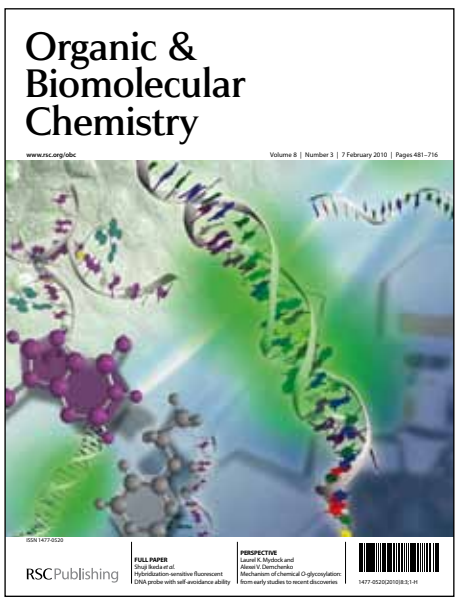
Organic & Biomolecular Chemistry

Accepted Manuscript



Organic
Biomolecular Chemistry

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

PAPER

A cascading reaction sequence involving ligand-directed azaelectrocyclization and autooxidation-induced fluorescence recovery enables visualization of target proteins on the surfaces of live cells

Katsunori Tanaka*[a], Masataka Kitadani[b], Ayumi Tsutsui[a], Ambara R. Pradipta[a], Rie Imamaki[c], Shinobu Kitazume[c], Naoyuki Taniguchi[c] and Koichi Fukase*[b]

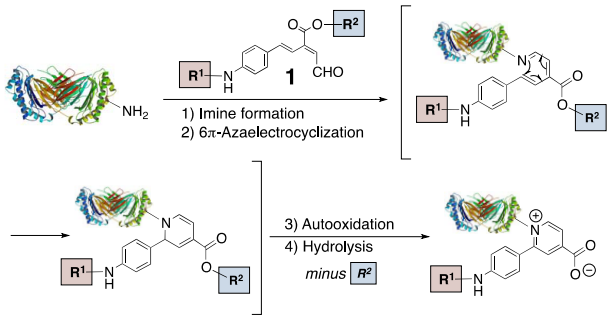
Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X
DOI: 10.1039/b000000x

A general probe designed to induce a cascading sequence of reactions on a target protein was efficiently synthesized. The cascading reaction sequence involved (i) ligand-directed azaelectrocyclization with lysine, and (ii) the autooxidation-induced release of a fluorescence quencher from the labeled protein. The probe was linked to a cyclic RGDyK peptide to enable the selective visualization of integrin $\alpha_V\beta_3$ on the surfaces of live cells.

Introduction

A variety of novel chemical methods have been recently combined with biological techniques to achieve protein labeling. Successful examples include the Cu(I)-mediated Huisgen [3+2] cycloaddition,[1] Staudinger ligation,[2] and the strain-promoted Cu(I)-free Huisgen cycloadditon[3] involving an azide that has been genetically, metabolically, or enzymatically introduced at a desired position within a protein or on a cell surface. Variants of the Cu(I)-free reaction,[4] e.g., the tetrazine/norbornene D-A reaction[4a-f] and the base- or photo-induced nitrile imine/norbornene cycloaddition reaction,[4g-i] have been examined in an effort to improve the reactivity, chemo-orthogonal, and bio-orthogonal profiles. Chemical methods in which a protein-specific ligand is used to directly introduce a probe molecule have been investigated by Hamachi and co-workers.[5] Their methods were successfully applied to the fluorescence labeling of proteins in cells, tissues, and living mice.[6]

Independently of these efforts, we have been investigating a lysine-based protocol for labeling peptides, proteins, and living cell surfaces (Scheme 1).[7,8] The labeling reaction involves Schiff base formation and a subsequent rapid 6π-azaelectrocyclization.[9] The 1,2-dihydropyridine products are then spontaneously oxidized in aqueous media, which accelerates the hydrolysis of the ester and provides a zwitterion as the amino modification product. This method was used to efficiently and selectively introduce DOTA (1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid) or fluorescent groups (substituents at **R[1]**, see Scheme 1) onto the lysine residues through a reaction with the unsaturated aldehyde probes **1** at very low concentrations (~10^{-8} M) within a short period of time (10–30 min) at room temperature. This electrocyclization protocol was used to enable the successful visualization of receptor-mediated accumulation, circulatory residence, and trafficking of glycoproteins and/or lymphocytes via PET and non-invasive fluorescence imaging.[7,8]

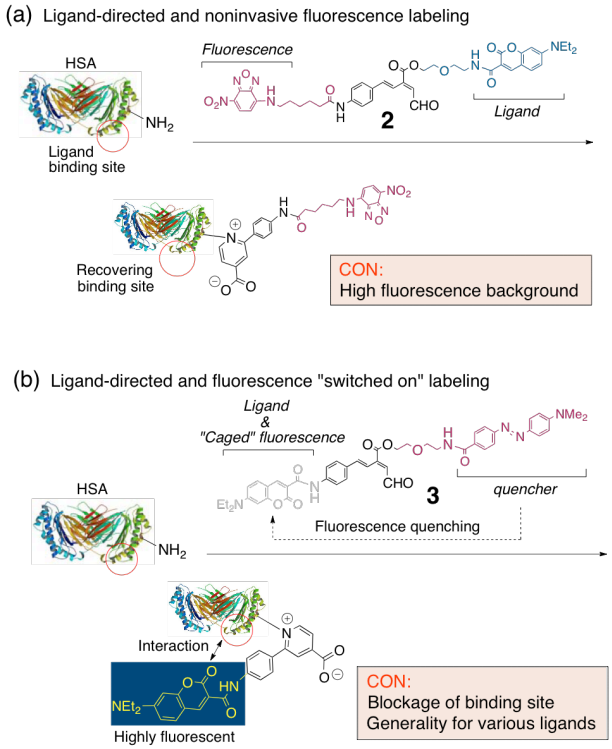


Scheme 1 Cascading reaction involving 6π-azaelectrocyclization, autooxidation, and hydrolysis.

