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Simple and efficient syntheses of Boc- and Fmoc-protected 4(*R*)- and 4(*S*)-fluoroproline solely from 4(*R*)-hydroxyproline

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Abstract—As building blocks of collagen model peptides, Boc- and Fmoc-protected 4(*R*)- and 4(*S*)-fluoroproline, which will be widely used in peptide synthesis including solid-phase strategy, were synthesized from the readily available 4(*R*)-hydroxyproline in higher yield than with conventional methods. To establish the stereospecificity of the Mitsunobu reaction and the subsequent fluorination that were presumed to cause the inversion of configuration at the C-4 position of a proline derivative, the absolute configuration of one of the key products, Boc-4(*S*)-fluoroproline, was determined by X-ray crystallography.

1. Introduction

Various polytripeptides have been synthesized as collagen model peptides on the hypothesis that the unique amino acid sequence of collagen, which has glycine in every third position and approximately 300 repeats of X-Y-Gly where X is often proline and Y is often 4(*R*)-hydroxyproline (4(*R*)-Hyp), should result in its unique triple helical structure.[1–3] Their physico-chemical properties have been intensively investigated.

For example, we synthesized a series of polytripeptide $(X\text{-}Y\text{-}Gly)_n$ with defined number of n by a solid phase polycondensation of tripeptides, X-Y-Gly.[4,5] The results showed that $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}4(R)\text{-}Hyp\text{-}Gly)_{10}$ have triple helical structures at lower temperature and undergo thermal transition to single random coil states.[6,7] The transition temperature of the latter was much higher than that of the former.[6–8] The additional thermal stabilities provided by the existence of Hyp residues are consistent with the co-relation found in naturally occurring collagen molecules themselves between their Hyp contents and thermal transition temperatures. However, $(4(R)\text{-}Hyp\text{-}Pro\text{-}Gly)_{10}$, $(4(S)\text{-}Hyp\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}4(S)\text{-}Hyp\text{-}Gly)_{10}$ do not form triple helices and exist as single coils.[9,10]

In order to clarify what forces are controlling the self-assembly of these chains and how a Hyp residue stabilizes or destabilizes the triple structure, X-ray analyses were carried out on crystals of these peptides.[11,12] Although intra- and inter-chain hydrogen bond formations were discussed extensively both directly and through water molecules, the contribution of Hyp residues were not well established.

Recently Raines and co-workers synthesized $(Pro\text{-}4(R)\text{-}fPro\text{-}Gly)_{10}$, where 4(*R*)-fPro is 4(*R*)-fluoroproline, and showed that it takes the most stable collagen mimic among the all polytripeptides mentioned here.[13,14] They explained the increased stability by the stereoelectronic effects coming from the electronegativity of fluorine atom which fixes the pyrrolidine ring pucker and makes all three main-chain torsion angles; ω, φ and ψ, favorable to fit the triple helix formation.[15] The inductive effects of hydroxyl group of Hyp were analogous to those of fluorine atom in the typical derivatives of fPro.[13–15] The different angles correlated with C^{γ} pucker of the *X*-position and the *Y*-position. This was also pointed out later by Zagari and co-workers with X-ray analysis on $(Pro\text{-}Pro\text{-}Gly)_{10}$ at higher resolution. Furthermore, the preferential ring pucker was applied to the case with 4(*S*)-Hyp analogues as well.[16]

Despite these explanations we seem to solve the long-unresolved questions regarding the factors to control the thermal stability of the collagen-like triple helical structure. To do this we would like to inspect them by systematic thermodynamic experiments on the series of polytripeptides

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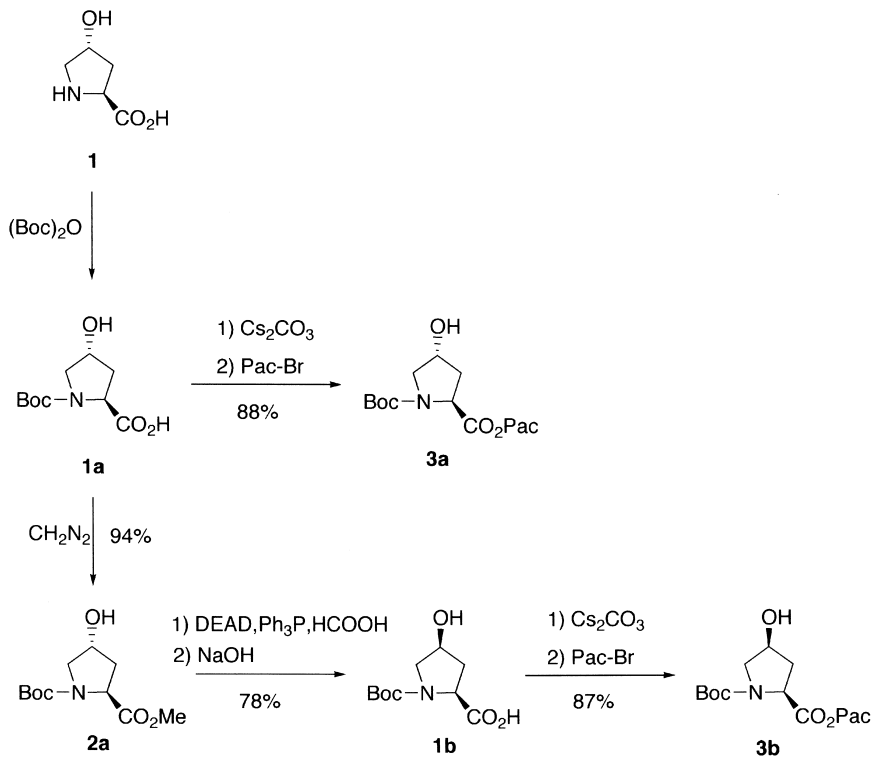


