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Synthesis and evaluation of two uncharged ^{99m}Tc-labeled derivatives of thioflavin-T as potential tracer agents for fibrillar brain amyloid

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Thioflavin-T is a fluorescent dye for *in vitro* detection of fibrillar amyloid β, a protein found in the brain of patients suffering from Alzheimer's disease. We synthesized and biologically evaluated two uncharged ^{99m}Tc-labeled derivatives of thioflavin-T. The precursors for labeling were synthesized by coupling an *S,S*′-bis-triphenylmethyl-*N*-*tert*-butoxycarbonyl bis-amino-bis-thiol tetradentate ligand via a propoxy spacer to 2-(4′-aminophenyl)-1,3-benzothiazole at the 6-position or the 2′-position. Deprotection and labeling with ^{99m}Tc were done via a one-pot procedure (15% yield) after which the labeled compound was isolated by high performance liquid chromatography (LC). LC in combination with mass spectrometry (MS) was used for identity confirmation of the labeled compounds. Results of electrophoresis and log *P* determination supported the assumption that the radiolabeled compounds could cross the blood–brain barrier by passive diffusion. However, in normal mice both compounds showed a low brain uptake 2 min post injection. They were mainly excreted through the hepatobiliary system, with some accumulation in the stomach. Sixty minutes after intravenous injection, 37% of the ^{99m}Tc-activity in the blood corresponded to the original compound. In view of the low brain uptake, it is concluded that the studied ^{99m}Tc-labeled derivatives of thioflavin-T are not suitable as tracer agents for *in vivo* visualization of amyloid in brain.

Keywords: amyloid; thioflavin-T; technetium-99m; SPECT; 2-phenylbenzothiazoles

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, which typically strikes the older age groups. As life expectancy of the population increases, an increasing number of people suffer from AD, placing a heavy burden on society for the health care of these patients. Nowadays, the diagnosis of AD is done by clinical assessment of the symptoms such as memory loss and by exclusion of systemic diseases and other brain diseases.[1] However, this kind of diagnosis is only a 'probable' diagnosis. Definite diagnosis is done by (immuno)histochemical staining of *post mortem* brain tissue.[2] Histochemical dyes, such as silver (Bielschowsky technique), Congo red, chrysamine G, thioflavin-S or thioflavin-T (ThT), bind specifically to fibrillar amyloid β (Aβ), a protein that is found in senile brain plaques in persons suffering from AD. Fibrillar Aβ may be a useful biomarker in the differential diagnosis of AD from other dementias and in the follow-up of future therapies directed against the Aβ deposition in the brain.

Several uncharged radiolabeled phenylbenzothiazoles based on the structure of thioflavin-T have already been reported, including the iodine-125 labeled compounds ^{125}I-TZDM[3] and 2-(3′-[^{125}I]iodo-4′-aminophenyl)-6-hydroxybenzothiazole[4] and the carbon-11 labeled BAT-derivatives.[5] Especially 6-OH-BTA-1, also known as Pittsburgh Compound-B or PIB, seems to be promising, as human studies in AD patients and healthy controls show that there is a marked retention of tracer agent in the brain regions rich in fibrillar Aβ in AD patients as compared to the control subjects.[6]

In view of the promising results obtained with radiolabeled phenylbenzothiazoles and the nearly optimal characteristics of technetium-99m as radionuclide for nuclear imaging purposes, several neutral ^{99m}Tc-labeled compounds were already developed and evaluated for their biological characteristics. Our group first investigated a ^{99m}Tc-labeled MAMA-derivative of chrysamine G.[7] However, brain uptake of this radiolabeled agent was minimal, probably because of its large size and ionized character at physiological pH. The group of Kung studied biphenyl derivatives containing N_2S_2 (BAT)-chelating groups.[8] One of these neutral and lipophilic ^{99m}Tc-labeled complexes showed good blood–brain barrier (BBB) penetration (1.18% ID

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2 min p.i.) with a reasonable brain wash-out in normal mice (Figure 1(A)). This complex, however, labeled amyloid deposits in transgenic APP mouse brain sections, but not in human AD brain sections. Our group also developed a ^{99m}Tc-labeled BAT-conjugate of 2-(4′-aminophenyl)-1,3-benzothiazole that binds *in vitro* to amyloid β (Figure 1(B)).[9] Despite its high lipophilicity and absence of charge, this radiolabeled conjugate did not cross the BBB in a sufficient way (0.1% ID 2 min p.i.) and thus is not useful for *in vivo* detection of AD. Recently, the group of Chen reported a ^{99m}Tc-MAMA-BTA complex in which the chelator MAMA was conjugated with 2-(4′-aminophenyl)-1,3-benzothiazole through a 5-carbon alkyl chain (Figure 1(C)).[10] They demonstrated that this ^{99m}Tc-MAMA-BTA complex, which showed brain uptake in normal mice (1.34% ID/g), binds to Aβ aggregates in brain sections of both transgenic APP mouse and an AD patient.

Here, we report the synthesis of two uncharged derivatives of 2-(4′-aminophenyl)-1,3-benzothiazole coupled via a linker at position 6 or 2′ to *S*,*S*′-bis-trityl-*N*-BOC protected BAT ligand, together with their deprotection, labeling with ^{99m}Tc, subsequent purification and identification (Figure 2). The log *P* value of the compounds was determined and a biodistribution and stability study in normal mice was performed.

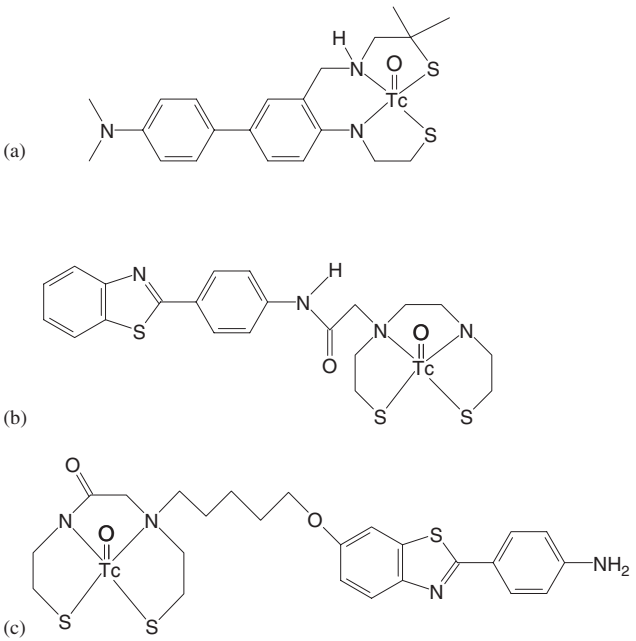


Figure 1. Structures of reported potential ^{99m}Tc-labelled tracer agents for imaging of fibrillar amyloid β.

