Synthesis of a new bifunctionalised fluorescent label and physical properties of the bound form on model peptide of troponin C†

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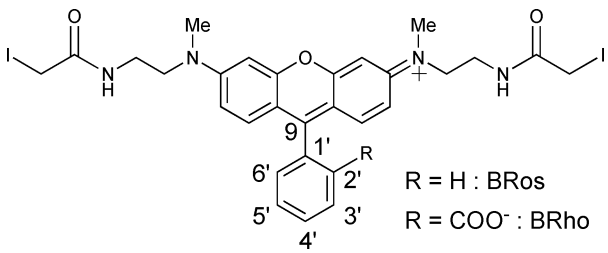
A new bifunctional fluorescent label, BRos, was synthesised in order to monitor protein dynamics using fluorescence microscopy, and the photophysical properties were compared with those of bifunctionalised rhodamine, BRho. In a labelling experiment with a model peptide of troponin C, which regulates muscle contraction and relaxation, it was found that BRos was bound to the peptide through two linkages and provided a homogeneous compound, whereas BRho gave a pair of diastereomers having different physical properties in NMR and HPLC analyses.

Introduction

Recently, single molecule analysis by fluorescence microscopy has become a powerful method for monitoring and clarifying protein dynamics.[1] In order to reveal the orientation of the target domain of the protein by analysing that of the fluorescent dipole, not only should the dye be fixed tightly but also the direction of the dipole relative to the target α-helix of the protein should be unambiguously determined. A number of fluorescent labels are now available and have been applied to protein labelling,[2] but most of them are monofunctionalised, which have one reactive linker arm to bind to a protein. It is impossible to estimate *a priori* their dipole orientation against an α-helix, because of free rotation around the linker, and thus the orientation should be clarified by some experimental methods. In order to overcome this problem, bifunctionalised rhodamine, BRho, was reported by Corrie and co-workers.[3] BRho binds to a protein through two linkages and immobilizes its fluorescent dipole parallel to the α-helix. They applied it to the myosin head domain and troponin C (TnC),[4,5] which play key roles in the regulation of muscle contraction and relaxation,[6] and discussed the mechanism of the lever arm movement of myosin light chain and the TnC orientation in the actin filament.[5] In the double binding, however, diastereomeric isomers are generated by the sterically restricted rotation of the carboxyphenyl ring around the 1′–9 bond of rhodamine.[7] The energy barrier of the rotation was estimated to be more than 20 kcal mol^{-1} by a DFT calculation.[7] When it binds to a chiral α-helix, the difference in the carboxyl group orientation relative to the xanthene plane provides two diastereomers, and their separation was reportedly difficult.[4,5] This problem does not occur when a fluorescent probe bound in a single manner is used. Herein, we report a new bifunctionalised fluorescent dye, BRos, for protein labelling based on a rosamine platform (Scheme 1). Since rosamine has no substituent on the phenyl ring attached to the 9-position of the xanthene ring, this fluorescent molecule should not generate any diastereomers of the labelled form. Furthermore, we performed a model peptide study[8] to show the structural differences between the two BRho-labelled peptide diastereomers, prepared as a model of labelled-TnC.



Scheme 1 Structures of bifunctionalised fluorescent labels.

Results and discussion

Both BRos and BRho were synthesised as depicted in Scheme 2. The aminophenol **5** is a key intermediate, as a common precursor for BRos and BRho. The previous reported synthesis of aminophenol **5** took 8 steps with 12% overall yield.[3] In this study, we established a simple and efficient synthetic route for the intermediate **5**. The hydrochloride salt of **2**, obtained by monomethylation of *m*-anisidine, was treated with 2-oxasolidinone to yield the amine **3**.[9] Cleavage of O–CH_3 with refluxing in HBr, followed by acetylation provided **4**, and selective hydrolysis afforded **5**, which was purified by silica gel column chromatography. Thus, we achieved the preparation of the aminophenol **5** in 25% overall yield with only 5 steps.

