Pseudomonic Acid Biosynthesis. The Putative Role of 3-Hydroxy-3-methylglutarate

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The putative role of 3-hydroxy-3-methylglutarate (HMG) as a precursor in pseudomonate biosynthesis has been investigated by the efficient ozonolysis of the Δ C2–C3 of methyl pseudomonate A to give a C_{16} ketone moiety, allowing comparison of the incorporation of small additions of [1-^{14}C]acetate, [3-^{14}C]HMG and [1,5-^{14}C]HMG to fermentations during pseudomonate biosynthesis. Half of the radioactivity of the pseudomonate from all precursors was in the C_{16} moiety, which would not be radiolabelled by [1,5-^{14}C]HMG if HMG is a direct precursor. Prior degradation of HMG to acetate, the interpretation of a previous ^{13}C experiment, is implied and further supported by the extent of incorporation of [3-^{14}C]HMG heavily diluted with unlabelled HMG. Although new hypotheses may be required to define pseudomonate biosynthesis, *Pseudomonas fluorescens* may degrade all exogenous HMG, however sparingly administered, and foil its use as an effective biosynthetic probe.

The pseudomonic acids are structurally unique antibiotics biosynthesised principally from acetate, only two of the 26 carbons being C_1 units derived from methionine.[1,2] From the labelling pattern of [^{13}C]acetate incorporated into pseudomonic acid A **1** (Fig. 1), it was concluded that the molecule may be constructed from three separate units: a C_{12} unit made up of a pentaketide substituted with two methionine-derived carbons, a branched C_5 unit (uniquely involved for a bacterial secondary metabolite) possibly derived from 3-hydroxy-3-methylglutarate (HMG) and a C_9 fatty acid in the biosynthesis of which HMG could also be involved with homo-HMG to account for only half enrichment of C7′ from [1-^{13}C]acetate.[2] HMG, normally an intermediate in isoprene biosynthesis in eucaryotic microorganisms, could satisfy the biosynthetic requirements of the pseudomonate carbon skeleton particularly with respect to the acetate-derived C(15) which with C(1)–C(4) appears like an isoprene unit. However, a role for mevalonic acid, which is biosynthetically nearer isoprene, could not be demonstrated,[2,3] probably because there is no uptake mechanism, and the initial biosynthetic study did not explore HMG. Subsequently, experimental evidence suggesting involvement of HMG in the biosynthesis of **1** was a 0.9–1.1% incorporation of [3-^{14}C]HMG,[3,4] contrasting with a lower (0.5%) value for [1-^{14}C]acetate,[2] and thus, however tenuously, implying a direct role for HMG as a biosynthetic precursor. Further, since most of the [^{14}C]HMG given to a *Pseudomonas fluorescens* fermentation was taken up into the cells, other more dominant competitive fates into poly-isoprenoid metabolites such as bactoprenols could be expected.[3–5] An HMG CoA lyase has been isolated from *P. mevalonii*[6] and therefore HMG could even be a normal intermediary metabolite in *P. fluorescens*.

Specific location of HMG-derived carbons in **1** may be expected from ^{13}C-precursor feeding followed by ^{13}C-NMR spectroscopy of enriched **1**. However, evidence can also be derived from ^{14}C-labelling strategies involving only trace amounts of putative precursor, followed by degradative chemistry. Results of the former approach have recently been published,[7] though without the experimental details. The clear conclusion was that, since [3,6-^{13}C]HMG added to the fermentation enhanced all acetate-derived carbons throughout **1**, HMG has no specific natural role in contributing the branched C_5 unit, nor is it a part of the C_9 unit. It was assumed that the [^{13}C]HMG given had been broken down to acetyl CoA before incorporation as such into **1**. However, this could have been a metabolic response to the abnormal, inevitably-large amount of [^{13}C]HMG given to the bacterium to enable sufficient specific ^{13}C enrichment in products for ^{13}C NMR spectroscopy. *Pseudomonas* spp. are well known to be especially versatile in metabolising organic xenobiotics and thus the ^{13}C experimental requirement may have obscured perception of a normal metabolic role of HMG.

