Total Synthesis of Human Insulin by Regioselective Disulfide Formation Using the Silyl Chloride–Sulfoxide Method

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Abstract: Total synthesis of human insulin, a two-chain peptide containing three disulfide bonds, was achieved unambiguously by sequential and selective formation of disulfide bonds in the protein for the first time. The key reaction in the synthesis is separate regioselective formation of three disulfide bonds using a silyl chloride method developed by us. Prior to the insulin synthesis, it was confirmed by the syntheses of double-disulfide peptides, conotoxin M1, β-hANP, and an unnatural parallel dimer of α-hANP, that no disulfide exchange occurred during the silyl chloride treatment. Using three orthogonal thiol-protecting groups, Trt, Acm, and *t*-Bu, the three disulfide bonds of human insulin were efficiently constructed by successive reactions using thiolysis, iodine oxidation, and the silyl chloride method. Each reaction for the stepwise disulfide formation proceeded within 15–60 min with no polymeric product and no solubility problem. The synthetic human insulin had the correct structure and was indistinguishable from natural human insulin.

Introduction

Human insulin is a globular protein of 51 amino acids composed of two polypeptide chains, the A-chain (21 residues) and the B-chain (30 residues)[1] (Figure 1). The molecule contains one intrachain and two interchain disulfide bonds, which play an essential role in the construction of the three-dimensional structure of human insulin and, hence, in its biological activity.[2] Thus, in the production of human insulin both by chemical synthesis[3] and by recombinant DNA techniques,[4] correct formation of the three disulfide bonds after the production of the A- and B-chains is a key step.

A general scheme for the correct pairing of the three disulfide bonds of insulin involves simultaneous folding and disulfide formation by random chain combination of the fully deblocked A- and B-chains in aqueous solution. For this purpose, air oxidation or the mixed disulfide interchange method is usually employed as a mild oxidation procedure.[5] However, the reaction is slow (1 day to several days for completion of the oxidation reaction) and allows equilibration of different conformers to produce a thermodynamically controlled product, which makes the yield of the desired insulin relatively low. In addition, the combination of the two chains has been found to be difficult when amino acid replacements or modifications are introduced. The production of certain insulin derivatives by synthesis of modified chains followed by combination with native counterparts was unsuccessful.[6] The same limitations have also been reported for insulin derivatives produced by recombinant DNA techniques.[7]

To avoid these disadvantages, regioselective separate formation of the three disulfide bonds of insulin would be a better approach. The only report of this approach is the synthesis of human insulin described by Sieber *et al.* in 1977.[8] They employed classical solution-phase peptide synthesis and iodine oxidation as the sole method to construct the three disulfide bonds of human insulin. To achieve separate formation of the three disulfide bonds, four protected fragments including two unsymmetrical cystine fragments prepared by iodine oxidation were selected as the building blocks. After these fragments were assembled, the third disulfide bond was also constructed by iodine treatment. In their scheme, the whole synthetic procedure needed carefully controlled conditions, since the unsymmetric cystine peptides easily underwent isomerization through disulfide exchange reactions in the basic pH range. To achieve the fragment condensation in solution, they had to employ three different N^{α} protecting groups, but all three groups were removed by the same mechanism using acid. These limitations make their elegant approach impractical. Thus, there has been no efficient procedure to regioselectively construct three disulfide bonds.

Recently, we have developed a new disulfide-bond-forming reaction using silyl chloride.[9] Methyltrichlorosilane, in the presence of diphenyl sulfoxide, can cleave various S-protecting groups of cysteine to form cystine directly (Scheme I). The reaction is rapid and is completed within 10–30 min in trifluoroacetic acid (TFA). During the silyl chloride treatment, no disulfide exchange occurred, and most of the nucleophilic amino acids were recovered unchanged. These features of the silyl chloride method appeared to satisfy the requirements for an oxidation method applicable to the separate formation of multiple disulfide bonds. In this paper, we provide a scheme for the regioselective stepwise formation of up to three disulfide bonds using silyl chloride and describe the total synthesis of human insulin.

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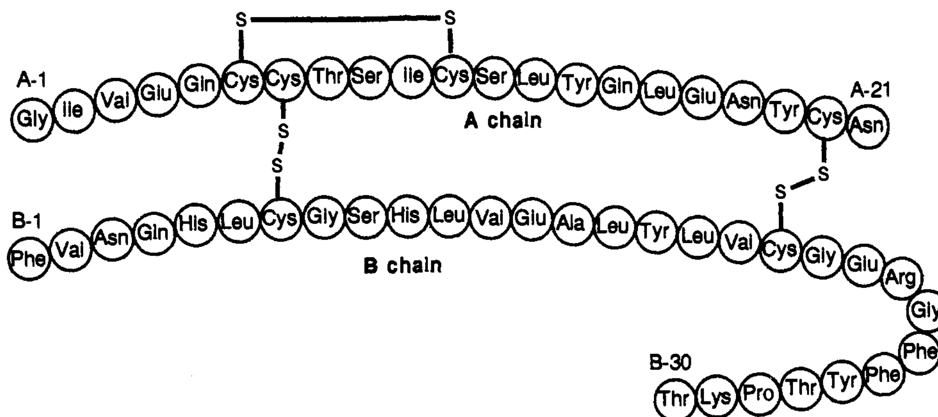
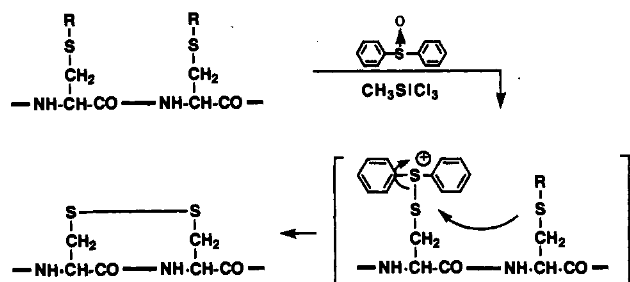


Figure 1. Amino acid sequence and disulfide pairing of human insulin.

Scheme I[a]



[a] R = Acm, Tacm, Bam, *t*-Bu, *i*-Pr, MeOBzl, MeBzl, and NO_2Bzl. Abbreviations: Acm = acetamidomethyl, Tacm = (trimethylacetamido)-methyl, Bam = benzamidomethyl, MeOBzl = 4-methoxybenzyl, MeBzl = 4-methylbenzyl, NO_2Bzl = 4-nitrobenzyl.

Results and Discussion

I. Directed Formation of Double Intrachain Disulfides. As a first step for the regioselective formation of three disulfide bonds, we have established a scheme for the directed formation of double intrachain disulfide bonds using silyl chloride. For this purpose,[10] successive formation of disulfide bonds by air oxidation and iodine oxidation,[11] as exemplified by the syntheses of endothelins,[12] conotoxins,[13] bee venom peptides,[14] and enterotoxins,[15] is the standard scheme, in which two orthogonal thiol-protecting groups (acid-stable Acm[16] and acid-labile MeBzl[17]) are employed. We have alternatively selected a combination of the silyl chloride method and iodine oxidation using two orthogonal S-protecting groups, Acm and *t*-Bu[18] groups.

Behavior of the *S-t*-Bu Group. The Acm group is stable against a strong acid such as HF,[19] but is cleavable with iodine to form cystine.[11] In contrast, the *t*-Bu group remains intact during iodine oxidation[20] or during weak acid treatment such as with TFA, but is cleavable with HBF_4[21] in the presence of scavengers. Concerning the stability of Cys(*t*-Bu) under strongly acidic conditions, it has been reported that the *t*-Bu group is resistant to usual HF treatment but is cleavable with a relatively high concentration of HF in the presence of a large excess of anisole.[22] However, the behavior of the *S-t*-Bu group in a peptide derivative is not well-known. Thus, as a preliminary stability test, we treated [Cys(*t*-Bu)^{1,6}]-oxytocin[9] with HF-scavenger and found that the *S-t*-Bu group is intact in the case of HF-*m*-cresol treatment but is cleavable with HF–anisole. Both Acm and *t*-Bu groups can be cleaved easily by treatment with silyl chloride–sulfoxide to form cystine.[9] Because of these properties of Acm and *t*-Bu groups, it appears feasible to construct two disulfide bonds of a peptide, having no protecting groups other than these two S-protecting groups, by successive treatment with iodine and silyl chloride. The S-protected peptide containing the Acm and *t*-Bu groups can be prepared by acid (TFA or HF) treatment of the corresponding fully protected peptide derivative constructed by standard solid-phase peptide synthesis.

