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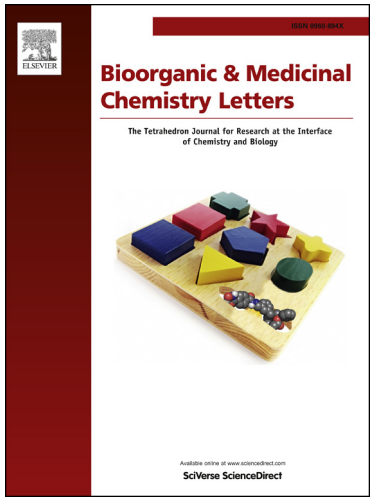

Tyrosine specific sequential labeling of proteins

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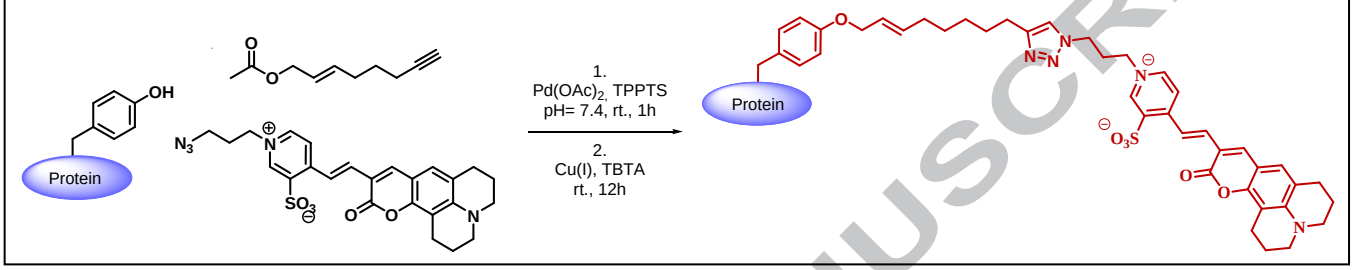

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Tyrosine specific sequential labeling of proteins

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ABSTRACT

We report (a) on the synthesis of a long-wavelength fluorescent coumarin containing an allyloxy acetate moiety, (b) the synthesis of two linkers containing an allyloxy acetate and an alkyne or azide function, respectively, and (c) the selective modification human serum albumin by a sequential method involving Pd(II) catalyzed modification of the phenolic side chain of tyrosine residues with an alkyne bearing linker and a subsequent azide-alkyne click reaction with an azide functionalized long-wavelength emitting coumarin dye. The method is likely to be applicable to various kinds of azido-modified fluorophores, and the Pd(II)-catalyzed modification of the tyrosines may also be used to introduce other kinds of tags. With these reagents, tyrosine specific modulation of proteins and peptides becomes possible either directly or in a sequential manner.

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In vitro and *in vivo* fluorescence imaging of biological species is superior due to its sensitivity, excellent temporal and spatial resolution, and its potential application to multicolor imaging. Fluorescent tags with longwave excitation and emission maxima are particularly useful for biolabeling because their excitation does not generate the strong autofluorescence of biomatter that is observed under shortwave (UV or blue) excitation.[1-4] Covalent labeling is expected to proceed rapidly, to give quantitative yields in aqueous solutions at near-neutral pH values, while not to affect any reactive sites of the biomolecule at the same time. Chemical reactions that meet all these criteria are collected under the term "bioorthogonal chemistry". Orthogonal modification of biomolecules is of great interest in chemical research, lately. Reactions that exclusively take place between two functional groups while not interfering with others are especially desirable in the labeling schemes of biomolecules. Up to now, a set of bioorthogonal schemes were delineated and applied e.g. for the fluorescent tagging of proteins, glycans and nucleotides.[5-9] Metal-free bioorthogonal transformations are preferred in case of *in vivo* labeling in order to avoid the use of toxic metal ion catalysts (for example by making use of the strain-promoted cycloaddition of azides and alkynes[8]), while in case of *in vitro* labeling, metal ion catalyzed methods are also adequate. Such metal catalyzed reactions are probably best represented by the robust Cu(I)-catalyzed azide-alkyne cycloaddition, which is probably the most widely used "click" reaction.[10,11]

