Supporting information

Peptide precursors that acquire denatured collagen-hybridizing ability by *O*-to-*N* acyl migration at physiological pH

Sayaka Kanai, Koshi Machida, Ryo Masuda, and Takaki Koide

General reagents

Resins, Fmoc-Hyp(tBu)-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-β-Ala-OH, and Fmoc-Cys(Acm)-OH were obtained from Novabiochem (San Diego, CA, USA). Fmoc-Lys(Mtt)-OH and Fmoc-(Pro-Hyp-Gly)-OH were obtained from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). Boc-Ser-(Fmoc-Gly)-OH was obtained from Iris Biotech GmbH (Marktredwitz, Germany). Boc-Thr-(Fmoc-Gly)-OH, ethanedithiol, and *m*-cresol were obtained from Sigma Aldrich (St. Louis, MO, USA). *N,N'*-diisopropylcarbodiimide (DIC), *N*-hydroxybenzotriazole (HOBt), piperidine, acetic acid (AcOH), acetic anhydrate, and pyridine were obtained from Wako Pure Chemical Industries (Osaka, Japan). D-biotin, *N, N*-dimethylformamide (DMF), and trifluoroacetic acid (TFA) were obtained from Nacalai Tesque (Kyoto, Japan). Fmoc-NH-PEG_2-CH_2CH_2-OH and thioanisole were obtained from Tokyo Chemical Industry (Tokyo, Japan).

Solid phase peptide synthesis

All peptides were synthesized by the Fmoc-based solid phase method on Rink amide resin. Fmoc amino acids were condensed in the presence of DIC and HOBt in DMF for 2 h. The protected amino acids used were Fmoc-Tyr(tBu)-OH, Fmoc-Pro-OH, Fmoc-Hyp(tBu)-OH, Fmoc-Gly-OH, Fmoc-Ser(tBu)-OH, Fmoc-Cys(Acm)-OH, and Fmoc-β-Ala-OH (5 eq); Boc-Ser-(Fmoc-Gly)-OH and Boc-Thr-(Fmoc-Gly)-OH (2.5 eq); and Fmoc-(Pro-Hyp-Gly)-OH and Fmoc-Lys(Mtt)-OH (3 eq). DIC and HOBt were used in equivalent amounts to each amino acid. The Fmoc group was removed with 20% or 30% (v/v) piperidine in DMF for 15 min. The *N*-terminus was acetylated with acetic anhydride (20 eq) and pyridine (20 eq) in DMF for 30 min. For the biotinylation of the *N*-terminus or side chain of Lys, Fmoc-NH-PEG_2-CH_2CH_2-OH (2.5 eq), used as a linker, was condensed by the same method used for the Fmoc amino acids. D-biotin was condensed in the presence of DIC and HOBt in dimethyl sulfoxide (DMSO) for 2 h. The Mtt group was removed from the side chain of Lys with dichloromethane (DCM)/1,1,1,3,3,3-hexafluoro-2-propanol/2,2,2-trifluoroethanol/triethylsilane (13/4/2/1, v/v) for 2 h. Peptides were cleaved from the resin with TFA/H_2O/*m*-cresol/thioanisole (85/5/5/5, v/v) for 2 h.

Cyclic CMPs were synthesized following a similar method to the single-strand CMPs. Boc-Ser-(Fmoc-Gly)-OH was condensed with DIC and HOBt in DCM. The Fmoc group of the second amino acid residue from the *N*-terminal end next to the ester bond was removed using 1-methylpyrrolidine (25% v/v)/hexamethyleneimine (2% v/v)/HOBt (3% w/v) in *N*-methylpyrrolidone (NMP): DMSO (1:1).[1] The

peptides were cleaved from the resin with TFA/H_2O/*m*-cresol/thioanisole/ethanedithiol (82.5/5/5/5/2.5, v/v) for 2 h. Cyclization of the peptides was performed using 1 mg/mL I_2/80% AcOH: 8 M guanidinium chloride (1:3) for 1 h at room temperature and the reaction was quenched by ascorbic acid.

The peptides were purified by reversed phase high-performance liquid chromatography (RP-HPLC) using a COSMOSIL 5C_{18}-AR-II column (Nacalai Tesque, 4.6 × 250 mm) at 60 °C with a linear gradient of 0.05% TFA/acetonitrile (CH_3CN) and 0.05% TFA/H_2O. Triple TOF 4600 (AB SCIEX, USA) electrospray ionization mass spectrometry (ESI) MS were used for mass spectrometric analysis.

Measurement of peptide concentration

The concentration of peptide in solution was determined based on the absorption at 280 nm.[2]

NMR analysis

1 (7.8 mg) and **2** (8.7 mg) were dissolved in methanol-d_4 (750 μL). NMR spectra were recorded at 298 K using an AVANCE 600 spectrometer (Bruker BioSpin, USA).

O-to-*N* acyl migration under physiological conditions

Compound **3** in 1 mM AcOH/H_2O was incubated in 90% fetal bovine serum (FBS) at 37 °C for 5 min. TFA (12.5%) was added to the solution to precipitate serum protein. Following centrifugation at 15,000 rpm for 10 min, the supernatants were analyzed by RP-HPLC.

Fourier transform infrared Spectroscopy (FT-IR)

FT-IR spectra of **1**, **2**, **3** and **4** were measured with a FT/IR-4200 (JASCO, Tokyo, Japan). The spectra of **8** and **9** were measured with an IRSpirit (Shimadzu, Kyoto, Japan). Peptide sample (approximately 500 μg) was mixed with KBr (approximately 120 mg) and then pressed at 3 tons for 1 min to obtain a pellet.

Circular dichroism (CD) spectroscopy

CD spectra were measured by a J-820 CD spectropolarimeter (JASCO) equipped with a Peltier thermo controller using a 0.5 mm quartz cuvette. Compounds **3**, **4**, **8** and **9** (0.5 mg/mL) in 0.05% TFA were heated at 85 or 95 °C for 5 min, incubated at room temperature for 10 min and stored at 4 °C overnight. The CD spectra from 260 to 190 nm of the CMP solutions were recorded at 4 °C. Temperature-dependent CD spectra measurements at 225 nm were performed from 4 to 85 °C. The obtained molar ellipticity was differentiated, and the temperature at which the maximum value was obtained was taken as the melting temperature (T_m).

Kinetics of *O*-to-*N* acyl migration

Compounds **3** and **8** (1.5 mg/mL) in 0.05% TFA/H_2O were incubated at 37 °C for 20 min. Phosphate buffer (100 mM; pH 7.4) was added to obtain a CMP concentration of 1.0 mg/mL. The solution was incubated at 37 °C and quenched by 0.05% TFA/H_2O at the desired time points. Then, the solution was analyzed using RP-HPLC.

The conversion rate was determined from:

$$\text{conversion rate} = \frac{A_{amide}}{A_{ester}+A_{amide}} \times 100 \quad \text{(Eq.1)}$$

where A_{amide} and A_{ester} represent the peak areas of the ester and amide forms, respectively. Data for initial 1 min were fitted to first-order kinetics, the reaction rate content (k_1) was determined from:

$$\ln[ester] = \ln[ester]_0 - k_1 t \quad \text{(Eq.2)}$$

where [ester] represents the concentration of the ester form.

Triple helical folding analysis of the CMPs

Compound **4** (1.0 mg/mL) in 33.3 mM phosphate buffer (pH 7.4)/0.05% TFA/H_2O was heated at 95 °C for 5 min, incubated at room temperature for 10 min, and stored at 4 °C overnight. The solution was incubated at 37 °C for 20 min and the CD spectra from 260 to 190 nm was measured and the θ_{225} was defined as θ_f. Then, the solution was incubated at 85 °C for 20 min and the CD spectra from 260 to 190 nm was measured and the θ_{225} was defined as θ_u. Time-dependent CD spectra measurements at 225 nm were performed at 37 °C for 2 h. Cooling from 85 to 37 °C took 2 min.

The kinetics of the triple helical folding of the CMPs were analyzed as follows[3]:

The fraction folded (F) was calculated from:

$$\mathrm{F} = \frac{\theta_{obs}-\theta_u}{\theta_f-\theta_u} \quad \text{(Eq.3)}$$

where θ_{obs} represents the observed folding ellipticity and [monomer] represents the concentration of monomer determined from:

$$[\text{monomer}] = (1-\mathrm{F})[\text{monomer}]_0 \quad \text{(Eq.4)}$$

The data from 2 to 45 min gave a good fit to Eq.5, the triple-helical folding of CMP was a third-order reaction under these conditions.

$$\frac{1}{[monomer]^2} = \frac{1}{[monomer]_0^2} + 6k_2t \quad \text{(Eq.5)}$$

Enzyme-linked immunosorbent assay

Type I collagen (10 μg/mL) (AteloCell I-PC, KOKEN, Tokyo, Japan) in 10 mM AcOH/H_2O was heated at 95 °C for 5 min to denature. 96-Well microplates (Thermo Fisher Scientific) were coated by the heat-denatured collagen solution and blocked with 0.5% skim milk/buffer (20 mM HEPES-Na (pH 7.5), 100 mM NaCl, and 0.005% Tween-20) at room temperature for 1 h. After washing with 1 mM AcOH/H_2O, compound **6** in PBS was added with or without heating at 95 °C for 5 min and chilled with ice water for 30 s. Compounds **5** and **10** in 1 mM AcOH/H_2O were added to the plate and 5 μL of 10 × PBS was added.

After incubation at 37 °C for 1.5 h and washing with buffer, horseradish peroxidase (HRP)-conjugated streptavidin (1:3000 dilution in 0.5% skim milk/buffer; Thermo Fisher Scientific) was added and the mixture was incubated on ice for 30 min and washed with buffer. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (0.5 mg/mL) in phosphate-citrate buffer [0.2 M phosphate and 0.1 M citrate (pH 5.0)] containing 0.05% H_2O_2 was added and the mixture was incubated at 37 °C for 30 min.

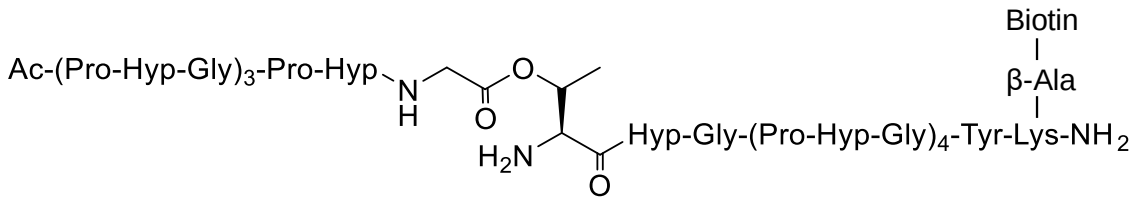

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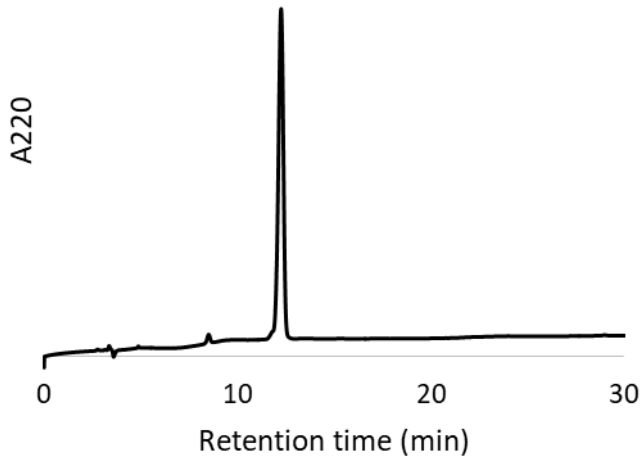
Figure S1. A structure of compound **7**.



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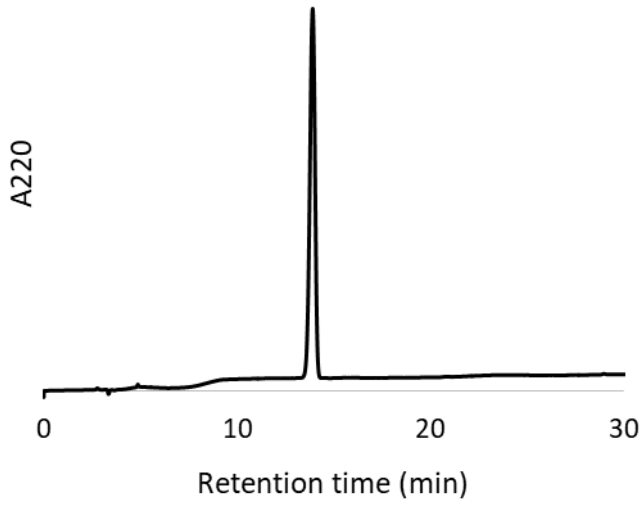

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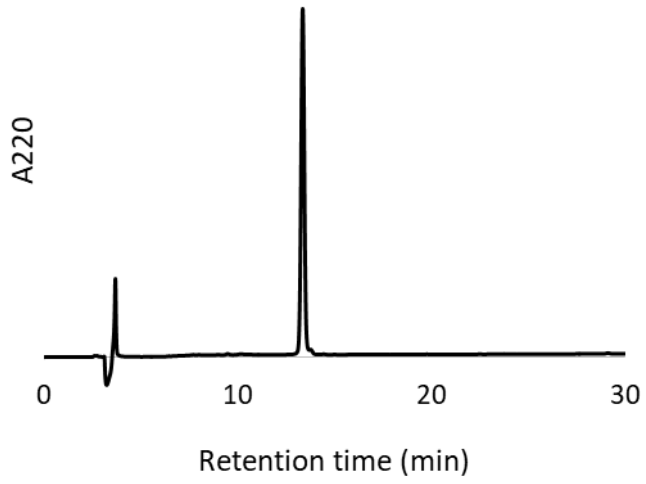
Figure S2. HPLC analysis of the purified peptides. COSMOSIL $5C_{18}$-AR-II column (size 4.6 × 250 mm), gradient 0%-20% for **1** and **2** MeCN/H_2O (0.05% TFA), or 10%–30% for other peptides, 30 min at 60 °C.



