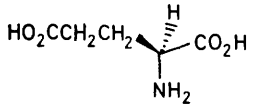
Stereochemical Course of the Decarboxylation of (*S*)-Glutamic Acid by Glutamate Decarboxylase from *Escherichia coli* (E.C. 4.1.1.15)

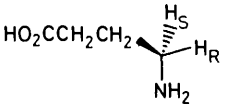
By **Enzo Santaniello,* Marzia Galli Kienle,** and **Ada Manzocchi,** Istituto di Chimica, Facoltà di Medicina, Università di Milano, Via Saldini, 50, I–20133 Milano, Italy
Enrica Bosisio, Istituto di Farmacologia e Farmacognosia, Facoltà di Farmacia, Università di Milano, I–20129 Milano, Italy

Decarboxylation of (*S*)-glutamic acid (1) in ^2H_2O, catalysed by glutamate decarboxylase from *Escherichia coli* E.C.4.1.1.15), afforded 4-[4-^{2}H]aminobutyric acid (2b). (+)-(*S*)-4-[4-^{2}H]aminobutyric acid (2c) was synthesized and the ^{1}H n.m.r. spectrum of the (1*S*,4*R*)-*O*-camphanoylamide of its methyl ester compared with the same derivative of (2b) in the presence of the shift reagent [Eu(dpm)$_3$]. It was shown that (2b) is in the (*R*) configuration and that glutamate decarboxylase catalyses the decarboxylation of (1) with retention of configuration.

DECARBOXYLATION of (*S*)-glutamic acid (1) to 4-aminobutyric acid (2a) (GABA) is catalysed by glutamate decarboxylase (E.C.4.1.1.15), a widely distributed enzyme in mammals, micro-organisms, and plants.[1] The increasing interest in the biochemistry and pharmacology of (2a) is well documented,[2] and is due to its behaviour as an inhibitory neurotransmitter within the central nervous system.[3] We thought that specifically labelled specimens of (*R*)- and (*S*)-(2b) and -(2c) could easily be prepared by enzymic decarboxylation of (1) using a commercially available, purified glutamate decarboxylase from *Escherichia coli*. Knowledge of the



(1)

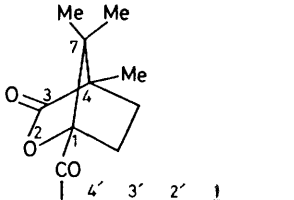


(2) a; $H_S = H_R = H$
b; $H_S = H$, $H_R = {}^2H$
c; $H_S = {}^2H$, $H_R = H$

stereochemistry involved in such decarboxylation would allow one to assign the right configuration to deuteriated (2b) or (2c) produced by incubation of (1) in ^2H_2O.

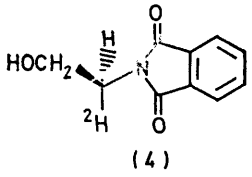
RESULTS AND DISCUSSION

Glutamate decarboxylase requires pyridoxal phosphate for its activity, and for two other pyridoxal phosphate-dependent decarboxylases it has been demonstrated that the process occurs with retention of configuration.[4,5] The most rational approach toward the determination of stereochemistry of the products of enzymic decarboxylation of (1), seemed to us to be the synthesis of (2b) [and/or (2c)] and comparison of ^{1}H n.m.r. of the (1*S*,4*R*)-ω-camphanoylamide of its methyl ester (3a) with that of the same derivative of (2b) [or (2c)] produced by enzymic decarboxylation of (1) in ^2H_2O.† The ^{1}H n.m.r. spectrum of (3b) confirmed the feasibility of such a plan, since in C^2HCl_3 the two



