Synthesis, Biodistribution and PET Studies in Rats of $^{18}$F-Labeled Bridgehead Fluoromethyl Analogues of WAY-100635

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Abstract

*In vitro* screening of fluoromethyl bridge-fused ring (BFR) analogues of WAY-100635 (5a, 5b and 5c) has shown a high binding affinity and a good selectivity for the 5-HT$_{1A}$ receptor. As these compounds were designed to provide PET ligands with high metabolic stability, they are now radiolabeled with fluorine-18 and investigated *in vivo*. BFR precursors were synthesized and reacted with fluorine-18 in dry MeCN in the presence of 2,2,2-kryptofix and K$_2$CO$_3$ as catalysts under standard conditions. In rats, biodistribution and PET studies were performed using [F$^{18}$]5a, [F$^{18}$]5b and [F$^{18}$]5c. The binding specificity was determined by administration of non-labeled WAY-100635 prior to the radiolabeled ligands. [F$^{18}$]5 ligands were synthesized in overall radiochemical yields of 24-45%, respectively with a radiochemical purity of >98%. Relatively good hippocampus to cerebellum ratios of 5.55, 4.79 and 5.45, respectively were reached at 45 minutes after injection. However, PET studies indicated defluorination of the radioligands by showing high accumulation of radioactivity in the bones in the order of [F$^{18}$]5a ≈ [F$^{18}$]5b > [F$^{18}$]5c.

In conclusion, the radioligands bind preferentially to the 5-HT$_{1A}$ receptor *in vivo*. Unfortunately, no metabolic stability with regard to defluorination was observed.
Introduction

The importance of the neurotransmitter serotonin (5-HT) in the pathophysiology of anxiety is well known.\textsuperscript{1,2} Also, imaging studies of the neuronal activity and the volume of 5-HT\textsubscript{1A} receptor in the brains of depressed patients were investigated\textsuperscript{1,2,3,4} as well as patients with Alzheimer’s disease (AD)\textsuperscript{5,6} and schizophrenia\textsuperscript{7,8,9}. In normal human brain, high 5-HT\textsubscript{1A} receptor concentrations are found in the brainstem raphe nuclei and high densities of postsynaptic 5-HT\textsubscript{1A} receptors are found in the limbic forebrain (hippocampus, entorhinal cortex and septum). Low densities are observed in the basal ganglia and in the adult cerebellum.\textsuperscript{1}

For positron emission tomographic (PET) imaging of these receptors, [carbonyl-\textsuperscript{11}C]WAY-100635 is the “gold standard”.\textsuperscript{10,11,12,13} Although the amide bond is rapidly hydrolyzed \textit{in vivo}, the resulting radioactive metabolite does not enter the brain and as such enables proper quantification of 5-HT\textsubscript{1A} receptor parameters. However, the introduction of the \textsuperscript{11}C-label requires a very complex reaction sequence; even an optimized automated one-pot synthesis gives a failure rate of nearly 10\%.\textsuperscript{14} Furthermore, due to the short physical half-life of 20 minutes, this radioligand is only available for hospitals having an in-house cyclotron and radiopharmacy. Taking this into consideration, studies have been directed toward \textsuperscript{18}F-labeled analogues of WAY-100635 (Figure 1).

\textbf{Figure 1.} WAY-100635 and WAY-100635 PET analogues; \textit{p}-MPPF, FCWAY, and MeFWAY.
The 5-HT₁A antagonist [¹⁸F]MPPF was found to be prepared easily, selective and with high affinity for the 5-HT₁A receptor, but lower than WAY-100635.¹⁵ Pre-treatment with non-labeled WAY-100635 showed only 60% reduction of brain uptake.¹⁶ Unfortunately, this radiotracer seems to be a substrate for P-glycoprotein (P-gp)¹⁷—probably due to the extra aromatic group¹⁸—implicating a relatively low amount reaching the brain. To circumvent the additional aromatic moiety, a variety of fluorocyclohexane derivatives (FCWAYs) have been synthesized and evaluated showing the best properties for trans-4-FCWAY¹⁹ to measure 5-HT₁A receptor density. However, the synthesis of the corresponding ¹⁸F-labeled compound is hampered by the formation of difficult to separate non-radioactive contaminants²⁰ such as the elimination product, which may act as a “pseudo-carrier”, but the major limitation is its in vivo defluorination. Placing a fluorine on a primary carbon as in [¹⁸F]MeFWAY greatly reduced this in vivo instability.²¹

Recently, we reported the synthesis and the in vitro screening of bridge-fused ring (BFR) analogues of WAY-100635 with a fluoromethyl group on the bridge-head, i.e. the non-labeled form of 5a, 5b and 5c (Figure 2). These analogues showed a high binding affinity (Ki = 0.86, 0.54 and 0.65 nM, respectively) and a good selectivity for the 5-HT₁A receptor over other related receptors.²² Furthermore, it was found that BFR analogues are more stable toward amide hydrolysis than [¹⁸F]MPPF.²²,²³ The aim of this study is to evaluate if the ¹⁸F-labeled 5a, 5b and 5c could serve as suitable PET ligands, circumventing the above mentioned disadvantages of the currently clinically used [carbonyl-¹¹C]WAY-100635, [¹⁸F]MPPF and [¹⁸F]FCWAY. In this chapter, the synthesis of these new radioligands, as well as their biodistribution and PET studies in rats are described.

![Bridgehead [¹⁸F]fluoromethyl analogues of WAY-100635](image_url)

**Figure 2.** Fluoromethyl BFR analogues of WAY-100635.
Results

Chemistry

A possible general route to the needed precursors 4 is depicted in Scheme 1. Formation of the acid chlorides of 1 was achieved with thionyl chloride in MeCN, which in all cases could effectively been coupled to WAY-100634 to give 2. Reduction of the ester group with NaBH₄-LiCl was surprisingly only applicable for the synthesis of 3a and 3b.

