# SYNTHESIS OF (2S,5R)-[5-$^2$H]PROLINE

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## SUMMARY

The title compound was obtained by preferential crystallisation of (2RS,5R)-[5-$^2$H]proline. The labelled racemic amino acid was synthesized in five steps from (5R)-[5-$^2$H]-2-pyrrolidone, which was prepared by cyclisation of (4R)-[4-$^2$H]GABA. The stereospecific label was introduced into GABA enzymatically decarboxylating in $^2H_2O$ (2S)-glutamic acid, in the presence of glutamate decarboxylase from E. coli.

Key Words: Proline, (5R)-[5-$^2$H]-2-pyrrolidone, (4R)-[4-$^2$H]-4-aminobutyric acid, glutamate decarboxylase.

## INTRODUCTION

In order to study the stereochemistry of some enzymatic reactions of proline, specimens of this amino acid stereospecifically labelled at 5-position with deuterium or tritium are desirable. To our knowledge, a method to prepare (2S,5R)-[5-$^2$H or $^3$H]proline (8) has not yet been reported in literature. We report here the preparation of the deuterated amino acid in satisfactory yields, good isotopic abundance and high optical purity, starting from (5R)-[5-$^2$H]-2-pyrrolidone (3). This stereospecifically labelled compound can be obtained by the well known enzymatic decarboxylation of (2S)-glutamic acid[1] in $^2H_2O$, followed by ring closure[2] (Scheme 1).

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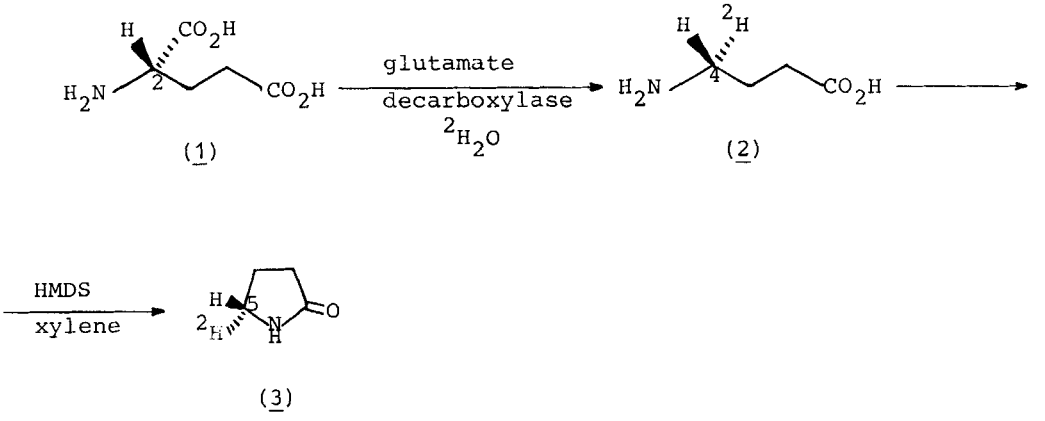
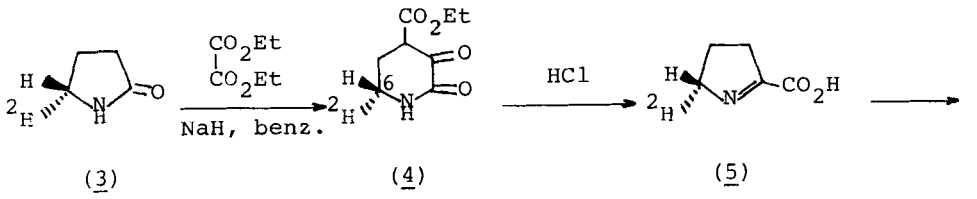
SCHEME 1

