Thioamide Substitution Selectively Modulates Proteolysis and Receptor Activity of Therapeutic Peptide Hormones

Xing Chen,[a] Elizabeth G. Mietlicki-Baase,[b,c] Taylor M. Barrett,[a] Lauren E. McGrath,[b] Kieran Koch-Laskowski,[b] John J. Ferrie,[a] Matthew R. Hayes,[b] and E. James Petersson[a,*]

[a]Department of Chemistry, University of Pennsylvania, 231 South 34th Street, Philadelphia, PA 19104

[b]Department of Psychiatry, Perelman School of Medicine, University of Pennsylvania, 125 South 31st Street, Philadelphia, PA 19104

[c]Current Address: Department of Exercise and Nutrition Sciences, State University of New York at Buffalo, G10G Farber Hall, Buffalo, NY 14214

General Information Boc-L-thionophenylalanine-1-(6-nitro)benzotriazolide and Ala-Pro-*p*NA (AP-*p*NA) were purchased from Bachem (Torrance, CA, USA). All Fmoc protected amino acids and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem (currently EMD Millipore; Billerica, MA, USA). Piperidine was purchased from American Bioanalytical (Natick, MA, USA). Sigmacote®, *N*,*N*-diisopropylethylamine (DIPEA), and human Dipeptidyl Peptidase 4 (DPP-4) (recombinant, expressed in Sf9 cells) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) unless otherwise specified. Milli-Q filtered (18 MΩ) water was used for all solutions (EMD Millipore). Luminescence and time-course UV-Vis absorbance data were obtained with a Tecan Infinite® M1000 PRO plate reader (San Jose, CA, USA). Peptides were purified with a Varian ProStar high performance liquid chromatography (HPLC) system with a diode array detector (currently Agilent Technologies; Santa Clara, CA, USA), and analyzed with an Agilent 1100 Series HPLC system. Peptide mass spectrometry data were collected with a Bruker Ultraflex III MALDI mass spectrometer (Billerica, MA, USA).

Peptide Synthesis and Purification All peptides were synthesized by a combination of automated and manual synthesis. GLP-$1_{10\text{-}37}$ and $GIP_{3\text{-}42}$ were either ordered from GenScript (Piscataway, NJ, USA) and delivered as protected peptides on dry resin, or synthesized on a CEM Liberty 1 automated microwave peptide synthesizer (Matthews, NC, USA) according to the company recommended protocol. The remaining residues were synthesized manually using our established protocols.[1-2] Specifically, for all GLP-1 and GIP related peptides, the following procedures were used. Prior to use, the glass peptide reaction vessel (RV) was coated with Sigmacote® (Sigma-Aldrich). Each reaction was repeated twice to ensure good yield. Between

each reaction, the resin was washed extensively with dimethylformamide (DMF). For deprotection, 20% piperidine in DMF was added to the RV for successive 5 min and 15 min incubation with resin under magnetic stirring. For normal amino acid coupling, 5 equiv. amino acid and 5 equiv. HBTU were dissolved in DMF, and then added to the RV, with 10 equiv. DIPEA. The reaction was allowed to proceed for 30 min with stirring. Thioamides were incorporated through pre-activated benzotriazole precursors. 2 equiv. thioamide precursor in dry dichloromethane (DCM) was added to the RV with 2 equiv. DIPEA, and stirred for 45 min. For cleavage, the resin was incubated with a fresh cleavage cocktail (95:2.5:2.5 v/v trifluoroacetic acid/water/ triisopropylsilane) on a rotisserie for 30 min then 45 min. After each cleavage, the cocktail solution containing peptides was expelled from the RV and mixed with cold ether on dry ice for 10 min. The mixture was centrifuged at 3000 rpm for 5 min, and the precipitates were collected and dissolved in CH_3CN/H_2O (1:1 v/v) for purification by HPLC. Peptides were purified on a SunFire C18 Prep column (Waters; Milford, MA, USA) using the solvent gradients described in **Table S1**.

Retention times and MALDI MS characterization of each peptide are shown in **Table S2**. Purified peptides were dried on a lyophilizer (Labconco; Kansas City, MO, USA) or in a vacuum centrifuge (Savant/Thermo Scientific; Rockford, IL, USA). If needed, peptides were subjected to multiple rounds of purification until 99% purity was achieved.

Table S1. HPLC Gradients for Peptide Purification.

Peptide	Time (min)	%B	Peptide	Time (min)	%B
GLP-1-A^{S}_{8}	0:00	2	GLP-1-E^{S}_{9}	0:00	2
	6:00	2		6:00	2
	8:00	25		8:00	20
	27:00	37		37:30	45
	30:00	100		40:00	100
	35:00	100		45:00	100
	40:00	2		50:00	2
GLP-1-F_7	0:00	2	GLP-1-F^{S}_{7}	0:00	2
GLP-1-$F_7A^{S}_{8}$	6:00	2		6:00	2
	8:00	20		8:00	25
	38:00	40		38:00	45
	40:00	100		40:00	100
	45:00	100		45:00	100
	40:00	2		40:00	2
GIP-Y^{S}_{1}	0:00	2	GIP-A^{S}_{2}	0:00	2
	6:00	2		6:00	2
	7:00	15		8:00	20
	37:00	35		37:00	40
	40:00	100		40:00	100
	45:00	100		45:00	100
	40:00	2		50:00	2

Solvent A: 0.1 % TFA in water; Solvent B: 0.1 % TFA in acetonitrile

Table S2. Retention Times and Masses of Purified Peptides.

Peptide	Retention Time (min)	Calculated $[M+H]^+$	Observed $[M+H]^+$
GLP-1-A^{S}_{8}	27	3313.65	3313.30
GLP-1-E^{S}_{9}	36	3313.65	3313.67
GLP-1-F_7	35	3307.68	3307.69
GLP-1-F^{S}_{7}	31	3323.66	3323.05
GLP-1-$F_7A^{S}_{8}$	36	3323.66	3323.40
GIP-Y^{S}_{1}	31	4999.46	4999.74
GIP-A^{S}_{2}	25	4999.46	4999.37

Peptide Secondary Structure A 40 μM solution of peptide in 10 mM potassium phosphate buffer (pH 7.2) containing 30 % trifluoroethanol was prepared in 1 mm quartz cuvettes for circular dichroism (CD) measurements of its secondary structure. Far-UV spectra were collected on a CD spectrometer (Aviv Biomedical, Inc.; Lakewood, NJ, USA) at 25 °C. Each scan was accumulated from 300 nm to 185 nm with a bandwidth of 1 nm and scanning speed of 0.1 nm/s. Three scans were repeated to ensure reproducibility, and the average was calculated. Final data in **Figure S1** are shown after solvent background subtraction.

