ChemPubSoc Europe

DOI: 10.1002/chem.201406093

CHEMISTRY
A European Journal
Full Paper

Fluorescent Probes

Intramolecular Long-Distance Nucleophilic Reactions as a Rapid Fluorogenic Switch Applicable to the Detection of Enzymatic Activity

Reisuke Baba,[a] Yuichiro Hori,[a, b, c] and Kazuya Kikuchi*[a, b]

Abstract: Long-distance intramolecular nucleophilic reactions are promising strategies for the design of fluorogenic probes to detect enzymatic activity involved in lysine modifications. However, such reactions have been challenging and hence have not been established. In this study, we have prepared fluorogenic peptides that induce intramolecular reactions between lysine nucleophiles and electrophiles in distal positions. These peptides contain a lysine and fluorescence-quenched fluorophore with a carbonate ester, which triggers nucleophilic transesterification resulting in fluorogenic response. Transesterification occurred under mild aqueous conditions despite the presence of a long nine-amino-acid spacer between the lysine and fluorophore. In addition, one of the peptides showed the fastest reaction kinetics with a half-life time of 3.7 min. Furthermore, the incorporation of this fluorogenic switch into the probes allowed rapid fluorogenic detection of histone deacetylase (HDAC) activity. These results indicate that the transesterification reaction has great potential for use as a general fluorogenic switch to monitor the activity of lysine-targeting enzymes.

Introduction

Intramolecular nucleophilic reactions have been adopted to design fluorogenic probes for the detection of enzymatic reactions. In these probes, nucleophiles generated after the reaction with the target enzymes attack internal electrophiles to form five- or six-membered cyclic intermediates.[1] Subsequent elimination of the cyclic structure in the parent probe results in the production of a fluorescent molecule, whereas the initial state of the probe is nonfluorescent. By using this self-immolative reaction, the fluorogenic detection of enzyme activity[2] and reductive environment[3] has been achieved, showing the benefit of this mechanism. However, the application of this fluorogenic switch to probe design is limited mostly to molecules with chemical structures that can form the above-mentioned cyclic intermediates after biological reactions, whereas the use of such a switch has been scarcely reported for intramolecular macrocyclization.[4] Thus, it is a great challenge to design the probes that induce a fluorogenic switch following long-distance nucleophilic reactions.

Lysine is an important target of post-translational modifications such as acetylation, methylation, and succinylation.[5] These reverse reactions are catalyzed by various "eraser" enzymes including histone deacetylases (HDACs),[6] lysine desuccinylases,[7] and lysine demethylases,[8] which play important roles in diverse pathological processes such as tumorigenesis, metabolic diseases, and neurodegenerative disorders.[9,10] Thus, fluorogenic probes for the detection of these enzymatic activities are valuable tools in biological and medical studies. However, the development of such probes has been considered unfeasible[11] because the lysine side chain contains a simple saturated aliphatic amine and does not allow π-conjugation of the amino group to a fluorophore for direct modulation of its electronic state. As a solution to this problem, a fluorogenic switch based on an intramolecular nucleophilic reaction can be utilized for the probe design,[12] since the nucleophilic amine in lysine is generated as a result of the "eraser" enzymatic reactions. In this study, we have synthesized peptides that can induce rapid long-distance intramolecular reactions between lysine nucleophiles and their distal target electrophiles and trigger a fluorogenic response.

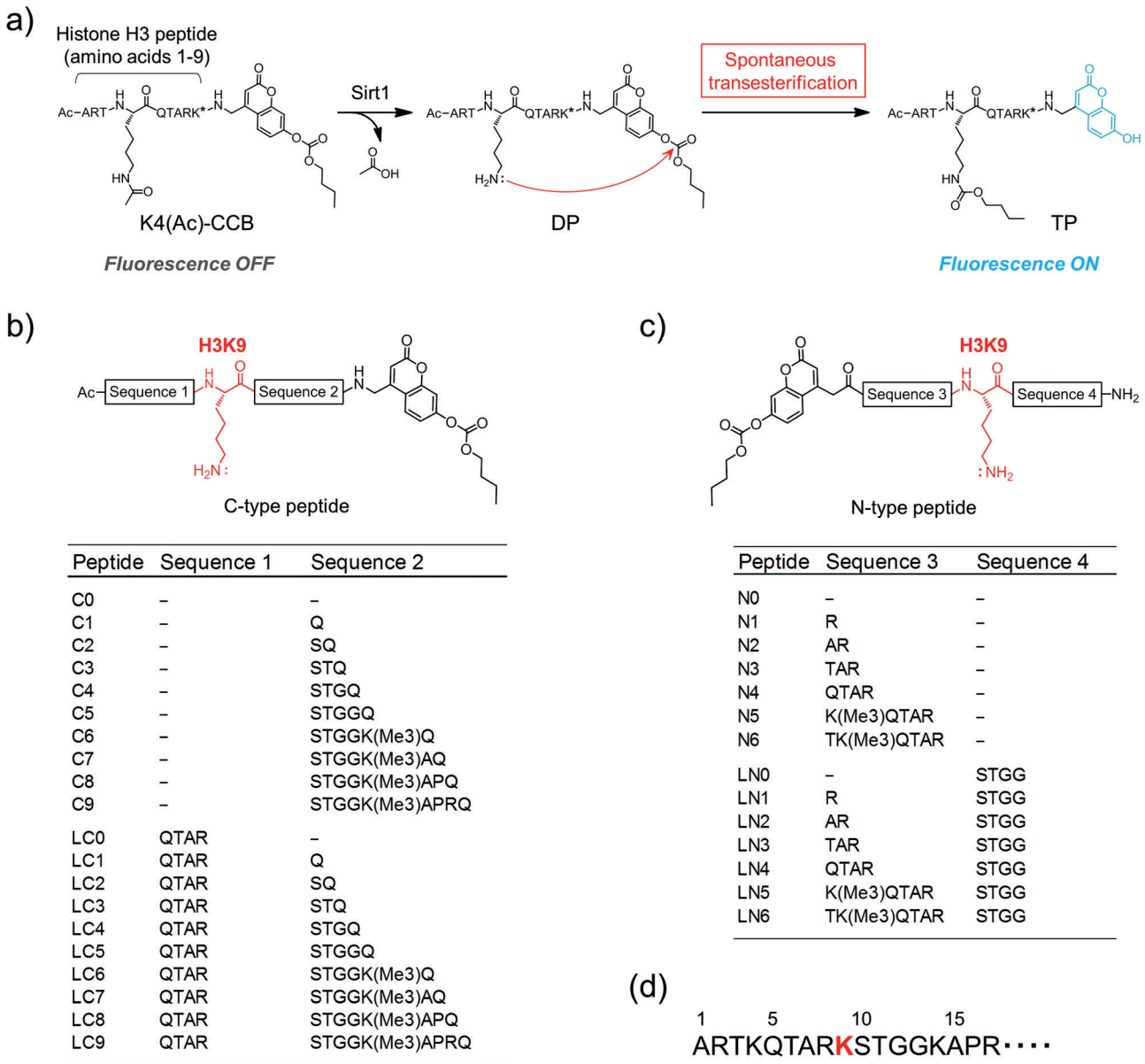
We have previously applied the intramolecular nucleophilic reaction to design a fluorogenic probe K4(Ac)-CCB for the detection of HDAC activity.[12] K4(Ac)-CCB consists of the histone H3 (1–9) peptide containing acetyl-lysine as an HDAC substrate and a fluorescence-quenched coumarin fluorophore with a carbonate ester (Figure 1a). HDAC activity is detected as a fluorescence signal produced by an intramolecular trans-