![Scheme 1](image)

**Scheme 1.** General synthetic pathway for the precursors 4: (i) thionyl chloride, MeCN, reflux; (ii) WAY-100634, NEt₃, MeCN, RT.; (iii) NaBH₄, LiCl, 1-methoxy-2-(2-methoxyethoxy)ethane, reflux; (iv) for BFR = bicyclo[2.2.1]heptane; 4-methylbenzene-1-sulfonyl chloride, pyridine, CH₂Cl₂, RT., for BFR = cubane; triphenyl phosphine, CBr₄, CH₂Cl₂, 0°C

Conversion of 2c to 3c was found to be non-selective leading to a reduction of the amide bond too. The primary alcohols (3a and 3b) needed to be converted into a good leaving group (LG) for the subsequent fluorination step. The tosylate 4a was synthesized in high yield (82%) under standard reaction conditions. As expected, tosylation of 3b resulted in a ring enlargement leading to the homocubyl derivative. Therefore, 3b was converted into the bromide 4b in moderate yield (49%) which was found to be stable when kept at ambient temperature.
Since a selective reduction of 2c or its free acid to 3c was unsuccessful, 4c was synthesized by the route shown in Scheme 2. Compound 6 was obtained by the reduction of 1c with borane dimethyl sulfide and subsequently converted to 7. Since it was impossible to saponify 7, this compound was first converted into 8 using NaI followed by saponification to give 9. To prevent chloro-deiodination by thionyl chloride, the acid chloride of 9 was obtained using dichloro(methoxy)methane in CH$_2$Cl$_2$ and was then coupled to WAY-100634 to afford 4c in good yield (63%).

**Scheme 2.** Synthetic pathway for the precursor 4c: (i) BH$_3$(CH$_3$)$_2$, THF, 0 °C-RT.; (ii) 4-methylbenzene-1-sulfonyl chloride, pyridine, RT.; (iii) NaI, 2-methoxyethanol, 130 °C; (iv) NaOH, MeOH:H$_2$O (10:1), RT.; (v) dichloro(methoxy)methane, CH$_2$Cl$_2$, 60 °C; (vi) WAY-100634, NEt$_3$, CH$_2$Cl$_2$, RT.

**Radiolabeling**

A general synthetic route to the desired radiofluorinated analogues of WAY-100635 is depicted in Scheme 3.

**Scheme 3.** Radiosynthesis of [${}^{18}$F]5a, [${}^{18}$F]5b and [${}^{18}$F]5c.
Radiofluorination of 4 using 2,2,2-kryptofix/K₂CO₃ in acetonitrile proceeded without extensive decomposition of the precursor. As an example, the radiolabeling of 4c for which the highest reaction temperature (150 °C) was needed is shown in Figure 3. Conversion of 4b proceeded already at 110 °C, presumably due to the pseudo-aromatic character of the cubyl moiety. Preparative HPLC afforded [¹⁸F]5 in fair to good overall radiochemical yields (24-45%) with acceptable SA’s (76-138 GBq/μL). Analytical HPLC revealed the presence of only very small amounts of unlabeled contaminants which are believed to hardly influence the biodistribution of [¹⁸F]5.

Figure 3. HPLC chromatogram of crude reaction mixture of [¹⁸F]5c (kromasil C18-10 μm column, 4.6 mm x 250 mm); eluent (MeOH/H₂O/DIPA, 80:20:0.02).
Bridghead $[^{18}\text{F}]$fluoromethyl analogues of WAY-100635

Biodistribution of $[^{18}\text{F}]$$5\text{a}$, $[^{18}\text{F}]$$5\text{b}$ and $[^{18}\text{F}]$$5\text{c}$ in rat

The results of the biodistribution of $[^{18}\text{F}]$$5\text{a}$, $[^{18}\text{F}]$$5\text{b}$ and $[^{18}\text{F}]$$5\text{c}$ are shown in Table 1.

### Table 1. Uptake of $[^{18}\text{F}]$$5\text{a}$, $[^{18}\text{F}]$$5\text{b}$ and $[^{18}\text{F}]$$5\text{c}$ in rats at 45 min p.i. $^a$

<table>
<thead>
<tr>
<th>Region of interest</th>
<th>$[^{18}\text{F}]$$5\text{a}$</th>
<th>$[^{18}\text{F}]$$5\text{b}$</th>
<th>$[^{18}\text{F}]$$5\text{c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.13 ± 0.02</td>
<td>0.16 ± 0.00</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Heart</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.00</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.18 ± 0.07</td>
<td>0.14 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>0.62 ± 0.02</td>
<td>1.34 ± 0.06</td>
<td>0.76 ± 0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.45 ± 0.04</td>
<td>0.55 ± 0.01</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>0.06 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.04 ± 0.00</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.00</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

$^a$Values are averaged ($n = 4$) %ID/g ± SD and decay corrected.

The degree of binding specificity in a brain area is often expressed as the ratio of radioactivity uptake in that area over the cerebellum, taken as a receptor-devoid reference tissue (Table 2).

### Table 2. The ratio of uptake of $[^{18}\text{F}]$$5\text{a}$, $[^{18}\text{F}]$$5\text{b}$ and $[^{18}\text{F}]$$5\text{c}$ in 5-HT$_{1A}$-rich brain regions over uptake in the cerebellum $^a$.

<table>
<thead>
<tr>
<th>Region of interest</th>
<th>$[^{18}\text{F}]$$5\text{a}$</th>
<th>$[^{18}\text{F}]$$5\text{b}$</th>
<th>$[^{18}\text{F}]$$5\text{c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>5.55 ± 0.91</td>
<td>4.79 ± 0.37$^*$</td>
<td>5.45 ± 0.31$^*$</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>1.89 ± 0.31</td>
<td>2.18 ± 0.31$^*$</td>
<td>3.36 ± 0.50</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>1.69 ± 0.01</td>
<td>1.66 ± 0.21$^<em>^</em>$</td>
<td>2.14 ± 0.08$^<em>^</em>$</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.20 ± 0.27</td>
<td>0.98 ± 0.17$^<em>^</em>$</td>
<td>1.09 ± 0.30$^<em>^</em>$</td>
</tr>
</tbody>
</table>

$^a$Results are presented as ratios ± SD (45 min after injection; $n = 4$) of uptake in specific brain regions over cerebellum $^*P \leq 0.01, ^*^*P \leq 0.05.$
PET studies

The brain uptake in time and the specific binding of the novel radioligands were determined using PET. As in the biodistribution study, brain uptake of $[^{18}\text{F}]5\text{a}$ and $[^{18}\text{F}]5\text{b}$ was almost the same, while that for $[^{18}\text{F}]5\text{c}$ was clearly higher (Figure 4).

