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Acetylenic aromatic compounds from *Stereum hirsutum*

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Abstract

Stereum hirsutum is a one of several fungi involved in a grapevine disease called esca. From the culture medium of this fungus, four new acetylenic compounds **1**–**3** and **6** have been isolated and identified. Structural elucidation and biological activity are reported.

Keywords: Esca; Grapevine; *Stereum hirsutum*; Frustulosin; Frustulosinol; Sterehirsutinal; Sterehirsutinol

1. Introduction

Esca is one of the most destructive disease of grapevine (Chiarappa, 1959). Because of the changes in cultural techniques and the gradual prohibition of sodium arsenite treatment, esca is increasing in Europe and California (Dubos & Larignon, 1988). Symptoms can appear in mild or severe form. The mild one is characterised by foliage deterioration, which later becomes necrotic. Sudden wilting and death of bearing vines or vine-parts in midsummer characterise the severe form, called apoplexy. Several micro-organisms (*Stereum hirsutum*, *Phellinus punctatus*, *Phaeoacremonium aleophilum*, *Phaeoacremonium chlamydosporum* and *Eutypa lata*) isolated from the woody stems of diseased grapevines, were shown to be involved in this complex disease (Larignon & Dubos, 1997). *Stereum hirsutum* is commonly isolated from decayed wood in several types of diseased trunks and its activity seems to be important. The aim of our work was to determine whether this disease could be related to the production, by these fungi, of some phytotoxic metabolites. In this paper, we report the investigation of *S. hirsutum* which seems to play an important role in the wood deterioration process (Larignon & Dubos, 1997). The investigation of the other fungi is in progress. From the EtOAc extract of the fungus culture medium, we isolated four new aromatic metabolites **1**–**3** and **6** containing 3-methylbut-3-en-1-ynyl and (or) isoprenyl substituents.

2. Results and discussion

Stereum hirsutum was grown in liquid culture on a synthetic medium (Eriksson & Petersson, 1975) for a period of four weeks. The culture broth was filtered to remove the orange-coloured mycelium. The orange filtrate was then extracted with ethyl acetate and the crude extract was chromatographed on a silica gel column using a hexane/EtOAc gradient. Several fractions of increasing polarity were collected. Screening of the mild-polar fractions for phytotoxic compounds using our bioassay (Section 3), led to the isolation of acetylenic aromatic metabolites **1**–**7**.

All compounds were identified by spectroscopic methods. Frustulosin (**4**) and Frustulosinol (**5**), isolated previously from cultures of *S. frustulosum*, exhibited antimicrobial activity at modest concentrations against a broad spectrum of pathogenic bacteria (Nair & Anchel, 1975, 1977). The respective structures were confirmed by regioselective total synthesis (Ronald &

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Table 1
^{1}H-NMR spectral data for compounds **1**–**3** and **6** (400 MHz; $CDCl_3$)

	1	**2**	**3**	**6**
ArOH	11.70 (*s*)	6.32 (*s*)	11.528 (*s*)	7.51 (*s*)
H-C(3)				6.81 (*d*)
H-C(4)	7.27 (*s*)	6.91 (*s*)	7.06 (*s*)	6.72 (*d*)
$ArCH_2O\underline{H}$		2.41 (*t*)		
$Ar\underline{CH_2}OH$		4.91 (*d*)		5.13 (*s*)
OCH_3				3.82 (*s*)
CHO	10.30 (*s*)		10.29 (s)	
Acetylenic chain	5.42 (*m*) (CH_2)	5.31 (*m*) (CH_2)	5.46 (m) (CH_2)	5.46 (*m*) (CH_2)
	2.02 (*t*) (CH_3)	5.41 (*m*) (CH_2)	2.03 (t) (CH_3)	2.02 (*s*) (CH_3)
	2.06 (*t*) (CH_3)	2.01 (*s*) (CH_3)		
Isoprenyl group			3.32 (*d*) (CH_2)	
			5.28 (*m*) (CH)	
			1.77 (*s*) (CH_3)	
			1.76 (*s*) (CH_3)	

Lausinger, 1979; Ronald, Lausinger, Lillie & Wheeler, 1982; Orr, 1979).

Compound **7** has been previously isolated from cultures of *S. hirsutum* (Kurazawa, Naganawa, Takeuchi & Umezawa, 1975a, Kurazawa, Takeuchi & Umezawa, 1975b; Nukamura, Iitaka, Kurazawa, Takeuchi & Umazawa, 1976). This metabolite was reported for its in vitro antitumour activity. The spectroscopic properties of **4**, **5** and **7** were identical to those in the literature.

In the ^{1}H-NMR spectra of compounds **1**–**3** (Table 1), the presence of only one aromatic proton suggested a pentasubstituted aromatic ring.

