Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

Total Chemical Synthesis of Human Proinsulin

Samuel Lusier, Michal Avital-Shmilovici, Michael A. Weiss,
Stephen B.H. Kent

Materials and Methods

Reagents: 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Nα-Boc protected D- and L-amino acids (Peptide Institute, Osaka) were obtained from Peptides International. Side-chain protecting groups used were Arg(Tos), Asn(Xan), Asp(OcHex), Cys(4-CH_3Bzl), Glu(OcHex), His(DNP), Lys(2Cl-Z), Ser(Bzl), Thr(Bzl), Tyr(2Br-Z). Aminomethyl-resin was prepared from Biobeads S-X1 (BioRad, California).[1] Boc-L-Asn-OCH_2-phenylacetic acid and Boc-L-Ala-OCH_2-phenylacetic acid were purchased from NeoMPS, Strasbourg France. Boc-D-Asn-OCH_2-phenylacetamidomethyl resin was obtained from Chem-Impex International Inc. (Wood Dale, IL). N,N-Diisopropylethylamine (DIEA) was obtained from Applied Biosystems Inc. (Foster City, California). N,N-Dimethylformamide (DMF) was purchase from Honeywell Burdick & Jackson). Dichloromethane, diethyl ether, HPLC-grade acetonitrile, and guanidine hydrochloride were purchased from Fisher. Trifluoroacetic acid (TFA) was obtained from Halocarbon Products (New Jersey). HF was purchased from Matheson. All other reagents were purchased from Sigma-Aldrich.

Peptide synthesis: The L-amino acid and D-amino acid containing Cys-peptide segments were synthesized on Boc-Asn-OCH_2-Pam-resin of the appropriate chirality, using manual in situ neutralization Boc chemistry protocols for stepwise SPPS.[2] The L-amino acid and D-amino acid containing peptide thioester segments were synthesized on trityl-SCH_2CH_2CO-Ala-OCH_2-Pam-resin. After removal of the last N^{α}-Boc group, peptides were cleaved from the resin and deprotected by treatment with anhydrous HF at 0°C for 1 h. The thioester peptides D- and L-proinsulin(Phe^{1}-Val^{18}) -$^{\alpha}$COSR, were treated *before* HF cleavage two times with 2-mercaptoethanol:DIEA:DMF 20%:10%:70% for 15 min each, in order to deprotect the DNP protection group from the His residue.[†] Then these peptides were cleaved with HF as described above.

LC-MS analysis and preparative HPLC: Peptide compositions were evaluated by analytical reverse phase LC-MS using a gradient of 0.08% TFA in acetonitrile (buffer B) versus 0.1% TFA in water (buffer A). For all the work reported in this paper, analytical

[†] Studies in our laboratory have shown that for a peptide-thioester with a C-terminal Val residue, only minimal loss of peptide through transthioesterification occurs under these conditions

HPLC was carried out using C4 (2.1 x 50 mm) column at 40 ºC with a flow rate of 0.5 mL/min. The eluent was monitored at 210 nm, with on-line electrospray mass spectrometry (MS) using an Agilent 1100 LC-ion trap. Peptides were purified on C8 silica using a column of dimensions 10 X 250 mm. The silica used was TP Vydac. Crude peptides were loaded onto the prep column in acid solution and eluted at a flow rate of 5 mL per minute with a shallow gradient (e.g. 15%B-35%B over 100 minutes) of increasing concentrations of solvent B in solvent A. Fractions containing the pure target peptide were identified by analytical LC, and were combined and lyophilized. Typical recovered yields of purified peptides were: L-proinsulin(Phe^{1}-Val^{18})-ᵅ$COSCH_2CH_2CO$-Ala (90 mg, 8% based on starting resin; observed mass 2201.2 ± 0.5 Da; calculated mass (average isotope composition) 2201.6 Da); L-proinsulin(Thz^{19}-Cys^{71})-ᵅ$COSCH_2CH_2CO$-Ala (99 mg, 11% based on starting resin; observed mass 5804.9 ± 0.7 Da; calculated mass 5805.5 Da); and, L-proinsulin(Cys^{72}-Asn^{86}) (80 mg, 9% based on starting resin; observed mass 1753.3 ± 0.7 Da; calculated mass 1753.9 Da).

