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Synthesis and biological evaluation of ^{11}C-labeled β-galactosyl triazoles as potential PET tracers for in vivo LacZ reporter gene imaging

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ABSTRACT

In our aim to develop LacZ reporter probes with a good retention in LacZ expressing cells, we report the synthesis and preliminary evaluation of two carbon-11 labeled β-galactosyl triazoles 1-(β-D-galactopyranosyl)-4-(*p*-[^{11}C]methoxyphenyl)-1,2,3-triazole ([^{11}C]-**6**) and 1-(β-D-galactopyranosyl)-4-(6-[^{11}C]methoxynaphthyl)-1,2,3-triazole ([^{11}C]-**13**). The precursors for the radiolabeling and the non-radioactive analogues (**6** and **13**) were synthesized using straightforward 'click' chemistry. In vitro incubation experiments of **6** with β-galactosidase in the presence of *o*-nitrophenyl β-D-galactopyranoside (ONPG) showed that the triazolic compound was an inhibitor of β-galactosidase activity. Radiolabeling of both precursors was performed using [^{11}C]methyl iodide as alkylating agent at 70 °C in DMF in the presence of a small amount of base. The log *P* values were −0.1 and 1.4, respectively, for [^{11}C]-**6** and [^{11}C]-**13**, the latter therefore being a good candidate for increased cellular uptake via passive diffusion. Biodistribution studies in normal mice showed a good clearance from blood for both tracers. [^{11}C]-**6** was mainly cleared via the renal pathway, while the more lipophilic [^{11}C]-**13** was excreted almost exclusively via the hepatobiliary system. Despite the lipophilicity of [^{11}C]-**13**, no brain uptake was observed. Reversed phase HPLC analysis of murine plasma and urine revealed high in vivo stability for both tracers. In vitro evaluation in HEK-293T cells showed an increased cell uptake for the more lipophilic [^{11}C]-**13**, however, there was no statistically higher uptake in LacZ expressing cells compared to control cells.

1. Introduction

With the advance of molecular biology, gene expression has been extensively studied using reporter genes.[1] One of the most widely used reporter genes is the LacZ gene which encodes the bacterial β-galactosidase (β-gal) enzyme. There are several chromogenic and fluorogenic reporter probes commercially available for detection of β-gal.[2,3] However, these optical-based reporter systems usually require histochemical staining or spectrophotometric assays of tissue acquired by invasive sampling or can only be used to image small animals that are transparent for light. Applications for in vivo monitoring of LacZ reporter gene expression with these techniques in larger living animals and humans is not possible due to the limited tissue penetration of visible light.

Nuclear medicine technology has been applied to monitor repetitively and quantitatively reporter gene expression in living systems using specific radiolabeled probes in combination with positron emission tomography (PET) or single photon emission computed tomography (SPECT).[4,5] Reporter genes that have been used for radionuclide imaging can be divided into three major categories.[6] The first category are reporter genes that lead to the production of an enzyme capable of metabolizing and trapping of the reporter probes. A theoretical advantage of these enzymatic reporter systems is that one molecule of enzyme can trap many molecules of reporter substrate, leading to amplification of the signal and an increased sensitivity. The most widely studied enzyme-based reporter system is the herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter gene for which radiolabeled substrate-probes are phosphorylated and trapped in HSV1-tk expressing cells. The amount of HSV1-tk expressing cells can thus be quantified in vivo by PET or SPECT.[7–9] The second class of reporter genes are those that encode proteins that act as a receptor for binding with the reporter probe. Since this is a one-to-one stoichiometric interaction between one ligand and one receptor, the receptor-based approach is less sensitive. An example is the human dopamine-2-receptor (hD2R) in combination with [^{18}F]fluoroethylspiperone. The third category of reporter genes are transporter proteins located in the cell membrane that actively pump the radiolabeled probes from the extracellular space into the cell. The

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human sodium iodide symporter (hNIS) has been studied as transporter reporter gene in combination with a wide range of iodine isotopes. However, the major problem encountered using this reporter system is that the radioactive iodide is not sequestered, leading to a rapid efflux of radioactivity out of the hNIS expressing cells.[6,10]

Concerning the LacZ reporter gene, which is an enzyme-based reporter system, little progress has been made in the development of suitable radiolabeled probes. Most problems encountered are poor cell membrane penetration and inefficient sequestering of the tracers in the target cells.

Bormans et al.[11] synthesized fluorine-18 labeled lactose using an enzyme-catalyzed reaction between fluorine-18 labeled glucose ([^{18}F]FDG) and galactose. Biodistribution studies in Rosa-26 mice (expressing LacZ in almost every tissue) compared to tissue distribution studies in normal control mice revealed that radiolabeled lactose was unable to cross the cell membrane. In *Escherichia coli,* cellular uptake of lactose, which is the natural substrate of β-galactosidase and other galactopyranosides, is mediated by lactose permease.[12,13] However, this transporter protein is not expressed in eukaryotic cells and since lactose is too hydrophilic for cell membrane penetration, no accumulation of radiolabeled lactose in the studied mammalian cells/tissue was observed.

Instead of radiolabeled substrates, Kim et al.[14] developed a radiolabeled inhibitor against β-gal activity by introducing iodine-123/iodine-125 in 2-phenylethyl 1-thio-β-D-galactopyranoside (PETG). PETG is a commercially available competitive inhibitor specific against *E. coli* β-galactosidase. In vivo imaging studies of [^{123}I]iodo-PETG injected intravenously in nude mice showed only a slightly improved visualization of a LacZ expressing tumor versus a control tumor. The low contrast was partly ascribed to poor transport across the cell membrane.[15] Non-modified D-galactose is avidly transported into mammalian cells and has been proposed to share a single transport mechanism with D-glucose and 2-deoxy-glucose.[16] Although structurally related to D-galactose, galactopyranosides derivatized at position C1 (like lactose and PETG) are not transported by the hexose transport system. Since in literature no evidence of active transport for C1-derivatized galactopyranosides is found, cell uptake of these probes is assumed to be mediated by passive diffusion. This implies that the developed LacZ tracers should be lipophilic enough to penetrate the cell membrane by passive diffusion.

Recently, we have developed fluorine-18 and carbon-11 labeled phenyl β-D-galactopyranosides[17] and 1-β-D-galactosyl esters (submitted). Although both tracer classes were good substrates of β-gal, in vitro evaluation in LacZ expressing HEK-293T (human embryonic kidney) cells and control HEK cells showed low cell uptake levels. This low uptake can be partly ascribed to the fact that these probes were still too hydrophilic for efficient cell entry via passive diffusion.

In our aim to synthesize LacZ reporter probes with a good retention in LacZ expressing cells, we have synthesized and evaluated radiolabeled inhibitors to probe β-galactosidase. In this case, no radiolabeled hydrolysis products are liberated and the radioactivity that probes the enzyme will remain localized in the target cells. From this point of view the inhibitor approach may have some advantages over the use of substrates of which the radiolabeled hydrolysis products may not be efficiently retained.

Glycosyl triazoles are nitrogen containing heterocyclic glycoconjugates that have been reported as inhibitors of glycosidase activity.[18,19] The triazoles function as rigid linking units that can mimic the atom placement and electronic properties of a peptide bond without the same susceptibility to hydrolytic and enzymatic cleavage, resulting in metabolically stable compounds.[20] Perhaps due in part to their ability to mimic certain aspects of a peptide bond, many 1,2,3-triazoles possess both anti-viral and anti-bacterial activity.[20] Furthermore, these molecules can be easily synthesized using reliable and efficient 'click' chemistry,[21,22] which offers the ability for rapid generation of combinatorial libraries for screening of the best inhibitory effect.

In this study we report the synthesis and preliminary evaluation of two carbon-11 labeled β-galactosyl triazoles [^{11}C]-**6** and [^{11}C]-**13**, containing respectively, a phenyl and naphthyl moiety in the aglycon. The naphthylic aglycon makes [^{11}C]-**13** more lipophilic, allowing to study the effect of increased lipophilicity on cell membrane penetration and cell uptake. We report the synthesis of stable and radiolabeled triazoles **6** and **13**, their biodistribution in normal mice and the study of the in vivo stability. Finally a cell uptake study was carried out in LacZ expressing human embryonic kidney (293T) cells.

