Supporting Information for

Synthetic Dityrosine-linked β-Amyloid Dimers Form Stable, Soluble, Neurotoxic Oligomers

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Experimental procedures

N,N'-Bis ([9*H*-fluoren-9-yl]methoxycarbonyl)dityrosine 3

Dityrosine[1] (0.43 g, 1.19 mmol), Fmoc-OSu (0.80 g, 2.38 mmol) and Na_2CO_3 (0.77 g, 7.14 mmol) were added to acetone/water (1:1, 15 mL) and stirred at room temperature overnight. The reaction mixture was then acidified to pH 2 with concentrated HCl, then concentrated *in vacuo* to remove the acetone. The resulting mixture was then extracted with DCM (2 × 6 mL), dried ($MgSO_4$) and concentrated *in vacuo* to give a beige solid. The crude product was chromatographed on reverse-phase HPLC with Phenomenex Synergi 4μ HYDRO-RP 80Å (50 × 21.20mm) on a linear gradient of 0–90% buffer B [buffer A: 0.1% TFA in water (v/v); buffer B: 0.1% TFA in acetonitrile (v/v)] over 45 min, with a flow rate of 5 mL/min, to yield a white solid **3** (0.67 g, 70%); m.p. 133–136ºC (decomp.); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.77 (4H, d, J = 7.2 Hz, ArH), 7.59 (4H, d, J = 7.6 Hz, ArH), 7.41 (4H, appt, J = 7.4 Hz, ArH), 7.32 (4H, appt, J = 7.4 Hz, ArH), 6.77–6.90 (6H, m, ArH), 5.18 (2H, d, J = 8.0 Hz, NH), 4.85 (2H, m, α-H), 4.54 (2H, dd, J = 6.8, 10.0 Hz, FmocH), 4.40 (2H, dd, J = 6.8, 10 Hz, FmocH), 4.23 (2H, t, J = 6.8 Hz, FmocH), 3.41 (2H, brd, J = 13.6 Hz, β-H), 3.05 (2H, dd, J = 6.4, 13.6 Hz, β-H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 178.2, 155.8, 152.3, 143.9, 143.8, 141.5, 132.5, 132.1, 128.0, 127.3, 125.3, 125.2, 120.2, 116.8, 67.3, 54.7, 47.4, 36.9; HRMS (ESI, +ve) *m/z* calc. for $(C_{48}H_{41}N_2O_{10})^+$, 804.268; found 804.262.

General amino acid coupling protocol

The synthesis of linear peptides from 9-fluorenylmethoxycarbonyl (Fmoc) protected L-amino acids was conducted using solid peptide synthesis techniques, either manually or in batch mode on a Liberty Microwave Peptide Synthesizer (CEM). All side chains were protected by standard trifluoroacetic acid (TFA)-labile protecting groups. Fmoc protected amino acids (5 molar excess) were activated with 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1 eq relative to amino acid) and *N,N'*-diisopropylethylamine (DIPEA) (0.5 eq relative to amino acid) in DMF (5 mL/g resin) for 30 min. The solution mixture was then added to the resin, and the reaction was allowed to proceed for 45 min, with occasional stirring. The resin was then washed with DCM (2 × 10 mL), followed by DMF (2 × 10 mL).

Coupling of Fmoc$_2$dityrosine to peptide

Fmoc$_2$dityrosine **3** (0.25 eq relative to resin loading), *N*-hydroxybenzotriazole (HOBt) (2 eq relative to amount of Fmoc$_2$dityrosine **3**) was dissolved in minimal volume of DMF. *N,N'*-diisopropylcarbodiimide (DIC) (4 eq relative to amount of Fmoc$_2$dityrosine **3**) was then added to the amino acid in solution. The mixture was stirred and added to the resin containing the linear peptide chain, and the reaction allowed to proceed overnight. The coupling reaction was monitored for completion with the qualitative TNBSA test. Further additions of Fmoc$_2$dityrosine **3** were performed until the reaction had proceeded to completion (typically 2–3 × 0.25 eq couplings required).

General deprotection protocol

The resin-bound peptide was treated with piperidine (20% v/v) in DMF (10 mL/g resin) for 10 min. The resin was then washed with DCM (2 × 10 mL), followed by DMF (2 × 10 mL).

Confirmation of coupling/deprotection reactions

A qualitative confirmation test was carried out after each coupling or deprotection reaction using the 2,4,6-trinitrobenzenesulphonic acid (TNBSA) test for free amino groups. The test involved the sampling of approximately twenty resin beads placed in a 1:1 mixture of 5% DIPEA in DMF, and 1% TNBSA in DMF. Clear beads indicated successful coupling, whilst red coloration confirmed the successful deprotection with the presence of free amino group.

Cleavage of peptide

Method A: The peptides were cleaved with an solution containing 2.5% triisopropylsilane (TIPS), 2.5% water, 95% TFA at room temperature for 3 h. Peptide **7c** was cleaved with a solution containing 2.5% TIPS, 2.5% TMSBr, 2.5% thioanisole, 92.5% TFA at room temperature for 3 h in order to effect reduction of any methionine sulfoxides. The TFA was removed under a stream of nitrogen gas, and peptides precipitated with diethyl ether, then centrifuged. The product was then redissolved in 30% acetonitrile in water (v/v), and lyophilised.

Method B: The oxidised peptide **7d** was cleaved with a solution (5 ml/100 mg resin) containing 2.5% 3,6-Dioxa-1,8-octane-dithiol (DODT), 2.5% triisopropylsilane (TIPS), 2.5% water and 92.5% TFA at room temperature for 2.5–3 h. The TFA was concentrated to

approx. 1 ml under a stream of nitrogen. The crude peptide was precipitated by addition of cold diethyl ether, and isolated by centrifugation. The solid material was washed with cold diethyl ether, dissolved in 30:69:1 acetonitrile/water/formic acid (v/v/v) solution and then lyophilised. When nessciatated, reduction of the crude, lypholised peptide was carried out in TFA (5 ml) and treated with an excess, approx. 30 equiv. of tetrabutylammonium bromide (TBAB) and DODT (100 μL) for 5 min. the resultant solution was concentrated to approx. 1 ml under a stream of nitrogen. The crude peptide was precipitated by addition of cold diethyl ether, and isolated by centrifugation. The solid material was washed with cold diethyl ether, dissolved in 30:69:1 acetonitrile/water/formic acid (v/v/v) solution and then lyophilised.

