Rotaxane-type resorcinarene tetramers as histone-sensing fluorescent receptors†

Osamu Hayashida*[a,c] and Masaki Uchiyama[b]

Received 14th March 2008, Accepted 6th June 2008
First published as an Advance Article on the web 8th July 2008
DOI: 10.1039/b804431g

Rotaxane-type receptors, which were composed of anionic cyclophane-based resorcinarene tetramers as the wheel and a 2,6-disubstituted naphthalene derivative having two fluorophore moieties, such as fluorescein and rhodamine residues, as the axle (**1** and **2**, respectively), were prepared. Rotaxane-type receptors **1** and **2** bound histone, a small basic protein component of eukaryotic chromatins, with binding constants of 2.3×10^6 and 9.0×10^5 M^{-1}, respectively. The rotaxane-type receptors showed fluorescence sensing ability with remarkable histone selectivity. Moreover, fluorescence resonance energy transfer (FRET) between the fluorescein residues of **1** and the rhodamine residues of **2** took place in the presence of histone, which was a useful method for the detection of histone.

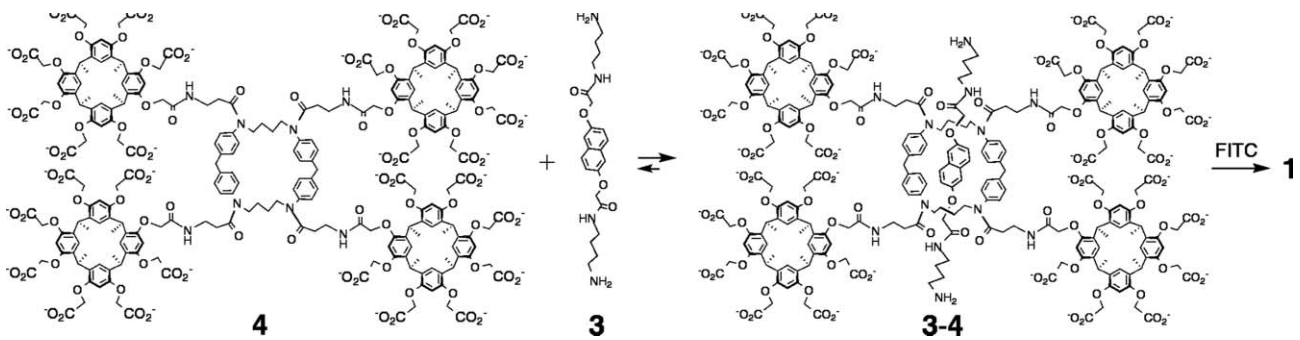
Introduction

Protein surface recognition by artificial receptors[1] is an important concept in chemical biology. Numerous attempts have been made to develop artificial receptors based on macrocycles[2] that can recognize and bind to specific protein surfaces. In particular, histones, a family of basic proteins that organize eukaryotic chromatins, are attractive as target proteins. Post-translational modifications of histones such as acetylation and methylation are claimed to play important roles in important biological processes such as gene regulation and chromosome condensation.[3] Histones have high concentrations of the amino acids lysine and arginine on their surfaces. On these grounds, we previously developed an anionic cyclophane-based resorcinarene tetramer (**4** in Scheme 1) as a histone binding receptor.[4] The binding constant of the resorcinarene tetramer **4** with histone was 31-fold larger than that of an untethered reference resorcinarene, reflecting the multivalency effect in resorcinarenes.[4] In addition, when a dansyl group was covalently attached to the macrocycle, the resulting fluorescent cyclophane-based resorcinarene was found to act as a fluorescent sensor for histone.[5]

On the other hand, a rotaxane is a typical interlocked compound consisting of wheel and axle components, which have unique functionality in the movement and rotation of the macrocycle (wheel).[6] In the course of our ongoing research on the surface recognition and fluorescence sensing of histones, we adopted rotaxane's functionality as molecular terms of the histone receptors. We have now designed novel rotaxane-type receptors composed of cyclophane **4** as the wheel and a 2,6-disubstituted naphthalene derivative having two fluorophore moieties, such as fluorescein and rhodamine residues, as the axle (**1** and **2**, respectively, see Fig. 1). The movable and rotatable cyclophane-based resorcinarene tetramer acts as a histone-binding site, whereas the fluorophore termini are expected to bring about desirable fluorescence spectral changes upon complexation with histone. We describe herein the synthesis of rotaxane-type receptors and their histone surface recognition behavior in an aqueous medium, with an emphasis on fluorescence detection and specificity.