2. Results and discussion

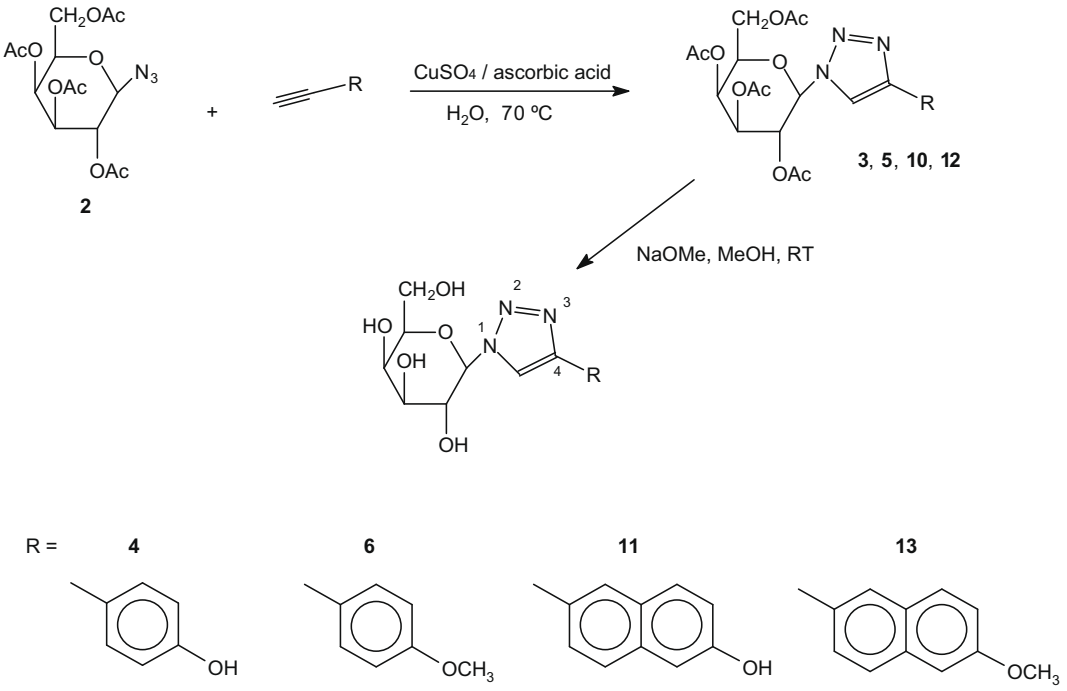
2.1. Chemistry

The precursors for the radiolabeling (**4** and **11**) and the non-radioactive reference compounds (**6** and **13**) are 4-substituted triazolyl galactopyranosides that were synthesized using straightforward 'click' chemistry (Scheme 1). 'Click' chemistry is a chemical philosophy that refers to a set of powerful and selective reactions that form heteroatom links using spring-loaded reactants. Reactions defined as 'click' reactions are high in yield and require only benign reaction conditions (e.g., water as solvent) with simple work-up and purification procedures.[21,22] The prototype 'click' reaction is the Huisgen 1,3-dipolar cycloaddition of azides with terminal alkynes to afford 1,2,3-triazoles. Further optimization by Sharpless and Meldal using Cu(I)-catalysis resulted in high regioselectivity affording the 1,4-regioisomer exclusively.[23,24] The Cu(I)-catalyzed 1,3-dipolar cycloaddition was used in this study to synthesize the precursors and cold reference compounds. The general reaction procedure applied to couple β-galactosyl azide with the terminal alkynes is depicted in Scheme 1 (Step 1).

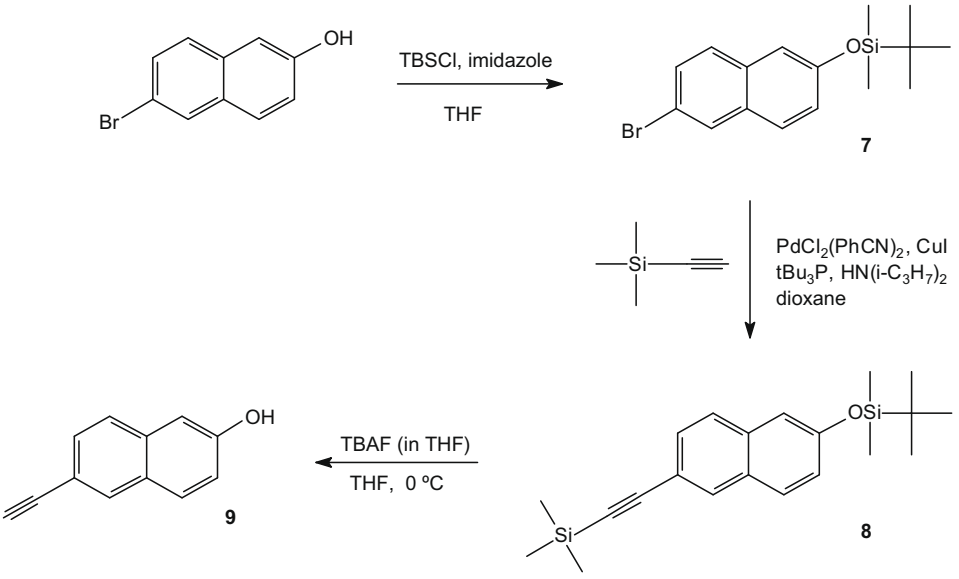
The acetyl-protected β-galactosyl azide (**2**) was synthesized in two steps according to reported procedures.[25,26] First, penta-acetylated β-D-galactose was converted into acetobromo-α-D-galactose (**1**). In a second step the bromine was replaced by an azide group using sodium azide. Inversion of the configuration at the anomeric site resulted in the formation of the protected β-galactosyl azide (**2**), as could be concluded from the large axial-axial coupling of the anomeric H-1 with H-2 ($^3J_{H1-H2}$ = 8.64 Hz) in the ^{1}H NMR spectrum. The alkynes used in this paper were commercially available except for 6-ethynyl-2-naphthol (**9**) that was synthesized in three steps (Scheme 2) starting from 6-bromo-2-naphthol.

In a first step the free hydroxyl function was protected with a *tert*-butyldimethylsilyl group to afford compound **7** according to a patented procedure.[27] Next, the bromine was converted into a trimethylsilyl-ethynyl group using a Sonogashira coupling reaction of **7** with trimethylsilylacetylene in 1,4-dioxane in the presence of diisopropylamine and catalytic amounts of $PdCl_2(PhCN)_2$, CuI and tri(*tert*-butyl)phosphine to obtain compound **8**.[28] Finally, removal of the silyl-protecting groups with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran afforded the desired 6-ethynyl-2-naphthol (**9**).[29]

For the catalysis of the cycloaddition between acetylated β-galactosyl azide (**2**) and the terminal alkynes (Scheme 1, Step 1) we preferred a mixture of Cu(II)SO_4 and a reducing agent (ascorbic acid) to produce Cu(I) in situ, instead of commercial sources of Cu(I), for example, CuI or CuBr, which are prone to oxidation. The reaction was carried out at elevated temperatures using water as solvent from which the crude reaction product could be easily recovered by filtration. Recrystallization from 95% ethanol afforded the desired acetylated cycloadducts, no further purification using column chromatography was necessary. Finally, deprotection un-



Scheme 1. Step 1: Cu(I)-catalyzed 1,3-dipolar cycloaddition of acetyl-protected β-galactosyl azide (**2**) with various terminal alkynes to afford the acetyl-protected 4-substituted 1,2,3-triazolyl β-D-galactopyranosides. Step 2: Deprotection under alkaline reaction conditions resulting in the radiolabeling precursors (**4** and **11**) and the cold references (**6** and **13**).



