Synthesis of Enkephalin Aminopeptidase Inhibitors

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ABSTRACT. In an effort to increase effective action of enkephalins, several peptide inhibitors of enkephalin aminopeptidase have been synthesized. The peptides contain 3-amino-2-hydroxy amino acid as a zinc binding site and side chains of substrate pattern. The peptides were synthesized in solution by chain elongation from C-terminal end using DCC/HOBt as coupling reagent. The peptides are shown to have very strong inhibitory activity against enkephalin aminopeptidase.

INTRODUCTION

The enkephalins, Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, are of great interest because of their opiate-like properties and their roles as neurotransmitters or neuromodulators in the central nervous system. It is well known that their physiological effects are transitory due to their rapid degradation by serum and brain enzymes. The principal inactivating events seem to be cleavage of the N-terminal Tyr-Gly bond by an aminopeptidase, although the possibility of carboxypeptidase activity cannot be excluded.

Most efforts directed at obtaining longer acting enkephalins have centered on the synthesis of enkephalin analogues resistant to enzymatic degradation. Another approach to increase the effective action of enkephalins would be to limit their rate of degradation by blocking enzymatic pathways associated with their catabolism. This approach has proven to be of therapeutic value in treating diseases such as myasthenia gravis, where specific inhibitors of acetyl cholinesterase are administered.

In a continuing search for enkephalin aminopeptidase inhibitors which give useful information on the mechanism of enkephalin aminopeptidase action and have new pharmacological properties, we have synthesized several peptide inhibitors. Recently, several enkephalin degrading aminopeptidases have been purified and characterized. As shown in several other aminopeptidases, the enkephalin aminopeptidase contains zinc and is inhibited by 1,10-phenanthroline. Therefore, the peptide inhibitors which contain a zinc binding site and side chains of substrate pattern were synthesized in solution by chain elongation from...
RESULTS AND DISCUSSION

Synthesis of 3-Amino-2-hydroxy Acids. Asmatatin and bestatin, which are strong inhibitors of several aminopeptidases, both have 3-amino-2-hydroxy acid as a zinc binding site. We, therefore, incorporated this novel amino acid as N-terminal residue in the inhibitor.

Following a modification of the reported procedure, 3-amino-2-hydroxy acid was prepared as outlined in Scheme 1. A protected amino acid methyl ester was reduced to aldehyde as described for the Boc derivative and the aldehyde was converted to the cyanohydrin by following the method of Nishizawa et al. Hydrolysis of the cyanohydrin gave the 3-amino-2-hydroxy acids as a mixture of diastereomers in 85~90% yield from amino acid methyl ester. Diastereomers can be separated readily by column chromatography over silica gel. Saponification of the ester gives amino protected acid in quantitative yields.

Synthesis of Peptides. The pentapeptides 1, 2, 3 were synthesized using optically pure Boc-AH-

\[
\begin{align*}
R_1 ^{-} & \text{NH} - \text{CH} - \text{CO}_2 \text{R}_1 \\
& \text{R}_1 ^{-} \text{NH} - \text{CH} - \text{CH} - \text{SO}_3 \text{Na}
\end{align*}
\]

\[
\begin{align*}
& \text{DIBAL} \quad \text{R}_1 ^{-} \text{NH} - \text{CH} - \text{CHO} \quad \text{NaHSO}_3 \quad \text{OH} \\
& \quad \text{Toluene} \quad \text{R}_1 ^{-} \text{NH} - \text{CH} - \text{CH} - \text{SO}_3 \text{Na}
\end{align*}
\]

\[
\begin{align*}
& \text{KCN} \quad \text{OH} \\
& \text{HC}L \quad \text{R}_1 ^{-} \text{NH} - \text{CH} - \text{CH} - \text{CN}
\end{align*}
\]

\[
\begin{align*}
& \text{OH} \quad \text{Boc-N}3
\end{align*}
\]

\[
\begin{align*}
& \text{B}0\text{C-N}3 \\
& \text{R}_1 = \text{CH}_2 \text{-C}_6\text{H}_s \text{=CH}_2 \text{-C} \text{a-Hs-OH}
\end{align*}
\]


\[
\begin{align*}
& \text{Boc-Gly-OH} \quad \text{HCl} \cdot \text{Phe} \quad \text{TEA} \quad \text{OMe} \quad \text{4N HCl/dioxane} \\
& \text{Boc} \quad \text{Boc} \quad \text{Boc} \quad \text{Boc} \quad \text{Boc} \quad \text{Boc} \quad \text{Boc}
\end{align*}
\]

\[
\begin{align*}
& \text{4N HCl/dioxane} \quad \text{OMe} \quad \text{4N HCl/dioxane} \quad \text{OMe} \quad \text{4N HCl/dioxane} \quad \text{OMe}
\end{align*}
\]

