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# Differentiating the Biosynthesis of Pseudomonic Acids A and B

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Pseudomonic acid A (**1**) has been the dominant commercial pseudomonate antibiotic produced by *Pseudomonas fluorescens*. In specific shaken flask conditions initial fermentation accumulation of **1** is followed by preferential accumulation of the 8-hydroxy derivative, pseudomonic acid B (**2**). Biosynthetic probing with a pulse of [1-$^{14}$C] acetate or L-[methyl-$^{14}$C] methionine at early, mid and late stages of the fermentation gave relative patterns of radioactivity in **1** and **2** that are inconsistent with an assumption that **2** arises by oxidation of **1**, or that **1** is formed by reduction of **2**. Since [methyl-$^{14}$C] methionine only labels carbons in the 12-carbon part of the pseudomonate molecule that is thought to be an early biosynthetic moiety, the evidence from radiolabelling experiments implies that preferential early oxidation of this biosynthetic intermediate causes the pathway diversion to accumulate **2** instead of **1**.

Pseudomonic acid antibiotics are a unique group with closely related structures, a 17-carbon skeleton being common to all seven derivatives. Acids A~E are produced by a *Pseudomonas fluorescens* strain[1~5], and two related nitrogen-containing compounds by a marine bacterium identified as *Altromonas* sp.[6]. As mupirocin, pseudomonic acid A (**1**, Fig. 1) has an important market niche as an antibiotic effective against Gram-positive pathogens and with a role against superficial methicillin-resistant *Staphylococcus aureus*. Structural variations in the *P. fluorescens* metabolites are partly in the 9 or 10-carbon aliphatic side chain esterified on the 17-carbon monate moiety, that varies only in oxygen substituents. Comprehensive analysis of the biosynthetic origin of **1**[7] firmly established the dominant role of acetate, with just two carbons arising from $C_1$ methylations by *S*-adenosyl methionine. Specifically, [methyl-$^{14}$C] methionine radiolabels C-16 and C-17 in the substituted pentaketide represented by carbons 5~14, 16 and 17. It was presumed that **1** was constructed from three sub-units, a 10-carbon polyketide chain with two $C_1$ substituents, a five-carbon branched unit, the origin of which from isoprene biosynthesis was an attractive idea, and a nine-carbon chain, part of which might also arise from an isoprene pathway. Invoking the isoprene pathway in the mid 1970's was a relatively bold proposal for procaryote secondary metabolite biosynthesis and this aspect has since stimulated several attempts to obtain definitive experimental evidence, without as yet any conclusive success[8].

Typically, relative amounts of pseudomonic acids A : B : C : D in commercial submerged fermentation are in the ratio 93 : 5 : 2 : 2, respectively[3], and pharmaceutical production has seemed to concentrate on **1** as the dominant product. The origin of pseudomonic acid B (**2**, Fig. 1), differing from **1** only by addition of one oxygen atom, has been considered only to the extent of expecting it to be formed by enzymic hydroxylation of **1**[2], though there has been no experimental evidence.

Development of shaken-flask fermentation, from methodology previously described[5] though in which **2** was recognised as the principal pseudomonate antibiotic, has facilitated study of the production dynamics of **1** and **2**, and has enabled acquisition of experimental evidence which suggests that their biosyntheses diverge rather early in the pathway.

Aspects of the biosynthesis, fermentative production and regulation of **1** have been important in the teaching of microbial biochemistry at Imperial College for many years, since the compounds were first studied there. The present pulse-labelling biosynthetic study is based on repeated,

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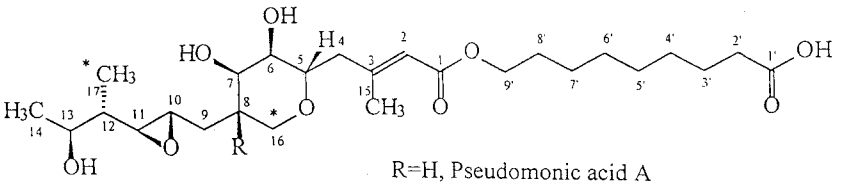
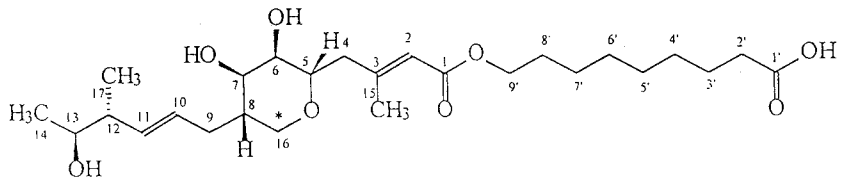
Fig. 1. Structures of pseudomonic acids A (**1**), B (**2**) and C.