Scheme 1.

in solution. Thus various collagen models containing diastereomers of fPro as well as those of Hyp need to be prepared for further comprehensive thermodynamic studies. For this purpose, solid phase peptide synthesis can provide us with model peptides with an appropriate quantity and quality if the requisite *tert*-butyloxycarbonyl (Boc) or 9-fluorenyl-methoxycarbonyl (Fmoc) derivative of fPro is available in large amounts. A diastereomer of fPro has been prepared by a stereospecific displacement of the hydroxyl group of Hyp by fluorine; this includes the synthesis of 4(*R*)-fPro from the *N*-protected-4(*S*)-Hyp derivatives,[17–19] and that of Boc-4(*R*)- and Boc-4(*S*)-fPro by the inversion of configuration at the C-4 atoms of the corresponding *N*-Boc-Hyp derivative.[17,18,20] In particular, Demange et al. employed the Mitsunobu reaction[21] to obtain *N*-Boc-4(*S*)-Hyp-OH from the 4(*R*) isomer in 58% yield, which successfully managed to alleviate the difficulty in relatively a lower availability of *N*-Boc-4(*S*)-Hyp-OH as the starting material for the synthesis of 4(*R*)-fPro-OH.[20] Perhaps this also serves to enhance the practicality of alternative methods that require the derivatives of *N*-Fmoc-4(*S*)-Hyp-OH for the synthesis of Fmoc-4(*R*)-fPro-OH.[22,23] However, there still remain a few problems to solve: (1) The reaction condition should be optimized so that the requisite 4(*R*)-fPro derivative may be obtained more easily in an appreciably improved overall yield. (2) Neither the Mitsunobu reaction nor the fluorination has been guaranteed to undergo in a manner of purely the S_N2 reaction or even an enantio-selective one with a certain extent of racemization, while the optical purity of an amino acid is of particular concern for use in solid-phase peptide synthesis.

We suggest here a simpler and more efficient method for the preparation of 4(*S*) and 4(*R*) diastereomers of Boc- and Fmoc-fPro starting solely from 4(*R*)-Hyp-OH. The X-ray diffraction analysis of Boc-4(*S*)-fPro-OH together with the analysis of all the reaction products using a chiral column in reversed phase high-performance liquid chromatography (RP-HPLC) demonstrates unambiguously that the method including the Mitsunobu reaction and the subsequent fluorination proceeds stereospecifically as we had expected.

2. Results and discussion

2.1. Synthesis of 4(*S*)-Hyp derivatives from 4(*R*)-Hyp-OH

In order to elaborate a simple and economical route for the preparation of Boc- and Fmoc-protected 4(*R*)- or 4(*S*)-fPro derivatives, we chose *N*-Boc-4(*R*)-Hyp-OH (**1a**) as the sole starting material because it is much less expensive than the 4(*S*) isomer and is readily available. For the large-scale preparation of 4(*S*)-fPro from the 4(*R*)-Hyp derivative, we basically followed the method involving the Mitsunobu reaction as reported by Demange et al.[20] with some modifications. After several trials with a variety of amino- and carboxyl-protected 4(*R*)-Hyp derivatives, the most satisfactory result was obtained in the one-pot synthesis of *N*-Boc-4(*S*)-Hyp-OH (**1b**) from *N*-Boc-4(*R*)-Hyp-OMe (**2a**) as shown in Scheme 1. The yield of **1b** in this reaction was 78%, which amounted to roughly 20% better than that of a conventional method[20] starting from *N*-trityl-4(*R*)-Hyp-OH. Obviously, this improvement in yield is due to the use of the less acid-labile protective group of Boc rather than the trityl group. By avoiding the need of exchanging these *N*-protective groups, we could carry out the reaction in one pot, which led to a substantially improved yield.

It seems convenient to use the phenacyl (Pac) ester,

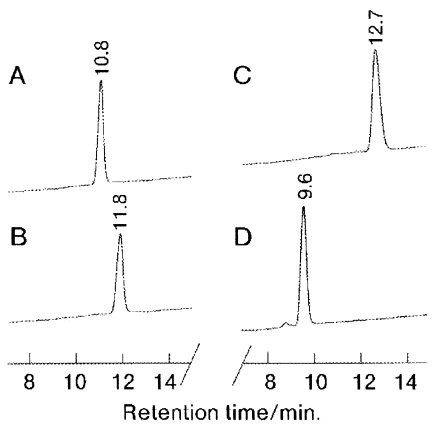


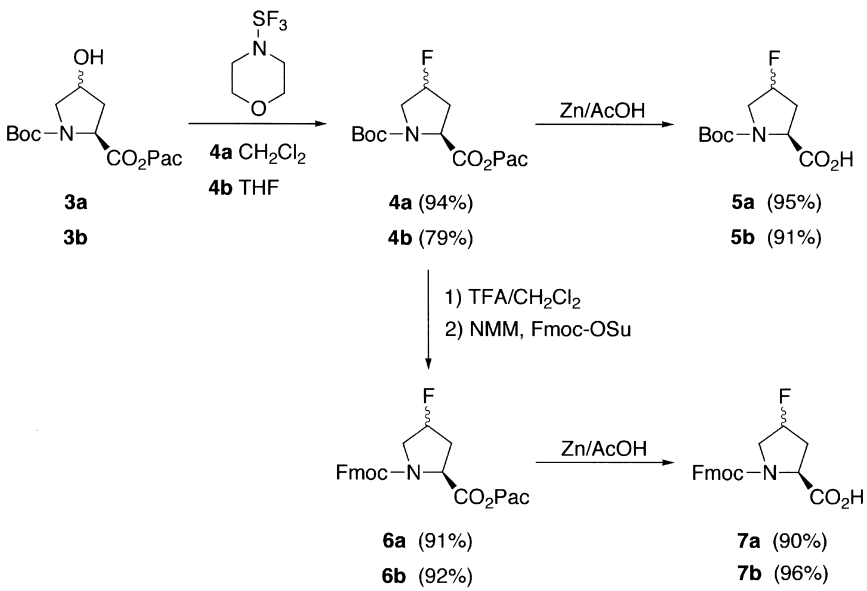
Figure 1. HPLC profiles of **3a** (A), **3b** (B), **4a** (C) and **4b** (D) at 20°C at a sample concentration of 0.2 mg/mL in ethanol. Column: Daicel CHIRALPAK AS (4.6×250 mm). Eluent: 0–40% hexane in ethanol in 20 min. Flow rate: 1.5 mL/min.

