

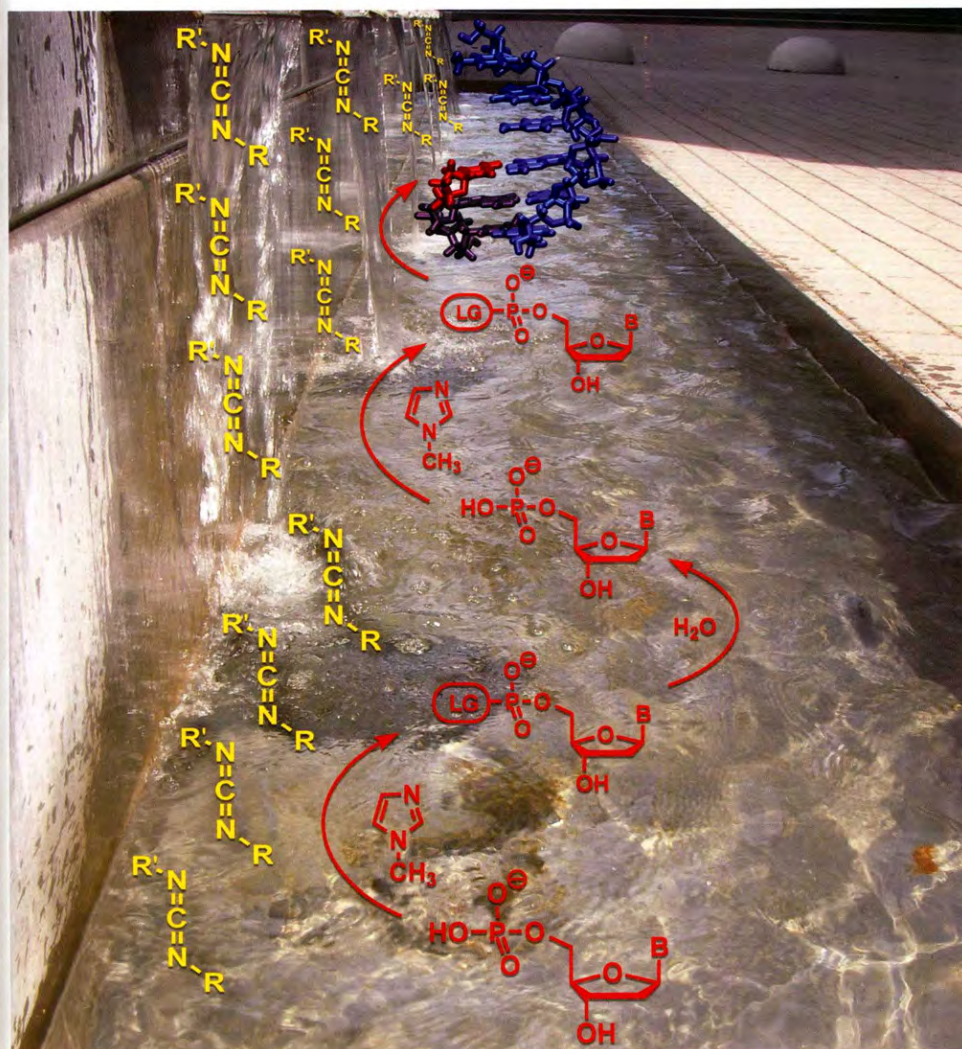
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7.71–7.55 (m, 9H), 7.29–7.25 (m, 3H), 7.17 (m, 1H), 2.56–2.53 (m, 12H). ^{13}C NMR (CD_3CN , 75 MHz) δ 157.1, 156.8, 156.7, 156.5, 153.7, 151.5, 151.4, 151.0, 150.9, 150.5, 150.4, 150.4, 150.2, 148.0, 140.4, 136.1, 132.7, 130.3, 130.1, 128.3, 128.2, 128.0, 127.5, 125.0, 124.9, 124.6, 124.2, 123.9, 122.2, 121.4, 120.7, 92.9, 88.2, 20.3, 20.3, 20.2. ESI-TOF MS m/z 982.17 ($[\text{M} - \text{PF}_6]^-$). ESI-HRMS calcd for $\text{C}_{48}\text{H}_{37}\text{F}_6\text{N}_5\text{O}_5\text{PRu}$ ($[\text{M} - \text{PF}_6]^-$) 982.1750, found 982.1776.

Bis(4,4'-dimethyl-2,2'-bipyridine)-1,2-bis(4-(4-nitrophenyl)-ethynylphenyl)-1*H*-1,2,3-triazol-4-ylpyridine/ruthenium(II) hexafluorophosphate (18). According to the above standing general procedure $\text{Ru}(\text{dmpp})_2\text{Cl}_2$ (25 mg, 0.046 mmol) and 2-(1-(4-(4-nitrophenyl)ethynylphenyl)-1*H*-1,2,3-triazol-4-yl)pyridine (15, 17 mg, 0.046 mmol) were reacted 2.5 h to yield the pure complex as red powder after washing twice with ethanol and diethyl ether (40 mg, 77%). ^1H NMR (CD_3CN , 300 MHz) δ 9.25 (s, 1H), 8.38–8.32 (m, 4H), 8.25–8.21 (m, 2H), 8.17–8.15 (m, 1H), 8.03–8.0 (m, 1H), 7.83–7.73 (m, 7H), 7.71–7.58 (m, 4H), 7.36–7.19 (m, 5H), 2.55–2.51 (m, 12H). ^{13}C NMR (CD_3CN , 75 MHz) δ 157.2, 156.8, 156.8, 156.5, 151.7, 151.3, 151.1, 150.8, 150.6, 150.4,