R=H, Pseudomonic acid A

R=OH, Pseudomonic acid B



Pseudomonic acid C

*Indicates carbons derived from the methyl of methionine. Carbons 1~17 constitute the monate moiety.

though unpublished, demonstration of rapid incorporation of $^{14}C$ from [methyl-$^{14}C$] methionine into **1** and excretion into the broth within 5 minutes in early idiophase. Further, incorporation of $^{14}C$ from the same precursor into **1** rather efficiently when given very early in a fermentation inferred that early steps in the pathway were operating several hours before any end-product accumulated.

## Materials and Methods

### Fermentation Production of Pseudomonic Acids

*Pseudomonas fluorescens* (NCIB 10586), maintained on nutrient agar, was used to inoculate 100 ml liquid seed medium in a 500 ml Erlenmeyer flask incubated on a rotary shaker (200 r.p.m., 10 cm eccentric throw) at 30°C.

The seed stage medium consisted of glucose 0.11%, yeast extract 2.85%, $Na_2HPO_4$ 0.26%, $KH_2PO_4$ 0.24%, $(NH_4)_2SO_4$ 0.5%. The production stage medium consisted of soya flour (Arkasoy-50) 0.2%, corn steep liquor solids 0.5%, glucose 6.0%, $Na_2HPO_4$ 0.1%, $KH_2PO_4$ 0.15%, KCl 0.1%, $(NH_4)_2SO_4$ 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.625%, pH 7 in distilled water. Sterilisation was at 121°C for 20 minutes.

Production stages were inoculated with a 5% volume of 24 hours seed stage culture, incubated for an accellerated trophophase for 6 hours at 30°C, during which neither **1** nor **2** accumulated, and then transferred to 24°C. The first experiment in 500 ml flasks used a shaker with a 10 cm eccentric throw. The second experiment in 250 ml conical flasks was performed in a shaking incubator with only a 5 cm eccentric throw. Maintaining liquid volume of about 100 ml in a 500 ml flask was important for allowing the switch in principal product formation from **1** to **2**. Fig. 2 illustrates the effect of progressive reduction of fermentation volume, giving **1** as the principal metabolite as occurs typically in fementations as originally developed[1].

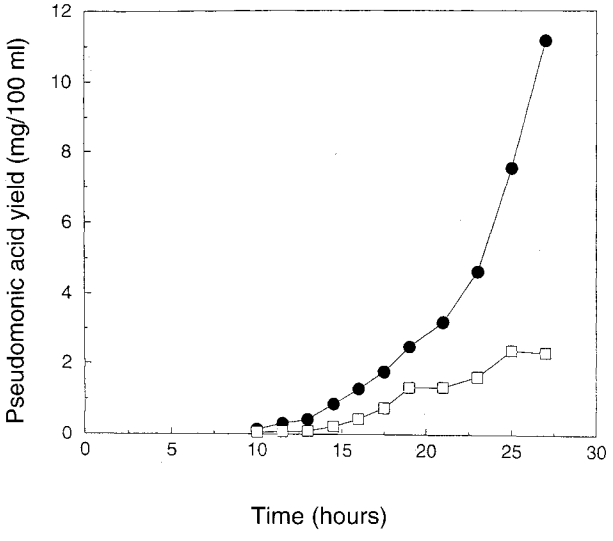
### Isolation of Pseudomonic Acids

Cultures were centrifuged to separate cells and medium debris from the broth which was adjusted to pH 4.5 with dilute HCl and extracted with an equal volume of isobutyl methylketone (IBMK). The extract was evaporated to dryness and residue solubles were taken up in methanol.

### High Performance Liquid Chromatography

Analytical HPLC used a Waters $C_{18}$ reverse phase cartridge in a Z-module, and methanol-ammonium acetate (50 mM in $H_2O$) [3 : 2, pH 4.5] as the mobile phase at a flow rate of 4 ml $min^{-1}$. Samples were injected in 20 $\mu$l methanol and eluate was monitored at 230 nm. For preparative

Fig. 2. Accumulation of **1** (●) and **2** (□) during a 105 ml fermentation in a 500 ml shaken flask, during which successive removal of 10 ml samples correspondingly depleted culture volume.




isolation of **2**, a Dynamax macro $C_{18}$ column (30×2.5 cm) was used at a solvent flow rate of 7 ml $min^{-1}$. Crude extract in methanol was injected (200 $\mu$l) repeatedly and eluates corresponding to **2** were combined and evaporated to small volume. This concentrated fraction, rich also in ammonium acetate, was shaken with an equal volume of IBMK; the organic layer was evaporated to dryness. The residue was taken up in methanol (400 $\mu$l) and re-purified (2×200 $\mu$l) by HPLC. The fractions containing **2** were combined, re-extracted into IBMK and evaporated to yield 30 mg of product for NMR spectroscopy.

