Synthesis of Two Possible Disulfide Bonds Containing Peptide Fragments (Cys^6–Cys^{47}, Cys^{48}–Cys^{52} (Type I), and Cys^6–Cys^{48}, Cys^{47}–Cys^{52} (Type II) of h-IGF-I) for the Identification of Disulfide Bond Linkage in Recombinantly Produced h-IGF-I

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(Received January 22, 1999)

The primary structure of human IGF-I, except for the disulfide bond system, has been reported by Rinderknecht and Humbel. IGF-I afforded the corresponding characteristic peptide fragments on V_8 protease digestion, which contained Cys^6, Cys^{47}, Cys^{48}, and Cys^{52}. Two possible fragments, Type I with Cys^6–Cys^{47} and Cys^{48}–Cys^{52} and Type II with Cys^6–Cys^{48} and Cys^{47}–Cys^{52} of h-IGF-I(4-9,47-53), were chemically synthesized. The disulfide bond system of IGF-I was unequivocally determined to be the Type-II form along with Cys^{18}–Cys^{61}. Interestingly, the Type-I system was included in the disulfide bond isomer produced as the main by-product in the refolding step on IGF-I synthesis by the recombinant DNA method.

The insulin-like growth factor I (IGF-I or somatomedin C) is a serum polypeptide (MW 7649) with growth-promoting and insulin-like hypoglycemic activities[1] by circulating through the body, bound by several IGF binding proteins (IGF-BPs). The primary structure of human IGF-I was determined chemically by Rinderknecht and Humbel in 1978;[2] it consists of 70 amino acid residues with 3 intermolecular disulfide bonds formed by 6 internal cysteines (Fig. 2). It was found that IGF-I shows 49% homology, including the cysteine positions, with insulin, whose disulfide bond linkages were chemically determined by Ryle et al.[3] Based on this homology, the disulfide linkage system of IGF-I was postulated to be Cys^6–Cys^{48}, Cys^{18}–Cys^{61}, and Cys^{47}–Cys^{52}.

Human IGF-I can now be obtained by means of a recombinant DNA method in quantity, and its identity to natural IGF-I has been reported.[4,5] However, the exact disulfide bond linkage system has never been determined by a chemical method.

Recently, we reported on the amino acid sequence of rat IGF-I and its weaker glucose stimulation activity in relation to [^{3}H]thymidine uptake stimulation, when compared with human IGF-I.[6] We have postulated that this weaker activity is due to a difference in the three-dimensional (3D) structures between the human and rat IGF-I. When the reduced form of IGF-I was refolded by air-oxidation, the formation of by-products with different disulfide bond linkages was noted.[5,7,8] Also, we have found that the disulfide bond isomers produced upon the refolding of IGF-I have weaker biological and immunological activities than the native form of IGF-I.[9] Thus, a direct determination of the disulfide linkages of IGF-I has become a prerequisite for discussing its 3D-structure-activity relationship.

We wish to report here on the synthesis of two possible disulfide bond-containing fragments (V_8-isomer peptides), one with Cys^6–Cys^{47} and Cys^{48}–Cys^{52} (Type I), and the other with Cys^6–Cys^{48} and Cys^{47}–Cys^{52} (Type II), and the chemical identification of the disulfide bond linkages of natural IGF-I as the Type-II form.

Materials and Methods

Abbreviations according to the IUPAC-IUB Commission *Eur. J. Biochem.*, **138**, 9 (1984) and J.H.Jones, "Amino Acids, Peptides and Proteins," Vol. 16, (The Royal Society of Chemistry,1985) are used throughout. All amino acid symbols denote the L-configuration, except for glycine. Additional abbreviations are: DCC, dicyclohexylcarbodiimide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HOBt, 1-hydroxybenzotriazole; HONb, 1-hydoxy-5-norbornen-2,3-dicarboxyimide; Boc, *t*-butoxycarbonyl; Bzl, benzyl ether; Tos, tosyl; Acm, acetamidomethyl; MBzl, *p*-methoxybenzyl; TFA, trifluoroacetic acid; DCM, dichloromethane; DMF, *N*, *N*-dimethylformamide; IGF-I, insulin like growth factor I.

Preparation of IGF-I and Its Disulfide Isomer. Recombinant IGF-I was produced by *Escherichia coli* and purified as reported.[5]

Deceased on Sep. 23, 1998.

IGF-I, a reduced form,[5] was dissolved in 6 M guanidine HCl–10 mM Tris-HCl buffer (pH 8.4) (1 M = 1 mol dm^{-3}) at 1.0 mg ml^{-1}, and then diluted with a 10-fold volume of 10 mM Tris-HCl buffer (pH 8.4) to allow the formation of intramolecular disulfide bonds. The mixture was analyzed by reverse-phase HPLC (Fig. 1). IGF-I and its disulfide bond isomer were isolated from the refolding mixture in a manner similar to the case of the reported IGF-I.[5]

Chymotrypsin–Trypsin Digestion for the Determination of the Disulfide Bond Linkage System between Cys^{18} and Cys^{61} in Both IGF-I Isomers. IGF-I or the isomer (2 mg ml^{-1}) in 50 mM PBS (pH 7.8) was digested with a mixture of chymotrypsin and trypsin (both from Sigma Chemical, St. Louis, Mo., U.S.A.), at a *S/E* ratio of 100/1 for each enzyme, at 37 °C for 2.5 h. All fragments were isolated by reversed-phase HPLC, and their structures were determined by amino acid analysis with a Hitachi Model 835 by the post-column ninhydrin assay method, with reference to the amino acid sequence reported in a previous paper.[5] The same fragment, IGF-I (17-24, 61-70), which is a dimer peptide comprising Val^{17}–Tyr^{24} and Cys^{61}–Ala^{70} connected through a disulfide bond, was obtained from both IGF-I and the isomer, which showed that both IGF-I isomers have a common disulfide bond between Cys^{18}–Cys^{61}, leaving the disulfide bond system comprising Cys^{6}, Cys^{47}, Cys^{48}, and Cys^{52} to be determined.

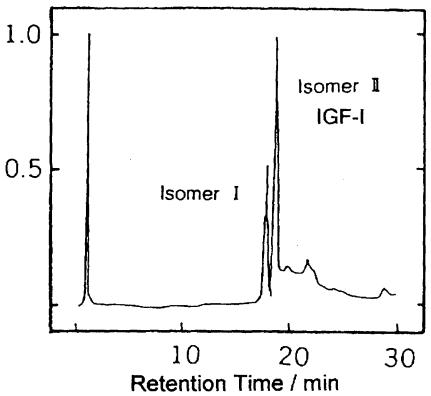

Fig. 1. HPLC profiles of refolding mixture of h-IGF-I by recombinant DNA method.[5] Conditions: column, Beckman RPSC (4.6×75 mm); detection, 210 nm; flow rate, 1.0 ml min^{-1}; elution solvent, 10 mM TFA with a 10—60% CH_3CN gradient.

