JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2003; **46**: 499–510.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.689

Research Article

[^{125}I], [^{127}I]-and [^{14}C]-Labelling of the GLP-1-(7-37) derivative NN2211

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Summary

Arg^{34}Lys^{26}(N^{ε}-(γ-L-glutamyl(N^{α}-palmitoyl)))-GLP-1(7-37) (NN2211) is currently in development as a diabetes type 2 drug. The fatty acid attached to the GLP-1(7-37) ensures a long and controlled duration of action. The synthesis of [^{125}I]NN2211, [^{127}I]NN2211 and [^{14}C]NN2211 used for preclinical ADME studies are described. NN2211 was iodinated using the lactoperoxidase/hydrogen peroxide method, and [^{14}C]NN2211 was synthesized in 4 steps by two routes both starting from an α-protected [U-^{14}C]glutamic acid. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: ^{125}I; ^{127}I; ^{14}C; GLP-1(7-37)

Introduction

Glucagon-like peptide-1 (GLP-1) is an intestinal hormone, which stimulates insulin secretion and inhibits glucagon release. Both of these effects are favourable in the treatment of hyperglucemia. However the short half life results from rapid degradation by the enzyme dipeptidyl peptidase and a rapid clearance by the kidney makes GLP-1 an unlikely drug candidate. With the aim of obtaining a drug candidate suitable for once a day administration, a series of GLP-1 analogs

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Received 26 September 2002
Revised 31 October 2002
Accepted 18 November 2002

were synthesized, all characterized by having fatty acids attached to lengthen their action.[1] A similar approach has been utilized to lengthen the action of insulin.[2,3] The mechanism involves increased binding to albumin and metabolic stability as well as a slower release from the injection site. One of the GLP-1 derivatives; $Arg^{34}Lys^{26}(N^{\varepsilon}$-(γ-L-glutamyl($N^{\alpha}$-palmitoyl)))-GLP-1 (7-37) (NN2211) was radio-labelled for absorption, distribution, metabolism, and excretion studies (ADME). Proteins are normally labelled with [^{125}I] for ADME studies. However, rapid de-iodination *in vivo* limits the amount of information, which can be obtained from these studies. Consequently, it was decided to label the compound with both [^{125}I] and [^{14}C]. In addition, [^{127}I]NN2211 was synthesized and used to adjust the specific activity of [^{125}I]NN2211, so LC-MS could be applied for metabolite identification.

Results and discussion

[^{125}I]NN2211

Direct iodination of proteins is normally accomplished by oxidation of iodide followed by electrophilic aromatic substitution in tyrosine (Y). Only one tyrosine exists in NN2211 placed in position 13 (Y^{19}) from the *N*-terminal end of the molecule (Figure 1). Iodination of NN2211 was performed using the lactoperoxidase method.[4] Iodide is oxidized in an enzymatic reaction avoiding use of powerful oxidizing agents, which could react with GLP-1(7-37) and thereby, produce impurities. To minimize the formation of di-iodinated NN2211, only one equivalent of [^{125}I] was used to ten equivalents of NN2211. The reaction was followed by protein precipitation of small aliquots using trichloroacetic acid (TCA). After 5 min, more than 90% of the radioactivity was attached to the protein. The reaction mixture was purified by RP-HPLC without further treatment. An excellent separation of the labelled from the unlabelled NN2211 was accomplished by careful optimizing the isocratic HPLC conditions (Figure 2). In a typical preparation, starting from 5 mCi of [^{125}I], the final radiochemical yield was $>50\%$, and the radiochemical purity was $>98\%$. Radiosequence analysis confirmed the position of labelling as (Tyr^{19}) in NN2211.[†]

[†] Unpublished results.

