SYNTHESIS OF SPECIFICALLY LABELLED TRYPTOPHANS: 5,7-$^3H_2$ AND 4,6-$^3H_2$ TRYPTOPHAN

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SUMMARY

Syntheses of 5,7-$^3H_2$ and 4,6-$^3H_2$ tryptophan, respectively from 2,4,6-$^3H_3$- and 3,5-$^3H_2$-aniline, were obtained by Fischer cyclization of suitable labelled phenylhydrazones.

Key Words: Aniline, Tryptophan, Tritium.

INTRODUCTION

Some specifically tritium and deuterium labelled tryptophans are known in literature[1,2], being mainly used for biogenetic studies and stereospecific enzymatic reaction mechanisms.

In our laboratory, we particularly needed tryptophans specifically tritiated at the 5,7 and 4,6 positions, in order to investigate the prenylation mechanism in the biosynthesis of indole metabolites produced by some strains of *Aspergillus amstelodami* and *Claviceps paspali*.[3]

In this report, we describe the synthetic pathway used to synthesize the above mentioned tryptophans.

RESULTS AND DISCUSSION

Different approaches were followed to obtain required products. At first, we studied the exchange reaction of 5- and 6-hydroxytryptophan with tritiated water. With this procedure tritium was

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not equally distributed in the required positions.[4] Moreover, product purification following dehydroxylation by hydrogenolysis of N-phenyl-tetrazolyl derivatives[5] proved to be very difficult, as a mixture of hardly separable compounds was obtained in the former step. Another problem arose from commercial unavailability of 6-hydroxytryptophan, otherwise difficult to synthesize.

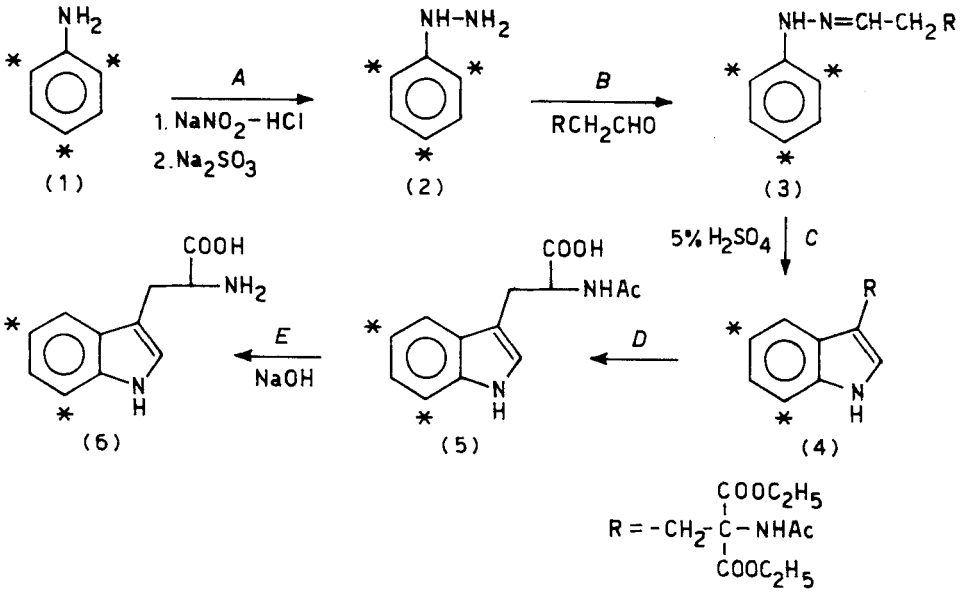
Reduction by tritium gas on bromotryptophans (bromine in the 4,6 or 5,7 positions respectively) was discarded owing to the troublesome synthesis of required starting bromoindoles.

A further attempt to synthesize 5,7-$^3H_2$-tryptophan through a Madelung condensation[6] was made starting from 3,5-$^3H_2$-*o*-toluidine; however, this method was also discarded on the basis of low yields and remarkable label losses during cyclization step. Better results were obtained following the tryptophan synthesis suggested by Warner and Moe[7] (Scheme 1); by this method we performed a satisfactory synthesis of both required specifically labelled tryptophans.

