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Fluorescent 2-styrylpyridazin-3(2*H*)-one derivatives as probes targeting amyloid-beta plaques in Alzheimer's disease

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ABSTRACT

Amyloid plaques, which are primarily composed of aggregated amyloid-beta (Aβ) peptide, are the neuropathological hallmarks of Alzheimer's disease (AD). Fluorescent markers containing 2-styrylpyridazin-3(*2H*)-ones were developed to detect intracellular aggregated Aβ peptides. Nine compounds exhibited a greater than 10-fold increase of in emission spectra before and after mixing with Aβ aggregates compared with before mixing. Among these compounds, compound **9n** exhibited the highest affinity for Aβ aggregates (K_d = 1.84 μM) and selectively stained both aggregated intracellular Aβ and Aβ plaques in the transgenic AD model mice (APP/PS1). These preliminary results indicate that 2-styrylpyridazin-3(2*H*)-one derivatives are promising alternative fluorescence imaging agent for the study of AD.

Alzheimer's disease (AD), the most common form of dementia, involves the progressive accumulation of amyloid-beta (Aβ) plaques in the brain parenchyma and neurofibrillary tangles (NFTs) in neurons.[1,2] Aβ peptides, which range in length from 39 to 42 amino acids, are the major metabolic peptides derived from the proteolytic cleavage of an endogenous transmembrane protein known as the amyloid precursor protein (APP). Recent reports have suggested that Aβ aggregates in the brain play a key role in the cascade of event leading to AD.[3,4] Thus, the development of diagnostic imaging agents targeting Aβ aggregates is important for the diagnosis and treatment of AD. Several imaging agents, such as the benzothiazole derivatives SB-13 ([^{11}C]4-*N*-methylamino-4′-hydroxystilbene),[5] PIB ([^{11}C]2-(4′-(methylaminophenyl)-6-hydroxybenzothiazole),[6] and AZD2184 ([^{11}C]-2-[6-(methylamino)pyridin-3-yl]-1,3-benzothiazol-6-ol),[7] have been studied as regents for the early diagnosis of AD by positron emission tomography (PET) imaging. More recently, the PIB analogue GE-067 (flutemetamol; 2-(3-[^{18}F]fluoro-4-methyaminophenyl)benzothiazol-6-ol),[8] the stilbene derivative BAY94-9172 (florbetaben; (*E*)-4-(*N*-methylamino)-4′-(2-(2-(2-[^{18}F]fluoroethoxy)ethoxy)ethoxy)stilbene),[9] and the styrylpyridine derivative AV-45 (florbetapir; (*E*)-4-(2-(6-(2-(2-(2-(-[^{18}F]fluoroethoxy)ethoxy)ethoxy)pyridyn-3-ylvinyl)-*N*-ethylbenzen-amine)[10] have proven useful for the imaging of Aβ plaques in living brain tissue. In addition, florescent probes that can stain Aβ deposits have also attracted increasing interest as potential tools for monitoring the progression of AD both in vitro and in vivo.[11] Fluorescent probes can be utilized as safer, faster, and less expensive alternatives to radiolabeled probes in detecting Aβ aggregates. Given their advantages, novel fluorescent probes are needed in many subfields of biomedical research. Figure 1 illustrates representative structures of several such fluorescent probes. Among the known amyloid-staining compounds, Congo red (CR) is the current standard for staining Aβ plaques. CR is thought to bind to Aβ aggregates due to the combination of hydrophobic interactions and electrostatic interactions between the negatively charged CR sulfonate groups and positively charged amino acid residues within Aβ.[12] In principle, an appropriate fluorescent probe for amyloid detection should have the following properties:[13,14] (1) specificity to Aβ plaques, (2) reasonable lipophilicity (log *P* value between 1 and 3), (3) molecular mass less than 600 Da, (4) emission wavelength above 450 nm and a large stokes shift, (5) high-affinity binding, (6) high quantum yield, (7) straightforward synthesis, and most importantly, (8) a significant change in fluorescent properties upon binding to Aβ deposits.