By following general procedures, successive Friedel–Crafts acylation of **5** with benzaldehyde and chloranil oxidation afforded the rosamine-acetamide **6a** in a yield of 12%. On the other hand, the rhodamine-acetamide **6b** was synthesised simply by heating a mixture of **5** and phthalic anhydride, without any solvent or catalyst. Although the rhodamine-acetamide **6b** was synthesised

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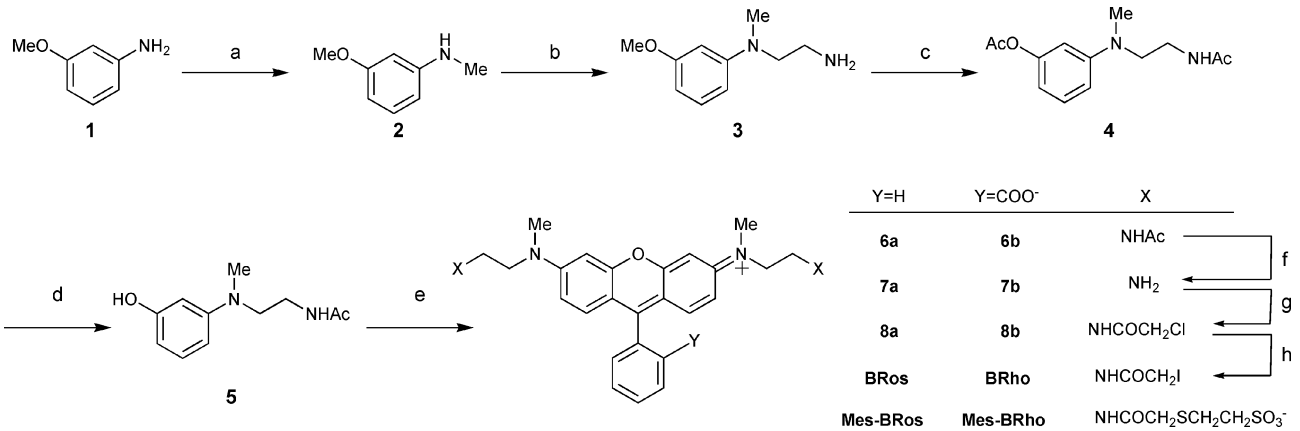

Scheme 2 Synthesis of BRos and BRho. *Reagents and conditions*: (a) formaldehyde, NaOMe, $NaBH_4$, 85 °C, overnight, quant.; (b) 6 M HCl, and then, 2-oxazolidinone, 150 °C, overnight; (c) conc. HBr, reflux, and then acetic anhydride, THF, 0 °C, 2 h, 28% for 2 steps; (d) KOH, MeOH, RT, 30 min, 91%; (e) **6a**: benzaldehyde, propionic acid, TsOH, 65 °C, overnight, and then chloranil, RT, 2 h, 12%; **6b**: phthalic anhydride, 143 °C, 20%; (f) 1.5 M HCl, reflux, 6 h, quant.; (g) chloroacetyl chloride, diisopropylethylamine, CH_2Cl_2, 0 °C, 2 h, 34%, (h) NaI, acetone, MeOH, $CHCl_3$, RT, 3 days, 68%.

through two Friedel–Crafts acylations in previous studies, one-step formation of the rhodamine platform was achieved in our method. Finally, according to the reported procedure,[3] the acetamide group was converted to the thiol-reactive iodoacetamide to give BRos and BRho. As a result, the overall yields were 0.8 and 1.4% for BRos and BRho, respectively, from the commercially available compound **1**.

The photophysical properties of the dyes were characterised with the 2-mercaptoethanesulfonate (MES) derivatives, MES-BRos and MES-BRho, respectively. The new fluorescent label, BRos, showed almost the same spectroscopic properties as BRho (Table 1). The quantum yields of MES-BRos and MES-BRho in water are sufficiently high for fluorescense microscopy analyses.

