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Labelling of the guanylate cyclase activator cinaciguat (BAY 58-2667) with carbon-14, tritium and stable isotopes

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For studies of pharmacokinetics and drug metabolism of the new soluble guanylate cyclase activator cinaciguat (BAY 58-2667) the ^{14}C-labelled compound was synthesized. The tritiated compound was required to elucidate the mode of action and the stable labelled compound was required for bio-analytical studies by quantitative mass spectrometry as well. Two radiosyntheses are described with different formation of the labelled intermediate 1-(chloro[^{14}C]methyl)-4-(2-phenylethyl)benzene. The first one started with ^{14}C-carboxylation of 1-bromo-4-(2-phenylethyl)benzene yielding the desired product in 5 steps. In the second synthesis intermediate 1-(chloro[^{14}C]methyl)-4-(2-phenylethyl)benzene was formed by chloromethylation of bibenzyl with [^{14}C]paraformaldehyde/hydrochloric acid subsequently resulting in the final product in three steps. Tritium labelling was performed by tritium exchange of the diester intermediate using an organo-iridium catalyst and subsequent saponification. The stable labelled compound was synthesized via a convergent synthesis starting with ^{13}C,^{15}N-cyanation of 1-(chloromethyl)-2-{[4-(2-phenylethyl)benzyl]oxy}benzene and ^{13}C-cyanation of methyl 4-bromobenzoate, respectively. The labelled product was obtained after 7 chemical steps.

Keywords: guanylate cyclase activator; carbon-14; carbon-11; tritium; carbon-13; nitrogen-15; synthesis

Introduction

Soluble guanylate cyclase (sGC) is a heterodimeric enzyme with a heme moiety as the prosthetic group. Activated by nitric oxide (NO), it generates the second messenger cGMP. Impaired bioavailability and/or responsiveness to endogenous NO have been implicated in the pathogenesis of especially cardiovascular diseases. Therapies with organic nitrites and other NO donors are limited by non-specific interactions of NO with various biomolecules, lack of response and development of tolerance.

The new guanylate cyclase activator cinaciguat (BAY 58-2667) acts in a NO-independent manner.[1] This provides considerable therapeutic advantages. Cinaciguat activates sGC with EC_{50} and K_d values in the low nanomolar range. This renders the compound the most potent NO-independent sGC activator reported to date[2] (Figure 1).

In contrast to hem-dependent sGC stimulators, cinaciguat produces an additive, non-synergistic effect when combined with NO donors. It relaxes blood vessels with potency that is several orders of magnitude greater than the NO donors sodium nitroprusside and 3-morpholinosydnonimine.[3] The compound reduces coronary perfusion pressure in the rat Langendorff heart preparation and remains active in the tissues made tolerant to gylceryl trinitrate.[4]

For studies of pharmacokinetics and drug metabolism of cinaciguat, the version with a metabolically stable carbon-14 label was required. Two carbon-14 labelling syntheses were performed using different synthetic routes to the carbon-14 labelled key intermediate 5. The objective of the second synthesis was to reduce the number of reaction steps and to avoid a critical by-product of the reduction step. Additionally for studies of the mode of action tritium-labelled substance was synthesized. The labelling with tritium was realized by an organo-iridium exchange using the ester form of cinaciguat. As an internal standard for quantitative mass spectral

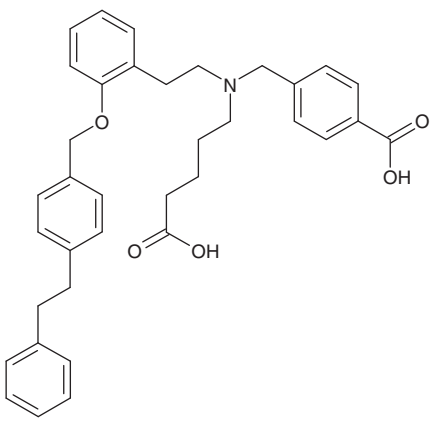

Figure 1. Structure of BAY 58-2667.

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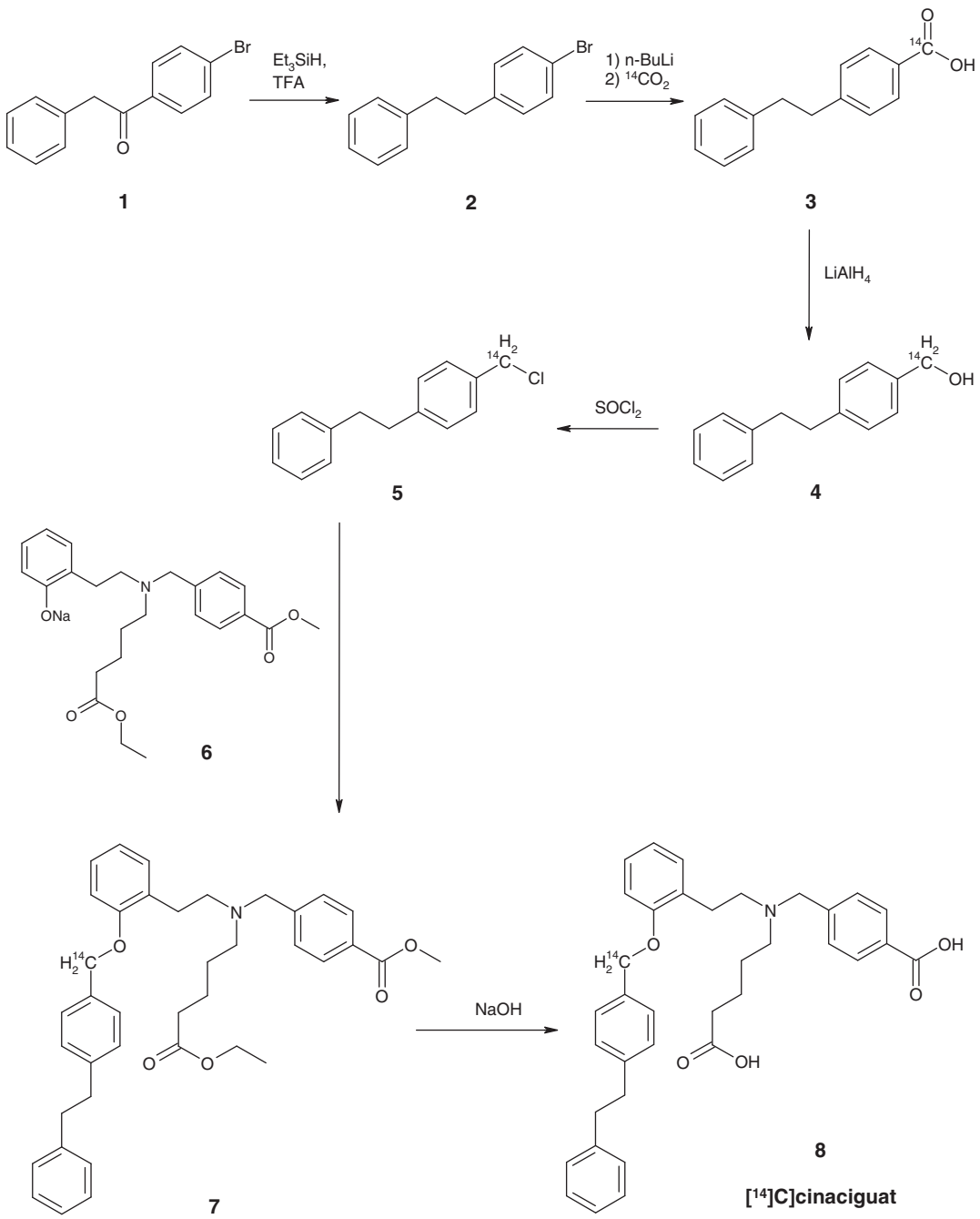
analysis a stable labelled compound was prepared as well. A convergent synthesis was performed to introduce carbon-13 and nitrogen-15.