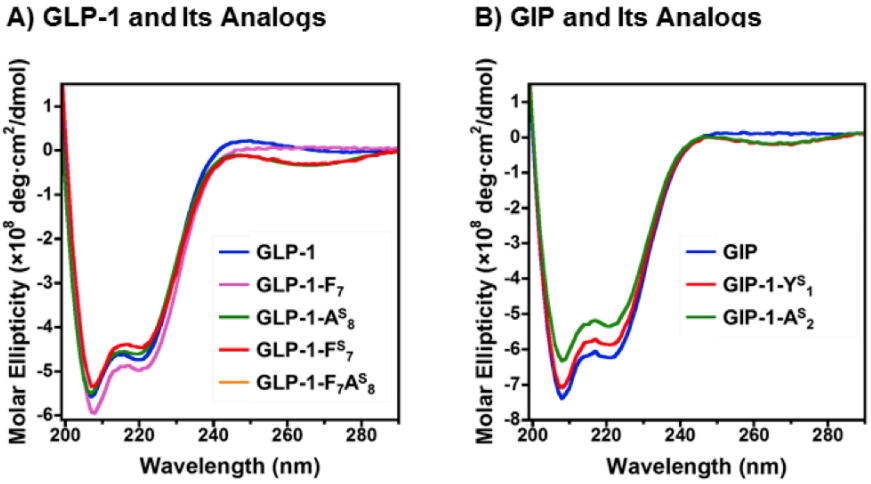

Figure S1. Secondary Structure Retained in Thioamide Analogs. **A)** CD spectra of GLP-1, GLP-1-F_7, GLP-1-A^S_8, GLP-1-$F_7A^S_8$, and GLP-1-F^S_7. **B)** CD spectra of GIP, GIP-Y^S_1 and GIP-A^S_2.

***In Vitro* Stability Assay** A 35 μM solution of the peptide of interest in Dulbecco's Phosphate-Buffered Saline (DPBS, Corning 21-031-CM) was incubated at 37 °C in the absence or presence of 2.5 ng/μL DPP-4. 50 μM L-tryptophan was also added to the mixture to serve as an internal standard for peptide quantification. After varying times of incubation, the reaction was quenched by an addition of 10% (v/v) 0.1 M HCl. Samples were kept at –80 °C if not analyzed immediately by HPLC. A Phenomenex Luna C8(2) analytical column (Torrance, CA, USA) was used to

analyze all samples using the gradients shown in **Table S4**. MALDI MS was used to check peptide identities (**Table S3**). The amount of intact peptide was quantified based on peak areas in HPLC chromatograms (**Figures S2**, **S3**, and **S4**). Specifically, GLP-1 and GIP peptides were monitored at 280 nm. In each chromatogram, the peak area of the intact peptide was normalized to that of the internal standard. For each time point, 3 runs were conducted and the average of the normalized peak areas was calculated. The average normalized peak area of the intact peptide at different time points compared to time 0 were calculated as intact peptide percentage. Intact peptide percentage data were fit to a single exponential equation to determine half-life values. In some cases, 37 μM (final concentration) bovine serum albumin (BSA; Fisher Scientific) was added to the reactions (**Figure S11**).

Table S3. Retention Times and Masses of Peptides from HPLC Analysis.

Peptide	Retention Time (min)	Calculated $[M+H]^+$	Observed $[M+H]^+$
GLP-1 (**S2**)	37.3	3297.67	3297.73
GLP-1-A^S_8 (**S1**)	37.4	3313.65	3313.18
GLP-1-E^S_9	37.5	3313.65	3313.11
GLP-1-F_7	39.0	3307.68	3307.44
GLP-1-F^S_7	39.3	3323.66	3323.84
GLP-1-$F_7A^S_8$	39.5	3323.66	3323.58
GLP-1_{9-37} (**S9**)	38.2	3089.58	3089.16
Cyclo GLP-1 (**S6**)	36.8	3279.67	3279.03
GIP	32.0	4983.48	4983.85
GIP-Y^S_1	32.2	4999.46	4999.79
GIP-A^S_2	32.4	4999.46	4999.94
GIP_{3-42}	31.4	4749.38	4749.58

Table S4. HPLC Gradients for Peptide Analysis.

Peptide	Time (min)	%B	Peptide	Time (min)	%B
GLP-1 and its analogs	0:00	2	GIP and its analogs	0:00	2
	5:00	2		5:00	2
	10:00	10		10:00	10
	40:00	40		35:00	35
	45:00	100		40:00	100
	50:00	100		45:00	100
	55:00	2		50:00	2

Solvent A: 0.1 % TFA in water; Solvent B: 0.1 % TFA in acetonitrile

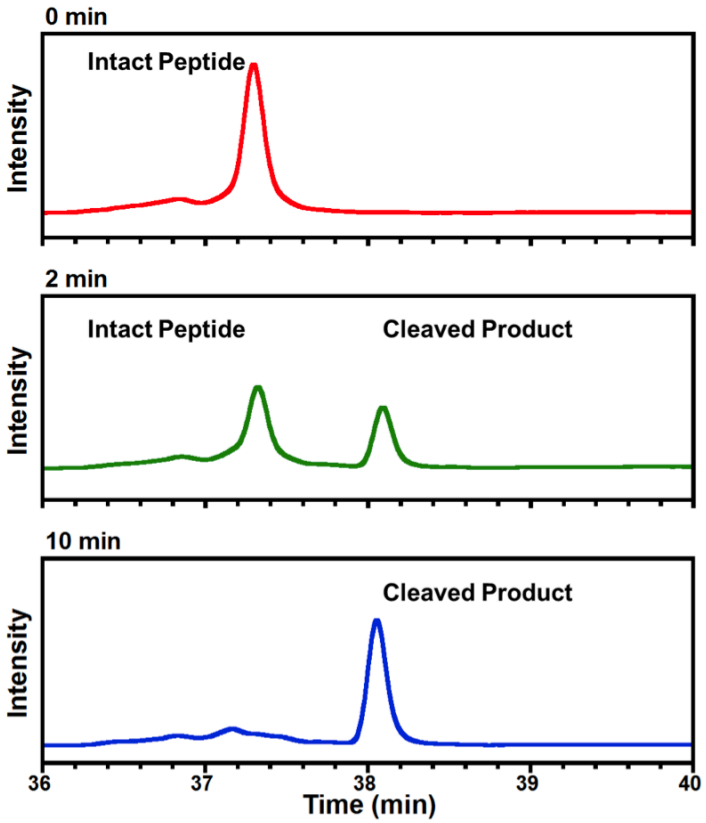

Figure S2. Representative HPLC Chromatogram of *In Vitro* DPP-4 Proteolysis. DPP-4 proteolysis of GLP-1-F_7 shown at select time points. Absorbance was monitored at 280 nm.