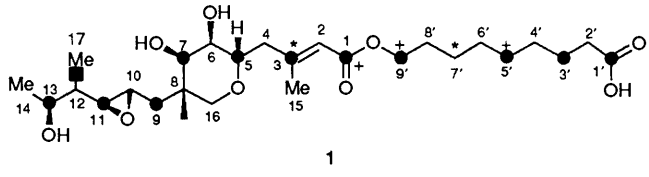

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Fig. 1 ●, Position of labelling from [1-^{14}C]acetate; ∗, putative position of labelling from [3-^{14}C]HMG; ■, position of labelling from [methyl-^{14}C]methionine; +, putative position of labelling from [1,5-^{14}C]HMG

We first explored the two obvious degradative strategies, following feeding with trace amounts of the commercially available radiochemicals [methyl-^{14}C]methionine, [1-^{14}C]-acetate and [3-^{14}C]HMG (specific radioactivities *ca.* 50 mCi mmol^{-1}), cleaving **1** by hydrolysis of the ester or by ozonolysis of the C(2)–C(3) double bond followed by measurement of radiolabel in the relevant moieties.

Saponification of **1**, with appropriate protection of *cis*-glycol functions, on a gram scale had been optimised as a source of monic acid A, **2**.[8] We have scaled down the process to the milligram scale to measure radiolabelling in the monic acid A moiety of **1** produced in 10 ml fermentations given radiolabelled precursors, but the technique proved insufficiently reliable in the recovery of deprotected **2** to be able to express with confidence its radioactivity with respect to that in the parent **1**. In comparing the fate of [1-^{14}C]acetate and [3-^{14}C]HMG, radioactivity was clearly evident in the monic acid A moiety but no valid comparison could be made (Table 1). Thus it was not possible to determine the effect of a large experimental

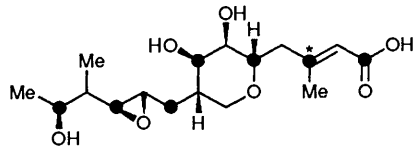

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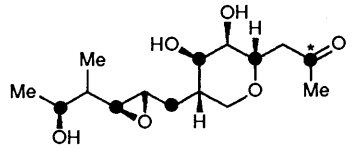
Table 1 Incorporation and distribution of radiolabel from [^{14}C]-labelled precursors into products of chemical degradation of pseudomonic acid A produced by *Pseudomonas fluorescens*

Fermentation volume (ml)	Precursor added to the culture	activity (μCi)	weight (mg)	Total pseudomonic acid A produced (mg)	Incorporation of [^{14}C]-labelled precursor into pseudomonic acid A (%)	Proporation of radiolabel in [^{14}C]monic acid A recovered from saponification of [^{14}C]pseudomonic acid A	Proportion of radiolabel in the ketone moiety after ozonolysis of [14C]pseudomonic acid A (%)
10	[1-^{14}C]acetate (Na salt)	10	0.012	0.41	1.58	13	—
10	[3-^{14}C]HMG	1	0.002	0.46	2.75	40	—
10	Unlabelled HMG	—	50	0.009	—	—	—
10	Unlabelled HMG (Na salt)	—	50	0.46	—	—	—
100 (×4)	L-[methyl-^{14}C]-methionine	8	0.021	12.4	17.8	—	99.7
100	[1-^{14}C]acetate (Na salt)	14	0.017	N.D.	N.D.	—	55.3 (54.4–56.7)[a]
100	[3-^{14}C]HMG	5	0.015	3.27	1.69	—	54.7
10	[1-^{14}C]acetate (Na salt)	7	0.008	0.79	2.9	—	54.3
10	[3-^{14}C]HMG	2	0.006	0.86	3.3	—	54.2 (54.1–54.3)[a]
10	[3-^{14}C]HMG + unlabelled HMG (Na salt)	27	0.084 50	0.46	0.76	—	53.3 (53.2–53.4)[a]
100	[1,5-^{14}C]HMG	2	0.005	2.97	1.85	—	53.4
100	[1,5-^{14}C]HMG	2	0.005	2.10	1.26	—	48.3

[a] Ozonolytic products divided into 2 aliquots and measured separately.

supplement of unlabelled HMG (mixed with a small amount of [^{14}C]HMG) in this way.

