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Fluorogenic Protein Labeling through Photoinduced Electron Transfer-Based BL-Tag Technology

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In recent years, protein labeling has been routinely performed with various fluorescent markers.[1] The labeling of proteins allows monitoring of the specific location, movement, and interaction of proteins with other intracellular components using fluorescence microscopy.[2] For several decades, the labeling strategy mostly dealt with green fluorescent protein (GFP) and its variants. In order to overcome the unaltered fluorescent property and uncontrolled expression time of GFP, small molecular probe-based labeling approaches for live-cell imaging methods have been developed.[3] However, most of the reported small-molecule labeling probes do not show different fluorescence properties for the labeled and unlabeled states. Fluorescein-based arsenical hairpin binder (FlAsH) and resorufin-based arsenical hairpin binder (ReAsH) are the pioneer techniques for fluorogenic protein labeling with small molecular probes.[4]

In our earlier studies, we developed a site-specific protein labeling technique that employs a genetically modified β-lactamase (BL-tag).[5] Mutation at a specific position in TEM-1 (class A β-lactamases) provides turn-on fluorogenic biosensors involving a β-lactam ring. The reaction of wild-type TEM-1 (WT TEM) with the β-lactam moiety involves acylation and deacylation steps.[6] We have utilized the BL-tag protein for covalent attachment with a substrate. Despite the similar molecular weights of the BL-tag and GFP, fluorogenicity can be introduced only through the BL-tag technology.

We have developed a fluorogenic mechanism based on aggregation–elimination processes for highly selective protein labeling with a fluorophore of desired color using the BL-tag technology. We have also shown a broad applicability of dinitrobenzene (DNB) as a quencher.[5c,d] However, the limitation of the technology is a slow fluorogenic response of the synthesized probes such as **CCDNB**, which carries a coumarin fluorophore moiety, a cephalosporin moiety, and DNB (Figure 1). The necessary incubation time for a cell imaging experiment using **CCDNB** was 60 minutes. To reduce the incubation time for the imaging experiments, we aimed at developing more sophisticated probes that possess the DNB quencher. Herein, we report two newly designed probes with shorter linkers compared to those of **CCDNB**. In addition, we modified slightly the quencher in one probe. The probe with the modified quencher showed a comparatively fast fluorogenicity in vitro and in live-cell imaging studies. Fluorescence lifetime measurements indicated a different quenching mechanism for the most active probe. A detailed analysis of this new fluorogenic mechanism revealed a photoinduced electron transfer (PET) process[7] from the fluorophore donor to the quencher acceptor.

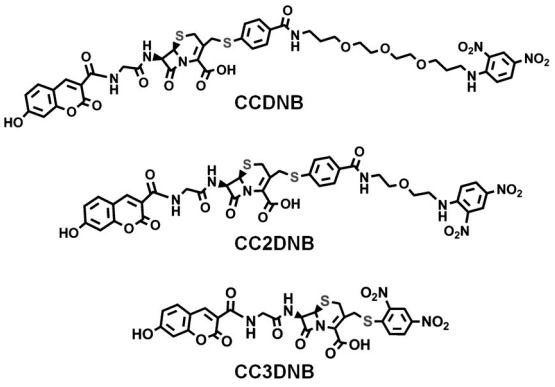

Figure 1. Chemical structures of the synthesized fluorescent probes used for protein labeling.

The two new probes **CC2DNB** and **CC3DNB** that have a comparatively short or no linker between the β-lactam and quencher, respectively, are depicted in Figure 1. 2-(2-Aminoethoxy)ethanamine was used as the linker between the cephalosporin part and the DNB quencher in **CC2DNB**. A coumarin derivative was attached to the opposite side of cephalosporin through a glycine linker to synthesize the **CC2DNB** probe. On the other hand, the synthesis of **CC3DNB** did not require any linker between the cephalosporin part and the quencher. 2,4-Dinitrothiophenol was directly introduced as a quencher to the cephalosporin part to synthesize **CC3DNB**.

