^{99m}Tc/Re complexes based on flavone and aurone as SPECT probes for imaging cerebral β-amyloid plaques

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Abstract

Two ^{99m}Tc/Re complexes based on flavone and aurone were tested as potential probes for imaging β-amyloid plaques using single photon emission computed tomography. Both ^{99m}Tc-labeled derivatives showed higher affinity for Aβ(1-42) aggregates than did ^{99m}Tc-BAT. In sections of brain tissue from an animal model of AD, the Re-flavone derivative **9** and Re–aurone derivative **19** intensely stained β-amyloid plaques. In biodistribution experiments using normal mice, ^{99m}Tc-labeled flavone and aurone displayed similar radioactivity pharmacokinetics. With additional modifications to improve their brain uptake, ^{99m}Tc complexes based on the flavone or aurone scaffold may serve as probes for imaging cerebral β-amyloid plaques

Key words: Alzheimer's disease, β-amyloid plaque, Tc-99m, single photon emission computed tomography (SPECT), imaging.

Alzheimer's disease (AD) is a neurodegenerative disorder of the brain associated with irreversible cognitive decline, memory impairment, and behavioral changes. Currently, the only definitive confirmation of AD is by postmortem histopathological examination of β-amyloid plaques in the brain. The early appraisal of clinical symptoms for a diagnosis of AD is often difficult and unreliable. Numerous reports suggest the accelerated accumulation of β-amyloid plaques in the brain to be a key risk factor associated with AD. Consequently, the detection of individual β-amyloid plaques *in vivo* by single photon emission tomography (SPECT) or positron emission tomography (PET) should improve the diagnosis of and also accelerate the discovery of effective therapeutic agents for AD[1-4]. Many PET/SPECT probes for imaging β-amyloid based on Congo Red, thioflavin T, and DDNP have been reported. Among them, [11C]PIB[5,6,] [11C]SB-13[7,8], [18F]BAY94-9172[9,10], [11C]BF-227[11], [18F]FDDNP[12,13], [123I]IMPY[14,16], and [18F]AV-45[17,18] have been tested clinically and demonstrated potential utility. There are more SPECT scanners than PET imaging devices installed for routine clinical imaging, which provides a certain advantage to using SPECT imaging agents. Since SPECT is more valuable than PET in terms of routine diagnostic use, the development of more useful Aβ imaging agents for SPECT has been a critical issue. Although many radioiodinated SPECT imaging agents for detecting β-amyloid plaques have been reported, there are few reports on the development of [99mTc] imaging agents.

^{99m}Tc ($T_{1/2}$ = 6.01 h, 141 keV) has become the most commonly used radionuclide in diagnostic nuclear medicine for several reasons: it is readily produced by an ^{99}Mo/^{99m}Tc generator, the gamma-ray energy it emits is suitable for detection, and its physical half-life is compatible with the biological localization and residence time required for imaging. Its ready availability, essentially 24 h a day, and easiness of use make it the radionuclide of choice. New ^{99m}Tc-labeled imaging agents will provide simple, convenient, and widespread SPECT-based imaging methods for detecting and eventually quantifying β-amyloid plaques in living brain tissue.

It has been reported that a dopamine transporter imaging agent, [^{99m}Tc]TRODAT-1, is useful to detect the loss of dopamine neurons in the basal ganglia associated with Parkinson's disease. This is the first example of a ^{99m}Tc imaging agent that can penetrate the blood-brain barrier via a simple diffusion mechanism and localize at sites in the central nervous system. Based on this success, efforts were made to search for comparable ^{99m}Tc imaging agents that target binding sites on β-amyloid plaques in the brain of AD patients. Several ^{99m}Tc-labeled imaging probes have been developed (Figure 1), but no clinical study of them has been reported[19-22].

Recently, we have reported that flavonoids including chalcone, flavone, and aurone serve as useful molecular scaffolds in the development of imaging agents for β-amyloid plaques in the brain[23-28]. Initially, we designed and synthesized four ^{99m}Tc-labeled

chalcone derivatives with monoamine-monoamide dithiol (MAMA) and bis-amino-bis-thiol (BAT) (Figure 1)[29]. MAMA and BAT were selected as a chelation ligand taking into consideration the permeability of the blood-brain barrier, because they form an electrically neutral complex with ^{99m}Tc[30]. ^{99m}Tc-BAT-chalcone (n=3) (Figure 1) showed good uptake into and rapid clearance from the brain in addition to high affinity for β-amyloid plaques, indicating it may be a promising probe for the detection of β-amyloid plaques in the brain[29]. Based on the positive results, we decided to further develop new ^{99m}Tc imaging agents based on the flavonoid scaffold.

In the present study, to develop more useful ^{99m}Tc imaging agents for the clinical diagnosis of AD, we synthesized two flavone and aurone derivatives with BAT as a chelation ligand. We then evaluated the biological potential of these compounds as probes by testing their affinity for Aβ aggregates and β-amyloid plaques in sections of brain tissue from *Tg2576* mice and their uptake by and clearance from the brain in biodistribution experiments using normal mice. Also, we compared their usefulness as Aβ imaging probes with a ^{99m}Tc-labeled chalcone derivative reported previously[31]. To our knowledge, this is the first time ^{99m}Tc/Re complexes based on flavone and aurone scaffolds have been proposed as probes for the detection of β-amyloid plaques in the brain.

The synthesis of the ^{99m}Tc/Re complexes based on flavone and aurone was outlined

in Schemes 1 and 2. The chelation ligand (BAT) were synthesized according to methods reported previously with some slight modifications (*26*). The most useful method of preparing flavones is known as the Baker-Venkataraman transformation[23]. A hydroxyacetophenone was first converted into a benzoyl ester (**1**) which was then treated with a base, forming a 1,3-diketone (**2**). Treatment of this diketone with acid led to the generation of the desired flavone (**3**). The free amino derivative **4** was readily prepared from **3** by reduction with $SnCl_2$ (92% yield). Conversion of **4** to the dimethylamino derivative **5** was achieved by a method reported previously (83% yield). **5** was converted to **6** by demethylation with BBr_3 in CH_2Cl_2 (40% yield). The reaction of dibromopentane with **6** produced the flavone derivative **7** with a trimethine group. Then, **7** was joined to Tr-Boc-BAT to generate **8** (the precursor of ^{99m}Tc/Re reaction). The target aurone derivatives were prepared as shown in Scheme 2. The synthesis of the aurone backbone was achieved via an Aldol reaction of benzofuranones with benzaldehydes using Al_2O_3. 5-Methozy-3-benzofuranone (**14**) was reacted with 4-dimethybenzaldehyde in the presence of Al_2O_3 in chloroform at room temperature to form **15** in a yield of 92%. The precursor of the reaction with ^{99m}Tc/Re, **18**, was obtained as described for the synthesis of the flavone derivative **8**. After deprotection of the thiol groups in **8** and **18** in TFA and triethylsilane, the Re complexes (**9** and **19**) were prepared through a reaction with $(PPh_3)_2ReOCl_3$. The corresponding ^{99m}Tc

complexes, **10** ([^{99m}Tc]BAT-FL) and **20** ([^{99m}Tc]BAT-AR), were prepared by a ligand

exchange reaction employing the precursor ^{99m}Tc-glucoheptonate (GH). The resulting

mixture was analyzed by reversed-phase HPLC, showing that a single radioactive

complex formed with radiochemical purity higher than 95% after purification by HPLC.

