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Tetrahedron Letters 46 (2005) 3745–3749
Tetrahedron Letters

Enforcing aggregation in a dipeptide conjugate

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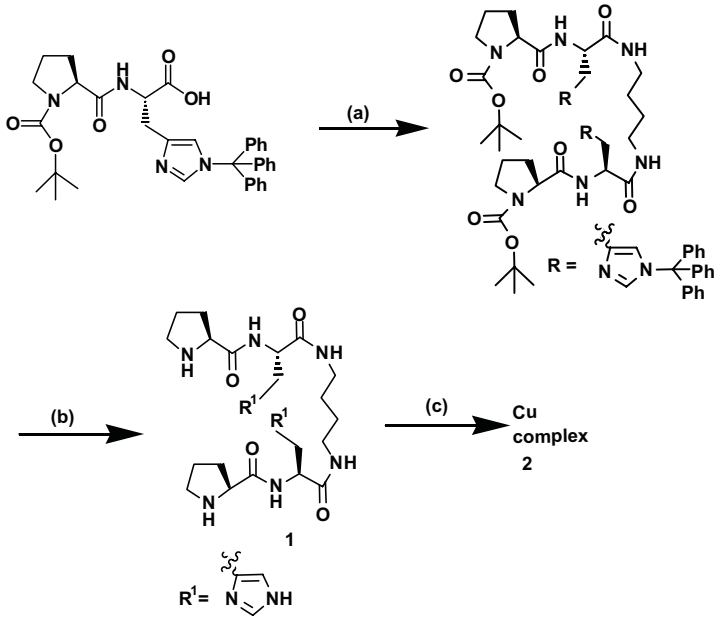
Received 23 January 2005; revised 15 March 2005; accepted 21 March 2005
Available online 6 April 2005

Abstract—A bis-dipeptide scaffold $(PH)_2$ DAB, derived from the N-terminal octa-repeats of the cellular prion protein, displayed remarkable nanoscopic self-assembly in solution. The ultrastructural details of the peptide filaments so obtained were probed by transmission electron microscopy and optical microscopy. Synthesis of the bis-dipeptide conjugate, its metalation and micrographs confirming the formation of fibrillar networks are presented.

Self-assembly is a prevalent concept in chemistry and biology, where the constituent components are invariably arranged in a 'bottom-up' approach. The foundation of assembly lies in the recruitment of nanoscopic building blocks for the construction of higher order structures through precisely directed molecular recognition.[1] Of the various natural building blocks available for such assemblies, amino acids, peptides and proteins, have received a considerable focus due to their side-chain functional diversity, potential to form hydrogen bonds and support for other non-covalent interactions.[2]



Scheme 1. Synthetic scheme for **1** and **2**: (a) HOBt, DCC, 1,4-diaminobutane, DCM, 8 h, yield 54%; (b) 95% TFA in DCM, 3 h, rt, yield 85%; (c) $Cu(OAc)_2{\cdot}H_2O$, aq. methanol.

Keywords: Peptide; Conjugate; Aggregation.
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0040-4039/$ - see front matter
doi:10.1016/j.tetlet.2005.03.145

A fascinating aspect of protein ordered assembly is observed in several neurological disorders that are caused by aggregating proteins, such as Alzheimer's disease, Huntington's diseases and prion based diseases, to name a few. For example, a conformational transition in the normal cellular prion protein (PrP^{C}) leads to its pathological isoform (PrP^{Sc}), which is known to be the causative agent for several neurodegenerative diseases.[3] The N-terminal region of PrP^{C} contains an octapeptide sequence (60–91) PHGGGWGQ, which repeats tandemly four times.[4] This octapeptide has been identified as the principal binding site for copper ions and this observation led to the proposition that the prion protein can act as a periplasmic copper transporter, as a sensor or reservoir of copper ions and can also exhibit antioxidant activity in the presence of copper ions, especially when the cells are under oxidative stress.[5]

In continuation with our studies with truncated prion peptides,[6] we wish to report filament formation by a dipeptide conjugate derived from the octa-repeat sequence. Solution phase methods were used to synthesize the protected dipeptide and it was tethered to 1,4-diaminobutane resulting in a bis conjugate, which was deprotected to yield the conjugate **1** (Scheme 1).† Conjugate **1** was subjected to FPLC and ESI-MS analysis to confirm its purity (Fig. 1).

