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Synthesis and evaluation of a ^{99m}Tc-BAT-phenylbenzothiazole conjugate as a potential in vivo tracer for visualization of amyloid β

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Abstract—We have conjugated *S*,*S*′-bis-trityl-*N*-BOC-*N*′-acetic acid-1,2-ethylenedicysteamine, a protected bis-amino-bis-thiol (BAT) tetraligand, with 2-(4′-aminophenyl)-1,3-benzothiazole, a derivative of thioflavin-T with known affinity for amyloid. The conjugate was efficiently labelled with ^{99m}Tc by heating of the protected precursor in diluted hydrochloric acid followed by neutralization and heating in the presence of ^{99m}Tc-tartrate. It was demonstrated that the ^{99m}Tc-BAT-phenylbenzothiazole conjugate binds in vitro to amyloid β present in postmortem brain slices of Alzheimer's patients. Despite its high lipophilicity and neutral character, the radiolabelled conjugate did not cross the blood–brain barrier to a sufficient degree and therefore is not useful for detection of Alzheimer's disease. Further evaluation of this ^{99m}Tc-labelled tracer agent could elucidate its potential usefulness to visualize amyloid plaques in peripheral amyloidosis.

The increasing incidence of dementia, in particular Alzheimer's disease (AD), is at the basis of a continuing search for a radioactive tracer which allows to definitely diagnose the disease in vivo in an early stage. AD is characterized by the presence of extensive, extracellular deposits of amyloid β (Aβ) in cortical brain tissue and blood vessels together with so-called neurofibrillar tangles which are found intracellularly.[1,2] The tangles consist of the microtubule-binding protein tau in a specific paired helical and straight filament form. The formation of Aβ probably leads to an abnormal cleavage of the tau protein, causing intracellular assemblage of tau into pathological filaments ending up with neuronal cell death and dementia.[3,4] Clinical diagnosis of AD is now done by neuro-psychological tests but yields only indirect information.[5] Immunohistochemical staining of brain tissue with polyaromatic dyes such as Congo red, chrysamine G, thioflavin-T (ThT, Fig. 1), thioflavin-S, or silver can provide direct information but only postmortem.[6] Therefore it would be useful to have a radiolabelled tracer agent which would allow non-invasive in vivo imaging and diagnosis of AD in an early stage and follow up of treatment. Such a radiotracer should have high affinity for the Aβ plaque deposits and a good blood–brain barrier (BBB) passage. To cross the BBB, the compound should have a neutral character, a partition coefficient between 1 and 2.5 and a molecular mass not exceeding 600.[7]

Systemic or peripheral amyloidosis (PA) is characterized by the presence of deposited proteins with the same properties, namely highly β-pleated Aβ plaques with a high propensity to aggregate, in liver, heart, kidneys, joints, etc.[1] So, radiolabelled compounds binding to Aβ may also be used to visualize the plaques in PA.

In the past years, radiolabelled derivatives of several of the higher mentioned dyes have been proposed and evaluated as potential diagnostic tracer agent for this purpose.[8] Our group studied a ^{99m}Tc-labelled conjugate of

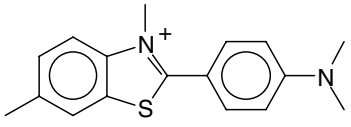

Figure 1. Structure of thioflavin-T.

Keywords: Amyloid β; Thioflavin-T; 2-Phenylbenzothiazoles; Technetium-99m.
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chrysamine G with a monoamide–monoaminedithiol (MAMA) chelating ligand.[9,10] However, brain uptake of this radiolabelled agent was minimal, probably because of its large size and ionized character at physiological pH. The most promising of the reported compounds are uncharged derivatives of thioflavin-T such as ^{125}I-TZDM[11] (Fig. 2A) and the carbon-11 labelled 2-phenylbenzothiazoles BTA (Fig. 2B) and 6-OH-BTA, also known as Pittsburgh Compound-B or PIB (Fig. 2C).[12] The latter has already been tested in several clinical studies and was shown to allow clear differentiation between AD and control subjects.[13]