The identity of the complex was established by comparative HPLC using the

corresponding Re complexes as a reference (Table 1). The retention times for

[^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR on HPLC (radioactivity) were 11.1 and 16.6 min,

respectively. The retention times of the corresponding Re complexes on HPLC (UV

detection) were 9.5 and 14.6 min, respectively.

In vitro binding experiments to evaluate the affinity of [^{99m}Tc]BAT-FL and

[^{99m}Tc]BAT-AR for Aβ(1-42) aggregates were carried out in solutions. The percent

radioactivity of [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR bound to aggregates increased

dependent on the dose of Aβ(1-42), while [^{99m}Tc]BAT showed no marked affinity for

the aggregates (Figure 2). At all concentrations of Aβ aggregates, [^{99m}Tc]BAT-AR

showed significantly greater affinity than [^{99m}Tc]BAT-FL. In these binding experiments,

the non-specific binding of [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR was estimated at

1.62-1.85%. The affinity of [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR was less than that of

^{99m}Tc-labeled chalcone derivatives reported previously (Figure 1)[31]. The order in terms

of strength of binding corresponded with that of radioiodinated flavonoids[23-25],

indicating that the scaffolds of the [^{99m}Tc]BAT complexes play an important role in the affinity for Aβ aggregates.

To confirm the affinity for β-amyloid plaques in the mouse brain, neuropathological fluorescent staining with Re derivatives (**9** and **19**) was carried out using *Tg2576* mouse brain sections (Figure 3). Many β-amyloid plaques were clearly stained with the derivatives (Figure 3-A and B), as reflected by the high affinity for Aβ aggregates in binding assays *in vitro*. The labeling pattern was consistent with that observed with thioflavin S (Figure 3-C and D). These results suggest that [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR would bind to β-amyloid plaques in the mouse brain in addition to having affinity for synthetic Aβ(1-42) aggregates. Although [^{99m}Tc]BAT-AR showed greater affinity than [^{99m}Tc]BAT-AR in the binding assays *in vitro*, no marked difference in binding between [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR was observed in the fluorescent staining experiments.

[^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR were examined as to their biodistribution in normal mice (Table 2). A biodistribution study provides important information on brain uptake. The ideal probe for imaging β-amyloid should penetrate the blood-brain barrier well enough to deliver a sufficient dose into the brain while clearing rapidly from normal regions so as to achieve a high signal to noise ratio in the AD brain. Previous studies suggest that the optimal lipophilicity for entry into the brain is obtained with log

P values of between 1 and 3. [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR had log P values of

2.77 and 2.23, respectively (Table 1), but showed less uptake, 0.64 and 0.79 %ID/g at 2

min postinjection, than expected. Thereafter, the radioactivity of [^{99m}Tc]BAT-FL and

[^{99m}Tc]BAT-AR that accumulated in the brain was rapidly eliminated (0.23 and

0.11%ID/g at 60 min postinjection). Recently, we have reported that the ^{99m}Tc-labeled

chalcone derivative showed high uptake (1.48%ID/g at 2 min postinjection) into and

rapid clearance (0.17%ID/g at 60 min postinjection) from the brain, a highly desirable

property for imaging agents for β-amyloid plaques[29]. The pharmacokinetics of the

^{99m}Tc-labeled chalcone derivative in the brain appears superior to that of any

^{99m}Tc-labeled probes reported previously, indicating that this compound should be

investigated further as a potentially useful probe for imaging β-amyloid. Compared with

that of the ^{99m}Tc-labeled chalcone[31], the radioactivity of [^{99m}Tc]BAT-FL and

[^{99m}Tc]BAT-AR appears insufficient for the imaging of β-amyloid plaques in the brain.

Since the affinity of [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR for Aβ aggregates was as

high as that of ^{99m}Tc-labeled chalcone derivatives[29], improvement of the uptake of

[^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR is an important prerequisite to developing more

useful ^{99m}Tc-labeled probes. Therefore, additional structural changes in the flavone and

aurone scaffold are needed to further improve the pharmacokinetics of [^{99m}Tc]BAT-FL

and [^{99m}Tc]BAT-AR *in vivo*.

In conclusion, we successfully designed and synthesized novel ^{99m}Tc/Re complexes based on flavone and aurone for the detection of β-amyloid plaques in the brain. Both [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR showed high affinity for synthetic Aβ(1-42) aggregates. In experiments *in vitro* using sections of brain from *Tg2576* mice, Re-complexes intensely stained β-amyloid plaques. In addition, [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR displayed good uptake into and a rapid washout from the brain after their injection in normal mice. This combination of affinity for β-amyloid plaques, and good uptake and clearance makes [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR promising probes for the detection of β-amyloid plaques in the brain, although additional modifications are required to enhance their uptake. The results of the present study should provide information useful for the development of ^{99m}Tc-labeled probes for the imaging of β-amyloid plaques in the brain.

Supporting Information

Procedure for the preparation of $_{99m}$Tc/Re complexes, *in vitro* binding assay, *in vitro* fluorescent staining using Tg2576 mouse brain sections, and biodistribution experiments.

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Table 1. HPLC retention times of ^{99m}Tc/Re compounds and log P of ^{99m}Tc compounds.

Re compounds	Retention time (min)[a]	^{99m}Tc compounds	Retention time (min)[a]	Log P of ^{99m}Tc compounds[b]
9	9.5	**10**	11.1	2.77 ± 0.04
19	14.6	**20**	16.6	2.23 ± 0.04

[a]Reversed-phase HPLC using a mixture of H_2O-acetonitrile (2:3) as a mobile phase.

[b]The measurement was done in triplicate and repeated three times. Each value represents the mean $\pm$ SD for three independent experiments.

Table 2. Biodistribution of radioactivity after injection of [^{99m}Tc]FL and [^{99m}Tc]AR in normal mice[a].

