Synthesis of L-Glutamic Acid Stereospecifically Labelled at C-4 with Tritium: Stereochemistry of Tritium Release Catalyzed by the Vitamin K-Dependent Carboxylase in the Absence of Carboxylation

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[4-$^3H_1$]-L-Glutamic acids have been synthesized by reduction of (2*S*,4*S*)- and (2*S*,4*R*)-*N*-benzyloxycarbonyl-4-halogenoglutamic acid dimethyl esters with [$^3H_4$]sodium borohydride in dimethylformamide. The degree of stereospecific labelling differed according to the diastereoisomeric configuration and the nature of the halogen substituent. Only the (2*S*,4*R*)-[4-$^3H_1$]-acid could be satisfactorily obtained by this method. An enzymatic method, using an isocitrate lyase–isocitrate dehydrogenase–glutamate dehydrogenase system, was successfully used to obtain (2*S*)-[2-$^3H_1$]succinate and to convert it into (2*S*,4*S*)-[4-$^3H_1$]glutamic acid. The introduction of the labelled glutamic acids into a peptide previously shown to be a substrate of the rat liver vitamin K-dependent carboxylase allowed us to demonstrate that the hydrogen exchange catalyzed by this preparation in the absence of CO_2 proceeds by a stereospecific abstraction of the *same* 4-*pro*-S-hydrogen atom of the glutamyl residue which is eliminated in the carboxylation reaction. A 4-methyl (*threo*) L-glutamic acid-containing peptide exerts the same competitive inhibition on the exchange and on the carboxylation reaction. These results ensure that the previously demonstrated hydrogen exchange is part of the carboxylation reaction.

Several studies on various stereochemical aspects of L-glutamic acid metabolism have involved the use of stereospecifically hydrogen isotope labelled derivatives.[1-3] Up to now, most of the efforts have been devoted to the preparation of 3-labelled molecules, using enzymic[3,4] or chemical methods.[3,5,6] Previously, we have described the synthesis of L-glutamic acids (partially) stereospecifically labelled at C-4 with deuterium.[7] These compounds were introduced in peptide substrates of the vitamin K-dependent carboxylase[8,9] and used to determine the stereochemistry of the hydrogen elimination associated with the carboxylation of the glutamyl residue to 4-carboxyglutamic acid.[10,11]

In an attempt to elucidate the as yet unknown mechanism of this reaction, Friedman and co-workers, using substrate peptides containing (2*S*,3*RS*,4*RS*)-[3,4-$^3H_2$]- or (2*S*, 4*RS*)-[4-$^3H_1$]-glutamic acid, have demonstrated, with a similar carboxylase preparation, a vitamin K-dependent tritium release *in the absence* of carboxylation.[12,13] Moreover, in the presence of tritiated water, tritium was incorporated into the glutamic acid residue(s) of unlabelled peptide, in a vitamin K-dependent reaction;[13-15] the localization of this exchange was found to be at the C-4 atom,[13,15] but no data about its stereochemistry was available. As it has not been demonstrated unequivocally that tritium release from tritiated peptides corresponds to the mandatory hydrogen abstraction preceding the formation of a 4-carboxylglutamic acid residue, in the absence of CO_2, it was thus desirable to repeat such experiments, using *stereospecifically* 4-tritiated glutamic acid-containing peptides, in order to verify that the tritium release exhibited the same 4-*pro*-*S* selectivity already demonstrated for the 4-hydrogen elimination associated with the carboxylation reaction.[10,11]

We report now the synthesis of both (4*R*)- and (4*S*)-tritiated L-glutamic acids and the use of peptides containing these amino acids to demonstrate a stereoselective hydrogen release, in the absence of carboxylation. Moreover, the inhibition of this tritium release by a 4-methyl-L-glutamic acid-containing peptide, a specific inhibitor of the carboxylation reaction,[16,17] has been examined.

In a previously described preparation of the correspondingly deuteriated glutamates, the [$^2H_3$]sodium cyanoborohydride reduction of 4-methylsulphonyloxyglutamate dimethyl ester derivatives was shown to occur with only 75% inversion of configuration, a result which was ascribed to the rather drastic conditions (100 °C; 18 h) necessary for an efficient reduction.[7] The same result had been previously reported for a similar reaction.[18] In a search for a more stereospecific introduction of tritium, we have investigated the milder [$^3H_4$]sodium borohydride reduction of 4-halogenoglutamate derivatives in dimethylformamide. According to kinetic data, this reaction is supposed to proceed mostly by an S_N2 mechanism,[19] but to our knowledge, no stereochemical analysis of the reduction products has been published.