Results and discussion

The first radiosynthesis of cinaciguat was performed as given in Scheme 1.[5] Intermediate 5 was prepared by a new synthetic route, different from the technical synthesis.[6]

Commercially available 1-(4-bromophenyl)-2-phenylethanone was reduced to the corresponding bibenzyl derivative. The conversion with red phosphorus in hydroiodic acid under drastic conditions was described earlier by Speer and Hill.[7] Common reduction with triethylsilane in trifluoroacetic acid performed in this synthesis led to 1-bromo-4-(2-phenylethyl)benzene in a better yield under mild conditions. In spite of the mild conditions, the dehydration of the hydroxyl intermediate could not be fully avoided giving 1-bromo-4-[(E)-2-phenylvinyl]benzene in a small amount as by-product which was very difficult to remove. The carbon-14 was introduced by halogen–metal exchange with *n*-butyllithium followed by carboxylation with $^{14}CO_2$. The obtained 4-(2-phenylethyl)[carboxy-^{14}C]benzoic acid (3) was then reduced to the alcohol derivative 4 with lithium aluminium hydride as described by M. Carrara et al. for the



Scheme 1. First radiosynthesis of [^{14}C]cinaciguat.

non-labelled methyl ester.[8] The direct reduction of the acid proceeded as good as in the case of the ester. By common chlorination with thionyl chloride intermediate 4 was converted to 1-[chloro[^{14}C]methyl]-4-(2-phenylethyl)benzene (5).

The last but one step of the radiosynthesis was the *O*–alkylation of intermediate 6 with the chloro[^{14}C]methyl derivative 5 using sodium methoxide as base. Intermediate 6 was prepared according to the technical synthesis.[6] Subsequent ester saponification yielded the carbon-14 labelled cinaciguat. Final analytics revealed a small contamination with the stilbene derivative of the product, which made the chromatographic purification difficult.

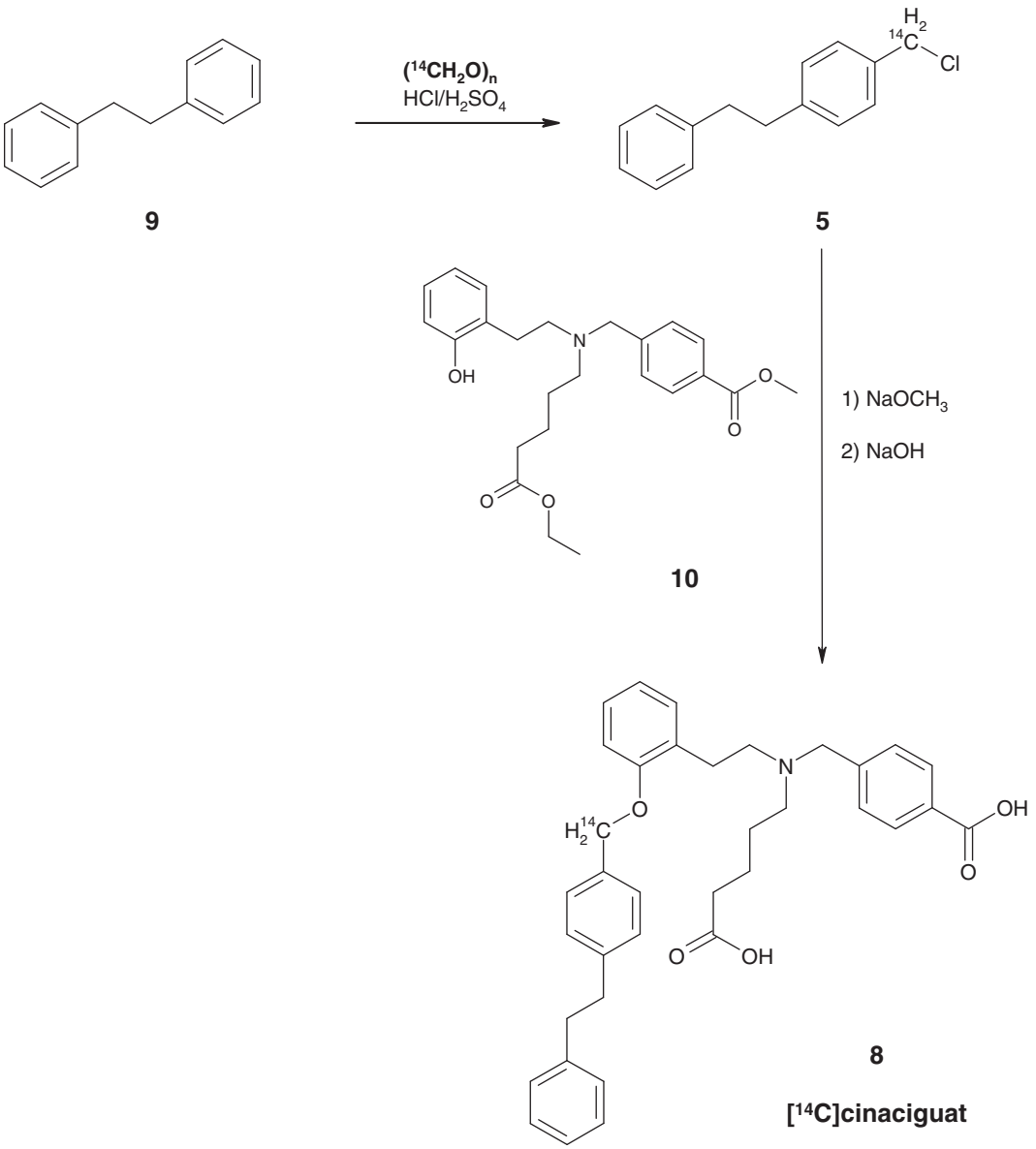
With the aim to shorten the radiosynthesis an alternative synthetic route to intermediate 5 was elaborated (Scheme 2).

In 1960 F. Vismara described the formation of intermediate 5 by chloromethylation of bibenzyl (9) with 37% formaldehyde solution (formalin).[9] Experiments with paraformaldehyde showed superiority over the solution. To keep the excess of formaldehyde in reasonable limits incomplete conversion was accepted. Nevertheless the reaction needed a long time (49 h at 60°C). The obtained labelled 1-[chloro[^{14}C]methyl]-4-(2-phenylethyl)benzene had to be purified intensively by repeated chromatography.