Purification of peptide dimers

Aβ16 dityrosine-linked dimer 7a

Purification of the dimer was carried out on the Phenomenex Synergi 4μ HYDRO-RP 80Å (50 mm × 21.2 mm) with a linear gradient of 0–75% buffer B [buffer A: 0.1% TFA in water (v/v); buffer B: 0.1% TFA in acetonitrile (v/v)] over 70 min, with a flow rate of 5 mL/min. Procedure was repeated to yield the purified dimer **7a**; MS (ESI, +ve) calc. mass 3906.0, found (after deconvolution) 3907.9.

Aβ28 dityrosine-linked dimer 7b

Purification of the dimer was carried out on the Agilent Eclipse XDB-C18 column (250 × 9.4 mm) with a linear gradient of 0–75% buffer B [buffer A: 0.1% TFA in water (v/v); buffer B: 0.1% TFA in acetonitrile (v/v)] over 70 min, with a flow rate of 5 mL/min. The dimer containing fractions were then further purified using the same conditions**,** to yield the purified dimer **7b**; MS (ESI, +ve) calc. mass 6522.0, found (after deconvoluting) 6523.2.

Aβ40 dityrosine-linked dimer 7c

Method A: Purification of the dimer was carried out first on a preparative Alltech preparative C8 polymeric reverse-phase column (5 μM, 300 Å pore size, 22 mm x 250 mm) heated in a water bath to 60°C, with a linear gradient of 10–90% buffer B [buffer A: 0.1% TFA in water (v/v); buffer B: 0.1% TFA in 1:1 acetonitrile:isopropanol (v/v)] over 40 min, with a flow rate of 5 mL/min. The dimer containing fractions were then eluted through an Alltech analytical C4 polymeric reverse-phase column (5 μ, 300 Å pore size, 4.6 mm × 240 mm) heated in a

water bath to 60°C using the same solvent system with a flow rate of 1 mL/min, yielding the pure dimer **7c**; MS (ESI, +ve) calc. mass 8658.1, found (after deconvoluting) 8658.1.

Method B: Purification of the dimer was carried out on a Phenomenex Jupiter C4 semi-preparative reverse-phase column (5 μM, 300 Å pore size, 10 mm x 250 mm), using a linear gradient of 30–45% buffer B [buffer A: 10 mM ammounium acetate in water (pH of 9.2); buffer B: 10 mM ammounium acetate in 80:20 acetonitrile:water (v/v) (pH of 9.2)] over 15 min, with a flow rate of 5 mL/min. MS (ESI, +ve) calc. mass 8658.1, found (after deconvoluting) 8658.2.

Aβ40 Met[O]^{35} dityrosine-linked dimer 7d

Purification of the dimer was carried out on a Phenomenex Jupiter C4 semi-preparative reverse-phase column (5 μM, 300 Å pore size, 10 mm x 250 mm), using a linear gradient of 25–40% buffer B [buffer A: 10 mM ammounium acetate in water (pH of 9.2); buffer B: 10 mM ammounium acetate in 80:20 acetonitrile:water (v/v) (pH of 9.2)] over 15 min, with a flow rate of 5 mL/min. MS (ESI, +ve) calc. mass 8090.1, found (after deconvoluting) 8090.3.

Aβ42-Met[O]^{35} dityrosine-linked dimer 7f

Purification of the dimer was conducted with the use of two connecting GE Healthcare Superdex^{TM} 75 (13 μM, 10 mm × 300 mm) columns attached on an AKTApurifier FPLC system, eluting with 70% formic acid / 30% water with a flow rate of 0.7 mL/min for 35 minutes. The dimer containing fractions were eluted through a Waters HLB Oasis Cartridge (to remove formic acid). The cartridge was first conditioned with methanol, and then equilibrated with water. Subsequent to the sample loading, a washing step was conducted with 5% methanol, followed by 30% methanol. Elution of the sample was conducted with a combination of 2% ammonium hydroxide and 90% methanol, to furnish the pure dimer **7f**; MS (ESI, +ve) calc. mass 9058.2, found (after deconvolution) 9058.6.

Dynamic Light Scattering

Lypholised peptides were dissolved at 4°C to the required concentration under a range of buffer conditions including water alone, 20 mM HEPES pH 7.0, and 1 x PBS pH 7.4. Samples were subject to centrifugation (15,000 g, 10 minutes, 4°C) to remove particulates immediately prior to analysis. DLS measurements were made with a Malvern Instruments Zetasizer Nano ZS instrument. Size distribution profiles were measured repeatedly over the

first 30 minutes following dissolution. All samples demonstrated some degree of polydispersity and no discernible difference was observed between buffer conditions. Peptide concentrations: WT Aβ(1–16), 0.26 mM; Aβ(1–16) DAP-linked dimer **15**, 0.13 mM; Aβ(1–16) dityrosine-linked dimer **56**, 0.013 mM and 1.02 mM; WT Aβ(1–28), 0.15 mM; Aβ(1–28) DAP-linked dimer **21**, 0.079 mM and 1.90 mM; Aβ(1–28) dityrosine-linked dimer **61**, 0.008 mM and 0.6 mM; WT Aβ(1–40), 0.11 mM; Aβ(1–40) DAP-linked dimer **29**, 0.059 mM; Aβ(1–40) dityrosine-linked dimer **68**, 0.006 mM and 0.12 mM.