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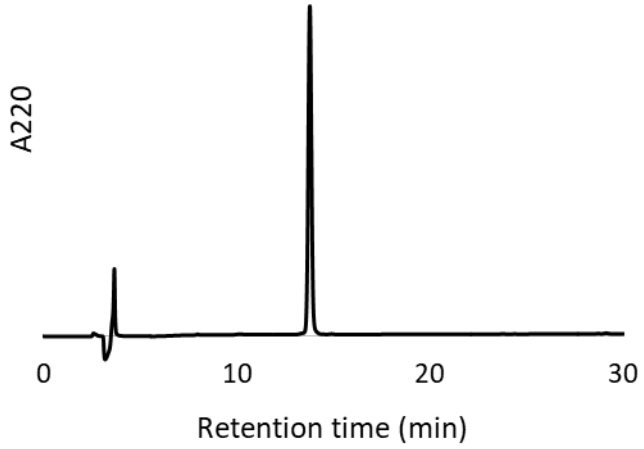

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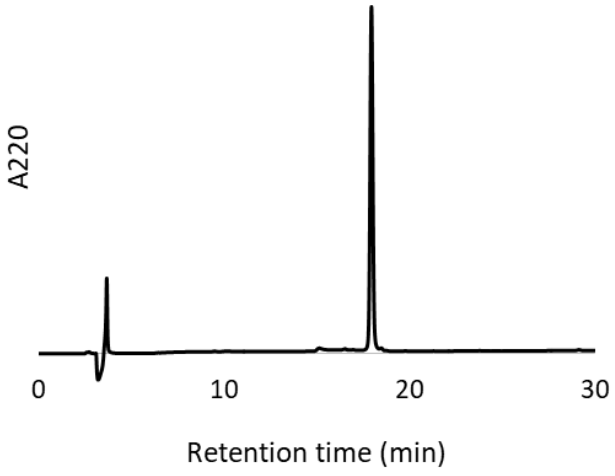

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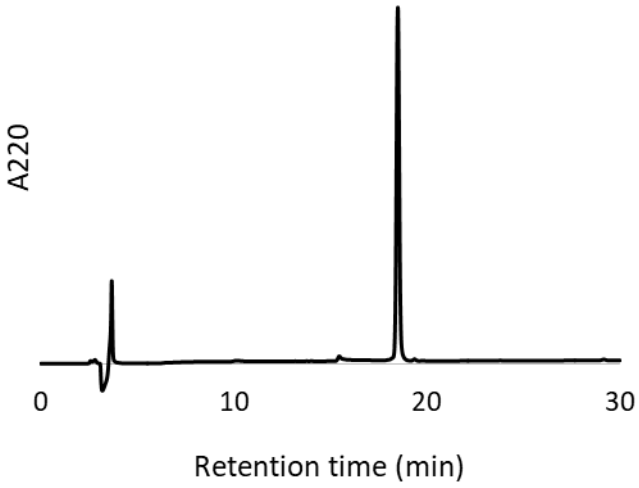
Figure S2. (Continued)



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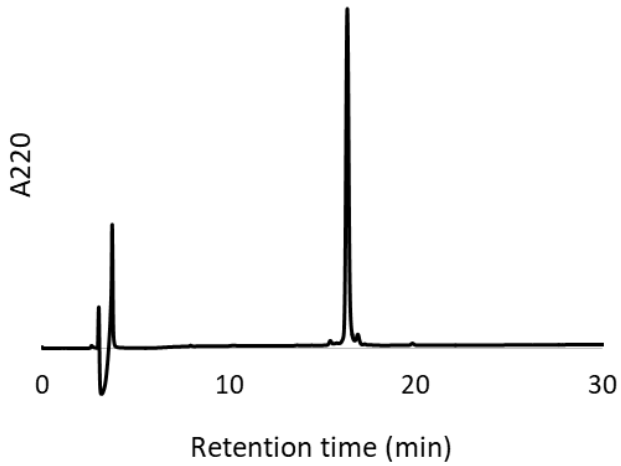

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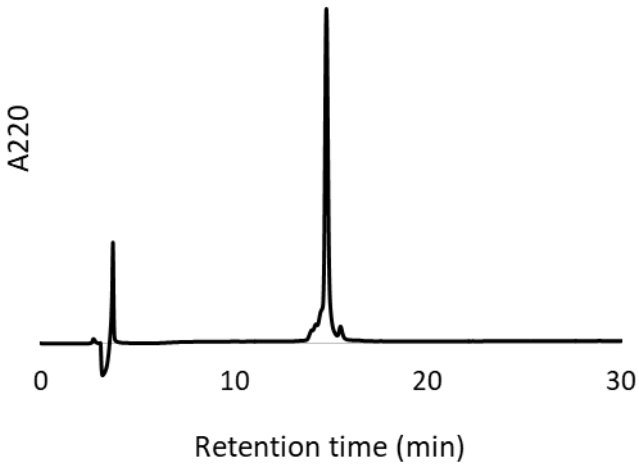

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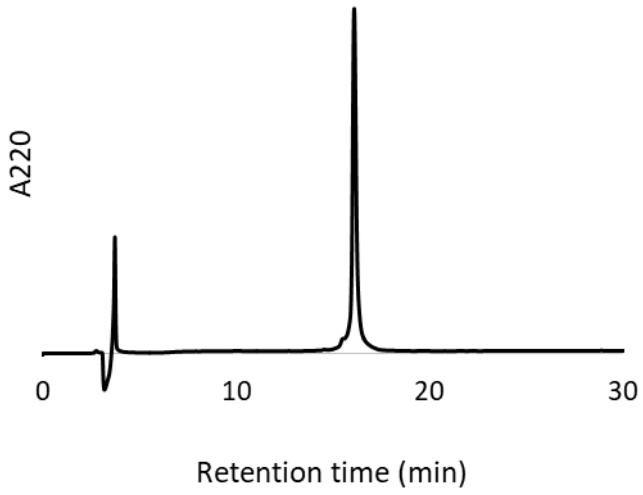
Figure S2. (Continued)



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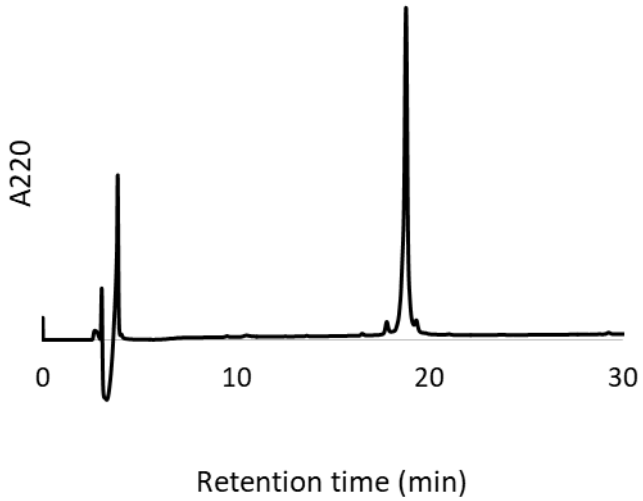


10

Figure S2. (Continued)

Table S1. HPLC retention time and analytical mass data of synthesized peptides.

peptide	t_R (min)	Calculated *m/z*	Found
1	12.3[a]	584.267[c]	584.272
2	13.9[a]	584.267[c]	584.273
3	13.4[b]	873.065[d]	873.068
4	13.8[b]	873.065[d]	873.068
5	18.0[b]	1040.481[d]	1040.482
6	18.5[b]	1040.481[d]	1040.482
7	16.3[b]	1019.474[d]	1019.478
8	14.8[b]	1245.559[e]	1245.561
9	16.1[b]	1245.559[e]	1245.559
10	18.8[b]	1381.623[e]	1381.622

HPLC conditions: COSMOSIL 5C_{18}-AR-II column (size 4.6 × 250 mm), 60 °C, 1 mL/min, gradient [a]0-20% or [b]10-30% MeCN/H_2O (0.05% TFA) over 30 min.

[c]$[M+H]^+$, [d]$[M+3H]^{3+}$, [e]$[M+4H]^{4+}$.

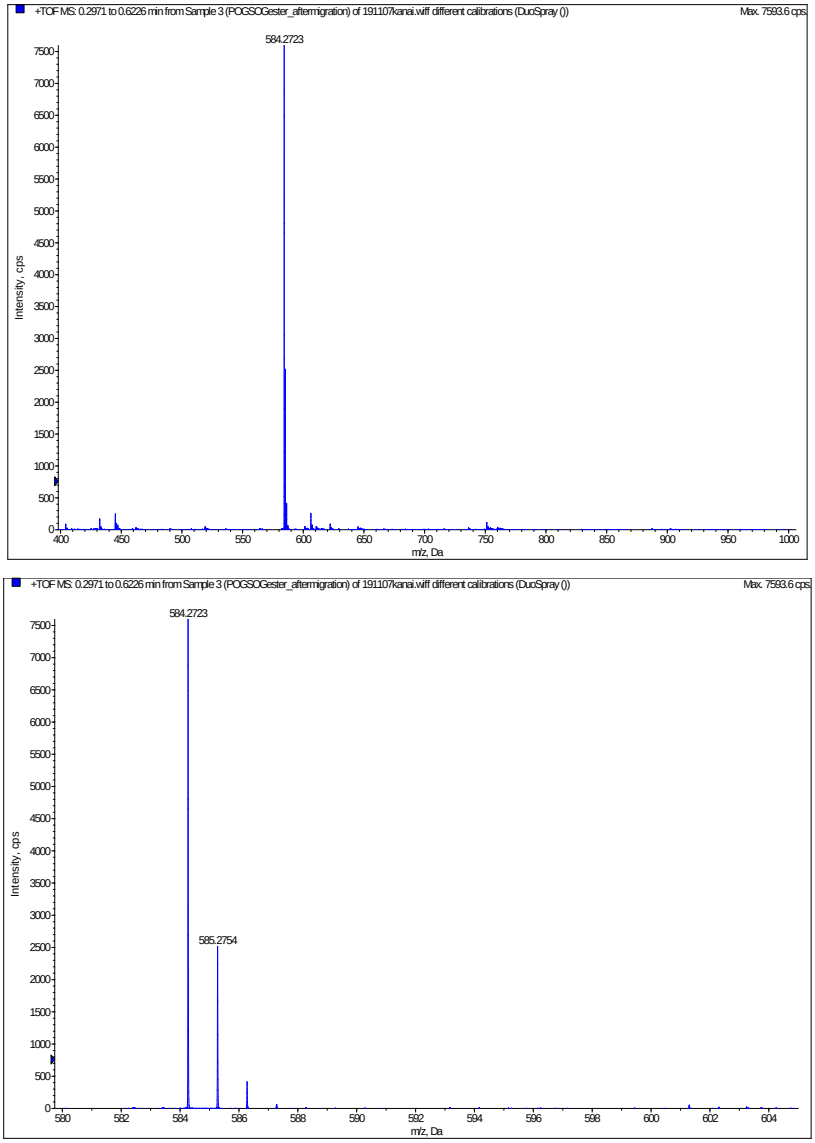


Figure S3. ESI-MS analysis of a converted compound of **1** in 0.1 M phosphate buffer (pH 7.4). The found *m/z* (584.272) corresponded with the calculated one of **2** ($[M+H]^+$: 584.267).

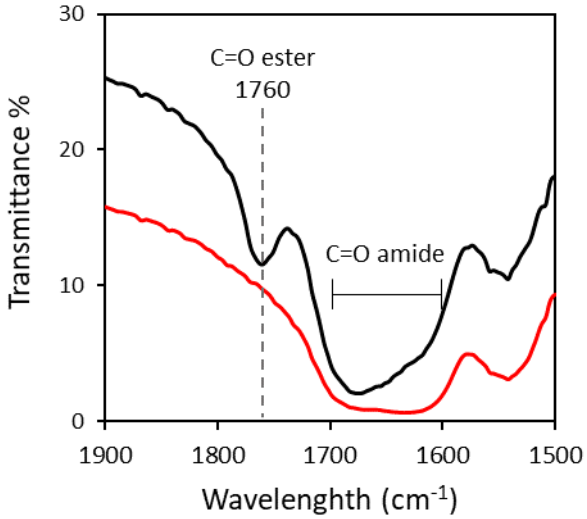
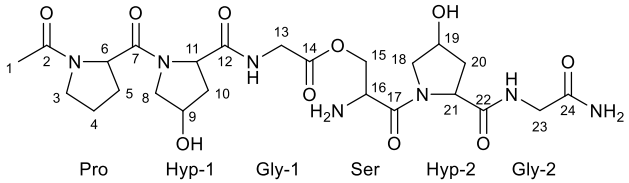


Figure S4. FT-IR spectra of **1** (black) and **2** (red).

Table S2. ^{1}H and ^{13}C NMR spectral data for **1** (methanol-d_4).



