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Research Article

Radiolabelling of NNC 05-1869, a compound for treatment of diabetic neuropathy

Jacob S. Valsborg* and Christian Foged
Novo Nordisk A/S, Isotope Chemistry, Novo Nordisk Park, DK-2760 Maaloev, Denmark

Summary

Synthesis of ^{3}H-, ^{14}C- and ^{125}I-labelled NNC 05-1869 is described. The ^{3}H-label was obtained by catalytic hydrogenation of a carbon–carbon double bond with tritium gas, which after purification yielded 470 μCi [^{3}H]NNC 05-1869 with a specific radioactivity of 36 Ci/mmol. ^{14}C-labelling was accomplished in three steps, including a chiral HPLC separation, starting from ethyl 3-[carboxyl-^{14}C]piperidinecarboxylate (1). The overall radiochemical yield was 1.5 mCi of [carboxyl-^{14}C]NNC 05-1869 (14%) with a specific radioactivity of 55 mCi/mmol. Electrophilic aromatic iododestannylation of the tin-precursor NNC 47-4310 with $Na^{125}I$ resulted in a *cis*/*trans* mixture, which was separated on RP-HPLC to give the two isomers in 20% and 22%, yield, respectively.

Key Words: ^{14}C; ^{125}I; ^{3}H; diabetic neuropathy; NNC 05-1869

Introduction

(*R*)-1-(3-(10,11-Dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-1-propyl)-3-piperidinecarboxylic acid[1] (= NNC 05-1869, Figure 1) has been shown to be an active compound in animal models of diabetic

*Correspondence to: J. S. Valsborg, Novo Nordisk A/S, Isotope Chemistry, Novo Nordisk Park, DK-2760 Maaloev, Denmark

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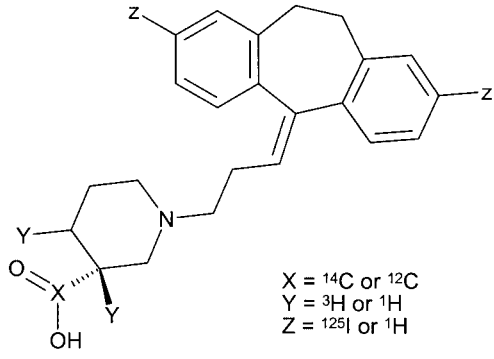


Figure 1. Structure of NNC 05-1869 showing the different labelling positions

neuropathy. In order to investigate the metabolic fate of this compound a ^{14}C-labelled version of NNC 05-1869 was required.

For receptor binding studies, a tritium-labelled form of NNC 05-1869 with a specific radioactivity of 36 Ci/mmol was prepared. However, for some receptor binding studies, a radioligand with a higher specific radioactivity was needed. The NNC 05-1869 structure was therefore labelled with ^{125}I in the tricyclic ring system resulting in [^{125}I]NNC 47-0264 and [^{125}I]NNC 47-0265 with a specific radioactivity of 2 Ci/μmol. The iodinated analogues NNC 47-0264 and NNC 47-0265 (Figure 1) have shown to retain their biological activity.[2] Both the *cis*-isomer (NNC 47-0265) and the *trans*-isomer (NNC 47-0264) are of interest in their ^{125}I-labelled form, since the difference in receptor selectivity of these two radioligands can provide important information about structure–activity relationship.

