Table II. Analytical Results of *Chlorella* and Tea Leaves[a]

| sample | metal | found, μg/g | cv, % | certified values, μg/g |
|---|---|---|---|---|
| *Chlorella* | Zn | 20.55 ± 0.24 | 1.1 | 20.5 ± 1.0 |
| | Cu | 3.50 ± 0.05 | 1.4 | 3.5 ± 0.3 |
| tea leaves (A)[b] | Ni | 6.28 ± 0.18 | 2.9 | 6.5 |
| ($HNO_3$ + $HClO_4$)[c] | Zn | 34.5 ± 2.5 | 7.2 | 34.1 |
| | Cu | 6.76 ± 0.26 | 3.8 | 7.1 |
| tea leaves (B)[b] | Ni | 6.34 ± 0.39 | 6.1 | 6.5 |
| ($HNO_3$ + $HClO_4$ + HF)[c] | Zn | 32.6 ± 1.6 | 4.9 | 34.1 |
| | Cu | 7.03 ± 0.24 | 3.4 | 7.1 |

[a] The values of the tea leaves are reference values. [b] The standard deviation (cv) was based on 6–12 replicate determinations including ashing, extraction, and HPLC steps. $N = 6$ for *Chlorella* and tea leaves (A). $N = 12$ for tea leaves (B). [c] Acids used in wet ashing method.

vitamin C was an effective masking agent for Mn in this biological sample.

In general, the results of *Chlorella* and tea leaves showed good agreement with the certified values reported by the NIES (Table I).

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# Structure–Activity Relationships of Cyclic and Acyclic Analogues of the Phytotoxic Peptide Tentoxin

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Structurally modified peptide analogues of the phytotoxin tentoxin demonstrated differences in biological activity as a result of changes in stereochemistry, amide bond N-methylation, and cyclic conformational constraint. Peptide analogues were prepared through a solution-phase peptide synthetic route. The bioactive properties of the compounds were determined in a lettuce seedling assay employed to measure chlorosis induction. A 12-atom ring of the peptide backbone and N-methylation at dehydrophenylalanine were required for full biological activity. Removal of the *N*-methyl group at alanine resulted in little change in chlorosis-inducing activity, but absence of both *N*-methyl groups at dehydrophenylalanine and alanine resulted in complete loss of activity in the analogues containing L-amino acids. A change in stereochemistry at alanine and leucine was used to evaluate biological activity upon conversion of cis–trans–cis–trans backbone conformation of the native sequence to cis–cis–cis–trans. Acyclic analogues having N-methylation at dehydrophenylalanine demonstrated low but significant chlorosis activity.

In recent years increasing attention has been given to small cyclic peptides demonstrating selective phytotoxic and growth-regulating activity in plants (Stoessel, 1981; Shimohigashi et al., 1978; Ueda et al., 1985; Walton et al., 1982). A number of these bioactive peptides display analogous structural moieties including the following: (1) cyclic, four amino acid containing sequences; (2) secondary amino acids (*N*-methylalanine and proline); (3) dehydro amino acids; and (4) alkyl and aromatic amino acid side chains. The non-host-specific cyclic tetrapeptide tentoxin (Figure 1) induces chlorosis in a broad range of plants (Fulton et al., 1965). The host-specific cyclic tetrapeptide AM toxin (Figure 2) that induces necrosis on apple leaves (Ueno et al., 1975) shares some of the similar structural features. In contrast the cyclic tetrapeptide *cyclo*(L-Pro-L-Val-L-Pro-L-Val-) and the D-Val-containing isomer show selective stem and root growth-regulating activity in rice seedlings (Ueda et al., 1985).

The present study focuses on tentoxin, a metabolite from the fungus *Alternaria alternata*, that has the sequence *cyclo*(-*N*-methylalanyl-L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanylglycyl-) (Figure 1) (Meyer et al., 1974a; Fulton et al., 1965). The peptide induces chlorosis in germinating seedlings of some dicotyledonous plants but not in corn, tomato, and members of the Cruciferae and Gramineae (Fulton, 1965; Templeton, 1967). Chlorosis, which is a diseased condition in plants characterized by yellowing, is determined by extracting tentoxin-treated seedlings with $Me_2SO$ and comparing measured chlorophyll levels with untreated controls. Early work attributed chlorosis to inactivation of the photosynthetic phosphorylation enzyme coupling factor 1 ($CF_1$) (Steele et al., 1976). Previous structure–function studies demonstrated that conformers of [D-MeAla[1]]-, [Sar[1]]-, and [Pro[1]]tentoxin were similar to native tentoxin in binding and inhibition of the $CF_1$ enzyme (Rich and Bhatnagar, 1978; Rich et al., 1980).

