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Total chemical synthesis and biophysical properties of a designed soluble 24 kDa amyloid analogue†

Régis Boehringer,^a Bruno Kieffer^b and Vladimir Torbeev^{*a}

Discovering molecular probes that specifically recognize distinct amyloid structures is highly important for physiological studies of protein-misfolding diseases as well as for the development of diagnostic reagents and inhibitors of amyloid self-assembly. Here, we demonstrate an approach that allows for identification of *N*-methylated peptides that are specific binders for a particular amyloid fiber subtype (or polymorph). Protein design and chemical synthesis were used to produce covalently tethered amyloid analogues with molecular masses approaching 24 kDa and containing nine copies of an amyloidogenic peptide. Such engineered constructs served as a molecular testing platform to evaluate the aggregation properties and solubility as a function of *N*-methylation pattern. An advantage of the method is the possibility of biophysical characterization of amyloid constructs in solution.

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Protein misfolding diseases are characterized by *in vivo* deposition of insoluble amyloid fibers resulting from aberrant conformational rearrangement and self-assembly of soluble monomeric peptide or protein precursors.¹ Inhibiting amyloid formation is considered to be a possible therapeutic strategy with some promising results reported for anti-amyloid antibodies.² Increasingly more structural information is available for amyloid fibers with several high-resolution structures recently reported based on solid-state NMR³ and cryo-electron microscopy studies.⁴ Remarkably, the same polypeptide sequence can result in amyloids with different structures, *i.e.* distinct polymorphs (also called “amyloid strains”).⁵ Moreover, for A β (1–40) and A β (1–42) peptides the correlation was established between different amyloid structures and the clinical subtypes characterized by altered rates of progression of Alzheimer’s disease.⁶ These results are highly important and will facilitate the development of molecular probes that can specifically recognize the distinct amyloid polymorphs *in vivo* for physiological studies and which can also be elaborated into diagnostic reagents and/or inhibitors.

However, such a task presents significant challenges. The major complications are (i) structural heterogeneity as well as compositional complexity in amyloid self-assembly mechanisms due to the presence of transient oligomers or intermediate protofibrils;⁷ and (ii) the insoluble nature of amyloids,

which prevents the use of biophysical tools such as solution NMR or isothermal titration calorimetry commonly applied for the analysis of compound binding to biomolecules.

To overcome these complications we considered elaborating a different approach. Starting from already available amyloid structures, protein design and total chemical synthesis can be applied in tandem to produce structural analogues of amyloids that are compositionally well-defined (as opposed to a mixture of oligomers) and behave as soluble species. Amyloids are multimeric supramolecular assemblies formed by amyloidogenic peptide or protein building blocks; hence, our goal here is to apply covalent tethering of several such peptide subunits into a construct in which the structure of a given amyloid polymorph is stabilized. In addition, modifications that prevent this construct from uncontrolled supramolecular polymerization need to be introduced, thus making it soluble. As a result, such amyloid analogues can be studied using various solution-phase biophysical methods.

In this study, the design of a covalently tethered construct is inspired by the structural model of the A β (1–40) amyloid with a 3-fold symmetric arrangement of peptide subunits.⁸ In this structure, three copies of A β (1–40) adopt a β -arch (β -strand-turn- β -strand) conformation in which β -strands interact *via* amino acid side chains and not through backbone hydrogen bonds. The amyloidogenic [20–41]-segment of human β 2-microglobulin ([20–41] β 2m) adopts a β -arch conformation similar to A β (1–40)⁹ but it represents a more tractable system to explore methodologies of chemical synthesis towards various multimeric constructs. Previously, we demonstrated that [20–41] β 2m covalently tethered into a “covalent trimer” aggregates into morphologically distinct amyloids.¹⁰ In this study, we engineered a covalently tethered oligomer composed of three units of the [20–41] β 2m covalent trimer. As a result, the final

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construct comprises nine copies of the progenitor [20–41] β 2m peptide in total (Fig. 1).

The design of this complex peptide architecture was realized with the help of molecular modelling taking into account the structural properties of the [20–41] β 2m covalent trimer.¹⁰ We envisaged three copies of covalent trimer [20–41] β 2m stacked in a parallel, in-register orientation, so that the internal covalent trimer being “sandwiched” by two external trimers has surroundings identical to those found in a complete amyloid fiber (Fig. 1). The desired arrangement of three covalent trimers is enabled by conformationally flexible tetra-glycine chains used as covalent tethers connecting the C- and N-termini of the corresponding peptide building blocks

(“C–N linkers”). To prevent uncontrolled spontaneous fibrillogenesis, a subset of solvent exposed amides in the external trimers is modified by *N*-methylation with the purpose of disrupting hydrogen bonding within β -sheets necessary for amyloid propagation (Fig. 1a).

Retrosynthetic analysis towards total synthesis of the designed peptide construct is outlined in Fig. 1b. The synthetic target represents a polypeptide with a non-linear topology. The sequential tethering of the covalent trimers is carried out by native chemical ligation;¹¹ therefore, one of the peptide subunits in each covalent trimer is modified with the required functionalities such as a Cys-residue at the N-terminus and an α hydrazide moiety at the C-terminus. α Hydrazides can efficiently be converted into α thioesters.¹² Cys is introduced in place of one of the Gly-residues in the tetra-glycine linker (hence the final linker sequence is Cys-Gly-Gly-Gly). As a result, three non-identical covalent trimers need to be prepared for the assembly of the desired covalently tethered construct starting from six modified [20–41] β 2m peptides (Fig. 1b and ESI Tables S1–S3[†]).

In our previous work on covalent trimer [20–41] β 2m amyloids, the trivalent linker *N,N,N'*-(benzene-1,3,5-triyl)-tris(acetamide) was used to join three copies of the [20–41] β 2m peptide.¹⁰ The conjugation was based on a nucleophilic displacement of a symmetrically substituted bromoacetamide derivative by a Cys6 residue of the [20–41] β 2m. In this work, according to our design objectives, the central linker needs to support the attachment of different peptides that would enable introduction of the additional C–N linkers. Thus, the central core linker was designed to support the synthesis of covalent trimers that are composed of two identical peptides and the one that is distinct. As required, the structure of the new central linker 3,5-bis((allyloxycarbonyl)amino)benzoic acid contains two amines protected by Alloc (allyloxycarbonyl) groups and one reactive carboxylic acid (ESI Fig. S1[†]). The unprotected carboxylic acid is coupled to the side chain amino-group of ornithine introduced at position 6 of the [20–41] β 2m peptide (Fig. 2). Ornithine was chosen because the length of the side chain is optimal and leads to the same number of atoms between the peptide backbone and the aromatic ring of the central linker. The corresponding peptide was synthesized with the side-chain of the ornithine protected by Alloc, which is orthogonal to the side chain protecting groups of the other amino acids used in Fmoc/*t*Bu-based solid phase peptide synthesis (SPPS). It was deprotected at the end of the chain assembly on the solid support and the central core linker was directly conjugated (Fig. 2). In the next step, the primary aromatic amino groups of the central linker were Alloc-deprotected and bromoacetylated. The final step is the cleavage of the modified peptide from the resin and purification by HPLC. The formation of the non-symmetric covalent trimers was then achieved by reaction with the required [20–41] β 2m-derived peptides having the Cys-residue at position 6 (Fig. 2 and ESI Fig. S2[†]).

Native chemical ligation was used to join the three non-symmetric covalent trimers in the required order (Fig. 3a and ESI Fig. S3[†]). *N*-methylated trimer A (NMT-A) was obtained as an α thioester and ligated to the central trimer (CT). Subsequently,

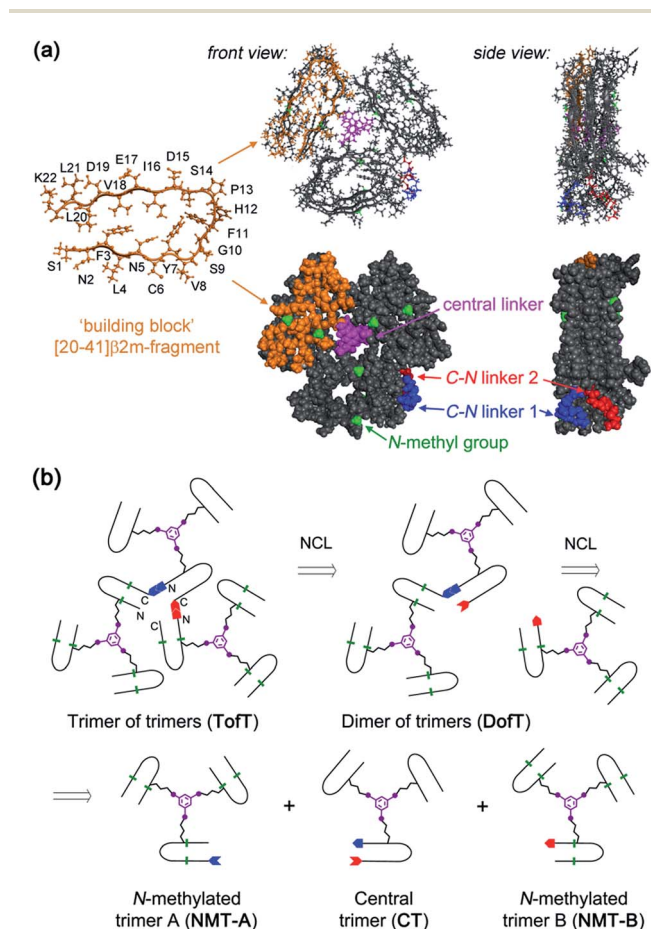


Fig. 1 Designed covalently tethered amyloid analogue. (a) Molecular model in ball-and-stick (top) and space-filling (bottom) representations. The construct is composed of nine subunits derived from the amyloidogenic peptide (in orange) adopting a β -arch conformation (based on PDB ID: 2E8D; amino acid residues are abbreviated as one-letter symbols). Each such subunit is joined via a central linker (in magenta) to form covalent trimers. Three covalent trimers are assembled with the help of two C–N linkers (in blue and red) connecting the C- and N-termini of the corresponding building blocks to form parallel β -sheets. The covalent trimers that are exposed to solvent are modified by *N*-methylation (in green) to prevent their aggregation into amyloids and to ensure a monomeric state of the species. (b) Retrosynthetic analysis for total chemical synthesis of the designed construct by sequential native chemical ligation (NCL) of three different covalent trimers.



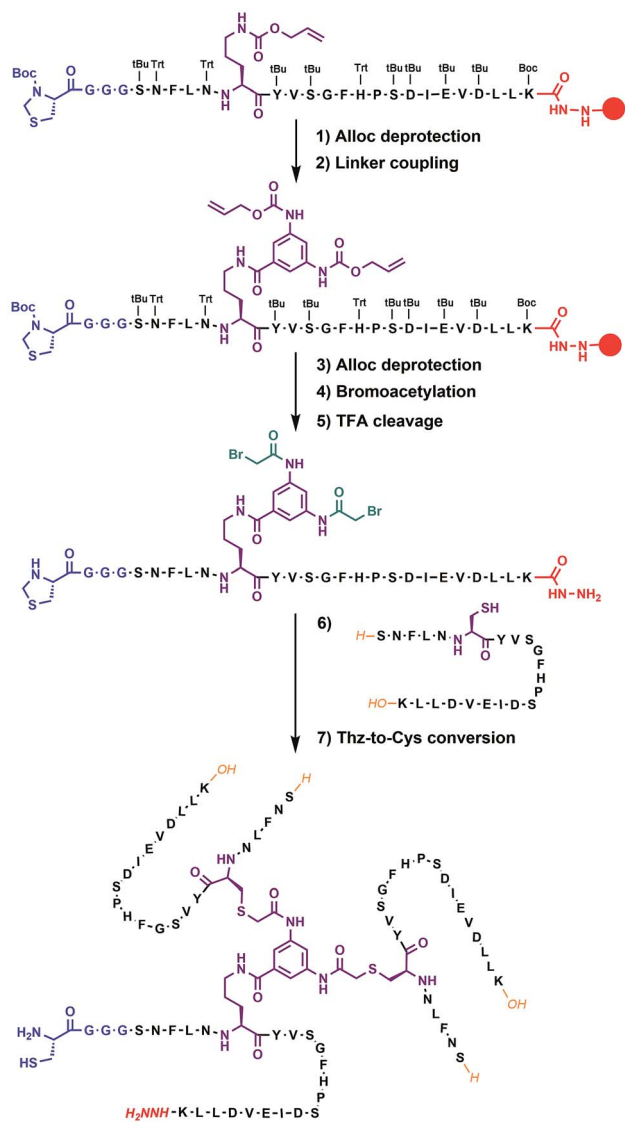


Fig. 2 Chemical synthesis of covalent trimer CT-1 (see also ESI Table S1†) illustrating the chemical steps in the synthesis of non-symmetric covalent trimers. Analytical monitoring of the reaction is provided in ESI Fig. S2.† Amino acid residues are shown as one-letter symbols. 'H' and 'OH' in orange stand for unmodified amino and carboxy functionalities of the N- and C-termini of the peptide chain, respectively. Red circles represent the polymer resin support. Abbreviations for protecting groups: tBu = *tert*-butyl, Trt = trityl, Boc = *tert*-butyloxycarbonyl, and Thz = thiazolidine.

the Cys-residue at the ligation junction was alkylated with 2-bromoacetamide to avoid undesirable Cys-oxidation and the α hydrazide group was converted into an α thioester followed by HPLC purification. Then, the second ligation was performed with *N*-methylated trimer B (**NMT-B**). After subsequent Cys capping with 2-bromoacetamide and HPLC purification, the desired product was obtained.

Several covalently tethered amyloid analogues were prepared according to this strategy (for more details, yields and analytical characterization, see Fig. 3 and ESI Fig. S4 and S5†). In the first construct **Toft-1** (*trimer of trimers 1*) only one *N*-methyl group

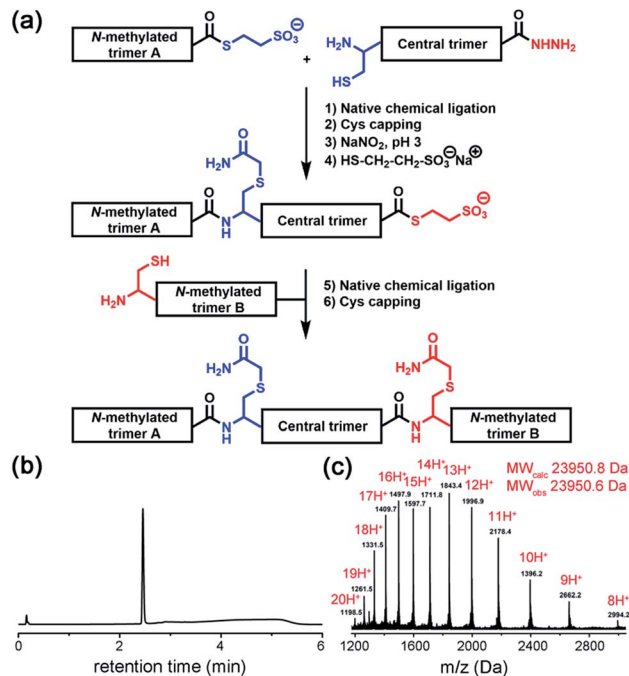


Fig. 3 (a) Native chemical ligation was used to join three covalent trimers. (b and c) Analytical HPLC and ESI-MS-Orbitrap analyses of the synthesized Toft-2.

per β -arch was introduced into external covalent trimers resulting in six *N*-methyl groups in total (**NMT-A-1**: *N*-Me-Ile16; **NMT-B-1**: *N*-Me-Glu17). In the second variant **Toft-2** (molecular model shown in Fig. 1a), two amino acids per β -arch (twelve *N*-methyl groups in total) were substituted by the corresponding *N*-methylated congeners (**NMT-A-2**: *N*-Me-Asn5 and *N*-Me-Val18; **NMT-B-2**: *N*-Me-Leu4 and *N*-Me-Asp19). Finally, the third construct **Toft-3** was identical to the second, except one of the β -arch peptides in the central trimer was 15 N-isotope labelled at five residues (Phe3, Val8, Gly10, Ile16 and Leu20).

To characterize the solubility and aggregation state of the synthesized constructs we used size-exclusion chromatography (Fig. 4a). We found that one *N*-methylated amino acid per β -arch in **Toft-1** is not sufficient to prevent the construct from aggregation. Oligomers of **Toft-1** formed instantaneously and upon longer incubation evolved into larger species. TEM analysis confirmed the presence of insoluble aggregates (ESI Fig. S6†). Strikingly, the introduction of two *N*-methylated amino acids per β -arch (one per β -strand) in **Toft-2** was sufficient to stabilize it from aggregation: monomeric species were observed upon prolonged (1 week) incubation (Fig. 4a).

The **Toft-1** and **Toft-2** constructs were tested for their ability to bind fluorescent dyes commonly used for the detection of amyloid fibers or solvent exposed hydrophobic pockets in proteins. We tested dyes such as thioflavin T (ThT), Nile red and bis-ANS. The dyes were added to solutions of covalently tethered constructs (*c* 30 μ M) at a concentration of 90 μ M giving a protein : fluorescent dye concentration ratio of 1 : 3. **Toft-1** gave strong ThT and Nile red responses compared to a weak signal observed for **Toft-2** (Fig. 4b). ThT and Nile red are typical dyes



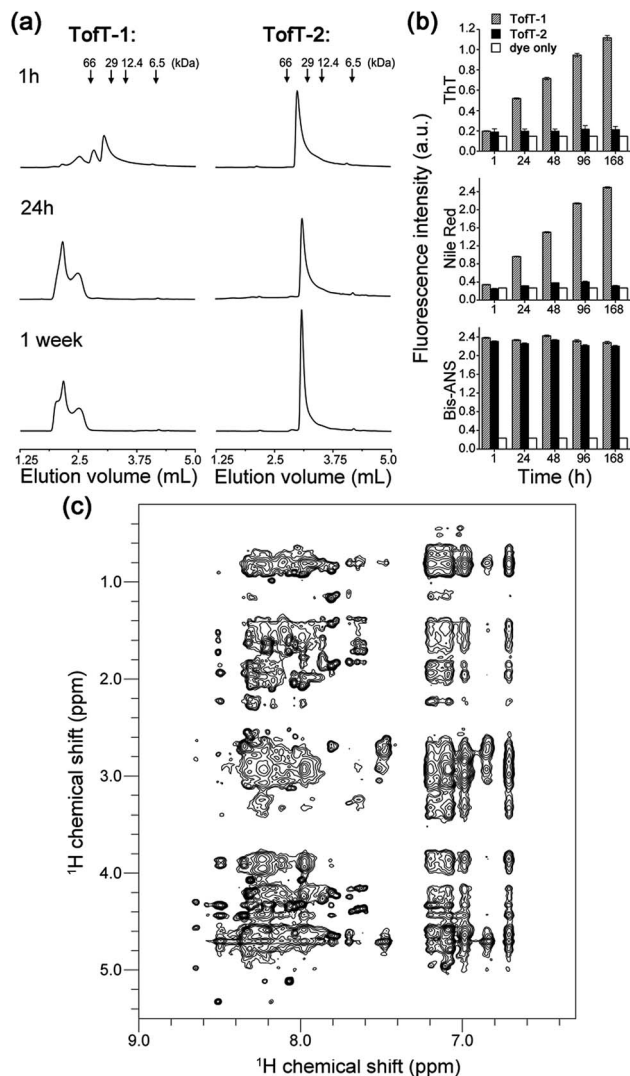


Fig. 4 Properties of the synthesized ToFT-1, ToFT-2 and ToFT-3 constructs. (a) Size-exclusion chromatography of aggregating ToFT-1 and monomeric ToFT-2. (b) Binding of ThT, bis-ANS and Nile red fluorescent dyes to ToFT-1, whereas for ToFT-2 only bis-ANS binding was detected. (c) ¹H-NOESY spectrum recorded for ToFT-3. See the ESI† for details.

used to detect the presence of larger aggregates such as amyloids; therefore, these results are in agreement with the enhanced aggregation propensity of ToFT-1 when compared to non-aggregating ToFT-2. However, with bis-ANS both ToFT-1 and ToFT-2 showed a strong fluorescence response (Fig. 4b). Bis-ANS can serve as a probe for amyloids but it is also known to bind to hydrophobic patches in soluble proteins.¹³ Therefore, efficient binding of bis-ANS to ToFT-2 suggests the presence of solvent exposed hydrophobic pockets.

