Biosynthesis of the Antibiotic Obafluorin from D-[U-^{13}C]Glucose and *p*-Aminophenylalanine in *Pseudomonas fluorescens*[1]

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The separate units which are used to construct the antibiotic obafluorin **1** in *Pseudomonas fluorescens* are defined by the results of D-[U-^{13}C]glucose incorporation. A key intermediate in the biosynthesis of **1** is established to be L-*p*-aminophenylalanine **8**; L-phenylalanine and L-*p*-nitrophenylalanine are very poor precursors. Results similar to those for obafluorin are obtained for *p*-nitrophenylacetic acid **20** which along with the derivative **4** and 2-(4-nitrophenyl)ethanol **21** are identified as new metabolites of *P. fluorescens*. Deuteriated samples of **20**, **22** and **23** are not precursors for obafluorin **1**. [2,3-$^{3}H_3$]-*p*-Aminophenylalanine **18** is incorporated into **1** with complete loss of deuterium from C-2 but retention of the deuterium present in both diastereotopic positions on C-3.

In the course of a screen for β-lactam antibiotics a unique β-lactone obafluorin **1** was isolated from *Pseudomonas fluorescens* (ATCC 39502).[2,3] The β-lactone functionality is rarely encountered in secondary metabolites[5] but, when present, it is usually associated with biological activity;[2-4] interestingly, the stereochemistry of the substituents on the β-lactone ring of **1** is the same as that of the corresponding penicillins and cephalosporins.

Additional uncommon features in the structure of obafluorin held out the promise, subsequently fulfilled, that study of the biosynthesis of the antibiotic would be most worthwhile: (i) the side chain of the α-amino acid **2** corresponding to **1** is C_4 as against C_3 in common aromatic amino acids such as phenylalanine **6**; (ii) a rare aromatic nitro group[5] is present in **1**/**2**. The structure of the amino acid **2** resembles that of the important antibiotic chloramphenicol **3** which is biosynthesised from L-*p*-aminophenylalanine **8** in *Streptomyces venezuelae*.[6]

Several biosynthetic mechanisms can be sketched to account for the fragment **2** in **1**, so we carried out an experiment with D-[U-^{13}C]glucose to narrow the number of possible paths to obafluorin, following the ingenious use of this labelled compound in several cases to define the separate units which are used to construct a secondary metabolite *in vivo*.[7,8] D-[U-^{13}C,^{14}G]Glucose was administered to cultures of *P. fluorescens*. The obafluorin which was isolated (1.86% total ^{14}C incorporation) was purified (HPLC) and subjected to ^{13}C NMR analysis at 100 MHz. From the couplings observed the separate units which constitute the antibiotic could be deduced (see Fig. 1). The patterns for the two aromatic rings are consistent with the expected biosynthesis by way of the shikimate pathway (Scheme 1).[7,9-11] The occurrence of an intact C_3 unit (see Fig. 1) for C-11 through C-13 of **1** confirmed that the dihydroxybenzoyl moiety **14** is formed by the known pathway from shikimic acid **11** *via* isochorismic acid **13**.[11]

Notably, C-3 and C-4 of obafluorin **1** (see Fig. 1) constitute a C_2 unit as (less clearly) do C-1 and C-2. The pattern for C-3 through C-10 is that expected of biosynthesis *via* an aromatic amino acid such as phenylalanine **6**. Chloramphenicol **3** is known to arise *via p*-aminophenylalanine **8** in a pathway which does not involve phenylalanine, *i.e.* the aromatic ring is aminated at a stage prior to aromatization (other evidence indicates amination occurs on chorsmic acid **12**).[6] A similar

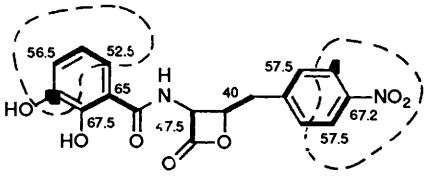
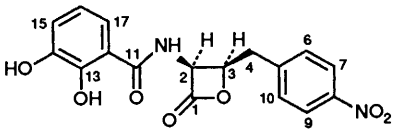
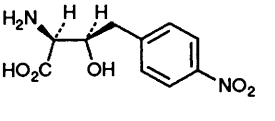


Fig. 1 Labelling of obafluorin **1** by D-[U-^{13}C]glucose; the numbers are the observed ^{13}C–^{13}C coupling constants in Hz



