Supporting Information

Engineering of Therapeutic Polypeptides Through Chemical Synthesis: Early Lessons from Human Parathyroid Hormone and Analogs

Suwei Dong,[†] Shiying Shang,[†] Jianfeng Li,[†] Zhongping Tan,[†] Thomas Dean [§], Akira Maeda[§], Thomas J. Gardella,[§] and Samuel J. Danishefsky[†,‡,]*

[†]*Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, NY 10065* [‡]*Department of Chemistry, Columbia University, Havemeyer Hall, 3000 Broadway, New York, NY 10027, (USA)* [§]*Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, 51 Blossom St. Boston, MA (USA)*

E-mail: s-danishefsky@ski.mskcc.org

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Materials and Methods

All commercially available materials (Aldrich®, Fluka®, Novabiochem®) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher®). Anhydrous THF, diethyl ether, CH_2Cl_2, toluene, and benzene were obtained from a dry solvent system (passed through column of alumina) and used without further drying. All reactions were performed under an atmosphere of pre-purified dry Ar(g). NMR spectra (^1H and ^{13}C) were recorded on a Bruker Advance II 600 MHz or Bruker Advance DRX-500 MHz, 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 flash column chromatography was performed on E. Merck silica gel 60 (40–63 mm). Yields refer to chromatographically pure compounds.

EDC (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide), TFE (trifluoroethanol), TIS (triisopropylsilane), Gn·HCl (guanidine hydrochloride), and TCEP·HCl [Tris(2-carboxyethyl)phosphine hydrochloride] were purchased from Sigma-Aldrich and used as received. HOOBt (Hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine) was purchased from Chem-Impex, VA-044 was from Wako, and Bond-Breaker was from ThermoScientific.

HPLC: All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A)/0.04% TFA in acetonitrile (solvent B). Analytical LC-MS analyses were performed using a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with Varian Microsorb 100-5, C18 150 × 2.0mm, and Varian Microsorb 300-5, C4 250 x 2.0mm columns at a flow rate of 0.2 mL/min.

UPLC-MS analyses were performed using a Waters Acquity™ Ultra Preformance LC system equipped with Acquity UPLC® BEH C18, 1.7µl, 2.1 x 100 mm, Acquity UPLC® BEH C8, 1.7µl, 2.1 x 100 mm, Acquity UPLC® BEH 300 C4, 1.7µl, 2.1 x 100 mm columns at a flow rate of 0.3 mL/min.

Preparative separations were performed using a Ranin HPLC solvent delivery system equipped with a Rainin UV-1 detector and Agilent Dynamax reverse phase HPLC column (Microsorb 100-8 C18 (250x21.4mm), or Microsorb 300-5 C8 (250x21.4mm), or Microsorb 300-5 C4

(250x21.4mm)) at a flow rate of 16.0 mL/min.

PTH binding and signaling assays: Binding to the RG and R^0 conformations of the human PTHR was assessed by competition reactions performed in 96-well plates using transiently transfected COS-7 cell membranes, as described

In brief, binding to R^0 was assessed using ^{125}I-PTH(1-34) as a tracer radioligand, and GTPgS ($1x10^{-5}$ M) was included in the reactions. Binding to RG was assessed using membranes containing a high affinity, negative-dominant Ga_S subunit (Ga_S^{ND}) and ^{125}I-M-PTH(1-15) as a tracer radioligand. Signaling via the cAMP/PKA pathway was assessed in HEK-293 cells transiently transfected in 96-well plates to express the hPTHR, and, for luciferase reporter assays, a cAMP-response-element/luciferase (Cre-Luc) reporter gene construct. For direct intracellular cAMP assessment, cells were treated with PTH in the presence of IBMX for 30 minutes at room temperature, the cells were then lysed with 50 mM HCl, and the cAMP content of the lysates were determined by radioimmunometric assay. For Cre-Luc assays, cells were treated with ligands in a 37˚C/CO_2 incubator for 4-hours, following which the SteadyGlo luciferase reagent (Promega) was added, and luminescence recorded using a PerkinElmer Envision plate reader.

Measurements of PTH analog effects in mice: Male mice, aged 9 weeks, of strain C57BL/6 were obtained from Charles River laboratory, and treated in accordance with the ethical guidelines adopted by the M.G.H. Mice were injected subcutaneously with vehicle (10 mM citric acid/150 mM NaCl/0.05% Tween-80, pH5.0) or vehicle containing a PTH analog. Peptides were injected at a dose of 20 nmol/kg of body weight. Tail vein blood was collected immediately prior to, and at times after injection for analysis. Blood Ca^{++} concentrations were measured using a Siemens RapidLab 348 Ca^{++}/pH analyzer.

Data calculations: Data were processed using Microsoft Excel and GraphPad Prism 4.0 software packages.

General Procedures:

A: Solid Phase Peptide Synthesis Using Fmoc-Strategy

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc protocols. The deblock mixture was a mixture of 100:2:2 of DMF/piperidine/DBU. The following Fmoc amino acids and pseudoproline dipeptides from Novabiochem® were employed: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Boc-Thz-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gln(Trt)-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(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Asn(Trt)-Ser($\psi^{Me,Me}$Pro)-OH, Fmoc-Gly-Ser($\psi^{Me,Me}$Pro)-OH, Fmoc-Leu-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Lys(Boc)-Ser($\psi^{Me,Me}$Pro)-OH. Pseudoproline dipeptides used in each sequence were highlighted in blue.

Upon completion of the automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was performed with TFA/H_2O/triisopropylsilane (95:2.5:2.5 v/v) solution or DCM/AcOH/TFE (8:1:1 v/v) for 45 min (x2). After filtering off the resin, the liquid was concentrated by a gentle stream of nitrogen. The oily residue was triturated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was lyophilized or purified for further use.

B: Preparation of Peptidyl Esters

The fully protected peptidyl acid (1.0 equiv) cleaved from resin using DCM/TFE/AcOH (8:2:2, v/v), and the amino acid ester hydrochloride (3.0 equiv) were dissolved in $CHCl_3$/TFE (3:1) and cooled to -10 °C. HOOBt (3.0 equiv) and EDCI (3.0 equiv) were then added. The reaction mixture was stirred at room temperature for 4 h. The solvent was gently blown off by a nitrogen stream and the residue was washed with H_2O/AcOH (95:5, v/v). After centrifugation, the pellet was dissolved in TFA/H_2O/TIS (95:2.5:2.5) and stirred at room temperature for 1 h. The solvent was removed and the residue was triturated with cold ether. The resulting solid was dissolved in MeCN/H_2O/AcOH (47.5:47.5:5, v/v) for further analysis and purification.

C: Native Chemical Ligation with Peptidyl 2-(ethyldithio)phenol ester

N-terminal peptide ester (1.5 equiv) and C-terminal peptide (1.0 equiv) were dissolved in ligation buffer (6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP·HCl, pH 7.2~7.3). The resulting solution was stirred at room temperature under an argon atmosphere, and monitored using LC-MS. The reaction was quenched with MeCN/H_2O/AcOH (47.5:47.5:5) and purified by HPLC.

D: Native Chemical Ligation with Peptidyl alkylthio ester

N-terminal peptide ester (1.5 equiv) and C-terminal peptide (1.0 equiv) were dissolved in ligation buffer (6 M Gdn·HCl, 300 mM Na_2HPO_4, 20 mM TCEP·HCl, 200 mM 4-mercaptophenylacetic acid (MPAA), pH 7.2~7.3). The resulting solution was stirred at room temperature under an argon atmosphere, and monitored using LC-MS. The reaction was quenched with MeCN/H_2O/AcOH (47.5:47.5:5) and purified by HPLC.

E: Metal-Free Dethiylation

To a solution of the purified ligation product in 0.2 ml of degassed buffer (6 M Gdn·HCl, 200 mM NaH_2PO_4) was added 0.2 ml of 0.5 M bond-breaker® TCEP solution (Pierce), 0.05 ml of 2-methyl-2-propanethiol and 0.1 ml of radical initiator VA-044 (0.1 M in H_2O). The reaction mixture was stirred at 37 °C under an argon atmosphere and monitored by LC-MS. Upon completion, the reaction was quenched by the addition of MeCN/H_2O/AcOH (47.5:47.5:5) and further purified by HPLC.

Experimental Procedures and Characterization of New Compounds:

Peptide 2

2

The fully deprotected peptidyl acid **2** was prepared by SPPS using the general procedure A described above. Peptide was cleaved from resin and deprotected using TFA/TIS/H_2O (95:2.5:2.5). The crude material was further purified using RP-HPLC (linear gradient 10-30% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 21.5-22.5 min. The fractions were collected, and concentrated via lyophilization to provide peptide **2** (28.1 mg, 11%) as a white solid.

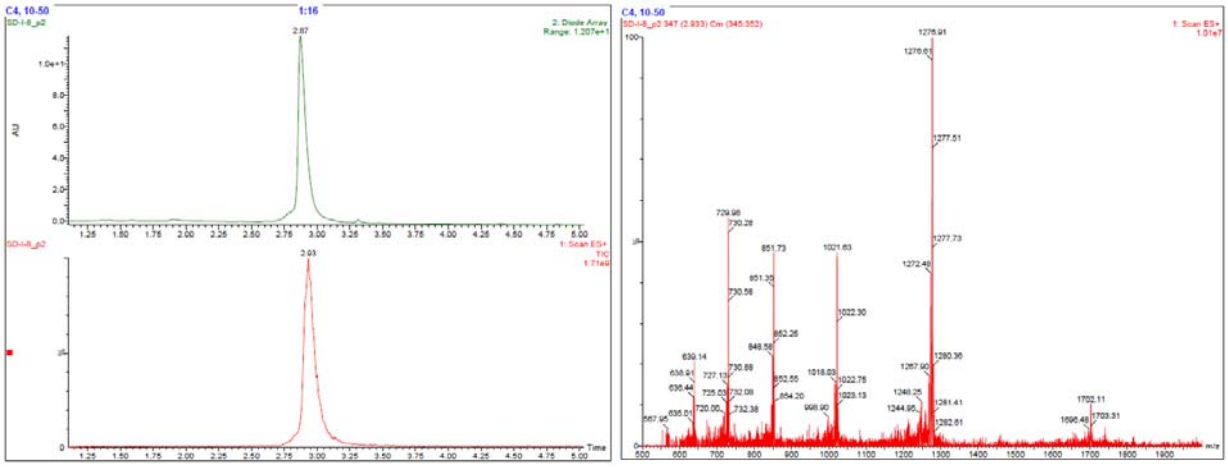
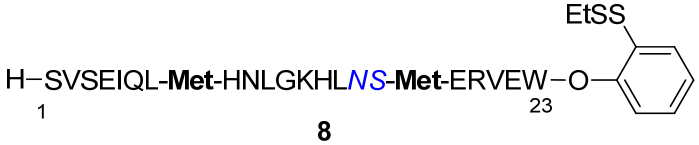


Figure S1. LC-MS traces and Mass-spec data for peptide **2**: Calcd for $C_{215}H_{365}N_{67}O_{72}S_2$: 5104.73 Da(average isotopes), $[M+3H]^{3+}$ *m/z* = 1702.58, $[M+4H]^{4+}$ *m/z* = 1277.18, $[M+5H]^{5+}$ *m/z* = 1021.95, $[M+6H]^{6+}$ *m/z* = 851.79, $[M+7H]^{7+}$ *m/z* = 730.25, $[M+8H]^{8+}$ *m/z* = 639.09; observed: $[M+3H]^{3+}$ *m/z* = 1702.11, $[M+4H]^{4+}$ *m/z* = 1276.91, $[M+5H]^{5+}$ *m/z* = 1021.63, $[M+6H]^{6+}$ *m/z* = 851.73, $[M+7H]^{7+}$ *m/z* = 729.98, $[M+8H]^{8+}$ *m/z* = 639.14.

Peptidyl phenol esters 8



8

The fully protected peptidyl acid (1-22) was prepared by SPPS using the general

procedure A described above. After cleavage using CH_2Cl_2/TFE/AcOH, 142.5 mg crude peptide was obtained (62% yield).

The fully protected peptidyl acid (132.3 mg, 28.8 μmol, 1.0 equiv) and HCl·H-Trp-O(EtSS)Ph (11.8 mg, 28.8 μmol, 1.0 equiv) in $CHCl_3$/TFE (v/v = 3:1, 1.5 mL) was cooled to –10 °C. HOOBt (4.7 mg, 28.8 μmol, 1.0 equiv) and EDC (5.10 μL, 28.8 μmol, 1.0 equiv) were added. The reaction mixture was stirred at room temperature for 3 h. The solvent was then blown off under a gentle N_2 stream and 8 mL of TFA/H_2O/TIS (95:2.5:2.5) was added. After deprotection for 45 min, TFA was blown off and the oily residue was triturated with 5 mL of diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 35-55% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 17-20 min. The fractions were collected, and concentrated via lyophilization to provide peptide **8 (**23.4 mg, 28%) as a white solid.

