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THE ISOLATION AND STRUCTURE OF 4-HYDROXYOCHRATOXIN A AND 7-CARBOXY-3,4-DIHYDRO-8-HYDROXY-3-METHYLISOCOUMARIN FROM PENICILLIUM VIRIDICATUM

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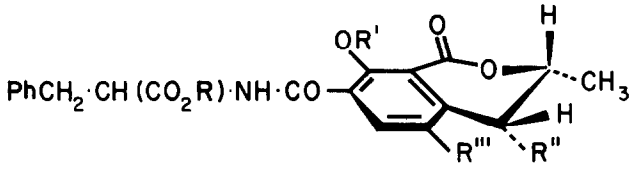
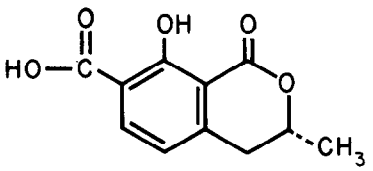
Ochratoxin A (1a), a hepato and nephrotoxin is produced by Aspergillus ochraceus[1] as well as by two other members of the A. ochraceus group, viz. Aspergillus melleus and Aspergillus sulphureus[2]. A report by van Walbeek[3] on Penicillium viridicatum Westling (ATCC 18411) as a new source of ochratoxin A therefore initiated our interest in cultures of this fungus.

P. viridicatum Westling (ATCC 18411) was cultivated on the YES medium under conditions similar to those described by van Walbeek[3]. Nine days after inoculation, the culture filtrate from 5 l. of medium was drained off the mycelial mat, acidified to pH 1.0 with 1N HCl and extracted with $CHCl_3$. The mycelium was separately extracted with $CHCl_3$/MeOH and this extract was combined with the above mentioned $CHCl_3$ layer. The organic acids (2.2 g) were obtained in the standard way and resolved by preparative SiO_2 TLC in C_6H_6:HOAc (4:1 v/v). Four fluorescent bands were isolated, viz. ochratoxin A (1a) (R_f 0.69, green, 750 mg), ochratoxin B (1b) (R_f 0.45, blue, 70 mg), 4-hydroxyochratoxin A (1c) (R_f 0.25, green, 60 mg) and 7-carboxy-3,4-dihydro-8-hydroxy-3-methylisocoumarin (II) (R_f 0.15, purple, 40 mg). Ochratoxin A and B were identified by a direct comparison with authentic samples. The identity of the compound at R_f 0.15, to 7-carboxy-3, 4-dihydro-8-hydroxy-3-methylisocoumarin (II) obtained by acid hydrolysis of ochratoxin B[1], was shown by mp, IR, UV, NMR and mass spectroscopy.

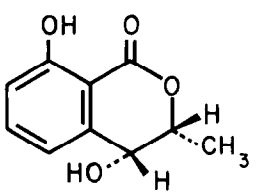
4-Hydroxyochratoxin A (1c), $C_{20}H_{18}ClNO_7$ is a colourless crystalline compound, mp 216-218° (benzene). The IR spectrum of 1c, revealed the presence of a carboxyl group ($\nu_{max}^{CHCl_3}$ 1723 cm^{-1} and a broad band between 2500 and 3000 cm^{-1}), a secondary amide group (ν_{max} 1655, 1535 and 3380 cm^{-1}) and a lactone group (ν_{max} 1678 cm^{-1}), and its UV spectrum showed absorption at λ_{max}^{EtOH} 213 and



I a R = R' = R'' = H, R''' = Cl
I b R = R' = R'' = R''' = H
I c R = R' = H, R'' = OH, R''' = Cl
I d R = R' = Me, R'' = OH, R''' = Cl
I e R = R' = H, R'' = OAc, R''' = Cl



II



III

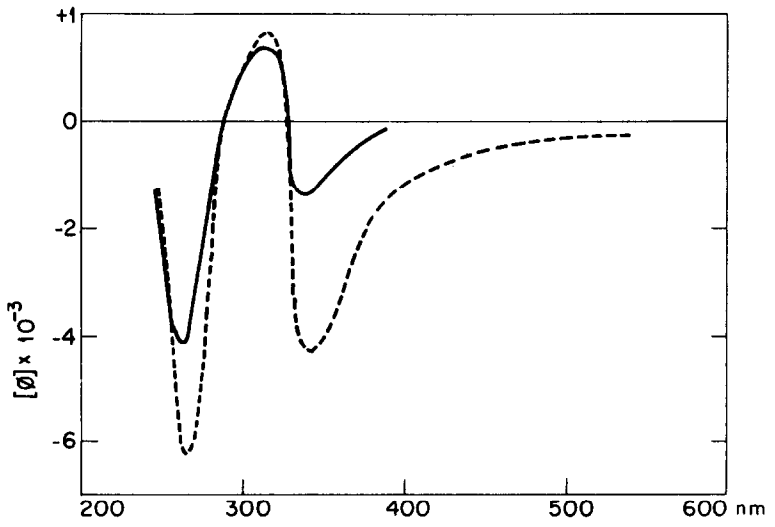


FIGURE 1

ORD curves of ochratoxin A (-----) and 4-hydroxy-ochratoxin A (——) in methanol

334 nm (ε, 32,500 and 6400, respectively). Ochratoxin A and 1c therefore contain the same chromophore, furthermore 1c gave the typical red colour with ethanolic $FeCl_3$.

