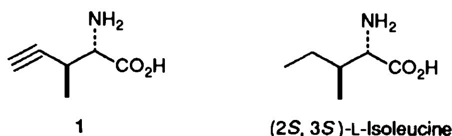
Synthesis of (2*S*,3*S*)-2-Amino-3-methylpent-4-ynoic Acid, a Precursor Amino Acid for the Preparation of Tritium- or Deuterium-Labelled Peptide in the Isoleucine Residue

Hiroshi Hasegawa, Shinichi Arai, Yoshihiko Shinohara and Shigeo Baba*
Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

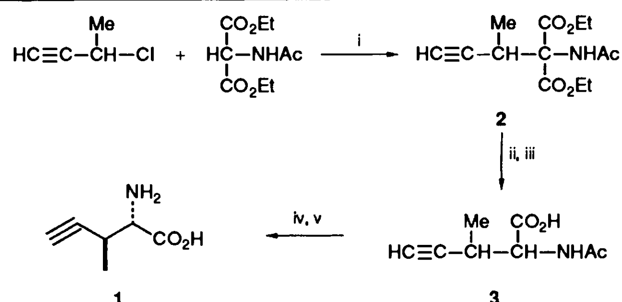
The synthesis of (2*S*,3*S*)-2-amino-3-methylpent-4-ynoic acid (Amp) **1**, its incorporation into a peptide chain, and the preparation of a deuterium-labelled peptide in the isoleucine residue are reported. Amino acid **1** was synthesized in three steps from 3-chlorobut-1-yne. The optical purity of amino acid **1** was determined by HPLC with a chiral stationary-phase column and was found to be more than 99% (ee). Tyr-Amp-Leu was synthesized by solid-phase synthesis based on Fmoc strategy. The tripeptide was deuteriated catalytically to yield Tyr-[^{2}H]Ile-Leu. The distribution of deuterium was investigated by fast-atom-bombardment mass spectrometry (FAB-MS) and ^{13}C NMR spectroscopy, which confirmed that the deuterium was located entirely at the isoleucine residue.

The availability of tritium- or deuterium-labelled peptides would be of obvious value for a variety of chemical and biological studies. If a labelled peptide is to be used for metabolic studies, the label must be placed at an appropriate location in the peptide chain. The specific tritiation of peptides has been carried out by catalytic dehalogenation of halogen-substituted precursor peptides or by catalytic reduction of unsaturated precursor peptides with tritium gas. Several investigators have prepared the precursor peptides containing halogenated tyrosyl,[1,2] phenylalanyl[3] or histidyl[4] residues and then obtained the tritium-labelled peptides by catalytic dehalogenation with tritium gas. If no aromatic amino acid residues are present to be labelled in the peptide, peptides containing unsaturated carbon–carbon bonds have been used as precursors for the incorporation of tritium by catalytic reduction. The alkene bond in L-3,4-didehydroproline can be readily tritiated, thus providing proline-labelled peptides.[5,6] The catalytic reduction of an alkyne bond is also possible and will, theoretically, lead to a higher specific activity. Kaspersen *et al.*[7] have prepared β-endorphin(6-17) containing L-2,6-diaminohex-4-ynoic acid[8] instead of lysine and obtained the [^{3}H-Lys^9]β-endorphin(6-17) with a specific activity of 1.4 TBq/mmol by catalytic reduction with tritium gas. We turned our attention to the aliphatic C(4)–C(5) bond of isoleucine for labelling. However, there are no reports concerning the synthesis of 2-amino-3-methylpent-4-ynoic acid, an acetylenic analogue of isoleucine.



In this study, we describe the synthesis of (2*S*,3*S*)-2-amino-3-methylpent-4-ynoic acid (Amp) **1**, its incorporation into a peptide chain using solid-phase synthesis based on Fmoc strategy[9] (Fmoc = fluoren-9-ylmethoxycarbonyl) and the preparation of a deuterium-labelled peptide in the isoleucine residue.

The synthetic route to Amp is illustrated in Scheme 1. Commercially available 3-chlorobut-1-yne was treated with sodium diethyl acetamidomalonate in ethanol to give the condensation product **2**. Treatment of compound **2** with Na_2CO_3 caused hydrolysis, followed by decarboxylation with HCl to give 2-acetamido-3-methylpent-4-ynoic acid **3**. ^{1}H NMR spectroscopy of compound **3** indicated a mixture of diastereoisomers, and analytical reversed-phase HPLC allowed excellent diastereoisomeric separation, as shown in Fig. 1 (**3a**: t_R 16.4 min, **3b**: 21.0 min). Compound **3** was purified and separated into its diastereoisomers by preparative HPLC. The ratio of the amount of compound **3a** to that of isomer **3b** was found to be ~1:1.7. These results apparently indicated that diastereoselective decarboxylation of the malonic acid derivative occurred similarly to Arnold's observation.[10,11] The optical purity of each diastereoisomer was determined by reversed-phase HPLC and was found to be more than 99% (de), respectively.



Scheme 1 *Reagents and conditions:* i, NaOEt–EtOH; ii, aq. Na_2CO_3, reflux; iii, HCl; iv, diastereoisomeric separation on reversed-phase HPLC [column μ-Bondasphere C-18; elution water–MeOH–TFA (94:6:0.1), flow rate 8.5 cm^3 min^{-1}; detection λ 200 nm]; v, acylase (from porcine kidney), pH 7.6, 37 °C, 5 days

It has been reported[12,13] that *erythro* and *threo* isomers in β-alkyl-substituted α-amino acids could easily be differentiated by means of a comparison of their ^{1}H NMR spectra. The coupling constant between C_α–H and C_β–H ($J_{\alpha\beta}$) was larger in DL-isoleucine (an *erythro* form) than in DL-alloisoleucine (a *threo* form). In the present study, $J_{\alpha\beta}$ of compound **3a** (6.4 Hz) was larger than that of compound **3b** (5.4 Hz), suggesting that compounds **3a** and **3b** belong to an *erythro* type and a *threo* type, respectively. However, the use of these differences would not be a reliable method for assignment of stereochemistry because the relationship in β-hydroxy α-amino acids was found to be reversed, that is, the *threo* form showed the relatively larger value over that of the *erythro* form.[14] The respective relative configurations of compounds **3a** and **3b** were assigned by reduction to the corresponding known amino acids. An aliquot of each diastereoisomer was catalytically hydrogenated and the product was analysed with reversed-phase HPLC to