ThT assay for fibril formation

Dry peptide was weighed and dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 mg/mL, then aliquotted and dried under vacuum and stored at –80 °C. The peptide concentration was determined from the absorbance at 214 nm using the following extinction coefficients; 91462 $M^{-1}cm^{-1}$ for Aβ40, 239706 $M^{-1}cm^{-1}$ for Aβ40 DAP-dimer **2c** and 197244 $M^{-1}cm^{-1}$ for Aβ40 dityrosine-dimer **7c**, as determined by amino acid analysis. Aliquots of HFIP-treated, dried peptide were dissolved in 20mM NaOH then diluted in deionized water and phosphate buffer (100mM potassium phosphate, pH 7.4) at a v/v/v ratio of 2:7:1. All solutions were sonicated at 0 °C for 10 min and filtered (20 μm) to ensure pre-formed aggregates were removed. Final concentrations: Aβ40 monomer = 14 μM, Aβ40 dimer **2c**, **7c** = 7 μM, Thioflavin-T (ThT) = 28 μM, in 1X PBS buffer (10mM sodium phosphate, 137mM NaCl, 2.7mM KCl at pH 7.4) to a final volume of 600 μL. ThT-induced fluorescence was measured using a Varian Cary Eclipse Fluorescence spectrophotometer fitted with a peltier-driven temperature controller and a multi-cell holder. Each sample was incubated at 37 °C. Excitation was at 444 nm and fluorescence emission measured at 480 nm. Readings were taken every 60 sec for the first 15 minutes, then every 15 minutes for the next 885 minutes. Slit widths were 5 nm for both excitation and emission.

Electron Microscopy

Transmission electron microscope (TEM) sample preparation was performed by absorbing a 3.5 μL aliquot of peptide prepared as for the ThT assay onto a carbon-coated Formwar film mounted on a 300 mesh copper grid (ProSciTech, Qld, AUS). Prior to adsorption, the grids were rendered hydrophilic by glow discharge *in vacuo* for 10 s. After 30 s adsorption, samples were blotted and negatively stained with 1.5% aqueous uranyl acetate. Investigations were undertaken using a FEI Company Technai TF30 transmission electron microscope

(FEI-Company, Eindhoven, The Netherlands) operated at 200 kV. Images were acquired digitally with a Gatan US1000 2kX2k CCD Camera (Pleasanton, Ca, USA).

Neuronal cell line toxicity assays

Dry peptide was weighed and dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to monomerize the peptide, then was then dispensed into small amounts and dried using a speed-vac and then stored at –80°C until use. Aβ peptides were initially dissolved in 20 mM NaOH, then diluted in water followed by the addition of 10x PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 138 mM NaCl, 8 mM Na_2HPO_4, at pH 7.4) in a ratio of 2:7:1 as previously described.[2] Peptide concentrations were determined from the absorbance value at 214 nm, using the calculated molar extinction coefficient values of 75887 $M^{-1}cm^{-1}$ for Aβ42, 91462 $M^{-1}cm^{-1}$ for Aβ40 and Aβ40Met$^{[O]}$, and 197,422 for Aβ40Met$^{[O]}$ dityrosine dimer **7d**.

SH-SY5Y human neuroblastoma cells (American Type Culture Collection, Rockville, MD, USA) were grown in DMEM supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS), 1 U/ml of penicillin, 1 μg/ml of streptomycin, and 2 mM glutamate (Gibco BRL; Invitrogen, Victoria, Australia) and maintained at 37°C and 5% CO_2 as previously described.[3] To obtain differentiated cells, 20,000 cells were plated per well of a 48-well plate and allowed to adhere for 24 h. Differentiation was started in DMEM supplemented with 1.5% FBS and 10 μM retinoic acid (RA). Fresh medium, containing RA and 1.5% FCS, was applied to the cells every 1–3 days. Experiments were typically performed on cells differentiated for at least 14 days and displaying a differentiated neuronal phenotype, including extensive neurites and branching, as evidenced by light microscopy. To determine the cell toxicity of the Aβ peptides, differentiated cells were treated with 0 and 5 μM concentration of Aβ peptides for 4 days in DMEM/1.5% FCS. The cell Counting Kit-8 (Auspep, Australia) was used to determine the cell viability at the end of the treatment. The measured cell viability values were normalised to vehicle-treated control groups (set to 100%) and each treatment was done in triplicate. Results are shown as mean ± S.E.M. The data were analyzed by two-tailed Student's *t*-test. Values of $p<0.05$ were considered significant.

[1] Skaff, O.; Jolliffe, K. A.; Hutton, C. A. *J. Org. Chem.* **2005**, *70*, 7353–7363; Hutton, C. A.; Skaff, O. *Tetrahedron Lett.* **2003**, *44*, 4895–4898.

[2] Ciccotosto, G. D.; Tew, D. J.; Drew, S. C.; Smith, D. G.; Johanssen, T.; Lal, V.; Lau, T.-L.; Perez, K.; Curtain, C. C.; Wade, J. D.; Separovic, F.; Masters, C. L.; Smith, J. P.; Barnham, K. J.; Cappai, R. *Neurobiol. Aging* **2011**, *32*, 235–248.

[3] Vella, L. J.; Cappai, R. *FASEB J.* **2012**, *26*, 2930–2940.