Compound **1**, obtained as a yellow solid, had a molecular formula of $C_{17}H_{14}O_3$ based on EI-mass spectra (M^+, 266) and ^{13}C-NMR spectral data. The ^{1}H-NMR spectrum revealed clearly the presence of the chelate hydroxyl group (11.6 ppm), the aldehyde (10.30 ppm) and two terminal methylvinyl groups. The latter substituent was previously observed in our laboratory in the spectra of eutypine and derivatives (Renaud, Tsoupras & Tabacchi, 1989; Defrancq, Zesiger & Tabacchi, 1993). The methyl group signals at 2.06 and 2.02 ppm are coupled with the signals at 5.42 ppm attributed to the olefinic protons (cf. Table 1). The IR spectra of these compounds contained a characteristic triple bond absorption band at 2190 cm^{-1}. Four singlets of low-intensity signals confirmed the presence of two acetylenic groups at $\delta = 82.91$, 98.70, 105.90 and 111.61 ppm in the ^{13}C-NMR spectra (Wehrli, Marchand & Wehrli, 1983). Compound **1** was characterised as 2,5-dihydroxy-3,6-bis(3-methylbut-3-en-1-ynyl)benzaldehyde.

Compound **2** had the molecular formula $C_{17}H_{16}O_3$, as deduced from the EI-mass spectrum ($[M]^+$ at m/z 268.2 amu more than that of compound **1**). The ^{1}H-NMR spectrum differed from that of compound **1** by the presence of a signal of a CH_2OH group at 4.91 ppm and the disappearance of the chelated hydroxyl proton and the aldehyde proton. The EI-mass spectrum revealed that the $[M]^+$ was fragmented through a process of water loss from the parent ion. Compound **2** was characterised as 3-(hydroxymethyl)-2,5-bis(3-methylbut-3-en-1-ynyl)benzene-1,4-diol.

Structures of **1** and **2** were confirmed by synthesis (Fkyerat, Dubin & Tabacchi, 1999); the spectroscopic properties of the synthetic and corresponding natural compounds were identical.

The molecular formula of compound **3** was found to be $C_{17}H_{16}O_3$, by EI-mass spectrum ($[M]^+$ at m/z 270) and ^{13}C-NMR spectral data. The ^{1}H-NMR spectrum revealed clearly the presence of chelated hydroxyl and the aldehyde protons. It showed the presence of an acetylenic and isoprenyl group [δ 1.76, 1.79 (2 × Me), 3.32 (1 × CH_2) and 5.18 (1 × CH)]. The long-range coupling experiment confirmed that the C-3 position was connected with the isoprenyl group. Therefore, the structure of **3** was established as 2,5-dihydroxy-3-isoprenyl-6-(3-methylbut-3-en-1-ynyl)benzaldehyde.

A typical pattern for 1-, 2-, 3-, 4-tetra substituted aromatic ring was observed in the ^{1}H-NMR spectra of compound **6**, showing two doublets of an AB spin system (J_{ortho} = 9 Hz). This compound appeared to the hydroxymethylated derivative of compound **5**, the methoxy signal appearing at 3.82 ppm. The structure of **6** was confirmed by regioselective synthesis (not published result).

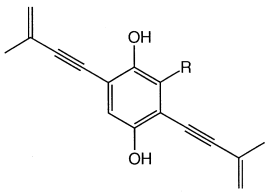
This series of new acetylenic compounds is structurally close to the previously reported frustulosin (Nair & Anchel, 1975, 1977) and eutypine (Renaud et al., 1989). All these compounds were bioassayed using tomato plants and compared to eutypine. In these standard bioassays (Renaud et al., 1989), compound **1** had the highest phytotoxic activity but acted differently compared to eutypine. This visual comparison showed that compound **1** induced a withering of the stem and a foliage wilting and eutypine induced only the necrosis of the leaves.

This result is in agreement with those obtained with the fungus on grapevine (Larignon & Dubos, 1997). *Stereum hirsutum* showed to be responsible for the typical wood degradation of esca (Larignon & Dubos, 1997). This rapid screening allows us to select compound **1** for further tests.

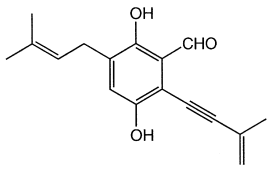
Sterehirsutinal **1** was then tested in callus of *Vitis vinifera cv* Gamay (grapevine) and its activity was compared to that of eutypine. *Vitis vinifera cv* Gamay was grown in standard culture media in the presence of sterehirsutinal or eutypine at different concentrations (100, 250 and 500 μM) for 28 days. The growth of the tissue was estimated by determination of the weight of callus, and expressed in percentage with the tissue cultivated without the presence of toxines.

Sterehirsutinal inhibits 50% of the growth of callus at 100 μM, and 100% at 500 μM.

