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Phenolic Oxime Oligomers Inhibit Alzheimer's Amyloid Fibril Formation and Disaggregate Fibrils In Vitro

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The formation of insoluble fibrils from misfolded proteins and their accumulation into amyloid plaques in the brain is an essential aspect of Alzheimer's, Parkinson's, and Huntington's diseases.[1–3] Amyloid fibrils, which induce neurotoxicity through a variety of mechanisms,[4–8] consist mainly of the amyloid-β (Aβ) peptides $Aβ_{1–40}$ and $Aβ_{1–42}$,[9] the formation of which is triggered by nucleation-dependent polymerization.[10,11] Therapeutic approaches aimed at reducing amyloid fibril formation include inhibition of Aβ production,[12] immune stimulation to remove Aβ from the brain,[13] and direct inhibition of Aβ self-assembly,[14,15] in particular by blocking the transition from monomeric to oligomeric and polymeric Aβ species with small molecule inhibitors. (This was initially discovered using the dye Congo red).[16] In the latter case the best small molecule inhibitors discovered to date are polyphenol-type compounds with potencies in the $IC_{50} = 5–10$ μM range,[17,21] such as the natural products nordihydroguaiaretic acid (NDGA),[18,22,23] myricetin,[21] or the yellow curry pigment curcumin (Figure 1).[18,24]

We recently reported an efficient high-throughput screen (HTS) for inhibitors of amyloid fibril formation using an engineered synthetic fibril building block $(Aβ_{16–37}Y_{20}K_{22}K_{24})_4$ displaying controllable kinetics of fibril formation.[25,26] The assay uses fluorescence detection of the characteristic cross-β sheet structure of amyloid fibrils by the dye thioflavin T (ThT).[27–29] In our effort to discover new inhibitors of Aβ fibril formation, we applied this HTS to a library of oxime oligomers prepared by an iterative two-step procedure for the oligomerization of hydroxy-aromatic aldehydes (Scheme 1),[30–32] and discovered of two novel inhibitors of Aβ fibril formation, **19** and **22** (Figure 1).

In the search for a suitable source of possible inhibitors of Aβ fibril formation, we noticed the structural analogy between known polyphenolic inhibitors and our recently reported collection of oxime oligomers.[30] Taking known inhibitors of Aβ-fibril formation into account, the library was extended by preparing the di- and trisubstituted phenolic oxime oligomers **3, 6, 7, 14**, **19**, as well as carbohydrate **27**, and **9**, which contains a dimethylamino group as found in the Aβ fibril formation inhibitors ThT and the dye methyl yellow (Figure 1).[19] The structures were confirmed by NMR, MS, and in selected cases by X-ray crystallography.[30] In total a series of 27 oxime oligomers spanning mono-, di-, and trimeric oximes were considered for inhibition screening (Figure 2).

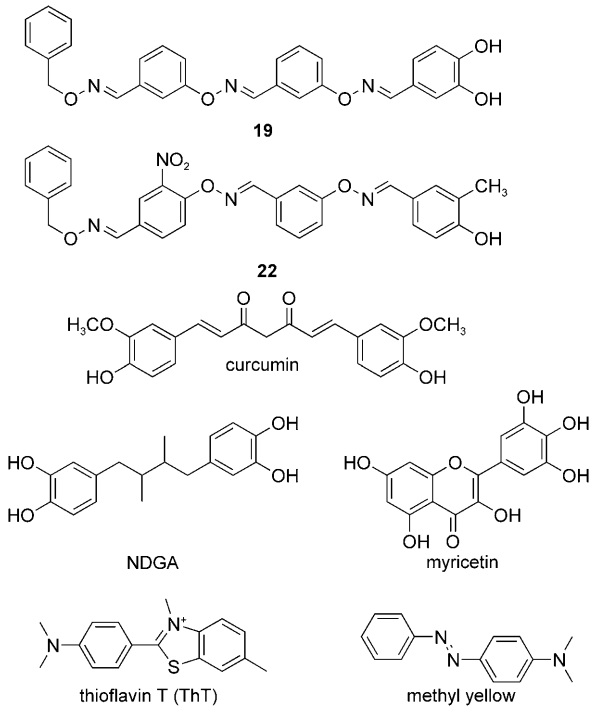
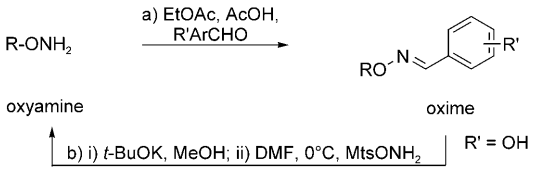


