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Synthesis of hydrophilic and flexible linkers for peptide derivatization in solid phase

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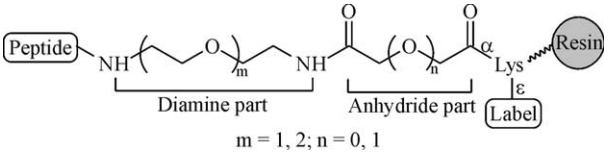
Abstract—Four *N*-Fmoc protected polyoxyethylene-based amino acid type linkers were designed and synthesized for peptide derivatization in solid phase. Three of them were obtained in a crystalline form. The crystallized linkers can be stored at 4 °C for 2 years without significant decomposition. Protocols for biotinylation and fluorescent labeling of peptides in solid phase were developed. The linkers also provide good ionization ability for single-bead mass spectrometry analysis of peptides.

Since 1963, the introduction of solid-phase synthesis has tremendously improved the synthesis of peptides and proteins, and even small organic molecule compounds.[1] Based on the solid-phase peptide synthesis (SPPS) technique, Lam et al. first reported the 'one-bead one-compound' (OBOC) combinatorial peptide library method in 1991.[2] In essence, when a split-mix synthesis method is used to generate a combinatorial library,[2,3] each resin bead expresses only one chemical entity.[2,4] We routinely use TentaGel to synthesize our OBOC libraries, in which library compounds are linked to the polystyrene scaffold via a poly(ethylene glycol) (PEG) linker.[4] The resin-supported libraries are screened with a high-throughput assay, and the discovered ligands are resynthesized and derivatized on a releasable resin for further biological characterization.

Using the OBOC combinatorial library method, a number of peptide or small molecule ligands for various targets have been identified in our laboratory.[4,5] To confirm the biological activities of the discovered ligands, they need to be resynthesized in a soluble form and derivatized with proper reporter groups. For example, biotinylation of protein binding ligands is required for Western blot analysis and fluorescent labeling of cell binding ligands is needed for flow cytometry. Preferably, the labeling reagent is connected to C-terminus of the peptide or ligand via a hydrophilic spacer to enhance water solubility and to simulate the presentation of ligands on TentaGel bead. For this purpose, a PEG linker would have been an ideal choice. However, the commercially available PEGs usually consist of a heterogenous mixture of PEG molecules with different molecular weights, thus causing problems in purification and characterization. There is a need for the development of a series of hydrophilic linkers with various length, but with a well-defined molecular masses.

In the present study, we designed four short hydrophilic linkers that can be conveniently used as building blocks in SPPS or solid phase organic synthesis (Scheme 1). Each linker consists of a diamine component and an anhydride component. We chose 2,2′-oxybis(ethylamine) and 2,2′-(ethylenedioxy)-bis(ethylamine) as the diamine components and succinic anhydride and diglycolic anhydride as the anhydride components. These



Scheme 1. Design of the linkers.

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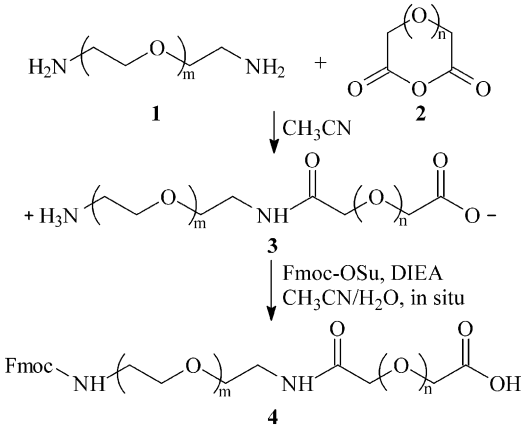
reagents are all commercially available. At the beginning of synthesis, a lysine derivative with an orthogonal side chain protecting group is coupled to the resin to provide an additional amino group for attachment of the reporter group such as fluorescent dyes or biotin. The linker is then built on the α-amino group of lysine and the peptide or small molecule ligand is synthesized on its amino terminus. The reporter groups can be coupled to the ε-amino group of lysine either prior to or after the synthesis of the ligands.

