Supplementary Information

General Information

All reactions were carried out under an atmosphere of argon in flame-dried or oven-dried glassware with magnetic stirring, unless otherwise noted. Air-sensitive reagents and solutions were introduced into the apparatus through rubber septa. When necessary, solvents and reagents were dried prior to use. Reagents were purified following the guidelines of Perrin and Armarego. Tetrahydrofuran (THF), dichloromethane (DCM) and diethyl ether (Et_2O) were filtered through a column of activated alumina under an argon atmosphere. Pyridine, *N, N*-diisopropylethylamine and triethylamine were distilled from calcium hydride. 1, 8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and piperidine were purchased from Aldrich and used without further purification. 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) was purchased from GenScript and used without purification.

Analytical Equipment

^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Bruker Advance DRX-600 MHz at ambient temperature. Chemical shifts are reported in parts per million relative to residual solvent CD_3OD (^{1}H, 3.31 ppm; ^{13}C, 49.15 ppm). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet, bs = broad singlet, dd = doublet of doublets, td = triplet of doublets. All ^{13}C NMR spectra were recorded with complete proton decoupling. 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 flash column chromatography was performed on E. Merck silica gel 60 (40–63 mm). Yields refer to chromatographically pure compounds.

Low resolution mass spectra (electronspray ionization) were acquired on a ZQ Micromass spectrometer. Samples were introduced by direct injection. LC-MS analysis were performed with a Waters Alliance analytical LC system in tandem with the ZQ Micromass measurement.

HPLC

All separations involved a mobile phase of 0.05% TFA (v/v) in Water (solvent A) /0.04% TFA in acetonitrile (solvent B). HPLC LC-MS chromatographic separations were performed using a Waters 2695 Separations Module and a Waters 2996 Photodiode Array Detector equipped with Varian Microsorb C18 column (150 x 2 mm) or Waters C8 X-Bridge column (150 x 2.1 mm) or Varian 300-5 C4 column (250 x 2 mm) at a flow rate of 0.2 mL/min. UPLC LC-MS chromatographic separations were performed using a Waters Acquity system equipped with an Acquity UPLC BEH C18 column (100 x 2.1 mm). HPLC separations were performed using a Rainin HPXL solvent delivery system equipped with a Rainin UV-1 detector using either Microsorb 100-5 C18 column (250 x 21.4 mm) at a flow rate of 16 mL/min or Waters C8 X-Bridge column (150 x 19 mm) at a flow rate of 16 mL/min or Varian 300-5 C4 column (250 x 4.6 mm) at a flow rate of 1.4 mL/min.

General procedure for Solid-phase peptide synthesis (SPPS) by Fmoc-strategy

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous S3 flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc protocols. The deblock mixture was a mixture of DMF/piperidine/DBU 98:2:2. The following Fmoc amino acids from NovaBiochem were employed: Fmoc-Ala-OH, Fmoc- Arg(Pbf)-OH, Fmoc- Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(*t*Buthio)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OAll)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Thz-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH. The following pseudoproline dipeptides from NovaBiochem were used: Fmoc-Tyr(*t*Bu)-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Glu(O*t*Bu)-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Lys(Boc)-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-Leu-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Ile-Thr($\psi^{Me,Me}$pro)-OH and Fmoc-Asp(O*t*Bu)-Ser($\psi^{Me,Me}$pro)-OH. Upon completion of automated synthesis on a 0.1 mmol scale, the peptide resin was washed into a peptide synthesis vessel with MeOH. The resin was subjected to a cleavage cocktail (1/1/8 of acetic acid/trifluoroethanol/methylene chloride, 10 ml) for 30 min and filtered. The process was repeated three times and the combined washings were evaporated *in vacuo*. The oily residue so obtained, was precipitated with cold ether and centrifuged. The precipitate was suspended in a mixture of acetonitrile and water, frozen in liquid nitrogen and subsequently lyophilized.

General procedure for removal of acid labile protecting groups with Cocktail B

Peptide were subjected to cocktail B consisting of trifluoroacetic acid (88% by volume), water (5% by volume), phenol (5% by weight), and *i*Pr_3SiH (2% by volume). The resulting solution was concentrated and the oily residue was triturated in ice cooled diethyl ether (3 x 45 ml) to give a white suspension, which was centrifuged. The precipitate was solubilized in water/acetonitrile (1:1) and lyophilized. The resulting material was subjected to HPLC purification.

Native Chemical Ligation buffer

The buffer required for native chemical ligation (NCL) was freshly prepared prior to the reaction. Guanidinium hydrochloride (5.73 g, 63.3 mmol) was solubilized in water (5 mL), followed by the addition of $Na_2HPO_4 \cdot 7H_2O$ (536 mg, 2.0 mmol) and TCEP·HCl (54.0 mg, 0.19 mmol). After complete solubilization, the pH was adjusted to 6.9 with solid Na_3PO_4. Thiophenol (0.3 ml, 3 mmol) was added, thoroughly mixed and the final volume was made up to 10 ml. The pH was then adjusted to pH = 7.4 by the addition of small portions of solid Na_3PO_4. The resulting solution was degassed by bubbling argon through the solution for 30 min. The solution so obtained was used as a buffer for NCL reactions.

Thz group deprotection buffer

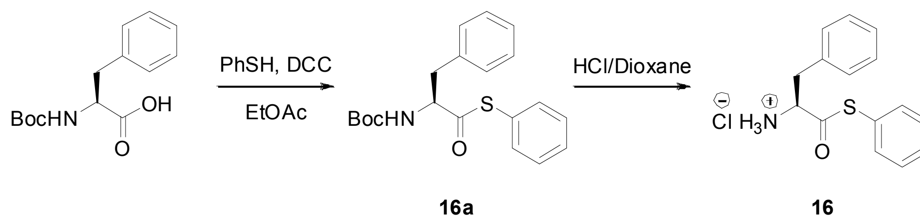
The buffer required for the deprotection of the Thz protecting group on terminal cysteines was freshly prepared prior to the reaction. $NH_2OMe \cdot HCl$ (501 mg, 6.0 mmol), Guanidinium hydrochloride (5.43g, 6.0 mmol), TCEP·HCl (50 mg, 0.18 mmol) were combined in a falcon tube and water (4.0 ml) was added and the contents were thoroughly solubilized. The final volume was made up to 10 ml and the solution was degassed by bubbling a stream of argon through the solution for 30 min.

Abbreviations

DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIEA	*N*,*N*-diisopropylethylamine
DMF	*N*, *N*-dimethylformamide
DMSO	Dimethylsulfoxide
HATU	2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate
HOOBt	3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-Mass spectroscopy
MeCN	Acetonitrile
NMP	*N*-methyl-2-pyrrolidone
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RT	Retention time
SPPS	Solid phase peptide synthesis
TCEP·HCl	*tris*(2-carboxyethyl)phosphine hydrochloride
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
THF	Tetrahydrofuran
UPLC	Ultra-performance liquid chromatography

For details regarding the synthesis and spectral data of compound **14** see P. N. Nagorny, B. Fasching, X. Li, G. Chen, B. Aussedat, S. J. Danishefsky, *J. Am. Chem. Soc.* **2009**, *131*, 5792–5799 [Reference 19 in main text].

H-Phe-SPh·HCl 16

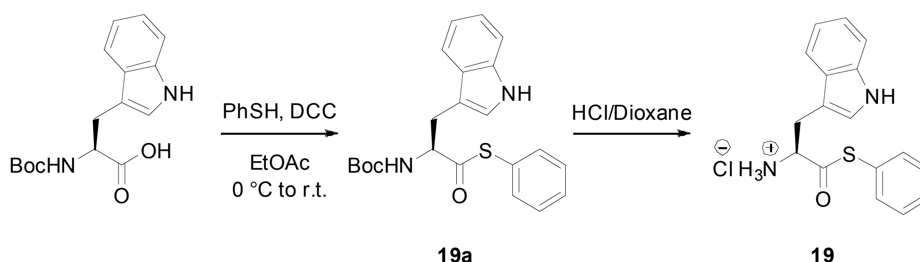


To a solution of Boc-Phe-OH (3.64 g, 13.7 mmol), thiophenol (1.68 ml, 98 mmol) in dry EtOAc (40 mL) at r.t. was added *N,N'*-Dicyclohexylcarbodiimide (DCC) (3.40 g, 16.4 mmol) and the resulting solution was stirred for 1 h. The reaction was checked for completion by TLC, then quenched with glacial acetic acid (165 µL), filtered through Celite and concentrated *in vacuo*. The crude material was purified by flash column chromatography (SiO_2, EtOAc/Hexanes 1:10) to provide 3.1 g (68% yield) of the pure product **16a**.

To **16a** (3.31 g, 9.26 mmol) was added a solution of HCl in dioxane (4 M, 10 ml) and the solution was stirred at r.t for 1 h. The mixture was concentrated *in vacuo* and the remaining solvent was azeotropically removed with dry toluene (2 x 20 mL) followed by DCM (2 x 20 mL) and dried under high vacuum to provide 2.74 g (99% yield) of the pure product **16**.

^{1}H NMR (600 MHz, CD_3OD): 7.46 (3H, m), 7.36 (7H, m), 4.86 (3H, bs), 4.61 (1H, t, J = 7.2 Hz), 3.33 (1H, dd, J = 7.2, 14.1 Hz), 3.26 (1H, dd, J = 7.2, 14.1 Hz). ^{13}C NMR (150 MHz, CD_3OD) 195.9, 135.9, 135.1, 131.6, 130.9, 130.9, 130.8, 130.4, 129.3, 126.7, 61.2, 38.9. Exact mass cald. for $C_{15}H_{16}NOSCl$ $[M\text{-}Cl^-]^+$ 258.0953, Found 258.0948.

