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The Anthelmintic Activity of the Cyclotides: Natural Variants with Enhanced Activity

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The cyclotides are a family of backbone-cyclised cystine-knot-containing peptides from plants that possess anthelmintic activity against Haemonchus contortus *and* Trichostrongylus colubriformis*, two important gastrointestinal nematode parasites of sheep. In the current study, we investigated the in vitro effects of newly discovered natural cyclotides on the viability of larval and adult life stages of these pests. The natural variants cycloviolacin O2, cycloviolacin O3, cycloviolacin O8, cycloviolacin O13, cycloviolacin O14, cycloviolacin O15, and cycloviolacin O16 extracted from* Viola odorata *showed up to 18-fold greater potency than the prototypic cyclotide kalata B1 in nematode larval development assays. Cycloviolacin O2 and cycloviolacin O14 were significantly more potent than kalata B1 in adult* H. contortus *motility assays. The lysine and glutamic acid residues of cycloviolacin O2, the most potent anthelmintic cyclotide, were chemically modified to investigate the role of these charged residues in modulating the biological activity. The single glutamic acid residue, which is conserved across all known cyclotides, was shown to be essential for activity, with a sixfold decrease in potency of cycloviolacin O2 following methylation. The three lysine residues present in cycloviolacin O2 were acetylated to effectively mask the positive charge, resulting in a 18-fold decrease in anthelmintic activity. The relative anthelmintic activities of the natural variants assayed against nematode larvae correlated with the number of charged residues present in their sequence.*

Introduction

Antiparasitic products are among the largest segments of both the livestock and companion animal markets for healthcare agents.[1] There is an ongoing need for the development of new anthelmintics as resistance develops to the current agents, particularly the recently introduced macrocyclic lactone group.[2] There is also increasing emphasis on the need for safe, convenient and environmentally friendly products for use in parasite control.

The cyclotides are a recently characterised family of cyclic, disulfide-rich peptides discovered in plants from the Violaceae and Rubiaceae families.[3] They incorporate a cystine knot motif[4] that interlocks three conserved disulfide bonds. Together with the head-to-tail cyclised backbone, this structural motif imparts exceptional stability to the cyclotides.[5] The cystine knot motif results in the exclusion of hydrophobic residues from the core of these peptidic molecules. These hydrophobic residues form a surface-exposed patch that may be involved in interactions with membranes or modulate self-association behaviour. Studies by analytical ultracentrifugation have demonstrated that cyclotides undergo self-association to form oligomers.[6] The hydrophobic face and in particular the exposed tryptophan of kalata B1 have been shown to penetrate membranes.[7] It has been proposed that the mode of action of the cyclotides is through self-association and membrane disruption.

Figure 1 shows the sequence alignment of a range of cyclotides isolated from *Viola odorata,*[8] *Viola yedoensis*[9] and *Viola hederaceae*.[10] These cyclotides were selected based on the net positive charge of the *V. odorata* peptides and the increased hydrophobicity of the *V. hederaceae/V. yedoensis* peptides relative to the prototypic cyclotide kalata B1. The number of cyclotides known is expanding rapidly and a recent investigation of diversity within Australian *Hybanthus* species estimates that there are >9000 cyclotides in the Violaceae family,[11] hence there is a huge potential for cyclotides with desirable biological activities to be uncovered.

The cyclotides exhibit a diverse range of biological activities, including anti-HIV,[12] antimicrobial,[13] cytotoxic,[14] haemolytic,[15] antifouling,[16] and antineurotensive[17] properties, although their natural role is postulated to be in plant defence.[18,19] The insecticidal activity of the prototypic cyclotide, kalata B1, was previously demonstrated against *Helicoverpa punctigera*[20] and *Helicoverpa armigera*[21] larvae, major pests of cotton. A recent examination of *H. armigera* mid-guts showed that cyclotides produced marked disruption of the microvilli, blebbing, swelling and the ultimate rupture of the gut epithelium.[22] Recently, we demonstrated that the cyclotides also show potent anthelmintic activity towards the gastrointestinal nematodes of sheep, *Haemonchus contortus* and *Trichostrongylus colubriformis*.[23]