In difference to the first synthesis alkylation and subsequent ester saponification were performed in a one-pot procedure.

This second synthesis proceeded much quicker than the first one and delivered the product in a radiochemical yield (9.6%) better than that of the first one (5.7%). Nevertheless due to new by-products the final purification was also problematic.

For research purposes a receptor binding study of cinaciguat was necessary. Therefore, the compound was labelled with tritium. Labelling was performed by an organo-iridium catalyzed hydrogen exchange.[10,11] The advantage of this method is the possibility of direct introduction of tritium into the final compound. Under several iridium catalysts such as (1,5–cyclooctadiene) bis-(triphenylphosphine)-iridium(I) tetrafluoroborate



Scheme 2. Second radiosynthesis of [^{14}C]cinaciguat.

and (1,5–cyclooctadien) bis-(methyldiphenylphosphine)-iridium(I) hexafluorophosphate (1,5-cyclooctadiene)(pyridine)(tricyclohexylphosphine)-iridium(I) hexafluorophosphate was the most suitable to get an acceptable labelling degree. The diester (11) of cinaciguat was used in the reaction due to the insufficient solubility of cinaciguat in dichloromethane. After the labelling process the tritiated diester 11 was saponified yielding [^{3}H]cinaciguat. Due to the small radioactive yield a ^{3}H NMR spectrum was not performed. The ^{3}H NMR spectrum of a very similar compound (the *O*-substituent at the phenol was 4-azidophenyl-benzyl instead of phenyl-ethyl-benzyl in compound (13), which was labelled with tritium under exactly the same conditions, showed the tritium in the ortho-position of the carboxyl group in the benzoic acid substituent. Because of the same aromatic methoxycarbonyl directing group in both of the molecules it was assumed that the labelling in compound (13) was in the ortho-position as well. The labelling degree was determined to be 18.7% by mass spectrometry (Scheme 3).

For quantitative mass spectrometry cinaciguat labelled with stable isotopes was required as internal standard (ISTD) with three mass units difference in comparison to the non-labelled compound.

For stable labelling a new synthetic way was developed. The convergent synthesis (Scheme 4) started from the substituted benzylchloride (14), which was converted to the ^{13}C and ^{15}N labelled amine (18) in 4 reaction steps.[6] The labelling positions were introduced by crown ether supported substitution of chlorine against [^{13}C, ^{15}N]cyanide which was reduced with hydrogen and raney nickel as catalyst under a pressure of 400 kPa.

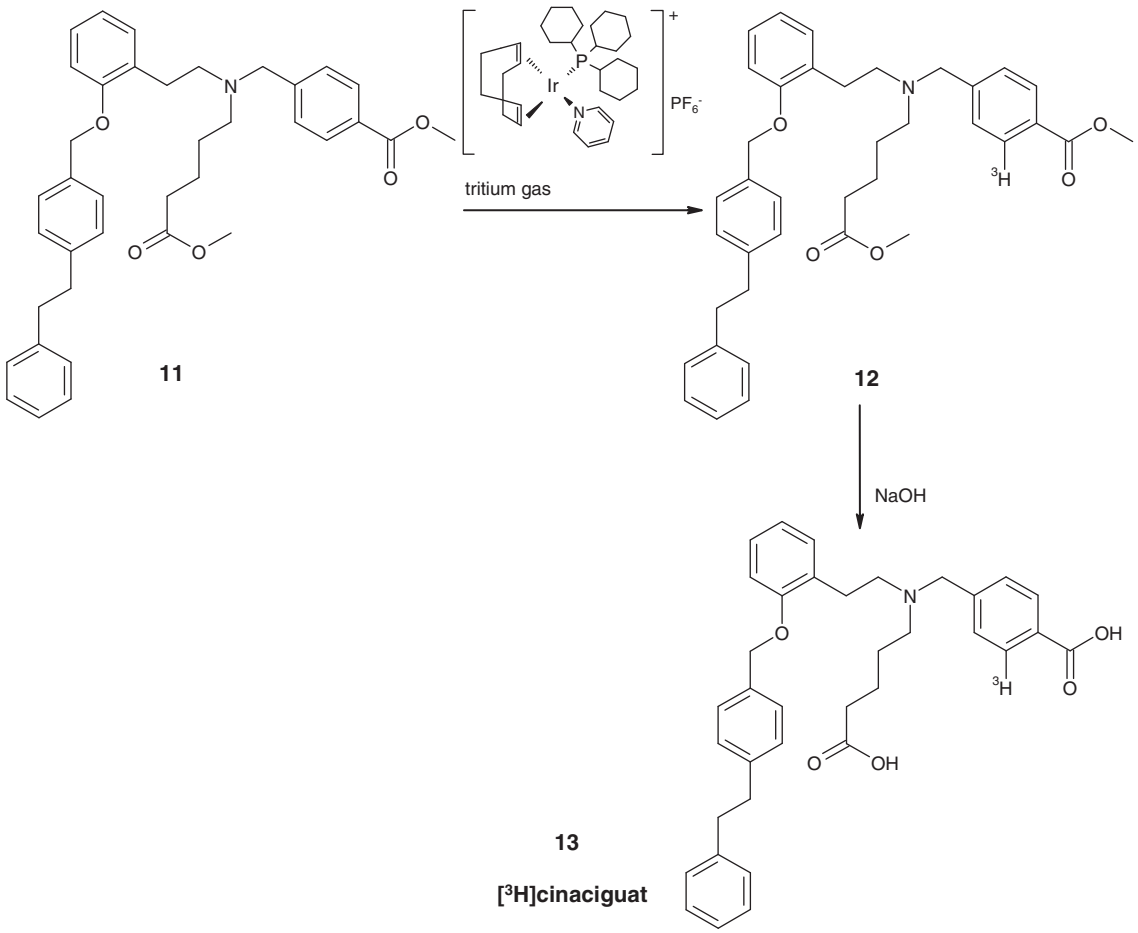
The synthesis of the second part of the labelled molecule started from 4-bromobenzoic acid (19). Under Rosenmund–von Braun conditions [^{13}C]cyanide was introduced. Boiling DMF and a reaction time of about 11 h were necessary to complete the reaction. The following reduction with Raney-nickel alloy in formic acid (75%) led to methyl 4-[^{13}C]formylbenzoate (21) in a yield of 42%.

Reductive amination starting from 18 to 21 was performed in boiling toluene giving the Schiff base derivative 22 in excellent yield. Platinum catalyzed hydrogenation lead to the secondary amine 23 in moderate yield. Alkylation of 23 with methyl 5-bromovalerate turned out to be the most critical step. The best conditions were found by using potassium carbonate as base in boiling acetonitrile. Nevertheless the yield was only 27%. Subsequent ester saponification and final purification by semi-preparative liquid chromatography yielded stable labelled cinaciguat in the desired quality (Scheme 4).