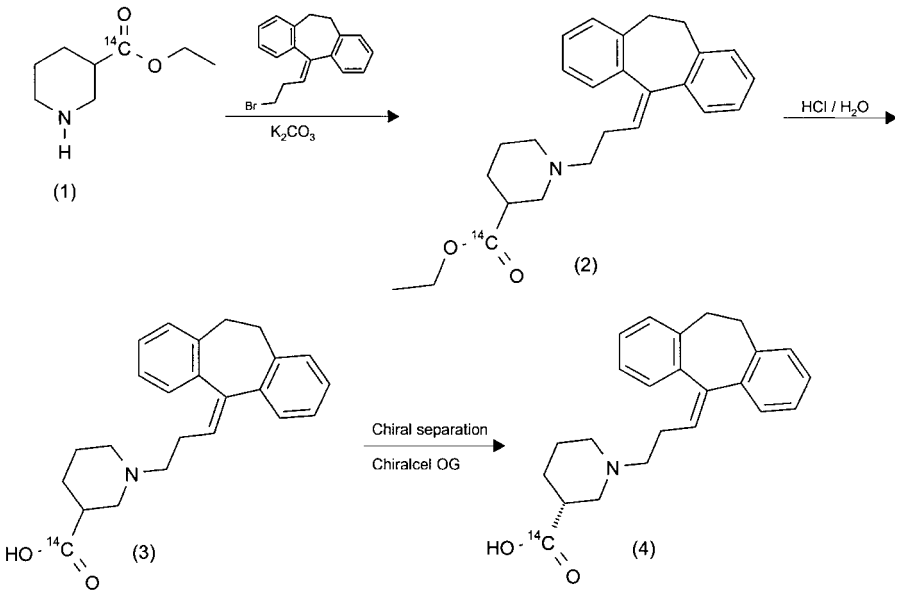
We report herein the synthesis of NNC 05-1869 labelled with ^{14}C, ^{3}H or ^{125}I.

Results and discussion

^{14}C-Labelling

^{14}C-Labelling of NNC 05-1869 was performed in the carboxylic part of the piperidine ring (Scheme 1). The starting materials in a three-step synthetic route were ethyl 3-[carboxyl-^{14}C]piperidinecarboxylate[3] and 5-(3-bromo-1-propylidene)-10,11-dihydro-5H-dibenzo[a,d]cycloheptene.

Reaction of ethyl 3-[carboxyl-^{14}C]piperidinecarboxylate (1) with 5-(3-Bromo-1-propylidene)-10,11-dihydro-5H-dibenzo[a,d]cycloheptene in ethyl acetate with potassium carbonate as base produced ethyl-1-(3-



Scheme 1. Synthetic route for preparation of ^{14}C-labelled NNC 05-1869

(10,11-dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-1propyl)-3-[carboxyl-^{14}C]piperidine-carboxylate (2) in a radiochemical yield of 73%. The overall reaction time for this step was approximately 94 h. During that period, 5-(3-bromo-1-propylidene)-10,11-dihydro-5H-dibenzo[a,d]-cycloheptene slowly degraded and therefore additional material was added.

Crude 2, with a radiochemical purity of 80%, was used directly without purification and hydrolysed with water and concentrated hydrochloric acid, leading to 1-(3-(10,11-dihydro-5H-dibenzo[a,d]-cycloheptene-5-ylidene)-1-propyl)-3-[carboxyl-^{14}C]piperidinecarboxylate (3). Radio-HPLC analysis showed a radiochemical conversion $>70\%$ after 20 h of hydrolysis. After neutralisation and work-up the raw material had a radiochemical purity $>94\%$ according to radio-HPLC. The material was used without further purification in the next step.

Crude 3 was dissolved in a mixture of 2-propanol and *n*-hexane. It was important to adjust the amount of 2-propanol, because preliminary studies had shown that the amount of 2-propanol was critical for the chromatography. The chiral radiochemical purity after the first separation was only approximately 90%. Therefore a second purification was carried out using the same procedure.

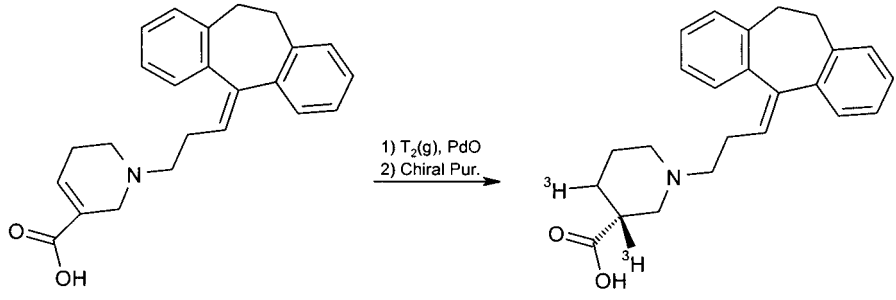
Due to the excess of 2-propanol and ethanol in the mobile phase, small impurities of ethyl and propyl esters were formed. These impurities were removed by a Sep-Pak purification. A gradient of water and MeCN was used as mobile phase for the Sep-Pak purification. The relevant fractions were combined and rotary evaporated. The residue was then dissolved and stored in a mixture of water and acetonitrile (1:1).