The (2*S*,4*RS*)-*N*-benzyloxycarbonyl-4-methylsulphonyloxyglutamate dimethyl ester[7] was easily converted into the 4-iodo or 4-bromo derivative by heating in toluene with tetrabutylammonium iodide or benzyltri(butyl)ammonium bromide respectively (Scheme 1). The resulting diastereoisomers were separated by column chromatography on silica gel† and then treated with 1 molar equivalent of NaB^3H_4 in dimethyl-

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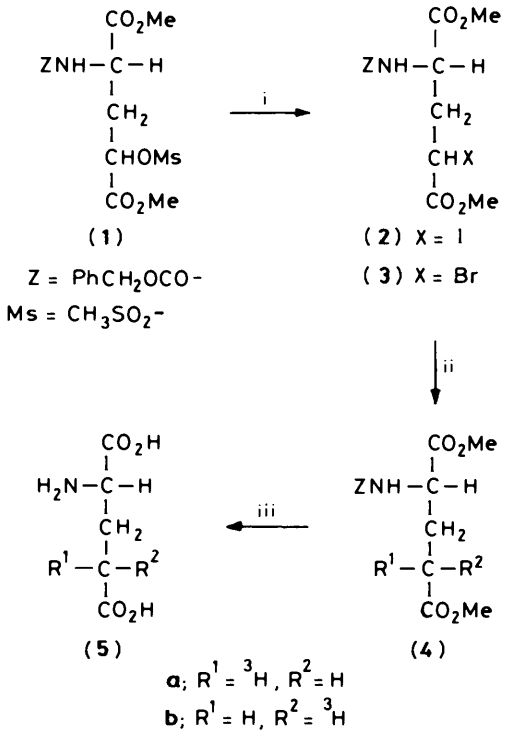
† The 4-bromo- and 4-iodo-L-glutamate diastereoisomers were identified by h.p.l.c. through comparison of their retention times with those of similar products prepared from the pure *N*-benzyloxycarbonyl (4*R*)- or (4*S*)-4-methylsulphonyloxyglutamic acid dimethyl esters[7] (assuming a likely inversion in the substitution reaction). The (4*R*) isomers were consistently eluted first from the column (Figure 1).



Scheme 1. *Reagents and conditions:* i, $R_4N^+I^-$ or Br^-/toluene; ii, diastereoisomer separation, NaB^3H_4/DMF; iii, 1.5M-LiOH/MeOH–H_2O, H_2–Pd/C in MeOH–H_2O

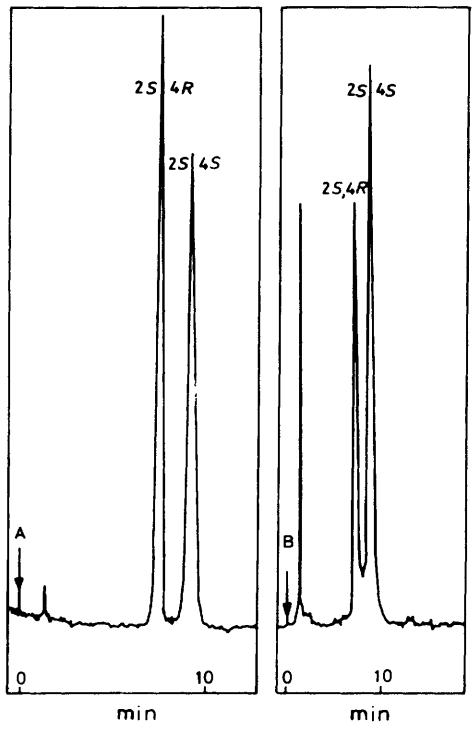
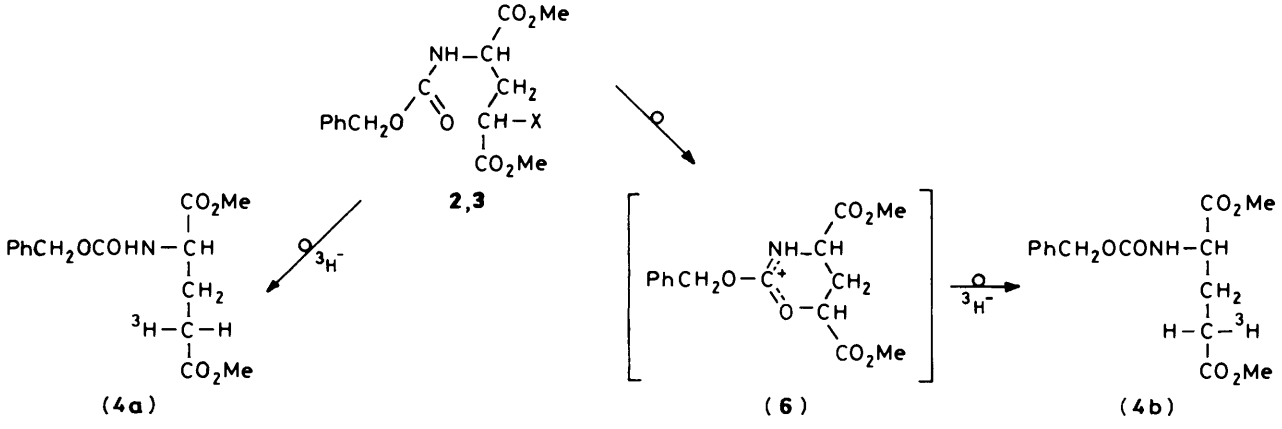


