Design of the Synthetic Route for Peptides and Proteins Based on the Solubility Prediction Method. I. Synthesis and Solubility Properties of Human Proinsulin C-Peptide Fragments[1)]

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The usefulness of the solubility prediction method is demonstrated using relatively small peptide fragments of human proinsulin C-peptide. The propriety of the solubility prediction method for peptides having polar side chains is also examined in the following respects: (1) Peptide intermediates smaller than a heptapeptide have high solubility regardless of their $<P_c>$ values; (2) the $<P_c>$ values of peptide intermediates are useful for judging solubility of peptide intermediates equal to or larger than an octapeptide level; (3) the Pro residue in a central position of a peptide chain is effective for increasing peptide solubility; and (4) there is critical chain length for peptide insolubility caused by a β-sheet aggregation. A strategy suitable for the design of the synthetic route for human proinsulin C-peptide is subsequently discussed on the basis of the solubility prediction of peptide intermediates.

To achieve total synthesis of large peptides and proteins, all of the components must be soluble in coupling solvents. In many cases of protein syntheses, indeed, avoidance of insolubility of peptide intermediates in coupling solvents is claimed to be a problem of utmost importance to achieve the protein syntheses favorably.[2)] Thus, the proper design of the synthetic route based on the solubility prediction of peptide intermediates is greatly required to avoid erroneous synthetic routes. Following this concept, we are pursuing the scope of the design of the synthetic routes for peptides and proteins based on a solubility prediction method.[3)]

So far, however, it has been widely recognized that the solubility prediction of protected peptide intermediates is difficult due to the diverse individuality of their amino acid sequences since their solubility is primarily dependent on their amino acid sequences. In our recent papers,[3-10)] however, we have demonstrated that insolubility of peptide intermediates is caused by a β-sheet aggregation of peptide intermediates equal to or larger than an octapeptide level and that the temporary protection of a N-H peptide bond in a central position of a peptide chain helps improve solubility to a remarkable extent.[4)] Furthermore, we have also demonstrated that, in hydrophobic sequences found in proteins, the β-sheet aggregation plays an important role in insolubility of hydrophobic peptides and that, contrary to the general recognition that solubility of peptide intermediates is primarily dependent on their amino acid sequences, their solubility is independent on their amino acid sequences.[10)] On the basis of these results, the solubility prediction of peptide intermediates was successfully performed by making use of both the randomness of peptide structures in the solid state and the existence of tertiary peptide bonds, that is, the disturbance of a β-sheet structure.[3)] The correctness of the concept of this solubility prediction method was furthermore verified by another evidence, that is, solubility improvement by promotion of helical folding in oligopeptides based on the restriction of the values of the backbone dihedral angles ϕ and ψ of the α-aminoisobutyric acid residue.[10-12)]

The usefulness of the solubility prediction method has been previously confirmed in hydrophobic oligopeptides.[3-12)] We here demonstrate its usefulness also for relatively small peptide fragments of human proinsulin C-peptide having polar side chains and we then propose a strategy suitable for the design of the synthetic routes for the C-peptide on the basis of the solubility prediction of peptide intermediates.

Experimental

General. The uncorrected capillary melting points will be reported. The amino acid compositions of acid hydrolysates were determined with a Shimadzu HPLC LC-3A all amino acid analysis system. The acid hydrolyses of the peptides containing the Ser residue were carried out with 3M† *p*-toluenesulfonic acid[13)] for 3 d at 115 °C in evacuated, sealed tubes, and those of other peptides, with propionic acid/12M HCl (vol. ratio, 2/1)[14)] for 2 d at 115°C. Other analytical instruments and conditions were described in the previous paper.[11)]

General Procedure for Removal of the Boc Group. Boc-amino acid Pac esters (10 mmol) and Boc-peptide Pac esters (10 mmol) in 3 M HCl/AcOEt (60 ml) were stirred in an ice-chilled bath for 1.5—3 h. Then, the mixture was concentrated in vacuo, followed by the repetition of the addition and removal in vacuo of dichloromethane. The residual amino component HCl salt was used for a subsequent coupling reaction without further purification.

