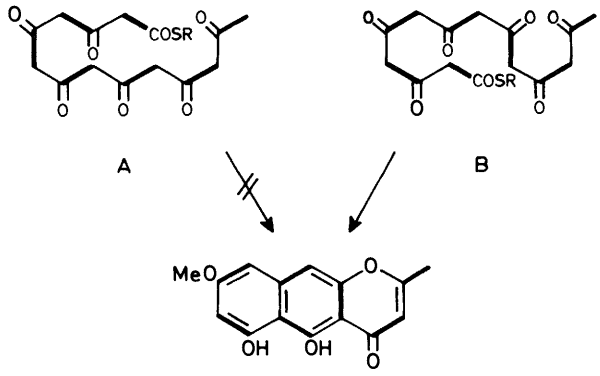
The Biosynthesis of Rubrofusarin, a Polyketide Naphthopyrone from *Fusarium culmorum*: ^{13}C N.M.R. Assignments and Incorporation of ^{13}C- and ^{2}H-Labelled Acetates

Finian J. Leeper and James Staunton *
University Chemical Laboratories, Lensfield Road, Cambridge CB2 1EW

The polyketide chain in rubrofusarin biosynthesis has been shown to adopt the folding pattern B by the incorporation of $^{13}CH_3{}^{13}CO_2Na$ and of $CD_3{}^{13}CO_2Na$, with observation of the β-isotopic shifts in the ^{13}C n.m.r. spectrum, in the rubrofusarin derivative (**5**). The extent of retention of deuterium at the various sites is interpreted in terms of an unusual sequence of biosynthetic steps. The assignment of the ^{13}C n.m.r. spectrum of rubrofusarin dimethyl ether (**2**) was made with the aid of specific deuteriation and ^{1}H–^{13}C n.O.e.'s as well as more standard techniques.

Rubrofusarin (**1**), a metabolite of *Fusarium culmorum*, was first isolated by Ashley, Hobbs, and Raistrick in 1937[1] and its structure finally established in 1961.[2] It has an unelaborated polyketide skeleton which could arise biosynthetically by appropriate folding and cyclisation of an enzyme-bound hexaoxoester. Several textbooks have suggested that the folding pattern is A (Scheme 1)[3] as did Mock and Robbers[4] who

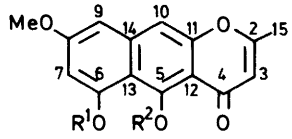


Scheme 1.

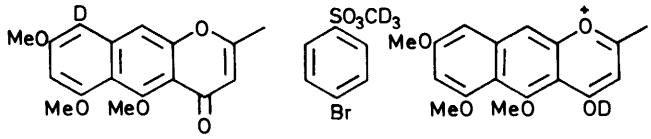
showed that [1-^{14}C]acetate is incorporated into the terminal C_2 unit in the manner expected for this biosynthetic scheme. There is, however, an alternative folding pattern, B, which is equally plausible. We decided to investigate the folding pattern not only for its intrinsic interest but also because a compound with the rubrofusarin skeleton has been proposed as a precursor to citromycetin (**18**) for which the intact acetate pattern is known[5] (see later), and because we recently reported a biomimetic synthesis of a methyl ether of rubrofusarin modelled on pathway B.[6] Some of the results have been reported in preliminary form.[7]

Results

When a strain of *F. culmorum* (C.M.I. 89364) was grown in still culture by the original method,[1] rubrofusarin was only produced in trace amounts, the major product being aurofusarin, an oxidised dimer of rubrofusarin. However in shaken culture the organism was found to produce a reasonable quantity of rubrofusarin (20—100 mg/l). Because of its low solubility, rubrofusarin was unsuitable for the proposed n.m.r. studies and so it was methylated with an excess of Me_2SO_4–K_2CO_3 in acetone to give the dimethyl ether (**2**).[8]



(**1**) $R^1 = R^2 = H$ (**4**) $R^1 = CD_3$, $R^2 = Me$
(**2**) $R^1 = R^2 = Me$ (**5**) $R^1 = H$, $R^2 = Me$
(**3**) $R^1 = R^2 = CD_3$



(**6**) (**7**) (**8**)

Spectroscopic Assignments.—To assist in the spectroscopic assignments, three deuteriated derivatives of rubrofusarin dimethyl ether (**3**), (**4**), and (**6**) were synthesised. In order to make a trideuteriomethylating agent, [$^{2}H_4$]methanol was converted into its 4-bromobenzenesulphonate (brosylate) (**7**) with 4-bromobenzenesulphonyl (brosyl) chloride and sodium hydride in tetrahydrofuran (THF).[9] Rubrofusarin was methylated with compound (**7**) and K_2CO_3 in acetone to give the bis(trideuteriomethyl) ether (**3**). Methylation of rubrofusarin with diazomethane on the other hand yields, as reported,[8] the monomethyl ether (**5**) which when further methylated with the brosylate (**7**) gave the mono(trideuteriomethyl) compound (**4**). In another deuteriation experiment, treatment of a solution of the dimethyl ether (**2**) in $CDCl_3$ with deuteriotrifluoroacetic acid (TFA) caused immediate protonation giving the deep red pyrylium salt (**8**); all the ^{1}H n.m.r. signals of this salt were shifted downfield and that at δ 5.95 [attributed to 3-H (see Table 1)] markedly so. Within a few minutes complete exchange of 9-H by deuterium had occurred. No other hydrogen atom suffered significant exchange even after several days.