Scheme 2. Synthesis of enkephalin aminopeptidase inhibitor by chain elongation form the C-terminal end.

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Table 1. Inhibition of enkephalin aminopeptidase by inhibitors

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds</th>
<th>$K_r (\times 10^{-4} M)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2S, 3R) AHPBA-Gly-Gly-Phe-Leu</td>
<td>9.77</td>
</tr>
<tr>
<td>2</td>
<td>(2S, 3R) AHPBA-Ala-Gly-Phe-Leu</td>
<td>7.15</td>
</tr>
<tr>
<td>3</td>
<td>(2S, 3R) AHPBA-D-Ala-Gly-Phe-Leu</td>
<td>4.54</td>
</tr>
<tr>
<td>4</td>
<td>(2S, 3R) AHpIBA-Gly-Gly-Phe-NH$_2$</td>
<td>4.30</td>
</tr>
<tr>
<td>5</td>
<td>(2S, 3R) AHpIBA-Gly-Gly-Phe</td>
<td>5.12</td>
</tr>
</tbody>
</table>

PBA-OH by the stepwise procedure in solution outlined in Scheme 2. Because of the danger of racemization in the activation of acyl peptides, chain elongation from the C-terminal end was undertaken. The Boc group was removed with 4 M hydrochloric acid in dioxane, the salt was neutralized with triethylamine before next condensation reaction. The condensation reaction was carried out by using DCC/HOBt in methylene chloride. The peptides were purified by column chromatography over silica gel eluting with gradient of methanol in chloroform.

In case of tetrapeptide 4,5, because the desired peptides contain Gly-Gly residues elongation from N-terminal end was applied without risk of racemization. Protected tetrapeptide 5 was prepared starting from Boc-AHpIBA-OH by stepwise addition of C-protected amino acids in 74% yield. Simultaneous removal of N-Boc and tert-butyl ester groups of the protected peptide with 4 M HCl in dioxan afforded peptide 5.

These synthetic peptides are shown to have relatively strong inhibitory activity against enkephalin aminopeptidase as shown in Table 1. The kinetics of inhibition of enkephalin aminopeptidase by these compounds will be reported separately.

**EXPERIMENTAL**

Melting points were determined on a Fisher-John's melting point apparatus and are corrected. The proton NMR spectra were recorded on a Bruker HX-90B pulse Fourier transform NMR spectrometer. Optical rotations were measured at the sodium D line by a Perkin-Elmer 241 polarimeter. Microanalyses were performed by Galbraith Laboratories in the U.S.A. Silica gel 60(70–230 mesh) was used for column chromatography. Thin-layer chromatography (TLC) was performed on silica gel G plates. TLC solvent systems used were: A. ethyl acetate-benzene (20:80); B. ethyl acetate benzene (10:90); C. methanol-chloroform (10:90); D. butanol-acetic acid-water (4:1:1).

**General Procedure A. Removal of the Boc Group.** Boc amino acid or peptide (1 mmol) in a solution of 4 N HCl in dioxane (3–5 ml) was stirred at room temperature for 30 min. After the removal of excess reagent under reduced pressure, the solid residue was triturated with ether several times to give a solid and dried in vacuo. All peptide hydrochlorides were prepared by this method.

