Pseudomonic Acid. Part 2.[1] Biosynthesis of Pseudomonic Acid A

By **Timothy C. Feline, Robert B. Jones, Graham Mellows,*** and **Lawrence Phillips,*** Departments of Biochemistry and Chemistry, Imperial College of Science and Technology, London SW7 2AY

The biosynthetic precursors of pseudomonic acid A (Ia) have been defined by using radioisotopically labelled substrates. Positions of labelled atoms and values of $^1J_{CC}$ couplings in (Ia) biosynthesised from [1-^{13}C]-, [2-^{13}C]-, and [1.2-$^{13}C_2$]-acetate, [1-^{13}C]- and [3-^{13}C]-propionate, and L-[*Me*-^{13}C]methionine have been determined by ^{13}C n.m.r. spectroscopy. A biosynthetic route to (Ia) is suggested which proceeds through C_{12}, C_9, and C_5 units.

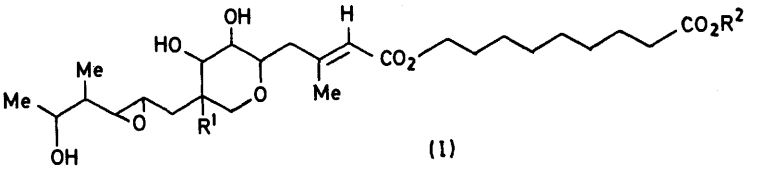
Pseudomonas fluorescens when grown in submerged culture produces a number of metabolites with antibiotic properties.[2,3] The structures of two of these substances, pseudomonic acids A (Ia) [1,4,5] and B (Ib) [6] have been established by chemical and spectroscopic methods. In the early phase of these studies the yield of the family of antibiotics, which reached a maximum after *ca.* 24 h fermentation time, was very low (<1 mg l^{-1} culture). Mutation and strain selection studies were therefore conducted concurrently which subsequently led to higher-yielding strains of the bacterium.[7] One such strain was selected for preliminary biosynthetic experiments.

The structure (Ia) may be considered as consisting of two parts, a 9-hydroxynonanoic acid moiety and a C_{17} fragment (see Figure 1). The former fragment comprises an odd-numbered carbon chain fatty acid which was at first thought to arise from the saturated fatty acid by terminal hydroxylation.[8] As far as we are aware, 9-hydroxynonanoic acid (VII) has not been isolated previously from natural sources, and its biosynthesis could be of relevance to the biogenesis of

[1] Part 1, E. B. Chain and G. Mellows, preceding paper.
[2] A. Baader and C. Garre, *Corresp.-Bl. Schweiz. Aerzte*, 1887, **17**, 385.
[3] A. T. Fuller, G. Mellows, M. Woolford, G. T. Banks, K. D. Barrow, and E. B. Chain, *Nature*, 1971, **234**, 416.
[4] E. B. Chain and G. Mellows, *J.C.S. Chem. Comm.*, 1974, 847.
[5] G. Mellows and K. D. Barrow, Beecham Group Ltd., 1971, Ger. P. 2,227,739.
[6] E. B. Chain and G. Mellows, following paper.
[7] G. T. Banks, E B. Chain, and G. Mellows, in preparation.
[8] T. C. Feline, Ph.D. Thesis, University of London, 1974.

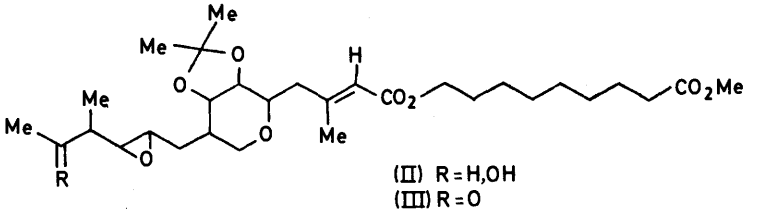
linear odd-numbered carbon chain fatty acids in general, about which relatively little is known.

In order to define the biological precursors of (Ia), a series of radioisotopically labelled substrates were fed to cultures of *Pseudomonas fluorescens* at the time of inoculation. Pseudomonic acid A was isolated in each case as its methyl ester (Ic) by dilution analysis, and the incorporation results are expressed in Table 1. The efficient incorporation of L-[*Me*-^{14}C]methionine suggested that (Ia) contains C_1 groups derived from the 'C_1 pool.' [^{14}C]Formate, an alternative 'C_1 pool' donor was poorly incorporated. Both [1-^{14}C]acetate and [2-^{14}C]propionate showed good incorporation. [2-^{14}C]Mevalonic acid lactone was not incorporated, implying that (Ia) does not contain an isoprenoid residue derivable from mevalonate. The non-incorporation of mevalonic acid was not surprising, since bacteria have so far not been shown to utilise this precursor.[9a]

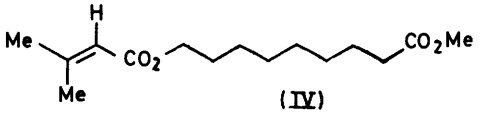


(I)

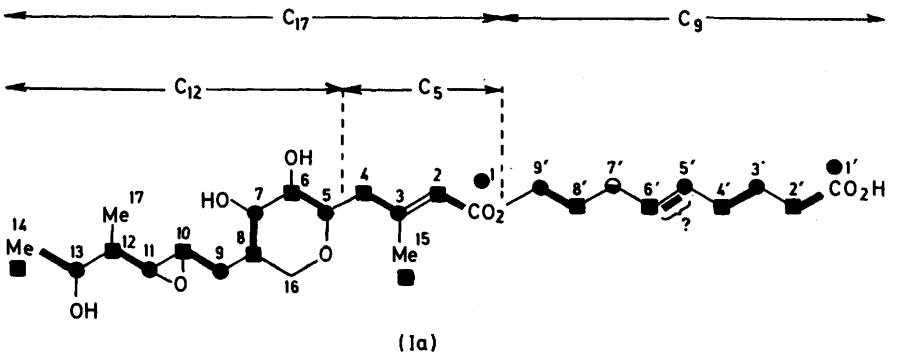
a; $R^1 = R^2 = H$
b; $R^1 = OH$, $R^2 = H$
c; $R^1 = H$, $R^2 = Me$



(II) R = H,OH
(III) R = O



(IV)



(Ia)

FIGURE 1 Labelling pattern of the acid (Ia) from Me—CO_2Na

TABLE 1

Incorporation of radiolabelled precursors into the acid (Ia)

Precursor	% Incorporation
Sodium [1-^{14}C]acetate	0.51
Sodium [2-^{14}C]propionate	0.36
Sodium [^{14}C]formate	0.004
DL-[*Me*-^{14}C]Methionine *	15.4
(*RS*)-[2-^{14}C]Mevalonic acid	0

* Allowing for the utilisation of the L-isomer only.