The site-selective and non-destructive modification of target proteins has been achieved by directing reactive groups to a specific site using a small molecule ligand of the protein (Scheme 2a).[10] After the selective azaelectrocyclization of the probe **2** with the target lysine in human serum albumin (HSA), the ligand connected to the ester linkage was then spontaneously cleaved from the conjugated albumin. This ligand-directed approach may be applied to the selective and noninvasive labeling of target proteins in cell lysates, on cell surfaces, or even in living animals. The selective labeling of a target protein in a mixture of biomolecules, e.g., using fluorescence reporter groups, usually yields poor fluorescence contrast between the labeled protein and the unreacted probes. The development of labeling probes that are fluorescently silent by default ("caged" fluorescence) but are "switched on" when they selectively react with the target proteins would go a long way toward removing the unlabeled background fluorescence signal.[6b,11]

We therefore incorporated a fluorescence quenching system into an unsaturated aldehyde probe **3** (Scheme 2b),[12] which could be unlocked only after an azaelectrocyclization-induced cascading reaction had proceeded in the presence of the target lysine. The coumarin moiety, which functioned as both a ligand of serum albumin and as a "caged" fluorescence reporter group,

was introduced at the left terminus of the probe **3**, and DABCYL (4-[4-(dimethylamino)phenylazo]benzoic acid) was loaded via an ester linkage. The structural arrangement shown in **3** achieved efficient quenching of the coumarin fluorescence by DABCYL so that the probe **3** itself was non-fluorescent. Incubation of albumin with the probe **3** selectively labeled the lysine moieties proximal to the ligand-binding site, and the subsequent autooxidation and hydrolysis reactions released the DABCYL quencher to selectively recover the coumarin fluorescence within the albumin. Although probe **3** blocked the protein-binding site after labeling (this technique could be characterized as invasive protein labeling), this strategy enabled the sensitive detection of the target albumin, even in a mixture of several proteins and peptides, by achieving a high fluorescence contrast.

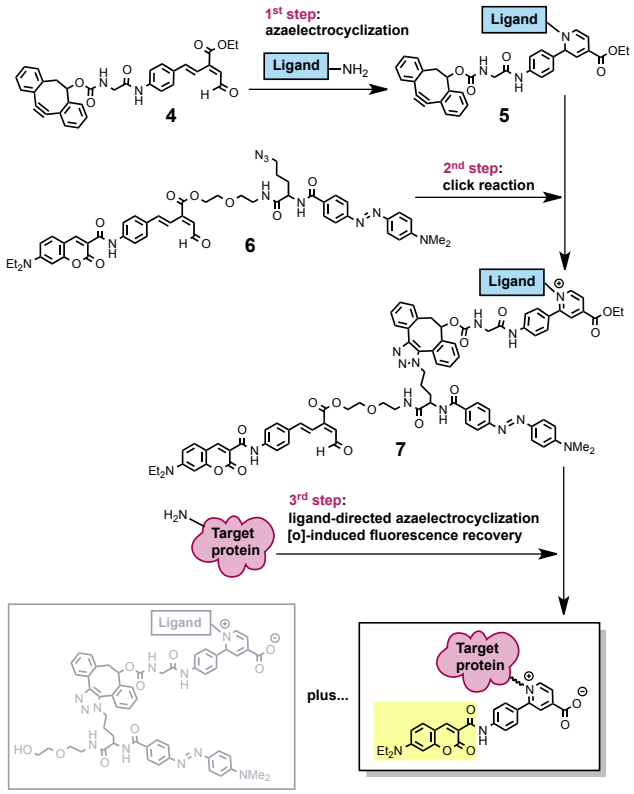


Scheme 2 Two previously reported ligand-directed strategies for protein labeling. (a) Lysine-selective and noninvasive fluorescence labeling. (b) Lysine-selective and invasive "switched on" labeling.

We envisioned that the simultaneous introduction of bioactive peptide- or glycan-based ligands bound to a fluorescence quencher and to probe **3** through an ester linkage could permit the "switched on" labeling and imaging of receptors in live systems, e.g., on the surfaces of live cells or in live animals. Here, we report a general route to the preparation of fluorescence "switch on" probes that can conveniently bind to a variety of amine-containing ligands during the last stage of synthesis through a combination of azaelectrocyclization and the strain-promoted click reactions. As a model case, the cyclic RGDyK peptide integrin $\alpha_V\beta_3$ ligand was loaded onto a new "switched on" probe, and the integrin $\alpha_V\beta_3$ was used to label live RAW264.7 cells (mouse leukemic monocyte macrophage cells). The cells were then selectively visualized through a cascading sequence of ligand-directed azaelectrocyclization and autooxidation-induced fluorescence recovery.

