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THE BIOSYNTHESIS OF THE *PSEUDOMONAS* ANTIBIOTIC OBAFLUORIN FROM *p*-AMINOPHENYLALANINE AND GLYCINE (GLYOXYLATE)

Richard B. Herbert* and Andrew R. Knaggs

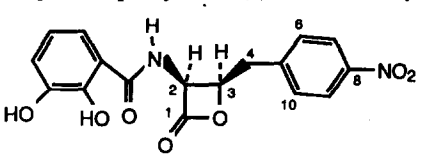
School of Chemistry, The University, Leeds, LS2 9JT.

Summary : DL-[2,3-$^2H_3$]-*p*-Aminophenylalanine is incorporated into obafluorin (1) with retention of C-3 deuterium and loss of deuterium from C-2. Labelled samples of *p*-nitrophenylacetic acid, *p*-aminophenylacetic acid and the N-acetylcysteamine thioester of the latter are not incorporated into obafluorin. Results of experiments with [2-^{13}C]-, [1-^{13}C]-, and [2-$^2H_2$]-glycine show that glyoxylic acid is specifically the source of C-1 and C-2 of the antibiotic. A mechanism is proposed for the biosynthesis of the unit (2) in obafluorin (1).

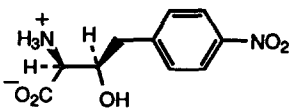
Obafluorin (1) is a unique antibiotic elaborated by *Pseudomonas fluorescens* (ATCC 39502). Results with D-[U-^{13}C]glucose and with phenylalanine and its *p*-nitro and *p*-amino derivatives establish that C-3 through C-10 are formed *via* L-*p*-aminophenylalanine (3) with loss of the carboxyl group of the amino acid and that the 2,3-dihydroxybenzoyl moiety of (1) has a conventional biosynthetic origin *via* chorismate/isochorismate.[1] We address here the origin of C-1 and C-2 of (1) and the mechanism whereby the peculiar C_4 side chain of the putative intermediate (2) is constructed.

DL-[2,3-$^2H_3$]-*p*-Aminophenylalanine [as (3)] (d_3 = 32%, d_2 = 68%, d_1 = 0) was synthesized by adaptation of a published procedure.[2] This material was an excellent precursor for obafluorin; deuterium at C-3 of (3) was incorporated equally at both diastereotopic positions on C-4 of obafluorin (^{2}H n.m.r.; m.s. : d_2 = 8.1%, d_1 = 6.1%) but the label on C-2 of (3) was completely lost. The label at C-3 of *p*-aminophenylalanine is thus secure throughout the course of biosynthesis. This has first importance in relation to the negative results which follow : deuterium label in intermediates cannot be lost by adventitious exchange.

p-Nitrophenylacetic acid (4) and 2-(4-nitrophenyl)ethanol (5) are co-metabolites of obafluorin in cultures of *Ps. fluorescens*.[1] This suggested that *p*-aminophenylacetic acid (6) or *p*-nitrophenylacetic acid (4) might be biosynthetic intermediates for (1) which lie between (3) and (2). However, we found that label in neither [2-$^2H_2$]-*p*-nitrophenylacetic acid [as (4)] (d_2 = 96%) nor [2-$^2H_2$]-*p*-aminophenylacetic acid [as (6)] (d_2 = 98%) was incorporated into obafluorin (1). Transamination of (3) would yield the keto-acid (15, X=H) and we considered that the coenzyme A ester of *p*-aminophenylacetic (6) formed directly from (15, X=H) might yet be involved in obafluorin biosynthesis.



(1) Obafluorin



(2)

N-Acetylcysteamine esters [as (7)] can conveniently be effective substitutes *in vivo* for coenzyme A esters.[3] We thus synthesized (7) dideuteriated on C-2. It was, however, not incorporated into obafluorin (it was stable to the conditions of culturing). From these, albeit negative, results we deduced that the keto-acid (15, X=H) and/or the aldehyde (8) were likely to be intermediates after (3) in obafluorin biosynthesis and (4) and (6) are not.

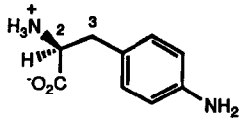
A remaining problem[1] in connection with obafluorin biosynthesis was the unknown origins of C-1 and C-2. A prime candidate to provide this C_2 unit was glycine. Under a variety of conditions for administering precursor to cultures of *Ps. fluorescens*, however, we observed no incorporation of [2-^{14}C]glycine. No incorporation of [U-^{14}C]serine, [U-^{14}C]acetate and [U-^{14}C]pyruvate was observed either. These results were actually not too surprising since the organism was still actively multiplying at the time the feeding experiments had to be done. But a complete change of conditions allowed the problem to be solved.

