solutions were then sized 19 times by extrusion through two stacked Nucleopore polycarbonate membranes (Avanti Polar Lipids). Vesicles loaded with CF were separated from nonentrapped dye on a Sephadex G-50 column (Amersham Pharmacia Biotech), using an isoosmolar buffer (10 mM HEPES, 5 mM NaOH, 1 mM EDTA, and 107 mM NaCl, pH 7.4).

*Vesicle Leakage Experiments.* Fluorescence emission spectra were measured between 460−600 nm on a Perkin-Elmer luminescence spectrometer with a scan speed of 140 nm/min. An excitation wavelength of 480 nm and a quartz cuvette with an optical pathwidth of 4 mm was used. The excitation and emission slit-widths were set to 4 nm. Experiments were performed at 25 °C. The initial fluorescence signal, $F_o$ (when vesicles are intact, i.e., no leakage), was measured and each run was started by the addition of 50 μL of a peptide solution. Fluorescence spectra were measured every minute for 20 min before the addition of 10% Triton X-100 solution, whereby the vesicles are completely perturbed giving rise to the formation of mixed

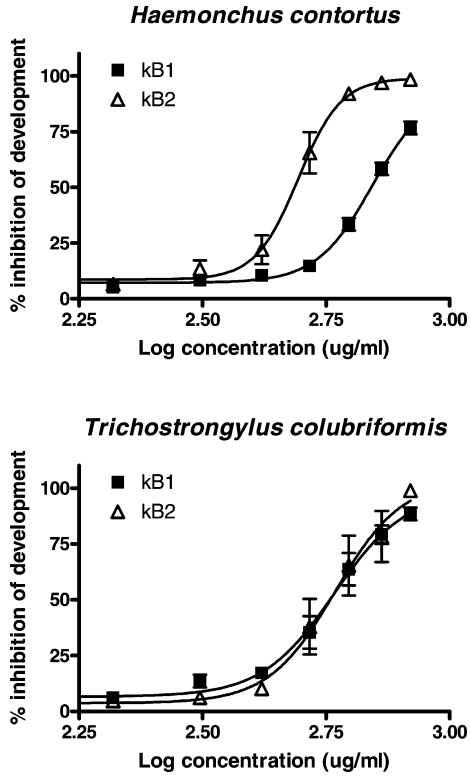


FIGURE 2: Effect of the cyclotides, kalata B1 and kalata B2, on the hatching of *H. contortus* and *T. colubriformis* eggs in vitro. Each data point represents the mean ± SE ($n = 2$ experiments, with three assay wells at each of a range of cyclotide concentrations).

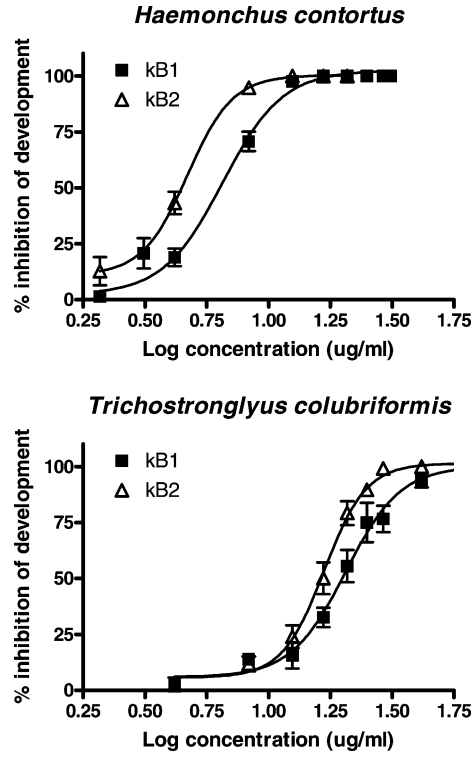


FIGURE 3: Effect of the cyclotides, kalata B1 and kalata B2, on the development of eggs to third instar (L3) larvae of *H. contortus* and *T. colubriformis*. Each data point represents the mean ± SE ($n = 2$ experiments, with three assay wells at each of a range of cyclotide concentrations).