We attempted to synthesize the linkers in solid phase directly, but failed. The attachment of succinic anhydride or diglycolic anhydride was very smooth and the reaction was usually completed in 30 min. However, the coupling of diamines to the resin-supported carboxyl group was problematic. First, the coupling reaction proceeded very slowly at a low diamine concentration, but a high diamine concentration was not compatible with base-labile resin linkers such as 4-hydroxymethylbenzoic acid (HMBA linker)[6] or 3-mercaptopropionic acid.[7] Second, the coupling results were inconsistent even with strong coupling reagents such as (*O* - 7 - azabenzotriazol - 1 - yl) - *N*,*N*,*N*′,*N*′ - tetramethyluronium hexafluorophosphate (HATU). Therefore, we decided to prepare the *N*-Fmoc protected linkers in solution phase so that they can be used as special amino acids in standard Fmoc SPPS.[8]

The synthetic route of *N*-Fmoc protected linkers is illustrated in Scheme 2. The diamine **1** was allowed to react with the cyclic anhydride **2** in acetonitrile to form the mono-acylated intermediate **3**, which was subsequently protected with *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide (Fmoc-OSu) in situ. Acetonitrile was chosen as the solvent for the first acylation step because the product precipitated immediately as an inner salt to avoid dual acylation and the waxy product could be easily separated from the unreacted starting materials. In a typical experiment, 10 mmol of the diamine was dissolved in 50 mL acetonitrile.[9] A solution of the anhydride (10 mmol) in 25 mL of acetonitrile was added dropwise under vigorous magnetic stirring over 1 h. The stirring was stopped after 3 h. After the waxy product settled down, the liquid phase was decanted and discarded. The waxy product was redissolved in 100 mL



Scheme 2. Solution-phase synthesis of *N*-Fmoc protected linkers.

50% acetonitrile/water and chilled in an ice bath for 30 min. A solution of Fmoc-OSu (4.4 g, 13 mmol) in 25 mL acetonitrile was added dropwise under vigorous magnetic stirring over 1 h. The pH of reaction mixture was maintained at 8–9 by adding diisopropylethylamine (DIEA) throughout the reaction. The reaction was allowed to proceed overnight at room temperature. The solvents were removed in vacuo. The residue was dissolved in 100 mL of 5% aqueous $NaHCO_3$ solution and washed with ethyl acetate. The aqueous phase was then acidified with 1 M HCl to pH 2 and extracted with ethyl acetate (50 mL×3). The combined organic phase was washed with brine, dried over anhydrous $MgSO_4$ and then concentrated to about 15 mL. Hexanes was slowly added to the solution until it became slightly turbid. Seed crystals were added. The resulting mixture was allowed to stand at room temperature for 24 h. The crystallized product was collected, washed with ethyl acetate/hexanes (v/v 3:2) and dried in vacuo. The yields of four *N*-Fmoc protected linkers are listed in Table 1.[10]

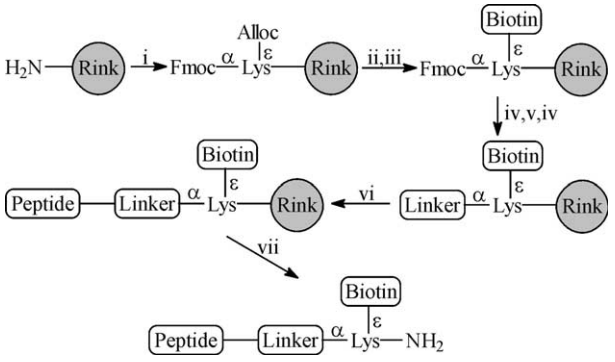
Since all of the *N*-Fmoc protected linkers have a hydrophobic Fmoc group and a long hydrophilic tail, they are very difficult to crystallize. We have previously reported another hydrophilic linker with a similar but longer structure,[11] which was used for preparing peptide microarrays.[12] We failed to crystallize that linker and it was used in a viscous oily form, which was difficult to handle. Furthermore, the oily linker was highly hygroscopic and unstable. The oil slowly changed from colorless to yellow and 9-fluorenylmethanol was found as the hydrolysis product, even though it was stored in the dark at 4 °C. In this work, we were able to crystallize three of the four *N*-Fmoc protected linkers. Compounds **4a**–**c** were obtained in crystalline forms with sharp melting points. The crystallized *N*-Fmoc protected linkers **4a**–**c** are quite stable. They can be stored at 4 °C for two years without significant decomposition according to HPLC and NMR analysis.

