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DIHYDROTENTOXIN AND A RELATED DIPEPTIDE PRODUCED BY *ALTERNARIA ALTERNATA*

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Key Word Index—*Alternaria alternata*; fungi; structure elucidation; cyclic peptides; dihydrotentoxin; cyclo-(L-leucyl-*N*-methyl-L-phenylalanyl).

Abstract—*Alternaria alternata* produces, besides tentoxin, two other cyclic peptides. The most abundant compound is dihydrotentoxin. Its structure was elucidated as cyclo-(L-leucyl-*N*-methyl-L-phenylalanyl-glycyl-*N*-methyl-L-alanyl). A related dipeptide was found to be cyclo-(L-leucyl-*N*-methyl-L-phenylalanyl). It is the first time that these compounds have been isolated as natural substances.

INTRODUCTION

The phytopathogenic fungus *Alternaria alternata* produces tentoxin, chemically it is cyclo-(L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl) [1,2]. Previously we reported a novel, more effective isolation procedure of this phytotoxin [3, 4], which has also proven to be a method to obtain related compounds. Our interest was especially focussed on cyclic peptides similar to tentoxin.

RESULTS AND DISCUSSION

In addition to tentoxin, separation of ether extracts on Sephadex-G-15 yielded several compounds detected by fluorescence quenching and/or positive tolidine reaction after TLC on silica gel. A typical Sephadex-G-15 elution profile, in which the substances were detected by absorbance at 286 and 222 nm is shown in Fig. 1. Further purification of each compound was accomplished by one or two steps of rechromatography on Sephadex-G-15 following a run on a silica gel column [3, 4]. The substances were eluted in a sequence comparable to the R_f-values obtained by TLC: C_3 (0.41), C_4 (0.41), C_1 (0.35), C_0 (0.24), C_2 = tentoxin (0.20), T_1 (0.15). With the aid of UV spectra several compounds were recognizable. The substances represented by the peaks C_1, C_3, and C_4 are nitrogen-free. They have no chemical relationship to tentoxin. Until now their structures have been only partially elucidated.

Substance C_0

Substance C_0 was isolated as a lyophilized white powder. The molecular formula was established as $C_{22}H_{32}O_4N_4$ by high resolution mass spectrometry. After acidic hydrolysis leucine, glycine, *N*-methylalanine, and *N*-methylphenylalanine were detected by GC. *N*-methylphenylalanine shows two peaks, the first originating from incompletely converted amino acid (*n*-butylester

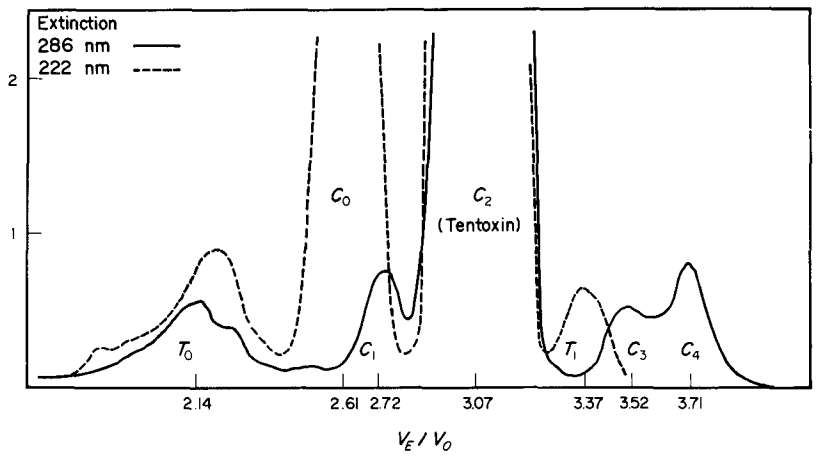


Fig. 1. Separation of tentoxin and other compounds on Sephadex-G-15-column (180 × 3.5 cm, 47 ml H_2O/hr). V_E = elution volume, V_0 = void volume.

only). The released amino acids were also identified as their dansyl derivatives by 2D TLC on micro polyamide layer plates. On the basis of these results we have presumed that C_0 is dihydrotentoxin (**1**).