**General Procedure B. Coupling Reactions Using Dicyclohexylcarbodiimide/1-Hydroxybenzotriazole.** The amino hydrochloride (1 mmol) was dissolved in methylene chloride (5 ml) and neutralized at 0°C with triethylamine. Boc amino acid (1 mmol) and HOBt (1.5 mmol) were added, followed by DCC (1.5 mmol). The reaction mixture allowed to stir at 4°C overnight and at room temperature for 2–3 h. DCU was filtered and the filtrate was evaporated under reduced pressure. The residue was dissolved in ethyl acetate, washed successively with water, cold 1 N HCl, saturated NaHCO$_3$, and brine. The ethyl acetate layer was dried (MgSO$_4$), filtered, and concentrated under reduced pressure. The peptide was purified by silica gel column chromatography, eluting with methanol in chloroform ethylacetate in toluene and crystallized from ethyl acetate or ethyl acetate-Skellysolve B, unless otherwise specified.

**General Procedure C. Saponification of N-(tert-Butoxycarbonyl) Amino Acid Esters in Aqueous Dioxane.** A stirred solution of Boc amino acid ester (2 mmol) in aqueous dioxane (4 ml of dioxane/2 ml of H$_2$O) was maintained at pH 10 with 2 N NaOH for 30 min. The reaction mixture was washed once with ether and then acidified to pH 2–3 with cold 1 N HCl. The aqueous layer was extracted with ethyl acetate. The organic layer was dried (MgSO$_4$) and evaporated to give Boc amino acid.

**General Procedure D. Synthesis of 3-Amino-2-hydroxy acid from N-(tert-Butoxycarbonyl) Amino
Acid Methyl Ester. Boc amino acid ester (50 mmol) was dissolved in dry toluene (170 mL). The solution was thoroughly flushed with dry nitrogen before cooling to -78°C. A solution of disobutylaluminium hydride in toluene (67 mL) was slowly added over 15 min under a nitrogen atmosphere with vigorous stirring. After stirring for an additional 15 min, the reaction was quenched with methanol (2.5 mL) and Rochelle salts solution (300 mL) was added immediately. The mixture was allowed to warm to room temperature and then extracted with ether (3 x 150 mL). The organic layer was washed with brine, dried (MgSO₄), filtered, and concentrated in vacuo to yield Boc amino aldehyde. To amino aldehyde (10 mmol) was added ice-cold solution of NaHSO₃ (8 mmol in 50 mL) and the mixture was stirred overnight at 5°C. To the resulting suspension of NaHSO₃ adduct was added ethyl acetate (150 mL) and a solution of KCN (10 mmol in 90 mL). The reaction mixture was stirred for 4 h at room temperature. The ethyl acetate layer was washed with water and brine, dried (MgSO₄) and concentrated in vacuo to give the cyanohydrin which was dissolved in dioxi- nane-concentrated HCl (1:1 (100 mL)) and hydrolyzed by reflux for 12 h. The hydrolyzate was washed with ether and concentrated under reduced pressure. The residue was dissolved in a small amount of water. Acetone (200 mL) was added and the mixture was adjusted to pH 5.5 with 2 N NaOH. Crystals which deposited after standing at 5°C overnight were filtered and washed with acetone. Alternately, the pure hydroxy amino acid could be isolated by Dowex 50W(H⁺) ion-exchange chromatography eluting with 2N H⁺OH.

(2S,3R)-Boc-3-amino-2-hydroxy-4-phenylbutanoic acid methyl ester. The title compound was prepared in 70% yield from Boc-D-Phe-OMe using general procedure D. The diastereomer of the title compound was separated by column chromatography over silica gel, eluting with gradient of 20-30% ethyl acetate in toluene. mp. 87~89°C; TLC Rf(B) 0.55; [α]D⁰ + 6.05 (c 1.0, MeOH); NMR (CDCl₃) δ 1.27 (s, 9H, (CH₂)₃C), 2.51~2.88 (AB, J₆₂=13.3 Hz, J₂₆=6.8 Hz, J₆₄=8.9 Hz, 2H), 3.55 (s, OCH₃), 3.83~4.12 (m, 2H), 5.41 (bs, OH), 6.53 (d, J=10 Hz, NH), 7.13~7.34 (m, 5H). Anal. Calc'd for C₁₀H₁₃NO₂: C, 62.12; H, 7.49; N, 4.53. Found: C, 62.12; H, 7.52; N, 4.50.