![Figure 4. PET data of brain uptake of $^{18}\text{F}$ radioligands.](image)

Pre-treatment with WAY-100635 showed for all three radioligands an increased washout from the brain (for an example see Figure 5). This indication of specific binding was confirmed by dissection of the rats directly after the PET study (60 minutes after injection). The hippocampus over cerebellum ratios of the non-pre-treated rats were slightly lower than those at 45 minutes ($[^{18}\text{F}]5\text{a}$: 3.72, $[^{18}\text{F}]5\text{b}$: 2.06 and $[^{18}\text{F}]5\text{c}$: 4.56) but after pre-injection with WAY-100635 all ratios went down to 1.

![Figure 5. PET data of brain uptake of $^{18}\text{F}$ without and with pretreatment with WAY-100635.](image)
However, at the end of the study radioactivity uptake in the skeleton was visible (Figure 6), indicating that all three radioligands defluorinated \textit{in vivo}.

![Image](image.png)

Figure 6. Cumulative uptake of $^{18}$F radioactivity at the end of the PET study.

The rate of bone uptake of radioactivity and thus the order of defluorination is $[^{18}\text{F}]5a \approx[^{18}\text{F}]5b >[^{18}\text{F}]5c$ (Figure 7).

![Image](image.png)

Figure 7. PET data of the $^{18}$F fluoride accumulation in bone.
Discussion

All the currently clinically used radiopharmaceuticals for imaging the 5-HT_{1A} receptor have some disadvantages. Thus, there is still a strong need for an effective PET ligand for this receptor preferentially labeled with fluorine-18. Analogues containing the moiety WAY-100634 have a good chance to display high 5-HT_{1A} receptor binding affinity. However, this moiety should not contain the radiolabel as it also enters the brain interfering with the scintigraphic measurements.\textsuperscript{19,25,26} Fluoromethyl-BFR-analogues were found to have a high affinity and good selectivity for the target 5-HT_{1A} receptor, at least comparable to that of WAY-100635.\textsuperscript{22} Since the three analogues 5a, 5b and 5c showed lipophilicities (log \(D_{7.4} = 2.70, 2.94\) and 3.37, respectively) that are close to that of WAY-100635 (log \(D_{7.4} = 3.03\))\textsuperscript{22} and they all fall within the range log \(D_{7.4} = 2-3.5\)\textsuperscript{27}, it is likely that these analogues will also penetrate the BBB and reach the target receptor in the brain. For a full \textit{in vivo} evaluation it was decided to synthesize \(^{18}\)F-labeled 5a, 5b and 5c.

The synthesis route of precursors 4a and 4b (Scheme 1) was not valid for 4c (Scheme 2). Reduction of the ester group of 2c with NaBH\(_4\)-LiCl found to be non-selective leading to a reduction of the amide bond too. The reason for this is not fully clear but might be caused by the difference in S-character of the exocyclic bond of the BFR’s. The calculated hybridization for cubane is sp\(^{2.2}\) compared to sp\(^{2.8}\) for bicyclo[2.2.1]heptane and sp\(^{3.2}\) for bicyclo[2.2.2]octane.\textsuperscript{28,29}

As expected, a nucleophilic radiofluorination of 4 as shown in Scheme 3, where X is a good leaving group, was proceeded cleanly and in fair to good overall radiochemical yields. Elimination of HX - which often occurs under the alkaline reaction conditions-, is herein not possible since HX is placed on a primary carbon, facilitating an easy HPLC separation.

In rats, all three radioligands ([\(^{18}\)F]5a, [\(^{18}\)F]5b or [\(^{18}\)F]5c) showed a higher uptake in the 5-HT_{1A} receptor containing regions (hippocampus and cortex) than in devoid regions (cerebellum and striatum) (P <0.01, Table 1). The uptake of [\(^{18}\)F]5a and [\(^{18}\)F]5b was almost identical except for a higher uptake in the liver, which might be due to the enhanced hepatic activity for the cubyl moiety in [\(^{18}\)F]5b.\textsuperscript{30,31} The uptake of [\(^{18}\)F]5c was higher than the uptake of [\(^{18}\)F]5a as well as of [\(^{18}\)F]5b in all brain tissues but not in the peripheral organs. The ratios of hippocampus over the cerebellum (Table 2) were higher
than those for $^{18}$F$\mu$-MPPF (hippocampus; 3, frontal cortex; 1.4 and striatum; 0.93)\textsuperscript{32}, while similar ratios were observed regarding frontal cortex and striatum. The observed ratios were lower than those reported for WAY-100635 (hippocampus; 14.3, frontal cortex; 7.7, and striatum; 1.6)\textsuperscript{12}. These low ratios of the novel radioligands compared to the WAY-100635 could be due to a non-specific binding in the cerebellum, however, a recent in vitro study suggests that the high ratios of WAY-100635 might also be due to its high binding affinity to the dopamine D$\textsubscript{4}$ receptor.\textsuperscript{33}

As shown in Figures 6 and 7, these radioligands were defluorinated in vivo. The rate of bone uptake of radioactivity $^{18}$F$^5\text{a}$ and $^{18}$F$^5\text{b}$ was almost identical. The rate of bone uptake of radioactivity of $^{18}$F$^5\text{c}$ was lower than that of $^{18}$F$^5\text{a}$ as well as $^{18}$F$^5\text{b}$. This was rather disappointing since these ligands were designed as the first WAY-100635 analogues, labeled with fluorine-18 on a primary carbon atom attached to the bridgehead of a BFR whereby HF elimination is chemically impossible. There are several enzymes by which fluorine containing ligands can be metabolized such as cytochrome P450 isozyme 2E1 (CYP2E1) and glutathione S-transferase (GST).\textsuperscript{34} CYP2E1 might be responsible for an $\alpha$-hydroxylation at the carbon to which fluorine is attached followed by an elimination of hydrofluoric acid, which will yield an aldehyde. In this case, defluorination can be reduced by pre-administration of CYP2E1 inhibitors like miconazol\textsuperscript{35} or disulfiram\textsuperscript{26}. Since a stability study of $^{18}$F$^5\text{b}$ in human hepatocytes\textsuperscript{22}, which should also contain CYP2E1, did not reveal the formation of free fluoride, this isozyme is probably not involved. However, $\alpha$-hydroxylation by another enzyme cannot be excluded. GST is known to dehalogenate mostly alkyl iodines, bromides, and chlorides by nucleophilic substitution. This has recently also been suggested for the CH$_2$F in SP203. GST shows species and tissue variations in its activity and is significantly lower in human subjects than in rats.\textsuperscript{36} In that case, $^{18}$F$^5$ ligands might be stable in human with regard to defluorination. It would be interesting, and for getting permission to study these radioligands in human even essential, to elucidate which of the above enzymes is responsible for the release of $^{18}$Ffluoride from the radioligands, or if there is maybe another pathway involved.

