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Supporting Information

Real-Time BODIPY-Binding Assay To Screen Inhibitors of the Early Oligomerization Process of Aβ1–42 Peptide

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Real-time BODIPY-binding assay to screen inhibitors of the early oligomerization process of Aβ 1-42 peptide

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Synthesis and methods.

Usual solvents were purchased from commercial sources and dried and distilled by standard procedures. Protected amino acids, 1-hydroxy-1H-benzotriazol (HOBt), N-N'-diisopropylcarbodiimide (DIC), and Oxyma Plus were purchased from commercial sources. Triazole-containing BODIPY dye was synthesized according to the literature protocol, described by Smith *et al.* [1] The synthesis of SEN304 was performed by adapting the protocol described by Kokkoni *et al.* [2] (solid-phase Fmoc chemistry using DIC/Oxyma Plus as the coupling reagents). The intermediate Fmoc cyclohexyl glycine was prepared according the procedure described by Bain *et al.* [3] All the other compounds were synthesized by following a manual solid-phase peptide synthesis protocol using Fmoc chemistry. Rink amide resin was used as solid support (300 mg for 0.1 mmol scale). Crude peptides were purified and were analysed for purity by reverse-phase high performance liquid chromatography system and characterized for identity by high resolution mass spectrometry.

General procedure: Resin was first swelled in DMF for 1 hour in a reaction vessel equipped with a sintered glass bottom. Fmoc group on the linker was then removed by using a solution of 0.1M HOBt and 20 % piperidine in DMF (8 mL) for 40 min (deprotection cycle was performed twice). Subsequently, Fmoc protected amino acid was first activated in situ by treatment with DIC (1.1 eq.) and Oxyma Plus (1.1 eq.) in DCM for 10 minutes and then transferred to the resin, dispersed in a solution of DMF (4 mL) and 2,4,6-trimethylpyridine (10 eq.). The amino acid was then let coupling by mechanical shaking at ambient temperature overnight. The coupling step was followed by a capping phase, using a solution of acetic anhydride (10 eq.) in DMF (8 mL) and stirring for one hour at ambient temperature. Successful completion of each coupling was monitored by UV spectroscopic method. The cycle of Fmoc removal and coupling was repeated with subsequent Fmoc protected amino acids to obtain resin-bound desired pentapeptides. Each coupling was followed by removal of Fmoc group, washing the resin by dimethylformamide (5x 8 mL), dichloromethane (3x 8 mL), methanol (1x 8 mL), dichloromethane (1x 8 mL) and dietylether (1x 8 mL) and then drying. Cleavage of peptides from resin was carried out by using TFA:triisopropylsilane:water (95:2.5:2.5) as cleavage cocktail (10 mL), stirred by mechanical shaking at ambient temperature for 2 hours. Filtration afforded the peptide in filtrate which was evaporated under vacuum to remove the excess of TFA. Then, the crude was dissolved in ACN/water 1:1 (v/v) and freeze-dried, before performing the purification by HPLC (0%-70% of ACN/H_2O/TFA 95:5:0.1 in 45 min).

Compound	Calculated ion mass	Measured ion mass	Retention time (0-98% in 4 min, Flow rate 0.25mL/min)
1	725.49601	725.4936	4.30 min
2	457.3133	457.3153	3.15 min
3	457.3133	457.3128	3.10 min
4	499.3286	499.3228	3.43 min
5	694.4287	694.4258	4.05 min

Preparation of the Aβ1-42. Aβ1-42 was obtained from Bachem (Bubendorf, Switzerland) or from American Peptide. To remove preformed aggregates, it dissolved during 15-20 minutes at room temperature in 0.16% NH_4OH to give a concentration of 2 mg/mL, followed by immediate lyophilisation. The dry samples were then stored at -20°C in aliquots of 40 μg each until use. [4] Milli-Q system was used to purify water that was used throughout this work.

A. **Fibrillation protocol.** The Aβ1-42 peptide was dissolved in an aqueous 1% ammonia solution to a concentration of 1 mM and then, just prior to use, was diluted to 0.2 mM with 10 mM Tris-HCl, 100 mM NaCl buffer (pH 7.4). [5-7]

B. **Oligomerization protocol.** The Aβ1-42 peptide was reconstituted first in 1% NH4OH (at 1 mg/mL) with sonication at 25°C for 1 minute before dissolving in 20 mM phosphate buffer pH 7.4 to provide the appropriate concentration. Under this preparation condition, fibrils were detected after 3 days with naked eye.

Circular dichroism. CD spectra (195-250 nm) were acquired on Jasco J-810 using 0.1 cm quartz cells. The spectra were recorded with 0.1 nm resolution at 25°C and a scan rate of 50 nm/min. Five scans were acquired and averaged for each sample; raw data were processed by background subtraction (or inhibitor subtraction when required), followed by smoothing (Savitzky-Golay meth-od) using the software Origin. The data are given as CD intensity in mdeg. Secondary

structure percentages were calculated from these spectra over the range 200-250 nm using the freely accessible algorithm BeStSel (best-sel.elte.hu) which includes independent basis spectra for both parallel and anti-parallel β-sheet. [8-9] The deconvolution was performed on the CD measurement, after subtraction of the corresponding buffer or the solution containing the 5/1 concentration of inhibitor.

Fluorescence spectroscopy. Fluorescence measurements were performed on FP-8300 fluorescence spectrometer from Jasco in a 1 cm quartz cell with a resolution of 0.5 nm. The bandwidth used was 5 nm for the excitation and emission slits. Samples were excited at the absorption maxima: 440 nm for ThT and 518 nm for BODIPY. Thioflavin T was obtained from Sigma and BODIPY was obtained from the synthesis described in literature. [10]

SDS-PAGE. SDS-PAGE analysis of the Aβ1-42 solution were performed by adapting the protocol described by Amijee et al. [11] Aβ1-42 was prepared as described in the oligomerization protocol (Protocol B). At various time points (0, 2, 4 and 8h), the incubated mixture was snap frozen over liquid nitrogen and stored at -80°C until required. Bio-Rad Mini-PROTEAN TGX precast gels and 1x Glycine SDS running buffer were used. The samples containing Aβ1-42 alone or Aβ1-42 and inhibitor were prepared in the following manner before loading into the gel: 1 part of the sample was diluted with 1 part 2x Laemmli sample buffer containing 5% of 2-mercaptoethanol, and boiled at 95° C for 2 min. 25 µL aliquots were placed on the gel as well as a 10 µL 1:10 dilution of Precision Plus Protein Dual Color Standards (Bio-Rad). Samples were subjected to electrophoresis at 80 V for 10 minutes and 200 V for 30-40 minutes. The gel staining was performed with SilverXpress Staining kit (Invitrogen) and documented by placing between two sheets of transparency and scanning at 600 dpi using a RICOH MP C306Z. The estimation of the intensity of the bands was performed with the free available software ImageJ.

