Biosynthesis of Austocystin D in *Aspergillus ustus*. A Carbon-13 Nuclear Magnetic Resonance Study

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The assignment of the natural-abundance ^{13}C n.m.r. spectrum of austocystin D, a mycotoxin produced by *Aspergillus ustus* MRC 1163, allowed a study of its biosynthetic origin. The complete ^{13}C–^{13}C coupling pattern in austocystin D enriched from [1,2-$^{13}C_2$]acetate has been determined and confirms that no randomisation of labelling in ring A occurs during the anthraquinone ⟶ xanthone conversion in the biosynthetic pathway.

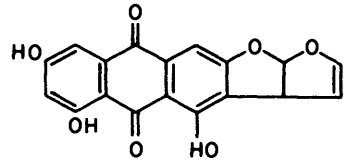
The acute awareness of the importance of mycotoxins to human and animal health arose from the discovery of a group of potent hepatocarcinogens (the aflatoxins) in 1960.[1] It subsequently became evident that the biological activity of the aflatoxins, their biosynthetic congeners, *e.g.* versicolorin A (1) and sterigmatocystin (2),[2] and the products of their *in vivo* metabolism, *e.g.* aflatoxin M_1, could be related to the presence of the bisdihydrofuran moiety.[3]

Steyn and Vleggaar [4,5] reported the structure elucidation of the austocystins A—I, dihydrofuro[3′,2′:4,5]furo[3,2-*b*]xanthone † metabolites isolated from cultures of *Aspergillus ustus*, CSIR 1128 grown on maize. These xanthones possess a linear fusion of the xanthone and bisdihydrofuran moieties in contrast to the closely related metabolite sterigmatocystin (2) which has an angular fusion. The strain of *A. ustus* MRC 1163 used in the present study proved a rich source of metabolites and, when grown on maize, produces the austalides [6] and the biosynthetically related compounds averufin, averufanin, versicolorin C, 8-deoxy-6-*O*-methylversicolorin A, three differently substituted 3a,12a-dihydro-5*H*-furo[3′,2′:4,5]furo-[3,2-*b*]xanthen-5-one compounds, 7-chloro-2-(3-furyl)-1,3,8-trimethoxyxanthone, and austocystin A, B (3), D (4), and H.[7]

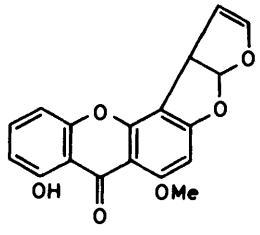
The unambiguous assignment of the signals in the proton-noise-decoupled (p.n.d.) natural-abundance ^{13}C n.m.r. spectrum of austocystin B (3) and D(4) and the biosynthetic origin of the carbon framework of this family of compounds are presented in this report. The ^{13}C n.m.r. data for the bisdihydrofuran moiety of austocystin D (4) serves as a unique model for the corresponding structure in the rare mycotoxins aflatoxin M_1[8] and aspertoxin.[9]

The assignments of the ^1H n.m.r. spectral signals of austocystin D (4) as presented in the Table are based on proton–proton coupling patterns, chemical shift considerations, and nuclear Overhauser enhancement (n.O.e.) experiments on 3a,4,6-tri-*O*-methylaustocystin D.[4] An unambiguous assignment of the 11- and 12a-proton in austocystin D could only be achieved by heteronuclear $^{13}C\{^1H\}$ selective population inversion (SPI) experiments.[10]

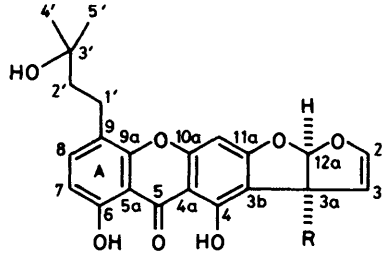
The results obtained from single-frequency n.O.e., p.n.d., and off-resonance proton-decoupled spectra, SPI experiments,[10] and the chemical shift values and (C,H)-coupling constants of related compounds, *viz.* tajixanthone (5),[11] sterigmatocystin (2),[12] and versicolorin A (1),[13] led to the assignment of the signals in the natural-abundance ^{13}C n.m.r. spectrum of austocystin D (see Table). The reported deuterium isotope shifts [14] are the separations between doubled signals observed in the p.n.d. ^{13}C n.m.r. spectrum when the exchangeable protons were partially exchanged with deuterium upon addition of a mixture of ^2H_2O–^1H_2O (1 : 1). The main parameters used in SPI studies [10] are (C,H)-couplings, and normally only one-, two-, and three-bond (C,H)-couplings are observed. The reported (C,H)-couplings over four bonds are *ca.* 1 Hz,[15] and need not be considered at the power levels used in the SPI experiments (*ca.* 5 Hz).