We previously employed a chicken skeletal TnC mutant (S94C) for labelling with the bifunctionalised spin labels.[10] In the present work, we chose an α-helical peptide, C8C15 (Ac-MKEDKAKCEEELANCFRIFDK-OH), as a model for the E-helix of TnC, to confirm the structural homogeneity of the BRos-labelled protein. The model peptide C8C15 has two cysteines, and the distance between them is appropriate for the present fluorescent labels. In this case, glycine-6 was replaced by alanine to stabilise the α-helical structure (Fig. 1). Labelling C8C15 with BRos or BRho was performed by the procedure reported previously,[10] except for the final purification using reverse phase HPLC. Fig. 2a shows part of the HPLC profile of the BRos-labelled peptide in the final purification, in which the major, single peak at 37 min, together with a broad shoulder, appeared (monitored at 550 nm, solid line). A small peak corresponding to unreacted peptide was also detected at 35 min (monitored at 214 nm, dashed line). The MALDI-TOF mass spectrum of the main peak indicated the 1 : 1 adduct (BRos-pep, mass = 3074) of the peptide labelled through two linkages. The formation of BRos-pep was also confirmed by UV-vis and emission spectra, in which the absorption and emission bands are similar to those of BRos, together with a 214 nm band in the UV-vis spectrum, due to the peptide absorption. By mass analysis, the shoulder component (retention time 41 min) that appeared after the main peak was assigned to the 1 : 2 adduct of the peptide with two dyes. Another undesired 1 : 1 adduct, bound through only one linkage, was not detected. In contrast to the labelling with BRos, the reaction of the same peptide with BRho produced two distinctly separated peaks with almost the same intensities (Fig. 2b). Their mass spectra were identical to each other and were consistent with the calculated mass of the 1 : 1 adduct (BRho-pep-1 and BRho-pep-2, mass = 3118) with two linkages. The UV-vis spectra of these two products showed the same features with absorption maxima at 214 nm and 551 nm, due to the peptide backbone and the dye, respectively. These results indicate that the reaction between BRho and C8C15 produces almost equal amounts of each of the two diastereomers, which are separable by HPLC. This suggests that the configuration of the label is determined by the first of the two consecutive reactions between the iodoacetamide groups and the cysteine residues. Thus, it is evident that labelling with BRos yields a structurally homogeneous product, while labelling with BRho affords a pair of diastereomers with different hydrophobicities.

Table 1 Photophysical properties of MES-BRos and MES-BRho

	$\lambda_{abs,max}$/nm	$\lambda_{em,max}$/nm[a]	ε/$M^{-1}cm^{-1}$[b]	Quantum yield
BRos	548	576	52 000	0.22
BRho	547	571	89 000	0.40

[a] Excited at 548 nm for BRos and at 547 nm for BRho. [b] Calculated at 548 nm for BRos and at 547 nm for BRho.

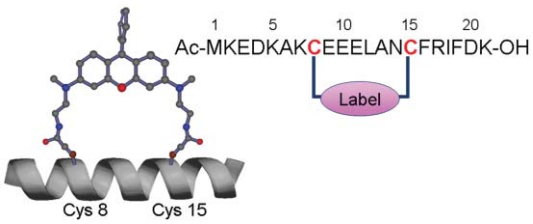

Fig. 1 Sequence and model of a labelled peptide.

The CD spectra of the labelled peptides (BRos-pep, BRho-pep1, and BRho-pep2) revealed Cotton effects at 194 nm (positive), 205 and 222 nm (negative), which are typical for an α-helix. The intensities of their Cotton effects were almost the same as those of the non-labelled peptide, and no spectral differences between