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Absorption spectra of **CC2DNB** and **CC3DNB** were recorded in 100 mM HEPES buffer (pH 7.4, Figure 2a) and methanol (Figure 2b), and compared with those of **CCDNB** under similar conditions. The reported absorption maxima of 348 nm for free *N*-ethyl-substituted 2,4-dinitroaniline[8]

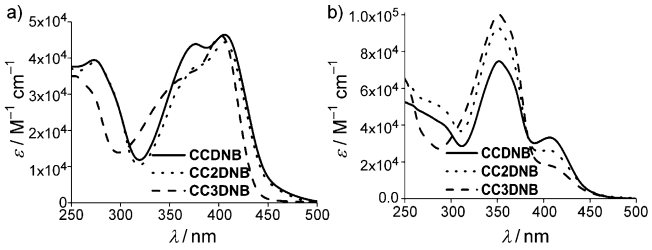

Figure 2. Absorbance spectra of **CCDNB**, **CC2DNB**, and C**C3DNB**. a) Spectra in 100 mM HEPES buffer (pH 7.4). b) Spectra in methanol.

and of 362 nm for 1-(methylthio)-2,4-dinitrobenzene[9] in methanol are quite similar to the observed absorption peaks at 350 nm in methanol for the synthesized probes containing the DNB part. In aqueous buffer, the absorption maxima for the coumarin and DNB parts of both **CCDNB** and **CC2DNB** were observed at 407 nm and 375 nm, respectively. Under the same conditions, the corresponding absorption peaks in **CC3DNB** were detected at 402 nm and 356 nm (shoulder band), respectively. The observed shift of the latter peak was due to the different DNB linkage in this probe. The slight blue shift in the absorption peak of coumarin in **CC3DNB** suggested a change in the interaction between the coumarin and DNB parts within this probe. Comparative measurements with the previously reported probe **CA**[5b] (Figures S3 and S4 in the Supporting Information) confirmed that the peaks around 356 nm or 375 nm in the new probes were due to the quencher DNB parts.

Next, we measured the fluorescence emission spectra of the free probes in 100 mM HEPES buffer of pH 7.4 (physiological pH) and compared them with those in methanol[10] (Figure S5 in the Supporting Information). The fluorescence signals of all the probes were sufficiently quenched in the aqueous buffer (Table 1). The strength of the interaction between the fluorophore and the quencher was comparable for **CCDNB** and **CC2DNB** due to the similar nature of the interacting parts. However, maximum fluorescence quenching was observed in the case of **CC3DNB**. The measured emission spectrum of **CA** in aqueous buffer (Figure S4 in the Supporting Information) confirmed the quenched state of the fluorophore in the newly synthesized probes.

Table 1. Fluorescence quantum yields of the synthesized probes in buffer of physiological pH and in methanol.

Solvent	Fluorescence quantum yield (Φ)		
	CCDNB[5d]	**CC2DNB**	**CC3DNB**
100 mM HEPES buffer (pH 7.4)	0.04	0.04	0.02
Methanol	0.19	0.19	0.19

The fluorogenicities of the synthesized probes were studied by measuring the enhancement of the fluorescence intensity of each probe in the presence of the BL-tag and WT TEM. The reaction rates in the presence of WT TEM were faster for all three probes as compared to the rates in the presence of the BL-tag (Figure 3). In our earlier study, we found that the quencher part was eliminated from the **CCDNB** probe in the presence of the BL-tag.[5d] To modulate the elimination kinetics, the **CC2DNB** probe was synthesized with a much shorter linker compared to the previous polyethylene glycol spacer. However, there was only a slight enhancement in the reaction rate in the presence of the BL-tag. The rate of fluorescence enhancement with the BL-tag was the fastest for **CC3DNB** (Figure 3).

