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^{14}C-Labelling of Ipamorelin, a Growth Hormone Secretagogue

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Summary

Ipamorelin (NNC 26-0161) labelled with ^{14}C has been synthesised from [^{14}C]α-aminoisobutyric acid in three steps using solid phase chemistry. The overall radiochemical yield was 31%. The radiochemical purity (RCP) was > 98% and the specific radioactivity was 57 mCi/mmol.

Key words: α-2-Amino[1-^{14}C]isobutyric acid, growth hormone, ^{14}C, ipamorelin, NNC 26-0161, solid phase chemistry.

Introduction

Human growth hormone (hGH) is used to treat children with growth hormone deficiency, as well as adults suffering from Turner syndrome, chronic renal disease and achondroplasia. hGH is administered by daily subcutaneous injections. However, injection is an unpleasant form of administration which causes compliance problems, especially with children. Thus, development of a non-injectable growth hormone secretagogue is of great interest

Novo Nordisk A/S has developed several peptidyl mimetics which possess such characteristics[1]. One of these compounds, ipamorelin (Figure 1), has entered clinical trials. In order to gain a better understanding of the metabolic fate of the compound a ^{14}C-labelled version of ipamorelin was needed. We here describe the three step solid phase synthesis of [^{14}C]ipamorelin using [^{14}C]α-amino-isobutyric acid as starting material.

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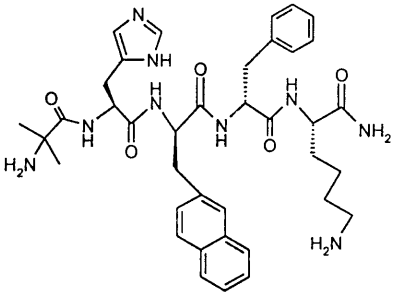


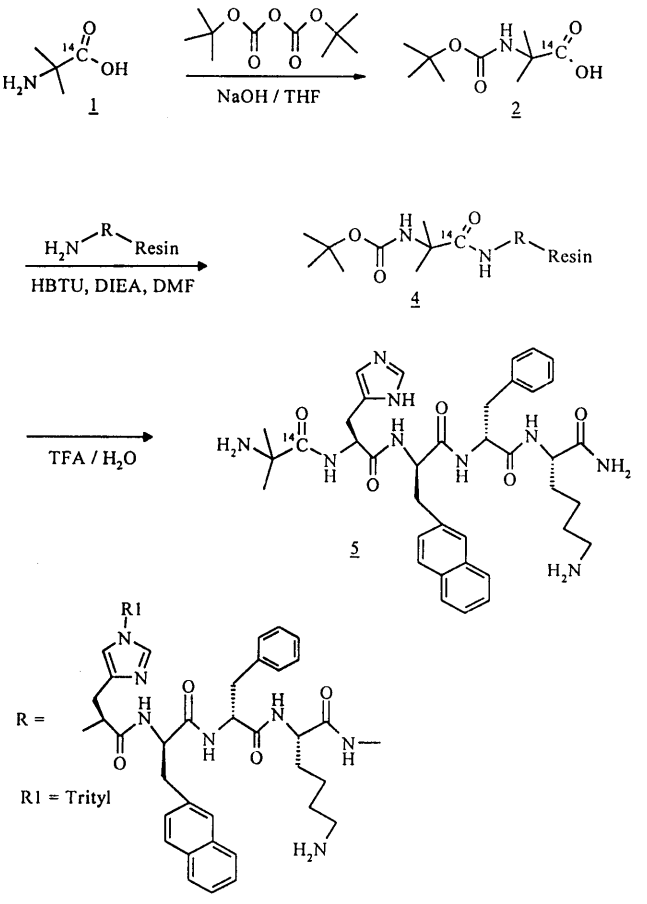
Figure 1: Structure of ipamorelin (α-Aib -His-D-2Nal-D-Phe-Lys-NH_2)

Results and Discussion

The synthesis of ipamorelin was performed using solid phase chemistry and the synthetic route is shown in Scheme 1. ^{14}C-labelling in the α-amino-isobutyric acid part of the molecule turned out to be suitable for the metabolism studies.