The ^{1}H n.m.r. data of compound (**2**) are given in Table 1; the assignment of 9-H to the more downfield of the two *meta*-coupled doublets was confirmed by n.O.e.'s observed between this proton and 10-H. N.O.e.'s were also seen from 15-H to 3-H, and from both 7-H and 9-H to methoxy resonances. The assignment of the methoxy resonances was based on the ^{1}H n.m.r. spectra of the trideuteriomethyl ethers (**3**) and (**4**).

The assignments of some of the resonances in the ^{13}C n.m.r. spectrum of the ether (**2**), shown in Table 2, were straightforward. Thus resonances could be assigned to C-4, C-14, and C-

Table 1. ^{1}H N.m.r. data[a] for rubrofusarin methyl ethers

Proton	Dimethyl ether (2) δ (p.p.m.)	Monomethyl ether (5) δ (p.p.m.)
10	7.34 (1 H, s)	7.40 (1 H, s)
9	6.61 (1 H, d, *J* 2 Hz)	6.61 (1 H, d, *J* 2 Hz)
7	6.42 (1 H, d, *J* 2 Hz)	6.53 (1 H, d, *J* 2 Hz)
3	5.95 (1 H, s)	5.99 (1 H, s)
5-OMe	3.96 (6 H, s)	4.12 (3 H, s)
6-OMe	3.96	
8-OMe	3.90 (3 H, s)	3.89 (3 H, s)
15	2.28 (3 H, s)	2.32 (3 H, s)

[a] Relative to Me_4Si in a $CDCl_3$ solution.

Table 2. ^{13}C N.m.r. data[a] for rubrofusarin methyl ethers

	Dimethyl ether (2)		Monomethyl ether (5)	
Carbon	δ (p.p.m.)	*J* (^{13}C–^{13}C) (Hz)[b]	δ (p.p.m.)	β-Shift (p.p.m.)[c]
4	177.88	57	177.37	
2	163.67	52	164.52	0.03
8	160.65	72	161.66	0.05
5	159.64[d]	70	157.56	
6	159.60	75	157.06	0.04
11	154.75	61	154.05	
14	139.76	59	138.78	0.07
13	114.42	70	111.34	
12	113.98	61	111.01	
3	110.50	57	109.99	
10	108.29	59	108.96	
7	98.94	75	102.08	
9	97.46	72	97.03	
5-OMe	62.98		64.55	
6-OMe	56.13			
8-OMe	55.18		55.27	
15	19.68	52	19.99	

[a] Relative to Me_4Si in a $CDCl_3$ solution containing tris(acetylacetonato)chromium. [b] In (2) derived from [1,2-$^{13}C_2$]acetate; confirmed by three separate runs at 100 MHz and two at 25 MHz. [c] In (5) derived from [1-^{13}C, 2-$^2H_3$]acetate. [d] Where necessary this resonance was shifted from that at δ 159.60 p.p.m. by addition of $Eu(fod)_3$ to the sample.

15 on the grounds of chemical shift alone. The three methoxy groups were each assigned on the basis of the spectra of the deuteriated compounds (3) and (4). Specific decoupling at 10-H, 9-H, 7-H, and 3-H in turn identified the carbon to which each proton is attached. These specific decoupling experiments also identified C-2 as it showed long-range coupling to both 15-H and 3-H; C-12 was distinguished from C-13 by its coupling to 3-H as well as 10-H.

The four remaining resonances, which arise from carbons of the naphthalene nucleus having an attached oxygen (C-5, C-6, C-8, and C-11), were more difficult to assign. All four showed some coupling to nearby protons, but only in that at δ 154.75 was the coupling clearly resolved (d, *J* 4 Hz coupled to 10-H). It was not certain at this stage that this peak is due to C-11 rather than C-5, firstly because C-14 shows no coupling to 10-H, and secondly because the coupling of an aromatic proton to a *para*-carbon is generally about the same size as that to an *ortho*-carbon.[10]

Next, the ^{13}C n.m.r. spectrum of the monodeuteriated compound (6) in a 1:1 mixture with the undeuteriated ether (2) was obtained. As expected the signal for C-9 was diminished in intensity. In addition, some peaks were seen to be shifted upfield, *viz.* those at δ 160.65 (0.05 p.p.m.), 159.60 (0.01 p.p.m.), 139.76 (0.06 p.p.m.), and 108.29 p.p.m. (0.06 p.p.m.). The latter two signals have already been assigned to C-14 and C-10 respectively and these isotopic shift results are consistent with that conclusion. The shifted signals associated with those at δ 160.65 and 159.60 p.p.m. establish that these two resonances arise from C-6 and C-8, though they have not been distinguished. It is more likely that the one with the larger isotopic shift arises from C-8 because a two-bond shift would be expected to be larger than a four-bond shift.

Further evidence for the assignments came from the use of paramagnetic shift reagents. In the ^{1}H n.m.r. spectrum of the (2), one methoxy ether signal showed a very large shift in the presence of europium tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl[$^2H_9$]octane-4,6-dionate) and this was assigned to the 5-OMe group by comparison with the spectra of the deuteriated compounds (3) and (4). Similarly in the ^{13}C n.m.r. spectrum the signals for the 5-OMe and the carbonyl (C-4) group showed large shifts. Therefore the shift reagent must bind between the carbonyl oxygen and the oxygen on C-5. One other ^{13}C signal shows a large shift, and could therefore be assigned to C-5. From this experiment alone it was not certain whether this peak was that originally at δ 159.64 or at 159.60 p.p.m. However, since the latter resonance has already been assigned to C-6 or C-8 on the basis of the isotopic shift experiments described above, the former can be assigned to C-5. This conclusion was confirmed by rerunning the isotopic shift experiment in the presence of a paramagnetic shift reagent; as expected for the two peaks under consideration, the one which was strongly shifted by the paramagnetic reagent did not show an isotopic shift, whereas the other one did.

