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Sulfa-Michael Addition on Dehydroalanine: A Versatile Reaction for Protein Modifications

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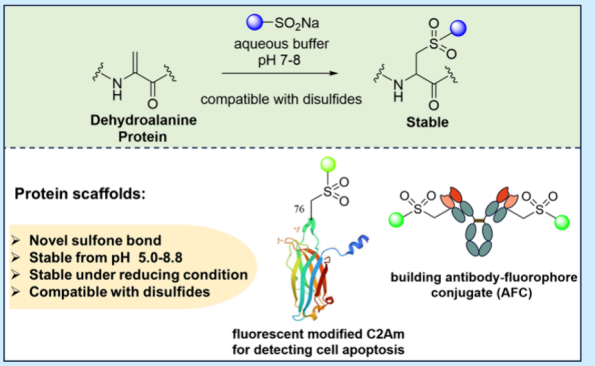
ABSTRACT: Chemical modification of proteins has been widely applied in diagnostic and therapeutic processes. Here, we report a novel bioconjugation between sulfinic acids and amino acid dehydroalanine (Dha) in the context of both small molecules and proteins. This conjugation enables the rapid formation of sulfone linkages in a chemoselective and disulfide-compatible manner under biocompatible conditions with Dha residues chemically installed in proteins and thus provides a robust tool that is simple and has exquisite site selectivity for protein functionalization in a wide range.



Chemical attachment of synthetic molecules to a specific site of a protein is an ongoing challenge in chemical biology and an essential tool for a plethora of applications in the life sciences, in particular for the investigation of biological processes, disease diagnosis, the construction of protein conjugates for targeted therapeutics, and their tracking in cells.[1−5] Therefore, a broad range of bioconjugation reactions for modifying and functionalizing peptides and proteins at the N-termini, C-termini, and side chains of proteinogenic residues such as lysine, cysteine, methionine, tyrosine, histidine, arginine, tryptophan, serine, etc., have been developed in recent years.

In addition to modifying natural amino acid residues on proteins, dehydroalanine (Dha), as an unnatural and unsaturated amino acid residue chemically installed by cysteine, which has a low natural abundance in its reduced form on accessible protein surfaces, possessed a unique electrophilic character.[6−13] As versatile synthetic precursor moieties, dehydroalanine can therefore be readily transformed into various natural and unnatural amino acid derivatives. The representative α,β-unsaturated carbonyl moiety of Dha could act as a Michael acceptor and undergo Michael addition with various nucleophiles, which is one of the most powerful and widely used synthetic tools. So far, several types of Michael addition reactions have been reported with different nucleophiles for site-specific modification of peptides and proteins at Dha residues in aqueous media at moderate pH values and temperatures of <40 °C, including thia-Michael-type (C−S),[14−20] aza-Michael-type (C−N),[10,21,22] selena-Michael (C−Se),[23] and phospha-Michael (C−P)[24−26] addition reactions. On the contrary, Dha could also act as a competent conjugated electrophile, as well as an efficient partner radical acceptor ("SOMO-phile") of carbon-centered C• radical additions to install a C−C bond on proteins.[27,28] The formation of novel C−X or C−C covalent bonds manipulates the attachment of affinity tags, fluorophores, cargoes, and secondary reaction sites such as click handles, which enabled the development of robust and reliable methods for variously functionalized proteins [e.g., mimicking post-translational modifications (PTMs), such as phosphorylation, acetylation, methylation, sulfonation or glycosylation, building ADCs, etc.] and promoted diagnosis of and therapeutics for various diseases.[29−31] However, concerns about these conjugations remained, including thiol exchange,[13,16] interfering with the stability of the structure,[13,16,23] changing the charge of the protein,[23] side reactions, etc. The thia-Michael-type bioconjugation still appeared to be the most commonly used reaction for Dha-based peptides and protein modification and/or functionalization. Although it is extremely powerful, a critical drawback of this method is the potential interference of existing disulfide bonds present in proteins by the small molecular sulfur nucleophile probes, which might form mixed disulfide to disrupt the native structures and functions of proteins.

To sidestep this drawback, we initiated a program to investigate the applicability of sulfinic acids in developing the

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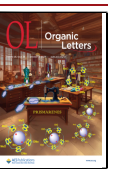

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disulfide-compatible sulfur nucleophile−Dha conjugation reaction, as sulfinates and sulfinic acids were generally thought to be alternative options of thiols for Michael addition to yield sulfones, but not to react with disulfide bonds.[32] Here, we report this simple and efficient methodology for Dha-specific protein modification. We proved that this reaction proceeded under mild conditions with no requirement of transition-metal catalysts, resulting in stable sulfonyl derivatives conjugated with a β,γ-C−S(O)$_2$ bond. We demonstrated the potential of the sulfinic acid−Michael ligation method for three different proteins [C2Am, β-lactoglobulin, and monoclonal antibodies (mAbs)]. We deemed that this novel, simple, and robust methodology with excellent disulfide compatibility is likely to find broad application for the modification of proteins used in diagnostic and therapeutic applications.

