8-HYDROXY-6-METHOXY-3-METHYLISOCOUMARIN AND OTHER METABOLITES OF CERATOCYSTIS FIMBRIATA[1]

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Abstract. The isolation of the title compound as a metabolite of the mold throws doubt on the role of the corresponding dihydro derivative as a carrot phytoalexin. The fungus also produces d-p-hydroxyphenyllactic acid and other compounds implicated in phenylpropanoid metabolism. The accumulation of large amounts of indole-3-acetic acid may be of significance in the pathogenicity of the mold.

INTRODUCTION

As part of a program on chemical aspects of host-pathogen interactions, we have investigated the metabolic products of Ceratocystis fimbriata Ell. & Halst. It was of particular interest to determine whether the mold produces compounds related to 8-hydroxy-6-methoxy-3-methyl-3,4-dihydroisocoumarin (I), a substance widely (1) but probably erroneously (2) regarded as an agent ("phytoalexin") produced by carrots as a defense against fungal infection. C. fimbriata was believed to be particularly effective in inducing carrots to synthesize the compound. When our study commenced, the only substances known to be produced by the mold itself were some simple volatile olefins, alcohols, esters and carbonyl compounds (3).

METHODS AND RESULTS

The mold (American Type Culture Collection No. 13323) was grown in still culture on Brian's Medium T (casein hydrolyzate, sucrose, and mineral salts (4) which had been modified by the addition of thiamine (0.1 mg/l) to facilitate fungal growth. The products, which were extracted from the culture filtrates

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by ethyl acetate, were chromatographed on a column of silica gel and further purified by preparative thin layer chromatography (t.l.c.), as summarized in the Table.

A phenolic substance, which could first be detected after about 6 days' growth, resembled (I) in chromatographic behaviour but differed from it in the quality of its fluorescence. It crystallized from ether-light petrol with m.p. 128-129°, and was characterized by M^+ 206, λ_{max} 323, 287, 277, 255 (inf), 242, and 238 nm (log ϵ 3.71, 3.62, 3.77, 3.99, 4.61, and 4.59), and $\nu_{max}(CCl_4)$ 3120, 2800-2400 (w), 1680, 1658, and 1624 cm^{-1} inter al. The nuclear magnetic resonance (n.m.r.) spectrum (in $CDCl_3$) was assigned as follows: δ 11.1 (1H, sharp, removed by D_2O, chelated phenol), 6.44 and 6.31 (1H each, pair of doublets, J 2.3 Hz, meta-coupled aromatic protons), 3.88 (3H, s, aromatic methoxyl), 6.18 and 2.26 (1H and 3H respectively, pair of doublets, J $\sim$ 1 Hz, vinylogously coupled olefinic proton and methyl group). These data indicated that the metabolite was 8-hydroxy-6-methoxy-3-methylisocoumarin (II). A direct comparison with an authentic specimen of (II) (5) confirmed this identity in all respects[2].

The isocoumarin was also isolated (Table) from still cultures of C. fimbriata on Raulin-Thom solution ((6), modified by the addition of thiamine as above) which is completely defined in chemical composition (glucose, tartaric acid, ammonia, and mineral salts). In supplementary tests, it was detected chromatographically in cultures of the fungus on a potato-dextrose medium, on malt extract, on unmodified Medium T and Raulin-Thom solution, and on Medium T which contained ammonium tartrate (10-200 mg/l) instead of the casein hydrolyzate.

In contrast to the isocoumarin (II) which is probably acetate-malonate derived, the other metabolites of the mold which were isolated in pure form,

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[2] Both the mold metabolite and the authentic isocoumarin are colourless. Prof. Hardegger has asked me to state that his earlier description of the compound as coloured arose from an error.

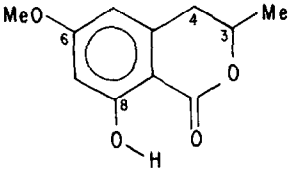
Table

Fractionation procedures and yields

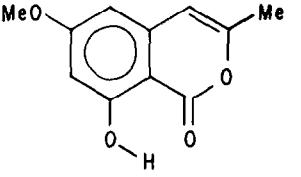
Fraction No.[a]	Eluant Composition[b]	Refractionation Method	Product	Yield[f] (mg) Medium T[h]	Medium T[i]	Raulin-Thom[j]
43-55	1:4	c	II	3.5	3.1	1.7
			VI	70	51	2
56-65	3:7	c	IV	4	19	0
72-84	1:1	d	III	269	310	k
			VII	39	44	
85-90	1:1	e	V	4	10	k
91-102	1:0		VIII	48	18	

