Fluorescent Probes

Solid-Phase Synthesis of Styryl Dyes and their Application as Amyloid Sensors**

*Qian Li, Jun-Seok Lee, Chanki Ha, Chan Beum Park, Guang Yang, Wen Biao Gan, and Young-Tae Chang**

Fluorescent sensors and probes have attracted attention because of their high sensitivity and exceptional ease of handling relative to their radioactive counterparts.[1–3] Combinatorial chemistry is now widely used in the medicinal/pharmaceutical field and in chemical biology for the discovery of novel biologically active molecules or drug candidates,[4,5] yet the application of this method to fluorescence dyes is only in its infancy. A few early examples include oligopyridines,[6] coumarins,[7,8] oligonucleotides,[9] and conjugated polymers.[10] We previously reported the first combinatorial wide-color-range fluorescent styryl library by solution-phase chemistry and their potential application as organelle-specific probes.[11] Here we report the first solid-phase synthesis of a styryl library and its application as amyloid sensors.

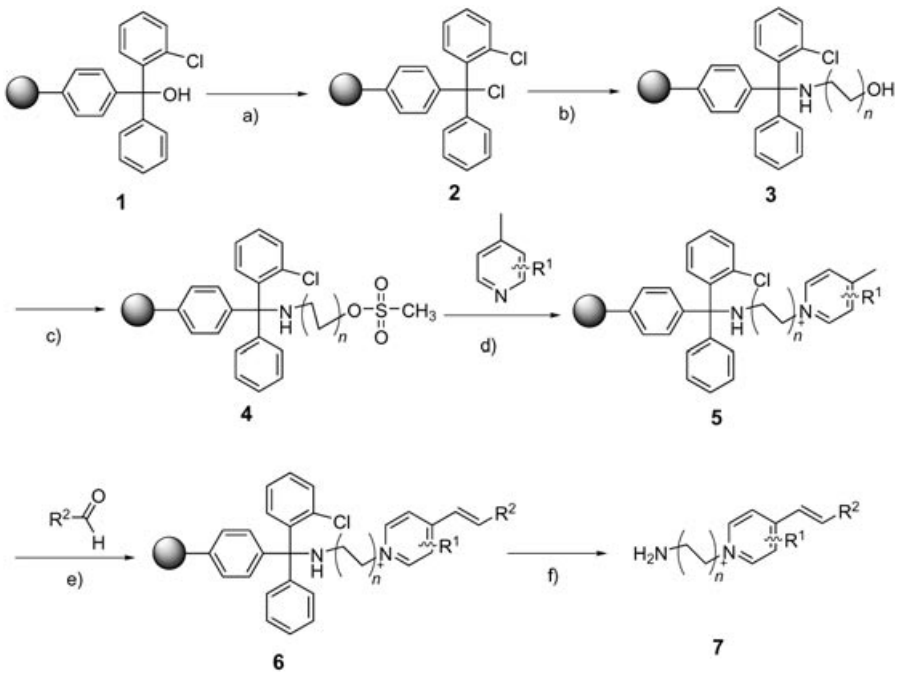
Amyloids result when protein misfolding leads to the formation of ordered secondary structures rich in cross-β-sheets, which are present as fibrillar deposits in tissues.[12] The formation of amyloids has been associated with a large number of protein-misfolding diseases including type II diabetes, Alzheimer's, Parkinson's, Huntington's, mad-cow disease, and others.[13,14] Of the many amyloidoses, Alzheimer's disease (AD) is the fourth leading cause of death in the United States and the most common cause of adult-onset dementia.[15,16] The deposition of β-amyloid (Aβ) aggregates in brain tissue is one of the hallmark characteristics of AD, and the histological staining of the deposits is crucial for the diagnosis of AD.[17] Whereas thioflavin T or S (ThT or ThS) and Congo red (CR) are widely used as detection agents for amyloids, neither of these are considered accurate enough for the quantitative estimation of fibril formation.[18–21] Congo red is not fluorescent (thus the sensitivity is low), whereas ThT (or S) is a blue-emission dye, which often interferes with autofluorescence from tissue or other small-molecule components in the assay system. Therefore, sensor dyes that are more sensitive and assay-friendly which can stain biological tissue have been sought.