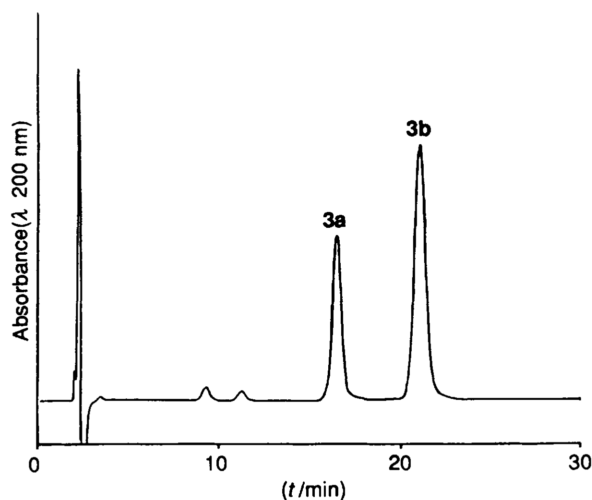
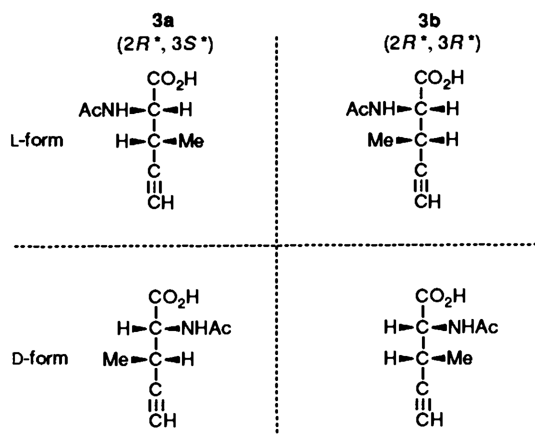


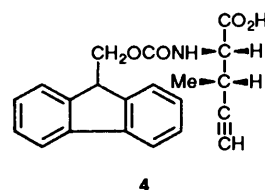
Fig. 1 Diastereoisomeric separation of 2-acetamino-3-methylpent-4-ynoic acid **3** on reversed-phase HPLC. HPLC conditions: column LiChrospher RP-18 (4 x 250 mm); elution water–MeOH–TFA (94:6:0.1), flow rate 1 $cm^3 min^{-1}$; detection λ 200 nm. **3a** = ($2R^*,3S^*$)-2-acetamino-3-methypent-4-ynoic acid, **3b** = ($2R^*,3R^*$)-2-acetamino-3-methylpent-4-ynoic acid

give single peak, respectively. Under these HPLC conditions, authentic ($2R^*,3S^*$)-*N*-acetyl-DL-alloisoleucine and ($2R^*,3R^*$)-*N*-acetyl-DL-isoleucine were eluted at 51.9 and 49.4 min, respectively. The reduction products eluted at the same retention times as those of the authentic samples. The ^1H NMR and ^{13}C NMR chemical shifts of the reduction products were also identical with those of ($2R^*,3S^*$)-*N*-acetyl-DL-alloisoleucine and ($2R^*,3R^*$)-*N*-acetyl-DL-isoleucine, respectively. These results showed that compounds **3a** and **3b** were identical with ($2R^*,3S^*$)-2-acetamido-3-methylpent-4-ynoic acid and ($2R^*,3R^*$)-2-acetamido-3-methylpent-4-ynoic acid, respectively.

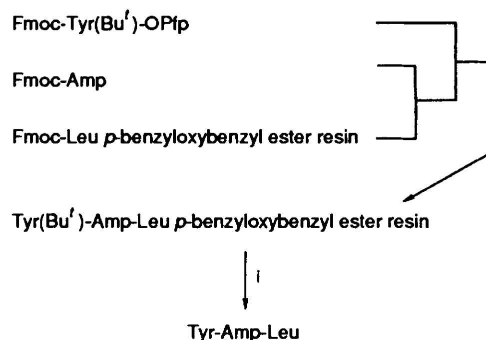


Stereospecific hydrolysis of compound **3b** with porcine kidney acylase gave a digested product **1**. The absolute configuration of amino acid **1** was confirmed by reduction to the corresponding known amino acid. The reduction product and authentic ($2S,3S$)-L-isoleucine and ($2R,3R$)-D-isoleucine were analysed by HPLC with chiral stationary-phase columns (Chiralpak WH and Crownpak CR). In each analysis, the retention time of the reduction product was identical with that of authentic ($2S,3S$)-L-isoleucine. These results showed that amino acid **1** was identical with ($2S,3S$)-2-amino-3-methylpent-4-ynoic acid (Amp). The enantiomeric purity of amino acid **1** was determined by HPLC with a chiral stationary-phase column (Chiralpak WH), and was found to be more than 99% (ee).

Amp **1** could be incorporated into peptides using standard



solid-phase synthesis by Fmoc strategy. As an example, Tyr-Amp-Leu **5** was synthesized (Scheme 2). Starting with Fmoc-Leu *p*-benzyloxybenzyl ester resin, the peptide chain was elongated manually, according to the sequence steps shown in the Table 1, *i.e.* removal of Fmoc group by 20% piperidine in dimethylformamide (DMF) and condensation of the respective amino acids by the active-ester procedure. Coupling of Fmoc-Amp **4** was carried out by the benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate (BOP) coupling procedure.[15] Fmoc-Tyr(Bu^t)was coupled with the pentafluorophenyl (Pfp) ester procedure.[16] The protected tripeptide resin was treated with trifluoroacetic acid (TFA)–water (9:1) to give tripeptide **5**, which showed a single peak on reversed-phase HPLC without purification. The assignment of all protons in tripeptide **5** was established by a combination of 2D-NMR experiments [COSY, HOHAHA (homonuclear Hartmann-Hahn spectroscopy) and NOESY] with the sequence-specific assignment procedure introduced by Wüthrich.[17] The FAB-mass spectrum of tripeptide **5** gave $[M + H]^+$, B_2, A_2, Y_2 and A_1 ions at *m/z* 404, 273, 245, 241 and 136, respectively.[18] These results showed that Amp could be incorporated into the peptide chain by using solid-phase synthesis based on standard Fmoc-strategy with no side reactions.



