Carbon-13 Nuclear Magnetic Resonance Assignments and Biosynthesis of the Mycotoxin Ochratoxin A

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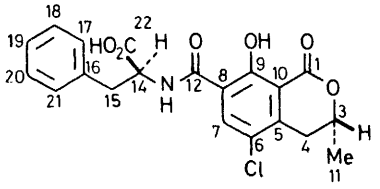
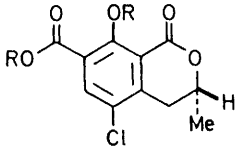
The ^{13}C n.m.r. spectrum of ochratoxin A has been assigned. The distribution of ^{13}C label and of intact acetate units, as determined from the ^{13}C n.m.r. spectra of ochratoxin A derived from singly and doubly labelled [^{13}C]acetate, defines the biosynthetic origin of the 3,4-dihydro-(3*R*)-methylisocoumarin moiety unequivocally.

THE discovery [1] of toxigenic strains of the fungus *Aspergillus ochraceus* Wilh. led to the isolation and structure elucidation of ochratoxin A (1), a potent nephro- and hepato-toxic metabolite.[2]

Ochratoxin A comprises a 3,4-dihydro-(3*R*)-methylisocoumarin moiety which is linked to L-β-phenylalanine through a carboxy-group. The intact incorporation of DL-[U-^{14}C]- [3,4] and [1-^{14}C]-β-phenylalanine [5] firmly established the direct role of this amino-acid as a precursor for the biosynthesis of ochratoxin A. The origin of the 3,4-dihydro-(3*R*)-methylisocoumarin *via* the acetate–polymalonate pathway was indicated by feeding experiments with [1-^{14}C]- [3] and [2-^{14}C]-acetate [4] and [2-^{14}C]-malonate.[4] The participation of a C_1 unit in the formation of the carboxy-group at C(8) was established through addition of DL-[*methyl*-^{14}C]methionine to a resting culture of *A. ochraceus*.[3] This result was subsequently verified by feeding experiments with sodium [^{13}C]formate.[4] The ^{13}C-enriched ochratoxin A was hydrolysed to the acid (2) which was converted to the ester (3) by treatment with diazomethane. The proton-noise-decoupled (p.n.d.) ^{13}C n.m.r. spectrum of (3) indicated that only the signal at δ 165.1 (relative to tetramethylsilane), which had been assigned to C(12), was enhanced.[4]



(1)



(2) R = H
(3) R = Me

The present biosynthetic study of ochratoxin A was undertaken to determine the labelling pattern of ochratoxin A derived from acetate as well as the folding of the original polyketide. At the same time the proposal of a phenylpropanoid precursor for the carbon atoms of the lactone ring as suggested by Searcy *et al.*[5] could be investigated.

The 100-MHz ^1H n.m.r. spectrum of ochratoxin A in $CDCl_3$ exhibited signals due to three exchangeable protons at δ 12.70 (s, 9-OH), 10.80 (s, CO_2H), and 8.55 (d, *J* 7.1 Hz, NH). The C(7) proton appeared as a singlet at δ 7.23 while the singlet at δ 7.15 was attributed to the five protons of the phenyl ring. The multiplets at δ 4.71 and 5.07 were assigned to the C(3) and C(14) methine protons, respectively. The C(4) methylene protons appeared as the AB part of an ABX system with δ_A 2.78 (J_{AB} 17.5, J_{AX} 11.3 Hz) and δ_B *ca.* 3.2, the latter overlapping with the signal due to the C(15) methylene protons. The protons of the methyl group appeared as a doublet at δ 1.55 (*J* 6.2 Hz).

The ^{13}C n.m.r. data for ochratoxin A obtained from p.n.d. and nuclear Overhauser enhanced (n.O.e.) spectra are given in the Table. In the assignment of the ^{13}C spectra use was made of the observed directly bonded [^1J(C,H)] and over more than one bond [$^{>1}J$(C,H)] carbon-13–proton coupling constants, deuterium isotope shifts,[6] and of techniques such as off-resonance proton

^{13}C Chemical shifts, directly bonded [^1J(C,H)] and over more than one bond [$^{>1}J$(C,H)] coupling constants of ochratoxin A (1), and coupling constants [^1J(C,C)] of [1,2-^{13}C]acetate-enriched ochratoxin A (*J*/Hz)

