Electronic Supplementary Information

Triblock Peptide-Linker-Lipid Molecular Design Improves Potency of Peptide Ligands Targeting Family B G Protein-Coupled Receptors

Yuting Liu,[†a] Yingying Cai,[†a] Wei Liu,[a] Xiao-Han Li,[a] Elizabeth Rhoades[bc] and Elsa C. Y. Yan[*a]

[a] Department of Chemistry, Yale University, New Haven, CT 06520;
[b] Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520;
[c] Department of Physics, Yale University, New Haven, CT 06520.
[†] These authors contributed equally to this manuscript.

Synthesis and Characterization of the PTH(1-14)-$(GS)_8$-lipid Construct.

The peptide, hPTH(1-14)-$(GS)_8$-Cys-$CONH_2$, was synthesized by GL Biochem (Shanghai). The lipid, 16:0 MPB PE with phosphatidylethanolamine headgroup functionalized with maleimide, was purchased from Avanti Polar Lipid. The peptide stock was prepared in 1X PBS (pH 7.4) and the lipid was dried under argon and then dissolved in 1X PBS/CH_3CN (3:1). The peptide (1 equiv.) and the lipid (3 equiv.) was mixed with TCEP (10 equiv.) added to prevent the formation of disulfide bonds between the peptides. The reaction was incubated at 37°C and monitored by LC-MS. The lipid-peptide conjugate was purified by HPLC using a reverse-phase C8 column (Vydac). The calculated mass of the peptide-lipid conjugate is 3778.83. The product fraction collected from HPLC had the molecular mass of 3779.82, corresponding to $[M+H]^+$.

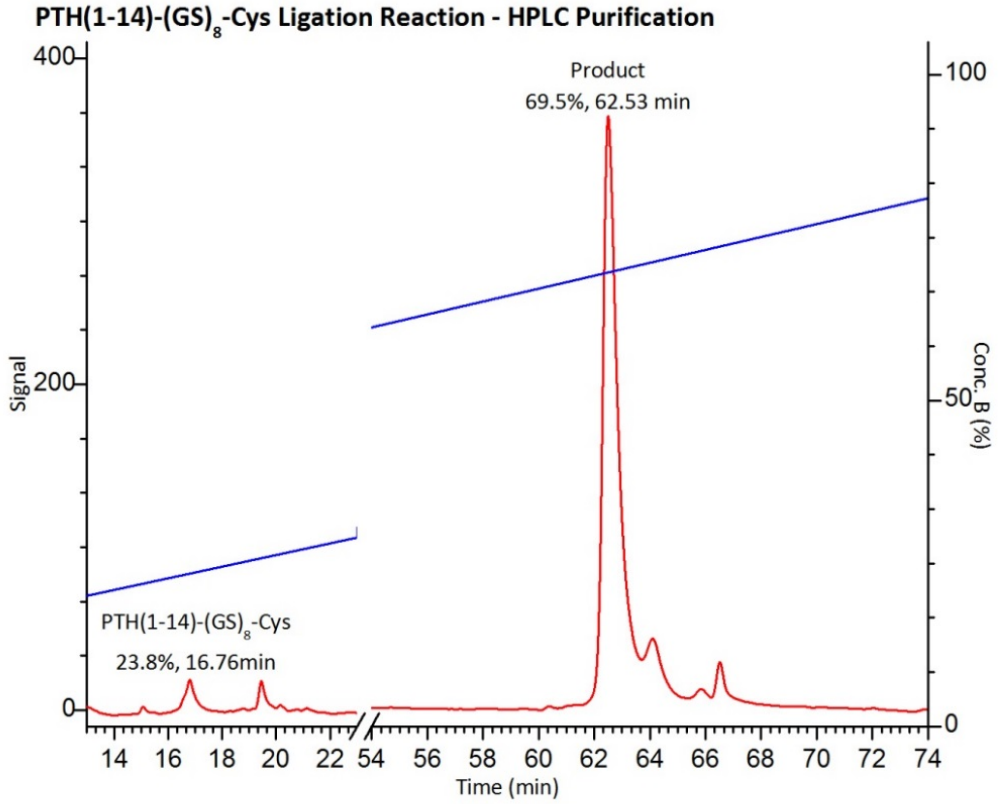
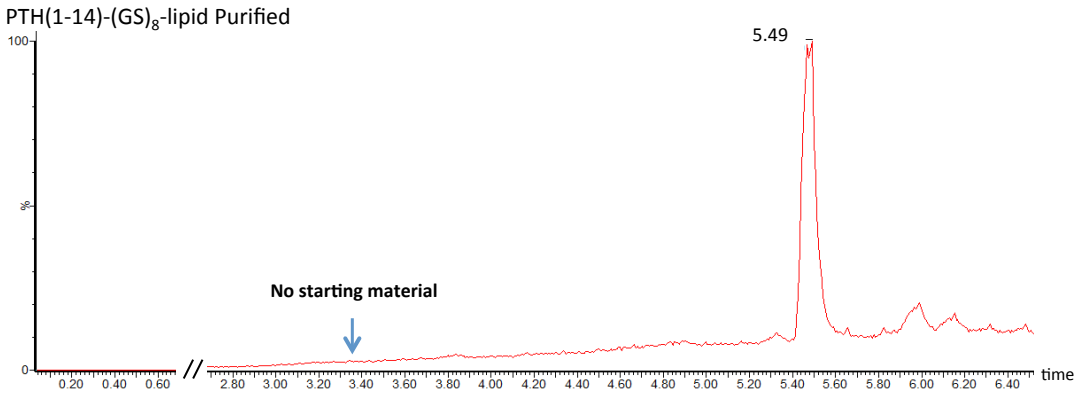


