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β-Cyclodextrin as a mimetic of the natural GFP-chromophore environment

Martina Cacciarini [a,*], Mogens Brøndsted Nielsen [b], Elisa Martinez de Castro [b], Lavinia Marinescu [b], Mikael Bols [b]

[a] Department of Chemistry, University of Florence, Via della Lastruccia 3-13, I-50019 Sesto F.no (FI), Italy
[b] Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark

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ABSTRACT

The green fluorescent protein (GFP) chromophore has been anchored to β-cyclodextrin (βCD) via a copper(I)-catalyzed azide-alkyne cycloaddition. The photophysical properties of this new GFP-CD derivative have been evaluated, showing the formation of a self-inclusion complex and enhancement of fluorescence of the GFP-chromophore covalently bound to the βCD. This enhancement of fluorescence by encapsulation and hence fixation of the chromophore in a binding pocket mimics the effect exerted by the natural protein environment.

In the last decade, green fluorescent protein (GFP) has changed from an almost unknown protein to a widely used tool as biological marker in molecular biology and medicine, because of the special fluorescent properties of its chromophore.[1–5] The chromophore of the wild type GFP (Fig. 1) shows peculiar spectroscopic properties: two absorption peaks at 479 nm and at 397 nm, usually ascribed, respectively, to a deprotonated chromophore (phenolate) and to a protonated chromophore (neutral phenol), and one emission peak at 508 nm. It is well documented that the photophysical properties of GFP are the results of interactions between the chromophore itself and its immediate protein environment, in fact the denatured GFP and the isolated chromophore loose totally their emissive properties.[6–8]

Previous gas-phase absorption studies of the isolated GFP phenolate chromophore revealed a similar absorption maximum (479 nm) to that of the chromophore inside the protein,[9] while the absorption is red-shifted relative to the chromophore in alcoholic solution. This observation can be explained in two ways; either the protein binding pocket does not exert any absorption tuning or it exerts as many red-shifting interactions as blue-shifting ones. To take the study a step further and evaluate the minimal environment required for the GFP-chromophore to show fluorescence and to display the photophysical properties shown in natural environment, we developed a system where the GFP-chromophore is anchored to a β-cyclodextrin (βCD, Fig. 1). This macrocycle has a hydrophobic cavity that should allow inclusion of the GFP-chromophore. The hydrophobic nature of this cavity presents an environment that is not likely to interfere with the absorption properties of the chromophore, hence resembling the natural environment, but may influence the fluorescence properties by shielding the chromophore from solvent molecules.

Macrocyclic hosts of different nature, shape, and characteristics from crown ethers, to cryptands, from cyclophanes, to calixarenes and cucurbiturils have received great attention as new supramolecular systems and materials.[10–14] Among them, cyclodextrins are the most important and promising macrocyclic hosts because of their many advantages: water solubility, low cost, commercial availability, and easy functionalization.[15,16] Besides, chromophore-modified CDs have been studied for a long time by many researchers and several sensors bearing different type of chromophores have been reported,[17–19] such as azobenzene and stilbene photoswitches.[20,21]

Herein we present the synthesis and characterization of a GFP-chromophore derivative that is covalently attached to β-cyclodextrin through a triazole linker, easily affordable using a 'click reaction'.[22] We show that there is an enhancement of fluorescence when the GFP-chromophore is covalently bound to the cyclodextrin enforcing encapsulation in its cavity.

The GFP-chromophore appended β-CD has been obtained through a procedure that identifies a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC)[23–25] as the key step. The synthetic pathway to obtain the GFP-chromophore bearing an alkyne moiety is depicted in Scheme 1. The synthesis starts from 4-[(*Z*)-4-acetoxybenzylidene]-2-methyloxazol-5(4*H*)-one **1**, which was prepared from *N*-acetylglycine and 4-hydroxybenzaldehyde.[25]

* Corresponding author. Tel.: +39 055 4573544; fax: +39 055 4573531.
E-mail addresses: martina.cacciarini@gmail.com, martina.cacciarini@unifi.it (M. Cacciarini).

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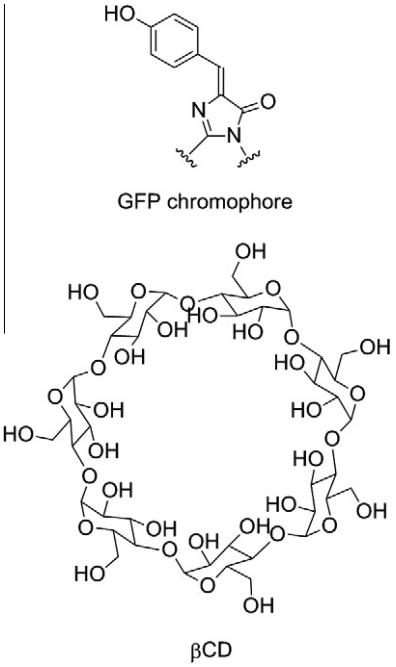


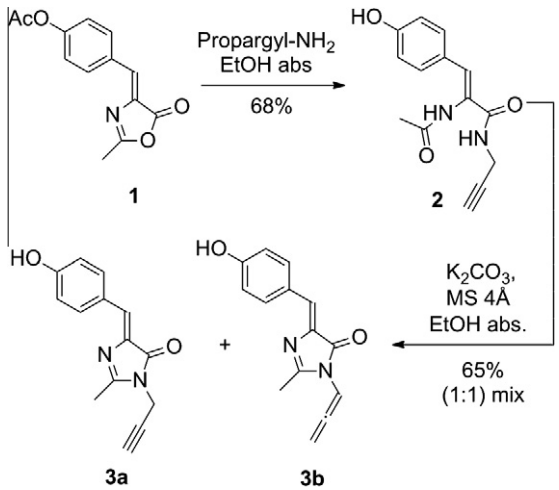
Figure 1. GFP-chromophore and βCD structures.