(3) a; R = Me, X = ^{2}H
b; R = Me, X = H

diastereotopic protons at C-4′ appear as a multiplet centred at δ 3.40 (*J* 7 Hz). Addition of the shift reagent [Eu(dpm)$_3$] shifted the resonances downfield, the C-4′ protons appearing as two separated multiplets centred at δ 4.70 and 5.02. An incubation carried out with (1) in the presence of glutamate decarboxylase from *E. coli* in ^2H_2O afforded a deuteriated sample of (2a). Examination of the ^{1}H n.m.r. spectrum of its amide (3a) in the presence of [Eu(dpm)$_3$] showed only a multiplet at δ 5.02 (integration 0.9 H) for the position C-4′. In order to assign the configuration [(*R*) or (*S*)] of the product obtained enzymatically from (1), we devised a synthesis of (2c). It was not considered worthwhile utilizing as starting material chiral [2-^{2}H]glycine, prepared by exchange of [2-$^2H_2$]glycine and water catalysed by serine hydroxymethyltransferase,[7] since it has recently been demonstrated that in such preparations a mixture of (*R*)- and (*S*)-[2-^{2}H]glycine is formed (*R* : *S* = 45 : 25).[8] We therefore preferred an entirely chemical

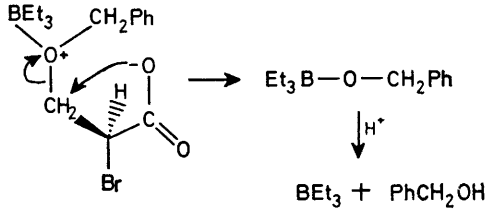


(4)

synthesis, taking advantage of the described procedure for the preparation of (+)-(*S*)-2-hydroxy-*N*-phthaloyl-[1-^{2}H]ethylamine (4).[8] However, in the preparation of (4) we encountered unexpected difficulties in the crucial

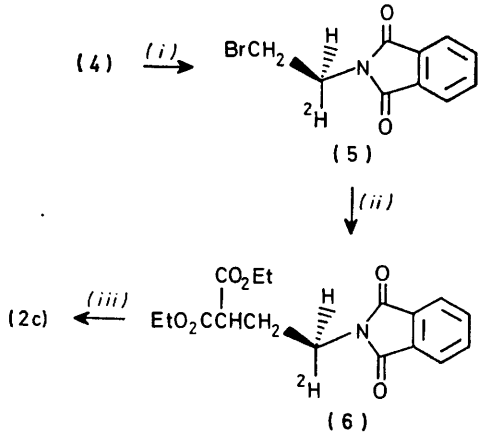
† When this work was close to conclusion, it came to our attention that the stereochemistry of reactions catalysed by glutamate decarboxylase had been determined,[6] but the method of these authors is different from ours. They prepared methyl (*S*)-4-phthalimido-[4-^{2}H]butyrate from (*S*)-[2-^{2}H]glycine (obtained by incubation of [2-$^2H_2$]glycine in water in the presence of serine hydroxymethyltransferase [7]). Comparison of the optical rotations of the above compound and the same derivative obtained from incubation of (1) in ^2H_2O allowed the assignment of the configuration.

step, *i.e.* the reduction of (—)-(*S*)-3-benzyloxy-2-bromopropionic acid to (—)-(*S*)-3-benzyloxy-[2-^{2}H]propionic acid by means of LiB^{2}HEt$_3$ (Superdeuteride),[9] reported to occur with retention of configuration (yield 50%).[8] In our hands, this reaction occurred with no more than 20% yield through several runs, the main product being benzyl alcohol; this can be explained by assuming that the commercial samples of Superdeuteride used by us were contaminated with BEt_3.* This Lewis acid might then



SCHEME 1

promote the reductive removal of the benzyloxy group *via* the anchimeric assistance of the carboxylate anion (Scheme 1). We infer such a participation, since (*R*)-3-benzyloxy-2-bromopropyl methanesulphonate is reduced with $LiBHEt_3$ to (*S*)-1-benzyloxy-2-bromopropane as the sole product (90% yield).[8] Steps following the reductive debromination of (*S*)-3-benzyloxy-2-bromopropionic acid did not present any further problems and (4) was synthesized in an overall yield of 5%. Finally, we prepared (2c) as described in Scheme 2.



