Chemical Synthesis of the β-subunit of Human Luteinizing (hLH) and Chorionic Gonadotropin (hCG) Glycoprotein Hormones

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SUPPLEMENTARY INFORMATION

I. GENERAL INFORMATION

A. Matherial and Methods

All commercially available materials (Aldrich, Fluka, Novabiochem) were used without further purification. *N*-α-Fmoc protected amino acids, pseudoproline dipeptides, Oxyma Pure and pre-loaded NovaSyn TGT resins were purchased from Novabiochem. 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) was purchased from Genscript. (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from Oakwood Products, Inc. TCEP solution (0.5 M, neutral pH) was purchased from ThermoScientific. Chitobiose octaacetate was purchased from Carbosynth Limited. All other reagents were purchased from Aldrich. All solvents were reagent grade or HPLC grade (Fisher Scientific). Anhydrous tetrahydrofuran, diethyl ether, dichloromethane, toluene, and benzene were obtained from a dry solvent system (passed through column of neutral alumina under an argon atmosphere) and used without further drying.

Reactions were performed under an atmosphere of pre-purified dry argon. Air- and moisture-sensitive liquids and solutions were transferred via syringe. The appropriate carbohydrate reagents were dried via azeotropic removal of water with toluene. Molecular sieves were activated at 350 °C and were crushed immediately prior to use, then flame-dried under vacuum. Organic solutions were concentrated under reduced pressure by rotary evaporation below 30 °C. NMR spectra (^{1}H and ^{13}C) were recorded on a Bruker Advance DRX-600 MHz spectrometer, and referenced to TMS or residual solvent. Low-resolution mass spectral analyses were performed with a JOEL JMS-DX-303-HF mass spectrometer or Waters Micromass ZQ mass spectrometer. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and visualized under UV light (254 nm) or by staining with cerium ammonium molybdenate (CAM) or 5% sulfuric acid in methanol. Silica flash column chromatography was performed on E. Merck 230-400 mesh silica gel 60.

UPLC/LC-MS analyses and RP-HPLC purification. All reverse-phase chromatographic separations involved a mobile phase consisting of 0.05% trifluoroacetic acid (TFA) (v/v) in water and 0.04% TFA in acetonitrile. Reaction progress was monitored by UPLC-MS analysis

on a Waters AcquityTM Ultra Preformance Liquid Chromatography system with a photodiode detector and single quadrupole mass detector, equipped with Acquity UPLC BEH C18/C8/C4 columns (1.7 μm, 2.1 × 100 mm), at a flow rate of 0.3 mL/min. Analytical LC-MS analyses were performed on a Waters 2695 Separations Module equipped with a Waters 2996 Photodiode Array Detector, using a Varian Microsorb C18 column (150 × 2.0 mm), a Varian Microsorb C8/C4 column (250 × 2.0 mm) or a Waters X-Bridge C18 column (150 × 2.1 mm), at a flow rate of 0.2 mL/min. Preparative scale HPLC purification was carried out on a Ranin HPLC solvent delivery system equipped with a Rainin UV-1 detector, using an Agilent Dynamax reverse phase HPLC Microsorb C18/C8/C4 column (250 × 21.4 mm), or a Waters X-Bridge C18 column (150 × 19.0 mm), at a flow rate of 16.0 mL/min.

II. General Experimental Procedures

A. Fmoc-based Solid Phase Peptide Synthesis (SPPS)

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous-flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc protocols, using 4 equiv of each amino acid and 3.8 equiv of HATU as a coupling reagent. The deblock solution consisted of a mixture of DMF/piperidine/DBU (96:2:2). The following pre-loaded NovaSyn TGT resins were used: Fmoc-Gly-TGT, Fmoc-Ser(*t*Bu)-TGT, Fmoc-Leu-TGT, Fmoc-Gln(Trt)-TGT. The following α-L-amino acids were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OMpe)-OH, Fmoc-Asp(OPp)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(*t*Butylthio)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(O*t*Bu)-OH, Fmoc-Thr(O*t*Bu)-OH, Boc-Thz-OH (Boc-*Z*-OH), Fmoc-Trp(Boc)-OH, Fmoc-Tyr(O*t*Bu)-OH, Fmoc-Val-OH. The following pseudoproline dipeptides were used: Fmoc-Ala-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Glu-Ser($\psi^{Me,Me}$Pro)-OH, Fmoc-Ile-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Leu-Ser($\psi^{Me,Me}$Pro)-OH, Fmoc-Ser-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Val-Ser($\psi^{Me,Me}$Pro)-OH.

Upon completion of automated synthesis on a 0.05–0.2 mmol scale, the peptide resin was washed into a peptide synthesis vessel with MeOH. After drying, the resin was subjected to a cleavage cocktail (2-8 mL) of acetic acid/trifluoroethanol/methylene chloride (1:1:6), 3 times for 45 min each. Following filtration, the resulting cleavage solutions were combined and concentrated. The oily residue was dissolved in a minimum amount of trifluoroethanol, precipitated with water (0.05% trifluoroacetic acid), and then lyophilized to give the crude protected peptide bearing the free carboxylic acid at the *C*-terminus.

B. Synthesis of *C*-terminal Peptide Thioesters

The fully protected peptidyl acid (1.0 equiv), obtained after resin cleavage as described in the previous section, was dissolved in chloroform, and EDC (3.0 equiv) and HOOBt (3.0 equiv) were then added. After 5 min, the corresponding amino thioester hydrochloride (1.5 equiv) was

added, and the reaction mixture stirred for 3 h to form the crude protected *C*-terminal peptide thioester.

C. Removal of Acid Labile Protecting Groups with Cocktail B

Protected peptides were subjected to Cocktail B (1 mL / 30 mg of peptide) consisting of trifluoroacetic acid (88% by volume), water (5% by volume), phenol (5% by weight), and *i*Pr$_3$SiH (2% by volume). After stirring for 2 h, the solution was evaporated under a stream of nitrogen to half of the initial volume. The residue was treated with ice-cold diethyl ether (40 mL), and the resulting suspension was centrifuged to give a white pellet. The supernant was decanted and the pellet was triturated with ice-cold diethyl ether (40 mL). This process was repeated three times in total, and the resulting precipitate was solubilized in water/acetonitrile (1:1, 0.05% trifluoroacetic acid) and lyophilized to give the deprotected crude peptide.

D. Native Chemical Ligation Buffer

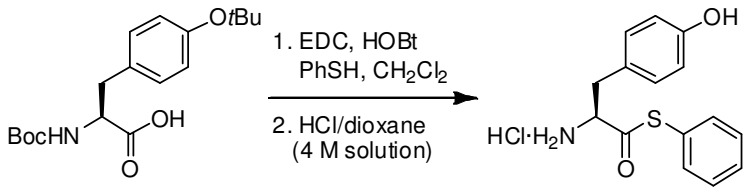
Native chemical ligation (NCL) buffer was freshly prepared prior to the reaction by dissolving Na_2HPO_4 (56.6 mg, 0.2 M) and guanidine·HCl (1.146 g, 6 M) in water. *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) (10.8 mg, 0.02 M) was then added, and the mixture was diluted to 2.0 mL. After complete solubilization, the pH was brought to ~7.2 by the addition of a NaOH solution (5.0 M). 4-mercaptophenylacetic acid (MPAA) (67 mg, 0.2 M) was then added and the pH of the mixture was adjusted to 7.2–7.4 with NaOH (5.0 M). The solution was sonicated and sparged with Ar for 30 min before use.

E. Size Exclusion Centrifugal Filtration

Centrifugal filtrations were performed using an Eppendorf 5804 R Centrifuge and Millipore Amicon Ultra-4 Centrifugal Filters (3 or 10 kD cut-off). Buffer solutions were diluted with acetonitrile/water (1:4, 0.05% trifluoroacetic acid) to 3.0 mL total volume in a Millipore centrifugal filter tube. The tube was centrifuged at 4500 rpm until residual volume was 0.25–0.5 mL. This volume was diluted to 3.0 mL, and the process was repeated three times. The residual solution was diluted with acetonitrile/water (1:1, 0.05% trifluoroacetic acid) and then lyophilized.

III. SYNTHESIS OF AMINO ACID BUILDING BLOCKS

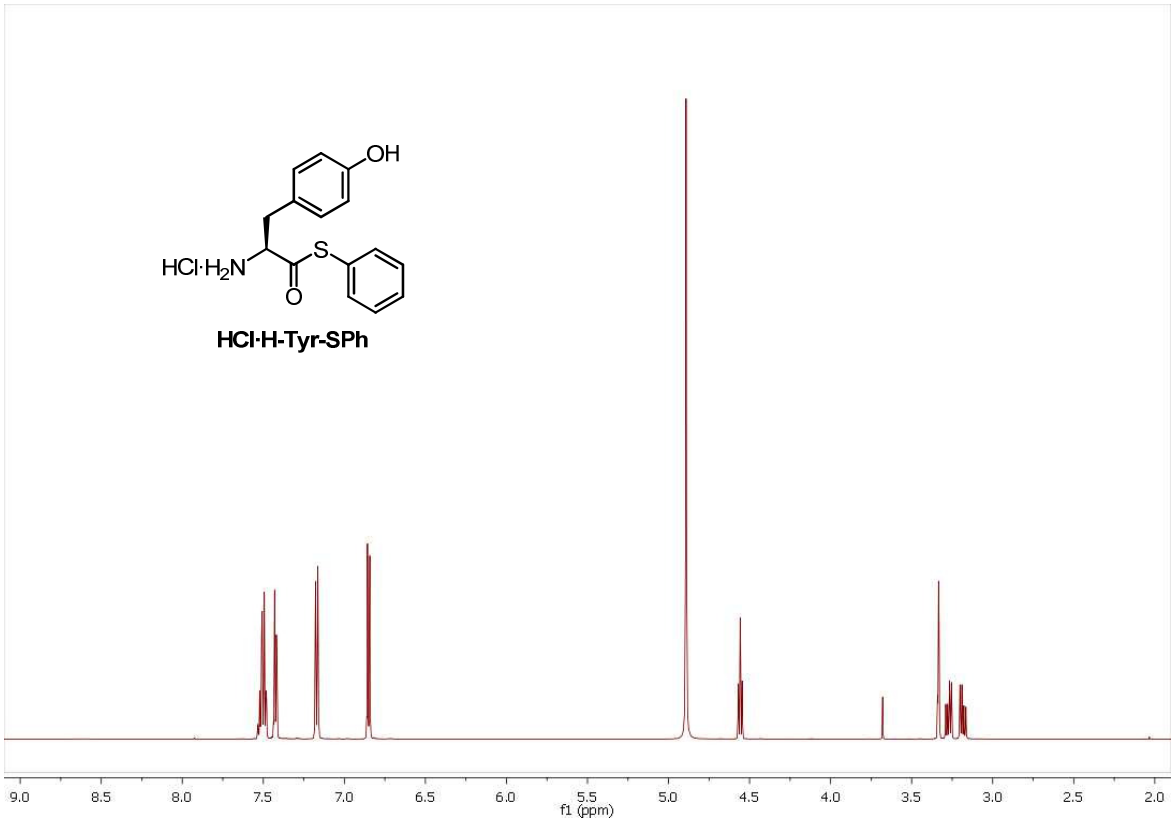
L-Tyrosine thiophenyl ester hydrochloride (HCl·H-Tyr-SPh)



To a solution of Boc-Tyr(*t*Bu)-OH (1.68 g, 5.0 mmol) in dichloromethane (25 mL) at 0º C, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) (1.0 mL, 6 mmol) and 1-hydroxybenzotriazole (HOBt) (2.0 g, 15 mmol) were added, followed by thiophenol (PhSH) (1.0 mL, 10 mmol). The reaction mixture was stirred overnight at 23 ºC and concentrated *in vacuo*. Purification by silica gel chromatography (hexanes/EtOAc, 95:5) gave Boc-Tyr(*t*Bu)-SPh (876 mg, 41% yield) as a colorless syrup.

This compound (876 mg, 2.0 mmol) was treated with a solution of HCl/dioxane (4 M, 5.0 mL) and stirred for 2 h. The solvent was removed by rotary evaporation and the residue triturated with diethyl ether (× 2) and dried under vaccum to afford **HCl·H-Tyr-SPh** (613 mg, 99% yield) as a white solid.

^{1}H NMR (600 MHz, CD_3OD): 7.55–7.47 (3H, m), 7.45–7.40 (2H, m), 7.21–7.14 (2H, m), 6.88–6.82 (2H, m), 4.89 (4H, bs), 4.56 (t, J = 7.2 Hz, 1H), 3.27 (dd, J = 14.3, 7.0 Hz, 1H), 3.18 (dd, J = 14.2, 7.3 Hz, 1H). **^{13}C NMR** (150 MHz, CD_3OD) 196.9, 159.4, 136.6, 132.7, 132.2, 131.5, 127.5, 126.1, 117.8, 62.1, 38.9. **MS (ESI)** m/z: Calcd. for $C_{15}H_{16}NO_2SCl$ $[M+Cl]^-$ 309.06, found 309.10.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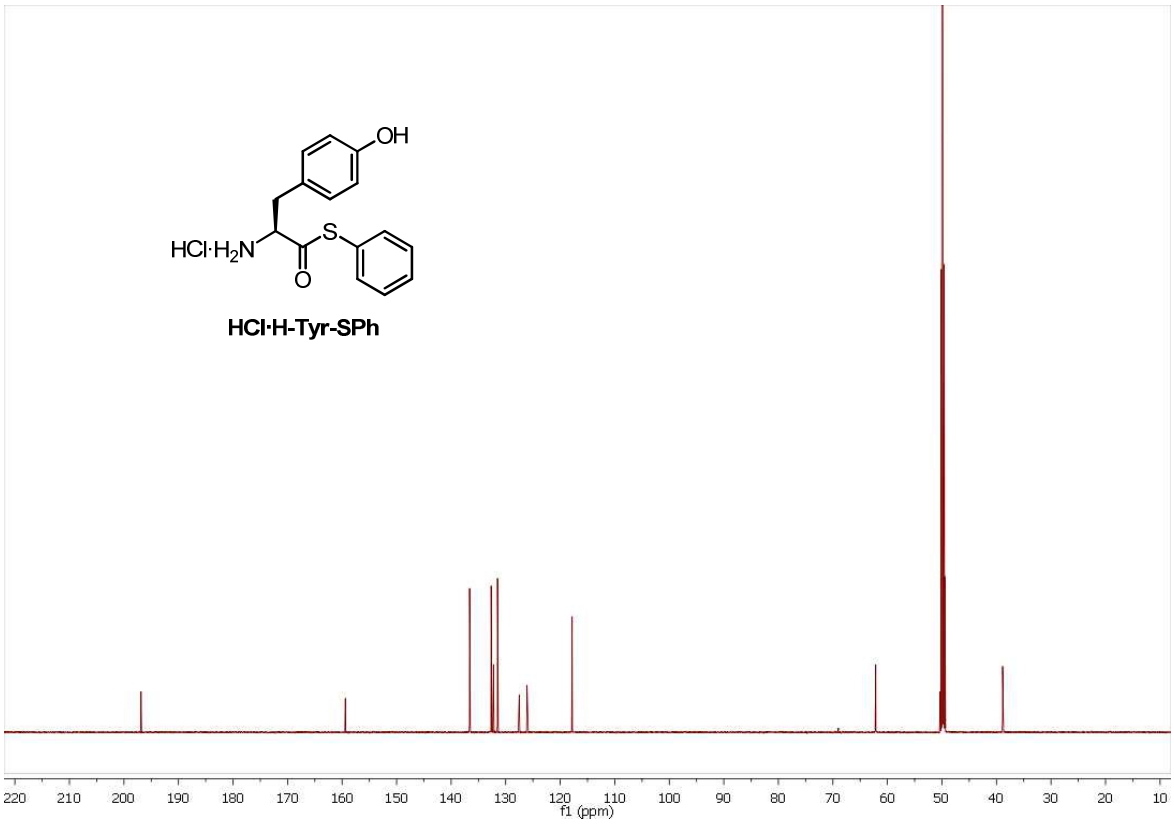
3.0
2.5
2.0
f1 (ppm)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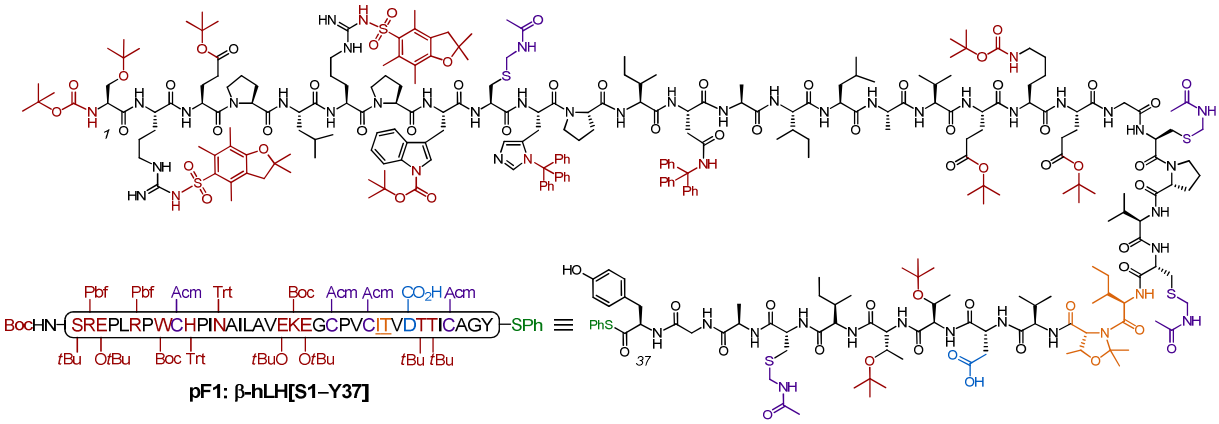
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

