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

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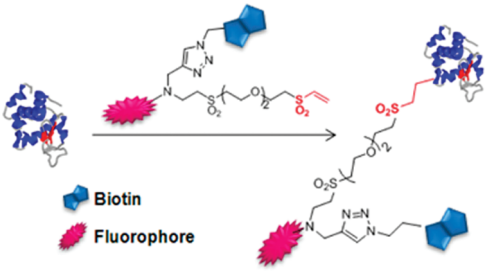

The introduction of multiple labels onto biomolecules is a challenge. We report herein the synthesis of vinyl sulfone derivatized bifunctional tag single-attachment-point reagents (BTSAP) bearing biotin and a fluorescent tag and their applications in proteins for the introduction of multiple labels by 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 attainment 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 catalysis, 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 chemistry (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's 1,3-dipolar cycloaddition of terminal alkynes and azides under copper(I)

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DOI: 10.1021/jo100324p

catalysis (CuAAC).[3,4] The copper catalyst is toxic for living organisms and induces degradation of the structure of the biomolecules in vitro.[5,6] These drawbacks have been overcome by developing copper-free azide−alkyne cycloaddition strategies[7] that represent a significant advance but still with limitations,[8,9] and it seems unlikely that they will constitute a general alternative to replace the CuAAC.[10] Additionally, click reactions involve groups not naturally occurring in biomolecules which must be introduced by a chemical or a biological strategy. The chemical approach is based on conventional reactions toward naturally occurring functional groups in macromolecules or, for short peptides and oligonucleotides, in vitro synthesis with building blocks bearing a clickable function. Alternatively, the cell's biosynthetic pathway has been exploited to introduce unnatural clickable amino acids or other clickable modified building blocks (i.e., nucleotides, glycans, lipids, and other biomolecules). One of the major advantages of this biological approach is that it may provide site-specificity but at the cost of using molecular biology techniques as well as the knowledge of the tertiary/quaternary structure of the protein to select the reaction point.

Regardless of the methodology, sequential approaches also have drawbacks. The need for several steps of protection−deprotection is time-consuming and reduces the overall yield. Moreover, the stoichiometric attachment of different labels is difficult to control and for molecules with a single reactive center or low reactivity multilabeling may not succeed. In this context, the synthesis of "multifunctional single-attachment-point" (MSAP) reagents which feature several tags and a single reactive group (NHS ester, maleimide, or thiol) to allow the introduction of various labels in one step is an attractive alternative that has been recently reported.[11,12] These reagents have been successfully applied for the preparation of multifunctional nanoparticles[11] and biotinylated versions of the monoclonal antibody cetuximab.[12]

Fluorescence and biotin are among the tags most commonly used in *omic* sciences. Fluorescence is the key element in many strategies that are not limited to passive detection but that comprises techniques aimed at studying conformational changes or interactions between molecules.[13,14] Biotin is the base of what has been coined as the *avidin−biotin technology*,[15−17] widely used to mediate between a recognition system (e.g., antibody−antigen or lectin−carbohydrate) and a reporter group (e.g., enzymatic activity or fluorescence). Herein, we report on the synthesis of bifunctional tag single-attachment-point reagents (BTSAP) bearing both biotin (affinity tag) and a fluorophore (fluorescent tag) and a vinyl sulfone function as a reactive linking group. As we have recently reported[18] vinyl sulfone is an appealing function for protein conjugation through the Michael-type addition with the amine and thiol groups present in the proteogenic residues under mild conditions that are compatible with their biological function. These new vinyl sulfone BTSAP reagents are easily prepared by a simple strategy that combines vinyl sulfone functionalization and CuAAC click chemistry.[19] Their applications for protein labeling are also reported.

Results and Discussion

1. Synthesis of Vinyl Sulfone-Based BTSAP Reagents. The synthesis of vinyl sulfone-based bifunctional tag single-attachment-point reagents (BTSAP) was approached with biotin and commonly used fluorophores (dansyl, fluorescein and rhodamine B) as tags by a two-step chemical strategy: (i) preparation of an alkyne−vinyl sulfone derivatized tag (AVST) and (ii) subsequent copper-mediated click reaction of the AVST intermediate with a complementary azide derivative of a second tag. For this purpose, compound **2** bearing three orthogonal functional groups (i.e., amine, vinyl sulfone, and terminal alkyne) was envisaged as a key starting material easily accessible by the reaction of the bis-vinyl sulfone **1**[18] with propargylamine. This compound is a suitable scaffold for the sequential introduction of two functionalized tags while leaving unreacted the vinyl sulfone group for the ulterior coupling with biomolecules (Scheme 1). The first tag was introduced by selective reaction of the amine group present in **2** with the acid chloride derivatives of biotin and rhodamine B and with dansyl chloride in an appropriate solvent to yield the AVST intermediates **3**−**5**, respectively (Scheme 1). The second tag was then introduced by CuAAC reaction of the **3** and **4** AVST intermediates with the clickable azide derivative of dansyl[20] (**6**), fluorescein (**7**), and biotin[21] (**8**) leading to the vinyl sulfone BTSAP reagents **9**−**11** (Scheme 2) in 70−90% yield. The reactions were carried out using $(EtO)_3P\cdot$CuI as catalyst.[22] These conditions were found to be optimal considering the possible reactivity of azide derivatives with the vinyl sulfone group[23−25] and the reactions finished in short reaction times (3.5 h).