To investigate the reactivity of Dha toward sulfinic acid, we first utilized the Ac-Dha methyl ester (**1**) as a Dha small molecular model to react with a series of sulfinic acid (or sodium sulfinate) nucleophiles, which were expected to form different sulfone hybridizations. The reactions were carried out in a 1:1 MeOH/50 mM sodium phosphate buffer mixture at near neutral pH 7.4 and 37 °C. As shown in Figure 1a, the

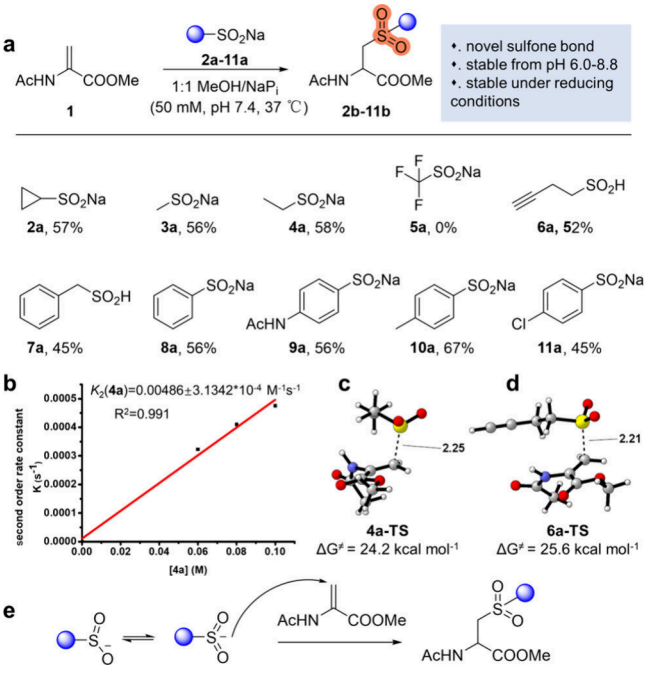

Figure 1. (a) Reaction of **1** with sulfinic acid nucleophiles. General conditions for sulfinic acid addition to Dha: **1** (1 equiv) and sulfinic acids **2a−11a** (10 equiv) in a 1:1 MeOH/sodium phosphate buffer (50 mM, pH 7.4) mixture at 37 °C for 24 h. All yields were calculated after SiO_2 flash column chromatography purification. (b) Experimental determination of the second-order rate constant for the addition of sodium ethyl sulfinate (**4a**) to **1**. (c and d) Calculated transition structures and associated activation free energies ($\Delta G^{\ddagger}$) of model reactions. (e) Mechanism of the sulfa-Michael reaction.

reactions performed well with the expected sulfone products obtained in moderate yields (45−67%), except for electron-deficient sulfonate **5a**, which had a much lower nucleophilicity and showed no reactivity toward Dha. To more accurately quantify sulfinic acid/sulfinate reactivity, a ^{1}H nuclear magnetic resonance (NMR) analysis was developed to monitor the kinetics of Dha conjugation by different sulfinic/sulfinate nucleophiles (Figure 1b). As demonstrated in Figure S1, the second-order constants (k_2) of these reactions were determined to range from 4.86 to 6.79 × 10^{-3} M^{-1} s^{-1} separately, with no significant difference observed between aryl and alkyl sulfinic acids. These values are approximately 1−2 orders of magnitude higher than that of another disulfide-compatible aza-Dha Michael ligation. These results were in good agreement with the results of M06-2X33 density functional theory calculations, revealing the higher nucleophilic character of **4a** and **6a**, compared to that of reactive N nucleophiles such as N-sp^3 in benzylamine and N-sp^2 in imidazole.[21]

As the conjugate stability is also an important characteristic for diagnostic and therapeutic applications of protein conjugation, we evaluated the stability of the newly formed sulfone linkage at different pHs for 24 h via high-performance liquid chromatography (HPLC) analysis. We demonstrated that the sulfone linkage was stable over a broad pH range (6.0−8.0) with no degradation observed (Figure S3). These findings inspired us to synthesize various functionalized sufinic electrophiles (Figure 2). Utilizing commercially accessible

