Pergamon

PII: S0031-9422(96)00789-3

Phytochemistry, Vol. 45, No. 1, pp. 37–40, 1997

0031-9422/97 $17.00+0.00

ISOLATION AND SYNTHESIS OF A HOST-SELECTIVE TOXIN PRODUCED BY *ALTERNARIA ALTERNATA*

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(*Received in revised form* 9 *October* 1996)

Key Word Index—phytotoxins; AS-I; *Alternaria alternata*; tetrapeptide; synthesis; HPLC.

Abstract—Two phytotoxins are isolated from culture filtrates of an *Alternaria alternata* pathogenic to sunflower. One was identified by chemical and physicochemical techniques as the tetrapeptide Ser-Val-Gly-Glu. This peptide, for which we suggested the name AS-I toxin, was further characterised by synthesis and by its phytotoxic effect on sunflower and other plants.

INTRODUCTION

Fungal toxins play an important role in pathogenesis of plants [1]. The fungus *Alternaria alternata* (Fries) Keissler has been reported to cause various phytotoxic effects during pathogenesis, including chlorosis or necrosis on leaves and other parts of the plant, as well as inhibition of seed germination [2–12]. At least seven host-specific phytotoxins, which the fungus produces while attacking respective hosts, have been reported [9, 13]. Host-specific phytotoxins, produced by more than 10 species of fungal pathogens, include relatively low M_r cyclodepsipeptides, terpenopeptides [14] and unbranched long chain polyketols and esters of organic acids with long chain alcohols [15].

Here, we report the isolation of two low M_r phytotoxins extracted from culture filtrates of *A. alternata* pathogenic to sunflower leaves [15, 16]. The structure of one of the two host selective toxins, deduced from chemical and physicochemical methods, mainly by plasma desorption mass spectrometry, and its synthesis and phytotoxicity on various plants are also described.

RESULTS AND DISCUSSION

Crude ethylacetate extract of *Alternaria alternata* culture filtrates caused, necrotic spots on sunflower leaves (Fig. 1). The crude EtOAc extract was evaporated to dryness and chromatographed on a Biogel P-4 column (110 × 1.5 cm). The phytotoxic fraction was eluted from the column as a rather broad peak indicating the presence of more than one low M_r compound, and TLC in systems 1 and 3 showed the presence of more than three spots. The fractions corresponding to peak B (Fig. 2) which were found to be toxic to sunflower leaves from Biogel P-4 were subsequently analysed by HPLC on a lichrosphere RP C8 column with the first gradient. Only fractions corresponding to peaks 9 and 11 (Fig. 3) were found to cause necrotic spots on sunflower leaves similar to

Fig. 1. Toxic effect of crude ethylacetate extract on sunflower leaves.

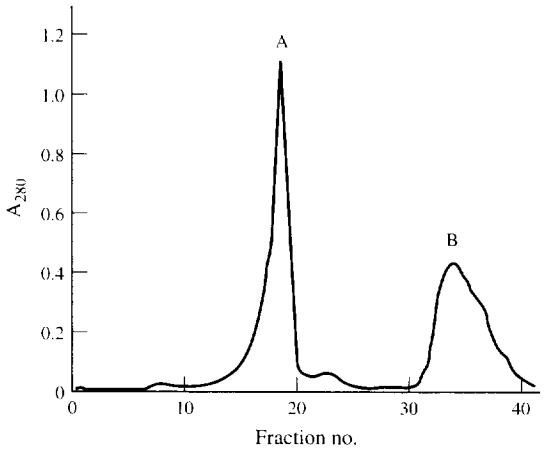

Fig. 2. Separation of crude ethylacetate extract on a Biogel P-4 column.

Table 1. Positive ion PD mass spectrum of AS-I toxin

Fragments	*m/z*
(MH^+)	391
Val (I_2)	72
Ser (I_1)	60
Glu (I_4)	57
Gly (I_3)	30
A_2	158
A_3	216
B_1	81
B_2	187

* Spectrum data are restricted to significant ions only.

those caused by the crude filtrate and EtOAc extract. Fractions from these two peaks were further purified on the same column with the second gradient. TLC of peak 9 R_f 0.5 proved its homogeneity. Five carbonyl groups (>C = 0) were found in the IR spectrum three amides (1675 and 1545 cm^{-1}) and two acids (1735 cm^{-1}). Secondary amide groups were found (at 3300 and 3280 cm^{-1}). UV-Vis spectrum of the compound showed a λ_{max}^{MeOH} nm: 210.