Scheme 2 *Reagents and conditions:* i, TFA–water (9:1)

Catalytic deuteriation of tripeptide **5** was carried out in the presence of platinum(IV) oxide in DMF to give the deuteriated tripeptide **6**. The distribution of the deuterium label was determined by FAB-MS and NMR spectroscopy because the catalytic hydrogen–deuterium exchange reaction might occur at unexpected positions during catalytic reduction. The FAB-mass spectrum of tripeptide **6** indicated a mixture of compounds having one to five deuterium atoms, the triply deuteriated species being the most abundant (Fig. 2). If the reduction is carried out using tritium gas instead of deuterium gas, about three tritium atoms will be incorporated and the reduction product will have (theoretically) a high radiospecific activity beyond 3 TBq/mmol. The location of deuterium was indicated by the mass shift of the fragment ions containing the isoleucine residue. This was demonstrated, for example, by the mass shift of the $[M + H]^+$ ion and the fragment ions from *m/z* 408, 277 (B_2), and 249 (A_2) of non-labelled tripeptide **7** to *m/z* 411, 280 and 252 of the labelled product **6**, respectively. The absence of deuterium incorporation in the tyrosine residue was indicated by the absence of a mass shift of fragment ion at *m/z* 136, which is attributed to the tyrosine $[HOC_6H_4CH_2CH{=}NH_2^+]$ portion of the molecule. However, the absence of deuterium incor-

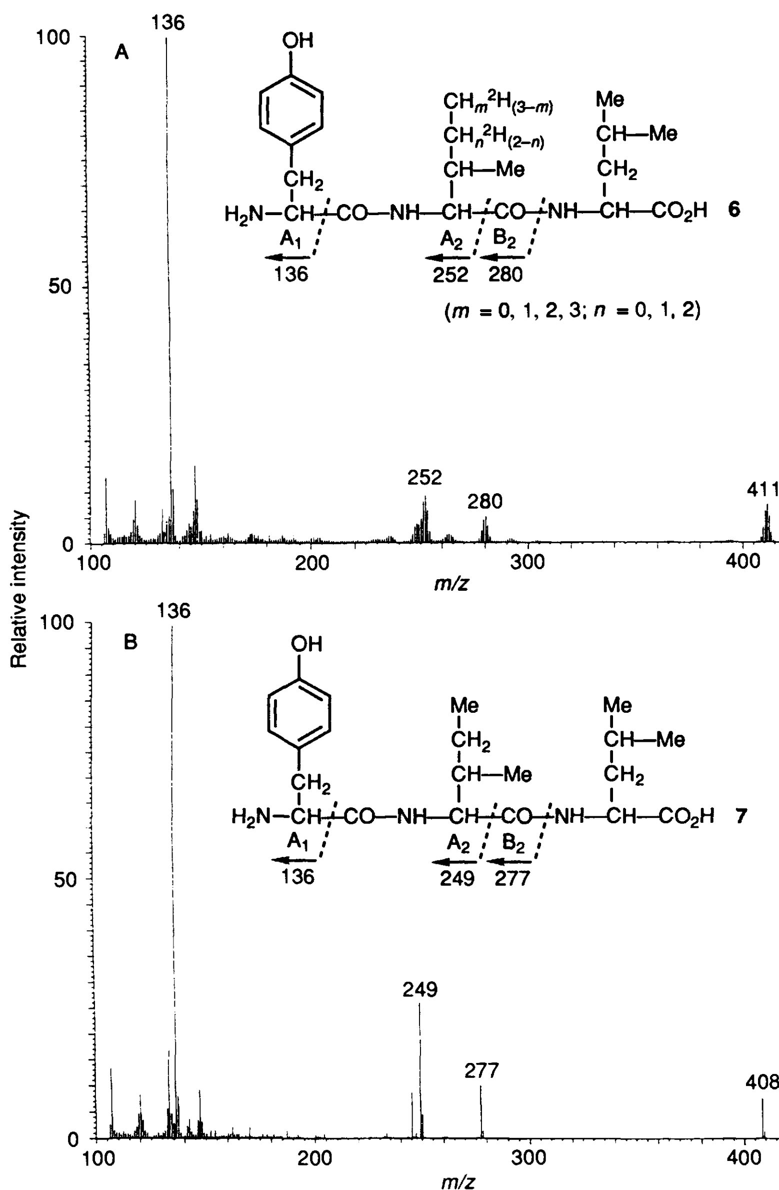


Fig. 2 FAB-mass spectra of (A) Tyr-[^{2}H]Ile-Leu **6** and (B) Tyr-Ile-Leu **7**

poration in the leucine residue could not be clarified in the FAB-mass spectrum since the fragment ions of both isoleucine and leucine had common mass numbers.

Deuterium incorporation can be monitored indirectly from isotope-induced shifts in ^{13}C NMR spectroscopy.[19,20] In the proton-decoupled ^{13}C NMR spectrum, the signal for the carbon directly attached to the deuterium shifts its centre of resonance by 0.3–0.6 ppm and spin–spin coupling ($^1J_{C^2H}$) produces a characteristic multiplet, hence C^2H appears as a triplet whereas C^2H_2 and C^2H_3 would give, respectively, a quintet and a septet. The proton-decoupled ^{13}C NMR spectrum of the deuteriated tripeptide **6** gave complex signals at δ_C 10.3–11.0 and 23.7–24.32 corresponding to C(5) and C(4) positions in the isoleucine residue, comprising lines from mixtures of C^2H_3-, CH^2H_2-, $CH_2{}^2H$- and CH_3-labelled species and C^2H_2-, CH^2H- and CH_2-labelled species, respectively (Fig. 3). In contrast, the other signals were observed as singlets. These results confirmed that the label had been located entirely at the C(4) and C(5) positions in the isoleucine residue, and that non-specific exchange labelling into the other residue had not occurred.

