Biosynthesis of the Antibiotic Obafluorin from *p*-Aminophenylalanine and Glycine (Glyoxylate)[1]

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Results of experiments using 'resting' cells of *Pseudomonas fluorescens* with [2-^{13}C]-, [1-^{13}C]- and [2-$^{2}H_2$]-glycine as precursors show that glyoxylic acid is specifically the source of C-1 and C-2 of the antibiotic obafluorin **1**. The ^{13}C labelling patterns are consistent with the passage of the precursors through the tartronic semialdehyde and glyoxylate pathways. The glycine derivative **6** is not a precursor for **1**. The analogues **24** and **25** of *p*-aminophenylalanine **3** are synthesised and tested as substrates for biosynthesis in *P. fluorescens*. With **24**, the metabolite **26** is detected; no metabolites of **25** are detected.

L-*p*-Aminophenylalanine **3** is a key precursor for the unit **2** in the biosynthesis of the unique β-lactone antibiotic obafluorin **1** in *Pseudomonas fluorescens* (ATCC 39502).[2] In the course of biosynthesis the carboxy group is lost (evidence from the incorporation of D-[U-^{13}C]glucose) as is a deuterium atom originally present at C-2; deuterium in both diastereotopic positions at C-3 of **3** is retained into C-4 of **1**. It is apparent that oxidation of the aromatic amino group occurs at a late stage of biosynthesis. On the basis of results with [U-^{13}C]glucose the 2,3-dihydroxybenzoyl moiety of obafluorin has an unexceptional origin through 2,3-dihydroxybenzoic acid.[2] This leaves the origin of a C_2–N unit (C-1 + C-2 + NH) to be accounted for, also the mechanism of formation of the fragment **2** in obafluorin **1**. We address these questions here and argue for a mechanism of formation which is readily extended to encompass, *inter alia*, the β-amino-α-hydroxy acid derivatives bestatin **4**[3] and amistatin **5**.[4]

The most obvious candidate to provide the C_2–N unit in obafluorin **1** is glycine. However, we failed to get an incorporation with [2-^{14}C]glycine at times during the growth of *P. fluorescens* when, for example, *p*-aminophenylalanine was well incorporated. Neither pulse-feeding, continuous slow addition of precursor, nor pulse-feeding in the presence of chloramphenicol (hopefully to suppress protein synthesis) were successful. Similar, negative results were obtained with L-[U-^{14}C]serine, [U-^{14}C]pyruvate and [U-^{14}C]acetate.

Unfortunately, under our culture conditions obafluorin production took place while the organism was still growing. Thus, these negative results could simply have been the result of the channelling of the precursors into primary metabolism. In order to try and overcome this problem we tested a potential, later intermediate which, if involved in obafluorin biosynthesis, should preferentially have been incorporated into the antibiotic: the dideuterio derivative **6** of *N*-(2,3-dihydroxybenzoyl)-glycine. It was fed to *P. fluorescens* but was not incorporated into obafluorin.

Another approach to the problem was clearly necessary and we chose to examine 'resting' cells of *P. fluorescens* for the incorporation of glycine (the precursor solution contained in addition 2,3-dihydroxybenzoic acid and *p*-aminophenylalanine). An excellent incorporation of [2-^{14}C]glycine was obtained (0.5% total incorporation, 12.4% specific incorporation). No secondary metabolites were produced in the absence of the three added precursors. Similar results were obtained with [2-^{13}C, ^{14}C]glycine (^{14}C: 0.7 and 24%) and [1-^{13}C, 2-^{14}C]glycine (^{14}C: 1.0 and 21%). However, no label from [2-$^{2}H_2$]glycine was incorporated into **1**.

