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STRUCTURE-ACTIVITY RELATIONSHIP OF LEPIDOPTERAN, A SELF-DEFENCE PEPTIDE OF BOMBYX MORI[1)]

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Abstract - Silkworm, Bombyx mori, produces self-denfense substance lepidopteran in a haemolymph when it is vaccinated with killed Escherichia coli. Lepidopteran family contains at least three congeners A, B and C, all of which were isolated and determined in amino acid sequences. Furthermore, total synthesis of lepidopteran A was achieved. For elucidation of structure-activity relationship of lepidopteran, twenty one different kinds of peptide fragments were then synthesized. From results of antibacterial tests for those peptides, it is concluded that N-terminal region particularly the Arg^1-Trp^2 sequence is very important for exhibition of the biological activity. The minimal sequence of lepidopteran providing significant bioactivity is the peptide fragment (1-18). α-Helix content in the peptide chain does not necessarily guarantee a manifestation of the activity. An important factor to strengthen the activity seems to be a hydrophobicity in the molecule in addition to the N-terminal region. The assumption was confirmed by preparation of long aliphatic amide of the fragment (1-18) which showed almost comparable activity to that of the original lepidopteran.

Introduction

Self-defense mechanisms of vertebrate animals based on immunoreactions either by humoral or cellular immunity have been extensively studied. On the contrary, there is relatively little known about an immune response mechanism of invertebrate animals lacking lymphocyte and immunoglobulin. In the case of insect, an alien substance is excluded mainly through phagocytosis by granular cell or plasmacyte. Another mechanism will be operated by production of antibacterial substance into body fluid of insect. It has been known since about thirty years ago that hemolymph of various insects exhibit antibacterial and antiviral activities. In a digest juice of silkworm larvae, an antivirus protein were found.[2,3)] Flesh fly induces antibacterial peptide called sarcotoxin in its hemolymph.[4)]

In 1979, Kikuchi observed an antibacterial activity induced in hemolymph of silkworm, Bombyx mori, according to self-defence mechanism when it was injected with killed vaccine of Escherichia coli.[5] In collaboration with his group, we isolated and purified the active substance called lepidopteran. Lepidopteran is comprised of mainly three peptide congeners A, B, and C, all of which involve 35 amino acid residues. The structures determined by sequential analysis is shown in Fig. 1.[6,7,8] Total synthesis of lepidopteran A as a major congener was performed and provided direct evidence for authenticity of the proposed structure.[9,10]

In parallel with our work, Boman and his collaborators isolated also antibacterial peptide called cecropin from cecropia moth as well as chinese oak silkworm and proposed structures of cecropin A, B, and D, all of which are comprised of 37 amino acid residues.[11,12] Amino acid sequences in cecropin show considerable similarity with those of lepidopteran although some of the latter contain δ-hydroxylysine (Hyl) as unique amino acid. Subsequently, the structure of cecropin was revised to that of 35 amino acid residues according to the result obtained from a cDNA clone.[13] Now, its length becomes to coincide with that of lepidopteran. Recently, Boman and co-workers have isolated attacin, another antibacterial protein from the cecropia moth too. However, attacin is quite different from cecropin or lepidopteran in its structure.[14,15]

The above studies prompted us more investigation on relationship between structure and biological activity of lepidopteran by means of preparation of many structural analogs and fragments of the natural peptide. In comparison of entire structures of three lepidopterans, a difference between A and B is found only at position-21, whereas A and C have 11 sites of amino acid differences which are located mostly in the C-terminal part. In view of extreme similarity in antibac-

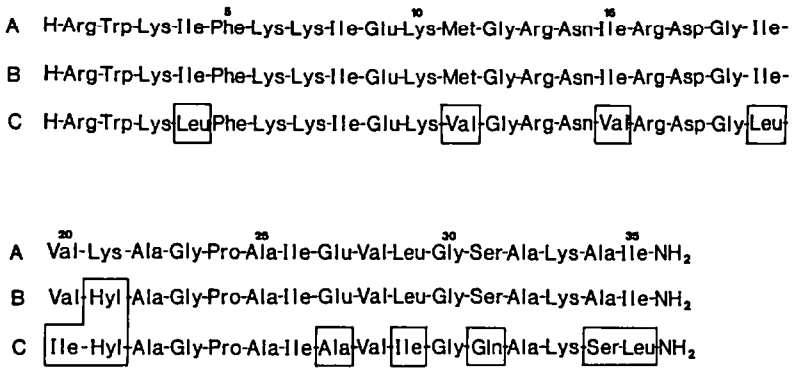

Fig. 1. Structure of Lepidopteran A, B, and C. Hyl refers to δ-hydroxylysine

Table 1. Antibacterial Activity of Lepidopteran A, B, and C (MIC†, μg/ml)

Test organism	Lepidopteran A	B	C
Proteus mirabilis 129	16	16	16
Proteus mirabilis 1287	16	16	16
Escherichia coli NIHJ	4	4	4
Escherichia coli NIHJ JC-2	2	2	2
Salmonella enteritidis IFO 3313	4	8	8
Shigella sonnei EW-33	4	4	4
Pseudomonas aeruginosa	16	8	8
Pseudomonas aeruginosa 99	8	8	8
Enterobacter aerogenes IFO 13534	8	8	8
Staphylococcus aureus FDA 209P	250	250	250
Bacillus subtilis PCI 219	16	16	16
Streptococcus hemolyticus	250	250	125

terial activities of three congeners as shown in Table 1, it may be suggested that the most important site for the activity is located at the N-terminus.

