Pergamon

S0031-9422(96)00194-X

Phytochemistry, Vol. 42, No. 6, pp. 1537–1540, 1996

0031-9422/96 $15.00 + 0.00

ISOTENTOXIN, A CONVERSION PRODUCT OF THE PHYTOTOXIN TENTOXIN

Bernd Liebermann, Renate Ellinger and Eric Pinet*

Biologisch-Pharmazeutische Fakultät der Friedrich-Schiller-Universität Jena, Institut für Pharmazie, Neugasse 23, D-07743 Jena, Germany; *Section de Bioénergétique, Département de Biologie Cellulaire et Moléculaire, CEA-Saclay, F-91191 Gif-sur-Yvette Cedex, France

(*Received* 8 *January* 1996)

Key Word Index—*Alternaria alternata*; tentoxin; isotentoxin; phytotoxins; UV irradiation; structure elucidation; bioassay; chlorosis; wilting.

Abstract—Isotentoxin, the *E*-isomer of the phytotoxin tentoxin, can be produced from tentoxin by UV irradiation. A clean separation of both cyclopeptides is possible using adsorption chromatography on Sephadex G-15. Since artificial daylight causes an inter-conversion of both compounds, special care should be taken in consideration for the interpretation of the chlorosis bioassays. Indeed, isotentoxin seems to have no chlorosis activity, but has stronger wilting effects than tentoxin against the problem weed *Galium aparine* L.

INTRODUCTION

The phytopathogenic deuteromycete *Alternaria alternata* (Fries) Keissler produces, among other secondary metabolites, the cyclic tetrapeptides tentoxin [1] and its natural precursor dihydrotentoxin [2–4]. Tentoxin as a non-host-specific toxin, causes chlorosis in the seedlings of many plants [5], other bioactivities are also well-known (for a review, see [6]). The most important effect of tentoxin may be its influence on the photophosphorylation by binding to the chloroplast coupling factor 1 (CF_1) [7–9]. To some extent concerning chlorosis a species specificity exists. Therefore, some authors discuss the possibility to use tentoxin as a potential biogenic herbicide [10].

In order to reveal the mode(s) of action, the conformational properties of both tentoxin and analogues in aqueous solution must be known. Using ^1H NMR spectroscopy, tentoxin was recently reported to present four conformations in water [11]. Tentoxin is chemically cyclo-[L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl] [12]. The presence of the styrene structure in the dehydrophenylalanyl residue is crucial for the chlorotic activity, since dihydrotentoxin has almost no chlorotic effect [2, 13]. Indeed this unsaturated bond governs the backbone geometry as already described [11]. In this context it should be interesting to investigate the influence of a configurational change in the unsaturated unit on both biological activities and the appearance of different conformers in aqueous solution. In this paper we report the preparation of isotentoxin and its chlorosis and wilting effects.

RESULTS AND DISCUSSION

Production and identification of isotentoxin

The conversion of tentoxin to isotentoxin (cyclo-[L-leucyl-*N*-methyl-(*E*)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl]) can be performed by UV-irradiation with a common thin layer chromatography detection lamp at 254 nm. Isotentoxin can be retrograde converted to tentoxin in the same manner (Fig. 1). For analytical scale under the conditions described in Experimental, an equilibrium state appears after 30 min. In both cases, the balance is only slightly in favour of isotentoxin (53%). The reaction courses were monitored by analytical HPLC, isotentoxin is eluated shortly before tentoxin (factor 0.93). In the preparative scale with higher concentrations, the time necessary for the establishment of the equilibrium state is about 2.5 hr. With longer irradiation time, both compounds were destroyed. We assume a dimerization reaction comparable with the formation of truxinic/truxillic acids from cinnamic acid by UV irradiation. Recently Bland *et al.* [13] reported the photoisomerization of tentoxin at longer wavelengths (>300 nm) for a long time (6–29 hr), but with incomplete experimental data (NMR, UV, optimal time of irradiation, stability of the compound).

