Phytochemistry, Vol. 23, No. 12, pp. 2781–2784, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

STRUCTURE–TOXICITY RELATIONSHIPS OF THE EREMOPHILANE PHOMENONE AND PR-TOXIN

RENATO CAPASSO, NICOLA S. IACOBELLIS,* ANTONIO BOTTALICO* and GIACOMINO RANDAZZO

Dipartimento di Chimica Organica e Biologica dell'Università, Napoli, Italy; *Istituto Tossine e Micotossine da Parassiti Vegetali del Consiglio Nazionale delle Ricerche, Bari, Italy

(*Revised received* 15 *May* 1984)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; phomenone; phomenone derivatives; PR-toxin; eremophilane sesquiterpenes; phytotoxins; mycotoxins.

Abstract—The structure–toxicity relationships of phomenone were studied on cuttings and seedlings of tomato, and on larvae of brine shrimp. Four derivatives prepared by chemical modification of phomenone were tested in comparison with PR-toxin. The toxicity to tomato cuttings (wilting and necroses of leaflets) appeared to be dependent on the integrity of the phomenone molecule, as any structural modification markedly reduced or completely abolished its phytotoxicity. By contrast, the toxicity to tomato seedlings (growth inhibition of shoots and rootlets) and to brine shrimp (larvae mortality) suggested a role for the epoxy rings in eremophilane molecules, which was enhanced by acetylation, as demonstrated by the progressive loss of activity for the sequence PR-toxin, phomenone and 9-methoxy-6,7-de-epoxyphomenone.

INTRODUCTION

Investigations on a wilt disease of tomato (*Lycopersicon esculentum* Mill.) caused by *Phoma destructiva* Plowr. led to the identification of phomenone (**1**) [1], a known phytotoxic metabolite produced by certain phytopathogenic *Phoma* species [2–4], whose role as a phytotoxin in wilted leaves of infected tomato plants has recently been demonstrated [5, 6]. Phomenone is a sesquiterpene [7] based on the same eremophilane ring system as PR-toxin (**6**) [8, 9], a mycotoxin produced by *Penicillium roqueforti* Thom [10, 11] which inhibits DNA, RNA and protein synthesis [10, 12–14] and is strongly toxic to mice and rats [10, 15].

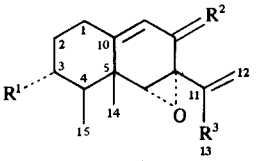
The biological activity of PR-toxin was attributed to the presence of the aldehyde group at C-12 [16, 17], but this was critically reviewed by Chu [18]. To obtain a better biological characterization of phomenone, and to obtain further data on the structure–toxicity relationships of the eremophilanes, four derivatives of phomenone, namely 13-aldophomenone (**2**), 8-β-hydroxyphomenone (**3**), acetylphomenone (**4**) and 9-methoxy-6,7-de-epoxyphomenone (**5**), were prepared by chemical modification of the parent compound, and their toxicities, compared to those of PR-toxin, assayed on cuttings and seedlings of tomato and on brine shrimp (*Artemia salina* L.).

RESULTS AND DISCUSSION

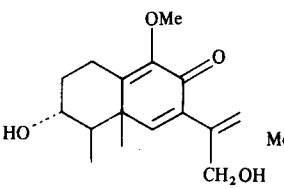
In the tomato cutting assay, besides phomenone, which was known to be toxic [1], only 13-aldophomenone was slightly toxic to leaflets. Both compounds caused the appearance of wilting and necroses: phomenone was scored as 3 and 13-aldophomenone as 0.5 [19].

Preliminary investigations on the mechanism of action of phomenone indicated that this phytotoxin induces an electrolyte loss from tomato leaf discs (Iacobellis, N. S., Bottalico, A. and Lippolis, R., unpublished results). This dysfunction of cell membrane permeability, which is characteristic of many wilt phytotoxins [20], could explain the appearance of wilting and necroses on leaves of *P. destructiva*-infected tomato plants [6], as well as on leaflets of tomato cuttings. It appears, from the above phytotoxicity data on tomato cuttings, that, for the eremophilanes tested, this biological property is limited to the integrity of the phomenone molecule, as any structural modification either markedly reduced or completely abolished its phytotoxicity.