## Biosynthesis Experiments

### Experiment 1

Fermentations (100 ml medium in 500 ml flasks) were given 5 $\mu$Ci sodium [1-$^{14}$C] acetate (50~60 $\mu$Ci $mmol^{-1}$) in water (1 ml) at either 6 hours (immediately after transferring from 30°C to 24°C), 24 hours or 32 hours. Fermentations were harvested at 50 hours and the pseudomonic acids extracted from the broth of each flask[8] were applied in IBMK as adjacent 5 cm strips on a PLC plate (20×20 cm) coated (2 mm) with silica gel $GF_{254}$ (Merck). The chromatogram was developed in chloroform-methanol (9 : 1) and resolved solutes were visualised under $UV_{254}$ light. Autoradiography with Fuji RX X-ray film was performed for 4 days. Silica gel regions visualised by absorbance under 254 nm light and corresponding to standard **1** and **2** were excised, suspended as a powder in methanol (1 ml) and mixed with 5 ml scintillant (Ecolume, ICN Biochemicals, Costa Mesa, California). $^{14}$C was measured (Kontron liquid scintillation counter) against standard $^{14}$C-hexadecane.

### Experiment 2

Fermentations of 150 ml medium in 250 ml flasks, incubated at 30°C for the first 6 hours and then at 24°C, were given 1.2 $\mu$Ci L-[methyl-$^{14}$C] methionine (50~60 $\mu$Ci $mmol^{-1}$) immediately after inoculation, or at 2, 6, 20 or 34 hours after inoculation. More detailed study of the early phase of fermentation was made also by administration of 20 $\mu$Ci [1-$^{14}$C] acetate or 7 $\mu$Ci [methyl-$^{14}$C] methionine to separate flasks at 2, 4, or 6 hours after inoculation. Samples (10 ml) were taken at intervals after administration of radiolabelled precursor to measure the total $^{14}$C in broth, the titre of **1** and **2** by HPLC analysis, and the amount of $^{14}$C by scintillation counting in each of these antibiotics separated by HPLC. All flasks were harvested at 48 hours and the end point titres of **1** and **2** were measured.

Small portions of culture extract were also examined by TLC (Polygram SIL G $UV_{254}$, developed in chloroform-methanol, 9 : 1), and the chromatograms were autoradiographed, first for 3 weeks and then for 2 months.

## Results

### Characterisation of Pseudomonic Acid B (2)

The surprising preferential fermentation accumulation of a *P. fluorescens* metabolite which was slightly more polar than **1** and suspected to be **2** required unequivocal identification. Electron impact mass spectrometry showed a weak molecular ion (*m/z* 516) and two fragment ions (*m/z* 498 and 480) which, from accurate mass measurement, arose by sequential loss of one or two molecules of water from the molecular ion (*m/z* 516.29266, $C_{26}H_{44}O_{10}$ [**2**] requires 516.29345). This fragmentation is a feature both of **1** and **2**[1,2]. **2** (30 mg, isolated by HPLC) gave $^{1}$H and $^{13}$C NMR spectra in $d_6$-acetone in which the C-8 proton ($\delta$ 1.95) of **1**, clearly evident in our reference spectra of **1** in $d_4$-methanol, was absent; a quaternary carbon signal at 71.9 ppm was attributed to C-8, there being no $^{13}$C-8 signal of **1** (41.5 ppm) present. Published data[9] for **1** was helpful in assigning the $^{13}$C NMR spectral data to the 26 carbons of **2**, and in making for the first time complete assignment of $^{1}$H and $^{13}$C NMR resonances (Table 1).

The UV absorption maximum for **2** (232 nm) was also at

Table 1. $^{13}C$ and $^{1}H$ NMR assignments for pseudomonic acid B.