Amino acid analysis of the compound showed the presence of Val, Ser, Gly, Glu in almost 1:1:1:1 ratio, whereas its PD mass spectrum gave a M_r of 390. Cationized species $[M+H]^+$ at 391, $[M+Na]^+$ at 413 and $[M+K]^-$ at 429 were observed. The fragmentation pattern of the positive ion mass spectrum (Table 1) showed the presence of Val (I_2) at 72, Ser (I_1) at 60, Glu (I_4) at 57 and Gly (I_3) at 30 *m/z*. Additional sequence fragments B_1 and B_2 at 88 and 187, respectively, were observed. Other sequence fragments A_2 and A_3 at 158 and 216 also appeared. The nomenclature used for designating sequence ions is that reported by Roepstorff and Fohlman [18] and Biemann [19, 20].

From the above data the structure of the toxin was deduced as the tetrapeptide Ser-Val-Gly-Glu. This peptide toxin, named henceforth AS-I (*Alternaria alternata*, Sunflower toxin I), was synthesized as described in the Experimental and identified by the same methods used for the isolated toxin AS-I. The synthesis of the tetrapeptide toxin was carried out in solution using *N*-*t*.Boc-amino acids and starting from Boc-Glu(OMe)-OMe as the C-terminal. The mixed anhydride and DCCI coupling methods were used. Synthetic tetrapeptide was eluted exactly at the same time (33 min) when analysed by HPLC on the same lichroshpere C8 column with the second gradient, and its PD mass spectrum gave the same fragmentation pattern as that reported above for the isolated AS-I toxin. Considering the name suggested for the isolated toxin, it should be noticed that names similar to the above were given to other isolated host-specific toxins of *Alternaria alternata* pathogenic to various plants ([13] and references therein).

Experiments were repeated twice by preparing new filtrate cultures and extracting them with EtOAc. It was found that ethylacetate extracts, submitted to the

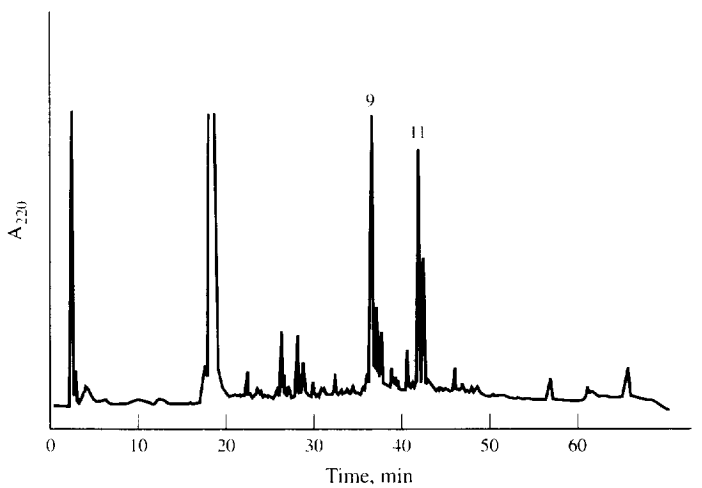

Fig. 3. HPLC separation of phytotoxic fraction B from Biogel P-4 on a lichrosphere RP C8 column.

protocol of purification reported in the Experimental, always gave the same results. Isolated toxin AS-I developed necrotic spots on sunflower leaves on the 3rd day after application. Necrosis was not developed on tobacco leaves even 2 weeks after application. Synthetic AS-I at 7×10^{-2} M to a threshold concentration of 10^{-2} M caused strong necrotic spots on young sunflower leaves, including veinal necrosis. At lower than 10^{-2} M the effect appeared the 2nd or 3rd day after the application of the solution to sunflower leaves. Synthetic AS-I was also tested, at the same concentrations as above, for toxicity in other plants. A very slight toxic effect was observed on tobacco and zucchini leaves only at the highest concentration tested (7×10^{-2} M). No toxicity could be detected in the other plants tested. This presents strong evidence that AS-I is a host specific toxin. The characterization of the other isolated toxin (peak 11, Fig. 3) is in progress and will be reported soon.

