Synthesis and photochemical properties of a light-activated fluorophore to label His-tagged proteins†‡

Clélia Orange,[ab] Alexandre Specht,*[a] David Puliti,[a] Elias Sakr,[a] Toshiaki Furuta,[c] Barbara Winsor[b] and Maurice Goeldner[a]

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Rapid and efficient light-induced fluorescence enhancement is demonstrated on a DMNPB-"caged" coumarin derivative carrying a His-tag recognition motif.

Studies of protein localization and movement in living cells are most commonly achieved by expressing the protein as a fusion to a fluorescent protein (FP).[1] This genetic targeting allows high sensitivity of detection with no background except intracellular autofluorescence. However, FP's are full size proteins of at least 220 aa, which may severely perturb folding, trafficking and functioning of the proteins studied.[2] This limitation can be overcome by using small genetically encoded peptide tags (20 aa) and complementary small organic fluorescent probes[3] with sufficient specificity and affinity to be used in living cells. Since the pioneering work reported by Tsien and co-workers, describing a tetracysteine motif (-Cys-Cys-X-X-Cys-Cys-)/bisarsenical ligand (FlAsH) pair and its application to bio-imaging experiments in living cells;[4] many peptide tag/probe pairs have been developed. For example, the oligo-aspartate sequence (D4 tag) and the corresponding multinuclear Zn(II) complexes (Zn(II)-DpaTyr) have been used for the labelling and fluorescent imaging of a membrane-bound receptor protein.[5] The conventional His tag -$(His)_n$-/Ni(II)-NTA pair was used for fluorescent labelling of a protein on a cell surface.[6] Light-activated fluorescent molecules (also called "caged" fluorophores), including photoactivatable GFP's,[7] are important research tools for tracking the spatiotemporal dynamics of molecular movements in biological systems.[8a] The general strategy in masking fluorescence is to perturb the electronic structure by attachment of the photoremovable group to make the molecule either non-fluorescent or very weakly so. Photoactivation removes the protecting group and abruptly switches on the fluorescence of the parental dye. Desirable properties for "caged" fluorophores include fast release of the fluorophore and a large enhancement of the fluorescence in response to brief irradiation with reasonable photostability of the released dye to resist photobleaching. "Caged" fluorophores initially described on fluorescein[8a] and rhodamine[8b] derivatives have been reviewed[8a] and prompted innovative cell biology work.[8c] Subsequently, 7-hydroxycoumarin derivatives were caged through Williamson coupling reactions as *o*-nitrobenzyl ether derivatives.[9] These molecules allowed a 200-fold fluorescence enhancement after UV photolysis and displayed an increased uncaging cross section over previously reported "caged" fluorophores.[9] Recently, novel *ortho*-nitrobenzyl fluorescein derivatives ("caged" Tokyo-Greens) displaying a large fluorescence enhancement were described.[10]

We report here a new class of "caged" 7-hydroxycoumarin fluorophores using a DMNPB ether as a photoremovable protecting group which was initially described for the caging of carboxylic acids.[11] This probe displayed high uncaging cross section at 365 nm and showed a rapid enhancement of fluorescence after irradiation. A Mitsunobu coupling reaction was used for an efficient incorporation of the caging group as an ether derivative and convenient chemical modifications allowed the incorporation of the Ni-nitrilotriacetic (Ni^{2+}-NTA) His-tag recognition motifs. Since the penetration assays of this molecule into HeLa cells remained inconclusive, we designed and synthesized a new cell-permeable and non-toxic coumarin derivative (7-acetylcoumarin-Ni-NDA, **8a**-Ni^{2+}) able to penetrate into HeLa cells and regenerate the coumarin-Ni-NTA.