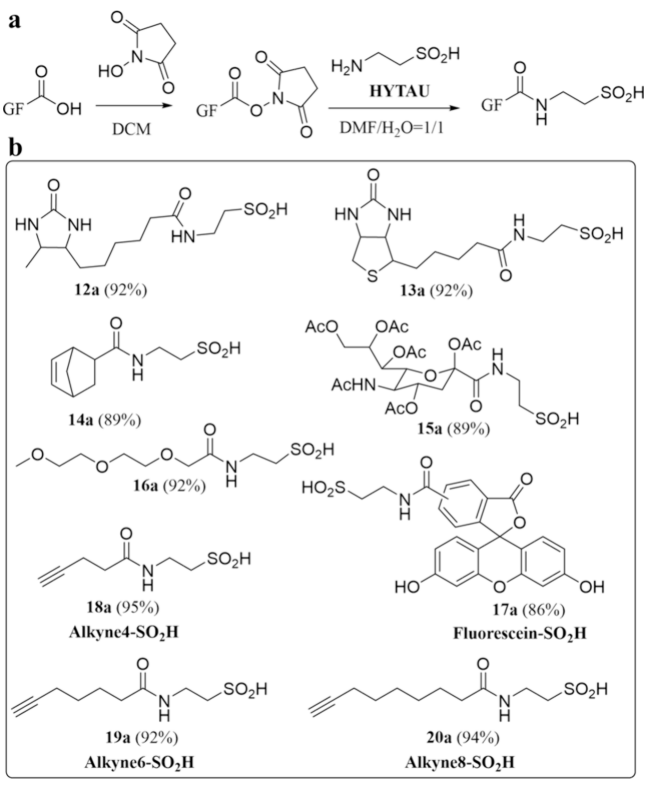

Figure 2. (a) Synthetic procedure for the generation of functionalized sulfinic acid nucleophiles. (b) Functional nucleophiles obtained via the synthetic procedure depicted in excellent yield (values represent isolated yields of SiO_2).

hypotaurine [2-aminoethylsulfinic acid (HYTAU)] as a precursor for the synthesis, we were able to easily synthesize a series of sulfinic acid reagents bearing a fluorophore (**17a**), hydrophilic tags (**16a**), lipid tags (**18a−20a**), affinity tags (**12a** and **13a**), bioorthogonal click handles (**14a** and **18a−20a**), and *N*-acetylneuraminic acid (**15a**) in excellent yields via amidation with functional group-tethered activated esters (NHS esters). Moreover, the purified sulfinic acids with a terminal alkyne warhead attached were quite stable in solution

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at neutral pH over several days and could be stored at 4 °C for several months without any observable decomposition.

Encouraged by these small molecule results, we next studied the application of this bioconjugation in recombinant proteins to evaluate its potential as a novel protein ligation strategy for the construction of a sulfone linkage with protein conjugates. To achieve this aim, we selected a single cysteine mutation of the C2A domain of synaptotagmin-I (C2Am) that could act as a diagnostically relevant apoptosis imaging agent with the accessibility of the engineered Cys residue on the protein surface.[33] First, we successfully generated Dha from the engineered Cys at position 76 on the C2Am protein via the previously reported procedure.[7] Next, we assessed the specificity and effectiveness of sulfinic acids bearing terminal alkynes (Alkyne4-SO_2H, Alkyne6-SO_2H, or Alkyne8-SO_2H) in C2Am-Dha76 modification by Western blot analysis. As shown in Figure 3, a significant signal of the C2Am band was

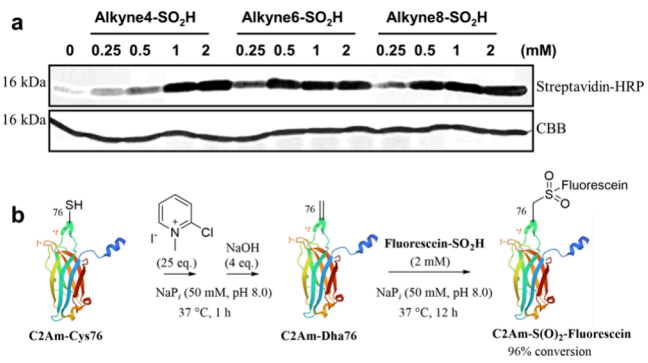

Figure 3. (a) Concentration-dependent covalent attachment of Alkyne4-SO_2H, Alkyne6-SO_2H, and Alkyne8-SO_2H with C2Am-Dha76, following the "CuAAC" reaction, and then Western blotting. CBB = Coomassie gel. (b) Synthetic scheme of chemically site-selective Dha modification with Flluorescein-SO_2H on C2Am-Dha76.

observed when labeled with 250 μM Alkyne4-SO_2H, Alkyne6-SO_2H, or Alkyne8-SO_2H (~7 equiv of protein Cys). The intensities of the signals increased with the labeling reagent concentration and reached a maximum with 0.5 mM Alkyne6-SO_2H (~14 equiv of protein Cys). Our results also revealed that only 120 min was required for labeling C2Am-Dha76 with Alkyne6-SO_2H (1 mM) (Figure S4). An increase in the labeling time did not trigger a significant increase in the intensity of the protein band. The presence of the expected sulfone linkage at position 76 was further confirmed by tryptic digestion and MS/MS analysis (Figure S11). According to the external calibration curve approach, the conversion of Fluorescein-SO_2H ligated to C2Am-Dha76 was calculated to be 96% (Figure 3 and Figure S10).

