ACTA CHEMICA SCANDINAVICA 17 (1963) 1771–1776

Toluquinones from *Aspergillus fumigatus*

GÖSTA PETTERSSON

Institute of Biochemistry, University of Lund, Sweden

From the metabolism solution of a strain of *Aspergillus fumigatus*, known to produce fumigatin only, five other quinones were isolated by chromatography and structurally determined. One of these is the well-known fungal metabolite spinulosin, and the others were shown to be 3-hydroxy-2,5-toluquinone, 3,6-dihydroxy-2,5-toluquinone, 3,4-dihydroxy-2,5-toluquinone and 4-hydroxy-3-methoxy-2,5-toluquinone.

During investigations on the biosynthesis of fumigatin,[1] produced by a strain of *Aspergillus fumigatus* Fresenius,[2] this mould was found to form several other quinonoid pigments when grown as surface culture on a modified Raulin-Thom solution. Paper and thin-layer chromatographic methods adapted to the analysis of benzoquinones were elaborated,[3] and made it possible to isolate five distinct compounds, besides fumigatin, from the culture medium. In Table 1 are listed the R_F values and colours of these compounds (numbered I—V) when chromatographed in propanol-butanol-ammonium hydroxide, a solvent system generally used for the separation. Table 1 also gives the average amounts present in the medium after two weeks' cultivation.

The compounds show typical quinone behaviour. For example they react with carbonyl reagents, take up bromine in carbon tetrachloride solution and are easily reduced by aqueous sodium dithionite to the corresponding hydroquinones, which can be reoxidized to quinones with air in a pH 8.0 phosphate buffer solution. They do not react with *o*-phenylenediamine, excluding an *o*-quinonoid structure. Both the ultra-violet (Table 2) and infra-red absorption curves show benzoquinonoid characteristics [4,5]. The presence of at least one hydroxyl group in all five compounds is strongly indicated by the infra-red absorption bands in the region of 3 μ.

The colour reactions in aqueous solution, as listed in Table 3, also are in consistence with a hydroxyquinonoid structure. Acid solutions of the compounds are brownish yellow, but on neutralization the colour changes to intense purple or red, due to the dissociation of one hydroxyl group (confirmed by titration). The presence of two dissociable hydroxyl groups in compound II and III is indicated by the rapid and reversible fading of the purple colour when the pH is further raised, and confirmatively both compounds titrated as dibasic acids.

Table 1. Yield and chromatographic behaviour of the isolated pigments. Solvent system: Butanol-propanol-2M ammonium hydroxide (6:1:3 by vol.).

Compound	mg/liter produced in two weeks	Paper chromatog.		Thin-layer chromatog.	
		R_F	colour	R_F	colour
I	2	decomp.		0.52	orange
II	1	0.19	red-violet	0.10	red-violet
III	1	0.25	blue-violet	0.00	blue-violet
IV	3	0.30	violet	0.30	violet
V	5	0.65	violet	0.60	violet
fumigatin	15	0.45	violet	0.49	violet

Table 2. Wave-length in mμ of UV-maxima. Solvent: Chloroform.

Compound	First maximum	Second maximum
I	270	380
II	282	420
III	294	460
IV	283	425
V	279	385
Fumigatin	285	450

Table 3. Colour reactions in aqueous solution.

pH	< 3	4—7	> 8
Compound			
I	yellow	red	irreversible fading
II	»	red-violet	reversible fading
III	»	blue-violet	» »
IV	»	violet	violet
V	»	»	irreversible fading
Fumigatin	»	»	violet

Table 4. Incorporation of radioactivity from different ^{14}C-labelled substrates added to the culture.

