Isolation, Characterization, and Bioactivity of Cyclotides from the Micronesian Plant *Psychotria leptothyrsa*

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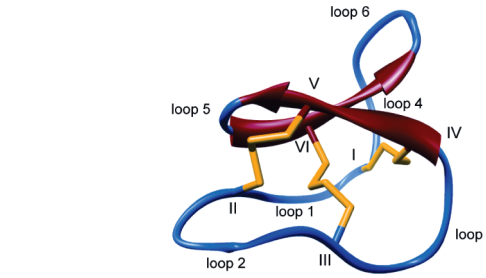
Cyclotides, the largest known family of head-to-tail cyclic peptides, have approximately 30 amino acid residues with a complex structure containing a circular peptide backbone and a cystine knot. They are found in plants from the Violaceae and Rubiaceae families and are speculated to function in plant protection. In addition to their insecticidal properties, cyclotides display cytotoxic, anti-HIV, antimicrobial, and inhibition of neurotensin binding activities. Although cyclotides are present in all violaceous species hitherto screened, their distribution and expression in Rubiaceae are not fully understood. In this study, we show that *Psychotria leptothyrsa* var. *longicarpa* (Rubiaceae) contains a suite of different cyclotides. The cyclotide fractions were isolated by RP-HPLC, and sequences of six new peptides, named psyles A−F, were determined by MS/MS sequencing. One of these, psyle C, is the first rubiaceous linear variant known. Psyles A, C, and E were analyzed in a fluorometric microculture assay to determine cytotoxicity toward the human lymphoma cell line U937-GTB. The IC_{50} values of psyles A, C, and E were 26, 3.50, and 0.76 μM, respectively. This study expands the number of known rubiaceous cyclotides and shows that the linear cyclotide maintains cytotoxicity.

Cyclotides have distinctive structural features including a head-to-tail cyclic peptide backbone and a compact cystine knot formed by three conserved disulfide bonds.[1−3] The prototypic cyclotide, kalata B1, was discovered by physician Lorents Gran during a Red Cross relief mission in the Congo during 1960 after he observed African women using a decoction of *Oldenlandia affinis* (Roem. & Schult.) D.C. (Rubiaceae) to hasten uterine contractions during childbirth. Subsequent investigations revealed that one active ingredient in that plant was a peptide that displayed powerful uterotonic activity.[4,5] Initial reports established that the peptide contained 30 amino acids, including six cysteine residues.[6] However, more than two decades elapsed before the full sequence and structure of kalata B1 was elucidated, in part because of the compound's unique complement of features.[7,8] During the 1990s, a series of peptides of similar size and structure to kalata B1 were discovered, which prompted the formal designation of the cyclotide peptide family.[1] The characteristic structural attributes contribute to the remarkable stability of cyclotides, which exhibit resistance to degradation by a variety of thermal, enzymatic, and chemical treatments.[9]

Figure 1 depicts the cyclic cystine knot (CCK) motif of kalata B1 and illustrates that cyclotides are divided into two main subfamilies (Möbius and bracelet) based on sequence similarities. Möbius cyclotides have a *cis*-peptide bond prior to the proline (Pro) in loop 5 and display lower net charges and less variation in loop size and content compared to bracelet cyclotides. Generally, cyclotides contain one glutamic acid (Glu) in loop 1 and have at least one cationic residue (Lys or Arg). While loops 1 and 4 are highly conserved, loops 3 and 6 are variable in terms of residue type and size.[10,11]

Although cyclotides may function in plant defense,[12,13] they have a wide range of bioactivities. They inhibit the replication and cytopathic effects of HIV,[14−16] display antibacterial and antifungal activity,[17] inhibit neurotensin binding to cell membranes,[18] and exhibit uterotonic activity in rat, rabbit, and human uteri.[4] Cyclotides demonstrate potent, dose-dependent cytotoxic activity in vitro against several cancer cell lines.[19,20] The disruption of lipid membranes via pore-formation and an intact disulfide network play critical roles in their cytotoxic activity,[11,21,22] while membrane interactions may explain their antimicrobial and hemolytic activities.[23,24] Their extraordinary stability and documented medicinal attributes make cyclotides promising candidates for drug design and protein engineering programs.[2,8,25]



Loop	6		1		2		3		4	5	6
Cys		I		II		III		IV	V		VI
Bracelet											
Cycloviolacin O1	GIP	C	AES	C	VYIP	C	TVTALLG	C	S	C SNRV	C YN
Cycloviolacin O2	GIP	C	GES	C	VWIP	C	ISSAIG	C	S	C KSKV	C YRN
Cyclopsychotride A	SIP	C	GES	C	VFIP	C	TVTALLG	C	S	C KSKV	C YKN
Möbius											
Kalata B1	GLPV	C	GET	C	VGGT	C	NTPG	C	T	C SWPV	C TRN
Kalata B2	GLPV	C	GET	C	FGGT	C	NTPG	C	S	C TWPI	C TRD
Varv A	GLPV	C	GET	C	VGGT	C	NTPG	C	S	C SWPV	C TRN
Trypsin Inhibitors											
MCoTI-I	GGV	C	PKILQR	C	RRDSD	C	PGA	C	I	C RGNGY	C GSGSD
MCoTI-II	GGV	C	PKILKK	C	RRDSD	C	PGA	C	I	C RGNGY	C GSGSD

Figure 1. Cyclotide structure and sequence. The three-dimensional structure of prototypic kalata B1 cyclotide (pdb 1NB1[71]) is shown at the top. The seamless peptide backbone and three disulfides (in yellow) define the cyclic cystine knot topology, which is associated with distorted *beta* sheets. Cysteine residues are marked with roman numerals.

