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Syntheses and Properties of Oligo-L-leucines Containing α-Aminoisobutyric Acid Residues. The Novel Strategy for Solubility Improvement in Helical Oligopeptides Based on the Restriction of the Values of the Backbone Dihedral Angles φ and ψ of α-Aminoisobutyric Acid Residues[1)]

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In order to provide a succinct demonstration of the usefulness of a new strategy for solubility improvement in protected peptide fragments included in α-helical regions of proteins, model oligo(Leu)s containing Aib or Ala residues were prepared by stepwise elongation and fragment condensation methods. The peptides prepared were the following: Boc-(Leu$_3$-Aib)$_n$-OBzl, Boc-(Leu$_4$-Aib)$_n$-OBzl, and Boc-(leu$_3$-Ala)$_n$OBzl (n=1—3). Carboxyl component peptides having Aib residues at the C-terminals smoothly reacted with amino component peptides in high yields with no care of racemization due to the absence of chiral centers in Aib residues. As expected, peptides containing Aib residues have high solubility in moderate- and high-polar organic solvents and are easily purified by recrystallization from aqueous ethanol. This is in remarkable contrast with the result that the octa- and dodecapeptides containing Ala residues are barely soluble or insoluble in these solvents. Conformational analyses by IR spectroscopies indicated that Boc-(Leu$_3$-Aib)$_n$-OBzl and Boc-(Leu$_4$-Aib)$_n$-OBzl (n=2 and 3) had helical conformations (3_{10}- or α-helices) in dichloromethane, whereas Boc-(Leu$_3$-Ala)$_n$-OBzl (n=2 and 3) had fully developed β-sheet structures in the solid state. The high solubility of the peptides containing Aib residues is explained by the observation that replacement of C$^\alpha$ hydrogen atoms with methyl groups greatly disturbs β-sheet structures, promoting helical folding in peptides. The implications of the new findings for the chemistry of peptides and proteins containing α,α-disubstituted α-amino acid residues is also discussed by building up a CPK model of an α-helical structure.

In our recent papers,[2-8)] we have demonstrated that a β-sheet aggregation plays an important role in the insolubility of oligopeptides larger than octa- or nonapeptide levels, and that the temporary protection of a NH peptide bond in a central position of a peptide chain helps improve solubility to a remarkable extent. The solubility improvement is attributed to the existence of tertiary peptide bonds of X-(Z)Y bonds, where X and Y are arbitrary amino acid residues and Z is a temporary protecting group for the X-Y peptide bond. The tertiary peptide bond causes disturbance of the β-sheet structure by the rotation of the tertiary peptide bond plane, and we called this segmentation of structures the "peptide segment separation."[2)] On the basis of these results, solubility prediction of protected polypeptides was also successfully performed by making use of both the randomness of peptide structures in the solid state and the existence of tertiary peptide bonds.[6)]

At this juncture, we would like to propose a novel strategy for improving solubility of protected oligo- and polypeptide fragments included in α-helical regions of proteins. The protected peptide fragments ranging from octa- to around pentadecapeptides have, in general, β-sheet structures and are less soluble in organic solvents.[2,6,9,10)] Insolubility of protected peptide fragments is one of serious problems in the efforts of protein syntheses, and it has been found very difficult to achieve further elongation of these peptide chains.[11-16)] Thus, solubility improvement in the peptides is one of the upmost important problems to be solved in protein syntheses. The strategy for solubility improvement as proposed in this paper is based on the restriction of the values around the helical regions of the backbone dihedral angles φ and ψ of Aib residues in partial replacement of Ala residues with Aib residues. The conformational space of an Aib residue is sterically severely restricted, and, φ,ψ torsional angles of the Aib residue in a linear polypeptide are mainly found in the region of conformational map which includes both the α-helix (φ=±57°, ψ=±47°) and 3_{10}-helix (φ=±60°, ψ=±30°).[17)] The conformational preferences of linear peptides containing α,α-disubstituted α-amino acids have recently been reviewed, and the ability of Aib residues to promote helical folding in peptides is well documented in the conformational analyses of membrane-channel-forming polypeptides like alamethicin and suzukacillin.[18)] Thus, partial replacement of Ala residues with Aib residues in protected polypeptide fragments included in α-helical regions of proteins is expected to disturb β-sheet structures, which are general conformations of the protected polypeptide fragments ranging from octa- to around pentadecapeptides included in α-helical regions of proteins, and to promote helical folding in peptides, providing solubility improvement in the peptide fragments (Fig. 1). In this connection, it is well recognized that the β→helical conformational transformation results in remarkable solubil-