Organ	Time after injection (min)			
	2	10	30	60
[^{99m}Tc]BAT-FL				
Blood	1.90 (0.08)	0.80 (0.16)	0.41 (0.06)	0.28 (0.06)
Liver	19.35 (1.30)	24.75 (3.45)	27.73 (3.30)	24.12 (3.08)
Kidney	9.70 (0.83)	5.56 (0.84)	2.38 (0.30)	1.40 (0.20)
Intestine [b]	4.54 (0.42)	11.36 (1.88)	26.61 (3.93)	42 67 (2.98)
Spleen	3.24 (0.61)	2.21 (0.31)	1.04 (0.42)	0.45 (0.07)
Lung	11.42 (2.10)	3.84 (0.57)	1.70 (0.24)	1.07 (0.16)
Stomach [b]	0.90 (0.15)	1.36 (0.55)	1.52 (0.67)	2.45 (1.04)
Pancreas	4.41 (0.29)	4.31 (0.35)	1.89 (0.15)	0.84 (0.17)
Heart	12.00 (1.16)	3.12 (0.51)	0.99 (0.18)	0.44 (0.09)
Brain	0.64 (0.07)	0.57 (0.14)	0.36 (0.01)	0.23 (0.04)
[^{99m}Tc]BAT-AR				
Blood	1.56 (0.16)	0.71 (0.07)	0.35 (0.04)	0.21 (0.04)
Liver	17.76 (1.51)	17.77 (1.70)	15.17 (0.95)	12.96 (1.48)

Kidney	11.50 (0.73)	8.77 (1.15)	4.83 (0.77)	3.28 (1.52)
Intestine [b]	6.78 (0.78)	26.20 (2.45)	46.06 (3.17)	55.33 (7.42)
Spleen	2.87 (0.30)	1.92 (0.47)	0.70 (0.07)	0.35 (0.15)
Lung	6.10 (1.15)	3.25 (0.78)	1.63 (0.42)	0.85 (0.18)
Stomach [b]	1.03 (0.13)	1.63 (0.25)	1.88 (0.11)	1.69 (0.49)
Pancreas	5.85 (1.09)	4.20 (0.68)	1.53 (0.54)	0.60 (0.30)
Heart	12.30 (1.21)	3.26 (0.43)	1.15 (0.30)	0.40 (0.09)
Brain	0.79 (0.12)	0.70 (0.05)	0.27 (0.06)	0.11 (0.04)