The styryl scaffold is formed by a condensation reaction of aldehydes and pyridinium salts, which are widely available commercially and easily prepared starting materials. A series of resins and reaction routes were tested, however, the best results, both in yield and purity, were obtained when 2-chlorotrityl resin **1** (BeadTech Inc., Korea) was used as the first linker-protecting group (Scheme 1). Two different aminoalcohols with 2- or 6-carbon-atom chains were loaded onto the 2-chlorotrityl resin, and the alcohol groups were mesylated for subsequent treatment with picoline moities. Four picoline and three quinoline derivatives were chosen as R^1 building blocks to give solid-supported pyridinium salts **5**. 64 aldehyde R^2 building blocks that contain various functionalities and have various lengths and electron-donating or withdrawing properties are outlined in Scheme 2 (page 6333). Condensation of the solid-supported pyridinium salts **5** and the aldehydes was effectively performed under microwave irradiation. The addition of diluted TFA (1%) then led to the facile release of the final styryl dye compounds **7** from the resin (10 min). All of the synthesized compounds were analyzed by LC/MS, and 320 components were selected based on their purity for further study (average purity: 82%; purity data for individual compounds are available in the Supporting Information). Also, the λ_{max} values of the fluorescence excitation and emission bands of the library compounds

[*] Q. Li, J.-S. Lee, Prof. Dr. Y.-T. Chang
Department of Chemistry
New York University
New York, NY 10003 (USA)
Fax: (+1) 212-995-4203
E-mail: yt.chang@nyu.edu
C. Ha, Prof. Dr. C. B. Park
Department of Chemical & Materials Engineering
Arizona State University
Tempe, AZ 85287-6006 (USA)
Fax: (+1) 480-965-0037
G. Yang, Prof. Dr. W. B. Gan
Skirball Institute of Biomolecular Medicine
New York University School of Medicine
540 First Avenue
New York, NY 10016-6481 (USA)
Fax: (+1) 212-263-8214

[**] We thank BeadTech Inc., Korea, for technical support of this work.
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200461600



Scheme 1. Solid-phase synthesis of the styryl dyes **7:** a) thionyl chloride, CH_2Cl_2, room temperature, 2 h; b) ethanolamine ($n=1$) or 6-amino-1-hexanol ($n=3$), CH_2Cl_2, room temperature, 3 h; c) methanesulfonyl chloride, NEt_3, CH_2Cl_2, room temperature, overnight; d) pyridine derivative (R^1), NMP, 80–90 °C, overnight; e) aldehyde (R^2), pyrrolidine, NMP, 80 W microwave, 6 min; f) TFA (1%) in CH_2Cl_2, 10 min. NMP = *N*-methylpyrrolidone.

were recorded with a Gemini XS fluorescent plate reader (data available in Supporting Information). The compounds in the library cover practically the whole color range from blue to red. These 320 compounds were tested for amyloid sensing without further purification.

Primary screening of amyloid sensing was carried out with insulin amyloid fibrils generated from fresh insulin which were induced to aggregate at low pH conditions to form a cross-β-sheet secondary structure.[22] (A representative atomic force microscope (AFM) picture of the insulin amyloid fibrils used in this work is provided in the Supporting Information). The formation of insulin amyloids, a characteristic of injection-localized amyloidosis, occurs through kinetic mechanisms that are similar to those for other amyloidogenic proteins such as Aβ peptides, α-synuclein, and transthyretin, and its rate is highly accelerated under stressful conditions such as elevated temperature or low pH.[23,24] 13 compounds from the 320 compounds in the library were selected based on the observed increases in their fluorescence intensities upon addition of insulin amyloid fibril (relative to the dye or fresh insulin alone; see Figure 1), and their spectroscopic properties are summarized in Table 1. Promisingly, some of the compounds, such as 2C32 and 2C9, displayed dramatic increases in fluorescence intensity (663- and 736-fold) that were much better than the known standard dye ThS (42-fold). The fluorescence of many of the compounds demonstrated a red shift after binding with insulin amyloid fibril (Figure 1) which is a favorable property for the reduction of background signals and also for ratiometric measurements. The longer emission wavelengths of 2C32 and 2C9 may also allow for the screening of blue-green fluorescent inhibitors of amyloid formation, which was not possible with ThS or ThT.