In order to separate isotentoxin from remaining tentoxin we used the adsorption effects on a Sephadex G-15 column caused by the different double binding systems. With this method a separation of tentoxin and dihydrotentoxin was also previously possible [14]: V_E/V_0 (tentoxin) = 3.07, (dihydrotentoxin) = 2.61, (iso-

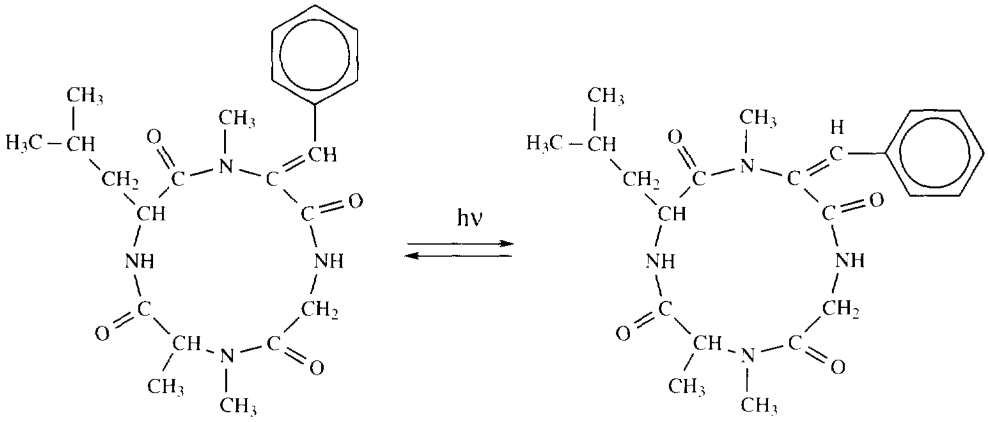

Fig. 1. Interconversion of tentoxin and isotentoxin under UV light.

tentoxin) = 2.12. This procedure is very cheap (elution with water) and yields an excellent purity of isotentoxin. In contrast to the HPLC method it is possible to quantify very small amounts of tentoxin or isotentoxin in the presence of a large quantity of the other.

Identity of istentoxin was proved by two-dimensional TLC of the hydrolysis products (leucine, glycine, *N*-methylalanine, sarcosine, and methylamine). As expected, the UV extinction coefficient is considerably lower in comparison to that of tentoxin (9.700 and 17.500, respectively, in H_2O). A comparable result is known with the compound Boc-MeAla-Leu-Phe(Δ)-Gly-OMe, an intermediate in tentoxin synthesis (Δ^E 9.080, Δ^Z 18.400) [15]. The estimation of the extinction coefficients of isotentoxin and tentoxin was carried out with compounds freshly separated on Sephadex G-15 being free of mutual impurities. Concerning the purity of both compounds, a long exposure to UV light detecting the substances on preparative TLC plates must also be avoided.

An additional proof for the identification of isotentoxin was supplied by ES mass spectrometry. The molecule peak was identical to the one of tentoxin, excluding a ring opening of the cyclopeptide.

The most important proof of the changed structure was shown by ^1H NMR spectroscopy. The signal of the vinyl proton $C^{\beta}H$ of *N*-methyl-Δ^Z-Phe-tentoxin (7.75 ppm) was shifted to 7.0 ppm for *N*-methyl-Phe-Δ^E-tentoxin (isotentoxin). In a NOE experiment, an excitation of this proton caused an intensification of the signal of the *N*-methyl group of dehydro-phenylalanine at 3.17 ppm indicating a spatial proximity of the two protons in isotentoxin.

A conversion of tentoxin into isotentoxin also occurs during the isolation procedure of the phytotoxin from the culture filtrate of *A. alternata*, obviously caused by the step of continuous ether extraction over 8 hr [3, 14]. To quantify this conversion effect we added radioactively labelled tentoxin to a 14-day-old culture filtrate with unlabelled phytotoxin and isolated tentoxin and isotentoxin as usual. By isotope dilution estimation we could calculate not only the initial content of the phytotoxin in the culture filtrate, but also that about 1.5% tentoxin was converted to its isomer.

At room temperature and under diffuse daylight both substances are relatively stable in aqueous solution. Equilibrium states were reached after three days and were constant after seven days: tentoxin with 3% isotentoxin, and isotentoxin with 7% tentoxin. However, artificial daylight or irradiation by a light heat lamp cause conversion effects. Boiling pure tentoxin in reflux with *n*-PrOH we reached 3.7% isotentoxin after 1.5 hr increasing continuously to 13% after 8 hr. We recognized a destruction of both compounds at the same time revealed by separation on Sephadex G-15, which showed additional decomposition products. These results must especially be taken into consideration in the performance of the bioassays (see below).