ity improvement. Furthermore, CPK models of α-helical structures suggest to us that the replacement takes place without difficulty keeping the nature of proteins intact. Therefore, replacement of a few Ala residues with Aib residues in proteins offers the prospect for creating novel proteins not found in nature.

In this paper, we report syntheses of oligo(Leu)s containing Aib or Ala residues and demonstrate the effect of the replacement of Ala residues with Aib residues on solubility and conformations of the oligo(Leu)s.

Results

Syntheses and Solubilities of Oligo(Leu)s Containing Aib or Ala Residues. In order to demonstrate the usefulness of the strategy for solubility improvement in helical oligopeptides, model oligo(Leu)s containing Aib or Ala residues were prepared by stepwise elongation and fragment condensation methods. The synthetic scheme is shown below. Boc-Leu$_3$-Aib-OBzl (**1**), Boc-Leu$_4$-Aib-OBzl (**4**), and Boc-Leu$_3$-Ala-OBzl (**7**) were prepared by a stepwise elongation, starting with H-Aib-OBzl and H-Ala-OBzl as amino components. The stepwise elongation was performed in dichloromethane using DCC activation. The liberation of the amino group from Boc-peptide Bzl esters in the stepwise elongation was carried out by treatment with 3 M HCl/AcOEt (1 M=1 mol dm^{-3}) followed by treatment with NMM. Removal of the

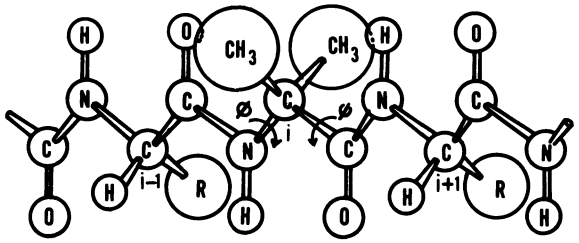


Fig. 1. Disturbance of a β-sheet structure by the restriction of the values of the backbone dihedral angles ϕ and Ψ of an Aib residue.

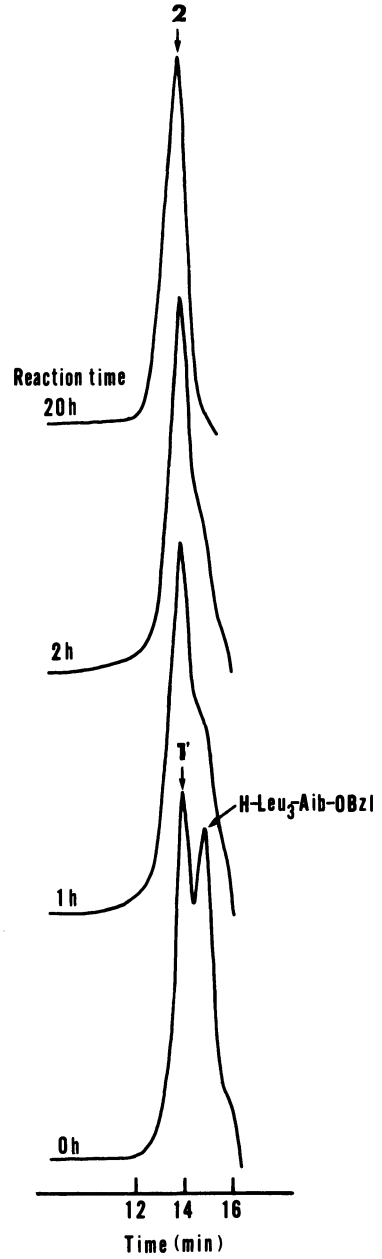
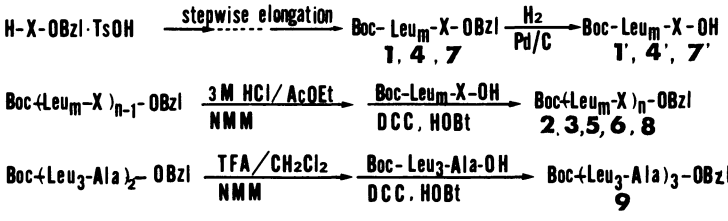