(1)



(2)



(3) R = H
(4) R = OH

Single-frequency n.O.e. and off-resonance proton-decoupled ^{13}C n.m.r. spectra revealed that the twenty-two carbon resonances observed in the p.n.d. ^{13}C n.m.r. spectrum of austocystin D (4) are due to two methyl, two methylene, six methine, and twelve quaternary carbon atoms.

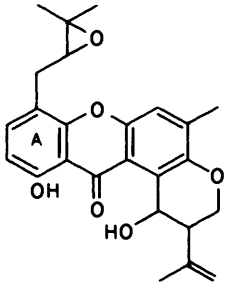
The residual splittings observed in a series of off-resonance proton-decoupled ^{13}C experiments enabled us to correlate the signals of the proton-bearing carbon atoms with specific proton resonances.[16] With this relationship the resonances due to C-3 and C-7 were unambiguously assigned.

† Xanthone is xanthen-9-one.

Table. N.m.r. data for austocystin B (3) and D (4)

Carbon atom	(4) δ_C[a]	^1J(CH)/Hz	$^{>1}J$(CH)/Hz	^1J(CC)/Hz[b]	δ_H	J(HH)/Hz	(3) δ_C[a]
2	148.8 Ddd	196.6	12.1, 5.1	75.2	6.79d	3.0	145.9
3	107.3 Dd (br)	180.3	11.9	75.9	5.76d	3.0	103.2
3a	89.8 Sd (br)		6.8	39.6			47.9
3b	112.3 S (br)			60.3			110.7
4	158.5 S (br)			63.0			157.7
4a	103.7 Sm			62.8			103.6
5	185.5 S			56.5			185.7
5a	107.3 Sd (br)		5.8	56.3			107.8
6	159.5 Sddd		10.7, 4.9, 2.6	66.4			159.9
7	111.0 Ddd	161.3	7.6, 3.7	66.4	6.70d	8.5	111.0
8	138.0 Dt	160.5	4.3	59.1	7.56d	8.5	138.2
9	121.3 Sm			59.1			121.7
9a	153.5 Sdt		11.3, 4.2				154.0
10a	158.9 Sd		3.9	73.5			158.8
11	90.9 D	170.3		74.4	6.53s		90.6
11a	167.4 St		3.8	60.1			166.6
12a	118.6 Dt	184.2	6.2	39.6	6.43s		114.5
1′	24.5 Tm	128.7		34.3	2.67—2.90m		24.8
2′	44.5 Tm	122.5		34.5	1.56—1.83m		44.9
3′	70.2 S (br)			39.1			70.1
4′[c]	29.3 Qm	125.0		39.3	1.27s		29.6
5′[c]	29.2 Qm	125.0		38.7	1.27s		29.6

[a] Recorded on a Varian XL-100 spectrometer at 25.1 MHz. Values are relative to internal Me_4Si; solvent $(CD_3)_2CO$. Capital letters refer to the pattern resulting from directly bonded (C,H) couplings [^1J(CH)] and small letters to that from (C,H) couplings over more than one bond [$^{>1}J$(CH)]. [b] Obtained from the p.n.d. ^{13}C n.m.r. spectrum of austocystin D derived from [1,2-$^{13}C_2$]acetate recorded on a Bruker WM-500 spectrometer at 125.76 MHz; solvent $(CD_3)_2CO$. [c] These resonances are obscured by the $(CD_3)_2CO$ signals. The chemical shift and ^1J(CC) values were obtained from the spectrum recorded in $CDCl_3$ on a Bruker WH-360 spectrometer at 90.56 MHz. The assignments may be interchanged.



