Studies in Mycological Chemistry. Part XXIV.[1] Synthesis of Ochratoxin A, a Metabolite of *Aspergillus ochraceus* Wilh.

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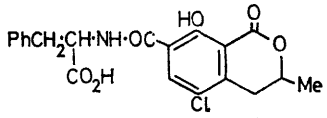
An alternative synthesis of ochratoxin A (*ex Aspergillus ochraceus* Wilh.) is described.

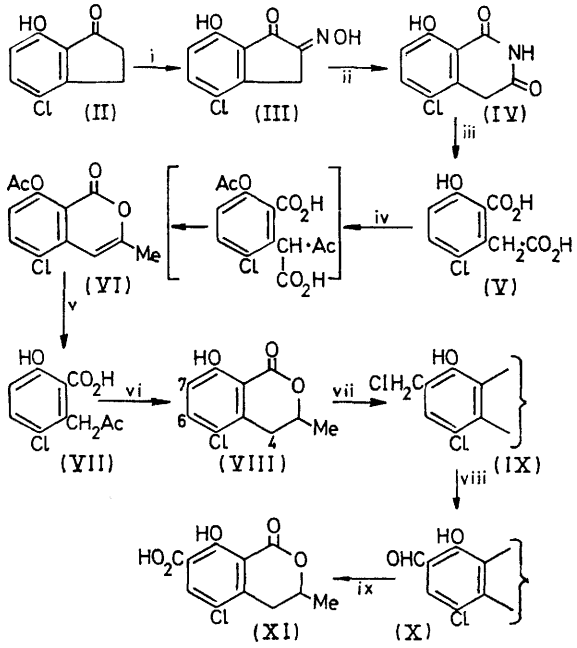
OCHRATOXIN A is a highly toxic metabolite[2] of some strains of *Aspergillus ochraceus* Wilh. Structure (I) was allocated[3] to the mycotoxin, and this was subsequently confirmed by synthesis.[4] We now describe an alternative, and substantially different, synthesis for this mycotoxin.†



(I)

We first looked for an efficient synthesis of the dihydroisocoumarin carboxylic acid (XI). We decided to start with 4-chloro-7-hydroxyindanone,[5] in which the phenolic hydroxy-group and chlorine function were already present and correctly orientated (*cf.* ref. 4). This was converted (see Scheme), *via* the oximino-compound (III), the homophthalimide (IV) (not isolated), and the homophthalic acid (V), into the isocoumarin (VI) by adaptations of known procedures.[6] Alkaline hydrolysis of the isocoumarin (VI) gave the benzoic acid (VII) which was converted, by successive treatment with sodium borohydride and acid, into the dihydroisocoumarin (VIII). It was then necessary to introduce a carboxy-group (or another group easily convertible into a carboxy-group) into the 7-position. This was achieved by successive application of the Rieche reaction[7] [chloromethyl methyl ether–titanium(IV) chloride], (VIII) → (IX), the Sommelet reaction,[8] (IX) → (X), and, finally, mild oxidation, (X) → (XI). The (±)-dihydroisocoumarin carboxylic acid (XI) was thus synthesised from compound (II) in eight steps (overall yield *ca.* 0·62%).

The amino-acid portion of natural ochratoxin A is in the L(−)-form and the dihydroisocoumarin carboxylic acid portion is the (−)-enantiomorph.[3] We therefore attempted next to obtain the (−)-dihydroisocoumarin carboxylic acid. The (±)-acid (XI) has been resolved[3] (giving acids of *ca.* 80% optical purity) by fractional crystallisation of the brucine salts, but in our hands this method did not give such a degree of resolution. Attempted fractional crystallisation of the (−)-menthylamine salts gave little or no resolution. The cinchonine and *N*-methylquininonium[9] salts formed intractable gums. Chromatography of the (±)-acid on



SCHEME

Reagents: i, C_5H_{11}·O·NO–HCl; ii, *p*-MeC_6H_4·SO_3Cl; iii, (*a*) NaOH, (*b*) HCl; iv, Ac_2O–AcONa (*cf.* ref. 6); v, (*a*) NaOH, (*b*) HCl; vi, (*a*) $NaBH_4$, (*b*) HCl; vii, $ClCH_2$·OMe–$TiCl_4$; viii, (*a*) $(CH_2)_6N_4$, (*b*) aqueous AcOH; ix, Ag_2O.

an optically active adsorbent (paper) again yielded no resolution. We therefore decided to condense L(−)-phenylalanine with the (±)-acid and to attempt a resolution of the resulting diastereoisomeric forms [(−, +) and (−, −)] of ochratoxin A.