| Carbon atom | $\delta^{13}C$ (in $CDCl_3$; Chain & Mellows 1977) | $\delta^{13}C$ (in $(CD_3)_2CO$) | DEPT 135 | $\delta^{1}H$ (in $(CD_3)_2CO$) | J($H_z$) |
|---|---|---|---|---|---|
| 1 | 166.8 | 166.8 | Q | - | |
| 2 | 117.7 | 117.9 | CH | 5.71 | 2,4 ax = 0.7 |
| 3 | 156.4 | 158.2 | Q | - | |
| 4eq | | | | 2.65 | 14.2 |
| | 42.9 | 43.5 | CH | | |
| 4ax | | | | 2.21 | 14.2 |
| 5 | 70.2 | 75.1 | CH | 3.61 | 5,4=9.3,2.6;5,6=9.3 |
| 6 | 72.9 | 70.3 | CH | 3.38 | 6,5=9.3; 6,7=3.1 |
| 7 | 74.6 | 74.3 | CH | 3.80 | 7,6=2.8 |
| 8 | 72.0 | 71.9 | Q | - | |
| 9a | | | | 2.42 | |
| | 37.1 | 38.2 | $CH_2$ | | |
| 9b | | | | 1.94 | 14.3; 9,10=5.3 |
| 10 | 51.8 | 52.4 | CH | 2.95 | 10,9=5.9;10,11=2.2 |
| 11 | 60.7 | 60.2 | CH | 2.73 | 11,10=2.3; 11,12=7.2 |
| 12 | 41.7 | 42.9 | CH | 1.33 | |
| 13 | 71.5 | 70.7 | CH | 3.77 | 13,14=6.4; 3,12=5.0 |
| 14 | 21.2 | 20.8 | $CH_3$ | 1.16 | 14,13=6.4 |
| 15 | 19.2 | 19.8 | $CH_3$ | 2.16 | 15,2 =1.2 |
| 16eq | | | | 3.49 | 11.0;16,9b=0.7 |
| | 68.5 | 69.3 | $CH_2$ | | |
| 16ax | | | | 3.43 | 11.0 |
| 17 | 12.1 | 12.0 | $CH_3$ | 0.88 | 17,12=7.0 |
| 1' | 174.4 | 174.8 | Q | - | |
| 2' | 34.1 | 34.1 | $CH_2$ | 2.29 | 2',3'=7.0 |
| 3' | 24.9 | 25.5 | $CH_2$ | 1.60 | |
| 4' | 29.0 | 29.7 | $CH_2$ | 1.33 | |
| 5' | 29.0 | 29.8 | $CH_2$ | 1.33 | |
| 6' | 29.0 | 29.8 | $CH_2$ | 1.33 | |
| 7' | 25.9 | 26.6 | $CH_2$ | 1.33 | |
| 8' | 28.7 | 29.4 | $CH_2$ | 1.60 | |
| 9' | 63.8 | 63.9 | $CH_2$ | 4.03 | 9',8'=6.6 |

slightly lower wavelength than that of **1** (235 nm), consistent with literature data[1,2].

## Biosynthetic Probing with $^{14}C$-Labelled Precursors

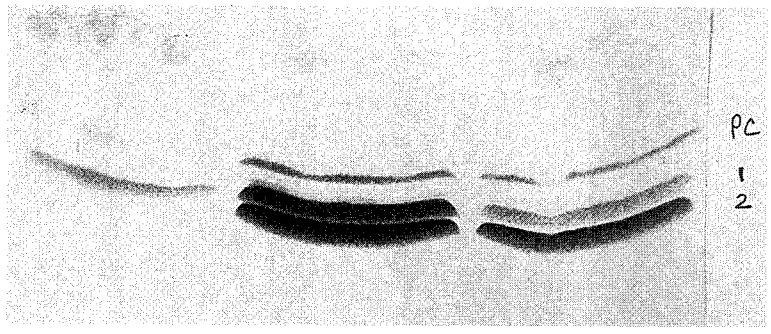
### Experiment 1

Accumulation of **1** and **2** followed separate dynamics; by 50 hours the fermentation had accumulated over 10 mg total antibiotics in a control flask in which pH was well buffered by the $CaCO_3$. Addition of $^{14}C$-acetate to another flask at 6 hours (Table 2) radiolabelled the three pseudomonic acids, though most prominently **1**, although the first-accumulated **1** did not become evident in the broth until several hours later. Following addition of $^{14}C$-acetate at 24 hours during rapid accumulation of pseudomonic acids, the total incorporation of $^{14}C$ into pseudomonic acids measured at 50 hours exceeded 4.5%, nine times higher than previously reported for **1**[7]. Notably, the highest percentage incorporation of $^{14}C$ was in **2**. At 32 hours, when the rate of accumulation of **1** and **2** was declining, total incorporation of radiolabel still exceeded 1.5%. However, incorporation into **2** had become a dominant feature so that the ratio of incorporation of $^{14}C$ into **1** and **2** at the three stages of the batch fermentation changed seven-fold in favour of **2** (Table 2), reflecting the changing relative rates of biosynthesis of **1** and **2** at the different stages of administration of $^{14}C$-acetate. Since the data concerns only the end stage status of radiolabelled pseudomonic acids in the fermentation, the results leave open the possibility either of a close pathway connection directly between **1** and