[a]*Institute for Materials Chemistry and Engineering, Kyushu University, Moto-oka 744, Fukuoka 819-0395, Japan. E-mail: ohaya@ms.ifoc.kyushu-u.ac.jp; Fax: (+81) 92 802 2503; Tel: (+81) 92 802 2503*
[b]*Department of Chemistry and Biochemistry, Kyushu University, Moto-oka 744, Fukuoka 819-0395, Japan*
[c]*PRESTO, JST, Sanbancho Bldg., 5-Sanbancho, Chiyodaku, Tokyo 102-0075, Japan*

† Electronic supplementary information (ESI) available: Computer-generated CPK models of **1**, and figures showing the concentration dependency of the fluorescence spectra of **1** and fluorescence titration experiments with other proteins. See DOI: 10.1039/b804431g



Scheme 1 Preparation of rotaxane-type resorcinarene tetramer **1**.

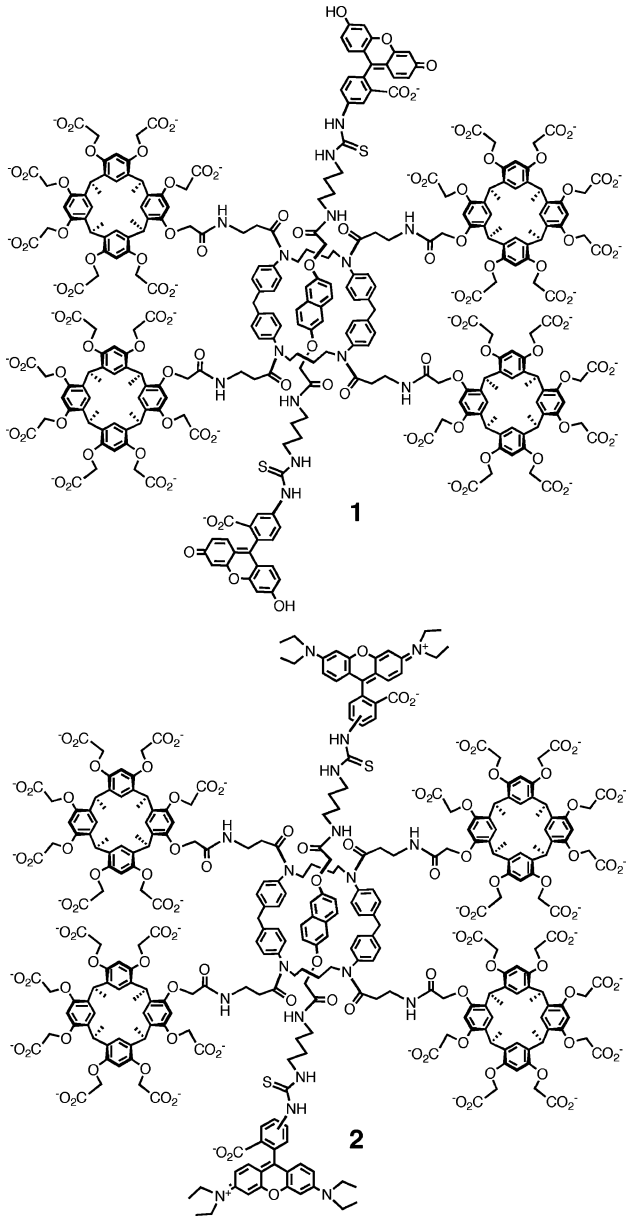


Fig. 1 Rotaxane-type resorcinarene tetramers.