Figure 1. Structures of inhibitors **19**, **22**, NDGA, curcumin, myricetin, ThT and methyl yellow.



Scheme 1. Iterative oxime bond formation.

Screening of the oxime oligomer library showed that several compounds were active in the 10 μM range (Figure 3). The inhibition observed with our HTS assay was also measured on native $Aβ_{1–40}$ fibril formation using the ThT assay (Figure S1 in the Supporting Information). To test if the observed effect was indeed due to fibril inhibition, amyloid fibrils were visualized directly by atomic force microscopy (AFM, Figure 4). All screen-

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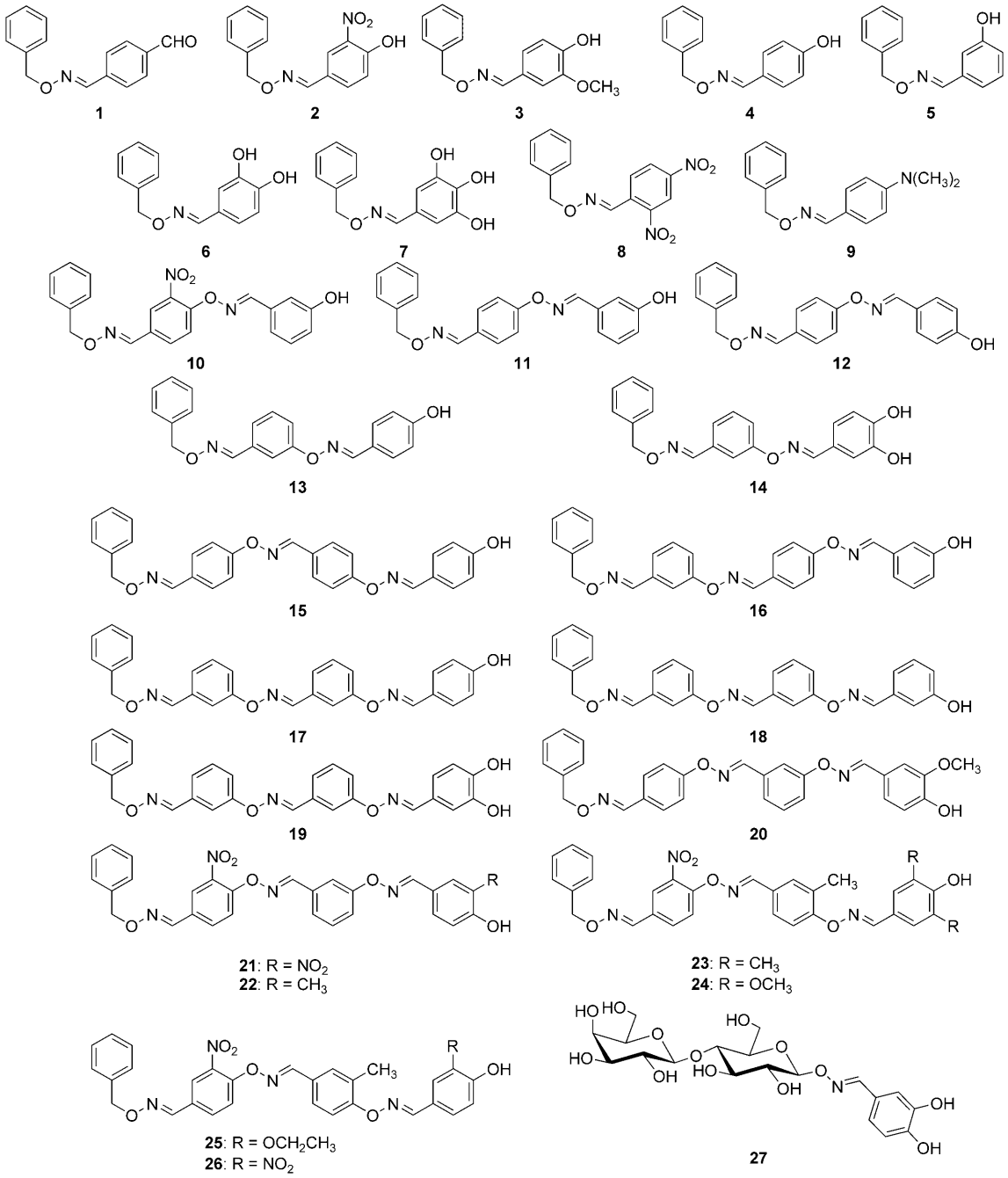

