Pergamon

0031-9422(95)00128-X

Phytochemistry, Vol. 39, No. 5, pp. 1033–1037, 1995

0031-9422/95 $9.50 + 0.00

DIBENZOFURAN PHYTOALEXINS FROM THE SAPWOOD TISSUE OF *PHOTINIA*, *PYRACANTHA* AND *CRATAEGUS* SPECIES

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(*Received in revised form* 26 *January* 1995)

Key Word Index—*Photinia davidiana*; *Pyracantha coccinea*; *Crataegus pontica*; *Crataegus monogyna*; Rosaceae; sapwood; phytoalexins; dibenzofurans; eriobofurans; fungistatic activities.

Abstract—Fungus-challenged sapwood of *Photinia davidiana* and *Pyracantha coccinea* produced two new dibenzofuran phytoalexins, 7-methoxyeriobofuran and 9-hydroxyeriobofuran, respectively. The known eriobofuran was also confirmed in both species. On the other hand two *Crataegus* species produced antifungal dibenzofurans constitutively in the bark; the major constituent is now identified as 2,8-dihydroxy-3,4,7-trimethoxydibenzofuran. The antifungal activities (ED_{50}) of the newly reported dibenzofurans against three plant pathogens were in the range of 55 to 328 $\mu g\,ml^{-1}$, comparable with those of the cotonefurans. The taxonomic aspects are discussed briefly.

INTRODUCTION

Our previous studies of phytoalexin synthesis in the Rosaceae have revealed the production of the biphenyl aucuparin in leaves of *Sorbus aucuparia* [1], of 2′,6′-dihydroxy-4′-methoxyacetophenone in roots of *Sanguisorba minor* [2] and of several novel benzofurans, α- to ε-cotonefurans, in the genus *Cotoneaster* [3]. Our continuing study of antifungal defence mechanisms in this family has now revealed three new dibenzofurans from several closely related plant species of the subfamily Maloideae. Their antifungal properties and identification are the subject of this paper.

RESULTS

Three phytoalexins, with the UV spectra and colour properties typical of dibenzofurans [3], were detected in *Photinia davidiana*, when challenged by *Nectria cinnabarina*. Two of them were unambiguously identified as eriobofuran (**1**) and its 7-methoxy derivative (**2**) (see below). The third compound is most probably 7-hydroxyeriobofuran, on the basis of the similarity in UV spectra with 7-methoxyeriobofuran and the measured molecular mass, 260. However, no further structural proof could be obtained due to the small amount of compound isolated.

A constitutive antifungal compound acetovanillone (4-hydroxy-3-methoxyacetophenone) was also identified in the sapwood of this plant. It probably occurs in conjugated form as the glucoside. The aglycone was detected in traces in healthy tissue, but a large amount was released when the healthy wood tissue was treated with hot acid (2 M HCl, 100°) for 30 minutes. Although precise quantification was not carried out, the dibenzofuran-type phytoalexins **1** and **2** seem to be more abundant than this simple phenolic compound.

Widyastuti *et al.* [4] isolated two aucuparin-related biphenyls from the leaves of *Photinia glabra*, infected with *Entomosporium mespili* or treated with $HgCl_2$. In regard to this, the leaves of *P. davidiana* were subjected to re-examination in the same procedure employed by these researchers, with 0.0015, 0.015 or 0.15% aqueous $HgCl_2$ solution; however, no trace of these compounds was detected even after the bands corresponding to aucuparins on the TLC were concentrated and analysed (data not shown). It seems that the ability to produce phytoalexins is virtually confined to the wood tissue of this plant.

Pyracantha coccinea, a plant of a genus closely related to *Photinia*, gave two phytoalexins. Eriobofuran was confirmed by direct comparison with an authentic sample obtained from *Photinia davidiana* and from the leaves of *Eriobotrya japonica*, infected naturally with an unidentified fungus, by the method described by Miyakado *et al.* [5]. The second phytoalexin (M_r 260) from this plant was thought to be a hydroxy derivative of eriobofuran. The hydroxy group could be located at the *m*-position to the ethereal oxygen in the central furan ring, i.e., at the 7- or 9-position, since it has previously been observed that in the presence of alkali the absorption peak around 270 nm is diminished when the free hydroxy is confined to either the 3- or 7-positions of dibenzofurans, as in eriobofuran and its derivatives above, β-cotonefuran and β-pyrufuran (Kokubun, T.,

unpublished result). Since this alkaline absorption band in **3** was not greatly diminished compared with the neutral spectrum, it suggests that the extra hydroxyl is located at position 9. The 9-substitution in **3** was confirmed by the NMR experiments described below.