The present study provides the first synthesis of (2*S*,3*S*)-2-amino-3-methylpent-4-ynoic acid and its incorporation into a peptide chain by solid-phase synthesis based on Fmoc strategy, followed by reductive deuteriation to yield the deuterium-labelled peptide in the isoleucine residue. This procedure is also applicable to the synthesis of tritium-labelled peptide in the isoleucine residue.

Experimental

M.p.s were determined on a Yamato MP-1 melting point apparatus and were uncorrected. ^1H NMR and ^{13}C NMR spectra were determined on a Varian Gemini 300, a Bruker AM-400 or a Bruker AM-500 spectrometer using tetramethylsilane (TMS) or sodium 3-(trimethylsilyl)propanesulfonate (DSS) as internal standard. *J*-values are given in Hz. Optical rotations were determined on a JASCO DIP-4 polarimeter; values for $[\alpha]_D$ are given in units of 10^{-1} deg cm^2 g^{-1}. The IR spectrum was determined on a Perkin-Elmer 240B spectrophotometer. Mass spectra were determined on a Hitachi double-focusing mass spectrometer M-80. FAB-mass spectra were determined on a VG Auto Spec. The two HPLC systems were used as follows. Preparative HPLC was performed on a Waters M600 multisolvent delivery system and a Shimadzu SPD-6A variable-wavelength UV detector using μ-Bondasphere C-18 (5 μm; 100 Å; 19 × 150 mm; Waters, Mass, USA) column in the following

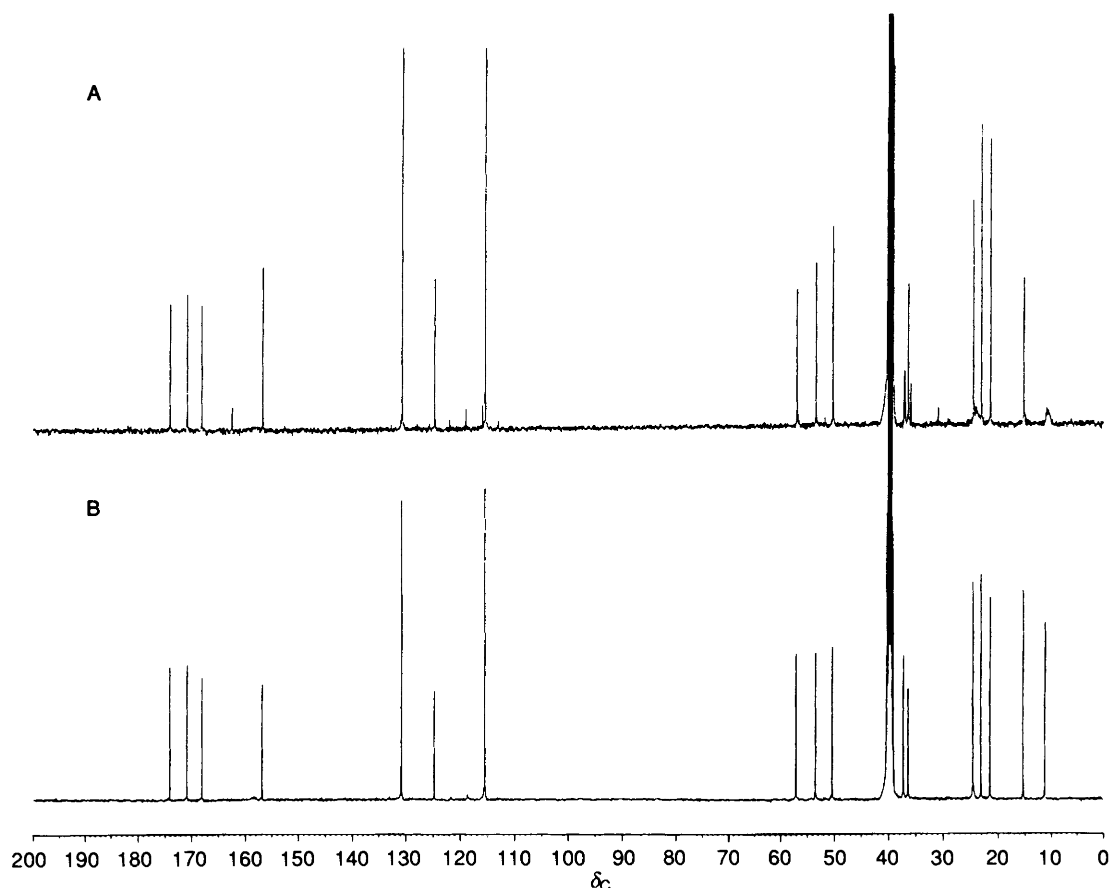


Fig. 3 Proton-decoupled ^{13}C NMR spectra of (A) Tyr-[^{2}H]Ile-Leu **6** and (B) Tyr-Ile-Leu **7** in $(CD_3)_2SO$

solvent system (v/v): water–MeOH–TFA (94:6:0.1). The flow rate was 8.5 cm^3 min^{-1}. Analytical HPLC was performed on a Shimadzu LC-3A pump and Otsuka Electronics photodiode array MCPD-3500 detector. Analytical HPLC was carried out on a LiChrospher RP-18 (5 µm, 4 × 250 mm; E. Merck, Darmstadt, Germany), a Crownpak CR (4 × 150 mm; Daicel Chemical, Tokyo, Japan) or a Chiralpak WH (4.6 × 250 mm; Daicel Chemical) column in the following solvent systems (all v/v): (a) water–MeOH–TFA (94:6:0.1); (b) 0.14% $HClO_4$–MeOH (9:1); (c) 0.25 mmol dm^{-3} $CuSO_4$; (d) 0.07% $HClO_4$; (e) water–MeCN–TFA (80:20:0.1).

