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Activation of lysine-specific demethylase 1 inhibitor peptide by redox-controlled cleavage of a traceless linker

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Abstract

We have previously employed cyclization of a linear peptide as a strategy to modulate peptide function and properties, but cleavage to regenerate the linear peptide left parts of the linker structure on the peptide, interfering with its activity. Here, we focused on cyclization of a linear peptide *via* a "traceless" disulfide-based linkage that would be cleaved and completely removed in a reducing environment, regenerating the original linear peptide without any linker-related structure. Thus, the linker would serve as a redox switch that would be activated in the intracellular environment. We applied this strategy to a lysine-specific demethylase 1 (LSD1) inhibitor peptide **1**. The resulting cyclic peptide **2** exhibited approximately 20 times weaker LSD1-inhibitory activity than peptide **1**. Upon addition of reducing reagent, the linker was completely removed to regenerate the linear peptide **1**, with full restoration of the LSD1-inhibitory activity. In addition, the cyclic peptide was far less susceptible to proteolysis than the linear counterpart. Thus, this switch design not only enables control of functional activity, but also improves stability. This approach should be applicable to a wide range of peptides, and may be useful in the development of peptide pharmaceuticals.

Keywords: Epigenetics; Histone demethylase; Inhibitor peptide; Cyclic peptide; Traceless linker; Redox-responsive linker

1. Introduction

Peptides are attractive drug candidates, since they show specific and potent biological activities [1] and can bind to protein surfaces with high specificity [2]. Consequently, they have the potential to inhibit protein-protein interactions, which mediate many important physiological pathways that may be altered in disease states. However, peptides are easily metabolized by proteases, and often have short half-lives in plasma, so that they are generally unsuitable for clinical use.

Many efforts have been made to develop general methods to improve the pharmacokinetic properties of peptides without reducing their activity. Representative methodologies include incorporation of non-natural amino acids such as D-amino acids [3, 4] or β-amino acids [5-8], changing the position of side chains, as in peptoids [3], *N*-methylation of backbone amide bond(s) [9], and covalent macrocyclization [10-12]. These approaches are effective, but tend to reduce the biological activity, although they may improve cell membrane permeability. As a general method to control peptide function, we have explored the cyclization of active linear peptides to restrict their conformation, so that an active conformation cannot be formed. Our strategy is based on the occurrence of a large structural change after photoirradiation, as illustrated in Figure 1, and is thus potentially versatile. The basic idea is that a stimulus-responsive linker is incorporated into the cyclic peptide sequence, and then when the stimulus is applied, the linker is cleaved to form a linear peptide, which can adopt the active conformation. This approach offers at least two advantages: firstly, the switch stimulus can be selected to enable targeting of the activation process to a desired environment (thereby reducing non-target-related side effects), and secondly, cyclic peptides are generally more metabolically stable than linear ones (thereby enabling use of lower doses). We have already employed this strategy to develop a photo-responsive matrix metalloproteinase-3 (MMP-3) inhibitor peptide whose K_i value was increased around 50 times upon photoirradiation [13]. However, portions of the linker moiety remain on the linear peptide after cleavage, and interfere with the activity, so that the full activity of the intact linear peptide is not regained. Therefore, the aim of the present work was to examine the feasibility of a macrocyclization strategy using a "traceless" disulfide-based linkage that would be cleaved and completely removed in a reducing environment, regenerating the original linear peptide without any linker-related structure. This linker would serve as a redox switch that would be activated in the intracellular environment.

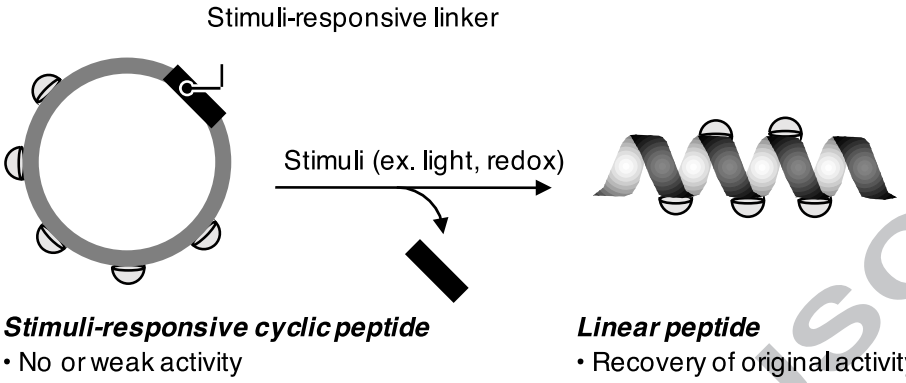


Figure 1. Concept of our cyclization strategy to control peptide activity.

As a model peptide for this study, we focused on lysine-specific demethylase 1 (LSD1/KDM1A), which is a flavin adenine dinucleotide (FAD)-dependent lysine demethylase that removes one or two methyl groups from histone H3 lysine 4 (H3K4) or lysine 9 (H3K9) in complexes with several nuclear proteins, such as the corepressor CoREST and the androgen receptor [14-19]. We chose LSD1 as a model protein for the following reasons: firstly, structural information for its complexes with several native peptide substrates such as histone H3 and SNAIL1 is available [20, 21]; secondly, since the N-terminal ends of peptide substrates are completely embedded within the enzyme's catalytic cavity, an additional moiety (such as a part of a linker) at the N-terminus might significantly impair the enzyme–substrate interaction; and thirdly, inhibitors of the enzyme are considered to be potential anti-cancer agents. In this study, we synthesized a series of cyclic and linear peptide derivatives based on SNAIL1 peptide sequence, and assessed their LSD1-inhibitory activity and proteolytic stability.

2. Results and Discussion

2.1. Design and synthesis of cyclic LSD1 inhibitor peptide activatable in a reducing environment.

For the present purpose, we selected a redox-activated switching moiety, because the concentration of cellular free thiols, such as glutathione (GSH), is markedly higher inside cells than outside cells. Thus, disulfide bonds are stable in the extracellular space, but are cleaved inside cells by cellular free thiols. Disulfide-based functionalization has widely been used in the development of chemosensors [22, 23], prodrugs [24-28], and so forth [29]. We also took account of reports that cyclic peptides with a disulfide bond linkage show superior stability to proteases [30-32].

In our previous study [13], we used a photocleavable linker, of which a part remained in the linear peptide after photocleavage. This impacted negatively on the activity, and the photocleaved peptide showed 5 to 10 times weaker activity than the parent linear peptide. To overcome this problem, in the present work we chose a traceless linker, which leaves no linker-related structures in the product after cleavage.

The chemical structures of peptides used in this study are shown in Figure 2. Linear peptide **1** is the N-terminal domain of transcription factors of the SNAIL1 family, and is known to bind to the active site of LSD1 and to potently inhibit its activity [33]. We therefore designed cyclic peptide **2**, in which the N-terminal secondary amine of Pro1 is linked to the primary amine of Lys8 side chain *via* a redox-activatable linker, as a candidate redox-responsive LSD1 inhibitor. Cyclization is expected to restrict the overall peptide conformation, so that the active conformation cannot be formed. The expected reaction mechanism in a reducing environment is shown in Scheme 2. We also synthesized the reducing conditions-stable cyclic peptide **3**, which contains a CH_2-CH_2 bond instead of the S-S bond, for comparison.

