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ANTIFUNGAL BIPHENYL COMPOUNDS ARE THE PHYTOALEXINS OF THE SAPWOOD OF *SORBUS AUCUPARIA*

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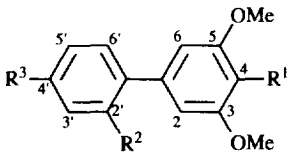
Key Word Index– *Sorbus aucuparia*; Rosaceae; sapwood; phytoalexins; aucuparins.

Abstract—An examination of the sapwood tissue of *Sorbus aucuparia* L. has revealed that aucuparin and its derivatives are essentially absent from healthy tissue, and are only produced as phytoalexins following fungal infection. Five biphenyls were identified: aucuparin, 2′-methoxyaucuparin, 4′-methoxyaucuparin, 2′-hydroxyaucuparin and isoaucuparin (2′-hydroxy-3,5-dimethoxybiphenyl). The latter is a new phytoalexin. A survey of 11 individual *Sorbus* trees showed that not all these compounds are necessarily produced in the phytoalexin response.

INTRODUCTION

The biphenyl derivatives aucuparin (**1**) and its 2′-methoxy (**2**) derivative have been known as constitutive components of the heartwood of *Sorbus aucuparia* since 1963 [1]. More recently, the same two compounds were detected in the sapwood or cortical tissue of *Eriobotrya japonica* and *Malus pumila* as their phytoalexins [2, 3]. Additionally these antifungal biphenyls can be induced when the leaves of *Photinia glabra* and *Raphiolepsis umbellata* are infected with fungi or treated with heavy metal ions [4, 5]. This is also true for the leaves of *Sorbus aucuparia* [6]. Our interest in the phytoalexins of the Rosaceae has now led us to confirm the formation of biphenyls as phytoalexins in the sapwood of *S. aucuparia*.



	R¹	R²	R³
1	OH	H	H
2	OH	OMe	H
3	OH	H	OMe
4	OH	OH	H
5	H	OH	H

RESULTS AND DISCUSSION

The sapwood of *Sorbus aucuparia* cv. 'Edulis' gave five such compounds two months after inoculation with *Nectria cinnabarina* (for methods see Experimental). Four of them were unambiguously identified as aucuparin, 2′- and 4′-methoxyaucuparin and 2′-hydroxyaucuparin (**1**–**4**), with reference to published data [1, 7]. The ^{1}H NMR of aucuparin (**1**), M_r 230, showed a simple resonance pattern indicating symmetrical substitution. The shielded, isolated singlet (H-2 and H-6) at δ6.81 indicates that the methoxyl groups should be placed at the 3- and 5-positions, rather than 2- and 6-positions. An ABCD coupling pattern in the aromatic region was observed in the second compound (**2**) whose M_r was 260. This can be explained by *o*-substitution of one ring; thus the methoxyl group must be placed at the 2′-position. The ^{1}H NMR of **3**, the isomer of 2′-methoxyaucuparin, gave a symmetrical AB resonance pattern in addition to the signal of the syringyl moiety, hence indicating 4′-substitution. 2′-Hydroxyaucuparin (**4**), which has been previously characterised from *Salix cuprea* (Salicaceae) [7] was identified by comparing its chromatographic, UV and MS spectral properties with the literature data. Methylation of **4** with dimethyl sulphate, potassium carbonate and acetone gave two intermediate monomethyl derivatives and then 3,4,5,2′-tetramethoxybiphenyl, which was compared directly with the same product produced by methylation of 2′-methoxyaucuparin (**2**). This dimethyl ether of **4** was very different in its properties from the isomeric 3,4,5,4′-tetramethoxybiphenyl, which was also prepared for comparative purposes. Although the amount of the fifth compound produced was very low, several NMR experiments revealed the structure to be 2′-hydroxy-3,5-dimethoxybiphenyl (**5**). Thus, in acetone-d_6, an ABCD pattern was observed for the aromatic protons of one ring, while three further aromatic protons, two equivalent and each of which showed *meta*-coupling, could be attributed to a 3,5-disubstituted ring.

The symmetrical nature of the latter was indicated by the occurrence of two equivalent aromatic and methoxyl proton resonances. The identity of this ring as 3,5-dimethoxyphenyl was confirmed by an NOE-difference experiment in which irradiation of the methoxyl resonance caused enhancement of H-2, H-4 and H-6. Here we propose isoaucuparin for its trivial name. All these five biphenyls were essentially absent from the healthy sapwood of the same plant (see Experimental).

