Supplementary Methods

1. General techniques

NMR spectra were recorded on a Bruker AVANCE NEO 600 (600 MHz for ^{1}H, 151 MHz for ^{13}C) instrument in the indicated solvent. Chemical shifts are reported in units of parts per million (ppm) relative to tetramethylsilane (0.00 ppm) for ^{1}H NMR and DMSO-d_6 (39.5 ppm) for ^{13}C NMR. Multiplicities were reported by using the following abbreviations: s; singlet, d; doublet, t; triplet, q; quartet, m; multiplet, br; broad, *J*; coupling constants in Hertz (Hz). IR spectra were recorded on a PerkinElmer Inc. FT-IR / NIR Spectrometer Frontier with CZITEK MicromATR. Only the strongest and/or structurally important peaks were reported as the IR data given in cm^{-1}. HRMS (ESI-TOF) were measured with a Waters SYNAPT G2-S. Automated column chromatography was performed using a Biotage Isolera One Flash Purification System. Analytical HPLC was carried out on Waters ACQUITY UPLC H-Class system with PDA Detector and SQ Detector 2.

2. System configuration

2-1. General information

PTFE tubes were purchased from Flon Industry Co., Ltd. Fittings were purchased from IDEX Health & Science LLC., Flon Industry Co., Ltd., Surpass Industry Co., Ltd., and GL Science Inc. Check valve was purchased from IDEX Health & Science LLC. Degassing unit (Gastorr AG-44), 1-6 way valve (VA-21-618), and 4 way valve (VA-21-610) were purchased from Flom, Inc. A 2 way electric valve (MTV-2R-N1/4UG) was purchased from Takasago Electric, Inc. A picture of the system is shown in Figure S1, and schematic of the developed continuous-flow peptide synthesizer is shown in Figure S2.

Figure S1: A picture of the system

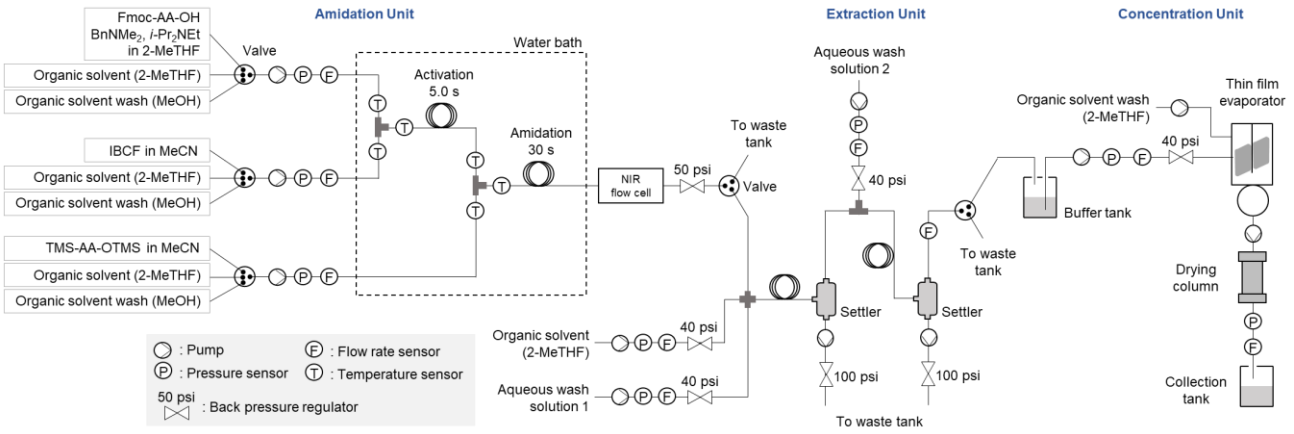


Figure S2: Schematic of the developed continuous-flow peptide synthesizer

2-2. Amidation unit

The employed amidation unit is shown in Figure S3. Degassed reagents, solvents or washing solvents (MeOH) were introduced to a flow system with HPLC pumps (PU-4086, purchased from JASCO Corporation). Pressure sensors (HPID, purchased from Surpass Industry Co., Ltd.) and flow rate sensors (mini CORI-FLOW M13, purchased from Bronkhorst Japan K.K.) were connected to the downstream of the HPLC pumps. Activation and amidation reactions were performed in PTFE tubes (inner diameter = 0.8 mm) immersed in water bath (CTB6A, purchased from Yamato Scientific co., ltd.). Reagents were mixed using stainless steel T-shape mixers (inner diameter: 0.25 mm), purchased from Sanko Seiki Co. Ltd. Temperature of water bath was controlled using constant temperature water circulator CTA802, purchased from Yamato Scientific co., ltd. Temperature of reagents and reaction mixtures was measured by using T-type thermocouples (ϕ = 0.5 mm, class 1, ± 0.5 °C), purchased from HAYASHI DENKO Co., LTD. The thermocouples were installed in the flow path using sleeves (purchased from M&S Instruments Inc.) and SUS joint, designed by our group (figure S4). Back pressure was regulated using BPR-10, purchased from Zaiput Flow Technologies.

NIR flow cell (FIA-USP-1000, optical path length = 1.0 mm, purchased from Ocean Insight, USA, FL) was installed at the position downstream of the amidation reaction tube. FT-NIR spectroscope (FT-NIR Rocket) and light source (ARCLIGHT-NIR) were purchased from ARCoptix, Switzerland. The spectroscope, the flow cell and the light source were connected using ZrF_4 optical fiber cable (MZ41L1, NA = 0.20, Operationg Wavelength Range = 0.3–4.5 µm), purchased from Thorlabs, Inc. (USA, NJ) The light guided to the flow cell was collimated with a ruby-sapphire ball lens (diameter = 3.18 mm, purchased from Edmund Optics, USA, NJ).

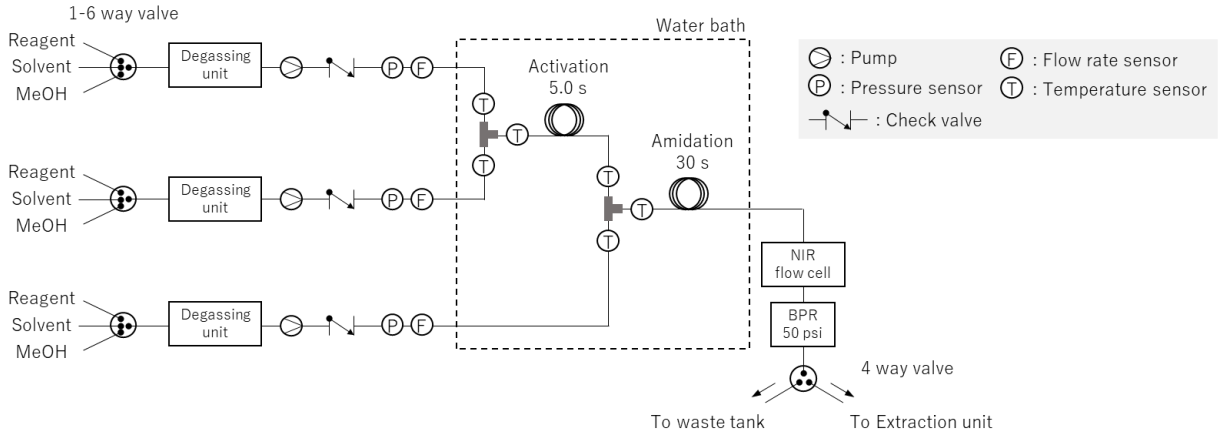


Figure S3: Diagram of Amidation unit

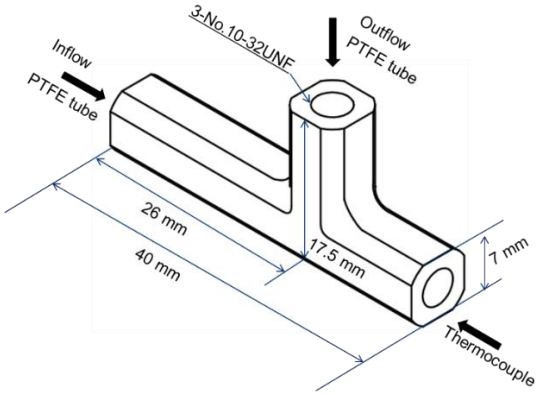


Figure S4: SUS joint for installation of thermocouple

2-3. Extraction unit

The employed extraction unit is shown in Figure S5. Degassed organic solvent and aqueous wash solutions were introduced to a flow system with diaphragm pumps (QI-30, purchased from TACMINA CORPORATION). Pressure sensors (HPID), flow rate sensors (NTFZ-3S-35, purchased from Surpass Industry Co., Ltd.) and back pressure regulators (40 psi, purchased from IDEX Health & Science LLC.) were connected to the downstream of the diaphragm pumps. Solutions were mixed using PTFE cross or T-shape mixer (inner diameter = 2.0 mm), purchased from Surpass Industry Co., Ltd. Extraction was performed in PTFE tubes (length = 3.0 m, inner diameter = 1.59 mm). Gravity separation of organic/aqueous phase was performed in grass settler, designed by our group (Figure S6). Aqueous waste was ejected from settler by using diaphragm pumps. After extraction, obtained organic layer was collected into a buffer tank.

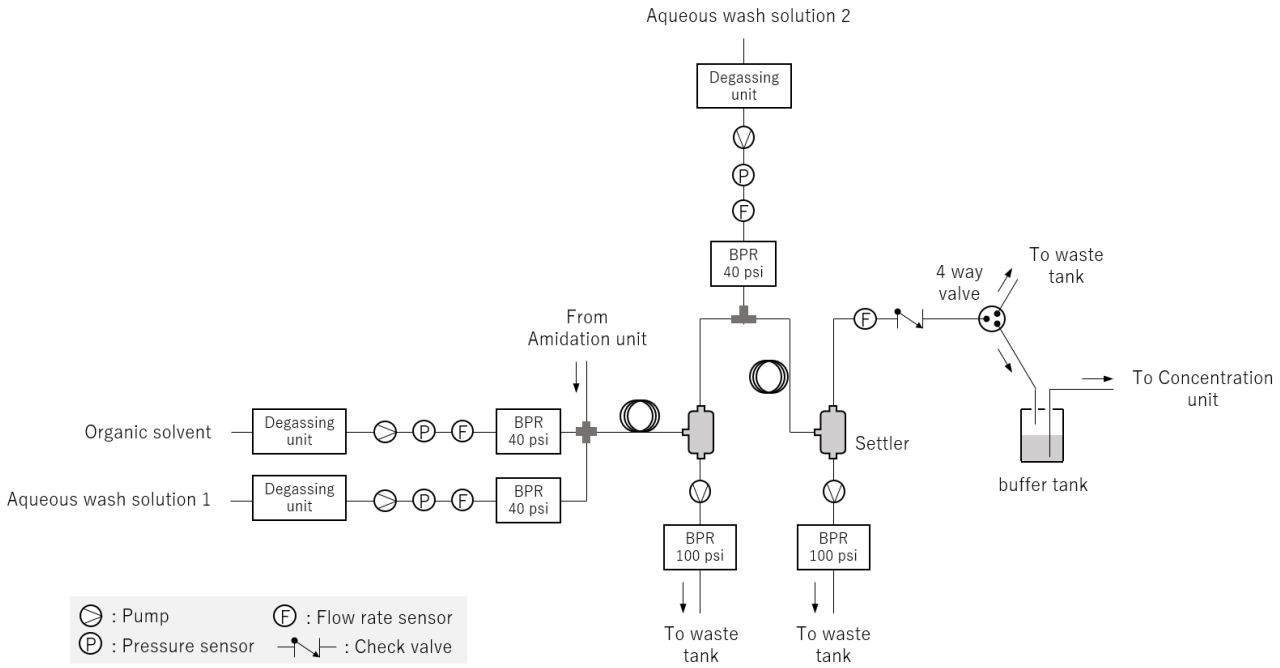


Figure S5: Diagram of extraction unit

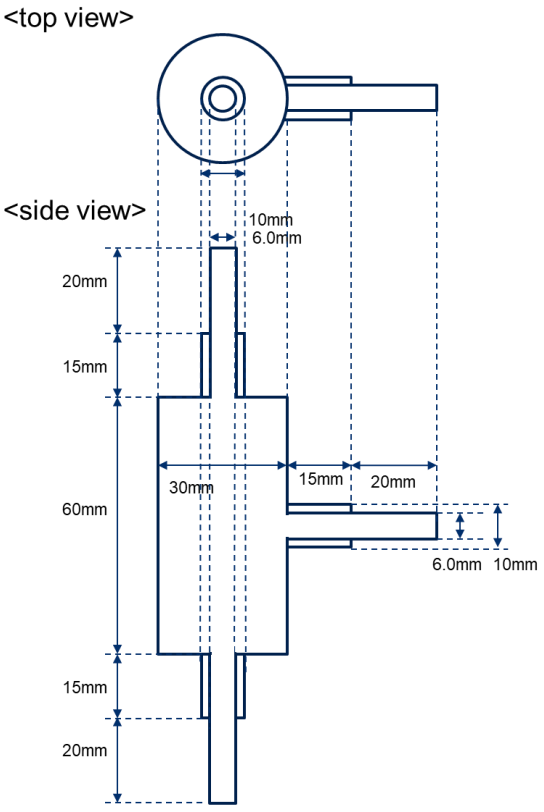


Figure S6: Glass settler for separation of organic/aqueous phase

2-4. Concentration unit

The employed concentration unit is shown in Figure S7. Extracted organic solution and wash solvent were introduced to a thin film evaporator system (MF-1000, purchased from TOKYO RIKAKIKAI CO, LTD.) with diaphragm pumps (QI-30). Pressure sensors (HPID), flow rate sensors (NTFZ-3S-35) and back pressure regulators (40 psi, purchased from IDEX Health & Science LLC.) were connected to the downstream of the diaphragm pumps. The thin film evaporator system has vacuum pump (NVP-1000), vacuum controller (NVC-3000), hot water circulation device (HS-1000) for evaporation promotion and chiller (NCA-1000) for collecting the distilled solvent. Concentrated solution was introduced to a drying column with diaphragm pump and collected in a collection tank. Pressure sensors (HPID) and flow rate sensors (NTFZ-3S-35) were connected to the downstream of the drying column.