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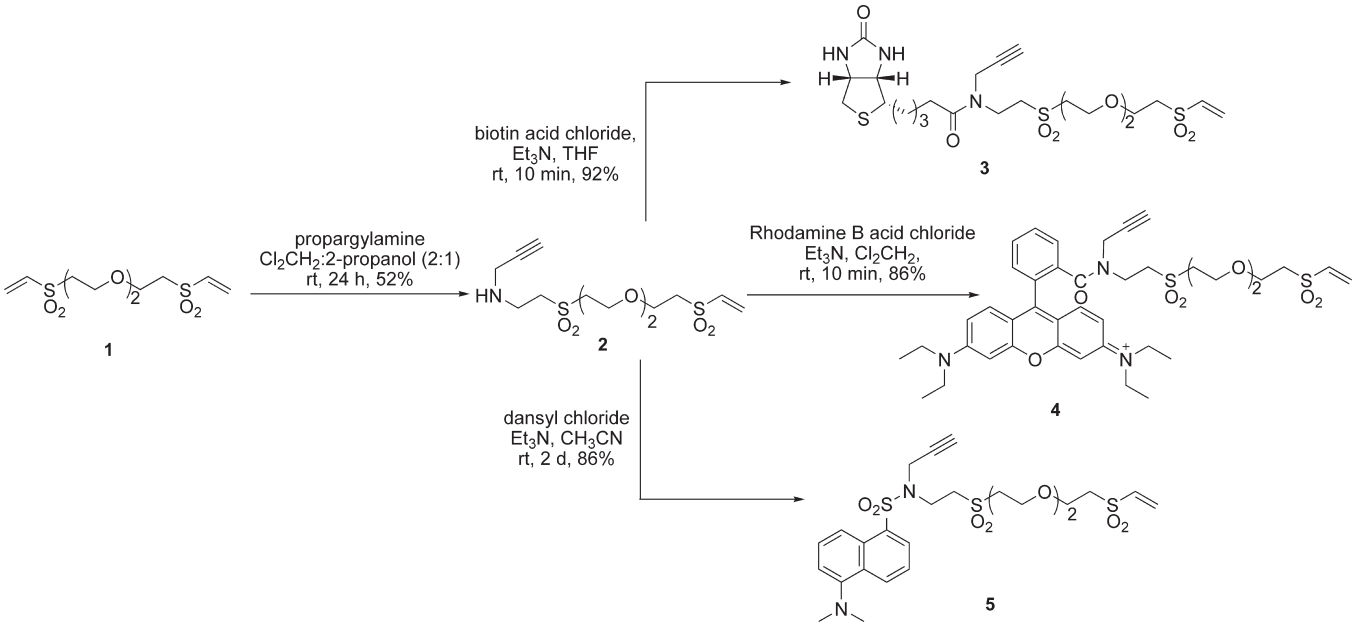
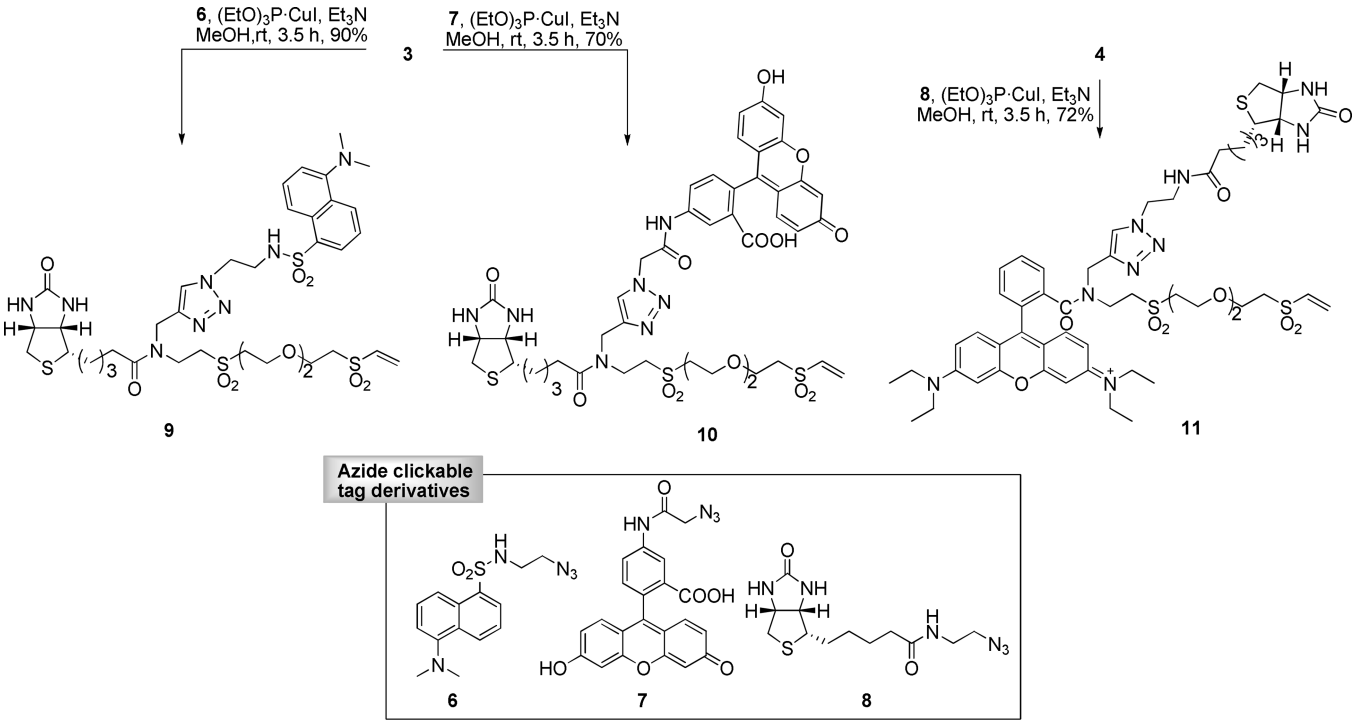
SCHEME 1. Synthesis of Alkyne Vinyl Sulfone Derivatized Tags (AVST) 3−5



