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α-Chymotrypsin-catalyzed peptide synthesis in frozen aqueous solution using N-protected amino acid carbamoylmethyl esters as acyl donors

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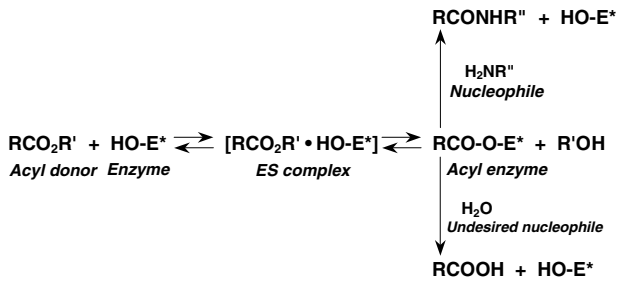
Abstract—A kinetically controlled peptide synthesis catalyzed by α-chymotrypsin was performed in frozen aqueous solution (ice, −24 °C). The yield of the peptide was significantly improved by the use of the carbamoylmethyl (Cam) ester as the acyl donor instead of the conventional ethyl ester. The peptide yield increased up to ca. 90% when *N*-benzyloxycarbonyl (CBZ)-Phe-OCam and H-Phe-NH_2 were used as the acyl donor and nucleophile, respectively. Such an improvement of the peptide yield in ice was also observed in the coupling of other CBZ-amino acid Cam esters as acyl donors. Furthermore, this approach was applied to the synthesis of peptides containing D-amino acids. The peptides such as CBZ-D-Phe-Phe-NH_2, CBZ-Phe-D-Phe-NH_2 and CBZ-D-Phe-D-Phe-NH_2 were also obtained in excellent to moderate yields in ice. A high diastereoselectivity towards the L–L peptide was observed when the racemic amino acid Cam ester was used as the acyl donor in ice.

1. Introduction

Protease-catalyzed peptide synthesis has attracted much attention because of several advantages over the chemical method, such as minimum protection of side chains, prevention of racemization and progress of the reaction under mild conditions.[1,2] In kinetically controlled peptide synthesis (Scheme 1), an ester substrate (acyl donor) first combines with the enzyme to form the acyl enzyme intermediate via the ES complex (acylation step). The acyl enzyme is then deacylated either by a nucleophilic amine (nucleophile) to give the desired peptide or by water present in the reaction medium to form the hydrolysis product of the ester substrate. Generally, the latter deacylation is a rate-determining step. Therefore, such protease-catalyzed peptide synthesis has often been performed in an organic solvent with low content of water, in order to suppress undesired hydrolysis of the acyl donor.[3] However, the peptide synthesis becomes time consuming because of the inhibition with a high concentration of the organic solvent.



Scheme 1. α-Chymotrypsin-catalyzed kinetically controlled peptide synthesis.

As an alternative approach, protease-catalyzed peptide synthesis in frozen aqueous solution (ice) has been proposed, where the hydrolysis of the donor ester decreases significantly compared with aminolysis (peptide synthesis).[4] This attractive method requires that a donor ester dissolves in aqueous solution before freezing. Consequently, the donor ester applicable is limited to that of a relatively hydrophilic amino acid, such as Arg, Tyr, Asp or Glu, when the N-terminal of the donor ester is protected with hydrophobic benzyloxycarbonyl (CBZ).[5,6] Alternatively, in order to make the donor ester of a hydrophobic amino acid water soluble, its N-termi-

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nal is masked with hydrophilic maleyl, acetyl or betainyl group instead of CBZ.[7] However, these N-protecting groups cannot be removed under mild conditions. It would be ideal for the N-terminal of the donor ester to be protected with CBZ, which has been used frequently in both chemical and enzymatic peptide syntheses and is known to be removed easily without side reactions.

An alternative and promising approach is to employ a hydrophilic ester moiety instead of the conventional ethyl or methyl ester. Previously, Jakubke et al.[8] have found that carbamoylmethyl (Cam) ester of CBZ-Phe has a better solubility in an aqueous solution and is also a good acyl donor for α-chymotrypsin-catalyzed peptide synthesis. Recently, Miyazawa et al.[9,10] have demonstrated the superiority of the Cam ester as the acyl donor for the α-chymotrypsin-catalyzed kinetically controlled peptide synthesis in acetonitrile with low content of water. Herein, we report the α-chymotrypsin-catalyzed peptide synthesis in ice, where the Cam esters of CBZ-amino acids were used as the acyl donors.

2. Results and discussion

2.1. Effect of ester moiety

Initially, we examined the α-chymotrypsin-catalyzed peptide synthesis between the ester of CBZ-Phe (CBZ-Phe-OR, 10 mM) and H-Phe-NH_2 (5-fold excess) in both aqueous solution (30 °C) and ice (−24 °C) (Scheme 2). We used a mixture of buffer solution (0.1 M, pH 8, 4.8 ml) and DMSO (0.2 ml) as the reaction medium. As shown in Table 1 (entries 1–4), a good coupling yield (81–85%) was obtained for the Cam, cyanomethyl (CM) and ethyl (Et) esters in aqueous solution after 0.5 h of incubation. In contrast, the hydrophobic 2,2,2-trifluoroethyl (Tfe) ester gave only 55% yield. In this case, a significant amount of the Tfe ester (28%) remained after 30 min of incubation, presumably due to its low solubility in aqueous solution.

Herein, the temperature of the ice was maintained at −24 °C because it was known that keeping the temperature between −10 and −25 °C was optimal.[4] As compared with peptide synthesis in aqueous solution, the coupling efficiency was improved for the Cam and CM esters in ice (Table 1, entries 5–8). Figure 1 shows the time course of α-chymotrypsin-catalyzed peptide synthesis in ice, using the Cam ester as the acyl donor. The

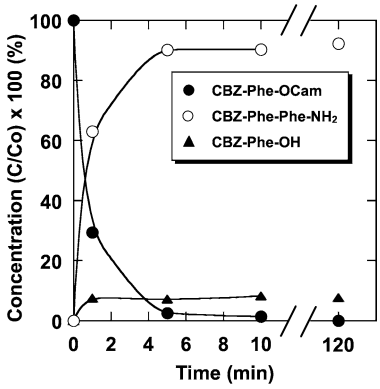
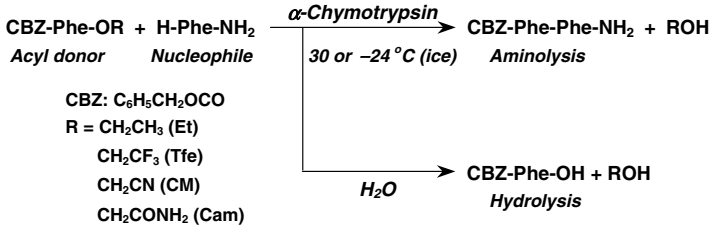


Figure 1. Time course of α-chymotrypsin-catalyzed peptide synthesis in ice (−24 °C) between CBZ-Phe-OCam and H-Phe-NH_2. CBZ-Phe-OCam, 10 mM; H-Phe-NH_2, 50 mM; enzyme, 2 mg ml^{-1}.