3-Chlorobut-1-yne and acylase (from porcine kidney, EC 3.5.1.14) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). L-Isoleucine, D-isoleucine, L-alloisoleucine and D-alloisoleucine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluoren-9-ylmethoxycarbonyl chloride (Fmoc-Cl) was purchased from Peptide Institute Inc. (Osaka, Japan). Fmoc-Leu *p*-benzyloxybenzyl ester resin (0.58 mmol Fmoc-Leu g^{-1} resin; styrene-1% divinylbenzene) was purchased from Kokusan Chemical Works Co. (Tokyo, Japan). *N*-Fmoc-*O*-*tert*-butyltyrosine pentafluorophenyl ester [Fmoc-Tyr(Bu^t)-OPfp] was purchased from Cambridge Research Chemicals (Cambridge, UK).

Diethyl 2-*Acetamido*-2-(1-*methylprop*-2-*ynyl*)*malonate* **2**.—Diethyl acetamidomalonate (12.27 g, 56.5 mmol) was added to a stirred solution of sodium ethoxide [sodium (1.30 g, 56.5 mmol) in absolute EtOH (80 cm^3)]. 3-Chlorobut-1-yne (5.00 g, 56.5 mmol) was added dropwise during 20 min to the mixture. After being refluxed for 17 h, the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water and extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and the $CHCl_3$ was evaporated under reduced pressure to give an oil. After silica gel column chromatography of the oil with $CHCl_3$–ethyl acetate (5 : 1) as eluting solvent, the purified *product* **2** (2.73 g, 17.9%) was obtained as an oil, δ_H(400 MHz; $CDCl_3$) 1.23 (3 H, t, *J* 7.1, CH_2Me), 1.27 (3 H, t, *J* 7.1, CH_2Me), 1.34 (3 H, d, *J* 7.0, CH*Me*), 2.05 (3 H, s, Ac), 2.08 (1 H, d, *J* 2.5, C≡CH), 3.66 (1 H, dq, *J* 2.5 and 7.0, C*H*Me), 4.17–4.31 (4 H, m, CH_2Me) and 6.70 (1 H, br, NH); *m/z* (EI) 269.1237 (M^+. $C_{13}H_{19}NO_5$ requires *M*, 269.1264).

(2R*,3R*)-2-*Acetamido*-3-*methylpent*-4-*ynoic Acid* **3b**.—A solution of compound **2** (1.76 g, 6.5 mmol) in 10% aq. Na_2CO_3 (17 cm^3) was refluxed for 3 h. After cooling, the solution was washed with $CHCl_3$ and was acidified with conc. HCl to pH 3. The water was evaporated off under reduced pressure to give a residue. Hot acetone (20 cm^3) was added to the residue and the remaining material was removed by filtration. Evaporation of the solvent gave 2-acetamido-3-methylpent-4-ynoic acid **3** (1.08 g) as an oil.

The diastereoisomers **3** (1.01 g) were purified and separated with preparative HPLC to afford (2*R**,3*S**)-2-acetamido-3-methylpent-4-ynoic acid **3a** (282 mg) as an oil which solidified on storage for 3 days over P_2O_5 and (2*R**,3*R**)-2-acetamido-3-methylpent-4-ynoic acid **3b** (502 mg) as an oil which solidified on storage for 7 days over P_2O_5. Each diastereoisomer gave the following physical characteristics: *Compound* **3a**; m.p. 123–123.5 °C (decomp.) (Found: C, 55.7; H, 6.4; N, 8.1. $C_8H_{11}NO_3 \cdot 0.2H_2O$ requires C, 55.61; H, 6.65; N, 8.10%); δ_H[400 MHz; $(CD_3)_2SO$] 1.13 (3 H, d, *J* 7.0, 3-Me), 1.89 (3 H, s, Ac), 2.87 (1 H, m, 3-H), 2.92 (1 H, d, *J* 2.5, 5-H), 4.36 (1 H, dd, *J* 6.4 and 8.7, 2-H) and 8.16 (1 H, d, *J* 8.7, NH); *m/z* (EI) 169.0749 (M^+. $C_8H_{11}NO_3$ requires *M*, 169.0739) and *compound* **3b**; m.p. 118–118.5 °C (decomp.) (Found: C, 55.8; H, 6.4; N, 8.1%); δ_H[400 MHz; $(CD_3)_2SO$] 1.11 (3 H, d, *J* 7.0, 3-Me), 1.91 (3 H,

s, Ac), 2.93 (1 H, d, *J* 2.5, (5-H), 2.99 (1 H, ddq, *J* 2.5, 5.4 and 7.0, 3-H), 4.37 (1 H, dd, *J* 5.4 and 8.8, 2-H) and 8.00 (1 H, d, *J* 8.8, NH); *m/z* (EI) 169.0754 (M^+. $C_8H_{11}NO_3$ requires *M*, 169.0739), respectively.

