THE SEQUENCE AND OPTICAL CONFIGURATION OF THE AMINO ACIDS IN TENTOXIN*

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SUMMARY

Tentoxin, a phytotoxic tetrapeptide, has been shown by sequencing and mass spectrometric techniques to have the structure cyclo [N-methyl-dehydrophenylalanyl-L-leucyl-glycyl-L-N-methylalanyl].

INTRODUCTION

The fungus Alternaria tenuis Auct. synthesizes a cyclic tetrapeptide, tentoxin, which causes chlorosis in germinating seedlings (1). Meyer et al. (2) found that it contained N-methylalanine (NMA), glycine (Gly), leucine (Leu), and N-methyl-dehydrophenylalanine (NMeDHPhe). However, the amino acid sequence and absolute configuration of leucine and N-methylalanine were not determined.

We observed that treatment of tentoxin with base alleviates chlorosis. Evidence to be presented showed that this converted tentoxin to a linear tetrapeptide, thus making possible application of classical sequencing procedures. A determination of the sequence of amino acids in this linear peptide and the optical configurations within tentoxin constitute the basis of this communication.

MATERIALS AND METHODS

Materials--The thin-layer chromatographic (TLC) solvent systems used were: 1, n-butanol:acetic acid:water (4:1:5, upper phase)(3); 2, chloroform:tert amyl alcohol:acetic acid (70:30:0.5)(4); 3, n-butanol:acetic acid:water (4:1:1)(5); 4, chloroform:acetic acid (70:1); 5, chloroform:formic acid (100:5)(6); 6, benzene:pyridine:acetic acid (80:20:2)(4); and 7, chloroform (6). Tentoxin was purified from filtrates of 28-day-old cultures of A. tenuis (7). The linear tetrapeptide was prepared by incubating tentoxin (3 mg/ml) in 0.4 N sodium hydroxide for 24 hr

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at 22°C. The reaction mixture was then chromatographed on Merck silica gel F-254, the fluorescence-quenching linear peptide (Rf_1, 0.43; tentoxin, 0.82) removed and subjected to further analysis.

Dansylchloride (DNS-Cl) as a 1% solution in acetone, and DNS-Leu and DNS-Gly were purchased from Pierce Chemical Co. Dansyl derivatives of N-methylalanine and methylamine were prepared by reaction with DNS-Cl and purified by TLC on 0.25 mm silica gel G (Rf_2, DNS-NMA, 0.6; DNS-methylamine, 0.9). The phenylthiohydantoins of leucine and glycine were obtained from Aldrich Chemical Co: phenyl isothiocyanate (PITC), obtained from the same source, and trifluoroacetic acid were dried over $CaCl_2$ and redistilled before use. Likewise, pyridine, triethylamine and hydrazine were dried over KOH and redistilled.

Peptides were hydrolyzed (6N HCl, 108°C for 16 hr) in evacuated flasks. The hydrolysates were evaporated in vacuo at 40°C, and residual HCl eliminated by thrice redrying from aqueous solution. Qualitative analyses were performed using TLC and solvent system 3. Standards were simultaneously chromatographed, and the spots visualized by spraying with 0.2% ninhydrin in acetone then heating at 105°C for 10 min. Quantitative analyses were obtained on a Beckman amino acid analyzer using accelerated elution schedules with a pH 3.95 (0.2 N) sodium citrate buffer for the PA-28 resin column (8, 9). However, N-methylalanine had a low color yield and was poorly resolved.

The C-terminal amino acid was determined by hydrazinolysis of the linear peptide using anhydrous hydrazine at 108°C for 8 hr (10). Excess hydrazine was removed by evaporation in vacuo at 40°C and the residue dansylated as described by Akabori et al. (8) for dinitrophenylation. After removal of the didansylamino acid hydrazides by ethyl acetate-extraction from alkaline solution, the DNS-amino acid was extracted with ethyl acetate from the acidified aqueous phase and chromatographed on silica gel G. Edman degradations were performed by the method of Eriksson and Sjoquist (11) except that anhydrous trifluoroacetic acid was used to effect release of the terminal amino acid. The PTH-derivatives were extracted with ethyl acetate and chromatographed on silica gel F-254 using solvent systems 5 or 7.

