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SYNTHESIS OF [4,5-^{3}H-LEU^4]SALMON CALCITONIN

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SUMMARY

The synthesis of salmon calcitonin, a C-terminal amide dotriacontapeptide, labelled with tritium in the leucine residue at position 4 to a specific activity of 100Ci mmole^{-1} is described. The peptide was assembled using the Fmoc-Polyamide solid-phase synthesis strategy incorporating tritium labelled leucine, in the form of Fmoc-4,5-^{3}H-leucine-pentafluorophenylester, at the appropriate stage. The Fmoc-4,5-^{3}H-leucine-pentafluorophenylester was prepared via a multistage synthesis from 4,5-dehydroleucine.

Key Words: Salmon Calcitonin, tritium

INTRODUCTION

The calcitonins are a class of peptides of 32 amino acids in length ending in a C-terminal amide. Calcitonins from several species - for example, chicken, eel, human, porcine, salmon and rat - are known. In each case the entire 32 amino acid chain appears to be required for biological activity, fragments of the molecule being totally inactive.[1] There is a marked enhanced biological potency of the piscine calcitonins over other types.

We required to synthesis salmon calcitonin (I) labelled with tritium at high specific activity.

Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2

(I) Salmon Calcitonin

The common method employed to obtain tritium labelled peptides is via the catalytic dehalogenation or reduction of suitable halogenated or unsaturated precursor peptides. However, salmon calcitonin contains two cysteine residues that form a

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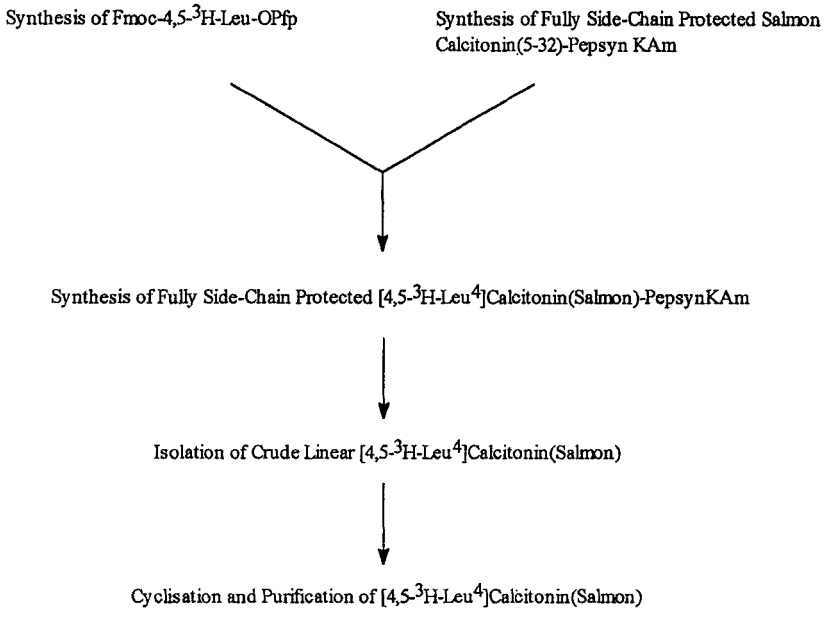
disulphide bridge between residues 1 and 7. Peptides that contain cysteine are known to be difficult to hydrogenate because of the combined phenomena of conversion of cysteine to DL-alanine and catalyst poisoning.[2] The former hydrogenolysis occurs whether the cysteine is side-chain protected as the S-trityl derivative[3] or in its reduced (cystine) form.[4,5] A claim that iodinated peptides can be cleanly dehalogenated in aqueous buffer[6] could not be successfully applied to the preparation of human calcitonin.[7] The partial conversion of two cysteine residues to DL-alanine in the hydrogenation of a halogenated or unsaturated salmon calcitonin derivative could potentially yield an extra eight peptides containing different permutations of cysteine and/or D- and L-alanine at positions 1 and 7 thereby presenting significant post-reduction purification difficulties. A further reason for seeking an alternative synthetic approach to tritium labelled salmon calcitonin was the reduction in specific activity that has been observed on reduction of halogenated and unsaturated cysteine containing peptides with tritium[3] since we desired to prepare material at a high specific activity.