Next, we sought to broaden the scope of this reaction to other proteins. Thus, we investigated the addition of Alkyne4-SO_2H, Alkyne6-SO_2H, and Alkyne8-SO_2H to engineered Dha residues in both β-lactoglobulin and trastuzumab. Prepared protein Dha was co-incubated with different concentrations of Alkyne4-SO_2H, Alkyne6-SO_2H, and Alkyne8-SO_2H separately for 2 h and further analyzed by Western blot assays. Consistent with C2Am-Dha76, the close labeling efficiencies of three probes were demonstrated with respect to β-lactoglobulin Dha in a concentration-dependent manner (Figure S5), while different labeling efficiencies were observed with these probes toward trastuzumab Dha. Alkyne8-SO_2H exhibited the highest labeling efficiency, followed by Alkyne6-SO_2H and Alkyne4-SO_2H with shorter alkane chain lengths, probably owing to the increased complexity and lipophilicity of trastuzumab compared to C2Am and β-lactoglobulin (Figure S7). Then, Alkyne6-SO_2H was chosen to perform the time-dependent labeling test with respect to β-lactoglobulin Dha and trastuzumab Dha (Figures S6 and S8), and a rapid coupling rate was observed with the labeling reaching equilibrium within 30 min. The presence of the expected sulfone linkage was further confirmed by tryptic digestion and MS/MS analysis (Figures S12 and S13). We analyzed the adjacent sequences of labeled peptides and unlabeled peptides with Dha residues in C2Am, β-lactoglobulins, and trastuzumab, and the results suggested no amino acid/sequence preference in this conjugation reaction on these three recombinant proteins. More proteins with Dha residues were still required to be tested to illuminate amino acids or sequence preference of our conjugation reaction (Figure S15). These chemical modifications proceed with minimal perturbation of the global structure of C2Am, as evidenced by circular dichroism (CD) analysis (Figure S16).

To verify the disulfide compatibility of this new sulfinate addition methodology, we synthesize a disulfide compound **37** as a small molecule disulfide model. **37** was treated with 10 equiv of sodium benzenesulfinate (**8a**) in a 1:1 PB/MeOH mixture at pH 7.4 and 37 °C for 24 h, and the reaction was analyzed by HPLC analysis. We were encouraged that no product was observed, and it suggested that the disulfide bond maintained its integrity in the presence of sodium sulfinate in small molecule mode. Next, we compared the disulfide compatibility of commonly used Dha modification ligations (thia-Michael-type, phospha-Michael ligation) and our sulfinate addition methodology by evaluating the addition of the thiol, phosphine, and sulfinate to a particularly relevant protein, trastuzumab, which contains 16 structural disulfides without a free cysteine thiol via Western blot analysis. Recombinant trastuzumab was treated with 4 mM β-mercaptoethanol (thiol model), TCEP (phosphine model), sodium ethanesulfinate (sulfinic acid model), or PBS (control) for 12 h at 37 °C, resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under reducing and nonreducing conditions, and analyzed using Coomassie blue staining. As shown in Figure 4 (lanes 7−10), two separate protein bands of the heavy (H) chain at 50 kDa and the light (L) chain at 25 kDa were observed under reducing conditions for all four samples, owing to the cleavage of protein disulfide bonds with DTT in loading buffer. However, under the nonreducing SDS−PAGE conditions (without DTT), the unreduced trastuzumab with intact interchain disulfide bonds theoretically displays a band at ~180 kDa, corresponding to the H2L2 form. Our results revealed that only protein bands of 180 kDa were detected for both control and ethanesulfinate-treated trastuzumab (lanes 2 and 4) under the nonreducing SDS−PAGE conditions, indicating the intact interchain disulfide bonds in trastuzumab are retained. In contrast, trastuzumab treated with β-mercaptoethanol (lane 3) or TCEP (lane 5) exhibited bands with a molecular weight of ~50 or 25 kDa, respectively, under nonreducing SDS−PAGE conditions, suggesting the cleavage of disulfides in the interchain disulfide bridges of trastuzumab. Importantly, we conclude that the new method for installing sulfone bonds on proteins via a new sulfinate−Michael ligation is fully compatible with disulfide bonds, whereas thia-Michael-type and phospha-Michael-type ligation might raise concerns about the destruction of protein structural disulfide bonds.

