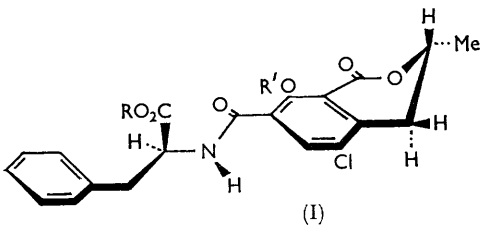
1304. *Mycotoxins. Part II.* The Constitution of Ochratoxins A, B, and C, Metabolites of* Aspergillus ochraceus *Wilh.*

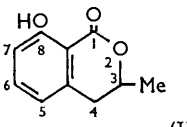
By K. J. van der Merwe, P. S. Steyn, and L. Fourie

Ochratoxin A, a toxic metabolite from *Aspergillus ochraceus* Wilh. is shown to be 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocoumarin, linked over its 7-carboxy-group to L-β-phenylalanine by an amide bond. Two further metabolites, ochratoxins B and C, have been isolated and characterised as the dechloro and ethyl ester derivatives of ochratoxin A, respectively. Full stereochemical formulæ are suggested.

The discovery [1] of toxigenic strains of the fungus *Aspergillus ochraceus* Wilh. led to the isolation of ochratoxin A, the toxic principle, for which structure (I; R = R′ = H) is suggested.[2] The structure of heterocyclic portion of ochratoxin A closely resembles that of mellein (II),[3] a known metabolite of *A. ochraceus*.



(I)



(II)

Ochratoxin A (I; R = R′ = H), $C_{20}H_{18}ClNO_6$, was isolated from a chloroform–methanol extract of fungal cultures by extraction with sodium hydrogen carbonate solution, and purified by chromatography on silica and ion-exchange (Dowex I) columns. It is a colourless crystalline compound containing a carboxy ($\nu_{max.}$ 1690 cm.$^{-1}$ and broad absorption between 2500 and 2700 cm.$^{-1}$), a secondary amide ($\nu_{max.}$ 1665, 1535, and 3430 cm.$^{-1}$), a lactone ($\nu_{max.}$ 1745 cm.$^{-1}$), and a phenolic hydroxyl group (red ferric chloride reaction in ethanol). The presence of both a carboxy and a phenolic hydroxyl group was confirmed by treatment of ochratoxin A with methanol–hydrochloric acid to give a methyl ester ($\nu_{max.}$ 1725 cm.$^{-1}$) still showing a positive ferric chloride reaction. Methylation of this ester, or ochratoxin A, with diazomethane gave the *O*-methyl methyl ester (I; R = R′ = Me). The negative ferric chloride reaction of this compound indicated that methylation of a phenolic hydroxyl group had occurred. Its mass spectrum showed, in accordance with the suggested formula, a molecular ion peak at *m/e* 431 and an isotope peak at *m/e* 433 with relative intensities 3 : 1.[4]

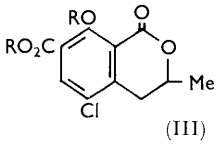
The nuclear magnetic resonance (n.m.r.) spectrum of the *O*-methyl methyl ester (I; R = R′ = Me) showed the peaks of 22 protons, *viz.*: a doublet at τ 8·46 (J = 7·0 c./sec., secondary *C*-methyl), a complex pattern centred around τ 6·8 (four protons), two *O*-methyl peaks (τ 6·21 and 6·28), two complex patterns centred around τ 4·98 and 5·45 (one proton each), a singlet at τ 2·74 (five protons, phenyl group), a singlet at τ 1·61 (one proton) and a doublet at τ 1·63 (J = 7·0 c./sec., one proton). Addition of deuterium oxide did not alter the spectrum, but subsequent addition of a trace of triethylamine caused the doublet at τ 1·63 to disappear during 36 hr. This behaviour is in accord with the exchange of an amide proton for deuterium.

* K. J. van der Merwe, L. Fourie, and De B. Scott, *Chem. and Ind.*, 1963, 1660, is regarded as Part I of this Series.