The above evidence points to the following assignments: δ 160.65 (C-8), 159.64 (C-5), 159.60 (C-6), and 154.75 p.p.m. (C-11). That these are correct was finally confirmed by the little used technique of heteronuclear n.O.e.[11] It was found that by using the right pulsing conditions, n.O.e.'s could be observed from a specific proton to nearby quaternary carbons only. Thus irradiating 10-H enhanced the signals for C-11 (δ 154.75 p.p.m.) and C-14. Similarly irradiation of 9-H enhanced C-8 (δ 160.65 p.p.m.) and C-14. When $Eu([^2H_9]fod)_3$ was used to separate the two peaks at δ 159.64 and 159.60 p.p.m., it could be seen that irradiation of 7-H enhanced C-6 (δ 159.60) as well as C-8. There was no significant enhancement of the C-5 signal (δ 159.64 p.p.m.) when any of the hydrogens was irradiated.

The conditions for acquiring the n.O.e.'s appeared to be critical as a compromise had to be made between long irradiation and relaxation times which would give a large effect, and rapid pulsing to obtain a good signal-to-noise ratio. The conditions which were used on the Bruker WH400 spectrometer were: low power irradiation of a specific proton for 1 s, then 15—20° pulse to the ^{13}C nuclei, followed by acquisition for 0.4 s (16K data points over 20 kHz); this sequence was repeated 32 or 64 times without any relaxation delays before changing to a new proton, and beginning the accumulation again. Included in the list of proton-decoupling frequencies was one corresponding to a blank part of the spectrum as a control. The whole cycle was repeated as many times as was necessary to obtain a satisfactory signal-to-noise ratio. Under these conditions it seems likely that the n.O.e. only builds up over the course of the 32 or 64 scan sequences.

The results of the heteronuclear n.O.e. experiments confirmed the assignments shown in Table 2. It was decided to investigate whether the trideuteriomethylation procedure described earlier could be useful in future as an assignment technique. In the ^{13}C n.m.r. spectrum of a 1:1 mixture of the mono(trideuteriomethylated) compound (4) and the undeuteriated ether (2), isotopic shifts were observed for C-7 (0.40 p.p.m.) and C-9 (0.022 p.p.m.). Using a narrow spectral width and hence greater digital resolution, shifts were also observed for C-5 (0.008

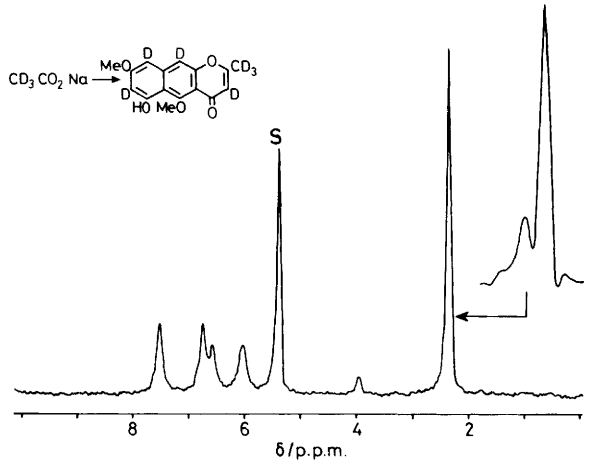


Figure 1. ^2H N.m.r. of rubrofusarin methyl ether (**5**) derived from CD_3CO_2Na. 2 K Data points over 1 000 Hz at 61.4 MHz. The peak marked S is due to the solvent, CH_2Cl_2. Inset: resolution enhancement of the methyl peak at δ 2.35

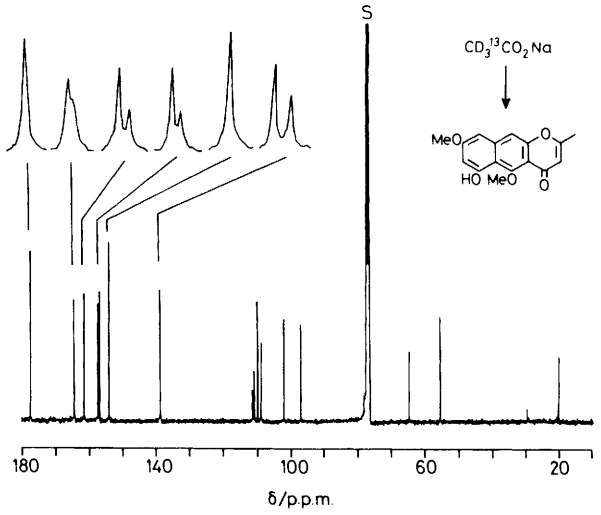


Figure 2. ^{13}C N.m.r. of rubrofusarin methyl ether (**5**) derived from $CD_3{}^{13}CO_2Na$. 32 K Data points over 20 kHz at 100.6 MHz. The peak marked S is due to the solvent, $CDCl_3$. Insets: expansions of each of the low-field peaks

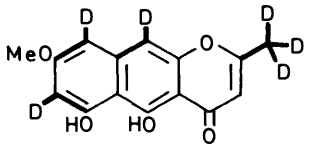
p.p.m.) and C-8 (0.003 p.p.m.), but C-6 appeared as a sharp singlet. When the bis(trideuteriomethyl) ether (**3**) was used, the signals for C-10 (0.024 p.p.m.) and C-6 (0.015 p.p.m.) were also shifted. The conclusion is that, paradoxically, deuteriation of an aromatic methoxy group causes some isotopic shifts to other carbons of the aromatic system, especially *ortho-* and *para*-proton bearing ones, but *not* to the carbon to which it is directly attached. Evidently, use of this technique as an aid to assignment requires a good deal of caution.