To explore these relative configurations, the following method was employed. Compound **3a** (55 mg) was dissolved in MeOH (1 cm^3) and was reduced with hydrogen in the presence of platinum(IV) oxide (10 mg) for 2 h. The catalyst was filtered off and the filtrate was evaporated under reduced pressure to afford a solid (46 mg, 81.7%), δ_H[400 MHz; $(CD_3)_2SO$] 0.847 (3 H, d, *J* 6.9, 3-Me), 0.851 3 H, t, *J* 7.3, 5-Me), 1.14 (1 H, m, C*H*H), 1.29 (1 H, m, CH*H*), 1.81–1.90 (1 H, m, 3-H), 1.91 (3 H, s, Ac), 4.34 (1 H, dd, *J* 4.7 and 8.8, 2-H) and 7.90 (1 H, d, *J* 8.8, NH); δ_C[100 MHz; $(CD_3)_2SO$] 11.69 (C-5), 15.00 (3-Me), 22.49 (CO*Me*), 25.90 (C-4), 36.41 (C-3), 54.89 (C-2) 169.75 (CONH) and 173.68 (CO_2H). Compound **3b** (55 mg) was also catalytically hydrogenated in the same manner as above to afford a solid (41 mg, 72.8%), δ_H[400 MHz; $(CD_3)_2SO$] 0.82–0.86 (6 H, 3- and 5-Me), 1.18 (1 H, m, C*H*H), 1.40 (1 H, m, CH*H*), 1.75 (1 H, m, 3-H), 1.87 (3 H, s, Ac), 4.17 (1 H, dd, *J* 6.0 and 8.4, 2-H) and 7.98 (1 H, d, *J* 8.4, NH); δ_C[100 MHz; $(CD_3)_2SO$] 11.47 (C-5), 15.77 (3-Me), 22.52 (CO*Me*), 24.92 (C-4, 36.55 (C-3), 56.52 (C-2), 169.53 (CONH) and 173.42 (CO_2H). An aliquot of each reduction product was analysed by reversed-phase HPLC [column LiChrospher RP-18; elution (b), flow rate 1 cm^3 min^{-1}; detection λ 200 nm]. The retention-time values of these reduction products were identical with those of (2*R**,3*S**)-*N*-acetyl-DL-alloisoleucine (t_R 51.9 min) and (2*R**,3*R**)-*N*-acetyl-DL-isoleucine (49.4 min), respectively.

(2S,3S)-2-*Amino*-3-*methylpent*-4-*ynoic Acid* **1**.—Compound **3b** (0.82 g, 4.8 mmol) was dissolved in water (50 cm^3) and the pH was adjusted to 7.6 with 3 mol dm^{-3} LiOH. Porcine kidney acylase (17.2 mg) was added and the mixture was incubated at 37 °C for 5 days. After incubation, acetic acid was added to adjust the pH to 5.0 and the protein was filtered off with the aid of charcoal. The filtrate was subjected to ion-exchange column chromatography (Dowex 50W X8, H^+-form). The column was washed with water until the washings were neutral, and then eluted with 10% NH_4OH. The eluate was evaporated under reduced pressure to give *amino acid* **1** (211 mg, 68%) as a solid, m.p. 209 °C (decomp.) (Found: C, 56.4; H, 7.0; N, 11.05. $C_6H_9NO_2$ requires C, 56.68; H, 7.13; N, 11.02%); $[\alpha]_D$ −33.5 (*c* 0.5 in water); ν_{max}(KBr)/cm^{-1} 3309 [C(5)–H] and 2109 (C≡C); δ_H(400 MHz; D_2O) 1.34 (3 H, d, *J* 7.2, 3-Me), 2.63 (1 H, d, *J* 2.4, 5-H), 3.29 (1 H, ddq, *J* 2.4, 4.5 and 7.2, 3-H) and 3.73 (1 H, d, *J* 4.5, 2-H); *m/z* (CI) 128 (M^+ + 1).

To explore the absolute configuration of amino acid **1**, the following method was employed. Amino acid **1** (1 mg) was dissolved in water (1 cm^3) and was reduced with hydrogen in the presence of platinum(IV) oxide (1 mg) for 2 h. The catalyst was filtered off and the filtrate was evaporated under reduced pressure. The residue was dissolved in water and was subjected to analytical HPLC on two chiral stationary columns [(A): column Chiralpak WH; elution (c), flow rate 2 cm^3 min^{-1}; temperature 40 °C; detection λ 230 nm; and (B): column Crownpak CR; elution (d), flow rate 0.3 cm^3 min^{-1}; temperature; 0 °C; detection λ 200 nm]. The retention time value of the reduction product was identical with that of (2*S*,3*S*)-L-isoleucine under both conditions [(A) t_R 28.9 min, (B) t_R 22.0 min].

The enantiomeric purity of amino acid **1** was analysed with HPLC by using a Chiralpak WH column [elution (c), flow rate 1.2 cm^3 min^{-1}; temperature 45 °C; detection λ 230 nm] and was found to be more than 99% (ee).

(2S,3S)-2-(*Fluoren-9′-ylmethoxycarbonylamino*)-3-*methylpent*-4-*ynoic Acid* (*Fmoc-Amp*) **4**.—Amino acid **1** (177 mg, 1.39 mmol) was dissolved in a mixture of 10% aq. Na_2CO_3 (3.7 cm^3) and 1,4-dioxane (1 cm^3) and the solution was stirred at 0 °C. To the stirred solution was added a solution of Fmoc-Cl (398 mg, 1.54 mmol) in 1,4-dioxane (3 cm^3) dropwise during 10 min. After being stirred at 0 °C for 1 h and then at room temperature for 2 h, the solution was poured into water (30 cm^3). The resulting solution was washed with Et_2O, acidified with 5% aq. $KHSO_4$ to pH 2, and extracted with ethyl acetate. The extracts were washed with water, dried over Na_2SO_4, and evaporated under reduced pressure to give the *title compound* **4** (407 mg, 83.5%) as a solid, m.p. 145–146.5 °C (Found: C, 72.45; H, 5.5 N; 4.0. $C_{21}H_{19}NO_4$ requires C, 72.19; H, 5.48; N, 4.01%); $[\alpha]_D$ + 9.9 (*c* 0.5 in MeOH); δ_H(300 MHz; $CDCl_3$) 1.30 (3 H, d, *J* 7.1, 3-Me), 2.20 (1 H, d, *J* 2.3, 5-H), 3.27 (1 H, m, 3-H, 4.26 (1 H, t, *J* 7.0, fluorene 9-H), 4.43 (2 H, d, *J* 7.0, fluorene 9-CH_2), 4.49 (1 H, dd, *J* 3.3 and 9.8, 2-H), 5.49 (1 H, d, *J* 9.8, NH) and 7.26–7.78 (8 H, m, fluorene); *m/z* (EI) 349 (M^+).