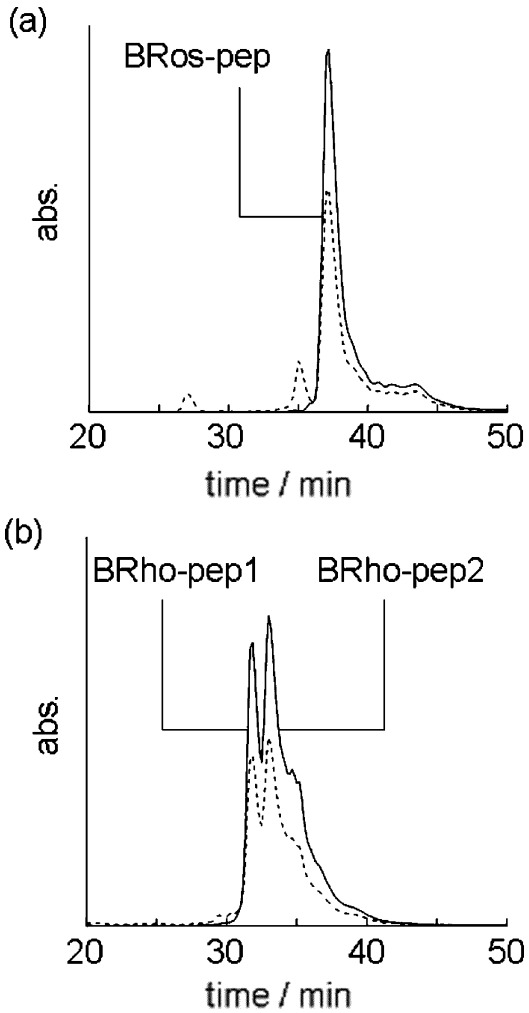

Fig. 2 HPLC charts of (a) BRos-peptide and (b) BRho-peptide at 550 nm (solid line) and 214 nm (dashed line).

BRho-pep-1 and BRho-pep-2 were detected at the region of dye absorption, despite their axial chirality. The structural difference between these diastereomeric peptides was also confirmed by ^{1}H NMR experiments. Significant chemical shift differences ($\Delta\delta$) were observed at the carboxylphenyl moieies of BRho-pep-1 and BRho-pep-2 (Fig. 3). That is, a large difference of $\Delta\delta_{3'} = 0.08$ ppm and medium differences of $\Delta\delta_{4',5'} = 0.03$ ppm were observed, respectively, while the hydrogens at the 6′-position were overlapped with other aromatic hydrogens and were not assigned. On the other hand, the peaks corresponding to the aromatic hydrogens of the xanthene and those of tyrosine-16 and -19 did not show meaningful differences. These results indicate that the carboxylate moieties of BRho on each labelled peptide are located in distinctly different environments, while the peptide backbone remains the same. The electrostatic interaction between the negatively charged carboxylate of the dye and the positively charged ammonium ion of lysine-7 is suggested to cause the down-field shifts of the aromatic hydrogens in BRho-pep-2 (Fig. 3, blue line; Fig. 4). Moreover, the fact that BRho-pep-2 elutes more slowly in the reverse phase HPLC indicates its lower hydrophilicity compared to that of the counterpart BRho-pep-1, and supports our interpretation (Fig. 2). The carboxylate group must be exposed to the solvent in BRho-pep-1, making it more hydrophilic (Fig 4). Thus, the HPLC and NMR results indicate that between the two diastereomers, the dye moiety must be in different environments which must influence the direction of the fluorescent dipole of the dye.

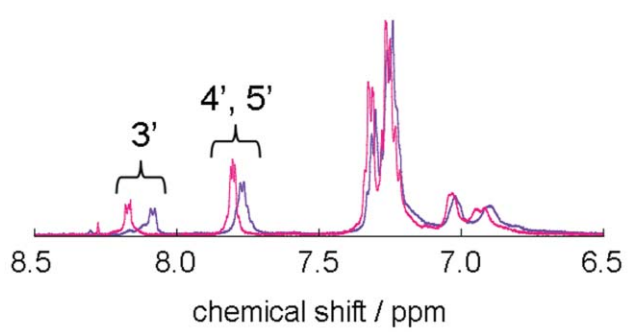

Fig. 3 NMR spectra of BRho-pep-1 (red line) and BRh-pep-2 (blue line).

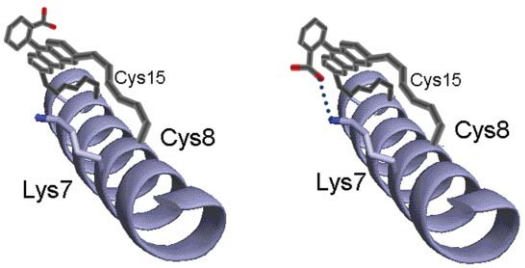

Fig. 4 Models of the interaction between the amino group of Lys7 and the carboxyl group of BRho, expected for BRho-pep-1 (left) and BRho-pep-2 (right).