Although the present study has shown that a rational design is not always a guarantee for success, at least in rats, we are currently focusing on derivatives with a CF$_3$
group instead of the CH$_2$F and on analogues which have the fluoro atom directly attached to the bridgehead. In both $\alpha$-hydroxylation is no longer possible whereas in the last also a nucleophilic attack by e.g. glutathione is precluded while HF elimination remains unlikely because this would lead to a highly strained ring system.

**Conclusion**

Three $^{18}$F-labeled radioligands, chemically stable against HF elimination, have been prepared in good radiochemical yields. They all showed a specific binding to the 5-HT$_{1A}$ receptor in rat brain. Unfortunately, these compounds appeared to be unstable toward *in vivo* enzymatic defluorination in rats, which suggest that they are not better radioligands than the already clinically used WAY-100635 analogues.

**Experimental section**

**Chemistry: General procedures.**

4-Methoxycarbonylcubane-carboxylic acid was purchased from Boron Molecular (North Caroline, USA). 4-(Methoxycarbonyl)bicyclo[2.2.2]octane-1-carboxylic acid was purchased from Atlantic research chemicals Ltd (Bude, UK). All other chemicals used were reagent grade and were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Citrate buffer (pH 4.8-5.0) was made in house (480 mg citric acid trisodium salt dihydrate, 240 mg citric acid and 210 mg sodium acetate in 30 mL water). SepPak C18 plus cartridges and SepPak tC18 plus cartridges were obtained from Waters Chromatography B.V. (Etten-Leur, The Netherlands). $^{18}$F (nca.) in [$^{18}$O]H$_2$O was produced by BV Cyclotron VU using proton bombardment on 99.9% enriched $^{18}$O. Nuclear magnetic resonance spectra ($^1$H NMR and $^{13}$C NMR) were determined in the indicated solvent using a Bruker AC 200 MHz (200.13 MHz and 50.32 MHz, respectively) or a Bruker Avance 250 MHz (250.13 and 62.90 MHz). Chemical shifts (δ) are given in ppm downfield from tetramethylsilane ($^1$H, $^{13}$C) and coupling constants (J) in Hz. High performance liquid chromatography (HPLC) was carried out using a kromasil C18 column (4.6 mm x 250 mm). Peaks were detected at 254 nm. The radioactivity of the eluate was monitored using an inline NaI(Tl) radiodetector.
Synthesis of the precursors

Methyl4-(N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamoyl)bicyclo[2.2.1]heptane-1-carboxylate (2a).

4-(methoxycarbonyl)bicyclo[2.2.1]heptane-1-carboxylic acid (1a) was synthesized as mentioned previously. In a flame dried flask under argon atmosphere, compound 1a (353 mg, 1.79 mmol) was dissolved in MeCN (10 mL) and thionyl chloride (3 mL) was added. The reaction mixture was refluxed at 70 °C for 1 hour, the excess of thionyl chloride was totally evaporated under reduced pressure and coevaporated with MeCN (3 x 3 mL). To the corresponding acid chloride, WAY-100634 (564 mg, 1.81 mmol) and NEt₃ (247 µL) in MeCN (10 mL) were added and stirred at room temperature overnight. The solvent was evaporated, the residue was dissolved in water and extracted with CH₂Cl₂ (3 x 10 mL). The organic phase was collected, dried over anhydrous Na₂SO₄ and evaporated to dryness. Column chromatography (EtOAc/NEt₃, 1:0.01) gave 2a; yield: 803 mg (90%) as a white solid. ¹H NMR (250.13 MHz, CDCl₃): δ 1.08-1.30 (m, 2H, CH₂), 1.40-1.60 (m, 2H, CH₂), 1.70-2.00 (m, 6H, 3 x CH₂), 2.48-2.70 (m, 6H, N(CH₂)₃), 2.80-3.10 (m, 4H, N(CH₂)₂), 3.60 (s, 3H, CH₃), 3.70-4.00 (m, 5H, NCH₂ and CH₃), 6.75-7.00 (m, 5H, 5 x CH), 7.22-7.30 (m, 1H, CH), 7.70 (dt, J = 7.6 Hz, 1H, CH), 8.54 (d, J = 4.9 Hz, 1H, CH).

4-(Hydroxymethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)bicyclo[2.2.1]heptane-1-carboxamide (3a).

In a flame dried flask under argon atmosphere, compound 2a (366 mg, 0.74 mmol) was dissolved in 1-methoxy-2-(2-methoxyethoxy)ethane (10 mL), NaBH₄ (30 mg, 0.79 mmol) and LiCl (36 mg, 0.85 mmol) were added. The reaction mixture was refluxed for 2 hours and allowed to reach room temperature and quenched with water (5 mL). The solvent was evaporated under reduced pressure, the aqueous layer was extracted with CH₂Cl₂ (3 x 10 mL). The organic phase was collected, dried over anhydrous MgSO₄ and evaporated in vacuo to dryness. Column chromatography (EtOAc/NEt₃, 1:0.01) gave 3a; yield: 190 mg (55%) as a colorless glass. ¹H NMR (250.13 MHz, CDCl₃): δ 1.00-1.90 (m, 10H, 5 x CH₂), 2.10 (bs, 1H, OH), 2.48-2.70 (m, 6H, N(CH₂)₃), 2.80-3.10 (m, 4H, N(CH₂)₂), 3.50 (s, 2H, CH₂OH), 3.70-4.00 (m, 5H, NCH₂ and CH₃), 6.75-7.00 (m, 4H, 4 x...
4-(N-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamoyl) bicyclo[2.2.1]heptan-1-yl)methyl 4-methylbenzenesulfonate (4a).