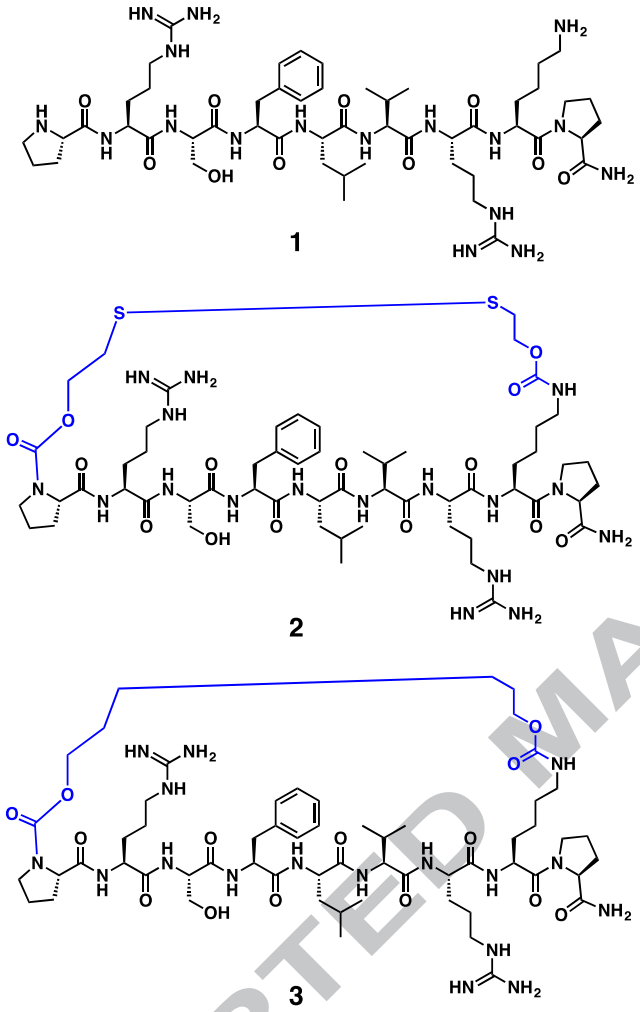
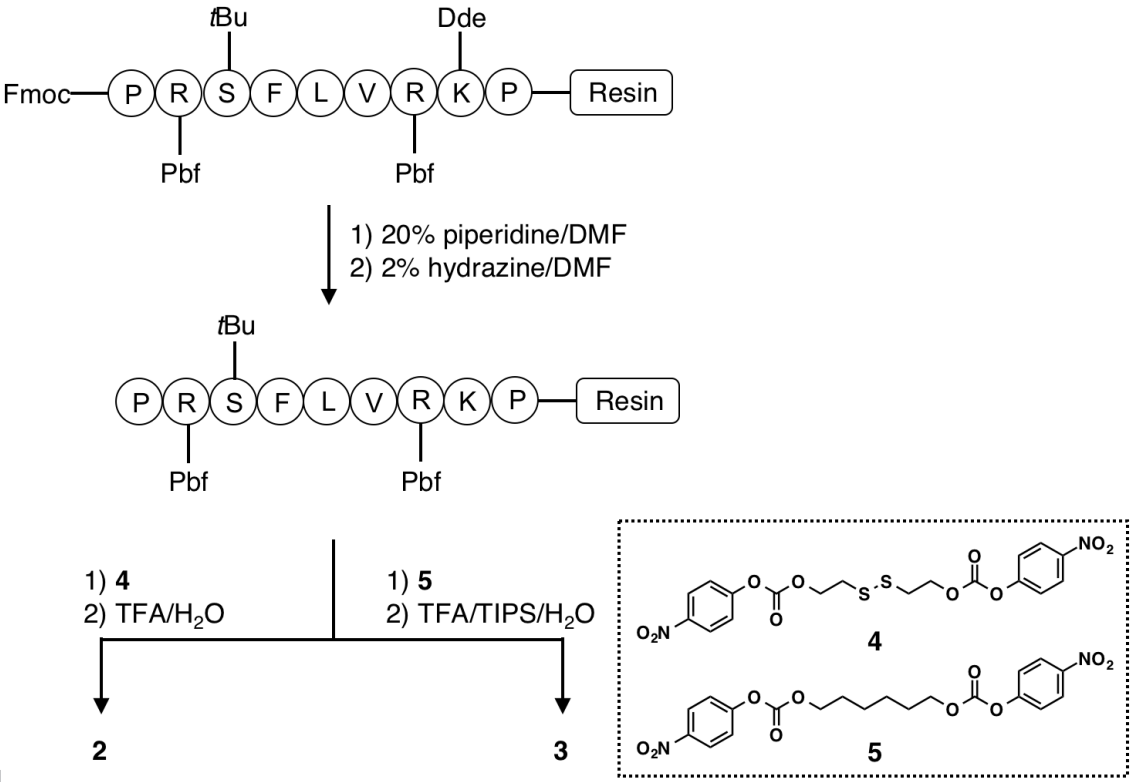


Figure 2. Chemical structures of peptides used in this study.

Linear peptide **1** was synthesized according to the standard Fmoc solid-phase peptide synthesis. The designed cyclic peptides **2** and **3** were synthesized according to Scheme 1. First, the linear peptide was synthesized on resin with the standard Fmoc protocol, using the Dde group [34, 35] as side chain-protecting group for Lys8 (for the linear peptide **1**, a Boc group was used). The Fmoc group at the N-terminus was cleaved with 20% piperidine in DMF and the Dde group on the Lys8 side chain was selectively cleaved with 2% hydrazine in DMF. Then, the resin-bound peptide was treated with activated linker **4** or **5**. Activated linkers **4** and **5** were synthesized in a single step from commercially available bis(2-hydroxyethyl) disulfide (for linker **4**) or 1,6-hexanediol (for