Conclusions

In summary, we have synthesised a new bifunctionalised dye, BRos, in which the synthetic route for the key intermediate amino phenol **5**, common to BRho, was improved. In addition, a one-step synthesis of the rhodamine platform was achieved. The photophysical properties of BRos and BRho were measured and found to be almost the same. A peptide-labelling experiment revealed that BRos provided the homogeneous 1 : 1 adduct (BRos-pep) through two linkages, while a pair of diastereomers separable by HPLC was obtained with BRho. On the other hand, we have carried out labelling the TnC mutant (S94C) with BRho, and the diastereomers could not be separated from each other. The different behavior of the diastereomeric BRho-peptides in NMR and HPLC analyses indicates that their physical properties are not identical, and this could possibly have adverse consequences for single molecular analyses. This problem may be avoided by selecting BRho-labelling sites that are far from the cationic side chains. As an alternative, BRos should be used, as this probe is more predictable for the analyses. Now, the labelling of TnC with BRos and its application in fluorescent analyses are in progress.

Experimental

General

^{1}H NMR spectra were recorded with a JEOL JNM-EX-270 spectrometer (at 270 MHz) and a JEOL alpha-500 spectrometer

(at 500 MHz) to ^{1}H, using TMS as the internal standard. HRMS was performed with a JEOL JMS-DX-300 spectrometer. BioRad Duoflow was employed for HPLC. MALDI-TOF mass spectra were measured by an Applied Biosystem Voyager-DE(tm) PRO with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. UV-vis spectra were measured with an Agilent 8453 spectrometer. Circular dichroism (CD) spectra were obtained with a JASCO J-715 spectropolarimeter. TLC analyses were carried out by using silica gel 60 F_{254} (Merck). Flash chromatography was carried out with Wakogel C-300 (silica gel, 100 ∼ 200 mesh, Wako Pure Chemical Industries, Ltd.). Most of the reagents were purchased from Wako Pure Chemical Industries, Ltd., Nacalai Tesque, and Aldich Chemical Company, Inc. The model peptide (Ac-MKEDKAKCEEELANCFRIFDK-OH) was purchased from Tufts University Core Factory, Boston, USA.

Synthesis

3-Methoxy-*N*-methylaniline (2). To a suspension of NaOMe (7.90 g, 146.2 mmol) in MeOH (37 mL) was added *m*-anisidine **1** (3.00 g, 24.4 mmol). The resulting brown solution was poured into a suspension of paraformaldehyde (1.02 g, 34.2 mmol) in MeOH (24 mL). After stirring at room temperature for 16 h, $NaBH_4$ (923 mg, 24.2 mmol) was added to the mixture. After refluxing for 2 h, the mixture was evaporated to 1/2 volume, treated with 1 M KOH (100 mL), and then extracted with ether (100 mL × 2). The combined organic extracts were dried over $MgSO_4$ and evaporated to afford **2** (3.20 g, 96%), which was used without further purification. TLC $R_f = 0.41$ (AcOEt–hexane, 1 : 1), ^{1}H NMR ($CDCl_3$, 270 MHz) δ 2.81 (3 H, s, –NCH_3), 3.77 (3 H, s, –OCH_3), 6.15–6.16 (1 H, m, Ar), 6.20–6.29 (2 H, m, Ar), 7.08 (1H, t, $J = 8.1$, Ar).

3-Acetoxy-*N*-(2-acetamidoethyl)-*N*-methylaniline (4). A mixture of aniline **2** (2.20 g, 16.0 mmol) and 6 M HCl (20 mL) was stirred at room temperature for 10 min, washed with ether (50 mL), and evaporated to dryness. The residue was mixed with 2-oxazolidone (1.39 g, 16.0 mmol), and the mixture was heated at 150 °C for 19 h. After cooling to room temperature, 10% NaOH (100 mL) was added to the mixture. The resulting solution was extracted with CH_2Cl_2 (100 mL × 2). The combined extracts were dried over $MgSO_4$, and then evaporated. The residue was dissolved in 48% HBr (40 mL), and the solution was refluxed under an argon atmosphere for 2 h and evaporated. The residue was dissolved in saturated aqueous sodium borate (138 mL). To the mixture was added acetic anhydride (10.4 mL, 110.4 mmol) in THF (70 mL) dropwise under an argon atmosphere at 0 °C. The mixture was stirred at 0 °C for 30 min, while the pH of the mixture was maintained at 9.0–9.5 by the addition of 2 M KOH, as required. A further portion of acetic anhydride (5.52 mL, 58.6 mmol) in THF (20 mL) was added to the mixture. Stirring was continued at 0 °C for 1 h, while the pH was maintained as described above. A final portion of acetic anhydride (3.45 mL, 36.6 mmol) in THF (12 mL) was added, and the mixture was stirred at room temperature. After stirring for 0.5 h, the mixture was extracted with CH_2Cl_2 (100 mL × 3). The combined organic extracts were washed with saturated $NaHCO_3$ (150 mL), dried over $MgSO_4$, and then evaporated. The residual gum was azeotroped with toluene (3 mL × 3). Purification by flash chromatography (AcOEt) afforded **4** (1.12 g, 28%) as a colorless gum. TLC $R_f = 0.30$ (AcOEt); ^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.86 (3 H, s, NHCOCH_3), 2.26 (3 H, s, OCOCH_3), 2.90 (3 H, s, NCH_3), 3.42–3.43 (4 H, m, CH_2CH_2), 6.23–6.33 (3 H, m, Ar), 7.06 (1 H, t, $J = 8.1$, Ar).

