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A synthetic ditryptophan conjugate that rescues bacteria from mercury toxicity through complexation

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ABSTRACT

The synthesis of ditryptophan–pyridine conjugates and their binding to mercury ions is described. Conjugate **3** shows an excellent ability to sequester mercury from solution and rescue bacterial growth in a concentration-dependent survival assay. It is proposed that such compounds, composed primarily of bioessential/biodegradable components, could be potentially used as sequestrating agents for the removal of Hg(II) ions in detoxification strategies.

Mercury is a highly toxic metal that has serious environmental and biological consequences.[1] Mercury accumulation in higher animals occurs in the form of methylmercury. Its uptake may lead to chronic nervous system defects, oxidative stress, and eventual kidney failure in mammals.[2] Detection and detoxification of mercury remains a challenge due to such health related concerns. In this regard, the use of fluorescent probes is an attractive strategy for facile mercury detection,[3] as opposed to other analytical techniques such as atomic absorption spectroscopy, induced coupled-plasma spectroscopy, and anodic stripping voltammetry, to name a few.[4]

The fluorescence property of tryptophan is well characterized and documented among the naturally occurring amino acids.[5] The interaction of heavy metal ions with tryptophan in peptide and proteins results in quenching—a property generally exploited for heavy metal detection.[6–8] However, selective and specific mercury sensing entities involving tryptophan moieties have seldom been explored.[9] We decided to explore mercury–tryptophan interaction toward Hg(II) ion sensing by fluorescence method, with an eventual goal of testing the efficacy of our approach in cell culture.

Three different tryptophan–pyridine conjugates (Fig. 1) were synthesized and their interaction with Hg(II) ions studied. The design of compounds **1**–**3** involved the conjugation of Trp or Trp–Trp with 2,6-pyridine dicarboxylic acid or 2-picolinic acid by using standard synthetic procedures (Scheme 1).[10]

All conjugates (10 μM) displayed a characteristic tryptophan emission band at 355 nm on excitation at 280 nm, in 1:1 methanol–water solvent. The inherent fluorescence of **1** and **2** was quite low as compared to **3** (Fig. 2a). This observation can perhaps be correlated to the presence of additional tryptophan units in **3**, compared to conjugates **1** and **2**. Therefore, we chose to work with

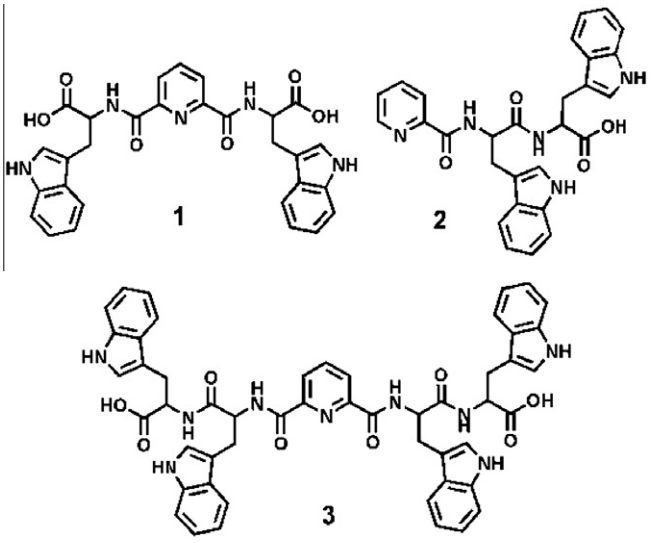

Figure 1. Pyridine–tryptophan conjugates, **1**–**3**.

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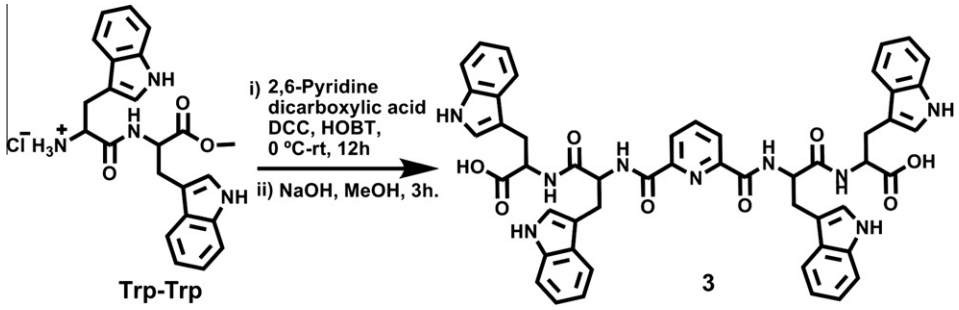

Scheme 1. Synthetic route for Trp–Trp conjugate **3**.

