On the Preparation of L-[2,3-$^3H_2$]Phenylalanine and L-[2,3-$^3H_2$]Tyrosine

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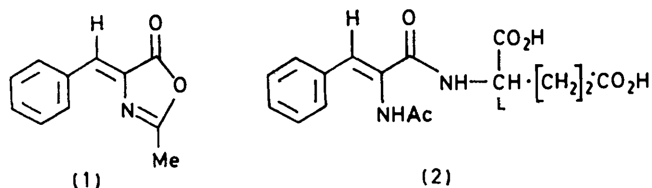
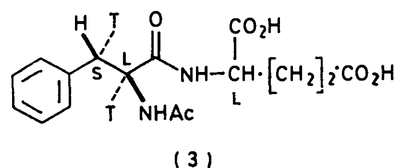
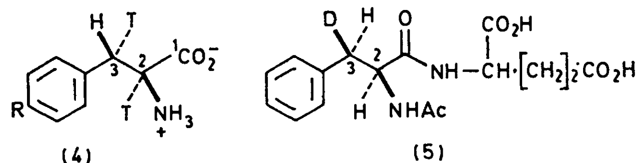
Specimens of L-[2,3-$^3H_2$]phenylalanine and L-[2,3-$^3H_2$]tyrosine, prepared *via* reduction of acylaminocinnamic derivatives with tritium gas, have been degraded to establish the tritium labelling patterns. Both amino-acids were found to be labelled approximately equally at C-2 and -3 with the tritium at C-3 in predominantly the *pro-S* position. Catalytic hydrogenation of (*Z*)-(α-acetamido[β-^{2}H]cinnamyl)-L-glutamic acid was used to demonstrate, by n.m.r. spectroscopy, the stereochemical course of the tritiation.

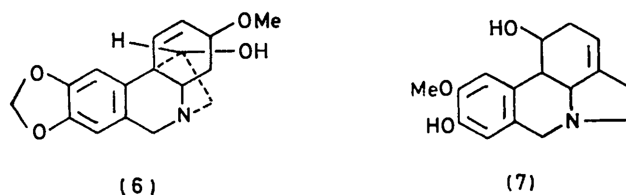
L-[2,3-$^3H_2$]PHENYLALANINE,† available commercially from the Radiochemical Centre, Amersham, is prepared [1,2] as follows. The oxazolinone (1) is treated with L-glutamic acid and the resulting olefin (2) reduced catalytically (palladium black) with tritium gas to give a mixture of diastereoisomers from which the tritiated acetyl-L-phenylalanyl-L-glutamic acid (3) can be obtained by repeated crystallisation. Hydrolysis of (3) gives L-[2,3-$^3H_2$]phenylalanine (4; R = H) which may alternatively [2] be obtained by hydrolysing the foregoing mixture of diastereoisomers and removing D-[2,3-$^3H_2$]phenylalanine with a D-amino-acid oxidase. The labelling pattern and, in particular, the configuration of tritium at C-3 implied in (4; R = H) have not till now been determined. Our interest in the synthesis of stereoselectively tritiated amino-acids [3] led us to examine the labelling pattern in (4; R = H) and the related L-[2,3-$^3H_2$]-tyrosine (4; R = OH).