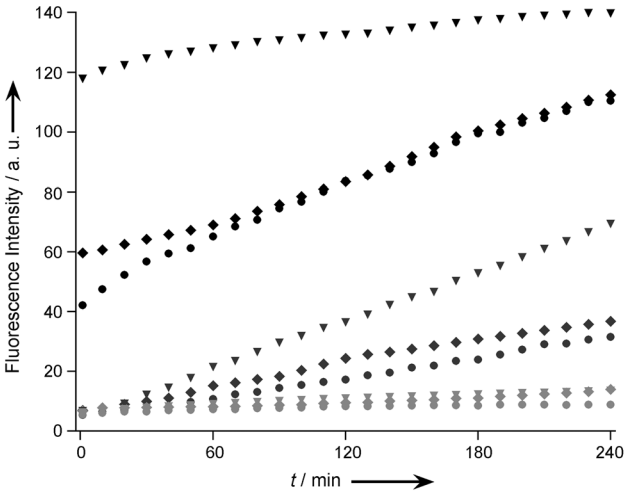

Figure 3. Change in the fluorescence intensities of **CCDNB**, **CC2DNB**, and **CC3DNB** (1 μM each) with time in the presence of WT TEM or BL-tag (0.5 μM each) in 100 mM HEPES buffer (pH 7.4) containing 0.1% DMSO at 25°C. Marker and color codes, circles: **CCDNB**, squares: **CC2DNB**, triangles: **CC3DNB**; black: WT TEM, dark grey: BL-tag, light grey: free probe.

The difference in the rates of fluorescence enhancement indicates that the removal of the quencher in **CC3DNB** is faster compared to the removal of the quencher in the other quenched probes. The overall stability of the eliminating groups partly controls the removal of quenchers from the probes. In the case of **CC3DNB**, the eliminating thiophenolate anion is sufficiently stabilized due to the presence of two highly electron-withdrawing nitro groups at the 2- and 4-positions. On the other hand, such stabilization was less pronounced in the **CCDNB** and **CC2DNB** probes owing to the less electron-withdrawing monosubstituted amide functional group at the 4-position of the thiophenol parts (Figure S6 in the Supporting Information).

The fluorescence enhancements of the different probes were not the same, even in the presence of WT TEM. Approximately 36% and 45% of the reactions were completed within 1 minute during the incubation of WT TEM with **CCDNB**[5d] and **CC2DNB**, respectively. The highest rate of

fluorescence enhancement was observed for the **CC3DNB** in the presence of WT TEM, with 88% of the catalytic reaction completed within 1 minute.

The labeling efficiency with each probe in the presence of the BL-tag was determined in vitro using SDS-PAGE. Incubation with the BL-tag protein for 30 minutes led to the elimination of the quencher in each probe and labeling of the tag protein with the same coumarin fluorophore through covalent linkage. Successful labeling of protein was verified for all three probes by visualization of protein bands in the gel upon irradiation with UV light ($\lambda_{ex}=350$ nm, see Figure 4). In each case, a fluorescent band at approximately

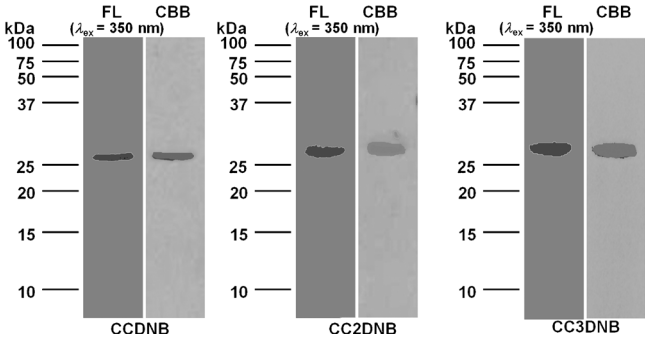

Figure 4. Gel images of BL-tag incubated with **CCDNB** (left), **CC2DNB** (middle), and **CC3DNB** (right) upon irradiation with UV light (FL) or after staining with Coomassie brilliant blue (CBB), as indicated on the top of each gel.

