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Hexafluoroisopropanol as a Bioconjugation Medium of Ultrafast, Tryptophan-Selective Catalysis

Mohammad Nuruzzaman, Brandon M. Colella, Chiamaka P. Uzoewulu, Alissa E. Meo, Elizabeth J. Gross, Seiya Ishizawa, Sravani Sana, He Zhang, Meredith E. Hoff, Bryce T. W. Medlock, Emily C. Joyner, Shinichi Sato, Elon A. Ison, Zibo Li, and Jun Ohata*

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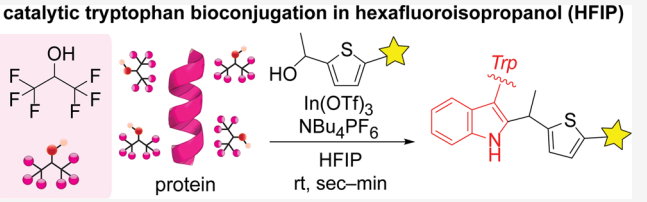
ABSTRACT: The past decade has seen a remarkable growth in the number of bioconjugation techniques in chemistry, biology, material science, and biomedical fields. A core design element in bioconjugation technology is a chemical reaction that can form a covalent bond between the protein of interest and the labeling reagent. Achieving chemoselective protein bioconjugation in aqueous media is challenging, especially for generally less reactive amino acid residues, such as tryptophan. We present here the development of tryptophan-selective bioconjugation methods through ultrafast Lewis acid-catalyzed reactions in hexafluoroisopropanol (HFIP). Structure−reactivity relationship studies have revealed a combination of thiophene and ethanol moieties to give a suitable labeling reagent for this bioconjugation process, which enables modification of peptides and proteins in an extremely rapid reaction unencumbered by noticeable side reactions. The capability of the labeling method also facilitated radiofluorination application as well as antibody functionalization. Enhancement of an α-helix by HFIP leads to its compatibility with a certain protein, and this report also demonstrates a further stabilization strategy achieved by the addition of an ionic liquid to the HFIP medium. The nonaqueous bioconjugation approaches allow access to numerous chemical reactions that are unavailable in traditional aqueous processes and will further advance the chemistry of proteins.



INTRODUCTION

Among many classes of chemical tools devised to date, protein bioconjugation technologies or protein labeling methods have proven exceptionally valuable in a wide variety of contexts. Functionalized antibodies can be elaborated and used for therapeutic,[1] diagnostic,[2] and analytical[3] purposes through installation of cytotoxins, radioisotopes, or detection handles, respectively. Other notable applications of protein bioconjugation include design of covalent inhibitors,[4] proteomics techniques,[3] and identification of new disease-related targets and detection of cellular information such as post-translational modification of proteins[4] as well as the roles of intracellular entities such as metal ions.[5] The backbone of bioconjugation is a chemical reaction that selectively installs functionality of interest to a target biomolecule. As a protein substrate comes into contact with various reactive nucleophilic functional groups in the presence of chemically inert side chains, protein bioconjugation presents many inherent challenges.[6] The demand for new chemical tools persists,[7] and there is always a need to improve the efficiency and kinetics of reactions, enhance chemoselectivity by suppressing side reactions, and avoid bioincompatible agents such as strong acids, oxidants, ultraviolet light, and transition metals.

The past decade has witnessed a substantial advance in useful bioconjugation technologies, although challenges of selective and efficient labeling have not been completely addressed to date for many of the 20 canonical amino acids, including tryptophan. Many sophisticated approaches have been developed through creation of intricate labeling reagents,[8] harnessing (photo)redox chemistry,[9] or control of pH,[10] for example. Such methods have facilitated targeting of various components in proteins such as tyrosine,[11] methionine,[12] C-terminus,[13] N-terminus,[14,15] and even post-translationally modified functional residues[16−18] as well as historically common targets such as cysteine[19] and lysine.[20] On the other hand, tryptophan-selective labeling has been a long-standing issue in the development of bioconjugation,[21−23] even though the low natural abundance of Trp[24] and its commonly low availability on a protein surface offers a better chance for more controlled, selective modification than other abundant, polar amino acid residues. There has been continuous interest in the utilization of indole rings in the organic and medicinal chemistry fields for over a hundred years,[25] and tryptophan-

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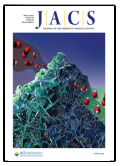

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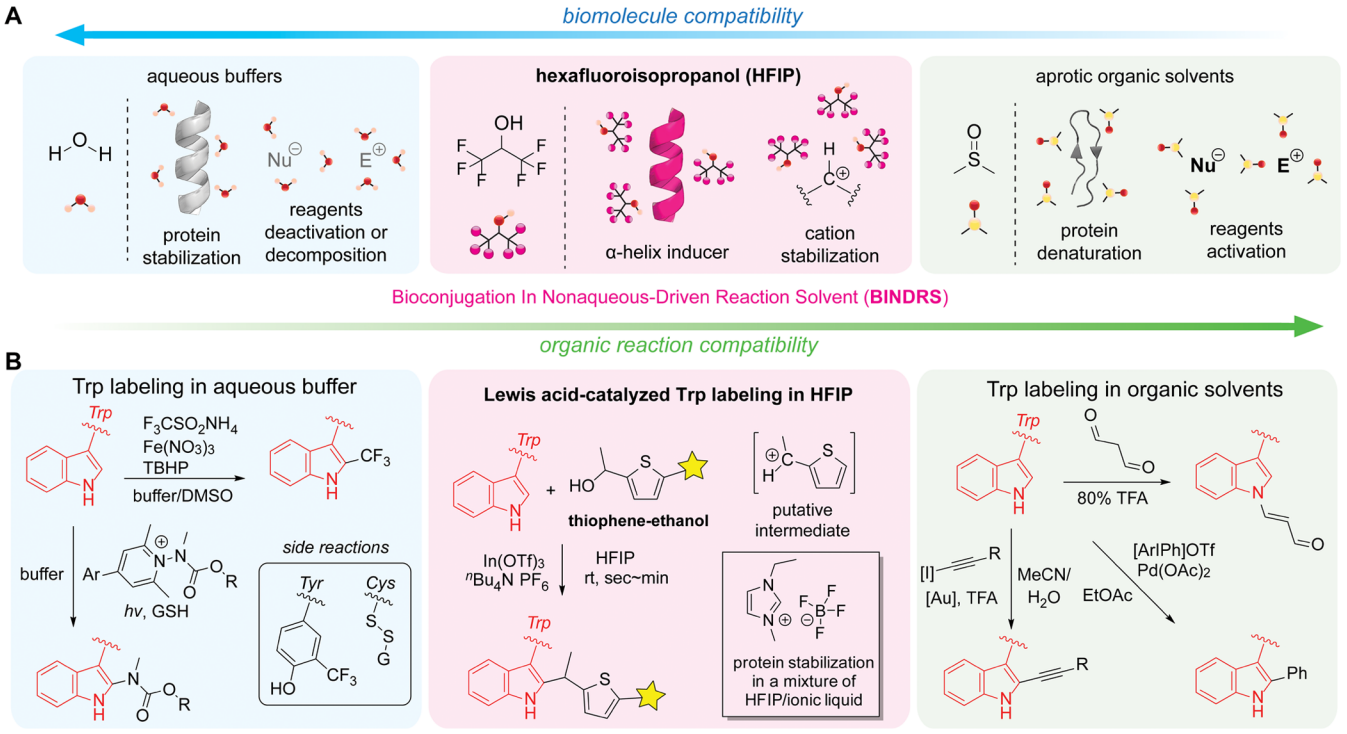