Tyr-Amp-Leu **5**.—The solid-phase synthesis was carried out manually. Fmoc-Leu *p*-benzyloxybenzyl ester resin (0.52 g; 0.58 mmol Fmoc-Leu g^{-1} resin; styrene–1% divinylbenzene) was loaded into a solid-phase-synthesis vessel. The tripeptide **5** was built up according to the sequence steps shown in Table 1. Fmoc deprotection was achieved by treatment with 20% piperidine in DMF. Compound **4** (0.25 g, 0.74 mmol) was coupled by means of BOP reagent (0.32 g, 0.74 mmol), 1-hydroxybenzotriazole (HOBT) (0.11 g, 0.74 mmol), and diisopropylethylamine (0.12 cm^3, 0.74 mmol). Coupling of Fmoc-Tyr(Bu^t) was carried out by the corresponding pentafluorophenyl ester [Fmoc-Tyr(Bu^t)-OPfp] (0.46 g, 0.74 mmol) in the presence of HOBT (0.11 g, 0.74 mmol). Coupling reactions were monitored by the ninhydrin test [21] and no recoupling was necessary. After the removal of the *N*-terminal Fmoc group, the protected tripeptide resin was treated with TFA–water (9:1; 15 cm^3) for 1 h at room temperature. After filtration, the resin was washed with TFA–water (1:9). The combined filtrates and washings were evaporated under reduced pressure to give tripeptide **5** (120 mg, 98%) as a solid, homogeneous by HPLC (column LiChrospher RP-18; elution (e), flow rate 1 cm^3 min^{-1}; detection λ 210 nm; t_R 12.8 min), *m/z* (FAB) 404 ([M + H]$^+$), 273 (B_2), 241 (A_2) and 136 (A_1); δ_H[500 MHz; $(CD_3)_2SO$] 0.86 (3 H, 5-H_3 of Leu), 0.92 (3 H, 5-H_3 of Leu), 1.18 (3 H, 3-Me of Amp), 1.55 (2 H, 3-H_2 of Leu), 1.66 (1 H, 4-H of Leu), 2.82 (1 H, 3-H of Tyr), 2.87 (1 H, 3-H of Amp), 2.91 (1 H, 5-H of Amp), 2.99 (1 H, 3-H of Tyr), 4.09 (1 H, 2-H of Tyr), 4.26 (1 H, 2-H of Leu), 4.44 (1 H, 2-H of Amp), 6.68 (2 H, Ar 3- and 5-H of Tyr), 7.02 (2 H, Ar 2- and 6-H of Tyr), 8.04 (3 H, NH_3^+ of Tyr), 8.40 (1 H, NH of Leu) and 8.79 (1 H, NH of Amp).

Tyr-Ile-Leu **7**.—Tripeptide **7** was synthesized in a similar manner to that described above. Starting from Fmoc-Leu *p*-benzyloxybenzyl ester resin (0.45 g; 0.58 mmol Fmoc-Leu g^{-1} resin; styrene–1% divinylbenzene), the tripeptide was built up according to the sequence steps shown in Table 1. Fmoc-Ile (0.26 g, 0.74 mmol) was coupled by means of BOP reagent (0.33 g, 0.74 mmol), HOBT (0.11 g, 0.74 mmol), and diisopropylethylamine (0.12 cm^3, 0.74 mmol). Coupling of Fmoc-Tyr(Bu^t) was carried out by the corresponding pentafluorophenyl ester [Fmoc-Tyr(Bu^t)-OPfp] (0.46 g, 0.74 mmol) in the presence of HOBT (0.11 g, 0.74 mmol). Coupling reactions were monitored by the ninhydrin test [21] and no recoupling was necessary. After removal of the *N*-terminal Fmoc group, the protected tripeptide resin was treated with TFA–water (9:1; 15 cm^3) for 1 h at room temperature. After filtration, the resin was washed with TFA–water (1:9). The combined filtrates and washings were evaporated under reduced pressure to give tripeptide **7** (99 mg, 93%) as a solid, homogeneous by HPLC (column LiChrospher RP-18; elution (e), flow rate 1 cm^3 min^{-1}; detection λ 210 nm; t_R

Table 1 Schedule for solid-phase synthesis (see Scheme 2)

		Reagent[a]	Time × Repeat
Wash	1	CH_2Cl_2	1 min × 4
	2	DMF	1 min × 4
Deprotection	3	20% Piperidine–DMF	2 min × 1
	4	20% Piperidine–DMF	30 min × 1
Wash	5	DMF	1 min × 4
	6	CH_2Cl_2	1 min × 4
	7	DMF	1 min × 4
Coupling	8	Fmoc-amino acid derivative +coupling reagents	90 min × 1
Wash	9	DMF	1 min × 4
	10	CH_2Cl_2	1 min × 4
	11	DMF	1 min × 4

[a] Solvent volume 15 cm^3.

17.6 min); δ_H[500 MHz; $(CD_3)_2SO$] 0.84 5-H$_3$ of Ile), 0.86 (5-H$_3$ of Leu), 0.90 (3-Me of Ile), 0.92 (5-H$_3$ of Leu), 1.09 (4-H of Ile), 1.51 (4-H of Ile), 1.54 (3-H of Leu), 1.57 (3-H of Leu), 1.66 (4-H of Leu), 1.73 (3-H of Ile), 2.80 (3-H of Tyr), 2.96 (3-H of Tyr), 4.04 (2-H of Tyr), 4.25 (2-H of Leu), 4.27 (2-H of Ile) 6.67 (Ar 3- and 5-H of Tyr), 6.99 (Ar 2- and 6-H of Tyr), 8.03 (NH of Tyr), 8.32 (NH of Leu) and 8.56 (NH of Ile); δ_C[100 MHz; $(CD_3)_2SO$] 11.18 (C-5 of Ile), 15.30 (3-Me of Ile), 21.37 (C-5 of Leu), 23.05 (C-5' of Leu), 24.46 (C-4 of Ile), 24.51 (C-4 of Leu), 36.34 (C-3 of Tyr), 37.24 (C-3 of Ile), 39.91 (C-3 of Leu), 50.34 (C-2 of Leu), 53.42 (C-2 of Tyr), 57.00 (C-2 of Ile), 115.45 (Ar C-3, -5 of Tyr), 126.70 (Ar C-1 of Tyr), 130.68 (Ar C-2, -6 of Tyr), 156.78 (Ar C-4 of Tyr), 167.94 (C-1 of Leu), 170.73 (C-1 of Ile) and 173.95 (C-1 of Tyr). The FAB-mass spectrum was measured and the result is shown in Fig. 2.