29 kDa was observed. Similar experiments carried out with WT TEM did not result in labeled protein. Because the relatively high rate of labeling with the **CC3DNB** probe compared to that with the other probes could not be completely understood from the in vitro analysis, further studies with living cells were performed.

For live-cell imaging experiments, we performed site-specific labeling of the transmembrane protein epidermal growth factor receptor (EGFR) fused with the BL-tag at the *N*-terminus, and observed labeling by confocal microscopy. To check the efficiencies of the synthesized probes, specific labeling of the cell membranes expressing the BL–EGFR fusion protein was assessed after 15 minutes of incubation with each probe (Figure 5). In the case of **CC3DNB**, sufficient protein labeling at the cell membrane was observed after this incubation period (Figure 5). However, for **CCDNB** and **CC2DNB**, cell membrane labeling was not sufficient after 15 minutes. Fluorescence images taken at 15-minute intervals confirmed sufficient labeling after 60 minutes of incubation with these probes (Figure S7 in the Supporting Information).

To check the origin of the different labeling efficiency among the probes, fluorescence decay profiles of the synthesized probes were obtained (Figure S9 in the Supporting Information). The data were compared with the fluorescence decay profile of **CA**. The **CA** probe, without any quencher, exhibited a single-exponential fluorescence decay. By contrast, the other fluorogenic probes exhibited biexponential decays due to the phenomenon of fluorescence quenching (Table S1 in the Supporting Information). The calculated average fluorescence lifetimes (τ) of the **CCDNB** and **CC2DNB** probes were very close to that of **CA** (Table 2).

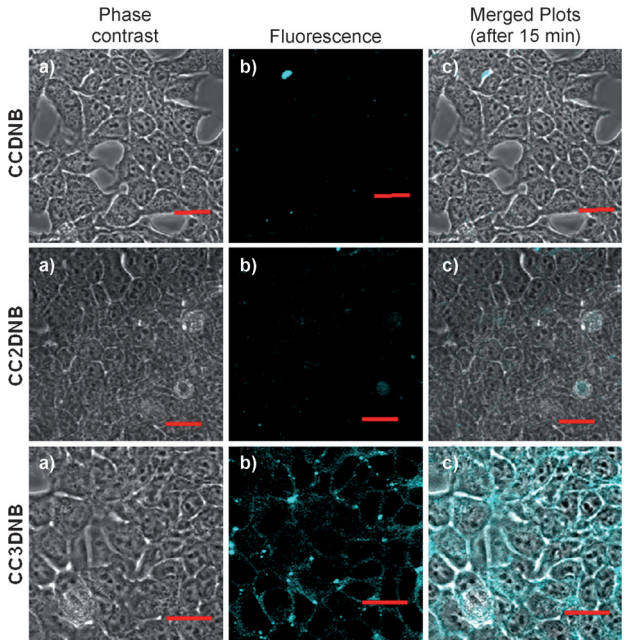

Figure 5. Confocal microscopic images of **CCDNB**-labeled (top), **CC2DNB**-labeled (middle), and **CC3DNB**-labeled (bottom) HEK293T cells expressing BL-tag–EGFR fusion protein after 15 min of incubation. a) Phase-contrast microscopic images, b) fluorescence microscopic images, and c) merged images. For fluorescence microscopic images, the cells were excited at 405 nm. Scale bars: 20 μm.

Table 2. Radiative and nonradiative rate constants of the synthesized probes in 100 mM HEPES buffer (pH 7.4) at 25 °C.

Probe	Φ	Avg τ [ns]	k_r [s^{-1}]	k_{nr} [s^{-1}]
CA	0.4[5b]	3.25	1.2×10^8	1.9×10^8
CCDNB	0.04[5d]	3.60	1.1×10^7	2.6×10^8
CC2DNB	0.04	3.00	1.3×10^7	3.2×10^8
CC3DNB	0.02	0.39	5.1×10^7	2.5×10^9

The radiative decay rate constants (k_r) were significantly lower in **CCDNB** and **CC2DNB** compared to that of the **CA**. This can be attributed to an aggregation between the coumarin fluorophore and the DNB quencher of these two probes, a phenomenon quite similar to static aggregation-based quenching,[11] which is probably due to the π–π stacking interaction between the fluorophore and quencher.