Scheme 2. 3-Step-synthesis of 6-ethynyl-2-naphthol (**9**) starting from 6-bromo-2-naphthol.

der alkaline reaction conditions (Scheme 1, Step 2) afforded the desired 4-substituted triazolyl galactopyranosides: two precursors **4** and **11** and two non-radioactive reference compounds **6** and **13**. High stereo- and regioselectivity was achieved as could be concluded from the NMR spectra. The stereoselectivity was proven by the large axial–axial coupling of the anomeric H-1 with H-2 ($^3J_{H1-H2}$ = >8 Hz) in the ^{1}H NMR spectra, showing that the galactosyl triazoles had the β-configuration. This is a necessary condition to have affinity for the β-galactosidase enzyme. The regiochemistry was unambiguously attested by performing a set of 2D-NMR experiments (COSY, HSQC and HMBC). The ^{1}H–^{13}C HMBC spectrum showed 3-J cross coupling between the anomeric C-1 and the H-5 of the phenyl ring and between the anomeric H-1 and the C-5 of the phenyl ring, proving that the 4-substituted triazole (**6**) was synthesized regioselectively. Formation of the 5-substituted triazole, which could sterically hinder binding to the active site of the enzyme, was prevented, presumably due to the presence of the Cu(I)-catalyst.[23]

2.2. Radiochemistry

Radiosynthesis of [^{11}C]-**6** and [^{11}C]-**13** was carried out using compounds **4** and **11**, respectively, as precursors and [^{11}C]methyl iodide ([^{11}C]MeI) as labeling agent (Scheme 3). [^{11}C]MeI was

synthesized following a reported procedure with some modifications.[30] The labeling was performed in DMF with NaOH as base. At 70 °C slightly higher yields were obtained compared to room temperature. Both tracers were purified using semi-preparative RP-HPLC and the chemical and radiochemical purity was found to be >99% using analytical HPLC. Co-elution with the authentic non-radioactive compounds (**6** and **13**) after co-injection on analytical RP-HPLC confirmed the identity of the desired tracers.

2.3. In vitro incubation with β-galactosidase

To validate whether the galactosyl triazoles act as inhibitors of β-galactosidase, some preliminary in vitro incubation experiments were performed. When a solution of 1 mg/mL of the non-radioactive triazole **6** was incubated with 10 U of β-galactosidase at 37 °C overnight and analyzed using RP-HPLC, no hydrolysis products were detected. The same observation was made when the radiolabeled compound [^{11}C]-**6** was incubated with the enzyme (10 U). These findings indicate that the triazolic compound is not cleaved by the enzyme. When the chromogenic substrate ONPG (1 mg/mL) was incubated under the same conditions for 15 min, complete conversion into the corresponding nitrophenol was observed. However, when ONPG (1 mg/mL) was added to an incubation mixture of triazole **6** (1 mg/1 mL) and 10 U β-galactosidase, the conversion of ONPG was slowed down. After 15 min, only 40% of ONPG was hydrolyzed, after 90 min, the conversion reached 70%. Addition of an excess of ONPG resulted in an immediate yellow color formation and HPLC analysis showed complete conversion into the nitrophenol. These incubation experiments indicate that the triazole binds to the enzyme and that it acts as a competitive inhibitor.

2.4. Partition coefficient

The log partition coefficient (log *P*) values of [^{11}C]-**6** and [^{11}C]-**13** were found to be −0.1 and 1.4, respectively. Since in literature no mammalian active transporters are described for this kind of tracers, we assume that uptake in cells and in the brain must occur by passive diffusion. According to Dischino et al.,[31] C-11 labeled compounds with log *P* values between 0.9 and 2.5 are able to pass freely across the blood–brain barrier (BBB). Extrapolating this to cell membrane penetration, we could postulate that the naphthylic tracer [^{11}C]-**13** could have a more efficient uptake in LacZ expressing cells due to its increased lipophilicity. [^{11}C]-**6** has a negative log *P* value and will presumably be too hydrophilic for good cell entry.

2.5. Biodistribution in normal mice

The results of the in vivo distribution studies of [^{11}C]-**6** and [^{11}C]-**13** in male NMRI mice at 2 min and 60 min p.i. are shown in Table 1 and Table 2. As described above, for a reasonable uptake in the brain via passive diffusion, the log *P* value of a compound should be between 0.9 and 2.5. Furthermore, the molecular mass should be less than 650 Da and the compound should be uncharged.[31,32] Both tracers [^{11}C]-**6** and [^{11}C]-**13** are uncharged at physiological pH and their molecular mass is 336 Da and 386 Da, respectively. [^{11}C]-**6** has a negative log *P* value (−0.1) and is probably too hydrophilic to cross the BBB freely, explaining its low concentration in the brain. The naphthylic tracer [^{11}C]-**13** on the other hand has an additional aromatic ring which makes it more lipophilic. Despite the presence of the polar sugar moiety, the addition of the naphthalene ring increases its lipophilicity to a log *P* value of 1.4. Although [^{11}C]-**13** fulfills all the above-mentioned theoretical requirements, it also shows negligible brain uptake. This implies that besides the general requirements of lipophilicity (log *P* estimation), molecular mass and charge, other factors or properties of the molecule do influence cell membrane penetration. Presumably, the presence of the polar hydroxyl-bearing sugar moiety is detrimental for BBB penetration of these galactose-derivatives, irrespective of their overall lipophilicity. Apart from the partition

Table 1
Biodistribution of [^{11}C]-**6** in NMRI mice 2 min and 60 min p.i.

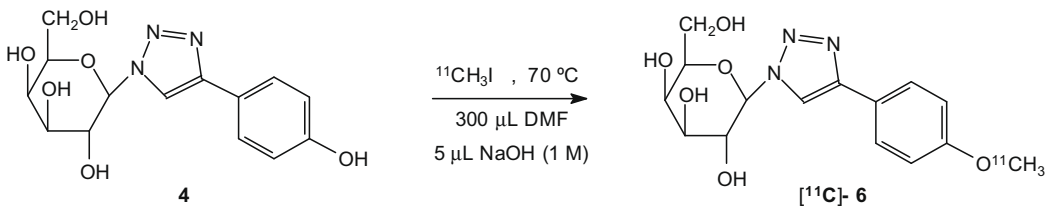
[^{11}C]-**6**	% ID[a]		% ID/g[b]	
	2 min	60 min	2 min	60 min
Urine	3.1 ± 1.2	47.5 ± 4.0		
Kidneys	16.1 ± 3.0	4.0 ± 1.5	19.3 ± 1.0	4.4 ± 1.6
Liver	18.7 ± 0.9	13.4 ± 2.1	8.7 ± 0.6	6.3 ± 1.1
Intestines	6.6 ± 0.4	9.7 ± 1.5	2.0 ± 0.3	3.0 ± 0.8
Spleen + pancreas	0.6 ± 0.1	0.6 ± 0.3	2.3 ± 0.2	1.7 ± 0.5
Lungs	1.5 ± 0.4	0.6 ± 0.1	6.2 ± 0.5	2.0 ± 0.3
Heart	0.6 ± 0.1	0.2 ± 0.1	2.8 ± 0.2	1.2 ± 0.2
Stomach	0.9 ± 0.2	0.9 ± 0.4	1.5 ± 0.7	1.4 ± 0.8
Brain	0.1 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	0.3 ± 0.1
Blood	27.0 ± 4.4	8.1 ± 0.4	8.9 ± 0.1	2.4 ± 0.2
Carcass	38.1 ± 3.0	19.6 ± 1.7		

Data are expressed as mean ± SD; $n = 4$ per time point; p.i. = post injection.
[a] Percentage of injected dose calculated as cpm in organ/total cpm recovered.
[b] Percentage of injected dose per gram tissue.

Table 2
Biodistribution of [^{11}C]-**13** in NMRI mice 2 min and 60 min p.i.

