The Journal of Organic Chemistry

Note

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J. Org. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.joc.5b02399 • Publication Date (Web): 08 Dec 2015

ACS Publications

The Journal of Organic Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Development of an intein-inspired amide cleavage chemical device

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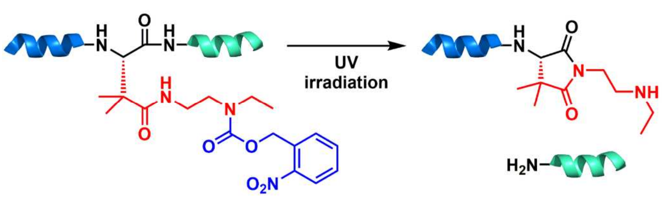


Abstract

A photo-responsive amide cleavage device was developed based on the asparagine imidation-mediated cleavage of peptide bonds during intein-mediated protein splicing. The chemical environment of the protein splicing process was mimicked by the incorporation of geminal dimethyl groups and a secondary amine unit in asparagine scaffold. Furthermore, the resulting photo-responsive device could induce the photo-triggered cleavage of an amide bond by the protection of the secondary amine unit with an *o*-nitrobenzyloxycarbonyl group.

Intein proteins,[1] which are found in a wide range of unicellular organisms,[2] mediate the self-splicing of intein-containing proteins to produce intein-removed splicing proteins through sequential *N*–*S*(or *O*), *S*(or *O*)–*S*(or *O*) and *S*(or *O*)–*N*-acyl transfers. The third *S*(or *O*)–*N*-acyl transfer step in this process starts from the imide cyclization of an asparagine (Asn) residue at the intein C-terminus, which is followed by the transfer of an *O*(or *S*)–peptidyl unit to the liberated amino group.[3] The progress of this sequence of reactions depends on several requirements, including (i) enhancement of the nucleophilicity of the amide side chain of the Asn residue; (ii) activation of the scission peptide bond; and (iii) appropriate arrangement of the functional groups involved in the reactions. An analysis of the structural basis for this reaction[4] indicated that the appropriate arrangement of several functional units, including a water molecule, assist in the cleavage of the amide via an acid-base-catalyzed mechanism (Figure 1).[4a]

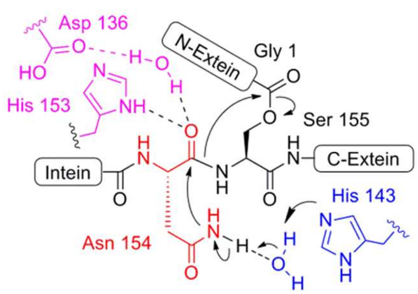


Figure 1. Mechanism of acid-base-catalyzed amide cleavage.

Methodologies for photo-induced amide bond cleavage[5] and conformational change[6] have provided powerful tools for the spatiotemporal control of the functions of peptide/proteins. We previously developed a stimulus-responsive processing residue (Spr)[7] based on a trimethyl-lock system.[8,9] This Spr system has shown utility in the field of chemical biology and has potential for real-life application.[7a,c,e] In conjunction with our studies on Spr, we also explored the development of an alternative new scaffold. In this context, the result of a mechanistic study[4] of the third step of the reaction mentioned above inspired us to design a new amide bond cleavage device with a modified Asn structure. In this way, it was envisioned that the modifications shown in Figure 2 would provide the structural features necessary to affect the cleavage of an amide bond. The incorporation of a pendant secondary amine would provide an intramolecular base,[10] which could enhance the nucleophilicity of the amide nitrogen. Furthermore, the incorporation of geminal dimethyl groups would lead to a Thorpe-Ingold effect,[11–13] which would fix the conformation of the intein system and assist in the formation of the succinimide ring. Lastly, the masking of the basic character of the secondary amine with a photo-sensitive *N*-protecting group,[14] such as *o*-nitrobenzyloxycarbonyl (*o*NBnoc), could provide a simple platform for the development of stimulus-responsive amide bond cleavage device.

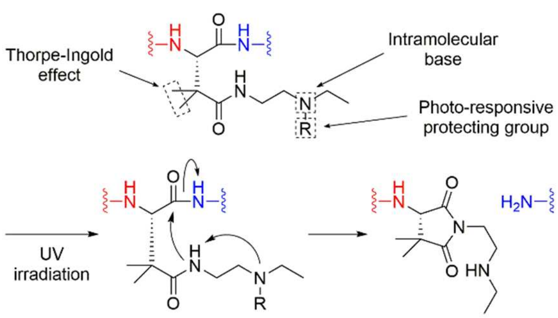
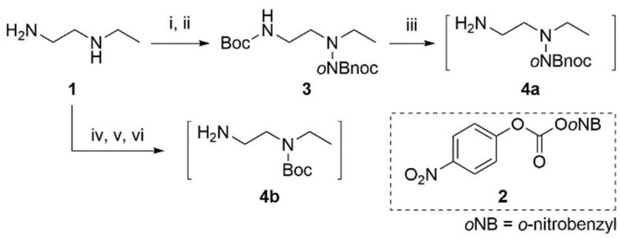


Figure 2. Design of an intein-mediated UV-responsive amide cleavage device.

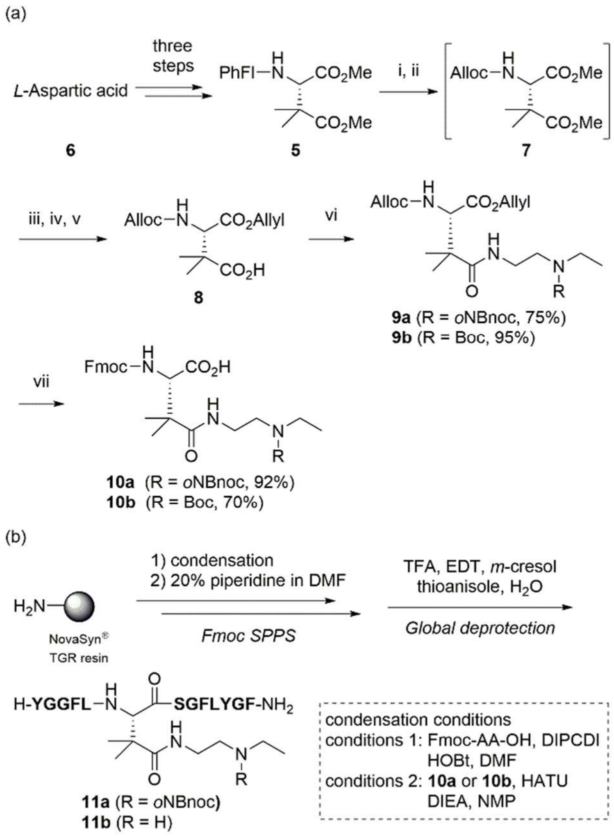
Our work toward preparation of a pendant secondary amine capable of responding to UV irradiation started from *N*-ethylethylenediamine (**1**) (Scheme 1). The reaction of **1** with *tert*-butyloxycarbonyl anhydride (Boc_2O) in THF allowed for the selective protection of the primary amino group. The subsequent reaction of the secondary amine group with *p*-nitroformate **2**[15] and triethylamine (Et_3N) in THF afforded the requisite compound **3** in quantitative yield (over two steps). The trifluoroacetic acid (TFA)-mediated removal of the Boc protecting group from **3** gave the pendant amine unit **4a**. The synthesis of the Boc-protected pendant unit **4b** proceeded via the trifluoroacetylation of the primary amino group of **1**, followed by the introduction of a Boc group and the subsequent hydrolysis of the trifluoroacetyl protecting group.

Scheme 1. Synthesis of the pendant secondary amine **4a** and **4b**



Reagents and conditions: (i) Boc_2O, THF; (ii) **2**, Et_3N, THF, quant. (two steps); (iii) TFA, CH_2Cl_2; (iv) ethyltrifluoroacetate, CH_2Cl_2; (v) Boc_2O, CH_2Cl_2; (vi) K_2CO_3, MeOH, H_2O

Scheme 2. (a) Synthesis of the Intein-inspired amide cleavage device **10a** and **10b** and (b) the preparation of a model peptide.



