Studies in Relation to Biosynthesis. Part 48.[1] Phomazarin. Part 2.[1] ^{13}C N.m.r. Spectra and Biosynthesis of Phomazarin

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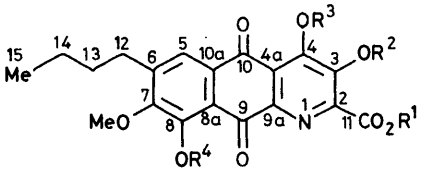
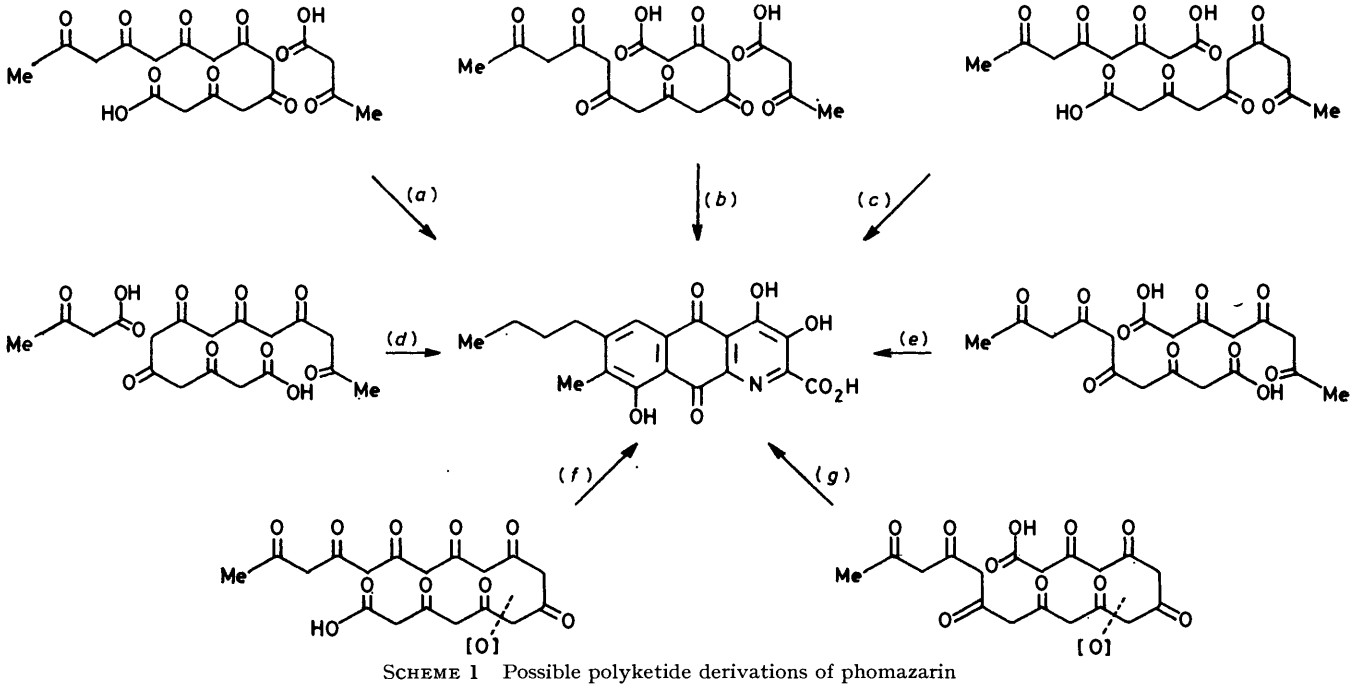
The ^{13}C n.m.r. spectra of phomazarin methyl ester and seven further derivatives of phomazarin have been assigned. Incorporations of [^{13}C]acetates and [^{13}C]- and [^{14}C]-malonates by cultures of *Pyrenochaeta terrestris* indicate that phomazarin is biosynthesised by condensation, followed by oxidative ring-fission, of a single nonaketide precursor chain.

PHOMAZARIN, an orange pigment isolated from the mycelium of *Pyrenochaeta terrestris* has been shown to



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

have the unique aza-anthraquinone structure (1).[1] The origin of the branched C_{18} skeleton of phomazarin is of interest as previous studies[2] have shown that [^{14}C]-formate is incorporated mainly into the 7-*O*-methyl group only, so the C_1-pool does not contribute to the branched structure; and the activities of the acetic acid and carbon dioxide obtained respectively from Kuhn–Roth oxidation and decarboxylation of phomazarin from feeding experiments with [2-^{14}C]acetate are consistent with an entirely polyketide origin for phomazarin from nine acetate molecules. However, the biosynthesis of phomazarin cannot be accounted for by a normal polyketide pathway[3] but must involve a condensation between two, or possibly more, preformed polyketide chains for which different possibilities exist (Scheme 1 indicates some of these), or by cleavage of a carbocyclic intermediate, which can itself be formed *via* either of the two possible condensations of a single polyketide chain shown in Scheme 1. This latter route would necessarily involve oxidation of a carbon derived from methyl of acetate to a carboxy, so accounting for the origin of the C-11 carboxy function. However, it must be noted that oxidation of methyl to higher levels is not an uncommon biosynthetic process.