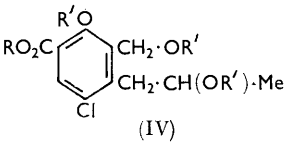
[1] De B. Scott, *Mycopathol. et Mycol. Appl.*, 1965, **25**, 213.
[2] K. J. van der Merwe, P. S. Steyn, L. Fourie, De B. Scott, and J. J. Theron, *Nature*, 1965, **205**, 1112 for a preliminary account.
[3] J. Blair and G. T. Newbold, *J.*, 1955, 2871.
[4] J. H. Beynon, "Mass Spectrometry and its Applications to Organic Chemistry," Elsevier Publishing Co., London, 1960, p. 298.

Acid hydrolysis of ochratoxin A gave L-β-phenylalanine and a crystalline lactone acid $C_{11}H_9ClO_5$ ($\nu_{max.}$ 1735 cm.$^{-1}$), formulated as the acid (III; R = H) on the basis of the following evidence. The acid reacted with diazomethane to give a dimethyl derivative $C_{13}H_{13}ClO_5$ which could not be acetylated. Reduction of the acid with lithium borohydride followed by brief methylation of the product with diazomethane and acetylation gave a derivative (IV; R = Me, R′ = Ac) showing n.m.r. signals for one *O*-methyl (τ 6·12) and three acetate methyl groups (τ 8·17, 7·98, and 7·64). Addition of dilute alkali to alcohol solutions of either the *O*-methyl methyl ester (III; R = Me), or *O*-methylochratoxin A methyl ester, caused a 10 mμ hypsochromic shift of their long-wavelength absorption maxima which indicates the direct linkage of the aromatic nucleus to the carbonyl group of the lactone ring.[5]

The presence of a 3,4-dihydroisocoumarin system with a methyl group at position 3 is evident from the n.m.r. spectra of the ester (III; R = Me) and its derived triacetate (IV; R = Me, R′ = Ac). In the former spectrum the methylene protons at position 4 occurred as an ABX pattern centred around τ 7·1 with $\Delta\nu_{AB} = 25{\cdot}8$; $J_{AB} = 17{\cdot}0$; $J_{AX} = 3{\cdot}4$; and $J_{BX} = 10{\cdot}8$ c./sec. The X proton gave rise to a complex pattern centred around τ 5·5, while a secondary methyl peak occurred as a doublet at τ 8·63 ($J = 7{\cdot}0$ c./sec.). In the spectrum of the triacetate a singlet at τ 4·72 (two protons) indicated the system $ArCH_2{\cdot}OAc$.[6] Complete analysis of the peaks of the methylene protons in the propyl side chain was not possible, but appeared to be $J_{AX} = 6$ and $J_{BX} = 8$ c./sec.



(III)



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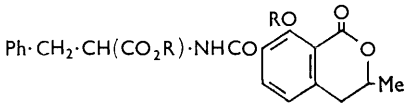

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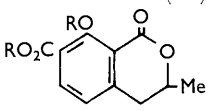

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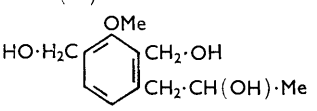

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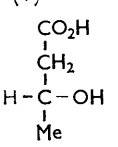

(VIII)

The substitution pattern on the aromatic ring of the 3,4-dihydroisocoumarin system was determined by u.v. spectroscopy and colour tests. Treatment of ochratoxin A with aluminium chloride resulted in a bathochromic shift of 33 mμ for its long-wavelength absorption maximum, indicating the presence of a hydroxyl group at position *peri* (position 8) to the lactone carbonyl.[7] The presence of a carboxy-group *ortho* to the phenolic hydroxyl group of the triol acid (IV; R = R′ = H) was evident from its u.v. spectrum, which showed a striking similarity to that of salicyclic acid, but differed from those of *m*- or *p*-hydroxybenzoic acid. Like salicyclic acid, this compound gave a violet ferric chloride reaction in ethanol. The chlorine atom is placed at position 5, since both the Gibbs and Liebermann colour tests on ochratoxin A indicated substitution *para* to the phenolic hydroxyl group. The above results confirm the suggested structure (I; R = R′ = H) for ochratoxin A.

Ochratoxin B, $C_{20}H_{19}NO_6$ is a colourless crystalline compound closely related to ochratoxin A, as indicated by the similarity of their u.v. spectra and the presence of the same oxygen functions (i.r. spectra, red ferric chloride reaction in ethanol). Ochratoxin B differs from the latter by the absence of halogen, suggesting that it is the dechloro-derivative (V; R = H) of ochratoxin A. This was confirmed by positive Gibbs and Liebermann colour tests and the n.m.r. spectrum of *O*-methylochratoxin B methyl ester (V; R = Me).