Results and discussion

Synthesis of rotaxane-type receptors

It is well known that water-soluble cyclophanes provide appropriate hydrophobic cavities in aqueous media for the inclusion of guest molecules.[7] Indeed, guest molecules such as 6-*p*-toluidinonaphthalene-2-sulfonate were effectively incorporated by cyclophane-based resorcinarene tetramer **4** through hydrophobic interactions.[4] Therefore, the rotaxane-type cyclophane-based resorcinarene tetramers **1** and **2** were prepared by following the reaction sequence given in Scheme 1. First, an aqueous solution containing **4** and the 2,6-disubstituted naphthalene derivative (**3**) having two side chains with terminal amine residues, gave host–guest complex **3**–**4**. Subsequently, the terminal amine residues of the host–guest complex were subject to reaction with fluorescein isothiocyanate (FITC) to afford the corresponding rotaxane **1**. In addition, a rotaxane-type cyclophane-based resorcinarene tetramer bearing rhodamine residues (**2**) was also prepared by using rhodamine isothiocyanate in place of FITC, in a manner similar to that applied to the synthesis of **1**.

Based on molecular mechanics studies[8] of **1**, followed by molecular dynamics simulations, **1** was found to provide four anionic resorcinarene derivatives suitable for histone binding as well as two fluorescein termini, separated by a reasonable distance (*ca.* 4.5 nm), as steric stoppers (see ESI†). These hydrophilic groups were expected to confer the advantage of enhanced solubility in neutral aqueous media. From a practical standpoint, fluorescent rotaxane **1** had good H_2O-solubility of >0.2 g ml^{-1}. At least at concentrations of **1** below 5.0 μM, the fluorescence intensity originating from **1** increased in a linear fashion as its concentration increased, without any change in its maximum (λ_{em}, 518 nm) (see ESI†), indicating that **1** is in a monomeric state under these conditions. In addition, the molecular size of **1** in its extended conformation is 3.3–4.5 nm, while histone has a diameter of *ca.* 7–10 nm (see ESI†).

Histone binding by rotaxane-type receptors

First, the binding behavior of **1** with histone was evaluated by fluorescence spectroscopy. Upon the addition of histone to an aqueous 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) buffer (0.01 M, pH 7.4, 0.15 M with NaCl) containing **1** (0.1 μM), the fluorescence intensity originating from the fluorescein groups of **1** increased, showing a saturation behavior for the complexation of **1** with histone as shown in Fig. 2(a). The quantum yield of **1** was increased from 0.23[9] to 0.42 in the absence or presence of histone (3.0 μM). The stoichiometry for the complexes formed with the receptor and histone was investigated

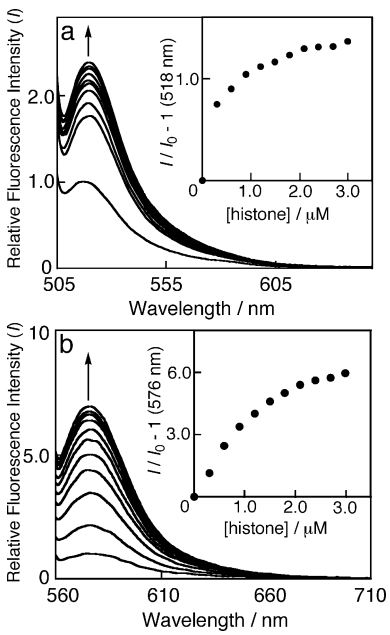


Fig. 2 Fluorescence spectral changes for an aqueous solution of **1** (0.1 μM) (a) or **2** (0.1 μM) (b) upon the addition of histone, [histone] = 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7, and 3.0 μM (from bottom to top). Ex. 488 and 540 nm, respectively.

by Job's continuous variation method. The obtained Job's plot for the complexes of **1** with histone is shown in Fig. 3. The result reveals that receptor **1** forms a complex with histone in a 3 : 1 molar ratio of receptor to histone. The 1 : 1 binding constant (K) of **1** toward histone (per binding site) was evaluated on the basis of the Benesi–Hildebrand relationship for the titration data under the condition of large excess amounts of histone. The K value was calculated to be 2.3×10^6 M^{-1}. The K value of rotaxane-type receptor **1** toward histone was somewhat smaller than that of a tetramer of resorcinarene **4** (1.3×10^7 M^{-1}),[4] reflecting the steric hindrance of the fluorescein groups of **1** for the histone binding.