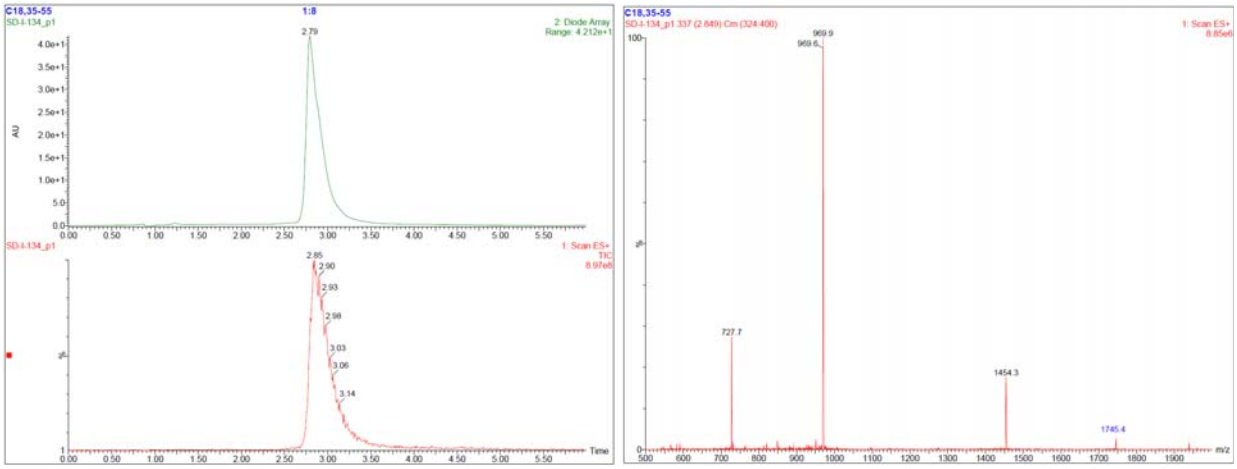
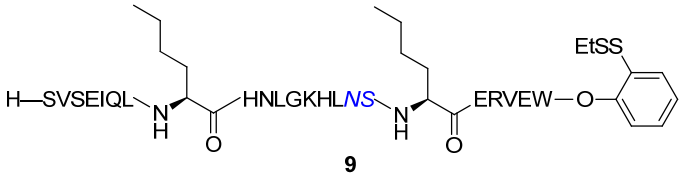


Figure S2. LC-MS traces and Mass-spec data for peptide **8**: Calcd for $C_{126}H_{197}N_{35}O_{36}S_4$: 2906.39 Da(average isotopes), $[M+2H]^{2+}$ *m/z* = 1454.20, $[M+3H]^{3+}$ *m/z* = 969.80, $[M+4H]^{4+}$ *m/z* = 727.60; observed: $[M+2H]^{2+}$ *m/z* = 1454.3, $[M+3H]^{3+}$ *m/z* = 969.9, $[M+4H]^{4+}$ *m/z* = 727.7.

Peptidyl phenol ester 9



9

The fully protected peptidyl acid (1-22) was prepared by SPPS using the general

procedure A described above. After cleavage, 151.0 mg crude peptide was obtained (66% yield).

The fully protected peptidyl acid (151.0 mg, 33.1 μmol, 1.0 equiv) and HCl·H-Trp-O(EtSS)Ph (13.5 mg, 33.1 μmol, 1.0 equiv) in $CHCl_3$/TFE (v/v = 3:1, 1.5 mL) was cooled to –10 °C. HOOBt (5.4 mg, 33.1 μmol, 1.0 equiv) and EDC (5.86 μL, 33.1 μmol, 1.0 equiv) were added. The reaction mixture was stirred at room temperature for 3 h. The solvent was then blown off under a gentle N_2 stream and 10 mL of TFA/H_2O/TIS (95:2.5:2.5) was added. After deprotection for 45 min, TFA was blown off and the oily residue was triturated with 6 mL of diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 35-55% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 22.5-26.5 min. The fractions were collected, and concentrated via lyophilization to provide peptide **9 (**23.8 mg, 25%) as a white solid.

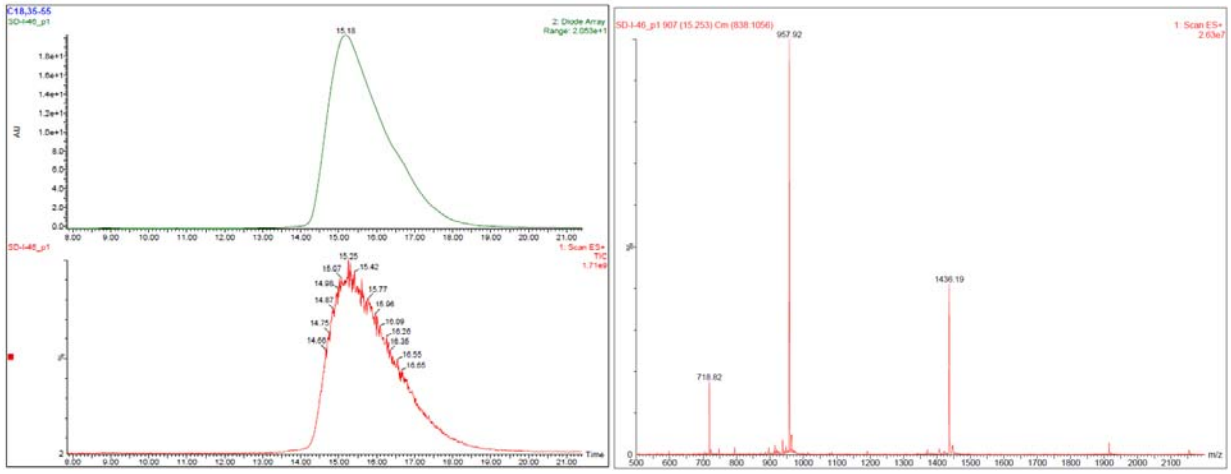
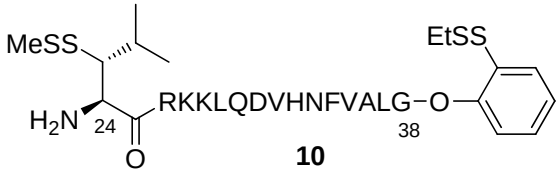


Figure S3. LC-MS traces and Mass-spec data for peptide **9:** Calcd for $C_{128}H_{201}N_{35}O_{36}S_2$: 2870.31 Da(average isotopes), $[M+2H]^{2+}$ *m/z* = 1436.16, $[M+3H]^{3+}$ *m/z* = 957.77, $[M+4H]^{4+}$ *m/z* = 718.58; observed: $[M+2H]^{2+}$ *m/z* = 1436.19, $[M+3H]^{3+}$ *m/z* = 957.92, $[M+4H]^{4+}$ *m/z* = 718.69.

Peptidyl phenol ester 10



10

The fully protected peptidyl acid (25-37) was prepared by SPPS using the general procedure A described above. The pre-leucine surrogate was installed manually using 1.7 equiv

of Boc-Leu(SSMe)-OH,[S1] 5 equiv of HATU, 6 equiv of DIEA in DMF, and the resin was gently shaken for 15 min. After wash with DMF, MeOH, and CH_2Cl_2, the side-chain protected peptide was further cleaved using CH_2Cl_2/TFE/AcOH, the resulting solution was concentrated to afford 110.4 mg crude peptide (76% yield).

The crude peptidyl acid (48.6 mg, 16.7 μmol, 1.0 equiv) and HCl·H-Gly-O(EtSS)Ph (13.5 mg, 16.7 μmol, 1.0 equiv) in $CHCl_3$/TFE (v/v = 3:1, 700 μL) was cooled to –10 °C. HOOBt (2.7 mg, 16.7 μmol, 1.0 equiv) and EDC (2.96 μL, 16.7 μmol, 1.0 equiv) were added. The reaction mixture was stirred at room temperature for 3 h. The solvent was then blown off under a gentle N_2 stream and 5 mL of TFA/H_2O/TIS (95:2.5:2.5) was added. After deprotection for 45 min, TFA was blown off and the oily residue was triturated with 3 mL of diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 31-51% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 22.5-24 min. The fractions were collected, and concentrated via lyophilization to afford peptide **10 (**13.4 mg, 40%) as a white solid.

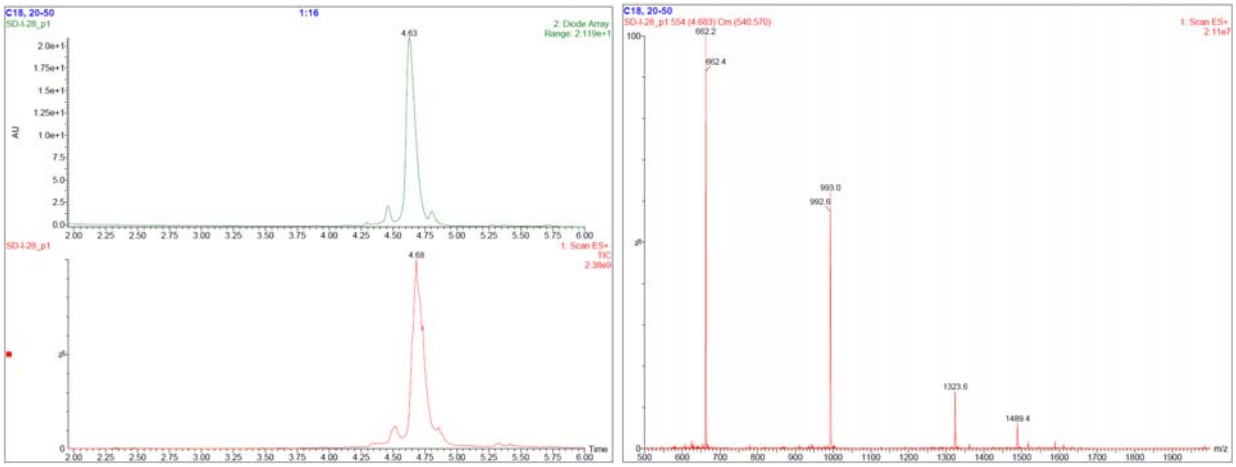
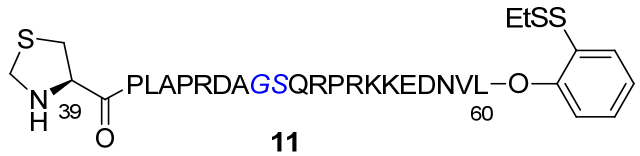


Figure S4. LC-MS traces and Mass-spec data for peptide **10:** Calcd for $C_{88}H_{142}N_{24}O_{20}S_4$: 1984.48 Da(average isotopes), $[3M+4H]^{4+}$ *m/z* = 1489.36, $[2M+3H]^{3+}$ *m/z* = 1323.99, $[M+2H]^{2+}$ *m/z* = 993.24, $[M+3H]^{3+}$ *m/z* = 662.49; observed: $[3M+4H]^{4+}$ *m/z* = 1489.4, $[2M+3H]^{3+}$ *m/z* = 1323.6, $[M+2H]^{2+}$ *m/z* = 993.0, $[M+3H]^{3+}$ *m/z* = 662.2.

[S1] Tan, Z.; Shang, S.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2010**, *49*, 9500-9503.

Peptidyl phenol esters 11



11

The fully protected peptidyl acid (39-59) was prepared by SPPS using the general procedure A described above. Boc-Thz-OH was used at the *N*-terminus, and the Boc group was removed in the TFA treatment step. In particular, Fmoc-Asp(ODie)-OH was used in this sequence to prevent the aspartimide formation. After cleavage, 123.3 mg crude peptide was obtained (60% yield).

The crude peptidyl acid (123.3 mg, 30.0 μmol, 1.0 equiv) and HCl·H-Leu-O(EtSS)Ph (10.1 mg, 30.0 μmol, 1.0 equiv) in $CHCl_3$/TFE (v/v = 3:1, 1.5 mL) was cooled to –10 °C. HOOBt (4.9 mg, 30.0 μmol, 1.0 equiv) and EDC (5.31 μL, 30.0 μmol, 1.0 equiv) were added. The reaction mixture was stirred at room temperature for 3 h. The solvent was then blown off under a gentle N_2 stream and 10 mL of TFA/H_2O/TIS (95:2.5:2.5) was added. After deprotection for 45 min, TFA was blown off and the oily residue was triturated with 6 mL of diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 27-47% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 19.5-21.5 min. The fractions were collected, and concentrated via lyophilization to provide peptide **11 (**27.4 mg, 36%) as a white solid.

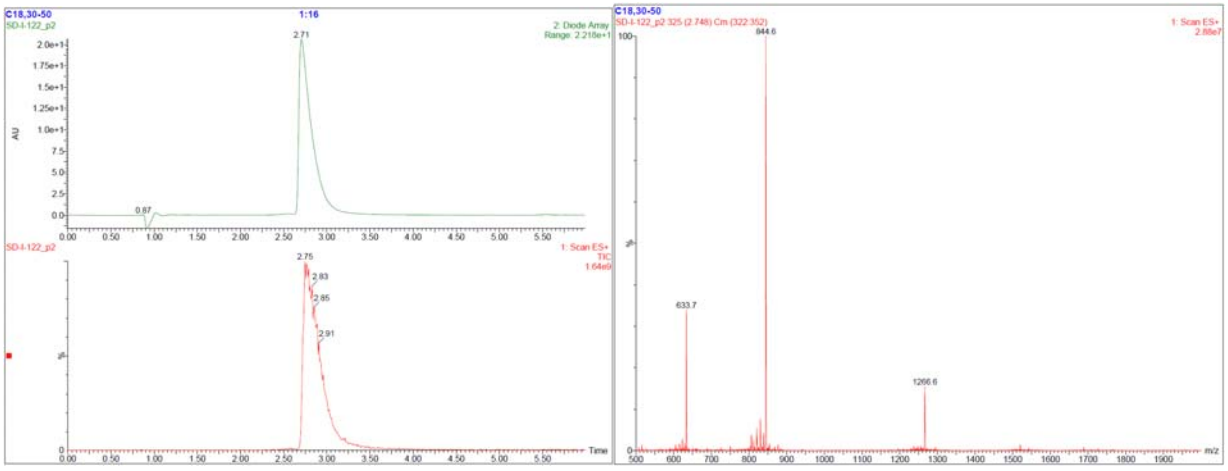


Figure S5. LC-MS traces and Mass-spec data for peptide **11:** Calcd for $C_{107}H_{176}N_{34}O_{31}S_3$: 2530.95 Da(average isotopes), $[M+2H]^{2+}$ *m/z* = 1266.48, $[M+3H]^{3+}$ *m/z* = 844.65, $[M+4H]^{4+}$ *m/z*

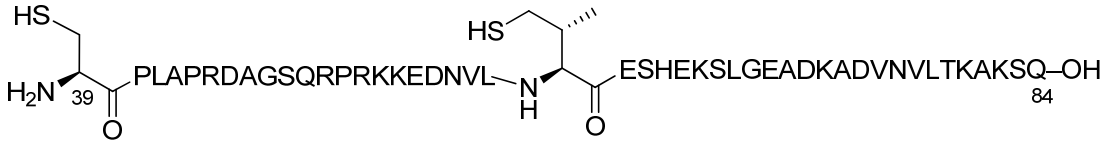
= 633.74; observed: $[M+2H]^{2+}$ *m/z* = 1266.6, $[M+3H]^{3+}$ *m/z* = 844.6, $[M+4H]^{4+}$ *m/z* = 633.7.