Reagents and conditions: (i) TFA, TES, CH_2Cl_2; (ii) AllocCl, $NaHCO_3$, THF, H_2O; (iii) LiOH, THF, H_2O; (iv) Ac_2O, THF; (v) allyl alcohol, 60% (five steps); (vi) **4a** or **4b**, PyBrop, DIEA, CH_2Cl_2; (vii) $Pd(PPh_3)_4$, *N*-methylaniline, THF then FmocOSu, DIPEA

PhFl-diMe-Asp(OMe)-OMe **5**[16] was synthesized over three steps from *L*-Aspartic acid (**6**) following Goodman's procedure (Scheme 2). The deprotection of the PhFl group in **5** with a mixture of TFA and triethyl silane (TES) in CH_2Cl_2, followed by the protection of the resulting amine with allylchloroformate (Alloc-Cl) gave Alloc-diMe-Asp(OMe)-OMe **7**. The subsequent hydrolysis of the two methyl esters of **7** with LiOH in a mixture of THF and H_2O gave the corresponding carboxylic acid, which was reacted with Ac_2O in THF at reflux temperature, followed by an alcoholysis reaction with allyl alcohol to give the *α*-allylester Alloc-diMe-Asp(OH)-OAllyl **8** in 60% isolated yield (over five steps). The reaction of the UV-responsive amine **4a** with the sterically-crowded *β*-carboxylic acid of **8** was accomplished using bromotripyrrolidinophosphonium hexafluorophosphate (PyBrop) and diisopropylethyl amine (DIPEA) in CH_2Cl_2 to yield the fully protected Asn derivative Alloc-diMe-Asn(Et-*N*-*o*NBnoc)-OAllyl **9a** in 75% isolated yield. The conversion of **9a** to the corresponding 9-fluorenylmethyloxycarbonyl (Fmoc)-protected derivative for Fmoc solid-phase peptide synthesis (SPPS) was achieved by the deprotection of the allyl and allyloxycarbonyl (Alloc) groups by the treatment of **9a** with $Pd(PPh)_4$ and *N*-methylaniline, followed by the Fmoc protection of the resulting amine to give Fmoc-diMe-Asn(Et-*N*-oNBnoc)-OH **10a** in 92% isolated yield. The Boc-protected material Fmoc-diMe-Asn(Et-*N*-Boc)-OH **10b** was also prepared in a similar manner in 70% isolated yield from **9b**.

With the requisite Asn derivatives in hand, we proceeded to synthesize two model peptides (H-**YGGFL-X-SGFLYGF**-NH_2 **11a** and **11b**: X = Asn derivatives) to examine the self-processing properties of the peptides. The Fmoc protected amino acids were condensed on NovaSyn® TGR resin using diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenztriazole (HOBt) in dimethylformamide (DMF) except for **10a** and **10b**. The condensation of compound **10a** was achieved using 1-[bis-(dimethylamino)methylene]1H-1,2,3-triazolo[4,5-β]pyridine-3-oxide hexafluorophosphate (HATU) and DIEA in *N*-methylpyrrolidone (NMP). The completed peptide resins were subsequently exposed to a mixture of TFA-ethanedithiol (EDT)-*m*-cresol-thioanisole-H_2O at room temperature for 2 h to give a mixture of two peptides with mass values identical to that of the desired material (Figure 3).

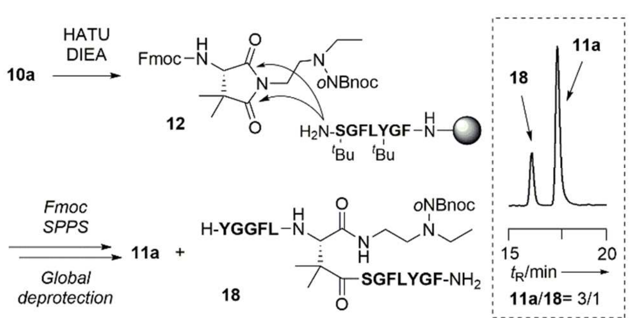
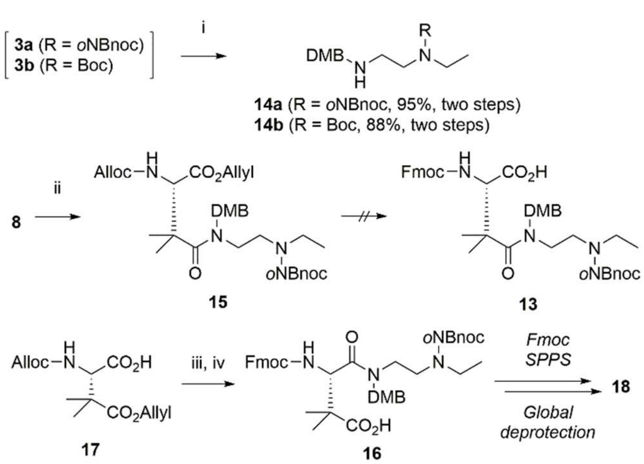


Figure 3. Possible mechanism for the generation of the byproduct **18**.

The origin of the two peptides was attributed to the formation of the succinimide species **12** during the activation of **10a**, followed by the aminolysis of the succinimide ring by the amino group of the growing peptide chain. Given that one of the two possible electrophilic sites in the succinimide ring of **12** was sterically crowded by the neighboring geminal dimethyl groups, it was anticipated that the major product of this reaction would be desired *α*-peptide **11a**. We envisaged that the benzyl protection of the amide nitrogen in **10a** would prevent the formation of the succinimide ring.[17] However, our attempts to synthesize the dimethoxybenzyl (DMB)-protected Asn derivative **13** resulted in failure (Scheme 3). Although the condensation of the *N*-dimethoxybenzyl diamine derivative **14a** with **8** gave the desired amide **15** in 50% yield, the subsequent exchange of the protecting groups resulted in the release of the pendant amine molecule **14a**. The failure of this reaction was attributed to the nucleophilic attack of the *α*-carboxylate on the substituted amide. To confirm the structure of peptide **18** as the iso-peptide form, the α-amide protected derivative **16**, suitable for the straightforward preparation of **18**, was prepared from the reaction of the *β*-allyl ester **17** with **14a** (Scheme 3).

Scheme 3. Synthetic approach to **13** and the synthesis of the isomeric peptide **18**



Reagents and conditions: (i) 2,4-dimethoxybenzaldehyde, Na_2SO_4, MeOH; $NaBH_4$; (ii) MsCl, Et_3N, CH_2Cl_2 then **14a**, 50%; (iii) MsCl, Et_3N, THF then **14a**, 60%; (iv) $Pd(PPh_3)_4$, *N*-methylaniline, THF then FmocOSu, DIPEA, quant.

The resulting imidation-tolerant *β*-carboxylic derivative **16** was also incorporated into a peptide resin in a manner similar to that employed for **11a**. The subsequent deprotection of the resin afforded the *β*-peptide **18**. This result clearly indicated that the major and minor products of the succinimide ring-opening reaction described above were **11a** and **18**, respectively. This result is shown in the HPLC chart in Figure 3 for the *α*- and *β*-peptides, respectively. A peptide sample without a UV-responsive group was also synthesized using the Boc-protected secondary amine **10b** in a manner similar to that used for **11a**. This peptide behaved in the same way as the corresponding system containing **10a**, in that the deprotection of the protected resin afforded a mixture of *α*- and *β*-peptides.

Peptides **19** and **20** were also prepared to determine the effects of the geminal dimethyl groups and secondary amine on the outcome of the transformation.[18] The Asn-protected derivative without geminal dimethyl groups was prepared by the coupling of the *β*-carboxylic acid of Fmoc-Asp(OH)-OAllyl[19] with *N*-DMB-*N'*-Boc-*N'*-ethyl ethylenediamine **14b**, followed by the removal of the allyl ester. The Fmoc-based incorporation of the resulting amino acid into the resin was followed by an acidic deprotection step to afford the desired peptide **19**, where the Asn residue had been successfully modified with a pendant secondary amine without the need for the protection of the secondary amine. Notably, no significant side reactions, including the hydrolysis of the peptide bonds, were observed during the acidic deprotection and HPLC purification stages. The Asn-incorporated peptide **20** was also synthesized as a separate reference compound using standard Fmoc protocols.

We initially investigated the self-processing of these synthetic peptides, as shown in Figure 4. Peptide samples were dissolved in a buffered solution (6 M guanidine hydrochloride (Gn·HCl)-0.2 M phosphate), where they were monitored for peptide bond cleavage by HPLC analysis. As expected, the presence of both the secondary amine as an intramolecular base and the geminal methyl groups as an inducer of cyclization greatly facilitated the cleavage of the peptide bond (Figure 4, Entries 1–3). When a mixture of the materials was held at pH 7.4 for 24 h at 37 °C, almost all of the samples went to completion to afford a mixture of split peptides consisting of N-half imide peptide **21**, C-half peptide **22** and the succinimide ring-opened peptides **23** and **24**. The results of these comparison experiments clearly show that modifications capable of mimicking the environments involved in the intein-induced cleavage of an amide bond were responsible for the envisioned artificial amide bond cleavage reaction (Figure 4, Entry 4).

