Biosynthesis of Pyoluteorin: A Mixed Polyketide-Tricarboxylic Acid Cycle Origin Demonstrated by [1,2-$^{13}C_2$]Acetate Incorporation

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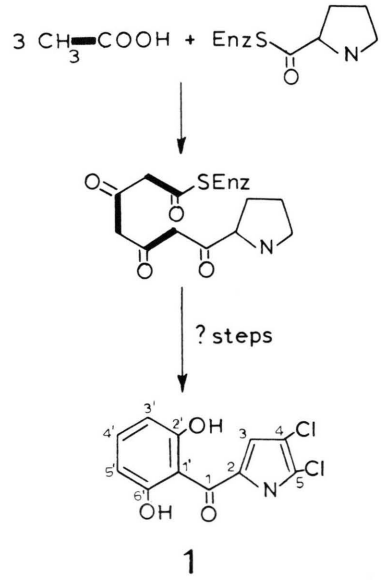
The incorporation of [1,2-$^{13}C_2$]acetate into the bacterial phytotoxin pyoluteorin (**1**) as determined by ^{13}C NMR spectroscopy occurs in a pattern consistent with the biosynthesis of the molecule as a tetraketide in which proline, or an equivalent precursor derived from the TCA cycle, serves as the starter unit.

Pyoluteorin (**1**) is an antibiotic and phytotoxic metabolite of several *Pseudomonas* spp. [1–3]. Its biosynthesis had not been studied previously but consideration of its structure suggested that it is a tetraketide with proline (or equivalent) as the starter unit. This has now been confirmed by an incorporation experiment employing [1,2-$^{13}C_2$]acetate as precursor and analysis of the isolated labelled product by ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR).

The basis for the experiment was the expectation that acetate would be incorporated into the resorcinol moiety by the usual acylphloroglucinol route (Scheme 1) while the acyl moiety, *i.e.*, the dichloropyrrole carboxylic acid residue, would be derived from the tricarboxylic acid (TCA) cycle as in Scheme 2. With the assumption that only the first two turns of the cycle would make a significant contribution, intact acetate units would be incorporated into two bonds in this fragments, C-1, -2 and C-4, -5, which would be revealed by the presence of the characteristic satellite patterns flanking the normal singlets for these four carbons in the ^{13}C NMR spectrum. Since



Scheme 1.

the first turn of the cycle may be expected to make a greater contribution than the second and only the C-4, -5 bond arises from an intact acetate unit in both turns, the incorporation level may be anticipated to be higher for the C-4, -5 bond than that for C-1, -2, which would be comparable to that for C-3. A few analogous experiments revealing the incorporation of acetate into complex molecules via the TCA cycle have been described recently [4–6].

Part 126 of ^{13}C magnetic resonance studies.

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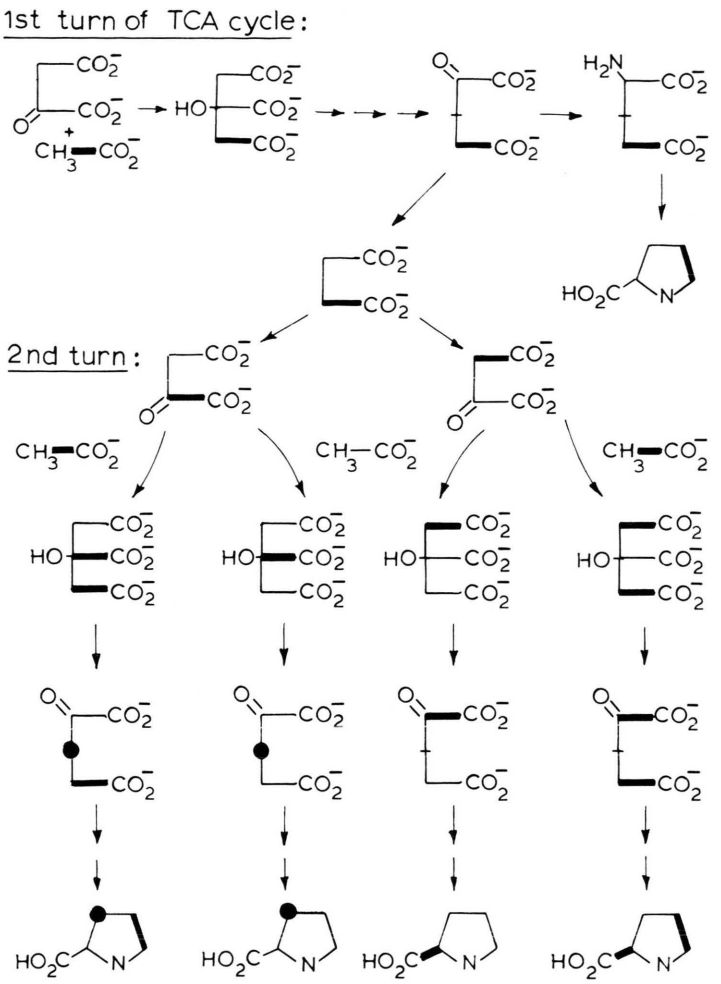