It is reported that the coupling between amino acids in peptide synthesis is facilitated in the presence of a tertiary base such as N-ethyldiisopropylamine and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphat (HBTU)[2]. Therefore HBTU was used in the coupling between Boc-α-2-amino[1-^{14}C] isobutyric acid and the unlabelled peptide fragment NNC 26-0080. Since the product of this reaction was bound to the resin moiety, it was difficult to follow the reaction kinetics through ordinary analyses. However, we developed a simple method that allowed us to determine to what extent the coupling was carried out. Centrifugation of the homogeneous mixture separated [^{14}C]Boc-α-Aib resin bound from the free compound. After centrifugation of the reaction mixture, the supernatant contained only the dissolved [^{14}C]Boc-α-Aib and the precipitate consisted of a mixture of the non-radioactive NNC 26-0080 and the resin bound [^{14}C]ipamorelin. Samples were taken from the supernatant and the amount of radioactivity was determined using liquid scintillation counting. The same method was used at the deprotection step.

The use of ammonium sulphate/acetonitrile as the HPLC-eluent was found to be preferable to TFA-/acetonitrile when separating the non-radioactive impurities from [^{14}C]ipamorelin. But



Scheme 1: Preparation of [^{14}C]ipamorelin (5)

evaporation of the HPLC fractions *in vacuo* resulted in large amounts of inorganic salts. Instead of evaporation *in vacuo* the HPLC fractions were eluted through a C-18 Sep-Pak thus avoiding salt contamination of the final product. The [^{14}C]ipamorelin was eluted from the Sep-Pak using 0.1% TFA/acetonitrile and freeze-dried overnight. The solid [^{14}C]ipamorelin was dissolved in Milli Q water.

The final radiochemical yield was 3.1 mCi (overall 31%). The radiochemical purity was >98%, as determined by radio-HPLC analysis and the specific radioactivity of [^{14}C]ipamorelin

was 57 mCi/mmol. [^{14}C]Ipamorelin was stored at -25°C in a radiochemical concentration of 2.3 mCi/ml. Stability studies performed after 8 months storage at -25°C showed only minor radiochemical degradation (less than 1%).

Conclusions

[^{14}C]-labelled ipamorelin, required for metabolism studies, has been prepared using solid phase chemistry. The radiochemical purity was >98% as determined by HPLC and the specific radioactivity was 57 mCi/mmol. The overall radiochemical yield was 31%. The radiochemical purity of [^{14}C]ipamorelin dissolved in Milli-Q water after 8 months storage at -25°C was > 98%.

Experimental

Reagents and chemicals

α-2-Amino[1-^{14}C]isobutyric acid dissolved in water containing 2% ethanol (2.5 mCi in 50 ml) was supplied by Amersham International plc, UK. The specific radioactivity as given by the supplier was 59 mCi/mmol.

The peptide resin NNC 26-0080 was synthesized according to the Fmoc strategy on an Applied Biosystems (Foster City, CA, USA) 431A peptide synthesizer. The following protected amino acid derivatives and resin were used: FMOC-Lys(BOC)-OH, FMOC-D-Phe-OH, FMOC-D-2-Nal-OH, FMOC-His(Trt)-OH and FMOC-Rink amide resin (Sub. 0,55 meq/g), all from BACHEM AG, Bubendorf, Switzerland. The final concentration of compound was 0.275 mmol compound/g Rink resin.

Ipamorelin reference material was synthesised by, Novo Nordisk A/S according to the procedure described above. All other reagents and solvents used were of analytical grade.

Apparatus

HPLC analyses were performed using a Merck HPLC pump L-6200 with a rheodyne injector (20 µl loop) and a Merck UV-detector L-4200 (operating at 214 nm).

Semi-Preparative HPLC separations were performed using a Merck HPLC pump L6200 with a rheodyne injector (200 µl and 1000µl loop) and a Merck UV-detector L-4200 (operating at 214 nm).