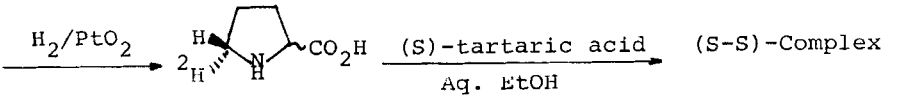



(1) (2) (3)

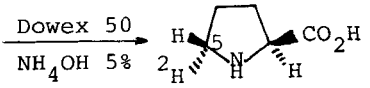
SCHEME 2




(3) (4) (5)




(6) (7)




(8)

## RESULTS AND DISCUSSION

(5R)-[5-$^2H$]-2-Pyrrolidone (3) was synthesized as reported in Scheme 1. (2S)-Glutamic acid (1) was decarboxylated to (4R)-[4-$^2H$]-4-aminobutyric acid (GABA) (2) by glutamate decarboxylase from Escherichia coli (EC 4.1.1.15) in deuterated water. The stereochemistry of such a reaction, which proceeds with retention of configuration, has been recently clarified in two independent ways[1,3].

Subsequent cyclisation of GABA with hexamethyldisilazane in xylene[2] afforded the desired pyrrolidone (3), with a total yield of 45% starting from glutamic acid. The retention of label in 3 was confirmed by spectroscopic methods (MS and NMR, $^2H_1$-species= 75±5%). (5R)-[5-$^2H$]-2-pyrrolidone was then transformed into (2S,5R)-[5-$^2H$]proline (8) by a modification of the method of Hasse and Wieland[4] (see Scheme 2)†.

Pyrrolidone (3) was condensed with diethyl oxalate (pyrrolidone-oxalate ratio 1:5) in benzene in the presence of sodium hydride to give (6R)-2,3-dioxo-4-ethoxycarbonyl-[6-$^2H$]piperidone (4) (55% yield). After acidic hydrolysis (HCl 6N) of 4, (5R)-[5-$^2H$]-$\Delta^1$-pyrroline-2-carboxylic acid (P2C, 5) so formed was hydrogenated with $H_2/PtO_2$. This hydrogenation was carried out by stepwise addition of fresh catalyst till complete reduction to proline. The reaction product was then purified by column chromatography on Dowex 50-X8 and crystallised from methanol-acetone to obtain pure (2RS,5R)-[5-$^2H$]proline (6) (65% yield). Proline yield was estimated by spectrophotometric analysis of its ninhydrine derivative[5] and the percentage of $^2H_1$-species (70±5%) by MS analysis based on the fragmentation ion at m/e 71 ($M^+-CO_2H$).

The racemic proline (6) was resolved by preferential crystallisation from aqueous ethanol of the diastereoisomeric solid complex (S)-proline-(S)-tartaric acid[6]. This complex (7),

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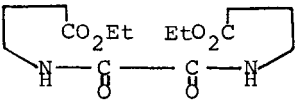
† When the reaction was carried out following the procedure described by Hasse and Wieland[4] we obtained N,N'-oxalyl-bis-(ethyl γ-aminobutyrate) (9) as the main product:



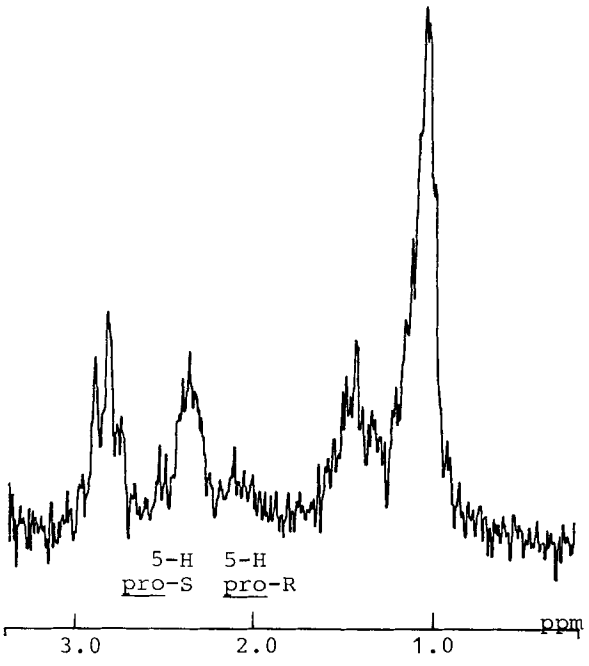
(9)