IV. Synthesis Of β-Human Luteinizing Hormone (β-LH)

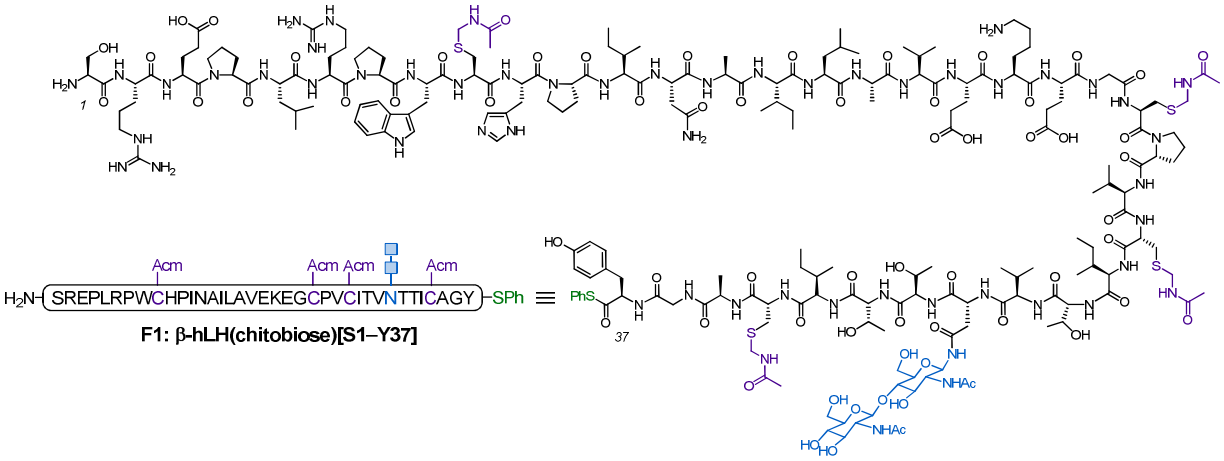
A. Synthesis of β-hLH Peptide Fragments

Side-chain protected fragment 1 peptide thioester (pF1:β-hLH[S1−Y37])



Crude protected peptide BocHN-Ser^{1}−Gly^{36}-OH, (prepared according to general experimental procedure A) (180 mg, 30 μmol) was dissolved in chloroform (3.0 mL) and reacted with HCl·H-Tyr-SPh (17 mg, 45 μmol) in the presence of EDC (16 μL, 90 μmol) and HOOBt (15 mg, 90 μmol), following general procedure B. After 3 h, the crude mixture was concentrated, treated with 2% trifluoroacetic acid (TFA) in dichloromethane (15 mL) and stirred for 1 h to selectively remove the 2-phenylisopropyl ester (2-Ph^{i}Pr, OPp) from the aspartic acid (Asp^{30}) side chain. The contents were divided into four conical tubes and treated with ice-cold diethyl ether as described above. The residue was dissolved in acetonitrile/water (1:1, 0.05% TFA) and lyophilized to give protected crude peptide thioester bearing the free carboxylic acid at Asp^{30} side chain (178 mg, 98% yield). This peptide **pF1:β-hLH[S1–Y37]** was used in the next step without further purification.

Chitobiose-bearing fragment 1 peptide thioester: β-hLH(chitobiose)[S1–Y37]



Peptide **pF1:β-hLH[S1-Y37]** (42 mg, 6.8 µmol) and **chitobiose** ($GlcNAc_2$) anomeric amine (8.5 mg, 20 µmol) were combined and dissolved in anhydrous DMSO (0.5 mL). HATU (7.6 mg, 20 µmol) and DIEA (3.0 µL, 17 µmol) were added and the golden-yellow mixture was stirred for 1.5 h, frozen and lyophilized. The protected glycopeptide was then subjected to cocktail B (2 mL) for 2 h, precipitated with ice-cold diethyl ether, centrifuged, resuspended, and lyophilized according to general procedure C. The crude peptide was dissolved in 30% acetonitrile/water (0.05% TFA) (8 mL) and purified by HPLC on a C18 column, using a linear gradient of 35–55% acetonitrile in water (0.05% TFA) over 30 min. The fractions containing the desired product, which eluted at 19 min, were collected and lyophilized to provide glycopeptide **β-hLH(chitobiose)[S1–Y37]** (7.4 mg, 22% yield) as a white solid.

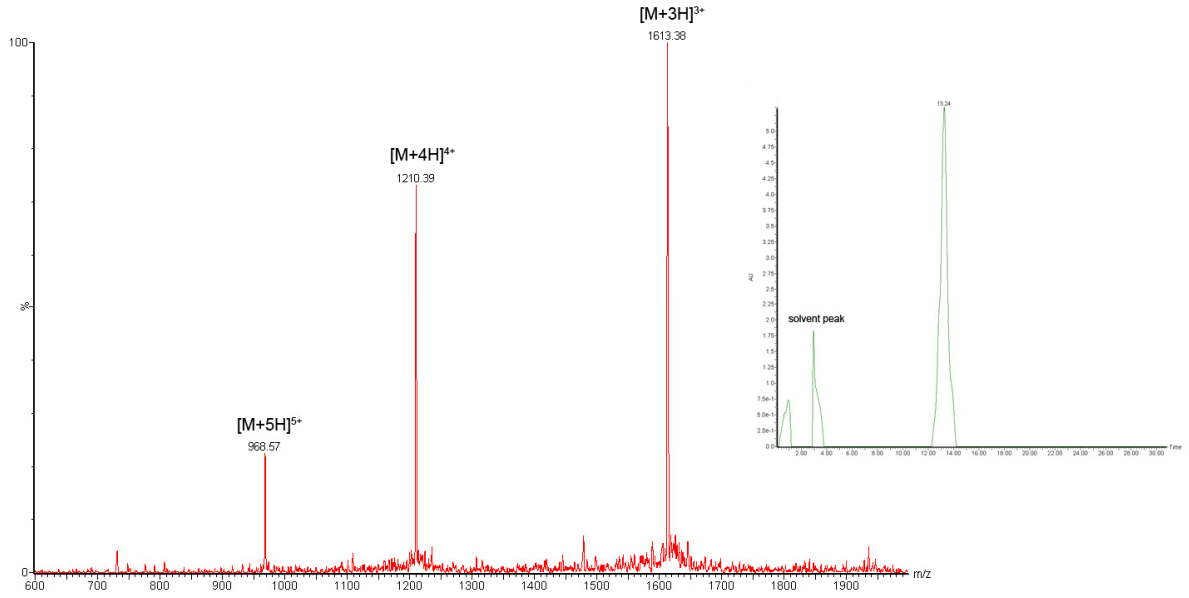
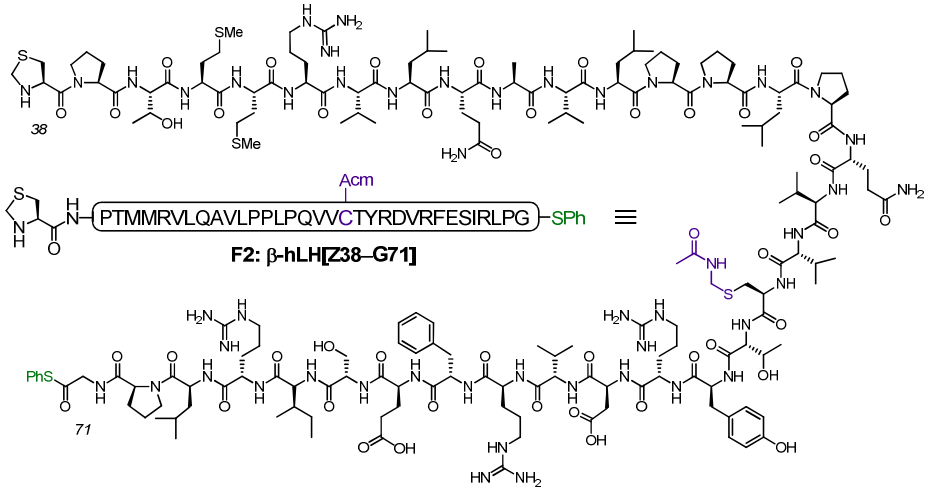


Figure S1. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hLH(chitobiose)[S1–Y37]**. Calcd. for $C_{212}H_{335}N_{55}O_{64}S_5$, 4838.58 (average isotopes) $[M+3H]^{3+}$ *m/z* 1613.86, found 1613.38; $[M+4H]^{4+}$ *m/z* 1210.65, found 1210.39; $[M+5H]^{5+}$ *m/z* 968.72, found 968.57. Waters X-Bridge C18 column, gradient: 25–60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Fragment 2 peptide thioester: β-hLH[Z38–G71]



To a solution of crude protected peptide BocN-Thz^{38}–Gly^{71}-OH, (prepared according to general experimental procedure A) (150 mg, 25 μmol) in chloroform (5.0 mL), benzotriazol-1-

yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (131 mg, 0.25 mmol) and DIEA (44 µL, 0.25 mmol) were added, followed by thiophenol (PhSH) (77 µL, 0.75 mmol). The reaction mixture was stirred for 8 h, concentrated, and the residue subjected to cocktail B (6.7 mL) following general procedure C. The crude peptide thioester was purified by HPLC on a C18 column, using a linear gradient of 40-60% acetonitrile in water over 30 min. The fractions containing the desired product (retention time ~16 min) were collected and lyophilized to give peptide thioester **β-hLH[Z38–G71]** (73 mg, 72% yield) as a white solid.

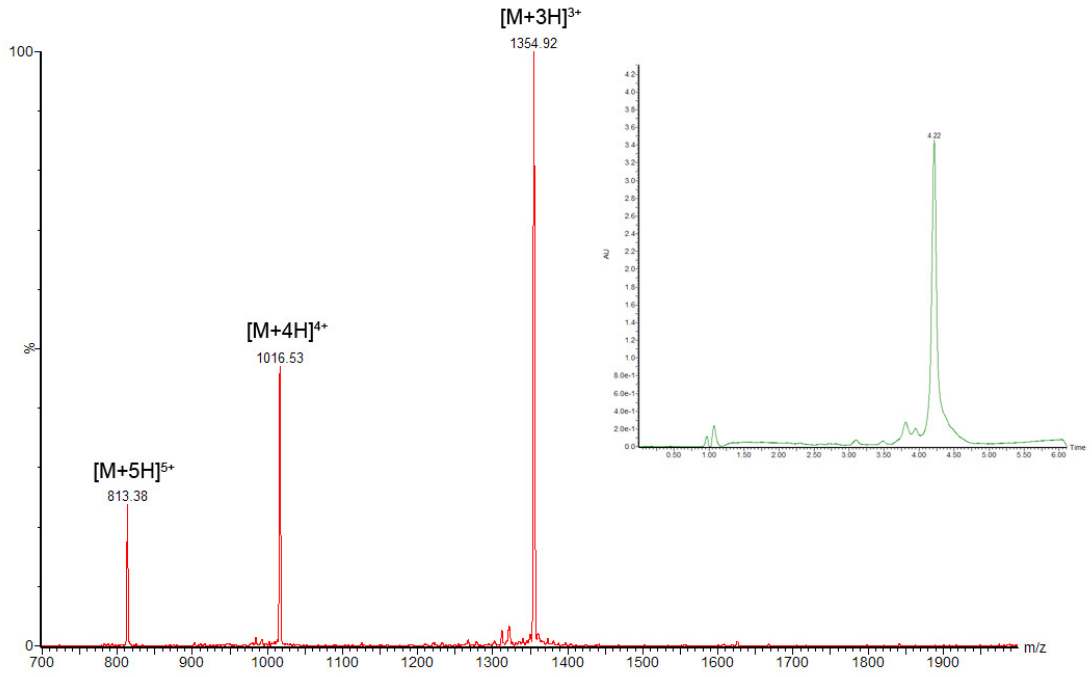
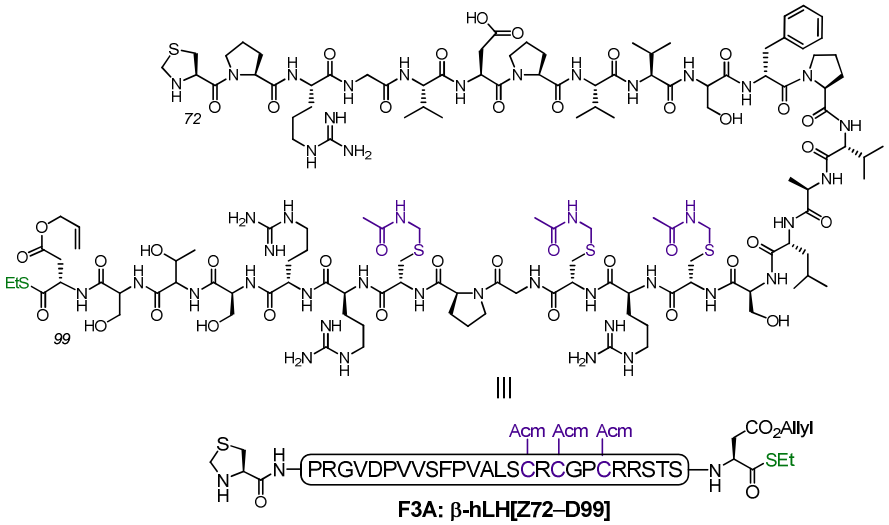


Figure S2. ESI-MS and UV traces from UPLC analysis for glycopeptide **β-hLH[Z38–G71]**. Calcd. for $C_{183}H_{295}N_{49}O_{45}S_5$, 4061.93 (average isotopes) $[M+3H]^{3+}$ *m/z* 1354.98, found 1354.92; $[M+4H]^{4+}$ *m/z* 1016.48, found 1016.53; $[M+5H]^{5+}$ *m/z* 813.38, found 813.38. BEH C4 column, gradient: 20–60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min.