In the case of the **CC3DNB** probe, both the average fluorescence lifetime and quantum yield decreased significantly compared to those of **CA**. This is due to the dynamic quenching process[11] operating in the **CC3DNB** probe. The radiative (k_r) and nonradiative (k_{nr}) decay rate constant values (Table 2) confirmed the existence of a different quenching mechanism in **CC3DNB** compared to that in

CCDNB and **CC2DNB**. The radiative decay rate constant of **CC3DNB** was slightly increased in comparison to those of the other quenched probes. This result confirmed that the aggregation between the quencher and the fluorophore in **CC3DNB** was not affected too much. However, the nonradiative decay rate constant in **CC3DNB** was much higher compared to those in all other probes. This dynamic quenching can be explained by an effective photoinduced electron transfer (PET) process from the coumarin donor to the DNB acceptor. This phenomenon is well known to occur with coumarin probes.[12]

According to the Rehm–Weller equation,[7] the rate of the PET reaction from coumarin to DNB is governed by the energy of the excited state species (ΔG_{00}), the ground state oxidation potential of the coumarin donor ($E_{D^+/D}$), the reduction potential of the DNB acceptor (E_{A/A^-}), and the coulombic attraction energy (E_{coul}). On the basis of the experimental data for the excited state energy ($\Delta G_{00} = 2.93$ eV) and the available data for the oxidation potential of 7-hydroxycoumarin[12b] ($E_{D^+/D} = 0.67$ V vs. SCE) and for the reduction potential of DNB,[13] ($E_{A/A^-} = -0.88$ V vs. SCE), the Gibbs energy (ΔG) for the electron transfer in a polar environment[7] ($E_{coul} = 0.03$ eV) was found to be approximately -1.35 eV. This value was sufficiently negative for operating the diffusion-controlled electron-transfer process at ambient temperature. The shortening of the linker chain length in **CC3DNB** led to an effective PET process. The quencher did not directly interact with the fluorophore in the PET process like it did in the previous aggregation mode. This phenomenon was reflected in the faster recognition of the tag protein in the new PET-based probe. The probability of fluorescence resonance energy transfer (FRET) from coumarin to the DNB group can be ruled out due to the small overlap integral ($J = 2 \times 10^{-23}\,\mathrm{M^{-1}\,cm^3}$, Figure S10 in the Supporting Information) between the coumarin donor emission in **CC3DNB** and the acceptor DNB absorbance of compound **12** (probe without coumarin, Scheme S2 in the Supporting Information).

In summary, we have developed new fluorogenic probes valuable for protein labeling through our developed BL-tag technology. We reduced the linker chain length and slightly modified the quencher with regard to the probe previously reported by us, **CCDNB**. The probe with the shortest linker, **CC3DNB,** was found to be most effective, resulting in fast kinetics. The quenching mechanism in this probe was demonstrated as a PET process, which is different from the FRET- or static aggregation-based quenching occurring in the other probes. Thus, the new probe overcomes the limitation of the previous coumarin probes that required a long incubation period. In principle, such PET-based probe design could lead to the development of multi-colored fluorogenic probes for successful use in next-generation live-cell imaging technologies.

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Keywords: biotechnology · electron transfer · fluorescence spectroscopy · fluorescent probesprotein labeling

