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FULL PAPER

Peptide Chain Elongation Using Unprotected Amino Acids in a Micro-flow Reactor

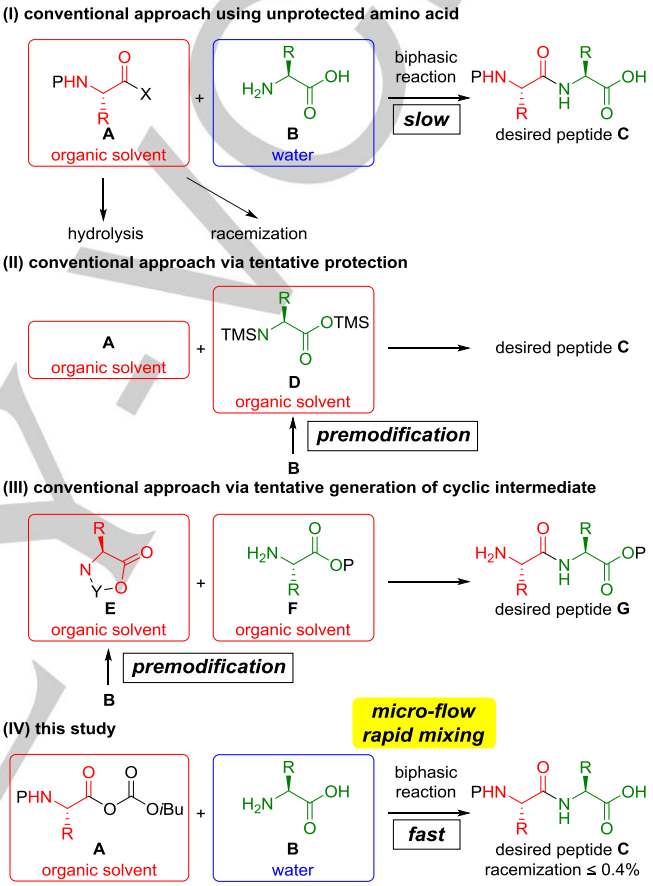
Shinichiro Fuse,*[a] Koshiro Masuda,[a, b] Yuma Otake,[a, b] and Hiroyuki Nakamura[a]

Abstract: Conventional peptide synthesis requires a deprotection step after each amidation step, which decreases synthetic efficiency. Therefore, peptide synthesis using unprotected amino acids is considered an ideal approach. Here we report peptide chain elongation using unprotected amino acids via mixed carbonic anhydride. Micro-flow technology enabled rapid mixing of an organic layer containing a protected amino acid or dipeptide and an aqueous layer containing an unprotected amino acid or dipeptide to accelerate the desired amidation, and this approach successfully suppressed undesired racemization/epimerization (≤ 0.4%). Various di-, tri-, and tetra-peptides were obtained in good to high yields. This is the first report of achieving peptide chain elongation without severe racemization from unprotected amino acids using inexpensive, non-explosive, less wasteful, and less toxic reagents.

Introduction

The realization of a less wasteful, low-cost, and scalable chemical synthesis of peptides is desperately required due to the increasing importance of peptide-based drugs. Peptide chain elongation is generally performed by repeating the following steps: 1) condensation of an *N*-protected amino acid with a peptide that has a free N-terminus, and 2) removal of the protecting group at the N-terminus. Although the average molecular weight of amino acids is *ca*. 110, the molecular weights of frequently used protecting groups (Fmoc: 223, Boc: 101, Cbz: 135) are comparable or larger. In addition, the condensation step usually requires an excess amount of low atom economy and expensive coupling agents.[1] The deprotection steps also require an excess amount of a strong acid, an organic base, or a palladium catalyst that causes concerns about the problem of residual heavy metals.

In order to reduce the number of synthetic steps, waste, and cost, peptide chain elongations using unprotected amino acids have been investigated. The most frequently investigated



Scheme 1. Conventional approaches for synthesis of peptides using unprotected amino acids and our approach developed in this study.

approach involves the coupling of unprotected amino acid **B** in water with activated acyl species **A** (X = O-succinimide,[2] pentafluorophenol,[3] O*p*-$C_6H_4NO_2$,[4] OCOR,[5] benzotriazole,[6] N_3,[7] F[8] or others[9]) in an organic solvent, as shown in Scheme 1 (I). Slow amidation in a biphasic solvent system tends to cause undesired racemization and/or hydrolysis of **A**. This is one of the most challenging issues for achieving peptide chain elongation using unprotected amino acids. In addition, the use of toxic, explosive, and/or expensive reagents for the preparation of **A** and/or necessity for the isolation of labile **A** decreases the usefulness of these approaches. Extensive attempts have been reported to improve the solubility of unprotected amino acids **B** either by using a large amounts of organic[3a, 7b, 10] and inorganic additives[11] or by irradiating ultrasound.[12] These additives, however, are expensive and/or sometimes insufficient to completely dissolve unprotected amino acids.

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Another approach is premodification of unprotected amino acids **B** to bistrimethylsilylated compounds **D** as shown in Scheme 1 (II). The amidation proceeds smoothly because **D** is soluble against an organic solvent and the trimethylsilyl (TMS) groups can be removed during the aqueous work-up process to afford **C**. Premodification, however, requires a stoichiometric amount of an expensive silylating agent.[13] Premodifications of unprotected amino acids **B** to cyclic compounds **E** using silyl[14], boron[15], phosphorous[16] phosgene equivalent reagents[17] or hexafluoroacetone[18] as shown in Scheme 1 (III) were also reported. The subsequent aqueous work-up process affords peptides with a free N-terminus. However, these approaches suffered from either an insufficient reactivity of **E**, undesired reactions or use of toxic and expensive reagents.