We therefore decided to attempt to prepare tritium labelled salmon calcitonin by conventional solid phase peptide synthesis incorporating the radioactivity into the elaborating sequence via a previously synthesised tritium labelled amino acid. As a high specific activity was a primary goal we chose to use the amino acid leucine, since a maximum of seven tritons can potentially be incorporated into this amino acid via reduction of dehydroleucine, as the vehicle for the tritium. Salmon calcitonin contains leucine residues at positions 4, 9, 12, 16 and 19; to reduce the number of peptide synthesis operations required on the radioactive solid support the leucine at position 4 was considered to be the most appropriate position for the tritium label. The Fmoc-Polyamide solid phase method[8] was employed for the synthesis because of its mild chemistry and operation.

RESULTS AND DISCUSSION

The general strategy employed for the synthesis of [4,5-^{3}H-Leu^4]Salmon Calcitonin is shown in Scheme 1. A derivative of 4,5-^{3}H-leucine, Fmoc-4,5-^{3}H-Leu-OPfp, suitably protected and activated for peptide synthesis was coupled to a pre-formed segment consisting of residues 5-32 of salmon calcitonin with all appropriate amino acid side chains still protected and the C-terminal residue attached to the solid support via a suitable linkage agent. The remaining four residues were then attached to the resulting radioactive peptide/support to complete the salmon calcitonin sequence. Cleavage of the peptide from the solid support, concurrent with the removal of the side-chain protecting group yielded the crude linear tritium-labelled salmon calcitonin which, after cyclisation and purification, yielded the desired [4,5-^{3}H-Leu^4]salmon calcitonin at 100Ci mmole^{-1}.

Scheme 1 **Strategy for the Synthesis of [4,5-^{3}H-Leu^4]Salmon Calcitonin**



1 Synthesis of Fully Side-Chain Protected Salmon Calcitonin(5-32)

The synthesis of the 5-32 residue segment of salmon calcitonin was accomplished using the Fmoc-Polyamide strategy[8]. All amino acids were protected at their α-amino group with the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group[9] and activated at the carboxyl function as either their *O*-pentafluorophenyl esters[10] or by in-situ activation with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate[11]. Those amino acids with reactive side-chains were protected with acid-labile groups as appropriate for the Fmoc-Polyamide strategy. Since the segment was assembled on a semi-automatic peptide synthesiser using a continuous flow method, the solid support produced by the polymerisation of dimethylacrylamide, bisacryolethylene diamine and acryloylsarcosine methyl ester within a kieselguhr matrix was employed[12] (Pepsyn K). The support was derivatised with a linkage agent which yields C-terminal amides directly on treatment with trifluoroacetic acid .[13]

The couplings of all the amino acids were conducted in dimethylformamide and catalysed by 1-hydroxybenzotriazole which has been demonstrated to both reduce racemisation and promote the rate of acylation.[14] Each coupling was allowed to continue for 60 minutes; after this period a portion of the support was removed and assayed for the presence of free amino groups which indicates incomplete coupling. If a

negative result was obtained then the acylating solution was removed and replaced with a fresh solution - residues 5, 6, 7, 8, 9, 10 and 26 required this further extra treatment. If a positive result was obtained then the acylating solution was removed and the Fmoc-group removed prior to the coupling of the next amino acid.

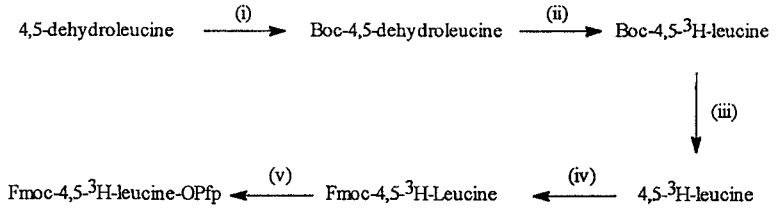
When the assembly was complete the final Fmoc group was left in place and the support kept in a dry form until required.

2 Synthesis of Fmoc-4,5-^{3}H-Leucine-*O*-Pentafluororophenyl Ester

The value of dehydroleucine in the synthesis of tritium-labelled peptides lies in its ability to incorporate a possible maximum of seven tritons, spread throughout the isopropyl group, during its reduction with tritium gas.[15] This allows for the preparation of tritium labelled peptides in excess of 100Ci mmole^{-1} by labelling within a single residue. For the reasons discussed above the synthesis of tritiated salmon calcitonin at a high specific activity necessitated the synthesis of Fmoc-4,5-^{3}H-Leucine-*O*-pentafluorophenyl ester and its subsequent incorporation into the salmon calcitonin chain rather than the reduction of a salmon calcitonin analogue containing 4,5-dehydroleucine.