V_8 Protease Digestion. It was expected from its amino acid sequence that IGF-I and the isomer would afford respective fragments (V_8-isomer peptides) containing these four cysteines on V_8 protease digestion, the expected structures being IGF-I (4-9, 47-53) Type I, and IGF-I (4-9, 47-53) Type II (Fig. 2).

IGF-I (4-9, 47-53) Type II was obtained upon V_8 protease digestion of recombinant IGF-I as follows. Recombinant IGF-I, 100 μg, was digested with 2 μg of *Staphylococcus* V_8 protease (Pierce Chemical, Rockford, Ill., U.S.A.) in 50 mM Tris buffer (pH 7.0) at 37 °C for 18 h. All fragments were isolated by reversed-phase HPLC and their structures were determined in a manner similar to that described above. IGF-I (4-9, 47-53) Type II was obtained in a pure state.

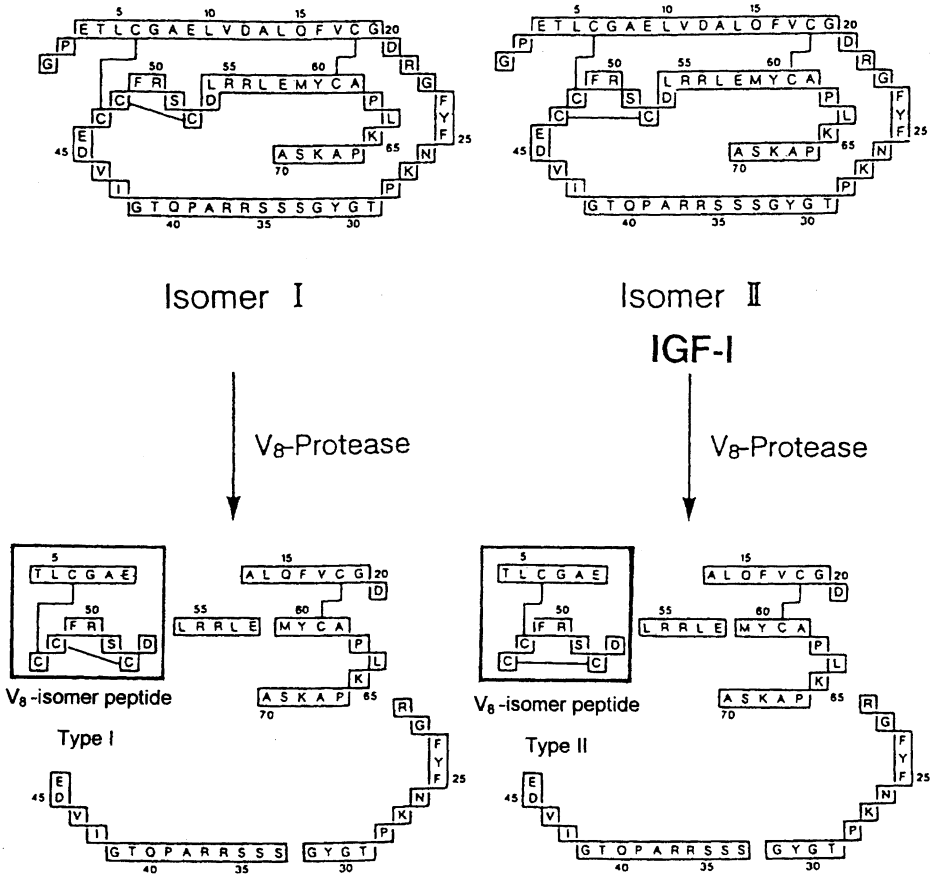

Fig. 2. The possible structures of h-IGF-I and V_8 protease digested peptides (V_8-isomer peptides) of recombinantly produced h-IGF-I (Isomer II) and its isomer (Isomer I).

The disulfide bond isomer of IGF-I was treated in the same way to afford IGF-I (4-9, 47-53) Type I.

Preparation of Two Types (I and II) of V_8 Protease Digested Peptides, h-IGF-I (4-9, 47-53) (V_8-Isomer Peptides). We tried to synthesize these two possible disulfide-containing fragments in order to determine which of these linkages is involved in natural IGF-I or the isomer. The strategy which we used to determine the disulfide bond linkage was also used by Nagasawa et al. to determine the disulfide bond linkage of Bombyxin, an insulin-like brain neuropeptide from silkworm.[10]

The key intermediates, 6-Acm IGF-I (4-9) (**10**), 47-Acm IGF-I (47-53) (**22**), and 48-Acm IGF-I (47-53) (**26**), for the synthesis of two types of V_8 protease digested peptides (V_8-isomer peptides), were synthesized in the liquid phase by the Boc strategy, as shown in Figs. 3, 4, and 5. The full protected peptides (**9**, **21**, and **25**) were assembled in a pure state according to stepwise elongation from their C-terminal by individually coupling 1.00 equiv of Boc amino acid in DCM or DMF using DCC (1.10 equiv) and HOBt (1.00 equiv) or EDC (1.00 equiv) and HONb (1.00 equiv) as coupling reagents. Each segment was protected at its C-terminus by benzyl ester. The functional side chains were protected by the following groups: benzyl ether (Bzl) for Ser and Thr, Tos for Arg, benzyl ester (Bzl) for Asp and Glu, MBzl for Cys of intramolecular disulfide bond position, Acm for Cys of intermolecular disulfide bond position.

The protected peptides were treated by the standard HF procedure (HF: anisole was 10 : 1, v/v) at 0 °C for 1 h to remove all of the protecting groups, except the Acm group.

HF-treated segments of **21** and **25** were subjected to an intramolecular oxidative folding reaction in a pH 8.0 solution at room temperature in the presence of 0.1 M $K_3[Fe(CN)_6]$ for 30 min, or air oxidation for 3 h, respectively.

The crude products of each segment were purified by preparative HPLC on a reversed-phase column, and the homogeneity was confirmed by RP-HPLC and amino acid analysis.

Heterodimerization reactions of **10** and **26**, and of **10** and **22**, were carried out under the Kamber's condition[11] (Fig. 6). Acm peptides were dissolved at 5×10^{-4} M concentration in a mixture of MeOH and H_2O (4 : 1, v/v), then mixed with the same volume of a mixture of MeOH and 1 M HCl (4 : 1, v/v) containing I_2 (40 equiv of the peptides), and kept for 15 min at room temperature to form disulfide bond dimers, IGF-I (4-9, 47-53) Type II, and IGF-I (4-9, 47-53) Type I. The reactions were stopped by adding L-ascorbic acid, and the reaction products were purified, as a single peak, on a reversed-phase HPLC column using an acetonitrile gradient, from 0 to 60% in 30 min, in 0.01 M TFA, as shown in Figs. 7 and 8.

