ChemComm

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COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2013, **49**, 11551

Received 29th July 2013,
Accepted 15th October 2013

DOI: 10.1039/c3cc45780j

www.rsc.org/chemcomm

[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]coumarin as a carbene-generating photocross-linker with masked fluorogenic beacon†

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A coumarin-fused diazirine photolabeling agent exhibited dramatic enhancement in fluorescence after cross-linking with the target protein. Fluorescence emission from the coumarin moiety was efficiently quenched by the diazirine group, and was then intensively recovered from photolysis treatment by irradiation with light at a wavelength of 365 nm.

Specific tagging of target molecules has enabled the visualization of cell functions based on molecular recognition. In particular, the selective incorporation of small fluorophores has progressed to reducing structural perturbations[1] of the labeled protein by using enzymatic systems,[2] affinity-based chemical methodologies,[3] and biological machineries such as metabolism[4] and translation.[5] Recently, bio-orthogonal ligation, one of the most widely used chemoselective tagging methods, has been significantly developed for the highly selective fluorescent labeling of biomolecules that cannot be genetically encoded with fluorescent protein or tagged using multi-step labeling procedures.[6] In addition to high target selectivity, a high signal-to-noise ratio and easy handling are necessary for site-specific imaging. Reaction-dependent on/off switching strategies for emissions, which trace the time-space distribution of target molecules, have been improved by coupling with a quenching system that contains a selective chemical- or enzyme-reactive group that can function in cleavage, capturing active species, and coordinating with protons or metal ions.[7] The fluorescence switching property was initially investigated by Kanaoka in studies of protein function, using maleimide reagents for thiols.[8] Given that ethynyl[9] and azide[10] groups efficiently quench the fluorescence of compounds such as coumarins,[11] naphthalimides,[12] boron–dipyrromethene,[13] and fluorescein,[14] these derivatives were investigated as ligation-dependent fluorogenic agents for the Staudinger, Huisgen, and other reactions.[15]

The photoaffinity labeling method (PAL) is a cross-linking technique that joins molecules by photo-irradiation, and has been used in the life sciences field for the specific imaging[16] and profiling[17] of binding proteins, including those previously unidentified. PAL enables specific labeling at the molecular interaction site without any modification of the protein in advance, resulting in the elucidation or mapping of the ligand-binding surface, and allowing for potent ligand screening. Since aryl azides are known to be nitrene precursors, some azide-incorporated fluorophores (coumarin[18] and nitrobenzoxadiazole[19]) have been examined as potential photocross-linkers. 3-Phenyl-3-trifluoromethyl diazirine, another photophore, has been recognized as a useful photocross-linker in biological systems because of its stability under general synthetic conditions and its ability to generate an electron-deficient carbene capable of forming a stable covalent bond with various substrates through rapid photolysis by long-wave ultraviolet light (365 nm).[20] Although these properties are attractive in practical use, radioisotope (RI)-coded cross-linkers have often been used to identify targets because of difficulties in handling the labeled protein in infinitesimal quantities. Generally, PAL-based fluorophore labeling agents are made up of a photophore, a ligand, and a fluorophore *via* a branching structure. However, a bulky and unstable fluorophore sometimes hinders the specificity of the ligand as well as preparation of the probe. Here, we describe a new fluorogenic diazirine-based cross-linker as a small, non-RI, and highly sensitive tagging agent (Fig. 1A).