[a]Each value represents the mean (SD) for 3–6 mice at each interval. Expressed as % injected dose per gram. [b]Expressed as % injected dose per organ.
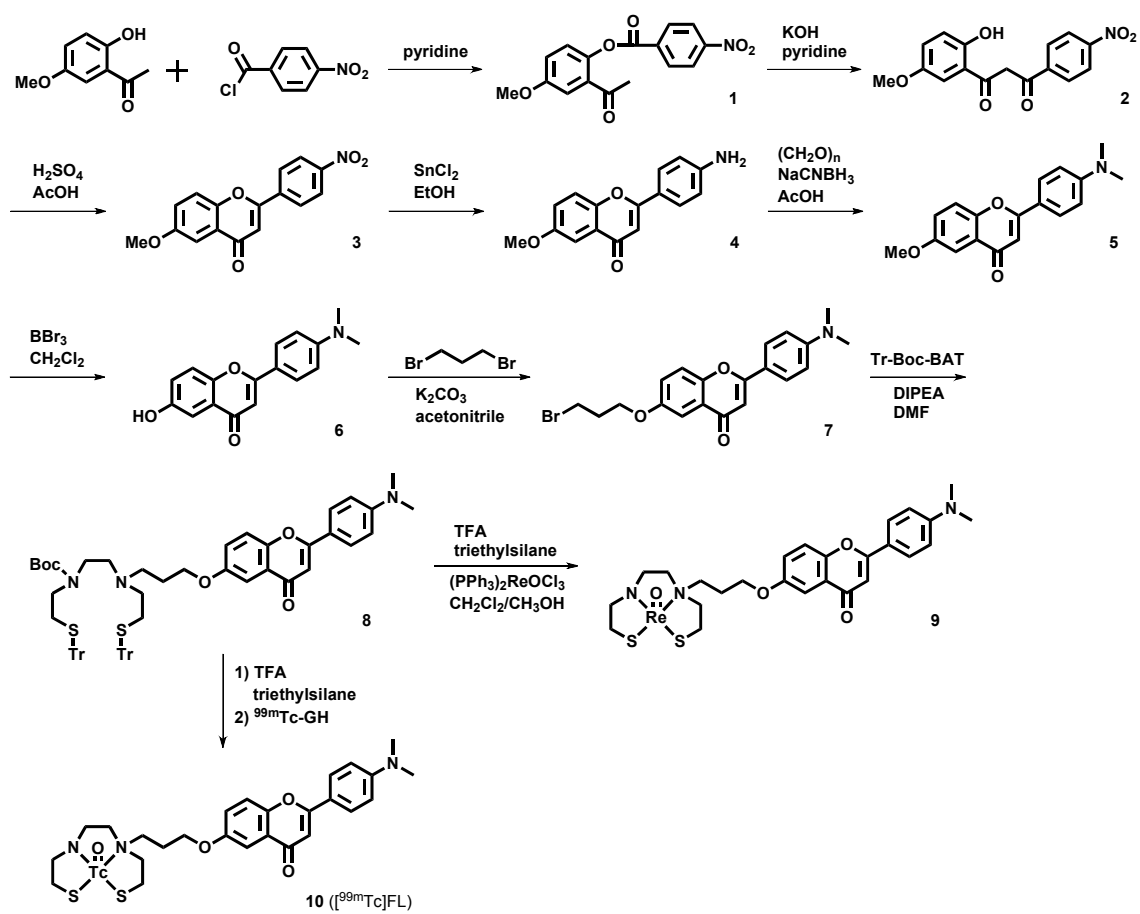
Scheme 1. Synthesis of flavone derivatives.
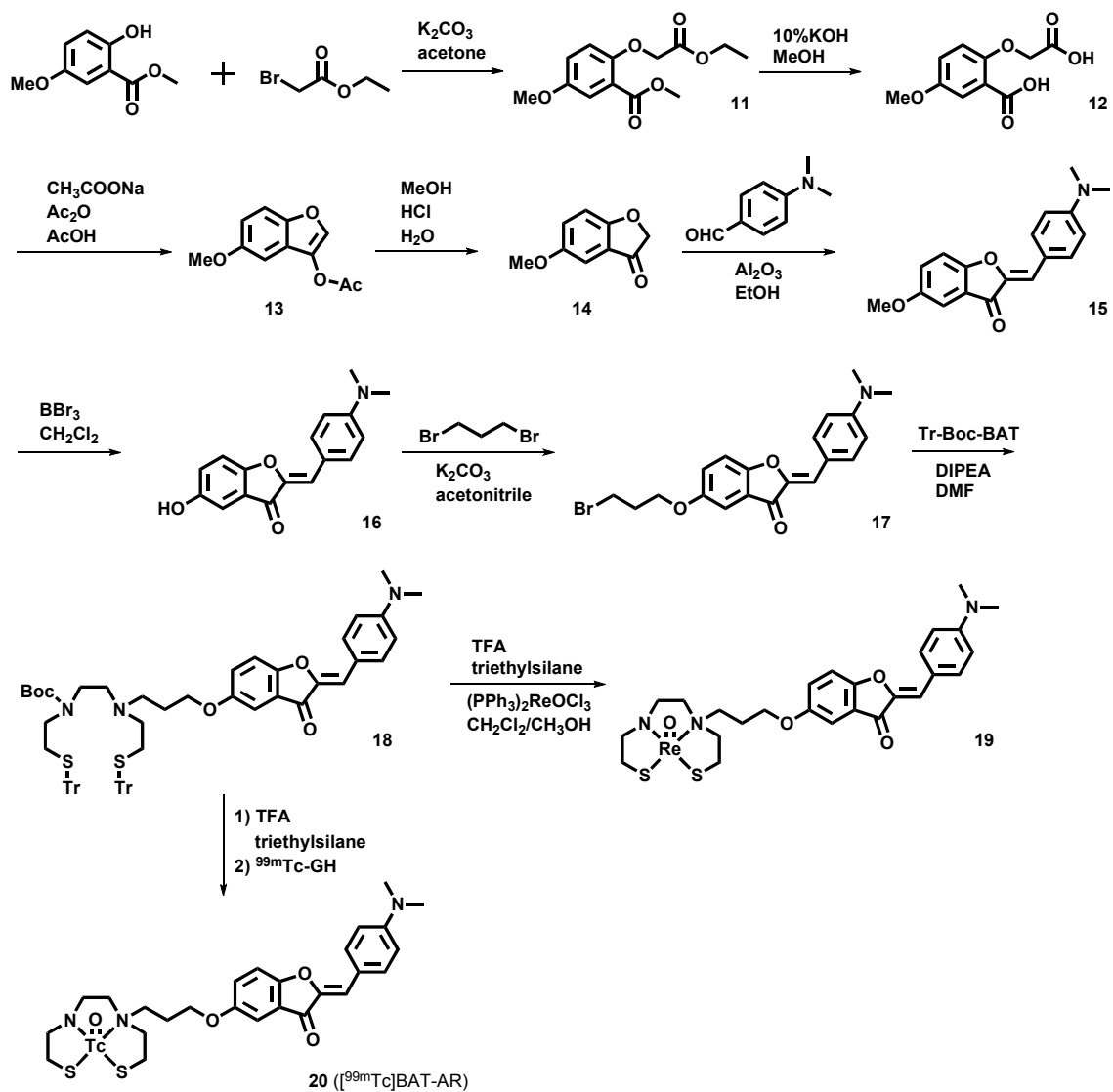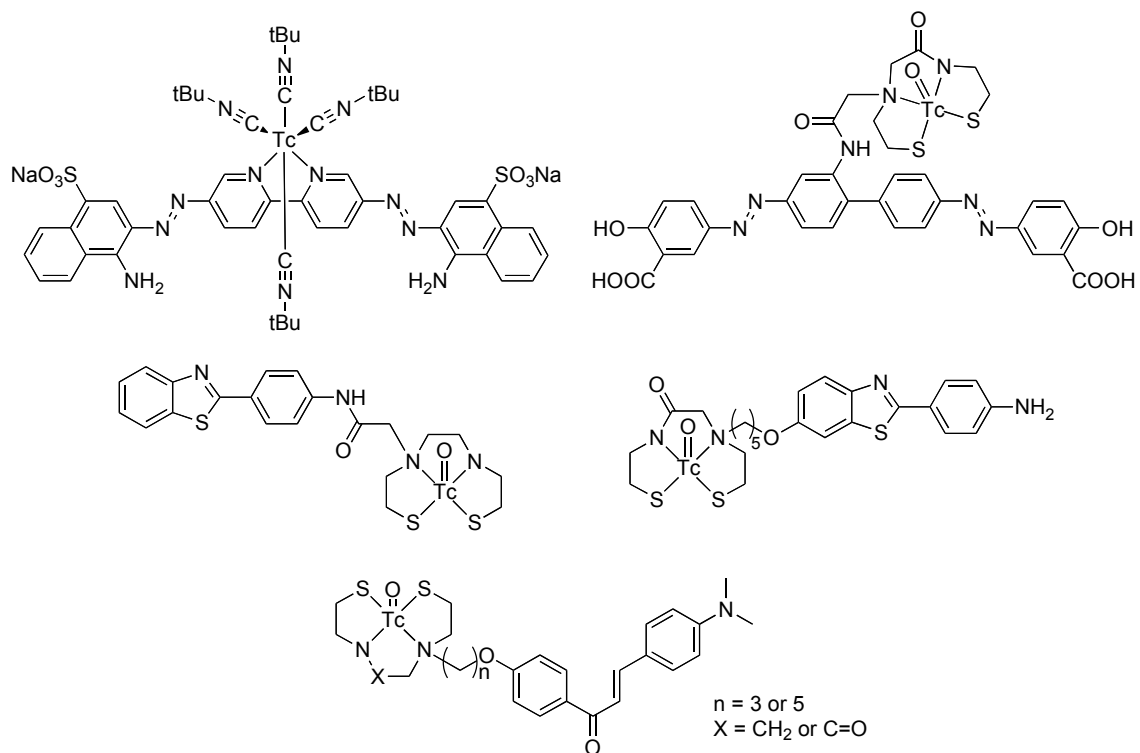
Scheme 2. Synthesis of aurone derivatives.