Scheme 2. α-Chymotrypsin-catalyzed peptide synthesis.

Table 1. α-Chymotrypsin-catalyzed peptide synthesis between CBZ-Phe-OR and H-Phe-NH_2[a]

Entry	R	Temperature (°C)	Time (h)	CBZ-Phe-OR[b] (%)	Yield (%)	
					Peptide[c]	CBZ-Phe-OH
1	Et	30	0.5	2.5	80.8	16.7
2	Tfe	30	0.5	28.1	54.9	17.0
3	CM	30	0.5	0	83.2	16.8
4	Cam	30	0.5	0	84.6	15.4
5	Et	−24	0.5	23.7	66.8	9.5
6	Tfe	−24	0.5	44.4	48.1	7.5
7	CM	−24	0.5	1.7	87.4	10.8
8	Cam	−24	0.5	1.2	88.2	10.6

[a] Reaction conditions: CBZ-Phe-OR, 10 mM; H-Phe-NH_2, 50 mM; enzyme, 2 mg ml^{-1}; solvent, phosphate buffer (0.1 M, pH 8)/DMSO [96/4 (v/v)].
[b] Remaining acyl donor.
[c] CBZ-Phe-Phe-NH_2.

Cam ester was consumed almost completely within 5 min to give the desired dipeptide amide in 88% yield. In this case, accumulation of the acyl enzyme brought about by the enhanced formation of the ES-complex through favourable hydrogen bonding using an amide proton is responsible for the rapid reaction, because the electron-withdrawing ability of the Cam ester moiety is not so large as compared with that of CM or Tfe.[9] The activated CM ester also gave the dipeptide in high yield (ca. 85%) within 10 min. In contrast, the Et and Tfe esters resulted in both low yield and slow reaction. This may be due to their incomplete dissolution in the reaction solution before freezing. We confirmed that no peptide synthesis took place in ice without α-chymotrypsin.

2.2. Effect of nucleophile concentration

In the kinetically controlled peptide synthesis, the yield of the peptide depends on the nucleophile concentration because the nucleophile and water (undesired nucleophile) attack the acyl enzyme competitively to form the peptide and the hydrolysis product of the acyl donor, respectively (Scheme 1). We then investigated the effect of the nucleophile concentration on the peptide synthesis between CBZ-Phe-OCam (10 mM) and the H-Phe-NH_2. In Figure 2, the peptide yields after 0.5 h of incubation at 30 °C and those after 2 h at −24 °C (after 4 h in the case of 10 mM of H-Phe-NH_2) were plotted. The acyl donor mostly disappeared within these reaction times in both aqueous solution and ice. As expected, the effect of the nucleophile concentration was more significant in ice than in aqueous solution, with the exception of the nucleophile concentration of 10 mM. The 5-fold excess of the nucleophile was used throughout for further experiments, because if the excess was too large, the nucleophile would often be wasted.

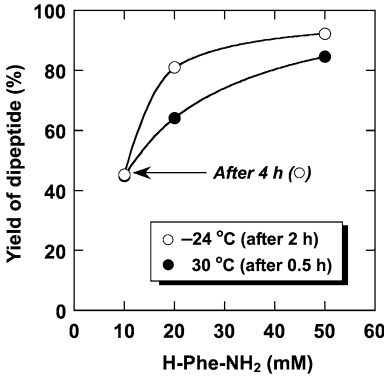


Figure 2. Effect of nucleophile concentration on α-chymotrypsin-catalyzed peptide synthesis between CBZ-Phe-OCam and H-Phe-NH_2 in both aqueous solution (30 °C) and ice (−24 °C). CBZ-Phe-OCam, 10 mM; enzyme, 2 mg ml^{-1}. Peptide yields after 0.5 h at 30 °C and those after 2 h at −24 °C (after 4 h in the case of 10 mM of H-Phe-NH_2) were plotted.

2.3. Synthesis of various peptides

The success of this approach prompted us to synthesize a variety of dipeptides in ice using the Cam esters as acyl donors. As shown in Table 2, the aminolysis (peptide synthesis) was profoundly improved and hydrolysis of the acyl donor was suppressed well in ice, although the reaction became somewhat slow. The high coupling yield can be explained in terms of the concentration effect of the nucleophile in the reduced liquid phase around the enzyme in frozen mixture, where the aminolysis versus hydrolysis ratio becomes higher (freeze-concentration model[4,11]). Therefore, the diverse peptides were obtained in good yields. In an aqueous solution, a rather low yield was observed when the Ala residue was used as the acyl

Table 2. α-Chymotrypsin-catalyzed peptide synthesis between CBZ-AA_1-OCam and H-AA_2-NH_2[a]

Entry	AA_1	AA_2	Temperature (°C)	Time (h)	CBZ-AA_1-OCam[b] (%)	Yield (%)	
						Peptide[c]	CBZ-AA_1-OH
1	Phe	Phe	30	0.5	0	84.6	15.4
2	Phe	Leu	30	0.5	10.8	67.8	21.5
3	Phe	Ala	30	0.5	0.4	40.6	59.1
4	Leu	Phe	30	0.5	10.6	66.3	23.1
5	Leu	Leu	30	0.5	9.7	73.2	17.2
6	Leu	Ala	30	0.5	2.3	41.8	55.9
7	Ala	Phe	30	0.5	0	71.7	28.3
8	Ala	Leu	30	0.5	1.4	75.4	23.2
9	Ala	Ala	30	0.5	0	36.9	63.1
10	Phe	Phe	−24	2	0	92.3	7.7
11	Phe	Leu	−24	2	0	87.7	12.3
12	Phe	Ala	−24	1	0	76.5	23.5
13	Leu	Phe	−24	5 d	25.3	68.2	6.5
14	Leu	Leu	−24	4	1.0	91.4	7.6
15	Leu	Ala	−24	6	2.8	72.4	24.9
16	Ala	Phe	−24	4 d	4.2	83.9	11.8
17	Ala	Leu	−24	5 d	2.1	91.2	6.7
18	Ala	Ala	−24	5 d	2.9	87.6	9.5