We further tested the 13 selected compounds in synthetic Aβ40 and Aβ42 aggregates. The two Aβ variants, Aβ40 and Aβ42, which differ by truncation at the carboxyl terminus, are the predominant plaque proteins in AD.[25] All of the dyes showed comparable increases in their fluorescence emissions. Interestingly, all of the styryl compounds showed higher sensitivities to fibril Aβ40 than to fibril Aβ42 (Aβ40:Aβ42 = 0.8–3.5), in contrast to ThS (Aβ40:Aβ42 = 0.4). Furthermore, the 13 candidates and ThS were tested for the in vitro staining of fixed AD mouse-brain sections. Images of the amyloid stained with the two most-effective compounds, 2C40 and 2E10, are shown in Figures 2 and 3. As 2C40 displays red

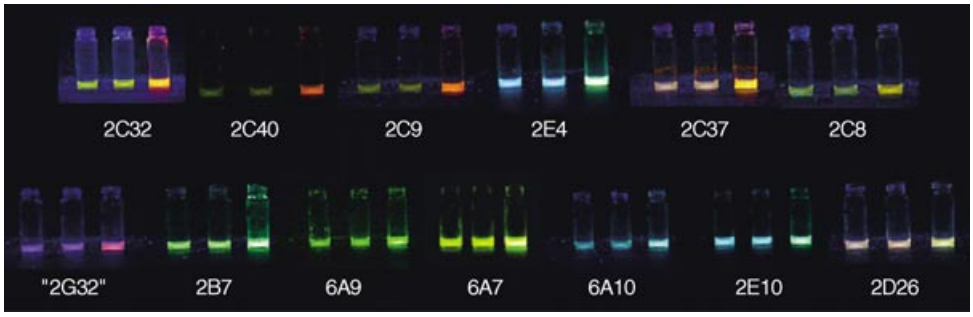
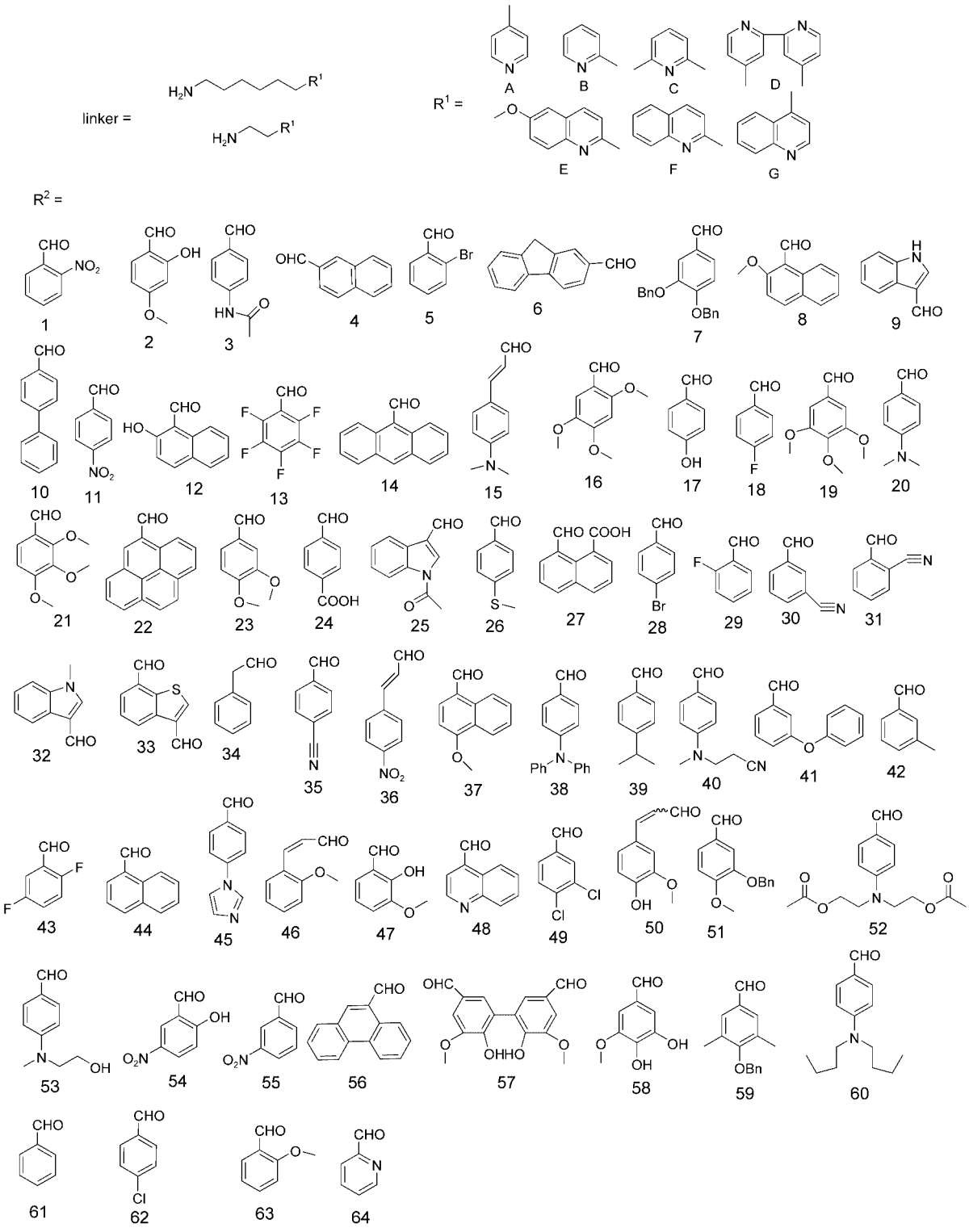