SCHEME 1 Possible polyketide derivations of phomazarin

^{13}C N.m.r. methods have proved to be very useful in biosynthetic studies.[4] In particular, intact acetate residues can be recognised by feedings with [$^{13}C_2$]acetate: the ^{13}C n.m.r. spectra of the enriched metabolites show ^{13}C–^{13}C couplings on adjacent carbons derived from the

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same acetic acid molecule, cleavage of an originally intact acetate derived unit during the biosynthetic pathway being revealed by a loss of the ^{13}C–^{13}C coupling. Studies of this type have been particularly useful in distinguishing between different foldings of polyketide chains,[5] and in elucidating pathways involving a ring-fission of a polyketide-derived intermediate,[6] molecular rearrangements of the polyketide itself [7] (or an intermediate [8]), or condensations to potentially equivalent positions.[9] Thus the incorporation of ^{13}C-enriched precursors into phomazarin has been studied.

RESULTS AND DISCUSSION

A prerequisite of ^{13}C biosynthetic studies is an unambiguous assignment of the ^{13}C n.m.r. spectrum of the metabolite. The ^{13}C n.m.r. spectra of phomazarin methyl ester (2) and the further derivatives (3)—(9) have been assigned and are summarised in Table 1. These assignments are based on standard chemical-shift data; single frequency off-resonance decoupled (S.F.O.R.D.) spectra; [10] comparison of chemical shifts in compounds (2)—(9); analysis of ^{13}C–^{1}H couplings in fully ^{1}H coupled spectra, making use of the recorded values of $^{2}J(^{13}C\text{–}CH_3)$ and $^{3}J(^{13}C\text{–}C\text{–}CH_3)$ and the observation that $|^{3}J(^{13}C\text{–}C\text{–}CH)| > |^{2}J(^{13}C\text{–}CH)|$ in aromatic systems; [10] and ^{15}N–^{13}C couplings in proton-noise-decoupled (p.n.d.) spectra.

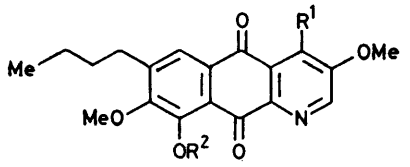
The aliphatic carbons of the n-butyl side chain and the methoxy groups are readily identified from their chemical shifts and multiplicities in S.F.O.R.D. spectra. In contrast to n-butylbenzene where the α and β carbons of the butyl side chain resonate at 36.0 and 34.0 p.p.m.,[11] C-12 and C-13 resonate at 30.2 and 31.7 p.p.m. in (2). This assignment was confirmed by a specific ^{1}H-decoupling experiment; irradiation of the benzylic methylene at τ 7.23 causing the resonance at 30.2 p.p.m. to collapse to a singlet. Presumably, the presence of the adjacent 7-*O*-methyl function moves C-12 upfield. The protonated aromatic carbon resonance, C-5, is readily assigned in p.n.d. spectra from its high intensity relative to the neighbouring resonances (due to non-protonated carbons) and from its multiplicity in S.F.O.R.D. spectra. In fully ^{1}H-coupled spectra it appears as a doublet of triplets, $^{1}J(^{13}C\text{–}H)$ 164.6 and $^{3}J(^{13}C\text{–}C\text{–}CH_2)$ 5.8 Hz, due to coupling to H-5 and the benzylic methylene protons of the adjacent n-butyl group. The C-5 resonance appears at 121—122 p.p.m. in compounds (2)—(4) and (7) but moves downfield by *ca.* 3 p.p.m. in the 8-*O*-methyl derivatives (5), (6), (8), and (9). In the decarboxy-compounds (7)—(9) the additional protonated aromatic carbon resonance at 137 p.p.m. is readily assigned to C-2.