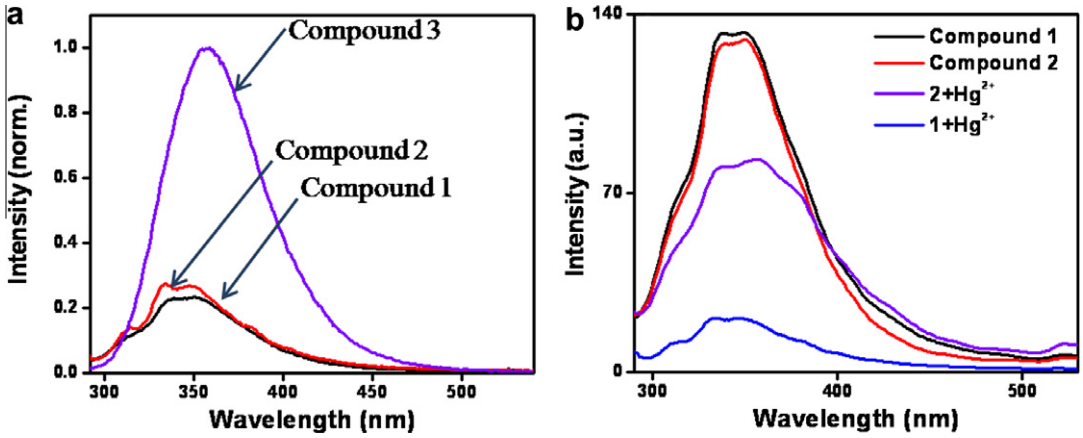

Figure 2. (a) Fluorescence spectra of **1**–**3** (10^{-5} M) and (b) fluorescence responses of **1**–**2** (1×10^{-5} M) with Hg^{2+} (10^{-4} M) in 1:1 methanol–water with an excitation at 280 nm.

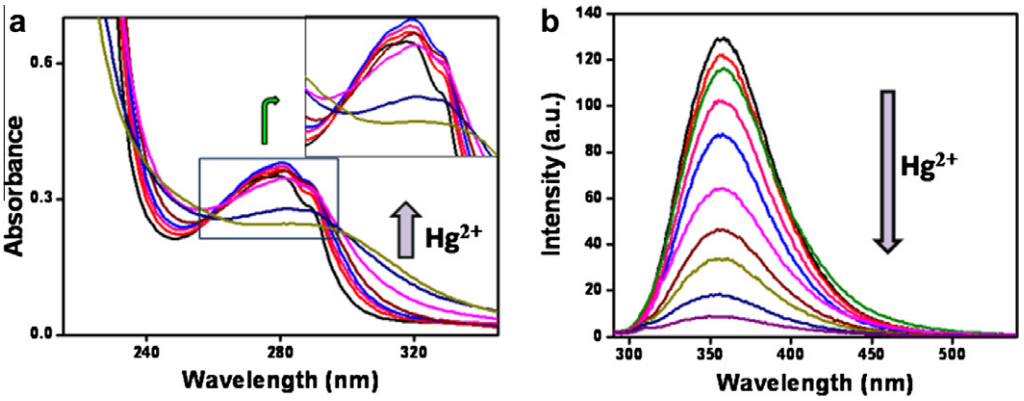

Figure 3. (a) Absorbance spectra of compound **3** (10^{-5} M) in 1:1 methanol–water in the presence of 0.5, 0.8, 1, 2, 3, 4 and 8 equiv Hg^{2+} ions. (b) Fluorescence spectra of compound **3** (1×10^{-5} M) in 1:1 methanol–water in the presence of various concentrations of Hg^{2+} (0, 5, 10, 20, 30, 40, 50, 60, 70, and 80 μM) with the excitation at 280 nm.

3 as it was expected to exhibit better sensitivity toward the detection of Hg^{2+}. We also tested the response of compounds **1** and **2** toward different metal ions (see Supplementary data). While the conjugates display good selectivity toward Hg^{2+}, as seen in Figure 2b, the extent of quenching is noticeably higher with **1** than **2** with identical concentrations of the metal ion. This implies that, of the compounds studied, 2,6-pyridine dicarboxylic acid scaffold is critical.