4,5-Dehydroleucine, like all amino acids, exists in a zwitterioinc form. It is therefore soluble in water and other protic solvents. These solvents are unsuited to the reduction of olefins with tritium gas where a high specific activity is required since the possibility of significant exchange between the solvent and the tritium gas exists thereby reducing the specific activity of the tritium gas and the resulting product. The aprotic solvent dimethylformamide is more suited as a solvent for tritium gas reductions since there are no protons available to reduce the specific activity. We therefore wished to use this solvent but needed to prepare a suitable soluble derivative of 4,5-dehydroleucine. Unfortunately, the obvious candidate - Fmoc-4,5-dehydroleucine - is unsuitable since the Fmoc-group is not stable to the catalytic hydrogenation.[16] The tributoxycarbonyl (Boc) group was chosen since this derivative is easily prepared, stable to catalytic hydrogenation, and thereafter easily removed. The reaction sequence is shown in Scheme 2. Reduction of the Boc-4,5-dehydroleucine with tritium gas followed

Scheme 2 **Synthesis of Fmoc-4,5-^{3}H-Leucine-*O*-pentafluorophenyl ester**



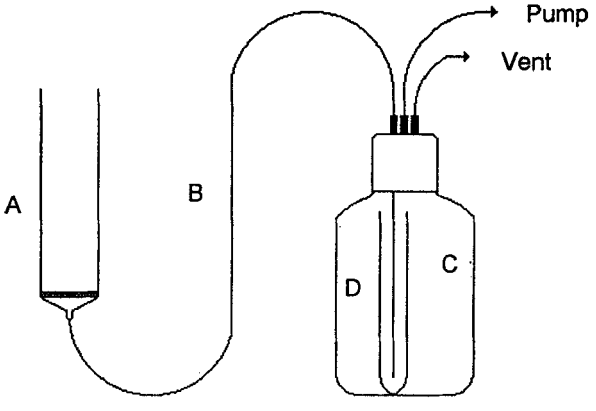
Reagents: (i) Boc_2O, aq NaOH, 1,4-dioxan; (ii) $^3H_2$, 10%Pd/C, DMF; (iii) 90% aq TFA; (iv) Fmoc-ONSu, aq Na_2CO_3, acetone; (v) HOPfp, DCC, EtOAc

by replacement of the Boc group by the Fmoc group gave Fmoc-4,5-^{3}H-leucine at 100Ci mmole^{-1}. The tritium labelled amino acid was activated as its *O*-pentafluorophenyl ester utilising pentafluorophenol and diisopropylcarbodiimide and purified by HPLC before use.

3. Synthesis of Fully Side-Chain Protected [4,5-^{3}H-Leu^4]Salmon Calcitonin-Pepsyn KAm

The coupling of the Fmoc-4,5-^{3}H-Leu-OPfp and the final three residues thereafter to the fully side-chain protected salmon calcitonin(5-32) segment were carried out in the apparatus shown in Figure 1. All operations on the resin were carried out in a disposable plastic vessel fitted with a porous sinter (A) and this was connected via PTFE tubing (B) to the vessel (C) in which was fitted a secondary vessel (D). Evacuation of vessel C via a PTFE tube leading to a vacuum pump allowed filtration of the resin. A third tube was present to permit the vacuum within C to be released. The function of the secondary vessel D was to allow for the facile collection of radioactive waste.

Figure 1 **Apparatus for Solid Phase Peptide Synthesis on Radioactive Resin**



The coupling of the Fmoc-4,5-^{3}H-Leucine-*O*-pentafluorophenyl ester to the deprotected fully side-chain protected salmon calcitonin(5-32)-Pepsyn KAm was performed in DMF. In solid phase peptide synthesis it is usual to employ a large, typically four-fold, molar excess of the acylating reagent over the free amino groups of the resin-bound peptide since it is usually the peptide which is the more valuable of the two commodities. In this case, however, we wished to maximise the amount of radioactivity incorporated into the peptide since the Fmoc-4,5-^{3}H-Leucine-*O*-pentafluorophenyl ester was of greater value - thus we chose to use equimolar amount of the Fmoc-4,5-^{3}H-Leucine-*O*-pentafluorophenyl ester and the resin-bound peptide. The principle reason for using a large excess of acylating reagent is to ensure that all of the