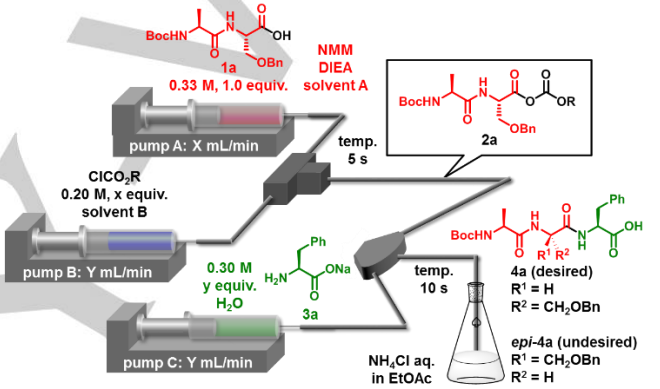
Among these approaches, a mixed carbonic anhydride-based approach (Scheme 1 (IV), X = OCOR) is attractive, because mixed carbonic anhydride can be readily generated *in situ* under mild conditions using inexpensive, non-explosive, less wasteful, and less toxic reagents.[19] Moreover, generated CO_2 and alcohol can be removed easily. Imai et al.[5b, 5c] reported an *in situ* preparation of mixed carbonic anhydride using $ClCO_2Et$, Et_3N, in THF, and aqueous $NaHCO_3$ and its use in amidation with free amino acids. A problem in a mixed carbonic anhydride-based amidation is racemization/epimerization.[5a] In particular, suppression of undesired epimerization in the synthesis of peptides (≥ 3 amino acids) is challenging because epimerization of activated peptides usually proceeds 35-110 times faster than racemization of the corresponding activated carbamate-protected amino acids.[20] In fact, Verardo et al. examined dipeptide synthesis using Imai's conditions and observed undesired racemization.[21] Therefore, they reported an improved mixed carbonic anhydride-based amidation using $ClCO_2iPr$ or $ClCO_2iBu$ and *N*-methylmorpholine (NMM) in dimethyl sulfoxide (DMSO) and water in the presence of a stoichiometric amount of nBu_4NOH. The nBu_4NOH improved the solubility of unprotected amino acids against DMSO. However, use of somewhat expensive nBu_4NOH and less easily removable DMSO remains a problem. In addition, even their improved method could not suppress undesired epimerization in the synthesis of tripeptides.

In recent years, micro- or continuous-flow[22] syntheses of peptides using protected amino acids have been reported.[23] We have also developed efficient synthetic approaches for peptides using protected amino acids.[24] In addition, a limited number of micro-flow syntheses of peptides using unprotected amino acids or peptides have been reported, although those approaches require premodification of substrates[25] or the use of a Cys residue[26]. We recently reported a highly efficient amino acid *N*-carboxyanhydride synthesis based on the rapid mixing[27] (< 0.1 s) of an MeCN/water biphasic system using micro-flow technology.[28] Here, we report mixed carbonic anhydride-based amidation with unprotected amino acids using micro-flow technology. Micro-flow technology enabled the rapid mixing of an organic layer containing a protected amino acid or dipeptide and an aqueous layer containing an unprotected amino acid or dipeptide to accelerate the desired amidation, and this approach successfully suppressed the undesired racemization/epimerization (≤ 0.4%). The developed approach afforded a variety of peptides without requiring the premodification of substrates.

Results and Discussion

We started by investigating the synthesis of tripeptide **4a**, as shown in Table 1. A challenge in the synthesis of a tripeptide is the suppression of undesired epimerization as previously described. We connected a T-shaped mixer and a V-shaped mixer[29] with Teflon tubing and immersed them in a water bath. A solution of dipeptide **1a**, *N*-methylmorpholine (NMM), and *N*,*N*-diisopropylethylamine (DIEA) in solvent A was introduced into the first mixer with a syringe pump. The solution of $ClCO_2R$ in solvent

Table 1. Micro-flow amide bond formation of dipeptide **1a** and unprotected amino acid **3a**.



entry	$ClCO_2R$	solvent	x, y (equiv.)	temp. (°C)	X, Y (mL/min)	yield[a]	
						4a	*epi*-**4a**
1	$ClCO_2iPr$	THF	1.0, 1.5	20	1.2, 2.0	72	<0.1
2	$ClCO_2iPr$	1,4-dioxane	1.0, 1.5	20	1.2, 2.0	62	0.3
3	$ClCO_2iPr$	MTBE	1.0, 1.5	20	1.2, 2.0	<0.1	<0.1
4	$ClCO_2iPr$	CPME	1.0, 1.5	20	1.2, 2.0	<0.1	<0.1
5	$ClCO_2iPr$	DMF	1.0, 1.5	20	1.2, 2.0	70	0.6
6	$ClCO_2iPr$	EtOAc	1.0, 1.5	20	1.2, 2.0	1	<0.1
7	$ClCO_2iPr$	MeCN	1.0, 1.5	20	1.2, 2.0	79	0.3
8[b]	$ClCO_2iPr$	MeCN	1.0, 1.5	0	1.2, 2.0	74	0.3
9	$ClCO_2iPr$	MeCN	1.0, 1.5	40	1.2, 2.0	58	2
10	$ClCO_2iPr$	MeCN	1.0, 1.5	20	2.4, 4.0	82	0.1
11	$ClCO_2iPr$	MeCN	1.0, 1.5	20	0.96, 1.6	62	0.3
12	$ClCO_2iBu$	MeCN	1.0, 1.5	20	1.2, 2.0	81	N.D.
13	$ClCO_2Et$	MeCN	1.0, 1.5	20	1.2, 2.0	65	0.1
14[c]	$ClCO_2iBu$	MeCN	1.0, 1.5	20	1.2, 2.0	78	0.6
15	$ClCO_2iBu$	MeCN	1.2, 1.7	20	1.2, 2.0	85-86[d] (78[e])	<0.1-0.3

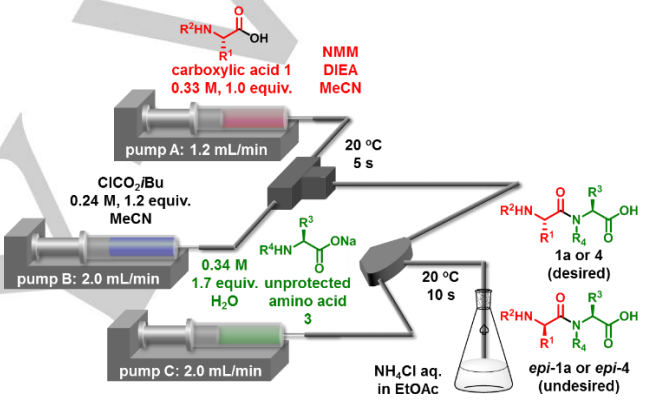
16	$ClCO_2iBu$	MeCN	1.2, 2.0	20	1.2, 2.0	85	0.7
17[f]	$ClCO_2iBu$	MeCN	1.2, 1.7	20	1.2, 2.0	66-76[d]	0.7-0.8

[a] Yield was determined by HPLC-UV analysis using a calibration curve (see supporting information Figure S-2). [b] Reaction time to generate **2a** was 10 s. [c] Amidation time was 30 s. [d] Three independent experiments were carried out. [e] Isolated yield. [f] This reaction was performed using a conventional flask.