SCHEME 2. Synthesis of Vinyl Sulfone Bifunctional Tag Single-Attachment-Point Reagents (BTSAP)



2. Labeling of HRP with the Vinyl Sulfone Based BTSAP Reagents. The BTSAP reagents **9**−**11** are large size compounds whose reactivity toward biomolecules may be compromised by their bulky nature. In order to assess their effectiveness in the labeling of proteins, they were assayed on a model system where the accessibility to the reactive groups is limited. The glycoprotein horseradish peroxidase (HRP) was the protein of choice because besides its biotechnological relevance (HRP has been coupled to other molecules to use the peroxidase activity as a reporter), its structure is known (PDB access code 6atj[26]) and the carbohydrate residues that may hinder the reactive sites are characterized.[27] The protein is glycosylated at eight sites, $(Xyl)Man_3(Fuc)GlcNAc_2$ being the major species, and contains 8 Cys, 6 Lys, and 3 His in its amino acidic sequence that may react with the vinyl sulfone group. However, the tertiary structure of HRP shows

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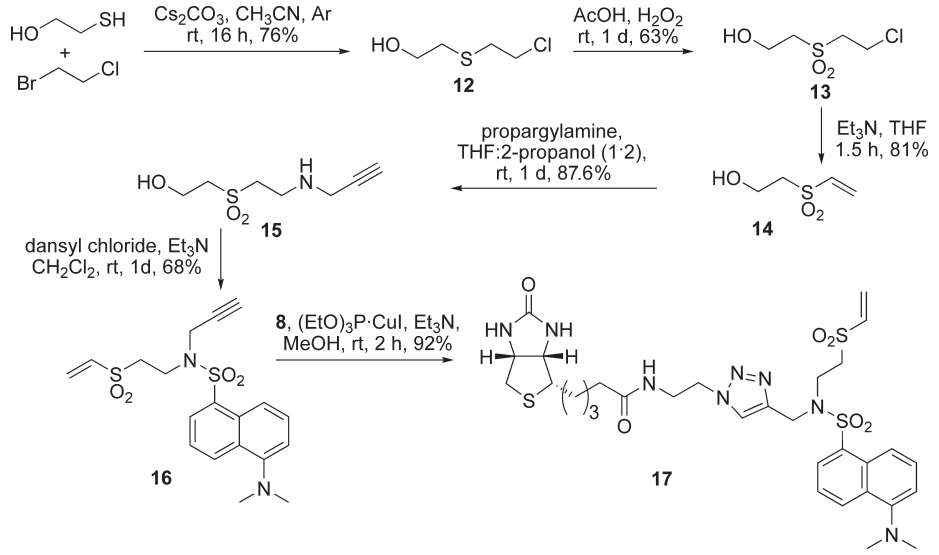
SCHEME 3. Synthesis of AVST 16 and BTSAP Reagent 17



the presence of four disulfide bonds, and the analysis of the accessible solvent area with ASA-view[28] 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]