The ^{13}C NMR spectrum of the obafluorin derivative **7**, which was obtained in the experiment with [2-^{13}C, ^{14}C]glycine, showed the expected enhancement of the natural abundance signal for C-2. This is consistent with specific utilisation 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 (Fig. 1); small amounts of enrichment (doublets) were also seen for C-3 plus C-4 and C-5 plus C-6 (*no* other signals were enriched, further attesting to the manifest specificity of the incorporation). Initially these results were 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 **8**, the tartronic semialdehyde pathway (Scheme 1) and the glyoxylate pathway (Scheme 2);[5] and relatively large amounts of glycine were present in our incubation mixture, sufficient to swamp any endogenous, unlabelled glycine present. Thus, to a large extent, the tartronic semialdehyde formed would be doubly labelled as shown in **9**. Glyoxylate subsequently generated in the glyoxylate pathway (Scheme 2) from acetyl coenzyme A **11** would also be doubly labelled. This accounts (Schemes 1 and 2; *cf.* ref. 5) for the equal intensity doublets associated with the signals for C-1 and C-2; also for C-3 plus C-4 and C-5 plus C-6 by incorporation through doubly labelled phosphoenol pyruvate **10** (Scheme 1).

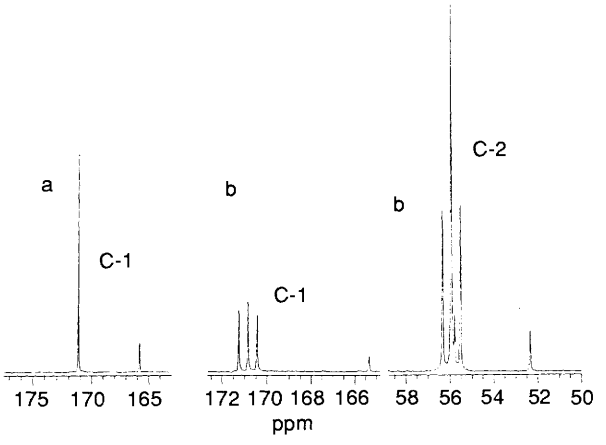
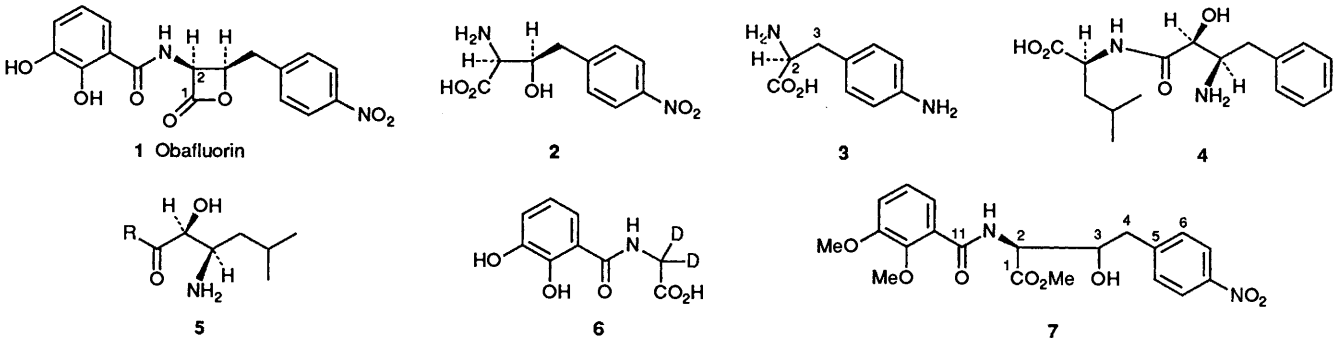
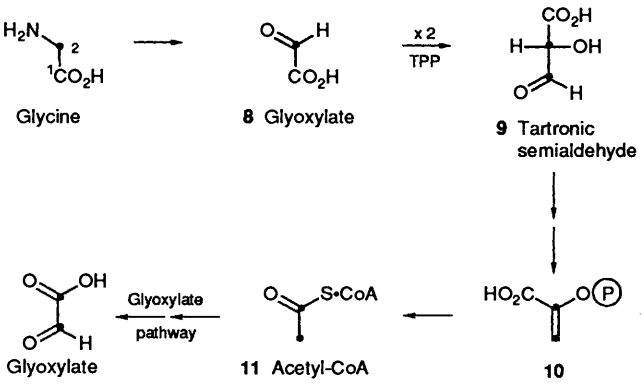