At this point many combinations of acetate, propionate, and C_1 units could be accommodated by the structure of pseudomonic acid A (Ia). The C_{17} fragment of (Ia) is composed of a continuous carbon chain extend-

[9] J. H. Richards and J. B. Hendrickson, 'Biosynthesis of Terpenes, Steroids, and Acetogenins,' Benjamin, New York, 1964, (*a*) pp. 177—188; (*b*) pp. 16—26.

ing from C-14 to C-1 with 'extra' C_1 units (C-15, -16, and -17), potentially of *S*-adenosylmethionine origin, attached (to C-3, -8, and -12, respectively). As a result of two degradations of (Ia) derived from DL-[*Me*-^{14}C]-methionine in the early stages of the investigation it was inferred that C-16 and -17 were the most likely labelled positions from this precursor.[8] To avoid the exhaustive chemical degradation of (Ia) necessary to confirm this result, and to evaluate the origin of the remaining carbon atoms, our biosynthetic studies were continued by use of the ^{13}C n.m.r. approach.

Initially the natural abundance, proton-noise-decoupled (p.n.d.) ^{13}C n.m.r. spectrum of (Ic) was assigned by comparison with the spectra of the acetonide derivative (II), its C-13 ketone analogue (III), and 8-methoxycarbonyloctyl 3-methylbut-2-enoate (IV) for comparison.

Comparison of the spectrum of the ester (IV) with that of (Ic) allowed the corresponding signals of (Ic) to be distinguished. The olefinic signals, due to C-2 and -3 in (Ic), (II), and (III), were assigned from their chemical shifts and multiplicities in single frequency off-resonance decoupled (s.f.o.r.d.) spectra. Of the two carboxylic carbon atoms in (Ic), C-1, showing a singlet in the s.f.o.r.d. spectrum, resonated at δ_C 166.8 [*cf.* C-1 in methyl 3-methylbut-2-enoate (δ_C 166.3) [10a]]. The other low field signal, δ_C 174.4, was assigned to C-1' [*cf.* C-1 in methyl heptanoate (δ_C 173.4) [10a]]. The aliphatic methyl signals (C-14, -15, and -17) were easily identified as quartets in the s.f.o.r.d. spectra, with the exception of C-14 in (III), where the signal overlapped with other lines. The C-15 signal remains almost invariant in all three models, and C-17 resonates in a similar position in (Ic) and (II) but shows a small upfield shift on oxidation of the C-13 alcohol group in (II) (to δ_C 12.4). This is similar to the small upfield shifts, on oxidation of 3-methylbutan-2-ol to its ketone analogue, observed for the resonances of the similarly disposed methyl groups which are non-equivalent in the alcohol (δ_C 18.3 and 18.5) but coalesce slightly to higher field in the ketone (δ_C 17.9).[10b] The C-14 signal occurs at δ_C 20.8 and 20.6 in (Ic) and (II), respectively and is expected to shift downfield on oxidation at C-13. A shoulder at δ_C 29.4 was seen on the side of the multiple peaks at δ_C 29.1 in the spectrum of (III). In the s.f.o.r.d. spectrum a sharp line was seen 6 Hz to low field of the position of this shoulder, which could not arise from any of the overlapping resonances contributing to the line at δ_C 29.1 (all of which should give poorly defined residual triplets). The expected residual ^{13}C–^{1}H coupling for this methyl group is 11—15 Hz and therefore this line is attributed to the lower field component of the two centre lines of the residual quartet arising from C-14. A further weak peak about 13 Hz to lower field was attributed to the lowest field component of the quartet, and hence the C-14 signal appears at δ_C 29.4 in (III).

The methyl ester carbon signal was identified by its low field residual quartet in the s.f.o.r.d. spectrum. The resonances arising from the isopropylidene groups in (III) and (II) were distinguished by comparison of these spectra with the spectrum of (Ic), the methyl groups being non-equivalent, occurring at δ_C 26.3 and 28.3 in both derivatives.

The signals for the carbon atoms in the methylene chain (C-2'—9') were assigned by comparison with models. Of the two methylene carbon atoms bonded to oxygen in (Ic), (II), and (III) (C-9' and C-16), C-9' was assigned from its position in the spectrum of (IV) and its relatively invariant position in the spectra of (Ic), (II), and (III). The C-16 signal therefore appears at δ_C 65.4 in (Ic) and δ_C 67.0 in (II) and (III). The invariant signals at δ_C 28.7 and 25.9 in the spectra of (Ic), (II), and (III) were assigned to C-8' and C-7', respectively [*cf.* chemical shifts of corresponding carbon atoms in heptyl acetate; C-2 and C-3, δ_C 28.6 and 25.8, respectively.] [11] The signal at δ_C 34.1 was assigned to C-2' and that at 24.9 to C-3' (*cf.* corresponding carbon atoms in methyl hexanoate: C-2, δ_C 33.9; C-3, δ_C 25.5).[10c] The signals for the central methylene carbon atoms of a long methylene chain tend to approach a constant chemical shift of δ_C 29—30,[10d] and the intense signals at δ_C 29.0 in (Ic) and (II) and δ_C 29.1 in (III) were therefore assigned to C-4', -5', and -6'. Further evidence for the presence of three overlapping signals accrues from the enhancement of this resonance in (Ic) derived from [1-^{13}C]- and [2-^{13}C]-acetate (see below).

The epoxide carbon atoms (C-10 and C-11) were distinguished from others bonded to oxygen by their relatively high field position, and from each other by the shifts in opposite direction seen on oxidation at C-13; the lower field signal at δ_C 61.3 in (Ic) and (II) moving 2.1 p.p.m. to higher field in (III) and the higher field signal at δ_C 55.6 in (Ic) moving 0.6 p.p.m. to lower field in (III). By using the oxidation of octan-2-ol to octan-2-one as a model,[10e] the effects on C-10 and C-11 (equivalent to C-4 and C-5 in octan-2-ol) were predicted to be shifts of 1.4 p.p.m. to high field and 0.8 p.p.m. to low field, respectively; C-11 was therefore assigned to the lower field and C-10 to the higher field resonance, respectively. This assignment is reasonable because of the greater degree of alkyl substitution at C-12 than at C-9.