Results and discussion

The application of the fluorescence "switched on" probes to imaging of various proteins in biological systems required that the synthetic route be general and practical, and that a variety of protein-selective ligands, i.e., peptides, could be installed during the last stages of synthesis. The introduction of complex and often hydrophilic molecules, e.g., peptides or glycans, to the relatively unstable (*E*)-3-alkoxycarbonyl-5-phenyl-2,4-dienal (see structure **3** in Scheme 2) through an appropriate ester linkage similar to that used to link the coumarin fluorophore and DABCYL quencher was not trivial. The conventional thiol-based or photo-induced ligation conditions led to the decomposition, polymerization, or isomerization of the (*E*)-carbonyl-2,4-dienal system. The dienals themselves were not appropriate substrates for the amide coupling reaction due to a rapid azaelectrocyclization reaction that occurred upon exposure to the free amino groups in the linker and/or the dienal molecules.



Scheme 3 One-pot sequence involving ligand functionalization with DIBO (1st step), loading onto a fluorescently "caged" probe (2nd step), and ligand-directed azaelectrocyclization and oxidation-induced fluorescence recovery (3rd step).

We eventually hit upon the idea that the unsaturated aldehydes with an azide functionality, such as **6** (Scheme 3), could be coupled to the acetylene-functionalized ligands **5** via a strain-promoted click reaction[3] during the last stage of synthesis. Previous investigations[13] indicated that the strain-promoted click reaction was chemoselective toward the azides and strained acetylenes and did not destroy the dienal system. In this work, dibenzocyclooctyne (DIBO), developed by Boons and co-workers,[3e] was used as the strained acetylene because this

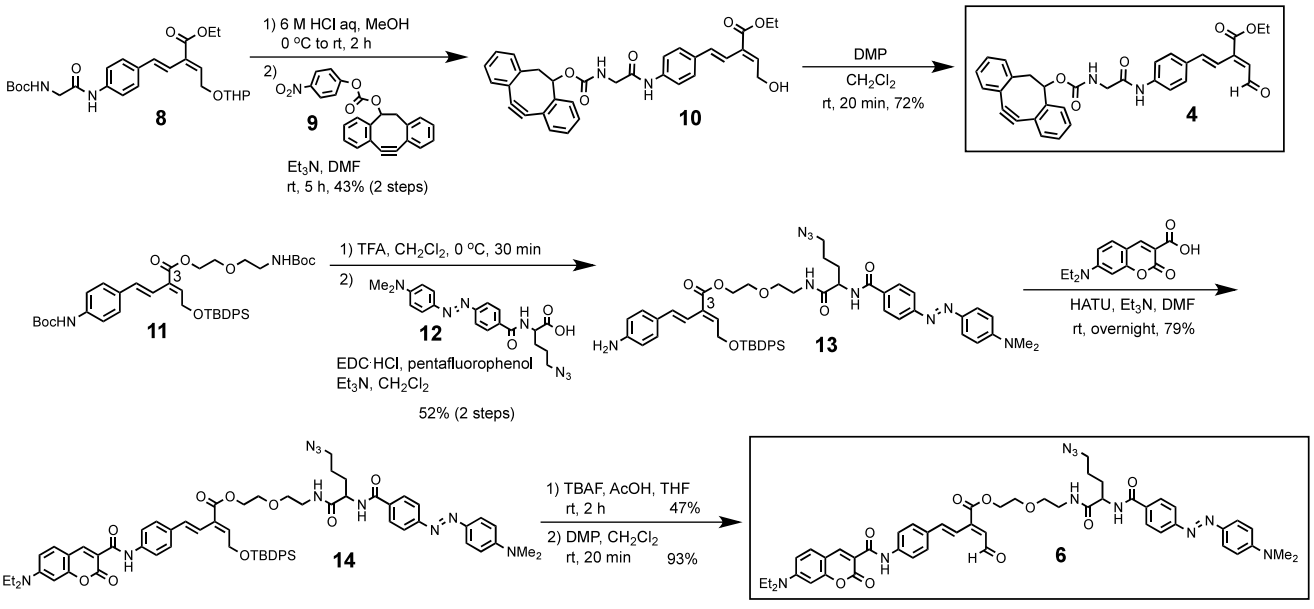
acetylene displays a relatively high reactivity and is readily prepared from a simple starting material.

We also envisioned that various bioactive ligands, e.g., peptides, could be readily functionalized with the strained acetylene, DIBO, by submitting the DIBO aldehyde **4** to a rapid azaelectrocyclization reaction with lysine amino groups (Scheme 3). The last two steps of the probe synthesis, i.e., peptide functionalization with DIBO (from **4** to **5**) and coupling to the azide-aldehyde (**5** + **6**), and the subsequent ligand-directed "switched on" labeling of the target proteins, could all be handled in a one-pot process without isolating the unstable and complex intermediates. The synthetic route shown in Scheme 3 is applicable to a variety of peptides and other amine-containing ligands, once the DIBO-aldehyde **4** and azide-aldehyde **6** has been prepared as the two key intermediates of the probe synthesis.

Throughout this study, we focused on the use of an RGD peptide as an $\alpha_V\beta_3$-integrin ligand. $\alpha_V\beta_3$-Integrin is a cell adhesion molecule and is highly expressed on endothelial and tumor cells. The cyclic RGDyK peptide (see the structure shown in Scheme 5) is used as a strong integrin agonists in breast, brain, and lung cancer models.[7b,14] This peptide presents an attractive model ligand for the selective imaging of cell surface integrin molecules via the fluorescence "switched on" strategy.