The stereochemical consequences of the reduction step, (2) ⟶ (3), were investigated first by using deuterium labelling. [*formyl*-^{2}H]Benzaldehyde [4] was converted [1] into the correspondingly deuteriated analogues of (1) and (2). Catalytic hydrogenation of [^{2}H]-(2) over palladium black in dioxan or palladium–carbon in ethanol gave, after fractional crystallisation of the product mixture, (3*R*)-[3-^{2}H]-L-phenylalanyl-L-glutamic acid (5), which was examined by n.m.r. spectroscopy. The unlabelled form (5; H in place of D) showed an ABX pattern for the protons at C-2 and -3 with additional splitting of the signal for H-2 by the neighbouring N–H: τ[$(CD_3)_2SO$] 5·44 (m, *J* 10, 8, and 4 Hz, 2-H), 7·00 (q, *J* 14 and 4 Hz, 3-H_S), and 7·29 (q, *J* 14 and 10 Hz, 3-H_R). The spectrum of the deuteriated derivative (5) showed a doublet (*J* 4 Hz) for the *pro-S* hydrogen atom at C-3 (τ 7·00) and no detectable signal for a *pro-R* hydrogen atom. Reduction had therefore involved highly stereoselective (>95%) *cis*-addition of hydrogen to the *Z*-olefin, [^{2}H]-(2); there is ample precedent for this.[3,5,6] It follows that the tritiated phenylalanine should have predominantly the stereochemistry shown (4; R = H); this was confirmed by degradation of a commercial sample.









Oxidation of (4; R = H) with alkaline permanganate gave benzoic acid which contained negligible (0·02%) amounts of tritium; the phenylalanine was therefore labelled only at C-2 and -3. The amount of tritium at C-2 was then determined by racemisation.‡ Treatment of the L-phenylalanine (4; R = H), mixed with L-[1-^{14}C]phenylalanine to provide a reference label, with 10N-hydrochloric acid at 170—180 °C gave DL-phenylalanine containing, in separate experiments, 49·9 and

† The side-chain numbering used by the Radiochemical Centre and earlier workers will be retained here.

‡ The tritiated amino-acids (4) have two chiral centres; for brevity the term 'racemisation' will be used throughout this paper to denote epimerisation at C-2 under equilibrating conditions without affecting the configuration at C-3.

[1] M. Bergman, F. Stern, and C. Witte, *Annalen*, 1926, **449**, 277; M. Winand, S. Bricteux-Gregoire, and W. G. Verly, 'Proceedings of the Symposium on the Preparation and Biochemical Application of Labelled Molecules.' Venice, 1964, p. 17.

[2] D. C. Warrell, personal communication.

[3] G. W. Kirby and J. Michael, *J.C.S. Perkin I*, 1973, 115.

[4] D. J. Bennett, G. W. Kirby, and V. A. Moss, *J. Chem. Soc.* (*C*), 1970, 2049.

[5] R. H. Wightman, J. Staunton, A. R. Battersby, and K. R. Hanson, *J.C.S. Perkin I*, 1972, 2355.

[6] P. G. Strange, J. Staunton, H. R. Wiltshire, A. R. Battersby, K. R. Hanson, and E. A. Havir, *J.C.S. Perkin I*, 1972, 2364; G. W. Kirby, J. Michael, and S. Narayanaswami, *ibid.*, p. 203; G. W. Kirby and M. J. Varley, *J.C.S. Chem. Comm.*, 1974, 833.

50·4% (see Table) of the original tritium, thereby demonstrating equal labelling at C-2 and -3 in (4; R = H). In confirmation, the *N*-acetyl derivative of (4; R = H) was racemised *via* the corresponding oxazolinone [7] and the process repeated until the product had a constant activity; again loss of *ca.* 50% tritium was observed. The configuration of tritium at C-3 in (4; R = H) was determined by using phenylalanine ammonia-lyase, which converts the amino-acid into cinnamic acid with stereospecific removal of the *pro-S* hydrogen atom from the methylene group.[5] The racemised form of phenylalanine gave cinnamic acid with a retention of tritium (16·5 and 17·0% in separate experiments) indicating predominant, but not exclusive, 3-*pro-S* labelling. Oxidative conversion of the cinnamic acid into benzaldehyde confirmed the amount and location of the tritium. The doubly labelled phenylalanine (4; R = H) was also treated with the ammonia-lyase before racemisation. It was apparent (see Table) that the crude enzyme, in producing cinnamic acid, had caused some loss of tritium from C-2 as well as the obligatory loss from C-3. However conversion of the cinnamic acid into benzaldehyde provided a direct measure of the tritium retained at C-3 during the enzymic elimination of ammonia; the results agreed well with those obtained with the racemised phenylalanine. Combining the data from several independent degradations (see Table) we conclude that the commercial L-[2,3-$^{3}H_{2}$]phenylalanine had the labelling pattern: [3-*pro-S*-^{3}H], 41·5 ± 0·5%; [3-*pro-R*-^{3}H], 8·5 ± 0·5%; [2-^{3}H], 50 ± 1%.