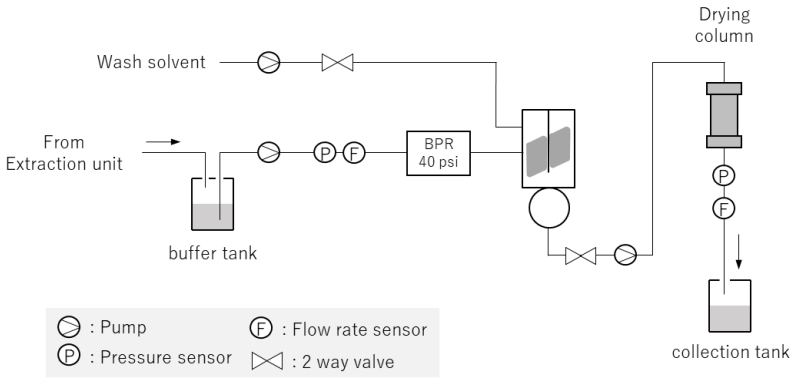


Figure S7: Diagram of concentration unit

2-5. Control unit

Diagram of our originally developed control unit is shown in Figure S8. Pumps, valves, sensors, and water circulator were connected to the subunits with various I/O interfaces (RS-232C, RS-485, 4–20 mA analog communication and thermocouple analog signal). Subunits were connected to a main controller that has a CPU for processing data from sensors and executing synthetic recipes. The main controller was connected to a desktop PC. We can monitor process parameters and edit synthetic recipes in it.

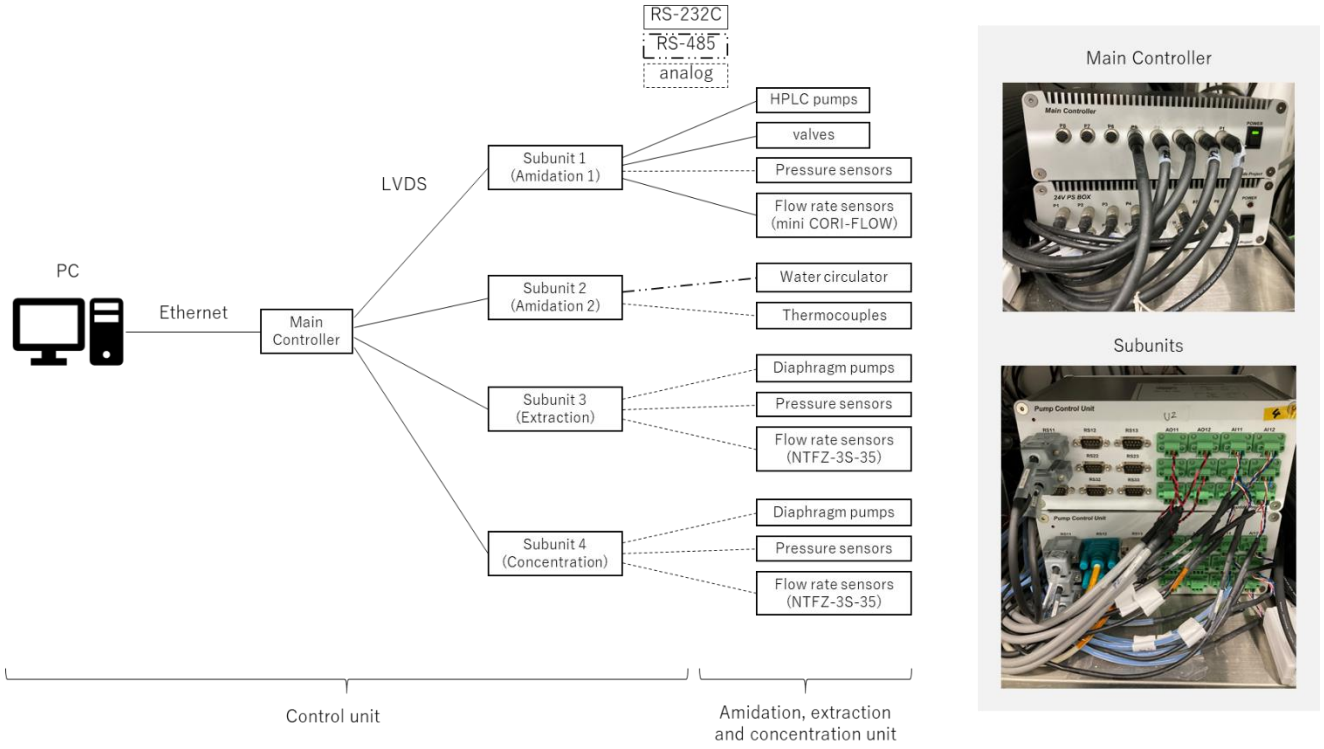
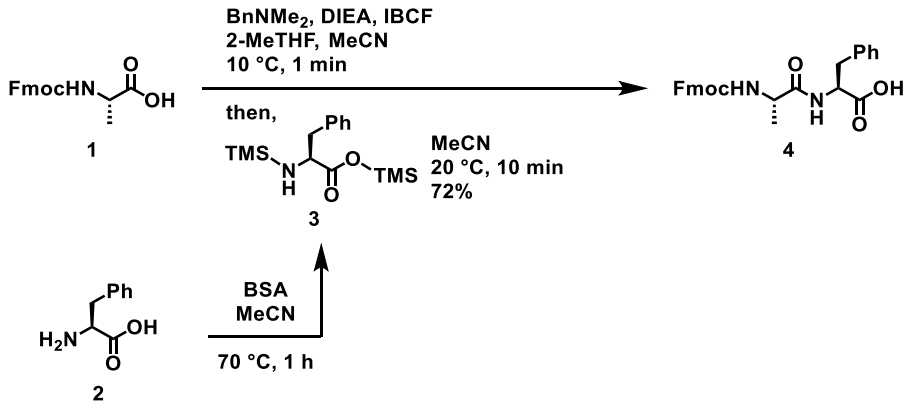


Figure S8: Diagram of control unit. LVDS: Low Voltage Differential Signaling

3. Optimization of amidation conditions

3-1. Synthesis of Fmoc-L-Ala-L-Phe-OH (4) in a batch reactor



Silylation (batch)

To a suspension of H-L-Phe-OH (**2**) (2.23 g, 13.5 mmol, 1.20 equiv.) in MeCN (47.5 mL), *N,O*-bis(trimethyl silyl)acetamide (BSA, 6.60 mL, 27.0 mmol, 2.40 equiv.) was added at room temperature. After being stirred at 70 °C for 1 h, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-OTMS (**3**) was directly used for next reaction.

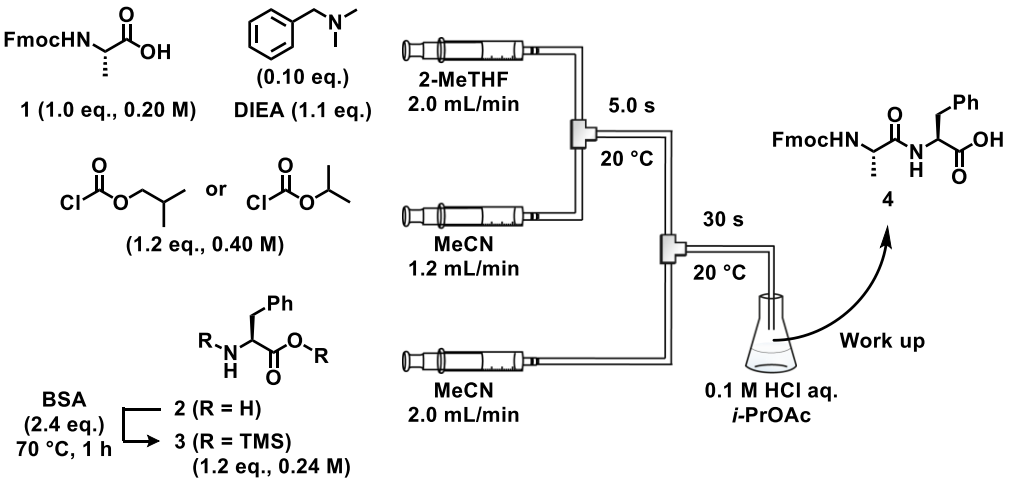
Amidation (batch)

To a solution of Fmoc-L-Ala-OH (**1**) (3.50 g, 11.2 mmol, 1.00 equiv.), Me_2NBn (169 µL, 1.12 mmol, 0.100 equiv.) and DIEA (2.15 mL, 12.3 mmol, 1.10 equiv.) in 2-MeTHF (50.5 mL), a solution of isobutyl

chloroformate (1.75 mL, 13.5 mmol, 1.20 equiv.) in MeCN (32.0 mL) was added at 10 °C. After being stirred at the same temperature for 1 min, a solution of TMS-L-Phe-OTMS (**3**) was added at 20 °C. After being stirred at the same temperature for 10 min, aqueous 10% citric acid (200 mL) and *i*-PrOAc (200 mL) were added. The aqueous layer was extracted twice with *i*-PrOAc. The combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by recrystallization from THF/heptane gave Fmoc-L-Ala-L-Phe-OH (**4**) (3.72 g, 8.11 mmol, 72%) as a white solid.

^{1}H NMR (600 MHz, DMSO-d_6): δ 12.74 (brs, 1H), 8.06 (d, J = 7.8 Hz, 1H), 7.89 (d, J = 7.2 Hz, 2H), 7.72 (t, J = 8.4 Hz, 2H), 7.47 (d, J = 7.8 Hz, 1H), 7.42 (t, J = 7.2 Hz, 2H), 7.33 (t, J = 7.2 Hz, 2H), 7.26–7.17 (m, 5H), 4.45–4.41 (m, 1H), 4.26–4.19 (m, 3H), 4.11–4.06 (m, 1H), 3.05 (dd, J = 5.4, 13.8 Hz, 1H) 2.92 (dd, J = 8.4, 13.8 Hz, 1H), 1.19 (d, J = 6.0 Hz, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): δ 172.8, 172.5, 155.6, 143.9, 143.8, 140.7, 137.4, 129.2, 129.1, 128.2, 127.6, 127.1, 126.4, 125.3, 125.3, 120.1, 65.6, 53.4, 49.8, 46.6, 36.6, 18.2. The observed spectral data were well consistent with those reported in the literature. [1)]

3-2. Preliminary examination of the comparison of the activating agent



*In this section, we used equipment consisting of syringe pumps. Solutions were introduced to a micro-flow system with syringe pumps (Harvard PHD ULTRA, purchased from Harvard Apparatus) equipped gastight syringes (10 mL, purchased from Trajan Scientific Australia Pty Ltd.). The gastight syringes and the PTFE tubes were connected with joints purchased from Flon Industry Co., Ltd. The gastight syringes and the T-shape mixers 1 and 2 were connected with the PTFE tubes. The T-shape mixers 1 and 2 were connected with the reaction tube 1 (PTFE tube, inner diameter = 0.8 mm). The T-shape mixer 2 was connected with the reaction tube 2 (PTFE tube, inner diameter = 0.8 mm). T-shape mixers and reaction tubes were immersed in water bath.

Silylation (batch)

To a suspension of H-L-Phe-OH (**2**) (476 mg, 2.88 mmol, 1.20 equiv.) in MeCN (10.1 mL), *N,O*-bis(trimethyl silyl)acetamide (BSA, 1.41 mL, 5.76 mmol, 2.40 equiv.) was added at room temperature. After being stirred at 70 °C for 1 h, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-OTMS (**3**) was directly used for next flow reaction.

Amidation (flow)

A solution of Fmoc-L-Ala-OH (**1**) (0.200 M, 1.00 equiv.), Me_2NBn (0.0200 M, 0.100 equiv.) and DIEA (0.220 M, 1.10 equiv.) in 2-MeTHF (flow rate: 2.00 mL/min) and a solution of **isobutyl or isopropyl chloroformate** (0.400 M, 1.20 equiv.) in MeCN (flow rate: 1.20 mL/min) were introduced to the T-shape mixer 1 at 20 °C with the syringe pumps. The resultant mixture was passed through the reaction tube 1 (length: 531 mm, volume: 267 μL, reaction time: 5.01 s) at the same temperature. Then, the resultant mixture and a solution of TMS-L-Phe-OTMS (**3**) (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to the T-shape mixer 2 at 20 °C with the syringe pumps. The resultant mixture was passed through the reaction tube 2 (length: 5.17 m, volume: 2.60 mL, reaction time: 30.0 s) at the same temperature. After being eluted for *ca.* 85 s to reach a steady state, the resultant mixture was poured into a solution of *i*-PrOAc (3.6 mL) and aqueous 0.1 M HCl (3.6 mL) for 30 s at room temperature.