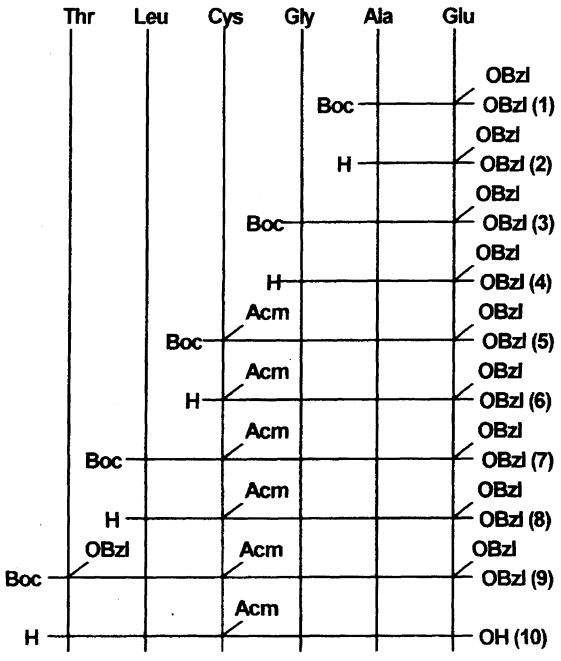

Fig. 3. Synthetic scheme for 6-Acm h-IGF-I(4-9) (**10**).

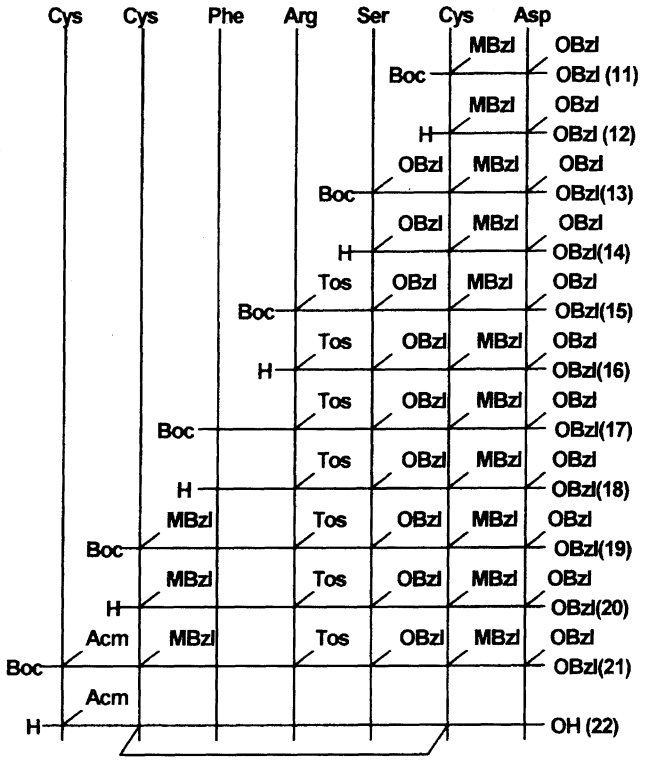

Fig. 4. Synthetic scheme for 47-Acm h-IGF-I(47-53) (**22**).

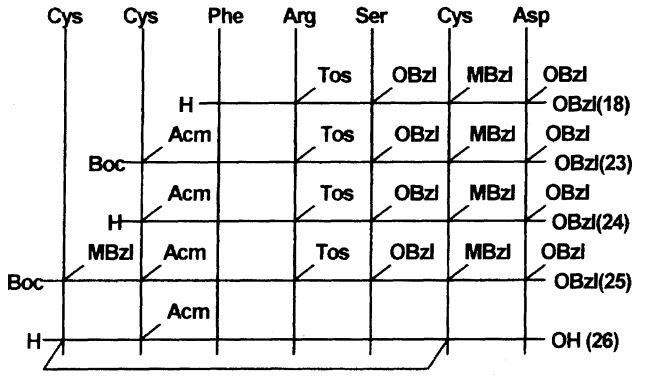

Fig. 5. Synthetic scheme for 48-Acm h-IGF-I(47-53) (**26**).

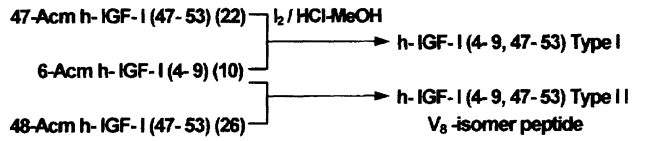

Fig. 6. A scheme for the preparations of two possible V_8 protease digested disulfide containing peptides (V_8-isomer peptides), h-IGF-I(4-9, 47-53) Type I and Type II (Fig. 2), of recombinantly produced h-IGF-I and its isomer.

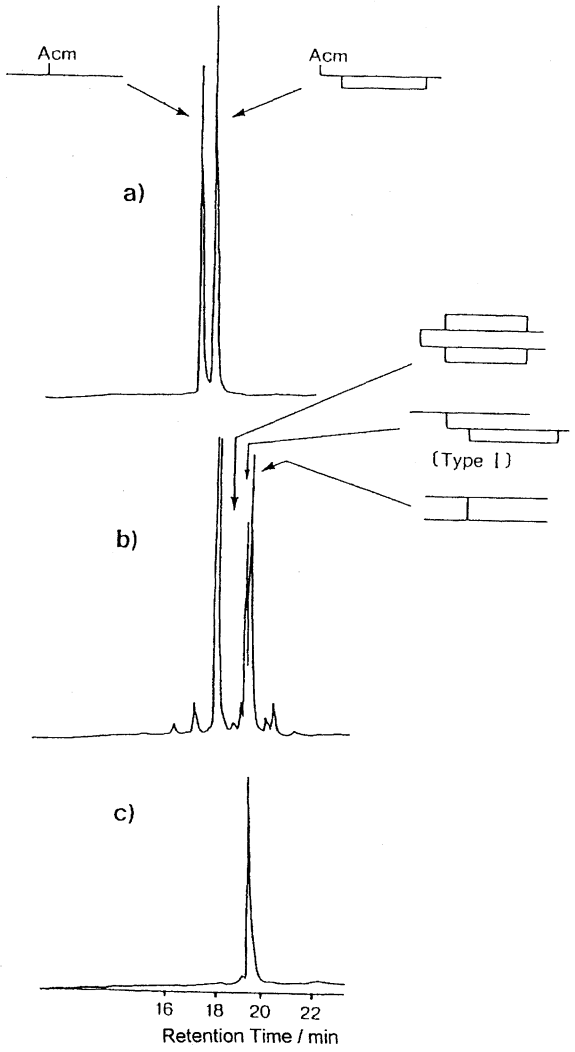

Fig. 7. HPLC analysis of synthetic h-IGF-I(4-9, 47-53) Type I by heterodimerization reaction. Peptide was eluted on YMC packed AP-302 200 Å ODS column (4.6×150 mm) with H_2O–CH_3CN gradient containing 0.01 M TFA (0 to 60% CH_3CN over a period of 0 to 30 min). Flow rate, 1 ml min^{-1}. Detection was 214 nm. a) 6-Acm h-IGF-I(4-9) (**10**) and 47-Acm h-IGF-I(47-53) (**22**). b) I_2-Oxidized solution of a). c) After purification with preparative HPLC (YMC packed AP-343-10 200 Å ODS, 20×250 mm).