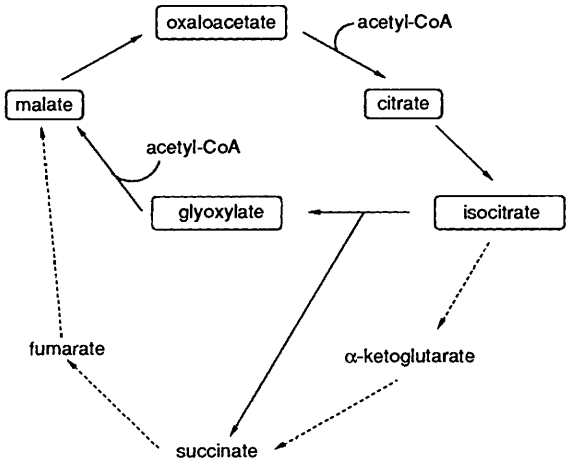
Fig. 1 Parts of the ^{13}C NMR spectra of the obafluorin derivative **7** following incorporation of: (a) [1-^{13}C]glycine; (b) [2-^{13}C]glycine, doublet: *J* 61.5

Combination of [2-^{13}C]glycine (glyoxylate) with endogenous *unlabelled* glyoxylate would give equal amounts of compound **12** and **13**. Glyoxylate subsequently generated would be

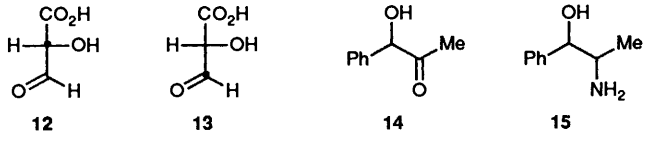




Scheme 1 The tartronic semialdehyde pathway



Scheme 2 The glyoxylate pathway



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 affirmed by results obtained for [1-^{13}C]glycine.

The ^{13}C NMR spectrum for **7** which was labelled by this material showed, as predicted, *only* simple enhancement (3.3 atom %) of the natural abundance signal for C-1 (Fig. 1). Label on the carboxy groups of glyoxylate is lost very rapidly on passage through the tartonic semialdehyde and glyoxylate pathways (compare particularly **8** and **11** in Scheme 1), 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 data not least the loss of all the deuterium from [2-$^{2}H_2$]glycine and loss from C-2 of deuteriated *p*-aminophenylalanine [2] leads to the process involving thiamine pyrophosphate (TPP) shown in Scheme 3. (We cannot exclude the possibility that deuterium loss from C-2 of deuteriated **3** occurs by adventitious transamination of **2** or **3** but since these are quite uncommon amino acids we do not think this is very likely.) Analogies are to be found in the TPP-mediated condensation in yeast of benzaldehyde with pyruvic acid which affords **14**, also the closely similar biosynthesis of ephedrine **15** in *Ephedra gerardiana*.[6] Further: (i) a similar pathway can be adduced (Scheme 4) for the biosynthesis of the β-lactone **18** which is elaborated like obafluorin by a *Pseudomonas* species; and (ii) the putative intermediates **19** and **20** (*cf.* **17**) can simply yield the β-amino-α-hydroxy acids which are, respectively, constituents of the antibiotics bestatin **4** [3] and amistatin **5**.[4] It is to be noted that, like the unit **2** in obafluorin, **4** and **5** have one more carbon atom than is present in the corresponding common α-amino acid. A similar origin may be adduced, with less immediate security, for the fragment **21** in the amiclenomycin antibiotics.[8]