ToFT-1 and ToFT-2 were also analyzed by ¹H NMR (ESI Fig. S7†). Very broad and weak signals were present in the spectra of ToFT-1, which suggests that the major part of the protein aggregated – herein the larger species are not detectable by solution NMR. In contrast, soluble non-aggregating ToFT-2 shows well detectable signals in the amide and aromatic

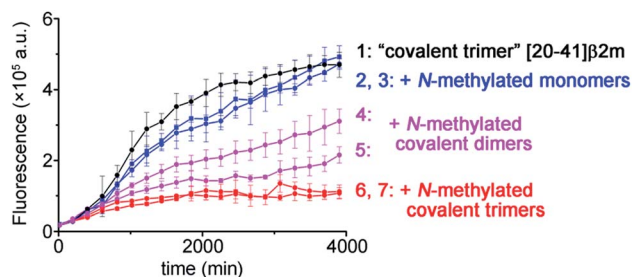


Fig. 5 Kinetics of the aggregation of covalent trimer [20–41]β2m monitored by ThT fluorescence specific to the cross-β amyloid structure and the effect of addition of the distinct N-methylated peptide inhibitors. Data points are averages of three experiments, and error bars depict the standard deviations. See ESI Fig. S16† for additional experimental information.

regions. Spectra were also recorded for ToFT-3 (¹⁵N-labelled version of ToFT-2) at 25, 30 and 35 °C (ESI Fig. S8†). The dispersion and line-width improve at 35 °C; therefore, subsequent 2D spectra were recorded at this temperature.

The ¹H-NOESY spectrum of ToFT-3 (Fig. 4c) shows some interesting features: (i) the presence of both sharp and broad resonances; and (ii) a large number of NOE cross-peaks (at ~7 ppm) presumably corresponding to the aromatic central linker inside a hydrophobic core. A ¹H-¹³C HSQC spectrum was also recorded, showing well-resolved cross-peaks with a large spread of peak intensities (ESI Fig. S9†). The ¹H-¹⁵N HSQC spectrum of ¹⁵N-labelled ToFT-3 displays five resonances as expected (ESI Fig. S10†). Two of those peaks with ¹⁵N chemical shifts at 121.5 ppm and 118.5 ppm are of similar and high intensity, while the other three signals at 118.7, 119 and 109.9 ppm are weaker. The latter cross-peak can be assigned to Gly10 based on the distinct chemical shifts for glycines, whereas for the other peaks, unambiguous assignment was not achieved. ¹H-¹⁵N relaxation experiments yielded ¹⁵N *T*₁ and *T*₂ relaxation times suggesting a uniform dynamic behaviour of the construct (ESI Fig. S11 and S12†). Furthermore, the *T*₁/*T*₂ ratio provides an estimate of the global correlation time of ToFT-3 between 9.8 and 13.2 ns in agreement with the expected correlation time of a protein composed of ~200 amino acids at 35 °C (11.5 ns is an estimated value from the model of Daragan¹⁴).

In addition, DOSY (diffusion ordered spectroscopy) experiments support the globular shape of the ToFT-3 species, where the measured diffusion coefficient displays the expected linear dependence when correlated to the variation of viscosity as a function of temperature and corresponds to a molecular weight of ~20 kDa, consistent with the size of ToFT-3 (ESI Fig. S13†). Overall, NMR data confirm a monomeric form for ToFT-2 and ToFT-3; however, their structural properties are similar to those of a molten globule rather than a well-structured protein. Binding of bis-ANS is in agreement with this conclusion and was directly confirmed *via* chemical shift changes in the NMR spectrum of ToFT-3 in the presence of bis-ANS (ESI Fig. S14†).

The finding that two methyl groups per β-arch in ToFT-2 are sufficient to prevent it from aggregation has important



implications. **Toft-2** (or **Toft-3**) essentially represents a fragment of an amyloid rendered soluble. The non-methylated central trimer **CT** when taken separately corresponds to the basic repeating unit of the amyloid polymorph with a trimeric core. We proved that central trimer **CT** is indeed amyloidogenic; moreover, the morphology of the resulting fibers is similar to that previously determined for covalent trimer [20–41] β 2m (ESI Fig. S15[†]). The two *N*-methylated trimers covalently tethered to **CT** prevent it from aggregation; thus, they can be regarded as amyloid growth inhibitors that recognize and bind at two non-equivalent ends of this minimalistic amyloid building block.

To illustrate the molecular recognition and potency of inhibition of amyloid growth by different *N*-methylated constructs we performed amyloid inhibition assays. By using two monomer peptides, two covalent dimers and two covalent trimers synthesized possessing the same *N*-methylation patterns to prevent aggregation on both sides of the putative amyloid fiber axis as in the **Toft-2** construct, we inspected which of them is the most efficient in inhibiting the growth of non-methylated covalent trimer [20–41] β 2m amyloids. As illustrated in Fig. 5, the *N*-methylated trimers are more efficient in suppressing the formation of the respective cross- β structures than the covalent dimers or monomers. This result highlights the significance of structural complementarity in the molecular recognition at the cross-section of amyloid fibers and feasibility for elaborating specific binders to distinct amyloid polymorphs.

Conclusions

In summary, in this study we proposed a novel approach for studying molecular interactions that involve amyloids. Commonly used random testing of various compounds that can interact with amyloids or influence their growth kinetics does not reveal the molecular details of a compound's interactions with fibers. By designing and synthesizing an amyloid analogue that can be studied in solution by various analytical techniques, we found that the *N*-methylation pattern critically influences the solubility properties of the construct. *N*-methylation is a promising strategy and needs to be explored further, because such peptides can be designed to be fully complementary to an amyloidogenic peptide (or protein) target and are, furthermore, membrane-permeable and protease-resistant.¹⁵ Thus, our proof-of-principle study outlines a conceptual workflow for future design and evaluation of peptide-based binders specific to a particular amyloid polymorph, which can then be developed into diagnostic reagents or inhibitors – an especially pressing and unresolved task in Alzheimer's disease research.

Conflicts of interest

There are no conflicts to declare.

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Total chemical synthesis and biophysical properties of a designed soluble 24 kDa amyloid analogue

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Materials and methods

Reagents: All solvents, chemicals, and reagents were purchased from commercial sources and used without further purification. Coupling reagents, Fmoc- α -L-amino acids, Fmoc-N-methyl- α -L-amino acids and resins for SPPS (solid-phase peptide synthesis) were purchased from Iris Biotech (Marktredwitz, Germany); diisopropylethylamine, DMF and 4-methylpiperidine were from Sigma-Aldrich; TFA (BioGrade) was from Halocarbon (Peachtree Corners, GA). ^{15}N -phenylalanine, ^{15}N -valine, ^{15}N -glycine, ^{15}N -leucine and ^{15}N -isoleucine were purchased from Cambridge Isotope Laboratories (Tewksbury, MA) and Fmoc-protected in-house. All other chemicals were purchased from Sigma-Aldrich.

Peptide synthesis: automated microwave peptide synthesis was performed on a Liberty Blue (CEM) synthesizer. Typically, syntheses were performed on a 0.1 mmol scale by Fmoc/tBu solid-phase peptide synthesis (Fmoc-SPPS; side-chain protection: Asn(Trt), Asp(OtBu), Cys(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Tyr(tBu)). Peptide- $^{\alpha}$ carboxylates were synthesized on a preloaded Fmoc-Lys(Boc)-Wang-PS-resin (loading: 0.68 mmol/g, mesh size: 180-333). Peptide- $^{\alpha}$ hydrazides were prepared on a 2-CT-NHNH₂ resin prepared in-house (loading: 0.4 mmol/g, mesh size: 200-400). The coupling reactions were performed by adding amino acids dissolved in DMF (2.5 mL, 0.2 M), the coupling reagent DIC in DMF (1.0 mL, 0.5 M) and Oxyma Pure in DMF (0.5 mL, 1 M) to the respective resin. Standard couplings were performed at 90 °C for 2 min. Standard deprotections were performed using 20% (v/v) 4-methylpiperidine in DMF at 90 °C for 1 min.

Prior to cleavage, peptidyl resin was washed with DCM and dried under vacuum. Peptides were cleaved and fully deprotected by treatment with TFA/DTT/TIPS/H₂O (92.5:2.5:2.5:2.5, v/w/v/v) for 2 h (1 mL of cleavage reagent for 100 mg of resin). The reaction mixtures were diluted 20-fold with diethyl ether (-20°C) for precipitation of the crude peptides. After centrifugation, the precipitates were dissolved in H₂O/CH₃CN (1:1, v/v) containing TFA (0.1%, v/v) and lyophilized.

Analytical HPLC: Analytical reversed-phase HPLC was performed on a Shimadzu Nexera XR UHPLC instrument. Alternatively, a Thermo Scientific Dionex 3000 was used. A Phenomenex Kinetex XB-C18 column (50×2.1 mm, 100 Å, 2.6 μm) was used at 1 mL/min with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v).

Preparative HPLC: Purifications of peptides were performed on a preparative Shimadzu HPLC instrument equipped with two LC-20AP pumps and an SPD-20A Prominence UV/vis detector connected to an FRC-10A fraction collector. Phenomenex Kinetex XB-C18 column (250×21.2 mm, 100 Å, 5 μm) was used at 10 mL/min with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v).

LC-MS: Peptide masses were measured on a LC/MS instrument equipped with a Thermo Scientific Accela UHPLC (Hypers II GOLD column, 50×2.1 mm, 1.9 μm) integrated with a Thermo Scientific LCQ Fleet ion-trap. Deconvolution of data was performed in MagTran 1.03 (Amgen, Thousand Oaks, CA). More precise MS measurements for larger protein products have been performed using a Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass-spectrometer. Direct infusion into electrospray ionization source was performed from solutions of proteins in H₂O/CH₃CN (1:1, v/v) containing formic acid (0.1%, v/v).

Size-exclusion chromatography: SEC was performed on a Shimadzu Nexera XR UHPLC instrument. A Phenomenex Yarra 3μm SEC 2000 column was used (300×4.6 mm, 2.8 μm) with an isocratic mobile phase composed of NaH₂PO₄ (10 mM), Na₂HPO₄ (40 mM), NaCl (100 mM) and NaN₃ (0.05%, w/v), pH 7.5, at a flow of 0.5 mL/min, at 27 °C, detected at 280 nm. A gel filtration markers kit for protein molecular weights (6,500-66,000 Da) was used for calibration. The kit was composed of Blue Dextran (for the determination of the void volume), aprotinin from bovine lung (6.5 kDa), cytochrome c from equine heart (12.4 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and albumin from bovine serum (66 kDa) and was purchased from Sigma-Aldrich.

NMR spectroscopy: 1D ¹H spectra shown in Figure S7 were recorded on a Bruker Avance III 600 MHz spectrometer. The spectra and data depicted in Figure 4c, Figures S8-S14 were recorded on a Bruker Avance III 700 MHz spectrometer equipped with a TCI cryoprobe. For 2D, relaxation and DOSY spectra acquisitions, a sample of **Toft-3** in a 3 mm tube was prepared at concentration of 200 μM (volume 180 μL) in 50 mM phosphate buffer at pH 7 (90:10% v/v H₂O/D₂O). The ¹H NOESY spectrum (2048 x 600 points) was recorded at 35 °C with a mixing time of 150 ms. 32 scans were acquired for each t1 increment leading to a total experimental time of 12 hours. The water signal was suppressed using a Watergate sequence. The ¹H-¹³C HSQC spectrum (2048 x 400 points) was recorded at 35 °C with 32 scans per t1 increment. The carbon bandwidth and carrier frequency were set to 70 ppm and 40 ppm, respectively and the quadrature detection was obtained using pulse field gradients selection of echo and anti-echo coherences. The experimental acquisition time was 5 hours. The ¹H-¹⁵N HSQC spectrum (2048 x 128 points) was recorded at 35 °C with 200 scans per t1 increment (6 hours experimental time). The nitrogen bandwidth and carrier frequency were set to 30 ppm and 116.5 ppm, respectively and the quadrature detection was obtained using a STATES-TPPI phase program. The ¹⁵N relaxation rates were measured from HSQC type experiments as described.⁽¹⁾ For ¹⁵N T₁ relaxation, intensities were extracted from a set of 9 spectra recorded with relaxation delays ranging between 20 ms and 1 s. For ¹⁵N T₂ relaxation, intensities were extracted from a set of 10 spectra recorded with relaxation delays ranging from 0 to 130 ms, with ¹⁵N 180° pulses applied every 0.9 ms at a field strength of

4.2 kHz. The 2D spectra were processed using nmrPipe program⁽²⁾ and the intensities were measured using CcpNmr.⁽³⁾ The relaxation rates were computed from non-linear fits of the time dependent intensities by either a single or double exponential decay function using in-house Python scripts. The values of the T_1 and T_2 relaxation times provide an estimate of the global correlation time (τ_c) of **ToFT-3** using the approximation provided by Farrow et al.⁽¹⁾

$$\tau_c = \sqrt{\frac{6\frac{T_1}{T_2} - 7}{4\omega_N^2}},$$

where T_1 = spin-lattice relaxation time constant, T_2 = spin-spin relaxation time constant and ω_N = ^{15}N Larmor frequency.

This gives a value between 9.8 and 13.2 ns, in agreement with the expected correlation time of a protein of ~200 amino-acids at 35 °C (11.5 ns as estimated from the model of Daragan).⁽⁴⁾

DOSY spectra were recorded for **ToFT-3** as a function of temperature to get an estimate of the hydrodynamic radius using TRIS as an internal reference.⁽⁵⁾ The DOSY spectrum was performed using the led_es sequence at 35 °C. The main diffusion time (Δ) was set to 200 ms. The diffusion pulse length was 1.8 ms.

Fluorescence spectroscopy: Fluorescence was recorded on a Victor X5 2030-0050 multi-label HTS plate-reader (PerkinElmer) with Nunc black standard 96-well plates sealed with transparent ThermalSeal RT film (Sigma-Aldrich). The excitation wavelength was 485 nm (ThT, 4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline), 405 nm (bis-ANS, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid), or 531 nm (Nile Red, 9-diethylamino-5-benzo[a]phenoxazinone). The emitted fluorescence was collected at 535 nm (ThT), 460 nm (bis-ANS), and 595 nm (Nile Red). Data are averages of three measurements. Concentrations of **ToFT-1** and **ToFT-2** were 10 μM and the concentration of dye (ThT, bis-ANS or Nile Red) was 30 μM in sodium phosphate buffer (100 μL , 50 mM, pH 7.5) with NaCl (100 mM) and NaN_3 (0.05%, w/v).

Kinetics of amyloid growth: Continuous amyloid growth assays were performed on a Victor X5 2030-0050 multi-label HTS plate-reader (PerkinElmer) with Nunc black standard 96-well plates sealed with transparent ThermalSeal RT film (Sigma-Aldrich). Fluorescence readings were taken at 30 °C, with shaking for 1 s every 10 min; reactions were monitored for up to five days. Excitation/emission wavelengths were set with optical filters. Samples had a volume of 100 μL of a sodium phosphate buffer (50 mM, pH 7.5) with NaCl (100 mM) and NaN_3 (0.05%, w/v) containing 33.3 μM of “covalent trimer” [20-41] β 2m, 100 μM of ThT and 11.1 μM of *N*-methylated peptide inhibitors. “Covalent trimer” [20-41] β 2m was dissolved

in DMSO as a 3.33 mM stock solution and was added to the phosphate buffer by 100-fold dilution. The *N*-methylated peptide inhibitors were directly dissolved in the buffer. The final concentration of DMSO in each sample was 1% (v/v).

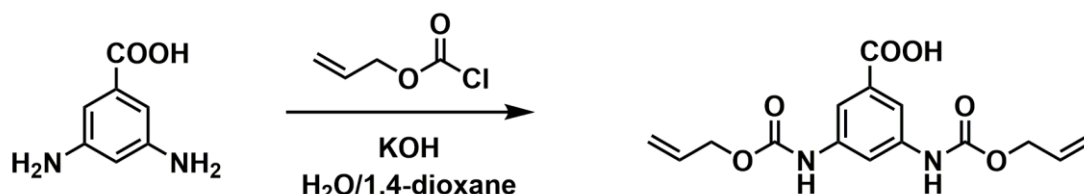
Transmission electron microscopy: Carbon Type B (15-25 nm) formvar-coated Cu electron microscopy grids (400 mesh; Ted Pella, Redding, CA) were placed coated-side-down for 60 s on the amyloid sample. The grids were washed with deionized water, stained with uranyl acetate (2%, w/v) for 20 s, and then air-dried. Images were taken on a Philips CM12 electron microscope (80 kV).