Transmission Electron Microscopy (TEM). The TEM-micrographs were recorded with a JEM 2100 micro-scope (JEOL, Eching, Germany), operating at an acceler-ation voltage of 200 kV in the bright-field mode. TEM specimens were prepared by putting ca. 5 µl of a sample solution on a TEM copper grid with a carbon support film (200 mesh, Science Services, Munich, Germany) at room temperature. The carbon coated copper grids have been pre-treated by glow discharge for 20 s. The excess of liquid was blotted off with a filter paper after ca. 2 min. Images were analysed using software ImageJ.

Fluorescence-detected Thioflavin-T binding assay. Aβ1-42 was prepared according protocol A (fibrillation) or protocol B (oligomerization) as required. Stock solutions of compounds (**1**-**5**) were dissolved in DMSO (20 mM). Thioflavin T fluorescence was optimized to evaluate the development of Aβ1-42 fibrils over time using a fluorescence plate reader (Fluostar Optima, BMG lab-tech) with standard 96-well black microtiter plates. The ThT fluorescence intensity was recorded with 440/480 nm excitation/emission filters set for 42 hours applying a double orbital shaking of 10 s before the first cycle. Experiments were started by adding Aβ1-42 reaching a final concentration of 10 µM into a mixture containing 40 µM Thioflavin T in 10 mM Tris-HCl, 100 mM NaCl buffer (pH 7.4) with and without the tested compounds at different concentrations (100 and 10 µM) at 25°C (maxi-mal DMSO concentration of 0.5% (v/v)). Each condition was recorded in triplicate and the kinetic curve was fitted to a Boltzman sigmoidal equation using GraphPad Prism 7 from which the time of the half-life of aggregation (t1/2) and the intensity of the experimental fluorescence plateau (F) were obtained. The ability of compounds to inhibit Aβ1-42 aggregation was assessed considering both the $t_{1/2}$ extension/reduction and the F re-duction and presented in Table 1. [12; 5-7] The $t_{1/2}$ extension is defined as the experimental $t_{1/2}$ in the presence of the tested compound relative to the one obtained without the compound and is evaluated as the following percent-age: $t_{1/2}$ (Aβ1-42+compound) – $t_{1/2}$ (Aβ1-42) / $t_{1/2}$ (Aβ1-42) X 100. The F reduction is defined as the intensity of experimental fluorescence plateau observed with the tested compound relative to the value obtained without the compound and is evaluated as the following percentage: (F Aβ1-42+compound – F Aβ1-42) / F Aβ1-42 X 100.

Fluorescence-detected BODIPY binding assay. Stock solutions of the BODIPY dye for spectroscopic measurements and for time-dependent kinetics were prepared in EtOH (0.0428 mM) and subsequently diluted to 5.3 µM in PBS buffer (stock solution). The Aβ1-42 peptide was prepared, following protocol A (fibrillation) or protocol B (oligomerization) as required. Stock solutions of compounds (**1**-**5**) were dissolved in DMSO (20 mM) and later diluted in PBS buffer to reach two different concentration (400 µM and 40 µM). BODIPY fluorescence was optimized to evaluate the development of Aβ1-42 oligomers over time using a fluorescence plate reader (Tecan i-control 200) with standard 96-well black microtiter plates. The BODIPY fluorescence intensity was recorded with 518/540 nm excitation/emission filters set for 15 hs (or 9hs) hours performing a double orbital shaking of 10 s before the first cycle. Experiments were started by adding Aβ1-42 to final concentration of 10 µM into a mixture containing 0.53 µM BODIPY in 20 mM buffer (pH 7.4) with and without the tested compounds at different concentrations (100 and 10 µM) at 25°C. The kinetic curves are the representative average of triplicate measurements and representative values of two independent

experiments; error bars (+/- standard error) for each measured point (every 10 min) are presented. The ability of compounds to inhibit Aβ1-42 aggregation is reported in Table 1 considering the time of the half-life of aggregation ($t_{1/2}$), the intensity of the experi-mental fluorescence plateau (F) and the slope of the linear part of the curve. The $t_{1/2}$ extension is defined as the experimental $t_{1/2}$ in the presence of the tested com-pound relative to the one obtained without the com-pound and is evaluated as the following percentage: $t_{1/2}$ (Aβ1-42+compound) – $t_{1/2}$ (Aβ1-42) / $t_{1/2}$ (Aβ1-42) X 100. The F reduction is defined as the intensity of experimental fluorescence plateau observed with the tested compound relative to the value obtained without the compound and is evaluated as the following percentage: (F Aβ1-42+compound – F Aβ1-42) / F Aβ1-42 X 100. The slope variation, which is correlated with the rate of the oligomerization process, was calculated by fitting the linear part of the curve between 0 and 4 hours by RMSD regression. The slope variation is defined as the slope of experimental linear curve observed with the tested com-pound relative to the value obtained without the compound and is evaluated as the following percentage: (Slope Aβ1-42+compound – Slope Aβ1-42) / Slope Aβ1-42 X 100.

