Fungal Products. Part 21.[1] Biosynthesis of the Fungal Metabolite, Wortmannin, from [1,2-$^{13}C_2$]-Acetate

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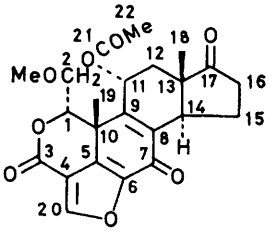
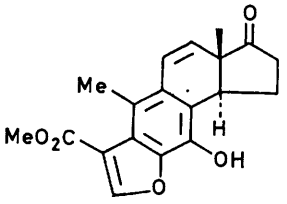
The ^{13}C-n.m.r. spectrum of wortmannin, enriched with sodium [1,2-$^{13}C_2$]-acetate by cultures of *Penicillium wortmannii*, is consistent with the biogenesis of this metabolite *via* triterpenoid intermediates. Although the overall incorporation of ^{13}C was low, ^{13}C–^{13}C coupling was observed between carbon atoms derived from adjacent acetate units in addition to the expected coupling between carbon atoms in intact acetate units.

WORTMANNIN, a metabolite isolated [2] from culture filtrates of *Penicillium wortmannii*, was shown to have structure (1) except for the C_1-stereochemistry by MacMillan *et al.*[3] This structure was confirmed, and the complete stereochemistry (1) was established, by *X*-ray



(1)



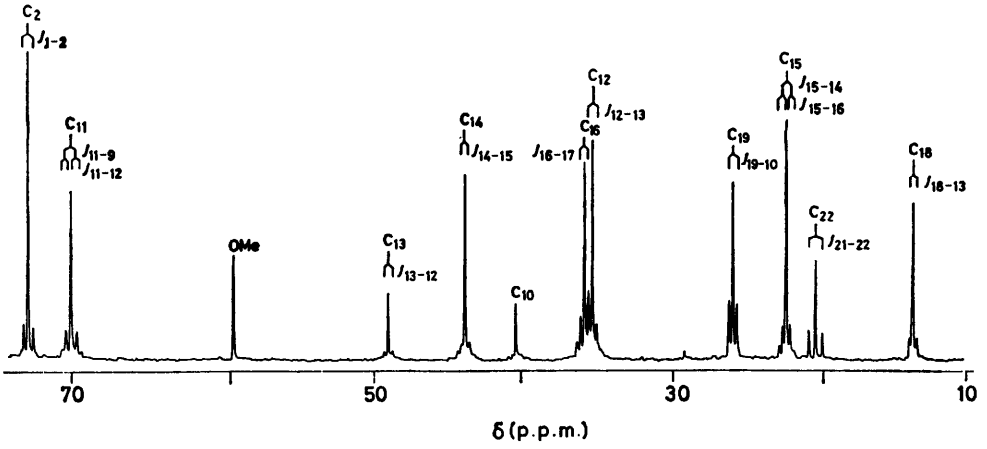
(2)

diffraction studies by Pecher *et al.*[4] Preliminary investigations [5] of the incorporation of [^{14}C]- and [^{3}H]-labelled precursors were consistent with a triterpenoid biogenesis for wortmannin. To provide further evidence for this pathway the incorporation of [1,2-$^{13}C_2$]-acetate into wortmannin has been studied.

The anticipated incorporation pattern of acetate *via* mevalonic acid, squalene, and lanosterol is shown in the Scheme. Thus the pairs of carbon atoms, 20 and 4, 19 and 10, 5 and 6, 9 and 11, 12 and 13, 16 and 17, 14 and 18, and 21 and 22, were expected to be derived from intact acetate units and show ^{13}C–^{13}C spin couplings. The remaining carbon atoms, derived from acetate residues which have been cleaved at various stages along the biosynthetic pathway, should appear as uncoupled resonances.