Although the first clinical results using [^{11}C]PIB seem to be very promising, its routine clinical use is limited because of the short half-life of carbon-11 and the high cost of production (cyclotron) and imaging (PET camera). Therefore, great effort has been made to develop ^{99m}Tc-labelled tracers for in vivo detection of amyloid β plaques. ^{99m}Tc has attractive nuclear-physical properties and is continuously available in the hospital at a relatively low cost. However, contrary to the labelling of a compound with ^{11}C, the transition metal ^{99m}Tc needs a chelating structure to become stably bound to an organic molecule.

The aim of this study was to synthesize a neutral technetium-99m labelled derivative of ThT, namely 2-(4′-aminophenyl)-1,3-benzothiazole conjugated to a bis-amine-bis-thiol (BAT) ligand, and to study its biological characteristics. Synthesis of the BAT type bifunctional chelating agent *S*,*S*′-bis-trityl-*N*-BOC-1,2-ethylenedicysteamine (Scheme 1, **1**) was done according to published data.[14] The thiol groups were protected with triphenylmethyl (trityl) groups to prevent oxidation (e.g., disulfide formation) and one amine was protected with a *tert*-butoxycarbonyl (BOC) group to allow introduction of a single ethyl acetate substituent on the other amine. The reaction with ethyl bromoacetate was done in CH_2Cl_2 with *N*,*N*-diisopropylethylamine (DIEA) as base and provided **2**[15] in 37.5% yield. In the next step, the ethyl ester was removed with NaOH in H_2O/EtOH under reflux, yielding the free acid **3**[16] (50% yield). 2-(4′-aminophenyl)-1,3-benzothiazole **4**[17] was synthesized according to a method described by Shi and coworkers[18] and was obtained in 64% yield after crystallization. Coupling of the aromatic amine of the phenylbenzothiazole with the carboxylic acid group of the protected BAT ligand was performed in the presence of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI·HCl) and hydroxybenzotriazole (HOBt), classical coupling reagents used in peptide chemistry. Although the reagents were added in excess and a long reaction time was used, only a moderate yield of 23% was obtained for precursor **5**.[19] No attempts were made to optimize the reaction because only low amounts of the metal complexing ligand are required for labelling with technetium-99m.

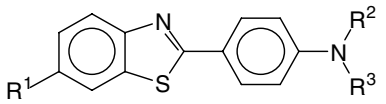
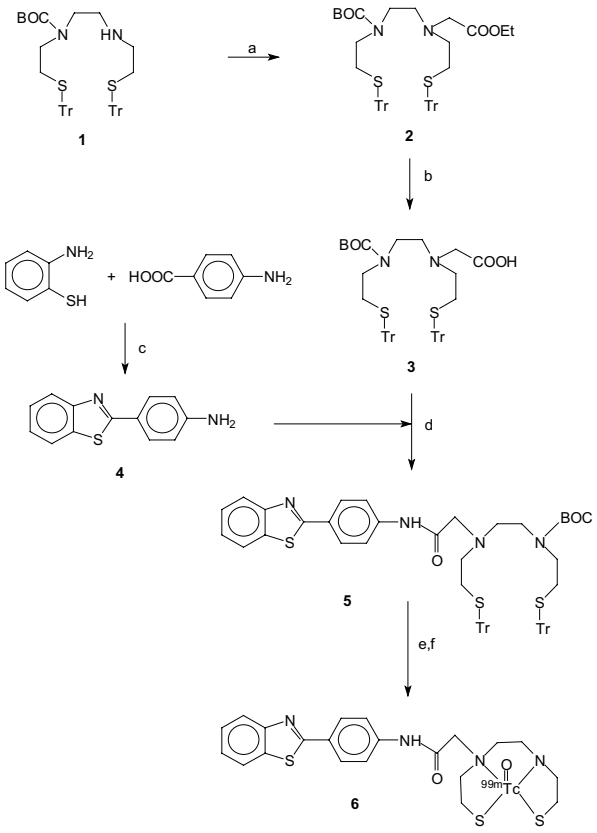


Figure 2. Structures of some proposed tracers for visualization of amyloid β plaques.