Figure 1. High pressure liquid chromatography of *N*-benzyloxycarbonyl-4-bromo (A) and 4-iodo (B) (2*S*)-glutamic acid dimethyl esters on Lichrosorb Si60; solvent (A) hexane–ethyl acetate (85:15), 3 ml/min. (B) hexane–ethyl acetate (8:2), 3 ml/min. Detection at 256 nm

formamide at 30—40 °C. The tritiated glutamic acids were obtained from the reduction products by mild alkaline hydrolysis and catalytic hydrogenolysis. Their stereochemistry was determined by oxidative conversion into the corresponding [2-$^3H_1$]succinic acids,[20] followed by equilibration with isocitrate lyase (from *Pseudomonas indigofera*) as previously described.[20,21] The results (Table 1) showed that only the borohydride reduction of the (2*S*,4*S*)-4-bromoglutamate derivative, which proceeded almost exclusively by an S_N2 mechanism, had practical interest; this method was used for the preparation of (2*S*,4*R*)-[4-$^3H_1$]glutamic acid.

In all other cases, the reduction proceeded with a lower degree of inversion, indicating a competitive mechanism. Either a cyclic, reducible intermediate such as (**6**), involving participation of the *N*-benzyloxycarbonyl group in the reduction is a possibility (Scheme 2),[22] or glycol methanesulphonate monoester substitution;[23] these results, and especially the fact that nonsymmetrical results were obtained with 4-halogenoglutamates of the reverse configuration, might reflect diastereoisomeric effects in the formation of such an intermediate, concurrently with a direct S_N2 reduction.

The diastereoisomeric (2*S*,4*S*)-[4-$^3H_1$]glutamic acid was prepared by an adaptation of the enzymatic method previously described to analyse the absolute configuration of tritiated succinic acids.[20] Isocitrate lyase (EC.4.1.3.1) catalyses, in the presence of Mg^{2+}, an equilibrated condensation of glyoxylate and succinate to *threo*-D_S-isocitrate. In tritiated water of high activity there is exclusive introduction of ^3H into the positions



Scheme 2.

Table 1. Diastereoisomeric analysis of the NaB^3H_4 reduction products of 4-halogeno-L-glutamic acids

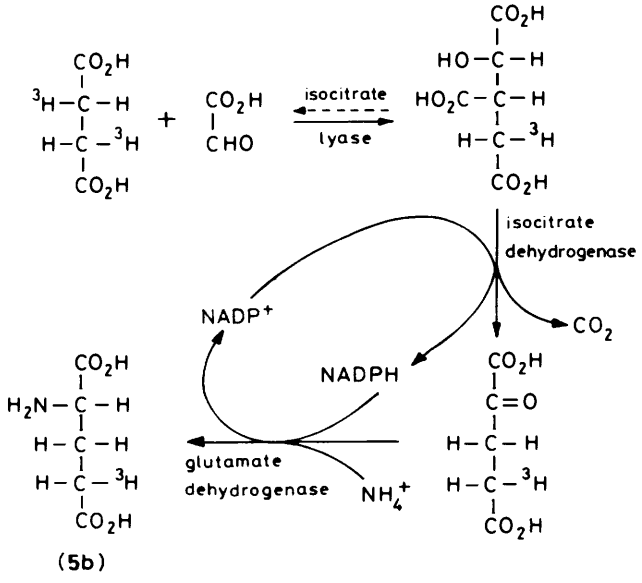
Halogen, temp. (°C)	From (4*R*)		From (4*S*)	
	%(4*S*)	%(4*R*)	%(4*S*)	%(4*R*)
I, 40	60	40	40	60
I, 30	60	40	25	75
Br, 40	65	35	5	95