Fragment 3A peptide thioester: β-hLH[Z72–D99]



Crude protected peptide BocN-Thz^{72}–Ser^{98}-OH (prepared according to general experimental procedure A) (226 mg, 50 μmol) was dissolved in chloroform (5.0 mL) and reacted with **HCl·H-Asp(OAllyl)-SEt**[1] (19 mg, 75 μmol) in the presence of EDC (27 μL, 0.15 mmol) and HOOBt (25 mg, 0.15 mmol), as described in general procedure B. After stirring for 3 h, the reaction mixture was concentrated and subjected to cocktail B following general procedure C. The crude peptide thioester was purified by HPLC on a C18 column, using a linear gradient of 20-50% acetonitrile in water over 30 min. The fractions containing the desired product, which eluted at 18 min, were collected and lyophilized to give peptide thioester **β-hLH[Z72–D99]** (97 mg, 58% yield) as a white solid.

(1) Aussedat, B .; Vohra, Y.; Park, P. K.; Fernández-Tejada, A.; Alam, S. M.; Dennison, S. M.; Jaeger, F. H.; Anasti, K.; Stewart, S.; Blinn, J. H.; Liao, H. X.; Sodroski, J. G.; Haynes, B. F.; Danishefsky, S. J. *J. Am. Chem. Soc*. **2013**, *135*, 13113–13120.

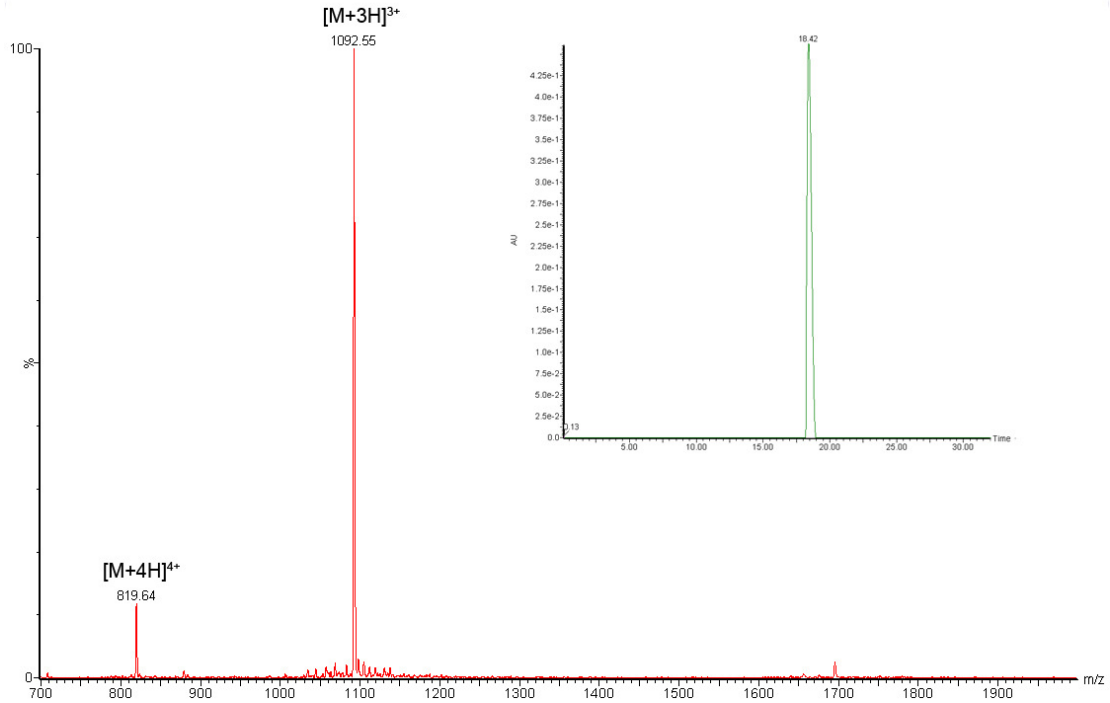
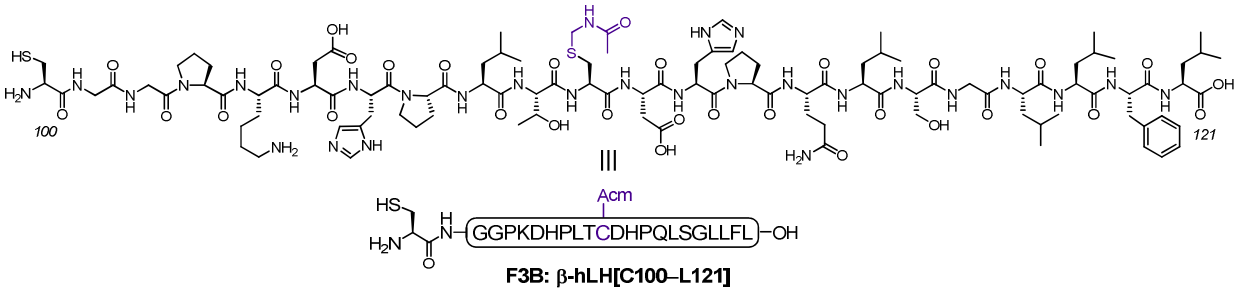


Figure S3. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hLH[Z72–D99]**. Calcd. for $C_{137}H_{225}N_{43}O_{40}S_5$, 3274.84 (average isotopes) $[M+3H]^{3+}$ *m/z* 1092.61, found 1092.55; $[M+4H]^{4+}$ *m/z* 819.71, found 819.64. Varian Microsorb 300-5 C4 column, gradient: 20–50% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Fragment 3B peptide: β-hLH[C100–L121]



Crude protected peptide BocN-Thz^{100}–Leu^{121}-OH (prepared according to general experimental procedure A) (150 mg, 0.04 mmol) was treated with cocktail B as described in general procedure C, and upon lyophilization the crude deprotected peptide (100 mg, 0.04 mmol, >99%) was directly subjected to thiazolidine (Thz) cleavage. The Thz-deprotection buffer was freshly prepared prior to the reaction by dissolving $MeONH_2$·HCl (100 mg, 0.3 M) and

guanidine·HCl (2.3 g, 6 M) in water (4.0 mL) and adjusting the pH of the mixture to ~4.0–4.5 by addition of 2 M HCl solution. After overnight treatment with this buffer (4.0 mL), the reaction mixture was diluted with 8 mL of 30% acetonitrile in water (0.05% TFA), and purified by HPLC (C18 column, linear gradient of 30–60% acetonitrile/water over 30 min). The desired product eluted at 12 min, and the collected fractions were lyophilized to give peptide **β-hLH[C100–L121]** (67 mg, 64% yield) as a white solid.

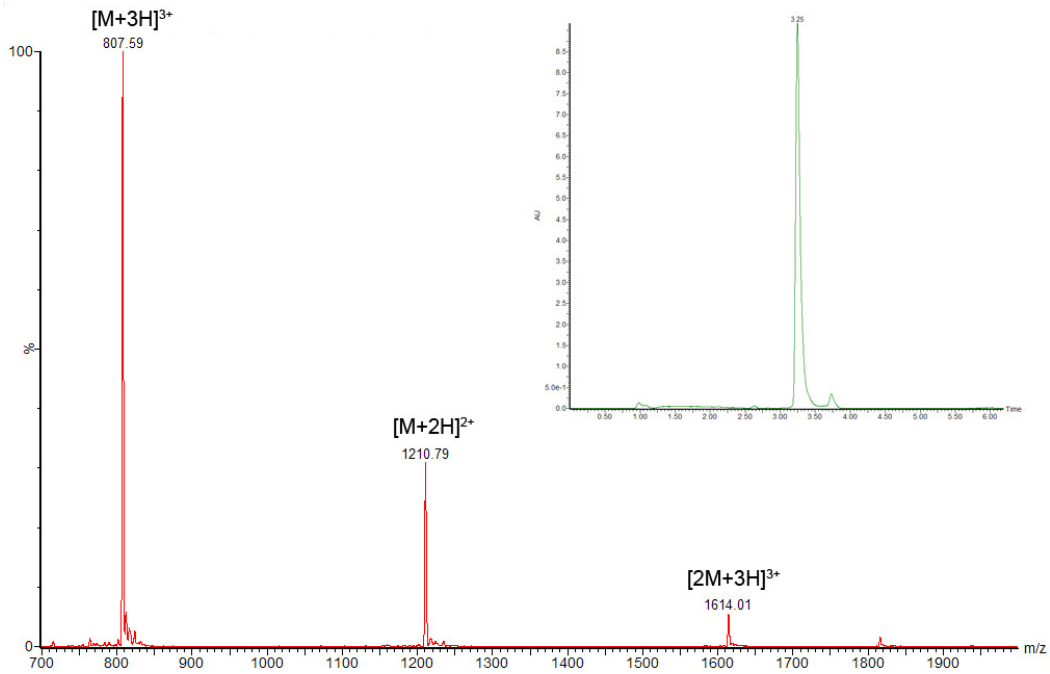
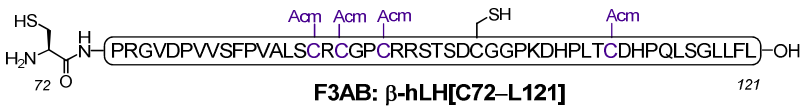


Figure S4. ESI-MS and UV traces from UPLC analysis for glycopeptide **β-hLH[C100–L121]**. Calcd. for $C_{107}H_{167}N_{29}O_{31}S_2$, 2419.78 (average isotopes) $[M+3H]^{3+}$ *m/z* 807.59, found 807.59; $[M+2H]^{2+}$ *m/z* 1210.89, found 1210.79; $[2M+3H]^{3+}$ *m/z* 1614.01, found 1613.18. BEH C4 column, gradient: 20–60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min.

B. Assembly of hLH Peptide Fragments

Fragment 3 [3AB] peptide: β-hLH[C72–L121]



F3AB: β-hLH[C72–L121]

Peptides **β-hLH[Z72–D99]** (16.4 mg, 5.0 μmol) and **β-hLH[C100–L121]** (12.2 mg, 5.0 μmol) were dissolved in NCL buffer (1.25 mL), prepared as described in general procedure D, and neutral TCEP solution (0.5 M, 125 μL) was added. The reaction mixture was stirred under Ar atmosphere for 4 h and then desalted by centrifugal filtration in a 3000 MW cut-off filter following general procedure E. The lyophilized peptide (28 mg, 5.0 μmol) was dissolved in DMSO (0.5 mL) and $PhSiH_3$ (55 μL, 0.44 mmol) was added, followed by a solution of $PdCl_2$(dppf) (9.8 mg, 13.3 μmol) in DMSO (0.5 mL). The mixture was stirred in the dark for 30 min, diluted with 30% acetonitrile/water (0.05% TFA) (2.5 mL) and purified by HPLC (C18 column, linear gradient of 30–70% acetonitrile/water over 30 min, product eluted at 12 min) to give β-hLH[Z72–L121] (20 mg, 67% yield) after lyophilization. This peptide (21.1 mg, 3.8 μmol) was finally treated with Thz deprotection buffer (1.0 mL) at pH ~4.0-4.5, prepared as described above, and the reaction mixture stirred overnight. The contents were diluted with 30% acetonitrile/water (0.05% TFA) (3 mL) and purified by HPLC (C18 column, linear gradient of 30–70% acetonitrile/water over 30 min, product eluted at 14 min) to provide **β-hLH[C72–L121]** as a white solid (16.1 mg, 55% yield over 3 steps).