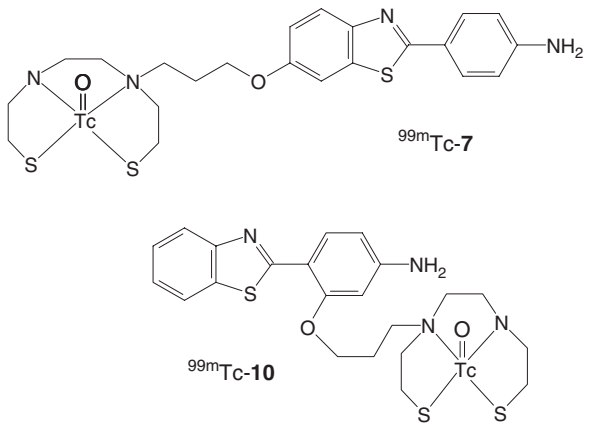
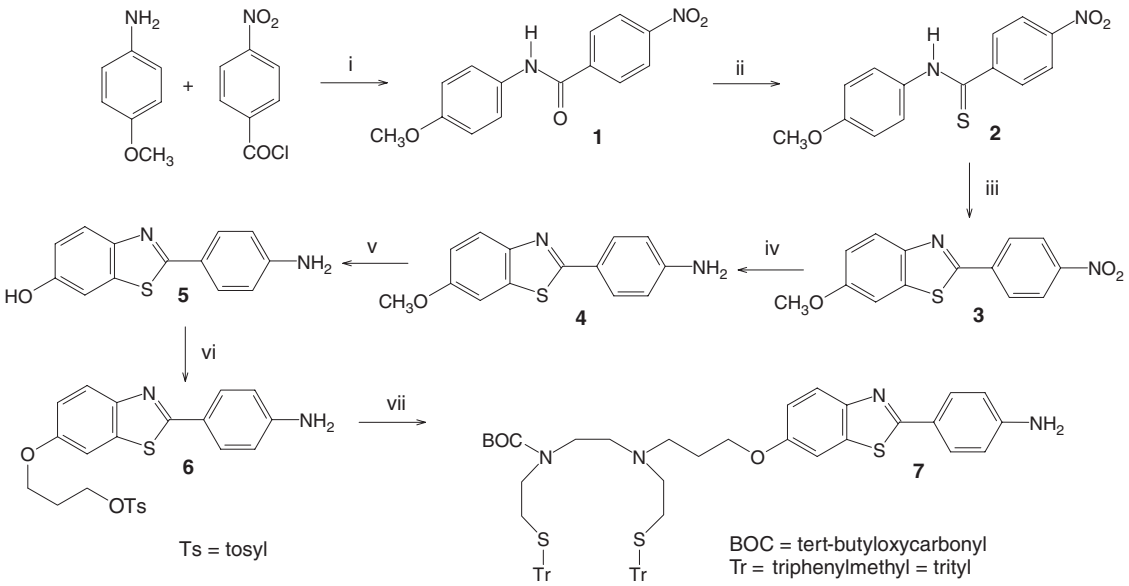


Figure 2. Structures of ^{99m}Tc-labelled tracer agents **7** and **10**.

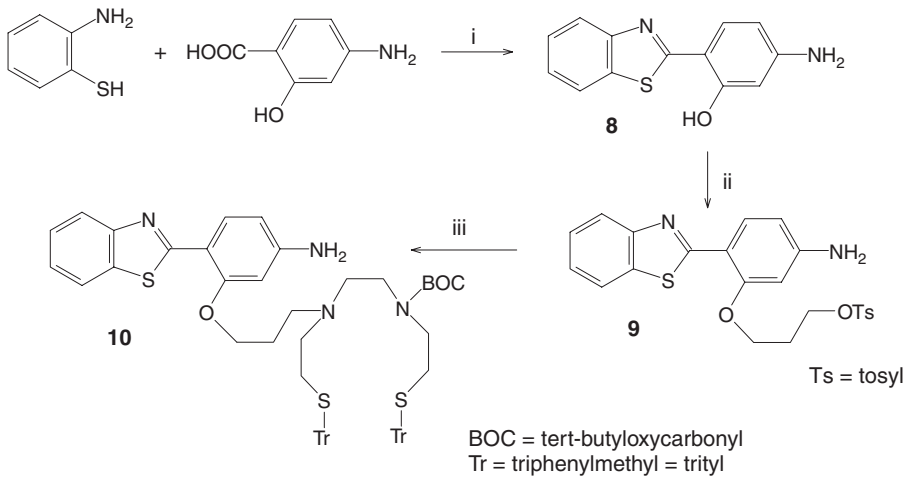
Results and discussion

Chemistry

As 2-(4′-aminophenyl)-1,3-benzothiazoles do not have the potential to chelate technetium-99m, we conjugated them with an *S*,*S*′-bis-trityl-*N*-BOC protected bis-amino-bis-thiol (BAT) tetradentate ligand via a propoxy linker. Depending on the intended position of derivatization of the phenylbenzothiazole (6-position or 2′-position), two different synthetic pathways have been followed. The preparation of 2-(4′-aminophenyl)-6-hydroxy-1,3-benzothiazole was based on the method described by Shi *et al.*,[11] but some modifications were introduced in several steps of the synthesis to improve the yields or the purity of the reaction products. A schematic representation of the reaction pathway to obtain the conjugation of the phenylbenzothiazole at position 6 with the protected BAT ligand is shown in Scheme 1. In the first step, *p*-anisidine and 4-nitrobenzoyl chloride were reacted in boiling pyridine to yield *N*-4′-methoxyphenyl-4-nitrobenzamide (**1**). According to literature, **1** should then be reacted with Lawesson's reagent in hexamethylphosphoramide, but we were not successful in producing *N*-4′-methoxyphenyl-4-nitrothiobenzamide (**2**) following this method. Changing the solvent to 1,4-dioxane and refluxing the reaction for 3 h resulted in a yield of 93% of **2** after recrystallization from methanol. Cyclization by Jacobson's method with the oxidizing agent potassium ferricyanide in alkaline solution produced 2-(4′-nitrophenyl)-6-methoxy-1,3-benzothiazole (**3**) in a yield of 70%. To convert **3** into 2-(4′-aminophenyl)-6-hydroxy-1,3-benzothiazole (**5**), the nitro group was first reduced to an amine using stannous chloride in ethanol and the methoxy group was then demethylated to a hydroxyl group using boron tribromide. Performing the reduction and demethylation reactions in the reverse order yielded a reaction mixture from which it was difficult to isolate **5**. For the preparation of 2-(4′-aminophenyl)-6-(3′′-*p*-tosyloxypropoxy)-1,3-benzothiazole (**6**), 1,3-propanediol di-*p*-tosylate was reacted with **5** in the presence of sodium methanolate. A two-fold excess of 1,3-propanediol di-*p*-tosylate was used to avoid reaction at both tosyl groups and this was further inhibited by adding **5** slowly to the solution of 1,3-propanediol di-*p*-tosylate. Final coupling of the *S*,*S*′-bis-trityl-*N*-BOC protected BAT to **6** was done in DMF in the presence of diisopropylethylamine as a base to sufficiently deprotonate the unprotected amine of the BAT precursor. A reaction time of 24 h and a temperature of 50°C were necessary to obtain 2-(4′-aminophenyl)-6-{3′′-[*N*-(*N*′-triphenylmethylmercaptoethyl-*N*′-*tert*-butoxycarbonyl)-aminoethyl-*N*-triphenylmethylmercaptoethyl]aminopropoxy}-1,3-benzothiazole (**7**) in a sufficient yield. Rising the temperature to 70°C had a negative influence on the yield, as qualitatively and quantitatively more impurities were formed. Several purification techniques, including silica gel column chromatography and preparative TLC were tried, but none of them allowed to separate the desired products from their impurities. Finally, purification was done with normal phase high performance liquid chromatography (HPLC) on a semi-preparative column. However, we were not able to completely eliminate in this way the *S*,*S*′-bis-trityl-*N*-BOC protected BAT ligand from the desired conjugate. Reversed



Scheme 1. Synthetic pathway to 6-{3′′-[*N*-(*N*′-triphenylmethylmercaptoethyl-*N*′-*tert*-butoxycarbonyl)aminoethyl-*N*-triphenylmethylmercaptoethyl]aminopropoxy}-2-(4′-aminophenyl)-1,3-benzothiazole (**7**). Reagents and conditions: (i) pyridine, reflux; (ii) Lawesson's reagent, 1,4-dioxane, N_2, reflux; (iii) $K_3Fe(CN)_6$, EtOH, 90°C; (iv) $SnCl_2 \cdot 2H_2O$, EtOH, reflux; (v) BBr_3, CH_2Cl_2; (vi) 1,3-propanediol di-*p*-tosylate, NaOMe, MeCN/MeOH 9:1 V/V; (vii) *S*,*S*′-bis-trityl-*N*-BOC-BAT, DIEA, DMF, 50°C.