Figure 2. Library of oxime oligomers and compounds tested for inhibition of $A\beta_{1-40}$ fibril formation and fibril destabilization.

ing hits were also tested for inhibition in the presence of preformed $A\beta_{1-40}$ fibrils as seeds (Figure 5). Furthermore, selected inhibitors were tested for their ability to degrade preformed $A\beta_{1-40}$ fibrils in vitro using the ThT assay, and the effect was confirmed in turn by AFM (Figure S2). The results of these experiments are summarized in Table 1.

The simple oximes **6** and **7** showed IC_{50} values near 20 μM. However, AFM images of the $A\beta_{1-40}$ assays showed no inhibition of fibril formation compared to the control (Figure 4A and B). Considering that filter-effects can be ruled out for **6** and **7** because they do not absorb at the wavelengths of fluorescence excitation or emission,[26] fluorescence inhibition is probably caused by direct competition against the fluorescent reporter ThT for binding to the fibrils, a typical feature of polyphenolic compounds.[26] The same effects were observed with the phenolic bis-oximes **10**, **11**, **14** with IC_{50} values in the 10–30 μM range, but no effective inhibition of fibril formation when tested by AFM.

By contrast to the smaller oximes and dioximes, the larger trioxime hits **15**, **16**, **17**, **18**, **19**, and **22** not only showed good inhibition in the screening, but also blocked fibril formation as observed by AFM, as exemplified by compound **19** (Figure 4C). The most effective compounds were the trioximes **22** and **19**, with IC_{50} values in the 10 μM range. The compounds exhibited

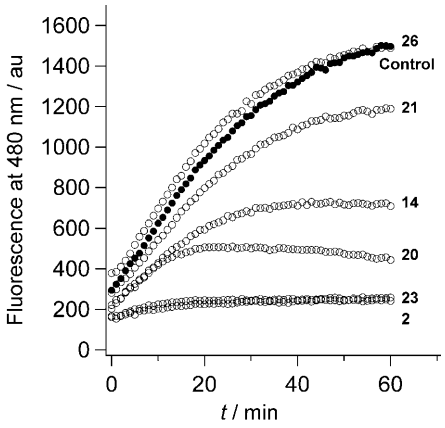

Figure 3. Initial HTS assay of fibril inhibition with selected oxime oligomers (10 μM) using the amyloid fibril model ($A\beta_{16-37}Y_{20}K_{22}K_{24})_4$. Fibril formation was triggered by adding phosphate buffer pH 7.1 at 25 °C (to a final concentration of 5 mM) to an aqueous solution of the Aβ model system (70 μL of 4 μM ($A\beta_{16-37}Y_{20}K_{22}K_{24})_4$)in the presence of 10 μM concentrations of test compounds and was followed in real time by monitoring the binding of the Aβ-sheet-specific dye thioflavin T (ThT, 10 μM) by following fluorescence at λ_{em} = 480 nm (λ_{ex} = 440 nm), which increases as a function of fibril growth. The assay was performed on a black 96-well micro plate and emission data were collected at 480 nm with one minute intervals with excitation at 440 nm.