1 Obafluorin



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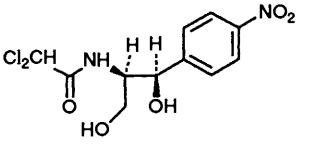
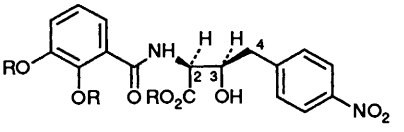
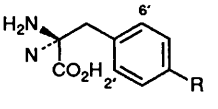


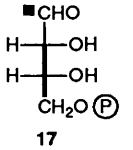
3 Chloramphenicol



4; R = H
5; R = Me



6; R = H
7; R = NO_2
8; R = NH_2



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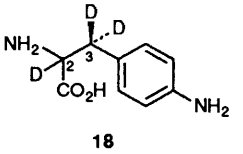
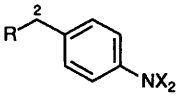


18



19; R = CHO, X = H
20; R = CO_2H, X = O
21; R = CH_2OH, X = O
22; R = CO_2H, X = H
23; R = $COSCH_2CH_2NHAc$, X = H

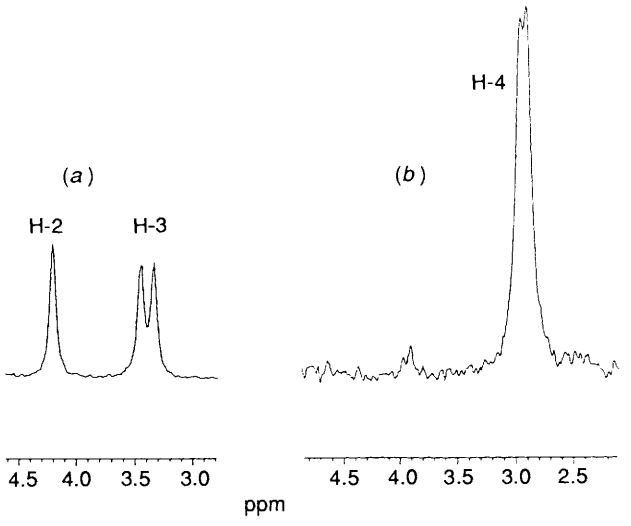


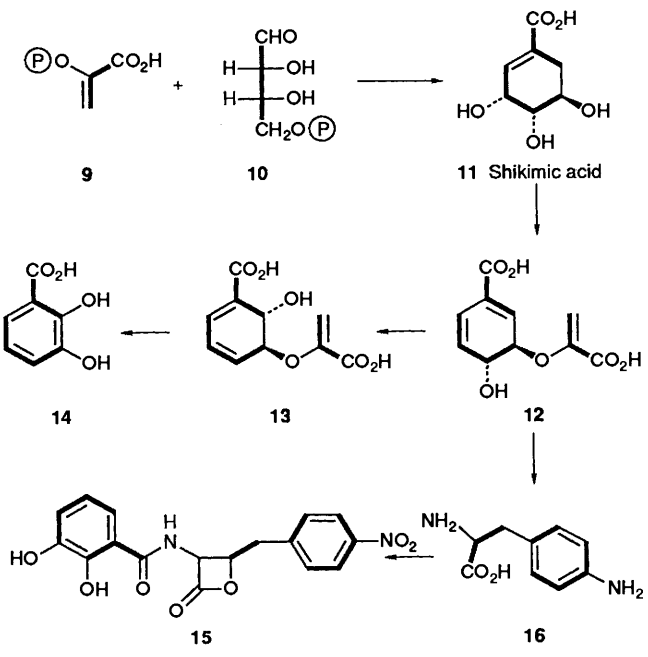
Fig. 2 ^2H NMR spectra of: (a) [2,3-$^2H_3$]-*p*-aminophenylalanine **18**; (b) the obafluorin derivative **5** after incorporation of **18**

pathway *via* *p*-aminophenylalanine **8** = **16** is thus suggested for obafluorin **1**.

Nitration[12] of L-[2′,6′-^{3}H]phenylalanine gave L-[2′,6′-^{3}H]-*p*-nitrophenylalanine, as **7**, which upon catalytic hydrogenation gave L-[2′,6′-^{3}H]-*p*-aminophenylalanine, as **8**. This latter compound was an excellent precursor (10.43% incorporation) for obafluorin **1**, whilst L-[2′,6′-^{3}H]-*p*-nitrophenylalanine, as **7**, and L-[2′,6′-^{3}H]phenylalanine, as **6**, were poor precursors (0.17 and 0.2%, respectively). We conclude therefore that L-*p*-aminophenylalanine **8** is a key precursor in the biosynthesis of obafluorin **1** and, as found for chloramphenicol **3**, neither phenylalanine nor *p*-nitrophenylalanine are involved in the antibiotic's biosynthesis. It is clear that, as for chloramphenicol: (i) oxidation of the aromatic amino group only occurs after modification elsewhere in **8**; (ii) amination occurs at the pre-aromatic stage (probably on chorismate **12**/isochorismate **13**). This congruence between the biosynthesis of a *Streptomyces* metabolite **3** and of a *Pseudomonas* metabolite **1** is notable.