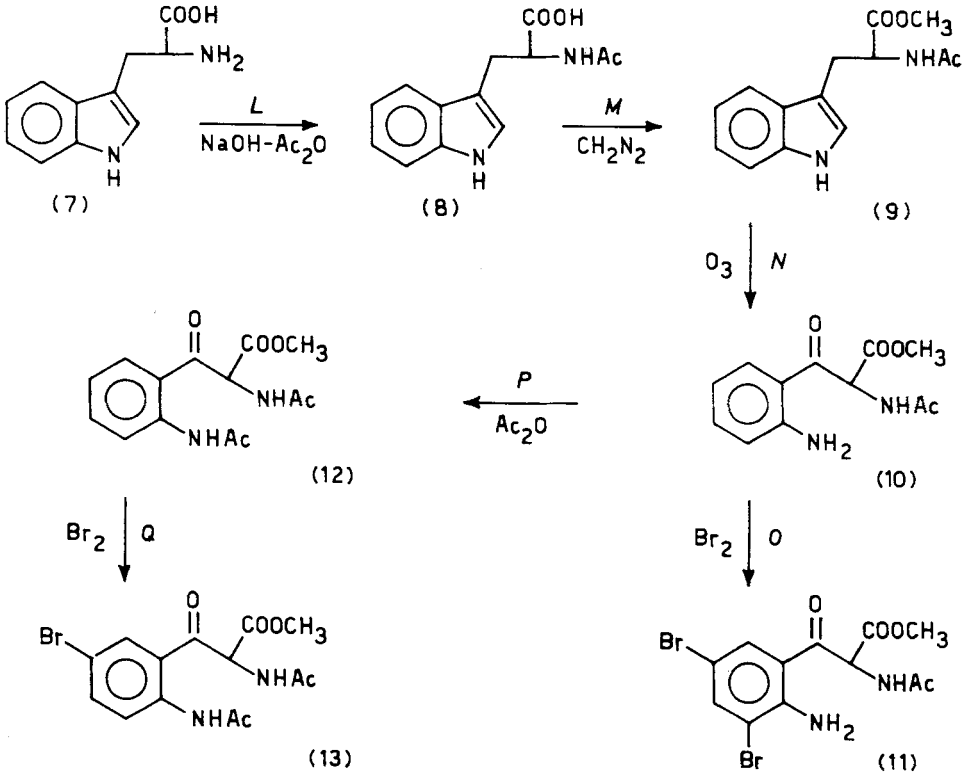
5,7-$^3H_2$-tryptophan (6) was achieved as final product, from 2,4,6-$^3H_3$-aniline (1) obtained by exchange of aniline hydrochloride with tritiated water: about 35% of the label split out during step C; moreover, in the subsequent alkaline hydrolysis (step D and E) further amounts of tritium were lost (10-20%). The experimental conditions were suitably arranged in order to minimize such label losses.

As the distribution of tritium between 5 and 7 positions of tryptophan was also needed, we performed the ozonolysis of 5,7-$^3H_2$-tryptophan and the bromination at the labelled positions of N-acetyl-kynurenine (10) and N,N'-diacetyl-kynurenine (12)(Scheme 2). In this way the actual label distribution was obtained, showing a higher label in 7 than in 5 position (ratio 3:2), while in the starting aniline the ratio was 1:1, as determined from specific molar activity of 2,6-$^3H_2$-4-bromo-acetanilide and 2,4,6-tribromo-aniline, synthesized from labelled aniline. The activity data after

Scheme 1



Scheme 2



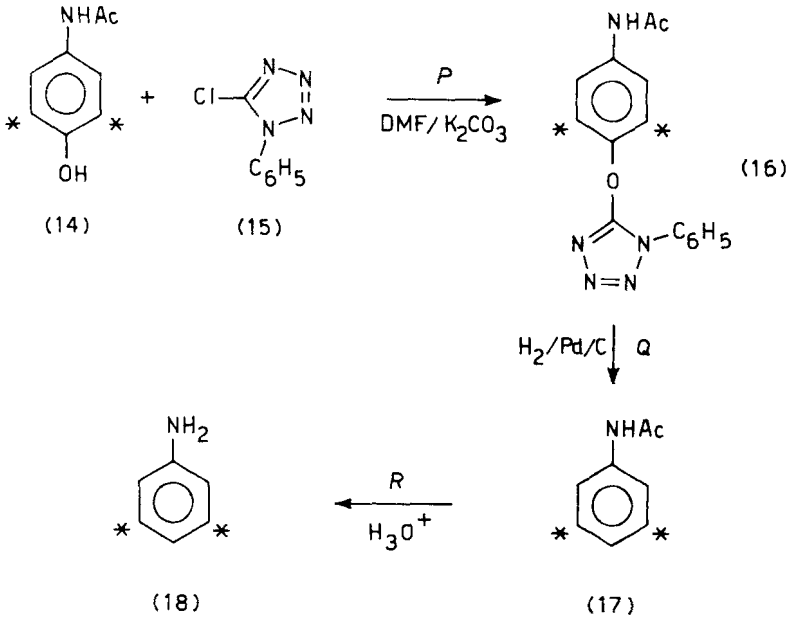
steps *D* and *E* showed some exchange not yet completely understood.[8]

