Med Chem Res
DOI 10.1007/s00044-012-0414-2

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and evaluation of thioflavin-T analogs as potential imaging agents for amyloid plaques

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Received: 19 September 2012 / Accepted: 11 December 2012

Abstract Thioflavin-T (ThT) is a benzothiazole dye that exhibits enhanced fluorescence upon binding to beta-amyloid (Aβ) plaques and is commonly used in the diagnosis of Alzheimer's disease (AD). In this study, the ThT analogs were designed and synthesized, and screening was conducted for the detection of $A\beta_{40}$ fibrils in vitro. Among these compounds, 2-(2′-methoxy-4′-methylaminophenyl)benzoxazole (2) meets two critical requirements for an imaging agent: high its higher fluorescent responsiveness and stronger binding affinity than those of ThT. This compound showed the highest binding affinity with a dissociation constant of 3.27 ± 0.29 μM, and selectively stained Aβ aggregated in SHSY5Y neuroblastoma cells. This finding demonstrates the compound's potential use as a brain-imaging agent for AD studies.

Keywords Alzheimer's disease (AD) · Amyloid plaques · Beta-amyloid peptide (Aβ) · Thioflavin-T (ThT)

Introduction

Alzheimer's disease (AD) is the most common form of dementia in elderly people. AD is a general term used for a loss of memory and other intellectual abilities that are serious enough to interfere with daily activities (Berchtold and Cotman, 1998). The accumulation of amyloid plaques and neurofibrillary tangles in brain tissue is one of the neuropathological hallmarks of AD (Luurtsema *et al*., 2008). Amyloid plaques are composed of a tangle of regularly ordered fibrillar aggregates, known as amyloid fibers. Beta-amyloid (Aβ) is the main component of amyloid plaques. Aβ is a peptide of 39–42 amino acids that is processed from the amyloid precursor protein (Kang *et al*., 1987; Lahiri and Maloney, 2010). The most common isoforms are $A\beta_{40}$ and $A\beta_{42}$; the shorter form is typically produced by cleavage that occurs in the endoplasmic reticulum while the longer form is produced by cleavage in the trans-Golgi network (Hartmann *et al*., 1997). Current drug research is mainly focused on decreasing Aβ production, blocking the formation of these plaques by preventing Aβ protofibril and fibril formation, and alleviating the toxic effects of neuritic plaque deposition (Xia, 2003). Therefore, assessing the degree of Aβ deposition is an important tool for AD diagnosis.

Aβ imaging pharmaceuticals have been developed with the foundation of a dye chemical structure that is used for Aβ pathological examination. Several imaging agents, such as the thioflavin-T derivative, [^{11}C]PIB ([*N*-methyl-^{11}C]-2-(4′-methylaminophenyl)-6-hydroxybenzothiazole), the stilbene derivative [^{11}C]SB-13 ([*N*-methyl-^{11}C]-4-methylamino-4′-hydroxytrilben), and [^{18}F]FDDNP (2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl)amino]2-naphthyl}ethylidene)malononitrile), have been reported for use in the positron emission tomography imaging of Aβ plaques in AD patients (Mathis *et al*., 2003). Among these agents, thioflavin-T (ThT) dye shows enhanced fluorescence upon binding to amyloids in tissue sections. Therefore, ThT dye fluorescence is widely used to quantify the presence of Aβ found in the brains of AD patients (Hudson *et al*., 2009). In addition, fluorescent probes can be utilized as a safer, faster, and less expensive

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Published online: 04 January 2013

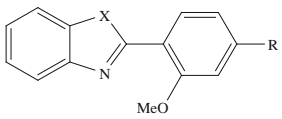


Fig. 1 Structure of thioflavin-T analogs. Compounds reported in this study include the following: X = O, S; R = NH_2, $NHCH_3$, $N(CH_3)_2$ (total of six ThT analogs)

alternative compared with radiolabeled probes for detecting Aβ plaques. In this study, we describe the optical properties of ThT analogs, the structures of which are shown in Fig. 1 and their biological properties as fluorescent probes.