The aqueous layer was extracted twice with *i*-PrOAc. The combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Obtained crude mixture was analyzed by HPLC.

Table S1: Comparison of the activating agent

Entry	activating agent	Area% of product 4 [%][1]
1	IBCF	94
2	IPCF	90

[1] HPLC conditions were shown below.

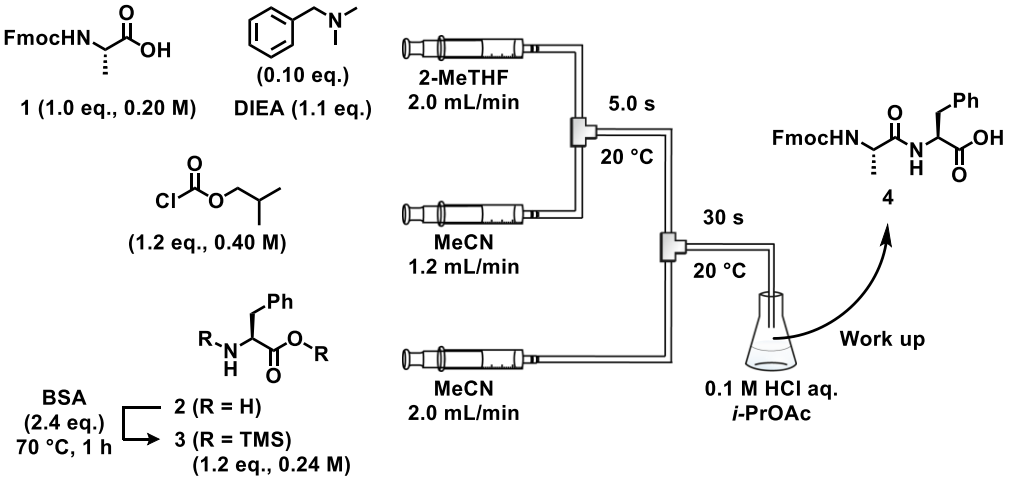
ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 100 mm

mobile phase A = H_2O + 0.025% TFA, mobile phase B = MeCN + 0.025% TFA

ratio of mobile phase B: 0–5.56 min: 5 to 95%, 5.56–7.22 min: 95%, 7.22–10 min: 5%

flow rate 0.6 mL/min, 60 °C, detection wavelength 215 nm, retention time 4.17 min

3-3. Synthesis of Fmoc-L-Ala-L-Phe-OH (4) in a flow reactor



*In this section, we used equipment consisting of syringe pumps. For detail, see section 3-2 in ESI.

Silylation (batch)

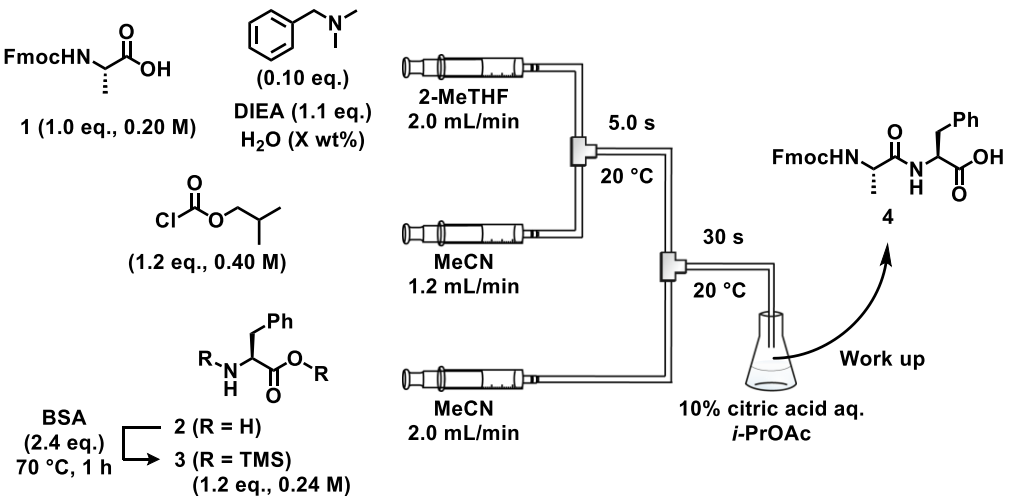
To a suspension of H-L-Phe-OH (**2**) (992 mg, 6.01 mmol, 1.20 equiv.) in MeCN (21.1 mL), *N,O*-bis(trimethyl silyl)acetamide (BSA, 2.93 mL, 12.0 mmol, 2.40 equiv.) was added at room temperature. After being stirred at 70 °C for 1 h, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-OTMS (**3**) was directly used for next flow reaction.

Amidation (flow)

A solution of Fmoc-L-Ala-OH (**1**) (0.200 M, 1.00 equiv.), Me_2NBn (0.0200 M, 0.100 equiv.) and DIEA (0.220 M, 1.10 equiv.) in 2-MeTHF (flow rate: 2.00 mL/min) and a solution of isobutyl chloroformate (0.400 M, 1.20 equiv.) in MeCN (flow rate: 1.20 mL/min) were introduced to the T-shape mixer 1 at 20 °C with the syringe pumps. The resultant mixture was passed through the reaction tube 1 (length: 531 mm, volume: 267 μL, reaction time: 5.01 s) at the same temperature. Then, the resultant mixture and a solution of TMS-L-Phe-OTMS (**3**) (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to the T-shape mixer 2 at 20 °C with the syringe pumps. The resultant mixture was passed through the reaction tube 2 (length: 5.17 m, volume: 2.60 mL, reaction time: 30.0 s) at the same temperature. After being eluted for *ca.* 85 s to reach a steady state, the resultant mixture was poured into a solution of *i*-PrOAc (75.6 mL) and aqueous 0.1 M HCl (75.6 mL) for 10 min 30 s at room temperature.

The aqueous layer was extracted with *i*-PrOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by recrystallization from THF/heptane gave Fmoc-L-Ala-L-Phe-OH (**4**) (1.65 g, 3.60 mmol, 86%) as a white solid.

3-4. Procedure for the examination of allowable amount of water contained



*In this section, we used equipment consisting of syringe pumps. For detail, see section 3-2 in ESI.

Silylation (batch)

To a suspension of H-L-Phe-OH (**2**) (1.16 g, 7.00 mmol, 1.20 equiv.) in MeCN (24.6 mL), *N,O*-bis(trimethyl silyl)acetamide (BSA, 3.42 mL, 14.0 mmol, 2.40 equiv.) was added at room temperature. After being stirred at 70 °C for 1 h, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-

OTMS (**3**) was directly used for next flow reaction.

Amidation (flow)

A solution of Fmoc-L-Ala-OH (**1**) (0.200 M, 1.00 equiv.), Me_2NBn (0.0200 M, 0.100 equiv.), DIEA (0.220 M, 1.10 equiv.) and H_2O in 2-MeTHF (flow rate: 2.00 mL/min, **X** wt% of water contained in this solution) and a solution of isobutyl chloroformate (0.400 M, 1.20 equiv.) in MeCN (flow rate: 1.20 mL/min) were introduced to the T-shape mixer 1 at 20 °C with the syringe pumps. The resultant mixture was passed through the reaction tube 1 (length: 531 mm, volume: 267 μL, reaction time: 5.01 s) at the same temperature. Then, the resultant mixture and a solution of TMS-L-Phe-OTMS (**3**) (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to the T-shape mixer 2 at 20 °C with the syringe pumps. The resultant mixture was passed through the reaction tube 2 (length: 5.17 m, volume: 2.60 mL, reaction time: 30.0 s) at the same temperature. After being eluted for *ca.* 85 s to reach a steady state, the resultant mixture was poured into a solution of *i*-PrOAc (3.6 mL) and aqueous 10% citric acid (3.6 mL) for 30 s at room temperature.

The aqueous layer was extracted twice with *i*-PrOAc. The combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Obtained crude mixture was analyzed by HPLC.

Table S2: Relationship between water content and purity of the amidation mixture

Entry	X [wt%][1]	Area% of product 4 [%][3]
1	0.072	96
2	0.11	95
3	0.20	93
4	0.51	90
5	1.1	-[2]

[1] The amount of water contained in the solution of Fmoc-Ala-OH was determined by Karl-Fischer method using CA-310 Convertible Moisture Meter (Nittoseiko Analytech Co., Ltd.).

[2] Flow reactor was clogged due to the generation of insoluble material.

[3] HPLC conditions were shown below.

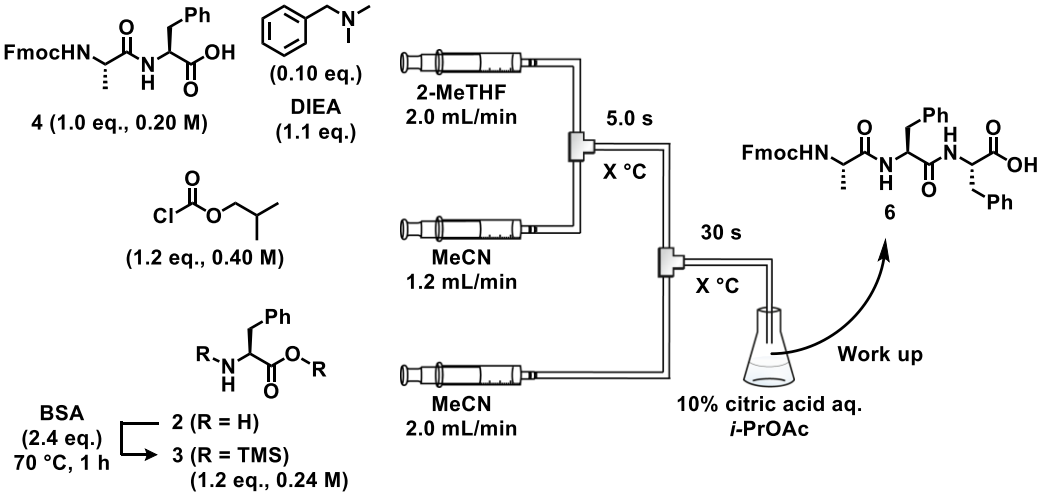
ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 100 mm

mobile phase A = H_2O + 0.025% TFA, mobile phase B = MeCN + 0.025% TFA

ratio of mobile phase B: 0–5.56 min: 5 to 95%, 5.56–7.22 min: 95%, 7.22–10 min: 5%

flow rate 0.6 mL/min, 60 °C, detection wavelength 215 nm, retention time 4.17 min

3-5. Preliminary examination for optimization of reaction temperature



*In this section, we used equipment consisting of syringe pumps. For detail, see section 3-2 in ESI.

Silylation (batch)

To a suspension of H-L-Phe-OH (**2**) (463 mg, 2.80 mmol, 1.20 equiv.) in MeCN (9.8 mL), *N,O*-bis(trimethyl silyl)acetamide (BSA, 1.40 mL, 5.73 mmol, 2.45 equiv.) was added at room temperature. After being stirred at 70 °C for 1 h, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-OTMS (**3**) was directly used for next flow reaction.

Amidation (flow)

A solution of Fmoc-L-Ala-L-Phe-OH (**4**) (0.200 M, 1.00 equiv.), Me_2NBn (0.0200 M, 0.100 equiv.) and DIEA (0.220 M, 1.10 equiv.) in 2-MeTHF (flow rate: 2.00 mL/min) and a solution of isobutyl chloroformate (0.400 M, 1.20 equiv.) in MeCN (flow rate: 1.20 mL/min) were introduced to the T-shape mixer 1 at **X** °C with the syringe pumps. The resultant mixture was passed through the reaction tube 1 (length: 531 mm, volume: 267 μL, reaction time: 5.01 s) at the same temperature. Then, the resultant mixture and a solution of TMS-Phe-OTMS (**3**) (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to the T-shape mixer 2 at **X** °C with the syringe pumps. The resultant mixture was passed through the reaction tube 2 (length: 5.17 m, volume: 2.60 mL, reaction time: 30.0 s) at the same temperature. After being eluted for *ca.* 85 s to reach a steady state, the resultant mixture was poured into a solution of *i*-PrOAc (3.6 mL) and aqueous 10% citric acid (3.6 mL) for 30 s at room temperature.

The aqueous layer was extracted twice with *i*-PrOAc. The combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Obtained crude mixture was analyzed by HPLC.

