S-Protected cysteine sulfoxide-enabled tryptophan-selective modification with application to peptide lipidation

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Supporting Information Placeholder

ABSTRACT: Lipidation of peptides is a promising means of modification that can improve the therapeutic character of biologically active peptides. Here a novel lipidation protocol for peptides is described. The C−H sulfenylation of indole in peptides using *S*-*p*-methoxybenzyl cysteine sulfoxide under acidic conditions in the presence of ammonium chloride, anisole and triisopropylsilane enables late-stage tryptophan-selective peptide lipidation. This developed protocol has been used successfully for the lipidation of glucagon-like peptides. Oral glucose tolerance tests in wild-type mice indicated that the resulting lipidated peptides stimulate insulin secretion and exhibit a more long-lasting blood-glucose-lowering effect than a parent non-lipidated peptide. KEYWORDS: C−H sulfenylation of indole, tryptophan-selective modification, S-protected cysteine sulfoxide, peptide lipidation, glucagon-like peptides

Site-selective modification of peptides/proteins using reactions specific to particular residues allows modulation of a peptide's chemical or biochemical character, and can improve their therapeutic effect.[1-4] Such modifications include lipidation which can improve the physical and pharmacological properties of peptides[5-6] through the binding of their lipid moieties with human serum albumin (HSA).[7] Long-acting insulin (Insulin Degludec®[8]) and the glucagon-like peptide-1 (GLP-1) (Liraglutide®[9] and Semaglutide®[10]) are lipidated peptide therapeutic agents currently in clinical use. General access to peptide analogues has benefitted from the acylative coupling of a fatty acid-containing unit with the side chain of a lysine (Lys) attached on a solid support. This process requires only one Lys residue in the substrate or manipulation of amine protections for the selective acylation. In the reaction of peptides with lipids in solution, there is also concern that the reaction conditions suitable for hydrophilic peptides are inappropriate for coupling of the hydrophobic lipid molecules. Innovative protocols[11-15] have been explored to address these problems.

Recently, we reported the tryptophan (Trp) indole selective C−H sulfenylation reaction using *S*-*p*-methoxybenzyl cysteine sulfoxide (Cys(MBzl)(O)) in a solution containing 1 M methanesulfonic acid (MSA) and 4 M guanidine hydrochloride (Gn·HCl) and trifluoroacetic acid (TFA) (Fig. 1).[16] The formation of *S*-chlorocysteine mediated by an ammonium chloride such as Gn·HCl under acidic conditions (Fig. 1(a)) and subsequent electrophilic aromatic substitution (S_EAr) of the indole in Trp by the *S*-chlorocysteine leads to production of the tryptathionine moiety (Fig. 1(b)).[16-20] The TFA employed for the reaction addresses the concern for the solubility of the peptides and lipids. Trp is an ideal modification residue due to its relatively low abundance in peptides,[21-33] and the applicability of Trp-selective sulfenylation as a residue selective lipidation reaction was evaluated.

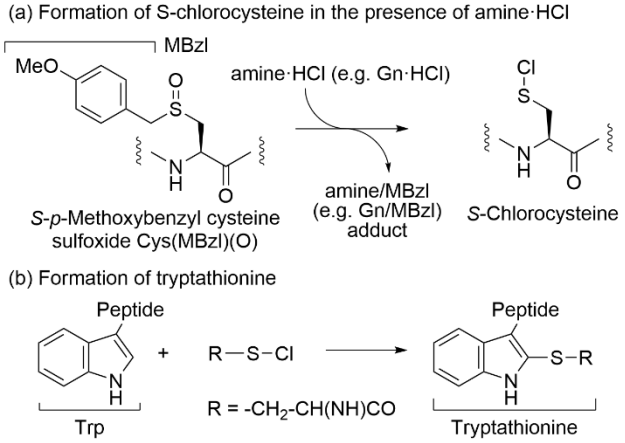
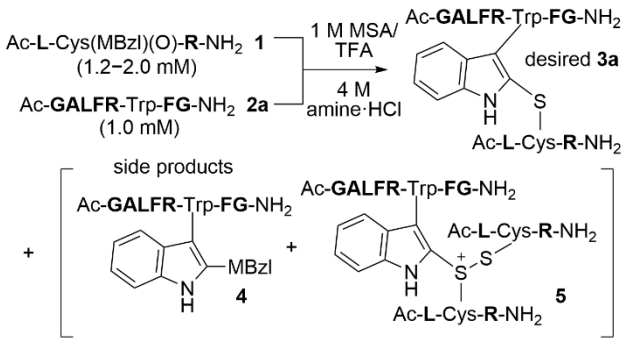