[1] a) A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, *Nat. Biotechnol.* **2003**, *21*, 86–89; b) L. W. Miller, J. Sable, P. Goelet, M. P. Sheetz, V. W. Cornish, *Angew. Chem.* **2004**, *116*, 1704–1707; *Angew. Chem. Int. Ed.* **2004**, *43*, 1672–1675; c) C. Hoffman, G. Gaietta, M. Bunemann, S. R. Adams, S. Oberdolff-Maass, B. Behr, J.-P. Vilardaga, R. Y. Tsien, M. H. Ellisman, *Nat. Methods* **2005**, *2*, 171–176; d) G. V. Los, A. Darzins, N. Karassina, C. Zimprich, R. Learish, M. G. McDougall, L. P. Encell, R. Friedman-Ohana, M. Wood, G. Vidugiris, K. Zimmerman, P. Otto, D. H. Klaubert, K. V. Wood, *Cell Notes* **2005**, *11*, 2–6; e) A. Ojida, K. Honda, D. Shinmi, S. Kiyonaka, Y. Mori, I. Hamachi, *J. Am. Chem. Soc.* **2006**, *128*, 10452–10459; f) S. R. Adams, R. Y. Tsien, *Nat. Protoc.* **2008**, *3*, 1527–1534; g) X. Zhou, X. Jin, D. Li, X. Wu, *Chem. Commun.* **2011**, *47*, 3921–3923.

[2] a) D. Summerer, S. Chen, N. Wu, A. Deiters, J. W. Chin, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 9785–9789; b) C. Smith, *Nat. Methods* **2007**, *4*, 755–761; c) Z. Zhou, A. Koglin, Y. Wang, A. P. McMahon, C. T. Walsh, *J. Am. Chem. Soc.* **2008**, *130*, 9925–9930; d) R. McRae, P. Bagchi, S. Sumalekshmy, C. J. Fahrni, *Chem. Rev.* **2009**, *109*, 4780–4827; e) H. Ren, F. Xiao, K. Zhan, Y.-P. Kim, H. Xie, Z. Xia, J. Rao, *Angew. Chem.* **2009**, *121*, 9838–9842; *Angew. Chem. Int. Ed.* **2009**, *48*, 9658–9662; f) R. W. Watkins, L. D. Lavis, V. M. Kung, G. V. Los, R. T. Raines, *Org. Biomol. Chem.* **2009**, *7*, 3969–3975; g) D. Maurel, S. Banala, T. Laroche, K. Johnsson, *ACS Chem. Biol.* **2010**, *5*, 507–516; h) C. Uttamapinant, K. A. White, H. Baruah, S. Thompson, M. Fernández-Suárez, S. Puthenveetil, A. Y. Ting, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 10914–10919; i) D. Srikun, A. E. Albers, C. I. Nam, A. T. Iavarone, C. J. Chang, *J. Am. Chem. Soc.* **2010**, *132*, 4455–4465.

[3] a) C. Szent-Gyorgyi, B. F. Schmidt, Y. Creeger, G. W. Fisher, K. L. Zakel, S. Adler, J. A. Fitzpatrick, C. A. Woolford, Q. Yan, K. V. Vasilev, P. B. Berget, M. P. Bruchez, J. W. Jarvik, A. Waggoner, *Nat. Biotechnol.* **2008**, *26*, 235–240; b) O. Tour, S. R. Adams, R. A. Kerr, R. M. Meijer, T. J. Sejnowski, R. W. Tsien, R. Y. Tsien, *Nat. Chem. Biol.* **2007**, *3*, 423–431; c) N. T. Calloway, M. Choob, A. Sanz, M. P. Sheetz, L. W. Miller, V. W. Cornish, *ChemBioChem* **2007**, *8*, 767–774; d) N. Johnsson, K. Johnsson, *ACS Chem. Biol.* **2007**, *2*, 31–38; e) G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R, Learish, R. F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D. H. Klaubert, R. F. Bulleit and K. V. Wood, *ACS Chem. Biol.* **2008**, *3*, 373–382; f) M. A. Brun, K.-T. Tan, E. Nakata, M. J. Hinner, K. Johnsson, *J. Am. Chem. Soc.* **2009**, *131*, 5873–5884; g) S. S. Gallagher, J. E. Sable, M. P. Sheetz, V. W. Cornish, *ACS Chem. Biol.* **2009**, *4*, 547–556; h) H. E. Rajapakse, D. R. Reddy, S. Mohandessi, N. G. Butlin, L. W. Miller, *Angew. Chem.* **2009**, *121*, 5090–5092; *Angew. Chem. Int. Ed.* **2009**, *48*, 4990–4992; i) M. Bannwarth, I. R. Corrêa Jr., M. Sztretye, S. Pouvreau, C. Fellay, A. Aebischer, L. Royer, E. Ríos, K. Johnsson, *ACS Chem. Biol.* **2009**, *4*, 179–190; j) K. K. Sadhu, S. Mizukami, Y. Hori, K. Kikuchi, *Chem-*