Carbon atom	δ_C [a]	^1J(C,H)	$^{>1}J$(C,H)	^1J(C,C)
1	169.6 S			67.0
3	75.9 Dqn	149.7	4.8	39.9
4	32.2br Td	131.9	4.6	42.1
5	141.0 Sqd		6.2, 2.1	42.5
6	123.2 Sq		3.4	67.5
7	138.9 D	168.9		67.3
8	120.3 Sdd		4.7, 3.0	68.9
9	159.1 Sdd		8.4, 3.5	68.7
10	110.1br S			67.3
11	20.6 Q	128.3		40.0
12	163.0br S			
14	54.3br D	144.5		
15	37.5br T	130.3		
16	135.9 Sm			
17, 21	129.4 Dt	158.9	5.7	
18, 20	128.6 Dd	159.5	5.4	
19	127.2 Dtt	160.8	8.0, 4.0	
22	174.9br S			

[a] Relative to internal Me_4Si. Capital letters refer to the pattern resulting from directly bonded protons and lower-case letters to (^{13}C,H) couplings over more than one bond. S = singlet, D or d = doublet, T or t = triplet, Q or q = quartet, qn = quintet, m = multiplet, br = broad.

decoupling, selective proton decoupling, and selective population inversion (SPI).[7]

Off-resonance proton decoupling experiments distinguished between the methyl, the two methylene, the two aliphatic methine, the six aromatic methine, and the nine quaternary carbon atoms.

The assignment of the signals at δ 37.5 and 32.2 to the methylene carbon atoms C(15) and C(4), respectively, follows from the chemical shift of the methylene carbon atom in phenylalanine (δ 37.5).[8] Selective decoupling of the methyl protons sharpened the C(4) signal (confirming the above assignments) and changed the signal at δ 75.9 from a doublet of quintets to a doublet of doublets. The aliphatic methine signals at δ 75.9 and 54.3 must therefore be assigned to C(3) and C(14), respectively, in agreement with chemical-shift considerations.

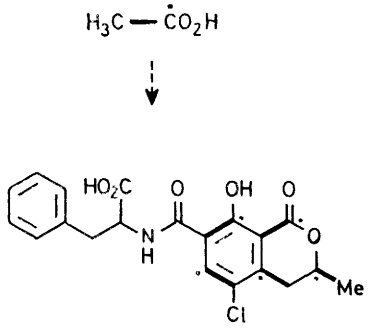
The six aromatic methine carbon resonances were assigned from the values of the observed (C,H) couplings over more than one bond. In aromatic systems these (C,H) couplings are usually in the order $^3J > {}^2J > J^4$ with values of 5—10, 1—4, and ≤1 Hz, respectively.[9] In the n.O.e. single frequency ^{13}C spectrum C(7) appeared as a doublet [δ 138.9; ^1J(C,H) 168.9 Hz], C(17) and C(21) as a doublet of triplets [δ 129.4; ^1J(C,H) 158.9, ^3J(C,H) 5.7 Hz]. C(18) and C(20) as a doublet of doublets [δ 128.6; ^1J(C,H) 159.5, ^3J(C,H) 5.4 Hz], and C(19) as a doublet of triplets of triplets [δ 127.2; ^1J(C,H) 160.8, ^2J(C,H) 4.0, ^3J(C,H) 8.0 Hz]. The resonances at δ 128.6 and 129.4 were approximately twice the intensities of the other proton-bearing aromatic resonances.

The chemical shifts of the three carbonyl atoms, C(1), C(12), and C(22) at δ 169.6, 163.0, and 174.9, respectively, were characteristic for these types of carbon atoms. Selective proton decoupling at the resonance position of the methylene protons changed the resonance at δ 174.9, assigned to C(22) to a doublet with $^{>1}J$(C,H) 5.5 Hz.