Tyr-[^{2}H]*Ile-Leu* **6**.—Tripeptide **5** (50 mg) was dissolved in distilled DMF (1 cm^3) and reduced using deuterium gas (99 atom-% ^{2}H) in the presence of platinum(IV) oxide (5 mg) for 2 h at room temperature. The catalyst was filtered off and washed with MeOH, and the combined filtrate and washings were evaporated under reduced pressure. The residue was redissolved in MeOH to remove the labile deuterium to give the reduction product **6** (46 mg) as a solid, δ_H[500 MHz; $(CD_3)_2SO$] 0.82 (5-H$_3$ of Ile), 0.83 (5-H$_3$ of Leu), 0.87 (3-Me of Ile), 0.90 (5-H$_3$ of Leu), 1.06 (4-H of Ile), 1.49 (4-H of Ile), 1.53 (3-H of Leu), 1.56 (3-H of Leu), 1.64 (4-H of Leu), 1.70 (3-H of Ile), 2.76 (3-H of Tyr), 2.93 (3-H of Tyr), 3.99 (2-H of Tyr), 4.23 (2-H of Leu), 4.25 (2-H of Ile), 6.65 (Ar 3- and 5-H of Tyr), 6.98 (Ar 2- and 6-H of Tyr), 8.29 (NH of Leu) and 8.51 (NH of Ile); δ_C[100 MHz; $(CD_3)_2SO$] 10.3–11.0 (C-5 of Ile), 15.26 (3-Me of Ile), 21.36 (C-5 of Leu), 23.04 (C-5' of Leu), 23.7–24.3 (C-4 of Ile), 24.49 (C-4 of Leu), 36.39 (C-3 of Tyr), 37.03 (C-3 of Ile), 39.89 (C-3 of Leu), 50.33 (C-2 of Leu), 53.43 (C-2 of Tyr), 56.93 (C-2 of Ile), 115.42 (Ar C-3, -5 of Tyr), 124.73 (Ar C-1 of Tyr), 130.65 (Ar C-2, -6 of Tyr), 156.73 (Ar C-4 of Tyr), 168.01 (C-1 of Leu), 170.70 (C-1 of Ile) and 173.93 (C-1 of Tyr). The FAB-mass spectrum was measured and the result is shown in Fig. 2. An aliquot of tripeptide **6** was analysed by reversed-phase HPLC (column LiChrospher RP-18; elution (e), flow rate 1 cm^3 min^{-1}; detection λ 210 nm). The retention time of tripeptide **6** was identical with that of tripeptide **7** (t_R 17.6 min).

References

1 D. E. Brundish and R. Wade, *J. Chem. Soc., Perkin Trans. 1*, 1973, 2875.
2 H. Hasegawa, N. Akagawa, Y. Shinohara and S. Baba, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2085.
3 D. E. Brundish and R. Wade, *J. Chem. Soc., Perkin Trans. 1*, 1976, 2186.
4 H. Levine-Pinto, P. Pradelles, J. L. Morgat and P. Fromageot, *J. Labelled Compd. Radiopharm.*, 1980, **17**, 231.
5 A. M. Felix, C. Wang, A. A. Liebman, C. M. Delaney, T. Mowles, B. A. Burghardt, A. M. Charnecki and J. Meienhofer, *Int. J. Pept. Protein Res.*, 1977, **10**, 299.
6 E. Klauschenz, M. Bienert, H. Egler, U. Pleiss, H. Niedrich and K. Nikolics, *Peptides*, 1981, **2**, 445.
7 F. M. Kaspersen, F. M. van Rooy, J. Wallaart and C. Funke, *Recl. Trav. Chim. Pays-Bas*, 1983, **102**, 450.
8 A. C. A. Jansen, K. E. T. Kerling and E. Havinga, *Recl. Trav. Chim. Pays-Bas*, 1970, **89**, 861.
9 E. Atherton and R. C. Sheppard, *Solid Phase Peptide Synthesis*, IRL Press, Oxford, 1989.
10 H. Arold, M. Eule and S. Reissmann, *Z. Chem.*, 1969, **9**, 447.
11 Y. Kataoka, Y. Seto, M. Yamamoto, T. Yamada, S. Kuwata and H. Watanabe, *Bull. Chem. Soc. Jpn.*, 1976, **49**, 1081.
12 J. Shoji, K. Tori and H. Otsuka, *J. Org. Chem.*, 1965, **30**, 2772.
13 K. Okubo and Y. Izumi, *Bull. Chem. Soc. Jpn.*, 1970, **43**, 1541.
14 S. Futagawa, T. Inui and T. Shiba, *Bull. Chem. Soc. Jpn.*, 1973, **46**, 3308.
15 D. L. Nguyen, R. Seyer, A. Heitz and B. Castro, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1025.
16 E. Atherton and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1985, 165.
17 K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, 1986.
18 Nomenclature for sequence ions in mass spectra of peptides: P. Roepstorff and J. Fohlman, *Biomed. Mass Spectrom.*, 1984, **11**, 601.
19 M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, **8**, 539.
20 T. J. Simpson, *Chem. Soc. Rev.*, 1987, **16**, 123.
21 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595.

Paper 2/04722E
Received 2*nd September* 1992
Accepted 15*th October* 1992