B was also introduced into the first mixer with a syringe pump to rapidly generate mixed carbonic anhydride **2a** *in situ*. This **2a** is highly epimerization prone, therefore, it was rapidly introduced into the second mixer. A solution of unprotected amino acid, H-Phe-ONa (**3a**) in H_2O, was then introduced into the second mixer with a syringe pump. The reaction was quenched by pouring the mixture into an aqueous solution of NH_4Cl and ethyl acetate. Solvent A for dissolving dipeptide **1a** was examined at first (Table 1, entries 1-7). The use of THF, 1,4-dioxane, DMF, and MeCN afforded the desired tripeptide **4a** in yields of more than 60%, whereas the use of methyl-*t*-butylether (MTBE), cyclopentylmethylether (CPME), and EtOAc did not produce a sufficient amount of **4a**. The MeCN and THF were the best solvents for this amide bond formation. This is good for industrial preparation of peptides. The use of DMF, which has previously been the first choice in peptide synthesis, should be avoided from the viewpoint of green chemistry.[30] MeCN and THF have been regarded as good alternatives to DMF.[31] Further optimization was carried out using MeCN solvent. The temperature of activation and amidation steps was examined (Table 1, entries 8 and 9). Lower temperature conditions afforded a slightly lower yield of desired **4a** (entry 7 vs. 8). On the other hand, higher temperature conditions caused undesired epimerization (entry 7 vs. 9). Then, flow rates were examined (entries 10 and 11). Higher flow rate conditions afforded a comparable yield of **4a** (entry 7 vs. 10). On the other hand, lower flow rate conditions resulted in a lower yield due to an insufficient conversion of **1a** (entry 7 vs. 11). These results indicated the importance of mixing efficiency (higher flow rates led to higher mixing efficiency). The use of $ClCO_2iBu$ instead of $ClCO_2iPr$ afforded a slightly better yield without the generation of epimer (entry 7 vs. 12), whereas the use of $ClCO_2Et$ resulted in decreased yield (entry 7 vs. 13). This was good because $ClCO_2iBu$ is more suitable for industrial processing due to its lower cost and higher stability compared with $ClCO_2iPr$.[32] The extension of amidation time (10 s to 30 s) did not improve the yield of **4a** (entry 7 vs. 14). The use of 1.7 equiv. of unprotected amino acid **3a** improved the yield of **4a** (entry 12 vs. 15) but a further increase of **3a** did not improve the yield (entry 15 vs. 16). We compared the developed best conditions (entry 15) with the corresponding batch conditions (entry 17). The batch conditions afforded less reproducible and worse results. Again, these results clearly indicated the importance of the rapid mixing of micro-flow technology.

The substrate scope was examined based on the established conditions (Table 2). Coupling of Boc-Ala-OH (**1b**) with H-Ser(Bn)-ONa (**3b**) afforded the desired dipeptide **1a** in a high yield (entry 1). This reaction could be carried out on a 2.2 gram scale without an obvious decrease in yield (entry 2). This result demonstrated the scalability of our developed, micro-flow approach. Coupling of Boc-Ser(Bn)-OH (**1c**) with H-Phe-ONa (**3a**) or H-Ile-ONa (**3c**) afforded the desired dipeptides **4b** and **4c** in high to excellent yields (entries 3 and 4). Coupling of **1c** with sterically hindered substrates H-Pro-ONa (**3d**) and H-Sar-ONa (**3e**) and coupling of **3a** with sterically hindered Boc-Val-OH (**1d**) resulted in slightly decreased yields (entries 5-7). Coupling of **3a** with Fmoc-Ser(Bn)-OH (**1e**), Cbz-Ser(Bn)-OH (**1f**), and Boc-Tyr(Bn)-OH (**1g**) afforded the desired peptides in high yields (entries 8-10), whereas coupling of **3a** with Fmoc-His(Trt)-OH (**1h**) did not afford the desired peptide, but afforded a complex mixture (entry 11). The coupling of **3a** with racemizable Fmoc-Cys(Trt)-OH (**1i**) and highly racemizable Cbz-Phg-OH (**1j**) afforded the desired peptides in good yields without obvious racemization (<

Table 2. Synthesis of various dipeptides and tripeptides using unprotected amino acids **3**.



entry	1	3	yield	
			1a or **4**[a]	*epi*-**1a** or *epi*-**4**[b]
1	Boc-Ala-OH (**1b**)	H-Ser(Bn)-ONa (**3b**)	**1a**: 87%	<0.1
2	Boc-Ala-OH (**1b**)	H-Ser(Bn)-ONa (**3b**)	**1a**: 85% (2.2 g scale)	<0.1
3	Boc-Ser(Bn)-OH (**1c**)	H-Phe-ONa (**3a**)	**4b**: 96%	<0.1
4	Boc-Ser(Bn)-OH (**1c**)	H-Ile-ONa (**3c**)	**4c**: 82%	<0.1
5	Boc-Ser(Bn)-OH (**1c**)	H-Pro-ONa (**3d**)	**4d**: 61%	<0.1
6	Boc-Ser(Bn)-OH (**1c**)	H-Sar-ONa (**3e**)	**4e**: 67%	<0.1
7	Boc-Val-OH (**1d**)	H-Phe-ONa (**3a**)	**4f**: 68%	<0.1
8	Fmoc-Ser(Bn)-OH (**1e**)	H-Phe-ONa (**3a**)	**4g**: 80%	<0.1
9	Cbz-Ser(Bn)-OH (**1f**)	H-Phe-ONa (**3a**)	**4h**: 79%	<0.1
10	Boc-Tyr(Bn)-OH (**1g**)	H-Phe-ONa (**3a**)	**4i**: 83%	<0.1
11	Fmoc-His(Trt)-OH (**1h**)	H-Phe-ONa (**3a**)	-	-
12	Fmoc-Cys(Trt)-OH (**1i**)	H-Phe-ONa (**3a**)	**4j**: 82%	<0.1
13	Cbz-Phg-OH (**1j**)	H-Phe-ONa (**3a**)	**4k**: 88%	<0.1