Table 2. Vitamin K-dependent tritium release from tritiated peptides. Incubations were for 1 h at 17 °C in air without shaking in a total volume of 125 µl containing 0.8mM-peptide. All results are corrected for a blank value (no vitamin K added) lower than 100 d.p.m. and correspond to the average of triplicate measurements

Substrate	Specific activity mCi mmol^{-1}	D.p.m. released in water	% Hydrogen released[a]
Boc(4*RS*)-[4-^{3}H]Glu-Glu-LeuOMe	16	81 800	4.6
Boc-[3,4-^{3}H]Glu-Glu-Val	10	23 560	4.3
Boc-(4*R*)-[4-^{3}H]Glu-Glu-Val	5	5 350	0.5
Boc-(4*S*)-[4-^{3}H]Glu-Glu-Val	8	54 700	3.1

[a] Calculated for 1 exchangeable hydrogen atom.

2-*pro-S* of succinate and 4-*pro-S* of isocitrate.[21] The labelled products were separated from excess of ^3H_2O and re-incubated in ordinary water–buffer containing glyoxylate, NH_4^+ and $NADP^+$ with isocitrate lyase, excess of isocitrate dehydrogenase (EC.1.1.1.42), and glutamate dehydrogenase (EC.1.4.1.4). Preliminary experiments revealed that, under these conditions, the isocitrate lyase equilibrium was completely shifted to isocitrate by the irreversible isocitrate dehydrogenase reaction (Scheme 3). Moreover, the isocitrate dehydrogenase–glutamate



Scheme 3.

dehydrogenase system constituted an efficient arrangement for the nicotinamide coenzyme recycling. As expected, about half of the specific activity of tritiated water was introduced into L-glutamate, with a nearly quantitative yield from succinate + isocitrate. The assignment of isotope labelling at C-4 was confirmed by ^3H n.m.r. spectroscopy (Figure 2); there is no reason to suspect any incorporation into the 4*R*-position, owing to the highly stereospecific procedure which was employed.

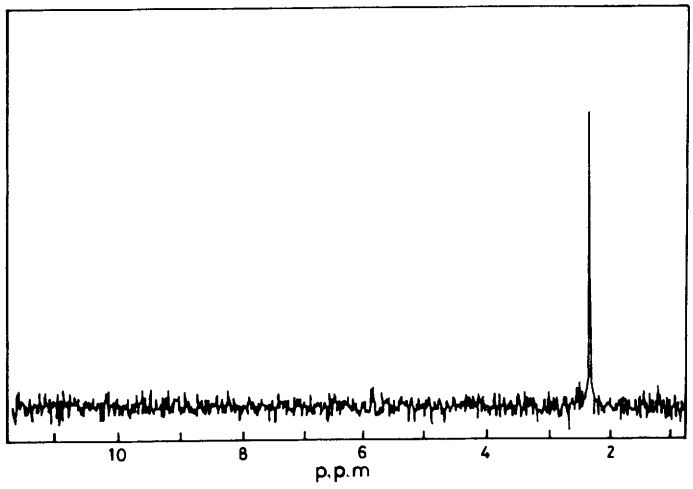


Figure 2. 106 MHz ^3H n.m.r. spectrometry of (2*S*,4*S*)-[4-$^3H_1$]glutamic acid in D_2O with broad band decoupling of proton resonance; δ in p.p.m. are externally referenced to tetramethylsilane protons