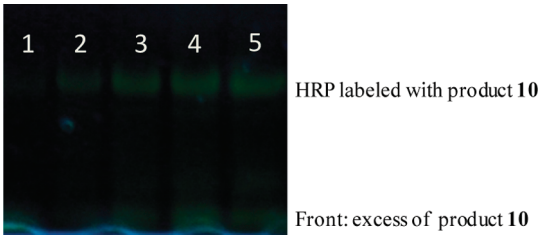


FIGURE 1. SDS−PAGE of HRP labeled with compound **10** at 37 °C illuminated at 365 nm: HRP/**10** stoichiometries are 1:10 (lane 1), 1:20 (lane 2), 1:30 (lane 3), 1:40 (lane 4), and 1:50 (lane 5).

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,[18] 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.

In order to gain additional insight into the influence of the steric impediment, the BTSAP reagent **17** bearing a shorter linker between the vinyl sulfone reactive group and the bulky dansyl was synthesized (Scheme 3). The comparison of the fluorescence from the conjugates **HRP-9** and **HRP-17** showed that the later yielded higher fluorescence despite the presence of a shorter linker. This unexpected experimental result may be rationalized on the basis of the higher solubility of compound **17** when compared to that of compound **9**, and it suggests a major contribution of the larger molar excess rather than a steric effect.

3. Functionality of the Labeled HRPs and the Biotin Tag. The rationale behind the introduction of biotin as the second tag in the BTSAP reagents was to create an anchor point to link a recognition system. The labeling of HRP with biotin-containing reagents is an attractive and general strategy to generate a dual reporter group (i.e., fluorescence and peroxidase activity) that may be also coupled to any recognition system such as antibody−antigen or lectin−carbohydrate via biotin−avidin affinity. Although the previous experiment (Figure 1) demonstrated that the use of high stoichiometries yields more intense fluorescence, overlabeling is not always beneficial and may lead to solubility problems and/or reduction of the functionality. Hence a new experiment was designed to evaluate the effect of labeling on the functionality of HRP. Thus, HRP was conjugated with the BTSAP reagents **10** and **11** under harsh conditions (stoichiometries 1:25 and 1:50, 37 °C, 24 h) to promote the negative effect of the overlabeling and then thoroughly dialyzed to remove the excess of BTSAP reagent. No solubility problems were detected, probably as a consequence of the reduced number of reactive groups present in HRP and the beneficial effect exerted by the carbohydrate moiety. When the specific activity of the labeled HRP was measured and compared to that of the unlabled HRP, the values were in the order of 60% for the HRP−biotin−fluorescein conjugate (**HRP-10**)

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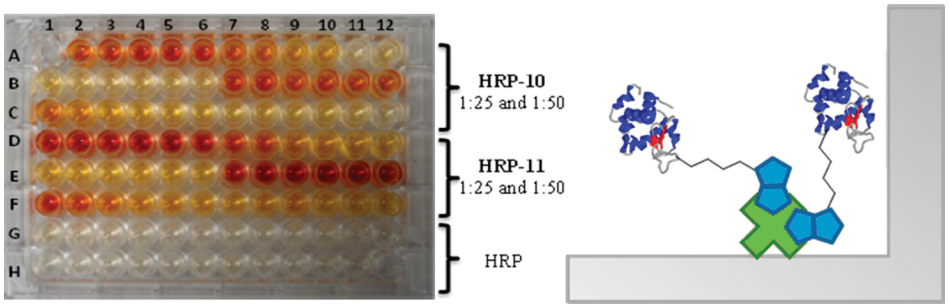


FIGURE 2. Assessment of the functionality of the biotin tag present in labeled **HRP-10** and **HRP-11** conjugates. Commercial avidin (green cross) was immobilized onto the surface of ELISA plate wells and then incubated with different concentrations of labeled HRP (**HRP-10** and **HRP-11**). After 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).