Scheme 1. Reagents and conditions: (a) DIEA, $BrCH_2COOEt$, CH_2Cl_2, room temperature (rt), overnight; (b) H_2O/EtOH, $NaOH_{sat}$, reflux, overnight; (c) polyphosphoric acid, 180 °C, 4 h; (d) EDCI·HCl, HOBt, CH_2Cl_2, rt, overnight; (e) HCl 0.5 M, 20 min, 100 °C; (f) NaKtartrate buffer, pH 7, $SnCl_2{\cdot}2H_2O$, $^{99m}TcO_4^-$, 15 min, 100 °C.

In precursor **5**, the two thiol groups are protected with a trityl group and the secondary amine with a BOC group. Removal of thiol protective trityl groups is usually done with trifluoroacetic acid and triisopropylsilane in an inert solvent. This procedure also removes the *N*-BOC protective group and allows performing deprotection and labelling with ^{99m}Tc as a one-pot reaction. Based on our previous work,[20] however, deprotection in aqueous medium using heating in the presence of hydrochloric acid was preferred. The latter method generates a low amount of the fully deprotected precursor, while the major part is converted to the mono-*S*-trityl or di-*S*-trityl form without *N*-BOC group as shown by mass spectrometry (MS) analysis. Such partial deprotection appears to be sufficient to allow successful exchange labelling of the BAT chelating structure with ^{99m}Tc. Such labelling can be done by heating in a boiling water bath in the presence of stannous ions and tartrate as a weak chelating agent (the formation of an intermediate weak Tc(V)O–tartrate complex prevents the formation

of $^{99m}TcO_2$ in colloidal form). The efficient labelling with ^{99m}Tc can be explained by the fact that only nanomolar amounts of ^{99m}Tc are present in the used activities (400–800 MBq) and that low amount of fully deprotected ligand is sufficient for chelation of the few nanograms of ^{99m}Tc. In addition, previous studies have shown that upon binding of technetium to one or more ligand atoms, the remaining protecting groups are cleaved off, apparently by the 'chelate effect'. The mean yield of the labelling reaction was 86% and the different products present in the labelling reaction mixture could be efficiently separated by reversed phase high performance liquid chromatography (RP-HPLC) (Fig. 3).[21,22]

Identification of the peak eluting at 15.3 min was done by comparing its retention time (t_R) with that of the complex formed between deprotected **3** and ^{99m}Tc (^{99m}Tc-BAT-CH_2-COOH). The presence of this complex as a radiochemical impurity is due to the presence of a low amount of BAT-CH_2-COOH as an impurity after heating of **5** in acidic conditions, and the fact that this small molecule can bind ^{99m}Tc more efficiently than the more sterically hindered **5**. The yield of the labelling reaction was, however, sufficient for further biological evaluation of **6**.

Due to the tiny amounts of technetium in a ^{99m}Tc labelled preparation (typically in the nano- to picomolar range), it is difficult to obtain useful mass spectra of technetium-99m labelled compounds in no-carrier-added form. In order to be able to determine the mass of **6**, we increased the amount of technetium in the radiolabelling mixture by addition of technetium-99 in the form of ammonium pertechnetate to obtain a carrier-added form. Technetium-99 emits β-rays and has a half-life of 2.14×10^5 year, so care should be taken when ^{99}Tc is used to prevent contamination. Figure 4b depicts the radiometric signal of the radio-LC–MS[23] analysis of carrier-added **6**, which shows the presence of different radioactive compounds. Figure 5a shows the background subtracted mass spectrum of the peak with t_R 18.78 min (summed mass spectra obtained prior and after the peak at 18.78 min subtracted from the summed mass spectrum over the peak at 18.78 min), showing a single molecular ion mass of 559.2326 Da, which corresponds to the theoretical ion mass of **6** (Fig. 5b; 559.0112 Da). Figure 4a shows the single ion mass chromatogram over the mass range 559.172–559.322 Da. As the peak on this single ion mass chromatogram has both an identical retention time and shape as the peak observed in the radiometric channel, the Tc-complex eluting at 18.78 min can be assumed to have with high probability the structure as depicted in **6** (Scheme 1). In the radiometric signal, the peak at 18.78 min corresponds to **6**, while ^{99m}Tc-tartrate elutes with the void volume at 1.64 min. Peaks at 2.95 and 6.82 min correspond to pertechnetate and ^{99m}Tc-BAT-CH_2-COOH, respectively. The radiochemical yield of **6** in carrier-added preparations was lower as compared to preparations using no-carrier-added technetium-99m (Fig. 3).