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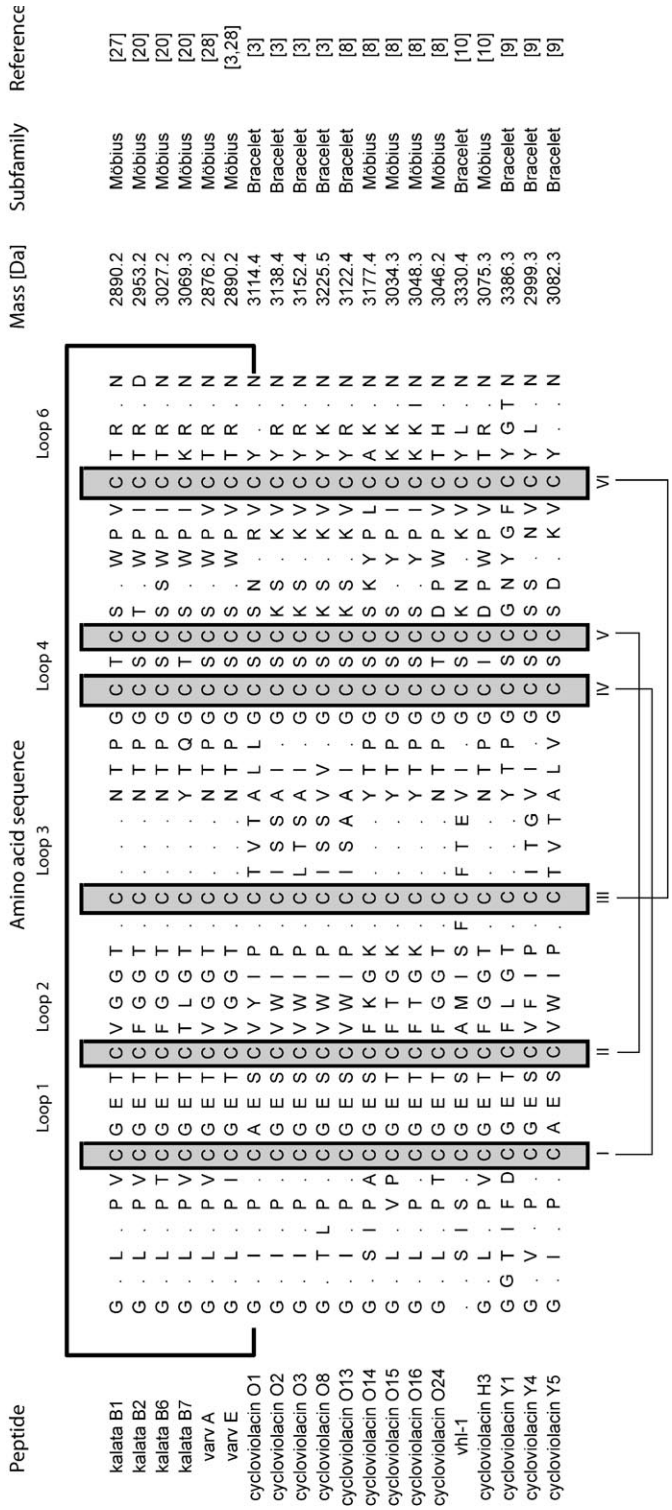

Figure 1. Sequence alignment of cyclotides tested in larval development assays. The kalata peptides isolated from *O. affinis* were the first known examples of cyclotides. The cycloviolacins from *V. odorata* contain multiple basic amino acids, whilst peptides isolated from *V. yedoensis* and *V. hederacede* are more hydrophobic in nature.

The aim of the current study was to identify natural variants of the cyclotides with increased toxicity towards these gastrointestinal parasites. Cycloviolacin O2 showed a 18-fold increase in activity in larval development assays and anthelmintic activity was demonstrated to correlate with the net peptide charge. 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. We also used chemical modification of key residues to determine the role of these residues and their associated charge in modulating the biological activity of the cyclotides.

Results

Anthelmintic activity was assessed by screening cyclotides in a larval development assay in which newly hatched first-stage larvae (L1) were cultured to fully developed third stage larvae (L3) in the presence of the cyclotides at a range of concentrations. Selected cyclotides that demonstrated increased potency in this assay system, relative to the prototypic cyclotide (kalata B1), were examined in adult worm motility assays. Finally, cycloviolacin O2, the most potent cyclotide tested, was chemically modified to investigate the role of charged residues in modulating anthelmintic activity.