SCHEME 2 (*i*) PBr_5; (*ii*) EtONa,$CH_2(CO_2Et)_2$; (*iii*) 2.5N HCl

Bromination of (4) to (5) was achieved using PBr_5 as brominating reagent, the reaction occurring with 65% yields.† Compound (6) was obtained from (5) using sodium ethoxide in ethanol and diethyl malonate [10] in 40% yields. All attempts to improve the yields with different bases (Na–toluene or NaH–tetrahydrofuran) or different leaving groups were unsuccessful. Compound (2c) was obtained from (6) by hydrolysis with 2.5N HCl for 3 h and purification on a cation-exchange resin.[11] Synthetic (*S*)-(2c) was transformed into its camphanoylamide methyl ester (3a) and the ^{1}H n.m.r. of this derivative was examined in the presence of [Eu(dpm)$_3$]. The resonance of the C-4′ H_R is at δ 4.70 (multiplet, 0.9 H). In contrast, the C-4′ hydrogen in the derivative (3a) for the 4-[4-^{2}H]aminobutyric acid produced enzymatically from (1) in ^2H_2O resonates at δ 5.02. Therefore, the abovementioned C-4′ hydrogen is necessarily H_S and the deuteriated 4-aminobutyric acid from the enzymic reaction is (*R*)-(2b). Consequently, decarboxylation of (1) in ^2H_2O in the presence of glutamate decarboxylase proceeds with retention of configuration. This result is in agreement with the reported data on the stereochemistry of pyridoxal phosphate-dependent decarboxylases.[4-6]

* Armarego *et al.*[8] did not report this reaction in the reductive process; this is presumably due to different amounts of BEt_3 in different preparations of Superdeuteride.

† The use of PBr_3,Br_2 and P_4, or PPh_3Br_2 in dimethylformamide, was unsatisfactory; chlorination of (4) with PCl_5 or $SOCl_2$ proceeded with similar yields to those of (5).

EXPERIMENTAL

I.r. spectra were obtained on Nujol mulls with a Perkin-Elmer 157 spectrometer. ^{1}H N.m.r. spectra (60 MHz) were determined in $CDCl_3$ solutions (tetramethylsilane as internal standard) on a Hitachi–Perkin-Elmer R-24 spectrometer; 100-MHz spectra were recorded on a Varian S-100 XL spectrometer, locked on the ^{2}H signal at 15.4 MHz. Mass spectra were performed on a Varian 11 spectrometer. The progress of reactions was monitored by t.l.c. on silica gel (HF 254) microplates; g.l.c. determinations were on a 2-m silanized-glass column of 1% SE 30 on Gas-Chrom Q operating at 150—250 °C. Distillations were performed on a Büchi GKR 500 glass tube oven. Glutamate decarboxylase was purchased from Fluka, and Superdeuteride from Aldrich.

Preparation of (2b) *from* (1) *by Enzymic Decarboxylation.*—The incubation mixture was prepared as follows: compound (1) (15 mg), sodium carbonate (5 mg), [U-^{14}C]-(1) (270 mCi mmol^{-1}) as tracer, and 1M phosphate buffer (pH 7; 25 μl) were mixed and brought to dryness. ^2H_2O (10 ml), pyridine (160 μl), and fatty-acid-free albumin (5 mg) were added to the above mixture and the pH was adjusted to 4.6. Addition of glutamate decarboxylase (2 mg) started the reaction which was kept at 35 °C (20 h). The incubation was stopped by addition of methanol. A cation-exchange column (AG 50W-X2, 100—200 mesh, previously washed with 1% HCl and 7% ammonia) was used to separate the product. The resin was equilibrated with 0.1M acetate buffer (pH 4) before use. The incubation mixture was dried, suspended in 0.1M acetate buffer (5 ml), and loaded onto the column. Unreacted (1) was eluted with distilled water (70 ml), and (2b) with 7% ammonia (50 ml). An aliquot of the ammonia fraction was evaporated to dryness and its content of (2b) examined by g.l.c.–mass spectrometry, as the tris(trimethylsilyl) derivative. Quantitative estimation of (2b) and its deuterium content was performed on a Finnigan 9000 mass spectrometer, using the peaks at *m/e* 304 {$[M - 15]^+$, tris(trimethylsilyl) derivative of unlabelled (2a)} and *m/e* 305 {$[M - 15]^+$, tris(trimethylsilyl) derivative of (2b)}. Under the conditions of incubation described there was 98% conversion of (1) into (2b), and the deuterium content in (2b) was 95—98%.