[^{11}C]-**13**	% ID[a]		% ID/g[b]	
	2 min	60 min	2 min	60 min
Urine	0.1 ± 0.0	2.8 ± 1.2		
Kidneys	5.7 ± 0.7	1.0 ± 0.2	7.2 ± 0.6	1.4 ± 0.4
Liver	25.8 ± 3.4	7.1 ± 0.4	12.1 ± 1.7	3.4 ± 0.5
Intestines	6.1 ± 1.2	73.4 ± 4.0	1.6 ± 0.2	19.8 ± 1.3
Spleen + pancreas	0.8 ± 0.2	0.4 ± 0.1	2.9 ± 0.4	1.2 ± 0.4
Lungs	2.6 ± 1.7	0.3 ± 0.1	8.5 ± 3.1	1.1 ± 0.4
Heart	0.9 ± 0.1	0.2 ± 0.0	5.1 ± 0.4	0.8 ± 0.2
Stomach	0.9 ± 0.1	0.4 ± 0.2	1.1 ± 0.3	0.2 ± 0.1
Brain	0.2 ± 0.0	0.0 ± 0.0	0.9 ± 0.1	0.2 ± 0.1
Blood	50.9 ± 7.0	4.2 ± 1.5	18.6 ± 1.2	1.6 ± 0.6
Carcass	34.3 ± 4.6	12.2 ± 2.1		

Data are expressed as mean ± SD; $n = 4$ per time point; p.i. = post injection.
[a] Percentage of injected dose calculated as cpm in organ/total cpm recovered.
[b] Percentage of injected dose per gram tissue.



Scheme 3. Radiolabeling of precursor **4** yielding 1-(β-D-galactopyranosyl)-4-(*p*-[^{11}C]methoxyphenyl)-1,2,3-triazole ([^{11}C]-**6**). The naphthylic tracer 1-(β-D-galactopyranosyl)-4-(6-[^{11}C]methoxynaphthyl)-1,2,3-triazole ([^{11}C]-**13**) was synthesized following the same labeling procedure with compound **11** as precursor.

coefficient, another important factor in the prediction of passive diffusion of a compound across membranes is the overall hydrogen bond capacity, which can be estimated by calculating the molecular polar surface area (PSA) of the compound.[33] This PSA is defined as the sum of surface contributions of polar atoms, usually oxygens, nitrogens and attached hydrogens, in a molecule[34] and has been shown to correlate well with BBB penetration.[35] Since our radiolabeled galactose-derivatives have four polar hydroxyl functions, the PSA will be large (130 Å^2 compared to e.g., 24 Å^2 for ethanol)[34] and thus the hydrogen bond interaction will probably be too high to allow efficient passive diffusion of these tracers across the BBB. Nevertheless, the sugar-hydroxyl functions should be present because they are essential for binding to the active site of the enzyme.[36,37] [^{11}C]-**6** was cleared from blood mainly via the renal pathway with 47.5% of the injected dose (%ID) in the urine at 60 min p.i. and to a lesser extent via the liver to the intestines. In accordance to its higher log *P* value, the more lipophilic [^{11}C]-**13** was excreted via the hepatobiliary system with 73.4% ID in the intestines. Only a negligible fraction (3% ID) was found in the urine at 60 min p.i.

2.6. Biostability in normal mice

The metabolic stability was assessed in plasma and urine collected from normal mice 30 min after injection of the tracer using RP-HPLC. The cold intact compound (**6** or **13**) was co-injected onto HPLC to identify the intact parent tracer. An example of the analysis of urine obtained 30 min p.i. of [^{11}C]-**6** and of a plasma sample obtained at 30 min p.i. of [^{11}C]-**13** is shown in Figures 1a and b. For [^{11}C]-**6**, in plasma as well as in urine, almost 100% of the recovered radioactivity was present as intact tracer. The same result was found for the plasma analysis of [^{11}C]-**13** (metabolite analysis of a urine sample was not performed), indicating that both tracers are very stable in vivo.

2.7. In vitro evaluation of the tracers

Both tracers were further evaluated in vitro by assessing their uptake and accumulation in β-gal expressing and VZV-TK expressing (control) HEK-293T cells. The results of these cell experiments

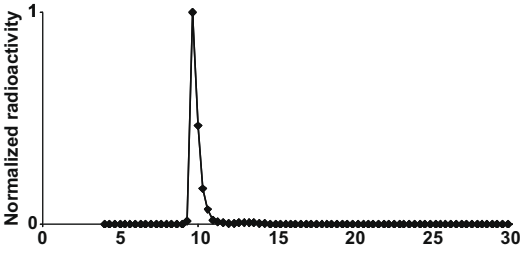


Figure 1a. Metabolite analysis of [^{11}C]-**6**: RP-HPLC radiochromatogram of a urine sample from a normal mouse collected 30 min p.i. Fractions were collected per 20 s.

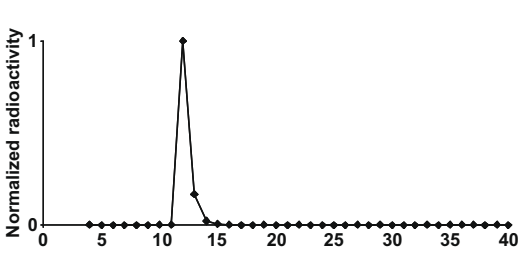


Figure 1b. Metabolite analysis of [^{11}C]-**13**: RP-HPLC radiochromatogram of a plasma sample from a normal mouse collected 30 min p.i. Fractions were collected per minute.

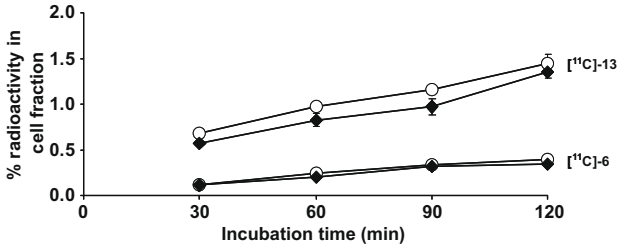


Figure 2. Time-dependent uptake levels of [^{11}C]-**6** and [^{11}C]-**13** in LacZ gene (♦)- and VZV-tk gene (○)-transduced 293T cells. Data are expressed as % radioactivity in the cell fraction and are mean values of triplicate samples.

are shown in Figure 2. The phenylic tracer ([^{11}C]-**6**) had a poor cell uptake: the percentage of radioactivity in the cell fraction did not exceed 0.4% (Fig. 2, 120 min incubation time point). Compared to [^{11}C]-**6** and the previously synthesized galactosyl ethers[17] and galactosyl esters (submitted), the more lipophilic napthylic compound [^{11}C]-**13** had an increased uptake of radioactivity in the cell fraction (not normalized to protein content) and the accumulation in the cells increased as a function of the incubation time from 0.6% after 30 min of incubation to 1.4% after 120 min of incubation.

However, there was no significant difference in cell uptake between the LacZ expressing cells and the control cells (Fig. 2). This can be due to the decreased sensitivity of the inhibitor approach compared to the use of radiolabeled substrates. According to literature, 1-(β-D-galactopyranosyl)-4-phenyl-1,2,3-triazole, which has a similar structure as compound **6** but without the methoxy group, has an inhibition constant that is situated in the high micromolar range ($K_i \sim 330$ μM).[18] PET imaging of gene expression using radiolabeled inhibitors is comparable to the use of radiolabeled PET ligands for imaging receptor occupancy, in which the interaction between receptor and ligand also has a 1:1 stoichiometry. However, to have a good target to background signal, PET imaging of receptors requires ligands with an affinity in the nanomolar range. Using 'click' chemistry, libraries of different variants of galactosyl triazoles can be prepared that can be used to identify inhibitors with higher affinity. Furthermore to increase the signal to noise ratio it could be preferred to synthesize ^{18}F-labeled galactosyl triazoles that, because of their longer half-life compared to ^{11}C-labeled tracers, allow to perform imaging up to several hours post injection. By this time, the tracer will probably be better cleared from cells and tissue that do not express the LacZ gene, resulting in better in vitro and in vivo contrast.

3. Conclusion

An efficient and convenient chemical and radiochemical synthesis of two 4-substituted 1,2,3-triazolyl β-D-galactopyranosides was developed. The two precursors **4** and **11** and the two non-radioactive reference compounds **6** and **13** were synthesized in good yields using a Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between acetylated β-galactosyl azide and the corresponding terminal alkynes. Radiolabeling produced [^{11}C]-**6** and [^{11}C]-**13** in amounts and purity suitable for PET studies. Both tracers were very stable in vivo. Cell uptake experiments in LacZ expressing and control 293T cells, revealed an increased cell uptake for the naphthylic tracer [^{11}C]-**13** compared to the phenylic triazole [^{11}C]-**6**. However, no difference in uptake was observed between LacZ expressing cells and control cells, which is presumably due to the decreased sensitivity using radiolabeled inhibitors instead of substrates. Development of lipophilic ^{11}C- and ^{18}F-labeled β-galactosyl triazoles with a higher binding affinity for LacZ may lead to higher cell uptake ratios and better in vivo imaging contrasts.