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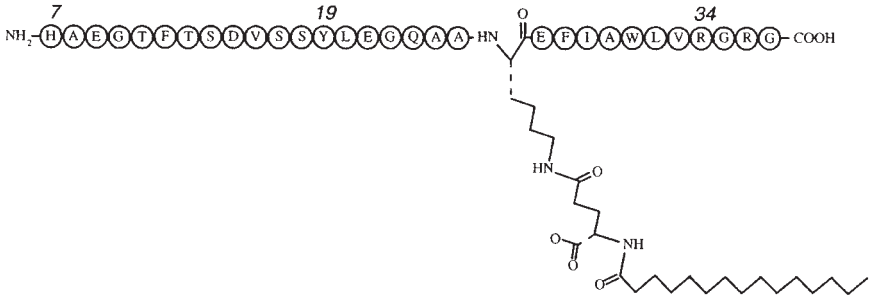

Figure 1. Structure of NN2211

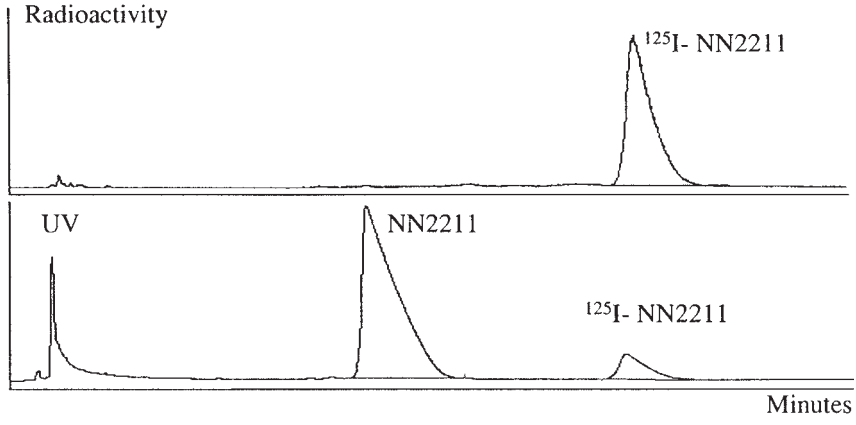

Figure 2. HPLC chromatogram of the iodination mixture of [^{125}I]NN2211

[^{127}I]NN2211

[^{127}I]NN2211 was prepared according to the method described above. However, to achieve the highest possible utilization of the protein, two equivalents of [^{127}I] were used to one equivalent of NN2211. By optimizing the quantity of hydrogen peroxide employed and the reaction time, it was possible to obtain up to 69% of the desired mono-iodinated NN2211. [^{127}I]NN2211 was purified by RP-HPLC. In a typical, preparation starting with 3.50 mg of NN2211, 50% of the mono iodinated product was isolated in $>96\%$ purity.

[^{14}C]NN2211

[^{14}C]NN2211 was synthesized in four steps from α-protected [U-^{14}C] labelled L-glutamic acids by two different routes. The protection groups

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were *t*-butyl and α-benzyl (Figure 3). The first two steps were identical in both routes. The first reaction was a coupling between the amine group in α-protected L-[U-^{14}C]glutamic acids **2A** and **2B** and palmitoyl-benzotriazole **1**, using triethylamine (TEA) as base (Figure 3).

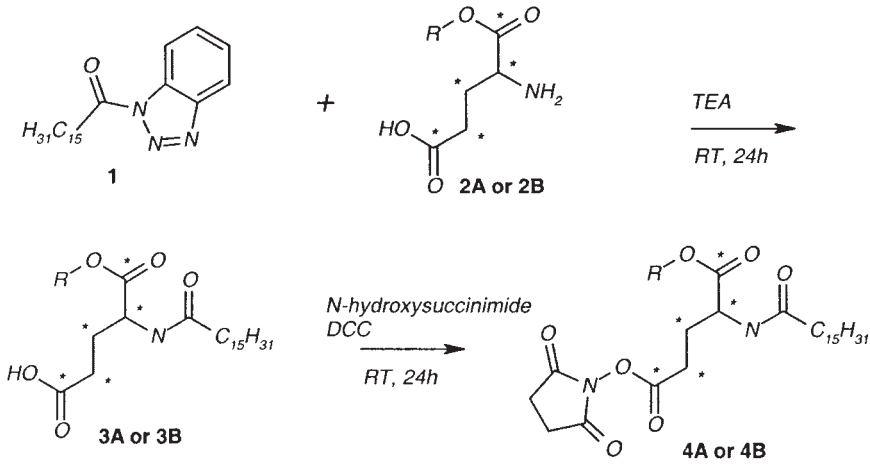

Figure 3. Synthesis of α-R-N^{α}-palmitoyl-γ-L-[U-^{14}C]glutamyl *N*-succinylester. A: R = t-butyl and B: R = benzyl