The simplest prediction of the labelling patterns in the two aromatic rings of obafluorin following the incorporation of [U-^{13}C]glucose is as shown in **15**; the aromatic, C_4 units are provided by way of an intact molecule of erythrose 4-phosphate **10**. Although the appropriate labelling could be discerned, the pattern observed was largely C_3 + C_1 for both aromatic rings (see dotted lines in Fig. 1). This could be attributed to cycling through the pentose–phosphate pathway,[10] but it is well established that *Pseudomonas* species catabolise glucose mainly *via* the Entner–Doudoroff pathway[13] which involves an obligatory split of glucose into C_3 units (glyceraldehyde 3-phosphate plus pyruvic acid). Subsequent synthesis of erythrose 4-phosphate from these C_3 units gives rise to a net C_3 + C_1 pattern in this intermediate, *i.e.* **17**; direct, minor, biosynthesis *via* the pentose–phosphate pathway yields erythrose 4-phosphate which when formed from [U-^{13}C]glucose would be contiguously labelled over all four carbon atoms, *i.e.* **10**. Our results then are consistent with the functioning of the Entner–Doudoroff pathway as a major route from glucose in *P. fluorescens*. C_3 units derived *via* phosphoenol pyruvate, as **9**, by either glycolysis or the Entner–Doudoroff pathway will be contiguously labelled by D-[U-^{13}C]glucose.

In order to probe how the side chain of **8** becomes that of obafluorin **1** we examined DL-[2,3-$^2H_3$]-*p*-aminophenylalanine **18** ($^2H_3$ = 32.2, $^2H_2$ = 67.8, $^2H_1$ = 0%) (details of its synthesis are given in the Experimental section) as a precursor. An excellent incorporation was observed with equal labelling of the



Scheme 1

diastereotopic protons on C-4 of **5** (^2H NMR; MS: $^2H_2$ = 8.1, $^2H_1$ = 6.1%), but the label on C-2 of **18** was completely lost (Fig. 2). This latter result points away from biosynthetic mechanisms yielding say **19** as an intermediate through direct, pyridoxal-phosphate mediated, decarboxylation of **18**. However, deuterium could be lost from this position by adventitious, facile transamination of **18**.

Since the deuterium at C-3 in **18** is retained in **1**, possible biosynthetic mechanisms are again limited, also deuterium label at this site in any intermediates cannot be lost by adventitious exchange. This is relevant to the negative results which are discussed below.

Examination of cultures of *P. fluorescens* showed the presence of three metabolites in addition to obafluorin: the ring-opened form **4** of **1** which appeared after **1** during culturing and is thus its hydrolysis product; *p*-nitrophenylacetic acid **20**; and 2-(4-nitrophenyl)ethanol **21**. The biosynthesis of *p*-nitrophenylacetic acid **20** is clearly closely related to that of obafluorin because the levels of incorporation into **20** with the tritiated compounds **8**, **7** and **6** were similar to those for obafluorin: 7.44, 0.29 and 0.01%, respectively. The failure of tritiated **4** (derived biosynthetically from [2′,6′-^{3}H]-*p*-aminophenylalanine) to be incorporated into **20** or **21**, ruled out the biosynthesis of these two metabolites being from **4**. The production of **20** and **21** by *P. fluorescens* obviously suggested that either **20** or **22** might be intermediates in the biosynthesis of obafluorin, but when [2-$^2H_2$]-*p*-nitrophenylacetic acid, as **20** ($^2H_2$ = 96.2, $^2H_1$ = 3.8%), and [2-$^2H_2$]-*p*-aminophenylacetic acid, as **22** ($^2H_2$ = 97.6, $^2H_1$ = 2.4%) were fed to our cultures no incorporation of either into obafluorin was observed. Deuteriated *p*-nitrophenylacetic acid **20** was recovered in the experiment with the labelled **20** and some deuterium loss by exchange was apparent ($^2H_2$ = 68.7, $^2H_1$ = 3.8%). The deuteriated *p*-aminophenylacetic acid also gave labelled **20**, which is the result of apparently ready enzymic oxidation of the aromatic amino group. Curiously, however, much more deuterium was lost from this precursor by exchange ($^2H_2$ = 12.7, $^2H_1$ = 31.2%) than with the nitro-substituted precursor. This was surprising since the C-2 protons (deuterons) are only accessibly acidic when the *p*-nitro-group is present. Exchange can only occur after oxidation of **22** to **20**, *i.e.* there should *a priori* be more exchange with **20** than with **22** as precursors. However, the

