Introducing D-Amino Acid or Simple Glycoside into Small Peptides to Enable Supramolecular Hydrogelators to Resist Proteolysis

Xinming Li,† Xuewen Du, Jiayang Li, , Yuan Gao, Yue Pan, Junfeng Shi, Ning Zhou and Bing Xu*

Supporting information

1. MATERIALS AND METHODS

1.1 Materials. 2-(Naphthalen-6-yl) acetic acid, N, N'-diisopropylcarbodiimide DIC), N-hydroxysuccinimide (NHS), L-phenylalanine, L-argnine, L-glycine, L-aspartic acid, D-phenylalanine, D-glucosamine and trifluoro acetic acid (TFA) were purchased from Sigma-Aldrich, and used without further purification unless otherwise stated. Chondrosine was prepared by following the procedure reported by Levene.[1] All the hydrogelators were synthesized via the combination of solid phase synthesis and simple coupling reactions in solution phase. ^{1}H spectra were obtained on a Varian Unity Inova 400 spectrometer, LC-MS on a Waters Acouity ultra Performance LC with Waters MICROMASS detector, and TEM on a Morgagni 268 transmission electron microscope.

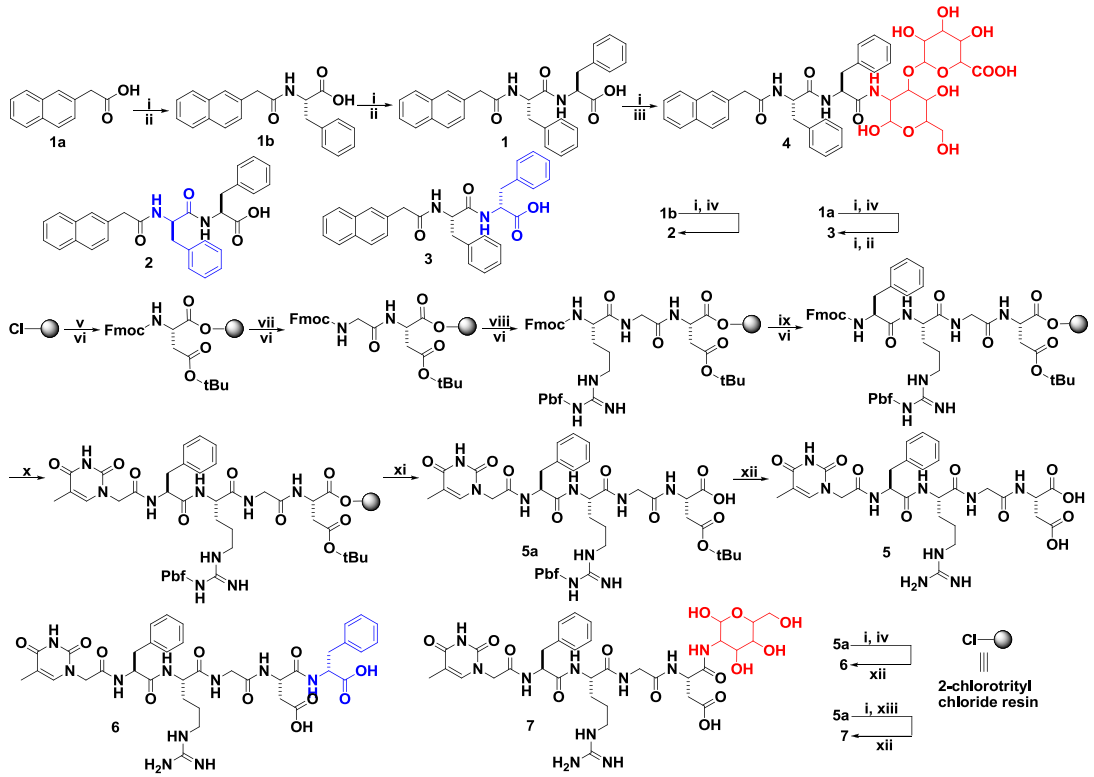

Figure S1. The synthetic routes and molecular structures of the hydrogelators which contain a D-amino acid or a glycoside at the C-terminal of the hydrogelators.