[a] R. Baba, Dr. Y. Hori, Prof. K. Kikuchi
Graduate School of Engineering
Osaka University, Suita
Osaka, 565-0871 (Japan)
Fax: (+81) 6-6879-7875
E-mail: kkikuchi@mls.eng.osaka-u.ac.jp

[b] Dr. Y. Hori, Prof. K. Kikuchi
Immunology Frontier Research Center
Osaka University, Suita
Osaka, 565-0871 (Japan)

[c] Dr. Y. Hori
JST, PRESTO
Suita, Osaka, 565-0871 (Japan)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201406093.



Peptide	Sequence 1	Sequence 2
C0	–	–
C1	–	Q
C2	–	SQ
C3	–	STQ
C4	–	STGQ
C5	–	STGGQ
C6	–	STGGK(Me3)Q
C7	–	STGGK(Me3)AQ
C8	–	STGGK(Me3)APQ
C9	–	STGGK(Me3)APRQ
LC0	QTAR	–
LC1	QTAR	Q
LC2	QTAR	SQ
LC3	QTAR	STQ
LC4	QTAR	STGQ
LC5	QTAR	STGGQ
LC6	QTAR	STGGK(Me3)Q
LC7	QTAR	STGGK(Me3)AQ
LC8	QTAR	STGGK(Me3)APQ
LC9	QTAR	STGGK(Me3)APRQ

Peptide	Sequence 3	Sequence 4
N0	–	–
N1	R	–
N2	AR	–
N3	TAR	–
N4	QTAR	–
N5	K(Me3)QTAR	–
N6	TK(Me3)QTAR	–
LN0	–	STGG
LN1	R	STGG
LN2	AR	STGG
LN3	TAR	STGG
LN4	QTAR	STGG
LN5	K(Me3)QTAR	STGG
LN6	TK(Me3)QTAR	STGG

Figure 1. a) A previously developed probe K4(Ac)-CCB and the principle of the fluorogenic switch. The trimethylated lysine at position 9 is marked with an asterisk; b, c) Structures of the peptides inducing intramolecular transesterification reactions; d) The histone H3 peptide (amino acids 1–17); lysine at position 9 is colored red.

esterification between the HDAC-deacetylated lysine and the carbonate ester (Figure 1 a). However, the peptide sequence inducing the transesterification reaction is currently limited to K4(Ac)-CCB. This limitation hinders the development of probes for the detection of enzymes with distinct substrate specificity, because the activity of most lysine-targeting enzymes depends on the recognition of long and diverse lysine-neighboring sequences.[7,13] It is therefore essential to clarify whether the peptides containing different or longer spacers between the target lysine and fluorophore can also induce transesterification. The variability in spacer amino acid composition would expand the applicability of the transesterification switch to probe design. Another problem for K4(Ac)-CCB is that the transesterification reaction is slow, requiring a long incubation time (2 h). Because the kinetics of intramolecular transesterification critically affect the fluorogenic response of a probe, its improvement would lead to a rapid detection of enzymatic activity. Therefore, the development of peptide probes with structures that can provide rapid transesterification is critical for monitoring enzymatic activity. To overcome the limitations and demonstrate a broad applicability of the fluorogenic switch, we designed and synthesized a series of lysine-containing peptides that exhibited transesterification, and investigated the effect of the peptide length on the kinetics of intramolecular transesterification. The results indicate that intramolecular reaction occurred in all the synthetic peptides under mild aqueous conditions despite the long distance between the lysine residue and fluorophore. Moreover, the optimization of the peptide structure

remarkably improved transesterification kinetics. By using the optimized peptides, we successfully designed new fluorogenic probes with rapid fluorogenic response for the detection of HDAC activity, and applied these probes to evaluate the potency of an HDAC inhibitor. Overall, our data indicate that the probes based on the intramolecular transesterification could be widely used as a general fluorogenic switch for the detection of various lysine-targeting enzymes.