Peptidyl acid 12

12

The fully protected peptidyl acid (62-84) was prepared by SPPS using the general procedure A described above. The pre-valine surrogate was installed manually using 1.0 equiv of Boc-Val(SSMe)-OH,[S2] 3 equiv of HATU, 6 equiv of DIEA in DMF, and the resin was gently shaken for 15 min. After wash with DMF, MeOH, and CH_2Cl_2, the peptide was cleaved from resin and deprotected using 10 mL of TFA/H_2O/TIS (95:2.5:2.5) for 45 min. TFA was blown off and the oily residue was triturated with 5 mL of diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 11-31% solvent B over 30 min, Microsorb 300-5 C8 column, 16 mL/min, 230 nm), and the product eluted at 19-21 min. The fractions were collected, and concentrated via lyophilization to afford peptide **12 (**45.2 mg, 33%) as a white solid. Mass-spec of peptidyl thioester **17** was identical to literature reported data.[S3]

Ligated Peptide 14



14

According to General Procedure C, reaction of peptide **11** (9.3 mg, 3.67 μmol, 1.3 equiv) and peptide **12** (7.8 mg, 2.82 μmol, 1.0 equiv) in 800 μL of ligation buffer at rt for 15 h, followed by the addition of $MeONH_2 \cdot HCl$ (20 mg) in one portion. The resulting mixture was further stirred for 3 h, then quenched with 3 mL of CH_3CN/H_2O/AcOH (15:80:5).

[S2] Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J. L.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2008**, *47*, 8521.
[S3] Shang, S.; Tan, Z.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4297.

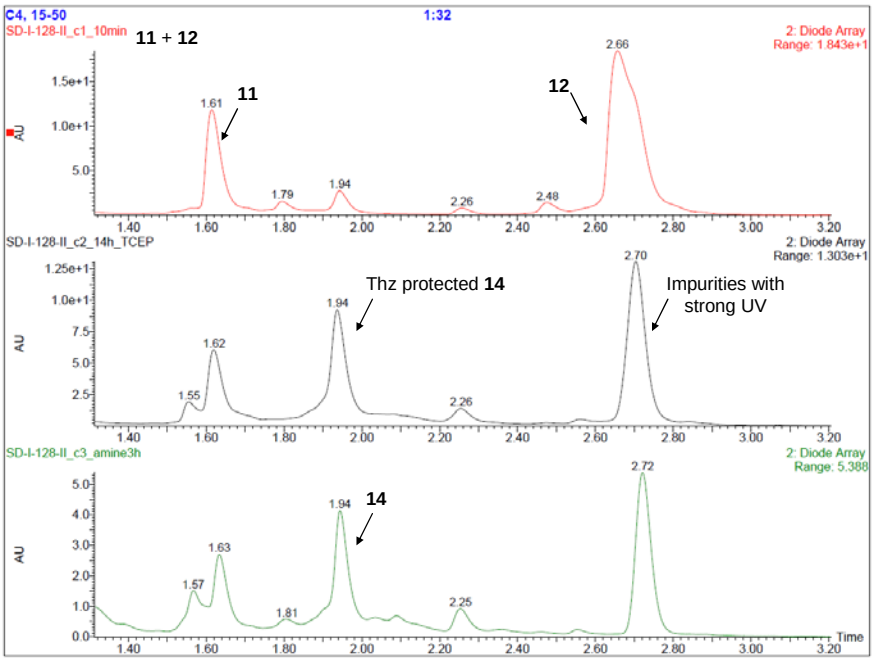


Figure S6. LC traces of crude reactions.

The crude mixture was purified using RP-HPLC (linear gradient 9-29% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 18.5-20.5 min. The fractions were collected, and concentrated via lyophilization to afford peptide **14 (**10.3 mg, 72%, two steps) as a white solid.

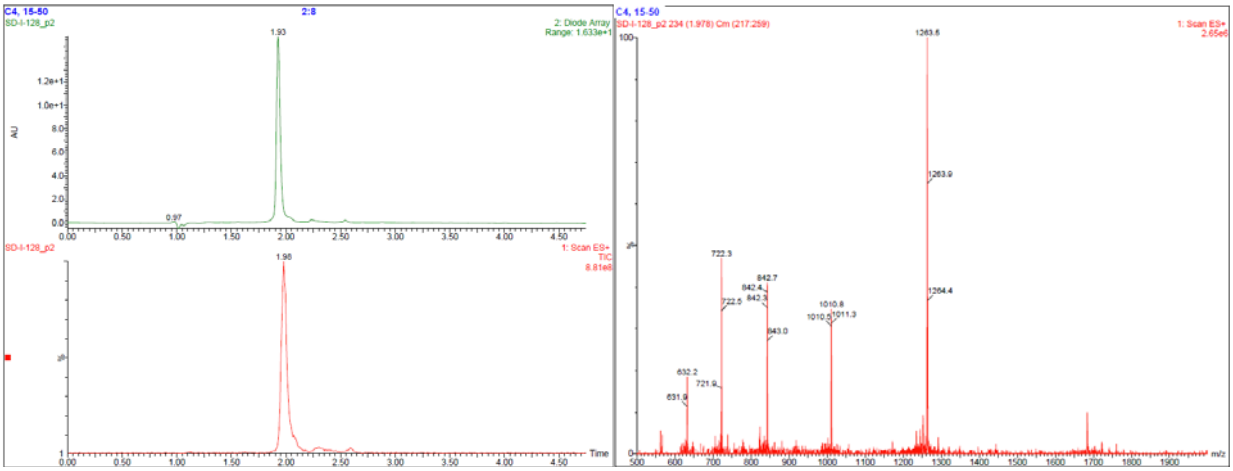


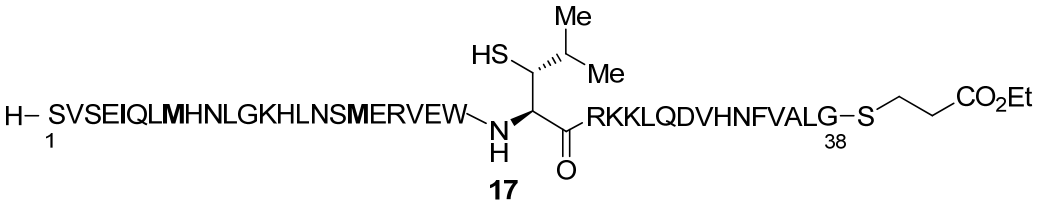
Figure S7. LC-MS traces and Mass-spec data for peptide **14:** Calcd for $C_{211}H_{357}N_{67}O_{72}S_2$: 5048.63 Da(average isotopes), $[M+3H]^{3+}$ *m/z* = 1683.88, $[M+4H]^{4+}$ *m/z* = 1263.16, $[M+5H]^{5+}$ *m/z* = 1010.73, $[M+6H]^{6+}$ *m/z* = 842.44, $[M+7H]^{7+}$ *m/z* = 722.23, $[M+8H]^{8+}$ *m/z* = 632.08; observed: $[M+4H]^{4+}$ *m/z* = 1263.5, $[M+5H]^{5+}$ *m/z* = 1010.8, $[M+6H]^{6+}$ *m/z* = 842.7, $[M+7H]^{7+}$ *m/z* = 722.3, $[M+8H]^{8+}$ *m/z* = 632.2.

Peptidyl alkylthio ester 16

16

The side-chain protected peptidyl acid (24-37) was prepared according to the procedure described for peptide **10**. The crude peptidyl acid (91.6 mg, 31.5 μmol, 1.0 equiv) and HCl·H-Gly-$SCH_2CH_2CO_2Et$ (7.2 mg, 31.5 μmol, 1.0 equiv) in $CHCl_3$/TFE (v/v = 3:1, 1.5 mL) was cooled to –10 °C. HOOBt (5.1 mg, 31.5 μmol, 1.0 equiv) and EDC (5.57 μL, 31.5 μmol, 1.0 equiv) were added. The reaction mixture was stirred at room temperature for 3 h. The solvent was then blown off under a gentle N_2 stream and 10 mL of TFA/H_2O/TIS (95:2.5:2.5) was added. After deprotection for 45 min, TFA was blown off and the oily residue was triturated with 7 mL of diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 23-43% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 19.5-23 min. The fractions were collected, and concentrated via lyophilization to afford peptide **16 (**35.4 mg, 58%) as a white solid. Mass-spec of peptidyl thioester **17** was identical to literature reported data.[S3]

Ligated Peptide 17



17

The synthesis of **17** was carried out under kinetically controlled ligation conditions. Following General Procedure C, Peptide **8** (4.7 mg, 1.62 μmol, 1.0 equiv) and peptide **16** (3.1 mg, 1.60 μmol, 1.0 equiv) were dissolved in 300 μL of ligation buffer. The reaction mixture was stirred at room temperature for 3 h. The reactions were monitored by LC-MS and diluted with 3 mL of CH_3CN/H_2O/AcOH (25:75:5) upon completion.

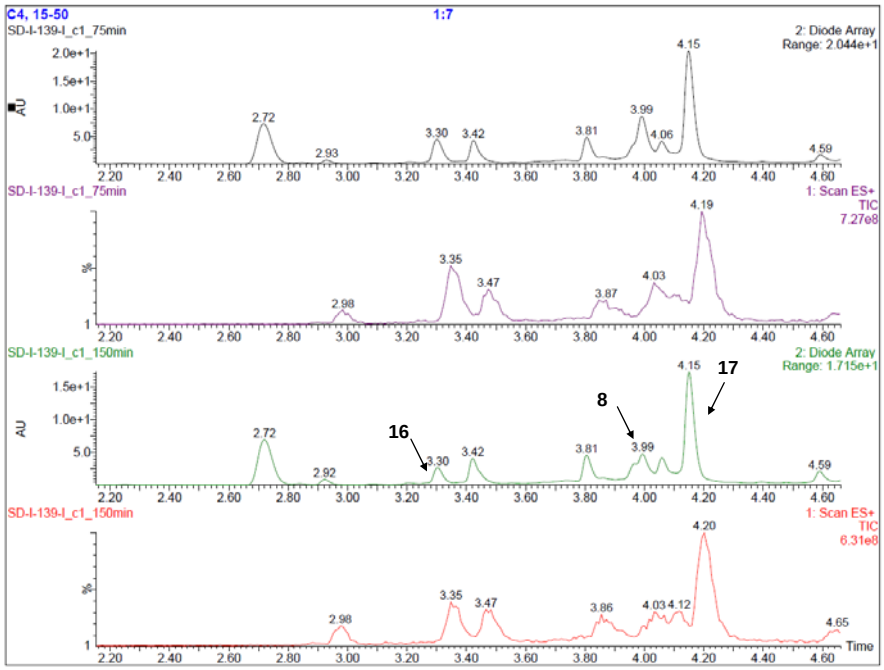
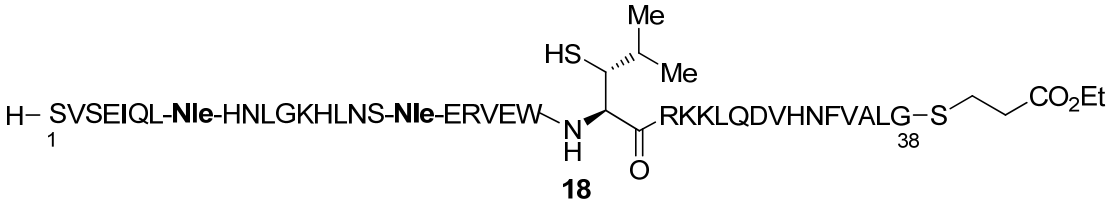


Figure S8. LC-MS traces of crude reactions.

The crude mixture was purified using RP-HPLC (linear gradient 25-45% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 18.5-20 min. The fractions were collected, and concentrated via lyophilization to afford peptide **17** (4.4 mg, 59%) as a white solid. Mass-spec of peptidyl thioester **17** was identical to literature reported data.[S3]

Ligated Peptide 18



18

The synthesis of **18** was carried out under kinetically controlled ligation conditions similar to that described for peptide **17**. Peptide **9** (4.3 mg, 1.50 µmol, 1.21 equiv) and peptide **16** (2.4 mg, 1.24 µmol, 1.0 equiv) were dissolved in 250 µL of ligation buffer. The reaction mixture was stirred at room temperature for 3.5 h. The reactions were monitored by LC-MS and diluted with 3 mL of CH_3CN/H_2O/AcOH (25:75:5) upon completion.

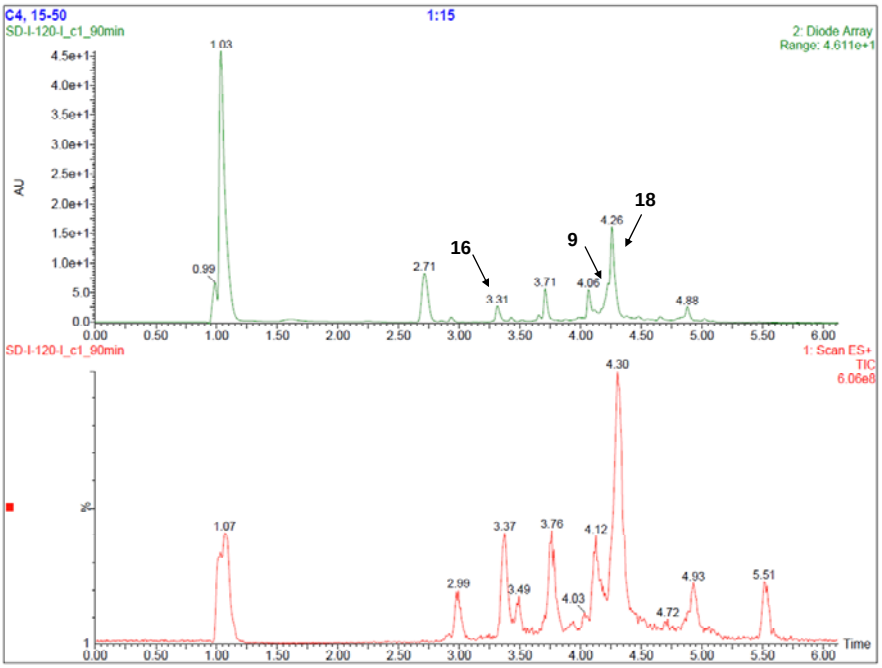


Figure S9. LC-MS traces of crude reactions.