Recent evidence suggests that tentoxin-induced chlorosis and impairment of chloroplast development is mediated through inhibiting transport of the nuclear-coded protein polyphenoloxidase (PPO) into the chloroplast (Vaughn and Duke, 1984; Duke and Vaughn, 1982; Duke et al., 1982; Lax et al., 1985). Earlier studies reporting impairment of starch degradation in cabbage (Halloin et al., 1970) where no chlorosis was observed further pose the possibility of more

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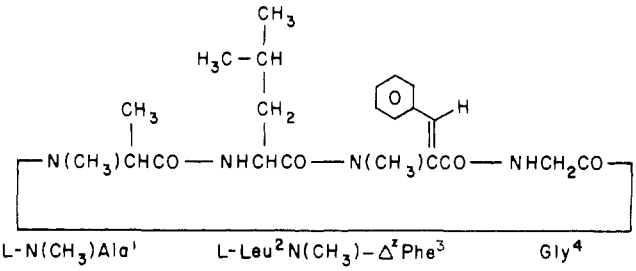



**Figure 1.** Structure of tentoxin (a cyclic tetrapeptide containing two *N*-methyl amino acids and one dehydro amino acid).

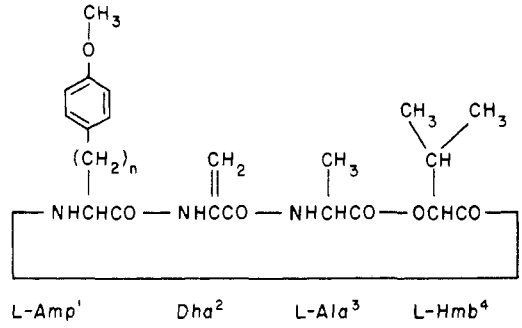



**Figure 2.** Structure of AM toxin. Abreviations: Amp, 2-amino-5-(*p*-methoxyphenyl)pentanoic acid; Dha, $\alpha,\beta$-dehydroalanine; Hmb, 2-hydroxy-3-methylbutanoic acid.

than one mode of action for tentoxin. Recent studies (Edwards et al., 1986) that compared the biological activity of the conformationally similar [Pro$^1$]tentoxin with that of tentoxin for transport of PPO vs. inhibition of $CF_1$ demonstrated differences in $CF_1$ inhibition while inhibition of the chloroplast import of PPO was maintained.

Prior structure–function work has shown the necessity of the *Z* configuration at the dehydrophenylalanine double bond for full activity (Rich and Mathiapuranam, 1974), and partial reduction of the dehydrophenylalanyl double bond results in apparent loss of activity (Woodhead et al., 1973). Further structure–activity studies are required to both elucidate the structural requirements of other side chain and peptide backbone elements required for chlorosis induction and provide insight into the biochemical mode of action and specificity involved in chlorosis.

The current study reports the structure–function relationships of a series of tentoxin analogues prepared through a solution-phase peptide synthetic route. The analogues were designed to evaluate stereochemical and structural peptide backbone requirements for chlorosis induction that include the following: (1) the effect of varying configuration at the $\alpha$-carbon of leucine and alanine, (2) the necessity for N-methylation at alanine and dehydrophenylalanine, and (3) the importance of the 12-atom peptide ring. With the recent disclosure that peptide backbone conformation of a cyclic tetrapeptide can be predicted on the basis of a set of sequence conformation empirical rules (Kato et al., 1985), it is possible to more readily evaluate the conformational effect of substituting a D-amino acid for an L-amino acid in a cyclic tetrapeptide. Alteration of sequence conformation and N-methylation has been utilized in this study to explore the relative roles of N-methylation vs. backbone conformation in the chlorosis induction activity of tentoxin.

## MATERIALS AND METHODS

All melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. Amino acid analysis were performed by hydrolyzing peptides in 6 N HCl for 24 h at 110 °C in deaerated vials. The evaporated residues were analyzed on a Beckman System 6300 utilizing conventional single-column methodology. Proton nuclear magnetic resonance ($^1$H NMR) spectra were determined on a CFT-20, 80-MHz spectrometer in the Fourier transform mode. $CDCl_3$ was employed as the NMR solvent. Chemical shifts were measured in $\delta$ relative to tetramethylsilane. Mass spectra were provided by Dr. Kenneth Tomer at the Midwest Center for Mass Spectrometry, University of Nebraska, using a Kratos MS-50 triple-analyzer mass spectrometer equipped with fast atom bombardment (FAB) ionization. The mass spectra samples were prepared by dissolving the sample (approximately 20 $\mu$g) in glycerol, and a small drop of the solution was placed on the copper target of a direct-insertion probe. Gel permeation chromatography was performed using a 3 cm × 62 cm column packed with Sephadex G-10 (Sigma) preswollen in 40% aqueous acetic acid. Normal workup refers to dissolution of the residue in a specified organic solvent, successive washings with 1 N HCl, saturated $NaHCO_3$, and saturated NaCl, drying over anhydrous $Na_2SO_4$, and removal of the solvent under reduced pressure. Acid workup refers to a normal workup with deletion of the saturated $NaHCO_3$ wash. Thin-layer chromatography was performed on 10-cm glass plates precoated with silica gel (0.25 cm, silica gel 60, F-254, E. Merck). Developing solvents were 1-butanol/acetic acid/water, 4:1:1 (A), and ethyl acetate/hexane/acetone, 2:1:1 (B). Visualization of the samples was accomplished by UV, iodine vapor, and a ninhydrin spray solution.