The quinonoid carbonyl carbons were expected to appear at lowest field and in phomazarin methyl ester (2), C-9 and C-10 appear at 184.7 and 187.6 p.p.m. respectively. They are readily distinguished by the three-bond coupling of C-10 to the aromatic proton, $^{3}J(^{13}C\text{–}C\text{–}CH)$ 4.4 Hz, and in ^{15}N-enriched phomazarin methyl ester by the coupling of C-9 to ^{15}N, $^{2}J(^{13}C\text{–}C\text{–}^{15}N)$ 7.8 Hz. In the fully ^{1}H-coupled spectrum of tri-*O*-methylphomazarin methyl ester (5) determined on a

TABLE 1

Carbon-13 chemical shifts of a series of phomazarin derivatives (in p.p.m. downfield from $SiMe_4$, for $CDCl_3$ solutions)

	Compound							
Carbon atom	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
C-2	132.2	148.2	149.2	149.7	148.6	137.1	137.4	137.3
C-3	152.5	147.0	151.7	151.4	146.1	148.3	147.5	154.7
C-4	158.3	161.2	161.0	160.6	160.8	156.4	156.9	131.8
C-4a	117.3	117.3	127.9	130.3	116.3	115.6	114.5	130.3
C-5	122.2	122.1	121.4	124.8	124.9	121.7	124.7	125.1
C-6	144.9	144.7	145.2	144.3	144.1	144.4	143.8	144.1
C-7	153.6	153.4	152.3	153.6	154.3	153.1	154.3	153.7
C-8	156.6	156.4	155.6	157.9	159.3	157.2	159.1	157.9
C-8a	115.8	115.3	114.9	125.0	125.4	115.6	125.4	124.7
C-9	184.7	184.8	185.0	178.9	178.2	185.6	179.1	178.9
C-9a	140.4	143.6	144.3	146.7	144.7	142.7	143.7	144.7
C-10	187.6	187.9	180.2	181.3	188.7	188.2	188.8	181.4
C-10a	126.1	125.7	126.1	125.1	127.8	126.1	128.2	127.0
C-11	169.5	164.3	164.0	164.4	164.3			
C-12	30.2	30.4	30.4	30.4	30.4	30.4	30.3	30.4
C-13	31.7	32.0	32.1	32.2	32.0	32.1	32.1	32.1
C-14	22.6	22.6	22.6	22.7	22.7	22.6	22.6	22.6
C-15	13.9	13.9	13.9	13.9	13.9	13.9	13.9	13.9
7-OMe	60.5	61.0	60.8	61.3	61.4	61.0	61.4	61.6
11-OMe	53.5	53.1	52.3	52.3	53.0			
3-OMe		61.8	62.5	62.5	61.7	56.0	57.0	57.5
4-OMe			62.5	62.5				
8-OMe				61.6	61.5		61.4	61.6



(7) R^1 = OH, R^2 = H
(8) R^1 = OH, R^2 = Me
(9) R^1 = Cl, R^2 = Me

1 kHz sweep width, C-9 also shows a small coupling, 0.9 Hz, which must be due to four-bond coupling to H-5 (see Figure 1). On methylation of the *peri*-hydroxy groups on either C-8 or C-4, the C-9 and C-10 resonances respectively move upfield by 6—7 p.p.m., due to removal of the deshielding chelation effect, to appear at *ca.* 179 p.p.m. in the 8-*O*-methyl and *ca.* 181 p.p.m. in the 4-*O*-methyl derivatives. Replacing the 4-hydroxy group in the decarboxy-derivative (8) by chlorine to give (9) likewise results in a 7 p.p.m. upfield shift of the C-10 resonance. As well as the shifts of the quinone carbonyl resonance, removal of the hydrogen-bonding by methylation also allows assignment of the C-4a and C-8a resonances, which show characteristic downfield shifts of *ca.* 10 p.p.m., *e.g.* C-4a moves downfield from 117.3 in (3) to 127.9 p.p.m. in the corresponding 4-*O*-methyl derivative (4), and C-8a moves downfield from

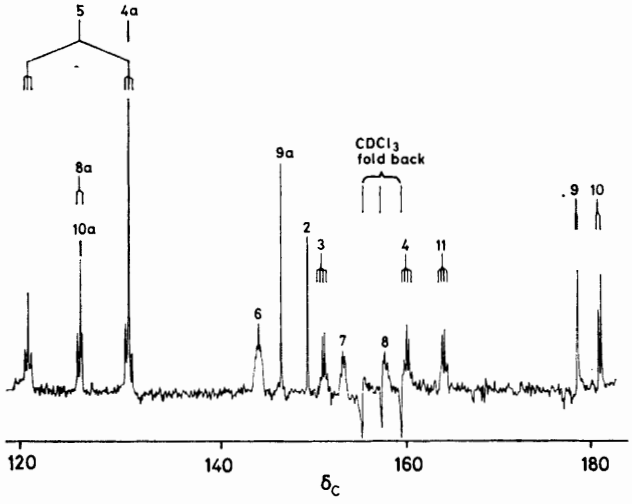


FIGURE 1 Low-field region (120—180 p.p.m.) of the fully ^{1}H-coupled ^{13}C n.m.r. spectrum of (5), 1 000 Hz sweep width