Synthesis and Biological Activity of Peptide Fragments Related to Lepidopteran

All peptide fragments prepared so far in our laboratory are summarized in Fig. 2. Six peptides, (1-6), (7-12), (13-18), (19-23), (24-30), and (31-35) cor-

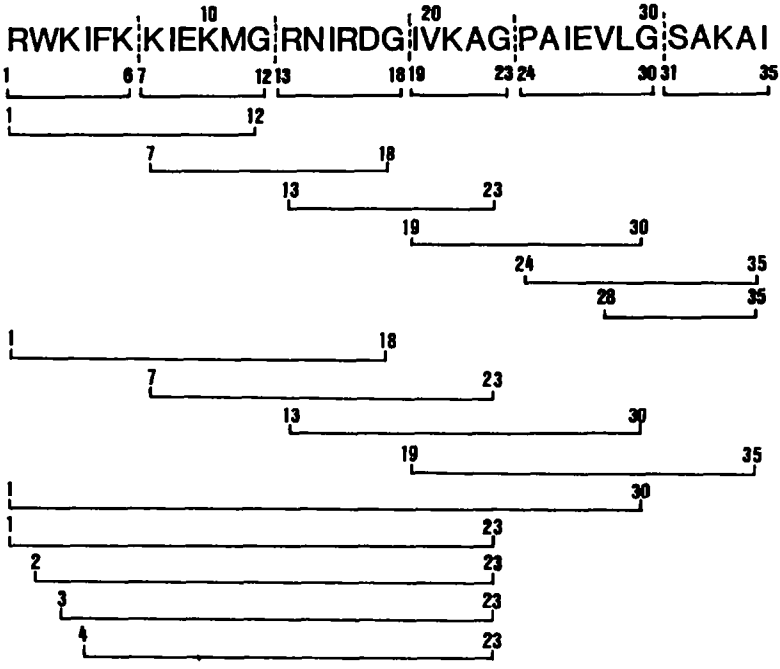

Fig. 2. Peptide Fragments Related to Lepidopteran

responding to building blocks for total synthesis of lepidopteran A[9,10] were prepared through deprotection of the respective protected derivatives by HF-anisole. Since those six smaller fragments show complete inactivity in biological test as shown in the footnote of Table 2, we then synthesized six longer peptides, (1-12), (7-18), (13-23), (19-30), (24-35), and (28-35) by coupling of two fragments mentioned above followed by deprotection. For coupling of each fragment, the water-soluble carbodiimide-hydroxybenzotriazole method was employed. For deprotections, phenacyl ester was removed by zinc-acetic acid, and all other protecting groups, except for the formyl group, were cleaved by HF-anisole. The formyl group was deprotected by dimethylsulfoxide-hydroxylamine-ammonia. Of the relative antibacterial activities tested for in the above six peptides, only minor activity was recognized on the peptide (1-12). The other five fragments were completely inactive as indicated in the footnote of Table 2.

Therefore, extensions of peptide length were attempted to synthesize four fragments, (1-18), (7-23), (13-30), and (19-35) to test for biological activity. Although the chemistry procedures were similar to those for preparation of the smaller fragments mentioned before, the synthetic scheme for these protected peptides is shown in Fig. 3. Based on the biological testing, weak but significant antibacterial activity was recognized in the peptide (1-18), while the other three peptides (7-23), (13-30) and (19-35) were quite inactive (Table 2). This result again suggested us that an active region should be present in the N-terminal

Table 2. Antibacterial Activity of Peptide Fragments Related to Lepidopteran (MIC, μg/ml)

Test organism	1-6	1-12	1-18	1-23	1-30	Lepidopteran A
Proteus mirabilis 129	-	250	-	-	250	16
Proteus mirabilis 1287	-	-	-	-	-	32
Escherichia coli NIHJ	-	250	125	8	4	4
Escherichia coli NIHJ JC-2	-	250	125	8	4	2
Salmonella enteritidis IFO 3313	-	250	250	16	8	4
Shigella sonnei EW-33	-	250	125	8	4	4
Pseudomonas aeruginosa	-	-	250	62	16	8
Pseudomonas aeruginosa 99	-	-	-	62	32	8
Enterobacter aerogenes IFO 13534	-	250	250	250	16	8
Staphylococcus aureus FDA 209P	-	-	-	-	250	250
Bacillus subtilis PCI 219	-	-	-	125	62	16
Streptococcus hemolyticus	-	250	-	-	-	-

- :>250

7-12, 13-18, 19-23, 24-30, 31-35
7-18, 13-23, 19-30, 24-35, 28-35
7-23, 13-30, 19-35