EXPERIMENTAL

General. IR spectra were measured in nujol. TLC was performed on silica gel G (Merck) using: (1) $CHCl_3$–MeOH (9:1); (2) $CHCl_3$–MeOH–HOAc (11:8:1); and (3) EtOAc–toluene (7:3). Spots were visualized by their fluorescence at 254 nm or by spraying with 1% ninhydrin in acetone. Analytical data were provided by the Microanalytical service laboratory at the Department of Chemistry Aristotle University.

Isolation and culture of the fungus. Alternaria alternata was isolated from diseased leaves of the sunflower (*Helianthus annuus* L.) cultivated in Northern Greece. Pure cultures of the fungus were obtained from single spore isolates. A virulent isolated [IMI 366417(1)] was grown in a modified Richard's soln (containing 37.5 g of sucrose per 1 of medium) for 14–17 days at 20 in the dark without agitation.

Separation of phytotoxic fraction. Culture filtrates of *A. alternata* (2 l of medium) were extracted with EtOAc (3×250 ml). Organic layers were concd to dryness and dissolved in the minimum vol. of $2\times$ distilled H_2O. The soln was centrifuged at 3500 *g* for 10 min and the supernatant was chromatographed on a Biogel P-4 column (110×1.5 cm). The column was eluted with H_2O, fractions of 2 ml were collected and their *A* was measured at 280 nm. Frs that developed necrotic spots on young sunflower leaves were collected and lyophilized. This fr., called henceforth phytotoxic fr., was further purified by HPLC.

HPLC analysis. Phytotoxic fr. was further analysed by HPLC on a lichrosphere RP-C8 column, 5 μm. Flow speed 0.6 ml min^{-1}, *A* 220 nm, paper speed 2 mm min^{-1}. Solvents: A: 0.1% TFA in H_2O, B: 0.1% TFA in 1 MeCr. 1. Gradient: 0 time (min) 100% A, 10 min 100% A, 60 min 20% A–80% B, 61 min 20% A–80% 70 min 100% A. 2. Gradient: 0 time (min) 1 min 100% 10 min 75% A–25% B, 50 min 50% A–50% B 55 min 20% A–80% B, 60 min 100% A.

Amino acid analysis was performed on a Beckman model C120 Analyser. The acid hydrolysis of the sample was carried out at 110 under N_2 for 22 hr. W-1 single column and AA15 for basic amino acids were used.

M_r and structure determination by plasma desorption mass spectrometry. The experiments were carried out on the time-of-flight mass spectrometer DEPIL-X built at the IPN (Institut de Physique Nucleaire, Orsay, France). The PD mass spectra were recorded in both positive and negative modes. Spectra were run at an acceleration voltage of +10 kV and collected over 600 sec. Mass assignments of the peaks were based on calcn of peak centroids using H^+ and Na^+ masses (H^+ 1.00073 u and Na^+ = 22.9892 u) for calibration of the positive PD mass spectra. The H^+ and CN^- ions were used to calibrate the negative PD mass spectra (H^+ = 1.0089 and CN^- = 26.0036 u).

Sample preparation. The isolated toxin and the synthetic one, respectively, were dissolved in a 1:1 mixt. of isoPrOH and ultra pure H_2O. For positive ion spectra, the solns were purified on Amberlyst 15 acidic resin in order to prevent formation of cationized molecules. The sample was transferred to the sample holder of the ^{252}Cf mass spectrometer system as reported in ref. [17].

Synthesis of AS-I. HClGlu(OMe)-OMe Thionyl chloride (4 ml) was added dropwise in MeOH (15 ml) chilled at 0 followed by addition of glutamic acid (2.94 g, 20 mmol). The soln was stirred for 10 min at 0 and 24 hr at room temp. The solvent was concd *in vacuo* and the oily residue was solidified by addition of dry Et_2O. The pptd product was filtered, washed on the filter with dry Et_2O and crystallized twice from MeOH–Et_2O. It was dried over NaOH–P_2O_5. Yield 3.8 g (89.8%), mp 92–93 , $[a]^{25}$ +17 (c1,EtOH), R_{f1} 0.39, R_{f2} 0.48.