The synthesis of both intermediates **4** and **6** was carried out from the Boc-protected amines **8** and **11**, which were previously synthesized through a key Stille coupling step (Scheme 4).[7a,10] Thus, the Boc and THP protecting groups of **8** were simultaneously removed by treatment with 6 M HCl in methanol. The resulting amino group was selectively acylated by DIBO **9** activated by the *p*-nitrophenylcarbonate to yield the alcohol **10** in 43% yield over two steps. Oxidation with the Dess Martin periodinane in CH_2Cl_2 for 20 min gave one of the key intermediates, the DIBO-aldehyde **4**.

The two Boc protecting groups in **11** were simultaneously removed by treatment with 20% TFA in CH_2Cl_2 to give the unstable diamine. The more reactive aliphatic amine at the C3 ester group was selectively reacted with the *in situ*–generated pentafluorophenyl ester of the DABCYL-azide acid **12** to provide **13** in a 52% yield in two steps. The remaining anilino nitrogen group in **13** was acylated with 7-diethylaminocoumarin-3-carboxylic acid in the presence of HATU and triethylamine in DMF to provide the coupling product **14** in a 79% yield. TBDPS deprotection was then achieved in a 47% yield by reaction with TBAF buffered with acetic acid in THF. Finally, the allylic alcohol was oxidized by the Dess Martin periodinane to afford the azide-aldehyde **6** as the second intermediate. The relatively unstable aldehyde **6**, the structure of which was confirmed by the presence of the characteristic ^{1}H NMR and MS signatures, was then immediately used in a strain-releasing click reaction with the acetylene-functionalized cRGDyK ligands to enable the labeling experiments (*vide infra*).



Scheme 4 Synthesis of the DIBO- and azide-containing unsaturated ester aldehydes. HATU: *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; TBAF: tetra-*n*-butylammonium fluoride.

Prior to performing the one-pot synthesis, "click" ligand loading, and cell surface "switched on" imaging studies, the efficiency of the cascade process involving the azaelectrocyclization and autooxidation-induced fluorescence recovery steps was determined using the azide-aldehyde **6** (Fig. 1). As designed, the fluorescence spectroscopic analysis of **6** (concentration of **6**: 1.0×10^{-6} M, 10% MeOH in PBS) detected a fluorescence signal intensity that was 10% of the fluorescence intensity due to the 7-diethylaminocoumarin carboxylic acid moiety at 480 nm (excitation at 420 nm) as a result of efficient quenching. The quenching resulted from fluorescence resonance energy transfer (FRET) between the two proximal dyes in **6** (Fig. 1a). Incubation at 25 °C of probe **6** with the *n*-octylamine (used as a model compound for lysine) resulted in a rapid two-fold increase in the coumarin fluorescence over 60 min (Fig. 1b). No

significant increase in fluorescence was observed in the absence of the amine (Fig. 1c), indicating that the DABCYL quencher had been released from the coumarin-modified octylamine through the azaelectrocyclization-initiated autooxidation and hydrolysis reactions. The formation of the zwitterion product was confirmed by MALDI-TOF-MS analysis (*m/z* = 570.3 (theoretical; 570.3 for $C_{34}H_{40}N_3O_5$ $(M+H)^+$); thus, the amino group could be sensitively detected using the new "switched on" probe. Because the 1,2-dihydropyridine could be rapidly oxidized and hydrolyzed during ionization by FAB, CI, ESI, and MALDI, an MS-based time-course analysis of the 1,2-dyhydropyridine formation, oxidation, and hydrolysis processes was not possible, as reported previously.[12]