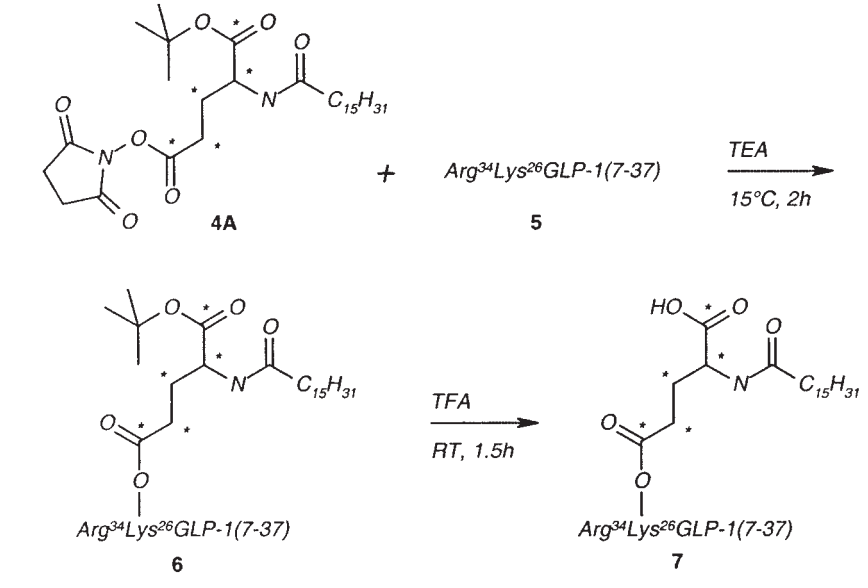

Figure 4. Synthesis of [^{14}C]NN2211 from α-t-butyl-N^{α}-palmitoyl-γ-L-[U-14C] glutamyl *N*-succinylester

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Formation of *N*-succinyl esters **4A** and **4B** was accomplished by reaction of the free γ-acids **3A**, **3B** and *N*-hydroxysuccinimide in the presence of the dehydrating agent *N,N′*-dicyclohexylcarbodimide (DCC). The following steps differed depending on the protection group employed.

t-Butyl protection group. α-*t*-Butyl N^{α}-palmitoyl-γ-L-[U-^{14}C]glutamyl *N*-succinylester **4A** was coupled to the Lys^{26} position of Arg^{34}Lys^{26}GLP-1(7-37) **5** (Figure 4). To separate off the 1-methyl-2-pyrrolidone (NMP) and TEA, the product **6** was precipitated by titration to the isoelectric point (pH 4.9). The solid was washed and freeze dried, so the final step could be performed anhydrously. The α-*t*-butyl protection group was cleaved with TFA to give [^{14}C]NN2211 **7**. Slowly adding the reaction mixture to a pre-cooled buffer solution quenched the reaction. To dispose of salts from the buffer, [^{14}C]NN2211 was precipitated by titration to the isoelectric point.

Benzyl protection group. This approach had the advantage of performing the deprotecting of the L-glutamic acid before the coupling to Arg^{34}Lys^{26}GLP-1(7-37). In this way, harsh treatment of the protein as described above was avoided. The benzyl group in **4B** was hydrogenated using Pd/C as catalyst. N^{α}-Palmitoyl-γ-L-[U-^{14}C]glutamyl *N*-succinylester **8** was coupled to the Lys^{26} position of Arg^{34}Lys^{26}GLP-1(7-37) **5** under basic conditions to give [^{14}C]NN2211 **7** (Figure 5).

Purification: The products were purified by RP-HPLC using an alkaline mobile phase. This approach was selected to avoid fibrilation of the product, which is more likely to occur under acidic or neutral conditions. The pronounced tendency of GLP-1 to fibrilate and the ensuing difficulties in handling the protein in solution, are well-known from the literature.[1,5] Therefore, it was unfortunate that a second purification was necessary to reach the required purity of >98%. The final purification was achieved by HPLC using a RP C18 column and an acidic mobile phase. Only a small amount of [^{14}C]NN2211 (25 μCi, 0.4 mg) could be purified in each HPLC run, and a substantial loss of product was observed, probably due to fibrilation. The overall yield was 5.5% (*t*-butyl protection group) and 4.0% (benzyl protection group).