3-Hydroxy-*N*-(2-acetamidoethyl)-*N*-methylaniline (5). The acetamide **4** (500 mg, 2.0 mmol) was dissolved in MeOH (20 mL) containing KOH (236 mg, 2.1 mmol), and the mixture was stirred at room temperature under an argon atmosphere for 0.5 h. After evaporation, the residue was diluted with 2 M aqueous potassium phosphate (pH 8.5, 30 mL). The mixture was extracted with CH_2Cl_2 (50 mL × 4). The combined organic extracts were dried over $MgSO_4$ and evaporated. The residual gum was azeotroped with toluene (3 mL × 3) to afford **5** (379 mg, 91%), which was used without further purification. TLC $R_f = 0.21$ (AcOEt); ^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.94 (3 H, s, NHCOCH_3), 2.91 (3 H, s, NCH_3), 3.42–3.43 (4 H, m, CH_2CH_2), 6.23–6.33 (3 H, m, Ar), 7.06 (1 H, t, $J = 8.1$, Ar).

Bis[*N*-(2-acetamidoethyl)-*N*-methyl)]-rosamine (6a). A mixture of phenol **5** (417 mg, 2.0 mmol), benzaldehyde (106 mg, 1.0 mmol), and *p*-TsOH (18 mg, 0.10 mmol) was stirred at 65 °C for 21 h. After cooling to room temperature, the mixture was poured into 3 M NaOAc (200 mL). The resulting suspension was extracted with $CHCl_3$ (100 mL × 3). The combined organic extracts were dried over $MgSO_4$ and then evaporated to give the crude dihydro-compound. This was dissolved in a mixture of MeOH (25 mL) and $CHCl_3$ (25 mL), and was treated with chloranil (197 mg, 0.8 mmol). After stirring vigorously for 2 h, the mixture was evaporated. The residue was purified by flash chromatography ($CHCl_3$–MeOH, 9 : 1) to give **6a** (116 mg, 12%) as a purple solid. TLC $R_f = 0.63$ ($CHCl_3$–MeOH, 2 : 1); ^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.95 (6 H, s, NHCOCH_3), 3.30 (6 H, s, NCH_3), 3.55 (4 H, br s, CH_2NH), 3.80 (4 H, br s, –CH_2N), 7.10 (4 H, br s, Ar), 7.33–7.36 (4 H, m, Ar), 7.62 (3 H, m, Ar).

Bis[*N*-(2-acetamidoethyl)-*N*-methyl]-rhodamine (6b). The mixture of phthalic anhydride (89 mg, 0.6 mmol) and phenol **5** (250 mg, 1.2 mmol) was heated at 150 °C for 19 h under an argon atmosphere. The resultant gum was purified by flash column chromatography (MeOH–$CHCl_3$ 1 : 1 → 1 : 0) to afford 64 mg (20%) of **6b** as a dark purple glass. TLC $R_f = 0.21$ ($CHCl_3$–MeOH, 1 : 1); ^{1}H NMR (CD_3OD, 500 MHz) δ 1.83 (6 H, s, NHCOCH_3), 3.20 (6H, s, NCH_3), 3.40 (4 H, t, $J = 6.4$, CH_2NH), 3.66 (4H, t, $J = 6.4$, –CH_2N), 6.91 (d, 2 H, $J = 2.4$, 4-, 5-H), 6.99 (dd, $J = 9.6$ and 2.4 Hz, 2-,7-H), 7.17–7.23 (3H, m, 1-,8-,6′-H), 7.60–7.72 (2H, m, 4′-,5′-H), 8.12 (d, $J = 7.6$ Hz, 1H, 3′-H).