150.3, 150.1, 148.7, 147.6, 138.1, 136.1, 133.4, 132.5, 129.0, 128.3, 128.2, 127.5, 126.2, 124.9, 124.8, 124.6, 124.2, 123.9, 123.8, 123.4, 122.7, 120.8, 92.1, 89.4, 20.3, 20.3. ESI-TOF MS m/z 982.17 ($[\text{M} - \text{PF}_6]^-$). ESI-HRMS calcd for $\text{C}_{48}\text{H}_{37}\text{F}_6\text{N}_5\text{O}_5\text{PRu}$ ($[\text{M} - \text{PF}_6]^-$) 982.1750, found 982.1759.

Acknowledgment. Financial support of this work by the Dutch Polymer Institute (DPI), the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, VICI award to U.S.S.), the Fonds der Chemischen Industrie (C.F.), and the Carl-Zeiss Stiftung (D.E.) is kindly acknowledged.

Supporting Information Available: UV–vis absorption and emission spectra for the ligands and corresponding ruthenium complexes, crystallographic data for 17, computational details of 16a and 16b (Cartesian coordinates and total energies), and ^1H and ^{13}C NMR spectra of all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

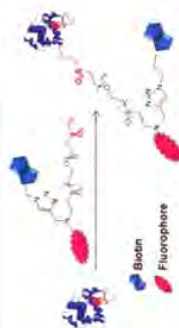
Vinyl Sulfone Bifunctional Tag Reagents for Single-Point Modification of Proteins

Julia Morales-Sanfrutos, Francisco Javier Lopez-Jaramillo, Fernando Hernandez-Mateo, and Francisco Santoyo-Gonzalez*

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The introduction of multiple labels onto biomolecules is a challenge. We report herein the synthesis of vinyl sulfone derivatized bifunctional tag single-attachment-point reagents (BTSAP) bearing biotin and a fluorescent tag and their applications in proteins for the introduction of multiple labels by means of the Michael-type addition of the electrophilic vinyl sulfone group. These BTSAP reagents were easily synthesized by a two-step chemical strategy involving the preparation of alkyne vinyl sulfone derivatized tags (AVST) and subsequent click CuAAC attachment of a second azide functionalized tag. The direct coupling of BTSAP reagents with the low reactive protein horseradish peroxidase (HRP) turned it into a dual reporter group (i.e., fluorescence and peroxidase activity) that may be coupled to any recognition system via biotin–avidin affinity. The AVST compounds are not mere synthetic intermediates for the preparation of BTSAP reagents but valuable *clickable self-reporting* compounds that allow the simultaneous introduction in proteins of an alkyne function and labeling when conjugated via the vinyl sulfone group. The implementation of these clickable AVST compounds in a CuAAC-based sequential approach also allows attachment of the dual labeling of HRP. This approach yields equivalent results in terms of fluorescent labeling, specific activity, and functionality of the biotin tag when compared with the direct bifunctional labeling by the BTSAP reagent. However, for life science this direct approach is more convenient since it avoids the use of copper catalysts, overcoming the toxicity drawback of this metal in biological systems.

Introduction

The covalent labeling of biomolecules is one of the cornerstones of *omic* sciences. In particular, the introduction of multiple labels onto biomolecules is still a challenging task that has been tackled by sequential approaches based on click chemistry. Thus, Kele et al.¹ addressed the dual labeling of bovine serum albumin by exploiting copper-free and copper-mediated click-chemistry reactions of cyclooctyne and terminal alkynes, respectively, that were previously introduced by conventional chem-

istry (i.e., derivatization with maleimide and succinimide ester). Additionally, the triple labeling of oligonucleotides was approached by Gramlich et al.² by incorporation of nucleotides bearing protected alkynes that were sequentially cleaved to react with the azide counterpart tags.

However, the use of click-chemistry in biological systems is not exempt from some limitations. The term *click-chemistry* is almost exclusively used to denote the Huisgen 1,3-dipolar cycloaddition of terminal alkynes and azides under copper(I)

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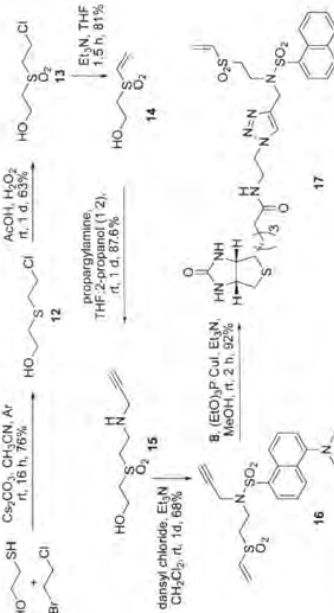
DOI: 10.1021/jo100324p

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(2) Gramlich, P. M.; E.; Wernicke, S.; Gierlich, J.; Currell, T. *Angew. Chem., Int. Ed.* **2008**, *47*, 3442–3444.