Figure 1. Color changes of the 13 "hit" compounds observed under irradiation with a Blak–Ray 365-nm UV lamp. Each set of 3 samples contains the dye (200 μM) alone in HCl (0.5 mL, pH 1.4; left), with fresh insulin (1 mg mL^{-1}, 0.5 mL, pH 1.4; center), and with insulin amyloid fibrils (1 mg mL^{-1}, 0.5 mL, pH 1.4; right). The dyes are numbered according to the length of the linker (2 or 6), the letter corresponding to R^1 (A–G), and the number corresponding to R^2 (1–64; see Scheme 2).



Scheme 2. Components (linkers, pyridine derivatives R^1, and aldehydes R^2) for the parallel syntheses of the styryl dyes. Bn = benzyl.

fluorescence and 2E10 displays green fluorescence, these compounds may be good complementary-color dye substitutes for ThS (blue). We observed that our new dyes are at least as sensitive as ThS in terms of labeling aggregates and showed 100% colocalization with ThS-labeling in 50 plaques assayed (two brain-slices each). Interestingly, compared to ThS, which stains the peripheral area of the plaque, our compounds mainly stained the core of the amyloid plaque.

In summary, we have reported the first solid-phase parallel synthesis of a styryl dye library that comprises 320 dye compounds. Through in vitro amyloid screening, 13 new amyloid sensors were identified of which two show outstand-

Table 1: Fluorescent properties of the 13 "hit" compounds from the styryl library.

Compound Code[a]	Buffer[b]		Fresh Insulin[b]		Insulin Amyloid Fibrils[b]		*n*-Fold Increase		
	λ_{ex} [nm]	λ_{em} [nm]	λ_{ex} [nm]	λ_{em} [nm]	λ_{ex} [nm]	λ_{em} [nm]	Insulin Amyloid Fibrils[c]	Aβ40 Fibrils[d]	Aβ42 Fibrils[d]
ThS	385	445	385	445	450	482	42	10	23
2C32	425	537	426	539	548	593	663	276	85
2C40	433	565	432	568	505	590	20	27	20
2C9	392	536	390	537	538	585	736	173	62
2E4	366	443	367	445	447	522	29	23	9
2C37	406	545	405	545	453	572	8	7	5
2C8	423	529	421	536	450	547	7	11	14
2G32	473	590	473	594	453	585	7	7	2
2B7	375	515	371	513	417	509	18	9	6
6A9	417	526	424	527	447	515	6	9	7
6A7	371	521	372	525	392	520	6	6	5
6A10	361	495	361	494	379	494	6	5	6
2E10	441	491	441	492	464	503	185	30	16
2D26	394	550	395	550	434	527	4	4	3