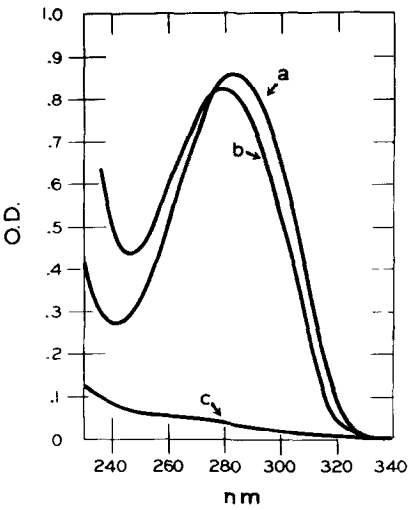

Fig. 1. The UV spectrum of tentoxin (a), linear tetrapeptide (b), and tripeptide (c).

The absolute configurations of N-methylalanine and leucine were determined by hydrolyzing tentoxin, then converting the resulting amino acids to their diasteriomeric dipeptides by reaction with L-leucine N-carboxyanhydride (10). The dipeptides formed were separated on the amino acid analyzer using a pH 4.25 (0.2 N) sodium citrate buffer at 60°C for 1 hr followed by pH 4.62 (0.35 N) buffer, and compared to the five possible product dipeptides.

Mass spectra were obtained on a CEC-110 high-resolution mass spectrometer. Exact masses were determined for all peaks.

RESULTS AND DISCUSSION

Tentoxin was converted to a linear tetrapeptide by treatment with base. Unlike tentoxin, it was ninhydrin positive, ethanol precipitable, and did not cause chlorosis. Acid hydrolysis yielded leucine, glycine, methylamine and N-methylalanine (mol. ratio, 1.01:1.0:0.36:1.37; tentoxin, 0.96:1.0:0.55:2.7). The UV absorption spectrum (λ max 279 nm)(Fig. 1) indicated that the aromatic nucleus was still present and, accordingly, that the compound was a linear tetrapeptide, A, derived from tentoxin. Dansylation of this compound followed by hydrolysis yielded a major fluorescent spot, not DNS-methylamine, which was indistinguishable from DNS-NMA (Rf_4, 0.21). However, the hydrolysate contained only leucine, glycine and N-methylalanine (mol. ratio, 0.97:1.0:1.8).

It is interesting to note that while acid hydrolysis of tentoxin yields methylamine from NMeDHPhe, hydrolysis of dansylated A did not yield DNS-methylamine, although spectral evidence suggests that DNS-NMeDHPhe was present before hydrolysis. Due to lack of authentic DNS-NMeDHPhe, it was not possible to check whether it, unlike NMeDHPhe is stable to acid hydrolysis.

An Edman degradation of the linear tetrapeptide yielded a ninhydrin-positive, UV-negative material B (Rf_1, 0.6-0.75) which contained N-methylalanine, glycine and leucine (Fig. 1). The PTH derivative did not correspond to PTH-Leu or PTH-Gly. It was therefore concluded that the aromatic amino acid derivative occupies the N-terminal position in the linear tetrapeptide.

Dansylation of B followed by hydrolysis yielded a fluorescent derivative with Rf values identical to DNS-Leu (Rf_2, 0.63; Rf_4, 0.11; Rf_6, 0.9). The hydrolysate contained glycine, N-methylalanine and traces of leucine. An Edman degradation of B yielded a ninhydrin-positive product C, which was not purified by TLC, and a PTH derivative chromatographically identical to PTH-Leu (Rf_5, 0.6; Rf_7, 0.4). These results indicated that leucine occupies the second position in the linear tetrapeptide.

The product C on hydrolysis yielded glycine, N-methylalanine and leucine (mol. ratio, 1.0:1.15:0.02). Dansylation of C, followed by hydrolysis yielded DNS-Gly. The hydrolysate also contained glycine and N-methylalanine. Thus, the data indicate that glycine occupies the third position.

The fourth amino acid was elucidated by determination of the C-terminal amino acid. A fluorescent dansyl derivative was obtained which migrated with DNS-NMA (Rf_4, 0.21).

Analysis of the product diasteriomeric dipeptides showed that leucine and N-methylalanine in tentoxin are of the L configuration.

The results presented are consistent with only one structure for the linear tetrapeptide. On the assumption that the peptide A merely represents an opened ring system, tentoxin would then have the structure: [NMeDHPhe-L-Leu-Gly-L-NMA].