results can be explained as follows: deuteriated **22**, but not deuteriated **20**, is transported through the cell envelope where, within the cytoplasm, it is enzymically oxidized to deuteriated **20** and where it then suffers extensive exchange before being transported out of the cell into the culture medium where little exchange occurs. This incidentally assuages doubts that the failure of **22** to act as an obafluorin precursor might have been because it was not transported to within the cell.

These results are corroborated by the observation that DL-[2,3-$^2H_3$]-*p*-aminophenylalanine **18** gave *p*-nitrophenylacetic acid **20** with a very low level of label ($^2H_2$ = 0.1, $^2H_1$ = 0.5%) in contrast to an excellent incorporation into obafluorin (above).

We considered that it was still possible that the coenzyme A ester of *p*-aminophenylacetic acid might be involved in obafluorin biosynthesis. A simple and convenient substitute is the *N*-acetylcysteamine[14] thioester **23**. No incorporation of the C-2 deuteriated compound into obafluorin was observed, however. Deuterium was incorporated into **20** at a very low level which is consistent with enhanced intra-cellular exchange of the putative intermediate thioester of **20**. Interestingly the amount of **20** and **21** present in the cultures was significantly enhanced in this experiment.

The above results indicate that the biosynthesis of obafluorin involves the condensation of 2,3-dihydroxybenzoic acid **14**, a C_2 unit which could, *a priori*, most simply be glycine, and a derivative of L-*p*-aminophenylalanine. Further results are given in the following paper.

Experimental

For general directions see ref. 8. All ^{13}C NMR spectra were obtained on a Bruker AM400 spectrometer. In each case $Cr(acac)_3$ (10 mg) was added to the solution. Pulse delay was 5 s and inverse-gated decoupling was used. ^2H NMR spectra were recorded at 61.4 MHz and 45 °C on the Bruker AM400 spectrometer; *J* values are recorded in Hz. Deuterium enrichments were determined by MS. The intensities in the M^+ region of the labelled compound were compared with those of unlabelled material; an average of 10 scans was used and the samples were run under the same conditions and one after the other. Deuterium on exchangeable groups was in all cases removed by exchange prior to measurements.

Centrifugation was with Wifug 4000E or MSE High Speed 18. HPLC was performed with a Gilson 303/802 or Varian 5000 and Polymer Laboratories reverse phase columns (PLRP-S 100, 10 μm, 300 × 7.5 mm and 300 × 25 mm).

Cultivation of Pseudomonas fluorescens (*ATCC 39502*).[3] The organism was grown in liquid culture consisting of Difco (not Sigma) yeast extract (0.5%), D-glucose (0.5%), $MgSO_4 \cdot 7H_2O$ (0.01%) $FeSO_4$ (0.01%), which were dissolved in the filtrate of a soil extract plus tap water (1 part filtrate: 4 parts water). Soil extract was obtained by gently boiling 1 volume of garden soil with 2 volumes of tap water for 1 h; filtration was through a pad of Celite. All media were sterilized before use. Agar slants were prepared using 2% Difco agar. Subcultures on agar slants were grown for 36 h at 25 °C and then stored at 5 °C. Culturing was from agar slants into 100 cm^3 of medium in 250 cm^3 conical flasks which were incubated at 25 °C at 300 rpm for 24 h (Gallenkamp INR-401). Innoculation from these was at 1% into 100 cm^3 of medium in 500 cm^3 conical flasks for growth and production at metabolites. Incubation was as above for up to 24 h.

The production of metabolites was monitored by UV spectroscopy (EtOAc extract; spectrum in MeCN, λ_{max} 268 nm) and by HPLC (EtOAc extract; chromatography on the 300 × 7.5 mm column in MeCN–H_2O–TFA, 45:55:0.1; detector: λ_{max} 258 nm).

In the course of incubation of the growth flasks over 24 h the cultures changed colour from an initial orange/brown to grey (10 or 11 h) to purple. Obafluorin production began after 8 h and reached a peak after 14 h. The amount of **4** increased with time as that of obafluorin decreased.

