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# The Influence of β-Alanine and 4-Aminobutyric Acid Residues on the Solubility of Peptides Containing Them[1)]

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The influence of unnatural amino acid residues, i.e., β-alanine (β-Ala) and 4-aminobutyric acid (γ-Aba) residues, on the solubility of peptides containing them was studied in organic solvents. The difference between the solubilities of peptides containing β-Ala, γ-Aba, Pro, Gly, Leu, and Asp(OBzl) was investigated by the solvent titration method by use of IR absorption spectra. The order of their solubilities is as follows, peptides containing Pro>β-Ala>γ-Aba>Asp(OBzl)>Leu>Gly. The extreme high solubility of peptides containing Pro residues is explained by the concept of "Peptide Segment Separation" caused by the tertiary peptide bond of the Pro residue. The high solubility of peptides containing β-Ala or γ-Aba residues is believed to be due to the difference of the geometries of the Gly, β-Ala, and γ-Aba residues. Their effective concentration seemed to be less important than their geometry. The role of β-Ala and γ-Aba residues in the solubility of peptides is similar to the role of Pro residues rather than Asp(OBzl), Gly, and Leu residues.

Several distinct approaches are being considered for the design and construction of synthetic peptides similar to those occurring in proteins.[2)] One of the essential factors of protein engineering is how to design the amino acid sequence of a de novo protein.[3)] We expect that the introduction of unnatural amino acids will be useful a method to enhance the stability of the structure and the activity of an artificial protein. A study of neurokinin A showed that the substitution of Gly with the more flexible β-alanine (abbreviated as β-Ala) residue lead to an analogue that was more active than the parent compound.[4)] The utilization of unnatural amino acids can offer the prospect for creating novel proteins, which can be produced by chemical synthesis.

On the other hand, one of the most serious obstacles in peptide and protein synthesis is the insolubility of protected peptides in organic solvents which causes difficulty in the successive reactions. As the insolubility is due to intermolecular hydrogen-bonded β-sheet aggregation, the disruption of the β-sheet structure caused by sufficient solvation of a peptide chain is significantly important to carry out successive reactions smoothly. Thus, the estimation of the β-sheet-structure disruption of protected peptides is essential for the design of synthetic routes for peptides and proteins. In previous papers, we demonstrated that the stability of the β-sheet structure in organic solvents is principally influenced by two factors, namely, the nature of the organic solvents and that of the protected peptides.[5—9)]

In a recent study, we proposed an estimation method for the β-sheet-structure stability of protected peptides using their <SPβ> values, which is defined as the arithmetic average of the β-sheet-structure-stabilizing potentials, SP$\beta_i$, of the amino acid residues composing the protected peptides.[10,11)] The stability of the β-sheet structure of protected peptides is dependent on the SP$\beta_i$ of the amino acid residues composing the protected peptides as well as their peptide chain lengths.[12,13)] In practice, the <SPβ> values of protected peptides were in harmony with their β-sheet-structure stability.[14)]

In the present study, considering the high flexibility of the peptide backbones of the protected peptides containing β-Ala and 4-aminobutyric acid (γ-Aba), we attempt an evaluation of the β-sheet-structure stabilizing potentials of the β-Ala and γ-Aba residues in protected peptides. The influence of the unnatural amino acid residues β-Ala and γ-Aba on the solubility of peptides containing them was studied. β-Ala and γ-Aba were inserted into the pentapeptide fragment, -Leu-Ala-Glu(OBzl)-Leu-Gly-, at intervals as shown in Fig. 1.

The protected peptides Boc–(X–Ala–Glu(OBzl)–Leu–Gly)$n$-OPac ($n$=1,2,3; X=Asp(OBzl), Gly, Leu, Pro) were synthesized for the evaluation of the β-sheet-structure-stabilizing potentials of the Asp(OBzl), Gly, Leu, and Pro residues and in protected large peptides. Particularly, the concept of "peptide segment separation" (PSS) in the peptides containing Pro residues was ascertained by use of the solvent titration method.[15)] On the basis of the results, the role of β-Ala and γ-Aba on the solubility of peptides is discussed. It is proposed that the replacement and introduction of these amino acids could be used for the design of highly functionalized proteins.