Fig. S1 Purification of PTH(1-14)-$(GS)_8$-lipid by HPLC.

(a)



(b)

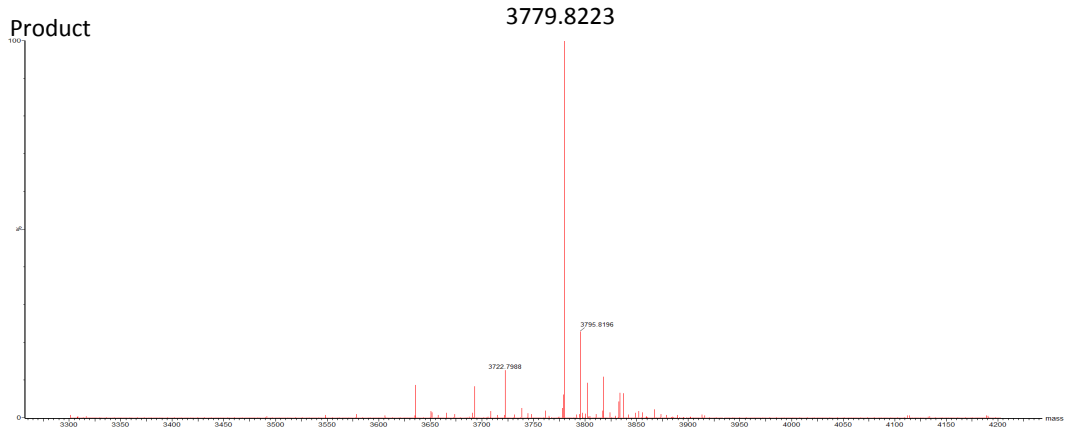


Figure S2. LC-MS characterization of purified PTH(1-14)-$(GS)_8$-lipid.
(a) Chromatography (b) Mass of the major peak

Synthesis and Characterization of the PTH(1-14)-PEG-lipid Construct.

The peptide, hPTH(1-14)-Cys-$CONH_2$, was synthesized by GL Biochem (Shanghai). The lipid, DSPE-PEG(2000) Maleimide, was purchased from Avanti Polar Lipid. The synthesis and purification procedure was similar to that described previously for the GS-linker construct, except that the dried lipid was dissolved in 1X PBS (pH 7.4). The calculated molecular mass of the peptide-lipid conjugate is 4616.62 and the purified product eluted from HPLC had the mass of 4617.58, corresponding to $[M+H]^+$. The presence of multi-peaks in the mass spectrum reflects the distribution of the molecule weight of the PEG molecule.