Fig. 2. Monitoring of a fragment condensation of Boc-Leu$_3$-Aib-OH with H-Leu$_3$-Aib-OBzl by HPLC.



n	1	2	3	1	2	3	1	2	3
X	Aib(m=3)			Aib(m=4)			Ala		
	1	2	3	4	5	6	7	8	9

Scheme

Bzl group from the peptides **1**, **4**, and **7** was performed by hydrogenolysis using Pd/C as a catalyst and the products **1'**, **4'**, and **7'** were used as carboxyl components in a subsequent fragment condensation. Boc-(Leu$_3$-Aib)$_n$-OBzl (n=2, **2**; n=3, **3**), Boc-(Leu$_4$-Aib)$_n$-OBzl (n=2, **5**; n=3, **6**), and Boc-(Leu$_3$-Ala)$_n$-OBzl (n=2, **8**; n=3, **9**) were prepared in dichloromethane, DMF, or HMPA by a fragment condensation method using DCC activation in the presence of HOBt. Removal of the Boc group from the peptides **1**, **2**, **4**, **5**, **7**, and **8** was carried out by treatment with 3 M HCl/AcOEt or TFA/CH_2Cl_2 (1/2, v/v). The coupling reactions were monitored by HPLC on a gel filtration column and a representative result was shown in Fig. 2. Boc-Leu$_3$-Aib-OH and Boc-Leu$_4$-Aib-OH were easily coupled with H-(Leu$_3$-Aib)$_n$-OBzl and H-(Leu$_4$-Aib)$_n$-OBzl (n= 1 and 2) in high yields, respectively, and the reactions could be performed with no care of racemization since Aib residues had no asymmetric carbon.

All the peptides containing Aib residues have high solubility in moderate- and high-polar organic solvents such as dichloromethane, chloroform, acetone, EtOH, MeOH, DMF, DMA, NMP, and HMPA (Table 1), and are easily purified by recrystallizations from aqueous ethanol. This is in remarkable contrast with the result that the peptides **8** and **9** are barely soluble or insoluble in these solvents except HMPA and are purified by washing with hot methanol. Homogeneity of the peptides except **8** and **9** was easily ascertained as the peptides had a single peak on HPLC. The synthetic results and physical properties of the peptides are assembled in Table 2. Elemental and amino acid analyses of the peptides shown in Table 3 are in good agreement with the calculated values.

IR Absorption Studies of Oligo(Leu)s Containing Aib or Ala Residues. Conformational analyses of the peptides **1**—**6** by IR absorption measurements were first carried out in dichloromethane. Figure 3 shows the most significant spectral regions for the conformational assignments (3600—3200 cm^{-1}, amide A; 1750—1600 cm^{-1}, amide I; 500—400 cm^{-1}, far-IR). As expected from solubility data and the nature of Aib residues, no band at around 3280 cm^{-1} and 1630 cm^{-1} is observed in all of the peptides **1**—**6**, indicating

Table 1. Solubility properties[a] of the peptides **1**—**9** (c=1.0 g/dl)

Compound	Solvent[b]												
	HMPA	NMP	DMA	DMF	DMSO	MeOH	EtOH	AC	CF	DCM	CT	BE	AcOEt
1	A	A	A	A	A	A	A	A	A	A	A	A	A
2	A	A	A	A	A	A	A	A	A	A	A	A	B
3	A	A	A	A	B	A	A	A	A	A	A	A	B
4	A	A	A	A	A	A	A	A	A	A	B	B	B
5	A	A	A	A	B	A	A	B	A	A	B	B	C
6	A	A	A	A	B	A	A	B	A	A	B	B	C
7	A	A	A	A	A	A	B	B	A	A	D	C	B
8	A	C	D	D	C	D	D	D	D	D	D	D	D
9	B	C	D	D	C	D	D	D	D	D	D	D	D

a) Solubility: A, soluble at room temperature; B, soluble at 80 °C or refluxing temperature; C, partially soluble at the same temperature; D, nearly insoluble at the same temperature. b) Abbreviations: AC, acetone; CF, chloroform; DCM, dichloromethane; CT, carbon tetrachloride; BE, benzene. Others, see text.