Figure 1. Chemical structure of ^{99m}Tc-labeled Aβ imaging probes reported previously.
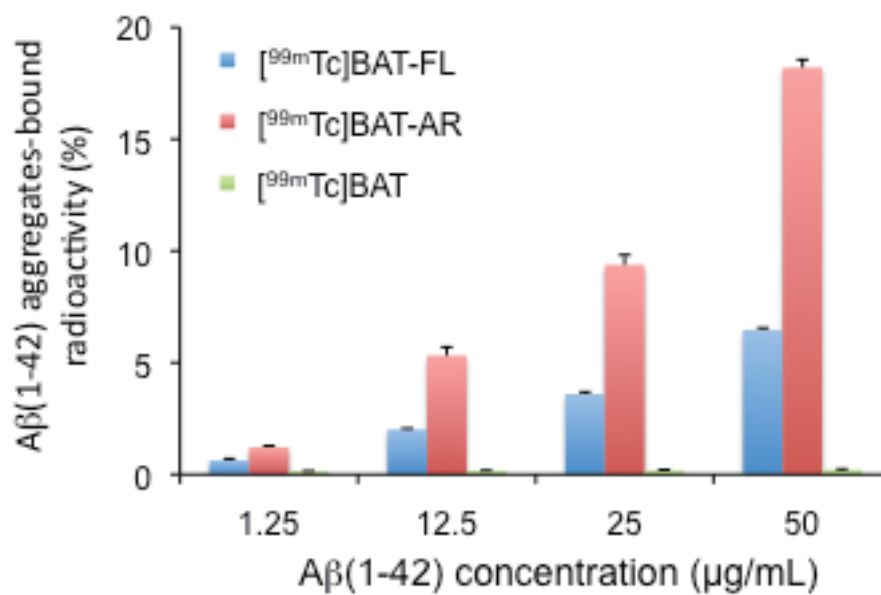
Figure 2. Binding assay of [^{99m}Tc]BAT-FL, [^{99m}Tc]BAT-AR, and [^{99m}Tc]BAT with Aβ(1-42) aggregates. Values are the mean ± standard error of the mean for three independent experiments.
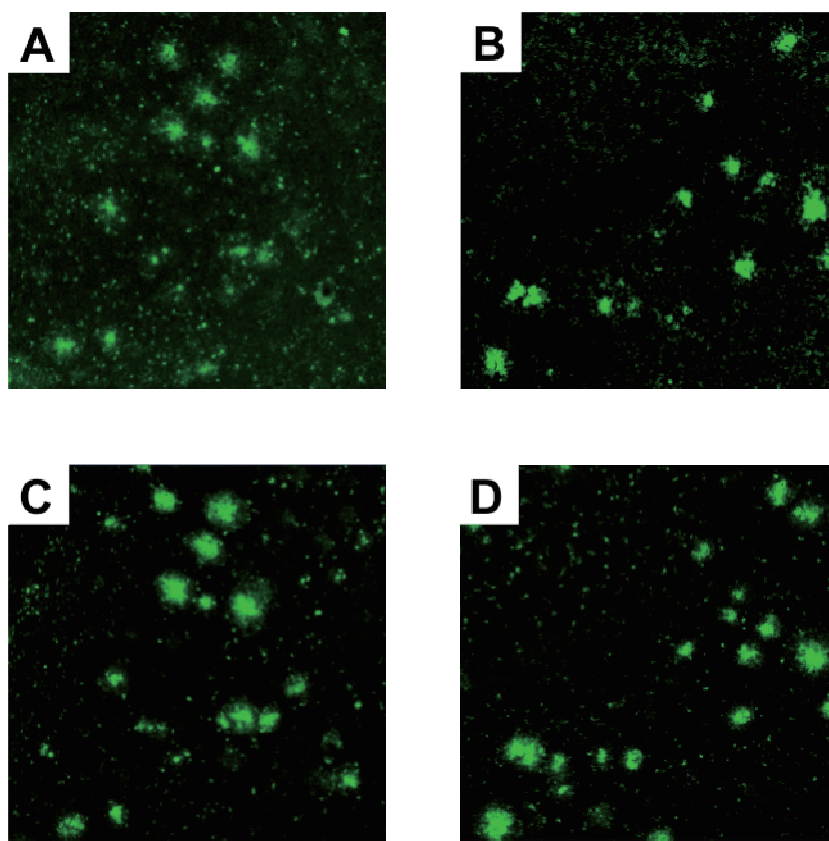
Figure 3. Fluorescent staining of the flavone derivative **9** (A), and aurone derivative **19** (B) in *Tg2576* mouse brain. Labeled plaques were confirmed by staining the adjacent sections with thioflavin-S (C and D).

Experimental

General

All reagents were obtained commercially and used without further purification unless otherwise indicated. ^{1}H NMR spectra were obtained on a Varian Gemini 300 spectrometer with TMS as an internal standard. Coupling constants are reported in hertz. Multiplicity was defined by s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), and m (multiplet). Mass spectra were obtained on a JEOL IMS-DX. HPLC was performed with a Shimadzu system (a LC-10AT pump with a SPD-10A UV detector, λ= 254 nm) using a Cosmosil C18 (Nakalai Tesque, 5C18-AR-II, 4.6×150 mm) and acetonitrile/water (3/2) as the mobile phase delivered at a flow rate of 1.0 mL/min. The ^{99m}Tc-chalcone derivative was synthesized according to a method reported previously. Compounds **9** and **19** were proven by this method to show >95% purity.

Chemistry

2-Acetyl-4-methoxyphenyl 4-nitrobenzoate (1).

To a stirring solution of 2'-hydroxy-5'-methoxyacetophenone (1.66 g, 10 mmol) in pyridine (25 mL) was added 4-nitrobenzoyl chloride (1.86 g, 10 mmol). The reaction mixture was stirred at room temperature for 3 h, and poured into a 1 M aqueous HCl / ice solution with vigorous stirring. The precipitate that formed was filtered and washed

with water to yield acetophenone **2** (2.66 g, 85% yield). ^{1}H NMR (300 MHz, CDCl$_3$) δ

2.54 (s, 3H), 3.89 (s, 3H), 7.14–7.16 (m, 2H), 7.38 (d, J = 2.7 Hz, 1H), 8.37 (s, 4H).

1-(2-Hydroxy-5-methoxyphenyl)-3-(4-nitrophenyl)propane-1,3-dione (2).

A solution of **1** (2.66 g, 8.45 mmol) and pyridine (25 mL) was heated to 50 , and

pulverized potassium hydroxide (2.37 g, 42.3 mmol) was added. The reaction mixture

was stirred for 90 min, and when it had cooled, a 10% aqueous acetic acid solution was

added. The yellow precipitate that formed was filtered to yield **2** (2.08 g, 78% yield). [1]H

NMR (300 MHz, CDCl$_3$) δ 3.85 (s, 3H), 6.84 (s, 1H), 6.99 (d, J = 8.7 Hz, 1H),

7.14–7.18 (m, 1H), 7.22 (d, J = 3.0 Hz, 1H), 8.10 (d, J = 9.0 Hz, 2H), 8.35 (d, J = 9.3

Hz, 2H), 11.50 (s, 1H).

6-Methoxy-2-(4-nitrophenyl)-4*H*-chromen-4-one (3).

A mixture of the diketone **2** (2.08 g, 6.59 mmol), concentrated sulfuric acid (2 mL), and

glacial acetic acid (30 mL) was heated at 100 for 2 h and cooled to room temperature.

The mixture was poured into cold water, and the resulting precipitate was filtered to

yield **3** (1.98 g, 100%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 3.92 (s, 3H), 6.91 (s, 1H),

7.33–7.37 (m, 1H), 7.55 (d, J = 9.0 Hz, 1H), 7.61 (d, J = 3.0 Hz, 1H), 8.11 (d, J = 9.0

Hz, 2H), 8.39 (d, J = 9.3 Hz, 2H).