Most proteins are randomly labeled, usually at the ε-amino group of lysine, the thiol group of cysteine, or after manipulation of N- and C-termini.[7,12] Selective modification of tyrosine (Tyr) residues is particularly attractive because Tyr is a fairly rare amino acid. Moreover, surface-accessible tyrosines are rather scarce in natural proteins so selective tagging of Tyr can lead to site-specific labeling. Tilley et al.[13] and Ma's group[14] employed Pd(II)-catalyzed reaction to label the phenolic side chain of Tyr with allyloxy acetates. The group of Barbas[15] have reported on the use of a stable formylbenzene diazonium salt to modify proteins through the phenolic side-chain of tyrosines.

Besides direct modification of Tyr side chains with e.g. a fluorescent label, the use of allyloxy-acetates offers the possibility to design bifunctional chemical reporters that combine Tyr selective and "clickable" moieties as well. Selective manipulation with such chemical reporters with subsequent further modification give rise to sequential approaches, a method that holds many benefits and numerous applications.[6,16-19] For example, well-accessible Tyr residues may be modified with such

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chemical linkers in a first step. This reporter tag is then further reacted in a subsequent step with a suitable label, for example certain cell-permeable fluorophores bearing complementary functions.[20-22]

We report here on the design and synthesis of a longwave emitting fluorescent allyloxy acetate functionalized coumarin displaing a large Stokes shift (**1**). We are also presenting two generally applicable bioorthogonalized chemical reporters (**2** and **3**) that combine the versatility of Tyr-specific labeling via an allyloxy-acetates moiety and the excellent performance of azide-alkyne click reaction-based fluorescent tagging (Figure 1).

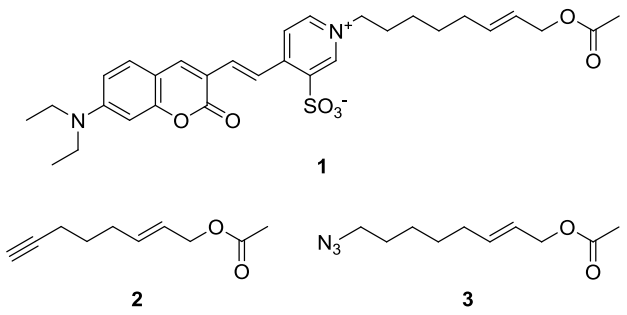
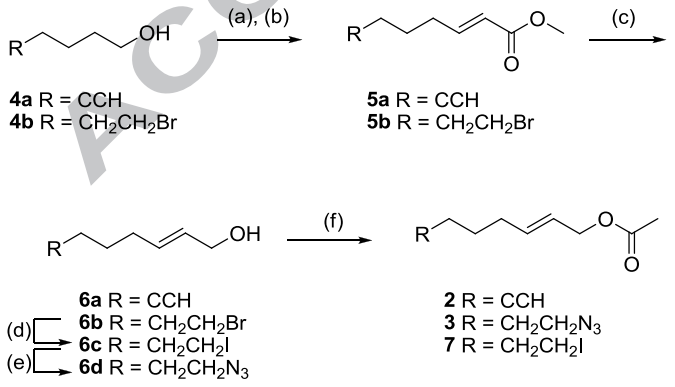


Figure 1. Structures of the Tyr-specific fluorescent coumarin (**1**) and the two bifunctional chemical linkers (**2**, **3**) used for sequential labeling.