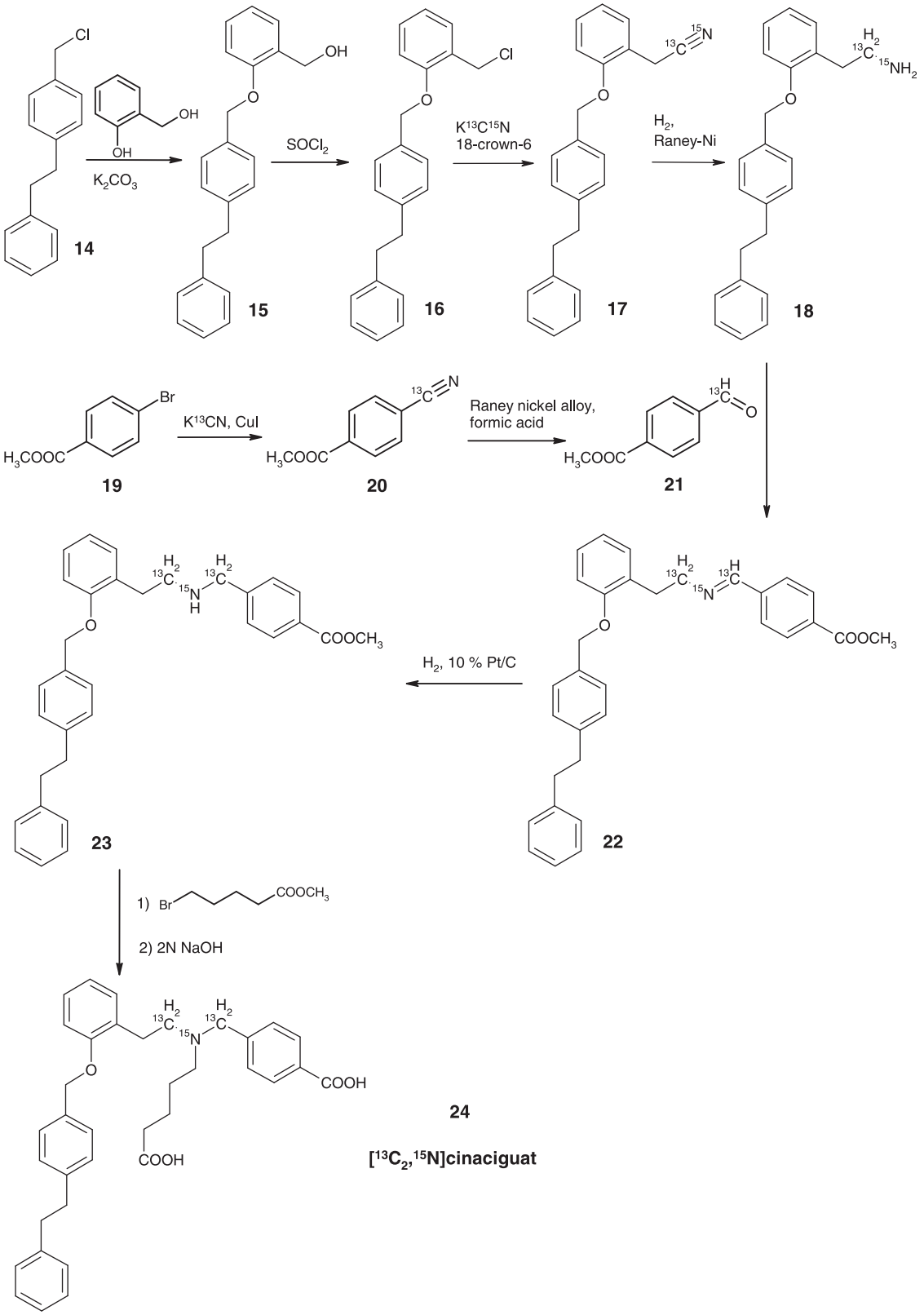
Experimental

Materials

Barium [^{14}C]carbonate was delivered by Izotop VO, Moscow, Russia. [^{14}C]Paraformaldehyde was obtained from GE Healthcare UK Ltd., Buckinghamshire, UK. Tritium was delivered by RC Tritec



Scheme 3. Tritium labelling of cinaciguat.



Scheme 4. Stable labelling of cinaciguat.

Ltd., Teufen, Switzerland (in uranium storage). Potassium [^{13}C,^{15}N]cyanide and potassium [^{13}C]cyanide were both obtained from Cambridge Isotope Laboratories, Inc., Andover, USA. All remaining solvents and reagents were obtained from commercial sources and used without further treatment unless indicated otherwise.

Liquid scintillation counting

Quantification of radioactivity was performed using a Perkin-Elmer TRI-CARB® 2500 TR liquid scintillation analyzer, with Ultima Gold™ cocktail used throughout.

High-performance liquid chromatography

[^{14}C]Cinaciguat (first radiosynthesis) and [$^{13}C_2$,^{15}N]cinaciguat were analyzed by HPLC using a HP 1050 system Series II (Hewlett-Packard, Waldbronn, Germany) with a Ramona® Cell 5 (Raytest, Straubenhardt, Germany) for radioactivity detection. The following HPLC system was used for the purity check: Prodigy® ODS (3) 100 A, 250 × 3.2 mm, 5 µm (Phenomenex; Hoesbach, Germany), column temperature: 45°C, flow rate: 0.6 mL/min, eluent; A: phosphate buffer (0.61 g KH_2PO_4 + 0.78 g Na_2HPO_4/L H_2O), B: CH_3CN, gradient: 0 min 30% B, 40 min 70% B, 50 min 80% B, UV 210 nm.

[^{14}C]Cinaciguat of the second radiosynthesis was analyzed by HPLC using a HP 1100 (Hewlett-Packard; Waldbronn, Germany) with a detector Ramona® Star (Raytest, Straubenhardt, Germany) for radioactivity detection. The following HPLC system was used for the purity check: Synergy® MAX-RP 250 × 4.6 mm, 4 µm (Phenomenex; Hoesbach, Germany), column temperature: 45°C, flow rate: 1.5 mL/min, eluent; A: ammonium acetate buffer pH 4.7, B: CH_3CN, gradient: 0 min 40% B, 15 min 90% B, 35 min 90% B, 50min 90% B, UV 230 nm.

[^{3}H]Cinaciguat was analyzed by HPLC using a HP 1050 system Series II (Hewlett-Packard, Waldbronn, Germany) with a Ramona® Cell 5 (Raytest, Straubenhardt, Germany) for radioactivity detection. The following HPLC system was used for the purity check: Phenomenex® Aqua C18 250 × 4.6 mm, 5 µm (Phenomenex; Hoesbach, Germany), flow rate: 1.0 mL/min, eluent; A: 0.05% $HClO_4$, B: CH_3CN, gradient: 0 min 30% B, 5 min 60% B, 20 min 60% B, 35 min 90% B, UV 210 nm.

Radio-TLC

[^{14}C]Cinaciguat (first radiosynthesis) was analyzed on silica gel plate 60 F_{254}, 10 × 20 cm, Merck; Darmstadt, Germany) with the eluent: dichloromethane/methanol/acetic acid = 85:10:5 (v/v) and radiodetection by a Raytest Rita®-3200 analyzer (Raytest, Straubenhardt, Germany). [^{14}C]Cinaciguat of the second radiosynthesis was analyzed under the same conditions with radiodetection by a Phosphorimager BAS 5000 (Fuji Photofilm Ltd., Tokyo, Japan).