Molecular modeling: Molecular graphics and analyses were performed using MOE software.⁽⁶⁾ Model 1 from PDB ID: 2E8D (solid-state NMR structure of [20-41] β 2m amyloid protofilament) was selected. A stack of three β -arch-containing trimers composed of nine copies of [20-41] β 2m was built along the fiber axis. Central linkers were attached to each of the three trimers. Energies of the models were minimized by using the minimization force field AMBER10:EHT, with R-Field as a solvation mode. Minimizations were done stepwise: first, backbones were fixed and models were minimized, thus allowing side-chain and central linker reorganization; and a second minimization afforded reorganization of peptide backbone and side chains simultaneously. *C-N* linkers 1 and 2 were then introduced and the models were minimized using the same protocol as before. Finally, *N*-methylations were inserted by replacing the hydrogen atom of the amide bonds by a methyl group and models were minimized.

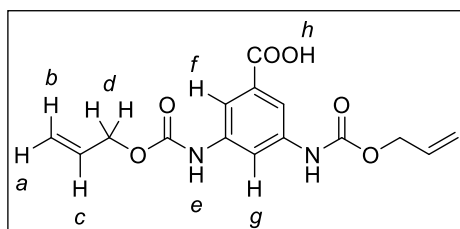
Chemical synthesis

Preparation of the hydrazide (2-CT-NHNH₂) resin:⁽⁷⁾ The 2-CT-OH resin (3 g, 1.6 mmol/g active groups, mesh size 200-400) was placed in a three-necked round bottom flask. It was flushed several times with argon. Dry dichloromethane (28 mL) was added. The mixture was gently stirred allowing the resin to swell. Thionyl chloride (450 μ L, 6.2 mmol, 1.3 equiv) was slowly added at 0°C. The suspension was stirred under argon for 2 h allowing it to slowly warm up to room temperature. The solvent was removed and the resin was washed with DCM and DMF. The 2-CT-Cl resin was then swollen for 20 min in DMF (18 mL). A mixture of hydrazine monohydrate (700 μ L, 14.4 mmol, 3 equiv) and DIEA (2 mL, 11.5 mmol, 2.4 equiv) in DMF (4 mL) was added to the resin at 0°C. The suspension was stirred at r.t. for 90 min. The reaction was quenched by the addition of methanol (300 μ L). The resin was washed with DMF, water, DMF, methanol and diethyl ether and dried under reduced pressure. Final mass of the resin: 3 g.

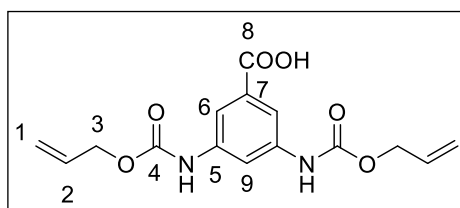
Synthesis of the central linker: 3,5-bis(((allyloxy)carbonyl)amino)benzoic acid



Potassium hydroxide (1.09 g, 19.5 mmol, 3 equiv) was added to a mixture of 3,5-diaminobenzoic acid (1.05 g, 6.5 mmol, 1 equiv) in water (40 mL). It was stirred until a clear solution was obtained. 1,4-dioxane (30 mL) was added to the reaction mixture followed by a dropwise addition of a solution of allyl chloroformate (1.52 mL, 14.3 mmol, 2.2 equiv) in 1,4-dioxane (10 mL). It was stirred at r.t. overnight. The volatiles were removed under reduced pressure. Water (100 mL) was added to the resulting paste and the pH was adjusted to 8-9 with sat. Na₂CO₃(aq). The aqueous solution was washed with ethyl acetate (2 x 50 mL). The aqueous layer was then treated with 6 M HCl(aq) to lower the pH to 3 and extracted with ethyl acetate (3 x 50 mL). The organic layers were gathered, washed with brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. The isolated yield was 2.0 g (95%). LC-MS (ESI): Average isotope calculated 320.3 Da [*M*]; found: 320.8 Da. Elemental analysis calculated for C₁₅H₁₆N₂O₆: C, 56.25; H, 5.04; N, 8.75; found: C, 55.69; H, 5.11; N 8.70.



¹H NMR (400 MHz, DMSO-*d*₆) δ, ppm: 4.61 (4H, ddd, H_d, ³J_{dc} = 5.4 Hz, ⁴J_{ad} = 1.5 Hz, ⁴J_{bd} = 1.5 Hz), 5.24 (2H, ddt, H_a, ²J_{ab} = 1.5 Hz, ³J_{ac} = 10.5 Hz, ⁴J_{ad} = 1.5 Hz), 5.36 (2H, ddt, H_b, ²J_{ab} = 1.5 Hz, ³J_{bc} = 17.2 Hz, ⁴J_{bd} = 1.5 Hz), 5.98 (2H, ddt, H_c, ³J_{bc} = 17.2 Hz, ³J_{ac} = 10.7 Hz, ³J_{dc} = 5.4 Hz), 7.73 (2H, d, H_f, ⁴J_{fg} = 2.0 Hz), 7.94 (1H, t, H_g, ⁴J_{fg} = 2.0 Hz), 9.91 (2H, s, H_e), 12.92 (1H, s, H_h).



¹³C NMR (101 MHz, DMSO-*d*₆) δ, ppm: 65.20 (C3), 112.72 (C9), 113.94 (C6), 118.06 (C1), 132.15 (C7), 133.71 (C2), 140.20 (C5), 153.63 (C4), 167.54 (C8). The assignment of the carbon signals to the appropriate proton resonances is based on DEPT, HSQC and HMBC measurements.

Procedures for the on-resin functionalization of the ornithine residue (0.1 mmol scale):

Boc protection of the last coupled residue (This step is only required for the synthesis of peptides B and H (see Tables S1 and S2)): After chain assembly and deprotection of the last Fmoc-group, the resin was swollen in DMF. *N*-terminal Boc protection of *N*-terminal serine was performed with the addition of Boc-anhydride (87.5 mg, 0.4 mmol, 4 equiv, in 1 mL of DMF) and DIEA (68 μ L, 0.4 mmol, 4 equiv). It was let to react at r.t. for 30 min with manual stirring. Kaiser test was negative.

Procedures below were employed for the synthesis of peptides B, D, F, H, J and K (see Tables S1-S3):

Alloc deprotection of ornithine: The resin was swollen in DCM purged with argon. A solution of phenylsilane (296 μ L, 2.4 mmol, 24 equiv) in DCM (1 mL) purged with argon was added to the resin. Alloc deprotection was carried out upon the addition of a solution of Pd(PPh₃)₄ (58 mg, 0.05 mmol, 0.5 equiv) in DCM (2 mL) purged with argon. It was let to react at r.t. for 30 min with manual stirring. Kaiser test was positive.

Linker coupling to ornithine side chain: The resin was swollen in DMF. A solution of 3,5-bis(((allyloxy)carbonyl)amino)benzoic acid (128 mg, 0.4 mmol, 4 equiv), HATU (144 mg, 0.38 mmol, 3.8 equiv) and DIEA (100 μ L, 0.6 mmol, 6 equiv) in 1 mL of DMF was prepared (2 min of pre-activation) and added to the resin. It was let to react at r.t. for 30 min. Kaiser test was negative.

Alloc deprotection of the linker: Same procedure as before (repeated twice). Chloranil test was positive.

Bromoacetylation of the linker: The resin was swollen in DMF. A solution of bromoacetic acid (167 mg, 1.2 mmol, 12 equiv) and *N,N*-diisopropylcarbodiimide (86 μ L, 1.1 mmol, 11.2 equiv) in 2 mL of DMF was prepared (2 min of pre-activation) and added to the resin. It was let to react at r.t. for 60 min. Chloranil test was negative.

Procedure for the synthesis of NMT-A-1:

Peptide **A** (30.1 mg, 12 μ mol) and peptide **B** (17.5 mg, 6 μ mol) (see Table S1) were dissolved in 5 mL of buffer ([Na₂HPO₄] = 0.1 M, [Gn·HCl] = 6 M, [Na₄-EDTA] = 5 mM, pH = 8.0). The pH was adjusted to 8.0 with a 1 M Na₂CO_{3(aq)} solution. It was let to react at 30°C for 2 h. The pH was then lowered to 3 with 6 M HCl(aq). Sodium nitrite, solubilized in argon purged pH 3 buffer (30 μ mol, c = 0.2 M, 150 μ L, 5 equiv), was added to the reaction mixture. It was let to react at -10°C for 15 min under argon. Sodium 2-mercaptoethanesulfonate, solubilized in argon purged pH 7 buffer (300 μ mol, c = 0.2 M, 1.5 mL, 50 equiv) was added.

It was let to react at -10°C for 2 h under argon. The reaction mixture was diluted and directly injected into preparative HPLC and the product was purified using a Phenomenex Kinetex XB-C18 (250 x 21.2 mm, 100 Å, 5 µm) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions of product were combined and lyophilized to give a white solid. *This procedure was also employed for the synthesis of NMT-A-2 (see Table S2).*

Procedure for the synthesis of CT-1:

Peptide **C** (34.8 mg, 13.9 µmol) and peptide **D** (22.0 mg, 6.9 µmol) (see Table S1) were dissolved in 9.2 mL of buffer ([Na₂HPO₄] = 0.1 M, [Gn·HCl] = 6 M, [Na₄·EDTA] = 5 mM, pH = 8.0). The pH was adjusted to 8.0 with a 1 M Na₂CO_{3(aq)} solution. It was let to react at 30°C for 2 h. Methoxyammonium chloride (154 mg, 16.7 mg/mL) was added to the reaction mixture. The pH was then lowered to 4 with 6 M HCl(aq). It was let to react at 30°C for 2 h. The reaction mixture was diluted and directly injected into preparative HPLC and the product was purified using a Phenomenex Kinetex XB-C18 (250 x 21.2 mm, 100 Å, 5 µm) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions were combined and lyophilized to give a white solid. *This procedure was also employed for the synthesis of NMT-B-1, NMT-B-2 and CT-2 (see Tables S1-S3).*

First ligation step in the synthesis of ToFT-2 construct:

NMT-A-2 (10.0 mg, 1.3 µmol) and **CT-1** (10.1 mg, 1.3 µmol) were dissolved in 1.0 mL of argon purged buffer 1 ([Na₂HPO₄] = 0.2 M, [Gn·HCl] = 6 M, pH = 7.0) containing MPAA (13 mM, 12.6 µmol, 10 equiv) and TCEP (20 mM, 20.2 µmol, 16 equiv). The pH was adjusted to 7.0 with a 1 M Na₂CO_{3(aq)} solution after peptide dissolution. It was let to react at 40°C for 2 h under argon. The reaction mixture was diluted with 2 mL of buffer 1. Bromoacetamide (158 µL, 0.2 M, 32.5 µmol, 25 equiv) was solubilized in buffer 1 and added to the reaction mixture. It was let to react at 40°C for 30 min. The pH was adjusted to 3.0 with a 6 M HCl_(aq) solution. At -10°C , sodium nitrite (64 µL, 0.2 M, 12.6 µmol, 10 equiv) was solubilized in buffer 2 ([Na₂HPO₄] = 0.2 M, [Gn·HCl] = 6 M, pH = 3.0) and was added to the reaction mixture. It was let to react for 20 min. At -10°C , sodium 2-mercaptoethanesulfonate (640 µL, 0.2 M, 126 µmol, 100 equiv) was solubilized in buffer 1 and added to the reaction mixture. It was let to react for 2 h. The reaction mixture was diluted and directly injected into preparative HPLC. The product was purified using a Phenomenex Kinetex XB-C18 (250 x 21.2 mm, 100 Å, 5 µm) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions were combined and lyophilized to give a white solid of the ligation product (**DofT-2** = “dimer of trimers”-2). Isolated yield: 7.0 mg (34 %). *This procedure was also employed for the synthesis of DofT-1 (product of ligation of NMT-A-1 and CT-1, isolated*

yield: 4.2 mg (54 %) and DofT-3 (product of ligation of NMT-A-2 and CT-2, isolated yield: 5.0 mg (29 %)).

DofT-1: LC-MS (ESI): $[M + 8H^+] = 1990.8$ m/z, $[M + 9H^+] = 1769.6$ m/z, $[M + 10H^+] = 1592.6$ m/z, $[M + 11H^+] = 1448.1$ m/z, $[M + 12H^+] = 1327.6$ m/z, $[M + 13H^+] = 1225.4$ m/z, $[M + 14H^+] = 1138.1$ m/z, $[M + 15H^+] = 1062.4$ m/z, $[M + 16H^+] = 996.3$ m/z, $C_{720}H_{1061}N_{173}O_{222}S_7$; Average isotope calculated 15916.8 Da $[M]$; found: 15919.2 Da. Here, there is a discrepancy between the calculated and experimental mass due to an incorrect calibration of the LC-MS apparatus. However, the final product **TofT-1** (after ligation of **DofT-1** and **NMT-B-1**) has correct mass (see Figure S4).

DofT-2: LC-MS (ESI): $[M + 9H^+] = 1773.9$ m/z, $[M + 10H^+] = 1596.6$ m/z, $[M + 11H^+] = 1451.6$ m/z, $[M + 12H^+] = 1330.8$ m/z, $[M + 13H^+] = 1228.5$ m/z, $[M + 14H^+] = 1140.8$ m/z, $[M + 15H^+] = 1064.9$ m/z, $[M + 16H^+] = 998.3$ m/z, $C_{723}H_{1067}N_{173}O_{222}S_7$; Average isotope calculated 15958.90 Da $[M]$; found: 15957.0 Da.

DofT-3: LC-MS (ESI): $[M + 8H^+] = 1996.5$ m/z, $[M + 9H^+] = 1774.5$ m/z, $[M + 10H^+] = 1597.4$ m/z, $[M + 11H^+] = 1452.1$ m/z, $[M + 12H^+] = 1331.3$ m/z, $[M + 13H^+] = 1229.0$ m/z, $[M + 14H^+] = 1141.2$ m/z, $[M + 15H^+] = 1065.2$ m/z, $[M + 16H^+] = 998.7$ m/z, $C_{723}H_{1067}N_{173}O_{222}S_7$; Average isotope calculated 15964.0 Da $[M]$; found: 15963.5 Da.

Second ligation step in the synthesis of TofT-2:

NMT-B-2 (6.5 mg, 0.8 μ mol) and **DofT-2** (6.6 mg, 0.4 μ mol) were dissolved in 200 μ L of argon purged buffer 1 ($[Na_2HPO_4] = 0.2$ M, $[Gn-HCl] = 6$ M, pH = 7.0) containing MPAA (11 mM, 2 μ mol, 5 equiv) and TCEP (34 mM, 6.6 μ mol, 16 equiv). The pH was adjusted to 6.8 with a 1 M $Na_2CO_{3(aq)}$ solution after peptide dissolution. It was let to react at 40°C for 30 min under argon. The reaction mixture was diluted with 400 μ L of buffer 1. Bromoacetamide (51 μ L, 0.2 M, 13.3 μ mol, 25 equiv) was solubilized in buffer 1 and added to the reaction mixture. It was let to react at r.t. for 30 min. The reaction mixture was diluted and directly injected into preparative HPLC using a Phenomenex Kinetex XB-C18 (250 x 21.2 mm, 100 Å , 5 μ m) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions were combined and lyophilized to give a white solid. Isolated yield: 3.7 mg (38 %). This procedure was also employed for the synthesis of TofT-1 (isolated yield: 3.2 mg (25 %)) and TofT-3 (isolated yield: 2.1 mg (40 %)).

TofT-1: ESI-MS-Orbitrap: $[M + 12H^+] = 1989.9$ m/z, $[M + 13H^+] = 1836.9$ m/z, $[M + 14H^+] = 1705.8$ m/z, $[M + 15H^+] = 1592.0$ m/z, $[M + 16H^+] = 1492.7$ m/z, $[M + 17H^+] = 1404.9$ m/z, $[M + 18H^+] = 1326.9$ m/z, $[M + 19H^+] = 1257.1$ m/z, $[M + 20H^+] = 1194.3$ m/z, $[M + 21H^+] =$

1137.6 m/z , $C_{1084}H_{1596}N_{262}O_{332}S_8$; Average isotope calculated 23866.7 Da [M]; found: 23866.4 Da.

ToF-2: ESI-MS-Orbitrap: [$M + 12H^+$] = 1996.9 m/z , [$M + 13H^+$] = 1843.3 m/z , [$M + 14H^+$] = 1711.8 m/z , [$M + 15H^+$] = 1597.6 m/z , [$M + 16H^+$] = 1497.9 m/z , [$M + 17H^+$] = 1409.9 m/z , [$M + 18H^+$] = 1331.6 m/z , [$M + 19H^+$] = 1261.6 m/z , [$M + 20H^+$] = 1198.5 m/z , [$M + 21H^+$] = 1141.5 m/z , $C_{1090}H_{1608}N_{262}O_{332}S_8$; Average isotope calculated 23950.8 Da [M]; found: 23950.6 Da.

ToF-3: ESI-MS-Orbitrap: [$M + 12H^+$] = 1997.1 m/z , [$M + 13H^+$] = 1843.8 m/z , [$M + 14H^+$] = 1712.1 m/z , [$M + 15H^+$] = 1597.9 m/z , [$M + 16H^+$] = 1498.2 m/z , [$M + 17H^+$] = 1410.2 m/z , [$M + 18H^+$] = 1331.9 m/z , [$M + 19H^+$] = 1261.8 m/z , [$M + 20H^+$] = 1198.8 m/z , [$M + 21H^+$] = 1141.7 m/z , $C_{1090}H_{1608}N_{262}O_{332}S_8$; Average isotope calculated 23955.9 Da [M]; found: 23955.5 Da.