The sharp lineshapes of the residual doublet seen in the s.f.o.r.d. spectrum for C-13 allowed it to be distinguished from the less well defined residual doublets arising from C-5, -6, and -7, as a result of the more nearly first-order character of the adjacent inter-proton spin system.[12] Further, on oxidation of C-13 the resonance disappeared from the aliphatic region.

Assignment of the remaining oxygenated carbon signals (C-5, -6, and -7) was more difficult because of the

[10] J. B. Stothers, 'Carbon-13 NMR Spectroscopy,' Academic Press, New York, 1972, (*a*) p. 296; (*b*) pp. 141, 146; (*c*) p. 150; (*d*) p. 56; (*e*) pp. 140, 146; (*f*) pp. 370—375.

[11] R. B. Jones, Ph.D. Thesis, University of London, 1976, p. 2.153.

[12] K. D. Barrow, R. B. Jones, P. W. Pemberton, and L. Phillips, *J.C.S. Perkin I*, 1975, 1405.

uncertainty of the stereochemical consequences of the substituted pyran ring particularly on acetal formation. Simple alkylation of a secondary alcohol usually causes a downfield shift of at least 3 p.p.m. (*e.g.* methylation of propan-2-ol moves the C-2 signal 3.7 p.p.m. downfield). If it is assumed that acetal formation across C-6 and C-7 will produce a similar effect in (V) and (III), then the signal at δ_C 74.8 in (Ic) may be assigned to C-5. In (II) and (III) the lowest field aliphatic signal occurred at δ_C 76.3 and was assigned to C-5. The resonances of C-6 and -7 could not be unambiguously assigned directly but were distinguished following the incorpor-

TABLE 2

^{13}C N.m.r.[a] and enrichment data

Carbon no.	(Ic) (l.i.s.*)	(II)	(III)	(IV) [b]	Enriched carbon atoms † in (Ic) from: [1-^{13}C]Acetate [c]	[2-^{13}C]Acetate [d]	[1-^{13}C]Propionate [e]	[3-^{13}C]Propionate [f]	[*Me*-^{13}C]Met [g]
1	166.8 (1.5)	166.7	166.7	166.7	+				
2	117.6 (17.9)	117.8	117.8	116.2		+		+	
3	156.7 (12.5)	156.1	156.1	156.0	+				
4	42.9 (32.2)	44.0	44.0			+		+	
5	74.8 (66.3)	76.3	76.3		+				
6	69.0 (236.4)	74.2	74.2			+		+	
7	70.4 (226.4)	75.7	75.6		+				
8	39.5 (31.8)	35.1	35.1			+		+	
9	31.6 (67.7)	33.8	33.7		+				
10	55.6 (212.1)	55.5	56.2			+		+	
11	61.3 (335.0)	61.3	59.2		+				
12	42.9 (121.7)	42.8	49.3			+		+	
13	71.4 (220.0)	71.0	209.3		+				
14	20.8 (60.9)	20.6	29.4			+		+	
15	19.1 (8.7)	18.9	19.0			+		+	
16	65.4 (53.3)	67.0	67.0						+
17	12.7 (73.6)	12.7	12.4						+
1′	174.4	174.2	174.2	174.0	+				
2′	34.1 (5.0)	34.0	34.1	34.1		+		+	
3′	24.9 (2.1)	24.9	24.9	24.9	+				
4′	29.0 (−0.4)	29.0	29.1	29.2		+		+	
5′	29.0 (−0.4)	29.0	29.1	29.2	+				
6′	29.0 (−0.4)	29.0	29.1	29.2		+		+	
7′	25.9 (−0.5)	25.9	25.9	26.0	+		+		
8′	28.7 (−0.2)	28.7	28.7	28.8		+		+	
9′	63.9 (4.9)	63.7	63.7	63.5	+				
Me_2C		28.3	28.3	(Me)					
		26.3	26.3	(Me)					
		108.8	108.8	(C)					
OMe	51.5 (2.5)	51.4	51.4	51.4					

[a] P.p.m. to low field of Me_4Si; solvent $CDCl_3$. [b] *cis*-3-Me 20.1; *trans*-3-Me 27.4.

* Lanthanide-induced shift (in Hz) observed after addition of $Eu(fod)_3$ (182 mg) to a solution of (Ic) (170 mg) in $CDCl_3$ (2 ml).

† Approximate enrichment factors: [c] 7, except for C-7′ which was enriched by half this amount; [d–f] 5; [g] 70.

Enrichment factor $= \frac{\text{signal intensity in natural abundance spectrum of (Ic)}}{\text{signal intensity in spectrum of } ^{13}\text{C-enriched (Ic)}}$; intensities are normalised onto C-16 (for *c–f*) or onto C-2′ (for *g*).

ation of the [^{13}C]acetate specimens described below. No attempt has been made to assign signals to C-5, -6, and -7 in the spectra of (II) and (III).

The remaining resonances are ascribed to the methylene (C-4 and -9) and methine (C-8 and -12) carbon atoms. One of the two overlapping signals at δ_C 42.9 in the spectrum of (Ic) is assigned to C-12; it remains essentially invariant on acetal formation, but moves significantly downfield (6.3 p.p.m.) on oxidation at C-13 [*cf.* the oxidation of 3-methylbutan-2-ol to 3-methylbutan-2-one: the C-3 signal (corresponding to C-12) then appears 5.4 p.p.m. downfield of its position in the alcohol].[10b] The remaining methine signal, which showed a significant upfield shift on acetal formation but was invariant on oxidation at C-13 was assigned to C-8. Of the two methylene signals (C-4 and -9), the one at lower field is consistantly better defined in the s.f.o.r.d. spectrum, and was assigned to C-4 [δ_C 42.9 in (Ic)], the higher field signal (δ_C 31.6) being assigned to C-9. This order of chemical shifts is expected because of the position of C-4 adjacent to both C–O and C=C, both of which have deshielding influences.

Details of the ^{13}C n.m.r. spectra of compounds (Ic), (II), (III), and (IV) are collected in Table 2.