Materials and methods

Chemistry

All commercial reagents and solvents were purchased from Aldrich at analytical grade and used as received without purification. The melting points were measured on a Mel-Temp. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA-500 NMR spectrometer, and chemical shift data for the proton resonances were reported at a parts per million scale with tetramethylsilane serving as an internal standard. Mass spectra were recorded on a JEOL JMS-AX505WA mass spectrometer, and the compounds were measured using the FAB method using *m*-nitrobenzyl alcohol as a matrix at the National Center for Inter-University Research Facilities. Gravity column chromatography was performed on Merck silica gel 60 (70–230 mesh ASTM). Thin layer chromatography was performed on Merck silica gel 60F-254 glass plates and visualized by UV light.

Synthesis of 2-(2′-methoxy-4′-aminophenyl)benzoxazole (1)

A mixture of 2-aminophenol (0.54 g, 5 mmol) and 4-amino-2-methoxybenzoic acid (0.83 g, 5 mmol) in polyphosphoric acid (10 mL) was heated at 200 °C for 4 h. After cooling, the reaction mixture was poured into 50 mL of 10 % sodium carbonate. The crude product was extracted using ethyl acetate and subsequently washed with brine. The ethyl acetate portion was dried over sodium sulfate and filtered. The filtrate was concentrated to dryness, and the residue was purified using column chromatography to yield 2-(2′-methoxy-4′-aminophenyl)benzoxazole (0.17 g, 14 %) as a light yellow solid. R_f: 0.34 (EA:Hx = 1:8). m.p.: 117–118 °C. ^{1}H NMR ($CDCl_3$) δ 7.78 (d, J = 8.8 Hz, 1H), 7.62 (d, J = 7.5 Hz, 1H), 7.52 (d, J = 7.5 Hz, 1H), 7.31 (t, J = 7.5 Hz, 1H), 7.27 (t, J = 7.5 Hz, 1H), 6.25 (s, 1H), 6.23 (d, J = 2.3 Hz, 1H), 2.90 (s, 3H).^{13}C NMR ($CDCl_3$) δ 163.95, 160.87, 153.93, 149.01, 140.63, 128.31, 124.62, 124.13, 118.39, 110.23, 105.80, 100.20, 98.14, 30.26. HRMS (FAB^+) *m/z*: 240.09 (100), 241.09 (16). Anal. calcd. for $C_{14}H_{12}N_2O_2$: 240.09: C, 69.99; H, 5.03; N, 11.66; O, 13.32. Found: C, 69.71; H, 5.01; N, 11.62;O, 13.28.

Synthesis of 2-(2′-methoxy-4′-methylaminophenyl)benzoxazole (2)

Sodium methoxide (27 mg, 0.5 mmol) and paraformaldehyde (13 mg, 0.5 mmol) were added to a solution of compound **1** (0.12 g, 0.5 mmol) in methanol (10 mL), and the mixture was refluxed for 2 h. The mixture was cooled to 0 °C, sodium borohydride (19 mg, 0.5 mmol) was added in portions, and the mixture was refluxed further for 1 h. Next, the mixture was poured into ice water and extracted with ethyl acetate. The combined extracts were dried over sodium sulfate and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography to yield 2-(2′-methoxy-4′-methylaminophenyl)benzothiazole (0.06 g, 28 %) as a light yellow solid. R_f: 0.55 (EA:Hx = 1:8). m.p.: 146-148 °C. ^{1}H NMR ($CDCl_3$) δ 7.82 (d, J = 8.9 Hz, 1H), 7.62, (d, J = 8.0 Hz, 1H), 7.52 (d, J = 7.5 Hz, 1H), 7.31 (t, J = 7.5 Hz, 1H), 7.28 (t, J = 7.5 Hz, 1H), 6.36 (d, J = 8.9 Hz, 1H), 6.33 (d, J = 2.6 Hz, 1H), 3.05 (s, 6H).^{13}C NMR ($CDCl_3$) δ 164.00, 160.31, 154.33, 149.06, 140.72, 128.17, 124.59, 124.06, 118.35, 110.22, 104.65, 99.37, 98.65, 40.19. HRMS (FAB^+) *m/z*: 254.11 (100), 255.11 (17). Anal. calcd. for $C_{15}H_{14}N_2O_2$: 240.09: C, 70.85; H, 5.55; N, 11.02; O, 12.58. Found: C, 70.57; H, 4.99; N, 10.98;O, 12.53.