1.2 Synthesis.

1.2.1 Synthesis of Nap-L-phe (1b). Compound **1b** was prepared by following the procedure reported by Yang Z. *et al.*.[2]

1.2.2 Synthesis of Nap-L-Phe-L-Phe (1). Compound **1** was synthesized according to the procedure published by Yang Z. *et al.*.[2]

1.2.3 Synthesis of Nap-L-Phe-L-Phe-chondrosine (4). Compound **1** (480 mg, 1 mmol) and NHS (126.5 mg, 1.1 mmol) were dissolved in 30 mL of THF, and DIC (140.8 mg, 1.1 mmol) was added to the above solution with stirring. After the reaction mixture was stirred at room temperature overnight, the resulted solid was filtered off, and the filtrate was evaporated under reduced pressure to dryness. The crude product was used in the next reaction without purification.

Chondrosine (355 mg, 1 mmol) and Na_2CO_3 (84.8 mg, 0.8 mmol) were dissolved in 20 mL of water with stirring, and the solution of the crude product (dissolved in 20 mL THF) was added. The resulted reaction mixture was stirred at room temperature for 24 hrs. The reaction mixture was vacuum-dried and purified by using HPLC with water-acetonitrile as eluent (from 8:2 to 2:8) to afford the product (**4**) in 41% yield. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.02-8.41 (m, 3H), 6.92-7.98 (m, 17H), 6.8 (s, 1H), 4.95 (s, 1H), 4.38-4.61 (m, 4H), 4.20 (s, 1H), 2.78-3.95 (m, 20H); ESI MS(m/z) $[M]^+$ calcd. for $C_{42}H_{47}N_3O_{14}$ 817.31, found $[M-H]^-$ 816.56.

1.2.4 Synthesis of Nap-L-phe-D-Phe (2). Compound **2** was synthesized following the procedures described in **1.2.2** for the synthesis of L-version compound **1** except replacing L-Phenylalanine with D-Phenylalanine. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 7.90–8.06 (m, 3H), 7.80 (s, 1H), 7.63–7.68 (m, 2H), 7.18–7.39 (m, 11H), 4.91–4.95 (q, 1H, *J* = 4 Hz), 4.64–4.67 (q, 1H, *J* = 4 Hz), 3.66–3.74 (q, 2H, *J* = 4 Hz), 2.75–3.07 (m, 4H); ^{13}C NMR (100MHz, DMSO-d_6) δ 172.92, 171.44, 169.89, 137.95, 137.84, 137.62, 134.12, 133.08, 129.44, 129.39, 129.32, 128.35, 128.09, 128.02, 127.77, 127.60, 127.52, 127.42, 126.64, 126.33, 126.27, 126.13, 125.60, 53.81, 53.73, 42.39, 37.78, 36.85; ESI MS(m/z) $[M]^+$ calcd. for $C_{30}H_{28}N_2O_4$ 480.20, found $[M-H]^-$ 479.53.

1.2.5 Synthesis of Nap-D-Phe-L-Phe (3). Compound **3** was prepared following the procedures described in **1.2.1** for the synthesis of L-version compound **1b** except replacing the L-Phenylalanine with D-Phenylalanine. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 7.89–8.05 (m, 3H), 7.80 (s, 1H), 7.62–7.68 (m, 2H), 7.18–7.40 (m, 11H), 4.90–4.95 (q, 1H, *J* = 4 Hz), 4.64–4.67 (q, 1H, *J* = 4 Hz), 3.65–3.74 (q, 2H, *J* = 4 Hz), 2.74–3.07 (m, 4H); ^{13}C NMR (100MHz, DMSO-d_6) δ 172.91, 171.48, 169.89, 137.93, 137.82, 137.56, 134.12, 133.09, 129.45, 129.39, 129.30, 128.37, 128.10, 128.02, 127.79, 127.61, 127.53, 127.43, 126.67, 126.35, 126.28, 126.14, 125.61, 53.76, 53.65, 42.39, 37.79, 36.84; ESI MS(m/z) $[M]^+$ calcd. for $C_{30}H_{28}N_2O_4$ 480.20, found $[M-H]^-$ 479.53.