Feeding of precursors. Aqueous solutions of precursors (pH 6) were added in equal portions to the cultures after 8 and 10 h. The following are the quantities of precursors fed and the volumes of culture used. [U-^{13}C, ^{14}C]Glucose (98.2 atom% ^{13}C), 100 mg, 2 μCi, to 24 × 100 cm^3; 2′,6′-tritiated samples of L-*p*-nitrophenylalanine (5.7 mg, 6.3 μCi), L-*p*-aminophenylalanine (7.3 mg, 8.1 μCi), L-phenylalanine (24 mg, 8.1 μCi), each to 10 × 100 cm^3; [2,3-$^2H_3$]-*p*-aminophenylalanine, 75 mg, 15 × 100 cm^3; deuteriated samples of *p*-nitrophenylacetic acid (28 mg), *p*-aminophenylacetic acid (17 mg), the *N*-acetylcysteamine ester of the latter (24 mg), each to 10 × 100 cm^3. Incorporation of D-[U-^{13}C]glucose into obafluorin **1**: C-1, 0.48; C-2, 0.23; C-3, 0.89; C-4, 0.86; C-5, 0.53; C-6/10, 0.83; C-7/9, 1.19; C-8, 0.52; C-11, 0.75; C-12, 0.94; C-13, 0.65; C-14, 0.87% total enrichment above natural abundance; overlapping signals made measurement of C-15 through C-17 enrichment particularly inaccurate but values were similar to those of other aromatic carbon atoms.

Isolation and Purification of Metabolites.—After 13 h incubation the culture broth (1 l) was centrifuged (Wifug, 3000 rpm, 30 min). The pH of the supernatant liquid was adjusted to 3 (dil. HCl). The resulting green solution was extracted with ethyl acetate (3 × 1 l), the combined extracts were dried ($MgSO_4$), and the solvent was removed under reduced pressure at 35 °C to leave a dark brown residue (149 mg). The residue was taken up in acetonitrile (4 cm^3) and the solution was filtered using Millipore 5 μm filter paper. The filtrate was evaporated to dryness. A solution in acetonitrile (1 cm^3) was chromatographed by HPLC (300 × 25 mm column, MeCN–H_2O–TFA, 60:40:0.1, 4 cm^3 min^{-1}; 5–10 separate injections) to yield obafluorin (17.5 mg) and a brown polar residue from which methyl *p*-nitrophenylacetate was isolated after treatment with an excess of diazomethane in MeCN. Purification was by column chromatography (ether–hexane, 1:4) to give white crystals of methyl *p*-nitrophenylacetate (4.5 mg).

Alternatively, metabolites were isolated in the same way after 24 h. The dark brown residue (157 mg) this time was treated with an excess of diazomethane in acetonitrile (24 h, room temp). The resulting material was chromatographed on silica with ether–hexane, 4:1 to give **5** plus non-polar material which contained methyl *p*-nitrophenylacetate; elution with ethyl acetate–methanol (1:1) gave impure 2-(4-nitrophenyl)ethanol. Compound **5** was purified by HPLC(MeCN–H_2O–TFA, 65:35:0.1, 4 cm^3 min^{-1} to give a pale yellow solid (14.5 mg).

Spectral Data on Metabolites.—Obafluorin has been well characterised.[2] We include our data for completeness; NMR assignments have been revised in a minor way on the basis of 2D ^1H-^1H COSY and 2D ^{13}C-^1H COSY NMR experiments and also the results of the D-[U-^{13}C]glucose incorporation; ν_{max}/cm^{-1} 3700–2700, 3510, 3310, 1835, 1640, 1520 and 1350; δ_H(400 MHz, $CD_3CO \cdot CD_3$) 3.39 (1 H, dd, *J* 15 and 5, 4a-H), 3.53 (1 H, dd, *J* 15 and 10, 4b-H), 5.21 (1 H, ddd, *J* 10, 6 and 5, 3-H), 6.02 (1 H, dd, *J* 8.5 and 6, 2-H), 6.80 (1 H, t, *J* 8, 16-H), 7.05 (1 H, dd, *J* 8 and 1.5, 17-H), 7.37 (1 H, dd, *J* 8 and 1.5, 15-H), 7.58 (2 H, d, *J* 8.5, 6/10-H), 8.15 (2 H, d, *J* 8.5, 7/9-H), 9.10 (1 H, br d, *J* 8.5, NH) and 11.91 (1 H, br s, OH); δ_C(100 MHz, CD_3COCD_3) 35.95 (C-4), 59.65 (C-2), 78.21 (C-3), 114.50 (C-12), 118.14 (C-15), 119.63 (C-16), 120.23 (C-17), 124.23 (C-7/9), 131.05 (C-6/10), 145.30 (C-5), 146.98 (C-14), 147.63

(C-13), 150.13 (C-8), 168.50 (C-1) and 170.86 (C-11); *m/z* 358.0801 (M^+, calc. for $C_{17}H_{14}N_2O_7$, 358.0806).