Cyclotides have been reported from Violaceae and Rubiaceae plant families; they are expressed in all violaceous species hitherto analyzed but are found in only a few species of Rubiaceae (Table 1). Recently, chemical profiling of >200 rubiaceous species

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Table 1. Cyclotides Isolated from Rubiaceae

plant source	cyclotide(s)	year[a]	reported bioactivity
Oldenlandia affinis (R. & S.) DC.	kalata B1	1973	antimicrobial, insecticidal, uterotonic, anti-HIV
	kalatas B2−B5	1999	antimicrobial, insecticidal, uterotonic
	kalatas B6, B7	2001	[b]
	kalatas B8	2006	antiviral
	kalatas B9−B17	2007	hemolytic
Psychotria vellosiana Benth.	cyclopsychotride A	1994	inhibition of neurotensin binding
Psychotria suterella Müll. Arg.	PS-1	2008	[b]
Chassalia parvifolia K. Schum.	circulins A, B	1994	anti-HIV, antimicrobial
	circulins C−F	2000	anti-HIV
Chassalia discolor K. Schum.	CD-1	2008	[b]
Palicourea condensata Jacq.	palicourein	2001	anti-HIV

[a] Year denotes first report of the cyclotide. [b] No reported bioactivity. References are as follows: kalatas B1−B5,[1,4,7,12,13,17,76] kalatas B6 and B7,[12] kalata B8,[77] kalatas B9−B17,[73] cyclopsychotride A,[56] PS-1 and CD-1,[26] circulins A and B,[14,17] circulins C−F,[78] palicourein.[16]

Table 2. Species of *Psychotria* (Rubiaceae) Screened for Cyclotides

species screened	collection site[a]	medicinal use	chemistry	plant part[c]
P. bacteriophila Valeton	Hawaii	[e]	β-sitosterol, ursolic acid	leaf, stem, berry
P. carthaginensis Jacq.	Hawaii	antiparasitic, emetic, hallucinogen (Amazon)	alkaloids, DMT[b]	leaf, stem, inflorescence
P. erythrocarpa Schldtl.	Mexico	[e]	[e]	leaf, stem, inflorescence
P. hobdyi S.H. Sohmer	Kauai	[e]	[e]	leaf, stem
P. insularum A. Gray	Samoa	cataracts, abdominal distress, infections (Samoa)	[e]	leaf, stem, seed, remedy[d]
P. leptothyrsa var. *longicarpa* Val.	Palau	[e]	cyclotides	leaf, stem
P. mariana Bartl. Ex. D.C.	Tinian, Mariana Island	[e]	[e]	leaf, stem, inflorescence
P. psychotrioides (D.C.) Roberty	Sri Lanka	[e]	[e]	leaf, stem
P. serpens L. Mant.	Hong Kong	antirheumetic, analgesic, muscle relaxant (Taiwan)	ursolic acid	leaf, stem
P. viridis Ruíz & Pavón	Peru	antiparasitic, emetic, hallucinogen (Amazon)	alkaloids, DMT	leaf, stem, seed
P. sp.	New Caledonia	[e]	[e]	leaf, stem

[a] Collection site is the original site of harvest. [b] DMT is dimethyltryptamine. [c] Plant part indicates aerial tissues screened for cyclotides. [d] The Samoan remedy was provided by Paul A. Cox. Accessions located at NTBG, and voucher specimens available at the PTBG. [e] No reported medicinal use or chemistry discovered during literature searches.

indicated the presence of cyclotides in 22 additional species; however, only two cyclotides were sequenced from this plants.[26] More than 140 cyclotides have been characterized, but many more await discovery. The number of cyclotides in Violaceae is estimated to exceed 9000,[27] and possible unique cyclotides in Rubiaceae are predicted to range between 10 000 and 50 000 peptides.[26] Individual plants may express just one cyclotide, but evidence increasingly suggests one species can contain a complex suite of up to 100 cyclotides.[27,28]

The distribution of cyclotides is not fully understood in Rubiaceae. Economically, the family is an important source of commercial coffee, dyes, ornamentals, native medicines, and prescription drugs.[29−31] In fact, Farnsworth[32] lists caffeine, emetine, and quinine among the 30 most important plant-derived drugs. However, few rubiaceous genera have been investigated for cyclotides. Until now they have been sequenced from just six species in Rubiaceae, displaying a diversity of bioactivity (Table 1). Two cyclotide-containing species of *Psychotria* L. (Rubiaceae) have been reported: neurotensin binding inhibitory cyclopsychotride A from *P. vellosiana* Benth.[18] and PS-1 from *P. suterella* Müll. Arg.[26] *Psychotria* has a long history of indigenous medicinal use; the corresponding chemistry has been revealed in some cases.

For instance, *P. viridis* Ruíz & Pavón contains alkaloids (notably dimethyltryptamine), which may explain its use throughout Amazonia in the hallucinogenic beverage ayahuasca.[33] Other species are actively employed in traditional remedies, although compounds producing therapeutic properties remain unknown. For example, Samoan healers use *P. insularum* A. Gray to treat microbial infections, complications of maternity, and various inflammation-related illnesses.[34−36] Crude extracts depress central nervous system activity and decrease the inflammatory action of cyclooxygenase-1. The bioactive compounds responsible for each activity have not been identified.[34,37,38]

Since relatively few species of *Psychotria* have been investigated for cyclotides, their generic distribution and activity leaves a number of questions. Do *Psychotria* express cyclotides as complex cocktails, as in violaceous species and *Oldenlandia affinis*? Do their sequences resemble those of other cyclotides, and do they have the same bioactivity? How often are cyclotides found in *Psychotria?* Could cyclotides be bioactive constituents in remedies of *P. insularum*? Triggered by these questions, 11 species of *Psychotria* were analyzed for cyclotides using LC-MS. Only one species was found to contain cyclotides, *P. leptothyrsa* Miq. var. *longicarpa* Valeton, and six novel cyclotides were sequenced by MS/MS. Highly abundant cyclotides were tested for cytotoxic activity in order to compare potency to that of known cytotoxic cyclotides.