Results and Discussion

Design of peptides with a fluorogenic switch

To analyze the peptide structures that trigger spontaneous intramolecular transesterification under mild aqueous conditions, we synthesized 34 lysine-containing peptides conjugated to coumarin derivatives (Figure 1 b, c). In these peptides, coumarin fluorescence is quenched by the incorporation of a carbonate ester into its 7-hydroxy group and is expected to be recovered by transesterification between the carbonate ester and lysine, as described earlier. A series of target peptides distinct from K4(Ac)-CCB were designed based on the histone H3 tail sequence containing lysine at position 9 (H3K9) and surrounding regions of different lengths. H3K9 is an important substrate of enzymatic acetylation/deacetylation or methylation/demethylation for epigenetic regulation of gene expression.[14] In addition, an enzyme that catalyzes desuccinylation or demalonylation of H3K9 has been recently identified.[7] H3K9 is therefore a suitable test substrate to monitor transesterification during the design of fluorogenic probes for the detection of lysine-targeting enzymatic activity. The peptides with C-terminal coumarin, C-type peptides, were designed to contain 0 to 9 amino acids between H3K9 and coumarin, and were designated as C0 to C9, respectively. Other C-type peptides named LC0–LC9 contained 4 additional residues at the N-terminal side of H3K9 to examine the influence of the neighboring sequence on transesterification. In all the C-type peptides except C0 and LC0, glutamine was the C-terminal residue that allowed the introduction of coumarin into the peptides by using a solid-phase procedure (see the Supporting Information). Similarly, the peptides with the N-terminal coumarin, N-type peptides, were designed by introducing 0 to 6 amino acids between H3K9 and coumarin, and LN0–LN6 were also obtained by adding four extra residues at the C-terminus of H3K9. Lysines other than K9 were trimethylated to prevent the possibility of undesirable transesterification reactions. All the peptides were synthesized using Fmoc solid-phase chemistry (the Supporting Information, Schemes S2–S5).

Intramolecular transesterification in the peptides

The intramolecular transesterification reactions were investigated by high-pressure liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS; see Figure 2 and the Supporting Information, Figure S2 and Table S1). After each peptide was incubated at 37 °C in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM), aliquots were injected into a reversed-phase HPLC column. The chromatograms for LN1 and LC8 are shown in Figure 2 and those for the other peptides in Figure S2 (see the Supporting Information). For all the peptides, with the exception of C0, LC0, N0, and LN0, the initial peaks were diminished within a 3 h incubation; instead, single peaks appeared at different retention times (Figure 2 and the Supporting Information, Figure S2). The eluted compounds represented by the newly generated peaks showed mass values of the transferred products, which were also the same as those of the intact peptides (the Supporting Information, Figure S2). The fluorescence chromatograms verified that the peaks observed after the peptide incubation exhibited fluorescence, whereas those without the incubation did not. The HPLC-purified compounds obtained after the incubation with LN6 and LC9, which contained the longest sequences among the N- and C-type peptides, were further analyzed using matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-LIFT-TOF/TOF MS).[15] It was demonstrated that transesterification in the peptides occurred at lysine 9 (the Supporting Information, Figures S3 and S4). It is notable that transesterification was detected even in LC9 despite the long distance between lysine 9 and coumarin. C0, LC0, N0, and LN0 also showed transesterification as evidenced by the results of the HPLC and ESI-MS experiments, but the reaction took nearly 10 h to complete (the Supporting Information, Figures S2 and S6). A significant difference in the reaction rate suggests that in the peptides lacking spacer amino acids between lysine 9 and coumarin, steric constraints may influence lysine attack of the carbonate ester. To investigate the occurrence of intermolecular reactions, LN1 was mixed with LN1(Ac), an acetylated form of LN1, and subjected to HPLC analysis. The intermolecular reaction product, in which both Lys and coumarin of LN1 were acylated,

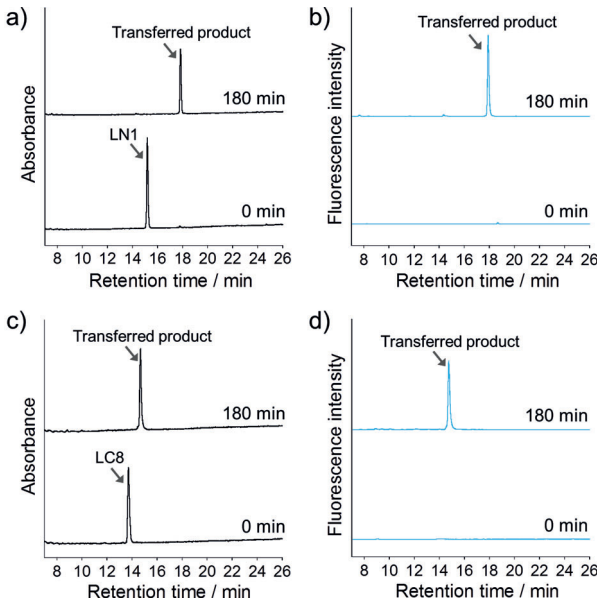


Figure 2. Reversed-phase HPLC analyses of the intramolecular transesterification reactions in LN1 (a, b) and LC8 (c, d). Each peptide (5 μM) was incubated for 180 min. The absorption wavelength was 305 nm (a, c), and the excitation and emission wavelengths were 338 and 458 nm (b, d), respectively.

was not detected (Figure S5 and the discussion in the Supporting Information). In addition, whereas the hydrolysis of the coumarin carbonate ester in LN1(Ac) was observed (the Supporting Information, Figure S5), such a reaction in LN1 was not detected. Similarly, no hydrolysis product was detected in the other peptides except C0, LC0, N0, and LN0 (Figure 2, and the Supporting Information, Figures S2 and S5). These results indicate that rapid intramolecular transesterification precedes carbonate ester hydrolysis, which is thus not detectable during transesterification. Taken together, these results clearly demonstrate that the intramolecular transesterification occurred in all the peptides under mild aqueous conditions regardless of the relative positions of lysine and coumarin. The data also provide evidence that peptide sequences other than that of K4(Ac)-CCB can induce transesterification, indicating the versatility of the lysine-based fluorogenic switch.

Effect of salt concentration on the transesterification reaction rate

The analysis of the peptide properties demonstrated that salt concentration in the reaction buffer affected the kinetics of transesterification. The reaction rate was evaluated by using two different reaction buffers: High salt (HEPES·NaOH (20 mM), pH 8.0, containing NaCl (150 mM), KCl (2.7 mM), $MgCl_2$ (1 mM), and NAD^+ (300 μM)), which is frequently used in the assays of NAD^+-dependent HDACs, and low salt (HEPES·NaOH (20 mM), pH 8.0, containing NAD^+ (300 μM)). For all the peptides except C0, LC0, N0, and LN0, intramolecular transesterification was monitored by fluorescence intensity. The enhancement of the peptide fluorescence under low-salt conditions was markedly faster than that under high-salt conditions, although in several peptides the kinetic differences were slight (the Supporting Information, Figure S7). These results show that the decrease in salt concentration in the reaction buffer leads to an increase in the rate of intramolecular transesterification. Therefore, the following experiments were conducted under low-salt conditions, unless otherwise noted.