Initially, and with moderate success, the (±)-acid (XI) was condensed with L(−)-phenylalanine by the acid azide method.[3] Subsequently, we employed a procedure which involved the use of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ).[10] Condensa-

† We had started our synthetic work before the publication of Steyn and Holzapfel's paper.[4] It was clear that our synthesis would differ significantly from theirs, so we decided, in agreement with Dr. Holzapfel, to complete our project.

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tion of the t-butyl ester of L(−)-phenylalanine with the (±)-acid (XI) by means of EEDQ, and subsequent removal of the protecting t-butyl group, led to a mixture of the diastereoisomeric forms of ochratoxin A.

Repeated preparative t.l.c. on silica gave a sample of ochratoxin A of *ca.* 90% isomeric purity. Lack of material precluded any attempt to improve this standard.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. Unless otherwise specified, u.v. spectra were recorded for solutions in ethanol with a Unicam SP 700 spectrophotometer (inflections are indicated in italics), and i.r. spectra were determined for potassium bromide discs with a Unicam SP 200 spectrophotometer. ^{1}H n.m.r. spectra were recorded with a Perkin-Elmer R10, 60 MHz spectrometer, with tetramethylsilane as internal reference. Optical rotations were measured for solutions in chloroform with an Ericsson E.T.L.–N.P.L. automatic polarimeter, type 143A, and o.r.d. spectra were recorded for solutions in methanol containing 6% acetic acid with a Bendix Polarmatic 62 spectropolarimeter.

The silica used for t.l.c. was ' Chromalay Silica Gel with Calcium Sulphate Binder ' (May and Baker); the silica used for column chromatography was Hopkin and Williams MFC.

4-Chloro-7-hydroxy-2-hydroxyimino-indan-1-one (III).—To a solution of 4-chloro-7-hydroxyindanone (II) [5] (200 g., 0·99 moles) and isopentyl nitrite (500 ml., 3·75 moles) in ethanol (4 l.) at 45—50° was added, dropwise, concentrated hydrochloric acid (50 ml.). The solution was kept at 45—50° for 45 min.; during this time the oxime began to crystallise. The mixture was cooled to 0° and the *product*, filtered off and dried *in vacuo*, gave yellow needles (147 g., 67%), m.p. 184·5—185·5° (from ethanol) (Found: C, 47·25; H, 3·45; N, 6·25. $C_9H_6ClNO_3,H_2O$ requires C, 47·1; H, 3·5; N, 6·1%). The product was dried at 100°/0·1 mm. for 24 hr. to give the *anhydrous form* as pale yellow needles, m.p. 184·5—185·5° (Found: C, 51·45; H, 2·8; N, 6·7. $C_9H_6ClNO_3$ requires C, 51·1; H, 2·85; N, 6·6%), $\lambda_{max.}$ 220, 249, 279, and 356 nm. (10^{-3} ε 10·81, 11·27, 12·95, and 4·6), $\nu_{max.}$ 925, 1045, 1300, 1335, 1620, and 1690 cm.$^{-1}$.

2-Carboxy-3-hydroxy-6-chlorophenylacetic acid (V).—The crude hydroxyimino-indanone (146 g.) was dissolved in 8% aqueous sodium hydroxide (3·0 l.) and toluene-*p*-sulphonyl chloride (368 g.) was added slowly, with stirring. The mixture was stirred at 35—40° for 20 min. and then stirred while the temperature was raised to 100°. It was allowed to cool to *ca.* 80°; sodium hydroxide (pellets) (440 g.) was added and the solution was heated under reflux for 5 hr. It was then cooled, filtered, acidified (concentrated hydrochloric acid), and extracted with ethyl acetate (4 × 1 l.). The organic solution was washed (10% brine), dried ($MgSO_4$), and evaporated to give a brown resin. This was warmed with toluene (200 ml.) and the solid residue was partially decolourised by rinsing with a small amount of acetone (*ca.* 10 ml.). The *product* was a buff solid (93 g., 55%), which gave colourless needles, m.p. 162—168° [from water (charcoal)] (Found: C, 46·7; H, 2·85. $C_9H_7ClO_5$ requires C, 46·9; H, 3·05%), $\lambda_{max.}$ 212, *217*, *233*, and 321 nm. (10^{-3} ε 27·8, 25·8, 6·31, and 3·69), $\nu_{max.}$ 685, 835, 925, 1210, 1310, 1440, 1600, 1650, and 1710 cm.$^{-1}$.