1

Residue	position	δ_H	δ_C
Ac	1	2.09	22.3
	2		172.2
Pro	3	3.63	49.5
	4	2.07	25.8
		1.97	
	5	2.27	29.8
		2.03	
	6	4.65	59.9
	7		173.4
Hyp-1	8	3.76	56.5
	9	4.50	71.1
	10	2.24	39.0
		2.07	
	11	4.56	60.7
	12		175.2
Gly-1	13	4.06	42.2
		3.98	
	14		170.5
Ser	15	4.62	62.5
		4.41	
	16	4.61	53.2
	17		166.4
Hyp-2	18	3.82	57.0
		3.73	
	19	4.56	71.1
	20	2.29	39.0
		2.10	
	21	4.59	61.4
	22		174.2
Gly-2	23	3.74	43.4
		3.95	
	24		174.4

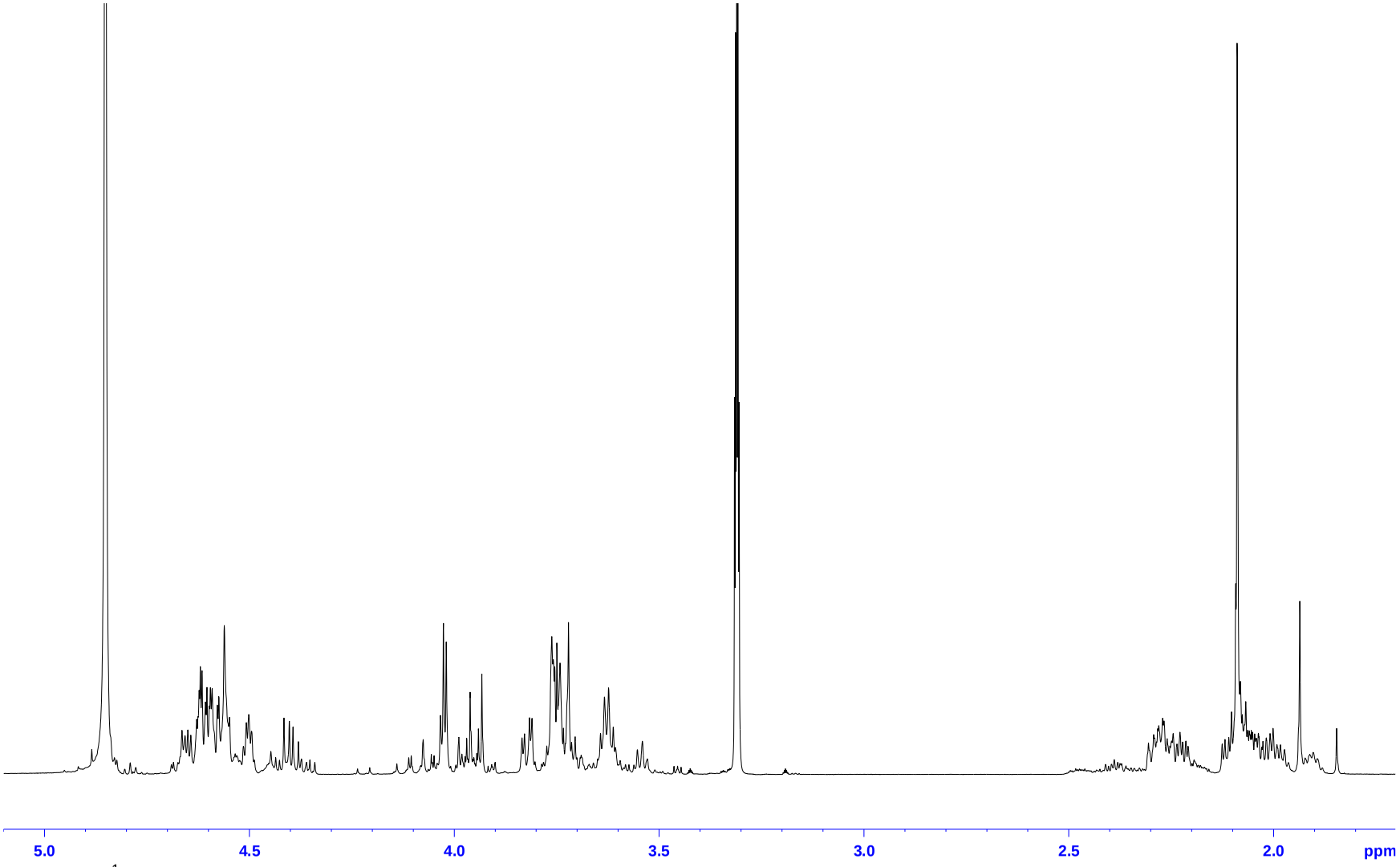


Figure S5. ^{1}H NMR spectrum of **1** (600 MHz, methanol-d_4).

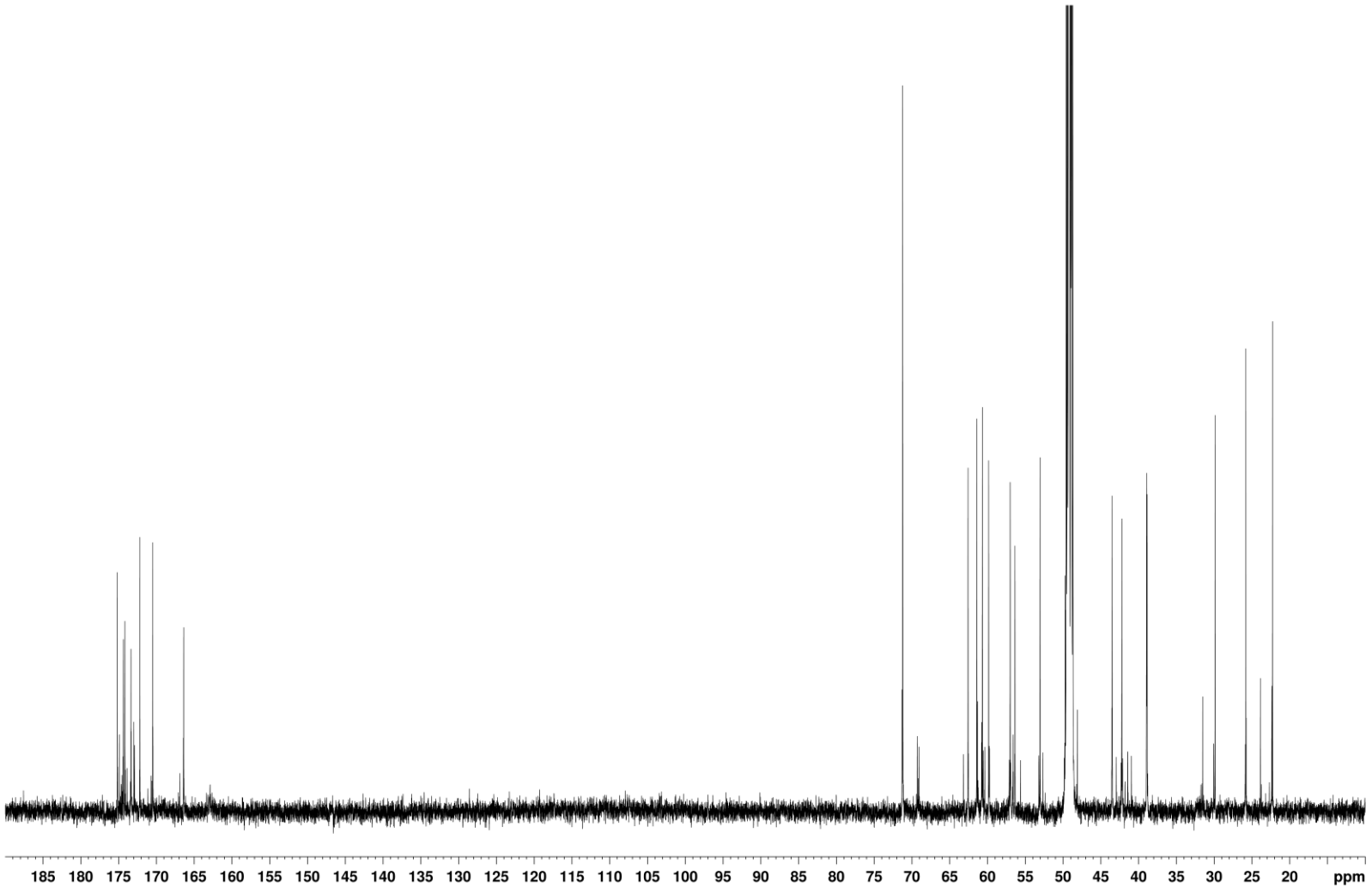


Figure S6. ^{13}C NMR spectrum of **1** (150 MHz, methanol-d_4).

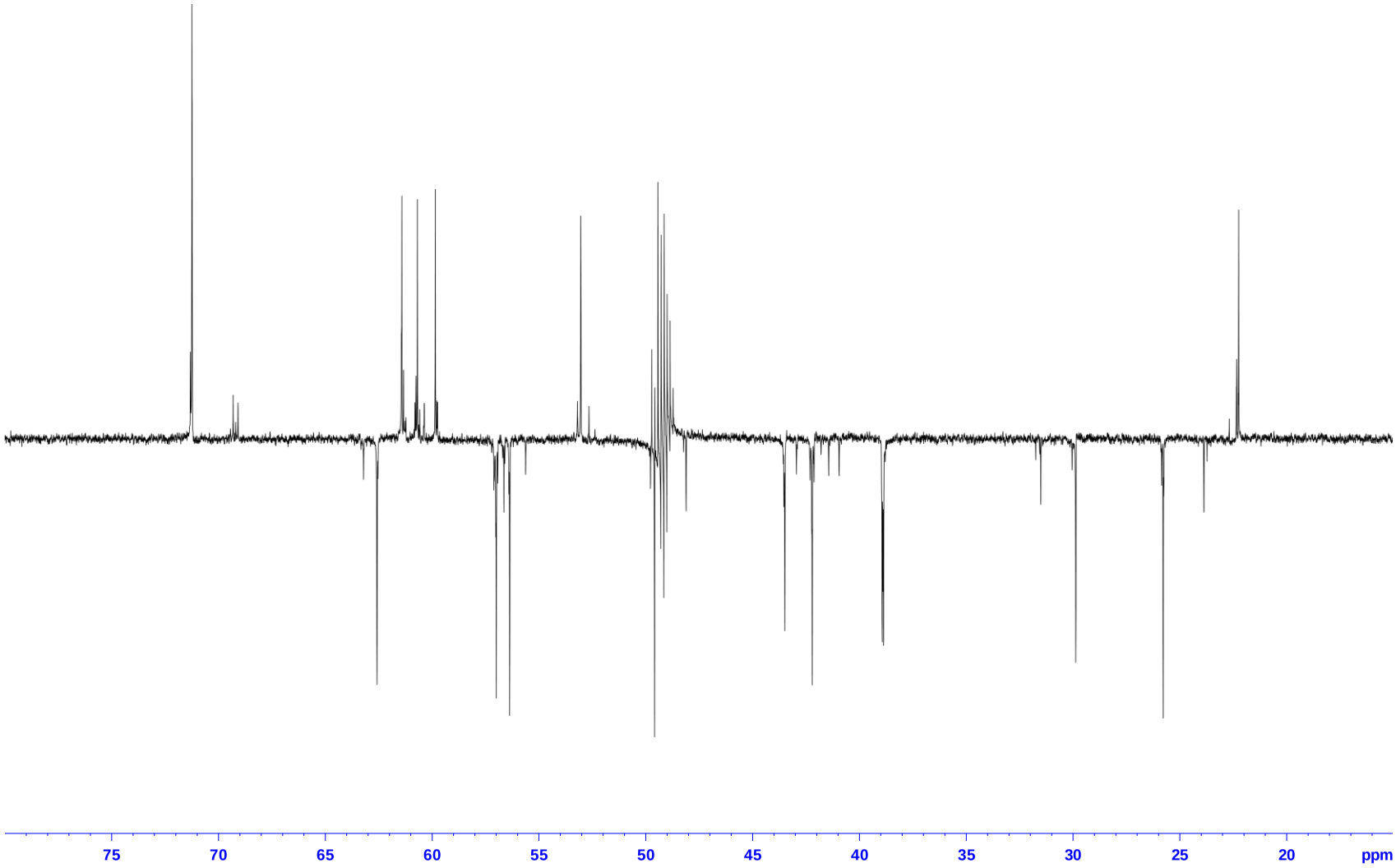


Figure S7. DEPT-135 spectrum of **1** (150 MHz, methanol-d_4).

