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THE BIOSYNTHESIS OF THE OCHRATOXINS, METABOLITES OF *ASPERGILLUS OCHRACEUS*

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Abstract—The biosynthesis of ochratoxin A (Ia) was studied in *Aspergillus ochraceus* Wilh. by experiments in which DL-phenylalanine [U-^{14}C], sodium acetate [1-^{14}C] and methionine [$^{14}CH_3$] were supplied to a resting culture of the fungus. The carboxyl carbon of the amide group is shown to be derived from the C_1-pool.

INTRODUCTION

THE FUNGUS *Aspergillus ochraceus* Wilh. elaborates a group of heterocyclic metabolites[1,2] including ochratoxin A (Ia) and ochratoxin B (Ib). Lai *et al.*[3] reported recently on two new fungal sources of ochratoxin A, viz. *A. sulphureus* and *A. melleus*, two members of the *A. ochraceus* group. Ochratoxin A was found to be produced on solid media,[3-7] semisynthetic media[5,8] and synthetic media.[3,9,10]

Ochratoxins comprise a 8,9-dihydro-9R-methylisocoumarin moiety which is linked to L-β-phenylalanine through a carboxyl group. The lactone acids IIa and IIb obtained on hydrolysis of ochratoxins A and B, respectively, are structurally and probably biogenetically closely related to mellein (III), a metabolite of both *A. ochraceus*[11] and *A. melleus*.[12]

It is probable that the phenylalanine portion of the ochratoxins is synthesized via the shikimic acid pathway. The possibility that this pathway may be involved also in the synthesis of the isocoumarin moiety as in coumarin synthesis in higher plants was considered unlikely by analogy with the results on the biosynthesis of structurally related fungal metabolites. Thus, according to Stickings,[13] the hydroxy-derivative (IVb) of 6-acetonyl-2,4-dihydroxybenzoic acid (IVa) arises by head-to-tail condensation of one acetate and four malonate units.

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[2] P. S. STEYN and C. W. HOLZAPFEL, *J. S. African Chem. Inst.* **20**, 186 (1967).
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[4] K. J. VAN DER MERWE, P. S. STEYN, L. FOURIE, DE B. SCOTT and J. J. THERON, *Nature* **205**, 1112 (1965).
[5] W. VAN WALBEEK, P. M. SCOTT and F. S. THATCHER, *Can. J. Microbiol.* **14**, 131 (1968).
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[8] N. D. DAVIS, J. W. SEARCY and U. L. DIENER, *Appl. Microbiol.* **17**, 742 (1969).
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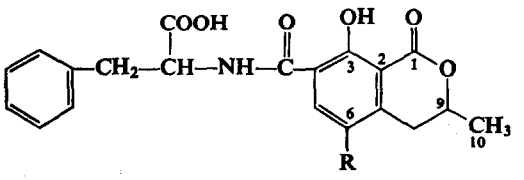
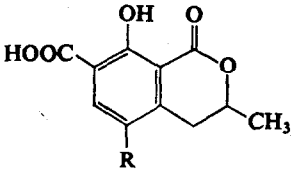


(Ia) R = Cl
(Ib) R = H



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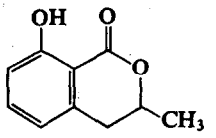
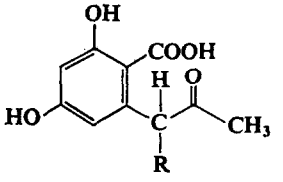
(IIb) R = H

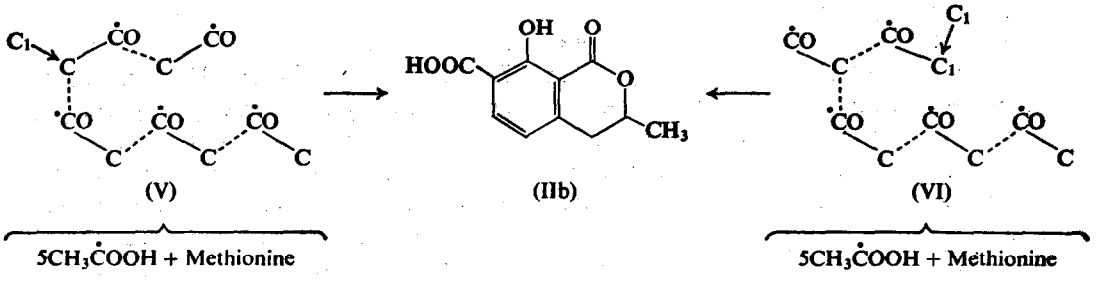


(III)