Using these *N*-Fmoc protected linkers, protocols for various peptide derivatization in solid phase have been developed. For example, biotinylation of peptides can be readily achieved in solid phase (Scheme 3). Fmoc-Lys(Alloc)-OH was used for this purpose. The lysine derivative was first attached to Rink resin and the allyloxycarbonyl (Alloc) group on side chain was removed. Biotin was then coupled to the ε-amino group of lysine. We found that biotin is poorly soluble in either *N*,*N*-dimethylformamide (DMF) or dichloromethane (DCM) at room temperature even with addition of DIEA and, therefore, prevents efficient coupling. This problem was solved by warming biotin in DMF at 60 °C. Biotin dissolved well in warm DMF and did not precipitate after cooling to room temperature. The linker was attached

Table 1. Synthesis of *N*-Fmoc protected linkers **4a**–**d** (Scheme 2)

Entry	*m*	*n*	Yield (%)	Mp (°C)
a	1	0	62	145–146
b	1	1	49	114–115
c	2	0	72	111–113
d	2	1	76	—

to the α-amino group of lysine after biotinylation and Fmoc deprotection. The peptide was then constructed on the linker using standard Fmoc chemistry, followed by trifluoroacetic acid (TFA) cleavage. A number of biotinylated peptides have been synthesized using this procedure and successfully applied to peptide microarrays.[5] A B-lymphoma cell ligand peptide, wGeyidvk (lowercase letters represent D-amino acids),[13] was selected as an example. The HPLC and MS analysis results of the crude biotinylated peptide after TFA cleavage are shown in Figure 1.



Scheme 3. Synthetic route for solid-phase peptide biotinylation. Reagents and conditions: (i) 2 equiv of Fmoc-Lys(Alloc)-OH, 2 equiv of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP®) and 4 equiv of DIEA in DMF, rt, 1 h; (ii) 0.25 equiv of $Pd(PPh_3)_4$, 24 equiv of $PhSiH_3$ in DCM, Ar, rt, 30 min×2; (iii) 2 equiv of biotin, 2 equiv of PyBOP® and 4 equiv of DIEA in DMF, rt, 1 h; (iv) 20% piperidine in DMF, rt, 15 min×2; (v) 2 equiv of linker, 2 equiv of PyBOP® and 4 equiv of DIEA in DMF, rt, 1 h; (vi) peptide synthesis; (vii) TFA/H_2O/TIS (v/v/v 95:2.5:2.5).

Fluorescent probes may be unstable under the conditions used for peptide synthesis. Therefore, they should be attached to the peptide after the synthesis to avoid undesired side reactions. In this case, Fmoc-Lys(Dde)-OH was used (Scheme 4). The peptide was synthesized on the linker at the α-amino group of lysine and its N-terminus was then protected with Boc anhydride. The 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) side-chain protecting group was removed by treatment with hydrazine. The fluorescent probe was attached to the ε-amino group of lysine, followed by TFA cleavage. As an example, a non-Hodgkin's lymphoma cell binding ligand peptide, pLDI,[14] was labeled with FITC using this procedure. The HPLC and MS spectra of the crude product are shown in Figure 2.

The hydrophilic linkers can also facilitate MS analysis of peptides by providing good ionization ability for poorly ionizable short peptides or small molecule compounds.[15] In single-bead MS analysis of combinatorial peptide libraries, an ionization linker consisting of several amino acids is usually needed.[16,17] Besides increasing the synthetic steps and steric hindrance, the ionization linker itself may interfere during the biological screening. To demonstrate the ionization ability of our linkers, a peptide ligand for anti-β-endorphin antibody, YGGFL,[2] was synthesized and encoded on TentaGel resin with linker **4c** using a laddering method.[16] In each coupling step, 10% of the corresponding Boc protected amino acid was added to effect partial termination. The single-bead MALDI-FTMS analysis result is shown in Figure 3. All of the fragments are clearly shown, indicating the good ionization ability of the linker.