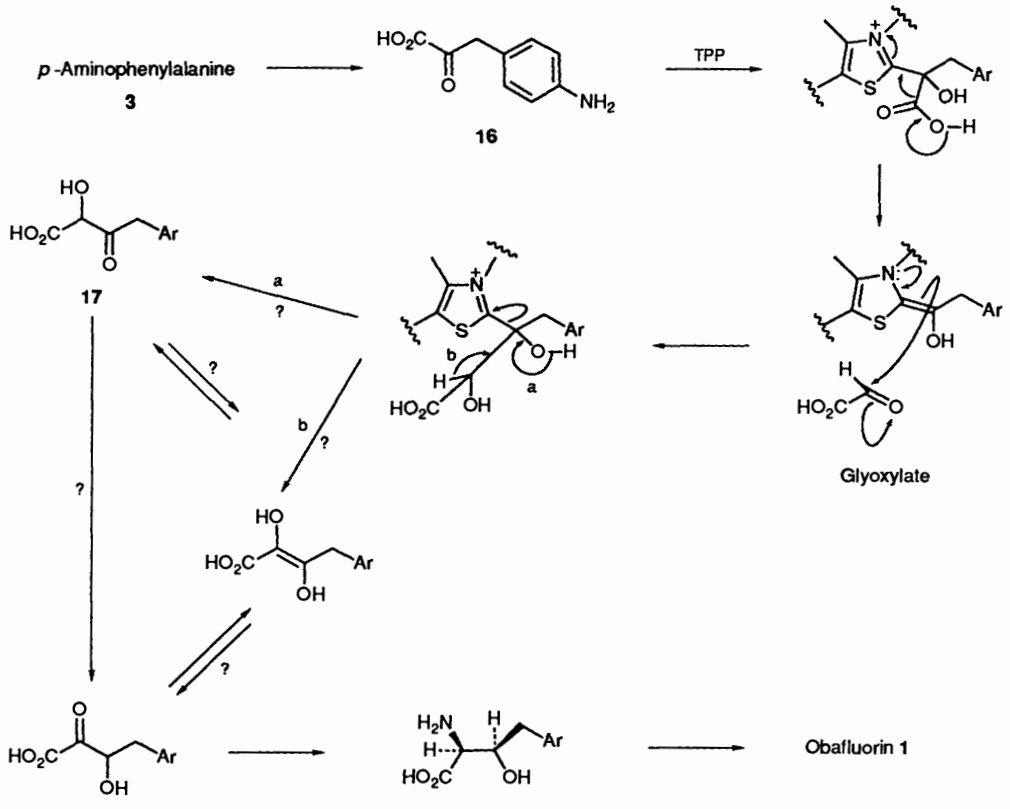
There are alternatives to Scheme 3 for obafluorin biosynthesis but they lack supporting analogies. The alternatives involve the aldehyde **22** instead of **16** or the TPP derivative of glyoxylate reacting with **16**/**22**.

A unit **23**, related to **2**, is found in the fungal antibiotic L-671,329 which is a cyclic hexapeptide. In **23** C-2 has been shown to arise from C-2 of acetate.[10] Our results do not permit derivation of **2** in obafluorin from glyoxylate/glycine by way of acetate [as discussed above good incorporation of label from C-1 of glycine was observed and this can only occur before any further metabolism to give, *e.g.* acetyl CoA (Scheme 1)]. However, incorporation of acetate *via* glyoxylate may be possible for **23** in L-671,329.

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 semialdehyde and glyoxylate pathways) for studying secondary metabolism.

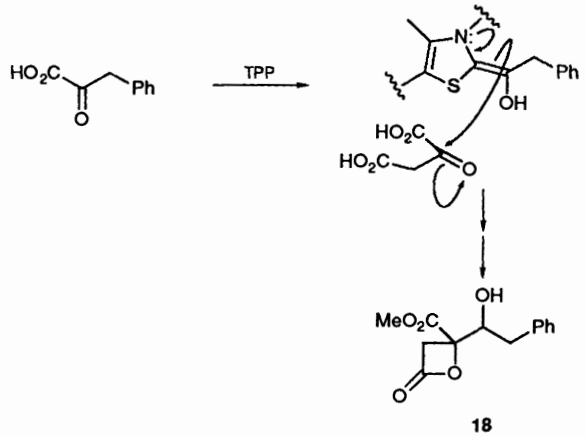
We considered that the analogues of **3**, namely DL-4-(4-aminophenyl)-2-aminobutanoic acid **24** and DL-(4-aminophenyl)-glycine **25**, could yield metabolites in *P. fluorescens* which might provide information on the biosynthesis of obafluorin **1**; the synthesis of these compounds is detailed in the Experimental section.

After feeding the amino acid **24** to cultures of *P. fluorescens* a small amount of a new metabolite was detected by HPLC; with 'resting' cells two minor components of very similar retention time could be detected. One compound in the two-component



Scheme 3

mixture was identified as **26** by ^{1}H NMR spectroscopy. It is clearly formed by condensation of **24** with 2,3-dihydroxybenzoic acid before or after oxidation of the aromatic amino group to a nitro group.