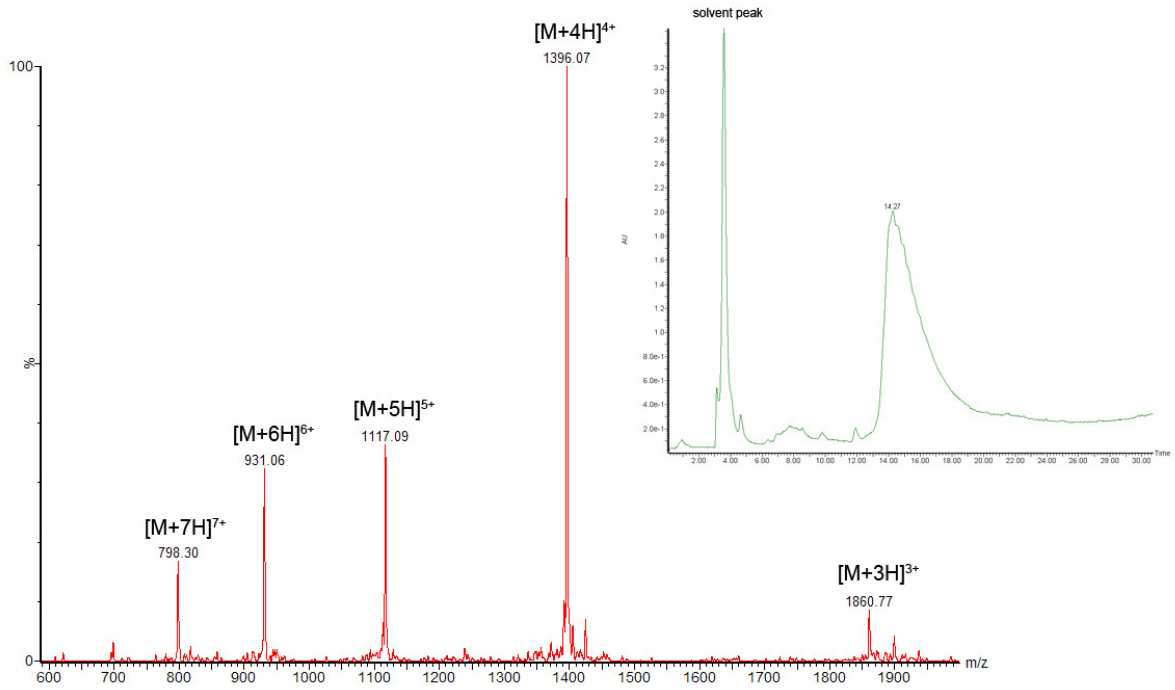
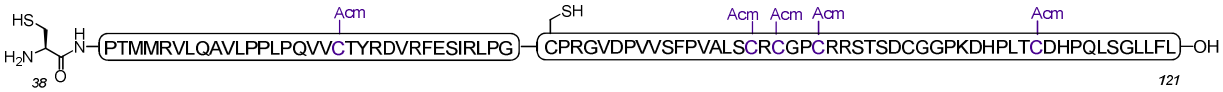


Figure S5. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hLH[C72–L121]**. Calcd. for $C_{238}H_{382}N_{72}O_{71}S_6$, 5580.41 (average isotopes) $[M+3H]^{3+}$ *m/z* 1861.14, found 1860.77; $[M+4H]^{4+}$ *m/z* 1396.10, found 1396.07; $[M+5H]^{5+}$ *m/z* 1117.08, found 1117.09; $[M+6H]^{6+}$ *m/z* 931.07, found 931.06; $[M+7H]^{7+}$ *m/z* 798.20, found 798.30. Varian Microsorb 300-5 C18 column, gradient: 30–60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Fragment [2.3] peptide: β-hLH[C38–L121]



F2.3: β-hLH[C38–L121]

Peptides **β-hLH[Z38–G71]** (7.2 mg, 1.8 μmol) and **β-hLH[C72–L121]** (10.0 mg, 1.8 μmol) were dissolved in NCL buffer (450 μL), prepared as described in general procedure D, and neutral TCEP solution (0.5 M, 45 μL) was added. The reaction mixture was stirred under Ar atmosphere for 4 h, and after completion of the ligation as assessed by UPLC, an aqueous solution of $MeONH_2$·HCl (45 μL, 0.6 M) was added followed by TCEP solution (45 μL, 0.5 M). The pH was adjusted to ~4.0–4.5 by addition of 2 M HCl solution and the turbid mixture was stirred for 18 h. After this time, the contents were diluted with 30% acetonitrile/water (0.05% TFA) (4.0 mL) and purified by HPLC (C4 column, linear gradient of 30–70% acetonitrile/water over 30 min, product eluted at 16 min). The collected fractions were lyophilized to afford **β-hLH[C38–L121]** (6.9 mg, 43% yield) as a white solid.

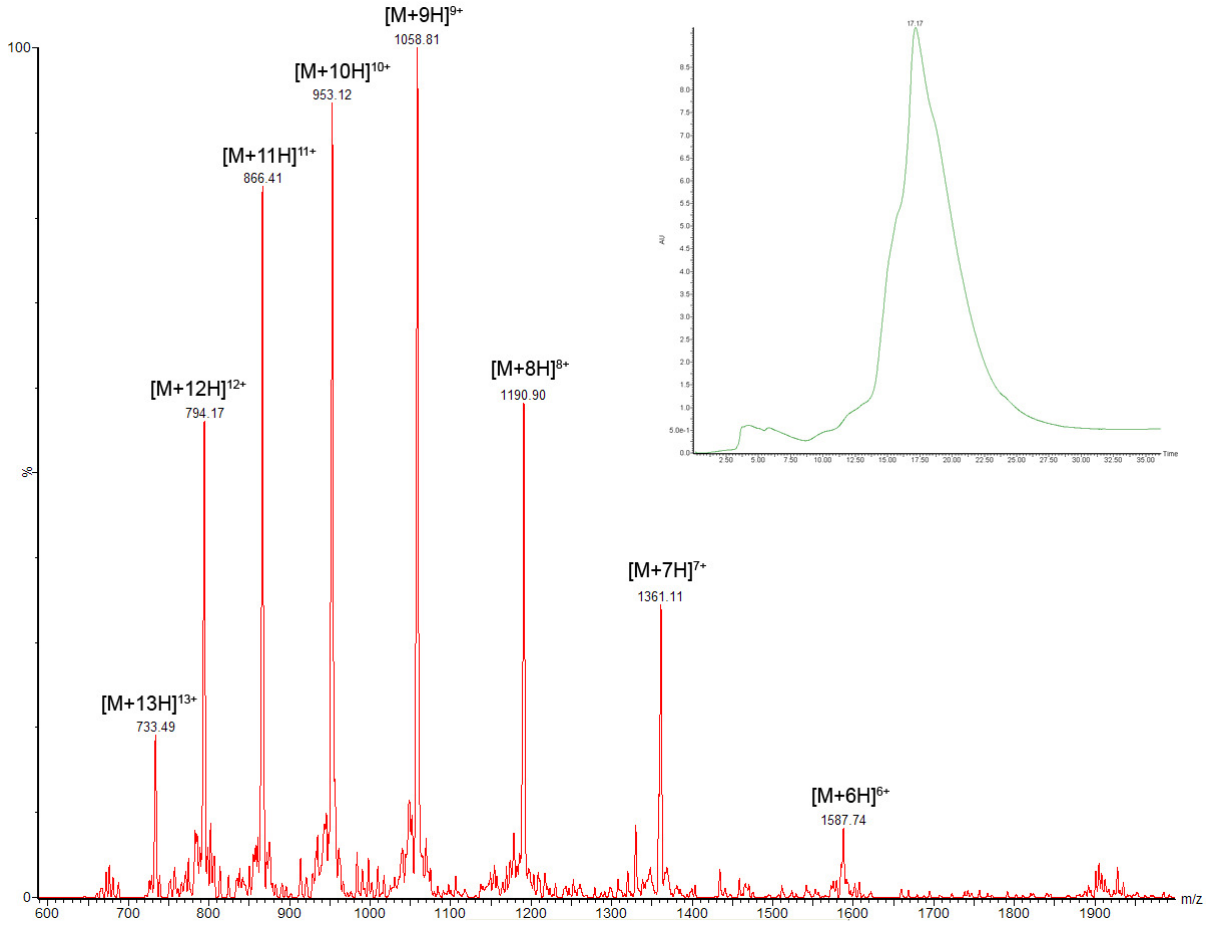
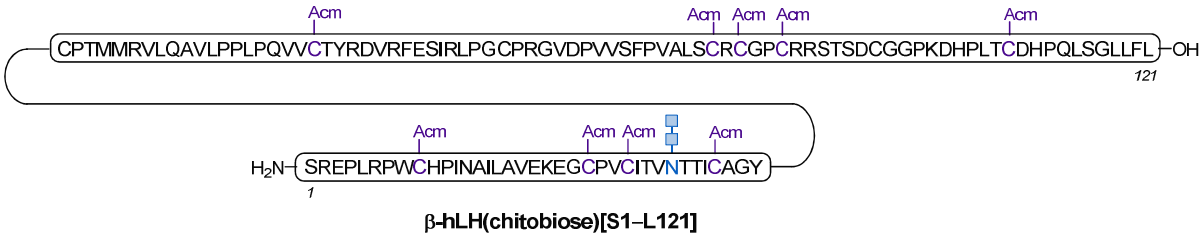


Figure S6. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hLH[C38–L121]**. Calcd. for $C_{414}H_{671}N_{121}O_{116}S_{10}$, 9520.15 (average isotopes) $[M+6H]^{6+}$ *m/z* 1587.69, found 1587.74; $[M+7H]^{7+}$ *m/z* 1361.02, found 1361.11; $[M+8H]^{8+}$ *m/z* 1191.02, found 1190.90; $[M+9H]^{9+}$ *m/z* 1058.79, found 1058.81; $[M+10H]^{10+}$ *m/z* 953.01, found 953.12; $[M+11H]^{11+}$ *m/z* 866.47, found 866.41; $[M+12H]^{12+}$ *m/z* 794.35, found 794.17; $[M+13H]^{13+}$ *m/z* 733.32, found 733.49. Varian Microsorb 300-5 C4 column, gradient: 30–70% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Full Sequence β-hLH(chitobiose)[S1–L121] Glycoprotein Hormone



β-hLH(chitobiose)[S1–L121]

Peptides **β-hLH(chitobiose)[S1–Y37]** (3.0 mg, 0.6 μmol) and **β-hLH[C38–L121]** (6.0 mg, 1.8 μmol) were dissolved in NCL buffer (160 μL), prepared as described in general procedure D, and neutral TCEP solution (0.5 M, 16 μL) was added. The reaction mixture was stirred under Ar atmosphere for 4 h, and then diluted with 30% acetonitrile/water (0.05% TFA) (2.0 mL). Purification by HPLC on a C4 column, using a linear gradient of 30–70% acetonitrile/water, over 30 min (product eluted at 15.5 min), followed by lyophilization of the collected fractions gave full sequence **β-hLH(chitobiose)[S1–L121]** glycoprotein hormone (3.2 mg, 35% yield) as a white solid.

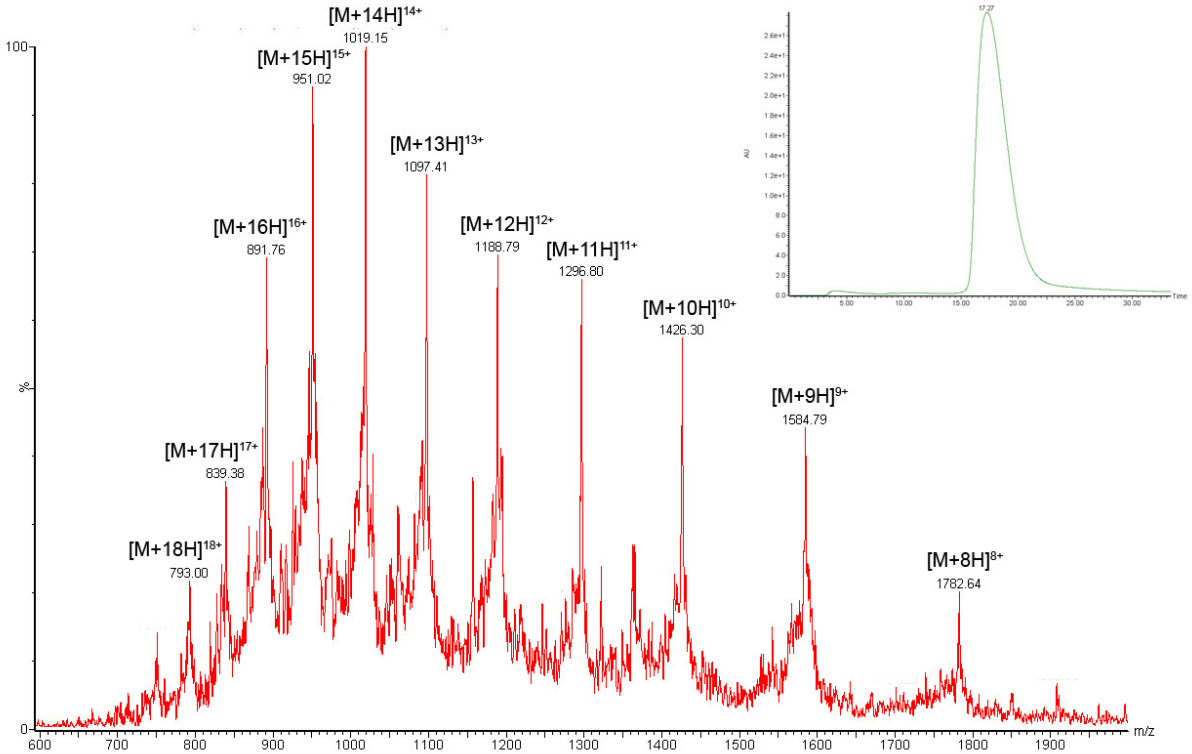
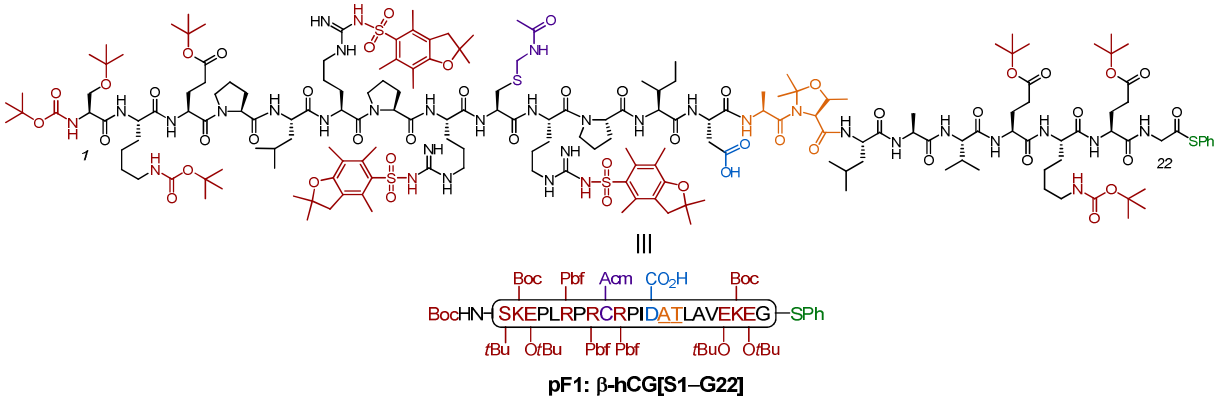


Figure S7. ESI-MS and UV traces from analytical HPLC analysis for glycoprotein **β-hLH(chitobiose)[S1–L121]**. Calcd. for $C_{620}H_{1000}N_{176}O_{180}S_{14}$, 14248.56 (average isotopes) $[M+8H]^{8+}$ *m/z* 1782.07, found 1782.64; $[M+9H]^{9+}$ *m/z* 1584.17, found 1584.79; $[M+10H]^{10+}$ *m/z* 1425.86, found 1426.30; $[M+11H]^{11+}$ *m/z* 1296.32, found 1296.80; $[M+12H]^{12+}$ *m/z* 1188.38, found 1188.79; $[M+13H]^{13+}$ *m/z* 1097.04, found 1097.41; $[M+14H]^{14+}$ *m/z* 1018.75, found 1019.15; $[M+15H]^{15+}$ *m/z* 950.90, found 951.02; $[M+16H]^{16+}$ *m/z* 891.53, found 891.76; $[M+17H]^{17+}$ *m/z* 839.15, found 839.38; $[M+18H]^{18+}$ *m/z* 792.59, found 793.00. Varian Microsorb 300-5 C4 column, gradient: 30–70% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

V. Synthesis Of β-Human Chorionic Gonadotropin Hormone (β-CG)

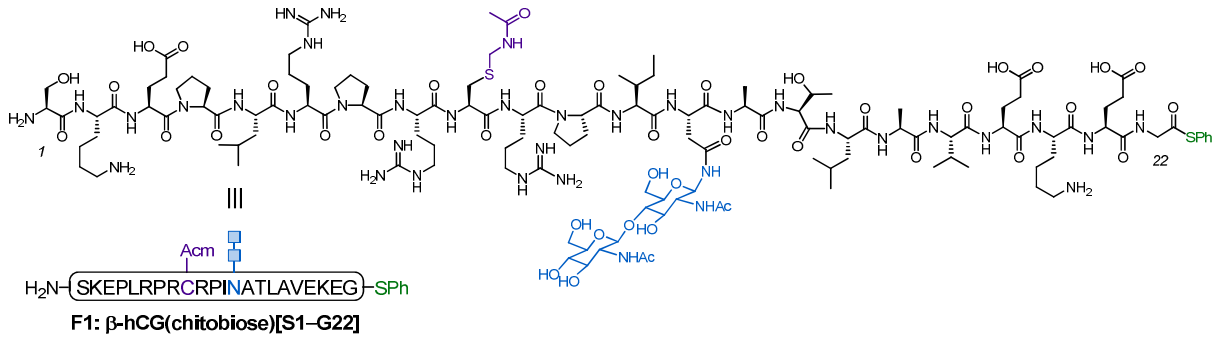
A. Synthesis of β-hCG Peptide Fragments

Side-chain protected fragment 1 peptide thioester (pF1:β-hCG[S1−G22])



Crude protected peptide BocHN-Ser[1]−Gly[22]-OH, (prepared according to general experimental procedure A) (100 mg, 25 μmol) was dissolved in chloroform (5.0 mL) PyBOP (131 mg, 0.25 mmol) and DIEA (44 μL, 0.25 mmol) were added, followed by PhSH (77 μL, 0.75 mmol). The reaction mixture was stirred for 8 h, concentrated, and then treated with 2% TFA in dichloromethane (15 mL). After 1 h, the contents were divided into four conical tubes and treated with ice-cold diethyl ether as described above. The residue was dissolved in acetonitrile/water (1:1, 0.05% TFA) and lyophilized to give protected crude peptide thioester bearing the free carboxylic acid at Asp[13] side chain (98 mg, >99% yield). This peptide **pF1:β-hCG[S1–G22]** was used in the next step without further purification.