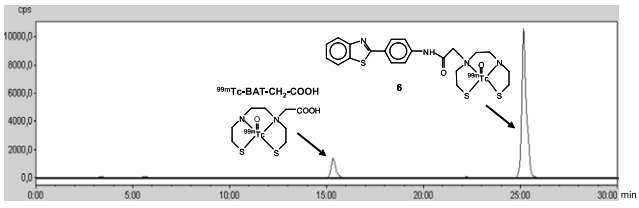


Figure 3. RP-HPLC chromatogram of the reaction mixture after labelling of **5** with ^{99m}Tc. Radiolabelled **6** elutes at 25 min and ^{99m}Tc-BAT-CH_2-COOH at 15.3 min.

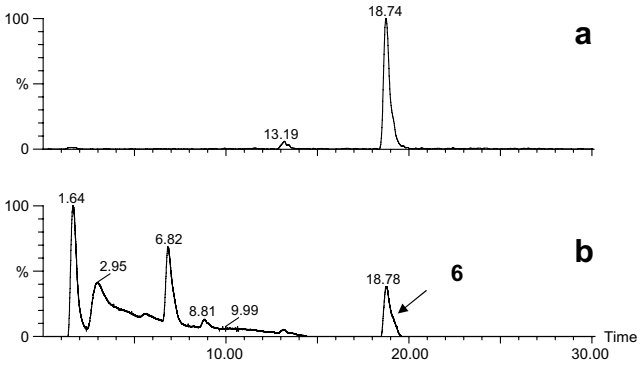


Figure 4. (a) Single ion mass chromatogram (ES^+, 559.172–559.322 Da) and (b) = radiometric chromatogram of **6**.

The molecular ion mass found for **6** (559.2 Da) is in agreement with a Tc(V)O-complex of a BAT chelating structure of **5**. Upon complexation of the $[Tc(V)O]^{3+}$ core, both thiols and one amine of the BAT ligand lose a proton. Three positive charges of the $[Tc(V)O]^{3+}$ core are balanced by the loss of these three protons and a neutral complex is thus formed. The partition coefficient was determined using a described procedure[24] and found to be 83.28 ± 2.35 ($\log P = 1.92 \pm 0.012$), which is in a good range for passive BBB diffusion. As its molecular mass does not exceed 600 Da, **6** has in principle favorable characteristics which allow passage through the BBB.

The affinity of ^{99m}Tc-labelled **6** for amyloid β was studied by incubation of postmortem brain slices of AD patients with **6** in the absence or in the presence of ThT (1 μM).[25,26] Figure 6a shows the visualization of Aβ plaques in the brain cortex (red areas). In Figure 6b, the binding of **6** on an adjacent slice was decreased in the presence of ThT (1 μM) showing that both molecules have the same binding sites.

Biodistribution of **6** was studied in normal mice,[25] which were sacrificed at 2 min or 60 min post-injection (pi). The results are shown in Table 1. At 2 min pi, the percentage of injected dose (ID) in the cerebrum and the cerebellum was only 0.09% and 0.11%, respectively. Activity in both organs decreased to 0.03% ID at 60 min pi, showing that brain uptake was minimal. Therefore the compound is not useful for detection of amyloid plaques in the living AD brain.