(IVa) R = H
(IVb) R = OH

Oosponol and oospolactone in *Oospora astringenes* also arise essentially from acetate units.[14] It was, therefore, considered likely that the biosynthesis of the lactone acids IIa and IIb involve head-to-tail condensation of five acetate (or one acetate and four malonate) units, with the introduction of a C_1-unit at position 4, as shown in V. This pathway would be analogous to the biosynthesis of citrinine in *A. candidus*[15] and oospolactone in *O. astringenes*.[14] A plausible alternative would, however, involve the introduction of a C_1-unit in a branched acetic acid derived chain as depicted in VI. A biogenetic scheme similar to VI which involved the condensation of two distinct polyketide chains was recently reported for the biosynthesis of sclerin by Kubota *et al.*[16]



(V) (IIb) (VI)

$5CH_3\dot{C}OOH$ + Methionine $5CH_3\dot{C}OOH$ + Methionine

In an attempt to distinguish between these possibilities, the biosynthesis of ochratoxin A was studied with the aid of radioactive precursors. Some progress had been made with this investigation when a paper by Searcy *et al.*[17] appeared on the biosynthesis of ochratoxin A. This prompts us to report our results on the biosynthesis of ochratoxin A and to compare this with the results obtained by Searcy *et al.*[17]

RESULTS AND DISCUSSION

A resting culture of *Aspergillus ochraceus* Wilh. was cultured for 24 hr on a resuspension medium containing universally labelled ^{14}C-DL-phenylalanine. Acid hydrolysis of the

[14] K. NITTA, Y. YAMAMOTO, T. INOUE and T. HYODO, *Chem. Pharm. Bull. Tokyo* **14**, 363 (1966).
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resulting labelled ochratoxin A yielded inactive IIa and labelled phenylalanine. Searcy *et al.*[17] showed that [1-^{14}C] phenylalanine was incorporated unaltered into the phenylalanine moiety of ochratoxin A. However, these workers found that the isocoumarin moiety of ochratoxin A contained approximately 1 per cent of the radioactivity of the added [1-^{14}C] phenylalanine. This was presumably due to indirect incorporation resulting from the long incubation (8 days of the growing fungus on medium containing the labelled substrate). Ferreira and Pitout[18] recently described the preparation of a crude enzyme fraction from *A. ochraceus* capable of effecting coupling of L-β-phenylalanine and IIa to give ochratoxin A.

Incubation of *A. ochraceus* in a replacement medium containing [1-^{14}C] sodium acetate yielded labelled ochratoxin A. A short incubation time (24 hr) in the presence of labelled substrate was chosen in order to minimize randomization. This, together with the fact that the toxin is produced exclusively during the log phase of growth, may account for the low extent (0·13%) of incorporation of the labelled substrate. The neutral fraction of the total extract accounted for 50% of the added activity while a further 20% of the added activity was located in the mycelium from which it could not be removed by extraction by organic solvents. The CO_2 formed during the incubation period accounted for 12% of the added activity.

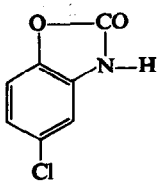
The labelled ochratoxin A was hydrolysed with hydrochloric acid. The isocoumarin moiety IIa accounted for all the activity of the original [1-^{14}C] acetate-labelled ochratoxin A. Searcy *et al.*[17] achieved a substantially better incorporation from [2-^{14}C] sodium acetate but in this case an actively growing culture was incubated for 8 days in a medium containing the labelled substrate. They report that the phenylalanine moiety contained at least 16·5% of the activity of the label. The long incubation time employed by Searcy *et al.*[17] may also account for the significant extent of randomization of the label from [2-^{14}C] sodium acetate. These workers degraded [2-^{14}C] sodium acetate labelled IIa and concluded that most of the incorporated radioactivity was located in carbons* 2, 4 and 6 with little or no activity in carbons 1, 3, 5, 9, 10 or 11. However, total activities of fragments were compared without regard to (weight) yield and radiopurity. It is therefore difficult to draw definite conclusions from these results regarding the pattern of labelling. In particular, the low total activity of the acetate obtained on melting IIa with potassium hydroxide was taken as evidence that carbons 9 and 10 were not derived from acetic acid. In the absence of evidence to the contrary, this low total activity may well be due to a low yield of acetate. In the experiments described herein, carbons 9 and 10 were obtained as acetic acid by Kuhn–Roth degradation of IIa. The corresponding *p*-bromophenacylacetate was crystallized to constant radioactivity. The isocoumarin IIa, biogenetically labelled with [1-^{14}C] sodium acetate, yielded *p*-bromophenacylacetate which had a specific molar activity one-fifth of that of the starting material. Schmidt decarboxylation of the acetic acid showed that all its activity was contained in the carboxyl group. This result is consistent with the theory that ten carbons of the isocoumarin moiety arises by condensation of five acetate units. However, confirmation of the correctness of this deduction requires accurate determination of the activity of the other carbon atoms of the isocoumarin acid IIa labelled from [1-^{14}C] and [2-^{14}C] sodium acetate.