H-Trp-SPh·HCl 19

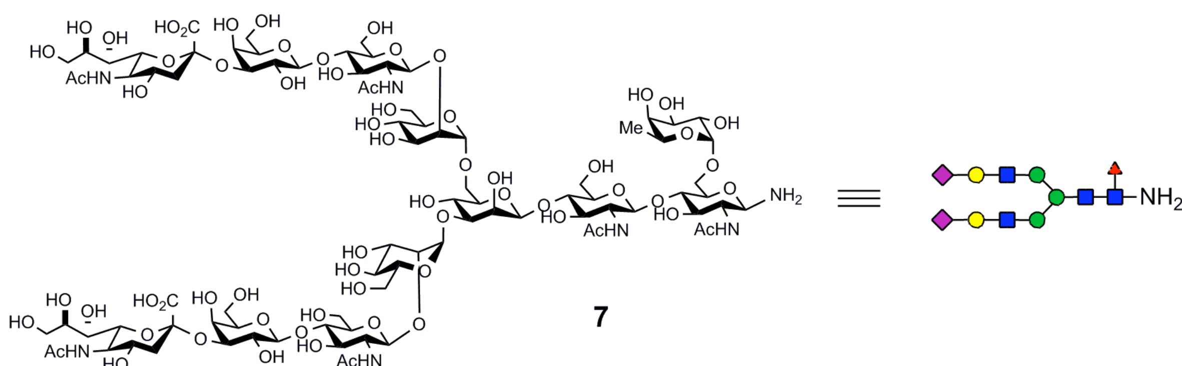


To a solution of Boc-Trp-OH (1.0 g, 3.29 mmol), thiophenol (403 μL, 3.95 mmol) in dry EtOAc (10 mL) at 0 °C was added *N,N′*-Dicyclohexylcarbodiimide (DCC) (814 mg, 3.95 mmol) and the solution was stirred at 0 °C for 30 min and at r.t for 1 h. The reaction was checked for completion by TLC and then quenched with AcOH (70 μL) filtered through Celite and concentrated *in vacuo*. The crude material was purified by flash column chromatography (SiO_2, EtOAc/Hexane 1:4 to 1:2) to provide 1.20 g (92% yield) of the pure product **19a**.

To **19a** (1.09 g, 2.75 mmol) was added a solution of HCl in dioxane (4 M, 10 ml) and the solution was stirred at r.t for 1 h. The mixture was concentrated *in vacuo* and the remaining solvent was azeotropically removed with dry toluene (2 x 20 mL) followed by DCM (2 x 20 mL) and dried under high vacuum to provide 870 mg (95% yield) of the pure product **19**.

^{1}H NMR (600 MHz, CD_3OD): 7.58 (1H, d, J = 7.9 Hz), 7.45 (1H, d, J = 7.3 Hz), 7.42 (3H, m), 7.27 (2H, d, J = 8.2 Hz), 7.26 (1H, s), 7.17 (1H, td, J = 0.9, 7.1 Hz), 7.08 (1H, td, J = 0.9, 7.1 Hz), 4.87 (4H, bs), 4.59 (1H, t, J = 7.1 Hz), 3.54 (1H, dd, J = 7.0, 14.8 Hz), 3.42 (1H, dd, J = 7.2, 14.8 Hz). ^{13}C NMR (150 MHz, CD_3OD) 196.5, 138.4, 135.9, 131.4, 130.7, 128.5, 126.8, 125.9, 123.1, 120.5, 119.2, 112.9, 107.5, 60.7, 29.2. $[\alpha]_D^{20}$ = +89.54 (c = 0.685 in TFE/DCM 1:1). Exact mass cald. for $C_{17}H_{17}N_2OSCl$ $[M-Cl^-]^+$ 297.1062, Found 297.1061.

Dodecasaccharide glycosylamine 7.

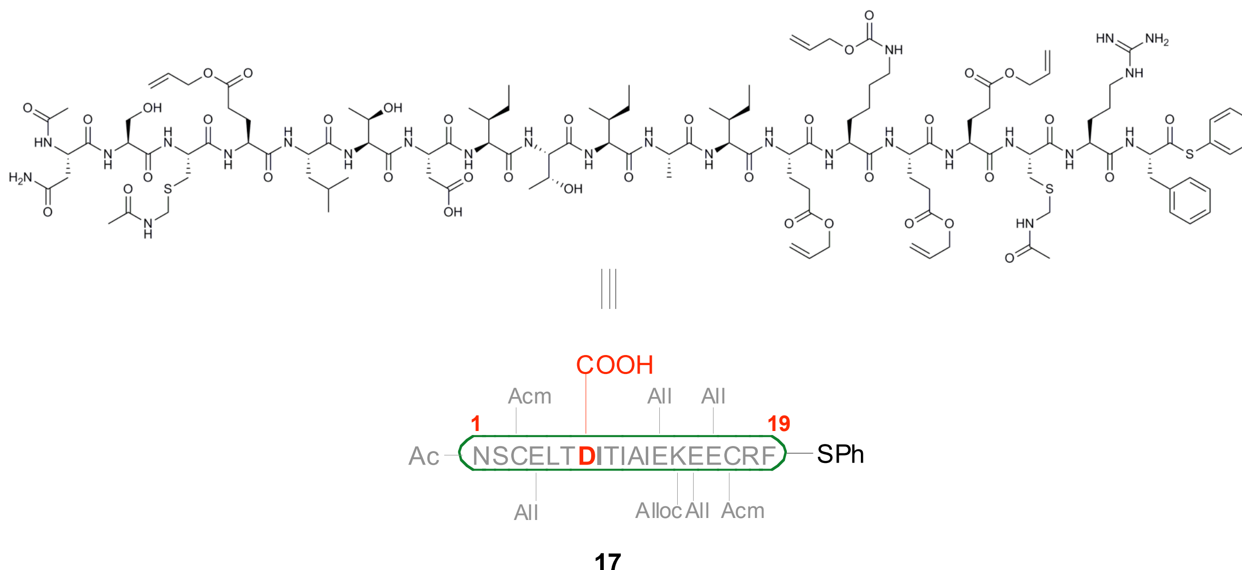


Dodecasaccharide **14** (11 mg, 4.23 μmol) was dissolved in water (5 mL) and $(NH_4)HCO_3$ (6g, BioUltra, 99.5% (T), Cat. No. 09830 Fluka) was slowly introduced in small portions while stirring. The resultant slurry was warmed to 40 °C and stirred for 3 days at this temperature. $(NH_4)HCO_3$ was periodically added to ensure a saturated content of ammonia in the solution. After three days, the stirring was turned off and the clear supernatant was transferred to a BD Falcon™ tube and frozen in liquid nitrogen. The remaining material was quickly dissolved in minimum amount of cold water, transferred to separate falcon tube and frozen in liquid nitrogen. The frozen material was lyophilized to remove the excess $(NH_4)HCO_3$ and water. The lyophilization was deemed complete until the mass of the product remained constant. This provided a white solid, the **dodecasaccharide glycosylamine 7** (11 mg, 99 % yield).

Exact mass calcd. for $C_{90}H_{149}N_7O_{65}$ $[M-2H]^{2-}$ 1182.9, Found 1182.8.

MALDI-TOF calcd. for $C_{90}H_{149}N_7O_{65}$ $[M+Na]^+$ 2391.85, Found 2391.44.

[βFSH$^{1-19}$] phenylthioester 17



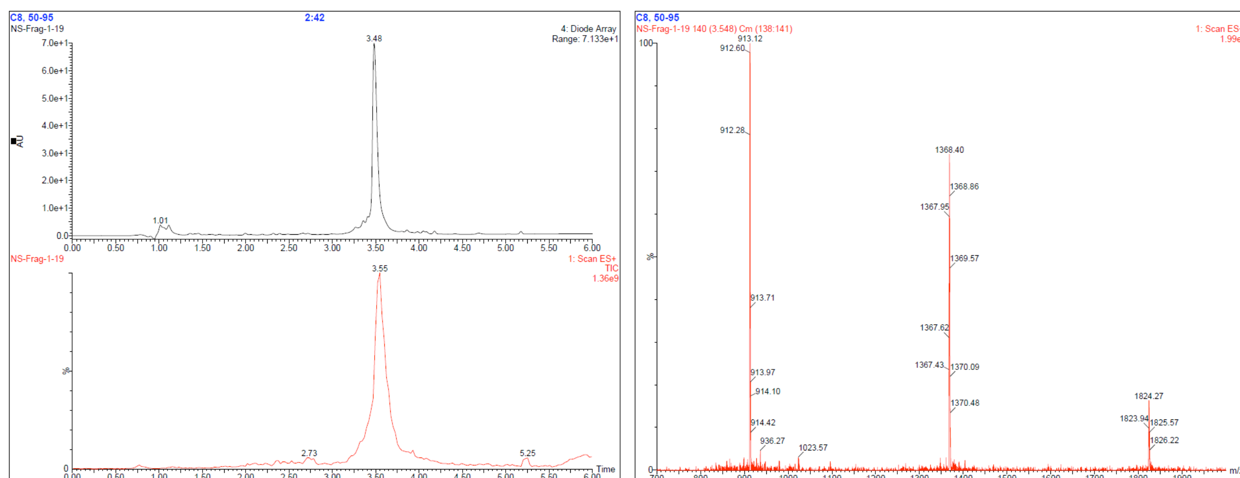
0.21 g (0.05 mmol) of Fmoc-Arg(Pbf)-Nova-TGT resin was subjected to continuous flow automated peptide synthesis following the general procedure for SPPS. The amino acids used in the sequence were as follows: Fmoc-Cys(Acm)-OH, Fmoc-Glu(OAllyl)-OH, Fmoc-Glu(OAllyl)-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Glu(OAllyl)-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Ile-OH, Fmoc-Ile-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Leu-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Glu(OAllyl)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asn(Trt)-OH. The resin from above was transferred to a manual peptide synthesis vessel, washed with DMF (10 mL), and treated with 2:2:1 DMF/Pyridine/Ac_2O (10 mL) for 45 min. The solvent was drained and the resin was washed with DMF and methanol, and treated with the cleavage solution (10 mL) consisting of 1:1:8 trifluoroethanol/acetic acid/dichloromethane for 1.5 h. The beads were filtered, rinsed with another 10 mL of cleavage solution, filtered again, and then treated for another 1 h with 10 mL of the cleavage solution. This process was repeated for a total of three times over 2 h cleavage cycles, and the combined filtrate was concentrated *in vacuo* to afford 80 mg of **[βFSH$^{1-18}$] peptide 15** after cleavage.