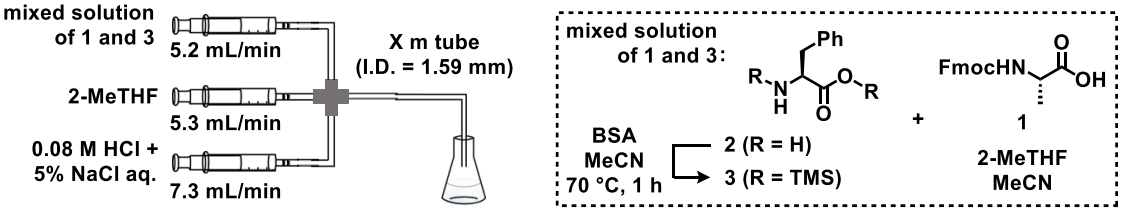
Table S3: optimization of reaction temperature

Entry	X [°C]	Area% [%][1]	
		Product 6	Starting material 4
1	20	89	4
2	60	63	27

[1] HPLC conditions were shown below.
ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 100 mm
mobile phase A = H_2O + 0.025% TFA, mobile phase B = MeCN + 0.025% TFA
ratio of mobile phase B: 0–5.56 min: 5 to 95%, 5.56–7.22 min: 95%, 7.22–10 min: 5%
flow rate 0.6 mL/min, 60 °C, detection wavelength 215 nm, retention time 4.51 min

4. Optimization of extraction conditions

Procedure for the optimization of extraction conditions



*In this section, we used equipment consisting of syringe pumps. Solutions were introduced to a micro-flow system with syringe pumps (Harvard PHD ULTRA, purchased from Harvard Apparatus) equipped gastight syringes (50 mL, purchased from Trajan Scientific Australia Pty Ltd.). The gastight syringes and the PTFE tubes were connected with joints purchased from Flon Industry Co., Ltd. The gastight syringes and the cross mixer were connected with the PTFE tubes. The cross mixer was connected to the extraction tube (PTFE tube, length = **X** m, inner diameter = 1.59 mm).

Flow condition

A solution of Fmoc-L-Ala-OH (**1**) (0.200 M, 1.00 equiv.) and TMS-L-Phe-OTMS (**3**) (0.240 M, 1.20 equiv., prepared by same procedure as section 3 in ESI) in a 2 : 3 mixture of 2-MeTHF and MeCN (flow rate: 5.20 mL/min), 2-MeTHF (flow rate: 5.30 mL/min) and aqueous solution of 0.08 M HCl and 5% NaCl (flow rate: 7.30 mL/min) were introduced to the cross mixer at room temperature with the syringe pumps. The resultant mixture was passed through the extraction tube (length: **X** m) at the same temperature. After being eluted to reach a steady state, the resultant mixture was collected in an empty test tube. Both organic layer and aqueous layer were analyzed by HPLC.

Batch condition (for reference)

A solution of Fmoc-L-Ala-OH (**1**) (0.200 M, 1.00 equiv.) and TMS-L-Phe-OTMS (**3**) (0.240 M, 1.20 equiv., prepared by same procedure as section 3 in ESI) in a 2 : 3 mixture of 2-MeTHF and MeCN (total 2.6 mL), 2-MeTHF (2.7 mL) and aqueous solution of 0.08 M HCl and 5% NaCl (3.7 mL) were mixed in the test tube at room temperature. After being stirred for 10 min, both organic layer and aqueous layer were analyzed by HPLC.

Table S4: optimization of extraction conditions

Entry	Tube length[1] X [m]	Organic layer[2]		Aqueous layer[2]	
		Fmoc-L-Ala-OH (1)	H-L-Phe-OH (2)	Fmoc-L-Ala-OH (1)	H-L-Phe-OH (2)

		Area% [%]	Area [μVsec]	Area% [%]	Area [μVsec]	Area% [%]	Area [μVsec]	Area% [%]	Area [μVsec]
1	1	99.7	1681584	0.3	5147	0.5	6692	99.5	1356628
2	3	99.7	1581454	0.3	5303	0.5	6789	99.6	1514964
3	5	99.5	1575581	0.5	8300	0.3	4157	99.7	1483064
4	Batch	99.5	1461927	0.5	7641	0.6	9321	99.4	1480293

[1] residence time of each tube: 1 m = 6.7 s, 3 m = 20.1 s, 5 m = 33.5 s

[2] HPLC conditions were shown below.

ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 100 mm

mobile phase A = H_2O + 0.025% TFA, mobile phase B = MeCN + 0.025% TFA

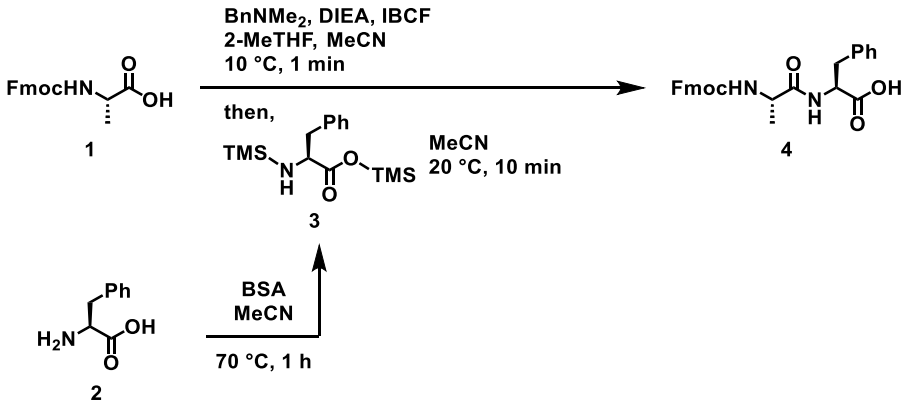
ratio of mobile phase B: 0–5.56 min: 5 to 95%, 5.56–7.22 min: 95%, 7.22–10 min: 5%

flow rate 0.6 mL/min, 60 °C, detection wavelength 215 nm, retention time H-L-Phe-OH: 0.87 min, Fmoc-L-Ala-OH: 3.70 min

Note: All tube length showed comparable result. We selected 3 m tube because it has moderate length to handle in our flow system.

5. Optimization of concentration conditions

5-1. Procedure for the preparation of crude dipeptide 4



Silylation (batch)

To a suspension of H-L-Phe-OH (**2**) (6.58 g, 39.8 mmol, 1.20 equiv.) in MeCN (140 mL), *N,O*-bis(trimethyl silyl)acetamide (BSA, 19.5 mL, 79.8 mmol, 2.40 equiv.) was added at room temperature. After being stirred at 70 °C for 1 h, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-OTMS (**3**) was directly used for next reaction.

Amidation (batch)

To a solution of Fmoc-L-Ala-OH (**1**) (10.3 g, 33.2 mmol, 1.00 equiv.), Me_2NBn (499 μL, 3.32 mmol, 0.100 equiv.) and DIEA (6.36 mL, 36.5 mmol, 1.10 equiv.) in 2-MeTHF (149 mL), a solution of isobutyl chloroformate (5.17 mL, 39.8 mmol, 1.20 equiv.) in MeCN (95 mL) was added at 10 °C. After being stirred at the same temperature for 1 min, a solution of TMS-L-Phe-OTMS (**3**) was added at 20 °C. After being stirred at the same

temperature for 10 min, aqueous 10% citric acid (600 mL) and *i*-PrOAc (600 mL) were added. The aqueous layer was extracted twice with *i*-PrOAc. The combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Obtained crude Fmoc-L-Ala-L-Phe-OH (**4**) was directly used for the next experiment.

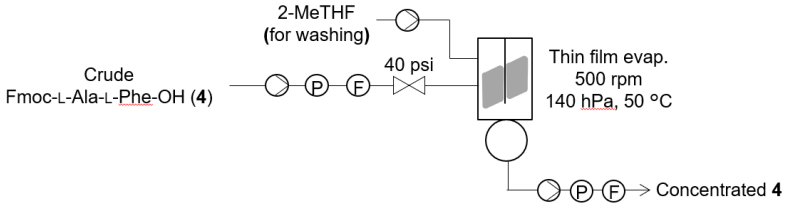
5-2. Preliminary examination for concentration of Fmoc-L-Ala-L-Phe-OH (4) in *i*-PrOAc and MeCN

A solution of crude Fmoc-L-Ala-L-Phe-OH (**4**) (21.1 mmol) in *i*-PrOAc and MeCN (total 490 mL) was introduced to the thin layer evaporator with pumps. The solution was continuously concentrated (rotor speed: 700 RPM, pressure: 100 hPa, temperature: 50 °C) for 45 min.

Note: Peptide **4** crystallized in the chamber due to its relatively low solubility in *i*-PrOAc and MeCN (Figure S9), and was hard to collect the concentrate. Therefore, we changed solvent.

Figure S9: A picture of the thin layer evaporator that have crystallized peptide **4** in the chamber

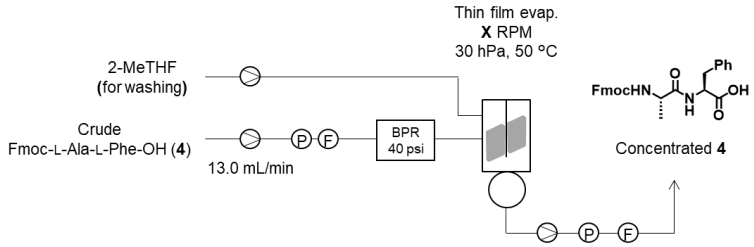
5-3. Preliminary examination for concentration of Fmoc-L-Ala-L-Phe-OH (4) in 2-MeTHF and MeCN



A solution of crude Fmoc-L-Ala-L-Phe-OH (**4**) (9.3 mmol) in 2-MeTHF and MeCN (total 270 mL) was introduced to the thin layer evaporator with pumps. The solution was continuously concentrated (rotor speed: 500 RPM, pressure: 140 hPa, temperature: 50 °C) for 27 min. After setting the pressure of the thin film evaporator to atmospheric pressure, thin film evaporator was washed twice with 2-MeTHF. The total volume of collected samples was 110 mL.

Note: In this experiment, peptide **4** did not crystalize in the chamber due to its relatively high solubility in 2-MeTHF. In the next experiment, we chose higher vacuum condition to get sufficient evaporation.

5-4. Procedure for the optimization of rotor speed at 30 hPa



A solution of crude Fmoc-L-Ala-L-Phe-OH (**4**) (39.8 mmol) in 2-MeTHF (625 mL) and MeCN (275 mL) was introduced to the thin layer evaporator at 13.0 mL/min with pumps. The solution was continuously concentrated (rotor speed: **X** RPM, pressure: 30 hPa, temperature: 50 °C) for 77 min. After setting the pressure of the thin film evaporator to atmospheric pressure, thin film evaporator was washed twice with 2-MeTHF. The total volume of collected samples are shown below.

Table S5: optimization of rotor speed

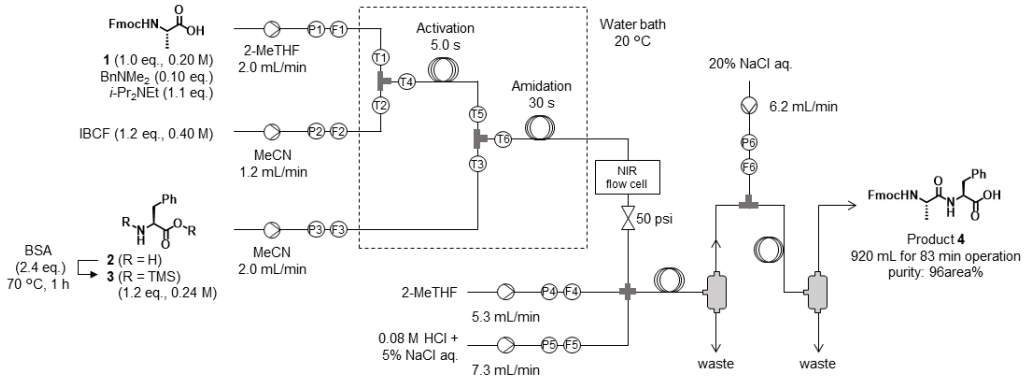
Entry	X [rpm]	Volume of collected sample [mL][1]
1	300	206
2	600	158
3	900	-[2]

[1] Target volume: < 159 mL (For the next amidation, 0.2 M solution of **4** is needed.)

[2] We stopped this experiment because high speed rotor made an abnormal noise.

6. Continuous flow synthesis of Fmoc-L-Ala-L-Phe-L-Phe-OH (6) using our developed system

6-1. Procedure for the synthesis and extraction of Fmoc-L-Ala-L-Phe-OH (4)



Silylation (batch)

To a suspension of H-L-Phe-OH (**2**) (8.29 g, 50.2 mmol, 1.19 equiv.) in MeCN (176 mL), *N,O*-bis(trimethylsilyl)acetamide (BSA, 24.6 mL, 100.4 mmol, 2.38 equiv.) was added at room temperature. After being stirred at 70 °C for 60 min, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-OTMS (**3**) was directly used for next flow reaction.