} >250 against all test organisms

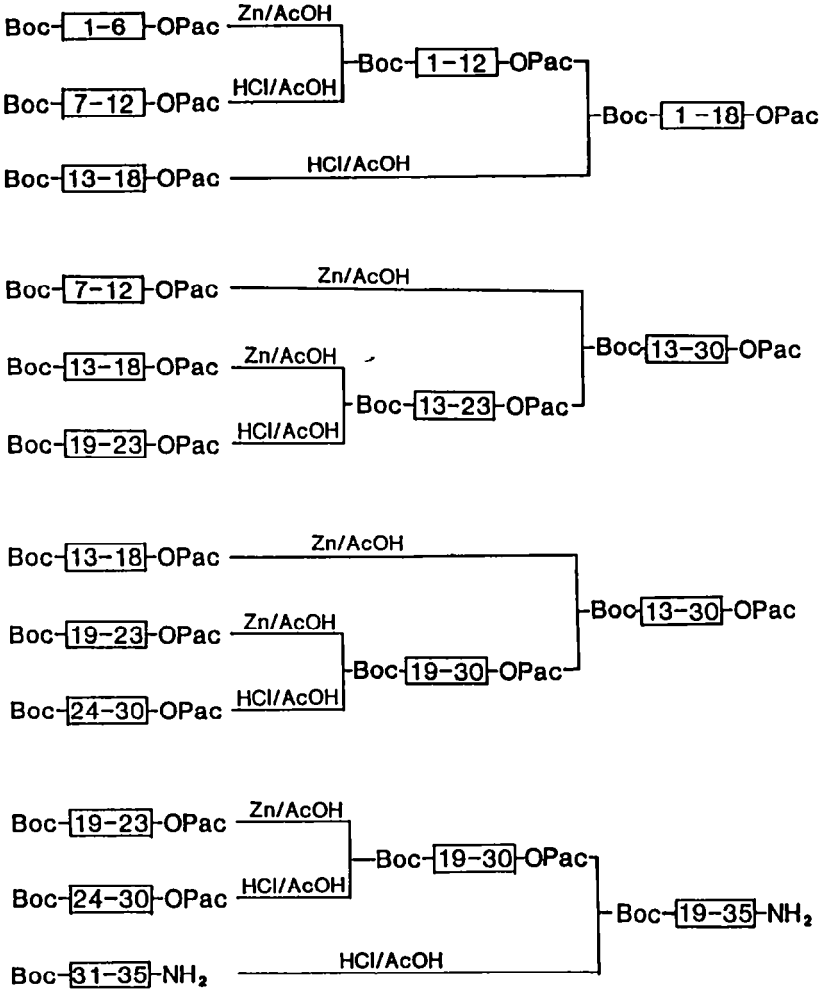

Fig. 3. Synthetic Scheme of Protected Peptide Fragments†

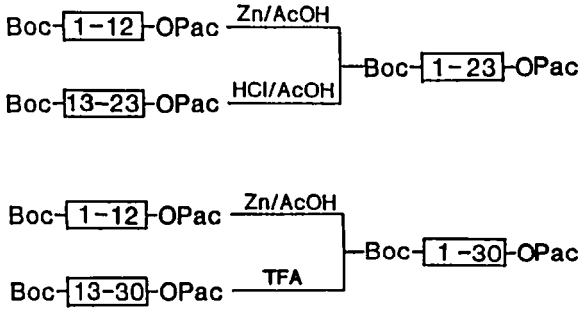

Fig. 4. Synthetic Scheme of Protected Fragments (1-23) and (1-30)

side rather than C-terminal part. However, it is obvious that a large difference in the intensity of activity exists between fragment (1-18) and the entire peptide (1-35).

Therefore, longer peptides extending to C-terminal side from the peptide (1-18) were expected to possess stronger activities. Thus, we prepared two longer peptides, (1-23) and (1-30), according to the synthetic scheme shown in Fig. 4. Both peptides now showed remarkable activities and approached in intensity to the natural peptide (Table 2). From these results obtained from our studies on structure-activity relationships, we were able to conclude that minimal structure exhibiting an activity comparable to lepidopteran seems to be the fragment (1-23). This supports a tentative suggestion for an importance of the N-terminal region and, perhaps, an auxiliary role of C-terminal region to strengthen the potency by addition of hydrophobicity to the molecule.

In order to determine more detailed information about a role of N-terminal part in the peptide (1-23), we then tried to synthesize three peptides lacking each N-terminal amino acid, (2-23),(3-23), and (4-23) as shown in Fig. 5. Dramatic loss of activity was recognized even by the peptide (2-23) which lacked only N-terminal Arg^1 residue. Removal of two or three amino acids from N-terminal region resulted in complete inactivation as shown in Table 3. Therefore the residues Trp^2 and Lys^3 may be indispensable for manifestation of activity.

Another discussion about length of active peptide relating to lepidopteran activity arises from the results obtained above. The peptides (3-23) and (4-23) were devoid of activity in spite of being longer peptides than peptide (1-18) which effected measurable activity. This indicates that peptide length did not necessarily guarantee the activity, but strongly suggests the extreme importance of N-terminal Arg^1-Trp^2 sequence.

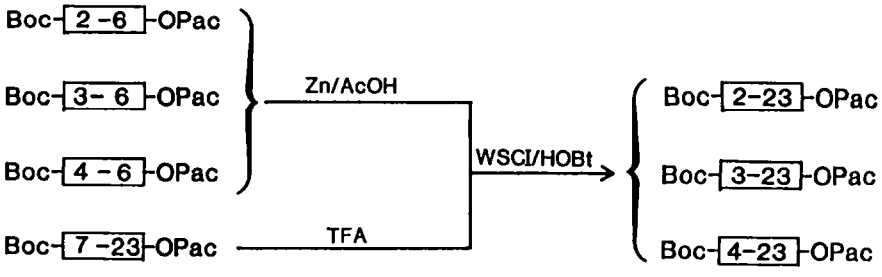

Fig. 5. Synthetic Scheme of Protected (2-23), (3-23), and (4-23)

Table 3. Antibacterial Activity of Peptides (1-23), (2-23), (3-23), and (4-23) (MIC, μg/ml)

Test organisms	1-23	2-23	3-23	4-23
Proteus mirabilis 129	- (-)	-	-	-
Proteus mirabilis 1287	- (-)	-	-	-
Escherichia coli NIHJ	8 (8)	64	250	-
Escherichia coli NIHJ JC-2	16 (8)	64	-	-
Salmonella enteritidis IFO 3313	64 (16)	250	-	-
Shigella sonnei EW-33	N.T (8)	N.T	N.T	N.T
Pseudomonas aeruginosa	125 (62)	-	-	-
Pseudomonas aeruginosa 99	125 (62)	-	-	-
Enterobacter aerogenes IFO 13534	250 (250)	-	-	-
Staphylococcus aureus FDA 209P	- (-)	-	-	-
Bacillus subtilis PCI 219	125 (125)	-	-	-
Streptococcus hemolyticus	- (-)	-	-	-

- :>250, N.T : not tested, ( ) : previous data

For elucidation of a relationship between a secondary structure and the biological activity of the peptide, we then measured their circular dichroism spectra. It has been assumed that the antibacterial activity may be exhibited by some inhibition of the membrane action through attachment of the peptide over cell surface. Therefore, a hydrophobic solvent system for CD measurement, such as