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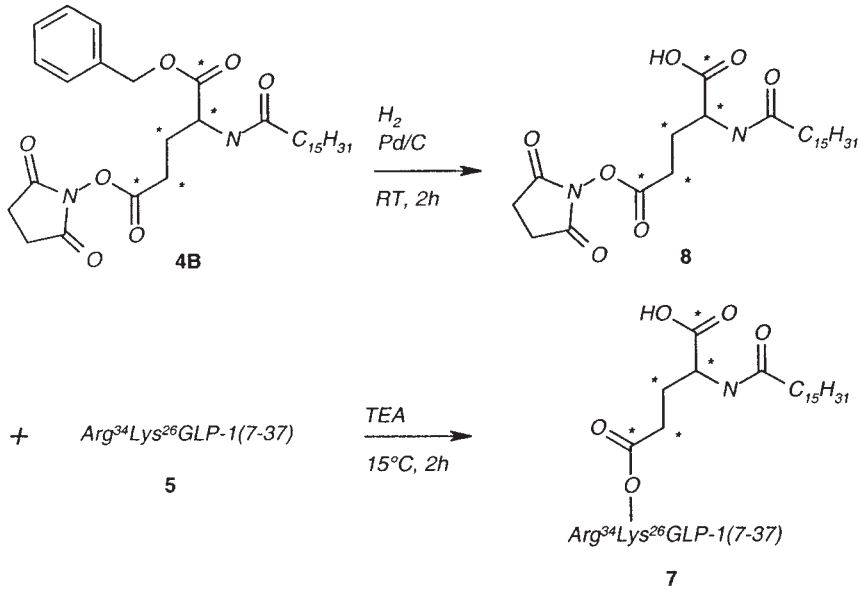

Figure 5. Synthesis of [^{14}C]NN2211 from α-benzyl-N^{α}-palmitoyl-γ-L-[U-^{14}C] glutamyl *N*-succinylester

Experimental

Materials and methods

Chemicals were purchased from Merck, Sigma, and Rathburn and used without further purification. Lactoperoxidase (Code No. 427488) was obtained from Calbiochem, USA and Human Serum Albumin (HSA) from Behringwerke, Germany. All reagents and solvents used were of analytical grade. [^{125}I]NaI (Code No. IMS 30), α-benzyl-L-[U-^{14}C]glutamic acid, and α-*t*-butyl-L-[U-^{14}C]glutamic acid were supplied by Amersham Bioscience, UK. NN2211, *N*-palmitoylbenzotriazol and [Arg^{34}Lys^{26}] GLP-1(7-37) were all supplied by Novo Nordisk A/S. The HPLC systems consisted of: Merck Hitachi Intelligent pump L-6200, Merck Hitachi Column Thermostat T6300 with a Rheodyne Injector, and Merck Hitachi UV/VIS detector L4000. Detection of [^{125}I] was performed on a Berthold HPLC radioactivity monitor LB 509, whereas [^{14}C] was detected using a Canbarra Packard Flow Detector 500 TR. The HPLC methods for [^{125}I]NN2211 and [^{127}I]NN2211 are shown in Table 1 and the methods used for [^{14}C]NN2211 are shown in Table 2.

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Table 1. HPLC Methods for [^{125}I] and [^{127}I] Iodination

Remarks	Column	Mobile Phase	Gradient
Method 1 Purification of [^{125}I] iodination mixture.	RP C18, OdDMeSi 5 µm YMC 120 Å, 250 × 4.6 mm Oven: 40°C; UV: 276 nm	0.1%TFA, 51.6% MeCN Flow: 1.0 ml/min	Isocratic 60 min Wash 60–70 min
Method 2 Analysis of [^{125}I] and [^{127}I] NN2211	RP C4, OdDMeSi 5 µm (YMC 120 Å, 250 × 4.6 mm) Oven: 40°C; UV: 276 nm	A: 10%MeCN, 0.1%TFA B: 45% MeCN, 45% 1-propanol, 0.1%TFA Flow: 1.0 ml/min	0–40 min.: 45% B - 48% B 41–50 min.: 100% B
Method 3 Purification of [^{127}I] iodination mixture	RP C4, OdDMeSi 5 µm (YMC 120 Å, 250 × 10 mm) Oven: 40°C; UV: 276 nm	A: 10% MeCN, 0,1% TFA B: 45% MeCN, 45% 1-propanol, 0.1% TFA Flow: 5.0 ml/min	0–70 min.: 43% B—44% B 71–80 min.: 100% B