BioChem **2011**, *12*, 1031–1034; k) C. Grunwald, K. Schulze, G. Giannone, L. Cognet, B. Lounis, D. Choquet, R. Tampé, *J. Am. Chem. Soc.* **2011**, *133*, 8090–8093; l) T. Komatsu, K. Johnsson, H. Okuno, H. Bito, T. Inoue, T. Nagano, Y. Urano, *J. Am. Chem. Soc.* **2011**, *133*, 6745–6751.

[4] a) B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269–272; b) S. R. Adams, R. E. Campbell, L. A. Gross, B. R. Martin, G. K. Walkup, Y. Yao, J. Llopis, R. Y. Tsien, *J. Am. Chem. Soc.* **2002**, *124*, 6063–6076.

[5] a) S. Mizukami, S. Watanabe, Y. Hori, K. Kikuchi, *J. Am. Chem. Soc.* **2009**, *131*, 5016–5017; b) S. Watanabe, S. Mizukami, Y. Hori, K. Kikuchi, *Bioconjugate Chem.* **2010**, *21*, 2320–2326; c) K. K. Sadhu, S. Mizukami, S. Watanabe, K. Kikuchi, *Chem. Commun.* **2010**, *46*, 7403–7405; d) K. K. Sadhu, S. Mizukami, S. Watanabe, K. Kikuchi, *Mol. BioSyst.* **2011**, *7*, 1766–1772; e) A. Yoshimura, S. Mizukami, Y. Hori, S. Watanabe, K. Kikuchi, *ChemBioChem* **2011**, *12*, 1031–1034; f) S. Watanabe, S. Mizukami, Y. Akimoto, Y. Hori, K. Kikuchi, *Chem. Eur. J.* **2011**, *17*, 8342–8349; g) S. Mizukami, T. Yamamoto, A. Yoshimura, S. Watanabe, K. Kikuchi, *Angew. Chem.* **2011**, *123*, 8909–8911; *Angew. Chem. Int. Ed.* **2011**, *50*, 8750–8752.

[6] a) G. Guillaume, M. Vanhove, J. Lamotte-Brasseur, P. Ledent, M. Jamin, B. Joris, J.-M. Frère, *J. Biol. Chem.* **1997**, *272*, 5438–5444; b) H. Adachi, T. Ohta, H. Matsuzawa, *J. Biol. Chem.* **1991**, *266*, 3186–3191.

[7] D. Rehm, A. Weller, *Isr. J. Chem.* **1970**, *8*, 259–271.

[8] G. Punte, B. E. Rivero, *Acta Crystallogr. Sect. C* **1989**, *45*, 1952–1957.

[9] R. L. Gupta, B. H. K. Saini, T. R. Juneja, *Mutat. Res.* **1997**, *381*, 41–47.

[10] S. V. Balasubramanian, J. L. Alderfer, R. M. Straubinger, *J. Pharm. Sci.* **1994**, *83*, 1470–1476.

[11] S. Doose, H. Neuweiler, M. Sauer, *ChemPhysChem* **2009**, *10*, 1389–1398.

[12] a) J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, *3rd ed.*, Springer Science+Business Media, LLC, **2006**; b) G. J. Smith, Y. Tang, J. M. Dyer, S. M. Scheele, *Photochem. Photobiol.* **2011**, *87*, 45–50.

[13] M. E. Peover, *Trans. Faraday Soc.* **1964**, *60*, 479–483.

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