(2*S*,4*R*)- and (2*S*,4*S*)-[4-$^3H_1$]Glutamic acid were introduced into the peptide Boc-Glu-Glu-Val,* in place of the usually carboxylated glutamyl residue,[9,24–26] by the liquid-phase methodology, involving protection of the amino group as t-butoxycarbonyl and the γ-carboxyl groups as benzyl esters. When these labelled peptides were incubated with the rat liver vitamin K-dependent carboxylase preparation in the absence of added hydrogen carbonate, a specific tritium release to water was demonstrated for the (4*S*) labelled peptide (Table 2). The 6-fold smaller tritium release measured for the (4*R*) labelled peptide cannot be entirely explained by the small amount of (4*S*) [4-$^3H_1$] contaminating isomer (less than 5%), but might be ascribed to some residual carboxylation resulting from traces of CO_2 in buffers and involving the partial exchange of the acidic γ-proton of the 4-carboxyglutamic acid residue formed. This ratio by itself has probably no significance because each exchange reaction may be affected by a very different isotope effect. However, the higher tritium release measured (Table 2) for the previously used unspecifically labelled peptides Boc-[3,4-$^3H_2$]Glu-Glu-Val or Boc-(4*RS*)-[4-$^3H_1$]Glu-Glu-Leu-OMe[12,13] nearly corresponds to the accumulated exchanges measured for the (4*R*)- and the (4*S*)-[4-$^3H_1$]-isomers. The apparent K_M value (0.9 mM) calculated from the tritium release of the (4*S*)-tritiated peptide (Figure 3) is very similar to the apparent K_M (0.8 mM) of the carboxylation reaction of the same peptide.[9–11]

It was recently demonstrated that the carboxylation reaction of synthetic peptide substrates was competitively inhibited by peptides containing L-4-methyl(*threo*)glutamic acid (MeGlu) in place of the usually carboxylated glutamyl residue.[16,17] As shown in Figure 4, the tritium release from Boc-(4*RS*)-[4-$^3H_1$]Glu-Glu-LeuOMe is also competitively inhibited by Boc-MeGlu-Glu-Val with a K_i (0.1 mM) very similar to that measured for the inhibition of the carboxylation reaction of various peptides (K_i = 0.08 mM).[16,27]

* Abbreviations used: Boc = t-butoxycarbonyl; OMe = methyl ester; OBn = benzyl ester; OSu = *N*-hydroxysuccinimide ester; *Z* = benzyloxycarbonyl.

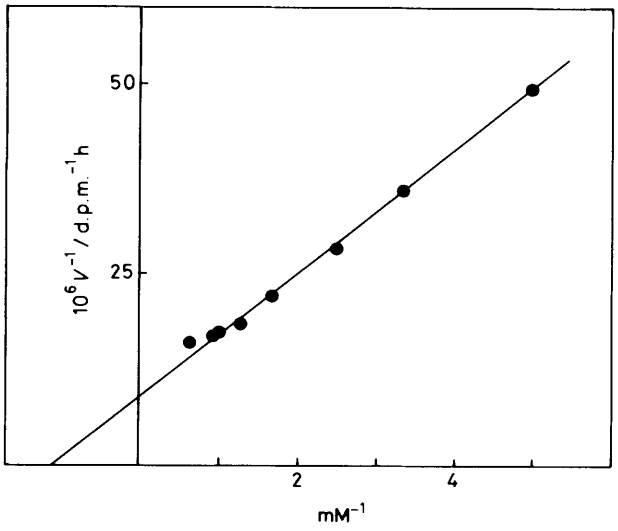


Figure 3. Double reciprocal plot of the rate of tritium release *versus* Boc-(4*S*)-[4-$^3H_1$]Glu-Glu-Val concentration. The results are the average of triplicate determinations from which controls without vitamin K have been subtracted

The data presented here show that the tritium release previously demonstrated from tritiated glutamic acid-containing peptides is *from the same hydrogen* which is eliminated in the carboxylation reaction. Moreover, the exchange is competitively inhibited by a specific competitive inhibitor of the carboxylation reaction, with the same inhibition constant. Hence, the hydrogen abstraction demonstrated in the absence of CO_2 undoubtedly constitutes the initial step of the mechanism of the vitamin K-dependent carboxylation reaction and appears to occur at the very site of binding of the peptide substrate on the carboxylase. Together with other results showing that glutamate analogues containing peptides act as carboxylase non-competitive inhibitors with respect to CO_2,[27,28] it follows that the mechanism of the vitamin K-dependent carboxylation is probably non-concerted, involving preliminary binding of the peptide substrate and hydrogen abstraction, before CO_2 approach.

Experimental

^{1}H N.m.r. spectra were recorded at 250 MHz on a Bruker WM-250 spectrometer and referenced to tetramethylsilane. Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter (10 cm cell). H.p.l.c. analyses were carried out on a 25 cm × 4.6 mm Lichrosorb Si60 (7 μm) column with hexane–ethyl acetate mixtures as solvent. Medium-pressure preparative column chromatography was carried out on Merck Silicagel H 60. Analytical t.l.c. was performed on Merck Silicagel 60 F_{254} pre-coated plates (0.2 mm) and spots were detected in u.v. light, with tetramethylamino(diphenyl)methane (TDM) reagent,[29] or by spraying with 10% phosphomolybdic acid in ethanol and heating at 120 °C. Radioactivity was measured in a Packard scintillation spectrometer using water–Instagel as liquid scintillator.