Cell viability assay. SH-SY5Y neuroblastoma cells were grown in low serum Optimem (Life Technologies) for 24 hours at 37 °C, 5% CO2 in a 96 well plate at 20 000 cells per well. Aβ1-42 was prepared as described above. The Aβ1-42 aliquot was prepared by dissolving in sterile PBS (20 mM phosphate buffer pH 7.4) reaching a final Aβ1-42 concentration of 50 µM in the presence of 250 and 50 µM of compounds **1**, **2**, **4**, **5** for 24 hours at 37 °C, along with a negative control without inhibitor and a positive control with resveratrol. After the 24-hour peri-od, media was removed from the cells and replaced with Optimem containing the pre-incubated Aβ1-42 solutions diluted one to ten (5 µM Aβ1-42 final concentration). The cells were incubated for a further 24 hours under the aforementioned conditions, performed the cell viability (MTS assay) using the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega). The assay was performed in two independent experiments with n=6 for each condition, representative results are shown in Figure 5 in the paper. The significant difference between the values from cells in the presence of Aβ1-42 and cells in the presence of Aβ1-42 and the inhibitor ($p < 0.05$, 0.01 and 0.001) was measured by statistical ANOVA test.

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Overtime comparison between ThT and BODIPY fluorescence emission in presence of Aβ42.

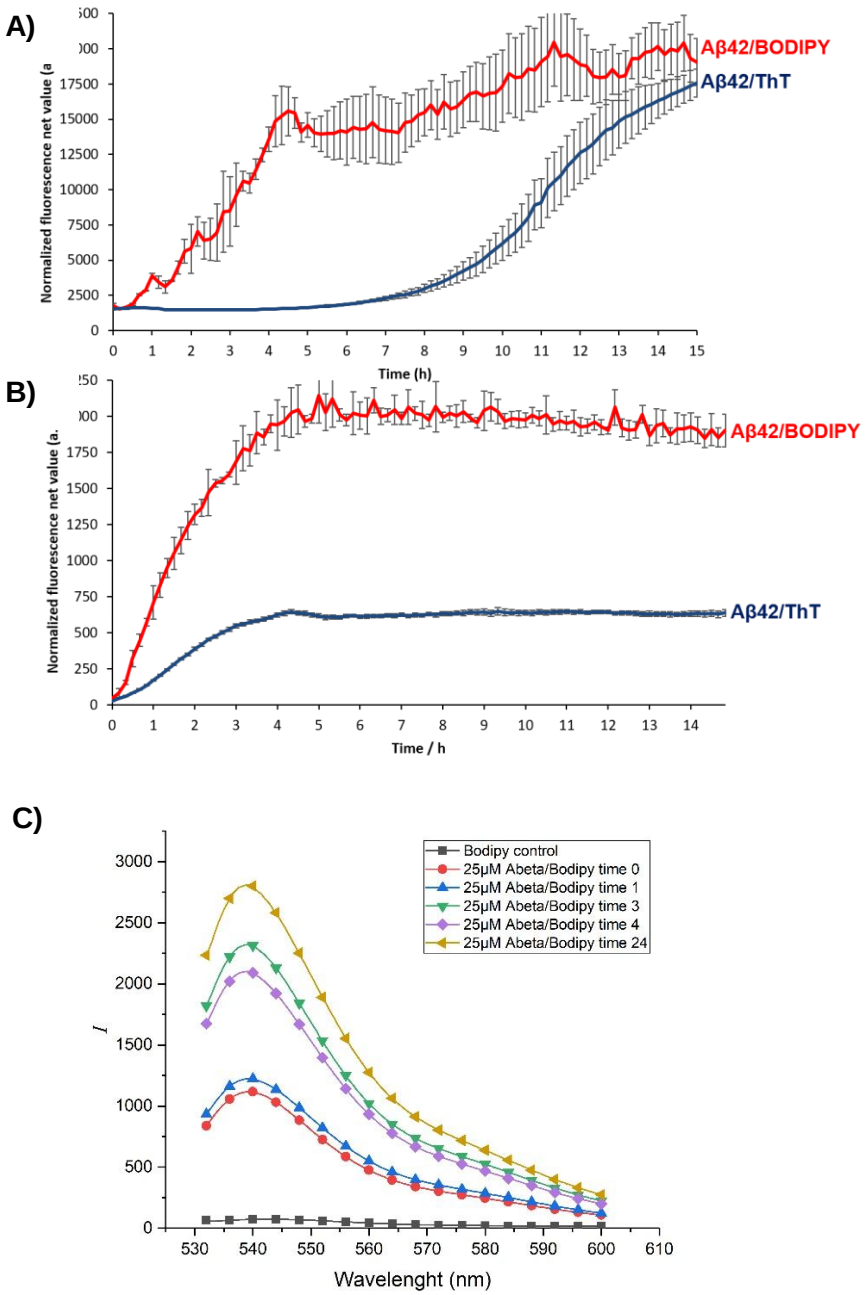


Figure S1: A) Comparison of BODIPY (red) and ThT (blue) fluorescence intensity over 15h in 96-well plate format, in TrisHCl buffer pH 7.4, in the presence of 10 μM Aβ42, prepared following the protocol A (fibrillization). The curves are provided as the average of triplicate measurements with error bars (+/- standard error) for each measured point (every 10 min). The measurements were done in triplicate at least in two different experiments. The data are presented as normalized fluorescence net values. B) Comparison of BODIPY (red) and ThT (blue) fluorescence intensity over 15h in 96-well plate format, in PBS buffer pH 7.4, in the presence of 10 μM Aβ42, prepared following the protocol B (oligomerization). The curves are provided as the average of triplicate measurements with error bars (+/- standard error) for each measured point (every 10 min). The measurements were done in triplicate at least in two different experiments. The data are presented as normalized fluorescence net values. C) BODIPY fluorescence intensity over 24h in PBS buffer pH 7.4, in the presence of 25 μM Aβ42, prepared following the oligomerization protocol.

Overtime monomer and oligomer distribution of 10 µM Aβ42 in 20 mM PBS buffer pH 7.4 (Protocol B: oligomerization) in Mini-PROTEAN TGX precast gel.

