101. Biologically Active Natural Acetylenic Compounds from *Eutypa lata* (Pers: F.) TUL.

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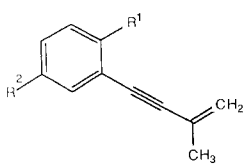
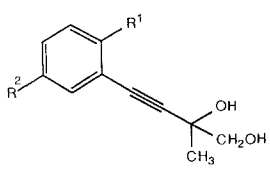
Eight new acetylenic compounds have been isolated and identified from the culture medium of the fungus *Eutypa lata*. Structural elucidation and biological activity are discussed.

Introduction. – Eutypiosis or 'dying arm' disease is responsible for the dieback of vineyards and woody species observed during the last few years in Switzerland, France, and many other countries of the world [1–3]. The pathogenic agent is the fungus *Eutypa lata* (Pers: F.) TUL., also named *Eutypa armeniacea*. Previous studies [4] [5] have shown that the disease is linked to a secondary phytotoxic fungal metabolite. The evaluation of the role and extent of mycotoxin required the isolation and chemical characterisation of the active agent from fungus culture medium. Screening the fractions of the Et_2O extract for phytotoxic compounds using our bioassay, led to the isolation of eight new aromatic metabolites with a 3-methylbut-3-en-1-ynyl substitution.

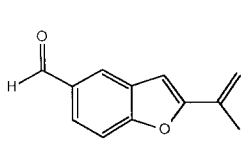
Results and Discussion. – *Eutypa lata* was grown *in vitro* for a period of six weeks. The phytotoxic activity of culture medium at pH 4.5 was transferred into the Et_2O phase on extraction. The crude Et_2O extract was subjected to silica-gel column chromatography ($CHCl_3$/MeOH gradient). Several fractions of increasing polarity were collected. Further purification was carried out by semi-preparative HPLC on a *RP-18* column. Biological activity was monitored on tomato plants and vine leaves. This process led to the isolation of aromatic acetylenic metabolites **1**–**8**.



1 $R^1 = OCH_3$, $R^2 = CH_2OH$
2 $R^1 = OH$, $R^2 = CH_2OH$
3 $R^1 = OCH_3$, $R^2 = CHO$
4 $R^1 = OH$, $R^2 = CHO$
5 $R^1 = OH$, $R^2 = COOH$



6 $R^1 = OH$, $R^2 = CH_2OH$
7 $R^1 = OH$, $R^2 = CHO$
8 $R^1 = OH$, $R^2 = COOH$



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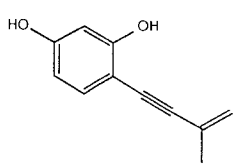

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A typical pattern for a 1,2,4-trisubstituted aromatic ring was observed in the ^{1}H-NMR spectra of these compounds; *i.e.* the three signals of an *AMX* spin system ($J_{ortho} = 8.5$, $J_{meta} = 2.4$, $J_{para} \approx 0$ Hz). For compounds **1** and **2**, the signal of a CH_2OH

group appeared at 4.60 ppm. This signal was absent in the spectra of compounds **3** and **4**, whilst a signal for an aldehydic proton appeared at 9.89 ppm. The presence of a COOH group in compound **5** shifted the signal of protons in *ortho*-position downfield; the proton in *meta*-position was also slightly shifted.

In the ^{1}H-NMR spectra of compounds **1**–**5**, the characteristic signals of a terminal methylvinyl group were also present. Decoupling experiments on the Me-group signal at 2.02 ppm revealed a coupling ($J = 1.2$ Hz) with the signals at 5.31 and 5.43 ppm attributed to the olefinic protons (*cf.* the *Table*). The IR spectra of these compounds contained a characteristic C≡C bond absorption band at 2190 cm^{-1}. The presence of the

Table. *^{1}H-NMR Spectral Data of Compounds* **1**–**8** (δ in ppm; $CDCl_3$ as solvent)

	1	**2**	**3**	**4**	**5**	**6**[a])	**7**[a])	**8**[a])
H–C(2)	7.44 (*d*)	7.36 (*d*)	7.90 (*d*)	7.95 (*d*)	8.15 (*d*)	7.30 (*d*)	7.90 (*d*)	8.14 (*d*)
H–C(5)	6.85 (*d*)	6.90 (*d*)	7.08 (*d*)	7.01 (*d*)	7.03 (*d*)	6.80 (*d*)	7.08 (*d*)	7.07 (*d*)
H–C(6)	7.29 (*dd*)	7.27 (*dd*)	7.81 (*dd*)	7.84 (*dd*)	8.01 (*dd*)	7.12 (*dd*)	7.82 (*dd*)	7.95 (*dd*)
CH_3O	3.91 (*s*)		3.97 (*s*)					
Ar–CH_2OH	4.61 (*s*)	4.60 (*s*)				4.45 (*s*)		
Ar–CHO			9.86 (*s*)	9.89 (*s*)			9.86 (*s*)	
$=CH_2$	5.31 (*m*)	5.37 (*m*)	5.44 (*m*)	5.35 (*m*)	5.43 (*m*)			
	5.43 (*m*)	5.45 (*m*)	5.51 (*m*)	5.46 (*m*)	5.55 (*m*)			
CH_2OH						3.60 (*s*)	3.60 (*s*)	3.60 (*s*)
CH_3	2.01 (*dd*)	2.01 (*dd*)	2.02 (*dd*)	2.01 (*dd*)	2.03 (*dd*)	1.50 (*s*)	1.51 (*s*)	1.50 (*s*)