(2S,3R)-Boc-3-amino-2-hydroxy-4(4'-hydroxyphenyl)-butanoic acid methyl ester. The title compound was prepared from N-Boc-O-benzyl-D-tyrosine methyl ester in 60% yield by the procedure described for the synthesis of N-Boc-APBA-OMe. mp. 136~138°C; TLC Rf(B) 0.43; [α]D⁰ + 85.76 (c 1.0, MeOH); NMR (CDCl₃) δ 1.32 (s, 9H, (CH₂)₃C), 2.44~2.75 (AB, J₆₂=13.1 Hz, J₂₆=6.8 Hz, J₆₂=7.6 Hz, 2H), 3.55 (s, 3H, OCH₃), 3.82~4.11 (m, 2H), 5.33 (d, J=8 Hz, OH), 6.46 (d, J=9 Hz, NH), 6.82~7.06 (AB'BB', 4H). Anal. Calc'd for C₇H₁₄NO₄: C, 59.02; H, 7.13; N, 4.30. Found: C, 58.82; H, 6.97; N, 4.19.

N-Boc-glycyl-L-phenylalanyl-L-leucine methyl ester. Leucine methyl ester (1.27 g, 7 mmol) and Boc-phenylalanine (1.86 g, 7 mmol) was coupled to give the protected dipeptide N-Boc-Phe-Leu-OMe using general procedure B. The protected dipeptide was purified by column chromatography over silica gel, eluting with a gradient of 0.5~1% methanol in chloroform (55% yield). After removal of the Boc group from N-Boc-Phe-Leu-OMe by general procedure A. The salt (1.1 g, 3.5 mmol) was coupled with Boc-glycine (0.7 g, 4 mmol) as described above to give protected tripeptide Boc-Gly-Phe-Leu-OMe: 62% yield; mp 115~118°C; TLC Rf(C) 0.66; NMR (CDCl₃) δ 0.83 (d, 6 H), 1.40 (s, 6H), 1.50~1.75 (m, 3H), 3.07 (d, 7H), 3.67 (s, 3H), 3.8 (s, 2H), 4.25~4.95 (m, 2H), 5.05~5.50 (m, 1H), 6.50~7.10 (m, 2H), 7.25 (s, 5H).

N-Boc-glycyl-L-phenylalanyl-L-leucine methyl ester. Boc group of Boc-Gly-Phe-Leu-OMe was removed by general procedure A and the tripeptide hydrochloride salt (0.19 g, 0.5 mmol) was coupled with Boc-glycine (0.09 g, 0.5 mmol) using general procedure B to give protected tetrapeptide Boc-Gly-Gly-Phe-Leu-OMe: 68.8% yield; mp 115~117°C; TLC Rf(C) 0.49; NMR (CDCl₃) δ 0.90 (bs, 6 H), 1.43 (s, 9 H), 1.50~1.85 (m, 3H), 3.07 (d, 7H), 3.70 (s, 3H), 3.80~4.15 (bm, 4H), 4.40~4.75 (6m, 1H), 4.80~5.15 (bm, 1H), 5.60~5.95 (bm, 1H), 7.20 (s, 5H), 7.40~7.85 (m, 3H).

N-Boc-L-alanyl-glycyl-L-phenylalanyl-L-leucine methyl ester. Boc-L-alanine (0.01 g, 0.5 mmol) was coupled with HCl-Gly-Phe-Leu-OMe (0.19 g, 0.5...
mmol) as described above to give protected tetrapeptide Boc-Ala-Gly-Leu-OMe: 61.5% yield; mp. 164~166°C; TLC Rf(C) 0.54: NMR(CDC13) δ 0.90(s, 6H), 1.22(d, 3H), 1.44(s, 9H), 1.48~1.85(m, 3H), 3.07(d, 2H), 3.68(s, 3H), 3.80~4.34 (m, 3H), 4.42~4.70(m, 1H), 4.80~5.14(m, 1H), 5.60~5.90(m, 1H), 7.20(s, 6H), 7.40~7.85(bm, 4 H).