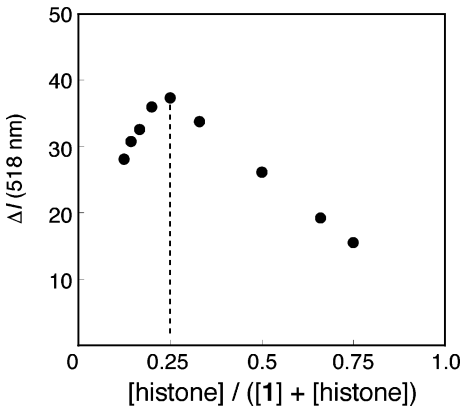


Fig. 3 Job's plot for the complex of **1** with histone: total concentration of **1** and histone, 0.2 μM.

Furthermore, relatively large fluorescence polarization values (P) were obtained for **1** in the presence of histone (0.30) and the obtained P value was much larger than that of **1** alone (0.12), reflecting tight interactions in the **1**–histone complexes.[10] A similar binding characteristic toward histone of another rotaxane-type cyclophane-based resorcinarene tetramer **2**, which has rhodamine residues, was also confirmed by identical methods, as shown in Fig. 2(b) (K, 9.0×10^5 M^{-1}).

The fluorescence intensity of **1** showed characteristic pH-dependence, reflecting the acid-dissociation equilibrium of a carboxylic acid of the fluorescein groups of **1** (pK_a,[11] 7.2[12]), as shown in Fig. 4. Interestingly, the apparent pK_a value of **1** was found to be 6.3 in the presence of histone, as shown in Fig. 4. These results imply that the micro-environmental pH around the **1**–histone complexes was somewhat basic in comparison with that for the bulk aqueous phase. That is, hydroxide ions were concentrated to some extent on the histone surfaces through electrostatic

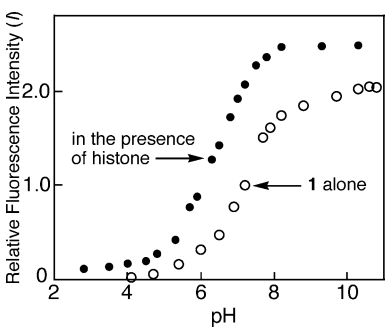


Fig. 4 pH-Dependency of the fluorescence intensity for an aqueous solution of **1** (0.1 μM) in the absence or presence of histone (3.0 μM).

interactions with residues of the amino acids lysine and arginine of the histone surfaces. The micro-environmental properties of the binding sites of histone were mainly responsible for the fluorescence spectral changes of **1** upon complexation with histone.

Histone selectivity of rotaxane-type receptors

We have previously clarified that cyclophane-based resorcinarene oligomers such as **4** bound histone selectively as confirmed by surface plasmon resonance measurements[4] as well as fluorescence spectroscopy.[5] The histone selectivity experienced by the rotaxane-type receptor **1** was also evaluated using fluorescence spectroscopy in a manner similar to that reported previously.[5] The fluorescence spectral changes of **1** were almost negligible upon the addition of other acidic and neutral proteins such as ovalbumin, peanut agglutinin, myoglobin, concanavalin A, and cytochrome c, to the aqueous HEPES buffer containing **1** (Fig. 5) (see ESI†). In addition, upon the addition of lysozyme having a pI of 11.0 to the aqueous solution of **1**, the fluorescence intensity of **1** decreased slightly. These results suggests that the histone selectivity of **1** was maintained even when the cyclophane-based resorcinarene tetramer was threaded by an axle. It was found that **1** exhibited histone selectivity and a fluorescence sensing capability, reflecting electrostatic interactions.

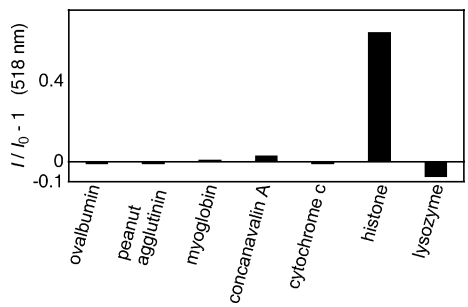


Fig. 5 Changes in fluorescence intensity at 518 nm for an aqueous solution of **1** upon addition of proteins (0.5 μM) such as ovalbumin, peanut agglutinin, myoglobin, concanavalin A, cytochrome c, histone, and lysozyme. Ex, 488 nm.