Recently, we reported the synthesis of several 2-styrylpyridazin-3(2*H*)-one derivatives and described their photophysical

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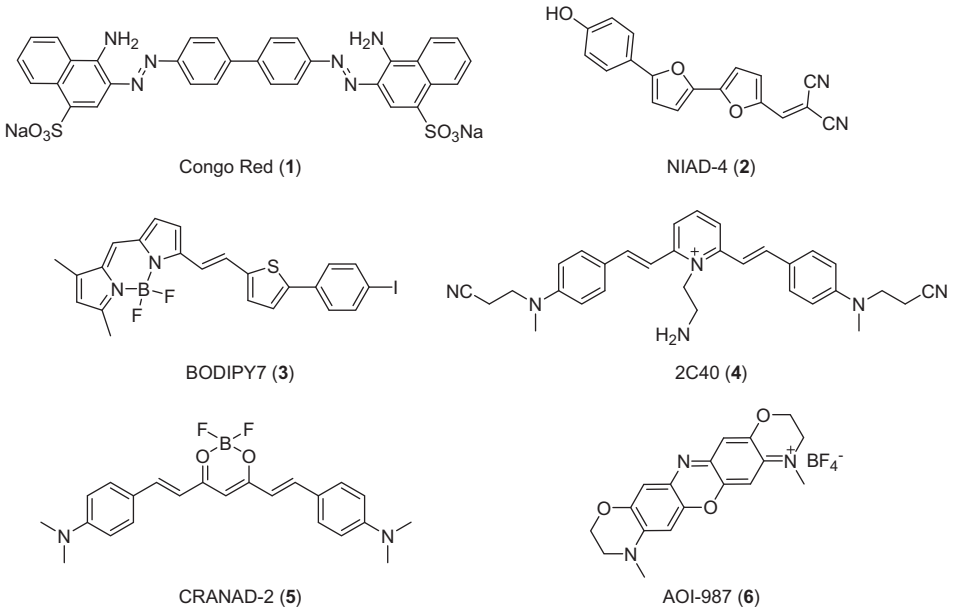


Figure 1. Sample structures of fluorescent probes that stain Aβ aggregates in tissue.

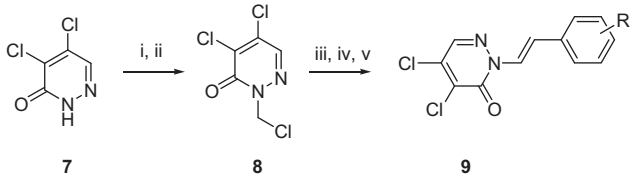
properties, and their potential for application as fluorescent probes.[15] We hypothesized that the structure of 2-styrylpyridazin-3(2*H*)-ones could be used to exploited to create a probe that exhibited certain changes in fluorescence properties upon binding to Aβ plaques. Furthermore, the hydrophobic planarized system of 2-styrylpyridazin-3(2*H*)-ones is an advantage, promoting the binding of Aβ aggregates through hydrophobic interactions. In the present study, we report the optical properties of several 2-styrylpyridazin-3(2*H*)-one derivatives and their biological properties as fluorescent probes.

Various 2-styrylpyridazin-3(2*H*)-one derivatives (**9a**–**9ab**, Table 1) were prepared as described previously[15] using the synthesis sequence outlined in Scheme 1. Compound **8** was reacted with benzaldehyde after treatment with potassium iodide and triphenylphosphine to produce trans-isomer **9**. The 28 synthesized compounds were mixed with Aβ aggregates to evaluate their fluorescence response; a fluorescent Aβ-binding probe should display a significant increase in fluorescence emission upon binding with the aggregates relative to their emission as free probe in the solution.[11] We evaluated the fluorescent properties of 28 compounds at

Table 1
Structure, fluorescence profile, and K_d of compounds with Aβ aggregates

Compound	R	λ_{ex} (nm)	λ_{em} (nm)	Fold increase[a] ($F_{Aβ}/F_0$)	K_d[b] (mean ± SD) (μM)
9a	H	350	496	4.72	—
9b	4-F	350	492	5.93	—
9c	4-Cl	380	495	3.03	—
9d	4-Br	370	495	2.12	—
9e	4-I	380	498	5.26	—
9f	4-CN	380	486	2.94	—
9g	4-CO_2Me	370	488	2.07	—
9h	2-NO_2	370	500	4.12	—
9i	3-NO_2	390	485	2.12	—
9j	4-NO_2	390	515	2.24	—
9k	4-Me	350	516	2.81	—
9l	4-C_6H_5	380	514	1.92	—
9m	4-CH=CHC_6H_5	380	510	2.04	—
9n	4-$N(Me)_2$	430	636	40.84	1.84 ± 0.31
9o	4-NH_2	400	598	12.15	2.95 ± 1.15
9p	2-OMe	380	492	8.24	—
9q	3-OMe	350	492	11.13	3.48 ± 1.14
9r	4-OMe	400	532	19.81	2.57 ± 0.54
9s	2,3-$(OMe)_2$	350	504	15.35	8.52 ± 2.48
9t	2,4-$(OMe)_2$	400	554	11.52	5.03 ± 3.48
9u	2,5-$(OMe)_2$	370	512	3.42	—
9v	2,6-$(OMe)_2$	350	545	4.53	—
9w	3,4-$(OMe)_2$	350	552	3.52	—
9x	3,5-$(OMe)_2$	350	492	5.37	—
9y	2,3,4-$(OMe)_3$	390	536	26.81	4.24 ± 0.98
9z	2,4,5-$(OMe)_3$	350	542	3.95	—
9aa	2,4,6-$(OMe)_3$	430	560	11.72	17.55 ± 2.49
9ab	3,4,5-$(OMe)_3$	390	580	26.12	4.14 ± 1.94