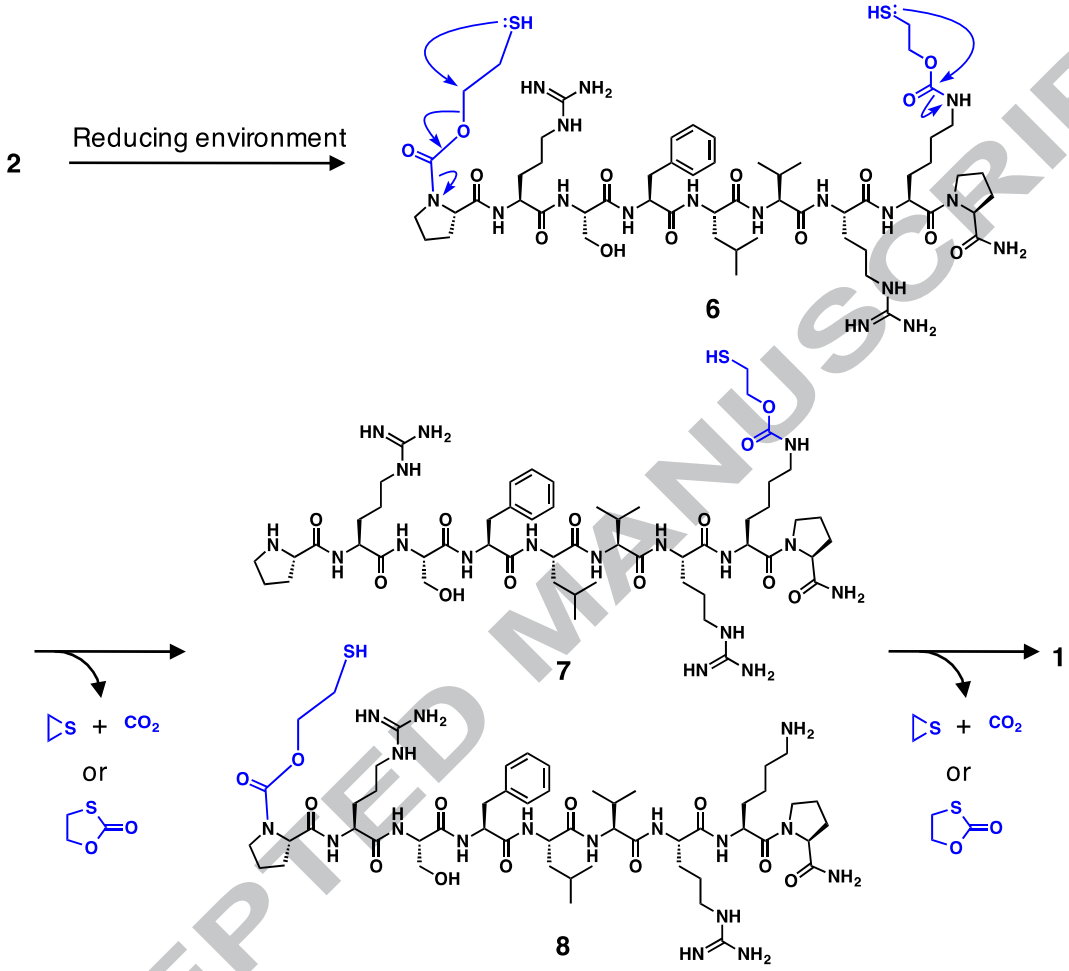
linker **5**) by treatment with *p*-nitrophenyl chloroformate. The progress of linker incorporation was monitored by means of the Kaiser test. This simple protocol proceeded moderately well, probably due to the pseudo-dilution effect on a solid support [36-38]. Cleavage and deprotection of remaining side chain protecting groups were achieved simultaneously with TFA/water (95:5). In general, cleavage cocktails contain a reducing reagent as a scavenger, such as triisopropylsilane (TIPS) or 1,2-ethanedithiol (EDT). However, a scavenger-free condition is critical in this case, since a reducing reagent would cleave the disulfide bond and result in decomposition of the cyclic peptide.



Scheme 1. Solid-phase synthesis of cyclic peptides **2** and **3**.

The proposed reaction mechanism of peptide **2** in a reducing environment is shown in Scheme 2. The disulfide bond is cleaved to form linear intermediate **6**, and the resulting thiolates react intramolecularly. Although two different reaction mechanisms can be considered (see intermediate **6** in Scheme 2; the two thiols react differently) [27,

39, 40], all linker-related substructure is completely eliminated in either case [22, 24, 27, 41], and the unmodified linear peptide **1** is formed.



Scheme 2. Plausible reaction mechanism of cyclic peptide **2** in a reducing environment to afford unmodified linear peptide **1**.

2.2. Reactivity of cyclic peptide 2 in a reducing environment.

The reactivity of cyclic peptide **2** was evaluated in the presence and absence of tris(2-carboxyethyl)phosphine hydrochloride salt (TCEP) as a reducing reagent. Three molar equivalent of TCEP relative to disulfide was added [42]. As shown in Figure 3, cyclic peptide **2** was converted to intermediate **6** within one minute in the presence of TCEP, that is, cleavage of the disulfide bond was rapid and clean. Then, the dithiol peptide **6** was converted to linear peptide **1** *via* peptide **7** or **8**. Each of these

intermediates (**6**, **7** or **8**) and product (**1**) was identified by MALDI-TOF-MS. The retention time of peptide **1** was identical with that of synthesized peptide **1**. The conversion proceeded cleanly without any prominent byproduct and was almost completed in 3 hours. As for the thiol-based reducing reagent, dithiothreitol (DTT) gave similar result to TCEP at the same concentration of reducing reagent, although the reduction of disulfide was slower than that with TCEP (see Supplementary material, Figure S1). As expected, control cyclic peptide **3** was stable under the same conditions (Figure 4). Also, peptide **2** was completely stable under the conditions in the absence of reducing reagent (Figure 5).

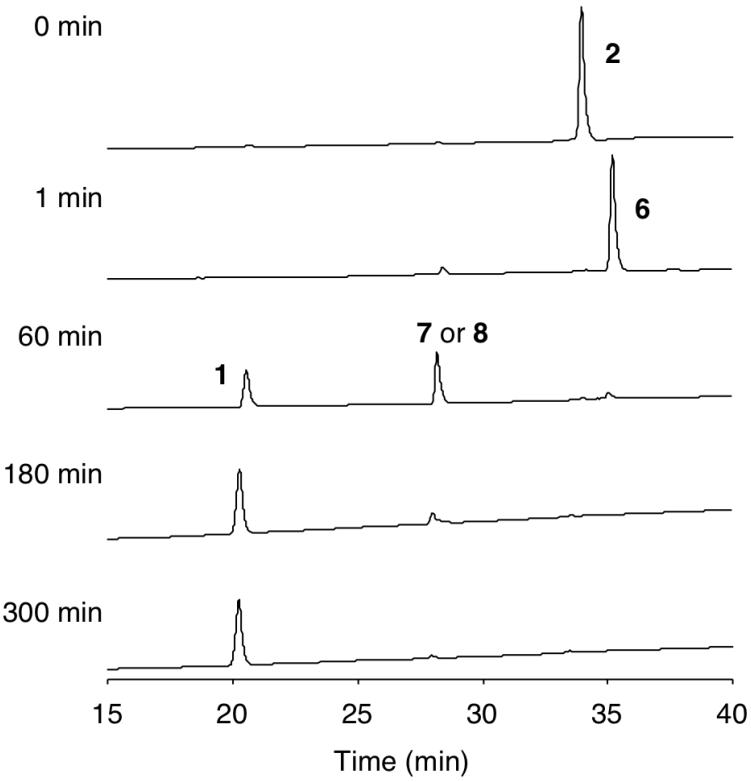


Figure 3. Conversion of cyclic peptide **2** to linear peptide **1** in the presence of TCEP in PBS (10 mM, pH 7.4; 3% DMSO) at 37 ºC. [**2**] = 100 μM, [TCEP] = 300 μM.