4. Experimental

4.1. General

4-Ethynylphenylacetate was purchased from GFS chemicals (Columbus, Ohio, USA). 4-Ethynylanisole was purchased from Maybridge (Cornwall, UK). All other reagents and solvents were obtained commercially from Acros Organics (Geel, Belgium), Aldrich, Fluka, Sigma (Sigma–Aldrich, Bornem, Belgium), Merck (Darmstadt, Germany) or Fischer Bioblock Scientific (Tournai, Belgium) and used as supplied. For ascending thin layer chromatography (TLC), pre-coated aluminum backed plates (Silica Gel 60 with fluorescent indicator, 0.2 mm thickness; Macherey-Nagel, Düren, Germany) were used and developed using mixtures of ethyl acetate and heptane, ethyl acetate and methanol or acetonitrile and water as mobile phase. After evaporation of the solvent, compounds were detected under UV light (254 nm). The sugar compounds were additionally visualized by spraying with an oxidating solution (5% H_2SO_4 in ether), followed by heating. ^{1}H NMR spectra were recorded on a Bruker AVANCE 300 MHz spectrometer (Bruker AG, Faellanden, Zwitserland) using $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts are reported in parts per million relative to tetramethylsilane ($\delta = 0$). Coupling constants are reported in hertz. Splitting patterns are defined by s (singlet), d (doublet), dd (double doublet), t (triplet) or m (multiplet). High performance liquid chromatography (HPLC) purification and analysis was performed either on a Merck Hitachi L6200 intelligent pump (Hitachi, Tokyo, Japan) or on a Waters 600 pump (Waters Corporation, Milford, USA) connected to a UV-spectrometer (Waters 2487 Dual γ absorbance detector) set at 254 nm. The output signal was recorded and analyzed using a RaChel data acquisition system (Lablogic, Sheffield, UK). For analysis of radiolabeled compounds, after passing through the UV detector, the HPLC eluate was led over a 2-in. NaI(Tl) scintillation detector connected to a single channel analyzer (Medilab-Select, Mechelen, Belgium). The radioactivity measurements during log *P* determinations, biodistribution studies, in vivo stability analysis and cell uptake studies were done using an automated gamma counter equipped with a 3-in. NaI(Tl) well crystal coupled to a multichannel analyzer (Wallac 1480 Wizard, Wallac, Turku, Finland). The values were corrected for background radiation and physical decay during counting. Mass measurements were performed on a time-of-flight spectrometer (LCT, Micromass, Manchester, UK) equipped with an orthogonal electrospray ionization (ESI) interface. Samples were infused in acetonitrile/water mixtures with a Harvard 22 syringe pump (Harvard Apparatus, Holliston, Massachusetts, USA). Accurate mass determination was done by co-infusion with a 10 μg/mL solution of *o*-nitrophenyl β-D-galactopyranoside (ONPG) in acetonitrile/water as an internal calibration standard in positive mode (ES+) and a 10 μg/mL solution of *meso*-2,3-dibromosuccinic acid in water/acetonitrile as internal calibration standard in negative mode (ES−). For compound **4**, kryptofix 222 was used as internal calibration standard in positive mode. Acquisition and processing of the data was done with MASSLYNX™ software (version 3.5, Waters). Melting points (mp) were determined using an IA9000 digital melting point apparatus (Electrothermal, Southend-on-Sea, England).

The animal studies were performed according to the Belgian code of practice for the care and use of animals, after approval from the university ethics committee for animals.

4.2. Synthesis

4.2.1. 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide (1)

Compound **1** was prepared from 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose according to the method of Hanessian et al.[25] and was used as such for the synthesis of compound **2** without further purification.

4.2.2. 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl azide (2)

Compound **2** was prepared from 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**1**) according to the method of Jarrahpour et al.[26] Yield: 41% of white crystals.

^{1}H NMR ($CDCl_3$) δ 1.99, 2.07, 2.1, 2.17 (12H, 4 × s, 4 × *CH*$_3$*CO*), 4.03 (1H, t, *H*-5), 4.16–4.19 (2H, m, 2 × *H*-6), 4.62 (1H, d, $^3J_{H1-H2}$ = 8.64 Hz, *H*-1), 5.05 (1H, dd, $^3J_{H3-H2}$ = 10.35 Hz, $^3J_{H3-H4}$ = 3.33 Hz, *H*-3), 5.17 (1H, dd, $^3J_{H2-H3}$ = 10.35 Hz, $^3J_{H2-H1}$ = 8.64 Hz, *H*-2), 5.42 (1H, d, $^3J_{H4-H3}$ = 3.33 Hz, *H*-4).

Accurate mass (ESI-MS) for $C_{14}H_{19}N_3O_9$ [M+Na]$^+$: found 396.1015, calcd 396.1013.

4.2.3. 1-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-4-(*p*-acetoxyphenyl)-1,2,3-triazole (3)

An amount of azide (**2**) (1.0 g, 2.7 mmol), $CuSO_4$ (10 mg, 0.04 mmol), ascorbic acid (0.1 g, 0.6 mmol) and 4-ethynylphenylacetate (0.43 g, 2.7 mmol) was stirred in water (15 mL) at 70 °C overnight. The mixture was cooled in an ice bath, the solid was filtered off and subsequently washed with water (10 mL) and methanol (10 mL). Recrystallization from 95% ethanol yielded 1.05 g (1.97 mmol, 73%) of a pale yellow solid.

^{1}H NMR ($CDCl_3$) δ 1.91, 2.04, 2.07, 2.26, 2.33 (15H, 5 × s, 5 × *CH*$_3$*CO*), 4.15–4.30 (3H, m, *H*-5; 2 × *H*-6), 5.29 (1H, dd, $^3J_{H3-H2}$ = 9.75 Hz, $^3J_{H3-H4}$ = 3.24 Hz, *H*-3), 5.59 (1H, d, $^3J_{H4-H3}$ = 3.24 Hz, *H*-4), 5.64 (1H, t, $^3J_{H2-H3}$ = 9.75 Hz, $^3J_{H2-H1}$ = 9.75 Hz, *H*-2), 5.9 (1H, d, $^3J_{H1-H2}$ = 9.75 Hz, *H*-1), 7.19 (2H, d, ^3J = 8.3 Hz, H_{ar}), 7.89 (2H, d, ^3J = 8.3 Hz, H_{ar}), 8.04 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{24}H_{27}N_3O_{11}$ [M+Na]$^+$: found 556.1584, calcd 556.1538.

4.2.4. 1-(β-D-Galactopyranosyl)-4-(*p*-hydroxyphenyl)-1,2,3-triazole (4)

To a solution of compound **3** (0.14 g, 0.3 mmol) in methanol (2 mL) was added dry NaOMe (1.4 mg, 0.03 mmol) and the reaction mixture was stirred overnight at room temperature. Neutralization of the solution with Amberlite IR-120 (H^+) ion-exchange resin, followed by filtration and evaporation of the filtrate to dryness, afforded a pale yellow solid. This solid was purified by flash chromatography on silica gel (ethyl acetate/methanol 9:1 v/v) to afford 32 mg (0.09 mmol, 38%) of a white solid.

^{1}H NMR (DMSO-d_6) δ 3.4–3.6 (3H, m, *H*-5; 2 × *H*-6), 3.7–3.79 (2H, m, *H*-3; *H*-4), 4.08 (1H, t, *H*-2), 5.47 (1H, d, $^3J_{H1-H2}$ = 9.06 Hz, *H*-1), 6.81 (2H, d, ^3J = 8.31 Hz, H_{ar}), 7.68 (2H, d, ^3J = 8.31 Hz, H_{ar}), 8.52 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{14}H_{17}N_3O_6$ [M+Na]$^+$: found 346.1027, calcd 346.1010. Mp 207–208 °C.