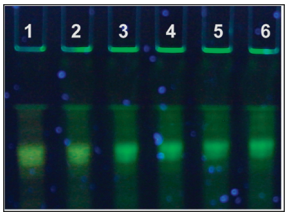
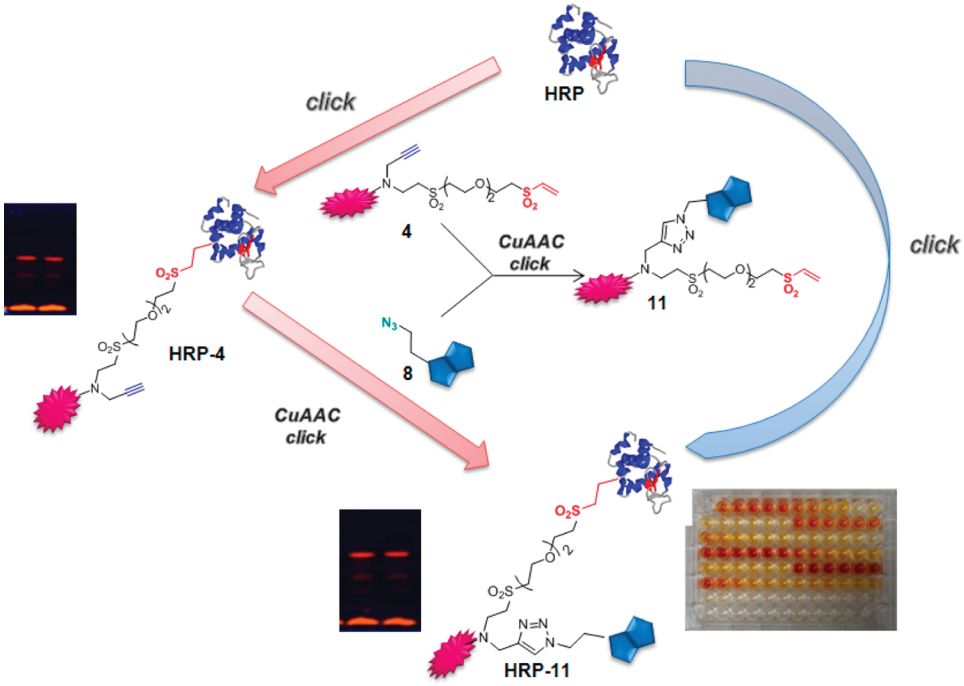


FIGURE 3. SDS−PAGE of the click reaction between fluorescein azide **7** and BSA labeled with AVST **5**. Aliquots were taken at 0, 1, 3, 5, 7, and 9 h of reactions (lanes 1−6).

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[5,6] 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

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



advantage of the vinyl sulfone stability in water over the activation chemistries used on those strategies.[31] Moreover, the vinyl sulfone group possess 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 point 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 alkyne−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. Their implementation in a two-step CuAAC-based sequential approach to attain the dual labeling of HRP is illustrative of the utility of these reagents. Although from a chemical point of view both approaches yield the dual labeling, in the context of life science the use of BTSAP is advantageous because it overcomes the drawbacks of copper catalysis in biological systems.

Finally, in the context of biomolecules labeling vinyl sulfone chemistry shares some of the attributes of click chemistry: (i) vinyl sulfone groups reacts selectively toward amines and/or thiols[32,33] to form a stable covalent link with (ii) outstanding efficiency, (iii) no byproduct, and (iv) mild reaction conditions compatible with the biological nature of biomolecules. In fact, the Michael-type addition between vinyl sulfone and thiols is accepted as a click reaction.[34] The reactivity of the vinyl sulfone group offers the additional advantage of being independent of the introduction of non-natural azide or alkyne click functions since amine and thiol groups are naturally present in biomolecules.[32,33,35,36]

Experimental Section

General Experimental Methods. TLC was performed on aluminum sheets. Reagents used for developing plates include potassium permanganate (1% w/v), ninhydrin (0.3% w/v) in ethanol, and UV light when applicable. Flash column chromatography was performed on silica gel (230−400 mesh, ASTM). Melting points are uncorrected. Optical rotations were recorded on a polarimeter at room temperature. ^{1}H and ^{13}C NMR spectra were recorded at room temperature. Chemical shifts are given in ppm and referenced to internal $CDCl_3$. *J* values are given in Hz.

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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 Cl_2CH_2−2-propanol 2:1 was added propargylamine (152 μL, 2.2 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 → EtOAc−MeOH 10:1) to give **2** as a syrup (411 mg, 52%): IR (film) γ_{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 = 2.3 Hz); ^{13}C 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 $C_{13}H_{23}NO_6S_2Na$ $[M + Na]^+$ 376.0864, found 376.0861.