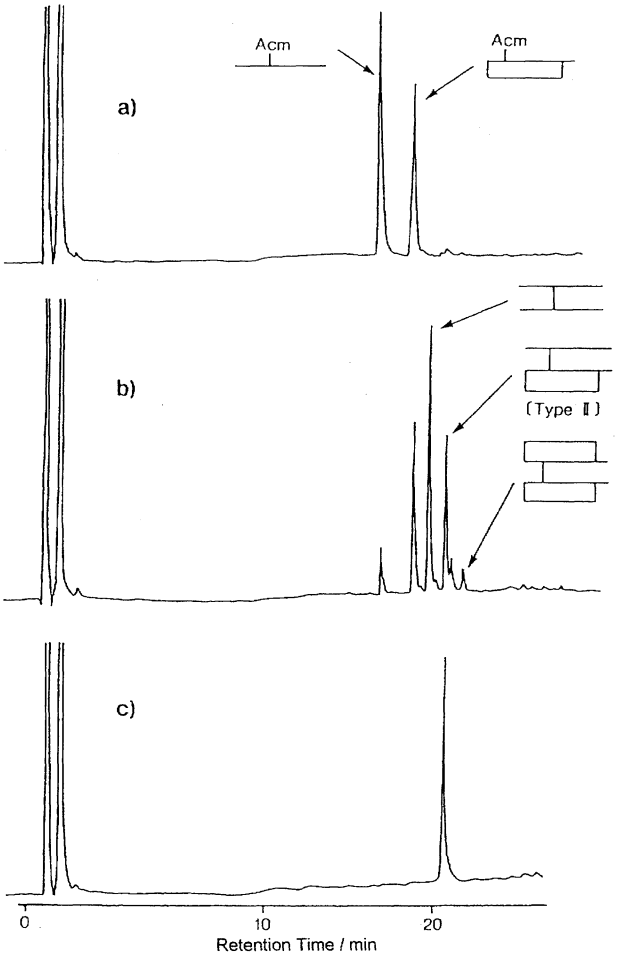

Fig. 8. HPLC analysis of synthetic h-IGF-I(4-9, 47-53) Type II by heterodimerization reaction. The conditions were the same as those shown in Fig. 7. a) 6-Acm h-IGF-I(4-9) (**10**) and 48-Acm h-IGF-I (47-53) (**26**). b) I_2-Oxidized solution of a). c) After purification with preparative HPLC (YMC packed AP-343-10 200 Å ODS, 20×250 mm).

Results and Discussion

When the V_8 protease-digested fragment from recombinant IGF-I was analyzed under the same HPLC conditions, it was eluted at the same retention time as the synthetic fragment, IGF-I (4-9, 47-53) Type II (Figs. 9a and 9b. A mixed preparation gave a single peak, supporting the identity of these peptides (Fig. 9c). The HPLC profiles of IGF-I (4-9, 47-53) Type I confirmed its identity with the characteristic fragment obtained on V_8 digestion of the IGF-I isomer (Figs. 10a, 10b, and 10c).

The amino acid composition was determined with a Waters Pico–Tag System; the results are presented in Table 1 together with those for fragments prepared from IGF-I and its isomer.

The possibility of disulfide reshuffling during V_8 protease digestion (pH 7.0, 37 °C for 18 h) was concluded to be negligible for the following two reasons. First, the Type-I or Type-II fragment was solely obtained from the isomer or native form of IGF-I, respectively. Second, the respective fragment was obtained in quantitative yield, and fragments containing Cys^6, Cys^{47}, Cys^{48}, and Cys^{52} other than the fragment (Type I from the isomer or Type II from the IGF-I) were not detected in the digestion mixture.

Thus, IGF-I was proved directly by chemical synthesis to have the disulfide bond linkage system of Cys^6–Cys^{48}, Cys^{18}–Cys^{61}, and Cys^{47}–Cys^{52}, and the isomer to have one of Cys^6–Cys^{47}, Cys^{18}–Cys^{61}, and Cys^{48}–Cys^{52}. It would be interesting to know whether or not the presently reported fragments of IGF-I (4-9, 47-53) Type I, and Type II exhibit any distinguishable activity in a radioreceptor assay for the IGF-I receptor or the insulin receptor, or in an immunocross-reactivity assay. Studies along these lines are now in progress in our labolatory.[12–19]

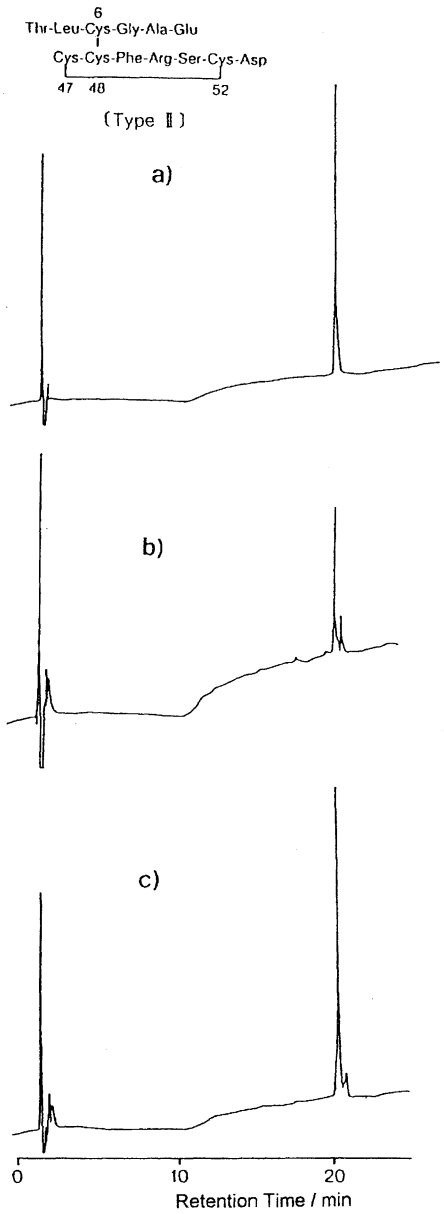

Fig. 9. HPLC profile of synthetic h-IGF-I(4-9, 47-53) Type II for the identification of the disulfide bond linkage system of h-IGF-I. The conditions were the same as those shown in Fig. 7. a) Synthetic. b) V_8-Protease digested fragment-(4-9, 47-53) of h-IGF-I produced by recombinant DNA method. c) Mixture of a) and b).