The remaining quaternary carbon resonances were assigned from SPI experiments and the $^{>1}J$(C,H) values in aromatic systems. Furthermore carbon–hydroxy-proton coupling constants [J(C,OH)] are dependent on the stereochemistry of the coupled nuclei. The three-bond coupling for a *trans*-arrangement is larger than for the *cis*-arrangement or than a two-bond (C,OH) coupling constant.[10] When a selective π-pulse with $\gamma H_2 = 5$ Hz was applied at *ca.* 5 Hz to high field of the hydrogen-bonded hydroxy-proton the carbon resonances at δ 159.1 {^2J[C(9),OH] 3.5 Hz}, 120.3 {^3J[C(8),OH] 4.7 Hz}, and 110.1 {^3J[C(10),OH] 7.3 Hz} were affected. Addition of D_2O–H_2O (1 : 1) to a compound with exchangeable protons will result in a doubling of specific ^{13}C resonances due to the isotope shifts large enough to be resolved (≥0.1 p.p.m.) provided that the hydrogen–deuterium exchange is sufficiently slow. An isotope shift of 0.17 p.p.m. was observed for the resonance at δ 159.1 after addition of D_2O–H_2O (1 : 1), thus assigning it to C(9). The signals at δ 120.3 and 110.1 were attributed to C(8) and C(10). The application of a selective π-pulse with $\gamma H_2 = 5$Hz to the high-frequency side (*ca.* 5 Hz) of the C(7) proton signal affected the resonances at δ 163.0 [C(12), ^3J(C,H) 5.2 Hz], 159.1 [C(9), ^3J(C,H) 8.4 Hz], 141.0 [C(5), ^3J(C,H) 6.2 Hz], 123.2 [C(6), ^2J(C,H) 3.4 Hz], and 120.3 [C(8), ^2J(C,H) 3.0 Hz]. The assignment of the signals due to C(5) and C(6) followed from the $^{>1}J$(C,H) values as well as chemical-shift considerations. The experiment also distinguished between C(8) and C(10) and the signal at δ 110.1 was thus assigned to C(10). The remaining quaternary carbon resonance at δ 135.9 was assigned to C(16).

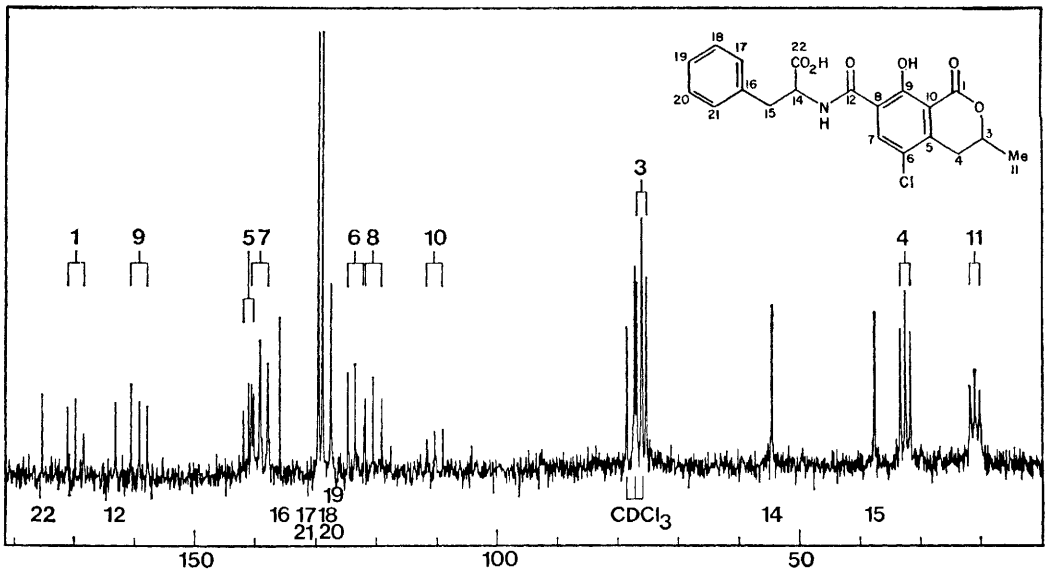
Cultures of *Penicillium viridicatum* (ATCC 18411) were grown on yeast extract-sucrose medium and supplemented with [1-^{13}C]acetate every 24 h from day 4 to day 6. The p.n.d. spectrum of the [1-^{13}]acetate-derived ochratoxin A showed five enhanced carbon signals *viz.* C(1), C(3), C(5), C(7), and C(9). The data support an acetate–polymalonate pathway with the labels occupying alternating positions for the 3,4-dihydro-(3*R*)-methylisocoumarin moiety of ochratoxin A.