In a flame dried flask under argon atmosphere, compound 3a (85 mg, 0.18 mmol) was dissolved in a mixture of pyridine (2 mL) and CH$_2$Cl$_2$ (10 mL), 4-methylbenzene-1-sulfonyl chloride (100 mg, 0.52 mmol) was added. This reaction mixture was stirred for 48 minutes at room temperature. The solvents were evaporated, the residue was dissolved in water and extracted with CH$_2$Cl$_2$ (3 x 5 mL). The organic phase was collected, dried over anhydrous Na$_2$SO$_4$ and evaporated under reduced pressure. Column chromatography (EtOAc/n-hexane/NEt$_3$, 1:0.10:0.01) afforded 4a; yield: 91 mg (82%) as a white solid. $^1$H NMR (250.13 MHz, CDCl$_3$): δ 1.00-1.90 (m, 10H, 5 x CH$_2$), 2.35 (s, 3H, CH$_3$), 2.48-2.70 (m, 6H, N(CH$_2$)$_3$), 2.80-3.10 (m, 4H, N(CH$_2$)$_2$), 3.70-4.00 (m, 7H, CH$_2$, NCH$_2$ and CH$_3$), 6.75-7.00 (m, 4H, 4 x CH), 7.10-7.30 (m, 2H, 2 x CH), 7.36 (d, $J = 8$ Hz, 2H, 2 x CH), 7.70 (dt, $J = 7.6$ Hz, 1H, CH), 7.77 (d, $J = 8$ Hz, 2H, 2 x CH), 8.54 (d, $J = 4.9$ Hz, 1H, CH). $^{13}$C NMR (62.90 MHz, CDCl$_3$): δ 21.47, 32.08, 34.01, 45.27, 45.75, 46.99, 50.41, 53.23, 55.16, 55.53, 111.01, 117.89, 120.74, 122.65, 122.89, 123.47, 127.63, 129.64, 132.69, 137.98, 141.09, 144.50, 148.93, 152.03, 155.42, 174.71. HRMS (EI) $m/z$ calcld for C$_{34}$H$_{42}$N$_4$O$_5$S, 618.2876; found, 619.2879 (M + H).

Methyl4-(N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamoyl)cubane-1-carboxylate (2b).

In a flame dried flask under argon atmosphere, 4-methoxycarbonylcubane carboxylic acid (1b) (300 mg, 1.45 mmol) was dissolved in MeCN (20 mL) and thionyl chloride (145 µL) was added. The reaction mixture was refluxed at 70 °C for 45 minutes, the excess of thionyl chloride was totally evaporated under reduced pressure and coevaporated with MeCN (3 x 3 mL). To the corresponding acid chloride, WAY-100634 (455 mg, 1.45 mmol) and NEt$_3$ (224 µL) in MeCN (15 mL) were added and stirred at room temperature overnight. The solvent was evaporated, the residue was dissolved in
water and extracted with CH$_2$Cl$_2$ (3 x 15 mL). The organic phase was collected, dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. Column chromatography (EtOAc/NEt$_3$, 1:0.01) gave 2b; yield: 581 mg (80%) as a white solid. $^1$H NMR (200.13 MHz, CDCl$_3$): $\delta$ 2.50-2.70 (m, 6H, N(CH$_2$)$_3$), 2.80-3.00 (m, 4H, N(CH$_2$)$_2$), 3.60 (s, 3H, CH$_3$), 3.75 (s, 6H, 6 x CH), 3.90-4.05 (m, 5H, NCH$_2$ and CH$_3$), 6.70-7.05 (m, 4H, 4 x CH), 7.10-7.30 (m, 2H, 2 x CH), 7.60 (dt, $J$ = 7.6 Hz, 1H, CH), 8.45 (d, $J$ = 4.9 Hz, 1H, CH).

**4-(Hydroxymethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl) cubane-1-carboxamide (3b).**

In a flame dried flask under argon atmosphere, compound 2b (393 mg, 0.786 mmol) was dissolved in (1-methoxy-2-(2-methoxyethoxy) ethane (20 mL), NaBH$_4$ (160 mg, 4.28 mmol) and LiCl (180 mg, 4.26 mmol) were added. The reaction mixture was refluxed overnight. After, the reaction mixture was quenched with water (15 mL), the solvent was evaporated under reduced pressure, the aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 10 mL). The organic layer was collected, dried over anhydrous MgSO$_4$ and evaporated in vacuo to dryness. Column chromatography (EtOAc/NEt$_3$, 1:0.01) gave 186 mg of 3b (50%) as a colorless glass. $^1$H NMR (200.13 MHz, CDCl$_3$): $\delta$ 2.50-2.80 (m, 6H, N(CH$_2$)$_3$), 2.90-3.00 (m, 4H, N(CH$_2$)$_2$), 3.50-3.75 (m, 9H, 6 x CH and CH$_3$), 3.80 (s, 2H, CH$_2$OH), 3.90-4.10 (m, 2H, NCH$_2$), 5.00 (bs, 1H, OH), 6.70-7.00 (m, 4H, 4 x CH), 7.05-7.25 (m, 2H, 2 x CH), 7.70 (dt, $J$ = 7.6 Hz, 1H, CH), 8.54 (d, $J$ = 4.9 Hz, 1H, CH).

**4-(Bromomethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl) cubane-1-carboxamide (4b).**

Compound 3b (180 mg, 0.38 mmol) was dissolved in CH$_2$Cl$_2$ (24 mL) and cooled to 0 °C, triphenyl phosphine (189 mg, 0.72 mmol) was added and followed by a dropwise addition of CBr$_4$ (142 mg, 0.43 mmol). The reaction mixture was stirred at 0 °C for 4 hours. The solvent was evaporated under reduced pressure at 0 °C. The residue was dissolved in eluent and purified with column chromatography (EtOAc/NEt$_3$, 1:0.01) to give 100 mg (49%) of 4b as a colorless glass. $\delta$ 2.50-2.80 (m, 6H, N(CH$_2$)$_3$), 2.90-3.10 (m, 4H, N(CH$_2$)$_2$), 3.50-3.90 (m, 11H, 6 x CH, CH$_3$ and CH$_2$Br), 4.00-4.20 (m, 2H, NCH$_2$), 6.80-7.10 (m, 4H, 4 x CH), 7.10-7.30 (m, 2H, 2 x CH), 7.65 (dt, $J$ = 7.6 Hz, 1H,
Methyl 4-(N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamoyl) bicyclo[2.2.2]octane-1-carboxylate (2c).