The high-resolution mass spectrum of tentoxin was consistent with this struc-

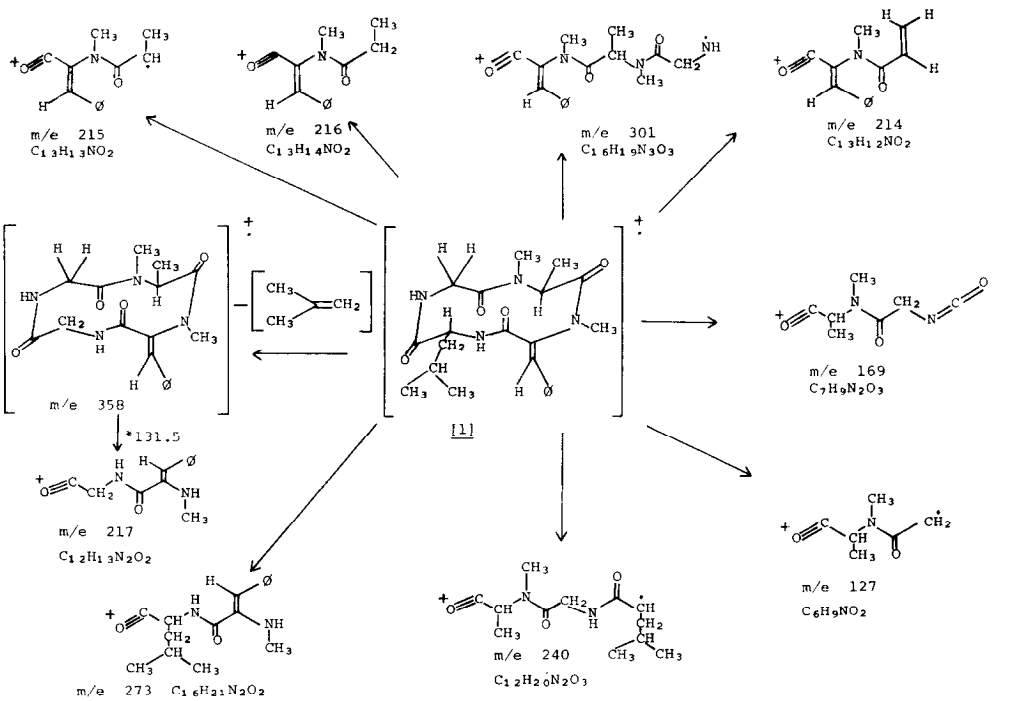

Fig. 2. The sequence specific fragment ions of tentoxin.

ture. As in other cyclic peptides, ring opening with subsequent multiple fragmentations may occur by several processes (13). Several sequence specific fragment ions are consistent with the structure proposed from the sequential analysis (Fig. 2). The ions at m/e 214, 215, and 216 indicate that N-methylalanine is adjacent to N-methyl-dehydrophenylalanine. The ion at m/e 273 shows that leucine and N-methyl-dehydrophenylalanine are adjacent. The ion at m/e 217 appears to be formed by a secondary fragmentation process from $[M-56]^{+\cdot}$ (14). The metastable peak (m/e 131.5) corresponding to this transformation has been observed. The presence of ions at m/e 127 and 169 indicates the adjacency of N-methylalanine to glycine. The possibility that the ion at m/e 127 may be formed by a secondary fragmentation from either m/e 183 or $[M-56]^{+\cdot}$ can be ruled out. The ion at m/e 183 does not correspond to a composition of $C_{10}H_{17}NO_2$, and the metastable peak (m/e 45) for the transformation $[M-56]^{+\cdot} \rightarrow$ m/e 127 has not been observed. These observations rule out the possibility of leucine being adjacent to N-methylalanine. Furthermore, ions at m/e 301 and 240 which include three amino acid residues can be

represented (Fig. 2). Peaks at m/e 273, 240 and 127 are consistent with the sequence for the ion [N-MeDHPhe-Leu-Gly-NMA]$^{+\cdot}$ and the peaks at m/e 301, 240 and 215 are consistent with the sequence for the ion [Leu-Gly-NMA-N-MeDHPhe]$^{+\cdot}$. Both sequences lead to the same cyclic structure (Fig. 2, structure I).

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