Effects of cyclotides on the development of sheep nematode eggs to L3 larvae

Development of larvae from the L1 stage to the fully developed L3 stage was $>90\%$ in the absence of cyclotides; this indicates that the different diluents used in this assay had no significant effect on larval development.

A range of natural variants were selected based on net charge and/or hydrophobicity and were tested alongside the prototypic cyclotide kB1 in larval development assays with *H. contortus* and *T. colubriformis*. Table 1 shows the IC_{50} and IC_{99} values for all peptides tested against both nematode species.

Figure 2 shows the dose-response curves for a number of cyclotides isolated from the sweet violet *V. odorata*. A number of these cyclotides were found to have larvicidal activities significantly greater than kB1, and cO2, cO3, cO8 and cO13—the most potent of the variants tested—had IC_{50} values of 0.12–0.24 μM for *H. contortus* and 0.19–0.24 μM for *T. colubriformis*. The peptides cO14, cO15 and cO16 also showed increased activity compared to kB1 (with IC_{50} values of 0.27–0.41 μM for *H. contortus* and 0.41–0.64 μM for *T. colubriformis*). The cyclotides, cO1 and cO24 (*V. odorata*), vhl-1 (*V. hederaceae*), and cY4 and cY5 (*Viola yedoensis*) showed comparable activities to kB1

Table 1. Effects of the cyclotides on development of *H. contortus* and *T. colubriformis* in vitro.

	H. contortus		*T. colubriformis*	
Cyclotide	IC_{50} [μM]	IC_{99} [μM]	IC_{50} [μM]	IC_{99} [μM]
kalata B1[a]	2.26 (2.20–2.33)	7.11 (7.16–8.23)	7.13 (6.99–7.23)	26.05 (24.29–27.99)
kalata B2[b]	1.59 (1.59–1.60)	5.32 (4.64–6.10)	5.69 (5.62–5.76)	15.03 (14.05–16.08)
kalata B6[c]	0.87 (0.86–0.87)	1.69 (1.56–1.84)	2.62 (2.54–2.69)	11.96 (11.03–12.98)
kalata B7[d]	6.29 (6.09–6.65)	12.58 (10.82–14.63)	5.64 (5.51–5.80)	13.13 (11.21–15.38)
varv A[d]	1.13 (1.07–1.20)	4.46 (3.13–6.36)	1.89 (1.61–2.23)	19.78 (12.19–32.08)
varv E[d]	0.90 (0.74–1.09)	4.19 (1.87–9.42)	3.75 (3.57–3.93)	9.36 (7.84–11.14)
cycloviolacin O1[d]	2.82 (2.49–3.19)	17.48 (10.76–28.49)	3.89 (3.19–4.75)	13.14 (7.76–22.21)
cycloviolacin O2[d]	0.12 (0.11–0.13)	0.35 (0.29–0.43)	0.24 (0.23–0.25)	0.51 (0.43–0.60)
cycloviolacin O3[d]	0.21 (0.18–0.24)	0.41 (0.24–0.71)	0.23 (0.19–0.27)	0.33 (0.22–0.49)
cycloviolacin O8[d]	0.24 (0.22–0.26)	1.24 (0.99–1.57)	0.22 (0.20–0.24)	1.13 (0.90–1.43)
cycloviolacin O13[d]	0.21 (0.19–0.22)	0.76 (0.55–1.05)	0.19 (0.17–0.21)	0.92 (0.73–1.15)
cycloviolacin O14[d]	0.41 (0.38–0.44)	0.91 (0.75–1.10)	0.64 (0.61–0.66)	1.38 (0.98–1.96)
cycloviolacin O15[d]	0.38 (0.35–0.40)	1.76 (1.23–2.51)	0.41 (0.38–0.44)	2.14 (1.45–3.17)
cycloviolacin O16[d]	0.27 (0.24–0.30)	2.56 (1.86–3.52)	0.45 (0.43–0.47)	1.43 (1.05–1.95)
cycloviolacin O24[d]	1.74 (1.65–1.82)	4.17 (3.55–4.91)	2.99 (1.72–5.19)	4.83 (1.24–18.81)
vhl-1[d]	2.06 (1.55–2.73)	50.52 (23.80–107.28)	5.78 (3.72–8.98)	42.32 (23.52–76.30)
cycloviolacin H3[d]	0.85 (0.77–0.94)	7.36 (4.47–12.14)	5.90 (5.23–6.67)	19.46 (12.83–29.52)
cycloviolacin Y1[d]	n.c.	n.c.	n.c.	n.c.
cycloviolacin Y4[d]	2.01 (1.77–2.28)	6.73 (4.44–10.21)	2.27 (2.04–2.54)	5.26 (4.12–6.71)
cycloviolacin Y5[d]	2.28 (1.88–2.76)	22.24 (9.29–53.35)	2.40 (2.00–2.88)	10.97 (5.48–21.94)