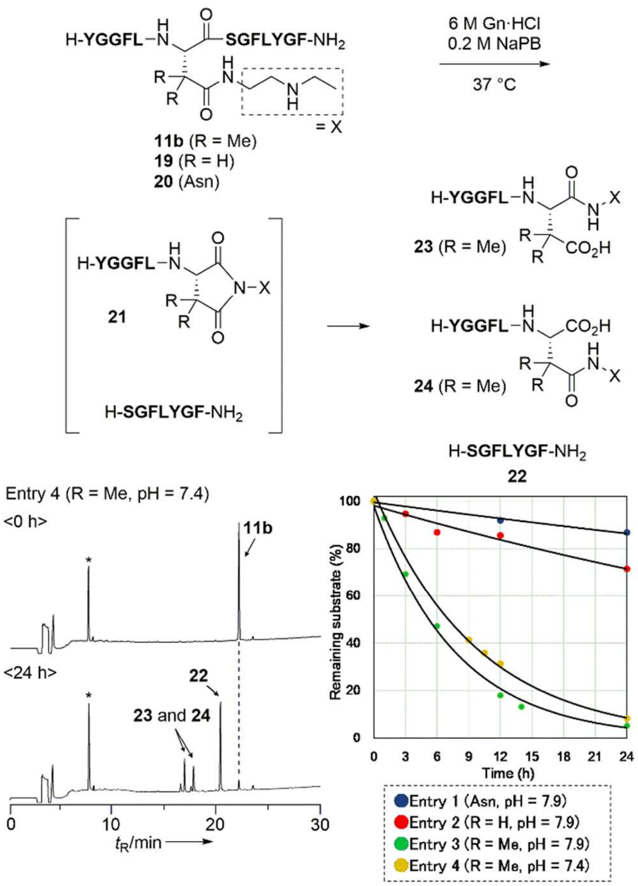


Figure 4. Self-processing reactions of the model peptides. *Internal standard.

Encouraged by the potential utility of **10a** as a stimulus-responsive processing device, we next examined the photo-responsive cleavage of the peptide bond in the synthetic peptide **11a** (Figure 5). When the *o*NBnoc-protected peptide **11a** was incubated for 24 h at 37 °C in a mixture of 6 M Gn·HCl and 0.2 M phosphate at pH 7.4 without UV irradiation, the material remained almost completely intact. The irradiation of the reaction mixture with UV light led to the removal of the *o*NBnoc group from the secondary amine unit to produce peptide **11b**, which was split to the processing peptides with about 80% cleavage after 24 h. These results clearly indicated that **11a** could serve as a stimulus-responsive processing device and an alternative to the Spr system based on a trimethyl lock.

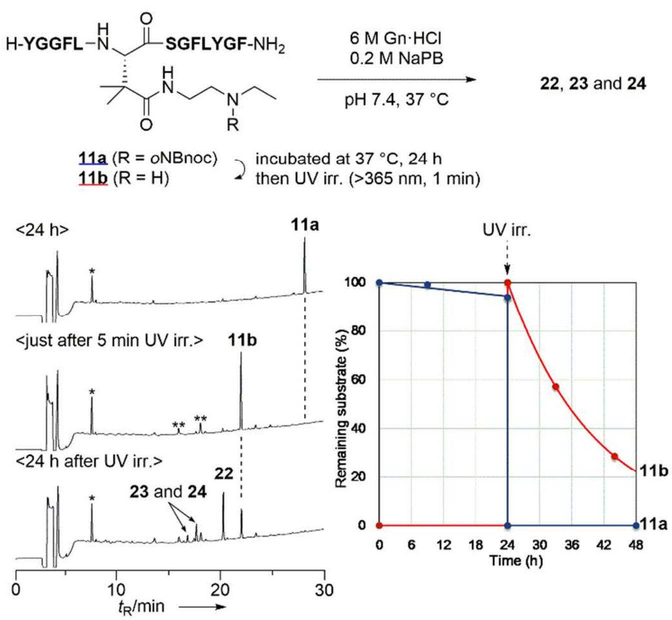


Figure 5. Photo-responsive peptide bond cleavage. *Internal standard. **Not peptidyl compounds, provably derived from deprotected *o*NBnoc group with UV irradiation.

In conclusion, we achieved the development of the new amide bond cleavage device modeled on the intein-mediated protein splicing. The design concept of the device is derived from the mimicking chemical environments involved in the protein splicing. Although preparation of the device and its incorporation into peptides are laborious, an important issue in this work is that the incorporation of geminal dimethyl groups and a secondary amine unit in asparagine scaffold well imitate the splicing system. Furthermore, protection of the secondary amine with the photo-removal group allowed the device to cleave the amide bond in response to photo-irradiation.

Experimental Section

General Information

All reactions were carried out under an atmosphere of argon. All commercial reagents were used without further purification. For column chromatography, silica gel (spherical, natural, 63-210 μm) was used. The progress of reactions was monitored by thin layer chromatography using precoated silica gel glass plates (0.25 mm) with F254 indicator. Mass spectra (ESI-MS) were obtained using a ToF mass spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured using a 300 or 400 MHz spectrometer at room temperature unless otherwise noted. Chemical shifts were calibrated to the solvent signal. Multiplicities are given as s (singlet), d (doublet), br d (broad doublet), t (triplet), br t (broad triplet), q (quartet), m (multiplet) or br m (broad multiplet). For HPLC separation, a Cosmosil 5C_{18}-AR-II analytical column (4.6 × 250 mm, flow rate 1.0 mL/min) or a Cosmosil 5C_{18}-AR-II semi-preparative column (10 × 250 mm, flow rate 3.0 mL/min) was employed and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution. IR spectra and optical rotations were measured using a polarimeter (concentration in g/100 mL), respectively. Photolysis was performed with the filtered output (>365 nm) of a 3000 mW/cm^2 HG-Xe lamp.

Synthesis of asparagine derivatives

2-Nitrobenzyl {2-[(tert-butoxycarbonyl)amino]ethyl}ethyl carbamate ***3***. To a solution of *N*-ethylethylenediamine (**1**) (5.37 mL, 50.0 mmol) in THF (100 mL) was added a solution of Boc_2O (3.27 g, 15.0 mmol) in THF (30 mL) dropwise at 0 °C. The reaction mixture was stirred at room temperature for 5 h, and then concentrated in vacuo. The obtained residue was subsequently diluted with EtOAc and sat. $NaHCO_3$ aq. The obtained mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude carbamate (2.82 g, 15.0 mmol, quant., pale yellow powder) was used for a next step without further purification.

The obtained carbamate (2.82 g) in THF (30 mL) was treated with Et_3N (1.62 mL, 11.6 mmol) followed by 2-nitrobenzyl 4-nitrophenyl carbonate **2**[15] (3.69 g, 11.6 mmol). The reaction mixture was stirred at room temperature for 4 h, and then concentrated in vacuo and diluted with EtOAc and 5% $KHSO_4$ aq. The obtained mixture was extracted three times with EtOAc. The combined organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The crude material was purified by column chromatography (*n*-hexane/EtOAc = 8/1 then 1/1) to afford *o*NBnoc-diamine **3** (4.26 g, 11.6 mmol, quant.) as yellow oil. IR ($CHCl_3$): ν_{max}, cm^{-1}: 1364, 1477, 1529, 1701, 2875, 2977, 3358; ^{1}H-NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 1.09 (3H, t, J = 7.0 Hz), 1.38 (9H, s), 3.11 (2H, dt, J = 6.6 and 6.6 Hz), 3.22–3.35 (4H, m), 5.39 (2H, s), 6.35–6.47 (1H, br m), 7.60 (1H, dd, J = 8.1 and 7.5 Hz), 7.67 (1H, d, J = 7.3 Hz), 7.76 (1H, dd, J = 7.3 and 7.5 Hz), 8.05 (1H, d, J = 8.1 Hz); ^{13}C-NMR (DMSO-d_6, 60 °C, 75 MHz) δ = 13.1, 28.0, 41.9, 62.7, 77.5, 124.3, 128.7, 128.8, 132.1, 133.6, 147.2, 154.5, 155.3; HRMS (ESI-TOF) m/z calcd for $C_{17}H_{25}N_3NaO_6$ ($[M + Na]^+$): 390.1641, found: 390.1643.

2-Nitrobenzyl (2-aminoethyl)(ethyl)carbamate ***4a***. Carbamate **3** (2.00 g, 2.72 mmol) in CH_2Cl_2 (1.36 mL) was treated with trifluoroacetic acid (1.36 mL). The reaction mixture was stirred at room temperature for 45 min and concentrated in vacuo. After dilution of the resulting residue with EtOAc and sat. $NaHCO_3$ aq, the solution was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. Crude *o*NBoc amine **4a** (1.45 g) was obtained as yellow powder. The obtained crude **4a** was used for preparation of **9a** and **14a** without further purification.