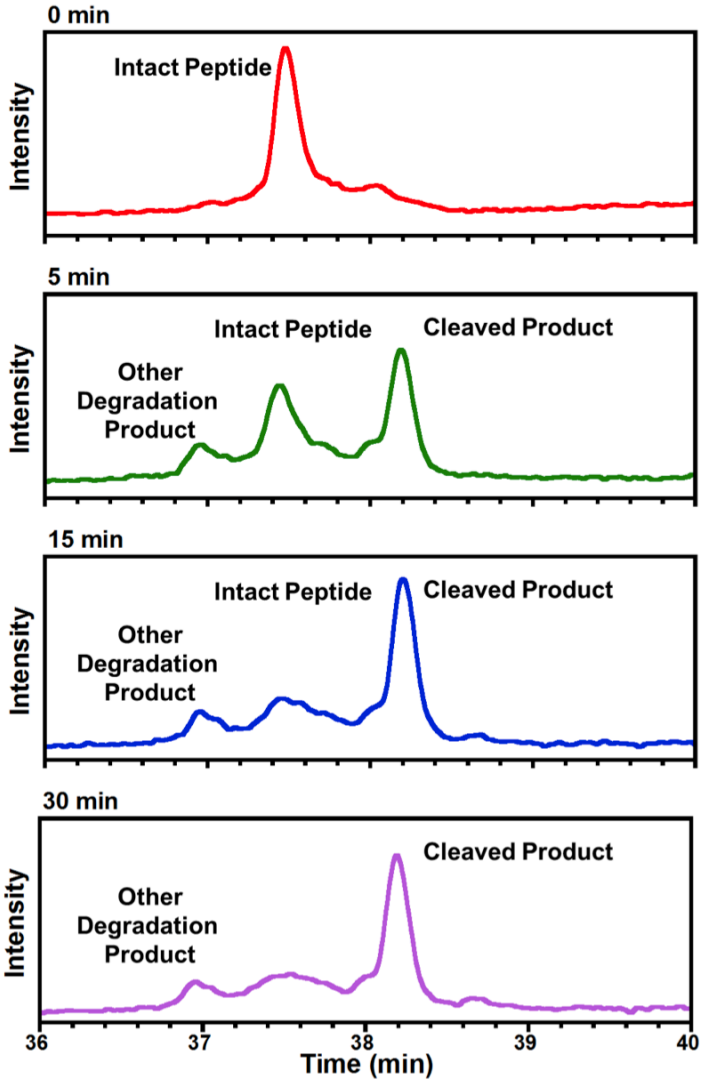

Figure S3. *In Vitro* DPP-4 Proteolysis Assay with GLP-1-E^{S}_{9}. GLP-1-E^{S}_{9} was fully degraded by 2.5 ng/μL DPP-4 in 30 min. Absorbance was monitored at 280 nm.

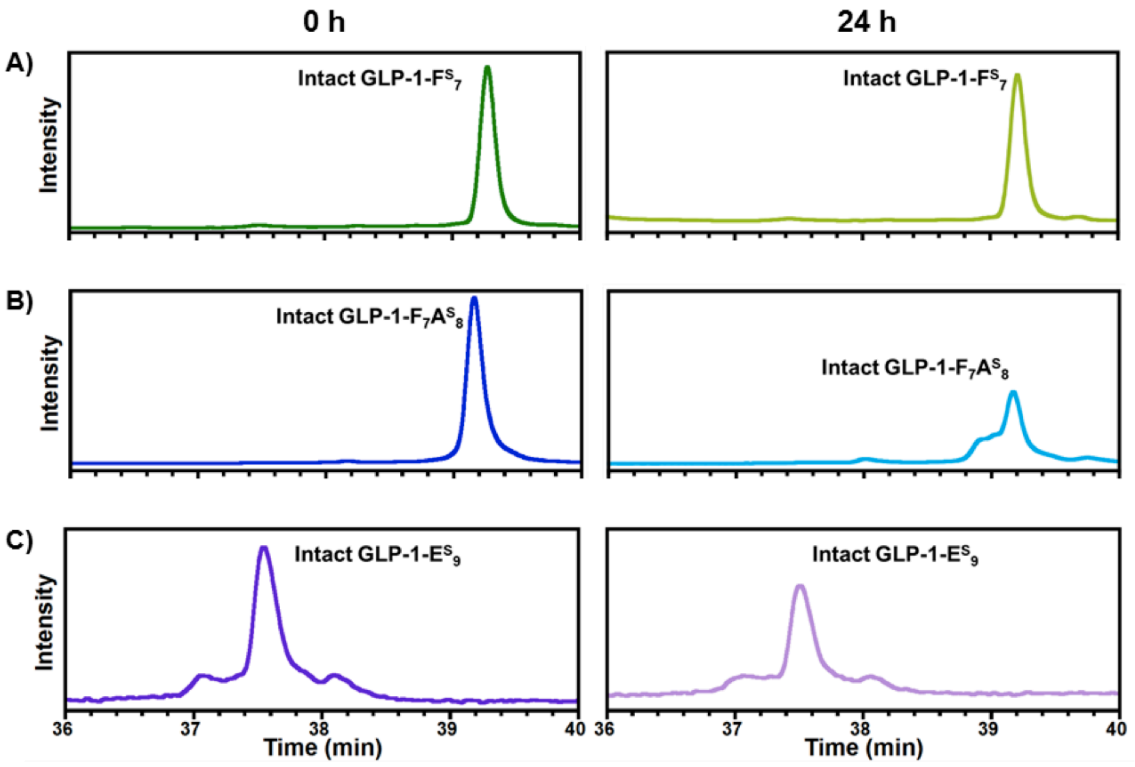

Figure S4. Buffer Stability of GLP-1 Thioamide Derivatives. Peptides were incubated in DPBS buffer at 37 °C for up to 24 h, then analyzed by HPLC. **A)** GLP-1-F$^{S}_{7}$; **B)** GLP-1-F$_{7}$A$^{S}_{8}$; **C)** GLP-1-E$^{S}_{9}$. Left: Intact peptide at time 0; Right: Remaining peptide after 24 h. Absorbance was monitored at 280 nm.

Auto-Degradation of GLP-1-A$^{S}_{8}$ GLP-1-A$^{S}_{8}$ was not stable in 100 mM Tris-HCl buffer, pH 8.0 at 37 °C, while GLP-1 was intact after 12 h (**Figure S5**). In fact, no significant difference was observed for the half-lives of GLP-1-A$^{S}_{8}$ in buffer only, 0.25 ng/mL DPP-4, or 2.5 ng/mL DPP-4 (**Figure S5**, **Figure 2**). In addition, the degradation of GLP-1-A$^{S}_{8}$ in buffer was dependent on the pH (**Figure S6**). In acidic environments, no obvious degradation was observed over 4 h. We hypothesize that GLP-1-A$^{S}_{8}$ underwent an auto-degradation process in which His$_{7}$ catalyzed cyclization of the thioamide and the terminal amine, by initial deprotonation of the amine as well as facilitating subsequent proton transfer steps (**Figure S7**). The identities of both **S2** and **S9** could be confirmed by HPLC retention time and MALDI MS since they correspond to oxoamide GLP-

1 and the DPP-4 degradation product GLP-$1_{9\text{-}36}$, respectively (**Figure S8**). Cyclic amidine **S6** could be isolated by HPLC, and upon further incubation, we observed formation of byproducts **S2** and **S9** (**Figure S9**). We observed much slower (24 h half-life) auto-degradation of GLP-1-$F_7A^S_8$, further supporting the role of the His_7 imidazole group in catalyzing the cyclization reaction (**Figure S4A** and **S5**). We note that this half-life is comparable to the half-life of GLP-1-$F_7A^S_8$ in the presence of DPP-4.