2-(4-Aminophenyl)-6-methoxy-4*H*-chromen-4-one (4).

A mixture of **3** (1.98 g, 6.67 mmol), SnCl$_2$ (6.33 g, 33.4 mmol), and EtOH (20 mL) was stirred at 100   for 1 h. After the mixture had cooled to room temperature, 1 M NaOH was added until the solution became alkaline. After extraction with ethyl acetate, the combined organic layers were dried over Na$_2$SO$_4$ and filtered. The solvent was removed to give 1.63 g of **4** (92% yield). ^{1}H NMR (300 MHz, CDCl$_3$) δ 3.91 (s, 3H), 4.08 (s, broad, 2H), 6.70 (s, 1H), 6.76 (d, *J* = 9.0 Hz, 2H), 7.24–7.28 (m, 1H), 7.47 (d, *J* = 9.3 Hz, 1H), 7.60 (d, *J* = 3.0 Hz, 1H), 7.75 (d, *J* = 9.0 Hz, 2H).

2-(4-(Dimethylamino)phenyl)-6-methoxy-4*H*-chromen-4-one (5).

To a mixture of **4** (1.63 g, 6.08 mmol) and paraformaldehyde (1.82 g, 60.8 mmol) in AcOH (25 mL) was added NaCNBH$_3$ (1.91 g, 30.4 mmol) in one portion at room temperature. The resulting mixture was stirred at room temperature for 1.5 h, and the addition of 1 M NaOH was followed by extraction with CHCl$_3$. The organic layer was dried over Na$_2$SO$_4$. The solvent was removed, and the residue was purified by silica gel chromatography (CHCl$_3$: MeOH = 49 : 1) to give 1.48 g of **5** (83% yield). ^{1}H NMR (300 MHz, CDCl$_3$) δ3.08 (s, 6H), 3.91 (s, 3H), 6.71 (s, 1H), 6.76 (d, *J* = 9.0 Hz, 2H), 7.23–7.27 (m, 1H), 7.47 (d, *J* = 9.3 Hz, 1H), 7.60 (d, *J* = 3.3 Hz, 1H), 7.82 (d, *J* = 9.3 Hz, 2H).

2-(4-(Dimethylamino)phenyl)-6-hydroxy-4*H*-chromen-4-one (6).

To a solution of **5** (905 mg, 3.06 mmol) in CH_2Cl_2 (20 mL) at 0 was added BBr_3 (12 mL, 1 M solution in CH_2Cl_2) dropwise in an ice bath. The mixture was allowed to warm to room temperature and stirred for 6 h. Water was added while the reaction mixture was cooled in an ice bath to keep the reaction temperature at 0 . After the addition of a 1 M aqueous HCl solution and extraction with $CHCl_3$, the combined organic layers was dried over Na_2SO_4. The filtrate was concentrated and the residue was purified by silica gel chromatography ($CHCl_3$: MeOH = 20 : 1) to give 342 mg of **6** (40% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.08 (s, 6H), 6.69 (s, 1H), 6.76 (d, *J* = 9.3 Hz, 2H), 7.25–7.27 (m, 1H), 7.46 (d, *J* = 9.0 Hz, 1H), 7.73 (d, *J* = 3.0 Hz, 1H), 7.82 (d, *J* = 9.0 Hz, 2H).

6-(3-Bromopropoxy)-2-(4-(dimethylamino)phenyl)-4*H*-chromen-4-one (7).

To a solution of **6** (342 mg, 1.22 mmol) in acetonitrile (20 mL) were added 1,3-dibromopropane (0.248 mL, 2.43 mmol) and K_2CO_3 (1 g). The reaction mixture was heated to reflux for 4 h. When the acetonitrile had evaporated, the residue was poured into a saturated NaCl solution. After extraction with $CHCl_3$, the organic layer was combined and dried with Na_2SO_4. The mixture was concentrated, and purified by silica gel chromatography ($CHCl_3$: CH_3OH = 199 : 1) to give 312 mg of **7** (64% yield). [1]H

NMR (300 MHz, CDCl$_3$) δ 2.36 (q, J = 6.2 Hz, 2H), 3.07 (s, 6H), 3.62 (t, J = 6.6 Hz, 2H), 4.22 (t, J = 5.9 Hz, 2H), 6.70 (s, 1H), 6.76 (d, J = 7.2 Hz, 2H), 7.22–7.30 (m, 1H), 7.47 (d, J = 9.3 Hz, 1H), 7.61 (d, J = 3.0 Hz, 1H), 7.81 (d, J = 9.0 Hz, 1H).

Compound 8 (Tr-BAT-C3-FL).

To a solution of **7** (529 mg, 1.27 mmol) and Tr-Boc-BAT (847 mg, 1.11 mmol) in DMF (20 mL) was added DIPEA (2 mL). The reaction mixture was heated to reflux for 12 h. After the solvent had evaporated, the residue was purified by silica gel chromatography (using CHCl$_3$ and then ethyl acetate : hexane of 1 : 1) to give 57 mg of **8** (5% yield). ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.37 (s, 9H), 1.71 (t, J = 6.5 Hz, 2H), 2.24–2.44 (m, 10H), 2.84-3.00 (m, 4H), 3.07 (s, 6H), 3.96 (t, J = 6.0 Hz, 2H), 6.70 (s, 1H), 6.76 (d, J = 9.3 Hz, 2H), 7.13–7.29 (m, 19H), 7.37–7.41 (m, 13H), 7.55 (d, J = 3.0 Hz, 1H), 7.82 (d, J = 9.0 Hz, 2H). MS m/z 1086 (MH$^+$).

Compound 10 (Re-BAT-FL).

To a solution of **8** (49 mg, 0.045 mmol) in TFA (2 mL) was added triethylsilane (0.1 mL) and mixed, and then the solvent was removed under a stream of nitrogen gas. The residue was resolved in a mixed solvent (22 mL, CH$_2$Cl$_2$:CH$_3$OH = 10:1), was added (Ph$_3$P)$_2$ReOCl$_3$ (75 mg, 0.090 mmol) and 1 M sodium acetate in methanol (4 mL). The

reaction mixture was heated to reflux for 6 h, after cooling to room temperature, mixed with ethyl acetate (60 mL) and filtered. Evaporation of the solvent gave a residue which was purified with silica gel chromatography ($CHCl_3$: CH_3OH = 49 : 1), and then RP-HPLC (acetonitorile : H_2O = 4 : 1) to give 15 mg (46%) of **10**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.75–1.85 (m, 1H), 2.26–2.32 (m, 2H), 2.76–2.81 (m, 1H), 2.96–3.05 (m, 2H), 3.08 (s, 6H), 3.24–3.49 (m, 4H), 3.75–3.98 (m, 3H), 4.17–4.39 (m, 5H), 6.71 (s, 1H), 6.76 (d, J = 8.7 Hz, 2H), 7.23–7.28 (m, 1H), 7.50 (d, J = 9.3 Hz, 1H), 7.57 (d, J = 3.0 Hz, 1H), 7.82 (d, J = 9.0 Hz, 2H). HRMS m/z $C_{26}H_{33}N_3O_4ReS_2$ found 702.1489, calcd 702.1470 (MH$^+$).