**Boc-(D(OBzl)AE(OBzl)LG)$_n$-OPac, D5 (n = 1), D10 (n = 2), D15 (n = 3)**

**Boc-(GAE(OBzl)LG)$_n$-OPac, G5 (n = 1), G10 (n = 2), G15 (n = 3)**

**Boc-(LAE(OBzl)LG)$_n$-OPac, L5 (n = 1), L10 (n = 2), L15 (n = 3)**

**Boc-(PAE(OBzl)LG)$_n$-OPac, P5 (n = 1), P10 (n = 2), P15 (n = 3)**

**Boc-LAE(OBzl)LG-β-Ala-LAE(OBzl)LG-OPac, β11**

**Boc-LAE(OBzl)LG-γ-Aba-LAE(OBzl)LG-OPac, γ11**

**Boc-LAE(OBzl)LG-β-Ala-LAE(OBzl)LG-β-Ala-LAE(OBzl)LG-OPac, β17**

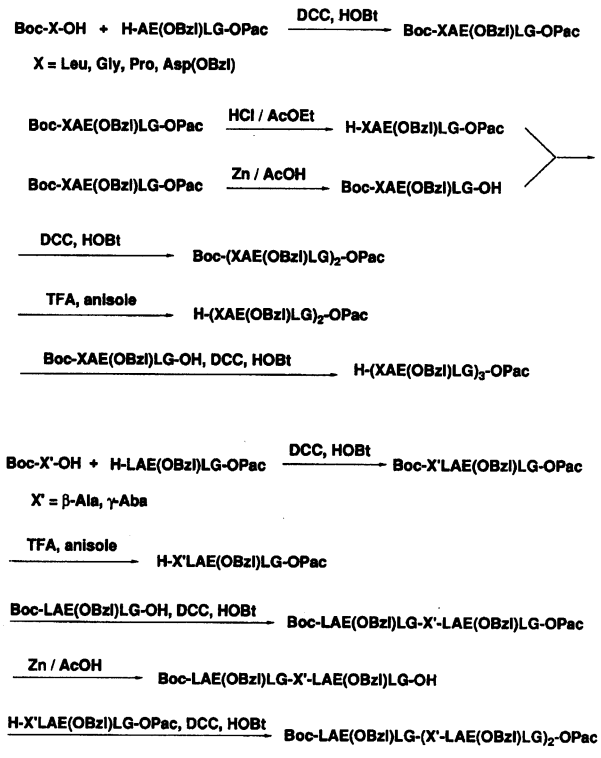
**Boc-LAE(OBzl)LG-γ-Aba-LAE(OBzl)LG-γ-Aba-LAE(OBzl)LG-OPac, γ17**

Fig. 1. The protected peptides used in this study.

## Experimental

**Materials.** The pentapeptides **D5**, **G5**, **L5**, and **P5** were prepared in $CH_2Cl_2$ by common stepwise elongation using DCC and HOBt as coupling reagents.[16] The other peptides were prepared by fragment condensation in a mixture of $CH_2Cl_2$ and DMF using DCC activation in the presence of HOBt as described before for fragment condensation.[13] The protected peptides containing β-Ala or γ-Aba were also prepared by stepwise elongation and fragment condensation, and the synthetic procedures were essentially the same as those described above. The synthetic procedure for protected peptides is illustrated in Scheme 1. In order to prepare **L15** by fragment condensation, removal of the Pac group in protected peptide **L5** was performed in Zn/AcOH. The removal of the Boc group was carried out by treatment with TFA/anisole (4/1, v/v) to give amino components. The coupling reactions were repeated until the Kaiser test became negative, using excess amounts of Boc-X–OH and DCC. After the usual work-up procedures, all products were purified by repeated washing with hot methanol. They gave a single peak on HPLC and were negative for the Kaiser test. Acid hydrolysis of the peptides was carried out with propionic acid/12 M HCl (2/1, v/v) at 115 °C for 5 days (1 M=1 mol dm$^{-3}$).[17] The amino acid ratios of the acid hydrolysates were in good agreement with the calculated values as shown in Table 1. The elemental analyses data are shown in Table 2.

**IR Absorption Spectra Measurements.** The IR absorption spectra of the peptides in solution or in the suspended state were recorded at room temperature with a JEOL Model JIR-100 FT-IR spectrometer by employing 0.5 mm-path length cells with sodium chloride windows or ditched sodium chloride plates. The peptides were dissolved or suspended in $CH_2Cl_2$ containing a variety of concentrations of DMSO. The peptides in the suspended state were recorded by putting them between ditched sodium chloride plates. The concentration of every peptide was kept around $1.6\times10^{-2}$ M.




Scheme 1.