The IR spectrum of the intact molecule shows typical amide bands and is very similar to that of tentoxin. Contrary to that there is a great difference in the UV spectra. While tentoxin offers a marked maximum at 286 nm (log ε 4.24), the lack of the double bond leads to a phenylalanine-like spectrum. In a relatively wide range of concentrations (10–100 μg/ml) it is possible to get a constant molar extinction coefficient at 222 nm (log ε 3.76), although there is no maximum, but only a rise of a peak, which is caused by the peptide bound. This value can be used for quantitative estimation.

1 was synthesized with both L- and D-configuration in the *N*-methylphenylalanine residue, although when in the L-configuration with a very low yield [5]. Also, in contrast to the D-compound, only an incomplete ^{1}H NMR spectrum has been published for the L-form [2, 5]. However, now we have obtained the lacking data. The signals were assigned to the respective protons by spin-decoupling and with the aid of Eu(FOD)$_3$ shift reagent. We could demonstrate that native **1** has the L-configuration in the part of *N*-methylphenylalanine. This result was confirmed by capillary GC of hydrolysate on a chiral phase, additionally we have obtained the proof of L-configuration of the other two optically active amino acids.

In the literature [2] there are few mass spectral data of **1** with D-configuration in the *N*-methylphenylalanine residue only. Nevertheless, we have found good conformity with our results. The electron impact mass spectrum of native **1** shows significant fragment ions corresponding to the loss of one amino acid residue from the molecular ion, of two complete residues, and many significant fragments which either contain or have lost part of one, two or three adjacent residues. The fragmentation process allows definite sequence determination of native **1** as cyclo-(L-leucyl-*N*-methyl-L-phenylalanyl-glycyl-*N*-methyl-L-alanyl) (Fig. 2). It is the first time that this compound has been isolated as a natural substance.

By quantitative GC we determined the concentration of **1** in the culture filtrate of the fungus *A. alternata*. The content was 75–90 mg per litre using a high tentoxin-producing strain corresponding 45–65% of the tentoxin amount.

Substance T_1

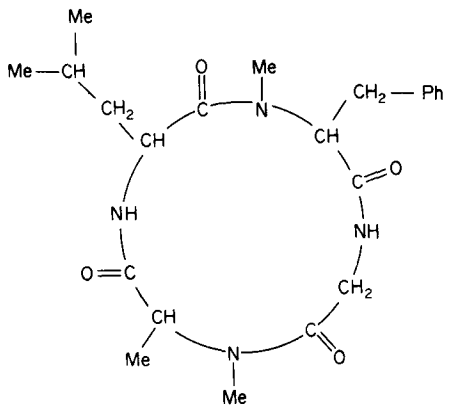
After acidic hydrolysis of the purified substance T_1 *N*-methylphenylalanine and leucine were detected by GC and by TLC of the dansyl derivatives. Both amino acids have L-configuration proved by capillary GC. The UV spectrum looks like that of **1**; a molar extinction coefficient (log ε 3.52) at 222 nm can be used for quantitative estimation as well (see native **1**). Like tentoxin and **1**, the intact molecule has not been retarded on ion exchange resins and has not reacted with dansyl chloride. Therefore T_1 was also thought to be a cyclic peptide.

The molecular formula of T_1 was established as $C_{16}H_{22}O_2N_2$ by high resolution mass spectrometry. The spectrum of T_1 exhibits some intensive characteristic fragment ions (see Experimental). It follows from all the data presented that T_1 is cyclo-(L-leucyl-*N*-methyl-L-phenylalanine) (**2**, Fig. 2). The content of this compound in the culture filtrate was calculated as 1.5–3 mg/l corresponding to 2–4% of tentoxin concentration.

EXPERIMENTAL

Strain and cultivation of *A. alternata*, and isolation procedure of tentoxin were reported elsewhere [3, 4, 6]. The evaporated fractions or the substances were chromatographed with EtOAc–Me_2CO–*n*-hexane (2:1:1) on Silufol UV 254 (detection: UV-quenching and/or tolidine reaction). After N_2-fumigation peptides were hydrolysed (6 M HCl, 100–110°, 24 hr) in ampoules. Amino acids were labelled with dansyl chloride and identified as described by ref. [7]. Derivative formation and GC analysis (3% OV 225, isothermal at 120°) were performed as described [8]. For qualitative estimation serine was used as the reference substance. *N*-methylphenylalanine was detected isothermally at 160°; for quantitative estimation aspartic acid was used as int. standard. Capillary GC was carried out with the same derivatives on a 20 m × 0.25 mm glass capillary (WCOT, Chirasil-Val, initial temp. 60°, delay 2 min, 2°/min, final temp. 210°, delay 5 min, injector temp. 63°, detector (FID) temp. 200°, flow rate (H_2) 0.4 ml/min, sample 0.1 μl, split ratio 1:20).