The ^{13}C n.m.r. spectrum of wortmannin is summarised in the Table and the region 70—10 p.p.m. downfield from internal tetramethylsilane is reproduced in the Figure. The assignments were based on (*a*) standard chemical shift data; [6] (*b*) off-resonance decoupling experiments using the graphical method devised by Birdsall *et al.*[7] which allowed the assignment of the protonated carbon resonances for wortmannin (1) and the degradation product (2); [8] (*c*) comparison of the resonances observed for wortmannin (1) and the degradation product (2); and (*d*) the resonances published [9] for furan derivatives.

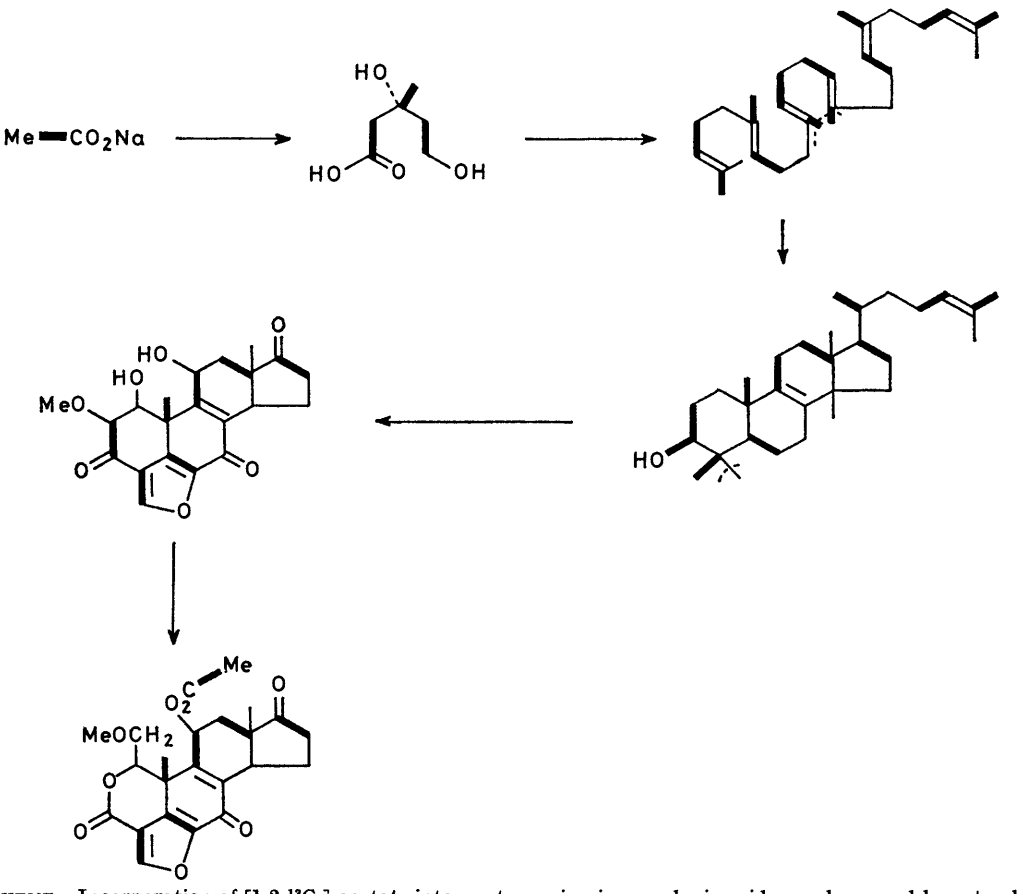
The amount of [^{13}C]-enriched precursor required to be fed to give satisfactory dilution values was determined by supplementing a series of shake cultures of *P. wortmannii* with sodium [1-^{14}C]-acetate and varying both the concentration of precursor and the time of addition. Optimum (minimum) dilution values were obtained by feeding acetate at a level of 200 mg l^{-1}. In this way dilution values of 13—21 were obtained and these values were not improved by adding higher concentrations of sodium acetate to the fermentations. These dilution values correspond to enrichments of 0.4 to 0.7% at each labelled site assuming 10 equally labelled sites (Scheme). Although such enrichment is low it was



High-field region (10—70 p.p.m.) of the proton-noise-decoupled ^{13}C n.m.r. spectrum of [1,2-$^{13}C_2$]-acetate-enriched wortmannin

anticipated that ^{13}C–^{13}C coupling satellites, 20—35% of the intensity of the natural abundance resonance, would be observed for the carbons derived from intact [1,2-$^{13}C_2$]-acetate units.