Circular dichroism (CD) is a powerful tool in measuring changes in the solution conformations of peptides and proteins. CD studies with conjugate **1** were performed in 50% aqueous trifluoroethanol (TFE). A freshly prepared solution of **1** (0.3 mM) displayed a random coil-like behavior, while aging the same sample for 30 days resulted in a major shift in the CD spectra indicating a more self-organized assembly as shown by a decrease in the CD signal observed at 196 nm (Fig. 2).‡

Encouraged by the shift in the CD signal between fresh and aged sample, we decided to elucidate the self-aggregational behavior of conjugate **1** via microscopic studies. Aggregation of **1** via self-assembly was evident on the basis of extensive fibril formation as probed by various microscopic techniques viz., transmission electron microscopy (TEM)§ and optical microscopy (OM).¶ TEM images of **1**, after 30 days aging, revealed the formation of fibrils having lengths of 25–40 nm range (Fig. 3a) and the extent of the fibrillation was high as such networks were observed in various grid regions. We were able to observe a meshed structure (Fig. 3a) as well as distinct single fibers as shown in Figure 3c. An optical microscopy technique was used as a tool to probe binding of a dye to an aged, fibrillar assembly of **1**. As discussed for amyloidogenic proteins, Congo red binding led to the detection of green birefringence, with a possibility of an amyloid nature associated with the ordered peptide aggregate obtained from **1**.[7] Similar observations have been reported for other synthetic models of aggregating peptides.[8–10] Control experiments with fresh samples of **1** did not reveal any fibril formation.

We have been interested in constructing copper-binding peptide scaffolds based on naturally occurring motifs.[11] In continuing these endeavors, we decided to study the copper coordination ability of **1** as this dipeptide has drawn inspiration from copper-binding prion octa-repeat. Copper metalation of **1** was achieved, its purity

† *Synthesis of 1*. To pre-cooled solution of Boc-Pro-N^{im}(trityl)histidine (3.35 g, 5.6 mmol) in dichloromethane (55 mL), 1-hydroxybenzotriazole (0.76 g, 5.6 mmol) followed by *N*,*N*′-dicyclohexylcarbodiimide (1.16 g, 5.6 mmol) in dichloromethane (1 mL) was added drop-wise. Stirring was continued for 15 min at 0 °C and then for 40 min at room temperature. Then 1,4-diaminobutane (0.28 mL, 2.8 mmol) in dichloromethane (0.5 mL) was added drop-wise and stirring was continued for 15 h at room temperature. After this time, the reaction mixture was filtered and the dichloromethane solution was washed with 2 N HCl (3 × 20 mL) followed by 10% sodium bicarbonate (3 × 20 mL) and finally with brine solution (1 × 50 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated. The crude compound was purified through silica gel column chromatography using dichloromethane and methanol (92:8) solvent system to give pure protected bis-dipeptide (2.16 g, 1.7 mmol, yield 62%, R_f [6.5% methanol in dichloromethane] = 0.77, mp 136–138 °C, $[\alpha]_D^{25}$ −30 [*c* 1, methanol]. The pure compound obtained (2.72 g, 2.2 mmol) was treated with 95% TFA in DCM for 2 h and the solvent evaporated under reduced pressure and triturated with ether. The resulted crude bis conjugate was dissolved in 50% aqueous methanol and passed through a strong anion exchange resin. The resin was washed with 50% aqueous methanol and the washings were concentrated in vacuo. The crude peptide was dissolved in isopropanol and reprecipitated by adding diethyl ether. Reprecipitation was repeated three times to give pure compound (0.88 g, 1.6 mmol, yield 72%, R_f [methanol/acetic acid/water, 1/0.25/0.42] = 0.45, mp 103–105 °C, $[\alpha]_D^{25}$ −25 [*c* 1, methanol]). Purity of **1** was checked by analytical FPLC (Akta Basic, Amersham Pharmacia) using a μRPC C2/C18 ST4.6/100 column with an applied gradient of 0.1% trifluoroacetic acid in water (**A**) to 0.05% trifluoroacetic acid in acetonitrile (**B**) (0–100% **B** in 30 min). Concentration of conjugate **1** for an analytical run was 1 mg/mL. Similar gradient was also applied to determine the purity of metalated conjugate **2**. Spectral features of **1**: ^{1}H NMR (400 MHz, D_2O, δ ppm): 1.12 (br s, 4 H, linker's –CH_2); 1.49–1.57 (m, 6 H, Pro γ, β H); 1.93–1.98 (m, 2 H, Pro β H); 2.74 (br d, 4 H, Pro δ H); 2.82–2.92 (m, 8 H, overlap signals for linker's –CH_2NH and His β H); 3.56 (br s, 2 H, pro α H); 4.34 (t, *J* = 8 Hz, 2 H, His α H); 6.80 (s, 2 H, His ring's H); 7.53 (s, 2 H, His ring's H). ^{13}C NMR (100 MHz, D_2O, δ ppm): 24.4, 26.3, 27.1, 30.5, 39.6, 47.2, 54.1, 60.2, 117.9, 128.9, 134.4, 170.2, 171.8. FT-IR (KBr, cm^{-1}) 1522 (amide II, –NH def); 1659 (amide I, –C=O str); 3320 (–NH str). FAB (M+1): 557, ESI-MS (M+1): 557.