[a] Reaction conditions: CBZ-AA_1-OCam, 10 mM; H-AA_2-NH_2, 50 mM; enzyme, 2 mg ml^{-1}; solvent, phosphate buffer (0.1 M, pH 8) /DMSO [96/4 (v/v)].
[b] Remaining acyl donor.
[c] CBZ-AA_1-AA_2-NH_2.

donor or nucleophile. For example, the yield of CBZ-Ala-Ala-NH_2 was as low as 37% (entry 9), but it increased up to 88% (after 5 d) in ice (entry 18). The significant increase in the peptide yield suggests that the hydrolysis of the acyl donor was suppressed quite well in ice.

2.4. The use of D-amino acid derivatives as either acyl donors or nucleophiles

The enzymatic incorporation of D-amino acids into peptides is of interest because they are often found in various biologically active compounds. In a previous communication,[12] we succeeded in incorporating a D-amino acid, such as D-Phe, at the N-terminus of the resulting dipeptide in aqueous solution by the use of the Cam ester of the D-amino acid as the acyl donor and α-chymotrypsin as catalyst. Therefore, we investigated the incorporation of a D-amino acid at either the P_1 or P_1' position or both positions of the resulting dipeptide in ice. As can be seen in Table 3, the coupling efficiency was generally improved in the reaction in ice.

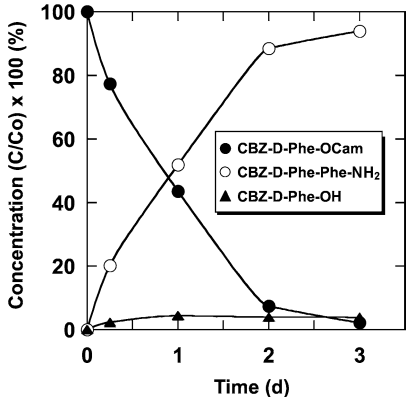


Figure 3. Time course of α-chymotrypsin-catalyzed peptide synthesis in ice (−24 °C) between CBZ-D-Phe-OCam and H-Phe-NH_2. CBZ-D-Phe-OCam, 10 mM; H-Phe-NH_2, 50 mM; enzyme, 4 mg ml^{-1}.

This can again be explained by the suppression of the competing hydrolysis of the acyl donor. Although the reaction became very slow when compared with that in an aqueous solution, the yield of CBZ-D-Phe-Phe-NH_2 increased from 81% to 94% in ice (entries 2 and 8). Figure 3 shows the time course of the α-chymotrypsin-catalyzed synthesis of CBZ-D-Phe-Phe-NH_2 in ice as a typical example.

However, in the case of CBZ-D-Leu-Leu-NH_2, the yield in ice decreased to some extent (from 79% to 70%) (entries 5 and 11). This is presumably due to the extremely slow reaction in ice, where a substantial amount of CBZ-D-Leu-OCam remained (27%) after 3 d, although the hydrolysis (3%) was quite well suppressed. The coupling of CBZ-Phe-OCam and H-D-Phe-NH_2 gave CBZ-Phe-D-Phe-NH_2 in 63% yield (entry 7). It is interesting to note that the D–D peptide such as CBZ-D-Phe-D-Phe-NH_2 was obtained in ice in moderate yield (54%) (entry 9), because there is only one example[13] for the enzymatic synthesis of a D–D dipeptide to the best of our knowledge.

2.5. The use of DL-amino acid derivatives as acyl donors or nucleophiles

Recently, Miyazawa et al.[10] performed an α-chymotrypsin-catalyzed peptide synthesis using the DL-amino acid Cam esters as the acyl donors in acetonitrile with low water content. Interestingly, they found that the peptide synthesis proceeds with a high diastereoselectivity to yield only the L–L dipeptide amide.

We also attempted to synthesize homochiral peptides (L–L diastereomers) in ice by the use of the DL-amino acid derivatives as the acyl donors or nucleophiles. The D–L (or L–D) and L–L diastereomers were separated from each other on a reversed phase HPLC column (ODS-80 A), which allowed us to determine the diastereomeric excess (de) accurately.

Table 3. α-Chymotrypsin-catalyzed synthesis of peptides containing D-amino acids between CBZ-AA_1-OCam and H-AA_2-NH_2[a]

Entry	AA_1	AA_2	Temperature (°C)	Time (h)	CBZ-AA_1-OCam[b] (%)	Yield (%)	
						Peptide[c]	CBZ-AA_1-OH
1	Phe	D-Phe	30	2 min	0	48.9	51.1
2	D-Phe	Phe	30	1	4.8	80.8	14.4
3	D-Phe	D-Phe	30	1	0	16.6	83.4
4	Leu	D-Leu	30	2 min	0.9	13.4	85.7
5	D-Leu	Leu	30	1	0	78.7	21.3
6	D-Leu	D-Leu	30	2	0	10.0	90.0
7	Phe	D-Phe	−24	4	0	62.5	37.5
8[d]	D-Phe	Phe	−24	3 d	2.2	93.9	3.9
9[d]	D-Phe	D-Phe	−24	8 d	10.3	54.3	35.4
10	Leu	D-Leu	−24	1 d	2.7	18.0	79.3
11[d,e]	D-Leu	Leu	−24	3 d	26.6	70.4	2.9
12[d]	D-Leu	D-Leu	−24	10 d	36.0	40.9	23.1

[a] Reaction conditions: CBZ-AA_1-OCam, 10 mM; H-AA_2-NH_2, 50 mM; enzyme, 2 mg ml^{-1}; solvent, phosphate buffer (0.1 M, pH 8) /DMSO [96/4 (v/v)].
[b] Remaining acyl donor.
[c] CBZ-AA_1-AA_2-NH_2.
[d] α-Chymotrypsin, 4 mg ml^{-1}.
[e] CBZ-D-Leu-OCam, 5 mM; H-Leu-NH_2, 25 mM.

