Synthesis of [^{14}C]Hippuryl-L-Histidyl-L-Leucine

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Summary

This paper describes the synthesis of [^{14}C]hippuryl-L-histidyl-L-leucine (hip-his-leu) from barium [^{14}C]carbonate. An overall radiochemical yield of 36% was obtained giving pure material with a specific activity of 17 mCi/mmol and a suitable substrate for the assay of angiotensin converting enzyme (ACE).

Keywords: Angiotensin Converting Enzyme, [^{14}C]hippuryl-L-histidyl-L-leucine, carbon-14

INTRODUCTION

Many peptides have been used as artificial substrates for Antiotensin Converting Enzyme (ACE) to measure levels of ACE in biological fluids and tissues. Both spectrometric and radiometric measurement methods have been employed. One of the most commonly used peptides is[^{14}C] hippuryl-histidyl-leucine and its use is well documented[1,4,5,8,9].

[^{14}C]Hip-his-leu is available commercially but is relatively expensive. The previously published synthesis[5] starts from

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[^{14}C]glycine which is more expensive than barium [^{14}C]carbonate as a source of carbon-14. A tritium labelled form has also been reported[6].

Two synthetic routes were explored as shown in Figures 1 and 2. Route 1 failed as we were unable to remove the Z protecting group selectively from the amino group of glycine when it was coupled to Z-histidine. This route would have been preferable as the label is inserted at a later stage. Route 2 was used successfully and produced [^{14}C]hip-his-leu with a radiochemical purity of 99% by tlc-radioscan. No chemical impurities could be detected by UV spectrophotometry.

The ACE assay is very sensitive to specific impurities and even a very small amount of [^{14}C]hippuric acid gives an unacceptably high blank in the assay. We have found that unidentified non-radioactive chemical impurities are potent inhibitors of ACE giving rise to low control values. [^{14}C]Hip-his-leu of acceptably high purity was achieved by preparative high performance liquid chromatography and stored in ethanol-water at 4°C to minimise decomposition.

EXPERIMENTAL

Materials and Methods Barium [^{14}C]carbonate was purchased from Amersham International plc. Organic solutions were dried over magnesium sulphate before evaporation. Tlc plates were scanned for radioactivity using a Panax Scanner.

Dicarbobenzoxy-L-histidine was prepared as described by Inouge and Otsuka[2] in 40% yield from L-histidine and benzylchloroformate. Mpt 87-90°C dec (lit.87-89°C).

Dicarbobenzoxy-L-histidyl-L-leucine, methyl ester was prepared[2] in 60% yield from dicarbobenzoxy-L-histidine and

Figure 1 Synthetic Route 1 (unsuccessful)