N-Boc-D-alanyl-glycyl-L-phenylalanyl-L-leucine methyl ester. Boc-D-alanine (0.10 g, 0.5 mmol) was coupled with HCl·Gly-Phe-Leu-OMe(0.19 g, 0.5 mmol) as described above to give protected tetrapeptide Boc-D-Ala-Gly-Leu-OMe: 58% yield; mp. 184~187°C; TLC Rf(C) 0.55; NMR(CDC13) δ 0.90(s, 6H), 1.22(d, 3H), 1.44(s, 9H), 1.48~1.85 (m, 3H), 3.07(d, 2H), 3.70(s, 3H), 3.80~4.34(m, 3H), 4.42~4.70(m, 1H), 4.8~5.11(m, 1H), 5.60~5.92(m, 1H), 7.22(s, 5H), 7.40~7.83(bm, 4H).

(2S, 3R)-N-Boc-3-amino-2-hydroxy-4-(4'-hydroxyphenyl)-butanoyl-glycyl-glycyl-L-phenylalanine ethyl ester. N-Boc-glycine(2.103 g, 12 mmol) was coupled to Gly-OEt hydrochloride (1.396 g, 10 mmol) using general procedure B except that a very small amount of HOEt(2 mmol) was used(92% yield). After purification of dipetide by column chromatography over silica gel, Boc group was removed from Boc-Gly-OEt using general procedure A and the salt(1.076 g, 0.89 mmol) was neutralized with triethylamine and coupled to(2S, 3R)-Boc-AHPhBA-CH2(0.279 g, 0.89 mmol) by general procedure B to give the title compound: 72.4% yield; TLC Rf(5.44~5.89(m, 1H), 6.56~7.56(AA'BB' + m. 11 H), 7.78(bs, 1H), Anal. Calcd for C26H34N3O3: C, 61.13; H, 7.50; N, 8.91. Found : C, 60.79 ; H, 7.24; N, 8.71.

(2S, 3R)-3-Amino-2-hydroxy-4-(4'-hydroxyphenyl)-butanoyl-glycyl-glycyl-L-phenylalanine hydrochloride. Removal of N-Boc-and tert-butyl-ester groups of the protected tetrapeptide N-Boc-AHPhBA-Gly-Gly-Phe-OMe was carried out using general procedure B. The product was purified by preparative TLC(n-butanol-acetic acid-water=4:1:1) 12.3% yield; TLC Rf(D) 0.29; NMR (DMSO-d6) δ 2.67~3.05(m, 4H) 3.37(s, 4H) 3.66~3.83(m, 5H) 3.93(bs, 1H) 4.22~4.62(m, 1H) 6.65~6.90(AA' BB', 4H) 6.88(s, 1H) 7.22(s, 5H) 7.83~8.45(m, 3H) 9.40(s, 1H).

(2S, 3R)-3-Amino-2-hydroxy-4-(4'-hydroxyphenyl)-butanoyl-glycyl-glycyl-L-phenylalanine hydrochloride. Removal of N-Boc-and tert-butyl-ester groups of the protected tetrapeptide N-Boc-AHPhBA-Gly-Gly-Phe-OMe was carried out using general procedure B. The product was purified by preparative TLC(n-butanol-acetic acid-water=4:1:1) 12.3% yield; TLC Rf(D) 0.29; NMR (DMSO-d6) δ 2.67~3.05(m, 4H) 3.37(s, 4H) 3.66~3.83(m, 5H) 3.93(bs, 1H) 4.22~4.62(m, 1H) 6.65~6.90(AA' BB', 4H) 6.88(s, 1H) 7.22(s, 5H) 7.83~8.45(m, 3H) 9.40(s, 1H).

(2S, 3R)-3-Amino-2-hydroxy-4-phenylbutanoyl-glucyl-glycyl-L-phenylalanine hydrochloride. Boc group of Boc-Gly-Gly-Phe-Leu-OMe was removed by general procedure A and the tetrapeptide hydrochloride salt(0.069 g, 0.2 mmol) was coupled with Boc-AHPhBA-Gly-Gly-Phe-Leu-OH using general procedure B to give protected pentapeptide Boc-AHPhBA-Gly-Gly-Phe-Leu-OMe. The protected peptide was purified by column chromatography over silica gel, eluting with a gradient 0%~1% methanol in chloroform: 61.0% yield; mp 90~92°C; TLC Rf(C) 0.34; NMR(CDC13) δ 0.90(s, 6H) 1.38(s, 9H) 1.50~1.85(m, 3H) 2.51~2.88(AB, 2 H) 3.08(s, 2H) 3.70(s, 3H) 3.80~4.12(bm, 6H) 4.40~4.75(m, 1H) 4.80~5.15(m, 1H) 5.41(bs, 1 H) 5.60~5.95(m, 1H) 6.53(bd, 1H) 7.20(s, 1

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By use of general procedure A and C, the protected pentapeptide was converted to the title compound (90% yield); TLC Rf(D) 0.40. Anal. Calcd for C_{22}H_{24}N_{4}O_{4}: C, 52.43; H, 7.60; N, 13.89. Found: C, 52.04; H, 7.56; N, 13.97.