Scheme 4

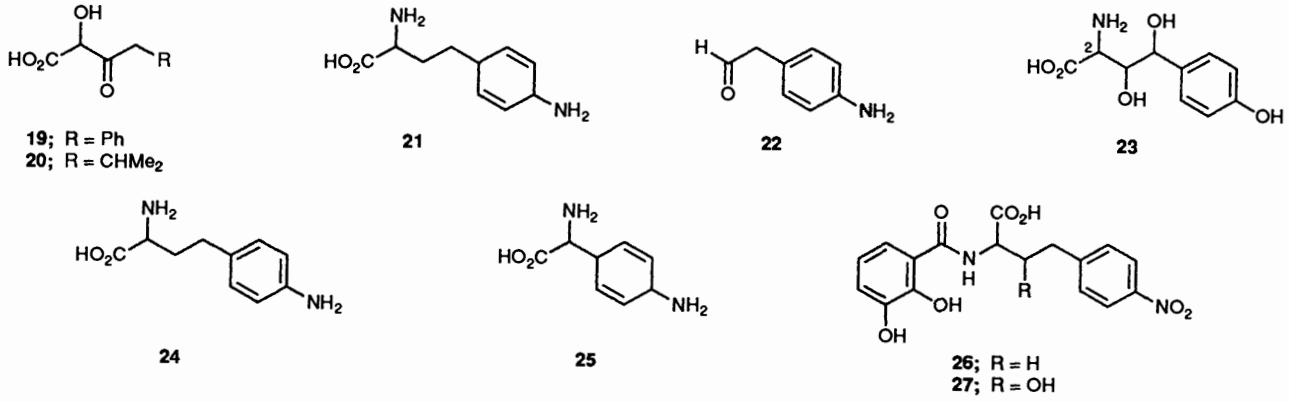
That the **27**, produced as a normal metabolite of *P. fluorescens*, does not derive from **24** by hydroxylation was confirmed when it was shown that the 2-deuterio derivative of **24** failed to label **27**.

The DL-(4-aminophenyl)glycine **25** failed to give any new, detectable metabolites in normal cultures or 'resting' cells.

Experimental

For general directions see refs. 9 and 2.

[2-$^2H_2$]-N-(2,3-*Dihydroxybenzoyl*)*glycine* **6**.—This compound was prepared[11] in three steps. (a) *Methyl* [2-$^2H_2$]*glycinate hydrochloride*.[11] To anhydrous methanol (10 cm^3) in ice was added dropwise thionyl chloride (1 cm^3). The solution was cooled to −10 °C and [2-$^2H_2$]glycine (473 mg) was added with stirring over 30 min. The mixture was stirred for a further 2.5 h at room temp., and then warmed at 50 °C for 30 min. The mixture was taken to dryness and the residue was recrystallised from water–ethanol; 63%, m.p. 176–182 °C (decomp.) (lit.,[13] 175 °C, decomp.); ν_{max}/cm^{-1} 3200–2700 and 1745 (Found: C, 28.55; H, 6.5; N, 10.9. Calc. for $C_3H_8ClNO_2$: C, 28.70; H, 6.42; N, 11.16%).



(b) *Methyl* [2-$^2H_2$]-N-(2,3-*dihydroxybenzoyl*)*glycinate*. To 2,3-dihydroxybenzoic acid (298 mg, 1.93 mmol) and the methyl glycinate hydrochloride (343 mg, 2.73 mmol) in dry THF (10 cm^3) under N_2, in ice, was added triethylamine (0.5 cm^3) and then over 5–10 min DCC (795 mg, 3.86 mmol) in dry THF (5 cm^3). The mixture was stirred for 18 h at 5 °C, and for 24 h at room temp. The mixture was then filtered and the precipitate was washed with THF (10 cm^3). The combined THF solutions were taken to dryness under reduced pressure and the residue was dissolved in chloroform (50 cm^3). The solution was washed with hydrochloric acid (0.5 mol dm^{-3}) and water, and then dried ($MgSO_4$) and evaporated to dryness under reduced pressure. The required product was obtained after purification by HPLC[2] (PLRP-S100, 300 × 25 mm, 4 cm^3 min^{-1}, 258 nm, MeCN–H_2O–TFA, 40:60:0.1) (41%); $\delta_{^2H}$(61.4 MHz, MeCN, 45 °C), 4.15 (br s, 2-D); on unlabelled sample: δ_H(90 MHz, CD_3COCD_3), 3.7 (3 H, s), 4.2 + 4.3 (2 H, conformational isomers), 6.9 (1 H, t, *J* 9), 7.1 (1 H, d, *J* 9), 7.4 (1 H, d, *J* 9), 8.6 (1 H, br s, NH) and 12.65 (1 H, br s, OH).