Kinetic analyses of the intramolecular transesterification reactions

The detailed comparison of the intramolecular transesterification kinetics was performed based on the fluorescence data shown in Figure S7 (the Supporting Information). Because all the peptides except C0, LC0, N0, and LN0 induced transesterification without any side reaction, the fluorescence data were fitted to a first-order reaction equation (Table 1, Figure 3, and the Supporting Information, Figure S8). The transesterification kinetic constant for DP, the deacetylated product of K4(Ac)-CCB, was also determined (Table 1, Figure 3, and the Supporting Information, Figure S9). Among all the peptides, LN1 showed the highest value ($k = 18.6 \times 10^{-2}\ min^{-1}$), which was 4.2-fold higher than that of DP ($k = 4.43 \times 10^{-2}\ min^{-1}$); under high-salt conditions, the value was 8.7-fold higher than that of DP ($k = 2.13 \times 10^{-2}\ min^{-1}$; Table 1 and the Supporting Information, Figure S10). Interestingly, all the N-type peptides showed k values higher than that of DP. In the C-type peptides, LC8 showed the highest value ($k = 7.36 \times 10^{-2}\ min^{-1}$), even though the spacer between lysine 9 and coumarin contained as many as eight amino acids. This result contrasts with the tendency observed for the N-type peptides, when the species with 1- and 2-amino acid spacers showed better transesterification kinetics than those with longer spacers. However, in both

Table 1. Transesterification kinetics in the peptides.

Peptide	$k \times 10^2$ [min^{-1}][a]	$t_{1/2}$ [min][b]	Peptide	$k \times 10^2$ [min^{-1}][a]	$t_{1/2}$ [min][b]
DP[c]	2.13	33	N0	n.d.[d]	>80[e]
DP	4.43	16	N1	17.4	4.0
C0	n.d.[d]	>100[e]	N2	10.4	6.7
C1	1.78	39	N3	6.24	11
C2	2.21	31	N4	6.47	11
C3	2.67	26	N5	7.91	8.8
C4	2.23	31	N6	5.92	12
C5	3.11	22	LN0	n.d.[d]	>80[e]
C6	4.77	15	LN1	18.6	3.7
C7	2.38	29	LN2	12.3	5.6
C8	1.95	36	LN3	6.81	10
C9	3.58	19	LN4	6.75	10
LC0	n.d.[d]	>100[e]	LN5	7.87	8.8
LC1	4.20	17	LN6	6.50	11
LC2	3.49	20			
LC3	3.71	19			
LC4	3.32	21			
LC5	4.65	15			
LC6	4.92	14			
LC7	5.26	13			
LC8	7.36	9.4			
LC9	4.74	15			

[a] All data were obtained in triplicated experiments. k = first-order rate constant. [b] $t_{1/2}$ = transesterification half-life time calculated using k value (except for C0, LC0, N0, and LN0). [c] k was determined using high-salt buffer (HEPES·NaOH (20 mM), pH 8.0, containing NaCl (150 mM), KCl (2.7 mM), $MgCl_2$ (1 mM), and NAD^+ (300 μM)). [d] Not determined. [e] $t_{1/2}$ was estimated from the data shown in Figure S6 (the Supporting Information).

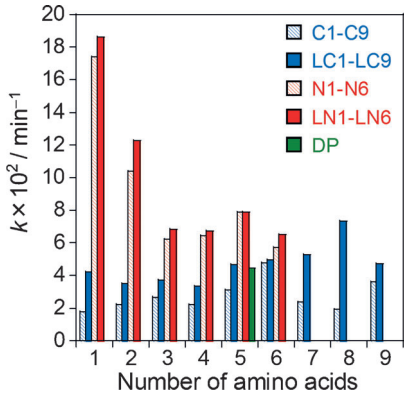


Figure 3. Changes in the first-order rate constants of intramolecular transesterification with the number of amino-acid residues between lysine (H3K9) and terminal coumarin in the tested peptides. Blue and red bars represent k for the C- and N-type peptides, respectively. Closed and hatched bars represent k for the peptides with and without, respectively, 4-amino acid sequences before or after H3K9. The green bar shows k for DP determined using low-salt buffer.

peptide types, spacer elongation did not cause significant decrease in the reaction kinetics (Figure 3), which is surprising because the accessibility of the nucleophile for the intramolecular electrophile is considered to depend on their mutual distance. Furthermore, the extra 4-amino acid sequence in the side opposite to coumarin tended to accelerate the reaction kinetics: For example, the rate for LC8 ($k = 7.36 \times 10^{-2}\ min^{-1}$) was 3.8-fold higher than that for C8 ($k = 1.95 \times 10^{-2}\ min^{-1}$) (Table 1 and Figure 3). These kinetic data demonstrate that the optimization of the peptide structures improved the transesterification kinetics compared to DP. In addition, these results also indicate that long amino acid sequences with a lysine in the middle can be used for fluorogenic switching. These structures can be applied to the design of fluorogenic probes for the detection of the enzymes such as histone deacetylases, desuccinylases, and demethylases that target modified lysines and require specific lysine-neighboring sequences for substrate recognition. Moreover, long spacers may be important when one needs to determine the amino acid sequence specific for substrate recognition by the target enzyme.

Design of fluorogenic probes for the detection of HDAC activity

Fluorogenic probes for the detection of HDAC activity were designed by applying the transesterification switch of the peptides described above. LN1(Ac) and LC8(Ac), which contain acetylated Lys, were selected as the substrate regions in the probes based on the transesterification kinetics (Figure 4): LN1 and LC8 showed the highest k values among the N- and C-type peptides, respectively. The reactivity of LN1(Ac) and LC8(Ac) with class III HDAC Sirt1[16] was investigated using HPLC and ESI-MS. Following the 10 min incubation of LN1(Ac) with the enzyme, the peak corresponding to the intact probe (retention time = 20.0 min) completely disappeared (Figure 5a). A new peak transiently detected at the retention time of 15.0 min, corresponded to LN1 (Figure 2 and the Supporting Information, Figure S11); this peak was not observed in the chromatogram of the probe incubated without Sirt1 (the Supporting Information, Figures S15 and S16). Thus, these results indicate that acetyl-lysine in LN1(Ac) was deacetylated by Sirt1. This peak was diminished after 20 min incubation. HPLC analysis also showed that, similar to LN1 (Figure 2), the peak at the retention time of 17.8 min increased with incubation and was fluorescent (Figure 5b). The eluate corresponding to this peak had the same mass as LN1 and its transferred product (the Supporting Information, Figure S11). Moreover, MALDI-LIFT-