General Procedure for Stepwise Elongation of Peptide Chains Using DCC Activation (Preparation of the Peptides 1—6 and 10). DCC (1.2 equiv) was added to an ice-chilled, stirred mixture of a carboxyl component (1.2 equiv) and an amino component (10 mmol) in dichloromethane, a mixture of dichloromethane and DMF, or DMF (100 ml). The amino components were obtained from the corresponding HCl salt with an equivalent of NMM. For incorpora-

† 1 M=1 mol dm^{-3}.

tion of the Gln residue, instead of DCC activation, Boc-Gln-ONp (1.5 equiv) was used for chain elongation. The reaction mixture was stirred in an ice-chilled bath for 3 h, at room temperature overnight, and then subjected to ninhydrin test. When the test was positive, double and triple couplings were carried out using the carboxyl component and DCC (each 0.3 equiv). After completion of the reaction, the reaction mixture was filtered. The subsequent work-up procedure was essentially the same as those described before.[11] The synthetic results and analytical data are shown in Tables 1—3.

General Procedure for Fragment Condensation Using DCC Activation in the Presence of HOBt (Preparation of the Peptides 7—9, 11, and 12). Assembly of peptide chains by fragment condensation was carried out in DMF or a mixture of DMF and NMP. DCC (1.2 equiv) was added to an ice-chilled, stirred mixture of a carboxyl component (1.2 equiv), HOBt (1.2 equiv), and an amino component (10 mmol) in solvent (100 ml). The amino components were obtained from the corresponding HCl salts with an equivalent of NMM. The reaction mixture was stirred in an ice-chilled bath for 3 h, at room temperature for 2 d, and then subjected to ninhydrin test. When the test was positive, double and triple couplings were carried out using the carboxyl component and DCC (each 0.3 equiv). After completion of the reaction, the reaction mixture was filtered. The subsequent work-up procedure was essentially the same as those described before.[11] The synthetic results and analytical data are also shown in Tables 1—3.

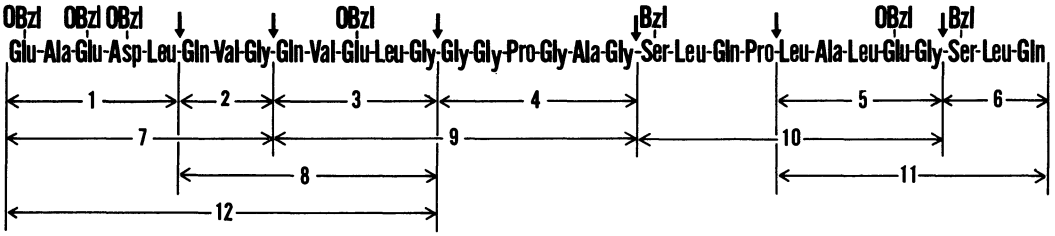
General Procedure for Removal of the Pac Group. Boc-peptide Pac esters were stirred in a mixture of AcOH using a several-fold molar excess of Zn dust at room temperature for several days. After removal of Zn residues, the filtrate was concentrated in vacuo. The residue was treated with aqueous citric acid, followed by washing with water and hexane. Completion of the reaction was examined by TLC. The residue was used for a subsequent coupling reaction without further purification.

Results

Fragmentation of Human Proinsulin C-Peptide Based on the Solubility Prediction Method. One of our purposes in this paper is to examine the propriety of the solubility prediction method for peptide intermediates having polar side chains in the following respects: (1) Peptide intermediates smaller than heptapeptides have high solubility regardless of their $<P_c>$ values; (2) the $<P_c>$ values of peptide intermediates are useful for judging solubility of peptide intermediates equal to or larger than an octapeptide level; (3) the Pro residue in a central position of a peptide chain is effective for increasing peptide solubility; and (4) there is critical chain length for peptide insolubility caused by a β-sheet aggregation.

To investigate these points, the primary structure[15] of human proinsulin C-peptide was divided into seven fragments at the points indicated by arrows in Fig. 1, where the primary structure includes side-chain-protecting Bzl groups in the Asp, Glu, and Ser residues. Figure 1 also illustrates the N- and C-terminal-protected peptide fragments **1—12** used in this study. The solubility properties of the peptides **1—12** will be discussed later on the basis of the solubility prediction method.[3]

Synthesis and Solubility Properties of Each Peptide Fragment. Starting from the Pac ester of each C-terminal amino acid residue as the amino component, the peptide fragments **1—6** and **10** were prepared by the usual stepwise elongation of the peptide chain using Boc-amino acids[16] as the acid components. The following side-chain-protected amino acids were used for the synthesis: Glu(OBzl), Asp(OBzl),