The O,O-*dimethyl ether methyl ester* **5**: $\lambda_{max}(CH_3CN)$/nm 203, 237 and 270; ν_{max}/cm^{-1} 3610, 3530, 3360, 1745 and 1655; δ_H(400 MHz, $CDCl_3$), 3.95 (2 H, m, 4-H), 3.77 (3 H, s, OCH_3), 3.95 (3 H, s, OCH_3), 4.05 (3 H, s, OCH_3), 4.50 (1 H, m, 3-H), 4.80 (1 H, dd, *J* 9 and 1.5, 2-H), 7.25 (2 H, m, 16/17-H), 7.55 (2 H, d, *J* 8, 6/10-H), 7.65 (1 H, m, 15-H), 8.20 (2 H, d, *J* 8, 7/9-H), and 8.88 (1 H, br d, *J* 9, NH); δ_C(100 MHz, $CDCl_3$) 40.22 (C-4), 52.75 (OMe), 56.14 (OMe), 56.23 (C-2), 61.75 (OMe), 72.51 (C-3), 115.94 (C-15), 122.79 (C-17), 123.70 (C-7/9), 124.47 (C-16), 125.54 (C-12), 130.31 (C-6/10), 145.34 (C-5), 146.85 (C-14), 147.85 (C-13), 152.70 (C-8), 165.65 (C-1) and 171.17 (C-11); *m/z* (CI-GC-MS, ammonia), 419 ($M^+ + 1$, 100%).

p-*Nitrophenylacetic acid.* M.p. 153–154 °C (from water); ν_{max}/cm^{-1} 3500–2200, 1700, 1505 and 1340 cm^{-1}; δ_H(90 MHz, $CD_3CO{\cdot}CD_3$) 3.83 (2 H, s), 7.60 (2 H, d, *J* 9) and 8.16 (2 H, d, *J* 9); δ_C(22.5 MHz, $CD_3CO{\cdot}CD_3$), 37 (C-2), 120.5 (C-5/7), 128.5 (C-4/8), 140 (C-3), 145 (C-6) and 169 (C-1); methyl ester: m.p. 54 °C (from benzene–hexane).

2-(4-*Nitrophenyl*)*ethanol.* M.p. 64 °C (from ether–hexane); δ_H(90 MHz, $CDCl_3$), 1.8 (1 H, s, OH), 2.95 (2 H, t, *J* 6), 3.95 (2 H, t, *J* 6), 7.4 (2 H, d, *J* 9) and 8.15 (2 H, d, *J* 9); δ_C(100 MHz, $CDCl_3$), 38.79, 62.73, 123.54, 129.77, 146.52 and 146.80.

HPLC retention times (min) with (a) 300 × 7.5 mm column, 2 cm^3 min^{-1}, 258 nm, MeCN–H_2O–TFA, 50:50:0.1, and (b) 300 × 25 mm column, 4 cm^3 min^{-1}, 258 nm, MeCN–H_2O–TFA, 60:40:0.1 (solvent variations noted): obafluorin: (a) 19.2, (b) 51; **5**: (a) 21.6, (b) 43 (65:35:0.1); *p*-nitrophenylacetic acid: (a) 6.3 (45:55:0.1), (b) 32; its methyl ester: (a) 30.1, (b) 53 (65:35:0.1); 2-(4-nitrophenyl)ethanol: (a) 9.3 (45:55:0.1), (b) 34.

L-p-*Nitrophenylalanine.*—This compound was prepared by adaptation of a published method.[12] L-Phenylalanine (5.7 g) was dissolved in sulfuric acid (18 mol dm^{-3}; 16.9 g) at 80 °C and then cooled, finally in ice. Nitric acid (10 mol dm^{-3}; 4.6 cm^3) was then added dropwise to the stirred mixture over 15 min. The yellow solution was left at room temperature for 24 h and then heated to 50 °C (bath temp.) for 2 h. The reaction mixture was cooled, finally in ice and then diluted with water (30 cm^3). The pH of the solution was adjusted to 4.5. The precipitate, collected and recrystallised from water, had m.p. 215–218 °C (decomp.), 44%; $[\alpha]_D^{24.5}$ +6.6 (2 mol dm^{-3} HCl); δ_H(90 MHz, NaOD/D_2O), 3.0 (2 H, m), 3.5 (1 H, m), 7.4 (2 H, d, *J* 9) and 8.15 (2 H, d, *J* 9) (Found: C, 51.45; H, 4.8; N, 13.2. Calc. for $C_9H_{10}N_2O_4$: C, 51.4; H, 4.8; N, 13.3%).