Figure 1. Formation of *S*-chlorocysteine from Cys(MBzl)(O) followed by C−H sulfenylation of Trp.

The lipidation of peptide substrates using a Cys(MBzl)(O)-incorporated lipid unit requires an intermolecular Trp-selective sulfenylation, which caused two concerns. One is the alkylation of the indole ring with MBzl cation resulting from the *S*-chlorocysteine-forming step.[34] The intramolecular C−H sulfenylation has no significant problem because the co-existing guanidine traps the transiently generated MBzl cation during the possible intermolecular indole alkylation. In contrast, the envisioned lipidation protocol includes the desired C−H sulfenylation and the undesired alkylation, both intermolecular reactions. Consequently, we surveyed various ammonium chlorides which are indispensable in the formation of *S*-chlorocysteine and evaluated their performance in trapping the MBzl cation (Table 1).

Table 1. Effects of scavengers for suppression of side reactions encountered in the intermolecular reaction



entry[a]	**1** (mM)	amine·HCl	additive	products conversion (%)[b]
1	1.2	Gn·HCl	–	**3a** (59), **4** (27), **5** (14)
2	1.2	DA·HCl	–	**3a** (86), **4** (7), **5** (7)
3	1.2	DA·HCl	anisole	**3a** (>95)
4	1.5	DA·HCl	anisole	**3a** (87), **5** (13)
5	2.0	DA·HCl	anisole	**3a** (62), **5** (38)
6[c]	1.2	DA·HCl	anisole, TIS	**3a** (>95, 94[d])
7[c]	1.5	DA·HCl	anisole, TIS	**3a** (>95)
8[c]	2.0	DA·HCl	anisole, TIS	**3a** (>95)

[a]Each reaction was conducted in 1 M MSA−4 M amine·HCl–(50 mM anisole)/TFA at 4 °C for 3 h. The reaction was diluted fivefold with H_2O and directly analyzed by HPLC. [b]Conversion (%) proportions were determined by HPLC analysis with UV detection at 220 nm and calculated using the equation: percent formation = 100 [(integ. **3a**, **4**, or **5**)/(integ. **3a** + **4** + **5**)], where integ. = integration of peak area of the UV absorption. [c]After 3 h reaction at 4 °C in the 1 M MSA system, 5% TIS was added to the reaction which was then stirred at 37 °C for 30 min. [d]Isolated yield.