The **[βFSH$^{1-18}$] peptide 15** from above (103 mg, 0.0322 mmol) was combined with HCl·H-Trp-SPh **16** (14.2 mg, 0.0483 mmol), HOOBt (7.9 mg, 0.0483 mmol), and dissolved in 1:3

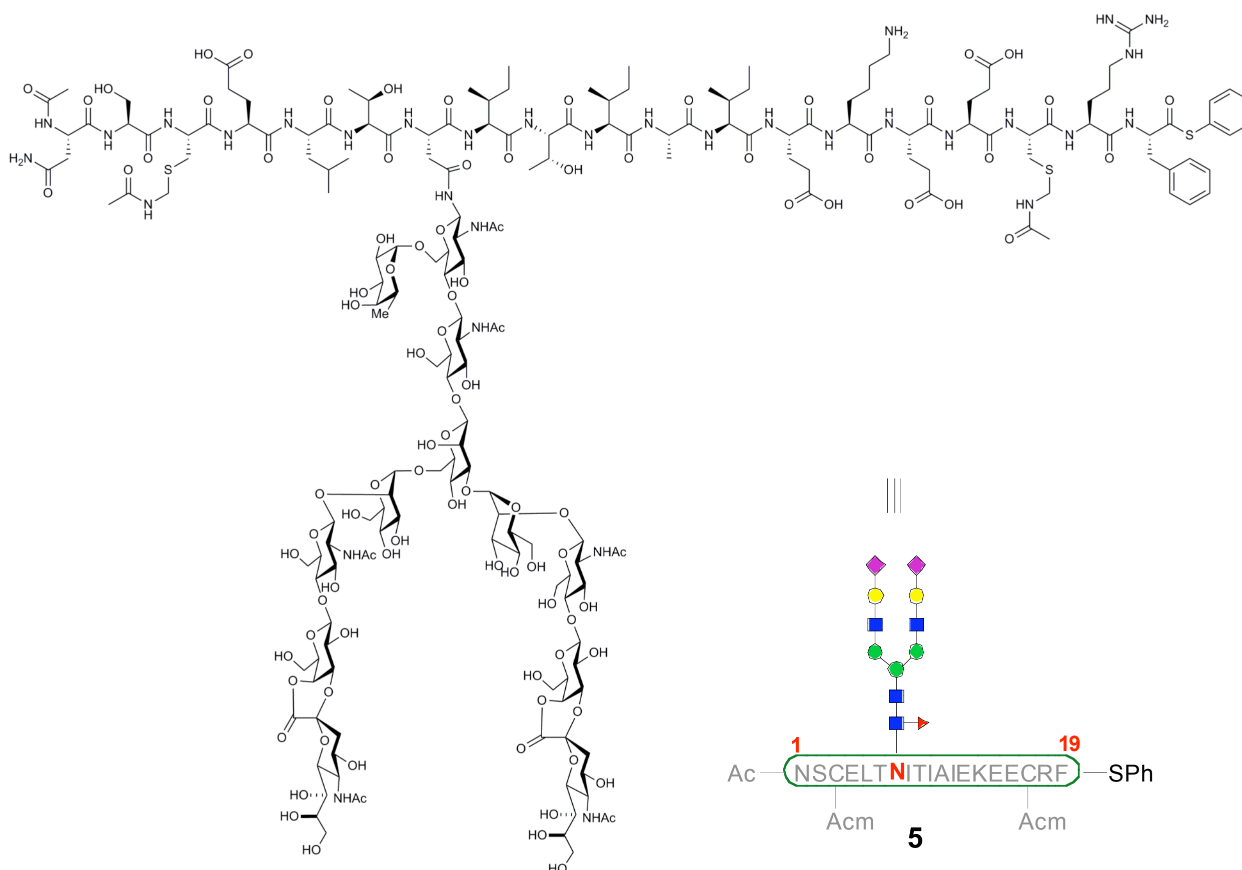
TFE/$CHCl_3$ (2.0 mL). To this solution, EDC (11 µL, 0.0483 mmol) was added and the resultant yellow solution was stirred for 2 h before being concentrated under reduced pressure.

The residue from above was treated with Cocktail B (3 mL) for 3.5 h, then diluted with dichloromethane, and concentrated. The residue was triturated with ether (2 x 15 mL), decanted, and dissolved in DMSO (3 ml) and acetonitrile/water (8:2, 10 mL) and purified by HPLC. (Microsorb C4 column, 30-90% MeCN in H_2O, 30 min, RT = 25.5 min) to afford 15.5 mg (21% yield, 2 steps) of pure **[βFSH$^{1-19}$] phenylthioester 17**.

Exact mass calcd for $C_{123}H_{188}N_{26}O_{38}S_3$ $[M+3H]^{3+}$ 912.1, Found 912.60; $[M+2H]^{2+}$ 1368.2, Found 1368.40.



[βFSH$^{1-19}$] glycopeptide phenylthioester 5



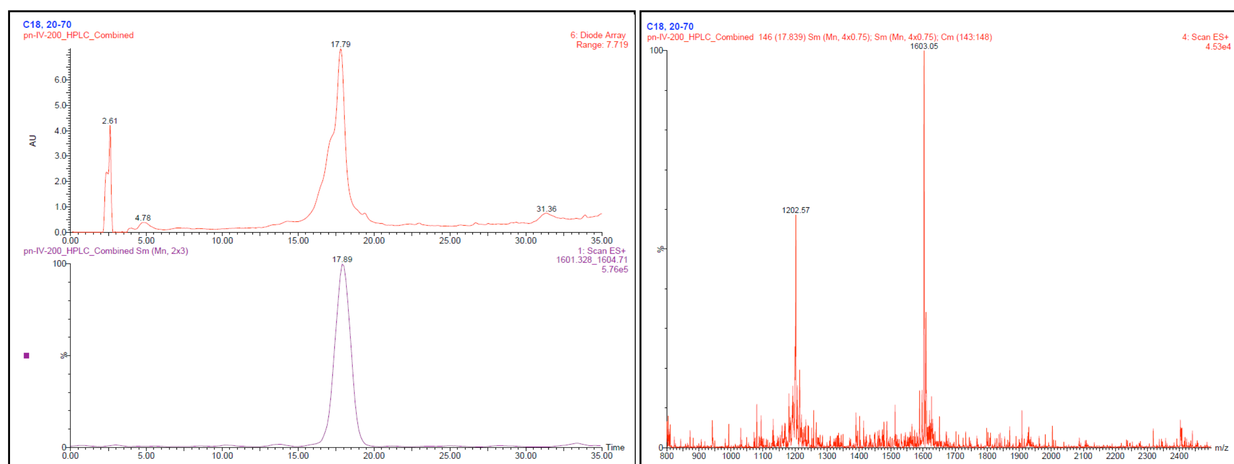
To a vial containing **[βFSH$^{1-19}$] phenylthioester 17** (2.8 mg, 1.02 µmol) and the dodecasaccharide **7** (2.02 mg, 0.85 µmol) under argon, was added a solution of HATU in DMSO (conc = 174 mg/ml, 18 µl) followed by a stock solution of DIEA in DMSO (conc. 40 µl/ml, 18 µl). The mixture was stirred for 1 h at r.t. at which time an aliquot of the reaction was monitored using LC-MS (Microsorb C4 column, 30-90% MeCN in H_2O, 30 min, RT = 19.8 min)

Exact mass calcd. for $C_{213}H_{331}N_{33}O_{100}S_3$: $[M+3H]^{3+}$ 1684.04, Found 1684.49.

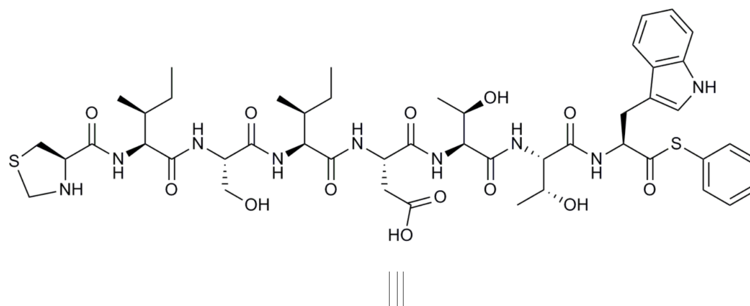
To a solution of $Pd(PPh_3)_4$ (0.5 mg) in NMP (1ml) was added phenylsilane (10 µl). The resulting solution (250 µl) was added to the previous reaction mixture and stirred for 1 h. The reaction was monitored by LC-MS. Additional 50 µl of the $Pd(PPh_3)_4$ solution (freshly prepared) was added and stirred for another 20 mins. The reaction mixture was then quenched with acetic acid (1 µl) and diluted with DMSO (2 ml). The resulting mixture was immediately purified by HPLC (Microsorb C18 column, 20-70% MeCN in H_2O, 30 min, RT = 16.7 min). The combined

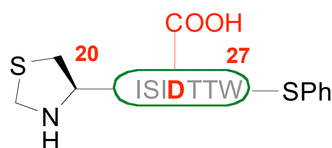
fractions of the pure product, upon lyophilization gave 1.0 mg (23% yield, 2 steps) of **[βFSH$^{1-19}$] glycopeptide phenylthioester 5**.

Exact mass calcd. for $C_{197}H_{311}N_{33}O_{98}S_3$: $[M+4H]^{4+}$ 1202.24, Found 1202.57; $[M+3H]^{3+}$ 1602.66, Found 1603.05.