L-[2,3-$^{3}H_{2}$]Tyrosine (4; R = OH), prepared at the Radiochemical Centre, Amersham, by a similar procedure, was also examined. Methylation followed by oxidation with alkaline permanganate gave 4-methoxybenzoic acid containing 0·84% of the original tritium and racemisation with 10N-hydrochloric acid gave DL-[3-^{3}H]tyrosine (50·9% retention of ^{3}H). The configuration of tritium at C-3 was determined by using 'Texas' daffodils since it is known that incorporation of tyrosine into haemanthamine (6) in these plants involves stereospecific removal of a *pro-R* hydrogen atom from the methylene group of the amino-acid.[3,8] A racemised sample of (4; R = OH) was mixed with DL-[2-^{14}C]tyrosine and fed to daffodils in the usual way.[3] Incorporation into haemanthamine (6) and norpluviine (7) occurred with tritium retentions of 72 and 105%, respectively. The tyrosine (4; R = OH), like the phenylalanine, therefore contained approximately equal amounts of tritium at C-2 and -3 and the configuration at C-3 was again predominantly *S*.

The commercial availability of stereoselectively tritiated phenylalanine and tyrosine makes their use in metabolic studies attractive. However the observed configurational purity at C-3 is, for reasons which are not clear, less than that expected from the method of preparation and may vary from batch to batch.

Degradation of L-[2,3-$^{3}H_{2}$]phenylalanine (4; R = H)

	Tritium retentions (%)			
	After racemisation [a]	After racemisation [b]	In cinnamic acid [c]	In benzaldehyde
L-[2,3-$^{3}H_{2}$; 1-^{14}C]Phenylalanine (2 independent experiments)	50·4	50·6	44·9	8·9
	49·9		47·1	8·7
L-[2,3-$^{3}H_{2}$]Phenylalanine		50·5		
DL-[3-^{3}H; 1-^{14}C]Phenylalanine [from racemisation of (4; R = H)			16·5	15·9
			17·0	

[a] With 10N-HCl. [b] *via* Oxazolinone. [c] From lyase incubation.

EXPERIMENTAL

General Methods.—^{3}H and ^{14}C Activities were determined by using a Beckman CPM-100 liquid scintillation spectrometer. N.m.r. spectra were measured at 100 MHz.

Labelled Phenylalanine and Tyrosine.—L-[2,3-$^{3}H_{2}$]phenylalanine (batch No. B9) and L-[2,3-$^{3}H_{2}$]tyrosine (batch No. B12) from the Radiochemical Centre, Amersham, were diluted with the appropriate radioinactive L-amino-acids before use. L-[1-^{14}C]Phenylalanine and DL-[2-^{14}C]tyrosine were also obtained from the Radiochemical Centre.

(2S,3R)-N-*Acetyl*[3-^{2}H]*phenylalanyl*-L-*glutamic Acid* (5).—[*formyl*-^{2}H]Benzaldehyde [4] was converted into (*Z*)-(α-acetamido[β-^{2}H]cinnamyl)-L-glutamic acid by established methods.[1] Catalytic hydrogenation either over palladium-black in dioxan or over palladium–carbon (10%) [3] in ethanol at ambient temperature and pressure gave a mixture of diastereoisomers from which the isomer (5) was obtained by fractional crystallisation from water; m.p. 141—143° (lit.,[1] 140° for the undeuteriated form), $[\alpha]_D$ +5·4° (in EtOH) (lit.,[1] +5·6° for the undeuteriated form).