free amino groups of the growing peptide chain are reacted within a reasonable time since even minor incomplete coupling will result in significant quantities of undesired products being produced when it occurs at every stage in an assembly. However, minor incomplete coupling at any one stage is not, in itself, damaging to the eventual successful outcome of the synthesis. Furthermore, one of the effects of incomplete coupling - the corresponding generation of a peptide differing from the desired target by a single deficiency of that amino acid in its sequence with its concomitant implications for the purification processes that invariably follow the assembly of most peptides - can be significantly negated by the process known as capping. This involves treating the resin-bound peptide, after removing the acylating solution, with a large excess of acetic anhydride. The result N-acetylated peptides then take no further part in the synthesis and hence may differ substantially from the target peptide thereby facilitating their separation from the target peptide.

The coupling of 1.75Ci (17.5mmole) of Fmoc-4,5-^{3}H-leucine-*O*-pentafluorophenyl ester to the fully side-chain protected salmon calcitonin(5-32)-polymer resulted in 1.2Ci of radioactivity being incorporated into the peptide. Since the resin gave a positive result to the test for free amino groups it appeared that the coupling had been completed successfully. It is likely that less than one equivalent of the peptide-polymer had been used in the reaction - this is probably the result of the inaccuracy of weighing out this material for which a calculated loading (in terms of mmole per unit weight) cannot take into account distortions created by solvent hold-up.

Nevertheless the polymer was treated with acetic anhydride as a precaution and the final three residues coupled as their pre-activated O-pentafluorophenyl esters in the presence of the additive 1-hydroxybenzotriazole. Each residue was double coupled to promote complete coupling.

4. Preparation of [4,5-^{3}H-Leu^{4}]Salmon Calcitonin

The separation of the assembled fully side-chain protected [4,5-^{3}H-Leu^{4}] salmon calcitonin from the solid support was effected with trifluoroacetic acid containing both phenol (2.5% w/v) and β-mercaptoethanol (2.5% v/v) as scavengers to protect the peptide from the removed side-chain protecting groups. After removal of the trifluoroacetic acid and scavengers the crude linear peptide was dissolved in water and the pH of the resulting solution adjusted to 9 with ammonia. The cyclisation was monitored by reverse phase HPLC and was complete on standing overnight at room temperature.

The cyclised tritium labelled salmon calcitonin was purified by reverse HPLC and isolated and stored as a solution in aqueous acetonitrile. The material was chromatographically equivalent to authentic unlabelled salmon calcitonin and had a radiochemical purity in excess of 98%.

EXPERIMENTAL

Materials

All reagents for peptide synthesis were obtained from Cambridge Research Biochemicals, Northwich, Cheshire, UK. Dimethylformamide (Analar grade), phenol (Analar grade) and HPLC solvents were obtained from Merck Ltd., Dorset, UK. Ethanedithiol (Puriss grade) and dicyclohexylcarbodiimide (Puriss grade) were obtained from Fluka Chemicals Ltd., Derbyshire, UK. Trifluoroacetic acid, pentafluorophenol, 10% palladium on carbon and piperdine were obtained from Aldrich.

Techniques

All HPLC was performed on a system comprising a Waters 600 Controller and associated pumps, a Waters 490 UV detector and a Canberra-Packard Radiomatic A120 radioactivity detector. Data was collected on a Waters 810 Baseline system. Peptides were detected at 230nm and Fmoc-derivatives at 266nm. Solvent A was 0.02% trifluoroacetic acid in water and Solvent B was 0.02% trifluoroacetic acid in acetonitrile. For analytical work a Vydac 218TP54 reverse phase C_{18} column was employed; for the purification of Fmoc-4,5-^{3}H-Leucine-*O*-pentafluorophenyl ester, a Vydac 218TP510 reverse phase C_{18} column was employed and for purification of the [4,5-^{3}H-Leu^4]Salmon Calcitonin a Vydac 218TP1022 reverse phase C_{18} column was employed. Amino acid analysis was performed by the Waters Pico-Tag technique. Peptide synthesis was conducted in the standard fashion for the Fmoc-Polyamide strategy.[8]

Synthesis of [Fmoc-Ser(But)^5, Ser(But)^{13,29}, Thr(But)^{6,21,25,27,31}, Cys(Trt)^7, Lys(Boc)^{11,18}, Glu(OBut)^{15}, His(Boc)^{17}, Tyr(But)^{22}, Arg(Mtr)^{24}]Salmon Calcitonin(5-32)-Pepsyn KAm(100)