Radioactivity in the column effluent during HPLC-analyses was monitored with a Radiomatic/ Canberra Flo-One beta detector (A515), using a 500 µl liquid flow cell. The ratio of column effluent to liquid scintillator (Ultima Flo-M™, Packard) was 1:2.

For liquid scintillation counting a Packard Tri-Carb 1000 or 2300 LSC was used.

Radio-TLC analyses were performed using a Bioscan Imaging Scanner System 200-IBM with an Autochager 1000. The collimator grid contains 10 strings/mm and the P10 gas (10% methane in argon) flow was 1.5 l/min.

HPLC and TLC-systems

HPLC System 1

Reverse phase C-18 separations were accomplished at room temperature with a column from Novo Nordisk A/S (250 x 4 mm, 5 mm, YMC 120AA), using an eluent of A: Ammonium sulphate (0.1 M buffer was prepared by dissolving 66.1 g of $(NH_4)_2SO_4$ in 5000 ml Millipore filtrated water, pH adjusted to 2.5 with 4.8M sulphuric acid) / acetonitrile (90/10) and B: 100% acetonitrile. The flow rate was 1.0 ml/min..

Table 1: Gradient Programme for HPLC-system 1

Time (min)	**Pump A:**	**Pump B:**	Flow (ml/min)
0	95	5	0.1
0.1	95	5	1.0
60	90	10	1.0
100	90	10	1.0

HPLC System 2

Reverse phase C-18 semi-preparative purifications were accomplished at room temperature with a column from Novo Nordisk A/S (250 x 20 mm, 10 mm, OdDeMeSi), using an eluent

of A: Ammonium sulphate (0.1 M, pH = 2.5 adjusted with 4.8 M sulphuric acid) / acetonitrile (90/10) and B: 100% acetonitrile. The gradient programme was the same as with HPLC System 1, except for the flow (Table 1). The flow rate was 9.0 ml/min.

TLC system 1

TLC was performed on glass plates coated with cellulose (10 x 10 cm, Merck Art 5787). The mobile phase was a mixture of butanol, pyridine and water (1:1:1, System 1). R_f [^{14}C]α-Aib ; 0.45, R_f [^{14}C]Boc-α-Aib ; 0.88.

Determination of specific radioactivity

Specific radioactivity of [^{14}C]ipamorelin was determined using MS FAB at a Fisons VG-Autospec Ultima. The sample was dissolved in water using glycerol as matrix. Values were compared with an unlabelled reference sample.

Preparation of [^{14}C]ipamorelin (Scheme 1)

Boc-α-2-amino[1-^{14}C] isobutyric acid (Boc-[^{14}C]α-Aib)(2).

α-2-Amino[1-^{14}C] isobutyric acid ([^{14}C]α-Aib) (1))(0.17 mmol, 10 mCi, 50 μCi/ml) was evaporated to dryness *in vacuo*. The residue was dissolved in water (1.5 ml). To the 1.5 ml radioactive solution was added sodium hydroxide (aq) (0.83 mmol, 830 μl) THF (3 ml) and di-tert-butyl-pyrocarbonate (1.7 mmol, 386 μl). The mixture was stirred at room temperature. After 4 hours (pH = 9-10) additional di-tert-butyl-pyrocarbonate (1.7 mmol, 386 μl) was added and the mixture was stirred overnight at room temperature. After 25 hours the pH was adjusted to 9-10 with 1.0M NaOH and an aliquot was taken from each of the two phases for radio-TLC analysis (TLC system 1). The analysis showed a 90 % radiochemical conversion of [^{14}C]α-Aib(1) to Boc-[^{14}C]α-Aib (2).

Subsequently diethyl ether (10 ml) was added and the layers were separated. In the aqueous fraction the pH was adjusted to pH=2 with sulphuric acid (1.0 M) and the solution was extracted with ethyl acetate (3 x 10 ml). The organic fraction (30 ml) was isolated and washed

with water (2 x 25 ml); the pH was about 7 in the final water fraction. The isolated ethyl acetate phase was dried (magnesium sulphate) and evaporated. The residue was dissolved in ethanol (1.0 ml) and used without further purification in the next step.