1. Boc-Glu(OBzl)Ala Glu(OBzl)Asp(OBzl)Leu-OPac
2. Boc-Gln Val Gly-OPac
3. Boc-Gln Val Glu(OBzl)Leu Gly-OPac
4. Boc-Gly Gly Pro Gly Ala Gly-OPac
5. Boc-Leu Ala Leu Glu(OBzl)Gly-OPac
6. Boc-Ser(Bzl)Leu Gln-OPac
7. Boc-Glu(OBzl)Ala Glu(OBzl)Asp(OBzl)Leu Gln Val Gly-OPac
8. Boc-Gln Val Gly Gln Val Glu(OBzl)Leu Gly-OPac
9. Boc-Gln Val Glu(OBzl)Leu Gly Gly Gly Pro Gly Ala Gly-OPac
10. Boc-Ser(Bzl)Leu Gln Pro Leu Ala Leu Glu(OBzl)Gly-OPac
11. Boc-Leu Ala Leu Gln(OBzl)Gly Ser(Bzl)Leu Gln-OPac
12. Boc-Glu(OBzl)Ala Glu(OBzl)Asp(OBzl)Leu Gln Val Gly Gln Val Glu(OBzl)Leu Gly-OPac

Fig. 1. The amino acid sequence of human proinsulin C-peptide and the N- and C-terminal protected peptide fragments **1—12** used in this study.

and Ser(Bzl).[17,18] The coupling reactions were mediated by DCC[19] throughout except for the Gln residue, which was incorporated by the active ester method[20] using Boc-Gln-ONp. Use of the Pac ester[21] at the C-terminus should have the advantage that Boc-peptide Pac ester thus prepared can be used not only as an amino component, but also as a carboxyl component since the stability of the side-chain-protecting Bzl group under removal conditions of Boc- and Pac groups has been well established.[17] Liberation of the amino groups from Boc-peptide Pac esters was performed by treatment with 3 M HCl/AcOEt, followed by treatment with NMM. Removal of the Pac group from the Boc-peptide Pac esters was performed by hydrogenolysis using Zn dust in AcOH.[22] The larger peptide fragments **7**—**9**, **11**, and **12** were subsequently prepared by the fragment condensation method after removal of the C- or N-terminal-protecting

Table 1. Synthetic Results and Physical Properties of the Peptides **1**—**12**

Compound	Yield/%[a]	Recrystallization solvent	Mp θ_m/°C	$[\alpha]_D^{19°}$ (c=1.0, DMF)	Retention time time/min
1	67	AcOEt/Hexane	102—104	−21.2	13.1
2	77	MeOH/Water	203—205	−21.0	13.5
3	95	EtOH/Water	216—220	−23.2	13.2
4	52	AcOEt/Hexane	104—109	−28.0	13.6
5	86	AcOEt/Hexane	179—183	−27.0	13.4
6	90	AcOEt/Hexane	157—160	−20.2	13.5
7	86	b)	235—237	−47.0[d]	13.0
8	56	b)	235—236	−41.0[d]	13.0
9	60	b)	203—205	−31.4	12.8
10	57	THF/Hexane	175—179	−37.8	13.1
11	92	c)	210—213	−25.9	13.0
12	72	b)	Over 250	e)	e)

a) Coupling yields in final steps. b) Purified by washing with hot MeOH. c) Purified by washing with hot EtOH. d) Measured in AcOH. e) Not obtained due to insolubility in DMF.

Table 2. Elemental Analyses of the Peptides **1**—**12**

Compound	Formula	Found (Calcd) (%)		
		C	H	N
1	$C_{57}H_{69}N_5O_{15}\cdot H_2O$	63.47 (63.26)	6.70 (6.61)	6.78 (6.47)
2	$C_{25}H_{36}N_4O_8$	57.95 (57.68)	7.11 (6.97)	10.72 (10.76)
3	$C_{43}H_{60}N_6O_{12}\cdot 0.5H_2O$	59.75 (59.92)	6.92 (7.13)	9.73 (9.75)
4	$C_{29}H_{40}N_6O_{10}$	55.43 (55.06)	6.60 (6.37)	12.88 (13.28)
5	$C_{42}H_{59}N_5O_{11}$	62.41 (62.28)	7.44 (7.34)	8.69 (8.65)
6	$C_{34}H_{46}N_4O_9\cdot 0.5H_2O$	61.69 (61.52)	6.91 (7.14)	8.57 (8.44)
7	$C_{69}H_{89}N_9O_{19}$	61.28 (61.46)	6.80 (6.65)	9.50 (9.35)
8	$C_{55}H_{80}N_{10}O_{16}\cdot H_2O$	57.41 (57.18)	7.15 (7.15)	12.07 (12.12)
9	$C_{59}H_{84}N_{12}O_{18}\cdot 2H_2O$	55.09 (55.13)	7.14 (6.90)	12.96 (13.08)
10	$C_{68}H_{96}N_{10}O_{17}\cdot 0.5H_2O$	61.19 (61.20)	7.42 (7.33)	10.52 (10.50)
11	$C_{63}H_{89}N_9O_{16}\cdot 2.5H_2O$	59.15 (59.42)	7.17 (7.44)	9.78 (9.90)
12	$C_{99}H_{133}N_{15}O_{27}\cdot 3H_2O\cdot 3DMF$	57.93 (57.95)	6.88 (7.20)	11.35 (11.26)