Values given are the mean IC_{50} or IC_{99} for [a] eight, [b] three, [c] two, or [d] one experiment(s), with 95% confidence intervals, with three assay wells at each of a range of cyclotide concentrations; n.c.: not calculated.

for *H. contortus* (1.74–2.82 compared to 2.26 μM), while vhl-1 had similar activity against *T. colubriformis* (with an IC_{50} of 5.78 compared to 7.13 μM for kB1). Cycloviolacins O24, Y4 and Y5 showed two- to threefold improved activity against *T. colubriformis* (with IC_{50} values of 2.99, 2.27 and 2.40 μg mL^{-1}, respectively). Cycloviolacin Y1 had markedly reduced activity (IC_{50} could not be calculated for either worm species). The cyclotides varv A and varv E extracted from *V. odorata,* and cycloviolacin H3 extracted from *V. hederaceae* showed similar activities, with IC_{50} values of 0.85–1.13 μM towards *H. contortus*, representing a twofold increase in toxicity compared to kB1.

In summary, the most potent cyclotides tested were all extracted from *V. odorata* and contained three or four basic amino acids. The IC_{50} values for this group were significantly lower than all other cyclotides that contained fewer (0–2) basic amino acids.

Effects of cyclotides on the motility of adult *H. contortus*

The worm motility assay involves scoring the nematodes on a 0–3 scale, in which 0 represents no movement and 3 represents normal sinusoidal motion.[24] Observations on worm motility in control assays (no peptide) showed that *H. contortus* adults cultured for 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 for control worms at 72 h was 2.6 ± 0.2 (mean $\pm$ SE, $n = 5$ separate control assay tubes). The effects on motility of the cyclotides cO2 and cO14 are shown in Figure 3. A range of concentrations of cO2 and cO14 were tested and scored after 24 and 48 h. After 24 h, motility scores of ≤ 0.5 were recorded for concentrations of ≥ 16 (cO2) and 31 μM (cO14), and the intermediate concentrations responded in a dose-dependent manner. At this time, worms exposed to kB1 at 35 μM showed motility scores > 1.5. After 48 h, there was a significant reduction in motility with all three peptides tested. Both cO2 and cO14 were significantly more potent and caused greater decreases in worm motility than kB1. Although the limited number of concentrations and the subjective "step-wise" nature of the scoring system does not allow ready quantification of differences between the activities of the peptides, it was apparent that after 48 h a motility score of 1 was associated with < 4, 4 and approximately 20 μM for cO2, cO14 and kB1, respectively.

Effects of chemical modifications on anthelmintic activity

Figure 4 shows dose-response curves that compare the anthelmintic activities of chemically modified cO2 analogues in the larval development assay against *H. contortus*. Acetylating the lysine side-chain amines or methylating the glutamic acid effectively removed the charge on the peptide. The sites of chemical modification were confirmed by tandem mass spectrometric sequencing following the reduction and enzymatic digestion of the cO2 analogues (results not shown). Acetylation of the lysine side chains dramatically decreased the an-