Figure 1. Protein bioconjugation in hexafluoroisopropanol (HFIP). (A) Schematic illustration of potential compatibility of HFIP with stabilization of proteins and chemical reactions. (B) Examples of previously reported Trp labeling in aqueous buffer (left, blue),[34,35] the current work on the Lewis acid-catalyzed reaction in HFIP (middle, red), and examples of previously reported Trp labeling in organic solvents (right, green).[27−29] TBHP: *tert*-butyl hydroperoxide. DMSO: dimethyl sulfoxide. GSH: reduced glutathione; GS-: oxidized glutathione; OTf: triflate; and TFA: trifluoroacetic acid.

targeted labeling in proteins having been attempted even in the early 20th century.[26] A set of indole-selective chemical reactions were examined as tryptophan-targeting methods for proteins in organic solvents,[27−31] although organic solvents are generally incompatible with protein substrates (Figure 1A,B). More recently, elegant tryptophan-selective chemical labeling in an aqueous environment was achieved through a rhodium-catalyzed diazo decomposition reaction,[32] amine oxide-based radical addition,[33] iron-assisted radical trifluoromethylation,[34] or photoinduced electron transfer-based carbamylation.[35] However, none of the aqueous tryptophan labeling methods have become a universal tryptophan labeling strategy to date for various reasons such as unavoidable side reactions (*e.g.*, undesired reactions at tyrosine and cysteine), insufficient reactivity or sluggish kinetics, technical demands (*e.g.*, degassed reaction conditions), need of transition metals (*e.g.*, rhodium and iron), or redox reagents causing protein oxidation (*e.g.*, sodium nitrite). Accordingly, there is still a need for development of protein-compatible, efficient, and rapid tryptophan-selective reactions.

The chemical attributes of hexafluoroisopropanol (HFIP) for enhancement of chemical reactions and stabilization of polypeptide structures could provide a new opportunity for the development of bioconjugation. While, intuitively speaking, aqueous media are the most benign solvents for biomolecules, the extremely polar and protic nature of water as a solvent severely limits the scope of applicable organic chemistry reactions. Despite the undeniable challenges of bioconjugation processes in aqueous media, efforts to discover alternative reaction media that are conducive to both bioconjugation reactions and stabilization of proteins have delivered little. Our research program, termed as Bioconjugation In Nonaqueous-Driven Reaction Solvent (BINDRS), has been pursuing novel bioconjugation technologies in nonaqueous media such as ionic liquids,[36−42] and we hypothesized that HFIP would be a suitable candidate as a biomolecule- and organic chemistry reaction-compatible solvent for nonaqueous bioconjugation strategies (Figure 1A). HFIP and other fluorinated alcohols such as trifluoroethanol have been known to induce a certain secondary structure in peptide (formation of a helicoidal structure)[43] due to their hydrogen-bonding capability, increased acidity of their hydroxyl group, and a high dielectric constant.[44] In addition, there has been considerable growth in synthetic chemistry using HFIP with its unique molecular capabilities,[45] which include stabilization of cationic species.[46,47]

This report presents a bioconjugation strategy to selectively target tryptophan residues of peptides and proteins through Lewis acid-mediated ultrafast catalysis in HFIP (Figure 1B, middle). We found that thiophene-ethanol reagents undergo a formal dehydration process in HFIP with a Lewis acid, such as an indium salt, and induce chemoselective alkylation of an indole ring of a tryptophan residue. Structural examination of this thiophene-based reagent provided a chemical probe bearing a bioorthogonal handle without compromising reactivity for catalytic labeling, which can be complete within minutes. Successful modification of proteins, including a monoclonal antibody, was accomplished, and the compatibility of HFIP with proteins has been demonstrated with the aid of an ionic liquid as a cosolvent.

RESULTS AND DISCUSSION

Alkylation of the indole in a tryptophan residue occurs with a thiophene-ethanol reagent in hexafluoroisopropyl alcohol