Synthesis of covalently tethered N-methylated inhibitors:

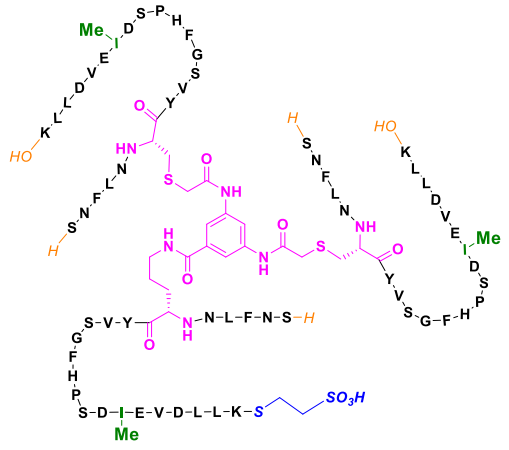
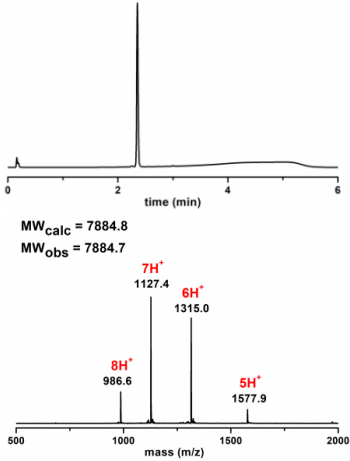
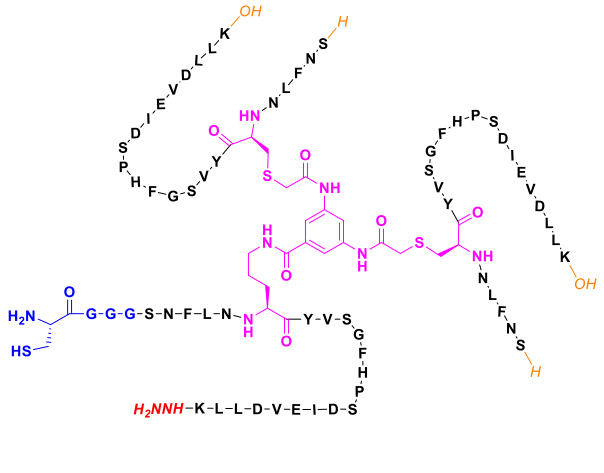
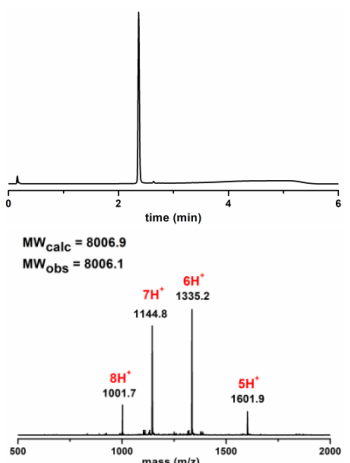
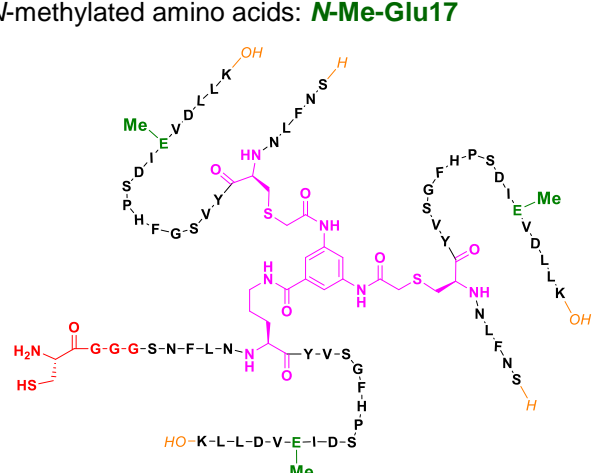
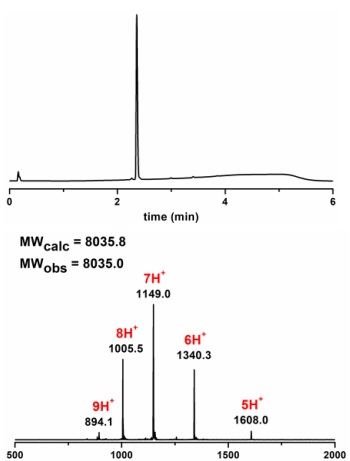
Covalent dimer [20-41] β 2m with N-Me-Asn5, N-Me-Val18: Peptide G (Table S2, 10.0 mg, 4.0 μ mol) were dissolved in 5 mL of buffer ($[Na_2HPO_4] = 0.1$ M, $[Gn \cdot HCl] = 6$ M, $[EDTA.Na_4] = 5$ mM, pH = 7.0). The pH was adjusted to 9.0 with 6 M $NaOH_{(aq)}$ after peptide dissolution. It was let to react at 30°C for 4 days. The crude peptide was purified by preparative HPLC using a Phenomenex Kinetex XB-C18 (100 x 21.2 mm, 100 Å, 5 μ m) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions were combined and lyophilized to give a white solid. The isolated yield was 3.2 mg (31%). LC-MS (ESI): [$M + 3H^+$] = 1684.4 m/z , [$M + 4H^+$] = 1263.7 m/z , [$M + 5H^+$] = 1011.1 m/z , [$M + 6H^+$] = 842.5 m/z , $C_{230}H_{344}N_{54}O_{70}S_2$; Average isotope calculated 5149.7 Da [M]; found: 5050.8 Da.

Covalent dimer [20-41] β 2m with N-Me-Leu4, N-Me-Asp19: Peptide I (Table S2, 10.0 mg, 4.0 μ mol) were dissolved in 5 mL of buffer ($[Na_2HPO_4] = 0.1$ M, $[Gn \cdot HCl] = 6$ M, $[EDTA.Na_4] = 5$ mM, pH = 7.0). The pH was adjusted to 9.0 with 6 M $NaOH_{(aq)}$ after peptide dissolution. It was let to react at 30°C for 4 days. The crude peptide was purified by preparative HPLC using a Phenomenex Kinetex XB-C18 (100 x 21.2 mm, 100 Å, 5 μ m) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions were combined and lyophilized to give a white solid. The isolated yield was 4.0 mg (39%). LC-MS (ESI): [$M + 3H^+$] = 1684.0 m/z , [$M + 4H^+$] = 1263.5 m/z , [$M + 5H^+$] = 1011.3 m/z , [$M + 6H^+$] = 842.5 m/z , $C_{230}H_{344}N_{54}O_{70}S_2$; Average isotope calculated 5149.7 Da [M]; found: 5049.8 Da.

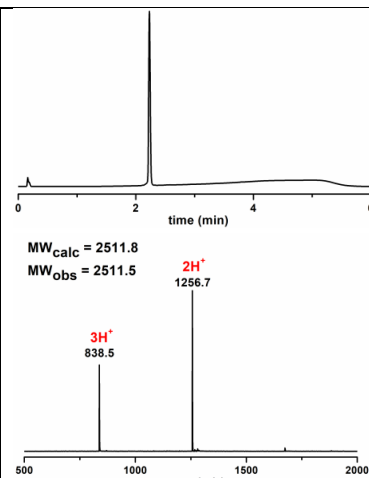
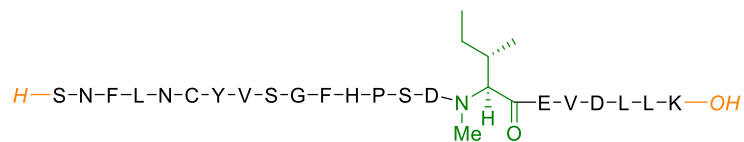
Covalent trimer [20-41] β 2m with N-Me-Asn5, N-Me-Val18: Peptide G (Table S2, 8.5 mg, 6 μ mol) was suspended in NH_4HCO_3 (3.4 mL, 200 mM, pH 8) containing EDTA (5 mM). Then, *N,N',N''*-(benzene-1,3,5-triyl)-tris(2-bromoacetamide) (840 μ L, 1 mM in acetonitrile, 1.5 μ mol) was added. The suspension was vortexed and sonicated until a clear solution was obtained. The reaction mixture was shaken on a ThermoMixer (600 rpm) at 30°C for 2 h and then lyophilized. The crude solid was dissolved in guanidine hydrochloride (Gn-HCl, 6 M, 1 mL per 10 mg of crude solid) and immediately purified by preparative HPLC using a Phenomenex Kinetex XB-C18 (100 x 21.2 mm, 100 Å, 5 μ m) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions were combined and lyophilized to give a white solid. The isolated yield based on the linker was 3.6 mg (55%). LC-MS (ESI): $[M + 4\text{H}^+] = 1956.2$ m/z, $[M + 5\text{H}^+] = 1565.3$ m/z, $[M + 6\text{H}^+] = 1304.2$ m/z, $[M + 7\text{H}^+] = 1118.2$ m/z, $[M + 8\text{H}^+] = 978.6$ m/z, $[M + 9\text{H}^+] = 869.8$ m/z, $\text{C}_{357}\text{H}_{528}\text{N}_{84}\text{O}_{108}\text{S}_3$; Average isotope calculated 7820.8 Da $[M]$; found: 7820.1 Da.

Covalent trimer [20-41] β 2m with N-Me-Leu4, N-Me-Asp19: Peptide I (Table S2, 8.5 mg, 3.4 μ mol) was suspended in NH_4HCO_3 (3.4 mL, 200 mM, pH 8) containing EDTA (5 mM). Then, *N,N',N''*-(benzene-1,3,5-triyl)-tris(2-bromoacetamide) (840 μ L, 1 mM in acetonitrile, 1.5 μ mol) was added. The suspension was vortexed and sonicated until a clear solution was obtained. The reaction mixture was shaken on a ThermoMixer (600 rpm) at 30°C for 2 h and then lyophilized. The crude solid was dissolved in guanidine hydrochloride (Gn-HCl, 6 M, 1 mL per 10 mg of crude solid) and immediately purified by preparative HPLC using a Phenomenex Kinetex XB-C18 (100 x 21.2 mm, 100 Å, 5 μ m) column with a gradient of water with TFA (0.1%, v/v) and acetonitrile with TFA (0.08%, v/v). Pure fractions were combined and lyophilized to give a white solid. The isolated yield based on the linker was 4.8 mg (73%). LC-MS (ESI): $[M + 4\text{H}^+] = 1956.4$ m/z, $[M + 5\text{H}^+] = 1564.9$ m/z, $[M + 6\text{H}^+] = 1304.4$ m/z, $[M + 7\text{H}^+] = 1118.2$ m/z, $[M + 8\text{H}^+] = 978.6$ m/z, $[M + 9\text{H}^+] = 869.8$ m/z, $\text{C}_{357}\text{H}_{528}\text{N}_{84}\text{O}_{108}\text{S}_3$; Average isotope calculated 7820.8 Da $[M]$; found: 7820.5 Da.

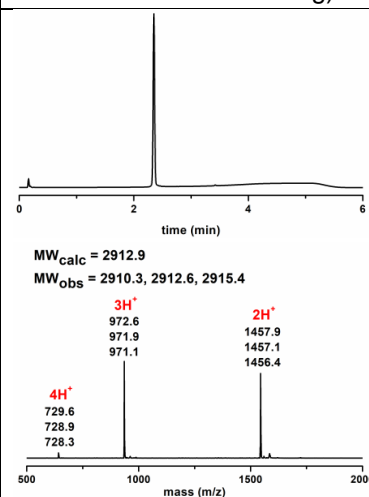
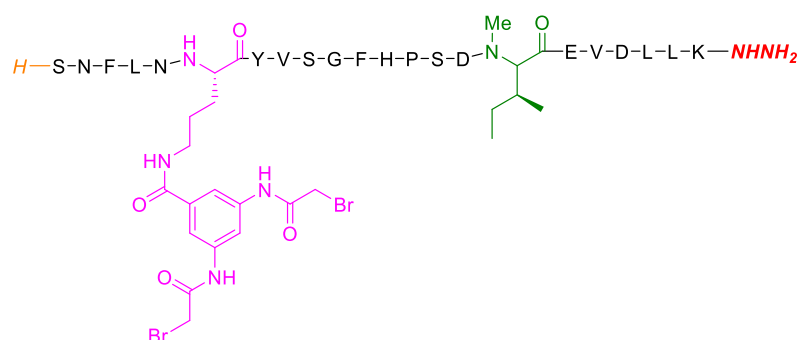
Table S1. Building blocks used to assemble **TofT-1** construct

Building block	Analytical characterization
Covalent trimers:	
<p>NMT-A-1</p> <p><i>N</i>-methylated amino acids: N-Me-Ile16</p> 	 <p>MW_{calc} = 7884.8 MW_{obs} = 7884.7</p> <p>7H⁺ 1127.4 6H⁺ 1315.0 8H⁺ 986.6 5H⁺ 1577.9</p> <p>The isolated yield: 32.4 mg (68%).</p>
<p>CT-1</p> 	 <p>MW_{calc} = 8006.9 MW_{obs} = 8006.1</p> <p>7H⁺ 1144.8 6H⁺ 1335.2 8H⁺ 1001.7 5H⁺ 1601.9</p> <p>The isolated yield: 22.5 mg (41%).</p>
<p>NMT-B-1</p> <p><i>N</i>-methylated amino acids: N-Me-Glu17</p> 	 <p>MW_{calc} = 8035.8 MW_{obs} = 8035.0</p> <p>7H⁺ 1149.0 6H⁺ 1340.3 8H⁺ 1005.5 9H⁺ 894.1 5H⁺ 1608.0</p> <p>The isolated yield: 20.3 mg (54%).</p>

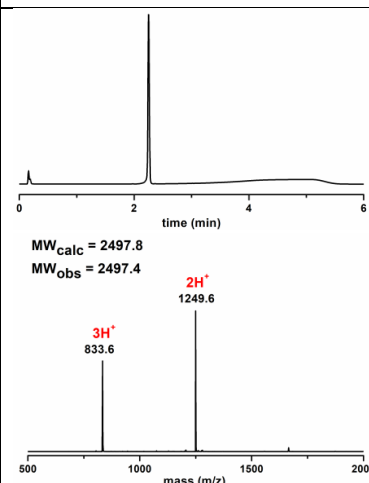
Continued on p. 13

Peptides used in the synthesis of covalent trimers:**peptide A:**N-methylated amino acids: **N-Me-Ile16**sequence: SNFLN**Cys**YVSGFHPSD**N-Me-Ile**EVDLLK

The isolated yield: 97 mg (37% based on the resin loading).

peptide B:N-methylated amino acids: **N-Me-Ile16**sequence:SNFLN**Orn(linker)**YVSGFHPSD**N-Me-Ile**EVDLLK-**NHNH₂**

The isolated yield: 84 mg (29% based on the resin loading).

peptide C: [20-41]β2m-fragmentsequence: H-SNFLN**Cys**YVSGFHPSDIEVDLLK-OH

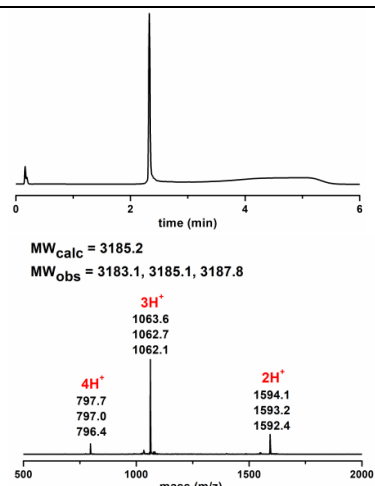
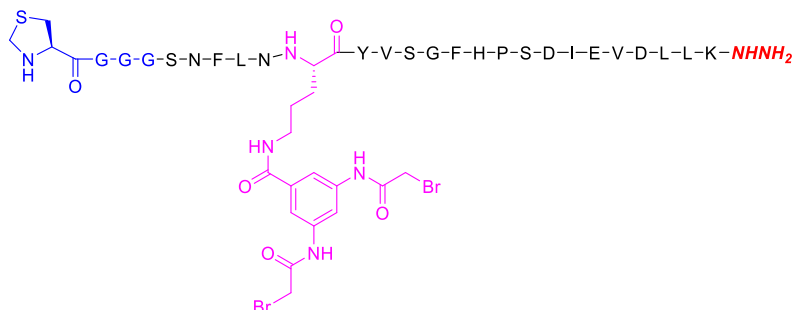
The isolated yield: 104 mg (42% based on the resin loading).

Continued from p. 13

peptide D:

sequence:

Thz-GGG-SNFLN*Orn(linker)***YVSGFHPSDIEVDLLK-NHNH₂**



MW_{calc} = 3185.2
MW_{obs} = 3183.1, 3185.1, 3187.8

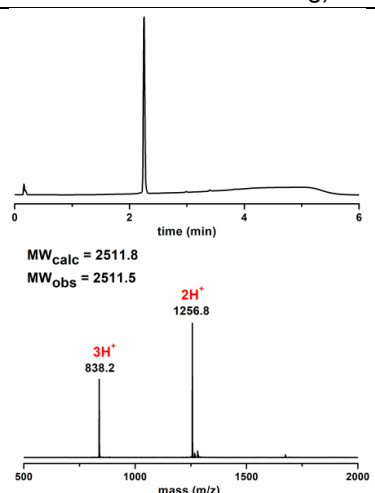
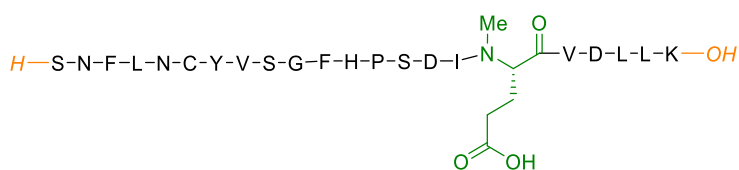
The isolated yield: 140 mg (44% based on the resin loading).

peptide E:

N-methylated amino acids: **N-Me-Glu17**

sequence:

H-SNFLN*Cys***YVSGFHPSD***N-Me-Glu***VDLLK-OH**



MW_{calc} = 2511.8
MW_{obs} = 2511.5

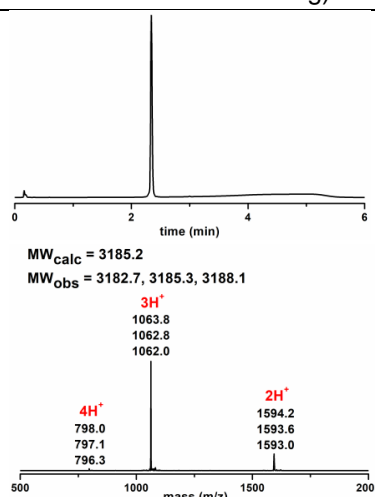
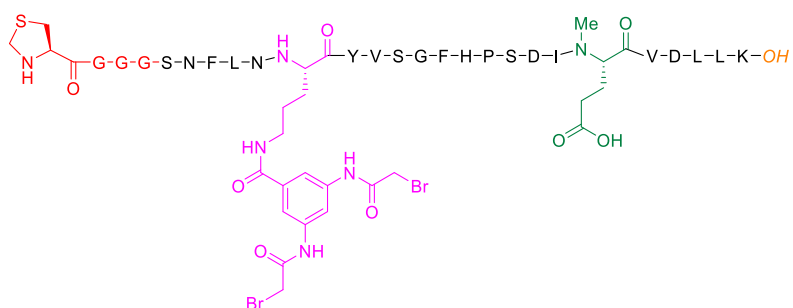
The isolated yield: 96 mg (38% based on the resin loading).

peptide F:

N-methylated amino acids: **N-Me-Glu17**

sequence:

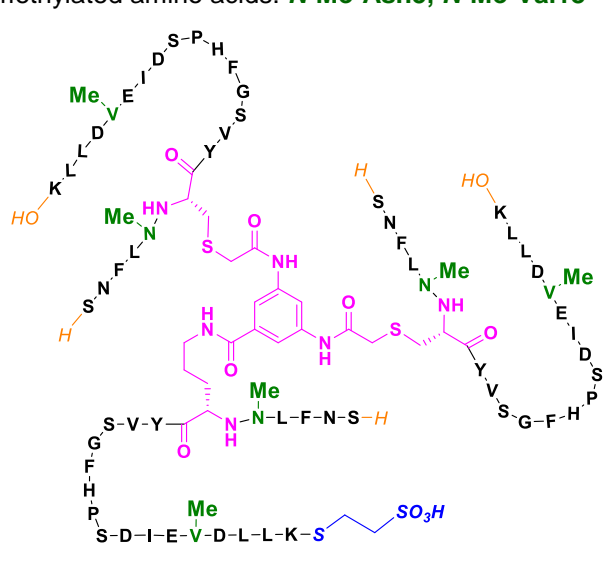
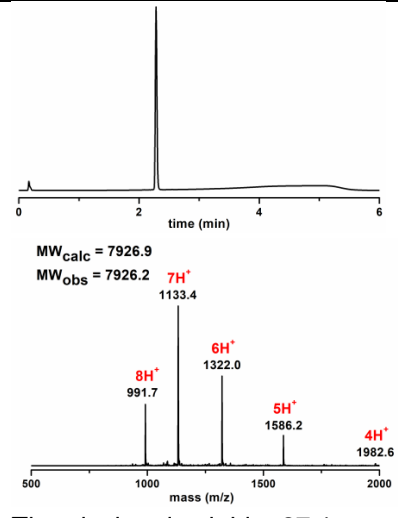
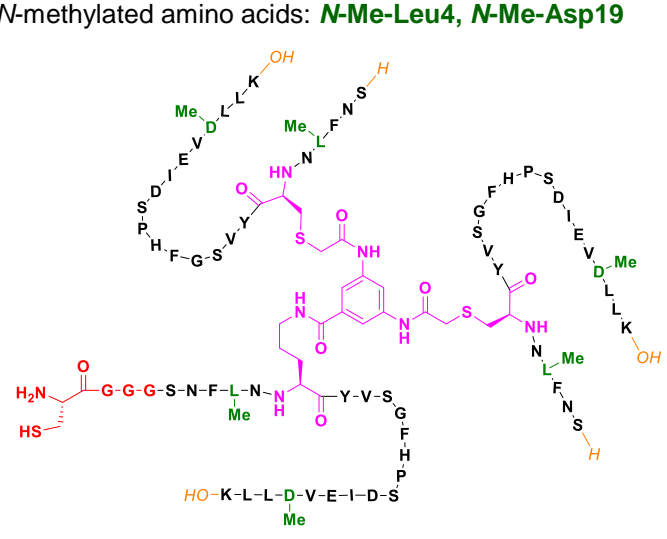
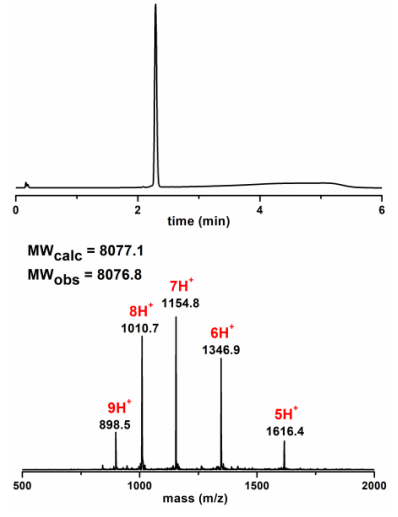
Thz-GGG-SNFLN*Orn(linker)***YVSGFHPSD***N-Me-Glu***VDLLK-OH**



MW_{calc} = 3185.2
MW_{obs} = 3182.7, 3185.3, 3188.1

The isolated yield: 85 mg (30% based on the resin loading).

Table S2. Building blocks used to assemble **TofT-2** construct

	<i>Building block</i>	<i>Analytical characterization</i>
Covalent trimers:		
NMT-A-2	<p><i>N</i>-methylated amino acids: N-Me-Asn5, N-Me-Val18</p> 	 <p>MW_{calc} = 7926.9 MW_{obs} = 7926.2</p> <p>7H⁺ 1133.4 8H⁺ 991.7 6H⁺ 1322.0 5H⁺ 1586.2 4H⁺ 1982.6</p> <p>The isolated yield: 27.1 mg (49%).</p>
	CT-1	Same as in the synthesis of TofT-1 (see Table S1)
NMT-B-2	<p><i>N</i>-methylated amino acids: N-Me-Leu4, N-Me-Asp19</p> 	 <p>MW_{calc} = 8077.1 MW_{obs} = 8076.8</p> <p>7H⁺ 1154.8 8H⁺ 1010.7 6H⁺ 1346.9 9H⁺ 898.5 5H⁺ 1616.4</p> <p>The isolated yield: 36.8 mg (66%).</p>
	Peptides used in the synthesis of covalent trimers:	
peptide C: same as in the synthesis of TofT-1 (see Table S1)		
peptide D: same as in the synthesis of TofT-1 (see Table S1)		

Continued on p. 16

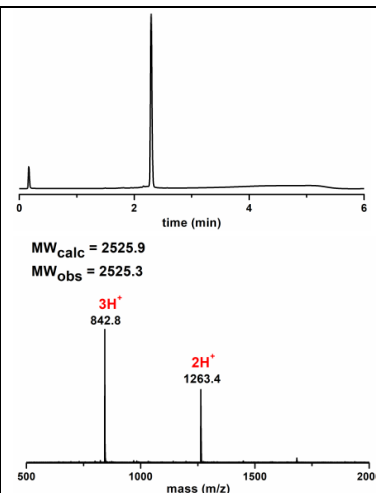
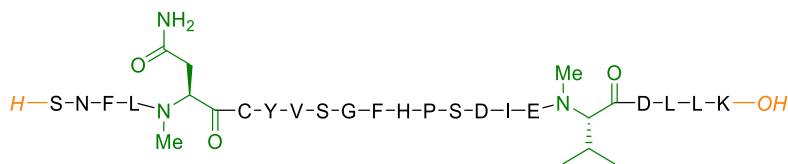
Continued from p. 15

peptide G:

N-methylated amino acids: **N-Me-Asn5**, **N-Me-Val18**

sequence:

H-SNFLN-Me-AsnCysYVSGFHPSDIE**N-Me-Val**DLK-OH



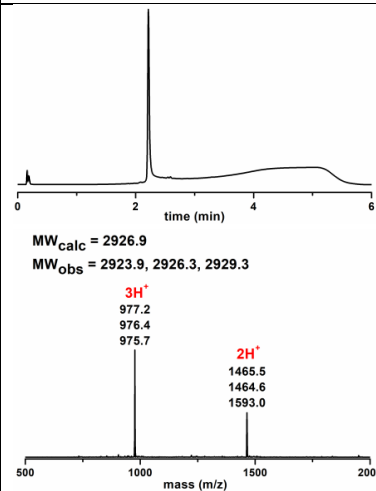
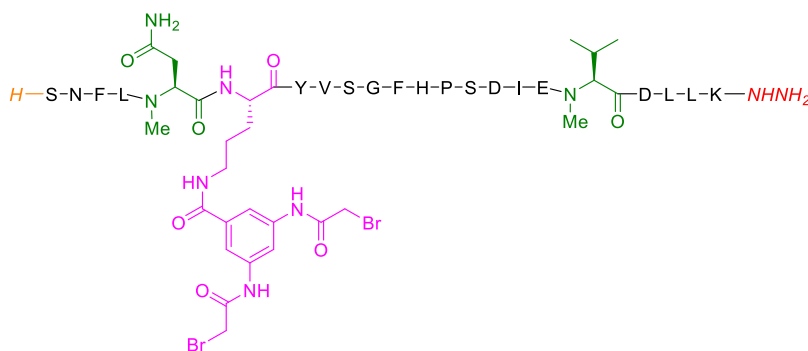
The isolated yield: 76 mg (30% based on the resin loading).

peptide H:

N-methylated amino acids: **N-Me-Asn5**, **N-Me-Val18**

sequence:

H-SNFLN-Me-AsnOrn(linker)YVSGFHPSDIE**N-Me-Val**DLK-NHNH₂



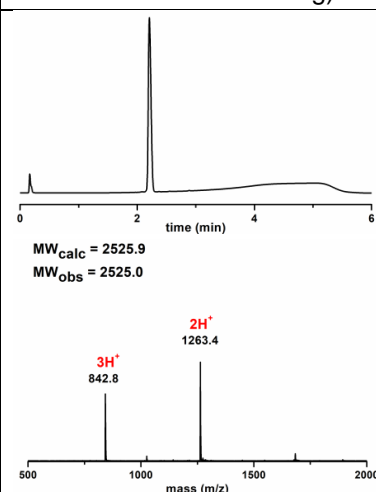
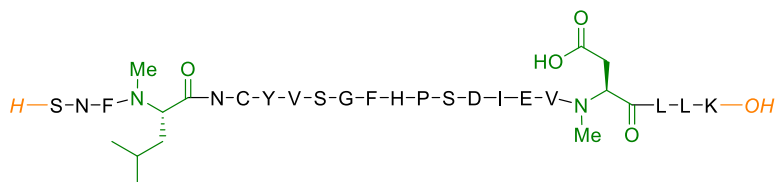
The isolated yield: 150 mg (33% based on the resin loading).

peptide I:

N-methylated amino acids: **N-Me-Leu4**, **N-Me-Asp19**

sequence:

H-SNFN-Me-LeuNCysYVSGFHPSDIEV**N-Me-Asp**LLK-OH



The isolated yield: 77 mg (30% based on the resin loading).

Continued on p. 17

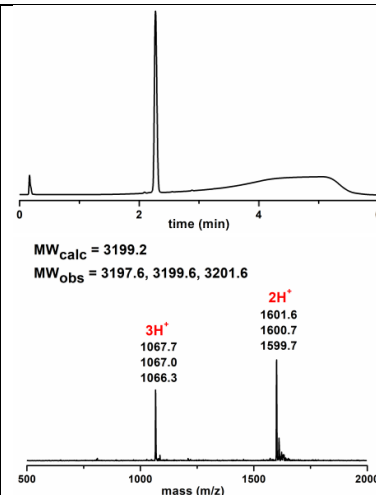
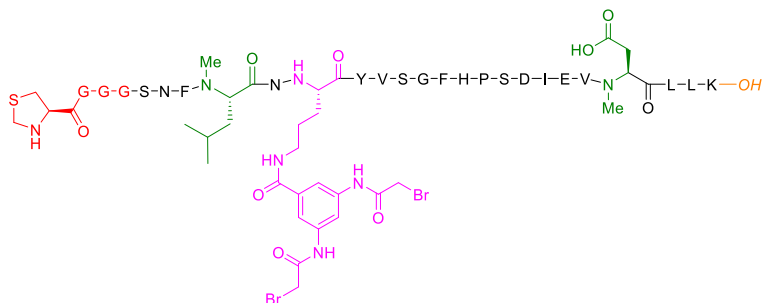
Continued from p. 16

peptide J:

N-methylated amino acids: **N-Me-Leu4**, **N-Me-Asp19**

sequence:

Thz-GGG-SNF-N-Me-Leu-N^{Orn}(linker)-YVSGFHPSDIEV-N-Me-Asp-LLK-OH

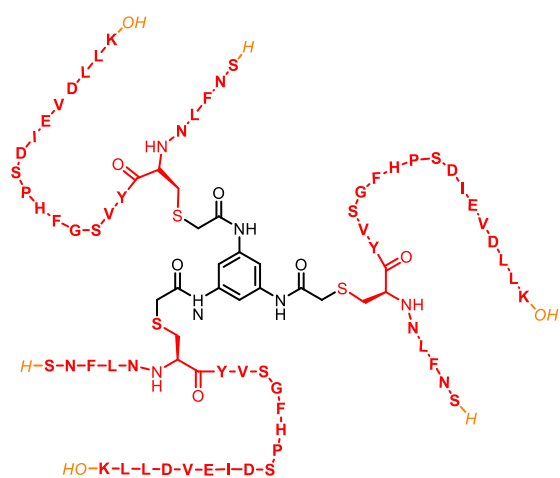
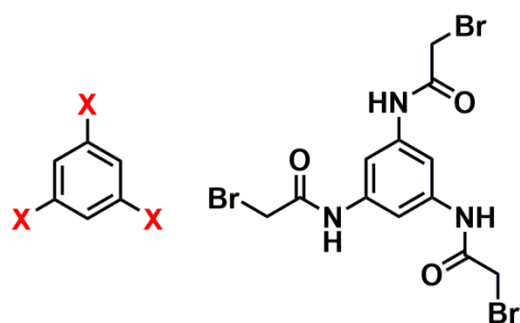


The isolated yield: 71 mg (22% based on the resin loading).

Table S3. Building blocks used to assemble **TofT-3** construct

	<i>Building block</i>	<i>Analytical characterization</i>
Covalent trimers:		
NMT-A-2	Same as in the synthesis of TofT-2 (see Table S2)	
CT-2	¹⁵ N-labeled amino acids: Phe3, Val8, Gly10, Ile16, Leu20	<p>MW_{calc} = 8011.0 MW_{obs} = 8011.4</p> <p>The isolated yield: 19.2 mg (35%).</p>
NMT-B-2	Same as in the synthesis of TofT-2 (see Table S2)	
Peptides used in the synthesis of covalent trimers:		
peptide C: same as in the synthesis of TofT-1 (see Table S1)		
peptide K:	¹⁵ N-labeled amino acids: Phe3, Val8, Gly10, Ile16, Leu20 <u>sequence:</u> Thz-GGG-SNFLN <i>Orn(linker)</i> YVSGFHPSDIEVDLLK-NHNH₂	<p>MW_{calc} = 3190.1 MW_{obs} = 3197.8, 3190.0, 3192.5</p> <p>The isolated yield: 54 mg (17% based on the resin loading).</p>
peptide G: Same as in the synthesis of TofT-2 (see Table S2)		
peptide H: Same as in the synthesis of TofT-2 (see Table S2)		
peptide I: Same as in the synthesis of TofT-2 (see Table S2)		
peptide J: Same as in the synthesis of TofT-2 (see Table S2)		

a)



b)

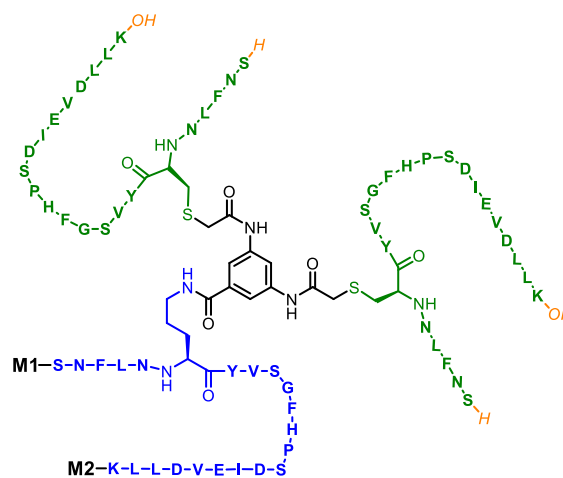
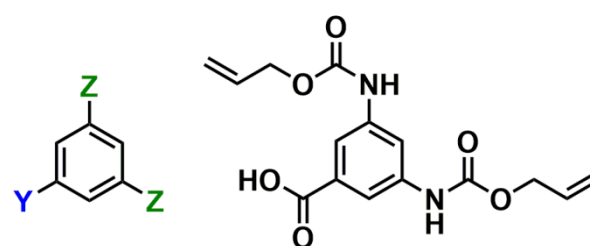
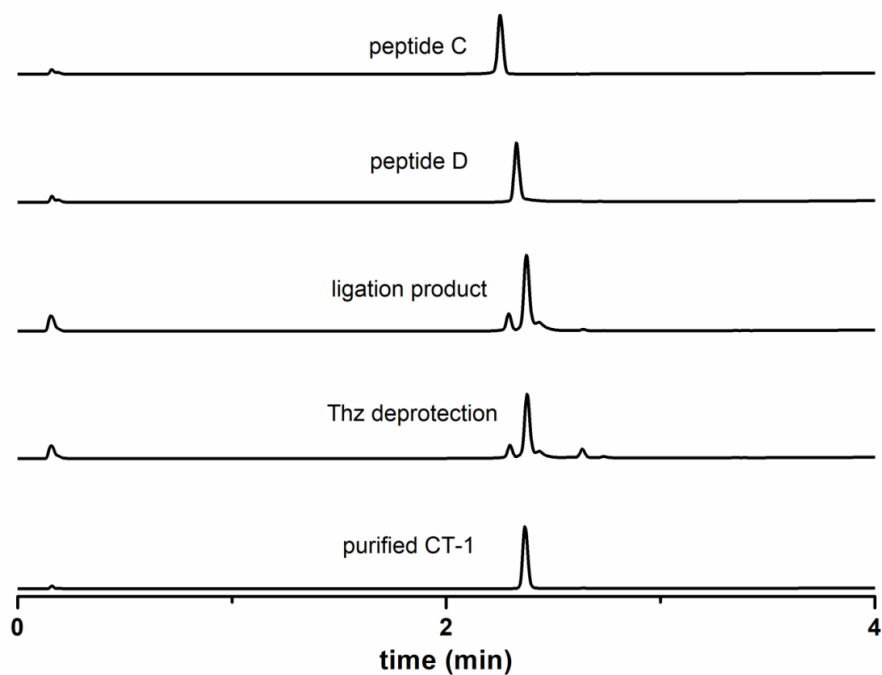


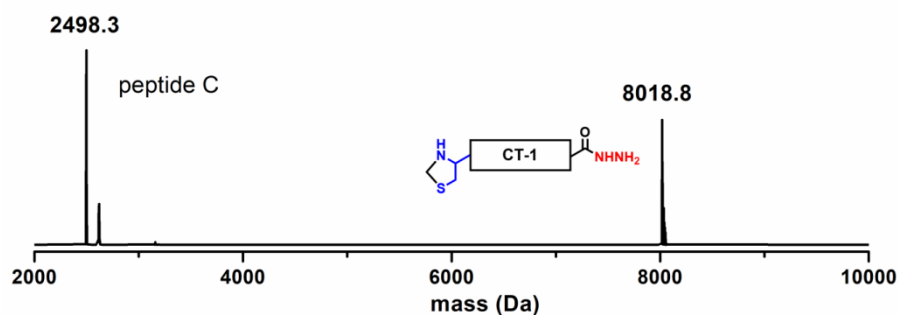
Fig. S1. Chemical structures of the central linkers. a) The linker used for the formation of symmetric trimers of [20-41]β₂m peptide (“covalent trimer”).⁽⁸⁾ b) The new central linker that allows for synthesis of covalent trimers with non-identical peptides attached, M1 = modification 1 and M2 = modification 2. ‘H’ and ‘OH’ in orange designate unmodified amino and carboxy functions of N- and C-termini of peptide chain, respectively.

a)



b)

Deconvoluted MS of the reaction between **peptides C and D**:



Deconvoluted MS after the conversion of Thz (thiazolidine) into Cys:

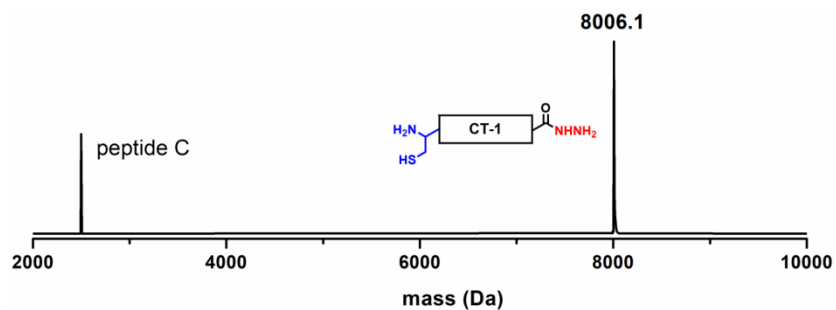


Fig. S2. a) Analytical HPLC and b) LC-MS monitoring of the reaction steps in the synthesis of non-symmetric covalent trimer **CT-1** (see Table S1).