(5)

The resonances of the carbon atoms of the bisdihydrofuran moiety were assigned by comparison of the chemical shift and one-bond (C,H) values with the corresponding carbon atoms in sterigmatocystin (2)[12] and versicolorin A (1).[13] The resonances at δ_C 148.8 [^1J(CH) 196.6 Hz] and 118.6 p.p.m. [^1J(CH) 184.2 Hz] could therefore be assigned to C-2 and C-12a, respectively. The directly bonded (C,H) coupling constant of 184.2 Hz for the δ_C 118.6 p.p.m. resonance distinguishes it from the aromatic proton-bearing carbon resonances. As a consequence the resonance at δ_C 90.9 p.p.m. [^1J(CH) 170.3 Hz] could be allocated to C-11. In an SPI experiment, a π-pulse with γH_2 5 Hz applied at a position 6 Hz downfield from the low-field transition of the doublet due to 2-H (δ_H 6.79) affected the signals at δ_C 107.3 (C-3) and 89.8 p.p.m. (C-3a).

Chemical shift considerations dictate that the resonance at δ_C 185.5 p.p.m. must be attributed to the C-5 carbonyl carbon atom whereas the six resonances in the δ_C 148—168 p.p.m. region are due to the six oxygen-bearing sp^2 carbon atoms. Application of a selective π-pulse (γH_2 5 Hz) to the low-field ^{13}C transition of 8-H (δ_H 7.56) affected the resonance at δ_C 138.0 p.p.m. thereby confirming the assignment of C-8. Similarly, the application of a π-pulse (γH_2 5 Hz) to the high-field transition of the 8-H doublet at δ_H 7.56 affected the resonances at δ_C 159.5 and 153.5 p.p.m. which are therefore due to C-6 and C-9a. The observed deuterium isotope shift of −0.20 p.p.m. for the signal at δ_C 159.5 p.p.m. assigns this resonance to C-6 and that at δ_C 153.5 p.p.m. is therefore due to C-9a.

When a π-pulse (γH_2 5 Hz) was applied at a position 2 Hz downfield from the singlet at δ_H 6.53 the resonances at δ_C 158.9 and 167.4 p.p.m. due to C-10a and C-11a, as well as those at δ_C 103.7 and 112.3 p.p.m. due to C-4a and C-3b, were affected. The transitions which were selectively pulsed must arise from 11-H as the carbon atoms are all either two or three bonds removed from this proton. Conversely, selective irradiation of the transitions of the proton at δ_H 6.43 in a $^{13}C\{^1H\}$ SPI experiment affected the resonance at δ_C 167.4 p.p.m. The results of the above two SPI experiments allow an unambiguous assignment of 11-H (δ_H 6.53) and 12a-H (δ_H 6.43) and show that the resonance at δ_C 167.4 p.p.m. must be due to C-11a as this carbon atom is both two bonds removed from 11-H and three bonds from 12a-H. As a consequence the resonance at δ_C 158.9 p.p.m. could be allocated to C-10a. The assignment of the resonances at δ_C 103.7 and 112.3 p.p.m. to C-4a and C-3b, respectively, follows from a

comparison of the chemical shift values of the corresponding carbon atoms in austocystin B (3) (see Table) and was confirmed by the ^1J(CC) values in the p.n.d. ^{13}C spectrum of austocystin D derived from [1,2-$^{13}C_2$]acetate (see Table).

The resonance of the remaining oxygen-bearing sp^2 carbon atom at δ_C 158.5 p.p.m. is, by elimination, assigned to C-4, an assignment which is confirmed by the observed deuterium isotope shift of 0.20 p.p.m. for this resonance upon addition of ^2H_2O–^1H_2O (1 : 1) to the sample.

Exchange of the phenolic hydroxy-group protons for deuterium caused the resonance at δ_C 111.0 p.p.m., due to C-7, to change its fine structure as the 7.6 Hz splitting was now absent. In addition the resonances at δ_C 107.3, 112.3 (C-3b), and 103.7 p.p.m. (C-4a) were simplified. The resonance at δ_C 107.3 p.p.m. must be due to C-5a and this was confirmed by an SPI experiment. Application of a π-pulse (γH_2 5.0 Hz) to the 7-H transitions affected *inter alia* the resonances at δ_C 107.3 and 121.3 p.p.m., which could thus be allocated to C-5a and C-9, respectively. This result shows that the assignment of the corresponding carbon atoms in tajixanthone [14] should be interchanged.

The resonances of the carbon atoms of the 3-hydroxy-3-methylbutyl side-chain were assigned on the basis of the chemical shift values and multiplicities in the single-frequency n.O.e. spectrum.