[βFSH[20-27]] phenylthioester 6





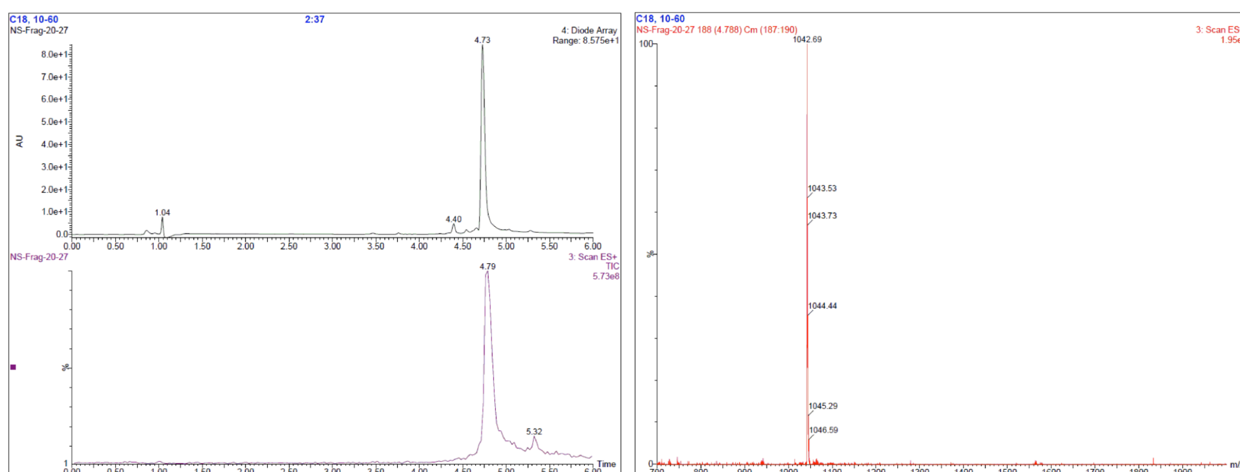
0.42 g (0.1 mmol) of Fmoc-Thr(*t*Bu)-Nova-TGT resin was subjected to continuous flow automated peptide synthesis following the general procedure for SPPS. The amino acids used were, in order of synthesis: Fmoc-Thr(*t*Bu)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-Ile-OH, Fmoc-Thz-OH. The resin was transferred to a manual peptide synthesis vessel, washed with methanol (10 mL), and treated with the cleavage solution (10 mL) consisting of 1:1:8 trifluoroethanol/acetic acid/dichloromethane for 1.5 h. The beads were filtered, rinsed with another 10 mL of cleavage solution, filtered again, and then treated for another 1 h with 10 mL of the cleavage solution. This process was repeated for a total of three 2-hour cleavage cycles, and the combined filtrate was concentrated in vacuo to afford 101 mg of peptide **[βFSH[20-26]]** after cleavage (99% yield). Exact mass calcd for $C_{51}H_{89}N_7O_{15}S$: $[M+H]^+$ 1072.6, Found 1072.8; $[M+Na]^+$ 1094.6, Found 1094.7; $[M+TFA]^-$ 1184.6, Found 1184.8; $[M-H]^-$ 1070.5, Found 1070.8.

Peptide **[βFSH[20-26]] peptide 18** (20 mg, 0.018 mmol) was combined with HCl·H-Phe-SPh **19** (11 mg, 0.0575 mmol), HOOBt (6 mg, 0.037 mmol), and dissolved in 1:3 TFE/$CHCl_3$ (1.0 mL). To this solution, EDC (6.5 µL, 0.037 mmol) was added and the resultant yellow solution was stirred for 2.5 h before and subsequently concentrated *in vacuo* to provide product (30 mg) of the crude product**.**

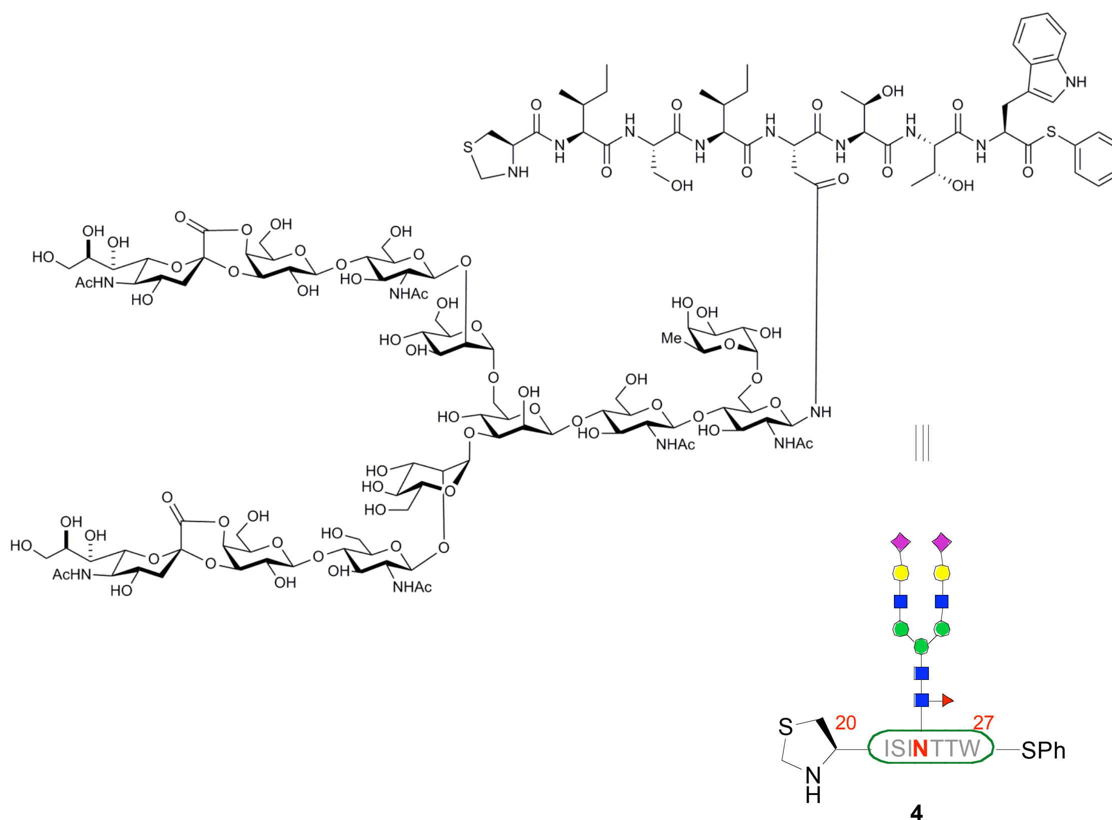
Exact mass calcd for $C_{68}H_{103}N_9O_{15}S_2$: [M+H$^+$] 1350.71, Found 1351.00

The peptide **[βFSH$^{20-27}$] phenylthioester** so obtained, was treated with cocktail B (2 mL) for 2 h, then diluted with dichloromethane, and concentrated. The residue was triturated with ice-cold ether (2 x 15 mL), centrifuged and decanted. The precipitate was re-dissolved in DMSO (3 ml) and acetonitrile/water (7:3, 10 mL), and purified by HPLC. (Microsorb C18 column, 30-60% MeCN in H_2O over 30 min, RT = 14.5 min) to afford 13 mg (66%, 3 steps) of pure **[βFSH$^{20-27}$] phenylthioester 6**.

Exact mass calcd for $C_{48}H_{68}N_9O_{13}S_2$: $[M+H]^+$ 1042.44, Found : 1042.69.