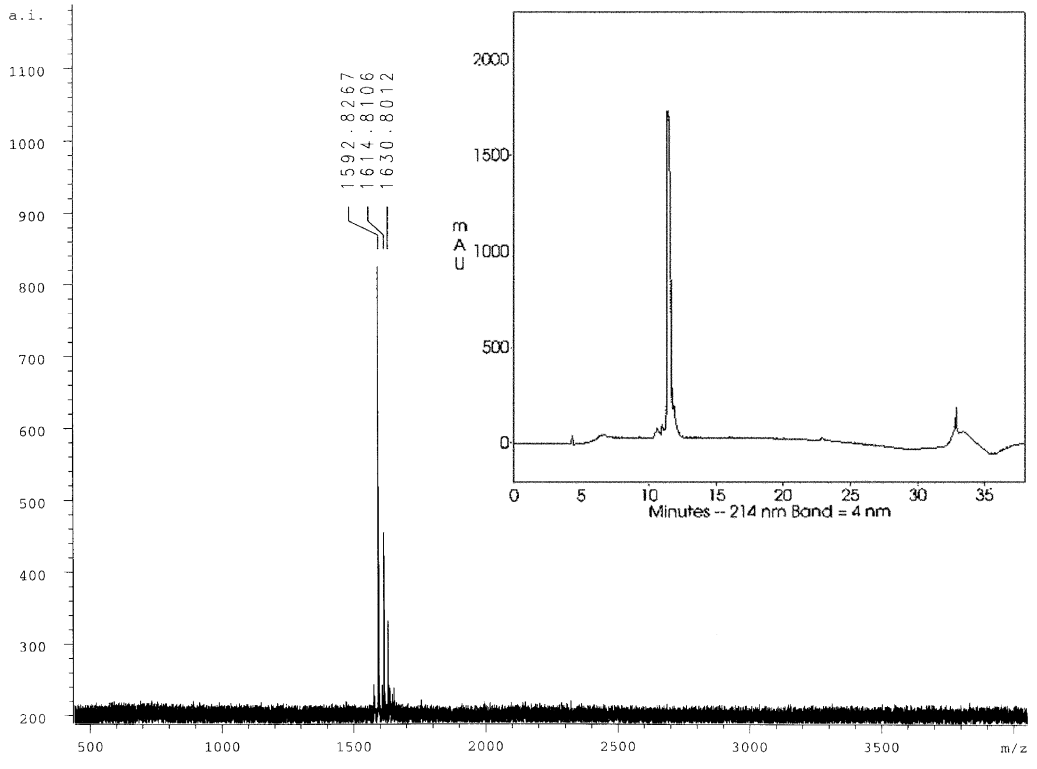
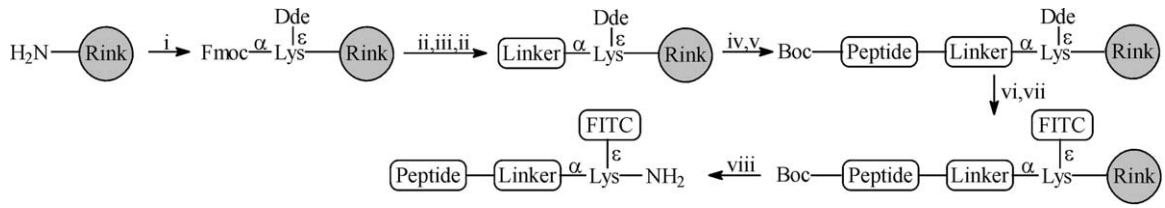


Figure 1. MALDI-TOF MS and HPLC (insert) spectra of crude biotinylated peptide wGeyidvk-linker(**4c**)-K(biotin)-NH_2 after TFA cleavage.



Scheme 4. Synthetic route for FITC labeled peptides. Reagents and conditions: (i) 2 equiv of Fmoc-Lys(Dde)-OH, 2 equiv of PyBOP® and 4 equiv of DIEA in DMF, rt, 1 h; (ii) 20% piperidine in DMF, rt, 15 min×2; (iii) 2 equiv of linker, 2 equiv of PyBOP® and 4 equiv of DIEA in DMF, rt, 1 h; (iv) peptide synthesis; (v) 20 equiv of Boc_2O and 4 equiv of DIEA in DCM, rt, 30 min; (vi) 2% hydrazine in DMF, rt, 5 min×2; (vii) 1.5 equiv of FITC, 3 equiv of DIEA in DMF, rt, overnight; (viii) TFA/H_2O/TIS (v/v/v 95:2.5:2.5).