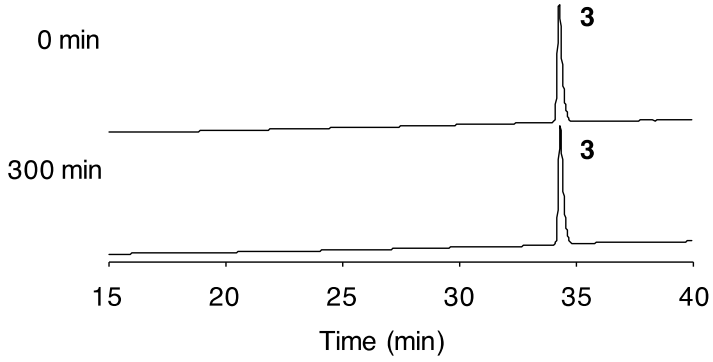


Figure 4. Reaction of cyclic peptide **3** in the presence of TCEP in PBS (10 mM, pH 7.4; 3% DMSO) at 37 ºC. [**3**] = 100 μM, [TCEP] = 300 μM.

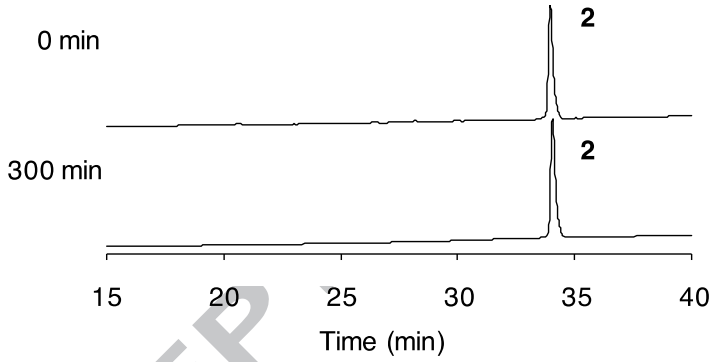


Figure 5. Reaction of cyclic peptide **2** in the absence of TCEP in PBS (10 mM, pH 7.4; 3% DMSO) at 37 ºC. [**2**] = 100 μM, [TCEP] = 0 μM.

2.3. LSD1-inhibitory activity.

The LSD1-inhibitory activity of the synthesized peptides was measured *in vitro* using the peroxidase-coupled assay system. The K_i values are summarized in Table 1. Linear peptide **1** showed potent activity (K_i = 1.9 ± 0.2 μM), although this value is about 10 times weaker than the reported value (K_i = 0.14 μM) [33]. However, this difference is likely due to differences in the assay conditions. Cyclic peptide **2** showed approximately 20 times weaker activity (K_i = 44 ± 10 μM) than linear peptide **1**, while cyclic peptide **3** was inactive (K_i > 300 μM). We suspected that peptide **2** might have been partially

activated by residual reducing reagent, since DTT was used for the preparation of human LSD1 protein (the final concentration of DTT was approximately 17 μM), and therefore we prepared human LSD1 without DTT and repeated the experiment. But, as shown in Table 1, the inhibitory activity was almost the same (K_i = 37 ± 8 μM), suggesting that the residual DTT hardly reduced peptide **2**, although the possibility still remains that peptide **2** was partially activated under the assay condition without DTT. To rule out any possible effect of the disulfide bond in peptide **2**, we examined the activity of the linker moiety, bis(2-hydroxyethyl) disulfide, but this compound did not perturb the assay or inhibit LSD1. These results suggested that cyclic peptide **2** was partially activated even under the assay condition without DTT. Otherwise, the difference in linker structure between peptides **2** and **3** (i.e. -S-S- vs. $-CH_2-CH_2-$) might influence the overall conformation and the LSD1-inhibitory activity.

Table 1. K_i values of synthesized peptides for LSD1 inhibition.

Peptide	Reducing reagent	K_i (μM)
1	-	1.9 ± 0.2
2	-	44 ± 10
3	-	>300
2 [a]	-	37 ± 8
3 [a]	-	>300
2	+ [b]	1.6 ± 0.1 [c]
9	-	190 ± 30

[a] LSD1 protein was prepared and handled without DTT. [b] After treatment with TCEP, resulting peptide was roughly purified by HPLC, since remaining TCEP interfered with the assay. [c] Calculated based on the concentration of the reaction product.

Next, we examined the activation of cyclic peptide **2** by reducing reagent. Since our assay measures H_2O_2 produced by LSD1-catalysed demethylation reaction, added reducing reagent might react with H_2O_2 and perturb the assay. Indeed, TCEP, DTT, and 2-mercaptoethanol were all incompatible with the assay. Thus, in order to remove any remaining reducing reagent, we purified the reaction product of cyclic peptide **2** with TCEP by HPLC. As shown in Table 1, the resulting peptide showed potent LSD1-inhibitory activity (K_i = 1.6 ± 0.1 μM), at essentially the same level as the linear peptide **1**. These data confirm that conversion of cyclic peptide **2** to linear peptide **1**, with full functional activation, can be achieved by redox switching.

In order to confirm the superiority of the traceless linker, we synthesized peptide **9** (Figure 6), which contains linker-related structures at the N- and C-termini of peptide **1**. Peptide **9** was originally designed for the other stimulus-responsive linkers (manuscript in preparation), but serves as a good control for this purpose. Peptide **9** showed considerably weaker LSD1-inhibitory activity (K_i = 190 ± 30 μM) than peptide **1** (K_i = 1.9 ± 0.2 μM), as shown in Table 1. The decrease of LSD1-inhibitory activity compared with peptide **1** is considered to be due to the presence of the linker-related hydroxyacetyl (-$COCH_2OH$) group at the N-terminus, because the N-terminal Pro1 of the native SNAIL1 substrate peptide is embedded within the LSD1 catalytic cavity in the crystal structure, whereas the C-terminal Pro9 is located outside the cavity [21]. The hydroxyacetyl group at Pro1 is expected to clash sterically with Ala539 of human LSD1 and disturb hydrogen bonding between Pro1 of the peptide and Ala539 of LSD1, which would account for the decreased LSD1-inhibitory activity. Peptide **9** (K_i = 190 ± 30 μM) showed even weaker activity than cyclic peptide **2** (K_i = 44 ± 10 μM), further suggesting the possibility of partial activation of cyclic peptide **2** under the assay condition. This result also suggests that even a slight chemical modification at the peptide termini can greatly impair the inhibitory activity, and highlights the advantage of our traceless linker for the present purpose.

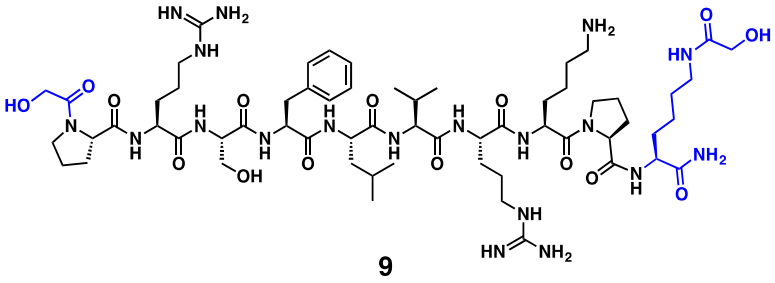


Figure 6. Chemical structure of peptide **9**. Linker-related structures are shown in blue.