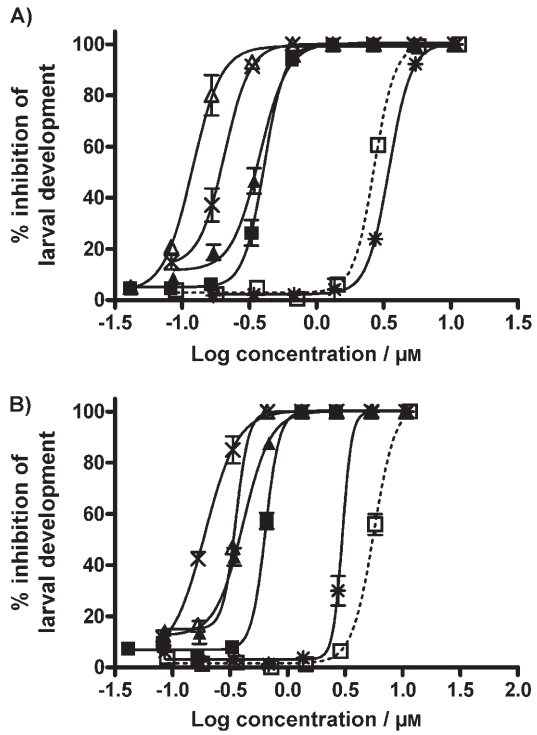

Figure 2. The effect of selected natural variants on the development of nematode eggs to third stage (L3) larvae of: A) *H. contortus*, and B) *T. colubriformis*. Cycloviolacin O2 (△), cycloviolacin O13 (×), cycloviolacin O14 (■), cycloviolacin O15 (▲) and cycloviolacin O24 (*) were compared to the prototypic cyclotide kalata B1 (□). Each data point represents the mean (± SE, with three assay wells at each of a range of cyclotide concentrations).

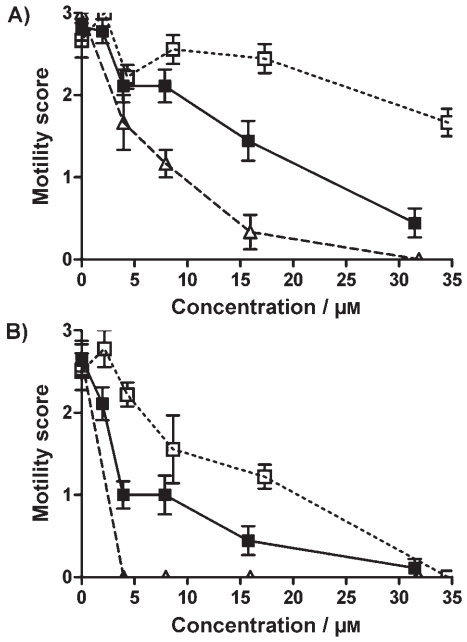

Figure 3. The effect of kalata B1 (□, dotted line), cycloviolacin O2 (△, dashed line) and cycloviolacin O14 (■, solid line) on motility of *H. contortus* adults in vitro after A) 24, and B) 48 h. Each data point represents mean ± SE ($n = 3$, separate assay tubes).

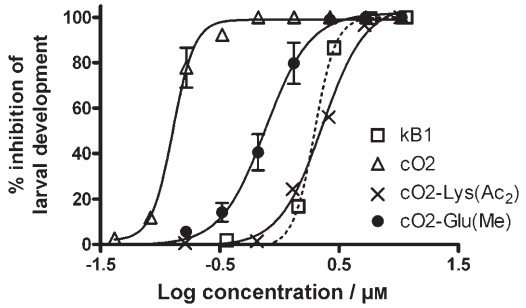

Figure 4. Chemical modification of key residues of cycloviolacin O2. The glutamic acid residue was modified with acetyl chloride in methanol (cO2-Glu(Me)), while the lysine residues were acetylated with acetic anhydride in ammonium bicarbonate buffer (cO2-Lys(Ac_2)). These modifications effectively removed the negative and positive charges, respectively.

thelmintic activity as evidenced by the IC_{50} value of 2.30 μM, compared to 0.13 μM for native cO2 (18-fold decrease). The IC_{50} value for cO2-Glu(Me) was 0.76 μM, representing an approximately sixfold decrease in potency. Similar decreases in activity were observed with *T. colubriformis* (Table 2).