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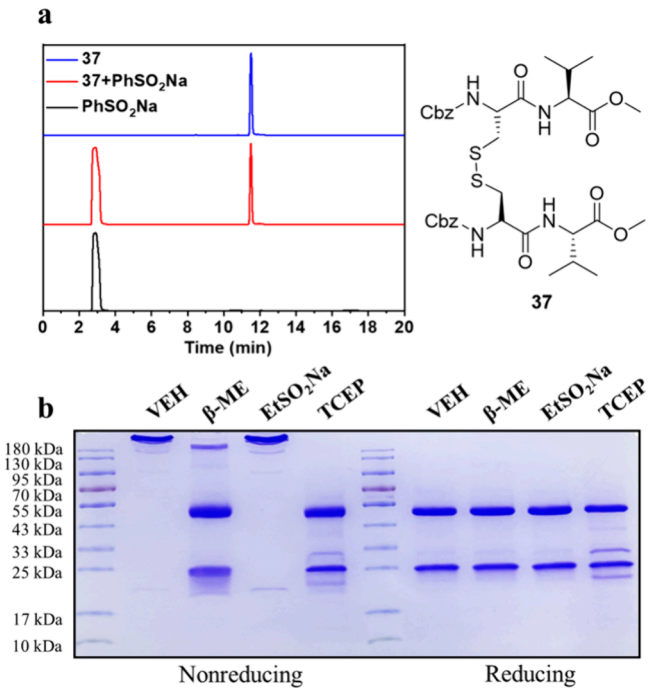

Figure 4. (a) HPLC analysis of the reaction between a disulfide compound (**37**) and model compound sodium benzenesulfinate (**8a**). (b) SDS−PAGE analysis of the compatibility of **4a** and disulfide bonds in the context of trastuzumab.

C2Am specifically labels apoptotic cells by binding to phosphatidylserine (PS) on their cell membrane and was therefore validated for in vivo detection of apoptotic tissue.[34,35] We investigate whether C2Am modified with a fluorescent dye through a sulfone linkage retains its biological function and thus enable the fluorescent detection of apoptotic cells. We equipped C2Am-Dha76 with Alkyne6-SO_2H, followed by the CuAAC reaction to install fluorophore 545 to form conjugate C2Am-Alkyne6-$S(O)_2$-Fluor545. Meanwhile, according to the procedure reported in the literature, we also generated the C2Am-AF647 and C2Am-$S(O)_2$-Alkyne6-AF647 conjugates that have been reported not to interfere with its binding to PS of apoptotic cells. SDS−PAGE analysis confirmed the successful preparation of these protein conjugates. The MAD-MB-231 cells were treated with different concentrations of doxorubicin (0, 1, 5, and 20 μg/mL) to induce cell apoptosis, labeled with C2Am-$S(O)_2$-Alkyne6-AF647 or C2Am-AF647 (positive control), and imaged by confocal microscopy.[36] Pleasingly, similar results were observed for C2Am-$S(O)_2$-Alkyne6-AF647/C2Am-AF647; i.e., both C2Ams accumulated in late apoptotic/necrotic MAD-MB-231 cells but not normal cells (Figure S18). We therefore verified that the C2Am-$S(O)_2$-Alkyne6-AF647 conjugate retained its ability to preferentially bind to apoptotic/necrotic cells over viable cells, at a level comparable to that of the unmodified protein, highlighting the potential of the sulfone linkage conjugation method for the construction of functional modified proteins.

To further demonstrate the applicability of our method to the construction of protein conjugates, we proceeded with a proof-of-principle study to investigate the potential of this reaction in the development of ADCs via the construction of antibody-fluorophore conjugates (AFCs). For antibody modification, we applied a previously described analogous protocol to prepare mAbs-Dha and co-incubated it with Flluorescein-SO_2H (**17a**) to generate an antibody-fluorophore conjugate (AFC) (Figures S19 and S20). The successful modification was confirmed by in-gel fluorescence measurements of the antibody's heavy and light chain (Figure 5b). Meanwhile, the