Synthesis of Conotoxin M1. According to the above scheme, we have synthesized conotoxin M1,[23] a neurotoxin peptide consisting of 14 amino acid residues and containing two disulfide bonds per molecule (Scheme II). Construction of the peptide chain was carried out by standard Fmoc-based solid-phase synthesis[24] starting from 4-[(2′,4′-dimethoxyphenyl)-(Fmoc-amino)methyl]phenoxy resin.[25] The fully protected peptide resin was treated with HF–*m*-cresol to yield [Cys(*t*-Bu)^{3,8},Cys-(Acm)^{4,14}]-conotoxin M1 (**1**). In this particular synthesis, HF–*m*-cresol was selected as a suitable deprotecting reagent instead of the standard TFA procedure since the reaction time for complete deprotection of the (4-methoxy-2,3,6-trimethylphenyl)sulfonyl (Mtr) group[26] from Arg was relatively long when TFA was employed. After fast protein liquid chromatography (FPLC) purification, the S-protected conotoxin M1 (**1**) was treated with iodine at 25 °C for 15 min to construct the first disulfide bond between the cysteine residues having the Acm groups, while the two *t*-Bu groups were left intact. The product **2** in TFA (peptide concentration; 1 μmol/mL) was then treated with CH_3SiCl_3 in the presence of PhS(O)Ph at 25 °C for 10 min to construct the second disulfide bond between the cysteine residues having the *t*-Bu groups (Figure 2). The crude product **3** gave a single main peak on high-performance liquid chromatography (HPLC), and

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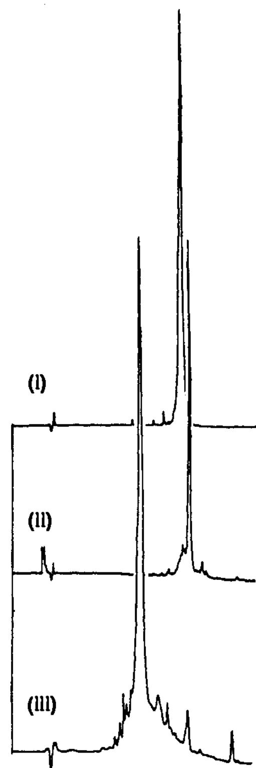
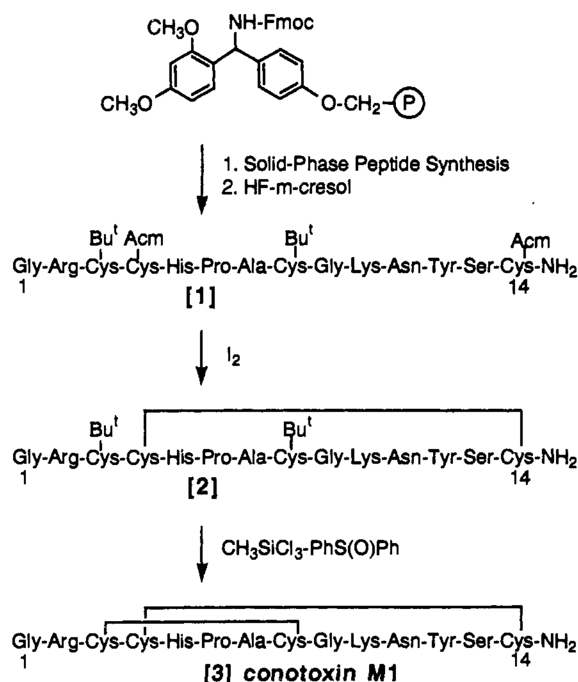


Figure 2. HPLC of the reaction mixture for the synthesis of conotoxin M1: (i) [Cys(t-Bu)[3,8],Cys(Acm)[4,14]]-conotoxin M1; (ii) reaction mixture after a 15-min treatment of i with iodine; (iii) reaction mixture after a 10-min treatment of ii with CH_3SiCl_3–PhS(O)Ph at 25 °C.

Scheme II



no marked disulfide exchange occurred during the silyl chloride treatment. After purification by FPLC, the homogeneous peptide had the same elution time on analytical HPLC as that of an authentic conotoxin M1 purchased from Peptide Institute Inc., Osaka, Japan. The integrity of the purified product was further determined by the amino acid analyses after acid hydrolysis and LAP digestion, sequencing, and FAB mass spectrometry.

II. Directed Formation of Double Interchain Disulfides. We then used the silyl chloride method for the stepwise formation of two interchain disulfide bonds. To achieve this, we employed

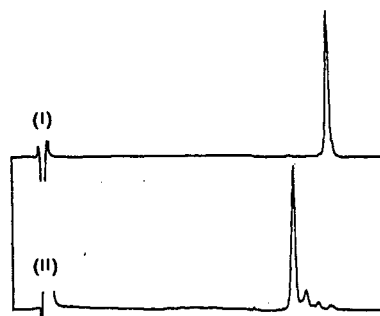


Figure 3. HPLC of the reaction mixture after a 10-min treatment of S-protected mono-disulfide β-hANP with CH_3SiCl_3–PhS(O)Ph at 25 °C: (i) mono-disulfide β-hANP (**8**); (ii) reaction mixture after treatment of i.

Acm and Trt[27] groups as the orthogonal thiol protecting groups of cysteine. The Trt group can be cleaved by a weak acid such as TFA or HBF_4[21] in the presence of scavengers,[28] leaving the Acm group intact. Subsequent treatment with 2,2′-dithiodipyridine (PySSPy)[29] converted the liberated thiol group to the corresponding S-(2-pyridylsulfenyl)cysteine derivative. Thus, to achieve the directed formation of double interchain disulfides, the silyl chloride method was combined with disulfide formation by the thiolysis of S-(2-pyridylsulfenyl)cysteine. According to this scheme, we synthesized β-human atrial natriuretic peptide (β-hANP),[30] an antiparallel dimer of α-hANP (Scheme III), and its parallel analogue (Scheme IV). The syntheses of β-hANP and its analogue appear to be suitable tests for the directed formation of double disulfides using silyl chloride, since it has been reported that α-hANP forms through disulfide exchange during the iodine treatment of a mono-disulfide β-hANP derivative.[31]

Synthesis of β-hANP. Two fully protected peptide resins, having the same amino acid sequence as α-hANP but differing in the positions of the S-protecting groups (the Acm and Trt groups), were prepared by the standard Fmoc-based solid-phase synthesis starting from 4-(benzyloxy)benzyl alcohol resin.[32] Each protected peptide resin was treated with 1 M HBF_4–thioanisole,[21] and the product was purified by FPLC to obtain partially protected α-hANP, i.e., [Cys(Acm)[7],Cys[23]]-α-hANP (**4**) or [Cys[7],Cys(Acm)[23]]-α-hANP (**5**). [Cys(Acm)[7],Cys[23]]-α-hANP (**4**) was reacted with 2,2′-dithiodipyridine to give [Cys(Acm)[7],Cys(S-Py)[23]]-α-hANP (**7**), which was then mixed with [Cys[7],Cys(Acm)[23]]-α-hANP (**5**) at pH 6.5 for 30 min to construct the first disulfide bridge. After FPLC purification, product **8** in TFA (peptide concentration 1.5 μmol/mL) was treated with CH_3SiCl_3–PhS(O)Ph at 25 °C for 10 min to obtain the second disulfide bond between the cysteine residues having the Acm groups. On analytical HPLC, the crude product had a single main peak and no α-hANP was detected (Figure 3); thus, no disulfide exchange occurred during the silyl chloride treatment. In addition, no additional peak corresponding to Met(O)-peptides was detected during the silyl chloride treatment, as expected from the previous synthesis of a single disulfide peptide containing Met residues.[9] The homogeneous product, obtained after preparative HPLC purification, had the same elution time on analytical HPLC as that of the authentic sample (Peptide Institute).

Synthesis of the Parallel Analogue. According to the above scheme, we synthesized the parallel α-hANP dimer having