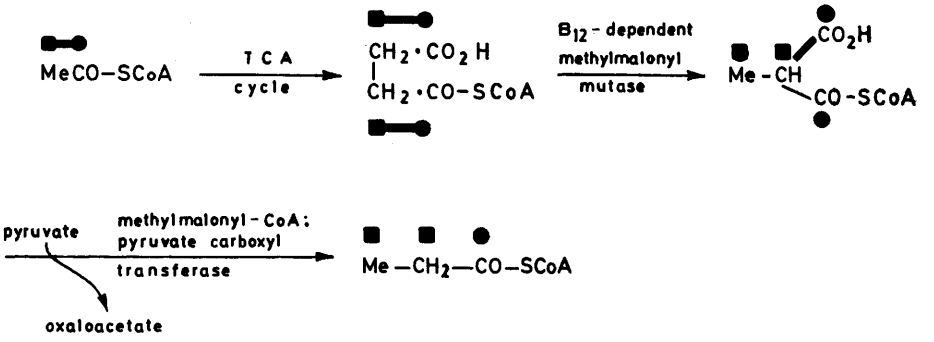
Early attempts to incorporate sufficient label from ^{14}C-labelled acetate and propionate into (Ia) at the necessary high dosage levels required to give enhancements in signal intensity in the ^{13}C n.m.r. spectrum of (Ic) derived from the corresponding ^{13}C labelled precursors failed. The high dosage levels of these precursors inhibited the growth of the bacterium and caused abnormal pH changes during the fermentation resulting in very low yields of (Ia). Considerable effort was expended devising a suitable high-yielding buffered medium giving adequate yields of (Ia) and also programming the time of addition of precursors to minimise the drop in production levels of (Ia). Only when the precursors were pulse-fed over a 10 h period commencing towards the end of the growth phase were suitable results obtained.

The sites of enrichment and the approximate enrichment factors in (Ia), isolated as (Ic) (50—60 mg l^{-1}) following the incorporation of (500 mg l^{-1} in each case) sodium [1-^{14}C]acetate (92.2 atom %), sodium [2-^{13}C]-acetate (90.2 atom %), sodium [1-^{13}C]propionate (89.1 atom %), sodium [3-^{13}C]propionate (92.4 atom %), and L-[*Me*-^{13}C]methionine (90.0 atom %) are recorded in Table 2. L-[*Me*-^{13}C]Methionine was most efficiently incorporated and exclusively enhanced the signals of C-16 and -17 in the p.n.d. spectrum of (Ic), in full agreement with the conclusions drawn from the earlier experiments with [*Me*-^{14}C]methionine. Because of the high level of incorporation into these two carbons, signals due to $^1J_{CC}$ couplings were clearly visible centred about the natural abundance ^{13}C resonances of C-8 and -12: $^1J_{CC}$ ($\pm$1 Hz) 34.9 Hz (C-8,C-16) and 34.4 (C-12,C-17). [1-^{13}C]Acetate clearly labelled C-1, C-3, C-5, C-9, C-11, C-13, C-1′, C-3′, C-7′, and C-9′. All the signals due to these carbon atoms were enhanced approximately 7-fold with the exception of that of C-7′, which was enhanced by approximately half this amount. [2-^{13}C]Acetate clearly labelled (5-fold signal enhancement) C-2, C-4, C-8, C-10, C-12, C-14, C-15, C-2′, and C-8′. It was apparent from the intensity of the overlapping signals of C-4′, -5′, and -6′ at 29.0 p.p.m. that [2-^{13}C]acetate enriched two of the carbon atoms and [1-^{13}C]acetate only one. In order to maintain the biogenetically acceptable alternate labelling pattern along the C_9 chain, C-4′ and C-6′ were assumed to be derived from [2-^{13}C]-acetate, whereas C-5′ would originate from [1-^{13}C]-acetate. [This was verified later by using (Ic) derived from [1,2-$^{13}C_2$]acetate, and is discussed below.] In the ^{13}C n.m.r. spectrum of (Ic) the signals at 69.0 and 70.4 p.p.m., due to C-6 and -7, could not be assigned unambiguously. However, the former signal was clearly derived from [2-^{13}C]acetate, and the latter from [1-^{13}C]-acetate. The signal at 69.0 p.p.m. was therefore assigned to C-6 and that at 70.4 p.p.m. to C-7, thereby maintaining an alternating labelling sequence in the carbon chain extending from C-1 to C-14. The labelling pattern observed in the C_{17} fragment of (Ic) (Figure 1) and in particular the derivation of C-15 from C-2 of acetate rather than the methyl group of methionine, strongly suggested at this point that this moiety was enzymically constructed from two different biounits, respectively containing twelve and five carbon atoms (see later). However, no discernible differences in the relative enhancements of signals in the C_{12} and C_5 fragments were evident. Although conclusions drawn from comparisons of signal intensity data can sometimes be misleading, it was clear from the spectrum of (Ic) derived from [1-^{13}C]acetate [and also in (Ic) derived from [1,2-$^{13}C_2$]acetate] that C-7′ was enriched to only approximately half the enrichment levels of the remaining carbon atoms enriched from this precursor. This anomaly was more apparent when the exclusive enrichment (5-fold) of C-7′ from [1-^{13}C]propionate was observed. At first sight this result and the labelling pattern of C-1′—6′ from [1-^{13}C]- and [2-^{13}C]-acetate suggested that the 9-hydroxynonanoate fragment of (Ia) was constructed from a propionyl-CoA primer and 3 malonyl-CoA residues in an analogous manner to the formation of even-numbered carbon chain fatty acids. The incorporation of propionic acid into odd-numbered carbon chain fatty acids has previously been demonstrated,[13] but the position(s) of incorporated label were not deduced. However, the labelling pattern observed for C-7′—9′ from [1-^{13}C]- and [2-^{13}C]-acetates did not agree with the expected labelling pattern for propionyl-CoA derived from these precursors. The pathway by which acetyl-CoA is converted into propionyl-CoA,[14] shown in Scheme 1, would necessitate the derivation of C-9′ in (Ic) from C-2 of acetate and not C-1 as observed.

[13] M. G. Horning, D. B. Martin, A. Karmen, and P. R. Vagelos, *J. Biol. Chem.*, 1961, **236**, 669; F. Lynen, *Fed. Proc.*, 1961, **20**, 941; A. T. James, G. Peeters, and M. Lawryssens, *Biochem. J.*, 1956, **64**, 726; J. Katz and J. Kornblatt, *J. Biol. Chem.*, 1962, **237**, 2466.

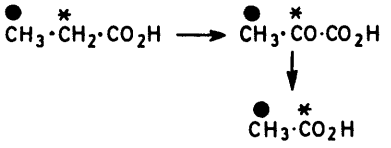
[14] Y. Kaziro and S. Ochon, *Adv. Enzymol.*, 1964, **26**, 283.