[a] Fold-change values were calculated using the fluorescence emission intensity at λ_{em} of compounds (10 μM compound and 10 μM Aβ40 aggregates were used for the measurements).
[b] The K_d values are expressed as the mean ± SD of values obtained from three independent replicates ($N = 3$).



Scheme 1. Reagents and conditions: (i) CH_2O, H_2O, 5–10 °C; (ii) $SOCl_2$, DMF, CH_2Cl_2, rt; (iii) KI, CH_3CN, reflux; (iv) PPh_3, CH_2Cl_2, reflux; (v) aldehyde, KO^tBu, CH_3CN, reflux.

10 μM in PBS before and after mixing with aggregated Aβ40 peptides (10 μM, aggregated in PBS buffer for 3 days at 35 °C). Nine compounds showed a greater than 10-fold fluorescence intensity increase in their emission spectra, indicating that these compounds bind to the Aβ aggregates (Table 1). For these nine compounds, the apparent binding constants (K_d) were measured with 10 μM aggregated Aβ40 peptide. The fluorescent intensity of each probe at concentrations of 0.1, 0.5, 1, 25, 5, and 10 μM in PBS and mixed with aggregated Aβ peptide was measured. Compound **9n** (Fig. 2A and B) exhibited the highest affinity for the Aβ40

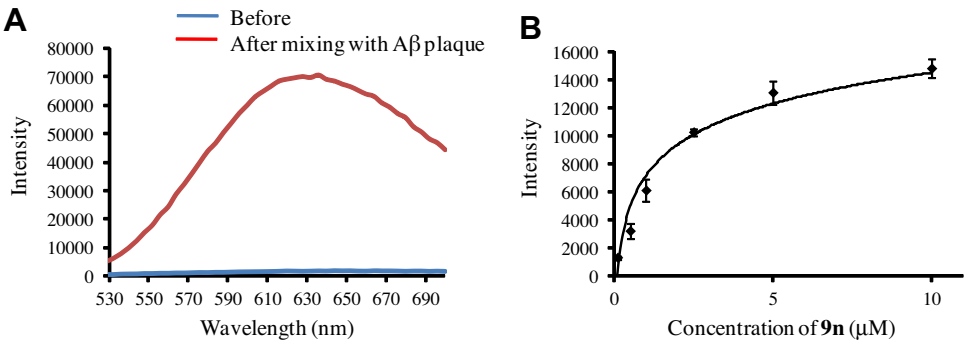


Figure 2. (A) Fluorescent emission of compound **9n** before (blue line) and after (red line) mixing with Aβ40 aggregates, (B) plot of the fluorescence intensity (at λ_{em} = 630 nm) as a function of the concentration of compound **9n** in the presence of Aβ40 aggregates (10 μM). The apparent dissociation constant (K_d) was 1.84 ± 0.31 μM.