Biological activities of isotentoxin

Bland *et al.* [13] described a chlorosis assay with lettuce seeds kept under continuous light at 28° for 48 hr, where tentoxin induced chlorosis at the lowest concentration tested ($10\,\mu g\ ml^{-1}$), isotentoxin was 150–200 times less active than the natural toxin. We used cucumber seedlings kept under continuous artificial daylight for 120 hr in a comparable bioassay [14], the limited concentration causing chlorosis was only $0.25\,\mu g$ tentoxin ml^{-1}. Table 1 shows the dose-response values both of tentoxin and isotentoxin. At first glance isotentoxin seems to cause a clear but weaker chlorosis effect. However, at the end of the isotentoxin bioassay we can detect a conversion to 40% tentoxin. We deduce, therefore, that isotentoxin has no or only a very small bioactivity in the chlorosis test.

In a control experiment (bioassay without plants) both tentoxin and isotentoxin were converted to the isomeric compounds with a ratio isotentoxin/tentoxin of 55/45. These results allow the conclusion that the real chlorosis-inducing activity of tentoxin is higher than hitherto reported. The conversion effects in this

Table 1. Chlorosis assay of tentoxin and isotentoxin with cucumber seedlings as described in ref. [14]

Concn μg ml^{-1}	Chlorosis %	
	By tentoxin	By isotentoxin
0	0	0
0.25	16.6	—
0.5	44.3	—
0.75	70.5	—
1.0	84.0	5.5
2.0	95.3	—
5.0	—	11.9
10.0	—	18.1
20.0	—	85.6
50.0	—	98.1
100.0	—	96.9

bioassay can be explained by looking at the absorption spectrum of tentoxin/isotentoxin, the usual spectrum of artificial daylight and the transmission of the used glassware. Since tentoxin is an important tool in the investigation of photophosphorylation, careful handling will be necessary in order to prevent an undesired conversion to isotentoxin.

Wilting effects of tentoxin and isotentoxin against the problem weed *Galium aparine* were studied under diffuse daylight for only 48 hr [16]. Therefore, only a slight conversion was detected as described above. Table 2 shows that isotentoxin causes wilting at a lower concentration than tentoxin. The existence of isotentoxin and its wilting activity rules out the presence of a postulated tentoxin conjugate reported in the past [16].

Conclusion

We describe here an easy and cheap method to produce a photoisomer of the natural phytotoxin tentoxin which exhibits a wilting effect better than tentoxin and probably a poorer chlorotic effect. Such an analogue will be very useful to understand the structure/function relationships. Our method could certainly be adapted to obtain Δ^E Phe isomers from other tentoxin analogues with ring enlargement.

Table 2. Wilting assay of tentoxin and isotentoxin with *Galium aparine* L. (represented by wilting factors as described in ref. [16])

Concn μg ml^{-1}	Wilting by tentoxin	Wilting by isotentoxin
0	0	0
50	0	1.0
100	0	1.75
150	0.5	1.75
300	2.0	—
600	2.6	2.25
1200	3.0	2.75
2400	3.5	3.25

EXPERIMENTAL

Tentoxin was produced by a strain of *Alternaria alternata* and isolated as described [3, 14]. UV irradiation was carried out with a detection lamp (DESAGA HP-UVIS, Hg low pressure lamp, irradiation power 4 W m^{-2}) at 254 nm without cooling. (a) Analytical scale for equilibrium characterization: 30 μg tentoxin or isotentoxin ml^{-1} MeOH, fill height: 1 cm. (b). Preparative scale: 2.5 mg tentoxin ml^{-1} MeOH, fill height: 0.5 cm.

The ratio tentoxin/isotentoxin was determined by HPLC (Shimadzu equipment LC-7A, UV detector SPD-7A, integrator C-R4AD; Hibar LiChrosorb RP-18 column, 250 × 4mm, with a guard column; MeOH–H_2O (13:7), 0.6 ml min^{-1}, isocratic, room temp.; detection at 286 nm, retention times: tentoxin 12.7 min, isotentoxin 11.8 min). Estimation of both extinction coefficients in MeOH–H_2O (13:7) at 286 nm allowed the calculation of the ratio.

Separation on Sephadex G-15 and 2D-TLC were reported elsewhere [3].

