Cyclopsychotride A, a Biologically Active, 31-Residue Cyclic Peptide Isolated from Psychotria longipes

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CYCLOPSYCHOTRIDE A, A BIOLOGICALLY ACTIVE, 31-RESIDUE CYCLIC PEPTIDE ISOLATED FROM *PSYCHOTRIA LONGIPES*

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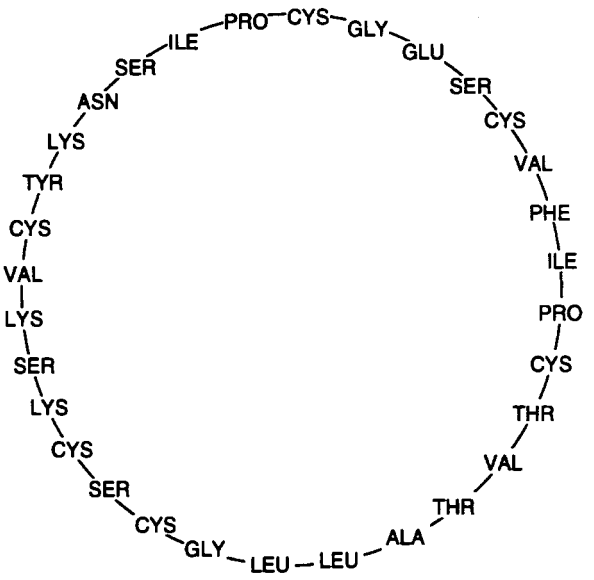
ABSTRACT.—A preliminary characterization is provided of a naturally occurring cyclic peptide with interesting and potent biological activity. A 31-residue cyclic peptide, designated cyclopsychotride A [**1**], was obtained from the organic extract of the tropical plant, *Psychotria longipes*. Compound **1** inhibited [^{125}I] neurotensin (NT) binding to HT-29 cell membranes (IC_{50} 3 μM) and also stimulated increased levels of cytosolic Ca^{2+} in two unrelated cell lines that do not express NT receptors. The peptide was found to dose-dependently increase intracellular Ca^{2+} at concentrations ranging from 3 to 30 μM, and this response was not blocked by a known NT antagonist. Cyclopsychotride A [**1**] possesses three disulfide linkages and is thought to be the largest cyclic peptide isolated from a natural source. Both ^{1}H-nmr and cd spectroscopy showed **1** to be highly structured.

The genus *Psychotria* (Rubiaceae) consists of 1400 tropical trees and shrubs, with most ethnobotanical interest ascribed to the dimethyltryptamine content of *Psychotria viridis*, one of the plants used to prepare hallucinogenic beverages by the indigenous peoples of the Amazon Basin (1). Some species of *Psychotria* contain pyrrolidinoindole polymers such as psychotridine, isopsychotridine, the quadrigemines, and hodgkinsine (2). At least two species in the Rubiaceae are known to produce cyclic peptides; cyclic hexapeptides with antitumor activity have been isolated from *Rubia cordifolia* (3) and *Bouvardia ternifolia* (4).

We became interested in the organic solubles obtained from *P. longipes* Muell. Arg. in the course of testing natural products for neurotensin (NT) antagonistic activity, which ultimately led to the isolation of **1**. The crude extract was found to selectively inhibit binding of [^{125}I] NT to membranes prepared from human HT-29 cells (5). The functional activity of NT inhibitors can be ascertained by examining their effects on NT-induced cytosolic Ca^{2+} levels in HT-29 cells (6).

RESULTS AND DISCUSSION

Hydrolysis of cyclopsychotride A [**1**] resulted in the generation of the amino acids shown in Table 1. Sequential Edman degradation of native **1** was unsuccessful, suggesting the starting material to be *N*-terminally blocked or cyclized. The typical increase in background with increasing number of cycles was noted during the sequencing trial. Treatment of **1** with proteolytic enzymes such as trypsin, chymotrypsin, and Glu-C also failed to produce any detectable peptide fragments. In order to obtain internal fragments, **1** was subjected to partial acid digestion with 0.015 M HCl at 110° *in vacuo* for 16 h (Figure 1). Peptide fragments labeled 1 and 2, with R_ts of 31.43 min and 39.69 min, respectively, were isolated and subjected to Edman degradation to determine their amino acid sequences. This resulted in the overlapping primary sequence as indicated by the underlined residues shown:



1

Fragment 1

Ser Ile Pro (X) Gly Glu Ser (X) Val Phe Ile Pro (X) Thr Val Thr
Ala Leu Leu Gly (X) Ser (X) Lys Ser Lys Val (X) Tyr Lys

Fragment 2

Ala Leu Leu Gly (X) Ser (X) Lys Ser Lys
Val (X) Tyr Lys Asn Ser Ile Pro (X) Gly

TABLE 1. Amino Acid Composition Analysis of Cyclopsychotride A [1].