**Syntheses.** *p-Toluenesulfonate* D,L-*3-Phenylserine Ethyl Ester* (*1*). D,L-3-Phenylserine hydrate (1.90 g, 10 mmol) (Aldrich, mp 186 °C) was refluxed with 3.80 g (20 mmol) of *p*-toluenesulfonic acid in 50 mL of ethanol for 12 h. The solvent was removed in vacuo and the residue repeatedly washed with ether to yield a white solid: 3.8 g (95% yield); $R_f$ (A) 0.63; mp 165–167 °C (Edwards et al., 1986).

*N-(tert-Butyloxycarbonyl)leucyl-*D,L*-phenylserine* (*3*). *N*-(*tert*-Butyloxycarbonyl)-L-leucine (1.7 g, 7.5 mmol) was combined with *N*-methylmorpholine (1 g, 8.9 mmol) in dry tetrahydrofuran and the resultant mixture chilled to −5 °C. Isobutyl chloroformate (1.0 g, 7.5 mmol) was added and the solution stirred at −5 °C for 1 h. Addition of D,L-3-phenylserine ethyl ester (*p*-tosyl) salt (1; 3.0 g, 7.5 mmol) in dioxane/water and 1 equiv of triethylamine, followed by stirring for 2 h and a subsequent normal workup in ethyl acetate, provided 2.9 g (7.0 mmol, 94%) of product **2**, $R_f$ (A) 0.91, (B) 0.83. Saponification with 20 mL of 1 N sodium hydroxide/methanol (1:1; v/v followed by a acid workup resulted in 2.7 g (95% yield) of compound **3**: $R_f$ (A) 0.79, (B) 0.83; mp 75–78 °C; $[\alpha]_D$ −22.2° (*c* 1, MeOH).

*Aza Lactone of Boc-Leu-*$\Delta^Z$*-Phe* (*4*). The N-terminal protected peptide **3** (2.3 g, 6 mmol) was stirred with acetic anhydride (6 mL) and sodium acetate (0.4 g) for 8 h. The reaction mixture was evaporated in vacuo followed by a normal workup in ethyl acetate. Recrystallization with ethyl acetate/hexane afforded the aza lactone of Boc-Leu-$\Delta^Z$-Phe (**4**): 1.86 g (yield 90%); TLC $R_f$ (A) 0.88, (B) 0.66; mp 118–120 °C; $[\alpha]_D$ −107.4 (*c* 1, MeOH) (Edwards et al., 1986).

*Boc-Leu-*$\Delta^Z$*-Phe-Gly-OMe* (*5*). Conversion of compound **4** to the protected tripeptide **5** was accomplished by refluxing **4** (5.3 mmol, 2.0 g) with 1.2 equiv of glycine methyl ester (6.36 mmol, 0.79 g) in ethyl acetate (25 mL) overnight. Following a normal workup in ethyl acetate product **5** was isolated in 84% yield: 2.0 g; $R_f$ (A) 0.87, (B) 0.68; mp 135–138 °C; $[\alpha]_D$ −42.95 (*c* 1, MeOH) (Edwards et al., 1986).

*Boc-N($CH_3$)Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-OMe (8).* Removal of the N-terminal protecting group of compound 5 by reaction with 30 mL of 40% trifluoroacetic acid in methylene chloride (30 min) followed by removal of solvent and trituration of the residue with ether yields the trifluoroacetate salt of the tripeptide methyl ester 6.

A solution-phase reaction of *N*-(*tert*-butyloxycarbonyl)-*N*-methylalanine (4.4 mmol, 0.89 g) (Shuman et al., 1983) in DMF with compound 6 was performed as follows. One equivalent of *N*-methylmorpholine was added to compound 6 followed by addition of 1 equiv each of dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt). The reaction was stirred in a ice bath at 0 °C and allowed to proceed at room temperature overnight. A normal workup gave compound 7: 0.8 mmol, 0.44 g (80% yield); $R_f$ (A) 0.86, (B) 0.88. N-Methylation of the Boc-protected tetrapeptide methyl ester 7 was accomplished as previously reported (Rich et al., 1975), through reaction of methyl iodide (10 equiv), potassium carbonate (8 equiv), and 18-crown-6 (0.05 equiv) in DMF (10 mL) at room temperature for 72 h. The reaction solvent was evaporated in vacuo, the resulting residue mixed with ethyl acetate (10 mL) and water (20 mL), and the solution subjected to a normal workup. The solvent was removed in vacuo, resulting in 0.044 g of 8: 0.72 mmol (90% yield); $R_f$ (A) 0.86, (B) 0.68; UV $\lambda_{max}$ 277 ($\epsilon$ 18400); $^1$H NMR ($CDCl_3$) $\delta$ 0.5–0.6, (m, 7 H, $CH_3CHCH_3$), 1.3 (d, 3 H, $CH_3$ ($\alpha$-C of Ala)) 1.4 (s, 9 H, Boc), 2.7 (s, 3 H, $NCH_3$ (Ala)), 3.0 (s, 3 H, $NCH_3$ (MePhe)), 3.7 (s, 3 H, $OCH_3$), 4.1 (d, 2 H, $CH_2CO$), 4.6 (m, 1 H, ($\alpha$-H, Ala)), 6.5 (br s, 1 H, NH), 8.1 (m, 1 H, NH), 7.35–7.7 (m, 6 H, PhCH=C) (Rich and Mathiaparanum, 1974); IR 3400, 3300, 2960, 1680–1650, 1525 $cm^{-1}$.