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14	Boc-Ala-Cys(Trt)-OH (**1k**)	H-Phe-ONa (**3a**)	**4l**: 60%[c]	0.4%[c]
15[d]	Boc-Ala-Phg-OH (**1l**)	H-Phe-ONa (**3a**)	**4m**: 68%	<0.1

[a] Isolated yield. [b] The yield was determined by the intensity of UV absorption relative to that of the desired product in HPLC analysis. [c] The mixture of **4l** and *epi*-**4l** was separated by GPC. Yields of **4l** and *epi*-**4l** were determined by the relative intensity of the UV absorption during HPLC analysis. [d] THF was used as an organic solvent instead of MeCN due to insufficient solubility of **1l** against MeCN.

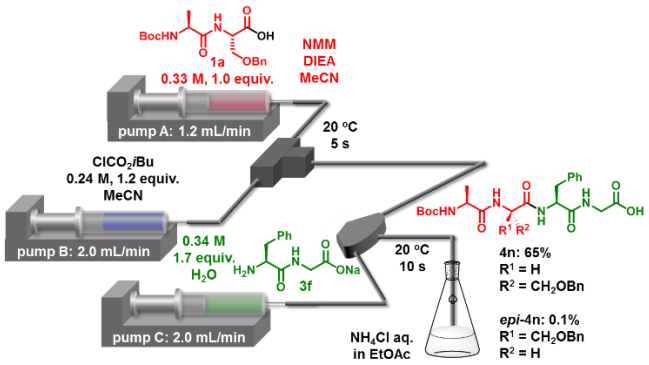

Figure 1. Synthesis of tetrapeptide **4n**

0.1%) (entries 12 and 13). This is notorious because Phg is 60 times more racemization prone compared with alanine.[33] To our delight, a more challenging coupling of **3a** with significantly racemizable dipeptide Boc-Ala-Cys(Trt)-OH (**1k**) and Boc-Ala-Phg-OH (**1l**) afforded the desired peptides without severe racemization (≤ 0.4%), although a slight decrease of yields was observed (entries 14 and 15). This result is significant because Verardo's approach could not suppress undesired racemization (9%) in the synthesis of a tripeptide from a dipeptide containing Phg at its C-terminus. Our conditions require neither expensive additives, a high boiling-point solvent, nor low-temperature conditions and allows a ready scale-up either by continuous running or by increasing the number of reactors.

Finally, we attempted a coupling between dipeptide Boc-Ala-Ser(Bn)-OH (**1a**) and unprotected dipeptide H-Phe-Gly-ONa (**3f**), as shown in Figure 1. The desired tetrapeptide **4n** was obtained in a 65% yield. This result shows the potential of our developed approach for the chemical ligation of peptides.

Conclusions

In summary, we demonstrated peptide chain elongation using unprotected amino acids. Micro-flow technology enabled the rapid mixing of an organic layer containing a protected amino acid or a protected dipeptide and an aqueous layer containing an unprotected amino acid or an unprotected dipeptide to accelerate the desired amidation. It successfully suppressed the undesired racemization/epimerization of activated acyl species. The 11 dipeptides were synthesized in high to excellent yields. The 3 tripeptides were synthesized in good to high yields without severe racemization (≤ 0.4%). In addition, tetrapeptide synthesis using an unprotected dipeptide was demonstrated. Our developed approach is potentially useful for peptide chemical ligation. This is the first report of peptide synthesis from unprotected amino acids or an unprotected peptide using inexpensive, non-explosive, less wasteful, and less toxic reagents and additives. This research should pave the way to the realization of truly efficient peptide synthesis.

Experimental Section

General procedure: Micro-flow amide bond formation using unprotected amino acid or peptide

A solution of carboxylic acid (0.333 M, 1.00 equiv.), NMM (0.333 M, 1.00 equiv.), and DIEA (0.333 M, 1.00 equiv.) in MeCN (flow rate: 1.20 mL/min) and a solution of isobutyl chloroformate (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to a T-shape mixer at 20 °C with syringe pumps. The resultant mixture was passed through a reaction tube (inner diameter: 0.800 mm, length: 531 mm, volume 267 µL, reaction time: 5.00 s) at the same temperature. Then, the resultant mixture and a solution of amino acid sodium salt (0.340 M, 1.70 equiv.) in H_2O (flow rate: 2.00 mL/min) were introduced to the V-shape mixer at 20 °C. The resultant mixer was passed through a reaction tube (inner diameter: 0.800 mm, length 1724 mm, volume: 867 µL, reaction time 10.0 s) at the same temperature. After being eluted for 50 s to reach a steady state, the resultant mixture was poured into a solution of saturated aqueous NH_4Cl, H_2O and EtOAc for 60 s. The aqueous layer was acidified to pH of 1 with aqueous 1 M HCl and extracted 3 times with EtOAc. The combined organic layer was washed with aqueous 1 M HCl and brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by recrystallization, silica-gel column chromatography, or GPC to afford the desired product.

