Phytochemistry, Vol 23, No 10, pp 2193–2198, 1984
Printed in Great Britain

0031–9422/84 $3 00 + 0 00

PHYTOTOXINS FROM *STEMPHYLIUM BOTRYOSUM*: STRUCTURAL DETERMINATION OF STEMPHYLOXIN II, PRODUCTION IN CULTURE AND INTERACTION WITH IRON

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(*Received* 8 *December* 1983)

Key Word Index—*Stemphylium botryosum*, phytotoxins, stemphyloxin I, stemphyloxin II, iron

Abstract—Stemphyloxin II, a new phytotoxic compound isolated from liquid cultures of *Stemphylium botryosum* f sp *lycopersici* has been identified as a tricyclic compound derived from stemphyloxin I Production of stemphyloxins I and II increases by 4–6 fold in the presence of succinate, fumarate or malonate The secretion of stemphyloxins is iron-regulated and both compounds act as ferric chelates The phytotoxicity of stemphyloxin I is approximately 100-fold higher than that of stemphyloxin II The possible role of stemphyloxins in iron acquisition by *S botryosum* is discussed

INTRODUCTION

Stemphylium botryosum Wallr f sp *lycopersici* is the causal agent of leaf spot and foliage blight disease of tomato Culture filtrates of this fungal pathogen have been reported to contain a phytotoxin, stemphyloxin I, capable of producing symptoms associated with this disease [1] Stemphyloxin I has been recently crystallized and its structure was fully determined by X-ray diffraction analysis [2] It is a highly functionalized β-ketoaldehyde *trans*-decalone [1] and a completely novel natural product When stemphyloxin I was bioassayed by injection into intact leaves, it exhibited a differential phytotoxicity towards various plants, with tomato being the most sensitive [1] A more sensitive measure of toxin activity was obtained by following the suppression of ^{14}C-labelled amino acids incorporation into proteins of exponentially growing tomato cell suspension [1]

In further investigations we have detected an additional phytotoxin in culture filtrate which was designated stemphyloxin II Stemphyloxin II reacts with iron to yield a coloured product as reported for stemphyloxin I [2] In the present study we describe (a) the structure determination of stemphyloxin II, (b) nutritional factors affecting the production of stemphyloxins I and II in culture, and (c) comparative phytotoxicity of stemphyloxins I and II in relation to their interactions with iron

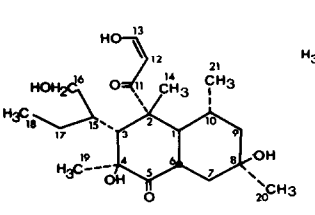
RESULTS AND DISCUSSION

Structural determination of stemphyloxin II

Stemphyloxin II was obtained as an amorphous material, mp 158–160°, *m/z* 364, $[M-H_2O]^+$, $C_{21}H_{32}O_5$ Since stemphyloxin I (**1**) can be easily converted, by acid or base catalysis, into stemphyloxin II(**2**), the close relationship between the two compounds is self-evident The two major changes in the structure of stemphyloxin II are the disappearance of the saturated ketone (ν_{max} 1705 cm^{-1}, δ216 5) and the alteration in the β-ketoaldehyde group A positive ferric chloride test and IR absorptions at 1665 and 1595 cm^{-1} clearly indicate the existence of a 1,3-dione in stemphyloxin II However, in contrast to stemphyloxin I, the β-ketoaldehyde of stemphyloxin II is now a 2,3-disubstituted group [δ7 16 *s* (1H) in II as compared to δ5 80 *d* (*J* = 4 9 Hz) and δ7 70 *d* (*J* = 4 9 Hz) in I] The foregoing changes are best explained by an aldol type condensation of the active methylene at C-12 with the carbonyl at C-5 in stemphyloxin I leading to a tricyclo [6 2 2 0^{2,7}] dodecane system