Figure 3a illustrates the change in absorption spectrum of **3** with increasing mercuric ion concentration. The absorbance reaches a maximum after the addition of one equivalent Hg^{2+}, and further incremental increase of Hg^{2+} concentration leads to a sharp decrease in the absorption maxima, and a red shift of the associated λ_{max} values. The literature reports confirmed a change in tryptophan absorbance upon complexation with Hg^{2+}.[8b] In accordance with the available data, the appearance of two isosbestic points, one associated with the each band, when **3** is treated with more than one equivalent of Hg^{2+}, clearly indicates direct associative interaction between the indole unit and Hg^{2+}. The appearance of charge transfer character on complexation and their steady increase with increasing Hg^{2+} concentration indicates a long range interaction.[11] Figure 3b shows the decrease in fluorescence emission intensity upon the addition of increasing amount of mercuric ions.

The fluorescence decay time of the complexes was measured to probe the quenching mechanism. It is known that decay time of

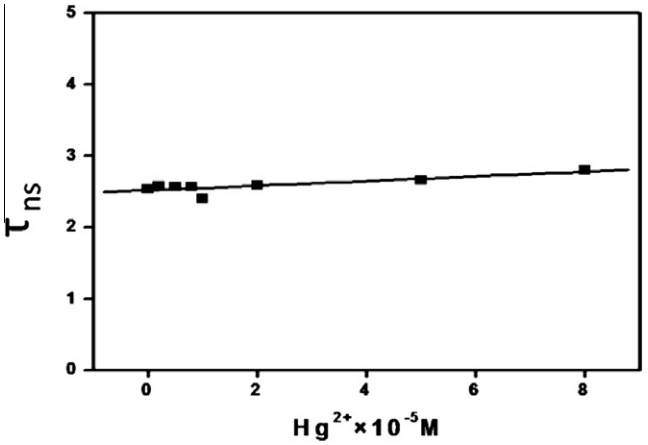

Figure 4. Fluorescence lifetime (τ) of compound **3** (1×10^{-5} M) with different Hg^{2+} concentrations.

the fluorophore remains constant in static quenching on increasing the quencher concentration but a decrease in decay time is noticed for dynamic quenching.[12] Figure 4 shows efforts to determine the decay time of **3** in the presence of increasing Hg^{2+} concentration. Compound **3** was found to possess a lifetime of 2.54 ns. No significant change in the decay time was observed on increasing the Hg^{2+} concentration, suggesting involvement of non-fluorescent ground states in the quenching process. The bimolecular quenching constant was estimated to be 9.62×10^{12} M^{-1} s^{-1}, which is in good agreement with the static quenching process (calculated from Fig. 3b). The binding constant was also determined from emission spectra using Benesei–Hildebrand equation and found to be 2.48×10^3 M^{-1}. The regeneration of fluorescence intensity of **3** in the presence of excess EDTA proves the reversibility of this system (see Supplementary data).

Excellent selectivity of **3** toward Hg^{2+} ions was demonstrated by conducting competitive experiments in the presence of different ions (see Supplementary data). It was further determined that a detection limit of 200 nM could be achieved for Hg^{2+} ions using **3** as a fluorescent sensor (see Supplementary data).[13]

We have worked on covalent linking strategies to maximize the non-covalent interactions in self-assembling of peptides and peptide-conjugates, thus providing an expeditious entry to the generation of peptide-based soft structures.[14] As reported earlier, ditryptophan units exhibit propensity to self-organize giving rise to nanoscopic architectures.[14] Thus, we decided to study the pattern of self-assembly of **3** in aqueous methanol (75:25). Instantaneous formation of nanoscale spherical structures was observed (Fig. 5a). These soft structures were co-incubated with Hg^{2+} ions in 1:1 molar ratio. The SEM micrographs of the latter incubation did not show any appreciable change in their morphology when imaged immediately after Hg^{2+} addition (Fig. 5a), but aging for longer periods of time afforded fusion of soft structures (Fig. 5b and c; shown by white arrow).