Amidation, extraction (flow)

A solution of Fmoc-L-Ala-OH (**1**) (0.200 M, 1.00 equiv.), Me_2NBn (0.0200 M, 0.100 equiv.) and DIEA (0.220 M, 1.10 equiv.) in 2-MeTHF (flow rate: 2.00 mL/min) and a solution of isobutyl chloroformate (0.400 M, 1.20 equiv.) in MeCN (flow rate: 1.20 mL/min) were introduced to the first T-shape mixer at 20 ℃ with the pumps. The resultant mixture was passed through the activation reaction flow channel (residence time = 5.01 s). Then, the resultant mixture and a solution of TMS-L-Phe-OTMS (**3**) (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to the second T-shape mixer at 20 °C with the pumps. The resultant mixture was passed through the amidation reaction flow channel (residence time = 30.0 s). Then, the resultant mixture, 2-MeTHF (flow rate: 5.33 mL/min) and aqueous 0.08 M HCl + 5 % NaCl (flow rate: 7.34 mL/min) were introduced to the cross mixer at room temperature with the pumps. The resultant mixture was passed through the extraction flow channel (inner diameter: 1.59 mm, length: 3.00 m) and separated by the first settler. Then, the resultant organic phase solution and aqueous 20% NaCl (flow rate: 6.17 mL/min) were introduced to the T-shape mixier at room temperature with the pumps. The resultant mixture was passed through the extraction flow channel (inner diameter: 1.59 mm, length: 3.00 m) and separated by the second settler. After being eluted for 16 min to reach a steady state, the resultant mixture was collected for 83 min at room temperature. The obtained crude solution of Fmoc-L-Ala-L-Phe-OH (**4**) (*ca.* 920 mL, water content: 5.5wt%, purity: 96Area%) was directly used for next concentration step.

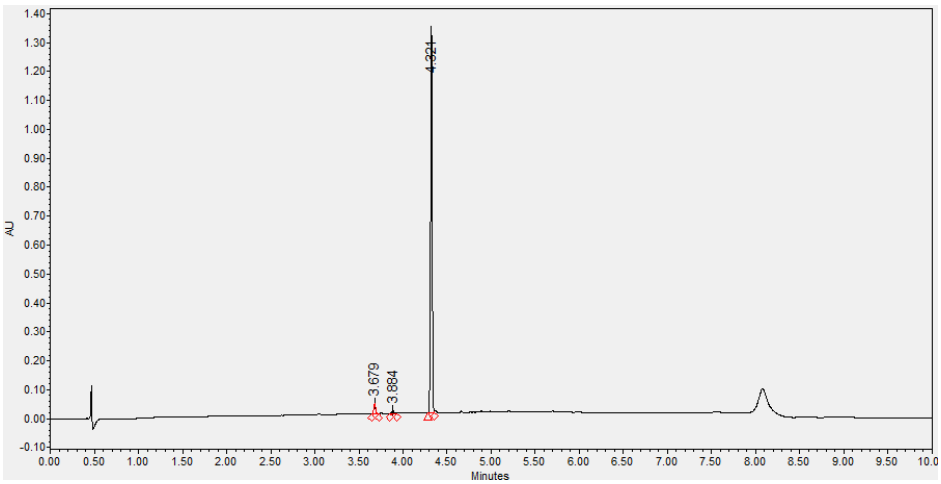


Figure S10: HPLC chart of crude **4** (after extraction)

HPLC conditions were shown below.

ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 100 mm

mobile phase A = H_2O + 0.025% TFA, mobile phase B = MeCN + 0.025% TFA

ratio of mobile phase B: 0–5.56 min: 5 to 95%, 5.56–7.22 min: 95%, 7.22–10 min: 5%

flow rate 0.6 mL/min, 60 °C, detection wavelength 215 nm

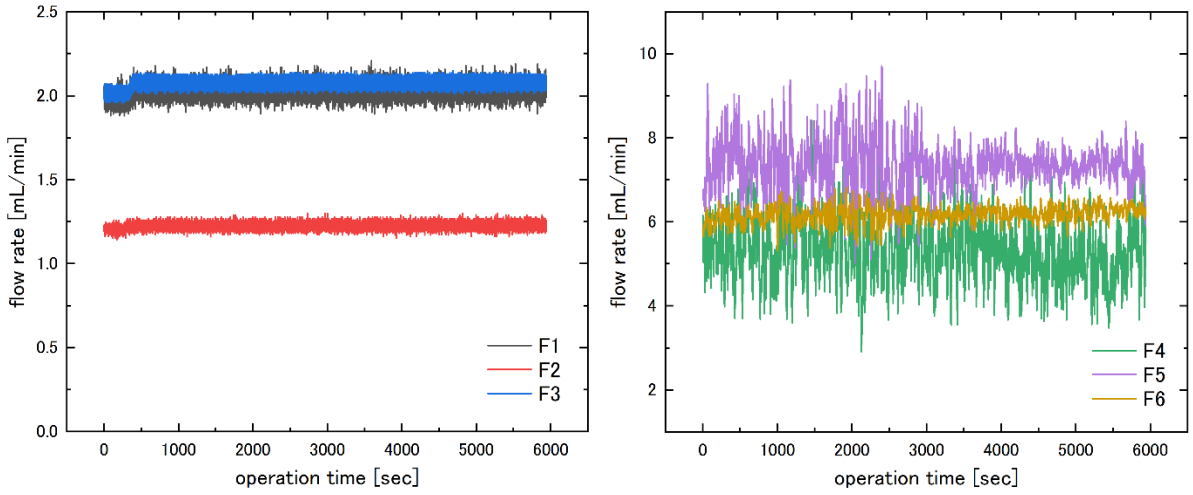
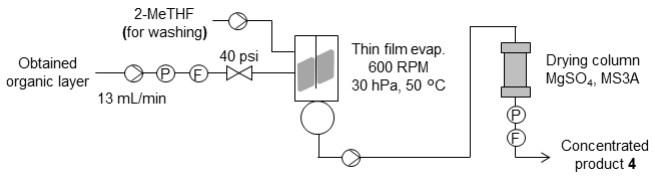


Figure S11: Flow rate sensing data

6-2. Procedure for the concentration of Fmoc-L-Ala-L-Phe-OH (4)



A crude solution of Fmoc-L-Ala-L-Phe-OH (**4**) obtained after extraction was introduced to the thin layer evaporator at 13.0 mL/min with pumps. The solution was continuously concentrated (stirring blade speed: 600

RPM, pressure: 30 hPa, temperature: 50 °C) for 75 min. After setting the pressure of the thin film evaporator to atmospheric pressure, the concentrated solution was introduced to the drying column (Biotage® Sfär DLV Empty 10 g Column packed with $MgSO_4$ and Empty 25 g Column packed with MS3A). Then, thin film evaporator and drying column were washed twice with 2-MeTHF. The combined solution of Fmoc-L-Ala-L-Phe-OH (**4**) (94 mL, water content: 0.14wt%, purity: 93Area%) was directly used for the next step.

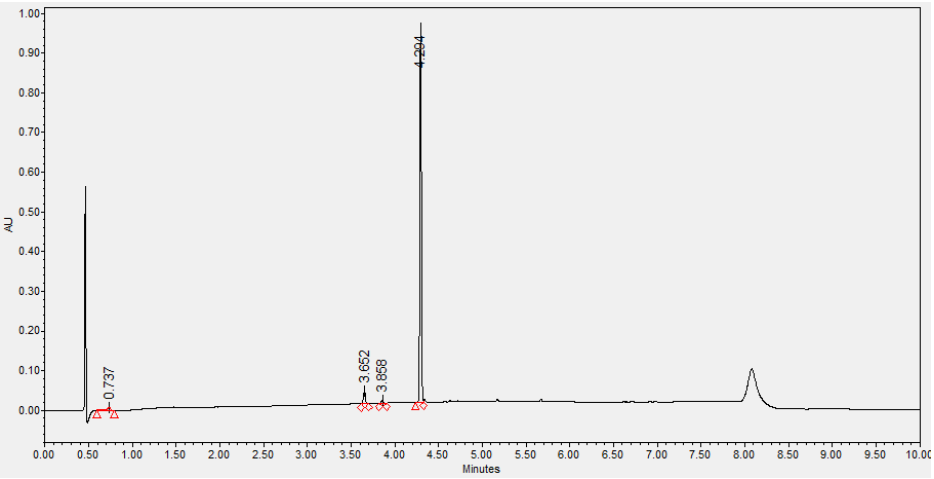
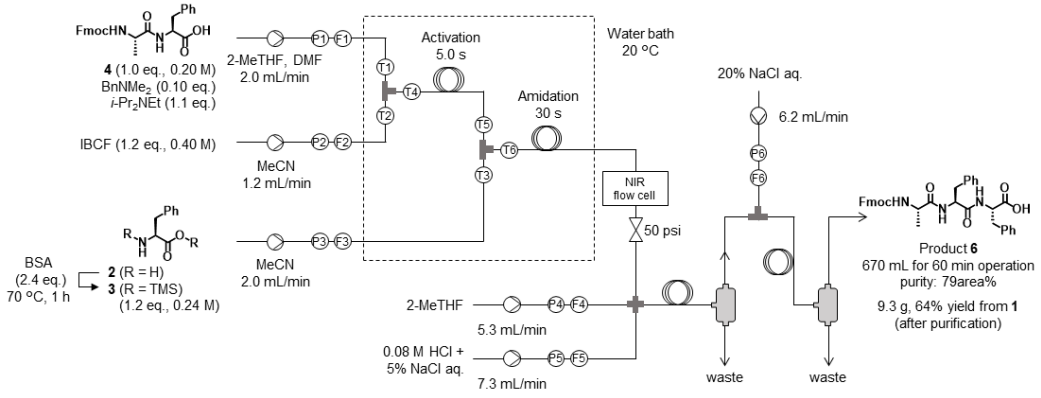


Figure S12: HPLC chart of crude **4** (after concentration)

HPLC conditions were shown below.
ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 100 mm
mobile phase A = H_2O + 0.025% TFA, mobile phase B = MeCN + 0.025% TFA
ratio of mobile phase B: 0–5.56 min: 5 to 95%, 5.56–7.22 min: 95%, 7.22–10 min: 5%
flow rate 0.6 mL/min, 60 °C, detection wavelength 215 nm

6-3. Procedure for the synthesis and extraction of Fmoc-L-Ala-L-Phe-L-Phe-OH (6)



Silylation (batch)

To a suspension of H-L-Phe-OH (**2**) (6.58 g, 39.8 mmol, 1.20 equiv.) in MeCN (140 mL), *N,O*-bis(trimethylsilyl)acetamide (19.5 mL, 79.6 mmol, 2.40 equiv.) was added at room temperature. After being

stirred at 70 °C for 60 min, the reaction mixture was cooled to room temperature. The obtained solution of TMS-L-Phe-OTMS (**3**) was used for next flow reaction.

Amidation, extraction (flow)

To a crude solution of Fmoc-L-Ala-L-Phe-OH (**4**), Me_2NBn (499 μL, 3.32 mmol, 0.100 equiv.), DIEA (6.21 mL, 36.5 mmol, 1.10 equiv.) and DMF (7.20 mL) were added. Then, 2-MeTHF was added to achieve a total volume of 166 mL. This solution (water content: 0.098wt%, flow rate: 2.00 mL/min) and a solution of isobutyl chloroformate (0.400 M, 1.20 equiv.) in MeCN (flow rate: 1.20 mL/min) were introduced to the first T-shape mixer at 20 °C with the pumps. The resultant mixture was passed through the activation reaction flow channel (residence time = 5.01 s). Then, the resultant mixture and a solution of TMS-L-Phe-OTMS (**3**) (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to the second T-shape mixer at 20 °C with the pumps. The resultant mixture was passed through the amidation reaction flow channel (residence time = 30.0 s). Then, the resultant mixture, 2-MeTHF (flow rate: 5.33 mL/min) and aqueous 0.08 M HCl + 5 % NaCl (flow rate: 7.34 mL/min) were introduced to the cross mixer at room temperature with the pumps. The resultant mixture was passed through the extraction flow channel (inner diameter: 1.59 mm, length: 3.00 m) and separated by the first settler. Then, the resultant organic phase solution and aqueous 20% NaCl (flow rate: 6.17 mL/min) were introduced to the T-shape mixier at room temperature with the pumps. The resultant mixture was passed through the extraction flow channel (inner diameter: 1.59 mm, length: 3.00 m) and separated by the second settler. After being eluted for 16 min to reach a steady state, the resultant mixture was collected for 60 min at room temperature.

The obtained crude solution of Fmoc-L-Ala-L-Phe-L-Phe-OH (**6**) (*ca.* 670 mL, purity: 79Area%) was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by recrystallization from MeCN and column chromatography (Biotage Isolera One flash purification system, Sfär HC Duo 100 g, heptane : THF = 75 : 25 to 20 : 80) gave Fmoc-L-Ala-L-Phe-L-Phe-OH (**6**) (9.30 g, 20.3 mmol, 64%) as a white solid.

mp 203–205 °C; IR (ATR): 3298, 1710, 1690, 1650, 1539, 1452, 1262, 1085, 741, 699 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): δ 12.78 (brs, 1H), 8.26 (d, J = 7.8 Hz, 1H), 7.90–7.87 (m, 3H), 7.72–7.69 (m, 2H), 7.46 (d, J = 7.8 Hz, 1H), 7.42 (t, J = 7.2 Hz, 2H), 7.34–7.14 (m, 12H), 4.55–4.51 (m, 1H), 4.47–4.43 (m, 1H), 4.28–4.19 (m, 3H), 4.03–3.98 (m, 1H), 3.06 (dd, J = 5.4, 13.8 Hz, 1H), 3.00 (dd, J = 4.8, 13.8 Hz, 1H), 2.93 (dd, J = 8.4, 13.8 Hz, 1H), 2.78 (dd, J = 9.0, 13.8 Hz, 1H), 1.11 (d, J = 7.2 Hz, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): δ 172.7, 172.1, 170.9, 155.6, 143.9, 143.8, 140.7, 137.5, 137.4, 129.3, 129.1, 128.2, 127.9, 127.6, 127.1, 126.5, 126.2, 125.3, 125.3, 120.1, 65.7, 53.5, 53.5, 50.1, 46.6, 37.5, 36.7, 18.2; HRMS (ESI-TOF): calcd for $[C_{36}H_{35}N_3O_6+H]^+$ 606.2604, found 606.2606.