Discussion

In a recent paper we described the anthelmintic activity of the prototypic cyclotide kalata B1 and a few selected cyclotides from *O. affinis* that differed by up to six single-point mutations (kalata B2, B6 and B7; Figure 1).[23] In the current work, we examined a wide range of cyclotides that vary dramatically in amino acid composition, net charge and hydrophobicity. The significantly increased activity detected in the variants examined here clearly highlights the potential for cyclotides as anthelmintics. There are currently ~100 published sequences of cyclotides although it is expected that >9000 exist in nature.[11]

The natural variants extracted from *V. odorata*, namely the cycloviolacins cO2, cO3, cO8, cO13, cO14, cO15 and cO16, possess up to 18-fold greater activity than the prototypic cyclotide kalata B1 against *H. contortus*. The activity of cO2 (IC_{50} of 0.12 μM) compares favourably with current commercial anthelmintics, such as thiabendazole (IC_{50} of 0.064 μM), levamisole (IC_{50} of 1.4 μM) and naphthalaphos (IC_{50} of 0.73 μM), but is significantly less than the macrocyclic lactone ivermectin (IC_{50} of 0.00076 μM) in this assay system.[23]

It is interesting to note that the increased anthelmintic activity observed for the natural variants compared to kB1 correlates with the number of charged residues present within each of the peptide sequences. Although peptides that contain one or two charged residues, such as varv A, cO1 and vhl-1, possess activity that is comparable to that previously reported for kB1, those that contain three or four charged residues showed a substantial improvement in activity that is independent of subfamily classification (i.e., high activity is seen in variants from both the Möbius and bracelet subfamilies). Interestingly, all of the natural variants showing substantial increases in anthelmintic activity eluted within a 25–33% (HPLC solvent B:

Table 2. Effects of chemically modified cyclotides on development of *H. contortus* and *T. colubriformis* in vitro.

	H. contortus		*T. colubriformis*	
Cyclotide	IC_{50} [μM]	IC_{99} [μM]	IC_{50} [μM]	IC_{99} [μM]
kalata B1	2.26 (2.20–2.33)	7.11 (7.16–8.23)	7.13 (6.99–7.23)	26.05 (24.29–27.99)
cycloviolacin O2	0.13 (0.12–0.14)	0.35 (0.29–0.43)	0.24 (0.23–0.25)	0.59 (0.49–0.70)
cO2-Lys(Ac_2)	2.30 (2.11–2.50)	11.64 (8.35–16.21)	3.17 (2.48–4.07)	158.42 (65.59–379.56)
cO2-Glu(Me)	0.76 (0.65–0.90)	5.92 (2.82–12.40)	0.91 (0.81–1.02)	8.66 (5.65–8.66)

Values given are the mean IC_{50} or IC_{99} for two experiments, with 95% confidence intervals, with three assay wells at each of a range of cyclotide concentrations.

90% CH_3CN, 0.05% TFA) window correlating with relatively lower hydrophobicity than kB1 (38% B).

The importance of charge was further examined by chemically modifying the charged residues within the peptide cO2 in an approach similar to that used by Herrmann et al.[25] Cycloviolacin O2 was selected for this analysis as it was the most potent of all cyclotides tested. Acetylation of the two lysine residues in loop 5 resulted in an 18-fold decrease in activity against *H. contortus*, and 13-fold decrease against *T. colubriformis*. Esterification of the conserved glutamic acid residue in loop 1 decreased activity sixfold for *H. contortus* and fourfold for *T. colubriformis*. In a previous study, a complete suite of alanine mutants of the prototypic cyclotide kalata B1 was examined for their anthelmintic activity relative to wild-type kB1.[23] Replacing the glutamic acid in loop 1 with alanine was shown to completely abolish the activity. In addition, replacing the sole positively charged residue (arginine in loop 6) with alanine also greatly decreased the anthelmintic activity. The charge-modification data shown in the present study correlates with previously reported data, in which decreased cytotoxicity was observed following methylation of the glutamic acid (48-fold decrease) and the acetylation of the lysine residues in cO2 (threefold decrease).[25]

Three-dimensional surface representations for two of the most potent anthelmintic cyclotides, cO2 and cO14, are shown in Figure 5. Hydrophobic residues, which are highlighted in green, cluster together to form a surface-exposed hydrophobic patch. Previous studies on kB1 in dodecylphosphocholine micelles have shown that residues involved in the formation of this patch penetrate the micellar surface and the charged residues, glutamic acid and arginine, are in contact with the polar head groups of the detergent.[7] The masking of the charge on the highly conserved glutamic acid (red) along with substitution by alanine[26] have a profound effect on activity. The cyclotides with multiple basic residues (blue) were observed to have increased anthelmintic activity. Figure 5 indicates that these residues are spatially removed from the hydrophobic patch. The increased activity can be attributed to the increased sequestering of these molecules on the negatively charged membrane surface. The removal of these basic residues by acetylation reduced the anthelmintic activity to a level comparable with kB1.