Scheme 2. Synthetic pathway to 2-{2′-[3′′-(*N*-(*N*′-triphenylmethylmercaptoethyl-*N*′-*tert*-butoxycarbonyl)aminoethyl-*N*-triphenylmethylmercaptoethyl)aminopropoxy]-4′-aminophenyl}-1,3-benzothiazole (**10**). Reagents and conditions: (i) polyphosphoric acid, 180°C; (ii) 1,3-propanediol di-*p*-tosylate, NaOMe, MeCN/MeOH 9:1 V/V; (iii) *S*,*S*′-bis-trityl-*N*-BOC-BAT, DIEA, DMF, 50°C.

phase-HPLC (RP-HPLC) showed that still 1.5% of this reagent was present in the final product. Evidently, this *S*,*S*′-bis-trityl-*N*-BOC protected BAT interferes with the radiolabeling, as it is also capable to form a complex with Tc after deprotection.

Introduction of the Tc-binding side chain on the 2′-position of the aminophenyl part was done by reaction of 1,3-propanediol di-*p*-tosylate with 2-(2′-hydroxy-4′-aminophenyl)-1,3-benzothiazole (**8**), which was obtained by reaction of 2-aminothiophenol with 4-aminosalicylic acid in polyphosphoric acid at 180°C (Scheme 2). After introduction of the propoxy linker, 2-[2′-(3′′-*p*-tosyloxypropoxy)-4′-amino]-1,3-benzothiazole (**9**) was coupled with *S*,*S*′-bis-trityl-*N*-BOC protected BAT and subsequent purification of the reaction mixture was done analogously as described for **7**. There was also contamination with *S*,*S*′-bis-trityl-*N*-BOC protected BAT ligand in purified 2-{2′-[3′′-(*N*-(*N*′-triphenylmethylmercaptoethyl-*N*′-*tert*-butoxycarbonyl)aminoethyl-*N*-triphenylmethylmercaptoethyl)aminopropoxy]-4′-aminophenyl}-1,3-benzothiazole (**10**) (1.5% as determined by RP-HPLC). Compounds **7** and **10** were purified using RP-HPLC resulting in sufficient amounts to perform labeling with ^{99m}Tc. Owing to the difficult purification, melting point determinations and nuclear magnetic resonance (NMR) spectra of these compounds could not be performed.

Labeling and characterization

For the radiolabeling of **7** and **10**, a one-pot procedure was developed. The deprotection of the precursor and consecutive radiolabeling of the deprotected compound was performed in the same vial without purification between the two steps.[12] In

order to obtain enough deprotected compound for radiolabeling, 50 µl 0.5 M HCl was added to a solution of the protected compound (**7** or **10**) in EtOH and the mixture was heated in a boiling water bath for 20 min. Liquid chromatography–mass spectrometry (LC–MS) analysis of the reaction mixture after deprotection of **10** showed that the BOC-group was easily removed, while it was much harder to remove the two trityl groups. Figure (3.1) shows a typical HPLC-chromatogram of the reaction mixture after deprotection of **10**, with at 1.95 min the fully deprotected compound with both thiols oxidized forming an intramolecular disulfide bridge (accurate MS ES^+ *m/z* $[M+H]^+$ 461.1468 Da, 6.5 ppm relative error, Figure (3.4)), at 12.07 min a peak corresponding to the compound without the BOC group but with the two trityl groups still present (accurate MS ES^+ *m/z* $[M+H]^+$ 947.3823, 2.4 ppm relative error, Figure (3.3)) and at 14.54 min the original fully protected compound **10**, (Figure (3.2)). No compound without the BOC and with only one trityl group was found, except after direct injection of the reaction mixture into the mass spectrometer without prior neutralization. In these conditions, also the fully deprotected compound was found in the free-thiol form. This suggests that neutralizing the reaction mixture results in an oxidation of the thiols, either in the reaction vessel or in the LC–MS system, with the completely deprotected compound forming an intramolecular disulfide bridge and the compound missing the BOC and one trityl group forming a compound with unknown structure. Apparently, only a small percentage of the precursor is fully deprotected and immediately available for complexation of reduced Tc. The fraction of compound missing the BOC and one trityl group can, however, also be further deprotected during the metal complexation process but this requires sufficient energy (heating) and time, which necessitates the temporary binding of reduced Tc in a weak complex such as TcO-tartrate. In addition, the phenylbenzothiazole linked via a propoxy spacer to the BAT tetradentate ligand constitutes a serious steric hindrance, which slows down the complexation kinetics, also a reason to apply an exchange labeling instead of a direct labeling. We obtained the highest labeling yield (15%) when the amounts of tartrate and $SnCl_2$ in the reaction mixture were 800 and 50 µg, respectively. The remainder of the ^{99m}Tc-activity was found under the form of colloidal $^{99m}TcO_2$, $^{99m}TcO_4^-$, ^{99m}Tc-tartrate and ^{99m}Tc-BAT. The ratios of these side products could be influenced by changing the amounts of $SnCl_2$ and tartrate, but changing these parameters did not increase the yield of the desired product. Although *S,S'*-bis-trityl-*N*-BOC protected BAT was only present in amounts as low as 1.5% in the purified precursors, ^{99m}Tc-BAT was present as a 6% impurity on the radiometric signal of the labeling mixtures. This is probably due to a more efficient deprotection and/or a better ability to form a complex with technetium in view of the absence of steric hindrance. Analysis using LC in combination with mass spectrometry (LC–MS) of the mixture obtained after labeling of **10** with eluate enriched in ^{99}Tc (because of the difficult detection of the tracer amounts of technetium in ^{99m}Tc-labelled preparations) showed that the main radiometric peak, eluting at 9.63 min (Figure 4(c)), is with high probability the desired compound, as the background subtracted mass spectrum summed over this peak shows a single peak with a mass close to the theoretical mass of the desired compound (Figure 4(b)). Performing an accurate mass assessment of the spectrum revealed that this peak has an accurate mass of 575.0370 Da (Figure 5(c)), or an error of only 9.6 ppm compared to the theoretical accurate mass of 575.0425 Da (Figure 5(a)). The peak eluting at 6.40 min corresponds to ^{99m}Tc-BAT, as we observed a single peak in the background subtracted mass spectrum summed over the radiometric peak at 6.39 min, with a