## Results

The stability of the β-sheet structure was examined for protected penta- to heptadecapeptides. It was evaluated by monitoring the β-sheet-structure-disrupted behaviors in $CH_2Cl_2$ using a solvent-titration method introduced by Toniolo et al.[15] The β-sheet structure of the protected peptides was disrupted in $CH_2Cl_2$ by adding increasing amounts of DMSO. The IR absorption spectra of the protected peptides, except for protected peptides **D5** and **P5** swollen in $CH_2Cl_2$ alone, showed strong bands around 3280 cm$^{-1}$ in the amide A region and around 1630 cm$^{-1}$ in the amide I region, assigned to a β-sheet structure. The behavior of the β-sheet-structure disruption was monitored in $CH_2Cl_2$ by a successive decrease in the intensity of the band around 1630 cm$^{-1}$ and an increase in the band around 1670 cm$^{-1}$, mainly assigned to an unordered structure,[18] resulting from successive addition of titrating solvent DMSO. Figure 2 shows the typical IR absorption spectra of Boc–Leu–Ala–Glu(OBzl)–Leu–Gly–OPac in $CH_2Cl_2$ containing a variety of molar concentrations of DMSO. The solvent-titration curves of the protected peptides (Figs. 3 and 4) are depicted using the relative intensities of the bands around 1630 cm$^{-1}$, which were determined by using the bands around 1760 cm$^{-1}$ and 1730 cm$^{-1}$ due to the ester carbonyl groups of Gly–OPac and Glu(OBzl), respectively, as a standard and normalizing to be 1.0 for each relative intensity in $CH_2Cl_2$ alone. As shown in Figs. 3 and 4, successive addition of titrating solvent induced a dramatic decrease in the strong band around 1630 cm$^{-1}$.

## Discussion

By using the IR absorption spectra of the swollen state of the protected peptides, the solvent-titration curves were obtained as shown in Figs. 3 and 4. On the basis of the solvent-titration curves of Boc–(Leu–Ala–Glu(OBzl)–Leu–Gly)$n$-OPac ($n$=1,2,3), the β-sheet-structure stability of the protected peptides containing β-Ala and γ-Aba could be estimated.

The β-sheet structure of protected peptides **L5**, **L10**, and **L15** became more stable with increasing peptide chain length up to the pentadecapeptide. In practice, it was difficult to destroy the β-sheet structure of the protected pentadecapeptide even in DMSO alone. In previous papers,[14] we proposed that the β-sheet-structure stability of protected peptides depended on the <SPβ> value and the peptide chain length. The protected peptide **L15** has many more peptide bonds than peptides **L5** and **L10**. Hence, peptide **L15** was considered to be more sensitive to the influence of effective

Table 1. Amino Acid Analyses of the Protected Peptides

| Protected peptide | Found (Calcd) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ala | Glu | Leu | Gly | Asp | Pro | β-Ala | γ-Ala |
| **D15** | 3.32(3) | 2.95(3) | 3.26(3) | 2.86(3) | 3.00(3) | — | — | — |
| **G15** | 3.00(3) | 2.69(3) | 3.04(3) | 5.76(6) | — | — | — | — |
| **L15** | 3.00(3) | 2.64(3) | 6.21(6) | 2.73(3) | — | — | — | — |
| **P15** | 2.93(3) | 2.83(3) | 3.22(3) | 3.00(3) | — | 3.30(3) | — | — |
| **β11** | 1.93(2) | 1.92(2) | 4.02(4) | 1.90(2) | — | — | 0.80(1) | — |
| **γ11** | 2.02(2) | 2.00(2) | 3.89(4) | 2.05(2) | — | — | — | 0.95(1) |
| **β17** | 2.97(3) | 2.88(3) | 6.10(6) | 3.00(3) | — | — | 1.88(2) | — |
| **γ17** | 3.10(3) | 2.88(3) | 6.10(6) | 3.00(3) | — | — | — | 2.08(2) |