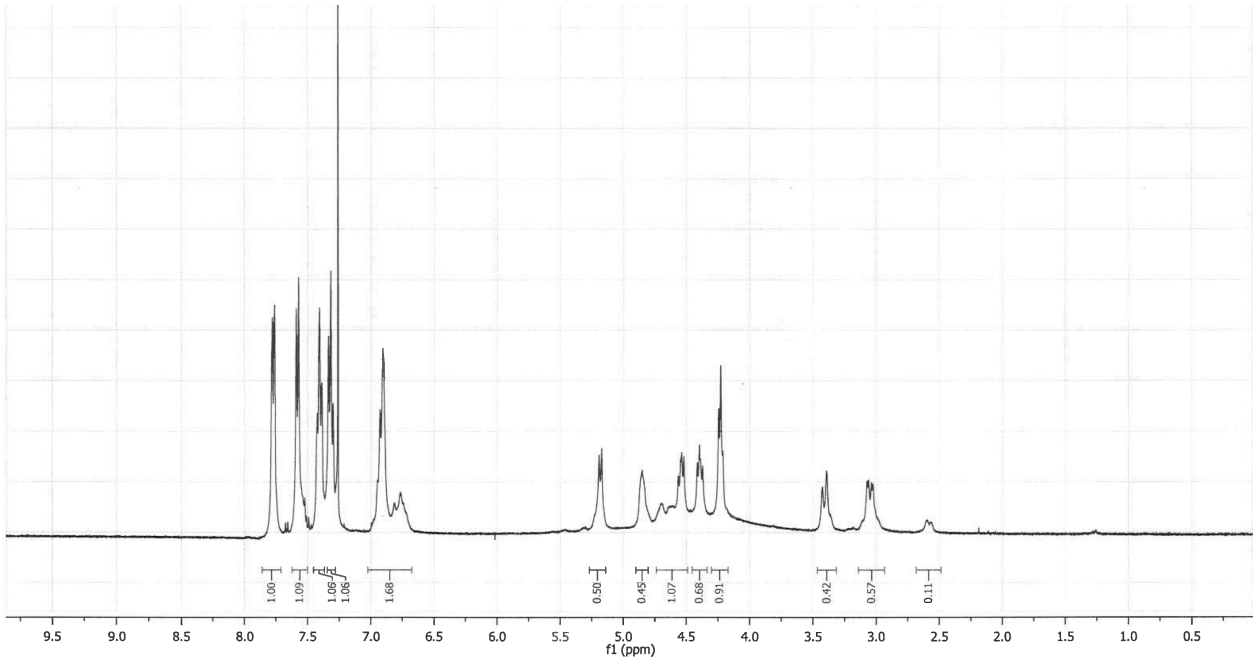


Figure S1: ^{1}H NMR of (Fmoc)$_2$-dityrosine **3** (400 MHz, $CDCl_3$).

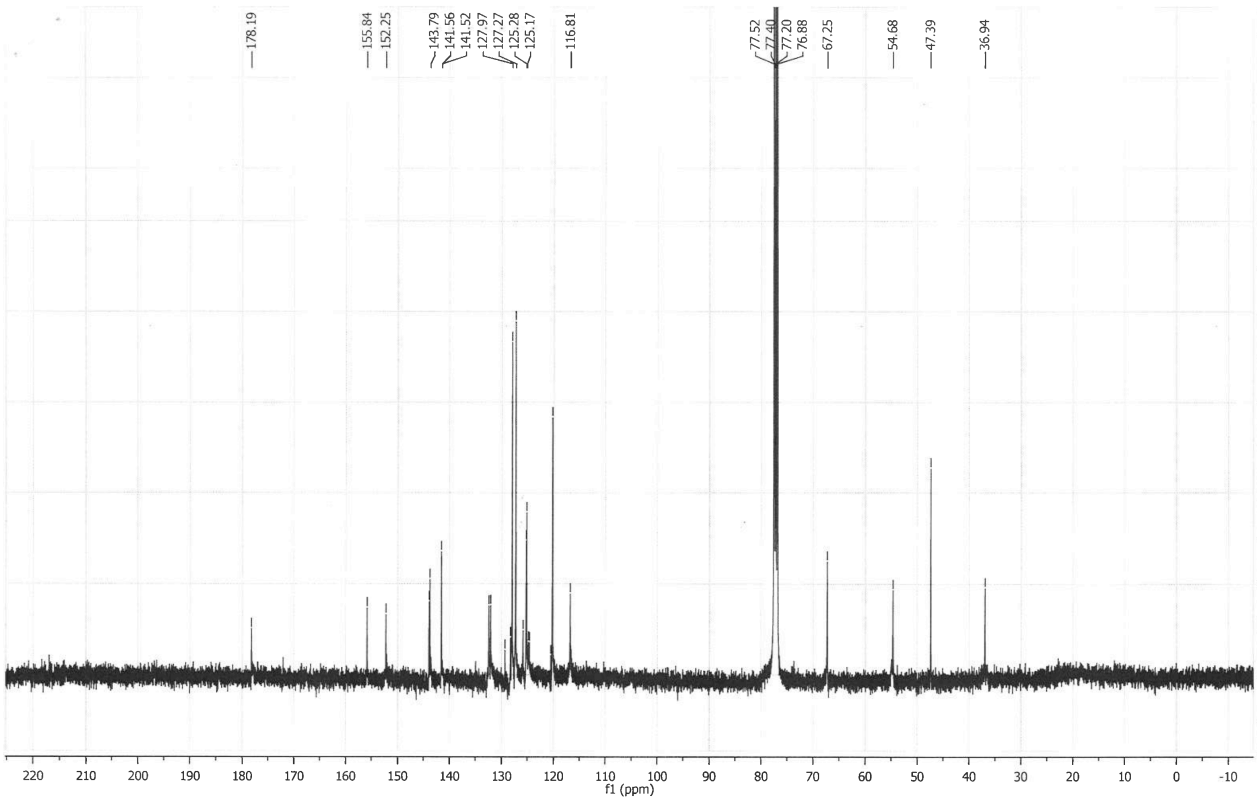


Figure S2: ^{13}C NMR of (Fmoc)$_2$-dityrosine **3** (100 MHz, $CDCl_3$).

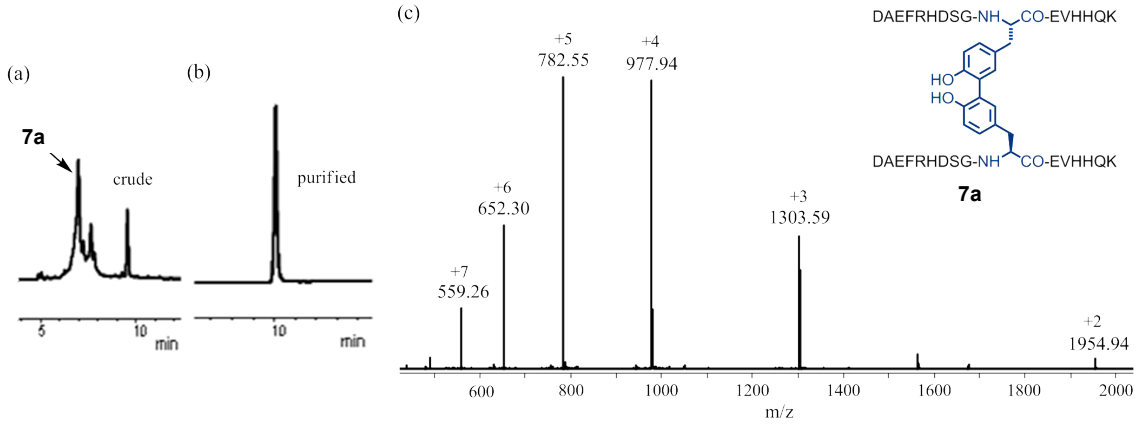


Figure S3: HPLC traces of (a) crude Aβ16 dityrosine dimer **7a**; (b) purified Aβ16 dimer **7a** following preparative HPLC Note: retention times altered due to different C18 columns used in the analytical HPLC; (c) QTOF MS of purified Aβ16 dityrosine-linked dimer **7a** (calcd. *m/z* 3906.0, found (after deconvolution) 3907.9).