Table 1: Effect of Cyclotides on the Hatching of Eggs of *H. contortus* and *T. colubriformis* In Vitro[a]

| cyclotide | *H. contortus* $IC_{50}$ (μg/mL) | *T. colubriformis* $IC_{50}$ (μg/mL) |
|---|---|---|
| kalata B1 | 699 (642−760) | 584 (527−646) |
| kalata B2 | 494 (472−518) | 587 (505−682) |

[a] Values given are the $IC_{50}$ with 95% confidence intervals, $n = 2$ experiments (data pooled), with three assay wells at each of a range of cyclotide concentrations.

micelles and yielding a fluorescence signal of maximum intensity, $F_x$. Control experiments were conducted with the addition of MilliQ $H_2O$ only.

## RESULTS

*Effects of Cyclotides on the Hatching of Nematode Eggs.* Egg hatch on agar in microtiter plate wells was very efficient, with at least 90% of the eggs hatching for both nematode species in control wells. kB1 and kB2 inhibited the hatching of *H. contortus* and *T. colubriformis* eggs in a concentration-dependent manner (Figure 2, Table 1). Kalata B2 was slightly more potent than kB1 in inhibition of egg hatching of *H. contortus*, while both peptides were equally potent toward *T. colubriformis*. $IC_{99}$ values could not be calculated for inhibition of egg hatching, as even at the highest cyclotide concentration, 100% inhibition was not observed. At concentrations >40 μg/mL, a larvicidal effect was apparent, as all larvae that had emerged from eggs were dead.

*Effects of Cyclotides on the Development of Nematode Eggs to L3 Larvae.* Development of larvae from the newly hatched L1 stage to the fully developed L3 stage was >90% in the absence of cyclotides, indicating that the different diluents used in this assay, including up to 20% ethanol, did not interfere with the natural development of larvae. The cyclotides caused a concentration-dependent response in the LDA with the two nematode species. Typical dose−response curves for the inhibition of development of eggs to L3 larvae by wild-type kalata B1 (kB1) and the natural variant kB2 are shown in Figure 3. $IC_{50}$ and $IC_{99}$ values for the natural variants tested are presented in Table 2 and were significantly lower (more potent) than observed in the EHA.

A range of cyclotides was tested, including the prototypic cyclotide kalata B1, as well as kalata B2, B3, B5, B6, and B7 isolated from *Oldenlandia affinis*. The natural variants were found to have differing larvicidal activities, with kalata B6 the most potent of the variants tested having an $IC_{50}$ of 2.6 μg/mL for *H. contortus* and 7.9 μg/mL for *T. colubriformis* kB1 and kB2 showed comparable activities (around 5−6 μg/mL for *H. contortus* and 17−21 μg/mL for *T. colubriformis*), while kB7 had markedly reduced activity (ca. 17−19 μg/mL for *H. contortus* and *T. colubriformis*) and kB3 showed very little activity ($IC_{50}$ could not be calculated). kB5 was only tested at three concentrations (owing to limited material) within the dose−response curve for kB1 and showed comparable activity at these concentrations. The percentage inhibition of development resulting from treatment with kB3 was <5% (*H. contortus*) at 12.5 μg/mL and only 18% (*T. colubriformis*) at 20 μg/mL, concentrations at which ~90% inhibition was observed for kB1 and 100% inhibition for kB6. Typically, *T. colubriformis* were 2-fold less sensitive to a given cyclotide than *H. contortus*.

*Effects of Cyclotides on the Motility of Adult Hemonchus contortus.* Observations on worm motility in control assays (no peptide) showed that *H. contortus* adults cultured for

Table 2: Effects of Cyclotides on Development of *H. contortus* and *T. colubriformis* Larvae In Vitro[a]

| | *H. contortus* | | *T. colubriformis* | |
|---|---|---|---|---|
| cyclotide | $IC_{50}$ ($\mu$g/mL) | $IC_{99}$ ($\mu$g/mL) | $IC_{50}$ ($\mu$g/mL) | $IC_{99}$ ($\mu$g/mL) |
| kalata B1 | 6.5 (6.4−6.7, $n = 8$) | 22 (21−24, $n = 8$) | 21 (20−21, $n = 7$) | 75 (70−81, $n = 7$) |
| kalata B2 | 4.7 (4.69−4.72, $n = 2$) | 16 (14−18, $n = 2$) | 17 (16−17, $n = 3$) | 44 (42−47, $n = 3$) |
| kalata B6 | 2.6 (2.61−2.63, $n = 2$) | 5.1 (4.7−5.6, $n = 2$) | 7.9 (7.7−8.2, $n = 2$) | 36 (33−39, $n = 2$) |
| kalata B7 | 19 (18−20, $n = 1$) | 39 (33−45, $n = 1$) | 17 (17−18, $n = 2$) | 40 (34−47, $n = 2$) |