Cyclic peptide **2** was designed as a prototypal compound that may exhibit biological function under reducing conditions such as inside cells although it would need further modification(s) to acquire cell permeability. Therefore, we assessed whether peptide **2** inhibits proliferation of human acute promyelocytic leukemia cell line HL-60 which is sensitive to LSD1 inhibition [43]. We found that peptide **2**, along with peptides **1** and **3**, did not show inhibitory effect on the proliferation of HL-60 cells at the final concentration up to 800 μM, suggesting that cyclic peptide **2** did not penetrate the cell membrane effectively. Additional modification(s) of peptide **2** should be necessary to obtain practical membrane permeability, which is under investigation in our laboratories.

2.4. Stability of the peptides to protease.

Cyclic peptides [10-12], including those containing a disulfide bond [30-32], often show enhanced stability to proteases. Here, we examined the stability of cyclic peptide **2** toward α-chymotrypsin as a representative protease. α-Chymotrypsin is a member of the serine protease family, which cleaves backbone amide bonds on the C-terminal side of hydrophobic and/or aromatic residues in peptides, such as Phe, Leu, Tyr, or Trp [44]. As shown in Figure 7, cyclic peptide **2** showed enhanced stability to α-chymotrypsin, compared with linear peptide **1**. This result confirmed that cyclization is effective to improve stability toward protease, and potentially supports the idea that our molecule would be effective to deliver the fully active linear inhibitor to target sites in a redox-dependent manner, although further modification of cyclic peptide **2** is necessary for membrane permeability.

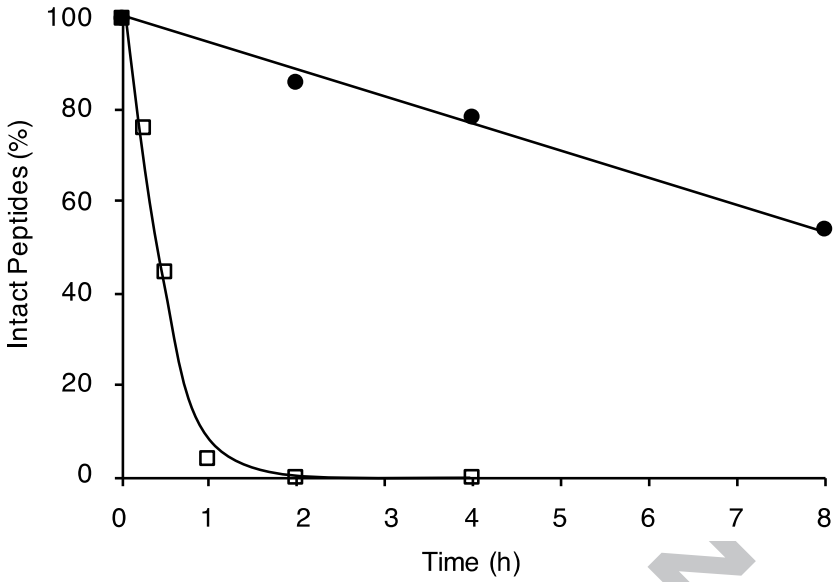


Figure 7. Proteolytic digestion of peptides **1** and **2** by α-chymotrypsin in Tris buffer (10 mM, pH 8.0; 0.5% DMSO) at 37 ºC; [peptide] = 20 μM, [α-chymotrypsin] = 0.1 μg/mL; closed circle: peptide **2**, open square: peptide **1**.

3. Conclusion

We constructed a redox-responsive cyclic peptide **2** with a traceless linker, and showed that it is cleanly converted to the unmodified linear peptide **1** in a reducing environment. Cyclic peptide **2** showed approximately 20 times weaker LSD1-inhibitory activity than peptide **1**, but upon addition of reducing reagent TCEP, the linker was cleaved and the LSD1-inhibitory activity increased, becoming essentially the same as that of peptide **1**. Also, cyclic peptide **2** was more stable to α-chymotrypsin than peptide **1**. These results suggest that the cyclization strategy used here, with a disulfide-based traceless linker, is effective. Thus, this switch design not only enables control of functional activity, but also improves stability to hydrolytic enzyme. The methodology presented here could be applicable to a wide variety of peptides, and may be advantageous in the development of fine-tuned peptide pharmaceuticals. Appropriate linker design should also be possible to design peptides responsive to other stimuli, such as light, pH, or enzymatic activity. Work to develop other stimulus-responsive peptides is in progress in our laboratories.

4. Experimental

4.1. Chemical synthesis

4.1.1. Materials

N-(9-Fluorenylmethyloxycarbonyl) (Fmoc)-protected amino acids, 1-hydroxybenzotriazole (HOBt), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Fmoc-NH-SAL-Resin (0.54 mmol/g), *N*,*N*-diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), and triisopropylsilane (TIPS) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Dichloromethane (DCM) and *N*,*N*-dimethyformamide (DMF) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and Merck Biosciences (Darmstadt, Germany). Unless otherwise stated, other chemicals and reagents were obtained commercially and used without further purification. Purified α-chymotrypsin (from bovine pancreas, Type II, lyophilized powder, ≥40 units/mg protein) was purchased from Sigma-Aldrich (St. Louis, USA).

4.1.2. Disulfide linker 4

To a stirred solution of bis(2-hydroxyethyl) disulfide (400 mg, 2.59 mmol) in DCM (12.0 mL) at 0 ºC were added pyridine (1.03 g, 13.0 mmol) and *p*-nitrophenyl chloroformate (1.31 g, 6.49 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 6 h. The mixture was diluted with DCM (50 mL), and washed sequentially with sat. $NaHCO_3$ (30 mL x 3), water (30 mL), 10% citric acid (30 mL x 2), water (30 mL), and brine (30 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by silica gel column chromatography (5% to 50% AcOEt/hexane) to afford disulfide linker **4** (1.26 g, quant) as a white powder. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.28 (d, J = 8.9 Hz, 4H), 7.39 (d, J = 8.9 Hz, 4H), 4.57 (t, J = 6.5 Hz, 4H), 3.08 (t, J = 6.5 Hz, 4H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 155.4, 152.4, 145.6, 125.4, 121.9, 66.9, 36.9. IR (KBr, cm^{-1}): 1770, 1520, 1348, 1260, 1213, 928, 860. m.p.: 74.0-74.5 ºC. HR-MS (*m/z*, EI): calcd for $C_{18}H_{16}N_2O_{10}S_2$ (M^+), 484.0246; found, 484.0255.

4.1.3. Methylene linker 5 [45]

To a stirred solution of 1,6-hexanediol (301 mg, 2.55 mmol) in DCM (11.7 mL) at 0 ºC were added pyridine (1.01 g, 12.7 mmol) and *p*-nitrophenyl chloroformate (1.28 g, 6.35

mmol). The reaction mixture was allowed to warm to room temperature and stirred at room temperature for 3 h. The mixture was diluted with DCM (20 mL), and washed sequentially with sat. $NaHCO_3$ (20 mL x3), water (20 mL), 10% citric acid (20 mL x2), water (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The resulting residue was purified by silica gel column chromatography (3% to 100% AcOEt/hexane) to afford desired linker **5** (995 mg, 87%) as a pale brown powder. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.28 (d, *J* = 9.1 Hz, 4H), 7.38 (d, *J* = 9.1 Hz, 4H), 4.31 (d, *J* = 6.6 Hz, 4H), 1.84-1.78 (m, 4H), 1.53-1.50 (m, 4H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 155.4, 152.4, 145.6, 125.4, 121.9, 66.9, 36.9. IR (KBr, cm^{-1}): 1766, 1525, 1347, 1255, 1213, 950, 862. m.p.: 112-114 ºC, HR-MS (*m/z*, ESI+) : calcd for $C_{20}H_{20}N_2NaO_{10}$ ($[M+Na]^+$), 471.1016; found, 471.1018.