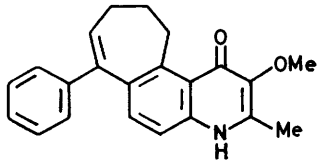
114.9 in (4) to 125.0 p.p.m. in (5). In 2-hydroxyacetophenone, C-1 moves downfield by 8.6 p.p.m. on conversion to the methyl ether, with a concomitant upfield shift, 4.3 p.p.m., by the carbonyl.[12] In compounds where both C-4a and C-8a have similar chemical shifts, *e.g.* (2), (3), (5), and (7), they can be distinguished by examination of the fully ^{1}H-coupled spectra where C-4a appears as a sharp singlet whereas C-8a gives a doublet due to coupling to H-5, $^3J(^{13}C\text{–}C\text{–}CH)$ 7.3 Hz. The C-11 methoxycarbonyl resonance at 169.5 p.p.m. in (2) also moves upfield to 164 p.p.m. on formation of the 3-*O*-methyl ethers (3)—(6). Correspondingly, the C-2 resonance moves downfield from 132.2 p.p.m. in (2) to *ca.* 149 p.p.m. in compounds (3)—(6). On decarboxylation, the ester carbonyl resonance is replaced by a protonated aromatic carbon resonance at 137 p.p.m. in (7)—(9). In ^{15}N-enriched (2) and (5),[1] C-11 is coupled to the ^{15}N, $^2J(^{13}C\text{–}C\text{–}^{15}N)$ 8.8 Hz.

Of the remaining carbons only C-6 and C-10a are not attached to either oxygen or nitrogen. They are assigned in phomazarin methyl ester (2) to the resonances at 144.9 and 126.1 p.p.m., respectively; in the fully ^{1}H-coupled spectrum C-6 appears as a partially resolved multiplet due to coupling to the butyl methylene protons whereas C-10a appears as a sharp singlet, there being no protons in a three-bond relationship to C-10a. In the p.n.d. spectrum of the tri-*O*-methylphomazarin methyl ester (5) only 12 of the 14 low-field resonances could be seen. On re-determining the spectrum on a 1 000 Hz sweep width the intense resonance at 125 p.p.m. was resolved into three closely spaced signals and in the fully ^{1}H-coupled spectrum, also determined on a 1 000 Hz sweep, these three signals appear as a doublet of triplets (J 164.6 and 5.7 Hz), a doublet (J 7.3 Hz), and a singlet (see Figure 1) allowing their assignment to C-5, C-8a, and C-10a, respectively. In Figure 1, the C-4a singlet, at 130.3 p.p.m., lies under the low-field half of the C-5 resonance.

The resonances still to be assigned are those of the phenolic carbons and the heteroaromatic carbons, C-2 and C-9a; C-2 is readily assigned in compounds (2) and (7)—(9), but is not so readily distinguished from the resonances in the 146—161 p.p.m. region in the remaining compounds. The resonances of methoxy-bearing carbons can be identified by their multiplicities in fully ^{1}H-coupled spectra; in the spectrum of (5) carbons 3, 4, 8, and 11 appear as quartets due to coupling to the methoxy protons, $^3J(^{13}C\text{–}C\text{–}CH_3)$ *ca.* 4 Hz, further splitting being observed in the C-7 resonance due to three-bond coupling to H-5 and the 12-methylene protons, whereas C-2 and C-9a appear as sharp singlets (see Figure 1). In the p.n.d. spectrum of ^{15}N-enriched (5), however, C-2, C-3, C-4, and C-9a all appear as doublets due to coupling (J 1.5, 2, 2, and 3.9 Hz, respectively) to ^{15}N, thus allowing C-3 and C-4 to be distinguished from C-7 and C-8, which are themselves differentiated by the further couplings, discussed above, displayed by the C-7 resonance. In the partially methylated derivatives, the free phenolic carbons are identified as they appear as doublets due to coupling to the hydroxy proton, $^2J(^{13}C\text{–}OH)$ *ca.* 5 Hz; this coupling is commonly observed in strongly hydrogen-bonded phenols [13] and can be removed by exchange with D_2O. On formation of the 8-*O*-methyl ether, the C-8 resonance is moved to lower field by 2—3 p.p.m. compared with the 8-hydroxy compounds. In contrast, formation of the 4-*O*-methyl ether has no effect on the C-4 resonance but does result in a downfield shift of the C-3 resonance from *ca.* 147 to *ca.* 151 p.p.m. On decarboxylation, the C-4 resonance is moved upfield by *ca.* 5 p.p.m. and in the chloro-compound (9), the C-4 resonance undergoes a large upfield shift to 131.8 p.p.m. On the basis of the above coupling and shift behaviour, a complete assignment of resonances in the 145—161 p.p.m. region can be made.