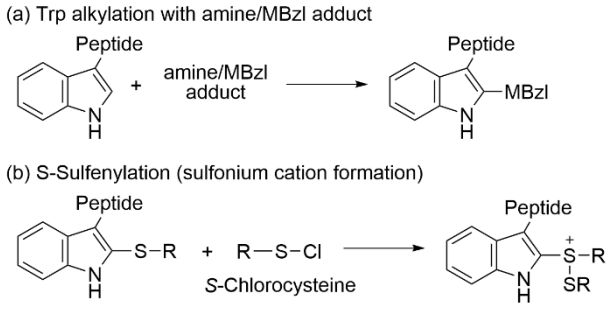
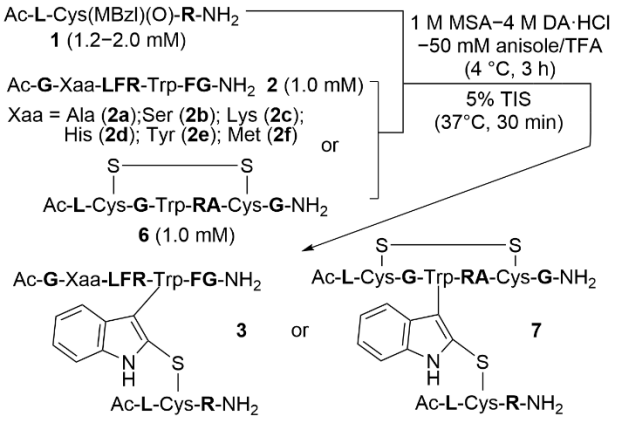


Figure 2. Reactions involved in the lipidation of Trp using a Cys(MBzl)(O) derivative.

Initially, the effect of Gn·HCl on suppression of side reactions induced by the MBzl cation was reevaluated. The intermolecular reaction of Ac-**GALFR**-Trp-**FG**-NH_2 (**2a**) (1.0 mM) with Ac-**L**-Cys(MBzl)(O)-**R**-NH_2 (**1**) (1.2 mM) in 1 M MSA−4 M Gn·HCl/TFA at 4 °C for 3 h proceeded less efficiently than the intramolecular reaction and afforded the desired tryptathionine peptide **3a** in 59% yield, but considerable amounts of the Trp-alkylated product **4** (27%) and the *S*-sulfenylated sulfonium peptide **5** (14%) were detected (Table 1, entry 1 and Fig. 2). Among the ammonium chlorides that were examined, diisopropylammonium chloride (DA·HCl) most efficiently suppressed the alkylation with only 7% of the side product **4** remaining (Table 1, entry 2 and Fig. S9). Although the amine/MBzl adducts resulting from the trapping of the cations retained the ability to transfer the MBzl cation to Trp under acidic conditions (Fig. 2(a)), the performance of DA·HCl is superior to that of Gn·HCl which can probably be attributed to the difference in the steric hindrance of the resulting amine/MBzl-adducts. The amine moiety of the DA/MBzl is more space-demanding than that of the Gn/MBzl adduct and this will hinder the amine/MBzl adducts from electrophilically attacking the Trp indole. The reaction in 1 M MSA−4 M DA·HCl/TFA in the presence of 50 mM anisole as a quencher of the amine/MBzl adduct led to the no formation of **4**, yielding instead the desired **3a** in a yield of over 95% (entry 3 and Fig. S10(a)). However, the increase in the concentration of **1** resulted in increasing formation of the *S*-sulfenylated material **5** (entries 4 and 5 and Fig. S10(b and c)).

Table 2. Trp-selectivity in the presence of various amino acids



entry	Trp peptide	**1** (mM)	products conversion (%)[a]
1	**2b**	1.2	**3b** (>95)
2	**2c**	1.2	**3c** (>95)
3	**2d**	1.2	**3d** (>95)
4	**2e**	1.2	**3e** (>95)
5	**2e**	2.0	**3e** (>95)
6	**2f**	1.2	**3f** (30)
7	**2f**	1.5	**3f** (23)
8[b]	**2f**	1.2	**3f** (88, 58[c])
9	**6**	1.2	**7** (>95)

[a]Conversion [%] proportions were determined by HPLC analysis with UV detection at 220 nm and calculated from the equation: percent formation = 100[(integ. **3** or **7**)/(integ. detected peptide materials)], where integ. = integration of peak area of the UV absorption. [b]Reaction with MSA was conducted at 37 °C for 3 h. [c]Isolated yield.