N-Boc-4(*R*)-Hyp-OPac (**3a**), as the starting material because **3a** is convertible not only to Boc-4(*S*)-fPro-OPac (**4a**) but also to Boc-4(*R*)-fPro-OPac (**4b**) through the fluorination of *N*-Boc-4(*S*)-Hyp-OPac (**3b**). The merits of the Pac group are that it is very stable to acidolytic conditions of Boc deprotection and that it can be removed by the reduction with Zn/AcOH in good yield (usually more than 90%),[24] keeping both Boc and Fmoc groups intact. However, the yield of **3b** directly from the 4(*R*) isomer (**3a**) was prohibitively low (35%) to be rated as practical. The steric hindrance of the Pac group against the Mitsunobu reagents and undesirable hydrolysis concomitant with the treatment of reaction intermediate with NaOH could possibly reduce the yield of **3b**. Instead of taking this route, we therefore chose the present one (Scheme 1) to obtain **1b**, from which the Pac ester (**3b**) was prepared by the standard procedure of esterification. Although the direct conversion from **3a** to **3b** was thus bypassed, the overall yield of **3b** from **1a** via **1b** was 64%, which still exceeded that attained through the shorter pathway.

The optical purity of **3a** and **3b** was checked by chiral HPLC analysis. In principle, an ordinary RP-HPLC would suffice for the analysis of diastereomers, but HPLC with a chiral column enabled us to distinguish these isomers more clearly so that even a minute content of racemization or epimerization product could be discerned. As shown in Fig. 1(A) and (B), each compound exhibited a single peak, resolved well enough to discriminate one from another; the retention times for **3a** and **3b** were 10.8 and 11.8 min, respectively. This result indicates that the Mitsunobu reaction leading to **1b** has undergone with virtually complete stereochemical inversion in a manner of the S_N2 reaction, rendering the C-4 position of **3b** the *S*-configuration.

2.2. Conversion of the Hyp to fPro derivative

We obtained Boc-4(*S*)-fPro-OPac (**4a**) and Boc-4(*R*)-fPro-OPac (**4b**) by the fluorination of **3a** and **3b**, respectively, with morpholinosulphur trifluoride (Scheme 2). The geometry of **4a** was tentatively assigned to the 4(*S*)-configuration by the assumption that the substitution of the hydroxyl group with fluorine is a typical S_N2 reaction; this finally proved to be correct by allowing for the results of the X-ray analysis of **5a** as described below. Note that the configuration at the C-4 atom is inverted during the fluorination, by which **3b** can also be converted to **4b** in the same way. The outcome of the reaction was, however, not quite the same for each product: when CH_2Cl_2 was used as a solvent, the yield of **4a** from **3a** was as high as 94%, while that of **4b** from **3b** was 56%. In contrast, the yield of **4b** from **3b** was improved up to 79% by changing the solvent from CH_2Cl_2 to tetrahydrofuran (THF). The products, **4a** and **4b**, were analyzed by chiral HPLC with respect to the optical purity. Similarly to the chromatograms of **3a** and **3b**, there appeared a single peak for each sample of **4a** and **4b** (Fig. 1C and D). The difference in retention times between the peaks of these diastereomers was more than 3 min, sufficient to rule out the possibility of overlooking the contamination of one component with the



Scheme 2.

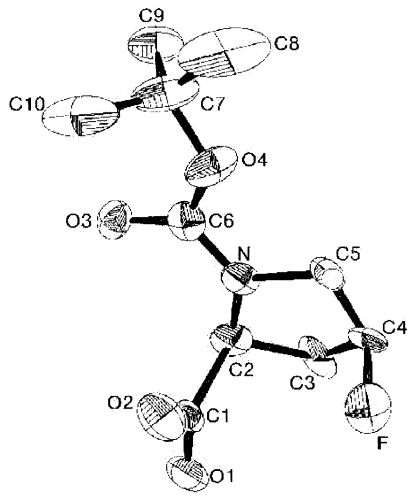


Figure 2. ORTEP view of the X-ray crystal structure of **5a**. Each atom is drawn with 50%-probability thermal ellipsoid.

counterpart. These results indicate that **4a** and **4b** have been obtained through an almost complete inversion of configuration at the C-4 position of their respective precursors. In the reaction of a derivative of 4(*R*)-*O*-tosyl-Hyp with fluoride ion, it was reported that 17% of the fluorination product retained the *R*-configuration at the C-4 atom.[19] The retention is considered to arise from the intra-molecular participation of the ester carbonyl in the displacement process. It is therefore remarkable that the reaction of **3a** with morpholinosulphur trifluoride appeared to proceed without retention of configuration, while the ester carbonyl group of **3a** is in the same disposition to the hydroxyl group as that of the 4(*R*)-*O*-tosyl-Hyp derivative.