A further experiment was conducted to confirm that this phytoalexin response is general in this plant species. The same procedure was applied on 11 individual plants, all raised from seeds, but not by grafting or division. Sampling was performed on trees growing in southern England. After two weeks incubation *in vitro*, the EtOAc-soluble fraction of the MeOH extract was analysed. The results obtained are presented in Table 1. It can be seen that 2′-methoxyaucuparin was found in all cases and only this compound could be detected in some individuals (plants 1, 4, 8 and 11). Isoaucuparin could not be confirmed in any plant, but this is probably due to the low concentration, and the shorter incubation period employed. From these data, at least five different phytoalexin patterns can be noted, indicating considerable genetic and/or physiological variation within the species. Variation in peroxidase isoenzymes have also been recorded in *Sorbus aucuparia* populations [8]. It may be noted that the two phytoalexins most frequently produced in *Sorbus aucuparia* trees (Table 1), namely 2′-methoxy- and 4′-methoxyaucuparin, are significantly more fungitoxic than either aucuparin or 2′-hydroxyaucuparin (Table 2).

These aucuparin derivatives were only found in fungus-challenged sapwood. They were not present in bacterium-infected twigs or in the cut stems, allowed to dry by leaving at room temperature for a few months. It seems that living tissue is essential for a genuine phytoalexin response.

Aucuparins have hitherto been isolated from the heartwood of several *Sorbus* species, belonging to the sub-genus *Sorbus* (*S. americana*, *S. aucuparia*, *S. decora* and *S. scopulina*) [9–11]. On the other hand, it was earlier noted that biphenyls could not be detected in *S. intermedia* (sub-genus *Aria*), and Erdtman remarks this is partially because this species does not form true heartwood [9]. However, our preliminary investigation of a wide range of *Sorbus* species, including *S. intermedia*, has uncovered both biphenyls and dibenzofurans as phytoalexins in the sapwood. The details of this survey will be published later.

EXPERIMENTAL

The general inoculation and incubation of wood tissue were performed as described earlier [12]. *Nectria cinnabarina* was used as the inducer fungus of phytoalexins. Silica gel TLC was used throughout the experiments. The R_fs (HPLC) are on a phenyl column (4 × 250 mm), MeOH–HOAc–H_2O (63:10:127), isocratic, 1 ml min^{-1}. NMR data was obtained on a Bruker AMX-400; the chemical shifts are in δ scale.

The necrotic zone underneath the bark was extracted with MeOH for 5 days and the extract was then filtered and concentrated *in vacuo* at the room temp. The EtOAc-soluble fraction from the residue was sepd over silica gel prep. TLC with hexane–EtOAc–MeOH (60:40:1). Guided by direct bioassay with *Cladosporium herbarum*, five antifungal bands were sepd. Aucuparin, 2′- and 4′-

Table 1. Phytoalexin production pattern in *S. aucuparia*

Compound	Plant number										
	1	2	3	4	5	6	7	8	9	10	11
Aucuparin (**1**)		+	+			+	+			+	
2′-Methoxyaucuparin (**2**)	+	+	+	+	+	+	+	+	+	+	+
4′-Methoxyaucuparin (**3**)			+		+	+	+		+	+	
2′-Hydroxyaucuparin (**4**)					+		+		+	+	
Isoaucuparin (**5**)											

Table 2. Inhibition of spore germination by biphenyls in three fungi

Compound	ED_{50}[μg ml^{-1}(μM)]		
	Alternaria alternata	*Botrytis cinerea*	*Fusarium culmorum*
Aucuparin	> 100 (> 430)	> 100 (> 430)	84 (360)
2′-Hydroxyacuparin	> 100 (> 410)	> 100 (> 410)	62 (250)
2′-Methoxyacuparin	42 (161)	17 (65)	29 (112)
4′-Methoxyaucuparin	60 (231)	66 (254)	16 (73)

methoxyaucuparin appeared to be pure at this stage (R_fs 0.60, 0.55 and 0.51, respectively). The least and the most polar phytoalexins (R_fs 0.68 and 0.36) were further purified on prep. TLC with $CHCl_3$–Me_2CO (49:1, R_f 0.52) and $CHCl_3$–Me_2CO (19:1, R_f 0.30), respectively, and isoaucuparin and 2′-hydroxyaucuparin obtained.

A blank test of the healthy tissue was performed as follows. The MeOH extract of corresponding tissue of similar size and age to fungus-inoculated twig was concd and extracted with EtOAc. This was chromatographed on TLC (0.5 mm thick, 20 cm in width and run 10 cm in hexane–EtOAc–MeOH, 60:40:1) and the bands corresponding area to aucuparin markers, though no quenching was recognised under UV light, were eluted with MeOH and brought to dryness. The residue was analysed with HPLC equipped with a diode-array detector.

Aucuparin (**1**). UV λ_{max}^{MeOH} (nm) (log ε): 228 (4.59), 274 (4.18); + NaOH: 314. MS: 230 (100, $[M]^+$); ^{1}H NMR: δ6.81 *s* (H-2, 6), 7.55 *d* (H-2′, 6′), 7.43 *t* (H-3′, 5′), 7.33 *t* (H-4′), 3.97 *s* (3,5-OMe). Rf: 0.58 ($CHCl_3$–Me_2CO, 19:1); 0.60 (hexane–EtOAc–MeOH, 60:40:1); R_t 13.2 min.