Synthesis of Compound 3. A solution of biotin (200 mg, 0.82 mmol) in Cl_2SO (5 mL) was kept at room temperature for 1 h. The reaction mixture was evaporated in vacuo and coevaporated with anhydrous toluene (3 × 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 Et_3N (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%): $[\alpha]_D$ +18.3 (*c* 1, DMSO); IR (film) γ_{max}/cm^{-1} 3368, 3261, 2923, 2870, 2115, 1696, 1644, 1461, 1288, 1120, 1034; ^1H NMR (MeOD-d_4, 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, 4H), 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); ^{13}C NMR (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 $C_{23}H_{37}N_3O_8S_3Na$ $[M + Na]^+$ 602.1640, found 602.1641.

Synthesis of Compound 4. A solution of rhodamine B (195 mg, 0.41 mmol) in Cl_2SO (5 mL) was kept at room temperature overnight. The reaction mixture was evaporated in vacuo and coevaporated with anhydrous toluene (3 × 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 Et_3N (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 (Cl_2CH_2−MeOH 20:1) giving **4** as a foam solid (272 mg, 86%): IR (film) γ_{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 (1H, d, J = 9.8 Hz), 3.88−3.62 (m, 18 H), 3.32−316 (m, 4H), 2.44(br s, 1H), 1.92 (br s, 4H), 1.42 (t, 12H, J = 7.3 Hz); ^{13}C 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 $C_{41}H_{52}N_3O_8S_2$ $[M]^+$ 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 Et_3N (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 → 3:1) giving **5** as a syrup (257 mg, 86%): IR (film) γ_{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); ^{13}C 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 $C_{25}H_{34}O_8N_2S_3Na$ $[M + Na]^+$ 609.1375, found 609.1368.

Synthesis of Azide−Fluorescein 7. To a suspension of chlorofluorescein[37] (400 mg, 0.94 mmol) in MeOH (20 mL) was added sodium azide (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 → MeOH) giving **7** as a solid (330 mg, 82%): mp > 270 °C dec; IR (KBr) γ_{max}/cm^{-1} 3388, 2119, 2036, 1680, 1628, 1588, 1465, 1397, 1330, 1270, 1169, 1109; ^1H NMR (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); ^{13}C NMR (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 $C_{22}H_{14}O_6N_4Na$ $[M + Na]^+$ 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**[20] (0.24 mmol), respectively, Et_3N (85 μL, 0.6 mmol), and $(EtO)_3P \cdot CuI$[22] (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%): mp >260 °C dec; $[\alpha]_D$ +25 (*c* 1, DMF); IR (KBr) γ_{max}/cm^{-1} 3398, 1689, 1635, 1457, 1310, 1136; ^1H NMR (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 = 7.5 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, 2H), 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); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 172.3, 162.6, 151.2, 143.0, 137.9, 135.4, 129.5, 128.9, 128.8, 128.5, 128.1, 127.8, 123.4, 118.8, 115.1, 69.3, 69.2, 66.2, 63.8 × 2, 60.9, 59.0, 55.3, 53.4, 52.6, 51.1, 49.2, 44.9 × 2, 42.2, 39.7, 38.9, 32.1, 28.1 × 2, 24.5; HRMS (NALDI-TOF) calcd for $C_{37}H_{54}O_{10}N_8S_4Na$ $[M + Na]^+$ 921.2743, found 921.2743.

Synthesis of BTSAP Reagent 10. Column chromatography (EtOAc−MeOH 3:1 → MeOH) gave **10** as a foam solid (140 mg, 70%): $[\alpha]_D$ +22 (*c* 0.25, DMF); IR (film) γ_{max}/cm^{-1} 3358, 2916, 1678, 1562, 1454, 1280, 1238, 1099; ^1H NMR (DMSO-d_6 × D_2O, 500 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 and 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); ^{13}C NMR (DMSO-d_6 × D_2O, 125 MHz) δ

(37) Aita, K.; Temma, T.; Kuge, Y.; Saji, H. *Luminescence* **2007**, *22*, 455–461.

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, 127.6, 124.8, 124.7, 118.4, 117.9, 110.5, 102.4, 69.4, 69.3, 63.9, 61.0, 59.1, 55.4, 53.5, 52.7, 51.2, 42.6, 40.1, 32.2, 28.2, 28.1 × 2, 24.6; HRMS (MALDI-TOF) calcd for $C_{45}H_{51}O_{14}N_7S_3$ $[M + H]^+$ 1010.2734, found 1010.2730.