4.1.4. Synthesis of Fmoc-Lys(Dde)-OH [34, 46, 47]

The synthesis of 2-acetyldimedone (Dde-OH) was performed according to the literature with slight modifications [46]. Dimedone (6.05 g, 43.2 mmol) and pyridine (3.83 mL, 47.5 mmol) were added to DCM (70 mL), and the mixture was cooled to 0 ºC. Acetyl chloride (3.73 mL, 10.1 mmol) was added, and stirring was continued at 0 ºC for 1 h. The reaction mixture was allowed to warm to room temperature, and stirred for 7 h. The mixture was diluted with DCM (50 mL), and the organic phase was washed with 1 M HCl (100 mL), sat. $NaHCO_3$ (100 mL), and brine (100 mL). The solution was dried over Na_2SO_4, filtered, and concentrated. The resulting residue was dissolved in DCM (97 mL), and $AlCl_3$ (12.21 g, 91.6 mmol) was added. The reaction mixture was stirred at room temperature for 7 h, then poured into 6 M HCl (70 mL). The aqueous phase was extracted with DCM (100 mL x 6) and AcOEt (100 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The resulting residue was purified by silica gel column chromatography (10% to 20% AcOEt/hexane) to give 2-acetyldimedone (6.05 g, 77%) as a pale yellow oil. Spectroscopic data were identical to the published data [47]. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.61 (s, 3H), 2.53 (s, 2H), 2.36 (s, 2H), 1.08 (s, 6H), MS (*m/z*, EI): 182 $[M]^+$

Fmoc-Lys(Boc)-OH (5.66 g, 12.1 mmol) was dissolved in 4 M HCl/dioxane (120 mL), and stirred at room temperature for 2 h to remove the side-chain Boc group. The solvent was removed under reduced pressure. The resulting residue was dissolved in EtOH (60 mL), and then 2-acetyldimedone (3.36 g, 18.4 mmol) and DIPEA (6.2 mL,

35.6 mmol) were added. The reaction mixture was refluxed for 17 h. After cooling to room temperature, the solvent was removed under reduced pressure. The residue was dissolved in AcOEt (300 mL) and washed with 1 M HCl (100 mL) and brine (100 mL), dried over Na_2SO_4, filtered, and concentrated. The resulting residue was purified by silica gel column chromatography (0.5% to 3% MeOH/DCM) to give Fmoc-Lys(Dde)-OH (2.64 g, 41%) as a white solid. Spectroscopic data are identical to the published data [34]. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 13.31 (brs, 1H), 7.75 (d, *J* = 7.8 Hz, 2H), 7.59 (t, *J* = 7.8 Hz, 2H), 7.38 (t, *J* = 7.8 Hz, 4H), 7.31-7.28 (m, 2H), 5.73 (d, *J* = 8.0 Hz, 1H), 4.48-4.45 (m, 1H), 4.37 (d, *J* = 7.1 Hz, 2H), 4.20 (t, *J* = 7.1 Hz, 1H), 3.43-3.40 (m, 2H), 2.55 (s, 3H), 2.36 (s, 4H), 2.00-1.50 (m, 6H), 1.01 (s, 6H). HR-MS (*m/z*, FAB): calcd for $C_{31}H_{37}N_2O_6$ ($[M+H]^+$), 533.2652; found, 533.2643.

4.1.5. Synthesis of linear peptide 1.

The linear peptide **1** was synthesized by standard Fmoc solid-phase peptide synthesis. Prior to the peptide synthesis, Fmoc-NH-SAL-Resin was washed with DMF 5 times. Three molar amounts of Fmoc amino acid, HBTU, HOBt and 6 molar amounts of DIPEA were used for each coupling. DMF was used as the reaction solvent. The reaction mixture was shaken for 30 min at room temperature for each coupling step. The completion of amino acid coupling was monitored with the Kaiser test. For Fmoc deprotection steps, the resin was treated twice with 20% (v/v) piperidine in DMF and shaken for 20 min at room temperature.

When peptide elongation was complete, the peptide was cleaved from the resin and side-chain protecting groups were removed by shaking with TFA/TIPS/H_2O (95:2.5:2.5, v/v/v) for 2 h. The resin was removed by filtration and rinsed with additional cleavage cocktail. The combined filtrate was concentrated by blowing Ar. The peptide was precipitated from cold diethyl ether, and collected by centrifugation/decantation. The crude peptide was purified by semipreparative, reversed-phase HPLC on an Inertsil ODS-3 column (GL Sciences, Tokyo, Japan) on a Shimadzu instrument with a linear gradient system of 10:90 to 50:50 CH_3CN/H_2O (0.1% TFA) over 40 min at the flow rate of 3.0 mL/min. The purity of peptide **1** was >99%, based on the peak areas in analytical HPLC traces at 220 nm. MS (*m/z*, MALDI-TOF): 1098.7 $[M+H]^+$.

4.1.6. Synthesis of cyclic peptides 2 and 3

Fmoc-Lys(Dde)-OH was used for Lys8, instead of Fmoc-Lys(Boc)-OH. After completion of linear peptide elongation, the resin was swollen with DMF, treated with 20% (v/v) piperidine in DMF for 20 min to remove N-terminal Fmoc protecting group, and washed with DMF (x 5). The resin was then treated twice with 2% hydrazine monohydrate (v/v) in DMF [34, 35] for 1 h to remove the Dde group on the Lys8 side chain, and washed with DMF (x 5).

To the resin-bound peptide with two free amino groups was added activated linker **4** (for peptide **2**, 1.5 equiv) or linker **5** (for peptide **3**, 1.5 equiv) in DMF and DIPEA (3 equiv). The mixture was shaken at room temperature for 3 h, and then washed with DMF (x 5) and DCM (x 5). Resin-bound cyclic peptide was cleaved by shaking with TFA/H_2O (for peptide **2**, 95:5, v/v) or TFA/TIPS/H_2O (for peptide **3**, 95:2.5:2.5, v/v/v) for 2 h. The resin was removed by filtration and rinsed with additional cleavage cocktail. The combined filtrate was concentrated by blowing Ar. To the mixture was added cold diethyl ether. The resulting precipitate was collected by centrifugation/decantation. The purification was performed by reversed-phase HPLC using a linear gradient system of 25:75 to 40:60 CH_3CN/H_2O (0.1% TFA) over 30 min at the flow rate of 3.0 mL/min. The purities of peptides **2** and **3** were >96% and >97%, respectively, based on the peak areas in analytical HPLC traces at 220 nm. MS (*m/z*, MALDI-TOF): 1304.7 $[M+H]^+$ (**2**), 1268.7 $[M+H]^+$ (**3**).