The radioactivity in [^{125}I] samples was determined using a Na/Tl crystal connected to a Gamma-analyser, Type 1600, model 1601-1, Moelsgaard Medical. A Packard Tri-Carb 1000 liquid scintillation analyser was employed for counting [^{14}C]-samples. TLC analyses were performed on glass plates coated with silica gel 60 (5 × 20 cm, Merck Art 5714). The eluents were: Dichloromethane/MeOH (80/20) for analysis of **3A** or **3B** and dichloromethane/MeOH (95/5) for analysis of **4A** or **4B.** Radio-TLC analyses were obtained using a Bioscan Imaging Scanner System 200-IBM with an Autochanger 1000. For the freeze drying of products, a Christ freeze dryer Alpha 1-2 was employed.

Chemistry: ^{125}I and ^{127}I

[^{125}I]NN2211. The following substances were added to a 11 × 70 mm MiniSorp test tube: NN2211 (27 nmol) dissolved in phosphate buffer (50 µl, 0.1 M $NaPO_4$, pH 7.2), $Na^{125}I$ (50 µl, 5 mCi), H_2O_2 (20 µl, 0.32 mM) and lactoperoxidase (10 µl, 0.1 mg/ml). The reaction mixture was gently shaken for 5 min at 25°C. HPLC buffer (370 µl: MeCN 10%,

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Table 2. HPLC Methods for analytical and preparative [^{14}C] synthesis

Remarks	Column	Mobile Phase	Gradient
Method 4	RPC18, OdDMeSi 5 µm YMC 120 Å, 250 × 4.6 mm Oven: 35°C, UV: 276 nm	90% MeCN, 0,1%TFA Flow: 1.0 ml/min	Isocratic 45 min
Method 5	RP C4, OdDMeSi, 5 µm YMC 120 Å, 125 × 4.6 mm Oven: 60°C; UV: 276 nm	A: 10% MeCN, 0,1% TFA B: 45% MeCN, 45%, 1-propanol, 0.1% TFA Flow: 1.0 ml/min	0–60 min.: 46%B - 56%B 61–75 min.: 100%B
Method 6 First purification	X terra, Waters 250 × 4.6 mm, 5 µm Oven: 40°C; UV: 276 nm	A: 10% MeCN, 0.1% TEA B: 90% MeCN, 0.1% TEA Flow: 1.0 ml/min	0–60 min.: 17%B - 27% B, Manuel wash
Method 7 Second purification	RP C18, OdMeSi 5 µm YMC 120 Å, 250 × 4.6 mm Oven: 40°C, UV: 276 nm	A: 10% MeCN, 0.1% TFA B: 45% MeCN, 45% 1-propanol, 0.1%TFA Flow: 1.0 ml/min	0–60 min.: 47%B - 53%B, Manuel wash

TFA 0.1%) was added and the mixture injected on preparative HPLC (product peak at 26 min, HPLC method 1). [^{125}I]NN2211 was collected in a MiniSorp tube containing rat serum albumin (0.5 ml, 10% w/w solution). NaOH (10 µl, 1.0 M) per ml pool volume was added to neutralize TFA from the HPLC buffer. The MeCN was evaporated off by vacuum centrifugation (until a $\frac{1}{4}$ of the original pool volume was reached). The radioactivity concentration was adjusted to 100 µCi/ml by dilution with rat serum albumin (1% w/w solution).

[^{127}I]NN2211. The following substances were added to a 10 ml glass tube: NN2211 (5.3 µmol) dissolved in phosphate buffer 400 µl, 0.1 M $NaPO_4$, pH 7.2), NaI (10 µmol in 100 µl), H_2O_2 (40 µmol in 220 µl), and lactoperoxidase (200 µl, 0.1 mg/ml). After 5-and 80 min standing at 25°C, another 110 µl of the H_2O_2 solution was added to the reaction mixture. After 2.5 h, HPLC buffer (2.8 ml: MeCN 10%, TFA 0.1%) was

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added, and pH 2 was reached by addition of diluted TFA (200 µl: 1% solution). [^{127}I]NN2211 was purified by preparative HPLC in 10 runs. In each HPLC run, 2 mg protein (410 µl of the reaction mixture) was purified. Before application of HPLC, the sample volume was adjusted to 1.0 ml by adding HPLC buffer (300 µl: MeCN 10%, TFA 0.1%) and DMSO (300 µl). [^{127}I]NN2211 was collected from approximately 23–to 31 min. MeCN and 1-propanol were evaporated off *in vacuo*. Finally, the product was solidified by freeze drying.