The 3-trifluoromethyldiazirinyl (DA) coumarin derivative **1a** is designed as a relatively small compound with a ring fused to the common phenyldiazirine cross-linker unit that forms a coumarin fluorophore. Compound **1a** was easily synthesized by a Knoevenagel condensation reaction from 2-hydroxy-6-methoxy-4-[(3-trifluoromethyl)-3*H*-diazirin-3-yl]benzaldehyde (see the ESI†). A carboxylate group has been introduced into the compound for attachment of the ligand, and a methoxy group has been included at position C-7 of coumarin to increase the compound's fluorescent property. Fig. 1B shows the fluorescence spectra of compound **1a** (1 μM in methanol) after irradiation with light at a wavelength of 365 nm, which can initiate the photolysis of the diazirine group, using a high-pressure mercury lamp (Asahi Spectra REX-250, Tokyo, Japan)

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† Electronic supplementary information (ESI) available: Materials and methods, and additional spectroscopic data. See DOI: 10.1039/c3cc45780j

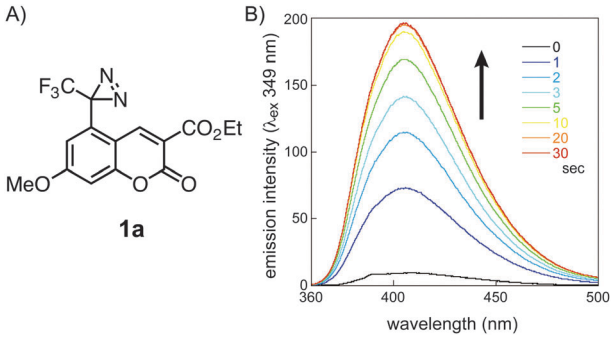

Fig. 1 (A) Structure of coumarin-fused diazirine cross-linker (**1a**) and (B) fluorescence spectra (λ_{ex} 349 nm) of 5-DA-7-OMe coumarin **1a** (1 μM in methanol) after irradiation with 365 nm light through a bandpass filter (full-width at half-maximum = 10 nm) using a 250 W high-pressure Hg lamp at 0 °C.

through a bandpass filter [full-width at half-maximum (fwhm) = 10 nm]. The spectra were measured at a scan rate of 500 nm min^{-1} with the excitation light at 349 nm. These data indicated that fluorescence emission from the coumarin moiety was initially quenched by the diazirine moiety, but was extensively recovered in response to its decomposition by irradiation with 365 nm light (fluorescence quantum yields: Φ_f = 0.03 for compound **1a** and Φ_f = 0.28 for the methanol adduct in hexane, respectively). The photoreaction was also monitored through NMR spectroscopy (20 mM in CD_3OD, Fig. 2). ^{1}H NMR signals from the aromatic protons of the photoproducts clearly distinguished the diazirine compound as a starting material, with signals observed at δ 9.12 (s), 7.35 (d), and 7.13 (d), and the methanol adduct, which showed signals at δ 9.06 (s), 7.15 (d), and 7.04 (d) (Fig. 2A). The results indicated that the photolysis of diazirine compound **1a** by irradiation with 365 nm light mainly produced the corresponding methanol adduct, whose structure was determined by NMR and HRMS (see the ESI†). Interestingly, the photolysis did not incorporate a pathway yielding a corresponding diazo intermediate. Diazo derivatives are usually produced in the photolysis of common phenyldiazirines,[20] and in the subsequent denitrogenation, to yield the corresponding carbene species; the reaction proceeds slowly under irradiation with 365 nm light, but proceeds rapidly under irradiation with 313 nm light. The spectra clearly show conversion among the three species: the peak at δ 7.36 (d) from the diazirine derivative, which decreased with irradiation with 365 nm light; the peak at δ 7.23 (d) from the diazo derivative, which increased with irradiation at 365 nm and then disappeared with irradiation at 313 nm; and the peak at δ 7.57 (d) from the methanol adduct, which increased constantly. Hence, in panel A (Fig. 2), the ^{1}H NMR spectra of compound **1a** do not show the signal from the corresponding diazo compound during photolysis. ^{19}F NMR signals from the CF_3 group[21] confirmed this result (see Fig. S1 in the ESI†). These data indicate efficient production of the carbene species from compound **1a**, and implicate it in forming a covalent bond with the target molecule without going through the rather slow photolysis of the diazo intermediate.