Lehn and co-workers have reported metal-induced fusion process of egg phosphatidylcholine vesicles when modified with metal coordinating lipoligand containing a 2,2′-bipyridine unit.[15] Similarly, fusion phenomenon observed for **3** may also involve stacking of individual vesicles assisted by metal ions. Interestingly, still higher concentrations of mercury (10 equiv) exhibited complete disruption of the spherical morphology (Fig. 5d). This outcome could be attributed to the interaction of tryptophan indole units

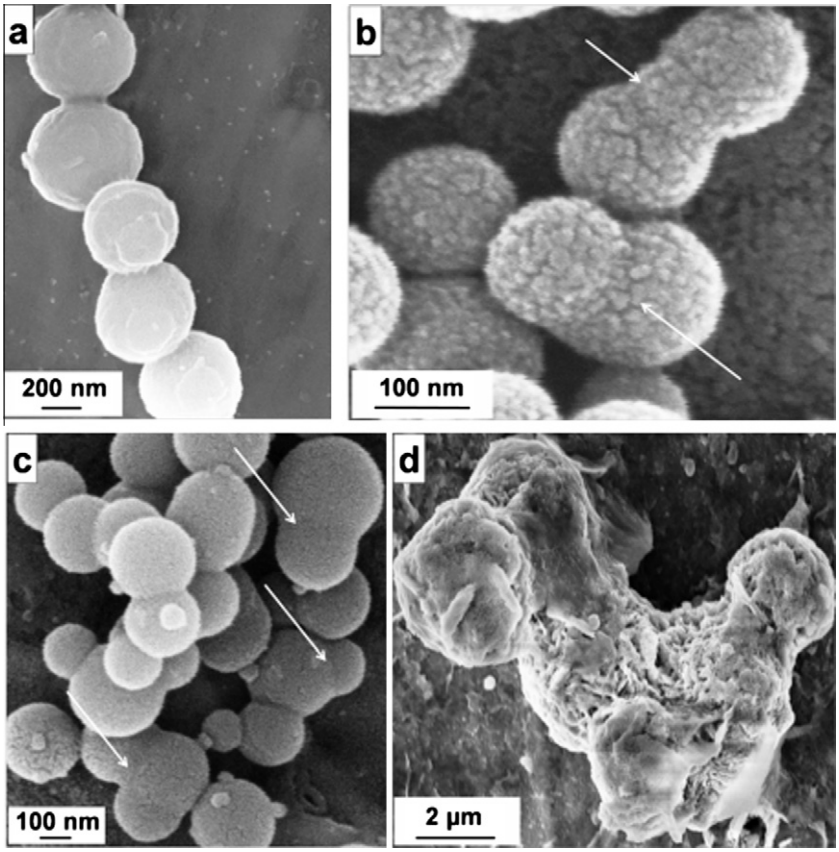

Figure 5. (a) Spherical morphology of compound **3**, in the presence of 1 equiv of mercury ion in fresh condition. (b) After 48 h aging. (c) After 20 days of aging. (d) SEM micrograph of compound **3** upon addition of 10 equiv of mercury ion.

with mercuric ions, thereby interfering with aromatic residue, a property that was previously reported.[16]

Mercury, its ionic forms and organomercurials are well known for extreme toxicity and pose a considerable hazard to the human life.[17] Nephrotoxicity, neurotoxicity, inhibition of protein synthesis, interference with microtubule assembly, increase of intracellular calcium levels, and oxidative stress are some of the manifestations of mercury toxicity. Recently, several reports have described that the exposure to mercury could result in brain and neuromotor dysfunctions similar to autism.[18] There are also reports suggesting the questionable role of an organomercurial, thimerosal, in therapeutic immunization and the possible subsequent development of autism.[19] Thus, small molecules, which are able to sequester or chelate mercury, could offer detoxification in selected conditions. A recent report has demonstrated this possibility by mimicking mercury–metallothionein interaction.[20]

We evaluated whether **3**-Hg^{2+} interaction could be used to rescue living cells from potential mercury toxicity. *E. coli* DH5α cells were employed for this study and their growth was followed in the presence of different Hg^{2+} concentrations that ranged from 0 to 2 μM. It was found that the lag phase of the cell culture became longer as Hg^{2+} concentration was increased until it became toxic for the cells at 2 μM, where growth was completely inhibited. The Hg^{2+} concentration was finally optimized at 1.8 μM and the cell growth was further monitored in the absence or presence of 10, 20, and 40 μM concentration of **3**. The addition of **3** showed a concentration-dependent survival of bacterial cells suggesting an efficient rescue by complexation of Hg^{2+} ions by **3** in the culture medium (Fig. 6).

In control experiments, bacterial cell culture was exposed to a pre-formed complex of **3** with Hg(II) ions and the viability of the cell culture was monitored for 25 h. It was found that the complex had no effect on cell viability and it was identical to what was observed for the culture alone or in the presence of **3** alone. It could be deduced from the experiments that Hg(II) did not leach into the solution from the complex over an extended period of time, which reflects favorably on the stability of this complex, and moreover points to the fact that **3** alone is benign toward the growth of bacterial cell culture (Fig. 7).

