Phytochemistry, 1974, Vol. 13, pp. 2567 to 2571. Pergamon Press. Printed in England.

PHENOLIC METABOLITES OF *CERATOCYSTIS ULMI*

NORMAN CLAYDON, JOHN FREDERICK GROVE and MARK HOSKEN

Agricultural Research Council, Unit of Invertebrate Chemistry and Physiology, The University of Sussex, Falmer, Brighton, Sussex, BN1 9QJ

(*Received* 10 *March* 1974)

Key Word Index—*Ceratocystis ulmi*; fungi; Dutch elm disease; secondary metabolites; C_{10} phenolic carboxylic acids.

Abstract—The C_{10} acid 2,4-dihydroxy-6-(1-hydroxyacetonyl) benzoic acid, together with the 6-acetonyl- and 6-pyruvyl-analogues, has been identified as a metabolic product of *Ceratocystis ulmi*, the causative agent of Dutch elm disease. In a comparison of aggressive "fluffy" and non-aggressive "waxy" strains of *C. ulmi*, the C_{10} acids were produced more rapidly and in greater yield by the former group.

INTRODUCTION

Ceratocystis ulmi, the causative agent of Dutch elm disease, disseminated in Europe by bark beetles of the genus *Scolytus*, is a phytopathogenic fungus which has not been investigated for the production of secondary metabolites of low MW.

After earlier work[1] had suggested that culture filtrates of *C. ulmi* contained phytotoxic material, Dimond[2] demonstrated that this consisted of two components, a high MW polysaccharide, precipitated by ethanol and considered to be responsible for the upcurling of leaves, and an ethanol-soluble substance responsible for the necrotic lesions which develop between the veins of leaves of infected trees. Following a controversy over the relation of the polysaccharide fraction to wilting,[3] attention was concentrated on the ethanol-soluble component. A mixture of glycoproteins of high MW, which were considered to induce disease symptoms similar to those produced by the fungus, was isolated[4] after dialysis and ultracentrifugation.

Although this evidence[4] appeared to be conclusive, the recent outbreak in this country of Dutch elm disease, arising probably from the reimportation from North America of a virulent "fluffy" strain of *C. ulmi*, more aggressive in character than the endemic "waxy" strain,[5,6] has led us to compare the ability of these strains to produce secondary metabolites of low MW. Our results have already been reported in summary.[7]

RESULTS AND DISCUSSION

Two "fluffy" strains, numbers 43 and 44 in our culture collection, and two "waxy" strains, numbers 45 and 46, kindly contributed by Dr. J. N. Gibbs of the Forestry Commis-

[1] ZENTMEYER, G. A. (1942) *Science* **95**, 512.
[2] DIMOND, A. E. (1947) *Phytopathology* **37**, 7.
[3] FELDMAN, A. W., CAVORELLI, N. E. and HOWARD, F. L., (1950) *Phytopathology* **40**, 341.
[4] SALEMINK, C. A., REBEL, H., KERLING, L. C. P. and TCHERNOFF, V. (1965) *Science* **149**, 202.
[5] BRASIER, C. M. and GIBBS, J. N. (1973) *Nature* **242**, 607.
[6] GIBBS, J. N. and BRASIER, C. M. (1973) *Nature* **241**, 381 and references therein.
[7] CLAYDON, N., GROVE, J. F., and HOSKEN, M. (1974) *Chem. Ind.* 344.

sion Research Station, Farnham, Surrey, were grown under identical conditions in surface culture at 25° on a chemically-defined medium.[4] The pH and optical rotation of the culture filtrate, and the UV absorption and behaviour on TLC of the crude products obtained by extraction of the culture filtrate with organic solvents, were determined at intervals over a period of 4 to 5 weeks.