Boc-L-Ala-L-Ser(O-Bn)-L-Phe-OH (4a)

Purification method: The crude product was recrystallized from EtOAc/hexane. 161 mg, 0.313 mmol, 78%, HPLC conditions: DAICEL CHIRALPAK ID 4.6 mm×25 cm, 20% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 259 nm, retention time 51.38 min (epimer 40.61 min). White solid; ^{1}H NMR ($CDCl_3$+CD_3OD, 400 MHz) δ 7.97 (s, 1H), 7.36-7.13 (m, 9H), 4.71-4.63 (m, 1H), 4.59-4.47 (m, 3H), 4.09 (d, *J* = 6.7 Hz, 1H), 3.77 (dd, *J* = 4.8, 9.6 Hz, 1H), 3.68 (dd, *J* = 5.1, 9.8 Hz, 1H), 3.18 (dd, *J* = 5.4, 13.9 Hz, 1H), 3.03 (dd, *J* = 7.4, 13.9 Hz, 1H), 1.42 (s, 9H), 1.27 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$+CD_3OD, 125 MHz) δ 173.6, 172.5, 169.6, 155.8, 137.1, 135.9, 129.0, 128.1, 128.1, 127.6, 127.5, 126.6, 79.8, 73.1, 69.1, 53.3, 52.6, 49.9, 37.0, 27.8, 17.6; IR (ATR): 3313, 2987, 1734, 1693, 1638, 1515, 1446, 1276, 1260, 1106, 1048, 749, 696 cm^{-1}; $[\alpha]^{30}_D$ = +27.1 (c 1.02, 25% MeOH in CH_2Cl_2); mp 160-162 °C, HRMS (ESI-TOF): calcd for $[C_{27}H_{35}N_3O_7\text{-}H]^-$ 512.2402, found 512.2403.

Boc-L-Ala-L-Ser(Bzl)-OH (1a)

<0.4 mmol scale> Purification method: The crude product was purified by GPC. 126 mg, 0.344 mmol, 87% (epimer: <0.1%). <7 mmol scale> Purification method: The crude product was recrystallized from EtOAc 2.17 g, 5.92 mmol, 85% (the reaction mixture was collected for 1050 s). HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 3% IPA (containing

0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 38.22 min (epimer 22.78 min). White solid; ^{1}H NMR ($CDCl_3$+CD_3OD, 400 MHz) δ 7.37-7.23 (m, 5H), 4.64 (t, *J* = 3.3 Hz, 1H), 4.54 (d, *J* = 12.0, 15.1 Hz, 2H), 4.19 (d, *J* = 6.3 Hz, 1H), 3.92 (dd, *J* = 4.0, 9.7 Hz, 1H), 3.75 (dd, *J* = 3.4, 9.7 Hz, 1H), 1.44 (s, 9H), 1.34 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$+CD_3OD, 125 MHz) δ 173.4, 171.5, 155.7, 137.2, 128.0, 127.4, 127.4, 79.6, 73.0, 69.4, 52.5, 45.7, 27.8, 17.7; IR (ATR): 3403, 1750, 1707, 1608, 1508, 1455, 1368, 1341, 1282, 1157, 1065, 848 cm^{-1}; HRMS (ESI-TOF): calcd for $[C_{18}H_{26}N_2O_6\text{-H}]^-$ 365.1718, found 365.1716.

Boc-L-Ser(O-Bn)-L-Phe-OH (4b)

Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK ID 4.6 mm×25 cm, 8% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 13.14 min (epimer 15.98 min). 170 mg, 0.384 mmol, 96% (epimer: <0.1%). White solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.53 (s, 1H), 7.35-7.22 (m, 5H), 7.19-7.07 (m, 6H), 5.48 (s, 1H), 4.83 (s, 1H), 4.46 (t, *J* = 15.4 Hz, 2H), 4.32 (s, 1H), 3.81 (s, 1H), 3.53 (dd, *J* = 6.2, 8.9 Hz, 1H), 3.17 (dd, *J* = 5.3, 14.0 Hz, 1H), 3.03 (dd, *J* = 6.1, 13.9 Hz, 1H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.1, 170.5, 155.7, 137.4, 135.8, 129.5, 129.3, 128.5, 128.0, 127.9, 127.0, 80.5, 73.5, 69.7, 54.0, 53.5, 37.4, 28.3; IR (ATR): 3402, 3250, 2974, 2360, 1706, 1660, 1496, 1452, 1259, 1107, 749 cm^{-1}; $[\alpha]^{30}_D$ = +33.1 (c 1.00, CH_2Cl_2); mp 109-110 °C; HRMS (ESI-TOF): calcd for $[C_{24}H_{30}N_2O_6\text{-H}]^-$ 441.2031, found 441.2028.

Boc-L-Ser(O-Bn)-L-Ile-OH (4c)

The reaction mixture was collected for 120 s. The crude product was used for HPLC analysis. Purification method: The residue was dissolved in EtOAc then mixed with cyclohexylamine (138 µL, 1.21 mmol) and concentrated *in vacuo*. The residue was recrystallized from 1,4-dioxane. The obtained solid was dissolved in CH_2Cl_2 and acidified by aqueous 1 M HCl to pH of 1. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. HPLC conditions: DAICEL CHIRALPAK ID 4.6 mm×25 cm, 20% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 9.52 min (epimer 12.09 min). 269 mg, 0.659 mmol, 82% (epimer: <0.1%). Colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.67 (s, 1H), 7.30-7.10 (m, 6H), 5.52 (s, 1H), 4.51 (dd, *J* = 4.3, 7.8 Hz, 1H), 4.45 (s, 2H), 4.29 (s, 1H), 3.80 (dd, *J* = 3.8, 9.2 Hz, 1H), 3.53 (t, *J* = 6.6 Hz, 1H), 1.92-1.78 (m, 1H), 1.35 (s, 10H), 1.10-0.95 (m, 1H), 0.79 (dd, *J* = 7.1, 16.5 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 175.1, 170.8, 155.8, 137.4, 128.5, 128.0, 127.9, 80.5, 73.6, 69.9, 56.8, 53.9, 37.7, 28.3, 24.9, 15.5, 11.6; IR (neat): 3419, 2967, 1715, 1540, 1456, 1368, 2350, 1166, 1106, 1025, 739, 699 cm^{-1}; $[\alpha]^{29}_D$ = +25.0 (c 1.01, $CHCl_3$); HRMS (ESI-TOF): calcd for $[C_{21}H_{32}N_2O_6\text{-H}]^-$ 407.2188, found 407.2185. Spectral data of ^{1}H NMR was identical to that of previously reported.[34]

Boc-L-Ser(O-Bn)-L-Pro-OH (4d)