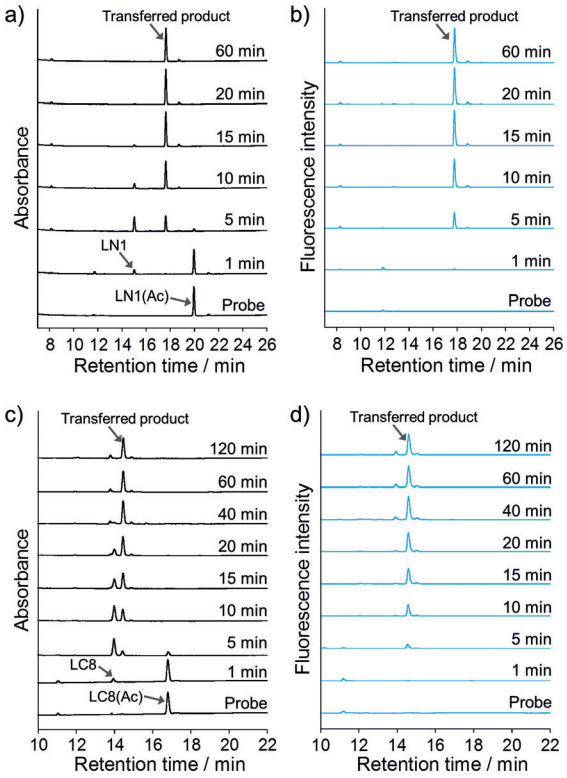


Figure 5. Reversed-phase HPLC analyses of the enzymatic reaction of LN1(Ac) and LC8(Ac) with Sirt1. Sirt1 (100 nM) was incubated with 5 μM LN1(Ac) (a,b) or LC8(Ac) (c,d) in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM) at 37 °C for the designated times. The absorption wavelength was 305 nm (a,c); the excitation and emission wavelengths were 338 and 458 nm, respectively (b,d).

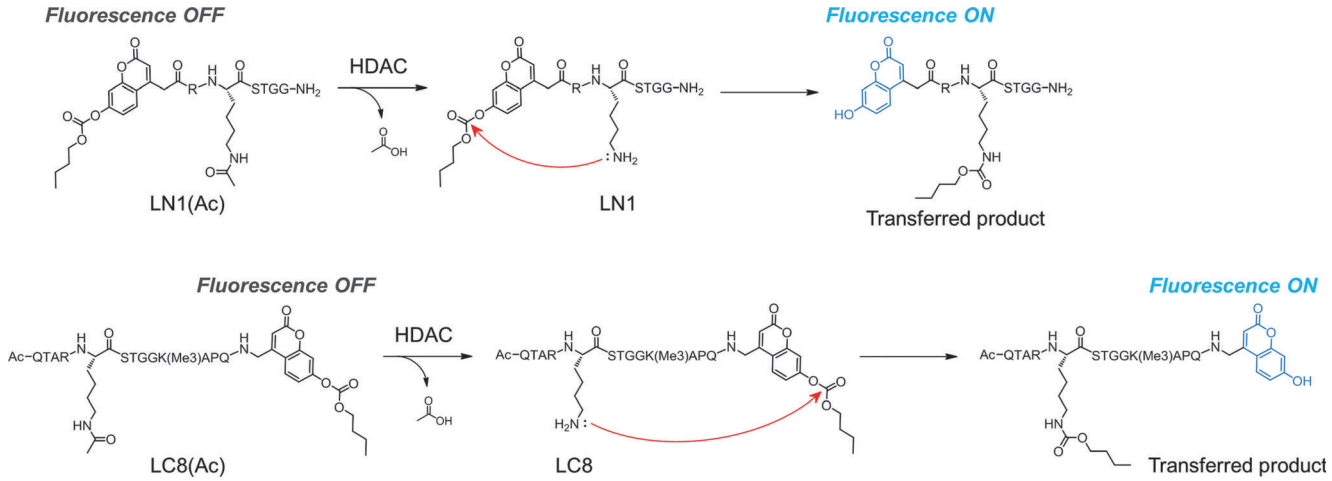


Figure 4. Detection of HDAC activity using LN1(Ac) and LC8(Ac).

TOF/TOF MS clearly detected acylation of the substrate lysine in the probe (the Supporting Information, Figure S12). Based on these analyses, this peak can be assigned to the transferred product of LN1.

Similar results were obtained for LC8(Ac) (Figure 5c,d). After the disappearance of the probe peak (retention time = 16.8 min), a new peak corresponding to the deacetylated product (LC8) was transiently generated (retention time = 14.0 min). Subsequently, a fluorescent peak representing the transferred product of LC8 was detected at the retention time of 14.7 min. For the Sirt1-free LC8(Ac), these peaks were not observed (the Supporting Information, Figure S15). Similar to LN1(Ac), each of the peaks was analyzed by ESI-MS (the Supporting Information, Figures S13 and S16) and MALDI-LIFT-TOF/TOF MS (the Supporting Information, Figure S14). In Figure 5b and 5d, several small fluorescent peaks, which most likely corresponded to reaction side products, were observed. However, these products were generated at the level below MS detection limit and therefore were not identified. These results clearly demonstrate that Sirt1 catalyzes the deacetylation of both LN1(Ac) and LC8(Ac), triggering subsequent intramolecular transesterification that switches on the fluorescence of the probe.

Rapid fluorogenic detection of HDAC activity

The time course for HDAC activity with LN1(Ac) and LC8(Ac) as substrates was monitored by fluorescence intensity. After adding LN1(Ac) or LC8(Ac) to Sirt1 solution, fluorescence intensity was recorded at the indicated incubation times (Figure 6).