In a flame dried flask under argon atmosphere, 4-(methoxycarbonyl)bicyclo[2.2.2]octane-1-carboxylic acid (1c) (250 mg, 1.18 mmol) was dissolved in MeCN (15 mL) and thionyl chloride (120 µL) was added. The reaction mixture was refluxed at 70 °C for 45 minutes, the excess of thionyl chloride was totally evaporated under reduced pressure and coevaporated with MeCN (3 x 3 mL). To the corresponding acid chloride, WAY-100634 (367 mg, 1.18 mmol) and NEt₃ (200 µL) in MeCN (10 mL) were added and stirred at room temperature overnight. The solvent was evaporated, the residue was dissolved in water and extracted with CH₂Cl₂ (3 x 15 mL). The organic phase was collected, dried over anhydrous Na₂SO₄ and evaporated to dryness. Column chromatography (EtOAc/NEt₃, 1:0.01) gave 2c; yield: 419 mg (70%) as a white solid. 

**Methyl 4-(hydroxymethyl)bicyclo[2.2.2]octane-1-carboxylate (6).**

In flame dried three-necked flask under argon atmosphere, 4-(methoxycarbonyl)bicyclo[2.2.2]octane-1-carboxylic acid (1c) (1.18 g, 5.23 mmol) was dissolved in freshly distilled THF (50 mL). This solution was cooled to 0 °C and a solution of 2 M borane dimethyl sulfide in THF (4.2 mL) was added dropwise. This reaction mixture was stirred at this temperature for 20 minutes then further stirred for 2.5 hours at room temperature, quenched with water (10 mL) and stirred for 2 hours at room temperature. The solvent was evaporated under reduced pressure and the residue was extracted with
EtOAc (3 x 15 mL). The organic layer was washed with unsaturated NaHCO₃ (2 x 10 mL), collected, dried over anhydrous MgSO₄ and evaporated in vacuo to dryness to give 1.061 g (96%) of 6 as a colorless oil. ¹H NMR (250.13 MHz, CDCl₃): δ 1.36-1.51 (m, 6H, 3 x CH₂), 1.72-1.86 (m, 6H, 3 x CH₂), 3.28 (s, 2H, CH₂OH), 3.50 (s, 3H, CH₃).

4-(Methoxycarbonyl)bicyclo[2.2.2]octan-1-yl)methyl 4-methylbenzenesulfonate (7).

In a flame dried flask under argon atmosphere, the appropriate alcohol 6 (2.11 g, 10.01 mmol) was dissolved in pyridine (15 mL) and 4-methylbenzene-1-sulfonyl chloride (1.91 g, 10.01 mmol) was added. This reaction mixture was stirred at room temperature overnight, the solvent was evaporated and the residue was dissolved in water and extracted with EtOAc (3 x 20 mL). The organic phase was separated, washed with unsaturated NaHCO₃ (2 x 10 mL), collected, dried over anhydrous Na₂SO₄ and evaporated under reduced pressure to dryness to give 3.60 g (98.5%) of 7 as white crystals. ¹H NMR (250.13 MHz, CDCl₃): δ 1.35-1.46 (m, 6H, 3 x CH₂), 1.68-1.84 (m, 6H, 3 x CH₂), 2.45 (s, 3H, CH₃), 3.50 (s, 3H, CH₃), 4.07 (q, J = 7 Hz, 2H, CH₂OTs), 7.34 (d, J = 8 Hz, 2H, 2 x CH), 7.77 (d, J = 8 Hz, 2H, 2 x CH).

Methyl 4-(iodomethyl)bicyclo[2.2.2]octane-1-carboxylate (8).

In closed vial, compound 7 (500 mg, 1.37 mmol) was dissolved in 2-methoxyethanol, also known as ‘methylcellosolve’ (25 mL), an excess of NaI (2.46 g, 16.42 mmol) was added and the reaction mixture was heated to 130 °C overnight. The solution was allowed to reach room temperature and the solvent was evaporated under reduced pressure. The residue was dissolved in water (10 mL), extracted with ether (3 x 10 mL). The organic layer was collected, dried (Na₂SO₄), filtered and evaporated to dryness to give 300 mg (68%) of 8 as a white solid after column chromatography (n-hexane/EtOAc, 1:0.04). ¹H NMR (250.13 MHz, CDCl₃): δ 1.43-1.55 (m, 6H, 3 x CH₂), 1.74-1.85 (m, 6H, 3 x CH₂), 3.05 (s, 2H, CH₂I), 3.50 (s, 3H, CH₃).

4-(Iodomethyl)bicyclo[2.2.2]octane-1-carboxylic acid (9).

Compound 8 (150 mg, 0.49 mmol) was dissolved in MeOH (10 mL), added to a solution of NaOH (19.5 mg, 0.49 mmol) in water (1 mL) and stirred at room temperature
overnight. Then, the solvent was evaporated under reduced pressure. The residue was dissolved in water and extracted with diethyl ether (3 x 5 mL). The organic phase was collected to get rid of any non-reacting starting material. The aqueous phase was collected and subsequently acidified with 6 N HCl to reach a pH 1, which was then extracted with EtOAc (3 x 5 mL), the organic phase was collected, dried (Na₂SO₄) and evaporated to dryness giving 74 mg (51%) of 9 as a white solid after column chromatography (n-hexane/EtOAc, 6:1). ¹H NMR (250.13 MHz, CDCl₃): δ 1.43-1.74 (m, 6H, 3 x CH₂), 1.80-1.85 (m, 6H, 3 x CH₂), 3.10 (s, 2H, CH₂I).

4-(Iodomethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)bicyclo[2.2.2]octane-1-carboxamide (4c).