Mass spectral analysis

[^{14}C]Cinaciguat (first radiosynthesis), [^{3}H]cinaciguat and [$^{13}C_2$,^{15}N]cinaciguat were analyzed by PE/API III with MacIntosh Quadra® 900 (Perkin-Elmer Sciex Instruments; Thornhill, Canada). [^{14}C]Cinaciguat of the second radiosynthesis) was analyzed by HP 1100 MSD (Agilant; Waldbronn, Germany).

Gas chromatography and GC-MS analysis

GC and GC-MS analyses were recorded on a HP 5890 equipped with MS HP 5970 (Agilent; Waldbronn, Germany).

NMR spectra

The NMR spectrum of [^{14}C]cinaciguat (first radiosynthesis) was recorded on a Bruker DRX 400 magnetic resonance spectrometer (Bruker, Rheinstetten, Germany). The NMR spectrum of [^{14}C]cinaciguat of the second radiosynthesis was recorded on a Bruker Avance 500 magnetic resonance spectrometer (Bruker, Rheinstetten, Germany).

First radiosynthesis of cinaciguat

1-Bromo-4-(2-phenylethyl)benzene (2)

Benzyl 4-bromophenyl ketone (1.88 g, 32.2 mmol) was dissolved in 28 mL of trifluoroacetic acid. Triethylsilane (28 mL) was added and the mixture was stirred for 42 h at ambient temperature. Then the solution was evaporated and the residue was dissolved in 50 mL of cyclohexane and washed with 50 mL of water. The organic layer was dried over sodium sulfate and evaporated to dryness. Flash chromatography on silica gel with cyclohexane as the mobile phase afforded 6.9 g (26.4 mmol) of 2 with a purity of 97.3% (GC). The yield was 82%.

4-(2-Phenylethyl)[carboxy-^{14}C]benzoic acid (3)

The non-labelled 2 (4.1 g, 15.7 mmol) was dissolved in 20 mL of diethyl ether (dried over sodium/lead alloy) and cooled in an ice bath. Butyl lithium solution (12 mL, 15% in *n*-hexane) was added. The solution was stirred for 30 min at ice bath temperature and 1.5 h at ambient temperature. Then the reaction flask was connected over a vacuum line with an equipment of a reaction flask with the carbon-14 labelled barium carbonate (3.1 g, 15.7 mmol, SA: 2080.6 MBq/mmol, corresponding to 32.67 GBq) and a dropping funnel with concentrated sulfuric acid (25 mL). The solution of the bromo derivative was frozen with liquid nitrogen and the equipment was evacuated. Then the equipment was closed and the carbon-14 labelled carbon dioxide was liberated by drop wise addition of the sulfuric acid and condensed to the frozen bromo derivative solution. For complete carbon dioxide liberation the carbonate mixture was heated after addition of the total amount of the sulfuric acid until a clear liquid was obtained. Then the vacuum was broken and the reaction mixture was stirred for 1 h at ice bath temperature and for 16 h at ambient temperature. The labelled acid 3 was liberated by addition of 20 mL of 2 M hydrochloric acid. The resulting suspension was diluted with 25 mL of water and the product was extracted with 125 mL of dichloromethane. The organic layer was washed with 25 mL of water. For formation of the sodium salt 2.5 mL of 45% sodium hydroxide solution were added and the mixture was stirred for 18 h at ambient temperature. The solid was filtered off and washed three times with each 10 mL of water. The solubility of the salt in water is very low. For liberation of the free acid the solid was stirred with 20 mL of 2 M hydrochloric acid. The obtained acid was filtered off, washed three times with each 10 mL of water and dried for 16 h in an evacuated desiccator over blue gel yielding 2.88 g of 3. This was dissolved in 25 mL of THF and filtered off from insoluble solid. By LS counting the total radioactivity of the solution was determined to 16.284 GBq. From the specific radioactivity of the starting $Ba^{14}CO_3$ (2080.6 MBq/mmol) the content of 3 was calculated to 1.776 g (7.8 mmol). The radiochemical yield was 49.7%. The purity was 99.2% (GC, after derivatization to the methyl ester). Product formation and labelling were confirmed by GC-MS with $m/z = 242$ $[M]^+$ (methyl ester).

[4-(2-Phenylethyl)phenyl][^{14}C]methanol (4)

The above solution of the labelled 3 was diluted with 10 mL of THF and heated to 50°C. Lithium aluminium hydride solution (16 mL, 1 M in THF) was added drop wise. The reaction mixture was stirred for 2 h at 50°C. After cooling in an ice bath 2 mL of water and 0.6 mL 10% potassium hydroxide solution were added drop wise followed by evaporation. The residue was suspended in 80 mL of boiling diisopropyl ether and filtrated over silica guhr. The filter cake was washed with 45 mL of diisopropyl ether. The mother liquor was evaporated to dryness under reduced pressure yielding 1.91 g of 4 (17% more than the theoretical yield). Product formation and labelling were confirmed by GC-MS with $m/z = 214$ $[M]^+$.

1-[Chloro[^{14}C]methyl]-4-(2-phenylethyl)benzene (5)

Intermediate 4 (1.91 g, maximal 7.86 mmol) was dissolved in 18 mL of chloroform. A solution of thionyl chloride (1.31 mL, 18.05 mmol) in 3 mL of chloroform was added. After stirring for 2 h at ambient temperature the solution was washed three times with each 10 mL of water and evaporated yielding 2.13 g of 5 (about 20% more than the theoretical yield).

Sodium 2-(2-{(5-ethoxy-5-oxopentyl)[4-(methoxycarbonyl)benzyl]-amino}ethyl)-phenolate (6)

Methyl 4-({(5-ethoxy-5-oxopentyl)[2-(2-hydroxyphenyl)ethyl]amino}-methyl)-benzoate[6] was purified by flash chromatography on silica gel using *n*-heptane/ethyl acetate = 1:1 (v/v) as the mobile phase before starting the formation of the sodium salt. Purified 6 (7.95 g, 19.2 mmol) was dissolved in 25 mL of methanol. Sodium methoxide solution (3.8 mL, 30% in methanol, 19.9 mmol) was added. The solution was stirred for 3 h at ambient temperature, evaporated and the residue was evaporated again from 25 mL of toluene. Then the oily residue was evaporated from 25 mL of acetonitrile and dried under oil pump vacuum yielding 8.12 g of 6. The substance was used without further characterization in the next chemical step.

Methyl 4-{[(5-ethoxy-5-oxopentyl){2-[2-({[4-(2-phenylethyl)phenyl]-[^{14}C]methyl}oxy)-phenyl]ethyl}amino]methyl}benzoate (7)

Non-labelled 6 (4 g) was added to a solution of 5 (2.12 g, maximal 7.86 mmol) in 25 mL of DMF. The reaction mixture was stirred for 3 h at 55°C. LC-MS revealed the presence of the expected ethylmethyl ester (7) and a greater part of dimethyl ester. Due to the subsequent ester hydrolysis this conversion was not critical. The reaction mixture was evaporated. Product formation and labelling were confirmed by LC-MS with *m/z* 596 $[M+H]^+$ (dimethylester) and 610 $[M+H]^+$ (7). The crude product mixture was used without purification in the next chemical step.