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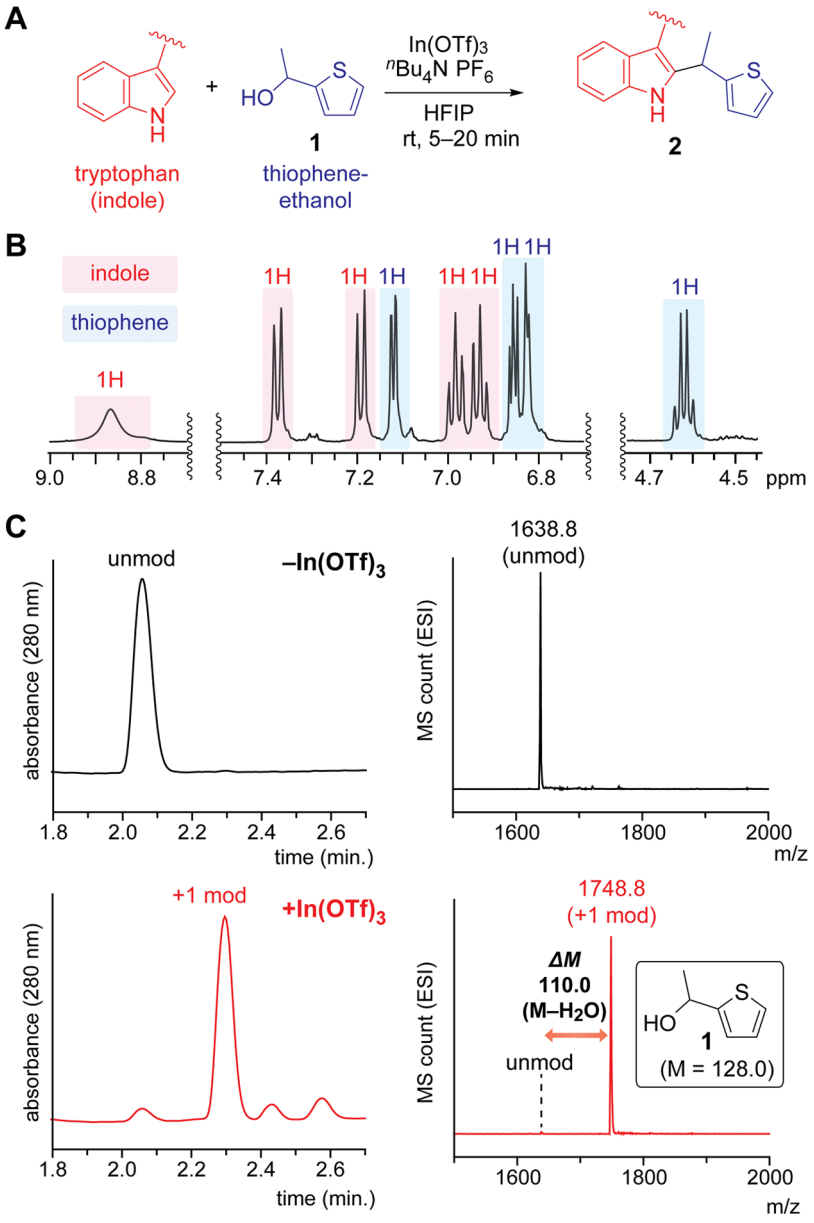

Figure 2. Characterization of the tryptophan alkylation reaction in hexafluoroisopropanol (HFIP). Typical conditions: tryptophan-containing molecule (0.1 mM), indium triflate (0.5 mM), $^{n}Bu_4N$ PF_6 (0.5 mM), and thiophene-ethanol 1 (3 mM) in HFIP at room temperature for 20 min. (A) General scheme of the indole/tryptophan alkylation; OTf: triflate. (B) ^{1}H NMR spectrum of the product of the reaction of 3-methylindole with thiophene-ethanol. The number of protons based on the peak area integration is shown at the top of each peak. The full spectrum including the two additional peaks for methyl groups at the upfield region is available in the Supporting Information together with its chemical structure. (C) Liquid chromatography–mass spectrometry (LC-MS) analysis of a reaction between a tryptophan-containing substrate (somatostatin) and thiophene-ethanol. Sequence of somatostatin (disulfide bond between the cysteine residues): H-AGCKNFFWKTFTSC-OH.

(HFIP) in the presence of a Lewis acid (Figure 2A). Published precedents[46,47] demonstrated the remarkably broad scope of heteroaryl group-targeting reactions in HFIP through stabilization of benzylic cationic intermediates, often with a main group metal salt catalyst and an ammonium salt. Thus, we performed an initial reaction screening process in HFIP and identified thiophene-ethanol as a potential tryptophan labeling reagent. The structure of the reaction product from a model substrate, 3-methylindole, was characterized by a set of analytical methods including ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, ^{1}H–^{1}H NOESY, and $^{1}H^{13}C$-HSQC nuclear magnetic resonance (NMR), liquid chromatography–mass spectrometry (LC-MS), and infrared (IR) spectroscopy (Figures 2B,C and S1–S9), suggesting that the 2 position of the indole ring was alkylated with the loss of H_2O (*i.e.*, dehydrative substitution of alcohol[48,49]). For instance, the ^{1}H NMR spectrum of the reaction product exhibited a loss of the hydrogen at the C2 position, while all of the other indole CH and NH protons remained present (Figure 2B). LC–MS analysis of the reaction mixture obtained from an indole-containing molecule showed the addition of the corresponding alkyl-thiophene moiety with the loss of H_2O in a Lewis acid-dependent manner (Figure 2C).

Further examination of the bioconjugation reaction showed its high dependence on the thiophene-ethanol scaffold for labeling as well as the nonaqueous reaction medium.

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Considering the breadth of the reactivity of the other heteroaryl groups in HFIP such as furan derivatives for aza-Piancatelli rearrangement,[50,51] a series of reagents with aryl or heteroaryl substituents has been examined (Figure S10), showing that replacement of the thiophene ring by a furan (**3a**), benzene (**3b**), pyridine (**3c**), pyrrole (**3d**), or thiazole (**3e**) ring significantly decreases the labeling efficiency. Furthermore, the ethanol moiety in the thiophene-ethanol reagent proved to be essential for efficient labeling, as a considerable decrease in conversion was observed with other compounds with analogous groups such as methanol (**4a**), propanol (**4b**), acetyl (**4c**), and ethyl (**4d**) groups (Figure S11). The observed reactivity trends may reflect the high electronic and steric sensitivity of the labeling reagents, as the reaction presumably proceeds through multistep processes where such electronic and steric influences may affect some steps but not others (*vide infra*). Consistent with our initial intention to develop a nonaqueous bioconjugation method, the sensitivity of the bioconjugation toward water proves the importance of the nonaqueous media (Figure S12). Alkylammonium salts play important roles in many reactions in HFIP,[46] and even though there were no observable effects of the salt on the tryptophan labeling of a certain peptide substrate (Figure S13), future studies may be necessary to properly understand the significance of the salt on various aspects during the process such as reaction kinetics.