These findings are in agreement with those of similar attempts to develop ^{99m}Tc-labelled N_2S_2-complexes for imaging amyloid plaques in brain.[27] One of the reasons

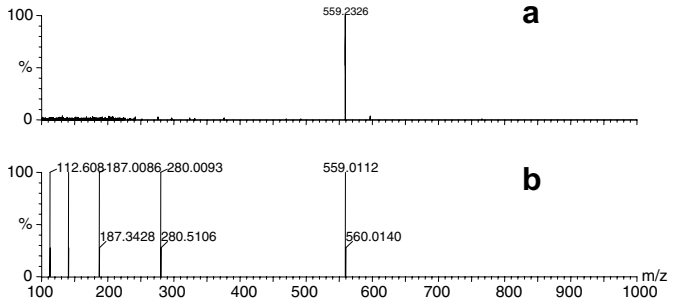


Figure 5. (a) Background subtracted mass spectrum of the chromatogram peak at 18.78 min (Fig. 4a) and (b) theoretical molecular ion mass of **6**.

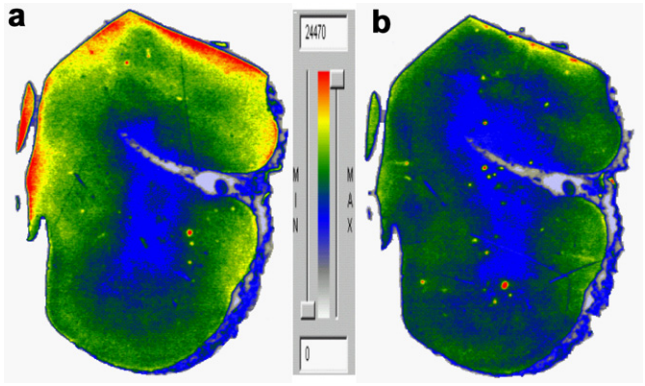


Figure 6. (a) Visualization of Aβ in postmortem brain slices of AD patients after incubation with **6** and (b) in the presence of ThT (1 μM).

Table 1. Biodistribution of **6** in normal mice at 2 min and 60 min pi ($n = 4$)

% ID	2 min pi	60 min pi
Kidneys	4.4	2.2
Liver	32.6	19.2
Lungs	1.8	0.4
Intestines	5.1	24.2
Blood	18.8	5.7
Cerebrum	0.09	0.03
Cerebellum	0.11	0.03

for the poor passage of the studied ^{99m}Tc-labelled phenylbenzothiazole through the BBB might be its low in vivo stability. We studied the percentage of intact tracer in plasma of normal mice ($n = 4$)[28] and found that at 2 min pi already 85% of the compound is converted into a polar radiolabelled metabolite, so only a small percentage of the intact tracer remains available for brain uptake. Surprisingly, the same percentage of intact tracer was found in plasma at 10, 30, and 60 min pi, indicating a very rapid initial metabolism but no further degradation of the residual intact fraction.

In summary, the synthesis of the newly developed ^{99m}Tc-[2-(4′-aminophenyl)-1,3-benzothiazole]-BAT conjugate was successful and its structure was confirmed using radio-LC–MS. It was demonstrated that the new radiolabelled conjugate binds in vitro to amyloid β. Despite its high lipophilicity and neutral character, the radiolabelled conjugate did not cross the BBB to a sufficient degree and thus is not useful for in vivo detection of AD. Further evaluation of this ^{99m}Tc-labelled tracer agent is required to investigate its potential usefulness to visualize amyloid plaques in peripheral amyloidosis.