The synthesis of 7-DMNPB-coumarin-NTA (**9**), coumarin-NTA (**10**), 7-acetylcoumarin-NTA (**8a**) and 7-acetylcoumarin-NDA (**8b**) are outlined in Fig. 1. Starting from 2,4-dihydroxy-5-chlorobenzaldehyde (**1**)[12] we prepared 6-chloro-7-hydroxycoumarin 3-carboxylate (**2**) in one step. Reaction using standard DCC/NHS coupling conditions between **2** and (*tert*-butoxy)-*N*,*N*-bis(*tert*-butoxycarbonylmethyl)-L-lysine (**4a**)[13] or methyl-*N*,*N*-bis(*tert*-butoxycarbonylmethyl)-L-lysine (**4b**), obtained in two steps from appropriately protected L-lysine derivatives (**3a,b**), gave the *tert*-butyl ester-protected NTA and NDA coumarins (**5a,b**). Coupling under Mitsunobu condition[14] of the *tert*-butyl ester-protected NTA-coumarin (**5a**) and the threo diastereoisomers of 3-(3,4-dimethoxyphenyl)butan-2-ol (DMNPB) (**11**),[11] a photoremoval protecting group,

[a] *Universtté Louis Pasteur/CNRS UMR 7175-Laboratoire de chimie bioorganique, Faculté de pharmacie, 74 route du rhin, 67400 Illkirch, France. E-mail: specht@bioorga.u-strasbg.fr; Fax: +33 390244306; Tel: +33 390244166*
[b] *Universtté Louis Pasteur/CNRS UMR7156 Génétique moléculaire, Génomique et Microbiologie, Dépt. Génétique moléculaire et cellulaire, IPCB, 21 rue René Descartes, 67084 Strasbourg, France*
[c] *Toho University, 2-2-1 Miyama, Funabashi, Chiba, 274-8510, Japan*

† Abbreviations: DMNPB, [3-(4,5-dimethoxy-2-nitrophenyl)but-2-yl]; DpaTyrs, (2,2′-dipicolylamine) complex based on the L-tyrosine scaffold; FCS, fluorescent correlation spectroscopy; FlAsH, fluorescein arsenical helix binder; NDA, nitrilodiacetic acid; NPE, 2-(nitrophenyl)-ethyl; NTA, nitrilotriacetic acid.

‡ Electronic supplementary information (ESI) available: chemical synthesis and characterization, one and two photon photolysis, quantum yield determination, laser flash photolysis, Cell membrane permeability studies and FCS studies. See DOI: 10.1039/b716486f