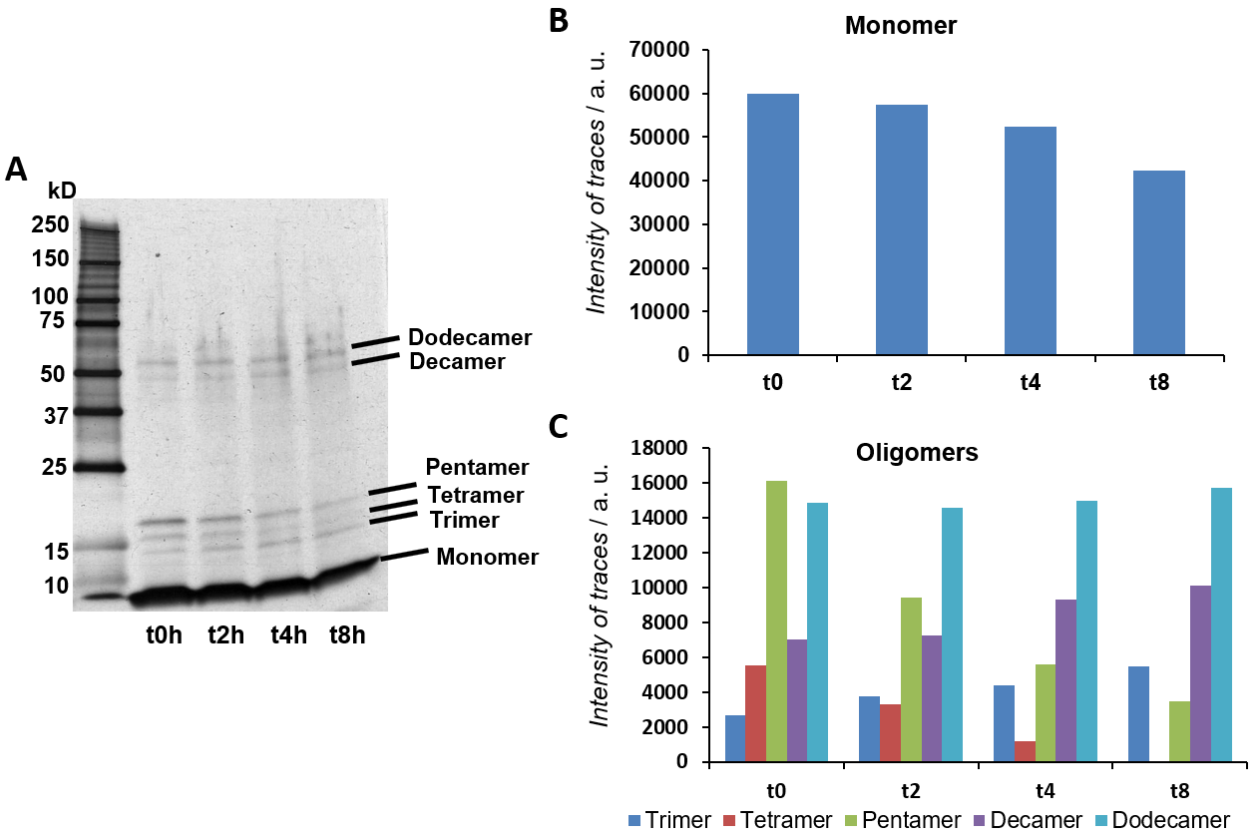


Figure S2: A) SDS-PAGE and silver stained glycine gel, showing the bands of different Aβ42 oligomeric species after 0, 2, 4, and 8h (10 µM Aβ42) B) Overtime estimation of the intensity of the monomer banding in Mini-PROTEAN TGX precast gel at various time points (0, 2, 4 and 8h) for 10 µM Aβ42 in 20mM PBS buffer; C) Overtime oligomer distribution of 10 µM Aβ42 in 20 mM PBS buffer in Mini-PROTEAN TGX precast gel, according to the estimation of the intensity of the traces, corresponding to the different size of oligomers, at various time points (0, 2, 4 and 8h).

Time-dependent changes in secondary structure of 25 µM Aβ42, using the oligomerization protocol (Protocol B).