The increased activity observed for cO2 and cO14 against larval life-stages of the parasite was also evident against adults. The degree of increase in activity relative to the proto-

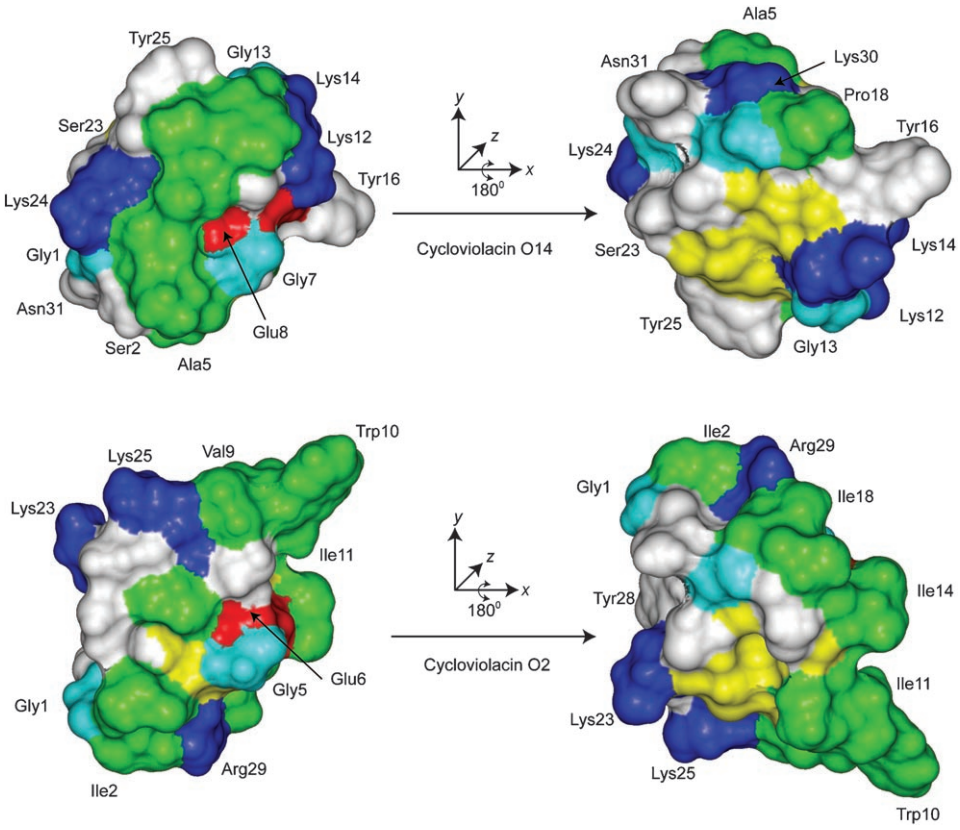

Figure 5. Structure of cycloviolacin O2 and O14 showing the Glu (red) and Lys (blue) residues that were found to have roles in modulating the anthelmintic activity. The hydrophobic residues are coloured green and are thought to play a role in membrane binding and disruption. The yellow residues represent the cysteines that are involved in the cyclic cystine knot motif that is responsible for the exceptional stability of this class of molecules.

typic peptides appeared to be less for adults than larvae, but this may be a reflection of the insensitivity of the adult assay as it is based on the scoring of worm motility using a limited number of scoring values rather than the more broad measurement of larval development percentages for the larval assay. Nevertheless, it was apparent that increased activity toward the larval life stages was also observed against the adult parasites. This suggests that structure–activity relationships established using the larval assay may also apply to establishing the factors important for optimising activity against the adult life stage, which will be the principal target of any cyclotide-based commercial anthelmintic.

Encouragingly, a number of the cycloviolacins show greater anthelmintic activity towards *H. contortus* than kalata B1 and they also showed decreased haemolytic activity.[8] Thus, given the extent of cyclotide occurrence in nature, there may be significant potential for identification of variants with desirable anthelmintic activity, while minimising undesirable characteristics such as haemolytic activity, in order to ensure a degree of specificity for the parasite alongside minimal toxic effects on the host animal.