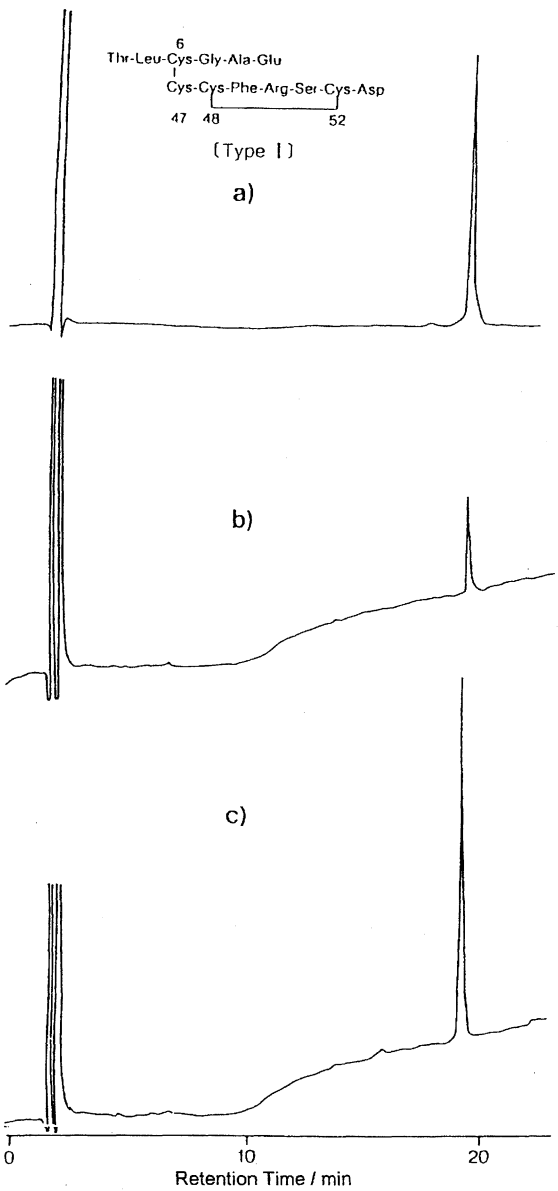

Fig. 10. HPLC profile of synthetic h-IGF-I(4-9, 47-53) Type I for the identification of the disulfide bond linkage system of Isomer I of h-IGF-I. The conditions were the same as those shown in Fig. 7. a) Synthetic. b) V_8-Protease digested fragment(4-9, 47-53) of Isomer I produced by recombinant DNA method. c) Mixture of a) and b).

Expermental

All melting points are uncorrected. TLC was performed on Merck silica-gel F_{254} plates with the following solvent systems (v/v): $R_f(1)$ = water saturated ethyl acetate, R_f (2) = *n*-BuOH–AcOH–Water(4 : 1 : 1), R_f (3) = $CHCl_3$–MeOH (6 : 1). Optical rotations were obtained using a Perkin–Elmer 141 polarimeter using DMF as the solvent at 24 °C. Amino acid analyses were carried out by a Hitachi 835 amino acid analyzer or Waters Pico–Tag System, after hydrolysis of the peptides in 6 M HCl at 110 °C for 24 h. HPLC was performed using a Waters M600 multisolvent delivery system and a Shimadzu LC-6A liquid chromatgraph system.

6-Acm h-IGF-I(4-9) [10]. Boc–Ala–Glu(OBzl)–OBzl-(1). H–Glu(OBzl)–OBzl *p*-toluenesulfonate (24.98 g, 0.05 mol) was dissolved in AcOEt (300 ml) and the solution was washed with Na_2CO_3 saturated water (300 ml×2) and water (300 ml×2), dried over anhydrous Na_2SO_4, and evaporated to dryness in vacuo. The resulting oily residue was dissolved in DCM (170 ml) together with Boc–Ala–OH (8.61 g, 0.05 mol) and HOBt (6.75 g, 0.05 mol), and the solution was cooled to 0 °C. To this solution was added dropwise the DCM solution (100 ml) of DCC (11.35 g, 0.055 mol) with stirring. After stirring at 0 °C for 2 h at room temperature for an additional 12 h, the reaction mixture was filtered to remove

Table 1. Amino Acid Compositions of h-IGF-I V_8-Protease Digestion Fragments (V_8-Isomer Peptides) from IGF-I and Its Isomer, and Synthetic IGF-I(4-9, 47-53) Type I and Type II

	Fragment from			Synthetic	
	Theoretical	IGF-I[a)]	Isomer-I[a)]	Type II[b)]	Type I[b)]
Asp	1	1.1	1.0	1.1	0.8
Glu	1	1.0	1.1	1.0	1.1
Ser	1	1.0	0.9	1.0	1.0
Gly	1	1.0	1.0	1.1	1.2
Arg	1	0.9	1.0	1.0	1.0
Thr	1	1.0	1.0	0.9	1.1
Ala	1	1.0	1.0	1.1	1.2
$(Cys)_2$	2	1.4	1.2	1.0	1.0
Leu	1	0.9	0.9	1.0	1.1
Phe	1	0.9	0.8	1.0	1.0

a) Amino acid analysis with a Hitachi Model 835. b) with a Waters Pico–Tag System.

the formed dicyclohexylurea (DCU), and the filtrate was evaporated to dryness in vacuo. The resulting oily residue was dissolved in AcOEt (170 ml) and cooled, and the thus-produced DCU was filtered off. After the filtrate had been washed with 1 M citric acid, a saturated aqueous $NaHCO_3$ solution and water successively, it was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. After the oily product had then been allowed to stand for 2 weeks at room temperature, **1** was obtained as a needle crystal. The pure product was recrystallized from the mixed solvent of AcOEt–ether–Hexane in 78.8% (19.65 g) yield. Mp 136—137 °C, $[\alpha]_D^{24}$ −18.50° (*c* 1.0, DMF), R_f (1) = 0.83. Found: C, 65.30; H, 6.57; N, 5.76%. Calcd for $C_{27}H_{34}N_2O_7$: C, 65.04; H, 6.87; N, 5.62%.