Synthesis of 2-(2′-methoxy-4′-dimethylaminophenyl)benzoxazole (3)

Methyl iodide (0.12 mL, 2 mmol) and K_2CO_3 (0.28 g, 2 mmol) were added to a solution of compound **1** (0.14 g, 0.58 mmol) in DMF (anhydrous, 3 mL). The reaction mixture was heated at 140 °C for 4 h. Upon cooling to room temperature, the reaction mixture was poured into water and extracted with ethyl acetate (3 × 10 mL). The organic layers were combined, and the solvent was evaporated. The residue was purified by column chromatography to yield 2-(2′-methoxy-4′-dimethylaminophenyl) benzoxazole (60 mg, 39 %) as a light yellow solid. R_f: 0.09 (EA:Hx = 1:8). m.p.: 62–63 °C. ^{1}H NMR ($CDCl_3$) δ 7.82 (d, J = 8.9 Hz, 1H), 7.62 (d, J = 7.5 Hz, 1H), 7.52 (d, J = 7.7 Hz, 1H), 7.30 (t, J = 7.8 Hz, 1H), 7.28 (t, J = 7.8 Hz, 1H), 6.36 (dd, J = 8.9 Hz, J = 2.6 Hz, 1H), 6.33 (d, J = 2.6 Hz, 1H), 4.00 (s, 3H), 3.07 (s, 6H).^{13}C NMR ($CDCl_3$) δ 162.71, 160.14, 153.82, 150.05, 142.77, 132.16, 123.91, 123.85, 119.52, 110.00, 104.65, 103.85, 95.04, 56.04, 40.28. HRMS (FAB^+) *m/z*: 268.13 (100), 269.13 (18). Anal. calcd. for $C_{16}H_{16}N_2O_2$: 268.12: C,

71.62; H, 6.01; N, 10.44; O, 11.93. Found: C, 71.55; H, 6.00; N, 10.43; O, 11.92.

Synthesis of 2-(2′-methoxy-4′-aminophenyl) benzothiazole (4)

A mixture of 2-aminothiophenol (0.5 mL, 5 mmol) and 4-amino-2-methoxybenzoic acid (0.83 g, 5 mmol) in phosphorus oxychloride (5 mL) was heated at 100 °C for 4 h. After cooling, the reaction mixture was carefully and slowly poured into a beaker of distilled water while stirring. The pH was adjusted to 7.0 by the addition of sodium hydroxide pellets. The crude product was extracted using dichloromethane and washed twice with distilled water. The combined extracts were dried over sodium sulfate and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography to yield yellow 2-(2′-methoxy-4′-aminophenyl)benzothiazole (0.14 g, 11 %). R_f: 0.07 (EA:Hx = 1:8). m.p.: 146–148 °C. ^{1}H NMR ($CDCl_3$) δ 8.31 (d, J = 8.6 Hz, 1H), 7.99 (d, J = 8.3 Hz, 1H), 7.86 (d, J = 8.6 Hz, 1H), 7.43 (t, J = 7.7 Hz, 1H), 7.29 (t, J = 8.0 Hz, 1H), 6.41 (dd, J = 8.6 Hz, J = 2.3 Hz, 1H), 6.29 (d, J = 2.0 Hz, 1H), 3.99 (s, 3H).^{13}C NMR ($CDCl_3$) δ 163.92, 158.94, 152.35, 150.41, 135.59, 131.02, 125.72, 123.86, 122.08, 121.10, 113.17, 108.05, 97.60, 55.58. HRMS (FAB^+) *m/z*: 256.07 (100), 257.07 (17). Anal. calcd. for $C_{14}H_{12}N_2OS$: 256.07: C, 65.60; H, 4.72; N, 10.93; O, 6.24; S, 12.51. Found: C, 65.53; H, 4.72; N, 10.92; O, 6.23; S, 12.50.

Synthesis of 2-(2′-methoxy-4′-methylaminophenyl)benzothiazole (5)