Synthesis of BTSAP Reagent 11. Column chromatography (EtOAc−MeOH 3:1 → MeOH) gave **11** as a foam solid (157 mg, 72%): IR (film) γ_{max}/cm^{-1} 1640, 1588, 1466, 1413, 1275, 1180, 1128; ^1H NMR (DMSO-d_6, 500 MHz) δ 8.09 (s, 1H), 7.90 (m, 1H), 7.75 (m, 2H), 7.55 (m, 1H), 7.17−7.08 (several m, 3H), 6.99−6.89 (several m, 3H),6.85 (dd, 1H, J = 16.6 and 9.9 Hz), 6.35 (br s, 2H), 6.13 (d, 1H, J = 16.3 Hz), 6.11 (d, 1H, J = 9.8 Hz), 4.51 (br s, 2H), 4.37 (t, 2H, J = 5.8 Hz), 4.26 (m, 1H), 4.08 (m, 1H), 3.77−3.28 (several m, 21H), 3.17 (m, 1H), 3.03 (m, 4H), 2.77 (dd, 1H, J = 12.4 and 5 Hz), 2.66 (m, 1H), 2.54 (d, 1H, J = 12.4 Hz), 2.02 (m, 2H), 1.56−1.38 (several m, 4H), 1.20 (several m, 14H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 177.7, 173.3, 167.8, 162.3, 162.1, 160.6, 160,3, 147.1, 143.2, 140.3, 136.8, 135.8, 135.1, 135.0, 133.8, 132.7, 132.6, 119.5, 118.2, 109.9, 101.1, 74.6, 74.5, 71.5, 69.0, 68.9, 66.2, 64.2, 60.5, 58.7, 57.4, 55.3, 54.2, 50.5, 50.4, 45.0, 43.9, 40.2, 33.3, 33.2, 30.3, 17.7, 13.7; HRMS (MALDI-TOF) calcd for $C_{53}H_{72}O_{10}N_9S_3$ $[M]^+$ 1090.456, found 1090.458.

Synthesis of Compound 15. To a solution of **14**[38] (577 mg, 4.24 mmol) in THF−2-propanol 1:2 (20 mL) was added propargylamine (212 mg, 3.85 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−MeOH 5:1) giving **15** as a solid (710 mg, 87.6%): mp 79−81 °C; IR (KBr) γ_{max}/cm^{-1} 3309, 3262, 3145, 1474, 1360, 1287, 1256, 1119, 1080, 1023; ^1H NMR (DMSO-d_6, 500 MHz) δ 5.08 (br s, 1H), 3.76 (t, 2H), 3.31 (d, 2H, J = 2.4 Hz), 3.23 (m, 4H), 3.07 (t, 1H, J = 2.4 Hz), 2.94 (t, 2H, J = 6.8 Hz), 2.23 (br s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 80.9, 72.5, 56.7, 56.2, 54.6, 41.7, 37.9.

Synthesis of Compound 16. To a solution of **15** (160 mg, 0.84 mmol) in CH_2Cl_2 (20 mL) were added dansyl chloride (680 mg, 2.52 mmol) and Et_3N (0.71 mL, 5.02 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−hexane 2:3) giving **16** as a syrup (230 mg, 68%): IR (film) γ_{max}/cm^{-1} 3273, 1568, 1454, 1387, 1309, 1135, 1069; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.62 (d, 1H, J = 8.5 Hz), 8.28 (d, 1H, J = 8.7 Hz), 8.24 (d, 1H, J = 7.4 Hz), 7.60 (m, 2H), 7.24 (d, 1H, J = 7.6 Hz), 6.65 (dd, 1H, J = 16.5 and 9.7 Hz), 6.45 (d, 1H, J = 16.5 Hz), 6.21 (d, 1H, J = 9.7 Hz), 4.23 (d, 2H, J = 2.3 Hz), 3.77 (m, 2H), 3.42 (m, 2H), 2.92 (s, 6H), 2.22 (t, 1H, J = 2.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 151.9, 135.9, 133.3, 131.2, 131.1, 130.1, 130.0, 128.5, 123.1, 119.0, 115.4, 76.8, 74.5, 52.5, 45.4, 41.0, 37.4; HRMS (FAB+) calcd for $C_{19}H_{22}O_4N_2S_2Na$ $[M + Na]^+$ 429.0919, found 429.0914.