Results and Discussion

Cyclotide Screening of *Psychotria* Species. Eleven species of *Psychotria* were selected for investigation on the basis of reported medicinal use or availability from collections at the National Tropical Botanical Garden (NTBG) in Hawaii. Aerial parts were collected, dried, and divided into different plant tissues (Table 2), then screened for cyclotide content. Screening followed previously outlined protocols for cyclotide extraction.[27] Extracts were subjected to SPE on reverse-phase material to effectively capture cyclotides based on their pronounced hydrophobic properties.[1] Resulting cyclotide-enriched fractions were analyzed by LC-MS, using late retention times and molecular masses in the range 2800−3500 Da as typical chemical markers for cyclotides.[3,7,39]

Only one of the 11 species analyzed, *P. leptothyrsa*, screened positive for cyclotides. As Figure 2 illustrates, LC-MS traces for stem and leaf tissue show psyle cyclotides elute between 19 and 40 min and have masses in the range 2840−3258 Da. The relative abundance of psyle cyclotides varies between these tissue types. The major peak in the stem extract, which consists of two peptides

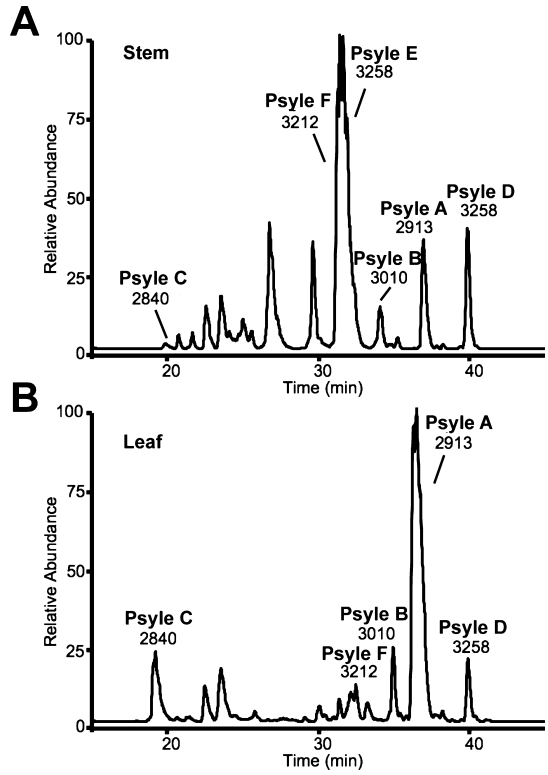

Figure 2. Liquid chromatography−mass spectrometry of *Psychotria leptothyrsa* var. *longicarpa* stem and leaf extracts. Cyclotides expressed had chemical markers [late retention time on RP-HPLC and LC-MS and masses in the range 2800−3500 Da] comparable to previously reported cyclotides. Retention times were in the range 19−40 min with a gradient from solvent A to B over 75 min at a flow rate of 0.3 mL/min.

with masses of 3212 and 3256 Da (later named psyles E and F), is practically absent in the leaf extract. In contrast, the peak eluting first in the leaf extract (psyle C, 2840 Da) occurs in barely detectable amounts in the stem extract.

Since retention time and molecular weight are strong but inconclusive indicators of cyclotides, *P. leptothyrsa* extracts were reduced and alkylated by iodoacetamide to lend further proof of identity. Using this strategy, each disulfide bond is identified by a mass shift of 116 Da (2 × 58 Da). All cyclotide peaks in each extract showed mass shifts of 348 Da (3 × 116 Da), which was consistent with the presence of three disulfide bonds in the CCK motif. Following alkylation, we turned to a larger extraction of stem and leaf material (∼10 g each) and the preparative isolation of individual cyclotides for determination of their primary sequence and cytotoxicity.

Sequencing of Novel *Psychotria leptothyrsa* (Psyle) Cyclotides. We used SPE to concentrate hydrophobic compounds, followed by preparative RP-HPLC to differentiate cyclotides from other compounds within the extract.[40,41] Mass spectrometry was used to rapidly identify fractions containing masses in the range of known cyclotide molecular masses,[20] and purification of individual cyclotides (named psyles A−F) was performed by sequential RP-HPLC.[21,40] After quantification with UV absorbance, 20 g of dried plant material yielded 8.50, 4.26, and 0.99 mg of psyles A, C, and E, respectively.

Due to the CCK motif, cyclotides must be reduced, alkylated, and cleaved before sequencing. A combination of enzymatic digests and nanospray MS/MS is the method of choice for their sequencing, and several protocols targeting cyclotide residues have been developed. One protocol introduces positive charges at each cysteine through aminoethylation of reduced cysteines, with bromoethylamine producing linear fragments of suitable size for analysis. Digestion with trypsin results in cleavage at the basic residue (Arg, Lys, and aminoethylated Cys), allowing the intercysteine loops to be sequenced.[3] A second protocol produces a single linear peptide when endoproteinase Glu-C cleaves the circular peptide at the Glu in loop 1. Cleavage is followed by digestion with trypsin, which cleaves at cationic residues, or chymotrypsin, which cleaves C-terminal to aromatic amino acids.[10] A combination of the above protocols was used in this work and is available as Supporting Information (Table 1).