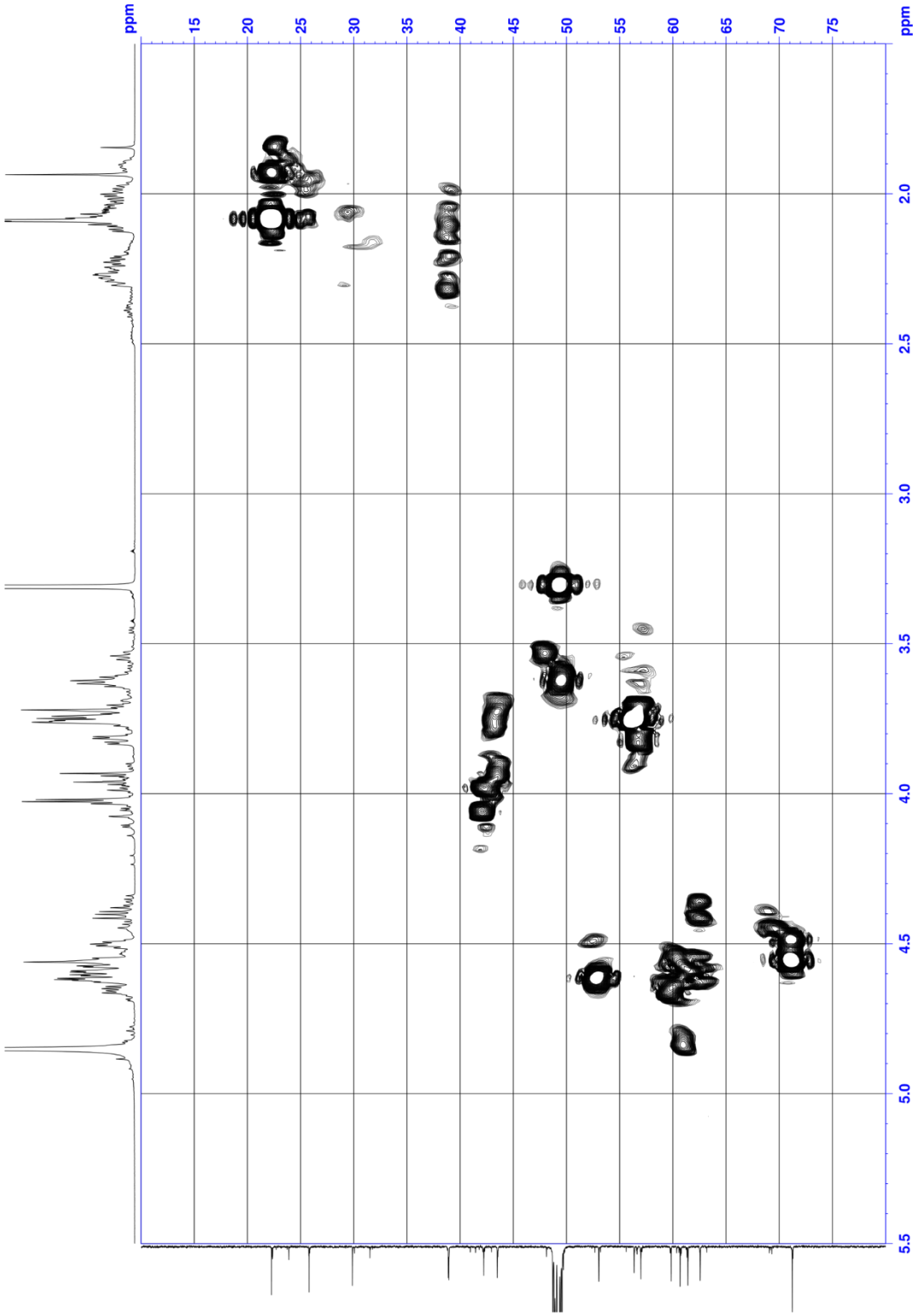


Figure S8. HMQC spectrum of **1** (600 MHz, methanol-d_4).

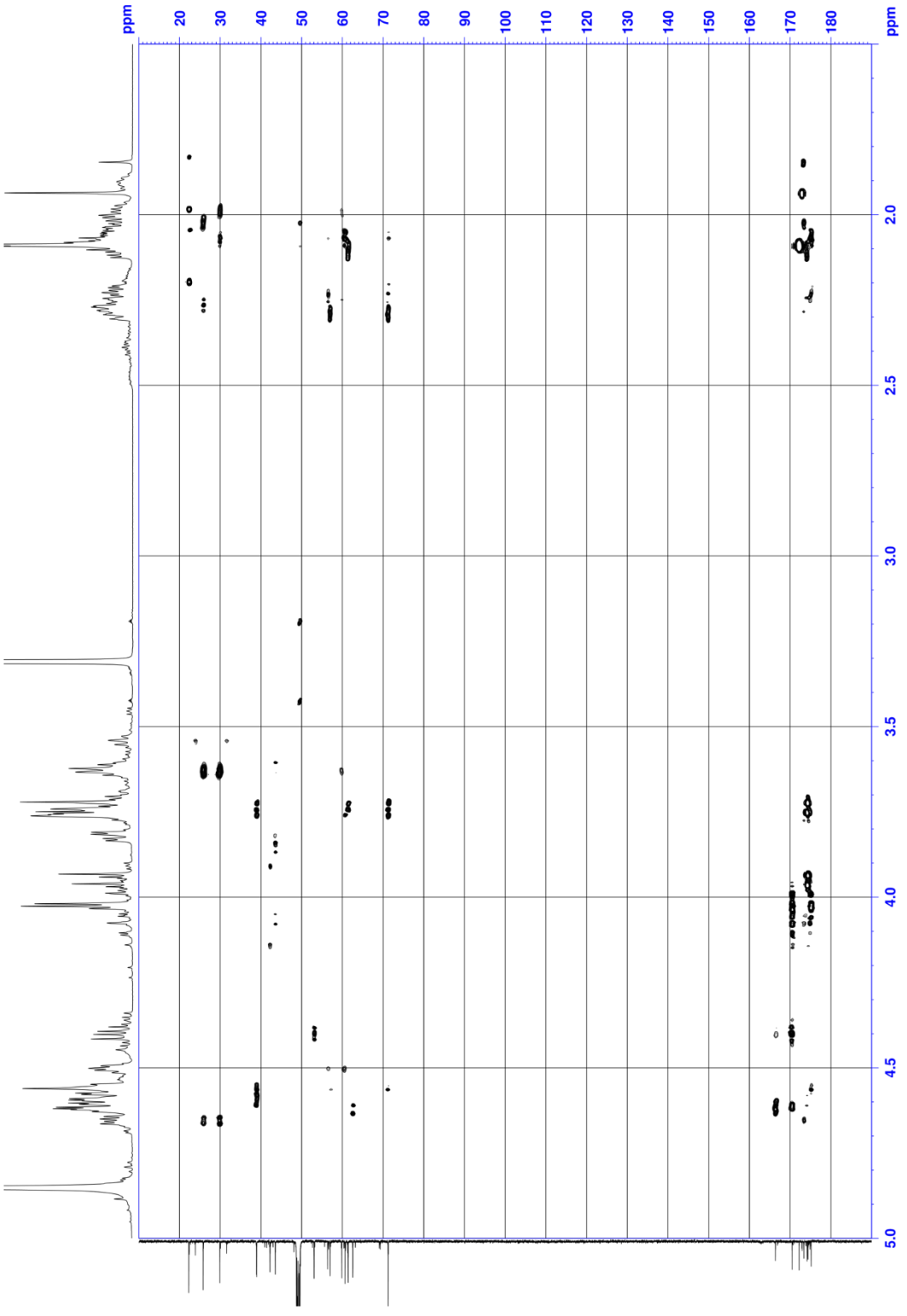


Figure S9. HMBC spectrum of **1** (600 MHz, methanol-d_4).

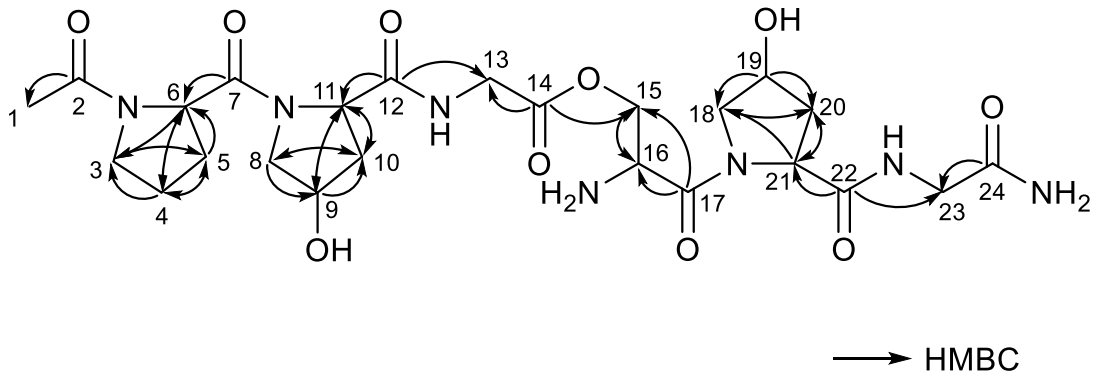


Figure S10. HMBC correlations for **1**.

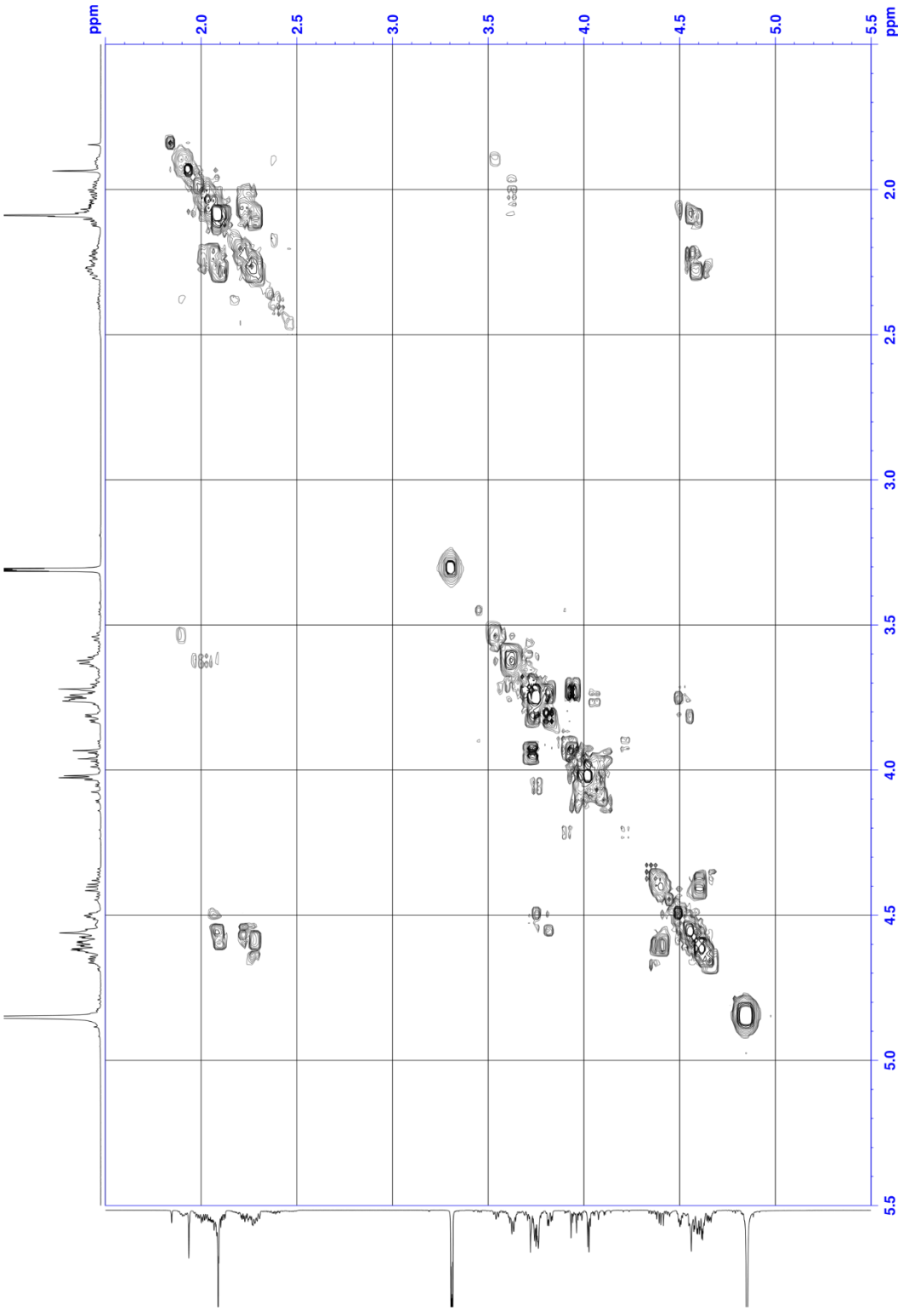
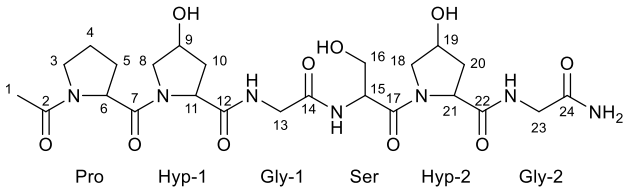


Figure S11. COSY spectrum of **1** (600 MHz, methanol-d_4).

Table S3. ^{1}H and ^{13}C NMR spectral data for **2** (methanol-d_4).