(S)-2-*Bromo*-N-*phthaloyl*-[1-^{2}H]*ethylamine* (5).—(+)-(*S*)-

2-Hydroxy-*N*-phthaloyl-[1-^{2}H]ethylamine (4) (1.9 g, 10 mmol) and phosphorus pentabromide (4.79 g, 11 mmol) were mixed with stirring at 100 °C. Reaction occurred instantly, and when effervescence and fume development ceased the reaction was cooled to room temperature. The residue was taken up in ether (3 × 20 ml), the ether washed with sodium bicarbonate solution and water, and then dried (Na_2SO_4). After evaporation of solvent, the residue was recrystallized from ethanol; m.p. 80—82 °C (lit.,[11] 83 °C); $\nu_{max.}$ 1 768, 1 710, and 1 240 cm^{-1}; δ 3.63 (d, CHDBr, *J* 7 Hz), 4.13 (d, CHDN, *J* 7 Hz), and 7.90 (4 H, m, aromatic) (Found: C, 46.70; H, 3.75; N, 5.30. $C_{10}H_7DBrNO_2$ requires C, 47.20; H, 3.55; N, 5.49%). 2-Bromo-*N*-phthaloylethylamine, m.p. 81 °C (lit.,[12] 83 °C) was prepared as above starting with 2-hydroxy-*N*-phthaloylethylamine; it had $\nu_{max.}$ 1 768, 1 710, and 1 240 cm^{-1}; δ 3.63 (t, CH_2Br, *J* 7 Hz), 4.13 (t, CH_2N, *J* 7 Hz), and 7.90 (4 H, m, aromatic) (Found: C, 46.80; H, 3.34; N, 5.34. $C_{10}H_8BrNO_2$ requires C, 47.30; H, 3.17; N, 5.51%).

(S)-*Ethyl* 4-(N-*Phthaloyl*)-[4-^{2}H]*amino*-2-*ethoxycarbonylbutanoate* (6).—Na (0.3 g, 13 mmol) was dissolved in absolute ethanol (3 ml), diethyl malonate (2 ml, 13 mmol) was added, and the mixture was warmed until clear. (5) (2.84 g, 11.5 mmol) was then added, the solution was stirred at room temperature for 8 h, and the crude reaction mixture was steam-distilled. The oily residue was triturated with ether, but no precipitation occurred, and the oily product was thus directly hydrolysed to (2c). Part of the residue, however, was purified by column chromatography on neutral alumina (Merck, grade II, 70—230 mesh). Elution with hexane–ether (20 : 80, v/v) gave (6) which was recrystallized from n-hexane at −20 °C, m.p. 40—42 °C (lit.,[10] 42—44 °C); $\nu_{max.}$ 1 750 and 1 715 cm^{-1}; δ 1.25 (6 H, t, 2 Me, *J* 7 Hz), 2.30 (2 H, br d, CH_2CH, *J* 7 Hz), 3.43 (1 H, t, CH, *J* 7 Hz), 3.82 (1 H, t, CHD, *J* 7 Hz), 4.3 (4 H, t, OCH_2, *J* 7 Hz), and 7.82 (4 H, m, aromatic); *m/e* 333 (M^+), 288, and 242 (Found: C, 60.90; H + D, 6.13; N, 3.90. $C_{17}H_{18}DNO_6$ requires C, 61.07; H + D, 6.03; N, 4.19%).

(+)-(S)-4-*Amino*-[4-^{2}H]*butanoic Acid* (2c).—Crude (6) was treated with 2.5N HCl (10 ml) at 170 °C for 3 h. On cooling phthalic acid separated out and was filtered off. The mother liquor was purified on a column (1 × 13 cm) of AG 50 W X2 (100—200 mesh, H^+ form) as follows. The pH was adjusted to 4 in an acetate buffer (0.1M) and the aqueous solution loaded on the column; purification was then carried out as described for (2b) from enzymic decarboxylation. The fractions eluted with 7% ammonia were collected and dried to give (2c) (200 mg). It was recrystallized from ethanol–water, m.p. 202—203 °C (lit.,[13] 202 °C). From (2c), methyl (+)-(*S*)-4-phthalimido-[4-^{2}H]-butanoate was prepared as described[6] and its optical rotation recorded (Perkin-Elmer 141 spectropolarimeter), $[\alpha]_{589}$ +0.72° (lit.,[6] +0.73°). In an analogous manner, the same derivative of (2b) gave $[\alpha]_{589}$ −0.83° (lit.,[6] −0.84°).