[a]From a column of silica gel (British Drug Houses; 150 g), fraction volume 50 ml.
[b]Ether: light petrol.
[c]T.l.c. on SiO_2 (Camag DF-5) in $CHCl_3$/HOAc 100:0.2.
[d]Crystallization from ether; t.l.c. of mother liquors on SiO_2 in MeOH/$CHCl_3$HOAc 2:98:1
[e]Crystallization of fractions 91-96 from ether; t.l.c. of mother liquors and remaining fractions on SiO_2 in MeOH/$CHCl_3$/HOAc 5:95:1 and on microcrystalline cellulose (FMC Corp., American Viscose Division) in NH_4OH/iPrOH/H_2O 1:8:1.
[f]Yield of essentially homogeneous substance.
[h]50 x 120 ml, incubated 15 days; crude extract 1.53 g.
[i]As h, incubated 21 days; crude extract, 1.56 g.
[j]100 x 120 ml, incubated 14 days; crude extract, 425 mg.
[k]Not investigated.

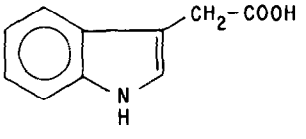
appear to be products of phenylpropanoid metabolism. They were readily characterized by n.m.r. spectroscopy and their identities were confirmed by direct comparison with authentic specimens by the usual criteria, except in the case noted. They were indole-3-acetic acid (III), phenylethanol (IV), p-hydroxyphenylethanol (tyrosol) (V), phenylacetic acid (VI), and p-hydroxyphenylacetic acid (VII). d-p-Hydroxyphenyllactic acid (VIII) was also isolated and identified by a comparison of its properties (m.p. 169-171°, $[\alpha]_D^{23^\circ}$ +18° (c, 1.0 in H_2O)) with those cited in the literature (7). Its infrared spectrum (Nujol) was almost identical with that of an authentic specimen of racemic p-hydroxyphenyllactic acid; the two specimens were indistinguishable chromatographically and in their ultraviolet and n.m.r. spectra.



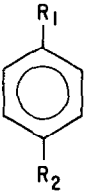
I



II



III



IV , R_1 = H , R_2 = $-CH_2-CH_2OH$
V , R_1 = OH , R_2 = $-CH_2-CH_2OH$
VI , R_1 = H , R_2 = $-CH_2-COOH$
VII , R_1 = OH , R_2 = $-CH_2-COOH$
VIII , R_1 = OH , R_2 = $-CH_2-CHOH-COOH$

DISCUSSION

Several different aspects require consideration. The isolation of the isocoumarin (II) from a chemically completely defined medium proves beyond doubt that it is a fungal metabolite. While the results of this study were being prepared for publication, the isolation of the substance was also reported by Curtis (8), who obtained it from cultures of C. fimbriata on corn-steep liquors. Curtis has briefly indicated the significance of the isolation but some amplification is in order. The isocoumarin (II) is so closely related to the dihydroisocoumarin (I) that it would be a strange coincidence if, as required by the phytoalexin concept, (I) were biosynthesized de novo by carrot tissue in response to fungal infection but not through the synthetic activity of the micro-organism. Conversely, it would be unexceptionable if contact with live carrot tissue induced the fungus to manufacture (I) instead of (II). The reported formation of very low amounts of (I) by apparently healthy carrot slices after treatment with certain chemicals (9) can probably be ascribed to low levels of fungal infection which may have eluded observation. Similarly, the apparent induction of (I) in carrots, by a few fungi other than C. fimbriata (9), represents a problem only if it can be shown that these fungi are incapable of synthesizing (I) or (II) or related compounds. One must conclude that a critical experimental re-examination is mandatory before the dihydroisocoumarin can be accepted as a carrot phytoalexin.

p-Hydroxyphenyllactic acid has importance as a postulated intermediate in the shikimic acid pathway of aromatic biosynthesis (10) but does not appear to have been recorded previously as a fungal product. The other phenylpropane-derived compounds which were obtained (III-VII) have been found as metabolites of other molds (10, 11). The compounds may accumulate together as products of C. fimbriata, because of a metabolic block for which evidence is also found in the growth habits of the mold. Growth on Raulin-Thom solution and on malt extract was poor at all times, while vigorous starts on Medium T and on potato-dextrose appeared to be aborted after about 2 weeks. The mold might thus prove a suitable subject for biogenetic studies.

Indoleacetic acid was isolated from the medium used for the main experiment in unusually high yield (ca 50 mg/l). It could also be identified, by chromatographic means, as a metabolite of the fungus in all of the other media which were examined but there was considerable variation in the amounts produced. Depending on the level of its production in vivo, the substance might account for some of the phytotoxic properties of the mold. It is noteworthy that C. fimbriata can give rise to cankers on trees (12), while indoleacetic acid has been discussed in relation to gall formation in other host-pathogen interactions (13). Further studies on this aspect appear to be warranted.

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