Distribution of Rubiaceous Cyclotides. This research explores the selective distribution of cyclotides in rubiaceous plants through the expression profiles of several psychotrian species. Our findings illustrate that only *P. leptothyrsa* contains detectable levels of cyclotides, making it the seventh rubiaceous and third psychotrian cyclotide-containing species. Moreover, it is the first rubiaceous species shown to express a complex cocktail of cyclotides. The question arises, why were cyclotides not found in every species of *Psychotria*? Psyle cyclotides were partially characterized by their hydrophobicity,[12,42−44] a method that could fail to identify some hydrophilic cyclotides and thereby provide an incomplete representation of cyclotide diversity.[42] However, we used LC-MS with a gradient covering a wide range of hydrophilic and hydrophobic properties; thus, we are confident the majority of cyclotides present in extracts were detected. Since the crude extracts of *P. insularum* stem, leaf, and Samoan remedy had no characteristic indicators of cyclotides, it is reasonable to conclude that cyclotides are not the main compounds responsible for the therapeutic activity of *P. insularum* Samoan remedies.

Taxonomic Complexity of *Psychotria*. *Psychotria* (subfamily Rubioideae, tribe Psychotrieae) are usually abundant flowering shrubs or small trees in understory vegetation throughout tropical lowland regions. They exhibit high levels of insular endemism and are taxonomically complex, in part due to the enormous number of species (∼1400−2000) in the genus,[45−53] but also from the absence of morphologically useful characters available to delineate groups.[49−53] A comprehensive phylogenetic analysis of *Psychotria* is lacking, although, several regional treatments have been undertaken in Mesoamerica,[48,54,55] Samoa,[56] Fiji,[45] New Guinea,[50] Micronesia,[49,57] and the Marquesas.[52]

Systematic investigations of *Psychotria* show that relationships within the genus are far from resolved. The genus has two complexes, *Palicourea* and *Psychotria*, and *Psychotria* can be subdivided into pantropical subgenus *Psychotria*, neotropical subgenus *Heteropsychotria*, and African subgenus *Tetramerae*, but Asian/Oceanic relationships are more complex.[51,58−62] All species in this study belong to the *Psychotria* complex; however, the three documented cyclotide-containing species are not simultaneously included in any morphological or molecular analyses. Andersson (2002) proposes that a more stable classification of the *Psychotria* complex requires subdivision into two clades based on pyrene morphology: *P. sensu stricto* is pantropical with most species distributed throughout the Neotropics and Africa, while the Pacific clade includes species of the Indian Ocean and Pacific Islands. The Pacific clade remains undivided; however, *P. sensu stricto* is partitioned into four supported geographically distinct subclades: neotropical subclade I, African subclade II, East Asian subclade III, and Australian/Asian subclade IV.[62]

Although some ambiguity remains over the typification of *P. sensu stricto*, Andersson's analysis illustrates that taxonomic and geographical considerations cannot yet account for cyclotide expression in *Psychotria*. The species screened for cyclotides were represented in each clade, except subclade II, as shown in Table 3.[62] It is tempting to hypothesize that since *P. leptothyrsa* is Micronesian,[49] it belongs to the Pacific clade and *P. suterella* and *P. vellosiana* with Brazilian origins would fall in *P. sensu stricto*, possibly neotropical subclade I. Thus, cyclotide expression in *Psychotria* would not be restricted to any clade, subclade, or

Table 3. Species of *Psychotria* Screened for Cyclotides and Included in Cladistic Analysis by Andersson[62]

plant species	origin	clade
P. carthagenensis Jacq.	French Guiana	*P. sensu stricto*, subclade I
P. erthrocarpa Schldtl.	Mexico	*P. sensu stricto*, subclade I
P. insularum A. Gray	Samoa	Pacific clade
P. mariana Bartl. Ex. D.C.	Mariana Islands	Pacific clade
P. psychotrioides (D.C.) Roberty	Congo	*P. sensu stricto*, subclade III
P. serpens L. Mant.	Hong Kong	*P. sensu stricto*, subclade IV
P. viridis Ruís & Pavón	Bolivia	*P. sensu stricto*, subclade I

geographical region. However, without an inclusive phylogenetic study, the only assertion possible is that cyclotide expression is absent in species included in each clade and all subclades examined to date.

Structure of Rubiaceous Cyclotides. The sequences of six novel cyclotides (psyles A−F) from *P. leptothyrsa* are summarized in Table 4. In congruence with previous cyclotide structure reports,[11,43] psyle cyclotides have six conserved cysteines, Glu in loop 1, at least one cationic residue, highly conserved loops 1 and 4, and variable loops 3 and 6 with respect to number and type of residue. Psyle C is the most hydrophilic cyclotide, as reflected by the earliest elution time (19 min) on RP-HPLC. With increasing hydrophobicity (psyles F, E, B, A, D, respectively), bracelet subfamily cyclotides are retained approximately 12−21 min after elution of psyle C. The bracelet psyle cyclotides may form a hydrophobic patch in loops 2 and 3, a common feature of the bracelet subfamily that influences bioactivity.[42−44] The longest psyle cyclotides are psyles E and F (each 31 amino acids), while psyle C consists of only 25 residues. Psyle C is the first acyclic variant found in Rubiaceae, in part because, unlike other psyle cyclotides, digestion with endoproteinase Glu-C failed to produce a linear peptide after cleavage at the Glu in loop 1. Psyle C is the second naturally occurring linear cyclotide known, as illustrated in a modeled structure (Figure 3B); the first, violacin A, was isolated from *Viola odorata* L.[28]