The NMR spectrum of 1c in C_5D_5N had the following characteristic features. Peaks attributable to the phenylalanine moiety were similar to those exhibited by ochratoxin A. A one-proton singlet at τ 1.3 was assigned to the aromatic proton at C_6. The remaining dihydroisocoumarin protons appeared as an AMX_3 system. The secondary methyl group at C_3 resonated as a doublet at τ 8.32 (J 7 Hz), the methine proton at C_3 appeared as a well defined quartet of doublets at τ 5.2 (J 2, 7 Hz) while the proton on the hydroxyl group bearing carbon atom resonated as a doublet at τ 4.89 (J 2 Hz). A coupling constant of 2 Hz is consistent with a dihedral angle of approximately 90° for the protons at C_3 and C_4, therefore these protons are cis-oriented[4]. Similar values were reported for cis-4-hydroxymellein (III)[5] in which the proton at C_4 appeared as a doublet at τ 5.53 ($J_{3,4}$ 2 Hz), while trans-4-hydroxymellein had $J_{3,4}$ 4 Hz.

Ochratoxin A and 4-hydroxyochratoxin A exhibited similar ORD curves, (see Fig. 1). The 3R configuration for ochratoxin A was established by degradative studies, while the conformation of the dihydroisocoumarin part was based on NMR data[1]. Arakawa[6] showed that dihydroisocoumarins containing the 3R configuration exhibited a negative Cotton effect near 260 nm. The negative Cotton effects of 1a and 1c near 260 nm prove the 3R configuration of these compounds. The stereochemistry of 1c can thus be represented as shown in the formula.

Treatment of 1c with ethereal diazomethane yielded O-methylhydroxyochratoxin A methyl ester (1d). It had M^+ 447.106, $C_{22}H_{22}ClNO_7$ requires M, 447.108; $\nu_{max}^{CHCl_3}$ 1740 (ester CO), 1730 (lactone CO), and 1660 cm^{-1} (amide CO); and λ_{max}^{EtOH} 217 and 308 nm (ε, 25,400 and 2400, respectively). Acetylation of 1c with acetic anhydride and perchloric acid at -80° gave 4-acetoxylochratoxin A (1e). Ochratoxin A showed no reaction under these conditions. 4-Acetoxylochratoxin A had M^+ 461.091, $C_{22}H_{20}ClNO_8$ requires M, 461.088; and $\nu_{max}^{CHCl_3}$ 1740 (acetate CO), 1720 (carboxyl CO), 1670 (lactone CO) and 1640 cm^{-1} (amide CO).

A comparison of the mass spectra of ochratoxin A (1a), 4-hydroxyochratoxin A (1c), O-methyl-4-hydroxyochratoxin A methyl ester (1d) and 4-acetoxylochratoxin A (1e) unambiguously confirmed that the additional oxygen function in (1c) was located on the dihydroisocoumarin moiety (see Table 1).

4-Hydroxyochratoxin A is probably derived from ochratoxin A rather than vice versa as the additional hydroxyl group present in this metabolite is attached to an acetate-methyl carbon[7].

Little is known about the sequence leading to the construction of ochratoxin A, thus the isolation of the isocoumarin acid of ochratoxin B only, is of interest, as it is still not clear at what stage chlorination occurs.

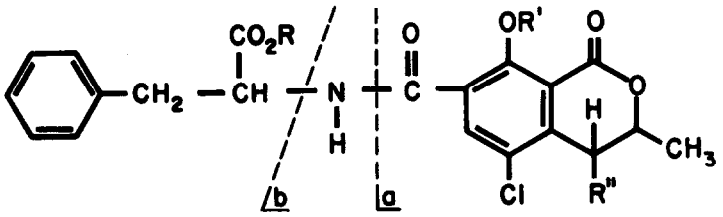


TABLE 1

	Compound	M^+	Fragment a	Fragment b + H
1a	R = H, R' = H, R" = H	403	239	255
1c	R = H, R' = H, R" = OH	419	255	271
1d	R = Me, R' = Me, R" = OH	447	269	285
1e	R = H, R' = H, R" = OAc	461	297	313

Intraperitoneal injection of male Wistar rats with ochratoxin A caused excretion in the urine of ochratoxin A, the isocoumarin acid of ochratoxin A[8] and a green fluorescent compound. The latter compound had an R_f value identical to 4-hydroxyochratoxin A in five different SiO_2 TLC systems. Ochratoxin A caused the mortality of all the male Wistar rats after 6 days at a dose of 40 mg/Kg. 4-Hydroxyochratoxin A had no effect at this level.

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