[a] The styryl dyes are numbered according to the length of the linker (2 or 6), the letter corresponding to R^1 (A–G), and the number corresponding to R^2 (1–64): see Scheme 2. [b] λ_{max} values for the excitation and emission bands of ThS and the "hit" compounds as solutions in aqueous HCl (pH 1.4) recorded with a Hitachi F-2500 FL Spectrophotometer. [c] *n*-fold increase in fluorescence intensity of the dye in insulin amyloid fibrils relative to the control (EDTA (1 mM) in PBS) at pH 4.0. [d] *n*-fold increase in fluorescence intensity of the dye in Aβ40 and Aβ42 fibrils relative to the control (EDTA (1 mM) in PBS, pH 7.4) measured with a Gemini XS plate reader.

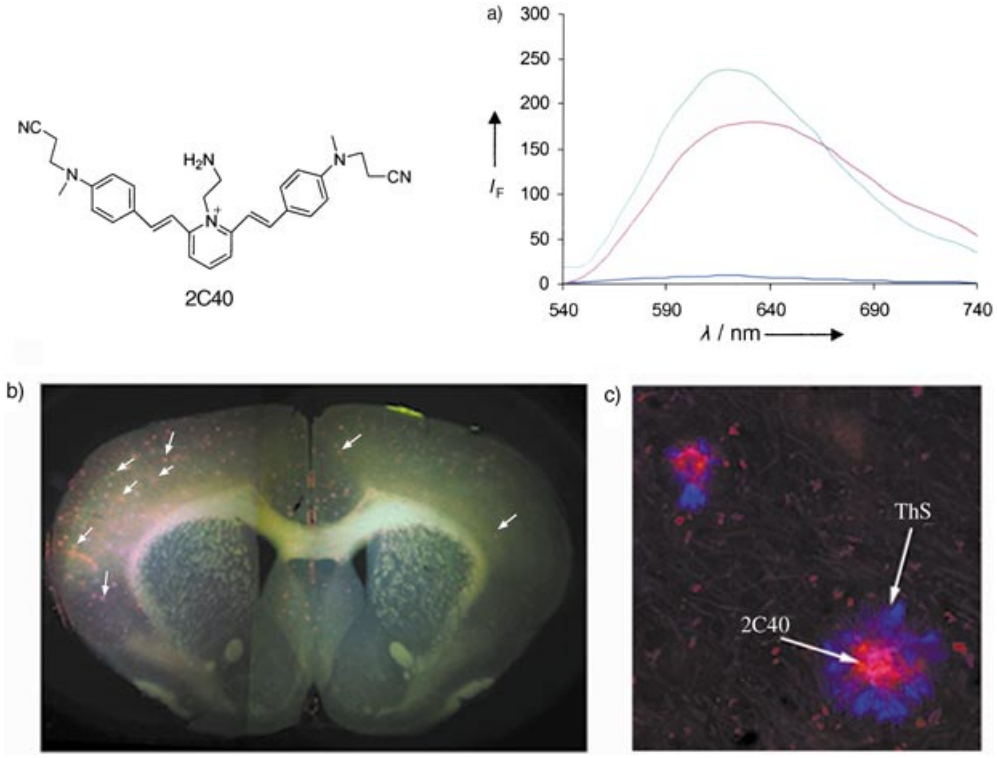


Figure 2. a) Fluorescence spectra of 2C40 (25 μM) with Aβ40 fibril (green line; 30 μL of a 0.5-mg mL^{-1} solution in PBS (pH 7.4) containing EDTA (1 mM)), and Aβ42 fibril (purple line; 30 μL of a 0.5-mg mL^{-1} solution in PBS solution (pH 7.4) containing EDTA (1 mM)) compared to a control buffer solution (dark blue line; 30 μL of a solution of PBS (pH 7.4) containing EDTA (1 mM)). b) Labeling of amyloid deposits with 2C40 in a slice of mouse brain (red, indicated by arrows). The image was taken with a charge-coupled device camera. c) Confocal microscopy image of amyloid deposits labeled with 2C40 (red) and ThS (blue); note that 2C40 preferentially labels the core of deposits.