The discovery of psyle C enhances our structural knowledge of linear cyclotides. By elucidating the cDNA clone of the linear violacin A, a point mutation introducing a stop codon was discovered that inhibited the translation of an aspartic acid, a residue potentially necessary for cyclization.[63] Although the mechanism by which cyclotides are cyclized in vivo is not fully described, the N and C termini of the precursor are joined in loop 6 at the highly conserved asparagine (Asn) (occasionally aspartic acid (Asp)) residue.[12,64,65] In fact, in vivo asparaginyl bond hydrolysis seems necessary for cyclization, and asparaginyl endopeptidase may catalyze the peptide bond cleavage and ligation of cyclotides in one event.[66] The bracelet psyle cyclotides and cyclopsychotride A all possess the Asn/Asp residue in loop 6, and although the cDNA clone of psyle C has not been elucidated, a Asn/Asp residue is absent in loop 6 of psyle C, as seen with the other known linear variant, violacin A. Psyle C and violacin A share other similarities. They contain a local hydrophobic region in loop 2 (two phenylalanine (Phe) residues) associated closely with hydrophobic residues in loop 5; both are more hydrophilic than other cyclotides with four positively charged residues, and neither contains the conserved Pro residue in loop 6 of Möbius cyclotides.

By comparing sequence data of psyle cyclotides (Table 4) to those of established cyclotides, we illustrate similarities of psychotrian cyclotides to each other and establish macrocyclic peptides. Fundamental cyclotide structural elements (cystine knot, Glu in loop 1, and a surface-exposed hydrophobic patch) are present in psyle cyclotides and cyclopsychotride A. Loops 1 and 4 are embedded in the ring of the CCK, are conserved, and play a significant role in the proper folding of cyclotides.[67−71] Psyle cyclotides and cyclopsychotride A conform to the conserved sequences in loop 1 (CGE (T/S) C) and loop 4 (C (T/S/L/I) C). All but six cyclotides recorded to date have a glycine (Gly) residue preceding Cys IV,[27] and data support that the conserved Gly residue readily adopts a positive φ angle required for the type II β-turn needed to connect loop 3 to the cystine knot.[70,71] The cyclotide profiles from *Psychotria* all have the Gly residue in loop 3.

This study demonstrates the diversity of cyclotide profiles and the suitability of the CCK motif as a scaffold for drug development since observed loop variability reflects the ability of the CCK framework to tolerate structural changes. Typically, loop 3 of bracelet cyclotides contains six to seven residues featuring a short helical segment, while Möbius cyclotides have only four residues and form a type II β-turn. The bracelet cyclotides, psyles A and B, have only four residues in their shortened loop 3, perhaps indicating that this region of each cyclotide forms a turn as seen in Möbius cyclotides. It has been suggested that the Pro residues in loop 2 of bracelet cyclotides and that of loop 3 in Möbius cyclotides play an important role in the turn geometry of macrocyclic peptides.[70] The shortened loop 3 of psyles A and B has a Pro residue in position 3, and the conserved Pro residue in loop 2 preceding Cys III in the bracelets is substituted with a Gly residue. This increases support for the assertion that psyles A and B contain a loop 3 comparable to Möbius cyclotides. A similar occurrence was seen in some cyclotide profiles of the Australian *Hybanthus*.[27] Therefore, loop 3 of psyles A and B may prove useful in protein engineering or drug design programs aimed at grafting foreign bioactive epitopes onto cyclotide sequences.[2,27]

Cytotoxic Activity of Psyle Cyclotides. To elucidate the bioactivity of novel peptides, the more abundant peptides psyles A, C, and E were tested in the fluorometric microculture cytotoxicity assay using the human lymphoma cell line U937-GTB. The concentration-dependent cytotoxicities are shown in Figure 4 as survival index (average fluorescence of treated cells relative to control cells minus the blank) expressed as percent. Sequence modifications affect the selectivity and bioactive potency of cyclotides, which could potentially control the therapeutic index (i.e., the ratio between therapeutic and toxic effects). The cytotoxic activity of cyclotides against several human tumor cell lines (lymphoma, leukemia, myeloma)[19,20] is influenced by the primary structure (eliminating the Glu in loop 1 decreases activity by 48-fold, and alkylation causes a complete loss of activity),[11] as well as by loop size and placement of charged residues.[28,63,69]

The IC_{50} values for psyles A, C, and E were 26, 3.50, and 0.76 μM, respectively. The most active cyclotide, psyle E, is equipotent to bracelet cyclotides cycloviolacin O2, vibi G, vibi H, and vitri A. They all have IC_{50} values around 1 μM, at least three positively charged residues, and a similar hydrophobic loop 3 with six to seven residues.[19,20,41] Loop 3 plays a role in maintaining activity, as illustrated by the drastically reduced activity of the bracelet psyle A, which has a shortened loop 3 more similar to that of Möbius cyclotides. Recent evidence suggests cyclotides may form multimeric pores with channel-like activity in cell membranes.[22] Since membrane-active peptides are considered promising leads toward novel anticancer agents, further studies exploring the mode of membrane necrosis are needed.[21] Interestingly, we provide new insight into the structure−activity relationship of linear cyclotides. Linearization of cyclotides results in loss of activity, and violacin A exhibits almost no activity in hemolytic assays.[63,71] However, psyle C maintains cytotoxic activity comparable to cyclic varv A and E; therefore, additional bioactivity and potency studies of psyle C are warranted.