Although the C-5 hydroxyl is β to two CO groups, its elimination under the reaction conditions is prevented due to its bridge head position The proposed vicinity of the latter hydroxyl functionality to the C-4 hydroxyl was confirmed by a microperiodate oxidation with $NaIO_4$ [3], in which one mole of the oxidizing reagent was consumed The close proximity of the activated C-12 methylene to the C-5 carbonyl in the boat conformation of the cyclohexanone ring is most likely responsible for the easy formation of the C_5–C_{12} bridge The possibility of the cyclohexanone ring occupying a boat conformation results from a lower activation energy of the chair-boat equilibrium of cyclohexanones as compared to cyclohexanes and from the multi-substitution pattern of the ring

The ^{1}H and ^{13}C NMR spectra of stemphyloxin II (Tables 1 and 2) are in full agreement with the suggested structure In addition, the NMR data of stemphyloxin II agrees with the spectrum of betaenone A, a tricyclic phytotoxin recently isolated from culture filtrate of



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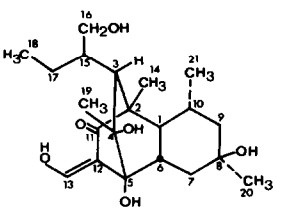

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Table 1 Comparison of the methyl chemical shifts of stemphyloxins I and II and betaenones A and B

Compound*	Me-18	Me-21	C-16	Me's 14 & 19	Me-20
Stemphyloxin II	0 89 *t*	0 97 *d*	3 80 AB quart	1 16 *s*, 1 17 *s*	1 25 *s*
Betaenone A	0 88 *t*	0 98 *d*	1 14 *d*	1 16 *s*, 1 17 *s*	1 25 *s*
Stemphyloxin I	0 83 *t*	0 75 *d*	3 4 and 3 87 AB quart	1 32 *s*, 1 62 *s*	1 26 *s*
Betaenone B	0 86 *t*	0 67 *d*	1 15 *d*	1 40 *s*, 1 57 *s*	1 26 *s*

*^{1}H NMR data for stemphyloxin I was obtained from Barash *et al* [1] and for betaenones A and B from Ichihara *et al* [4]

Table 2 Comparative ^{13}C NMR data of stemphyloxins I and II and betaenones A and B

Group	Stemphyloxin I	Betaenone B	Stemphyloxin II‡	Betaenone A
	19 7 (17)*	25 1 (17)	21 45 (17)	27 1 (17)
	41 6 (7)	41 5 (7)	39 90 *dd* (7)	40 0 (7)
CH_2		43 9 (12)		
	47 6 (9)	47 7 (9)	52 24 *dd* (9)	52 6 (9)
	66 1 (16)	58 4 (13)	63 75 (16)	
	31 1 (10)	29 1 (10)	31 75 (10)	32 1 (10)
	40 5 (1)	40 3 (1)	37 27 (1)	37 6 (1)†
	43 0† (15)	35 8 (15)	44 52 (15)	36 2 (15)†
CH	45 0† (3)	46 6 (3)	46 52 (3)	52 9 (3)
	55 0 (6)	57 3 (6)	57 69 (6)	57 4 (6)
	101 7 (12)			
	172 2 (13)		158 23 (13)	158 1 (13)
	49 9 (2)	52 9 (2)	50 09 (2)	50 8 (2)
	68 7 (8)	68 7 (8)	71 21 (8)	71 5 (8)
C	75 9 (4)	77 3 (4)	74 35 (4)	74 0 (4)
	207 2 (11)	217 6 (11)	208 23 (11)	207 8 (11)
	216 5 (5)	216 9 (5)	80 16 (5)	80 6 (5)
			112 91 (12)	112 5 (12)

*Numbers in parentheses are tentative line assignments based on δ-values, substituent effects and comparisons of the chemical shifts of stemphyloxins I and II and betaenones A and B