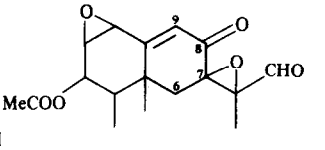
Phomenone, acetylphomenone and PR-toxin supplied to tomato seedlings at 10^{-4} M elicited the same strong activity. At this concentration, they halved the growth of shoots and completely inhibited the growth of rootlets. Among the other compounds tested, 13-aldophomenone



	R^1	R^2	R^3
1	OH	O	CH_2OH
2	OH	O	CHO
3	OH	OH, H	CH_2OH
4	OAc	O	CH_2OAc



5



6 PR-toxin

and 9-methoxy-6,7-de-epoxyphomenone were inactive, whereas only 8-β-hydroxyphomenone was slightly toxic (Table 1). Proof that the latter compound was not a mixture of stereoisomers, i.e. that its formation by the reduction of **1** had proceeded in a stereospecific manner, was provided by ^{1}H NMR data. Thus the ^{1}H NMR signals of 8-β-hydroxyphomenone were characteristic of the presence of a single isomer. In addition, the coupling constants between H-8 and H-9 (1.96 Hz) corresponded, according to a Karplus curve [21], to a dihedral angle of 60°, formed by the equatorial α-oriented H-8 with H-9, as shown in the Drieding model of **3**; therefore the hydroxyl group at C-8 is β axially oriented. Moreover, the acetyl derivative of **3** is described [7] as showing the same configuration at C-8.

In the brine shrimp assay, the most toxic eremophilanes were PR-toxin and acetylphomenone, which caused 100% larvae mortality at 10^{-4} M. The activity of phomenone was 16% at 10^{-4} M and 100% at 10^{-3} M. At 10^{-3} M, toxicity was shown also by 13-aldophomenone (100%) and 8-β-hydroxyphomenone (16%), whereas 9-methoxy-6,7-de-epoxyphomenone was inactive (Table 2).

Moulé *et al.* [17] reported that some biological properties of PR-toxin are dependent on the presence of the aldehyde function at C-12. They suggested that the presence of the epoxide group is not accompanied by significant biological activity, and that acetylation of the eremophilane ring is not connected with toxic properties. As pointed out by Chu [18], these structure–toxicity relationships based on an aldehyde function with a non-specific inhibitory effect do not account for the overall toxicity of the PR-toxin.

The data reported here on the toxic effects of eremophilanes on tomato seedlings and on brine shrimp (Tables 1 and 2) show that structural modifications of the eremophilane ring arising from oxidation or reduction reactions have variable effects on the toxicity, whereas the de-epoxidation reaction abolishes the biological activity. Moreover, acetylation gives a strongly active compound. Therefore, the above results suggest that the epoxy ring of eremophilanes could play a role in the biological activity (the preparation of either 9-methoxyphomenone or 6,7-de-epoxyphomenone should confirm this hypothesis) and that acetylation is connected with the toxic properties. Such a feature, which is characteristic of other biologically active sesquiterpenes like the trichothecenes [18, 22], led us to allot more specific toxic properties to eremophilanes, and increase their significance in nature.

Table 1. Effect of phomenones and PR-toxin on the growth of tomato seedlings*

Phomenone and derivatives	Average length (mm)†					
	Shoot			Main rootlet		
Control (0.1% methanol)	48.5	a	A	47.4	a	A
Phomenone (**1**)	22.6	c	B	6.6	c	C
13-Aldophomenone (**2**)	45.3	ab	A	49.3	a	A
8-β-Hydroxyphomenone (**3**)	40.9	b	A	35.6	b	B
Acetylphomenone (**4**)	27.1	c	B	7.0	c	C
9-Methoxy-6,7-de-epoxyphomenone (**5**)	46.9	a	A	45.8	a	A
PR-toxin (**6**)	25.1	c	B	5.9	c	C

*Dose of 2 ml 10^{-4} M toxin in 0.1% methanol/10 pre-germinated tomato seedlings in 5 cm diameter Petri dish.