The constancy of the C-4 chemical shift in both the methylated and free hydroxy compounds is in agreement with the predominance of the pyridol tautomer in phomazarin, as established previously.[1] In 4-pyridone and 4-quinolone, C-4 resonates at 175.7 [14] and 177.2 p.p.m.,[15] respectively. In the recently isolated quinolone

derivative (10), C-4 resonates at 175.8 p.p.m. and so must exist as the keto-tautomer. The authors experi-



(10)

enced some difficulty in recognising the presence of the 4-quinolone moiety,[16] but it would appear that ^{13}C n.m.r. provides a good method for indicating the presence of the system. The relatively large two-bond ^{13}C–^{15}N couplings to C-9 and C-11 have been observed in ^{15}N-enriched phomazarin methyl ester (2) and tri-*O*-methylphomazarin methyl ester (5) and these couplings are most useful in both assignment and structural studies. The magnitude of the coupling depends on the interaction of the carbon with the nitrogen lone-pair of electrons as $^2J(^{13}C–^{15}N)$ is much larger when the carbon is *cis* to the lone-pair than when it is *trans*, thus the two-bond coupling to C-3 is only 2 Hz. In [^{15}N]-quinoline the two-bond couplings to C-3 and C-8 are 2.7 and 9.3 Hz, respectively.[17] The remaining ^{13}C–^{15}N couplings observed in phomazarin, particularly the small one-bond couplings, are consistent with observations on [^{15}N]pyridine [18] and [^{15}N]quinoline.[17] On protonation the large two-bond coupling in [^{15}N]-quinoline is reduced to 1.0 Hz, and the couplings to C-2 and C-9 increase from 1.4 and 0.6 Hz (CCl_4) to 15.9 and 13.8 Hz (H_2SO_4 solution).[17] Similarly in [^{15}N]pyridine the coupling to C-2 increases from 0.6 Hz to 11.9 Hz on protonation and to 15.2 Hz on *N*-oxide formation.[18]

As previously described for other fungal metabolites,[19] feedings of sodium [1-^{14}C]acetate to *P. terrestris* were used to establish conditions which would give a suitable abundance of ^{13}C in the individual acetate-derived atoms of phomazarin on feeding of [^{13}C]acetate. Studies of the course of fermentation indicate that phomazarin production commences after 5 d growth maximising after a further 7 d, and optimum dilution values were obtained by separate additions of sodium acetate on days 5, 6, and 7 to a total level of 0.5 g l^{-1}; feeding to four flasks, *i.e.* 400 ml of culture medium, gave sufficient phomazarin for spectral determinations. Despite its rather low solubility, *ca.* 30 mg ml^{-1} phomazarin methyl ester (2), was used for the study of ^{13}C enrichments as the overlap of signals in its ^{13}C n.m.r. spectrum makes the more soluble tri-*O*-methylphomazarin methyl ester (5) unsuitable. The natural abundance ^{13}C n.m.r. spectrum of (2) determined under normal conditions displays an extremely wide range of resonance intensities which, despite high enrichments, makes identification and comparison of enriched resonances somewhat difficult. However, if the spectrum is determined in the presence of the relaxation agent [20] [$Cr(acac)_3$] these intensity differences are removed and all the non-protonated carbons display almost identical intensities, the protonated carbons also giving similar intensities to one another but at a slightly higher overall intensity than the non-protonated carbons. It is suggested that, solvent considerations permitting, this procedure should be standard practice in ^{13}C-enrichment studies, as it largely removes the uncertainties that can arise in ^{13}C biosynthetic studies.

The ^{13}C n.m.r. spectra of phomazarin methyl ester enriched from feedings of [1-^{13}C]- and [2-^{13}C]-acetate showed the alternate labelling pattern anticipated, carbons 2, 4, 5, 7, 8a, 9a, 10, 12, and 14 being derived from the carboxy of acetate and carbons 3, 4a, 6, 8, 9, 10a, 11, 13, and 15 being derived from the methyl carbon of acetate (Table 2) so confirming an entirely

TABLE 2

^{13}C N.m.r. enrichment data for phomazarin methyl ester (2)

Carbon atom	Chemical shift (p.p.m.)	[1-^{13}C]-acetate [a]	[2-^{13}C]-acetate [a]	$J(^{13}C–^{13}C)$ [b]	[2-^{13}C]-malonate enrichment [c]
C-2	132.2	*		82	1.2
C-3	152.5		*	71	1.5 (+)
C-4	158.3	*		73	1.1
C-4a	117.3		*	53	1.7 (+)
C-5	122.2	*		60	0.9
C-6	144.9		*	68	1.8 (+)
C-7	153.6	*		68	0.9
C-8	156.6		*	64	1.6 (+)
C-8a	115.8	*		62	1.0
C-9	184.8		*	62	1.5 (+)
C-9a	140.4	*		64	1.1
C-10	187.6	*		53	0.8
C-10a	126.1		*	60	1.5 (+)
C-11	169.5		*	84	1.8 (+)
C-12	30.2	*		31	1.0
C-13	31.7		*	31	1.6 (+)
C-14	22.6	*		36	1.0
C-15	13.9		*	37	1.3
7-OMe	60.5			—	0.8
11-OMe	53.5			—	0.8

