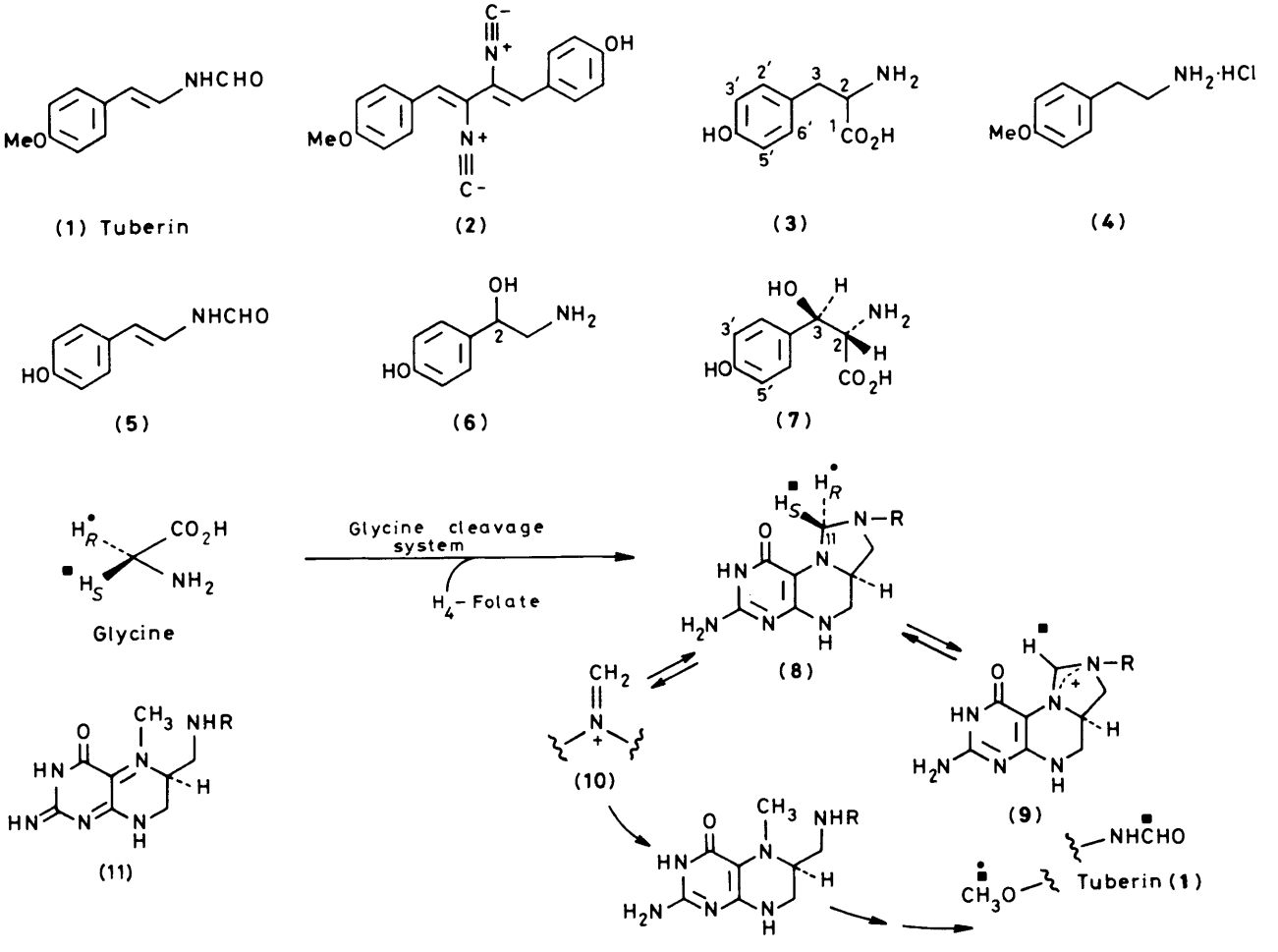
The Biosynthesis of Tuberin from Tyrosine and Glycine; Observations on the Stereochemistry Associated with the Conversion of Glycine through Methylenetetrahydrofolate into Methenyltetrahydrofolate

Karl M. Cable, Richard B. Herbert,* and Jonathan Mann
Department of Organic Chemistry, The University, Leeds LS2 9JT

Tuberin (**1**) is shown to derive from glycine by way of tetrahydrofolate metabolism and from tyrosine. Glycine is incorporated into the *N*-formyl group of tuberin with (partial) stereospecific retention of the 2-*pro-S* proton; there is also some non-stereospecific loss of deuterium label from [2-^{13}C, 2-$^{2}H_2$]glycine on incorporation into the C_1 units present in tuberin. [2-^{14}C]-*threo*-3-Hydroxytyrosine [as (**7**)] is only incorporated into tuberin after degradation; exclusive labelling of the two C_1 units was observed. [2-^{3}H]Octopamine [as (**6**)] is a poor precursor for tuberin.

Tuberin (**1**) is a metabolite of *Streptomyces amakusaensis*.[1-3] Its structural resemblance to some naturally occurring isonitriles, *e.g.* xanthocillin-X-monomethyl ether (**2**),[4] suggested that knowledge of the biosynthesis of tuberin (**1**) might provide insight into the intriguing mystery of microbial isonitrile biosynthesis.[5-8] We have also used tuberin as a convenient substrate for studying aspects of stereochemistry associated with microbial C_1-tetrahydrofolate metabolism. Some of our results have appeared in preliminary form.[5,6]

L-[U-^{14}C]Tyrosine [as (**3**)] served as an outstandingly good precursor for tuberin but [1-^{14}C]tyrosine was poorly incorporated. It follows that tyrosine is used to construct the major part of the tuberin skeleton with loss of the carboxy group. In spite of the high incorporation of [U-^{14}C]tyrosine we found to our surprise, in experiments with L-[U-^{14}C, 3′,5′-$^{3}H_2$]- and L-[U-^{14}C, 2′,6′-$^{3}H_2$]-tyrosine [as (**3**)], that some degradation of the precursor had occurred: there was a decrease in the ratio of ^{3}H to ^{14}C of 17—45% in the tuberin isolated (in



Scheme 1.