Methyl 2-((ethoxycarbonyl)methoxy)-5-methoxybenzoate (11).

To a solution of methyl 5-methoxysalicylate (1.49 mL, 10 mmol) in acetone (10 mL) were added K_2CO_3 (1 g) and ethyl bromoacetate (1.49 mL, 13.5 mmol). The mixture was stirred for 4 h under reflux. When the solvent had evaporated, the residue was dissolved in water. After extraction with ethyl acetate, the organic layer was dried over Na_2SO_4. Evaporation of the solvent gave 2.90 g of **11** (100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30 (t, J = 7.1 Hz, 3H), 3.80 (s, 3H), 3.91 (s, 3H), 4.26 (q, J = 7.1 Hz, 2H), 4.65 (s, 2H), 6.92 (d, J = 9.0 Hz, 1H), 6.98–7.02 (m, 1H), 7.35 (d, J = 3.0 Hz, 1H).

2-(Carboxymethoxy)-5-methoxybenzoic acid (12).

To a solution of **11** (2.90 g, 10.8 mmol) in methanol (10 mL) was added 10% aqueous

KOH (10 mL). The mixture was stirred for 2 h at room temperature. The product

formed by adding 1 M HCl was filtered to give 2.25 g of **12** (100%). ^{1}H NMR (300

MHz, CD$_3$OD) δ 3.79 (s, 3H), 4.77 (s, 2H), 7.08 (s, 1H), 7.10 (d, J = 3.0 Hz, 1H), 7.41

(d, J = 3.0 Hz, 1H).

5-Methoxybenzofuran-3-yl acetate (13).

A mixture of acetic anhydride (30 mL), acetic acid (6 mL), anhydrous sodium acetate

(1.5 g), and **12** (865 mg, 3.82 mmol) was heated to reflux for 6 h. Water was added, and

after extraction with ethyl acetate and drying of the organic layer over Na$_2$SO$_4$, the

solvent was removed, and the residue was purified by silica gel chromatography (ethyl

acetate : hexane = 1 : 4) to give 687 mg of **13** (87%). ^{1}H NMR (300 MHz, CDCl$_3$) δ

2.38 (s, 3H), 3.86 (s, 3H), 6.91–6.95 (m, 1H), 6.97 (d, J = 2.1 Hz, 1H), 7.34 (d, J = 9.0

Hz, 1H), 7.98 (s, 1H).

5-Methoxybenzofuran-3(2*H*)-one (14).

A mixture of **13** (815 mg, 3.95 mmol), methanol (10 mL), water (5 mL), and 1 M HCl

(2 mL) was heated to reflux for 5 h, and allowed to cool to room temperature. Water

was then added, and following extraction with CHCl$_3$, the combined organic layers were dried over Na$_2$SO$_4$ and filtered. The solvent was removed to give 621 mg of **14** (96%). ^{1}H NMR (300 MHz, CDCl$_3$) δ3.81 (s, 3H), 4.65 (s, 2H), 7.06 (s, 1H), 7.08 (d, *J* = 5.4 Hz, 1H), 7.24 (d, *J* = 3.0 Hz, 1H).

(*Z*)-2-(4-(Dimethylamino)benzylidene)-5-methoxybenzofuran-3(2*H*)-one (15).

To a solution of **14** (246 mg, 1.50 mmol) and 4-dimethylaminobenzaldehyde (224 mg, 1.50 mmol) in EtOH (20 mL) was added Al$_2$O$_3$ (2.7 g). The mixture was stirred for 24 h at room temperature. After filtration of the reaction mixture, the solvent of the filtrate was removed, and the residue was purified by silica gel chromatography (ethyl acetate : hexane = 1 : 2) to give 405 mg of **15** (92%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 3.08 (s, 6H), 3.84 (s, 3H), 6.75 (d, *J* = 9.0 Hz, 2H), 6.92 (s, 1H), 7.23 (s, 3H), 7.84 (d, *J* = 9.0 Hz, 2H).

(*Z*)-2-(4-(Dimethylamino)benzylidene)-5-hydroxybenzofuran-3(2*H*)-one (16).

BBr$_3$ (7 mL, 1 M solution in CH$_2$Cl$_2$) was added to a solution of **15** (405 mg, 1.37 mmol) in CH$_2$Cl$_2$ (10 mL) dropwise in an ice bath. The mixture was allowed to warm to room temperature and stirred for 12 h. Water was added while the reaction mixture was cooled in an ice bath to keep the reaction temperature at 0 . The mixture was poured

into a 1 M aqueous HCl solution and after extraction with CHCl$_3$, the combined organic phase was dried over Na$_2$SO$_4$. The filtrate was concentrated and the residue was purified by silica gel chromatography (CHCl$_3$: MeOH = 20 : 1) to give 186 mg of **16** (48%). ^{1}H NMR (300 MHz, CD$_3$OD) δ 3.08 (s, 6H), 6.81 (d, J = 9.3 Hz, 2H), 6.87 (s, 1H), 7.07 (d, J = 2.7 Hz, 1H), 7.16–7.20 (m, 1H), 7.27 (d, J = 9.0 Hz, 1H), 7.85 (d, J = 9.0 Hz, 2H).

(*Z*)-5-(3-Bromopropoxy)-2-(4-(dimethylamino)benzylidene)benzofuran-3(2*H*)-one (17).

To a solution of **16** (432 mg, 1.54 mmol) in acetonitrile (25 mL) were added 1,3-dibromopropane (0.313 mL, 3.07 mmol) and K$_2$CO$_3$ (1 g). The reaction mixture was heated to reflux for 4 h. When the acetonitrile had evaporated, the residue was poured into a saturated NaCl solution. After extraction with CHCl$_3$, the organic layer was combined and dried with Na$_2$SO$_4$. The mixture was concentrated, and purified by silica gel chromatography using CHCl$_3$ to give 410 mg of **17** (66% yield). ^{1}H NMR (300 MHz, CDCl$_3$) δ 2.33 (q, J = 6.1 Hz, 2H), 3.05 (s, 6H), 3.61 (t, J = 6.5 Hz, 2H), 4.12 (t, J = 5.9 Hz, 2H), 6.73 (d, J = 9.0 Hz, 2H), 6.90 (s, 1H), 7.21–7.23 (m, 3H), 7.83 (d, J = 9.0 Hz, 2H).