The thiophene-ethanol labeling in HFIP has superior selectivity toward a tryptophan residue over that of other amino acids. To assess the chemoselectivity for tryptophan in our labeling method, the thiophene-ethanol labeling of a tryptophan-containing model substrate (somatostatin) was performed in the presence of an excess amount of an amino acid as an inhibitor, which would indicate the chemoselectivity of a particular amino acid by a decrease of the modification of the model substrate (Figure S14). Indeed, the inhibition experiment showed an obvious decrease in the modification caused by the addition of a tryptophan-based inhibitor. On the other hand, other nucleophilic amino acids including cysteine and tyrosine did not display such an extent of decrease of the modification. A simpler system with a 3-methylindole substrate as a tryptophan model and a *p*-cresol inhibitor as a tyrosine model also showed similar results (Figure S15). Modification of peptide substrates with various amino acid types also achieved high tryptophan chemoselectivity as well (Table 1 and Figure S16). The site of the tryptophan modification was confirmed by tandem mass spectrometry (MS/MS) analysis (Figures S17−S21). The lack of significant reactivity of nucleophiles with heteroatoms bearing lone pairs, such as SH and NH, may be due to the reversible nature of the addition reaction of the heteroatom to a putative cationic species, as even the activation of the thiophene-ethanol reagent occurs through the cleavage of the carbon−heteroatom (C−OH) bond. Suppression of the tyrosine reactivity may be a result of the exceptional hydrogen-bonding donor capability of HFIP. While the indole can behave only as a hydrogen bond donor, the hydroxyl group of tyrosine could behave as either a hydrogen bond donor or an acceptor, and HFIP may cause significant stabilization of the phenol group of tyrosine. Possibly, indoles may be simply more nucleophilic than phenols in certain conditions, as a recent DFT study indicates higher nucleophilicity of a pyrrole than that of a phenol.[52]

A structure−reactivity relationship study revealed that the modification efficiency of tryptophan labeling can be profoundly influenced by substitution patterns of thiophene-ethanol derivatives. The successful labeling results of the model substrates led us to the fabrication of a thiophene-ethanol reagent with a useful functionality such as a biorthogonal handle. In order to understand which position of the parent thiophene-ethanol can be suitable for the installation of additional groups, the modification efficiencies of four types of methyl-substituted thiophene-ethanol derivatives were compared, and this screening showed a decrease in the reaction conversion by the introduction of a methyl group at any position other than the 5 position (Figures 3A and S22). Based on this observation, six other thiophene derivatives with various electron-donating and -withdrawing groups at position 5 were synthesized and tested for tryptophan labeling to find the optimal molecular structure (Figures 3B and S23). Both strong electron-donating and -withdrawing groups (*i.e.*, methoxy, carbamyl, sulfonyl, and nitrile) were incapable of effective tryptophan modification, and only groups inducing subtle electronic perturbation such as alkyl and thioether groups proved to be useful as labeling reagents. The high sensitivity toward the alteration of the electronic status may be consistent with the varied reactivities of a series of heteroaryl-ethanol molecules as well as different substitution on the parent thiophene molecule, shown in Figures S10 and S11. Finding the optimal linkage position and structure, two types of thiophene-ethanol reagents with a bioorthogonal (alkyne) handle on the 5 position with an alkyl and thioether linker have been designed and synthesized (Figure 3C,D).

The optimized thiophene-ethanol compounds with substitutions on the 5 positions are capable of ultrafast labeling of tryptophan with a Lewis acid-based catalyst, and this capability enabled radiofluorination. The reaction kinetics of two alkyne-tagged thiophene-ethanol was compared with the parent reagent (R = H at the 5 position) for the tryptophan bioconjugation process in the standard conditions used above (500 mol % indium salt), and the resulting LC-MS analysis showed completion of all of the reactions within 10 s (Figures 3E, S24 and S25). To our surprise, the extraordinary reaction efficiency and speed were observed even with a catalytic amount of the indium salt (10 mol %) for the alkyl-substituted and parent thiophene derivatives (Figure S26). The structure of the reaction product and the need for the catalytic amount

Table 1. Scope of the Labeling of Peptide Substrates[a]

name	sequence	conversion (%)
somatostatin	H-AGCKNFFWKTFTSC-OH	94
α-MSH	Ac-SYSMEHFRWGKPV-NH_2	93
IDR-1018	H-VRLIVAVRIWRR-NH_2	98
LHRH	Glp-HWSYGLRPG-NH_2	97
C3a (63−77)	H-WWGKKYRASKLGLAR-OH	82
substance P	H-RPKPQQFFGLM-NH_2	0
OGP	H-ALKRQGRTLYGFGG-OH	0

[a]Typical modification conditions: peptide (0.1 mM), indium triflate (0.5 mM), NBu_4 PF_6 (0.5 mM), and thiophene-ethanol **1** (3 mM) in HFIP at rt for 20 min. The conversions were calculated by dividing the product peak area by the sum of the product peak area and starting material peak area of ultraviolet (UV) chromatograms (liquid chromatography). Somatostatin: cyclic somatostatin-14 with a disulfide bond between the two cysteine residues; α-MSH: an α-melanocyte stimulating hormone; IDR-1018: innate defense regulator 1018; LHRH: luteinizing hormone releasing hormone; C3a (63−67): (Trp^{63},Trp^{64})-C3a (63−77), superagonist analog of complement 3a; and OGP: osteogenic growth peptide.