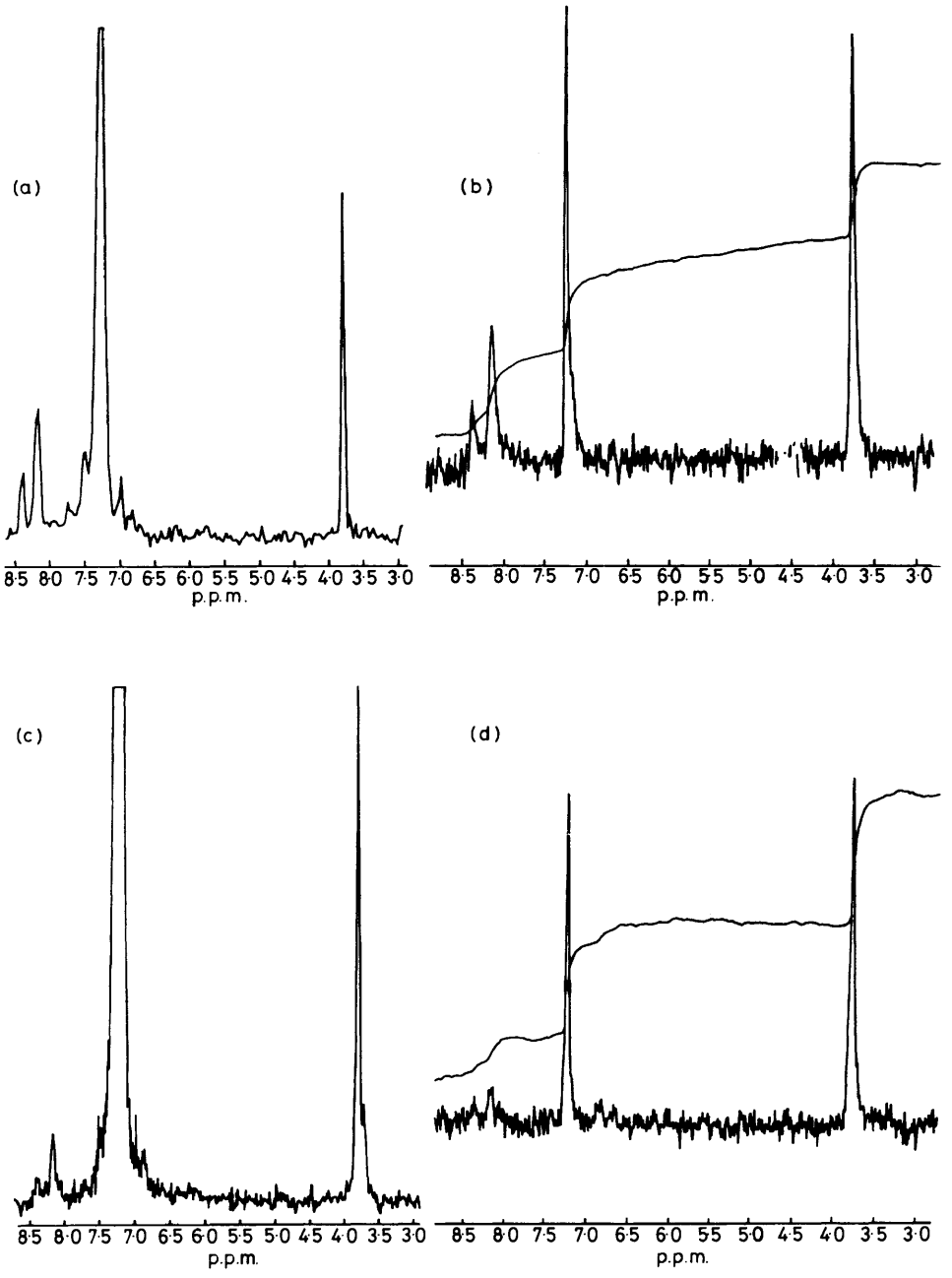


Figure. ^{2}H N.m.r. spectra of tuberin (**1**) in $CHCl_3$ ($CDCl_3$ reference) at 61.4 MHz and at 45 °C; (a) and (b) material obtained from (2*S*)-[2-^{2}H]glycine; (c) and (d) material obtained from (2*R*)-[2-^{2}H]glycine in two of three parallel sets of experiments.

calculations here, and below, associated with mixed isotopic labelling of tyrosine, allowance was made for the loss of one ninth of the ^{14}C activity arising from loss of the carboxy carbon atom). That tritium was not being lost by exchange *ortho* to the phenolic hydroxy group in [3′,5′-$^{3}H_2$]tyrosine during biosynthesis was confirmed in a feeding experiment with [U-^{14}C, 3′,5′-$^{2}H_2$]tyrosine (84% $^{2}H_2$ species). The tuberin obtained was shown by mass spectrometry to contain a significant amount of dideuteriated species but $\leqslant 1\%$ of monodeuteriated species.