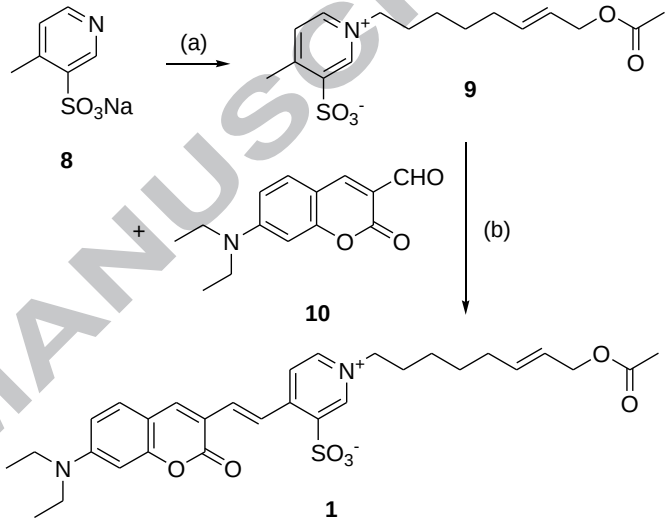
The synthesis of **2** and **3** is outlined in Scheme 1. The procedure was adapted from Tilley's work[13] and started with the transformation of the primary alcohols **4a** and **4b** to aldehydes **5a**, **5b** *via* Swern oxidation, followed by a Wittig reaction with freshly prepared phosphorane to from the desired acrylate derivatives. These intermediates were then reduced to allyl alcohols with excess amount of DIBAL-H. The bromine substituent of **6b** was exchanged for iodine and subsequently for azide. In the last step, acylation of the hydroxy group by acetic anhydride gave the desired products in moderate to good yields.

Fluorescent label **1** was prepared as shown in Scheme 2. N-alkylation of 4-methylpyridine-3-sulfonate (whose sulfo group improves water solubility) with **7** gave the zwitterionic intermediate **9**, which was then condensed with 3-formylcoumarin (**10**). Both the alkylation and the condensation step usually require higher temperature,[23] however, due to the sensitive nature of the allyloxy acetate moiety, the temperature was kept below 60 °C in this case.



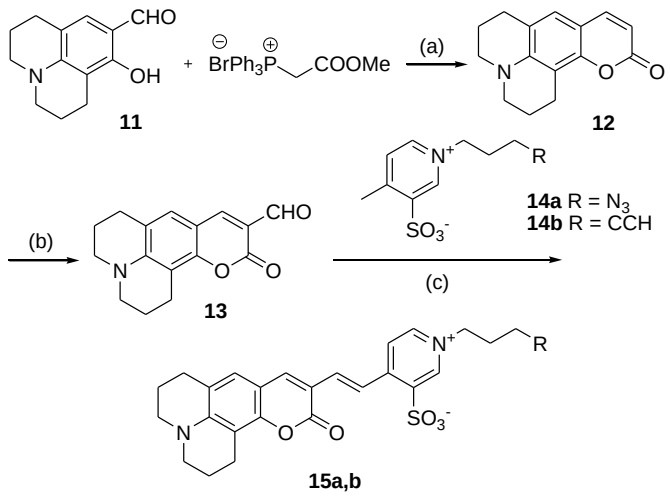
Scheme 1. Synthesis of chemical linkers **2** and **3**. Reagents and conditions: (a) oxalyl chloride, DMSO, DCM, -78 °C; (b) appropriate phosphorane, DCM, rt., Ar-atm.; (c) DIBAL-H, DCM, -78 °C; (d) NaI, acetone, Δ; (e) NaN_3, acetone, Δ; (f) Ac_2O, cat. DMAP, pyridine:DCM 1:1; rt. (yields: **5a**: 92%, **5b**: 78%, **6a**: 86%, **6b**: 98%, **6c**: 76%, **6d**: 40%, **2**: 80%, **3**: 60%, **7**: 46%).

To evaluate the possibility of the sequential tyrosine-labeling concept, we have designed and synthesized new, "clickable" fluorescent dyes as well (**15a,b**; Scheme 3). Synthesis of these "clickable dyes started from salicylaldehyde **11**.[24] Wittig reaction with phosphonium salt resulted in coumarin **12**, which was subjected to Vilsmeyer-Haack formylation to furnish **13**. Condensation reaction with betains **14a,b**[3] resulted in the clickable labels **15a**,**b**. These fluorophores have a purple color and display a Stokes shift (i.e., the difference between emission and excitation maxima) of ~150 nm, with excitation and emission maxima of 564 and 714 nm, respectively. Its purple color and dark-red fluorescence make these dyes particularly suitable for imaging applications as they are less interfered by biological auto-fluorescence. The large Stokes shift, in turn, makes the probe more resistant to interferences by scattered light.