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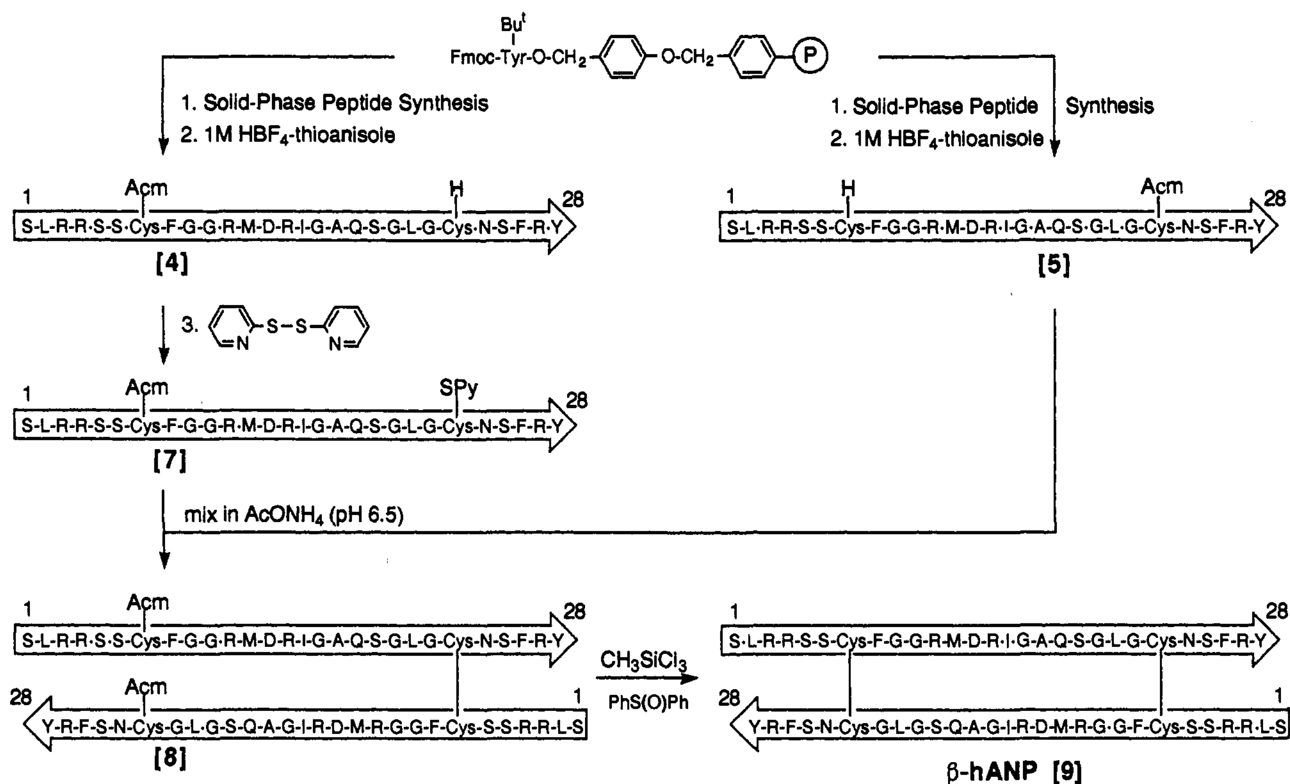
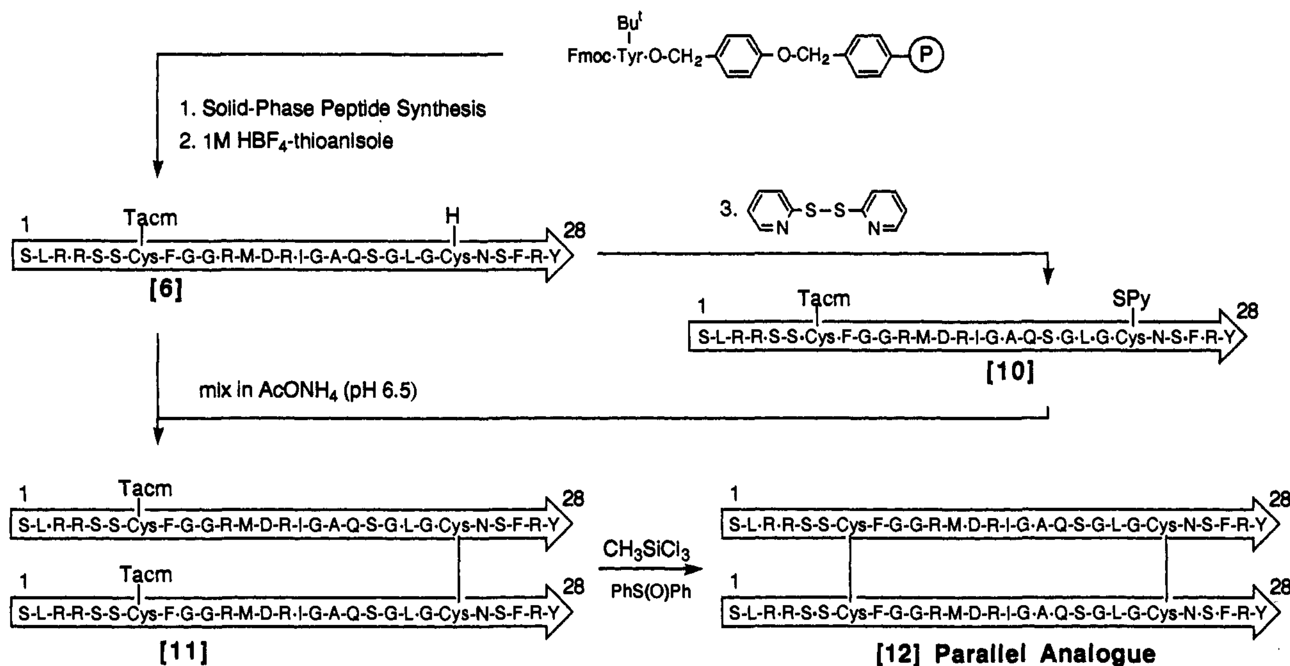
Scheme III



Scheme IV



disulfide positions different from those of β-hANP (Scheme IV). In this synthesis, we employed the more lipophilic Tacm group[33] instead of the Acm group, since the *S*-Tacm mono-disulfide analogue (**11**) was better separated from the parallel analogue (**12**) on HPLC than the corresponding *S*-Acm mono-disulfide derivative. The mono-disulfide parallel analogue (**11**) in TFA (peptide concentration 0.1 μmol/mL) was treated with CH_3SiCl_3–PhS(O)Ph at 25 °C for 20 min, and the resulting crude product had a sharp main peak on analytical HPLC (Figure 4). During the silyl chloride treatment, no α-hANP or β-hANP was detected; this shows that no disulfide exchange occurred even during the construction of an unnatural disulfide bridge, which is thermodynamically less stable than that of the natural peptide.

Both dimers (**9** and **12**) and α-hANP can be separated on HPLC, and the three peptides gave the same reduced thiol-peptide after dithiothreitol treatment. The synthetic β-hANP and its parallel analogue were shown to be dimers of α-hANP by FAB mass spectrometry. Trypsin digestion of the synthetic β-hANP gave a single cystine-containing fragment, whereas that of the parallel analogue gave two cystine-containing fragments (Figure 5). These findings indicate that the synthetic β-hANP is an antiparallel dimer and the analogue is a parallel dimer. In the chick rectum relaxant assay, the relative potency of the β-hANP

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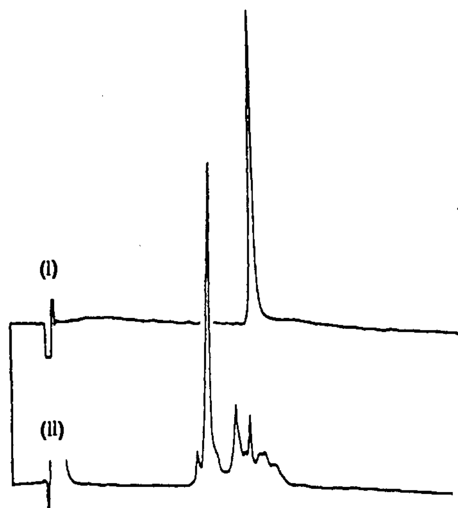

Figure 4. HPLC of the reaction mixture after a 20-min treatment of the S-protected mono-disulfide parallel analogue with CH_3SiCl_3–PhS(O)-Ph at 25 °C: (i) the mono-disulfide parallel analogue (**11**); (ii) reaction mixture after treatment of i.

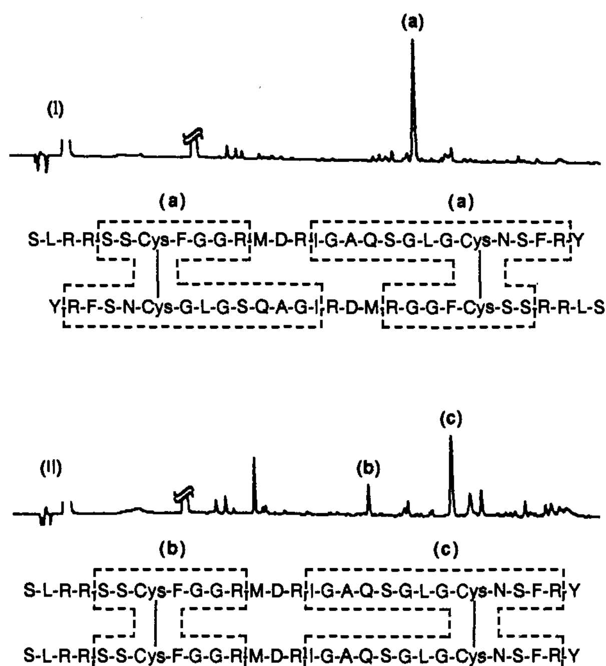

Figure 5. HPLC of tryptic digests derived from the synthetic β-hANP and its parallel analogue. The structures of fragment peptides are also shown under each chromatogram: (i) tryptic digest of β-hANP; (ii) tryptic digest of the parallel analogue.

to rat ANP was 0.71 and that of the analogue was 0.35; these values are in good agreement with published values.[31]

III. Regioselective Formation of the Three Disulfide Bonds of Human Insulin. By combination of the above two schemes for the directed double-disulfide formation, we have established a regioselective scheme to form the three disulfide bonds of human insulin. The three disulfide bonds are constructed stepwise by the successive reactions of thiolysis, iodine oxidation, and the silyl chloride method using three orthogonal thiol-protecting groups of cysteine: Trt, Acm, and *t*-Bu (Scheme V). As confirmed in both double-disulfide formations, the Trt group is selectively cleaved with an acid in the presence of the Acm and *t*-Bu groups, and the Acm group is oxidatively cleaved with iodine to form cystine, leaving the *t*-Bu group intact.

According to this novel scheme, cleavage of the Trt group with a weak acid to liberate the cysteine side chain at position A20 and the subsequent thiolysis of the *S*-(2-pyridylsulfenyl)cysteine at position B19 direct the formation of the first interchain disulfide bond A20–B19. The second interchain disulfide bond A7–B7 is formed between the cysteine residues having the Acm groups by the treatment of the resulting [di-Acm,di-*t*-Bu,mono-disulfide]-human insulin (**16**) with iodine. Finally, product **17** was treated with silyl chloride to construct the intrachain disulfide bond A6–A11 between the cysteine residues having the *t*-Bu groups to give human insulin.

Preparation of the Thiol-Protected A- and B-Chains. The fully protected B-chain was constructed on 4-(benzyloxy)benzyl alcohol resin by the standard Fmoc-based solid-phase synthesis. The cysteine thiol group at position B7 was protected by the acid-stable Acm group, and that at position B19 was protected by the acid-labile Trt group. The protected B-chain resin was treated with 1 M HBF_4–thioanisole, and the product, after FPLC purification, was then treated with 2,2′-dithiodipyridine to yield the [Cys(Acm)^{B7},Cys(*S*-Py)^{B19}]-B-chain derivative (**15**).