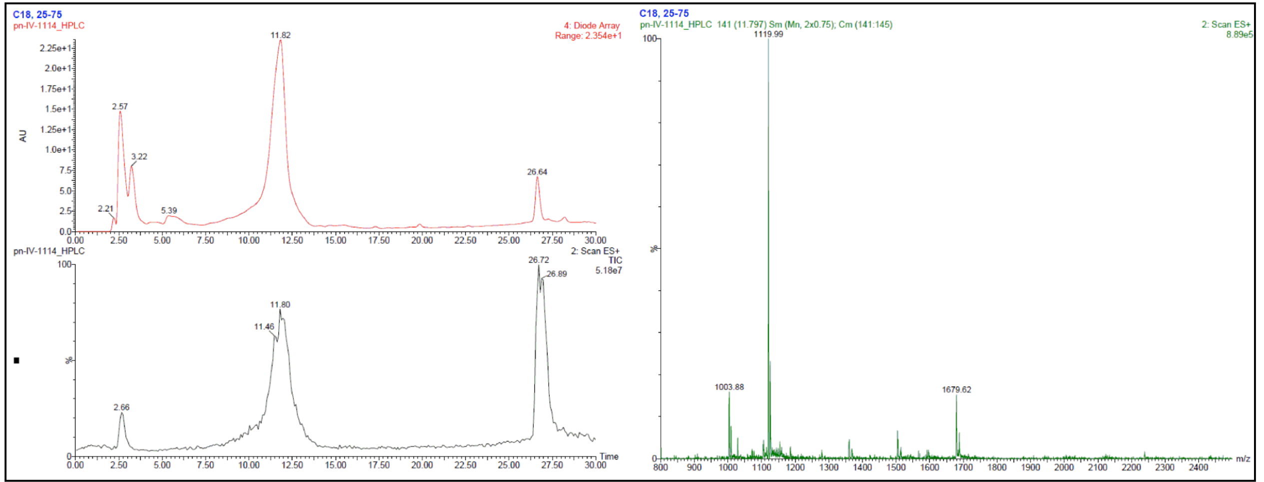


[βFSH$^{20\text{-}27}$] glycopeptide phenylthioester 4

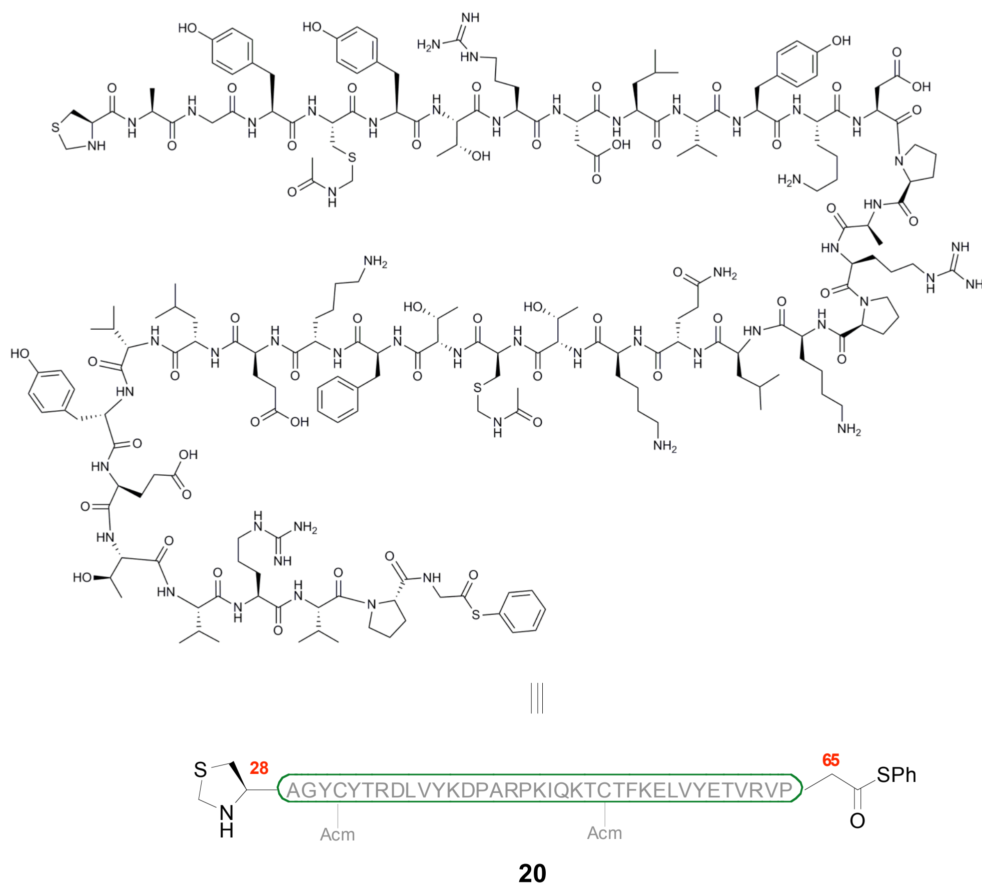


To a vial containing **[βFSH$^{20\text{-}27}$] phenylthioester 6** (660 μg, 0.63 μmol) and the dodecasaccharide **7** (1.0 mg, 0.42 μmol) was flushed with argon. To this was added a premixed solution of DIEA (0.32 mg, 2.52 μmol) in DMSO (20 μl) followed by a premixed solution of HATU (1.92 mg, 5 μmol). The mixture turned yellow and this solution was stirred at r.t. for 1 h. An aliquot of the reaction mixture was analyzed by LC-MS and indicated complete conversion to the desired product. After 2 h of stirring, the reaction mixture was quenched with acetic acid (2 μl) and diluted with DMSO (200 μl) and water (400 μl). This was immediately purified by HPLC (Microsorb C18 column, 20-70% MeCN in H_2O over 30 min, RT = 14.9 min) to provide 400 μg (28% yield) of the pure product **4**.

Exact mass calcd. for $C_{138}H_{210}N_{16}O_{75}S_2$: $[M+2H]^{2+}$ 1679.14, Found 1679.62; $[M+3H]^{3+}$ 1119.76, Found 1119.99.


C18, 25-75
pn-IV-1114_HPLC
4: Diode Array
Range: 2.354e+1
AU
11.82
2.57
3.22
2.21
5.39
26.64
pn-IV-1114_HPLC
2: Scan ES+
TIC
5.18e7
26.72
26.89
11.80
11.46
2.66
Time
C18, 25-75
pn-IV-1114_HPLC 141 (11.797) Sm (Mn, 2x0.75); Cm (141:145)
2: Scan ES+
8.89e5
1119.99
1003.88
1679.62
m/z

[βFSH$^{28\text{-}65}$] phenylthioester 20



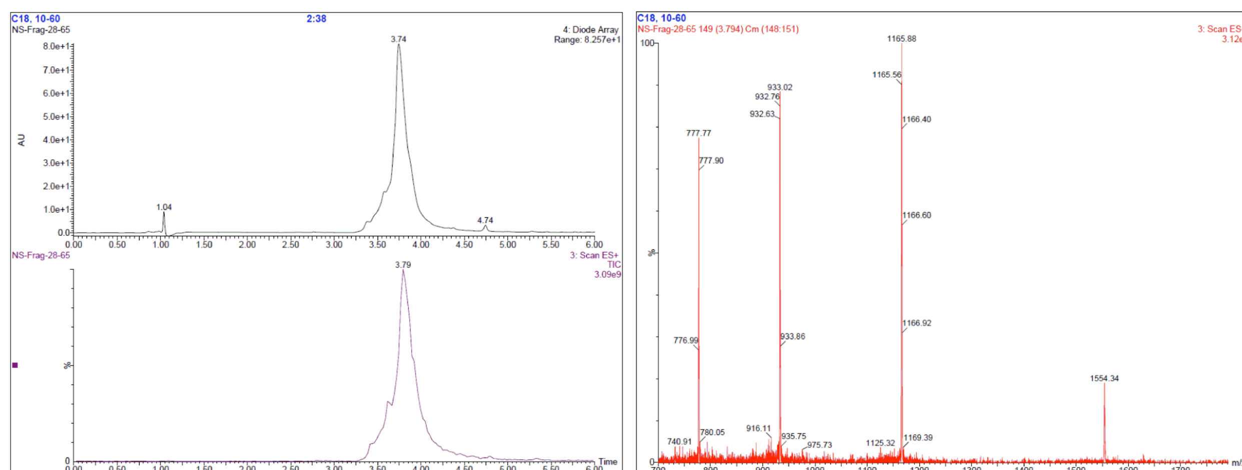
0.56 g (0.10 mmol) of Fmoc-Gly-Nova-TGT resin was subjected to continuous flow automated peptide synthesis following the general procedure for SPPS. Fmoc-Thz-OH was used for position 28 and Fmoc-Glu(O*t*Bu)-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Lys(Boc)-Thr($\psi^{Me,Me}$pro)-OH and Fmoc-Tyr(*t*Bu)-Thr($\psi^{Me,Me}$pro)-OH dipeptides were used to substitute for the corresponding amino acids at positions 59-60, 54-55 and 33-34 respectively. Upon cleavage from the resin the procedure afforded 520 mg (79% yield) of **[βFSH$^{28\text{-}65}$] peptide**.

Exact mass calcd for $C_{333}H_{508}N_{54}O_{77}S_6$: $[M+5H]^{5+}$ 1339.13, Found 1339.52; $[M+4H]^{4+}$ 1673.65, Found 1673.92.

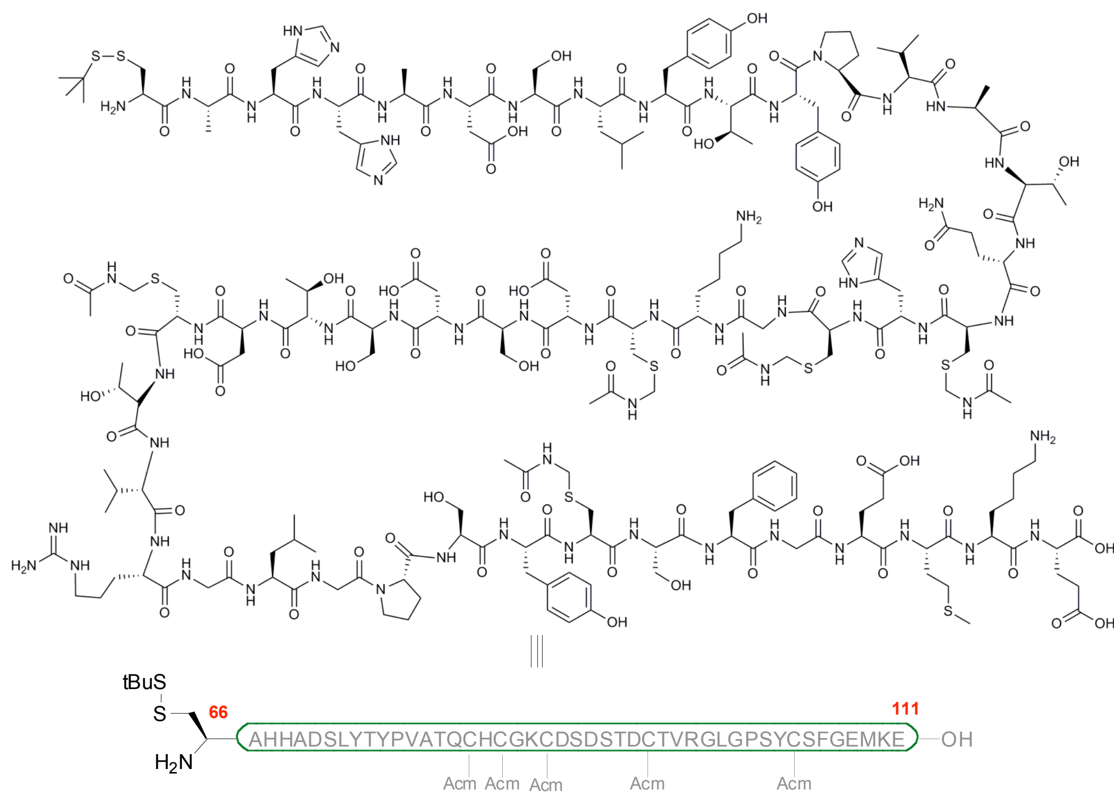
The product from above (190 mg, 28.8 μmol) was combined with thiophenol (88 μL, 0.86 mmol), PyBOP (150 mg, 0.29 mmol), and DIEA (49 μL, 0.29 mmol) in dichloromethane (3mL) and THF (3 mL). The reaction mixture was stirred for 3 h before being concentrated and used

without further purification in the next reaction. The prepared above thioester was dissolved in 6.5 mL of Cocktail B and stirred for 3 h at rt before being concentrated, triturated with ether (2 x 15 mL), decanted. The crude product was redissolved in acetonitrile/water (1:1, 15 mL), frozen in liquid nitrogen and lyophilized. The crude mass so obtained was purified by HPLC (Microsorb C18 column, 10-60% MeCN in H_2O, 30 min, RT = 23.3 min) to afford 89 mg (62 % over 2 steps) of pure product **[βFSH$^{28-65}$] phenylthioester 20**.

Exact mass calcd. for $C_{211}H_{326}N_{54}O_{57}S_4$: $[M+6H]^{6+}$ 777.39, Found 777.77; $[M+5H]^{5+}$ 932.67, Found 932.76; $[M+4H]^{4+}$ 1165.59, Found 1165.88; $[M+3H]^{3+}$ 1553.78, Found 1554.34.