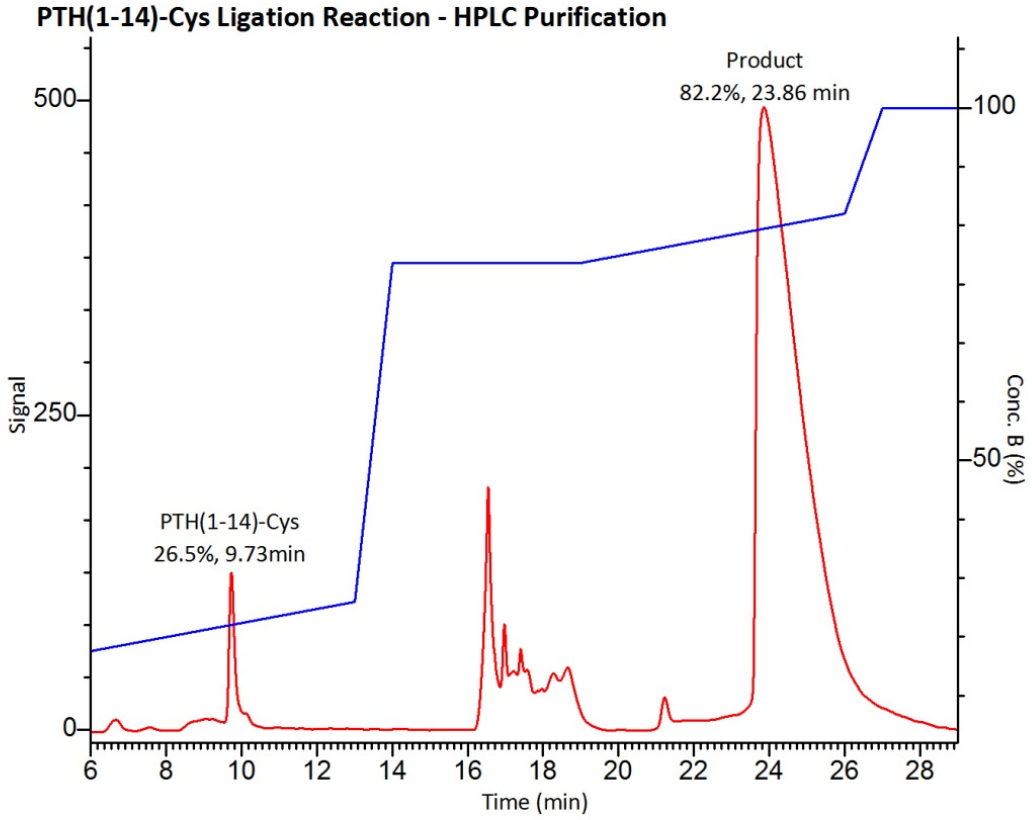
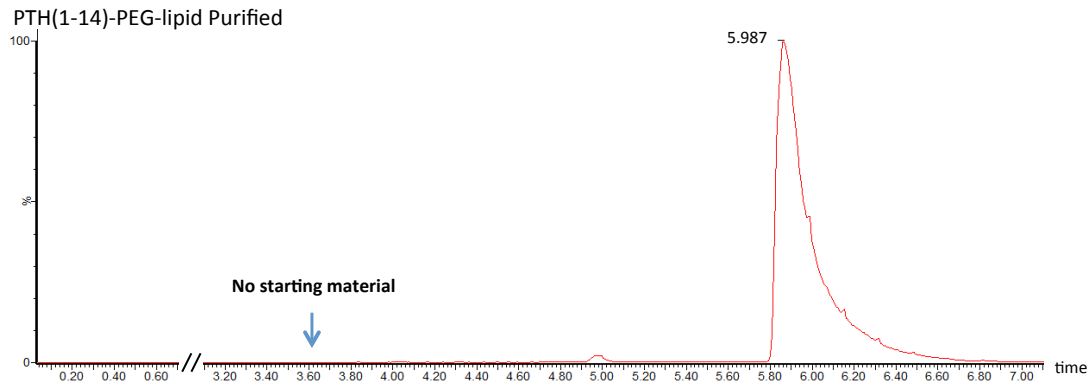


Fig. S3 Purification of PTH(1-14)-PEG-lipid by HPLC.