Conclusions

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 suffer from problems of resistance, contamination and environmental pollution. The cyclotides show promising activity. In this work, we have tested several natural variants of cyclotides and found examples with increased nematocidal activity. Further work is required to evaluate their toxicity towards livestock and companion-animals, as well as to determine adequate in vivo dose levels for nematode control.

Experimental Section

Cyclotide isolation: Kalata B1 and other natural variants were isolated from the above ground parts of *Oldenlandia affinis,*[20,27,28] *Viola odorata,*[3,8] *Viola yedoensis*[9] and *Viola hederaceae.*[10] Fresh plant material (500 g) was ground and extracted with DCM/MeOH (2 L, 1:1, *v/v*), and the crude extract was partially purified by RP flash chromatography to yield a fraction that predominantly contained 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.[3]

Preparation of nematode eggs: The eggs of *H. contortus* and *T. colubriformis* were recovered from faeces collected from sheep housed at the McMaster Laboratory, CSIRO Livestock Industries, Armidale, NSW. The parasites were from the drug-susceptible McMaster isolate of *T. colubriformis*, with no history of exposure to anthelmintics, and the Kirby isolate of *H. contortus*, isolated from the field at the University of New England Kirby Research Farm in 1986,[29] and known to be susceptible to all commercial anthelmintics (unpublished data). The nematode eggs were isolated from the faeces by passing the faeces through a series of fine sieves (250 and 75 μm) 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 μm sieve with water to remove residual sucrose and diluted in order to obtain 50–60 eggs per 30 μL after the addition of amphotericin B (37.5 $\mu g\,mL^{-1}$).

Adult nematodes: The adult worm recovery and culture methods were as described previously.[30] Briefly, adult nematodes were removed from sheep abomasa 10–20 weeks post infection and placed into RPMI-1640 medium containing glucose (1%), amphotericin B (0.25 $\mu g\,mL^{-1}$), penicillin (10 $U\,mL^{-1}$), streptomycin (10 $\mu g\,mL^{-1}$), and HEPES buffer (10 mM, pH 6.8), at approximately 37 °C. The only significant change from the previously described method[30] was the inclusion of newborn bovine serum (20%) 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). Nematodes were removed from digesta material by using forceps, and were held in the medium for 3–4 h whilst removal from digesta continued. They were then transferred to medium containing the antimicrobial and antifungal agents described above at tenfold higher concentrations, and left for 1–2 h. Groups of ten females were then placed into 0.5 mL of the culture medium described above in separate glass vials and held at 37 °C before use in adult motility assays.

Larval development assay (LDA): LDAs were conducted in a microtitre plate format as described by Lacey et al.[31] Briefly, 200 μL of agar (2%) was deposited into each well of a 96-well microtitre plate. After this had solidified, nematode egg solution (30 μL) was added into each well. Water (10 μL) was added to the control wells and cyclotide solution (10 μL, varying concentrations) was added into the treatment wells. The final concentration range was 0.3–33.3 $\mu g\,mL^{-1}$ (0.09–11.53 μM). After 24 h, growth medium (20 μL) 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*).[32] 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 and/or ethanol (20%) 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.[24] The cyclotides were added at various concentrations to assay tubes containing adult worms 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.[24] Briefly, the worms were scored as: 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; 0: no movement.

Calculations and statistical analysis: The percentage inhibition of larval development was calculated by using Equation (1):

$$\% \text{ inhibition} = (A-B)/A \times 100 \quad (1)$$

where A is the number of larvae that had developed into the L3 stage (LDA) in control incubations, and B is the number of L3 larvae in incubations that contained different concentrations of cyclotides. To provide parameters for comparison of the test compounds, we calculated the concentration at which 50 and 99% of larvae failed to achieve full development to L3 larvae as IC_{50} and/or IC_{99} values. The larvicidal IC_{50} and IC_{99} values were calculated by using nonlinear regression (sigmoidal dose-response, GraphPad Prism). In order to determine whether there was significant variation between the different cyclotides tested, the 95% confidence intervals (CIs) were calculated. The IC_{50} and IC_{99} values were considered to be significantly different if their 95% CI values did not overlap.

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