SCHEME 3. Synthesis of AVST 16 and BTSAP Reagent 17



the presence of four disulfide bonds, and the analysis of the accessible solvent area with ASA-view²⁸ reveals that at least four out of the nine His and Lys residues are buried (Figure S1, Supporting Information). Thus, coupling of compounds such as the vinyl sulfone based BTSAP reagents that react with thiol and primary amine groups is not a trivial task, and the introduction of more than one label per attachment site is relevant. In fact, literature comparing glutaraldehyde and periodate HRP conjugation reports the latter as more efficient despite the fact that it also provokes the larger extent of denaturation.^{29,30}

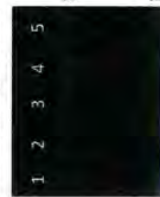


FIGURE 1. SDS-PAGE of HRP labeled with compound 10 at 37 °C illuminated at 365 nm: HRP 10 stoichiometries are 1:10 (lane 1), 1:20 (lane 2), 1:30 (lane 3), 1:40 (lane 4), and 1:50 (lane 5). A first experiment to analyze the reactivity of the BTSAP reagents toward the low reactive HRP was carried out with compound 10. HRP was incubated overnight at pH 8.5 either at room temperature or at 37 °C and protein 10 stoichiometries ranging from 1:10 to 1:50. Results were analyzed by SDS-PAGE and showed that the fluorescence of the HRP-10 conjugates obtained by reaction at room temperature was very weak, and only those that reacted at 37 °C could be detected by the naked eye under a conventional UV illuminator, the higher stoichiometries yielding the stronger fluorescence (Figure 1). Despite our previous experience that proteins are labeled with structurally simpler vinyl sulfone derivatized reagents at room temperature within 30 min,¹⁹ the results with HRP were expected, and they are in full agreement with the limited number of reactive groups and the hindrance effect of the glycan fraction.

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FIGURE 2. Assessment of the functionality of the biotin tag present in labeled HRP-10 and HRP-11 conjugates. Commercial avidin (green cross) was immobilized onto the surface of ELISA plate wells and then incubated with different concentrations of labeled HRP (HRP-10 and HRP-11). After washing to remove the unbound enzyme the peroxidase activity was assayed. Samples are as follows: A1, blank (no avidin); A2–B6, HRP-10 stoichiometry 1:25; B7–C12, HRP-10 stoichiometry 1:50; D1–E6, HRP-11 stoichiometry 1:25; E7–F12, HRP-11 stoichiometry 1:50; G1–H6, control (unlabeled HRP).

and 70% for the HRP–biotin–rhodamine B conjugate (HRP-11), with minor differences between the two stoichiometries (see the Supporting Information, Table S1). These results demonstrate that despite the forced reaction conditions, the concomitant reduction of the enzymatic activity is not incompatible with the use of the dual-labeled HRPs for enzymatic detection purposes.

On the other hand, the functionality of the biotin moiety as an anchor is of primary importance in order to use the labeled HRP as a dual reporter. The interaction of conjugates HRP-10 and HRP-11 with avidin was confirmed by adsorbing commercial avidin onto ELISA plate wells and detecting the peroxidase activity resulting from the incubation with labeled and unlabeled HRP. Only those wells incubated with labeled HRP yielded enzymatic activity since the enzyme remained anchored to the well by interaction between the biotin tag from the BTSAP reagent and the avidin immobilized in the well, confirming the functionality of the biotin (Figure 2).

4. One-Step versus Sequential Approach for Bifunctional Labeling. The above results demonstrate the feasibility of the HRP-BTSAP conjugates as dual reporter groups ready to be linked to a recognition system via avidin–biotin affinity. However, besides the plethora of applications of the BTSAP reagents by themselves, the AVST compounds 3 and, particularly, 4 and 5, which bear a fluorescent tag, are also of interest. These compounds are not simple synthetic intermediates for the synthesis of the BTSAP reagents but also *clickable self-reporting reagents* that allow the simultaneous introduction of an alkyne function and a tag (biotin or fluorophore) into a biomolecule when conjugated through the vinyl sulfone group, facilitating the isolation/complexation of the resulting system with avidin when AVST 3 is used, or its detection/quantification by fluorescence when the reaction is with AVST 4 or 5. The application of the fluorescent tags to monitor the introduction of alkyne groups and their ulterior click reaction of biomolecules was demonstrated by the reaction of bovine serum albumin (BSA) with AVST 5 and then reaction with azide fluorescein (7) in presence of copper(I) as catalyst. The first reaction turned the protein fluorescent, confirming the introduction of the clickable function and allowing its quantification. The second reaction led to a shift in the color of fluorescence emission detectable by naked eye, corroborating that the click reaction took place (Figure 3).



FIGURE 3. SDS-PAGE of the click reaction between fluorescein azide 7 and BSA labeled with AVST 5. Aliquots were taken at 0, 1, 3, 5, 7, and 9 h of reactions (lanes 1–6).