Biosynthetic Results.—Sodium [1,2-$^{13}C_2$]acetate (200 mg) was administered in equal batches during 4 days, starting when the organism had begun to turn brown (day 10—14), and the culture was harvested after one additional day of incubation. After isolation, the rubrofusarin was methylated with Me_2SO_4. The ^{13}C n.m.r. spectrum of the resulting dimethyl ether (**2**) showed doublets symmetrically disposed about every signal except those of the methoxy groups. The coupling constants (Table 2) all paired up unambiguously, and with the spectroscopic assignment complete, the pattern of intact acetate units was apparent (Scheme 1). This pattern can only be derived from a linear heptaketide by folding pattern B, and the previously favoured folding pattern A is ruled out.

$^{13}CH_3{}^{13}CO_2Na$ is not the only labelled acetate which can demonstrate the incorporation of intact units. In principle, any double-labelling experiment is suitable, as long as the labels are on different ends of the C_2-unit used, and both labels are retained in the final metabolite. We have investigated the incorporation of $CD_3{}^{13}CO_2Na$ into rubrofusarin in order to demonstrate this point. First it was necessary to show that sufficient deuterium survives in the final metabolite. Therefore CD_3CO_2Na was administered to *F. culmorum* as in the previous feeding experiment. It was not possible to obtain a good signal-to-noise ratio in the ^2H n.m.r. spectrum of the isolated rubrofusarin because of its low solubility. Unfortunately, methylation by the normal procedure using Me_2SO_4–K_2CO_3 in acetone resulted in considerable loss of deuterium, as could be seen in the ^2H n.m.r. spectrum of the dimethyl ether (**2**). Therefore the rubrofusarin was methylated with diazomethane instead to give the monomethyl ether (**5**). The ^1H n.m.r. spectrum of compound (**5**) is very similar to that of the ether (**2**), and was assigned accordingly (Table 1). The ^2H n.m.r. spectrum of (**5**) derived from CD_3CO_2Na is shown in Figure 1. Deuterium is retained at all the possible sites (*viz.* C-10, -9, -7, -3, and -15), and 50% or more is estimated to be retained at the malonate-derived sites relative to the starting methyl group. The spectrum illustrated was recorded without proton decoupling, so the fact that the methyl group is a singlet demonstrates that most of the molecules have retained three deuterium atoms at this site. In fact, with resolution enhancement a second peak is apparent downfield of the main peak (see inset) which is half of the doublet due to a CHD_2 group (the other half is obscured under the CD_3 singlet).

$CD_3{}^{13}CO_2Na$ was fed to *F. culmorum* in the usual way and the isolated rubrofusarin was methylated with diazomethane to give compound (**5**). The ^{13}C n.m.r. spectrum of the ether (**5**) is given in Table 2; it was assigned by comparison with the ^{13}C n.m.r. spectrum of the ether (**2**), and by the ^1H–^{13}C couplings. The assignments were confirmed by heteronuclear n.O.e. experiments, which gave results analogous to those described earlier for the ether (**2**). In the ^{13}C n.m.r. spectrum of compound (**5**) derived from $CD_3{}^{13}CO_2Na$, seven carbon atoms were enriched (C-2, -4, -5, -6, -8, -11, and -14) and four of these showed isotopically shifted peaks due to a deuterium atom on an adjacent carbon (β-shifts[12]) (see Table 2 and Figure 2). The signal for C-2 was a broad envelope due to ^{13}C nuclei with one, two, or three deuterium atoms next to them. Unexpectedly, no shift was observed for C-4 despite the fact that the previous experiment had indicated that deuterium is retained at C-3. However negative β-shifts to carbonyl groups are common, so almost no shift at this site is reasonable. Carbon C-6 shows a β-shift, which must be due to a deuterium atom on C-7. As no carbon showed more than one shifted peak, it must be assumed that the incidence of simultaneous labelling of adjacent acetate units is negligible. Therefore the shift of C-6 due to a deuterium on C-7 implies that these two carbons derive from a single intact acetate. Carbon C-8 shows a shifted signal which must be due to a deuterium on C-9 (because C-7/C-6 was an intact unit). It follows that C-8/C-9 is an intact unit, and similarly C-14 shows a β-shift indicating that C-14/C-10 is an intact unit too. As required C-11 shows no β-shift because its partner C-12 has lost all its deuterium. These results, then, support the findings from the $^{13}CH_3{}^{12}CO_2Na$ feeding and indicate that the bonds shown in structure (**9**) have remained unbroken throughout the course of the biosynthesis.