The assembly of residues 5-32 of the Salmon Calcitonin were carried out on a Cambridge Research Biochemicals Mark II Pepsythesiser. The solid-phase resin employed was Fmoc-Pepsyn KAm(100), a polyamide/kieselguhr support functionalised with 4-(α-Fmoc-amino-2',4'-dimethoxybenzyl)phenoxyacetic acid, an acid labile linking agent which liberates C-terminal amide peptides on treatment with trifluoroacetic acid. All coupling were performed in dimethylformamide which had previously been treated with 4Å molecular sieve for a minimum of 24 hours. Residues 5 to 25 were coupled as their pentafluorophenyl esters, whilst residues 26-32 were coupled after pre-activation with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.[10] Couplings involving pentafluorophenyl esters were conducted in the presence of four equivalents of 1-hydroxybenzotriazole. Coupling times were 60 minutes; after this period the efficiency of the coupling was determined using the Kaiser test[17]. Where incomplete coupling was indicated the acylating solution was washed from the resin with dimethylformamide, replaced with a fresh acylating solution and the coupling continued for a further 60 minutes; this was found necessary for residues 5 to 10 and residue 26.

Removal of the Fmoc-groups was accomplished with a 10 minute treatment of the peptide/solid support with 20% piperidine/dimethylformamide solution; the final Fmoc-group was not removed until immediately before the coupling of the Fmoc-4,5-^{3}H-leucine-*O*-pentafluorophenyl ester.

Synthesis of Fmoc-[4,5-^{3}H]-Leucine-*O*-pentafluorophenyl ester

(i) Preparation of Boc-4,5-dehydroleucine

4,5-Dehydroleucine (15mg, 0.1mmole) was dissolved in water (2mL) and 1,4-dioxan (2mL). After addition of aqueous sodium hydroxide (1N, 0.1mL), di-tert-butylcarbonate (28.8mg, 0.13mmole) was added as a solution in 1,4-dioxan (0.7mL) and the mixture stirred at room temperature for 150 minutes whilst maintaining the pH at 10 with further additions of aqueous sodium hydroxide (1N, 0.03mL required). The solution was washed with diethyl ether (3 x 5mL), the pH reduced to 2.5 with aqueous potassium hydrogen sulphate (1N) and the solution extracted with ethyl acetate (3 x 5mL). The extracts combined, washed with saturated aqueous sodium chloride and dried with magnesium sulphate. Evaporation of the ethyl acetate under reduced pressure resulted in Boc-4,5-dehydroleucine (23mg; 100%).

(ii) Preparation of Boc [4,5-^{3}H-Leucine]

Boc-4,5-dehydroleucine (23mg, 0.1mmole) was dissolved in dimethylformamide (1.5mL) and 10% palladium on carbon (15mg) added. Tritium gas (20Ci, >98% isotopic purity) was introduced and the mixture stirred at room temperature for 18 hours. The catalyst was removed by filtration (Millex 5μ) and labile tritium removed by evaporation to yield Boc-4,5-^{3}H-leucine (8.6Ci).

(iii) Preparation of Fmoc 4,5-^{3}H-leucine

Boc-4,5-^{3}H-leucine (8.6Ci) was dissolved in trifluoroacetic acid (5mL) and allowed to stand at room temperature for 60 minutes. The trifluoroacetic acid was removed by evaporation under reduced pressure and the resulting solid dissolved in water (2mL) and the pH adjusted to 10 with aqueous sodium carbonate (1N). Fmoc-N-hydroxysuccinimide ester (34mg, 0.1mmole) was added and the resulting solution stirred at room temperature for 75 minutes, maintaining the pH at 10 with aqueous sodium carbonate (1N). The pH of the solution was reduced to 2.5 with aqueous potassium hydrogen sulphate (1N) and then extracted with ethyl acetate (3 x 5mL). The extracts were combined and dried with magnesium sulphate to yield a solution of Fmoc-4,5-^{3}H-leucine in ethyl acetate (7.6Ci). The specific activity of the Fmoc-4,5-^{3}H-leucine was determined to be 100Ci mmole^{-1}.