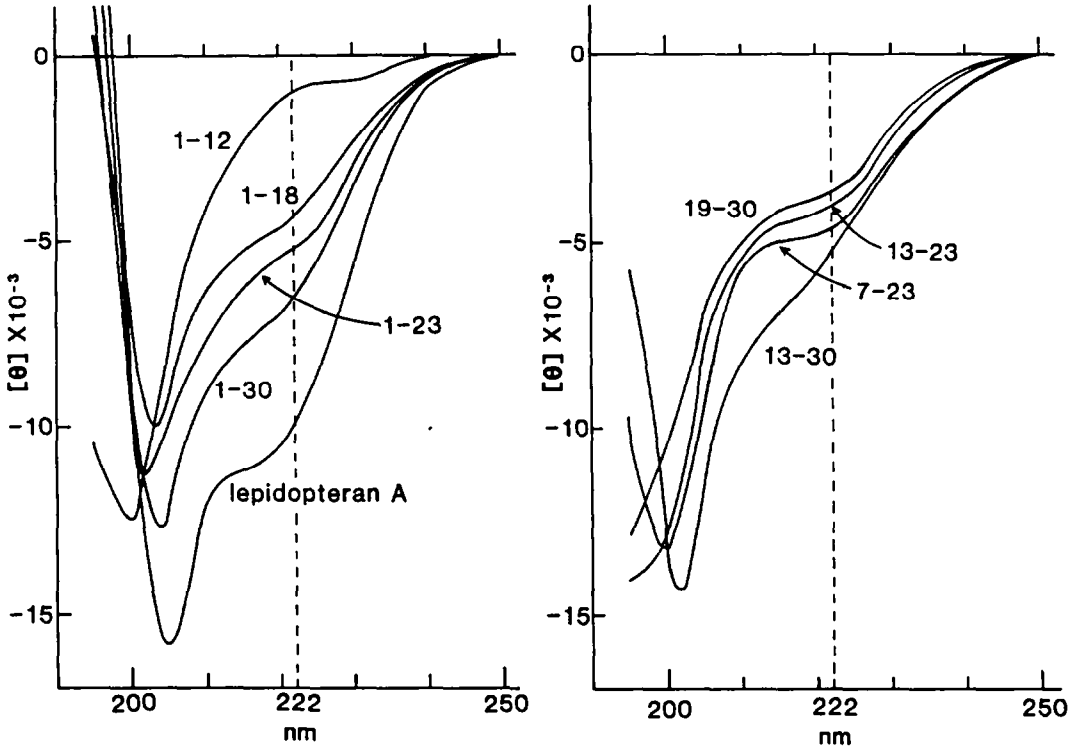

Fig. 6. CD Spectra of Peptide Fragments related to Lepidopteran in 30% CF_3CH_2OH

aqueous trifluoroethanol, was employed as a model for environment of cell surface.

A summary of the CD spectra for the natural lepidopteran A as well as several other peptide fragments are shown in Fig. 6. The values in ordinates represent a mean residue ellipticity which was obtained by dividing a molecular ellipticity by the number of amino acid residues.[16] Those values at 222 nm, in which a contribution either from β-structure or random coil structure may be neglected, correspond to α-helix content. The CD spectra for all substances showing significant biological activities are depicted in a chart on left hand side in Fig. 6, whereas those for inactive compounds are summarized in a right hand side chart. As seen in the left-hand side of Fig. 6, the values $[\theta]_{222}$ increased in order of the peptides (1-12),(1-18),(1-23), and (1-30). This result may indicate a possibility that the strength of antibacterial activity would require α-helix structure. However, when values $[\theta]_{222}$ for active and inactive substances are compared, significant differences between them can not be definitively recognized. Therefore, in conclusion, a presence of α-helix structure in the peptide molecule is not enough to provide for exhibition of the antibacterial activity.

Synthesis and Biological Activity of Alkylamide Derivatives of Peptide Fragment of Lepidopteran

The problem of what structural features are major factors controlling the antibacterial activity of lepidopteran still exists. We know that the N-terminal part can not be removed without loss of the activity, and at least the (1-18) sequence is required for manifestation of the activity. In addition, the α-helix does not always assure the activity. What can we conclude about the structure-activity relationship of lepidopteran?

When we noticed the amphiphilic character of lepidopteran, its strong activity might be realized in alkyl amide derivatives of smaller N-terminal peptide fragments connected to lipophilic alkylamine group at their C-termini. This modification would provide hydrophobicity to the molecule in replacement of the C-terminal part in the original molecule.

According to this assumption, several kinds of long aliphatic amide derivatives of the peptide (1-18) were prepared as shown in Fig. 7. Results of the antibacterial tests of the aliphatic amide derivatives against various microorganism are summarized in Table 4. Among five amide derivatives, the octadecylamide of the peptide (1-18) showed no activity, the hexadecyl- and ethylamide showed some

activity, and the tetradecyl- and dodecylamides showed remarkable enhancement of activities in comparison to that of the original peptide (1-18). It is particularly noteworthy to know that the activity of the dodecylamide is almost comparable to the natural lepidopteran, and even extraordinarily higher against some of the bacteria, such as Staphylococcus aureus and Bacillus subtilis. Again, the compounds lack the C-terminal heptadecapeptide sequence. An interaction of alkyl chain of the dodecylamide with the cellular membrane on the microorganism seems to play a characteristic and important role for enhancement of the biological activity.

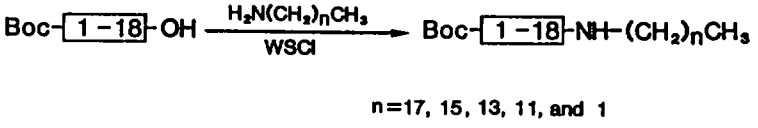

Fig. 7. Synthetic Scheme of Protected Alkyl Amide Derivatives

Conclusively, the present study on structure-activity relationship of lepidopteran may provide a way to exploit novel compounds which may be expected to yield promising antibacterial drugs. The balance of active peptide sequence and an appropriate length of the hydrophobic carbon chain remains an interesting and challenging target for future investigation in this field.