*Cyclic Peptides.* The Boc-protected linear peptide methyl ester was dissolved in methanol (20 mL), 1 N sodium hydroxide (20 mL) was added, and the reaction mixture was stirred for 3 h. The methanol was evaporated in vacuo and 10 mL of water added. The aqueous mixture was cooled on an ice bath and acidified with 4 N HCl. The solution was extracted with ethyl acetate, washed with saturated sodium chloride, and dried over $Na_2SO_4$ and the solvent removed in vacuo. The resulting residue was dried in vacuo under $P_2O_5$ and then mixed for 30 min with 40% TFA in methylene chloride (0.5 g of peptide/20 mL of TFA solution). The solvent was then evaporated in vacuo. Following evaporation of the solvent the trifluoroacetate salt of the tetrapeptide was dried in vacuo under $P_2O_5$. The residue was then triturated with ether and homogeneous purity established on two TLC systems and amino acid analysis prior to cyclization. Listed below are the amino acid analyses and the $R_f$ values for the acyclic analogues.

18 [H-N($CH_3$)Ala-Leu-$\Delta^Z$-Phe-Gly-OH]: TLC, $R_f$ (A) 0.30, (B) 0.60; amino acid analysis, N($CH_3$)Ala 0.97, Leu 1.01, Gly 0.99.

19 [H-N($CH_3$)Ala-D-Leu-$\Delta^Z$-Phe-Gly-OH]: TLC, $R_f$ (A) 0.30, (B) 0.60; amino acid analysis, N($CH_3$)Ala 0.99, Leu 1.02, Gly 0.97.

20 [H-Ala-D-Leu-$\Delta^Z$-Phe-Gly-OH]: TLC, $R_f$ (A) 0.25, (B) 0.55; amino acid analysis, Ala 1.00, Leu 0.98, Gly 1.02.

21 [H-N($CH_3$)Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-OH]: N-($CH_3$)-$\Delta^Z$-Phe-Gly-OH]: TLC, $R_f$ 0.39, 0.71; amino acid analysis, N($CH_3$)Ala 0.94, Leu 1.01, Gly 0.99.

22 [H-Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-OH]: TLC, $R_f$ 0.35, 0.65; amino acid analysis, Ala 1.01, Leu 1.00, Gly 1.04.

The trifluoroacetate salt of the tetrapeptide was dissolved in dry degassed DMF (40 mM), and the solution was adjusted to pH 7.2. The solution was chilled to −25 °C under argon. A dilute DMF solution of diphenylphosphoryl azide was added dropwise, as were solutions of hydroxybenzotriazole (1 equiv) and (dimethylamino)-pyridine (0.1 equiv). The cyclization solution was stored at −5 °C and the pH was adjusted periodically. The reaction was monitored by TLC and in most cases was observed to be complete within 48 h. Completion of the reaction was signaled by disappearance of a ninhydrin and UV-positive response corresponding to the appropriate $R_f$ value. The reaction solution was stirred with 10-mL portions each of mixed-bed ion-exchange resin (Bio-Rad AG501-X8 D) and water for a period of 3–4 h. The solution was then filtered with a sintered-glass funnel and the solvent evaporated in vacuo. The resulting residue was dissolved in ethyl acetate and subject to a normal workup. Following evaporation of ethyl acetate the resulting oil was triturated with ether and the residue directly committed to gel permeation (G-10 Sephadex) chromatography eluted with 40% distilled acetic acid. Typically the column was eluted at a flow rate of 16 mL/h, and fractions of 5.5 mL were collected, resulting in elution of the cyclic tetrapeptide between fractions 20 and 25. Column fractions were pooled based on absorption at 280 nm, homogeneity as observed on TLC, and comparison of UV scans (330–220 nm).

11 [*cyclo*(-D-Ala-Leu-$\Delta^Z$-Phe-Gly-)]: 65% yield; $R_f$ (A) 0.71, (B) 0.43; amino acid analysis, Ala 1.01, Leu 0.98, Gly 1.00; FABMS, 387 (calcd $MH^+$ 387).