FRET study for histone detection

Fluorescein and rhodamine form an archetypical donor–acceptor pair in fluorescence resonance energy transfer (FRET)[13] experiments. The supramolecular assembly of two or more individual rotaxane-type receptors on the histone surfaces was also easily monitored by means of the FRET technique. That is, upon addition of **2** to an aqueous solution containing histone and **1**, the fluorescence intensity originating from **1** at 518 nm decreased along with a concomitant increase in the fluorescence intensity of **2** bound to histone at 576 nm by excitation at 430 nm[14] of the fluorescein residues of **1**, as shown in Fig. 6. The FRET between fluorescein residues of **1** and rhodamine residues of **2** did take place under the conditions described above, but in the absence of histone. These results suggest that both receptors **1** and **2** were, to some extent, assembled simultaneously on the histone surfaces, which brought two-wavelength fluorescence detection. In addition, the histone selectivity of the rotaxane-type receptors was also confirmed by the FRET assay (see ESI†).

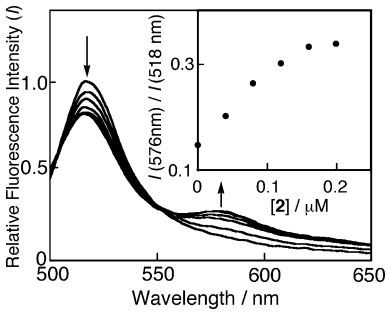


Fig. 6 Fluorescence spectral changes for an aqueous solution containing **1** (0.2 μM) and histone (0.6 μM) upon addition of **2**, [**2**] = 0, 0.04, 0.08, 0.12, 0.16, and 0.20 μM (from bottom to top at 576 nm). Ex, 430 nm. Inset: corresponding titration curve.

Conclusions

In conclusion, fluorescent rotaxanes, composed of a cyclophane-based resorcinarene tetramer and a 2,6-disubstituted naphthalene derivative having two fluorophore termini, were prepared as histone-sensing receptors. These rotaxanes performed histone binding as a result of the presence of four anionic resorcinarene derivatives. The micro-environmental pH experienced by the bound rotaxane-type receptors provided a fluorescence sensing ability with remarkable histone selectivity. In addition, to the best of our knowledge, the pair of rotaxanes **1** and **2** are the first examples of compounds enabling the FRET detection of histones. These rotaxane-type histone receptors may be quite promising to use as long-wavelength fluorescent probes for the fluorescent imaging of living cells. These subjects of interest will be explored further in the future.

Experimental

Elemental analyses were performed at the Microanalysis Center of Kyushu University. ^{1}H and ^{13}C NMR spectra were taken on Bruker DRX 600 and JEOL JNM-EX400 spectrometers. Fluorescence spectra were recorded on a Perkin Elmer LS55 spectrophotometer. The following proteins were obtained from commercial sources and used without further purification: calf thymus histone, egg white ovalbumin (both from Worthington), egg white lysozyme, horse heart cytochrome c (both from Wako Pure Chemical), peanut agglutinin (from Vector), concanavalin A, and myoglobin (both from Nacalai Tesque).

Naphthalene derivative having terminal amines (3)