Chitobiose-bearing fragment 1 peptide thioester: β-hCG(chitobiose)[S1–G22]



Peptide **pF1: β-hCG(S1−G22)** (13.0 mg, 3.3 μmol) and chitobiose anomeric amine (4.2 mg, 10 μmol) were combined and dissolved in anhydrous DMSO (0.3 mL). HATU (3.8 mg, 10 μmol) and DIEA (1.4 μL, 8.3 μmol) were added and the golden-yellow mixture was stirred for 1.5 h, frozen and lyophilized. The protected glycopeptide was then subjected to cocktail B (1.0 mL) for 2 h, precipitated with diethyl ether, centrifuged, resuspended, and lyophilized according to general procedure C. The crude peptide was dissolved in 20% acetonitrile/water (0.05% TFA) (2.5 mL) and purified by HPLC on a C18 column, using a linear gradient of 20–50% acetonitrile in water over 30 min. The fractions containing the desired product, which eluted at 13 min, were collected and lyophilized to provide glycopeptide **β-hCG(chitobiose)[S1–G22]** (2.8 mg, 28% yield) as a white solid.

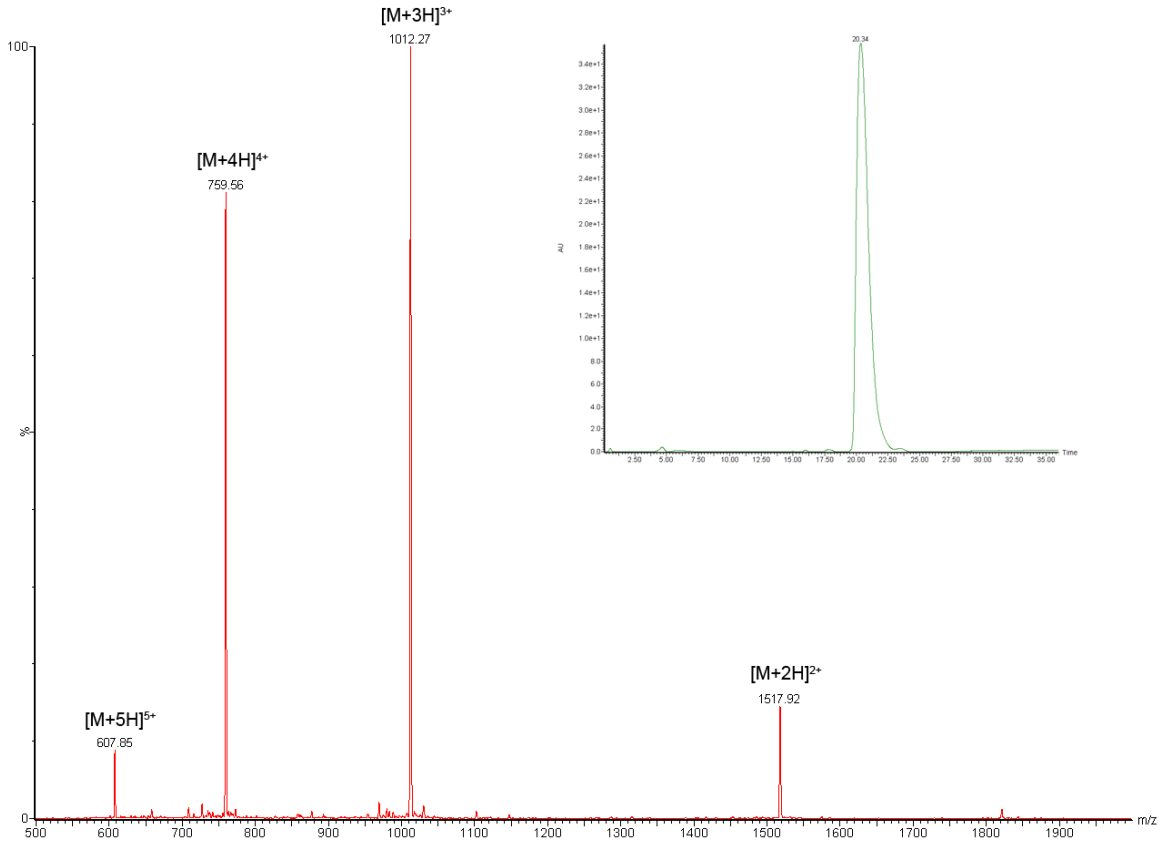
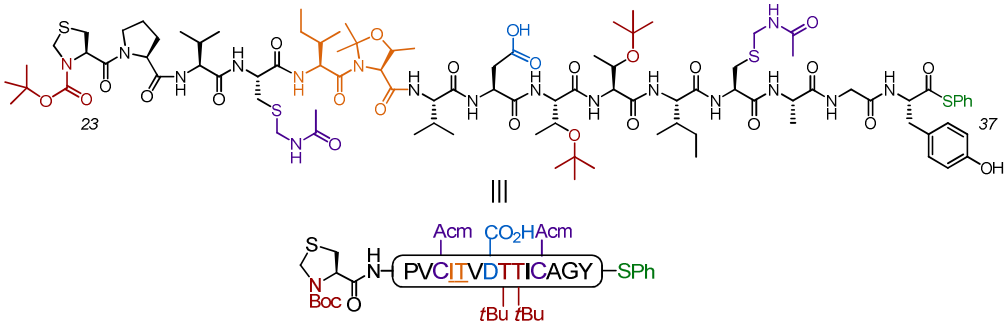


Figure S8. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hCG(chitobiose)[S1–G22]**. Calcd. for $C_{130}H_{217}N_{37}O_{42}S_2$, 3034.47 (average isotopes) $[M+2H]^{2+}$ *m/z* 1518.23, found 1517.92; $[M+3H]^{3+}$ *m/z* 1012.49, found 1012.27; $[M+4H]^{4+}$ *m/z*

759.62, found 759.56; $[M+5H]^{5+}$ *m/z* 759.62, found 607.89. Varian Microsorb 300-5 C8 column, gradient: 10–50% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

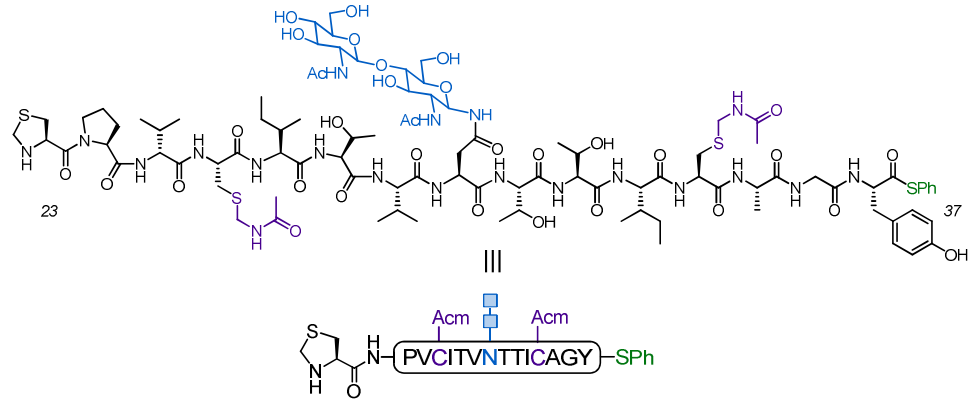
Side-chain protected fragment 2 peptide thioester (pF2:β-hCG[Z23−Y37])



pF2: β-hCG[Z23–Y37]

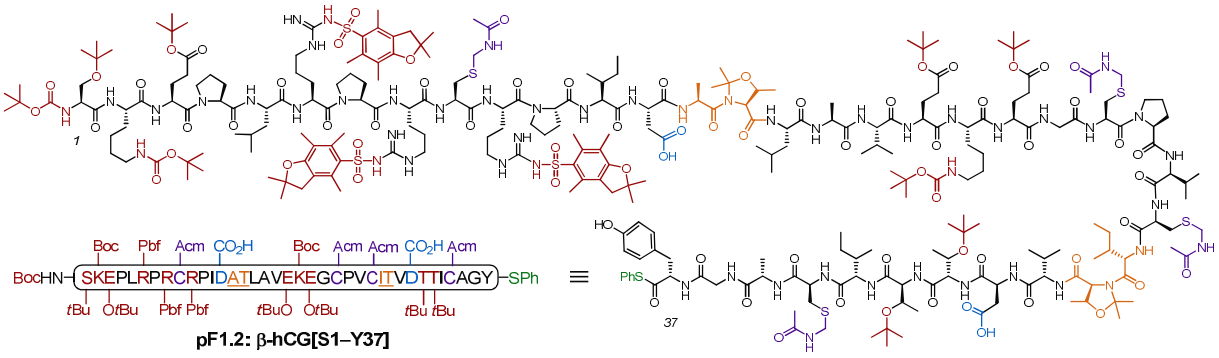
Crude protected peptide BocN-Thz^{23}–Gly^{36}-OH, (prepared according to general experimental procedure A) (48 mg, 25 μmol) was dissolved in chloroform (2.5 mL) and reacted with HCl·H-Tyr-SPh (14 mg, 38 μmol) in the presence of EDC (13 μL, 75 μmol) and HOOBt (12 mg, 75 μmol), following general procedure B. After 3 h, the crude mixture was concentrated, treated with 2% TFA in dichloromethane (12 mL) and stirred for 1 h to selectively remove the OPp ester from the aspartic acid (Asp^{30}) side chain. The contents were divided into four conical tubes and treated with ice-cold diethyl ether as described above. The residue was dissolved in acetonitrile/water (1:1, 0.05% TFA) and lyophilized to give protected crude peptide thioester bearing the free carboxylic acid at Asp^{30} side chain (49 mg, 98% yield). This peptide **pF2:β-hCG[Z23−Y37]** was used in the next step without further purification.

Chitobiose-bearing fragment 2 peptide thioester: β-hCG(chitobiose)[Z23–Y37]



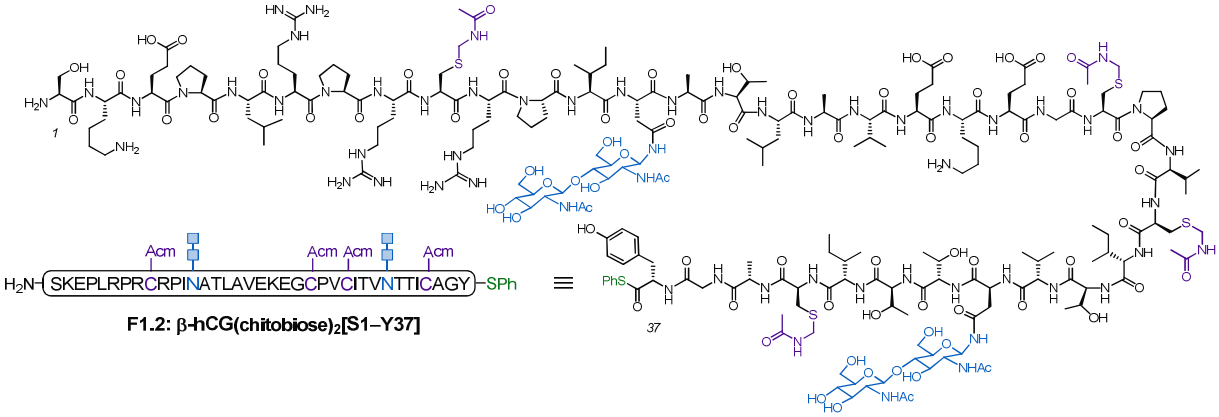
Peptide **pF2:β-hCG[Z23–Y37]** (7.0 mg, 3.4 µmol) and **chitobiose** (4.7 mg, 11 µmol) were combined and dissolved in anhydrous DMSO (0.3 mL). HATU (4.2 mg, 11 µmol) and DIEA (1.5 µL, 8.5 µmol) were added and the golden-yellow mixture was stirred for 1.5 h, frozen, and lyophilized. The protected glycopeptide was then subjected to cocktail B (1 mL) for 2 h, precipitated with ice-cold diethyl ether, centrifuged, resuspended and lyophilized according to general procedure C. The crude peptide was dissolved in 30% acetonitrile/water (0.05% TFA) (2.5 mL) and purified by HPLC on a C18 column using a linear gradient of 30–50% acetonitrile in water over 30 min. The fractions containing the desired product were collected and lyophilized to provide glycopeptide **β-hCG(chitobiose)[Z23–Y37]** (1.9 mg, 25% yield) as a white solid.

Side-chain protected fragment [1.2] peptide thioester (pF1.2:β-hCG[S1−Y37])



Crude protected peptide BocHN-Ser^{1}–Gly^{36}-OH, (prepared according to general experimental procedure A) (175 mg, 30 μmol) was dissolved in chloroform (3.0 mL) and reacted with HCl·H-Tyr-SPh (17 mg, 45 μmol) in the presence of EDC (16 μL, 90 μmol) and HOOBt (15 mg, 90 μmol), following general procedure B. After 3 h, the crude mixture was concentrated, treated with 2% TFA in dichloromethane (15 mL) and stirred for 1 h to selectively remove the OPp esters from the two aspartic acid (Asp^{13} and Asp^{30}) side chains. The contents were divided into four conical tubes and treated with ice-cold diethyl ether as described above. The residue was dissolved in acetonitrile/water (1:1, 0.05% TFA) and lyophilized to give protected crude peptide thioester bearing the two free carboxylic acids at Asp^{13} and Asp^{30} side chain (174 mg, 99% yield). This peptide **pF1.2:β-hCG[S1−Y37]** was used in the next step without further purification.