Compound 18 (Tr-BAT-AR).

To a solution of **17** (410 mg, 1.02 mmol) and Tr-Boc-BAT (728 mg, 0.952 mmol) in DMF (20 mL) was added DIPEA (0.4 mL). The reaction mixture was heated to reflux for 12 h. When the solvent had evaporated, a saturated NaCl solution was added and after extraction with $CHCl_3$, the organic layers were combined and dried with Na_2SO_4. Evaporation of the solvent gave a residue which was purified by silica gel chromatography (using $CHCl_3$ and then ethyl acetate : hexane of 1 : 2) to give 176 mg of **18** (16% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ1.38 (s, 9H), 1.69 (quin, $J = 6.3$ Hz, 2H), 2.22–2.37 (m, 10H), 2.81–2.99 (m, 4H), 3.04 (s, 6H), 3.88 (t, $J = 6.0$ Hz, 2H), 6.73 (d, $J = 9.3$ Hz, 2H), 6.90 (s, 1H), 7.12–7.28 (m, 21H), 7.36–7.41 (m, 12H), 7.83 (d, $J = 9.0$ Hz, 2H). MS m/z 1086 (MH^+).

Compound 20 (Re-BAT-AR).

To a solution of **18** (54 mg, 0.050 mmol) in TFA (2 mL) was added triethylsilane (0.1 mL) and mixed, and then the solvent was removed under a stream of nitrogen gas. The residue was resolved in a mixed solvent (22 mL, CH_2Cl_2:CH_3OH = 10:1), and $(Ph_3P)_2ReOCl_3$ (83 mg, 0.100 mmol) and 1 M sodium acetate in methanol (4 mL) were added. The reaction mixture was heated to reflux for 6 h, then cooled to room temperature. Ethyl acetate (60 mL) was added and the mixture was filtered. Evaporation

of the solvent gave a residue which was purified with silica gel chromatography

(CHCl$_3$: CH$_3$OH = 49 : 1), and then RP-HPLC (acetonitorile : H$_2$O = 4 : 1) to give 10

mg (29%) of **20**. ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.75–1.81 (m, 1H), 2.31–2.36 (m, 2H),

2.76–2.81 (m, 1H), 2.96–3.04 (m, 2H), 3.08 (s, 6H), 3.25–3.49 (m, 4H), 3.78–3.90 (m,

3H), 4.09–4.36 (m, 5H), 6.75 (d, J = 9.0 Hz, 2H), 6.93 (s, 1H), 7.20–7.29 (m, 3H), 7.84

(d, J = 9.0 Hz, 2H). HRMS m/z C$_{26}$H$_{33}$N$_3$O$_4$ReS$_2$ found 702.1442, calcd 702.1470 (MH

$^+$).

^{99m}Tc labeling reaction.

To a solution of sodium heptonate dehydrate (2 g, 7.04 mmol) in nanopure water (25

µL) was added 0.75 µL of a SnCl$_2$·2H$_2$O solution [12 mg of Tin ( ) chloride dehydrate

(53.2 mmol) dissolved in 15 µL of 0.1 M HCl]. This solution was adjusted to pH

8.5–9.0 using a small amount of 0.1 M NaOH, and then lyophilized to give Sn

glucoheptonate (SnGH) kit. SnGH kit (1 mg) was added to a Na^{99m}TcO$_4$ solution (200

µL) and reacted at room temperature for 10 min to give a ^{99m}TcGH solution. To

solutions of **8** and **18** (0.5 mg) in TFA (200 µL) was mixed in triethylsilane (10 µL),

and mixed, and the solvents were removed under a stream of nitrogen gas. The residues

were resolved in acetonitrile (200 µL), and 0.1 M HCl (15 µL) and the ^{99m}TcGH

solution (200 µL) were added. The reaction mixtures were heated to 80–90 ˚C for 10

min. After cooling to room temperature, the mixtures were purified with RP-HPLC to give [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR. [^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR were analyzed by analytical RP-HPLC on a Cosmosil C$_{18}$ column with an isocratic solvent of H$_2$O/acetonitrile (2/3) at a flow rate of 1.0 mL/min. The absorption of the complexes was measured at 254 nm and the radioactivity of the ^{99m}Tc-labeled form was recorded for 30 min.

Binding assays using the aggregated Aβ peptide in solution

A solid form of Aβ(1–42) was purchased from Peptide Institute (Osaka, Japan). Aggregation was achieved by gently dissolving the peptide (0.25 mg/mL) in a buffer solution (pH 7.4) containing 10 mM sodium phosphate and 1 mM EDTA. The solution was incubated at 37 ˚C for 42 h with gentle and constant shaking. The binding assay was performed by mixing 50 µL of the Aβ(1–42) aggregates (0–100 µg/mL), 50 µL of an appropriate concentration of the ^{99m}Tc-labeled form ([^{99m}Tc]BAT-FL and [^{99m}Tc]BAT-AR), and 900 µL of 30% ethanol in 12 × 75 mm borosilicate glass tubes. Nonspecific binding was defined in the presence of 1 µM of a chalcone derivative (4-dimethylamino-4'-iodo-chalcone). After incubation for 3 h at room temperature, the binding mixture was filtered through GF/B filters (Whatman, Kent, UK) using a M-24 cell harvester (Brandel, Gaithersburg, MD). Filters containing the bound ^{99m}Tc-labeled

form were measured for radioactivity in a gamma counter (Perkin Elmer, WIZARD^2 2470).

In vivo biodistribution in normal mice

The experiments with animals were conducted in accordance with our institutional guidelines and approved by the Nagasaki University Animal Care Committee. A saline solution (100 µL) of radiolabeled agents (1.0×10^7 cpm/100 µL) containing ethanol (30 µL) was injected intravenously directly into the tail of ddY mice (5 weeks old, 22–25 g). The mice were sacrificed at various time points postinjection. The organs of interest were removed and weighed, and radioactivity was measured with an automatic gamma counter (Perkin Elmer, WIZARD^2 2470).

Neuropathological staining of model mouse brain sections

The *Tg2576* transgenic mice (female, 30-month-old) were used as the Alzheimer's model. After the mice were sacrificed by decapitation, the brains were immediately removed and frozen in powdered dry ice. The frozen blocks were sliced into serial sections, 10 mm thick. Each slide was incubated with a 50% EtOH solution (2.5-10 µM) of **10** and **20** for 2–9 h. The sections were washed in 50% EtOH for 5 min two times, and **10** was examined with excitation at 458 nm and **20** with excitation at 488 nm under

a microscope (Carl Zeiss, LSM710). Thereafter, the sections were stained with thioflavin S, a pathological dye commonly used for staining Aβ plaques in the brain.

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