Chemistry: ^{14}C

*α-t-Butyl N$^{\alpha}$- palmitoyl-*L-*[U-^{14}C]glutamic acid* ***3A***. α-*t*-Butyl-L-[U-^{14}C]-γ-glutamate (15 mCi, 60 µmol) **2A** in water: MeOH (4:1 v/v) was concentrated to dryness. The residue was dissolved in DMF (1 ml). TEA (10 µl, 72 µmol) and palmitoylbenzotriazol (25 mg, 72 µmol) **1** were added to the reaction mixture. After stirring overnight at room temperature the product was employed in the next step without further purification. (> 90% product **3A**, analyzed with TLC).

*α-t-Butyl N$^{\alpha}$-palmitoyl-γ-*L-*[U-^{14}C]glutamyl N-succinylester* ***4A***. *N*-hydroxysuccinimide (21 mg, 180 µmol), DCC (37 mg, 180 µmol), and THF (2 ml) were added to **3A** and the mixture was stirred overnight at room temperature. The product was employed in the next step without further purification. (> 80% product **4A** analyzed with TLC).

*Arg^{34}Lys^{26} (N$^{\varepsilon}$-(γ-*L-*[U-^{14}C](α-t-butyl glutamyl)(N$^{\alpha}$-palmitoyl)))-GLP-1(7-37)6*. Arg^{34}Lys^{26}GLP-1(7-37) **5** (152 mg, 45 µmol) was suspended in water (4 ml). NaOH (1 µM) was slowly added until dissolution of the protein (pH 9.5). To defibrilate the protein, the solution was cooled (4°C), and the pH adjusted to 11.5 by dropwise addition of NaOH (1 M). After 4 min, the pH adjusted back to 9.5 by dropwise addition of acetic acid (1 M). NMP (5 ml) was added carefully, and the pH adjusted to 11.5 by dropwise addition of TEA. α-*t*-Butyl *N*$^{\alpha}$-palmitoyl-γ-L-[U-^{14}C]glutamyl *N*-succinylester **4A** (8 mCi, 32 µmol) dissolved in 3 ml NMP was slowly added to the solution of Arg^{34}Lys^{26}GLP-1(7-37). The reaction mixture was gently stirred (3 h, 15°C). Water (12 ml) was added followed by titration to the isoelectric point (pH 4.9) with HCl (2 M). The mixture was allowed to precipitate overnight (4°C). The solid was isolated by centrifugation and washed with water (4 ml). The crude

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product was freeze-dried overnight before the final step (50% product analyzed with HPLC method 5).

[^{14}C]NN2211 (t-butyl Route) **7**. Arg^{34}Lys^{26}(N^{ε}-(γ-L-[U-^{14}C](α-t-butyl glutamyl)(N^{α}-palmitoyl)))-GLP-1 (7-37) **6** was dissolved in TFA (2 ml) and stirred for 1.5 h at room temperature. The reaction was quenched by dropwise addition of the reaction mixture to a phosphate buffer (Na_2HPO_4 2.8 g in 15 ml water) at 0°C. [^{14}C]NN2211 was precipitated and isolated as described in the previous step. (45% product, analyzed with HPLC method 5).

α-*Benzyl N^{α}-palmitoyl*-L-*[U-^{14}C]glutamic acid* **3*B***. α-Benzyl-L-[U-^{14}C]-glutamic acid **2B** (5 mCi, 30 μmol) was dissolved in NMP (0,9 ml). TEA (7 μl, 49 μmol) and palmitoylbenzotriazole **1** (16 mg, 45 μmol) was added. The reaction mixture was stirred overnight at room temperature. The mixture was diluted with water (25 ml) and HCl (100 μl, 2 N) was added. The mixture was extracted with ethylacetate (2 × 25 ml). The combined organic layers were used in the following step without further purification (> 90% product, analyzed with TLC).