For the production of the partially protected A-chain derivative, the cysteine thiol groups were protected by the TFA-labile Trt group at position A20 and the TFA-stable groups, Acm at A7 and *t*-Bu at A6 and A11. To avoid side reactions during direct anchoring of the C-terminal asparagine residue to the standard alkoxyl resin,[24] the α-*t*-Bu ester of N^{α}-Fmoc-aspartic acid was coupled at the β-carboxyl group to 4-[(2′,4′-dimethoxyphenyl)-aminomethyl]phenoxy resin using DIPCDI as a coupling reagent to obtain the starting resin. The C-terminal asparagine can be generated by the final acidolytic cleavage of the β-carboxyl linkage to the amide resin. Subsequent condensation was conducted using TBTU[34] as a coupling reagent, since the standard procedure using the carbodiimide in DMF or in NMP[35] gave unsatisfactory coupling yields. Nearly 90% incorporation up to the N-terminal Gly residue was achieved with TBTU activation, while the carbodiimide procedure resulted in 50–60% incorporation of the several C-terminal residues, probably caused by aggregation of the growing peptide chains. Deprotection of the protected peptide resin with TFA in the presence of scavengers yielded an A-chain derivative with a free thiol group, [Cys(Acm)^{A7},Cys(*t*-Bu)^{A6,11},-Cys^{A20}]-A-chain (**14**), which was purified by FPLC.

Synthesis of Human Insulin. The formation of the first interchain disulfide bond was achieved by mixing the above [Cys-(Acm)^{A7},Cys(*t*-Bu)^{A6,11},Cys^{A20}]-A-chain (**14**) and the [Cys-(Acm)^{B7},Cys(*S*-Py)^{B19}]-B-chain (**15**) in 8 M urea, pH 8, at 25 °C. The absence of precipitation of insoluble product during the reaction indicates that neither marked formation of the polymeric product nor aggregation occurred. After 50 min, both the starting A- and B-chain derivatives disappeared on HPLC, and a mixture consisting of the desired product **16** and [Cys(Acm)^{B7},Cys^{B19}]-B-chain (**13**), derived from the competitive hydrolysis of the starting *S*-Py-B-chain derivative (**15**), was obtained. The desired product was isolated and purified by gel-filtration followed by preparative HPLC to give the mono-disulfide insulin **16** having a sharp single peak on analytical HPLC (Figure 6i). To construct the second interchain disulfide bond, the mono-disulfide insulin (**16**) in 80% aqueous AcOH was treated with iodine at 25 °C to oxidatively cleave the Acm groups. The starting peptide (**16**) disappeared on HPLC after 60 min. The crude product, having a single main peak (Figure 6ii), was purified by preparative HPLC to give a homogeneous di-disulfide insulin (**17**). The third disulfide bond was constructed by the treatment of the di-disulfide insulin (**17**) in TFA (peptide concentration 0.3 μmol/mL) with CH_3-$SiCl_3$–PhS(O)Ph at 25 °C for 15 min. After the reaction, the starting peptide **17** disappeared to give a crude product having a sharp main peak (Figure 6iii), which was easily purified by preparative HPLC. No marked formation of isomers and polymers was detected on HPLC during the silyl chloride treatment. The synthetic human insulin gave a sharp single peak

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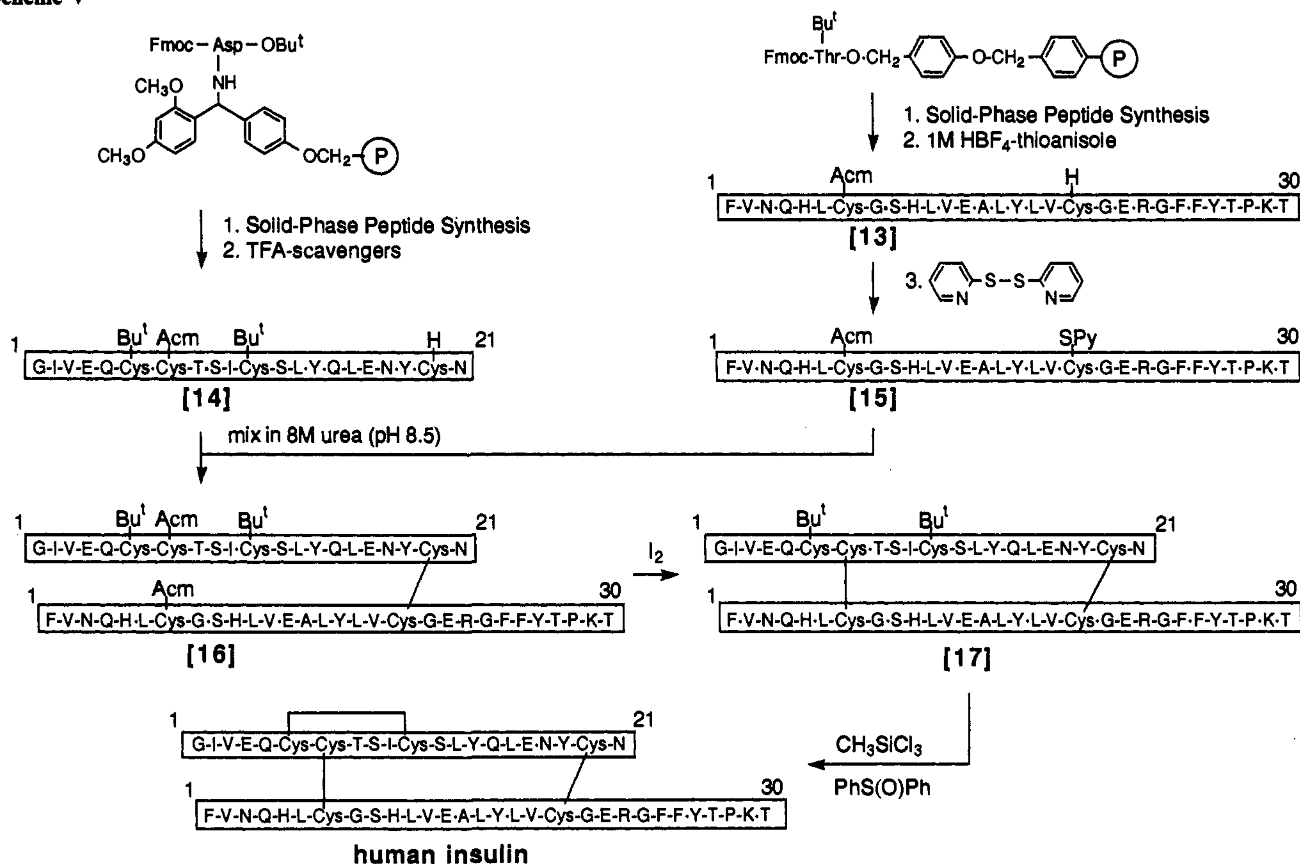
Scheme V



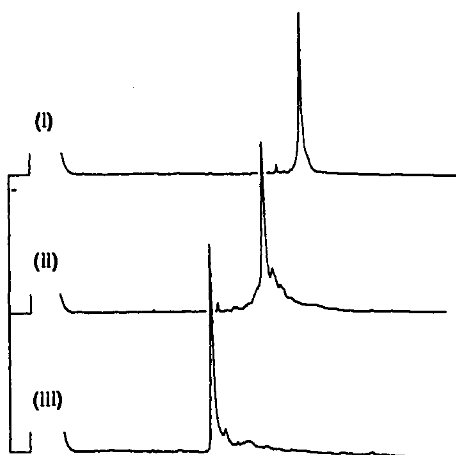


Figure 6. HPLC of the reaction mixture after successive disulfide bond formation for the synthesis of human insulin: (i) mono-disulfide human insulin (**16**); (ii) reaction mixture after a 60-min treatment of i with iodine; (iii) reaction mixture after a 15-min treatment of ii with CH_3SiCl_3–PhS(O)Ph at 25 °C.

on analytical HPLC and had the same retention time as that of an authentic sample prepared by a semisynthetic method.[36]

The observed mass values of the synthetic human insulin as well as its intermediates (**16** and **17**) were in accordance with the expected values of the respective $[M + H]^+$ ions. Acid hydrolysates of the synthetic human insulin, **16** and **17** gave amino acid ratios in good agreement with the theoretical values. The positions of three disulfide bridges in the synthetic human insulin were identical to those of the natural insulin by enzymatic digestion; the same digestion mixture was obtained after thermolysin digestion of the synthetic insulin and the authentic sample prepared by a semisynthetic method. In addition, all the expected

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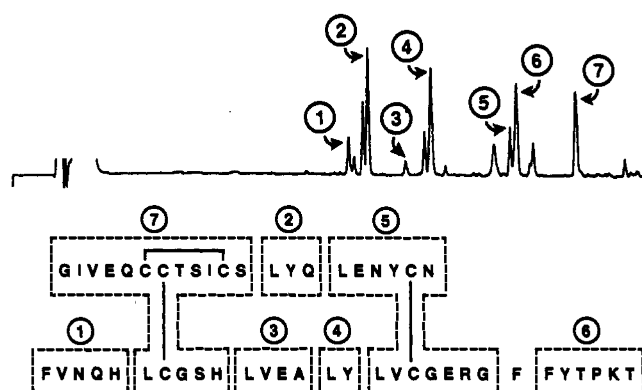

Figure 7. HPLC of thermolysin digest derived from the synthetic human insulin. The structures of fragment peptides are also shown.

peptide fragments were recovered from the digest of the synthetic human insulin (Figure 7). In the hypoglycemia test in rats, the activities of the synthetic insulin were of the same order of magnitude as those of the authentic sample (Figure 8). These results show that the synthetic human insulin prepared by regioselective disulfide formation has the correct structure and is indistinguishable from natural human insulin.