ing promise as assay probes (2C32 and 2C9) and two others show promise as brain-imaging agents (2C40 and 2E10). This report demonstrates the high potential of the combinatorial approach in the development of novel sensors as well as in the established medicinal chemistry field. The study of systematic structure–property relationships of the library compounds, and in vivo amyloid imaging studies are currently underway.[26]

Received: August 10, 2004
Revised: August 30, 2004

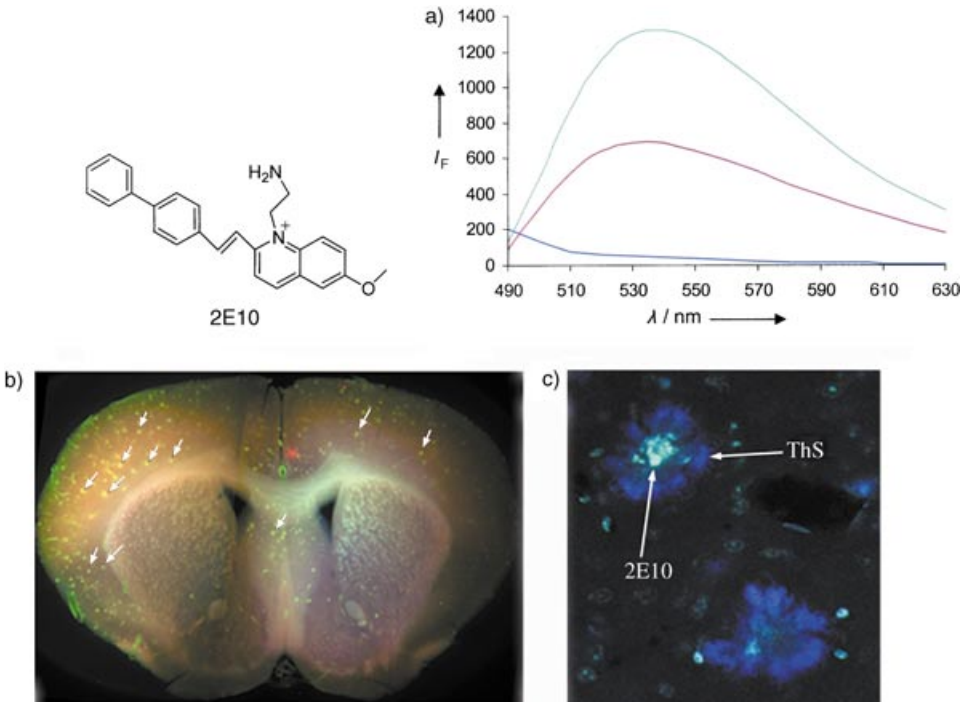


Figure 3. a) Fluorescence spectrum of 2E10 (25 μM) with Aβ40 fibril (green line; 30 μL of a 0.5 mg mL^{-1} solution in PBS solution (pH 7.4) containing EDTA (1 mM)), and Aβ42 fibril (purple line; 30 μL of a 0.5 mg mL^{-1} solution in PBS solution (pH 7.4) containing EDTA (1 mM)) compared to a control buffer solution (dark blue line; 30 μL of a solution in PBS (pH 7.4) containing EDTA (1 mM)). b) Labeling of amyloid deposits in a slice of mouse brain with 2E10 (green, indicated by arrows). c) Confocal microscopy image of amyloid deposits labeled with 2E10 (green) and ThS (blue); note that 2E10 preferentially labels the core of deposits.