[5] A. I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products," Pergamon Press, London, 1964, p. 115.
[6] K. Nukada, O. Yamamoto, T. Suzuki, M. Takenchi, and M. Ohnishi, *Analyt. Chem.*, 1963, **35**, 1892.
[7] T. A. Geissman, "The Chemistry of Flavonoid Compounds," Pergamon Press, London, 1962, p. 119.

In addition to peaks attributable to the phenyl group and non-aromatic protons, it contained the doublets of two *ortho* aromatic protons (τ 1·78 and 2·93, J = 8·0 c./sec.). The methylene protons at position 4 gave rise to essentially two peaks (τ 7·02 and 7·15) compared with the ABX pattern observed in the case of *O*-methylochratoxin A methyl ester. These results confirm the previous deduction that the chlorine atom in ochratoxin A is situated at position 5.

Ochratoxins A and B were inter-related by the following procedure. Acid hydrolysis of *O*-methylochratoxin B methyl ester (V; R = Me) and methylation with diazomethane gave a compound $C_{13}H_{14}O_5$. Chlorination of this compound with sulphuryl chloride and remethylation gave the ester (III; R = Me) identical in all respects with the compound previously obtained from ochratoxin A. The above methyl derivative is therefore (VI; R = Me) and establishes the constitution of ochratoxin B as (V; R = H).

Ochratoxin C, $C_{22}H_{22}ClNO_6$, is the amorphous ethyl ester of ochratoxin A. It gave a red ferric chloride reaction in ethanol and on methylation with diazomethane a monomethyl derivative. The n.m.r. spectrum of *O*-methylochratoxin C showed the same peaks as that of *O*-methylochratoxin A methyl ester except for the replacement of one *O*-methyl peak by the peaks of an *O*-ethyl group (triplet of three protons at τ 8·73, quartet of two protons at τ 5·77, J = 7·5 c./sec.). Confirmation was obtained by brief treatment of ochratoxin A with diazoethane to give ochratoxin C (I; R = Et, R′ = H). Ochratoxin C is not considered an artifact, since methanol (and not ethanol) was used in its isolation.

The stereochemistry of the asymmetric carbon atom at position 3 was determined by oxidative degradation of the triol (VII), obtained from the ester (VI; R = Me) by reduction with lithium borohydride. Ozonolysis of the crude product, treatment with water, and oxidation with neutral silver oxide gave β-hydroxybutyric acid $[\alpha]_D$ −19·7°, identified by paper chromatography, the infrared spectrum of its sodium salt, and by oxidation to acetoacetic acid. D-β-Hydroxybutyric acid, $[\alpha]_D$ −24·3°, is known to have the Fischer projection formula (VIII).[8] Since alkali treatment of mellein (II) caused no racemisation or inversion[3] and reduction of a lactone with complex hydrides is known not to affect an adjacent asymmetric centre,[9] these results indicate the 3*R* configuration[10] shown in (I; R = R′ = H) for ochratoxin A. The steric conformation of the lactone ring in this formulation was deduced from the diaxial relationship[11] of protons at positions 3 and 4 (J_{BX} = 10·8 c./sec.) found in the n.m.r. spectrum of (III; R = Me).

The strongly negative optical rotations of the ester (III; R = Me) and *O*-methyl mellein (−230° and −245°, respectively),[3] the occurrence of ochratoxin A and mellein in the same fungus, and the fact that the 3-methyl group of ochratoxin A is in the favoured equatorial position, suggest that the stereochemistry of the lactone ring of mellein (II) is the same as that of ochratoxin A.

The fragmentation pattern observed in the mass spectrum of *O*-methylochratoxin A methyl ester agrees with the suggested structure (I; R = R′ = Me). The interpretation of this pattern was confirmed by the mass spectrum of the deuterium-labelled analogue (I; R = R′ = CD_3), prepared by a method previously described.[12] The significant peaks and their suggested assignments are as follows (the corresponding peaks of the deuterated compound are shown in parentheses): *m/e* 431 (437), molecular ion; *m/e* 253 (256), base peak due to loss of Ph·CH_2·CH·CO_2Me·NH; *m/e* 400 (403), loss of OMe; *m/e* 372 (375), loss of ·CO_2Me; *m/e* 269 (272), loss of Ph·CH:CH·CO_2Me; *m/e* 239 (240), loss of Ph·CH:CH·CO_2Me and formaldehyde (from methyl ether group); *m/e* 223 (224), loss of Ph·CH_2·CH·CO_2Me·NH and formaldehyde; *m/e* 209 (212) loss of Ph·CH_2·CH·CO_2Me·NH and carbon dioxide (from lactone ring).