Scheme 2.

Materials

Pseudomonas fluorescens Pf5, available from earlier work [2], was maintained on nutrient broth-yeast extract (NBY) agar slants [7] at 4 °C as well as in NBY broth +15% glycerol at −73 °C. Sodium [1,2-$^{13}C_2$]acetate (90 atom% ^{13}C) was obtained from MSD Isotopes, Pointe Claire-Dorval, P.Q.

Methods

*Production of pyoluteorin (**1**) in King's B medium*

A *P. fluorescens* Pf5 culture grown overnight in King's B medium [8] at 24 °C on a rotary shaker served as inoculum. Two 250 ml flasks each containing King's B broth (40 ml) were inoculated with *P. fluorescens* to a A_{600} of 0.05 and incubated at 24 °C on a rotary shaker set at 180 rpm. At 4, 7, 20, 24, 28, 32, 44, 48, 52, and 68 h, the A_{600} of the cultures was recorded and a 2 ml sample was removed from one of the flasks for estimation of pyoluteorin concentration. After centrifugation (3000 × *g* for 10 min at 4 °C) to remove the bacterial cells, the sample was acidified (5 N HCl) and extracted with ethyl acetate. The pyoluteorin content was estimated spectrophotometrically at λ_{max} 308 in ethanol (ϵ 13,000) [3].

The same experiment was repeated with sodium acetate added at 20 h after inoculation to a final concentration of 0.005 M.

Incorporation of doubly-labelled acetate

Sodium [1,2-$^{13}C_2$]acetate (249.4 mg, 2.97 mmol) in water (6.4 ml) was added in equal portions to sixteen 40 ml cultures 15 h after inoculation (culture A_{600} = 5.0). Another 249.4 mg was added after an additional 6 h of incubation (culture A_{600} = 7.5). After incubation for 44 h, the cells were removed from the culture by centrifugation (4300 × *g* at 4 °C for 15 min) and the supernatant was acidified (32 ml 5 N HCl) and extracted with ethyl acetate. The residue (216 mg) obtained by evaporation of the extracts was chromatographed over silica gel (SiO_2; Camag DF 0, 100 g) in methanol/chloroform/acetic acid (10:90:1 by volume, 5 ml fractions). Fractions 27–31 (17.4 mg) were purified further by preparative thin layer chromatography (TLC) on SiO_2 (10 plates, Baker 7001-4) in the same solvent. Natural abundance pyoluteorin was obtained similarly from cultures without added sodium acetate. Diacetylpyoluteorin was prepared from the natural and ^{13}C-enriched samples by treatment with acetic anhydride/pyridine (2:3) at room temperature overnight, evaporation and purification by preparative TLC as above with recrystallization from aqueous ethanol.