In the event, the noise-decoupled ^{13}C n.m.r. spectrum of wortmannin, derived from a [1,2-$^{13}C_2$]-acetate feed, was not as simple as anticipated (see Figure). Extensive spin coupling was observed between carbons derived from adjacent acetate units in addition to the expected coupling between carbons derived from intact acetate units. [^{13}C]-enrichment. The addition of a large amount of [1,2-$^{13}C_2$]-acetate to the culture probably suppresses the formation of endogenous acetate. Thus, for a brief period most of the mevalonate was being formed from added acetate so that the wortmannin was produced from mevalonate in which all six carbons were highly enriched. When the exogenous acetate pool fell below a low threshold level, the production of endogenous acetate, and hence of unlabelled mevalonate and of wortmannin, recommenced to give the final



SCHEME Incorporation of [1,2-$^{13}C_2$]-acetate into wortmannin *via* mevalonic acid, squalene, and lanosterol

Additional coupling of this type has previously been observed [10,11] in several biosynthetic studies using [1,2-$^{13}C_2$]-acetate in which high incorporation efficiencies result in an increased probability of adjacent acetate-derived units being labelled. It should be noted, however, that the overall enrichments are not, *per se*, sufficient to account for the intensity of the observed additional couplings. Also, in some cases where the overall enrichment levels are much too low to allow for any significant inter-acetate coupling, additional couplings have been observed to a minor extent [12] and, in one recent study,[13] to a major extent. Considering the present case the additional couplings indicate that the wortmannin was being produced, over a limited period, mainly from the added [1,2-$^{13}C_2$]-acetate to give a small proportion of the total wortmannin with very high low overall level of enrichment. That there was some dilution of the exogenous acetate (and consequent mevalonate) by endogenous metabolic pools was apparent from the relative intensities of the ^{13}C–^{13}C satellites arising from intra-acetate, inter-acetate and inter-mevalonate couplings (see Table and Figure). As a consequence of the inter-acetate couplings, the intensities of the primary satellites arising from intra-acetate couplings are reduced, the ' lost ' intensities appearing as secondary satellites to the primary satellites. For some quaternary carbons (*e.g.* C-5, C-8, and C-9) the primary satellites were not observed due to the additional coupling to several attached carbon atoms and to the inherently low intensity of these quaternary resonances.

However, on detailed examination of the spectrum of the [1,2-$^{13}C_2$]-acetate-enriched wortmannin, the

primary couplings of intact acetate units could be distinguished readily by their greater intensities. These couplings, summarised in the Table, are entirely in agreement with the biogenetic pathway in the Scheme. Thus, where there is no possibility of additional coupling to adjacent carbons (C-19, C-20, C-21, and C-22), the intensities of the primary satellites are *ca.* 30% of those of the uncoupled resonances, as predicted from the [^{14}C]-dilution factors. For the remaining carbons, derived from the incorporation of intact acetate units (C-4, C-6, C-10, C-11, C-12, C-13, C-16, and C-17), the intensities of the primary satellites are lower due to additional coupling to adjacent carbons, but they are sufficiently intense to be distinguished easily from the other couplings.

^{13}C N.m.r. chemical shifts and coupling constants observed in [1,2-$^{13}C_2$]-acetate-enriched wortmannin

Carbon atom	δ_C (p.p.m.)	Intra-acetate	Inter-acetate	Inter-mevalonate
1	88.5		35	(42) [a]
2	72.9			42
3	157.6		85	
4	114.2	75		
5	140.3 [c]	* [b]		
6	144.8 [c]	60		
7	172.6		* [b]	
8	149.5 [d]		* [b]	
9	142.8 [d]	* [b]		
10	40.7	* [b]		
11	70.1	48		(35)
12	35.7	35		(35)
13	49.2	35		
14	44.1		30	
15	22.9		33	(30)
16	36.2	37	(30)	
17	216.1	37		
18	14.6		30	
19	26.4	33		
20	150.1	75		
21	169.4	59		
22	21.0	59		
MeO	59.4			

[a] Values in parentheses observed as secondary coupling satellites. [b] No coupling observed due to low intensity of resonance. [c,d] Assignments may be reversed.