Finally, regeneration of the desired **3a** from **5** was achieved by adding triisopropylsilane (TIS)[35] during the treatment of the mixture of **1** and **2a** with 1 M MSA−4 M DA·HCl–50 mM anisole/TFA at 4 °C for 3 h, followed by an additional 30 min stirring in the presence of 5% TIS. The result was almost

quantitative (94%) production of **3a** (entries 6-8, Fig. S10(d-f), Fig. S11). Consequently, the treatment with 1 M MSA−4 M DA·HCl−50 mM anisole/TFA and the subsequent reaction in the presence of TIS were adopted as the standard reaction conditions for the intermolecular lipidation. In addition to the tolerance of the formed Trp-Cys linkage under acidic conditions, the robustness of the linkage under basic conditions was confirmed by incubation of **3a** in an aqueous buffer (pH 9.0) at 37 °C for 24 h (Fig. S12).

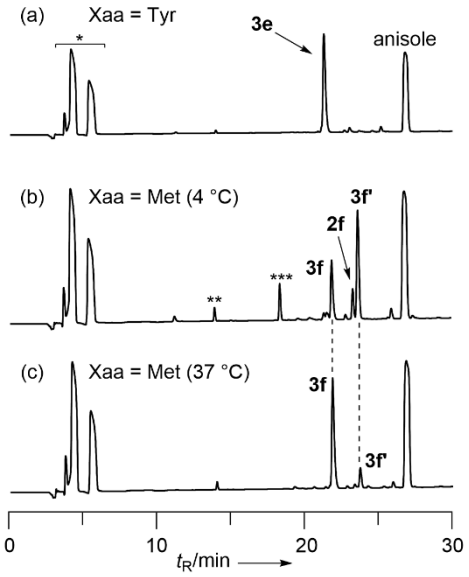


Figure 3. HPLC analyses of crude materials obtained from the reactions of **1** with **2e** (a: Table 2, entry 4) or **2f** (b and c: entries 6 and 8, respectively). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 65% over 30 min. UV detection at 220 nm. *non-peptidic matrials. **disulfide peptide derived from **1** and ***sulfide form of **1**: plausible mechanism for forming these peptidic materials, see Fig. S22.

A further concern is that the Trp-selective reaction for the lipidation requires stricter residue specificity than is required for its intramolecular use because of the expected excess use of the Cys(MBzl)(O) derivative for quantitative lipidation. The Trp-selectivity in the intermolecular reaction was evaluated by the sulfenylation of the Trp-containing disulfide peptides **6** or the linear peptides **2** (Ac-**G**-Xaa-**LFR**-Trp-**FG**-NH_2: Xaa = Ala (**2a**), Ser (**2b**), His (**2c**), Lys (**2d**), Tyr (**2e**), Met (**2f**)). This sulfenylation uses from 1.2 to 2.0 equivalents of **1** under the optimized conditions at 4 °C and the results are summarized in Table 2 and Fig. S13. With the exception of the Met-containing peptide **2f**, almost quantitative conversion of the Trp-containing peptides (**2b**−**2e** and **6**, both 1 mM) was achieved with excess sulfoxide **1** (Fig. 3(a) for **2e** and Fig. S13(a−e, i)) as was the case for **2a** (Table 1, entries 6−8). No significant reduction of the disulfide in peptide **7** was observed under the attempted conditions, even in the presence of TIS (Figs. S14, S15). The reaction of **2f** with 1.2 or 1.5 equiv. of **1** at 4 °C remained incomplete even though the desired product **3f** was obtained. The side product (**2f** + 35 Da (**3f'**) chlorination material) was observed as a main component (Figs. 3(b), S13(f and g)). In contrast, the reaction at 37 °C dramatically improved the reaction profile and the desired **3f** was obtained with >88% conversion and 58% isolated yield (Fig. S16) with 12% of the side product **3f'** remaining (Table 2, entry 8, Figs. 3(c), S13(h)). We hypothesized that the dramatic change that was observed could be attributed to the *S*-chlorocysteine species reacting preferentially with Met rather than Trp to afford the *S*-sulfenylated Met sulfonium cation. This cation then electrophilically attacks Trp at 37 °C to give the desired **3f**.[36] Alternatively, the sulfonium cation is thought to participate in forming **3f'** at the low reaction temperature. The reactions of Ac-Nle-Cys(MBzl)(O)-NH_2 with Bz-Met-OMe in the presence or absence of Ac-Trp-OMe contributed to the identification of **3f'** (Fig. S18). The formation of a chlorination material requires the presence of Ac-Trp-OMe which is converted to the corresponding 2-chloro-Trp derivative (Figs. S19−21). It was deduced from the observed results that the generated *S*-sulfenylated Met sulfonium cation should be converted to the corresponding *S*-chlorosulfonium cation, and this then reacts with the indole[37] or with a chloride anion to form chlorine (Cl_2)[38] enabling the chlorination of Trp (Fig. S22). These findings indicate that the side product **3f'** is the 2-chloro-Trp congener of **2f**.