After an initial fall,[3] consistent with the formation of acidic metabolites, the pH of the culture filtrate from strain 44 rose rapidly (Table 1) as soon as the sugar had been consumed (16 days). The formation of material with λ_{max} 265, 300 nm commenced after about 7 days, increased rapidly during the 6 days after day 10, and then levelled off when the carbon source was exhausted. The formation of this UV absorbing material in the ethyl acetate extract was correlated with the appearance on TLC of a series of spots at R_f 0·05 0·15 in chloroform–methanol. Very similar results were obtained with strain 43.

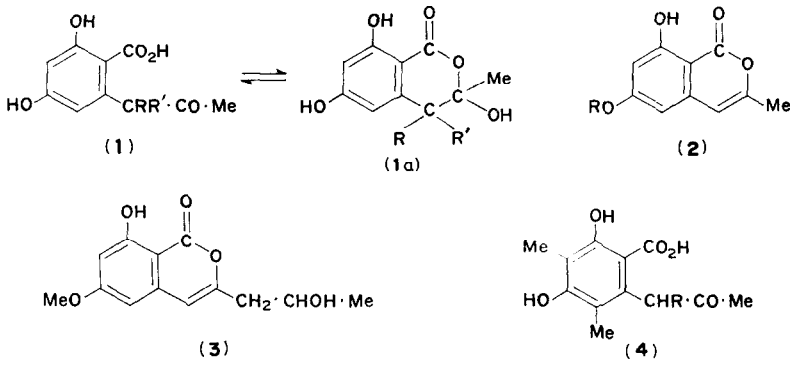
TABLE 1. COURSE OF TYPICAL FERMENTATIONS WITH THE *C. ulmi* STRAINS: CHANGE WITH TIME IN THE pH AND OPTICAL ROTATION OF THE CULTURE FILTRATE AND IN THE YIELD OF C_{10} ACIDS

Strain	Days	0	7	10	13	16	23	27	30	34
43	pH	4·2	3·6	3·7	3·7	4·4	7·0	7·5	7·5	7·0
	Optical rotation (% value at day 0)	100	59	26	0		0	0	0	
	C_{10} acids (mg/l.)	0	0		15	63	82			
44	pH	4·2	3·6	3·7	3·8	4·5	7·4	7·5	7·8	7·6
	Optical rotation (% value at day 0)	100	63	37	9	1	0	0	0	0
	C_{10} acids (mg/l.)	0	5		32	79		76	82	83
45	pH	4·2	3·6	3·7	3·7	3·7	4·5	6·0	6·7	6·8
	Optical rotation (% value at day 0)	100	85	80	46	15	1	0	0	
	C_{10} acids (mg/l.)	0	0	0	0	5	24	51	64	67
46	pH	4·2	3·7	4·0	4·1	4·1	4·5	6·5	7·2	7·5
	Optical rotation (% value at day 0)	100	79	70	39	26	7		1	1
	C_{10} acids (mg/l.)	0	0	0	3	12	26	50	59	50
52	pH	4·2	3·7		3·8	4·1	4·1			
	Optical rotation (% value at day 0)	100	86		49	6	1			
	C_{10} acids (mg/l.)	0	0		3	12				

With the "waxy" strains 45 and 46 the fermentation took the same general course, but with a much expanded time scale (30 days). The pattern of metabolic activity presented by these results is consistent with the differences in growth rate previously[6] correlated with differences in pathogenicity of the "fluffy" and "waxy" strains.

The major component, m.p. 202° (decomp.) of the material responsible for the UV absorption was extracted at pH 2 by ethyl acetate, but not by chloroform. Particularly with the waxy strains, an initial extraction of the culture filtrate with chloroform gave a cleaner product. It was purified by repeated recrystallization from ethyl acetate and its composition was determined by microanalysis and high resolution MS. It was an optically inactive phenolic carboxylic acid, $C_{10}H_{10}O_6$, identified as 2,4-dihydroxy-6-(1-hydroxyacetonyl) benzoic acid (**1**; RR′=H, OH) or its 3,4-dihydro-3-hydroxy-isocoumarin tautomer (**1a**; RR′=H, OH). It was accompanied by smaller amounts of the $C_{10}H_{10}O_5$ 6-acetonyl-analogue (**1**; RR′=H_2) or lactol tautomer and, particularly in the later stages of the fermentation, by the $C_{10}H_8O_6$ 6 pyruvyl-analogue (**1**; RR′=O) or lactol tautomers. With strain 44 this was apparent after day 30, characterized by the appearance of an additional band at 350 nm in the UV spectrum of the extract.