H–Ala–Glu(OBzl)–OBzl(2) Hydrochloride. **1** (7.00 g, 14 mmol) was dissolved in 5 M HCl/AcOEt (70 ml) at 0 °C. After the reaction mixture had been allowed to stand at room temperature for 20 min, the solution was concentrated in vacuo. The residual oil was washed three times with anhydrous ether. **2** Hydrochloride was obtained in the theoretical yield as an oily product and used in the next procedure without further purification. R_f (1) = 0.00.

Boc–Gly–Ala–Glu(OBzl)–OBzl (3). Boc–Gly–OH (316 mg, 2 mmol) and **2** [prepared from hydrochloride (870 mg, 2 mmol) with triethylamine (0.28 ml, 2 mmol) in DCM were treated with DCC (413 mg, 2 mmol) and HOBt (270 mg, 2 mmol) in DCM. **3** was obtained in a similar manner as in the synthesis of **1**. The product was recrystallized from AcOEt–ether–Hexane; yield, 1.16 g, (100%). Mp 101.5—102 °C, $[\alpha]_D^{24}$ −15.50° (*c* 1.0, DMF), R_f (1) = 0.75, R_f(2) = 0.80. Found: C, 63.08; H, 6.72; N, 7.64%. Calcd for $C_{29}H_{37}N_3O_8$: C, 62.69; H, 6.71; N, 7.56%.

H–Gly–Ala–Glu (OBzl)–OBzl(4) Hydochloride. **3** (1.11 g, 2 mmol) was dissolved in 5 M HCl/AcOEt (10 ml) and left to stand at room temperature for 30 min. The solution was evaporated to dryness in vacuo and the residue triturated with anhydrous ether. The solid product was disintegrated under anhydrous ether, filtered, washed with ether and dried in vacuo; it was then used in the next procedure without further purification; yield, 980 mg (99.7%). R_f (1) = 0.00.

Boc–Cys(Acm)–Gly–Ala–Glu(OBzl)–OBzl (5). Boc–Cys(Acm)–OH (409 mg, 1.38 mmol) and **4** [prepared from hydrochloride (680 mg, 1.38 mmol) with triethylamine (0.20 ml, 1.38 mmol) in DMF] were treated with DCC (289 mg, 1.38 mmol) and HOBt (189 mg, 1.38 mmol) in DMF. **5** was obtained in a similar manner as in the synthesis of **1**. The product was recrystallized from AcOEt; yield 808 mg (80.2%). Mp 154.5—155.5 °C, $[\alpha]_D^{24}$ −25.70° (*c* 1.0, DMF), R_f (1) = 0.49. Found: C, 57.50; H, 6.35; N, 9.49; S, 4.30%. Calcd for $C_{35}H_{47}N_5O_{10}S$: C, 57.60; H, 6.49; N, 9.60; S, 4.39%.

Boc–Leu–Cys(Acm)–Gly–Ala–Glu(OBzl)–OBzl (7). Boc–Leu–OH monohydrate (274 mg, 1.1 mmol) and **6** [prepared from hydrochloride (660 mg, 0.99 mmol), which were obtained in the same way (5 M HCl/AcOEt with **5**) as in the synthesis of compound **4**], were treated with DCC (227 mg, 1.1 mmol) and HOBt (149 mg,1.1 mmol) in DMF. **7** was obtained in a similar manner as in the synthesis of **1**. The product was collected as an amorphous solid from a mixed solvent of $CHCl_3$–ether; yield, 669 mg (80.1%). $[\alpha]_D^{24}$ −21.40° (*c* 1.0, DMF), R_f (1) = 0.40, R_f (2) = 0.75. Found: C, 58.24; H, 7.02; N, 10.03; S, 3.68%. Calcd for $C_{41}H_{58}N_6O_{11}S$: C, 58.42; H, 6.93; N, 9.97; S, 3.80%.

Boc–Thr(OBzl)–Leu–Cys(Acm)–Gly–Ala–Glu(OBzl) –OBzl (9). Boc–Thr(OBzl)–OH (371 mg, 1.21 mmol), HONb (217 mg,1.21 mmol), and **8** [prepared from hydrochloride (856 mg, 1.10 mmol) with triethylamine (0.15 ml, 1.10 mmol) in DMF (10 ml), which was obtained in the same way (5 M HCl/AcOEt with **7**) as in the synthesis of compound **4**] were dissolved in DMF (10 ml) and EDC (186 mg, 1.21 mmol) was added at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and at room temperature overnight. The solvent was removed in vacuo and the residue was dissolved in DCM. After the solution was washed with 1 M citric acid, water, a saturated aqueous $NaHCO_3$ solution, and water successively, it was dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and the residue was solidified with the mixed solvent of AcOEt–ether. **9** was collected as an amorphous solid from AcOEt–ether; yield 1.095 g (96.2%). $[\alpha]_D^{24}$ −24.70° (*c* 1.0, DMF), R_f (1) = 0.29, R_f (2) = 0.46. Found: C, 60.24; H, 6.67; N, 9.56; S, 3.11%. Calcd for $C_{52}H_{71}N_7O_{13}S$: C, 60.39; H, 6.92; N, 9.48; S, 3.10%. Ami-

no acid analysis: Thr 0.80, Leu 0.90, $(Cys)_2$ 0.40, Gly 1.00, Ala 1.00, Glu (1.00).

H–Thr–Leu–Cys(Acm)–Gly–Ala–Glu–OH(10)·AcOH. (6-Acm h-IGF-I(4-9) [10]) **9** (790 mg, 0.76 mmol) was dissolved in anhydrous hydrogen fluoride (15 ml) together with anisole (1.5 ml) at −70 °C, and the mixture was stirred at 0 °C for 70 min. Hydrogen fluoride was evaporated in vacuo at room temperature, and the residue was dissolved in water (150 ml). The solution was washed with ether (50 ml×3) and the aqueous layer was applied to a column of Dowex 1×2 (AcO^-, 2×10 cm). The passed solution and washings (1 M AcOH, 100 ml) were combined and lyophilized to give crude **10** (441 mg). The crude product was purified by HPLC on a reversed-phase column (YMC-packed AP-343-10, 2×25 cm, ODS 200 Å). The peptide (150 mg) was eluted with a linear gradient of 0—100% CH_3CN in 20 min containing 0.01 M trifluoroacetic acid at a flow rate of 5 ml min^{-1}. A fraction of **10** was monitored for absorbance at 214 nm and concentrated in vacuo, and then lyophilized; yield, 110 mg. R_f (2) = 0.09. Amino acid analysis: Thr 0.97, Leu 0.96, $(Cys)_2$ 0.35, Gly 0.99, Ala 0.98, Glu (1.00). $[\alpha]_D^{24}$ −26.0° (*c* 1.0,1 M–AcOH).