Inter-acetate couplings are clearly observed on the resonances of C-3 (to C-4), C-1 (to C-10), C-14 (to C-15), and C-15 (to C-14). In addition C-18 is coupled to C-13. Carbon-18 is known to arrive at C-13 by a 1,2-methyl migration from C-14 with which it originally formed an intact acetate unit; however the biosynthetically significant two-bond ^{13}C–^{13}C coupling between C-18 and C-14 was not resolved.

Inter-mevalonate couplings were observed between C-1 and C-2, C-11 and C-12, and on C-15. That there was some dilution by an endogenous metabolic pool of mevalonate is evidenced by examination of the couplings displayed by C-1 and C-2. The C-2 resonance showed satellites corresponding to the inter-mevalonate coupling to C-1 (see Figure). This coupling of 42 Hz is rather large for coupling between sp^3-hybridised carbons, due to the presence of oxygen substituents on both carbons. The primary satellites on C-1, however, correspond to the inter-acetate coupling (35 Hz) to C-10, the 42 Hz coupling to C-2 appearing as secondary satellites. A similar inter-mevalonate coupling was observed as secondary satellites on the C-15 and C-16 resonances and a secondary coupling was observed between C-11 and C-12 as a result of the head-to-head linkage of farnesyl units.

In conclusion the results, though more complex than expected, are in full agreement with the biogenesis of wortmannin as outlined in the Scheme.

EXPERIMENTAL

^{13}C *N.M.R. Determinations.*—Natural abundance ^{13}C spectra were determined for solutions in $CDCl_3$ or $(CD_3)_2CO$ with Me_4Si as internal standard on a JEOL PFT-100 instrument operating at 25.2 MHz in the Fourier transform mode. Sweep-widths of 6.25 KHz with 4 K data points were used to give chemical shifts with an accuracy of ± 1.52 Hz (± 0.06 p.p.m.). A proton-noise-decoupled spectrum of [1,2-$^{13}C_2$]-acetate-enriched wortmannin was recorded with a Bruker WF-270 F.T. spectrometer operating at 67.89 MHz. A sweep width of 15 KHz with 16 K data points was used to give chemical shift values accurate to ± 0.9 Hz ($\pm$ 0.01 p.p.m.).

^{14}C-*Enriched Wortmannin.*—Forty 250 ml baffled conical flasks, each containing 50 ml Czapek-Dox medium were innoculated with mycelium from pre-grown 5-day old shake cultures of *Penicillium wortmannii.* Sodium [1-^{14}C]-acetate (20 μCi) and unlabelled sodium acetate (80 mg) were distributed equally among 8 flasks in two portions on the third and fourth day after inoculation. After a further 4 days the flasks were removed and wortmannin isolated as usual [2] and crystallised to constant activity (2.11×10^6 disint. min^{-1} mmol^{-1}; yield 5.1 mg). This corresponds to a dilution factor of 21 and on this basis equivalent feedings of [^{13}C]-acetate (90%) would give wortmannin with an excess of 0.4% ^{13}C at each labelled position (assuming 10 labelled positions). A second experiment gave wortmannin (3.4×10^6 disint. min^{-1} mmol^{-1}; yield 7.3 mg). This corresponds to a dilution factor of 13 and an 0.7% excess of ^{13}C.

Incorporation of Sodium [1,2-$^{13}C_2$]-*Acetate.*—Sodium [1,2-$^{13}C_2$]-acetate (800 mg) was distributed among 80 flasks after 3 and 4 days' growth as above and the enriched [^{13}C]-wortmannin (56 mg) isolated after a further 4 days' growth.

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