2.3. Syntheses of Boc and Fmoc-fPro-OH and X-ray diffraction analysis of 5a

By removing the Pac group with Zn/AcOH, we obtained Boc-4(*S*)-fPro-OH (**5a**) and Boc-4(*R*)-fPro-OH (**5b**) in 90–94% yields. A drawing of the crystal structure revealed by X-ray diffraction analysis shows the geometry at the C-4 position of **5a** to be the *S*-configuration (Fig. 2). Referring to this configuration of **5a**, we can now unambiguously determine the geometry of every reaction product obtained in the present method. This also confirms that both the Mitsunobu reaction and the substitution of the hydroxyl group with fluorine occur almost exclusively with inversion of configuration at the C-4 position.

For the solid phase synthesis of collagen mimics based on the Fmoc chemistry, each isomer of Fmoc-fPro-OH (**7a** and **7b**) was also derived from the corresponding isomer of Boc-fPro-OPac (**4a** and **4b**). The procedure consists of: (1) Boc removal in trifluoroacetic acid (TFA), (2) amino protection by Fmoc-OSu, and (3) deprotection of the Pac group (Scheme 2). To prevent a serious lowering of yield owing to extremely high solubility of carboxyl-free fPro derivatives in water, we deliberately avoided removing the Pac group before acylation that reportedly has a likelihood of causing racemization at the C^{α} position.[25] Judged from the results of HPLC analysis with a chiral column that can distinguish between a pair of enantioners as well as diastereomers, it was fortunate that contamination of **7a** and **7b** with their epimerization products turned out to be negligible. Even though there should arise a further need to purify these products, a task of removing such a diastereomer contaminant in a small amount is much less laborious compared to that of resolving a mixture of amino-acid enantiomers in general. Therefore a small extent of epimerization at this stage, if any, may not seriously degrade the efficacy of our method as a whole.

2.4. Stereochemistry of the Mitsunobu reaction and the fluorination

The Mitsunobu reaction of **2a** to **1b** appeared to proceed with a complete inversion of the configuration as revealed by the HPLC analysis of the reaction product. We also found that **4a** and **4b** were produced by the S_N2 reaction of **3a** and **3b**, respectively. There arose, however, a problem of a much poorer performance of the reaction from **3b** to **4b** than that from **3a** to **4a** under the same reaction conditions. In unfavorable cases, poor yield can often be associated with low stereoselectivity of the reaction.

Fluorination of **3b** gave **4b** in various yields ranging from 56 (in CH_2Cl_2) to 79% (in THF) depending on solvent conditions. It is likely that the cause of variation in these yields is attributable to a solvent-dependent conformational change of **3b**. When IR spectra of **3a** and **3b** were compared in CH_2Cl_2 (Fig. 3(A) and (B)), there appeared noticeable differences in the amide A region and the amide I region. In the amide A region, **3a** had one sharp absorption band at 3603 cm^{-1}, while **3b** had one additional broad band around 3508 cm^{-1} to a sharp one at 3602 cm^{-1}. In the amide I region, **3a** had two bands at 1759 and 1704 cm^{-1}, while **3b** had three at 1762, 1746 and 1702 cm^{-1}. We regard these additional bands of **3b** as the clue to indicate the presence of intra-molecular hydrogen bond between the hydroxyl group and the carbonyl group of the ester moiety. This is because hydrogen bonding tends to alter the stretching frequencies of bands due to both the proton donor and acceptor

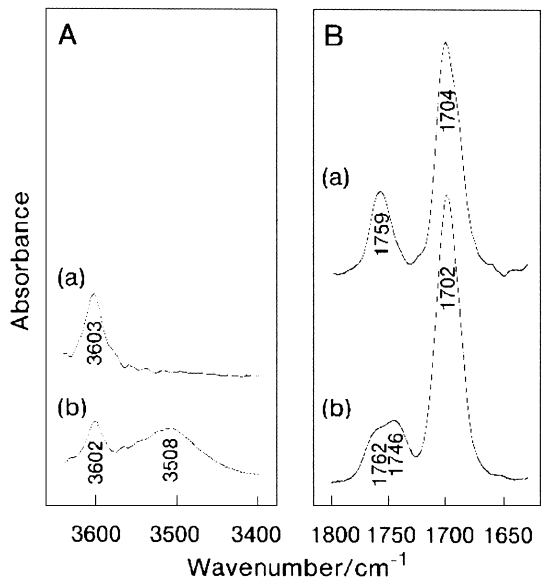


Figure 3. FT-IR spectra of **3a** (a) and **3b** (b) at a sample concentration of 1.0 mM in CH_2Cl_2. Parts of the spectra of the amide A region and the amide I region were shown in (A) and (B), respectively.

groups.[26,27] The relevant bands in the present case are those with wavenumbers at 1746 cm^{-1} ($\nu_{C=O}$ of the ester carbonyl) and at 3508 cm^{-1} (ν_{O-H}). If this hydrogen bonding interferes with the attachment of hydroxyl oxygen to the sulphur atom of morpholinosulphur trifluoride, activation of the hydroxyl group into the better leaving group and the subsequent nucleophilic attack of fluoride ion become less likely to occur. Note that only the 4(*S*)-configuration of **3b** permits the intra-molecular hydrogen bonding possible (see, for example, the structure shown in Scheme 2). In THF, where the reaction with the fluorinating reagent appeared to proceed without such an interference and eventually gave **4b** in considerably enhanced yield (79%), which is roughly comparable with the case affording **4a** (89%), the hydrogen bond in **3b** could be very weak or absent altogether.

Because the yield of **4a** is indistinctly lower in THF (89%) than in CH_2Cl_2 (94%), it is unlikely that THF plays some intrinsic role in facilitating the reaction. The improved efficiency in fluorination to give **4b** by using THF in place of CH_2Cl_2 is thus regarded as a result of releasing the 4(*R*)-Hyp derivative from forming an undesirable conformation in which an intra-molecular hydrogen bond is assumed to interfere with the reaction. Allowing for a remarkably high yield of **4a** achieved in CH_2Cl_2, we expect to be able to improve the yield of **4b** a little further by optimizing the reaction conditions with respect to the choice of the protective group as well as solvent.