After ozonolysis the theoretical[2] distribution of radiolabel from [1-^{14}C]acetate in **1** would give six labelled carbons in the ketone moiety **3** and, using comparable criteria, 5.5 carbons in the aldehyde moiety **4** or 4.5 carbons in the more stable alcohol form **5**. Thus the proportion of the total radioactivity in the three radiolabelled products of ozonolysis attributed to **3** would be in the range 52.2–57.1% depending on the stability of **4**. In practice on a microgram scale, ozonolysis of **1** was highly efficient. From both the 10 ml and 100 ml fermentations given [1-^{14}C]acetate of [3-^{14}C]HMG, similar values were obtained for the proportion of total radiolabel in **3** (Table 1), well within the theoretical range and consistent with partial degradation of **4** to **5**.



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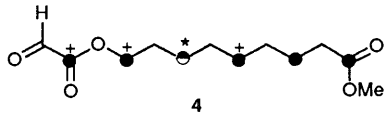

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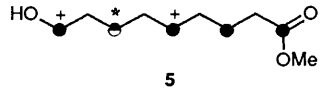

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Unlabelled HMG administered throughout the fermentation idiophase completely inhibited synthesis of **1** (Table 1); the broth pH value was 3.5 at the end of the fermentation implying that *P. fluorescens* was unable to buffer the added acid. However, addition of the sodium salt of HMG, presumably the form used by Martin and Simpson,[7] allowed a normal yield of **1** at the usual neutral end-point.

Efficient incorporation of [methyl-^{14}C]methionine into **1** was shown to be located almost exclusively in **3** after ozonolysis (Table 1), consistent with the theoretical expectation; the extent of metabolic scrambling of the label of the precursor into acetate was negligible.

In both 10 and 100 ml fermentations, distribution of radiolabel from [3-^{14}C]HMG into the two moieties of **1** after ozonolysis was indistinguishable from that of [1-^{14}C]acetate (Table 1), but the values could equally be interpreted as consistent with one HMG-derived unit contributing to each moiety of **1**. When the [3-^{14}C]HMG was diluted by addition of unlabelled sodium salt of HMG, increasing the amount of HMG (over that used as [3-^{14}C]HMG) by a factor of *ca.* 10^4 to mimic the magnitude of [^{13}C]HMG which would be necessary for a ^{13}C-NMR experiment, the distribution after ozonolysis was again similar. However, it was notable that 380 μg HMG (0.76% incorporation of 50 mg HMG) gave rise to the 460 μg of **1**, requiring that the HMG was metabolised to be widely distributed, as concluded by Martin and Simpson,[7] rather than located in one or two specific regions.

Recognising that the evidence from [3-^{14}C]HMG experiments was equivocal, and that of Martin and Simpson[7] could be criticised as having been obtained under abnormal conditions, we have synthesised [1,5-^{14}C]HMG of high specific radioactivity and administered it in the smallest amount consistent with reliable measurement of the distribution of radiolabel in the fermentation product. Maximum broth concentration, at each of 10 hourly additions of [1,5-^{14}C]HMG, of *ca.* 0.5 μg ml^{-1} could hardly be expected to present a xenobiotic challenge to *P. fluorescens* still in trophophase, but, if an uptake mechanism were available, could facilitate access of the putative precursor to the enzymes of pseudomonate biosynthesis. ^{14}C located specifically *via* ^{14}CN in both 1 and 5 positions of HMG would be retained only in the pyrophosphorylated carbon of the conventional isoprene

precursor or in the CoA-linked carbon in dimethylacryloyl–CoA.[2] ^{14}C released by decarboxylation of [1,5-^{14}C]HMG in contributing a branched C_5 unit during aerobic respiration would be expected to be dissipated mainly as CO_2, though some might be fixed into oxaloacetate. If ^{14}C of [1,5-^{14}C]HMG is incorporated directly, as suggested,[2] only into C(1) and, possibly, C(5′) and C(9′) of **1**, and without scrambling of the radiolabel into acetate or methionine, the ketone moiety **3** from ozonolysis of the [^{14}C] **1** would contain no radiolabel. Even partial expression of such biosynthesis using the present probe would cause a substantial reduction in the *ca.* 50% ^{14}C distribution in **3** occurring when all HMG is degraded to acetate.