Also, during the formation of methylmalonyl-CoA from succinyl-CoA one of the two C–C bonds derived from intact acetate residues would be broken. The C–C bond of the remaining intact acetate residue in methylmalonyl-CoA would be broken during its enzymic decarboxylation to propionyl-CoA. In (Ic) derived from [1,2-$^{13}C_2$]acetate no $^1J_{CC}$ couplings would therefore be expected for carbon atoms 7′—9′ if propionyl-CoA 'primes' the biosynthesis of the C_9 unit. A $^1J_{CC}$ coupling was however observed between C-8′ and -9′ twice natural abundance) produced satellite lines centred about natural abundance signals of approximately half their height. Signals arising from C-16, C-17, and the methoxy-carbon atom of the methyl ester were not enhanced and appeared without $^1J_{CC}$ coupling satellites as expected. Signals due to C-7′ and -15, whilst showing enhancement, appeared as singlets, indicating that although these carbon atoms have been proven to be of acetate origin, their acetate units are not incorporated intact. In order to simplify and confirm aspects of the



SCHEME 1

when [1,2-$^{13}C_2$]acetate was incorporated into (Ic) (see below) and the above mechanism can therefore be excluded. It was clear from our earlier radiolabelling studies that C-2 of propionate is incorporated into (Ia). To determine whether the methyl carbon atom (C-3) of propionate was incorporated, [3-^{13}C]propionate was fed to the bacterium. Surprisingly, this was incorporated (5-fold signal enhancement) into all the carbon atoms in (Ic) which had previously been shown to be derived from C-2 of acetate. This presumably had taken place by the prior degradation of [3-^{13}C]propionate, *via* [3-^{13}C]pyruvate, to [2-^{13}C]acetate before assimilation (Scheme 2). Importantly, C-9′ was not labelled.



SCHEME 2

Although the sites of incorporation of label in (Ia) derived from [2-^{14}C]propionate were not located, the above result strongly suggests that they correspond to those positions labelled from C-1 of acetate.

In order to throw further light on the biogenesis of the C_5 and C_9 units of (Ia), [1,2-$^{13}C_2$]acetate (1 part) mixed with unlabelled acetate (3 parts) was incorporated into (Ia). The p.n.d. spectrum of the enriched ester (Ic) obtained from (Ia) made possible a study of the incorporation of intact acetate units into (Ia) and distinguished those carbon atoms which had been assimilated from acetate subsequent to carbon–carbon bond fission.[15] The incorporation level achieved (about spectrum it was necessary to add the shift reagent, Eu(fod)$_3$, to the sample. The effect on the natural abundance spectrum of (Ic) was studied initially; shifts (in Hz) observed after the addition of five portions of Eu(fod)$_3$ (total 182 mg) to (Ic) [170 mg in $CDCl_3$ (2 ml)] are shown in Table 2.

The incorporation of doubly labelled acetate units into (Ia) is shown in Figure 1, and observed $^1J_{CC}$ values are listed in Table 3. With the exception of the coupling

TABLE 3

Carbon–carbon coupling constants ($^1J_{x,y}$) observed in the ^{13}C n.m.r. spectrum of ^{13}C-enriched ester (Ic)

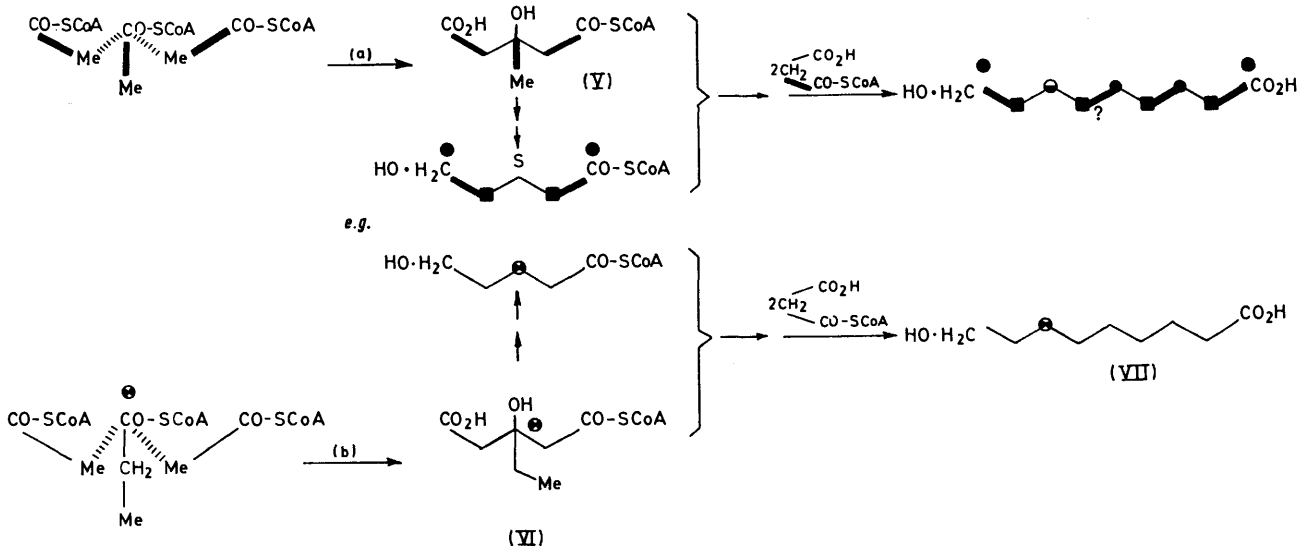
x, *y*	*J*/Hz *	*x*, *y*	*J*/Hz
1, 2	65.7	13, 14	38.9
3, 4	41.1	12, 17	34.4
5, 6	41.1	8, 16	34.9
7, 8	35.9	1′, 2′	57.0
9, 10	44.3	3′, 4′	34.6
11, 12	44.0	8′, 9′	38.4

* Obtained from (Ic) enriched from [1,2-^{13}C]acetate, except $J_{13,17}$ and $J_{8,16}$ which were obtained from (Ic) enriched from L-[*Me*-^{13}C]methionine.

observed at C-1, where spectral 'noise' hides the doublet splitting due to coupling with C-2, and coupling between C-5′ and -6′, which would not be visible because of the accidental equivalence of these resonances (leading to an effective A_2 system), all couplings were measurable from either the original spectrum of the doubly labelled sample or the lanthanide-shifted spectrum [addition of 9.5 mg of Eu(fod)$_3$ to 17 mg of (Ic) in 1.8 ml of $CDCl_3$].