Amino Acid	Residues/mol[a]	
	Experimental	Theoretical
Asp+Asn	0.96	1
Thr	1.77	2
Ser	3.56	4
Glu+Gln	0.95	1
Pro	2.40	2
Gly	1.90	2
Ala	1.00	1
Cys	ND[b]	6
Val	2.73	3
Met	0.00	0
Ile	1.82	2
Leu	1.86	2
Tyr	0.86	1
Phe	0.91	1
His	0.00	0
Lys	2.66	3
Arg	0.00	0

[a]Normalized to 1 residue of Ala.
[b]Not determined

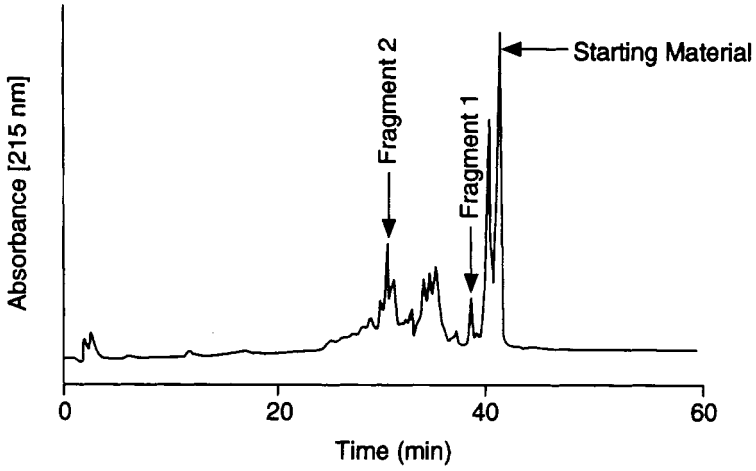


FIGURE 1. Hplc analysis of the partial acid hydrolysis of **1** [ABI C_8, 0.2 mm×3.0 mm column, 150 µl/min flow, 0% to 70% linear gradient H_2O (0.1% TFA)/90% MeCN (0.09% TFA)].

Because cysteine residues do not result in identifiable PTH derivatives during Edman reaction [indicated above by (X)], the peptide was carboxamidomethylated (CAM) as described earlier (7). The CAM-modified peptide was purified by reversed-phase hplc. Glu-C treatment, which cleaves at the *C*-terminus of Glu-residues, cleaved the cyclic peptide into a linear peptide. The linear peptide was sequenced, confirming the above primary sequence, including positive identification of the CAM-cysteine residues. Based on these results it is clear that **1** is a cyclic 31-residue peptide. To our knowledge, **1** represents the largest cyclic peptide isolated from a natural source.

To determine the stereochemical structure of the respective amino acid residues, the peptide was hydrolyzed and the resultant mixture of residues was derivatized with Marfey's reagent (8). Comparison of R_fs via reversed-phase for the derivatized mixture and L- and D-amino acid standards, respectively, indicates that all residues are L-isomers (data not shown).

Hrfabms (glycerol, 0.1% TFA) gave an M+H ion, *m/z* 3229.490417, $C_{139}H_{222}O_{41}N_{35}S_6$ requires 3229.4685. No other diagnostic peaks were noted. The ms data coupled with a negative Ellman's test for sulfhydryls suggest the presence of three disulfide linkages in the peptide (9). Treatment of **1** with dithiothreitol prior to analysis by reversed-phase hplc, showed the decrease in R_t expected for the reduced form (data not shown). Because of the difficulties associated in cleaving **1** with proteolytic enzymes, the exact positions of the disulfide bridges are not assigned.

The 500 MHz ^{1}H-nmr spectra of **1** recorded on samples dissolved in MeOH-d_3 and MeOH-d_4 are shown in Figure 2. The spectra are indicative of a highly structured polypeptide with numerous resonances significantly shifted from the values expected for unstructured peptides. Nmr spectra acquired on the peptide dissolved in MeOH-d_4 (Figure 2 inset) displayed a subset of approximately 20 amide protons (out of a possible 29) that are slow to exchange with solvent deuterons; many remained unexchanged for days at 25°. Slowly exchanging amide protons are often involved in intramolecular hydrogen bonding and are suggestive of stable secondary structure. The majority of these resonances displayed large vicinal coupling constants of 8–10 Hz, also indicative of a structured polypeptide backbone.