4,6-$^3H_2$-tryptophan synthesis from 3,5-$^3H_2$-aniline (18) occurred with slight label losses (5-6%); (18) was prepared according to the sequence shown in Scheme 3, whereas (14) was obtained by exchange with tritiated water in basic medium; such exchange is specific in ortho position with respect to the hydroxy group, as determined by conversion of (18) to 2,4,6-tribromoaniline, which occurred without appreciable label losses (less than 2%).

Actually we found some label losses in the whole sequence from (14) to 4,6-$^3H_2$-tryptophan (about 30%), which were due mainly to step *R*,[9] thus indicating marked labelling in the $COCH_3$ group.

Anyway, both label positions being symmetrical, any loss from the aromatic ring would not influence the uniformity of label distribution.

Scheme 3



EXPERIMENTAL

Radioactivity assays: measures were taken with a Beckmann L.S. liquid scintillation counter; the scintillation mixture was: Permafluor (Packard) ml 55, naphthalene g 150, 2-ethoxyethanol ml 300, toluene ml 600. ^{3}H-hexadecane and ^{14}C-hexadecane were added to each sample as internal standards. Each step described (see infra) has been carefully checked with unlabelled and/or deuterium labelled compounds. All reagents (Merck) were carefully purified before use.

5,7-$^{3}H_2$-tryptophan

Aniline hydrochloride (200 mg) was dissolved in tritiated water (300 mCi, 0.5 ml) and heated at 100° in a sealed tube for two days. The solvent was removed and the residue exchanged twice with MeOH and diluted with 1.3 g of unlabelled product. The sample (1.5 g, 320 μCi/mM) was dissolved in conc HCl (3.5 ml) and added with 30% aqueous sodium nitrite (3 ml) at 0° under stirring. The diazonium salt solution was quickly poured in a refrigerated Na_2SO_3 solution (20% w/w, 20 ml), freshly prepared from aqueous NaOH and SO_2. The reaction mixture was then slowly heated to 70°, kept at this temperature for 30 min, filtered and chilled at 0°; cold conc HCl (30 ml) was then added to precipitate the phenylhydrazone hydrochloride. The salt was recovered, dissolved in cold water and treated with 10% NaOH; the free base was extracted four times with benzene and evaporated to dryness at reduced pressure, yielding 1.1 g of (2) without appreciable tritium losses (315 μCi/mM). (2) and acetic acid (0.2 ml) were added to a benzene solution of γ-acetamido-γ,γ-dicarbethoxybutyraldehyde, freshly prepared from ethyl-acetamidomalonate (2.5 g) and acrolein (0.9 ml).[10] The solution was then slowly concentrated at room temperature to allow precipitation of labelled (3) (2.8 g, m.p. 142°, 310 μCi/mM), which was suspended in 5% H_2SO_4 (15 ml) and heated at 100° under N_2, with

efficient stirring, until the oily suspension solidified (about 1hr). The cyclization product (4) (2.1 g, m.p. 155°, 206 μCi/mM) was crystallized from methanol, suspended in 10% NaOH (10 ml), and refluxed for 2 hrs under N_2. The reaction mixture was then acidified to pH 1-2 with 20% H_2SO_4 and exhaustively extracted with ether. The dicarboxylic acid thus separated (1.4 g, m.p.144°, 185 μCi/mM) was quickly decarboxylated to (5) by refluxing for 1 hr its aqueous solution, under N_2. Finally, the hydrolysis of (5) (1.0 g, m.p. 206°, 183 μCi/mM) was accomplished by refluxing in 10% NaOH (6 ml) for 8 hrs under N_2. After neutralization, precipitated tryptophan (6) was repeatedly crystallized from water. Yield 530 mg of pure (6) (m.p. 280-82°, 173 μCi/mM). A portion of (6) was added with 3'-^{14}C-tryptophan and crystallized twice more without modification of $^3H/^{14}C$ ratio.