[15] A. G. McInnes, D. G. Smith, C.-K. Wat, L. C. Vining, and J. L. C. Wright, *J.C.S. Chem. Comm.*, 1974, 281; A. G. McInnes, D. G. Smith, J. A. Walter, L. C. Vining, and J. L. C. Wright, *ibid.*, p. 282.

The observed couplings followed established trends.[10f] The $^1J_{CC}$ values observed were additionally useful in unambiguously assigning the signals due to C-5, -6, and -7 in (Ic). The $^1J_{CC}$ value observed (41.1 Hz) for the signals at δ_C 69.0 and 74.8 in (Ic) was in excellent agreement with one-bond couplings between two sp^3 carbon atoms where each carbon carries an oxygen substituent (*ca.* 41 Hz).[10f] The assignment of the signal at 74.8 to C-5 follows from the previous discussion and its derivation from C-1 of acetate. Similarly the signal at δ_C 70.4 was ascribed to C-7 because of the observed $^1J_{CC}$ (35.9 Hz) with C-8, which had been unambiguously assigned. This $^1J_{CC}$ value was in good agreement with those reported [10f] (36—39 Hz) for two sp^3 carbon atoms only one of which carries an oxygen substituent.



SCHEME 3

The foregoing observations add further weight to the assumption that pseudomonic acid A is derived from three different biounits.

The 9-Hydroxynonanoate Moiety.—The possibilities that this unit is biosynthesised from (i) a preformed longer chain fatty acid, such as oleic acid, by oxidative degradation [*cf.* the biosynthesis of the diacetylenic triol $MeCH_2{\cdot}CH(OH){\cdot}C{\equiv}C{\cdot}C{\equiv}C{\cdot}CH(OH){\cdot}CH_2{\cdot}OH$]; [16] (ii) an even-numbered carbon chain fatty acid such as decanoic acid, by α-oxidation; and (iii) propionyl-CoA and malonyl-CoA, by a process similar to that leading to even-numbered carbon chain fatty acids, can be eliminated since none of these alternatives would lead to the ^{13}C labelling and ^{13}C–^{13}C coupling patterns observed in (Ic). The possibility that the 9-hydroxynonanoate moiety arises through an enzyme-mediated Baeyer-Villiger-type oxidation [17] of a preformed acetate-derived aliphatic chain can also be discounted, since C-1 and C-9′ are both derived from C-1 of acetate. The incorporation of intact acetate units into C-8′ and -9′, C-3′ and -4′, and C-1′ and -2′ (and most probably C-5′ and -6′), and the incorporation of only C-1 of a separate acetate unit into C-7′ of (Ia) suggests that the ' primer ' for the formation of the 9-hydroxynonanoate moiety could be β-hydroxy-β-methylglutaryl-CoA(HMG-CoA) (V) or a C_5 precursor derived from it [Scheme 3(a)]. The labelling pattern of HMG-CoA from acetate is well established,[18] and this substance is an important intermediate in the biosynthesis of mevalonate, the precursor of the isoprene unit in terpene biosynthesis. The formation of 9-hydroxynonanoic acid would necessitate the addition of two malonyl-CoA residues to (V), or a C_5 ' primer ' (*e.g.* 5-hydroxypentanyl-CoA) derived from it. In either case the methyl group derived from the β-position of (V) would be removed and the terminal carboxy-group reduced.

This theory alone does not account for the incorporation of label from [1-^{13}C]propionate solely into C-7′ and the reduced enrichment of this carbon from [1-^{13}C]- and [1,2-$^{13}C_2$]-acetate. These observations suggest the existence of two competing mechanisms for the formation of the 9-hydroxynonanoate moiety, involving a second ' primer ' having a different origin. This might be homo-HMG-CoA (VI) or a C_5 unit, possibly the same as that derived from HMG-CoA [Scheme 3(b)]. Although homo-HMG-CoA (or the free acid) has not previously been isolated from natural sources, the recent finding that homomevalonate, which is presumably formed from (VI), is incorporated into juvenile hormone,[19] provides evidence for its possible role as an intermediate in metabolic pathways. Although these

[16] G. C. Barley, A. C. Day, V. Graf, Sir E. R. H. Jones, I. O. Neill, R. Tachikawa, V. Thaller, and R. A. Vere Hodge, *J. Chem. Soc.* (*C*), 1971, 3308.

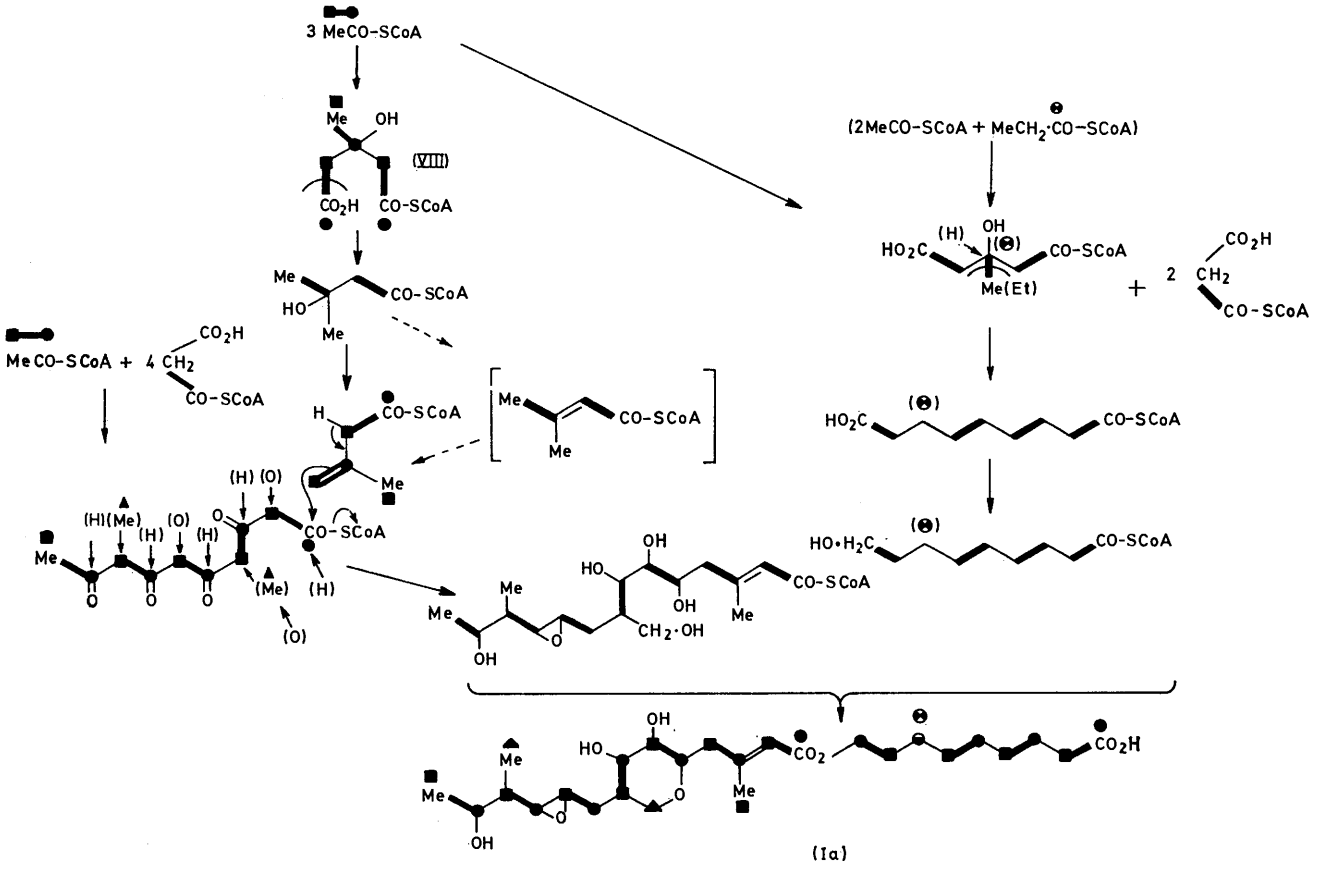
[17] G. S. Fonken and R. A. Johnson, ' Chemical Oxidations with Microorganisms,' Dekker, New York, 1972, pp. 157—164.