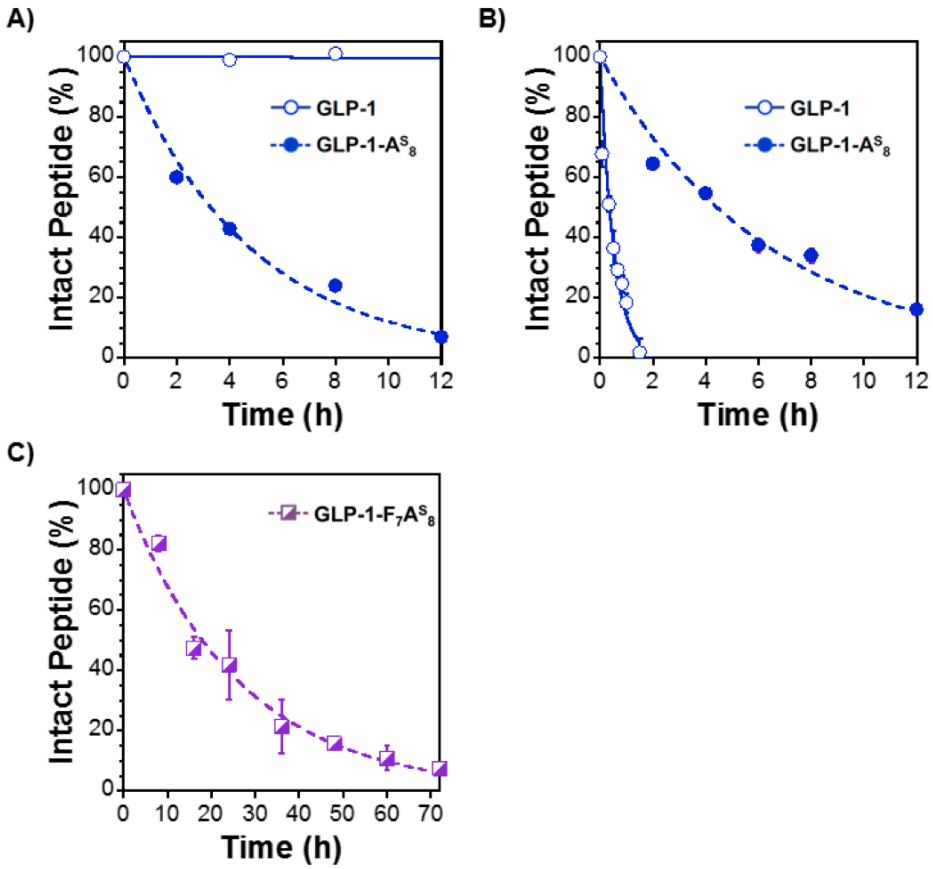

Figure S5. Degradation of GLP-1 and GLP-1-A^S_8 in Buffer and by DPP-4 Proteolysis. **A)** 35 μM GLP-1 or GLP-1-A^S_8 was incubated in 100 mM Tris-HCl buffer, pH 8.0 at 37 °C. **B)** 35 μM GLP-1 or GLP-1-A^S_8 was incubated with 0.25 ng/μL DPP-4 in 100 mM Tris-HCl buffer, pH 8.0 at 37 °C. **C)** 35 μM GLP-1-$F_7A^S_8$ was incubated in 100 mM Tris-HCl buffer, pH 8.0 at 37 °C. Bars indicate standard error.

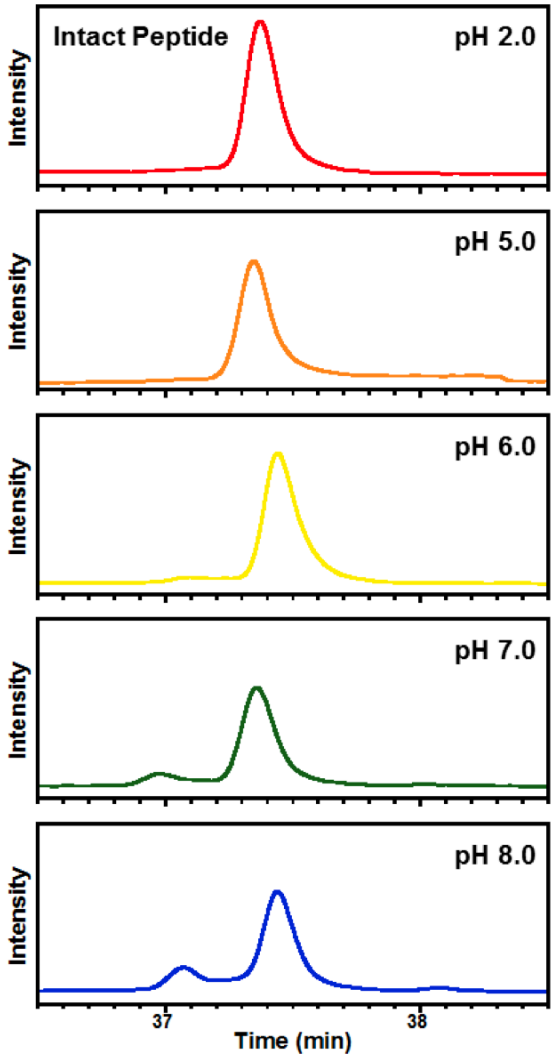

Figure S6. pH Dependence of GLP-1-A^{S}_{8} Degradation in Buffer. 35 μM GLP-1-A^{S}_{8} was incubated in 10 mM potassium phosphate buffer of various pH at 37 °C for 4 h. Absorbance was monitored at 280 nm.

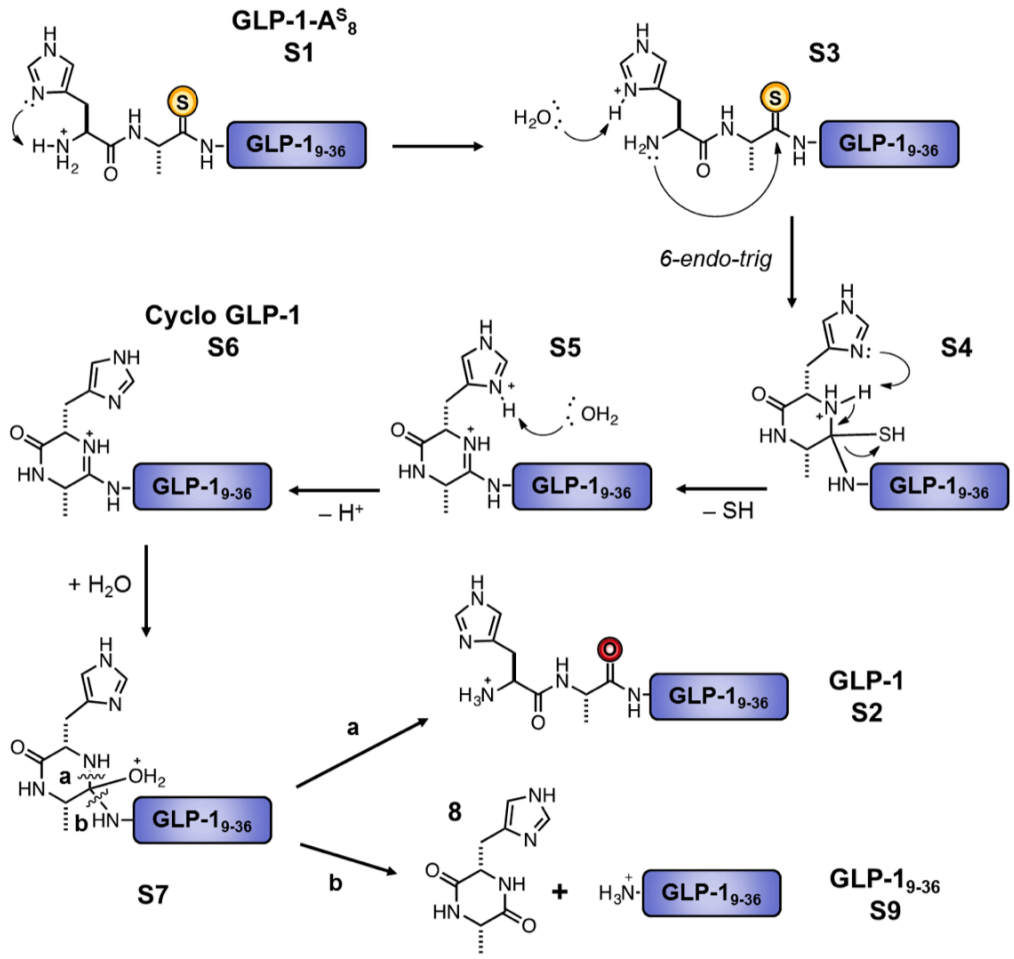

Figure S7. Proposed Mechanism of GLP-1-A$^S_8$ Auto-Degradation in Buffer.