2′-Methoxyaucuparin (**2**). UV λ_{max}^{MeOH} (nm) (log ε): 268 (4.10), 288sh (4.02); + NaOH: 304; MS: 260 (100, $[M]^+$); ^{1}H NMR: δ6.78 *s* (H-2, 6), 6.99 *d* (H-3′), 7.31 *t* (H-4′), 7.03 *t* (H-5′), 7.32 *d* (H-6′), 3.92 *s* (3,5-OMe), 3.83 *s* (2′-OMe). R_f: 0.58 ($CHCl_3$–Me_2CO, 19:1); 0.55 (hexane–EtOAc–MeOH, 60:40:1); R_t 14.1 min.

4′-Methoxyaucuparin (**3**). UV λ_{max}^{MeOH} (nm) (log ε): 267 (4.14), 292sh (3.99); + NaOH: 298; MS: 260 (100, $[M]^+$); ^{1}H NMR: 6.73 *s* (H-2, 6), 7.23 *d* (H-2′, 6′), 6.56 *d* (H-3′, 5′), 3.91 *s* (3,5-OMe), 3.86 *s* (4′-OMe); ^{13}C NMR: 129.7 (C-1), 106.8 (C-2, 6), 148.9 (C-4), 123.9 (C-1′), 157.6 (C-2′), 99.3 (C-3′), 160.3 (C-4′), 104.8 (C-5′), 131.3 (C-6′), 56.6 (3,5-OMe), 55.7 (4′-OMe). R_f: 0.58 ($CHCl_3$–Me_2CO, 19:1); 0.51 (hexane–EtOAc–MeOH, 60:40:1); R_t 14.9 min.

2′-Hydroxyaucuparin (**4**). UV λ_{max}^{MeOH} (nm) (log ε): 266 (4.07), 292 (4.00); + NaOH: 310; MS: 246 (100, $[M]^+$). R_f: 0.30 ($CHCl_3$–Me_2CO, 19:1); 0.36 (hexane–EtOAc–MeOH, 60:40:1); R_t 8.39 min.

Isoaucuparin (**5**). UV λ_{max}^{MeOH} (nm) (log ε): 248sh (3.64), 291 (3.52); + NaOH; MS: 230 (100, $[M]^+$); ^{1}H NMR: δ3.81 (6H, *s*, 3,5-OMe), 6.45 (1H, *t*, J = 2.3 Hz, H-4), 6.72 (2H, *d*, J = 2.3 Hz, H-2, H-6), 6.91 (1H, *td*, J = 7.5, 1.2 Hz, H-5′), 6.97 (1H, *dd*, J = 8.1, 1.2 Hz, H-3′), 7.18 (1H, *td*, J = 7.7, 1.2 Hz, H-4′), 7.28 (1H, *dd*, J = 7.6, 1.7 Hz, H-6′). R_f: 0.63 ($CHCl_3$–Me_2CO, 19:1); 0.68 (hexane–EtOAc–MeOH, 60:40:1); R_t 14.6 min.

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REFERENCES

1. Erdtman, H., Eriksson, G. and Norin, T. (1963) *Acta Chem. Scand* **17**, 1151.
2. Watanabe, K., Ishiguri, Y., Nonaka, F. and Morita, A. (19823) *Agric. Biol. Chem.* **46**, 567.
3. Kemp, M. S., Holloway, P. J. and Burden, R. S. (1985) *J. Chem. Res. M.* 1848.
4. Watanabe, K., Widyastuti, S. M. and Nonaka, F. (1990) *Agric. Biol. Chem.* **54**, 1861.
5. Widyastuti, S. M., Nonaka, F., Watanabe, K., Sako, N. and Tanaka, K. (1992) *Ann. Phytopath. Soc. Jpn* **58**, 228.
6. Kokubun, T. and Harborne, J. B. (1994) *Z. Naturforsch.* **49c**, 628.
7. Malterud, K. E. and Sandanger Dugstad, E. K. (1985) *Z. Naturforsch.* **40B**, 853.
8. Proctor, M. C. F., Proctor, M. E. and Groenhof, A. C. (1984) *New Phytol.* **112**, 569.
9. Erdtman, H. (1963) In *Chemical Plant Taxonomy* (Swain, T., ed.), pp. 89–125. Academic Press, London.
10. Narasimhachari, N. and von Rudloff, E. (1962) *Can. J. Chem.* **40**, 1118.
11. Narasimhachari, N. and von Rudloff, E. (1973) *Phytochemistry* **12**, 2551.
12. Kokubun, T., Harborne, J. B., Eagles, J. and Waterman, P. G. (1995) *Phytochemistry* **38**, 57.