In conclusion, we have demonstrated that a pyridine–ditryptophan conjugate can efficiently bind Hg^{2+} ions in aqueous medium.

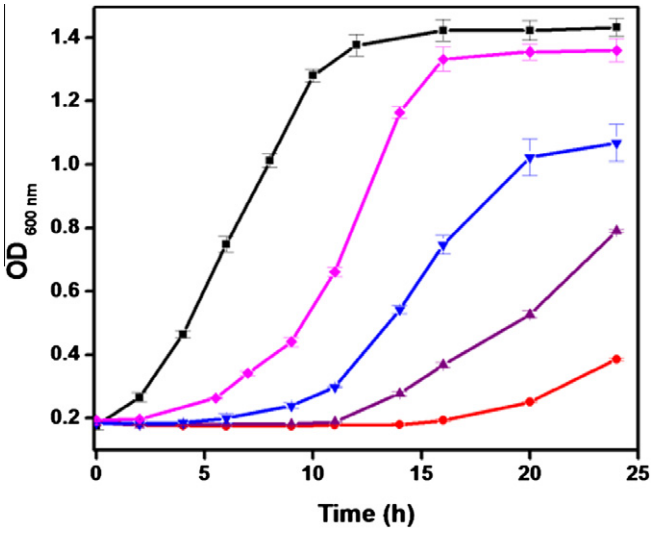

Figure 6. Rescue of *E. coli* DH5α cells from mercury toxicity. (■) Normal growth; cells pre-treated with 1.8 μM Hg^{2+} followed by addition of (♦) 40 μM, (▼) 20 μM and (▲) 10 μM of **3**; (●) cells treated with 1.8 μM Hg^{2+}. Optical density was measured at 600 nm.

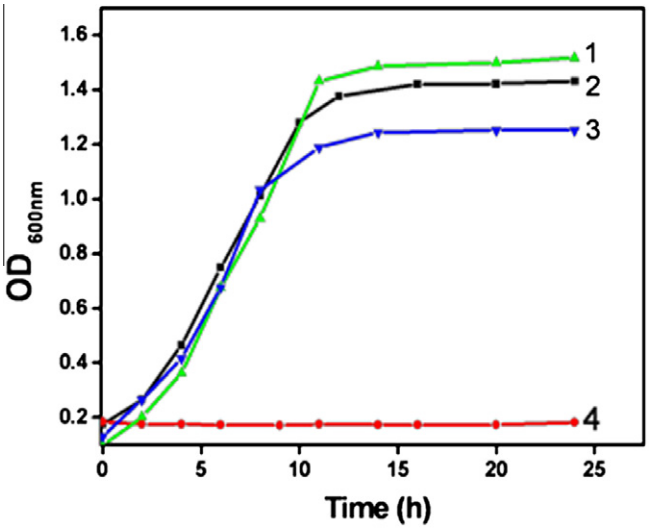

Figure 7. Control experiments of *E. coli* DH5α cell growth in culture media. (1) Culture media containing 1.8 μM Hg^{2+} and 40 μM compound **3** (complexed before); (2) normal cell growth; (3) cells treated with compound **3** (40 μM); (4) cells treated with 2 μM Hg^{2+}. Optical density was measured at 600 nm.

The interaction between Hg^{2+} and conjugate **3** was further established by studying the self-assembly behavior of the conjugate by microscopy and fluorescence quenching. Furthermore, **3** was able to rescue *E. coli* DH5α cells from mercury toxicity. Such an approach is worthy of further investigation as a mercury sequestration agent as it is an amide conjugate and therefore biodegradable.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures, detail of microscopic studies, additional fluorescence data, additional cell culture data, and additional microscopic images) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.09.047.