(iv) Preparation of Fmoc-4,5-^{3}H-leucine-*O*-pentafluorophenyl ester

To an ice-cold solution of Fmoc-4,5-^{3}H-leucine (7.6Ci, 100Ci mmole^{-1}) in ethyl acetate (5mL) was added diisopropylcarbodiimide (48μL, 0.3mmole) and pentafluorophenol (45mg, 0.25mmole). The resulting solution was stirred at room temperature for 4

hours. The Fmoc-4,5-^{3}H-leucine-*O*-pentafluorophenyl ester was purified by reverse-phase HPLC to yield 3.3Ci at 100Ci mmole^{-1}.

Synthesis of [Fmoc-Cys(Trt)^1, Ser(Bu^t)^{2,13,29}, 4,5-^{3}H-Leu^4, Thr(Bu^t)^{6,21,25,27,31}, Cys(Trt)^7, Lys(Boc)^{11,18}, Glu(OBu^t)^{15}, His(Boc)^{17}, Tyr(Bu^t)^{22}, Arg(Mtr)^{24}]Salmon Calcitonin-Pepsyn KAm(100)

[Fmoc-Ser(Bu^t)^5, Ser(Bu^t)^{13,29}, Thr(Bu^t)^{6,21,25,27,31}, Cys(Trt)^7, Lys(Boc)^{11,18}, Glu(OBu^t)^{15}, His(Boc)^{17}, Tyr(Bu^t)^{22}, Arg(Mtr)^{24}]Salmon Calcitonin (5-32)-Pepsyn KAm(100)(0.5g at about 0.035mmole g^{-1} [18]; approximately 0.0175mmole) placed in the reaction vessel depicted in Figure 1. After removal of the Fmoc group the resin was coupled overnight with Fmoc-4,5-^{3}H-leucine-O-pentafluorophenyl ester (1.75Ci at 100Ci mmole^{-1}; 0.0175mmole) in the presence of 1-hydroxybenzotriazole (9mg, 0.07mmole). The following morning the resin gave a positive result to the Kaiser test and 0.55Ci of radioactivity were found to remain in the coupling solution; therefore 1.2Ci of Fmoc-4,5-^{3}H-leucine had coupled to the resin. The resin was treated with acetic anhydride (6 equivalents) in dimethylformamide for 1 hour before the Fmoc group was removed and the remaining three residues double coupled as their pentafluorophenyl esters with catalysis by 1-hydroxybenzotriaxole (four equivalents).

Cleavage and Cyclisation of [4,5-^{3}H-Leu^4]Salmon Calcitonin

[Fmoc-Cys(Trt)^1, Ser(Bu^t)^{2,13,29}, 4,5-^{3}H-Leu^4, Thr(Bu^t)^{6,21,25,27,31}, Cys(Trt)^7, Lys(Boc)^{11,18}, Glu(OBu^t)^{15}, His(Boc)^{17}, Tyr(Bu^t)^{22}, Arg(Mtr)^{24}] Salmon Calcitonin-Pepsyn KAm(100)(1.2Ci) was treated with 20%v/v piperidine /dimethylformamide to remove the N-terminal Fmoc protecting group and then successively washed with dimethylformamide, dichloromethane and diethyl ether and allowed to dry at room temperature. The resin was then suspended in trifluoroacetic acid (12mL) containing phenol (300mg) and ethanedithiol (0.3mL). The mixture was filtered after 90 minutes and the filtrate allowed to stand overnight at room temperature. The solution was evaporated in vacuo at 40^o to yield 357mCi of crude linear [4,5-^{3}H-Leu^4]salmon calcitonin. The crude material was dissolved in water (7mL), the pH adjusted to 9 with aqueous ammonia and left for two hours at room temperature and then overnight at 0^o. The solution was reduced in volume and then purified by HPLC to yield 52mCi of [4,5-^{3}H-Leu^4]Salmon Calcitonin at 100Ci mmole^{-1} with a radiochemical purity in excess of 98%. The material was chromatographically equivalent to authentic unlabelled salmon calcitonin (CRB Ltd., Northwich, United Kingdom) and had the following amino acid analysis: Asx 1.3 (2), Glx 3.3 (3), Ser 3.8 (4), Gly 2.9 (3), His 1.2 (1), Arg 1.3 (1), Thr 6.0 (5), Pro 2.5 (2), Tyr 1.0 (1), Leu 4.8 (5), Lys 2.4 (2).

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17 The loading of the polyamide resin was reduced from its original value of 0.1mmole g^{-1} by the additional mass resulting from the assembled protected peptide