UV spectra were recorded on a Shimadzu spectrophotometer UV-160 A: isotentoxin [λ_{max} (MeOH) nm (log ε): 281 (4.05); (H_2O) nm (log ε): 281 (3.99)]. ES-MS spectra were recorded on a VG Quattro (Fisons), ^{1}H NMR spectra on a Unity plus 600 (Varian, 600 MHz).

Estimation of isotentoxin formation during the isolation procedure of tentoxin: 18.8 mg tentoxin-[U-^{14}C-leucine] with a specific radioactivity of 1.3×10^6 Bq mmol^{-1} were added to 1.4 l culture filtrate, tentoxin was isolated by a run on Sephadex G-15 as described [3], the range of isotentoxin was rechromatographed twice, in order to purify it from the remaining radioactively labelled tentoxin. The isotentoxin radioactivity curve was recorded by measurement of scintillation count in dioxan cocktails [4]. The radioactively labelled tentoxin was produced by feeding [U-^{14}C]-L-leucine into a tentoxin biosynthesizing culture of *A. alternata* [17].

Chlorosis and wilting bioassays were carried out as described ([14] and [16], respectively).

Acknowledgements—We are grateful to Dr W. Ihn, Hans-Knöll-Institut für Naturstofforschung Jena for performing the ES-MS, and to Mr Leppert, Institut für Molekulare Biotechnologie Jena for performing the NMR, we also thank Dr W. Günther, Institut für Organische Chemie und Makromolekulare Chemie for critical discussion and Miss E. Hänert for reliable technical assistance. This work was supported by a grant of the Federal Ministry of Research and Technology (BMFT).

REFERENCES

1. Templeton, G. E., Meyer, W. L., Grable, C. I. and Sigel, C. W. (1967) *Phytopathology* **57**, 516.
2. Kono, Y., Gardner, J. M. and Takeuchi, S. (1986) *Agric. Biol. Chem.* **50**, 2401.

3. Liebermann, B., Ihn, W., Baumann, E. and Tresselt, D. (1988) *Photochemistry* **27**, 357.
4. Liebermann, B. and Ihn, W. (1988) *J. Basic Microbiol.* **28**, 63.
5. Durbin, R. D. and Uchytil, T. F. (1977) *Phytopathology* **67**, 602.
6. Dahse, I. (1992) in *Vom Organismus zum Molekül. Physiologische Prozesse, ihre Modellierung und Beeinflußbarkeit auf verschiedenen Ebenen* (Dahse, I., ed.), p. 155, Friedrich-Schiller-Universität Jena, Germany.
7. Hu, N., Mills, D. A., Huchzermeyer, B. and Richter, M. L. (1993) *J. Biol. Chem.* **268**, 8536.
8. Ohta, Y., Yoshioka, T., Mochimaru, M., Hisabori, T. and Sakurai, H. (1993) *Plant Cell Physiol.* **34**, 523.
9. Dahse, I., Pezennec, S., Girault, G., Berger G., André, F. and Liebermann, B. (1994) *J. Plant Physiol.* **143**, 615.
10. Lax, A. R., Shepherd, H. S. and Edwards, J. V. (1988) *Weed Technol.* **2**, 540.
11. Pinet, E., Neumann, J. M., Dahse, I., Girault, G. and André, F. (1995) *Biopolymers*, **36**, 135.
12. Meyer, W. L., Templeton, G. E., Grable, C. I., Jones, R., Kuyper, L. F., Lewis, R. B., Sigel, C. W. and Woodhead, S. H. (1975) *J. Am. Chem. Soc.* **97**, 3802.
13. Bland, J. M., Edwards, J. V., Eaton, S. R. and Lax, A. R. (1993) *Pestic. Sci.* **39**, 331.
14. Liebermann, B. and Oertel, B. (1983) *Ztschr. Allgem. Mikrobiol.* **23**, 503.
15. Rich, D. H., Bhatnagar, P., Mathiaparanam, P., Grant, J. A. and Tam, J. P. (1978) *J. Org. Chem.* **43**, 296.
16. Liebermann, B. and Kölblin, R. (1992) *J. Phytopath.* **135**, 245.
17. Liebermann, B., Schuffenhauer, S. and Ihn W. (1991) *J. Basic Microbiol.* **31**, 51.