At this point, it is important to recall that our strategy to achieve the bifunctional labeling in a single-attachment-point involves the reaction of vinyl sulfone BSTP reagents obtained by CuAAC click chemistry with naturally occurring functional groups in biomolecules. The successful result in the labeling of BSA described above indicates that the bifunctional labeling of proteins is also attainable in a two-step sequence: introduction of a first label by means of the AVST reagents and click ligation of a second label in the resulting clickable biomolecule (Scheme 4). To further prove the feasibility of this alternative strategy, HRP was reacted with the AVST compound 4 yielding the fluorescent HRP-4 conjugate bearing alkyne groups that after reaction with biotin azide 8 led to a product that is fluorescent, recognizable by avidin, and shows the same peroxidase specific activity as the bifunctional labeled HRP-11 conjugate obtained by direct reaction with the BTSAP reagent 11 (see the Supporting Information, Table S2 and Figure S2). Although both approaches are feasible and yield equivalent results, the one-step approach is preferable because (i) from a synthetic point of view the click reaction between organic compounds is more convenient than that between the label and a macromolecule and (ii) it circumvents the problem of the use of copper in bioconjugation whose toxicity for living organisms and potential to induce degradation of the structure of the biomolecules *in vitro*³⁶ are important drawbacks in life science. In addition, it should be also remarked that in the context of click bioconjugation the assays described with the AVST compounds clearly establish that the vinyl sulfone chemistry is a valuable methodology for the introduction of clickable groups with similar characteristics to other traditional bioconjugation strategies (i.e., activated esters–lysine or maleimide–Cys) used for this same purpose with the major

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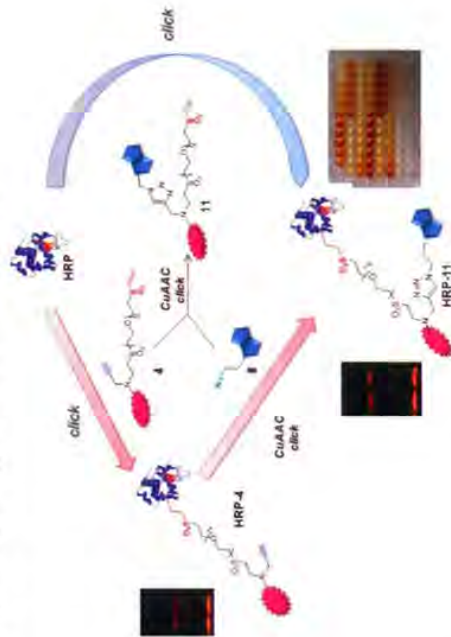
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(30) Trecea, J. P.; Ricoux, R.; Pontet, M.; Engler, R. *Ann. Biol. (Paris)* 1995, 33, 227–231.

SCHEME 4. One-Step versus Sequential Approach for Bifunctional Labeling of HRP



advantage of the vinyl sulfone stability in water over the activation chemistry used on those strategies.³¹ Moreover, the vinyl sulfone group possesses a broader reactivity as they react with the amine, thiol, and imidazole groups naturally present in proteins.

Conclusions

The combination of biotin as an anchor compound and a fluorophore as a reporter group in a single compound leads to versatile bifunctional labeling reagents, especially appealing when functionalized with the vinyl sulfone group, whose reactivity toward proteins in "green" conditions has been previously reported.¹⁸ The synthesis of the vinyl sulfone BTSAP reagents bearing a fluorophore and biotin is achieved in a straightforward two-step strategy that implies the preparation of an alkyl-vinyl sulfone derivatized tag (AVST) from simple starting materials and the subsequent CuAAC reaction with a second azide functionalized tag. The use of BTSAP reagents leads to the simultaneous introduction of two tags via a single attachment point in a single reaction (one-step approach) under conditions that are compatible with the biological nature of proteins. For the particular case of HRP, the conjugation yields a dual reporter group (fluorescence and peroxidase activity) that can be coupled to any recognition system via biotin-avidin affinity. It has been also demonstrated that the AVST compounds are not mere synthetic intermediates but also advanced reagents for click functionalization with applications in any scenario based on click chemistry. The AVTS compounds act as clickable self-reporter reagents that enable the simultaneous introduction of an alkyne function and a label that allows monitoring the introduction of the alkyne function and the latter click reaction of the resulting clickable biomolecule.

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FAB mass spectra were recorded using *m*-nitrobenzyl alcohol or thioglycerol as matrix. MALDI-TOF and NALDI-TOF mass spectra were recorded using HCCA and NaI, respectively, as matrix.