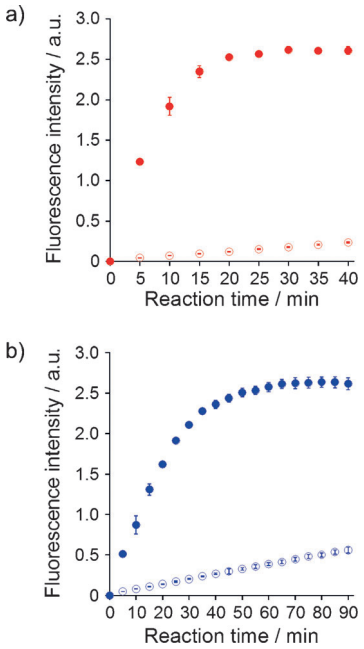


Figure 6. Time course of a) LN1(Ac) and b) LC8(Ac) fluorescence intensity in the presence (●) or absence (○) of Sirt1. The reaction was performed with LN1(Ac) or LC8(Ac) (5 μM) and Sirt1 (100 nM) in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM). Fluorescence was measured at 37 °C. The excitation and emission wavelengths were (350 ± 20) and (450 ± 20) nm, respectively.

The fluorescence intensity of LN1(Ac) increased during incubation, reaching saturation after 20 min period, which was significantly shorter than that for K4(Ac)-CCB (120 min)[12] (Figure 6a). For LC8(Ac), the increase in fluorescence intensity was observed up to 60 min after the probe addition (Figure 6b). As expected from the transesterification kinetics of LN1 and LC8 (Table 1 and Figure 3), saturation for LN1(Ac) was reached faster than for LC8(Ac). Nevertheless, the incubation time required for the saturation of fluorescence intensity was much shorter for LC8(Ac) than for K4(Ac)-CCB. A slight increase in the fluorescence of each probe in the absence of the enzyme was due to some hydrolysis of the carbonate ester (Figure 6). To suppress the hydrolysis, a more stable functional group such as carbamate ester or imide ester can be utilized instead of carbonate ester. These structural optimizations are now under investigation. However, the hydrolysis did not prevent the clear detection of HDAC activity because the increase in fluorescence due to the enzymatic reaction was considerably greater than that resulting from the probe hydrolysis. To confirm that the enzymatic deacetylation of the probes was caused by Sirt1, the probes were incubated with Sirt1 denatured at 95 °C for 5 min, which reduced the fluorescence to the background level (the Supporting Information, Figure S17). HPLC analyses of these reaction mixtures demonstrated that no compounds corresponding to the deacetylation products of the probes were generated (the Supporting Information, Figure S18). These results demonstrate that the probes can specifically and rapidly detect Sirt1 activity.

We also examined the reactivity of the probes with HDAC isoforms other than Sirt1: HDACs of class I (HDAC1, 2, 3/NCOR1, and 8), class II (HDAC4, 5, 6, 7, 9, and 10), class III (Sirt2, 3, 4, 5, 6, and 7), and class IV (HDAC11).[17] For both probes, a significant fluorescence enhancement was observed in the reactions with HDAC3/NCOR1 and Sirt3 (the Supporting Information, Figures S19 and S20).

Determination of the IC50 value for an HDAC inhibitor

Finally, we applied LN1(Ac), which showed the fastest fluorogenic response to enzymatic deacetylation, for a quantitative evaluation of the potency of a Sirt1 selective inhibitor EX-527.[18] In this assay, Sirt1 was mixed with the probe in the presence (1 nM to 20 μM) or absence of EX-527. EX-527 inhibited Sirt1 activity in a concentration-dependent manner (the Supporting Information, Figure S21). Using the fluorescence data, 50% inhibitory concentration (IC50) was calculated as 0.52 μM, which is within the reported range of 0.1–1 μM.[19] Thus, LN1(Ac) is demonstrated to be a practical tool to investigate the potency of HDAC inhibitors by a simple procedure.

Conclusion

We developed 34 peptides that exhibit a fluorogenic switch due to intramolecular transesterification. The reaction was triggered by the nucleophilic amine of a lysine residue, leading to a fluorogenic response. In all the synthesized peptides, the transesterification proceeded under mild aqueous conditions.

The reaction kinetics of the new peptides were greatly improved compared with the case of a previously developed peptide DP: One of the peptides showed the highest reaction rate, with a transesterification half-life time of 3.7 min. Importantly, the reaction also occurred in the peptides containing a long 9-amino acid spacer between the lysine residue and fluorophore. Although nucleophilic amino acids such as cysteine, histidine, and tyrosine might affect the intramolecular transesterification, this would not be a serious problem because the histone tails contain no cysteine or tyrosine, and only one histidine residue exists at the end of the H4 tail. Based on the structure of the peptides inducing rapid transesterification, we designed fluorogenic probes LN1(Ac) and LC8(Ac) for monitoring HADC activity. The fluorogenic responses of the new probes after enzymatic deacetylation were much faster than that of a previously reported probe, K4(Ac)-CCB. We also demonstrated that LN1(Ac) can be used to evaluate the potency of an HDAC inhibitor.

Currently, two-step enzyme-coupled methods for the detection of HDAC activity are commercially available.[20] In these methods, peptide sequences in the probe are restricted because of direct conjugation of the carboxyl group in the substrate lysine to a fluorophore. In contrast, the fluorogenic probes developed in this study have a relatively high versatility in the spacer amino acids between the lysine and fluorophore. Moreover, the probes detect HDAC activity by using a one-step procedure, in which the probe is simply mixed with the enzyme. Thus, the fluorogenic switch based on long-distance intramolecular transesterification would be useful for the investigation of the substrate specificity of histone deacetylases, and will be a valuable tool in the development of HDAC-targeting drugs.