12 [*cyclo*(-Ala-Leu-$\Delta^Z$-Phe-Gly-)]: 45% yield; $R_f$ (A) 0.79, (B) 0.43; amino acid analysis, Ala 1.00, Leu 1.02, Gly 0.99; FABMS, 387 (calcd $MH^+$ 387).

13 [*cyclo*(-N($CH_3$)-Ala-Leu-$\Delta^Z$-Phe-Gly-)]: 35% yield; $R_f$ (A) 0.81, (B) 0.50; amino acid analysis, N($CH_3$)Ala 0.97, Leu 1.00, Gly 0.98; FABMS, 402 (calcd $MH^+$ 402).

14 [*cyclo*(-Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-)]: 42% yield; $R_f$ (A) 0.88, (B) 0.67; amino acid analysis, Ala 1.01, Leu 0.97, Gly 1.02; FABMS, 401 (calcd $MH^+$ 401).

15 [*cyclo*(-Ala-D-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-)]: 40% yield; $R_f$ (A) 0.87, (B) 0.67; amino acid analysis Ala 1.01, Leu 0.96, Gly 1.02; FABMS, 401 (calcd $MH^+$ 401).

16 [*cyclo*(-Ala-D-Leu-$\Delta^Z$-Phe-Gly-)]: 47% yield; $R_f$ (A) 0.79, (B) 0.43; amino acid analysis, Ala 1.00, Leu 0.97, Gly 1.00; FABMS, 387 (calcd, $MH^+$ 387).

17 [*cyclo*(-N($CH_3$)-Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-)]: 30% yield; $R_f$ (A) 0.59, (B) 0.22; amino acid analysis, N($CH_3$)Ala 1.01, Leu 0.99, Gly 1.00; FABMS, 415 (calcd $MH^+$ 415).

**Bioassay.** Solutions of the tentoxin analogues (100 and 10 $\mu$M) were prepared in ethyl acetate. Samples of 1 mL were pipetted onto a 4.25-mm filter disk placed in a Petri plate. The ethyl acetate was allowed to evaporate, and 1 mL of distilled water was pipetted into the Petri dish. Approximately 25 lettuce seeds were uniformly distributed on the surface of the filter paper and imbibed in the dark 24 h. The samples were placed in a growth chamber under continuous light at 28 °C for 48 h. The samples were then dried in vacuo and weighed. The weighed samples were extracted with $Me_2SO$, and chlorophyll was determined per gram dry weight by an established spectrophotometric method (Hiscox and Israelstam, 1978). Chlorophyll concentrations were calculated from the equation of Arnon (1949).

## RESULTS AND DISCUSSION

Several modifications were introduced into the six cyclic peptide analogues including (1) elimination of the *N*-methyl groups at alanine and/or dehydrophenylalanine and (2) substitution using the D-amino acids of alanine and/or leucine. The modifications were introduced at varied points during the synthesis (Scheme I).

**Table I. Results of Lettuce Seedling Chlorosis Assay of Cyclic Tentoxin Analogues**

| no. | cyclic analogue [100 μM][a] | μg chlorophyll/ g plant tissue | rel pot. | % chlorophyll rel to control |
|---|---|---|---|---|
| 11 | *cyclo*(-D-Ala-Leu-$\Delta^Z$-Phe-Gly-)[b] | 7.3 ± 1.5 | 0.43 | 34 |
| 12 | *cyclo*(-Ala-Leu-$\Delta^Z$-Phe-Gly-)[b] | 20.4 ± 1.5 | 0.16 | 95 |
| 13 | *cyclo*(-N($CH_3$)Ala-Leu-$\Delta^Z$-Phe-Gly-)[b] | 8.6 ± 2.1 | 0.37 | 40 |
| 14 | *cyclo*(-Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-)[b] | 2.0 ± 0.5 | 1.00 | 15 |
| 15 | *cyclo*(Ala-D-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-)[c] | 8.1 ± 1.1 | 0.40 | 37 |
| 16 | *cyclo*(Ala-D-Leu-$\Delta^Z$-Phe-Gly-)[c] | 12.8 ± 2.0 | 0.25 | 60 |
| 17 | *cyclo*(N($CH_3$)Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-),[b] tentoxin | 3.2 ± 1.0 | 1.00 | 15 |
| | control | 21.4 ± 2.0 | | 100 |

[a] Assays were done in triplicate. [b] Denotes cis–trans–cis–trans conformation (predicted based on empirical rules for cyclic tetrapeptides (Kato et al., 1985)). [c] Denotes cis–cis–cis–trans conformation (predicted based on empirical rules for cyclic tetrapeptides (Kato et al., 1985)).