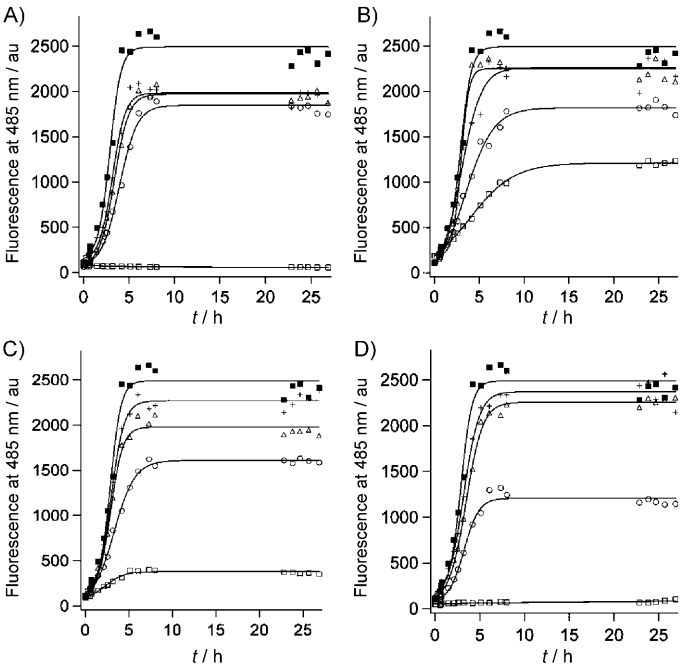

Figure 5. Inhibition studies of $A\beta_{1-40}$ (50 μM) fibril formation with the oxime compounds, in the presence of sonicated preformed $A\beta_{1-40}$ fibrils (2.5 μM) as seeds. A) **19**, B) **20**, C) **22**, D) NDGA. Concentrations of the oxime compounds are: 0 μM (■), 0.1 μM (+), 1 μM (△), 10 μM (○) and 100 μM (□). Solid lines are a nonlinear fitting of the data with a sigmoid function to aid the eye (Igor Pro, WaveMetrix). Experimental details are in Figure 3. Data are representative of two independent experiments.

comparable potencies under conditions of fibril formation and in the presence of seeds. In these two cases the compounds also triggered the deaggregation of established Aβ-fibrils with potencies comparable to the reference natural product NDGA.