α-*Benzyl N^{α}-palmitoyl-γ*-L-*[U-^{14}C]glutamyl N-succinylester* **4*B***. α-Benzyl N^{α}-palmitoyl-L-[U-^{14}C]glutamic acid **3B** (25 ml, 6.75 mCi, 40 μmol) was concentrated to dryness. The residue was dissolved in THF (2.5 ml). *N*-hydroxysuccinimide (11.5 mg, 100 μmol) and DCC (20.6 mg, 100 μmol) were added. The reaction mixture was stirred overnight at room temperature. The solvent was evaporated *in vacuo*, and the residue was dissolved in dichloromethane (2 ml). The resulting solution was applied to a Waters Sep-Pak silica cartridge (0.5 g, activated with 10 ml dichloromethane). The product was extracted with dichloromethane/acetone 90%/10% (4 ml). (> 89% product, analyzed by TLC).

N^{α}-Palmitoyl-γ-L-*[U-^{14}C]glutamyl N-succinylester* **8**. α-Benzyl N^{α}-palmitoyl-γ-L-[U-^{14}C]glutamyl *N*-succinylester **4B** (4 ml, 2.2 mCi, 13 μmol) was concentrated to dryness *in vacuo*. The residue was dissolved in 2.5 ml acetone and a stream of H_2 was passed through the solution. Pd/C (2.5 mg) was added, and the reaction mixture was stirred at room temperature (2 h) under H_2 (1 atm). The reaction mixture was filtered, and the catalyst was washed with acetone (4 × 1 ml). The combined filtrates were evaporated to dryness and used immediately in the last step. (> 72% product, analyzed by the HPLC method 4).

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[^{14}C]NN2211 (Benzyl route) **7**. Arg^{34}Lys^{26}GLP-1(7-37) **5** (67.7 mg, 20 μmol) was suspended in water (2 ml) and defibrilated as described previously. NMP (4.5 ml) was added, and the pH adjusted to 11.5 by addition of TEA. The solution was transferred to a round-bottom flask containing 1.9 mCi N^{α}-palmitoyl-γ-L-[U-^{14}C]glutamyl *N*-succinylester **8**. The reaction mixture was stirred for 4 hours at 15°C. Water (2 ml) was added, and the reaction quenched by addition of acetic acid (1 M), until pH 9.5 was reached. (> 50% product analyzed using HPLC method 5).

[^{14}C]NN2211 Purification. [^{14}C]NN2211 was purified using two different preparative RP-HPLC methods (method 6 and 7). The crude product was dissolved in HPLC buffer (MeCN 10%, TEA 0,1%) to a radiochemical concentration of 60–100 μCi/ml. 800 μl of this solution was injected on the preparative HPLC in each separation (HPLC method 6). The MeCN was evaporated *in vacuo*, and the remaining solution was freeze-dried. [^{14}C]NN2211 was dissolved in ethanol and then prepared for the second purification by adjusting the concentration with HPLC buffer (MeCN 10%, TFA 0.1%) to 25–30 μCi/ml. 850 μl of this solution was subsequently purified in each HPLC run (method 7). Fractions were collected in a round bottom flask between approximately 30–40 min runtime. Evaporation and freeze-drying was performed as described above. The [^{14}C]NN2211 was formulated at a concentration of 19 μCi/ml in 80% ethanol and 20% phosphate buffer (0.25 mM Na_2HPO_4, pH 7.4) and stored at –20°C. The overall yield was 5.5% and 4.0%, respectively and radiochemical purity > 97% by both routes (analyzed by HPLC method 5).

Conclusion

[^{125}I]NN2211 and [^{127}I]NN2211 were synthesised by iodination using the lactoperoxidase method. [^{14}C]NN2211 was prepared in four steps applying two different routes with overall yields of 5.5% (with t-butyl protection group) and 4.0% (with benzyl protection group).

References

1. Knudsen LB, Nielsen PF, Huusfeldt PO, *et al. J Med Chem* 2000; **43:** 1664–1669.

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2. Markussen J, Havelund S, Kurtzhals P, *et al. Diabetologia* 1996; **39:** 281–288.
3. Shäffer L, Jonassen I, Markussen J, *et al. Diabetes* 1996; **45** (**Nsuppl 2**): 139A.
4. Regoeczi E. *Iodine-labeled Plasma Proteins*, vol 1. CRC Press, Inc.: Boca Raton, 1984.
5. Clodfelter DK, Pekar AH, Rebhun DM, *et al. Pharm Res* 1998; **15:** 254–262.

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