The Incorporation of [1,2-$^{13}C_2$] Acetate into Pisatin to Establish the Biosynthesis of Its Polyketide Moiety *

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The biosynthesis of the polyketide moiety in pisatin has been examined by incorporation experiments with [1,2-$^{13}C_2$] acetate and ^{13}C-NMR measurements. The results establish that the C-5 carbonyl group in the enzyme-bound, open-chain polyketide precursor is reduced before cyclization to form the aryl ring. The specific incorporation attained in these experiments was considerably higher than that reported for earlier studies using singly labelled, radioactive acetate, emphasizing the importance of the timing of precursor addition.

Pisatin (**1**), the classical phytoalexin [1, 2] from *Pisum sativum*, had been shown to incorporate [^{14}C] acetate [3] as would be expected from its probable biosynthesis [3, 4] by the polymalonate-shikimate pathway. However, the incorporation level was relatively low and there was no evidence provided to prove that the labelled carbons were located at the predicted positions. The present study was undertaken not only to furnish formal proof of the labelled positions but also to verify that reduction of a carbonyl group in the polyketide derived portion of the presumed precursor takes place *before* closure [5] to ring A. The well-established ^{13}Cmr methodology based on the use of [1,2-$^{13}C_2$] acetate [6–8] seemed eminently suited to the realization of both objectives and this proved to be the case.

Experimental

Solvents were redistilled before use. Light petrol was of b. p. 30–60 °C; ethanol contained 4% of water. Evaporations were done *in vacuo*. Precoated silica gel plates (Polygram Sil N-HR/UV$_{254}$; Macherey Nagel) were used for thin-layer chromatography (TLC) with ether or ether/light petrol (1 : 1) as irrigant and visualization by examination under UV light (254 nm) and exposure to I_2 vapor. UV spectra were determined using a Beckman DK-1 instrument. ^{13}Cmr spectra were recorded with a Varian XL-100-15 system operating in the Fourier transform mode. The pisatin samples were examined as 10% solutions (w/v) in CD_3OD.

Locally grown peas (1.8 kg), purchased in the market in early summer, were shelled. The halfpods (416) obtained were placed on moistened filter paper in glass incubation trays and each charged with 2–3 ml of 3×10^{-3} M $CuCl_2$ solution [9], using 1.0 l total. After one day's incubation at room temperature in the covered trays, the $CuCl_2$ solution was drained from the pods and the trays and the halfpods, many of which had badly curled resulting in spillage into the trays, were refilled to the extent possible with a solution (305 ml) containing [1,2-$^{13}C_2$] NaOAc (430 mg; 91% labelled at each carbon atom) in 500 ml. After another day's incubation, the solution was collected from the pods and the trays and extracted with light petrol (3×200 ml) to afford extract 1. Pods and trays were then rinsed three times with small volumes of methanol to give extract 2. Finally, the rinsed pods were steeped for 3 days in methanol to furnish extract 3 which was found to contain more pisatin by TLC but was not examined further.

On evaporation extract 1 gave a gum (11 mg) containing 9.3 mg of pisatin as estimated by TLC and UV spectrometry at 280, 286 and 309 nm [2]. The ^{13}C spectrum of this material showed only the signals of pisatin and was essentially identical to that described below.

Extract 2 was concentrated until mainly aqueous ($\sim$50 ml) and then was extracted with light petrol (3×100 ml) affording a colorless solid (46 mg) containing 43 mg of pisatin by UV analysis. After

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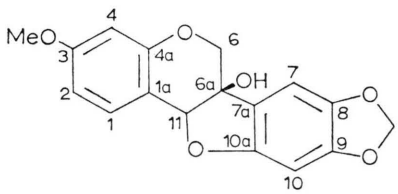
recrystallization from light petrol, this furnished the ^{13}C spectrum illustrated in Fig. 1 a, from which the data are collected in Table I. The assignments listed agree nicely with those reported for pterocarpin [10] with due allowance for the additional hydroxyl group in **1**.