The same mixture of C_{10} acids, together with mycophenolic acid and 3,5-dihydroxyphthalic acid has previously been isolated from a number of strains of *Penicillium brevicompactum*.[8,9] The closely related isocoumarins (**2**; R=H and Me) are known metabolic products of another species of *Ceratocystis*, *C. fimbriata*.[10,11]



The C_{10} acids are related to the isocoumarin (**3**),[12] the phytotoxin of *Endothia parasitica*, a pathogen of chestnut, and to the sclerotinins (open form: **4**; R=H or Me), plant growth-promoting metabolic products of *Sclerotinia sclerotiorum*.[13]

The C_{10} acids form a solid solution from which the individual components were initially[8,9] obtained by a complex and tedious fractional crystallization involving, at one stage, the separation of the lead salts. Prepared in this way, each acid contained (by TLC) traces of the other two components. Subsequently, the mixture was separated by column chromatography on cellulose.[14] We find that preparative TLC on silica gel in diisopropyl ether–formic acid–water is satisfactory on a small scale and gives a series of well-separated bands from which the individual C_{10} acids can be recovered by extraction with water and re-extraction of the acidified aqueous solution with ethyl acetate.

With the major metabolite identified as the ketol (**1**; RR'=H, OH), a spectrophotometric assay for the C_{10} acids, expressed as "apparent ketol", was developed, based on the intensity of absorption at 302 nm (ε 6900). The ketone (**1**; RR'=H$_2$) and the ketol (**1**; RR'=H,OH) have essentially identical UV absorption curves. Although the introduction of a second carbonyl group in the diketone (**1**; RR'=O) produces a bathochromic shift of the first absorption band to 348 nm, by coincidence the second band falls close to 302 nm and the extinction coefficient is not so different from 6900 as to cause any major error when amounts of the diketone are small. In more accurate work the diketone can be estimated separately from the intensity at 348 nm and the appropriate correction made.

The yield, expressed in this way, of the C_{10} acids from the "fluffy" strain 44 was *ca* 70 mg/l after 16 days when the yield from the "waxy" strain 45 was only 5 mg/l. At the point where the total yield of C_{10} acids from strain 44 was approaching its maximum, the yield,

[8] CLUTTERBUCK, P. W., OXFORD, A. E., RAISTRICK, H. and SMITH, G. (1932) *Biochem. J.* **26**, 1441.
[9] OXFORD, A. E. and RAISTRICK, H. (1933) *Biochem. J.* **27**, 634.
[10] CURTIS, R. F., (1968) *Experientia* **24**, 1187.
[11] STOESSL, A., (1969) *Biochem. Biophys. Res. Commun* **35**, 186.
[12] HARDEGGER, E., RIEDER, W., WALSER, A. and KUGLER, F. (1966) *Helv. Chim Acta.* **49**, 1283.
[13] SASSA, T., AOKI, H., NAMIKI, M. and MUNAKATA, K., (1968) *Agr. Biol. Chem.* **32**, 1432.
[14] GODIN, P. (1955) *Antonie van Leeuwenhoek* **21**, 362.

by preparative TLC, of the ketone (**1**; RR′=H_2) was only 5 mg/l, but could have been greater earlier in the fermentation since the biosynthesis of this group of polyketides via the acetate-polymalonate pathway clearly proceeds in the sequence (**1**: RR′=H_2)→(**1**; RR′=H,OH)→(**1**; RR′=0). Very much higher yields of the C_{10} acids were obtained in shake culture (4–6 days), 250 mg/l. being obtained from strain 43.