Table 2. Elemental Analyses of the Protected Peptides

| Protected peptide | Formula | Found (Calcd) | | |
|---|---|---|---|---|
| | | C/% | H/% | N/% |
| **D15** | $C_{47}H_{59}N_5O_{13}$ | 61.82 (62.58) | 6.69 (6.59) | 7.73 (7.76) |
| **G15** | $C_{38}H_{51}N_5O_{11}$ | 59.55 (60.54) | 6.91 (6.82) | 9.23 (9.29) |
| **L15** | $C_{42}H_{59}N_5O_{11}$ | 62.05 (62.28) | 7.41 (7.34) | 8.65 (8.65) |
| **P15** | $C_{41}H_{55}N_5O_{11}$ | 60.47 (62.03) | 7.08 (6.98) | 9.04 (8.82) |
| **β11** | $C_{74}H_{107}N_{11}O_{19}$ | 60.73 (61.10) | 7.47 (7.41) | 10.50 (10.59) |
| **γ11** | $C_{75}H_{109}N_{11}O_{19}$ | 60.39 (61.33) | 7.65 (7.48) | 10.79 (10.49) |
| **β17** | $C_{106}H_{155}N_{17}O_{27}$ | 59.24 (60.64) | 6.63 (7.44) | 11.49 (11.34) |
| **γ17** | $C_{108}H_{159}N_{17}O_{27}$ | 60.22 (60.97) | 7.85 (7.53) | 11.11 (11.19) |

concentration[19] and the β-sheet structure of **L15** became more stable than that of **L5** and **L10**.

Nevertheless, when a β-Ala residue was inserted in the sixth and twelfth positions from the N-terminal in peptides **L10** and **L15**, the β-sheet structures of protected peptides **β11** and **β17** were easily disrupted by titrating solvent in spite of having a longer peptide chain length than peptides **L10** and **L15**. Also, when γ-Aba was inserted in the sixth and twelfth positions from the N-terminal inpeptides **L10** and **L15**, the β-sheet structures of protected peptides **γ11** and **γ17** were more easily disrupted than peptides **L10** and **L15**. This means that regarding these peptides, the influence of effective concentration on the β-sheet-structure stability is not very strong.

The secondary and tertiary structure of the protein is based on the restriction of the values of the backbone dihedral angles $\phi$ and $\psi$.[20] Due to the replacement of a $C^\alpha$ hydrogen atom of Ala with a methyl group, an Aib (α-aminoisobutyric acid) residue disturbs the β-sheet structure and promotes helical folding in peptides.[12,13] The stabilizing effect of the Aib residue on the helical structure is due to the restriction of the values of the backbone dihedral angle $\phi$ and $\psi$ of the Aib residue. On the other hand, the Aib and γ-Aba residues are isomeric with respect to each other, but γ-Aba has a greater freedom of peptide backbone dihedral angles than Aib. In a previous paper,[21] we found that a protected homooligo peptide of Gly has a high potential for β-sheet-structure formation because of the freedom of the backbone dihedral angles of Gly residues in the β-sheet structure. The flexibility of the Gly residue is attributed to the fact that there is no substituent on the $C^\alpha$ atom of the Gly residue. Actually, the protected peptides containing Gly residues, **G5**, **G10**, and **G15**, have a high potential for β-sheet-structure formation as shown in Fig. 4. The β-sheet structure of protected pentadecapeptide **G15** is retained even in a high-polar solvent, such as DMSO alone.

However, the β-sheet structure of protected peptides containing β-Ala and γ-Aba residues is easily disrupted by titrating solvent in spite of containing β-Ala and γ-Aba residues that have free backbone dihedral angles $\phi$ and $\psi$. The flexibility of the protected peptide containing β-Ala and γ-Aba residues is much greater than that of the peptides containing Gly residues, because of the fact that there is no substituent on the $C^\alpha$ and $C^\beta$ atoms of the β-Ala and the $C^\alpha$, $C^\beta$, and $C^\gamma$ atoms of the γ-Aba residues. These facts indicate that the potential for β-sheet-structure formation of Gly, β-Ala, and γ-Aba is independent of the freedom of the backbone dihedral angles. When the β-sheet structure is formed by the protected peptide containing β-Ala and γ-Aba residues, the hydrogen-bonding formation of the peptide backbone changes into an irregular style as shown in Fig. 5. Using CPK models of the antiparallel β-sheet structure of protected peptides **β11**, **γ11**, **β17**, and **γ17**, we actually confirmed the change of the hydrogen-bonding form. We proposed that the irregular hydrogen bond form of the peptides was the reason that the β-sheet structure of protected peptides **β11**, **γ11**, **β17**, and **γ17** was disrupted easily. Therefore, the effective concentration and the flexibility of the backbone dihedral angles of the peptides are thought to be less important than the regularity of the hydrogen-bonding form.