Decarboxylation of the isocoumarin acid (IIa) gave carbon dioxide in poor yield. As the Schmidt reaction is known to involve an acid azide intermediate, this reaction was investigated with the azide of 5-chlorosalicylic acid. Heating of this azide in benzene resulted in the formation of a benzoxazolidone derivative (VII) in high yield. The rearrangement was

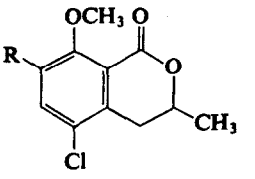
* The numbering of the isocoumarin nucleus introduced by Searcy *et al.*[17] is retained in order to facilitate direct comparison of results.

[18] N. P. Ferreira and M. J. Pitout, *J. S. African Chem. Institute*, **22**, S1 (1969)

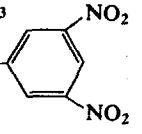
presumably due to attack by the *ortho*-phenolic hydroxyl group on the isocyanate grouping formed on decomposition of the acid azide. Aliphatic 2-oxazolidones were prepared[19] in an analogous manner from β-hydroxyisocyanates. It was, therefore, clear that the phenolic hydroxyl-group of IIa would have to be protected in order to carry out a Schmidt decarboxylation.



(VII)



(VIIIa) R = $COOCH_3$
(VIIIb) R = COOH
(VIIIc) R = CON_3
(VIIId) R = NH_2
(VIIIe) R = NH—



Treatment of IIa with diazomethane gave VIIIa which, on alkaline hydrolysis, yielded VIIIb. Treatment of the corresponding acid azide with sulphuric acid gave the amine (VIIId) and carbon dioxide in good yield. Starting from the [1-^{14}C] acetate-labelled isocoumarin acid (IIa), the carbon dioxide obtained in this way was inactive while the amine (VIIId), isolated as its 3,5-dinitrobenzamide-derivative, had the same specific molar activity as the starting material. This result shows clearly that the carboxyl group at position 4 is not derived from an acetate carboxyl group. It follows that the isocoumarin acid IIa is not constructed according to the biogenetic scheme VI.

In order to establish whether the carboxyl group at position 4 was derived from the C_1-pool, a resting culture of *A. ochraceus* Wilh. was incubated in a medium containing ($^{14}CH_3$)-methionine. The resulting radioactive ochratoxin A was hydrolysed and the isocoumarin acid (IIa) converted into VIIIb. Schmidt decarboxylation of this acid yielded active carbon dioxide (counted as barium carbonate) and the amine (VIIId) which contained no radioactivity. This result provides direct evidence that the carboxyl group at position 4 is derived from methionine.

The simplest interpretation of the above results is that the isocoumarin acid IIa is derived from five acetate units by head-to-tail condensation with the introduction from the C_1-pool of a methyl group which is subsequently oxidized. Searcy *et al.*[17] however, interpreted their results as indicating that only carbons 2, 3, 4, 5, 6 and 7 are derived directly from acetate. It is clear that further radiolabelling experiments are required to distinguish between these two schemes which have now been suggested for the biosynthesis of the isocoumarin acid (IIa).

EXPERIMENTAL

U.v. absorption refers to EtOH (Unicam Model S.P. 800 Spectrometer) and i.r. absorption to KBr discs (Perkin-Elmer Model 237 Spectrometer). Mass spectra were taken on a MS-9 double-focusing mass spectrometer. Radioactivity was assayed on a Packard Tri-Carb Liquid Scintillation Spectrometer Model 574, organic compounds were analysed in toluene as scintillation solvent containing PPO and DM-POPOP as scintillation solute.* ^{14}C-$BaCO_3$ was assayed by suspension scintillation counting[20] in the same scintillator

* Packard Scintillation Grade.

[19] N. E. DYEN and D. SWERN, *Chem. Rev.* 197 (1967).
[20] H. J. CLULEY, *Analyst* **87**, 170 (1962).

mixture which contained *ca.* 4 per cent (w/v) of Cab-o-sil as gelling agent. All samples were counted for a minimum of 10^4 counts.

For preparative TLC, 2·0 mm silica gel G was used. The compounds were detected on the chromatoplates by exposure to long wave-length u.v. illumination (366 nm) or by spraying with a 1% solution of $Ce(SO_4)_2$ in 6 N H_2SO_4.