4.1.7. Synthesis of *O*-tritylglycolic acid[48]

To a solution of glycolic acid (620 mg, 8.15 mmol) in pyridine (30 mL) was added TrtCl (2.71 g, 9.72 mmol). The mixture was stirred at room temperature for 21 h, then diluted with AcOEt (100 mL), washed with brine (60 mL x 3), dried over Na_2SO_4, and concentrated. The resulting residue was purified by silica gel column chromatography (0% to 1.5% MeOH/DCM) to give *O*-tritylglycolic acid (270 mg, 10%) as a white solid. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.42 (d, J = 7.5 Hz, 6H), 7.34 (t, J = 7.5 Hz, 6H), 7.27 (t, J = 7.5 Hz, 3H), 3.91 (s, 2H). HR-MS (*m/z*, ESI-): calcd for $C_{21}H_{17}O_3$ ($[M-H]^-$), 317.1178; found, 317.1172.

4.1.8. Synthesis of peptide 9

Fmoc-Lys(Dde)-OH was used for C-terminal Lys (Lys10). For Lys8, Fmoc-Lys(Boc)-OH was used. After completion of peptide elongation, the N-terminal

Fmoc group and Dde group on the Lys10 side chain were sequentially removed as described for the synthesis of cyclic peptides **2** and **3**. The resin-bound peptide with two free amino groups was treated with 6 molar amounts of *O*-tritylglycolic acid, HBTU, HOBt and 12 molar amounts of DIPEA (3 and 6 molar amounts for each amino group, respectively). The reaction mixture was shaken for 3.5 h at room temperature, and then washed with DMF (x 5) and DCM (x 5). Then, the peptide was cleaved from the resin according to the same protocol described for the synthesis of linear peptide **1**. The crude peptide was purified by reversed-phase HPLC with a linear gradient system of 10:90 to 40:60 CH_3CN/H_2O (0.1% TFA) over 30 min at the flow rate of 3.0 mL/min. The purity of peptide **9** was >99%, based on the peak areas in analytical HPLC traces at 220 nm. MS (*m/z*, MALDI-TOF): 1342.8 $[M+H]^+$.

4.2. Reaction of peptides 2 and 3 with TCEP or DTT

The reaction of the cyclic peptides (final concentration: 100 μM) with TCEP (final concentration: 300 μM) was monitored by HPLC. Peptide **2** or **3** was dissolved in DMSO (10 mM) and diluted with PBS (pH 7.4) to prepare peptide solution (102 μM, 10.2 mM PBS, 1.02% DMSO v/v). To the peptide solution (49 μL), TCEP•HCl in DMSO (15 mM, 1 μL) was added. For the TCEP-free control, DMSO was added instead. The reaction mixture was shaken at 37 ºC. At each time point, the reaction mixture (50 μL) was diluted with CH_3CN/H_2O (1:4, v/v, 450 μL), and injected into the RP-HPLC. A linear gradient system of 10:90 to 50:50 CH_3CN/ H_2O (0.1% TFA) over 40 min at the flow rate of 1.0 mL/min was used for separation, with monitoring at 220 nm. Essentially same procedure was used for the reaction of peptide **2** (final concentration: 100 μM) with DTT (final concentration: 300 μM). Peptide **2** was dissolved in DMSO (3.3 mM) and diluted with PBS (pH 7.4) to prepare peptide solution (102 μM, 10.2 mM PBS, 3.06% DMSO v/v). To the peptide solution (49 μL), DTT in water (15 mM, 1 μL) was added.

4.3. LSD1 inhibition assay

In vitro LSD1-inhibitory activity was measured by the peroxidase-coupled reaction method as described previously [49]. Briefly, human LSD1 protein (2.8 μM) was incubated with serial dilutions of LSD1 inhibitor peptides in 50 mM HEPES-Na (pH 7.5) buffer containing 400 μM 4-aminoantipyrine, the modified Trinder's reagent

TOOS (*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate) and 40 μg/mL horseradish peroxidase at room temperature for 10 min. Then, the reaction mixture was incubated with 83 μM H3 (1–20) K4me2 peptide for 30 min. Absorption of the peroxidase byproduct generated by LSD1 demethylation was measured at 562 nm with a microplate reader (Ultrospec Visible Plate Reader II 96; GE Healthcare). The results (determined in triplicate) were fitted with the Morrison equation to obtain K_i using Prism 6 software (version 6.0e). LSD1 was buffered in 20 mM HEPES (pH 7.5), 300 mM NaCl, 2 mM DTT. This sample was loaded onto a HiPrep 26/10 desalting column, and eluted with 20 mM HEPES (pH 7.5), 300 mM NaCl in order to remove DTT. LSD1 samples with or without DTT were each used for the assays. Regarding the cell-based assay, proliferation inhibition was assessed by alamarBlue cell viability assay (Bio-Rad Laboratories), using the human acute promyelocytic leukemia cell line HL-60. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich) which was supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin (Sigma-Aldrich) and 0.1 mg/mL streptomycin (Sigma-Aldrich). The medium contained only 1 mg/L glutathione (reduced). The cultured cells were seeded at a density of 4×10^3 cells per well in a 96-well plate. After 4 h incubation, peptides **1**–**3** were added and the cells were incubated at 37 °C for 72 h in a humidified 5% CO_2 incubator. Then, alamarBlue reagent was added and cells were treated at 37 °C for 2 h. The fluorescence (excitation: 535 nm, emission: 600 nm) was measured with EnVision (PerkinElmer) and cell viability was calculated.

4.4. Proteolytic assay [6]

The stability of peptides **1** and **2** to α-chymotrypsin was evaluated by conducting protease reactions at 37 ºC. The reaction was performed in 10 mM Tris-HCl buffer (pH 8.0) containing 0.5% DMSO (v/v). Tris-HCl buffer (477 μL; pH 8.0) was added to each polypropylene tube and gently shaken at 37 ºC for 15 min for temperature equilibration. Then, a solution of peptide (3.0 μL; 4.0 mM in DMSO), and a solution of α-chymotrypsin in 10 mM Tris-HCl buffer (120 μL; 0.5 μg/mL, pH 8.0) were added sequentially. The final concentrations of peptides and enzyme were 20 μM and 0.1 μg/mL, respectively. The reaction mixtures were gently shaken at 37 ºC, and aliquots were taken and quenched with a solution of TFA in water (5%, v/v, 200 μL) at the

selected time points. The progress of proteolysis was quantified by HPLC analysis of the quenched reaction solutions on an Inertsil ODS-3 column.