8-Acetoxy-5-chloro-3-methylisocoumarin (VI).—A solution of the crude phenylacetic acid (93 g.) and fused sodium acetate (9 g.) in acetic anhydride (1200 ml.) was heated under reflux for 12 hr. Most of the acetic anhydride was then removed *in vacuo*, and water (1 l.) was slowly added to the remaining solution. When reaction had ceased the mixture was cooled and extracted with ether (4 × 1 l.). The extract was washed several times with water, then with sodium hydrogen carbonate solution until the washings had pH 9, and finally with water. It was dried ($MgSO_4$) and evaporated to give a brown resin, from which the isocoumarin sublimed at 90°/0·1 mm. as colourless needles (10·1 g., 10%). The *product* was crystallised from carbon tetrachloride and resublimed to give colourless needles, m.p. 138·5—141° (Found: C, 57·0; H, 3·45. $C_{12}H_9ClO_4$ requires C, 57·05; H, 3·6%), $\lambda_{max.}$ 206, *229*, 235, *243*, 253, *262*, 272, 282, and 341 nm. (10^{-3} ε 14·0, 19·2, 23·1, 16·7, 16·9, 9·44, 9·57, 7·93, and 4·71), $\nu_{max.}$ 820, 905, 995, 1030, 1120, 1210, 1370, 1460, 1590, 1660, 1730, and 1755 cm.$^{-1}$, τ ($CDCl_3$) 2·3 (1H, d, *J* 9 Hz, *H ortho* to Cl), 3·0 (1H, d, *J* 9 Hz, *H ortho* to OH), 3·4 (1H, q, *J* 1 Hz, *H*C=C), 7·6 (3H, s, Ac), 7·7 (3H, d, *J* 1 Hz, *Me*C=C).

2-Acetonyl-3-chloro-6-hydroxybenzoic Acid (VII).—The sublimed isocoumarin (10·1 g.) was heated under reflux with sodium hydroxide solution [5·2 g. in water (3 l.)] for 1 hr. The resulting solution was cooled, filtered, acidified (concentrated hydrochloric acid), and extracted with ether. The extract was washed (water) and extracted with sodium hydrogen carbonate solution. This extract was acidified (concentrated hydrochloric acid) and extracted with ether, and the ether extract was washed (water), dried ($MgSO_4$), and evaporated to give colourless crystals (8·2 g., 88%), which yielded the *acid* as colourless needles, m.p. 192—194·5° (from ethanol) (Found: C, 52·55; H, 3·95; Cl, 15·7. $C_{10}H_9ClO_4$ requires C, 52·5; H, 3·95; Cl, 15·5%), $\lambda_{max.}$ 215, *219*, *234*, *246*, and 325 nm. (10^{-3} ε 25·3, 24·4, 5·48, 4·86, and 4·05) $\nu_{max.}$ 695, 745, 875, 1065, 1180, 1260, 1380, 1460, 1480, 1580, 1600, and 1660 cm.$^{-1}$.