(c) [2-$^2H_2$]-N-(2,3-*Dihydroxybenzoyl*)*glycine*. A mixture of the methyl ester above (24.6 mg) in THF (2 cm^3) plus hydrochloric acid (2 mol dm^{-3}; 5 cm^3) was heated at 70 °C for 10 h and then allowed to cool to room temp. Extraction (EtOAc), drying and evaporation yielded the required product which was purified by HPLC (as above, MeCN–H_2O–TFA, 55:45:0.1), m.p. 209–211 °C; 60%; $\delta_{^2H}$(61.4 MHz, MeCN, 45 °C), 4.1 (br s, 2-D); deuterium content: $^2H_2$ = 100%.

Precursor Feeding Experiments.—Negligible incorporation was observed into obafluorin of the following precursors which were fed as aqueous solutions after 8 and 10 h growth of the *Pseudomonas fluorescens* cultures (10 × 100 cm^3): [2-$^2H_2$]-*N*-(2,3-dihydroxybenzoyl)glycine (26 mg); [2-^{14}C]glycine (6 μCi, 107 mg; 10 μCi, 1 g); L-[U-^{14}C]serine (12 μCi); [U-^{14}]pyruvic acid (28 μCi); [U-^{14}C]acetic acid (10 μCi, 96 mg). Negative results were obtained also with continuous addition of [2-^{14}C]glycine (10 μCi, 100 mg) over the period of metabolite production from 8 to 13 h and with an 8.5 and 10.5 h feed of [2-^{14}C]glycine (15 μCi, 95 mg) in the presence of chloramphenicol added after 8 h (2.5 mg per 100 cm^3) and 10 h (2.5 mg per 100 cm^3) with 10 × 100 cm^3 of culture.

In the experiments with **24** and **25** each compound (200 mg) was fed to 10 × 100 cm^3 of culture. In all cases metabolites were isolated after a total of 24 h growth except in the case of the continuous feed of glycine when this was after 13 h.

Preparation of Resting Cells of P. fluorescens.[14]—Growth/production cultures[2] of *P. fluorescens* (8 × 100 cm^3) were incubated for 10 h (25 °C, 300 rpm). The cells were sedimented by centrifugation (4 °C, 3000 rpm, 20 min) and the supernatant liquid was carefully decanted. The cell pellet was washed with MES buffer [4 °C, 200 cm^3, 50 mmol dm^{-3} MES = 2-(*N*-morpholino)ethanesulphonic acid, pH 6.5]. After centrifuging (4 °C, 3000 rpm, 20 min) the supernatant liquid was removed. The washing and centrifuging was repeated. Then the cell pellet was resuspended in MES buffer (pH 6.5, 400 cm^3), and aqueous solutions (pH 6.5) of the labelled samples of glycine plus 2,3-dihydroxybenzoic acid (37 mg) and DL-*p*-aminophenylalanine (*ca.* 100 mg) were added. Incubation (300 rpm, 25 °C) in 4 flasks was for 13 h and then the metabolites were isolated as before[2] for cultures grown for 24 h. The samples of glycine used were: [2-^{14}C] (14 μCi, 37.2 mg); [2-$^2H_2$] (98 atom %, 92.2 mg); [2-^{13}C, ^{14}C] (14.6 μCi, 95 atom %, 95.7 mg); [1-^{13}C, 2-^{14}C] (7.3 μCi, 99 atom %, 98.8 mg). Total atom % enrichment of **7** by [2-^{13}C]glycine: C-1, 6.9; C-2, 12.9; C-3, 2.1; C-4, 0.6; intensities for C-5 and C-6 too low for meaningful measurement.

In the work with **24** and **25** each (200 mg) was added in separate experiments plus 2,3-dihydroxybenzoic acid (47 mg) plus glycine (111 mg).