The reaction mixture was collected for 120 s. Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK ID 4.6 mm×25 cm, 20% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 15.88 min (epimer 20.26 min). 191 mg, 0.487 mmol, 61% (epimer: <0.1%). White amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz, major rotamer) δ 8.39 (brs, 1H), 7.34-7.22 (m, 5H), 5.57 (d, *J* = 8.6 Hz, 1H), 4.74 (q, *J* = 6.9 Hz, 1H), 4.60-4.41 (m, 3H), 3.76-3.59 (m, 4H), 2.19-2.10 (m, 2H), 2.04-1.90 (m, 2H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.2, 170.6, 155.5, 137.8, 128.3, 127.7, 127.5, 79.9, 73.3, 70.3, 59.3, 52.0, 47.3, 28.6, 28.3, 24.7; HRMS (ESI-TOF): calcd for $[C_{20}H_{28}N_2O_6\text{-H}]^-$ 391.1875, found 391.1875. Spectral data of ^{1}H NMR was identical to that of previously reported.[35]

Boc-L-Ser(O-Bn)-Sar-OH (4e)

The reaction mixture was collected for 120 s. Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK ID 4.6 mm×25 cm, 20% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 9.33 min (epimer 16.54 min). 196 mg, 0.535 mmol, 67% (epimer: <0.1%). Colorless oil; ^{1}H NMR ($CDCl_3$+CD_3OD, 500 MHz, major rotamer) δ 7.33-7.20 (m, 5H), 4.87 (t, *J* = 4.8 Hz, 1H), 4.56-4.45 (m, 2H), 4.17-3.92 (m, 2H), 3.69-3.56 (m, 2H), 3.12 (d, 3H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz, major rotamer) δ 171.8, 171.6, 155.6, 137.7, 128.4, 127.7, 127.7, 80.1, 73.3, 70.3, 50.3, 49.8, 36.7, 28.3; IR (neat): 3430, 2976, 2533, 1712, 1652, 1496, 1407, 1167, 868, 741, 699 cm^{-1}; $[\alpha]^{31}_D$ = +14.60 (c 0.98, CH_2Cl_2); HRMS (ESI-TOF): calcd for $[C_{18}H_{26}N_2O_6\text{-H}]^-$ 365.1718, found 365.1711.

Boc-L-Val-L-Phe-OH (4f)

Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 3% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 9.33 min (epimer 13.06 min). 98.8 mg, 0.271 mmol, 68% (epimer: <0.1%). White amorphous solid; ^{1}H NMR ($CDCl_3$+CD_3OH, 500 MHz) δ 7.34-7.10 (m, 5H), 4.77 (t, *J* = 6.6 Hz, 1H), 3.88 (d, *J* = 6.7 Hz, 1H), 3.19 (dd, *J* = 5.2, 13.9 Hz, 1H), 3.01 (dd, *J* = 7.2, 13.8 Hz, 1H), 2.03-1.91 (m, 1H), 1.45 (s, 9H), 0.88 (dd, *J* = 6.7, 15.0 Hz, 6H); ^{13}C NMR ($CDCl_3$＋CD_3OD, 100 MHz) δ 173.0, 172.0, 156.1, 136.2, 129.1, 128.2, 126.6, 79.8, 59.5, 53.1, 37.4, 30.9, 27.9, 18.8; IR (neat): 3339, 2968, 1716, 1660, 1523, 1367, 1170, 1018, 876, 700 cm^{-1}; HRMS (ESI-TOF): calcd for $[C_{19}H_{28}N_2O_5\text{-H}]^-$ 363.1925, found 363.1919. Spectral data of ^{1}H NMR was identical to that of previously reported.[36]

Fmoc-L-Ser(Bzl)-L-Phe-OH (4g)

Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 8% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 52.40 min (epimer 41.51 min). 181 mg, 0.320 mmol, 80% (epimer: <0.1%). White solid; ^{1}H NMR ($CDCl_3$+CD_3OD, 400 MHz) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.1 Hz, 2H), 7.45-7.36 (m, 3H), 7.35-7.26 (m, 6H), 7.23-7.06 (m, 5H), 4.84-4.69 (m, 1H), 4.42-4.32 (m, 5H), 4.21 (t, *J* = 6.7 Hz, 1H), 3.87-3.73 (m, 1H), 3.65-3.53 (m, 1H), 3.21 (dd, *J* = 5.3, 13.9 Hz, 1H), 3.07 (dd, *J* = 6.5, 14.0 Hz, 1H); ^{13}C NMR ($CDCl_3$+CD_3OD, 100 MHz) δ 172.8, 170.0, 156.4, 143.6, 141.2, 137.2, 136.0, 129.3, 128.4, 128.3, 127.8, 127.8, 127.7, 127.0, 126.8, 125.0, 119.9, 73.3, 69.6, 67.1, 54.2, 53.4, 47.0, 37.3; IR (neat): 3326, 3063, 2947, 2361, 1716, 1669, 1522, 1452, 1219, 1196, 1031, 740 cm^{-1}; $[\alpha]^{30}_D$ = +32.2 (c 1.06, 25% MeOH in CH_2Cl_2); mp 158-160 °C; HRMS (ESI-TOF): calcd for $[C_{34}H_{32}N_2O_6\text{-H}]^-$ 563.2188, found 563.2188.

Cbz-L-Ser(Bzl)-L-Phe-OH (4h)

The reaction mixture was collected for 120 s. Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 12% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 19.88 min (epimer 14.38 min). 301 mg, 0.631 mmol, 79% (epimer: <0.1%). White solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.59 (s, 1H), 7.40-7.00 (m, 16H), 5.82 (s, 1H), 5.05 (d, *J* = 4.4 Hz, 2H), 4.82 (q, *J* = 6.4 Hz, 1H), 4.50-4.31 (m, 3H), 3.77 (s, 1H), 3.51 (t, *J* = 7.1

Hz, 1H), 3.15 (dd, *J* = 5.2, 14.0 Hz, 1H), 3.00 (dd, *J* = 6.4, 14.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.5, 170.3, 156.3, 137.2, 136.0, 135.7, 129.4, 128.7, 128.6, 128.6, 128.4, 128.2, 128.1, 128.0, 127.2, 73.6, 69.6, 67.4, 54.2, 53.5, 37.4; IR (ATR): 3279, 3059, 1724, 1696, 1656, 1537, 1496, 1238, 1043, 694 cm^{-1}; $[\alpha]^{30}_D$ = +48.2 (c 1.01, CH_2Cl_2) ; mp 119-121 °C; HRMS (ESI-TOF): calcd for $[C_{27}H_{28}N_2O_6\text{-}H]^-$ 475.1875, found 475.1876.