Bis[*N*-(2-chloroacetamidoethyl)-*N*-methyl]-rosamine (8a). A mixture of the acetamide-rosamine **6a** (116 mg, 0.24 mmol) and 1.5 M HCl (10 mL) was refluxed for 6 h and evaporated to give the crude product **7a**. It was azeotroped with toluene (5 mL) and diluted with CH_2Cl_2 (10 mL). Chloroacetyl chloride (0.077 mL, 0.96 mmol) and diisopropylethylamine (0.33 mL, 1.9 mmol) were added to the solution with ice-cooling. After stirring at 0 °C for 2 h and at room temperature for 1 h, a mixture of $CHCl_3$ (135 mL) and MeOH (15 mL) was added. The resulting solution was washed with 0.5 M HCl (30 mL). The aqueous layer was extracted with $CHCl_3$–MeOH (9 : 1, 30 mL × 2), and the combined organic layers were washed with 10% $NaHCO_3$ (20 mL) and saturated NaCl (20 mL). The solution was dried over $MgSO_4$ and then evaporated.

Purification by flash chromatography (MeOH–$CHCl_3$, 9 : 1 → 4 : 1) afforded **9a** (46 mg, 34%), TLC $R_f = 0.70$ ($CHCl_3$–MeOH, 2 : 1); ^{1}H NMR (CD_3OD, 500 MHz) δ 3.27 (6 H, s, NCH_3), 3.60 (4 H, br s, CH_2NH), 3.82 (4H, br s, $-CH_2N$), 3.96 (4 H, s, $-CH_2Cl$), 7.03 (4 H, br s, Ar), 7.30–7.34 (4 H, m, Ar), 7.54–7.58 (3 H, m, Ar); ^{13}C-NMR (CD_3OD, 126 MHz) : δ 39.5, 42.8, 51.9, 53.7, 97.4, 129.0, 129.4, 130.5, 131.8, 131.9, 157.4, 158.0, 158.1, 167.7; HRMS (FAB) : m/z calcd for $C_{29}H_{31}Cl_2N_4O_3$ (M^+) 553.1773, found 553.1775.

Bis[*N*-(2-chloroacetamidoethyl)-*N*-methyl]-rhodamine (8b). The rhodamine **8b** was obtained from 105 mg (0.20 mmol) of **6b** by a similar procedure as for **8a** in 37% yield (44 mg). TLC R_f = 0.79 ($CHCl_3$–MeOH, 1 : 1); ^{1}H-NMR (CD_3OD, 500 MHz) δ 3.23 (6H, s, NCH_3), 3.51 (4 H, t, J = 6.4 Hz, CH_2NH), 3.75 (4 H, t, J = 6.4, $-CH_2N$), 3.94 (4 H, s, $-CH_2Cl$), 6.96 (2 H, d, J = 2.5, 4-, 5-H), 7.02 (2 H, dd, J = 9.6 and 2.5 Hz, 2-,7-H), 7.20–7.23 (3 H, m, 1-,8-,6′-H), 7.63–7.67 (2 H, m, 4′-, 5′-H), 8.12 (1 H, d, J = 7.6, 3′-H); ^{13}C-NMR (CD_3OD, 126 MHz) : δ 38.3, 39.6, 43.1, 52.3, 97.7, 97.8, 114.6, 114.7, 130.0, 130.7, 130.9, 131.1, 132.6, 135.5, 158.0, 158.1, 158.8, 172.8, 173.8.