The same compounds, **1** and **3**, were also confirmed in the naturally diseased wood tissue of different specimens of *Photinia davidiana* and of *Pyracantha coccinea*. In all cases, these antifungal dibenzofurans were not found in the extracts of healthy tissue. On the other hand, as was found previously in the case of *Cotoneaster* spp., and a few related plants [3], these dibenzofuran-producing plants failed to yield any biphenyl compounds, such as aucuparin, even in trace amounts.

By contrast, a constitutive antifungal dibenzofuran was isolated from the wood tissue of *Crataegus pontica*, and its structure shown to be 2,8-hydroxy-3,4,7-trimethoxydibenzofuran (**4**). This compound was mainly located in the bark, but an identical compound was also present in the sapwood. Constitutive production of dibenzofurans in this plant was, however, expected because it was previously found that *Crataegus monogyna* produced α- and γ-cotonefurans in the wood tissue, constitutively [3]. An examination of several different cultivars of the latter species revealed that these cotonefurans were most often, but not always, encountered in the bark. There seems little qualitative difference between sapwood and bark of a single plant and, occasionally, a few additional unknown, but presumably dibenzofuran (UV absorption), compounds were found.

Ultraviolet, mass spectral and chromatographic data for the dibenzofurans mentioned above are collected in Table 1. Identification of the dibenzofurans **1**–**4** was either confirmed or established by means of high-field NMR studies. Eriobofuran (**1**) was analysed by ^1H and ^{13}C NMR and resonances unambiguously assigned (Table 2) by reference to ^1H–^1H COSY, ^1H–^1H NOESY, ^1H–^{13}C ^1J coupling (HC-COBIdec) and ^1H–^{13}C $^{2/3}J$ coupling (HMBC) experiments [6]. For 7-methoxyeriobofuran (**2**) critical features were the ABD coupling pattern for H-6, H-8 and H-9; the highly shielded resonance for C-6, indicative of both *ortho*-positions being oxygenated; the occurrence of two methoxyl resonances at δ56–57, necessitating at least one unsubstituted *ortho*-position for each [7]; and the non-equivalence of both ^1H and ^{13}C spectra with those of the isomeric dibenzofuran δ-cotonefuran (**5**) [3]. The second novel dibenzofuran from *Pyracantha coccinea* exhibited resonances for only two methoxyl substituents and three protons with an ABC spin–spin coupling pattern for one of the aromatic rings, so requiring a single substituent. The placement of that substituent at C-9 rather than C-6 followed from the ^{13}C NMR spectrum where highly deshielded resonances at δ153.9 and 158.7 precluded *ortho*-oxygenation at C-5a and C-6. As the methoxyl substituents could be attributed to C-2 and C-4 through NOESY and HMBC experiments, this isolate could be assigned as 9-hydroxyeriobofuran (**3**).

The constitutive dibenzofuran from *Crataegus pontica* was available for NMR analysis only in trace amounts

Table 1. Ultraviolet, mass spectral and chromatographic data for dibenzofurans **1**–**4**

Compound	λ_{max}^{MeOH} (nm)	log ε	$\lambda_{max}^{MeOH+NaOH}$ (nm)	EIMS	R_f (×100)* CA5	HEM	R_t† (min)
Eriobofuran (**1**)	260 294 302 317 sh	4.09 3.96 4.12 3.94	333	244 (100) 230 (42) 229 (52)	66	72	8.49
7-Methoxyeriobofuran (**2**)	229 262 sh 307 316	4.28 4.18 4.23 4.22	260 277 sh 331	274 (100) 259 (47)	57	64	8.92
9-Hydroxyeriobofuran (**3**)	238 268 286 306 318	4.19 4.09 4.06 3.93 4.01	280 324 335	260 (100) 245 (55)	46	42	6.15
2,8-Dihydroxy-3,4,7-trimethoxy-dibenzofuran (**4**)	225 sh 260 309		303 sh 326	290 (91) 275 (100)	28	23	5.93

*On silica gel in CA5 = $CHCl_3$–Me_2CO (19:1) and HEM = hexane–EtOAc–MeOH (60:40:1).
†HPLC on a phenyl column (4 mm × 250 mm) isocratic with MeOH–HOAc–H_2O (27:2:20) at 1 ml min^{-1}.