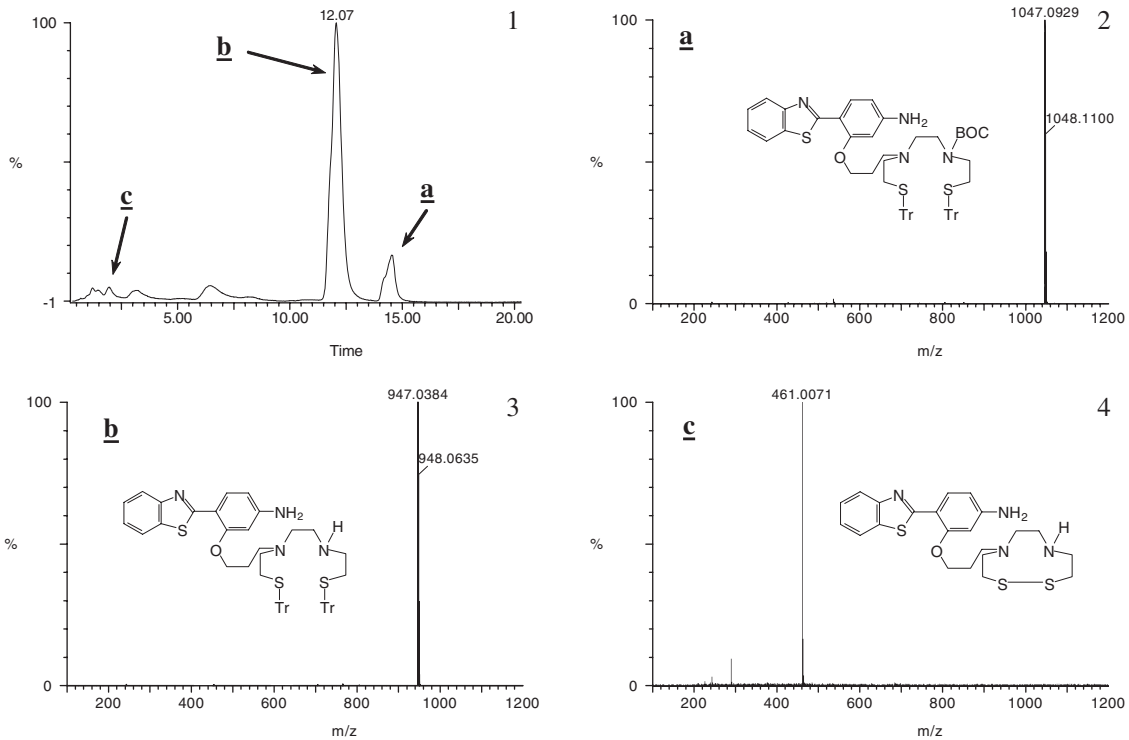


Figure 3. LC-MS analysis of **10** after deprotection. (1): UV chromatogram (365 nm); (2, 3 and 4): Background subtracted mass spectra summed over the different peaks. The spectra are not accurate mass spectra as the signal of the lock mass is lost when background substracted mass spectra are used.

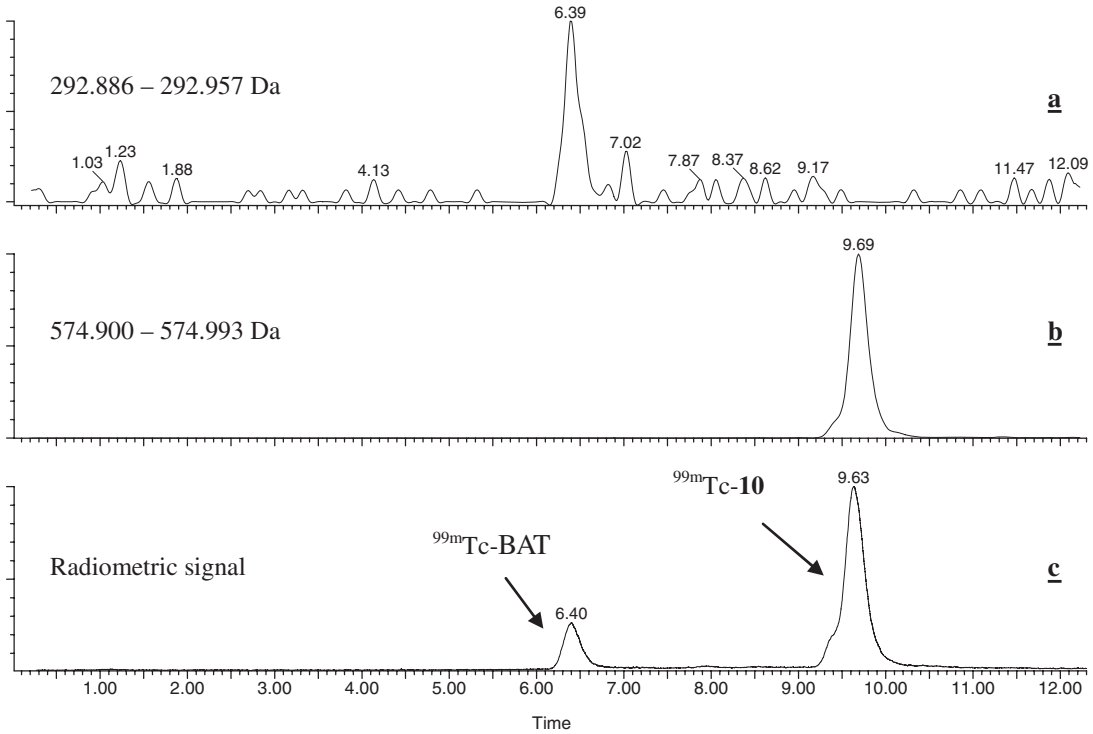


Figure 4. LC–MS analysis of the reaction mixture after labelling of **10** with ^{99m}Tc spiked with ^{99}Tc (after Seppak® Light C18 purification). (**a**) Ion mass chromatogram (range 292.886–292.957 Da) corresponding to the molecular ion mass of ^{99m}Tc-BAT; (**b**) ion mass chromatogram (range 574.900–574.993 Da) corresponding to the molecular ion mass of ^{99m}Tc-**10**; (**c**) radiometric signal.

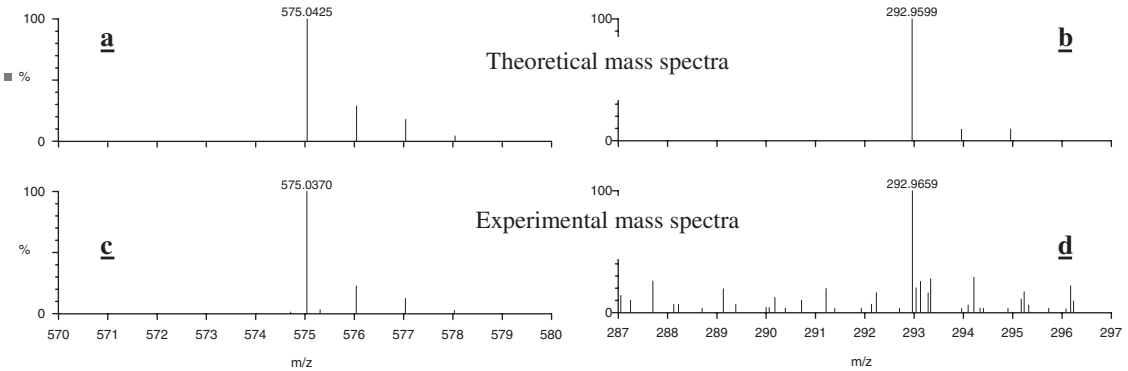


Figure 5. Accurate mass spectra of ^{99m}Tc-**10** (left) and ^{99m}Tc-BAT (right). The top spectra (**a** and **b**) show the theoretical mass spectra of the labelled compounds. The bottom spectra (**c** and **d**) the obtained mass spectra with kryptofix® 222 as lock mass.

mass close to theoretical mass (Figure 4(a)). The observed accurate mass was 292.9659 (Figure 5(d)), or an error of 20.5 ppm compared to the theoretical accurate mass of 292.9599 (Figure 5(b)). We found similar results for ^{99m}Tc-**7**, also supporting the assumed structure of the labeled compounds.