*tert-Butyl (2-aminoethyl)(ethyl)carbamate **4b***. To a stirred mixture of *N*-ethylethylenediamine **1** (2.39 mL, 22.7 mmol) in CH_2Cl_2 (50 mL) was added ethyl trifluoroacetate (3.46 mL, 22.7 mmol) in CH_2Cl_2 (50 mL) dropwise over 40 min at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then concentrated in vacuo. After dilution of the resulting residue with CH_2Cl_2 (100 mL), to the solution was added Boc_2O (4.95 g, 22.7 mmol) in CH_2Cl_2 (5.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h and then diluted with EtOAc and sat. $NaHCO_3$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. The obtained crude material in MeOH (90 mL) and H_2O (10 mL) was treated with K_2CO_3 (2.00 g). The reaction mixture was refluxed for 2 h and then concentrated in vacuo. The mixture was extracted three times with EtOAc. The combined organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Crude Boc amine **4b** (4.27 g) was obtained as pale yellow oil. The obtained crude **4b** was used for preparation of **9b** and **14b** without further purification.

*(S)-3-{[(Allyloxy)carbonyl]amino}-2,2-dimethylsuccinic acid 4-(allyl)ester **8** and (S)-4-(allyloxy)-2-{[(allyloxy)carbonyl]amino}-3,3-dimethyl-4-oxobutanoic acid **17*** To a solution of PhFl-diMe-Asp(OMe)-OMe **5**[16] (1.83 g, 4.05 mmol) in CH_2Cl_2 (10.2 mL) was added triethyl silane (1.43 mL, 14.2 mmol) followed by trifluoroacetic acid (10.2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h and concentrated in vacuo. The obtained mixture was diluted with 1 M HCl aq. The precipitate was filtrated and washed with MeOH. The filtrate was concentrated in vacuo and the resulting crude amine was used for a next step without further purification.

The crude amine in THF (12.5 mL) and H_2O (8.96 mL) was treated with $NaHCO_3$ (2.51 g, 29.9 mmol) followed by allylchloroformate (63.6 μL, 5.98 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 9 h and diluted with H_2O and EtOAc. The mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude Alloc-diMe-Asp(OMe)-OMe **7** was used for next step without further purification.

The crude **7** in THF (10.9 mL) and H_2O (30 mL) was treated with 1 M LiOH aq. (17.4 mL, 17.4 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 10 h and diluted with CH_2Cl_2. The aqueous layer was washed three times with CH_2Cl_2 and then acidified (pH ≈ 3) with 3 M HCl aq. To the aqueous layer was added EtOAc and NaCl. The mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. Crude carboxylic acid (1.22 g) was obtained as colorless oil. 600 mg of it was used for next step without further purification.

The stirred mixture of obtained crude carboxylic acid (600 mg) in THF (2.43 mL) was treated with Ac_2O (616 μL, 6.56 mmol). The reaction mixture was refluxed for 18 h and then concentrated in vacuo. To the obtained crude anhydride was added allylalcohol (7.5 mL). The reaction mixture was stirred at room temperature for 23 h and concentrated in vacuo. The obtained crude material was purified by column chromatography (chloroform/MeOH = 400/1 then 50/1) to afford **8** (449 mg, 1.47 mmol, 61% over five steps from **5**) as colorless oil and **17** (131 mg, 0.459 mmol, 19% over five steps from **5**) as colorless oil. **8**: $[\alpha]^{28}_{D}$ -12.3 (*c* 1.56, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1330, 1519, 1713, 2886, 2942, 3084, 3349; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ = 1.26 (3H, s), 1.37 (3H, s), 4.58–4.67 (5H, m), 5.21–5.27 (2H, m), 5.29–5.36 (2H, m), 5.66 (br d, J = 9.6 Hz), 5.85–5.98 (2H, m); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ = 22.2, 23.3, 45.7, 59.8, 66.0, 66.4, 118.2, 118.7, 131.9, 132.5, 156.5, 175.1, 175.3; HRMS (ESI-TOF) m/z calcd for $C_{13}H_{19}N_1NaO_6$ ($[M + Na]^+$): 308.1110, found 308.1115. **17**: $[\alpha]^{28}_{D}$ -11.8 (*c* 2.40, $CHCl_3$), IR ($CHCl_3$): ν_{max}, cm^{-1}:932, 1251, 1525, 1724, 2886, 2944, 2984, 3088, 3350; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ = 1.23 (3H, s), 1.34 (3H, s), 4.57–4.67 (5H, m), 5.20–5.26 (2H, m), 5.28–5.36 (2H, m), 5.68 (1H, br d, J = 10.4), 5.81–5.98 (2H, m); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ = 22.1, 23.3, 45.6, 59.9, 66.3, 66.4, 118.2, 119.3, 131.3, 132.6, 156.4, 170.2, 181.5; HRMS (ESI-TOF) m/z calcd for $C_{25}H_{19}N_1NaO_6$ ($[M + Na]^+$): 308.1110, found: 308.1121.

Allyl (S)-2-{[(allyloxy)carbonyl]amino}-4-{[2-ethyl(2-nitrobenzyloxycarbonyl)aminoethyl]amino}-3,3-dimethyl-4-oxobutanoate ***9a***. To a solution of **8** (34.5 mg, 0.121 mmol) in CH_2Cl_2 (605 µL) were added crude **4a** (93.8 mg), bromotri(pyrrolidino)phosphonium hexafluorophosphate (PyBrop) (152 mg, 0.454 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (77.2 µL, 0.454 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 34 h and then diluted with EtOAc and 5% $KHSO_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with sat. $NaHCO_3$ aq., dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 1/1 then 1/2) to afford amide **9a** (48.0 mg, 90.4 µmol, 75%) as colorless oil. $[\alpha]^{28}_D$ -2.9 (c 1.25, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1268, 1342, 1427, 1526, 1650, 1703, 2875, 2939, 2973, 3079, 3361; ^{1}H-NMR (DMSO-d_6, 70 °C, 300 MHz) δ = 1.08 (3H, t, J = 7.1 Hz), 1.13 (3H, s), 1.14 (3H, s), 3.19–3.35 (6H, m), 4.46–4.59 (5H, m), 5.13–5.24 (2H, m), 5.24–5.36 (2H, m), 5.4 (2H, s), 5.81–5.98 (2H, m), 7.16 (1H, br d, J = 2.9 Hz), 7.46–7.55 (1H, br m), 7.60 (1H, dd, J = 7.1, 8.0 Hz), 7.69 (1H, br d, J = 7.2 Hz), 7.78 (1H, dd, J = 7.1, 7.2 Hz), 8.07 (1H, d, J = 8.0 Hz); ^{13}C-NMR (DMSO-d_6, 70 °C, 75 MHz) δ =13.0, 21.1, 22.6, 37.7, 41.8, 44.1, 45.3, 59.7, 62.7, 64.4, 64.5, 116.6, 117.4, 124.2, 128.7, 128.9, 131.9, 132.0, 133.1, 147.3, 154.5, 155.7, 169.7, 174.6; HRMS (ESI-TOF) m/z calcd for $C_{25}H_{34}N_4NaO_9$ ($[M + Na]^+$): 557.2223, found: 557.2244.

(S)-2-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2-ethyl(2-nitrobenzyloxycarbonyl)aminoethyl]amino}-3,3-dimethyl-4-oxobutanoic acid ***10a***. To a stirred mixture of **9a** (20.2 mg, 37.4 µmol) in

THF were added $Pd(PPh_3)_4$ (6.49 mg, 5.61 μmol) and *N*-methylaniline (40.8 μL, 0.374 mmol). The reaction mixture was stirred at room temperature for 6 h. To the reaction mixture were added DIPEA (15.3 μL, 89.8 μmol) and FmocOSu (15.1 mg, 44.9 μmol) at 0 °C. The reaction mixture was stirred at room temperature for 10 h and diluted with EtOAc and 5% $KHSO_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography ($CHCl_3$/MeOH = 150/1 then 30/1) to afford carboxylic acid **10a** (21.7 mg, 34.3 μmol, 92%) as pale yellow oil. $[\alpha]^{28}_{D}$ -1.1 (*c* 1.30, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 930, 1249, 1367, 1530, 1672, 1726, 2875, 2934, 2975, 3079, 3350; ^{1}H-NMR ($CDCl_3$, 50 °C, 300 MHz) δ = 1.03–1.20 (6H, m), 1.25 (3H, s), 3.30 (2H, q, *J* = 7.1 Hz), 3.34–3.57 (4H, m), 4.18 (1H, t, *J* = 6.8 Hz), 4.37 (2H, d, *J* = 6.8 Hz), 4.42–4.58 (1H, m), 5.47 (2H, s), 6.03–6.13 (1H, m), 6.79–6.94 (1H, m), 7.24–7.61 (9H, m), 7.71 (2H, d, *J* = 7.5 Hz), 7.98 (1H, d, *J* = 7.9 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ =13.9, 23.2, 23.6, 40.7, 43.0, 45.5, 45.9, 47.3, 60.0, 64.7, 67.5, 120.1, 125.2, 125.3, 127.2, 127.9, 128.9, 129.1, 129.3, 132.3, 133.8, 141.4, 143.8, 143.9, 147.9, 149.0, 156.9, 157.6, 172.3; HRMS (ESI-TOF) m/z calcd for $C_{33}H_{37}N_4O_9$ ($[M + H]^+$): 633.2561, found: 633.2549.