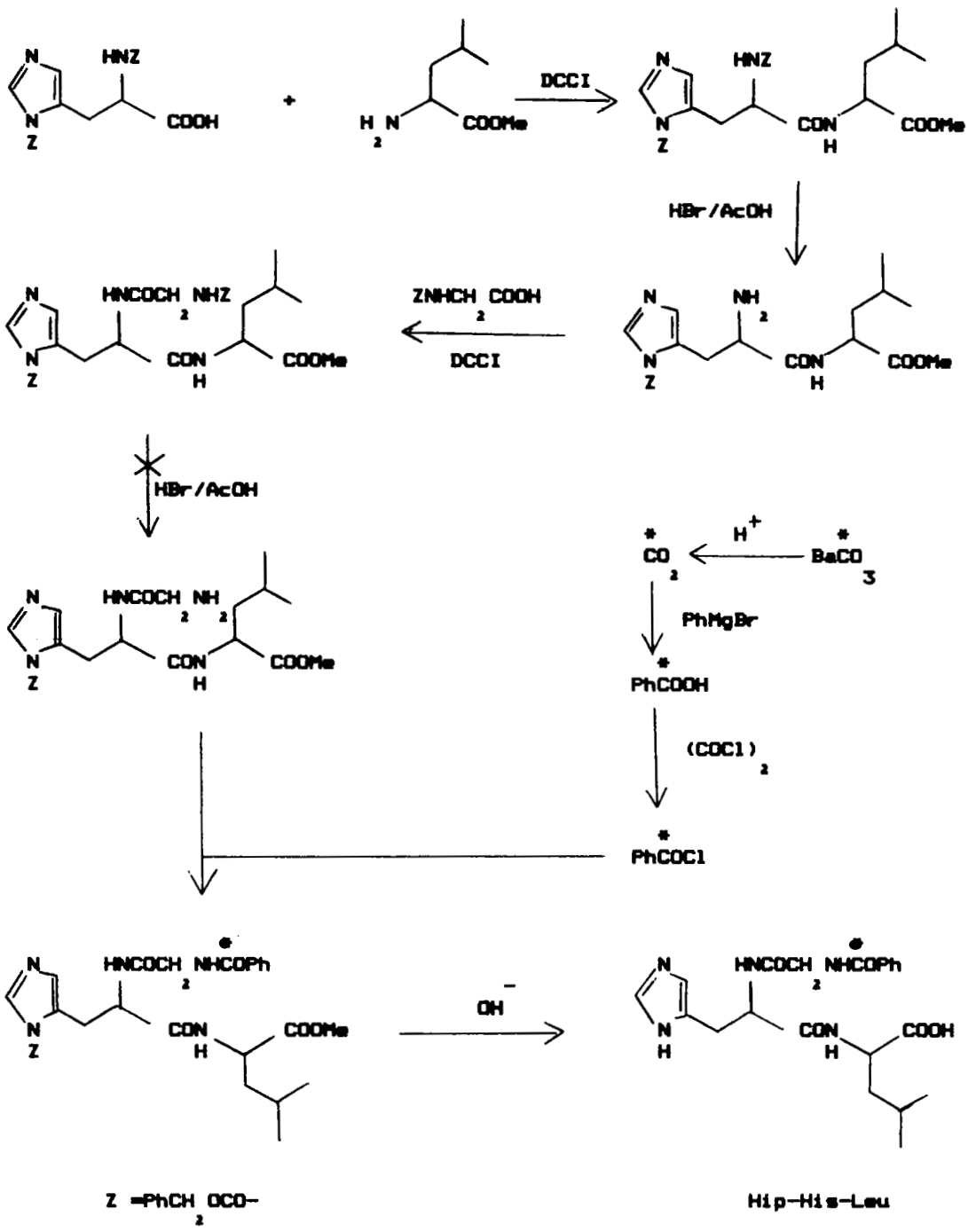
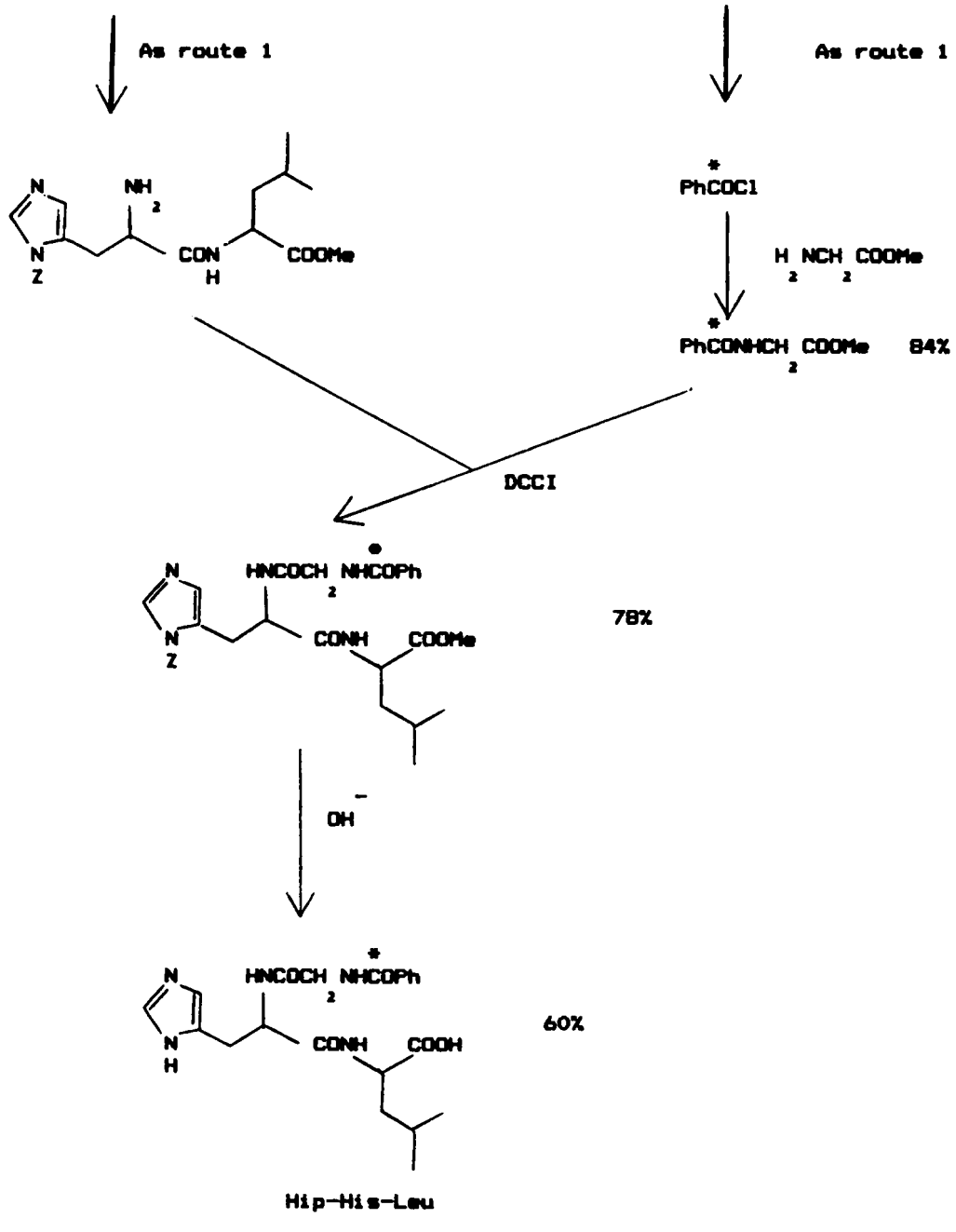