2.5. Syntheses of 4(*R*) and 4(*S*) isomers of Fmoc-fPro-OH

For the syntheses of Fmoc-fPro-OH (**7a**: 4(*R*) and **7b**: 4(*S*)), it would also be envisaged that an appropriate ester of Fmoc-Hyp-OH might serve for direct fluorination as suggested by other authors.[22,23] Although our method does have the option to utilize **1b** as a precursor to *N*-Fmoc-4(*R*)-Hyp-OH, the yield of 83%[23] in the conversion of *N*-Fmoc-4(*S*)-Hyp-OBzl (Bzl: benzyl) to Fmoc-4(*R*)-fPro-OBzl is not impressive enough to let us prefer this reaction to the corresponding pathway leading to **4b** from **3b** in 79% yield. One of the main features of the present method is that it can provide both 4(*S*) and 4(*R*) diastereomers of fPro derivatives starting solely from 4(*R*)-Hyp-OH with a modest improvement in overall yield for each compound, making them more accessible to the studies of collagen.

3. Experimental

3.1. General methods

The preparative column chromatography of synthetic materials was carried out on a column (20×300 mm) of Wakogel® C-300 (45~75 μm, Wako Pure Chemical Industries, Ltd, Osaka, Japan). Analytical RP-HPLC was performed with a CHIRALPAK AS (4.6×250 mm, Daicel, Osaka, Japan) at 20°C and a flow rate of 1.5 mL/min. A solution containing 0.2 mg/mL in ethanol was loaded onto the column and eluted with a linear gradient concentration of hexane from 0% (0 min) to 40% (20 min) in ethanol. Chromatograms were monitored by UV-detection at 220 nm. FT-IR spectra were recorded on a Shimadzu 8100 FT-IR spectrometer (Shimadzu Co., Ltd, Kyoto, Japan). Measurements were performed using a liquid cell (KBr, 5 mm-path length) containing samples at a concentration of 1.0 mM in CH_2Cl_2 at room temperature. ^1H and ^{13}C NMR spectra were recorded on a Varian UNITY-plus 300 spectrometer (Varian Inc., Palo Alto, CA, USA). Chemical shifts for ^1H and ^{13}C spectra are reported relative to tetramethylsilane (TMS) at 0 ppm. Assignments were made using two-dimensional correlation spectroscopy (COSY), nuclear Overhauser enhancement spectroscopy (NOESY) and $^1H-^{13}C$ hetero-scalar correlation spectroscopy (HETCOR). Concentration of samples was approximately 30 mM in $CDCl_3$. Measurements of NOESY and HETCOR spectra (data not shown) were indispensable for the assignment of ^1H signals, especially of C^β and C^δ protons, unambiguously. In many cases, a couple of spin systems were located at a particular site of the proline resonance by COSY cross-peaks that were considered to represent a pair of *trans*- and *cis*-conformers.[28] The results of these assignments are reported only for the major conformer (probably the *trans* form) unless the signals of the minor component are clearly recognizable. Molecular weights were determined by electron-spray-ionization mass spectrometry (ESI-MS) using an Applied Biosystems Mariner™ instrument (Applied Biosystems Inc., Foster City, CA, USA) operating in the mode that detects positive ions.

3.1.1. *N*-Boc-4(*R*)-Hyp-OMe (2a). Boc-4(*R*)-Hyp-OH (**1a**; mp 125~126°C, $[\alpha]_D^{22}=-84.4°$ at $c=1.00$ in $CHCl_3$) was prepared from 4(*R*)-Hyp-OH (**1**) and $(Boc)_2O$ (purchased from Peptide Institute Inc. Osaka, Japan) by the standard protocol using $(Boc)_2O$.[29] To a solution of **1a** (15.0 g, 64.9 mmol) in diethyl ether (200 mL) was added an excess of diazomethane in diethyl ether at 0°C, and then the reaction mixture was allowed to stand overnight. After removing the solvent and diazomethane under reduced pressure, the oily residue dissolved in ethyl acetate was applied onto a silica gel column, and **1a** was eluted with ethyl acetate/hexane (1:2, v/v). Yield: 15.0 g (94.3%).

3.1.2. *N*-Boc-4(*S*)-Hyp-OH (1b). To a solution of **2a** (7.04 g, 28.7 mmol), triphenylphosphine (15.1 g, 57.4 mmol), and formic acid (2.20 mL, 57.4 mmol) in THF (75 mL) was added dropwise 40% diethyl azodicarboxylate (25.0 g, 57.4 mmol) in toluene at room temperature under a nitrogen atmosphere. The reaction mixture was stirred overnight, and then 1 M NaOH (94.8 mL) was added to the solution. After stirring for 1 h at room temperature, most of organic solvent was removed in vacuo. The aqueous residue diluted by addition of H_2O (50 mL) was washed with ethyl acetate, and acidified with 1 M HCl, followed by extraction with ethyl acetate. The extract was washed with water and saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated in vacuo. The residue was triturated with diethyl ether, and the powdery product was collected by filtration. The thus-obtained crude product was recrystallized from ethyl acetate and hexane. Yield: 5.18 g (78.1%).

3.1.3. *N*-Boc-4(*R*)-Hyp-OPac (3a). To a solution of **1b** (5.75 g, 24.9 mmol) in methanol (100 mL) was added Cs_2CO_3 (4.05 g, 12.5 mmol) in water (65 mL) at 0°C, and the mixture was concentrated in vacuo. The residue was

dissolved in *N*,*N*-dimethylformamide (DMF) (125 mL), and then phenacyl bromide (Pac-Br) (4.95 g, 24.9 mmol) was added to the solution at 0°C. After being stirred for 30 min, the reaction mixture was filtered, and the filtrate was concentrated in vacuo. The residue was extracted with ethyl acetate, and the extract was washed with water and saturated aqueous $NaHCO_3$ (2×100 mL). The organic layer was dried over Na_2SO_4, and then the solvent was evaporated to dryness. The residue was triturated with diethyl ether, and the powdery product was collected by filtration. The thus-obtained crude product was recrystallized from ethyl acetate and hexane. Yield: 7.66 g (88.0%).