Chemical synthesis of D- human proinsulin: Chemical synthesis of D- human proinsulin was performed following the same procedures as described for the synthesis of L- human proinsulin. The analytical data are shown in Figures S1-S4 (below).

CD Spectra of D- and L-human proinsulin: Far-ultraviolet (UV) CD was employed to probe protein conformation. Spectra were recorded using an Aviv spectropolarimeter equipped with thermister-based temperature control (Aviv Biomedical, Inc., Lakewood, NJ) in 10 mM potassium phosphate and 50 mM KCl at pH 7.4. The protein concentration was *ca.* 25-50 μM. Data were acquired with an optical pathlength of 1 mm at 25° C. (Figure S5).

1D & 2D ^{1}H-NMR spectra of D- and L-human proinsulin: Spectral data and full experimental details are given in Figures S6 & S7 (below).

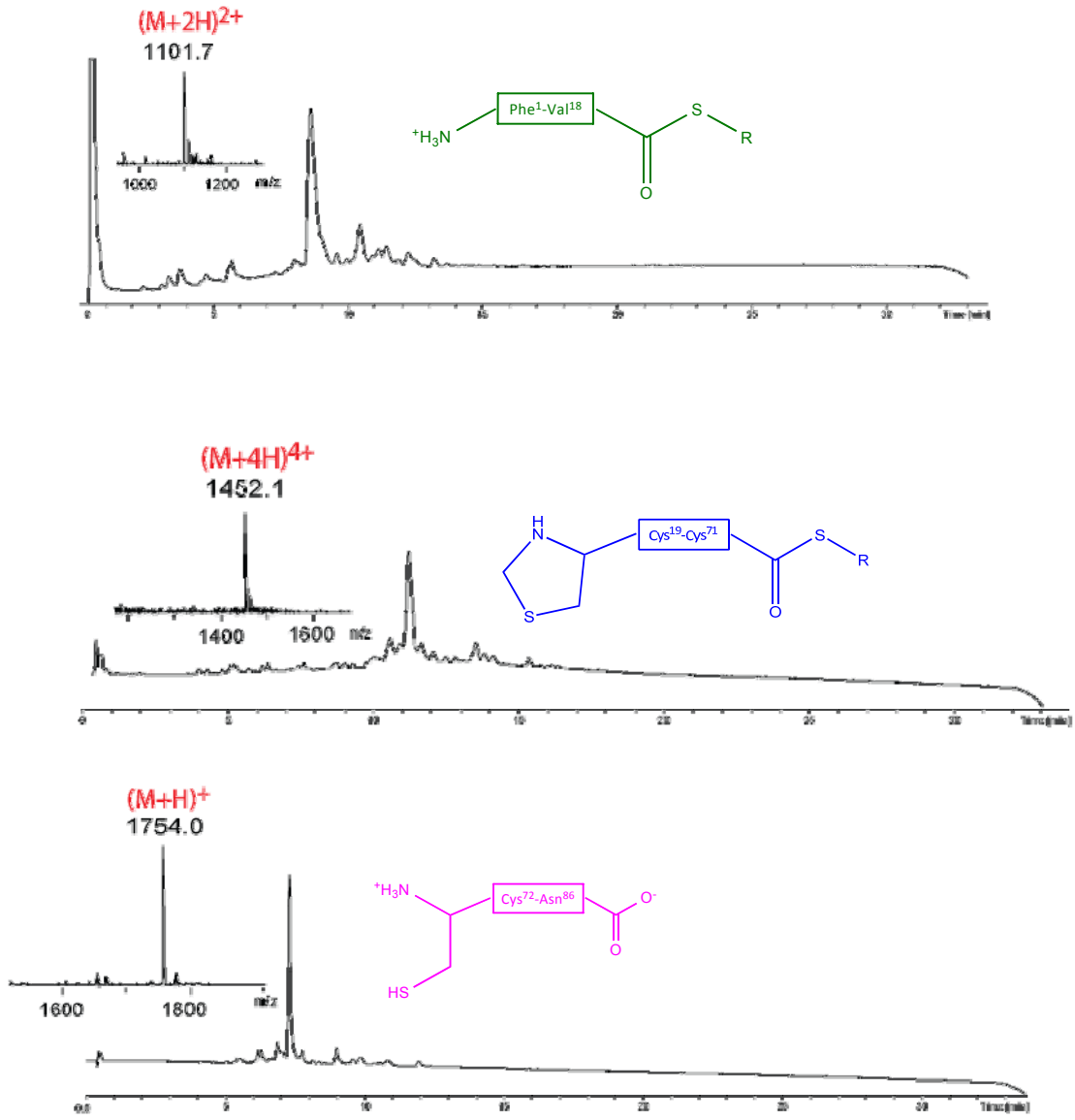

Figure S1: Analytical HPLC profiles (λ = 210 nm) and ESI MS data (inset) corresponding to each major product of crude synthetic peptides of D- human proinsulin. The chromatographic separations were performed using linear gradient (20%-45%) of buffer B in buffer A over 25 min.