Table 2. Radioactivity (dpm) in pseudomonic acids A, B and C in a shaken flask culture after 50 hours fermentation during which 5 $\mu$Ci [1-$^{14}$C] acetate was administered at 6 hours, 24 hours or 32 hours. Percentage incorporations of $^{14}$C are given in parentheses.

| Pseudomonic acid | 6 h | 24 h | 32 h |
|---|---|---|---|
| A (**1**) | 35811 (0.33) | 203061 (1.89) | 60716 (0.19) |
| B (**2**) | 11082 (0.1) | 285019 (2.66) | 141784 (1.32) |
| C | 2698 (0.02) | 26804 (0.19) | 17861 (0.13) |
| Ratio $^{14}$C-**1**:$^{14}$C-**2** | 1:0.3 | 1:1.4 | 1:2.3 |

Fig. 3. Autoradiograph of a silica gel chromatogram separating **1**, **2** and pseudomonic acid C extracted from 50 hours shaken flask fermentations fed [1-$^{14}$C] acetate at 6 hours (left), 24 hours (centre) or 32 hours (right).



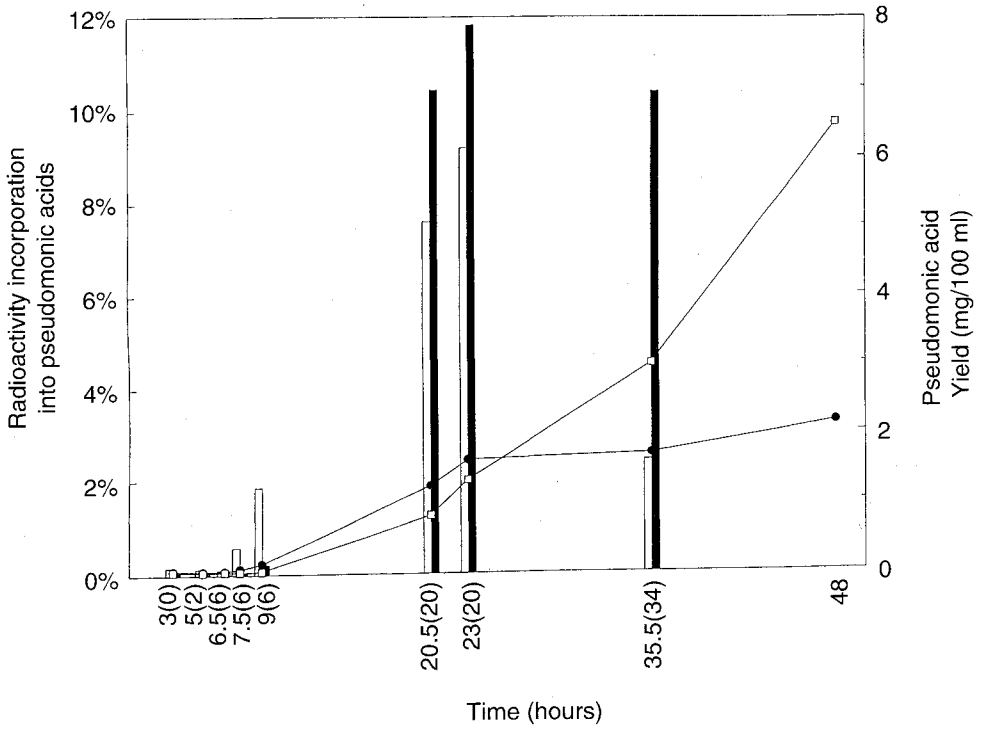

**2**, or of two separate pathways diverging at an early stage.

The numerical data was derived from the PLC plate after autoradiography, by which time some coloured changes in certain metabolites had occurred on the silica surface. These changes and the evidence from viewing the chromatograms under $UV_{254}$ light enabled quite accurate and conservative excission of silica gel regions corresponding to the autoradiograph. Consequently, the striking differences seen in the autoradiograph (Fig. 3) are reflected in the numerical data. There was also a discrete radiolabelled band, less polar than **1**, which was attributed to pseudomonic acid C (Fig. 1). Its radioactivity followed the pattern in **2** so that both differed markedly from **1**.