†Assignments may be interchanged

‡Recorded in a Bruker 300 MHz instrument

Phoma betae the structure of which was determined by X-ray analysis [4] Comparison of the ^{13}C NMR spectra of stemphyloxins I [1] and II with those of betaenones A and B [4] enabled us to partially interpret the spectra of the 4 compounds as summarized in Table 2 The methyls 14 and 19 of the cyclohexanone ring are expected to be significantly affected during the cyclization process Indeed, the two methyl groups are strongly shifted upfield due to the disappearance of the neighbouring CO group in the case of C-19, and a change in the nature and spatial orientation of the β-ketoaldehyde moiety in the case of C-14 Stemphyloxin II can be obtained from stemphyloxin I by a 'bio-aldol' condensation, as discussed previously

Both stemphyloxins I and II were detected in culture filtrates of *S botryosum* f sp *lycopersici* However, since stemphyloxin I can easily undergo an acid or base catalysed transformation into stemphyloxin II, it is difficult to determine whether the latter substance is indeed a true natural compound

Production of stemphyloxins I and II in culture

Stemphyloxins production begins early and quickly becomes proportional to the growth rate of the fungus [2] Sucrose or glucose were found to serve as optimal carbon sources, and L-glutamate as an optimal nitrogen source for stemphyloxin secretion The effects of various concentrations of either sucrose or L-glutamate on the formation of stemphyloxins is illustrated in Fig 1 When sucrose concentration was elevated from 0 5 to 1 5% at a constant level of L-glutamate (1 3%) the amount of stemphyloxins increased by approximately 10-fold At concentrations higher than 2%, sucrose caused repression of toxins production Similarly, when sucrose level was kept at 2% and L-glutamate varied from 0 3 to 1 5%, stemphyloxins production increased about 10 times but diminished sharply thereafter at higher glutamate concentration Information on the effect of carbon concentration on toxin production by plant pathogens is very scanty

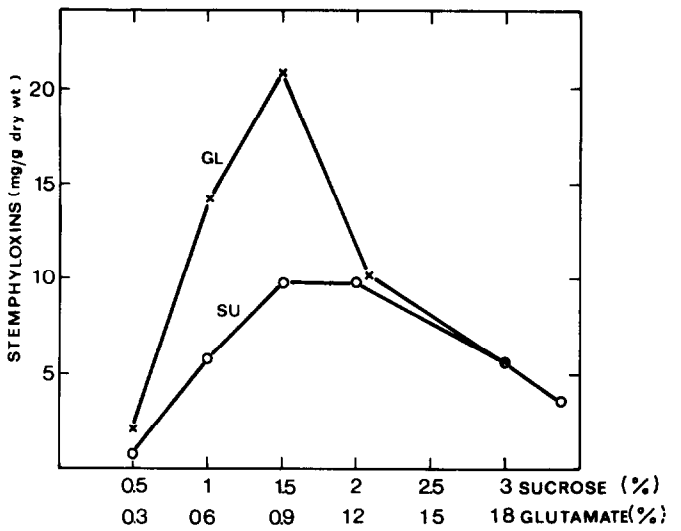

Fig 1 Stemphyloxins I and II formation as a function of sucrose and L-glutamate concentration Cultures were grown on the sucrose–glutamate medium as described in the Experimental in which the concentration of either sucrose or glutamate was varied SU, Different concentrations of sucrose with a constant level of L-glutamate (1 3%) GL, Different concentrations of L-glutamate with a constant level of sucrose (2%) Results are an average of three different experiments

The effect of sucrose or glucose concentration on toxin formation by *Alternaria dauci* [5] and *Corynospora cassiicola* [6] has been studied in chemically defined media In both cases toxin production was maximal at 3% carbon source and was not affected by elevating the sugar concentration In contrast, the repression of stemphyloxins at high carbon concentrations suggests that in addition to serving as a source for synthesis of cellular materials, it may act as a metabolic regulator and hence have a profound effect on toxin production The significant reduction in stemphyloxin formation at high glutamate concentration may be ascribed to a decrease in carbon–nitrogen ratio The latter is known to play a major role in the biosynthesis of secondary metabolites in fungi [5, 7]