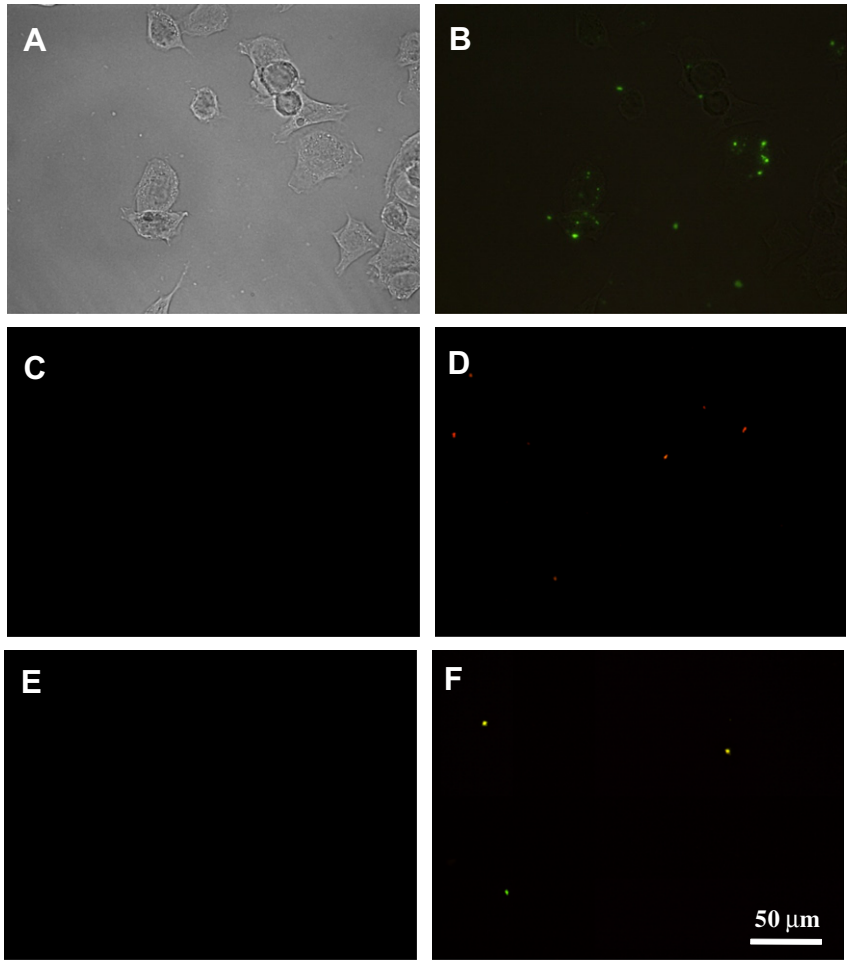


Figure 3. Cell uptake of FITC-Aβ42: (A) SHSY5Y cells, (B) SHSY5Y cells cultured with 1 μM FITC-Aβ42 for 3 days and then imaged using fluorescence microscopy. (C–F) Cell extracts from SHSY5Y cells cultured in the absence (C, E) or presence (D, F) of 1 μM Aβ42 for 3 days and then imaged to observe intercellular Aβ aggregates. Extracts from cells cultured with Aβ42 were fluorescently stained with both **9n** (D) and thioflavin T (F), while extracts from control cells grown in the absence of Aβ42 did not exhibit any **9n** (C) or thioflavin T (E) fluorescence.

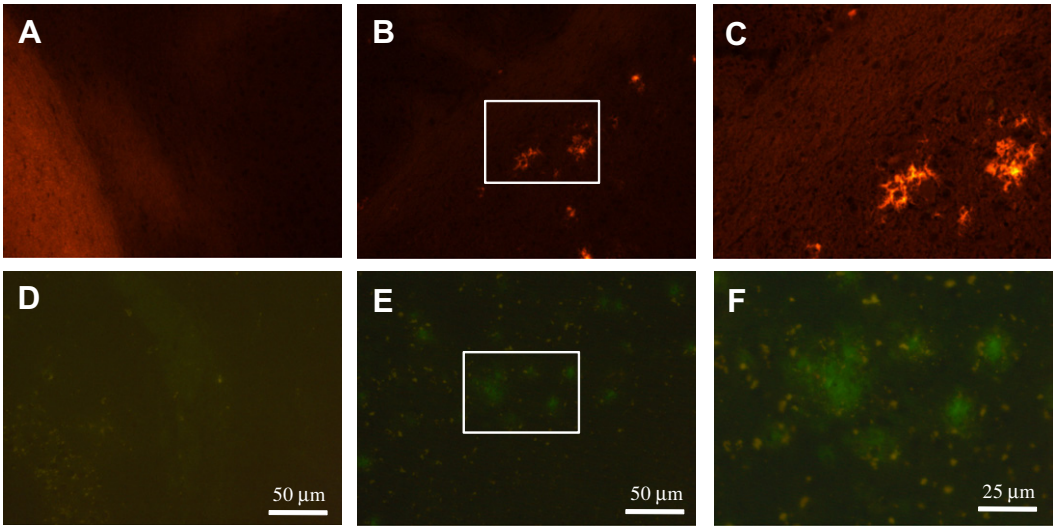


Figure 4. Histological staining of the mouse brain sections. Staining with **9n** (B and C) and staining with thioflavin T (E and F) in APP/SP1 transgenic AD model mice. Staining with **9n** (A) and thioflavin T (D) in control normal mice.