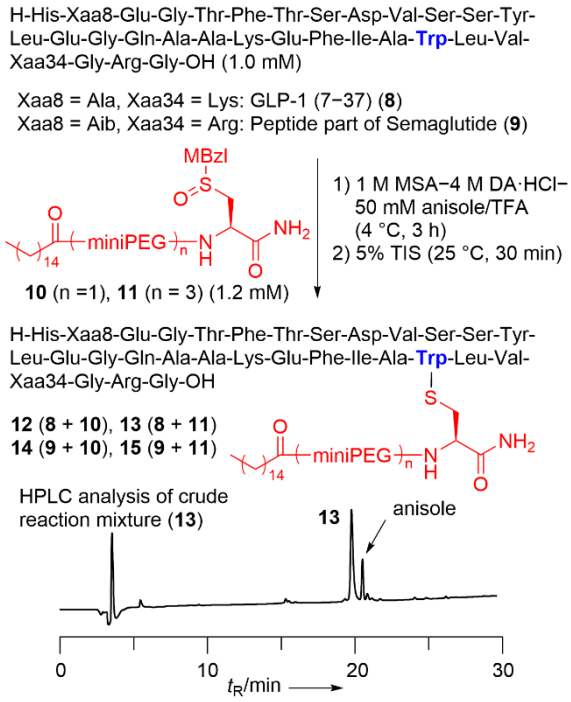


Figure 4. Lipidation of GLP-1 peptides (**8** and **9**) and HPLC analysis of crude **13**. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 95% over 30 min. UV detection at 220 nm. Aib = 2-aminoisobutyric acid

Having established the optimum conditions for the C-H sulfenylation of Trp residues, we next sought to incorporate a lipid unit into GLP-1 (7−37) (**8**) and the peptide segment **9** of Semaglutide® in clinical use (Fig. 4).[39] Requirement of an appropriate choice of the suitable linker between the peptide and lipid molecule prompted us to incorporate two different lengths of 8-amino-3,6-dioxaoctanoic acid (miniPEG) linker.[10] The necessary Cys(MBzl)(O)-incorporated lipid units (**10** and