Cyclotide Cocktails. A study of Australian *Hybanthus* deduced that, on average, 10 to 20 cyclotides were found per species.[27] Cyclotide diversity in Rubiaceae also fluctuates from one to at least 17 macrocyclic peptides per species (Table 1). Only one cyclotide

Table 4. Sequences of Cyclotides from *Psychotria leptothyrsa*

			sequence[c]												
			Loop 6												
cyclotide	MW[a]	AA[b]	Cys	I	1	II	2	III	3	IV	4	V	5	VI	6
							Bracelet								
psyle A	2913	28	GIA	**C**	GES	**C**	VFLG	**C**	FIPG	**C**	S	**C**	KSKV	**C**	YFN
psyle B	3010	28	GIP	**C**	GET	**C**	VAFG	**C**	WIPG	**C**	S	**C**	KDKL	**C**	YYD
psyle D	3258	28	GIP	**C**	GES	**C**	VFIP	**C**	TVTALLG	**C**	S	**C**	QNKV	**C**	YRD
psyle E	3258	31	GVIP	**C**	GES	**C**	VFIP	**C**	ISSVLG	**C**	S	**C**	KNKV	**C**	YRD
psyle F	3212	31	GVIP	**C**	GES	**C**	VFIP	**C**	ITAAVG	**C**	S	**C**	KNKV	**C**	YRD
vibi G	3226	31	GTFP	**C**	GES	**C**	**VFIP**	**C**	LTSAIG	**C**	S	**C**	KSKV	**C**	YKN
vibi H	3270	31	GLLP	**C**	AES	**C**	VYIP	**C**	LTTVIG	**C**	S	**C**	KSKV	**C**	YKN
vitri A	3152	30	GIP	**C**	GES	**C**	VWIP	**C**	ITSAIG	**C**	S	**C**	KSKV	**C**	YRN
							Linear								
psyle C	2840	25	KL	**C**	GET	**C**	FKFK	**C**	YTPG	**C**	S	**C**	SYPF	**C**	K
violacin A	3004	27	SAIS	**C**	GET	**C**	FKFK	**C**	YTPR	**C**	S	**C**	SYPV	**C**	K

[a] Molecular weight (MW) reflects the monoisotopic mass. [b] Number of amino acids (AA). [c] The number of I and L in the peptides is based on quantitative amino acid analyses; their position is based on sequence homology with previously described cyclotides (psyle F contains all I).

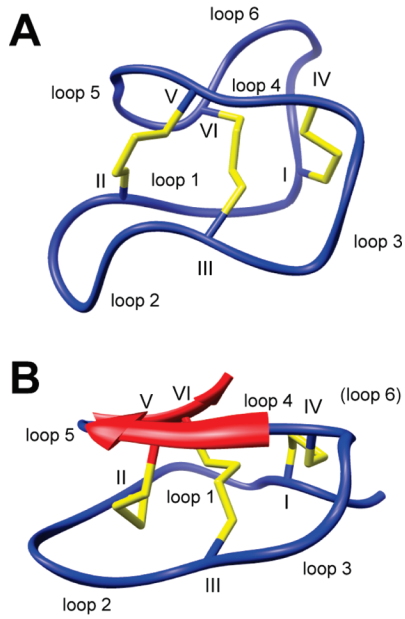

Figure 3. Modeled structures of psyle A and psyle C. Cysteine residues are numbered I−VI. (A) Backbone structure of psyles A. (B) Linear psyle C. Loop 6 in psyle C is in parentheses since it is open with two loose ends. Modeler 8v3 was used to make structures; cycloviolacin O1 (1NBJ) and violacin A (2FQA) were templates.[79]

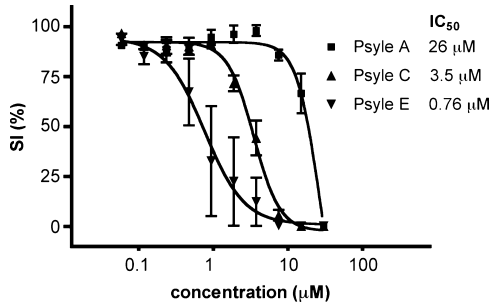

Figure 4. Cyclotide cytotoxicity on the human lymphoma cell line U-937 GTB. The concentration−response curves with IC_{50} values in parentheses for psyles A, C, and E. Survival index (SI) and IC_{50} values are calculated as described in Experimental Section. Each point represents the mean and the error bars ± SEM.

was isolated from *P. vellosiana*; however, extraction and sequencing methods differed from those used to isolate other rubiaceous cyclotides.[18] In contrast, *P. leptothyrsa* has at least six novel cyclotides (Table 4). Why are cyclotides sometimes expressed as complex cocktails?

Cyclotides decrease proliferation of bacteria and fungi,[17] inhibit growth and survival of lepidopteron larvae,[12,13,67] and inhibit development of nematode larvae and mobility of adult worms.[68] Thus, a growing body of evidence indicates that cyclotides function in plant protection; however, most of the material examined has been of cultivated origin, and few studies address geographical variations and possible abiotic events triggering expression.[27,69] Trabi and colleagues[69] analyzed seasonal and geographical variations in cyclotide diversity. The African violet (*Viola hederacea* Labill.) has a set of cyclotides consistently expressed throughout the year and produces additional cyclotides at various times in the year that do not correlate with temperature, number of daylight hours, or location. In contrast, cyclotide expression was significantly increased in the Swedish violet (*V. odorata*) during the warmest months of the year. An examination of Australian *Hybanthus* revealed a latitudinal pattern; the number of unique cyclotides increased as latitude decreased.[27] On the basis of these reports, it is tempting to postulate that psyle cyclotides are plant defenses expressed as complex cocktails in response to specific conditions (i.e., herbivore pressure, light intensity, and nutrient or water availability); however, further studies providing supporting data are necessary.