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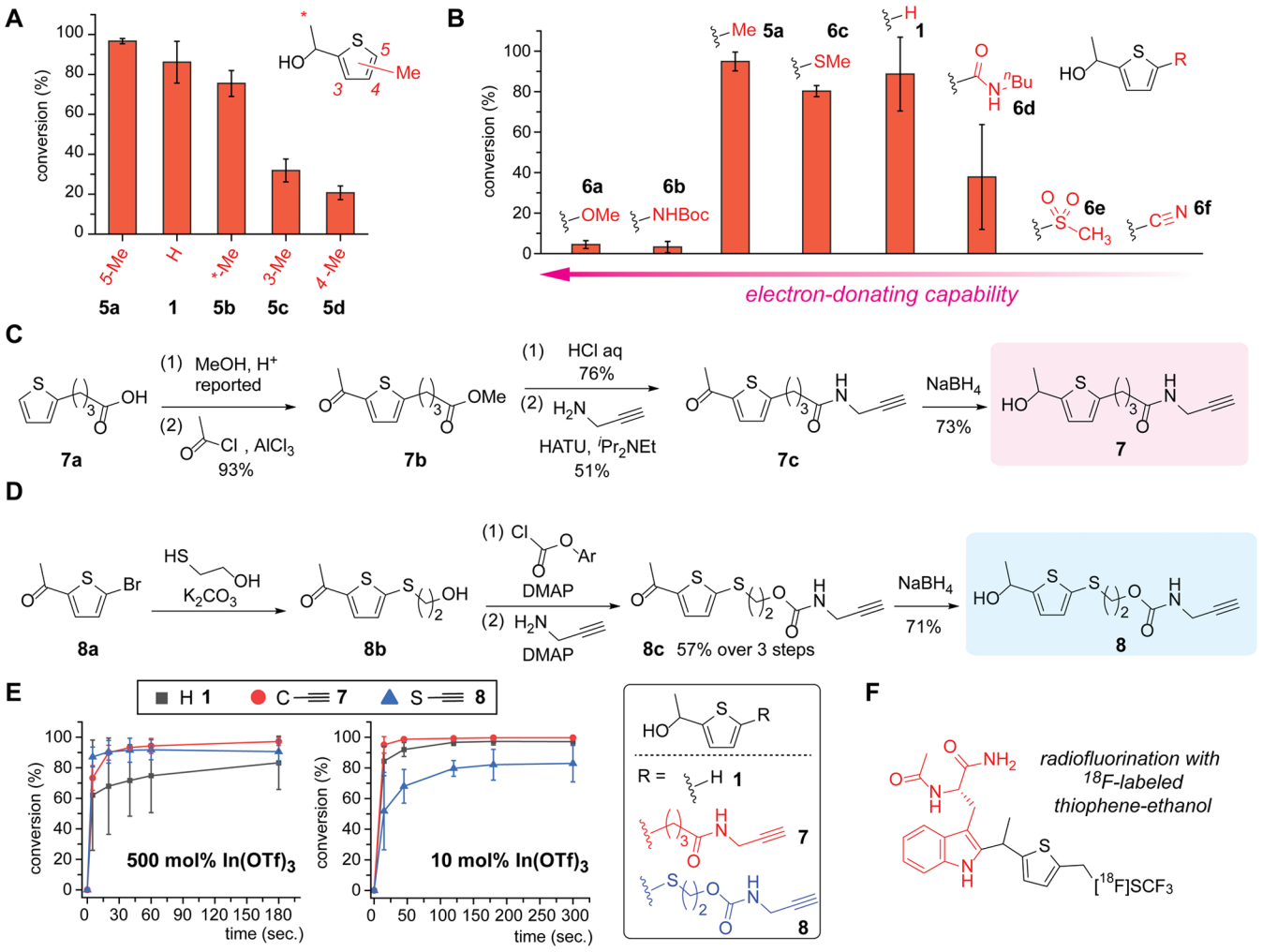

Figure 3. Structural optimization and bioorthogonal handle installation on the thiophene-ethanol scaffold. Typical modification conditions: tryptophan-containing molecule (0.1 mM), indium triflate (0.5 mM), $^{n}Bu_4N$ PF_6 (0.5 mM), and thiophene-ethanol derivatives (3 mM) in HFIP at rt for 20 min or a given time. Error bars represent the standard deviation (n = 3). (A) Liquid chromatography (LC)-based analysis of modification of tryptophan with thiophene-ethanol bearing a methyl group on possible substitution positions (*, 3, 4, and 5) compared to (nonmethylated, R = H) parent thiophene-ethanol. (B) LC-based analysis of modification of tryptophan with thiophene-ethanol bearing a functional group at C5. (C) Synthetic scheme for alkyl-substituted thiophene-ethanol with an alkyne handle. The methyl ester intermediate was synthesized according to a published report.[58] (D) Synthetic scheme for thioether-substituted thiophene-ethanol with an alkyne handle. Ar: *p*-nitrophenyl. DMAP: 4-dimethylaminopyridine. (E) LC-based kinetic analysis of modification of tryptophan with three different thiophene-ethanol derivatives at two different concentrations of indium triflate. (F) Radiofluorination of a model tryptophan substrate using a fluorinated thiophene reagent. Radiolabeling details are described in the Supporting Information.

of Lewis acid suggest that the reaction is proceeding through a Friedel−Crafts alkylation-type mechanism. Unlike the acylation variants,[53] the alkylation reaction is known to need only a catalytic amount of a Lewis acid such as $AlCl_3$, and there indeed have been reports of HFIP-facilitated Friedel−Crafts alkylation reactions.[54−56] We tested the stability of the linkage between the tryptophan and labeling reagent overnight at 37 °C in different pH conditions, and no noticeable decomposition of the modified tryptophan-containing molecules was observed (Figure S27), also supporting the formation of the stable C−C bond by the alkylation reaction. Taking advantage of the exceptional kinetics, radiolabeling of a model tryptophan substrate was examined using a thiophene reagent with fluorine atoms on its 5 position (Figure 3F). The fluorinated thiophene-ethanol reagent with nonradioactive isotope (^{19}F) was first prepared through 3-step synthesis and was found to be effective for the tryptophan labeling (Figure S28). The translation of the result to the radiofluorination experiment with ^{18}F was successful through a series of rapid chemical transformations (Supporting Information), showing the utility of the ultrafast tryptophan bioconjugation method for time-sensitive labeling experiments. It is noteworthy that previously reported radiofluorination methods could display reactivity toward other aromatic amino acid residues such as tyrosine,[34,57] and the high selectivity toward tryptophan of this method would be of great value.

The tryptophan-selective bioconjugation can cause the rapid and efficient labeling of natural proteins. Streptavidin contains six tryptophan residues in its monomeric unit, and the model protein was successfully labeled almost quantitatively within 5 min, giving the expected mass shifts in a Lewis acid-dependent fashion, based on mass spectrometric analysis (Figure 4A left). Importantly, all of the substrates lacking tryptophan residues (insulin, ubiquitin, and RNase) showed no observable sign of labeling even in the presence of the active reagent and Lewis acid (Figure 4A right). As all the 19 other canonical amino