4,6-$^3H_2$-tryptophan

4-acetamidophenol (0.2 g) was dissolved in dioxane (0.5 ml) and trimethylamine (0.2 ml), and exchanged with tritiated water (0.5 ml, 200 mCi) at 90° in a sealed tube for 3 days. The solvent was then removed and labelled acetamidophenol was repeatedly crystallized from aqueous MeOH to remove exchangeable tritium. 2,6-$^3H_2$--4-acetamidophenol (14) (11.5 mCi) was diluted with unlabelled product (0.8 g), dissolved in dimethylformamide (10 ml), added with dry K_2CO_3 (0.2 g) and 5-chloro-1-phenyl-tetrazole (15) (1.2 g) and heated at 100° for 30 min. After filtration and distillation of the solvent, (16) was purified by column chromatography (SiO_2, eluent: ethyl acetate), crystallized from ethanol (yield: 1.5 g, m.p. 166°, 1.6 mCi/mM), and dissolved in 1:1 MeOH/dioxane (20 ml); the solution was hydrogenated on 5% Pd/C (30°, 35 atm.) following the reaction by t.l.c. (SiO_2, eluent: 4:1 ethyl acetate/hexane). When the spot due to (16) disappeared (4 hrs) the solution was filtered, diluted with H_2O (50 ml), acidified with 5% H_2SO_4 and extracted with ether.

3,5-$^{3}H_2$-acetanilide (17) (450 mg, 1.5 mCi/mM) was recovered from the ethereal phase and hydrolyzed to (18) by refluxing in 20% H_2SO_4 for 20 min. (18) (310 mg, 1.05 mCi/mM) was diluted to 1.2 g with unlabelled product and used for synthesizing 4,6-$^{3}H_2$-tryptophan by the same procedure used for the 5,7-$^{3}H_2$-tryptophan. From 1.2 g of (18) (270 μCi/mM), 480 mg of required labelled tryptophan were obtained (m.p. 280-83°, 255 μCi/mM), after crystallization.

LABELLING PATTERN DETERMINATION

3,5-$^{3}H_2$-aniline (1)

(1) (10 mg) was mixed with unlabelled material (1.0 g), dissolved in ether and precipitated with dry HCl. Labelled hydrocloride (300 mg, m.p. 198°, 3.2 μCi/mM) was dissolved in water (10 ml), shaked with acetic anhydride (0.4 ml) and quickly poured in chilled aqueous sodium acetate (30%, 5 ml). The precipitated acetanilide (200 mg, m.p. 113°, 3.15 μCi/mM) was dissolved in acetic acid (3 ml) treated with bromine (1.2 mol. eq.) and poured in ice water (50 ml). Precipitated 4-bromo-acetanilide was crystallized from aqueous MeOH. Yield 250 mg, m.p. 167°, 2.1 μCi/mM.

Aniline hydrochloride (300 mg) dissolved in water (10 ml) was treated dropwise with bromine in acetic acid (3.5 mol. eq.) at 0° with efficient stirring. Precipitated 2,4,6-tribromoaniline was crystallized twice from MeOH. Yield 500 mg, m.p. 120°, 0.03 μCi/mM.