The outcome of this study is a better understanding of structural diversity and structure−activity relationships of cyclotides in *Psychotria*. However, not every cyclotide in *P. leptothyrsa* was fully sequenced, and an exhaustive screening of *P. suterella* and *P. vellosiana* for additional cyclotides has not been undertaken. A recent study successfully characterizing the complete cyclotide fingerprint of *Oldenlandia affinis* provides new knowledge of structure−activity relationships in rubiaceous cyclotides.[73] Future cyclotide diversity research on *Psychotria* should focus on completing the fingerprints of cyclotide-producing *Psychotria*, as well as fully exploring the possible cyclotide expression of closely related species, to more fully understand the evolutionary distribution of cyclotides in Rubiaceae.

Experimental Section

General Experimental Procedures. Acetonitrile, MeOH, HCO_2H, and chymotryspin were purchased from Merck (Darmstadt, Germany). Iodoacetamide, $(NH_4)HCO_3$, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl), EDTA, guanidine-HCl, citric acid, 1,4-dithiothreitol (DTT), and sequencing grade endoproteinase Glu-C derived from *Staphylococcus aureus* strain V8 were supplied by Sigma Chemical Co. (St. Louis, MO). All other digestion enzymes were purchased from Porcine Promega (Madison, WI). The U-937 GTB human lymphoma cell line was purchased from American Type Culture Collection (ATCC), The Global Bioresource Center (Manassas, VA).

Mass spectra were acquired on a Finnigan LCQ ion trap mass spectrometer and obtained in positive ion mode over a range 800−2000 mass-to-charge ratio (*m*/*z*) using Xcalibur from Finnigan/Thermo Electron. The capillary temperature was set at 220 °C with a spray voltage of 4.5 kV and a flow rate of 0.3 mL/min (solvent B, 60% MeCN, 0.1% formic acid in Millipore water).

Preparative RP-HPLC was performed on extracts of *P. leptothyrsa*. An LDC Milton Roy gradient HPLC system was coupled to a Shimadzu SPD-6A UV detector collecting data at 280 nm. Extracts were injected onto a ReproSil-Pur C18-AQ column (250 × 20 mm, 10 μm, 300 Å with 30 × 20 mm guard column) with a linear gradient from solvent A (10% MeCN, 0.1% HCO_2H in Millipore H_2O) to solvent B over 60 min at a flow rate of 5 mL/min. Fractions were collected every 90 s and analyzed by ESMS to identify fractions with masses between 2800 and 3500 Da. Samples derived from large-scale extractions were preloaded onto the column and fractions collected every 60 s. Fraction purity was checked using analytical RP-HPLC. When further purification was needed, RP-HPLC was repeated using an Amersham Pharmacia Biotech ÄKTA basic system with a UV-900 detector collecting data at 215, 254, and 280 nm using the same solvents until pure cyclotides were obtained. After freeze-drying, samples were quantified by absorbance with a UV Visible Recording Shimadzu UV-160 spectrophotometer set at 280 nm. A blank of 10% MeCN, 0.1% HCO_2H was used, and concentration (*C*) was calculated as follows: $C = A$ (absorbance)/extinction coefficient (ε).

LC-MS was performed on samples derived from the small-scale extraction using an Amersham Pharmacia Biotech ÄKTA basic system coupled online to a Finnigan LCQ ion trap MS. Mass spectra were obtained in positive ion mode over the range 800−2000 *m*/*z*, the capillary temperature was set at 220 °C and the spray voltage to 4.5 kV. Samples were injected onto a Vydac C18 column (250 × 4.6 mm, 5 μm, 300 Å) with a gradient from solvent A to B over 31 min and a flow rate of 0.7 mL/min. The flow was split, and 25% of the flow was directed to the MS. LC-MS was repeated for leaf and stem fractions of *P. leptothyrsa* using the same system and detector with a modified gradient over 75 min using a Grom C18 column (100 × 2.1 mm, 5 μm, 300 Å) with a flow rate of 0.3 mL/min.

The digestion products were sequenced by nanospray MS/MS using a Finnigan LCQ mass spectrometer coupled with a Protana NanoES source. The spray voltage was set at 0.5 kV and capillary temperature was 150 °C. Spectra were obtained in positive ion mode over the range 100−2000 *m*/*z*. Xcalibur software from Finnigan/Thermo Electron was used for data acquisition and processing. Amino acid composition was independently verified at the Amino Acid Analysis Centre (Department of Biochemistry, Biomedical Center, Uppsala University, Sweden) and is available as Supporting Information Table 2. Each peptide was hydrolyzed for 24 h at 110 °C with 6 N HCL containing 2 mg/mL phenol, and hydrolysates were analyzed by ninhydrin detection using a LKB model 4151 Alpha Plus amino acid analyzer.[3]

Plant Material. Eleven species of *Psychotria* were selected from collections at NTBG (Agreement MM-4/20/05, Table 2) for cyclotide characterization. Voucher specimens (Lorence 7678, 8243, 8339, 8386, 8793, 9196; Flynn 1716, 1721, 6544, 8386) are retained at the Pacific Tropical Botanical Garden Herbarium (PTBG), Kalaheo, Hawaii. A preserved remedy used for treating cataracts was prepared by Samoan healer Fa' ifau with young leaves of *P. insularum* and collected on July 26, 1997, in Tafua during an investigation of indigenous medicinal plant use. Voucher specimens are deposited at Brigham Young and Harvard Universities.