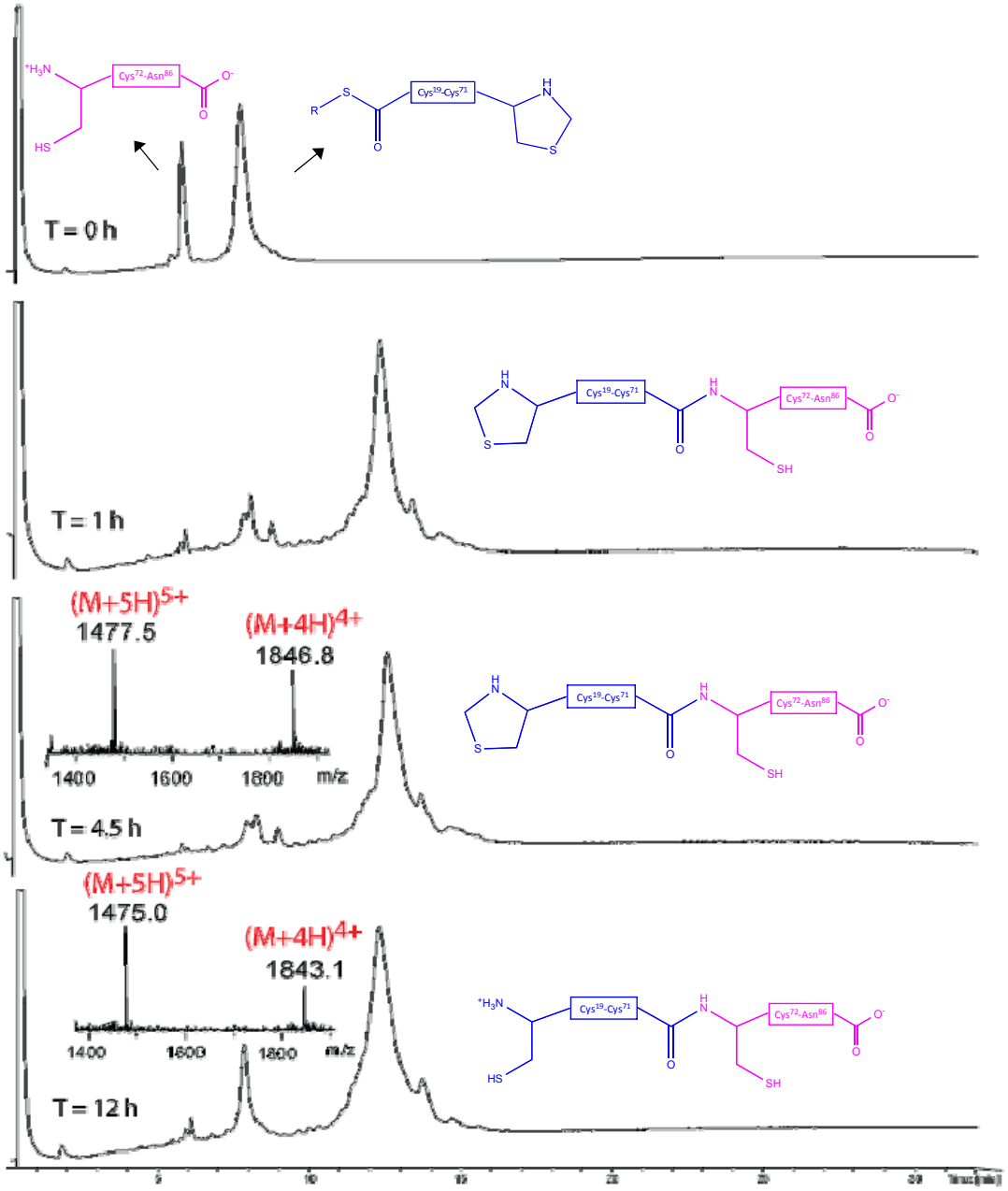

Figure S2: LC-MS analytical data for the first ligation between D-proinsulin(Thz^{19}-Cys^{71})-$^{\alpha}$COSR and D-proinsulin(Cys^{72}-Asn^{86}) following Thz- deprotection; the times shown refer to overall elapsed times for the synthesis. The chromatographic separations were performed using linear gradient (20%-45%) of buffer B in buffer A over 25 min.

