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DIBENZOFURAN PHYTOALEXINS FROM THE SAPWOOD OF *COTONEASTER ACUTIFOLIUS* AND FIVE RELATED SPECIES

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Key Word Index—*Cotoneaster acutifolius*; *C. divaricatus*; *C. henryanus*; Rosaceae; sapwood; phytoalexins; dibenzofurans; cotonefurans; fungistatic activities.

Abstract—Five dibenzofuran phytoalexins were isolated and characterized by NMR techniques from the fungal-inoculated sapwood tissue of *Cotoneaster acutifolius*. One is the known cotonefuran, here renamed α-cotonefuran. The other four are new dibenzofurans: β-cotonefuran (7-hydroxy-2,3,4,6-tetramethoxy), γ-cotonefuran (2,7-dihydroxy-3,4-dimethoxy), δ-cotonefuran (2-hydroxy-3,4,7-trimethoxy) and ε-cotonefuran (2,6-dihydroxy-3,4,7-trimethoxy). One or more of these cotonefurans were detected as phytoalexins in sapwoods of four other *Cotoneaster* species and *Mespilus germanica*. Their activities were measured against pathogenic and non-pathogenic fungi, and they gave ED_{50} values in the range of 14–90 ppm. They proved to be fungistatic on spore germination rather than fungitoxic.

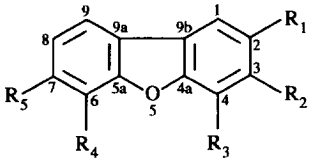
INTRODUCTION

Living wood tissues, besides other parts of the plants, are known to produce antimicrobial compounds as a part of their dynamic defence systems. A recent review has shown that phenolic and terpenoid phytoalexins are variously formed in inoculated sapwood tissues of both gymnosperms and angiosperms [1]. As part of a survey for phytoalexins in the plants of the Rosaceae, we have now found four novel dibenzofuran phytoalexins in the wood tissue of *Cotoneaster acutifolius*, together with the known cotonefuran, previously obtained from *C. lactea* by Burden *et al.* [2]. Dibenzofurans are of relatively rare occurrence as secondary metabolites. Here we report the isolation and characterization of these phytoalexins induced by the challenge inoculation of a fungus.

RESULTS

Five phytoalexins were isolated from the sapwood of *C. acutifolius* following inoculation with the fungus *Nectria cinnabarina*, the 'coral spot' pathogen of *Acer* and *Betula* spp. They were separated and purified by TLC, being monitored by bioassay (see Experimental).

The five substances **1**–**5** were readily recognized as closely related phenolics, since they gave purple or red colours with Gibbs reagent, and brown colours with diazotized *p*-nitroaniline. On UV spectral comparison with published data, it became apparent that **1** is identical to the dibenzofuran cotonefuran, previously isolated as a phytoalexin of *C. lactea* [2]. UV, MS and chromatographic measurements (Table 1) then showed that the other four phytoalexins were also dibenzofurans with



	R_1	R_2	R_3	R_4	R_5
1	OH	OMe	OMe	OMe	OH
2	OMe	OMe	OMe	OMe	OH
3	OH	OMe	OMe	H	OH
4	OH	OMe	OMe	H	OMe
5	OH	OMe	OMe	OH	OMe

similar substitution patterns to cotonefuran (2,7-dihydroxy-3,4,6-trimethoxydibenzofuran). Thus, from M_r analysis, **2** is a methyl ether of **1**, **3** a demethoxy derivative, **4** a demethoxy methyl ether and **5** an isomeric dihydroxy-trimethoxy derivative. The UV spectral maxima and alkaline shifts (Table 1) suggested that the basic hydroxylation substitution was the same (2, 3, 4, 6, 7) in all five compounds, and this was confirmed by NMR measurements. NMR data, derived from several NMR experiments, showed that **1**–**5** have the structures assigned below. Compound **1** has been renamed α-cotonefuran, to allow the use of other greek substituents for the other four compounds. Then **2** is named β-cotonefuran, with the 7-hydroxy-2,3,4,6-tetramethoxy structure. Compound **3** is γ-cotonefuran (2,7-dihydroxy-3,4-dimethoxy), **4** is δ-cotonefuran (2-hydroxy-3,4,7-trimethoxy) and **5** is ε-cotonefuran (2,6-dihydroxy-3,4,7-trimethoxybenzofuran).