(a)



(b)

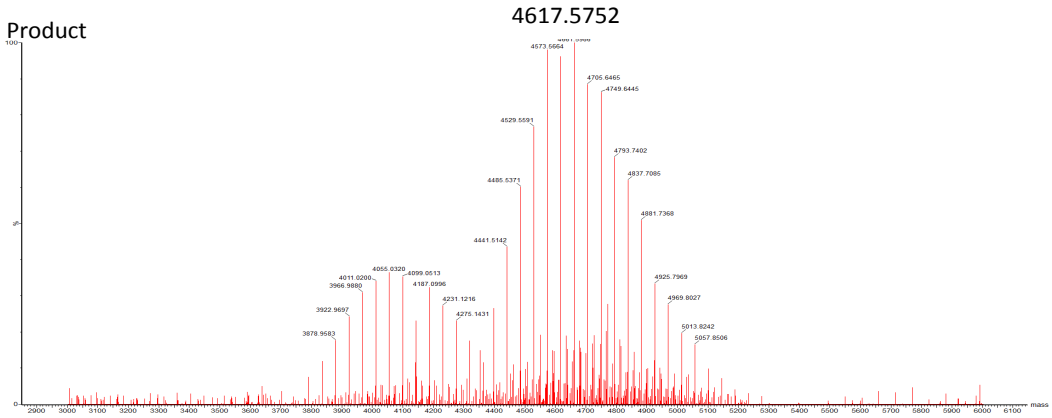


Fig. S4 LC-MS characterization of purified PTH(1-14)-PEG-lipid.
(a) Chromatography (b) Mass of the major peak

Cell cAMP ELISA.

HEK293 *GnTI*- cells were stably transfected with the tetracycline-inducible vector pACMV-tetO containing human PTH1R gene. The stable cells were grown in 48-well plated and induced with 0.55 mg/mL sodium butyrate and 2 µg/mL tetracycline and maintained under 5% CO_2/ 95% air in 1:1 DMEM/F12 supplemented with 10% FBS for ~40h for receptor expression. The induced cells were treated with 240 µL of cAMP assay buffer (DMEM containing 200 µM IBMX, 1 mg/mL BSA, 35 mM HEPES, pH 7.4), followed by the addition of 120 µL of binding buffer containing the peptides or constructs (concentrations indicated). The binding buffer contains 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5% FBS and 5% heat-inactivated FBS. The cells were then stimulated with peptides at various concentrations for 25 min and lysed with the lysis buffer (0.1 M HCl and 0.5% Triton X-100). The cAMP levels were quantified using direct cAMP enzyme-linked immunosorbent assay (ELISA) according to the protocol accompanying the commercial kits (Enzo Life Science). Each point was measured in duplicate in each experiment and each curve represented an average of three experiments.

Size Exclusion Chromatography.

Both the GS-linker and the PEG-linker constructs at a final OD_{254} of 0.2 were resolved using a

Superdex 200 10/300 GL column at a flow rate of 0.4 mL/min. The corresponding concentrations for the GS-linker and the PEG-linker constructs were 0.65 mM and 2.9 mM, respectively. The running buffer was 1X PBS (pH 7.4). Five protein standards were used to calibrate the column: thyroglobulin (17.2 nm), apoferritin (12.2 nm), alcohol dehydrogenase (9.1 nm), bovine serum albumin (7.2 nm), and carbonic anhydrase (4 nm). The sizes of the nanoparticles formed by the constructs were calculated using the calibration curve.

Electron Microscopy.

A 4 μL of sample at indicated concentration was applied to a glow-discharged carbon-coated EM grid and negatively stained with 2% uranyl acetate (w/v) solution. The specimen was subsequently examined in a FEI Tecnai-12 electron microscope operated at 120 kV. Images were collected as 4096 × 4096-pixel 8-bit gray scale Gatan Digital Micrograph 3 (DM3) files on a Gatan Ultrascan 4000 CCD camera. Particle sizes were estimated using ImageJ.