Addition of dicarboxylic acids, such as succinate, fumarate or malonate to the sucrose–glutamate medium at 0 017 M further increased the accumulation of the secreted phytotoxins by 4–5 fold as compared to the control whereas the growth rate of the fungus remained unaffected (Fig 2) These results suggest that the precursors for the biosynthesis of the stemphyloxins are closely linked to compounds derived from the tricarboxylic acid cycle It has been previously postulated that propionyl and acetyl units through their coenzyme A intermediates are the most likely precursors [2]

From the various divalent cations which were examined for an effect on toxin production, iron appeared to induce stemphyloxin accumulation by approximately 5-fold (Fig 3A) The iron effect reached an optimum at 2 mg/l, but decreased significantly in the presence of higher iron concentrations (Fig 3B) In contrast to the inducing effect of iron on stemphyloxin production, the presence of 0 1 mg/l ferric ion in the medium completely repressed the secretion of three hydroxamate siderophores which have been identified in *S botryosum* [Manulis, S, unpublished results]

Iron binding and phytotoxicity of stemphyloxins

Addition of an aqueous solution of ferric ion to stemphyloxin I produced a stable reddish complex with a maximal absorption at 480 nm (Fig 4A) The absorption spectrum of Fe(III)–stemphyloxin II complex has a maximum at 510 nm Stemphyloxins I and II show high affinity for ferric but not for ferrous ion The stoichiometry of Fe(III)–stemphyloxin I complex that was

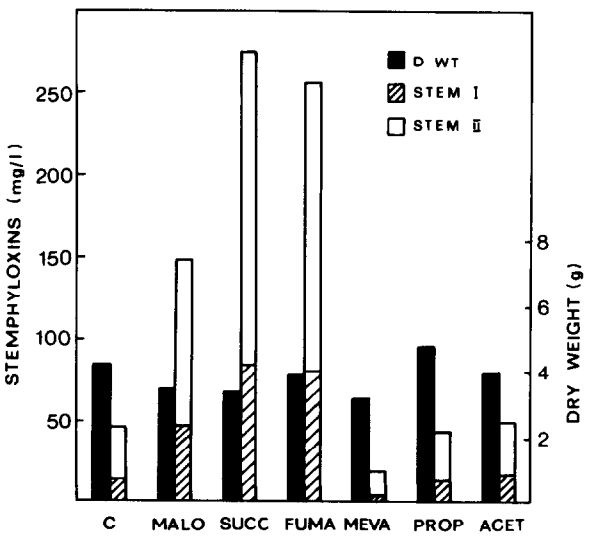

Fig 2 Effect of mono- and dicarboxylic acids on stemphyloxins production The cultures were grown on sucrose–glutamate medium (C) with the addition of each of the following organic acids at a concentration of 0 017 M malonate (MALO), succinate (SUCC), fumarate (FUMA), mevalonate (MEVA), propionate (PROP) and acetate (ACET)

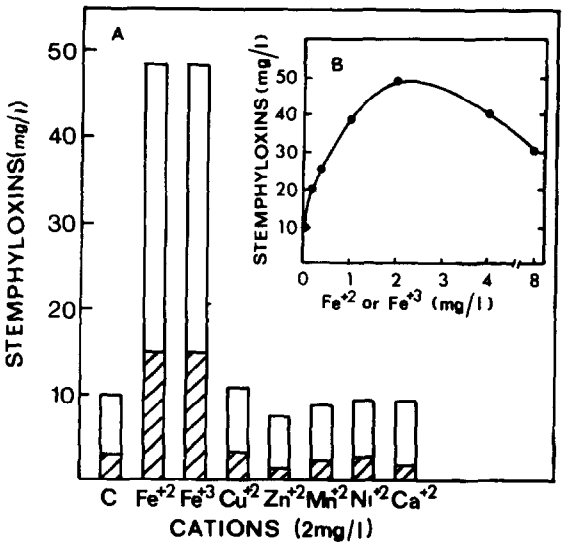

Fig 3A Effects of divalent cations on stemphyloxin production The cultures were grown on sucrose–glutamate medium but without iron Cations were added as indicated in the diagram ▨, Stemphyloxin I, □, stemphyloxin II B Secretion of the two stemphyloxins as a function of iron concentration Growth conditions as described above

calculated from changes in the absorption spectrum at 480 nm as a function of Fe^{3+} added (Fig 4A, B) is 1 3 An identical stoichiometry was obtained for Fe(III)–stemphyloxin II complex