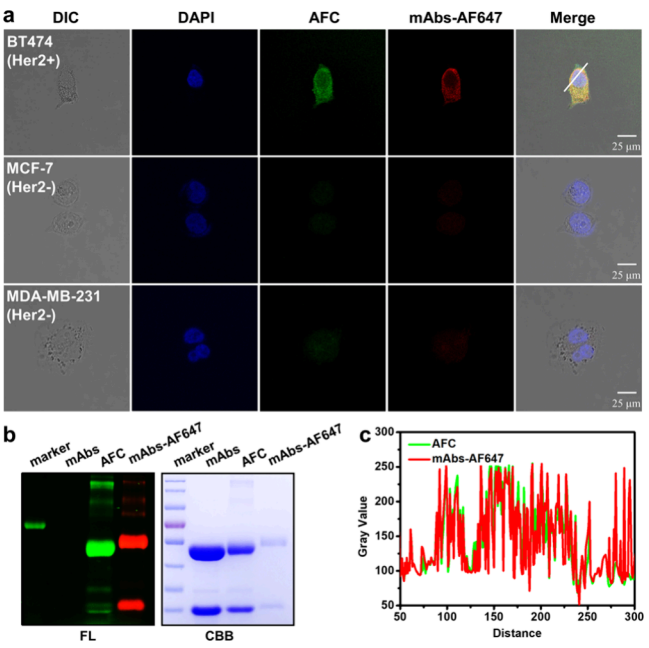

Figure 5. (a) Immunostaining of fixed cells, either overexpressing cell surface receptor Her2 (BT474) or exhibiting low Her2 expression levels (MCF-7 and MDA-MB-231). The nucleus stained with DAPI and AFC signal is colored green, and the mAbs-AF647 signal is colored red. The scale bar is 25 μm. (b) SDS−PAGE of trastuzumab (lane 2), AFC (lane 3), and trastuzumab nonspecifically modified with AF647 (mAbs-AF647) (lane 4). (c) Normalized fluorescence profiles of AFC and mAbs-AF647 along the white line in the merge panel.

functionalized trastuzumab showed excellent Her2 selectivity and clearly stained the outer membrane-bound Her2 on BT474 cells, while no fluorescence signal could be observed with the Her2-negative cell line (MAD-MB-231 and MCF-7) (Figure 5a). This experiment clearly showed that our modification strategy does not affect the antibody's performance and provides a simple conjugation approach for diagnostic reagents. Furthermore, the stability of AFC was performed by in-gel fluorescence measurements, and the result demonstrated that the newly formed sulfone linkage of the AFC was stable under different pH conditions (5.0−8.8), 10% FBS, or reductive conditions (1 or 10 mM GSH) for 24 h (Figure S21). These results indicated the potential of our strategy for targeted therapy.

In conclusion, we have developed a sulfinic acid-Michael addition to internal Dha residues as a chemoselective and biocompatible protein site-selective modification methodology for the construction of homogeneous protein conjugates. The pseudo-second-order rate constants of our conjugation were 1−2 orders of magnitude faster than the rates of previously reported N-nucleophile additions to Dha residues, and the sulfone linkage of small molecule or protein conjugates showed excellent stability, possessing great compatibility with all proteinogenic amino acids and disulfide of proteins. In addition, our modification strategy has been verified not to

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affect the proteins' performance and could provide a simple conjugation approach for multifunctionality, including as diagnostic reagents, targeted therapy, or simulated post-translational modification.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.4c02970.

Experimental procedures and characterization of products, including NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Professors Yong Tang and Zuowei Xie on the occasion of their 60th birthdays.

■ REFERENCES

(1) Sletten, E. M.; Bertozzi, C. R. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974−6998.

(2) Hoyt, E. A.; Cal, P. M. S. D.; Oliveira, B. L.; Bernardes, G. J. L. Contemporary approaches to site-selective protein modification. *Nat. Rev. Chem.* **2019**, *3*, 147−171.

(3) Krall, N.; da Cruz, F. P.; Boutureira, O.; Bernardes, G. J. L. Site-selective protein-modification chemistry for basic biology and drug development. *Nat. Chem.* **2016**, *8*, 103−113.

(4) Row, R. D.; Prescher, J. A. Constructing new bioorthogonal reagents and reactions. *Acc. Chem. Res.* **2018**, *51*, 1073−1081.

(5) Wang, C.; Zhao, Z.; Ghadir, R.; Yang, D.; Zhang, Z.; Ding, Z.; Cao, Y.; Li, Y.; Fassler, R.; Reichmann, D.; Zhang, Y.; Zhao, Y.; Liu, C.; Bi, X.; Metanis, N.; Zhao, J. Peptide and protein cysteine modification enabled by hydrosulfuration of ynamide. *ACS Cent. Sci.* **2024**, DOI: 10.1021/acscentsci.4c01148.

(6) Bogart, J. W.; Bowers, A. A. Dehydroamino acids: chemical multi-tools for late-stage diversification. *Org. Biomol. Chem.* **2019**, *17*, 3653−3669.

(7) Chalker, J. M.; Gunnoo, S. B.; Boutureira, O.; Gerstberger, S. C.; Fernández-González, M.; Bernardes, G. J. L.; Griffin, L.; Hailu, H.; Schofield, C. J.; Davis, B. G. Methods for converting cysteine to dehydroalanine on peptides and proteins. *Chem. Sci.* **2011**, *2*, 1666−1676.