Allyl (S)-2-{[(allyloxy)carbonyl]amino}-4-{[2-(tert-butoxy carbonyl)(ethyl)aminoethyl]amino}-3,3-dimethyl-4-oxobutanoate ***9b***. Amide **9b** was prepared from carboxylic acid **8** (72.0 mg, 0.252 mmol) and crude **4b** (96 mg) in a manner similar to that described for preparation of **9a**. **9b** (109 mg, 0.239 mmol, 95%) was obtained as colorless oil. $[\alpha]^{28}_{D}$ -6.7 (*c* 2.18, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1341, 1431, 1525, 1709, 2880, 2934, 2975, 3317; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ = 1.10 (3H, t, *J* = 7.0 Hz), 1.25 (3H, s), 1.35 (3H, s), 1.46 (3H, s), 3.21 (2H, q, *J* = 7.0 Hz), 3.28–3.50 (4H, br m), 4.32 (2H, d, *J* = 9.2 Hz), 4.57 (2H, ddd, *J* = 1.6, 1.6, 5.6 Hz), 4.60 (2H, ddd, *J* = 1.6, 1.6, 5.6 Hz), 5.16–5.25 (2H, m), 5.26–5.35 (2H, m), 5.82–5.97 (2H, m), 6.38 (2H, br d, *J* = 9.2 Hz), 7.05–7.20 (1H, br m); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ =13.9, 23.5, 24.6, 28.6, 41.1, 43.0, 44.5, 45.5, 61.3, 65.9, 80.3, 117.6, 118.6, 131.8, 132.9, 156.5, 157.6, 170.6, 176.3; HRMS (ESI-TOF) m/z calcd for $C_{22}H_{37}N_3NaO_7$ ($[M + Na]^+$): 478.2529, found: 478.2539.

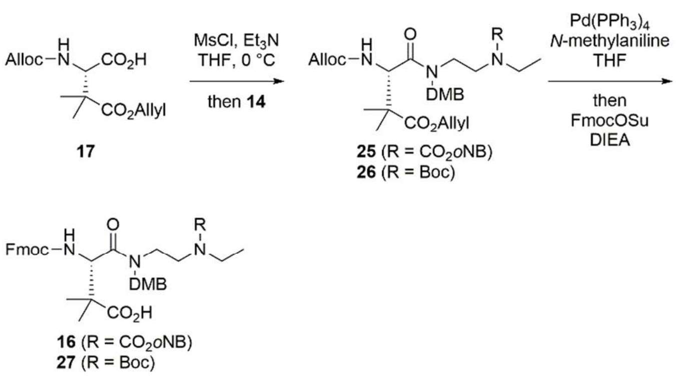
*(S)-2-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2-(tert-butoxycarbonyl)(ethyl)aminoethyl] amino}-3,3-dimethyl-4-oxobutanoic acid **10b***. Carboxylic acid **10b** was prepared from amide **9b** (88.8 mg, 0.195 mmol) in a manner similar to that described for **10a**. **10b** (72.2 mg, 0.136 mmol, 70%) was obtained as pale yellow oil. $[\alpha]^{28}_D$ -1.0 (*c* 2.16, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1366, 1450, 1479, 1531, 1709, 2875, 2934, 2975, 3329; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ = 1.11 (3H, t, J =7.2 Hz), 1.24 (3H, s), 1.36 (3H, s), 1.46 (9H, s), 3.22 (2H, q, J = 7.2 Hz), 3.3–3.47 (4H, m), 4.22 (1H, t, J = 7.2 Hz), 4.30 (2H, d, J = 7.2 Hz), 4.56 (1H, br d, J = 8.0 Hz), 6.11–6.26 (1H, br m), 7.31 (2H, dd, J = 7.6, 7.6 Hz), 7.39 (2H, dd, J = 9.2, 7.6 Hz), 7.57–7.65 (2H, m), 7.75 (2H, d, J = 9.2 Hz), 7.77–7.85 (1H, br m); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ =13.8, 23.4, 23.7, 28.5, 41.5, 43.2, 45.3, 45.5, 47.3, 60.1, 67.4, 80.8, 120.1, 125.3, 127.2, 127.8, 141.4, 143.8, 144.0, 156.8, 157.8, 172.1, 178.9; HRMS (ESI-TOF) m/z calcd for $C_{30}H_{39}N_3NaO_7$ ($[M + Na]^+$): 576.2686, found: 576.2672.

*2-Nitrobenzyl {2-[(2,4-dimethoxybenzyl)amino]ethyl}(ethyl)carbamate **14a***. Crude amine **4a** (1.24 g) in MeOH (8.9 mL) was treated with 2,4-dimethoxybenzaldehyde (1.23 g, 7.41 mmol), AcOH (278 µL, 4.86 mmol) and Na_2SO_4 (3.29 g, 46.3 mmol). The reaction mixture was stirred at room temperature for 2 h. To the reaction mixture was added $NaBH_4$ (700 mg, 18.5 mmol) at 0 °C. The reaction mixture was additionally stirred at room temperature for 1 h and then diluted with sat. $NaHCO_3$ aq. The mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 2/1 then EtOAc/MeOH 3/1) to afford DMB-*o*NBnoc amine **14a** (1.84 g, 4.41 mmol, 95% over two steps) as light brown oil. IR ($CHCl_3$): ν_{max}, cm^{-1}: 1343, 1423, 1465, 1529, 1613, 1701, 2836, 2935, 3340; ^{1}H-NMR (DMSO-d_6, 80 °C, 300 MHz) δ =1.09 (3H, t, J = 7.0 Hz), 1.38 (9H, s), 3.11(2H, dt, J = 6.6, 6.6 Hz), 3.21-3.37 (4H, m), 5.39 (2H, s), 6.33-6.48 (1H, br m), 7.60 (1H, dd, *J* = 8.1, 7.6 Hz), 7.68 (1H, d, *J* = 7.3 Hz), 7.76 (1H, dd, *J* = 7.3, 7.5 Hz), 8.05 (1H, d, *J* = 8.1 Hz); ^{13}C-NMR (DMSO-d$_6$, 60 °C, 75 MHz) δ =13.1, 28.0, 38.5, 41.9, 46.1, 62.7, 77.5, 124.3, 128.7, 128.8, 132.1, 133.6, 147.2, 154.5, 155.3; HRMS (ESI-TOF) m/z calcd for $C_{21}H_{28}N_3O_6$ ([M + Na]$^+$): 418.1978, found: 418.1988.

tert-Butyl {2-[(2,4-dimethoxybenzyl)amino]ethyl}(ethyl)carbamate ***14b***. DMB-Boc amine **14b** was prepared from amine crude **4b** (500 mg) and 2,4-dimethoxybenzaldehyde (221 mg, 1.33 mmol) in a manner similar to that described for **14a**. **14b** (396 mg, 1.17 mmol, 88%) was obtained as yellow oil. IR (CHCl$_3$): ν_{max}, cm^{-1}: 1156, 1366, 1463, 1507, 1613, 1690, 2837, 2933, 2973, 3342; ^{1}H-NMR (DMSO-d$_6$, 60 °C, 300 MHz) δ = 1.02 (3H, t, *J* = 7.0 Hz), 1.38 (9H, s), 2.69 (2H, t, *J* = 6.8 Hz), 3.17 (2H, q, *J* = 7.0 Hz), 3.25 (2H, t, *J* = 6.8 Hz), 3.70 (2H, s), 3.76 (3H, s), 3.78 (3H, s), 6.59 (1H, br s), 6.48 (1H, dd, *J* = 8.3, 2.2 Hz), 6.55 (1H, d, *J* = 2.2 Hz), 7.18 (1H, d, *J* = 8.3 Hz); ^{13}C-NMR (CDCl$_3$, 75 MHz) δ = 13.6, 28.5, 42.5, 46.6, 47.3, 48.7, 55.4, 55.5, 70.6, 77.2, 79.4, 98.6, 103.8, 120.2, 130.6, 158.7, 160.3; HRMS (ESI-TOF) m/z calcd for $C_{18}H_{31}N_2O_4$ ([M + H]$^+$): 339.2284, found: 339.2281.