Biological evaluation

The 1-octanol/buffer partition coefficient of ^{99m}Tc-**7** and ^{99m}Tc-**10** was found to be 160.8 ± 32.8 ($\log P = 2.20 \pm 0.10$) and 115.5 ± 8.3 ($\log P = 2.06 \pm 0.03$), respectively. These values are within the optimal range for passive diffusion over the BBB.[13] In electrophoresis experiments with ^{99m}Tc-**7** and ^{99m}Tc-**10**, 88% of the applied radioactivity remained at the application point, indicating that the compounds are uncharged at physiological pH. For biodistribution studies in normal mice, the RP-HPLC-isolated peaks were collected in a vial containing a solution of ascorbic acid and polysorbate 80. The purpose of the ascorbic acid was to prevent re-oxidation of the oxotechnetium(V) to pertechnetate while the polysorbate 80 was added to prevent sticking of the compound to the wall of the vial after evaporation of the organic solvent prior to injection in mice. The results of the biodistribution studies of ^{99m}Tc-**7** and ^{99m}Tc-**10** in healthy mice at 2 and 60 min p.i. are shown in Table 1. The first requirement for a suitable tracer agent for detection of amyloid plaques in brain is the ability to cross the BBB. As both compounds show a negligible brain uptake, this important prerequisite is not fulfilled. The reason for their limited passage over the BBB is probably the relatively high molecular mass of 574 Da rather than an insuitable lipophilic character. Excretion of the compounds proceeds mainly through the hepatobiliary pathway and to a lesser extent through the renal pathway, which

Table 1. Tissue distribution expressed as percentage of the injected dose (% ID) and percentage of the injected dose per gram tissue (% ID/g) after i.v. injection of ^{99m}Tc-**7** and ^{99m}Tc-**10** in normal mice at 2 and 60 min p.i. ($n = 4$ at each time point)

	% ID ± s.d.		% ID/g ± s.d.	
	^{99m}Tc-**7**			
	2 min p.i.	60 min p.i.	2 min p.i.	60 min p.i.
Urine	0.2 ± 0.1	0.9 ± 0.5	–	–
Kidneys	6.9 ± 0.9	2.9 ± 0.5	10.0 ± 0.9	4.2 ± 0.7
Liver	37.0 ± 4.7	34.4 ± 7.8	16.2 ± 2.6	4.6 ± 0.3
Intestines	6.9 ± 1.3	32.6 ± 8.5	–	–
Stomach	1.6 ± 0.9	5.8 ± 0.5	–	–
Cerebrum	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.1	0.3 ± 0.1
Cerebellum	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.2	0.4 ± 0.2
Blood	25.3 ± 7.7	8.8 ± 1.3	9.1 ± 2.5	3.3 ± 0.2
	^{99m}Tc-**10**			
Urine	0.2 ± 0.2	1.0 ± 0.6	–	–
Kidneys	5.1 ± 1.7	4.2 ± 0.8	7.7 ± 2.5	7.7 ± 1.6
Liver	43.3 ± 4.9	30.7 ± 10.9	18.8 ± 4.3	14.4 ± 4.8
Intestines	5.4 ± 1.9	44.2 ± 5.2	–	–
Stomach	1.3 ± 0.4	4.7 ± 1.1	–	–
Cerebrum	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.2
Cerebellum	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.3 ± 0.4
Blood	21.2 ± 4.3	4.1 ± 1.6	8.4 ± 1.9	1.6 ± 0.4

is rather logic in view of the lipophilic nature of the compounds. The relatively high uptake in the stomach, 60 min p.i., suggests a partial re-oxidation of the complexed technetium into pertechnetate *in vivo*. In view of the faster blood clearance of ^{99m}Tc-**10**, a biostability study in normal mice was performed. Metabolism of ^{99m}Tc-**10** leads to formation of both hydrophilic ^{99m}Tc-labeled compounds with unknown structures as $^{99m}TcO_4^-$. To separate the ^{99m}Tc-labeled hydrophilic compounds from $^{99m}TcO_4^-$, a TLC-system with acetone as mobile phase was used. The relative amounts of ^{99m}Tc-labeled compounds in blood at 60 min p.i. calculated from both the TLC-data and RP-HPLC data are about 37% original intact compound, 33% pertechnetate and 30% other ^{99m}Tc-labeled hydrophilic metabolites. RP-HPLC analysis of the urine showed the presence of hydrophilic radiolabeled metabolites, which was confirmed by TLC-analysis. RP-HPLC analysis of the bile shows that most activity is found under the form of ^{99m}Tc-labeled hydrophilic metabolites. TLC analysis of the bile is more difficult to interpret, as the results from paper chromatography eluted with acetone are not consistent with the results from paper chromatography eluted with saline. It may be hypothesized that some metabolites are bound to bile salts, which do not migrate with acetone or saline. As the results of the biodistribution study of ^{99m}Tc-**7** are similar to that of ^{99m}Tc-**10**, it can be assumed that the metabolism of ^{99m}Tc-**7** will also be similar.

Experimental

Materials and methods

All reagents and solvents used in synthesis were purchased from Acros Organics (Geel, Belgium), Aldrich, Fluka or Sigma (Sigma-Aldrich, Bornem, Belgium) and were used without further purification. $MgSO_4$ was used as drying agent. pH values of non-radioactive solutions were measured with a P600 pH-meter (Consort, Turnhout, Belgium) equipped with a glass electrode, whereas pH values of radioactive solutions were measured using pH strips (universal indicator pH 0–14, Merck, Darmstadt, Germany). Evaporation of organic solvents under reduced pressure was done with a Büchi Rotovapor (Büchi, Flawil, Switzerland). Column purification was done using silica gel with a particle size varying between 0.04 and 0.063 mm (230–400 mesh) (MN Kieselgel 60 M, Macherey Nagel, Düren, Germany). Thin layer chromatography (TLC) was carried out using precoated silica TLC plates (DC-Alufolien-Kieselgel, Fluka, Buchs, Switzerland). The structure of the synthesized products was confirmed with ^{1}H-NMR spectroscopy on a Gemini 200 MHz spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts are reported as δ-values (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) and m (multiplet). For melting point (mp) determination, an Electrothermal IA9000 digital melting point apparatus was used (Electrothermal, Southend-on-Sea, UK).

Generator eluate containing ^{99m}Tc in the form of sodium pertechnetate was obtained from an Ultratechnekow generator (Tyco Healthcare, Petten, The Netherlands). $NH_4^{99}TcO_4$ was a gift from Tyco Healthcare.

Quantitative determination of radioactivity in samples was done using an automatic γ counter coupled to a multi-channel analyzer (Wallac 1480 Wizard® 3″, Wallac, Turku, Finland). The values were corrected for physical decay and background radiation.

LC–MS was performed on a system consisting of a Waters Alliance 2690 separation module (Waters, Milford, MA, USA) containing an Xterra® MS C18 3.5 µm (2.1 mm × 50 mm) RP-HPLC column (Waters). The mobile phase consisted of a mixture of acetonitrile (MeCN, A) and 0.1% ammonium formate (B) with either a gradient α (from 50 A:50 B V/V to 100 A:0 B V/V in 10 min followed by 10 min 100% A at a flow rate of 300 µl/min) for the unlabeled compounds or a gradient β (0 A:100 B V/V–100 A:0 B V/V in 10 min followed by 10 min 100% A at a flow rate of 300 µl/min) for the radiolabeled compounds. The eluate was first led through a UV-spectrometer (Waters 2487 Dual λ absorbance detector) and subsequently over a 3 in NaI(Tl)-crystal coupled to a single channel analyzer (The Nucleus, Oak Ridge, TE, USA) for radiometric detection before it was fed into the ESI source of the time-of-flight mass spectrometer (LCT, Micromass, Manchester, UK). Accurate mass determination was done by co-injection of a kryptofix® 222-solution as an internal calibration mass when electrospray ionization was in positive mode and by co-injection of a meso-2,3-dibromosuccinic acid solution when electrospray ionization was in negative mode. Acquisition and processing of the data were done with Masslynx software (Version 3.5).