2

Residue	position	δ_H	δ_C
Ac	1	2.07	22.1
	2		172.4
Pro	3	3.67	49.5
		3.59	
	4	2.07	25.8
		1.95	
	5	2.27	29.7
		2.00	
	6	4.65	59.8
	7		173.2
Hyp-1*	8	3.86	57.0
	9	4.56	71.3
	10	2.24	38.8
		2.04	
	11	4.57	61.2
	12		174.9
Gly-1	13	4.10	43.6
		3.65	
	14		171.8
Ser	15	4.74	55.9
	16	3.82	63.0
	17		172.1
Hyp-2*	18	3.78	56.7
	19	4.56	71.5
	20	2.24	38.8
		2.04	
	21	4.57	61.1
	22		175.0
Gly-2	23	3.85	43.5
		3.75	
	24		174.6

*interconvertible

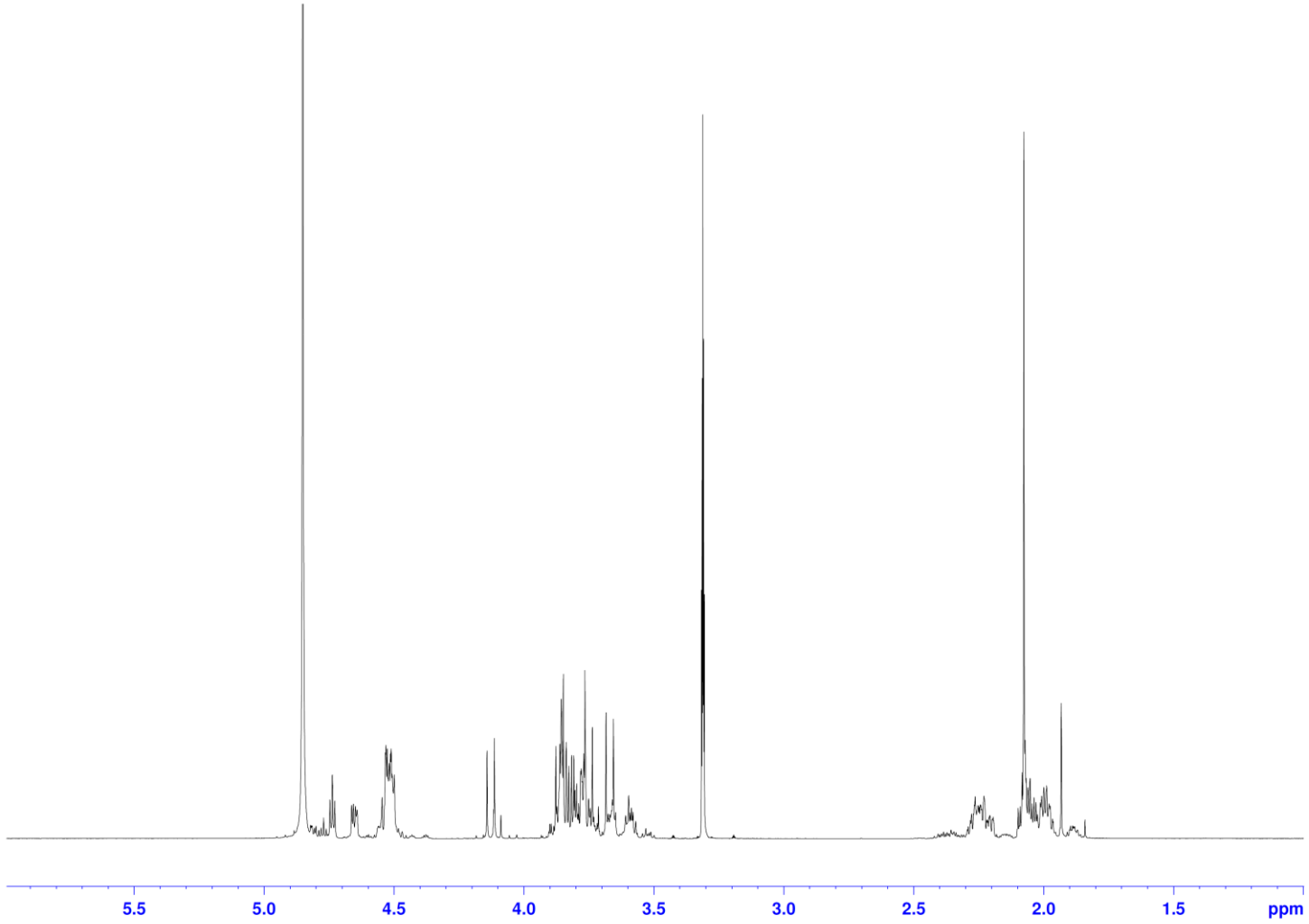


Figure S12. ^{1}H NMR spectrum of **2** (600 MHz, methanol-d_4).

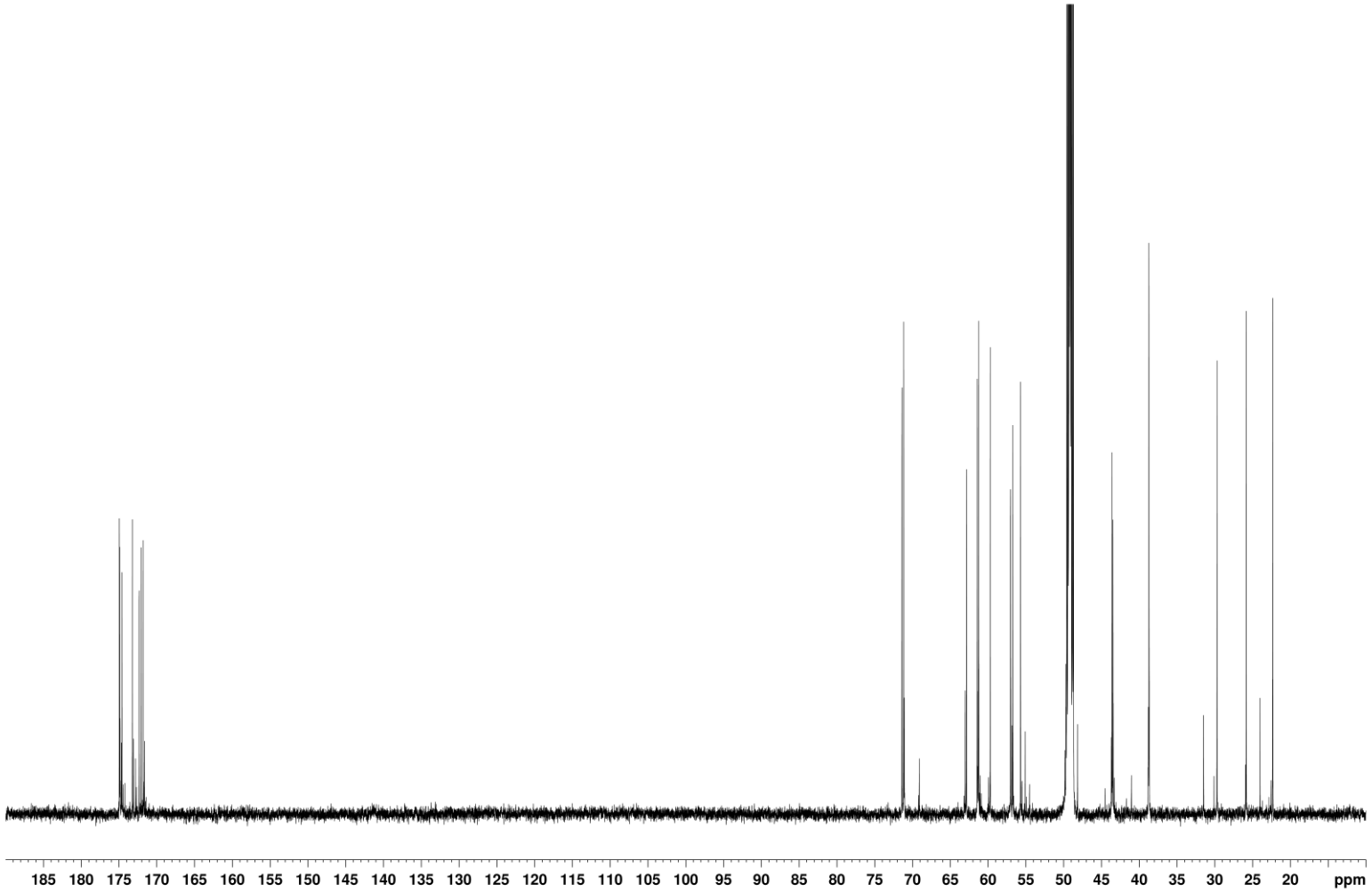


Figure S13. ^{13}C NMR spectrum of **2** (150 MHz, methanol-d_4).

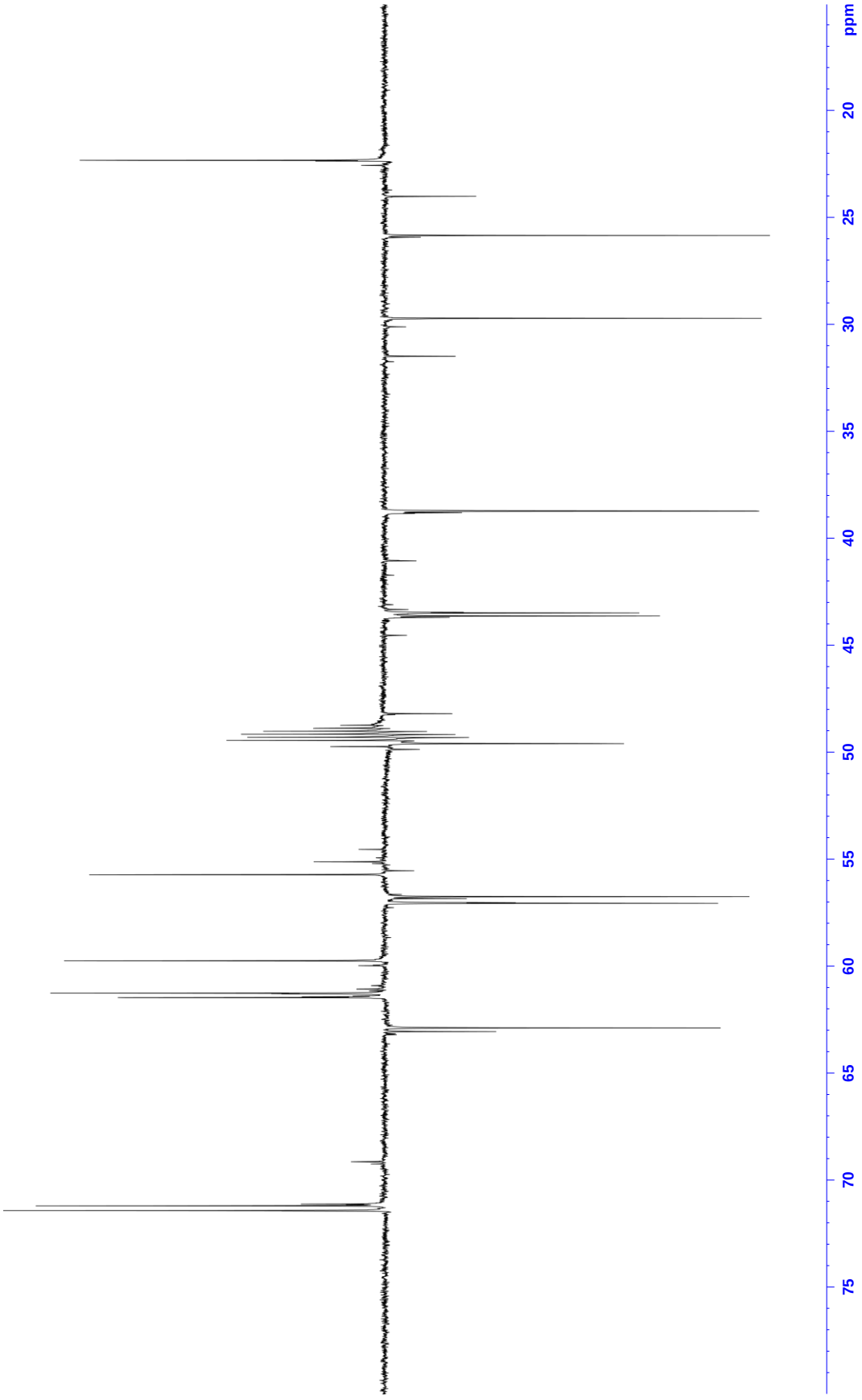


Figure S14. DEPT-135 spectrum of **2** (150 MHz, methanol-d_4).

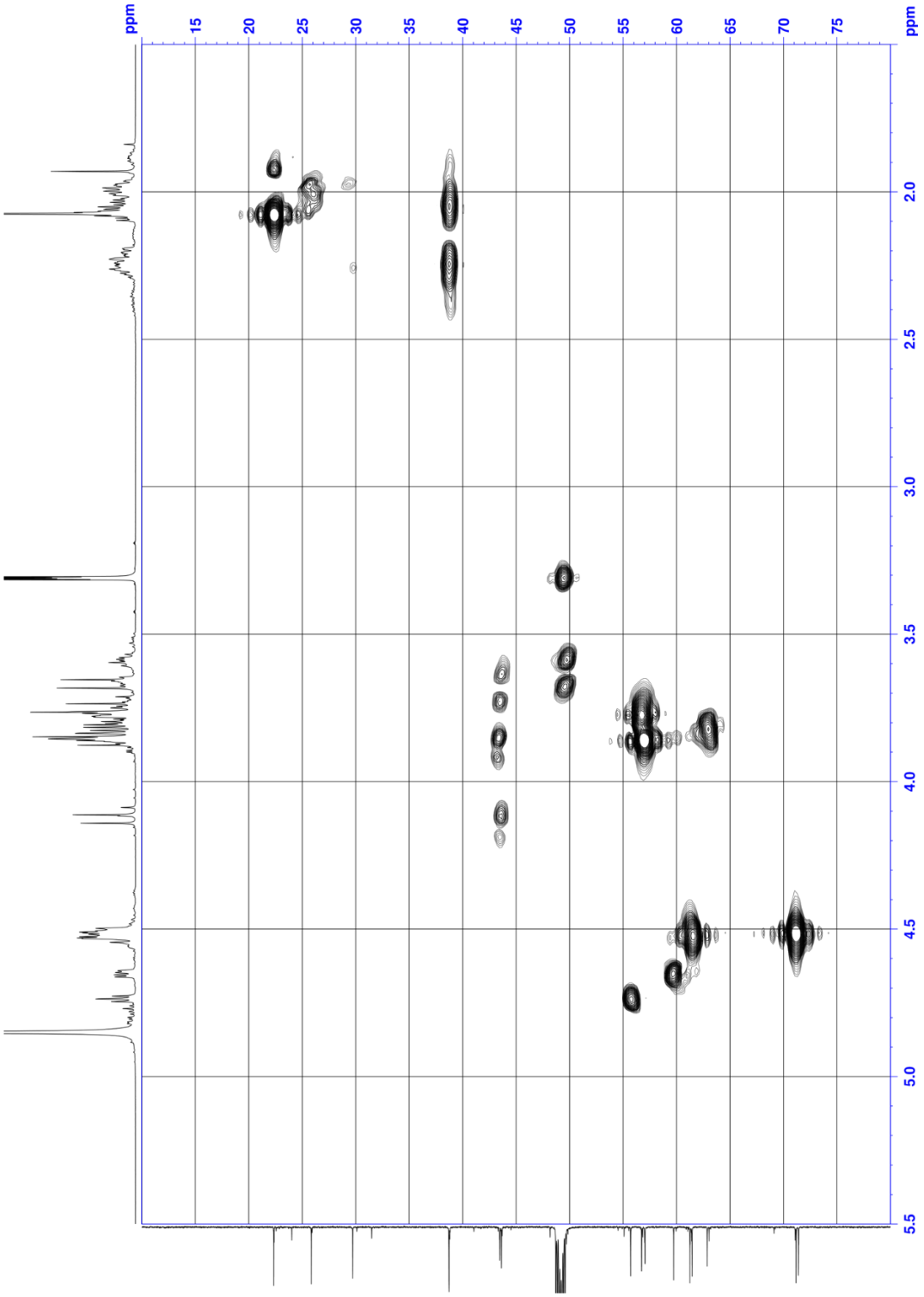


Figure S15. HMQC spectrum of **2** (600 MHz, methanol-d_4).