"Resting" cells of *Ps. fluorescens* in MES buffer were prepared.[4] An aqueous solution of [2-^{14}C]glycine (38mg, 14.3μCi), supplemented with the precursors 2,3-dihydroxybenzoic acid (37 mg) and *p*-aminophenylalanine (100 mg), pH 6.5, was added. This mixture was then incubated under similar conditions to those used for culturing the organism. An excellent incorporation of radioactivity into obafluorin (1) was observed (0.5% total incorporation, 12.4% specific incorporation). No secondary metabolites were produced in the absence of added precursors. Similar results were obtained for [2-^{14}C]glycine plus [2-^{13}C]glycine (^{14}C : 0.7% and 24%) and [2-^{14}C]glycine plus [1-^{13}C]glycine (^{14}C : 1.0% and 21%). However, no label from [2-$^{2}H_2$]glycine was incorporated into (1).

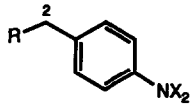
The ^{13}C n.m.r. spectrum of the obafluorin derivative (9), which was obtained in the experiment with [2-^{13}C]glycine, showed the expected enhancement of the signal for C-2. This is consistent with specific utilization of glycine as the source for C-1 and C-2. But some enhancement of the signal for C-1 was also observed and both the signals for C-1 and C-2 had doublets associated with them; a small amount of enrichment (doublets) was also observed for C-3 and C-4 (no other signals were enriched). Initially this was surprising but it is well known that *Pseudomonas* species are able to use glycine as a growth source with metabolism occurring by way of glyoxylic acid and the tartronic-semi-aldehyde (Scheme 1) and glyoxylate pathways.[5] Relatively large amounts of [2-^{13}-C]glycine were present in our incubation mixture. Thus, to a large extent, the tartronic semi-aldehyde (10) formed would be doubly labelled as shown. Glyoxylate subsequently generated in the glyoxylate pathway would also be doubly labelled. This accounts for the equal intensity doublets associated with the signals for C-1 and C-2; also for C-3 and C-4 by incorporation of doubly labelled phosphoenolpyruvate (PEP) (Scheme 1).

Combination of [2-^{13}C]glycine (glyoxylate) with endogenous unlabelled glyoxylate would give equal amounts of (11) and (12). Glyoxylate subsequently generated would be similarly labelled. This accounts for the enhancement of the natural abundance signal for C-1 and some of the singlet for C-2. There is additional and substantial enhancement, though, for C-2 (6.4 atom % compared to 1.6 atom % for C-1) and this must be due to *intact* utilization of glyoxylate in obafluorin biosynthesis before cycling. The correctness of these deductions was proved by the results for [1-^{13}C]glycine.

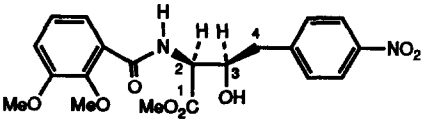
The ^{13}C n.m.r. for (9) labelled by this material showed *only* simple enhancement (3.3 atom %) of the



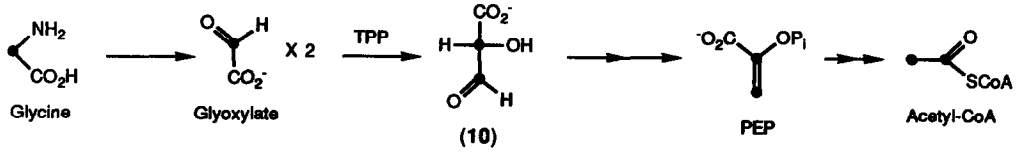
(3)



(4) R = CO_2H, X = O
(5) R = CH_2OH, X = O
(6) R = CO_2H, X = H
(7) R = $CO.SCH_2CH_2NHAc$, X = H
(8) R = CHO, X = H

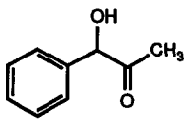


(9)

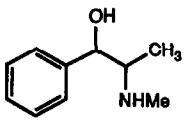


Glycine — Glyoxylate — (10) — PEP — Acetyl-CoA

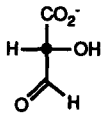
Scheme 1



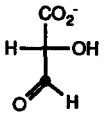
(13)