When the monomethyl ether (**5**) was treated with [^2H]-TFA in $CDCl_3$, exchange of both 7-H and 9-H occurred as shown by the ^1H n.m.r. spectrum. Therefore, when the sample of (**5**)

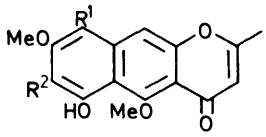


(9)

derived from $CD_3{}^{13}CO_2Na$ was treated with TFA in $CHCl_3$ the deuterium at C-7 and C-9 exchanged out, and consequently in the ^{13}C n.m.r. spectrum of the product the β-shifts of C-6 and C-8 disappeared, while the one of C-14 remained as the deuterium at C-10 was not affected. This is further proof that the intact units are as illustrated in structure (**9**).

Later experiments showed that very brief treatment of compound (**5**) with [^2H]-TFA causes exchange of only 9-H. If this procedure had been adopted with the biosynthetic sample, the location of the deuterium causing the shifts to C-6, C-8, and C-14 could have been identified one by one. It is obvious that the selective exchange of hydrogen atoms can greatly increase the amount of information from a β-shift feeding such as this.

Using the acidic exchange with TFA described above, the monodeuterio derivatives (**10**) and (**11**) were prepared. The ^{13}C

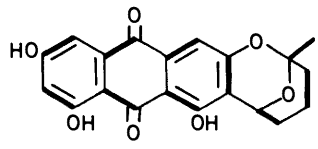


(**10**) $R^1 = D$, $R^2 = H$
(**11**) $R^1 = H$, $R^2 = D$

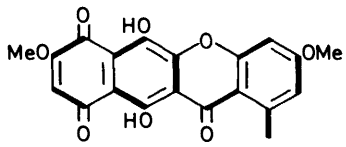
n.m.r. spectra of these compounds in a 1:1 mixture with undeuteriated compound (**5**) were used not only to confirm the assignments of the ^{13}C n.m.r. resonances, but also to demonstrate that the β-shifts observed in the biosynthetic experiment were of the correct magnitude. The β-shifts from 9-^2H to C-8 and from 7-^2H to C-6 matched very well with those observed in compound (**5**) derived from $CD_3{}^{13}CO_2Na$, but, interestingly, the β-shift from 7-^2H to C-8 was smaller than from 9-^2H to C-8 and so there is no doubt that the β-shift at C-8 observed in the biosynthetic experiment is caused by deuterium attached to C-9.

Discussion

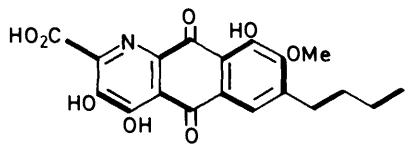
The chain folding pattern B for rubrofusarin, now amply substantiated, is of interest for several reasons. Firstly it is the less obvious one, and contrary to the folding assumed in several textbooks.[3,4] Secondly, a few other metabolites have been studied which could have alternative foldings analogous to A and B, including averufin (**12**) (the aflatoxin precursor),[13] bikaverin (**13**),[14] phomazarin (**14**),[15] and *O*-methylasparvenone (**15**).[16] All of these been shown to follow pathways of type B. It is possible that this is no coincidence, but that it reflects a generally preferred pattern of cyclisation to produce polyketide naphthalenes and anthracenes. A third reason for interest in the folding pattern of rubrofusarin is that we have reported a biogenetically inspired synthesis of a rubrofusarin methyl ether from an orsellinic acid derivative *via* 2-benzyl-γ-pyrone (**16**) (Scheme 2).[6] This synthesis is based on pathway B, and has therefore now been proved to be truly biomimetic in its general strategy. Lastly, a compound with the rubrofusarin skeleton, *e.g.* (**17**), has been suggested as a possible precursor of citromycetin (**18**) by the route outlined in Scheme 3.[17] The intact acetate pattern of compound (**18**) has been determined[5]



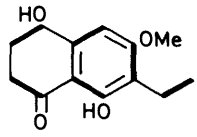
(12)



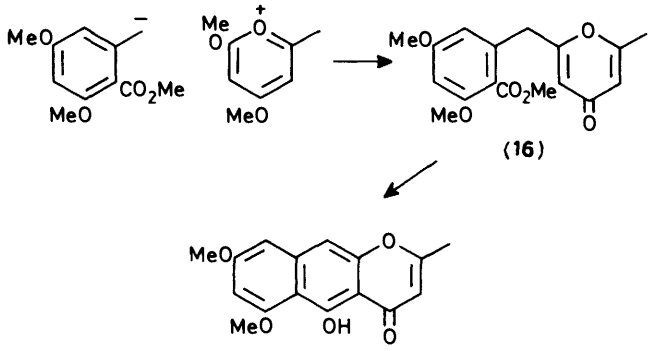
(13)



(14)



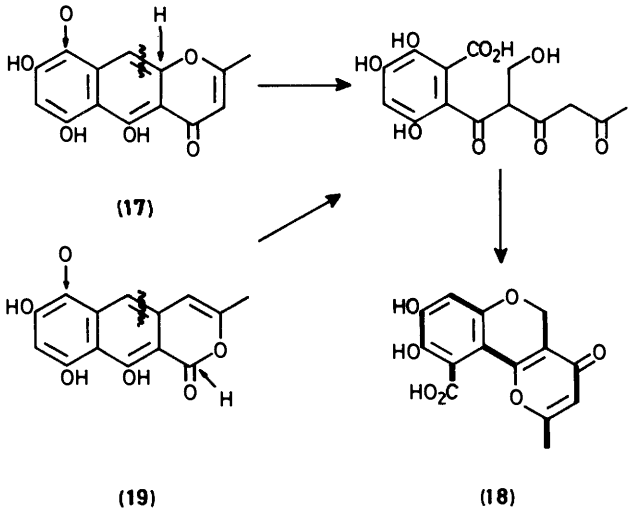
(15)