3.1.4. *N*-Boc-4(*S*)-Hyp-OPac (3b). Esterification of **1b** (3.50 g, 15.1 mmol) with Pac-Br (3.00 g, 15.1 mmol) was carried out in a similar manner as described above. The crude product was purified by recrystallization from ethyl acetate and hexane. Yield: 4.59 g (86.8%).

3.1.5. Boc-4(*S*)-fPro-OPac (4a). To a solution of **3a** (5.98 g, 17.1 mmol) in CH_2Cl_2 (80 mL) was added morpholinosulphur trifluoride (15.0 g, 85.5 mmol) dropwise over a 30 min period at −78°C under a nitrogen atmosphere, and the mixture was then stirred for 48 h at room temperature. The reaction mixture was concentrated in vacuo, and to the residue was added water (50 mL). The resulting oily product was extracted with ethyl acetate, and the extract was washed with water and saturated aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4, and then the solvent was evaporated to dryness. The crude product was purified by silica-gel column chromatography with ethyl acetate/hexane (1:2, v/v) eluents. Yield: 5.65 g (94.0%).

3.1.6. Boc-4(*R*)-fPro-OPac (4b). Procedures for the preparation of **4b** were carried out in a similar manner as those for **4a** except that **3b** (6.13 g, 17.5 mmol) was dissolved in THF (80 mL) instead of CH_2Cl_2. Yield: 4.88 g (79.4%).

3.1.7. Boc-4(*S*)-fPro-OH (5a). To a solution of **4a** (0.800 g, 2.28 mmol) in acetic acid (20 mL) was added zinc dust (7.44 g, 114 mmol), and the mixture was stirred overnight at room temperature. The reaction mixture was filtered, and the filtrate was concentrated in vacuo. The residue was extracted with ethyl acetate, and the extract was washed with water (2×30 mL) and 10% aqueous citric acid (2×30 mL). The organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo. The thus-obtained crude product was purified by silica-gel column chromatography with ethyl acetate/hexane (1:2, v/v) eluents. The oily product was solidified by trituration with diethyl ether, and collected by filtration. Yield: 0.503 g (94.5%). The thus-obtained **5a** was dissolved in ethanol, and colorless rectangular crystals for X-ray diffraction analysis were prepared by evaporating the solvent slowly at room temperature. $[\alpha]_D^{22}=-73°$ (c=0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$, ppm) 7.83 (bs, 1H, COOH), 5.22 (m, J_{H-F}=53 Hz, 1H, $C^{\gamma}H$), 4.56 (d, J=10 Hz, 1H, $C^{\alpha}H$; minor isomer at 4.46), 3.77 (m, J=13 Hz, J_{H-F}=26 Hz, 1H, $C^{\delta}H$; minor isomer at 3.85), 3.63 (m, J_{H-F}=36 Hz, 1H, $C^{\delta}H'$), 2.71 (m, J_{H-F}=21 Hz, 1H, $C^{\beta}H$; minor isomer at 2.55), 2.31 (m, J_{H-F}=41 Hz, 1H, $C^{\beta}H'$; minor isomer at 2.41), 1.44 (s, 9H, Boc; minor isomer at 1.49); ^{13}C NMR ($CDCl_3$, ppm) 177.12 (CO; minor isomer at 174.70), 155.53 (Boc-CO; minor isomer at 153.86), 91.91 (C^{γ}, J_{C-F}=177 Hz; minor isomer at 91.19), 81.73 (Boc-C; minor isomer at 80.97), 57.61 (C^{α}; minor isomer at 57.45), 53.57 (C^{δ}, J_{C-F}=25 Hz; minor isomer at 52.98), 37.36 (C^{β}, J_{C-F}=22 Hz; minor isomer at 35.65), 28.35 (Boc-CH_3; minor isomer at 28.26). Analysis calcd for $C_{10}H_6O_4NF$: C, 51.50; H, 6.91; N, 6.01. Found: C, 51.36; H, 6.79; N, 5.97; ESI-MS (*m*/*z*): 256.04 $[M+Na]^+$ (calcd 256.10) and 272.00 $[M+K]^+$ (calcd 272.07). Crystal data: orthorhombic, $P2_12_12_1$, a=9.654, b=18.70, c=6.455 Å, Z=4, λ (Mo Kα)=0.71069 Å, T=296 K, 1562 unique reflections, R=0.094. Crystals contained one molecule in each asymmetric unit.