Analysis of the cd spectrum (Figure 3) in H_2O by the variable selection method suggests that **1** is comprised of approximately 29% β-sheet, 23% β-turns, 14% helix, and 28% disordered structure (9).

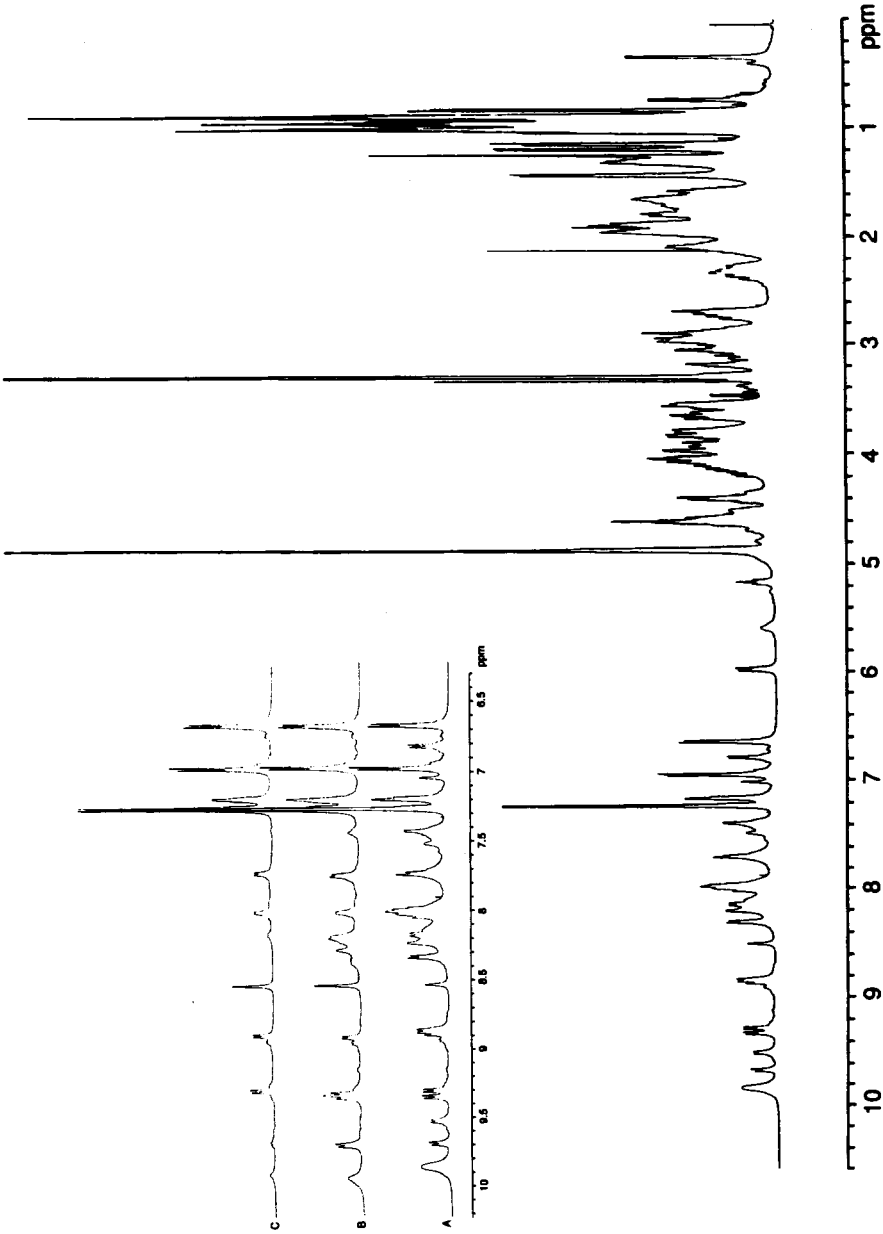


FIGURE 2. ^{1}H-Nmr spectrum of **1** (500 MHz in MeOH-d_3). Inset shows expansions of amide/aromatic proton spectra. A, sample dissolved in MeOH-d_3; B, spectrum acquired on **1** dissolved in MeOH-d_4 at t=15 min; and C, As B, but t=24 h.