The radiochemical yield was 5.8 mCi (58%) and the radiochemical purity as determined by radio-TLC analysis was >98%, (TLC System 1).

[^{14}C]α-Aib-His-D-2Nal-D-Phe-Lys-NH$_2$ ([^{14}C]ipamorelin) (5).

5.8 mCi Boc-[^{14}C]α-Aib (2) (0.01 mmol in 1.0 ml ethanol) was evaporated to dryness under a gentle flow of nitrogen. The residue was dissolved in DMF (3 ml) and NNC 26-0080 (3) (0.14 mmol, 526 mg), HBTU (0.482 mmol in 731 µl DMF) and N-ethyldiisopropylamine (0.963 mmol, 155 µl) were added; the pH was 9-10. The grey suspension was stirred overnight at room temperature. After 19 hours an extra portion of HBTU (0.482 mmol in 731 µl DMF) was added, the pH being 9-10.

To follow the incorporation of Boc-[^{14}C]α-Aib (2) to the solid phase material, the reaction vial was centrifuged at different time points (3500rpm in 3 min) and radioactivity in the supernatant, representing unbound Boc-[^{14}C]α-Aib (2), was measured. After 24 hours liquid scintillation counting of an aliquot from the supernatant revealed that around 95% of the radioactivity was incorporated in the solid phase material.

The supernatant was separated from the brown resin moiety. DMF (4 ml) was added to the solid phase material and swirled followed by centrifugation (3500rmp in 3 min). The supernatant was removed and the procedure repeated with diethyl ether/propanol (4 ml, 50/50), DMF (4 ml), propanol (4 ml), methanol (4 ml) and diethyl ether (4 ml). When the last diethyl ether portion was removed the resin material was dried and ready for deprotection.

TFA/water (7 ml, 95/5) was then added to the resin material. The colour of the suspension turned deep yellow instantaneously. After 15 minutes the colour was reddish and after another 15 minutes the colour was deep red. The suspension was kept at room temperature while stirring. After 90 minutes the reaction vial was centrifuged (3500 rpm in 3 min) and an aliquot

was taken for liquid scintillation counting. The results showed that only 35% of the radioactivity was in the TFA/water solution and around 65% was still bound to the resin material. The supernatant was isolated and stored at - 25°C. The deprotection procedure was repeated and the two supernatant fractions were combined. HPLC-analysis (HPLC System 1) of the crude solution showed 96% radiochemical purity, and the total radioactivity determined by liquid scintillation counting was 4.0 mCi. The TFA/water solution was evaporated to dryness. The resulting grey/brown mass was dissolved in Milli-Q (3 ml) and filtered through a glass filter (Whatman GF/B, micro fibre). The filter was washed with ammonium sulphate / acetonitrile (4 ml, 0.1M, pH = 2.5, 90/10), without loss of radioactivity.

Purification of the crude product was performed on a semi-preparative HPLC (HPLC System 2) by injections onto the HPLC. An analysis (System 1) of the pooled main fractions showed a radiochemical purity > 99%. The acetonitrile was removed in vacuo and the remaining solvent (800 ml) was applied to a C-18 Sep-Pak.

[^{14}C]Ipamorelin (5) was eluted from the Sep-Pak with 0.1% TFA in water/0.1 % TFA in acetonitrile (4 + 2 ml, 30/70), and frozen. Using a Heto-Vac vacuum centrifuge the frozen sample containing [^{14}C]ipamorelin was taken to dryness. Water was added to the dry residue (2 x 2 ml), and centrifugation under vacuum was continued overnight. The dry material was dissolved in 2.5 ml water, and samples were taken for HPLC (HPLC System 1) and liquid scintillation counting.

The final radiochemical yield was 3.1 mCi (54%, overall 31%). RCP was >98% and the specific radioactivity for [^{14}C]ipamorelin was 57 mCi/mmol. The radiochemical concentration was 2.3 mCi/ml. Stability studies performed after 8 months storage at -25°C showed only minor radiochemical degradation (less than 1%).

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