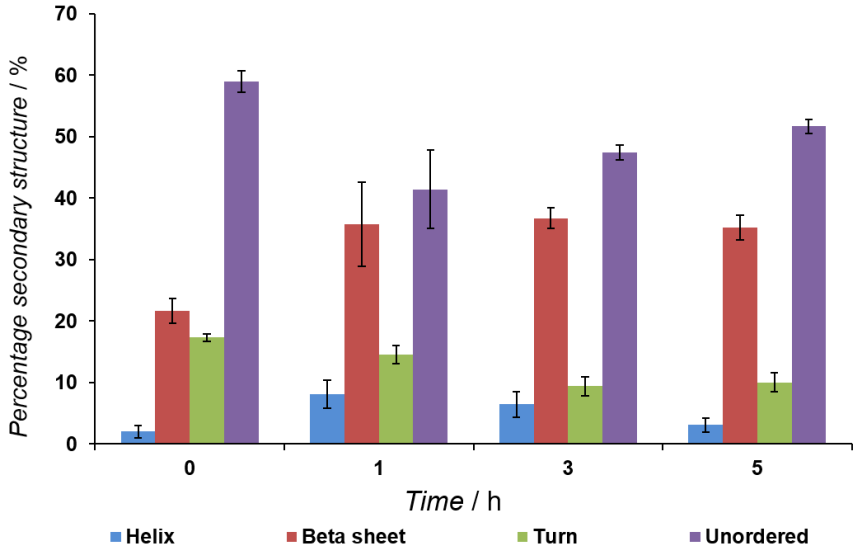
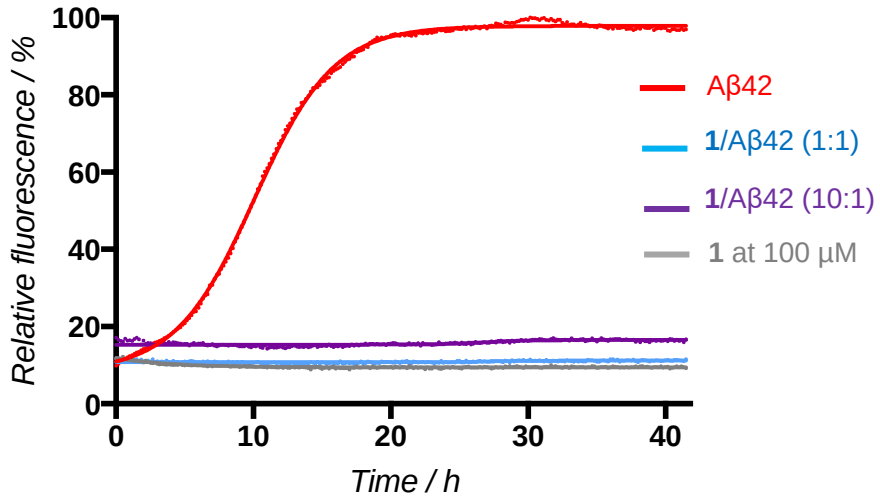
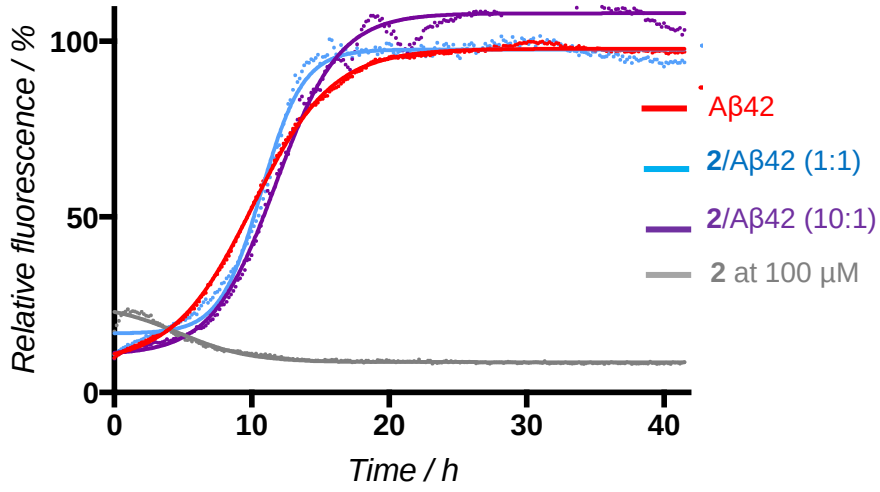
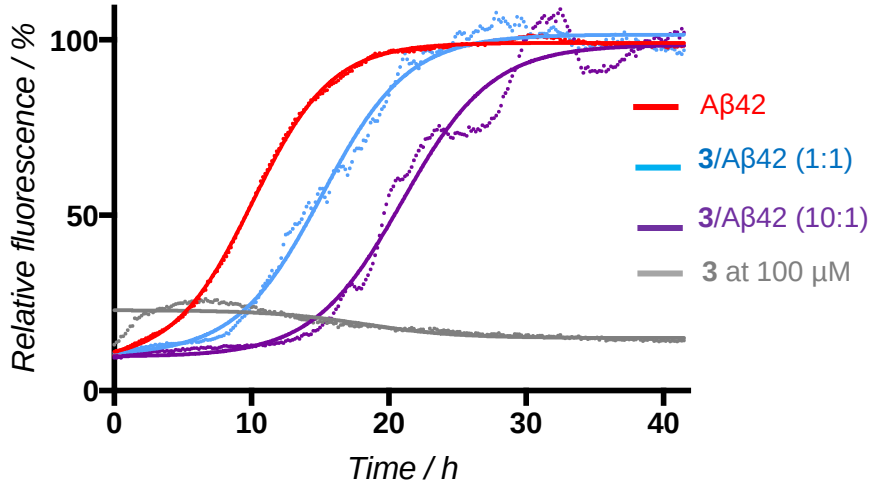


Figure S3: Estimated secondary structure content from CD analysis of 25 µM Aβ42 in 20 mM PBS buffer pH 7.4 (oligomerization protocol). Data are provided with error bars calculated from four independent analyses.

Representative real-time curves of Tht fluorescence assays, showing Aβ42 fibrillation in the presence of compounds 1-5 (Protocol A: fibrillation).







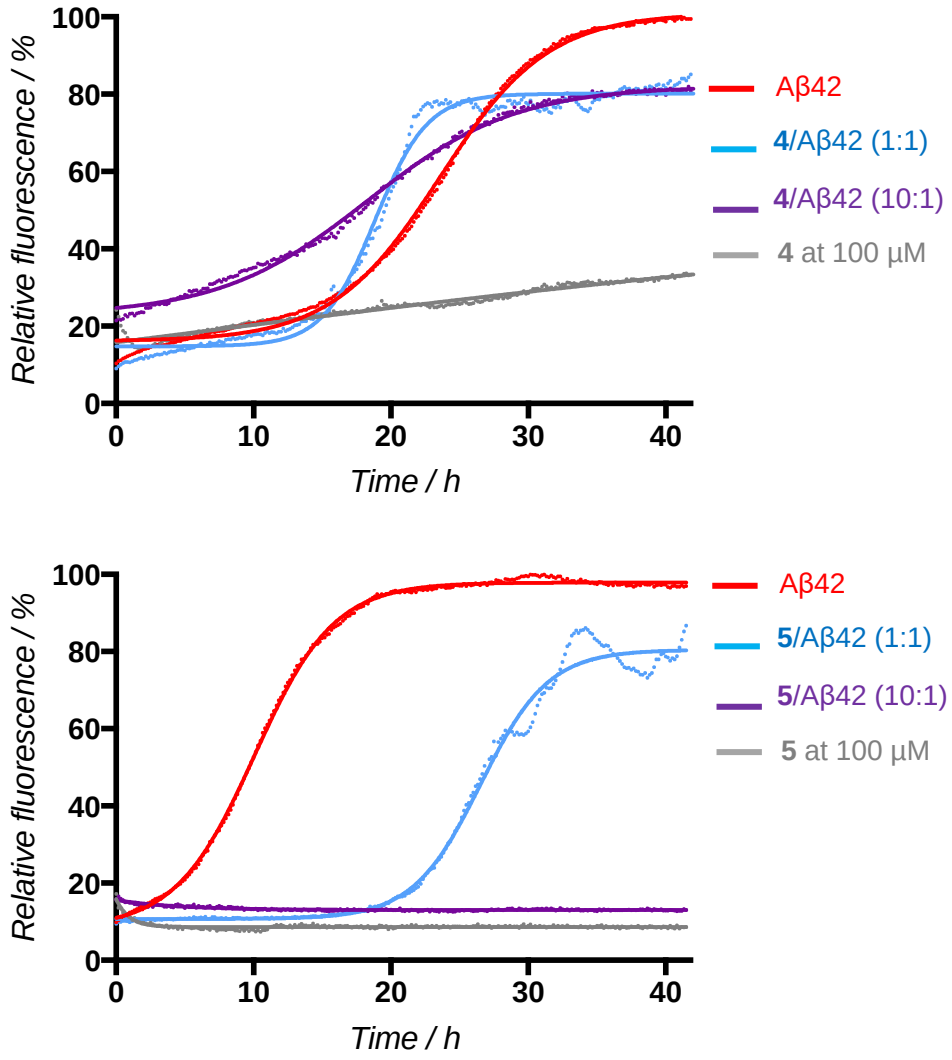
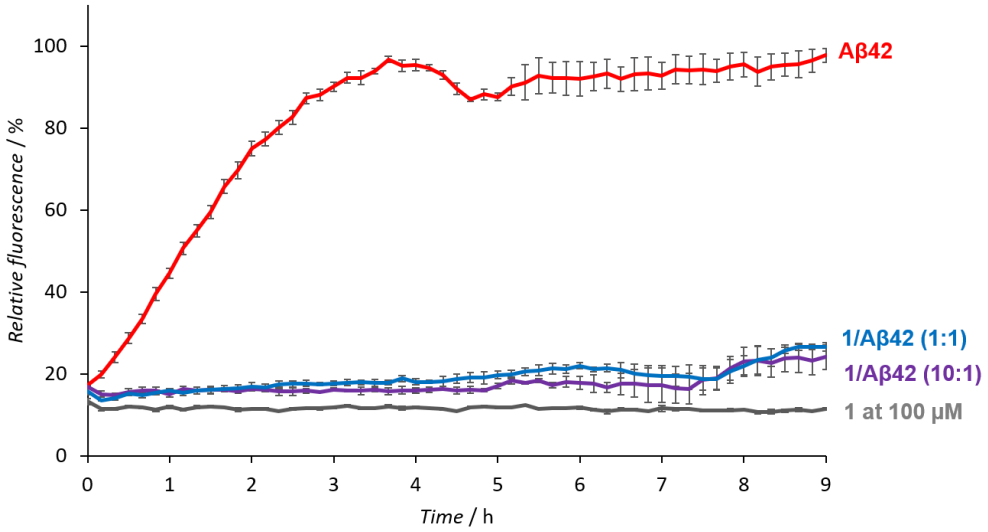