[a]) CD_3OD as solvent.

acetylenic group in our molecules was confirmed by the two *singlets* of low-intensity signals at 77.60 and 98.25 ppm in the ^{13}C-NMR spectra of compound **2** [6]. In the mass spectra of compounds **1**–**5**, the highly conjugated system led to little fragmentation of the molecular ions (base peak). Other fragment ions due to the elimination of a Me group and of the aromatic substituents were nevertheless present. A detailed study of the fragmentation pathway of these compounds by MS-MS experiments will be described later [7]. The structures **1**–**5** were confirmed by synthesis [8]; the spectroscopical properties of synthetic and corresponding natural compounds were identical. Compounds **6**, **7**, and **8** appeared to be products of hydroxylation of the terminal vinyl group of compounds **2**, **4**, and **5** respectively. In the ^{1}H-NMR spectra, no change in the aromatic region was observed; in the resulting glycol system the CH_2OH signal appeared as *singlet* at 3.60 ppm and the Me signal was shifted upfield to 1.50 ppm. The relatively polar compounds **6**, **7**, and **8** were analysed by GC/MS after trimethylsilylation. The presence of the glycol moiety was verified by partial synthesis of **7**. Treatment of compound **4** (which could be considered as a biogenetical precursor of **7**) with OsO_4 in Et_2O gave a compound having an identical mass spectrum to that of compound **7**.

Compound **4**, named *eutypine*, had the highest phytotoxic activity in our bioassay. An aldehyde group and a free OH group in *para*-position seem to be necessary for biological activity. The presence of the C≡C bond in *ortho*-position to the OH group can lead to benzofuran derivatives *via* a cyclisation process. Eutypine (**4**) gives under mildly acidic conditions the 5-formyl-2-(methylvinyl)[1]benzofuran (**9**), which is not a biologically active compound. The benzofuran derivative **9** was also detected in the culture medium;

therefore, care must be taken in extraction procedure in order to avoid lost of biological activity. These results suggest that phenols containing an acetylenic substitution in *ortho*-position could be biogenetic precursors of naturally occurring benzofurans.

This series of new natural acetylenic compounds is structurally close to the previously reported *frustulosin* and the 4-(3-methylbut-3-en-1-ynyl)benzene-1,3-diol (**10**) [9] [10]. The biogenetic origin of aromatic ring of acetylenic compounds *via* shikimic-acid pathway has been proposed; 3-methylbut-3-en-1-ynyl substituent linked to the aromatic ring probably arises from an isopropenoid group. The presence of eutypine in the infected material and/or in the rising sap of infected vines has been confirmed [7]. Investigation with a pure synthetic sample of compound **4** in more specific test (protoplast test) is necessary for the definitive proof of the role played by eutypine in the pathogenesis of eutypiosis. Biosynthetic studies using labelled precursors of these compounds are also in progress.

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Experimental Part

General. All solvents were distilled before use. Column chromatography (CC): *Merck* silica gel *60* (0.04–0.06 mm). IR spectra: *Perkin-Elmer 521* in KBr, in cm^{-1}. NMR spectra in ppm relative to TMS; solvent $CDCl_3$ or CD_3OD. ^{1}H-NMR (200 MHz): *Bruker WP-200*. ^{13}C-NMR (50.29 MHz): *Bruker WP-200*. EI-MS (70 eV): *Nermag R30-10*, in *m/z* (rel-%).

Material. The culture medium was prepared according to *Pezet* [11]. The fungus was grown during six weeks at 25° under normal light conditions. Bioassays were undertaken on small tomato plants, *Bonny Best* variety (5–8 cm, 200–300 mg, two leaves) and/or on young vine leaves. Evaluation of the biological activity was accomplished by visual estimation of withering after immersion into the solution of about 50 µg/ml of tested compound during 4 h and/or by determination of the weight loss of the plants. In all cases, blank tests (pure H_2O) were carried out.

Isolation. The mycelium was removed by filtration and the culture medium (4 l) extracted with Et_2O after adjustment to pH 4.5. The org. phase was concentrated *in vacuo* and the residue purified by CC (silica gel; $CHCl_3$/MeOH gradient). Further purification was achieved by semi-prep. HPLC on *RP-18* column (MeOH/H_2O gradient).