The tuberin from this last experiment was degraded to (**4**). The specific incorporation of ^{14}C label into (**1**) was higher (10.9%) than that of deuterium (7.5%) but the specific incorporation of ^{14}C measured on (**4**) was similar (7.8%) to that of deuterium in (**1**). These differences in specific incorporation are accounted for by catabolism of some of the tyrosine leading to incorporation of ^{14}C-label (15%) into the *N*-formyl group of (**1**). Degradation of tuberin to (**5**) was without loss of radioactivity, *i.e.* there was no detectable radioactivity in the *O*-methyl group of tuberin (**1**).

A reasonable hypothesis for the introduction of the double bond which is present in tuberin (**1**) could involve introduction of a hydroxy group at C-3 of tyrosine or tyramine. To this end we tested [2-^{3}H]octopamine [as (**6**)] and (2*SR*,3*RS*)-[2-^{14}C, 3′,5′-$^{2}H_2$]-*threo*-3-hydroxytyrosine [as (**7**)] as precursors for tuberin. The former compound was not significantly incorporated but radioactivity in the latter was satisfactorily incorporated into (**1**) albeit at a lower level than tyrosine. However, the tuberin isolated was devoid of deuterium and degradation to (**5**) and to (**4**) revealed that all the radioactivity was present in the *O*-methyl (40%) and the *N*-formyl (60%) groups. Thus the *threo*-3-hydroxytyrosine serves only as a source for the two C_1 units in tuberin.

It is known that glycine can serve as a C_1 source through methylenetetrahydrofolate[9,10] and has been noted, for example, to do so recently in the biosynthesis of another *Streptomyces* metabolite, naphthyridinomycin.[11] This suggests that labelled *threo*-3-hydroxytyrosine (**7**) is degraded by a reverse-aldol reaction to give [2-^{14}C]glycine which then labels the C_1 units in (**1**). This hypothesis is supported by other results discussed below and also by the observed conversion of *threo*-3-hydroxytyrosine (**7**) (but not the *erythro*-isomer) into *p*-hydroxybenzaldehyde with a cell-free preparation of *S. amakusaensis*.[7] (3-Hydroxytyrosine is chemically stable under the conditions of the feeding experiment.) The partial degradation of tyrosine noted above may also be explained as being by catabolism through *threo*-3-hydroxytyrosine and glycine into methylenetetrahydrofolate. The apparent failure of the [U-^{14}C]tyrosine to label the *O*-methyl group, in contrast to the *N*-formyl group in (**1**), is, however, puzzling.

[2-^{14}C]Glycine was a satisfactory precursor for tuberin. Assay of the radioactivity in the degradation products (**4**) and (**5**) showed that 38% of the radioactivity was present in the *O*-methyl group and 56% in the *N*-formyl group of (**1**), which is a very similar distribution to that found for the tuberin derived from *threo*-3-hydroxytyrosine (above); this strengthens the conclusions regarding catabolism of this compound (**7**). The result with [2-^{14}C]glycine was confirmed in a feeding experiment with [2-^{14}C, 2-^{13}C]glycine. The ^{13}C n.m.r. spectrum of the tuberin isolated showed enrichment of the signals for the *O*-methyl and *N*-formyl groups (3.1 and 3.4%, respectively); the extent of total labelling by ^{14}C and ^{13}C was similar.

Although formic acid can also serve as a C_1 source linked to tetrahydrofolate metabolism exogenous [^{14}C]formate failed to act as a precursor for tuberin. Neither [U-^{14}C]pyruvate nor [2-^{14}C]acetate were significantly incorporated into tuberin either.

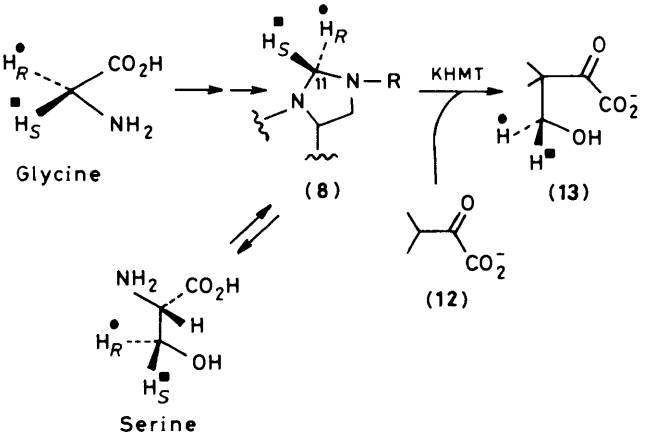
The foregoing results with labelled glycine indicate firmly that the two C_1 units present in tuberin originate in glycine by way of normal metabolism involving the important coenzyme, tetrahydrofolic acid. The *O*-methyl group of (**1**) is at the lowest oxidation level associated with tetrahydrofolate metabolism, whilst the *N*-formyl group is at the highest oxidation level. Tuberin provides then a useful metabolite with which to explore aspects of tetrahydrofolate metabolism. In particular, we were interested to observe the fate of the two enantiotopic protons on C-2 of glycine during the overall conversion of glycine into methylenetetrahydrofolate[9,10] and the subsequent biosynthesis of tuberin (**1**).