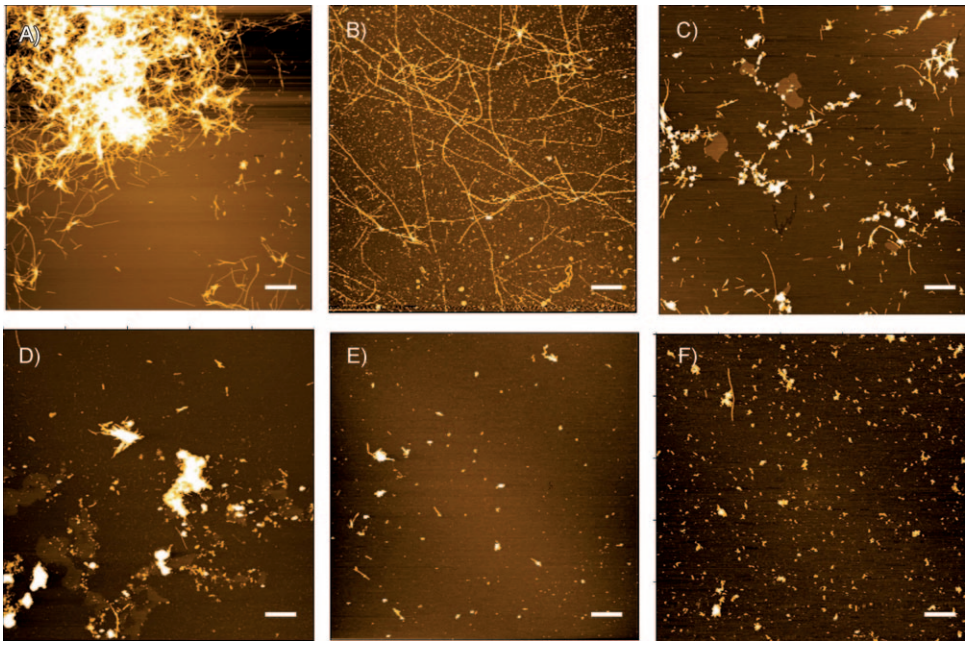

Figure 4. Representative AFM images after $A\beta_{1-40}$ fibril formation in the presence of different oxime compounds and NDGA (scan size 10×10 μm). The concentration of added compounds is 100 μM. A) Control with no added compounds. Large aggregates of fibrils are observed, the height of individual fibrils is 4 nm. B) Mono-oxime **6**, long fibrils cross the image. C) Diphenolic trioxime **19**, aggregates and a few short fibrils. D) Trioxime **22**, small aggregates. E) Trioxime **23**, small aggregates. F) Control inhibitor, NDGA, small aggregates are seen. Scale bars indicate a length of 1 μm.

The trioximes tested differ by the substitution patterns of the hydroxyaldehyde building block. The stronger inhibitory potency of **19** is probably related to the catechol group in the last building block, as its mono-deoxy analogues **17** and **18** are inactive; the catechol group is also found in the known inhibitor NGDA. Inhibition by the trioxime inhibitor **22** suggests that an *ortho*-methyl phenol is also suitable, although in this case the difference in the second building block (3-nitro-4-hydroxy-benzaldehyde instead of 3-hydroxy-benzaldehyde) might also be important. Aβ fibril inhibition by **19** and **22** probably involves hydrogen bonding of the aromatic hydroxyl group; this leads to extended hydrophobic contacts that disrupt the Aβ fibril. The smaller analogues such as **7** and **14** seem to engage in similar interactions with the fibrils as seen

Table 1. IC_{50} in the ThT assay with $A\beta_{1-40}$ and AFM observations of $A\beta_{1-40}$ fibril formation, extension, and destabilization.

Compounds	IC_{50} [μM][g]		
	Inhibition[a] (AFM)[b]	Seed[c]	Degradation[d] (AFM)[e]
2	100	100	–
6	17 (F.)[f]	85	–
7	25 (F.)	12	–
10	10 (F.)	27	–
11	8 (F.)	>100	–
14	30 (D.F.)[f]	16	30 (D.F.)
15	15 (Few F.)[f]	70	100 (F.)
16	100 (Few F.)	100	>100
17	100 (Few F.)	60	100 (F.)
18	100 (Few F.)	100	>100
19	17 (Few F.)	30	30 (No F.)
20	100 (F.)	100	>100 (F.)
21	10 (F.)	4	–
22	12 (No F.)[f]	20	30 (No F.)
23	35 (No F.)	55	100 (F.)
24	23 (F.)	60	–
26	80 (F.)	3	–
25	38 (F.)	60	–
27	4 (D.F.)	>100	50 (D.F.)
NDGA	14 (No F.)	10	10 (No F.)
	0.16[18]	0.14[18]	1.00[18]
curcumin	5 (No F.)	4	1 (D.F.)
	0.19[18]	0.19[18]	0.42[18]