The procedure described above resulted in [carboxyl-^{14}C]NNC 05-1869 with a chiral purity of $>98\%$ as determined by chiral radio-HPLC analysis. The enantiomeric purity showed less than 0.5% of the *S*-form enantiomer to be present. Reverse-phase (RP) HPLC showed a radiochemical purity of $>98\%$.

The identity of the radioligand was confirmed by HPLC and MS: [Carboxyl-^{14}C]NNC 05-1869 eluted with the same retention time as a standard reference sample on reverse phase HPLC. The specific radioactivity was 55 mCi/mmol as determined by MS using a reference standard. The decrease in radiochemical purity after 2 months of storage in water and acetonitrile (1:1) at $+5^{\circ}$C was less than 1%.

^{3}H-Labelling

^{3}H-Labelling was performed by treating 1-(3-(10,11-dihydro-5H-dibenzo[a,d]-cycloheptene-5-ylidene)-1-propyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (5) with tritium gas and catalytic amount of PdO (Scheme 2). This resulted in a product which after HPLC purification had a radiochemical purity $>98\%$ and a specific radioactivity of 36 Ci/mmol.



(5) (6)

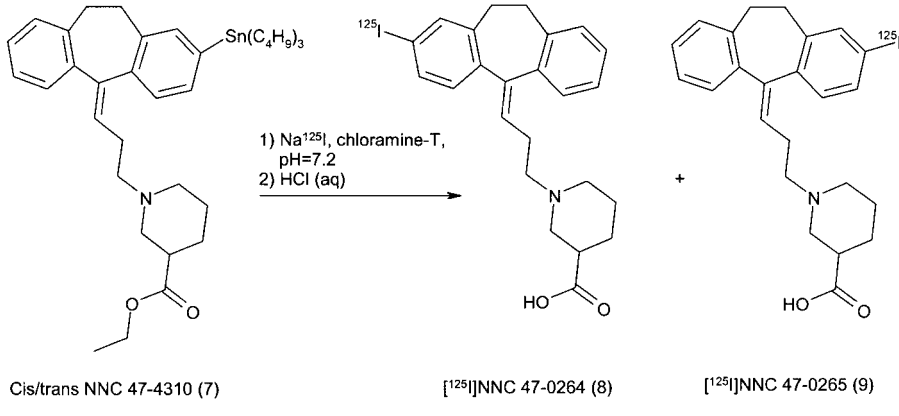
Scheme 2. Synthetic route for preparation of ^{3}H-labelled NNC 05-1869

^{125}I-Labelling

^{125}I-Labelling was performed from the tin-precursor by an electrophilic aromatic iododestannylation reaction (Scheme 3). In our laboratories, we have experienced destannylation to be a very suitable method for radiohalogenation.[4,5] The ester, (*R*)-1-(3-(2-Tributylstannyl-10,11-dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-1-propyl)-3-piperidine-carboxylic acid ethyl ester (=NNC 47-4310) was readily available and was stable both as a solid and in solution. Since the hydrolysis of the ester after iododestannylation was easily accomplished, there was no need for the tributyltin-precursor as a free acid. We chose to use the *cis*/*trans* mixture of the tin-precursor, since we needed both the *cis*- and *trans*-isomers of the radioiodinated product. The two forms were easily separated on HPLC. The labelled products could be separated from the tin-precursor using RP-HPLC. The radioactive yield of [^{125}I]NNC 47-0265 and [^{125}I]NNC 47-0264 was 20% and 22%, respectively. The specific radioactivity was assumed to be the same as that of Na^{125}I, i.e. 2 Ci/μmol which was considered to be satisfactory for receptor binding studies *in vitro*.

Conclusions

In conclusion, NNC 05-1869 has been labelled with ^{14}C, ^{3}H and ^{125}I. All three labelling procedures gave products with a radiochemical purity >98% and the specific radioactivity was 55 mCi/mmol, 36 Ci/mmol and 2 Ci/μmol, respectively.