The apparent stability constants (k_2) for the Fe(III)–stemphyloxin complexes were measured and calculated according to Rosotti and Rosotti [8] and Meyer and Abdallah [9] Using EDTA as a competitive chelator of Fe^{3+}, the stability constants for stemphyloxins I and II at pH 7 were found to be 1.7×10^{24} and 1.6×10^{24} respectively

The observations that the biosynthesis of stemphyloxins is iron-regulated and that these ligands act as chelates of ferric ion, suggest that they might play a significant role in the aquisition of iron by *S botryosum* Iron is an essential nutrient for most living cells and ferric ion is the predominant oxidation state prevailing in aerobic environment [10] In spite of its abundance in nature, the availability of iron is extremely low, due to its profound insolubility [10] Therefore, most microbes developed sequestering systems for ferric ion to secure its utilization [11] Hydroxamates have been demonstrated to function as siderophores in fungi [10] Their properties include specific derepression under conditions of Fe^{3+} deficiency and a very high affinity to ferric ion Stemphyloxins share some properties with siderophores, namely, regulation by iron and preferred binding of ferric ion However, they differ from siderophore compounds in their dependence on low iron concentration for biosynthesis and a distinctive lower affinity for ferric ion ($K_2 = 10^{24}$ as compared to $K_2 = 10^{30}$–10^{34} of hydroxamate siderophores) Therefore it can be postulated that stemphyloxins may act as harvesting ligands of Fe^{3+} in the presence of low iron concentration as opposed to hydroxamate siderophores, whose function is aimed at extreme conditions of iron deficiency The possibility that stemphyloxins might be involved in membrane mediated transport of ferric ion as demonstrated for some hydroxamate siderophores [10] remains to be investigated

The ability of microbial pathogens to remove iron from

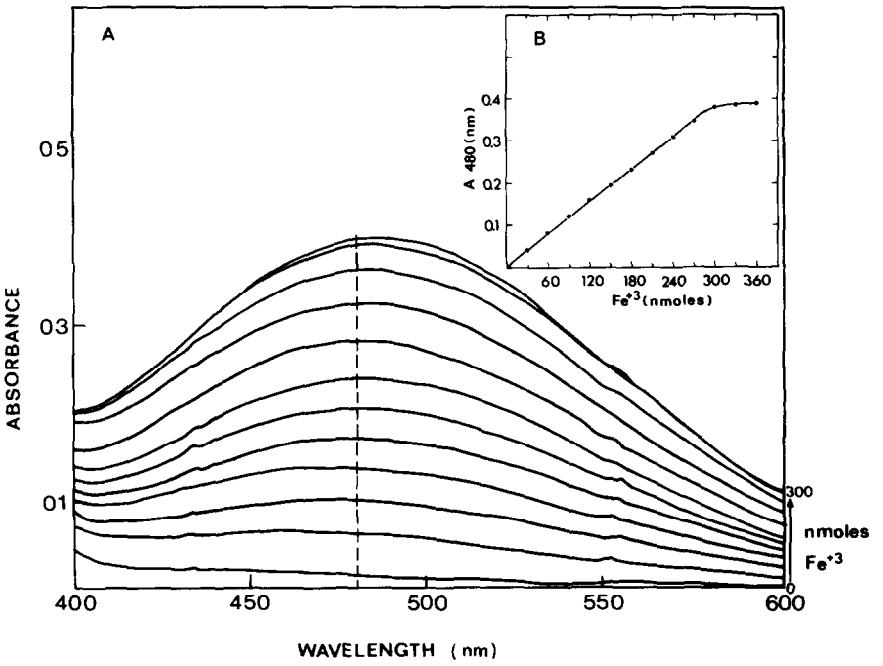

Fig 4 Determination of the stoichiometry of the Fe(III)–stemphyloxin I complex (A) Changes in the absorption spectrum of stemphyloxin I as a function of the amount of ferric ion added A cuvette was supplied with 1 5 ml of an aqueous solution (890 nmoles) of the iron-free stemphyloxin I Successive additions (5 μl) of a freshly prepared aqueous solution (6 μM) of $FeCl_3$ were then made and the absorption spectrum was measured after each addition (B) Increase in absorption at 480 nm (derived from Fig 4A) as a function of added Fe^{+3}