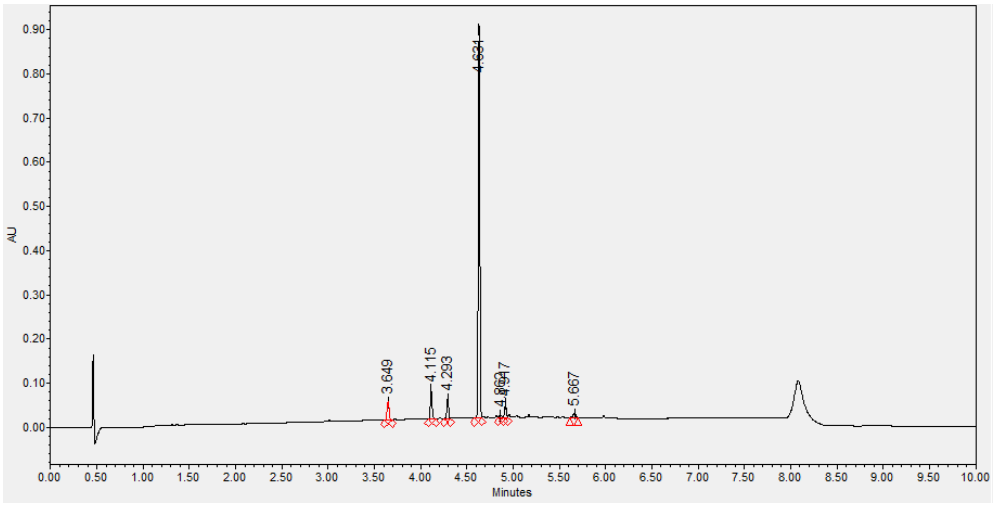


Figure S13: HPLC chart of crude **6** (after extraction)

HPLC conditions were shown below.

ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 100 mm

mobile phase A = H_2O + 0.025% TFA, mobile phase B = MeCN + 0.025% TFA

ratio of mobile phase B: 0–5.56 min: 5 to 95%, 5.56–7.22 min: 95%, 7.22–10 min: 5%

flow rate 0.6 mL/min, 60 °C, detection wavelength 215 nm

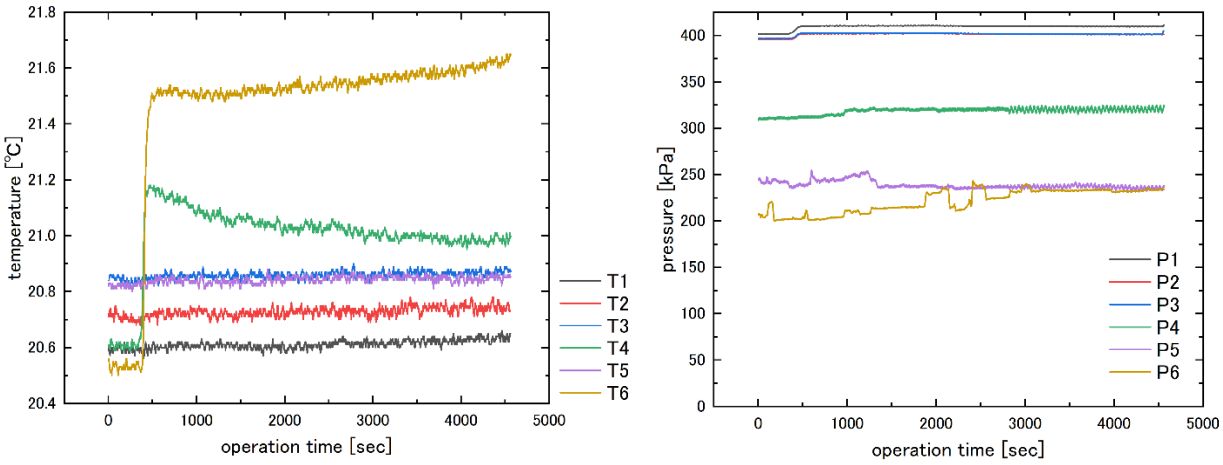


Figure S14: Temperature and pressure sensing data

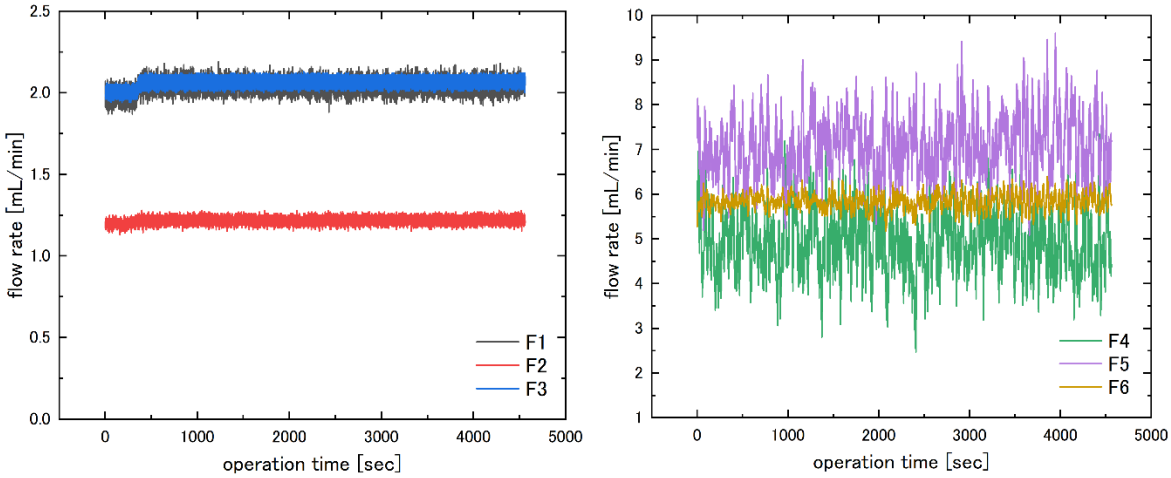


Figure S15: Flow rate sensing data

7. In-line NIR sensing of amide contained in peptide

7-1. General information

For the Near Infrared (NIR) spectroscopic measurements, Fourier Transfer (FT) NIR spectrometer (FT-NIR Rocket, ARCoptix, Switzerland), light source (ARCLIGHT-NIR, ARCoptix, Switzerland) and NIR flow cell (FIA-USP-1000, optical path length = 1.0 mm, Ocean Insight, USA, FL) were used. The spectrometer, the flow cell and the light source were connected using ZrF_4 optical fiber cable (MZ41L1, NA = 0.20, Operating Wavelength Range = 0.3–4.5 µm), purchased from Thorlabs, Inc. (USA, NJ). The light guided to the flow cell was collimated with a ruby-sapphire ball lens (diameter = 3.18 mm, purchased from Edmund Optics, USA, NJ). The measured wavenumber region was 10000−4000 cm^{-1}, the spectral resolution was 4 cm^{-1}. Second derivative spectra were calculated using the Savitzky−Golay (SG) method with the second order polynomial setting the window size as 15 (totally 31) after smoothening with the same SG conditions. For the spectral analysis, the chemometrics software Unscrambler 11 (Camo Analytics, Norway) and OriginPro 2021 (OriginLab Corporation, USA, MA) were used.

7-2. NIR measurement of *N*-methylacetamide in various solvents

Solutions of *N*-methylacetamide (Tokyo Chemical Industry Co., Ltd., Japan) or Fmoc-L-Ala-L-Phe-OH (**4**) (synthesized by us, see section 3-1 in ESI) were prepared as 100 mM by dissolving in various solvents (indicated below). Each solution was kept in the flow cell and 128 spectra were accumulated. The processed spectra (5100–4800 or 5000–4750 cm^{-1}) were shown in Figure S16 and S17. These spectra were obtained by subtracting the second derivative spectrum of each solvent from that of *N*-methylacetamide or Fmoc-L-Ala-L-Phe-OH solution (average of 3 measurements). The obtained data were summarized on Table S7.

Table S6: list of the solvents used in this NIR measurement

Solvent	**Supplier**
2-MeTHF + MeCN (2 : 3.2)	Kanto Chemical Co., Inc. / NACALAI TESQUE, INC.

$CHCl_3$	FUJIFILM Wako Pure Chemical Corporation
DMSO	FUJIFILM Wako Pure Chemical Corporation

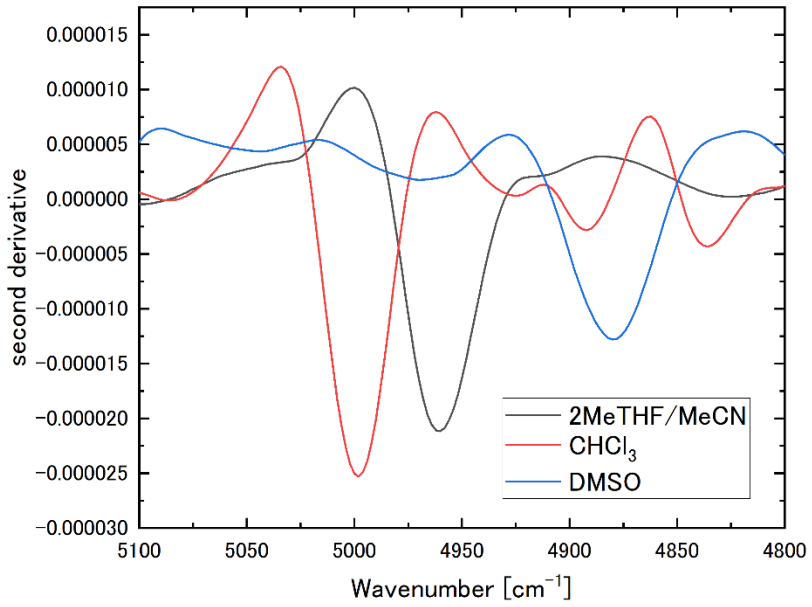


Figure S16: Spectra of *N*-methylacetamide in various solvents

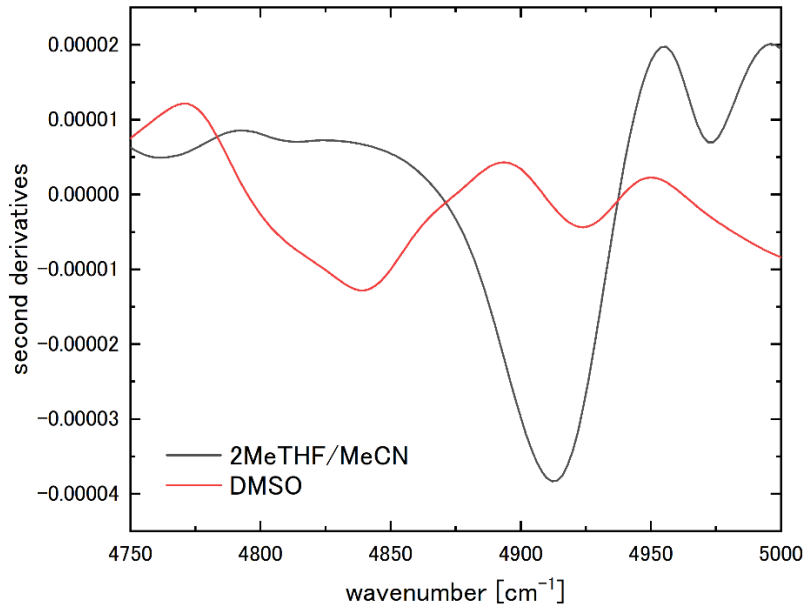


Figure S17: Spectra of Fmoc-L-Ala-L-Phe-OH (**4**) in various solvents

Table S7: Amide A/II bands of *N*-methylacetamide and peptide **4** in various solvents

Solvent	Band of Amide A/II (cm^{-1})	
	N-methylacetamide	Fmoc-Ala-Phe-OH (**4**)
DMSO	4880	4836 (4840[a])

2-MeTHF/MeCN	4960	4910
1,4-dioxane	(4950[b])	-
chloroform	(5000[b])	-

[a]Reported data for Fmoc-Gly-OH in DMSO.[2] [b]Reported data for *N*-methylacetamide in the indicated solvent.[3]

Note: According to a previous report,[2] the Amide A/II band appears at approximately 4840 cm^{-1} in DMSO. However, the desired band appeared at 4910 cm^{-1} for a sample of compound **4** in a mixture of 2-MeTHF and MeCN. We attributed this 70 cm^{-1} shift from 4840 cm^{-1} to the solvent (2-MeTHF + MeCN) in our system. Krikorian *et al.* reported that the amide A/II band of *N*-methylacetamide shifted by 50 cm^{-1} when 1,4-dioxane was used as the solvent instead of chloroform.[3] Therefore, we recorded the NIR spectra of *N*-methylacetamide and Fmoc-Ala-Phe-OH **4** in various solvents (Table S7). The amide A/II band of *N*-methylacetamide shifted 80 cm^{-1} when 2-MeTHF/ MeCN was used instead of DMSO, which confirmed our hypothesis. Furthermore, we recorded the NIR spectra of other amino acids and peptides in 2-MeTHF/ MeCN and observed that their bands also appeared at ~4910 cm^{-1} (see the next section for details).