4-{[(4-Carboxybutyl)(2-{2-4-phenethylphenyl[^{14}C]methoxy]phenyl}-ethyl)-amino]-methyl}benzoic acid, [^{14}C]cinaciguat (8)

The amount of 7 was added with 50 mL of 10% sodium hydroxide solution. The reaction mixture was stirred for 4 h at 100°C. Then the solution was cooled in an ice bath and 30 mL of 37% hydrochloric acid were added adjusting the pH to 4. The solid precipitate was filtered off, washed with 20 mL of water and dried in an evacuated desiccator over blue gel yielding 4.98 g of crude 8. The radiochemical purity was 87.2%. Purification by re-crystallization from methanol or ethanol failed.

The final purification was performed by semi-preparative HPLC. The total amount of crude 8 (1.68 g) was dissolved in 66 mL of acetonitrile and 7 mL of concentrated hydrochloric acid. Liquid chromatography was performed on a Nucleosil® C 18 phase (7 μm, 250 × 25 mm) with acetonitrile/0.1 M hydrochloric acid 55:45 (v/v) as mobile phase, a flow of 12 mL/min and UV-detection at 230 nm. The chromatographic separation was difficult due to near running by-products. The product was isolated and characterized as the hydrochloride. The yield was 547 mg of 8 as hydrochloride (0.9 mmol). The radiochemical purity was 98.9%, the chemical purity (210 nm) was 99.0%. Radio-TLC revealed a purity of 99.0% (total). The specific radioactivity was 3.41 MBq/mg (2062 MBq/mmol) determined by LC counting/UV quantification. The total radiochemical yield was 5.7% based on the starting barium [^{14}C]carbonate. ^{1}H NMR (400MHz, DMSO-d_6) $\delta = 7.94$ (d, 2 H), 7.63 (d, 2 H), 7.33 (t, 4 H), 7.29–7.16 (m, 7 H), 7.04 (d, 1 H), 6.89 (t, 1 H), 5.03 (s, 2 H), 4.12 (s, 2 H), 2.95 (m, 10 H), 2.14 (m, 2 H), 1.60 (m, 2 H), 1.43 (m, 2 H).

Second radiosynthesis of [^{14}C]cinaciguat

1-[Chloro[^{14}C]methyl]-4-(2-phenylethyl)benzene (5)

Bibenzyl (9, 2.08 g, 11.43 mmol) was submitted in a two-necked 100 mL-reaction tube. The tube was transferred into a single-use glove box. The total amount of [^{14}C]paraformaldehyde (29.6 GBq, 0.444 g, 14.29 mmol, at a specific radioactivity of 2.07 GBq/mmol) was added. One neck of the tube was closed tightly with a cold finger combined with an orifice for gas emission. The second neck was closed with a septum. Then the single-use glove box was evacuated to remove the volatile radioactivity. Acetic anhydride (5.23 mL, 91.45 mmol) was added with a syringe through the septum to the reactants in the reaction tube leading to a suspension. Then 4.26 mL (51.44 mmol) of 37% hydrochloric acid were added with a syringe through the septum giving a very bulky suspension. On heating the reaction mixture to 65°C, an emulsion was obtained. Concentrated sulfuric acid (3.51 mL, 62.87 mmol) was added with a syringe through the septum at 65°C within 1 h. The reaction mixture was stirred for 20 h at 60°C. HPLC analysis revealed 26.4% of product and 65.1% of bibenzyl. Additional concentrated hydrochloric acid (2.15 mL, 25.96 mmol) was added followed by the addition of 1.72 mL (30.81 mmol) of concentrated sulfuric acid within 10 min. HPLC analysis after a total time of 49 h at 60°C revealed 36.8% of product and 48.7% of bibenzyl. After cooling to ambient temperature a slight stream of air was passed for 1 h through the reaction mixture to remove volatile radioactivity. Then the reaction mixture was cooled in an ice sodium chloride cooling bath and 15 mL of ice-cold water and subsequently 22 mL of dichloromethane were added. After intensive stirring the layers were separated. The organic layer was washed with half-saturated sodium bicarbonate solution (18 mL) and subsequently with 10 mL of water and evaporated to dryness yielding 2.95 g of crude product 5. HPLC analysis revealed 41.6% of product and 41.2% of bibenzyl. Separation from bibenzyl was performed by low liquid pressure chromatography on a pre-packed column Lobar® LiChroprep® Si 60 (440 × 37 mm) with *n*-heptane as mobile phase, a flow of 20 mL/min and UV-detection at 254 nm yielding 1.20 g. The purity was 84.8% (GC). The second chromatographic

purification was performed on Nucleosil® C 18 (7 μm, 250 × 21 mm) with acetonitrile/water 45:55 (v/v) as mobile phase, a flow of 20 mL/min and UV-detection at 210 nm yielding 0.698 g. As the purity was not sufficient, a third chromatography was performed on Synergy Polar-RP 8 (4 μm, 150 × 21.2 mm) with methanol/water 65:35 (v/v) as mobile phase, a flow of 20 mL/min and UV-detection at 210 nm yielding 4349.9 MBq of 5 (2.10 mmol calculated with the starting specific radioactivity of 2.07 GBq/mmol) The radiochemical purity (radio-HPLC) was 96.8%. Product formation and carbon-14 labelling were confirmed by GC/MS with $m/z = 232$ $[M(^{14}C)]^+$ and the chlorine isotope pattern. The radiochemical yield was 14.6% based on the amount of [^{14}C]paraformaldehyde. The excess of 25% for the labelled paraformaldehyde in the chloromethylation was disregarded in the yield calculation. The chemical yield was 18.4% based on bibenzyl.

4-{[(4-Carboxybutyl)(2-{2-4-phenethylphenyl[^{14}C]methoxy]phenyl}-ethyl)amino]-methyl}benzoic acid, [^{14}C]cinaciguat (8)