5-Chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin (VIII).—To a solution of the benzoic acid (VII) (8·2 g.) in ethanol (400 ml.) was added a solution of sodium borohydride (16·5 g.) in water (1 l.). The combined solution was kept at room temperature for 3 hr. and was then acidified (concentrated hydrochloric acid) and extracted with chloroform. The extract was washed with water, sodium hydrogen carbonate solution, and water, dried ($MgSO_4$), and evaporated to give colourless plates (5·8 g., 79%), m.p. 87—93°, which were sublimed at 100°/0·1 mm., recrystallised from light petroleum (b.p. 80—100°), and resublimed to give the *dihydroisocoumarin* as colourless plates, m.p. 97·5—100° (Found: C, 56·6; H, 4·3; Cl, 16·7. $C_{10}H_9ClO_3$ requires C, 56·5; H, 4·25; Cl, 16·7%), $\lambda_{max.}$ 216, *235*, *245*, and 326 nm. (10^{-3} ε 22·3, 5·16, 4·5, and 3·79), $\nu_{max.}$ 695, 750, 800, 1115, 1205, 1225, 1465, 1610, and 1670 cm.$^{-1}$.

5-Chloro-7-chloromethyl-3,4-dihydro-8-hydroxy-3-methylisocoumarin (IX).—The crude 5-chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin (5·8 g.) was dissolved in chloromethyl methyl ether (100 ml.) and the solution was cooled to 0°. Titanium(IV) chloride (13 ml.) was added, and the resulting reddish-brown solution was stirred at 0° for 1 hr. It was then allowed to warm to room temperature during 1 hr., and heated under reflux for 6 hr. Most of the solvent was removed under reduced pressure. To the remaining solution was added 2N-hydrochloric acid (in excess). The resulting mixture was extracted with ether and the extract was washed with water, sodium hydrogen

carbonate solution, and water, dried ($MgSO_4$), and evaporated to give a brown solid. This was sublimed at 90°/0·1 mm. to give colourless needles (5·6 g., 79%). The product was recrystallised from light petroleum (b.p. 80—100°) and resublimed to give the *chloromethyl compound* as colourless crystals, m.p. 110—113° (Found: C, 50·85; H, 3·5; Cl, 27·1. $C_{11}H_{10}Cl_2O_3$ requires C, 50·6; H, 3·85; Cl, 27·15%), $\lambda_{max.}$ 215, *246*, and 330 nm. (10^{-3} ε 26·8, 4·69, and 4·98), $\nu_{max.}$ 730, 770, 1040, 1125, 1170, 1315, 1430, 1610, and 1680 $cm.^{-1}$, τ ($CDCl_3$) −1·6 (1H, s, OH), 2·3 (1H, s, ArH), 5·3 (2H, s, $ClCH_2$), *ca.* 7·0 (2H, q, $ArCH_2{\cdot}CHMe$), and 8·4 (3H, d, *J* 6 Hz, Me); the signal for the single methine proton was not observed.

5-Chloro-7-formyl-3,4-dihydro-8-hydroxy-3-methylisocoumarin (X).—A solution of 5-chloro-7-chloromethyl-3,4-dihydro-8-hydroxy-3-methylisocoumarin (5·6 g.) in chloroform (10 ml.) was added to a solution of hexamine (4·8 g.) in chloroform (80 ml.) and the combined solution was heated under reflux for 2 hr. During this time the hexaminium salt was precipitated. The chloroform was removed *in vacuo* and a solution of the residue in 50% aqueous acetic acid (60 ml.) was heated under reflux for 1 hr. To the cooled solution was added 2N-hydrochloric acid (50 ml.). The resulting mixture was extracted with ether and the extract was washed with water, sodium hydrogen carbonate solution, and water, dried ($MgSO_4$), and evaporated to give a yellow resin. This was sublimed at 120°/0·1 mm. to give colourless crystals (2·5 g., 48%). The *aldehyde* was purified by recrystallisation from benzene–light petroleum (b.p. 60—80°) and a second sublimation to give colourless prisms, m.p. 142·5—147° (decomp.) (Found: C, 54·95; H, 3·8; Cl, 14·7. $C_{11}H_9ClO_4$ requires C, 54·9; H, 3·75; Cl, 14·75%), $\lambda_{max.}$ 210, 223, *240*, 343, *347*, and 412 nm. (10^{-3} ε 20·6, 26·1, 8·7, 5·21, 5·1, and 0·132), $\nu_{max.}$ 680, 810, 825, 1035, 1140, 1180, 1260, 1300, 1390, 1435, 1610, and 1670 $cm.^{-1}$; *phenylhydrazone* [crystals from methanol–ethanol (1 : 1)], m.p. 212—213° (Found: C, 61·65; H, 4·5; N, 8·3. $C_{17}H_{15}ClN_2O_3$ requires C, 61·75; H, 4·55; N, 8·45%).