Figure S4: Representative real-time curves of ThT fluorescence assays showing Aβ1-42 (10 µM) fibrillation (Protocol A) in the absence (red curves) and in the presence of compounds **1**-**5** at compound/Aβ42 ratios of 10/1 (purple curves) and 1/1 (light blue curves). The control curves are represented in grey. Data were fitted with a Boltzmann sigmoidal curve using GraphPad Prism.

Representative real-time curves of BODIPY fluorescence assays showing Aβ42 oligomerization in the presence of compound 1-5 (Protocol B: oligomerization).

A



B

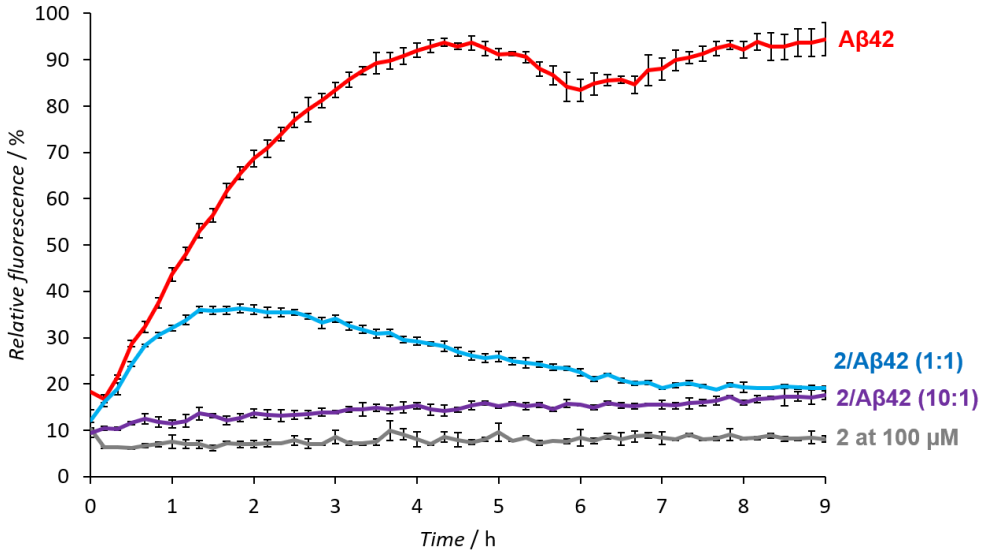


Figure S5: Representative real-time curves of BODIPY fluorescence assays showing 10 µM Aβ42 oligomerization in the absence (red curve) and in the presence of compound **1** (A) and **2** (B) at compound/ Aβ42 ratios of 10/1 (purple curve) and 1/1 (light blue curve). The control curve is represented in grey line. The curves are provided as the average of triplicate measurements with error bars (+/- standard error) for each measured point (every 10 min). The measurements were done in triplicate in two different experiments.