Biosynthetic Studies.—Cultures of *A. ustus* (MRC 1163) were grown in static culture on a modified Czapek–Dox medium. Studies on the course of fermentation indicated that austocystin D production commenced on day 7 and reached a maximum fourteen days after inoculation of the medium. Preliminary feeding experiments with [1-^{14}C]-acetate as the precursor established the optimum conditions for ^{13}C enrichment at each individual acetate-derived carbon atom of austocystin D on feeding ^{13}C-labelled acetate, as well as the yield of the metabolite. A good yield of austocystin D and a good incorporation (0.17%) of precursor but high dilution values (105, assuming eleven labelled positions) were obtained by feeding cultures of *A. ustus* every 12 h from day 7 to day 14 with sodium acetate to a total amount of 1.0 g l^{-1}.

The p.n.d. spectrum of [1-^{13}C]acetate-derived austocystin D showed eleven enhanced carbon signals, *viz.* those due to C-3, -4, -5, -6, -8, -9a, -10a, -11a, -12a, -1′, and -3′. The arrangement of intact acetate units in austocystin D was studied by addition of [1,2-$^{13}C_2$]acetate to cultures of *A. ustus*. The measured ^1J(CC) values obtained from the p.n.d. ^{13}C n.m.r. spectrum of austocystin D derived from [1,2-$^{13}C_2$]acetate are given in the Table and prove the presence of the following intact acetate units: C-2–C-3, C-3a–C-12a, C-3b–C-11a, C-4–C-4a, C-5–C-5a, C-6–C-7, C-8–C-9, C-10a–C-11, C-1′–C-2′, and C-3′–C-4′. It is of interest to note that the C-5′ resonance also exhibits low-intensity satellite signals due to (C,C)-coupling with C-3′ as a result of multiple labelling.

The resonances of the carbon atoms of ring A show only a single one-bond (C,C)-coupling apart from C-9a which appears as a single, enriched resonance with no evidence of (C,C)-coupling. This result implies that no randomisation of labelling occurs in ring A during the biosynthesis of austocystin D and that a symmetrical intermediate in the formation of the xanthone system from an anthraquinone precursor can be excluded. A similar conclusion has been reported for sterigmatocystin (2).[2,17] Randomisation of labelling in the anthraquinone ⟶ xanthone conversion has been observed for both tajixanthone (5) [18] and ravenelin [19] and means that ring A must have been symmetrical and free to rotate at some stage during the biosynthesis of these compounds.

Experimental

M.p.s were determined on a Kofler hot-stage apparatus. ^{13}C N.m.r. spectra were recorded, unless stated otherwise, on a Varian XL-100-15 F.T. spectrometer equipped with a 16K Varian 620i computer and a gated gyrocode decoupler. ^1H N.m.r. spectra (90 MHz) were recorded on a Varian EM 390 spectrometer.

Incorporation of Labelled Precursors.—Preliminary experiments on cultures of *A. ustus* (MRC 1163) grown in static culture on a modified Czapek–Dox medium [20] showed that austocystin D production commenced on day 7 and reached a maximum 14 d after inoculation of the medium.

To each of ten 500-ml Erlenmeyer flasks containing the 7-day old growth of *A. ustus* on the modified Czapek—Dox medium (100 ml) was added sodium [1-^{13}C]acetate (90 atom% ^{13}C; 1.0 g in total) each 12 h over a period of 6 d from day 7 to day 13. The mycelium was harvested on day 14, macerated with acetone in a Waring blender, and filtered. The filtrate was evaporated and the residue partitioned between aqueous methanol (90%) and n-hexane. The methanol solution was concentrated and the residue was partitioned between chloroform and water. The chloroform solution was evaporated to give a residue (524 mg) which contained austocystin D. The austocystin D was separated from the crude material by column chromatography on silica gel with chloroform–methanol (98 : 2 v/v) as eluant. Crystallization from benzene gave austocystin D (4) (14 mg), m.p. 113—115 °C (lit.,[4] 114—116 °C).

In a separate experiment when sodium [1,2-$^{13}C_2$]acetate (C-1: 93.1 atom%; C-2: 92.0 atom%) (1.0 g) was used as precursor, 10.5 mg of austocystin D were obtained.

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