[18] H. Rudney, *J. Amer. Chem. Soc.*, 1954, **76**, 2595.

[19] R. C. Jennings, K. J. Judy, and D. A. Schooley, *J.C.S. Chem. Comm.*, 1975, 21.

observations are concerned with the 9-hydroxynonanoate moiety in (Ia) they may have an important bearing on the biosynthesis of odd-numbered carbon chain fatty acids in general, which are currently assumed to be synthesised from a propionyl-CoA ' primer.'

The C_{12} *Moiety.*—This part of the molecule comprises a C_{10} chain, extending from C-5 to C-14, to which two ' extra ' carbon atoms (C-16 and -17) are attached (at C-8 and C-12, respectively). The alternate labelling sequence from [1-^{13}C]- and [2-^{13}C]-acetate along this chain and its formation from five intact acetate units and -3, and the assimilation of two intact acetate molecules into positions C-1 and -2 and C-3 and -4, could also be explained by the derivation of the C_5 moiety from HMG-CoA. It is well established that in rat liver HMG-CoA can undergo enzymic decarboxylation, producing hydroxyvaleryl-CoA, which in turn can undergo dehydration, generating dimethylacryloyl-CoA. The latter molecules have previously been shown to have the same labelling pattern from acetate as the C_5 moiety in (Ic).[20] A plausible route for the attachment of the C_5 unit to the polyketide thioester is shown in Scheme 4,



SCHEME 4

accords with its derivation from an acetyl-CoA primer (providing C-13 and -14) and four malonyl-CoA units (Scheme 4). The insertion of the two ' extra ' C_1 groups from *S*-adenosylmethionine would take place at saturated positions in the resulting polyketide chain, in accord with the acetate–malonate hypothesis.[9b] Further oxidative and reductive modifications would then lead to the functionalities present in the corresponding part of (Ia). The pyran ring in (Ia) is probably generated from an open-chain diol intermediate, as depicted in Scheme 4, by the elimination of water.

The C_5 *Moiety.*—The incorporation of C-2 of acetate into C-2, -4, and -15, with C-1 of acetate providing C-1 summarising our findings to data, in which 3-methylbut-1-enyl-CoA might serve as the more appropriate condensing unit.

EXPERIMENTAL

^{1}H N.m.r. spectra were recorded with a Varian T60 spectrometer. ^{13}C Spectra were recorded at 25.16 MHz with a Varian XL-100-12–Varian Data Machines 16K 620-L spectrometer system; 8 K sampling points were used to give 4 K plotted data points after Fourier transformation. Spectral widths of 4 307 Hz were used to give

[20] H. Rudney, *J. Amer. Chem. Soc.*, 1955, **77**, 1698; J. L. Rabinowitz, *ibid.*, p. 1295.

1.06 Hz per point, the resonances being deliberately broadened by multiplication of the FID by an exponentially decreasing function (0.5 s) to minimise intensity errors associated with digitisation. Samples were dissolved in $CDCl_3$ (*ca.* 2 ml) in a 10 mm n.m.r. tube, the solvent deuterium being used to provide a field-frequency lock.

Culture of Pseudomonas fluorescens (N.C.I.B. 10586).—*Pseudomonas fluorescens* was maintained on 2% malt agar at 24 °C and subcultured every 4 weeks. A loopful of bacteria was inoculated into primary stage medium (100 ml) comprising Oxoid nutrient broth (13 g), peptone (5 g), Na_2HPO_4 (2.6 g), KH_2PO_4 (2.4 g), and glucose monohydrate (1.1 g), made up to 1 l with distilled water (the pH of the medium was adjusted to 7.0 with 10% KOH before sterilisation at 121 °C and 15 lb in^{-2} pressure for 20 min). The culture was incubated on a rotary shaker at 30 °C for 24 h. A sample (10 ml, *ca.* 10^{11} cells) of the primary stage culture served as inoculum for the secondary stage medium (100 ml), which consisted of KH_2PO_4 (0.4 g), Na_2HPO_4 (0.65 g), KCl (0.5 g), $MnCl_2,2H_2O$ (0.003 g), $MgSO_4,7H_2O$ (0.375 g), and ground nut meal (21 g) made up to 1 l with distilled water (the pH was adjusted to 7.0 prior to sterilisation at 121 °C and 15 lb in^{-2} for 17.5 min). Immediately after inoculation a sample (10 ml) of 5% glucose in water (separately sterilised at 15 lb in^{-2} for 15 min) was added. Incubation was carried out at 24 °C for 24 h on a rotary shaker.