**Table II. Results of Lettuce Seedling Chlorosis Assay of Acyclic Tentoxin Analogues**

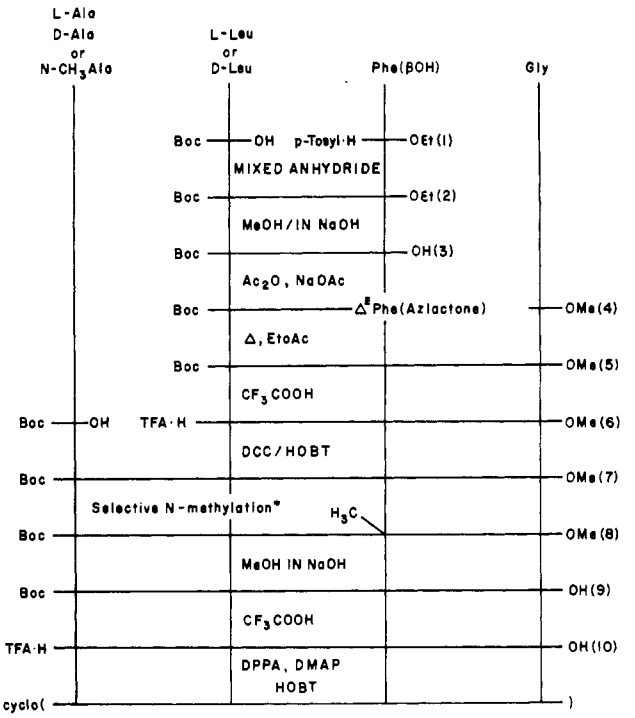
| no. | acyclic analogues (100 μM)[a] | μg chlorophyll/ g plant tissue | rel pot. | % chlorophyll rel to control |
|---|---|---|---|---|
| 18 | H-N($CH_3$)Ala-Leu-$\Delta^Z$-Phe-Gly-OH | 19.8 ± 1.1 | 0.16 | 93 |
| 19 | H-N($CH_3$)Ala-D-Leu-$\Delta^Z$-Phe-Gly-OH | 20.3 ± 2.2 | 0.16 | 95 |
| 20 | H-Ala-D-Leu-$\Delta^Z$-Phe-Gly-OH | 21.4 ± 1.2 | 0.15 | 100 |
| 21 | H-N($CH_3$)Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-OH | 13.1 ± 2.0 | 0.24 | 61 |
| 22 | H-Ala-Leu-N($CH_3$)-$\Delta^Z$-Phe-Gly-OH | 12.8 ± 2.0 | 0.25 | 60 |
| | control | 21.4 ± 2.0 | | 100 |

[a] Assays were done in triplicate.

The syntheses of the modified tentoxin analogues outlined in Scheme I were accomplished through employment of a variety of solution-phase peptide synthetic techniques. The details of the tentoxin synthesis have been reported elsewhere (Edwards et al., 1986). The physical constants for the D-Leu-containing intermediates are listed in Table II. Preparation of acyclic analogues of tentoxin involved syntheses of compounds of types **1–10** (Scheme I) wherein modifications at the 1–3-positions of tentoxin were introduced. The modifications were incorporated through substituting D-amino acids at the 1- and 2-positions for the native L-amino acids and deletion of each *N*-methyl group at positions 1 and 3, which was provided for by substituting alanine for *N*-methylalanine at position 1 and eliminating the selective N-methylation step at dehydrophenylalaine (position 3). The synthetic steps employed, with the exception of those used for compounds **4**, **5**, and **8**, are procedures typically used for the preparation of synthetic peptides [for details see Meienhoffer (1979)]. Incorporation of dehydrophenylalanine was achieved through the Bergmann method (Bergmann et al., 1943) which uses the spontaneous dehydration of β-hydroxy amino acid aza lactones to introduce the double bond. Conversion of the aza lactone of dehydrophenylalanine to the tripeptide **5** was accomplished through reflux of **4** with glycine methyl ester in ethyl acetate. Following selective methylation (Rich et al., 1975) at the 3-position and completion of the tetrapeptide sequence, compounds of type **10** (tetrapeptides) were subjected to ring closure. Tetrapeptides were assessed for their homogeneity prior to ring closure by thin-layer chromatography and amino acid analysis. Yields of the cyclic tetrapeptides ranged from 65 to 30%. The highest yield was realized with D-alanine at the N-terminus of the tetrapeptide. This is consistent with prior reports (Brady et al., 1979) of increased yields in peptide cyclizations where D-amino acids occur at the N-terminus. Overall increased yields using these methods were observed when compared with those of previous reports (Rich and Mathiapuranam, 1974).

Biological activity (chlorosis) was determined for six cyclic and five acyclic analogues, using a lettuce seedling chlorosis bioassay. Since both tentoxin and the analogues tested in this study contain several nonprotein amino acids, it was of interest to assay each amino acid individually to assess the singular effect of the *N*-methyl amino acids and dehydrophenylalanine. *N*-Methylalanine, leucine, dehydrophenylalanine, and glycine did not induce chlorosis. The results of the chlorosis assay for the acyclic and cyclic analogues are shown in Tables I and II. The amount of chlorosis was determined through comparison of percent chlorophyll in a treated seedling relative to that of a control. Relative potencies are expressed as (percent chlorosis of sample solution/percent chlorosis for tentoxin solution). Analogues demonstrating full potency at 100 μM were also tested at 10 μM.