A solution of *N*-1-Boc-1,4-diaminobutane hydrochloride (230 mg, 1.0 mmol) in dry *N*,*N*-dimethylformamide (DMF, 3 ml) was added dropwise to a solution of 2,2′-(naphthalene-2,6-diyl-bis(oxy))diacetic acid[15] (100 mg, 0.34 mmol), benzotriazole-1-yloxytris(pyrrolidino)-phosphonium hexa-fluorophosphate (Py-BOP, 1.6 g, 1.0 mmol) and triethylamine (0.28 ml, 1.5 mmol) in dry DMF (5 ml) under nitrogen at room temperature, and the resulting mixture was stirred for 5 days at room temperature. The precipitates were collected by filtration, and dried *in vacuo*. The residue was dissolved in dry dichloromethane (10 ml). Trifluoroacetic acid (4.0 ml) was added to the solution and the mixture was stirred for 3 h at room temperature. After the solvent was evaporated off under reduced pressure, dichloromethane (20 ml) was added to the residue, and this procedure was repeated three times to remove the remaining trifluoroacetic acid. Evaporation of the solvent under reduced pressure gave a white solid as the trifluoroacetic acid salt (170 mg, 95%): ^{1}H NMR (400 MHz, D_2O, 298K) δ 1.5 (m, 8H), 2.8 (m, 4H), 3.2 (m, 4H), 4.4 (m, 4H), 7.0 (m, 2H), 7.1 (m, 2H) and 7.6 (m, 2H). This compound was subjected to the next step without further purification.

Rotaxane-type receptor (1)

A mixture of **4** (40 mg, 7.4 μmol) and **3** (40 mg, 65 μmol) in H_2O (1 mL) was stirred for 1 h at room temperature. Fluorescein isothiocyanate (FITC) (80 mg, 205 μmol) and aqueous sodium hydroxide (1 ml, 0.1 mmol) were added to the solution, and the mixture was stirred for 24 h at room temperature. The crude product was purified by gel filtration chromatography on a column of Toyopearl HW-40 with H_2O as the eluant, followed by HPLC (YMC Pack ODS-A) with acetonitrile–H_2O as the eluant. Evaporation of the solvent under reduced pressure gave a yellow solid (3 mg, 6%): ^{13}C NMR (150 MHz, D_2O, 298K) δ 20, 24, 26, 31, 34, 36, 40, 45, 47, 49, 56, 67, 69, 100, 128, 129, 130, 131, 137, 139, 140, 153, 154, 155, 173 and 178. MALDI-TOF MS (matrix; CHCA): *m*/*z*, 6180 [M + H + matrix]$^+$. Found: C, 53.10; H, 4.26; N, 2.67. Calcd for $C_{302}H_{268}N_{14}Na_{30}O_{110}S_2$ $10H_2O$: C, 53.44; H, 4.28; N, 2.89.

Rotaxane-type receptor (2)

This compound was prepared by using rhodamine isothiocyanate in place of FITC in a manner similar to that applied to the synthesis of **1**. The crude product was purified by gel filtration chromatography on a column of Toyopearl HW-40 with H_2O as the eluant, followed by HPLC (YMC Pack ODS-A) with acetonitrile–H_2O as the eluant. Evaporation of the solvent under reduced pressure gave a dark brown solid (4 mg, 14%): ^{13}C NMR (100 MHz, D_2O, 298K) δ 12, 17, 20, 24, 26, 31, 39, 45, 58, 66, 68, 99, 108, 114, 115, 118, 129, 130, 135, 139, 141, 154, 155, 170 and 177. MALDI-TOF MS (matrix; CHCA): *m*/*z*, 6265 [M + Na]$^+$. Found: C, 50.54; H, 5.18; N, 3.62. Calcd for $C_{318}H_{306}Cl_2N_{18}Na_{30}O_{106}S_2 \cdot$ $34H_2O$: C, 50.84; H, 5.02; N, 3.36.

Binding constants of rotaxane-type receptors with histone

To a solution of **1** and **2** (0.1 μM) in HEPES buffer (0.01 M, pH 7.4, with 0.15 M NaCl) were added increasing amounts of histone at 298K, and the fluorescence intensity originating from the receptors was monitored after each addition, by excitation at 488 and 540 nm for **1** and **2**, respectively. The *K* values of the receptors toward histone (per binding site) were evaluated based on the Benesi–Hildebrand method applied to the fluorescence titration data.

Acknowledgements

The present work is partially supported by a Grant-in-Aid for the Global COE Program, "Science for Future Molecular Systems", no. 18655074, from the Ministry of Education, Culture, Science, Sports and Technology of Japan.