[a] Values given are the $IC_{50}$ or $IC_{99}$ with 95% confidence intervals, $n = 1-8$ experiments (data pooled), with three assay wells at each of a range of cyclotide concentrations.

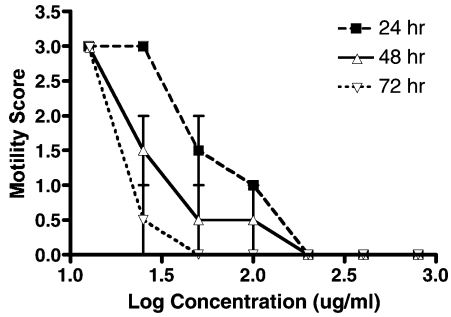


FIGURE 4: Effect of kalata B1 on motility of *H. contortus* adults in vitro at 24 h (dashed line), 48 h (solid line), and 72 h (dotted line). Each data point represents mean ± SE ($n = 2$ separate assay tubes).

72 h were able to maintain a degree of motility comparable to that seen at the commencement of the assay period. The mean motility score at 72 h was 2.8 ± 0.2 (mean ± SE, $n = 5$ separate control assay tubes).

The effects on motility of the cyclotides are shown in Figure 4. A range of concentrations of kB1 was tested and scoring was conducted after 24, 48 and 72 h. After 24 h, motility scores of 0 were recorded at the two highest concentrations tested and the intermediate concentrations responded in a dose-dependent manner. After 48 h, there was a significant reduction in motility and, after 72 h, motility scores of 0 were recorded for all concentrations tested except the lowest two (<25 $\mu$g/mL). It was also noted that while in the control assay tubes, a large proportion of laid eggs hatched to L1 larvae after 24 h, very few eggs hatched in the kB1 treated assay tubes above 400 $\mu$g/mL and nearly all newly hatched larvae were dead above kB1 concentrations of 50 $\mu$g/mL.

*Effects of Alanine Mutants on the Development of Nematode Eggs to L3 Larvae.* A complete suite of alanine mutants of kalata B1 was synthesized and oxidatively folded (*29*). Of the 23 possible non-Cys mutants, 21 folded to native-like structures, with $^1$H NMR utilized to monitor chemical shift differences between the mutants and kB1. The two mutants that failed to fold under the standard conditions were [W19A]-kB1 and [P20A]-kB1, and the W19 and P20 residues are postulated to play an important role in the stabilization of the hydrophobic patch during the folding process. The folded mutants were tested using LDAs with both *H. contortus* and *T. colubriformis* at two doses corresponding to high and intermediate concentrations within the range used for kB1. A synthetic all-D-isomer of kB1 was also tested.

Figure 5 shows the survival rates for the various mutants at the two doses: 20 and 10 $\mu$g/mL for *H. contortus* and at 40 and 20 $\mu$g/mL for *T. colubriformis*. Each dose was examined in triplicate and the error bars represent the