Table 1. UV, MS and chromatographic data for *Cotoneaster* benzofurans

Compound	UV maxima (nm): log ε*	Alkaline maxima		MS Data [M] (*m/z*) [M − 15] (*m/z*)	TLC R_f in Solvent A†	TLC R_f in Solvent B†	HPLC (min)‡
1	221, 244sh, 264, 304, 316sh 4.57, 4.37, 4.25, 4.22, 4.05	273, 330	**1**	290 (100) 275 (94)	0.36	0.56	7.4
2	224, 245sh, 264, 304, 316sh 4.19, 4.02, 3.88, 3.86, 3.71	250sh, 268, 326	**2**	304 (100) 289 (80)	0.38	0.67	12.1
3	222, 261, 290sh, 308 4.24, 3.92, 3.76, 3.83	273, 330	**3**	260 (89) 245 (100)	0.18	0.59	6.7
4	225, 254sh, 261, 302sh, 308 4.11, 3.82, 3.87, 3.84, 3.85	266, 309, 331sh	**4**	274 (87) 259 (100)	0.57	0.67	15.3
5	220, 245sh, 268, 254, 307sh, 319 4.37, 4.20, 4.00, 3.95, 3.82, 3.88	250, 302, 330	**5**	290 (97) 275 (100)	0.36	0.44	7.2

*UV spectra were measured in methanol and in methanol containing trace amounts of 2 N NaOH.
†Solvent A: 5% acetone in $CHCl_3$, silica gel; solvent B: hexane–EtOAc–MeOH (60:40:1), silica gel.
‡Isocratic separation on a phenyl column (4 × 250 nm) with MeOH–HOAc–H_2O (63:10:127) at the rate of 1 ml min^{-1}.

The ^1H and ^{13}C NMR data for the five isolated cotonefurans, all run in acetone-d_6, are listed in Table 2. The known α-cotonefuran (**1**) revealed signals for three aromatic protons, as a singlet and an AB spin system for two ortho-coupled protons, and three methoxyls. The ^{13}C NMR spectrum of **1** showed, in addition to the three methine carbons, nine quaternary's of which seven (resonating between 133.8 and 149.5 ppm) were for oxygen-bearing carbons adjacent to either one or two other oxygenated carbons. Signals for three methoxyls were observed between 61.2 and 61.8 ppm which is indicative of the presence of steric hindrance on both *ortho* positions [3]. These requirements can be met by the structure **1**.

β-Cotonefuran (**2**) exhibited the same substitution pattern as **1**, but revealed the presence of an additional methoxyl (δ3.92, 57.0 ppm). This was assigned to C-2 by means of a NOESY experiment which revealed interaction between the methoxyl and the aromatic singlet at δ7.28.

The ^1H NMR spectrum of **3** differed in revealing an ABD spin system for aromatic methines, as well as an isolated proton, indicating a single substituent on one ring. Only two methoxyls were present, each with ^{13}C resonances that required both adjacent carbons to be substituted. These must, therefore, be placed at C-3 and C-4. Insufficient material was available to allow an exhaustive study in order to fix the position of the single substituent at C-7, but this is assumed by comparison with **4** which exhibited the same substitution pattern but with an additional methoxyl (56.2 ppm). The position of this methoxyl was identified as being on the singly substituted ring by means of a NOESY study. An investigation of direct (^1J) and long-range (^2J, ^3J) heteronuclear interactions was undertaken by means of HC-COBI [4] and HMBC [5] techniques. This permitted unambiguous assignment of all carbon resonances, except for C-3/C-4 and C-9a/C-9b.

ε-Cotonefuran (**5**) had the same substitution pattern as **1** and was obviously an isomer. However, in this case one methoxyl must be adjacent to a proton and has to be placed at either C-2 or C-7. Confirmation of the 7-methoxyl came from a NOESY spectrum while placement of the other two methoxyls at C-3 and C-4 came from the HMBC spectrum which revealed that C-6 had a hydroxyl and not a methoxyl substituent.

Relatively few dibenzofurans have been characterized as secondary metabolites [6] and as far as we can ascertain, **2**–**5** are new natural products. A survey of some related species (Table 3) showed that *C. acutifolius* is unique at present in synthesizing all five cotonefurans as phytoalexins. The five other *Cotoneaster* species that have been examined produce fewer compounds. *Mespilus germanica*, which is also in the same subfamily Maloideae, produces α-cotonefuran, but none of the other compounds. It may also be noted that during our survey of rosaceous plants, *Crataegus monogyna* proved to contain two of these cotonefurans (**1** and **3**) as constitutive agents, predominantly in bark but in sapwood as well. Although plants of the Rosaceae are also known to produce related