Bis[*N*-(2-iodoacetamidoethyl)-*N*-methyl]-rosamine, BRos. Sodium iodide (2.08 mg, 13.9 mmol) was dissolved in a mixture of dry acetone (13 mL), methanol (5 mL), and chloroform (2 mL). The solution was stirred under an argon atmosphere for 10 min and was added to **8a** (46 mg, 0.083 mmol). The reaction vessel was flushed with argon and was kept in the dark at room temperature for 3 days. After adding $CHCl_3$ (180 mL), the mixture was washed with 5% aqueous sodium ascorbate (100 mL) and water (30 mL × 3). The solution was dried over $MgSO_4$ and evaporated to give **BRos** (42 mg, 68%). The product was dissolved in DMF and stored in aliquots (10 mM) at −80 °C.

Bis[*N*-(2-iodoacetamidoethyl)-*N*-methyl]-rhodamine, BRho. A similar procedure for **BRos** production yielded **BRho** (35 mg, 70%). The product was dissolved in DMF and stored in aliquots (10 mM) at −80 °C.

MES-BRos and MES-BRho

For photophysical measurements, BRos and BRho were converted to the mercaptoethanesulfonate(MES) forms (MES-BRos and MES-BRho), in order to inactivate the highly reactive iodoacetamide groups and to imitate a cysteine-bound form. To a mixture of a portion of a 10 mM solution of the dye in DMF (40 μL) and 200 mM potassium phosphate buffer (pH 7.2, 0.35 mL) was added 200 mM aqueous sodium 2-mercaptoethanesulfonate (80 μL). After stirring overnight in darkness at room temperature, the solution was diluted with 25 mM phosphate buffer containing 150 mM NaCl (pH 7.2) to an appropriate concentration for photophysical measurements.

Labelling experiment

The purchased crude model peptide was dissolved (final conc. 1 mM) in 10 mM MOPS (pH 8.5), and was purified by reverse phase HPLC (Resource RPC, 0.64 × 10 cm, GE-Healthcare) with a linear gradient from 20% solvent A (0.1% TFA) and 80% B (0.1% TFA, 90% acetonitrile) to 30% A and 70% B at 2 mL min^{-1} over 30 min. After lyophilisation, the pure model peptide was obtained, which was confirmed by its MALDI-TOF mass spectrum (m/z 2589.1, calcd 2590.0). To a solution of 100 μM purified model peptide, in 10 mM MOPS containing 0.1 M KCl (pH 8.5), was added the BRos solution (10 mM stock solution in DMF, final concentration of 100 μM). After a 1 h incubation, the unreacted iodoacetamide groups were quenched by the addition of 0.1 M aqueous sodium 2-mercaptoethanesulfonate (final conc. 2 mM). Removal of the unreacted dye by chromatography on Sephadex G-25 (GE-Healthcare) provided the crude labelled peptide. Reverse phase HPLC (Resource-RPC, GE-Healthcare) with a linear gradient from 20% solvent A (0.1% TFA) and 80% B (0.1% TFA, 90% acetonitrile) to 30% A and 70% B at 1 mL min^{-1} over 30 min, followed by lyophilisation, yielded BRos-pep (21 mg, 84%). The same procedure was applied to BRho-labelling and afforded BRho-pep1 (10 mg, 40%) and BRh-pep2 (9.0 mg, 36%). These peptides were all identified by MALDI-TOF mass spectra (BRos-pep: m/z 3074.4, calcd for the 1 : 1 adduct 3072.4, BRho-pep-1: m/z 3117.7 and BRho-pep-2: m/z 3118.1 calcd for the 1 : 1 adduct 3115.4).

NMR measurements of the labelled peptides

NMR measurements of each labelled peptides were done with the samples containing 2 mg of the labelled peptide (BRos-pep, BRho-pep-1, BRho-pep-2) dissolved in 2 mL of D_2O at 25 °C.

Fluorescent spectroscopy

Fluorescent spectra were recorded with a Hitachi F-4500 fluorophotometer with MES-BRos and MES-BRho in 25 mM phosphate buffer (pH 7.2), prepared as described above. For all measurements, the pathlength was 1 cm, with a cell volume of 3.0 mL. The quantum yields for fluorescence were obtained by comparison with fluorescein in 0.1 M NaOH, which has a quantum efficiency of 0.95.[11] The concentration of the reference sample was adjusted to match the test sample. The quantum efficiencies of MES-BRos and MES-BRho were obtained by using diluted samples (absorbance <0.02) in 25 mM phosphate buffer (pH 7.2).

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