Table 4. α-Chymotrypsin-catalyzed peptide synthesis between CBZ-DL-AA_1-OCam and H-AA_2-NH_2[a]

Entry	DL-AA_1	AA_2	Temperature (°C)	Time (h)	Acyl donor[b] (%)	Hydrolysis[c] (%)	Peptide[d] (%)		de[e] (%)
							L–L	D–L	
1	DL-Phe	Phe	30	1 min	43.2	13.5	43.3	0.07	99.7
2				1	28.0	20.9	44.0	7.1	72.2
3	DL-Leu	Leu	30	5 min	45.1	14.2	40.6	0.05	99.8
4				1	32.5	22.4	41.3	3.8	83.1
5	DL-Phe	Phe	−24	1	45.7	11.3	42.9	0.04	99.8
6				3	45.9	10.4	43.5	0.18	99.2
7	DL-Leu	Leu	−24	1	61.8	7.0	31.2	0	>99.9
8				3	49.8	6.2	44.0	0	>99.9

[a] Reaction conditions: CBZ-DL-AA_1-OCam, 10 mM; H-AA_2-NH_2, 50 mM; enzyme, 0.1 mg ml^{-1} (30 °C) or 0.4 mg ml^{-1} (−24 °C); solvent, phosphate buffer (0.1 M, pH 8)/DMSO [96/4 (v/v)].
[b] Remaining CBZ-(D/L)-AA_1-OCam.
[c] Yield of CBZ-(D/L)-AA_1-OH.
[d] Yields of CBZ-AA_1-AA_2-NH_2 and CBZ-D-AA_1-AA_2-NH_2.
[e] Diastereomeric excess.

At first, we performed the α-chymotrypsin-catalyzed coupling using the DL-amino acid Cam ester as the acyl donor. The results are summarized in Table 4. As expected, the de towards the L–L peptide in aqueous solution was very high at the early stage of reaction (entries 1 and 3), which reflects the high L-enantioselectivity of α-chymotrypsin at the P_1-position. However, it became significantly poor (entries 2 and 4) after a prolonged reaction time, indicating that the reaction of D-enantiomer took place gradually after the L-enantiomer was consumed because of high reactivity of the Cam ester. In contrast, in ice the de towards the L–L peptide proved to be quite high (entries 5–8), suggesting that the formation of the D–L peptide became significantly slow when compared with that of the L–L peptide at a low temperature. Figure 4 shows the time course of the α-chymotrypsin-catalyzed diastereoselective coupling between CBZ-DL-Leu-OCam and H-Leu-NH_2 in ice as a typical example. Interestingly, the yield of the L–L peptide reached 44% after 3 h of incubation, while the D–L peptide was not detected by HPLC.

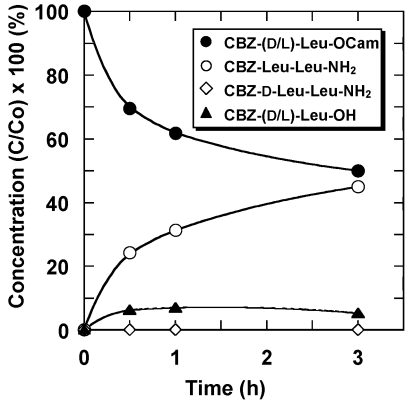


Figure 4. Time course of α-chymotrypsin-catalyzed peptide synthesis in ice (−24 °C) between CBZ-DL-Leu-OCam and H-Leu-NH_2. CBZ-DL-Leu-OCam, 10 mM; H-Leu-NH_2, 50 mM; enzyme, 0.4 mg ml^{-1}.

Next, we performed the α-chymotrypsin-catalyzed coupling using the DL-amino acid amide as the nucleophile. As shown in Table 5, the diastereoselectivity towards the L–L peptide did not improve in ice. A high diastereoselectivity was observed in the coupling between CBZ-Leu-OCam and H-DL-Leu-NH_2, while the diastereoselectivity became rather poor in the coupling between CBZ-Phe-OCam and H-DL-Phe-NH_2. The results are consistent with the fact that CBZ-Phe-D-Phe-NH_2 was

Table 5. α-Chymotrypsin-catalyzed peptide synthesis between CBZ-AA_1-OCam and H-DL-AA_2-NH_2[a]

Entry	AA_1	DL-AA_2	Temperature (°C)	Time (h)	CBZ-AA_1-OCam[b] (%)	CBZ-AA_1-OH[c] (%)	Peptide[d] (%)		de[e] (%)
							L–L	L–D	
1	Phe	DL-Phe	30	1 min	0.1	26.1	59.3	14.5	60.7
2	Leu	DL-Leu	30	1 min	3.2	31.3	63.1	2.3	93.0
3	Phe	DL-Phe	−24	0.5	4.4	21.0	61.2	13.3	64.3
4				4	0.9	22.4	62.2	14.6	62.0
5	Leu	DL-Leu	−24	2	18.9	9.8	68.7	2.5	93.0
6				4	3.3	10.8	82.8	3.1	92.8

[a] Reaction conditions: CBZ-AA_1-OCam, 10 mM; H-DL-AA_2-NH_2, 50 mM; enzyme, 1 mg ml^{-1}; solvent, phosphate buffer (0.1 M, pH 8)/DMSO [96/4 (v/v)].
[b] Remaining acyl donor.
[c] Yield of hydrolysis.
[d] Yields of CBZ-AA_1-AA_2-NH_2 and CBZ-AA_1-D-AA_2-NH_2.
[e] Diastereomeric excess.

formed more easily than CBZ-Leu-D-Leu-NH_2 (Table 3, entries 7 and 10). The results also indicate that the stereospecificity of the P'_1-position of α-chymotrypsin is rather flexible and depends on the nature of the nucleophile.

2.6. Preparative synthesis of peptides in ice

Preparative peptide syntheses (CBZ-Phe-Phe-NH_2, CBZ-Phe-Ala-NH_2 and CBZ-D-Phe-Phe-NH_2) were then successfully performed in ice. The pure product was easily isolated in good yield (68–83%) by filtration of the precipitates in the reaction solution after melting the ice followed by recrystallization from methanol–diethyl ether. The dipeptide amides obtained were characterized by spectroscopic methods.