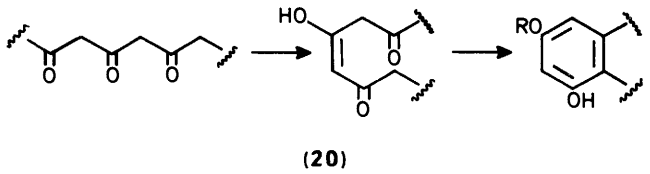
Scheme 2.

and is only consistent with derivation from a rubrofusarin skeleton biosynthesised by pathway B. Thus the present results do not exclude this derivation of citromycetin. However, as has been pointed out,[18] an alternative derivation from a toralactone skeleton (**19**) appears equally attractive in view of the structure of the related metabolites, fusarubin and fulvic acid.

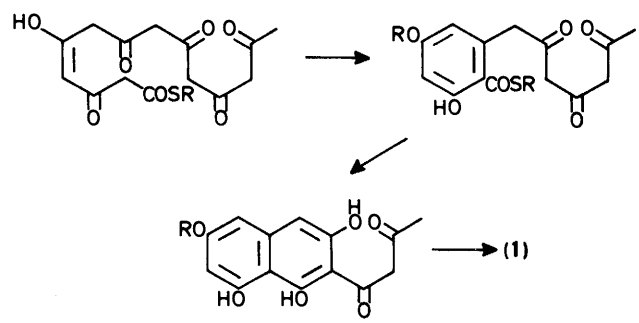
The distribution of deuterium in rubrofusarin derived from CD_3CO_2Na as revealed by the ^2H n.m.r. spectrum of the monomethyl ether (**5**) is worthy of comment. Firstly the deuterium is not distributed evenly over the four malonate-derived sites (Table 3), nor is there a regular progression in the retention values along the polyketide chain in the final metabolite. It might be expected that sites nearest the chain starter unit might suffer the most exchange, but in fact it is at C-



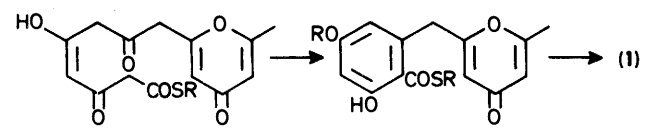
Scheme 3.



Scheme 4.



Scheme 5.



Scheme 6.

Table 3. Retention of deuterium (from ^2H n.m.r.) in compound (**5**)

	C-15	C-3	C-10	C-9	C-7
From CD_3CO_2Na	300[a]	65	80	75	60
From CDH_2CO_2Na	100[a]	28	35	32	26

[a] Figures quoted are percentages relative to the starter methyl group assuming no exchange at that position. The absolute values would be lower because some exchange at the starter group did occur but could not be measured by this technique.

7, nearest the chain terminus, that the most exchange has occurred. Taking this result with others obtained in Cambridge, it has been postulated [19] that this unusual amount of exchange at C-7 is due to directed enolization across the C(7)–C(8) bond during the cyclisation process. This enol, as in structure (**20**), would help to direct the subsequent aldol reaction which forms the first ring. However, for some molecules non-stereospecific reversion to the ketone, followed by regeneration of the enol, would result in extra loss of deuterium at C-7 (Scheme 4). The deuterium retention value at C-9 may in fact be greater than even the relatively high value shown in Table 3, because exchange at C-9 of compound (**5**) occurs very readily in the presence of traces of acid. Some loss of deuterium from this position might therefore have occurred after the biosynthesis was complete, either in the culture medium or in the process of isolation.

The second noteworthy feature of the ^2H n.m.r. spectrum (Figure 1) is that deuterium retention at C-3 is unexpectedly high. Thus if the biosynthesis follows the route shown in Scheme 5, then C-3 remains in a form susceptible to exchange of deuterium from the very first condensation of malonyl CoA with acetyl CoA up to the very last step, the closure of the pyrone ring. In view of this the relatively low degree of exchange at this site is surprising. Indeed, no deuterium was retained at the similar γ-pyrone position of citromycetin (**18**) when it was biosynthesised from CD_3CO_2Na by *Penicillium frequentans*.[20] It seems possible, therefore, that closure of the γ-pyrone ring in rubrofusarin biosynthesis occurs much earlier as shown in Scheme 6. This earlier formation of the pyrone would effectively prevent any further exchange at C-3. It would also simplify the task of controlling the direction of the further cyclisations on the enzyme because closure of ring A is now the only remaining fully active aldol reaction. The final step would then be a Claisen condensation onto the thioester. Our biomimetic synthesis of rubrofusarin methyl ether from compound (**16**)[6] has proved that such a reaction is chemically feasible. Further experiments are planned to test this hypothesis *in vivo*.

To gain further insight into the enzymic processes, CDH_2CO_2Na (made by the reduction of bromoacetic acid with zinc in D_2O [21]) was fed to *F. culmorum* in parallel with the feeding of CD_3CO_2Na. In the ^2H n.m.r. spectrum of the rubrofusarin monomethyl ether thus obtained, the methyl group (C-15) gave rise to a triplet, indicating the presence of CH_2D groups, but the relative intensities of the aromatic peaks were virtually identical with those resulting from in the CD_3CO_2Na feeding (Table 3). On the basis of similar results from studies of the biosynthesis of 6-methylsalicylic acid,[22] it has been suggested that the removal of the extra protons from the methylene sites during the aromatisation process must be enzymically mediated, because otherwise an isotope effect would have produced different distributions in the two experiments. In this case also it would seem that the deprotonations at C-3, C-7, C-9, and C-10 are all enzymically controlled.