aggregate (K_d = 1.84 μM). In principle, a good fluorescent probe for Aβ plaques should have high fluorescence responsiveness and a strong binding affinity for aggregated Aβ peptide.[16] Upon binding to Aβ plaques, the fluorescent probe should undergo a significant change in its fluorescent properties because an increase in fluorescence intensity means that the probe will be 'turned on' upon interacting with its target. The probe **9n** meets these requirements, detecting Aβ aggregates with an $F_{Aβ}/F_0$ = 40.84 and a K_d = 1.84 μM.

Encouraged by these promising properties, we further tested the applicability of compound **9n** for the fluorescent imaging of intracellular aggregated Aβ in SHSY5Y neuroblastoma cells. To determine the intracellular uptake and aggregation of Aβ, Aβ42 (1 μM) labeled with fluorescein isothiocyanate (FITC-Aβ42) was incubated with SHSY5Y cells for 3 days and then imaged with fluorescence microscopy. The uptake of FITC-Aβ42 was observed in SHSY5Y cells (Fig. 3B). Previous reports have suggested that extracellular soluble Aβ is taken up and then forms high-molecular-weight aggregates of Aβ42 (>200 kDa).[17] To determine whether compound **9n** could be used as the basis of a fluorescence probe for detecting intercellular Aβ aggregates, cells loaded with unlabeled Aβ42 were sonicated with SDS, and homogenates were incubated with 10 μM of **9n** for 10 min. Fluorescent precipitates were visible by microscopy in the Aβ-loaded cell extracts (Fig. 3D). The labeling pattern was consistent with that observed with thioflavin T (Fig. 3F). Extracts from cells grown in the absence of Aβ failed to show fluorescent staining (Fig. 3C and E). These staining experiments strongly suggest that 2-styrylpyridazin-3(2*H*)-one-based probes are capable of staining intercellular Aβ aggregates.

To assess whether the 2-styrylpyridazin-3(2*H*)-one-based probe **9n** could stain amyloid deposits in AD mouse brain tissue, we tested **9n** in brain sections from 15-month-old transgenic AD model mice (APP/PS1). Figure 4 shows representative fluorescence micrographs of these tissue samples. Aβ plaques were clearly stained with **9n**, consistent with its high affinity for Aβ aggregates in in vitro assay (Fig. 4B and C). The labeling pattern was consistent with the signal obtained from standard thioflavin T-stained sections (Fig. 4E and F). In contrast, tissue from normal mice did not stain with **9n** or thioflavin T (Fig. 4A and D). These results suggest that **9n** can function as a probe for detecting Aβ plaques in the brain.

Previously, stilbene derivatives were identified as ligands with high binding affinities to Aβ aggregates.[18] Currently, two structurally similar compounds are being developed as commercial probes, BAY 94–9172 and AV-45, and both these molecules possess rigid structures of stilbene (BAY 94–9172) and styrylpyridine (AV-45). We planned to develop fluorescent markers for detecting Aβ aggregates by displacing the phenyl group in stilbene with pyridazin-3(2*H*)-one to create styrylpyridazin-3(*2H*)-ones. Furthermore, all of the PET imaging probes in clinical trials (BAY 94–9172, AV-45, and GE067) contain a monomethylamino or dimethylamino group that confers binding specificity to Aβ aggregates.

In conclusion, we show that dimethylamino group-containing the 2-styrylpyridazin-3(2*H*)-one derivative (**9n**) has fluorescence characteristics appropriate for imaging Aβ aggregates. Compound **9n** exhibited a strong fluorescence response ($F_{Aβ}/F_0$ = 40.84) and binding affinity (K_d = 1.84 μM) to Aβ aggregates, and it clearly stained both intracellular Aβ aggregates and Aβ plaques in the transgenic AD model mice (APP/PS1). Thus, 2-styrylpyridazin-3(2*H*)-one derivatives may be good candidates as alternative fluorescence imaging agents for the study of AD. Based on the above results, our next study will focus on radiolabeled 2-styrylpyridazin-3(2*H*)-one derivatives as potentially useful PET imaging agents for cerebral Aβ plaques.

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