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15. Compound **2**: ^{1}H NMR ($CDCl_3$): δ 1.3 (3H, t); δ 1.38 (9H, s); δ 2.27–2.58 (8H, m); δ 2.90–3.04 (6H, m); δ 4.1 (2H, q); δ 7.1–7.4 (30H, m). Mass: $[M+H]^+$ 851 (calcd: 851).
16. Compound **3**: ^{1}H NMR ($CDCl_3$): δ 1.35 (9H, s); δ 2.25–2.32 (8H, m); δ 2.81–2.97 (6H, m); δ 7.16–7.42 (30H, m). Mass: $[M+H]^+$ 823 (calcd: 823).
17. Compound **4**: ^{1}H NMR (DMSO): δ 5.8 (2H, s); δ 6.68 (2H, d); δ 7.32 (1H, t); δ 7.45 (1H, t); δ 7.76 (2H, d); δ 7.89 (1H, d); δ 7.98 (1H, d). Mass: $[M-H]^-$ 225 (calcd: 225).
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19. Compound **5**: ^{1}H NMR ($CDCl_3$): δ 1.38 (9H, s); δ 2.27–2.58 (8H, m); δ 2.97 (6H, m); δ 7.1–7.6 (36H, m); δ 7.95 (1H, d); δ 8.05 (1H, d). Mass: $[M+H]^+$: 1031 (calcd: 1031).
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21. Compound **6**: A stock solution of **5** (1 mg/ml in acetonitrile) was prepared and also a labelling buffer solution consisting of a mixture of 5 ml 0.5 M phosphate buffer, pH 7, 2.5 ml 0.1 M disodium ethylenediaminetetraacetate (Na_2EDTA), and 2.5 ml NaKtartrate solution (40 mg/ml). A mixture of 200 μl of the stock solution and 60 μl of 0.5 M HCl in a labelling vial was heated in a boiling water bath for 20 min. After cooling, 15 μl $SnCl_2{\cdot}2H_2O$ solution (20 mg/5 ml 0.05 M HCl), 200 μl of labelling buffer solution, and 1 ml generator eluate containing 400–800 MBq of $^{99m}TcO_4^-$ were added. The mixture was heated again in a boiling water bath for 15 min. After cooling to room temperature, RP-HPLC analysis was performed.
22. RP-HPLC analysis of **6** was carried out using an Xterra™ RP C18 column (5 μm, 250 mm × 4.6 mm, Waters, Milford, MA, USA) with gradient mixtures of CH_3CN and 0.05 M ammonium acetate as eluent at a flow rate of 1 ml/min.
23. Combined liquid chromatography and mass spectrometry (LC–MS) was performed on a system consisting of a Waters Alliance 2690 separation module (Waters) coupled to a reverse phase Xterra™ MS C18 3.5 μm column (2.1 mm × 50 mm, Waters). The eluent was a gradient mixture of CH_3CN and 0.05 M ammonium acetate at a flow rate of 0.3 ml/min. The eluate was monitored for UV-absorbance (Waters 2487 Dual wavelength absorbance detector) and radioactivity (3-in. NaI(Tl)-crystal coupled to a single channel analyzer, The Nucleus, Oak Ridge, TE, USA) and then analyzed using a time-of-flight mass spectrometer (LCT, Micromass, Manchester, England) equipped with an electrospray ionization (ESI) source. Acquisition and processing of data was performed with Masslynx software (version 3.5).
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25. All animal experiments and the use of AD brain slices were approved by the local Ethical Committee.
26. Paraffinized postmortem brain sections of AD patients were incubated for 1 h with radiolabelled HPLC-purified **6** in the absence or presence of ThT (1 μM) after deparaffinization (toluene 3 × 3 min, ethanol 3 × 3 min). After rinsing three times with 40% ethanol and once with water, the sections were allowed to dry at room temperature and exposed overnight to a high-performance storage phosphor screen (Packard, Meriden, CT, USA) which was analyzed in a phosphor imaging system (Cyclone™, Packard) using Packard Optiquant software (Packard). Non-specific binding was determined in the presence of 1 μM ThT.
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28. Mice were injected iv with intact HPLC-purified radiolabelled **6** and sacrificed by decapitation at 2, 10, 30 or 60 min pi. Blood was collected and centrifuged. Plasma was injected on a Chromolith™ column (Merck, Darmstadt, Germany), eluted with a gradient mixture of 0.05 M NH_4OAc and CH_3CN at a flow rate of 1 ml/min, and the HPLC eluate was collected in 1 ml fractions of which the radioactivity was measured using a gamma counter.