Figure 2 Synthetic Route 2 (successful)



L-leucine methyl ester using dicyclohexylcarbodiimide as coupling reagent giving white crystals, mpt 105-6°C (lit 102-3.5°C).

N-(im)-Carbobenzoxy-L-histidyl-L-leucine, methyl ester dihydrobromide The dicarbobenzoxy-L-histidyl-L-leucine methyl ester (300 mg: 0.55 mmol) was stirred in 40% hydrogen bromide in glacial acetic acid (1.6 ml) for 30 minutes. Sodium-dried ether (10 ml) was added dropwise and the suspension stirred for 15 minutes. Most of the supernatant was removed and the residue triturated with more ether (10 ml) before filtration. The solid was quickly dried in vacuo at 40°C to give a pale pink solid (286 mg: 91%).

N-(im)-Carbobenzoxy-L-histidyl-L-leucine methyl ester The above dihydrobromide salt (286 mg: 0.49 mmol) without further purification was partitioned between ice-cold dichloromethane (4 ml) and cold 5% sodium bicarbonate solution (5 ml). The layers were separated and the aqueous phase saturated with sodium chloride before extraction with more dichloromethane (4 x 3 ml). The combined organic extracts after drying and evaporation gave 199 mg (97%) of the ester as a gum. Tlc in butanol/acetic acid/water (4:1:1) showed a single spot that was UV and ninhydrin positive and which had a R_f of 0.50.

[1-^{14}C]Benzoic acid was prepared by carboxylation[3] of phenylmagnesium bromide using [^{14}C]carbon dioxide generated from barium [^{14}C]carbonate (54 mCi/mmol).

[^{14}C]Hippuric acid, methyl ester [^{14}C]Benzoic acid and unlabelled benzoic acid (total 431 mg: 3.5 mmol, 60 mCi) in dry dichloromethane (4 ml) was treated with oxalyl chloride (2.0 ml: 23 mmol) followed by dimethylformamide (1 drop). The solution was stirred for 1 hour at room temperature and the solvent removed by evaporation under reduced pressure at 25°C. The residual light brown oil in dry dichloromethane (8 ml) was added

to a stirred suspension of glycine methyl ester hydrochloride (910 mg: 7.2 mmol) in dichloromethane (10 ml), dimethylformamide (1.8 ml) and N-ethylmorpholine (1.37 ml: 10.8 mmol). The resulting solution was stirred at room temperature for 2 hours before dilution with toluene (40 ml) and washing with N hydrochloric acid (3 x 10 ml). The combined aqueous washes were extracted with toluene (3 x 10 ml) and the combined organic layers washed once with water (10 ml) before drying. Evaporation of the solvents left a gum (626 mg: 92%). Tlc (silica plate eluted with 10% methanol in dichloromethane) showed a single radioactive spot (R_f 0.60).