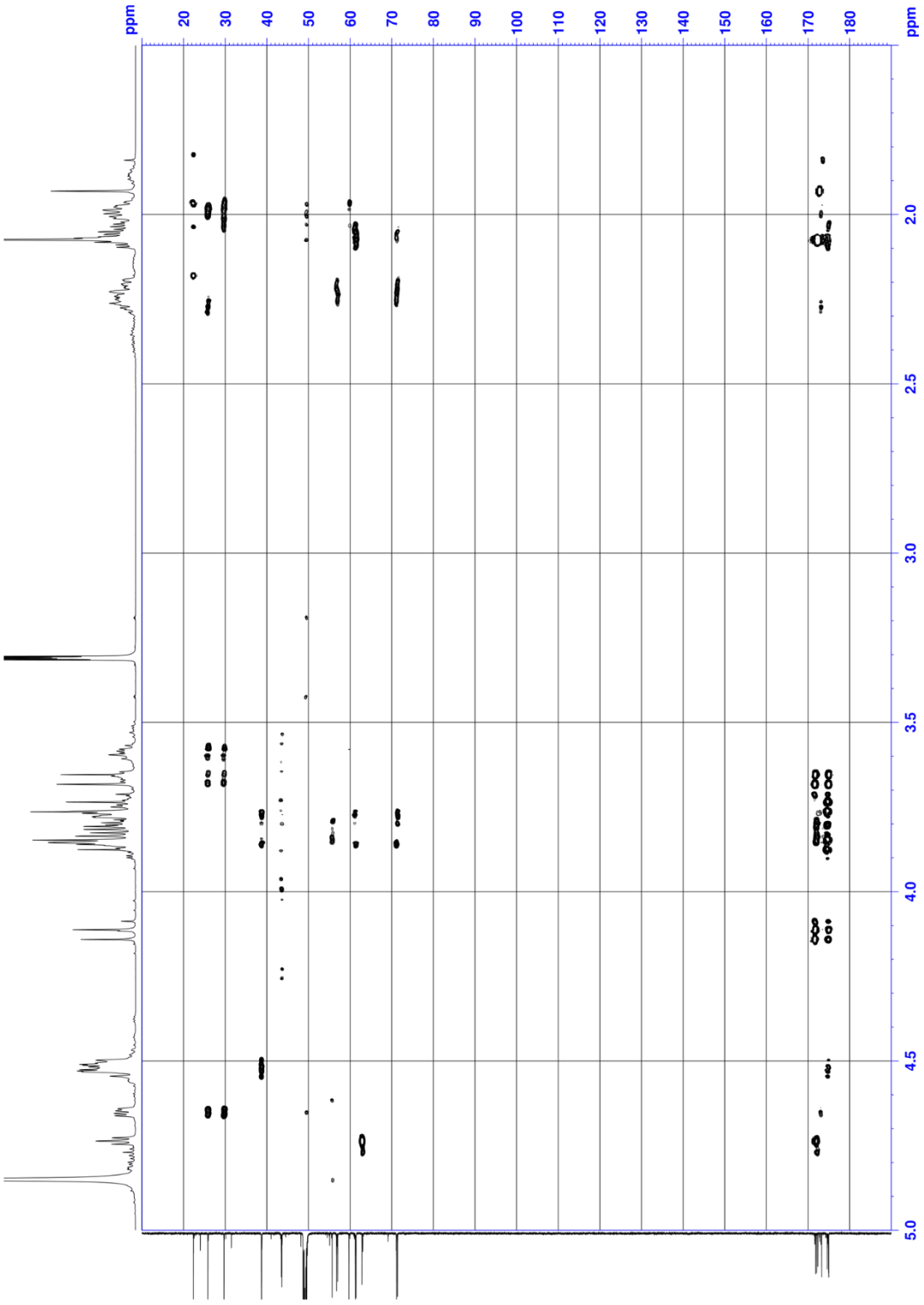


Figure S16. HMBC spectrum of **2** (600 MHz, methanol-d_4).

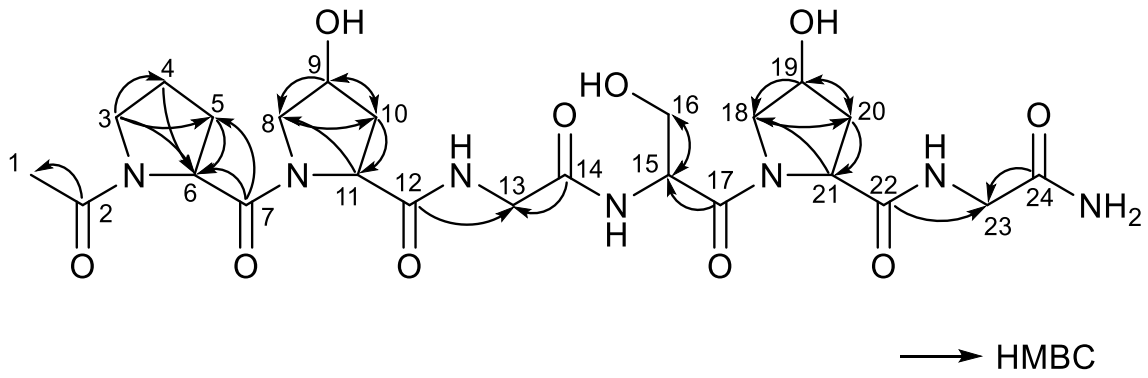


Figure S17. HMBC correlations for **2**.

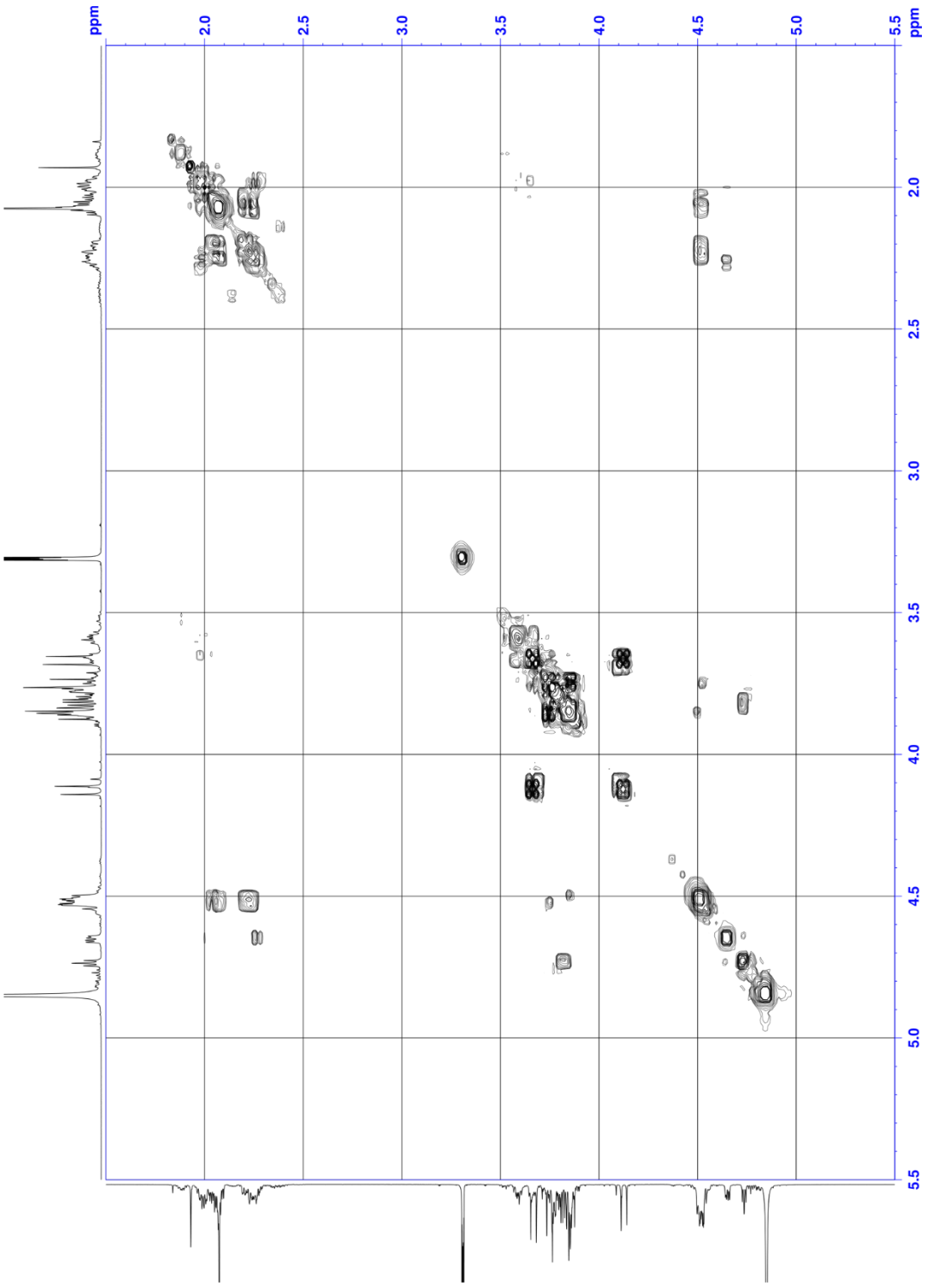
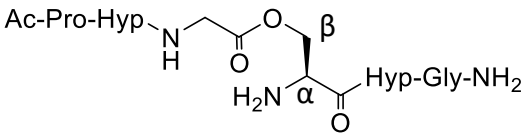
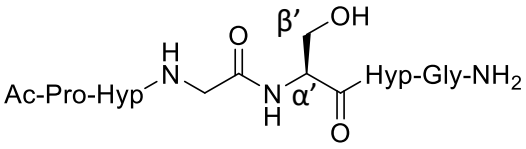


Figure S18. COSY spectrum of **2** (600 MHz, methanol-d_4).



1



2

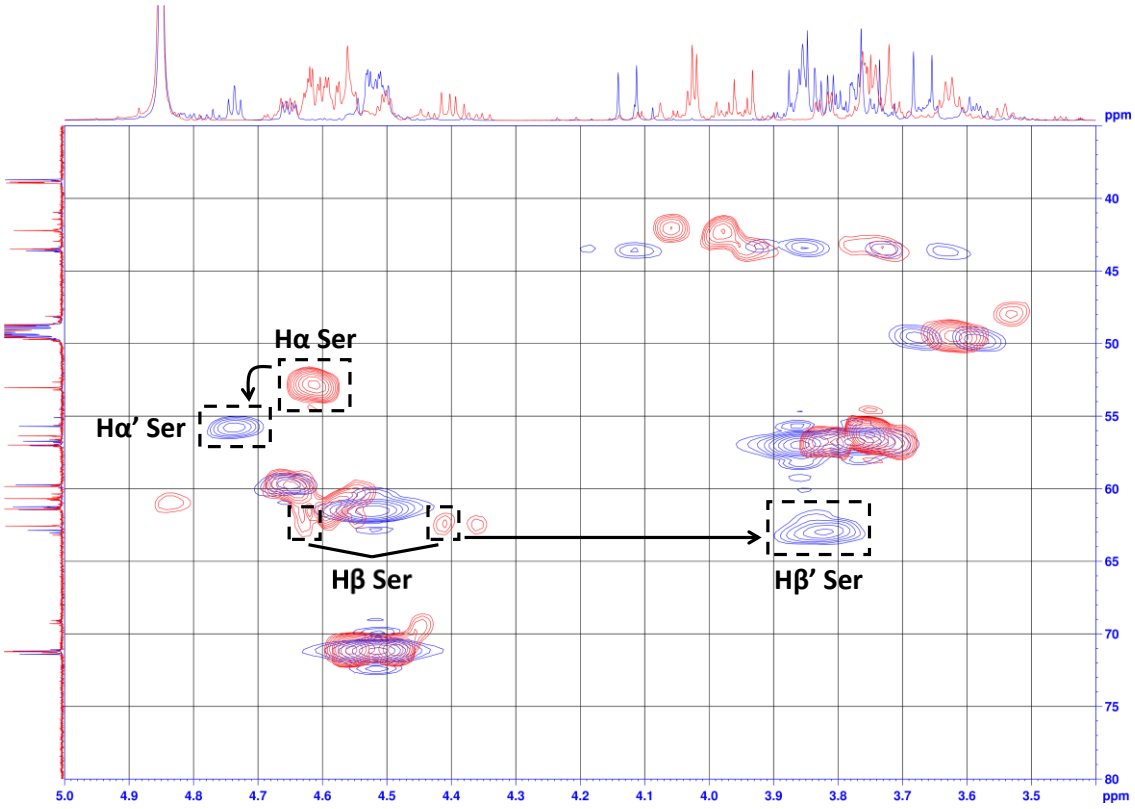
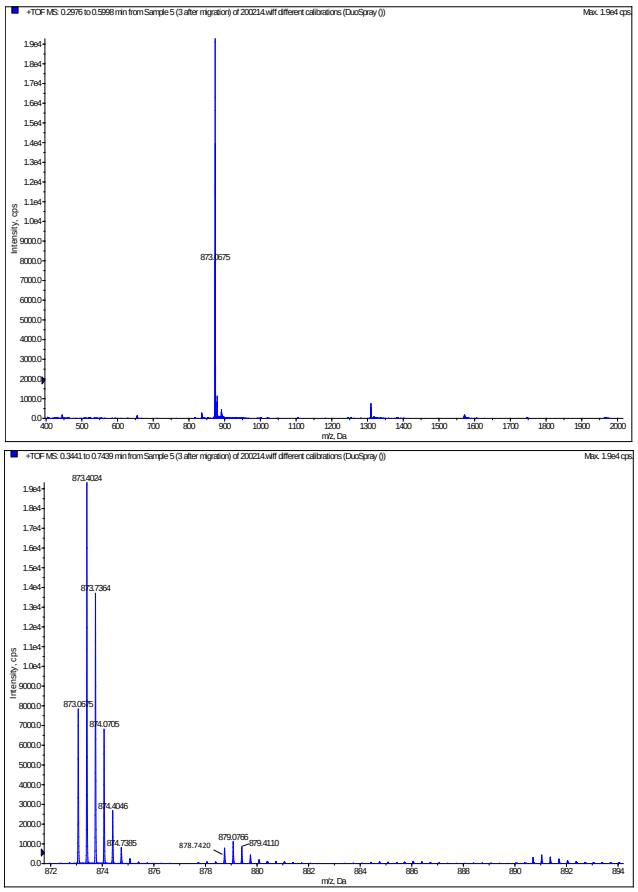


Figure S19. ^{1}H-^{13}C HMQC spectra of **1** (red) and **2** (blue) (600 MHz, methanol-d_4).