Chitobiose-bearing fragment [1.2] peptide thioester: β-hCG(chitobiose)$_2$[S1–Y37]



Peptide **pF1.2:β-hCG[S1−Y37]** (30 mg, 5.0 μmol) and **chitobiose** (13 mg, 30 μmol) were combined and dissolved in anhydrous DMSO (0.5 mL). HATU (12.5 mg, 33 μmol) and DIEA (4.4 μL, 25 μmol) were added and the golden-yellow mixture was stirred for 2.5 h. The reaction was quenched by addition of ice-cold water (0.05% TFA) (2 mL), and the precipitate formed was isolated by centrifugation, resuspended in water/acetonitrile (0.05% TFA) and lyophilized. The protected glycopeptide was then subjected to cocktail B (2 mL) for 2 h, precipitated with ice-cold diethyl ether, centrifuged, dissolved and lyophilized according to general procedure C. The crude peptide was dissolved in 25% acetonitrile/water (0.05% TFA) (5 mL) and purified by HPLC on a C18 column using a linear gradient of 30–35% acetonitrile in water over 30 min. The fractions

containing the desired product, which eluted at 16.5 min, were collected and lyophilized to provide glycopeptide **β-hCG(chitobiose)$_2$[S1–Y37]** (4.7 mg, 18% yield) as a white solid.

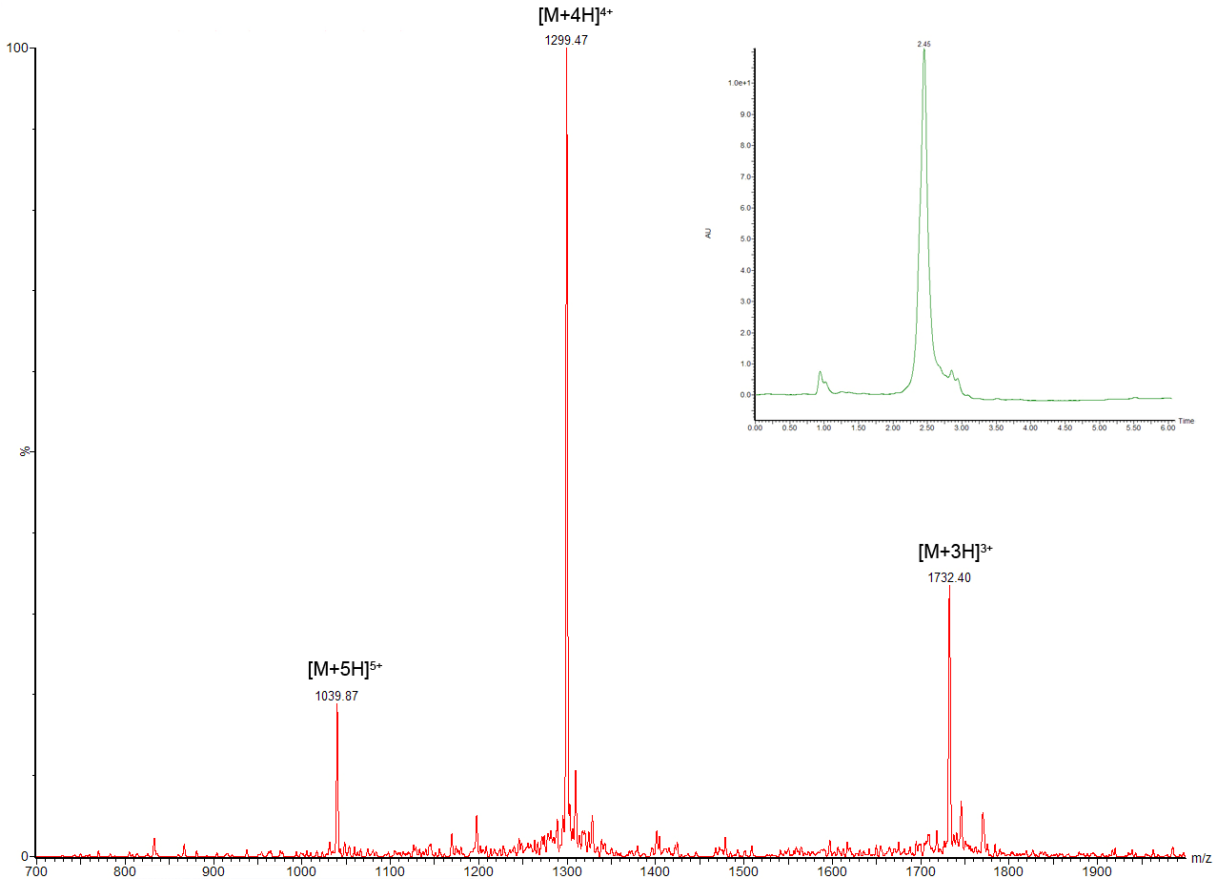
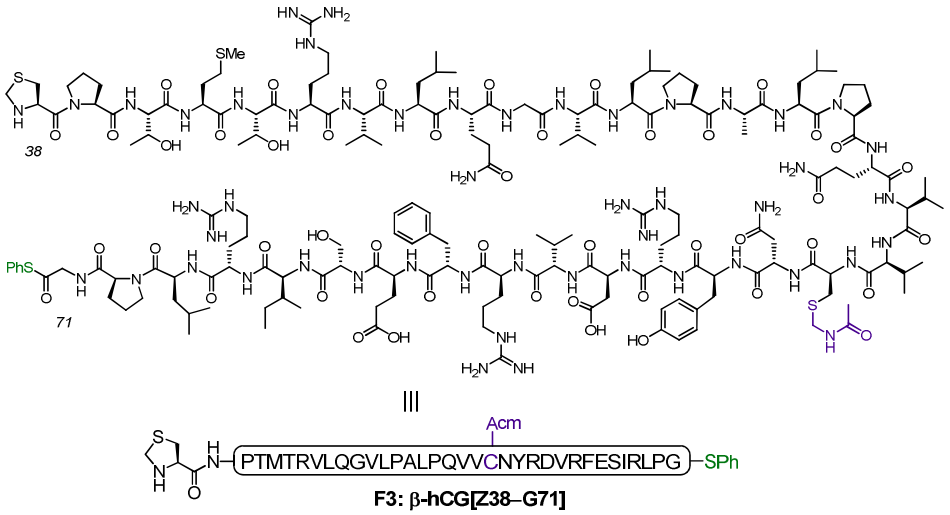


Figure S9. ESI-MS and UV traces from UPLC analysis for glycopeptide **β-hCG(chitobiose)$_2$[S1–Y37]**. Calcd. for $C_{221}H_{364}N_{58}O_{75}S_5$, 5193.92 (average isotopes) $[M+3H]^{3+}$ *m/z* 1732.31, found 1732.40; $[M+4H]^{4+}$ *m/z* 1299.48, found 1299.47; $[M+5H]^{5+}$ *m/z* 1039.78, found 1039.87. BEH C4 column, gradient: 20–60% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min.

Fragment 3 peptide thioester: β-hCG[Z38–G71]



To a solution of crude protected peptide BocN-Thz^{38}–Gly^{71}-OH, (prepared according to general experimental procedure A) (153 mg, 25 μmol) in chloroform (5.0 mL), PyBOP (131 mg, 0.25 mmol) and DIEA (44 μL, 0.25 mmol) were added, followed by PhSH (77 μL, 0.75 mmol). The reaction mixture was stirred for 4 h and concentrated, and the residue was subjected to cocktail B (6.7 mL) for 2 h following general procedure C. The crude peptide thioester was purified by HPLC on a C18 column using a linear gradient of 35–55% acetonitrile in water over 30 min. The fractions containing the desired product (retention time ~16 min) were collected and lyophilized to give peptide thioester **β-hCG[Z38–G71]** (75 mg, 75% yield).

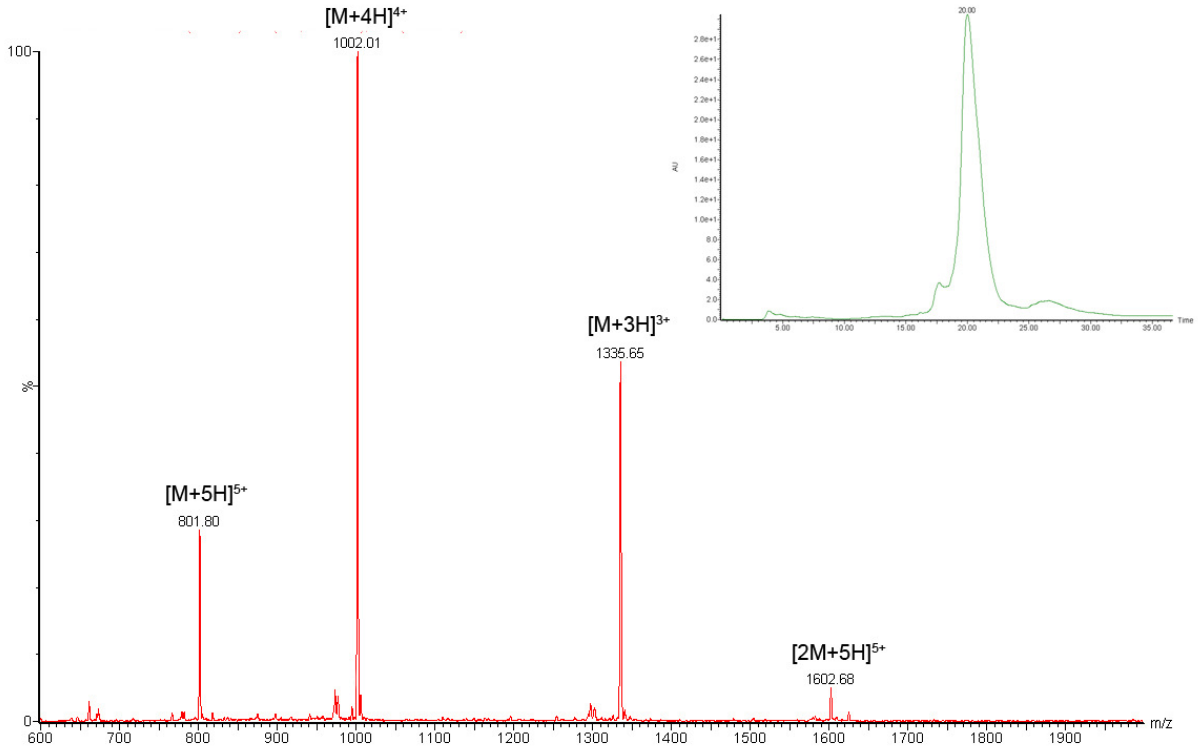
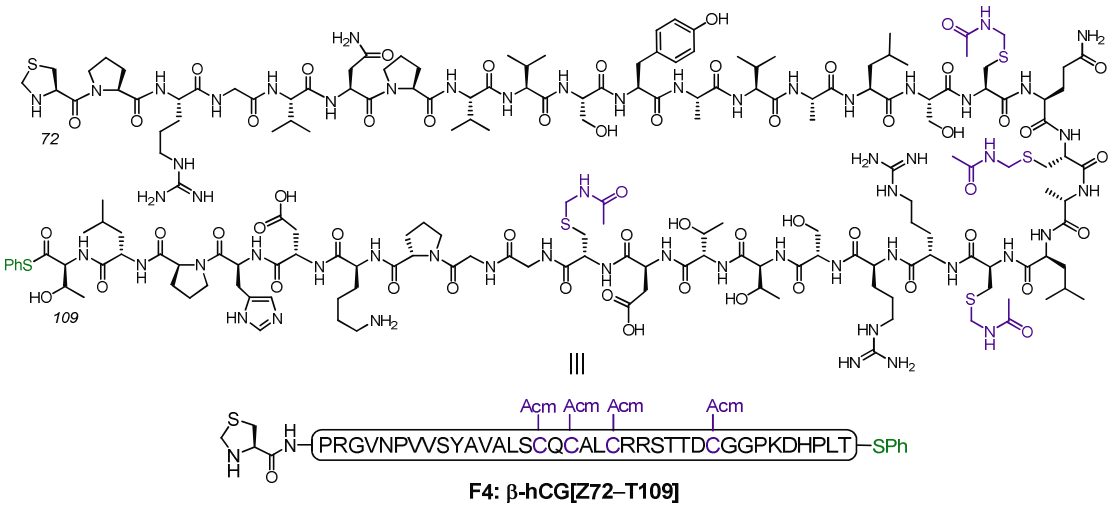


Figure S10. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hCG[Z38–G71]**. Calcd. for $C_{179}H_{288}N_{50}O_{46}S_4$, 4004.77 (average isotopes) $[2M+5H]^{5+}$ *m/z* 1602.91, found 1602.68; $[M+3H]^{3+}$ *m/z* 1335.92, found 1335.65; $[M+4H]^{4+}$ *m/z* 1002.19, found 1002.01; $[M+5H]^{5+}$ *m/z* 801.95, found 801.80. Varian Microsorb 300-5 C8 column, gradient: 25–60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Fragment 4 peptide thioester: β-hCG[Z72–T109]



Crude protected peptide BocN-Thz^{72}–Leu^{108}-OH, (prepared according to general experimental procedure A but using a solution of Oxyma Pure (0.1 M) in 20% piperidine/DMF as a deblock mixture) (126 mg, 20 μmol) was dissolved in chloroform (2.0 mL) and reacted with 3 equiv of **HCl·H-Thr-SPh**[2] (15 mg, 60 μmol) in the presence of EDC (11 μL, 60 μmol) and HOOBt (9.8 mg, 60 μmol), as described in general procedure B. After stirring for 3 h, the reaction mixture was concentrated and subjected to cocktail B (5 mL) following general procedure C. The crude peptide thioester was purified by HPLC on a X-Bridge C18 column using a linear gradient of 25–35% acetonitrile in water over 30 min. The fractions containing the desired product, which eluted at 15.5 min, were collected and lyophilized to give peptide thioester **β-hCG[Z72–T109]** (40 mg, 45% yield).

(2) Aussedat, B.; Fasching, B.; Johnston, E.; Sane, N.; Nagorny, P.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2012**, *134*, 3532−3541.