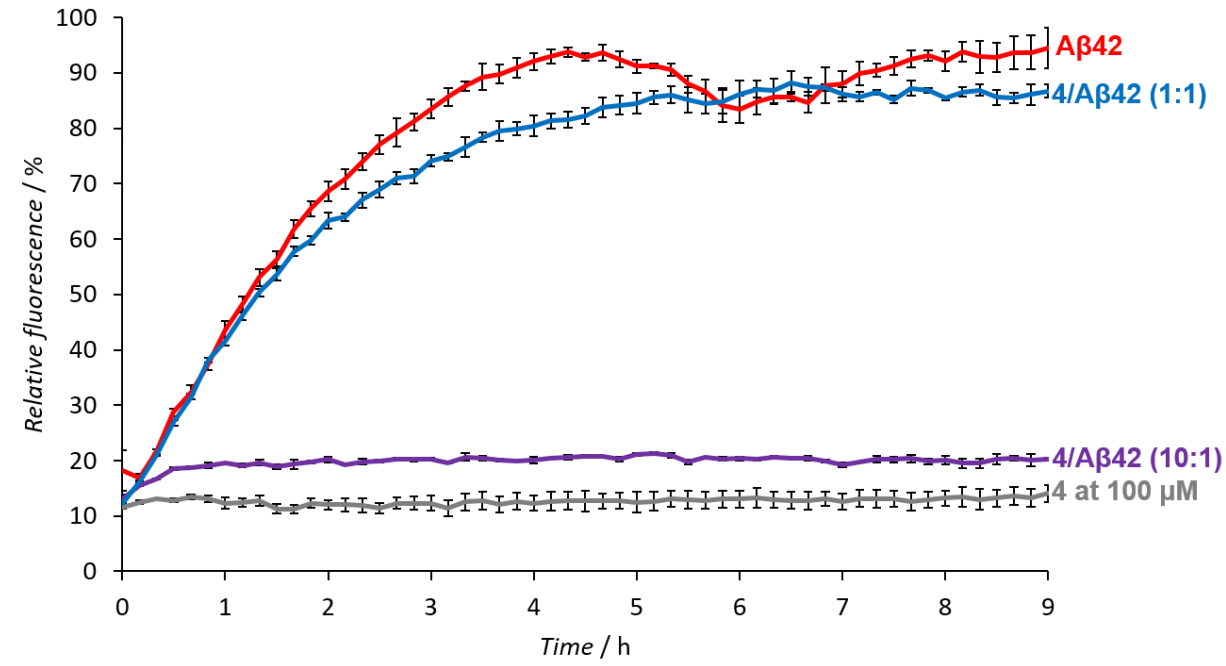


Figure S6: Representative real-time curves of BODIPY fluorescence assays showing 10 µM Aβ42 oligomerization in the absence (red curve) and in the presence of compound **4** at compound/ Aβ42 ratios of 10/1 (purple curve) and 1/1 (light blue curve). The control curve is represented in grey line. The curves are provided as the average of triplicate measurements with error bars (+/- standard error) for each measured point (every 10 min). The measurements were done in triplicate in two different experiments.

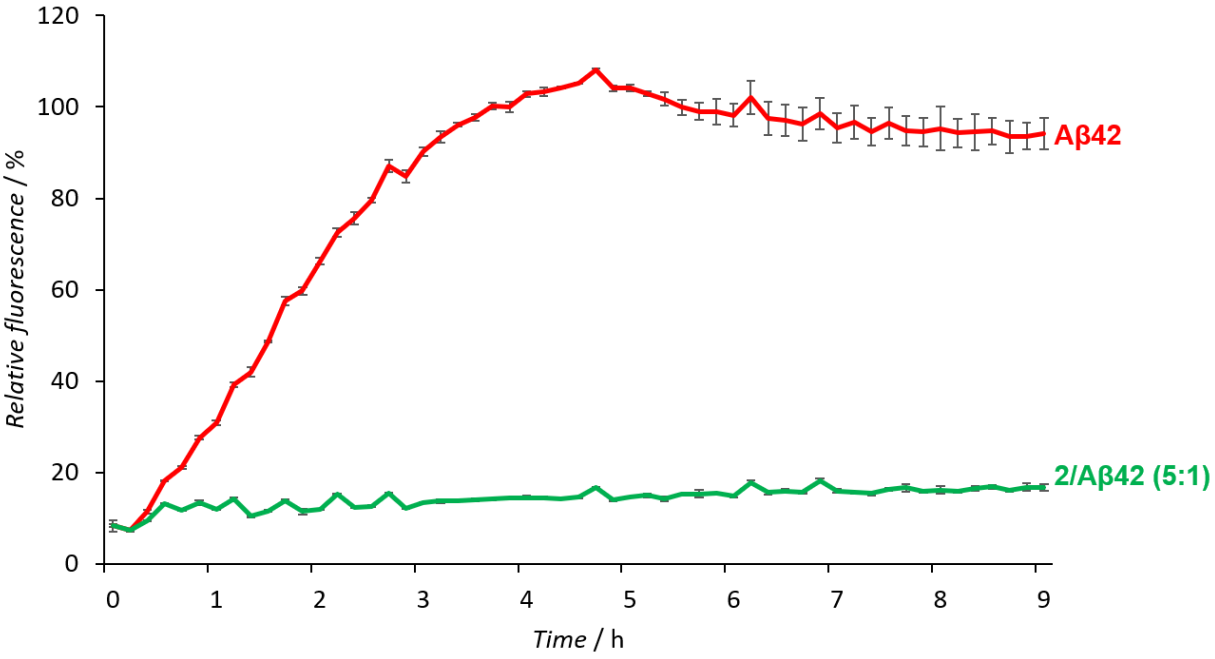


Figure S7: Representative real-time curves of BODIPY fluorescence assays showing 10 µM Aβ42 oligomerization in the absence (red curve) and in the presence of compound **2** at compound/ Aβ42 ratios of 5/1 (green curve). The curves are provided as the average of triplicate measurements with error bars (+/- standard error) for each measured point (every 10 min). The measurements were done in triplicate in two different experiments.