Surface Tension Isotherm.

The adsorption of both constructs at air-water interface at different concentrations was measured in a 5 mL Langmuir trough using a Langmuir-Wilhelmy film balance (KSV, Finland) with a piece of filter paper as a surface plate. Before the addition of construct stock solution, the surface tension of the 1X PBS (PH 7.4) buffer was set to zero. The stock solution was then added in increments onto the buffer subphase with a Hamilton microsyringe. The surface tension was monitored in real time during the process. For each increment, adsorption equilibrium was reached before the reading on the balance was taken as the surface tension for the corresponding concentration.

Preparation of Nanodisc Samples.

Nanodiscs were prepared following an established protocol with slight modifications.[1] The lipid molecule and membrane scaffold protein used in this preparation were 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and MSP1E3D1[2], respectively. Preparations of solubilized POPC and MSP1E3D1 were described in detail in the previous publication.[1] The nanodiscs were assembled by first mixing MSP1E3D1 and POPC at a ratio of 1:120 in a buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 4 mM EDTA, and 4% glycerol, and then adding Bio-Beads SM-2 (Bio-Rad) beads to remove the detergents. After shaking overnight at 4°C, the assembled nanodiscs were further purified to homogeneity by passing a Superdex 200 10/300 GL column, using a running buffer of 1X PBS (pH 7.4).

Fluorescence Correlation Spectroscopy.

Measurements of fluorescence correlation spectroscopy were made on a lab-built instrument based on an inverted Olympus IX71 microscope which has been described previously,[3] using a 561 nm DPSS laser for excitation. Samples were placed in a well of an 8-well NUNC chamber treated with PEG-PLL to prevent non-specific adsorption of molecules onto the chamber surface.[4] For each measurement, 20 autocorrelation curves were collected and averaged. The averaged curves were fit to Equation 1 using lab-written software in MATLAB to compare the diffusion times of the labeled species.[4-5]

$$G(\tau) = \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1}\left(1 + \frac{\tau}{s^2\tau_D}\right)^{-\frac{1}{2}} \quad (1),$$

where $G(\tau)$ is the autocorrelation as a function of time τ, N is the average number of fluorescent molecules in the probing beam, τ_D is the mean diffusion time of labeled species, s is the ratio of the axial to radial dimensions of the focal volume.

Protease Stability Assay.

In 1X PBS buffer, either 50 μM of PTH(1-14)-$(GS)_8$-Cys or 250 μM of PTH(1-14)-$(GS)_8$-lipid were added to bovine pancreatic trypsin at a molar ratio of 100:1 and incubated for 10 min at room temperature. After incubation, the mixture were immediately analyzed using HPLC with an analytical reverse phase C8 column, at a flow rate of 1 mL/min, with linear gradients of 15-65% acetonitrile for PTH(1-14)-$(GS)_8$-Cys and 40-90% for PTH(1-14)-$(GS)_8$-lipid in 45 min, using UV-visible detection at 214 nm. The chromatographic profiles were compared to those of the samples without trypsin treatment.

References

1 N. Mitra, Y. Liu, J. Liu, E. Serebryany, V. Mooney, B. T. DeVree, R. K. Sunahara and E. C. Yan, *ACS Chem. Biol.*, 2013, **8**, 617.

2 I. G. Denisov, B. J. Baas, Y. V. Grinkova and S. G. Sligar, *J. Biol. Chem.*, 2007, **282**, 7066.

3 A. Nath, A. J. Trexler, P. Koo, A. D. Miranker, W. M. Atkins and E. Rhoades, *Method Enzymol.*, 2010, **472**, 89.

4 S. Elbaum-Garfinkle, G. Cobb, J. T. Compton, X. H. Li and E. Rhoades, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 6311.

5 S. Elbaum-Garfinkle, T. Ramlall and E. Rhoades, *Biophys. J.*, 2010, **98**, 2722.