Synthesis of BTSAP Reagent 17. To a solution of **16** (128 mg, 0.31 mmol) in MeOH (15 mL) were added **8** (89 mg, 0.29 mmol), Et_3N (122 μL, 0.87 mmol), and $(EtO)_3P\cdot CuI$ (10 mg, 0.0.29 mmol) as catalyst. The reaction mixture was stirred at room temperature for 2 h. Evaporation of the solvent yielded a crude that was purified by column chromatography (EtOAc−MeOH 4:1) giving **17** as a foam solid (188 mg, 92%): IR (film) γ_{max}/cm^{-1} 3390, 3297, 1694, 1569, 1456, 1311, 1139, 1074; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.55 (d, 1H, J = 8,6 Hz), 8.21 (d, 1H, J = 8.5 Hz), 8.15 (d, 1H, J = 7.2 Hz), 7.64 (s, 1H), 7.54 (m, 2H), 7.17 (d, 1H, J = 7.4 Hz), 7.03 (br s, 1H), 6.58 (m, 2H), 6.28 (d, 1H, J = 16.4 Hz), 6.09 (d, 1H, J = 9.8 Hz), 5.86 (br s, 1H), 4.59 (s, 2H), 4.45 (s, 2H), 4.30 (br s, 1H), 3.63 (m, 4H), 3.26 (m, 2H) 3.10 (br s, 1H), 2.87 (s, 6H), 2.69 (d, 1H, J = 12.5 Hz), 2.13 (br s, 2H), 1.80−1.30 (several m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 174.1, 164.3, 152.0, 142.7, 135.8, 133.7, 131.2, 130.1, 129.9, 129.8, 128.7, 124.6, 123.3, 118.8, 115.5, 61.7, 60.2, 55.8, 53.9, 52.3, 49.8, 45.4, 43.1, 41.3, 40.7, 39.4, 35.7, 29.7, 28.2, 28.1, 25.5; HRMS (NALDI-TOF) calcd for $C_{31}H_{42}O_8N_6S_3Na$ $[M + Na]^+$ 741.2287, found 741.2281.

Labeling of HRP with Vinyl Sulfone Based BTSAP Reagent 10. HRP (2 mg/mL, 300 μL) was incubated with vinyl sulfone derivatized bifunctional tag reagent **10** (25 mM in DMSO, 5.5, 11, 16.5, 22, and 27.5 μL, 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 °C) and 37 °C for 1 day to yield the corresponding labeled **HRP-10**.

Labeling of HRP with Vinyl Sulfone Based BTSAP 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 μL, stoichiometries 1:25 and 1:50, respectively) and with **9** and **17** (25 mM in DMSO, 138 μL, stoichiometry 1:50) in 100 mM HEPES buffer pH 8.5 at 37 °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 μL, 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 °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 μL, 2 mg/mL in 50 mM phosphate buffer, pH 8) were added $CuSO_4\cdot 5H_2O$ (10 μL, 10 mM stock solution in deionized water), DTE (10 μL, 50 mM stock solution in deionized water), 1,10-phenanthroline (10 μL, 20 mM stock solution in DMSO), **7** (5 μL, 30 mM stock solution in DMSO), and deionized water (15 μL). 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 μL, 2 mg/mL in 100 mM HEPES buffer, pH 8.5) was added a solution of **11** (69 μL, 25 mM in DMSO). The reaction was incubated at 37 °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.[39]

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 μL, 25 mM in DMSO). The reaction was incubated at 37 °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 μL, 1.25 mg/mL in 50 mM phosphate buffer, pH 8) were added $CuSO_4\cdot 5H_2O$ (20 μL, 30 mM stock solution in deionized water), DTE (20 μL, 150 mM stock solution in deionized water), 1,10-phenanthroline (20 μL, 60 mM stock solution in DMSO), **8** (50 μL, 25 mM stock solution in DMSO), and deionized water (100 μL). The coupling reaction was carried out at room temperature for 7 h, and a 300 μL of aliquot was dialyzed in 50 mM phosphate buffer pH 8 (**HRP-SA7h**). After 21 h, another 300 μL of aliquot was also dialyzed (**HRP-SA21h**). The protein concentration was established by Bradford.[37]

HRP Activity Assays. Labeled-HRP (10 μL, ~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 μL of H_2O_2 (10 vol)/50 mL), and the absorbance was measured at 492 nm in kinetic mode for 1 min.

Interaction Assays between Biotin−HRP and Avidin. Microtitration plates were coated with avidin at 200 μL/well of a solution of 2 μg/mL in 100 mM carbonate buffer pH 9.6 overnight at 4 °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 μL/well of BSA in PBS (0.1% w/v) for 1 h at 37 °C. Each biotin-labeled HRP and nonlabeled HRP were added in serial dilution (200 μL/well) in 50 mM phosphate buffer pH 7.5, 100 mM NaCl, and the plates were incubated for 1 h at 37 °C. After that, 200 μL/well of a solution of *o*-phenylenediamine dihydrochloride (20 mg/50 mL) in 150 mM citrate buffer (pH 5.0 with 100 μL H_2O_2 (10 vol/50 mL) was added. The plates were incubated for 20 min at 37 °C, and the reactions were stopped by addition of aqueous H_2SO_4 (50 μL/well, 1.25 M).

Electrophoresis of the Labeled Proteins. Samples analyzed by SDS−PAGE[40] in a 12% polyacrylamide gel (12%T/1.3% C) in a MiniProtean 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.