[8] R. S. Cahn and C. K. Ingold, *J.*, 1951, 612.
[9] D. S. Noyce and D. B. Denney, *J. Amer. Chem. Soc.*, 1950, **72**, 5743; N. G. Gaylord, "Reduction with Complex Metal Hydrides," Interscience Publishers Ltd., London, 1956, ch. 9.
[10] R. S. Cahn, C. K. Ingold, and V. Prelog, *Experientia*, 1956, **12**, 81.
[11] R. U. Lemieux, R. K. Kulling, H. J. Bernstein, and W. G. Schneider, *J. Amer. Chem. Soc.*, 1957, **79**, 1005.
[12] K. J. van der Merwe, P. S. Steyn, and S. H. Eggers, *Tetrahedron Letters*, 1964, **52**, 3923.

The toxicity of ochratoxin A has previously been described.[2] In similar toxicity tests, ochratoxins B and C proved non-toxic at a thousand-fold higher dose-level.

Experimental

Unless otherwise stated, $[\alpha]_D$ and u.v. absorption spectra refer to ethanol, i.r. spectra to chloroform, and n.m.r. spectra to deuterochloroform solutions. I.r. absorption spectra were determined on a Perkin-Elmer model 21 spectrometer, u.v. absorption spectra on Unicam model S.P. 500 and 800 spectrometers, and n.m.r. spectra on a Varian A-60 spectrometer. Chemical shifts were measured on the τ scale relative to tetramethylsilane as internal standard (τ 10·0); τ-values are estimated to be accurate to $\pm$0·01 p.p.m., coupling constants to $\pm$0·2 c./sec. Mass spectra were determined on an Atlas CH4 mass spectrometer. M. p.s were corrected.

Isolation.—*A. ochraceus* Wilh. (strain K-804) was grown in bulk on sterilised wet maize meal as previously described.[1] The dried mouldy meal (23 kg.) was extracted with 1 : 1 chloroform–methanol, and the solvent removed *in vacuo*. The toxic extract (2·5 kg.) in chloroform (5 l.) was extracted with 0·5M-aqueous sodium hydrogen carbonate (3 l.) the acidified aqueous phase continuously extracted with chloroform, and the isolated acids (250 g.) chromatographed on acidic silica (3 kg.). Elution with 3 : 1 benzene–chloroform gave a colourless band, showing a bright green fluorescence under u.v. light and containing all the toxicity (42 g.).

Paper chromatography (3 : 1 propanol–3N-aqueous ammonium carbonate) (system A) and thin-layer chromatography on silica (12 : 2 : 1 benzene–methanol–acetic acid) (system B) of this band revealed the presence of several fluorescent components, which were quantitatively separated by ion-exchange chromatography [13] on " Dowex 1 × 8 " (1 kg., 200—400 mesh, formate form) in 1 : 1 aqueous methanol. The column was developed by gradient elution with 3·5M-formic acid in 1 : 1 aqueous methanol and 200 × 100 ml. fractions collected.

Chloroform extraction of the combined fractions 135 to 180 and fractional crystallisation from benzene gave pure crystalline ochratoxin A (950 mg.), m. p. *ca.* 90°, containing one mol. benzene of crystallisation. After removal of the solvent at 120°/10^{-4} mm. and crystallisation from xylene, it had m. p. 169°, $[\alpha]_D$ −118° (*c* 1·1 in chloroform); $\lambda_{max.}$ 215 and 333 mμ (ε 34,000 and 2400, respectively); $\nu_{max.}$ 1745, 1690, 1665, 1535, and 3430 $cm.^{-1}$ (Found: C, 59·5; H, 4·7; Cl, 8·5; N, 3·5. Calc. for $C_{20}H_{18}ClNO_6$: C, 59·5; H, 4·5; Cl, 8·8; N, 3·5%).