In two experiments with a mixture of [2-^{14}C]- and [2-$^{2}H_2$]-glycine the specific incorporation of ^{14}C into tuberin was higher than that of deuterium which was also somewhat variable (^{14}C: 7.4 and 6.8%; ^{2}H: 4.3 and 1.7%), *i.e.* more deuterium than expected was being lost from C-2 of glycine during biosynthesis. That this was not due to a deuterium isotope effect, which would favour consumption of ^{14}C-labelled material, was apparent in experiments with [2-^{13}C, 2-$^{2}H_2$]glycine (90% $^{2}H_2$, 90% ^{13}C). The ^{13}C n.m.r. spectrum of the tuberin which was obtained showed: (*a*) substantially enhanced natural abundance singlets for the carbon atoms of the *N*-formyl and *O*-methyl groups, and for these atoms only; (*b*) a triplet associated with each of these signals shifted upfield by 0.16 and 0.27 p.p.m., respectively, *i.e.* for each group there was a ^{13}C species with a single deuterium attached: (*c*) a quintet for the *O*-methyl group (upfield shift 0.54 p.p.m.), *i.e.* ^{13}C species with two deuterium atoms attached. Thus, deuterium was lost during biosynthesis. Samples of (2*R*)- and (2*S*)-[2-^{2}H]glycine mixed with [2-^{14}C]glycine each gave labelled tuberin (**1**). The ^{2}H n.m.r. spectrum of tuberin derived from (2*S*)-[2-^{2}H]-glycine showed signals for the *O*-methyl and *N*-formyl groups (Figure 1a and 1b). The tuberin derived from (2*R*)-[2-^{2}H]glycine showed ^{2}H n.m.r. signals for the *O*-methyl, and at low intensity, for the *N*-formyl group (Figure 1c and 1d); the label in the *S*-isomer was incorporated at least 3 to 4 times better than that in the *R*-isomer. This indicates that in the transformation of glycine through methylenetetrahydrofolate (**8**) into methenyltetrahydrofolate (**9**) and thence into the *N*-formyl group of tuberin the 2-*pro-S* proton of the amino acid is retained with (partial) stereospecificity. (We had previously reported[6] that the 2-*pro-R* proton of glycine was retained in this process. Results of further experiments revealed that we had made a mistake in the interpretation of the original ^{2}H n.m.r. spectra. With acetone as solvent a so-far inexplicable signal very close to that expected for the *N*-formyl group appeared on long accumulation of f.t. data. This problem does not arise with chloroform as solvent and all our later data have been obtained using this solvent.)

A principal source of C_1 units in a wide variety of organisms is provided through catabolism of glycine by the glycine cleavage system.[9,10] Three proteins are involved in the process whereby C-2 of the amino acid is transferred to tetrahydrofolic acid to form 5,10-methylenetetrahydrofolate (**8**) [C-2 of glycine becomes C-11 of (**8**)]. Further metabolism then affords C_1 units at different levels of oxidation. Dehydrogenation of 5,10-methylenetetrahydrofolate (**8**) gives 5,10-methenyltetrahydrofolate (**9**) which provides C_1 units at the formic acid level of oxidation. The 11-*pro-R* proton in (**8**) is removed in this dehydrogenase reaction (in liver).[12] The partially stereospecific retention of the 2-*pro-S* proton of glycine in the *N*-formyl group of tuberin (**1**) in *S. amakusaensis* shows that this proton becomes the 11-*pro-S* proton in (**8**). (In this conclusion the reasonable assumption is made that the stereochemical course in liver and *Streptomyces* is the same.)

Serine hydroxymethyltransferase (SHMT) catalyses the (reversible) conversion, in the presence of tetrahydrofolic acid, of serine into glycine which also affords 5,10-methylenetetrahydrofolate (**8**) [C-3 of serine becomes C-11 of (**8**)]. With SHMT from liver, the reaction is only partially stereospecific and under conditions of multiple turnover complete loss of stereochemical identity of the chiral label originally present at C-3 of serine may occur.[13]

Ketopantoate hydroxymethyltransferase (KHMT) catalyses the transfer of a methylene group from (**8**) to α-ketoisovaleric acid to give ketopantoate (**13**). Serine chirally deuteriated on C-3 has been found to undergo reaction sequentially in *Escherichia coli* with SHMT and KHMT to give (**13**) with partial stereospecificity (*ca.* 75%); loss of stereochemical identity apparently occurs again in the SHMT reaction. Overall the reaction has the same stereochemical course as the one catalysed by liver SHMT, *i.e.* as shown in Scheme 2,[14] provided that the stereochemistry associated with SHMT in liver and *E.*



Scheme 2.

coli is the same. Glycine also serves (*via* the glycine cleavage system and KHMT) as a source of the hydroxymethyl group in ketopantoate. Experiments in *E. coli* with chirally deuteriated glycine show that the overall reaction is ⩾78% stereospecific.[15] Based on the conclusions reached with the serine to ketopantoate conversion the stereochemistry of the glycine conversion follows as that shown in Scheme 2.[15] It can be seen that the 2-*pro-S* proton in glycine becomes the 11-*pro-S* proton in (**8**), which is the same conclusion reached in the study of tuberin biosynthesis using the chirally deuteriated glycines, above. A self-consistent pattern is thus apparent from the combined results.

In the conversion of glycine into tuberin (*S. amakusaensis*) and into ketopantoate (*E. coli*) some loss of stereochemical identity at C-2 of the amino acid was observed. Presumably these observations are again to be associated with the action of SHMT (*cf.* ref. 15). In addition other reactions leading to exchange of the C-2 protons of glycine were observed. In the case of *E. coli*[15] this involved loss of the 2-*pro-S* proton in glycine. In the case of *S. amakusaensis* our results with [2-^{13}C, 2-$^{2}H_2$]-glycine show that both protons may be lost from C-2 of glycine during metabolism culminating in tuberin biosynthesis. The glycine cleavage system has been reported to function without loss of the C-2 hydrogens in glycine,[10] so this does not account for what must then be reactions not directly involved in the conversion of glycine into methylenetetrahydrofolate (**8**). We tentatively concluded previously that exchange could occur through the equilibration of (**10**) and (**11**) on the basis of earlier information[16] but this possibility is excluded by the results of later work.[17]

The conclusion above, that the 2-*pro-S* proton in glycine is retained with (partial) stereospecificity through the tetrahydrofolate derivatives (**8**) and (**9**) into the *N*-formyl group of tuberin (**1**), is not affected by the observation of additional, stereorandom exchange at C-2 of glycine. This is because the comparison of the incorporations of deuterium label from the (2*R*)- and (2*S*)-[2-^{2}H]glycine samples into the *N*-formyl group of (**1**) was made by reference in each case to the level of deuterium labelling in the *O*-methyl group (the two C_1 units were shown, above, to be labelled to a similar extent by [2-^{13}C]- and [2-^{14}C]-glycine). A further check was provided by reference to the natural abundance signal for deuterium in the chloroform solutions used to obtain the ^{2}H n.m.r. spectra: in each pair of experiments the signal due to the methoxy group was of similar relative intensity (Figure 1b and 1d) when compared with that due to $CDCl_3$ (this is, further, consistent with stereorandom exchange).