Scheme 3. Synthetic route for preparation of ^{125}I-labelled NNC 05-1869

Experimental

Ethyl 3-[carboxyl-^{14}C]piperidinecarboxylate was synthesised in a four-step procedure as described previously.[3] The material obtained had a radiochemical purity of >90% by radio-TLC. The specific radioactivity of the material was 57 mCi/mmol. NNC 47-0264, NNC 47-0265 and the *cis*/*trans*-precursor NNC 47-4310 were synthesised at Novo Nordisk A/S, Maaloev, Denmark. Other chemicals were obtained from commercial sources and all solvents were of analytical grade. Carrier-free Na^{125}I was obtained from Amersham Denmark. Specific radioactivity of [carboxyl-^{14}C]NNC 05-1869 was determined using a Finnigan-MAT TSQ 70B, SP/MS system. Values were compared with an unlabelled reference sample. TLC was performed on glass plates coated with 0.25 mm silica gel 60 F_{254} (Merck). Radio-TLC analysis was performed using a Bioscan Imaging Scanner System 200-IBM with an Autochanger 1000. The collimator grid contained 10 strings/mm and the P10 gas (10% methane in argon) flow was 1.5 l/min. Determination of total radioactivity was carried out on a Packard 1000 CA Tri-carb liquid scintillation analyser.

HPLC analyses were performed using a Merck HPLC pump L-6200 with a rheodyne injector (100 µl loop) and a Merck UV-detector L-4000 (operating at 214 and 240 nm). Radioactivity in the column effluent was monitored with a Radiomatic/Canberra Flo-One beta detector (A-515), using a 500 ml liquid flow cell. Preparative HPLC separations were performed using a Merck HPLC pump L6200 with a rheodyne injector (1000 ml loop) and a Merck UV-detector L-4000 (operating at 280 nm).

Four different HPLC systems were used:

System A: Reverse phase C-18 separations were accomplished at room temperature with a column (250 × 4 mm, 5 mm) from Novo Nordisk A/S, using a mixture of TFA (0.1%) and MeCN. The flow rate was 1.0 ml/min.

System B: Chiral HPLC analysis was accomplished at room temperature with a Chiralcel OD-H column (250 × 4.6, 10 mm) from Chiralcel Industries. The mobile phase was a mixture of *n*-hexane, 2-propanol, ethanol and TFA (74/14/12/0.5). The flow rate was 0.8 ml/min.

System C: Preparative chiral HPLC was accomplished with a Chiralcel OG column (250 × 4.6, 10 mm) from Chiralcel Industries. The mobile phase was a mixture of *n*-hexane, 2-propanol, ethanol and TFA (74/14/12/0.5). The flow rate was 0.8 ml/min.

System D: Sep-Pak purification was accomplished with Waters™ Sep-Pak Vac RC Cartridges packed with 500 mg C-18 material. The mobile phase was a mixture of water and acetonitrile.

Ethyl-1-(3-(10,11-dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-1-propyl-3-[carboxyl-^{14}C]piperidinecarboxylate (2)

5-(3-Bromo-1-propylidene)-10,11-dihydro-5H-dibenzo[a,d]cycloheptene (87.98 mg, 0.28 mmol) and ethyl 3-[carboxyl-^{14}C]piperidinecarboxylate (1, 11 mCi; specific activity 57 mCi/mmol) were dissolved in ethyl acetate (7 ml). While stirring, potassium carbonate was added until the solution was alkaline (approx. 3 g added). The mixture was filtered and the filtrate rotary evaporated to a pale yellow oil. The oil was dissolved in ethyl acetate (7 ml) and potassium carbonate (112 mg) added. The mixture was stirred under reflux for 22 h. TLC on silica in heptane:ethyl acetate (1:1) showed a 70% conversion to the desired product. More bromo compound (200 mg) and ethyl acetate (7 ml was added and refluxing continued. TLC analysis at 94 h showed 93% conversion. The reaction mixure was cooled to room temperature, filtered and the filtrate rotary evaporated to give a yellow oil.

Radiochemical yield: 8.0 mCi (73%). Radiochemical purity >80%, determined by radio-HPLC (System A) and radio-TLC analysis (System I).