Compound **1** was treated with an excess of propargylamine (5 equiv) to give the ring-opened propargyl amide **2** in a 68% yield (Scheme 1). As expected,[26] under the described conditions deacetylation occurred to afford directly **2** with the unprotected phenol group. On the contrary use of minor amounts of propargylamine led to a mixture of **2** and its direct precursor, acetylated on the phenolic moiety. Formation of the imidazolone ring on **3** was accomplished by treatment of **2** with potassium carbonate in refluxing ethanol in the presence of molecular sieves (4 Å). Unfortunately, heating under basic conditions provoked a well-known[27] base-catalyzed rearrangement of the acetylene through a prototropic propargylic rearrangement and led to a hardly separable mixture of alkyne **3a** and allene **3b**. The allene structure was assigned by means of the proton and the carbon NMR spectra, which reflect typical signals in terms of resonances and multiplicity (see Supplementary data). In fact, a doublet at 5.51 ppm and a triplet at 6.73 ppm corresponding to the terminal CH_2 and the CH–N of the allene moiety, respectively, were detected, and a resonance at 205.8 ppm corresponding to the central *sp* carbon supported and confirmed the structure of **3b**. Numerous attempts to change the reaction conditions to avoid the formation of the allene **3b** were not successful.

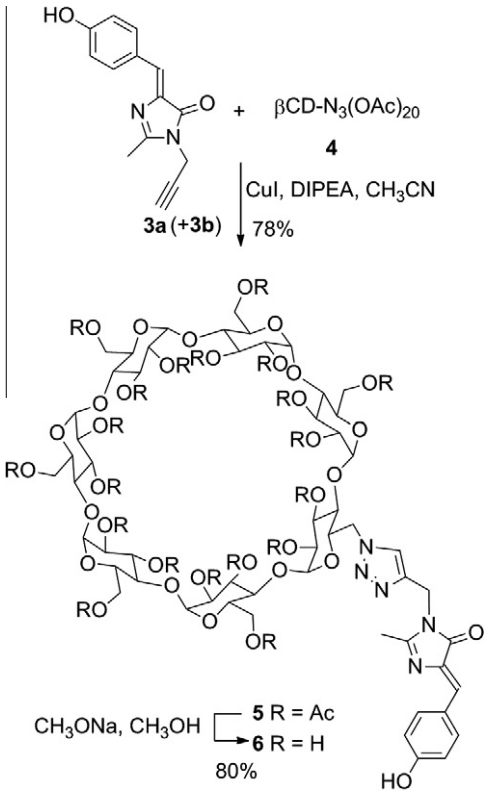
The per-*O*-acetylated-6-azido-6-deoxy-β-cyclodextrin **4** (Scheme 2) was prepared following a three-step procedure previously reported[28] starting from commercial β-cyclodextrin. The copper-catalyzed cycloaddition was performed between the azide **4** and the mixture **3a–b**[29] (enriched in **3a**, 2:1 ratio) in the presence of copper iodide and diisopropylamine in acetonitrile at room temperature for 65 h to give **5** (78% yield),[30] which was in turn deprotected under basic conditions to furnish the final GFP-CD **6**.[31]

Furthermore the synthesis of compound **7** (Scheme 3),[32] bearing the GFP-chromophore linked to a triazole ring but missing the CD, has been accomplished for direct comparison of the fluorescence properties of **6**.

The absorption and emission spectra of **6** (0.03 mM) were recorded in different buffer solutions. The absorption spectra (Fig. 2) at pH 4 and 7 are characterized by a maximum absorption at 371 and 372 nm, respectively, and the one at pH 10 by a λ_{max} at 432 nm. The redshift is determined by the deprotonation of the phenol group on the chromophore of **6** and this is confirmed by the absorption spectrum of **7** (Fig. 3) that presents an analogous redshift at pH 10. Since **7** is soluble in water only at pH 10, absorption spectra were measured in the three selected buffered solutions with the addition of commercial βCD. Indeed, βCD can include in its cavity the aromatic moiety of **7** forming a complex, hence bringing partially **7** in solution. This property has been evidenced at first by proton NMR spectra (see Supplementary data). In fact, addition of solid **7** to a 5 mM solution of βCD allowed us to register a spectrum that contains the signals of the model compound **7**, moreover a shift of the inner protons (H-3 and H-5) of βCD indicates the presence of a guest in the hydrophobic cavity



Scheme 1. Synthesis of the GFP-chromophore alkyne **3a**.



Scheme 2. Synthesis of GFP-CD **6**.