[βFSH$^{66-111}$] peptide 21

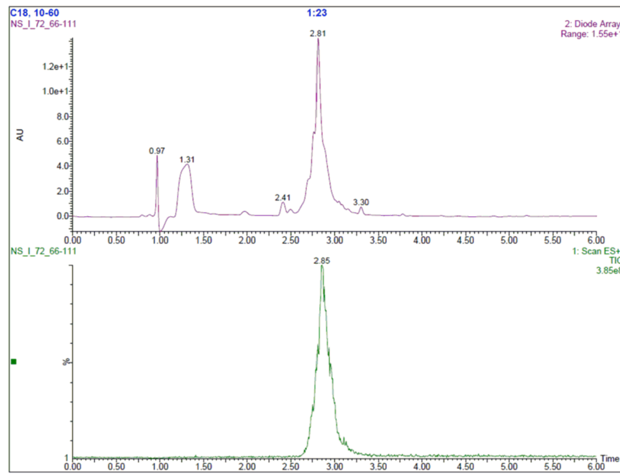


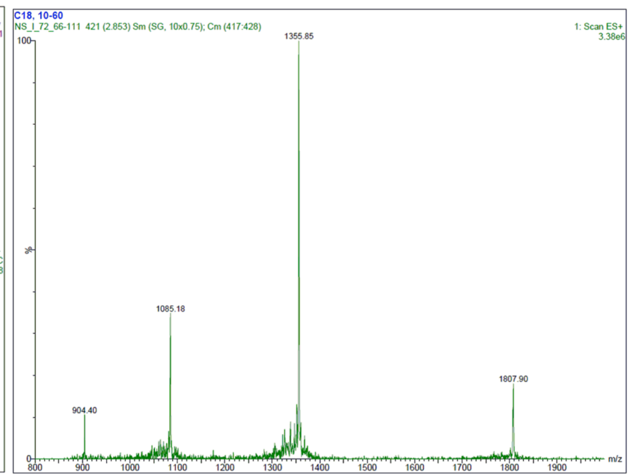
21

0.435 g (0.10 mmol) of Fmoc-Glu(O*t*Bu)-Nova-TGT resin was subjected to continuous flow automated peptide synthesis following the general procudure. Fmoc-Asp(O*t*Bu)-Ser($\psi^{Me,Me}$pro)-OH dipeptide was used to substitute for the corresponding amino acids at positions 90-91 and 71-72 respectively. Upon cleavage from the resin the procedure afforded 790 mg of peptide.

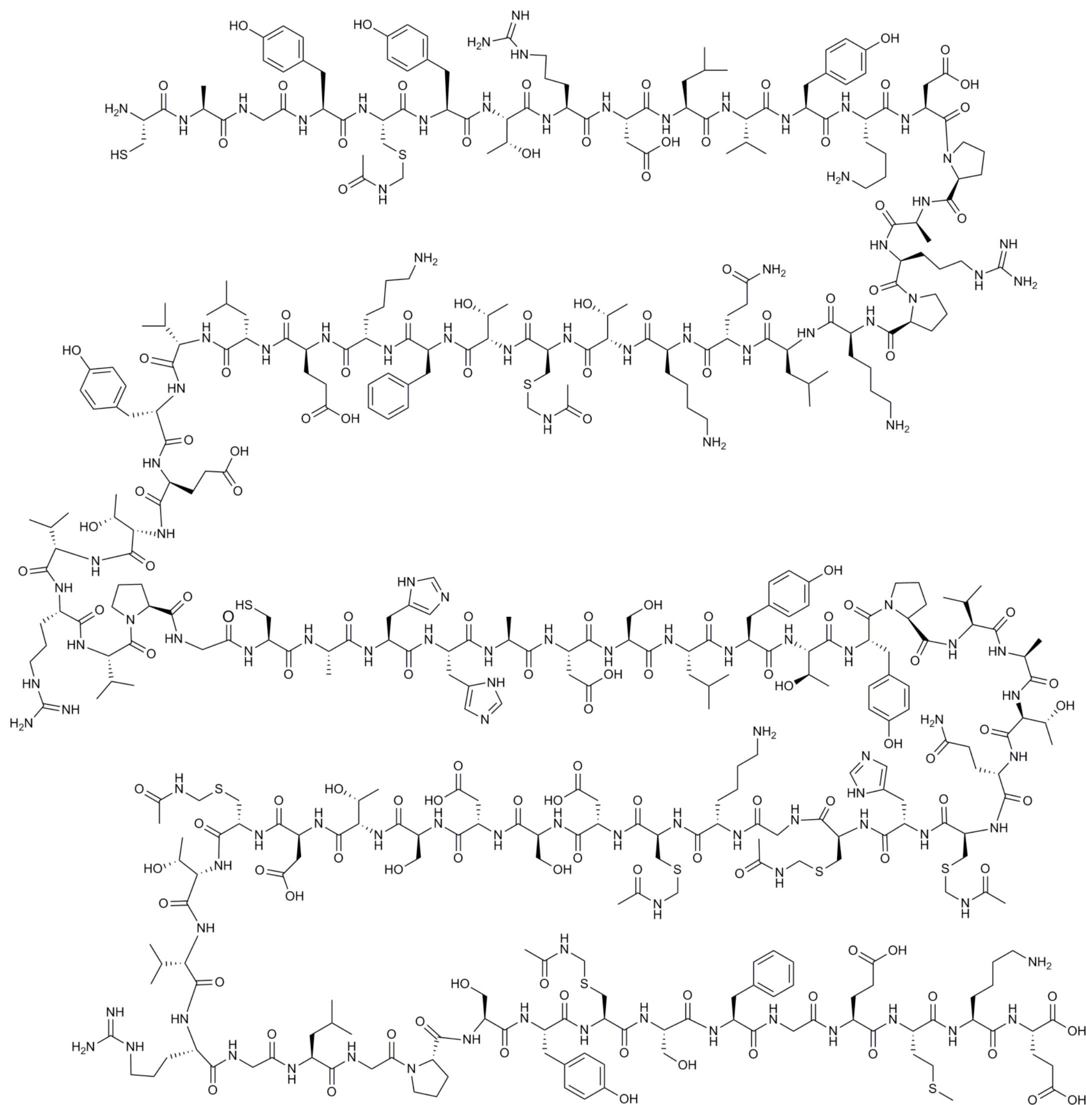
The above fragment (790 mg) was dissolved in 12 mL of Cocktail B and stirred for 4 h at rt before being concentrated, triturated with ether (2 x 15 mL), decanted and redissolved in acetonitrile/water (1:1, 15 mL). This solution was purified by HPLC (Microsorb C18 column, 10-60% MeCN in H_2O, 30 min, RT = 17.55 min) to afford 166 mg (31 % over two steps) of pure product **[βFSH$^{66-111}$] peptide 21**.

Exact mass calcd. for $C_{225}H_{343}N_{63}O_{77}S_8$: $[M+6H]^{6+}$ 903.89, Found 904.40; $[M+5H]^{5+}$ 1084.66, Found 1085.18; $[M+4H]^{4+}$ 1355.58, Found 1355.85; $[M+3H]^{3+}$ 1806.76, Found 1807.90.


C18, 10-60
1:23
NS_I_72_66-111
2: Diode Array
Range: 1.55e+1
AU
2.81
0.97
1.31
2.41
3.30
NS_I_72_66-111
1: Scan ES+
TIC
3.85e8
2.85
%
Time


C18, 10-60
NS_I_72_66-111 421 (2.853) Sm (SG, 10x0.75); Cm (417:428)
1: Scan ES+
3.38e6
1355.85
1085.18
904.40
1807.90
%
m/z

[βFSH$^{28\text{-}111}$] peptide 3



|||

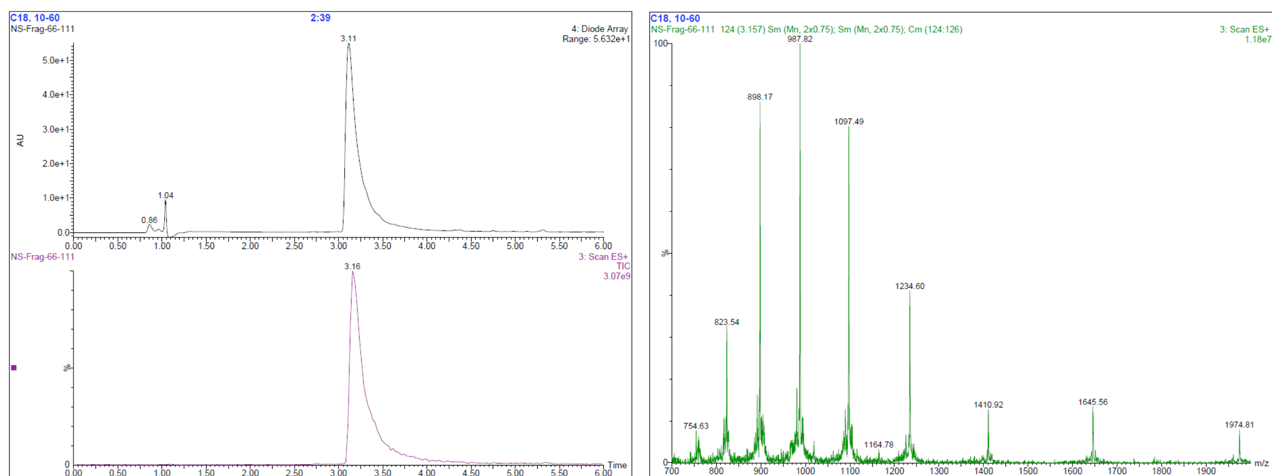
3

To a mixture of 28-65 **[βFSH$^{28\text{-}65}$] phenylthioester 20** (16.4 mg, 3.52 μmol), and **[βFSH$^{66\text{-}111}$] peptide 21** (20.0 mg, 3.69 μmol), was added freshly prepared degassed NCL buffer (pH ~ 7.4; 1.0 ml) and the reaction mixture was stirred at r.t. for 24 h. After 24 h, the reaction was checked for completion by LC-MS: (C18 Microsorb column, 10-60% MeCN in H_2O, 30 min, RT = 19.16 min).