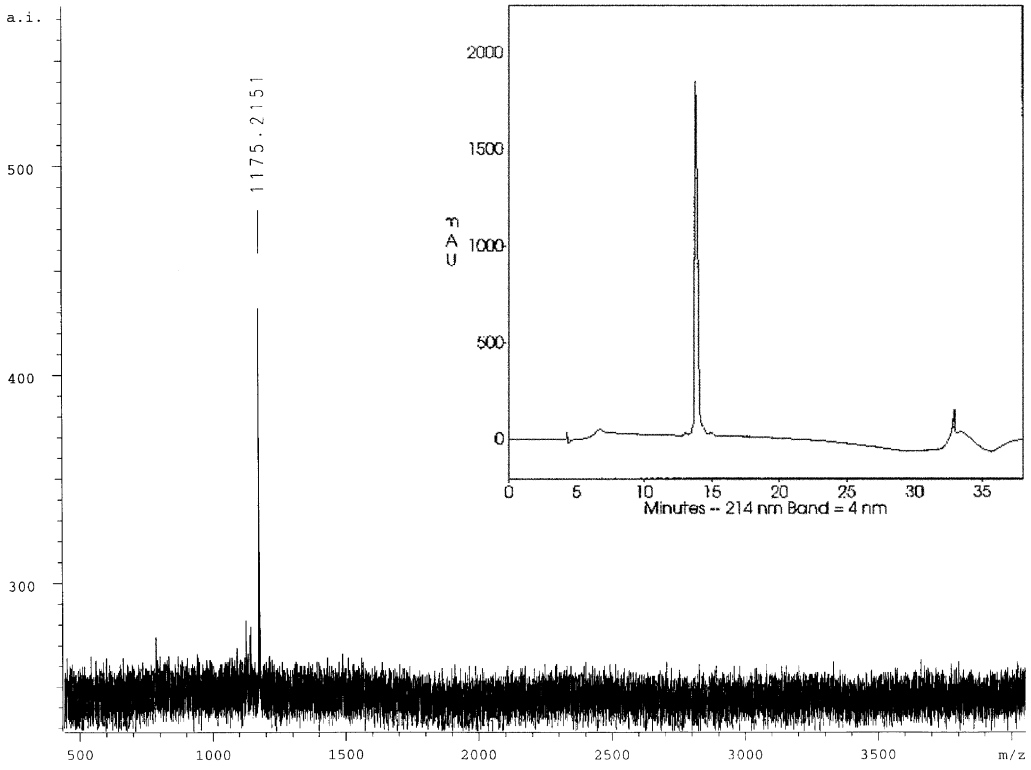


Figure 2. MALDI-TOF MS and HPLC (insert) spectra of crude FITC labeled peptide pLDI-linker(**4b**)-K(FITC)-NH_2 after TFA cleavage.

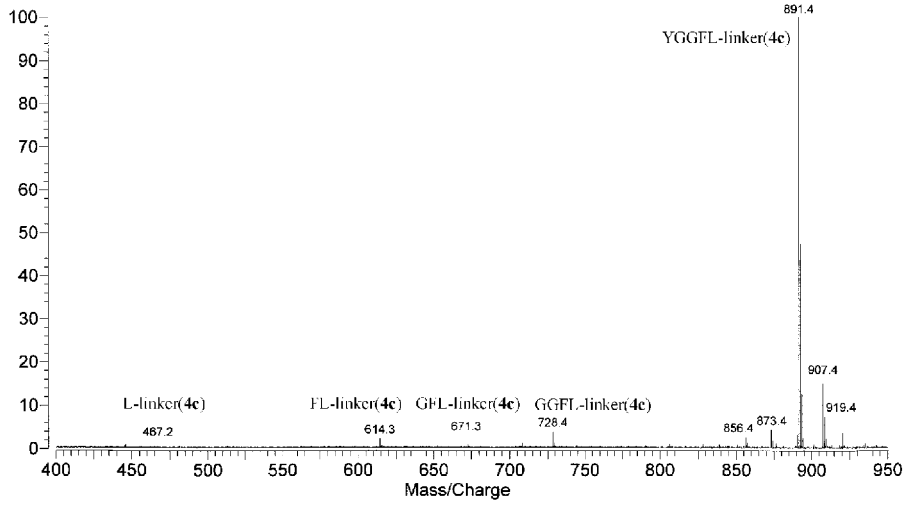


Figure 3. Single-bead MALDI-FTMS analysis result of YGGFL encoded by a laddering method.

In conclusion, four flexible and hydrophilic linkers for peptide derivatization in solid phase were designed and synthesized. Three of the *N*-Fmoc protected linkers were obtained in a crystalline form. They can be prepared in large scale and readily used in standard Fmoc SPPS. Protocols for biotinylation and fluorescent labeling of peptides using these linkers have been developed. Various biological assays using these peptide derivatives are currently underway in our laboratory. The linkers also provide good ionization ability for single-bead mass spectrometry analysis of peptides.