Isolation and Purification of Methyl Pseudomonate A (Ic)—The cells were separated from the whole broth (1 l) by centrifugation (10 000 *g*) at 2 °C. The supernatant liquor was extracted with isobutyl methyl ketone (2 × 200 ml) after adjustment of pH to 8.0 (2N-NaOH). The isobutyl methyl ketone extract was discarded. The aqueous layer was adjusted to pH 4.5 (2N-HCl) and re-extracted with isobutyl methyl ketone (2 × 200 ml). Emulsions were broken by centrifugation. The combined extracts were washed with distilled water (100 ml), dried, and evaporated to dryness. The residue was dissolved in methanol (10 ml) and treated with an excess of ethereal diazomethane, overnight. The mixture was evaporated and the residue was purified by p.l.c. [propan-2-ol–chloroform (1 : 9)]. The band at R_F *ca.* 0.35 afforded methyl pseudomonate A (*ca.* 80—90 mg) (Ic), which crystallised from benzene–light petroleum (b.p. 60—80 °C) with m.p. 77—78°, $[\alpha]_D$ −9°.

The yields of total pseudomonic acids under these conditions, as measured by bioassay, against growth of the bacterium are shown in Figure 2. The bioassay was performed by the plate assay method, with *Staphylococcus aureus* (N.C.T.C. 6571) as test organism. Units are expressed on an arbitrary scale where 1 000 units $ml^{-1} \cong 55$ mg of pseudomonic acid. Cells were counted with a haemocytometer.

Incorporation of ^{14}*C-Labelled Precursors into Pseudomonic Acid A* (Ia).—In separate experiments, sodium [1-^{14}C]-acetate (2.56 μCi; 56 mCi $mmol^{-1}$), sodium [2-^{14}C]propionate (5.0 μCi; 14 mCi $mmol^{-1}$), sodium [^{14}C]formate (12.5 μCi; 56 mCi $mmol^{-1}$), (*RS*)-[2-^{14}C]mevalonic acid (MVA) (10 μCi; 17.5 mCi $mmol^{-1}$), and DL-[*Me*-^{14}C]methionine (Met) (15.4 μCi; 60 mCi $mmol^{-1}$), as solutions in water (5 ml), were filtered through a Sartorius membrane filter (0.2 μ) into a growing secondary stage culture (100 ml) of *Pseudomonas fluorescens*, 5 h after inoculation. After 24 h the culture was worked up as described above. To each extract, prior to methylation, was added cold methyl pseudomonate A (150 mg). After reisolation by p.l.c., the methyl pseudomonate A was crystallised to constant specific activity. The results are collected in Table 4.

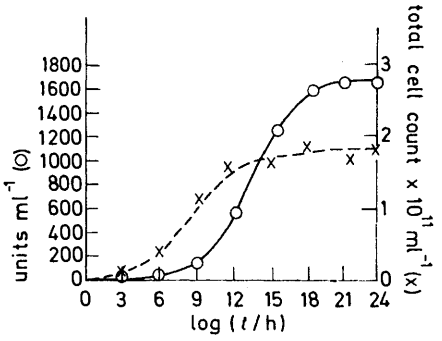


FIGURE 2 Yields of total pseudomonic acids against growth of the bacterium

^{13}C *Feeding Experiments.*—In separate experiments the following precursors (500 mg in each case, except where indicated) in water (50 ml) were fed hourly (0.45 ml sample per flask) over 10 h, to ten 100 ml secondary stage cultures of *Pseudomonas fluorescens*,[7] which had been incubating for 9 h: L-[*Me*-^{13}C]methionine (90.0 atom % ^{13}C), sodium [1-^{13}C]propionate (89.1 atom % ^{13}C), sodium [1-^{13}C]acetate (92.2 atom % ^{13}C) (760 mg fed to 15 × 100 ml cultures), sodium [3-^{13}C]propionate (92.4 atom % ^{13}C), sodium [2-^{13}C]-acetate (90.2 atom % ^{13}C), sodium [1,2-$^{13}C_2$]acetate (91.7 atom % ^{13}C at C-1 and 93.3 atom % ^{13}C at C-2) (120 mg) diluted with unlabelled sodium acetate (380 mg). The feedings were harvested after 24 h and the pseudomonic acid A was isolated and purified as its methyl ester (Ic) (respective amounts obtained: 63.6, 51.4, 87.2, 51.8, and 41.0 mg) as previously described. The s.f.o.r.d. spectra of the methyl pseudomonate A samples were recorded; the results are collected in Tables 2 and 3.

TABLE 4

Specific activity (disint. min^{-1} mg^{-1}) of methyl pseudomonate A

Cryst. no	[2-^{14}C]MVA	[1-^{14}C]AcONa	[2-^{14}C]$EtCO_2Na$	[*Me*-^{14}C]Met	[^{14}C]HCO_2Na
1	0	197	284	9 680	30
2		187	255	7 585	30
3		188	255	7 613	30
4		186	251	7 529	
5				7 579	
Mean	0	187	254	7 578	30
Incorporation (%)		0.51	0.36	15.41 *	0.004

* Allowing for incorporation of L-isomer only.

8-Methoxycarbonyloctyl 3-Methylbut-2-enoate (VII).—Thionyl chloride (0.5 ml) and 3-methylbut-2-enoic acid

(0.5 g) were stirred at 50 °C for 30 min. The excess of thionyl chloride was removed by flash distillation under vacuum, and the mixture was cooled to 0 °C. Methyl 9-hydroxynonanoate (1 g) was added dropwise over 15 min. After a further 30 min the residue was purified directly by p.l.c. [propan-2-ol–chloroform (1.5 : 98.5)]. The band at R_F 0.8 afforded the oily *ester* (VII) (1.32 g), $\lambda_{max.}$ 218 nm (ε 10 600); $\nu_{max.}$ (film) 1 725, 1 710, 1 650, and 1 155 cm^{-1}; τ 8.65 (10 H, s), 8.36 (2 H, m), 8.10 (3 H, d, *J ca.* 1 Hz, *trans*-Me), 7.84 (3 H, d, *J ca.* 1 Hz, *cis*-Me), 7.68 (2 H, t, *J* 7 Hz, $CH_2 \cdot CO_2Me$), 5.93 (3 H, t, *J* 7 Hz, $O \cdot CH_2$), 6.34 (3 H, s, OMe), and 4.31 (1 H, m, olefinic) (Found: C, 66.8; H, 9.6. $C_{15}H_{26}O_4$ requires C, 66.6; H, 9.7%).

We thank Sir Ernst Chain and Dr. P. Clayton for their interest in this work, and the S.R.C. for financial support toward the purchase of the Varian XL-100-12 spectrometer and maintenance awards (to T. F. and R. B. J.). Financial assistance from the Beecham Research Laboratories is gratefully acknowledged, and we thank R. Freer for technical assistance.

[6/923 *Received*, 14*th May*, 1976]