Compound	Substrate added		
	acetate	L-methionine	orsellinic acid
I	+	—	+
II	+	—	+
III	+	+	+
IV	+	+	+
V	+	—	+
Fumigatin	+	+	+

In the investigation of the biosynthesis of fumigatin [1] *Aspergillus fumigatus* was cultivated on solutions containing 1-^{14}C-acetate, $^{14}CH_3$-L-methionine and biologically ^{14}C-labelled orsellinic acid, which all were shown to be incorporated into fumigatin. These experiments were now repeated, the ethereal extract of the culture medium chromatographed, and the incorporation of radioactivity in the different compounds determined. The results, shown in Table 4, give valuable information on the structures. Both acetate and orsellinic acid are

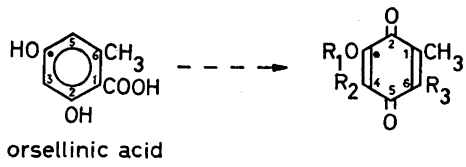

Fig. 1. Probable structural unit of the quinones produced by *Aspergillus fumigatus*.

incorporated in all the quinones, the specific activity of compound I—V being of the same order as that of fumigatin. The acetate and orsellinic acid origin of fumigatin has been proved,[1] and it is thus highly probable that all the quinones are acetate-derived with orsellinic acid as a common intermediate. In the case of fumigatin it was also proved that the hydroxyl group in position 4 of orsellinic acid appears in position 3 of fumigatin. Presumably compound I—V are formed in a similar manner, without any reduction of the hydroxyl group in position 4 of orsellinic acid, and a common feature should then be a 2,5-toluquinonoid structure with a hydroxyl (or alkoxyl) group in position 3, as shown in Fig. 1.

O- and C-methylation from the C_1-pool are well recognized processes in fungal metabolism. Confining the examples to benzoquinones O-methylation has been shown to occur in fumigatin,[1] aurantiogliocladin [6] and 4-methoxy-2,5-toluquinone,[7] C-methylation in aurantiogliocladin [6]. The incorporation of radioactivity from $^{14}CH_3$-L-methionine into compound III and IV must be interpreted as a methylation from the C_1-pool. To decide whether O-methylation is a possible alternative the compounds were submitted to a qualitative and quantitative Zeisel determination of methoxyl groups. The results, given in Table 5, are calculated on a molecular weight of 170, approximately correct for all the compounds according to cryoscopic determinations.

The quinones are fairly unstable, and for example decompose to some extent when treated with dilute alkali (the chromatographic separations had to be performed in alkaline solvent systems) and when heated in solid form (sublimation) or in solutions (recrystallization), making it impossible to get crystalline substances to work with. Furthermore only small amounts were at disposal, as the yield from the mould is poor and at least 50 % lost in the isolation procedures. Consequently, the quantitative Zeisel determination

Table 5. Zeisel analysis of methoxyl groups.

Compound	Qualitative	Quantitative Number of methoxyl groups *
I	—	
II	—	
III	+	0.8
IV	+	0.9
V	—	0.0
Fumigatin	+	1.0

* calculated on a molecular weight of 170.

performed could only be a rough one, but nevertheless it is obvious from Table 5 that compound III and IV contain one methoxyl group each.

As the quinones could not be obtained in a sufficient amount and purity, elementary analysis did not give quantitative information precise enough to decide the structures, but qualitatively C, H, and O were shown to be the only elements present, in proportions corresponding to the approximative molecular formula $C_{7-9}H_{5-10}O_{3-5}$, covering all the compounds. The molecular weight determinations performed exclude dibenzoquinonoid structures like phoenicin [8,9] and oosporein [10,11].

For the final identification of the compounds, which will now be described, chromatographic techniques were extensively used. When a compound is claimed to be identical with a known test substance, this means that both give the same R_F values and colours in several different solvent systems, both on paper and thin-layer chromatograms, and that no discrepancies in general behaviour and properties (*e.g.* colour reactions, UV- and IR-spectra) are observed.

Compound I. Being very unstable in alkaline solution, compound I could only be isolated from thin-layer chromatograms, where it appeared with orange coloured spots. The orange colour indicates that the quinonoid nucleus is not as heavily substituted as in for instance fumigatin, which is also clear from the ultra-violet spectrum, where maximum absorption occurs at shorter wavelengths than observed for fumigatin and the other compounds (Table 2). Chromatographically compound I showed identical with 3-hydroxy-2,5-toluquinone ($R_1 = R_2 = R_3 = H$ in Fig. 1). Treatment with diazomethane in ethereal solution gave a product chromatographically identified as 3-methoxy-2,5-toluquinone, and reduction of compound I with aqueous sodium dithionite yielded a hydroquinone identical with 2,3,5-trihydroxytoluene.