Acknowledgements

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9. 2,2′-Oxybis(ethylamine) which came as dihydrochloride salt, (1.77 g, 10 mmol) was dissolved in 25 mL of methanol. To the solution 4.59 mL of 25% sodium methoxide solution in methanol was added dropwise under magnetic stirring. The resulting mixture was stirred at room temperature for 30 min. The solvent was removed in vacuo. The residue was suspended in 50 mL of acetonitrile for subsequent acylation in situ.
10. Spectral data for synthesized compounds. Linker **4a**: ^{1}H NMR (DMSO-d_6) δ 12.06 (s, broad, 1H), 7.88 (m, 3H), 7.69 (d, $J=7.2$ Hz, 2H), 7.41 (t, $J=7.2$ Hz, 2H), 7.33 (m, 3H), 4.31 (d, $J=6.5$ Hz, 2H), 4.22 (t, $J=6.5$ Hz, 1H), 3.39 (m, 4H), 3.20 (m, 2H), 3.16 (m, 2H), 2.43 (t, $J=6.8$ Hz, 2H), 2.33 (t, $J=6.8$ Hz, 2H). ^{13}C NMR (DMSO-d_6) δ 174.6, 171.8, 156.9, 144.6, 141.5, 128.3, 127.8, 125.9, 120.8, 69.7, 69.6, 66.1, 47.5, 40.8, 39.3, 30.7, 29.8. ESI-MS m/z 427.2 (MH^+). Linker **4b**: ^{1}H NMR (DMSO-d_6) δ 12.82 (s, broad, 1H), 7.88 (d, $J=7.2$ Hz, 2H), 7.83 (t, $J=5.7$ Hz, 1H), 7.69 (d, $J=7.2$ Hz, 2H), 7.41 (t, $J=7.2$ Hz, 2H), 7.33 (m, 3H), 4.30 (d, $J=6.5$ Hz, 2H), 4.22 (t, $J=6.5$ Hz, 1H), 4.11 (s, 2H), 3.97 (s, 2H), 3.42 (m, 4H), 3.27 (m, 2H), 3.14 (m, 2H). ^{13}C NMR (DMSO-d_6) δ 172.1, 169.6, 156.9, 144.6, 141.5, 128.3, 127.8, 125.9, 120.8, 70.8, 69.6, 69.4, 68.5, 66.1, 47.5, 40.8, 38.8. ESI-MS m/z 443.2 (MH^+). Linker **4c**: ^{1}H NMR (DMSO-d_6) δ 12.69 (s, broad, 1H), 7.90 (t, $J=5.7$ Hz, 1H), 7.88 (d, $J=7.2$ Hz, 2H), 7.69 (d, $J=7.2$ Hz, 2H), 7.41 (t, $J=7.2$ Hz, 2H), 7.33 (m, 3H), 4.29 (d, $J=6.5$ Hz, 2H), 4.21 (t, $J=6.5$ Hz, 1H), 4.12 (s, 2H), 3.50 (m, 4H), 3.39 (m, 4H), 3.18 (m, 2H), 3.13 (m, 2H), 2.40 (t, $J=6.8$ Hz, 2H), 2.31 (t, $J=6.8$ Hz, 2H). ^{13}C NMR (DMSO-d_6) δ 174.6, 171.9, 156.9, 144.6, 141.5, 128.3, 127.8, 125.9, 120.8, 70.2, 69.8, 66.0, 47.4, 40.8, 39.3, 30.8, 30.1. ESI-MS m/z 471.2 (MH^+). Linker **4d**: ^{1}H NMR (DMSO-d_6) δ 12.51 (s, broad, 1H), 7.88 (d, $J=7.2$ Hz, 2H), 7.82 (t, $J=5.7$ Hz, 1H), 7.70 (d, $J=7.2$ Hz, 2H), 7.41 (t, $J=7.2$ Hz, 2H), 7.33 (m, 3H), 4.30 (d, $J=6.5$ Hz, 2H), 4.21 (t, $J=6.5$ Hz, 1H), 4.12 (s, 2H), 3.98 (s, 2H), 3.51 (m, 4H), 3.42 (m, 4H), 3.28 (m, 2H), 3.15 (m, 2H). ^{13}C NMR (DMSO-d_6) δ 172.1, 169.6, 156.9, 144.6, 141.5, 128.3, 127.8, 125.9, 120.8, 70.8, 70.2, 69.8, 69.6, 68.5, 66.1, 60.5, 47.5, 40.9, 38.8. ESI-MS m/z 487.2 (MH^+).
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