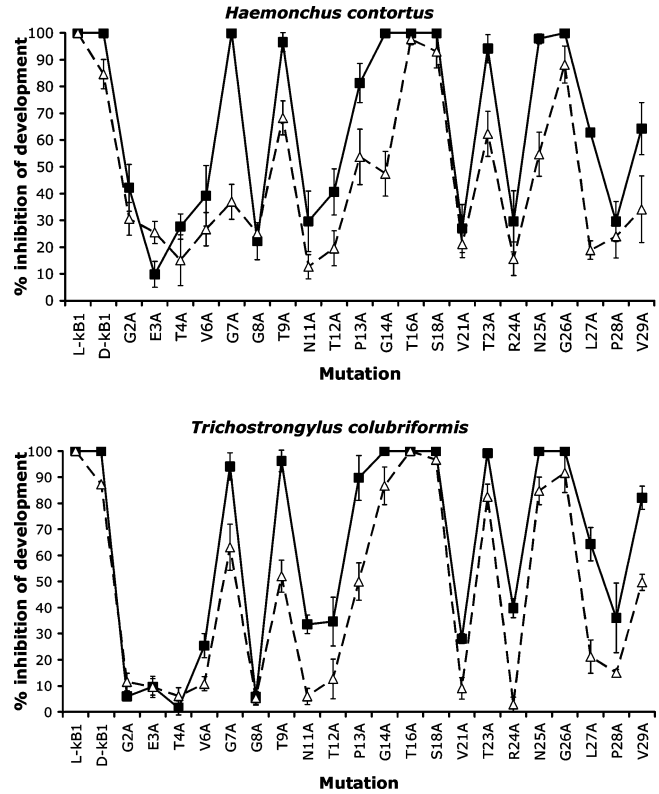


FIGURE 5: Effect of the alanine mutants of kalata B1 on the development of eggs to third instar (L3) larvae of *H. contortus* (A) and *T. colubriformis* (B). The various cyclotides were used at two concentrations: 20 $\mu$g/mL (solid line) and 10 $\mu$g/mL (Δ, dashed line) for *H. contortus* and 40 $\mu$g/mL (solid line) and 20 $\mu$g/mL (Δ, dashed line) for *T. colubriformis*. Each dose was conducted in triplicate and the error bars represent the standard deviation per treatment. The first two points represent wild-type (L-kB1) and the all-D-kB1 analogue and show close to 100% inhibition of larval development at the concentrations tested.

standard deviation per treatment. At these doses, the wild-type kB1 and synthetic D-kB1 were at least 90% effective in killing nematodes.

The Ala-scan study showed that the cyclotide mutants could be subdivided into groups according to their potency as inhibitors of larval development. The data used to delineate groups was derived from assays with the higher level of cyclotide with each nematode species, but the general pattern was the same at the lower concentration. The groupings were (1) inhibition similar to the wild-type (kB1): G7A, T9A in loop 2; G14A in loop 3; T16A in loop 4; S18A in loop 5; and T23A, N25A, G26A in loop 6; (2) inhibition at 65−90%: P13A in loop 3; and L27A, V29A in loop 6; (3) inhibition at 25−40%: N11A, T12A in loop 3; V21A in loop 5; and R24A and P28A in loop 6; and (4) inhibition < 25%: G2A, E3A, T4A in loop 1; and V6A, G8A in loop 2.

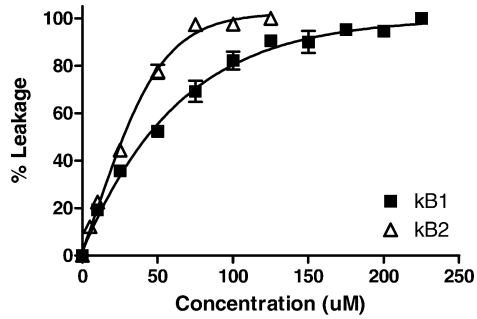


FIGURE 6: Cyclotide-induced leakage of CF from 100 $\mu$M POPC vesicles. The maximum fluorescence intensity after 20 min from individual experiments using a range of kB1 and kB2 concentrations were converted to percentage leakage values and plotted as a function of cyclotide concentration ($\mu$M).

Overall, these results showed that a range of residues play an important role in determining anthelmintic activity.