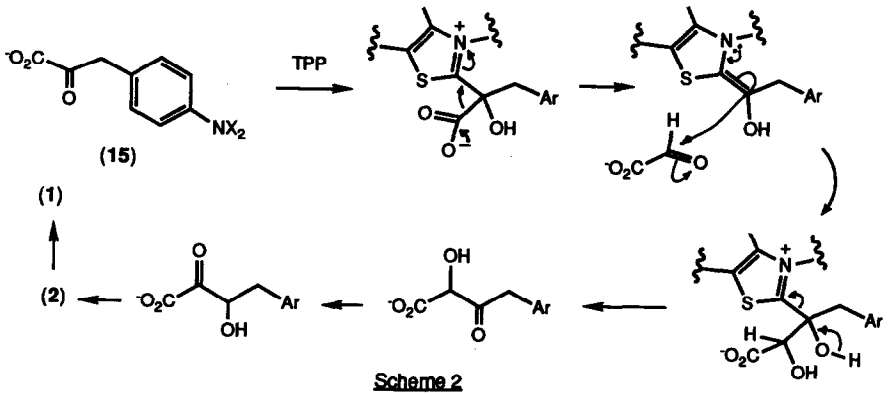
(14)



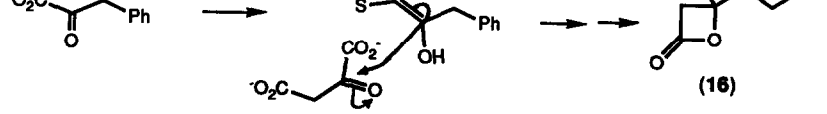
(11)



(12)



Scheme 2



Scheme 3

natural abundance signal for C-1 as predicted. Label on the carboxyl groups of glyoxylate is lost very rapidly on passage through the tartronic-semi-aldehyde and glyoxylate pathways, so significant incorporation of C-1 label can only occur before cycling.

We conclude that an intact molecule of glyoxylate specifically provides C-1 and C-2 of obafluorin (1) and that glycine serves to provide this more immediate precursor. Consideration of reasonable mechanisms for the biosynthesis of the fragment (2) of obafluorin, in the light of all the above data not least the loss of all the deuterium from C-2 of deuteriated glycine and loss of deuterium from C-2 of deuteriated *p*-aminophenylalanine,[6] leads through to the process involving thiamine pyrophosphate (TPP) shown in Scheme 2. Analogies are to be found in the TPP-medited condensation in yeast of benzaldehyde with pyruvic acid which affords (13) and the closely similar biosynthesis of ephedrine (14) in *Ephedra gerardiana*.[7] Further, a similar pathway can be adduced for the biosynthesis of the β-lactone (16) which is elaborated like obafluorin by a *Pseudomonas* species.[8] The proposed mechanism for the biosynthesis of the obafluorin fragment (2) may have general applicability in the biosynthesis of aromatic and related amino acids with a C_4 side chain *e.g.* the amiclenomycin antibiotics[9] [There are alternatives to Scheme 2, but they lack supporting analogies. The alternatives involve the aldehyde (8) instead of (15) or the TPP derivative of glyoxylate reacting with (15)/(8)].

Finally, we would like to draw fresh attention to the use of "resting" cells in biosynthetic studies and to the general opportunity this provides for specific induction of particular enzymes (in this case those of the tartronic-semi-aldehyde and glyoxylate pathways) for studying aspects of secondary metabolism.

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4. Cultures of *Ps. fluorescens* were grown normally for 10 hours; obafluorin production begins after *ca.* 8 hours. The cells were harvested by centrifugation and washed with 50 mM 3-(*N*-morpholino)ethanesulphonic acid (MES) buffer, pH 6.5. The cells were suspended in this buffer at double the original concentration. Incubation was at 25°C for 13 hours at 300 r.p.m., and this was followed by isolation and analysis of the metabolites. *cf.* D. R. Houck, J. Ondeyka, D.L. Zink, E. Inamine, M.A. Goetz, and O.D. Hensens, *J. Antibiot.*, 1988, **41**, 882.
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6. We cannot exclude the possibility that deuterium loss occurs by adventitious transamination reactions on (3) and (2) but since these are quite uncommon amino acids we do not think this is very likely.
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