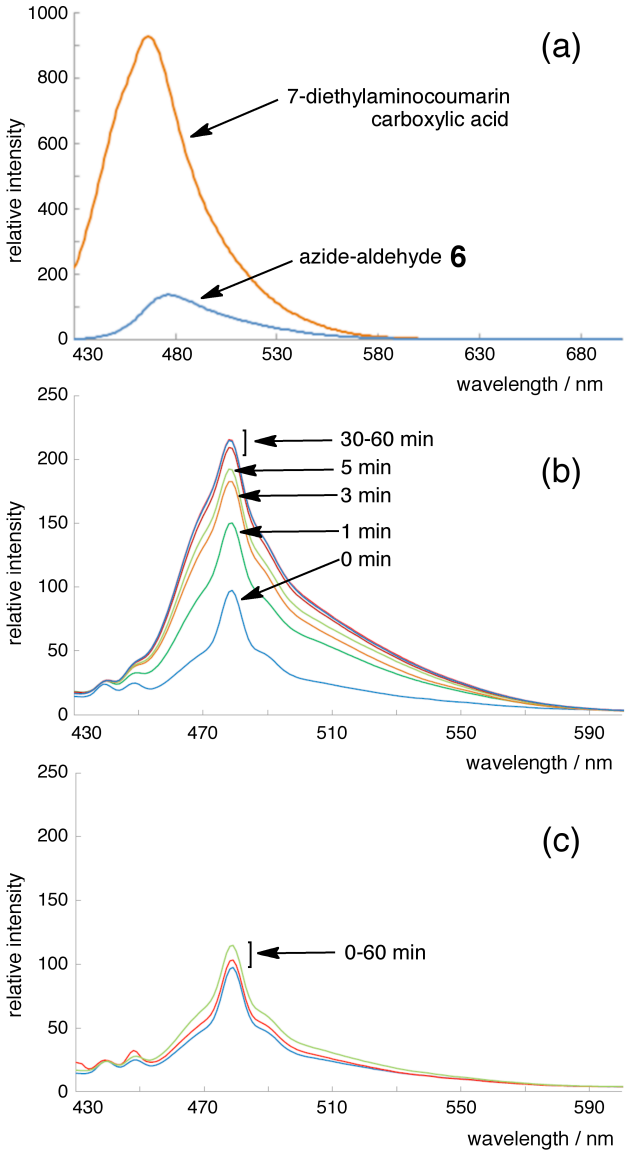
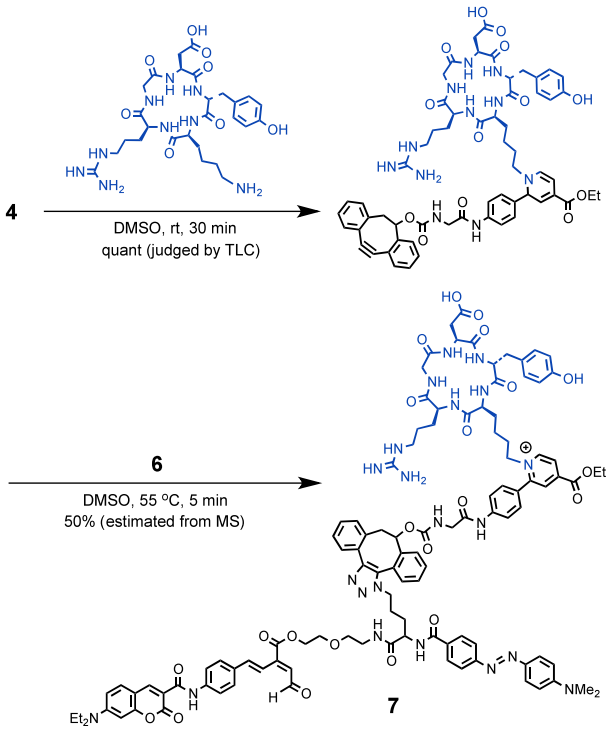


Fig. 1 Monitoring the azaelectrocyclization-induced fluorescence recovery (10% MeOH in PBS, rt). (a) Comparison of the fluorescence intensities of the azide-aldehyde **6** and the 7-diethylaminocoumarin carboxylic acid. Time-course fluorescence spectra of **6** (b) in the presence of *n*-octylamine, and (c) in the absence of *n*-octylamine.

The promising fluorescence results led to an examination of the one-pot sequence of ligand-directed "switched on" labeling reactions, including (1) peptide modification by the strained acetylene, (2) "click" ligand loading of the probe **6**, and (3) selective fluorescence "switched on" imaging of the integrins expressed on the cell surfaces (Scheme 5). The lysine amino group in the cyclic RGDyK peptide initially reacted with an equimolar amount of the DIBO-aldehyde **4** at room temperature. The peptide ligand was functionalized with the strained acetylene in quantitative yield over 30 min, as judging from the TLC and MS analysis. The reaction mixture was then directly treated with the azide-aldehyde **6** at 55 °C for 5 min, providing the desired RGDyK-loaded aldehyde **7** in approximately 50% yield based on the direct MS analysis of the reaction mixtures (Supplementary Information). **7** was found to decompose under either prolonged reaction times (in an attempt to improve the conversion to **7**) or HPLC purification. The decomposition reaction resulted from the instability of the densely functionalized dienal moiety in **7**. Nevertheless, the cell surface reaction of the azide-aldehyde **6** was much slower than the reaction involving the integrin ligand in **7** (*vide infra*), and the mixture could be used directly in labeling experiments.



Scheme 5 One-pot DIBO functionalization of the cyclic RGDyK ligand via (1) azaelectrocyclization and (2) loading onto a "switched on" probe by means of a strain-promoted click reaction.

Thus, the one-pot solution of the probe **7**, as prepared in Scheme 5, was diluted to a concentration of 1×10^{-5} M in PBS (containing 0.05% DMSO) and was applied directly to RAW264.7 cells, which express high levels of $\alpha_V\beta_3$-integrin on the cell surfaces, and Hep3B cells, which express lower levels of $\alpha_V\beta_3$-integrin. The cells were cultured on glass cover slips (2×10^5/50 μL) to facilitate imaging. Control experiments, in which the cells were treated with the azide-aldehyde **6** without the

cyclic RGDyK ligand, were performed under identical conditions. After treatment with the probes, the live cells were immediately analyzed (within 1 min)[15] by confocal microscopy.

The green fluorescence derived from the coumarin fluorophore was clearly observed on the RAW264.7 cells treated with probe **7** (Fig. 2a, middle panel), whereas no significant fluorescence was detected in the control cells treated with the azide-aldehyde **6** (Fig. 2a, left panel). Fluorescence intensity (averaged at each cell) was 5.7 times higher for the cells treated by the probe **7**, than those treated by the control **6**. The fluorescence signals on the Hep3B cells treated with probes **6** or **7** were indistinguishable (Fig. 2b). These results indicated that the selective "switched on" labeling of the cell surface $\alpha_V\beta_3$-integrin was efficiently achieved through the strong RGD–integrin interaction. The results were further supported by treating the RAW264.7 cells with probe **7** in the presence of an excess of the acetylated RGDyK peptide (1×10^{-4} M). The fluorescence intensity was significantly reduced by the presence of the competitive inhibitor (Fig. 2a, right panel). Despite repeated trials, the fluorescently labeled $\alpha_V\beta_3$-integrin could not be detected by SDS-PAGE analysis of the **7**-treated RAW264.7 cell lysate, presumably due to the presence of small amounts of the $\alpha_V\beta_3$-integrin and/or instabilities in the **7**-integrin conjugates during the lysis procedure. The data shown in Fig. 2 suggested that the selective fluorescence recovery on the RAW264.7 cells was derived from the integrin-selective "switched on" labeling procedure.