DL-4-(4-*Aminophenyl*)-2-*aminobutanoic Acid* **24**.—(a) 2-(4-*Nitrophenyl*)*ethanol*. To *p*-nitrophenylacetic acid (670 mg, 3.4 mmol) in dry carbon tetrachloride (10 cm^3) was added thionyl chloride (3 cm^3). The mixture was refluxed for 4 h after which it was cooled and the excess of thionyl chloride and carbon tetrachloride were removed under reduced pressure to leave a pale yellow solid (ν_{max}/cm^{-1} 1790, 1520, 1350). This was dissolved in dry THF (30 cm^3) and to the stirred solution was added, over 10 min, lithium borohydride (150 mg, 6.8 mmol). The mixture was stirred at room temperature for 1 h and then cooled in ice; the excess of reagent was then destroyed by the dropwise addition of water, followed by 2 mol dm^{-3} hydrochloric acid. The required alcohol was extracted into CH_2Cl_2 and the solution was washed with water, dried ($MgSO_4$) and evaporated. The crude product was purified by preparative HPLC[2] (Me_3CN–H_2O–TFA, 55:45:0.1) (85%). For spectral data see ref. 2.

(b) 2-(4-*Nitrophenyl*)*ethyl bromide* (*cf.* ref. 2). To a solution of the alcohol above (2.1 g, 12.3 mmol) in dry benzene (50 cm^3) was added carbon tetrabromide (4.5 g, 13.6 mmol), followed over 10 min by triphenylphosphine (7.1 g, 27 mmol). After being stirred for 30 min at room temperature the mixture was taken to dryness under reduced pressure. The bromide was purified by chromatography on silica (ether–hexane, 1:4) (69%); it had m.p. 65 °C (from benzene–hexane) (lit.,[15] 69–70 °C); δ_H(90 MHz, $CDCl_3$) 3.3 (2 H, m), 3.6 (2 H, m), 7.38 (2 H, d, *J* 9) and 8.18 (2 H, d, *J* 9) (Found: C, 41.75; H, 3.45; N, 5.95%. Calc. for $C_8H_8BrNO_2$: C, 41.77; H, 3.50; N, 6.09%).

(c) *Diethyl* 2-*acetamido*-2-(4-*nitrophenyl*)*malonate*. A mixture of diethyl acetamidomalonate (1.15 g, 5.3 mmol) in ethanolic sodium ethoxide (27 cm^3, 5.5 mmol) was brought to reflux. The heat was removed and a solution of the above bromide (1.15 g, 5 mmol) in dry dioxane (15 cm^3) was added rapidly. The mixture was refluxed for 90 min and allowed to cool to room temperature. The solvent was then removed under reduced pressure and the product was purified by chromatography on silica (ether) (85%), m.p. 115–115.5 °C (from benzene–hexane); ν_{max}/cm^{-1} 3300, 1760, 1660, 1535 and 1370; δ_H(400 MHz, CD_3OD), 1.28 (6 H, t, *J* 7), 2.03 (3 H, s), 2.66 (4 H, m), 4.93 (1 H, br s, NH), 7.44 (2 H, d, *J* 9) and 8.1 (2 H, d, *J* 9) (Found: C, 55.5; H, 5.95; N, 7.65. $C_{17}H_{22}N_2O_7$ requires C, 55.7; H, 6.05; N, 7.64%).

(d) DL-4-(4-*Nitrophenyl*)-2-*aminobutanoic acid*. A mixture of the malonate above (1.46 g, 4 mmol) in glacial acetic acid (5 cm^3) and 6 mol dm^{-3} hydrochloric acid (10 cm^3) was carefully refluxed for 4.5 h. The mixture was allowed to cool to room temperature and then washed twice with diethyl ether. The pH of the aqueous solution was adjusted to 5 and the volume of the solution was reduced under reduced pressure to 10 cm^3. The precipitate was collected, m.p. 207–208 °C (decomp.) (from water) (36%); ν_{max}/cm^{-1} 3300–2300, 1595, 1515 and 1350; δ_H(300 MHz, NaOD/D_2O), 1.72 (2 H, m), 2.56 (2 H, m), 3.17 (1 H, m), 7.25 (2 H, d, *J* 10) and 7.95 (2 H, d, *J* 10); δ_C(100 MHz, NaOD/D_2O, TMSP as ref.) 34.30, 38.93, 58.42, 126.31, 132.01, 148.32, 153.55 and 185.15 (Found: C, 53.7; H, 5.35; N, 12.55. $C_{10}H_{12}N_2O_4$ requires C, 53.6; H, 5.39; N, 12.49%).