Boc-L-Tyr(O-Bn)-L-Phe-OH (4j)

Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 5% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 10.74 min (epimer 15.40 min). 172 mg, 0.332 mmol, 83% (epimer: <0.1%). White solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.40 (brs, 1H), 7.34-7.03 (m, 9H), 7.03-6.90 (m, 4H), 6.85-6.67 (m, 3H), 5.25 (s, 1H), 4.86 (s, 2H), 4.69 (s, 1H), 4.33 (s, 1H), 3.04 (dd, *J* = 4.8, 13.6 Hz, 1H), 2.95-2.68 (m, 3H), 1.28 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.8, 171.7, 157.9, 155.8, 137.1, 136.0, 130.5, 129.5, 128.8, 128.6, 128.5, 127.9, 127.4, 127.1, 115.0, 80.5, 70.0, 58.2, 55.7, 53.4, 37.6, 28.3; IR (neat): 3332, 2978, 2930, 1723, 1512, 1367, 1242, 1175, 1024, 861, 737, 699 cm^{-1}; $[\alpha]^{30}_D$ = +27.4 (c 1.12, CH_2Cl_2); mp 121-123 °C; HRMS (ESI-TOF): calcd for $[C_{30}H_{34}N_2O_6\text{-}H]^-$ 517.2344, found 517.2344.

Fmoc-L-Cys(Trt)-L-Phe-OH (4j)

Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 8% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 24.50 min (epimer 21.53 min). 239 mg, 0.326 mmol, 82% (epimer: <0.1%). White solid; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.55 (brs, 1H), 7.77-7.65 (m, 2H), 7.52 (s, 1H), 7.40-7.30 (m, 8H), 7.26-7.11 (m, 12H), 7.10-7.00 (m, 5H), 6.51 (d, *J* = 7.7 Hz, 1H), 5.20 (d, *J* = 7.9 Hz, 1H), 4.73 (q, *J* = 6.2 Hz, 1H), 4.36 (t, *J* = 6.8 Hz, 1H), 4.27 (t, *J* = 7.1 Hz, 1H), 4.13 (t, *J* = 7.0 Hz, 1H), 3.72 (d, *J* = 6.0 Hz, 1H), 3.11 (dd, *J* = 5.1, 13.9 Hz, 1H), 2.96 (dd, *J* = 6.4, 14.0 Hz, 1H), 2.65-2.51 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.2, 170.4, 156.2, 144.4, 143.7, 141.3, 135.6, 129.6, 129.5, 128.6, 128.2, 127.9, 127.2, 127.0, 125.2, 125.1, 120.1, 67.5, 67.3, 54.0, 53.3, 47.1, 37.4, 33.5; IR (neat): 3402, 3059, 2947, 1715, 1695, 1520, 1489, 1216, 1032, 738, 699, 620 cm^{-1}; $[\alpha]^{30}_D$ = +7.20 (c 0.96, CH_2Cl_2); mp 115-117 °C; HRMS (ESI-TOF): calcd for $[C_{46}H_{40}N_2O_5S\text{-}H]^-$ 731.2585, found 731.2581.

Cbz-L-Phg-L-Phe-OH (4k)

The reaction mixture was collected for 120 s. Purification method: The crude product was purified by column chromatography on silica gel (MeOH : $CHCl_3$ = 1 : 99 to 15 : 95). HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 10% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 12.14 min (epimer 15.75 min). 306 mg, 0.707 mmol, 88% (epimer: <0.1%). White solid; ^{1}H NMR ($CDCl_3$+CD_3OD, 400 MHz) δ 7.43-7.06 (m, 15H), 5.31 (s, 1H), 5.11-5.01 (m, 2H), 4.71 (t, *J* = 6.9 Hz, 1H) 3.19 (dd, *J* = 5.3, 13.4 Hz, 1H), 3.00 (dd, *J* = 7.3, 13.7 Hz, 1H); ^{13}C NMR ($CDCl_3$+CD_3OD, 125 MHz) δ 175.2, 170.1, 156.1, 136.9, 136.8, 135.8, 128.9, 128.1, 127.8, 127.6, 127.5, 127.5, 127.3, 126.6, 125.8, 66.4, 58.7, 55.1, 37.0; IR (neat): 3417, 2109, 1692, 1644, 1549, 1404, 1252, 1107, 1014, 699 cm^{-1}; $[\alpha]^{30}_D$ = +55.6 (c 1.03, 25% MeOH in CH_2Cl_2); mp 220-222 °C; HRMS (ESI-TOF): calcd for $[C_{25}H_{24}N_2O_5\text{-}H]^-$ 431.1612, found 431.1612.

Boc-L-Ala-L-Cys(Trt)-L-Phe-OH (4l)

Purification method: The crude product was purified by GPC. HPLC conditions: DAICEL CHIRALPAK ID 4.6 mm×25 cm, 10% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 22.30 min (epimer 28.68 min). 165 mg, 0.242 mmol, 60% (epimer: 0.4%). White solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.0 (brs, 1H), 7.41-7.35 (m, 6H), 7.27-7.14 (m, 13H), 7.12-7.08 (m, 1H), 6.83-6.70 (m, 2H), 5.22 (s, 1H), 4.70 (s, 1H), 4.14-3.95 (m, 2H), 3.22-3.09 (m, 1H), 3.02 (dd, *J* = 6.6, 13.9 Hz, 1H), 2.76 (dd, *J* = 6.5, 12.4 Hz, 1H), 2.51 (s, 1H), 1.37 (s, 9H), 1.18 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.4, 173.1, 169.9, 155.6, 144.4, 136.1, 129.6, 129.4, 128.5, 128.1, 128.0, 126.9, 80.5, 67.3, 53.6, 52.4, 50.2, 37.2, 33.1, 28.4, 18.4; IR (neat): 3310, 3030, 2931, 1715, 1682, 1506, 1445, 1367, 1249, 1166, 741, 701 cm^{-1}; $[\alpha]^{30}_D$ = +4.82 (c 1.05, CH_2Cl_2); mp 114-116 °C; HRMS (ESI-TOF): calcd for $[C_{39}H_{43}N_3O_6S\text{-}H]^-$ 680.2800, found 680.2801.