In summary, this investigation has not only revealed an unexpected folding pattern of the heptaketide chain in rubrofusarin, but it has also demonstrated the application of novel techniques using deuterium-labelled precursors for probing the mechanisms of polyketide folding and cyclisations *in vivo*. These techniques are potentially applicable to a wide range of metabolites in this class.

Note Added in Proof.—In a recent preliminary report[23] it is claimed that the naphthalene nucleus of fonsecin arises by the alternative folding pattern A to that established here for rubrofusarin. We prefer to reserve comment until the full details

of the methods used to assign the ^{13}C n.m.r. spectrum of fonsecin have been published.

Experimental

M.p.s were determined on a Kofler hot-stage apparatus and are uncorrected. ^1H N.m.r. spectra were recorded on a Varian CFT20 (80 MHz) or a Bruker WH400 (400 MHz) spectrometer. ^{13}C N.m.r. spectra were recorded on a Varian XL100A12 (25 MHz), a Bruker WM250 (62.5 MHz), or a Bruker WH400 (100 MHz) spectrometer. All β-shifts were measured on the WH400 instrument.

Fusarium culmorum (C.M.I. 89364) was grown on agar slopes and stored at 5 °C. For the production of rubrofusarin, the medium was prepared as follows: glucose (75 g), tartaric acid (4 g), diammonium tartrate (4 g), $(NH_4)_2HPO_4$ (0.6 g), K_2CO_3 (0.6 g), $MgCO_3$ (0.4 g), $(NH_4)_2SO_4$ (0.25 g), $ZnSO_4 \cdot 7H_2O$ (0.07 g), $FeSO_4 \cdot 7H_2O$ (0.07 g), and distilled water (1 500 ml). This medium was divided among four 500-ml conical flasks and autoclaved at 115 °C for 1 h. Immediately before inoculation the pH was adjusted to *ca.* 7.9 with sterile 2.7M-NaOH (3.7 ml). The flasks were inoculated with some mycelium scraped off the surface of the agar slope, and then shaken in an incubator at 28 °C in the dark. After 10—14 days the suspension turned reddish-brown, probably owing to the production of rubrofusarin and aurofusarin. After a further 5 days the cultures were acidified with 50% H_2SO_4 (1.5 ml per flask) and filtered. The mycelium was dried in a high vacuum, and the filtrate was extracted with diethyl ether (3 times) and the combined extracts dried (Na_2SO_4). When dry, the mycelium was ground up in a blender and extracted in a Soxhlet apparatus with light petroleum (b.p. 30—40 °C) for 2 h, and then with the above ethereal extract for 18 h. The ethereal solution was cooled, decanted from the insoluble material, and evaporated to leave a red oil containing a precipitate of crude rubrofusarin. Light petroleum (b.p. 40—60 °C) and a little ethanol were added to dissolve the oil and the precipitate was filtered off and washed. The crude rubrofusarin was dissolved in dichloromethane leaving a small amount of insoluble material behind. It was generally methylated without further purification, but if necessary it could be purified on a column of silica gel impregnated with 10% oxalic acid, eluting first with dichloromethane–light petroleum (b.p. 40—60 °C) (1:1) then with dichloromethane, and finally with dichloromethane–diethyl ether (3:1). The rubrofusarin solution eluted from the column was washed several times with water to remove oxalic acid, dried and evaporated under reduced pressure to give rubrofusarin as orange-red crystals, m.p. 215—217 °C (from light petroleum) (lit.,[1] m.p. 210—211 °C). For the purpose of analytical t.l.c. rubrofusarin could be run on silica gel t.l.c. plates which had been dipped into a saturated solution of oxalic acid in ethyl acetate, and then left to dry, R_F 0.6 (CH_2Cl_2–Et_2O, 1:1); δ ($CDCl_3$) 15.9 (1 H, s, OH), 9.6 (1 H, s, OH), 6.95 (1 H, s), 6.56 (1 H, d, *J* 2 Hz), 6.46 (1 H, d, *J* 2 Hz), 6.01 (1 H, s), 3.90 (3 H, s), and 2.39 (3 H, s).

Rubrofusarin Dimethyl Ether (**2**).[8]—Crude rubrofusarin was methylated with an excess of Me_2SO_4–K_2CO_3 in acetone heated under reflux for 2 days. The product was purified by chromatography on a column of silica gel eluting with CH_2Cl_2–Et_2O to give the blue-green fluorescent dimethyl ether, R_F 0.24 (CH_2Cl_2–Et_2O, 1:1), m.p. 191—192 °C (lit.,[8] m.p. 186—187 °C).

Rubrofusarin Monomethyl Ether A (**5**).—Rubrofusarin was methylated with an excess of diazomethane (from *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide) in THF–Et_2O at room temperature overnight. The fluorescent product was purified by chromatography on a silica gel column eluting with CH_2Cl_2–Et_2O to give the blue-green fluorescent monomethyl ether, m.p. 202—204 °C (lit.,[8] m.p. 203—204 °C); R_F 0.39 (CH_2Cl_2–Et_2O, 1:1).