obtained in 65% yield after recrystallisation, was checked for its chemical purity by m.p., elemental analysis, IR and NMR, and for its optical purity[6]. Pure (2S,5R)-[5-$^2$H]proline (8) ($^2H_1$-species= 70±5%) was isolated after column chromatography of 7 on Dowex 50-X8, eluting with 5% $NH_4OH$.

Positional and configurational retention of deuterium from 2 to 8 was proved by comparing the $^1$H-NMR spectrum of 8 in $^2H_2O$-$NaO^2H$ (pH=13) with that of unlabelled (2S)-proline. The signals of the 5-protons of (2S)-proline in such a basic medium appear as two well separated multiplets, the lowest-field signals being due to the pro-S proton[7]. Deuterated proline (8) exhibited a multiplet centered at 2.10$\delta$ corresponding only to 0.3 proton (in addition to the expected multiplet at 2.40$\delta$ (1H)), thus indicating a complete stereospecific deuteration (pro-R) (Fig.1). The same conclusions were drawn comparing the NMR spectra of (S)-proline-(S)-tartaric acid complexes in $^2H_2O$-$NaO^2H$.

The method described here can, of course, be extended to the preparation of both the stereoisomers of proline, stereospecifically deuterated or tritiated at 5-position.

FIGURE 1



## EXPERIMENTAL

IR spectra were recorded on a Perkin-Elmer 257 spectrometer, $^1$H-NMR spectra on a Varian XL-100 spectrometer, using TMS as internal standard for spectra in $C^2HCl_3$ and DSS for spectra in $^2H_2O$. Mass spectra were performed on a Varian Mat 112 mass spectrometer and UV-VIS spectra on a Perkin-Elmer 551 spectrophotometer.

Elemental analyses were consistent with calculated values. TLC were carried out on silica gel plates (Merck), using the following developing systems: i) n-BuOH-$H_2O$-AcOH 6:2:2 for compounds 1,2 and 5-8 (saturated solution of ninhydrine in acetone as spraying mixture for spots detection); ii) $CHCl_3$-MeOH 8:2 for compounds 3 and 4 (spots detection by exposure to iodine vapors).

(5R)-[5-$^2$H]-2-pyrrolidone (3). (4R)-[4-$^2$H]-4-aminobutyric acid (2) was obtained from enzymatic decarboxylation of (2S)-glutamic acid (1) (880 mg, 6 mmol) in $^2H_2O$ according to the general procedure described in ref. 1. To eliminate proteins, the reaction mixture (100 ml) was boiled for 15 min and centrifuged for 15 min at 1200g. Most $^2H_2O$ was distilled from the supernatant and used for others incubations. After methanol addition (100 ml), a second centrifugation (15 min at 1200g) separated almost completely proteins still present. The supernatant was eventually evaporated to dryness and dried in a store at 100° C.

The dry residue (760 mg) was refluxed for 6 h in xylene (150 ml) containing hexamethyldisilazane (19 ml) and a few drops of dimethyldichlorosilane[2], diluted with ethanol (400 ml) and evaporated. The crude pyrrolidone so obtained was taken up with chloroform, filtered through a celite pad, evaporated under vacuum and chromatographed on a silica gel column (Merck, 70-230 mesh) collecting fractions eluted with $CHCl_3$-MeOH 9:1. Evaporation of these fractions afforded pure pyrrolidone (260 mg, 45% yield).