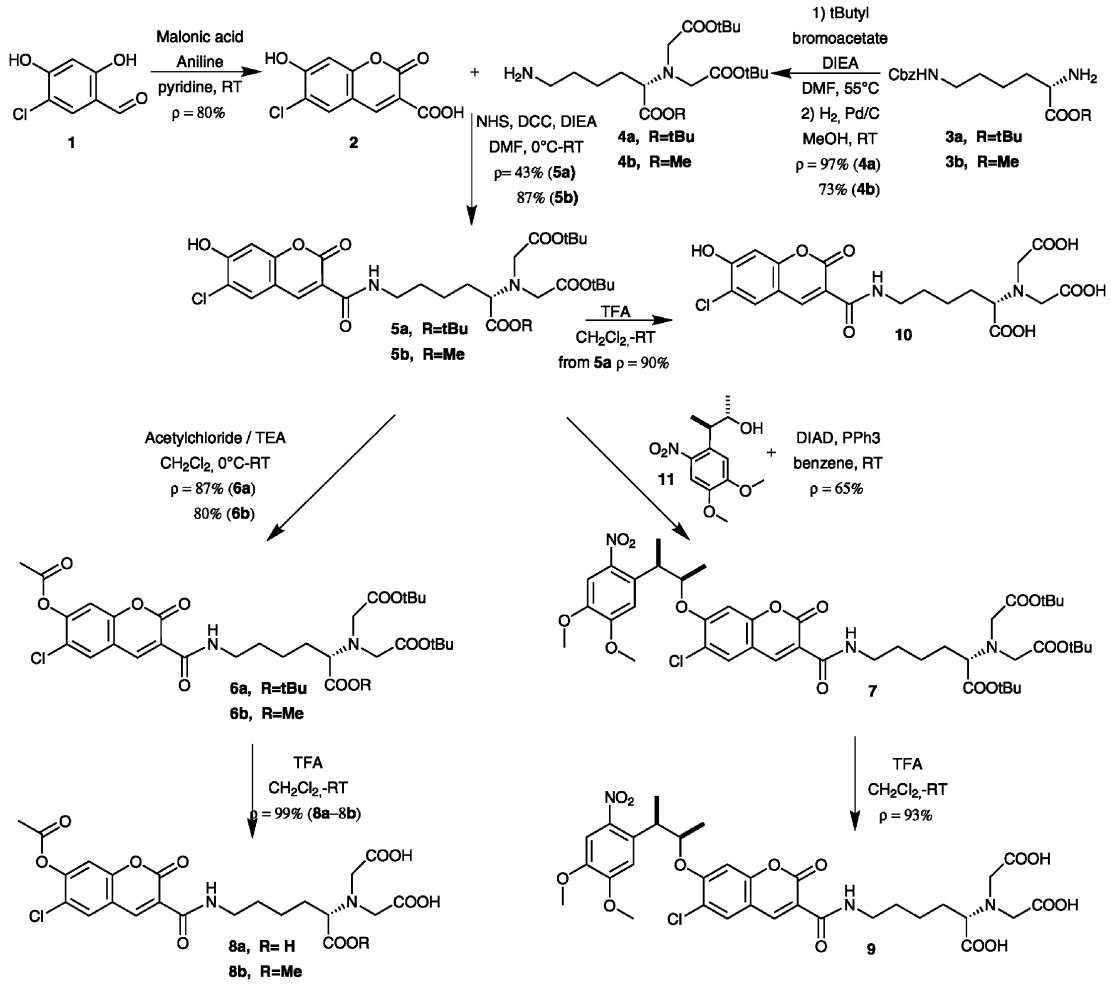

Fig. 1 Synthesis of 7-DMNPB-coumarin-NTA, 7-acetylcoumarin-NTA and 7-acetylcoumarin-NDA.

plus a final deprotection of the *tert*-butyl protecting groups, gave our new "caged" fluorophore: 7-DMNPB-coumarin-NTA (**9**) with good yield. It is noteworthy that our coupling reaction avoids synthetic problems due to the use of strong basic conditions which do not accommodate the halogenated *o*-nitro benzyl derivatives under Williamson reaction.[15] To test cell permeability to alkylated coumarin-NTA-Ni^{2+} probes, 7-acetylcoumarin-NTA (**8a**) and 7-acetylcoumarin-NDA (**8b**) were synthesized by acylation followed by *tert*-butyl deprotection of (**5a**) and (**5b**), respectively. The coumarin NTA dye (**10**) was obtained after removal of the *tert*-butyl protecting groups of **5a**.

This dye absorbs maximally at 408 nm with an extinction coefficient of 25 000, and a fluorescent emission at 450 nm with a quantum yield of 0.95 measured in an aqueous buffer (0.1 M phosphate buffer pH 7.4).

The photochemical properties of **9** and **10** are summarized in Table 1. The photolytic release of **10** from precursor **9** was analyzed by UV and HPLC (see ESI,† Fig. S1) after irradiation at 315, 365 or 403 nm in neutral buffered media. The UV analysis showed a decrease in absorbance at 355 nm and an increase at 406 nm, corresponding to the disappearance of **9** and the formation of **10**. The presence of an isosbestic point at 375 nm was indicative of a homogeneous photolytic reaction. An almost quantitative ($\geq 95\%$) formation of **10** after full conversion of **9** was demonstrated by HPLC. The quantum yield for coumarin-NTA (**10**) release from **9** was determined by competition with the 2-(nitrophenyl)ethyl-ATP[16] reference molecule. A disappearance quantum yield of 0.49 could be determined from this experiment by HPLC analysis. Our new "caged"-coumarin derivative displayed high quantum yields for uncaging, which conferred a high photolytic efficiency to this molecule in the near-UV, generating robust change in fluorescence quantum yield and conferring a high contrast optical signal with minimum background after irradiation.