Table 2. Synthetic results and physical properties of the peptides **1**—**9**

Compound	Yield/%	Recrystallization solvent	Mp θ_m/°C	$[\alpha]_D^{20}$ (c=1.0, MeOH)	Retention time/min
1	92[a]	EtOH/H_2O (6/5)	121—123	−63.0	14.0
1'	96	EtOH/H_2O (6/5)	91— 92	−70.5	13.8
2	89	EtOH/H_2O (3/2)	199—203	−52.5	13.6
3	84	EtOH/H_2O (3/2)	227—228	−28.5	13.5
4	94[a]	EtOH/H_2O (6/5)	231—232	−58.5	13.7
4'	95	EtOH/H_2O (6/5)	198—199	−70.5	13.7
5	74	EtOH/H_2O (3/2)	227—228	−31.5	13.4
6	81	EtOH/H_2O (3/2)	236—239	−15.0	13.3
7	89[a]	EtOH	201—203	−85.5	13.9
8	90	b)	over 250	—	—
9	82	b)	over 250	—	—

a) Final coupling step. b) Washing with hot methanol.

that the restricted torsional angles of ϕ and ψ of the Aib residues efficiently hinder development of a β-sheet structure. The IR spectrum of the peptide **1** has two strong bands at 3430 cm^{-1} and 3340 cm^{-1} in the amide A region, indicating the existence of both the nonhydrogen bonding NH and hydrogen bonding NH bonds.[19] The IR spectrum of the peptide **1** has also a medium-intensity shoulder band at 1700 cm^{-1} and a strong band at 1675 cm^{-1} in the amide I region, assigned to the hydrogen-bonded urethane and free amide carbonyl groups, respectively.[20] Due to a strong preference of the Aib residues to form a helical

TABLE 3. ELEMENTAL AND AMINO ACID ANALYSES OF THE PEPTIDES **1**—**9**

Compound	Formula	Found (Calcd)			Found (Calcd)
		C/%	H/%	N/%	Leu/Aib
1	$C_{34}H_{56}N_4O_7$	64.37 (64.53)	9.28 (8.92)	8.82 (8.85)	2.91 (3)
1'	$C_{27}H_{50}N_4O_7$	59.80 (59.76)	9.50 (9.29)	10.10 (10.32)	3.13 (3)
2	$C_{56}H_{96}N_8O_{11}0.5H_2O$	63.18 (63.07)	9.47 (9.17)	10.45 (10.51)	3.11 (3)
3	$C_{78}H_{136}N_{12}O_{15}H_2O$	62.79 (62.45)	9.64 (9.27)	11.02 (11.21)	3.04 (3)
4	$C_{40}H_{67}N_5O_8$	64.65 (64.40)	9.51 (9.05)	9.35 (9.39)	3.99 (4)
4'	$C_{33}H_{61}N_5O_8$	60.59 (60.43)	9.57 (9.05)	10.24 (10.68)	4.19 (4)
5	$C_{68}H_{118}N_{10}O_{13}H_2O$	62.90 (62.74)	9.51 (9.29)	10.76 (10.76)	4.06 (4)
6	$C_{96}H_{169}N_{15}O_{18}1.5H_2O$	62.41 (62.38)	9.30 (9.38)	10.95 (11.37)	4.00 (4)
7	$C_{33}H_{54}N_4O_7H_2O$	64.13 (63.84)	9.03 (9.09)	9.00 (9.02)	3.13 (3) [a]
8	$C_{54}H_{92}N_8O_{11}H_2O$	62.78 (62.87)	9.15 (9.19)	10.82 (10.86)	3.06 (3) [a]
9	$C_{75}H_{130}N_{12}O_{14}1.5H_2O$	61.97 (62.09)	9.12 (9.24)	11.61 (11.58)	3.04 (3) [a]

a) Leu/Ala.