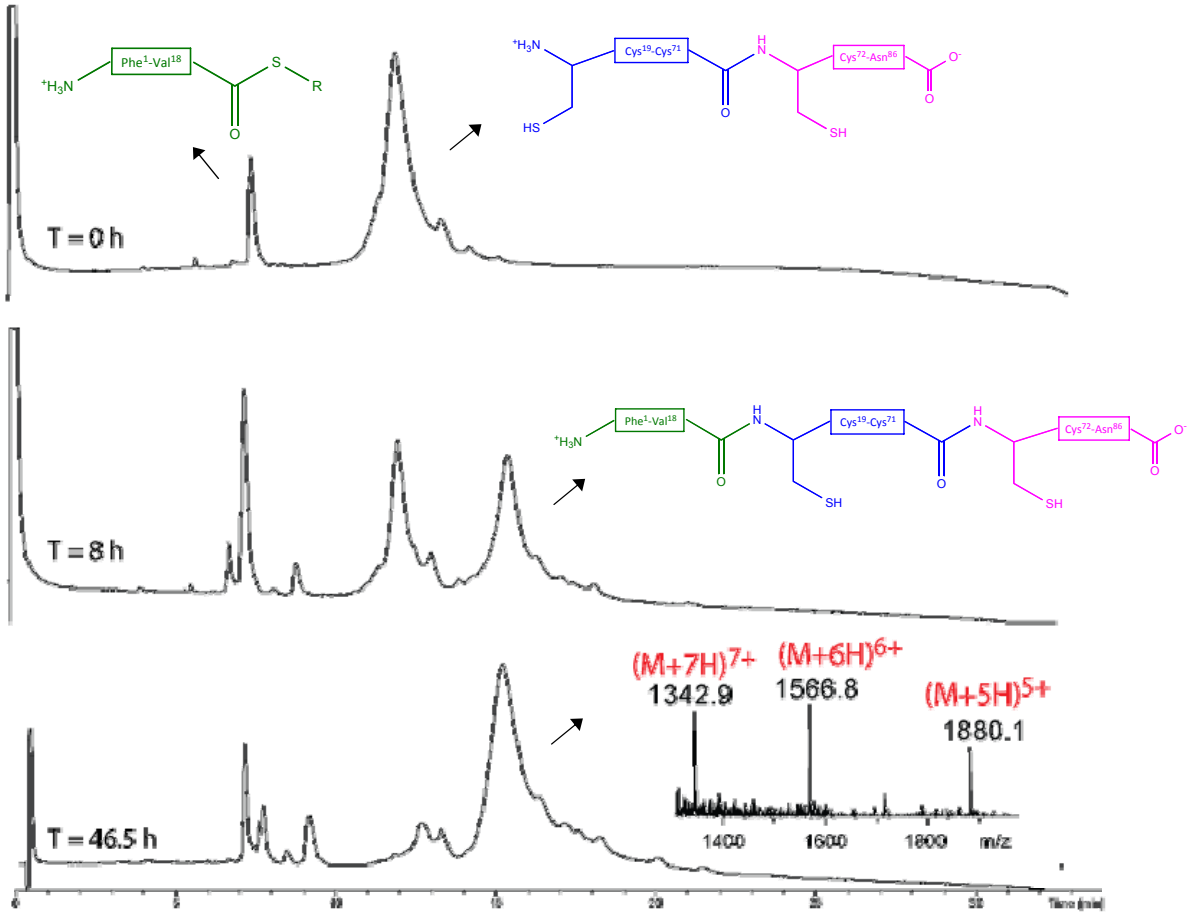

Figure S3: LC-MS analytical data for the second ligation between D-proinsulin(Phe[1]-Val[18]) -ᵅCOSR and D-proinsulin(Cys[19]-Asn[86]). The chromatographic separations were performed using linear gradient (20%-45%) of buffer B in buffer A over 25 min.