The replicated experimental finding of ^{14}C from [1,5-^{14}C]HMG distributed *ca.* 50% (0.01 μCi counted) in **3** cannot, therefore, sustain a putative direct role for HMG in pseudomonate biosynthesis. Thus the weight of experimental evidence suggests that new hypotheses are required which define the obscure aspects of pseudomonate biosynthesis. However, it is still possible that *P. fluorescens* so efficiently catabolises any exogenously supplied HMG that experimental probes never reach sub-cellular sites of HMG biosynthesis which contribute this precursor to the pseudomonate pathway.

Further, the consistent incorporation into **1** of [^{14}C]HMG at least as efficiently as [^{14}C]acetate is not easy to reconcile with the metabolic dilution during HMG catabolism to acetate. Even in an extreme case of indirect incorporation of a putative precursor, tyrosine, into the naphthoquinone plumbagin in *Drosophilum lusitanicum* the percentage incorporation value was lower than that of acetate to which tyrosine was degraded.[11] A search for complementary evidence of other more vital HMG pathways in *P. fluorescens* may indirectly encourage, or otherwise, the plausibility of diversion to secondary metabolites from pathways leading to bactoprenol or analogous unsaponifiable polyprenols.[5]

Experimental

Production and Isolation of [^{14}C]*Pseudomonic Acid A.*—*P. fluorescens* (NCIB 10586) fermentations[3,4] on 100 or 10 ml scales were established from a 20% (v/v) inoculum of seed stage culture. Flasks were incubated on a rotary shaker for 5 h at 30 °C and thereafter at 24 °C until harvest at 30 h. Cultures were given radiolabelled precursors in equal amounts hourly from 6–16 h after inoculation. Culture broth, separated from the cells by centrifugation, was adjusted to pH 4.5 with hydrochloric acid and extracted with isobutyl methyl ketone (2 × ½ vol.) with centrifugation to break emulsions. The combined crude organic extracts were taken to dryness *in vacuo*.

Where the extract was used to obtain **2**, **1** was first isolated from the crude extract by preparative HPLC[4] in methanol–ammonium acetate (60:40) and extraction of combined eluates with isobutyl methyl ketone.

Monic Acid A from Pseudomonic Acid A.—Preparation of **2** from **1** was modified from Clayton *et al.*,[8] for sub-milligram amounts. [^{14}C] **1** (*ca.* 0.4 mg), the radioactivity of which had been measured, was dissolved in trimethyl orthoformate (1 ml). Toluene-*p*-sulphonic acid (2 crystals) was added and the solution stood at room temperature for 15 min. The solvent was evaporated, aqueous NaOH (1 ml; mol dm^{-3}) was added immediately and the mixture was incubated at 65 °C for 3 h. The solution was cooled, adjusted to pH 7 with HCl, MeOH (1 ml) was added and the solution was adjusted to pH 2 with HCl. After 15 min, the solution was adjusted to pH 9.5 and set aside for 3 h at room temperature. Liquid–liquid extraction with ethyl acetate gave inefficient partition of **2** into the organic solvent. Thus, the methanolic NaOH solution was evaporated to dryness, dissolved in H_2O (150 μl) and resolved in 20 μl amounts by HPLC[4] in methanol–ammonium acetate (30:70) collecting **2** with baseline separation from other components absorbing at 230 nm. Radioactivity in collected eluates was measured by liquid scintillation counting.