Preparative thin-layer chromatography of the mother-liquors on silica (system B) gave ochratoxin A (50 mg.) and *ochratoxin C* (500 mg.). Ochratoxin C (I; R = Et, R′ = H), which could not be crystallised, had $[\alpha]_D$ −100° (*c* 1·2); $\lambda_{max.}$ 214 and 333 mμ (ε 30,000 and 7000, respectively); $\nu_{max.}$ 1730 and 1680 $cm.^{-1}$ (Found: C, 61·2; H, 5·3. $C_{22}H_{22}ClNO_6$ requires C, 61·2; H, 5·1%).

Fractions 110 to 130 contained a single fluorescent component, called *ochratoxin B*, which crystallised directly from the acidic aqueous methanol medium in a yield of 500 mg. Ochratoxin B (V; R = H) from methanol had m. p. 221°, $[\alpha]_D$ −35° (*c* 0·15); $\lambda_{max.}$ 218 and 318 mμ (ε 37,200 and 6900, respectively); $\nu_{max.}$ (KBr disc) 1730, 1680, and 1535 $cm.^{-1}$ (Found: C, 65·0; H, 5·3; N, 3·6. $C_{20}H_{19}NO_6$ requires C, 65·0; H, 5·2; N, 3·8%).

Ochratoxins A, B, and C appeared under u.v. light as bright-blue fluorescent spots (R_F 0·65, 0·68, and 0·8, respectively) on paper chromatograms in the alkaline system A and as bright green fluorescent spots (R_F 0·57, 0·55, and 0·73, respectively) on silica thin-layer chromatography in the acidic system B.

O-Methylochratoxin A Methyl Ester (I; R = R′ = Me).—Ochratoxin A (I; R = R′ = H) (50 mg.) in chloroform (10 ml.) was treated with an excess of ethereal diazomethane at room temperature for 16 hr. Decomposition of the excess of diazomethane with formic acid, column chromatography of the reaction product on neutral silica, and elution with benzene gave the *O-methyl methyl ester* (I; R = R′ = Me) as an oil (55 mg.). The compound had $[\alpha]_D$ −160° (*c* 0·3); $\lambda_{max.}$ 214 and 312 mμ (ε 33,900 and 2350, respectively); $\nu_{max.}$ 1740, 1725, and 1665 $cm.^{-1}$ (Found: C, 61·1; H, 4·9. $C_{22}H_{22}ClNO_6$ requires C, 61·2; H, 5·1%).

Acid Hydrolysis of Ochratoxin A (I; R = R′ = H).—Ochratoxin A (I; R = R′ = H) (420 mg.), suspended in 6N-hydrochloric acid (200 ml.), was heated under reflux for 30 hr. Chloroform extraction of the cooled homogeneous mixture and removal of the solvent from the dried (Na_2SO_4) organic phase gave 5-chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin-7-carboxylic acid (III; R = H) (250 mg.). After sublimation at 155°/5 × 10^{-4} mm., it had m. p.

[13] H. Busch, R. B. Hulbert, and V. R. Potter, *J. Biol. Chem.*, 1952, **196**, 717.

239° (decomp.), $[\alpha]_D$ −230° (*c* 0·14); $\lambda_{max.}$ 212 and 338 mμ (ε 30,000 and 5600, respectively); $\nu_{max.}$ (KBr disc) 1735 and 1680 cm.$^{-1}$ (Found: C, 51·8; H, 3·9. Calc. for $C_{11}H_9ClO_5$: C, 51·5; H, 3·5%).

Butanol extraction of the above aqueous layer, adjusted to pH 5·85 with sodium hydroxide, gave β-phenylalanine (100 mg.), identified by paper chromatography (4 : 1 : 1 butanol–acetic acid–water) and by means of a Beckman Amino Acid Analyser model 120B. The amino-acid had $[\alpha]_D$ −35° (*c* 1·7 in H_2O), the same as that reported for L-β-phenylalanine.[14]

Methyl 5-Chloro-3,4-dihydro-8-methoxy-3-methylisocoumarin-7-carboxylate (III; R = Me).—5-Chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin-7-carboxylic acid (50 mg.) in chloroform (30 ml.) was treated with an excess of ethereal diazomethane at room temperature for 20 hr. Decomposition of the excess of diazomethane with formic acid and removal of the solvent *in vacuo* gave O-*methyl methyl ester* (III; R = Me) (55 mg.). After sublimation at 105°/10^{-3} mm. the compound had m. p. 66°, $[\alpha]_D$ −226° (*c* 0·7); $\lambda_{max.}$ 216 and 309 mμ (ε 39,500 and 3900, respectively, $\nu_{max.}$ (KBr disc) 1740 cm.$^{-1}$ (Found: C, 55·2; H, 4·8. $C_{13}H_{13}ClO_5$ requires C, 54·9; H, 4·6%).