4.2.5. 1-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-4-(*p*-methoxyphenyl)-1,2,3-triazole (5)

The title compound was prepared from acetylated β-D-galactopyranosyl azide (**2**) and 4-ethynylanisole according to the method described for the synthesis of compound **3**. Yield: 0.57 g (1.1 mmol, 42%) of a fluffy, white solid.

^{1}H NMR ($CDCl_3$) δ 1.90, 2.02, 2.05, 2.25 (12H, 4 × s, 4 × *CH*$_3$*CO*), 3.85 (3H, s, *CH*$_3$*O*), 4.15–4.29 (3H, m, *H*-5; 2 × *H*-6), 5.28 (1H, dd, $^3J_{H3-H2}$ = 9.9 Hz, $^3J_{H3-H4}$ = 3.27 Hz, *H*-3), 5.57 (1H, d, $^3J_{H4-H3}$ = 3.27 Hz, *H*-4), 5.64 (1H, t, $^3J_{H2-H3}$ = 9.9 Hz, $^3J_{H2-H1}$ = 9.9 Hz, *H*-2), 5.9 (1H, d, $^3J_{H1-H2}$ = 9.9 Hz, *H*-1), 6.97 (2H, d, ^3J = 8.64 Hz, H_{ar}), 7.78 (2H, d, ^3J = 8.64 Hz, H_{ar}), 7.97 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{23}H_{27}N_3O_{10}$ [M+Na]$^+$: found 528.1608, calcd 528.1589.

4.2.6. 1-(β-D-Galactopyranosyl)-4-(*p*-methoxyphenyl)-1,2,3-triazole (6)

To a stirring solution of compound **5** (0.13 g, 0.3 mmol) in methanol (2 mL) was added dry NaOMe (1.4 mg, 0.03 mmol). After 30 min, colorless crystals were formed. After stirring overnight at room temperature, the crystals were filtered off, washed with cold methanol and dried. Yield: 70 mg (0.21 mmol, 80%).

^{1}H NMR (DMSO-d_6) δ 3.52–3.58 (3H, m, *H*-5; 2 × *H*-6), 3.73–3.77 (2H, m, *H*-3; *H*-4), 3.79 (3H, s, CH_3O), 4.07–4.11 (1H, m, *H*-2), 4.71 (t, *OH*), 4.74 (d, *OH*), 5.08 (d, *OH*), 5.28 (d, *OH*), 5.49 (1H, d, $^3J_{H1-H2}$ = 9.15 Hz, *H*-1), 7.02 (2H, d, ^3J = 8.8 Hz, H_{ar}), 7.82 (2H, d, ^3J = 8.8 Hz, H_{ar}), 8.64 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{15}H_{19}N_3O_6$ [M+H]$^+$: found 338.1329, calcd 338.1347. Mp 240–241 °C.

4.2.7. 6-Bromo-2-*tert*-butyldimethylsilyloxy-naphthalene (7)

The title compound was synthesized according to the method described in patent WO 2007/028104[27] with minor adaptations. To a solution of 6-bromo-2-naphthol (10 g, 44.6 mmol) in anhydrous DMF (75 mL) was added, under nitrogen atmosphere, imidazole (4.24 g, 62.4 mmol) followed by *tert*-butyldimethylsilylchloride (TBSCl, 9.36 g, 62.4 mmol). After stirring at room temperature for 18 h, the reaction mixture was poured into ice water (300 mL) followed by an extraction with diethylether (100 mL, 4×). The combined organic extracts were washed with brine (100 mL), dried over $MgSO_4$ and the solvent was removed under vacuo. Yield: 12.25 g (36.3 mmol, 81.3%) of colorless crystals.

^{1}H NMR ($CDCl_3$) δ 0.24 (6H, s, $OSi(CH_3)_2$), 1.01 (9H, s, $C(CH_3)_3$), 7.09 (1H, d, ^3J = 8.82 Hz, H_{ar}), 7.15 (1H, s, H_{ar}), 7.47 (1H, d, ^3J = 8.76 Hz, H_{ar}), 7.55 (1H, d, ^3J = 8.76 Hz, H_{ar}), 7.62 (1H, d, ^3J = 8.82 Hz, H_{ar}), 7.91 (1H, s, H_{ar}).

4.2.8. 6-Trimethylsilyl ethynyl-2-*tert*-butyldimethylsilyloxy-naphthalene (8)

Compound **8** was prepared according to the method of Carpita et al.[28] with minor adaptations. To a mixture of bis(benzonitrile)-palladium(II)chloride ($PdCl_2(PhCN)_2$, 0.16 g, 0.4 mmol) and CuI (54 mg, 0.29 mmol) in 1,4-dioxane (5 mL) was added a solution of compound **7** (4.82 g, 14.3 mmol) and tri(*tert*-butyl)phosphine (214 μL, 0.86 mmol) in 1,4-dioxane (5 mL), followed by the addition of trimethylsilylacetylene (2.35 mL, 17.1 mmol) and diisopropylamine (2.4 mL, 17.1 mmol). The resulting mixture was stirred overnight at room temperature under nitrogen. The black reaction mixture was diluted with ethyl acetate (100 mL), filtered over Celite and the filtrate was concentrated under reduced pressure to give a red oil. This oil was purified by flash column chromatography on silica gel with *n*-heptane as eluent. Yield: 1.2 g (3.4 mmol, 23.7%) of orange crystals.

^{1}H NMR ($CDCl_3$) δ 0.25 (6H, s, $OSi(CH_3)_2$), 0.27 (9H, s, $CSi(CH_3)_3$), 1.01 (9H, s, $C(CH_3)_3$), 7.06 (1H, dd, ^3J = 8.79 Hz, ^4J = 2.4 Hz, H_{ar}), 7.13 (1H, d, ^4J = 2.4 Hz, H_{ar}), 7.43 (1H, d, ^3J = 8.79 Hz, H_{ar}), 7.62 (1H, d, ^3J = 8.97 Hz, H_{ar}), 7.66 (1H, d, ^3J = 8.97 Hz, H_{ar}), 7.91 (1H, s, H_{ar}).

4.2.9. 6-Ethynyl-2-naphthol (9)

The title compound was synthesized according to the method of Pirali et al.[29] with minor adaptations. A volume of tetrabutylammonium fluoride solution (TBAF, 1 M in THF, 2.35 mL) was added slowly to a solution of compound **8** (0.71 g, 2 mmol) in THF (5 mL) at 0 °C and was further stirred at this temperature for 30 min. Next, a saturated solution of NH_4Cl (15 mL) was added and the mixture was extracted with ethyl acetate (15 mL, 3×). The combined organic fractions were washed with water (15 mL) and brine (15 mL) and subsequently dried with $MgSO_4$. An amount of silica gel was added and the solvent was evaporated. The silica gel impregnated with the crude product was applied on a silica gel column that was eluted with *n*-heptane. Yield: 0.33 g (1.96 mmol, 98%) of a brown solid.

^{1}H NMR ($CDCl_3$) δ 3.1 (1H, s, $C{\equiv}CH$), 7.1–7.13 (2H, m, H_{ar}), 7.45 (1H, d, ^3J = 8.52 Hz, H_{ar}), 7.58 (1H, d, ^3J = 9.57 Hz, H_{ar}), 7.69 (1H, d, ^3J = 9.57 Hz, H_{ar}), 7.94 (1H, s, H_{ar}).

Accurate mass (ESI-MS) for $C_{12}H_8O$ [M−H]$^-$: found 167.0495, calcd 167.0502.

4.2.10. 1-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-4-(6-hydroxynaphthyl)-1,2,3-triazole (10)

The title compound was prepared from acetylated β-D-galactopyranosyl azide (**2**) and 6-ethynyl-2-naphthol (**9**) according to the method described for the synthesis of compound **3**. Yield: 0.22 g (0.4 mmol, 19%) of a yellow solid.