Pseudomonic Acid A Ozonolysis.—Crude *P. fluorescens* culture extract, dissolved in MeOH, was methylated with an excess of ethereal diazomethane and chromatographed over silica gel (SIL GF_{254}; Camlab) in $CHCl_3$–MeOH (90:7) and autoradiographed (Fuji X-ray film) at −70 °C for 1–2 d. The principal radioactive component, methyl pseudomonate (R_f 0.35) absorbing strongly under UV light (λ 254 nm) was eluted with propan-2-ol and the eluate evaporated to dryness. [^{14}C]Methyl pseudomonate was dissolved in MeOH, pyridine (2 drops) was added and ozonised oxygen was bubbled into the solution at −78 °C until it remained persistently blue. The solution was purged with oxygen until colourless, triethyl phosphite (20 μl) was added and the mixture was brought slowly to room temperature. The solvent was evaporated under reduced pressure and residual triethyl phosphite removed in a stream of N_2. The residue was chromatographed over silica gel in $CHCl_3$–MeOH (90:4) and the chromatogram autoradiographed as above. Radioactivity was confined to three bands. The most radioactive and the most polar compound (R_f 0.11) co- chromatographed with the ketone **3** {3*R*,4*R*-dihydroxy-5*S*-[(2*S*,3*S*-epoxy-5*S*-hydroxy-4*S*-methyl)hexyl]tetrahydropyran-2*S*-ylacetone} prepared similarly from authentic methyl pseudomonate and having an electron impact mass spectrum fragmentation [*m/z* 302 (M^+), 284, 269, 266, 259, 253, 244, 241, 227, 154] identical to that reported.[9] The other two bands were well resolved (R_f 0.75 and 0.96) when chromatographing microgram amounts of ozonolysis products. The EI mass spectrum of the combined components obtained preparatively showed important ions at *m/z* 182, consistent with a fragment of the aldehyde **4** (M^+ 228 − CO − H_2O, 182), and *m/z* 172 equivalent to the molecular ion of the alcohol **5** to which **4** may degrade spontaneously. The silica gel of the relevant radiolabelled regions of the chromatogram was suspended in Packard Scintillator 199 and the radiolabel measured by liquid scintillation counting. It was evident that the compound with R_f 0.75 carried more radiolabel than the compound with R_f 0.96, by a factor of 1.5–2.2, implying a standard pattern of ozonolytic products from methyl pseudomonate.

[1,5-^{14}C]-3-*Hydroxy*-3-*methylglutaric Acid.*—Synthesis of HMG from 1-chloro-2,3-epoxy-2-methylpropane[10] was modified to a one-pot process at the microgram scale to give [1,5-^{14}C]HMG of high specific radioactivity. To $Na^{14}CN$ (0.5 mg; specific activity 50 mCi $mmol^{-1}$) was added acetic acid (2% v/v, 40 μl) and 1-chloro-2,3-epoxy-2-methylpropane (5% v/v in DMSO, 10 μl) and the mixture was stirred for 5 h. NaOH (17% w/v, 5 μl) and H_2O_2 (6% v/v, 10 μl) were added and the mixture was set aside overnight. H_2O_2 (30% v/v, 10 μl) was added and the solution incubated in steam for 4 h. The mixture was cooled, concentrated HCl (15 μl) was added and the solution loaded, and evaporated, along a 35 cm line on Whatman No. 1 paper. Descending paper chromatography (18 h) in ethanol–ammonia–water (20:1:4) resolved the products to yield [1,5-^{14}C]HMG, located autoradiographically (R_f 0.47), which was eluted in dilute acetic acid, repurified in the same chromatography system and shown by autoradiography to co-chromatograph with [3-^{14}C]HMG (Amersham International).

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References

1 E. B. Chain and G. Mellows, *J. Chem. Soc., Perkin Trans. 1*, 1977, 294.
2 T. C. Feline, R. B. Jones, G. Mellows and L. Phillips, *J. Chem. Soc., Perkin Trans. 1*, 1977, 309.
3 P. G. Mantle and E. A. Somner, *FEMS Microbiol. Lett.*, 1988, **49**, 117.
4 P. G. Mantle and K. M. Macgeorge, *FEMS Microbiol. Lett.*, 1988, **59**, 55.
5 D. P. Gough, A. L. Kirby, J. B. Richards and F. W. Hemming, *Biochem. J.*, 1970, **118**, 167.
6 D. S. Scher and V. W. Rodwell, *Biochim. et Biophys. Acta*, 1989, **1103**, 321.
7 F. M. Martin and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1989, 207.
8 J. P. Clayton, K. Luk and N. H. Rogers, *J. Chem. Soc., Perkin Trans. 1*, 1979, 308.
9 R. G. Alexander, J. P. Clayton, K. Luk, N. H. Rogers and T. J. King, *J. Chem. Soc., Perkin Trans. 1*, 1978, 561.
10 A. H, Yavrouian, R. A. Sanchez, J. K. Pollard and E. K. Metzner, *Synthesis*, 1981, 791.
11 R. Durand and M. H. Zenk, *Phytochemistry*, 1974, **13**, 1483.

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