[a] Approximately five-fold enrichment at each labelled site, denoted by an asterisk. [b] Feeding with [$^{13}C_2$] acetate. [c] See Experimental section for details of calculation; enriched (+) average 1.6, unenriched average 1.0.

polyketide origin of the phomazarin skeleton. An approximately five-fold enrichment at each labelled site was observed with no significant difference in the levels of enrichment at each labelled site being observed. The interpretation of the 15 MHz spectrum of phomazarin methyl ester from the [$^{13}C_2$]acetate feeding was extremely difficult due to (*a*) extensive overlapping of the signals and (*b*) the similarity of chemical shifts between coupled pairs of carbons causing non-first-order ^{13}C–^{13}C coupling effects to be observed; Figure 2(*a*) shows how AB coupling causes the outer satellites in some cases to be almost indistinguishable above the spectral noise level. However on re-determining the spectrum at 67 MHz, the greatly increased dispersion at high field overcame both these problems [Figure 2(*b*)] and the following ^{13}C–^{13}C couplings were observed (Table 2); C(11)–C(2), C(3)–C(4), C(4a)–C(10), C(10a)–C(5), C(6)–C(7), C(8)–C(8a), C(9)–C(9a), C(12)–C(13), and C(14)–C(15), so that the phomazarin skeleton is derived from nine intact acetate units assembled as shown in Scheme 2.

This assembly pattern eliminates some biosynthetic

possibilities *e.g.* (*a*), (*c*), and (*f*) in Scheme 1, but it is not at this stage possible to differentiate between a two-chain [*e.g.* (*b*), (*d*), or (*e*)] or a single chain [*e.g.* (*g*)] origin for phomazarin. However, if a two-chain pathway is operative then both C-15 and C-11 are derived from the methyl carbon of an acetate 'starter' unit, whereas if a single-chain route [path (*g*)] operates then only C-15 is derived from a 'starter' acetate, C-11 being formed from a chain-propagating malonate molecule, following cleavage of a pre-formed cyclic intermediate. Thus, by feeding of labelled malonate, it should be possible to distinguish between these two pathways.

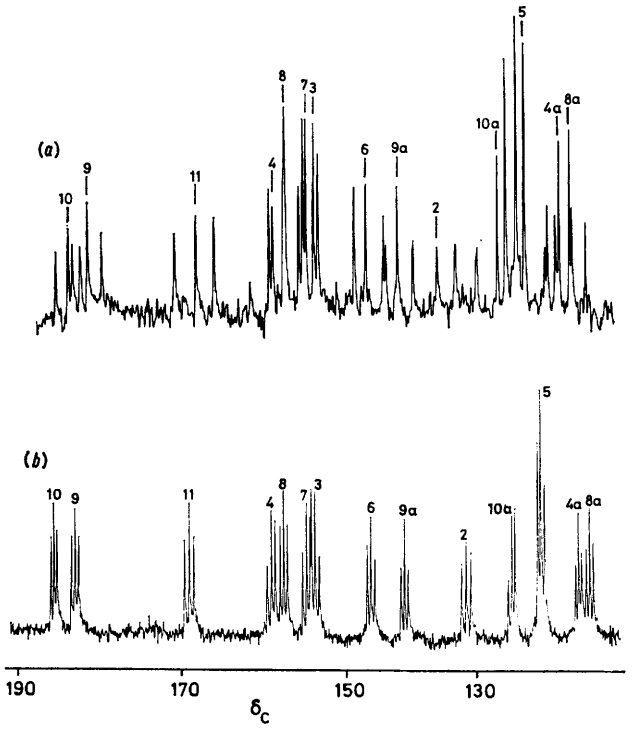
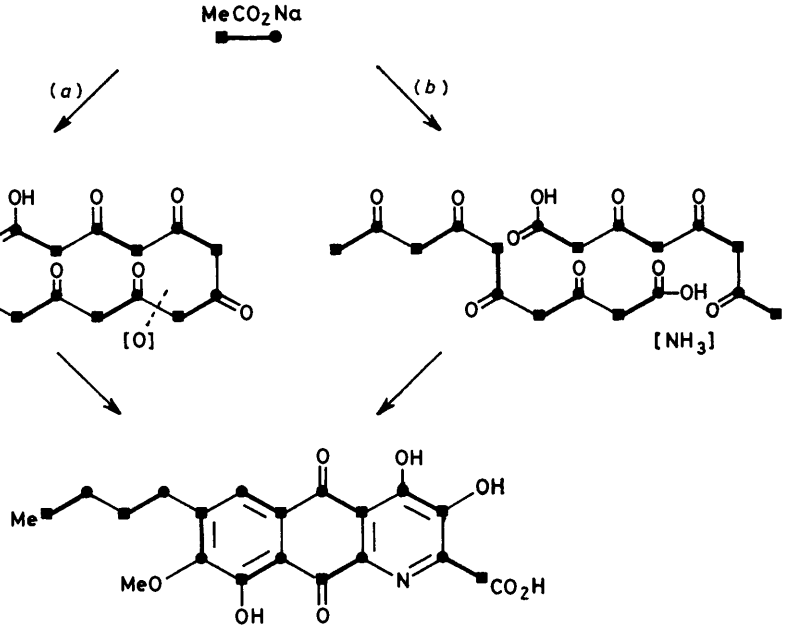