HPLC was done with a system consisting of a TSP SpectraSeries P4000 pump (Thermo Separations Products, San Jose, CA, USA) connected to a TSP SpectraSeries UV100 detector and a 3 in NaI(Tl) crystal connected to a Medi-Lab Select SC7II single channel analyzer (Medi-Lab Select, Mechelen, Belgium). Data were collected with a RaChel data acquisition system (Version 1.49, Lablogic, Sheffield, UK).

Gas chromatography was performed on a DI 200 gas chromatograph (Delsi Instruments, Suresnes, France) with a Porapak® QS 80/100 column (Alltech, Deerfield, IL, USA) of 180 cm × 0.25 in.

Synthesis of 2-(4′-aminophenyl)-6-{3′′-[*N*-(*N*′-triphenylmethylmercaptoethyl-*N*′-*tert*-butoxycarbonyl)aminoethyl-*N*-triphenylmethylmercaptoethyl]aminopropoxy}-1,3-benzothiazole (7)

*N-4′-methoxyphenyl-4-nitrobenzamide (**1**)*

A solution of *p*-anisidine (24.63 g, 0.2 mol) and 4-nitrobenzoyl chloride (37.11 g, 0.2 mol) in 250 ml pyridine was refluxed for 2 h. After cooling to room temperature, the mixture was poured into 1.5 l of cold water and the precipitate was filtered off, washed with water and dried in a vacuum oven. Crystallization from methanol (MeOH) yielded 0.169 mol (46.02 g, 84.5%) of **1** as yellow-green needles.

^{1}H-NMR (DMSO-d6, 200 MHz): δ 3.75 (3H, s, OCH_3); δ 6.94 (2H, d, ^3J = 8.8 Hz, 3′-H 5′-H); δ 7.67 (2H, d, ^3J = 8.8 Hz, 2′-H 6′-H); δ 8.16 (2H, d, ^3J = 8.8 Hz, 3-H 5-H); δ 8.35 (2H, d, ^3J = 8.8 Hz, 2-H 6-H). Accurate MS ES$^-$ *m/z* [M−H]$^-$ 271.0724 (calculated for $C_{14}H_{11}N_2O_4$ 271.0724). Mp: 196–198°C.

*N-4′-methoxyphenyl-4-nitrothiobenzamide (**2**)*

A solution of **1** (54.46 g, 0.2 mol) and 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide (Lawesson's reagent, 44.49 g, 0.11 mol) in 350 ml 1,4-dioxane was refluxed for 3 h, after which it was cooled to room temperature and poured into 1.5 l of cold water. The precipitate was filtered off, washed with water and dried in a vacuum oven. After recrystallization from MeOH, 0.187 mol (53.80 g, 93.3%) of **2** was obtained as orange-red flakes.

^{1}H-NMR (DMSO-d6, 200 MHz): δ 3.78 (3H, s, OCH_3); δ 7.01 (2H, d, ^3J = 8.8 Hz, 3′-H 5′-H); δ 7.75 (2H, d, ^3J = 8.8 Hz, 2′-H 6′-H); δ 7.98 (2H, d, ^3J = 8.4 Hz, 3-H 5-H); δ 8.29 (2H, d, ^3J = 8.8 Hz, 2-H 6-H). Accurate MS ES$^-$ *m/z* [M−H]$^-$ 287.0495 (calculated for $C_{14}H_{11}N_2O_3S$ 287.0496). Mp: 174.5–175.5°C.

*2-(4′-Nitrophenyl)-6-methoxy-1,3-benzothiazole (**3**)*

To a solution of potassium ferricyanide (210.7 g, 0.64 mol) in 400 ml water at 90°C was slowly added over a period of 2 h a solution of **2** (45.70 g, 0.16 mol) in a mixture of 50 ml ethanol (EtOH) and 300 ml 10% NaOH. After the addition, the obtained suspension was stirred for 2 h at 90°C and then cooled in an ice bath to 4°C. The precipitate was filtered off, washed with water and dried in a vacuum oven. The dry residue was dispersed in 3 l of a mixture of CH_2Cl_2 and EtOH (75:25 V/V). The dispersion was filtered off and the filtrate was concentrated by vacuum evaporation. The residue was purified on a silica gel column with ethyl acetate/hexane 50:50 V/V as the eluent. After recrystallization from EtOH/ethyl acetate 50:50 V/V, 0.113 mol (32.35 g, 70.5%) of **3** was obtained as yellow needles.

^{1}H-NMR (DMSO-d6, 200 MHz): δ 3.92 (3H, s, OCH_3); δ 7.16 (1H, d, ^3J = 9.4 Hz, 5-H); δ 7.38 (1H, s, 7-H); δ 8.00 (1H, d, ^3J = 9.0 Hz, 4-H); δ 8.21 (2H, d, ^3J = 8.4 Hz, 2′-H 6′-H); δ 8.34 (2H, d, ^3J = 8.8 Hz, 3′-H 5′-H). Accurate MS ES$^+$ *m/z* [M+H]$^+$ 287.0488 (calculated for $C_{14}H_{10}N_2O_3S$ 287.0485). Mp: 212.5–214°C.

*2-(4′-Aminophenyl)-6-methoxy-1,3-benzothiazole (**4**)*

A dispersion of **3** (14.32 g, 50 mmol) and $SnCl_2 \cdot 2H_2O$ (33.84 g, 150 mmol) in 400 ml EtOH was refluxed under nitrogen for 4 h. After cooling to room temperature, the EtOH was removed by vacuum evaporation. The residue was dispersed in 1.5 l ethyl acetate and the mixture was extracted with 500 ml 2 M NaOH. The organic layer was washed two times with 250 ml water and then with 250 ml brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated by vacuum evaporation to leave a residue of **4** (0.49 mmol, 12.58 g, 98.1%), which was used in subsequent reactions without further purification.

^{1}H-NMR (DMSO-d6, 200 MHz): δ 3.83 (3H, s, OCH_3); δ 6.67 (2H, d, ^3J = 8.0 Hz, 3′-H 5′-H); δ 7.06 (1H, d, ^3J = 8.8 Hz, 5-H); δ 7.60 (1H, s, 7-H); δ 7.71 (2H, d, ^3J = 8.2 Hz, 2′-H 6′-H); δ 7.80 (1H, d, 3 J = 8.8 Hz, 4-H). Accurate MS ES$^+$ *m/z* [M+H]$^+$ 257.0767 (calculated for $C_{14}H_{13}N_2OS$ 257.0743). Mp: 142–144°C.

*2-(4′-Aminophenyl)-6-hydroxy-1,3-benzothiazole (**5**)*

A dispersion of **4** (12.82 g, 50 mmol) in 180 ml of CH_2Cl_2 under nitrogen atmosphere was cooled to −70°C in an acetone/solid CO_2 bath. Over a period of 1 h, BBr_3 (0.106 mol, 106 ml of a 1 M solution in CH_2Cl_2) was added slowly, after which the mixture was stirred for another hour at −70°C. The suspension was allowed to warm up to room temperature and was then stirred for 12 h. The suspension was again cooled to −70°C and MeOH was slowly added until no more gas evolved. After warming up to room temperature, 400 ml of 2 M NaOH was added. The aqueous layer was separated and neutralized with 3 M HCl (final pH = 7.5). The precipitate formed was filtered off and dried in a vacuum oven to yield 36.07 mmol of **5** (8.74 g, 72%) as a brownish powder.