L-[2′,6′-^{3}H]-*p*-Nitrophenylalanine was prepared from L-[2′,6′-^{3}H]phenylalanine; activity: 1.1 μCi mg^{-1}.

L-p-*Aminophenylalanine.*—L-*p*-Nitrophenylalanine (100 mg) was dissolved in water (5 cm^3) and conc. hydrochloric acid (6 drops) was added. This solution was hydrogenated at atmospheric pressure for 4 h in the presence of Pd–$BaSO_4$ (5% Pd; 50 mg). After being filtered (Celite), the pH of the solution was adjusted to 3 and then lyophilised to give the dihydrochloride of L-*p*-aminophenylalanine; *m/z* (CI, NH_3): 181 (M^+, 100%); δ_H(90 MHz, D_2O, pH 3) 3.3 (2 H, m), 4.2 (1 H, m) and 7.4 (4 H, m); δ_C(22.5 MHz, NaOD/D_2O with TMSP as reference) 49, 59, 127, 132, 134, 139 and 176. L-[2′,6′-^{3}H]-*p*-Aminophenylalanine was prepared similarly.

[2-$^2H_2$]-p-*Nitrophenylacetic Acid and* [2-$^2H_2$]-p-*Aminophenylacetic Acid.*—A solution of *p*-nitrophenylacetic acid (600 mg) and triethylamine (5 cm^3) in deuterium oxide (5 cm^3) was heated at 70 °C (bath temp.) for 3 days. The deuteriated *p*-nitrophenylacetic acid was isolated and recrystallised from water (81%); δ_{2H}(61.4 MHz, MeCN, 45 °C), 3.8 (br s, 2-D); deuterium content: $^2H_2$ = 97.6; $^2H_1$ = 2.4; $^2H_0$ = 0%.

Hydrogenation at atmospheric pressure in the presence of Pd–$BaSO_4$ for 3 h gave [2-$^2H_2$]-*p*-aminophenylacetic acid, m.p. 192–193 °C (decomp.; from water), lit.,[15] 199–200 °C (decomp.); $\delta_{^2H}$(61.4 MHz, MeCN, 45 °C), 3.5 (br s, 2-D); deuterium content: $^2H_2$ = 97.6; $^2H_1$ = 2.4; $^2H_0$ = 0%.

N-*Acetylcysteamine Thioester of* [2-$^2H_2$]-p-*Aminophenylacetic Acid.*—To a suspension of [2-$^2H_2$]-*p*-aminophenylacetic acid (0.6 mmol; 85 mg) in dry THF (5 cm^3) was added *N*-acetylcysteamine (220 mg, 1.85 mmol), followed by DCC (270 mg, 1.3 mmol) in THF (2 cm^3) (*cf.* ref. 14). The mixture was stirred at room temperature under nitrogen for 14 h. It was then filtered and the filtrate was evaporated to dryness under reduced pressure. A pale yellow solid was obtained and the required thioester was purified by HPLC [MeCN–H_2O (pH 7), 30:70]; 20%; $\delta_{^2H}$(61.4 MHz, MeCN, 45 °C), 3.66 (br s, 2-D). Data on the unlabelled compound: ν_{max}/cm^{-1} 3300, 1695 and 1655; δ_H(300 MHz, CD_3COCD_3), 1.8 (3 H, s), 2.95 (2 H, t, *J* 6), 3.25 (2 H, t, *J* 6), 3.7 (2 H, s), 4.65 (1 H, br s, NH), 6.65 (2 H, d, *J* 6), and 6.97 (2 H, d, *J* 6). For feeding experiments the thioester was dissolved in HPLC grade acetonitrile. The solution was diluted 20 times with water and the acetonitrile was removed under reduced pressure.