(a)



(b)

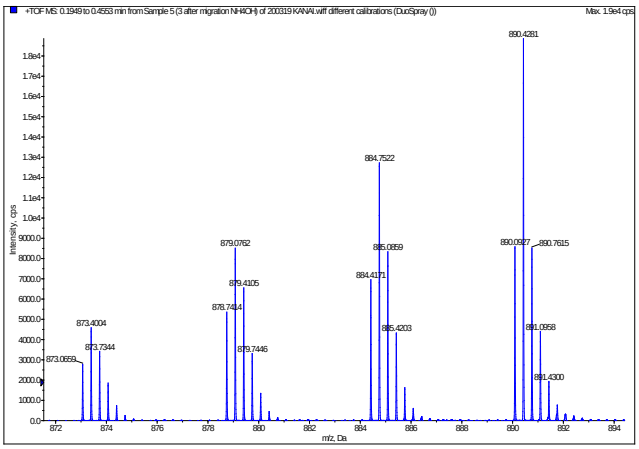


Figure S20. (a) ESI-MS analysis of a converted compound of **3** in PBS. The found *m/z* (873.068 and 878.742) corresponded with the calculated one of **4** ($[M+3H]^{3+}$: 873.065 and $[M+2H+NH_4]^{3+}$: 878.741). (b) ESI-MS analysis of the converted compound of **3** containing 0.005% aqueous ammonia. The found *m/z* (873.066, 878.741, 884.417 and 890.093) corresponded with the calculated ones of **4** ($[M+3H]^{3+}$: 873.065, $[M+2H+NH_4]^{3+}$: 878.741, $[M+H+2NH_4]^{3+}$: 884.416, $[M+3NH_4]^{3+}$: 890.092). Since the relative signal intensity of a minor peak of (a) increased in the presence of NH_4OH, the presence of a NH_4^+ adduct was indicated.

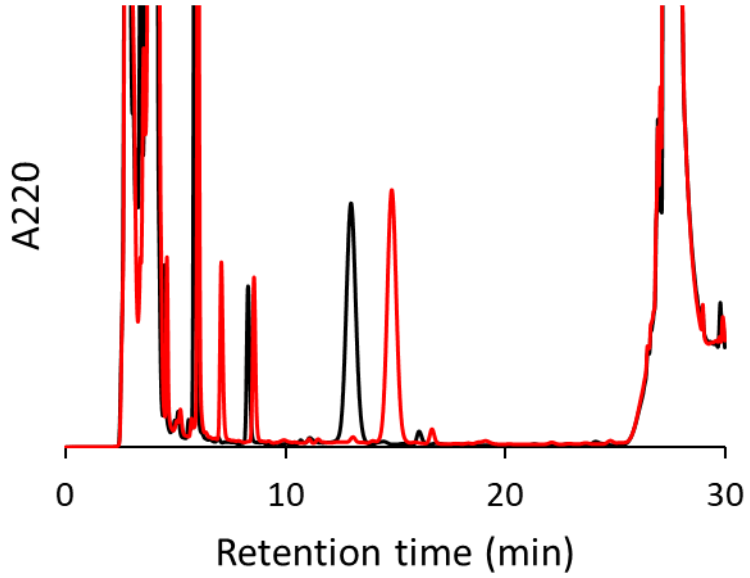


Figure S21. HPLC analysis of the *O*-to-*N* acyl migration of compound **3** in FBS for 0 (black) and 5 (red) min. COSMOSIL 5C_{18}-AR-II column (size 4.6 × 250 mm) at 60 °C, gradient 12%–14% MeCN/H_2O (0.05% TFA) (0–20 min), 14%–90% (20–25 min) and 90% (25–30 min).

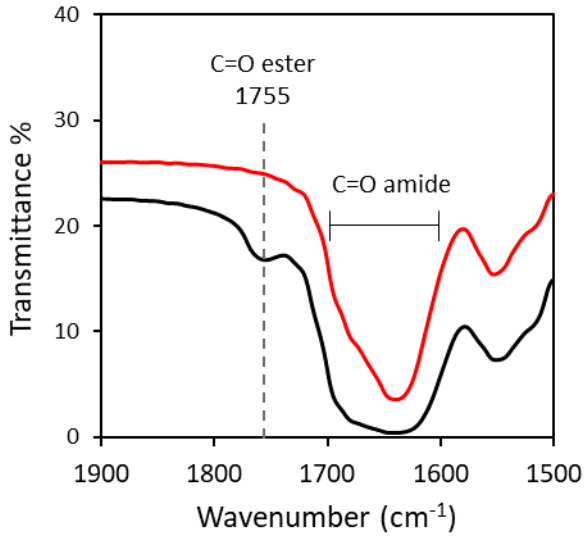
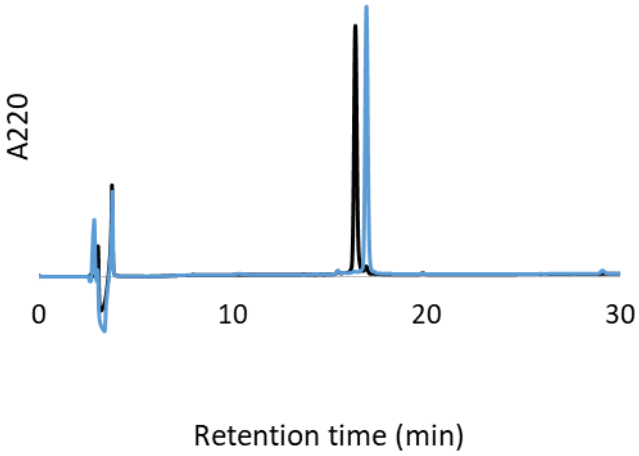


Figure S22. FT-IR spectra of **3** (black) and **4** (red).

(a)



(b)

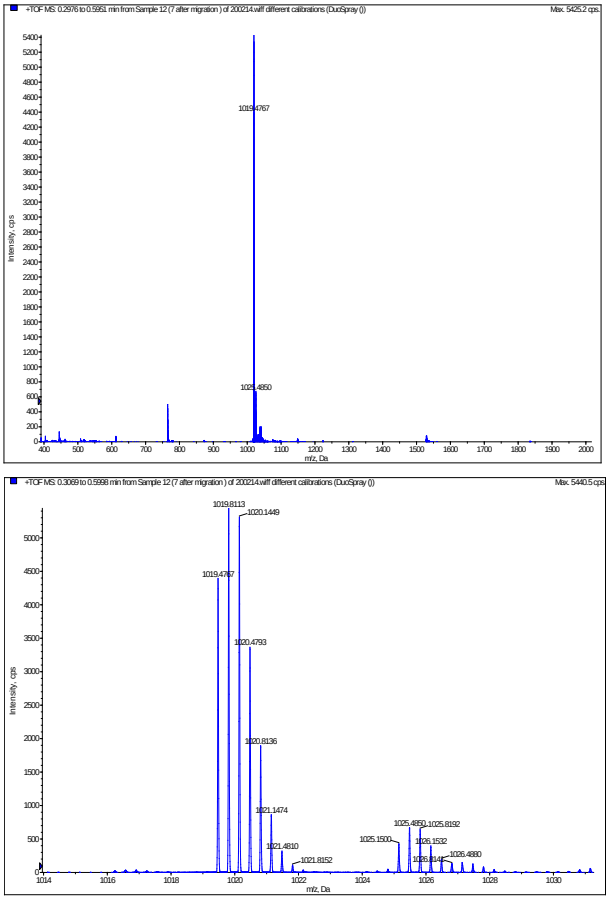


Figure S23. (a) HPLC analysis of the *O*-to-*N* acyl migration of compound **7** in 0.05% TFA/H_2O (black) and in PBS for 10 min (blue). COSMOSIL $5C_{18}$-AR-II column (size 4.6 × 250 mm), gradient 10%–30% MeCN/H_2O (0.05% TFA), 30 min at 60 °C. (b) ESI-MS analysis of a converted compound of **7** in PBS. The retention time of HPLC was different from **7** in 0.05% TFA/H_2O and the found *m*/*z* (1019.477 and 1025.150) corresponded with the calculated ones of the converted compound ($[M+3H]^{3+}$: 1019.474 and $[M+2H+NH_4]^{3+}$: 1025.149).

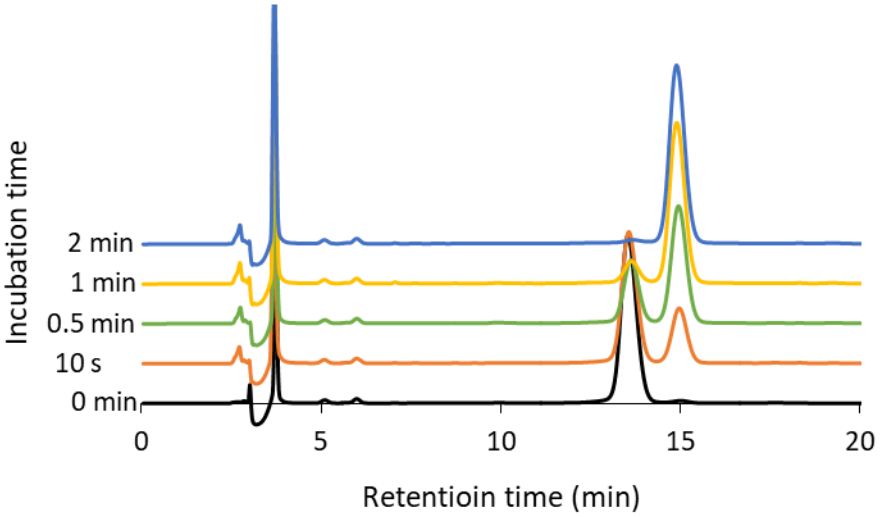
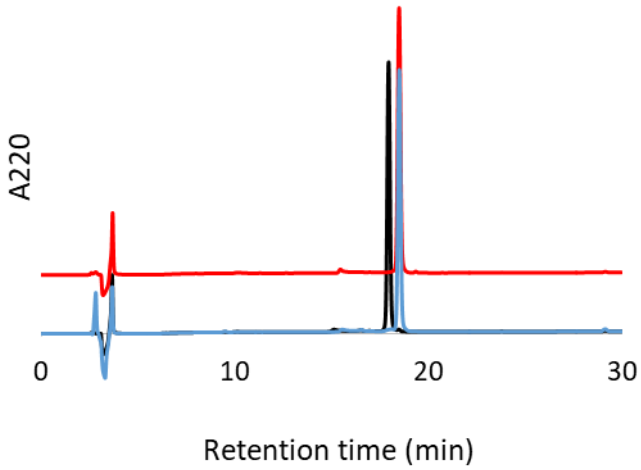


Figure S24. Kinetics of the *O*-to-*N* acyl migration of compound **3** monitored by RP-HPLC. COSMOSIL $5C_{18}$-AR-II column (size 4.6 × 250 mm), gradient 12%–14% MeCN/H_2O (0.05% TFA), 20 min at 60 °C.