Reduction of the Acid (III; R = H) *with Lithium Borohydride.*—A mixture of the acid (III; R = H) (200 mg.), anhydrous lithium chloride (200 mg.), and potassium borohydride (200 mg.) in dry tetrahydrofuran (200 ml.) was heated under reflux (anhydrous conditions) for 16 hr. Dilution with water, acidification with 4N-hydrochloric acid, and isolation with chloroform gave the *trihydroxy-acid* (IV; R = R′ = H) as an oil with $\lambda_{max.}$ 214 and 317 mμ (ε 13,000 and 1800, respectively) and $\nu_{max.}$ (KBr disc) 1680 cm.$^{-1}$. Treatment of this product (160 mg.) in methanol (30 ml.) with an excess of ethereal diazomethane for 3 min., acidification with formic acid, and removal of the solvent gave the crude *trihydroxy-ester* (IV; R = Me, R′ = H). The crude product (165 mg.) in acetic anhydride (25 ml.) was cooled to −50°, a trace of perchloric acid added, and left at room temperature for 5 hr. Treatment of the mixture with water (250 ml.) for 12 hr., isolation with chloroform and column chromatography on neutral silica in 99 : 1 chloroform–benzene gave the *triacetoxy-ester* (IV; R = Me, R′ = Ac) as an oil (170 mg.), $[\alpha]_D$ −20° (*c* 1·4), $\nu_{max.}$ 1750 and 1740 cm.$^{-1}$ (Found: C, 54·4; H, 5·3. $C_{18}H_{21}ClO_8$ requires C, 53·9; H, 5·3%).

O-*Methylochratoxin B Methyl Ester* (V; R = Me).—Ochratoxin B (96 mg.) was methylated with diazomethane as described for the preparation of *O*-methylochratoxin A methyl ester to give O-*methylochratoxin B methyl ester* (V; R = Me) as an oil (102 mg.), $[\alpha]_D$ −65° (*c* 0·4); $\lambda_{max.}$ 215 and 298 mμ (ε 32,000 and 2100, respectively); $\nu_{max.}$ 1730 and 1660 cm.$^{-1}$ (Found: C, 66·4; H, 5·9. $C_{22}H_{23}NO_6$ requires C, 66·5; H, 5·8%).

Acid Hydrolysis of O-*Methylochratoxin B Methyl Ester.*—The ester (V; R = Me) (100 mg.), suspended in 6N-hydrochloric acid (150 ml.), was heated under reflux for 40 hr. Isolation into chloroform, methylation with diazomethane, and removal of the solvents gave *methyl 3,4-dihydro-8-methoxy-3-methylisocoumarin-7-carboxylate* (VI; R = Me) (50 mg.). After sublimation at 120°/10^{-3} mm. it had m. p. 103°, $[\alpha]_D$ −170° (*c* 0·1); $\lambda_{max.}$ 218 and 310 mμ (ε 23,000 and 3000, respectively), $\nu_{max.}$ (KBr disc) 1735 cm.$^{-1}$ (Found: C, 62·3; H, 5·7. $C_{13}H_{14}O_5$ requires C, 62·4; H, 5·6%).

A small portion of the aqueous phase was evaporated to dryness and the presence of phenylalanine indicated with a Beckman Amino Acid Analyser model 120B.

Chlorination of Methyl 3,4-Dihydro-8-methoxy-3-methylisocoumarin-7-carboxylate (VI; R = Me).—A mixture of the *O*-methyl methyl ester (VI; R = Me) (10 mg.) and sulphuryl chloride (1 ml.) was kept at 90° for 70 min., and then evaporated to dryness, the residue re-dissolved in chloroform (10 ml.), and treated with ethereal diazomethane at room temperature for 15 hr. Acidification with formic acid, purification by thin-layer chromatography on silica in chloroform, and sublimation at 105°/10^{-3} mm. gave the *5-chloro-derivative* (III; R = Me) (5 mg.), identified by mixed m. p. (66°) and i.r. spectroscopy.