The percentage of deuteration was 75±5% by MS and NMR analyses.
IR (liquid film): 3250 $cm^{-1}$ (N-H amide); 2200 (C-$^2$H); 1685 (C=O amide).
$^1$H-NMR ($C^2HCl_3$): 6.4 $\delta$ (1H, broad s, exchangeable with $^2H_2O$, N-H), 3.56 (ca. 1H, t broad for H-$^2$H geminal coupling, H-5), 2.6-2.1 (4H, complex m, 2 $CH_2$).
MS (d.i.s.) m/e (I%): 86 ($M^+$, 61), 85 (20), 57 (10), 56 (13), 43 (88), 42 (100), 41 (35).

(6R)-2,3-dioxo-4-ethoxycarbonyl-[6-$^2$H]piperidone (4). Ethyl oxalate (1 ml) and (5R)-[5-$^2$H]pyrrolidone (3, 260 mg) in dry

benzene (5 ml) were slowly added to a suspension of sodium hydride (200 mg) in dry benzene (30 ml) in Ar atmosphere under stirring. The reaction mixture was warmed at 90°C for about 20 h (the initial yellow color changed to red). After addition of HCl 0.01 N and then HCl 6 N, the two layers were separated without preliminary cooling and the aqueous phase extracted with chloroform (4 x 20 ml). The organic solutions were then collected and evaporated under vacuum to give an oil residue (450 mg), which, when washed with ethyl ether, afforded pure 4 (55% yield).
IR (nujol): 3300 $cm^{-1}$ (N-H); 1660 (C=O cyclic amide); 1620 (C=O ketoester in the enolic form).
$^{1}$H-NMR ($C^{2}HCl_3$): 11.36δ (1H, broad s, exchangeable with $^{2}H_2O$, enolic OH), 6.76 (1H, broad s, exchangeable with $^{2}H_2O$, N-H), 4.3 (2H, q, J 7 Hz, $OC\underline{H}_2CH_3$), 3.4 (ca. 1H, t broad for coupling with $^{2}$H, J 7 Hz, $C\underline{H}^{2}H$-NH), 2.6 (2H, d, J 7 Hz, $C\underline{H}_2$-$CH^{2}H$-NH), 1.34 (3H, t, J 7 Hz, $OCH_2CH_3$).
MS (d.i.s.) m/e (I%): 186 ($M^+$, 73), 158 (38), 141 (63), 112 (100), 84 (57), 55 (53).

When the condensation reaction was carried out as reported in ref. 4 the main product was N,N'-oxalyl-bis(ethyl γ-amino-butyrate) (9), which was identified by spectroscopic methods.
M.p. 105-106°C (lit.[4] 106°C).
IR (nujol): 3300 $cm^{-1}$ (N-H); 1735 ($CO_2Et$); 1660 (CONHR).
$^{1}$H-NMR ($C^{2}HCl_3$): 7.75δ (1H, broad t, NH), 4.05 (2H, q, J 7 Hz, $OC\underline{H}_2CH_3$), 3.34 (2H, dt, $J_1$=$J_2$ 6.5 Hz, $C\underline{H}_2$-NHCO), 2.3 (2H, t, J 6.5 Hz, $C\underline{H}_2$-$CO_2Et$), 1.84 (2H, tt, $J_1$=$J_2$ 6.5 Hz, $C\underline{H}_2$-$CH_2CO_2Et$), 1.2 (3H, t, J 7 Hz, $OCH_2C\underline{H}_3$).
(2RS,5R)-[5-$^{2}$H]proline (6). The hydrolysis of 4 was performed with HCl 6 N (4.6 ml) at 155°C under stirring for 6 min. The formation of $\Delta^1$-pyrroline-2-carboxylic acid (5, P2C) was detected only by TLC (a single orange spot when sprayed with ninhydrine reagent), HCl was then eliminated by careful evaporation (35-40°C) under vacuum. After dilution with water (5 ml), hydrogenation over platinum oxide (50 mg initially and additional 20 mg after one day) gave 6 (pure on TLC). The catalyst was filtered off and the solution was chromatographed on a Dowex 50-X8 column (15 ml) using $NH_4OH$ 1 N as eluent. Each fraction was examined for its proline content by qualitative ninhydrine assay as reported in ref. 8. Final lyophilisation afforded 100 mg of (2RS,5R)-[5-$^{2}$H]-proline (65% yield). Quantitative photometric estimation at 515 nm of the ninhydrine derivative of proline was also performed as indicated in ref. 5. Before resolution, proline was crystallised