Conclusion

Selectivity is the major requirement for the efficient and unambiguous formation of multidisulfides in a protein. Directed formation of double disulfides has been achieved by successive oxidation with air and iodine using two orthogonal thiol-protecting groups, Acm and MeBzl. Basically, the same method has been employed for the separate formation of three disulfide bonds, which makes it necessary to use cystine-containing peptides as the building blocks for further peptide synthesis. Thus, in the previous syntheses of human insulin[8] or insulin-related peptides,[37] cystine-containing peptides had to be treated with a strong acid or coupled under slightly basic conditions at least twice.

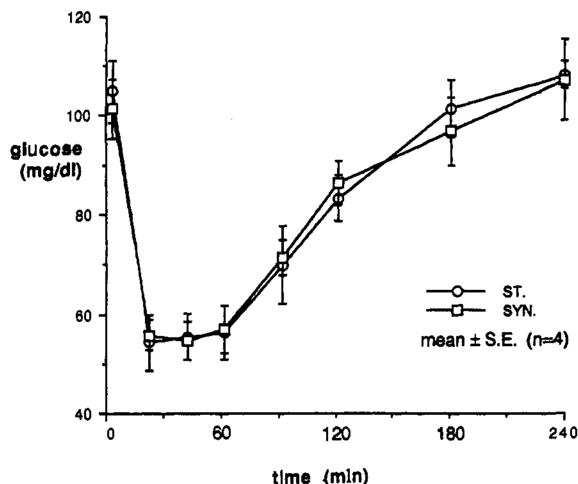

Figure 8. Time course of the blood glucose level in rats after injection of the synthetic human insulin: ST = standard human insulin prepared by a semisynthetic method; SYN = the human insulin synthesized by regioselective stepwise disulfide formation. Each symbol with a vertical bar represents the mean ± standard error of the mean of four animals.

We have introduced a silyl chloride method, which is another novel orthogonal reaction, for disulfide formation. This method enables sequential and selective formation of up to three disulfide bonds. Prior to insulin synthesis, we confirmed that no disulfide exchange occurs during the silyl chloride treatment by the model syntheses of double-disulfide peptides: conotoxin M1 and β-hANP. This important aspect makes the silyl chloride method applicable for the directed formation of unnatural disulfide bonds, as shown in the synthesis of the parallel dimer of α-hANP. The three disulfide bonds of human insulin were constructed regioselectively and efficiently by successive reactions using thiolysis, iodine oxidation, and the silyl chloride method. Each reaction for the stepwise disulfide formation proceeded within 15–60 min without the formation of polymers or isomers to give a single major product. The present regioselective method may be useful for the preparation of synthetic unnatural isomers which have disulfide bonds at different positions and, hence, have lower thermodynamical stability than the corresponding natural peptide.

Experimental Section

General. Amino acid analysis was conducted with a Hitachi L8500 amino acid analyzer utilizing postcolumn ninhydrin detection. Analytical HPLC characterization was carried out on a YMC AM302 column (4.6 × 150 mm), which was eluted with a linear gradient of CH_3CN (gradient A, 10–60%, 30 min; gradient B, 20–25%, 30 min; gradient C, 20–40%, 30 min; gradient D, 25–50%, 30 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min. FPLC (Pharmacia, Uppsala, Sweden) was carried out with a YMC ODS-AQ300 column (1.5 × 50 cm) eluted with a linear gradient of 60% CH_3CN–0.1% aqueous TFA (0–100%, 400 min) in 0.1% aqueous TFA at a flow rate of 3.0 mL/min. Semipreparative HPLC purification was carried out on a YMC AM323 column (10 × 250 mm), which was eluted with a linear gradient of CH_3CN (gradient E, 10–60%, 60 min; gradient F, 25–50%, 60 min) in 0.1% aqueous TFA at a flow rate of 2.5 mL/min. Preparative HPLC purification was carried out on a YMC D-ODS-5 column (20 × 250 mm), which was eluted with a linear gradient of CH_3CN (gradient G, 20–25%, 90 min; gradient H, 20–40%, 70 min) in 0.1% aqueous TFA at a flow rate of 5.0 mL/min. The eluate was monitored by measuring the UV absorption at 254 nm. The solvent of desired fractions was removed by lyophilization. Sequence analysis was carried out by using an Applied Biosystems 470A gas-phase protein sequencer equipped with a 120A PTH analyzer. FAB mass spectra were obtained on a JEOL JMS-HX110 spectrometer equipped with the JMA-DA5000 data system or on a VG Analytical 2AB-2SEQ spectrometer equipped with the 11-250J data system. Fmoc amino acid derivatives, 4-(benzyloxy)benzyl alcohol resin (Wang resin), and 4-[(2',4'-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxy resin (Rink resin) were purchased from Nova Biochem (Läufelfingen, Switzerland). Leucine aminopeptidase (LAP, microsomal type VI-S, lot no. 117F-8085) and trypsin (type XIII from bovine pancreas, lot no. 38F8140) were purchased from Sigma (St. Louis, MO). Thermolysin (from *Bacillus thermoproteolyticus Rokko*, lot no. WDP7126) was purchased from Wako Chemical Ind. (Osaka, Japan). Blood glucose was assayed using a Glucose B-Test Kit purchased from Wako Chemical Ind. (Osaka, Japan).

Standard Solid-Phase Peptide Synthesis. All amino acids were protected with N^{α}-Fmoc. Side-chain protecting groups were Asp(O-*t*-Bu), Glu(O-*t*-Bu), Ser(*t*-Bu), Thr(*t*-Bu), Tyr(*t*-Bu), Lys(Boc), Arg(Mtr), His(Bum) (Bum = (*tert*-butyloxy)methyl),[38] Cys(*t*-Bu), Cys(Trt), Cys(Acm), and Cys(Tacm). Peptide chains were constructed manually according to the published cycle[39] consisting of (i) a 20-min deprotection with 20% piperidine–DMF and (ii) a 2-h coupling of the Fmoc amino acid derivative (2.5 equiv) with diisopropylcarbodiimide (DIPCDI, 2.5 equiv)–HOBt (2.5 equiv) in DMF. The coupling reaction was repeated when the resin became positive to the Kaiser test.[40] The satisfactory incorporation of the respective amino acids was further confirmed by an amino acid analysis after acid hydrolysis of the assembled peptide resin.

Stability of Cys(*t*-Bu) to HF. [Cys(*t*-Bu)^{1,6}]-oxytocin (50 mg, 41 μM) was treated with HF (2 mL) in the presence of *m*-cresol (29 μL, 10 equiv) or anisole (44 μL, 10 equiv) at 0 °C for 60 min. Excess HF was removed in vacuo, and dry ether was added to the residue. The precipitate was dissolved in 1 N AcOH (2 mL), and the solution was applied to a column (3 × 40 cm) of Sephadex G-15, which was eluted with 1 N AcOH. The solvent of the desired fractions was removed by lyophilization to give a white powder (yield 48 mg each). The composition of each product was examined by analytical HPLC (gradient A). HF–*m*-cresol: [Cys(*t*-Bu)^{1,6}]-oxytocin 80%, [mono-Cys(*t*-Bu)$^{1\ \mathrm{or}\ 6}$]-oxytocin 20%. HF–anisole: reduced oxytocin 70% [mono-Cys(*t*-Bu)$^{1\ \mathrm{or}\ 6}$]-oxytocin 30%. Retention times of the oxytocin derivatives are as follows: oxytocin 15.68 min, reduced oxytocin 16.13 min, [mono-Cys(*t*-Bu)$^{1\ \mathrm{or}\ 6}$]-oxytocin 19.36 and 20.53 min, [Cys(*t*-Bu)^{1,6}]-oxytocin 22.89 min.

[Cys(*t*-Bu)^{3,8},Cys(Acm)^{4,14}]-Conotoxin M1 (1). Fully protected conotoxin M1-resin [H-Gly-Arg(Mtr)-Cys(*t*-Bu)-Cys(Acm)-His(Bum)-Pro-Ala-Cys(*t*-Bu)-Gly-Lys(Boc)-Asn-Tyr(*t*-Bu)-Ser(*t*-Bu)-Cys-(Acm)-Rink resin] was constructed according to the general procedure starting from Rink resin (0.3 g, 0.12 mM). The protected peptide resin (250 mg) was treated with HF (4 mL) in the presence of *m*-cresol (365 μL) at 0 °C for 20 min. The excess HF was removed in vacuo, and dry ether was added. The precipitate was extracted with 6 M guanidine·HCl (5 mL), and the resin was removed by filtration. The filtrate was purified by FPLC to afford a white powder showing a sharp single peak on analytical HPLC: yield 22 mg (24%, calculated from the starting resin), retention time on analytical HPLC (gradient A) 13.61 min. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 1.01 (nd); Ser × 1, 0.58 (1.01); Pro × 1, 1.05 (1.03); Gly × 2, 2.13 (1.98), Ala × 1, 1.07 (1.00); Cys(Acm) × 2, nd (1.95); Cys(*t*-Bu) × 2, nd (2.02); Tyr × 1, 1.02 (1.02); Lys × 1, 1.00 (1.00); His × 1, 1.01 (0.96); Arg × 1, 0.96 (0.97). The predicted sequence for conotoxin M1, except for Cys derivatives, was confirmed by sequencing analysis. FAB-MS (obtained on a JEOL JMS-HX110): m/z 1752.0 for $[M + H]^+$ (calcd 1751.8 for $C_{72}H_{119}N_{24}O_{19}S_4$).