Allyl (S)-2-[(allyloxycarbonyl)amino]-4-[(2,4-dimethoxybenzyl)(2-ethyl-2-nitrobenzyloxycarbonyl aminoethyl)amino]-3,3-dimethyl-4-oxobutanoate ***15***. To a solution of Carboxylic acid **8** (118 mg, 0.413 mmol) in CH_2Cl_2 (1.5 mL) was added Et$_3$N (173 µL, 1.24 mmol) followed by the addition of MsCl (38.4 µL, 0.496 mmol) in CH_2Cl_2 (100 µL) at 0 °C. The reaction mixture was stirred at same temperature for 2 h. After addition of **14a** (199 mg, 0.476 mmol) in CH_2Cl_2 (1.5 mL) to the reaction mixture at 0 °C, The resulting mixture was stirred at room temperature for additional 17 h and then diluted with EtOAc and 5% KHSO$_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with sat. NaHCO$_3$ aq. and brine, dried over Na$_2$SO$_4$, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 13/7 then 3/2) to afford amide **15** (140 mg, 0.204 mmol, 50%) as colorless oil. $[\alpha]^{29}_D$ -0.4 (*c* 0.80, CHCl$_3$); IR (CHCl$_3$): ν_{max}, cm^{-1}:1208, 1423, 1477, 1528, 1614, 1707, 2838, 2939, 2972, 3084, 3314, 3443; [1]H-NMR (DMSO-d$_6$, 100 °C, 300 MHz) δ = 1.04 (3H, t, J = 7.0 Hz), 1.27 (3H, s), 1.33 (3H, s), 3.21(3H, q, J = 7.0 Hz), 3.26–3.44 (4H, m), 3.76 (3H, s), 3.78 (3H, s), 4.50–4.64 (7H, m), 5.12–5.24 (2H, m), 5.26–5.38 (4H, m), 5.77–6.11 (2H, m), 6.49 (1H, dd, J = 8.4, 2.0 Hz), 6.56 (1H, d, J = 2.0 Hz), 6.85 (1H, br d, J = 9.3 Hz), 6.99 (1H, d, J = 8.4 Hz), 7.54–7.63 (2H, m), 7.71 (1H, t, J = 7.5 Hz), 8.03 (1H, d, J = 8.1 Hz); [13]C-NMR (CDCl$_3$, 75 MHz, rotamer)[20] δ = 13.2, 13.9, 24.2, 25.3, 25.5, 29.6, 42.4, 43.1, 43.6, 43.7, 44.1, 44.3, 45.5, 46.4, 47.6, 55.1, 55.3, 63.2, 63.5, 63.7, 65.7, 98.4, 104.0, 116.6, 117.0, 117.4, 117.5, 117.9, 118.1, 124.8, 128.2, 128.3, 128.4, 128.5, 128.7, 131.8, 131.9, 132.7, 133.1, 133.2, 133.5, 133.6, 155.0, 155.4, 156.8, 157.9, 160.2, 160.3, 170.7, 176.2, 176.3, 176.4; HRMS (ESI-TOF) m/z calcd for $C_{34}H_{44}N_4NaO_{11}$ ([M + Na]$^+$): 707.2904, found: 707.2924.

Synthesis of α-amide protected derivatives 16 and 27



Allyl (S)-3-(allyloxycarbonyl)amino-4-{[2,4-dimethoxybenzyl][2-(ethyl-2-nitrobenzyloxycarbonyl amino)ethyl]amino}-2,2-dimethyl-4-oxobutanoate ***25***. Carboxylic acid **17** (90.6 mg, 0.318 mmol) in THF (3.1 mL) was treated with Et$_3$N (133 μL, 0.953 mmol). Following to addition of MsCl (29.5 μL, 0.381 mmol) at 0 °C, the reaction mixture was stirredat same temperature for 30 min. After addition of **14a** (199 mg, 0.476 mmol) to the reaction mixture at 0 °C. The resulting solution was stirred at room temperature for additional 20 h and then diluted with EtOAc and 5% KHSO$_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with sat. NaHCO$_3$ aq. and brine, dried over Na$_2$SO$_4$, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 2/1 then 1/1) to afford amide **25** (130 mg, 0.190 mmol, 60%) as colorless oil. $[\alpha]^{28}_{D}$ -16.8 (*c* 1.03, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1209, 1343, 1426, 1509, 1529, 1645, 1709, 2939, 2976, 3196; ^{1}H-NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 1.04 (3H, br t, J = 6.1 Hz), 1.15 (3H, s), 1.24 (3H, s), 3.13–3.62 (6H, br m), 3.76 (6H, s), 4.33–4.64 (6H, m), 4.74–4.97 (1H, br m), 5.08–5.23 (2H, m), 5.23–5.34 (2H, m), 5.37 (2H, s), 5.78–6.02 (2H, m), 6.45 (1H, d, J = 8.2 Hz), 6.55 (1H, s), 6.85 (1H, br d, J = 9.0 Hz), 7.03 (1H, d, J = 8.2 Hz), 7.52–7.67 (2H, m), 7.73 (1H, dd, J = 7.4, 7.4 Hz), 8.04 (1H, dd, J = 8.1 Hz); ^{13}C-NMR (DMSO-d_6, 100 °C, 75 MHz) δ = 12.7, 21.0, 22.5, 41.6, 43.3, 43.4, 44.9, 45.5, 54.8, 55.1, 55.8, 62.4, 64.1, 64.4, 98.4, 104.7, 116.5, 116.8, 123.9, 128.4, 128.7, 131.6, 132.2, 132.8, 133.1, 147.3, 154.2, 155.1, 157.9, 159.8, 169.3, 174.6; HRMS (ESI-TOF) m/z calcd for $C_{34}H_{44}N_4NaO_{11}$ ($[M + Na]^+$): 707.2904 found: 707.2933.

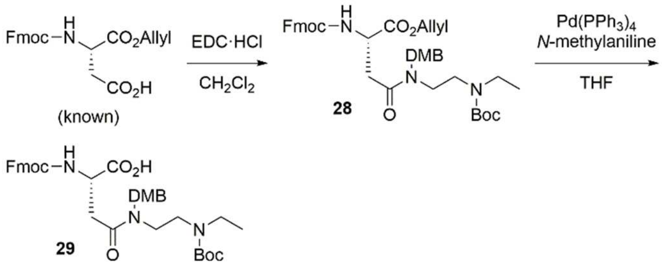
(S)-3-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2,4-dimethoxybenzyl][2-ethyl(2-nitrobenzyl oxycarbonyl)aminoethyl]amino}-2,2-dimethyl-4-oxobutanoic acid ***16***. Carboxylic acid **16** was prepared from amide **25** (55.5 mg, 81.1 μmol) in a manner similar to that described for **10a**. **16** (63.0 mg, 80.5 μmol, quant.) was obtained as pale yellow amorphous. $[\alpha]^{28}_{D}$ -8.2 (*c* 1.49, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}:1342, 1452, 1508, 1526, 1613, 1645, 1708, 2853, 2931, 2961, 3068, 3421; ^{1}H-NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 0.97 (3H, t, J = 7.0 Hz), 1.04 (3H, s), 1.14 (3H, s), 3.12–3.43 (6H, br m), 3.66 (3H, s), 3.68 (3H, s), 4.01–4.57 (5H, br m), 4.75 (1H, br d, J = 1.8 Hz), 5.31 (2H, s), 6.34 (1H, dd, J = 8.3, 2.0 Hz), 6.47 (1H, d, J = 2.0 Hz), 6.84–7.07 (2H, m), 7.16–7.29 (2H, m), 7.34 (2H, dd, J = 7.5, 7.1 Hz), 7.46–7.72 (5H, m), 7.79 (2H, d, J = 7.5 Hz), 7.97 (1H, 7.9 Hz); ^{13}C-NMR (DMSO-d_6, 75 MHz, rotamer)[20] δ = 13.1, 13.7, 20.8, 21.0, 24.3, 42.2, 42.3, 43.1, 44.9, 45.8, 45.9, 46.7, 55.0, 55.4, 55.6, 55.9, 63.2, 65.9, 98.1, 98.3, 104.3, 104.5, 116.4, 117.0, 120.2, 124.7, 125.4, 127.0, 127.7, 128.7, 129.0, 132.3, 132.6, 134.1, 134.2, 140.7, 143.5, 143.7, 143.8, 154.3, 154.7, 156.0, 156.3, 158.0, 158.2, 159.7, 160.2, 169.9, 177.5, 177.7; HRMS (ESI-TOF) m/z calcd for $C_{42}H_{46}N_4NaO_{11}$ ($[M + Na]^+$): 805.3061, found: 805.3063.