[a] IC_{50} values were determined from the ThT assay with 50 μM Aβ1–40. Compounds **1**, **3**, **4**, **5**, **8**, **9**, **12**, and **13** were also tested and showed no activity at 100 μM. [b] AFM was performed after the inhibition study with 100 μM of oligomers. [c] IC_{50} values in the seeding study are determined from the ThT assay with 50 μM Aβ1–40 in the presence of 2.5 μM preformed fibrils (see Figure 5). [d] IC_{50} values in the degradation study are determined from the ThT assay with 25 μM Aβ1–40 preformed fibrils (see Figure S2). [e] AFM was performed after the destabilization study with 100 μM of oligomers. [f] Observation in AFM study: (F.) = fibrils, (D. F.) = distorted fibrils, (No F.) = no fibrils. [g] Measurement error is ±10%.

in the ThT assay, but this binding only displaces the fluorescent reporter without blocking Aβ fibril formation itself. Although molecular size seems to be essential for activity in the oxime oligomers, it should be noted that screening of a related series of ether oligomers[34] assembled from the same building blocks with the ThT assay gave no hits, excluding a size-only effect.

In summary, screening for inhibition of amyloid fibril formation using our recently reported microtiterplate fluorescence assay[26] was implemented for the first time to discover new inhibitors in a library of oxime oligomers. Investigation of initial hits by AFM revealed that the smaller oxime and dioxime hits inhibited fluorescence without blocking amyloid formation, presumably by direct competition with the fluorescence label ThT for binding to the fibrils. This finding confirms previously reported difficulties with ThT assays,[33] which could potentially be circumvented by using assays not relying on ThT.[35,36] On the other hand the longer trioxime oligomers identified by screening indeed showed effective reduction in amyloid fibril formation. Two of these oxime oligomers, compounds **19** and **22**, were also active in fibril deaggregation assays both under fluorescence and by AFM, and thus represent a new class of Aβ fibril formation inhibitors. Optimization of their activity might be possible by further structural modifications.

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Keywords: amyloid fibrils · assays · combinatorial chemistry · inhibitors · oximes