Sodium methoxide (27 mg, 0.5 mmol) and paraformaldehyde (13 mg, 0.5 mmol) were added to a solution of compound **4** (0.13 g, 0.5 mmol) in methanol (10 mL), and the mixture was subsequently refluxed for 2 h. The mixture was cooled to 0 °C, sodium borohydride (19 mg, 0.5 mmol) was added in portions, and the mixture was refluxed further for 1 h. Next, the mixture was poured into ice water and extracted with ethyl acetate. The combined extracts were dried over sodium sulfate and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography to yield orange 2-(2′-methoxy-4′-methylaminophenyl)benzothiazole (0.06 g, 43 %). R_f: 0.16 (EA:Hx = 1:8). m.p.: 146–147 °C. ^{1}H NMR ($CDCl_3$) δ 8.33 (d, J = 8.8 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.85 (d, J = 7.5 Hz, 1H), 7.42 (t, J = 7.7 Hz, 1H), 7.28 (t, J = 8.0 Hz, 1H), 6.35 (dd, J = 8.6 Hz, J = 2.3 Hz, 1H), 6.18 (d, J = 2.0 Hz, 1H), 4.02 (s, 3H), 2.92 (s, 3H).^{13}C NMR ($CDCl_3$) δ 164.16, 159.16, 152.77, 152.44, 135.53, 130.80, 125.65, 123.66, 121.92, 121.05, 111.92, 105.77, 94.50, 55.54, 30.46. HRMS (FAB^+) *m/z*: 270.09 (100), 271.09 (18). Anal. calcd. for $C_{15}H_{14}N_2OS$: 270.08: C, 66.64; H, 5.22; N, 10.36; O, 5.92; S, 11.86. Found: C, 66.31; H, 5.19; N, 10.31; O, 5.89; S, 11.80.

Synthesis of 2-(2′-methoxy-4′-dimethylaminophenyl)benzothiazole (6)

A solution of compound **4** (0.13 g, 0.5 mmol) in tetrahydrofuran (5 mL) was slowly added to a stirred mixture of 37 % formaldehyde (0.5 mL, 5 mmol) and 4 M H_2SO_4 (0.4 mL, 1.6 mmol). Powdered iron (0.21 g, 4 mmol) was added to the mixture, and the mixture was stirred mechanically for 30 min. The iron precipitate was removed by filtration and washed with ethyl acetate. The filtrate was made strongly alkaline (pH > 11) with 10 % NaOH and was extracted with ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate and concentrated. The oily residue was subsequently purified with column chromatography to yield yellow 2-(2′-methoxy-4′-dimethylaminophenyl)benzothiazole (0.05 g, 35 %). R_f: 0.30 (EA:Hx = 1:8). m.p.: 95–96 °C. ^{1}H NMR ($CDCl_3$) δ 8.36, (d, J = 8.9 Hz, 1H), 7.98 (d, J = 8.3 Hz, 1H), 7.85 (d, J = 7.8 Hz, 1H), 7.42 (t, J = 7.2 Hz, 1H), 7.27 (t, J = 8.3 Hz, 1H), 6.45 (dd, J = 8.9 Hz, J = 2.3 Hz, 1H), 6.24 (d, J = 2.3 Hz, 1H), 4.04 (s, 3H), 3.07 (s, 6H).^{13}C NMR ($CDCl_3$) δ 164.15, 158.94, 153.36, 152.54, 135.55, 130.52, 125.61, 123.59, 121.89, 121.02, 111.10, 105.36, 94.65, 55.53, 40.35. HRMS (FAB^+) *m/z*: 284.11 (100), 285.11 (19). Anal. calcd. for $C_{16}H_{16}N_2OS$: 284.10: C, 67.58; H, 5.67; N, 9.85; O, 5.63; S, 11.28. Found: C, 67.38; H, 5.65; N, 9.82; O, 5.61; S, 11.25.