Allyl (S)-3-[(allyloxycarbonyl)amino]-4-{[2-ethyl(tert-butoxycarbonyl)aminoethyl][2,4-dimethoxybenzyl]amino}-2,2-dimethyl-4-oxobutanoate ***26***. Amide **26** was prepared from **17** (63.5 mg, 0.223 mmol) and **14b** (113 mg) in a manner similar to **25**. **26** (74.6 mg, 0.122 mmol, 55%) was obtained as colorless oil. $[\alpha]^{28}_{D}$ -1.8 (*c* 1.07, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1366, 1507, 1646, 1693, 1723, 2832, 2875, 2934, 2975; ^{1}H-NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 0.88–1.11 (3H, m,), 1.16 (3H, s), 1.13 (3H, s), 1.38 (9H), 3.03–3.60 (6H, br m), 3.77(3H, s), 3.79 (3H, s), 4.38–4.65 (6H, m), 4.84 (1H, br d, *J* = 9.0 Hz), 5.11–5.24 (2H, m), 5.24–5.41 (2H, m), 5.75–6.06 (2H, m), 6.48 (1H, br d, *J* = 7.7 Hz), 6.57 (1H, s), 6.83 (1H, br d, *J* = 9.0 Hz), 7.03 (1H, br d, *J* = 7.7 Hz); ^{13}C-NMR (DMSO-d_6, 100 °C, 75 MHz) δ = 12.8, 21.1, 22.6, 27.6, 41.3, 43.5, 44.8, 45.5, 54.8, 55.0, 55.8, 64.1, 64.4, 78.1, 98.3, 104.7, 116.4, 116.7, 128.7, 132.2, 132.8, 153.9, 155.0, 157.8, 159.8, 169.2, 174.6; HRMS (ESI-TOF) m/z calcd for $C_{31}H_{47}N_3NaO_9$ ([M + Na]$^+$): 628.3210, found: 628.3234.

(S)-3-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2-ethyl(tert-butoxycarbonyl)aminoethyl][2,4-dimethoxybenzyl]amino}-2,2-dimethyl-4-oxobutanoic acid ***27***. Carboxylic acid **27** was prepared from amide **26** (55.9 mg, 92.3 μmol) in a manner similar to that described for **10a**. **27** (64.7 mg, 92.0 μmol, quant.) was obtained as pale yellow amorphous. $[\alpha]^{25}_{D}$ -1.5 (*c* 0.80, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1160, 1210, 1455, 1508, 1616, 1643, 1692, 1718, 2928, 2973, 3277; ^{1}H-NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 0.10 (3H, t, *J* = 7.1 Hz), 1.12 (3H, s), 1.22 (1H, s), 1.38 (9H, s), 3.10–3.44 (6H, br m), 3.72 (3H, s), 3.77 (3H, s), 4.07–4.25 (1H, br m), 4.25–4.41 (2H, br m), 4.41–4.61 (2H, br m) 4.81 (1H, br d, *J* = 6.2 Hz), 6.42 (1H, dd, *J* = 8.3, 2.0 Hz), 6.55 (1H, d, *J* = 2.0 Hz), 7.25–7.36 (2H, m), 7.40 (2H, dd, *J* = 7.3, 7.5 Hz), 7.67 (2H, d, *J* = 7.0 Hz), 7.84 (2H, d, *J* = 7.3 Hz); ^{13}C-NMR (DMSO-d_6, 75 MHz, rotamer)[23] δ =13.5, 20.8, 20.9, 24.1, 24.3, 28.0, 42.8, 43.6, 44.8, 45.8, 46.6, 46.7, 55.0, 55.2, 55.3, 55.7, 65.9, 66.0, 78.5, 78.7, 98.2, 98.3, 104.3, 104.4, 116.4, 116.9, 120.1, 125.3, 125.4, 127.0, 127.3, 127.7,

128.9, 140.7, 143.5, 143.6, 143.8, 156.0, 156.3, 157.8, 158.0, 159.6, 159.9, 169.9, 177.6, 177.7; HRMS (ESI-TOF) m/z calcd for $C_{39}H_{49}N_3NaO_9$ ($[M + Na]^+$): 726.3367, found: 726.3365.

Synthesis of 29 for preparation of peptide 19



Allyl N[2]*-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-N*[4]*-{2-[(tert-butoxycarbonyl)(ethyl)amino]ethyl}-N*[4] *-(2,4-dimethoxybenzyl)-L-asparaginate* ***28***. To a stirred mixture of Fmoc-$_L$-Asp(OH)-OAllyl[19] (350 mg, 0.886 mmol) and **14b** (250 mg, 0.739 mmol) in CH_2Cl_2 (10 mL) was added EDC·HCl (170 mg, 0.886 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 1 h and diluted with EtOAc and 5% $KHSO_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with brine, sat. $NaHCO_3$ aq. and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 1/1) to afford amide **28** (500 mg, 0.698 mmol, 95%) as pale yellow oil. $[\alpha]^{28}_D$ 22.8 (*c* 2.13, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1289, 1454, 1506, 1642, 1690, 1725, 2838, 2934, 2972, 3438; ^{1}H-NMR (DMSO-d_6, 100 °C, rotamer, 300 MHz) δ =1.01 (3H, t, J = 7.0 Hz), 1.39 (9H, s), 2.89–2.95 (2H, m), 3.09–3.23 (4H, m), 3.33 (2H, br t, J = 6.3 Hz), 3.75 (3H, s), 3.79 (3H, s), 4.20–4.28 (1H, m), 4.30–4.36 (2H, m), 4.38–4.51 (2H, m), 4.56–4.63 (3H, m), 5.18 (1H, dd, J = 10.4, 1.5 Hz), 5.31 (1H, dd, J = 17.2, 1.5 Hz), 5.89 (1H, ddt, J = 17.2, 10.4, 5.3 Hz), 6.47 (1H, br d, J = 8.2 Hz), 6.58 (1H, s), 7.02 (1H, d, J =, 8.2 Hz), 7.05–7.21 (1H, m), 7.31 (2H, dd, J = 7.5, 7.1 Hz), 7.41 (2H, dd, J = 7.5, 7.1 Hz), 7.67 (2H, d, J = 7.3 Hz), 7.85 (2H, d, J = 7.5 Hz); ^{13}C-NMR (DMSO-d_6, 75 MHz, rotamer)[20] δ =13.3, 13.8, 28.0, 34.0, 34.7, 41.5, 42.1, 42.7, 43.6, 44.7, 44.9, 46.3, 46.6, 50.5, 50.9, 55.1, 55.2, 55.3, 55.4, 64.9, 65.0, 65.8, 78.4, 78.9, 98.2, 98.5, 104.3, 104.5, 116.3, 117.4, 117.6, 120.1, 125.2, 127.1, 127.6, 128.2, 128.4, 128.9, 132.3, 132.4, 140.7, 143.7, 155.7, 155.8, 157.9, 159.7, 160.2, 169.1, 169.6, 171.1, 171.3; HRMS (ESI-TOF) m/z calcd for $C_{40}H_{49}N_3NaO_9$ ($[M + Na]^+$):738.3367, found: 738.3389.

*N^2-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-N^4-{2-[(tert-butoxycarbonyl)(ethyl)amino]ethyl}-N^4-(2,4-dimethoxybenzyl)-L-asparagine **29***. To a solution of amide **28** (450 mg, 0.629 mmol) in THF (6.0 mL) was added $Pd(PPh_3)_4$ (72.7 mg, 62.9 μmol) and *N*-methylaniline (685 μL, 6.29 mmol). The reaction mixture was stirred at room temperature for 1 h and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 1/1 then EtOAc/MeOH = 10/1) to afford carboxylic acid **29** (394 mg, 0.583 mmol, 93%) as pale yellow amorphousness. $[\alpha]^{28}_D$ 39.5 (*c* 1.26, $CHCl_3$); IR ($CHCl_3$): ν_{max}, cm^{-1}: 1289, 1506, 1610, 1643, 1690, 1718, 2843, 2972, 3314, 3427; ^{1}H-NMR (DMSO-d_6, 120 °C, rotamer, 300 MHz) δ =1.02 (3H, t, J = 7.0 Hz), 1.40 (9H, s), 2.86–2.96 (2H, m), 3.15 (2H, q, J = 7.0 Hz), 3.19–3.27 (2H, br m), 3.30–3.42 (2H, br m), 3.75 (3H, s), 3.80 (3H, s), 4.20–4.29 (1H, m), 4.29–4.35 (2H, m), 4.42–4.58 (3H, m), 6.47 (1H, dd, J = 8.4, 2.2 Hz), 6.58 (1H, d, J = 2.2 Hz), 6.86 (1H, br d, J = 7.5 Hz), 7.04 (1H, d, J = 8.4 Hz), 7.31 (2H, dd, J = 7.5, 7.0 Hz), 7.41 (2H, dd, J = 7.5, 7.0 Hz), 7.68 (2H, d, J = 7.5 Hz), 7.84 (2H, d, J = 7.5 Hz); ^{13}C-NMR (DMSO-d_6, 75 MHz, rotamer)[20] δ =13.2, 13.8, 28.0, 33.9, 34.6, 41.5, 42.2, 42.8, 43.6, 44.0, 44.4, 44.9, 45.3, 46.4, 46.6, 50.5, 50.8, 55.1, 55.2, 55.3, 65.8, 66.3, 78.4, 78.7, 78.9, 98.1, 98.5, 104.3, 104.5, 116.4, 117.1, 120.1, 125.2, 127.1, 127.6, 128.2, 128.4, 128.7, 140.7, 143.8, 154.1, 154.6, 155.7, 155.8, 157.9, 159.7, 160.1, 169.4, 169.8, 172.9, 173.2; HRMS (ESI-TOF) m/z calcd for $C_{37}H_{45}N_3NaO_9$ ($[M + Na]^+$): 698.3054, found: 698.3051.