Scheme 2. Synthesis of the fluorescent tag **1**. Reagents and conditions: (a) **7**, MeCN:DMF 1.6:1, 55 °C; (b) **10**, cat. piperidine, EtOH, 40 °C (yields: **9**: 53%, **1**: 33%).

With these compounds in hands, we have selected compounds **2** and **15a** to fluorescently modulate human serum albumin (HSA) as a model protein in a sequential manner. First, HSA was modified with bifunctional chemical reporter **2**, in the presence of Pd catalyst at room temperature under similar conditions as described in the work of Tilley *et al*. (pH 7.4 phosphate buffer, 0.2 eq. catalyst, 5 eq. **2**).



Scheme 3. Synthesis of clickable fluorophores. Reagents and conditions: (a) DBU, DMSO, Δ; (b) $POCl_3$, DMF, 60 °C; (c) cat. piperidine, EtOH, Δ (yields: **12**: 63%, **13**: 32%, **15a**: 80%, **15b**: 48%).

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After 1 h reaction, label **15a** was added to the reaction mixture along with a catalytic quantity of Cu(I), and the mixture was then stirred overnight. The mixture was then subjected to size exclusion chromatography using Sephadex G-25 to remove unreacted linkers and labels from tagged HSA.

The excitation and emission spectra of the free dye and the fluorescently modified HAS are shown in Figure 2. The spectra show substantial differences: a) the emission maximum of the labeled HSA is shortwave-shifted by 40 nm relative to that of the free dye (from 714 nm to 673 nm); b) the excitation maxima remained the same (564 nm), however, the shape of the band is significantly altered. Such changes are in accordance with the rules of solvatochromism and suggest that the overall microenvironment of the label on the Tyr is less polar than the bulk of the buffer.

In order to exclude non-selective physical adsorption of the fluorophore on the surface of the protein, we also performed the experiments either without the chemical reporter, the Pd(II) catalyst or the Cu(I) catalyst. In none of these cases did we observe the formation of fluorescent HSA. This indicates that covalent binding of the chemical linkers and subsequent click labeling are mandatory for successful tagging of the Tyr residues of HSA.

In conclusion, we have shown that the use of our new linkers and appropriately functionalized fluorescent labels with a large Stokes shift enables specific labeling of proteins directly at Tyr units. The sequential tagging scheme reported here combines the selectivity of the Pd(II) catalyzed reaction between an aromatic hydroxy group and allyloxy acetates with the robustness of the Cu(I) catalyzed azide-alkyne dipolar cycloaddition reaction. We beleive that (a) the method is likely to be applicable to various kinds of azido / alkyne-modified fluorophores, and that (b) the Pd(II)-catalyzed modification of the tyrosine hydroxy group may also enable the introduction of other kinds of tags, e.g. biotin or nonfluorescent labels, provided that they carry a complementary function.

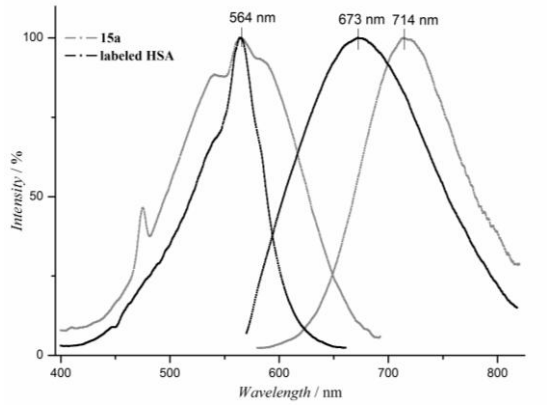

Figure 2. Excitation and emission spectra of the free dye **15a** (grey) and the labeled HSA protein (black) in water.

Acknowledgments

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Supplementary Material

Supplementary data (experimental procedures and characterization data for all new compounds associated with this article can be found, in the online version, at http://dx.doi.org/

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