†Values scored by the same letters are not statistically different at $P = 0.05$ (lower case letters) or at $P = 0.01$ (capital letters).

Table 2. Toxicity of phomenones and PR-toxin on larvae of brine shrimp(*A. salina*)*

Phomenone and derivatives	Larvae mortality (%)†		
	10^{-5} M	10^{-4} M	10^{-3} M
Control‡	0	0	0
Phomenone (**1**)	0	16	100
13-Aldophomenone (**2**)	0	0	100
8-β-Hydroxyphomenone (**3**)	0	0	16
Acetylphomenone (**4**)	0	100	100
9-Methoxy-6,7-de-epoxyphomenone (**5**)	0	0	0
PR-toxin (**6**)	0	100	100

*In 0.5 ml artificial sea water solution (containing 0.1% methanol).

†At 25° for 24 hr, according to the procedure of Eppley [26].

‡Artificial sea water solution (containing 0.1% methanol).

EXPERIMENTAL

Chemical methods. Mps are uncorr.; ^{1}H NMR: 270 MHz, TMS as internal standard; MS: 70 eV; TLC spots were visualized with 10% H_2SO_4 in MeOH and 3% phosphomolybdic acid in MeOH and then heating at 105°.

Phomenone (**1**). This toxin was purified from 7-day-old shake cultures of *P. destructiva*, according to a published procedure [1].

13-*Aldophomenone* (**2**). To a soln of phomenone (60 mg) in dry $CHCl_3$ (6 ml) was added MnO_2 (270 mg) and the mixture was then stirred overnight at room temp. More reagent was then added (70 mg) and after a further 2 hr at room temp., the mixture was filtered. The filtered soln was evapd and the residue was purified by CC (silica gel). Elution with EtOAc–*n*-hexane gave a homogeneous substance, which crystallized from EtOAc–*n*-hexane as white crystals (40 mg), mp 157–159°; $[\alpha]_D^{20} + 60.5°$ ($CHCl_3$; *c* 1); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450 and 3600 (OH), 1690 (conjugated aldehyde), 1670 (conjugated ketone), 1620 (C=C); UV λ_{max}^{MeOH} nm (ε): 244 (19 000) and 226 (17 000); MS *m/z* (rel. int.): 262 $[M]^+$ (100), 245 (40); ^{1}H NMR ($CDCl_3$): δ 9.65 (1H, *s*, aldehydic proton), 6.33 and 6.55 (2H, 2*s*, 12-CH_2), 5.79 (1H, *d*, $J_{9,1a}$ = 1.72 Hz, H-9), 3.65 (1H, *m*, $J_{3,2e}$ = 4.3 Hz, $J_{3,2a}$ = 10.32 Hz and $J_{3,4}$ = 10.32 Hz, H-3), 3.27 (1H, *s*, H-6), 2.57 (1H, *m*, $J_{1,1}$ = 12.89 Hz, $J_{1a,2a}$ = 14.61 Hz, $J_{1a,2e}$ = 5 Hz and $J_{1a,9}$ = 1.72 Hz, H_a-1), 2.37 (1H, *m*, $J_{1,1}$ = 12.89 Hz, $J_{1e,2a}$ = 3.44 Hz, H_e-1), 2.16 (1H, *m*, $J_{2e,1a}$ = 5 Hz and $J_{2e,3}$ = 4.3 Hz, H_e-2), 1.81 (1H, *m*, $J_{4,3}$ = 10.32 Hz and $J_{4,Me}$ = 6.88 Hz, H-4), 1.48 (1H, *m*, $J_{2a,1a}$ = 14.61 Hz and $J_{2a,3}$ = 10.32 Hz, H_a-2), 1.29 (3H, *s*, 14-Me), 1.24 (3H, *d*, $J_{Me,4}$ = 6.88 Hz, 15-Me).