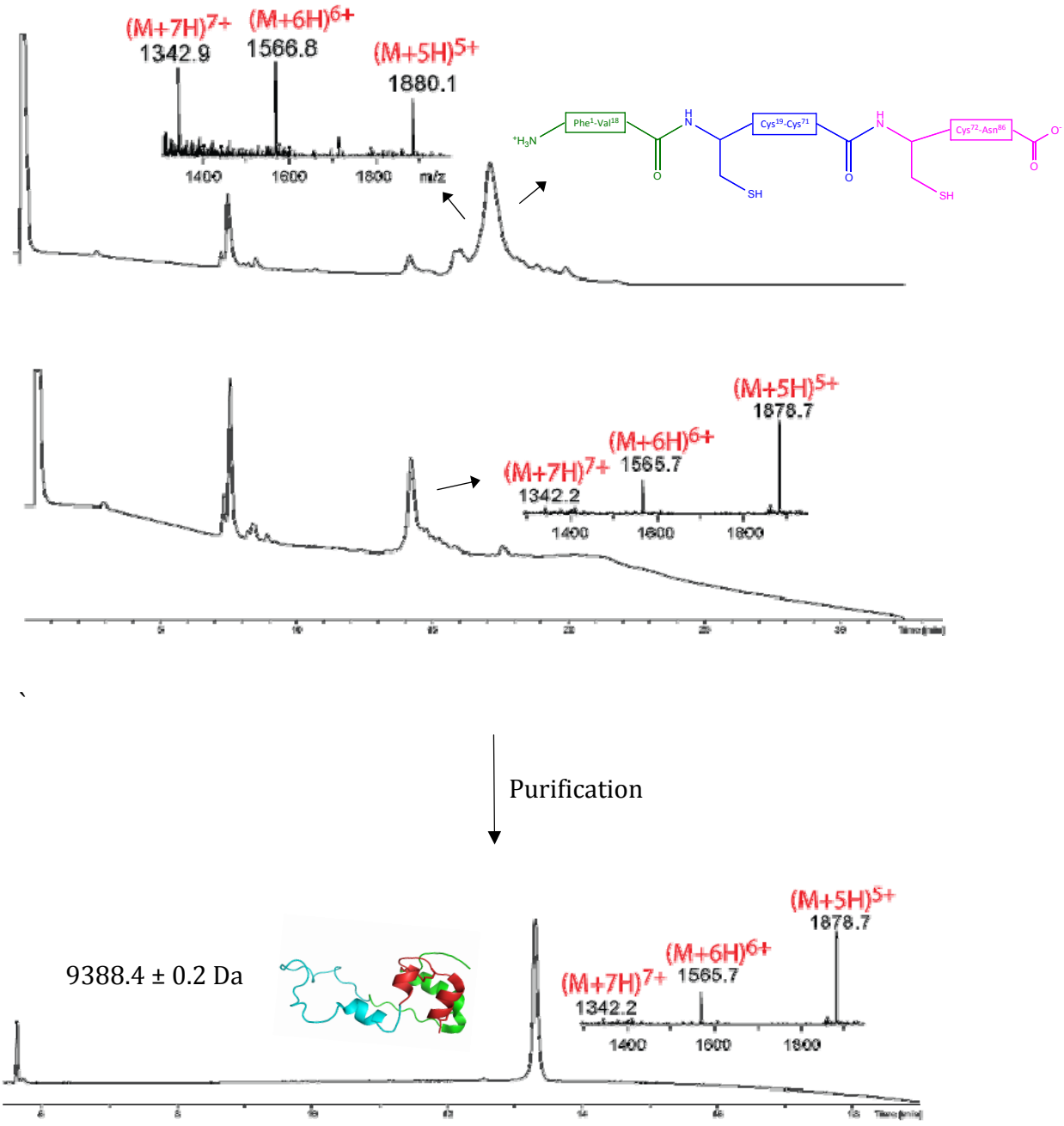

Figure S4: Analytical LC-MS analysis (λ = 210 nm) of D-proinsulin's folding (obs. = 9388.4±0.2 Da, calc. 9388.5 Da). Conditions: 50 mM glycine/NaOH pH 10.5, 1mM EDTA, 1mM reduced glutathione (GSH), 1 mM oxidized glutathione (GSSG) at peptide concentration of 0.1 mg/mL The chromatographic separations of the folding reaction were performed using linear gradient (20%-45%) of buffer B in buffer A over 25 min. The chromatographic separation of the purified folded D-proinsulin was performed using linear gradient (9%-53%) of buffer B in buffer A over 22 min.