1-(3-(10,11-Dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-1-propyl)-3-[carboxyl-^{14}C]piperidinecarboxylate (3)

Crude 2 was refluxed in a mixture of concentrated hydrochloric acid (0.2 ml) and water (4 ml) for 20 h with stirring. The reaction mixture was cooled to room temperature and rotary evaporated at 35°C. To the residue was added water (1 ml) and saturated aqueous sodium hydrogen carbonate (2 ml). A sample of this mixture was run at silica TLC in methanol against a non-radioactive marker showed 95% radiochemical purity. The mixture was rotary evaporated to a brown oil/white solid mixture.

Radiochemical yield: 5.9 mCi (74%). Radiochemical purity >94%, determined by radio-HPLC (System A).

(R)-1-(3-(10,11-Dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-1-propyl)-3-[carboxyl-^{14}C]piperidinecarboxylic acid (=[^{14}C]NNC 05-1869) (4)

Crude 3 was agitated in a sonicator bath with 2-propanol (3 ml). The resultant partial solution was filtered through a 0.45 μm PTFE

membrane and to the yellow filtrate was added *n*-hexane (7 ml) to give a total volume of approximately 10 ml. The resulting mixture was purified on a Chiralcel OG column using a mixture of *n*-hexane, 2-propanol, ethanol and TFA as mobile phase (System C). Two chiral purifications were needed to get an enantiomeric purity larger than 98%. Furthermore, a final Sep-Pak purification was also necessary to remove small impurities of ethyl and propyl esters. A gradient of water and MeCN was used as mobile phase for the Sep-Pak eluation (System D). The relevant fractions were combined and rotary evaporated. The residue was dissolved and stored in a mixture of water and MeCN (5.75 ml; 1:1).

Radiochemical yield: 1.5 mCi (25%). Radiochemical purity >98%, determined by radio-HPLC (System A). Radiochemical chiral purity >98%, determined by radio-HPLC (System B). Specific radioactivity 55 mCi/mmol as determined by MS.

[^{3}H]NNC 05-1869 (6)

1-(3-(10,11-Dihydro-5H-dibenzo[a,d]-cycloheptene-5-ylidene)-1-propyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (10.08 mg, 0.028 mmol) was dissolved in methanol (500 µl) with PdO (3.38 mg) as catalyst. The tritiation was run for 2 h using 7 Ci tritium gas. The catalyst was removed by filtration through celite and the solvent was removed by lyophilisation. HPLC analysis (System B) of the mixture (70 mCi) indicated a radioactivity profile in which 20% of the mixture was due to the desired product. Purification (System C) of a portion of the crude reaction mixture (4 mCi) afforded radiochemically pure [^{3}H]NNC 05-1869.

Radiochemical yield: 470 µCi (12%). Radiochemical purity >98%, determined by radio-TLC ($CHCl_3$:EtOAc:MeOH:1-Butanol:AcOH = 4:1:2:1:2; $R_f = 0.72$). Radiochemical chiral purity >98%, determined by radio-HPLC (System C). Specific radioactivity 36 Ci/mmol as determined by HPLC.

[^{125}I]NNC 47-0264 (8) and [^{125}I]NNC 47-0265 (9)

NNC 47-4310 (50 µg in 10 µl of acetonitrile), sodium phosphate buffer (20 µl, 0.5 M, pH 7.2) and Na^{125}I (2 mCi in 20 µl of 0.01N NaOH) were mixed in a small vial. Chloramine-T (10 µg in 10 µl of water) was added to the mixture, which was stirred for 10 min, concentrated HCl (20 µl)

was added and the reaction vial was heated at 60°C for 1 h. The reaction mixture was injected onto the HPLC column. [^{125}I]NNC 47-0265 eluted after 39–40 min and [^{125}I]NNC 47-0264 eluted after 41–42 min with a rentention time identical to that of non-radioactive standard reference samples. [^{125}I]NNC 47-0264 and [^{125}I]NNC 47-0265 were purified using a C-18 column (Novo Nordisk 250 × 4 mm), using a mixture of 0.1% TFA and acetonitrile (64:36) as a mobile phase. The flow rate was 1.0 ml/min. The yield after purification was 400 μCi of [^{125}I]NNC 47-0265 and 430 μCi of [^{125}I]NNC 47-0264 (overall radioactive yield of 42%) with a radiochemical purity of > 98%. The specific radioactivity was assumed to be the same as that of Na^{125}I, i.e. 2000 Ci/mmol.

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