The crude mixture was purified using RP-HPLC (linear gradient 26-46% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 18.5-22.5 min. The fractions were collected, and concentrated via lyophilization to afford peptide **18** (3.5 mg, 62%) as a white solid.

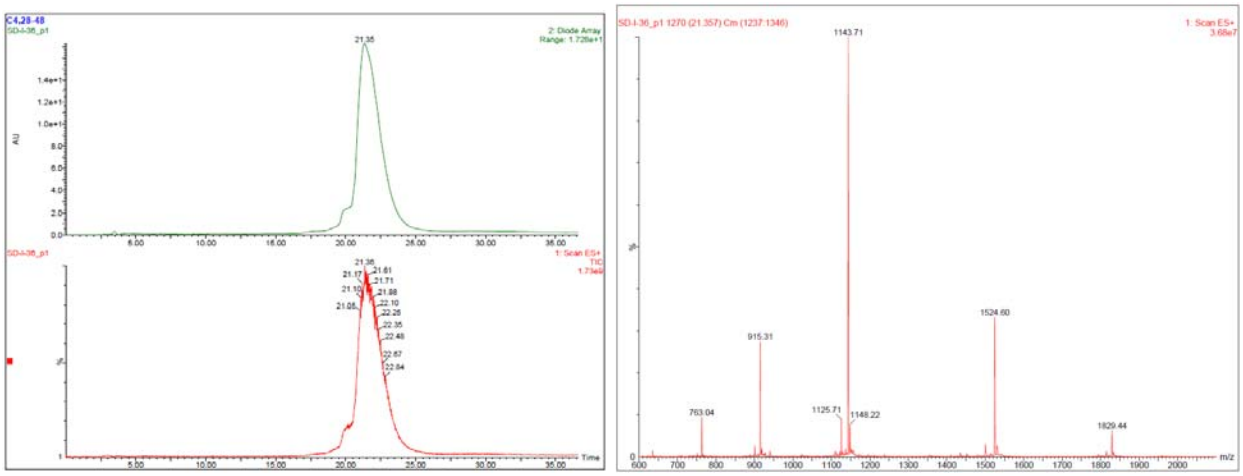
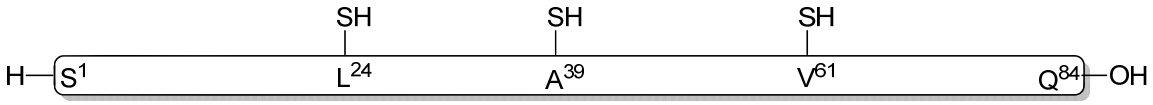


Figure S10. LC-MS traces and Mass-spec data for peptide **18:** Calcd for $C_{204}H_{331}N_{59}O_{56}S_2$: 4570.30 Da(average isotopes), $[M+3H]^{3+}$ *m/z* = 1524.43, $[M+4H]^{4+}$ *m/z* = 1143.58, $[M+5H]^{5+}$ *m/z* = 915.06, $[M+6H]^{6+}$ *m/z* = 762.72; observed: $[M+3H]^{3+}$ *m/z* = 1524.60, $[M+4H]^{4+}$ *m/z* = 1143.71, $[M+5H]^{5+}$ *m/z* = 915.31, $[M+6H]^{6+}$ *m/z* = 763.04.

Ligated Peptide 19



19

According to the General Procedure D, peptide **17** (3.4 mg, 0.74 μmol, 1.0 equiv) and peptide **14** (3.7 mg, 0.74 μmol, 1.0 equiv) were dissolved in 350 μL of ligation buffer. The reaction mixture was stirred at room temperature for 3 h. The reactions were monitored by LC-MS and diluted with 2 mL of $CH_3CN/H_2O/AcOH$ (25:75:5) upon completion.

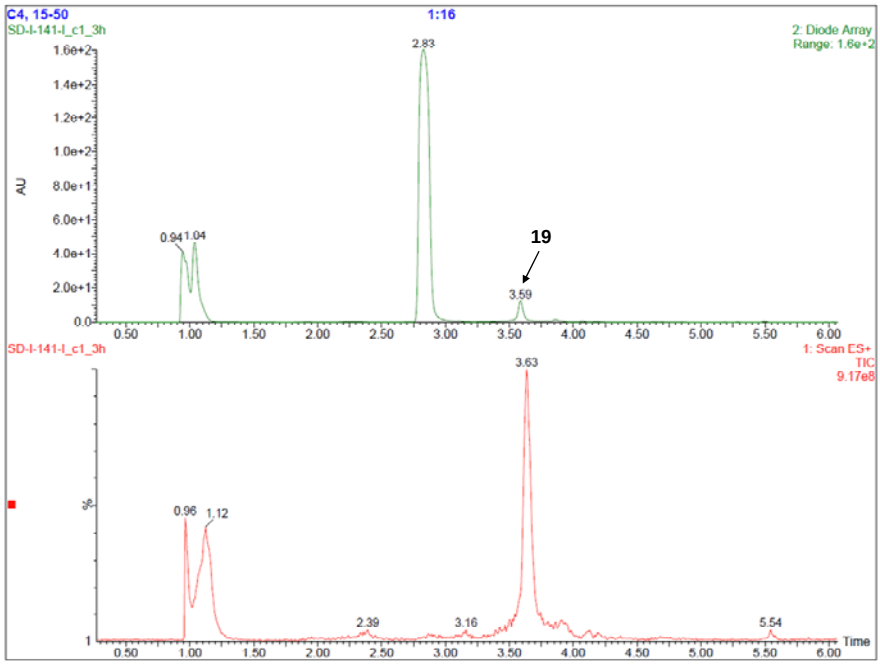
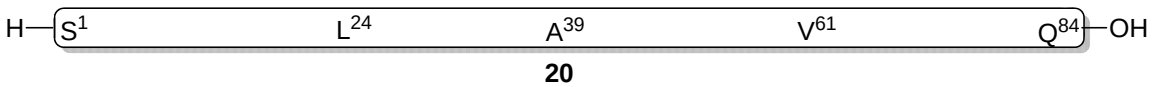


Figure S11. LC-MS traces of crude reactions.

The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 19.5-21 min. The fractions were collected, and concentrated via lyophilization to afford peptide **19** (4.4 mg, 63%) as a white solid. Mass-spec of peptidyl thioester **19** was identical to literature reported data.[S3]

hPTH(1-84) (20)



20

According to the General Procedure E, to a solution of peptide **19** (4.4 mg, 0.46 μmol) in

0.5 mL of degassed CH_3CN/H_2O (1:1) was added 0.5 mL of 0.5 M bond-breaker® TCEP solution (Pierce), 50 µL of 2-methyl-2-propanethiol and 0.5 mL of radical initiator VA-044 (0.1 M in degassed H_2O). The reaction mixture was stirred at 37 °C and monitored by LC-MS.

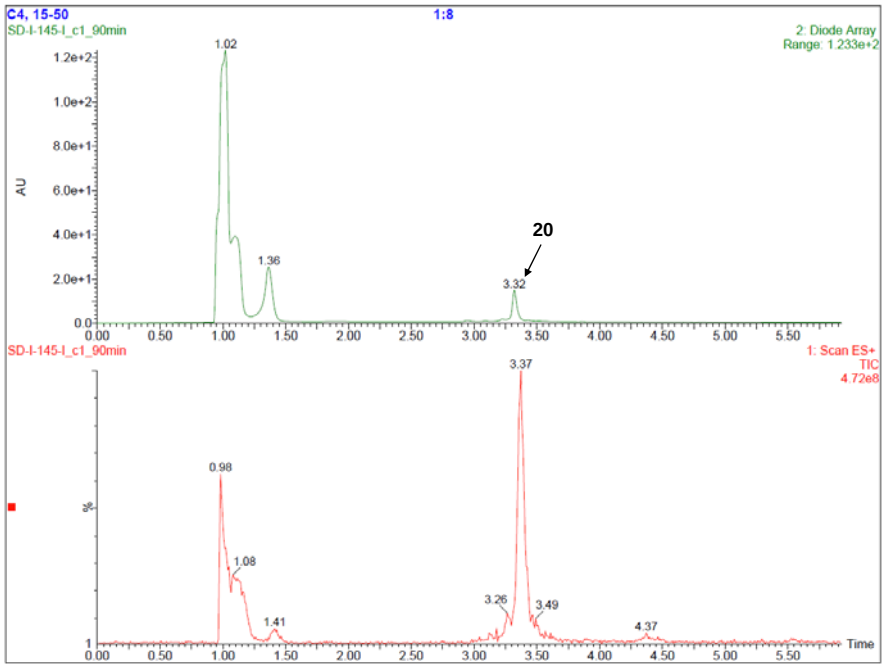


Figure S12. LC-MS traces of crude reactions.

Upon completion, the reaction was diluted with 3 mL of CH_3CN/H_2O/AcOH (25:75:5). The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 19-21 min. The fractions were collected, and concentrated via lyophilization to afford hPTH(1-84) (**20**, 3.7 mg, 86%) as a white solid.

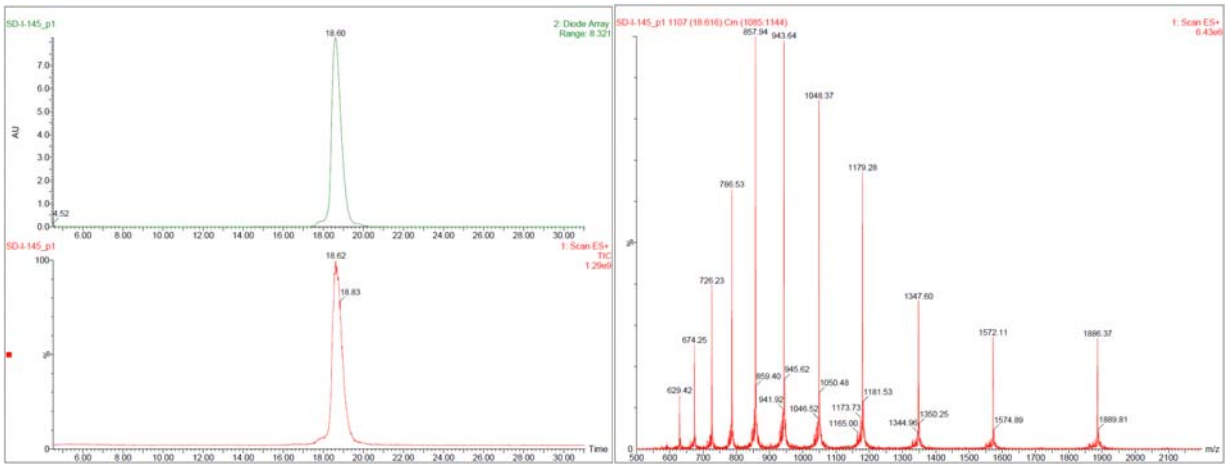
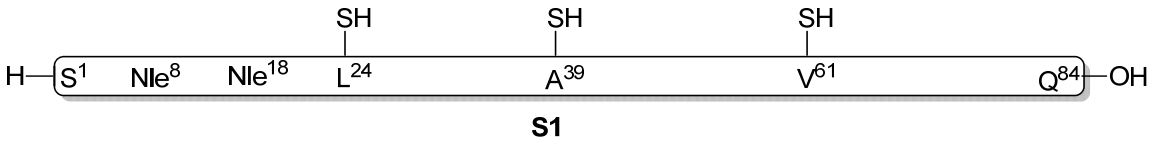


Figure S13. LC-MS traces and Mass-spec data for **20:** Calcd for $C_{408}H_{674}N_{126}O_{126}S_2$: 9424.62

Da(average isotopes), $[M+5H]^{5+}$ *m/z* = 1885.92, $[M+6H]^{6+}$ *m/z* = 1571.77, $[M+7H]^{7+}$ *m/z* = 1347.37, $[M+8H]^{8+}$ *m/z* = 1179.08, $[M+9H]^{9+}$ *m/z* = 1048.18, $[M+10H]^{10+}$ *m/z* = 943.46, $[M+11H]^{11+}$ *m/z* = 857.78, $[M+12H]^{12+}$ *m/z* = 786.38, $[M+13H]^{13+}$ *m/z* = 725.97, $[M+14H]^{14+}$ *m/z* = 674.19, $[M+15H]^{15+}$ *m/z* = 629.31; observed: $[M+5H]^{5+}$ *m/z* = 1886.37, $[M+6H]^{6+}$ *m/z* = 1572.11, $[M+7H]^{7+}$ *m/z* = 1347.60, $[M+8H]^{8+}$ *m/z* = 1179.28, $[M+9H]^{9+}$ *m/z* = 1048.37, $[M+10H]^{10+}$ *m/z* = 943.64, $[M+11H]^{11+}$ *m/z* = 857.94, $[M+12H]^{12+}$ *m/z* = 786.53, $[M+13H]^{13+}$ *m/z* = 726.23, $[M+14H]^{14+}$ *m/z* = 674.25, $[M+15H]^{15+}$ *m/z* = 629.42.