Boc-Gly-Glu(OMe)-OMe. To a stirred soln of Boc-Gly-OH (0.58 g, 3.3 mmol) prepd as described previously [18] and *N*-methyl morpholine (0.35 ml, 3.3 mmol) in dry tetrahydrofuran (5 ml), cooled to −15 , isobutyl chloroformate (0.42 ml, 3.3 mmol) was added. The mixt. was stirred for 2 min and a neutralized soln of H-Glu(OMe)-OMe (0.64 g, 3 mmol) was added into the mixed anhydrite. The stirring was continued for 2 hr at 15 and 1 hr at room temp. Subsequently the solvent was evapd *in vacuo* and the oily residue was subjected to flash column chromatography on silica gel (230–400 mesh) using EtOAc toluene (7/3) as eluent. The fragments containing the product were pooled and the solvent evapd *in vacuo* provided an oil. Yield 0.75 g (77%), R_{f2} 0.33, R_{f3} 0.59.

TFA.Gly-Glu(OMe)-OMe. Boc-Gly-Glu(OMe)-OMe (650 mg, 1.95 mmol) was deprotected by TFA/CH_2Cl_2 30% (3 ml). The mixt. was stirred for 30 min at room temp. Addition of Et_2O afforded a ppt which was collected, after 24 hr at 0 , by filtration. Yield 615 mg (95%) (R_{f2} 0.66, and R_{f1} 0.07).

Boc-Val-Gly-Glu(OMe)-OMe. To a stirred soln of TFA.Gly-Glu(OMe)-OMe (0.615 g, 1.78 mmol) in dry DMF (5 ml) neutralized with TEA, Boc-Val (0.39 g,

1.78 mmol) and DCCI (0.36 g, 1.78 mmol) were added at 0°. The mixt. was stirred for 2 hr at 0° and 20 hr at room temp. After the extraction of the reaction mixt. with EtOAc and washes with citric acid, brine and $NaHCO_3$ soln, the tripeptide Boc-Val-Gly-Glu-(OMe)-OMe was obtained. Yield 0.45 g (58%) R_{f1} 0.9.

TFA.Val-Gly-Glu(OMe)-OMe. Boc-Val-Gly-Glu-(OMe)-OMe (300 mg, 0.7 mmol) was treated with TFA/CH_2Cl_2 30% (2 ml) as described above. Yield 350 mg (79%) R_{f1}0.24.

Boc-Ser-Val-Gly-Glu(OMe)-OMe. To a stirred soln of TFA.Val-Gly-Glu(OMe)-OMe (0.3 g, 0.67 mmol) in dry DMF (3 ml) neutralized with equimolar amount of TEA, Boc-Ser (0.16 g, 0.8 mmol) and DCCI (0.16 g, 0.8 mmol) were added at 0°. The reaction mixt. treated as in the previous case, afforded a solid material which after purification on silica gel column chromatography with $CHCl_3$ and MeOH gave the desired product. Yield 260 mg (50%) R_{f1}0.86, R_{f2}0.95.

Ser-Val-Gly-Glu. Boc-Ser-Val-Gly-Glu(OMe)-OMe (200 mg, 0.38 mmol) was saponified with 2 ml 1N NaOH at 50° for 2 hr. The process of the reaction was followed by TLC in systems 1 and 2. The pH of the soln was adjusted to 4.5 and extracted with EtOAc (2 × 50 ml). The organic layers was evapd to dryness. TLC of the solid material showed the presence of starting material which was sepd off by silica gel column chromatography to give 60 mg, yield 30% of Boc-Ser-Val-Gly-Glu. This was subsequently treated with 1.6 M HCl/HOAc (1 ml) for 15 min to give the final product after pptn and recrystallization with dry Et_2O. Yield 38 mg 80%. Analytical data (Found: C46.22; H, 6.57 cal. for $C_{15}H_{26}O_8N_4$: C, 46.15; H, 6.66%).

Toxicity bioassay. The presence of host-selective or non-selective toxins was detected by bioassays on leaves attached to plants. All bioassays for phytotoxicity were done on leaves of very young sunflower plants. Bioassays for selectivity of the isolated and synthetic toxin AS-I were done on leaves of various other plants including tobacco, beans, melon, zucchini, beetroot and cotton. Samples of 20 μl drop (from each fraction) were applied on the leaves and the plants were incubated at 25° for 48 hr.

Acknowledgements—The authors are grateful to Dr Th. Choli, Dr G. Kyriakou and G. Chalavatzis for their assistance and Dr M. F. Gonnord (Ecole Polytechnique, Palaiseau Cedex, France) for taking PD mass spectra.

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