[1] P. T. Lansbury, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3342.
[2] R. M. Murphy, *Annu. Rev. Biomed. Eng.* **2002**, *4*, 155.
[3] C. M. Dobson, *Sem. Cell Devel. Biol.* **2004**, *15*, 3.
[4] M. P. Mattson, *Nature* **2004**, *430*, 631.
[5] A. Ferreira, Q. Lu, L. Orecchio, K. S. Kosik, *Mol. Cell. Neurosci.* **1997**, *9*, 220.
[6] X. Huang, C. S. Atwood, M. A. Hartshorn, G. Multhaup, L. E. Goldstein, R. C. Scarpa, M. P. Cuajungco, D. N. Gray, J. Lim, R. D. Moir, R. E. Tanzi, A. I. Bush, *Biochemistry* **1999**, *38*, 7609.
[7] M. A. Smith, C. A. Rottkamp, A. Nunomura, A. K. Raina, G. Perry, *Biochim. Biophys. Acta, Mol. Basis Dis.* **2000**, *1502*, 139.
[8] J. P. Blass, *J. Neurosci. Res.* **2001**, *66*, 851.
[9] G. G. Glenner, C. W. Wong, *Biochem. Biophys. Res. Commun.* **1984**, *120*, 885.
[10] J. T. Jarrett, E. P. Berger, P. T. Lansbury, *Biochemistry* **1993**, *32*, 4693.
[11] J. T. Jarrett, P. T. Lansbury, *Cell* **1993**, *73*, 1055.
[12] I. Dewachter, F. Van Leuven, *Lancet Neurol.* **2002**, *1*, 409.
[13] D. Schenk, R. Barbour, W. Dunn, G. Gordon, H. Grajeda, T. Guido, K. Hu, J. Huang, K. Johnson-Wood, K. Khan, D. Kholodenko, M. Lee, Z. Liao, I. Lieberburg, R. Motter, L. Mutter, F. Soriano, G. Shopp, N. Vasquez, C. Vandevert, S. Walker, M. Wogulis, T. Yednock, D. Games, P. Seubert, *Nature* **1999**, *400*, 173.
[14] F. E. Cohen, J. W. Kelly, *Nature* **2003**, *426*, 905.
[15] M. A. Findeis, *Biochim. Biophys. Acta, Mol. Basis Dis.* **2000**, *1502*, 76.
[16] A. Lorenzo, B. A. Yankner, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 12243.
[17] Y. Porat, A. Abramowitz, E. Gazit, *Chem. Biol. Drug Des.* **2006**, *67*, 27.
[18] K. Ono, K. Hasegawa, H. Naiki, M. Yamada, *J. Neurosci. Res.* **2004**, *75*, 742.
[19] S. Taniguchi, N. Suzuki, M. Masuda, S.-I. Hisanaga, T. Iwatsubo, M. Goedert, M. Hasegawa, *J. Biol. Chem.* **2005**, *280*, 7614.
[20] G. Forloni, L. Colombo, L. Girola, F. Tagliavini, M. Salmona, *FEBS Lett.* **2001**, *487*, 404.
[21] K. Ono, Y. Yoshiike, A. Takashima, K. Hasegawa, H. Naiki, M. Yamada, *J. Neurochem.* **2003**, *87*, 172.
[22] J. Luo, T. Chuang, J. Cheung, J. Quan, J. Tsai, C. Sullivan, R. F. Hector, M. J. Reed, K. Meszaros, S. R. King, T. J. Carlson, G. M. Reaven, *Eur. J. Pharmacol.* **1998**, *346*, 77.
[23] Y. Goodman, M. R. Steiner, S. M. Steiner, M. P. Mattson, *Brain Res.* **1994**, *654*, 171.
[24] F. Yang, G. P. Lim, A. N. Begum, O. J. Ubeda, M. R. Simmons, S. S. Ambegaokar, P. P. Chen, R. Kayed, C. G. Glabe, S. A. Frautschy, G. M. Cole, *J. Biol. Chem.* **2005**, *280*, 5892.
[25] G. T. Dolphin, P. Dumy, J. Garcia, *Angew. Chem.* **2006**, *118*, 2765; *Angew. Chem. Int. Ed.* **2006**, *45*, 2699.
[26] G. T. Dolphin, M. Ouberai, P. Dumy, J. Garcia, *ChemMedChem* **2007**, *2*, 1613.
[27] I. H. LeVine, *Methods Enzymol.* **1999**, *309*, 274.
[28] I. H. LeVine, *Protein Sci.* **1993**, *2*, 404.
[29] R. J. Chalifour, R. W. McLaughlin, L. Lavoie, C. Morissette, N. Tremblay, M. Boule, P. Sarazin, D. Stea, D. Lacombe, P. Tremblay, F. Gervais, *J. Biol. Chem.* **2003**, *278*, 34874.
[30] O. Renaudet, J.-L. Reymond, *Org. Lett.* **2003**, *5*, 4693.
[31] E. Lustig, *J. Phys. Chem.* **1961**, *65*, 491.
[32] S. M. Johnson, H. M. Petrassi, S. K. Palaninathan, N. N. Mohamedmohaideen, H. E. Purkey, C. Nichols, K. P. Chiang, T. Walkup, J. C. Sacchettini, K. B. Sharpless, J. W. Kelly, *J. Med. Chem.* **2005**, *48*, 1576.

[33] M. A. Findeis, G. M. Musso, C. C. Arico-Muendel, H. W. Benjamin, A. M. Hundal, J.-J. Lee, J. Chin, M. Kelley, J. Wakefield, N. J. Hayward, S. M. Molineaux, *Biochemistry* **1999**, *38*, 6791.
[34] O. Renaudet, J.-L. Reymond, *Org. Lett.* **2004**, *6*, 397.
[35] W. Kim, Y. Kim, J. Min, D. J. Kim, Y.-T. Chang, M. H. Hecht, *ACS Chem. Biol.* **2006**, *1*, 461.
[36] *Methods in Molecular Biology, Vol. 299: Amyloid Proteins* (Ed.: E. M. Sigurdsson), Humana, Totowa, **2005**.

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