Mono-Disulfide [Cys(*t*-Bu)^{3,8}]-Conotoxin M1 (2). The S-protected conotoxin M1 (1) (20 mg, 11.4 μM) was dissolved in 80% aqueous CH_3OH (11.4 mL) in the presence of 2 equiv of HCl. To this solution was added in one portion 0.29 mL of 20% iodine in CH_3OH, and the mixture was stirred at 25 °C for 15 min. The excess iodine was reduced with 1 M ascorbic acid in water. Diaion HP20 (ca. 10 g) in 0.1% aqueous TFA (100 mL) was added, and the mixture was stirred for 15 min at 25 °C. The adsorbed peptide was eluted with 80% CH_3CN in 0.1% aqueous TFA. The solvent of the eluate was removed by lyophilization to give [Cys(*t*-Bu)^{3,8}]-conotoxin M1 (**2**) as a white powder: yield 18.3 mg (100%), retention time on analytical HPLC (gradient A, a single main peak) 14.66 min. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 0.98 (nd); Ser × 1, 0.74 (1.01); Pro × 1, 1.08 (1.02); Gly × 2, 1.95 (2.00); Ala × 1, 1.00 (1.00); Cys × 1, 1.12 (0.88); Cys(*t*-Bu) × 2, nd (2.04); Tyr × 1, 1.01 (1.02); Lys × 1, 0.98

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(1.01); His × 1, 0.99 (1.00); Arg × 1, 0.94 (0.98). The predicted sequence for conotoxin M1, except for Cys derivatives, was confirmed by sequencing analysis. FAB-MS (obtained on a JEOL JMS-HX110): m/z 1607.8 for $[M + H]^+$ (calcd 1607.7 for $C_{66}H_{107}N_{22}O_{17}S_4$).

Conotoxin M1 (3). The Cys(*t*-Bu)-conotoxin M1 (**2**) (17.3 mg, 10.8 μM) in TFA (10.8 mL) was treated with CH_3SiCl_3 (190 μL, 150 equiv) in the presence of PhS(O)Ph (21.9 mg, 10 equiv) and anisole (117 μL, 100 equiv) at 25 °C for 10 min. Dry ether (100 mL) and 1 N AcOH (100 mL) were added to the reaction mixture. The analytical HPLC pattern of the product in the aqueous phase is shown in Figure 2. The crude product was purified by FPLC to give a sample having a sharp single peak on analytical HPLC: yield 6.5 mg (41%), retention time on analytical HPLC (gradient A) 10.54 min (same as the authentic sample purchased from Peptide Institute Inc.). Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 1.02 (nd); Ser × 1, 0.88 (1.01); Pro × 1, 0.97 (0.81); Gly × 2, 2.00 (2.26); Ala × 1, 1.00 (0.91); Cys × 2, 1.25 (1.60); Tyr × 1, 1.01 (1.04); Lys × 1, 0.98 (1.00); His × 1, 1.00 (0.82); Arg × 1, 0.96 (1.28). The predicted sequence for conotoxin M1, except for Cys, was confirmed by sequencing analysis. FAB-MS (obtained on a JEOL JMS-HX110): m/z 1493.7 for $[M + H]^+$ (calcd 1493.6 for $C_{58}H_{88}N_{22}O_{17}S_4$).

S-Protected α-hANP Compounds 4, 5, and 6. Each protected resin [H-Ser(*t*-Bu)-Leu-Arg(Mtr)-Arg(Mtr)-Ser(*t*-Bu)-Ser(*t*-Bu)-Cys(X)-Phe-Gly-Gly-Arg(Mtr)-Met-Asp(O-*t*-Bu)-Arg(Mtr)-Ile-Gly-Ala-Gln-Ser(*t*-Bu)-Gly-Leu-Gly-Cys(X)-Asn-Ser(*t*-Bu)-Phe-Arg(Mtr)-Tyr(*t*-Bu)-Wang resin] was prepared by the standard solid-phase method starting from Fmoc-Tyr(*t*-Bu)-Wang resin (200 mg, 0.1 mM). Fully protected [Cys(Acm)^7,Cys(Trt)^{23}]-α-hANP resin (300 mg) was treated with 1 M HBF_4–thioanisole in TFA (14 mL) in the presence of *m*-cresol (0.5 mL) and EDT (1.2 mL) in an ice-bath for 90 min. The crude product was extracted with 6 M guanidine·HCl (5 mL). The solution was applied to a Sephadex G-15 column (32 × 55 cm), which was eluted with 1 N AcOH. The solvent of the fractions corresponding to the main peak was removed by lyophilization to give a powder (108 mg). The product was further purified by FPLC to yield a white fluffy powder. Similarly, fully protected [Cys(Trt)^7,Cys(Acm)^{23}]-α-hANP resin (300 mg) and fully protected [Cys(Tacm)^7,Cys(Trt)^{23}]-α-hANP resin (200 mg) were deprotected with 1 M HBF_4–thioanisole, and the products were purified. [Cys(Acm)^7,Cys^{23}]-α-hANP (**4**): yield 57 mg (39%), retention time on analytical HPLC (gradient A, single peak) 16.50 min. Amino acid ratios after LAP digesion: Asp × 1, 0.93; Ser × 5, 4.66; Gly × 5, 4.70; Ala × 1, 0.95; Met × 1, 0.98; Ile × 1, 1.00; Leu × 2, 2.01; Tyr × 1, 1.08; Phe × 2, 2.00; Arg × 5, 4.58; Cys(Acm) × 1, 1.03. [Cys^7,Cys(Acm)^{23}]-α-hANP (**5**): yield 53 mg (36%), retention time on analytical HPLC (gradient A, single peak) 16.93 min. Amino acid ratios after LAP digestion; Asp × 1, 1.04; Ser × 5, 5.05; Gly × 5, 5.11; Ala × 1, 1.05; Met × 1, 0.97; Ile × 1, 1.01; Leu × 2, 2.00; Tyr × 1, 0.95; Phe × 2, 2.00; Arg × 5, 4.51; Cys(Acm) × 1, 0.81. [Cys(Tacm)^7,Cys^{23}]-α-hANP (**6**): yield 18 mg (24%), retention time on an analytical HPLC (gradient A, single peak) 17.82 min. Amino acid ratios after LAP digestion: Asp × 1, 0.99; Ser × 5, 4.48; Gly × 5, 4.86; Ala × 1, 1.02; Met × 1, 1.16; Ile × 1, 0.94; Leu × 2, 1.82; Tyr × 1, 0.97; Phe × 2, 2.00; Arg × 5, 4.31; Cys(Tacm) × 1, 0.99.

Mono-Disulfide β-hANP (8). [Cys(Acm)^7,Cys^{23}]-α-hANP (**4**) (37 mg, 11.7 μM) was dissolved in 2-propanol–2 N AcOH (1:1, 15 mL). To this solution was added in one portion 2,2′-dithiodipyridine (8 mg, 3 equiv) in 2-propanol–2 N AcOH (1:1, 12 mL), and the mixture was stirred for 30 min at 25 °C. The solution was applied to a Sephadex G-15 column (32 × 550 mm), which was eluted with 1 N AcOH. The solvent of the desired fraction was removed by lyophilization to give [Cys(Acm)^7,-Cys(*S*-Py)^{23}]-α-hANP (**7**) as a white powder: yield 36 mg (93%), retention time on analytical HPLC (gradient A, single main peak) 17.70 min. Product **7** (33.8 mg, 10.4 μM) and [Cys^7,Cys(Acm)^{23}]-α-hANP (**5**) (32.7 mg, 10.4 μM) were dissolved in 1 N AcOH (25 mL), and the pH of the solution was adjusted to 6.5 with 5% NH_4OH. The mixture was stirred at 25 °C for 30 min. The pH of the mixture was adjusted to 4 with AcOH, and the solvent of the mixture was removed by lyophilization. The product was purified by FPLC to yield the title compound, having a single main peak on analytical HPLC: yield 41 mg (62%); retention time on analytical HPLC by gradient A 17.02 min and by gradient B 26.16 min. Amino acid ratios after LAP digestion: Asp × 2, 1.91; Ser × 10, 9.62; Gly × 10, 9.83; Ala × 2, 1.94; Met × 2, 2.18; Ile × 2, 1.92; Leu × 4, 3.89; Tyr × 2, 1.98; Phe × 4, 4.00; Arg × 10, 9.10; Cys(Acm) × 2, 2.15; Cys × 1, 0.98. FAB-MS (obtained on a VG Analytical 2AB-2SEQ): m/z 6305.8 for $[M + H]^+$ (calcd 6306.2 for $C_{260}H_{418}N_{92}O_{80}S_6$).