Experimental Section

HPLC analyses

HPLC separation was carried out with an increasing ratio of B buffer (0.1% HCOOH in acetonitrile) to A buffer (0.1% HCOOH in H_2O). All samples were analyzed using a linear gradient of 3–48% B buffer over 30 min. To evaluate the intramolecular transesterification, the peptides (5 μM) were incubated in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM) at 37 °C. For C1–C9, LC1–LC9, N1–N6, and LN1–LN6, aliquots were taken at 0 and 180 min, mixed with trifluoroacetic acid (TFA; final concentration: 0.2%) to stop the reaction, and analyzed by using HPLC. For C0, LC0, N0, and LN0, aliquots were taken at 0 min and 18 h (1,080 min), mixed with TFA, and analyzed by HPLC. To investigate the intermolecular reaction, LN1 (5 μM) was incubated with LN1(Ac) (5 μM) in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM) at 37 °C for 60 min. The reaction was stopped by TFA, and analyzed by HPLC. The enzymatic reactions were conducted by incubating LN1(Ac) or LC8 (Ac) (5 μM) with Sirt1 (100 nM) in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM) at 37 °C. Aliquots were taken at 1, 5, 10, 15, 20, 40, 60, and 120 min, mixed with TFA, and analyzed by HPLC. To prepare the samples before the enzyme reaction, each of the probes (5 μM) was dissolved in an aqueous solution of 0.2% TFA, and then immediately analyzed by HPLC. For the analyses of Sirt1-free LN1(Ac) and Sirt1-free LC8 (Ac), the probes (5 μM) were incubated in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM) at 37 °C. Aliquots were taken at 0, 20, and 60 min, treated with TFA to minimize the hydrolysis of the carbonate ester in the probe, and analyzed by HPLC. Heat-denatured Sirt1 (100 nM) was prepared by heating at 95 °C for 5 min and mixed with each of the probes (5 μM) in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM) at 37 °C. After 90 min of incubation, the mixture was treated with TFA, and analyzed by HPLC.

Fluorescence spectroscopy

The fluorescence intensity of the peptides and probes was measured every 5 min using the excitation and emission wavelengths of (350 ± 20) and (450 ± 20) nm, respectively. To investigate the effect of salt concentration, C1–C9, LC1–LC9, N1–N6, LN1–LN6, and DP were incubated in high-salt buffer (HEPES·NaOH (20 mM), pH 8.0, NaCl (150 mM), KCl (2.7 mM), $MgCl_2$ (1 mM), and NAD^+ (300 μM)) or in low-salt buffer (HEPES·NaOH (20 mM), pH 8.0, NAD^+ (300 μM)) at 37 °C, and the fluorescence intensity was measured. The fluorescence data were normalized according to Equation (1):

$$[\text{Normalized fluorescence intensity}] = (F_t - F_0)/(F_{max} - F_0) \quad (1)$$

in which F_t, F_{max}, and F_0 represent observed, maximum, and initial fluorescence intensity, respectively.

To evaluate the reactivity of LN1(Ac) or LC8(Ac) with class I, II, or IV HDACs, the probes (5 μM) were incubated with and without 200 nM HDAC1 or HDAC2, or 100 nM HDAC3/NCOR1, HDAC4–HDAC11 in HEPES·NaOH buffer (20 mM, pH 8.0). For class III HDACs (Sirt1–7) each of the probes (5 μM) was incubated with or without 100 nM enzyme in HEPES·NaOH buffer (20 mM, pH 8.0) containing NAD^+ (300 μM) at 37 °C, then the fluorescence intensity of the probe was measured for 90 min. The first-order rate constant, k, was obtained by fitting the fluorescence data shown in Figure S7 (the Supporting Information) to Equation (2):

$$\text{Normalized fluorescence intensity} = 1 - \exp(-kt) \quad (2)$$

The half-life time, $t_{1/2}$, was calculated using Equation (3):

$$t_{1/2} = \ln 2/k \quad (3)$$

Acknowledgements

This work was supported by MEXT of Japan (Grants 25220207, 26102529, and 25620133 to K.K., 26282215 and 25560403 to Y.H., and 26·565 to R.B.), by CREST from JST, by JST, PRESTO, and by Asahi Glass Foundation.

Keywords: enzymes · fluorescence · fluorescent probes · kinetics · peptides

[1] a) W. S. Saari, J. E. Schwering, P. A. Lyle, S. J. Smith, E. L. Engelhardt, *J. Med. Chem.* **1990**, *33*, 97–101; b) P. Gomes, N. Vale, R. Moreira, *Molecules* **2007**, *12*, 2484–2506.

[2] a) Y. Meyer, J. A. Richard, M. Massonneau, P. Y. Renard, A. Romieu, *Org. Lett.* **2008**, *10*, 1517–1520; b) E. Kisin-Finfer, S. Ferber, R. Blau, R. Satchi-Fainaro, D. Shabat, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 2453–2458.

[3] M. M. Pires, J. Chmielewski, *Org. Lett.* **2008**, *10*, 837–840.

[4] a) D. Ye, G. Liang, M. L. Ma, J. Rao, *Angew. Chem. Int. Ed.* **2011**, *50*, 2275 – 2279; *Angew. Chem.* **2011**, *123*, 2323 – 2327; b) D. Ye, A. J. Shuhendler, L. Cui, L. Tong, S. S. Tee, G. Tikhomirov, D. W. Felsher, J. Rao, *Nat. Chem.* **2014**, *6*, 519 – 526.

[5] a) J. A. Latham, S. Y. Dent, *Nat. Struct. Mol. Biol.* **2007**, *14*, 1017 – 1024; b) A. J. Bannister, T. Kouzarides, *Cell Res.* **2011**, *21*, 381 – 395; c) Z. Zhang, M. Tan, Z. Xie, L. Dai, Y. Chen, Y. Zhao, *Nat. Chem. Biol.* **2011**, *7*, 58 – 63.

[6] a) C. M. Grozinger, S. L. Schreiber, *Chem. Biol.* **2002**, *9*, 3 – 16; b) B. C. Smith, W. C. Hallows, J. M. Denu, *Chem. Biol.* **2008**, *15*, 1002 – 1013.

[7] a) J. Du, Y. Zhou, X. Su, J. J. Yu, S. Khan, H. Jiang, J. Kim, J. Woo, J. H. Kim, B. H. Choi, B. He, W. Chen, S. Zhang, R. A. Cerione, J. Auwerx, Q. Hao, H. Lin, *Science* **2011**, *334*, 806 – 809; b) B. He, J. Du, H. Lin, *J. Am. Chem. Soc.* **2012**, *134*, 1922 – 1925.

[8] N. Mosammaparast, Y. Shi, *Annu. Rev. Biochem.* **2010**, *79*, 155 – 179.