Further evidence on the biosynthesis of tuberin *via* tetrahydrofolate metabolism was obtained in an experiment with L-[3-^{14}C]serine. This precursor was well incorporated and all the activity was confined to the *O*-methyl and *N*-formyl groups, being approximately equally distributed between them.

The above results with deuteriated glycine establish incidentally that the *N*-formyl group cannot arise *via* an isonitrile function.

Experimental

M.p.s were measured on a hot stage and are uncorrected. N.m.r. spectra were measured either on a JEOL FX90Q (90 MHz) or on a Bruker AM400 (400 MHz) spectrometer, with tetramethylsilane as an internal standard. ^{13}C N.m.r. spectra were obtained with added chromium acetylacetonate (20 mg ml^{-1}) to assist relaxation. ^{2}H N.m.r. spectra were obtained at 61.4 MHz in chloroform ($CDCl_3$ reference) at 45 °C.

All solvents were dried over magnesium sulphate. Chromatography was carried out on Merck Kieselgel 60G.

Compounds labelled with stable isotopes were purchased either from MSD Isotopes or the Aldrich Chemical Co. Ltd. Deuterium oxide contained 99.8 atom % deuterium (Aldrich). Radioactive compounds were purchased from Amersham International. Radioactivity was assayed using a Packard 300CD scintillation counter. Radioactive samples of tuberin were crystallized to constant activity. Enzymes were bought from the Sigma Chemical Co. Ltd.

Culturing of Streptomyces amakusaensis, *Isolation of Tuberin, and Method of Feeding.*—A culture of *Streptomyces anakusaensis*[1] (ATCC 23876) was obtained from The American Type Culture Collection, 12301, Parklawn Drive, Rockville, Maryland 20852-1776, U.S.A. It was maintained at 0—5 °C on slants containing: Difco Bacto yeast extract (4 g), Difco Bacto malt extract (10 g), glucose (4 g), distilled water (1 l), and agar (20 g), pH 7.3 (*cf.* ref. 18). Innoculation was into the same medium (50 ml in 250 ml conical flasks) without agar. The culture was incubated at 27 °C and 160 r.p.m. for 72 h. This culture was used to inoculate, at 2.5%, the production medium (200 ml in 500 ml conical flasks): soluble starch (**15** g), glucose (5 g), soyabean meal (10 g), Oxoid Lab-Lemco powder (5 g), sodium chloride (5 g), dipotassium hydrogen phosphate (400 mg), and distilled water (1 l), pH 7.0. Incubation was at 27 °C and 160 r.p.m. Assay for tuberin was by extraction of the culture with ethyl acetate; the extract was dried and evaporated; the initiation of tuberin production was marked by the appearance of u.v. absorption at 285 nm in ethanol. Typically the production of tuberin commenced after *ca.* 45 h, reaching a maximum after *ca.* 62 h. The cultures were harvested after *ca.* 68 h. Centrifugation of the cultures gave a clear supernatant liquid which was extracted thrice with 1/5 volumes of ethyl acetate; the solid residue contained no tuberin. The extracts were dried, and evaporated to dryness to leave a brown oil which gave tuberin (80 mg l^{-1}) on chromatography (1% methanol in chloroform). The tuberin was crystallized (benzene), m.p. 132—133 °C (lit.,[1] m.p. 132—133 °C); $\lambda_{max.}$(EtOH) 219 and 285 nm; $\nu_{max.}$(Nujol) 3 200, **1** 640, 1 600, and 962 cm^{-1}; δ_H [400 MHz; $CDCl_3$; because two isomers of tuberin are present resulting from restricted rotation associated with the amide function two resonances were observed for some of the protons; coalescence was observed at 90 °C; the isomers are present in an approximate ratio of 3 to 1; the minor isomer showed (*trans*) coupling between NH and CHO; *cf.* ref. 19] 3.80 (3 H, s), 6.05 and 6.15 (1 H, each a doublet, *J* **14**.5 H), 6.85 and 7.26 (4 H, aromatic AA′BB′ system), 7.40 and 7.43 (1 H, each a doublet, *J* **14**.5 Hz), and 8.19 (s) and 8.40 (d, *J* **11**.6 Hz) (1 H, CHO); δ_C (hexadeuterioacetone) 55.8 (OCH_3), 115.2, 116.0, **120**.0, 127.9, 129.9, 160.3, and 160.9 (CHO); *m/z* 177.0789 (M^+) ($C_{10}H_{11}NO_2$ requires *M*, 177.0789) 148, 134, and 121.

Precursors were dissolved in water (pH 7) and administered to production cultures of *S. amakusaensis* (3 flasks) in five batches over 15 h commencing when tuberin production had just begun. Isolation of tuberin was as above.