Intermediate 5 (2.10 mmol), intermediate 10 (1.042 g, 2.52 mmol) and 2.5 mL of dry *N*-methylpyrrolidinone were heated to 55 °C. Then 0.48 mL of sodium methoxide solution (30% in methanol, 2.52 mmol) was added. The reaction mixture was stirred for 1 h at 55°C. Additional compound 10 (0.26 g, 0.63 mmol) was added and stirring at 55°C was continued for 1 h. Additional sodium methoxide solution (0.072 mL, 0.38 mmol) was added and stirring at 55°C was continued for 3 h. Then 3 mL of 10% sodium hydroxide solution were added and stirring at 55°C was continued for 1 h. The milky suspension gave a clear yellow solution after 30 min. Work-up was performed on a pre-packed column Lobar® LiChroprep® RP–18 (440 × 37 mm) with acetonitrile/2.5% ammonium chloride solution 40:60 (v/v) as mobile phase, a flow of 20 mL/min and UV-detection at 230 nm yielding 0.792 g of 8. Due to a very near eluting by-product a second chromatography was necessary. This was performed on Synergy® MAX-RP (4 μm, 250 × 21.1 mm) with acetonitrile/acetate buffer (8.2 g NaOAc+acetic acid/L $H_2O \rightarrow$ pH 4.75) 30:70 (v/v) as mobile phase, a flow of 18 mL/min and UV-detection at 240 nm yielding 0.792 g of 8. The radiochemical purity and the chemical purity (230 nm) both were determined to 99.8%. Radio-TLC revealed a purity of 98.7%. The specific radioactivity of 3.60 MBq/mg (2039 MBq/mmol) was determined by LC counting/UV quantification. The total radiochemical yield was 9.6% based on the starting [^{14}C]paraformaldehyde. LC-MS: m/z 568 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) $\delta = 7.84$ (d, $J = 8.2$ Hz, 2 H), 7.34 (d, $J = 8.2$ Hz, 2 H), 7.27 (t, $J = 7.3$ Hz, 4 H), 7.25–7.20 (m, 4 H), 7.20–7.10 (m, 3 H), 7.00 (d, $J = 8.2$ Hz, 1 H), 6.85 (t, $J = 7.4$ Hz, 1 H), 4.99 (s, 2 H), 3.62 (s, 2 H), 2.86 (s, 4 H), 2.77–2.70 (m, 2 H), 2.62–2.55 (m, 2 H), 2.43 (t, $J = 6.8$ Hz, 2 H), 2.12 (t, $J = 7.1$ Hz, 2 H), 1.55–1.33 (m, 4 H).

Tritiation of cinaciguat

Methyl 4-({(5-methoxy-5-oxopentyl)[2-(2-{[4-(2-phenylethyl)benzyl]-oxy}phenyl)ethyl]-amino}methyl)[2-^{3}H]benzoate (12)

To a solution of diester 11 (3.8 mg, 6.4 μmol) in 1.0 mL of dichloromethane 3.5 mg of [(cod)Ir(pyridine)(tricyclohexylphosphine)] PF_6 were added. This mixture was stirred in a tritium labelling apparatus[12] for 1 h with 9.1 Ci (337 GBq) of tritium gas at a partial pressure of about 118 kPa (1188 mbar). After freezing of the reaction mixture with liquid nitrogen the non-reacted tritium and the solvent was adsorbed in a trap filled with platinum oxide and charcoal at the temperature of liquid nitrogen. The residue was dissolved in 1.0 mL of dichloromethane/methanol 4:1 and evaporated to dryness. This procedure was repeated three times with 1.0 mL of the same mixture of dichloromethane/methanol in order to remove the labile tritium. The dry crude product was taken up in 1 mL of acetonitrile. Due to the huge amount of labile radioactivity the crude material was dissolved with a mixture of acetonitrile/water and subsequently lyophilized twice resulting in 166 mCi (6 GBq) of crude product 12. Chromatographic purification was performed on Phenomenex® Aqua C18 (5 μm, 250 × 10 mm) with acetonitrile/0.05 M hydrochloric acid 65:35 (v/v) as mobile phase, a flow of 4 mL/min and UV-detection at 206 nm yielding intermediate 12 as hydrochloride. The amount was not determined.

4-({(4-Carboxybutyl)[2-(2-{[4-(2-phenylethyl)benzyl]oxy}phenyl)-ethyl]amino}methyl)-[2-^{3}H]benzoic acid (13)

To the hydrochloride of 12 a mixture of 1 mL of dioxane, 0.15 mL of water and 0.15 mL of 1 M sodium hydroxide solution were added and refluxed for 2 h. The pH of the mixture was adjusted to pH 3 with 25% hydrochloric acid. The solution was evaporated to dryness and the residue was dissolved with a mixture of 1 mL acetonitrile/0.05 M hydrochloric acid 1:1. Chromatographic purification was performed on Phenomenex® Aqua C18 (5 μm, 250 × 10 mm) with acetonitrile/0.05 M hydrochloric acid 50:50 (v/v) as mobile phase, a flow of 4 mL/min and UV-detection at 206 nm yielding the 510 MBq of [^{3}H]cinaciguat as hydrochloride. The radiochemical purity was > 99%. The specific radioactivity was 200 GBq/mmol determined by mass spectrometry under the following conditions; mass spectrometer: PE/Sciex/API III with MacIntosh Quadra® 900, infusion with acetonitrile/0.2% trifluoroacetic acid 9:1 (v/v), interface: 4.8 kV, nebulizing nitrogen pressure: 265 kPa, ion source in positive ion mode, scan rate: 1.0 s per scan, data collection: 0.2 amu steps from 560 to 580 amu.

Stable labelling of cinaciguat

(2-{[4-(2-phenylethyl)benzyl]oxy}phenyl)methanol (15)

2-Hydroxybenzyl alcohol (1.76 g, 14.2 mmol), 1-[chloromethyl]-4-(2-phenylethyl)benzene (14, 3.26 g, 14.2 mmol) and potassium carbonate (1.95 g, 14.2 mmol) were dissolved/suspended in 240 mL of acetonitrile and refluxed for 16 h. Water (100 mL) was added and exhaustive extraction was performed with dichloromethane. The extracts were dried over sodium sulfate and evaporated to dryness. The crude material was purified by flash chromatography on silica gel with cyclohexane/ethanol 9:1 (v/v) as mobile phase resulting in 3.68 g (11.6 mmol) of 15 with a purity of > 99% (GC) and a yield of 82%.

This step was repeated under the same conditions.

2-(Chloromethyl)phenyl 4-(2-phenylethyl)benzyl ether (16)

Thionyl chloride (1.38 mL, 19.0 mmol) was added to a solution of 15 (4.3 g, 13.5 mmol) in 70 mL of dichloromethane at 0°C. The mixture was stirred for 1 h at this temperature. Ice water (50 mL) was added and the organic phase was washed repeatedly with water and subsequently with sodium hydrogencarbonate solution. The organic layer was dried over sodium sulfate and evaporated to dryness yielding 4.3 g (12.8 mmol) of 16. The purity was > 96% (GC) and the yield was 95%.

(2-{[4-(2-Phenylethyl)benzyl]oxy}phenyl)[1-^{13}C,^{15}N]acetonitrile (17)

Intermediate 16 (2.0 g, 5.9 mmol), 18-crown-6 (95 mg) and potassium [^{13}C,^{15}N]cyanide (597 mg, 8.9 mmol) were submitted in 25 mL of acetonitrile and refluxed for 3 h. The suspension was filtered of and the filtrate was evaporated. The chromatographic purification was performed on a pre-packed column Lobar® LiChroprep® Si 60 (310 × 25 mm) with cyclohexane/ethanol 98.5:1.5 (v/v) as mobile phase, a flow of 8 mL/min and UV-detection at 254 nm yielding 1.57 g of 17 with a purity of >98% (GC) and a yield of 80%.

2-(2-{[4-(2-Phenylethyl)benzyl]oxy}phenyl)[1-^{13}C]ethan[^{15}N]amine (18)

Raney-nickel (4 g) was washed several times with THF to remove water, then added to a solution of 17 (830 mg, 2.5 mmol) in 45 mL of THF. Hydrogenation was performed in an autoclave at a hydrogen pressure of 4.0 bar for 4 h. The mixture was filtered off over silica guhr. The obtained clear product solution was evaporated giving 830 mg (2.4 mmol) of 18. The purity was >94% (GC) and the yield was 96%.