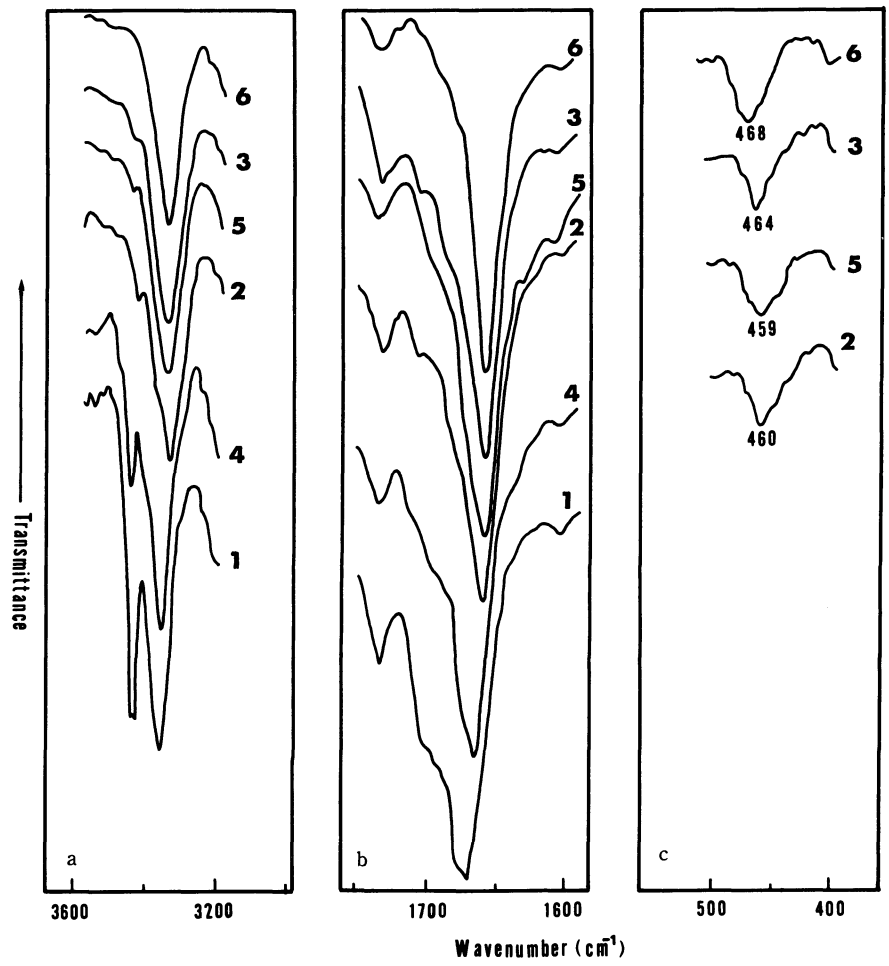


Fig. 3. IR absorption spectra of the peptides **1**—**6**. a: The amide A region, in methylene chloride; b: the amide I region, in methylene chloride; c: the far-IR, in a solid state.

conformation (3_{10}- or α-helix), the peptides **2**—**6** show strong intensity bands at 3340—3320 cm^{-1} and 1665—1659 cm^{-1}, indicating a helical structure (3_{10}- or α-helix), although the frequencies of the amide I region are rather high.[21] Weak or medium-intensity bands at 3430—3420 cm^{-1} of the peptides **2**—**6** are also assigned to the nonhydrogen bonding NH bonds, which are attributed to the NH bonds of two or three Leu residues at the N-terminal portions. The helical structure in the solid state also seems to be evidenced by a far-IR band at 468-459 cm^{-1} (Fig. 3c), which is characteristic of Leu residues with an α-helical conformation.[22] Figure 4 shows IR spectra of the peptides **8** and **9** in the solid state. Both peptides are barely soluble or insoluble in dichloromethane, and their IR measurement can not be performed in dichloromethane. In contrast with the peptides containing Aib residues, the peptides containing Ala residues (**8** and **9**) show strong bands at 3270 cm^{-1} and 1628 cm^{-1} and a weak band at 1693 cm^{-1}, assigned to antiparallel β-sheet structures.[21]