Experiment 2

In the first broad phase of the experiment, in which $^{14}$C-methionine was administered at various times up to 34 hours, a representative selection of results in Fig. 4 illustrate graphically the typical distinctive accumulation patterns for **1** and **2**, superimposed upon bars showing the percentage incorporation of $^{14}$C from methionine into **1** and **2** measured shortly (0.5~3 hours) after administration of radiolabelled precursor. Feeds up to and including the 6 hours stage mainly labelled **1**, but the 20 hours feed most prominently labelled **2**. The rapid rate of uptake of radiolabelled methionine is indicated by 18% of radiolabel already excreted as **1** and **2** 30 minutes after administration. The 34 hours feed, given when **2** was the product accumulating at the greater rate, also mainly labelled **2**. The full results (Fig. 5), arranged to show the fate of each label addition over the 48 hours duration of each fermentation, show that early additions (0, 2, 6 hours) led to persistent dominance of $^{14}$C-**1**. Further, the inverting pattern arising progressively from the 20 and 34 hours feeds similarly maintain the $^{14}$C-**1** : $^{14}$C-**2** ratio already established within 0.5~1.5 hours of giving the precursor.

Fig. 4. Dynamics of accumulation in biosynthetic experiment 2 of **1** (●) and **2** (□) in shaken flask fermentations to which [methyl-$^{14}$C] methionine was added either immediately after inoculation or at 2, 6, 20 or 34 hours.




Selected percentage incorporation values for $^{14}$C into **1** (white bars) or **2** (black bars), measured at various sampling times, are superimposed on the pseudomonic acid production curves at the relevant times.

Fig. 5. Groups of data of Fig. 5 arranged to show the percentage incorporation of $^{14}$C from [methyl-$^{14}$C] methionine, added to shaken flask fermentations at various stages, into **1** (white bars) and **2** (black bars) in the context of the titre of **1** (●) and **2** (□) in the fermentations.