Notes and references

1 (*a*) M. W. Peczuh and A. D. Hamilton, *Chem. Rev.*, 2000, **100**, 2479; (*b*) A. Ojida, Y. Miyahara, T. Kohira and I. Hamachi, *Biopolymers*, 2004, **76**, 177; (*c*) S. Fletcher and A. D. Hamilton, *Curr. Opin. Chem. Biol.*, 2005, **9**, 632; (*d*) S. Fletcher and A. D. Hamilton, *J. R. Soc., Interface*, 2006, **3**, 215; (*e*) S. M. Biros and J. Rebek, Jr, *Chem. Soc. Rev.*, 2007, **36**, 93.

2 (*a*) Y. Hamuro, M. C. Calama, H. S. Park and A. D. Hamilton, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2680; (*b*) Q. Lin, H. S. Park, Y. Hamuro, C. S. Lee and A. D. Hamilton, *Biopolymers*, 1998, **47**, 285; (*c*) O. Hayashida, M. Kato, K. Akagi and Y. Aoyama, *J. Am. Chem. Soc.*, 1999, **121**, 11597; (*d*) H. S. Park, Q. Lin and A. D. Hamilton, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5105; (*e*) H. C. Zhou, W. De-An, L. Baldini, R. K. Jain, A. Carie, S. M. Sebti and A. D. Hamilton, *Org. Biomol. Chem.*, 2006, **4**, 2376; (*f*) A. W. Coleman, F. Perret, S. Cecillon, A. Moussa, A. Martin, M. Dupin and H. Perron, *New J. Chem.*, 2007, **31**, 711.

3 P. Cheung and P. Lau, *Mol. Endocrinol.*, 2005, **19**, 563.

4 (*a*) O. Hayashida and M. Uchiyama, *Tetrahedron Lett.*, 2006, **47**, 4091; (*b*) O. Hayashida and M. Uchiyama, *J. Org. Chem.*, 2007, **72**, 610.

5 O. Hayashida, N. Ogawa and M. Uchiyama, *J. Am. Chem. Soc.*, 2007, **129**, 13698.

6 (*a*) M. C. T. Fyfe and J. F. Stoddart, *Acc. Chem. Res.*, 1997, **30**, 393; (*b*) I Aprahamian, O. Š. Miljanic, W. R. Dichtel, K. Isoda, T. Yasuda, T. Kato and J. F. Stoddart, *Bull. Chem. Soc. Jpn.*, 2007, **80**, 1856.

7 (*a*) K. Odashima, A. Itai, Y. Iitaka and K. Koga, *J. Org. Chem.*, 1985, **50**, 4478; (*b*) O. Hayashida, T. Ito, Y. Hisaeda and Y. Murakami, *Pure Appl. Chem.*, 1993, **65**, 551; (*c*) Y. Murakami, O. Hayashida and Y. Nagai, *J. Am. Chem. Soc.*, 1994, **116**, 2611; (*d*) O. Hayashida, K. Ono and Y. Murakami, *Supramol. Chem.*, 1995, **6**, 109.

8 The calculations were carried out by using MacroModel 7.5 software (Infocom, Inc., Tokyo, 2005).

9 The quantum yield of **1** was smaller than that of fluorescein itself (0.85). Modification of fluorescein by introducing carboxylic acid residues reduces the quantum yield: Q. He, E. W. Miller, A. P. Wong and C. J. Chang, *J. Am. Chem. Soc.*, 2006, **128**, 9316.

10 Neither fluorescein alone nor FITC-labeled glutamic acid showed meaningful fluorescence spectral changes upon the addition of histone.

11 The pK_a value was evaluated by using the Henderson-Hasselbach equation: M. J. Geisow, *Exp. Cell Res.*, 1984, **150**, 29.

12 The obtained pK_a value was larger than that of fluorescein itself (pK_a 6.4) and was almost comparable to that of a FITC-labeled glutamic acid that has carboxylic acids (pK_a 6.9–7.0): E. Glassker, W. Konings and B. Poolman, *Mol. Membr. Biol.*, 1996, **13**, 173.

13 T. Förster, *Ann. Phys.*, 1948, **2**, 55.

14 The fluorescence emission of **2** alone in aqueous media by excitation at 430 nm is almost negligible.

15 A. B. Kytr, K. A. Owens, L. O. Sutherland and R. F. Newton, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1921.