Ligated Peptide S1



S1

According to the General Procedure D, peptide **18** (2.1 mg, 0.46 μmol, 1.0 equiv) and peptide **14** (2.3 mg, 0.46 μmol, 1.0 equiv) were dissolved in 200 μL of ligation buffer. The reaction mixture was stirred at room temperature for 2.5 h. The reactions were monitored by LC-MS (*cf.* Figure S15) and diluted with 2 mL of CH_3CN/H_2O/AcOH (25:75:5) upon completion. The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 21-23 min. The fractions were collected, and concentrated via lyophilization to afford peptide **S1** (3.0 mg, 69%) as a white solid.

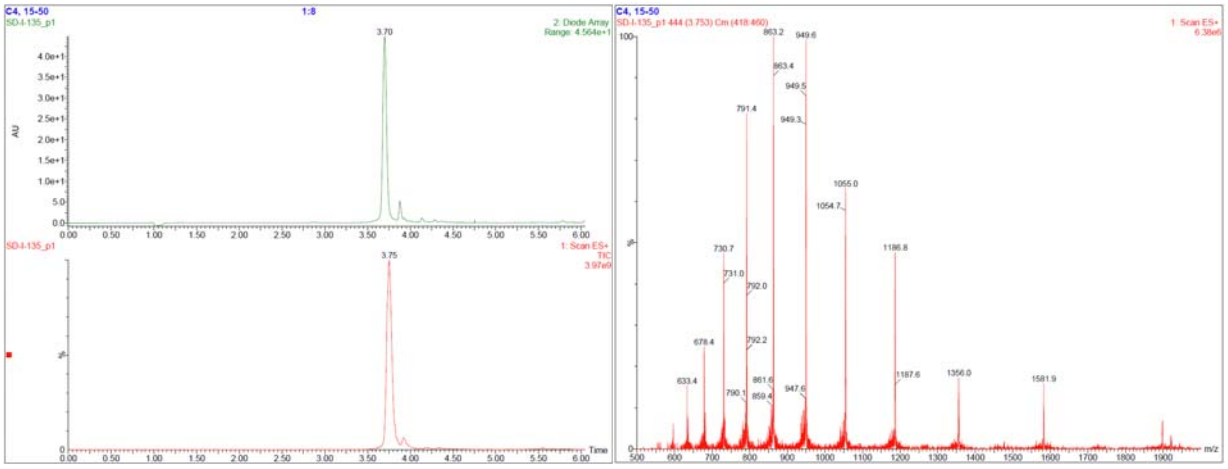
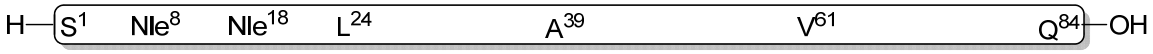


Figure S14. LC-MS traces and Mass-spec data for **S1:** Calcd for $C_{410}H_{678}N_{126}O_{126}S_3$: 9484.73 Da(average isotopes), $[M+6H]^{6+}$ *m/z* = 1581.79, $[M+7H]^{7+}$ *m/z* = 1355.96, $[M+8H]^{8+}$ *m/z* =

1186.59, $[M+9H]^{9+}$ *m/z* = 1054.86, $[M+10H]^{10+}$ *m/z* = 949.47, $[M+11H]^{11+}$ *m/z* = 863.25, $[M+12H]^{12+}$ *m/z* = 791.39, $[M+13H]^{13+}$ *m/z* = 730.59, $[M+14H]^{14+}$ *m/z* = 678.48, $[M+15H]^{15+}$ *m/z* = 633.32; observed: $[M+6H]^{6+}$ *m/z* = 1581.9, $[M+7H]^{7+}$ *m/z* = 1356.0, $[M+8H]^{8+}$ *m/z* = 1186.8, $[M+9H]^{9+}$ *m/z* = 1055.0, $[M+10H]^{10+}$ *m/z* = 949.6, $[M+11H]^{11+}$ *m/z* = 863.2, $[M+12H]^{12+}$ *m/z* = 791.4, $[M+13H]^{13+}$ *m/z* = 730.7, $[M+14H]^{14+}$ *m/z* = 678.4, $[M+15H]^{15+}$ *m/z* = 633.4.

[Nle^{8,18}]hPTH(1-84) (21)



21

According to the General Procedure E and the procedure described for the preparation of peptide **20**, to a solution of peptide **S1** (2.3 mg, 0.24 μmol) in 0.3 mL of degassed CH_3CN/H_2O (1:1) was added 0.3 mL of 0.5 M bond-breaker® TCEP solution (Pierce), 30 μL of 2-methyl-2-propanethiol and 0.3 mL of radical initiator VA-044 (0.1 M in degassed H_2O). The reaction mixture was stirred at 37 °C and monitored by LC-MS (Figure S15).

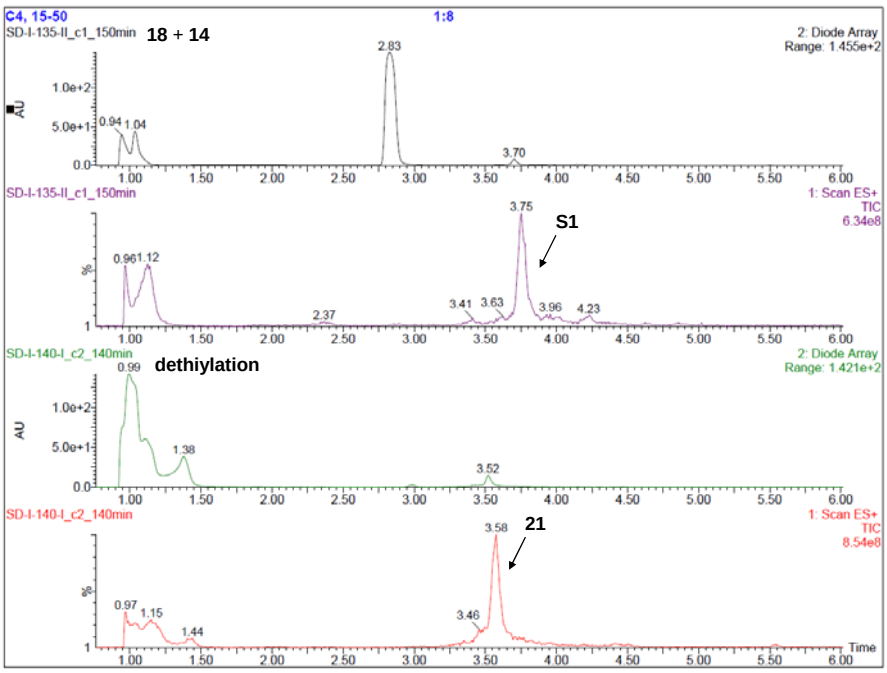


Figure S15. LC-MS traces of crude reactions.

Upon completion, the reaction was diluted with 2 mL of CH_3CN/H_2O/AcOH (25:75:5). The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 21-23 min. The

fractions were collected, and concentrated via lyophilization to afford [Nle^{8,18}]hPTH(1-84) (**21**, 1.7 mg, 75%) as a white solid.

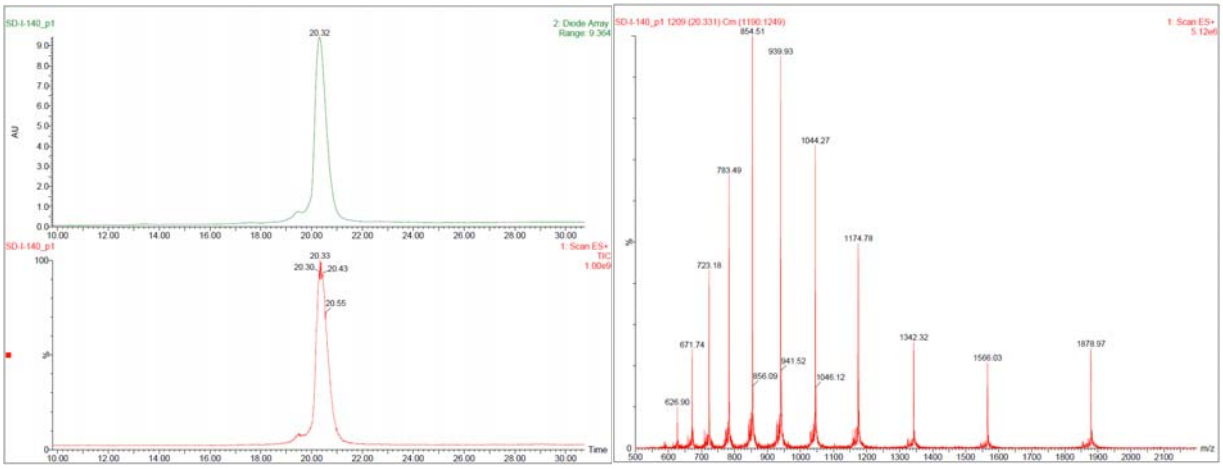
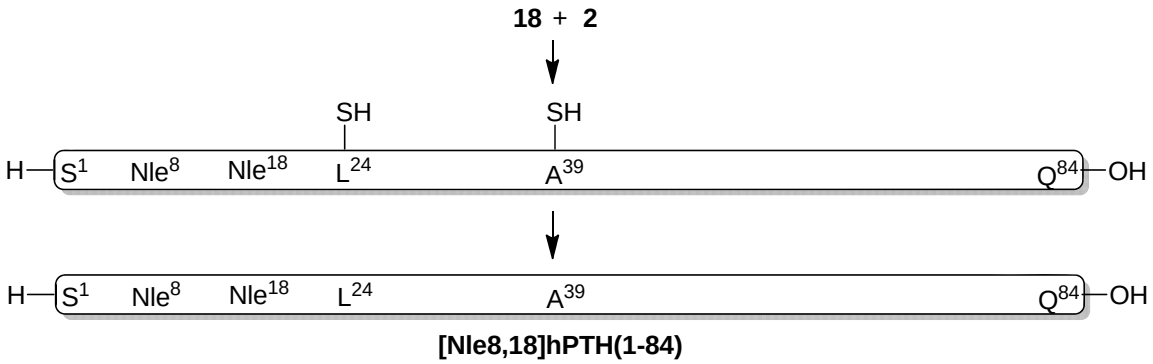


Figure S16. LC-MS traces and Mass-spec data for peptide **21:** Calcd for $C_{410}H_{678}N_{126}O_{126}$: 9388.54 Da(average isotopes), $[M+5H]^{5+}$ *m/z* = 1878.71, $[M+6H]^{6+}$ *m/z* = 1565.76, $[M+7H]^{7+}$ *m/z* = 1342.22, $[M+8H]^{8+}$ *m/z* = 1174.57, $[M+9H]^{9+}$ *m/z* = 1044.17, $[M+10H]^{10+}$ *m/z* = 939.85, $[M+11H]^{11+}$ *m/z* = 854.50, $[M+12H]^{12+}$ *m/z* = 783.38, $[M+13H]^{13+}$ *m/z* = 723.20, $[M+14H]^{14+}$ *m/z* = 671.61, $[M+15H]^{15+}$ *m/z* = 626.90; observed: $[M+5H]^{5+}$ *m/z* = 1878.97, $[M+6H]^{6+}$ *m/z* = 1566.03, $[M+7H]^{7+}$ *m/z* = 1342.32, $[M+8H]^{8+}$ *m/z* = 1174.78, $[M+9H]^{9+}$ *m/z* = 1044.27, $[M+10H]^{10+}$ *m/z* = 939.93, $[M+11H]^{11+}$ *m/z* = 854.51, $[M+12H]^{12+}$ *m/z* = 783.49, $[M+13H]^{13+}$ *m/z* = 723.18, $[M+14H]^{14+}$ *m/z* = 671.74, $[M+15H]^{15+}$ *m/z* = 626.90.

Alternative Preparation of [Nle^{8,18}]hPTH(1-84)



According to the procedure described for the preparation of peptide **S1**, peptide **18** (1.2 mg, 0.26 μmol, 1.1 equiv) and peptide **2** (1.2 mg, 0.24 μmol, 1.0 equiv) were dissolved in 200 μL of ligation buffer (*c.f.* General Procedure D). The reaction mixture was stirred at room temperature for 2.5 h and monitored by LC-MS. Upon completion, the reaction mixture was diluted with 3

mL of guanidine buffer (6 M Gn·HCl, 200 mM Na_2HPO_4), and then concentrated using an Amicon® ultra-4 centrifugal filter (ultracel-3 membrane, MWCO 3000). The dilution/centrifugation process was repeated two more times to remove most of the MPAA. The resulting buffer solution (~300 µL) containing ligated peptide was transferred to a small reaction vessel, degassed for 15 min using an argon stream, followed by the addition of 0.3 mL of 0.5 M bond-breaker® TCEP solution (Pierce), 30 µL of 2-methyl-2-propanethiol and 0.3 mL of radical initiator VA-044 (0.1 M in degassed H_2O). The reaction was stirred at 37 °C under an argon atmosphere and monitored by LC-MS. Upon completion, the reaction was diluted with 2 mL of CH_3CN/H_2O/AcOH (25:75:5). The crude mixture was purified using RP-HPLC (linear gradient 23-43% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 19-21 min. The fractions were collected, and concentrated via lyophilization to afford [$Nle^{8,18}$]hPTH(1-84) (1.4 mg, 63%, two steps) as a white solid.