Table 2. ^1H and ^{13}C NMR chemical shift data for **1–5**

	^1H					^{13}C				
H/C	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
1	7.10 *s*	7.28 *s*	7.09 *s*	7.13 *s*	7.11 *s*	100.0			100.2	100.3
2						148.5			148.4	148.4
3						139.7*			139.7*	139.8*
4						139.3*			139.5*	139.7*
4a						142.5			142.0	143.0
5a						148.4			158.8	147.0
6			7.02 *d*	7.17 *d*		133.8			97.3	133.7
7						149.5			160.8	148.0
8	6.89 *d*	6.91 *d*	6.86 *dd*	6.93 *dd*	7.02 *d*	113.2			111.9	109.2
9	7.44 *d*	7.49 *d*	7.72 *d*	7.82 *d*	7.35 *d*	115.2			118.4	110.9
9a						119.4**			121.6**	120.7
9b						122.0**			121.7**	121.4
2-OMe		3.92					57.0			
3-OMe	3.90	3.84	3.90	3.90	3.90	61.8	61.6	61.8	61.8	61.8
4-OMe	4.22	4.18	4.18	4.18	4.19	61.2	61.2	61.2	61.2	61.2
6-OMe	4.13	4.14				61.2	61.4			
7-OMe				3.90	3.93				56.2	57.2

All spectra run in acetone-d_6. The same number of (*) in the same column indicates signals can be assigned to either position. All aromatic coupling constants normal.
For **2** and **3** insufficient material was available to obtain a full ^{13}C spectrum.

Table 3. Distribution of cotonefuran phytoalexins in *Cotoneaster* and *Mespilus* species

	Production of cotonefuran*				
	α	β	γ	δ	ε
Cotoneaster acutifolius Turcz.	+	+	+	+	+
C. divaricatus Rehd. & Wils.	+	+	+	—	—
C. henryanus (Schneid.) Rehd. & Wils.	+	+	+	—	—
C. horizontalis Decne.	+	+	+	—	+
C. lacteus W. W. Sm.†	+	—	—	—	—
C. splendens Flink & Hylmö	—	—	+	—	—
Mespilus germanica L.‡	+	—	—	—	—

*α- and γ-Cotonefurans were also obtained as constitutive antifungal agents from bark and sapwood of *Crataegus monogyna* Jacq.
†Data from Burden *et al.* [2].
‡Also produces other phytoalexins in addition to the α-cotonefuran.

biphenyls as phytoalexins in wood or leaf [1], we could find no evidence that biphenyls were produced with the dibenzofurans in these plants.

The five cotonefurans were tested for their antifungal activity against three representative plant fungi *Alternaria alternata*, *Botrytis cinerea* and *Fusarium culmorum* (Table 4). The ED_{50} values ranged from 14 to 90 ppm and were similar to those values obtained earlier for isoflavonoid phytoalexins from the Leguminosae; values for pisatin range from 7 to 78 ppm [7]. The results show that α- and δ-cotonefuran are more inhibitory to spore germination than the other three compounds. Also, *F. culmorum* is more sensitive to inhibition than the other two fungi tested. The mode of action of the dibenzofurans appears to be fungistatic on spore germination. This was observed with all three fungi tested (Table 4). Following the removal of phytoalexins, no morphological abnormality e.g. distorted germ tubes or high degree of ramification of hyphae could be recognized. Spores previously incubated in higher concentrations of phytoalexins exhibit shorter hyphal growth, but this is probably due to the delay in germination. In conclusion, there seems to be no direct correlation between structure and activity among the five dibenzofuran phytoalexins. This is perhaps not surprising, since they all share a high degree of lipophilicity.

EXPERIMENTAL

The NMR data were obtained on a Bruker AMX 400 spectrophotometer.

Plant material and inoculation procedures. The wood material of *C. acutifolius* was supplied from the Harris Garden, the botanical garden of the University of Reading. Mature branches of *ca* 2 cm in diameter were cut off and were further cut into *ca* 40 cm in length. After washing briefly to remove moss on the bark, several narrow slits were engraved longitudinally with a knife (*ca* 2 mm in depth, 5–7 mm in circumference). A dense spore suspension of *Nectria cinnabarina* was immediately injected into this slit and spread by means of capillary action. This inoculated wood was loosely packed in a plastic bag and partially sealed to ensure the path of air.

Table 4. Inhibition of spore germination in three fungi by α- to ε-Cotonefuran (1–5)

Concentration (ppm)	% Spore germination in presence of				
	1	2	3	4	5
Alternaria alternata					
0	100	100	100	100	100
20	61	75	88	85	83
50	15	48	51	36	57
100	0	47	12	6	33
ED_{50} (ppm)	27	48	52	42	48
Botrytis cinerea					
0	100	100	100	100	100
20	84	102	67	98	97
50	14	81	83	30	77
100	0	28	33	0	27
ED_{50} (ppm)	35	80	84	44	76
Fusarium culmorum					
0	100	100	100	100	100
20	26	39	65	46	76
50	0	5	12	0	9
100	0	0	0	0	0
ED_{50} (ppm)	14	16	29	19	32

During incubation in the dark for 2 months at 22–24°, the progress of disease was monitored every 7 days visually, and occasionally de-ionized H_2O was applied to prevent drying out.