[3,4-$^3H_2$]-L-Glutamic acid and NaB^3H_4 were purchased from C.E.A. (Saclay, France). Isocitrate lyase was purified from *Pseudomonas indigofera* as previously described.[20] Isocitrate dehydrogenase (lyophilized, from pig heart) and glutamate dehydrogenase (saturated ammonium sulphate suspension, from beef liver) were obtained from Boehringer. α-Chymotrypsin (Type II) was obtained from Sigma. Boc-(4*RS*)-[4-$^3H_1$]Glu-Glu-LeuOMe[13] and (2*S*,4*S*)-4-methyl(*threo*)glutamic acid[30]

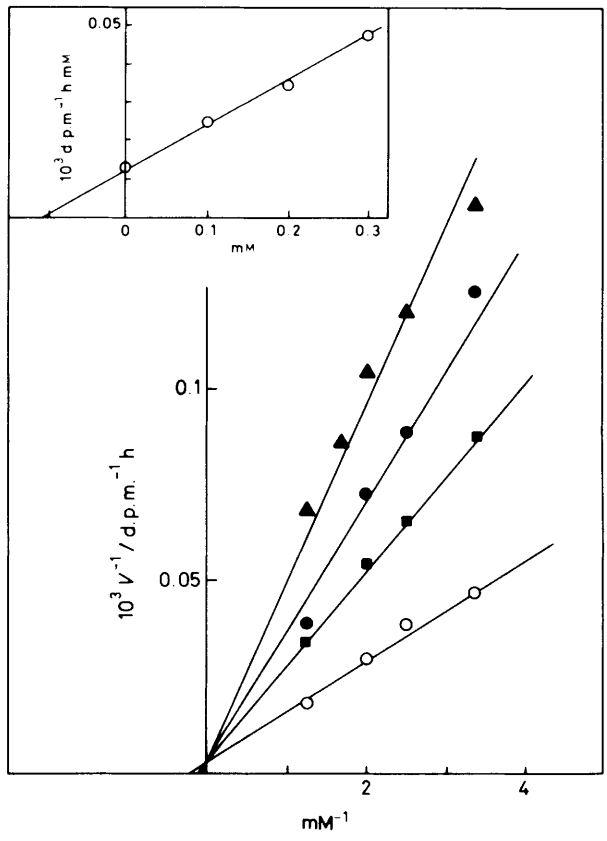


Figure 4. Double reciprocal plot of the rate of tritium release *versus* Boc-(4*RS*)-[4-$^3H_1$]Glu-Glu-LeuOMe concentration in the absence (○——○) or in the presence of Boc-MeGlu-Glu-Val (■——■: 0.1 mM; ●——●: 0.2 mM and △——△: 0.3 mM). The results are the average of triplicate determinations from which controls without vitamin K have been subtracted. The insert represents a replot of the slopes *versus* inhibitor concentrations

were prepared as previously described. Purity of peptides was checked by t.l.c. and high voltage paper electrophoresis.

(2S,4RS)-N-*Benzyloxycarbonyl*-4-*iodoglutamic Acid Dimethyl Ester* (**2**).—A solution of (2*S*,4*RS*)-*N*-benzyloxycarbonyl-4-methylsulphonyloxyglutamic acid dimethyl ester (40 mg) and tetrabutylammonium iodide (40 mg) in toluene (2 ml) was heated at 70 °C for 3 h. The mixture was cooled, diluted with water, and the organic layer submitted to column chromatography on silica gel (10 g) to give, on elution with hexane–ethyl acetate (8:2) the pure diastereoisomers (64% yield): (2S,4R)-(**2a**) was eluted first, $[\alpha]_D^{20}$ +32.1° (*c*, 1 in MeOH); δ($CDCl_3$) 7.36 (5 H, s, Ph), 5.56 (1 H, d, *J* 8, NH), 5.11 (2 H, s, CH_2Ph), 4.50 (1 H, dd, $J_{4,3}$ 9, $J_{4,3'}$ 5, 4-H), 4.38 (1 H, m, 2-H), 3.74 (6 H, s, 2 × OCH_3), 2.79 (1 H, ddd, $J_{3,3'}$ 15, $J_{3,4}$ 9, $J_{3,2}$ 5, 3-H), and 2.27 (1 H, m, 3'-H). (2S,4S)-(**2b**), $[\alpha]_D^{20}$ −54.3° (*c*, 1 in MeOH); δ($CDCl_3$) 7.36 (5 H, s, Ph), 5.35 (1 H, d, *J* 8, NH), 5.16 (2 H, s, CH_2Ph), 4.50 (2 H, 2 m, 4-H and 2-H), 3.78—3.73 (6 H, 2 s, 2 × OCH_3), 2.50 (2 H, m, 3-H).