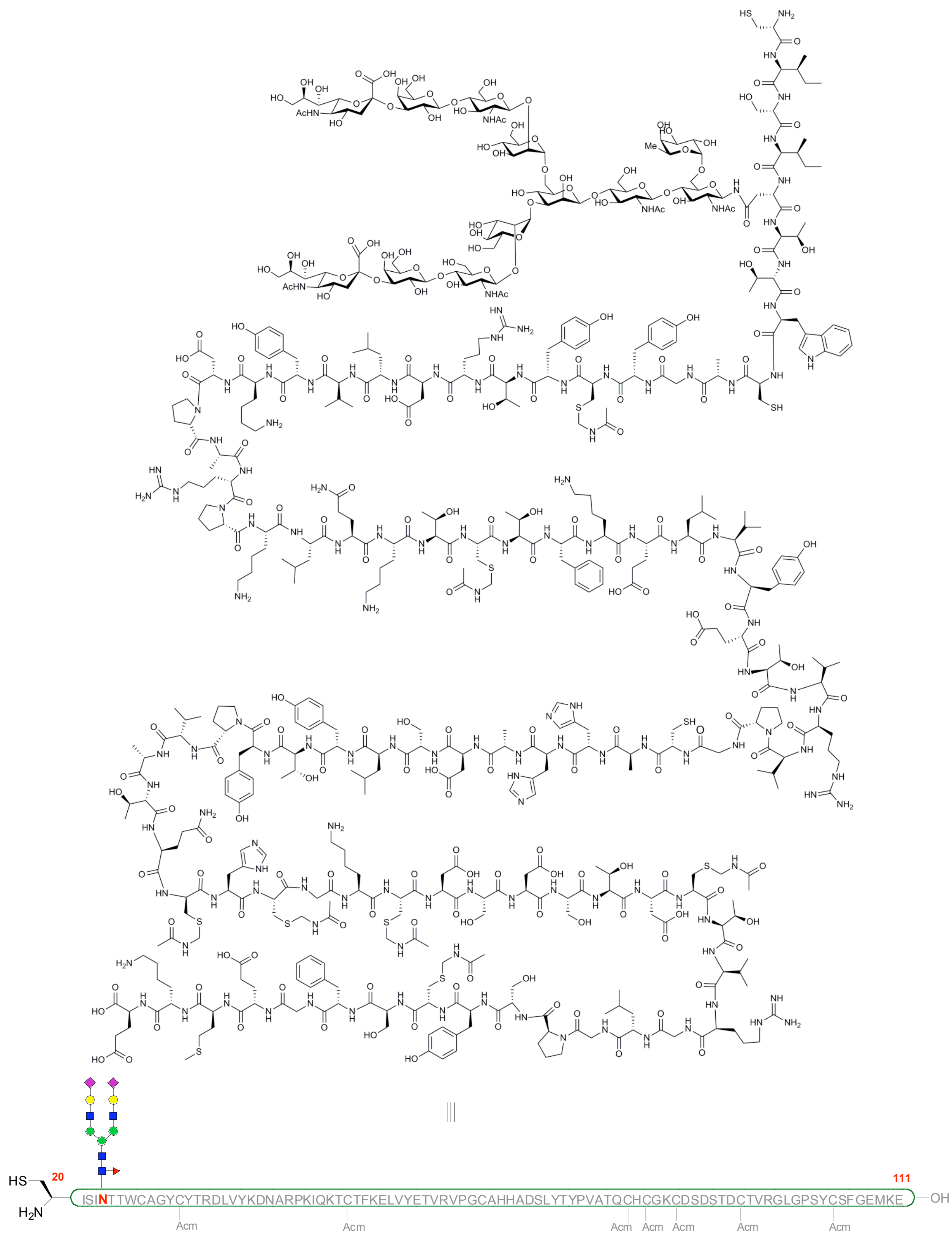
Exact mass calcd for $C_{426}H_{655}N_{117}O_{134}S_{10}$: $[M+9H]^{9+}$ 1098.62, Found 1098.92; $[M+8H]^{8+}$ 1235.82, Found 1236.12; $[M+7H]^{7+}$ 1412.22, Found 1412.36.

Upon completion of the previous step, the reaction mixture from above was diluted with Thz deprotection buffer (see general information, 600 μl), and the pH of the resultant solution was adjusted to 4.8 by addition of ultrapure conc. HCl. The reaction mixture was stirred for 20 h, and then purified by HPLC (Microsorb C18 column, 10-60% MeCN in H_2O, 30 min, RT = 20.5 min) to afford 7.7 mg (22 %) of pure product **[βFSH$^{28\text{-}111}$] peptide 3**.

Exact mass calcd. for $C_{425}H_{655}N_{117}O_{134}S_{10}$: $[M+5H]^{5+}$ 1974.32, Found 1974.81; $[M+6H]^{6+}$ 1645.43, Found 1645.56; $[M+7H]^{7+}$ 1410.51, Found 1410.92; $[M+8H]^{8+}$ 1234.32, Found 1234.60; $[M+9H]^{9+}$ 1097.29, Found 1097.49; $[M+10H]^{10+}$ 987.66, Found 987.82; $[M+11H]^{11+}$ 897.97, Found 898.1; $[M+12H]^{12+}$ 823.22, Found 823.54.



[βFSH$^{20\text{-}111}$] glycopeptide 22

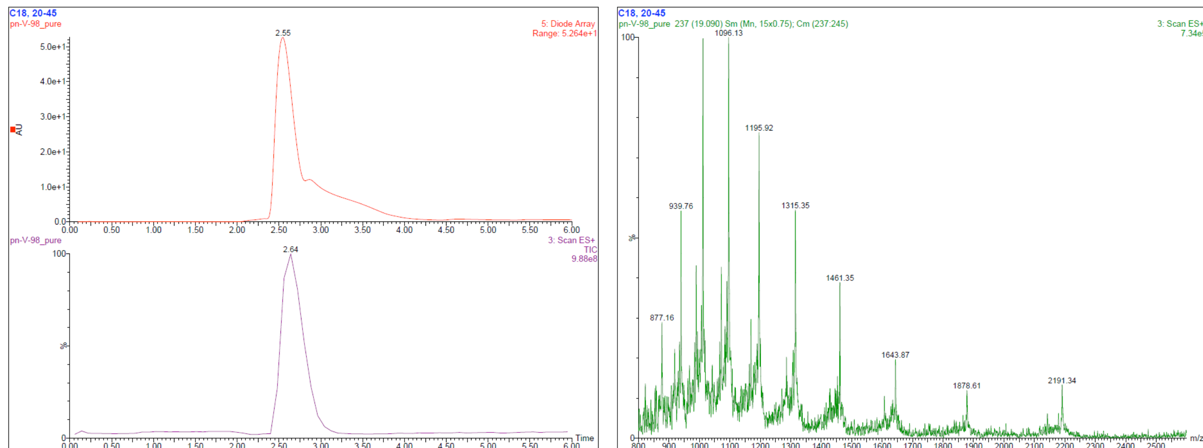


22

To a vial containing **[βFSH$^{20-27}$] glycopeptide 4** (0.6 mg, 0.18 μmol) and **[βFSH$^{28-111}$] 3** (1.76 mg, 0.178 μmol) under argon, was added the degassed NCL buffer (pH = 7.4, 40 μl) and the contents were thoroughly mixed and stirred at r.t for 24 h. An aliquot of the reaction mixture was analyzed by LC-MS (Microsorb C18 column, 20-45% MeCN in H_2O, 30 min, RT = 18.8 min) indicated complete conversion to the desired product. Mass calcd. for $C_{557}H_{863}N_{133}O_{211}S_{11}$: $[M+10H]^{10+}$ 1315.79, Found 1316.09; $[M+8H]^{8+}$ 1644.48, Found 1643.28.

To the above reaction mixture was added the Thz deprotection buffer (see general information, 600 μl) and the pH of the resulting solution was adjusted to 4.8 by the addition of ultrapure conc. HCl. The resulting solution was stirred for 24 h. This mixture was purified by HPLC (Microsorb C18 column, 20-45% MeCN in H_2O, 30 min, RT = 18.8 min) to provide 800 μg (34% yield, 2 steps) of the pure **[βFSH$^{20-111}$] glycopeptide 24**.

Exact mass calcd for $C_{557}H_{859}N_{133}O_{209}S_{11}$: $[M+14H]^{14+}$ 939.27, Found 939.76; $[M+13H]^{13+}$ 1011.45, Found 1012.22; $[M+12H]^{12+}$ 1095.66, Found 1096.13; $[M+11H]^{11+}$ 1195.17, Found 1195.92; $[M+10H]^{10+}$ 1314.59, Found 1315.35; $[M+9H]^{9+}$ 1460.54, Found 1461.35; $[M+8H]^{8+}$ 1642.98, Found 1643.87; $[M+7H]^{7+}$ 1877.55, Found 1878.61; $[M+6H]^{6+}$ 2190.31, Found 2191.34.



[βFSH1-111] glycoprotein 2

1 ... 111

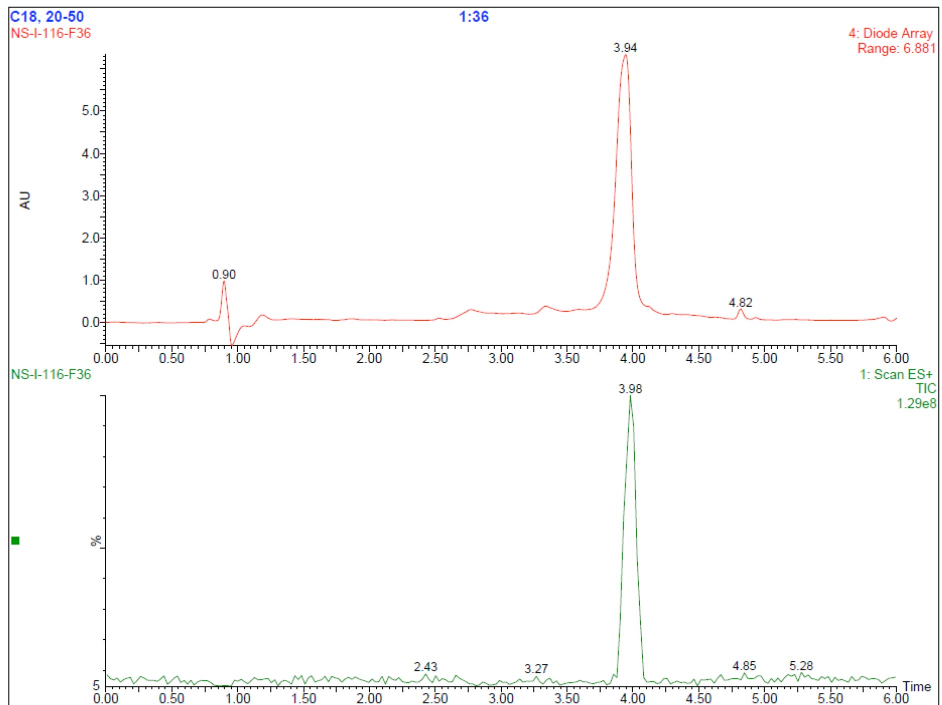
Ac—NSCELTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDNARPKIQKTCTFKELVYETVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE—OH