The Preparation of Labelled Ochratoxin A

Aspergillus ochraceus Wilh. strain K804 from the culture collection of the Microbiological Research Group, Council for Scientific and Industrial Research, Pretoria, was used in the investigation.

Precursor substrates were added in H_2O to resting cultures of the fungus. The resting cultures were prepared by cultivating the fungus for 84 hr in 500-ml conical flasks with 100 ml of the complete medium described by Ferreira.[10] The mycelium was washed aseptically and resuspended in a replacement medium devoid of a N source. The resuspended mycelium was incubated in 100-ml aliquots of the replacement medium on a rotary shaker (180 rev/min) at 25° for a total period of 48 hr. Precursor substances (e.g. for acetate labelling [1-^{14}C]-acetate 500 μc was added to 1 l. of the resuspension medium) were added to the resting cultures when ochratoxin A synthesis commenced, usually 18–24 hr subsequent to resuspension. The culture was filtered, the filtrate acidified and extracted with $CHCl_3$. The mycelium was continuously extracted with $CHCl_3$ and combined with the previous $CHCl_3$ extract. The organic layer was extracted with $NaHCO_3$ aq. (3 × 200 ml). The aqueous phase was acidified with 4 N HCl and extracted with $CHCl_3$ (4 × 200 ml). The combined organic layers were washed with water (2 × 100 ml) and dried ($CaCl_2$), filtered and evaporated to yield a residue 70·0 mg. This material was separated on preparative SiO_2 TLC in the system C_6H_6–CH_3COOH (4:1), v/v. The band at R_f 0·50 consisted of ochratoxin A (34 mg). M.p. 92–94° (from C_6H_6). Lit.[1] *ca.* 90°.

The results for the three different substrates are summarized in Table 1.

In each case the labelled material was diluted with inactive ochratoxin A as to obtain sufficient material for chemical degradation work. Specific activity (SA) of ochratoxin A: acetate, $1·362 \times 10^6$ counts/min m-mole^{-1}; methionine, $9·55 \times 10^5$ counts/min m-mole^{-1}; phenylalanine, $5·775 \times 10^5$ counts/min m-mole^{-1}.

Acid Hydrolysis of ^{14}C-Ochratoxin A

Ochratoxin A (Ia), suspended in 6 N HCl, was heated under reflux for 72 hr. $CHCl_3$ extraction of the mixture and removal of the solvent gave 6-chloro-8,9-dihydro-3-hydroxy-9-methylisocoumarin-4-carboxylic acid (IIa) (220 mg). M.p. 238–239° (from MeOH), lit.[1] m.p. 239°; λ_{max} 212 and 338 nm (ϵ 26,000 and 5200, respectively). (Accurate mass M^+ 256·013. Calc. for $C_{11}H_9ClO_5$: 256·013.) Acetate-labelled, SA $1·467 \times 10^{6*}$ counts/min m-mole^{-1}; methionine-labelled SA $1·024 \times 10^{6*}$ counts/min m-mole^{-1} phenylalanine, inactive-labelled. The purified L-β-phenylalanine had, from this latter experiment, a SA of $5·65 \times 10^5$ counts/min m-mole^{-1}, corresponding to 97·8 per cent of the activity of ochratoxin A.

Kuhn–Roth Degradation[21] of Compound IIa Labelled with ^{14}C-acetate

Compound IIa (53·6 mg); in 4 N chromic acid–conc. H_2SO_4 (4:1), v/v (4·5 ml), was heated under reflux for 2 hr. The acetic acid was collected (92%) and part converted into the *p*-bromophenacylacetate[22] which, after recrystallization to constant activity, had SA $2·947 \times 10^5$ counts/min m-mole^{-1}, corresponding to 20·1 per cent of the activity of compound IIa. A second portion as the dry $NaOCOCH_3$ (10·0 mg) was submitted to the Schmidt decarboxylation[23] at 40° in conc. H_2SO_4 (0·6 ml) with NaN_3 (40 mg). The CO_2 formed trapped as $BaCO_3$ had SA $2·195 \times 10^5$ counts/min m-mole-1 (uncorrected), corresponding to 74·4 per cent of the activity of the acetic acid. The methylamine from the reaction was trapped as the picrate and contained no radioactivity.