Table 4. Antibacterial Activity of Alkyl Amide Derivatives (MIC μg/ml)

Test organism	H-(1-18)-NH-R					Lepidopteran A
R:	C_{18}	C_{16}	C_{14}	C_{12}	C_2	
Proteus mirabilis 129	-	-	-	-	-	32
Proteus mirabilis 1287	-	-	-	-	-	32
Escherichia coli NIHJ	-	125	32	16	16	4
Escherichia coli NIHJ JC-2	-	64	32	8	16	4
Salmonella enteritidis IFO 3313	-	125	32	8	8	4
Pseudomonas aeruginosa	-	250	64	16	250	8
Pseudomonas aeruginosa 99	-	64	32	8	64	8
Enterobacter aerogenes IFO 13534	-	-	125	16	125	8
Staphylococcus aureus FDA 209P	-	64	32	8	16	125
Bacillus subtilis PCI 219	-	32	8	2	32	32
Streptococcus hemolyticus	-	-	-	-	-	-

-: >250, C_{18}:$(CH_2)_{17}CH_3$, C_{16}:$(CH_2)_{15}CH_3$, C_{14}:$(CH_2)_{13}CH_3$, C_{12}:$(CH_2)_{11}CH_3$, C_2: CH_2CH_3

EXPERIMENTAL

All melting points are uncorrected. Amino acid analysis was carried out by Hitachi KLA-5 amino acid analyzer, and optical rotation was obtained by a Perkin-Elmer 141 polarimeter. Fast atom bombardment mass spectrum (FAB-MS) was obtained with the Matsuda type mass spectrometer of Osaka University, and CD spectrum was measured with JASCO J-500A CD spectrometer.

General principle for preparation of protected fragment peptides

A solution method applying a maximum protection procedure was adopted for peptide synthesis. Protecting groups of functional groups in side chain of amino acid residues were as follows: p-toluenesulfonyl for Arg, formyl for Trp, 2-chlorobenzyloxycarbonyl for Lys, benzyl for Glu and Ser, and cyclohexyl for Asp. Protected fragment peptides (1-6), (7-12), (13-18), (19-23), (24-30), and (31-35) used to the following fragment condensation, were respectively synthesized by stepwise elongation from C-terminal amino acids.

Coupling of Boc amino acid succinimido active ester (Preparation of Boc-Ile-Phe-Lys(ClZ)-OPac)† To a solution of HCl•H-Phe-Lys(ClZ)-OPac (10.5 g, 17 mmol) and triethylamine (2.39 ml, 17 mmol) in DMF†(90 ml) was added Boc-Ile-OSu (5.59 g, 17 mmol) at 0°C under stirring. After stirring for 2 h at 0°C, the reaction mixture was stirred overnight at room temperature. N-(2-aminoethyl)-piperazine (0.45 ml, 3.4 mmol) was added at 0°C to decompose the unreacted active ester, and brine was added to the reaction mixture to give precipitate. The solid was filtered and washed with 10% citric acid, sat. $NaHCO_3$ solution, water, and ethyl acetate (or ether) successively. The product thus obtained was used to the next reaction, and reprecipitated from methanol-ether-hexane for elemental analysis. Yield 9.15 g (67.8%), mp 178-179°C, $[\alpha]_D^{17}$-14.4° (c 0.98, DMF), Found: C, 63.50; H, 6.75; N, 7.02; Cl, 4.44%. Calcd for $C_{42}H_{53}N_4O_9Cl$: C, 63.59; H, 6.73; N, 7.06; Cl, 4.47%.

Deblocking of Boc group by HCl in acetic acid (Preparation of HCl•H-Ile-Phe-Lys(ClZ)-OPac) Boc-Ile-Phe-Lys(ClZ)-OPac (7.93 g, 10 mmol) was dissolved in 1.15 M HCl in acetic acid (175 ml) and stirred for 40 min at room temperature. Ether was added to the reaction mixture to give a solid which was reprecipitated from methanol-ether. Yield 7.29 g (100%).

Deblocking of Boc group by TFA† (Preparation of HCl•H-Ile-Val-Lys(ClZ)-Ala-

Gly-Pro-Ala-Ile-Glu(OBzl)-Val-Leu-Gly-OPac) Boc-Ile-Val-Lys(ClZ)-Ala-Gly-Pro-Ala-Ile-Glu(OBzl)-Val-Leu-Gly-OPac (1.29 g, 0.782 mmol) was dissolved in TFA (20 ml), and stirred for 1 h at room temperature. The reaction mixture was concentrated in vacuo to one-third of its original volume, and 4M HCl in THF† (10 ml) was added. Ether was added to the solution to separate out precipitate. Yield 1.17 g (94.5%).

Deblocking of Pac ester (Preparation of Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-OH) To a solution of Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-OPac (699 mg, 0.433 mmol) in DMSO†-acetic acid (1:1) (20 ml) was added zinc powder (1.45 g, 22 mmol), and the reaction mixture was stirred for 2 h at room temperature. After filtration of insoluble material, water was added to the filtrate to give precipitate. The precipitate was collected by filtration or centrifugation, and washed with ether. Yield 609 mg (94%).

General procedure of the fragment condensation (Preparation of Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-OPac) To a solution of Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-OH (578 mg, 0.386 mmol), HCl• H-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-OPac (497 mg, 0.386 mmol), and HOBt† (52.2 mg, 0.386 mmol) in DMF (15 ml) was added WSCI† (60 µl, 0.386 mmol)at -70°C with stirring, and the reaction mixture was stirred overnight at room temperature. After addition of brine, the precipitate separated out was filtered and washed with 10% citric acid, sat. $NaHCO_3$ solution, water, and ethyl acetate (or ether) successively. The solid thus obtained was reprecipitated from DMF-methanol. Yield 812 mg (77.0%).

Final deprotection of the peptide fragment containing Trp residue

4HCl• H-Arg-Trp-Lys-Ile-Phe-Lys-OH (1-6) In the reaction tube of HF apparatus, a mixture of Boc-Arg-Trp-Lys-Ile-Phe-Lys-OH (101 mg, 67.3 µmol), dimethylsulfide (11.8 ml), p-cresole (1.4 ml), and p-thiocresole (0.5 ml) was allowed to react with HF (4.5 ml) at 0°C for 2 h. After HF and dimethylsulfide were evaporated in vacuo, HF (20 ml) was introduced at -70°C, the reaction mixture was stirred at 0°C for 1 h. The solution was concentrated in vacuo, and the aqueous solution of the residue was neutralized with $NaHCO_3$. The solution was washed with ether, and lyophilized. The powder thus obtained was desalted by Diaion HP-20 column (1.4 x 45 cm). The product was eluted with 50% methanol, and purified by

HPLC (Cosmosil $5C_{18}$, 6 x 250 mm, linear gradient from 12% CH_3CN-0.01M HCl to 45% CH_3CN-0.01M HCl, Flow rate 2 ml/min). Yield 24 mg (35%). UV:λ_{max} 280 nm (ε 5.6 x 10^3).