(8) Dadová, J.; Galan, S. R. G.; Davis, B. G. Synthesis of modified proteins via functionalization of dehydroalanine. *Curr. Opin. Chem. Biol.* **2018**, *46*, 71−81.

(9) Chen, H.; Zhang, Q.; Li, Q. Q.; Zhao, Y. F.; Chen, Y. X.; Li, Y. M. De novo design to synthesize lanthipeptides involving iascade cysteine reactions: SapB synthesis as an example. *J. Org. Chem.* **2018**, *83*, 7528−7533.

(10) Dadová, J.; Wu, K. J.; Isenegger, P. G.; Errey, J. C.; Bernardes, G. J. L.; Chalker, J. M.; Raich, L.; Rovira, C.; Davis, B. G. Precise probing of residue roles by post-translational β, γ-C, N aza Michael mutagenesis in enzyme active sites. *ACS Cent. Sci.* **2017**, *3*, 1168−1173.

(11) Key, H. M.; Miller, S. J. Site- and stereoselective chemical editing of thiostrepton by Rh-catalyzed conjugate arylation: new analogues and collateral enantioselective synthesis of amino acids. *J. Am. Chem. Soc.* **2017**, *139*, 15460−15466.

(12) Jones, L. H. Dehydroamino acid chemical biology: an example of functional group interconversion on proteins. *RSC Chem. Biol.* **2020**, *1*, 298−304.

(13) Lai, K.-Y.; Galan, S. R. G.; Zeng, Y.; Zhou, T. H.; He, C.; Raj, R.; Riedl, J.; Liu, S.; Chooi, K. P.; Garg, N.; Zeng, M.; Jones, L. H.; Hutchings, G. J.; Mohammed, S.; Nair, S. K.; Chen, J.; Davis, B. G.; van der Donk, W. A. LanCLs add glutathione to dehydroamino acids generated at phosphorylated sites in the proteome. *Cell* **2021**, *184*, 2680−2695.

(14) Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. Facile conversion of cysteine and alkyl cysteines to dehydroalanine on protein surfaces: versatile and switchable access to functionalized proteins. *J. Am. Chem. Soc.* **2008**, *130*, 5052−5053.

(15) Chalker, J. M.; Lercher, L.; Rose, N. R.; Schofield, C. J.; Davis, B. G. Conversion of cysteine into dehydroalanine enables access to synthetic histones bearing diverse post-translational modifications. *Angew. Chem., Int. Ed.* **2012**, *51*, 1835−1839.

(16) Meledin, R.; Mali, S. M.; Singh, S. K.; Brik, A. Protein ubiquitination via dehydroalanine: development and insights into the diastereoselective 1,4-addition step. *Org. Biomol. Chem.* **2016**, *14*, 4817−4823.

(17) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. Allyl sulfides are rivileged substrates in aqueous cross-