(2S,4RS)-N-*Benzyloxycarbonyl*-4-*bromoglutamic Acid Dimethyl Ester* (**3**).—A similar reaction to that described above was conducted with benzyltributylammonium bromide in toluene. After 7 h at 75 °C and a similar treatment of the reaction mixture the residual oil was chromatographed to give 99% pure diastereoisomers (60% yield): (2S,4R)-(**3a**), $[\alpha]_D^{20}$

+4.5° (*c*, 1 in MeOH); δ($CDCl_3$) 7.32 (5 H, s, Ph), 5.43 (1 H, d, *J* 7, NH), 5.10 (2 H, s, CH_2Ph) 4.49 (1 H, dt, $J_{2,NH}$ 7, $J_{2,3}$ 7, 2-H), 4.36 (1 H, dd, $J_{4,3}$ 8, $J_{4,3'}$ 7, 4-H), 3.75 (6 H, s, 2 × OCH_3), 2.78 (1 H, ddd, $J_{3,3'}$ 14, $J_{3,4}$ 8, $J_{3,2}$ 7, 3-H), 2.34 (1 H, ddd, $J_{3',3}$ 14, $J_{3',4}$ 7, $J_{3',2}$ 7, 3'-H). (2S,4S)-(**3b**), $[\alpha]_D^{20}$ −51.5° (*c*, 1 in MeOH); δ($CDCl_3$) 7.32 (5 H, s, Ph), 5.22 (1 H, d, *J* 8, NH), 5.10 (2 H, s, CH_2Ph), 4.58 (1 H, m, 2-H), 4.34 (1 H, dd, $J_{4,3}$ 8, $J_{4,3'}$ 7, 4-H), 3.77—3.73 (6 H, 2 s, 2 × OCH_3), and 2.50 (2 H, m, 3-H).

(2S)-[4-$^3H_1$]*Glutamic Acid* (**4**).—The halogenide (**2**) or (**3**) (0.2 mmol) and NaB^3H_4 (0.2 mmol, 17 mCi) were mixed in freshly distilled dimethylformamide (0.5 ml) and stirred and heated at 30 or 40 °C for 1 h. The mixture was poured into ice–water, acidified with 1M-HCl and then extracted with methylene chloride. The combined extracts were evaporated to leave an oil which was chromatographed on a silica gel column with hexane–ethyl acetate (85:15) as eluant. (2*S*)-[4-$^3H_1$]-*N*-Benzyloxycarbonylglutamic acid dimethyl esters were obtained in 85% yield (radioactivity yield: 15%). After hydrolysis in 1.5M-LiOH in methanol–water and 10% Pd-on-charcoal catalysed hydrogenolysis,[7] [$^3H_1$]glutamic acids (**4**) were obtained without measurable decrease in specific activity. For the determination of ^3H distribution between positions 4*S* and 4*R*, these glutamic acids were diluted to about 0.5 μCi/mmol and samples were converted into succinic acid by chloramine T oxidation and then analysed as previously described.[20]