Demethyltuberin (**5**).—To a stirred solution of tuberin (50 mg) in dry dichloromethane (2 ml) at −78 °C was added boron tribromide (250 mg). The mixture was stirred at −78 °C for 30 min and then allowed to warm to room temperature over 30 min. It was added to ice-cold water (5 ml) and this mixture was extracted with ethyl acetate (3 × 5 ml). The combined extracts were dried and the solvent was removed under reduced pressure. The residue was crystallized from ethyl acetate to give *demethyltuberin* (**5**) (43 mg, 95%) as colourless plates, m.p. 175—177 °C; $\lambda_{max.}$(EtOH) 219 and 285 nm; $\nu_{max.}$(Nujol) 3 200, 1 640, 1 610, and 1 590 cm^{-1}; δ_H(CD_3OD) 6.2 (1 H, d, *J* 15 Hz), 6.7 (2 H, d, *J* 9 Hz), 7.1 (2 H, d, *J* 9 Hz), 7.25 (1 H, d, *J* 15 Hz), and 8.0 (1 H, s); *m/z* 163.0631 (M^+) ($C_9H_9NO_2$ requires *M*, 163.0633),

135, 118, 107 and 77. (Found: C, 66.3; H, 5.45; N, 8.7. $C_9H_9NO_2$ requires C, 66.24; H, 5.56; N, 8.58%).

p-*Methoxyphenethylamine Hydrochloride* (**4**).—This compound was prepared from tuberin (50 mg) as described [2] in 68% yield, m.p. 213—214 °C (methanol–ether) (lit.,[2] 216—217.5 °C) (Found: C, 57.55; H, 7.75; Cl, 18.9; N, 7.35. Calc. for $C_9H_{14}ClNO$: C, 57.6; H, 7.52; Cl, 18.89; N, 7.47%).

(R,S)-[2-^{3}H]*Octopamine Hydrochloride*.—Bromination of 4-hydroxyacetophenone gave ω-bromo-4-hydroxyacetophenone [20] which on treatment with hexamethylenetetramine [21] gave ω-amino-4-hydroxyacetophenone hydrochloride, m.p. 248—250 °C in 7.5% overall yield; $\delta_H(CD_3OD)$ 4.1 (2 H, s) and 7.5 (4 H, m) (Found: C, 51.3; H, 5.3; Cl, 18.7; N, 7.5. Calc. for $C_8H_{10}ClNO_2$: C, 51.1; H, 5.35; Cl, 18.9; N, 7.8%).

ω-Amino-4-hydroxyacetophenone hydrochloride (50 mg, 0.265 mmol) was dissolved in methanol (2 ml) and the pH was adjusted to 7 with aqueous ammonia. The mixture was stirred and sodium borotritide (2.5 mg, 0.065 mmol, 592 mCi mmol^{-1}) was added under nitrogen. After 30 min sodium borohydride (25 mg, 0.65 mmol) was added; stirring was continued for a further 1.5 h. The mixture was acidified with conc. hydrochloric acid and then evaporated to dryness. The residue was dissolved in ethanol (1 ml) and the solution was filtered. Addition of ether to the filtrate gave (*R,S*)-[2-^{3}H]octopamine hydrochloride. The product was purified on a 3MM paper chromatogram (9:4:2:0.2, butanol:ethanol:water:2M aqueous ammonia) using authentic (*R,S*)-octopamine hydrochloride (Sigma Chemical Co. Ltd.) as reference (purple colour with ninhydrin). The band of radioactive octopamine hydrochloride (25 mg, 56%) was eluted from the chromatogram with water. Its R_F on paper chromatography (above solvent) and m.p. (169—171 °C) were identical with those of authentic material. Autoradiography showed that all the radioactivity was associated with the octopamine hydrochloride (13.7 μCi mg^{-1}).

(2SR,3RS)-threo-3-*Hydroxytyrosine* (**7**) *and its* erythro-*Isomer*.—The *threo*-3-hydroxytyrosine was prepared following a published procedure [22] which was reported to give only the *threo*-isomer. We found that the *erythro*-isomer was also produced in this reaction sequence. A solution of *p*-benzyloxybenzaldehyde (4 g) in ethanol (10 ml) was added to a solution of glycine (0.71 g) and potassium hydroxide (1.06 g) in ethanol (4 ml). The mixture was stood at room temperature for 3 h with occasional swirling. The precipitate of Schiff's base (A) was collected. Cooling the filtrate gave a second precipitate (B) which was collected. Reduction of the volume of the filtrate gave a third precipitate (C). Each of these three precipitates was separately and rapidly stirred in 1M hydrochloric acid (20 ml) and the mixture was then filtered. The pH of the filtrate was adjusted to 7.0 and the precipitated product was collected.

The Schiff's base (A) gave a precipitate of *p*-benzyloxyphenylserine (230 mg) which was almost pure *threo* isomer by ^{1}H n.m.r. The Schiff's base (B) gave *p*-benzyloxyphenylserine (200 mg) which was 90% *threo* and 10% *erythro* by ^{1}H n.m.r. The Schiff's base (C) gave product (200 mg) which was 60% *threo* and 40% *erythro*. Reducing the volumes of the filtrates obtained after collecting the three samples of *p*-benzyloxyphenylserine and cooling the solutions to 0 °C gave further crops of *p*-benzyloxyphenylserine (total mass: 425 mg) which were 60% *threo* and 40% *erythro* by ^{1}H n.m.r. The *threo*-isomer had m.p. 189—190 °C (decomp.) (lit.,[22] 195—197 °C decomp.); δ_H (CF_3CO_2D) 4.64 (1 H, d, *J* 3.5 Hz), 5.2 (2 H, s), 5.67 (1 H, d, *J* 3.5 Hz), 7.3 (4 H, m), and 7.43 (5 H, s). The *erythro*-isomer gave $\delta_H(CF_3CO_2D)$ 4.84 (1 H, d, *J* 4.5 Hz), 5.2 (2 H, s), 5.67 (1 H, d, *J* 4.5 Hz), 7.3 (4 H, m), and 7.43 (5 H, s).