[^{14}C]Hippuric acid [^{14}C]Hippuric acid methyl ester (182 mg: 0.94 mmol) was dissolved in methanol (2 ml) and 0.1N sodium hydroxide solution (10 ml) and stirred at room temperature overnight. The solution was concentrated to *ca* 2 ml before acidification with concentrated hydrochloric acid (0.5 ml). After standing at 4°C for 1 hour, the solid was collected by filtration and washed with water (1 ml) and ether (3 ml). After drying *in vacuo* the solid weighed 66 mg (37%). The filtrate was concentrated to 1 ml and on standing at 0°C gave a second crop of 85 mg (47%). The total yield of [^{14}C]hippuric acid was 84% at a specific activity of 17 mCi/mmol. Further crops of lower specific activity [^{14}C]hippuric acid could be extracted from the mother liquors by adding non-radiolabelled hippuric acid and concentrating to low volume. Tlc (silica plate eluted with chloroform/methanol/acetic acid/water 120:15:3:2) showed a single UV spot coincident with the radioactivity (R_f 0.28). After standing at -20°C for some months, some deterioration in the purity was noted. Before further syntheses were undertaken, it was necessary to purify the [^{14}C]hippuric acid by preparative high performance liquid chromatography using a Whatman Magnum Partisil column (50 x 2.5 cm) eluting with 7.5% methanol in dichloromethane.

N-[^{14}C]Hippuryl-N-(im)-carbobenzoxy-L-histidyl-L-leucine, methyl ester N-(im)-Carbobenzoxy-histidyl-leucine methyl ester (174 mg: 0.42 mmole) in dry dichloromethane (8 ml) was added to the [^{14}C]hippuric acid (46 mg: 0.256 mmol). Dicyclohexyl-carbodiimide (57 mg: 0.28 mmol) was added in dry dichloromethane (2 ml) and the mixture stirred at room temperature for 6 hours. The resulting suspension was shaken with ice-cold N sulphuric acid (2 ml) and filtered. The aqueous layer was separated from the filtrate and the organic solutions washed once more with cold N sulphuric acid (2 ml) followed by cold 5% sodium bicarbonate solution (2 x 5 ml) and finally water (1 x 5 ml). The organic solution was dried and after evaporation gave 122 mg (78%) of white solid.

[^{14}C]Hippuryl-L-histidyl-L-leucine The protected [^{14}C]-hip-his-leu (135 mg: 0.22 mmol) was stirred in methanol (5 ml), water (2 ml) and N sodium hydroxide solution (1 ml) for 2 hours, when no starting material remained (tlc in chloroform/methanol/acetic acid/water 120:15:3:2). The suspension was filtered to remove some remaining dicyclohexyl urea, which was washed with water (2 ml). The filtrate was acidified with 2N acetic acid (1 ml) and the hazy solution purified by reverse phase preparative hplc to give 56 mg of pure compound with a specific activity of 17 mCi/mmol.

Column:	Whatman Magnum ODS3 (reverse phase, 50 x 2.5 cm)
Eluent:	20% isopropanol/water + 0.05% acetic acid. pH adjusted to 4.5 with 2M ammonia.
Flow rate:	5.5 ml/min
Detection:	UV at 250 nm, 2 AUFS
Retention time:	63 minutes

ACE Assay (Based on that of Cushman & Cheung[8])

The [^{14}C]hip-his-leu was diluted with unlabelled peptide and dissolved in pH 8.3 phosphate buffer (0.1M with 0.3M chloride) to give a solution that was 9 mmolar with respect to hip-his-leu and contained approximately 10^5 dpm per 20 ul of solution. Both blank and control groups were used.

20 ul of the above solution was added to 40 ul of phosphate buffer saline for both groups. A diluted solution (1:50) of exogenous ACE was added to the controls and both groups incubated at 37°C for 90 minutes. 0.2M Sulphuric acid was added to both groups and the diluted ACE (50 ul) added to the blanks. Both groups were then vortexed with 1 ml of pentyl acetate before counting 800 ul of the pentyl acetate layer.

Typical values:	Controls	6000 dpm
	Blanks	100 dpm

Results and Discussion

[^{14}C]Hippuryl-L-histidyl-L-leucine made by the above method has proved to be suitable in ACE assays for measuring concentrations of angiotensin converting enzyme inhibitors and has remained usable for at least six months when stored as a 5 mg/ml solution in ethanol-water (6:4) at 4°C.

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