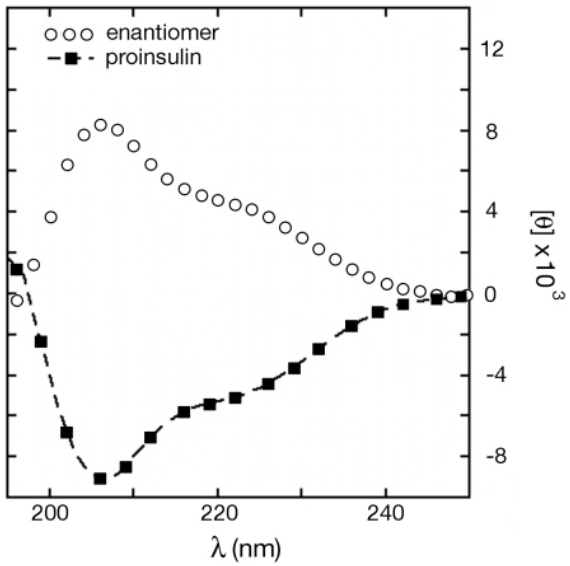

Figure S5: CD spectra of synthetic L- proinsulin vs. D- proinsulin.

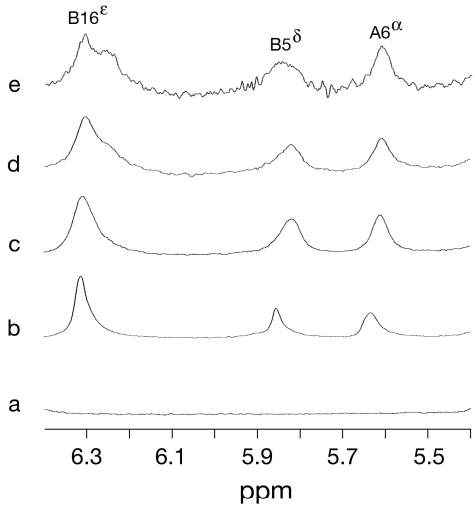

Figure S6: **1D ^{1}H-NMR spectra exhibit similar R_6 hexamer-related chemical shifts**. Stacked plot of spectra in upfield portion of aromatic region and downfield H_α region: (a) engineered insulin monomer DKP-insulin, (b) biosynthetic R_6 zinc insulin hexamer, (c) biosynthetic R_6 zinc proinsulin hexamer, (d) synthetic R_6 zinc proinsulin hexamer, and