In a dried closed reaction vial and under argon atmosphere, the reaction mixture of 9 (35 mg, 0.12 mmol) and dichloro(methoxy)methane (10.6 µL, 0.12 mmol) in CH₂Cl₂ (4 mL) was heated for four days at 60 °C. The volatile products (HCl and methyl formate) were removed. Then WAY-100634 (37.17 mg, 0.12 mmol) in CH₂Cl₂ (1 mL) and NEt₃ (24.1 mg, 0.24 mmol) were added to the corresponding acid chloride. The new reaction mixture was stirred for 1 hour at room temperature. The solvent was evaporated and the residue was dissolved in water (3 mL) and extracted with EtOAc (2 x 10 mL). The organic phase was collected, dried over anhydrous Na₂SO₄ and solvent was evaporated under reduced pressure to give 44 mg (63%) of 4c as a colorless glass after column chromatography (CH₂Cl₂/MeOH, 20:1). ¹H NMR (250.13 MHz, CDCl₃): δ 1.25-1.45 (m, 6H, 3 x CH₂), 1.60-1.80 (m, 6H, 3 x CH₂), 2.55-2.80 (m, 6H, N(CH₂)₃), 2.95 (s, 2H, CH₂I), 3.00-3.15 (m, 4H, N(CH₂)₂), 3.80-4.00 (m, 5H, CH₃ and NCH₂), 6.80-7.05 (m, 4H, 4 x CH), 7.22-7.33 (m, 2H, 2 x CH), 7.74 (dt, J = 7.6 Hz, 1H, CH), 8.51 (d, J = 4.9 Hz, 1H, CH). ¹³CNMR (100.62 MHz, CDCl₃): δ 22.90, 29.32, 30.72, 31.38, 42.36, 48.86, 50.56, 53.48, 55.37, 55.71, 111.34, 118.12, 120.98, 122.68, 122.82, 123.34, 138.21, 141.37, 149.01, 152.28, 156.84, 177.76. HRMS (EI) m/z calcd for C₂₈H₃₇IN₄O₂, 588.1961; found, 589.1972 (M + H).
Radiotracers

Radiofluorination:

$[^{18}\text{F}]^–$ was produced by the $^{18}\text{O}(p,n)^{18}\text{F}$ nuclear reaction with an IBA 18/9 cyclotron. After irradiation, $[^{18}\text{F}]^–$ was trapped on a Waters QMA SepPak column (carbonate form). It was eluted from the anion exchange column with 1 ml of acetonitrile:water (9:1 v:v) containing Kryptofix[2.2.2] and potassium carbonate. The solution was dried under helium flow and reduced pressure at 90 °C for 15 minutes. To remove residual water, 0.5 ml of acetonitrile was added and the solution was dried again. At room temperature, the precursor (4a, 4b or 4c) in 0.5 ml of acetonitrile was added. Test reactions were performed with small amounts of activity (0.5-1 GBq) to find conditions for which the labelling yield was at least 50%. All reactions were carried out utilizing homemade, remotely controlled equipment.

The yields were monitored by radio-thin-layer chromatography (radio-TLC) using Merck precoated silica gel F-254 plates (thickness 0.3 mm) and dichloromethane/methanol (95:5 v:v) as eluent, and by HPLC (kromasil C18-10 μm column, 4.6 mm x 250 mm; eluent (MeOH/H$_2$O/DIPA, 80:20:0.02). Radio thin-layer chromatography (radio-TLC) plates were exposed for 5 minutes to a Molecular Dynamics phosphor imager plate and subsequently scanned with a Storm 820 imager (Molecular Dynamics). Obtained images were analyzed with ImageQuant 5.2. Analytical HPLC was performed with a Jasco PU1580 pump, an in-line Jasco UV2075 UV detector (wavelength 254 nm), a flow through NaI (Tl) crystal scintillation detection system and Raytest Gina-NT for data acquisition.

For determining the specific activity (SA) at the end of synthesis (EOS), the UV absorbance on HPLC was calibrated using a 50 μl injection of the cold compounds. For all three compounds a linear correlation ($R^2>$0.995) was obtained in a concentration range of 0.5-10 nmol/mL.

$[^{18}\text{F}]4$-(Fluoromethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)bicyclo[2.2.1]heptane-1-carboxamide ($[^{18}\text{F}]5a$).

Good results for the labelling were found when 4 mg (6.5 μmol) of precursor 4a in dry MeCN (500 μL) was added to the dried radiofluoride, 2,2,2-kryptofix (13 mg) and
K₂CO₃ (2 mg), and heated at 130 °C for 30 minutes in a closed vial. Under these conditions the reaction was performed twice, first for the biodistribution study and a second time for the PET study with ¹⁸F amounts of 26 c.q. 24 GBq. After the addition of water (0.5 mL), the solution was injected on HPLC using a C18-10 μm column (kromasil, 4.6 mm x 250 mm) and (MeOH/H₂O/DIPA, 60:40:0.02) as eluent with a flow of 1 mL/min ($t_R = 24-28$ minutes). The collected radioactive fraction (4 mL) was diluted with water (30 mL) and the product was trapped on a SepPak C18 plus cartridge. The SepPak was rinsed with water (20 mL) and inversely eluted with EtOH (1 mL). The ethanolic fraction was further formulated with 9 mL of citrate buffer (pH 4.8) to give $[¹⁸F]5a$ in a concentration of 496 c.q. 433 MBq/mL. The total synthesis time was about 105 minutes. So, the decay corrected overall yields were 37 c.q. 35%. Analytical HPLC (kromasil C18-10 μm column, 4.6 mm x 250 mm); eluent (MeOH/H₂O/DIPA, 65:35:0.02), of a 50 μL sample revealed a radiochemical purity of $[¹⁸F]5a$ of 99.1 c.q. 99.8% and a SA at EOS of 132 c.q. 76 GBq/µmol. The UV channel showed besides $5a$ ($t_R = 17.2$ minutes, 75 c.q. 55%) some other detectable signals at $t_R = 13.8$ minutes (15 c.q. 20%), $t_R = 14.5$ minutes (0 c.q. 15%) and $t_R = 16.4$ minutes (10 c.q. 10%).


Formation of $[¹⁸F]5b$ already proceeded smoothly when 2 mg (3.7 µmol) of precursor $4b$ in dry MeCN (500 µL) was added to the dried radiofluoride, 2,2,2-kryptofix (13 mg) and K₂CO₃ (2 mg), and heated at 110 °C for 15 minutes in a closed vial. ¹⁸F amounts used for both the biodistribution and the PET study were 22 GBq. After the addition of water (0.5 mL) the solution was injected on HPLC using a C18-10 μm column (kromasil, 4.6 mm x 250 mm); eluent (MeOH/H₂O/DIPA, 60:40:0.02) with a flow of 1 mL/min and product was collected at ($t_R = 23-27$ minutes). The radioactive fraction (4 mL) was diluted with water (30 mL) and the product was trapped on a SepPak tC18 plus cartridge. The SepPak was rinsed with water (20 mL) and inversely eluted with EtOH (1 mL). The ethanolic fraction was further formulated with 9 mL of citrate buffer (pH 5.0) to give $[¹⁸F]5b$ in a concentration of 299 c.q. 474 MBq/mL. With a total synthesis time of about 90 minutes, the decay corrected yield was 24 c.q. 38%. Analytical HPLC (kromasil
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C18-10 μm column, 4.6 mm x 250 mm; eluent (MeOH/H2O/DIPA, 65:35:0.02), of a 50 μL sample revealed a radiochemical purity of $[^{18}\text{F}]5\text{b}$ of 98.1 c.q. 98.6% and a SA at EOS of 119 c.q. 138 GBq/μmol. The UV channel showed besides $5\text{b}$ ($t_R = 16.9$ minutes, 45 c.q. 50%) two other detectable signals at $t_R = 13.8$ minutes (30 c.q. 15%) and $t_R = 15.9$ minutes (25 c.q. 35%).