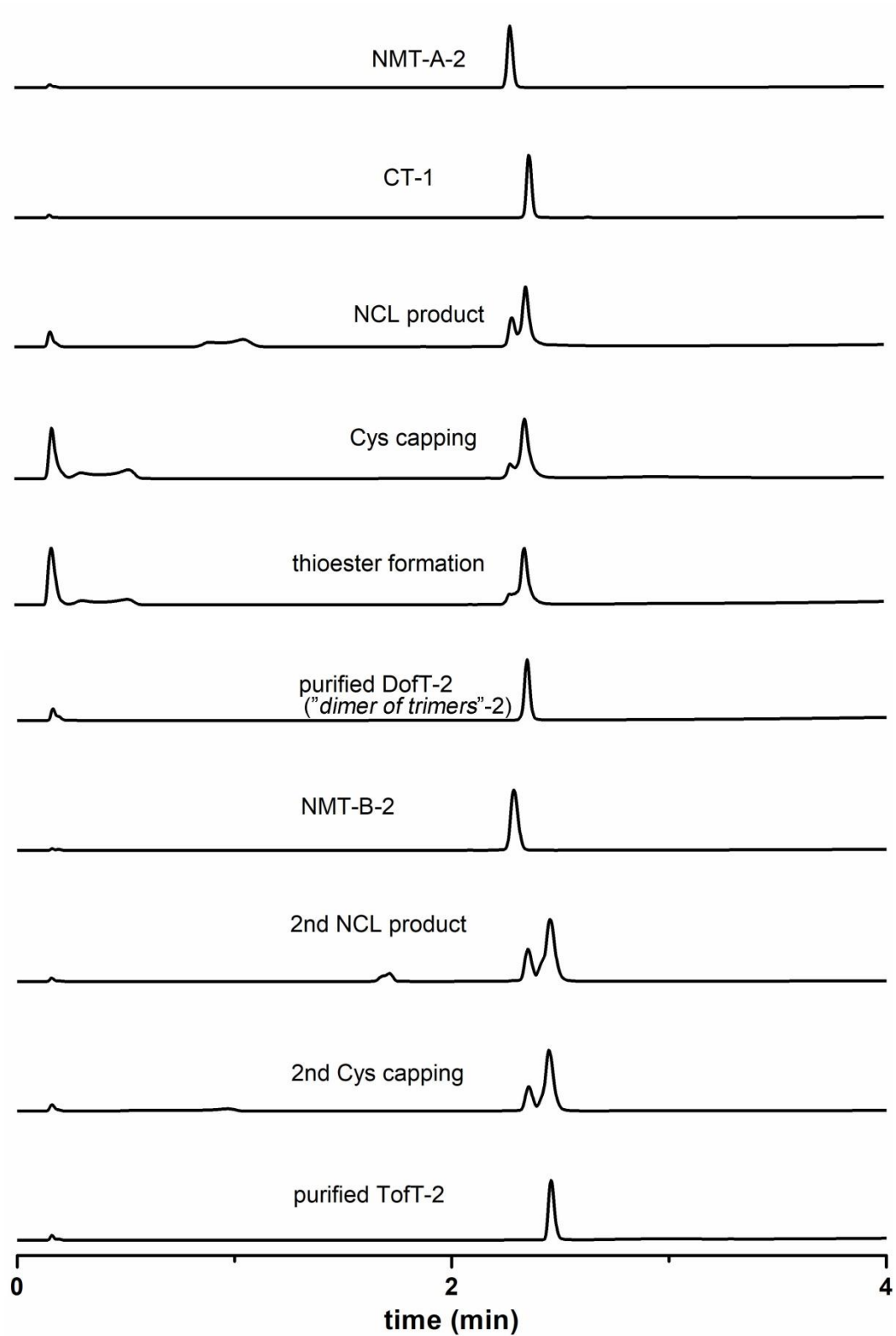
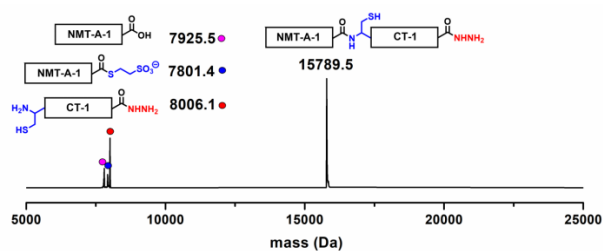
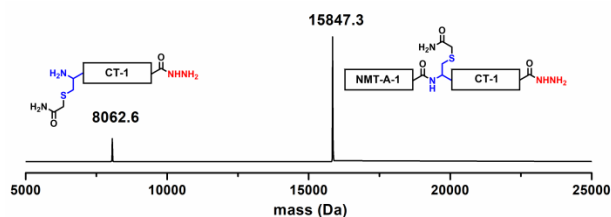


Fig. S3 (continued on p. 22). Analytical HPLC and LC-MS monitoring of the reaction steps in the sequential native chemical ligation in the total synthesis of **ToFT-2** construct.

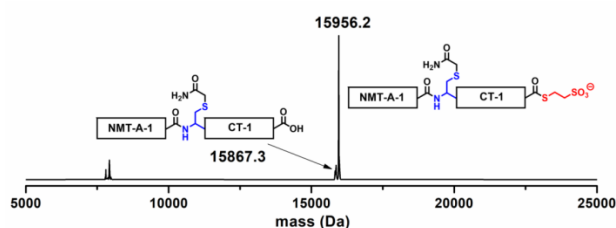
Deconvoluted MS after the native chemical ligation between **NMT-A-1** and **CT-1**:



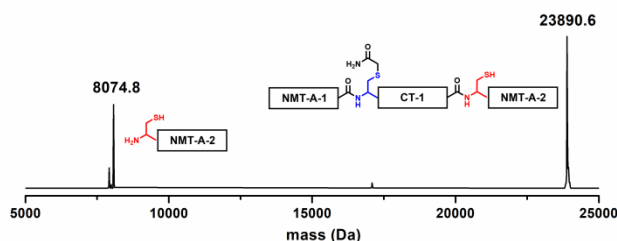
Deconvoluted MS after Cys capping at the ligation site:



Deconvoluted MS after conversion of peptide- α hydrazide into peptide- α thioester:



Deconvoluted MS after native chemical ligation between **DoT** (“*dimer of trimers*”)-2 and **NMT-A-2**:



Deconvoluted MS after Cys capping at the ligation site:

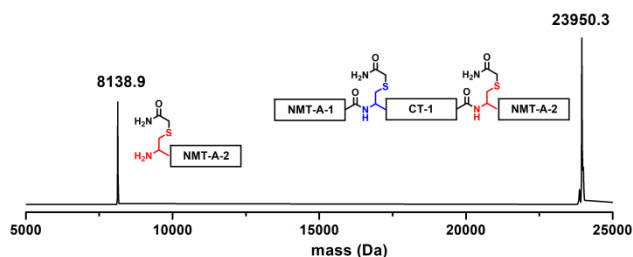


Fig. S3 (continued from p. 21). Analytical HPLC and LC-MS monitoring of the reaction steps in the sequential native chemical ligation in the total synthesis of **ToFT-2** construct.

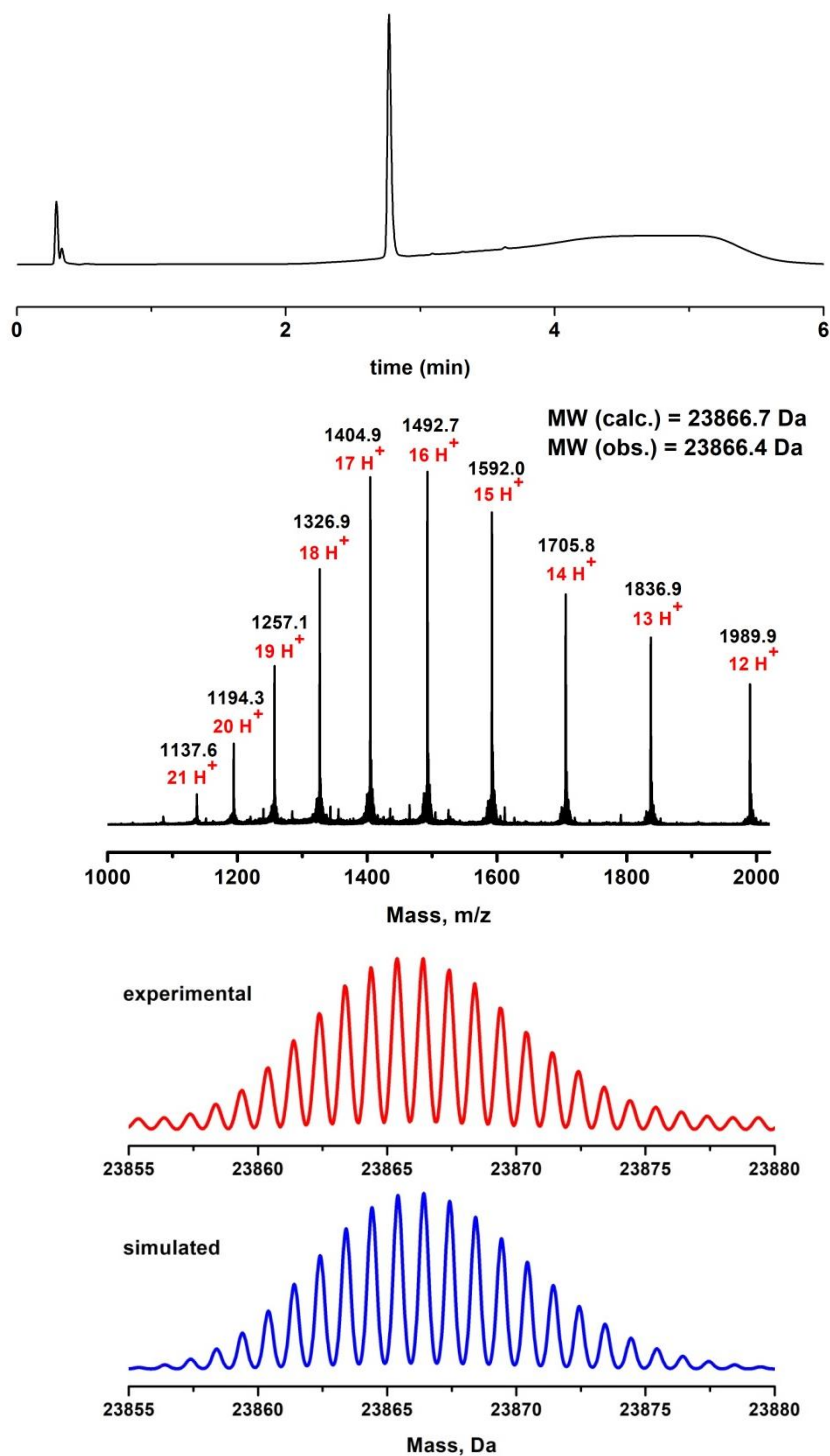


Fig. S4. Characterization of **ToFT-1**. Top: Analytical HPLC ($\lambda=220$ nm); Middle: ESI-MS-Orbitrap mass spectrum; Bottom: Deconvolution of the experimental mass spectrum in red and theoretical simulation in blue.

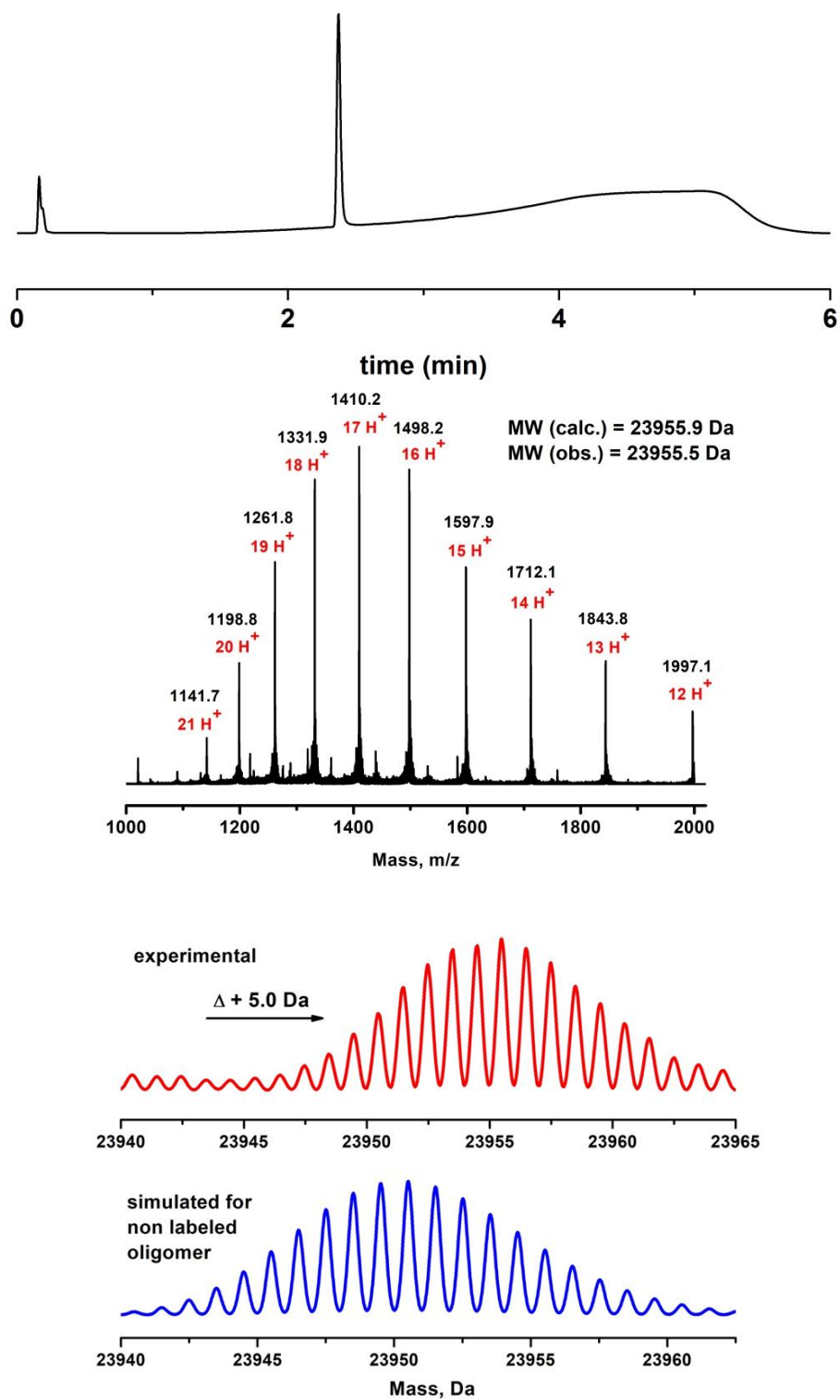


Fig. S5. Characterization of **ToFT-3**. Top: Analytical HPLC ($\lambda=220$ nm); Middle: ESI-MS-Orbitrap mass spectrum; Bottom: Deconvolution of the experimental mass spectrum in red and theoretical simulation in blue.

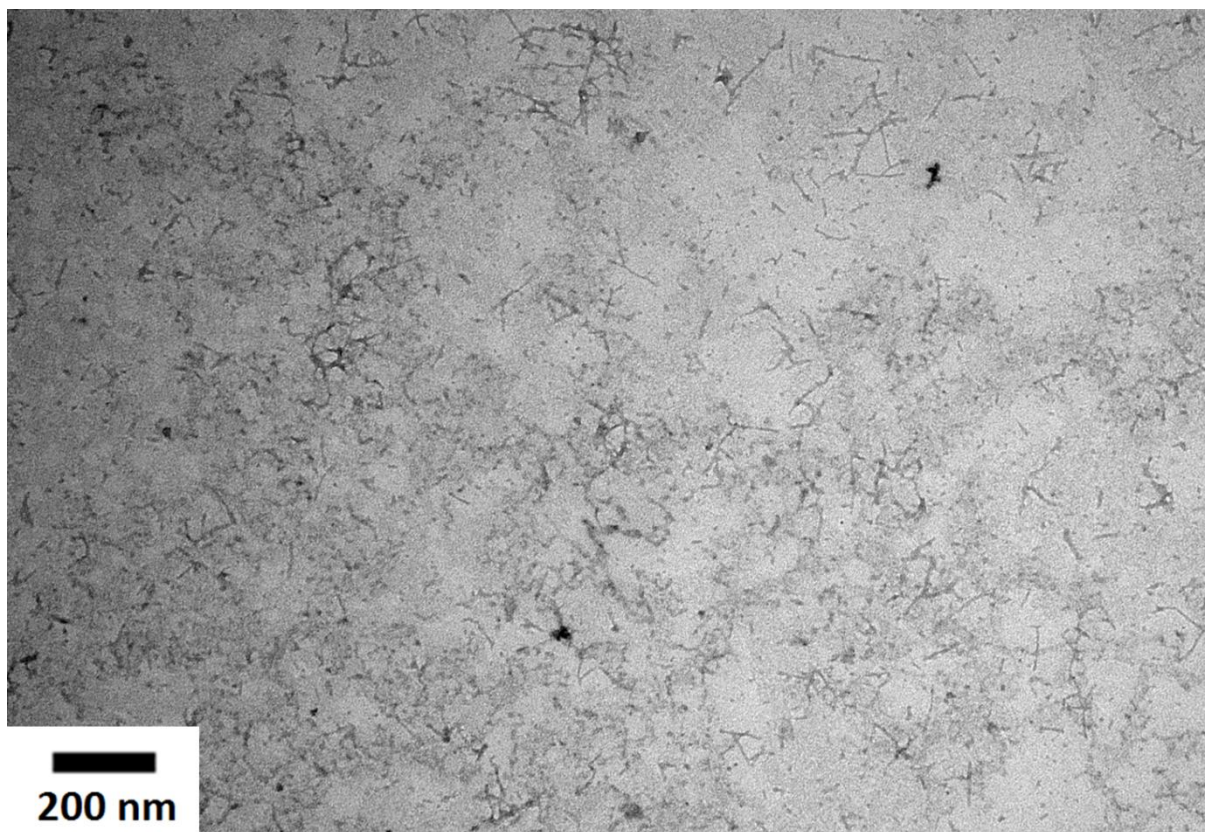


Fig. S6. TEM image of short fibers observed in **ToFT-1** sample.