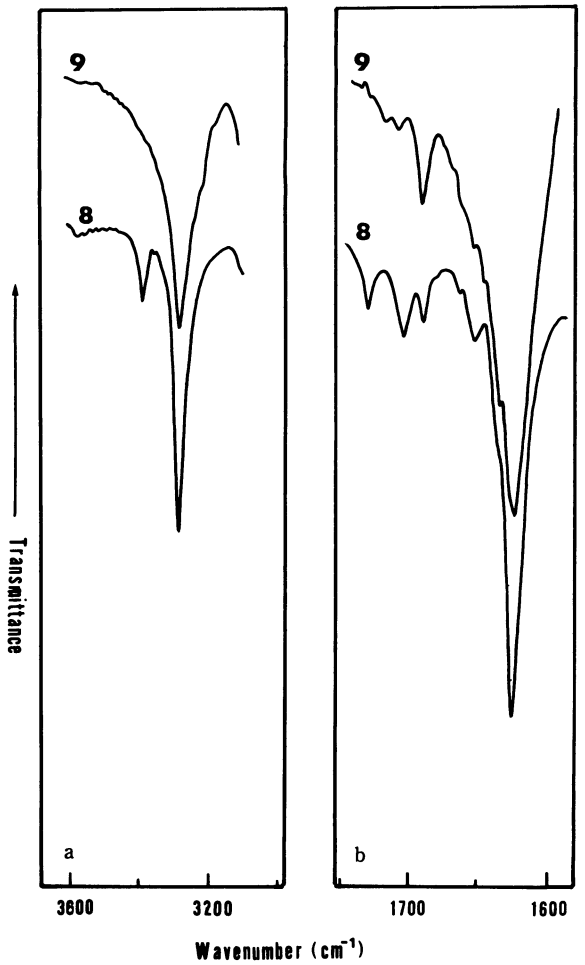


Fig. 4. IR absorption spectra of the peptides **8** and **9** in a solid state. a: The amide A region; b: the amide I region.

Discussion

As shown in Fig. 2, the carboxyl component peptides having Aib residues at the C-terminals react smoothly with amino component peptides in high yields, although the C-terminal Aib residues are sterically hindered.[23] The reaction of H-Aib-OBzl with Boc-Leu-OH is also performed by the usual stepwise method. Extreme difference in solubility between the peptides containing Ala residues and those containing Aib residues is readily explained by the conformational transformation of the peptides from β-sheet to helical structures. The ability of Aib residues to promote helical folding in peptides is outstanding in this study. The replacement of two Ala residues in the peptide **8** with two Aib residues constrains helical folding in the peptide **2**, and dramatic change takes place in solubility. Helical folding in the peptide **4** having the only one Aib residue at the C-terminal is also remarkable. Details of IR absorption conformational analyses of the peptides **1** and **4** will be presented in the following paper.[20] In the relation with this structure, the structures of the pentapeptides of Boc-Leu-Aib-Pro-Val-Aib-OMe and Boc-Ala-Aib-Ala-Aib-Aib-OMe, which were fragments of alamethicin and suzukacillin, were determined in the solid state, by X-ray diffraction analyses, to be a 3_{10}-helix.[24,25] The conformational-energy calculations for an Aib residue have demonstrated that only two small regions of the Ramachandran map are available.[26,27] The regions correspond to less than 1.0% of the total surface, where the right- and left-handed α-helices ($\phi=\pm57°$, $\psi=\pm47°$) and 3_{10}-helices ($\phi=\pm60°$, $\psi=\pm30°$) are found. 3_{10}- and α-helical conformations, however, could not be distinguished by the present IR analyses. Therefore, ^1H NMR analyses of the peptides are necessary to characterize their intramolecular hydrogen bonding patterns (3_{10}-helix, 4→1; α-helix, 5→1).[28]

In order to evaluate the influence on the nature of proteins of the replacement of a few Ala residues with Aib residues, a CPK model of the α-helical structure of Boc-$(Leu_3$-Aib$)_3$-OBzl is shown in Fig. 5. Figure 5 indicates that the replacement of C^α hydrogen atoms of Ala residues with methyl groups would have no or a little influence on the total feature of the α-helical regions of proteins and that the nature of proteins would be kept intact. The C^β_D methyl groups are identified by arrows in the model, where the C^β_D carbon atom that substitutes for the C^α hydrogen atom in Ala is designated as C^β_D. Figure 5 also indicates that the replacement of C^α hydrogen atoms of Leu residues with methyl groups is sterically permitted. The C^α hydrogen atoms are also identified by H^α in the model.