(a)



(b)

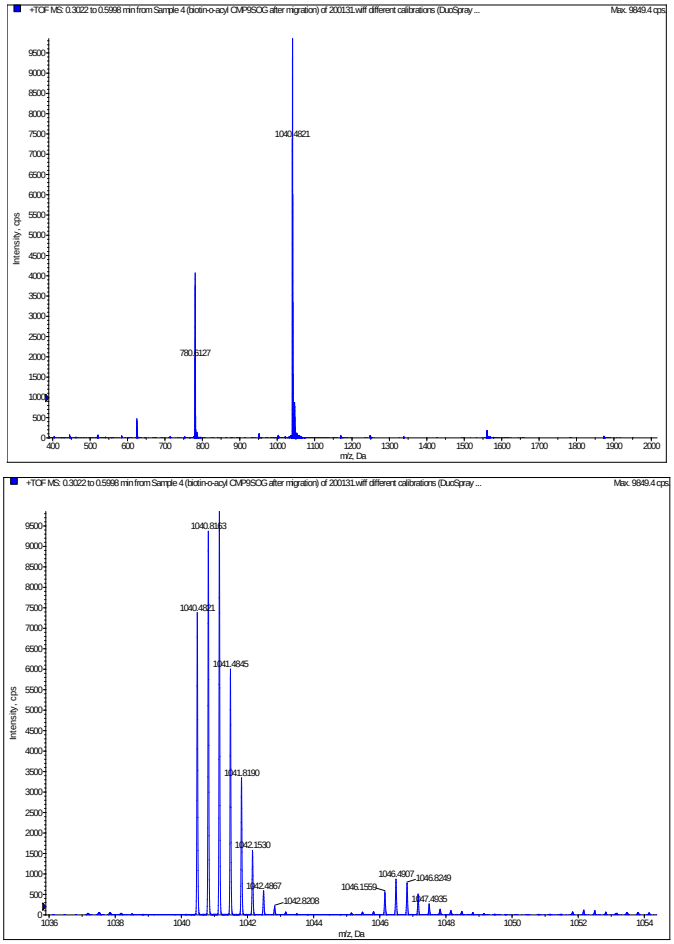
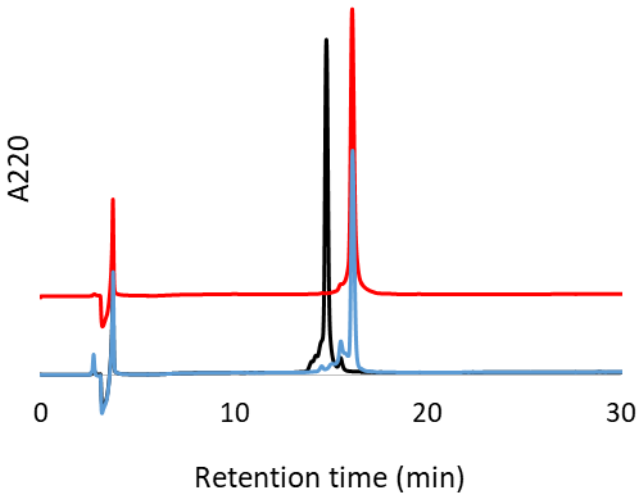


Figure S25. (a) HPLC analysis of the *O*-to-*N* acyl migration of compound **5** in 0.05% TFA/H_2O (black) and in PBS for 10 min (blue), and compound **6** in 0.05% TFA/H_2O (red). COSMOSIL 5C_{18}-AR-II column (size 4.6 × 250 mm), gradient 10%–30% MeCN/H_2O (0.05% TFA), 30 min at 60 °C. (b) ESI-MS analysis of a converted compound of **5** in PBS. The retention time of HPLC was different from **5** in 0.05% TFA/H_2O and the found *m/z* (1040.482 and 1046.156) corresponded with the calculated ones of **6** ($[M+3H]^{3+}$: 1040.481 and $[M+2H+NH_4]^{3+}$: 1046.156).

(a)



(b)

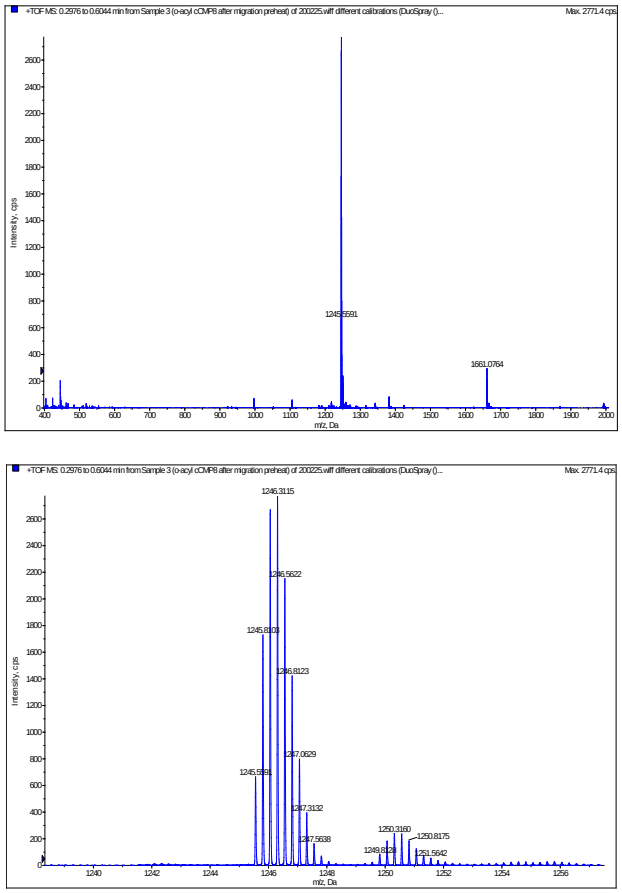


Figure S26. (a) HPLC analysis of the *O*-to-*N* acyl migration of compound **8** in 0.05% TFA/H_2O (black) and in PBS for 10 min (blue), and compound **9** in 0.05% TFA/H_2O (red). COSMOSIL 5C_{18}-AR-II column (size 4.6 × 250 mm), gradient 10%–30% MeCN/H_2O (0.05% TFA), 30 min at 60 °C. (b) ESI-MS analysis of a converted compound of **8** in PBS. The retention time of HPLC was different from **8** in 0.05% TFA/H_2O and the found *m/z* (1245.559 and 1249.813) corresponded with the calculated ones of **9** ($[M+4H]^{4+}$: 1245.559 and $[M+3H+NH_4]^{4+}$: 1249.816).

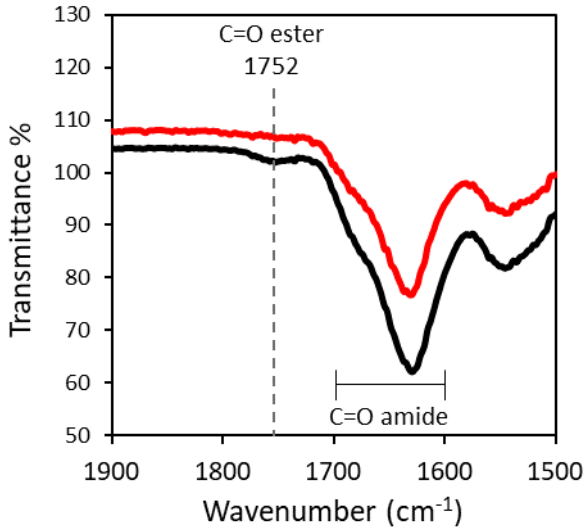


Figure S27. FT-IR spectra of **8** (black) and **9** (red).

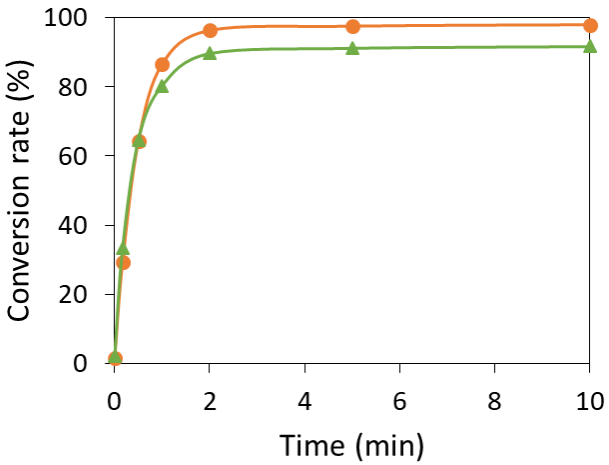


Figure S28. Comparison of the kinetics of the conversion from **3** to **4** (orange) and from **8** to **9** (green).

(a) (b)

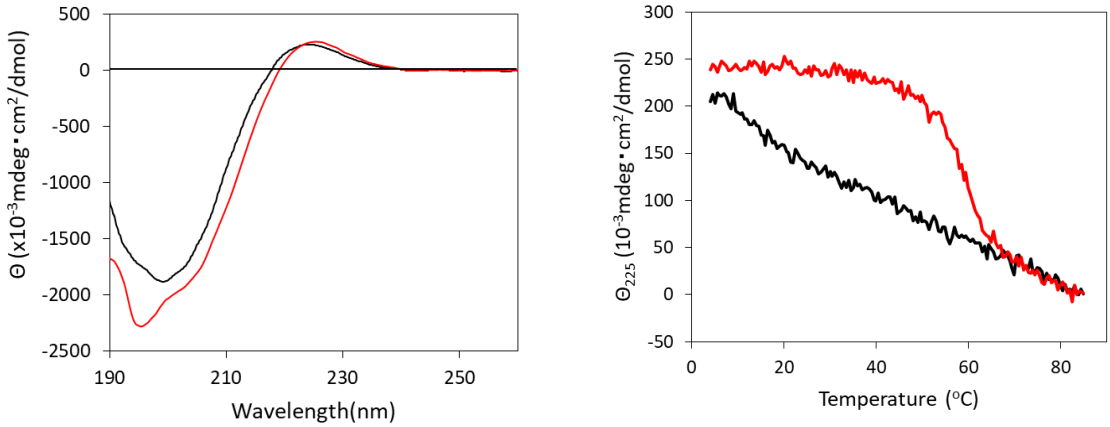
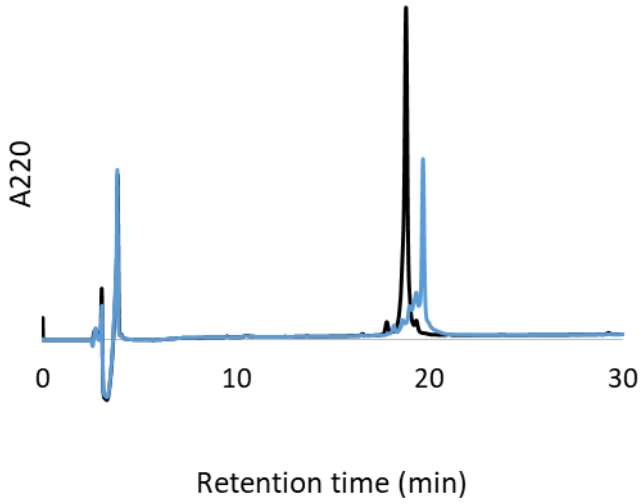


Figure S29. (a) CD spectra recorded in 0.05% TFA/H_2O at 4 °C. (b) Thermal melting curves of the triple helices. The black and red lines represent compounds **8** and **9**, respectively.

(a)



(b)

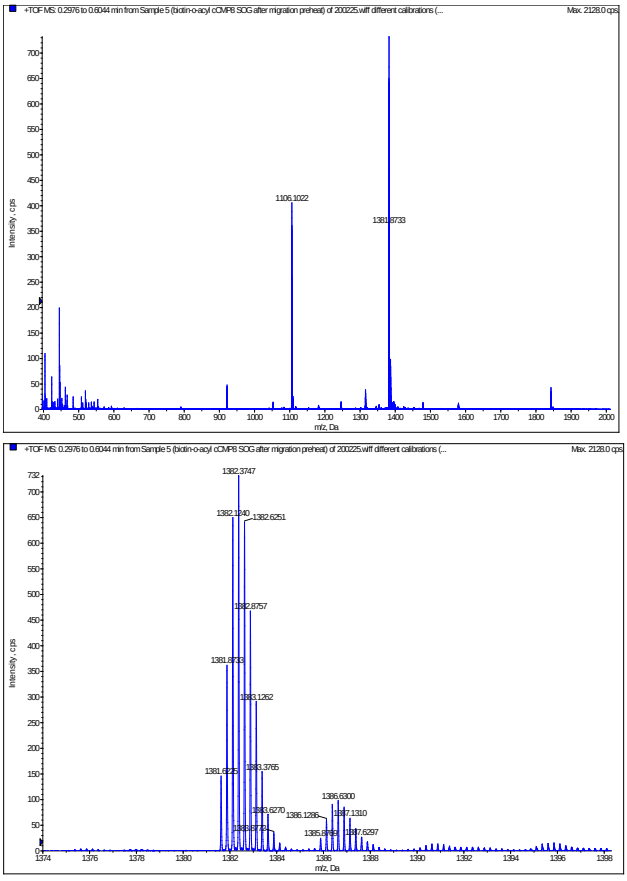


Figure S30. (a) HPLC analysis of the *O*-to-*N* acyl migration of compound **10** in 0.05% TFA/H_2O (black) and in PBS for 10 min (blue). COSMOSIL $5C_{18}$-AR-II column (size 4.6 × 250 mm), gradient 10%–30% MeCN/H_2O (0.05% TFA), 30 min at 60 °C. (b) ESI-MS analysis of a converted compound of **10** in PBS. The retention time of HPLC was different from **10** in 0.05% TFA/H_2O and the found *m*/*z* (1381.623 and 1385.877) corresponded with the calculated ones of the converted compound ($[M+4H]^{4+}$: 1381.623 and $[M+3H+NH_4]^{4+}$: 1385.880).

Reference

1. T. Yoshiya, H. Kawashima, Y. Sohma, T. Kimura, and Y. Kiso, *Org. Biomol. Chem.*, 2009, **7**, 2894-2904.
2. C. N. Pace, F. Vajdos, L. Fee, G. Grimsley, and T. Gray, *Protein Sci.*, 1995, **4**, 2411-2423.
3. M. S. Ackerman, M. Bhate, N. Shenoy, K. Beck, J. A. M. Ramshaw, and B. Brodsky, *J. Biol. Chem.*, 1999, **274**,7668-7673.