Synthesis of Compound 2. To a solution of **1**¹⁸ (1.0 g, 3.3 mmol) in CH_2Cl_2 -2-propanol (2:1) was added propylamine (152 μL , 2.3 mmol). The reaction mixture was kept at room temperature for 1 day. Evaporation of the solvent yielded a crude that was purified by column chromatography (EtOAc $\rightarrow$ EtOAc-MeOH 10:1) to give **2** as a syrup (411 mg, 52%). IR (film) ν_{max} (cm⁻¹): 3588, 3264, 1633, 1470, 1281, 1116; ¹H NMR (CDCl_3 , 400 MHz) δ 6.76 (dd, 1H, $J = 16.6$ and 10 Hz), 6.42 (d, 1H, $J = 16.6$ Hz), 6.12 (d, 1H, $J = 10$ Hz), 3.91 (t, 2H, $J = 5.5$ Hz), 3.89 (t, 2H, $J = 5.7$ Hz), 3.64 (s, 4H), 3.45 (d, 2H, $J = 2.3$ Hz), 3.35 (t, 2H, $J = 5.4$ Hz), 3.29 (m, 4H), 3.20 (m, 2H), 2.26 (t, 1H, $J = 7.3$ Hz); ¹³C NMR (CDCl_3 , 75 MHz) δ 137.9, 129.2, 81.5, 72.1, 70.4, 70.3, 64.9, 64.6, 54.9, 54.7, 54.6, 41.9, 37.8; HRMS (FAB+) calcd for $\text{C}_{14}\text{H}_{21}\text{NO}_5\text{Na}$ [M + Na]⁺ 376.0864, found 376.0861.

Synthesis of Compound 3. A solution of biotin (200 mg, 0.82 mmol) in CH_2Cl_2 (5 mL) was kept at room temperature for 1 h. The reaction mixture was evaporated in vacuo and coevaporated with anhydrous toluene (3 $\times$ 15 mL) to give biotin acid chloride. The crude product was dissolved in anhydrous THF (15 mL) and added dropwise to a solution of **2** (353 mg, 1 mmol) and Et₃N (223 μL , 1.6 mmol) in anhydrous THF (10 mL). The reaction mixture was kept at room temperature for 10 min. Evaporation of the solvent yielded a crude that was purified by column chromatography (EtOAc-MeOH 5:1) giving **3** as a syrup (438 mg, 92%). IR (film) ν_{max} (cm⁻¹): 3388, 3261, 2923, 2870, 2115, 1696, 1644, 1420, 1288, 1210, 1034; ¹H NMR (CDCl_3 , 400 MHz) δ 6.91 (dd, 1H, $J = 16.1$ and 10.5 Hz), 6.33 (d, 1H, $J = 16.4$ Hz), 6.18 (d, 1H, $J = 9.8$ Hz), 4.49 (m, 1H), 4.31 (m, 1H), 4.27 (br s, 2H), 3.99 (m, 1H), 3.88 (m, 4H), 3.66 (m, 1H), 3.5 (m, 1H), 3.49–3.20 (several m, 8H), 2.93 (dd, 1H, $J = 12.9$ and 5 Hz), 2.85 (br s, 1H), 2.70 (d, 1H, $J = 12.6$ Hz), 2.50 (m, 3H), 1.76–1.46 (several m, 6H); ¹³C NMR ($\text{MeOD}-d_4$, 75 MHz) δ 175.6, 166.1, 139.4, 129.6, 79.7, 74.7, 71.4, 71.3, 65.8, 65.6, 63.3, 61.6, 56.9, 55.6, 54.8, 53.3, 41.3, 41.1, 39.1, 33.8, 29.7, 29.5, 25.9; HRMS (MALDI-TOF) calcd for $\text{C}_{23}\text{H}_{31}\text{N}_2\text{O}_8\text{Na}$ [M + Na]⁺ 602.1640, found 602.1641.

Synthesis of Compound 4. A solution of rhodamine B (195 mg, 0.41 mmol) in CH_2Cl_2 (5 mL) was kept at room temperature overnight. The reaction mixture was evaporated in vacuo and coevaporated with anhydrous toluene (3 $\times$ 15 mL) to give rhodamine B acid chloride. The crude product was dissolved in anhydrous CH_2Cl_2 (10 mL) and was added dropwise to a solution of **2** (174 mg, 0.49 mmol) and Et₃N (116 μL , 0.81 mmol) in anhydrous CH_2Cl_2 (15 mL). The reaction mixture was kept at room temperature for 10 min. Evaporation of the solvent yielded a crude that was purified by column chromatography (CH_2Cl_2 -MeOH 20:1) giving **4** as a foam solid (272 mg, 86%). IR (film) ν_{max} (cm⁻¹): 2170, 1640, 1588, 1463, 1412, 1339, 1273, 1181, 1130, 1073; ¹H NMR (CDCl_3 , 300 MHz) δ 7.82–7.72 (several m, 3H), 7.40 (m, 1H), 7.23 (d, 2H, $J = 9.6$ Hz), 7.06 (dd, 2H, $J = 9.6$ and 2 Hz), 6.81–6.79 (several m, 3H), 6.36 (d, 1H, $J = 16.7$ Hz), 6.11 (d, 1H, $J = 9.8$ Hz), 3.88–3.62 (m, 18H), 3.32–3.16 (m, 4H), 2.44 (br s, 1H), 1.92 (br s, 4H), 1.42 (t, 12H, $J = 7.3$ Hz); ¹³C NMR (CDCl_3 , 75 MHz) δ 168.3, 157.8, 155.7, 137.9, 134.9, 131.7, 131.1, 130.5, 130.3, 129.1, 127.8, 114.7, 113.8, 96.5, 74.5, 70.3, 64.6, 64.5, 54.9, 53.7, 51.7, 46.3, 40.4, 39.1, 29.8, 12.8; HRMS (MALDI-TOF) calcd for $\text{C}_{41}\text{H}_{52}\text{N}_2\text{O}_8$ [M]⁺ 778.3195, found 778.3196.