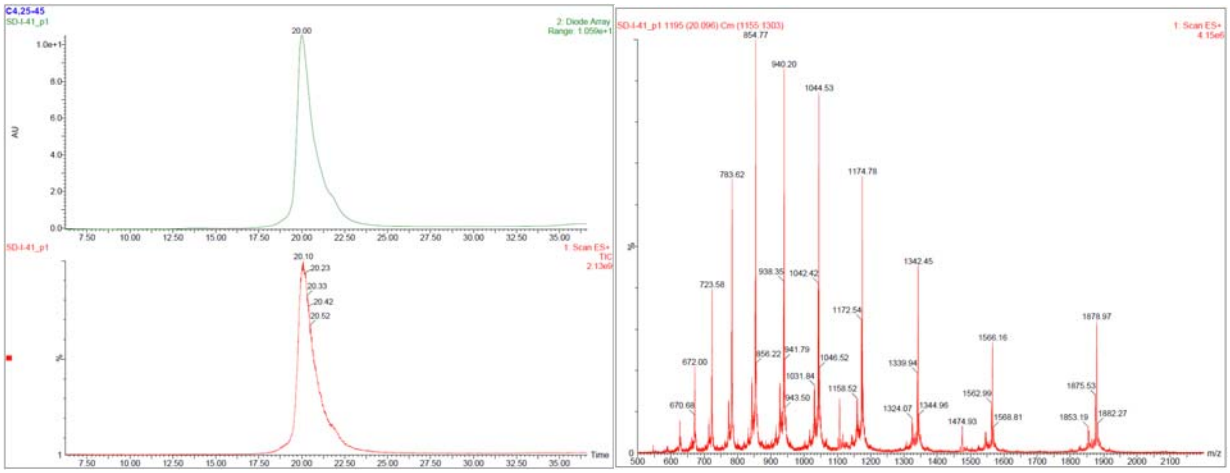
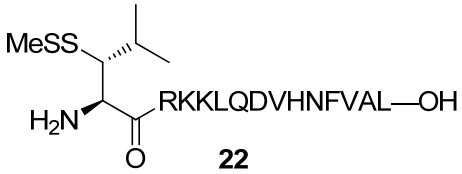


Figure S17. LC-MS traces and Mass-spec data.

Thioleucine-containing Peptide 22



22

The peptide resin from the Fmoc SPPS (9.12 µmol, 1.0 equiv) was mixed with Boc-Leu(SSMe)-OH (4.8 mg, 15.50 µmol, 1.7 equiv), HATU (17.3 mg, 45.6 µmol, 5.0 equiv) and DIEA (15.9 µL, 91.2 µmol, 10.0 equiv) in DMF (500 µL) and stirred at room temperature for 10

min. The resin was washed with DMF, DCM and MeOH several times and dried under vacuum. The dried resin was treated with TFA/TIS/H_2O (95:2.5:2.5) for 40 min, TFA was blown off by N_2 and the oily residue was triturated with diethyl ether. The precipitate was pelleted and the ether was subsequently decanted. The resulting solid was purified using RP-HPLC (linear gradient 19-39% solvent B over 30 min, Microsorb 100-8 C18 column, 16 mL/min, 230 nm), and the product eluted at 15.5-20 min. The fractions were collected, and concentrated via lyophilization to afford peptide **22** (8.2 mg, 51% based on 0.05 mmol of resin) as a white solid.

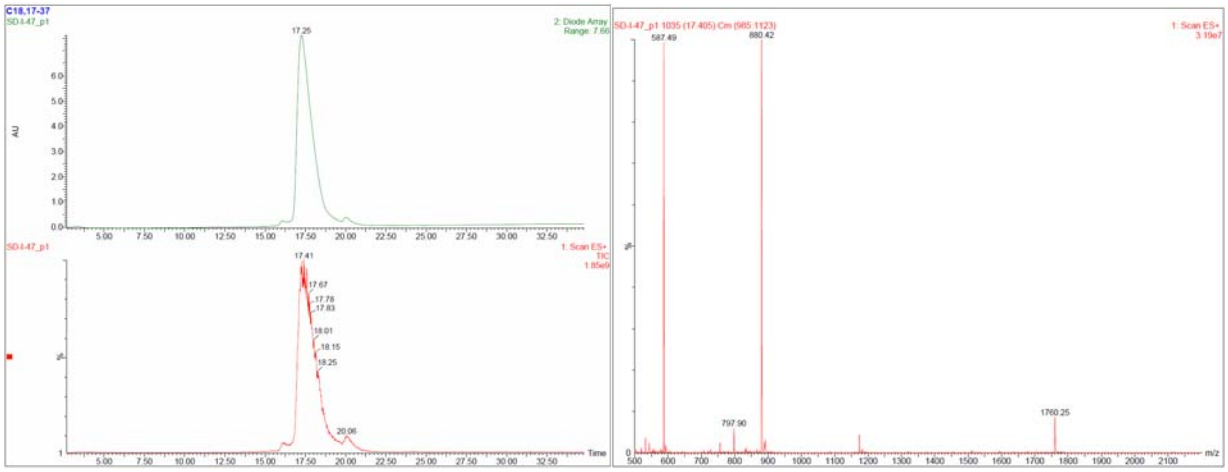
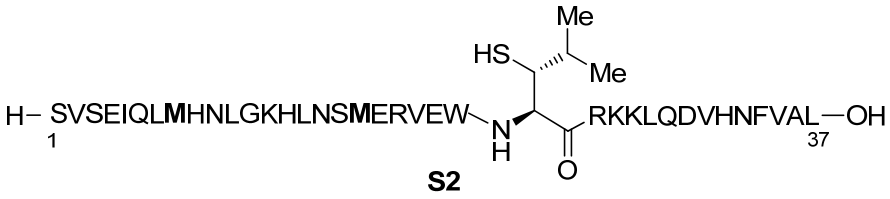


Figure S18. LC-MS traces and Mass-spec data for peptide **22:** Calcd for $C_{78}H_{131}N_{23}O_{19}S_2$: 1759.15 Da(average isotopes), $[M+2H]^{2+}$ *m/z* = 880.58, $[M+3H]^{3+}$ *m/z* = 587.38; observed: $[M+H]^{+}$ *m/z* =1760.25, $[M+2H]^{2+}$ *m/z* = 880.42, $[M+3H]^{3+}$ *m/z* = 587.49.

Ligated Peptide S2



S2

According to the General Procedure C, peptide **8** (2.5 mg, 0.86 μmol, 1.3 equiv) and peptide **22** (1.2 mg, 0.68 μmol, 1.0 equiv) were dissolved in 300 μL of ligation buffer. The reaction mixture was stirred at room temperature for 4 h. The reactions were monitored by LC-MS and diluted with 2 mL of CH_3CN/H_2O/AcOH (25:75:5) upon completion. The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 19-21 min. The fractions were

collected, and concentrated via lyophilization to afford peptide **S2** (2.0 mg, 66%) as a white solid.

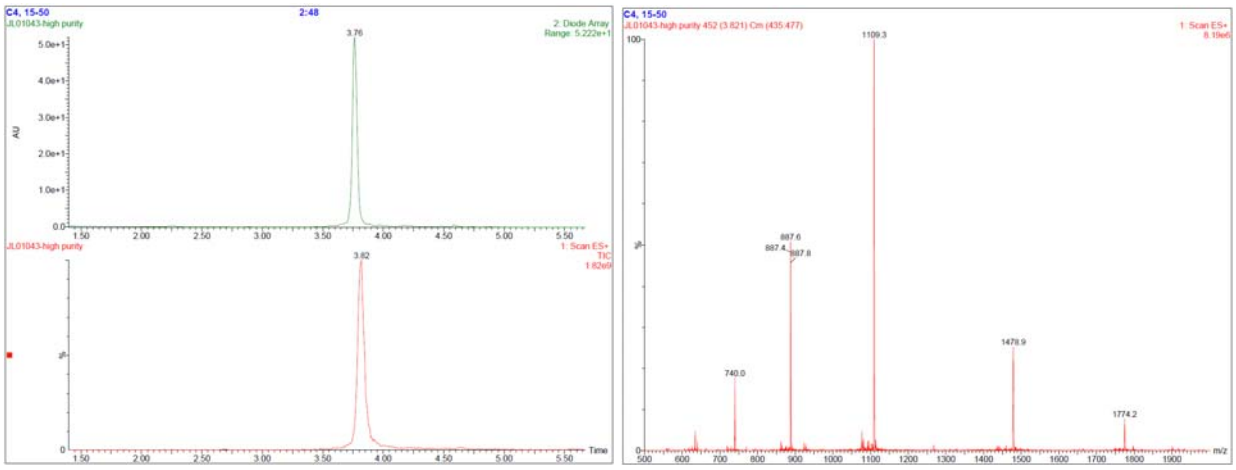
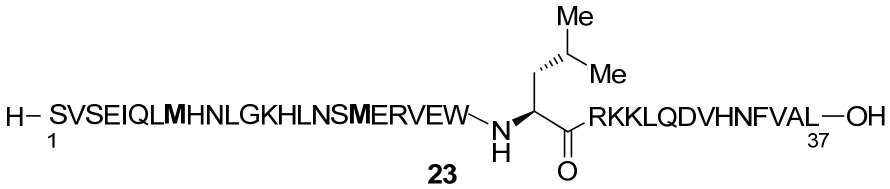


Figure S19. LC-MS traces and Mass-spec data for peptide **S2:** Calcd for $C_{195}H_{316}N_{58}O_{54}S_3$: 4433.15 Da(average isotopes), $[2M+5H]^{5+}$ *m/z* = 1774.26, $[M+3H]^{3+}$ *m/z* = 1478.72, $[M+4H]^{4+}$ *m/z* = 1109.29, $[M+5H]^{5+}$ *m/z* = 887.63, $[M+6H]^{6+}$ *m/z* = 739.86; observed: $[2M+5H]^{5+}$ *m/z* = 1774.2, $[M+3H]^{3+}$ *m/z* = 1478.9, $[M+4H]^{4+}$ *m/z* = 1109.3, $[M+5H]^{5+}$ *m/z* = 887.6, $[M+6H]^{6+}$ *m/z* = 740.0.

hPTH(1-37) (23)



23

According to the General Procedure E and the procedure described for the preparation of peptide **20**, to a solution of peptide **S2** (2.0 mg, 0.45 μmol) in 0.6 mL of degassed CH_3CN/H_2O (1:1) was added 0.6 mL of 0.5 M bond-breaker® TCEP solution (Pierce), 60 μL of 2-methyl-2-propanethiol and 0.6 mL of radical initiator VA-044 (0.1 M in degassed H_2O). The reaction mixture was stirred at 37 °C and monitored by LC-MS.

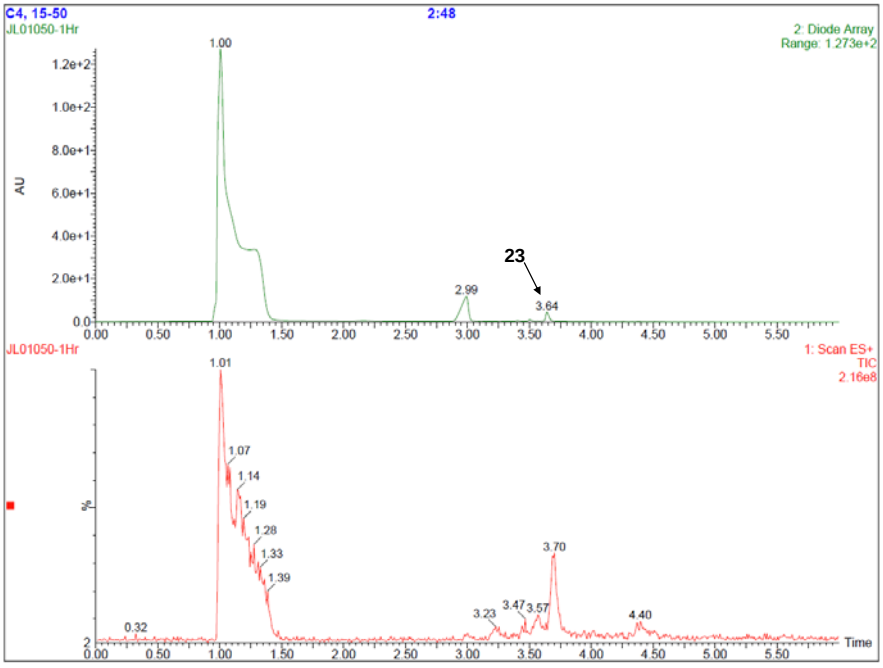


Figure S20. LC-MS traces of crude reactions.

Upon completion, the reaction was diluted with 3 mL of CH_3CN/H_2O/AcOH (25:75:5). The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 19-21 min. The fractions were collected, and concentrated via lyophilization to afford peptide hPTH(1-37) (**23**, 1.6 mg, 81%) as a white solid.

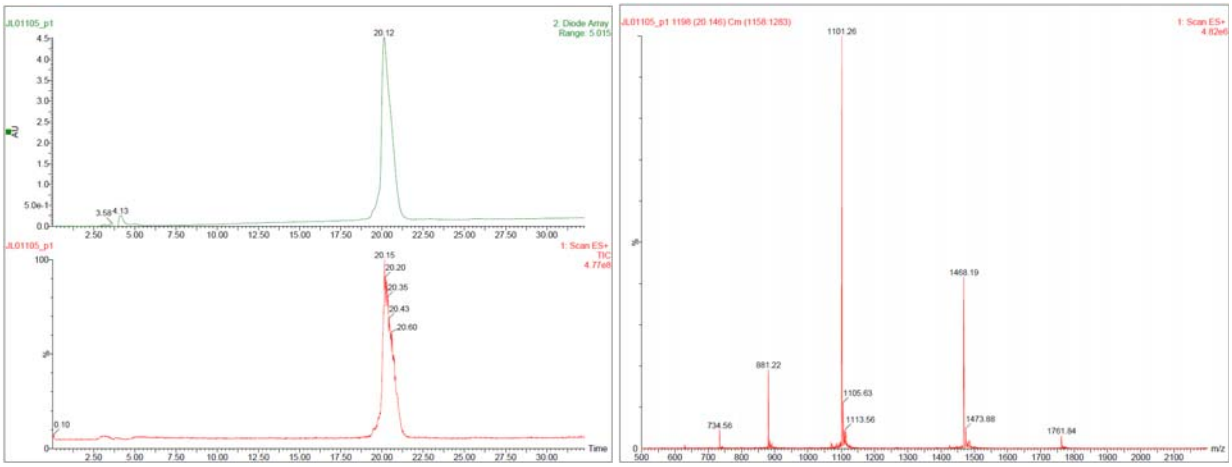
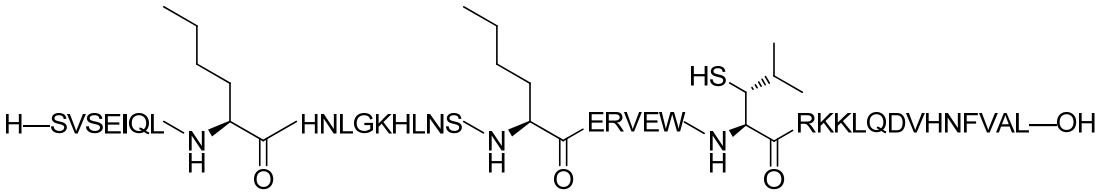


Figure S21. LC-MS traces and Mass-spec data for peptide **23:** Calcd for $C_{195}H_{316}N_{58}O_{54}S_2$: 4401.08 Da(average isotopes), $[2M+5H]^{5+}$ *m/z* = 1761.43, $[M+3H]^{3+}$ *m/z* = 1468.03, $[M+4H]^{4+}$ *m/z* = 1101.27, $[M+5H]^{5+}$ *m/z* = 881.22, $[M+6H]^{6+}$ *m/z* = 734.51; observed: $[2M+5H]^{5+}$ *m/z* = 1761.84, $[M+3H]^{3+}$ *m/z* = 1468.19, $[M+4H]^{4+}$ *m/z* = 1101.26, $[M+5H]^{5+}$ *m/z* = 881.22, $[M+6H]^{6+}$

m/z = 734.56.