6HCl•H-Arg-Trp-Lys-Ile-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-OH (1-12) In the reaction tube of HF apparatus, a mixture of Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-OH (23.9 mg,9.15 μmol), Met (27.3 mg, 183 μmol) and anisole (250 μl) was allowed to react with HF (2.5 ml) at 0°C for 1 h. Met was added as a scavenger against t-butylation on sulfide linkage of Met residue. After HF was evaporated at 0°C, an aqueous solution of the residue was neutralized with $NaHCO_3$. The solution was acidified with acetic acid, and washed with ether. The aqueous layer was acidified with 6M HCl and lyophilized. The powder obtained was purified by HPLC (Nucleosil $7C_{18}$, 8 x 250 mm, linear gradient from 10% CH_3CN-0.01M HCl to 35% CH_3CN-0.01M HCl, Flow rate 3ml/min). Yield 10.7 mg. The powder thus obtained (9.0 mg) was dissolved in DMSO-aq. solution of NH_2OH and NH_3, pH 9(3:1) (4.5 ml) and allowed to stand for 3 h. After lyophilization, the product was purified by HPLC (Nucleosil $7C_{18}$, 8 x 250 mm, linear gradient from 15% CH_3CN-0.01M HCl to 27% CH_3CN-0.01M HCl, Flow rate 3ml/min). Yield 8.2 mg (50%). UV: λ_{max} 279 nm (ε 2.8 x 10^3).

8HCl•H-Arg-Trp-Lys-Ile-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asp-Gly-OH (1-18) Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-Arg(Tos)-Asn-Ile-Arg(Tos)-Asp(OcHex)-Gly-OH was deprotected by the same manner as that of (1-12).

9HCl•H-Arg-Trp-Lys-Ile-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asp-Gly-Ile-Val-Lys-Ala-Gly-OH (1-23) Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-Arg(Tos)-Asn-Ile-Arg(Tos)-Asp(OcHex)-Gly-Ile-Val-Lys(ClZ)-Ala-Gly-OH (135 mg, 31 μmol) was dissolved in TFA (3 ml), and stirred at room temperature for 1h. The solution was evaporated *in vacuo*. After addition of anisole (560 μl), the residue was allowed to react with HF (5 ml) at 0°C for 1 h. After HF was evaporated, ethanedithiol (2.5 ml) was added to the residue. HF (2.5 ml) was again introduced and stirred at 0°C for 30 min. The solution was concentrated *in vacuo* to the residue which was then dissolved in 4% acetic acid and washed with ether. Aqueous layer was passed through a column of Dowex 1 (12 x 280 ml), and the eluate was purified by HPLC (Nucleosil 300-$7C_{18}$ 6 x 250 ml, linear gradient from 20% CH_3CN-0.01M HCl to 45% CH_3CN-0.01M HCl, Flow rate 3ml/min). Yield 25.6 mg (26.9%). UV: λ_{max} 280 nm (ε 2.7x10^3)

9HCl•H-Arg-Trp-Lys-Ile-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asp-Gly-Ile-Val-Lys-Ala-Gly-Pro-Ala-Ile-Glu-Val-Leu-Gly-OH (1-30) Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-Arg(Tos)-Asn-Ile-Arg(Tos)-Asp(OcHex)-Gly-Ile-Val-Lys-Ala-Gly-Pro-Ala-Ile-Glu(OBzl)-Val-Leu-Gly-OH was deblocked by the same manner as that of (1-23).

8HCl•H-Trp-Lys-Ile-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asp-Gly-Ile-Val-Lys-Ala-Gly-OH (2-23) Boc-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-Arg(Tos)-Asn-Ile-Arg(Tos)-Asp(OcHex)-Gly-Ile-Val-Lys-Ala-Gly-OH was deprotected by the same manner as that of (1-23).

General procedure of deprotection of the fragment peptide without Trp residue (Preparation of 6HCl•H-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asp-Gly-Ile-Val-Lys-Ala-Gly-OH (7-23)) A mixture of Boc-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-Arg(Tos)-Asn-Ile-Arg(Tos)-Asp(OcHex)-Gly-Ile-Val-Lys-Ala-Gly-OH (58.3 mg, 19.6 μmol), Met (51 mg, 389 μmol), and anisole (555 μl) was allowed to react with HF (5 ml) at 0°C for 1 h. After HF was evaporated in vacuo, the solution of the residue in water was neutralized with $NaHCO_3$ and washed with ether. The aqueous layer was acidified with 6M HCl and then lyophilized. The powder thus obtained was purified by HPLC (Nucleosil $7C_{18}$, 8 x 250 mm, linear gradient from 1% CH_3CN-0.01M HCl to 25% CH_3CN-0.01M HCl. Flow rate 3 ml/min). Yield 21.1 mg (51.1%).

General procedure of synthesis of the alkylamide derivative (Preparation of 8HCl•H-Arg-Trp-Lys-Ile-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asp-Gly-NH-$(CH_2)_{11}CH_3$) To a solution of Boc-Arg(Tos)-Trp(CHO)-Lys(ClZ)-Ile-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OBzl)-Lys(ClZ)-Met-Gly-Arg(Tos)-Asn-Ile-Arg(Tos)-Asp(OcHex)-Gly-OH (200 mg, 53.9 μmol), $CH_3(CH_2)_{11}NH_2$ (10.0 mg, 53.9 μmol), and HOBt (7.3 mg, 53.9 μmol) in DMSO-NMP†-DMF (1:1:1)(4 ml) was added WSCI•HCl (10.3 mg, 53.9 μmol) at 0° C with stirring, and the reaction mixture was stirred at room temperature for 18 h. After the ninhydrin test of the reaction mixture turned to negative, CH_3-$(CH_2)_{11}NH_2$ (2.0 mg, 10.8 μmol) and WSCI•HCl (2.1 mg, 10.8 μmol) were again added. After addition of brine, the precipitate separated out was filtered and washed with 10% citric acid, sat. $NaHCO_3$ solution, water and ethyl acetate successively. Yield 155 mg (74.1%). The product thus obtained (70 mg, 18.0 μmol) was dissolved in TFA (5 ml), and stirred for 1 h at room temperature. The reaction mixture was concentrated in vacuo, and the residue was allowed to react with HF (5ml) at 0°C