living cells may be a virulence factor during infection of animals [12] or plants [13] For example, addition of iron-chelating agents to inoculum of *Glomerella cingulata* stimulated the rate of progressive lesion development on *Capsicum frutescens* fruit [14] Consequently, it is attractive to postulate that the phytotoxic activity of stemphyloxins may be related to their ability to chelate iron Results shown in Table 3 indicate that the phytotoxicity of stemphyloxin I is higher than stemphyloxin II by at least 100-fold in spite of the very close similarity in their iron-binding constants It is possible that the variation in toxicity between stemphyloxins I and II results from differential transport rate into plant cells It is, however, quite likely that the reduced toxicity of stemphyloxin II may be accounted for by the structural change of the β-ketoaldehyde group which has been described earlier The latter group has already been implicated in the biological activity of stemphyloxin I [1]

EXPERIMENTAL

Culturing The culture of *S botryosum* f sp *lycopersici* used in this study has been previously described [1] The fungus was grown in 1-l Roux bottles containing 100 ml of a defined sucrose–glutamate medium composed of the following compounds (gl^{-1} of distilled water), monosodium glutamate, 9, sucrose, 20; $MgSO_4$ $7H_2O$, 0 5, KCl, 0 5, K_2HPO_4, 0 3, $FeSO_4$, 0 01, thiamine HCl, 0 005, pyridoxine, 0 005 The liquid cultures were incubated for 16 days at 25° under continuous illumination of 4 W M^{-2}

Bioassay Phytotoxicity of stemphyloxins I and II was determined by the following procedures (a) symptoms appearance after toxins injection into tomato leaves [1], (b) inhibition of protein synthesis as measured by incorporation of radioactive amino acids into protein of exponentially growing tomato cells [1], (c) growth inhibition of the duckweed *Spirodella oligorrhiza* Two-week-old fronds were transferred aseptically into 100 ml flasks containing 25 ml of sterilized Hoagland's medium enriched with 0 5% sucrose at pH 5 8 Fifteen fronds were placed in each flask Stemphyloxins were dissolved in EtOH and added to the medium to give a final volume of 0 1% EtOH The flasks were incubated under illumination of fluorescent and incandescent light (36 W M^{-2}) Growth was measured by the percentage increase in frond number after 4 days, (d) inhibition of rootlet elongation Germinated tomato seeds with 1–2 mm rootlets were selected and transferred into petri dishes (5 cm diam) containing 2 ml of a stemphyloxin solution The minimal toxic dose required for inhibition of rootlet elongation was determined by a dilution end point Fifty seeds were used for each treatment

Isolation and purification of stemphyloxins I and II The procedure for isolation and purification of the stemphyloxins was essentially as previously described [1] The culture filtrate (1500 ml) was separated from the mycelial mats by straining through Whatman No 1 filter paper The filtrate was adjusted to pH 2 5 with HCl and extracted with $CHCl_3$ ($\times 3$) The $CHCl_3$ extracts were taken to dryness and applied to silica gel H (type 60) column as described [1] The fractions containing stemphyloxin I or stemphyloxin II were combined and dried *in vacuo* below 40° The residue of each compound was dissolved in 1 ml MeOH–CH_2Cl_2 (1 1) and applied to a Sephadex LH-20 (Pharmacia) column (2 5 × 25 cm) and eluted with the same solvent The fractions containing the toxin were further purified by preparative TLC, using EtOAc–EtOH (97 3) The stemphyloxins were detected on the chromatograms by spraying with either ethanolic solution of 5% $FeCl_3$ or vanillin–H_2SO_4 [1] The R_f values for stemphyloxin I and II were 0 48 and 0 30 respectively Purity of the compounds was established as previously described [1]