threo-p-Benzyloxyphenylserine (1 g) was hydrogenated in methanol (20 ml) containing 2M hydrochloric acid (1.75 ml) with 10% palladium-on-charcoal (200 mg) at room temperature and atmospheric pressure until the required volume of hydrogen had been adsorbed. The solution was filtered and its volume was reduced to 1 ml. Water (5 ml) was added and the solution was neutralized with 2M sodium hydrogen carbonate. The mixture was set aside overnight at 0 °C after which the 3-hydroxytyrosine (415 mg, 62%) which had precipitated was collected; it was made up of 80% *threo* and 20% *erythro* by ^{1}H n.m.r.; m.p. 202—206 °C; $\nu_{max.}$(Nujol) 3 500, 3 200—2 300, 1 625, 1 250, 1 060, 850, and 695 cm^{-1}; $\delta_H(CF_3CO_2D)$ 4.67 (0.8 H, d, *J* 3 Hz, *threo*), 4.88 (0.2 H, d, *J* 4 Hz, *erythro*), 5.75 (1 H, two doublets, *J* 3 and 4 Hz), and 7.25 (4 H, m) (*cf.* ref. 23). The bistrimethylsilyl derivative gave *m/z* 398 (M^+, 15), 269, 268, 267 (base peak), and 73 (Found: C, 54.9; H, 5.7; N, 7.1. Calc. for $C_9H_{11}NO_4$: C, 54.82; H, 5.62; N, 7.11%).

Hydrogenolysis of a 1:1 mixture of *threo*- and *erythro-p*-benzyloxyphenylserine (310 mg) gave 3-hydroxytyrosine (154 mg, 72%) which was a similar mixture of isomers. Recrystallization of this material from water (7 ml) gave a first crop of crystals (30 mg), m.p. 210—212 °C (lit.,[24] for *erythro*-3-hydroxytyrosine: 215—220 °C) which was 80% *erythro* by ^{1}H n.m.r. The *erythro*-3-hydroxytyrosine was also prepared by an alternative route,[24] m.p. 216—220 °C; $\delta_H(CF_3CO_2D)$ 4.87 (1 H, d, *J* 4 Hz), 5.75 (1 H, d, *J* 4 Hz), and 7.24 (4 H, m). Samples of *erythro*-3-hydroxytyrosine prepared by the two routes were identical by ^{1}H n.m.r. and mass spectrometry (tristrimethylsilyl derivative). The mass spectra and microanalytical data for the two samples of *erythro*-3-hydroxytyrosine and the *threo*-3-hydroxytyrosine were the same.

Labelled threo-3-*Hydroxytyrosine*.—(a) [3,5-$^2H_2$]-4-*Hydroxybenzaldehyde*. 4-Hydroxybenzaldehyde (600 mg), triethylamine (194 mg), and deuterium oxide (3 ml) were heated *in vacuo* in a boiling water-bath for 3.5 days (*cf.* ref. 25). The solution was acidified with concentrated hydrochloric acid and then extracted with ethyl acetate (3 × 10 ml). The combined extracts were dried and evaporated to dryness. The residue was dissolved in methanol and taken to dryness to give [3,5-$^2H_2$]-4-hydroxybenzaldehyde; $\delta_H(CD_3OD)$ 7.32 (2 H, s) and 9.28 (1 H, s); *m/z* 124 (M^+), 123 (base peak), 122, 95, and 67 (75% dideuteriated by comparison with unlabelled material).

(b) (2SR,3RS)-[2-^{14}C]-threo-3-*Hydroxytyrosine*. This compound was prepared as described above for unlabelled material from 4-benzyloxybenzaldehyde (850 mg) and [2-^{14}C]glycine (150 mg; 50 μCi). The [2-^{14}*C*]-*threo*-3-hydroxytyrosine (65 mg; 158 μCi mmol^{-1}) was contaminated by 20% of the *erythro*-isomer, m.p. 203—206 °C. Recrystallization from water gave a sample (15 mg) (second crop) which was pure *threo*-isomer by ^{1}H n.m.r.

(c) (2SR,3RS)-[3′,5′-$^2H_2$,2-^{14}*C*]threo-3-*Hydroxytyrosine*. The deuteriated 4-hydroxybenzaldehyde from (a) above was converted into deuteriated 4-benzyloxybenzaldehyde (*cf.* ref. 26). Reaction of this material with [2-^{14}C]glycine, isolation, and purification of the product as described under (b) above gave pure [3′,5′-$^2H_2$, 2-^{14}C]*threo*-3-hydroxytyrosine (149 μCi mmol^{-1}).

[2-$^2H_2$; 2-^{13}*C*]*Glycine*. [2-^{13}C]Glycine (75 mg; 90 atom % ^{13}C) and potassium hydroxide (56 mg) were dissolved in deuterium oxide (1 ml) and the solution was evaporated to dryness. The residue was taken into deuterium oxide (1 ml) and the solution was evaporated to dryness. The residue was dissolved in deuterium oxide (2 ml) and the solution was refluxed for 48 h. The solution was evaporated and the residue was dissolved in deuterium oxide (2 ml) and refluxed for 24 h. Evaporation gave a residue which contained the [2-$^2H_2$, 2-^{13}C]glycine. It was 88% dideuteriated by assay using ^{1}H n.m.r.

(KOD, D_2O) (initially there was a doublet at δ 3.1, $J(^{13}C, ^{1}H)$ 135 Hz which decreased as the reaction proceeded) measured against an internal ethanol standard. The ^{13}C n.m.r. (KOD, D_2O) of the sample showed only a 1:2:3:2:1 quintet, δ 45, *J* 21 Hz, *i.e.* no triplet or singlet was apparent.