The cell morphologies were not observed to vary during the labeling process. Thus, the "switched on" imaging of live cell surfaces under mild conditions, i.e., an exposure time of less than 1 min at 37 °C, did not detectably affect the cell viability. Figure 2a suggested that not all fluorescent probes appeared to be present on the cell surfaces. The azaelectrocyclization reaction with the unsaturated aldehydes proceeded smoothly at the lysine amino groups of the $\alpha_V\beta_3$-integrin on the cell surface, and it is possible that the labeled integrin was rapidly endocytosed within the experimental timeframe, as has been observed previously.[8,13]

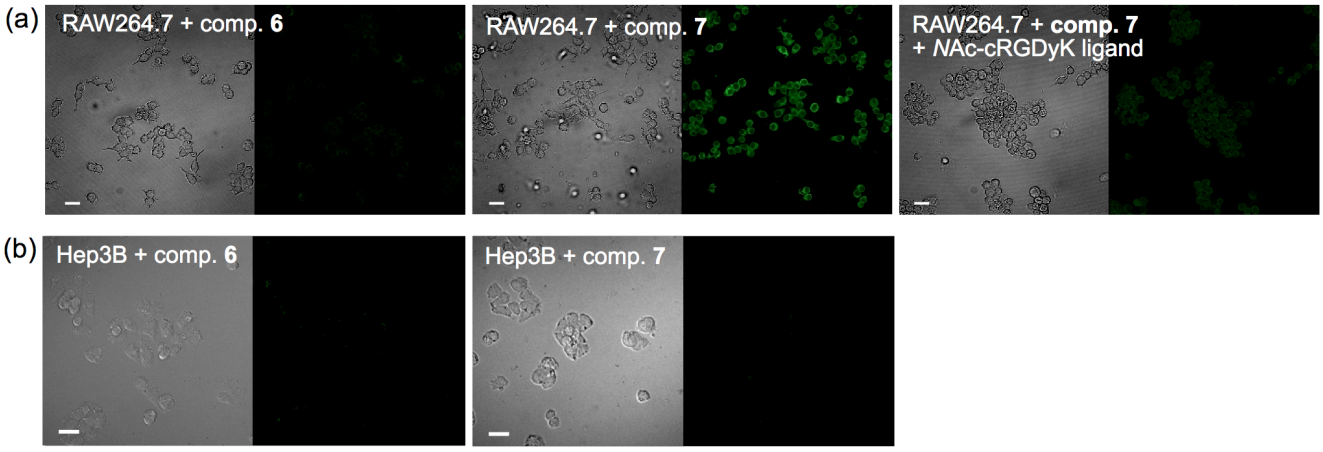


Fig. 2 Optical microscopy images of RAW264.7 and Hep3B cells treated with the fluorescence "switched on" probes **6** and **7**. The bars indicate 20 μm. (a) RAW264.7 cells treated with the probes **6** (left panel), **7** (middle panel), and **6** in the presence of the *N*Ac-cyclic RGDyK peptide (right panel). (b) Hep3B cells treated with **6** (left panel) and **7** (middle panel).

Conclusions

In conclusion, we established a method for preparing a general "switched on" labeling probe for the detection of target proteins using a fluorescence quenching system. A variety of amine-containing high-affinity ligands of target proteins, e.g., peptides or oligosaccharides, may be readily loaded onto the fluorescently "caged" probe **6** via azaelectrocyclization and a strain-releasing click reaction. The method was demonstrated using the cyclic RGDyK peptide as an integrin $\alpha_V\beta_3$ ligand loaded onto the "switched on" probe. The cell surface $\alpha_V\beta_3$-integrins were selectively imaged after carrying out a cascading sequence of ligand-directed azaelectrocyclization and autooxidation-induced fluorescence recovery reactions in the presence of the cells. Although many fluorogenic protocols have been combined with biological techniques in the past, purely chemical methods that employ novel reactivity strategies are quite rare.[5,6] The results described here are applicable to the efficient imaging of target proteins in cell lysates or on live cell surfaces without the need for isolation and/or washing procedures. Direct labeling in living animals could enable *in vivo* molecular imaging immediately following injection of the probe. These studies are currently in progress in our laboratory.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, 23681047, 23241074, and 25560410, by the New Energy and Industrial Technology Development Organization (NEDO), by a research grant from the Mizutani Foundation for Glycoscience, and by a MEXT Grant-in-Aid for Scientific Research on the Innovative Areas "Organic Synthesis Based on Reaction Integration, Development of New Methods and Creation of New Substances" and "Chemical Biology of Natural Products: Target ID and Regulation of Bioactivity".