for 1 h after addition of anisole (560 µl). After HF was evaporated, ethanedithiol (2.5 ml) was added to the residue which was allowed to react again with HF (2.5 ml) at 0°C for 30 min. the reaction mixture was concentrated in vacuo, and the solution of the residue in 4% acetic acid was washed with ether. Aqueous layer was passed through a column of Dowex 1 (12 x 280 mm). The eluate was purified by HPLC (Nucleosil 300-7C_{18} 6 x 250 ml, linear gradient from 30% CH_3CN-0.1% TFA to 60% CH_3CN-0.1% TFA, Flow rate 2 ml/min). Yield 12.8 mg (26.0%).

Amino acid analyses and optical rotations of deblocking peptides were listed in Table 5-9.

Table 5. Amino Acid Analysis and Optical Rotation of the Fragment Peptides

	(1-6)[a]	(7-12)	(13-18)[b]	(19-23)	(24-30)	(31-35)[c]
Asp	-	-	2.07(2)	-	-	-
Ser	-	-	-	-	-	1.00(1)
Glu	-	1.02(1)	-	-	1.03(1)	-
Pro	-	-	-	-	0.97(1)	-
Gly	-	1.00(1)	1.00(1)	1.00(1)	1.00(1)	-
Ala	-	-	-	0.98(1)	0.97(1)	2.00(2)
Val	-	-	-	1.02(1)	0.98(1)	-
Met	-	0.93(1)	-	-	-	-
Ile	0.96(1)	0.88(1)	0.92(1)	0.86(1)	0.86(1)	1.04(1)
Leu	-	-	-	-	0.93(1)	-
Phe	1.00(1)	-	-	-	-	-
Lys	2.12(2)	1.92(2)	-	0.99(1)	-	0.94(1)
Trp	0.98(1)	-	-	-	-	-
Arg	1.07(1)	-	2.03(2)	-	-	-
$[\alpha]_D^{20}$ [d]	-16.2	-48.6	-47.6	-54.0	-102.5	-68.9

Hydrolysis condition: TFA-cHCl (1:2), 166°C, 60 min.
a) 6M HCl containing 4% thioglycolic acid, 110°C, 24 h.
b) 6M HCl, 110°C, 24 h.
c) 6M HCl, 110°C, 35 h.
d) (c 1, 5% CH_3COOH)

Table 6. Amino Acid Analysis and Optical Rotation of the Fragment Peptides

	(1-12)	(7-18)[a]	(13-23)	(19-30)	(24-35)[a]	(28-35)[a]
Asp	-	2.13(2)	2.05(2)	-	-	-
Ser	-	-	-	-	0.95(1)	1.00(1)
Glu	0.97(1)	1.01(1)	-	1.02(1)	0.85(1)	-
Pro	-	-	-	0.97(1)	1.11(1)	-
Gly	1.00(1)	2.00(2)	2.00(2)	2.00(2)	1.00(1)	1.00(1)
Ala	-	-	1.04(1)	1.98(2)	2.99(3)	2.14(2)
Val	-	-	0.59(1)	1.51(2)	0.75(1)	0.59(1)
Met	0.92(1)	0.81(1)	-	-	-	-
Ile	1.92(2)	1.32(2)	1.53(2)	1.50(2)	1.79(2)	0.96(1)
Leu	-	-	-	0.94(1)	0.74(1)	0.66(1)
Phe	0.95(1)	-	-	-	-	-
Lys	4.13(4)	2.09(2)	1.07(1)	1.03(1)	1.06(1)	1.10(1)
Trp[b]	0.91(1)	-	-	-	-	-
Arg	1.07(1)	1.85(2)	2.01(2)	-	-	-
$[\alpha]_D^{28}$ c)	-37	-86	-72	-133	-16	-70

Hydrolysis condition: 6M HCl, 110°C, 90 h.
a) 6M HCl, 110°C, 24 h.
b) 6M HCl containing 4% thioglycolic acid, 110°C, 24 h.
c) (c 0.1, 4% CH_3COOH)

Table 7. Amino Acid Analysis and Optical Rotation of the Fragment Peptides

	(1-18)	(7-23)	(13-30)	(19-35)	(1-30)
Asp	2.08(2)	2.03(2)	2.07(2)	-	2.12(2)
Ser	-	-	-	0.86(1)	-
Glu	0.97(1)	1.12(1)	1.12(1)	1.07(1)	1.97(2)
Pro	-	-	1.20(1)	1.34(1)	1.45(1)
Gly	2.00(2)	3.00(3)	3.00(3)	2.00(2)	4.00(4)
Ala	-	-	2.04(2)	3.83(4)	2.11(2)
Val	-	1.05(1)	1.63(2)	1.51(2)	1.65(2)
Met	0.96(1)	0.66(1)	-	-	1.06(1)
Ile	2.94(3)	0.92(1)	2.62(3)	2.49(3)	4.79(5)
Leu	-	2.51(3)	1.03(1)	1.01(1)	1.03(1)
Phe	1.09(1)	-	-	-	0.95(1)
Lys	4.31(4)	-	1.05(1)	1.97(2)	5.76(5)
Trp[a]	0.67(1)	3.16(3)	-	-	1.19(1)
Arg	3.45(3)	2.15(2)	1.95(2)	-	3.03(3)
$[\alpha]_D^{28}$ b)	-48	-79	-106	-29	-85

Hydrolysis condition: 6M HCl, 110°C, 90 h.
a) 6M HCl containing 4% thioglycolic acid, 110°C, 24 h.
b) (c 0.1, 4% CH_3COOH)