(2S,4S)-[4-^3H]*Glutamic Acid* (**4b**).—A solution of succinic acid (30 mg, 0.3 mmol) dissolved in 10mM-MOPS (3-morpholinopropanesulphonic acid) buffer pH 7.7 (1 ml) was dried under reduced pressure. To the residue, glyoxylic acid (30 mg, 0.33 mmol) in MOPS buffer (30 μl), dithiothreitol (25 mg), isocitrate lyase (700 μl, 0.6 U) and ^3H_2O (1 ml, 10 Ci) were added and the solution incubated at 36 °C overnight. Excess of tritiated water and exchangeable ^3H were removed by repeated lyophilisation. The residue (16 mCi) was taken up in water, filtrated on Celite, and the filtrate was adsorbed on a Dowex-1X8 (100—200 mesh, HCO_2^-) column; after washing with water, [2-$^3H_1$]succinic acid (14 mCi) was eluted with 0.2M-HCO_2H, and then [4-$^3H_1$]isocitric acid (1.5 mCi) with 2M-HCO_2H. After evaporation of the solvent, both ^3H compounds were dissolved together in 10mM-MOPS buffer pH 7.5 (1.5 ml) containing glyoxylic acid (30 mg, 0.33 mmol). NH_4Cl (53 mg in 1 ml of water), dithiothreitol (12 mg), $NADP^+$ (2.5 mg), isocitrate dehydrogenase (5 mg, 20 U), glutamate dehydrogenase (50 μl, 120 U), and isocitrate lyase (250 μl, 0.15 U) were added and the solution was incubated again at 36 °C overnight. The incubation mixture was heated for 3 min in a boiling water bath, filtered on Celite, and the filtrate, diluted to 50 ml, was adjusted to pH 3.0 and adsorbed on a Dowex-50X2 (H^+) column. After washing with water and elution with 0.25M-HCl, the pooled radioactive fractions were evaporated, diluted to 250 ml, and the solution was adjusted to pH 7.5 and adsorbed on a Dowex-1X8 ($CH_3CO_2^-$) column. (2*S*,4*S*)-[4-$^3H_1$]glutamic acid (23 mg, 0.16 mmol, 8.6 mCi) was eluted with 0.25M-acetic acid.

Synthesis of Boc-[4-$^3H_1$]Glu-Glu-Val.—[4-$^3H_1$]Glutamic acid dibenzyl ester toluene-*p*-sulphonate[31] (90 mg, 1.5 mCi) was treated with t-butyl dicarbonate in the presence of triethylamine. The oily Boc-[4-$^3H_1$]Glu dibenzyl ester (54 mg) was regioselectively hydrolysed[32,33] by stirring overnight in dimethyl sulphoxide (2.5 ml) and 0.05M-phosphate buffer pH 7.0 (25 ml) with α-chymotrypsin (80 mg) at 35 °C. Boc-[4-$^3H_1$]Glu γ-benzyl ester was extracted in ether after acidification to pH 3.0 with $KHSO_4$, purified by chromatography on a silica gel column with hexane–EtOAc–acetic acid (1:1:0.002) as solvent (colourless oil, 45 mg), and converted into Boc-[4-$^3H_1$]Glu γ-benzyl α-*N*-hydroxysuccinimide ester.[34] To the crude OSu-ester (0.13 mmol), Glu(OBn)-Val(OBn) (0.15 mmol), and *N*-methylmorpholine (0.16 mmol) in DMF (2 ml) were added and the solution left overnight at 25 °C. After evaporation under reduced pressure and silica gel column chromatography of the residue in CH_2Cl_2–EtOAc (85:15), pure Boc-[4-$^3H_1$]-Glu(OBn)-Glu(OBn)-Val(OBn) was recovered (58 mg, 0.65 mCi) and hydrogenolysed with 10% Pd-on-charcoal (50 mg) in EtOAc (5 ml); yield 32 mg (8.2 mCi/mmol).

Synthesis of Boc(2S,4S)-4-methylGlu-Glu-Val.—This peptide was prepared by the same methodology from Boc(2*S*,4*S*)-4-methylglutamic acid γ-benzyl ester (identically obtained by α-chymotrypsin hydrolysis of the corresponding dibenzyl ester).

Tritium Release Assays.—Liver microsomes were prepared from warfarin-treated rats as previously described[13] and solubilized in 0.25M-sucrose–0.025M-imidazole–0.5M-KCl pH 7.2 (SIK buffer) containing 1% Triton X-100. No special precaution was taken for eliminating CO_2 from incubation media, except that buffers and solutions were prepared in freshly boiled water. Under these conditions, the hydrogen carbonate concentration in the incubations was between 0.5 and 1 mM with no more than 20% of hydrogen exchange events being followed by carboxylation.[12,35] Incubations at 17 °C in a final volume of 125 μl contained 100 μl of solubilized microsomes (20 mg prot./ml), peptide substrate (and inhibitor), 100 μg vitamin K_1 hydroquinone solubilized in isopropyl alcohol (5 μl), and 65 μg pyridoxal phosphate. Reaction was stopped by 10% perchloric acid (300 μl) and after centrifugation, the tritium release was determined in water aliquots obtained by lyophilisation of the supernatants.

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