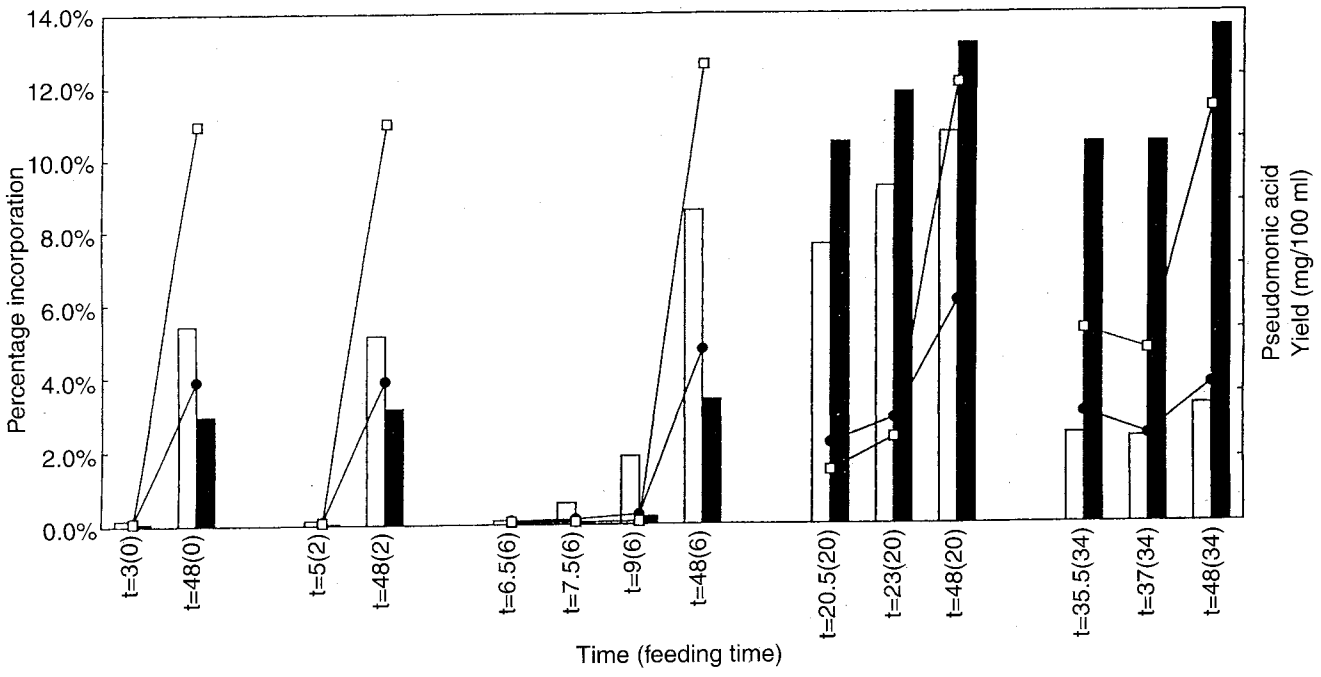
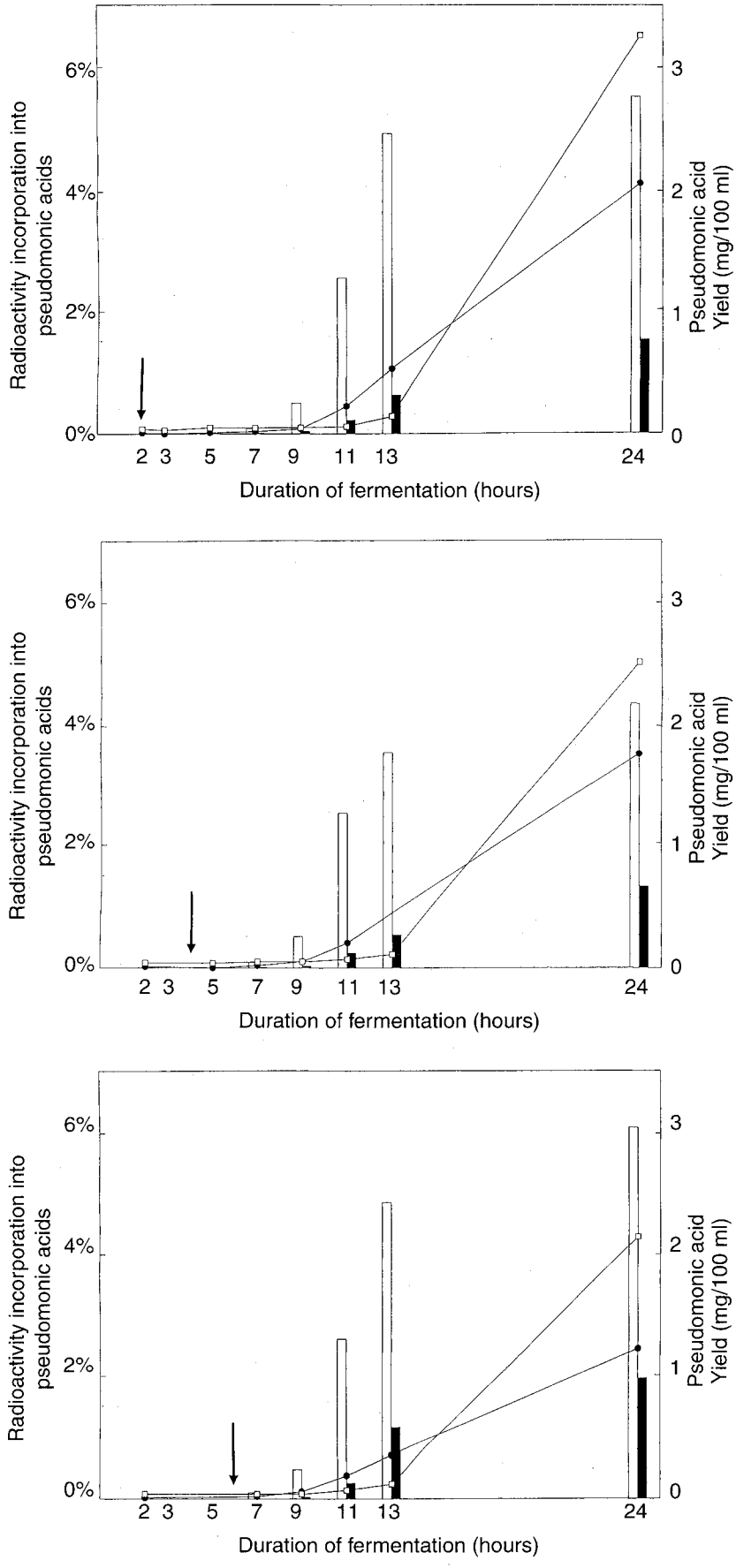


Fig. 6. Comparative incorporation of $^{14}C$, from radiolabelled methionine added at 2, 4 or 6 hours of fermentation (arrows), into **1** (white bars) and **2** (black bars) measured at various stages through early idiophase of fermentation.