Another derivative, the 7-DA-5-OMe coumarin derivative (**1b**), showed different phenomena. It emitted longer fluorescence at 469 nm with a large Stokes shift (λ_{ex} 329 nm), and its intensity

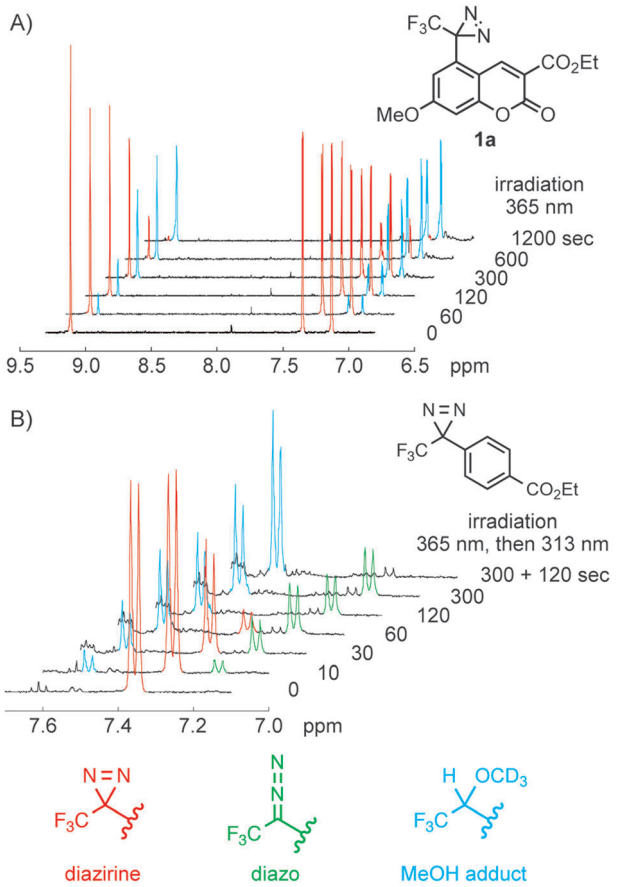

Fig. 2 ^{1}H NMR spectra (aromatic protons) of compound **1a** (A) and ethyl 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoate (B), respectively. The irradiation was performed in a CD_3OD solution of (20 mM) with 365 nm light through a bandpass filter at 0 °C over various periods of time. Panels A and B show the aromatic signals of diazirine compounds (red signals), the corresponding diazo compounds (green), and methanol adducts (blue).

was increased by irradiation. The photolysis of the diazirine group of compound **1b** was as rapid as that of compound **1a**. However, its fluorescence was weak and was not completely quenched by the diazirine group, which had an initial intensity of about 62% of the photoproduct in methanol (see Fig. S2 in the ESI†). On the other hand, the 5-DA-7-OH coumarin derivative (**2**, 1 μM in methanol), a hydroxyl derivative, exhibited a fluorogenic phenomenon similar to that of compound **1a** in response to photolysis. Hence, the rate constant of photolysis was calculated as less than one twenty of the value of compound **1a** (see Fig. S3 in the ESI†). On the basis of these data, compound **1a** was employed as a cross-linker unit for the further fluorogenic labeling of proteins (Fig. 3).

Finally, compound **1a** was installed at position C-17 of geldanamycin (GA), a potent inhibitor of heat shock protein 90 (Hsp90), to yield a photo-activatable GA probe **3a** according to the synthetic method in a previous report.[22] Hsp90 is a molecular chaperone responsible for managing the conformational maturation of multiple client proteins upon ATP binding and hydrolysis,[23] GA inhibits ATPase activity through competition for ATP binding (dissociation constant (K_d) = 1.2 μM).[24] A PAL of Hsp90 (5 μM) with probe **3a** (10 μM) was performed by