(e) DL-4(4-*Aminophenyl*)-2-*aminobutanoic acid* **24**. This compound was prepared by hydrogenation of the nitro compound (262 mg) in water (20 cm^3) plus conc. hydrochloric acid (10 drops) in the presence of 5% palladium on barium sulfate (150 mg) at atmospheric pressure and room temperature for 5 h; ν_{max}/cm^{-1} 3550–1990 and 1600; δ_H(300 MHz, D_2O, TMSP as ref.) 2.13 (2 H, m), 2.68 (2 H, m), 3.78 (1 H, t, *J* 6), 7.03 (2 H, d, *J* 7.5) and 7.25 (2 H, d, *J* 7.5); δ_C(100 MHz, D_2O, TMSP as ref.), 32.82, 35.34, 57.33, 121.93, 132.36, 138.10, 141.22 and 177.38; *m*/*z* (CI, NH_3), 197 (M^+ + 3), 196 (M^+ + 2) and 195 (M^+ + 1, 100%).

The 2-^{2}H-labelled compound could be prepared by use of 48% DBr in dioxane (1:1) in step d ($^2H_1$ = 88%).

D-(4-*Aminophenyl*)*glycine* **25**.—(a) *Ethyl* 2-(4-*nitrophenyl*)-2-*oximinoacetic Acid*.—To methyl *p*-nitrophenylacetate (1.32 g, 6.3 mmol) in THF–ethanol (1:2; 15 cm^3, anhydrous), was added ethyl nitrite (15% solution in ethanol; 5 cm^3) followed by the rapid addition of ethanolic sodium ethoxide (13 mmol). The coloured mixture was stirred at room temperature for 30 min and then acidified with dilute sulfuric acid. The product was extracted into ether and the solution was worked up, m.p. 181.9–182.5 °C (from benzene–methanol) (65%); ν_{max}/cm^{-1} 3240, 1715, 1515 and 1345 cm^{-1}; δ_H(90 MHz, NaOD/D_2O, TMSP as ref.) 1.05 (3 H, t, *J* 8), 3.5 (2 H, q, *J* 8), 7.3 (2 H, d, *J* 9) and 8.15 (2 H, d, *J* 9); *m/z* 238.059 24 (M^+; $C_{10}H_{10}N_2O_5$ requires 238.058 97).

(b) *Ethyl* DL-(4-*aminophenyl*)*glycinate*. The above oxime (414 mg) in ethanol–conc. hydrochloric acid (96:4; 50 cm^3) was hydrogenated at atmospheric pressure and room temperature in the presence of 10% palladium on charcoal (210 mg) for 2 h. The desired product as the dihydrochloride crystallised from solution after filtration, reduction in volume to 5 cm^3 and overnight refrigeration (68%), m.p. 192–196 °C (from water); ν_{max}/cm^{-1} 3100 and 1750 cm^{-1}; δ_H(300 MHz, D_2O), 1.1 (3 H, t, *J* 7), 4.2 (2 H, m), 5.3 (1 H, s), 7.48 (2 H, d, *J* 10) and 7.57 (2 H, d, *J* 10); δ_C(75 MHz, D_2O, TMSP as ref.) 15.96, 58.66; 67.04, 126.58, 132.82, 134.01, 135.97 and 171.40; *m/z* 194 (M^+, 1.6%) and 121 ($M^+ - CO_2Et$, 100%).

(c) DL-(4-*Aminophenyl*)*glycine* **25**. The above ester dihydrochloride (99.5 mg) was dissolved in 2 mol dm^{-3} hydrochloric acid (5 cm^3) and the solution was kept at 50 °C for 24 h. The pH was adjusted to 7 and the solution was lyophilized; ν_{max} 3700–2700 and 1620; δ_H(300 MHz, D_2O, pH 7), 5.01 (1 H, s), 7.18 (2 H, d, *J* 7.5) and 7.52 (2 H, d, *J* 7.5); δ_C(75 MHz, D_2O, TMSP as ref.), 61.18, 119.20, 129, 89, 131.31, 149.21 and 179.01.

Acknowledgements

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