3. Conclusion

Herein, we have found that α-chymotrypsin-catalyzed peptide synthesis in ice (−24 °C) gives the desired peptide in high yield by suppressing the undesirable competing hydrolysis, when the Cam ester of the CBZ-amino acid is used as the acyl donor. This method was successfully applied to the peptide syntheses using various CBZ-amino acid Cam esters containing D-amino acids as the acyl donors. Consequently, the combined use of the hydrophilic Cam ester as the acyl donor and the freezing of the aqueous reaction solution, offers new possibilities in the kinetically controlled peptide synthesis mediated by α-chymotrypsin.

4. Experimental

α-Chymotrypsin (EC 3.4.21.1, type II) was obtained from Sigma Chemical Co. and used for reaction as received. Amino acids (D-, L- and DL-) were purchased from Wako Pure Chemicals or Peptide Research Institute. ^1H and ^{13}C NMR spectra were recorded on a JEOL JMN-EX400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C) using TMS or DMSO-d_6 as an internal standard. Optical rotations were measured with a JASCO DIP-360 digital polarimeter. FT-IR spectra were measured with a BioRad FTS-3000MX spectrometer as KBr tablets and selected bands were listed. High resolution mass spectra were recorded using a JEOL JMS-SX102A spectrometer (FAB^+).

4.1. Preparation of CBZ-amino acid esters

CBZ-Phe-OEt was prepared by esterification of CBZ-Phe-OH with $BF_3{\cdot}OEt_2$ in ethanol. CBZ-Phe-OTfe and CBZ-Phe-OCM were prepared by the reported methods.[14,15] The purity of these esters was checked by reversed phase HPLC.

CBZ-Phe-OEt: oil; $[\alpha]_D^{26} = -13.8$ (c 1.07, MeOH) {lit.:[16] oil; $[\alpha]_D^{20} = -12.7$ (c 1, MeOH)}. ^1H NMR ($CDCl_3$) δ 1.22 (3H, t, $J = 7.1$ Hz, CH_2–CH_3), 3.06–3.12 (2H, m, CH_2–C_6H_5), 4.16 (2H, q, $J = 7.1$ Hz, CH_2–CH_3), 4.61–4.66 (1H, m, NH–CH), 5.10 (2H, s, O–CH_2–C_6H_5), 5.24 (1H, d, $J = 8.1$ Hz, NH–CH), 7.09–7.37 (10H, m, $2 \times C_6H_5$).

CBZ-Phe-OTfe: mp 78–79 °C; $[\alpha]_D^{26} = -20.0$ (c 1.05, MeOH) {lit.:[3] mp 80–80.5 °C; $[\alpha]_D^{25} = -20.8$ (c 1.0, MeOH)}.

CBZ-Phe-OCM: mp 51–52 °C (lit.:[17] mp 55 °C); $[\alpha]_D^{26} = -25.7$ (c 1.02, MeOH).

4.2. Preparation of CBZ-amino acid Cam esters

The CBZ-amino acid Cam esters were prepared according to the Cs salt method of Capellas et al.[18]

CBZ-Phe-OCam: mp 89–90 °C (lit.:[8] mp 87–88 °C); $[\alpha]_D^{26} = -24.0$ (c 1.02, MeOH) {lit.:[19] $[\alpha]_D^{20} = -24$ (c 3.05, EtOH)}.

CBZ-D-Phe-OCam: mp 87–88 °C; $[\alpha]_D^{26} = +25.4$ (c 1.05, MeOH). Found: C, 63.90; H, 5.73; N, 7.67. Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N, 7.86. ^1H NMR (DMSO-d_6) δ 2.85–3.21 (2H, m, CH_2–C_6H_5), 4.39–4.48 (3H, m, CH_2CONH_2+NH–CH), 4.98 (2H, s, O–CH_2–C_6H_5), 7.20–7.40 (12H, m, $2 \times C_6H_5$+$CONH_2$), 7.88 (1H, d, $J = 8.3$ Hz, NH–CH). IR (ν/cm^{-1}) 3447, 3362, 1749, 1703, 1533.

CBZ-DL-Phe-OCam: mp 114–117 °C. Found: C, 64.26; H, 5.80; N, 7.76. Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N, 7.86. ^1H NMR (DMSO-d_6) δ 2.85–3.20 (2H, m, CH_2–C_6H_5), 4.38–4.48 (3H, m, CH_2CONH_2+NH–CH), 4.98 (2H, s, O–CH_2–C_6H_5), 7.20–7.40 (12H, m, $2 \times C_6H_5$+$CONH_2$), 7.88 (1H, d, $J = 8.3$ Hz, NH–CH). IR (ν/cm^{-1}) 3391, 3186, 1740, 1703, 1659, 1537.

CBZ-Leu-OCam: mp 82–85 °C; $[\alpha]_D^{26} = -25.6$ (c 1.03, MeOH) {lit.:[20] mp 90–90.5 °C; $[\alpha]_D^{25} = -27.2$ (c 1.0, MeOH)}.

CBZ-D-Leu-OCam: mp 86–88 °C; $[\alpha]_D^{26} = +26.4$ (c 1.03, MeOH). Found: C, 59.57; H, 6.91; N, 8.70. Calcd for $C_{16}H_{22}N_2O_5$: C, 59.62; H, 6.88; N, 8.69. ^1H NMR (DMSO-d_6) δ 0.86–0.90 [6H, m, $2 \times CH(CH_3)_2$], 1.56–1.70 [3H, m, CH_2–$CH(CH_3)_2$], 4.15–4.21 (1H, m, NH–CH), 4.45 (2H, s, CH_2CONH_2), 5.04 (2H, s, O–CH_2–C_6H_5), 7.28–7.37 (7H, m, C_6H_5+$CONH_2$), 7.79 (1H, d, $J = 8.0$ Hz, NH–CH). IR (ν/cm^{-1}) 3420, 3375, 2961, 1732, 1680, 1516.

CBZ-DL-Leu-OCam: mp 103–105 °C. Found: C, 59.45; H, 6.66; N, 8.66. Calcd for $C_{16}H_{22}N_2O_5$: C, 59.62; H, 6.88; N, 8.69. ^1H NMR (DMSO-d_6) δ 0.85–0.90 [6H, m, $2 \times CH(CH_3)_2$], 1.55–1.71 [3H, m, CH_2–$CH(CH_3)_2$], 4.14–4.20 (1H, m, NH–CH), 4.44 (2H, s, CH_2CONH_2), 5.04 (2H, s, O–CH_2–C_6H_5), 7.27–7.39 (7H, m, C_6H_5+$CONH_2$), 7.79 (1H, d, $J = 8.0$ Hz, NH–CH). IR (ν/cm^{-1}) 3413, 3377, 2963, 1732, 1695, 1674, 1520.