5,7-$^{3}H_2$-tryptophan (6)

(6) (20 mg, 17.3 μCi) and 3'-^{14}C-tryptophan (10 mg, 2 μCi) were dissolved in ammonia (2% w/w, 20 ml), added with unlabelled tryptophan (1.0 g); the solution was slowly evaporated at reduced pressure and the residue crystallized from water, yielding (7) (950 mg, $^{3}H/^{14}C$ =8.65). (7) (900 mg) were dissolved in N NaOH (5 ml), chilled and treated under stirring with a mixture of acetic anhydride (0.5 ml) and N NaOH (5 ml). The reaction mixture was kept at room temperature for 1 hr, then added slowly with dil HCl; precipitated (8) was cry-

stallized from aqueous MeOH (m.p. 206°, $^{3}H/^{14}C$ = 8.55). (8) (700 mg) was dissolved in ether and treated with a slight excess of CH_2N_2 yielding (9), which was isolated, dissolved in MeOH and subjected to ozonolysis.[11] The end of the reaction was monitored by a KI trap. The solvent was removed and row N-acetyl-kynurenine methylester (10) (700 mg) was purified on SiO_2 column (eluent: ethyl acetate). A portion of (10) (200 mg) was dissolved in acetic acid (3 ml) and treated at 0° with bromine (2.2 mol. eq.) for 1 hr; the reaction mixture was then poured in ice water and the precipitate (11) was crystallized from aqueous MeOH (150 mg, m.p. 190°, $^{3}H/^{14}C$ = 0.16).

Another portion of (10) (300 mg) was dissolved in acetic acid and pyridine and reacted with acetic anhydride (0.2 ml). The mixture was evaporated slowly at reduced pressure and the acetyl derivative (12) purified on SiO_2column (eluent: ethyl acetate), then dissolved in acetic acid and treated with bromine (1.2 mol.eq.) at 20° for 1 hr. The mixture was poured in water and the precipitate was purified on SiO_2 column and crystallized from MeOH (m.p. 178°, $^{3}H/^{14}C$ = 5.1). Physical, nmr and mass spectral data agreed with structure of products (10)-(13).

3,5-$^{3}H_2$-aniline (18)

(18) (10 mg) was mixed with unlabelled product (0.5 g), dissolved in ether and precipitated with dry HCl as labelled hydrochloride, which (300 mg, m.p.198°, 5.4 μCi/mM) was dissolved in water (10 ml) and treated with bromine (3.5 mol.eq.) in acetic acid at 0°, yielding 2,4,6-tribromoaniline, which was crystallized from MeOH (m.p.120°, 5.3 μCi/mM).

4,6-$^{3}H_2$-tryptophan

Labelling pattern determination was carried out in the same way as previously described for 5,7-$^{3}H_2$-tryptophan, starting from a ratio $^{3}H/^{14}C$ = 10.3, which did not change appreciably during steps *F, G, H, L*. The dibromoderivative (11) had $^{3}H/^{14}C$ = 9.8).

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REFERENCES

1 Plieninger H., Fischer R., Keilich G., and Orth H.D., *Ann. Chem.*, 642: 214 (1961).

2 Renson J., Daly J. W., Weissbach H., Witkop B., and Undenfriend S., *Biochem.Biophys. Res. Comm.*, 25: 504 (1966).

3 Casnati G., Gardini G. P., Palla G., and Fuganti C., *J.C.S. Perkin I*, 2397 (1974); Marchelli R., Dossena A., and Casnati G., *J.C.S. Chem.Comm.* 779 (1975).

4 Daly J. W., Witkop B., *J. Am.Chem.Soc.*, 89: 1023 (1967).

5 Musliner W. J. and Gates W. J. jr, *J. Am. Chem. Soc.*,88: 4271 (1966).

6 Verley M. A., *Bull. Soc. Chim. Fr.*, 35: 1039 (1924).

7 Warner D. T. and Moe O. A., *J. Am. Chem. Soc.*,70: 2765 (1948).

8 In our opinion during cyclization step tritium losses were more than 35%, as H in 5-position is also splitted out.[12] This loss would be masked by some tritium label on the methyl group, which was lost during steps *D* and *E*; in this way we could explain why tryptophan did not exchange in basic medium[13] and why we did not notice exchange in steps *D* and *E* of 4,6-$^3H_2$-tryptophan synthesis.

9 We found, however, that keeping temperature below 90° during tritiation of (14), tritium losses in step *R* were reduced, due to lower labelling of methyl group.

10 Moe O. A. and Warner D. T., *J. Am. Chem. Soc.*, 70: 2763 (1948).

11 Previero A., Scoffone E., Pajetta P., and Benassi C. A., *Gazz. Chim. Ital.*, 93: 841 (1963).

12 Bak. B., Dambmann C., and Nicolaisen F., *Acta Chem. Scand.*, 21 : 1674 (1967).

13 Unpublished work from our laboratory.