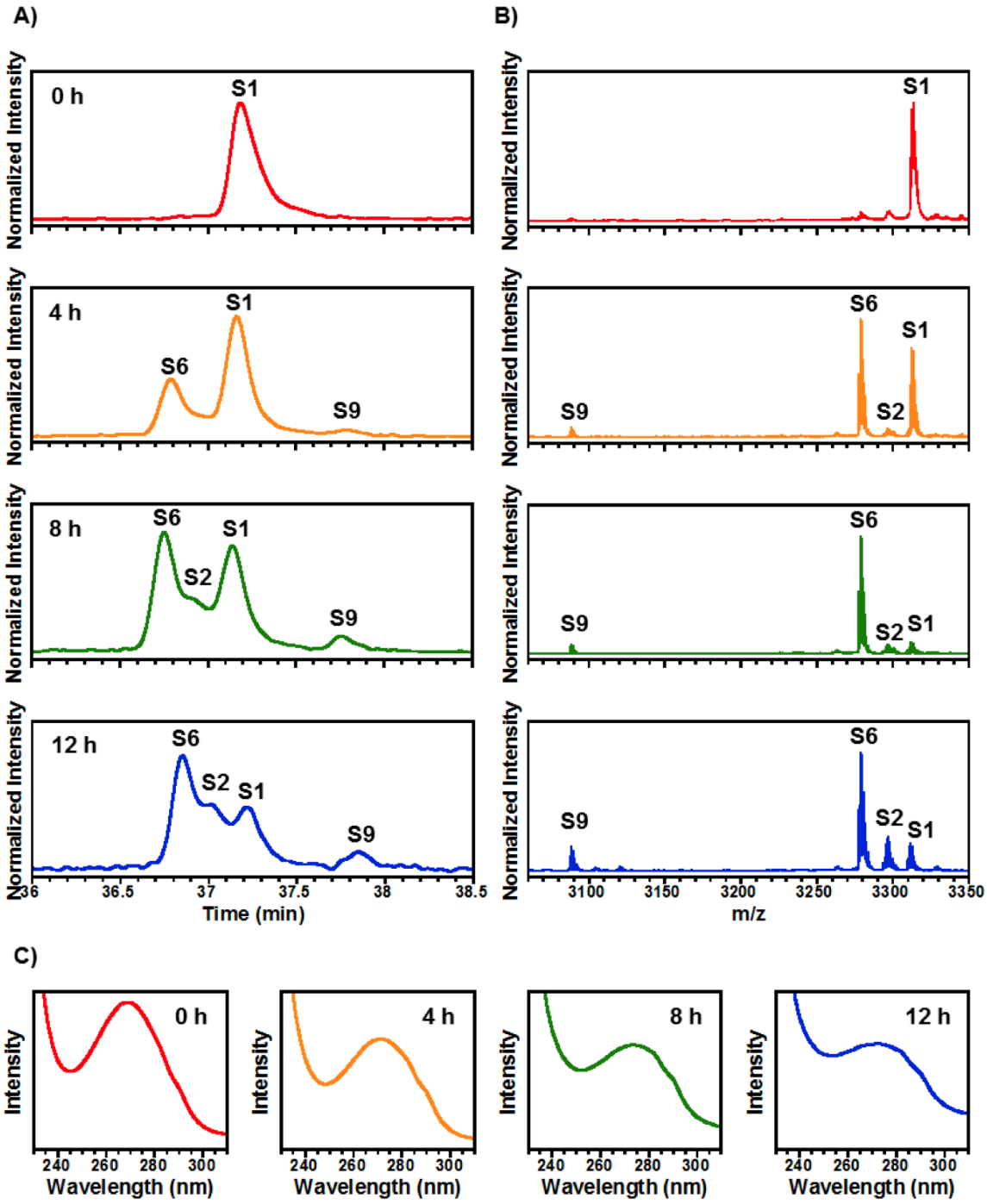

Figure S8. Analysis of GLP-1-A^{S}_{8} Degradation in Buffer. GLP-1-A^{S}_{8} was incubated in 100 mM Tris-HCl buffer, pH 8.0 at 37 °C for 0 h, 4 h, 8 h or 12 h. **A)** HPLC analysis of the reactions. Absorbance was monitored at 280 nm. **B)** MALDI MS analysis of the reactions. **C)** UV-Vis absorption of the reactions. Peaks are labelled corresponding to species in **Figure S7**.

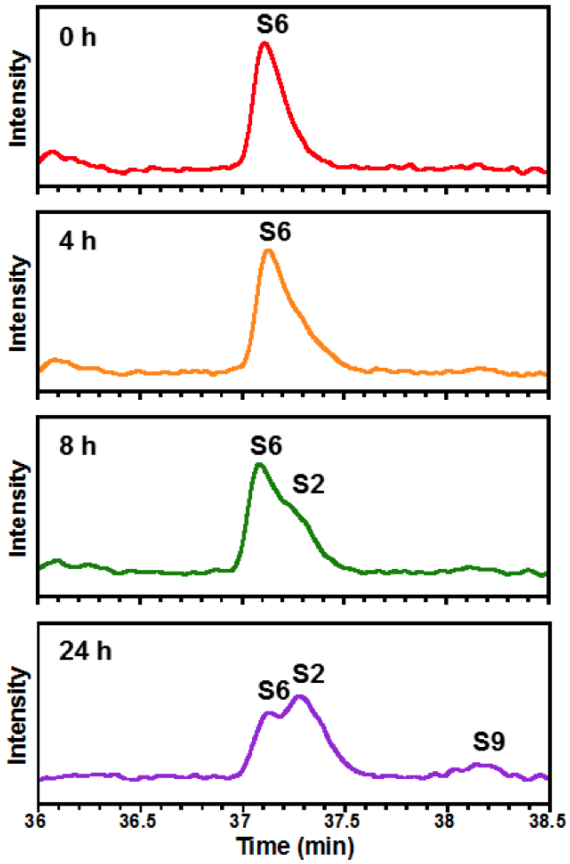

Figure S9. Degradation of Cyclo GLP-1 in Buffer. Isolated cyclo GLP-1 (**S6**) was incubated in 100 mM Tris-HCl buffer, pH 8.0 at 37 °C for 0 h, 4 h, 8 h or 24 h. Absorbance was monitored at 280 nm.

Table S5. Peptide Cleavage Rates.