Synthesis of Compound 5. To a solution of dansyl chloride (165 mg, 0.61 mmol) in anhydrous acetonitrile (15 mL) were added **2** (180 mg, 0.51 mmol) and Et₃N (150 μL , 1.02 mmol). The reaction mixture was kept at room temperature for 2.5 days. Evaporation of the solvent yielded a crude that was purified by column chromatography (EtOAc-hexane 1:1 $\rightarrow$ 3:1) giving **5** as a syrup (257 mg, 86%). IR (film) ν_{max} (cm⁻¹): 3262, 1568, 1453, 1388, 1310, 1124; ¹H

NMR (CDCl_3 , 400 MHz) δ 8.57 (d, 1H, $J = 8.4$ Hz), 8.28 (d, 1H, $J = 8.8$ Hz), 8.20 (d, 1H, $J = 7.2$ Hz), 7.55 (m, 2H), 7.19 (d, 1H, $J = 7.4$ Hz), 6.75 (dd, 1H, $J = 16.6$ and 10 Hz), 6.38 (d, 1H, $J = 16.6$ Hz), 6.08 (d, 1H, $J = 9.8$ Hz), 4.22 (s, 2H), 3.89 (m, 4H), 3.83 (m, 2H), 3.64 (s, 4H), 3.55 (m, 2H), 3.26 (t, 2H, $J = 5.8$ Hz), 3.21 (t, 2H, $J = 5.2$ Hz), 2.88 (s, 6H), 2.16 (s, 1H), ¹³C NMR (CDCl_3 , 75 MHz) δ 152.0, 137.9, 133.6, 131.2, 130.2, 130.1, 130.0, 129.1, 128.5, 123.2, 119.2, 115.5, 77.0, 74.5, 70.5, 70.2, 64.8, 64.6, 54.8, 54.2, 53.5, 45.4, 40.8, 37.2; HRMS (NALDI-TOF) calcd for $\text{C}_{33}\text{H}_{40}\text{O}_8\text{S}_2\text{Na}$ [M + Na]⁺ 609.1375, found 609.1368.

Synthesis of Azide-Fluorescein 7. To a suspension of chloro-sulfonium azide (400 mg, 0.94 mmol) in MeOH (20 mL) was added fluorescein (306 mg, 4.7 mmol). The reaction mixture was irradiated at 500 W and 65 °C in a Milestone Star Microwave Labstation for 10 h. Evaporation of the solvent yielded a crude that was purified by column chromatography (EtOAc-EtOAc-MeOH 1:1 $\rightarrow$ MeOH) giving **7** as a solid (330 mg, 82%). IR (KBr) ν_{max} (cm⁻¹): 3388, 2119, 2036, 1680, 1628, 1588, 1465, 1397, 1330, 1270, 1169, 1109; ¹H NMR ($\text{MeOD}-d_4$, 300 MHz) δ 8.12 (d, 1H, $J = 2.1$ Hz), 8.00 (dd, 1H, $J = 8.2$ and 2.2 Hz), 7.22 (d, 1H, $J = 8.4$ Hz), 7.16 (d, 2H, $J = 9.3$ Hz), 6.62 (dd, 1H, $J = 8.2$ and 2.2 Hz), 6.60 (s, 3H), 4.08 (s, 2H), ¹³C NMR ($\text{MeOD}-d_4$, 100 MHz) δ 179.6, 168.7, 159.7, 140.2, 132.5, 131.2, 122.9, 121.9, 121.8, 114.5, 104.2, 53.3; HRMS (FAB+) calcd for $\text{C}_{23}\text{H}_{14}\text{O}_8\text{N}_3\text{Na}$ [M + Na]⁺ 453.0811, found 453.0819.

General Procedure for the Synthesis of Vinyl Sulfone Derivatized Bifunctional Tag Single-Attachment-Point Reagents (BTSAP) (9–11). To a solution of **3** or **4** (0.2 mmol) in MeOH (15 mL) were added the corresponding azide derivative **6**¹⁹ and **7** or **8** (0.24 mmol), respectively. Et₃N (85 μL , 0.6 mmol), and (EtO)₂P-CuI²⁰ (10 mg, 0.03 mmol) as catalyst. The reaction mixture was stirred at room temperature for 3.5 h. Evaporation of the solvent yielded a crude that was purified by column chromatography.