Compound II. The extinction in aqueous solution at 525 mμ was measured as a function of pH (Fig. 2) and showed a maximum at pH 4.7. This type of extinction/pH curves are given by 2,5-dihydroxy-1,4-benzoquinones, and compound II showed identical with 3,6-dihydroxy-2,5-toluquinone ($R_1 = R_2 = H$; $R_3 = OH$). After methylation 3,6-dimethoxy-2,5-toluquinone could be identified, and reduction gave 2,3,5,6-tetrahydroxytoluene.

Compound III. The bluish colour in alkaline solutions, and the position of the UV-maxima (Table 2), indicate a heavily substituted quinonoid struc-

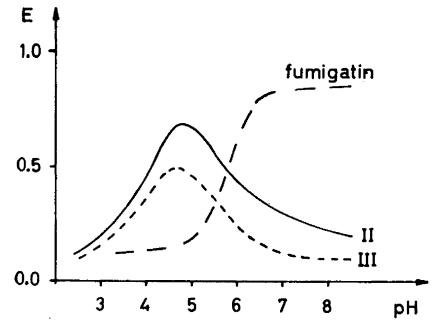

Fig. 2. Extinction at 525 mμ as a function of pH for aqueous solutions of fumigatin, compound II and compound III.

ture. One of the substituents is a methoxyl group (Table 5), and evidence for two hydroxyl groups *para* to each other is given by the extinction/pH curve (Fig. 2), which is similar to that obtained for compound II. Considering the structural unit in Fig. 1, the identity of compound III and spinulosin (R_1= H; R_2 = OCH_3; R_3 = OH) is probable, and was proved by chromatography. Confirmatively, methylation of compound III yielded 3,4,6-trimethoxy-2,5-toluquinone.

Spinulosin was the first mould product to be recognized as a quinone, obtained from strains of *Penicillium spinulosum* Thom [12]. It has also been isolated from an unidentified *Penicillium* [13] (possibly *P. spinulosum*), *P. cinerascens* Biourge,[14] and a strain of *Aspergillus fumigatus*,[15] different from the strain used in this investigation.

Compound IV and V: These two compounds and fumigatin are closely related. On methylation they all give the same derivative, identical with 3,4-dimethoxy-2,5-toluquinone. Demethylation of compound IV and fumigatin (see Table 5) with hydrogen iodide yielded the same polyphenol (2,3,4,5-tetrahydroxytoluene), which was also obtained by reduction of compound V. Thus compound V must be 3,4-dihydroxy-2,5-toluquinone (R_1 = R_3 = H; R_2 = OH) and compound IV 3-methoxy-4-hydroxy-2,5-toluquinone (R_1 = CH_3; R_2 = OH; R_3 = H), which was also confirmed chromatographically.

EXPERIMENTAL

Isolation and purification. Aspergillus fumigatus Fresenius, L.S.H.T.M. Cat. No. A 46, was cultivated on a modified Raulin-Thom solution as described by Anslow and Raistrick [2]. After 2–3 weeks the metabolic liquid was extracted with ether, and the crude extract chromatographed on Whatman No. 1 paper after evaporation of most of the ether. The chromatographic zones were cut out of the paper, moistened with dilute hydrochloric acid, and the different pigments eluted with acetone and rechromatographed. This procedure was repeated until the quinones showed chromatographically pure (2–3 times), when they were dried in the desiccator over concentrated sulfuric acid. For further purification the quinones were dissolved in a minimal volume of anhydrous ether and slowly poured into a large volume of boiling petroleum ether (b.p. 40°–60°). The amorphous, dark-brown precipitate obtained was filtered off and dried in the desiccator.

3-Hydroxy-2,5-toluquinone, which decomposed on paper chromatograms, was isolated in a similar way from thin-layer plates. In both cases propanol-butanol-2 M ammonium hydroxide (6:1:3 by vol.) was used to develop the chromatograms.

Chromatography. R_F values and colours of 52 benzoquinones in six different solvent systems, and the general techniques in chromatographic analysis of benzoquinones has been described elsewhere [3]. The same methods were used in this investigation for identification and quantitative estimation of the mould products and their methylated derivatives.