The subsequent addition of [1,2-^{13}C]acetate to cultures of *P. viridicatum* proved a failure as the fungus inexplicably lost its ability to produce ochratoxin A. A culture of *A. sulphureus* (NRRL 4077) was found to produce ochratoxin A in excellent yield on a modified Czapek medium. Cultures of *A. sulphureus* were supplemented continuously for 144 h from day 4 with [1,2-^{13}C]acetate (diluted with unlabelled acetate) by using motor-driven syringes. This method of precursor addition ensures a more stable precursor pool in the medium and should result in a product with a higher specific activity. A considerable saving in precursor should also be effected. The p.n.d. ^{13}C n.m.r. spectrum of ochratoxin A derived from [1,2-^{13}C]acetate is shown in the Figure and exhibits satellite resonances due to (^{13}C–^{13}C) spin–spin couplings. The observed spin–spin coupling data (Table) indicated that C(11)–C(3), C(4)–C(5), C(6)–C(7), C(8)–C(9), and C(10)–C(1) originated from five intact acetate units arranged as shown in (4).



(4)

The above data, in conjunction with the published results of Steyn *et al.*[3] and Maebayashi *et al.*,[4] provide conclusive evidence for the biosynthesis of the 3,4-dihydro-(3*R*)-methylisocoumarin moiety of ochratoxin A *via* the acetate–polymalonate pathway, whereas the carbonyl carbon atom, C(12), is derived from the C_1 pool. The results indicate that the biosynthesis suggested by Searcy *et al.*[5] is no longer tenable. Incubation of *A.*



Proton-noise-decoupled 25.2-MHz ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived ochratoxin A in $CDCl_3$ (spectral width 5 000 Hz; 70° radiofrequency pulse of 40 μs duration; pulse delay 1 s, transients 16 461; acquisition time 0.8 s)

ochraceus in a medium containing sodium [^{36}Cl]chloride resulted in the incorporation (0.75%) of ^{36}Cl into ochratoxin A.[11] It has not been established, however, at what point in the biosynthesis the chlorine atom is introduced. It is of interest to note that 8-carboxy-3,4-dihydro-9-hydroxy-3-methylisocoumarin has been isolated together with ochratoxin A, ochratoxin B, and 4-hydroxyochratoxin A from cultures of *P. viridicatum*.[12]

EXPERIMENTAL

For instrumental data see ref. 13.

Incorporations of Sodium [1-^{13}C]- *and* [1,2-^{13}C]-*Acetate.*—Preliminary experiments on cultures of *P. viridicatum* (ATCC 18411) on yeast extract–sucrose (2% : 15%) medium (YES medium) and of cultures of *A. sulphureus* (NRRL 4077) on modified Czapek medium * showed that ochratoxin A production started on day 3 and reached a maximum after 10 days.

To each of ten 500-ml Erlenmeyer flasks containing a 4 day growth of *P. viridicatum* on YES medium (100 ml) was added [1-^{13}C]acetate (500 mg; 90% ^{13}C) every 24 h from day 4 to day 6. The mycelium was removed by filtration on day 10 and extracted with chloroform–methanol (1 : 1 v/v) in a Soxhlet apparatus. The residue from this extract was partitioned between hexane and 90% methanol. The residue from the 90% methanol layer together with the ochratoxin A containing material obtained from the chloroform extract of the culture medium were separated by preparative layer chromatography on silica gel with benzene–acetic acid (7 : 3 v/v). Crystallisation from benzene gave ochratoxin A (93 mg), m.p. 90—92° (lit.,[3] 92—94°).

To each of four 500-ml Erlenmeyer flasks containing the 4-day old growth of *A. sulphureus* (NRRL 4077) on the modified Czapek medium (100 ml) was added continuously by four motor-driven syringes a solution containing [1,2-^{13}C]acetate (150 mg) and sodium acetate (350 mg) over 144 h. On day 10 the cultures were processed as described above to give ochratoxin A (60 mg).

* Modified Czapek medium (per litre): sucrose (30.0 g), sodium nitrate (2.0 g), potassium dihydrogenphosphate (1.0 g), magnesium sulphate (0.5 g), potassium chloride (0.5 g), urea (30 g), corn steep liquor (10 ml), iron(II) sulphate (10 mg).

We thank Dr. E. B. Lillehoj for a culture of *A. sulphureus*.

[9/140 *Received*, 30*th January*, 1979]

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