1.2.6 Synthesis of Thy-L-phe-L-Arg(pbf)-L-Gly-L-Asp(Otbu) (5a). Compound **5a** was synthesized following typical solid phase synthesis protocols.[18] Starting from 2-chlorotrityl resin, the peptide chain is extended from the C-terminal towards the N-terminal through step-by-step peptide chain elongation procedure with Fmoc-amino acids. After capping with thymine acetic acid at the N-terminal, the fully protected nucleopeptides fragment was cleaved from the resin by using TFE/DCM (2:8) solution in 45 min. The final product was used in the next reaction without purification.

1.2.7 Synthesis of Thy-L-Phe-L-Arg-L-Gly-L-Asp (5). Compound **5a** was dissolved and stirred in cleavage solution that contains 95% trifluoroacetic acid, 2.5% triispropylsilane and 2.5% water for 3 hrs. Then the mixture was concentrated by vacuum and purified by HPLC using water-acetonitrile as eluent (from 9:1 to 7:3). The product (**5**) was obtained in 56% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.43 (d, 1H, J = 8 Hz), 8.28 (t, 2H, J = 8 Hz), 8.07 (d, 1H, J = 8 Hz), 7.42 (s, 1H), 7.16-7.29 (m, 5H), 4.56 (m, 2H,), 4.18-4.34 (m, 3H), 3.72 (d, 2H, J = 4 Hz), 2.53-3.13 (m, 6 H), 1.72 (s, 3H), 1.49 (m, 4H); ^{13}C NMR (100MHz, DMSO-d_6) δ 172.46, 171.82, 171.54, 171.07, 168.67, 167.05, 164.59, 156.97, 151.18, 142.37, 137.76, 129.51, 129.45, 128.28, 128.23, 126.45, 108.21, 54.19, 52.45, 51.03, 49.31, 48.79, 41.76, 36.26, 29.27, 25.12, 12.10; ESI MS(m/z) $[M]^+$ calcd. for $C_{28}H_{37}N_9O_{10}$ 659.27, found $[M-H]^-$ 658.66.

1.2.8 Synthesis of Synthesis of Thy-L-Phe-L-Arg-L-Gly-L-Asp-D-phe (6). Compound **5a** (968.08 mg, 1 mmol) and NHS (126.5 mg, 1.1 mmol) were dissolved in THF (30 mL), and DIC (140.8 mg, 1.1 mmol) was added to the THF solution with stirring. After the reaction, the mixture was stirred at room temperature for 12 hrs, and the resulted solid was filtered. The filtrate was evaporated under reduced pressure to dryness. The crude product was used for the next reaction without purification.

D-Phenylalanine (166 mg, 1 mmol) and Na_2CO_3 (84.8 mg, 0.8 mmol) were dissolved in water (20 mL) with stirring, and the solution of the crude product (dissolved in 30 mL of THF) was added. The resulted reaction mixture was stirred at room temperature for 24 h. After evaporation of the organic solvent, the residue was treated with cleavage solution that contains 95% trifluoroacetic acid, 2.5% triispropylsilane and 2.5% water for 3 hrs. Then the mixture was concentrated by vacuum and purified by HPLC using water-acetonitrile as eluent (from 9:1 to 7:3). The product (**6**) was obtained in 51% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.43 (d, 1H, J = 8 Hz), 8.28 (t, 2H, J = 8 Hz), 7.95-8.07 (m, 3H), 7.16-7.29 (m, 11H), 4.56 (m, 2H,), 4.18-4.34 (m, 4H), 3.75 (d, 2H, J = 8 Hz), 2.53-3.13 (m, 8 H), 1.72 (s, 3H), 1.49 (m, 4H); ^{13}C NMR (100MHz, DMSO-d_6) δ 172.94, 172.06, 171.69, 171.11, 170.63, 167.09, 166.20, 164.60, 157.70, 156.93, 151.26, 151.20, 142.86, 142.40, 137.84, 137.70, 129.49, 129.40, 128.35, 128.28, 126.49, 114.70, 108.22, 61.99, 54.26, 51.66, 49.32, 41.41, 37.69, 36.88, 32.02, 29.35, 28.73, 12.12; ESI MS(m/z) $[M]^+$ calcd. for $C_{37}H_{46}N_{10}O_{11}$ 806.33, found $[M-H]^-$ 805.75.