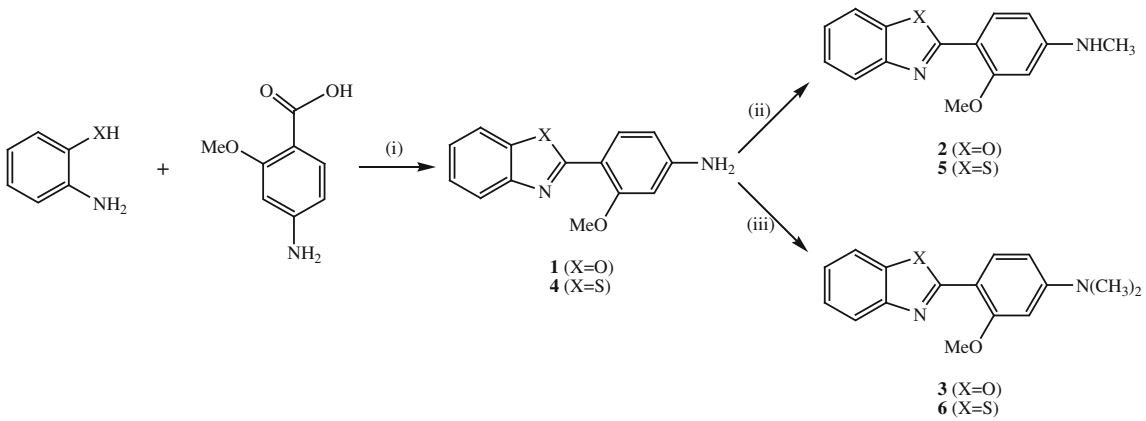
Biological procedures

General method for Aβ_{40} fibrils preparation

Purified Aβ_{40} peptides were dissolved in phosphate-buffered saline (PBS, pH 7.4) to obtain a final concentration of 200 μM. The solution was incubated by gentle shaking for 2 days at 37 °C. The formation of Aβ_{40} fibrils was confirmed using a ThT assay (Klunka *et al.*, 2001).

Fluorescence spectra measurement

The excitation and emission λ_{max} of each compound was measured with a TECAN 200 Pro. The final concentrations of 10–50 μM of the Aβ_{40} fibrils in the PBS buffer solution were used in the experiments. Generally, the λ_{max} of the emission was determined by scanning a fixed excitation first, and the λ_{max} of the excitation was determined using a scanning emission spectrum with a fixed λ_{em} (Hong *et al.*, 2010).



Scheme 1 Synthesis of thioflavin-T analogs. Reagents and conditions: (*i*) X = O: polyphosphoric acid, 200 °C, 4 h/X = S: phosphorus oxychloride, 100 °C, 4 h; (*ii*) $NaOCH_3$, paraformaldehyde, $NaBH_4$; (*iii*) X = O: CH_3I/K_2CO_3/X = S: H_2SO_4, Fe, formaldehyde

Table 1 Fluorescence profile and Aβ_{40} binding affinities (K_D)

Compound	λ_{ex} (nm)	λ_{em} (nm)	Fold ($F_{A\beta}/F_0$)[a]	K_D (mean ± SD) (μM)[b]	Log P^c_{oct}
1	340	400	23.3	6.03 ± 0.47	3.8
2	340	410	36.1	3.27 ± 0.29	3.7
3	340	410	6.4	17.56 ± 0.38	4.7
4	350	410	3.3	34.33 ± 1.53	4.8
5	350	430	4.2	7.33 ± 0.58	4.7
6	350	440	5.3	12.05 ± 0.67	4.6

[a] Fold change values were calculated using the fluorescence emission intensity at λ_{em} of each compound (10 μM compound and 10 μM Aβ_{40} aggregates were used for the measurement)

[b] K_D values were obtained from three independent replicates ($N = 3$)

[c] The numbers in parentheses were log P_{oct} values determined by conventional octanol–buffer partitioning

Measurement of binding affinity (K_D)

Aβ_{40} fibrils of 10 μM concentration solutions were titrated by a series concentration of the compounds **1**, **2**, **3**, **4**, **5**,, and **6**. The dissociation constants (K_D) were measured from the double reciprocal of the fluorescence maximum (F_{max}) and the concentration of the probe (Sutharsan *et al*., 2010).

In vitro seeding

SHSY5Y neuroblastoma cell pellets were suspended in 2 % SDS in PBS, sonicated and diluted 1:40 in PBS with 0.02 % sodium azide, and loaded into Labtek 8-well chambers to a final SDS concentration of 0.05 %. SHSY5Y cells, which were incubated with or without (control) 1 μM Aβ_{42} for 3 days at 37 °C, were homogenized. Culture wells were washed and incubated with 10 μM of compound **2** and 10 μM of ThT for 10 min, rinsed with 50 % ethanol and water, and cover slipped for imaging. The images were acquired by fluorescence microscopy (Hu et al., 2009).