β-hANP (9). Mono-disulfide β-hANP (**8**) (11.3 mg, 1.8 μM) in TFA (1.8 mL) was treated with CH_3SiCl_3 (55 μL, 250 equiv) in the presence of PhS(O)Ph (3.6 mg, 10 equiv) and anisole (19.4 μL, 100 equiv) at 25 °C for 10 min. NH_4F (40 mg) was added to the reaction mixture. Dry ether (30 mL) was added, and the precipitate was dissolved in 4 N AcOH (5 mL). The solution was applied to a Sephadex G-15 column (22 × 160 mm), which was eluted with 4 N AcOH. After lyophilization, the crude product was purified by preparative HPLC (gradient G) to give a product showing a sharp single peak on analytical HPLC: yield 2.4 mg (22%), retention time on analytical HPLC (gradient B) 25.61 min (same as the authentic sample purchased from Peptide Institute Inc.). Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 4, 4.07 (2.02); Ser × 10, 8.59 (9.93); Glu × 2, 2.11 (nd); Gly × 10, 10.17 (10.06); Ala × 2, 2.09 (2.06); Met × 2, 1.94 (2.35); Ile × 2, 1.99 (2.01); Leu × 4, 4.09 (4.06); Tyr × 2, 2.05 (2.05); Phe × 4, 4.00 (4.00); Arg × 10, 9.42 (9.52); Cys × 2, 0.45 (1.87). FAB-MS (obtained on a VG Analytical 2AB-2SEQ): m/z 6161.7 for $[M + H]^+$ (calcd 6162.0 for $C_{254}H_{406}N_{90}O_{78}S_6$). The purified β-hANP (20 μg, 3.3 nM) in 1 N AcOH (100 μL) was treated with DTT (100 μg, 0.65 μM) at 37 °C for 60 min. A single peak (retention time 18.66 min) was detected by analytical HPLC (gradient B).

Mono-Disulfide Parallel Analogue (11). [Cys(Tacm)^7,Cys^{23}]-α-hANP (**6**) (33 mg, 10.3 μM) in 2-propanol–2 N AcOH (1:1, 15 mL) was treated with 2,2′-dithiodipyridine (6.6 mg, 3 equiv) in 2-propanol–2 N AcOH (1:1, 10 mL), and the product was gel-filtered as described for compound **7** to give [Cys(Tacm)^7,Cys(*S*-Py)^{23}]-α-hANP (**10**) as a white powder: yield 33 mg (97%), retention time on analytical HPLC (gradient A, single main peak) 18.66 min. Product **10** (28.0 mg, 8.5 μM) and [Cys(Tacm)^7,Cys^{23}]-α-hANP (**6**) (27 mg, 8.5 μM) were reacted as described for mono-disulfide β-hANP (**8**). The product was purified by FPLC to yield the title compound, having a single main peak on analytical HPLC: yield 42 mg (78%), retention time on analytical HPLC by gradient A 18.20 min and by gradient C 17.21 min. Amino acid ratios after LAP digestion: Asp × 2, 2.04; Ser × 10, 9.63; Gly × 10, 10.00; Ala × 2, 2.09; Met × 2, 2.59; Ile × 2, 2.02; Leu × 4, 4.01; Tyr × 2, 2.14; Phe × 4, 4.00; Arg × 10, 9.34; Cys(Tacm) × 2, 1.99; Cys × 1, 1.02. FAB-MS (obtained on a VG Analytical 2AB-2SEQ): m/z 6389.6 for $[M + H]^+$ (calcd 6390.3 for $C_{266}H_{430}N_{92}O_{80}S_6$).

Parallel Analogue (12). The mono-disulfide parallel analogue (**11**) (10.0 mg, 1.6 μM) in TFA (15.7 mL) was treated with CH_3SiCl_3 (55 μL, 100 equiv) in the presence of PhS(O)Ph (3.2 mg, 10 equiv) and anisole (16.9 μL, 100 equiv) at 25 °C for 20 min. NH_4F (40 mg) was added to the reaction mixture. The product was isolated by gel-filtration as described for β-hANP synthesis and was purified by preparative HPLC (gradient H) to give a product showing a sharp single peak by analytical HPLC: yield 2.6 mg (27%), retention time on analytical HPLC (gradient C) 13.58 min. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 4, 4.27 (2.06); Ser × 10, 9.00 (9.82); Glu × 2, 2.12 (nd); Gly × 10, 10.45 (10.36); Ala × 2, 2.14 (2.13); Met × 2, 1.96 (2.53); Ile × 2, 1.98 (2.07); Leu × 4, 4.03 (4.04); Tyr × 2, 2.11 (2.11); Phe × 4, 4.00 (4.00); Arg × 10, 9.74 (9.70); Cys × 2, 0.38 (1.81). FAB-MS (obtained on a VG Analytical 2AB-2SEQ): m/z 6162.2 for $[M + H]^+$ (calcd 6162.0 for $C_{254}H_{406}N_{90}O_{78}S_6$). The purified analogue (20 μg, 3.3 nM) in 1 N AcOH (100 μL) was treated with DTT (100 μg, 0.65 μM) at 37 °C for 60 min. A single peak (retention time 18.66 min) was detected on analytical HPLC (gradient B).

Trypsin Digestion. Tryptic digestion of synthetic β-hANP (100 μg) and the parallel analogue (100 μg) were carried out with trypsin (1.0-2.0 μg) in 100 μL of 0.1 N ammonium acetate (pH 7.0) at 37 °C for 60 min. Tryptic peptides were separated by analytical HPLC using a YMC AM 302 column (4.6 × 150 mm), which was eluted with a linear gradient of CH_3CN (0–40%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min. Fragment peptides were collected manually from each peak. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses) of fragment peptides: fragment a, retention time 25.07 min; Asp × 1, 1.03 (nd); Ser × 4, 3.75 (3.86); Glu × 1, 1.04 (nd); Gly × 5, 4.99 (4.85); Ala × 1, 1.03 (1.14); Ile × 1, 0.98 (1.03); Leu × 1, 1.02 (1.22); Phe × 2, 2.00 (2.00); Arg × 2, 1.93 (1.84); Cys × 1, 0.88 (0.96); fragment b, retention time 21.98 min; Ser × 4, 3.53 (3.63); Gly × 4, 3.92 (3.55); Phe × 2, 2.00 (2.00); Arg × 2, 1.80 (1.65); Cys × 1, 0.44 (0.81); fragment c, retention time 27.08 min; Asp × 2, 1.92 (nd); Ser × 4, 3.45 (3.95); Glu × 2, 1.86 (nd); Gly × 6, 5.56 (5.71); Ala × 2, 1.90 (2.04); Ile × 2, 1.80 (1.94); Leu × 2, 1.88 (2.04); Phe × 2, 2.00 (2.00); Arg × 2, 1.77 (1.85); Cys × 1, 0.73 (0.93).

[Cys(Acm)^{B7},Cys^{B19}]-B-Chain (13). Protected peptide resin [H-Phe-Val-Asn-Gln-His(Bum)-Leu-Cys(Acm)-Gly-Ser(*t*-Bu)-His(Bum)-Leu-Val-Glu(O-*t*-Bu)-Ala-Leu-Tyr(*t*-Bu)-Leu-Val-Cys(Trt)-Gly-Glu(O-*t*-Bu)-Arg(Mtr)-Gly-Phe-Phe-Tyr(*t*-Bu)-Thr(*t*-Bu)-Pro-Lys(Boc)-Thr(*t*-

Bu)-Wang resin] was prepared by the standard solid-phase method starting from Fmoc-Thr(*t*-Bu)-Wang resin (140 mg, 0.1 mM). The fully protected [Cys(Acm)^{B7},Cys(Trt)^{B19}]-B-chain resin (230 mg) was treated with 1 M HBF_4–thioanisole in TFA (12 mL) in the presence of *m*-cresol (0.4 mL) and EDT (1.0 mL) in an ice-bath for 120 min. The crude product was extracted with 6 M guanidine·HCl (5 mL). The solution was applied to a Sephadex G-15 column (3 × 60 cm), which was eluted with 4 N AcOH. The solvent of the desired fractions was removed by lyophilization to give a powder. The product was further purified by FPLC to yield the title peptide as a white fluffy powder: yield 40 mg (30%), retention time on analytical HPLC (gradient A, a single peak) 23.76 min. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 0.99 (nd); Thr × 2, 1.88 (nd); Ser × 1, 0.91 (0.97); Glu × 3, 3.02 (2.04); Pro × 1, 1.02 (0.87); Gly × 3, 3.01 (2.73); Ala × 1, 1.01 (0.95); Val × 3, 2.96 (2.81); Leu × 4, 4.04 (3.65); Tyr × 2, 2.03 (1.81); Phe × 3, 2.94 (2.86); Lys × 1, 1.00 (1.00); His × 2, 1.95 (1.73); Arg × 1, 0.96 (0.85); Cys(Acm) × 1, nd (0.84).