3.1.8. Boc-4(*R*)-fPro-OH (5b). In a fashion similar to the preparation of **5a** from **4a**, **5b** was prepared from **4b** (0.500 g, 1.42 mmol). The oily product of **5b** was solidified by triturating with hexane, and collected by filtration to give amorphous powder. Yield: 0.300 g (90.6%). $[\alpha]_D^{22}=-62°$ (c=1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) 7.29 (brs, 1H, COOH), 5.21 (m, J_{H-F}=53 Hz, 1H, $C^{\gamma}H$; minor isomer at 5.32), 4.54 (t, J=8 Hz, 1H, $C^{\alpha}H$; minor isomer at 4.43), 3.90 (m, J_{H-F}=21 Hz, 1H, $C^{\delta}H$; minor isomer at 3.93), 3.52 (m, J=12 Hz, J_{H-F}=37 Hz, 1H, $C^{\delta}H'$; minor isomer at 3.63), 2.58 (m, J_{H-F}=20 Hz, 1H, $C^{\beta}H$; minor isomer at 2.65), 2.40 (m, J_{H-F}=40 Hz, 1H, $C^{\delta}H'$; minor isomer at 2.17), 1.50 (s, 9H, Boc; minor isomer at 1.44); ^{13}C NMR ($CDCl_3$) 178.04 (CO; minor isomer at 174.01), 153.58 (Boc-CO; minor isomer at 156.40), 91.21 (C^{γ}, J_{C-F}=178 Hz; minor isomer at 91.01), 82.36 (Boc-C; minor isomer at 81.15), 57.70 (C^{α}; minor isomer at 57.48), 53.47 (C^{δ}, J_{C-F}=23 Hz; minor isomer at 52.95), 35.60 (C^{β}, J_{C-F}=22 Hz; minor isomer at 37.43), 28.29 (Boc-CH_3; minor isomer at 28.18). Analysis calcd for $C_{10}H_6O_4NF{\cdot}0.1H_2O$: C, 51.10; H, 6.96; N, 5.96. Found: C, 50.78; H, 6.61; N, 5.46; ESI-MS (*m*/*z*): 256.06 $[M+Na]^+$ (calcd 256.10).

3.1.9. Fmoc-4(*S*)-fPro-OPac (6a). The solution of **4a** (4.40 g, 12.5 mmol) in 40 mL of TFA/CH_2Cl_2 (1:2, v/v) was stirred at room temperature for 1 h, and then concentrated in vacuo. The oily residue was triturated with diethyl ether, and the powdery product was collected by filtration and dried in vacuo. To the crude product was added Fmoc-OSu (4.64 g, 13.8 mmol) and *N*-methylmorpholine (1.26 g, 12.5 mmol) in CH_2Cl_2 (50 mL) at room temperature. The mixture was stirred for 6 h at room temperature, and the solvent was removed in vacuo. The oily residue was triturated with water, and the powdery substance was collected by filtration, and washed with ethanol. Yield: 5.41 g (91.2%). The thus-obtained **6a** was used for the next reaction without further purification.

3.1.10. Fmoc-4(*R*)-fPro-OPac (6b). Preparation of **6b** from **4b** (1.79 g, 5.10 mmol) was carried out in a similar manner as described above. Yield: 2.22 g (91.9%).

3.1.11. Fmoc-4(*S*)-fPro-OH (7a). 7a was prepared from **6a** (3.80 g, 8.03 mmol) in a similar manner as described in the preparation of **5a**. Yield: 2.56 g (89.8%). $[\alpha]_D^{22}=-55°$ (c=0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) 8.11 (brs, 1H, COOH), 7.27–7.76 (m, 8H, Fmoc-ArH), 5.24 (m,

J_{H-F}=53 Hz, 1H, $C^{\gamma}H$; minor isomer at 5.18), 4.63 (d, J=10 Hz, 1H, $C^{\alpha}H$; minor isomer at 4.37), 4.45 (d, 2H, Fmoc-CH_2; minor isomer at 4.44), 4.26 (t, J=7 Hz, H, Fmoc-CH; minor isomer at 4.16), 3.85 (dd, J=13 Hz, J_{H-F}=26 Hz, 1H, $C^{\delta}H$), 3.66 (m, J=13 Hz, J_{H-F}=35 Hz, 1H, $C^{\delta}H'$; minor isomer at 3.69), 2.63 (dd, J=16 Hz, J_{H-F}=23 Hz, 1H, $C^{\beta}H$; minor isomer at 2.57), 2.34 (m, J_{H-F}=41 Hz, 1H, $C^{\beta}H'$); ^{13}C NMR ($CDCl_3$) 175.22 (CO; minor isomer at 176.09), 155.07 (Fmoc-CO; minor isomer at 154.42), 91.96 (C^{γ}, J_{C-F}=178 Hz; minor isomer at 90.90), 67.95 (Fmoc-CH_2; minor isomer at 67.75), 57.66 (C^{α}, J_{C-F}=22 Hz; minor isomer at 57.16), 53.53 (C^{δ}, J_{C-F}=22 Hz; minor isomer at 53.22), 47.16 (Fmoc-CH), 36.26 (C^{β}; minor isomer at 37.55). Analysis calcd for $C_{20}H_{18}O_4NF{\cdot}H_2O$: C, 64.34; H, 5.41; N, 3.75. Found: C, 64.88; H, 5.35; N, 3.72; ESI-MS (m/z): 378.03 $[M+Na]^+$ (calcd 378.11) and 394.00 $[M+K]^+$ (calcd 394.09).

3.1.12. Fmoc-4(*R*)-fPro-OH (7b). 7b was prepared from **6b** (2.00 g, 4.22 mmol) in a similar manner as described in the preparation of **5b**. Yield: 1.43 g (95.5%). $[\alpha]_D^{22}=-63°$ (c=1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) 7.92 (brs, 1H, COOH), 7.26–7.78 (m, 8H, Fmoc-ArH), 5.25 (m, J_{H-F}=53 Hz, 1H, $C^{\gamma}H$; minor isomer at 5.20), 4.59 (t, J=8 Hz, 1H, $C^{\alpha}H$; minor isomer at 4.45), 4.47 (d, 2H, Fmoc-CH_2; minor isomer at 4.47), 4.28 (t, J=7 Hz, H, Fmoc-CH; minor isomer at 4.16), 3.77 (dd, J=14 Hz, J_{H-F}=21 Hz, 1H, $C^{\delta}H$), 3.62 (m, J=13 Hz, J_{H-F}=36 Hz, 1H, $C^{\delta}H'$), 2.65 (m, J_{H-F}=20 Hz, 1H, $C^{\beta}H$; minor isomer at 2.69), 2.34 (m, J_{H-F}=39 Hz, 1H, $C^{\beta}H'$; minor isomer at 2.17); ^{13}C NMR ($CDCl_3$) 176.81 (CO; minor isomer at 175.59), 155.49 (Fmoc-CO; minor isomer at 154.57), 91.43 (C^{γ}, J_{C-F}=179 Hz; minor isomer at 90.79), 68.13 (Fmoc-CH_2; minor isomer at 67.99), 57.13 (C^{α}; minor isomer at 57.80), 53.22 (C^{δ}, J_{C-F}=23 Hz; minor isomer at 53.62), 47.07 (Fmoc-CH; minor isomer at 47.12), 36.25 (C^{β}, J_{C-F}=23 Hz; minor isomer at 37.68). Analysis calcd for $C_{20}H_{18}O_4NF{\cdot}0.1H_2O$: C, 67.25; H, 5.10; N, 3.92. Found: C, 66.81; H, 5.40; N, 3.49; ESI-MS (m/z): 378.03 $[M+Na]^+$ (calcd 378.11) and 394.00 $[M+K]^+$ (calcd 394.09).