Results and discussion

Chemistry

Compounds based on uncharged ThT cause an increase in affinity for Aβ fibrils as well as greater lipophilicity, and therefore an easier crossing of the blood–brain barrier (BBB) can be achieved (Yona *et al*., 2008). The synthesis of the ThT analogs is shown in Scheme 1, producing the desired products with a 11–43 % yield. 2-(2′-Methoxy-4′-aminophenyl)benzoxazole (**1**) was synthesized by the condensation of 2-aminophenol and 4-amino-2-methoxybenzoic acid at 200 °C in polyphosphoric acid. Next, 2-(2′-methoxy-4′-methylaminophenyl)benzoxazole (**2**) was monomethylated using the deprotonating agent, sodium methoxide, paraformaldehyde, and the reducing agent, sodium borohydride, in methanol. Also, 2-(2′-methoxy-4′-dimethylaminophenyl)benzoxazole (**3**) was synthesized starting from compound **1**, which was followed by dimethylation using methyl iodide and potassium carbonate.

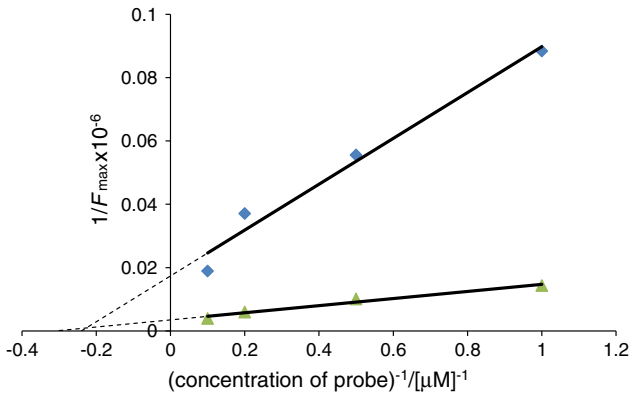


Fig. 2 Determination of the apparent binding constant (K_D) of probes compound **2** (*filled triangle* $R^2 = 0.97$) and ThT (*filled hexagon* $R^2 = 0.97$) to pre-aggregated Aβ fibrils

Subsequently, 2-(2′-methoxy-4′-aminophenyl)benzothiazole (**4**) was prepared through the conjugation of 2-aminothiophenol and 4-amino-2-methoxybenzoic acid in phosphorus oxychloride. Next, 2-(2′-methoxy-4′-methylaminophenyl)benzothiazole (**5**) was monomethylated using a deprotonating agent sodium methoxide, paraformaldehyde, and a reducing agent, sodium borohydride, in methanol. In addition, 2-(2′-methoxy-4′-dimethylaminophenyl)>benzothiazole (**6**) was synthesized starting from compound **4**, which was followed by dimethylation using formaldehyde in the presence of sulfuric acid and powdered iron.

Screening against Aβ plaques

The six synthesized ThT analogs were tested with synthetic Aβ_{40} aggregates for their fluorescence response. The aggregated fibrils of synthetic Aβ_{40}, and their methods of utilization were previously reported (Klunka *et al*., 2001). We evaluated the fluorescent properties of the six compounds at 10 μM concentrations in PBS before and after mixing with aggregated Aβ peptide (20 μM, aggregated in a PBS buffer for 2 days at 37 °C). Among the six tested compounds, compounds **1** and **2** showed a greater than 20-fold increase at 10 μM Aβ_{40} fibrils, and were further tested for their binding constants against Aβ_{40}. The binding constants (K_D) were measured using 10 μM aggregated Aβ_{40} fibrils. The fluorescent intensity of each probe was measured at concentrations of 1, 2, 5, and 10 μM mixed with pre-aggregated Aβ_{40} fibrils. The spectroscopic properties of the compounds are summarized in Table 1.

Promisingly, compound **2** meets the following two critical requirements for a fluorescence imaging probe: high fluorescence responsiveness ($F_{A\beta}/F_0 = 36.1$) and a strong binding affinity ($K_D = 3.27 \pm 0.29$ μM). Compared with the conventional Aβ probes, compound **2** exhibited stronger bindings than ThT ($F_{A\beta}/F_0 = 33.1$, $K_D = 4.12 \pm 0.47$) in our measurement. A double reciprocal plot of the fluorescence intensity versus the concentration of probes in compound **2** and ThT is shown in Fig. 2. The K_D corresponds to the -1/(x-intercept) of the linear regression. Table 1 also