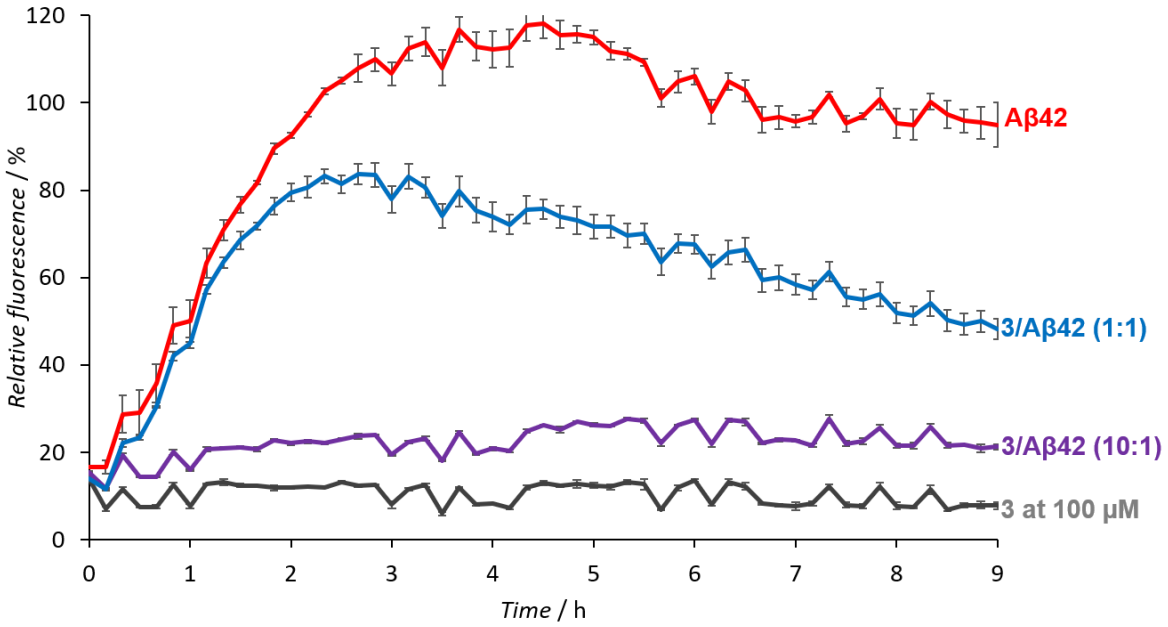


Figure S8: Representative real-time curves of BODIPY fluorescence assays showing 10 µM Aβ42 oligomerization in the absence (red curve) and in the presence of compound **3** at compound/ Aβ42 ratios of 10/1 (purple curve) and 1/1 (light blue curve). The control curve is represented in grey line. The curves are provided as the average of triplicate measurements with error bars (+/- standard error) for each measured point (every 10 min). The measurements were done in triplicate in two different experiments.

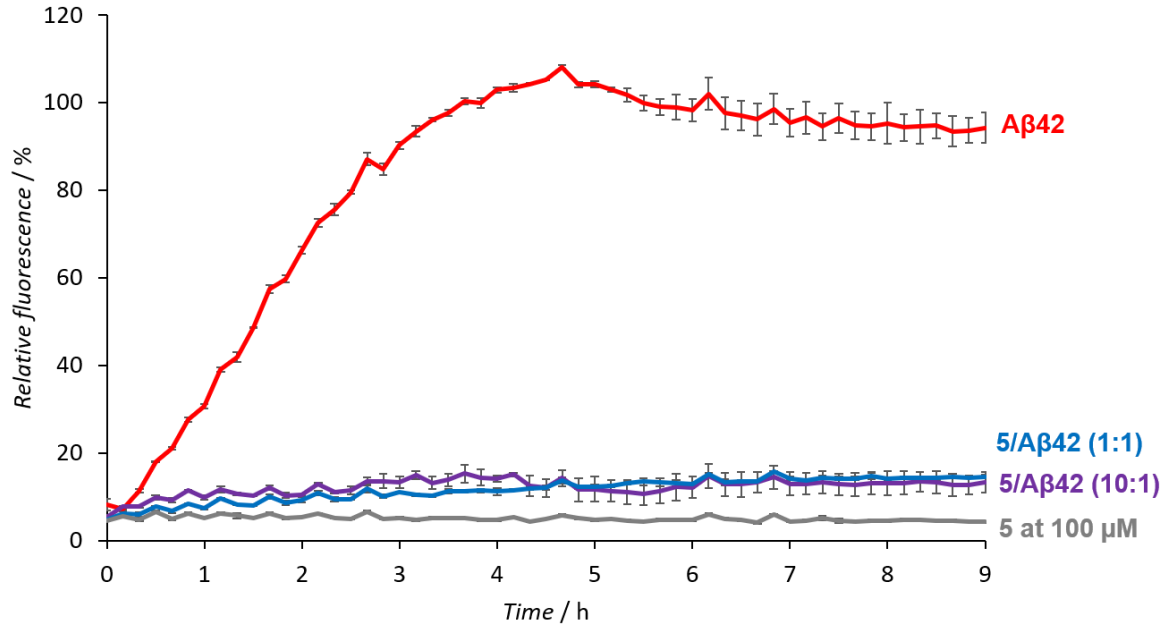


Figure S9: Representative real-time curves of BODIPY fluorescence assays showing 10 µM Aβ42 oligomerization in the absence (red curve) and in the presence of compound **5** at compound/ Aβ42 ratios of 10/1 (purple curve) and 1/1 (light blue curve). The control curve is represented in grey line. The curves are provided as the average of triplicate measurements with error bars (+/- standard error) for each measured point (every 10 min). The measurements were done in triplicate in two different experiments.

β-sheet structure content from CD deconvolution of 20 µM Aβ42 in absence and in presence of 100 µM compound 2.

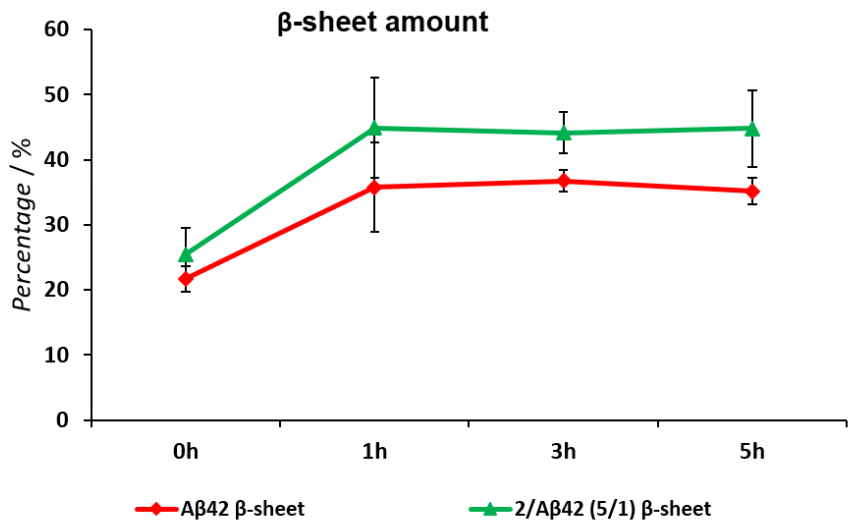


Figure S10: Time-dependent changes in β-sheet structure content from CD analysis of 20 µM Aβ42 in absence and in presence of 100 µM compound **2** (compound/ Aβ42, 5/1). Data are provided as an average of three independent analyses.