^{1}H NMR ($CDCl_3$) δ 1.9, 2.04, 2.06, 2.27 (12H, 4 × s, 4 × CH_3CO), 4.21–4.27 (3H, m, *H*-5; 2 × *H*-6), 5.3 (1H, dd, $^3J_{H3-H2}$ = 10.23 Hz, $^3J_{H3-H4}$ = 3.33 Hz, *H*-3), 5.59 (1H, d, $^3J_{H4-H3}$ = 3.33 Hz, *H*-4), 5.67 (1H, t, $^3J_{H2-H3}$ = 9.45 Hz, $^3J_{H2-H1}$ = 9.45 Hz, *H*-2), 5.93 (1H, d, $^3J_{H1-H2}$ = 9.45 Hz, *H*-1), 6.24 (s, *OH*), 7.1–7.14 (2H, m, H_{ar}), 7.65 (1H, d, ^3J = 8.64 Hz, H_{ar}), 7.7 (1H, d, ^3J = 8.64 Hz, H_{ar}), 7.82 (1H, d, ^3J = 8.52 Hz, H_{ar}), 8.14 (1H, s, H_{ar}), 8.23 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{26}H_{26}N_3O_{10}Na$ [M+Na]$^+$: found 564.1664, calcd 564.1589.

4.2.11. 1-(β-D-Galactopyranosyl)-4-(6-hydroxynaphthyl)-1,2,3-triazole (11)

The title compound was prepared by deprotection of compound **10** according to the method described for the synthesis of compound **4**. Yield: 0.03 g (0.08 mmol, 31%) of a pale yellow solid.

^{1}H NMR (DMSO-d_6) δ 3.52–3.6 (3H, m, *H*-5; 2 × *H*-6), 3.74–3.81 (2H, m, *H*-3; *H*-4), 4.12 (1H, m, *H*-2), 5.52 (1H, d, $^3J_{H1-H2}$ = 9.12 Hz, *H*-1), 7.08–7.11 (2H, m, H_{ar}), 7.71 (1H, d, ^3J = 8.64 Hz, H_{ar}), 7.78 (1H, d, ^3J = 8.64 Hz, H_{ar}), 7.89 (1H, d, ^3J = 8.52 Hz, H_{ar}), 8.3 (1H, s, H_{ar}), 8.77 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{18}H_{19}N_3O_6$ [M+Na]$^+$: found 396.1156, calcd 396.1166. Mp 216–217 °C.

4.2.12. 1-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-4-(6-methoxynaphthyl)-1,2,3-triazole (12)

The title compound was prepared from acetylated β-D-galactopyranosyl azide (**2**) and 2-ethynyl-6-methoxy-naphthalene according to the method described for the synthesis of compound **3**. Yield: 0.6 g (1.1 mmol, 41%) of a white solid.

^{1}H NMR ($CDCl_3$) δ 2.03, 2.04, 2.05, 2.26 (12H, 4 × s, 4 × CH_3CO), 3.94 (3H, s, CH_3O), 4.19–4.27 (3H, m, *H*-5; 2 × *H*-6), 5.3 (1H, dd, $^3J_{H3-H2}$ = 10.2 Hz, $^3J_{H3-H4}$ = 3.21 Hz, *H*-3), 5.58 (1H, d, $^3J_{H4-H3}$ = 3.21 Hz, *H*-4), 5.68 (1H, t, $^3J_{H2-H3}$ = 9.6 Hz, $^3J_{H2-H1}$ = 9.6 Hz, *H*-2), 5.92 (1H, d, $^3J_{H1-H2}$ = 9.6 Hz, *H*-1), 7.15–7.19 (2H, m, H_{ar}), 7.79 (2H, d, ^3J = 9.24 Hz, H_{ar}), 7.91 (1H, d, ^3J = 7.5 Hz, H_{ar}), 8.13 (1H, s, H_{ar}), 8.3 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{14}H_{19}N_3O_9$ [M+Na]$^+$: found 578.1834, calcd 578.1745.

4.2.13. 1-(β-D-Galactopyranosyl)-4-(6-methoxynaphthyl)-1,2,3-triazole (13)

The title compound was prepared by deprotection of compound **12** according to the method described for the synthesis of compound **6**. Yield: 0.31 g (0.8 mmol, 80%) of a fluffy, white solid.

^{1}H NMR (DMSO-d_6) δ 3.54–3.63 (3H, m, *H*-5; 2 × *H*-6), 3.77–3.82 (2H, m, *H*-3; *H*-4), 3.89 (3H, s, CH_3O), 4.1–4.18 (1H, m, *H*-2), 4.72–4.76 (m, 2 × *OH*), 5.09 (d, *OH*), 5.32 (d, *OH*), 5.56 (1H, d, $^3J_{H1-H2}$ = 9.12 Hz, *H*-1), 7.2 (1H, d, ^3J = 8.88 Hz, H_{ar}), 7.34 (1H, s, H_{ar}), 7.88 (2H, d, ^3J = 8.61 Hz, H_{ar}), 8.01 (1H, d, ^3J = 8.52 Hz, H_{ar}), 8.4 (1H, s, H_{ar}), 8.83 (1H, s, *H*-5 *triazole*).

Accurate mass (ESI-MS) for $C_{19}H_{21}N_3O_6$ [M+Na]$^+$: found 410.1363, calcd 410.1323. Mp 272–274 °C.

4.3. Radiochemistry

4.3.1. Production of [^{11}C]methyl iodide ([^{11}C]MeI) and radiosynthesis of [^{11}C]-6

Carbon-11 was produced by a $^{14}N(p,\alpha)^{11}C$ nuclear reaction. The target gas (a mixture of 95% N_2 and 5% H_2) was irradiated using 18-MeV protons at a beam current of 25 μA for about 30 min, to yield [^{11}C]CH_4. The [^{11}C]CH_4 was then reacted with vaporous I_2 at 650 °C in a home-built recirculation synthesis module to convert it to [^{11}C]MeI. The resulting volatile [^{11}C]MeI was bubbled with a flow of helium through a solution of 0.8 mg precursor (**4**) in 0.3 mL DMF containing 5 μL NaOH 1 M. When the radioactivity in the vial had stabilized, the reaction mixture was heated at 70 °C for 3 min. Subsequently, unreacted [^{11}C]MeI was evaporated by heating the mixture at 70 °C for 2 min with a flow of helium. After cooling, the resulting mixture was diluted with 1.2 mL water and purified by semi-preparative HPLC on an XTerra™ RP C_{18} column (5 μm, 7.8 mm x 150 mm; Waters), eluted with 0.1 M ammonium acetate buffer pH 7.1/ethanol (85:15 v/v) at a flow rate of 2 mL/min. The radiolabeled compound [^{11}C]-**6** eluted after 14 min with a radiochemical yield of approximately 51% (relative to starting activity of [^{11}C]MeI). Radiochemical purity and specific activity were assayed using HPLC on an analytical XTerra™ RP C_{18} column (5 μm, 4.6 mm × 250 mm; Waters) eluted with gradient mixtures of 0.1 M ammonium acetate buffer pH 7.1/acetonitrile (0 min: 90:10 v/v, 20 min: 30:70 v/v, linear gradient) at a flow rate of 1 mL/min. [^{11}C]-**6** eluted after 9.5 min and was obtained with an average specific activity of 118 GBq/μmol at the end of synthesis (EOS) and a radiochemical purity of >99%.

4.3.2. Radiosynthesis of [^{11}C]-13 using [^{11}C]MeI

Radiosynthesis of [^{11}C]-**13** was performed following the same procedure as described for [^{11}C]-**6** with compound **11** as precursor. Purification of the crude radiolabeling mixture was done by semi-preparative HPLC on an XTerra™ RP C_{18} column (5 μm, 7.8 mm × 150 mm; Waters), eluted with 0.1 M ammonium acetate buffer pH 7.1/ethanol (70:30 v/v) at a flow rate of 2 mL/min. The radiolabeled compound [^{11}C]-**13** eluted after 16 min with a radiochemical yield of approximately 48% (relative to starting activity of [^{11}C]MeI). The purified tracer was further analyzed by HPLC on an analytical XTerra™ RP C_{18} column (Waters) eluted with gradient mixtures of 0.1 M ammonium acetate buffer pH 7.1/acetonitrile (0 min: 90:10 v/v, 20 min: 30:70 v/v, linear gradient) at a flow rate of 1 mL/min. [^{11}C]-**13** eluted after 13.5 min and was obtained with an average specific activity of 61 GBq/μmol at the end of synthesis (EOS) and a radiochemical purity of >99%.