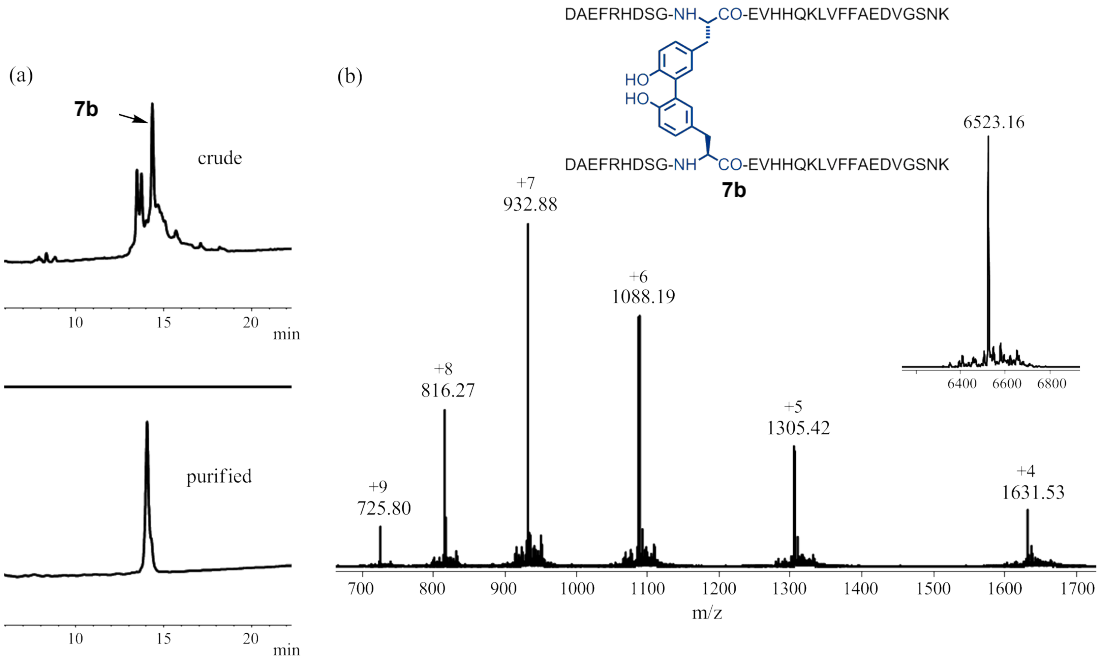
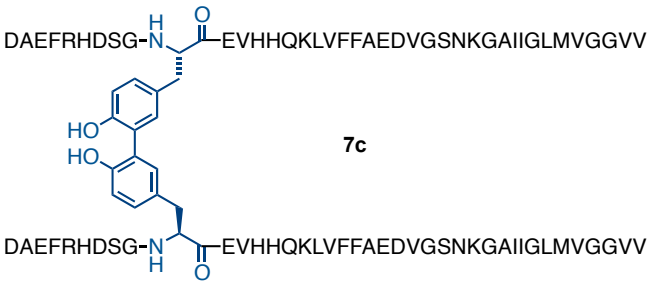


Figure S4: (a) HPLC traces of crude Aβ28 dityrosine dimer **7b** (top), and purified Aβ28 dimer **7b** following preparative RP and size exclusion HPLC (bottom); (b) QTOF MS of purified Aβ28 dimer **7b** (inset; deconvoluted spectrum) (calcd. *m/z* 6522.0, found (after deconvolution) 6523.2).