by dissolution in methanol (0.3 ml) followed by addition of acetone (1.25 ml) at 5°C. The percentage of deuteration was confirmed to be 70±5% by MS analysis on the fragmentation ion at m/e 71 ($M^+$-$CO_2H$).

(S)-proline-(S)-tartaric acid complex (7). (2RS,5R)-[5-$^2$H]proline (6) was resolved by using 0.5 molar amount of (S)-tartaric acid. A mixture of 6 (80 mg) and (S)-tartaric acid (52 mg) in water (0.07 ml) was gradually diluted with ethanol (0.42 ml) under stirring. This solution was then seeded with a small amount of (S-S)-complex previously prepared. Incipient crystallisation was further stimulated by ethanol addition (0.63 ml) and the mixture allowed to stand overnight at -5°C. Crystals so formed were filtered, washed with ethanol and dried (60 mg, 65% yield).

M.p. 153-154°C (lit.[6] 154°C).

$[\alpha]_D$ = -23.97° (c=1, water) optical purity 99%.

IR (nujol): as reported in ref. 6.

$^1$H-NMR ($^2H_2O$, pH 7): 4.25$\delta$ (2H, s, CHOH tartaric acid), 3.75 (1H, m, 2-H proline), 3.0 (1.3H, m, 5-H proline), 1.95 (1H, m, 3-H pro-S proline), 1.8-1.45 (3H, m, 3-H pro-R and 4-$H_2$ proline).

($^2H_2O$ + Na$O^2$H, pH 13): 3.65$\delta$ (2H, s, CHOH tartaric acid), 2.8 (1H, m, 2-H proline), 2.4 (1H, m, 5-H pro-S proline), 2.1 (0.3H, m, residual 5-H pro-R proline), 1.45 (1H, m, 3-H pro-S proline), 1.35-0.95 (3H, m, 3-H pro-R and 4-$H_2$ proline).

(2S,5R)-[5-$^2$H]proline (8). The title compound was recovered from its complex with tartaric acid by column chromatography on Dowex 50-X8 ion exchange resin. The complex (60 mg) dissolved in water (1 ml) was passed through the resin (5 ml) and (S)-proline was eluted with 5% $NH_4OH$ (20 ml). The collected eluates were concentrated, treated with charcoal and concentrated again to dryness. The residual crystals were recrystallised as indicated above for racemic proline to give pure 8 (90% yield).

$[\alpha]_D$ = -85° (c=1, water) optical purity 99.4%.

$^1$H-NMR ($^2H_2O$, pH 7): 3.74$\delta$ (1H, m, 2-H), 2.99 (1.3H, m, 5-H), 1.95 (1H, m, 3-H pro-S), 1.8-1.45 (3H, m, 3-H pro-R and 4-$H_2$).

($^2H_2O$ + Na$O^2$H, pH 13): 2.81$\delta$(1H, m, 2-H), 2.4 (1H, m, 5-H pro-S), 2.1 (0.3H, m, residual 5-H pro-R), 1.45 (1H, m, 3-H pro-S), 1.35-0.95 (3H, m, 3-H pro-R and 4-$H_2$).

MS (d.i.s.) m/e (I%): 71 ($M^+$-$CO_2H$, 100).

The percentage of deuteration was estimated 70±5% by MS and NMR.

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