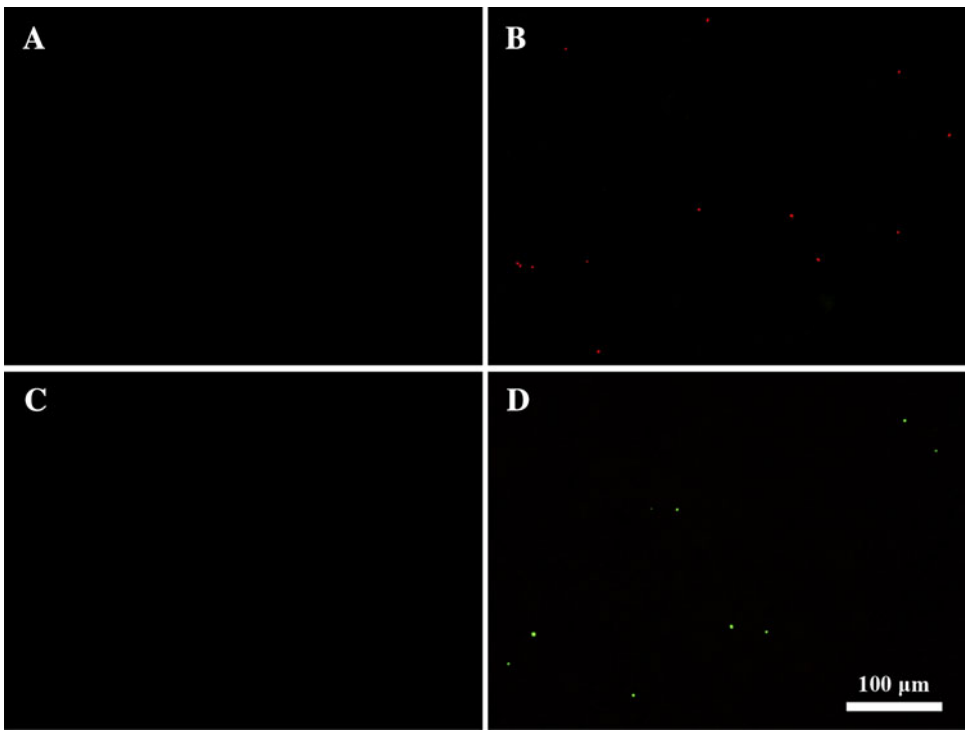


Fig. 3 Cell extracts from Aβ_{42}-loaded cells seed the formation of amyloid fibrils. Extracts from control cells (grown in the absence of Aβ_{42}) did not exhibit compound **2** (**a**) or ThT staining (**c**); Aβ_{42}-loaded cell extracts developed compound **2** precipitates (**b**) which stained for ThT (**d**)

shows the lipophilicities (log P_{oct}) of the six compounds for aggregated Aβ_{40} fibrils. The log P_{oct} values of the six compounds were determined using standard shaken-flask methods (Takács-Novák and Avdeef, 1996). The log P_{oct} values of the six compounds spanned and exceeded a range of approximately 3–4.

Encouraged by these predominant properties, we further tested the applicability of compound **2** to the fluorescence imaging of intracellular aggregated Aβ_{42} fibrils using SHSY5Y cells. To determine the intracellular uptake aggregation of Aβ_{42}, SHSY5Y cells, which were incubated with or without 1 μM Aβ_{42} for 3 days, were homogenized and subsequently incubated with 10 μM of compound **2** for 10 min. Aβ_{42}-loaded cell extracts induced the appearance of fluorescent precipitates that were visible by microscopy (Fig. 3b), while extracts from cells grown in the absence of Aβ_{42} (Fig. 3a) did not induce the appearance of fluorescent precipitates. These same precipitates were stained with ThT suggesting the formation of fibrils (Fig. 3d). Again, extracts from cells grown in the absence of Aβ_{42} (Fig. 3c) failed to develop fluorescent staining. These results suggest that compound **2** can be a useful marker of intercellular aggregated Aβ.

Conclusions

We synthesized six thioflavin-T analogs of the Aβ plaque ligand, and we identified 2-(2′-Methoxy-4′-methyl-amino-phenyl)benzoxazole (compound **2**) which exhibited a strong fluorescence response ($F_{A\beta}/F_0 = 36.1$) and binding affinity ($K_D = 3.27 \pm 0.29$ μM) to Aβ_{40} aggregates and SHSY5Y neuroblastoma cells. Therefore, compound **2** may be a good alternative candidate as a fluorescence imaging agent for studying Alzheimer's disease.

Acknowledgments This research was supported by Nuclear R&D Program through the Korean Ministry of Education, Science and Technology.

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