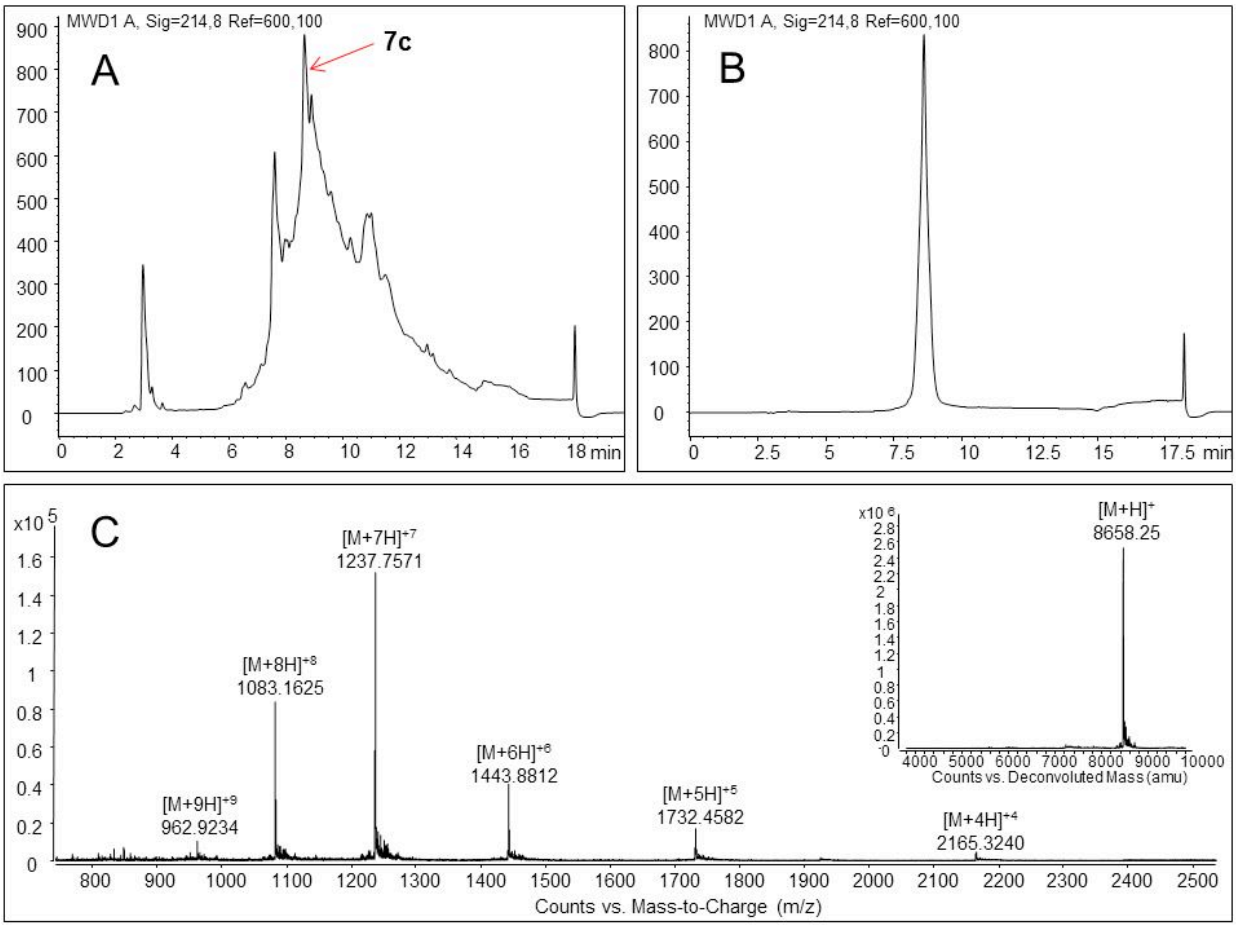
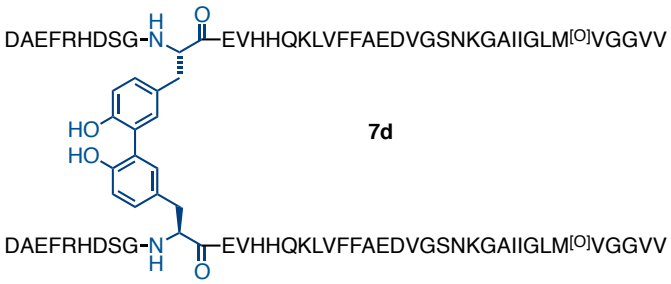


Figure S5: Characterisation of Aβ40 dityrosine-linked dimer **7c** (A) HPLC trace of crude Aβ40 dityrosine-linked dimer **7c**; (B) HPLC trace of purified Aβ40 dityrosine-linked dimer **7c** (C) ESI MS of purified Aβ40 dityrosine-linked dimer **7c** (inset; deconvoluted spectrum). Calcd. for $C_{388}H_{588}N_{106}O_{116}S_2$: 8657.61 (average isotopes); observed: *m/z* 2165.32 ($[M+4H]^{+4}$), 1732.46 ($[M+5H]^{+5}$), 1443.88 ($[M+6H]^{+6}$), 1237.76 ($[M+7H]^{+7}$), 1083.16 ($[M+8H]^{+8}$) and 962.92 ($[M+9H]^{+9}$); observed: (after deconvolution) 8658.25($[M+H]^{+}$). Data in this figure correlates to the purification of **7c** using purification Method B (refer S5).