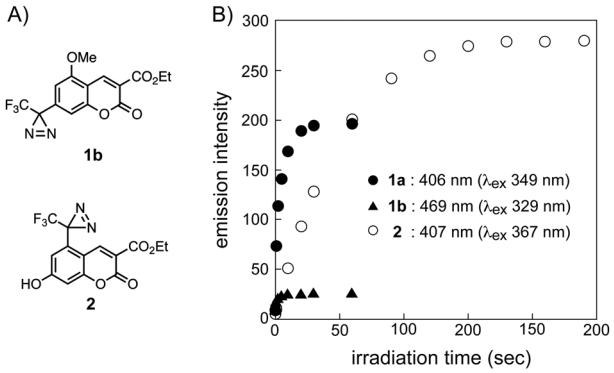

Fig. 3 (A) Structure of 7-DA-5-OMe coumarin (**1b**) and 5-DA-7-OH coumarin derivatives (**2**), and (B) emission recovery of coumarin derivatives (1 μM in methanol for compound **1a** (filled circles), **1b** (filled triangles), and **2** (open circles), respectively) with 365 nm light through a bandpass filter using a 250 W high-pressure Hg lamp at 0 °C.

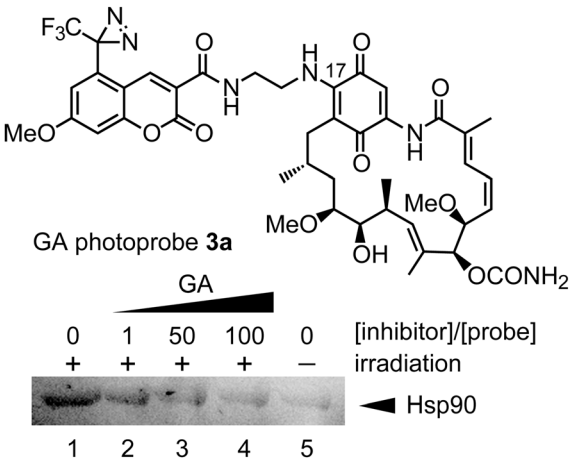

Fig. 4 Structure of geldanamycin (GA) photoprobe **3a** and the competitive inhibition assays for the photolabeling of Hsp90 (2 μM) with probe **3a** (10 μM) in the absence (lane 1) or presence of GA (lanes 2–5) at various concentrations. The bands were detected by a fluorescence method (λ_{ex} = 320 nm, λ_{em} = 420 nm, through bandpath filters (fwhm = 10 nm)).

irradiation with 365 nm light in a 20 mM Tris-HCl (pH 7.5) solution containing 50 mM KCl and 5 mM $MgCl_2$. Fig. 4 shows the structure of the GA photoprobe **3a** and part of the 10% SDS-PAGE of the photoproducts. Labeled Hsp90 was detected by measuring fluorescence, which increased in an irradiation-time- or probe-concentration-dependent manner (see Fig. S4 in the ESI†), at 420 nm (fwhm = 10 nm). Since the labeled Hsp90 was clearly inhibited in the presence of GA as a competitor (lanes 2–4), the probe can be considered to selectively bind to the target Hsp90. The HPLC profile of photoproducts showed the same inhibition manner as PAL (see Fig. S5 in the ESI†).

In conclusion, diazirinylcoumarin derivatives were synthesized as a new class of small fluorogenic cross-linkers. A photoactivatable GA probe was specifically labeled, and was able to visualize Hsp90 as a binding protein by brief irradiation. The photocross-linker provided cross-link-dependent fluorogenic labeling at the binding interface of the target protein. This should be an important feature for fluorescent imaging of a target protein without the necessity of pre-installing a large, unstable fluorophore in the probe. We recently reported a fluorogenic cross-linking method using a diazirinylcinnamate derivative capable of creating a coumarin molecule at the interacting protein by a two-step photochemical reaction, which was useful for both protein identification[25] and imaging.[26] Therefore, this technique of installing a fluorophore at the interacting interface should also be useful in identifying a ligand-binding domain within the target protein by simplifying the purification processes for analysis.

This research was supported by Grants-in-Aid for Scientific Research (20390032, 21310138) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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