Based on the above discussion,[29] the perspective in

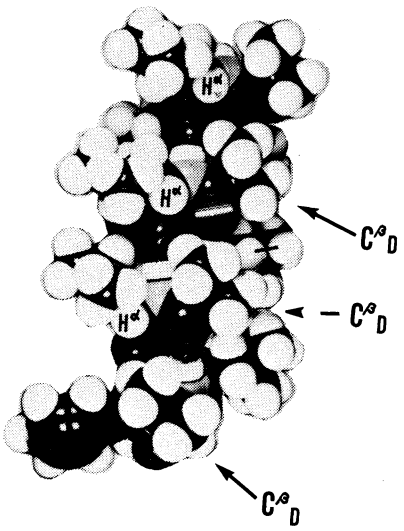


Fig. 5. The CPK model of the α-helical structure of the peptide 3. The arrows in the model indicate C^{β}_{D} methyl groups of Aib residues. The H^{α} atoms in the model indicate C hydrogen atoms of Leu residues.

the chemistry of peptides and proteins containing α,α-disubstituted α-amino acid residues gets into shape as follows: (1) Carboxyl component peptides having Aib residues at the C-terminals react with amino component peptides with no care of racemization due to the absence of chiral centers in Aib residues, and the reaction is carried out in high yields in common organic solvents using usual coupling reagents, (2) partial replacement of C^{α} hydrogen atoms with methyl groups remarkably helps improve solubility of intermediate peptide fragments included in α-helical regions of natural proteins and make further chain elongation easy in the modified protein syntheses, and (3) the replacement in α-helical regions of natural proteins keeps the nature of proteins intact.

Conclusion

The novel strategy for solubility improvement based on the restricted values of the backbone dihedral angles ϕ and ψ of Aib residues was successfully applied to model oligo(Leu)s. Partial replacement of C^{α} hydrogen atoms with methyl groups in α-helical regions of proteins is estimated, by building up the CPK model of the α-helical structure, to give no or a little influence on the nature of proteins. Thus, this strategy opens up a new breakthrough in creating proteins with novel properties which can not be achieved by genetic engineering technology.

Experimental

General. The uncorrected capillary melting points were reported. The optical rotations were taken in a 1 cm cell on a JASCO model ORD/UV-5 optical rotatory dispersion recorder. 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 were carried out with propionic acid/12 M HCl (2/1, v/v) for 2 d at 115 °C in sealed tubes. Analytical HPLC of the peptides was performed on a column packed with an acrylonitrile-divinylbenzene-styrene copolymer (7.5×500 mm)[30] using a mini micro pump NSP-800-5DX (Nihon Seimitsu Kagaku) and a refractive index detector model RI-3H (Japan Analytical Industry). The operating conditions of HPLC were the following: Column with exclusion limit of 5000; solvent, DMF; flow rate, 1 ml/min; chart speed, 0.5 cm/min; temperature, room temperature. The IR spectra in the solid state were recorded for nujor mulls with a JASCO model DS-701G spectrometer. Those in solution were recorded employing 0.025 mm pathlength cells with potassium bromide windows. The concentration of the solution was kept near 3.0 g/dl.