CBZ-Ala-OCam: mp 109–110 °C (lit.:[9] mp 71.5–72.5 °C); $[\alpha]_D^{26} = -28.0$ (c 1.03, MeOH).

4.3. Preparation of amino acid amides

Amino acid amides (H-AA$_2$-NH$_2$) were prepared by ammonolysis of the corresponding free ethyl or methyl esters in methanol–NH_3.[21] Recrystallization was performed from methanol–diethyl ether. The amino acid amides were identified by elemental analyses.

4.4. Preparation of authentic dipeptide amides for HPLC analysis

The preparation of CBZ-Phe-D-Phe-NH$_2$ is described as a typical example. A mixture of CBZ-Phe-OSu[22] (2.38 g, 6 mmol), HCl·H-D-Phe-OMe (2.59 g, 12 mmol) and triethylamine (2 ml, 14.4 mmol) in DMF (15 ml) was stirred at room temperature for 2 d. After completion of the reaction, the solution was evaporated to dryness and the residue taken up in ethyl acetate (AcOEt, 50 ml). The organic layer was washed successively with 5% (w/w) citric acid, 10% (w/w) $NaHCO_3$ and saturated NaCl solution, and then dried over anhydrous Na_2SO_4. After evaporation of the AcOEt layer, the residue was recrystallized from ethyl acetate–petroleum ether to give CBZ-Phe-D-Phe-OMe: yield, 2.13 g (77%); mp 146–147 °C (lit.:[23] mp 132–134 °C); $[\alpha]_D^{26} = -2.7$ (c 1.05, MeOH). The dipeptide ester (1.16 g, 2.5 mmol) was dissolved in methanol and then the resulting solution saturated with NH_3 under cooling on an ice bath by introducing dry ammonia gas. The saturated solution was allowed to stand at room temperature for 2 d. After evaporation of the solvent, the residue was recrystallized from methanol to give CBZ-Phe-D-Phe-NH$_2$: yield, 0.895 g (80%); mp 207–208 °C (lit.:[24] mp 202–204 °C); $[\alpha]_D^{26} = +2.9$ (c 0.70, MeOH). HRMS (FAB$^+$) MH$^+$ calcd for $C_{26}H_{28}N_3O_4$: 446.2080; found: 446.2098.

CBZ-D-Phe-D-Phe-NH$_2$: mp 233–236 °C; $[\alpha]_D^{26} = +30.9$ (c 0.83, DMF). Found: C, 69.99; H, 6.14; N, 9.39. Calcd for $C_{26}H_{27}N_3O_4$: C, 70.09; H, 6.11; N, 9.43. ^{1}H NMR (DMSO-d_6) δ 2.65–3.05 (4H, m, 2 × CH_2–C_6H_5), 4.20–4.26 (1H, m, NH–CH), 4.45–4.50 (1H, m, NH–CH), 4.94 (2H, s, O–CH_2–C_6H_5), 7.11 (1H, s, CONH), 7.19–7.34 (15H, m, 3 × C_6H_5), 7.39 (1H, s, CONH), 7.48 (1H, d, $J = 8.8$ Hz, NH–CH), 8.02 (1H, d, $J = 8.0$ Hz, NH–CH). IR (ν/cm^{-1}) 3377, 3319, 3213, 1695, 1643, 1528. HRMS (FAB$^+$) MH$^+$ calcd for $C_{26}H_{28}N_3O_4$: 446.2080; found: 446.2093.

CBZ-Leu-D-Leu-NH$_2$: mp 177–181 °C; $[\alpha]_D^{26} = +6.4$ (c 0.91, MeOH). Found: C, 63.53; H, 8.17; N, 11.28. Calcd for $C_{20}H_{31}N_3O_4$: C, 63.64; H, 8.28; N, 11.13. ^{1}H NMR (DMSO-d_6) δ 0.80–0.88 [12H, m, 2 × CH(CH_3)$_2$], 1.36–1.60 [6H, m, 2 × CH_2–CH(CH$_3$)$_2$], 4.01–4.07 (1H, m, NH–CH), 4.15–4.21 (1H, m, NH–CH), 5.01 (2H, s, O–CH_2–C_6H_5), 7.02 (1H, s, CONH), 7.23 (1H, s, CONH), 7.30–7.38 (5H, m, C_6H_5), 7.48 (1H, d, $J = 7.6$ Hz, NH–CH), 8.12 (1H, d, $J = 8.3$ Hz, NH–CH). IR (ν/cm^{-1}) 3383, 3329, 3283, 2955, 1674, 1643, 1547. HRMS (FAB$^+$) MH$^+$ calcd for $C_{20}H_{32}N_3O_4$: 378.2393; found: 378.2408.

CBZ-D-Leu-D-Leu-NH$_2$: mp 217–219 °C; $[\alpha]_D^{26} = +39.8$ (c 0.83, MeOH). Found: C, 63.39; H, 8.08; N, 10.99. Calcd for $C_{20}H_{31}N_3O_4$: C, 63.64; H, 8.28; N, 11.13. ^{1}H NMR (DMSO-d_6) δ 0.82–0.88 [12H, m, 2 × CH(CH_3)$_2$], 1.42–1.50 [4H, m, 2 × CH_2–CH(CH$_3$)$_2$], 1.55–1.63 [2H, m, 2 × CH(CH$_3$)$_2$], 4.00–4.06 (1H, m, NH–CH), 4.21–4.27 (1H, m, NH–CH), 5.03 (2H, s, O–CH_2–C_6H_5), 6.97 (1H, s, CONH), 7.28–7.38 (6H, m, C_6H_5+CONH), 7.44 (1H, d, $J = 8.0$ Hz, NH–CH), 7.77 (1H, d, $J = 8.3$ Hz, NH–CH). IR (ν/cm^{-1}) 3391, 3321, 3298, 2957, 1674, 1641, 1535. HRMS (FAB$^+$) MH$^+$ calcd for $C_{20}H_{32}N_3O_4$: 378.2393; found: 378.2384.