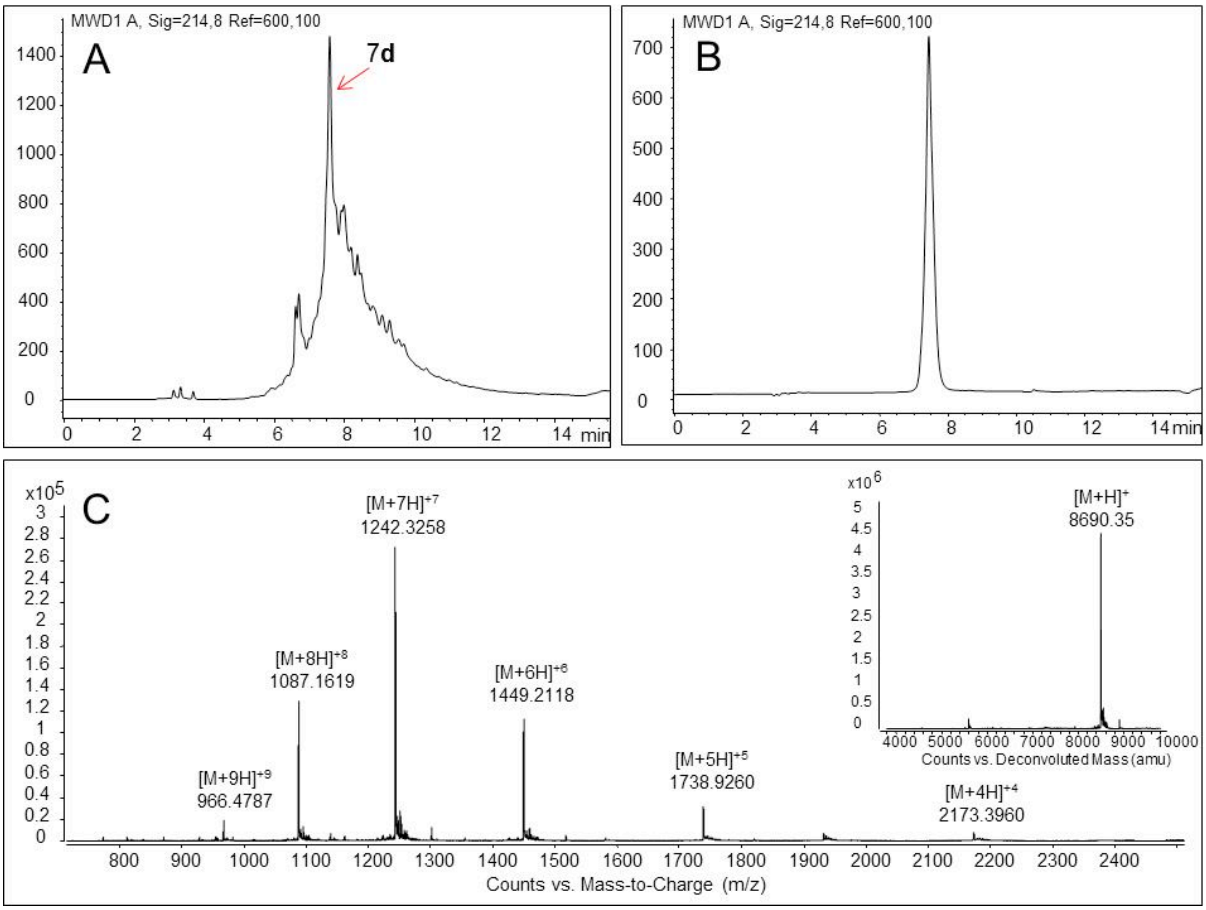


Figure S6: Characterisation of Aβ40 Met[35][O] dityrosine-linked dimer **7d** (A) HPLC trace of crude Aβ40 Met[35][O] dityrosine-linked dimer **7d**; (B) HPLC trace of purified Aβ40 Met[35][O] dityrosine-linked dimer **7d** (C) ESI MS of purified Aβ40 Met[35][O] dityrosine-linked dimer **7d** (inset; deconvoluted spectrum). Calcd. for $C_{388}H_{588}N_{106}O_{118}S_2$: 8689.61 (average isotopes); observed: *m/z* 2173.40 ($[M+4H]^{+4}$), 1738.93 ($[M+5H]^{+5}$), 1449.21 ($[M+6H]^{+6}$), 1242.33 ($[M+7H]^{+7}$), 1087.16 ($[M+8H]^{+8}$) and 966.48 ($[M+9H]^{+9}$); observed: (after deconvolution) 8690.35 ($[M+H]^{+}$).

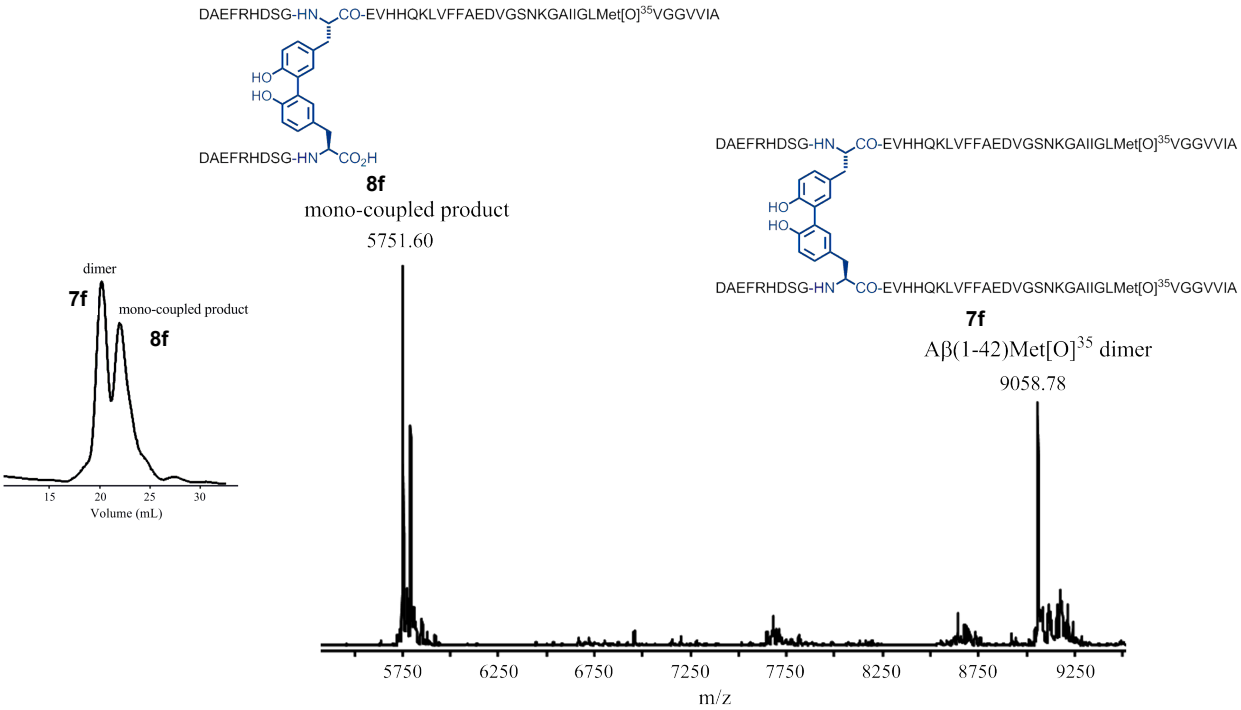


Figure S7: (a) HPLC (eluted with 70% formic acid through two connected Superdex 75 columns), and (b) QTOF MS of crude Aβ42Met[O]^{35} dimer **7f** (containing mono-coupled product **8f**).