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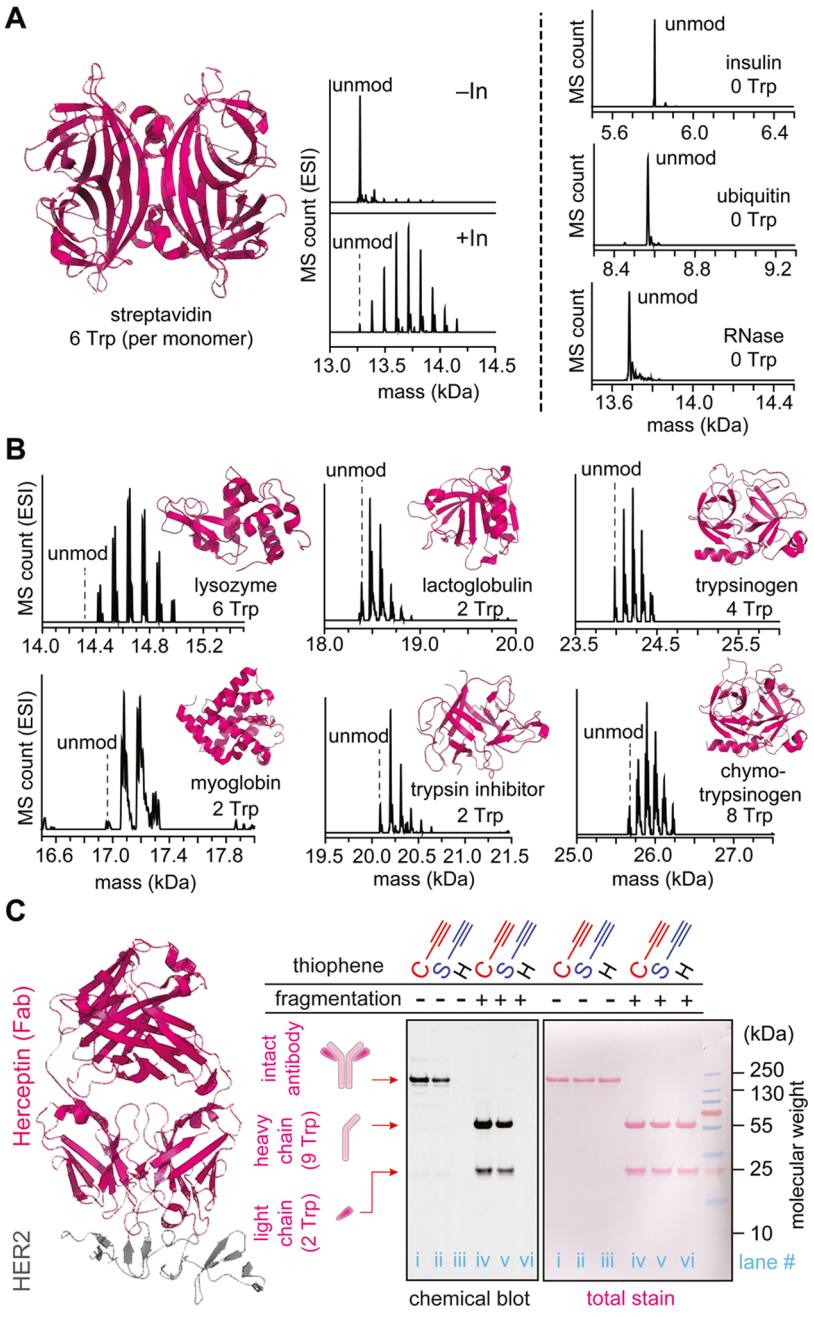

Figure 4. Tryptophan-targeting modification of proteins with thiophene-ethanol derivatives in hexafluoroisopropanol (HFIP). Typical modification conditions: protein (0.025 mM), indium triflate (0.5 mM), $^{n}Bu_4N\ PF_6$ (0.5 mM), and thiophene-ethanol derivatives (3 mM) in HFIP at room temperature for 5 min; MES: (*N*-morpholino)ethanesulfonic acid. The raw mass spectra were deconvoluted to show a single charge state. (A) Electrospray ionization mass spectrometry (ESI-MS) analysis of the modification of streptavidin with thiophene-ethanol in the presence and absence of indium triflate. (B) ESI-MS analysis of the modification of various proteins with thiophene-ethanol in the presence of indium triflate. (C) Chemical blot analysis (detection of an alkyne handle in proteins on a blot membrane)[59] of the modification of Herceptin (trastuzumab) with three different thiophene-ethanol derivatives 1 (H), 7 (C-≡), and 8 (S-≡). Fragmentation of Herceptin was caused by the treatment of the sample with tris(2-carboxyethyl)phosphine (TCEP) after the labeling of HFIP.

acids (*e.g.*, tyrosine, histidine, cysteine, lysine, methionine, and arginine) are present in one or some of the three peptides/proteins that showed no detectable product formation, those results further confirmed the remarkable chemoselectivity of the bioconjugation process for tryptophan over other amino acids. Six other model proteins with a varied number of tryptophan residues including an antimicrobial enzyme (lysozyme), a metalloprotein (myoglobin), a globulin with an exposed, reduced cysteine residue (lactoglobulin), a trypsin inhibitor, and protease precursors (trypsinogen and chymotrypsinogen) were also subjected to thiophene-ethanol labeling conditions, which showed the corresponding mass shift with the expected number of modified sites (Figures 4B and S29). While 16 Da mass adducts, probably due to oxidation processes, are observed for some of those protein labeling examples in the mass spectra, such adducts were not observed for the proteins without tryptophan residues (Figure 4A right), implying that the potential oxidation processes may not have