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10. *Synthesis of (pyridine–Trp–Trp–OMe)$_2$*: pyridine dicarboxylic acid (0.15 g, 0.89 mmol) and 1-hydroxybenzotriazole (0.302 g, 1.97 mmol) was dissolved in dichloromethane (10 mL) and dimethylformamide (5 mL) and the reaction mixture was cooled to 0 °C under N_2 atmosphere which was followed by the addition of dicyclohexylcarbodiimide (0.426 g, 2.06 mmol) dissolved in dichloromethane (20 mL). The reaction mixture was stirred at 0 °C for 1 h, and then tryptophanyl tryptophan methyl ester hydrochloride (0.791 g, 1.79 mmol) was added to the reaction mixture which was followed by the addition of triethylamine (0.62 mL, 4.48 mmol). The reaction mixture was stirred for 12 h at room temperature. The precipitated dicyclohexylurea was filtered off and the residue was washed with dichloromethane twice (2×5 mL). The combined organic layer was evaporated under reduced pressure and the residue was dissolved in dichloromethane. The organic layer was washed with 10% $NaHCO_3$ (2×10 mL) and brine solution (2×10 mL), followed by the drying of the organic layer over anhydrous sodium sulfate. Dichloromethane was evaporated and the crude solid product was purified by silica gel column chromatography (pure compound was eluted with 1.5% methanol in dichloromethane) to yield Py(WWOMe)$_2$ as a white solid (0.471 g, yield 56%). R_f = 0.5 (10% methanol in dichloromethane). Mp = 160–162 °C; $[\alpha]_D^{25} = -43$ (*c* 0.5, methanol); ESI-HRMS: $[M+H]^+$, calcd ($C_{53}H_{50}N_9O_8$) = 940.3782, found = 940.3782; ^{1}H NMR (500 MHz, $CDCl_3$, TMS, δ ppm): 3.22–3.34 (m, 6H); 3.42–3.49 (m, 2H); 3.73 (s, 6H); 4.95–4.98 (dd, 2H, *J* = 5.5 Hz, 6.4 Hz); 5.16–5.21 (dd, 2H, *J* = 6.5 Hz, 7.1 Hz); 6.64–6.66 (d, 4H, *J* = 5.5 Hz); 6.90–6.96 (m, 6H); 6.99–7.02 (t, 2H, *J* = 7.2 Hz, 7.3 Hz); 7.06–7.09 (t, 2H, *J* = 7.2 Hz, 7.2 Hz); 7.14–7.22 (m, 4H); 7.25–7.26 (2H, merged with $CDCl_3$ signal); 7.36–7.38 (d, 2H, *J* = 6.5 Hz); 7.55–7.58 (t, 1H, *J* = 7.5 Hz, 7.7 Hz); 7.61–7.63 (d, 2H, *J* = 7.6 Hz); 7.98–8.02 (m, 2H); 8.92 (br s, 2H). ^{13}C NMR (125 MHz; $CDCl_3$, TMS, δ ppm): 27.61, 28.47, 52.45, 52.70, 53.26, 109.29, 111.24, 111.67, 118.23, 119.48, 122.10, 122.33, 123.43, 124.21, 125.27, 127.24, 135.99, 136.42, 147.74, 163.33, 172.18, 172.40.
Py(WWOMe)$_2$ (0.1 mg, 0.106 mmol) was dissolved in methanol (5 mL)and 1 N NaOH in water (0.26 mL) was added. The reaction mixture was stirred for 4 h at room temperature. The reaction mixture was diluted by adding methanol (50 mL) and subsequently it was passed through Amberlite IR 120. The solvent was evaporated under reduced pressure and the residue was washed with diethyl ether, and dried under high vacuum to get compound **3** as a white solid (0.7 g, yield 72%). R_f = 0.5 (30% methanol in dichloromethane). Mp = decomposition occurs at >185 °C; $[\alpha]_D^{25} = -65$ (*c* 0.5, methanol); ESI-HRMS: $[M+H]^+$, calcd ($C_{51}H_{46}N_9O_8$) = 912.3469; found = 912.3463; ^{1}H NMR (500 MHz, CD_3OD, TMS, δ ppm):3.14–3.24 (m, 4H); 3.30–3.38 (m, 4H, merged with CD_3OD signal); 4.64–4.68 (m, 2H); 4.76–4.79 (m, 2H); 6.79–6.96 (m, 8H); 6.97–7.05 (m, 3H); 7.13–7.15 (d, 2H, *J* = 8.4 Hz); 7.18–7.20 (d, 2H, *J* = 8.4 Hz); 7.26–7.30 (t, 1H, *J* = 8.0 Hz, 8.0 Hz); 7.41–7.43 (d, 2H, *J* = 8.0 Hz); 7.53–7.54 (d, 2H, *J* = 7.6 Hz); 7.96–8.00 (t, 1H, *J* = 8.4 Hz, 8.6 Hz); 8.06–8.09 (m, 2H); 8.42–8.44 (br s, 4H). ^{13}C NMR (125 MHz; CD_3OD, TMS, δ ppm): 26.98, 54.73, 64.99, 110.20, 111.39, 118.18, 118.49, 120.69, 123.58, 124.62, 127.23, 127.63, 136.07, 139.35, 148.82, 163.22, 171.08, 173.80.
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