Results and Discussion

The ^{13}Cmr spectrum (Fig. 1 A) of the ^{13}C-enriched **1** from extract 2 demonstrates unambiguously that three intact acetate units, indicated by heavy bonds, were incorporated into ring A of **1**. By comparison with the spectrum of **1** containing only ^{13}C in natural abundance (Fig. 1 B) it is apparent that



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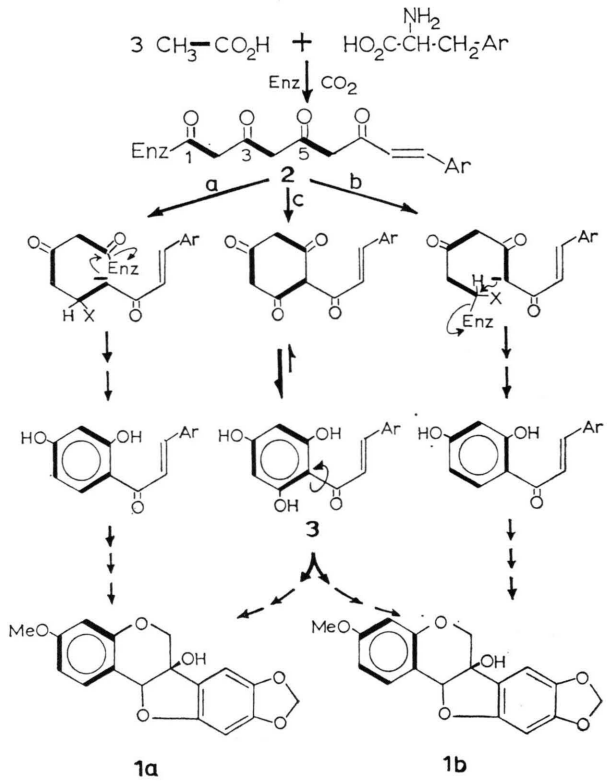
six of the signals are bracketed by "satellite" patterns arising from ^{13}C-^{13}C coupling in the enriched sample. The satellites arise from the doubly-labelled acetate units which have been incorporated and the separation between each of the more intense satellite lines flanking each of these six signals is the coupling constant. For the protonated centres the two intense satellite lines are also flanked by weak satellite signals because of the relatively high incorporation level. At this level some molecules contain both an intact labelled acetate unit and are enriched at an adjacent centre by incorporation of a second unit. The separation between these outermost satellites is the sum of the ^{13}C-^{13}C coupling constants for the two C-C bonds involved. Hence one-bond ^{13}C-^{13}C couplings for the A ring in **1** are provided by Fig. 1 A. From the relative intensities of the satellites to that of the central signal in each pattern the enrichment level was found [11] to be $2.3 \pm 0.2\%$. Since the remaining carbons in **1** arise from phenylalanine, none was enriched in these experiments.

Table I. ^{13}Cmr parameters [a] for **1**.

Carbon	δ_C	$^1J_{CC}$	Carbon	δ_C
1 a	114.1		6	70.7
1	133.1	60	6 a	77.7
2	110.2	(60)	7 a	121.4
3	162.4	66	7	104.3
4	102.4	(70)	8	143.6
4 a	157.3	72	9	150.8
OCH_3	55.8		10	94.6
$O\text{-}CH_2\text{-}O$	102.7		10 a	155.7
			11	85.9

[a] Shieldings (δ_C) given in ppm from internal TMS for CD_3OD solution. One-bond ^{13}C-^{13}C coupling constants ($^1J_{CC}$) given in Hz; J values from measurements of satellites of satellites (see text) given in parentheses.

Upon formation of the open-chain cinnamoylpolyketide precursor **2**, there are three possible pathways to pisatin which could generate labelling patterns **1a** and/or **1b**, differing in the disposition of intact acetate units in ring A and depending, essentially, on the timing of the required reduction of a carbonyl group. If reduction of **2** occurs *before*



ring closure at either C-5 (route a) or C-1 (route b), a single, but different, labelling pattern results in each case, namely **1a** and **1b**, respectively. In contrast, however, if reduction occurs *after* ring closure (route c), the intermediate chalcone **3** can be expected [8] to produce a 1 : 1 mixture of **1a** and **1b** because of free rotation about the acyl bond as indicated in the Scheme, *i.e.* cyclization to the dihydropyran ring could involve either ortho hydroxyl group in **3**. In such a case, the ^{13}C patterns of the