1.2.9 Synthesis of Synthesis of Thy-L-Phe-L-Arg-L-Gly-L-Asp-glucosamine (7). Compound **5a** (968.08 mg, 1 mmol) and NHS (126.5 mg, 1.1 mmol) were dissolved in THF (30 mL), and DIC (140.8 mg, 1.1 mmol) was added to the above solution with stirring. After the reaction, the mixture was stirred at room temperature for 12 hrs, and the resulted solid was filtered. The filtrate was evaporated under reduced pressure to dryness. The crude product was used for the next reaction without purification.

D-glucosamine hydrochloride (215.64 mg, 1 mmol) and Na_2CO_3 (212 mg, 2 mmol) were dissolved in water (20 mL) with stirring, and the solution of the crude product (dissolved in 30 mL of THF) was added. The resulted reaction mixture was stirred at room temperature for 24 hrs. After evaporation of the organic solvent, the residue was treated with cleavage solution that contains 95% trifluoroacetic acid, 2.5% triispropylsilane and 2.5% water for 3

hrs. Then the mixture was concentrated by vacuum and purified by HPLC using water-acetonitrile as eluent (from 9:1 to 7:3). The product (**7**) was obtained in 45% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δδ 8.43 (d, 1H, J = 8 Hz), 8.28 (t, 2H, J = 8 Hz), 8.07 (d, 1H, J = 8 Hz), 7.42 (s, 1H), 7.16-7.29 (m, 5H), 6.40-6.45 (s, 1H), 4.92-5.01(m, 3H), 4.18-4.62 (m, 5H), 3.80-3.90 (d, 2H, J = 8 Hz), 2.95-3.60 (m, 13H), 1.72 (s, 3H), 1.49 (m, 4H); ^{13}C NMR (100MHz, DMSO-d_6) δ 172.37, 171.08, 167.00, 164.61, 157.30, 151.17, 142.42, 139.11, 137.81, 134.47, 129.49, 128.26, 126.48, 119.34, 108.17, 82.89, 72.37, 71.90, 71.06, 69.62, 61.30, 59.07, 54.13, 51.40, 49.29, 47.37, 12.12; ESI MS(m/z) $[M]^+$ calcd. for $C_{34}H_{48}N_{10}O_{14}$ 820.34, found $[M+H]^+$ 819.72.

1.3 Transmission Electron Microscopy (TEM) Characterization. A carbon pre-coated copper grid (400 mesh, Pacific Grid-Tech) was glow discharged for 30 s and then incubated with 10 μL of sample. After removing excessive samples with filter paper, the loaded grid was washed in double distilled water. The sample was then stained with 2% (w/v) uranyl acetate. Data were collected at high vacuum on Morgagni 268 transmission electron microscope.

1.4 Biostability Test. 1 mg of each compound was dissolved in 5 mL (0.02 wt %) of HEPES buffer at pH 7.5. Then proteinase K were added at the concentration of 3.2 units/mL and incubated at 37 ^{o}C for 24 hrs, then 100 μL of the samples were taken out at 2, 4, 8, 12, and 24h and analyzed by HPLC.