Table 3. Amino Acid Analyses of the Peptides **1**—**12**

Compound	Found (Calcd)							
	Gly	Ala	Val	Leu	Ser	Pro	Asp	Glu[a]
1	—	1.05 (1)	—	0.85 (1)	—	—	1.15 (1)	2.00 (2)
2	1.05 (1)	—	1.00 (1)	—	—	—	—	0.90 (1)
3	1.08 (1)	—	1.05 (1)	1.00 (1)	—	—	—	1.80 (2)
4	4.44 (4)	1.03 (1)	—	—	—	1.00 (1)	—	—
5	1.00 (1)	0.89 (1)	—	2.04 (2)	—	—	—	0.98 (1)
6	—	—	—	1.00 (1)	0.98 (1)	—	—	1.02 (1)
7	1.00 (1)	1.10 (1)	1.00 (1)	0.95 (1)	—	—	1.21 (1)	2.92 (3)
8	2.08 (2)	—	2.00 (2)	0.99 (1)	—	—	—	2.70 (3)
9	4.58 (5)	0.89 (1)	1.05 (1)	1.00 (1)	—	1.21 (1)	—	2.05 (2)
10	1.06 (1)	1.09 (1)	—	2.98 (3)	0.94 (1)	1.00 (1)	—	1.89 (2)
11	1.05 (1)	1.00 (1)	—	3.03 (3)	0.91 (1)	—	—	1.93 (2)
12	2.12 (2)	0.93 (1)	2.08 (2)	2.00 (2)	—	—	1.02 (1)	4.51 (5)

a) Included Gln.

Table 4. Solubility Properties[a] of the Peptides **1**—**12** (c=1.0 g/dl)

Compound ( )[c]	$<P_c>$	Solvent[b]								
		High-polar solvent[d]	AcOH	MeOH EtOH	DCM	Dioxane, THF, $CHCl_3$	AC	AN	AcOEt	Benzene CCl_4
1 (5)	0.85	A	A	A	A	A	A	B	B	B
2 (3)	0.82	A	A	B	C	C	C	C	D	D
3 (5)	0.80	A	A	B	B	C	D	C	D	D
4 (6)	1.22	A	A	A	A	A	A	A	B	C
5 (5)	0.78	A	A	A	A	A	A	A	B	B
6 (3)	0.95	A	A	A	A	A	A	B	B	D
7 (8)	0.84	A	A	C	C	C	D	D	D	D
8 (8)	0.88	A	A	D	D	D	D	D	D	D
9 (11)	1.12	A	A	B	D	D	D	D	D	D
10 (9)	0.93	A	A	A	A	A	B	A	D	D, B[e]
11 (8)	0.91	A	A	B	C	D	C	D	D	D
12 (13)	0.86	D	C	D	D	D	D	D	D	D

a) Solubility: A, soluble at room temperature; B, soluble at 80° or refluxing temperature; C, partially soluble at 80° or refluxing temperature; D, practically insoluble at 80° or refluxing temperature. b) Abbreviations: DCM, dichloromethane; THF, tetrahydrofuran; AC, acetone; AN, acetonitrile. Others, see Ref. 1). c) Number of amino acid residues of the peptide. d) DMF, NMP, DMA, DMSO, and HMPA. e) Solubility in benzene.

group of the corresponding fragment. Each fragment was coupled mainly in DMF with least danger of racemization using DCC activation in the presence of HOBt.[23] Completion of every coupling reaction was monitored by checking the reaction product with the sensitive ninhydrin test.