CBZ-D-Leu-Leu-NH$_2$ was prepared enzymatically according to the reported method:[12] mp 179–181 °C; $[\alpha]_D^{26} = -7.7$ (c 0.83, MeOH). Found: C, 63.43; H, 8.13; N, 11.09. Calcd for $C_{20}H_{31}N_3O_4$: C, 63.64; H, 8.28; N, 11.13. ^{1}H NMR (DMSO-d_6) δ 0.80–0.88 [12H, m, 2 × CH(CH_3)$_2$], 1.36–1.60 [6H, m, 2 × CH_2–CH(CH$_3$)$_2$], 4.01–4.07 (1H, m, NH–CH), 4.15–4.21 (1H, m, NH–CH), 5.02 (2H, s, O–CH_2–C_6H_5), 7.03 (1H, s, CONH), 7.24 (1H, s, CONH), 7.29–7.38 (5H, m, C_6H_5), 7.48 (1H, d, $J = 7.3$ Hz, NH–CH), 8.12 (1H, d, $J = 8.3$ Hz, NH–CH). IR (ν/cm^{-1}) 3387, 3329, 3285, 2957, 1674, 1643, 1549. HRMS (FAB$^+$) MH$^+$ calcd for $C_{20}H_{32}N_3O_4$: 378.2393; found: 378.2381.

CBZ-Leu-Leu-NH$_2$ was also prepared enzymatically according to the reported method:[12] mp 222–223 °C; $[\alpha]_D^{26} = -40.2$ (c 0.63, MeOH) {lit.:[20] mp 212–213 °C; $[\alpha]_D^{25} = -39.1$ (c 1.0, MeOH)}.

4.5. α-Chymotrypsin-catalyzed peptide synthesis

4.5.1. In aqueous solution (30 °C). CBZ-AA$_1$-OCam (0.05 mmol) and H-AA$_2$-NH$_2$ (0.25 mmol) were dissolved in DMSO (0.2 ml) and in a phosphate buffer (0.1 M, pH 8, 4.8 ml), respectively. The reaction was started by dissolving α-chymotrypsin (10 mg) into the mixture of the above two solutions, and the resulting reaction mixture was reciprocally shaken on a water bath (30 °C, 150 strokes min^{-1}). At appropriate time intervals, an aliquot (0.1 ml) of the reaction mixture was withdrawn and a mixture of 50% TFA (v/v, 0.1 ml) and DMSO (0.4 ml) was added to stop the reaction.

4.5.2. In ice (−24 °C). A solution of H-AA$_2$-NH$_2$ (0.25 mmol) and α-chymotrypsin (10 mg) in a phosphate buffer (0.1 M, pH 8, 4.8 ml) was precooled to 0 °C. This enzyme solution was added to DMSO (0.2 ml) dissolving CBZ-AA$_1$-OCam (0.05 mmol). The resulting solution was rapidly shaken and shock frozen in liquid nitrogen for 20 s. After shock freezing, the frozen reaction mixture was incubated in a freezer (−24 °C). At appropriate time intervals, ca. 0.1–0.2 g of the frozen mixture was taken out and a mixture of 50% TFA (v/v, 0.1 ml) and DMF (0.4 ml) was added to stop the reaction.

4.6. Preparative peptide synthesis in ice

The preparation of CBZ-Phe-Phe-NH$_2$ is described as a typical example. The solution of H-Phe-NH$_2$ (163 mg, 1 mmol) and α-chymotrypsin (20 mg) in a phosphate buffer (0.1 M, pH 8, 9.6 ml) was precooled to 0 °C. This

enzyme solution was added to DMSO (0.4 ml) dissolving CBZ-Phe-OCam (72 mg, 0.2 mmol). The resulting solution was rapidly shaken and shock frozen in liquid nitrogen. After shock freezing, the frozen reaction mixture was incubated in a freezer (−24 °C). After 2 h, the ice was melted at room temperature and the precipitates collected by filtration and washed with water (2 × 50 ml). The precipitates were dried in vacuum, and then recrystallized from methanol–diethyl ether: yield, 74 mg (83%); mp 237–238 °C (lit.:[20] mp 228–228.5 °C); $[\alpha]_D^{26} = -32.4$ (*c* 0.82, DMF). ^{1}H NMR (DMSO-d_6) δ 2.64–3.04 (4H, m, 2 × CH_2–C_6H_5), 4.19–4.25 (1H, m, NH–C*H*), 4.44–4.49 (1H, m, NH–C*H*), 4.93 (2H, s, O–CH_2–C_6H_5), 7.11 (1H, s, CONH), 7.18–7.34 (15H, m, 3 × C_6H_5), 7.40 (1H, s, CONH), 7.49 (1H, d, $J = 8.5$ Hz, N*H*–CH), 8.03 (1H, d, $J = 8.3$ Hz, N*H*–CH). ^{13}C NMR (DMSO-d_6) δ 37.3, 37.7, 53.6, 56.2, 65.2, 126.2, 127.4, 127.6, 128.0, 128.3, 129.1, 129.2, 137.0, 137.7, 138.0, 155.7, 171.1, 172.6. HRMS (FAB$^+$) MH$^+$ calcd for $C_{26}H_{28}N_3O_4$: 446.2080; found: 446.2078.

CBZ-Phe-Ala-NH_2: yield, 68%; mp 212–214 °C; $[\alpha]_D^{26} = -11.6$ (*c* 0.53, MeOH) {lit.:[20] mp 205–205.5 °C; $[\alpha]_D^{25} = -9.8$ (*c* 1.0, MeOH)}. ^{1}H NMR (DMSO-d_6) δ 1.23 (3H, d, $J = 7.1$ Hz, CH_3), 2.70–3.06 (2H, m, CH_2–C_6H_5), 4.19–4.29 (2H, m, 2 × NH–C*H*), 4.94 (2H, s, O–CH_2–C_6H_5), 7.00 (1H, s, CONH), 7.18–7.34 (11H, m, 2 × C_6H_5+CONH), 7.50 (1H, d, $J = 8.5$ Hz, N*H*–CH), 8.03 (1H, d, $J = 7.6$ Hz, N*H*–CH). ^{13}C NMR (DMSO-d_6) δ 18.5, 37.3, 48.0, 56.1, 65.2, 126.2, 127.4, 127.6, 128.0, 128.3, 129.2, 137.0, 138.1, 155.8, 171.0, 174.0. HRMS (FAB$^+$) MH$^+$ calcd for $C_{20}H_{24}N_3O_4$: 370.1767; found: 370.1798.