1.5 Rheological Measurement. Rheological tests were conducted on a TA ARES G2 rheometer (with Trios Software). 25 mm parallel-plates were used during the experiment. 0.3 mL of the hydrogel sample was placed on the parallel-plate by a spatula.
i) Dynamic strain sweep Test. Test range (0.1 to 100 % strain, frequency =1 Hz), 10 points per decade. Sweep mode is "log" and temperature was at 25 ^{o} C.
ii) Critical strain determination. The critical strain (γ_c) value was determined from the storage-strain profiles of the hydrogel sample. The strain applied to the hydrogel sample increased from 0.1 to 100 % (1 Hz and 25 ^{o}C). Over a certain strain, a drop in the elastic modulus was observed, and the strain amplitude at which storage moduli just begins to decrease by 5 % from its maximum value was determined and taken as a measure of the critical strain of the hydrogels.
iii) Dynamic frequency sweep test. Test range (0.1 to 200 rad/s, strain = 0.4%), 10 points per decade. Sweep mode is "log" and temperature was at 25 ^{o} C.

1.6 The typical procedure for hydrogelation.
1.6.1 hydrogel 2: 3 mg of hydrogelator **2** dissolves in 300 μL water at pH 9.0 with gentle heating to make clear solution, respectively, followed by the adjustment of the pH to 7.0 with 1N HCl would allow the formation of a hydrogel at room temperature.

1.6.2 hydrogel 3: Hydrogel **3** was prepared by following the procedures described in **1.6.1** for the preparation of hydrogel **2** except replacing 3 mg of hydrogelator **2** with 4.5 mg of hydrogelator **3**.

1.6.3 hydrogel 4: hydrogel **4** was prepared by following the procedures described in **1.6.1** for the preparation of hydrogel **2** except using 6 mg of hydrogelator **4** and adjusting pH to 4.0.

1.6.4 hydrogel 5: 9 mg of hydrogelator **5** dissolves in 300 μL water at pH 7.0 with gentle heating to make clear solution, respectively, followed by the adjustment of the pH to 4.0 with 1N HCl would allow the formation of a hydrogel at room temperature.

1.6.5 hydrogel 6: hydrogel **6** was prepared by following the procedures described in **1.6.4** for the preparation of hydrogel **5** except replacing hydrogelator **5** with hydrogelator **6**.

1.6.6 hydrogel 7: hydrogel **7** was prepared by following the procedures described in **1.6.4** for the preparation of hydrogel **5** except replacing hydrogelator **5** with hydrogelator **7**.

Table S1. Summary of the conditions and properties of the hydrogelators containing D-amino acid or glycoside and the corresponding supramolecular nanofibers and hydrogels

Sample	**2**	**3**	**4**	**5**	**6**	**7**
wt %	1.0	1.5	2.0	3.0	3.0	3.0
pH	7.0	7.0	4.0	4.0	4.0	4.0
morphologies of nanostructure	single fibers, double parallel fibers	single fibers, double parallel fibers	single fibers, helical fibers	ribbons	fibers , bundle of fibers	single fibers ribbons, helical fibers
width of nanofibers (nm)	8	11	15	35	8	15
critical strain (%)	– [a]	– [a]	1.3	0.2	0.45	0.2
G' (kPa)	– [a]	– [a]	3.3	40	0.4	35
biostability (compound remained (%) after 24 hrs)	43	91	57	0	15	45

[a] These compounds fail to afford hydrogels that are stable enough for rheological characterization.

Reference:

[1] Levene, P. A., On Chondrosin. *J. Biol. Chem.* **1941,** *140*, 267-277.

[2] Yang, Z.; Gu, H.; Du, J.; Gao, J.; Zhang, B.; Zhang, X.; Xu, B., Self-Assembled Hybrid Nanofibers Confer a Magnetorheological Supramolecular Hydrogel. *Tetrahedron* **2007,** *63* (31), 7349-7357.