Ligated Peptide S3



S3

According to General Procedure C and the procedure described for the preparation of peptide **S2**, Peptide **9** (2.3 mg, 0.80 μmol, 1.1 equiv) and peptide **22** (1.3 mg, 0.68 μmol, 1.0 equiv) were dissolved in 300 μL of ligation buffer. The reaction mixture was stirred at room temperature for 4 h. The reactions were monitored by LC-MS and diluted with 2 mL of CH_3CN/H_2O/AcOH (25:75:5) upon completion.

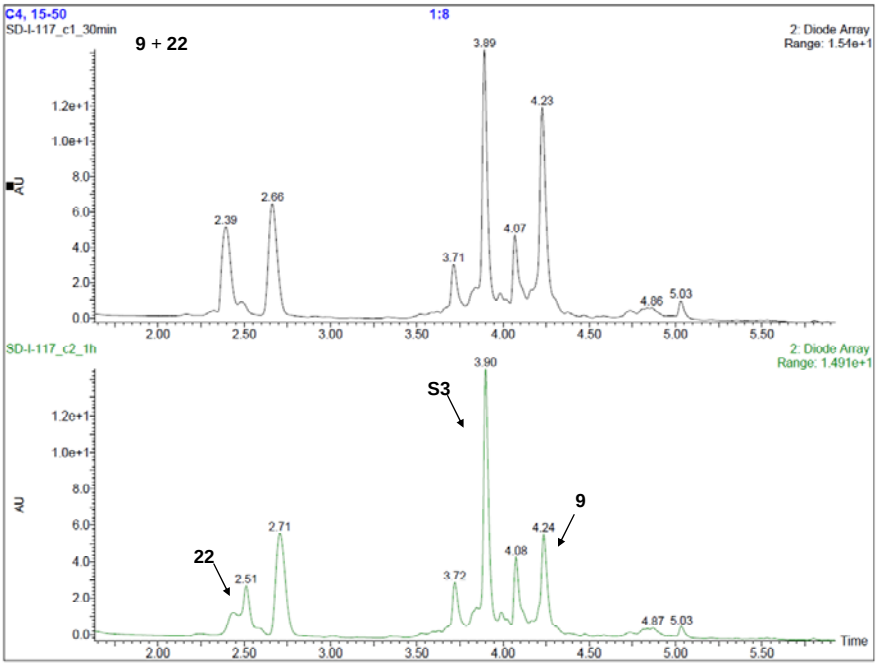


Figure S22. LC traces of crude reactions.

The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 19.5-21 min. The fractions were collected, and concentrated via lyophilization to afford peptide **S3** (2.2 mg, 68%) as a white solid.

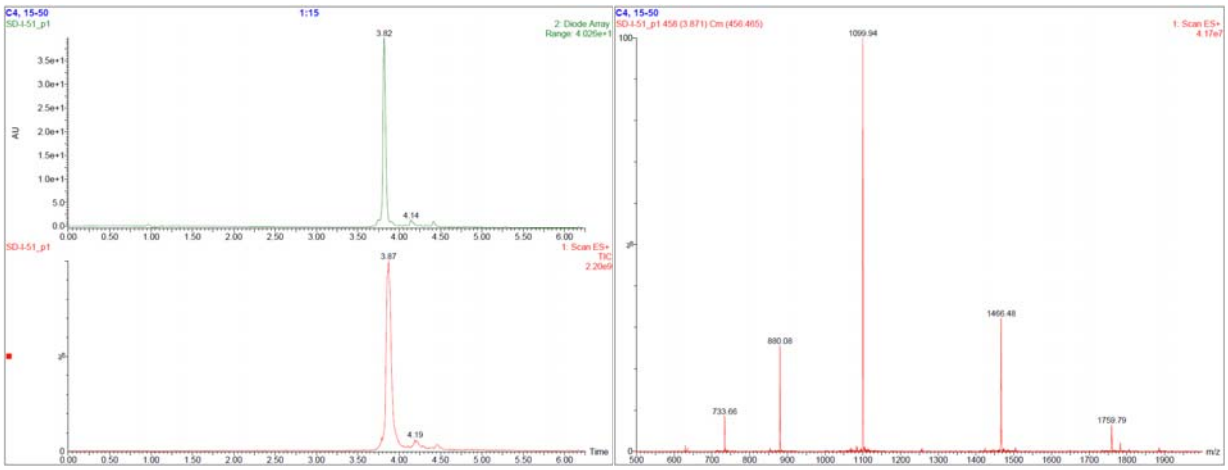
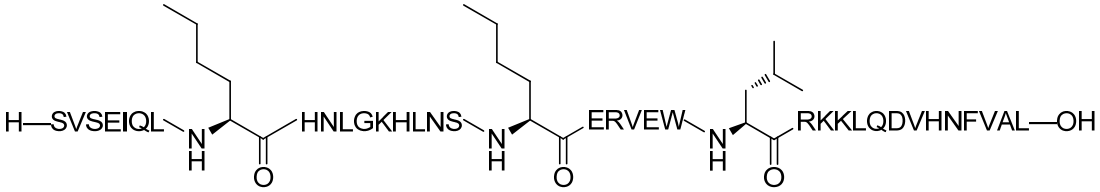


Figure S23. LC-MS traces and Mass-spec data for peptide **S3:** Calcd for $C_{197}H_{320}N_{58}O_{54}S$: 4397.07 Da(average isotopes), $[2M+5H]^{5+}$ *m/z* = 1759.83, $[M+3H]^{3+}$ *m/z* = 1466.69, $[M+4H]^{4+}$ *m/z* = 1100.27, $[M+5H]^{5+}$ *m/z* = 880.41, $[M+6H]^{6+}$ *m/z* = 733.84; observed: $[2M+5H]^{5+}$ *m/z* = 1759.79, $[M+3H]^{3+}$ *m/z* = 1466.48, $[M+4H]^{4+}$ *m/z* = 1099.94, $[M+5H]^{5+}$ *m/z* = 880.08, $[M+6H]^{6+}$ *m/z* = 733.66.

[Nle^{8,18}]hPTH(1-37) (24)



24

According to the General Procedure E and the procedure described for the preparation of peptide **23**, to a solution of peptide **S3** (2.0 mg, 0.45 μmol) in 0.6 mL of degassed CH_3CN/H_2O (1:1) was added 0.6 mL of 0.5 M bond-breaker® TCEP solution (Pierce), 60 μL of 2-methyl-2-propanethiol and 0.6 mL of radical initiator VA-044 (0.1 M in degassed H_2O). The reaction mixture was stirred at 37 °C and monitored by LC-MS.

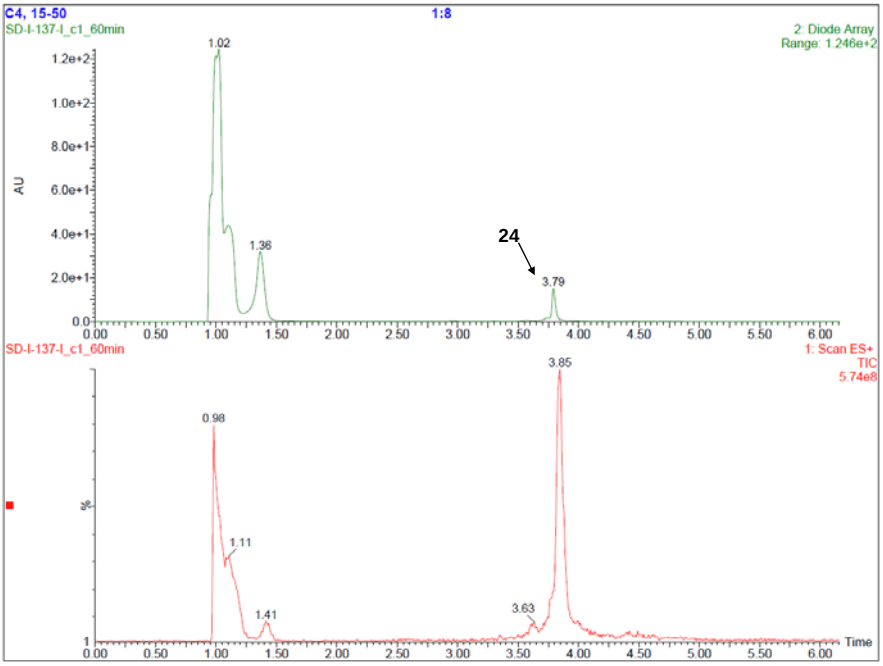


Figure S24. LC-MS traces of crude reactions.

Upon completion, the reaction was diluted with 3 mL of CH_3CN/H_2O/AcOH (25:75:5). The crude mixture was purified using RP-HPLC (linear gradient 22-42% solvent B over 30 min, Microsorb 300-5 C4 column, 16 mL/min, 230 nm), and the product eluted at 21-22.5 min. The fractions were collected, and concentrated via lyophilization to afford peptide [Nle^{8,18}]hPTH(1-37) (**24**, 1.7 mg, 86%) as a white solid.

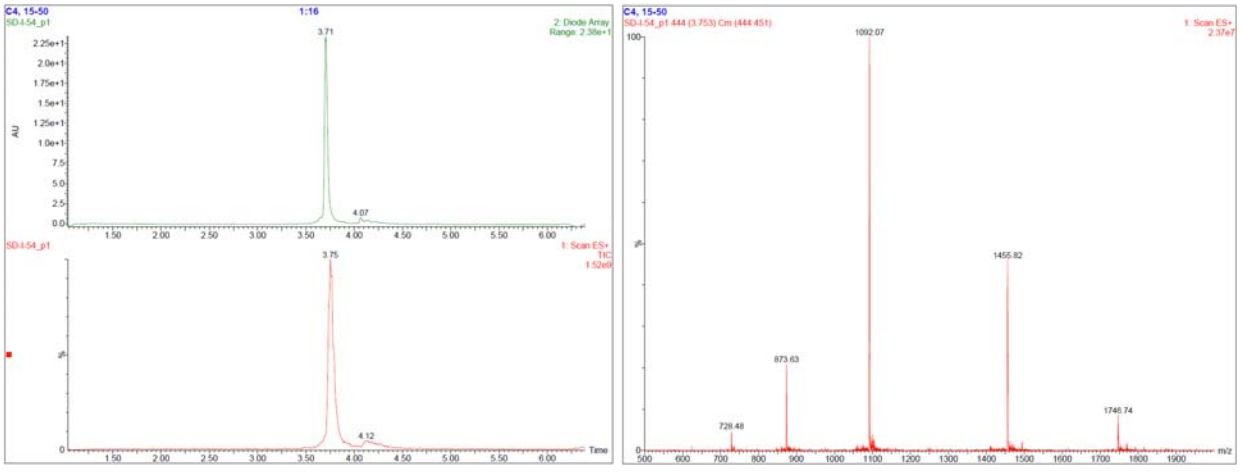


Figure S25. LC-MS traces and Mass-spec data for peptide **24:** Calcd for $C_{197}H_{320}N_{58}O_{54}$: 4365.00 Da(average isotopes), $[2M+5H]^{5+}$ *m/z* = 1747.00, $[M+3H]^{3+}$ *m/z* = 1456.00, $[M+4H]^{4+}$ *m/z* = 1092.25, $[M+5H]^{5+}$ *m/z* = 874.00, $[M+6H]^{6+}$ *m/z* = 728.50; observed: $[2M+5H]^{5+}$ *m/z* = 1746.74, $[M+3H]^{3+}$ *m/z* = 1455.82, $[M+4H]^{4+}$ *m/z* = 1092.07, $[M+5H]^{5+}$ *m/z* = 873.63, $[M+6H]^{6+}$

m/z = 728.48.

CD Spectra:

CD spectra were obtained on an Aviv 410 circular dichroism spectropolarimeter. Protein concentrations were determined based on the extinction coefficient, calculated according to the number of Trp residue.[S4] Sample was prepared as H_2O solution and the spectra were collected using a 1 mm pathlength cuvette.

[S4] Edelhoch H. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* **1967**, *6*, 1948.