(2S)-*and* (2-R)-[2-^2H]*Glycine*.—These compounds were conveniently prepared following a published procedure.[27] The samples of deuteriated glycine obtained were checked for integrity and purity by conversion into their camphanoate derivatives. ^2H N.m.r. analysis showed that each isomer was stereochemically pure: δ(^2H) ($CHCl_3$; $CDCl_3$ reference; 61.4 MHz) 4.14 (*S*) and 4.01 (*R*) (*cf.* ref. 27). ^1H N.m.r. showed that the (2*R*)-isomer was 85% labelled, the (2*S*)-isomer was 75% labelled.

Results of Feeding Experiments.—% Incorporation is 100 × (total radioactivity in tuberin isolated)/(total radioactivity in precursor). Specific incorporation as used here for ^{14}C, ^2H, and ^{13}C is: 100 × (amount of label per mmol of tuberin or degradation product)/(amount of label per mmol of precursor). In the following % incorporation is used unless otherwise stated.

(a) [^{14}C]Formate (814 μCi mg^{-1}; 3.2 μCi) (incorporation into tuberin: 0.02%); [2-^{14}C]acetate (59 μCi $mmol^{-1}$; 5 μCi) (0.01%); [U-^{14}C]pyruvate (169 μCi mg^{-1}; 4 μCi) (0.02%); [*Me*-^{14}C]methionine (371 μCi mg^{-1}; 18 μCi) (21.5%); L-[1-^{14}C]tyrosine (56 mCi $mmol^{-1}$; 10 μCi) (0.007%); (*RS*)-[2-^3H]octopamine (1.9 mg; 26 μCi) (0.05%); (2*SR*, 3*RS*)-[2-^{14}C]-*threo*-3-hydroxytyrosine (24 mg; 2 μCi) (3.1%); (2*SR*, 3*RS*)-[2-^{14}C; 3′,5′-$^2H_2$]-*threo*-3-hydroxytyrosine (11 mg; 3.15 μCi; 78.6% $^2H_2$) (^{14}C: 1.04% and 0.88% in two experiments; no deuterium incorporation by mass spectrometry) [60% and 40% of the ^{14}C activity lost on degradation to (**4**) and (**3**), respectively]; [2-^{14}C]glycine (10 μCi; 49.5 mCi mmol) (7.4%) [56 and 42% of activity lost on degradation to (**4**) and (**3**), respectively]; [2-^{13}C, 2-^{14}C]glycine (60 mg; 90% ^{13}C; 5 μCi) [3.1 and 3.4% enrichment (specific incorporation) at δ_C 54.7 and 158.3, respectively; value for ^{14}C: 6.5% specific incorporation]; [2-^{14}C; 2-$^2H_2$]glycine (60 mg; 8.3 μCi) (^2H: 4.3%; ^{14}C: 7.4% specific incorporation) (103 mg; 11.3 μCi) (^2H: 1.7%; ^{14}C: 6.8%) L-[3-^{14}C]serine (9.5 Ci) (8.7%) [52% and 48% of activity lost on degradation to (**4**) and (**3**), respectively].

(b) Incorporation into tuberin (1) of [U-^{14}C]tyrosine also labelled with ^2H or ^3H:

Precursor labelling	Activity fed (μCi)	$^{13}H/^{14}C$	% Incorporation into (**1**)[a]	$^3H/^{14}C$
1 U-^{14}C	15.4	1.04	51.1	0.58
3′,5′-$^3H_2$	14.2		28.7	
2 U-^{14}C	17.5	3.39	14.4	2.87
2′,6′-$^3H_2$	59.3		10.8	
3 U-^{14}C	0.9	13.7	23.6	11.4
2′,6′-$^3H_2$	11.5		20.6	
4 U-^{14}C	0.8		10.9[b]	
3′,5′-$^2H_2$[c]	(10 mg)		7.5[b]	

[a] ^{14}C Values allow for loss of 1/9th of the activity due to loss of the carboxy group in tyrosine. [b] These values are calculated as specific incorporation. Deuterium content determined by mass spectrometry on labelled tuberin: *m/z* 179 (8.7%), 178 (12.4%), and 177 (100.0%); unlabelled material: *m/z* 179 (0.6%), 178 (12.3%), and 177 (100.0%). [c] Prepared following ref. 25.

(c) Incorporation of glycine labelled with ^{13}C and/or ^2H: [2-^{13}C, 2-$^2H_2$]glycine [δ_c at 100.6 MHz (hexadeuterioacetone)] *ca.* 4.6% enhancement of natural abundance singlet at δ 54.7; 1:1:1 triplet, *J* 22 Hz, 0.27 p.p.m. upfield shift; 1:2:3:2:1 quintet *J* 22 Hz, 0.54 p.p.m. upfield shift; *ca.* 4.7% enhancement of natural abundance singlet at 158.3; triplet *J* 30 Hz, 0.16 p.p.m. upfield shift (the upfield shifts agree with literature values[28]); (2*R*)- and (2*S*)- [2-^2H]glycines (50 mg each): ^2H n.m.r. spectra in chloroform ($CDCl_3$ reference) signals at δ 3.79 (*O*-methyl group), 8.18 and 8.39 (*N*-formyl group); *N*-formyl intensity/*O*-methyl intensity in three experiments: 0.76, 0.95, and 0.92 (*S*), <0.25, <0.26, and <0.30 (*R*), respectively [^{14}C incorporation in third experiment: 2.6% (*R*-isomer) and 3.1% (*S*-isomer)].

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