All of the soluble peptides were purified by repeated recrystallization, and, as a result of this purification process, each peptide gave a single peak on HPLC. The peptides **7**, **8**, and **12** which were insoluble in MeOH were purified by repeated washing with hot MeOH. Synthetic and analytical results of the peptides **1**—**12** are assembled in Tables 1—3. The amino acid and elemental analyses of the peptides shown in Tables 2 and 3 are in good agreement with the calculated values.

Solubility properties of the peptides **1**—**12** in organic solvents are also summarized in Table 4. The $<P_c>$ values of peptides shown in Table 4 indicate the randomness of each peptide fragment and they are obtained using P_c value for each amino acid residue.[3] For example, a peptide intermediate with the $<P_c>$ value above 1.00 is predicted to have a random structure in the polar solvents such as DMA, DMF, DMSO, NMP, and HMPA. According to the rule 1 of the solubility prediction method,[3] regardless of its peptide chain length, it is soluble in the polar solvents sufficiently enough to be susceptible to subsequent coupling reactions. In fact, the undecapeptide **9** has high solubility in the polar solvents, while the tridecapeptide **12** having the $<P_c>$ value of 0.86, as expected on the basis of the rule 3 of the solubility prediction method,[3] is extraordinarily less soluble in the solvents such as DMA, DMF, DMSO, NMP, HMPA, MeOH, and AcOH. With respect to the oligopeptides smaller than a heptapeptide, except for the peptides **2** and **3** containing the Gln residue, the oligopeptides **1** and **4**—**6** are easily soluble even in the moderate-polar solvents such as dichloromethane, chloroform, dioxane, and THF.

Conformations of Each Peptide Fragment in the Solid State. Conformational analysis of each peptide fragment in the solid state is interesting for the purpose of elucidating the relationship between the conformation and solubility of peptides having polar side chains. All of the peptides show strong bands at 3290—3270 cm^{-1} and 1635—1625 cm^{-1}, assigned to a typical β-sheet structure.[24] The IR absorption spectra of the peptides **4**, **9**, and **10**, which contain the Pro residue in central positions of the peptides, have strong broad shoulders at 1700—1650 cm^{-1}, indicating the disturbance of the β-sheet structure by rotation of the tertiary peptide bond plane as reported in previous papers.[3-9] The peptides **10** and **11** happen to have the very close amino acid sequences except for the Pro residue and the peptide **10** clearly shows the effect of the Pro residue in a central position of the peptide chain on the disturbance of the β-sheet structure. Analytical details will be reported elsewhere.[25,26]

Discussion

As expected from the solubility prediction of peptide intermediates, all peptide fragments **1**—**12** of human proinsulin C-peptide were prepared without difficulty by the usual stepwise elongation and fragment condensation methods, and, during the formation of the peptide fragments **8** and **12**, gelation eventually took place in a mixture of DMF and NMP along with the coupling reaction.

Regardless of the $<P_c>$ values of peptide fragments, in actuality, the peptide fragments **1**—**6** smaller than a heptapeptide are readily soluble in the polar solvents (Table 4). Most of them, except for the peptides **2** and **3** containing the Gln residue, have high solubility even in moderate-polar solvents. As reported

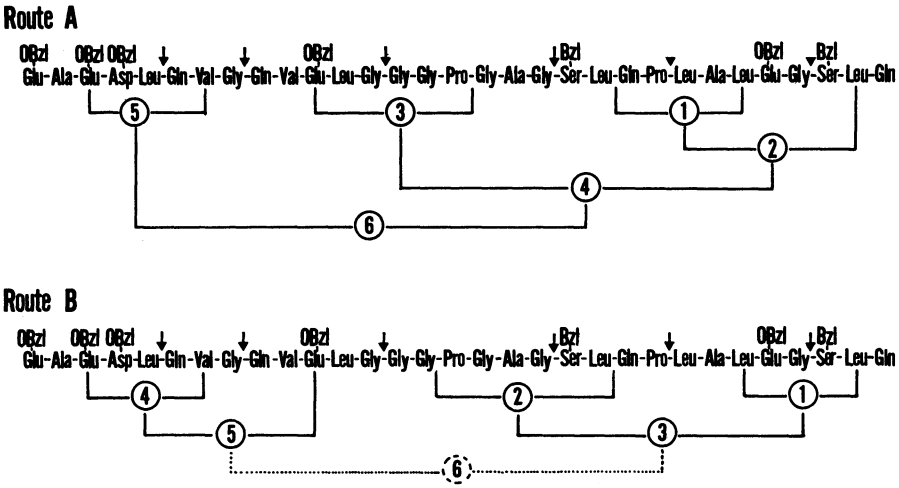


Fig. 2. Design of the synthetic routes for human proinsulin C-peptide. The number in a circle represents the order of each fragment condensation reaction.