Storage stability test

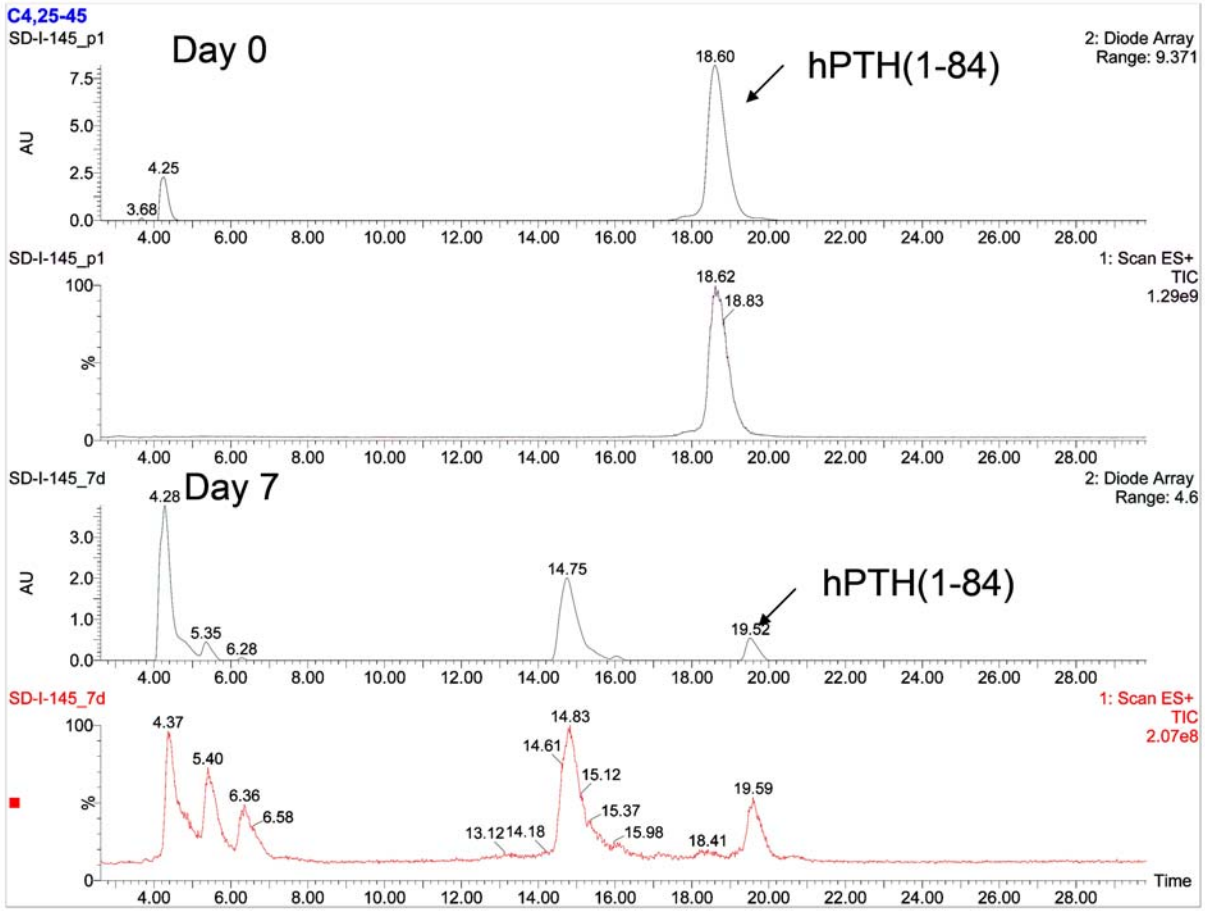


Figure S26. LC traces of hPTH(1-84) in water solution under ambient condtions.

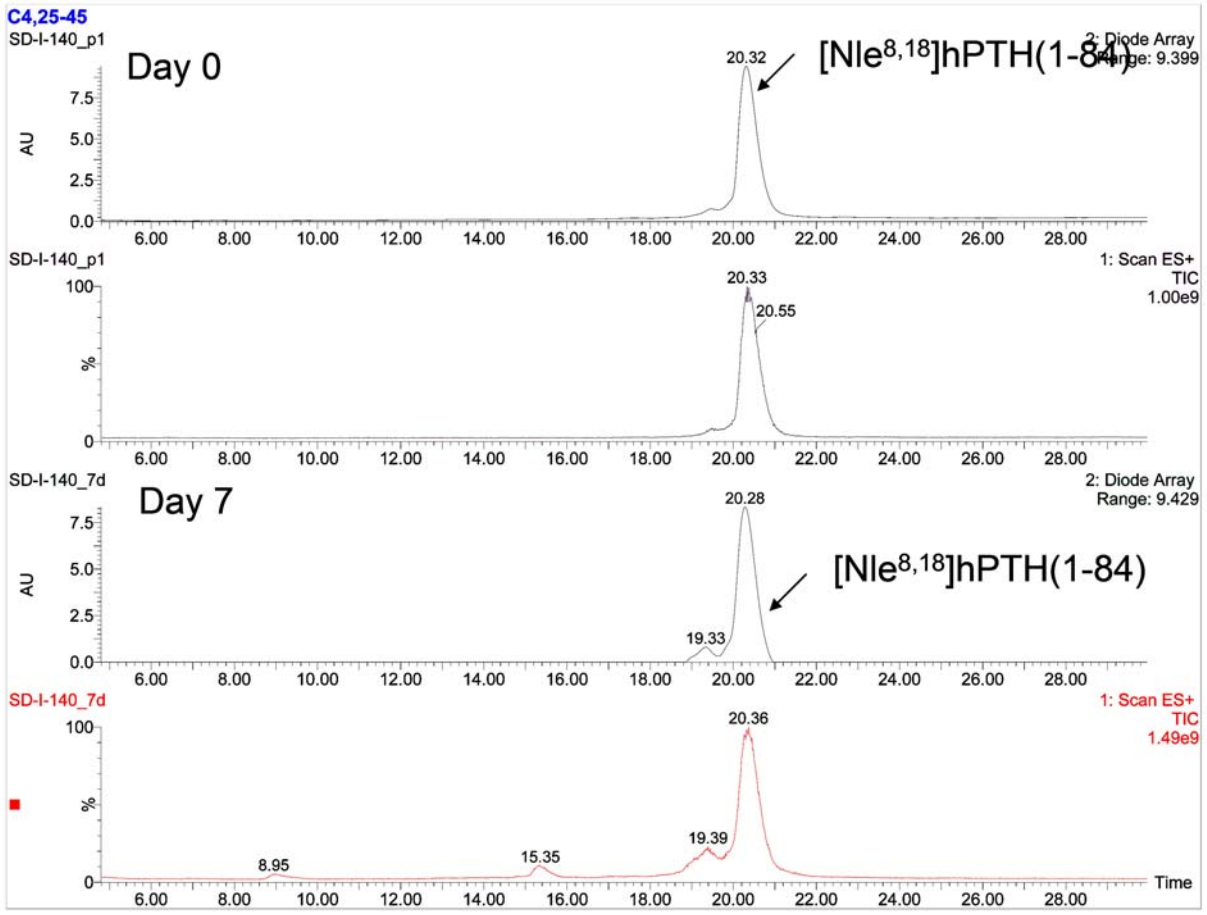


Figure S27. LC traces of [Nle^{8,18}]hPTH(1-84) in water solution under ambient condtions.

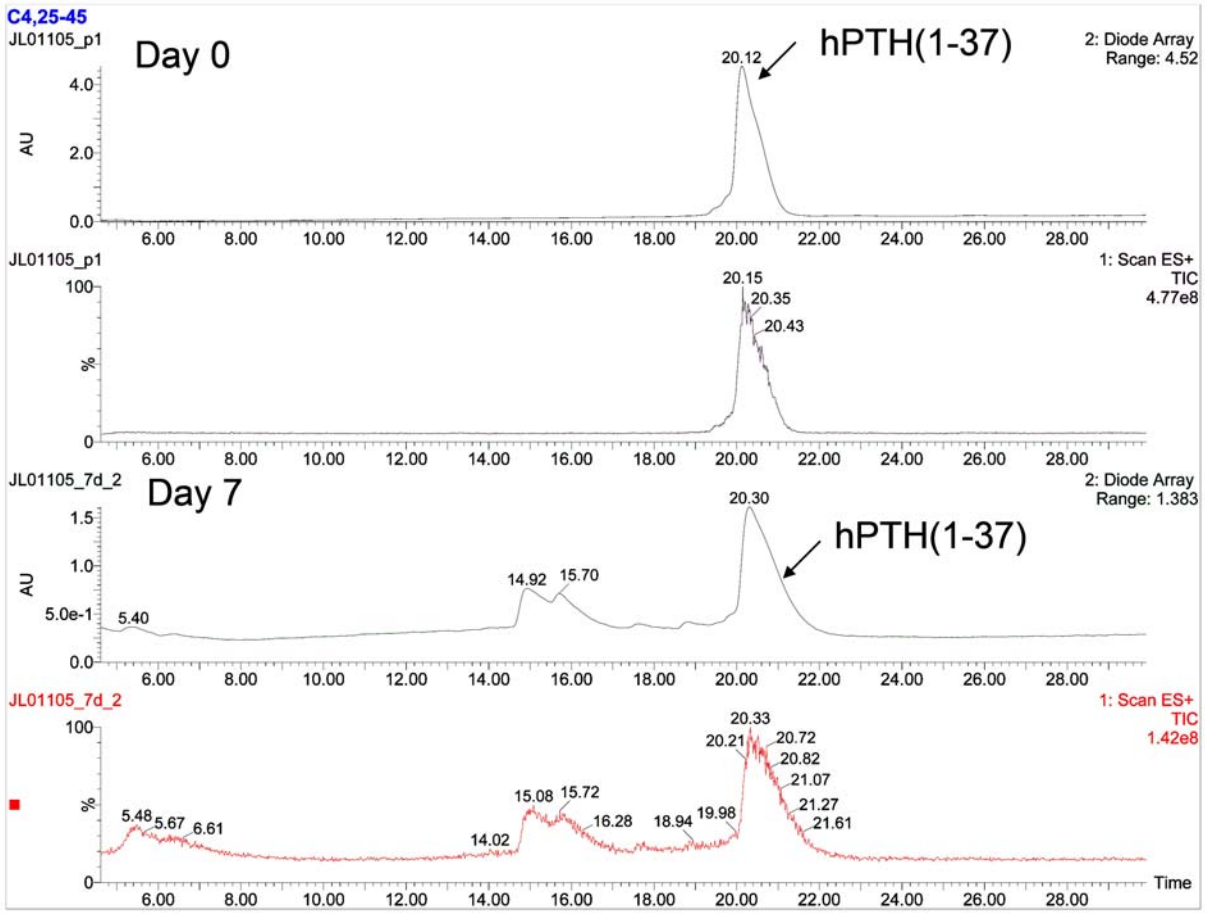


Figure S28. LC traces of hPTH(1-37) in water solution under ambient condtions.

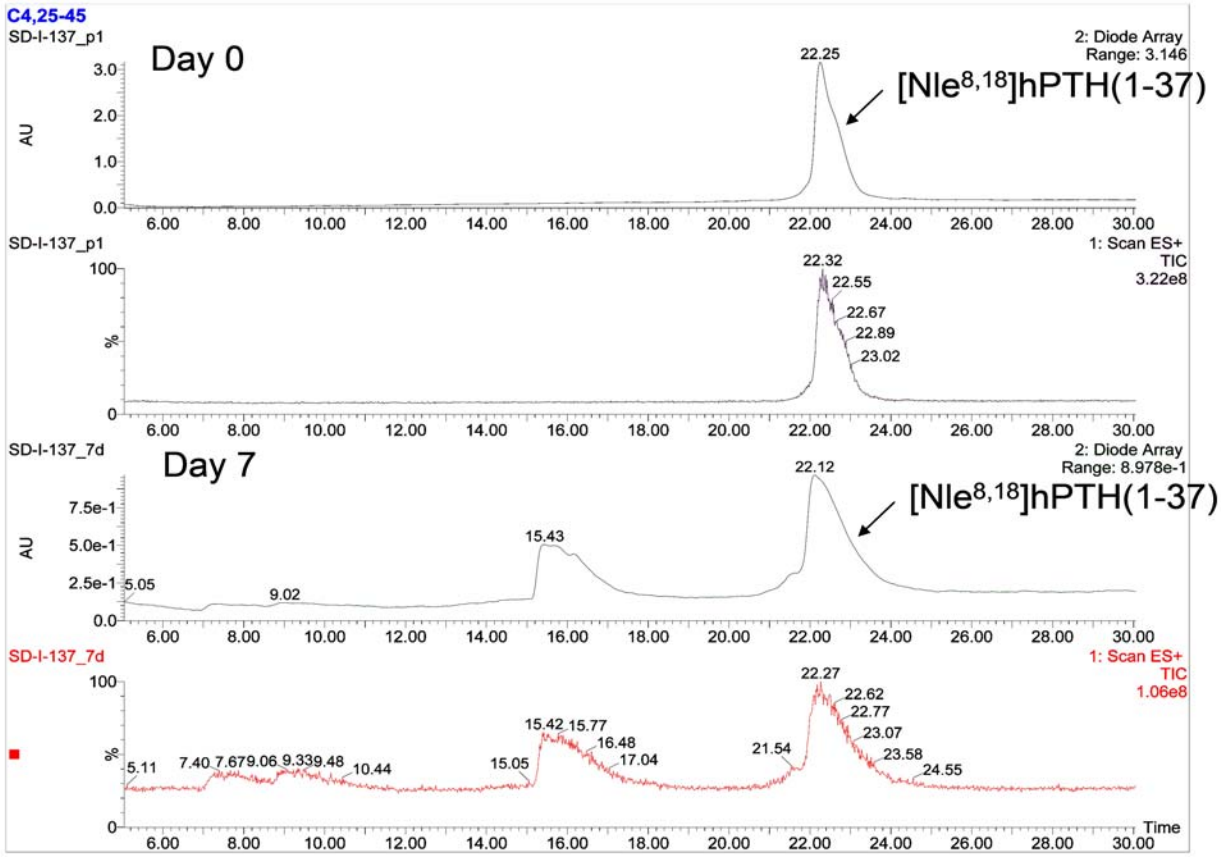


Figure S29. LC traces of [Nle^{8,18}]hPTH(1-37) in water solution under ambient condtions.