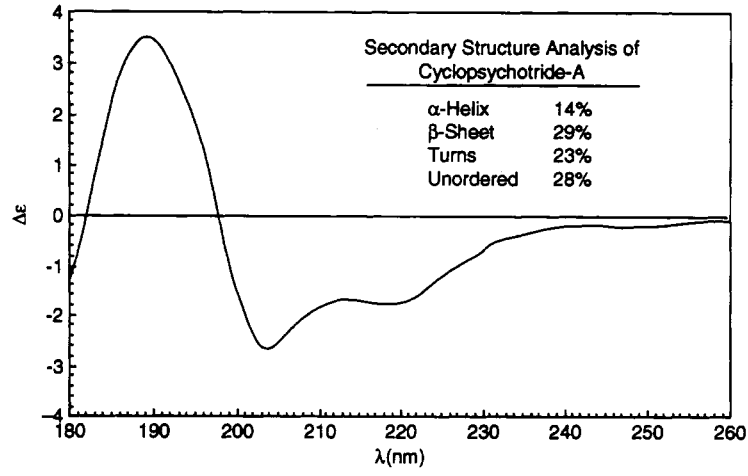


FIGURE 3. Cd spectrum of **1**.

The cyclic peptide had an IC_{50} of 3 μM in the NT binding assay. To determine whether **1** possessed functional antagonist activity, its effects on NT-induced elevation of HT-29 cytosolic Ca^{2+} levels were examined (Figure 4). However, 3–30 μM of **1** were found to dose-dependently increase intracellular Ca^{2+} concentrations and this response was not blocked by a known NT antagonist. Also, the compound increased cytosolic Ca^{2+} in two unrelated cell lines that do not express NT receptors. This activity precluded a demonstration that **1** is a NT antagonist, but suggests the peptide is acting through another receptor(s). The mechanism by which **1** increases cytosolic Ca^{2+} levels in cells is under investigation. Studies of additional biological activities are in progress. Any attempt to ascribe the medicinal ramifications of the observed pharmacological activities would be highly speculative at this point in time.

In summary, from an extract of *Psychotria longipes*, we have isolated the largest naturally occurring cyclic peptide found to date. The primary sequence has 31 residues, and preliminary characterization by cd and nmr spectroscopy reveals a highly compact and folded, globular-like structure, not frequently observed in peptides of this size. The peptide has interesting biological activities, inhibiting NT binding to its receptor and stimulating intracellular Ca^{2+} by an as yet uncharacterized mechanism which is under

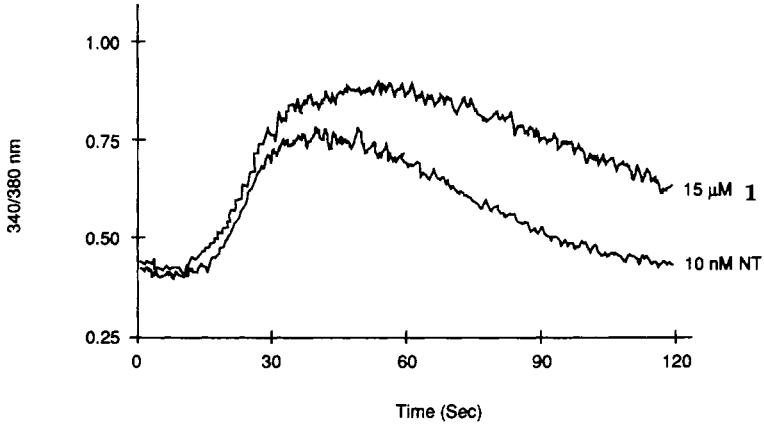


FIGURE 4. Mobilization of $[Ca^{2+}]$ in fura-2 loaded HT-29 cells (see Experimental).