‡ *CD studies*. **1** (0.3 mM as final concentration in 50% aqueous TFE) was used fresh and after 30 days of incubation at 30 °C. Far-UV CD measurements were performed with JASCO spectropolarimeter (J-810 Model), which is monitored by J-810 program running on Windows 95/NT. The curves were smoothened using Microcal Origin 6 software with a curve-fitting program. 0.1 cm path length cuvettes were used for this purpose. The measurements were taken at 0.5 nm wavelength intervals, 1 nm spectral bandwidth and five sequential scans were recorded for each sample.

§ *Transmission electron microscopy*. 30 days aged solutions of **1** and **2** (8 μL, 1 mM) were transferred onto Formvar coated TEM grids and dried. Grids were stained with 2% uranyl acetate, dried and examined under a JEOL 2000FXII electron microscope, at an operating voltage of 100 kV.

¶ *Optical microscopy*. Congo Red solution (2 μL, 150 mM) was added to 30 day aged solutions of **1** and **2** (98 μL, 1 mM) and the mixture was left for 6 h at room temperature. 50 μL of this solution were transferred on to a glass slide, dried and then viewed with an optical microscope (LaboMed, Digi 3) with cross-polarized light (100×), interfaced with a PC. Images were obtained by using a digital camera (Digi 3 1500) and processed by using Digipro V 2.0 software.

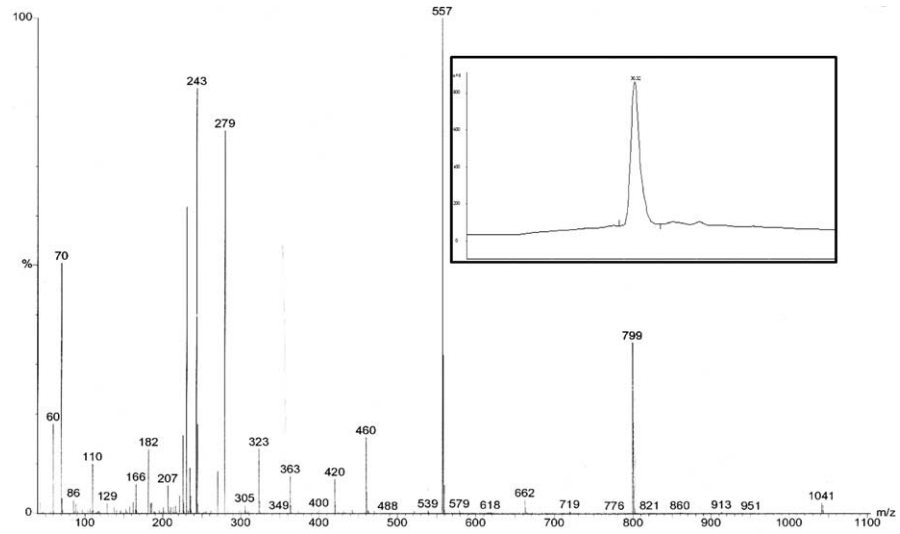

Figure 1. The ESI mass spectrum of **1** showing the molecular ion peak (M+1, 557) and the FPLC trace of pure **1** (inset).