**Scheme I. Synthesis of Tentoxin and Analogues of Tentoxin[a]**




[a] Abbreviations: $Ac_2O$, acetic anhydride; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMAP, (dimethylamino)pyridine; DPPA, diphenylphosphoryl azide; EtOAc, ethyl acetate; HOBT, hydroxybenzotriazole; *p*-tosyl, *p*-toluenesulfonic acid; TFA, trifluoroacetic acid; D,L-Phe(βOH), D,L-β-phenylserine; $\Delta^Z$-Phe, dehydrophenylalanine of the *Z* configuration. Asterisk: Rich et al. (1975).

**Table III. D-Leucine Derivatives of Synthetic Intermediates 3–5**

| compound | yield, % | TLC, $R_f$ | mp, °C | $[\alpha]_D$ |
|---|---|---|---|---|
| *N*-(*tert*-butyloxycarbonyl)-D-leucyl-D,L-phenylserine | 95 | 0.83 (A)<br>0.16 (B) | 58–61 | −1.98 (*c* 1, MeOH) |
| aza lactone of Boc-D-Leu-$\Delta^Z$-Phe | 90 | 0.92 (A)<br>0.75 (B) | 121–122 | +97.8 (*c* 1, MeOH) |
| Boc-D-Leu-$\Delta^Z$-Phe-Gly-OMe | 84 | 0.90 (A)<br>0.58 (B) | 125–130 | −67.19 (*c* 1, MeOH) |

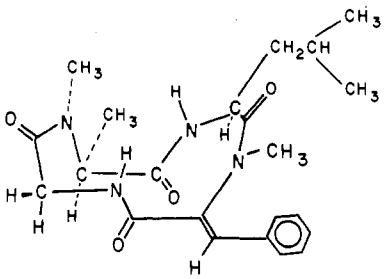


**Figure 3.** Peptide backbone conformation of tentoxin (cis–trans–cis–trans).

The peptide analogues in this study are designed to study important structural features of tentoxin necessary for the induction of chlorosis. This structure–function study assesses the importance of the *N*-methyl peptide backbone modifications persent at the 1- and 3-positions in the biological activity of tentoxin. Assessment of the importance of the conformational constraint resulting from the 12-atom peptide ring and N-methylation was done by comparing the biological activity of acyclic analogues with that of cyclic analogues. An assessment of the relative role of sequence backbone conformation to the N-methylated amide bonds in chlorosis induction was made by incorporation of D-amino acids at the 1- and 2-positions. Due to the predictable conformation relationship of cyclic tetrapeptides as proposed by Kato et al. (1985), it was possible to study the effect of stereochemical changes at the 1- and 2-positions on the resulting biological activities. Tentoxin has a cis–trans–cis–trans conformation (Figure 3) (Rich et al., 1978), which is predictably alterable to a cis–cis–cis–trans conformation upon substitution of a D-leucine for the naturally occuring L-leucine (2-position). Analogue 11 (Table I) is useful as an example of an approach of this type in assessing peptide backbone N-methylation vs. sequence conformation through substitution of D-Ala at the 1-position. Analog 11, which has the sequence *cyclo*(D-Ala-Leu-$\Delta^Z$-Phe-Gly), would predictably retain the cis–trans–cis–trans backbone conformation adopted by tentoxin, since a carbonyl group acylating a D-amino acid residue is oriented to the upper side of the main ring, as shown by Kato et al. (1985). Analogue 11 also lacks *N*-methyl groups at the 1- and 3-positions. Assaying this analogue for chlorosis induction makes possible the evaluation of the effect of a proteolytically resistant amide bond. (D-Amino acids confer resistance to proteolysis at their amide bonds similar to *N*-methyl amino acids (Roemer et al., 1977) at the 1-position while deleting both *N*-methyl groups at the 1- and 3-positions and retaining the native backbone conformation of tentoxin.) Analogue 11 induced significantly higher levels of chlorosis when compared with 12. This suggests that analogue 11 may be a better inducer of chlorosis as a result of its increased resistance to proteases.

Assessment of the importance of N-methylation at both alanine and dehydrophenylalanine in chlorosis induction was accomplished in compounds 13 and 14 (Table I). Compound 13, having an *N*-methyl group at alanine but not dehydrophenylalanine, retained approximately 40% of full activity at 100 μM. Compound 14 with an *N*-$CH_3$ group at dehydrophenylalanine (the 3-position, but not the 1-position) demonstrated full activity at both 100 and 10 μM concentrations. This demonstrates the necessity for N-methylation at the 3-position in retaining full biological activity. However, retention of biological activity in 14 indicates that N-methylation at position 1 is not necessary for the biological activity observed in tentoxin (19). When *N*-methyl groups were absent at both positions 1 and 3, as in compound 12, there was no significant biological activity.