NMR spectroscopy

The ^1H NMR and ^{13}C NMR spectra were recorded with Varian XL-200 and -300 systems, respectively, using acetone-d_6, methanol-d_4 and chloroform-d_1 with tetramethylsilane as internal reference. The ^{13}C spectra were obtained with 20° pulses, 3 s repetition rates, 30000 points with double precision across 200 ppm spectral widths and 65 K transforms. The relative intensities of the absorption bands were estimated by electronic integration and by the "cut and weigh" method.

Results and Discussion

Preliminary time-course experiments, both in the presence and absence of sodium acetate, indicated that pyoluteorin production commenced ca. 12 h after initiation of the cultures, with concentrations reaching a plateau at ca. 48 h. The amounts of pyoluteorin produced varied to some extent with state of inoculum, culture volume and the rate of shaking (aeration).

The protocol of the incorporation experiment was based on these results and yielded essentially pure, but oily, pyoluteorin (12 mg) which was identical with an authentic sample [2] by TLC and ^1H NMR. The observed ^{13}C shieldings (Table I) differed somewhat from those reported earlier for acetone-d_6 solutions [2], presumably because of different water content in the solvents employed but the assignments were straightforward and were substantiated by the observed coupling constants for directly bonded pairs of carbons in the labelled sample (Table I). ^{13}C spectra for methanol-d_4 solutions were also recorded; this clarified some regions of partial overlap of absorption patterns but, *vice versa,* obscured others. This, however, inadvertently introduced a minor complication since the meta-protons on the aryl ring (H-3′, 5′) slowly exchanged with deuterium in the solvent. Consequently the patterns for C-4′,

Table I. ^{13}C data[a] for **1** and **2**.

Cpd.	Solvent	C-1	C-2	C-3	C-4	C-5	C-1′	C-2′, 6′	C-3′, 5′	C-4′	Ac
1	Acetone-d_6[b]	n.r.[c]	131.0	117.5	113.5	119.9	110.6	157.3	107.5	132.5	–
	Acetone-d_6	187.0	135.5	117.3	110.6	123.1	112.2	163.9	108.3	134.6	–
		[73.2]			[81.5]		[62.7]	[67.3]	[57.5]		
	Methanol-d_4	185.4	132.0	119.0	111.7	121.5	115.1	157.6	107.9	132.5	–
		[69.1]			[88.3]		[69.5]	[64.8]	[58.2]		
2	Acetone-d_6	179.0	132.3	118.5	111.7	122.0	126.1	149.5	121.7	131.5	169.0, 20.5
		[71.6]			[89.0]		[73.0]	[68.2]	[56.5]		
	Methanol-d_4	179.9	130.7	119.6	112.3	23.3	126.5	149.7	121.8	131.7	170.1, 20.7
	Chloroform-d_1	178.5	129.0	118.5	112.6	122.0	124.3	148.4	120.6	131.0	168.5, 20.8
		[71.0]			[89.0]		[71.5]	[69.0]	[57.0]		

[a] ^{13}C shieldings in ppm from internal TMS; J_{cc} values (in Hz) for directly bonded carbons listed in square brackets between the coupled nuclei. *J* values for **2** in methanol-d_4 were not measured.
[b] Data from ref. [2].
[c] Not reported.

C-2′, 6′ and C-1′ gained isotope-shifted components induced by deuterium two and three bonds away. The uptake of deuterium was confirmed by a ^{2}H NMR spectrum which contained a single singlet at δ 7.48. After several weeks in methanol-d_4 solution, the deuterium content was found to be approximately 80 atom% in the ^{13}C-enriched sample from the relative intensities of the H-4′ and H-3′, 5′ absorption patterns and by comparison of the intensities of the isotope-shifted components with those for the protio species in the C-4′ absorption. The deuterium content of the natural abundance sample, which had been kept in methanol-d_4 for a significantly shorter period, was correspondingly lower at ca. 40 atom%.