The second phase of the experiment focussed on radiolabel additions early in fermentations giving yields of **1** and **2** similar to those in the first phase above. The fate of $^{14}C$ from $^{14}C$-methionine given at 2, 4 or 6 hours followed the pattern in Fig. 6, the data of which was derived from actual radioactivity measurements of, for example, as high as $10^4$ dpm for the 48 hours sample from the 6 hours administration. The data of Fig. 6 show a strong tendency for $^{14}C$-labelled precursor, administered early in the fermentation, to be preferentially incorporated into the **1** which appears as the first formed end product, as might be expected. However, this situation persisted in spite of the subsequent switch to accumulate mainly **2**. Autoradiography of TLC separation of fermentation time-course samples confirmed the pattern of this persistence of radiolabel in **1** which had been shown by the numerical data derived from HPLC analysis. In the flasks fed at 2, 4 or 6 hours there was a small increase in overall yield of **1** (to 2.84~3.28 mg 100 $ml^{-1}$) during the period from 24 hours to 48 hours (data shown in Fig. 6) and, as expected, a much larger increase in **2** (to 10~11.44 mg 100 $ml^{-1}$). Percentage incorporation of $^{14}C$ into **1** hardly changed during the period and, more importantly, incorporation into **2** also remained at low values (1.46, 1.56 and 3.04% for the 2, 4 and 6 hours additions of $^{14}C$-methionine, respectively), lower than those for **1**. This experiment therefore gave ample time for $^{14}C$-**1** in the broth to be biotransformed into **2**, but there was no evidence that this had occurred.

Maximum percentage incorporation of $^{14}C$-acetate into pseudomonic acids of only 0.15% was also achieved following addition at 6 hours, there being apparently no advantage in using a rather larger amount of radiolabeled precursor (20 $\mu$Ci) than was used in experiment **1**. The intracellular demand of primary metabolism on added acetate during early trophophase precludes more than a very small proportion going into secondary pathways. However, the inevitably weaker numerical data from [1-$^{14}C$] acetate still followed the same pattern as that from [methyl-$^{14}C$] methionine and was similarly confirmed by autoradiography.

## Discussion

Irrespective of why particular medium composition and volume in shaken flasks so greatly influences the relative dominance of **1** or **2** as the fermentation products of an isolate of *P. fluorescens*, the combination used in the present experiments gave pulse labelling data which is most consistent with biosynthesis of **1** and **2** from separate pathways diverging rather early from a common polyketide precursor, oxidation of which at C8 would make the pathway to **2**.

Radiolabelling experiments showed quick incorporation of added precursors into **1** and **2** (and probably into pseudomonic acid C) in relative proportion to the dynamics of their biosynthesis at the time of administration, irrespective of whether this was before the end-product had yet accumulated or shortly before its accumulation ceased. Also, pseudomonate degradative processes did not seem to occur during the fermentations.

Definitive interpretation of the present biosynthetic data is difficult, particularly with regard to intracellular processes. The experiments are the first to address the biosynthetic interrelationship between **1** and **2**, mainly because significant occurrence of **2** has not previously been reported. Biosynthesis of **1** *via* **2**, consistently involving reversal of an oxidation, is energetically rather unattractive. More plausibly, **2** might arise by oxidation of **1**, but **2** has apparently never been observed to accumulate towards the end of any fermentation idiophase, when primary metabolic demand on dissolved oxygen would be declining and oxidative process might be favoured. Therefore there seems to be no natural tendency for **1** to become oxidised to **2** in fermentation broth by either chemical or enzymic processes. In other words, activity of a PA hydroxylase for **1** has never been apparent.

Another interpretation might involve **2** arising, uniquely to a rather prominent extent in the system herein described, from **1** by enhanced activity of a hydroxylase for **1** during the latter part of a fermentation. However, this interpretation would require additional specific enzyme synthesis during a declining trophophase which seems rather improbable, especially if only to cause accumulation of another rather similar pseudomonic acid. However, since the switch to dominant accumulation of **2** was detected rather early in the idiophase for completed pseudomonate molecules, we prefer an interpretation of the experimental findings as indicating the natural occurrence of **2** being determined by hydroxylation of an early biosynthetic intermediate, the enzymology for which would need to have been in place several hours before end-product is first seen to accumulate. Over-production of a hydroxylase enzyme, specific for an intermediate rather than for the end-product, is more plausible during trophophase when extensive protein synthesis is occurring. In the present experiments temporal overlap of accumulation first of **1** and then mainly of **2** at least allowed the significant occurrence of $^{14}C$-**1** in the broth from mid fermentation onwards after early administration of [methyl-$^{14}C$] methionine. Nevertheless,

there was no evidence of $^{14}$C-**1** being significantly transformed to **2** at any stage thereafter, and this favours an interpretation of differentiation of a pseudomonate intermediate into a precursor for **1** or **2** rather early in their biosynthesis.

Understanding regulation of pseudomonic acid biosynthesis may therefore have to recognise that the oxidative status of the $C_{12}$ unit can change as a batch fermentation progresses from the epoxide (in the 10, 11 position of the pseudomonate carbon skeleton), leading to **1**, to the additional 8-OH form (leading to **2**).

Acknowledgements

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