General procedures for peptide synthesis

Fmoc-based solid-phase peptide synthesis (Fmoc SPPS). On NovaSyn® TGR resin (0.22 mmol amine/g) were coupled Fmoc protected naturally occurring amino acid derivatives (5.0 eq., a protective group of a side chain: *t*-Bu for serine and tyrosine) in the presence of *N,N'*-diisopropylcarbodiimide

(DIC, 5.0 eq.) and 1-hydroxybenzotriazole hydrate (HOBt·H_2O, 5.5 eq.) in DMF for 2 h. Coupling of asparagine derivatives **10a**, **16**, **10b** or **27** (2 eq.) was performed using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (1.95 eq.) and *N*,*N*-diisopropylethyamine (4.0 eq.) for 2 h. For Fmoc removal of the peptide resin, 20% (v/v) piperidine in DMF (10 min) was employed.

TFA cleavage. The resulting completed resin was treated with TFA/*m*-cresol/1,2-ethanedithiol/thioanisole/H_2O (80/5/5/5/5 (v/v)) for 2 h at room temperature otherwise noted. After filtration of the resin, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and was purified by semi-preparative HPLC to give peptides.

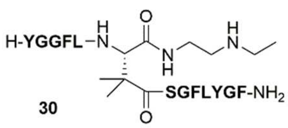
Preparation of model peptides 11a, 18, 11b, 19 and 20

Peptides were synthesized according to the section "Fmoc-based solid-phase peptide synthesis"

*Fmoc SPPS using **10a***. Peptide **11a** (major peak): a white lyophilized powder (2.08 mg, 1.19 μmol, 6.2%); retention time = 16.2 min (Analytical HPLC conditions: linear gradient of solvent B in solvent A, 38 to 50% over 30 min); retention time = 24.2 (Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 38 to 50% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{86}H_{113}N_{17}O_{21}$ ([M + 2H]$^{2+}$): 859.9, found: 859.7. Peptide **18** (minor peak): a white lyophilized powder (1.30 mg, 0.699 μmol, 3.9%); retention time = 17.6 min (Analytical HPLC conditions: linear gradient of solvent B in solvent A, 38 to 50% over 30 min); retention time = 22.9 (Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 38 to 50% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{86}H_{113}N_{17}O_{21}$ ([M + 2H]$^{2+}$): 859.9, found: 859.8.

*Fmoc SPPS using **16** isomer*. Peptide **18**: retention time = 16.3 min (Analytical HPLC conditions: linear gradient of solvent B in solvent A, 38 to 50% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{86}H_{113}N_{17}O_{21}$ ($[M + 2H]^{2+}$): 859.9, found: 859.8.

*Fmoc SPPS using **10b***. Peptide **11b** (major peak): a white lyophilized powder (0.75 mg, 0.411 μmol, 8.2%); retention time = 18.9 min (Analytical HPLC conditions: linear gradient of solvent B in A, 25 to 40% over 30 min); retention time = 21.4 (Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 27 to 42% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{78}H_{108}N_{16}O_{17}$ ($[M + 2H]^{2+}$): 770.4, found: 770.2.



30

Peptide **30** (minor peak): retention time = a white lyophilized powder (0.39 mg, 0.214 μmol, 4.3%); 20.3 min (Analytical HPLC conditions: linear gradient of solvent B in solvent A, 25 to 40% over 30 min); retention time = 23.0 (Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 27 to 42% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{78}H_{108}N_{16}O_{17}$ ($[M + 2H]^{2+}$): 770.4, found: 770.3.

*Fmoc SPPS using **27***. Peptide **30**: retention time = 20.3 min (Analytical HPLC conditions: linear gradient of solvent B in solvent A, 25 to 40% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{78}H_{108}N_{16}O_{17}$ ($[M + 2H]^{2+}$): 770.4, found: 770.2.

*Fmoc SPPS using **29***. Peptide **19**: a white lyophilized powder (12.0 mg, 6.70 μmol, 50%); retention time = 20.4 min (Analytical HPLC conditions: linear gradient of solvent B in solvent A, 30 to 40% over 30 min); retention time = 24.3 (Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 27 to 41% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{76}H_{104}N_{16}O_{17}$ ($[M + 2H]^{2+}$): 756.4, found: 756.3.

Fmoc SPPS using Fmoc-Asn(OtBu)-OH Peptide **20**: a white lyophilized powder (8.32 mg, 5.26 μmol, 53%); retention time = 23.5 min (Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5 to 60% over 30 min); retention time = 28.0 (Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 28 to 42% over 30 min); LRMS (ESI-TOF) m/z calcd for $C_{72}H_{95}N_{15}O_{17}$ ([M + H]$^+$): 1440.7, found: 1441.0.

Self-processing of peptide 11b, 19 and 20

*Self-processing of peptide **11b***. A solution of model peptide **11b** (45.0 μg, 25.9 nmol) and benzene sulfonic acid sodium salt (internal standard, 20.7 ng, 0.115 nmol) in phosphate buffer (0.2 M, pH 7.4 and 7.9, 550 μL) containing 6 M guanidine hydrochloride was incubated at 37 °C and the reaction was monitored by analytical HPLC. Analytical HPLC conditions: a linear gradient of solvent B in solvent A, 1 to 60% over 30 min.

The remaining substrate was calculated based on peak areas (= *A*) of HPLC as follow. $A^{t=0}$ indicates peak areas at beginning of the reaction ($t = 0$).

$$\text{Remaining substrate (\%)} = \frac{A_{\text{substrate}} \,/\, A_{\text{internal standard}}}{A^{t=0}_{\text{substrate}} \,/\, A^{t=0}_{\text{internal standard}}} \times 100$$

11b: retention time = 21.9 min.

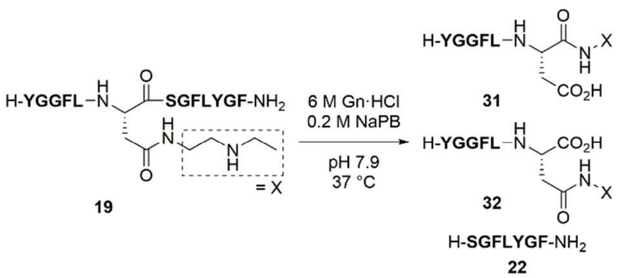
23 or **24**: retention time = 16.4 min; LRMS (ESI-TOF) m/z calcd for $C_{38}H_{57}N_8O_9$ ([M + H]$^+$): 769.4, found: 769.3.

23 or **24**: retention time = 17.4 min; LRMS (ESI-TOF) m/z calcd for $C_{38}H_{57}N_8O_9$ ([M + H]$^+$): 769.4, found: 769.3.

22: retention time = 20.1 min; LRMS (ESI-TOF) m/z calcd for $C_{40}H_{53}N_8O_9$ ([M + H]$^+$): 789.4, found: 789.2.

Benzene sulfonic acid sodium salt (internal standard): retention time = 7.5 min.

*Self-processing of peptide **19***



Procedure of self-processing of **19** was conducted as similar to that described for **11b** (pH = 7.9).

19: retention time = 21.9 min.

31 or **32**: retention time = 15.6 min; LRMS (ESI-TOF) m/z calcd for $C_{36}H_{53}N_8O_9$ ([M + H]$^+$): 741.4, found: 741.3.

31 or **32**: retention time = 16.3 min; LRMS (ESI-TOF) m/z calcd for $C_{36}H_{53}N_8O_9$ ([M + H]$^+$): 741.4, found: 741.3.

*Self-processing of peptide **20***. Procedure of self-processing of **20** was conducted as similar to that described for **11b** (pH = 7.9). Almost no splitted peptide was observed within 24 h.

20: retention time = 23.5 min.

Photo-responsible amide bond cleavage of peptide 11a

Photo-responsible peptide **11a** (45.0 μg, 24.2 nmol) and benzene sulfonic acid sodium salt (internal standard, 3.00 ng, 16.7 pmol) in phosphate buffer (0.2 M, pH 7.4, 515 μL) containing 6 M guanidine hydrochloride was incubated at 37 °C for 24 h, and the reaction mixture was then irradiated by UV (>365 nm) for 1 min. The resulting solution was incubated at 37 °C. The reaction was monitored by analytical HPLC. Analytical HPLC conditions: a linear gradient of solvent B in solvent A, 1 to 60% over 30 min.

11a: retention time = 28.2 min.

Supporting Information Available:

This material is available free of charge via the Internet at http://pubs.acs.org. ^{1}H and ^{13}C NMR spectra for new compounds.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI).

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