Preparation of the Peptides 1 and 4. DCC (11.3 g, 1.0 equiv) was added to an ice-chilled, stirred mixture of Boc-Leu-OH·H_2O (13.7 g, 1.0 equiv), H-Aib-OBzl·TsOH (20.0 g, 54.8 mmol), and NMM (5.6 g, 1.0 equiv) in dichloromethane (500 ml). The reaction mixture was stirred in an ice-chilled bath for 3 h, at room temperature overnight, and then filtered. The filtrate was washed with water, 10% citric acid, and 5% sodium hydrogencarbonate, dried over sodium sulfate, using charcol for decolorization, filtered, and concentrated *in vacuo*. The residue was used for the subsequent coupling reaction as an amino component after removal of the Boc group by treatment with 3 M HCl/AcOEt. The preparative procedure of Boc-Leu_2-Aib-OBzl, Boc-Leu_3-Aib-OBzl, and Boc-Leu_4-Aib-OBzl were essentially the same with that of Boc-Leu-Aib-OBzl. The peptides **1** and **4** were purified by recrystallization from aqueous ethanol.

Preparation of the Peptide 7. Preparation of the peptide **7** was carried out in dichloromethane (200 ml) in the same manner described above, using Boc-Leu-OH·H_2O (6.4 g, 1.1 equiv), H-Leu_2-Ala_2-OBzl·HCl (11.7 g, 23 mmol), and NMM (2.3 g, 1.0 equiv). The peptide **7** was purified by recrystallization from AcOEt.

General Procedure for Hydrogenolyses of the Peptides 1, 4, and 7. Hydrogenolyses of the peptides **1**, **4**, and **7** were performed in a mixture of acetic acid and methanol (1/5, v/v) at atmospheric pressure overnight using 10% Pd/C (10 wt.%) as a catalyst. After removal of Pd/C by filtration, the filtrate was concentrated *in vacuo*, and the residue was triturated with water. After filtration and drying, the products were purified by recrystallization from aqueous ethanol.

General Procedure for Removal of the Boc Group from the Peptides 1, 2, 4, 5, and 7. Each peptide (5 mmol) in 3 M HCl/AcOEt (30 ml) was stirred in an ice-chilled bath for 1.5 h. The mixture was concentrated *in vacuo*, followed by repetition of addition and removal, *in vacuo*, of dichloromethane. The residue was used with NMM (5 mmol) as an amino component for the subsequent coupling.

Removal of the Boc Group from the Peptide 8. The peptide **8** (5 mmol) in TFA/dichloromethane (1/2, v/v) (70 ml) was stirred at room temperature for 1 h. The solution was concentrated *in vacuo*, and the residue was dis-

solved in methanol containing an excess of NMM. The solution was poured into water to precipitate crystalline materials, which were used as an amino component for the subsequent coupling reaction.

Preparation of the Peptides 2, 3, 5, 6, 8, and 9. DCC (1.1 equiv) and HOBt (1.1 equiv) were added to an ice-chilled, stirred mixture of a carboxyl component (1.1 equiv), NMM (1.0 equiv), and an amino component. In the preparation of the peptides **2**, **3**, **5**, and **6**, the coupling reactions were carried out in dichloromethane. The reaction mixture was stirred in an ice-chilled bath for 3 h, at room temperature overnight, and then filtered. The filtrate was subjected to the same work-up procedure described above. The products were purified by recrystallization from aqueous ethanol. In the preparation of the peptides **8** and **9**, the coupling reaction was carried out in DMF and HMPA, respectively. The reaction mixture was stirred in an ice-chilled bath for 3 h, at room temperature for 2 d, and filtered. The filtrate was concentrated *in vacuo*, and the crystalline residue was purified by washing with hot methanol. The synthetic results and analytical data were shown in Tables 2 and 3.

References

1) Part I of "Peptides and Proteins Containing α,α-Disubstituted α-Amino Acid Residues." 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 denote the L-configuration. Additional abbreviations used are the following: Aib, α-aminoisobutyric acid residue; Boc, *t*-butoxycarbonyl; OBzl, benzyl ester; TsOH, *p*-toluenesulfonic acid; AcOEt, ethyl acetate; NMM, *N*-methylmorpholine; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxy-1*H*-benzotriazole; DMF, *N,N*-dimethylformamide; HMPA, hexamethylphosphoric triamide; TFA, trifluoroacetic acid; DMA, *N,N*-dimethylacetamide; NMP, *N*-methylpyrrolidone; DMSO, dimethyl sulfoxide.

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