for oligopeptides containing the Asn residue,[27] the peptides **2** and **3** have low solubility in the moderate-polar solvents probably due to hydrogen bonds through the Gln side-chain amide. The hexapeptide **4** and nonapeptide **10** are readily soluble even in the moderate-polar solvents such as dichloromethane, chloroform, dioxane, THF, and acetonitrile. The result apparently indicates that the tertiary peptide bonds (X–Pro) cause the disturbance of the β-sheet structures by the rotation of the tertiary peptide bond planes, bringing about their high solubility. The disturbance of the β-sheet structures is clearly verified by broadening of IR absorption bands in the amide I region. The solubility difference between the peptides **10** and **11** also conspicuously exhibits the effect of the Pro residue in a central position of the peptide chain on increasing peptide solubility (Table 4). Actually both the peptides have the very close amino acid sequences except for the Pro residue. On the other hand, solubility properties of the octapeptides **7**, **8**, and **11** are on a boundary. Although the peptides **7**, **8**, and **11** are soluble in the polar solvents, the peptides **7** and **8** are practically insoluble in MeOH and EtOH, and the peptide **11** is soluble only in the hot alcohols. These solubility behaviors indicate that, for the peptides containing polar side chains, critical chain length for peptide insolubility caused by the β-sheet aggregation is larger than an octapeptide level. With respect to larger peptides, the undecapeptide **9** is sufficiently soluble in the polar solvents due to the presence of the Pro residue in a central position of the peptide chain, while the tridecapeptide **12** has the $<P_c>$ value of 0.89, indicating that it is extraordinarily less soluble in the polar solvents. The peptide **12**, indeed, is actually insoluble in the polar solvents shown in Table 4 and difficult to remove the Pac group using Zn dust in AcOH. In terms of the relationship between the conformation and solubility of the peptides **7**, **8**, **11**, and **12**, the β-sheet aggregation clearly plays an important role in reducing these solubility properties.

The results and discussion mentioned above thoroughly make clear that the solubility prediction of peptide intermediates is very useful for the design of the synthetic routes for peptides and proteins. In general, assembly of the target structure by fragment condensation allows for great flexibility in the choice of the synthetic routes. In most cases of peptide and protein syntheses, for example, the Gly and Pro residues are recommended to be placed at C-termini of peptide intermediates since they are free from racemization in fragment condensation reactions. Our results clearly show that, for solving the insolubility problem of the peptide intermediates, the Pro residue should be placed in central positions of peptide intermediates. Thus, on the basis of the solubility prediction method, principally, we can design the proper synthetic route A of human proinsulin C-peptide as shown in Fig. 2. Assembled peptide intermediates in the route A are predicted to be sufficiently soluble in the polar solvents and to be susceptible to the subsequent fragment condensations **1**—**6**. All acid component candidates are also predicted to be sufficiently soluble in AcOH and to be converted to the corresponding acid components by removal of the Pac group from the peptide esters using Zn dust in AcOH. Contrary to the proper route A, if we choose the erroneous route B in Fig. 2, the acid component candidate obtained by the fragment condensation **5** is confronted with the insolubility problem on removal of the Pac group from the peptide ester using Zn dust in AcOH.

References

1) The abbreviations for amino acids are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature *J. Biol. Chem.*, **247**, 977 (1972). Amino acid symbols except for Gly denote the L-configuration. Addi-

tional abbreviations used are the following: Boc, *t*-butoxycarbonyl; Pac, phenacyl; AcOEt, ethyl acetate; DCC, dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; NMM, *N*-methylmorpholine; ONp, *p*-nitrophenyl ester; HOBt, *1*-hydroxy-1*H*-benzotriazole; NMP, *N*-methylpyrrolidone; AcOH, acetic acid; TLC, thin-layer chromatography; Bzl, benzyl; DMA, *N,N*-dimethylacetamide; DMSO, dimethyl sulfoxide; HMPA, hexamethylphosphoric triamide; HPLC, high-performance liquid chromatography; MeOH, methanol; THF, tetrahydrofuran; EtOH, ethanol; IR, infrared.

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