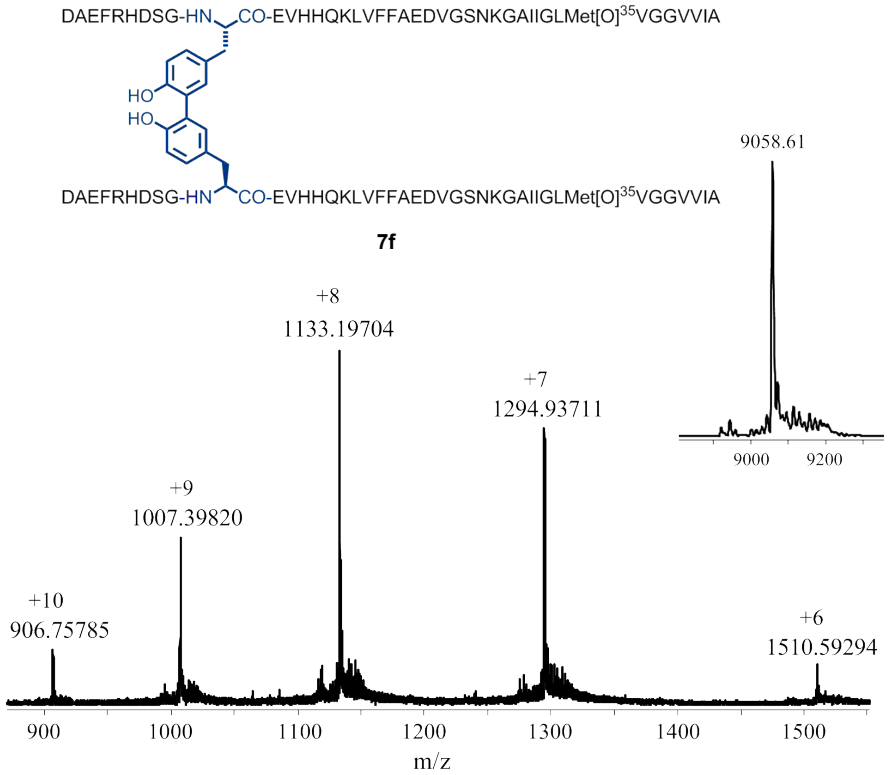


Figure S8: QTOF MS of purified Aβ42Met[O]^{35} dityrosine-linked dimer **7f** (inset; deconvoluted spectrum) (calcd. *m/z* 9058.2, found (after deconvolution 9058.6).

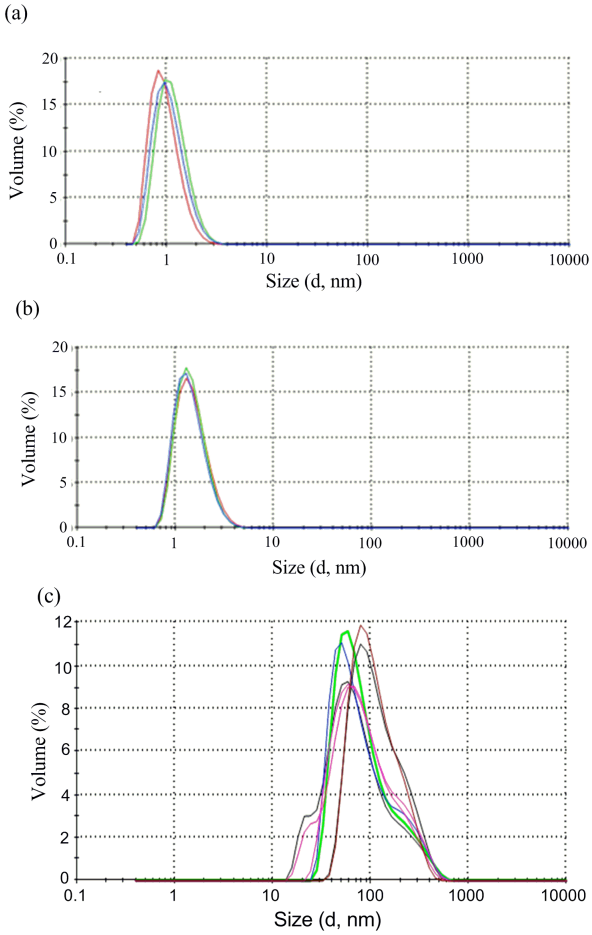


Figure S9: DLS data for Aβ16 peptides (repeat measurements in 20 mM HEPES, 0.5 mg/ml, pH7.0):
(a) Aβ16 monomer (0.26 mM),
(b) Aβ16 DAP-linked dimer **2a** (0.13 mM) and
(c) Aβ16 dityrosine-linked dimer **7a** (0.13 mM).

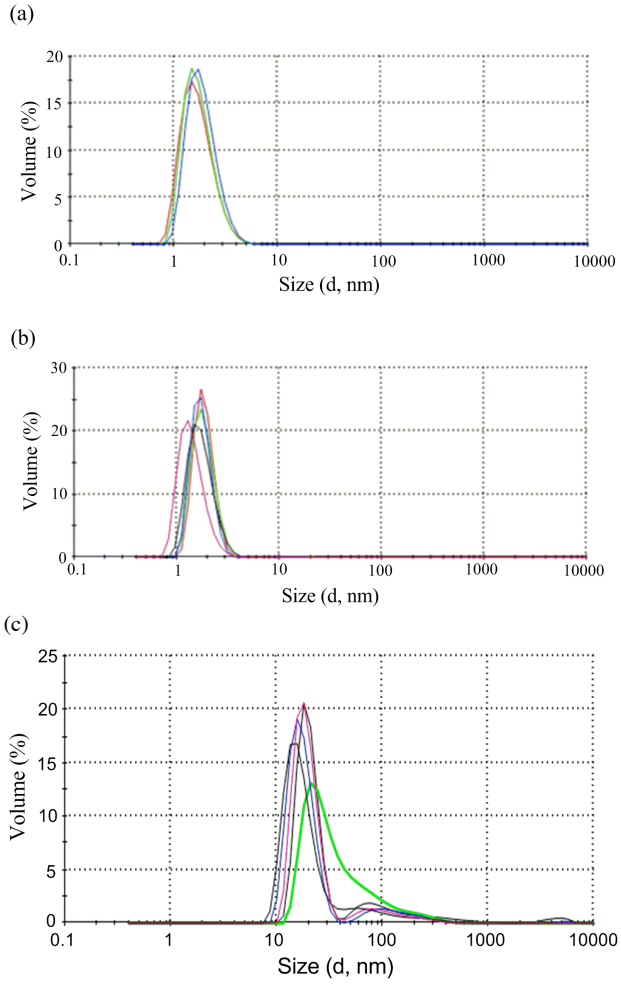


Figure S10: DLS data for Aβ28 peptides (repeat measurements in 20 mM HEPES, 0.5 mg/mL, pH 7.0):
(a) Aβ28 monomer (0.15 mM),
(b) Aβ28 DAP-linked dimer **2b** (0.08 mM)
(c) Aβ28 dityrosine-linked dimer **7b** (0.08 mM).