*Effects of Cyclotides on the Leakage of Vesicle Contents.* Because the data from the studies reported above are consistent with a mechanism of action involving membrane interaction, we investigated the effect of the prototypic cyclotides, kB1 and kB2, on leakage of a vesicle-entrapped dye in a model membrane system. Leakage of POPC vesicle contents to the external medium was monitored using a carboxyfluorescein (CF)-based assay by addition of increasing amounts of cyclotide from a stock solution to unilamellar vesicles suspensions. Background fluorescence of the vesicles was measured at 0 min and subsequent fluorescence spectra were recorded from time 0−20 min after the addition of a given cyclotide solution. After 20 min, 10% Triton X-100 was added, corresponding to complete disruption of the membrane and, thus, 100% leakage of the vesicle contents. The fluorescence maxima occurred at 518 nm and these data were then converted to percentage leakage values. Leakage occurred rapidly with >50% of the total fluorescence intensity observed after one minute followed by a gradual increase for up to 10 min (data not shown). As leakage was effectively complete after 10 min, the carboxyfluorescein fluorescence intensity 20 min after peptide addition was used as a measure of the total peptide-induced dye leakage. Figure 6 shows the percentage leakage versus concentration for kB1 and kB2. The calculated $EC_{50}$ of kB1 was 61 $\mu$M compared to 35 $\mu$M for kB2. These experiments showed that the cyclotides kB1 and kB2 have the capability to alter membrane permeability leading to the loss of the internal vesicle contents to the external medium.

## DISCUSSION

The present study has shown that the cyclotides are potent inhibitors of larval development in nematode parasites and that they are lethal to adult parasites. Cyclotides also show a low level of ovicidal activity. Although the larval development activity clearly indicates the anthelmintic activity of the cyclotides and allows ready comparisons with existing compounds; it is the activity against adult parasites that is of most interest in indicating that the cyclotides are lethal toward the parasitic stage to which anthelmintics are most readily directed in any control strategy.

In the LDAs, kB6 was the most potent of the cyclotides tested, followed by kB1, kB2 and kB5, while kB7 had markedly reduced activity, and little or no activity was observed for kB3. A comparison of LDA data for commercial anthelmintic drugs and cyclotide kB6 indicates that the cyclotide shows comparable activity to two widely used anthelmintics, levamisole, and naphthalophos in this assay system (Table 3). This indicates some promise for the cyclotide family of peptides as anthelmintic agents. Activity of kB6 is, however, significantly less than that shown by thiabendazole (a member of the benzimidazole group) and ivermectin (a member of the highly potent macrocyclic lactone group of anthelmintics). Table 3 also shows that activity of kB6 in this assay is comparable to that shown by a number of nematocidal compounds recently described from marine organisms, mostly sponges. These include the cyclic peptides phoriospongin A and B (*30*). Some of these experimental compounds are peptidic in nature but contain many unusual or modified amino acids and are side chain-to-backbone cyclized.

The cyclotides also showed low levels of ovicidal activity, with an $IC_{50}$ of approximately 500 $\mu$g/mL for kB2. This is significantly greater than the $IC_{50}$ of 0.052 $\mu$g/mL for thiabendazole against *H. contortus* in this EHA system (*31*). However, while the egg hatch inhibition by kB2 may not be viewed on its own as an indicator of promising anthelmintic

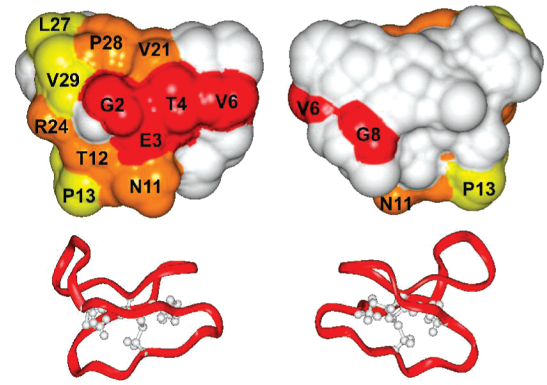


FIGURE 7: Three-dimensional structure of the prototypic cyclotide kalata B1. The residues that are important for anthelmintic activity are clustered together on one surface. The residues colored red are critical for activity (mutation at these points reduced anthelmintic activity to <25% inhibition in LDA), while those in orange and yellow decrease inhibition to 25−40% and 65−90%, respectively. Residues in white are those that had no effect on larval development along with the six conserved cysteines and the two residues shown to be important for correct folding (W19 and P20). Ribbon diagrams show the peptide backbone and disulfide bonds that occupy the core of the molecule in the same orientation as the surface representations.