4.4. In vitro incubation experiments with purified β-gal

4.4.1. Incubation of non-radioactive compound 6 with β-gal

An amount of 1 mg of compound **6** was dissolved in 1 mL of a solution containing HEPES buffer pH 7.0 (120 mM), $MgSO_4$ (4 mM) and NaCl (180 mM). A volume of 67 μL of a 1 mg/mL HEPES buffered solution of β-gal (*E. coli*, E.C. 3.2.1.23, 149 U/mg, Fluka) was added (=10 U) and the solution was incubated at 37 °C (n = 1). After 60 min of incubation, the mixture was analyzed for the presence of metabolites using RP-HPLC coupled to UV detection using an XTerra™ RP C_{18} column (5 μm, 4.6 mm × 250 mm; Waters) eluted with a mixture of 30% acetonitrile in 0.1 M ammonium acetate buffer pH 7.1 at a flow rate of 1 mL/min. After 20 min the percentage acetonitrile was increased to 70% in order to elute possible lipophilic hydrolysis products. The same HPLC analysis was performed after incubation overnight. Next, the experiment was repeated, but 1 mg ONPG in 1 mL HEPES buffered solution was added to the incubation mixture of compound **6** with 10 U β-gal at 37 °C. 15 min and 90 min after the addition of ONPG, a 10-μL sample was collected and analyzed for the presence of *o*-nitrophenol (hydrolysis product of ONPG) using the same RP-HPLC method.

4.4.2. Incubation of [^{11}C]-6 with β-gal

An aliquot of 0.2 mL HPLC purified [^{11}C]-**6** (10 MBq) was added to 0.4 mL water and 0.2 mL of a HEPES buffered solution. A volume of 67 μL of a 1 mg/mL HEPES buffered solution of β-gal (*E. coli*, E.C. 3.2.1.23, 149 U/mg, Fluka) was added (=10 U) and the solution was incubated for 60 min at 37 °C (n = 1). A 10-μL sample was collected and analyzed using the same RP-HPLC method (coupled to radiometric detection) as described for the incubation experiment with non-radioactive triazole.

4.5. Partition coefficient (log $P_{n\text{-octanol/phosphate buffer pH 7.4}}$)

An aliquot of 30 μL of RP-HPLC purified tracer solution containing approximately 555 kBq of [^{11}C]-**6** was added to a tube containing 2 mL 0.025 M sodium phosphate buffer pH 7.4 and 2 mL *n*-octanol (density = 0.827 g/mL). The tube was vortexed at room temperature for 2 min followed by centrifugation at 3000 rpm (1837 g) for 5 min (Eppendorf centrifuge 5810, Eppendorf, Westbury, USA). Aliquots of 900 μL and 100 μL were drawn from the *n*-octanol and aqueous phases, respectively, taking care to avoid cross-contamination between the two phases. The samples were weighed and their radioactivity was counted using an automated gamma counter. After correcting for density and mass difference between the two phases, the partition coefficient (P) was calculated as [radioactivity (cpm/mL) in *n*-octanol]/[radioactivity (cpm/mL) in phosphate buffer pH 7.4]. The experiments were performed at least in triplicate.

The same procedure was followed for [^{11}C]-**13** except that the volumes were switched: 100 μL was taken from the octanol layer and 900 μL was taken from the buffer layer.

4.6. Biodistribution study in normal mice

Biodistribution of both tracers was studied in male NMRI mice (body mass 35–45 g). These mice were anesthetized with 2.5% isoflurane in 100% O_2 at a flow rate of 1 L/min. Solutions of [^{11}C]-**6** and [^{11}C]-**13** obtained after RP-HPLC purification were diluted using 0.9% NaCl for injection to obtain an ethanol concentration < 10%, and further to a concentration of about 37 MBq/mL. A volume of 0.1 mL of the diluted purified tracer solution was then injected via a tail vein in the anesthetized mice and the mice were sacrificed by decapitation at 2 or 60 min post injection (p.i., n = 4 per time point). Blood and major organs were collected in tared tubes and weighed. The radioactivity in blood, organs and other body parts was counted using a 3-in. NaI(Tl) well counter, corrected for background radioactivity and expressed as percentage of the injected dose (% ID) or as percentage of the injected dose per gram tissue (% ID/g). For the calculation of total radioactivity in blood, blood mass was assumed to be 7% of the body mass.

4.7. Biostability in normal mice

Metabolic stability of [^{11}C]-**6** and [^{11}C]-**13** was studied in NMRI mice by determination of the relative amounts of the parent tracer and radiolabeled metabolites in plasma and in urine. After intravenous administration of about 18 MBq of [^{11}C]-**6** or [^{11}C]-**13** into anesthetized mice (isoflurane), the animals were sacrificed by decapitation at 10 or 30 min p.i. (n = 2), blood was collected into a BD vacutainer™ (containing lithium heparin; BD, Franklin Lakes, USA) and stored on ice. The samples were then centrifuged at 3000 rpm (1837*g*) for 5 min (Eppendorf centrifuge 5810) to separate plasma. A volume of 0.5 mL of plasma sample was isolated,

mixed with 15 μL of a 1 mg/mL solution of authentic non-radioactive **6** or **13** and injected onto a Chromolith Performance RP C_{18} column (3 mm × 100 mm; Merck), that was eluted with gradient mixtures of 0.05 M ammonium acetate buffer pH 6.8/acetonitrile (0 min: 100:0 v/v, 4 min: 100:0 v/v, 20 min: 30:70 v/v, 30 min: 10:90 v/v, linear gradient for [^{11}C]-**6** and 0 min: 100:0 v/v, 4 min: 100:0 v/v, 25 min: 10:90 v/v, 35 min: 10:90 v/v, linear gradient for [^{11}C]-**13**) at a flow rate of 1 mL/min. After passing through an in-line UV detector, the HPLC-eluate was collected. The first 4-min eluate, containing proteins of the biological matrix, was collected in one fraction. The rest of the eluate was collected in 0.33-mL fractions (fraction collection each 20 s) for [^{11}C]-**6** and in 1-mL fractions for [^{11}C]-**13** using an automatic fraction collector. The radioactivity in all fractions was measured using a gamma counter. Urine samples were collected from mice sacrificed at 30 min p.i. by manual voiding of the bladder, mixed with authentic **6** and analyzed following the same procedure without any pre-treatment or work-up. The whole eluate was collected in 0.33-mL fractions and counted for radioactivity.

4.8. In vitro evaluation of the tracers

A lentiviral vector (LV) encoding the cDNA of β-gal linked to the puromycin-*N*-acetyl-transferase (*pac*) gene from *Streptomyces alboniger* through an encephalomyocarditis virus internal ribosome entry site (IRES) was produced as described[38] and denominated LV-LacZ-I-P. A similar vector encoding the varicella zoster virus thymidine kinase (VZV-tk) was used as control (LV-VZV-tk-I-P).

Human embryonic kidney cells (293T) transduced with LV-LacZ-I-P and with LV-VZV-tk-I-P were maintained in culture in medium (Dulbecco's modified Eagle's medium (DMEM) with Glutamax supplemented with 10% heat-inactivated fetal calf serum) containing 1 μg/mL puromycin. They were plated in triplicate at a density of 200,000 cells per well in 24-well plates. After 24 h, the medium was discarded and 0.25 mL of fresh culture medium with HPLC-purified tracer (1.1 MBq per well) was added. The cells were then incubated at 37 °C in a 5% CO_2 atmosphere for time intervals of 30, 60, 90 or 120 min (n = 3 per time point, per vector). Following incubation and removal of the medium, the cells were washed three times with 0.4 mL of ice-cold phosphate-buffered saline (PBS). The cells were then lysed with 0.25 mL of Cell Culture Lysis Reagent 1× solution (Promega Corporation, Madison, USA) for 10 min after which the lysate was collected, followed by a 0.125-mL rinse using the same solution. The cell fractions (lysate and rinse) as well as the supernatant fractions (medium and PBS) were collected separately for each well and the radioactivity was measured using a gamma counter. This procedure was repeated for each incubation period. Normalization for protein content was not performed.

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