Synthesis of BTSAP Reagent 9. Column chromatography (EtOAc-MeOH 3:1) gave **9** as a solid (162 mg, 90%). IR (KBr) ν_{max} (cm⁻¹): 3398, 1689, 1635, 1457, 1310, 1316; ¹H NMR ($\text{DMSO}-d_6$, 600 MHz) δ 8.43 (d, 1H, $J = 8.5$ Hz), 8.18 (br s, 1H), 8.11 (d, 1H, $J = 8.6$ Hz), 8.01 (d, 1H, $J = 7.3$ Hz), 7.86 (s, 1H), 7.59–7.53 (several m, 2H), 7.22 (d, 1H, $J = 7.3$ Hz), 6.85 and 6.84 (2 dd, 1H, $J = 16.5$ and 9.8 Hz), 6.38 (br s, 1H), 6.33 (s, 1H), 6.17 (d, 1H, $J = 16.6$ Hz), 6.14 (d, 1H, $J = 9.9$ Hz), 4.48 and 4.42 (2s, 2H), 4.3–4.31 (m, 2H), 4.28 (m, 1H), 4.11 (m, 1H), 3.73 (m, 1H), 3.69 (m, 3H), 3.61 (m, 1H), 3.48 (m, 4H), 3.44 (m, 1H), 3.33–3.29 (several m, 5H), 3.24–3.19 (several m, 2H), 3.07 (m, 1H), 2.79 (s, 6H), 2.77 (m, 1H), 2.55 (d, 1H, $J = 12.5$ Hz), 2.40–2.34 (several m, 2H), 1.59–1.25 (several m, 6H); ¹³C NMR ($\text{DMSO}-d_6$, 75 MHz) δ 172.3, 162.6, 151.2, 143.0, 137.9, 135.4, 129.5, 128.9, 128.5, 128.1, 127.8, 123.4, 118.8, 115.1, 69.3, 69.2, 66.2, 63.8 $\times$ 2, 60.9, 59.0, 55.3, 53.4, 52.6, 51.1, 49.2, 44.9 $\times$ 2, 42.2, 39.7, 38.9, 32.1, 28.1 $\times$ 2, 24.5; HRMS (NALDI-TOF) calcd for $\text{C}_{34}\text{H}_{40}\text{O}_{10}\text{S}_2\text{Na}$ [M + Na]⁺ 921.2743, found 921.2743.

Synthesis of BTSAP Reagent 10. Column chromatography (EtOAc-MeOH 3:1 $\rightarrow$ MeOH) gave **10** as a foam solid (140 mg, 70%). IR (film) ν_{max} (cm⁻¹): 3358, 2916, 1678, 1562, 1454, 1280, 1238, 1099; ¹H NMR ($\text{DMSO}-d_6$, 600 MHz) δ 8.20 (s, 1H), 8.16 and 7.97 (2s, 1H), 7.80 (d, 1H, $J = 7.9$ Hz), 7.10 (d, 1H, $J = 8.2$ Hz), 6.90 and 6.89 (2dd, 1H, $J = 16.6$ and 9.9 Hz), 6.64 (d, 2H, $J = 8.9$ Hz), 6.42 (br s, 1H), 6.32 (m, 4H), 6.18 (d, 1H, $J = 16.6$ Hz), 6.16 (d, 1H, $J = 9.9$ Hz), 5.38 (m, 4H), 5.34 (2 br s, 2H), 4.65 and 4.56 (2 br s, 2H), 4.27 (m, 1H), 4.12 (m, 1H), 3.77 (m, 3H), 3.69 (m, 3H), 3.53 (m, 4H), 3.40–3.30 (several m, 6H), 3.09 (m, 1H), 2.78 (dd, 1H, $J = 12.3$ and 4.9 Hz), 2.55 (d, 1H, $J = 12.6$ Hz), 2.50–2.39 (several m, 2H), 1.61–1.43 (several m, 6H); ¹³C NMR ($\text{DMSO}-d_6$, 125 MHz) δ

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172.5, 171.8, 168.7, 164.5, 162.7, 155.0, 143.3, 143.2, 138.9, 138.0, 129.8, 129.6, 128.7, 128.6, 124.7, 118.4, 117.9, 110.5, 102.4, 69.4, 69.3, 63.9, 61.0, 59.1, 55.4, 52.7, 51.2, 42.6, 40.1, 32.2, 28.2, 28.1, 24.2, 6 HRMS (MALDI-TOF) calcd for $C_{24}H_{19}N_3O_5S_2Na$ [M]⁺ 741.2287, found 741.2281.

Labeling of HRP with Vinyl Sulfone Based BSA Reagent
 10. HRP (2 mg/mL, 300 μ L) was incubated with vinyl sulfone derivatized bifunctional tag reagent 10 (25 mM in DMSO, 5.5, 11, 16.5, 22, and 27.5 μ L, stoichiometries 1:10, 1:20, 1:30, 1:40, and 1:50, respectively) in 100 mM HEPES buffer pH 8.5 at both room temperature (22 $^{\circ}$ C) and 37 $^{\circ}$ C for 1 day to yield the corresponding labeled HRP-10.
Labeling of HRP with Vinyl Sulfone Based BSA Reagents
 9–11 and 17. HRP (2 mg/mL, 1.5 mL) was incubated with vinyl sulfone derivatized bifunctional tags 10 and 11 (25 mM in DMSO, 69 and 138 μ L, stoichiometries 1:25 and 1:50, respectively) and with 9 and 17 (25 mM in DMSO, 138 μ L, stoichiometry 1:50) in 100 mM HEPES buffer pH 8.5 at 37 $^{\circ}$ C for 1 day. The resulting labeled proteins (HRP-9, HRP-10, HRP-11, and HRP-12) were dialyzed in 50 mM phosphate buffer pH 7.5. A nonlabeled HRP sample was also included in the dialysis.
Labeling of BSA with 5. Compound 5 (50 μ L, 30 mM in DMSO) was added to a solution of BSA (1 mL, 2 mg/mL in 100 mM HEPES buffer pH 8). The reaction mixture was kept at 37 $^{\circ}$ C for 18 h and centrifuged at 14000 rpm for 2 min to remove the nondissolved labeling reagent. The supernatant was dialyzed against 50 mM phosphate buffer pH 8 to remove the excess of labeling reagent yielding labeled BSA-5.