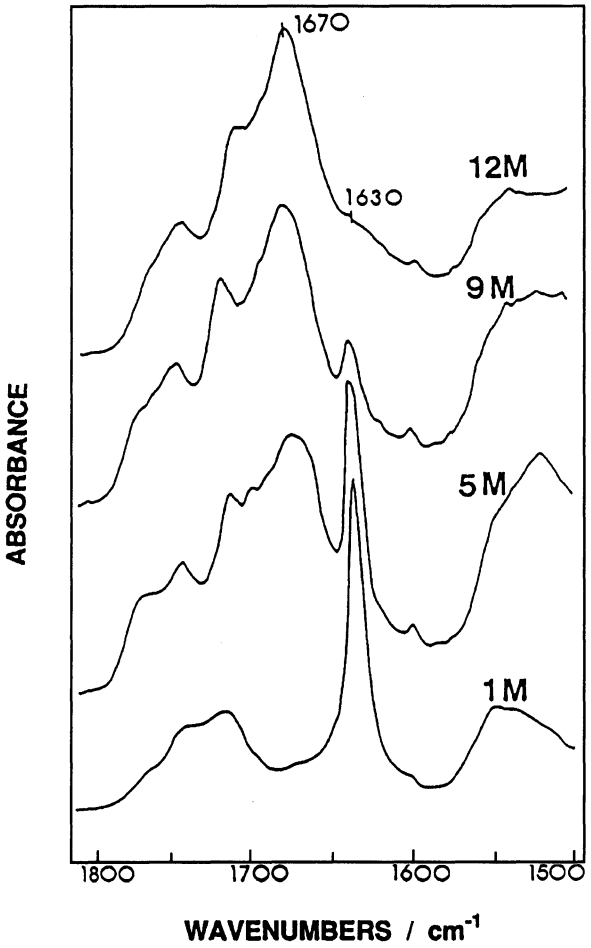



Fig. 2. Typical IR absorption spectra in the amide I region of protected peptide **L10** in $CH_2Cl_2$ containing a variety of molar concentrations of DMSO. The numerals in the figure indicate the molar concentrations of DMSO.

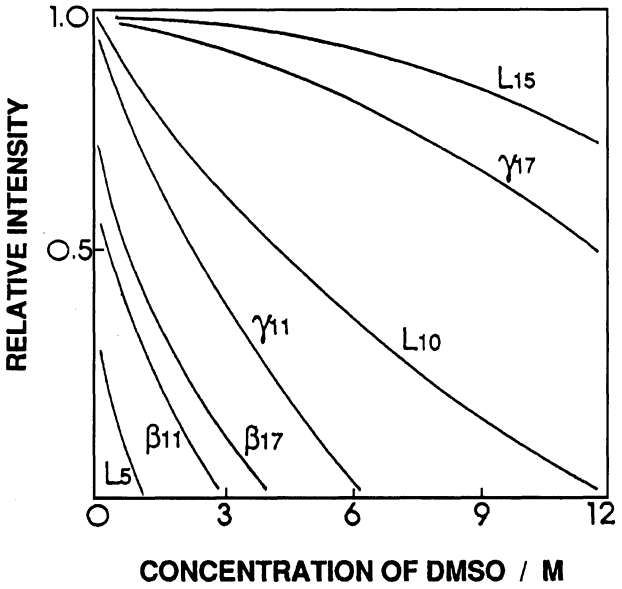



Fig. 3. The solvent titration curves of protected peptides **L5**, **L10**, **L15**, **β11**, **β17**, **γ11**, and **γ17** in $CH_2Cl_2$ using DMSO as a titration solvent.

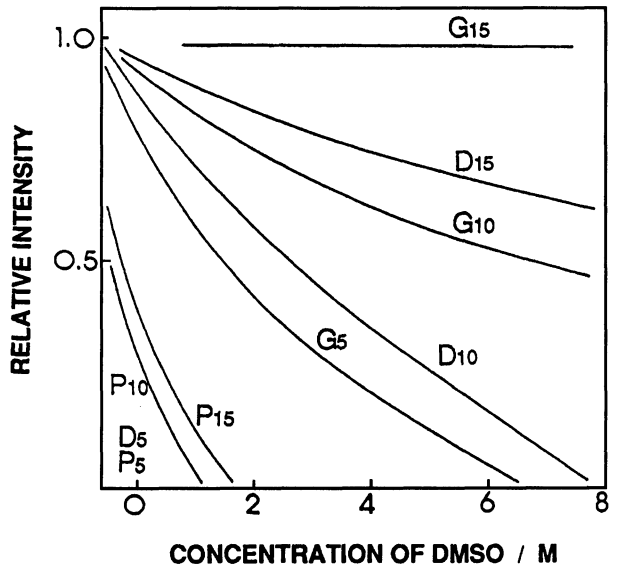



Fig. 4. The solvent titration curves of protected peptides **D5**, **D10**, **D15**, **G5**, **G10**, **G15**, **P5**, **P10**, and **P15**, in $CH_2Cl_2$ using DMSO as a titration solvent.