$[^{18}\text{F}]4$-(Fluoromethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)bicyclo[2.2.2]octane-1-carboxamide ($[^{18}\text{F}]5\text{c}$).

For the formation of $[^{18}\text{F}]5\text{c}$ the reaction temperature had to be raised. The amounts of 2,2,2-kryptofix and $\text{K}_2\text{CO}_3$ were reduced to prevent decomposition of the starting material. Best results were found when 4 mg (6.8 μmol) of precursor $4\text{c}$ in dry MeCN (500 μL) was added to the dried radiofluoride, 2,2,2-kryptofix (3.25 mg) and $\text{K}_2\text{CO}_3$ (0.5 mg), and heated at 150 °C for 15 minutes in a closed vial. The $^{18}$F amounts used for the biodistribution study and for the PET study was 24 c.q. 29 GBq. After the addition of water (0.5 mL) the solution was injected on HPLC using a C18-10 μm column (kromasil, 4.6 mm x 250 mm) and (MeOH/H2O/DIPA, 65:35:0.02) as eluent with a flow of 1 mL/min ($t_R = 22$-26 minutes). The collected radioactive fraction (4 mL) was diluted with water (30 mL) and the product was trapped on a SepPak C18 plus cartridge. The SepPak was rinsed with water (20 mL) and inversely eluted with EtOH (1 mL). The ethanolic fraction was further formulated with 9 mL of citrate buffer (pH 4.8) to give $[^{18}\text{F}]5\text{c}$ in a concentration of 531 c.q. 740 MBq/mL. The total synthesis time was 90 minutes and the decay corrected yield 39 c.q. 45%. Analytical HPLC (kromasil C18-10 μm column, 4.6 mm x 250 mm); eluent (MeOH/H2O/DIPA, 70:30:0.02), of a 50 μL sample revealed a radiochemical purity of $[^{18}\text{F}]5\text{c}$ of 99.8 for both productions and a SA at EOS of 90 c.q. 120 GBq/μmol. The UV channel showed besides $5\text{c}$ ($t_R = 16.0$ minutes, 80 c.q. 80%) some other signals at $t_R = 12.6$ minutes (0 c.q. 20%), $t_R = 14.2$ minutes (15 c.q. 0%) and $t_R = 19.4$ minutes (5 c.q. 0%).

**Biodistribution studies**

Animal experiments were performed with Wistar rats (male, body weight around 230 g, Harlan, Horst, The Netherlands). Approval of the institutional animal ethics
committee was obtained and all experiments were carried out in accordance with both the Dutch Law on Animal Experimentation and guidelines of the institutional committee on animal experimentation. Rats (four per study) were anesthetized with 2% isoflurane, 0.5 oxygen L/min and received an intravenous injection of 200 µL of the sterilized radiolabeled compound via the tail vein. Rats were sacrificed at 45 minutes after injection and tissues of interest were removed, weighed and measured for radioactivity using a LKB Wallac 1282 CompuGamma CS.

For calculation of the injected dose, five aliquots of the injected solution were weighed and counted for radioactivity. Results were decay corrected and expressed as percentage of injected dose per gram of tissue ± standard deviation (%ID/g ± S.D.). Data were analyzed by ANOVA (one-way analysis of variance) followed by Dunnett’s test to correct for multiple comparison. P ≤0.05 indicate significant difference.

**PET studies**

Additional *in vivo* PET studies of [18F]5a, [18F]5b and [18F]5c were performed using a double LSO/LYSO layer High Resolution Research Tomograph (HRRT; CTI/Siemens, Knoxville, TN, USA). Performance characteristics of this scanner have been reported previously. The combination of high spatial resolution and high sensitivity makes the HRRT an ideal PET scanner for dynamic studies in small laboratory animals. First, for attenuation and scatter correction, a transmission scan was acquired using a 740 MBq 2-dimensional (2D) fan-collimated $^{137}$Cs (662 keV) moving point source (9). Next, a dynamic emission scan of 60 minutes was acquired following administration of the radiotracer. Data were acquired in list mode and rebinned into the final frame sequence: 6 x 10, 1 x 20, 2 x 30, 2 x 60 seconds, 2 x 2.5, 2 x 5 and 4 x 10 minutes. Following corrections for decay, dead time, scatter and randoms, scans were reconstructed using an iterative 3D ordered-subsets weighted least-squares (3D-OSWLS) method.

Prior to the PET scan, rats (n = 2 for each study group) were anesthetized (isoflurane 2%, oxygen 0.45 volume %) and cannulated via the jugular vein for venous access. After cannulation, rats were positioned in the PET scanner (2 rats placed side by side on the camera bed and kept under anesthesia breathing through a ventilation mask
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(Isofluran 1-2%, oxygen 0.45 volume %) and body temperature was kept within normal range (37-38 °C)).

Rats were injected through the cannula with sterile 0.9% NaCl or WAY-100635 (2 mg/kg) 5 minutes prior to the radioligand (20 μL) injection. Rats were also sacrificed and tissues dissected immediately after the PET study. Both studies were done with the same batch of the radioligand with a time interval of 150 minutes; the blocking study the last.

Time activity curves of the radiotracer, reflecting delivery of the tracer to the brain, were derived for predefined volumes of interest (VOIs), in rats, the VOIs were drawn in ellipsoids over the whole brain. The position of all VOI was defined visually on a summation image of time frames from 0.5-60 minutes. Next, this VOI was projected onto all dynamic frames, resulting in a brain time-activity curve of the tracer, using the software package AMIDE (Amide 0.9.1 http://amide.sourceforge.net). The standardized uptake value (SUV = radioactivity per cm$^3$ / injected radioactivity per gram of bodyweight) was calculated based on the VOI activity, injected activity dose and animal weight.
References