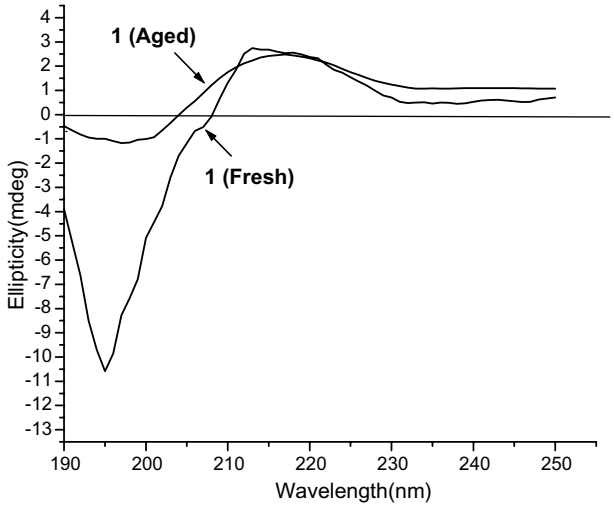

Figure 2. CD spectra of conjugate **1** as fresh and aged solutions.

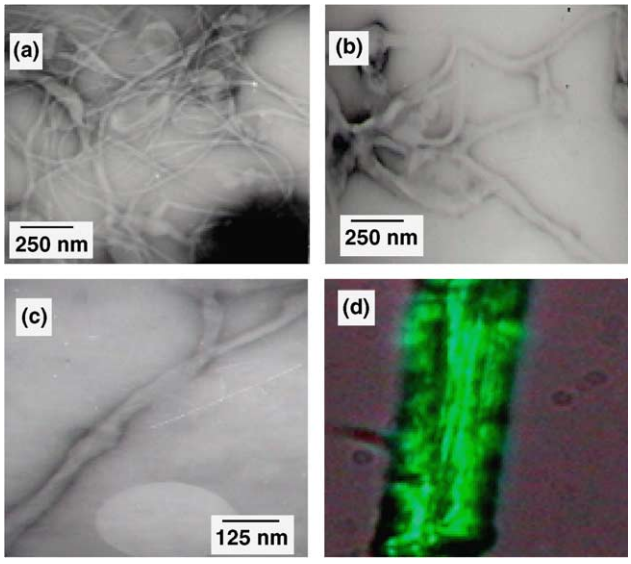

Figure 3. TEM images of **1** (a–c) and Congo red birefringence after 30 days incubation in 50% aqueous TFE (d).

confirmed by FPLC analysis, atomic absorption analysis and ESI-MS data, which suggested that two copper ions were bound per bis-dipeptide (Fig. 4).[‖] Complex **2** exhibited a λ_{max} at 590 nm with an extinction coefficient of 116 M/cm, which is similar to the reported spectroscopic data for copper complexes, with d–d transitions occurring at 597 nm.

An EPR evaluation of **2** resulted in the following spectroscopic data: $g_{\|} = 2.225$ and $A_{\|} = 192$, which was found to be similar to the literature data of $g_{\|} = 2.23$ and $A_{\|} = 187$ G, signifying a 3-N species (NH, N^-, N^{im}) binding mode.[12] Owing to the similarity of the values, participation of two peptide backbone nitrogens and an imidazole nitrogen can be predicted to be the main atoms stabilizing copper binding in **2**. A shift towards a lower frequency was observed for the amide I band (–C=O str vibrations weakly coupled with C–N str and in-plane N–H bending), while a shift towards a higher frequency number was evident for the amide II band (in-place N–H bending strongly coupled with C–N str), in the IR spectrum of **2** (data not shown). In the absence of a crystal structure, the spectroscopic data suggest that all the amide nitrogens were not deprotonated before participating in copper coordination. A

[‖] *Synthesis of 2*. The bis-peptide conjugate was treated with 2 equivalents of copper acetate monohydrate in 50% aqueous methanol for 6 h at room temperature. After this time, the solvent was evaporated under reduced pressure. The residue was washed with hot 1,4-dioxane, acetone and then dried in vacuo. Metalated peptide conjugates were characterized through EPR, UV–Visible spectra. The amount of copper present in the metalated conjugate **2** as determined by atomic absorption spectroscopic measurements was 0.14 g/g of bis-peptide, which corresponded to two copper atoms in **2**. ESI-MS analysis also displayed an (M+2) peak at 921.