^{1}H-NMR (DMSO-d6, 200 MHz): δ 6.67 (2H, d, ^3J = 8.4 Hz, 3′-H 5′-H); δ 6.92 (1H, d, ^3J = 8.8 Hz, 5-H); δ 7.32 (1H, s, 7-H); δ 7.68 (2H, d, ^3J = 8.8 Hz, 2′-H 6′-H); δ 7.71 (1H, d, ^3J = 8.8 Hz, 4-H). Accurate MS ES$^+$ *m/z* [M+H]$^+$ 243.0619 (calculated for $C_{13}H_{11}N_2OS$ 243.0587). Mp : 262–263°C.

*2-(4′-Aminophenyl)-6-(3′′-p-tosyloxypropoxy)-1,3-benzothiazole (**6**)*

To a dispersion of **5** (1.817 g, 7.5 mmol) in 15 ml of a mixture of acetonitrile (MeCN) and MeOH (9:1 V/V) was added Na (190 mg, 8.25 mmol) dissolved in a mixture of 3 ml MeOH and 12 ml MeCN. The resulting mixture was added to a dispersion of 1,3-propanediol di-*p*-tosylate (768 mg, 15 mmol) in 75 ml of a mixture of MeCN and MeOH (9:1 V/V) and the reaction mixture was heated to 50°C for 12 h. The formed suspension was filtered off and the solvent removed from the filtrate by vacuum evaporation. The residue was purified by silica gel column chromatography with a mixture of CH_2Cl_2 and MeOH (99:1 V/V) as eluent to obtain **6** (896 mg, 1.97 mmol, 26.3%) as a yellow-brown powder.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.14 (2H, m, $CH_2CH_2CH_2$); δ 2.30 (3H, s, CH_3); δ 3.98 (2H, t, $OCH_2CH_2CH_2$); δ 4.27 (2H, t, CH_2CH_2OTs); δ 6.73 (2H, d, ^3J = 8.8 Hz, 3′-H 5′-H); δ 6.87 (1H, d, ^3J = 8.8 Hz, 5-H); δ 7.16 (1H, s, 7-H); δ 7.20 (2H, d, ^3J = 8.0 Hz, 2′′-H 6′′-H); δ 7.75 (2H, d, ^3J = 7.6 Hz, 3′′-H 5′′-H); δ 7.83 (1H, d, ^3J = 9.0 Hz, 4-H); δ 7.84 (2H, d, ^3J = 8.8 Hz, 2′-H 6′-H). Accurate MS ES$^+$ *m/z* [M+H]$^+$ 455.1067 (calculated for $C_{23}H_{23}N_2O_4S_2$ 455.1094). Mp: 129.5–130.5°C.

*2-(4′-aminophenyl)-6-{3′′-[N-(N′-triphenylmethylmercaptoethyl-N′-tert-butoxycarbonyl)aminoethyl-N-triphenylmethylmercaptoethyl]-aminopropoxy}-1,3-benzothiazole (**7**)*

To a solution of **6** (114 mg, 0.25 mmol) and *N*,*N*-diisopropylethylamine (DIEA, 32 mg, 0.25 mmol) in 1 ml *N*,*N*-dimethylformamide (DMF) was added *S*,*S*′-bis-triphenylmethyl-*N*-(*tert*-butoxycarbonyl)-1,2-ethylenedicysteamine (*S*,*S*′-bis-trityl-*N*-BOC-BAT, 191 mg, 0.25 mmol) in 0.5 ml DMF.[14,15] The mixture was stirred for 24 h at 50°C. Purification was done with HPLC using a HS Hyper Prep

100 Å silica 8 μm column (10 mm × 250 mm, Alltech) and a mixture of CH_2Cl_2 and MeOH (99:1 V/V) as eluent at a flow rate of 3 ml/min. Accurate MS ES$^+$ *m/z* $[M+H]^+$ 1047.4365 (calculated for $C_{65}H_{67}N_4O_3S_3$ 1047.4370).

Synthesis of 2-{2′-[3″-(*N*-(*N*′-triphenylmethylmercaptoethyl-*N*′-*tert*-butoxycarbonyl)aminoethyl-*N*-triphenylmethylmercaptoethyl)aminopropoxy]-4′-aminophenyl}-1,3-benzothiazole (10)

*2-(2′-Hydroxy-4′-aminophenyl)-1,3-benzothiazole (**8**)*

A dispersion of 4-aminosalicylic acid (1.534 g, 10 mmol) in 50 g polyphosphoric acid was heated to 180°C and 2-aminothiophenol (1.252 g, 10 mmol) was added. After stirring for 4 h at 180°C, the solution was cooled to room temperature and the pH was adjusted to 5.0 using a 10% Na_2CO_3 solution. The precipitate was filtered off, washed with water, dried in a vacuum oven and then chromatographed on silica gel using CH_2Cl_2/hexane (80:20 V/V) as eluent. 1.79 g of **8** was obtained as a brownish powder (7.42 mmol, 74.2%).

^{1}H-NMR (DMSO-d6, 200 MHz): δ 6.16 (1H, s, 3′-H); δ 6.24 (1H, d, ^3J = 8.8 Hz, 5′-H); δ 7.32 (1H, dd, ^3J = 7.2 Hz, ^3J = 7.8 Hz, 6-H); δ 7.45 (1H, dd, ^3J = 7.45, ^3J = 7.0 Hz, 5-H); δ 7.61 (1H, d, ^3J = 8.4 Hz, 6′-H); δ 7.87 (1H, d, ^3J = 7.8 Hz, 7-H); δ 8.01 (1H, d, ^3J = 7.6 Hz, 4-H). Accurate MS ES$^+$ *m/z* $[M+H]^+$ 243.0601 (calculated for $C_{13}H_{11}N_2OS$ 243.0587). Mp: 201–203°C.

*2-[2′-(3″-p-Tosyloxypropoxy)-4′-amino]-1,3-benzothiazole (**9**)*

The same procedure as for the synthesis of **6** was used starting from 1.21 g (5 mmol) **8**. Compound **9** was obtained with a yield of 64.4%.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.17 (3H, s, CH_3); δ 2.31 (2H, m, $CH_2CH_2CH_2$); δ 4.01 (2H, t, $OCH_2CH_2CH_2$); δ 4.43 (2H, t, CH_2CH_2OTs); δ 6.16 (1H, s, 3′-H); δ 6.40 (1H, d, ^3J = 8.0 Hz, 5′-H); δ 6.98 (2H, d, ^3J = 8.2 Hz, 3″-H 5″-H); δ 7.31 (1H, dd, ^3J = 8.4 Hz, ^3J = 6.8 Hz, 6-H); δ 7.45 (1H, dd, ^3J = 7.0 Hz, ^3J = 6.8 Hz, 5-H); δ 7.64 (2H, d, ^3J = 8.4 Hz, 2″-H 6″-H); δ 7.81 (1H, d, ^3J = 7.8 Hz, 6′-H); δ 7.99 (1H, d, ^3J = 7.6 Hz, 7-H); δ 8.30 (1H, d, ^3J = 8.4 Hz, 4-H). Accurate MS ES$^+$ *m/z* $[M+H]^+$ 455.1064 (calculated for $C_{23}H_{23}N_2O_4S_2$ 455.1094). Mp: 130.5–132°C.