Table 3: In Vitro Toxicity of Commercial Anthelmintics and Experimental Compounds to *H. contortus* in the Larval Development Assay

| | $IC_{99}$ ($\mu$M) | $IC_{50}$ ($\mu$M) | reference |
|---|---|---|---|
| Commercial Anthelmintics | | | |
| ivermectin | 0.00076 | 0.0025 | (*46*) |
| thiabendazole | 0.064 | 0.10 | (*46*) |
| levamisole | 1.4 | 8.9 | (*46*) |
| naphthalaphos | 0.73 | 7.5 | (*47*) |
| Experimental Compounds with Reported Anthelmintic Activity | | | |
| kalata B6 | 0.85 | 1.7 | this work |
| convolutindole A, H | | 0.80, 0.46 | (*48*) |
| thiocyantin | | 3.7 | (*49*) |
| onnamide F | | 8.7 | (*50*) |
| phorboxazole A | | 0.49 | (*51*) |
| phoriospongin A, B | | 7.3 | (*30*) |

activity, it may be an important consideration when addressing the mode of action of these compounds in indicating that functioning of their molecular target is required for the larvae to emerge from the egg as normal. The low level of ovicidal activity may be due to an inability of the compound to penetrate the egg shell rather than low activity toward its particular target site in this life stage. Relative abilities to affect nematode egg hatch and larval development in vitro show significant variation within the benzimadazole class of compounds, and between chemical groups, most likely due to differences in hydrophobicity (*32*). Relative potency in the two assay systems is therefore most likely related largely to hydrophobicity rather than the absolute anthelmintic potency. For example, the highly hydrophobic macrocyclic lactone group of drugs are highly potent in the LDA, but do not have any inhibitory effect on egg hatch. On the other hand, the benzimidazoles, a class of drugs of intermediate hydrophobicity (e.g., thiabendazole), are active in both the LDA and EHA (*32*).

Cyclotide kB1 completely inhibited motility of adult *H. contortus* in the in vitro assay system, but this activity was significantly less than that shown by ivermectin, thiabendazole, and levamisole (*26*). For example, the minimum concentration required to give a motility score of $\leq 1$ at 72 h was approximately 0.02 $\mu$g/mL for levamisole and thiabendazole (*26*) compared to approximately 25 $\mu$g/mL for kB1. On the other hand, kB1 showed similar activity to naphthalophos, which required a concentration of 20 $\mu$g/mL to reduce adult worm motility scores at 72 h to $\leq 1$ (*26*). Toxic effects of kB1 are noted within 24 h at concentrations that completely inhibit motility, in a similar pattern to the activity shown in this assay system previously by ivermectin, levamisole, and naphthalophos (*26*). In contrast, the benzimidazole drugs (thiabendazole, albendazole) act more slowly in this assay system as drug concentrations (over a 1000-fold concentration range) that result in reduced motility at 48 and 72 h exert no effect at the 24 h time point (*26*). Further work is required to determine the minimum exposure period required for cyclotides to exert their toxic effects in solution. The length of time for which peptide concentrations need to be maintained in the immediate environment of the worm will be important for assessing the therapeutic potential of the peptide group.

The cyclotides possess extraordinary stability to temperature, acid, denaturants, and the action of proteases (*33*). Experiments on rats show that kB1 is excreted unmodified after both intravenous and oral administration (unpublished data), indicating that the cyclotides are not degraded in the stomach or blood stream. This stability offers important advantages to this group of compounds as any orally administered anthelmintic needs to be able to withstand microbial degradation in the rumen as well as the acidic pH and protease-rich environment of the abomasum to reach the sites of nematode infection. Hence, the exceptional stability of the cyclotides compared to conventional linear or non-disulfide containing peptides makes them amenable for use in gastrointestinal parasite control. Over recent years, a range of other gene-coded circular proteins have been discovered in bacteria, plants, and animals, and it is likely that further examples of the use of nature's cyclization strategy will emerge in coming years, as there are clear stability advantages of circular proteins (*34, 35*). As the cyclotides are plant-based gene products, there is the potential for their delivery to livestock via genetically modified forage crops. Besides, the extraordinary stability displayed by the cyclotides, an anthelmintic mode of action involving interaction with membranes rather than a specific receptor would have important implications for drug resistance. Such a mode of action is unlikely to suffer from a build up of resistance, as is seen with the benzimidazoles, levamisole, and ivermectin in which resistance is at least partly due to changes in their specific target proteins or receptors (*36*).