Table 8. Amino Acid Analysis and Optical Rotation of the Fragment Peptides

	(1-23)	(2-23)	(3-23)	(4-23)
Asp	1.96(2)	2.01(2)	1.97(2)	1.97(2)
Ser	-	-	-	-
Glu	1.13(1)	1.05(1)	1.01(1)	1.06(1)
Pro	-	-	-	-
Gly	3.00(3)	3.00(3)	3.00(3)	3.00(3)
Ala	1.02(1)	0.94(1)	0.92(1)	1.00(1)
Val	0.67(1)	0.67(1)	0.58(1)	0.71(1)
Met	1.01(1)	0.92(1)	0.95(1)	0.94(1)
Ile	3.52(4)	3.72(4)	3.59(4)	3.73(4)
Leu	-	-	-	-
Phe	1.00(1)	0.87(1)	0.73(1)	0.90(1)
Lys	5.45(5)	6.06(5)	5.72(5)	4.56(4)
Trp[a)]	0.80(1)	0.84(1)	-	-
Arg	2.94(3)	1.89(2)	1.79(2)	1.88(2)
$[\alpha]_D^{16}$ b)	-67	-70	-31	-49

Hydrolysis condition: 6M HCl, 110°C, 90 h.
a) 6M HCl containing 4% thioglycolic acid, 110°C, 24 h.
b) (c 0.1, 4% CH_3COOH)

Table 9. Amino Acid Analysis, Optical Rotation, and FAB-MS of Alkyl Amides of Peptide (1-18)

		H-(1-18)-NH-R				
	R:	n-$C_{18}H_{37}$	n-$C_{16}H_{33}$	n-$C_{14}H_{29}$	n-$C_{12}H_{25}$	C_2H_5
Asp	(2)	1.97	1.85	1.95	1.82	1.91
Glu	(1)	1.05	1.00	1.16	1.04	1.00
Gly	(2)	1.93	1.96	1.99	1.98	2.08
Met	(1)	1.00	1.05	1.17	1.11	0.96
Ile	(3)	2.96	2.92	3.15	2.77	2.88
Phe	(1)	1.00	1.04	1.00	1.00	1.02
Lys	(4)	4.32	4.33	4.52	3.96	4.35
Trp[a)]	(1)	0.59	0.81	1.02	0.92	0.94
Arg	(3)	2.94	3.46	3.06	3.40	3.24
$C_2H_5NH_2$	(1)	-	-	-	-	1.05
FAB-MS(M+H)		2524[b)]	2497	2469	2441	-
$[\alpha]_D^{27}$ c)		-45[d)]	-12	-30	-15	-45[d)]

Hydrolysis condition: 6M HCl, 110°C, 90 h.
a) 6M HCl containing 4% thioglycolic acid, 110°C, 24 h.
b) Experimental error range is ±2
c) (c 0.1, 4% CH_3COOH)
d) 17°C

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References

1) This work was presented in a preliminary form at the 19th European Peptide Symposium. Peptides 1986, **1987**, 651. Walter de Gruyter & Co., Berlin.
2) K. Hayashiya, Ent. Exp. & Appl. **1978**, 24, 228.
3) Y. Uchida, F. Kawamoto, M. Himeno, and K. Hayashiya, J. Invertebr. Pathol. **1984**, 43, 182.
4) M. Okada, and S. Natori, J. Biol. Chem., 1985, 260, 7174.
5) M. Kikuchi, The Third Naito Symposium "Defense Mechanism of Living Body", Abstract 1979, 7, Proceeding 1980, 19. The Naito Foundation, Tokyo Univ. Publ. Co., 1980, Tokyo.
6) T. Shiba, Y. Ueki, I. Kubota, T. Teshima, Y. Sugiyama, Y. Oba, and M. Kikuchi, Peptide Chemistry 1983, **1984**, 209, Protein Research Foundation, Osaka.
7) T. Teshima, Y. Ueki, T. Nakai, and T. Shiba, Tetrahedron, **1986**, 42, 829.
8) T. Teshima, T. Nakai, Y. Ueki, and T. Shiba, Tetrahedron, **1987**, in press.
9) T. Nakai, M. Kitazawa, T. Teshima and T. Shiba, Peptide Chemistry 1985, **1986**, 207, Protein Research Foundation, Osaka.
10) T. Teshima, T. Nakai, M. Kitazawa, and T. Shiba, Tetrahedron Lett., **1987**, 28, 4705.
11) H. Steiner, D. Hultmark, Å. Engström, H. Bennich, and H. G. Boman, Nature, **1981**, 292, 246.
12) X.-M Qu, H. Steiner, Å. Engström, H. Bennich, and H. G. Brown, Eur. J. Biochem., **1982**, 127, 219.
13) P. van Hofstein, I. Faye, K. Kockum, J.-Y. Lee, K. G. Xanthopoulos, I. A. Boman, H. G. Boman, Å. Engström, D. Andreu, and R. B. Merrifield, Proc. Natl. Acad. Sci. USA, 1985, 82, 2240.
14) D. Hultmark, Å. Engström, K. Andersson, H. Steiner, H. Bennich, and H. G. Boman, EMBO J., 1983, 2, 571.
15) K. Kockum, I. Faye, P. V. Hofsten, J.-Y. Lee, K. G. Xanthopoulos, and H. G. Boman, EMBO J., **1984**, 3, 2071.
16) Y.-H. Chen, J. Y. Yang, and K. H. Chau, Biochemistry, **1974**, 13, 3350.

† Abbreviations according to IUPAC-IBU joint commission on Biochemical Nomenclature (JCBN), Eur. J. Biochem., **1984**, 138, 9, were used. DMSO: dimethylsulfoxide, DMF: N,N-dimethylformamide, THF: tetrahydrofuran, NMP: N-methylpyrrolidone, TFA: trifluoroacetic acid, HOBt: 1-hydroxybenzotriazole, WSCI: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Pac: phenacyl, ClZ: 2-chlorobenzyloxycarbonyl, cHex: cyclohexyl, MIC: minimum inhibitory concentration.