*2-{2′-[3″-(N-(N′-triphenylmethylmercaptoethyl-N′-tert-butoxycarbonyl)aminoethyl-N-triphenylmethylmercaptoethyl)aminopropoxy]-4′-aminophenyl}-1,3-benzothiazole (**10**)*

The same procedure as for the preparation of **7** was used starting from 228 mg (0.5 mmol) **9**. Purification was done with HPLC using a HS Hyper Prep 100 Å silica 8 μm column (10 mm × 250 mm, Alltech) and a mixture of CH_2Cl_2 and MeOH (99:1 V/V) as eluent at a flow rate of 3 ml/min. Accurate MS ES$^+$ *m/z* $[M+H]^+$ 1047.4314 (calculated for $C_{65}H_{67}N_4O_3S_3$ 1047.4370).

Radiolabeling

To a vial containing 200 μl of a 1 mg/ml solution of **7** or **10** in EtOH was added 50 μl of 0.5 M HCl. The mixture was heated in a boiling water bath for 20 min. After cooling to room temperature were added consecutively 50 μl 0.5 M NaOH, 30 μl 0.5 M phosphate buffer pH 6.0, 20 μl of a 40 mg/ml aqueous solution of sodium potassium tartrate, 200 μl generator eluate (370–740 MBq ^{99m}Tc) and 5 μl of a freshly prepared 10 mg/ml solution of $SnCl_2 \cdot 2H_2O$ in 0.05 M HCl. The suspension was heated in a boiling water bath for 10 min, cooled to room temperature, diluted with 2 ml water and passed over a Seppak® Light C18 cartridge (Waters), conditioned with 5 ml EtOH and 5 ml H_2O. The cartridge was washed with 3 ml H_2O and the labeled compounds were eluted with 1 ml MeCN. This eluate was concentrated by passing a gentle flow of nitrogen while heating the solution to 40°C until approximately 300 μl solution was left. Before RP-HPLC purification, the mixture was diluted with 300 μl 0.1% ammonium acetate solution. Purification using RP-HPLC was done on a Hypersil BDS C18 5 μm column (4.6 mm × 250 mm, Alltech) with a mixture of MeCN and 0.1% ammonium acetate (50:50 V/V) as the mobile phase at a flow rate of 1 ml/min. For mass spectrometry a mixture of 100 μl generator eluate and 100 μl of an 83 μM solution of $NH_4^{99}TcO_4$ was used instead of 200 μl generator eluate. Radio-LC–MS was carried out using an Xterra C18 column (3.5 mm, 50 mm × 2.1 mm, Waters) with gradient mixtures of MeCN and 0.05 M ammonium formate as eluent at a flow rate of 0.3 ml/min.

Partition coefficient

The lipophilicity of the RP-HPLC-isolated ^{99m}Tc-complexes was determined using a modification of the method described by Yamauchi *et al.*[16] A 25 μl aliquot of the RP-HPLC isolated ^{99m}Tc-complex was added to a test tube containing 3 ml of 1-octanol and 3 ml of 0.025 M phosphate buffer pH 7.4. The test tube was vortexed at room temperature for 2 min and then centrifuged at 2700*g* for 10 min. A 100 μl aliquot was taken from the 1-octanol phase and a 900 μl aliquot from the aqueous phase, taking care to avoid cross contamination between the phases and weighed. The radioactivity of the aliquots was counted using an automatic γ counter and the partition coefficient *P* was calculated using the following equation:

$$P = \frac{\text{cpm/ml octanol}}{\text{cpm/ml buffer}}$$

with cpm = counts per minute.

Experiments were performed at least in triplicate.

Electrophoresis

A 5 μl aliquot of the RP-HPLC-purified compounds was applied on a paper strip (3.5 cm × 13 cm, Whatman 1 chromatography paper, Whatman, Maidstone, England) wetted with a mixture of 0.025 M phosphate buffer pH 7.4 and methanol (50:50 V/V). Electrophoresis was performed during 15 min using the described mixture as the electrolyte solution and an applied voltage of 300 V. As reference for migration, 5 μl of a 2 mM solution of thioflavin-T was spotted on the same paper. After drying, the paper was cut into 0.5 cm strips and the activity on each strip was counted using an automatic γ counter.

Biological evaluation in mice

The peak containing the desired labeled compound was isolated using RP-HPLC and collected in a vial containing 250 μl of a 1 mg/ml solution of ascorbic acid in saline and 250 μl of a 10 mg/ml solution of polysorbate 80 in water. The organic solvent from the HPLC-eluate was removed at 40°C with a stream of nitrogen for 20 min. The residual aqueous solution was diluted with saline to a concentration of 370 kBq/ml. Gas chromatography was used to verify that the concentration of

acetonitrile was below 0.1%. Male NMRI mice were sedated by intraperitoneal injection of 0.75 µl of a 1 to 4 dilution of Hypnorm® (fentanyl citrate 63 µg/ml + fluanisone 2 mg/ml after dilution, Janssen-Cilag, Beerse, Belgium). Each of the eight mice was injected via a tail vein with a 0.1 ml aliquot (37 kBq) of one of the ^{99m}Tc-complexes. The mice were sacrificed by decapitation at 2 or 60 min post injection (p.i.; four mice at each time point). Blood was collected in tared tubes and weighed. All organs and other body parts were dissected and weighed and their activity was counted using an automatic γ counter. Results were corrected for background activity and are expressed as percentage of the injected dose (% ID), which is the sum of the activities in all organs except the activity in the tail, and as percentage of the injected dose per gram tissue (% ID/g). For calculation of the total activity in blood, blood mass was assumed to be 7% of the body mass.[17]

For biostability studies, a mouse was injected with 1.85 MBq of ^{99m}Tc-**10** and sacrificed by decapitation 60 min p.i. Blood was collected and centrifuged at 2700*g* for 10 min. The plasma was transferred to a tube and mixed with an equal volume of MeCN. The tube was centrifuged again at 2700*g* for 10 min and the supernatant was analyzed by RP-HPLC (Hypersil BDS C18 5 µm column (4.6 mm × 250 mm) as stationary phase; MeCN/0.1% ammonium acetate (50:50 V/V) as mobile phase at a flow rate of 1 ml/min). In addition, the urine and bile were analyzed on HPLC using the same conditions. The HPLC-eluate was collected in 1 ml aliquots and their radioactivity was counted using an automatic γ counter. TLC analysis was done by applying a drop of plasma, urine or bile on Whatman 1 chromatography paper and eluting the paper strip in a TLC-tank with either saline or acetone as elution solvent. After elution, the TLC-strips were cut 2 cm above the application point and the activity of the upper and lower parts was counted using an automatic γ counter.

Conclusion

Two 4′-aminophenylbenzothiazoles have been derivatized at the 6 or 2′-position with an *S*,*S*′-bis-trityl-*N*-BOC protected BAT group via a propoxy linker. After deprotection of the compounds, they were successfully labeled with ^{99m}Tc. Analysis of the technetium-99 enriched reaction mixture on LC–MS showed that the main peak in the chromatogram is the proposed radiolabeled compound. Impurities in the reaction mixture were $^{99m}TcO_4^-$, $^{99m}TcO_2$, ^{99m}Tc-tartrate and ^{99m}Tc-BAT. Log *P* determination and electrophoresis proved that the compounds are uncharged and have potential to cross the BBB. Biological evaluation in healthy mice showed however a low uptake in the brain, which renders these compounds inappropriate for visualization of Aβ plaques in the brain of AD patients. The reason for the low brain uptake is probably the high molecular mass, a drawback that will remain a challenge for the future development of technetium-99m labeled tracers using the bifunctional ligand approach. In mice, the compound with the radioactive label at the 6-position was partially reoxidized *in vivo* and partially transformed into more hydrophilic compounds. A similar result can be assumed for the compound conjugated with ^{99m}Tc-BAT at the 2′-position.

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