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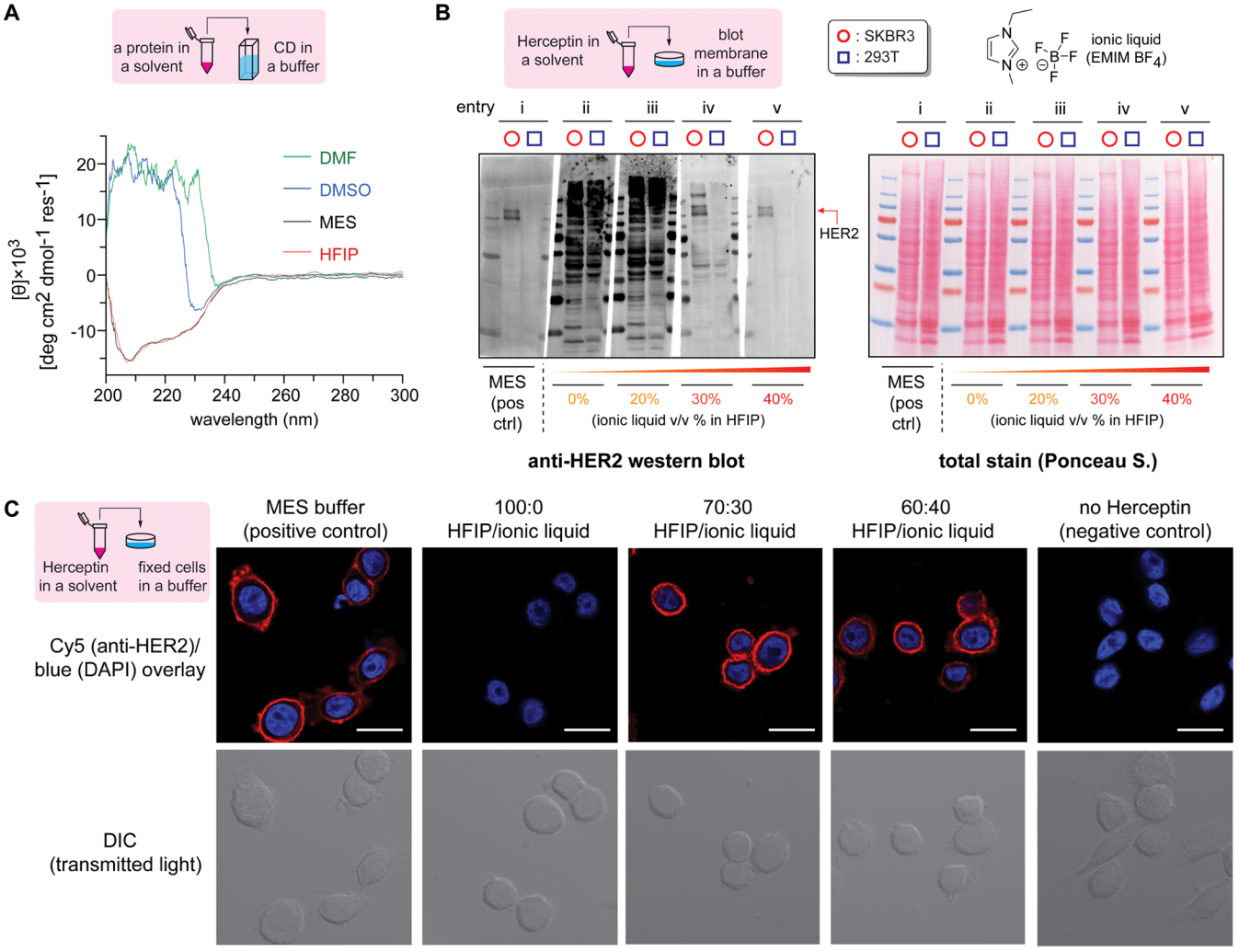

Figure 5. Effects of hexafluoroisopropanol (HFIP) or a mixture of HFIP and an ionic liquid on the structure and activity of proteins. (A) Circular dichroism (CD) analysis of lysozyme in phosphate-buffered saline (PBS) solution after treatment in HFIP, (*N*-morpholino)ethanesulfonic acid (MES) buffer, dimethyl sulfoxide (DMSO), or dimethylformamide (DMF). The MES (black) and HFIP (red) curves have significant overlaps with each other. (B) Anti-HER2 Western blot analysis of SK-BR-3 (HER2-overexpressing cell line) or HEK293T (human embryo kidney cell line) cells using Herceptin treated in different media: MES buffer (lane (i)), HFIP (lane (ii)), 20:80 mixture of ionic liquid/HFIP (lane (iii)), 30:70 mixture of ionic liquid/HFIP (lane (iv)), and 40:60 mixture of ionic liquid/HFIP (lane (v)). After the treatment of Herceptin in different solvents, the Herceptin was used for anti-HER2 Western blot in a typical aqueous solution (tris-buffered saline Tween 20 containing 5% bovine serum albumin). Ionic liquid: 1-ethyl-3-methylimidazolium tetrafluoroborate or EMIM BF_4 (structure is shown on the right top). Multiple bands by anti-HER2 Western blot of SK-BR-3 cell lines with an intact antibody was also observed in a previous report.[69] (C) Confocal microscopy analysis of SK-BR-3 cells stained using Herceptin treated in different media: MES buffer and a mixture of ionic liquid/HFIP. Cells were visualized by antihuman secondary antibody−Cy5 conjugate (red) and nuclear stain (DAPI, blue). DIC: differential interference contrast. Scale bars: 20 μm.

occurred at amino acid residues other than tryptophan. The tryptophan selectivity of the modification process was also confirmed by analysis through nanoLC-MS/MS after trypsin digestion of modified proteins (Figures S30−S35). Modification of a monoclonal antibody, Herceptin (trastuzumab), was also confirmed through an azide−alkyne cycloaddition reaction by installing a fluorophore on a blot membrane (Figure 4C),[59] displaying alkyne-dependent fluorescence signals originating from the alkyne-tagged thiophene reagents **7** and **8**. Furthermore, reductant-induced fragmentation of the antibody by tris(2-carboxyethyl)phosphine (TCEP) prior to the gel/blot analysis showed a higher degree of fluorescence/labeling on the heavy chain (9 Trp residues) over the light chain (2 Trp residues), which is also corroborative evidence of the tryptophan-dependent labeling process even in this large protein substrate.

Compatibility of the structure and activity of a protein after the temporary HFIP treatment may depend heavily on the type of protein and can be enhanced by the introduction of an ionic liquid to an HFIP solution. While the enhancement of α-helices of the peptide structure in HFIP is suggested by precedents,[60−62] there have been reports describing aggregation or denaturation of certain peptides/proteins by HFIP.[63,64] At the same time, however, successful use of some proteins or biological materials after the HFIP treatment has also been proved to be feasible,[65,66] and there have been only a few efforts to understand protein activity and the structure in aqueous buffer after treatment with HFIP. To this end, we first conducted a structural investigation of a model protein, lysozyme, which has both α-helix and β-sheet structures, using circular dichroism (CD) in an aqueous buffer after treatment in different media (Figure 5A). HFIP-treated lysozyme indeed