[9] a) J. E. Bolden, M. J. Peart, R. W. Johnstone, *Nat. Rev. Drug Discovery* **2006**, *5*, 769 – 784; b) H. Hess-Stumpp, *Eur. J. Cell Biol.* **2005**, *84*, 109 – 121.

[10] S. M. Kooistra, K. Helin, *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 297 – 311.

[11] a) K. Dhara, Y. Hori, R. Baba, K. Kikuchi, *Chem. Commun.* **2012**, *48*, 11534 – 11536; b) C. Yu, Y. Wu, F. Zeng, X. Li, J. Shi, S. Wu, *Biomacromolecules* **2013**, *14*, 4507 – 4514; c) H. V. Diyabalanage, G. C. Van de Bittner, E. L. Ricq, J. M. Hooker, *ACS Chem. Biol.* **2014**, *9*, 1257 – 1262; d) M. Minoshima, T. Matsumoto, K. Kikuchi, *Anal. Chem.* **2014**, *86*, 7925 – 7930.

[12] R. Baba, Y. Hori, S. Mizukami, K. Kikuchi, *J. Am. Chem. Soc.* **2012**, *134*, 14310 – 14313.

[13] a) F. Forneris, C. Binda, M. A. Vanoni, E. Battaglioli, A. Mattevi, *J. Biol. Chem.* **2005**, *280*, 41360 – 41365; b) F. Forneris, C. Binda, A. Dall'Aglio, M. W. Fraaije, E. Battaglioli, A. Mattevi, *J. Biol. Chem.* **2006**, *281*, 35289 – 35295; c) B. C. Smith, B. Settles, W. C. Hallows, M. W. Craven, J. M. Denu, *ACS Chem. Biol.* **2011**, *6*, 146 – 157; d) R. Meledin, A. Brik, A. Aharoni, *ChemBioChem* **2013**, *14*, 577 – 581; e) H. Y. Kuo, T. A. DeLuca, W. M. Miller, M. Mrksich, *Anal. Chem.* **2013**, *85*, 10635 – 10642.

[14] a) A. Lennartsson, K. Ekwall, *Biochim. Biophys. Acta* **2009**, *1790*, 863 – 868; b) A. Dhall, C. Chatterjee, *ACS Chem. Biol.* **2011**, *6*, 987 – 999.

[15] D. Suckau, A. Resemann, M. Schuerenberg, P. Hufnagel, J. Franzen, A. Holle, *Anal. Bioanal. Chem.* **2003**, *376*, 952 – 965.

[16] a) L. Bordone, L. Guarente, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 298 – 305; b) J. C. Milne, P. D. Lambert, S. Schenk, D. P. Carney, J. J. Smith, D. J. Gagne, L. Jin, O. Boss, R. B. Perni, C. B. Vu, J. E. Bemis, R. Xie, J. S. Disch, P. Y. Ng, J. J. Nunes, A. V. Lynch, H. Yang, H. Galonek, K. Israelian, W. Choy, A. Iffland, S. Lavu, O. Medvedik, D. A. Sinclair, J. M. Olefsky, M. R. Jirousek, P. J. Elliott, C. H. Westphal, *Nature* **2007**, *450*, 712 – 716; c) M. T. Borra, B. C. Smith, J. M. Denu, *J. Biol. Chem.* **2005**, *280*, 17187 – 17195.

[17] a) I. V. Gregoretti, Y. M. Lee, H. V. Goodson, *J. Mol. Biol.* **2004**, *338*, 17 – 31; b) R. H. Houtkooper, E. Pirinen, J. Auwerx, *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 225 – 238.

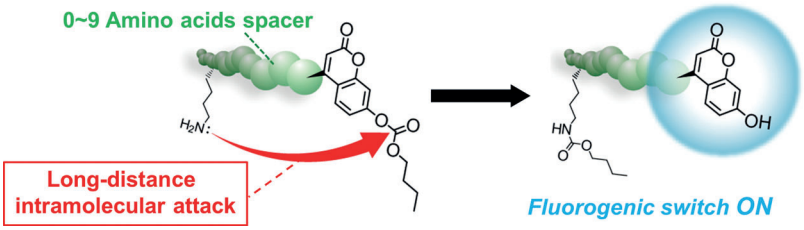
[18] a) A. D. Napper, J. Hixon, T. McDonagh, K. Keavey, J. F. Pons, J. Barker, W. T. Yau, P. Amouzegh, A. Flegg, E. Hamelin, R. J. Thomas, M. Kates, S. Jones, M. A. Navia, J. O. Saunders, P. S. DiStefano, R. Curtis, *J. Med. Chem.* **2005**, *48*, 8045 – 8054; b) J. M. Solomon, R. Pasupuleti, L. Xu, T. McDonagh, R. Curtis, P. S. DiStefano, L. J. Huber, *Mol. Cell. Biol.* **2006**, *26*, 28 – 38.

[19] a) P. H. Kiviranta, T. Suuronen, E. A. Wallén, J. Leppänen, J. Tervonen, S. Kyrylenko, A. Salminen, A. Poso, E. M. Jarho, *J. Med. Chem.* **2009**, *52*, 2153 – 2156; b) M. Lawson, U. Uciechowska, J. Schemies, T. Rumpf, M. Jung, W. Sippl, *Biochim. Biophys. Acta* **2010**, *1799*, 726 – 739.

[20] a) K. Hoffmann, G. Brosch, P. Loidl, M. Jung, *Nucleic Acids Res.* **1999**, *27*, 2057 – 2058; b) D. Wegener, F. Wirsching, D. Riester, A. Schwienhorst, *Chem. Biol.* **2003**, *10*, 61 – 68.

Received: November 14, 2014
Published online on ■■ ■■, 2015

FULL PAPER



Fluorogenic switch for lysine: A series of fluorogenic peptides that contain a lysine and a fluorescence-quenched fluorophore were developed. In all the peptides, intramolecular nucleophilic transesterification occurred between the lysine and fluorophore triggering fluorescence (see figure). This fluorogenic mechanism is useful for detecting the activity of lysine-targeting enzymes.

Fluorescent Probes

*R. Baba, Y. Hori, K. Kikuchi**

■■ – ■■

Intramolecular Long-Distance Nucleophilic Reactions as a Rapid Fluorogenic Switch Applicable to the Detection of Enzymatic Activity