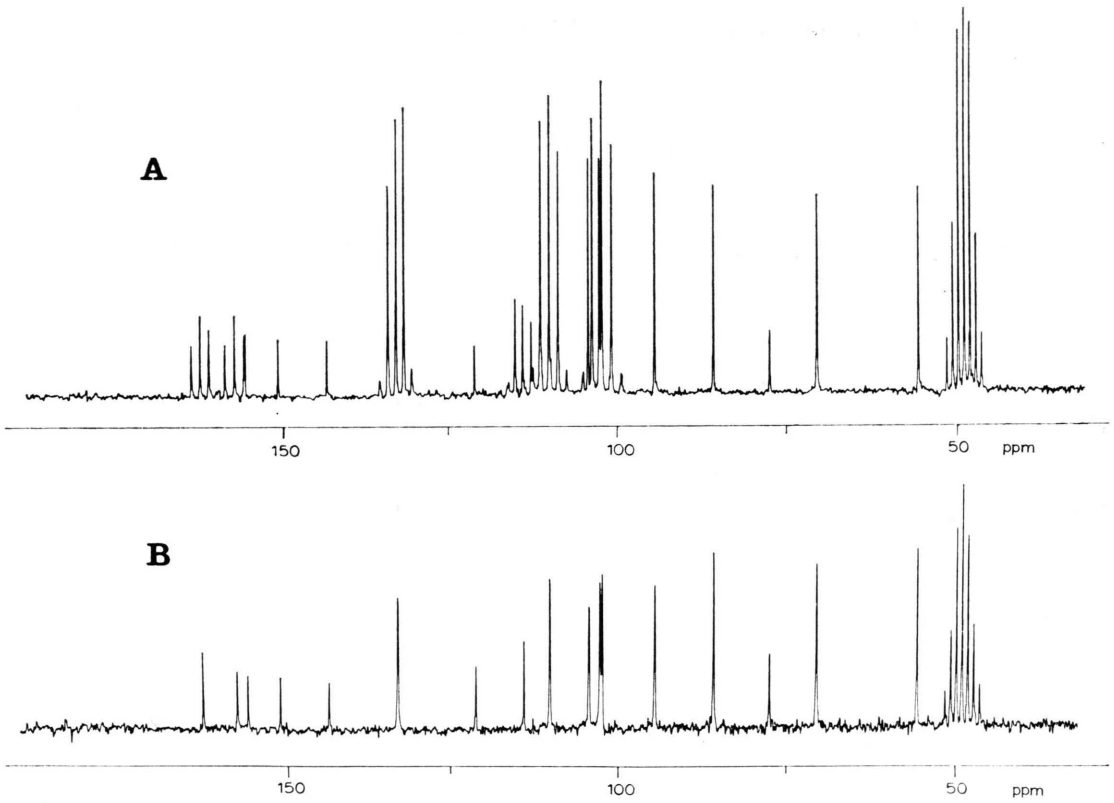


Fig. 1. ^{13}C spectra of **1**: (A) the spectrum of the enriched product from the incorporation experiment, (B) natural **1**. Both spectra were obtained for CD_3OD solutions.

enriched carbons would contain two sets of satellites rather than the single set observed. For example, the C-2 absorption at 110.2 ppm would exhibit a pair of satellite signals separated by 60 Hz as well as a pair separated by 66 Hz each of which would be half as intense as the single pair observed, separated by 66 Hz (Fig. 1 A). Thus the ^{13}C spectrum readily eliminates route c as a viable pathway to **1**. A decision between routes a and b is equally straightforward since the spacings between the pairs of prominent satellites for each of the protonated aryl carbons are different. The **1 b** labelling pattern requires these spacings to be the same for C-1 and C-2. Therefore, only **1 a** fits the observations. Accordingly it is the C-5 carbonyl in **2** which has undergone reduction before generation of the aryl ring A.

The incorporation level in these experiments is apparently much higher than that obtained in the earlier work [3] and must be due to the different methodology. The different results illustrate once again the importance, in biosynthetic studies, of experimental procedure; in this instance, the optimal timing of the addition of the labelled precursor is probably the major factor.

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