This reaction was repeated under the same conditions.

Methyl 4-[^{13}C]cyanobenzoate (20)

Methyl 4-bromobenzoate (2.15 g, 10.0 mmol), potassium [^{13}C]-cyanide (726 mg, 11.0 mmol) and cuprous iodide (2.09 g, 11 mmol) were submitted in 14 mL of DMF and refluxed for 11 h. The clear solution was evaporated. Dichloromethane (150 mL) was added to the residue yielding a suspension which was filtered off. The filtrate was washed with 150 mL of water, dried over sodium sulfate and evaporated to dryness resulting in 1.48 g (9.1 mmol) of 20. The purity was >97% (GC) and the yield was 73%.

Methyl 4-[^{13}C]formylbenzoate (21)

Intermediate 20 (1.48 g, 9.1 mmol) and nickel-aluminium-alloy (1.68 g) were refluxed in 29 mL of 75% formic acid for 3 h. The alloy was filtered off, washed with ethanol and the filtrates were evaporated. The residue was refluxed for 1.5 h in 14.8 mL of water and 7 mL of methanol. The solution was evaporated to remove the methanol, then 50 mL water were added and extracted with dichloromethane several times. The combined organic layers were dried over sodium sulfate and evaporated. Chromatographic purification was performed on a pre-packed column Lobar® LiChroprep® Si 60 (440 × 37 mm) with dichloromethane as mobile phase, a flow of 17 mL/min and UV-detection at 254 nm yielding 0.63 g of 21. The purity was >99% and the yield was 42%.

Methyl 4-[(E)-{[2-(2-{[4-(2-phenylethyl)benzyl]oxy}phenyl)[1-^{13}C]ethyl]-[^{15}N]imino}-[^{13}C]methyl]benzoate (22)

A solution of 18 (1.37 g, 3.8 mmol) and 21 (0.63 g, 3.8 mmol) in 15 mL of toluene was refluxed for 4 h. Then the solution was evaporated yielding 1.99 g of 22 (9% more than the theoretical yield). The purity was 94% (GC). The crude material was used without purification in the next step.

Methyl 4-[{[2-(2-{[4-(2-phenylethyl)benzyl]oxy}phenyl)[1-^{13}C]ethyl][^{15}N]-amino}-[^{13}C]methyl]benzoate (23)

The total amount of 22 (1.99 g) was dissolved in 60 mL of ethyl acetate and hydrogenated in an autoclave using 400 mg of platinum on charcoal (10 % Pt) at a hydrogen pressure of 4 bar for 22 h. The catalyst was removed by filtration over silica guhr. The filtrate was evaporated yielding 1.88 g of 23 with a purity of 87% (GC).

4-{[(4-Carboxybutyl)[2-(2-{[4-(2-phenylethyl)benzyl]oxy}phenyl)}-[1-^{13}C]ethyl]-[^{15}N]amino][^{13}C]methyl}benzoic acid (24)

Intermediate 23 (1.88 g), methyl 5-bromovalerate (694 mg, 3.5 mmol) and potassium carbonate (2.2 g, 16.0 mol) were refluxed in 15 mL of acetonitrile for 17 h. Remaining solid was filtered off and the filtrate was evaporated to dryness giving an oily residue (1.67 g). Chromatographic purification was performed on a Hibar® RP18 (7 µm, 250 × 25 mm) with acetonitrile/0.1% triethylamine 90:10 (v/v) as mobile phase, a flow of 15 mL/min. and UV-detection at 230 nm yielding 529 mg of the dimethyl ester of the labelled product. For saponification 2N sodium hydroxide solution (14 mL) was added and the reaction mixture was stirred for 14 h at a temperature of 100°C. Later 190 mg of sodium hydroxide and 1 mL of dioxane were added. Stirring was continued for 2 h at 130°C. The almost clear solution was filtrated and the filtrate was acidified with concentrated hydrochloric acid to pH 2–3. The precipitate was finely dispersed with the aid of ultrasonic, filtered off, washed and dried. Purification was performed by conversion to the hydrochloride. Therefore the crude product was suspended in 40 mL of a mixture of acetonitrile/0.1% hydrochloric acid 55:45 (v/v). The solution was filtrated to remove small amounts of insoluble solids. The filtrate was evaporated until a complete precipitation was observed. The solid was filtered off, washed with water and dried in a desiccator yielding 428 mg (0.71 mmol) of 24 as hydrochloride. Product formation and labelling were confirmed by LC-MS with $m/z = 569$ $[M+H]^+$. The molecular ion of the non-labelled molecule was not found in detectable amounts. The total yield was 18.6% referred to the reaction of the compounds 18 and 21 to the Schiff base.

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References

[1] O. V. Evgenov, P. Pacher, P. M. Schmidt, K. György, H. H. H. W. Schmidt, J. P. Stasch, *Nat. Rev. Drug Discov.* **2006**, *5*, 755–768. J. P. Stasch *et al.*, *J. Clin. Invest.* **2006**, 116, 2552–2561. P. M. Schmidt, M. Schramm, H. Schröder, F. Wunder, J. P. Stasch, *J. Biol. Chem.* **2004**, *279*, 3025–3032.
[2] P. M. Schmidt, M. Schramm, H. Schröder, J. P. Stasch, *Eur. J. Pharmacol.* **2003**, *468*, 167–174.
[3] A. J. Hobbs, *Br. J. Pharmacol.* **2002**, *136*, 637–640.
[4] J. P. Stasch *et al. Br. J. Pharmacol* **2002**, *136*, 773–783.
[5] U. Pleiss, D. Seidel, *J. Labelled Comp. Radiopharm* **2002**, *45*, 1172–1174.
[6] Patent WO. 01/19780 (Novel derivatives of Dicarboxylic Acid having Pharmaceutical Properties).
[7] J. H. Speer, A. J. Hill, *J. Chem. Soc.* **1937**, *2*, 139–147.
[8] M. Carrara, S. Zampiron, D. Pittarello, L. Cima, A. Rampa, P. Valenti, P. Da Re, P. Giusti, *ArzneimittelForsch./Drug Res.* **1997**, *47*, 803–809.
[9] Patent US 2,960,537 (4–Diphenylethane–Aldehyde).

[10] J. R. Heys, *J. Label. Comp. Radiopharm.* **2007**, *50*, 770–778.
[11] A. Y. L. Shu, J. R. Heys, Synthesis and applications of Isotopically labelled compounds, 2000, Proceedings of the Seventh International Symposium, Dresden, Germany, (Eds: U. Pleiss and R. Voges), Wiley, New York, p. 68.
[12] U. Pleiss, Synthesis and applications of isotopically labelled compounds, 1991, Proceedings of the Fourth International Symposium, Toronto, Kanada (Eds.: by E. Buncel, G. W. Kabalka), Elsevier Science Publishing Company, Amsterdam, pp. 34–39.