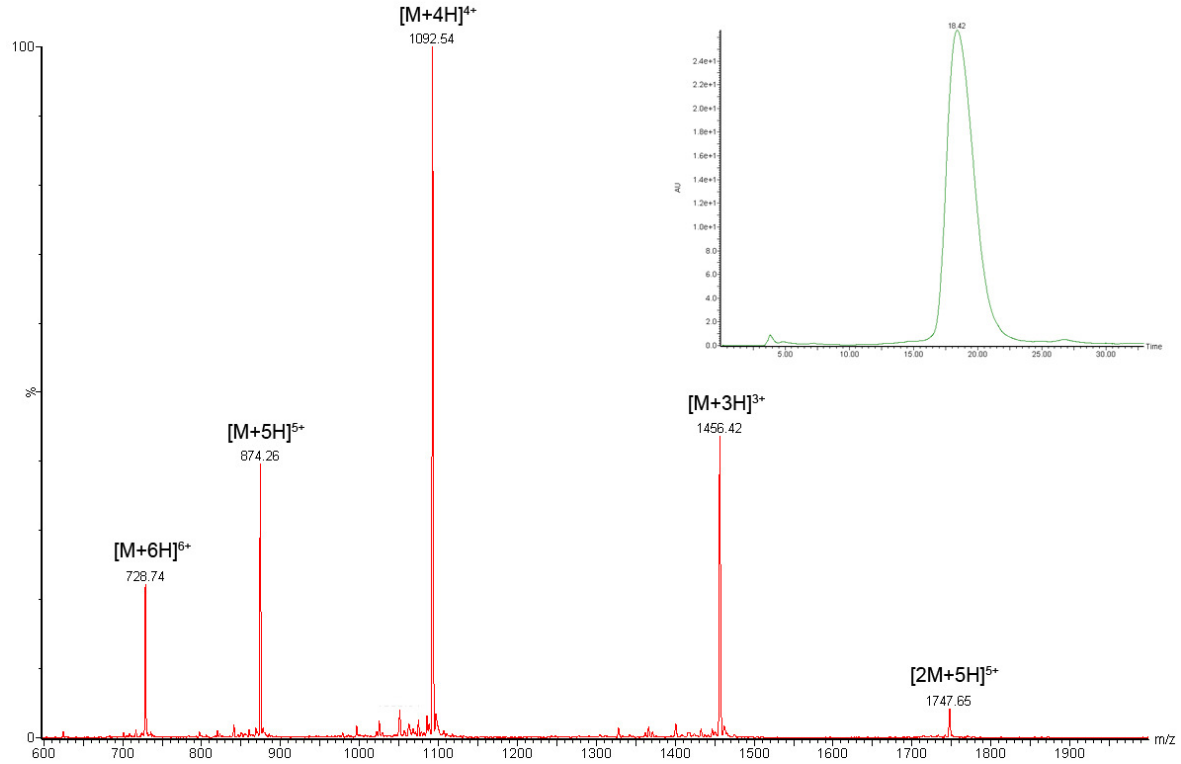
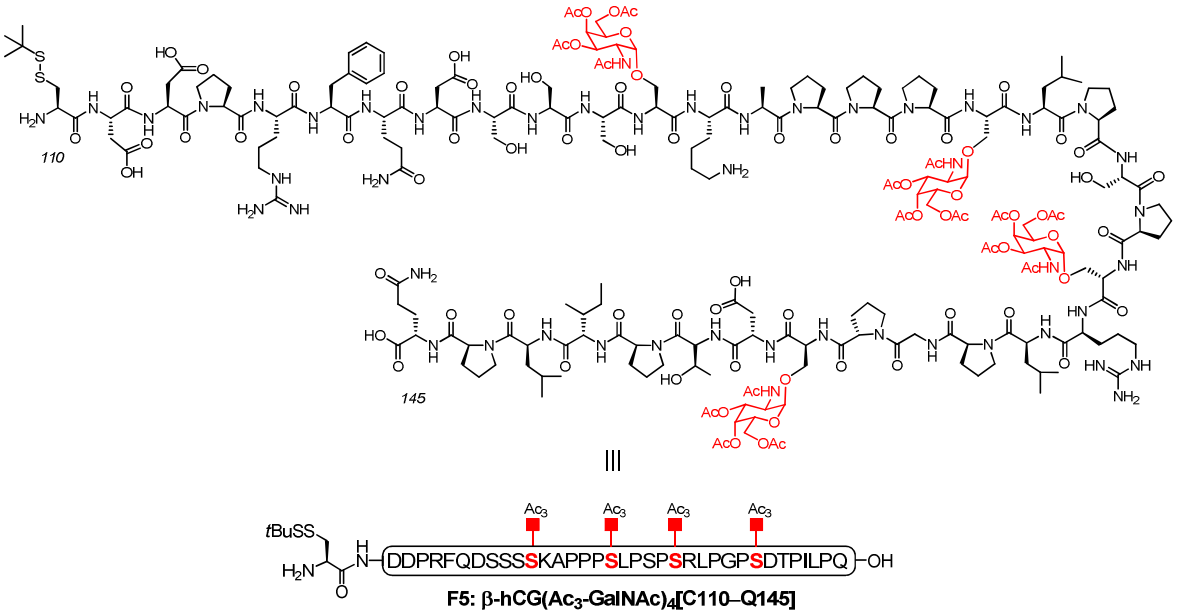


Figure S11. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hCG[Z72–T109]**. Calcd. for $C_{184}H_{296}N_{56}O_{55}S_6$, 4365.05 (average isotopes) $[2M+5H]^{5+}$ *m/z* 1747.02, found 1747.65; $[M+3H]^{3+}$ *m/z* 1456.02, found 1456.42; $[M+4H]^{4+}$ *m/z* 1092.26, found 1092.54; $[M+5H]^{5+}$ *m/z* 874.01, found 874.26; $[M+6H]^{6+}$ *m/z* 728.51, found 728.74. Varian Microsorb 300-5 C8 column, gradient: 25–45% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Fragment 5 glycopeptide: β-hCG(Ac$_3$-GalNAc)$_4$[C110–Q145]



Crude protected peptide β-hCG(Ac$_3$-GalNAc)$_4$[C110–Q145] was prepared by SPPS according to general procedure A with manual coupling of the glycosylated serine residues as follows.

Fmoc-Ser(Ac$_3$-α-GalNAc)-OH[3] (100 mg, 0.15 mmol), PyAOP (78.4 mg, 0.15 mmol), and HOAt (27.2 mg, 0.2 mmol) were dissolved in DMF (2.5 mL), and DIEA (36 μL, 0.22 mmol) was then added. After 5 min, the bright yellow solution was added to the resin (0.1 mmol) and the mixture was shaken in the reaction vessel by mild nitrogen bubbling for 12 h. The resin was then washed with DMF (5 × 3 mL), DCM (5 × 3 mL), and DMF (5 × 3 mL) and transferred to the peptide column, and the synthesis continued on the automated synthesizer. The progress of the synthesis after manual coupling of each glycosylamino acid was monitored by subjecting a small fraction of the resin to cleavage cocktail (acetic acid/trifluoroethanol/methylene chloride, 1:1:6) and analyzing the resulting peptide fragment by UPLC.

Upon completion of the automated synthesis, crude protected glycopeptide β-hCG(Ac$_3$-GalNAc)$_4$[C110–Q145] was cleaved from the resin as described by treatment with a mixture of acetic acid/trifluoroethanol/methylene chloride (1:1:6), 3 times × 45 min, and subjected to

(3) Kuduk, S. D.; Schwarz, J. B.; Chen, X.-T.; Glunz, P. W.; Sames, D.; Ragupathi, G.; Livingston, P. O.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 12474–12485.

cocktail B (10 mL) for 2 h following general procedure C. Half of the crude material was pre-purified by size-exclusion chromatography (Bio-Gel P-4 fine, 20% acetonitrile/water with 0.05% TFA), and the fractions were lyophilized. The resulting peptide was purified to homogeneity by HPLC (X-Bridge C18 column, 25–35% acetonitrile in water, over 30 min, product eluted at 15 min). The collected fractions were lyophilized to give glycopeptide **β-hCG(Ac$_3$-GalNAc)$_4$[C110–Q145]** (56 mg, 22% yield).

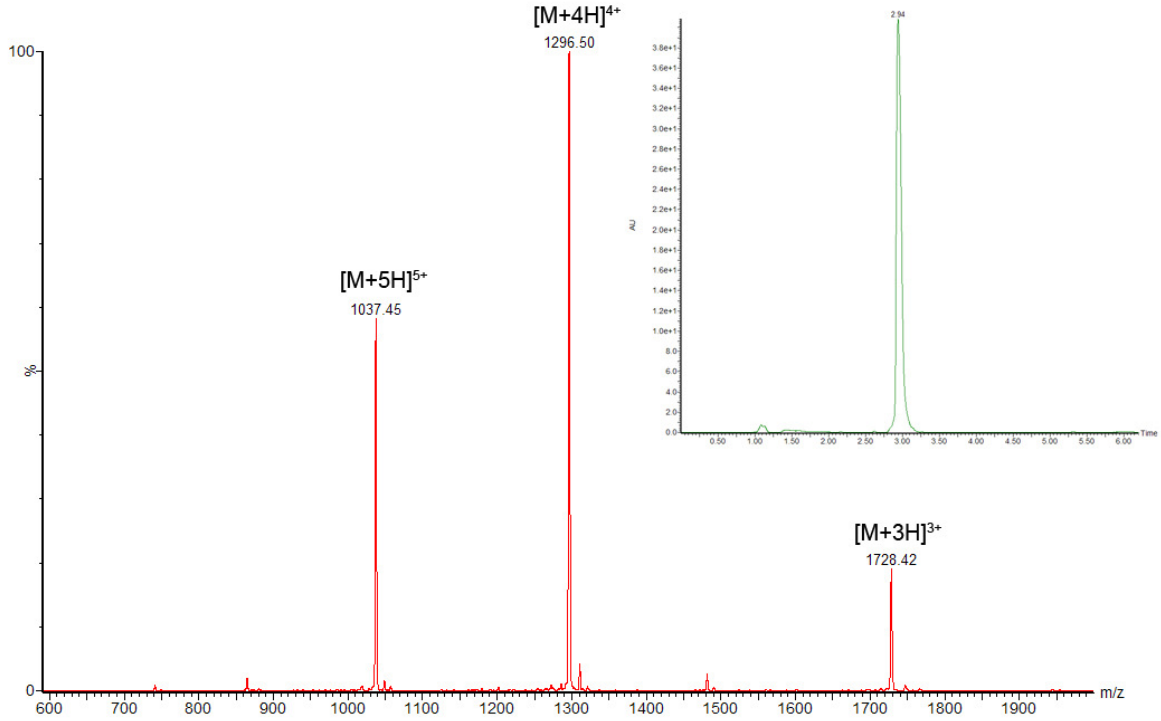
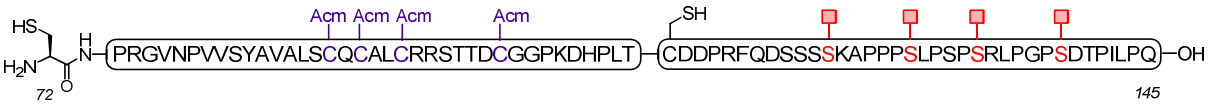


Figure S12. ESI-MS and UV traces from UPLC analysis for glycopeptide **β-hCG(Ac$_3$-GalNAc)$_4$[C110–Q145]**. Calcd. for $C_{223}H_{341}N_{49}O_{88}S_2$, 5180.50 (average isotopes) $[M+3H]^{3+}$ *m/z* 1727.83, found 1728.42; $[M+4H]^{4+}$ *m/z* 1296.12, found 1296.50; $[M+5H]^{5+}$ *m/z* 1037.10, found 1037.45. BEH C4 column, gradient: 30–70% acetonitrile/water over 6 min at a flow rate of 0.3 mL/min.

B. Assembly of β-hCG Peptide Fragments

Fragment [4.5] peptide: β-hCG(GalNAc)$_4$[C72–Q145]



F4.5: β-hCG(GalNAc)$_4$[C72–Q145]

Peptides **β-hCG[Z72–T109]** (4.4 mg, 1.0 μmol) and **β-hCG(Ac$_3$-GalNAc)$_4$[C110–Q145]** (5.2 mg, 1.0 μmol) were dissolved in NCL buffer (250 μL), prepared as described in general procedure D, and neutral TCEP solution (0.5 M, 25 μL) was added. The reaction mixture was stirred under Ar atmosphere for 3 h, and after completion of the ligation as assessed by UPLC, an aqueous solution of MeONH$_2$·HCl (250 μL, 0.6 M) was added followed by TCEP solution (25 μL, 0.5 M). The pH was adjusted to ~4.0–4.5 by addition of 2 M HCl solution and the turbid mixture was stirred for 18 h. After this time, the contents were diluted with 20% acetonitrile/water (0.05% TFA) (2.5 mL), and the crude peptide was isolated by centrifugal filtration in a 3000 MW cut-off filter, following general procedure E. Upon lyophilization, the resulting glycopeptide was treated with 5% aqueous hydrazine (1.6 mL, using hydrazine solution 35% wt in H$_2$O), and the solution stirred for 1 h. The reaction was quenched by addition of 1.6 mL of acetic acid and the mixture subjected to size exclusion chromatography (Bio-Gel P-6 fine, 20% acetonitrile/water with 0.05% TFA) to give glycopeptide **β-hCG(GalNAc)$_4$[C72–Q145]** (4.4 mg, 50% yield) as a white solid.