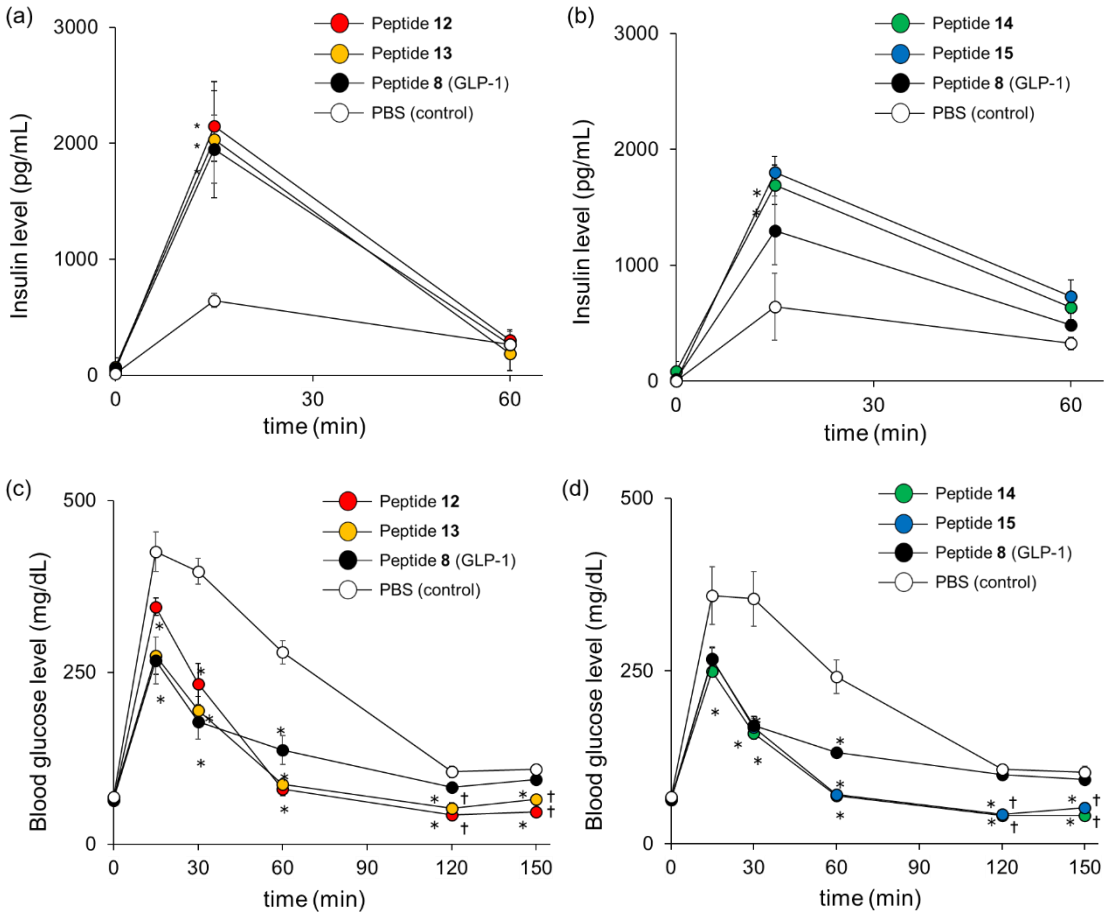


Figure 5. Blood insulin (a and b) and glucose concentration (c and d) during an OGTT in 10-week-old male WT mice (n = 4-5 mice in each group). White circles and bars indicate PBS, black circles and bars indicate **8**, red circles and bars indicate **12**, orange circles and bars indicate **13**, green circles and bars indicate **14**, and blue circles and bars indicate **15**, respectively. Statistical significance was calculated by one-way analysis of variance (ANOVA) with Tukey's test using statistical package for social science (SPSS) statistics. *P<0.05 vs. PBS, #P<0.05 vs. **8**.

11) and the acceptor peptides (**8** and **9**) were prepared by 9-fluorenylmethyloxycarbonyl (Fmoc)-solid-phase peptide synthesis (SPPS) (Figs. S23−26). Modification of peptides (1.0 mM) with the lipid units (1.2 mM) in 1 M MSA−4 M DA·HCl−50 mM anisole/TFA, with an additional 30 min treatment in the presence of TIS, resulted in the disappearance of parent peptides. HPLC analysis of the acidic quenched solution of the reaction showed that the crude reaction mixture has major and minor components with the same mass. However, dissolving the acid-treated samples in a buffer at pH 7.0 led to the disappearance of the minor components, indicating that N−O acyl shift in the Gly-Thr sequence of the peptides occurs under acidic conditions (Fig. S27).[40] All the attempted lipidation reactions proceeded efficiently, affording the corresponding lipidated peptides in good isolated yields after HPLC purification (**12** (**8** + **10**): 64%; **13** (**8** + **11**): 67%; **14** (**9** + **10**): 59%; **15** (**9** + **11**): 53%) (Figs. S28, S29). Peptide mapping of the lipidated material **13** using chymotrypsin and trypsin showed that the Trp residue was selectively modified by the lipid unit (Figs. S30, S31). Furthermore, the lipidation on the indole 2-position was confirmed by NMR analysis of the lipidated peptide fragment obtained from the chymotrypsin digestion (Figs S32−35).