5-Chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin-7-carboxylic Acid (XI).—A suspension of the formylisocoumarin (2·5 g.) in an aqueous solution (300 ml.) of silver nitrate (4·4 g.) was heated under reflux and 2N-sodium hydroxide solution (170 ml.) was added slowly. The resulting mixture was heated under reflux for 1 hr., cooled, and filtered. The residue was washed with water and then 2N-sodium hydroxide, and the combined filtrate and washings were acidified (concentrated hydrochloric acid) and extracted with chloroform. The extract was washed with water, dried ($MgSO_4$), and evaporated to give a buff solid, which gave the *acid* as colourless prisms (1·7 g., 64%), m.p. 246—249° (decomp.) (from acetone–ethanol) (Found: C, 52·0; H, 3·7; Cl, 13·25. Calc. for $C_{11}H_9ClO_5$: C,51·5; H, 3·55; Cl, 13·8%), $\lambda_{max.}$ 217, *227*, *246*, and 336 nm. (10^{-3} ε 32·09, 15·87, 5·63, and 6·44), $\nu_{max.}$ 720, 820, 1120, 1170, 1190, 1210, 1355, 1395, 1430, 1600, 1665, 1700, and 1730 $cm.^{-1}$.

A sample of the (−)-acid, m.p. 237—239°, was obtained [3] by acid hydrolysis of natural ochratoxin A. The synthetic (±)-acid and the (−)-acid had virtually identical i.r. spectra (Unicam SP 100) in chloroform solution ($\nu_{max.}$ 1135, 1440, 1610, 1677, and 1744 $cm.^{-1}$), and the same R_F values (in parentheses) when chromatographed on thin layers of silica in the following systems: (i) toluene–ethyl acetate–formic acid (5 : 4 : 1 v/v) (0·49); (ii) ethyl methyl ketone–formic acid (9 : 1) (0·52); (iii) ethyl acetate–formic acid (9 : 1) (0·56). The acid spots were detected by their blue-green fluorescence in u.v. light.

(−,±)*Ochratoxin A*.—The (±)-isocoumarin carboxylic acid (26 mg.) was dissolved in tetrahydrofuran (20 ml.) and L-(−)-phenylalanine t-butyl ester [11] (22 mg.) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (25 mg.) were then added. The solution was stirred at room temperature for 15 hr., filtered, and evaporated *in vacuo* at room temperature. The residue was dissolved in glacial acetic acid (0·5 ml.) and the solution was cooled to 10°. A cooled solution of 45% hydrogen bromide in acetic acid was then added and the mixture was shaken at this temperature for 5 min. Cold ethyl acetate (100 ml.) was added and the resulting solution was washed with cold sodium acetate solution (until the pH of the washings was >4) and then with water (2 × 20 ml.). The solution was dried ($MgSO_4$) and evaporated *in vacuo* at room temperature to give a colourless glass which was dissolved in benzene at 30°. (Since it has been reported [12] that solutions of ochratoxin A, when overheated, give rise to insoluble material, care was taken to keep temperatures below 35°.) The solution was filtered and most of the solvent was removed at 30° in a stream of nitrogen. Sometimes *unsolvated* (−,±)*-ochratoxin A* crystallised spontaneously as colourless prisms (20 mg., 50%), m.p. 240—243° (decomp.) (Found: C, 59·3; H, 4·5; N, 3·1%; M^+, 403/405. Calc. for $C_{20}H_{18}ClNO_6$: C. 59·5; H, 4·5; N, 3·65%; *M*, 403·8) $\lambda_{max.}$ (MeOH), 214, 334, and 373 (disappeared in acid) nm. (10^{-3} ε 36·2, 5·6, and 1·27), $\nu_{max.}$ ($CHCl_3$) (SP 100 instrument) 1138, 1429, 1537, 1612, 1676, and 1736 $cm.^{-1}$. The corresponding values for unsolvated natural ochratoxin A were: m.p. 169—172°, $\lambda_{max.}$ (MeOH) 211, 334, and 379 (disappeared in acid) nm. (10^{-3} ε 34·65, 4·0, and 3·3), $\nu_{max.}$ ($CHCl_3$) (SP 100), 1146, 1445, 1542, 1613, 1678, and 1737 $cm.^{-1}$.