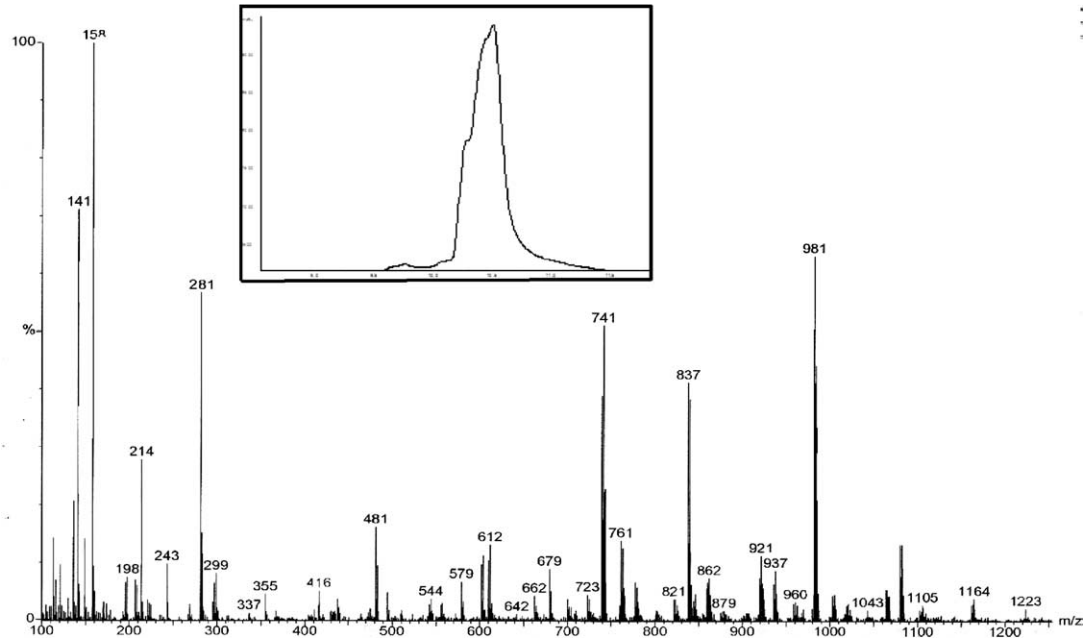

Figure 4. The ESI mass spectrum of 2 showing an (M+2) molecular ion peak and the FPLC trace of pure 2 (inset).

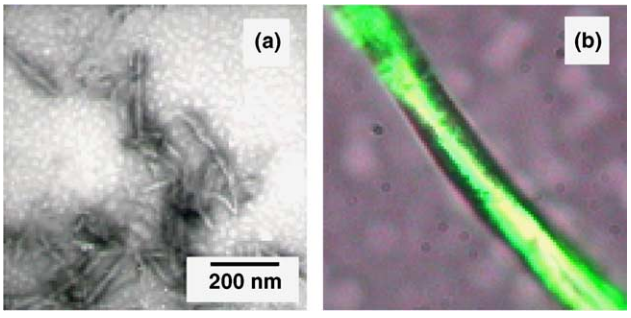

Figure 5. (a) TEM and (b) OM images of **2** after 30 days incubation in water.

crystal structure is needed, however, for a precise comment concerning the exact copper-binding mode in **2**.

We focused our attention on the ability of the metalated conjugate **2** to form fibrils. Due to the limited solubility of **2** in aqueous trifluoroethanol, the metalated peptide was aged in water for 30 days and TEM and OM images were recorded for the aged conjugate. Electron micrographs revealed nanorod-like structures with a mean length of 200 nm and a cross-sectional diameter of 25 nm, while the fibers displayed green birefringence in bright-field OM when stained with the Congo red dye (Fig. 5). This distinct change in the morphology of fibers of **2** suggests an alternative self-assembly and ordering driven by contributions from metal ion coordination as well. Further experiments are being conducted to understand clearly the coordination behavior of metal ions in aggregates and will form the basis of future reports.

Interestingly, the Pro-His and Boc-protected Pro-His dipeptides failed to aggregate in solution despite being aged at least 30 days or more. This clearly shows that the presence of a hydrophobic and bulky t-Boc group alone is not sufficient in enforcing aggregation in this dipeptide, instead an increase in hydrogen bonding interactions present in the extended bis conjugate structure, together with marginal hydrophobic contributions from the butane linker, enforce aggregation in **1** and **2**. These results will be of interest while formulating design paradigms for self-assembling peptides and may lead to the generation of novel peptide-based architectures and materials.

Acknowledgements

C.M. and K.K.P. would like to thank CSIR for research fellowships.

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