The polyphenols obtained by reduction or demethylation of the pigments were generally chromatographed in an atmosphere of nitrogen, using chloroform-methanol-acetic acid (10:3:1 by vol.) as solvent. The spots were detected with alcoholic ferric chloride, or by exposing the chromatograms to ammonia vapour in air, by which treatment the hydroquinones were oxidized to the corresponding, strongly coloured, quinones.

Reduction. The quinones were shaken with excess sodium dithionite in water for a few moments, when an almost colourless solution was obtained. This was extracted twice with an equal volume of ether, and the ether removed, leaving a syrupy residue, which was immediately chromatographed for identification of the hydroquinones formed.

Demethylation. The compound to be demethylated was dissolved in hydrogen iodide (d = 1.7), a little red phosphorus added, and the solution boiled for 5 min. After neutralization with solid sodium carbonate the polyphenol formed could be extracted with

ethyl acetate and submitted to immediate chromatographic analysis, or first oxidized to the corresponding quinone. The yields were fairly poor (10–30 %).

Oxidation. The hydroquinones were dissolved in a pH 8.0 phosphate buffer solution (1 M potassium dihydrogen phosphate, 50 ml; 1 M sodium hydroxide, 46.8 ml; water to 100 ml) and aerated for 2 min. The solution, which quickly became intense purple, was acidified with concentrated hydrochloric acid and the quinones extracted with ether.

Methylation. The quinones, dissolved in ether, were treated with ethereal diazomethane. There was immediate and vigorous evolution of nitrogen, and the brownish red solutions lightened in colour to orange or yellow. Great care was taken to avoid excess diazomethane to be added, as specially compound IV and V then gave a poor yield of the methylated derivatives, probably due to the simultaneous formation of pyrazole complexes, as described for fumigatin [2].

Light absorption measurements. The ultra-violet absorption spectra of the quinones were measured in a Perkin-Elmer Model 137 UV Spectrophotometer, with chloroform as solvent, and the infra-red spectra in a Perkin-Elmer Infracord Spectrophotometer, the quinones being dissolved in carbon tetrachloride, ethylene chloride, or anhydrous ether.

The extinction/pH curves were determined in a Beckman DU Spectrophotometer. The pH was regulated by the use of different standard buffers and measured electrometrically.

Radioactive assay. After chromatographic separation on paper, a Baird-Atomic Paper Chromatogram Scanner was used to determine the radioactivity of the different compounds. These could also be eluted from thin-layer chromatograms, dissolved in a toluene solution of 2,5-diphenyloxazol, and the radioactivity measured in a Baird-Atomic Liquid Scintillation Counter.

REFERENCES

1. Pettersson, G. *Acta Chem. Scand.* **17** (1963) 1323.
2. Anslow, W. K. and Raistrick, H. *Biochem. J.* **32** (1938) 687.
3. Pettersson, G. *J. Chromatog. In press.*
4. Flaig, W., Salfeld, J. C. and Baume, E. *Ann.* **618** (1958) 117.
5. Flaig, W. and Salfeld, J. C. *Ann.* **626** (1959) 215.
6. Birch, A. J., Fryer, R. I. and Smith, H. *Proc. Chem. Soc.* **1958** 343.
7. Birch, A. J. *CIBA Foundation Symposium on Quinones in Electron Transport*, London 1961, p. 233.
8. Friedheim, E. A. H. *Helv. Chim. Acta* **21** (1938) 1464.
9. Chatin, T., Fitzgerald, G. and Reilly, J. *Biochem. J.* **34** (1940) 1605.
10. Kögl, F. and van Wessem, G. C. *Rec. Trav. Chim.* **63** (1944) 5.
11. Smith, J. and Thomson, R. H. *Tetrahedron* **10** (1960) 148.
12. Birkinshaw, J. H. and Raistrick, H. *Phil. Trans. Roy. Soc. London* **B220** (1931) 245.
13. Hoshishima, K. *Tohoku J. Exptl. Med.* **52** (1950) 273.
14. Bracken, A. and Raistrick, H. *Biochem. J.* **41** (1947) 569.
15. Anslow, W. K. and Raistrick, H. *Biochem. J.* **32** (1938) 2288.

Received April 3, 1963.