[Cys(Acm)^{A7},Cys(*t*-Bu)^{A6,11},Cys^{A20}]-A-Chain (14). Protected peptide resin [H-Gly-Ile-Val-Glu(O-*t*-Bu)-Gln(Trt)-Cys(*t*-Bu)-Cys(Acm)-Thr-(*t*-Bu)-Ser(*t*-Bu)-Ile-Cys(*t*-Bu)-Ser(*t*-Bu)-Leu-Tyr(*t*-Bu)-Gln(Trt)-Leu-Glu(O-*t*-Bu)-Asn(Trt)-Tyr(*t*-Bu)-Cys(Trt)-Asp(α-O-*t*-Bu)-Rink resin] was prepared starting from Fmoc-Asp(α-O-*t*-Bu)-Rink resin (300 mg, 0.09 mM) according to the Fmoc-based solid-phase method. Each synthetic cycle consisted of (i) a 20-min deprotection with 20% piperidine–DMF and (ii) a 2-h coupling of the Fmoc amino acid derivative (3 equiv) with benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 3 equiv)–HOBt (3 equiv) in the presence of diisopropylethylamine (DIEA, 9 equiv) in DMF. The coupling reaction was repeated when the resin became positive to the Kaiser test. The fully protected A-chain resin (150 mg) was treated with TFA (7 mL) in the presence of phenol (0.28 mL), EDT (0.13 mL), and thioanisole (0.19 mL) at 25 °C for 180 min. Dry ether was added to the reaction mixture. The precipitate was extracted with TFA (5 mL), and the resin was removed by filtration. The filtrate was concentrated in vacuo. Dry ether was added to the residue. The resulting precipitate was dissolved in 8 M urea, and the pH of the mixture was adjusted to 8.5. DTT (0.75 g) was added to the solution, and the mixture was stirred under an Ar atmosphere at 25 °C for 5 h. The mixture was applied to FPLC to yield the title peptide as a white powder: yield 13 mg (26%), broad main peak around 26 min on analytical HPLC (gradient A). Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 2, 1.94 (nd); Thr × 1, 0.89 (nd); Ser × 2, 1.65 (1.87); Glu × 4, 3.55 (1.98); Gly × 1, 0.88 (0.86); Val × 1, 0.68 (0.88); Ile × 2, 1.35 (1.70); Leu × 2, 2.00 (2.00); Tyr × 2, 1.75 (1.98); Cys(Acm) × 1, nd (0.80); Cys(*t*-Bu) × 2, nd (1.67); Cys × 0.5, nd (0.32).

Mono-Disulfide Human Insulin (16). [Cys(Acm)^{B7},Cys^{B19}]-B-chain (**13**) (37 mg, 10.7 μM) in 2-propanol–2 N AcOH (1:1, 8 mL) was treated with 2,2′-dithiodipyridine (24 mg, 10 equiv) in 2-propanol–2 N AcOH (1:1, 1 mL) at 25 °C for 40 min, and the product was gel-filtered as described for compound **7** to give [Cys(Acm)^{B7},Cys(*S*-Py)^{B19}]-B-chain (**15**) as a white powder: yield 31 mg (80%), retention time on analytical HPLC (gradient A, a single peak) 24.78 min. Product **15** (7.7 mg, 2.2 μM) and [Cys(Acm)^{A7},Cys(*t*-Bu)^{A6,11},Cys^{A20}]-A-chain (**14**) (11 mg, 4.3 μM) were dissolved in 8 M urea (5 mL, pH 8.5), and the mixture was stirred at 25 °C for 50 min. The pH of the mixture was adjusted to 4 with AcOH. The solution was applied to a Sephadex G-25 column (2 × 15 cm), which was eluted with 50% AcOH. The solvent of the desired fractions was removed by lyophilization. The product was further purified by semipreparative HPLC (gradient E) to yield the title peptide, having a sharp single peak on analytical HPLC: yield 2.0 mg (15%), retention time on analytical HPLC by gradient A 25.66 min and by gradient D 23.64 min. Amino acid ratios after 6 N HCl hydrolysis: Asp × 3, 3.23; Thr × 3, 2.72; Ser × 3, 2.85; Glu × 7, 7.16; Pro × 1, 1.02; Gly × 4, 3.99; Ala × 1, 1.08; Val × 4, 3.79; Ile × 2, 2.05; Leu × 6, 5.89; Tyr × 4, 3.91; Phe × 3, 2.68; Lys × 1, 1.00; His × 2, 1.63; Arg × 1, 0.86. FAB-MS (obtained on a VG Analytical 2AB-2SEQ): *m/z* 6067.2 for $[M + H]^+$ (calcd 6067.1 for $C_{271}H_{414}N_{67}O_{79}S_6$).

Di-Disulfide Human Insulin (17). A 120-μL amount of 20% iodine in CH_3OH was added in one portion to an 80% aqueous AcOH (5 mL) solution of the mono-disulfide human insulin (**16**) (5.8 mg, 0.96 μM), and the mixture was stirred at 25 °C for 60 min. The excess iodine was reduced with 1 M ascorbic acid in water. The solution was applied to a Sephadex G-25 column (2 × 15 cm), which was eluted with 50% AcOH. The solvent of the desired fractions was removed by lyophilization. The product was further purified by semipreparative HPLC (gradient F) to yield the title peptide, having a sharp single peak on analytical HPLC: yield 2.3 mg (42%), retention time on analytical HPLC (gradient D) 20.75 min. Amino acid ratios after 6 N HCl hydrolysis: Asp × 3, 2.87; Thr × 3, 2.61; Ser × 3, 2.81; Glu × 7, 6.65; Pro × 1, 1.08; Gly × 4, 4.04; Ala × 1, 1.05; Val × 4, 3.59; Ile × 2, 1.58; Leu × 6, 5.88; Tyr × 4, 3.73; Phe × 3, 2.25; Lys × 1, 1.00; His × 2, 1.86; Arg × 1, 0.99. FAB-MS (obtained on a VG Analytical 2AB-2SEQ): *m/z* 5922.8 for $[M + H]^+$ (calcd 5922.9 for $C_{265}H_{402}N_{65}O_{77}S_6$).

Human Insulin. Di-disulfide human insulin (**17**) (1.0 mg, 0.17 μM) in TFA (0.6 mL) was treated with CH_3SiCl_3 (5 μL, 250 equiv) in the presence of PhS(O)Ph (0.7 mg, 20 equiv) at 25 °C for 15 min. NH_4F (3 mg) was added to the reaction mixture, and the solvent was removed in vacuo. The residue was dissolved in 50% AcOH (1 mL), and the solution was applied to a Sephadex G-25 column (1.5 × 5 cm), which was eluted with 50% AcOH. The solvent of the desired fractions was removed by lyophilization. The product was further purified by semipreparative HPLC (gradient F) to yield human insulin, having a sharp single peak on analytical HPLC: yield 0.6 mg (61%), retention time on analytical HPLC (gradient D) 16.43 min. Amino acid ratios after 6 N HCl hydrolysis: Asp × 3, 2.73; Thr × 3, 2.59; Ser × 3, 2.27; Glu × 7, 7.04; Pro × 1, 1.07; Gly × 4, 3.92; Ala × 1, 1.05; Val × 4, 3.74; Ile × 2, 1.51; Leu × 6, 5.74; Tyr × 4, 3.80; Phe × 3, 2.87; Lys × 1, 1.00; His × 2, 1.94; Arg × 1, 0.99. FAB-MS (obtained on a VG Analytical 2AB-2SEQ): *m/z* 5808.5 for $[M + H]^+$ (calcd 5808.7 for $C_{257}H_{384}N_{65}O_{77}S_6$).

Thermolysin Digestion. Synthetic human insulin (50 μg) was dissolved in 150 μL of 0.1 M MES–NaOH buffer (pH 6.5). To this solution was added 10 μL of thermolysin solution (0.5 μg/μL in 0.05 M Tris–HCl buffer, pH 8.0), and the solution was incubated at 45 °C for 120 min. The mixture was separated by analytical HPLC using a YMC AM 302 column (4.6 × 150 mm), which was eluted with a linear gradient of CH_3CN (0–40%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min. Fragment peptides were collected manually from each peak. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses) of fragment peptides: fragment 1, retention time 14.27 min; Asp × 1, 1.11; Glu × 1, 1.21; Val × 1, 1.00; Phe × 1, 0.84; His × 1, 0.81; fragment 2, retention time 15.08 min; Glu × 1, 0.97; Leu × 1, 1.00; Tyr × 1, 0.96; fragment 3, retention time 16.71 min; Glu × 1, 1.03; Ala × 1, 1.07; Val × 1, 0.96; Leu × 1, 1.00; fragment 4, retention time 17.75 min; Leu × 1, 1.00; Tyr × 1, 0.87; fragment 5, retention time 21.11 min; Asp × 2, 2.00 (nd), Glu × 2, 2.02 (1.47); Gly × 2, 2.46 (1.88); Val × 1, 1.04 (1.41); Cys × 0.5, nd (1.06); Leu × 2, 1.75 (2.44); Tyr × 1, 0.86 (1.12); Arg × 1, 0.99 (1.00); fragment 6, retention time 21.38 min; Thr × 2, 1.80; Tyr × 1, 1.02; Phe × 1, 1.00; Lys × 1, 1.09; Pr × 1, 1.05; fragment 7, retention time 23.88 min; Thr × 1, 0.74 (1.07); Ser × 3, 1.89 (1.57); Glu × 2, 1.78 (0.81); Gly × 2, 2.05 (1.67); Val × 1, 0.87 (0.95); Cys × 1, nd (1.10); Ile × 2, 1.25 (1.33); Leu × 1, 1.00 (1.00); His × 1, 0.71 (0.66).

Hypoglycemia Test. Synthetic human insulin and an authentic sample (3.7 μg each) in 0.01 M phosphate-buffered saline were intravenously injected into groups ($n = 4$) of rats fasted for 20 h. Blood samples (50 μL) were obtained at 0, 20, 40, 60, 90, 120, 180, and 240 min postadministration, and blood glucose was assayed enzymatically (glucose oxidase/peroxidase method[41]). The results are summarized in Figure 8.

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