The levels of ^{13}C enrichment at the various carbons except C-1 were determined initially from the spectra obtained in acetone-d_6 solution, from the relative intensities of the satellite signals and the central absorption [9]. For C-1 the satellites were partially obscured by instrumental artifacts. The enrichment of C-3 also could not be determined in this way since, as expected, its signal was a broad singlet flanked by weak satellites, $J \sim 61$ Hz, presumably arising from adventitious coupling with C-2 and/or C-4. It may be noted that very weak satellites corresponding to those just observable for C-3 were not detected in the C-2 or -4 absorption patterns, which, in principle, will introduce a small and almost certainly negligible error in the calculated enrichments for these carbons but will not affect the results for C-1 and C-5. For confirmation of these results for the specific enrichments, the spectra of methanol-d_4 solutions were analyzed similarly. This exercise gave equal enrichments for C-1 and C-2, which are the same as that found for C-2 from the acetone-d_6 spectra. However, the inadvertent ^{1}H/^{2}H exchange of the *meta*-protons, noted above, precluded the obtention of a reliable result for C-3′, 5′ because the fifteen-line pattern of relatively weak signals for the deuterated species lacks the Overhauser enhancement of the five-line pattern for the protio species thereby rendering intensity measurements suspect.

To confirm and extend the results for **1**, the partially deuterated samples were recovered from the methanol-d_4 solutions and converted to the known diacetyl derivative **2** [1]. ^{13}C NMR spectra of **2** were obtained in acetone-d_6, methanol-d_4 and deuteriochloroform solutions (Table I) which showed the latter to be the best solvent for integration purposes since the patterns for C-2 and C-4′ were clearly separated while these overlap in the other solvents. Thus, the C-4′ absorption represented the best internal standard for comparison with the C-3 signal, the enrichment of which can only be assessed by its intensity relative to those of the other centres. Since C-4′ and C-3 are protonated, while the others are fully substituted, these can be expected to exhibit comparable Overhauser enhancements. Careful measurements of the intensities of the absorptions for C-3 and C-4′ in both the normal and ^{13}C-enriched samples permitted a reliable assessment of the enrichment level at C-3. In the event, the value obtained was corroborated by closely similar estimates obtained from the analogous intensity measurements for C-2, -4 and -5. The pertinent results are collected in Table II from which it is apparent that the aryl ring carbons are most highly enriched (~ 2.7%) and the pairwise coupling of these confirms their polyacetate origin. While C-4, -5 and C-1, -2 also arise in a pairwise fashion from intact acetate units the enrichment levels differ by a factor of ca 3.5 and the enrichment of C-3 is comparable to that found for C-1, -2. This is

Table II. Enrichment levels[a] determined for the various sites in **1** and **2**.

Cpd.	Solvent	C-1	C-2	C-3	C-4	C-5	C-1′	C-2′, 6′	C-3′, 5′	C-4′
1	Acetone-d_6	[b]	0.7 ± 0.1		2.2 ± 0.1	2.3 ± 0.2	2.85 ± 0.1	2.65 ± 0.1	2.9 ± 0.2	2.85 ± 0.1
	Methanol-d_4	0.5 ± 0.1	0.5 ± 0.1		2.5 ± 0.2	2.4 ± 0.1				
2	Chloroform-d	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	[c]	2.8 ± 0.1

[a] Atom% ^{13}C estimated from the relative intensities (see text).
[b] Partially obscured by instrumental artifacts.
[c] Omitted because of inadvertent deuterium exchange (see text).

entirely consistent with Scheme 2 having the major incorporation arising from the first turn of the TCA cycle.

In summary, the results in Table II are in excellent agreement with the predictions represented in Schemes 1 and 2, and substantiate the origin of pyoluteorin as a tetraketide whose starter group is derived from the TCA cycle. The exact nature of the starter unit is unknown at this time but it may be proline itself which undergoes oxidation and chlorination at some stage in the sequence. Whether these elaborations occur before or after the primary condensation step is an open question which may deserve further attention.

Acknowledgements

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