(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanoyl-L-alanyl-glycy-L-phenylalanyl-L-leucine hydrochloride. Boc group of Boc-Ala-Gly-Phe-Leu-OMe was removed by general procedure A and the tetrapeptide hydrochloride salt (0.11g, 0.2 mmol) was coupled with Boc-AHPBA (0.09g, 0.2 mmol) using general procedure B to give protected pentapeptide Boc-AHPBA-Aly-Gly-Phe-Leu-OMe. The product was purified by column chromatography over silica gel, eluting with a gradient 0.5~1% methanol in chloroform: 66% yield: mp. 101~104°C; TLC Rf(C) 0.35; NMR(CDCl_3) δ 0.90(s, 6H); 1.22 (d, 3H); 1.38(s, 9H); 1.50~1.85(m, 3H); 2.51~2.88(m, 2H); 3.05(d, 2H); 3.70(s, 3H); 3.83~4.12 (m, 5H); 4.42~4.70(bm, 1H); 4.80~5.14(m, 1H); 5.41(bs, 1H); 6.54(bd, 1H); 6.71~6.90(m, 1H); 7.20(s, 1OHz); 7.40~7.85(bm, 4H). Anal. Calcd for C_{22}H_{24}N_{4}O_{4}: C, 60.04; H, 6.45; N, 13.62. Found: C, 59.88; H, 6.45; N, 13.42.

By use of general procedure A and C, the protected pentapeptide was converted to the title compound (85% yield); TLC Rf(D) 0.44. Anal. Calcd for C_{22}H_{24}N_{4}O_{4}: C, 61.15; H, 6.90; N, 12.29. Found: C, 59.83; H, 6.45; N, 13.69.

(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanoyl-D-alanyl-glycyl-L-phenylalanyl-L-leucine hydrochloride. Boc group of Boc-D-Ala-Gly-Phe-Leu-OMe was removed by general procedure A and the tetrapeptide hydrochloride salt (0.11g, 0.2 mmol) was coupled with Boc-AHPBA (0.09g, 0.2 mmol) using general B to give protected pentapeptide Boc-AHPBA-D-Ala-Gly-Phe-Leu-OMe. The product was purified by column chromatography over silica gel, eluting with a gradient 0.5~5% methanol in chloroform (66% yield): mp. 112~115°C; TLC Rf(C) 0.35; NMR(CDCl_3) δ 0.90(s, 6H); 1.23(d, 3H); 1.35(s, 9H); 1.50~1.85(m, 3H); 2.50~2.88(m, 2H); 3.05 (d, 2H); 3.70(s, 3H); 3.83~4.12(m, 5H); 4.42~4.70(bm, 1H); 4.80~5.14(m, 1H); 5.40(bs, 1H); 6.54(bd, 1H); 6.70~6.90(m, 1H); 7.20(s, 1OHz); 7.40~7.85(bm, 4H). Anal. Calcd for C_{22}H_{24}N_{4}O_{4}: C, 61.15; H, 6.90; N, 12.29. Found: C, 60.04; H, 6.72; N, 12.09.

ACKNOWLEDGEMENT

We are grateful to the Korea Science and Engineering Foundation for support of this work.

REFERENCES

18. The abbreviations used are as follows: AHPBA, 3-amino-2-hydroxy-4-phenylbutanoic acid; AHpHBA, 3-amino-2-hydroxy-4(4'-hydroxyphenyl)butanoic acid; Boc, tertiarybutyloxycarbonyl; DCC, N,N'-dicyclohexylcarbodiimide; HOBt 1-hydroxybenzotriazole; TEA, triethylamine; K, inhibitory binding constant.