Peptide	Cleavage Rate*
GLP-1	5.11
GLP-1-A^{S}_{8}	4.70 X 10^{-2}
GLP-1-E^{S}_{9}	1.94
GLP-1-F_{7}	3.73
GLP-1-F^{S}_{7}	1.34 X 10^{-2}
GLP-1-$F_{7}A^{S}_{8}$	6.289 X 10^{-3}
GIP	1.76
GIP-Y^{S}_{1}	2.39 X 10^{-3}
GIP-A^{S}_{2}	2.69 X 10^{-3}

* μmol peptide/min/mg DPP-4

Mouse Serum Stability Assay A 50 μM solution (final concentration) of the peptide of interest in Dulbecco's Phosphate-Buffered Saline (DPBS, Corning 21-031-CM) was incubated at 37 °C after the addition of 10 μL mouse serum (Sigma Aldrich M5905) to a final volume of 20-30 μL. 37 μM L-tryptophan (final concentration) was also present in the mixture to serve as an internal standard for peptide quantification. After incubating for the desired time, the serum proteins were precipitated by addition of 80 μL of acetone and chilling at -20 ºC for 10 min. Following precipitation with acetone, the samples were pelleted by spinning in an Eppendorf 5415R centrifuge at 13,000 RPM for 10 min at 4 ºC. Next, 80 μL of supernatant was diluted to 800 μL with Milli-Q water and analyzed by HPLC. A Phenomenex Luna C8(2) analytical column (Torrance, CA, USA) was used to analyze all samples using the gradients shown in **Table S6**. MALDI MS was used to check peptide identities (**Table S7**). The amount of intact peptide was quantified based on peak areas in HPLC chromatograms (**Figure S10**). GLP-1-F_7 was monitored at 215 nm, while GLP-1-F^S_7 was monitored at 280 nm. The internal Trp standard was used to normalize the amount of intact peptide in each sample. For each time point, samples were run in triplicate, and the data averaged. To determine percent intact peptide, the average ratio of intact peptide to internal standard was compared to t = 0 for all samples. Percent intact peptide over four time points is shown in **Figure S10** for both GLP-1-F_7 and GLP-1-F^S_7.

Table S6. HPLC Gradient for Serum Stability Assay*.

Peptide	Time (min)	%B
GLP-1-F_7 and GLP-1-F^S_7	0:00	2
	5:00	2
	10:00	30
	45:00	45
	50:00	100
	55:00	100
	60:00	2

Solvent A: 0.1 % TFA in water; Solvent B: 0.1 % TFA in acetonitrile

Table S7. Retention Times and Masses of GLP-1 Analogs from the Serum Stability Assay.

Peptide/Standard	Retention Time (min)	Calculated $[M+H]^+$	Observed $[M+H]^+$
Tryptophan	9.8		
GLP-1-F_7	25.3	3307.68	3306.89
GLP-1-F^S_7	25.9	3323.66	3323.17

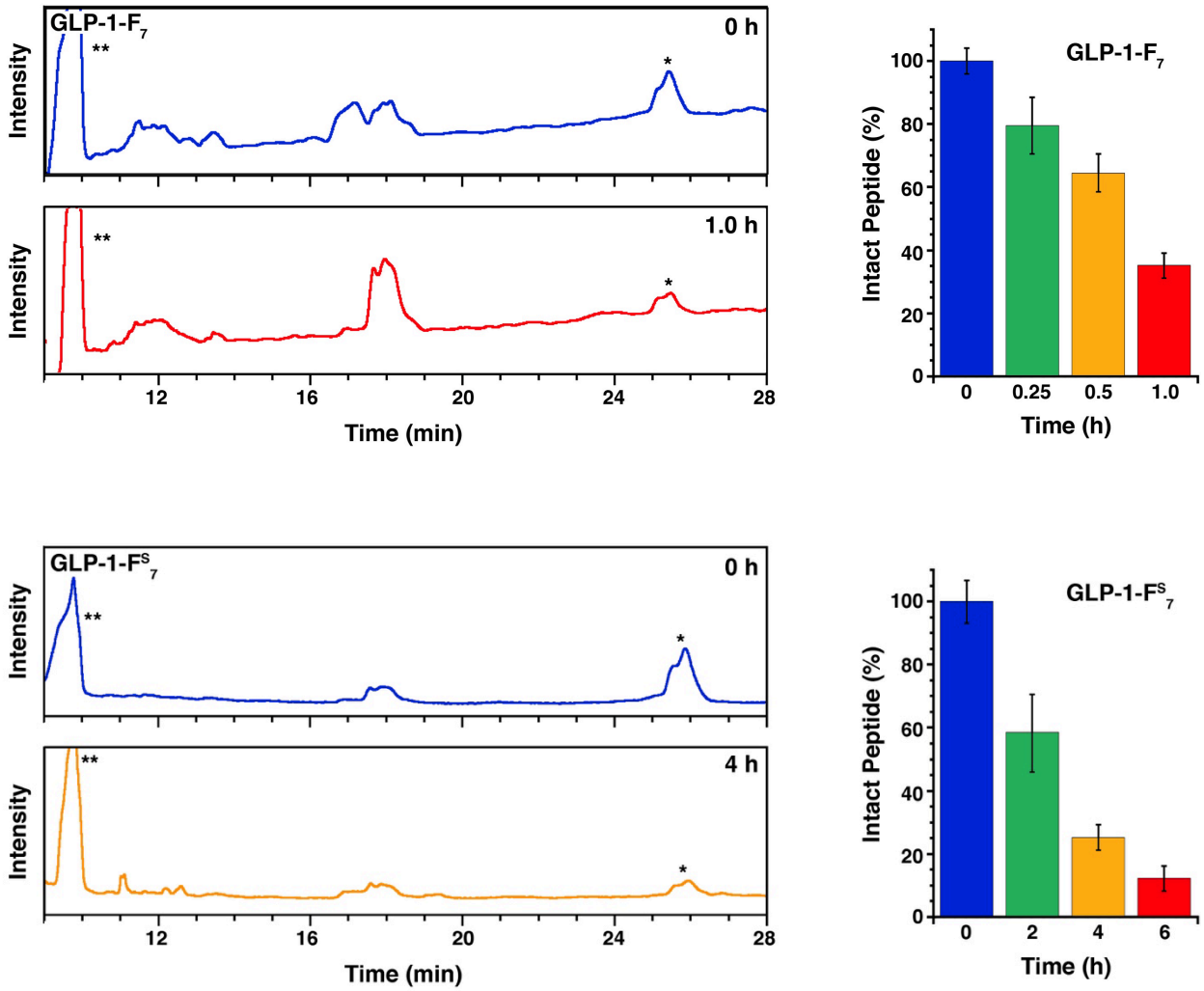

Figure S10. Serum Stability of GLP-1 Analogs. GLP-1-F_7 (top) or GLP-1-F^S_7 (bottom) were incubated in DPBS with mouse serum for 0, 0.25, 0.5, 1 , 2, 4, or 6 h. **Left:** HPLC analysis of the reactions. Absorbance was monitored at 215 nm (GLP-1-F_7) or 280 nm (GLP-1-F^S_7). * indicates intact peptide, ** indicates Trp standard. **Right:** Intact peptide as a function of time, quantified relative to the Trp standard. Bars indicate standard error.