Notes and references

[a]*Biofunctional Synthetic Chemistry Laboratory, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan. Fax: +81-48-467-9405; Tel: +81-48-467-9379; E-mail: kotzenori@riken.jp*

[b]*Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka-shi, Osaka 560-0043, Japan.*
[c]*Disease Glycomics Team, Systems Glycobiology Research Group, RIKEN-Max Planck Joint Research Center for Systems Chemical Biology, RIKEN Global Research Cluster, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*

† Electronic Supplementary Information (ESI) available: Experimental details, characterization data and selected copies of NMR spectra. See DOI: 10.1039/b000000x/

1 (*a*) Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless and M. G. Finn, *J. Am. Chem. Soc.*, 2003, **125**, 3192–3193; (*b*) A. E. Speers, G. C. Adam and B. F. Cravatt, *J. Am. Chem. Soc.*, 2003, **125**, 4686–4687; (*c*) A. J. Link and D. A. Tirrell, *J. Am. Chem. Soc.*, 2003, **125**, 11164–11165; (*d*) A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson and P. G. Schultz, *J. Am. Chem. Soc.*, 2003, **125**, 11782–11783; (*e*) A. E. Speers and B. F. Cravatt, *Chem. Biol.*, 2004, **11**, 535–546; (*f*) J. Gierlich, G. A. Burley, P. M. E. Gramlich, D. M. Hammond and T. Carell, *Org. Lett.*, 2006, **8**, 3639–3642; (*g*) P. V. Chang, X. Chen, C. Smyrniotis, A. Xenakis, T. Hu, C. R. Bertozzi and P. Wu, *Angew. Chem. Int. Ed.*, 2009, **48**, 4030–4033.

2 (*a*) E. Saxon and C. R. Bertozzi, *Science*, 2000, **287**, 2007–2010; (*b*) K. L. Kiick, E. Saxon, D. A. Tirrell and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A.*, 2002, **99**, 19–24; (*c*) D. J. Vocadlo, H. C. Hang, E.-J. Kim, J. A. Hanover and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A.*, 2003, **100**, 9116–9121; (*d*) P. V. Chang, J. A. Prescher, M. J. Hangauer and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2007, **129**, 8400–8401; (*e*) M. J. Hangauer and C. R. Bertozzi, *Angew. Chem. Int. Ed.*, 2008, **47**, 2394–2397.

3 (*a*) N. J. Agard, J. A. Prescher and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2004, **126**, 15046–15047; (*b*) M. Fernández-Suárez, H. Baruah, L. Martínez-Hernández, K. T. Xie, J. M. Baskin, C. R. Bertozzi and A. Y. Ting, *Nat. Biotechnol.*, 2007, **25**, 1483–1487.; (*c*) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A.*, 2007, **104**, 16793–16797.; (*d*) S. T. Laughlin, J. M. Baskin, S. L. Amacher and C. R. Bertozzi, *Science*, 2008, **320**, 664–667; (*e*) X. Ning, J. Guo, M. A. Wolfert and G.-J. Boons, *Angew. Chem. Int. Ed.*, 2008, **47**, 2253–2255; (*f*) B. C. Sanders, F. Friscourt, P. A. Ledin, N. E. Mbua, S. Arumugam, J. Guo, T. J. Boltje, V. V. Popik and G.-J. Boons, *J. Am. Chem. Soc.*, 2011, **133**, 949–957; (*g*) J. Z. Yao, C. Uttamapinant, A. Poloukhtine, J. M. Baskin, J. A. Codelli, E. M. Sletten, C. R. Bertozzi, V. V. Popik and A. Y. Ting, *J. Am. Chem. Soc.*, 2012, **134**, 3720–3728.

4 (*a*) K. Lang, L. Davis, J. Torres-Kolbus, C. Chou, A. Deiters and J. W. Chin, *Nat. Chem.*, 2012, **4**, 298–304; (*b*) E. Kaya, M. Vrabel, C. Deiml, S. Prill, V. S. Fluxa and T. Carell, *Angew. Chem. Int. Ed.*, 2012, **51**, 4466–4469; (*c*) L. I. Willems, N. Li, B. I. Florea, M. Ruben, G. A. van der Marel and H. S. Overkleeft, *Angew. Chem. Int. Ed.*, 2012, **51**, 4431–4434; (*d*) H.-S. Han, N. K. Devaraj, J. Lee, S. A. Hilderbrand, R. Weissleder and M. G. Bawendi, *J. Am. Chem. Soc.*, 2010, **132**, 7838–7839; (*e*) A. Niederwieser, A.-K. Späte, L. D. Nguyen, C. Jüngst, W. Reutter and V. Wittmann, *Angew. Chem. Int. Ed.*, 2013, **52**, 4265–4268; (*f*) R. Rossin, P. R. Verkerk, S. M. van den Bosch, R. C. M. Vulders, I. Verel, J. Lub and M. S. Robillard, *Angew. Chem. Int. Ed.*, 2010, **49**, 3375–3378; (*g*) W. Song, Y. Wang, J. Qu, M. M. Madden and Q. Lin, *Angew. Chem. Int. Ed.*, 2008, **47**, 2832–2835; (*h*) W. Song, Y. Wang, J. Qu and Q. Lin, *J. Am. Chem. Soc.*, 2008, **130**, 9654–9655; (*i*) J. Wang, W. Zhang, W. Song, Y. Wang, Z. Yu, J. Li, M. Wu, L. Wang, J. Zang and Q. Lin, *J. Am. Chem. Soc.*, 2010, **132**, 14812–14818; Representative review on Cu(I)-free reactions, see: (*j*) J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272–1279.