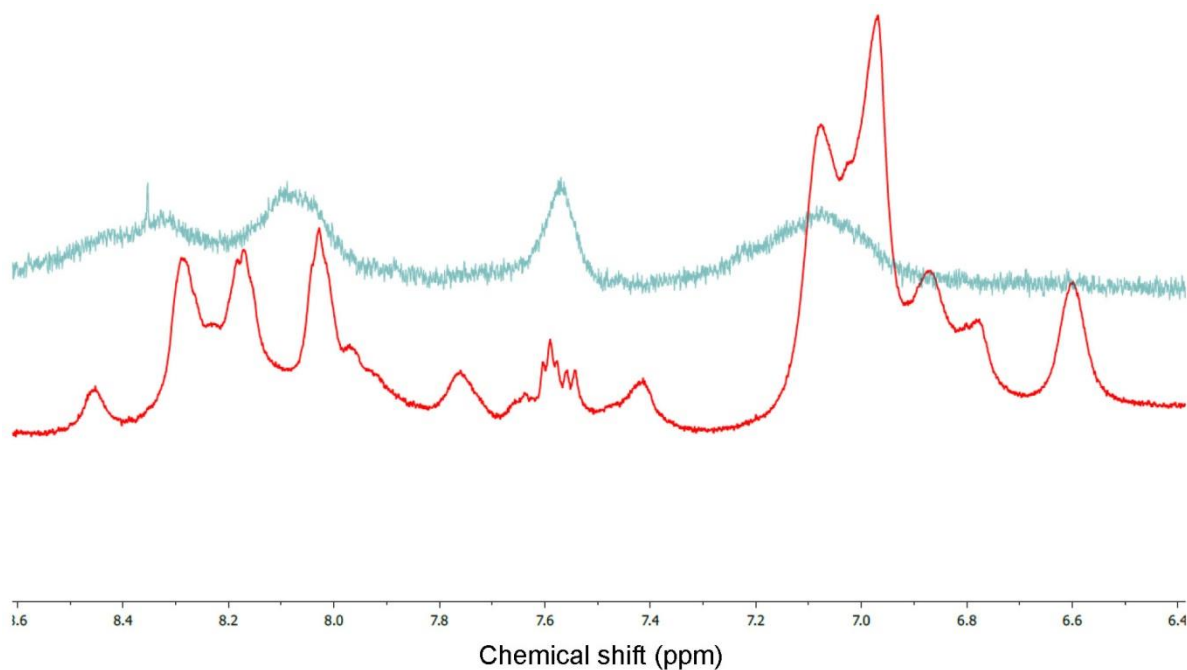


Fig. S7. ¹H NMR (amide and aromatic region) of **TofT-1** (in cyan) showing only broad peaks of peptide construct indicating an extensive aggregation. ¹H NMR of **TofT-2** (in red) shows well dispersed peaks coming from peptide construct, which indicates smaller size and high solubility of the species.

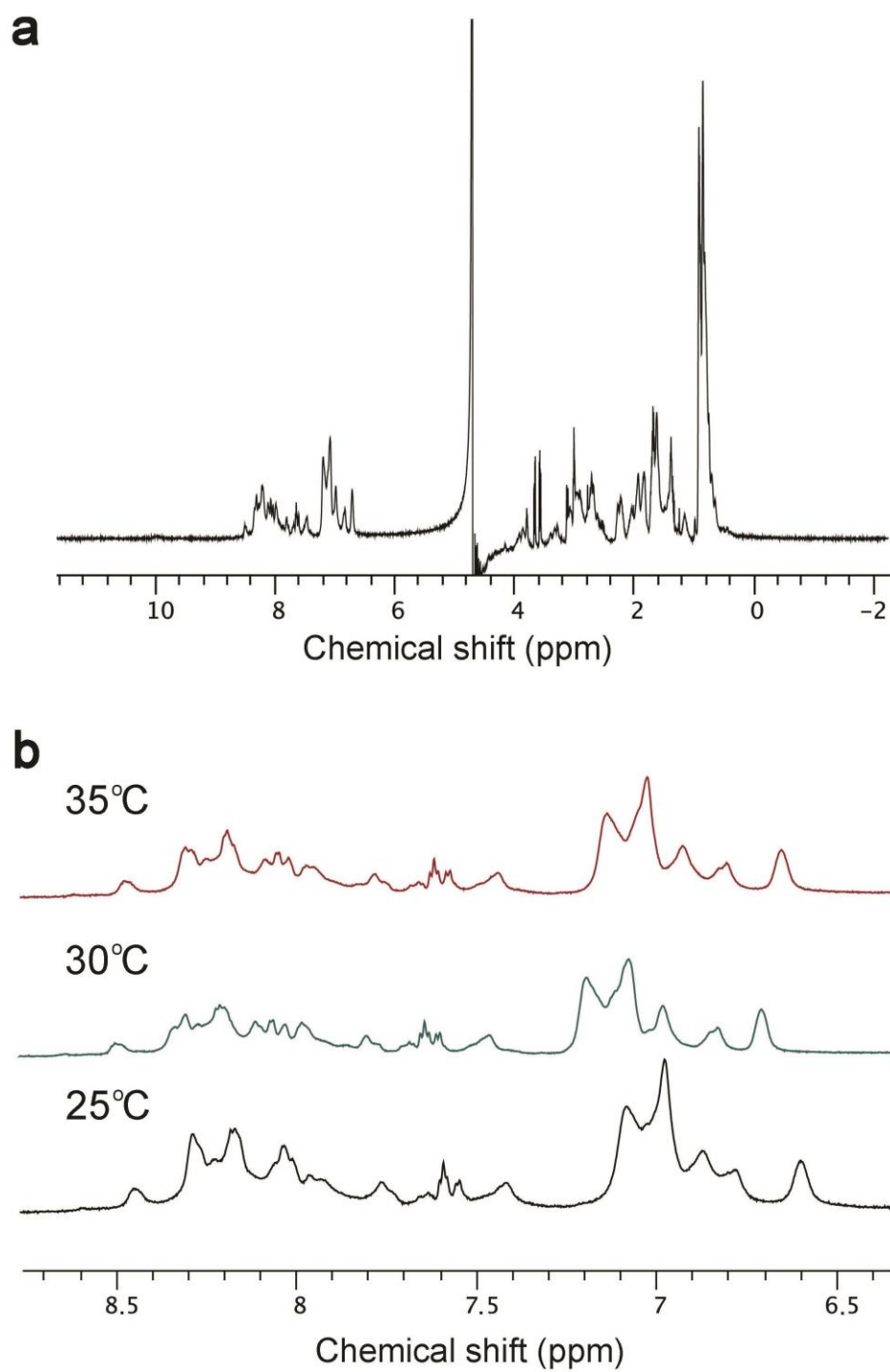


Fig. S8. a) ^1H NMR of **ToFT-3** (200 μM in 50 mM phosphate buffer, pH 7) at 35 $^\circ\text{C}$. b) Temperature dependence of amide and aromatic region of the ^1H NMR spectrum of **ToFT-3** recorded at 25, 30 and 35 $^\circ\text{C}$.

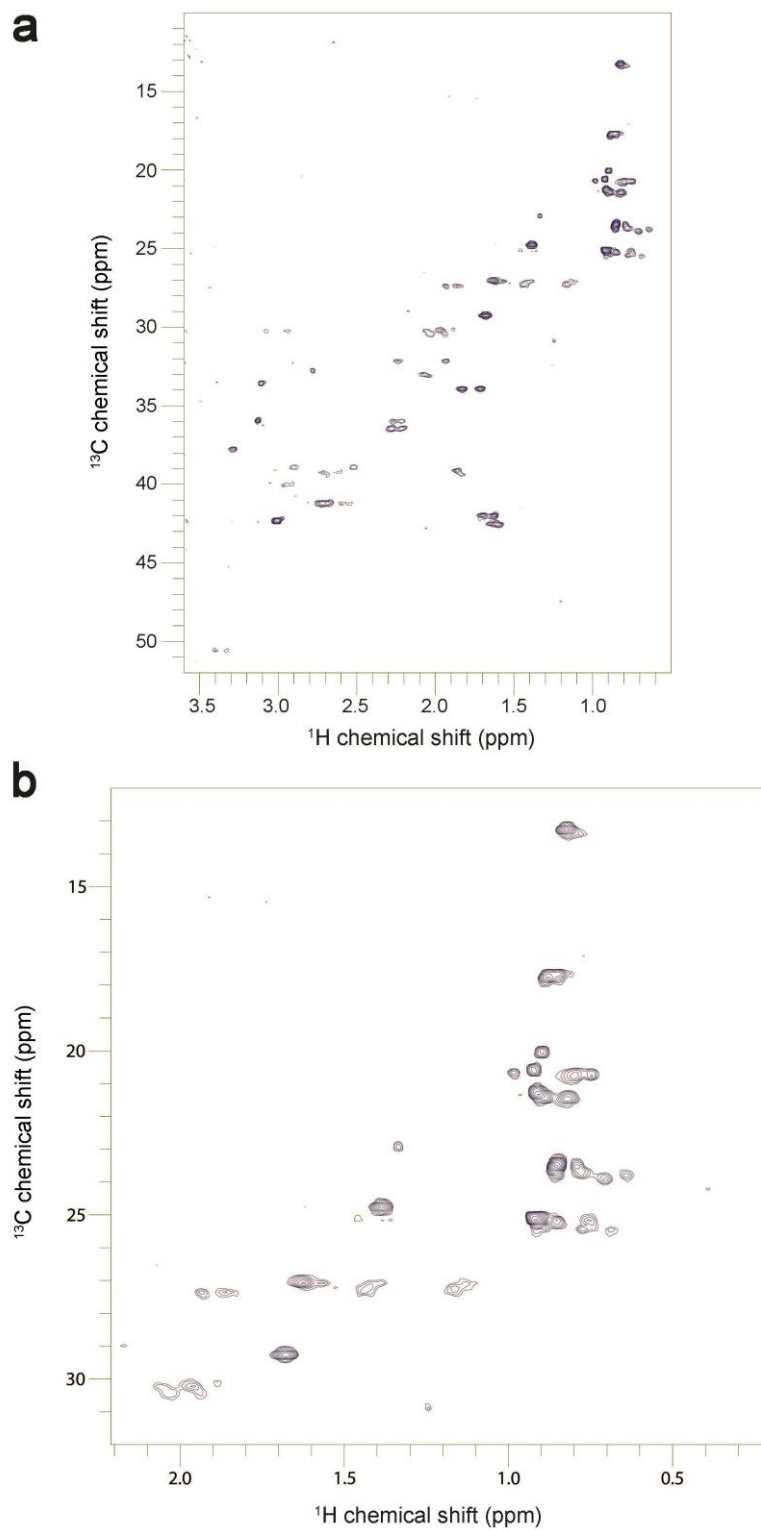


Fig. S9. a) ^1H - ^{13}C HSQC NMR spectrum of **ToFT-3**. b) Part in the spectrum that corresponds to methyl groups. Methyl resonances of different intensities suggest the presence of regions that differ in their conformational dynamics.

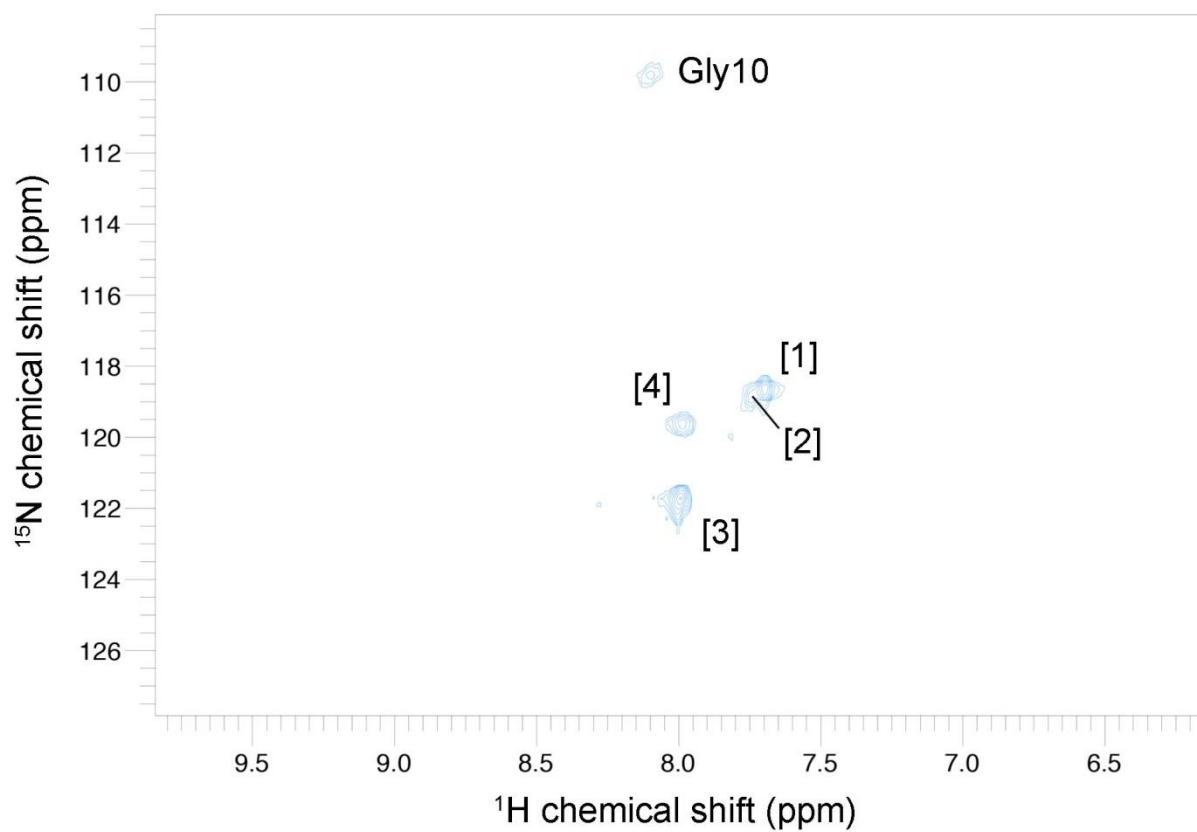
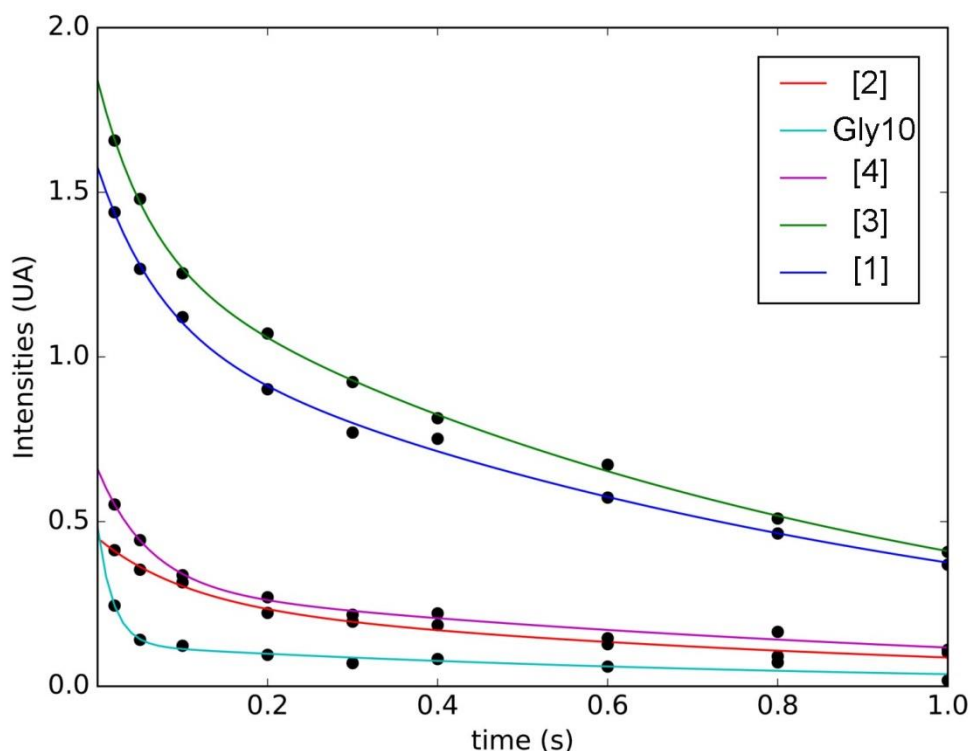
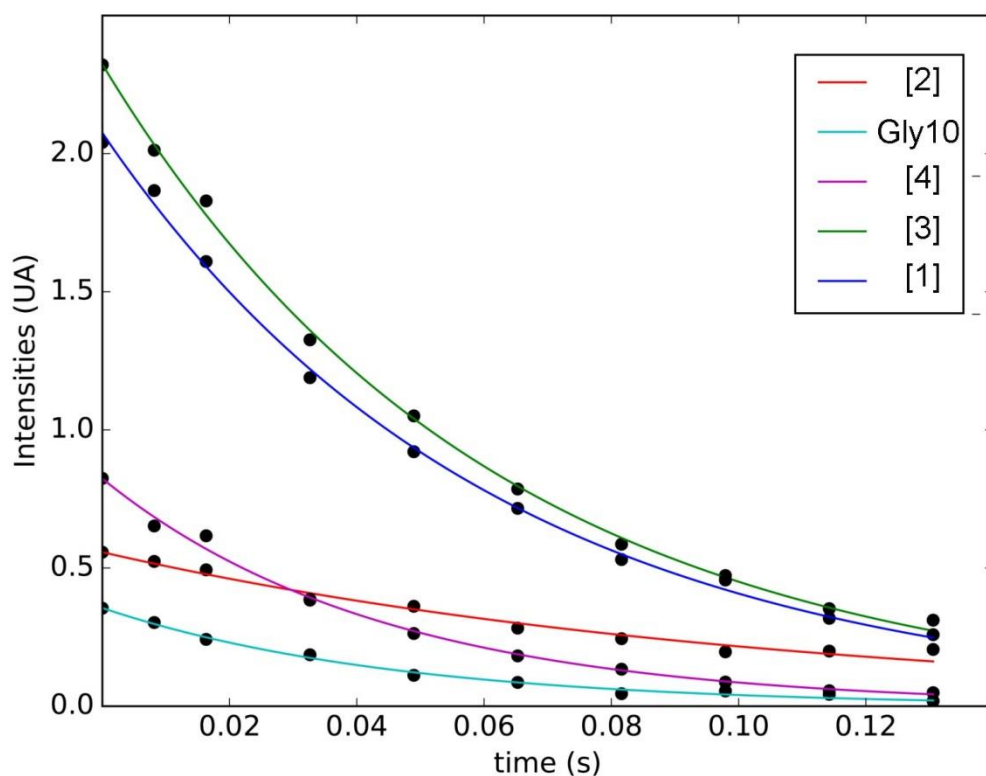


Fig. S10. ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled **ToFT-3** construct at 35 °C.