8-β-*Hydroxyphomenone* (**3**). To a soln of phomenone (20 mg) in dry MeOH (2 ml), $NaBH_4$ (50 mg) was added. The mixture was stirred for 30 min at room temp. then neutralized (0.1 M HCl), diluted with H_2O (4 ml) and, after MeOH evapn under reduced pressure, extracted with EtOAc (4 × 6 ml). The residue was purified by CC (silica gel: $CHCl_3$–MeOH, 7:3) to give an uncrystallizable colourless substance (18 mg); IR ν_{max}^{Nujol} cm^{-1}: 3500 and 3600 (OH); UV λ_{max}^{MeOH} nm: < 230; MS *m/z* (rel. int.): 248 $[M - H_2O]^+$ (33), 233 (100), 215 (50); ^{1}H NMR (CD_3OD): δ 5.23 (2H, *m*, 12-CH_2), 5.21 (1H, *t*, *J* = 1.96 Hz, H-8), 4.56 (1H, *q*, *J* = 1.96 Hz and *J* = 3.52 Hz, H-9), 4.28 and 4.15 (2H, 2*d*, J_{AB} = 13.75 Hz, H_A-13, H_B-13), 3.47 (1H, *m*, H-3), 3.37 (1H, *s*, H-6), 2.3 (1H, *m*, H_a-1), 2.15 (1H, *m*, H_e-1), 2.0 (1H, *m*, H_e-2), 1.6 (1H, *m*, H-4), 1.28 (1H, *m*, H_e-2), 1.28 (3H, *s*, 14-Me), 1.12 (3H, *d*, $J_{Me,4}$ = 8 Hz, 15-Me).

Acetylphomenone (**4**). Phomenone (15 mg) was acetylated with a mixture of C_5H_5N (300 μl) and Ac_2O (300 μl) to give a compound (12 mg) with spectroscopic and physical properties identical to those reported in the literature [7].

9-*Methoxy*-6,7-*de-epoxyphomenone* (**5**). Phomenone (40 mg) was treated with NaOH in MeOH (10 ml) for 20 hr at room temp. to give an uncrystallizable colourless residue (20 mg) with spectroscopic and physical properties identical to those reported in the literature [23].

Biological methods. Each compound was dissolved in a minute amount of MeOH and brought up to the required concn with H_2O, or with artificial sea water soln (brine shrimp assay).

Tomato cutting assay: The usual technique [24, 25] was followed with some modifications. Cv Supermarmande test plants were grown in a room at 27 ± 1.5° and 60 ± 5% relative humidity with a 12 hr photoperiod (14 W/m^2, 2100 lx by Sylvania Grolux F40T12/GRO fluorescent lamps). Tests were carried out on four-leaved cuttings from 25-day-old plants, in a room maintained at 22 ± 1° and 50 ± 3% relative humidity, under continuous illumination (4000 lx, by Philips TLF 40W/33 white fluorescent lamps). The toxicity was estimated on a 0–4 assessment scale according to Graniti [19]. Each compound was assayed at a dose level of 0.25 ml 10^{-3} M in 1% MeOH g fr. wt of tomato cuttings.

Tomato seedling assay: Seeds of cv Supermarmande were allowed to germinate on filter paper wetted with sterile H_2O for 3 days at 27°, and then transferred to filter paper wetted with 2 ml 10^{-4} M toxin in 0.1% MeOH, in plastic Petri dishes (5 cm diameter). Ten pre-germinated seedlings were used for each dish. After 4 days at 27°, the shoot and the main rootlet length of each seedling were recorded.

Brine shrimp assay: Brine shrimp eggs were hatched in artificial sea water for 24 hr at 25° (eggs and sea salt from Euraquarium S.p.A., Bologna, Italy). Aliquots of 30–50 shrimp larvae were transferred to 0.5 ml 10^{-4} M toxin and put in the wells of a sterile culture plate (Costar 3524, cluster 24 wells of 16 mm diameter, from Costar, Cambridge, U.S.A.). After 24 hr at 25°, the larvae mortality was recorded according to the Eppley procedure [26].