As a corollary to these results it was of interest to ascertain whether the C_{10} acids were also produced by strains of *C. ulmi* isolated during earlier epidemics of Dutch elm disease. When such a strain (number 52), which had been maintained in a culture collection for 25 yrs, was grown under the same conditions, the rate of consumption of carbon source was almost identical to that of the "waxy" strains 45 and 46 (Table 1) and UV absorbing secondary metabolites, formally identified as the C_{10} acids, were produced in yields similar to those from strains 45 and 46, and at the same rate. It seems probable, therefore, that pathogenic strains of *C. ulmi* have always been capable of the biosynthesis of the C_{10} acids.

Whilst this work was in progress the ketone (**1**; R=H_2) has been isolated from two other, quite unrelated, plant pathogens *Alternaria kikuchiana*[15] and *Pyricularia oryzae*[16] and has been shown to be moderately phytotoxic.

EXPERIMENTAL

M.ps were taken on a Kofler hot-stage apparatus and are corrected. Light petroleum had b.p. 60–80°. Merck silica gel H_{254} was used for TLC. MW were taken from M^+ in the low resolution MS. IR spectra were determined for mulls in Nujol and UV spectra for solns in MeOH.

C. ulmi cultures. Five strains of *C. ulmi* were examined. No's 43–46 inclusive, were obtained from Dr. J. N. Gibbs; two, No's 43 and 44 were his "fluffy" strains W2 and W4, respectively, and two, No's 45 and 46 were his "waxy" strains W7 and W9; No. 52 was CBS 115·47. They were maintained on malt agar slopes.

Fermentations with the C. ulmi strains: (a) *Surface culture.* Conical flasks (6 × 1 litre) containing the medium (250 ml) used by Salemink[4] (pH 4·2) were inoculated with a spore suspension (1 ml) of the *C. ulmi* strain prepared from a 4-day shake culture. The flasks were incubated at 25° in artificial light. At intervals aliquots (usually 10 ml) of the culture fluid were removed under sterile conditions from beneath the mycelial felts from selected flasks for determination of the pH and optical rotation (10 cm path), and for spectrophotometric and TLC assays for secondary metabolic products. Relevant data are presented in Table 1. After 4–5 weeks the fermentations were harvested and the culture filtrate was adjusted to pH 7 and extracted with $CHCl_3$ (2 × ⅓ vol.). The acidic metabolic products were then obtained by readjustment of the culture filtrate to pH 2 with HCl followed by extraction with EtOAc (2 × ⅓ vol.). (b) *Shake culture* Conical flasks (250 ml) containing inoculated medium (100 ml) were shaken at 140 rpm. on a rotary shaking machine and incubated under the above conditions. After 5–6 days they were harvested and the culture filtrates extracted as before.

Spectrophotometric and TLC assays for secondary metabolites. A portion (5 ml) of the filtered culture fluid was adjusted to pH 2 with HCl and extracted with EtOAc (2 × 2 ml). The residue obtained on recovery was dried *in vacuo* and dissolved in MeOH (10 ml). The A of this soln, with dilution as required, was recorded (1 cm cell) between 200 and 390 nm, and the total concentration of C_{10} acids in the culture fluid calculated as "apparent ketol (**1**; RR′=H, OH)" from the A at 302 nm, taking ϵ302 as 6900 (see Table 1). The solute was then recovered and examined by TLC in $CHCl_3$–MeOH (95:5). The R_f values of spots visible in UV light and in I_2 vapour were recorded. There were few significant changes in the pattern of metabolic products during the course of the fermentation as revealed by this test, and no significant differences between "fluffy" and "waxy" strains other than in the region $R_f < 0{\cdot}15$ (see text).

Separation of the C_{10} acids by preparative TLC. The crude mixture (25 mg) in Me_2CO was separated on silica gel (20 × 20 × 0·05 cm) using diisopropyl ether–HCOOH–H_2O (90:7:3). The air-dried plate was examined in UV light (254 nm) and the dark bands (quenching of the fluorescent indicator) marked and removed in the usual way. R_f values were calculated relative to that of the diketone (**1**; RR′=0) 0·67. The silica from each band was extracted with warm H_2O (2 × 4 ml) and the aq. extract (pH 3) was then extracted with EtOAc (2 × 2 ml). The UV absorption of a portion of the recovered fraction was examined in MeOH and the remainder of the product was then purified further as described below.