Acm Acm Acm Acm Acm Acm Acm Acm Acm

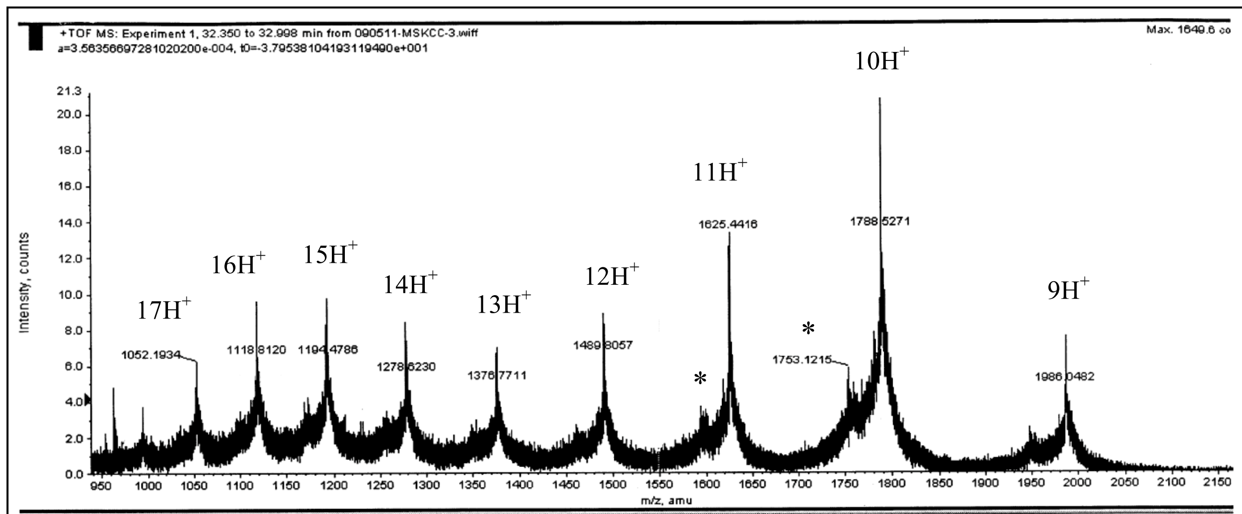
2

To a vial containing **[βFSH$^{20-111}$] glycopeptide 22** (2.0 mg, 0.152 µmol) and **[βFSH$^{1-19}$] glycopeptide thioester 5** (1.4 mg, 0.291 µmol) under argon, was added the degassed NCL buffer (pH = 7.3, 90 µl) and the contents were thoroughly mixed and stirred at r.t for 4 h. An aliquot of the reaction mixture was analyzed by LC-MS and indicated partial conversion to the desired product. Additional buffer (90 µl) was added and the solution was stirred for an additional 2 h. The mixture was diluted with additional buffer (400 µl) and purified by HPLC (Microsorb C18 column, 20-45% MeCN in H_2O, 30 min, RT = 24.8 min) to provide 700-800 µg (28 % yield) pure product **[βFSH$^{1-111}$] glycoprotein 2**.

Exact mass calc. for $C_{747}H_{1172}N_{166}O_{311}S_{13}$: $[M+17H]^{17+}$ 1052.05, Found 1052.19; $[M+16H]^{16+}$ 1117.74, Found 1118.81; $[M+15H]^{15+}$ 1192.19, Found 1192.47; $[M+14H]^{14+}$ 1277.28, Found 1278.62; $[M+13H]^{13+}$ 1375.29, Found 1376.77; $[M+12H]^{12+}$ 1489.99, Found 1489.80; $[M+11H]^{11+}$ 1625.35, Found 1625.44; $[M+10H]^{10+}$ 1787.78, Found 1788.52; $[M+9H]^{9+}$ 1986.31, Found 1986.05. Reconstructed mass calcd. for $[M+H]^{+}$ 17868.77, Found 17869.00. Some peaks corresponding to the loss of sialic acid residues were found in the MS (marked as *) and were attributed to ionization.

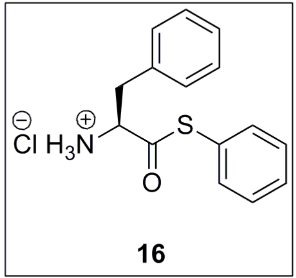


C18, 20-50
1:36
NS-I-116-F36
4: Diode Array
Range: 6.881
AU
3.94
0.90
4.82
NS-I-116-F36
1: Scan ES+
TIC
1.29e8
3.98
%
2.43
3.27
4.85
5.28
Time



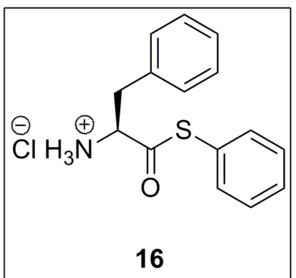
+TOF MS: Experiment 1, 32.350 to 32.998 min from 090511-MSKCC-3.wiff
a=3.56356697281020200e-004, t0=-3.79538104193119490e+001
Max. 1649.6 co
Intensity, counts
m/z, amu
17H+
16H+
15H+
14H+
13H+
12H+
11H+
10H+
9H+
*
*
1052.1934
1118.8120
1194.4786
1278.6230
1376.7711
1489.8057
1625.4416
1753.1215
1788.5271
1986.0482

group Danishefsky
.* sanen NS_I_Phe-SPh (10 1) MeOD 24.0C November_14.2011_12:30:20 Bruker AVIII 600MHz BBL1326: janggeum zg30 : 1H 7.500 ppm
_ProtonBC MeOD /opt/users/sanen sanen 18



[ppm]

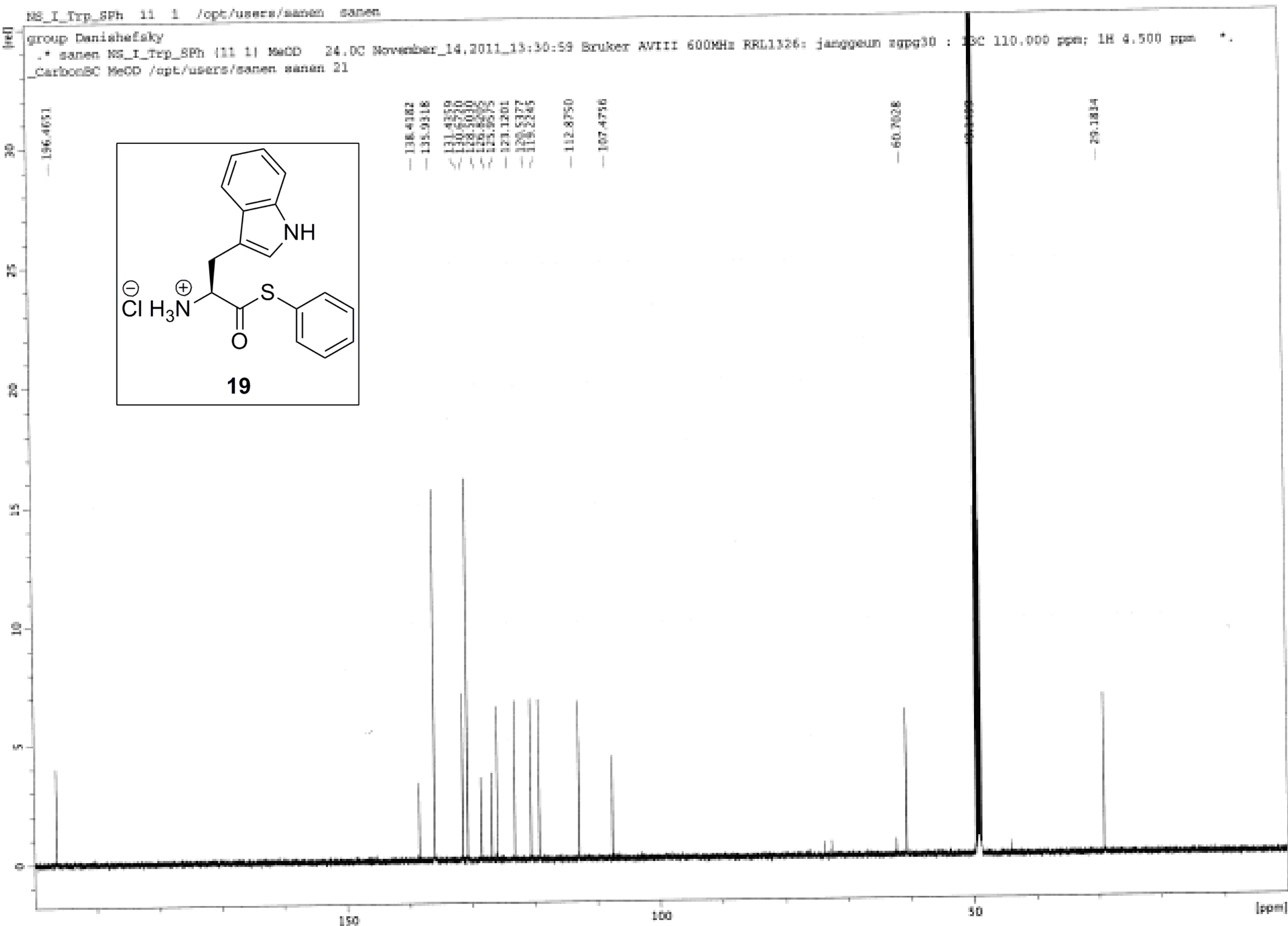
group Danishefsky
.* sanen NS_I_Phe-SPh (11 1) MeOD   24.0C November_14,2011_12:34:58 Bruker AVIII 600MHz RRL1326; janggeun zgpg30 : 13C 110.000 ppm; 1H 4.500 ppm   *.
_Carbon13C MeOD /opt/users/sanen sanen 18



195.9887
61.2069
38.8888
126.7236

150 100 50 [ppm]

group Danishefsky
.* sanen NS_I_Trp_SPh (10 1) MeOD   24.0C November_14,2011_14:21:11 Bruker AVIII 600MHz RRL1326: janggeum zg30 : 1H 7.500 ppm   *.
_ProtonBC MeOD /opt/users/sanen sanen 21


NS_I_Trp_SPh 11 1 /opt/users/sanen sanen
group Danishefsky
.* sanen NS_I_Trp_SPh (11 1) MeOD 24.0C November_14.2011_13:30:59 Bruker AVIII 600MHz RRL1326: janggeun zgpg30 : 13C 110.000 ppm; 1H 4.500 ppm *.
_CarbonBC MeOD /opt/users/sanen sanen 21
196.4651
138.4182
135.9318
123.1201
120.5377
119.2245
112.8750
107.4756
60.7028
29.1834
Cl H3N
NH
S
O
19
[rel]
[ppm]