Preparation of the Amide (VIIIe) from Compound (IIa), Labelled with ^{14}C-acetate

Compound IIa (170 mg, SA $1·467 \times 10^6$ counts min m-mole^{-1}) was methylated with CH_2N_2 at room temperature for 24 h, saponified and the product separated on preparative TLC in C_6H_6–CH_3COOH (4:1), v/v. Elution of the band at R_f 0·60 gave compound VIIIb. M.p. 168° (from MeOH). Lit.[24] m.p. 153° after sublimation. λ_{max} 215 and 310 nm (ϵ 25,000 and 2650, respectively). (Accurate mass M^+ 270·028. Calc. for $C_{12}H_{11}O_5Cl$, 270·029.)

This acid (25 mg) was heated under reflux with $SOCl_2$ (4·0 ml) for 2 hr and the acid chloride converted to the azide (VIIIc) with NaN_3 which showed characteristic i.r. bands, $\nu_{max\ CHCl_3}$ at 2255 and 2150 cm^{-1} (—CON_3).

* The difference in radioactivity is due to the fact that ochratoxin A contained some benzene of crystallization.

[21] E. Wiesenberger, *Mikrochim. Acta* 33, 51 (1948).
[22] A. I. Vogel, *Practical Organic Chemistry*, p. 362, Longmans, London (1961).
[23] E. F. Phares, *Arch. Biochem. Biophys.* 33, 173 (1951).
[24] P. S. Steyn and C. W. Holzapfel, *Tetrahedron* 23, 4449 (1967).

TABLE 1. THE INCORPORATION OF PRECURSORS INTO OCHRATOXIN A IN A TYPICAL EXPERIMENT

Precursor	Activity of precursor added (μc)	Volume of culture fluid (ml)	Yield of ochratoxin A (mg)	Total activity of ochratoxin A (μc)	Incorporation (%)
[1-^{14}C]-Acetate	500	1000	34	0·66	0·132
[^{14}C-CH_3]-DL-Methionine	150	500	20	0·55	0·37
DL-β-Phenylalanine [U-^{14}C]	50	100	7·6	0·50	1·00

Conc. H_2SO_4 (0·5 ml) was added to the azide in trichloroethylene (3·5 ml) at 35° over 10 min and the CO_2 collected and assayed as $BaCO_3$. The $BaCO_3$ was inactive. Standard work-up of the residue gave the *amine* (VIIId) (20 mg), (ν_{max} 3400 (NH) and 1725 cm^{-1} lactone CO). On treatment with 3,5-dinitrobenzoylchloride (45 mg) it gave an *amide* (VIIIe), purified on TLC, which had m.p. 195–196°. λ_{max} 220 and 315 nm (ϵ 20,000 and 2900, respectively). ν_{max} 3420 (NH), 1726 (lactone CO), 1690 (amide CO), 1550 and 1520 cm^{-1} (NO_2-grouping). (Accurate mass M^+ 435·047. $C_{18}H_{14}N_3O_8Cl$ requires 435·047.) SA $1{\cdot}480 \times 10^6$ counts/min m-mole^{-1}, corresponding to 100% of the activity of the lactone acid (IIa).

Preparation of the Amine (VIIId) from Compound (IIa) Labelled with Methionine

The chlorolactone acid (IIa) (140 mg, SA $1{\cdot}024 \times 10^6$ counts/min m-mole^{-1}), was converted into the amine (VIIId) and $BaCO_3$ as described above. The amine was crystallized from EtOH–H_2O and had m.p. 126–127°; λ_{max} 210, 234, 266 (sh), and 344 nm (ϵ 15,000, 19,800, 5600 and 3200, respectively), on addition of HCl the long wave-length band (344 nm) shifted to 308 nm; $\nu_{max\ CHCl_3}$ 3400 (NH) and 1725 cm^{-1} (lactone CO). (Accurate mass, M^+ 241·051, $C_{11}H_{12}NO_3Cl$ requires, 241·050.) This amine was inactive. The ^{14}C-$BaCO_3$ represented approximately 70 per cent of the activity of the chlorolactone acid (IIa).

Preparation of Compound (VII)

The azide of 5-chlorosalicylic acid (300 mg) in C_6H_6 (15 ml) was heated under reflux (anhyd. conditions) for 24 hr. Crystalline material formed during the reaction. The reaction mixture was allowed to cool and filtered to furnish compound VII (120 mg). M.p. 186° (from C_6H_6). λ_{max} 208, 232, 282, and 289 nm (ϵ 15,400, 4200, 4330, and 3516, respectively); ν_{max} 1783 cm^{-1} (CO). (Found: C, 49·60; H, 2·37; N, 8·20 and Cl, 21·15. $C_7H_4NO_2Cl$ required: C, 49·52; H, 2·37; N, 8·25, and Cl, 20·91%.)

Acknowledgement—We wish to thank Dr. I. F. H. Purchase for a generous sample of inactive ochratoxin A.