Keywords: combinatorial chemistry · dyes/pigments · fluorescent probes · imaging agents · solid-phase synthesis

[1] *Fluorescent Chemosensors for Ion and Molecule Recognition* (Ed.: A. W. Czarnik), American Chemical Society, Washington DC, **1992**.
[2] W. Rettig, B. Strehmel, J. Schrader, H. Seifert, *Applied Fluorescence in Chemistry, Biology, and Medicine*, Springer, Berlin, **1999**.
[3] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Klewer Academic/Plenum Publishers, New York, **1999**.
[4] K. C. Nicolaou, R. Hanko, W. Hartwig, *Handbook of Combinatorial Chemistry: Drugs, Catalysts, Materials*, Wiley-VCH, Weinheim, **2002**.
[5] S. Miertus, G. Fassina, *Combinatorial Chemistry and Technology: Principles, Methods, and Applications*, Book News, New York, **1999**.
[6] J. C. Loren, J. S. Siegel, *Angew. Chem.* **2001**, *113*, 776–779; *Angew. Chem. Int. Ed.* **2001**, *40*, 754–757.
[7] M. S. Schiedel, C. A. Briehn, P. Bauerle, *Angew. Chem.* **2001**, *113*, 4813–4816; *Angew. Chem. Int. Ed.* **2001**, *40*, 4677–4680.
[8] A. M. Song, J. H. Zhang, K. S. Lam, *J. Comb. Chem.* **2004**, *6*, 112–120.
[9] J. M. Gao, C. Strassler, D. Tahmassebi, E. T. Kool, *J. Am. Chem. Soc.* **2002**, *124*, 11590–11591.
[10] O. Lavastre, I. Illitchev, G. Jegou, P. H. Dixneuf, *J. Am. Chem. Soc.* **2002**, *124*, 5278–5279.
[11] G. R. Rosania, J. W. Lee, L. Ding, H. S. Yoon, Y. T. Chang, *J. Am. Chem. Soc.* **2003**, *125*, 1130–1131.
[12] J. D. Sipe, A. S. Cohen, *J. Struct. Biol.* **2000**, *130*, 88–98.
[13] J. W. Kelly, *Curr. Opin. Struct. Biol.* **1996**, *6*, 11–17.
[14] R. M. Murphy, *Annu. Rev. Biomed. Eng.* **2002**, *4*, 155–174.
[15] C. M. Clark, *Neurodegenerative Dementias: Clinical Features and Pathological Mechanisms*, McGraw–Hill, New York, **2000**.
[16] T. A. Bayer, O. Wirths, K. Majtenyi, T. Hartmann, G. Multhaup, K. Beyreuther, C. Czech, *Brain Pathol.* **2001**, *11*, 1–11.
[17] J. Hardy, D. J. Selkoe, *Science* **2002**, *297*, 353–356.
[18] J. D. Harper, S. S. Wong, C. M. Lieber, P. T. Lansbury, *Chem. Biol.* **1997**, *4*, 119–125.
[19] J. D. Harper, P. T. Lansbury, *Annu. Rev. Biochem.* **1997**, *66*, 385–407.
[20] G. T. Westermark, K. H. Johnson, P. Westermark, *Methods Enzymol.* **1999**, *309*, 3–25.
[21] B. Urbanc, L. Cruz, R. Le, J. Sanders, K. H. Ashe, K. Duff, H. E. Stanley, M. C. Irizarry, B. T. Hyman, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13990–13995.
[22] A. Arora, C. Ha, C. B. Park, *FEBS Lett.* **2004**, *564*, 121–125.
[23] L. Nielsen, R. Khurana, A. Coats, S. Frokjaer, J. Brange, S. Vyas, V. N. Uversky, A. L. Fink, *Biochemistry* **2001**, *40*, 6036–6046.
[24] A. Ahmad, I. S. Millett, S. Doniach, V. N. Uversky, A. L. Fink, *Biochemistry* **2003**, *42*, 11404–11416.
[25] G. Bitan, M. D. Kirkitadze, A. Lomakin, S. S. Vollers, G. B. Benedek, D. B. Teplow, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 330–335.
[26] R. H. Christie, B. J. Bacskai, W. R. Zipfel, R. M. Williams, S. T. Kajdasz, W. W. Webb, B. T. Hyman, *J. Neurosci.* **2001**, *21*, 858–864.