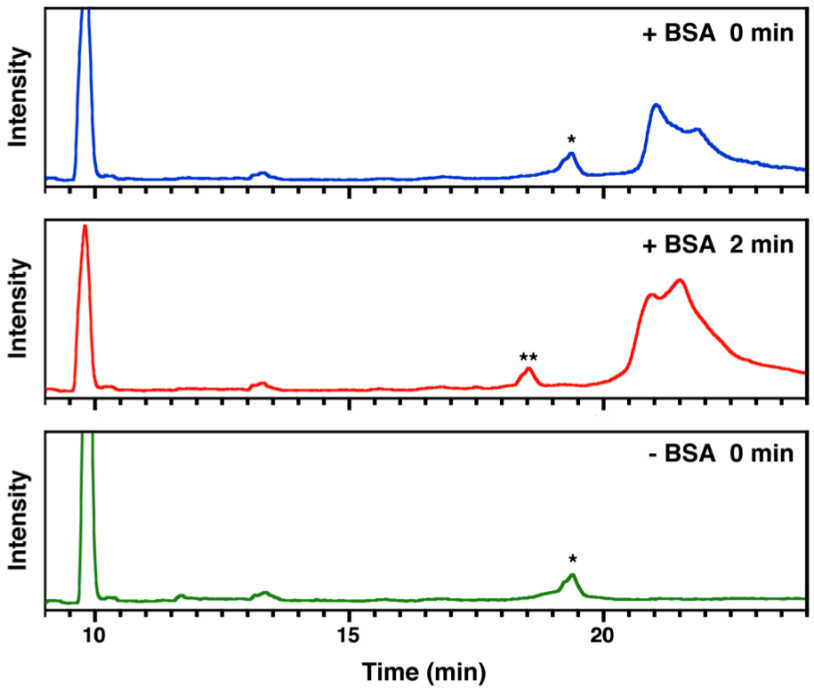

Figure S11. DPP-4 Proteolysis of GLP-1-F_7 is Not Affected by Albumin. GLP-1-F_7 was incubated in DPBS for 0 or 2 min in the presence or absence of 37 μM BSA. * indicates the GLP-1-F_7 and ** indicates the DPP-4 proteolysis product.

Competitor Assay A 70 μM solution of the peptide of interest was mixed with 2.5 ng/μL DPP-4 in 100 mM Tris buffer (pH 8.0), and incubated for 15 min at 25 °C. 100 μM Ala-Pro-*p*NA was added to the mixture, and the absorbance change at 410 nm was monitored for 30 min at 25 °C using the Tecan M1000 plate reader (**Figure S12**). The reaction of 100 μM Ala-Pro-*p*NA cleaved by 2.5 ng/μL DPP-4 was also monitored using the same protocol.

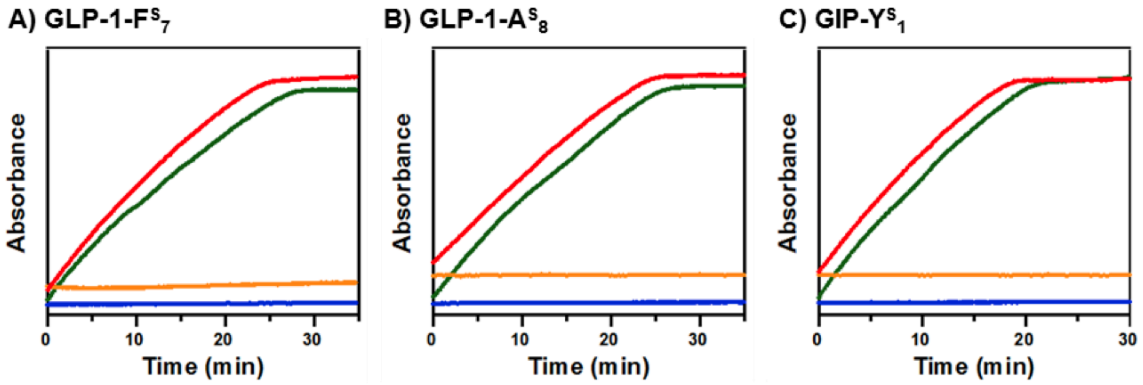

Figure S12. No Inhibition of DPP-4 Activity from Thiopeptides. When 70 µM of **A)** GLP-1-F$^{S}_{7}$, or **B)** GLP-1-A$^{S}_{8}$, or **C)** GIP-Y$^{S}_{1}$ was incubated with 2.5 ng/mL of DPP-4, activity of DPP-4 to cleave substrate Ala-Pro-*p*NA was not affected. Red trace: Ala-Pro-*p*NA cleaved by DPP-4 in the presence of thiopeptides; Orange trace: Ala-Pro-*p*NA alone in the presence of thiopeptides; Green trace: Ala-Pro-*p*NA cleaved by DPP-4 in the absence of thiopeptides; Blue trace: Ala-Pro-*p*NA alone.

Cellular Receptor Activation Assay The agonist activities of all peptides were measured using GPCR assay kits purchased from DiscoveRx (Fremont, CA, USA) following the manufacturer's protocols. Specifically, the cAMP Hunter™ eXpress GLP1R CHO-K1 GPCR Assay Kit was used to determine the cAMP activation by GLP-1 related peptides; and the cAMP Hunter™ eXpress mGIPR CHO-K1 GPCR Assay Kit was used determine the cAMP activation by GIP related peptides. Cells were incubated for 24 h, then treated with the peptide of interest for 30 min at 37 °C. For β-arrestin activation by GLP-1 related peptides, the PathHunter® eXpress GLP1R CHO-K1 Beta-Arrestin-1 GPCR Assay Kit and PathHunter® eXpress GLP1R CHO-K1 Beta-Arrestin GPCR Assay Kit were purchased. Cells were incubated for 48 h, then treated with the peptide of interest for 90 min at 37 °C. In all cases, luminescence signals were collected on the Tecan M1000 plate reader, and fitted to sigmoidal curves to determine the EC_{50} values. cAMP data are shown in **Figure 2**, **Figure 4**, and **Table 1** in the main text, β-arrestin data are shown in **Figure S13** and **Table 2** in the main text.

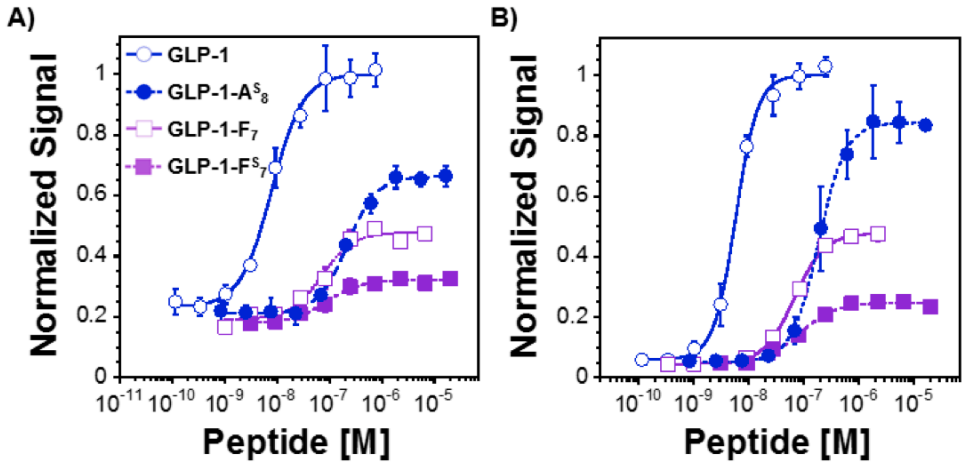

Figure S13. β-Arrestin Activation by GLP-1 and Its Analogs. Dose response curves for **A)** β-Arrestin-1 Activation and **B)** β-Arrestin-2 Activation by GLP-1, GLP-1-F_7, GLP-1-F^{S}_{7} and GLP-1-A^{S}_{8}. Bars indicate standard error.