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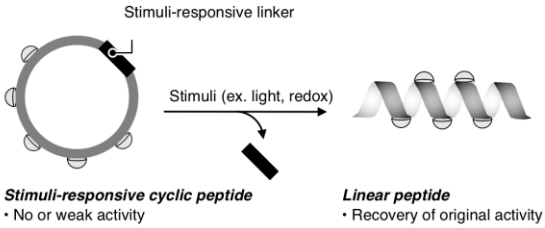


Figure 1

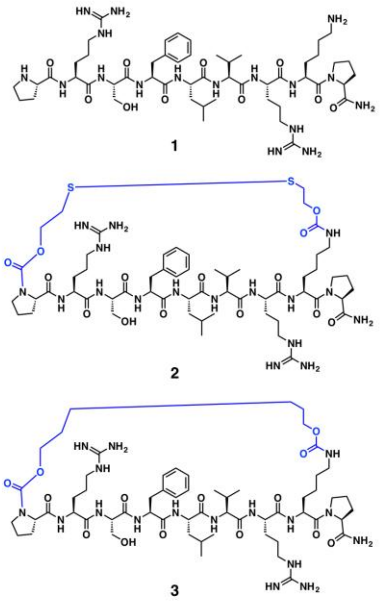
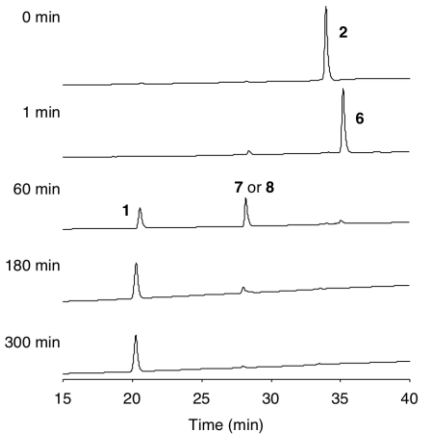


Figure 2



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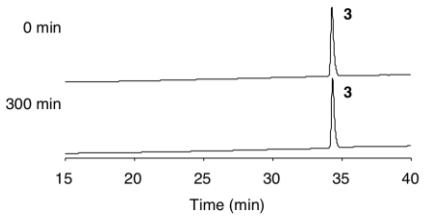

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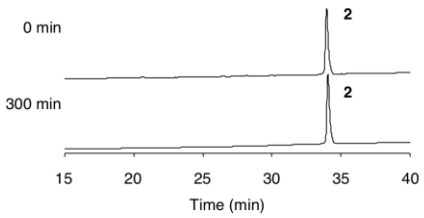

Figure 5.

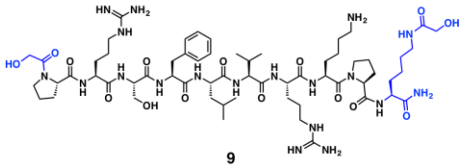

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Figure 6.

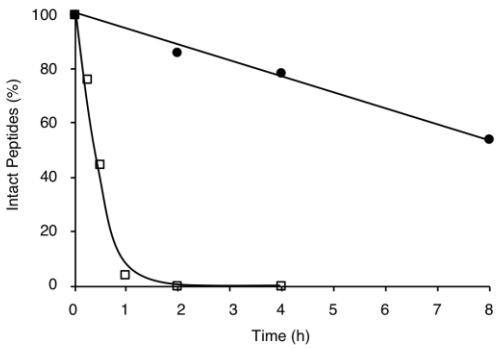
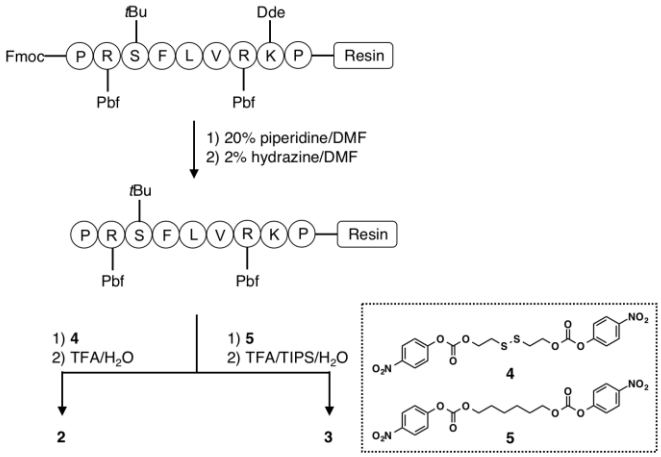
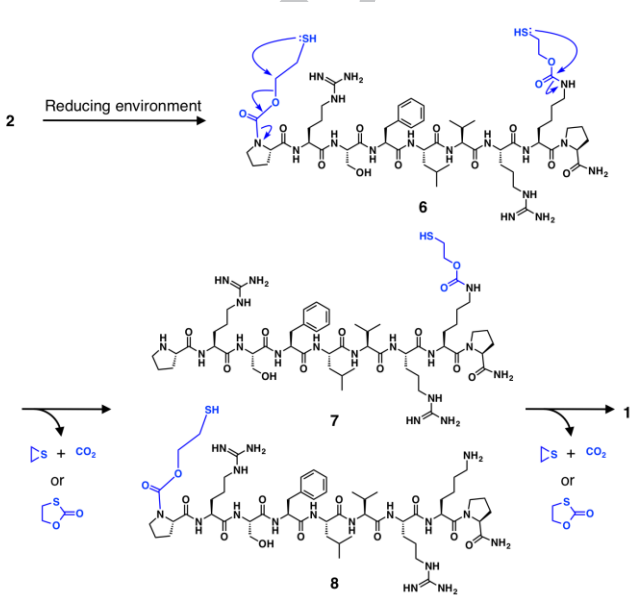


Figure 7.



Scheme 1

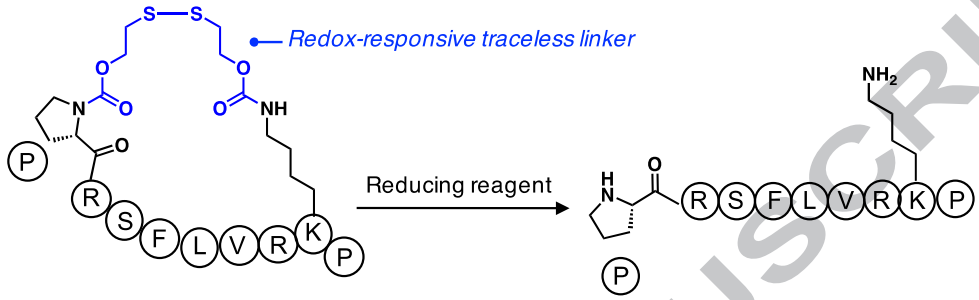


Scheme 2.

Graphical Abstract

Activation of lysine-specific demethylase 1 inhibitor peptide by redox-controlled cleavage of a traceless linker

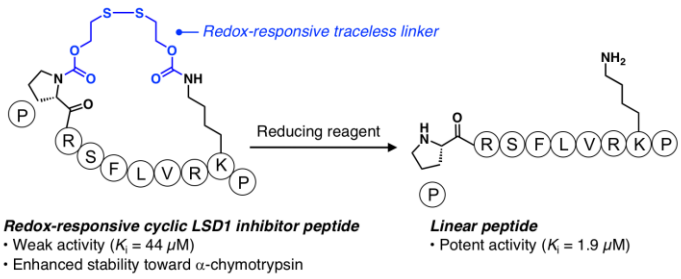
Yuichi Amano, Naoki Umezawa*, Shin Sato, Hisami Watanabe, Takashi Umehara*, Tsunehiko Higuchi*



Redox-responsive cyclic LSD1 inhibitor peptide
• Weak activity (K_i = 44 μM)
• Enhanced stability toward α-chymotrypsin

Linear peptide
• Potent activity (K_i = 1.9 μM)

Graphical abstract



Graphical Abstract