Acknowledgements—We wish to thank Prof. R. D. Wei, Department of Biochemistry of National Yang-Ming Medical College, Taipei, Taiwan, Republic of China, and Prof. Y. Moulé, CNRS 'Institut de Recherches Scientifiques sur le Cancer', Villejuif, France, for standards of PR-toxin; Euraquarium S.p.A., Bologna, for brine shrimp eggs and sea salt; and Mr. V. Ricci and Ms. N. Giannino for valuable technical assistance. This work was supported by the Italian Research Council, Special ad hoc Programme, Chimica Fine e Secondaria Subproject Ca.

REFERENCES

1. Bottalico, A., Frisullo, S., Lerario, P., Randazzo, G. and Capasso, R. (1982) *Phytopathol. Medit.* **21**, 39.
2. Bousquet, J. F. and Barbier, M. (1972) *Phytopathol. Z.* **75**, 365.
3. Bousquet, J. F. (1973) *Ann. Phytopathol.* **5**, 289.
4. Bottalico, A., Frisullo, S., Iacobellis, N. S., Capasso, R., Evidente, A., Iasiello, I. and Randazzo, G. (1983) *Phytopathol. Medit.* **22**, 120.
5. Capasso, R., Evidente, A., Iasiello, I., Randazzo, G., Bottalico, A. and Iacobellis, N. S. (1983) *Phytopathol. Medit.* **22**, 114.
6. Bottalico, A., Frisullo, S., Iacobellis, N. S., Capasso, R., Corrado, E. and Randazzo, G. (1983) *Phytopathol. Medit.* **22**, 116.
7. Riche, C., Pascard-Billy, C., Devys, M., Gaudemer, A., Barbier, M. and Bousquet, J. F. (1974) *Tetrahedron Letters* 2765.
8. Wei, R. D., Schnoes, H. K., Hart, P. A. and Strong, F. M. (1875) *Tetrahedron* **31**, 109.
9. Baert, F., Foulon, M., Odou, G. and Moreau, S. (1980) *Acta Crystallogr. Sect. B* **36**, 402.
10. Wei, R. D., Still, P. E., Smalley, E. B., Schnoes, H. K. and Strong, F. M. (1973) *Appl. Environ. Microbiol.* **25**, 111.
11. Polonelli, L., Morace, G., Delle Monache, F. and Samson, R. A. (1978) *Mycopathologia* **66**, 99.
12. Moulé, Y., Jemmali, M. and Rousseau, N. (1976) *Chem.-Biol. Interact.* **14**, 207.
13. Moulé, Y., Jemmali, M. and Darracq, N. (1978) *FEBS Letters* **88**, 341.
14. Tashiro, F., Hirai, K. and Ueno, Y. (1979) *Appl. Environ. Microbiol.* **38**, 191.
15. Chen, F. C., Chen, C. F. and Wei, R. D. (1982) *Toxicon* **20**, 433.
16. Moreau, S., Moulé, Y. and Bousquet, J. F. (1977) *Ann. Nutr. Alim.* **31**, 881.
17. Moulé, Y., Moreau, S. and Bousquet, J. F. (1977) *Chem.-Biol. Interact.* **17**, 185.
18. Chu, F. S. (1977) *Adv. Appl. Microbiol.* **22**, 83.
19. Graniti, A. (1964) *Phytopathol. Medit.* **3**, 125.

20. Daly, J. M. (1981) in *Toxins in Plant Disease* (Durbin, R. D., ed.), p. 359. Academic Press, London.
21. Karplus, M. (1959) *J. Chem. Phys.* **30**, 11.
22. Ballio, A. (1981) in *Toxins in Plant Disease* (Durbin, R. D., ed.), p. 395. Academic Press, London.
23. Devys, M., Ferezan, J. P., Gaudemer, A., Bousquet, J. F. and Barbier, M. (1976) *C. R. Acad. Sci., Ser. C* **283**, 45.
24. Gäumann, E., Naef-Roth, S. and Miescher, G. (1950) *Phytopathol. Z.* **16**, 257.
25. Gäumann, E., Naef-Roth, S., Reusser, P. and Amman, A. (1952) *Phytopathol. Z.* **19**, 160.
26. Eppley, R. M. (1974) *J. AOAC* **57**, 618.