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References

1. Bornstein, P.; Traub, W.; 3rd ed. *The Proteins*, Neurath, H., Hill, R. L., Eds.; Academic: New York, 1979; Vol. IV, pp 411–632.
2. Ramachandran, G. N. In *Aspects of Protein Structure*. Ramachandran, G. N., Ed.; Academic: New York, 1963; p 39.
3. Baum, J.; Brodsky, B. In *Mechanisms of Protein Folding*. 2nd ed. Pain, R. H., Ed.; Oxford University: Oxford, 2000; pp 330–351.
4. Sakakibara, S.; Kishida, Y.; Kikuchi, Y.; Sakai, R.; Kakiuchi, K. *Bull. Chem. Soc. Jpn* **1968**, *41*, 1273.
5. Sakakibara, S.; Inouye, K.; Shudo, K.; Kishida, Y.; Kobayashi, Y.; Prockop, D. *J. Biochim. Biophys. Acta* **1973**, *303*, 198–202.
6. Kobayashi, Y.; Sakai, R.; Kakiuchi, K.; Isemura, T. *Biopolymers* **1970**, *9*, 415–425.
7. Berg, B. A.; Prockop, D. *J. Biochem. Biophys. Res. Commun.* **1973**, *52*, 115–120.
8. Uchiyama, S.; Kai, T.; Kajiyama, K.; Kobayashi, Y.; Tomiyama, T. *Chem. Phys. Lett.* **1997**, *281*, 92–96.
9. Inouye, K.; Sakakibara, S.; Prockop, D. *J. Biochim. Biophys. Acta* **1976**, *420*, 133–141.
10. Inouye, K.; Kobayashi, Y.; Kyogoku, Y.; Kishida, Y.; Sakakibara, S.; Prockop, D. *J. Arch. Biochem. Biophys.* **1982**, *219*, 198–203.
11. Bella, J.; Eaton, M.; Brodsky, B.; Berman, H. M. *Science* **1994**, *266*, 75–81.
12. Bella, J.; Brodsky, B.; Berman, H. M. *Structure* **1995**, *3*, 893–906.
13. Holmgren, S. K.; Taylor, K. M.; Bretscher, L. E.; Raines, R. T. *Nature* **1998**, *392*, 666–667.
14. Holmgren, S. K.; Bretscher, L. E.; Taylor, K. M.; Raines, R. T. *Chem. Biol.* **1998**, *6*, 63–70.
15. Bretscher, L. E.; Jenkins, C. L.; Taylor, K. M.; DeRider, M. L.; Raines, R. T. *J. Am. Chem. Soc.* **2001**, *123*, 777–778.
16. Vitagliano, L.; Berisio, R.; Mazzarella, L.; Zagari, A. *Biopolymers* **2001**, *58*, 459–464.
17. Panasik, Jr. N.; Eberhardt, E. S.; Edison, A. S.; Powell, D. R.; Raines, R. T. *Int. J. Pept. Protein Res.* **1994**, *44*, 262–269.
18. Eberhardt, E. S.; Panasik, Jr., N.; Raines, R. T. *J. Am. Chem. Soc.* **1996**, *118*, 12261–12266.
19. Gottlieb, A. A.; Fujita, Y.; Udenfriend, S.; Witkop, B. *Biochemistry* **1965**, *4*, 2507–2513.
20. Demange, L.; Menez, A.; Dugave, C. *Tetrahedron Lett.* **1998**, *39*, 1169–1172.
21. Mitsunobu, O. *Synthesis* **1981**, 1–28.
22. Tran, T. T.; Patino, N.; Condom, R.; Frogier, T.; Guedj, R. *J. Fluorine Chem.* **1997**, *82*, 125–130.
23. Malkar, N. B.; Lauer-Fields, J. L.; Borgia, J. A.; Fields, G. B. *Biochemistry* **2002**, *41*, 6054–6064.
24. Takai, M.; Kurano, Y.; Kimura, T.; Sakakibara, S. In *Peptide Chemistry 1979*. Yonehara, H., Ed.; Protein Research Foundation: Osaka, 1980; p 187.
25. Jones, J. *The Chemical Synthesis of Peptides*. Oxford University: Oxford, 1994; pp 37–38.
26. Narita, M.; Doi, M.; Nakai, T. *Bull. Chem. Soc. Jpn* **1987**, *60*, 3255–3259.
27. Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*. 5th ed. Wiley: New York, 1991; pp 95–96.
28. Hondrelis, J.; Lonergan, G.; Voliotis, S.; Matsoukas, J. *Tetrahedron* **1990**, *46*, 565–576.
29. Moroder, L.; Hallet, A.; Wünsch, E.; Keller, O.; Wersin, G. *Hoppe Seyler's Z. Physiol. Chem.* **1976**, *357*, 1651–1653.