(e) synthetic enantiomer of R_6 zinc proinsulin hexamer. The downfield chemical shift of the alpha resonance of Cys^{A6} and upfield chemical shift of the delta resonance of His^{B5} are characteristic of the phenol-stabilized R_6 hexamer. Spectra were acquired at 700 MHz and 50° C in 50 mM deuterated Tris-DCl (pD 7.6, direct meter reading) and 12.5 mM deuterated phenol in D_2O. The nominal ratio of zinc ions per hexamer was ca. 2.5. Protein concentrations were ca. 0.5 mM (biosynthetic insulin and proinsulin), 0.4 mM (synthetic proinsulin), and 0.3 mM (proinsulin enantiomer). Biosynthetic human proinsulin and biosynthetic human insulin were kindly provided by Lilly Research Laboratories (Indianapolis, IN).

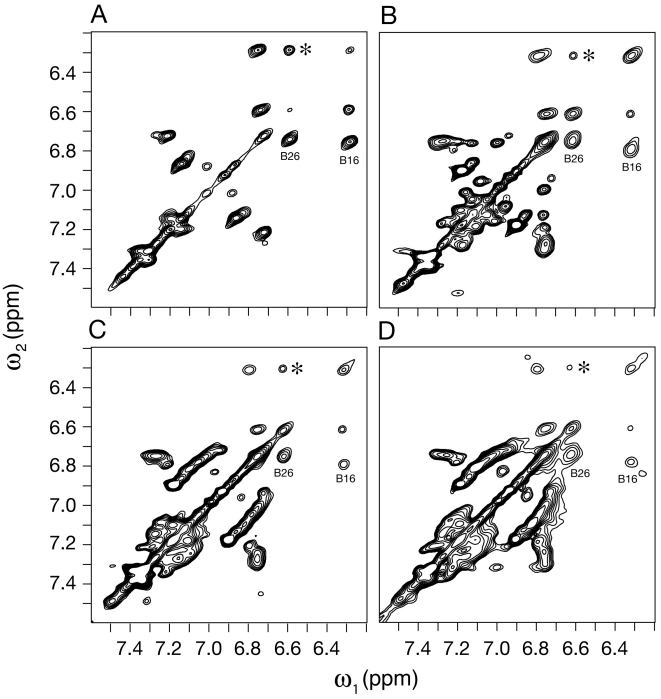

Figure S7: 2D ^{1}H-NMR spectra exhibit similar dimer-related contact and chemical shifts. Nuclear Overhauser spectroscopy (NOESY) showing aromatic spin systems in spectra of (A) biosynthetic human insulin, (B) biosynthetic proinsulin, (C) synthetic proinsulin, and (D) proinsulin enantiomer. Asterisk indicates diagnostic dimer-related NOE between the side chains of Tyr^{B16} in one subunit and Tyr^{B26} in another subunit within the R_6 zinc-stabilized hexamer. Although the synthetic samples are broader than those of the biosynthetic material, the unusual upfield chemical shifts of residues B16 and B26 are maintained. Spectra were acquired at 700 MHz and 50° C in 50 mM deuterated Tris-DCl (pD 7.6, direct meter reading) and 12.5 mM deuterated phenol in D_2O. The nominal ratio of zinc ions per hexamer was ca. 2.5. Protein concentrations were ca. 0.5 mM (biosynthetic insulin and proinsulin), 0.4 mM (synthetic proinsulin), and 0.3 mM (proinsulin enantiomer). The NOESY mixing time was 200 ms. Biosynthetic human proinsulin and biosynthetic human insulin were kindly provided by Lilly Research Laboratories (Indianapolis, IN).

References

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