Table 2. NMR spectral data dibenzofurans **1–5**

	Proton shifts					Carbon-13 shifts			
C/H	**1**	**2**	**5**	**3**	**4**	**1**	**2**	**5**	**3**
1	7.39	7.30	7.13	7.39	7.08	98.4	98.0	100.2	100.4
2						147.0	146.9	148.4	146.6
3						140.5	139.4	139.7	139.6
4						134.2	134.2	139.5	134.0
4a						144.5	144.4	142.0	143.6
5a						157.0	158.3	158.8	153.9
6	7.61	7.16	7.17	7.05	7.22	112.2	97.4	97.3	103.7
7	7.38			7.18		126.5	160.1	169.8	121.7
8	7.30	6.92	6.93	6.83		123.6	111.7	111.9	109.4
9	7.95	7.79	7.82		7.33	120.8	121.0	118.4	158.7
9a						126.0	119.0	121.6	113.0
9b						116.2	116.5	121.7	115.9
2-OMe	3.95	3.93		3.94		57.2	57.2		57.2
3-OMe			3.90		3.89			61.8	
4-OMe	4.14	4.10	4.18	4.10	4.16	61.1	61.1	61.2	61.0
7-OMe		3.89	3.90		3.96		56.2	56.2	

and in an impure form. The ^{1}H NMR spectrum (Table 2) revealed the presence of three methoxyl groups and three aromatic protons, each of which occurred as a singlet. The methoxyl resonance at δ3.96 showed an NOE interaction with the aromatic proton at δ7.22 but neither of the other methoxyls showed any NOE. This suggested that they should be placed at C-3 and C-4 of a 2,3,4,7,8-pentahydroxylated dibenzofuran, a hypothesis supported by the chemical shift values for these methoxyls which were nearly identical to those reported for **5** [3]. While a satisfactory ^{13}C NMR spectrum could not be obtained, the resonances of certain carbons could be inferred from the H-domain detected HMBC experiment. This confirmed ^3J couplings between the methoxyl protons at δ3.89 and 4.16 and carbons at *ca* 140 ppm (comparable to **5** in Table 2). The remaining methoxyl showed ^3J coupling to a carbon at *ca* 149 ppm, which is more deshielded than would be expected for C-2 (cf. **1–3** in Table 2) but would be acceptable for C-7 or C-8. On the spectral data available, an unambiguous assignment of the remaining methoxyl to C-7 or C-8 could not be made. However, the more shielded of the two aromatic protons (δ7.22) would seem likely to be placed at C-6, between two oxygen functions and, as that is the proton that shows the NOE, then it follows that the methoxyl should be placed at C-7. On this basis the dibenzofuran can be tentatively identified as 2,8-dihydroxy-3,4,7-trimethoxydibenzofuran (**4**).

Three of the four dibenzofurans isolated during the present study were tested for their antifungal activity against three pathogenic plant fungi, *Alternaria alternata*, *Botrytis cinerea* and *Fusarium culmorum* (Table 3). The ED_{50} values ranged from 55 to 328 $\mu g\,ml^{-1}$ and were similar to those obtained earlier for the cotonefurans from *Cotoneaster* [3] and for isoflavonoid phytoalexins from the Leguminosae [8]. Results for two other Rosaceae phytoalexins, aucuparin and α-cotonofuran, are included for comparison. There are no significant differences in antifungal activity between biphenyls and dibenzofurans or between the differently substituted dibenzofurans that have been tested so far. As noted earlier [3], *Fusarium culmorum* is more sensitive to these phytoalexins than the other two fungi tested. As with the *Cotoneaster* phytoalexins, the mode of action of the dibenzofurans **1–3** appears to be fungistatic rather than fungitoxic, in that inhibition was not accompanied by any morphological abnormalities in hyphal growth.

DISCUSSION

The subfamily Maloideae are taxonomically difficult and are subject to continuous revision from both chemical and morphological points of view [9, 10]. The boundaries of genera are often obscure because of the continuity of species in the subfamily. Thus, although *Photinia*, *Stranvaesia* and *Crataegus* are closely related (e.g. sometimes treated as the '*Photinia* complex' [11]), *Crataegus* is usually regarded as a member of the tribe Crataegeae, while the former two genera are included in the tribe Maleae [12].

It was previously shown that *Photinia glabra* produces biphenyls as its phytoalexins, but we show here that *Photinia davidiana* gives dibenzofurans. This is particularly interesting because the genus *Stranvaesia* is often included in *Photinia* in modern taxonomy [13]; *Photinia davidiana* (Decne.) Cordot., employed in the current study, was in fact traditionally treated as *Stranvaesia davidiana* Decne., while *P. glabra* has always been a member of *Photinia*. The different phytoalexin patterns revealed here may raise the question whether *Photinia* and *Stranvaesia* should occupy the same genus or not, although the organs examined are different. No record is

Table 3. Inhibition of spore germination in three fungi by dibenzofurans **1**–**3**