7-3. NIR measurements of various peptides and amino acids

Solutions of amino acids or peptides (indicated below) were prepared as 25, 50, 100 mM by dissolving in 2-MeTHF + MeCN (2 : 3.2). Each solution was kept in the flow cell and 128 spectra were accumulated. The processed spectra (5000–4800 cm^{-1}) were shown in Figure S18. These spectra were obtained by subtracting the second derivative spectrum of solvent from that of amino acids or peptides solution (average of 3 measurements).

Table S8: list of the amino acids and peptides used in this NIR measurement

Amino acids/peptides	**Supplier**
Fmoc-L-Ala-OH (1)	WATANABE CHEMICAL INDUSTRIES, LTD.
Fmoc-L-Phe-OH	WATANABE CHEMICAL INDUSTRIES, LTD.
Fmoc-L-Ala-L-Phe-OH (4)	synthesized by us, see section 3-1 in ESI
Fmoc-L-Ala-L-Ser(*t*-Bu)-OH	GL Biochem（Shanghai）Corporation.Ltd. (purity: 92%)
Fmoc-L-Phe-L-Tyr(*t*-Bu)-OH	GL Biochem（Shanghai）Corporation.Ltd. (purity: 98%)

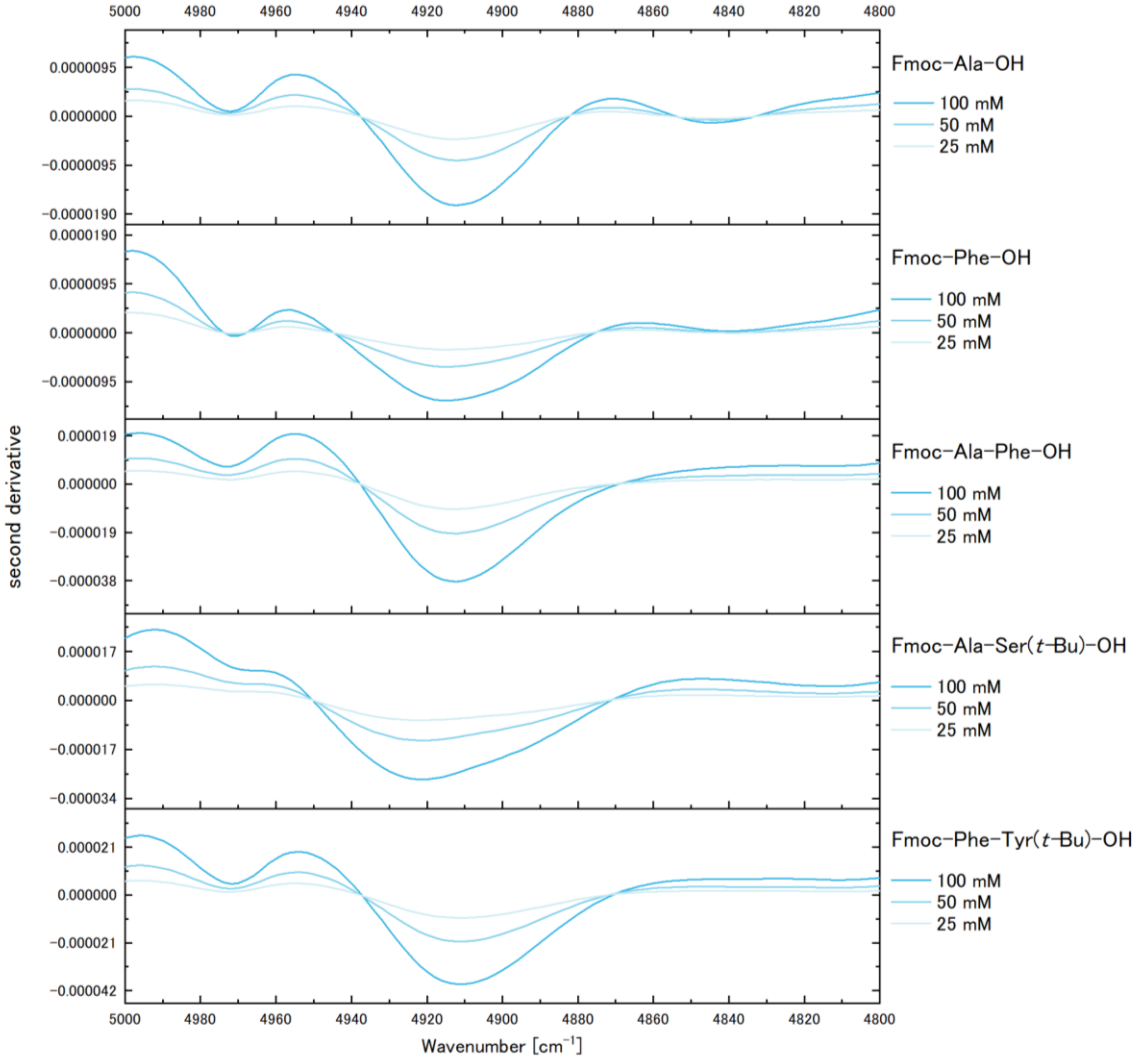


Figure S18: Spectra of amino acids and peptides

7-4. NIR measurements of reaction mixture and reactant/reagent solutions

Fmoc-L-Ala-OH (200 mM), Me_2NBn (20.0 mM) and DIEA (220 mM) were dissolved in 2-MeTHF (solution A). Isobutyl chloroformate (400 mM) was dissolved in MeCN (solution B). A solution C; TMS-L-Phe-OTMS (**3**) (240 mM) was prepared by same procedure as section 3-1 in ESI. The solution A was mixed with the solution B in a test tube. After being stirred for 30 sec, the solution C was added to afford reaction mixture. Each solution was kept in the flow cell and 128 spectra were accumulated. The processed spectra (5000–4800 cm^{-1}) were shown in Figure S19. These spectra were obtained by subtracting the second derivative spectrum of each solvent from that of reaction mixture or reactant/reagent solution (average of 3 measurements).

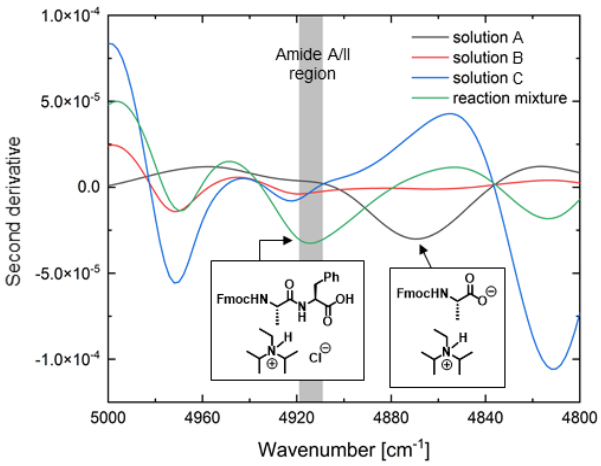


Figure S19: Near infrared spectra of reactant, reagents, and reaction mixture. Solution A: **1** and *i*-Pr_2NEt in 2-MeTHF, Solution B: IBCF in MeCN, Solution C: **3** in MeCN.

Note: The absorbances of the reactant, reagents, and reaction mixture were recorded to identify possible overlaps. The solutions of IBCF in MeCN (solution B) and **3** in MeCN (solution C) exhibited no absorption at ~4910 cm^{-1} (Figure S19). Although **1** has an amide bond in the carbamate moiety of the Fmoc group, the solution of **1** and *i*-Pr_2NEt in 2-MeTHF (solution A) showed no absorption at ~4910 cm^{-1}. However, another band appeared at 4870 cm^{-1} for solution A, which could be attributed to the shift from ~4910 cm^{-1} owing to the deprotonation of carboxylic acid **1** by *i*-Pr_2NEt (experiment and quantum calculation supported this hypothesis; see section 7-5 and -6). The reaction mixture of solutions A, B, and C prepared in a batch reactor exhibited a band at 4910 cm^{-1} in the NIR spectra. Because *i*-Pr_2NEt in solution A trapped HCl generated during the reaction, carboxylic acid **4** in the reaction mixture was not deprotonated. Therefore, the absorption of the reactant and reagents did not prevent the quantitative measurement of the peptide product.

7-5. Measurements and comparison of NIR spectra w/wo organic base

Solutions of Fmoc-L-Ala-OH (**1**) or Fmoc-L-Ala-L-Phe-OH (**4**), with or without DIEA (100 mM) were prepared as 100 mM by dissolving in 2-MeTHF + MeCN (2 : 3.2). Each solution was kept in the flow cell and 128 spectra were accumulated. The processed spectra (5000–4800 cm^{-1}) were shown in Figure S20. These spectra were obtained by subtracting the second derivative spectrum of solvent from that of amino acid/peptide solution (average of 3 measurements).

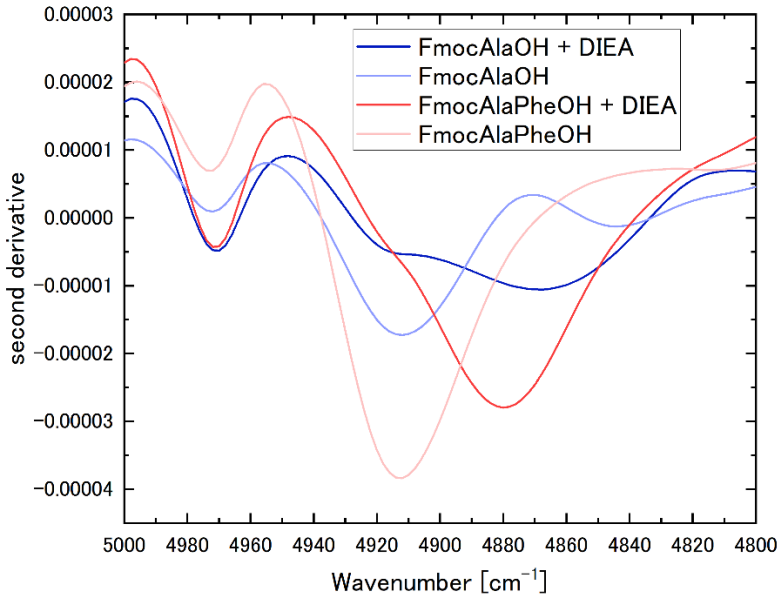


Figure S20: Spectra of amino acid/peptide solutions w/wo organic base

7-6. DFT calculation of NIR spectra w/wo base

One hundred each of Fmoc-Ala-OH and Fmoc-Ala-O$^-$Na$^+$ conformers were exported using Balloon[4, 5] in the Winmostar V9 program (X-Ability Co. Ltd., Tokyo, Japan, 2019). The Na$^+$ ion was put instead of the organic base for simplification of the system. An anharmonic DFT study of the NIR spectra was performed using the Gaussian 16 program[6] and the conformers were screened based on Gibbs free energies. Each of the most stable conformers was selected based on B3LYP/6-31+G(d) level, and the second-order vibrational perturbation theory (VPT2) model was applied to conduct the anharmonic vibrational analysis for these conformers (Table S9). We also considered an implicit solvation model using IEFPCM with model solvent, MeCN (Table S10).

Table S9: NIR spectra analysis in vacuum condition

Conditions	**Calculated wavenumber of Amide A/II [cm^{-1}]**
(1) Fmoc-Ala-OH	4936.87
(2) Fmoc-Ala-ONa	4889.20
(2) – (1) peak shift	-47.67

Table S10: NIR spectra analysis using IEFPCM solvation model with MeCN

Conditions	**Calculated wavenumber of Amide A/II [cm^{-1}]**
(1) Fmoc-Ala-OH	4931.13
(2) Fmoc-Ala-ONa	4823.96
(2) – (1) peak shift	-107.17

7-7. Continuous measurement of NIR spectra in the developed flow system

Reaction conditions were shown in section 6-1 in ESI. Sixteen spectra were repeatedly accumulated. Some parts of the processed spectra (380–1003 s, 5000–4800 cm^{-1}) were shown in Figure 7. These spectra were obtained by subtracting the second derivative spectrum of solvent from that of reaction solution.

NIR Spectrum of isolated Fmoc-Ala-Phe-OH (**4**) were shown in section 7-3 in ESI. These data were used for calibration curve. We used simple linear regression model. A plot of concentration vs. NIR peak height at 4914 cm^{-1} were shown in Figure S21. Based on this calibration curve, yields of Fmoc-Ala-Phe-OH were calculated (Figure 7).