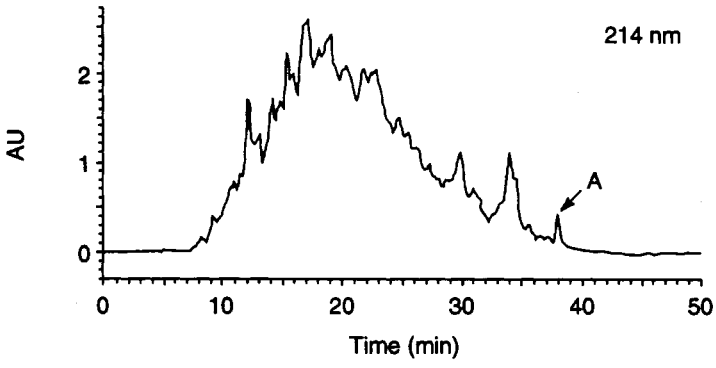


FIGURE 5. Prep. hplc fractionation of MeOH-soluble residue containing cyclopsychotride A [**1**]. Fraction A (peak) corresponds to **1**.

investigation. These characteristics prompted us to examine the peptide further; more rigorous studies concerned with the assessment of biophysical properties and biological activities are ongoing. Elucidation of the tertiary structure assignments is in progress.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-nmr spectra were acquired on a Varian Unity-500 NMR spectrometer operating at 499.843 MHz. Spectra were acquired on samples containing approximately 6 mg of peptide dissolved in 0.65 ml of MeOH-d_3 or MeOH-d_4 (99.9 atom % D, Merck Isotopes) at 25°. Proton chemical shifts were referenced internally to 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid at 0.00 ppm. Mass spectroscopy data were obtained on a VG ZAB-HF spectrometer. The cd spectrum was recorded on a Jasco J-720 spectropolarimeter.

PLANT MATERIAL.—The whole plant was collected in Brazil in June 1972, under the auspices of Dr. Robert E. Perdue, Medicinal Plant Laboratory, USDA, Beltsville, Maryland, where a voucher specimen is maintained (B824510).

EXTRACTION AND ISOLATION.—The CH_2Cl_2-MeOH (1:1) extraction of 800 g of *P. longipes* resulted in 17.3 g of organic solubles. A 12.47-g portion was passed through a 100-g plug of Vydac C_{18} adsorbent (15–30 μm, 300 Å), eluting first with 800 ml H_2O (0.2% TFA), then by an equal volume of MeOH (0.2% TFA). Solvent was removed under vacuum from the MeOH fraction to give 4.46 g dry residue. The residue was redissolved in MeOH (1 g/4 ml) and chromatographed (4×1 g) on a DeltaPak C_{18}, 300 Å, 15 μm, 5 cm×30 cm column (Waters), employing a H_2O (0.3% TFA)/MeCN (0.3% TFA) linear gradient at 80 ml/min, 0% to 55% MeCN over 40 min, to give fraction A (Figure 5). Fraction A (101 mg) was redissolved in MeOH (25 mg/ml), then chromatographed (25 mg×4) on a Spherisorb phenyl, 80 Å, 5 μm, 2.2 cm×25 cm column (Phenomenex) using the same linear gradient as above with a 12 ml/min flow rate, to yield 19.5 mg of **1** (0.002 dry wt %).

AMINO ACID AND SEQUENCE ANALYSIS.—For the total amino acid analysis, the peptide was incubated in constantly boiling 6 N HCl containing 0.2% phenol for 24 h at 110° *in vacuo*. The resulting hydrolysates were analyzed by post-column derivatization with ninhydrin using a Beckman System 6300 high-performance amino acid analyzer. The reversed-phase hplc-purified peptide samples were subjected to Edman degradation using an Applied Biosystems model 470A protein/peptide sequencer (7). The phenylthiohydantoin amino acids were analyzed by an on-line Applied Biosystems model 120A analyzer.

BIOLOGICAL ASSAYS.—Competition binding studies were performed by incubating crude HT-29 carcinoma cell membranes with 6 pM [^{125}I]NT for 30 min in a final volume of 1 ml. Assays were terminated by vacuum filtration over Whatman GF/B fiber filtermats (presoaked for 2 h in 0.2% polyethyleneimine) using a Brandel M24 cell harvester. Filter-bound radioactivity was quantitated using a Packard Multi-Prias gamma counter. Nonspecific binding was determined using 1 μM NT and represented less than 15% of total bound radioactivity. NT-induced increases in cytosolic Ca^{2+} in HT-29 cells were determined using methods modified from Turner *et al.* (5). Cells were loaded with 2 μM Fura-2/AM for 40 min at 37° and then washed three times with 37° buffer by centrifugation at 200×g for 10 min followed by resuspension. Changes in cytosolic Ca^{2+} levels in cell suspensions (2×10^6 cells/ml) were monitored using a Delta-Scan Spectrofluo-

rometer (Photon Technology Int.). The ratio of emitted fluorescence at 510 nM was measured using excitation wavelengths of 340 nM and 380 nM (6).

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