Dihydrotentoxin (native). Melting range: 146–149°, uncorr. UV $\lambda_{max}^{H_2O}$ nm: 258. IR ν_{max}^{KBr} cm^{-1}: 1660 *vs*, 1530 *s*, 1260 *m* (amide), 1640 *vs* (*tert.* amide). EIMS (75 eV), *m/z* (rel. int.): 416.2412 $[M]^+$ (3), 331.1523 $[M-NH-CH-C_4H_9)^+$ (13), 274.1337 $[C_6H_5-CH_2-CH-CO-NH-CH_2-CO-N(Me)-CH(Me)-CO]^+$ (17), 259.1538 $[C_4H_9-CH-CO-N(Me)-CH(CO)-CH_2-C_6H_5]^+$ (2),



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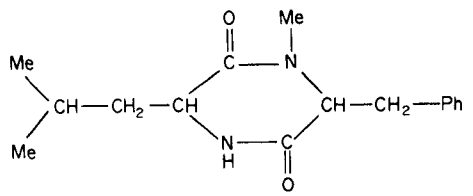

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Fig. 2. 1: Substance C_0 = native dihydrotentoxin = cyclo-(L-leucyl-*N*-methyl-L-phenylalanyl-glycyl-*N*-methyl-L-alanyl). 2: Substance T_1 = cyclo-(L-leucyl-*N*-methyl-L-phenyl-alanyl).

240.1461 $[CH_2–CO–N(Me)–CH(Me)–CO–NH–CH(CO)–C_4H_9)]^+$ (6), 218.1055 $(M-Leu–NMeAla)^+$ (1), 216.0996 $(CH_2–CH–CO–N(Me)–CH(CO)–CH_2–C_6H_5)^+$ (12), 212.1044 $[m/z\ 259-C_3H_7]^+$ (13), 142.0734 $[M-Leu–NMePhe]^+$ (4), 134.0975 $[Me–NH–CH–CH_2–C_6H_5]^+$ (100), 127.0618 $(CO–CH(Me)–N(Me)–CO–CH_2)^+$ (23). ^1H NMR (100.028 MHz, $CDCl_3$, TMS): glycine unit: δ3.47 (1H, *d*, J = 14.5 Hz, $C_\alpha H_a$), 4.97 (1H, *dd*, J = 14.5 and 10 Hz, $C_\alpha H_b$), 7.87 (1H, *d*, J = 10 Hz, NH); alanine unit: δ1.57 (3H, *d*, J = 7 Hz, C_α–Me), 2.78 (3H, *s*, NMe), 4.28 (1H, *q*, J = 7 Hz, $C_\alpha H$); phenylalanine unit: δ2.84 (3H, *s*, NMe), 2.86 (1H, *dd*, J = 14.5 and 11 Hz, $C_\beta H_a$), 3.70 (1H, *dd*, J = 14.5 and 3.5 Hz, $C_\beta H_b$), 4.62 (1H, *dd*, J = 11 and 3.5 Hz, $C_\alpha H$), 7.28 (5H, *m*, C_6H_5); leucine unit: δ0.78 (3H, *d*, J = 6 Hz, C_γ–Me), 0.83 (3H, *d*, J = 6 Hz, C_γ–Me), 1.10–1.80 (3H, *m*, $C_\beta H_a$, $C_\beta H_b$, $C_\gamma H$), 4.62 (1H, *m*, $C_\alpha H$), *ca* 7.30 (1H, *d*, J *ca* 10 Hz, NH).

Cyclo-(L-*leucyl*-N-*methyl*-L-*phenylalanyl*). Melting range: 164–166°, uncorr. UV $\lambda_{max}^{H_2O}$ nm (log ε): 258 (2.67). EIMS (75 eV) *m/z* (rel. int.): 274.1653 $[M]^+$ (23), 218.1049 $[M-C_4H_8]^+$ (14), 183.1116 $[M-C_7H_7]^+$ (80), 155.1191 $[m/z\ 183-CO]^+$ (100), 127.1250 $[m/z\ 155-CO]^+$ (31), 91.0546 $(C_7H_7)^+$ (27).

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