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exhibited signature curves of a typical lysozyme in aqueous buffer, but common aprotic organic solvents such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) crucially impaired its structure.[67] This observation suggests that short-term treatment of a certain protein in HFIP may not be detrimental to its structure. The activity of Herceptin, a larger and more intricate protein target with mostly β-sheet structures, was tested after short-term treatment (1 h) with HFIP. The binding specificity and strength of Herceptin against its antigen target, a HER2 receptor, was tested using SK-BR-3 cells known to possess the overexpressed receptor.[68] Anti-HER2 Western blot analysis in aqueous buffer treated with Herceptin in pure HFIP showed a myriad of protein bands even from a negative control cell lysate without the overexpressed HER2 receptor (HEK293T cell line), presumably reflecting nonspecific binding without any HER2 specificity (Figure 5B, lane (ii)). We discovered that the addition of an ionic liquid, 1-ethyl-3-methylimidazolium tetrafluoroborate (Figure 5B), to the HFIP solution prior to the Western blotting improved the antibody specificity dramatically (Figure 5B, lanes (iii−v)). Similar trends have also been observed in immunofluorescence experiments of SK-BR-3 cells treated with Herceptin in different media (Figure 5C), in which the fluorescence intensity through the binding of Herceptin treated in HFIP with an increased concentration of an ionic liquid was virtually identical to that of Herceptin treated in an aqueous buffer. It is curious that no detectable fluorescence signals of the antibody binding were observed from Herceptin treated in HFIP without the ionic liquid although significant nonspecific binding was observed in the Western blot experiments. This discrepancy between the immunofluorescence and blot experiments may imply that the nonspecific binding may be caused by intracellular targets inaccessible in this immunofluorescence setup. Overall, the data suggest the reasonable possibility of conserving structures and activities of other proteins after temporary treatment in HFIP or a HFIP mixture and suggest that the HFIP bioconjugation could be potentially applicable as a protein labeling method in a broader sense.

Screening of reaction solvents and Lewis acid types was performed to obtain more insight into the reaction mechanism of the Lewis acid-catalyzed bioconjugation in HFIP. First, the importance of the solvent was investigated by testing modification of a tryptophan-containing substrate in a wide variety of alcohol solvents (Figure S36). No meaningful product formation was observed in any nonfluorinated alcohol, including glycerol, a biomolecule-compatible alcohol solvent. The highest reactivity of HFIP compared to other fluorinated solvents was confirmed, indicating that the increased acidity of the hydroxyl group may play a pivotal role in the labeling process, possibly related to the unique behavior of the solvent molecule such as its strong hydrogen-bonding capability and cation stabilization. To obtain experimental insights of the catalyst into the reaction mechanism, a series of metal triflate salts were also screened for tryptophan labeling (Figure S37). Many types of metal-based Lewis acids with the exception of alkaline earth metals Ca^{2+} and Mg^{2+} were found to be able to facilitate the reaction, though alkaline earth cations are also known to be catalysts of many HFIP chemical transformations.[46] Plausibly, a certain strength of Lewis acidity is necessary for the current reaction system,[70,71] as such a tendency is often true in other Lewis acid-mediated reactions such as Friedel−Crafts reactions. Triflic acid was able to induce the labeling process as well (Figure S38). Other indium salts such as $InBr_3$ and $In(NO_3)_3$ also showed a peak corresponding to the expected product (Figure S39), and therefore, the reactions are probably not dependent on triflate ions. Preliminary computational studies using density functional theory (DFT) calculations implied the potential importance of HFIP and Lewis acid (Figure S40−42), though more detailed calculations would be necessary to fully understand the roles of each component in the future.

CONCLUSIONS

This study has demonstrated that hexafluoroisopropanol (HFIP) can serve as an alternative bioconjugation medium for the selective, efficient labeling of tryptophan residues. The use of a biomolecule-compatible nonaqueous medium has been found to address the longstanding challenges of previous tryptophan labeling, such as poor chemoselectivity, use of harsh organic solvents/oxidants, and sluggish reaction kinetics. The present method is operationally simple and does not need degassing or large, intricate labeling reagents/mediators, which require substantial synthetic efforts. Synthesis of the thiophene derivatives described here required a combination of straightforward reactions such as acylation and reduction with a borohydride reagent and used commercially available, inexpensive starting materials. The viability of several types of metal Lewis acid catalysts for this bioconjugation also provides experiment-specific advantages to avoid a certain metal source including a scandium salt which could cause peptide backbone cleavage[72] and lanthanide ions with their affinity to a class of proteins.[73] The attributes of the HFIP protein labeling method may be crucially important for future applications to create useful bioconjugates such as antibody−drug conjugates and radioisotope-labeled agents, which necessitate a rapid labeling method. HFIP is increasingly studied for synthetic organic chemistry, and a wealth of HFIP-based reactions could be of importance to developing different types of bioconjugation processes targeting other amino acid residues. Moreover, even a century after the original discovery, Friedel−Crafts reactions still have been attracting considerable interest in synthetic chemistry fields producing effective asymmetric and catalytic variants,[74] and this report would potentially be conducive to that end, as while the stereochemistry of the benzylic position of the thiophene moiety in the reaction product may not have significant influence for protein bioconjugation purposes, an exact stereochemistry of the position would be critical for small molecule substrates. Beyond the labeling purposes, this report also presented a first example of ionic-liquid-mediated conservation of the protein activity in HFIP, which offers new capabilities in the field of protein science. Such a stabilization strategy of proteins in a mixture of nonaqueous media could expand the repertoire of bioconjugation tools in the future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c13447.

^{1}H NMR spectrum of **2**, the product of a reaction between 3-methylindole and thiophene-ethanol in CD_3CN; infrared (IR) spectrum of **2**, the product of a reaction between 3-methylindole and thiophene-ethanol; tandem mass spectrum (MS/MS) analysis of LHRH

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modified with thiophene-ethanol; general information; experimental procedures; proteomics analysis; radiolabeling; computational analysis; and small molecule synthesis/characterization (PDF)

AUTHOR INFORMATION

Corresponding Author

Jun Ohata − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0002-3614-7472; Email: johata@ncsu.edu

Authors

Mohammad Nuruzzaman − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0003-1525-2887

Brandon M. Colella − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0009-0004-2539-2634

Chiamaka P. Uzoewulu − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0002-7687-384X

Alissa E. Meo − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Elizabeth J. Gross − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0003-2153-5381

Seiya Ishizawa − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0003-1925-031X

Sravani Sana − *Department of Radiology and Biomedical Research Imaging Center, and UNC Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Raleigh, North Carolina 27599, United States*

He Zhang − *Department of Radiology and Biomedical Research Imaging Center, and UNC Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Raleigh, North Carolina 27599, United States;* orcid.org/0000-0003-1165-6702

Meredith E. Hoff − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Bryce T. W. Medlock − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0009-0002-6827-1328

Emily C. Joyner − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0009-0005-6268-9362

Shinichi Sato − *Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Sendai 980-8578, Japan;* orcid.org/0000-0002-8563-1658

Elon A. Ison − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Zibo Li − *Department of Radiology and Biomedical Research Imaging Center, and UNC Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Raleigh, North Carolina 27599, United States;* orcid.org/0000-0002-1063-0298

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.3c13447

Notes

The authors declare no competing financial interest.

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