The mirror-image stereoisomer, D-kalata B1, showed comparable activity to WT-kB1, providing a clear indication that there was no chiral selectivity in the anthelmintic activity. This suggests that the mechanism of action may be via membrane interaction and disruption rather than by a specific receptor interaction as receptor interactions would most likely require a specific chirality in the peptide. Studies involving the use of all-D-amino acid containing antimicrobial peptides such as melittin, cecropin A, and magainin 2 (*37, 38*) have shown that these peptides interact with bacterial membranes via the formation of ion-channel pores, without specific interaction with chiral receptors or enzymes.

Testing of several alanine mutants showed that the residues G2A, E3A, T4A, V6A, and G8A (and N11A, T12A, P13A, L27A, and V29A to a lesser extent) are responsible for the anthelmintic activity. Figure 7 shows a surface representation of kalata B1 highlighting the residues that are responsible for anthelmintic activity. Nearly all of these residues are clustered together on one face of the molecule to form a large patch, suggesting that rather than a receptor-based mechanism, a membrane-interaction most likely involving oligomerization is responsible for the observed activity. The only residue not forming part of the patch is G8 in loop 2, which is important for the correct conformation of the turn in this region of the molecule (*39, 40*) and, hence, may play a structural role rather than be involved in binding. Two recent studies have demonstrated the binding of kB1 and kB7 to the surface of dodecylphosphocholine micelles mainly via two hydrophobic loops (2 and 5) with relatively little perturbation of the structure during incorporation (*41, 42*). The residues found to be involved in the micelle interaction (W19−V21 of loop 5 and L27−V29 of loop 6 for kB1 and W19−I21 of loop 5 and T6−L7 of loop 2 for kB7)) comprise the hydrophobic face, which interestingly is spatially resolved from the functional face responsible for insecticidal and hemolytic activities as reported in a recent paper by Simonsen et al. (*29*). The alanine mutants that resulted in decreased anthelmintic activity (G2A, E3A, T4A, G8A, N11A, T12A, and L27A) of kalata B1 in the current study are essentially the same as those described by Simonsen (*29*), suggesting that the anthelmintic activity of cyclotides arises from a similar membrane binding mechanism to that implicated in insecticidal and hemolytic activity. The similarity in the recently reported structure of kalata B2 (*15, 43*) with that of kalata B1 (*40*) suggests that it likely has a similar membrane binding orientation. Consistent with the membrane binding hypothesis, cycloviolacin O2 from *Viola odorata* was shown to cause leakage from liposomes as well as from whole cells (*7*), suggesting that a range of cyclotides act via this mechanism. NMR studies show that the divalent manganese cations coordinate to the conserved glutamic acid side chain (*29*) and are in direct contact with the polar head groups of

the detergent in DPC micelles (*42*). It has been postulated that this peptide/cation interaction plays a major role in membrane perturbation and, hence, biological activity.

In this study, we have demonstrated the membrane lytic ability of two prototypic cyclotides (kB1 and kB2) in a model membrane system (POPC vesicles). The peptides induce leakage of an entrapped self-quenching dye in a concentration-dependent manner. kB2 showed greater potency, which correlates with the data collected for anthelmintic activity. It will be of interest in future work to investigate the membrane-disrupting ability of the alanine mutants and additional natural variants in this model system to further the understanding of the mode of action of cyclotides.

The cyclotides are expressed in both leaves and in roots (*12*) as well as other tissues and although some cyclotides are common to all tissue types, recently, examples of tissue-specific-expressed cyclotides have been identified (*44, 45*). Given the anthelmintic properties of the peptides demonstrated in the present study, it is possible that those expressed preferentially in the roots may play a role in protection against plant parasitic nematodes.

The investigation of compounds obtained from natural sources such as plants is fundamentally important for the development of new anthelmintic drugs. Peptides offer a potential alternative to current methods of chemical control of gastrointestinal nematodes, which may suffer from problems of resistance, contamination, and environmental pollution. The cyclotides show some promise in this field. Further work is required to evaluate their precise mechanism of action and their toxicity toward ruminants, as well as to determine adequate in vivo dose levels for nematode control.

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