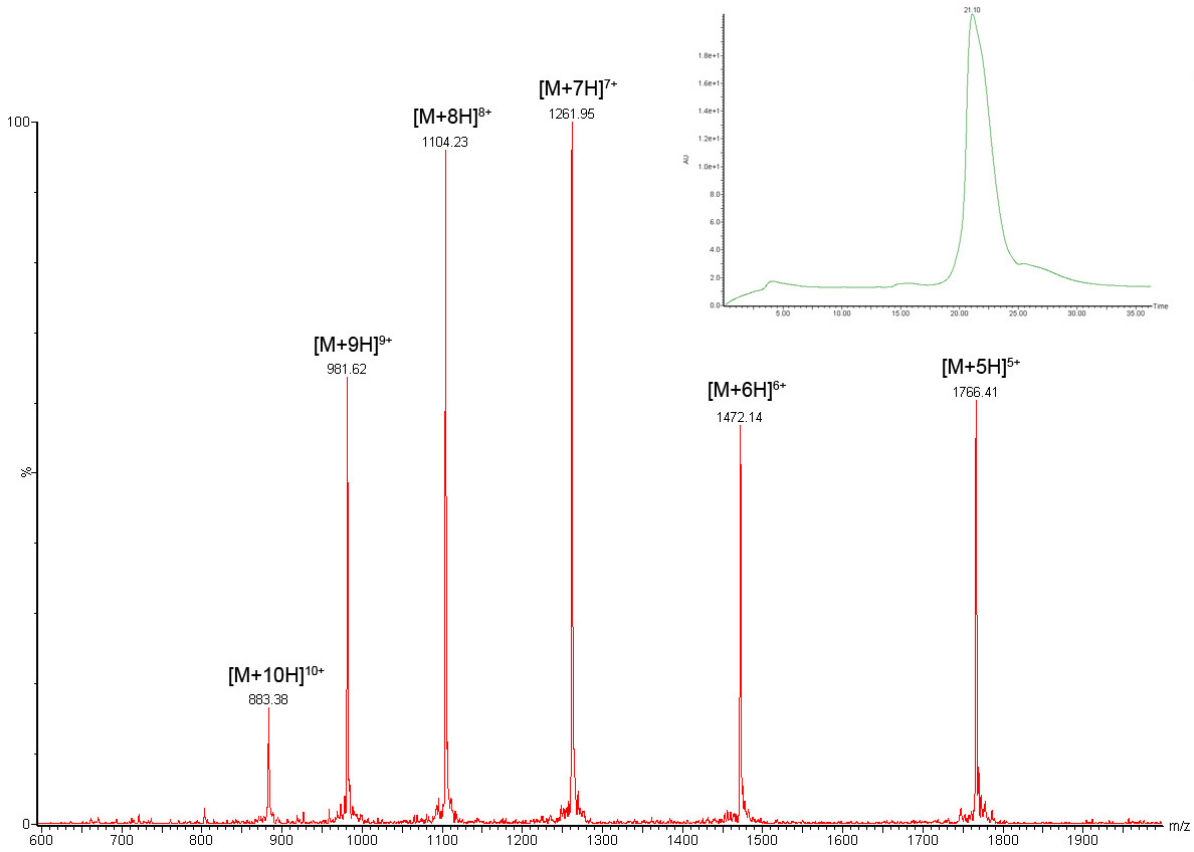
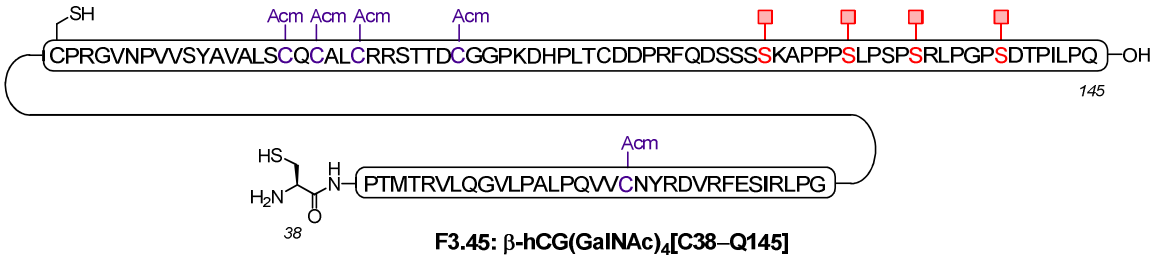


Figure S13. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hCG(GalNAc)$_4$[C72–Q145]**. Calcd. for $C_{372}H_{599}N_{105}O_{131}S_6$, 8830.75 (average isotopes) $[M+5H]^{5+}$ *m/z* 1767.15, found 1766.41; $[M+6H]^{6+}$ *m/z* 1472.79, found 1472.14; $[M+7H]^{7+}$ *m/z* 1262.53, found 1261.95; $[M+8H]^{8+}$ *m/z* 1104.84, found 1104.23; $[M+9H]^{9+}$ *m/z* 982.19, found 981.62; $[M+10H]^{10+}$ *m/z* 884.08, found 883.38. Varian Microsorb 300-5 C4 column, gradient: 20–40% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Fragment [3.45] peptide: β-hCG(GalNAc)$_4$[C38–Q145]



F3.45: β-hCG(GalNAc)$_4$[C38–Q145]

Peptides **β-hCG[Z38–G71]** (1.5 mg, 0.37 μmol) and **β-hCG(GalNAc)$_4$[C72–Q145]** (3.25 mg, 0.37 μmol) were dissolved in NCL buffer (92 μL), prepared as described in general procedure D, and neutral TCEP solution (0.5 M, 9.2 μL) was added. The reaction mixture was stirred under Ar atmosphere for 3 h, and after completion of the ligation as assessed by UPLC, an aqueous solution of $MeONH_2$·HCl (92 μL, 0.6 M) was added followed by TCEP solution (9.2 μL, 0.5 M). The pH was adjusted to ~4.4 by addition of 2 M HCl solution, and the mixture was stirred for 18 h. After this time, the contents were diluted with 20% acetonitrile/water (0.05% TFA) (1.0 mL), and the desired glycopeptide **β-hCG(GalNAc)$_4$[C38–Q145]** was isolated by centrifugal filtration (10000 MW cut-off filter) as a white solid following lyophilization (4.0 mg, 85% yield).

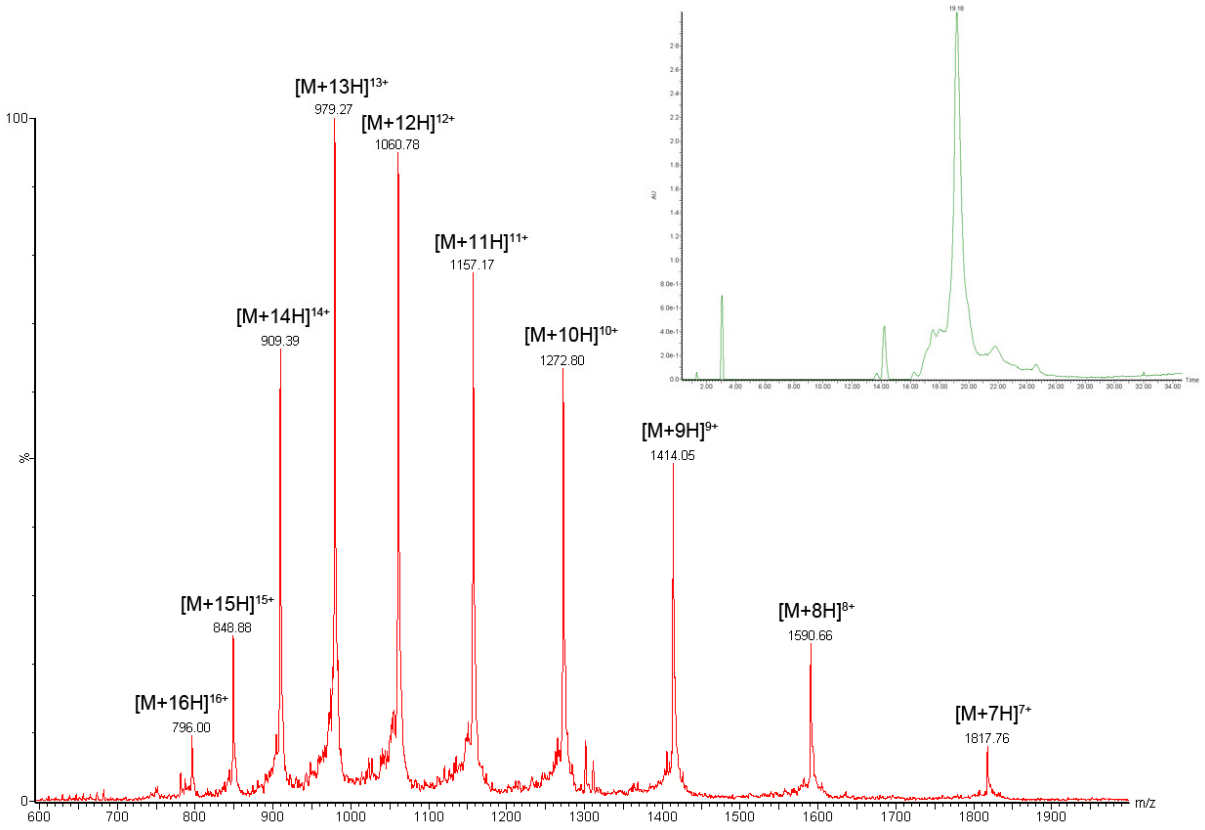
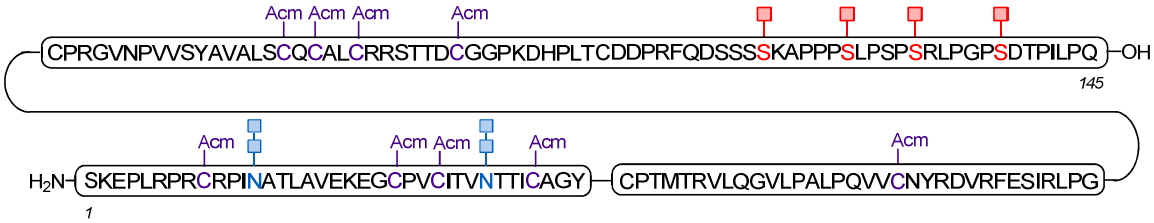


Figure S14. ESI-MS and UV traces from analytical HPLC analysis for glycopeptide **β-hCG(GalNAc)$_4$[C38–Q145]**. Calcd. for $C_{544}H_{881}N_{155}O_{177}S_9$, 12713.33 (average isotopes) $[M+7H]^{7+}$ *m/z* 1817.19, found 1817.76; $[M+8H]^{8+}$ *m/z* 1590.17, found 1590.66; $[M+9H]^{9+}$ *m/z*

1413.59, found 1414.05; $[M+10H]^{10+}$ *m/z* 1272.33, found 1272.80; $[M+11H]^{11+}$ *m/z* 1156.76, found 1157.17; $[M+12H]^{12+}$ *m/z* 1060.44, found 1060.78; $[M+13H]^{13+}$ *m/z* 978.95, found 979.27; $[M+14H]^{14+}$ *m/z* 909.10, found 909.39; $[M+15H]^{15+}$ *m/z* 848.56, found 848.88; $[M+16H]^{16+}$ *m/z* 795.58, found 796.00. Waters X-Bridge C18 column, gradient: 20–50% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.

Full Sequence β-hCG(chitobiose)$_2$(GalNAc)$_4$[S1–Q145] Glycoprotein Hormone



β-hCG(chitobiose)$_2$(GalNAc)$_4$[S1–Q145]

Peptides **β-hCG(chitobiose)$_2$[S1–Y37]** (1.3 mg, 0.25 μmol) and **β-hCG(GalNAc)$_4$-[C38–Q145]** (3.2 mg, 0.25 μmol) were dissolved in NCL buffer (85 μL), prepared as described in general procedure D, and neutral TCEP solution (0.5 M, 8.5 μL) was added. The mixture was stirred under Ar atmosphere for 4 h, and after completion of the ligation as assessed by UPLC, the reaction was quenched by addition of 50% acetonitrile/water (0.05% TFA) (2 mL), followed by TCEP solution (0.5 M, 15 μL). Upon lyophilization, the crude peptide was redissolved in 25% acetonitrile/water (2 mL), TCEP (0.5 M, 15 μL) added, and then purified by HPLC (X-Bridge C18 column, 20– 50% acetonitrile in water, over 30 min, product eluted at 15.5 min). The fractions containing the desired product were collected and lyophilized to afford full sequence **β-hCG(chitobiose)$_2$(GalNAc)$_4$[S1–Q145]** glycoprotein hormone (1.6 mg, 36%) as a white solid.

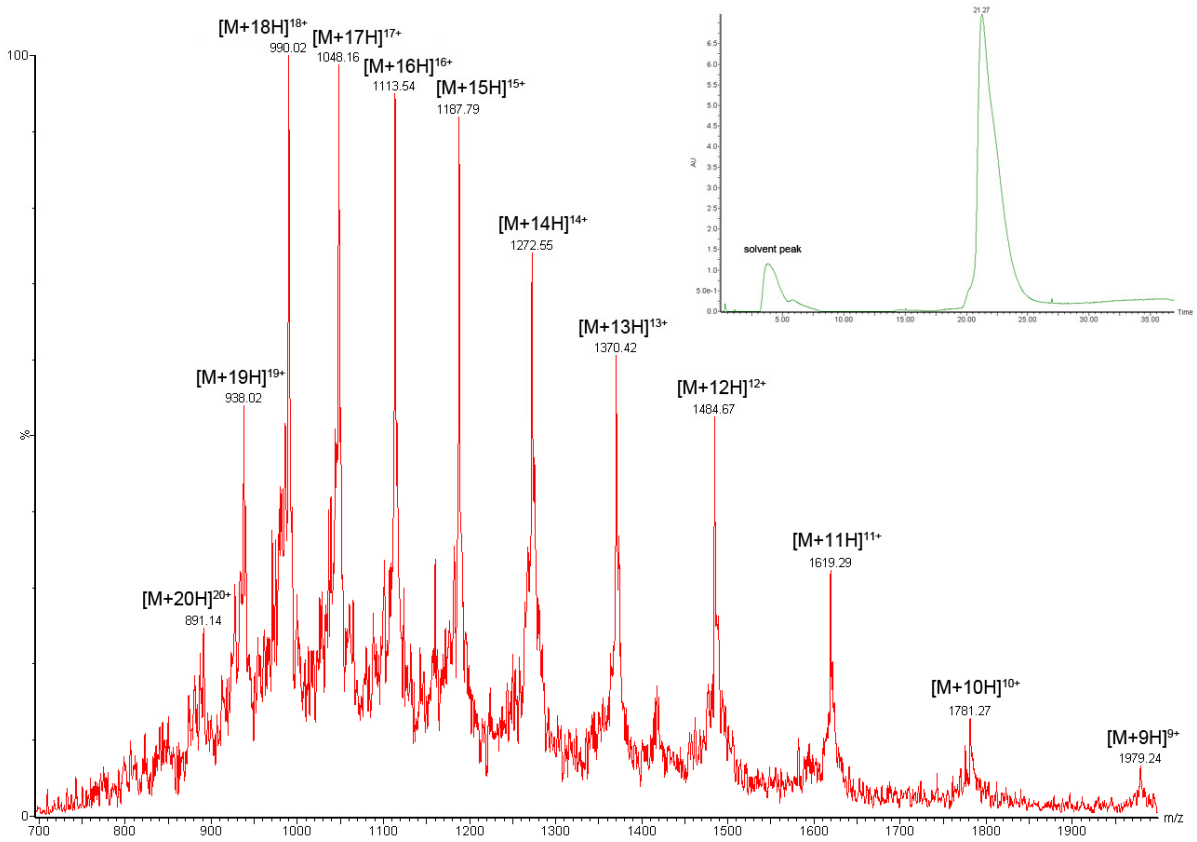


Figure S15. ESI-MS and UV traces from analytical HPLC analysis for glycoprotein **β-hCG(chitobiose)$_2$(GalNAc)$_4$[S1–Q145]**. Calcd. for $C_{759}H_{1239}N_{213}O_{252}S_{13}$, 17797.08 (average isotopes) $[M+9H]^{9+}$ *m/z* 1978.45, found 1979.24; $[M+10H]^{10+}$ *m/z* 1780.71, found 1781.27; $[M+11H]^{11+}$ *m/z* 1618.92, found 1619.29; $[M+12H]^{12+}$ *m/z* 1484.09, found 1484.67; $[M+13H]^{13+}$ *m/z* 1370.01, found 1370.42; $[M+14H]^{14+}$ *m/z* 1272.22, found 1272.55; $[M+15H]^{15+}$ *m/z* 1187.47, found 1187.79; $[M+16H]^{16+}$ *m/z* 1113.32, found 1113.54; $[M+17H]^{17+}$ *m/z* 1047.89, found 1048.16; $[M+18H]^{18+}$ *m/z* 989.73, found 990.02; $[M+19H]^{19+}$ *m/z* 937.69, found 938.02; $[M+20H]^{20+}$ *m/z* 890.85, found 891.14. Varian Microsorb 300-5 C4 column, gradient: 20–60% acetonitrile/water over 30 min at a flow rate of 0.2 mL/min.