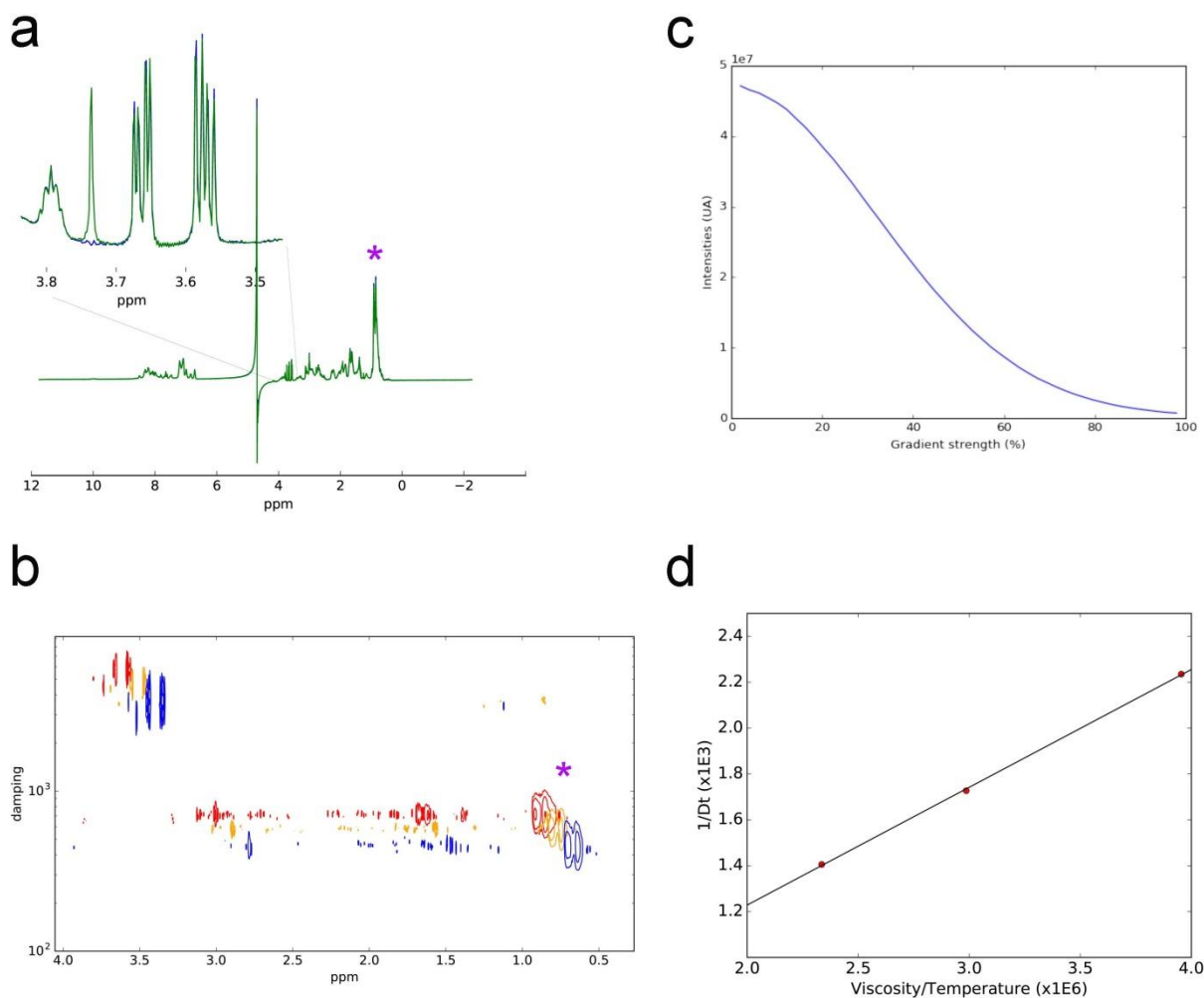
[1]	T1 69.1% :	938.7 ± 76.1 ms	T1 30.9% :	73.1 ± 19.3 ms
[2]	T1 56.6% :	934.7 ± 280.1 ms	T1 43.4% :	104.8 ± 50.0 ms
[3]	T1 71.3% :	860.7 ± 31.7 ms	T1 28.7% :	60.4 ± 9.1 ms
[4]	T1 45.5% :	1067.9 ± 223.1 ms	T1 54.5% :	59.9 ± 15.7 ms
[Gly10]	T1 26.1% :	810.1 ± 250.9 ms	T1 73.9% :	18.5 ± 10.7 ms

Fig. S11. T_1 relaxation times of **ToFT-3** measured at 35 °C. The T_1 relaxation curves display clear bi-exponential decay behavior with a contribution of a very short T_1 (in the range of 20 to 100 ms) and a contribution of a long T_1 (in the range of 1 s). The occurrence of a fast component for the T_1 relaxation suggests the presence of a supplementary relaxation mechanism, in addition to the dipolar and chemical shift anisotropy mechanisms that usually dominate the relaxation of an isolated ^{15}N . A possible explanation is the occurrence of a slow exchange between soluble **ToFT-3** and minute amounts of high molecular weight aggregates with the rate slower than the T_2 (100 ms).



[1]	T ₂	61.5 ± 1.2 ms
[2]	T ₂	105.7 ± 6.5 ms
[3]	T ₂	61.0 ± 1.1 ms
[4]	T ₂	44.2 ± 1.7 ms
[Gly10]	T ₂	46.0 ± 2.0 ms

Fig. S12. The T₂ relaxation curves displayed expected mono-exponential decays for all five resonances, including the low intensity GLY10. Most amide nitrogens display fast T₂ relaxation times between 40 and 60 ms, in agreement with homogeneous dynamic behavior of the construct. Only the peak [2] is characterized by a longer T₂, suggesting a faster correlation time for this site.



Ratios of the TRIS/**Toft-3** translational diffusion coefficients:

35 °C (308 K)	0.158
25 °C (298 K)	0.165
15 °C (288 K)	0.153

Fig. S13. DOSY measurements. a) Spectrum of **Toft-3** without (blue) and with TRIS (tris(hydroxymethyl)aminomethane) added (green). b) DOSY spectra recorded at temperatures of 15 °C (blue), 25 °C (orange) and 35 °C (red). The asterisk in purple indicates the methyl peaks used for calculation of diffusion coefficient. c) The diffusion coefficients were obtained after the Laplace transform of the DOSY spectra using the PALMA server (<https://arxiv.org/abs/1608.07055>). d) As a function of temperature, the diffusion coefficient displays the expected linear dependence when corrected to the variation of viscosity and corresponds to molecular weight of ~20 kDa.⁽⁵⁾

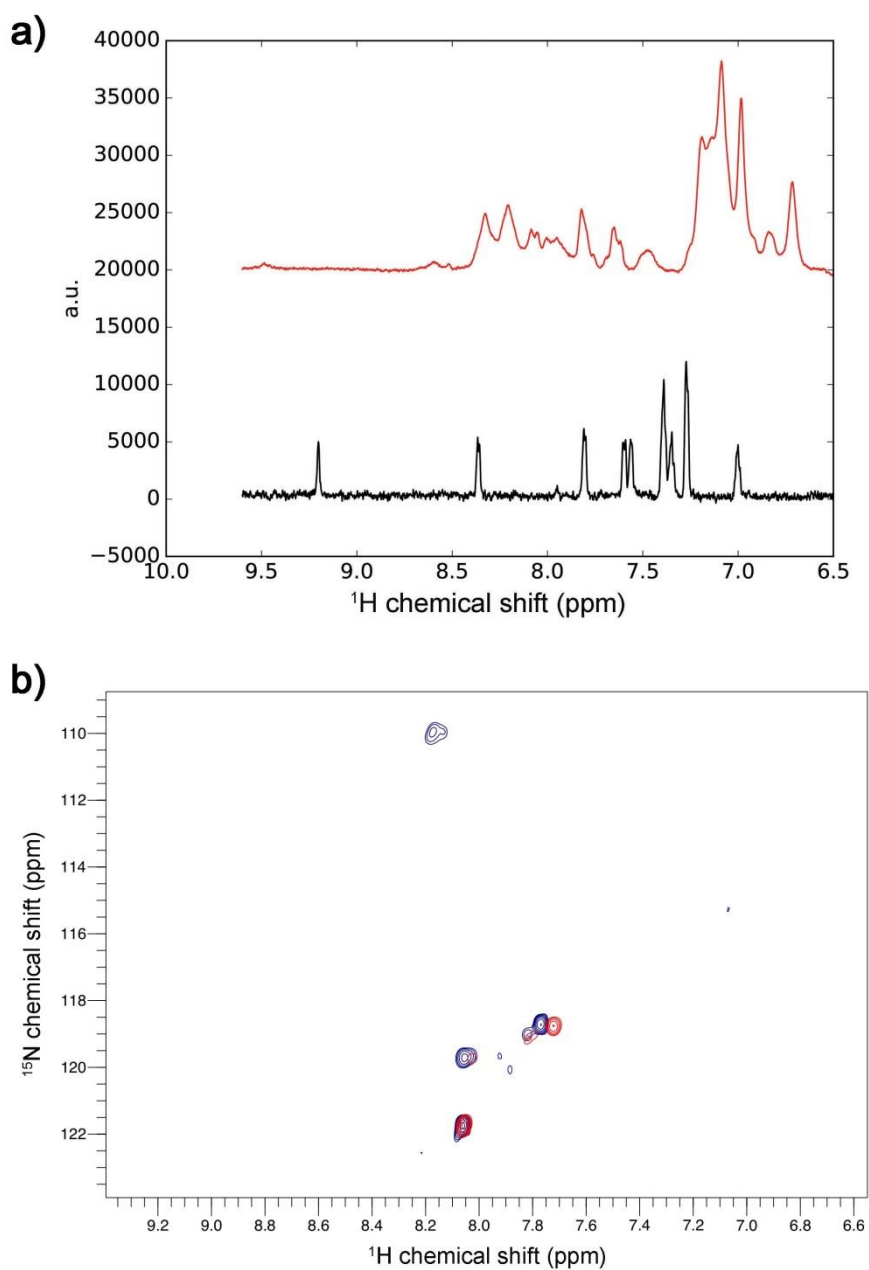


Fig. S14. Binding of bis-ANS to **ToFT-3** was directly verified by solution NMR. a) ^1H NMR spectra of bis-ANS in the presence of equimolar quantities **ToFT-3** (in red) and free bis-ANS (in black). b) Overlay of ^1H - ^{15}N HSQC spectra of **ToFT-3** (in blue) and upon addition of bis-ANS (in red).

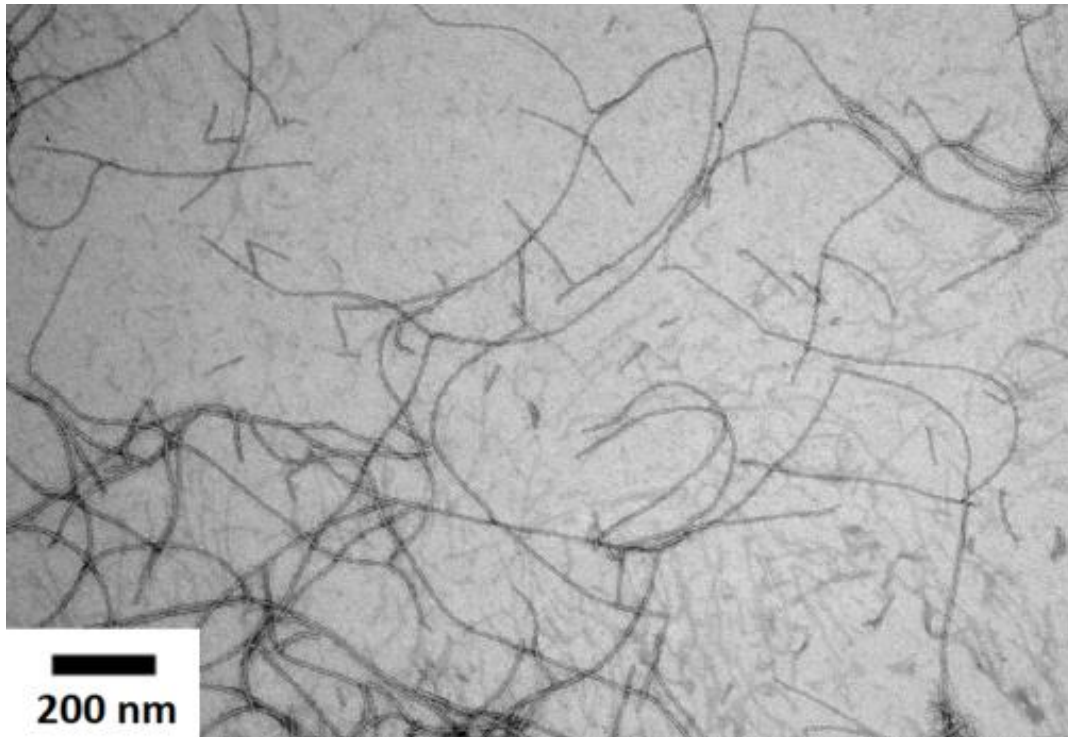


Fig. S15. TEM image of amyloid fibers obtained from central trimer **CT** have elongated morphology similar to “covalent trimer” [20-41] β 2m amyloids.⁽⁸⁾ This also indicates that the structures of the central linker (in **CT** it is different from “covalent trimer” [20-41] β 2m, see Figure S1) are not detrimental in the amyloid self-assembly mechanism.

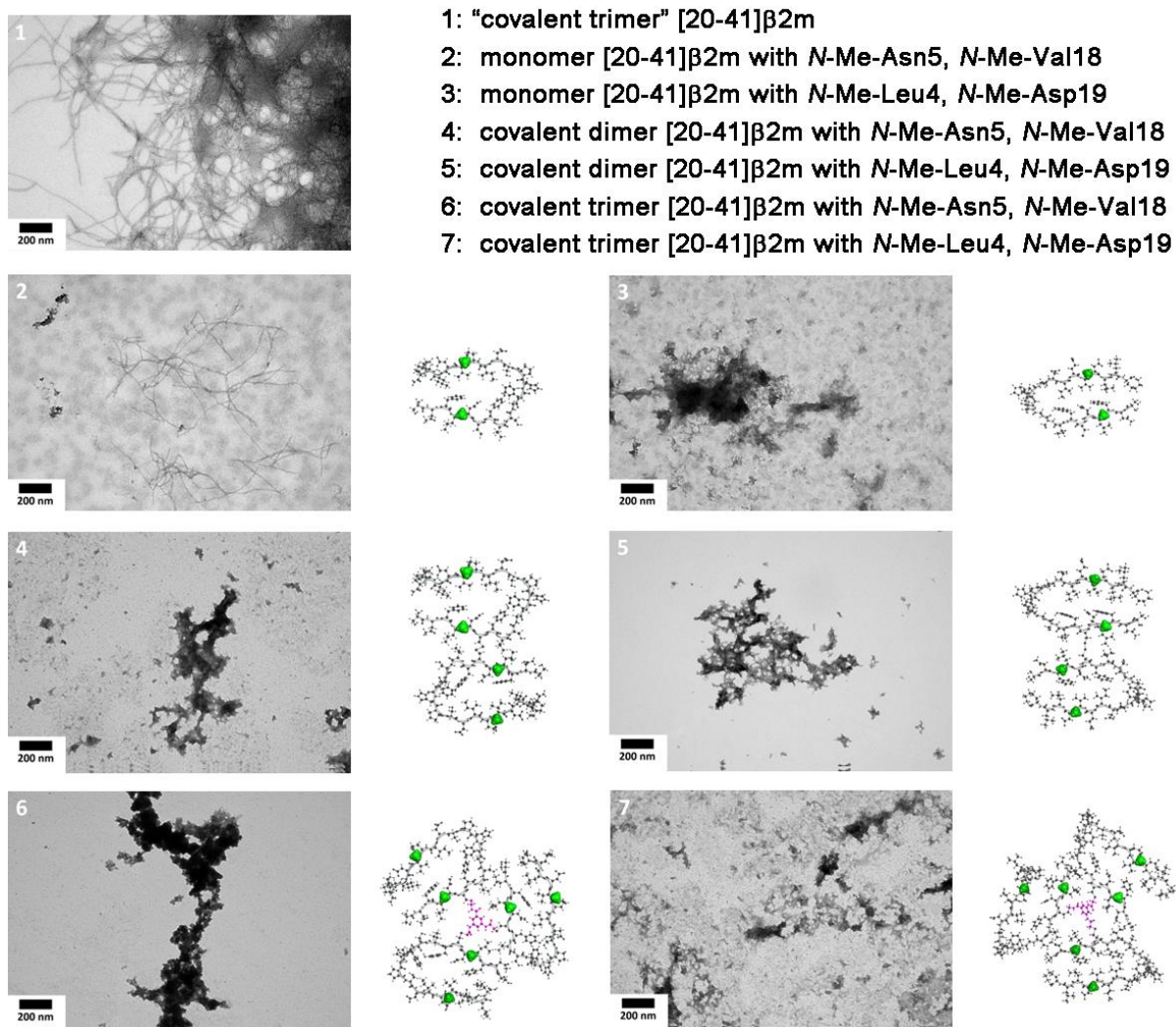


Fig. S16. Transmission electron microscopy (TEM) analysis of the aggregates obtained upon completion of the experiment described in Figure 5 in the main manuscript. *Panel 1* depicts the long fibers observed for the "covalent trimer" [20-41] β 2m (reference construct). *Panels 2-7* show morphologies of different aggregates obtained in the presence of *N*-methylated peptide inhibitors (their molecular structures are shown to the right of the corresponding TEM images). In the presence of covalent dimers and trimers, the fibrillar morphology was not observed indicating their higher potency to modify the amyloid self-assembly.

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