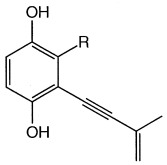
The presence of Sterehirsutinal (**1**) in the infected wood has been confirmed (Dubin, 1998). Investigation with a pure synthetic sample of **1** in more specific biological test is necessary for the definitive proof of the role played by Sterehirsutinal in the pathogenesis of esca.



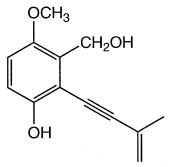
1 R = CHO
2 R = CH_2OH



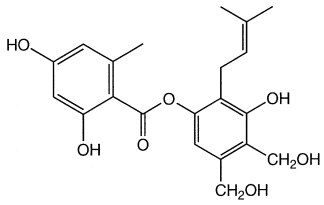
3



4 R=CHO Frustulosin
5 R=CH_2OH Frustulosinol



6



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3. Experimental

3.1. General

The culture medium was prepared according to Eriksson and Petersson (1975). The fungus was grown during 4 weeks at 25°C under normal light conditions. Bioassays were undertaken on small tomato plants, *Bonny Best* variety (5–8 cm, 200–300 mg, two leaves). Evaluation of the biological activity was accomplished by visual estimation after immersion into a solution of 0.5 or 1 mg/ml of tested compound during 24 h. In all cases, blank tests were carried out in the same conditions. In order to increase the solubility of the products, 5% of methanol (perfectly tolerated by the plant) is added to both the metabolite and the blank test.

3.2. Isolation

The mycelium was removed by filtration and the orange culture medium extracted with ethyl acetate. The organic phase was concentrated *under vacuum* and the residue purified by CC (silica gel; hexane/EtOAc gradient). Further purification was achieved by semipreparative HPLC on RP-C-18 column (MeOH/H_2O gradient).

3.2.1. 2,5-Dihydroxy-3,6-bis(3-methylbut-3-en-1-ynyl)benzaldehyde (*1*)

Yellow solid. R_f (silica gel; hexane/EtOAc 2/1): 0.46. UV: λ_{max} (MeOH, H_2O) (nm) 260, 310, 425. ^{1}H-NMR: see Table 1. ^{13}C-NMR: δ 23.86 (CH_3), 23.95 (CH_3), 82.91, 98.71, 105.90, 111.61 (acetylenic carbons), 115.43, 118.40, 124.01 (CH_2), 125.52 (CH_2), 126.08, 127.17 (C-4), 150.22 and 157.84 (C-2 and C-5), 196.47 (CHO). EI-MS: 266 (100, M^+), 251 (18), 238 (22), 195 (43), 165 (51), 152 (18).

3.2.2. 3-(Hydroxymethyl)-2,5-bis(3-methylbut-3-en-1-ynyl)benzene-1,4-diol (*2*)

R_f (silica gel; hexane/EtOAc 2/1): 0.30. UV: λ_{max} (MeOH, H_2O) (nm): 218, 296, 355. ^{1}H-NMR: see Table 1. ^{13}C-NMR: δ 149.70 and 148.98 (C-2, C-5), 126.31, 126.03 and 125.75 (C-1, C-3, and C-6), 123.63 and 123.31 ($2CH_2$), 116.30 (C-4), 111.74, 110.26, 102.75, 98.49, 82.06, 79.83, 60.66 (CH_2OH), 23.38 ($2CH_3$). EI-MS: 268 (62, M^+), 250 (100), 235 (7), 221 (7), 207 (8), 193 (6), 179 (25), 178 (32).

3.2.3. 2,5-Dihydroxy-3-isoprenyl-6-(3-methylbut-3-en-1-ynyl)benzaldehyde (*3*)

R_f (silica gel; hexane/EtOAc 2/1): 0.26. UV: λ_{max} (MeOH, H_2O) (nm) 248, 290, 405. ^{1}H-NMR: see Table 1. EI-MS: 270 (75, M^+), 255 (18), 186 (74), 85 (66), 83 (100).

3.2.4. 2-hydroxy–5-methoxy-6-(3-methylbut-3-en-1-ynyl) benzylalcohol (*6*)

R_f (silica gel; hexane/EtOAc 2/1): 0.27. UV: λ_{max} (MeOH, H_20) (nm) 240, 270, 330. ^{13}C-NMR: δ 24.18

(CH_3), 57.23 (OCH_3), 63.56 (CH_2OH), 82.59 and 100.21 (acetylenic carbons), 111.41 (alcene carbon), 112.25 and 118.09 (C-3 and C-4), 122.80 ($=CH_2$), 127.41 (C-1), 127.45 (C-6), 150.89 and 154.67 (C-2 and C-5). EI-MS: 218 (38, M^+), 200 (100), 185 (12), 172 (6), 157 (16), 128 (29), 115 (6), 91 (9), 77 (12).

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