If crystallisation did not occur spontaneously, the solution of ochratoxin in benzene was seeded with the natural toxin; *solvated* (−,±)*-ochratoxin* A then crystallised as colourless needles (16 mg., 35%), m.p. 71—75°, $[\alpha]_D^{22}$ +38° (*c* 0·1) (Found: C, 63·0; H, 5·25; N, 2·95. Calc. for $C_{20}H_{18}ClNO_6,C_6H_6$: C, 63·3; H, 5·25; N, 3·05%), $\lambda_{max.}$ *216*, *245*, and 332 nm. (10^{-3} ε 39·8, 8·9, and 5·9), $\nu_{max.}$ ($CHCl_3$) (SP 100) 1139, 1428, 1536, 1610, 1651, 1676, 1728, and 3389 $cm.^{-1}$. The corresponding values for natural ochratoxin A benzene solvate were: m.p. 86—89°, $\nu_{max.}$ ($CHCl_3$) (SP 100) 1140, 1428, 1537, 1611, 1651, 1676, 1735, and 3390 $cm.^{-1}$.

The t.l.c. behaviour of the (−, ±)- and (−, −) forms of ochratoxin A (detectable by their green fluorescence under u.v. light) is recorded in the Table.

Adsorbent	Solvent	R_F of synthetic (−,±)-ochratoxin A	R_F of natural (−,−)-ochratoxin A
Silica	Benzene–ethyl acetate–formic acid (70 : 30 : 1)	0·50 0·43	0·50
Silica	Benzene–acetone–formic acid (80 : 20 : 1)	0·49 0·46	0·46
Silica	Chloroform–acetone–formic acid (80 : 20 : 1)	0·65 0·61	0·65
Silica prepared with 0·25 M-oxalic acid	Di-isopropyl ether	0·35 0·27	0·35

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Separation of the (−, −)- *and* (−, +)-*Forms of Synthetic Ochratoxin A*.—Twelve t.l.c. plates (20 × 20 cm.) were each coated with a slurry of silica (8 g.) in ethanol–water (9 : 1), dried at room temperature, and then dried at 100° for 15 min. A solution of (−, ±)-ochratoxin A (2 mg.) in chloroform was applied to each plate and the chromatograms were developed with benzene–ethyl acetate–formic acid (70 : 30 : 1). The plates were dried at room temperature and the bands at R_F 0·50 and 0·45 were removed and extracted by shaking the adsorbent with acetone–acetic acid (10 : 1; 25 ml.) for 30 min. The solutions were filtered and evaporated *in vacuo* at room temperature. The extract of the upper (−, −) band was applied to a second set of plates and the process was repeated. The residue left after evaporation of the solvents was red. A solution of this residue in benzene was decolourised by chromatography on a short column of silica, the toxin being eluted with benzene–chloroform–acetic acid (12 : 3 : 1). The eluate was evaporated and the residue was crystallised from benzene, as already described, to give benzene-solvated (−, −)-ochratoxin A (5 mg.), m.p. 82—89° (unaltered by admixture with benzene-solvated natural toxin), $[\alpha]_D^{22}$ −66° (*c* 0·055), o.r.d. (*c* 0·010): [ϕ] values at 416, 370, 344, 323, 307, 288, and 266 nm. −400, −1000, −2800, 0, +1600, 0, and −5700°, respectively. Natural ochratoxin A (benzene-solvate) had m.p. 86—89°, $[\alpha]_D^{22}$ −93° (*c* 0·1), o.r.d. (*c* 0·007): [ϕ] values at 416, 370, 344, 321, 307, 292, and 266 nm. −600, −1300, −3200, 0, +1200, 0, and −7000°, respectively.

The sample of synthetic ochratoxin A may be calculated, from a knowledge of its specific rotation and of the specific rotations (all D-line) of (i) natural ochratoxin and (ii) the mixture of diastereoisomeric ochratoxins A, to be about 90% purity. (The validity of this calculation depends on the assumption that the diastereoisomeric forms had been produced in essentially equimolecular quantities.)

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