		Germination rate at ($\mu g\,ml^{-1}$) in per cent				ED_{50}	
	Compound*	10	20	50	100	$\mu g\,ml^{-1}$	μM
Alternaria alternata	Aucuparin	98	—	86	75	>100	>430
	α-Cotonefuran	—	61	57	0	27	93
	Eriobofuran **1**	—	85	60	37	80	328
	7-Methoxyeriobofuran, **2**	85	68	66	7	63	230
	9-Hydroxyeriobofuran **3**	100	87	40	19	44	269
Botrytis cinerea	Aucuparin	100	100	—	63	>100	>430
	α-Cotonefuran	—	97	14	27	35	121
	Eriobofuran **1**	76	56	41	—	32	131
	7-Methoxyeriobofuran, **2**	58	34	10	0	15	55
	9-Hydroxyeriobofuran **3**	100	86	32	—	39	150
Fusarium culmorum	Aucuparin	98	68	59	46	84	360
	α-Cotonefuran	—	76	0	0	14	48
	Eriobofuran **1**	61	39	35	23	18	74
	7-Methoxyeriobofuran, **2**	70	35	4	0	16	58
	9-Hydroxyeriobofuran **3**	98	95	42	1	49	188

* Aucuparin (4-hydroxy-3,5-dimethoxybiphenyl) and α-cotonefuran (2,7-dihydroxy-3,4,6-trimethoxydibenzofuran) included for comparison.

available of the phytoalexin of the stem tissue of *P. glabra* while the leaves of *P. davidiana* did not give any phytoalexin.

The closest allies of *Pyracantha* are often thought to be *Cotoneaster* and *Crataegus*. However, the dibenzofuran pattern of *Pyracantha* is very interesting; it produces them as phytoalexins, while *Crataegus* contains them as constitutive constituents. Also, the compounds they produce possess different substitution patterns; *Pyracantha*, *Eriobotrya* and *Stranvaesia* share the common phytoalexin eriobofuran but none of the phytoalexins produced by *Cotoneaster* and *Crataegus*. Conversely, cotonefuran producers have never been observed to give eriobofurans. Hence, the affinity of *Pyracantha* seems closer to *Eriobotrya* and *Stranvaesia*, in terms of phytoalexin production, than to the other two genera.

EXPERIMENTAL

The NMR data were obtained on a Bruker AMX 400 spectrometer. Plant material examined was collected from the Harris Garden, the botanical garden of the University of Reading. The mature stem tissue was treated as described earlier [3]. The ED_{50} wad determined in the same manner described therein.

Photinia davidiana (Decne.) Cordot. (*Syn* Stranvaesia davidiana *Decne.*). A MeOH extract (*ca* 400 ml) of thinly shaven necrotic tissue just beneath the bark (45 g) was concd and the EtOAc-soluble fr. was passed through a short silica gel column (9 cm × 1.2 cm, i.d.) with hexane–$CHCl_3$–Me_2CO (6:4:1) as the preliminary purification. The eluate was sepd with prep. TLC irrigated in $CHCl_3$–Me_2CO (49:1). Two major antifungal bands A (R_f 0.74) and B (0.44) were further sepd with prep. TLC. From band A, eriobofuran (**1**) (R_f 0.61) and 7-methoxyeriobofuran (**2**) (R_f 0.51) were sepd with TLC run in hexane–EtOAc (3:2). Band B was chromatographed over TLC in toluene–HOAc (4:1) and the probable 7-hydroxyeriobofuran (R_f 0.36) obtained.

Pyracantha coccinea Roem. The EtOAc-soluble fr. of concd MeOH extract from necrotized tissue (150 g) was passed through a short silica gel column (11 cm × 2 cm, i.d.) with hexane–$CHCl_3$–Me_2CO (6:4:1). The eluate was concd and applied on to silica gel CC eluted with $CHCl_3$ and increasing amount of Me_2CO. Frs containing fungitoxins were combined and further chromatographed on prep. TLC. Two dibenzofuran phytoalexins eriobofuran (CH_2Cl_2–Me_2CO, 49.1, R_f 0.62) and 9-hydroxyeriobofuran (**3**) (CH_2Cl_2–Me_2CO, 9:1, R_f 0.72, 18 mg) were obtained.

Crataegus pontica C. Koch. Healthy wood tissue, both bark and sapwood, were shaven thinly and extracted with MeOH for 5 days. This extract was filtered, concd and the $CHCl_3$-soluble fr. was loaded on a short silica gel column (10 cm × 1.2 cm, i.d.) and eluted with hexane–$CHCl_3$ –Me_2CO (6:4:1). The eluate was concd *in vacuo* and sepd over prep. TLC, with CH_2Cl_2–Me_2CO (49:1, R_f 0.42) and hexane–EtOAc–MeOH (60:40:1, R_f 0.36) to give **4**.

Acknowledgements—The specimen of diseased *Stranvaesia davidiana* Decne. (syn. *Photinia davidiana* (Decne.) Cordot.) was supplied by Dr H.A. McAllister, the Ness Botanic Gardens, S. Wirral, England. NMR experiments were performed in the Strathclyde University NMR Laboratory.

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