In Vivo Activity Assay Adult male Sprague Dawley rats (Charles River) were individually housed in a temperature- and humidity-controlled environment on a 12h/12h light/dark cycle. Standard rodent chow (Purina 5001) and water were available *ad libitum* except as noted. All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Oral glucose tolerance testing (OGTT) was used to compare the efficacy of GLP-1-F^S_7 with that of native GLP-1 on glycemic control. Rats (n=16) were deprived of food overnight. Shortly after lights-off the next day, water bottles were removed. A small sample of blood was collected from the tail tip of each rat (~10µl), and baseline blood glucose (BG) levels (t=-20 min) were measured using a standard glucometer (Accucheck). Rats were then given an intraperitoneal (IP) injection of either vehicle (1 ml/kg sterile 0.9% NaCl, pH 7.0-7.4), GLP-1 (Bachem; 0.50 mg/kg), or GLP-1-F^S_7 (0.50 mg/kg). Twenty minutes later (t=0 min), another BG reading was taken, and each rat received an oral gavage of glucose (2 g/kg). Subsequent BG readings were taken at 20, 40, 60, and 120 min post-gavage from the tail tip. After the final BG reading was taken, food and water were returned.

The same rats (n=16) were subsequently used for a dose-response analysis of the effects of GLP-1-F^S_7 on glycemic control in an OGTT. Methods of this dose-response study were similar to the first OGTT, except the IP injections consisted of either vehicle (1 ml/kg sterile 0.9% NaCl, pH 7.0-7.4) or one of three doses of GLP-1-F^S_7 (0.25, 0.50, or 1.00 mg/kg).

Both experiments were conducted using a counterbalanced within-subjects design, with treatments separated by approximately one week. Data were analyzed via repeated measures ANOVA (Statistica), accounting for the within-subjects factors of drug and time, with $p<0.05$ considered statistically significant. Significant results from an ANOVA were probed using Student-Newman-Keuls post hoc analyses.

Computational Modelling From the crystal structure (PDB: 1R9N), chains B (DPP4 protein) and F (NPY peptide fragment) were extracted and all water molecules and surface glycosylations were removed.[3] The NPY_{1-6} fragment peptide (chain F) in the modified 1R9N structure was then mutated to generate GLP-1_{7-12} using the PyRosetta mutate_residue tool. Following residue mutation, the side-chain rotamers were optimized through packing using FlexPepDock in Rosetta via the command ($ROSETTA/bin/FlexPepDocking.default.linuxgccrelease@prepack_flags).[4] Extra rotamers were extracted from the B chain of the unbound DPP4 crystal structure PDB:1PFQ.[5] Following packing, DPP4 structures containing NPY and GLP-1 fragments were subjected to refinement consisting of a Monte-Carlo based search of backbone rotamers and rigid body motions along with on-the-fly side chain optimizations. This was run in Rosetta using the command ($ROSETTA/bin/FlexPepDocking.default.linuxgccrelease@refine_flags) in parallel to generate a total of 1000 structures per peptide fragment. The resultant structures for each peptide fragment were sorted based on total energy and the lowest 1% of structures were selected for analysis. A representative DPP-4/GLP-1 structure of a cleavage competent conformation was selected from the lowest 1% of outputs and is shown in **Figure 5** in the main text. Similar analysis of the 1R9N structure itself is shown in **Figure S14**.

```
prepack_flags:
-s MUTATED_INPUT.pdb
-ex1
-ex2aro
-use_input_sc
-unboundrot 1pfq.pdb
-flexpep_prepack
-nstruct 1
-scorefile ppk.score.sc
-flexpep_score_only
-out:path:pdb
-out:path:score
```

```
refine_flags:
-s PACKED_INPUT.pdb
-ex1
-ex2aro
-use_input_sc
-unboundrot 1pfq.pdb
-pep_refine
-lowres_preoptimize
-nstruct 100
-ignore_zero_occupancy false
-flexpep_score_only
-scorefile refined_peptide.sc
```

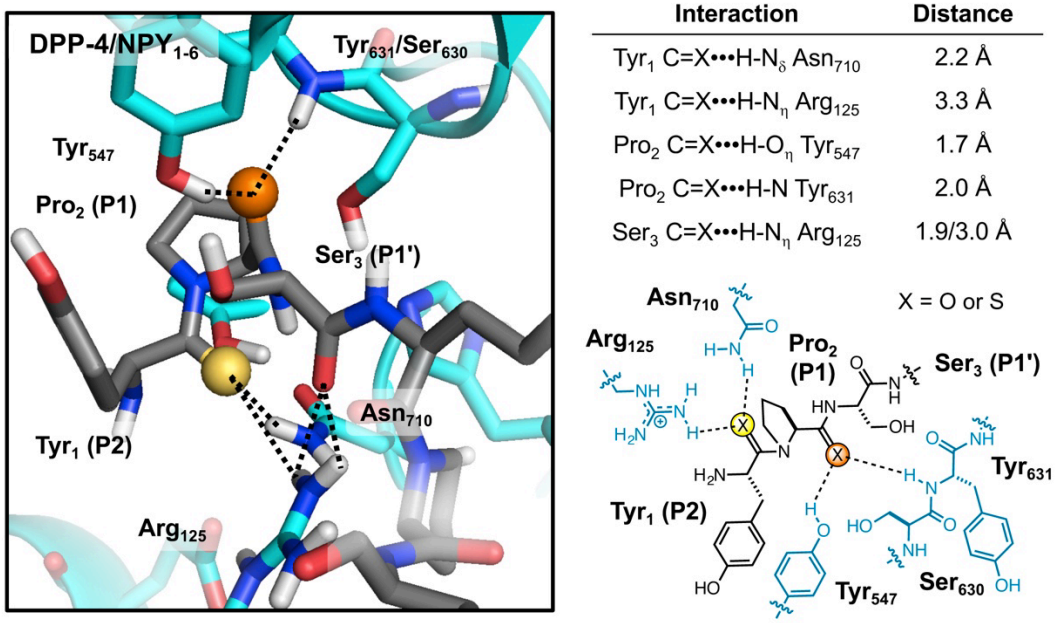

Figure S14. Structural Analysis of the Impact of Thioamide Substitution on DPP-4 Substrate Recognition. Left: An image of the DPP-4 (cyan) active site with an NPY N-terminal fragment (grey) bound rendered from PDB entry 1R9N. The P2 and P1 carbonyl oxygens are highlighted as yellow and orange spheres, respectively. Key interactions with DPP-4 and within NPY are shown as dashed lines. Right: Distances for the interactions shown at left with a schematic representation of the P2 and P1 binding site.

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