Inoculum preparation. Inoculum spore suspension was prepd prior to harvesting the wood material. Conidial pustules that were formed on a branch of an ornamental hybrid of *Cotoneaster* sp., showing typical symptoms of coral spot disease, were mechanically removed and suspended in deionized H_2O for 1 hr. The clump of spores was then ground with a mortar and pestle, and passed through 3 layers of lens tissue to remove debris of unground fruiting body. The spore density was kept at more than 10^5 ml^{-1}.

Isolation of cotonefurans. Incubation was terminated 2 months after inoculation when the bark was completely covered with white hyphae. The hyphal mat was removed together with bark tissue that could be peeled off readily. The necrotic tissue, exhibiting a dark brown to black colour, was scraped off (70 g) and soaked in 500 ml 90% MeOH for 5 days. The extract was then filtered and concd *in vacuo* below 35°, yielding 1.04 g of syrup. The $CHCl_3$-soluble fr. of this syrup was subjected to silica gel CC (25 × 2 cm, i.d.) and eluted by step gradient *n*-hexane with increasing amounts of EtOAc. The active frs were monitored by direct bioassay on the TLC plates run in $CHCl_3$–Me_2CO (19:1) [7]. Biologically active frs were combined according to the appearance of fungitoxic spots, and further sepd over silica gel prep. TLC (1 mm). From fr. 1 of CC δ-cotonefuran (8 mg) was obtained following a 2-step sepn. in $CHCl_3$–Me_2CO (19:1), R_f 0.57, and toluene–EtOAc (2:1), R_f 0.70. The 2nd combined fr. was chromatographed in $CHCl_3$–MeOH (24:1), R_f 0.90 followed by toluene–EtOAc (2:1), R_f 0.65 to give β-cotonefuran (13.1 mg). γ-Cotonefuran (3.1 mg) was isolated and purified from the 3rd fr. chromatographed in $CHCl_3$–Me_2CO (9:1), R_f 0.70. Finally the 4th combined fr. gave the mixt. of α- and ε-cotonefurans in $CHCl_3$–MeOH (24:1) as 1 band (R_f 0.71) and they were further sepd in *n*-hexane–EtOAc–MeOH (60:40:1), yielding 69.2 mg (R_f 0.56) and 13.2 mg (R_f 0.44), respectively.

Phytoalexins were isolated and identified from other *Cotoneaster* species and from *Mespilus germanica* (Table 3) after fungal inoculation of sapwood by similar procedures as described above for *C. acutifolius*. The other phytoalexins of *M. germanica* will be described in a later publication.

Antifungal assay. The toxicity of cotonefurans was tested on spore germination in Czapek-Dox liquid medium supplemented with 2% glucose and 0.5% yeast extract, pH 5.6. The spores of test fungi (*A. alternata*, *B. cinerea* and *F. culmorum*) were obtained by flooding 5–8-day-old culture with Czapek-Dox medium. Spore density was adjusted to 5.0×10^5 ml^{-1} and 100 μl of this suspension was mixed to 100 μl of the same media containing 0, 40, 100 or 200 ppm of test compound and 3.2% EtOH. The resulting spore suspension was placed on cavity microscope slides and incubated at 22° for 6 hr, 100% relative humidity. The germ tube growth was halted with 1 drop of cotton blue–lactophenol soln (0.05%) and germination rate determined. It was defined as 'germinated' when the length of germ tube equalled or exceeded the diameter of spore. In the case of *A. alternata* and *F. culmorum*, where multiple germination occurs, each event was counted as 1 spore and 1 germination. Meanwhile, the 2nd set of similarly prepd slide glass was treated as follows to determine if the effects were fungistatic or fungicidal. After 6 hr incubation, the medium was removed with a piece of filter paper and fresh Czapek-Dox medium without phytoalexins was applied. Following incubation for 6 hr, spore behaviour was observed microscopically.

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REFERENCES

1. Gottstein, D. and Gross, D. (1992) *Trees* **6**, 55.
2. Burden, R. S., Kemp, M. S., Wiltshire, C. A. and Owen, J. D. (1984) *J. Chem. Soc. Perkin Trans. I* 1445.
3. Panichpol, K. and Waterman, P. G. (1978) *Phytochemistry* **17**, 1363.
4. Bax, A. (1983) *J. Mag. Res.* **53**, 517.
5. Bax, A. and Summers, M. F. (1986) *J. Amer. Chem. Soc.* **108**, 2093.
6. Buckingham, J. (1993) *Dictionary of Natural Products*. Chapman and Hall, London.
7. Harborne, J. B. and Ingham, J. L. (1978) in *Biochemical Aspects of Plant and Animal Coevolution*, pp. 343–405. Academic Press, London.