Our previous studies on the conformational analysis of human proinsulin C-peptide fragments indicated that the estimation of β-sheet-structure stability could be independently applied to each peptide segment separated by tertiary peptide bonds such as X–Pro bonds, in which X stands for an arbitrary amino acid residue.[22,23] In practice, the concept of PSS is demonstrated by using peptides **P5**, **P10**, and **P15**. It is estimated that the β-sheet-structure stability of protected peptide **P15** is like that of peptide **P10** and the influence of the effective concentration on the β-sheet-structure stability is weak in peptides containing a Pro residue. The β-sheet-structure stability of protected peptides containing Pro residues can be treated separately for each peptide segment. In conclusion, the order of the β-sheet-structure-stabilizing potential of Asp(OBzl), Gly, Leu, Pro, β-Ala, and γ-Aba residues in the peptide is derived as follows: Gly>Leu>Asp(OBzl)>γ-Aba>β-Ala>Pro. The result obtained here for Asp(OBzl), Gly, Leu, and Pro residues is also in agreement with the SPβ values estimated in a previous paper.[11] The β-sheet-structure-disrupting behavior of peptides **β11** and **β17** containing β-Ala is quite similar to that of peptides containing Pro residues. This result further indicates that the β-sheet structure of the peptide segment is separated by the insertion of β-Ala residues. The insertion of the β-Ala residue plays the role of "peptide segment separation" effectively to improve the solubility of protected peptides. The use of this new methodological idea can eliminate solubility problems caused by β-sheet formation.

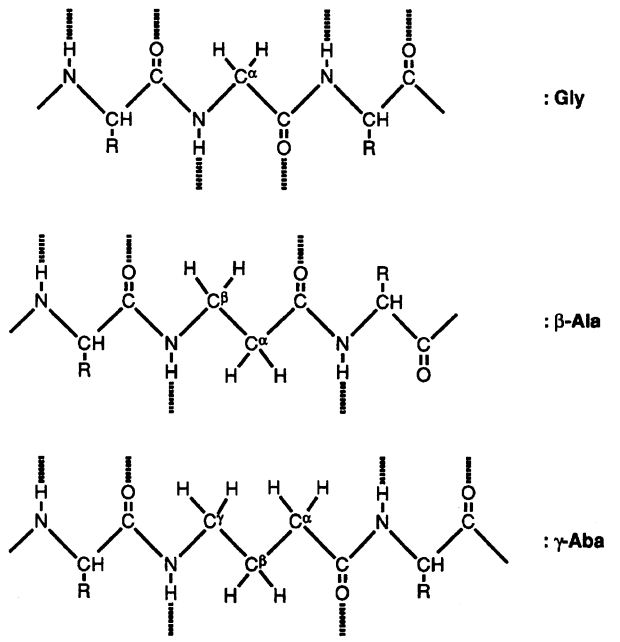



Fig. 5. The hydrogen-bonding form of the peptide backbone containing Gly, β-Ala, and γ-Aba residues.

The Gly residues in globular proteins have various of the backbone dihedral angles $\phi$ and $\psi$ as shown by X-ray diffraction studies.[24] Thus, Gly residues often appear in turn structures, such as the surface region of globular proteins.[25] Since the β-Ala residue has a low β-sheet-structure-stabilizing potential and is more flexible than the Gly residue, it is estimated that we can suitably replace the Gly residue with the β-Ala residue in the surface region of globular proteins without changing the protein structure dramatically.[4] The replacement of a Gly residue with a β-Ala residue offers the prospect of eliminating the insolubility problems of peptide and protein intermediates in synthetic procedures.

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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. Additional abbreviations used are the following: β-Ala, β-alanine; γ-Aba, 4-aminobutyric acid; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; TFA, trifluoroacetic acid; AcOEt, ethyl acetate; Boc, *t*-butoxycarbonyl; Pac, phenacyl; OBzl, benzyl ester; AcOH, acetic acid; IR, infrared; DCC, dicyclohexylcarbodiimide; HOBt, 1*H*-1,2,3-benzotriazol-1-ol.

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