FIGURE 2 Low-field region of the p.n.d. ^{13}C n.m.r. spectrum of [$^{13}C_2$]acetate enriched spectra of phomazarin methyl ester (2) determined at (*a*) 15.04 MHz and (*b*) 67.89 MHz

On feeding diethyl [2-^{14}C]malonate and isolation of C-15 as acetic acid by Kuhn–Roth oxidation of tri-*O*-methylphomazarin methyl ester (5), and C-11 as barium carbonate from sulphuric acid degradation [1] of (5) to give (8), it was found that both C-15 and C-11 had 11.1%, *i.e.* one-ninth, of the total activity and so no starter effect was being observed. However when the feeding was repeated with the addition of a large amount of inactive sodium acetate [21] it was found that C-15 carried only 8.4% of the activity whereas C-11 contains 11.4% of the activity, and so is labelled to the same extent as the remaining malonate derived carbons.

[^{13}C]Malonate has been used successfully to detect acetate starter effects in the fungal metabolites deoxyherqueinone [5] and sclerin,[22] and indeed it has been demonstrated that feeding the large amounts of labelled substrate necessary in ^{13}C studies can inhibit the randomisation and equilibration of labels sometimes observed in studies with the trace amounts of substrate used in ^{14}C studies.[23] This effect has not been entirely accounted for but is probably due to either inhibition or overloading of the enzyme systems responsible for randomisation. On feeding diethyl [2-^{13}C]malonate to *P. terrestris*, the overall enrichment of the resultant phomarazin was low. Absolute measurement of ^{13}C-enrichment levels is difficult; however, by the use of relaxation agents and multiple determinations of natural-abundance and enriched spectra to give average enrichment values, satisfactory results can be ob-

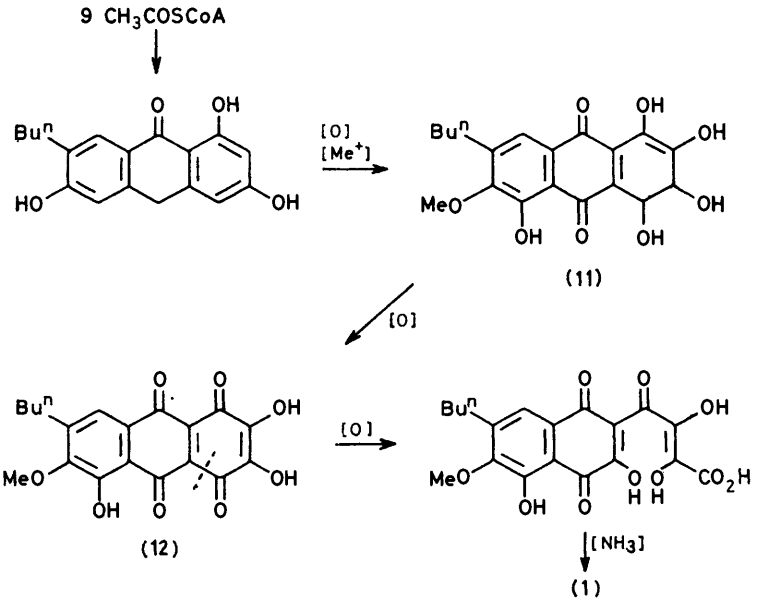


SCHEME 2 Assembly pattern of acetate units in phomazarin

tained.[5,24] The enrichments observed in phomazarin methyl ester (Table 2) indicate that C-15, but not C-11, is labelled to a much lower extent than the remaining [2-^{13}C]malonate-labelled carbons and so confirm the ^{14}C results. Thus phomazarin contains only one acetate starter molecule, and so must be formed from a single nonaketide chain folded as shown in path (*a*), Scheme 2, probably *via* an anthraquinone, *e.g.* (11), Scheme 3.