Isolation and identification of C. ulmi metabolic products: Surface culture (a). *Strain* 45. The fermentation was harvested after 34 days. Extraction of the culture filtrate (1030 ml) from 5 flasks gave a neutral fraction (37 mg)

[15] KAMEDA, K., AOKI, H., TANAKA, H. and NAMIKI, M. (1973) *Agr. Biol Chem.* **37**, 2137.
[16] IWASAKI, S., MURO, H., SASAKI, K., NOZOE, S., OKUDA, S. and SATO, Z. (1973) *Tetrahedron Letters* 3537.

and a brown resinous acid fraction (124 mg) which deposited an amorphous powder (10 mg) on trituration with EtOAc. Recrystallization of this from EtOAc afforded the ketol (**1**; RR′=H, OH), identified by IR (see strain 43 below). A portion (45 mg) of the recovered EtOAc soluble resin was subjected to preparative TLC (2 plates) as described above and the materials contained in bands at R_f 0·85, 0·67, 0·34, 0·33 and 0·20 were recovered. The fraction R_f 0·67 had λ_{max} 300, 350 nm and was sublimed at 140°/10^{-2} mm giving a sticky solid, which, after exposure to moist air, crystallized from EtOAc-light petrol in prisms, m.p. 166°, of the hydrate of the diketone (**1**; RR′=0) (Found: M^+ 224. Calc. for $C_{10}H_8O_6$: M^+ 224) ν_{max} 3465, 3315, 3150 (br), 1740, 1615 cm^{-1}, λ_{max} 296, 348 nm ϵ 4610, 1650, identified by comparison of the IR with that of an authentic specimen, m.p. 166–168°.[8] The fraction R_f 0·54 had λ_{max} 265, 300 nm and crystallized from EtOAc-light petrol in rhombs, m.p. 154° decomp. of the ketone (**1**: RR′=H_2) (Found: M^+ 210. Calc. for $C_{10}H_{10}O_5$: M^+ 210) ν_{max} 3150 (br), 1645, 1630, 1595, 1505 cm^{-1}, λ_{max} 265, 300 nm, ϵ 10·500 6000 (lit.[16] m.p. 156° decomp.) identified by comparison of the IR with that of an authentic specimen recrystallized in the same way from the same solvent system. This compound was sometimes obtained in a different crystalline modification, m.p. 154° decomp., ν_{max} 3380, 3220, 1665, 1630, 1590, 1510 cm^{-1}. The fractions R_f 0·85 and 0·33 showed only end-absorption in the UV and were not examined further. The fraction R_f 0·20 had λ_{max} 257, 294 nm. It was sublimed at 100°/10^{-2} mm, but the amount of material obtained was too small for unequivocal identification. (b) *Strain* 46. After 34 days the yields of neutral and acidic extracts were 48 mg and 157 mg respectively from 1140 ml culture filtrate. The ketol (**1**; RR′=H, OH) (19 mg, 17 mg/l.) was obtained by EtOAc trituration of the acidic extract and a portion (42 mg) of the recovered residue was then subjected to preparative TLC giving material R_f 0·79, 0·67, 0·54, 0·35, 0·19 and 0·13. The fractions R_f 0·67, 0·54 and 0·35 were shown to contain the diketone (**1**; RR′=0), the ketone (**1**; RR′=H_2) and the ketol (**1**; RR′=H, OH) respectively on spectroscopic evidence (UV and IR). (c) *Strain* 43. After 23 days extraction of the culture filtrate (900 ml) from 4 flasks gave neutral (77 mg) and brown acidic (65 mg) fractions. Trituration of the latter with EtOAc afforded a buff powder (12 mg, 13 mg/l.), m.p. ~ 170° decomp., which was recrystallized twice from the same solvent giving prisms m.p. 195° decomp. of the ketol (**1**; RR′=H, OH) (Found: C, 53·6; H, 4·8%. M^+ 226. Calc. for $C_{10}H_{10}O_6$: C, 53·1; H, 4·5%, M^+ 226) $[\alpha]_D$ = 0 at 589, 546 and 365 nm. C = 0·21; ν_{max} 3400, 3300, 3120 (br), 1640, 1625, 1590 cm^{-1}; λ_{max} 268, 302 nm, ϵ 11·400, 6900 (lit.