CBZ-D-Phe-Phe-NH_2: yield, 79%; mp 212–213 °C; $[\alpha]_D^{26} = -3.8$ (*c* 0.80, MeOH). Found: C, 69.81; H, 6.20; N, 9.46. Calcd for $C_{26}H_{27}N_3O_4$: C, 70.09; H, 6.11; N, 9.43. ^{1}H NMR (DMSO-d_6) δ 2.42–3.06 (4H, m, 2 × CH_2–C_6H_5), 4.20–4.25 (1H, m, NH–C*H*), 4.47–4.52 (1H, m, NH–C*H*), 4.91 (2H, s, O–CH_2–C_6H_5), 7.15–7.34 (16H, m, 3 × C_6H_5+CONH), 7.38 (1H, d, $J = 8.5$ Hz, N*H*–CH), 7.46 (1H, s, CONH), 8.35 (1H, d, $J = 8.5$ Hz, N*H*–CH). ^{13}C NMR (DMSO-d_6) δ 37.3, 37.8, 53.6, 56.1, 65.2, 126.1, 126.2, 127.4, 127.7, 127.9, 128.3, 129.2, 136.9, 137.9, 138.0, 155.8, 171.2, 172.9. IR (ν/cm^{-1}) 3427, 3291, 3204, 1692, 1670, 1639, 1543. HRMS (FAB$^+$) MH$^+$ calcd for $C_{26}H_{28}N_3O_4$: 446.2080; found: 446.2084.

4.7. HPLC analyses

A JASCO instrument BIP-I equipped with a JASCO UV-1575 monitor was used for HPLC. The amounts of the acyl donor, peptide and the hydrolysis product of the acyl donor were determined by reversed phase HPLC analysis under the following conditions: column, Inertsil ODS-80A (4.6 ϕ × 250 mm, GL Sciences); eluent, aq CH_3CN containing a small amount of TFA (0.1%, v/v); flow rate, 0.5 ml min^{-1}; detection, UV at 254 nm. A Shimadzu C-R6A data processor was used for the integration of the peak areas. The determination of each component was based on the fact that only benzene ring absorbs at 254 nm in proportion to its number.

The diastereomers [D–L (or L–D) and L–L] were also separated on the same column. The retention times of the relevant diastereomers were as follows. CBZ-Phe-Phe-NH_2 and CBZ-D-Phe-Phe-NH_2 (or CBZ-Phe-D-Phe-NH_2) were eluted after 18.3 and 19.3 min, respectively, when 50% (v/v) aq CH_3CN was used as eluent. CBZ-Leu-Leu-NH_2 and CBZ-D-Leu-Leu-NH_2 (or CBZ-Leu-D-Leu-NH_2) were eluted after 31.1 and 36.1 min, respectively, with an eluent of 40% (v/v) aq CH_3CN.

References

1. Drauz, K.; Waldmann, H. *Enzyme Catalysis in Organic Synthesis*; Wiley-VCH: Weinheim, Germany, 2002.
2. Bordusa, F. *Chem. Rev.* **2002**, *102*, 4817–4867.
3. Miyazawa, T.; Nakajo, S.; Nishikawa, M.; Hamahara, K.; Imagawa, K.; Ensatsu, E.; Yanagihara, R.; Yamada, T. *J. Chem. Soc., Perkin Trans. 1* **2001**, 82–86.
4. Hänsler, M.; Jakubke, H.-D. *J. Pept. Sci.* **1996**, *2*, 279–289.
5. Schuster, M.; Aaviksaar, A.; Jakubke, H.-D. *Tetrahedron* **1990**, *46*, 8093–8102.
6. Haensler, M.; Wissmann, H.-D.; Wehofsky, N. *J. Pept. Sci.* **2000**, *6*, 366–371.
7. Ullmann, D.; Bordusa, F.; Salchert, K.; Jakubke, H.-D. *Tetrahedron: Asymmetry* **1996**, *7*, 2047–2054.
8. Kuhl, P.; Zacharias, U.; Burckhardt, H.; Jakubke, H.-D. *Monatsh. Chem.* **1986**, *117*, 1195–1204.
9. Miyazawa, T.; Tanaka, K.; Ensatsu, E.; Yanagihara, R.; Yamada, T. *J. Chem. Soc., Perkin Trans. 1* **2001**, 87–93.
10. Miyazawa, T.; Ensatsu, E.; Yabuuchi, N.; Yanagihara, R.; Yamada, T. *J. Chem. Soc., Perkin Trans. 1* **2002**, 390–395.
11. Schuster, M.; Aaviksaar, A.; Haga, M.; Ullmann, U.; Jakubke, H.-D. *Biomed. Biochim. Acta* **1991**, *50*, 84–89.
12. Salam, S. M. A.; Kagawa, K.; Kawashiro, K. *Biotechnol. Lett.* **2005**, *27*, 1199–1203.
13. Margolin, A. L.; Tai, D.-F.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 7885–7887.
14. Kawashiro, K.; Sugahara, H.; Sugiyama, S.; Hayashi, H. *Biotechnol. Bioeng.* **1997**, *53*, 26–31.
15. Schwyzer, R.; Iselin, B.; Rittel, W.; Sieber, P. *Helv. Chim. Acta* **1956**, *39*, 872–883.
16. Moriniere, J. L.; Danree, B.; Lemoine, J.; Guy, A. *Synth. Commun.* **1988**, *18*, 441–444.
17. Fulcrand, V.; Jacquier, R.; Lazaro, R.; Viallefont, P. *Int. J. Pept. Protein Res.* **1991**, *38*, 273–277.
18. Capellas, M.; Benaiges, M. D.; Caminal, G.; Gonzalez, G.; Lopez-Santín, J.; Clapés, P. *Biotechnol. Bioeng.* **1996**, *50*, 700–708.
19. Martinez, J.; Laur, J.; Castro, B. *Tetrahedron Lett.* **1983**, *24*, 5219–5222.
20. Miyazawa, T.; Hiramatsu, M.; Murashima, T.; Yamada, T. *Biocatal. Biotransform.* **2003**, *21*, 93–100.
21. Yang, P. S.; Rising, M. M. *J. Am. Chem. Soc.* **1931**, *53*, 3183–3184.
22. Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839–1842.
23. Hill, R. R.; Birch, D.; Jeffs, G. E.; North, M. *Org. Biomol. Chem.* **2003**, *1*, 965–972.
24. Chen, S.-T.; Chen, S.-Y.; Chen, H.-J.; Huang, H.-C.; Wang, K.-T. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 727–733.