Click-Chemistry on BSA-5 with 7. To a solution of labeled BSA-5 (50 μ L, 2 mg/mL in 50 mM phosphate buffer, pH 8) were added $CuSO_4 \cdot 5H_2O$ (10 μ L, 10 mM stock solution in deionized water), DTE (10 μ L, 50 mM stock solution in deionized water), 1,10-phenanthroline (10 μ L, 20 mM stock solution in DMSO), 7 (5 μ L, 30 mM stock solution in DMSO), and deionized water (15 μ L). The click-chemistry reaction was allowed to proceed at room temperature for 9 h.
One-Step Bifunctional Labeling of HRP with DTSAP Reagent
 11. To a solution of HRP (750 μ L, 2 mg/mL in 100 mM HEPES buffer, pH 8.5) was added a solution of 11 (69 μ L, 25 mM in DMSO). The reaction was incubated at 37 $^{\circ}$ C for 1 day. The sample was dialyzed in 50 mM phosphate buffer pH 8 to remove the excess labeling reagent. The labeled HRP-11 concentration was established by Bradford.³⁷

Sequential Approach (SA) for Bifunctional Labeling of HRP.
 To a solution of HRP (1.5 mL, 2 mg/mL in 100 mM HEPES buffer, pH 8.5) was added a solution of 4 (138 μ L, 25 mM in DMSO). The reaction was incubated at 37 $^{\circ}$ C for 1 day. The sample was dialyzed in 50 mM phosphate buffer pH 8 to remove the excess labeling reagent. The labeled HRP-4 concentration was established by Bradford.³⁷ To a solution of HRP-4 (400 μ L, 1.25 mg/mL in 50 mM phosphate buffer, pH 8) were added $CuSO_4 \cdot 5H_2O$ (20 μ L, 30 mM stock solution in deionized water), DTE (20 μ L, 150 mM stock solution in deionized water), 1,10-phenanthroline (20 μ L, 60 mM stock solution in DMSO), 8 (50 μ L, 25 mM stock solution in DMSO), and deionized water (100 μ L). The coupling reaction was carried out at room temperature for 7 h, and a 300 μ L of aliquot was dialyzed in 50 mM phosphate buffer pH 8 (HRP-SA7b). After 21 h, another 300 μ L of aliquot was also dialyzed (HRP-SA21b). The protein concentration was established by Bradford.³⁷

HRP Activity Assays. Labeled-HRP (10 μ L, ~0.015 mg/mL) was added to a solution (1 mL) of *o*-phenylenediamine dihydrochloride (20 mg/50 mL) in 150 mM citrate buffer (pH 5.0) with 100 μ L of H_2O_2 (10 vol/30 mL), and the absorbance was measured at 492 nm in kinetic mode for 1 min.
Interaction Assays between Biotin–HRP and Avidin. Micro-tiltation plates were coated with avidin at 200 μ L/well of a solution of 2 μ g/mL in 100 mM carbonate buffer pH 9.6 overnight at 4 $^{\circ}$ C. The wells were washed twice with PBST buffer (10 mM phosphate

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buffer, pH 7.4, 100 mM NaCl (PBS) containing 0.05% (v/v) Tween 20 and twice with PBS. This washing procedure was repeated after each incubation step. Wells were then blocked with 380 μ L/well of BSA in PBS (0.1% w/v) for 1 h at 37 $^{\circ}$ C. Each biotin-labeled HRP and nonlabeled HRP were added in serial dilution (300 μ L/well) in 50 mM phosphate buffer pH 7.5, 100 mM NaCl, and the plates were incubated for 1 h at 37 $^{\circ}$ C. After that, 200 μ L/well of a solution of *o*-phenylenediamine dihydrochloride (20 mg/50 mL) in 150 mM citrate buffer (pH 5.0 with 100 μ L H_2O_2 (10 vol/50 mL) was added. The plates were incubated for 20 min at 37 $^{\circ}$ C, and the reactions were stopped by addition of aqueous H_2SO_4 (50 μ L/well, 1.25 M).

Electrophoresis of the Labeled Proteins. Samples analyzed by SDS–PAGE⁴⁶ in a 12% polyacrylamide gel (12% T/1.3% C) in

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a MiniProtein 3 cell. The fluorescence was detected by excitation at 365 nm with a conventional UV transilluminator.

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Supporting Information Available: Solvent accessibility of the residues comprising HRP, enzymatic activity of the labeled-HRP, and spectra for compounds 2–5, 7, 9–11, and 15–17. This material is available free of charge via the Internet at <http://pubs.acs.org>.