47-Acmh-IGF-I(47-53)[22]. Boc–Cys(MBzl)–Asp(OBzl)–OBzl (11). Boc–Cys(MBzl)–OH (3.41 g, 10 mmol), H–Asp(OBzl)–OBzl [prepared from *p*-toluenesulfonate (5.33 g, 11 mmol) with triethylamine (1.54 ml, 11 mmol) in $CHCl_3$ (50 ml)], and HOBt (1.48 g, 11 mmol) were treated with DCC (2.27 g, 11 mmol) in $CHCl_3$ (60 ml). **11** was obtained in a similar manner as in the synthesis of **1**. The product was recrystallized from AcOEt–ether–Hexane; yield, 6.22 g (97.6%). Mp 97.5—98 °C, $[\alpha]_D^{24}$ −28.40° (*c* 1.0, DMF), R_f (1) = 0.81, R_f (2) = 0.89. Found: C, 64.18; H, 6.40; N, 4.61; S, 5.22%. Calcd for $C_{34}H_{40}N_2O_8S$: C, 64.13; H, 6.33; N, 4.40; S, 5.03%.

Boc–Ser(OBzl)–Cys(MBzl)–Asp(OBzl)–OBzl (13). Boc–Ser(OBzl)–OH (2.36 g, 8 mmol), HOBt (1.081 g, 8 mmol), and **12** [prepared from hydrochloride (4.58 g, 8 mmol) with triethylamine (1.12 ml, 8 mmol) in DMF, which was obtained in the same way (5 M HCl/AcOEt with **11**) as in the synthesis of compound **2**] were treated with EDC (1.242 g, 8 mmol) in DMF (50 ml). **13** was obtained in a similar manner as in the synthesis of **9**. The product was recrystallized from cold AcOEt; yield 5.562 g (85.4%). Mp 114—115 ° C, $[\alpha]_D^{24}$ −19.50° (*c* 1.0, DMF), R_f (1) = 0.90. Found: C, 64.50; H, 6.51; N, 5.58; S, 3.98%. Calcd for $C_{44}H_{51}N_3O_{10}S$: C, 64.93; H, 6.32; N, 5.16; S, 3.94%.

Boc–Arg(Tos)–Ser(OBzl)–Cys(MBzl)–Asp(OBzl)–OBzl (15). Boc–Arg(Tos)–OH (2.799 g, 5.62 mmol), HONb (1.007 g, 5.62 mmol), and **14** [prepared from hydrochloride (3.83 g, 5.11 mmol) with triethylamine (0.715 ml, 5.11 mmol) in DMF (40 ml), which was obtained in the same way (5 M HCl/AcOEt with **13**) as in the synthesis of compound **4**] were treated with EDC (872 mg, 5.62 mmol) in DMF. **15** was obtained in a similar manner as in the synthesis of **9**. The product was recrystallized from AcOEt–ether–Hexane; yield 5.361 g (93.3%). Mp 95—98 °C, $[\alpha]_D^{24}$ − 18.30° (*c* 1.0, DMF), R_f (1) = 0.82, R_f (2) = 0.79. Found: C, 61.07; H, 6.34; N, 8.77; S, 5.50%. Calcd for $C_{57}H_{69}N_7O_{13}S_2$:C, 60.89; H, 6.19; N, 8.72; S, 5.70%.

Boc–Phe–Arg(Tos)–Ser(OBzl)–Cys(MBzl)–Asp(OBzl)–OBzl (17). Boc–Phe–OH (1.39 g, 5.25 mmol), HONb (941 mg, 5.25 mmol), and **16** [prepared from hydrochloride (5.059 g, 4.77 mmol) with triethylamine (0.67 ml, 4.77 mmol) in DMF (50 ml), which was obtained in the same way (5 M HCl/AcOEt with **15**) as in the synthesis of compound **2**] were treated with EDC (815 mg, 5.25 mmol) in DMF. **17** was obtained in a similar manner as in the synthesis of **9**. The product was recrystallized from AcOEt–ether; yield, 5.624 g (92.7%). Mp 122 °C (decomp), $[\alpha]_D^{24}$ −20.40° (*c* 1.0, DMF), R_f (1) = 0.83. Found: C, 62.14; H, 6.21; N, 9.01; S, 5.19%. Calcd for $C_{66}H_{78}N_8O_{14}S_2$:C, 62.34; H, 6.18; N, 8.81; S, 5.04%. Amino acid analysis: Phe 0.8, Arg (1.0), Ser 0.7, Asp 1.1.

Boc–Cys(MBzl)–Phe–Arg(Tos)–Ser(OBzl)–Cys(MBzl)–Asp(OBzl)–OBzl (19). Boc–Cys(MBzl)–OH (341 mg, 1.0 mmol), HONb (197 mg, 1.1 mmol), and **18** [prepared from hydrochloride (1.250 g, 1.03 mmol) with triethylamine (0.154 ml, 1.1 mmol) in DMF (15 ml), which was obtained in the same way (5 M HCl/AcOEt with **17**) as in the synthesis of compound **4**] were treated with EDC (197 mg, 1.1 mmol) in DMF. **19** was obtained in a similar manner as the synthesis of **9**. The product was recrystallized from MeOH; yield 1.236 g (82.7%). Mp 165—166 °C, $[\alpha]_D^{24}$ −22.0° (*c* 1.0, DMF), R_f (3) = 0.89. Found: C, 61.77; H, 6.21; N, 8.56; S, 6.55%. Calcd for $C_{77}H_{91}N_9O_{16}S_3$:C, 61.87; H, 6.15; N, 8.43; S, 6.43%.

Boc–Cys(Acm)–Cys(MBzl)–Phe–Arg(Tos)–Ser(OBzl)–Cys(MBzl)–Asp(OBzl)–OBzl (21). Boc–Cys(Acm)–OH (164 mg, 0.56 mmol), HONb (111 mg, 0.62 mmol), and **20** [prepared from hydrochloride (801 mg, 0.56 mmol) with triethylamine (0.08 ml, 0.56 mmol) in DMF (15 ml), which was obtained in the same way (5 M HCl/AcOEt with **19**) as in the synthesis of compound **4**] were treated with EDC (96 mg, 0.62 mmol) in DMF. **21** was obtained in a similar manner as in the synthesis of **9**. The product was recrystallized from MeOH; yield, 911 mg (97.4%). Mp 150—151 °C, $[\alpha]_D^{24}$ −21.90° (*c* 1.0, DMF), R_f (3) = 0.73. Found: C, 59.68; H, 5.93; N, 9.35; S, 7.31%. Calcd for $C_{83}H_{101}N_{11}O_{18}S_4$:C, 59.73; H, 6.10; N, 9.23; S, 7.68%.