The effect of singularly substituting a D-amino acid at positions 1 or 2 in analogues lacking N-methylation was evaluated in compounds 11 and 16 (Table I). Though the relative potency of the des-N-methylated analogues was much lower than for tentoxin, biological activities significantly above the all L-amino acid containing des-N-methylated analogue were observed. A relatively higher biological activity was observed in analogue 11 than in 12 or 16. This increased biological activity observed with the D-amino acid substituted des-N-methylated analogue suggests that either a more preferred receptor-bound conformation may occur with compound 11 than with compound 12 or the D-alanine introduces enzymatic resistance to seed proteases (Vodkin and Scandalios, 1981; Lichtenfeld et al., 1979). Low resistance to proteolytic seed proteases may in part explain the dramatic drop in activity observed with the all L-amino acid des-N-methylated compound 12 when compared with tentoxin and similar N-methylated analogues (13, 14, 17). This explanation is plausible, since N-methylation at the amide nitrogen of peptides has been previously shown to give substantial resistance to enzymatic degradation in mammalian systems (Roemer et al., 1977). Furthermore, in view of the resistance to proteolysis conferred by D-amino acids in peptides it is interesting to compare analogues 11 and 13. Both analogues 11 and 13 contain a proteolytically resistant amide bond at the 1-position with absence of N-methylation at the 3-position. The relative potencies of these two analogues are similar and yet significantly above the all L-amino acid analogue 12, which is completely inactive.

Other reports of D-amino acid replacement at the 3-position have revealed important aspects of tentoxin structure–activity relations. Previous accounts of loss in activity upon reduction of the dehydrophenylalanine of tentoxin to a D-phenylalanine residue (Woodhead et al., 1973; Meyer, 1974b) argue in favor of a preferred orientation of the phenyl ring at the 3-position, through fixation of the χ angle of the phenyl side chain (Hruby, 1982) for full activity.

Demonstration that a change in sequence conformation has a more decided effect on biological activity when compared with selective N-methylation is possible by comparing two 3-position N-methylated analogues which have different backbone conformations. Analogues 14 and 15 are compared for this purpose. Analogue 14 (cis–trans–cis–trans conformation) showed full retention of biological activity (relative potency 1.00) whereas compound 15 (cis–cis–cis–trans conformation) with D-leucine

substituted at the 2-position had a relative potency of only 0.40. The relative importance of both N-methylation and a cis–trans–cis–trans conformation for optimal biological activity is demonstrated in analogue **16** (cis–cis–cis–trans), which adopts a backbone conformation similar to **15** (cis–cis–cis–trans) but does not have an *N*-methyl group at the 3-position. A nearly twofold decrease in biological activity is observed between **15** and **16**. It is interesting to note that a similar twofold decrease in activity occurs on going from analogue **11** (cis–trans–cis–trans) to analogue **16** (cis–cis–cis–trans) with complete absence of N-methylation.

The chlorosis induction activities of the acyclic analogues were obtained in order to assess the effect of conformational constraint conferred by the 12-atom peptide ring of tentoxin and to examine this feature of the cyclic peptide relative to N-methylation. The biological activities of the acyclic analogues are shown in Table II. Analogues **18–20** with no N-methylation at the 3-position demonstrated no chlorosis activity. However those (**21**, **22**) where the 3-position was N-methylated did induce a significant but low level of chlorosis.

## CONCLUSION

The current study provides insight into the structure–function relations of key peptide backbone features in the lettuce seedling chlorotic activity observed with tentoxin. Three structural and conformational features were examined. These included amide bond N-methylation, sequence backbone conformation, and cyclic ring constraint. This study provides insight into important aspects of tentoxin structure and its influence on the induction of chlorosis in lettuce seedlings. The cyclic nature of tentoxin is critical for biological activity. However, acyclic analogues can mimic the action of tentoxin at greatly reduced potencies if the dehydrophenylalanine residue is N-methylated. Further, N-methylation at this position is more important than at the Ala residue. Both the cyclic nature of tentoxin and the N-methylations may be as important for the prevention of the degradation of the peptide as for the direct induction of chlorosis. Finally, tentoxin needs to be in a cis–trans–cis–trans conformation to produce the maximum loss of chlorophyll in lettuce seedlings.

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**Registry No.** **1**, 108340-82-5; **3**, 107694-12-2; **4**, 107794-92-3; **5**, 92455-70-4; **6**·$CF_3CO_2H$, 108233-93-8; **7**, 55478-19-8; **8**, 55478-20-1; **11**, 108233-94-9; **12**, 108340-83-6; **13**, 108340-84-7; **14**, 108233-95-0; **15**, 108340-85-8; **16**, 108340-86-9; **17**, 28540-82-1; **18**, 108233-96-1; **19**, 108340-87-0; **20**, 108233-97-2; **21**, 107694-13-3; **22**, 108233-98-3; DL-$NH_2CH(CO_2H)CH(OH)Ph$, 69-96-5; Boc-L-Leu, 13139-15-6; Gly-OMe, 616-34-2; Boc-$NCH_3$-Ala, 16948-16-6.

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