Oxidation of Phenylalanine and Tyrosine.—Phenylalanine (165 mg) in water (6 ml) containing potassium hydroxide (100 mg) was treated with potassium permanganate (240 mg) in portions. The mixture was heated under reflux for 2 h then more potassium permanganate (110 mg) was added and the heating continued. Benzoic acid (64 mg) was isolated and purified by crystallisation from water. Tyrosine (50 mg) in 4N-sodium hydroxide (2 ml) was treated with dimethyl sulphate (1 ml) with stirring at 0 °C. Sodium hydroxide (250 mg) was added and the mixture heated under reflux for 3 h. Oxidation was then carried out (as above) with potassium permanganate (500 mg), after addition of 4N-sodium hydroxide (1 ml), to give 4-methoxybenzoic acid (11 mg), which was crystallised from water.

Racemisation of Phenylalanine and Tyrosine.—The amino-acids were racemised [3] (see footnote in main text) by heating in 10N-hydrochloric acid in sealed tubes at 170—180 °C. Temperature control was important since extensive darkening of tyrosine occurred at temperatures $>$ 180 °C. Racemisation of L-[2,3-$^{3}H_{2}$]tyrosine in this way removed the same amount of tritium in 48 h as in 72 h. Alternatively,[7] phenylalanine was acetylated (Ac_2O–aq. NaOH) and the derivative converted into the corresponding oxazolinone

[7] H. Matsuo, Y. Fujimoto, and T. Tatsuno, *Tetrahedron Letters*, 1965, 3465.

[8] A. R. Battersby, J. E. Kelsey, J. Staunton, and K. E. Suckling, *J.C.S. Perkin I*, 1973, 1609.

with dicyclohexylcarbodi-imide in acetonitrile. The oxazolinone was treated with aqueous pyridine to regenerate *N*-acetylphenylalanine with removal of tritium from C-2. Tritium removal occurred, owing to the kinetic isotope effect, more slowly than racemisation as judged by optical rotation. The racemisation cycle was therefore repeated until no further tritium loss occurred: typically, L-[2,3-$^{3}H_2$]phenylalanine gave DL-[3-^{3}H]*N*-acetylphenylalanine with 65·5, 59·3, 50·5, and 50·6% retention of tritium after successive racemisation cycles.

Phenylalanine Ammonia-lyase.—The enzyme was obtained from potato tubers and purified as far as the first ammonium sulphate fractionation.[9] Incubation of phenylalanine with the enzyme gave cinnamic acid which was purified by sublimation before being counted for radioactivity. Conversion of L-[2,3-$^{3}H_2$]phenylalanine into cinnamic acid by the crude enzyme preparation involved some loss of tritium from C-2, as well as from C-3 (see text). A control experiment with DL-[2-^{3}H; 1-^{14}C]phenylalanine showed 13·9% loss of tritium during formation of cinnamic acid but the % loss may vary with different batches of enzyme.

Oxidation[10] *of Cinnamic Acid to Benzaldehyde.*—Cinnamic acid (144 mg) in water (10 ml) containing sodium hydrogen carbonate (82 mg) was treated with sodium periodate (214 mg) in water (30 ml) then, with stirring, with potassium permanganate (4 mg) in portions. After 10 h at room temperature benzaldehyde was extracted with dichloromethane and converted into the semicarbazone (66 mg).

Feeding Experiments with Daffodils.—DL-[3-^{3}H; 2-^{14}C]-Tyrosine was fed to 'Texas' daffodils in the usual way[3] and the major alkaloids were isolated. Norpluviine (7) (0·13% incorporation of ^{14}C; 105% retention of ^{3}H) was purified *via* the diacetate and haemanthamine (6) (0·01% incorporation; 72% retention) by chromatography and crystallisation from ethyl acetate.

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[9] E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1896.
[10] R. U. Lemieux and E. von Rudloff, *Canad. J. Chem.*, 1955, **33**. 1701.