DL-[2,3-$^2H_3$]-p-*Aminophenylalanine.*—This compound was prepared by adaptation of a published procedure.[16]

(a) [1-$^2H_2$]-p-*Nitrobenzyl alcohol.*[17] To a solution of a methyl *p*-nitrobenzoate (8.5 g, 46.7 mmol) in dry THF (25 cm^3) was added sodium borodeuteride (1 g, 23.8 mmol). The mixture was cooled in ice and aluminium trichloride (1.5 g) was added. The mixture was refluxed for 24 h, cooled, finally in ice, and the excess of reagents was destroyed by careful addition of 2 mol dm^{-3} hydrochloric acid. Extraction with ether (3 × 250 ml), drying ($MgSO_4$), and evaporation gave [1-$^2H_2$]-*p*-nitrobenzyl alcohol which was purified by chromatography (ether–hexane, 1:1), 27%; m.p. 87–89 °C (from benzene–hexane) (lit.,[18] 92–94 °C) [Found (for unlabelled sample): C, 55.05; H, 8.85; N, 4.55. Calc. for $C_7H_7NO_3$: C, 54.90; H, 9.15; N, 4.61%].

(b) [1-$^2H_2$]-p-*Nitrobenzyl bromide.*[19] To a solution of [1-$^2H_2$]-*p*-nitrobenzyl alcohol (1.0 g, 6.65 mmol) in dry benzene (40 cm^3) was added carbon tetrabromide (2.5 g, 7.5 mmol) and triphenylphosphine (3.9 g, 14.9 mmol). The mixture was stirred at room temp. until all the alcohol had reacted. The solvent was removed under reduced pressure and the residue was chromatographed (ether–hexane, 1:3) to give the bromide (56%), m.p. 97–99 °C (from benzene–hexane) (lit.,[20] 98 °C); deuterium content: $^2H_2$ = 92.3; $^2H_1$ = 7.7; $^2H_0$ = 0%.

(c) *Diethyl* [3-$^2H_2$]-N-*acetyl*-2-(4-*nitrobenzyl*)*aminomalonate.* To a stirred and refluxing solution of diethyl acetamidomalonate (850 mg, 2.41 mmol) in ethanol (35 cm^3) containing sodium ethoxide (1 equiv.) was added rapidly a solution of [1-$^2H_2$]-*p*-nitrobenzyl bromide (813 mg, 3.75 mmol) in dry dioxane (10 cm^3). The reaction mixture was stirred at reflux for a further 90 min. The cooled solution was left at 5 °C for 18 h after which the precipitated product was collected (53%), m.p. 196–197 °C (from glacial acetic acid); deuterium content: $^2H_2$ = 64.1; $^2H_1$ = 31.8; $^2H_0$ = 4.1%. Data on unlabelled material: ν_{max}/cm^{-1} 3260, 1750, 1650, 1525 and 1360 cm^{-1}; δ_H(90 MHz, CF_3CO_2D with TMSP as reference) 1.35 (6 H, t, *J* 8), 2.2 (3 H, s), 3.85 (2 H, s), 4.4 (4 H, q, *J* 8), 7.25 (2 H, d, *J* 9) and 8.2 (2 H, d, *J* 9); δ_C(22.5 MHz, CF_3CO_2D) 7.19, 15.23, 32.38, 60.04, 63.02, 118.71, 125.75; 137.57, 142.14, 163.54 and 170.06.

(d) DL-[2,3-$^2H_3$]-p-*Nitrophenylalanine.* The malonate above (707 mg, 2 mmol) was suspended in CH_3CO_2D (2–3 cm^3, formed by reaction of Ac_2O and D_2O) and DBr (5 cm^3, 48%) was added. The mixture was carefully refluxed for 4.5 h after which it was cooled, finally in ice. The precipitate of the

nitrophenylalanine hydrobromide was collected; $\delta_{^2H}$(61.4 MHz, H_2O, 45 °C), 4.51 (br s, 2-D), 3.64 and 3.55 (br s, 3-D).

(e) DL-[2,3-$^2H_3$]-p-*Aminophenylalanine*.[21] A solution of the nitro compound above (89.7 mg, 0.31 mmol) in D_2O (5 cm^3) plus 5 drops of DBr (48%) was hydrogenated at atmospheric pressure in the presence of Pd–$BaSO_4$ (5% Pd; 160 mg) for 4 h. The catalyst was filtered off (Celite) and the pH of the filtrate adjusted to 3. The filtrate containing the dihydrobromide could be used directly in a feeding experiment or lyophilised; $\delta_{^2H}$(61.4 MHz, H_2O, 45 °C), 4.20 (br s, 2-D) and 3.45 and 3.33 (br s, 3-D); deuterium content: $^2H_3$ = 32.2; $^2H_2$ = 67.8; $^2H_1$ and $^2H_0$ = 0%.

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