5 (*a*) I. Hamachi, T. Nagase, S. Shinkai, *J. Am. Chem. Soc*., 2000, **122**, 12065-12066; *(b)* T. Nagase, S. Shinkai, I. Hamachi, *Chem. Commun*., 2001, 229-230; *(c)* T. Nagase, E. Nakata, S. Shinkai, I. Hamachi, *Chem. Eur. J*., 2003, **9**, 3660-3669; *(d)* E. Nakata, Y. Koshi, E. Koga, Y. Katayama, I. Hamachi, *J. Am. Chem. Soc*., 2005, **127**, 13253-13261; *(e)* Y. Takaoka, H. Tsutsumi, N. Kasagi, E. Nakata, I. Hamachi, *J. Am. Chem. Soc*., 2006, **128**, 3273-3280; *(f)* A. Ojida, H. Tsutsumi, N. Kasagi, I. Hamachi, *Tetrahedron Lett*., 2005, **46**, 3301-3305; *(g)* Y. Koshi, E. Nakata, M. Miyagawa, S. Tsukiji, T. Ogawa, I. Hamachi, *J. Am. Chem. Soc*., 2008, **130**, 245-251.

6 *(a)* S. Tsukiji, M. Miyagawa, Y. Takaoka, T. Tamura, I. Hamachi, *Nat. Chem. Biol*., 2009, **5**, 341-343; *(b)* S. Tsukiji, H. Wang, M. Miyagawa, T. Tamura, Y. Takaoka, I. Hamachi, *J. Am. Chem. Soc*., 2009, **131**, 9046-9054.

7 (*a*) K. Tanaka, T. Masuyama, K. Hasegawa, T. Tahara, H. Mizuma, Y. Wada, Y. Watanabe and K. Fukase, *Angew. Chem. Int. Ed.*, 2008, **47**, 102–105; (*b*) K. Tanaka and K. Fukase, *Org. Biomol. Chem*, 2008, **6**, 815–828; (*c*) K. Tanaka, E. R. O. Siwu, K. Minami, K. Hasegawa, S. Nozaki, Y. Kanayama, K. Koyama, W. C. Chen, J. C. Paulson, Y. Watanabe and K. Fukase, *Angew. Chem. Int. Ed.*, 2010, **49**, 8195–8200; (*d*) K. Tanaka, K. Fukase and S. Katsumura, *Chem. Rec.*, 2010, **10**, 119–139; (*e*) K. Tanaka, K. Fukase and S. Katsumura, *Synlett*, 2011, **2011**, 2115–2139.

8 Discussions of live cell labeling and modification using small molecules *via* a 6π-azaelectrocyclization reaction are provided in: (*a*) K. Tanaka, K. Minami, T. Tahara, Y. Fujii, E. R. O. Siwu, S. Nozaki, H. Onoe, S. Yokoi, K. Koyama, Y. Watanabe and K. Fukase, *ChemMedChem*, 2010, **5**, 841–845; (*b*) K. Tanaka, K. Minami, T. Tahara, E. R. O. Siwu, K. Koyama, S. Nozaki, H. Onoe, Y. Watanabe and K. Fukase, *J. Carbohydr. Chem.*, 2010, **29**, 118–132; (*c*) K. Tanaka, S. Yokoi, K. Morimoto, T. Iwata, Y. Nakamoto, K. Nakayama, K. Koyama, T. Fujiwara and K. Fukase, *Bioorg. Med. Chem.*, 2012, **20**, 1865–1868; *(e)* K. Tanaka, K. Moriwaki, S. Yokoi, K. Koyama, E. Miyoshi and K. Fukase, *Bioorg. Med. Chem.*, 2013, **21**, 1074–1077.

9 (*a*) K. Tanaka, M. Kamatani, H. Mori, S. Fujii, K. Ikeda, M. Hisada, Y. Itagaki and S. Katsumura, *Tetrahedron*, 1999, **55**, 1657–1686; (*b*) K. Tanaka, H. Mori, M. Yamamoto and S. Katsumura, *J. Org. Chem.*, 2001, **66**, 3099–3110; (*c*) K. Tanaka and S. Katsumura, *J. Am. Chem. Soc.*, 2002, **124**, 9660–9661; (*d*) K. Tanaka, T. Kobayashi, H. Mori and S. Katsumura, *J. Org. Chem.*, 2004, **69**, 5906–5925.

10 K. Tanaka, Y. Fujii and K. Fukase, *ChemBioChem*, 2008, **9**, 2392–2397.

11 S. Mizukami, S. Watanabe, Y. Hori, K. Kikuchi, *J. Am. Chem. Soc*., 2009, **131**, 5016-5017.

12 K. Tanaka, M. Kitadani and K. Fukase, *Org. Biomol. Chem.*, 2011, **9**, 5346–5349.

13 K. Tanaka, Y. Nakamoto, E. R. O. Siwu, A. R. Pradipta, K. Morimoto, T. Fujiwara, S. Yoshida, T. Hosoya, Y. Tamura, G. Hirai, M. Sodeoka and K. Fukase, *Org. Biomol. Chem*., **2013**, *11,* 7326-7333.

14 X. Chen, R. Park, M. Tohme, A. H. Shahinian, J. R. Bading and P. S. Conti, *Bioconjug. Chem*., 2004, **15**, 41-49.

15 Due to the non-specific reaction on the cell surface, i.e., cRGDyK ligand-independent reaction, the microscopic analysis should be performed as soon as possible after the probe treatment. When the microscopic analysis was performed after 10 min, the significant fluorescence contrasts could not be detected between the cells treated by **7** and the control **6**.