4-[(1S,4R)-ω-*Camphanoylamido*]*butanoic Acid*.—(1*S*,4*R*)-ω-Camphanoyl chloride [350 mg, 1.615 mmol (from Chemipan, Warsaw)] was sublimed just prior to use (70 °C, 5 mmHg). A solution of this sublimed camphanoyl chloride (300 mg, 1.38 mmol) in toluene (0.54 ml) at 0 °C was treated simultaneously with a solution of (2a) in 2N sodium hydroxide (0.52 ml) and 3N sodium hydroxide (0.52 ml), and stirred at room temperature for 3 h, keeping the pH above 7 by dropwise addition of 2N sodium hydroxide. The mixture was washed with ether and then acidified, extracted with ether (3 × 5 ml), dried (Na_2SO_4), and solvent evaporated off to give an oily residue. Treatment of this residue with diazomethane in ether afforded *methyl* 4-[(1S,4R)-ω-*camphanoylamido*]*butanoate* (3a), which was purified on a silica gel column (Merck, 70—200 mesh). The product was eluted with benzene–ethyl acetate (1 : 1, v/v), yield 280 mg, and recrystallized from ether, m.p. 52—54 °C; $\nu_{max.}$ 3 320, 1 798, 1 735, 1 660, and 1 538; δ 0.92 (s, C-4 Me), 1.12 (s, C-7 Me_2), 1.6—2.1 (m, C-3′ methylene, C-5 methylene, and C-6 H), 2.3—2.7 (m, C-2′ methylene and C-6 H), 3.40 (m, C-4′ methylene, *J* 7 Hz), 3.72 (s, OMe), and 6.75 (broad, NH) (Found: C, 61.10; H, 8.20; N, 4.40. $C_{15}H_{23}NO_5$ requires C, 60.59; H, 7.8; N, 4.71%). Addition of 0.3—0.5 mol. equiv. of [Eu(dpm)$_3$] solution to a solution of (3a) caused a shift of the two C-4′ hydrogen resonances, to become two multiplets centred at δ 4.70 and 5.02.

The camphanoyl derivatives of (2b) and (2c) were prepared in a similar manner. After column chromatography, additional purification was achieved by preparative t.l.c. The ^{1}H n.m.r. pattern was as described in the text.

We thank the Italian National Council for Research (C.N.R.) for support, Professor A. Fiecchi for helpful discussions, and Professor P. Gariboldi for the 100-MHz deuterium-decoupled ^{1}H n.m.r. spectra.

[8/1074 *Received*, 8*th June*, 1978]

REFERENCES

[1] E. A. Boeker and E. E. Snell, 'The Enzymes,' Academic Press, New York, 1972, vol. VI, pp. 217—253.
[2] J. F. Collins, 'Annual Reports (B),' The Chemical Society, London, 1976, vol. 73, p. 427.
[3] K. Krnjevic, *Nature*, 1970, **228**, 119.
[4] B. Bellau and J. Burba, *J. Amer. Chem. Soc.*, 1960, **82**, 5751.
[5] E. Lestner and I. D. Spencer, *J.C.S. Chem. Comm.*, 1977, 378.
[6] H. Yamada and M. O'Leary, *Biochemistry*, 1978, **17**, 669.
[7] P. M. Jordan and M. Akhtar, *Biochem. J.*, 1970, **116**, 227.
[8] W. L. Armarego, B. A. Milloy, and W. Pendergast, *J.C.S. Perkin I*, 1976, 2229.
[9] H. C. Brown and S. Krishnamurthy, *J. Amer. Chem. Soc.*, 1973, **95**, 1669.
[10] S. Gabriel, *Ber.*, 1890, **23**, 1768.
[11] Purification on a small scale following the procedure described in *Org. Synth., coll. vol. II*, p. 25, was cumbersome and unsatisfactory.
[12] T. R. Seshadri, *J. Chem. Soc.*, 1929, 2952.
[13] J. Tafel and M. Stern, *Chem. Ber.*, 1900, **33**, 2230.