Reduction of the O-*Methyl Methyl Ester* (VI; R = Me) *with Lithium Borohydride.*—A mixture of the *O*-methyl methyl ester (VI; R = Me) (110 mg.), anhydrous lithium chloride (100 mg.), and potassium borohydride (100 mg.) in dry tetrahydrofuran (100 ml.) was heated under reflux (anhydrous conditions) for 20 hr. Treatment with 4N-hydrochloric acid and isolation with chloroform gave the triol (VII) as an oil (80 mg.), which was not further purified but used as such in the next experiment.

Ozonolysis of the Triol (VII).—The crude triol (VII) (80 mg.) in chloroform (25 ml.) was

[14] K. Freudenberg, "Stereochemie," Franz Deuticke, Leipzig, 1933, p. 682.

ozonised at 0° for 2 hr. After removal of the solvent and unchanged ozone *in vacuo* at 20°, the residue in water (10 ml.) was treated with silver oxide (1 g.) at 90° for 30 min. Acidification of the filtered aqueous solution with hydrochloric acid and isolation with ether gave a residue, showing a main spot at the same R_F value (0·55) as β-hydroxybutyric acid on paper chromatograms (system 4 : 1 tetrahydrofuran–3N-aqueous ammonia, with methyl red as indicator [15]). Purification of the residue by paper chromatography on Whatman No. 3MM in the above system and distillation at 80°/10 mm. gave β-hydroxybutyric acid (6 mg.), identified by the i.r. spectrum of its sodium salt and by oxidation with alkaline silver oxide to acetoacetic acid, identified by the i.r. spectrum of its ethyl ester. The β-hydroxybutyric acid had $[\alpha]_D$ −19·7° (*c* 0·6 in H_2O). D-β-Hydroxy-butyric acid has $[\alpha]_D$ −24·3° (*c* 1·0 in H_2O).[8,14]

O-*Methylochratoxin C* (I; R = Et, R′ = Me).—Ochratoxin C (I; R = Et, R′ = H) (190 mg.) in chloroform (50 ml.) was treated with an excess of ethereal diazomethane at room temperature for 16 hr. Decomposition of the excess of diazomethane with formic acid and filtration through neutral silica gave amorphous O-*methylochratoxin C* (I; R = Et, R′ = Me) (200 mg.) $[\alpha]_D$ −110° (*c* 1·0); $\lambda_{max.}$ 216 and 312 mμ (ε 32,000 and 1900, respectively); $\gamma_{max.}$ 1730 and 1665 cm.$^{-1}$ (Found: C, 62·3; H, 5·4. $C_{23}H_{24}ClNO_6$ requires C, 61·9; H, 5·4%).

Acid Hydrolysis of O-*Methylochratoxin C* (I; R = Et, R′ = Me).—The methyl ether (I; R = Et, R′ = Me) (200 mg.), suspended in 6N-hydrochloric acid (170 ml.), was heated under reflux for 55 hr. Extraction into chloroform and treatment with ethereal diazomethane at room temperature for 16 hr. gave the *O*-methyl methyl ester (III; R = Me) (70 mg.), identified by its mixed m. p. (66°) and i.r. spectrum. The presence of phenylalanine in the aqueous phase was verified by means of a Beckman Amino Acid Analyser model 120B.

Correlation of Ochratoxins A and C.—Ochratoxin A (I; R = R′ = H) (10 mg.) in chloroform (10 ml.) was treated with an excess of ethereal diazoethane at room temperature for 1 min. Acidification with formic acid and purification by thin-layer chromatography on silica in 12 : 2 : 1 benzene–methanol–acetic acid gave ochratoxin C (I; R = Et, R′ = H) (10 mg.), identified by its infrared spectrum.

We thank Dr. G. Spiteller, Institute of Organic Chemistry, University of Vienna, and Dr. S. H. Eggers of this laboratory for mass spectra, and the South African Oil Seeds Control Board, Pretoria, for financial support.

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South African Council for Scientific and Industrial Research,
Pretoria. [*Received, May 11th,* 1965.]

[15] H. Kalbe, *Z. physiol. Chem.*, 1954, **297**, 19.