Extraction. Dried samples (~0.5−2.0 g) were ground into a fine powder and extracted into 50 or 100 mL of solvent B (60% MeCN, 0.1% HCO_2H in Millipore H_2O) for 24 h with continuous shaking. Extractions were centrifuged for 5 min, and MeCN concentration of decanted supernatant was reduced to 10% by evaporation. The preserved remedy was diluted to 10% EtOH with Millipore H_2O and 0.1% HCO_2H. The resulting extracts were subjected to SPE on Isolute columns (c18-EC 500 mg/10 mL or 5 g/25 mL from Argonaut Technologies, Redwood City, CA) that were soaked in 100% MeOH and equilibrated with solvent A. Hydrophilic compounds were eluted with solvent A and hydrophobic compounds (including cyclotides) eluted with solvent B followed by reduction in MeCN concentration to 10% under heat with a SpeedVacPlus. Larger scale extractions of *P. leptothyrsa* (~11 g leaf and ~9 g stem tissue) were slightly modified with dried, ground tissues extracted into 400 mL of solvent B with continuous shaking for 24 h followed by collection of the aqueous layer and overnight extraction using 200 mL of solvent B. The decanted supernatants were centrifuged, reduced to 10% MeCN, and subjected to SPE as previously described.

Alkylation and Enzymatic Digestion. To reduce cysteines of each cyclotide-containing fraction, solution A (250 μL of 1 M Tris-HCL, pH 8.5, containing 4 mM EDTA and 750 μL of 8 M guanidine-HCl) and solution B (3 mg of DTT in 500 μL of solution A) were prepared. After adding 90 μL of solution A and 10 μL of solution B to purified, dried peptides (1 to 5 nmol) they were incubated in the dark under nitrogen for 1 h. Two methods of alkylation were employed. For alkylation with iodoacetamide, 250 μL of solution C (1 M Tris-HCL, pH 8.5, containing 4 mM EDTA and 250 μL of Millipore H_2O containing 50 mg of iodoacetamide) was added to each reduced peptide; the alkylation reaction was terminated after 2 min by adding 200 μL of 0.5 M citric acid. Alkylation with bromoethylamine was performed by adding solution D (2 mg of bromoethylamine dissolved into 10 μL of solution B) to each peptide, incubating overnight in a warm H_2O (37 °C) bath, and terminating the reaction by injection on RP-HPLC. Following isolation by RP-HPLC and mass identification by MS, each alkylated peptide fraction was dried without heat on a SpeedVacPlus for subsequent enzymatic digest with trypsin, chymotrypsin, and endoproteinase Glu-C. For trypsin and chymotrypsin cleavage, a buffer of 40 μL of 1 M ammonium hydrogen bicarbonate and 710 μL of Millipore water was vortexed with 6 mg of trypsin or chymotrypsin; 50 μL of this solution was added to each fraction. For endoproteinase Glu-C digestion, 2 μg of Glu-C was dissolved in 1 mL of 50 mM $(NH_4)HCO_3$; 200 μL of the solution was added to each fraction. Samples were incubated for 48 h at 37 °C and dried on a SpeedVacPlus.

Cytotoxicity Screening. A nonclonogenic fluorometric microculture cytotoxicity assay,[74,75] which measured cell integrity based on the fluorescence generated from hydrolysis of fluorescein diacetate to fluorescein by cells with intact membranes, was completed with psyles A, C, and E. The U-937 GTB human lymphoma tumor cell line was chosen since previous screens show cyclotides actively inhibit the growth of this cell line.[20] The peptides were dissolved in 10% EtOH, and 20 μL/well was dispensed onto 96-well microtiter plates (10 concentrations ranging from 30 to 0.5 μM) in triplicate with the experiment repeated in triplicate. Phosphate buffer solution (PBS) and Triton were included as negative and positive controls, respectively. To each well, 180 μL of media (RPMI-1640, 10% heat-inactivated fetal bovine serum, 60 μg/mL penicillin, 50 μg/mL streptomycin, and 2 mM glutamine) containing U-937 GTB human lymphoma tumor cells (2×10^4 cell/well) was added, and microtiter plates were incubated (37 °C, 5% CO_2) for 72 h. Following Q2-buffer preparation (40 mL of 125-NaCl, 10 mL of 25 mM Hepes, 400 mL of Millipore H_2O, 4 M NaOH), plates were centrifuged at 1000 rpm for 5 min and cells washed with PBS. After fluorescein diacetate (FDA) was added to preheated Q2-buffer (1 μL/mL), 100 μL of FDA/Q2-buffer was added to each well using the Multidrop 384 Labsytem followed by plate incubation (37 °C, 5% CO_2) for 40 min. Fluorescence was measured on a Labsystems Flouroskan II at 538 nm, following excitation at 485 nm, and inhibition of growth was quantified as a survival index (average fluorescence of test wells relative to control wells minus the blank) expressed as percent.

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Supporting Information Available: The following information is available free of charge via the Internet at http://pubs.acs.org: Table 1 (Fragments obtained by enzyme digestion and nanospray MS/MS sequencing) and Table 2 (Amino acid composition of psyle A−F).

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