A large variety of natural products appear to be formed *via* oxidative fission of the quinonoid ring of anthraquinones, *e.g.* sulochrin,[25] geodin,[26] ravenelin,[9] and tajixanthone;[19] and it has been shown that quinones or quinols are intermediates in the biosynthesis of patulin[27] and penicillic acid[28] from benzenoid precursors. Thus the bis(quinone) (12), Scheme 3, is a possible precursor for the ring-cleavage step in phomazarin biosynthesis. There is no other established precedent for the cleavage of the benzenoid ring of an anthraquinone, but the fungal metabolites lambertellin[29] and purpurogenone[30] may well be formed by such a process. The secalonic acids, known to be formed by quinonoid ring-cleavage of cynodontin, have been isolated from *P. terrestris*.[31]

Nonaketides are relatively uncommon metabolites in fungi.[32] The main group, which comprise the versicolorin anthraquinones and their related metabolites, are almost certainly derived from C_{20}-precursors. A group of nonaketide metabolites, ligustronones A, B, and C, have recently been isolated from *Cercospora lingustrina*.[33]



SCHEME 3 Proposed biosynthetic pathway to phomazarin from acetate *via* anthraquinone intermediates

EXPERIMENTAL

The culture of *P. terrestris*, the preparation of all derived compounds and the ^{14}C radioactivity assays were carried out as previously described.[1,9]

Carbon-13 N.m.r. Determinations.—The ^{13}C n.m.r. spectra were obtained for samples in acid-free deuteriochloroform with tetramethylsilane as internal reference. Proton noise-decoupled spectra, single frequency off-resonance decoupled spectra and fully proton-coupled spectra were determined on a JEOL JNM FX-60 spectrometer operating at 15.04 MHz. Fully proton-coupled spectra were determined under gated decoupling conditions to retain nuclear Overhauser enhancements. The spectrum of [$^{13}C_2$]acetate-enriched phomazarin methyl ester was determined on a Bruker WH-270 spectrometer operating at 67.89 MHz. Trisacetylacetonatochromium [Cr(acac)$_3$] (0.1M) was used as a relaxation agent.

Feeding Experiments.—(*a*) Sodium [1-^{14}C]acetate (200 mg; 4.1 μCi mmol^{-1}) was added in portions on days 5, 6, and 7 to four shake-culture flasks. After 11 d growth, the mycelium was filtered off and dried. The dried mycelium was exhaustively extracted with ether and methanol and then was refluxed with 10% dry hydrogen chloride in methanol for 5 h. After cooling to 0 °C, the mycelium was filtered off and extracted with chloroform. The resulting crude red solid was methylated and tri-*O*-methylphomazarin methyl ester (60 mg; 1.47 μCi mmol^{-1}) isolated as previously described.[1] On the basis of this dilution factor (2.8) it was anticipated that feedings of [^{13}C]acetate (95 atom-%) would give phomazarin with an excess 3.8% of ^{13}C label over natural abundance at each labelled portion (assuming formation from nine acetate units).

(*b*) Accordingly sodium [1-^{13}C]- [2-^{13}C]-, and [$^{13}C_2$]-acetates were fed as above. The resultant labelled phomazarins were isolated as the methyl ester (2) and their p.n.d. ^{13}C n.m.r. spectra determined. Comparison of the labelled and unlabelled resonance intensities indicated an approximately 4% excess ^{13}C abundance at each labelled position.

(*c*) Diethyl [2-^{13}C]malonate (250 mg) in ethanol (6 ml) was fed to four flasks on days 6, 7, and 8, and after 11 d growth phomazarin methyl ester (65 mg) was isolated and its ^{13}C n.m.r. spectrum determined three times, as was the natural abundance spectrum, and the average intensities after normalisation were used to calculate the enrichment

values given in Table 2, using the method reported previously.[24]

(*d*) Diethyl [2-^{14}C]malonate (100 μCi) in ethanol (1 ml) was distributed among five flasks on day 6 along with inactive sodium acetate (100 mg) in water (5 ml). After 11 d growth tri-*O*-methylphomazarin methyl ester (103 mg; 2.403 μCi mmol^{-1}) was isolated as above. For degradation, the [^{14}C]compound (100 mg) was diluted with unlabelled material (200 mg).

Tri-*O*-methylphomazarin methyl ester (80 mg; 0.801 μCi mmol^{-1}) was oxidised under standard conditions to acetic acid; this was then converted to *p*-bromophenacyl acetate, which was crystallised to constant activity (0.067 μCi mmol^{-1}). A second portion of tri-*O*-methylphomazarin methyl ester (100 mg; 0.801 μCi mmol^{-1}) was converted to di-*O*-methyldecarboxyphomazarin (8) as described previously.[1] Crystallisation to constant activity gave (8) with an activity of 0.710 μCi mmol^{-1}. Thus the activity of CO_2 (by difference) liberated in the decarboxylation is 0.091 μCi mmol^{-1}.

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