[Me-$^2H_3$]*Methyl 4-Bromobenzenesulphonate* (**7**).—[$^2H_4$]-Methanol (0.4 ml, 8.9 mmol) and 4-bromobenzenesulphonyl chloride (2 g, 7.84 mmol) were dissolved in dry THF (10 ml) and oil-free sodium hydride (0.25 g, 10.4 mmol) was added. The suspension was stirred for 7 h, poured into water and extracted with diethyl ether. The organic layers were washed with aqueous sodium hydrogen carbonate, dried (Na_2SO_4) and evaporated to dryness under reduced pressure to yield the bromobenzenesulphonate (**7**) (1.28 g, 65%) which crystallized when seeded, m.p. 59—61 °C (lit.,[9] m.p. 60 °C); R_F 0.52 (CH_2Cl_2); δ($CDCl_3$) 7.66 (4 H, s) [methyl 4-bromobenzenesulphonate also shows δ 3.77 (3 H, s)].

[5,6-OMe-$^2H_6$]*Rubrofusarin Dimethyl Ether* (**3**).—Crude rubrofusarin (61 mg), [Me-$^2H_3$]methyl 4-bromobenzenesulphonate (200 mg), anhydrous potassium carbonate (560 mg), and acetone (8 ml) were heated under reflux with stirring for 18 h. After work-up as above, the dimethyl ether (24 mg) was obtained. The n.m.r. spectrum was as in Table 1 except that the peak at δ 3.96 was absent.

[6-OMe-$^2H_3$]*Rubrofusarin Dimethyl Ether* (**4**).—Rubrofusarin monomethyl ether (34 mg, 0.12 mmol), [Me-$^2H_3$]methyl 4-bromomethanesulphonate (150 mg, 0.59 mmol), anhydrous potassium carbonate (500 mg), and acetone (6 ml) were heated under reflux with stirring for 18 h then poured into dilute aqueous sodium hydrogen carbonate and extracted with dichloromethane. The organic layers were dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The residue was purified by p.l.c. to yield the dimethyl ether (32 mg, 90%). The n.m.r. spectrum was as in Table 1 except the peak at δ 3.96 was reduced to 3 H.

Acknowledgements

We would like to thank St. John's College, Cambridge for a fellowship to F. J. L. and Dr. C. Abell for helpful discussions.

References

1 J. N. Ashley, B. C. Hobbs, and H. Raistrick, *Biochem. J.*, 1937, **31**, 385.
2 G. H. Stout, D. L. Dreyer, and L. H. Jensen, *Chem. Ind.*, 1961, 289; H. Tanaka and T. Tamura, *Tetrahedron Lett.*, 1961, **4**, 151.
3 W. B. Turner and D. C. Aldridge, 'Fungal Metabolites II,' Academic Press, London, 1983, 129; P. Manitto, 'Biosynthesis of Natural Products,' Ellis Horwood, Chichester, 1981, 185; U. Weiss and J. M. Edwards, 'The Biosynthesis of Aromatic Compounds,' Wiley-Interscience, New York, 1980, 331; J. D. Bu'Lock in 'Comprehensive Organic Chemistry,' eds. D. Barton and W. D. Ollis, Pergamon, Oxford, 1979, vol. 5, 958.
4 B. H. Mock and J. E. Robbers, *J. Pharm. Sci.* 1969, **58**, 1560.
5 G. E. Evans and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1976, 760.
6 F. J. Leeper and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1978, 406.
7 F. J. Leeper and J. Staunton, *J. Chem., Chem. Commun.*, 1982, 911.
8 S. Shibata, E. Morishita, and Y. Arima, *Chem. Pharm. Bull.*, 1967, **15**, 1757.
9 F. Krafft and A. Roos, *Ber.*, 1892, **25**, 2255.
10 F. W. Wehrli and T. Wirthlin, 'Interpretation of Carbon-13 NMR Spectra,' Heyden, London, 1980, 86.
11 J. Uzawa and S. Takeuchi, *Org. Magn. Reson.*, 1978, **11**, 502.
12 C. Abell and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 856.
13 C. P. Gorst-Allman, K. G. R. Pacher, P. A. Steyn, P. L. Wessels, and De B. Scott, *J. Chem. Soc., Perkin Trans. 1*, 1977, 2181.

14 A. G. McInnes, D. G. Smith, J. A. Walter, L. C. Vining, and J. L. C. Wright, *J. Chem. Soc., Chem. Commun.*, 1975, 66.
15 A. J. Birch and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 816.
16 T. J. Simpson and D. J. Stenzel, *J. Chem. Soc., Chem. Commun.*, 1981, 239.
17 W. B. Whalley in 'The Chemistry of Natural Phenolic Compounds,' ed. W. D. Ollis, Pergamon, London, 1961, ch. 3.
18 (*a*) T. Money, *Nature*, 1963, **199**, 592; (*b*) I. Kurobane, C. R. Hutchinson, and L. C. Vining, *Tetrahedron Lett.*, 1981, **22**, 493.
19 C. Abell, M. J. Garson, F. J. Leeper, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1982, 1011.
20 F. J. Leeper and J. Staunton, unpublished results.
21 D. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1974, 345.
22 C. Abell and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1984, 1005.
23 J. L. Bloomer, T. J. Caggiano, and C. A. Smith, *Tetrahedron Lett.*, 1982, **23**, 5103.

Received 4*th May* 1984; *Paper* 4/713