H–Cys(Acm)–Cys–Phe–Arg–Ser–Cys–Asp–OH (22)·AcOH. (47-Acm h-IGF-I(47-53) [22]) **21** (407 mg, 0.24 mmol) was dissolved in anhydrous hydrogen fluoride (15 ml) together with anisole (1.5 ml) at −70 °C, and the mixture was stirred at 0 °C for 70 min. Hydrogen fluoride was evaporated in vacuo at room temperature, and the residue was dissolved in water (50 ml). The solution was washed with ether (50 ml×3) and the aqueous layer was applied to a column of Dowex 1×2 (AcO^-, 2×10 cm). The passed solution (60 ml) and washings (1 M AcOH, 50 ml) were combined and after being adjusted pH 8.0 by 0.1 M NaOH followed by intramolecular disulfide bond formation by 0.1 M $K_3[Fe(CN)_6]$ (5 ml) for 30 min at room temperature. The reaction mixture was acidified at pH 5 by AcOH treated with SEP-

PAK(C_{18}), and lyophilized to give crude **22** (200 mg). The crude product was purified by HPLC on a reversed-phase column (YMC-packed AP-343-10, ODS 200 Å, 2×25 cm). The peptide (150 mg) was eluted with a linear gradient of 0—100 % CH_3CN in 20 min containing 0.01 M trifluoroacetic acid at a flow rate of 5 ml min^{-1}. A fraction of **22** was monitored for absorbance at 214 nm, concentrated in vacuo, and then lyophilized; yield, 105 mg. $[\alpha]_D^{24}$ −23.5° (*c* 1.0, H_2O). Amino acid analysis: Phe (1.00), Arg 1.00, Ser 1.40, Asp 1.00.

48-Acm h-IGF-I(47-53) [26]. Boc–Cys(Acm)–Phe–Arg (Tos)–Ser(OBzl)–Cys(MBzl)–Asp(OBzl)–OBzl (23). Boc–Cys(Acm)–OH (389 mg, 1.33 mmol), HONb (238 mg, 1.33 mmol), and **18** [prepared from hydrochloride (1.461 g, 1.21 mmol) with triethylamine (0.169 ml, 1.21 mmol) as mentioned above] were treated with EDC (206 mg, 1.33 mmol) in DMF (20 ml). **23** was obtained in a similar manner as in the synthesis of **9**. The product was collected by filtration as an amorphous solid from cold ethyl acetate solution; yield, 1.323 g (75.6%). $[\alpha]_D^{24}$ −20.20 ° (*c* 1.0, DMF), R_f (1) = 0.28, R_f (2) = 0.85, R_f (3) = 0.91. Found: C, 59.44; H, 5.94; N, 9.80; S, 6.77%. Calcd for $C_{72}H_{88}N_{10}O_{16}S_3$:C, 59.82; H, 6.14; N, 9.69; S, 6.65%.

Boc–Cys(MBzl)–Cys(Acm)–Phe–Arg(Tos)–Ser(OBzl) –Cys(MBzl)–Asp(OBzl)–OBzl (25). Boc–Cys-(MBzl)–OH(341 mg, 1.00 mmol), HONb (179 mg, 1.00 mmol), and **24** [prepared from hydrochloride (1.265 g, 0.915 mmol) with triethylamine (0.13 ml, 0.915 mmol) in DMF (10 ml), which was obtained in the same way (5 M HCl/AcOEt with **23**) as in the synthesis of compound **4**] were treated with EDC (155 mg, 1.00 mmol) in DMF. **25** was obtained in a similar manner as the synthesis of **9**. The product was cllected by filtration as the amorphous solid from MeOH; yield 1.184 g (77.5%). $[\alpha]_D^{24}$ −53.5° (*c* 1.0, DMF), R_f (1) = 0.73, R_f (3) = 0.53. Found: C, 59.29; H, 5.89; N, 9.37; S, 7.96%. Calcd for $C_{83}H_{101}N_{11}O_{18}S_4$: C, 59.73; H, 6.10; N, 9.23; S, 7.68%.

H–Cys–Cys(Acm)–Phe–Arg–Ser–Cys–Asp–OH (26)· AcOH. (48-Acm h-IGF-I(47-53) [26]) **25** (500 mg, 0.30 mmol) was dissolved in anhydrous hydrogen fluoride (20 ml) together with anisole (2.0 ml) at −70 °C, and the mixture was stirred at 0 °C for 60 min. Hydrogen fluoride was evaporated in vacuo at room temperature, and the residue was dissolved in water (100 ml). The solution was washed with ether (100 ml×2) and the aqueous layer was applied to a column of Dowex 1×2 (AcO^-, 2×10 cm). The passed solution (100 ml) and washings (1 M AcOH, 100 ml) were combined, adjusted to pH 8.0 by 0.1 M NaOH, and allowed to stand at room temperature for 3 h for intramolecular disulfide bond formation. The reaction mixture was acidified at pH 5 by AcOH, treated with SEP–PAK (C_{18}), and lyophilized to give the crude **26** (231 mg, 75.5%). The crude product was purified by HPLC on a reversed-phase column (YMC-packed AP-343-10, ODS 200 Å, 2×25 cm). The peptide (150 mg) was eluted with a linear gradient of 0—100% CH_3CN in 20 min containing 0.01 M trifluoroacetic acid at a flow rate of 5 ml min^{-1}. A fraction of **26** was monitored for absorbance at 214 nm and concentrated in vacuo, and then lyophilized; yield 115 mg. $[\alpha]_D^{24}$ −76.2° (*c* 1.0, H_2O). Amino acid analysis: Phe 1.00, Arg (1.00), Ser 1.10, Asp 1.00.

h-IGF-I(4-9,47-53) (V_8-Isomer Peptide) [Type I] and [Type II]. 6–Acm IGF-I(6-9) (**10**) (1.2 mg) and 48-Acm IGF-I(47-53) (**26**) (1.8 mg) for Type II or 6-Acm IGF-I(6-9) (**10**) (1.2 mg) and 47-Acm IGF-I(47-53) (**22**) (2.0 mg) for Type I were dissolved in a mixture(40 μl) of MeOH and H_2O (4 : 1, v/v), respectively. To these solutions, a mixture (40 μl) of MeOH and 1 M HCl (4 : 1, v/v) containing I_2 (40 equiv of the peptides) were added with stirring. After incubation at room temperature for 15 min, 0.25 M L-ascorbic acid in H_2O was added to stop the iodine oxidation reaction. The resulting solutions were subjected to a reversed-phase HPLC column using an acetonitrile gradient, from 0 to 60% in 30 min, in 0.01 M TFA, as shown in Figs. 7 and 8. The amino acid analyses of synthetic h-IGF-I(4-9, 47-53) [Type I] and [Type II] were determined with a Waters Pico–Tag System; the results are presented in Table 1 together with those for fragments prepared from recombinant IGF-I and its isomer.

The authors would like to thank Professor Koichi Kawasaki and Dr. Mitsuko Maeda of Kobe Gakuin University for using of HF-reaction apparatus.

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