[9] m.p. 202–206° decomp.) identified by comparison of IR with that of an authentic sample. The residue showed material R_f 0·95, 0·75, 0·67, 0·42, 0·31 and 0·18 on TLC, but was not examined further. After 34 days the culture filtrate (400 ml) from 2 flasks afforded neutral (14 mg) and acidic (46 mg) fractions. The ketol (**1**; RR′=H, OH) (6 mg) was isolated from the latter and a portion of the residue (26 mg) was subjected to preparative TLC Material in bands R_f 0·94, 0·76, 0·67, 0·60, 0·42, 0·31 and 0·14 was recovered. The fractions R_f 0·67, 0·42 and 0·31 were shown to contain the diketone (**1**; RR′=0), the ketone (**1**; RR′=H_2) and the ketol (**1**; RR′=H, OH) respectively by sublimation *in vacuo* and/or crystallization from EtOAc-light petroleum as described above. The fractions R_f 0·94 and 0·76 had no specific UV absorption and were not examined further. The fraction R_f 0·14 had λ_{max} 257, 294 nm, but proved to be intractable. (d) *Strain* 44. After 23 days neutral (85 mg) and acidic (100 mg) extracts were obtained from the culture filtrate (800 ml). The acidic extract afforded the ketol (**1**; RR′=H, OH) (19 mg, mg/l.) After 34 days the yields of neutral and acidic extracts were 13 mg and 70 mg respectively from 340 culture filtrate. The ketol (**1**; RR′=H, OH) (12·5 mg, 35 mg/l.) was obtained from the acidic extract. TLC of the residue showed material at R_f 0·79, 0·67, 0·52, 0·32, 0·19 and 0·13. (e) *Strain* 52. Preparative TLC of the black oil (109 mg) from 1·21. culture filtrate from a fermentation harvested after 21 days gave bands at R_f 0·94, 0·82, 0·67, 0·57, 0·50, 0·41, 0·31 and 0·17. The material R_f 0·50 crystallized from EtOAc-light petroleum in prisms m.p. 152° decomp. (6 mg) of the ketone (**1**; RR′=H_2) identified by the IR. The ketol (**1**; RR′=H, OH) m.p. 202° decomp. (8 mg) was obtained from the band R_f 0·31. The band R_f 0·67 had the UV absorption of the diketone (**1**; RR′=0) but the amount present (< 1 mg) was too small for formal identification.

Shake culture: *Strain* 43. The crude semi-solid acidic extract (3·15 g) from 8·1 l. culture filtrate was crystallized from EtOAc giving the ketol (**1**; RR′=H, OH) (1·36 g, 168 mg/l.). A portion (52 mg) of the residue was subjected to preparative TLC and material R_f 0·77, 0·67, 0·50, 0·30 and 0·13 was collected. The diketone (**1**; RR′=0) and the ketol (**1**; RR′=H, OH) were obtained from the fractions R_f 0·67 and 0·30 respectively by crystallization from EtOAc. Although the fraction R_f 0·50 had the correct UV absorption (λ_{max} 265, 300 nm) no crystalline ketone (**1**; RR′=H_2) could be obtained from it, and only a trace could have been present.

Acknowledgements—We thank Dr. F. A. Mellon for the MS. Prof. Sir Derek Barton for providing specimens of the C_{10} acids prepared by the late Prof. H. Raistrick and Dr. J. N. Gibbs for helpful discussion.