metathesis: application to site-selective protein modification. *J. Am. Chem. Soc.* **2008**, *130*, 9642−9643.
(18) Naidu, B. N.; Sorenson, M. E.; Bronson, J. J.; Pucci, M. J.; Clark, J. M.; Ueda, Y. Synthesis, in vitro, and in vivo antibacterial activity of nocathiacin I thiol-Michael adducts. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2069−2072.
(19) Naidu, B. N.; Li, W.; Sorenson, M. E.; Connolly, T. P.; Wichtowski, J. A.; Zhang, Y.; Kim, O. K.; Matiskella, J. D.; Lam, K. S.; Bronson, J. J.; Ueda, Y. Corrigendum to "organic reactions in frozen water: Michael addition of amines and thiols to the dehydroalanine side chain of nocathiacins. *Tetrahedron Lett.* **2004**, *45*, 1059−1063.
(20) Schoof, S.; Pradel, G.; Aminake, M. N.; Ellinger, B.; Baumann, S.; Potowski, M.; Najajreh, Y.; Kirschner, M.; Arndt, H. D. Antiplasmodial thiostrepton derivatives: proteasome inhibitors with a dual mode of action. *Angew. Chem., Int. Ed.* **2010**, *49*, 3317−3321.
(21) Freedy, A. M.; Matos, M. J.; Boutureira, O.; Corzana, B. F.; Guerreiro, A.; Akkapeddi, P.; Somovilla, V. J.; Rodrigues, T.; Nicholls, K.; Xie, B.; Jiménez-Osés, G.; Brindle, K. M.; Neves, A. A.; Bernardes, G. J. L. Chemoselective installation of amine bonds on proteins through aza-michael ligation. *J. Am. Chem. Soc.* **2017**, *139*, 18365−18375.
(22) Naidu, B. N.; Sorenson, M. E.; Connolly, T. P.; Ueda, Y. Michael addition of amines and thiols to dehydroalanine amides: a remarkable rate acceleration in water. *J. Org. Chem.* **2003**, *68*, 10098−10102.
(23) Lin, Y. A.; Boutureira, O.; Lercher, L.; Bhushan, B.; Paton, R. S.; Davis, B. G. Rapid cross-metathesis for reversible protein modifications via chemical access to Se-allyl-selenocysteine in proteins. *J. Am. Chem. Soc.* **2013**, *135*, 12156−12159.
(24) He, P. Y.; Chen, H.; Hu, H. G.; Hu, J. J.; Lim, Y. J.; Li, Y. M. Late-stage peptide and protein modifications through phospha-Michael addition reaction. *Chem. Commun.* **2020**, *56*, 12632−12635.
(25) Liu, M.; Sovrovic, M.; Suga, H.; Jongkees, S. A. K. Phosphine addition to dehydroalanine for peptide modification. *Org. Biomol. Chem.* **2022**, *20*, 3081−3085.
(26) Yu, C.; E, R.; An, Y.; Guo, X.; Bao, G.; Li, Y.; Xie, J.; Sun, W. Michael addition reaction between dehydroalanines and phosphites enabled the introduction of phosphonates into oligopeptides. *Org. Lett.* **2024**, *26*, 4767−4772.
(27) Yang, A.; Ha, S.; Ahn, J.; Kim, R.; Kim, S.; Lee, Y.; Kim, J.; Söll, D.; Lee, H. Y.; Park, H. S. A chemical biology route to site-specific authentic protein modifications. *Science* **2016**, *354*, 623−626.
(28) Wright, T. H.; Davis, B. G. Post-translational mutagenesis for installation of natural and unnatural amino acid side chains into recombinant proteins. *Nature Protoc.* **2017**, *12*, 2243−2250.
(29) Josephson, B.; Fehl, C.; Isenegger, P. G.; Nadal, S.; Wright, T. H.; Poh, A. W. J.; Bower, B. J.; Giltrap, A. M.; Chen, L.; Batchelor-McAuley, C.; Roper, G.; Arisa, O.; Sap, J. B. I.; Kawamura, A.; Baldwin, A. J.; Mohammed, S.; Compton, R. G.; Gouverneur, V.; Davis, B. G. Light-driven post-translational installation of reactive protein side chains. *Nature* **2020**, *585*, 530−537.
(30) Mollner, T. A.; Isenegger, P. G.; Josephson, B.; Buchanan, C.; Lercher, L.; Oehlrich, D.; Hansen, D. F.; Mohammed, S.; Baldwin, A. J.; Gouverneur, V.; Davis, B. G. Post-translational insertion of boron in proteins to probe and modulate function. *Nat. Chem. Biol.* **2021**, *17*, 1245−1261.
(31) Kaplaneris, N.; Akdeniz, M.; Fillols, M.; Arrighi, F.; Raymenants, F.; Sanil, G.; Gryko, D. T.; Noël, T. Photocatalytic functionalization of dehydroalanine derived peptides in batch and flow. *Angew. Chem., Int. Ed.* **2024**, *63*, e202403271.
(32) Lu, G. P.; Cai, C.; Chen, F.; Ye, R. L.; Zhou, B. J. Facile sulfa-michael reactions with sodium arylsulfinates in water: the promotion of water on the reaction. *ACS Sustainable Chem. Eng.* **2016**, *4*, 1804−1809.
(33) Alam, I. S.; Neves, A. A.; Witney, T. H.; Boren, J.; Brindle, K. M. Comparison of the C2A domain of Synaptotagmin-I and Annexin-V as probes for detecting cell death. *Bioconjugate Chem.* **2010**, *21*, 884−891.
(34) Krishnan, A. S.; Neves, A. A.; de Backer, M. M.; Hu, D. E.; Davletov, B.; Kettunen, M. I.; Brindle, K. M. Detection of cell death in tumors by using MR imaging and a gadolinium-based targeted contrast agent. *Radiology* **2008**, *246*, 854−862.
(35) Neves, A. A.; Xie, B.; Fawcett, S.; Alam, I. S.; Witney, T. H.; de Backer, M. M.; Summers, J.; Hughes, W.; McGuire, S.; Soloviev, D.; Miller, J.; Howat, W. J.; Hu, D. E.; Rodrigues, T. B.; Lewis, D. Y.; Brindle, K. M. Rapid imaging of tumor cell death in vivo using the C2A domain of Synaptotagmin-I. *J. Nucl. Med.* **2017**, *58*, 881−887.
(36) Eliaz, R. E.; Nir, S.; Marty, C.; Szoka, F. C. Determination and modeling of kinetics of cancer cell killing by doxorubicin and doxorubicin encapsulated in targeted liposomes. *Cancer Res.* **2004**, *64*, 711−718.

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