Quantitative determination of stemphyloxins Fifty to 250 μg of pure stemphyloxin I or II were dissolved in 50 μl EtOH After addition of 50 μl of 5% ethanolic $FeCl_3$, the volume was adjusted to 1 ml with EtOH and the colour was read at 480 nm for stemphyloxin I and at 530 nm for stemphyloxin II Linearity was obtained between 50–500 μg for each of the two compounds Results presented were an average of at least three different experiments

Spectral data Mps are uncorrected ^{1}H NMR spectra were recorded on Bruker WH-270 and Bruker WH-90 spectrometers, and ^{13}C NMR spectra were recorded on a Bruker WH-90 (22 63 MHz) and on a 300 MHz NMR spectrometers, all chemical shifts are reported with respect to TMS (δ 0)

Stemphyloxin II, IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3400, 2960, 2930, 2850, 1665, 1595, 1380, 1320, 1215, 1200, 1070, 1045, 1025, MS *m/z* (rel int) 364 $[M-H_2O]^+$ (0 1), 236 (18), 203 (17), 192 (24), 175 (15), 154 (45) and 136 (100), λ_{max}^{EtOH} nm (log ε) 276 (3 66), ^{1}H NMR (300 MHz, $CDCl_3$) δ 0 89*t* (3H), 0 97*d* (3H), 1 25*s* (3H), 1 16*s* (3H), 1 17*s*(3H), 3 80 AB quart (2H) and 7 16*s* (1H), ^{13}C NMR

Table 3 Potency and differential toxicity of stemphyloxins I and II as measured by various bioassay procedures

	Minimal effective concentration (μM)	
Bioassay procedure*	Stemphyloxin I	Stemphyloxin II
Symptom development on tomato leaves	2 6	500
Inhibition of rootlet elongation	6 5	500
Inhibition of protein synthesis	0 05	13
Growth inhibition of *Spirodella oligorrhiza*	2 6	275

*Bioassay procedures as described in the Experimental

data is given in Table 2 and methyls appeared at δ14 02, 16 54, 21 09, 22 47 and 31 45

REFERENCES

1 Barash, I, Pupkin, G, Netzer, D and Kashman, Y (1982) *Plant Physiol* **69**, 23
2 Barash, I, Manulis, S, Kashman, Y, Springer, J P, Chen, M H M, Clardy, J and Strobel, G A (1983) *Science* **220**, 1065
3 Marinette, G Z and Rouser, G (1955) *J Am Chem Soc* **77**, 3545
4 Ichihara, A, Oikawa, H, Hayashi, K and Sakamura, S (1983) *J Am. Chem Soc* **105**, 2907
5 Barash, I, Mor, H, Netzer, D and Kashman, Y (1981) *Physiol Plant Pathol* **19**, 7
6 Onesirosan, P, Mabuni, C T, Durbin, R D, Morin, R B, Rich, D H and Army, D C (1975) *Physiol Plant Pathol* **5**, 289
7 Turner, W B (1971) *Fungal Metabolites* Academic Press, London
8 Rosotti, J C and Rosotti, H (1961) *The Determination of Stability Constants* McGraw-Hill, New York
9 Meyer, J M and Abdallah, M A (1978) *J Gen Microbiol* **107**, 329
10 Neilands, J B (1981) *Ann Rev Nutr* **1**, 27
11 Neilands, J B (1981) *Ann Rev Biochem* **50**, 715
12 Weinberg, E D (1978) *Microbial Rev* **42**, 45
13 Ong, S A, Peterson, T and Neilands, J B (1979) *J Biol Chem* **254**, 1860
14 Adikaram, N K B, Brown, A E and Swinburne, T R (1982) *Physiol Plant Pathol* **21**, 171