4-Methoxy-3-(3-methylbut-3-en-1-ynyl)benzyl Alcohol (**1**): 1 mg of colourless oil. R_f (silica gel; $CHCl_3$/AcOEt 5:1): 0.44. IR: 3400 (OH), 2195 (C≡C), 1600, 1465 (arom.). ^{1}H-NMR: see the *Table*. EI-MS: 202 (100, M^+), 185 (25), 171 (5), 161 (30), 141 (10), 129 (10), 115 (20), 77 (6).

4-Hydroxy-3-(3-methylbut-3-en-1-ynyl)benzyl Alcohol (**2**): 1 mg of amorphous white solid. R_f (silica gel; $CHCl_3$/AcOEt 5:1): 0.30. IR: 3400 (OH), 2190 (C≡C), 1600, 1495 (arom.), 1425, 885. ^{1}H-NMR: see the *Table*. ^{13}C-NMR: 23.78 (*q*); 65.15 (*t*); 77.66 (*s*); 98.25 (*s*); 110.20 (*s*); 115.33 (*d*); 123.32 (*t*); 126.67 (*s*); 129.90 (*d*); 130.90 (*d*), 133.59 (*s*); 156.58 (*s*). EI-MS: 188 (100, M^+), 187 (50), 171 (65), 158 (27), 141 (30), 131 (55), 128 (50), 115 (40), 91 (30), 77 (25), 63 (20).

4-Methoxy-3-(3-methylbut-3-en-1-ynyl)benzaldehyde (**3**): 0.5 mg of colourless oil. R_f (silica gel, $CHCl_3$/AcOEt 5:1): 0.82. IR: 2820, 2720, 2200 (C≡C), 1690 (C=O), 1595, 1500, 1460 (arom.). ^{1}H-NMR: see the *Table*. EI-MS: 200 (100, M^+), 199 (5), 185 (8), 171 (4), 159 (20), 128 (50), 115 (26), 91 (19), 77 (25).

4-Hydroxy-3-(3-methylbut-3-en-1-ynyl)benzaldehyde (*Eutypine*; **4**): 2 mg of white solid. R_f (silica gel; $CHCl_3$/AcOEt 5:1): 0.58. IR: 3400 (OH), 2820, 2720, 2200 (C≡C), 1685 (C=O), 1590, 1450 (arom.). ^{1}H-NMR: see the *Table*. ^{13}C-NMR: 23.26 (*q*); 80.55 (*s*); 99.11 (*s*); 110.92 (*s*); 115.56 (*d*); 123.95 (*t*); 125.93 (*s*); 130.28 (*d*); 131.95 (*d*); 134.31 (*s*); 161.35 (*s*); 189.94 (*s*). EI-MS: 186 (100, M^+), 185 (80), 171 (5), 157 (45), 128 (40), 115 (35), 89 (8), 77 (10).

4-Hydroxy-3-(3-methylbut-3-en-1-ynyl)benzoic Acid (**5**): 0.7 mg of white solid. R_f (silica gel; $CHCl_3$/AcOEt 5:1): 0.30. IR: 3400 (OH), 2880, 1700 (COOH), 2200 (C≡C), 1600, 1400 (arom.). ^{1}H-NMR: see the *Table*. EI-MS: 202 (100, M^+), 201 (15), 187 (10), 185 (30), 175 (8), 162 (5), 157 (25), 145 (5), 128 (24), 115 (20), 77 (7).

4-Hydroxy-3-(3,4-dihydroxy-3-methylbut-1-ynyl)benzyl Alcohol (**6**): 0.5 mg. ^{1}H-NMR: see the *Table*. EI-MS (per-trimethylsilylated compound): 510 (1, M^+), 495 (3), 407 (100), 275 (30), 259 (10), 203 (7), 187 (5), 147 (18), 73 (60), 45 (10).

4-Hydroxy-3-(3,4-dihydroxy-3-methylbut-1-ynyl)benzaldehyde (**7**): 0.3 mg. ^{1}H-NMR: see the *Table*. EI-MS (per-trimethylsilylated compound): 436 (2, M^+), 421 (10), 346 (1), 333 (100), 291 (50), 147 (23), 73 (75), 45 (20).

Silyl Derivatives. Aliquots of compounds **6**, **7**, and **8** were treated in a vial with BSTFA/pyridine 5:1 for trimethylsilylation. The per-trimethylsilylated compounds **6**, **7**, and **8** were analysed by GC/MS.

4-Hydroxy-3-(3,4-dihydroxy-3-methylbut-1-ynyl)benzoic Acid (**8**): 0.3 mg. ^{1}H-NMR: see the *Table*. EI-MS (per-trimethylsilylated compound): 524 (3, M^+), 509 (20), 434 (1), 421 (100), 279 (3), 363 (6), 289 (25), 273 (8), 147 (20), 73 (70), 45 (10).

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