Boc-L-Ala-L-Phg-L-Phe-OH (4m)

THF was used for solvent instead of MeCN. Purification method: The crude product was purified by column chromatography on silica gel (MeOH : $CHCl_3$ = 1 : 99 to 3 : 97). HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 8% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 13.01 min (epimer 20.28 min). 128 mg, 0.272 mmol, 68% (epimer <0.1%). White solid; ^{1}H NMR ($CDCl_3$+CD_3OD, 400 MHz) δ 7.26-7.02 (m, 10H), 5.38 (s, 1H), 4.59 (t, *J* = 6.4 Hz, 1H) 4.06 (d, *J* = 5.7 Hz, 1H), 3.11 (dd, *J* = 5.2, 13.9 Hz, 1H), 2.93 (dd, *J* = 7.5, 13.8 Hz, 1H), 1.32 (s, 9H), 1.20 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$+CD_3OD, 125 MHz) δ 173.1, 172.5, 169.8, 155.8, 136.4, 136.1, 128.8, 128.2, 127.9, 127.7, 126.7, 126.3, 79.5, 56.6, 53.4, 49.6, 36.8, 27.5, 17.1; IR (neat): 3307, 2978, 1717, 1649, 1521, 1367, 1166, 698 cm^{-1}; $[\alpha]^{30}_D$ = +44.6 (c 1.00, 25% MeOH in CH_2Cl_2); mp 136-138 °C; HRMS (ESI-TOF): calcd for $[C_{25}H_{31}N_3O_6\text{-}H]^-$ 468.2140, found 468.2143.

Boc-L-Ala-L-Ser-L-Phe-L-Gly-OH (4n)

A solution of Boc-L-Ala-L-Ser(O-Bn)-OH (**1a**) (0.333 M, 1.00 equiv.), NMM (0.333 M, 1.00 equiv.), and DIEA (0.333 M, 1.00 equiv.) in MeCN (flow rate: 1.20 mL/min) and a solution of isobutyl chloroformate (0.240 M, 1.20 equiv.) in MeCN (flow rate: 2.00 mL/min) were introduced to the T-shape mixer at 20 °C with the syringe pumps. The resultant mixture was passed through a reaction tube (inner diameter: 0.800 mm, length: 531 mm, volume 267 µL, reaction time: 5.00 s) at the same temperature. Then, the resultant mixture and a solution of H-L-Phe-Gly-ONa[37] (**3f**) (0.340 M, 1.70 equiv.) in H_2O (flow rate: 2.00 mL/min) were introduced to the V-shape mixer at 20 °C. The resultant mixer was passed through a reaction tube (inner diameter: 0.800 mm, length 1724 mm, volume: 867 µL, reaction time 10.0 s) at the same temperature. After being eluted for 50 s to reach a steady state, the resultant mixture was poured into a solution of saturated aqueous NH_4Cl, H_2O and EtOAc for 60 s. The aqueous layer was acidified to pH of 1 with aqueous 1 M HCl and extracted 3 times with EtOAc. The combined organic layer was washed with aqueous 1 M HCl and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by GPC to afford **4o** (149 mg, 0.261 mmol, 65%, epimer: <0.1%). White solid. HPLC conditions: DAICEL CHIRALPAK IB 4.6 mm×25 cm, 8% IPA (containing 0.1% formic acid) in hexane (containing 0.1% formic acid), 40 °C, flow rate 1 mL/min, detection wavelength 254 nm, retention time 41.50 min (epimer 23.69 min). ^{1}H NMR ($CDCl_3$+CD_3OD, 500 MHz) δ 7.33-7.27 (m, 2H), 7.27-7.23 (m, 3H), 7.19-7.11 (m, 5H), 4.75 (q, *J* = 4.38 Hz, 1H), 4.51-4.42 (m, 3H), 4.11 (d, *J* = 6.5 Hz, 1H), 3.87-3.79 (m, 1H), 3.77-3.68 (m, 2H), 3.65-3.58 (m, 1H), 3.24 (dd, *J* = 4.7, 15.1 Hz, 1H), 2.96 (dd, *J* = 9.0, 14.1 Hz, 1H), 1.38 (s, 9H), 1.25 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$+CD_3OD, 100 MHz) δ 174.2, 171.7, 171.4, 170.0, 156.1, 137.0, 136.5, 128.7, 128.1, 128.0, 127.6, 127.4, 126.3, 79.9, 73.0, 68.7, 57.1,

54.0, 50.2, 40.9, 36.9, 27.7, 17.1; IR (neat): 3420, 2360, 1647, 1522, 1249, 1167, 752 cm^{-1}; $[\alpha]^{30}_D$ = -8.10 (c 1.12, 25% MeOH in CH_2Cl_2); mp 123-124 °C; HRMS (ESI-TOF): calcd for $[C_{29}H_{38}N_4O_8\text{-}H]^-$ 569.2617, found 569.2616.

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Conflicts of interest

The authors declare no conflicts of interest.

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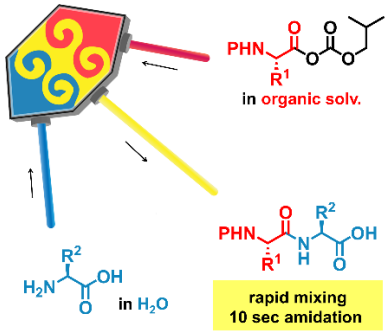
FULL PAPER

Peptide synthesis using unprotected amino acids via mixed carbonic anhydride was demonstrated. Micro-flow technology enabled rapid mixing of an organic layer containing a protected amino acid or dipeptide and an aqueous layer containing an unprotected amino acid or dipeptide to accelerate desired amidation without severe racemization/epimerization (≤ 0.4%). Various peptides were obtained in good yields using inexpensive, less toxic, and less wasteful reagents.



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Peptide Chain Elongation Using Unprotected Amino Acids in a Micro-flow Reactor