Table 1 Summary of the photochemical properties of **9** and **10** in 0.1 M phosphate buffer pH 7.4 (ϕ_f and ϕ_u fluorescence and uncaging quantum yield, δ_u two-photon uncaging action cross section, k first-order uncaging rate constant, GM, Goeppert–Mayer)

Compound	**9**	**10**
λ_{max} (excitation)/nm	355	408
$\varepsilon(\lambda_{max})/M^{-1}\ cm^{-1}$	15 000	25 000
λ_{max} (emission)/nm	450	450
ϕ_f	<0.009	0.95
ϕ_u	0.49	—
$\delta_{u\ (740\ nm)}$/GM	0.21	—
k/s^{-1}	1.7×10^5	—

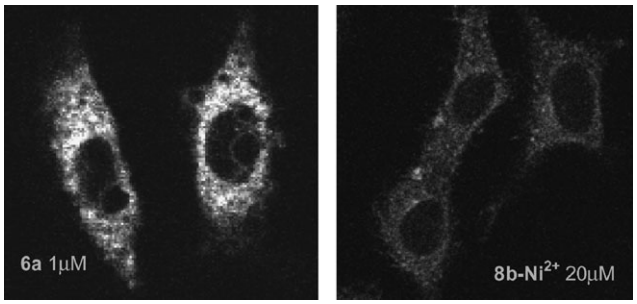

Fig. 2 Fluorescence imaging of HeLa cells loaded with cell permeable coumarins **6a** and **8b**-Ni^{2+} ($\lambda_{emission}$: 410–510 nm).

We checked the two-photon sensitivity of **9** by irradiating the sample with a femtosecond-pulsed mode-locked Ti-sapphire laser using a previously described method,[17] and a cross section of 0.21 GM was measured at 740 nm. Surprisingly, this value remained lower than the value described for NPE "caged" coumarin[9] despite a perfect overlap of the absorbances of the coumarin and caging group. Fragmentation kinetics were determined after laser photolysis at 350 nm by analysis of coumarin **10** formation at 410 nm. A monoexponential absorbance increase at 410 nm in the microsecond time range ($t_{1/2}$ = 4 μs) was observed, showing very fast release of **10** and corresponding presumably to the rate-limiting step of *o*-quinonoid *aci*-nitro decay.[18]

To test the cell-membrane permeability of these derivatives for further imaging applications in living cells, we selected the 7-acylated-coumarins as model compounds given their rapid cellular hydrolysis into the corresponding fluorescent 7-hydroxycoumarin derivatives. Effectively, after 30 min incubation of 1 μM of the tris-*tert*-butoxyesters **6a** on a HeLa cell culture, a strong coumarin fluorescent signal was observed inside the cytoplasm of cells with 405 nm excitation, confirming that **6a** entered into the cell and that intracellular hydrolysis released the fluorescent dye **5a** (Fig. 2). A similar experiment using the **8a**-Ni^{2+} complex, did not reveal any cell permeation since no cytoplasmic fluorescent signal was observed. Because this NTA-Ni^{2+} complex carries a negative charge, we synthesized the NDA-series, a neutral precursor complex of the NTA-Ni^{2+} motif by masking one carboxylic acid function of NTA as a methyl ester group. Following hydrolysis of the methyl ester by cellular esterases, the His-tag recognition NTA-Ni^{2+} motif would be regenerated *in situ*. Indeed, after 30 min incubation with 20 μM of the **8b**-Ni^{2+} complexes on a HeLa cell culture, a coumarin fluorescent signal was observed with 405 nm excitation (Fig. 2). This fluorescent signal, although weaker than that obtained with **6a**, was stable over time. Fluorescence measured after a long UV excitation time (1 h), demonstrated the non-toxicity and the fluorescence stability of our probe (data not shown). Finally a fluorescence correlation spectroscopy (FCS) experiment was performed to determine whether the Ni^{2+}-NTA-coumarin (**10**) was able to bind to a His-tagged protein, Bzz1p. For the Ni^{2+}-NTA-coumarin alone, the measured time of diffusion was 0.08 ms and when a His-tagged protein was added, this time increased to 0.156 ms. This indicates that the Ni^{2+}-NTA-coumarin bound to the His-tagged protein and changed the physical properties of diffusion of the fluorescent probe.

In conclusion, we have described here the synthesis and the characterization of a new "caged"-coumarin bearing NTA, the His tag recognition motif. Our new "caged" fluorophore showed a rapid and large fluorescence enhancement after near-UV activation (315–406 nm). For biological application we also developed a strategy to obtain cell membrane permeable Ni^{2+}-NTA dyes.

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