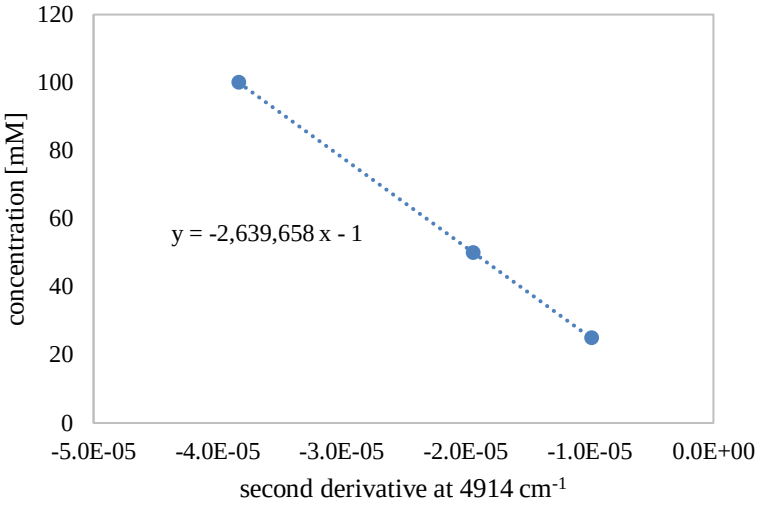
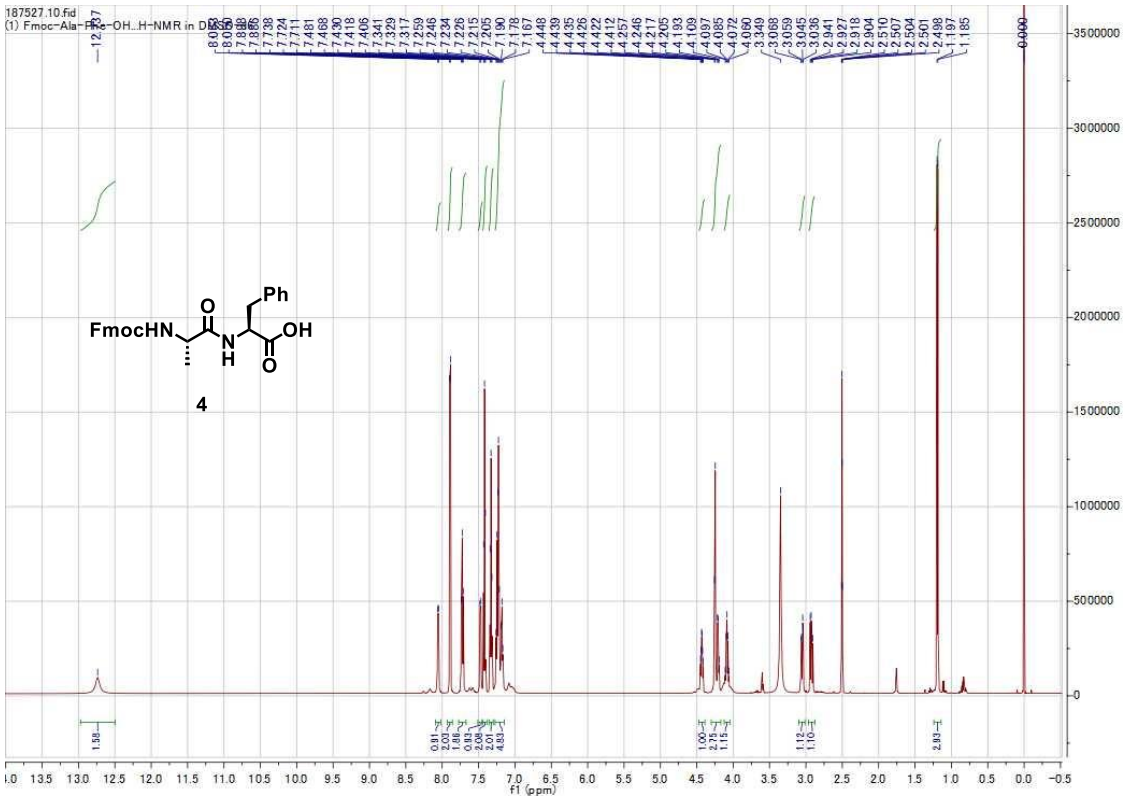


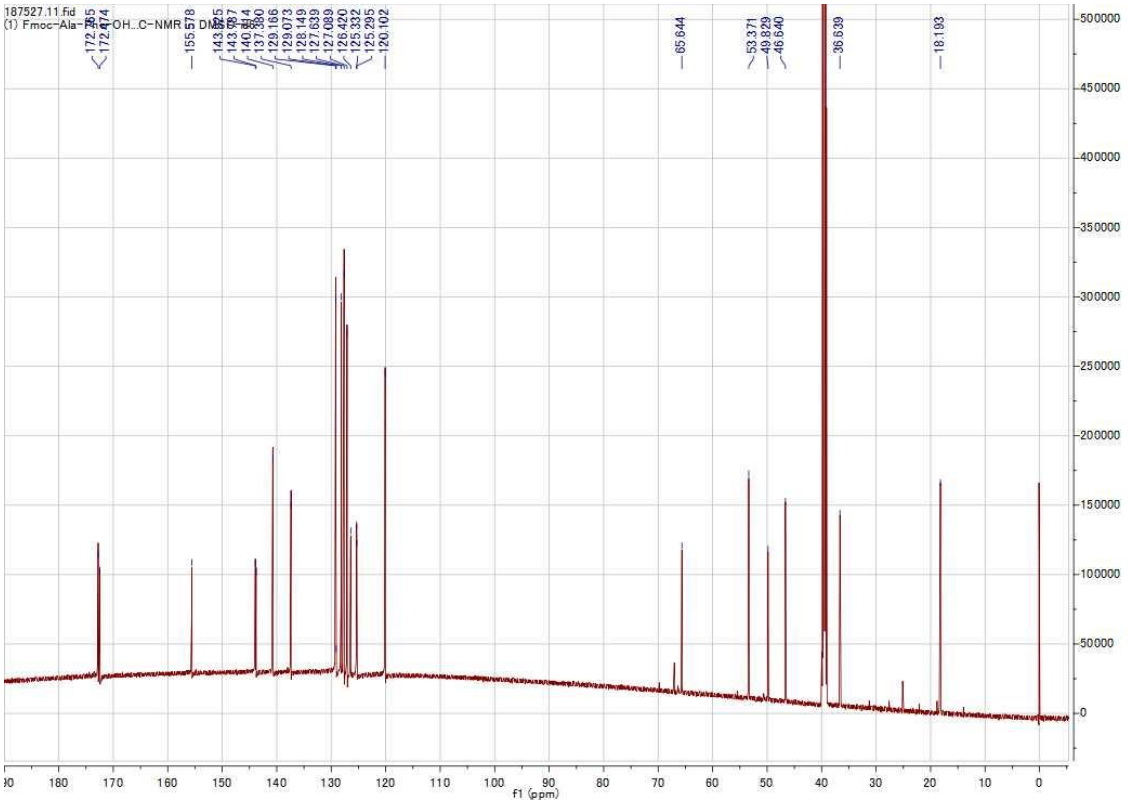
Figure S21: Calibration curve

8. NMR spectrum

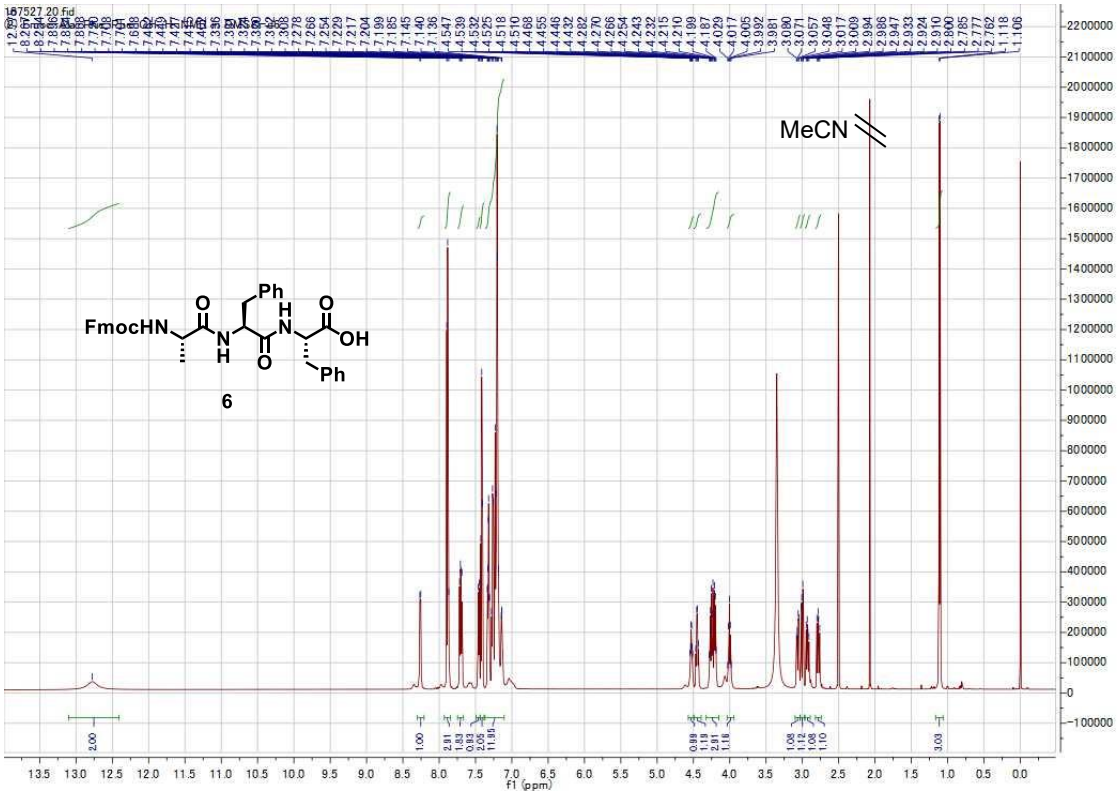
Fmoc-L-Ala-L-Phe-OH (**4**) (^{1}H, 600 MHz, DMSO-d_6)



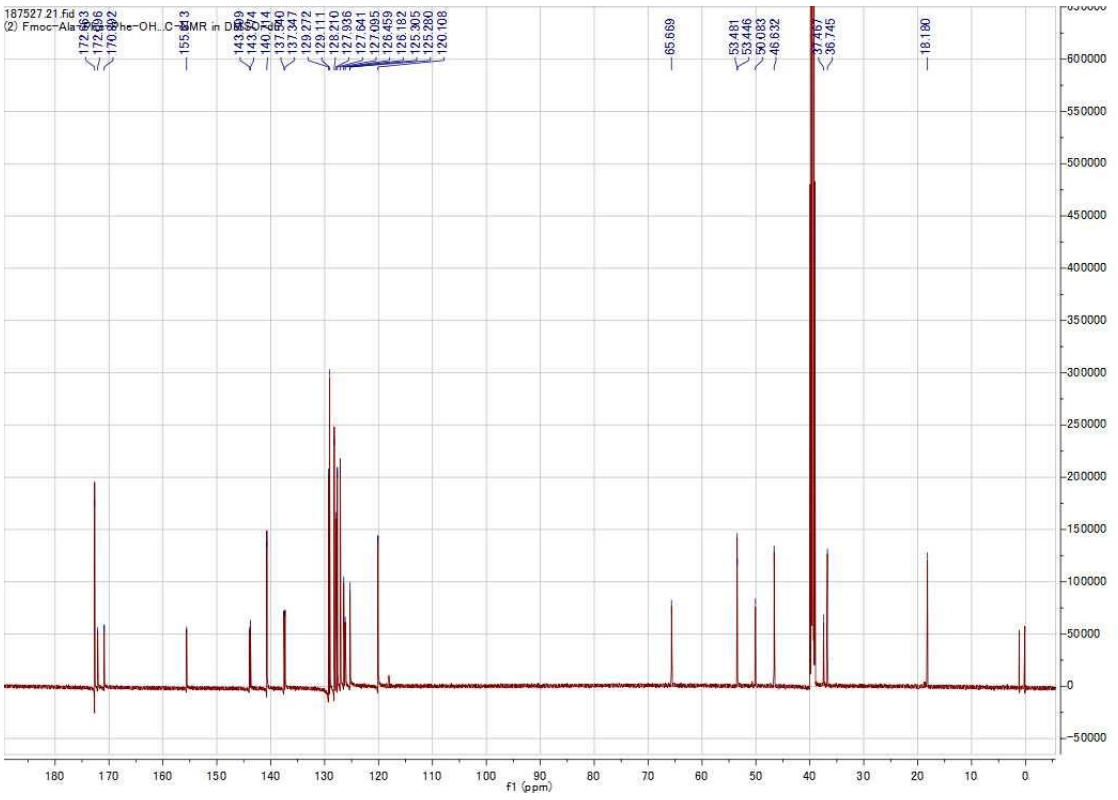
Fmoc-L-Ala-L-Phe-OH (**4**) (^{13}C, 151 MHz, DMSO-d_6)



Fmoc-L-Ala-L-Phe-L-Phe-OH (**6**) (^{1}H, 600 MHz, DMSO-d_6)



Fmoc-L-Ala-L-Phe-L-Phe-OH (**6**) (^{13}C, 151 MHz, DMSO-d_6)



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