Subsequently, we performed an oral glucose tolerance test (OGTT) in wild-type (WT) mice to evaluate the effect of the lipidated GLP-1 peptides **(12, 13, 14,** and **15)** on glucose tolerance. After intraperitoneal administration of phosphate-buffered saline (PBS) or GLP-1 peptides, insulin concentrations and blood glucose were measured during the OGTT (Fig. 5). After glucose ingestion and compared to PBS, the GLP-1 peptides including non-lipidated **8** were found to increase the insulin concentration (Fig. 5(a and b)) and reduce the glucose concentration (Fig. 5(c and d)). Blood glucose levels at 120 and 150 min were significantly lower in **12**, **13, 14** and **15** than in **8**.

In conclusion, the C-H sulfenylation of indole in peptides using S-protected cysteine sulfoxide, Cys(MBzl)(O), under the acidic conditions allows highly efficient and selective lipidation of Trp residues. Attempted lipidation of GLP-1 peptides showcases the high performance of the developing toolbox for bioconjugation. Further applications of the protocol to other peptide substrates and *in vivo* and *in vitro* evaluation compared to a launched lipidated GLP-1 analogue are under review in our laboratories.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed experimental procedures and charts for the HPLC analyses of the attempted reactions or purified peptide samples (Figs. S1 for **1**; S2 for **2a**; S3 for **2b**; S4 for **2c**; S5 for **2d**; S6 for **2e**; S7 for **2f**; S8 for **6**).
^{1}H-NMR and HRMS spectra (Figs. S17 for **S2**; S20 for **S6**). Comparison of ^{1}H NMR (Figs. S21 for **S4** vs **S6**; S35 for **frg. D** vs **S7**) (PDF).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HAS: human serum albumin; GLP-1: glucagon-like peptide-1; MBzl: *p*-methoxybenzyl; Cys(MBzl)(O): *S*-*p*-methoxybenzyl cysteine sulfoxide; MSA: methanesulfonic acid; Gn · HCl: guanidine hydrochloride; TFA: trifluoroacetic acid; S_EAr: electrophilic aromatic substitution; DA · HCl: diisopropylammonium chloride; TIS: triisopropylsilane; mini-PEG: 8-amino-3,6-dioxaoctanoic acid; Fmoc: 9-fluorenylmethyloxycarbonyl; SPPS: solid-phase peptide synthesis; OGTT: oral glucose tolerance test; WT: wild-type; PBS: phosphate-buffered saline; ANOVA: analysis of variance; SPSS: statistical package for social science; Ac-**L**-Cys(MBzl)(O)-**R**-NH_2: Ac-Leu-Cys(MBzl)(O)-Arg-NH_2; Ac-**GALFR**-Trp-**FG**-NH_2: Ac-Gly-Ala-Leu-Phe-Arg-Trp-Phe-Gly-NH_2; Ac-**G**-Xaa-**LFR**-Trp-**FG**-NH_2,: Ac-Gly-Xaa-Leu-Phe-Arg-Trp-Phe-Gly-NH_2 (Xaa = Ala, Ser, His, Lys, Tyr, or Met); Ac-**L**-Cys-**G**-Trp-**RA**-Cys-**G**-NH_2: Ac-Leu-Cys-Gly-Trp-Arg-Ala-Cys-Gly-NH_2.

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