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Two Rapid Catalyst-Free Click Reactions for In Vivo Protein Labeling of Genetically Encoded Strained Alkene/Alkyne Functionalities

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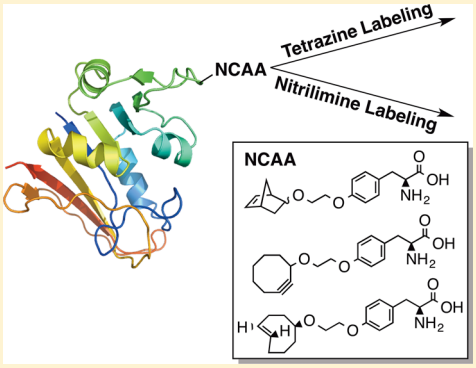
Supporting Information

ABSTRACT: Detailed kinetic analyses of inverse electron-demand Diels–Alder cycloaddition and nitrilimine-alkene/alkyne 1,3-diploar cycloaddition reactions were conducted and the reactions were applied for rapid protein bioconjugation. When reacted with a tetrazine or a diaryl nitrilimine, strained alkene/alkyne entities including norbornene, *trans*-cyclooctene, and cyclooctyne displayed rapid kinetics. To apply these "click" reactions for site-specific protein labeling, five tyrosine derivatives that contain a norbornene, *trans*-cyclooctene, or cyclooctyne entity were genetically encoded into proteins in *Escherichia coli* using an engineered pyrrolysyl-tRNA synthetase-tRNA$^{Pyl}_{CUA}$ pair. Proteins bearing these noncanonical amino acids were successively labeled with a fluorescein tetrazine dye and a diaryl nitrilimine both in vitro and in living cells.



INTRODUCTION

First described by Sharpless in 1999, the term "click chemistry" defines chemical reactions that are selective, rapid, and highly efficient.[1,2] Early focus of click chemistry centered on the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction.[3,4] The CuAAC reaction is highly bioorthogonal due to the biologically inert nature of the azide and alkyne functional groups. Applications of this reaction in chemical biology include activity-based protein profiling and selective biomolecule labeling.[5−9] One potential pitfall of using CuAAC in living systems is the cytotoxic effects of Cu(I) catalysts, which can be avoided using the copper-free azide-cyclooctyne cycloaddition reaction.[10,11] Due to ring strain, the cyclooctyne triple bond has an elevated energy state and therefore reacts spontaneously with an organic azide under mild aqueous conditions, bypassing the use of Cu(I) catalysts. Although originally described as a relative slow reaction, cyclooctyne derivatives that show fast reaction kinetics with organic azides have been developed.[12−16] The pursuit of other fast catalyst-free click reactions that can be applied in living systems for the labeling of biomolecules under biologically relevant conditions has recently revitalized two other reactions, namely, the inverse electron-demand Diels–Alder cycloaddition (IEDDAC) and the nitrilimine-alkene/alkyne 1,3-dipolar cycloaddition (NADC) (Scheme 1).[17−21] Both reactions display rapid kinetics when a strained alkene/alkyne is involved. Applications of these two reactions include labeling biomolecules in cells and cancer diagnostics.[22−24] Here, we report kinetic investigations of IEDDAC and NADC reactions that involve strained alkene/alkyne functionalities including norbornene, *trans*-cyclooctene, and cyclooctyne, and their applications in labeling proteins that are genetically modified with these functional groups.

RESULTS AND DISCUSSION

Kinetic Characterization of IEDDAC Reactions of Norbornene, *trans*-Cyclooctene, And Cyclooctyne. Kinetic investigations of reactions between strained alkene/alkyne dienophiles and tetrazines have been reported.[17,25,26] However, solvent systems, tetrazines, dienophiles, and methods of kinetic determination varied in previous reports, making it challenging to compare relative reactivities of dienophiles with tetrazines of interest. We chose to characterize and compare IEDDAC reactions of strained alkene/alkyne dienophiles in PBS buffer at pH 7.4 to mimic physiological conditions. For our kinetic studies, we selected three strained alkenes, 5-norbornen-2-ol and two diastereomers of (*E*)-2-(cyclooct-4-en-1-yloxyl)-ethanol, and cyclooct-2-ynol (NOR, DS1, DS2, and COY in Scheme 2) and a fluorescein tetrazine (FITC-TZ in Scheme 2). DS1 and DS2 are two diasteromeric products (ratio close to 1:2) of the UV-induced isomerization of (*Z*)-2-(cyclooct-4-en-1-yloxyl)ethanol. As minor products, DS1-like molecules were mostly dismissed in previous reports.[18] Since structural differences between DS1 and DS2 may lead to different reactivities toward tetrazines, both were included in our studies.

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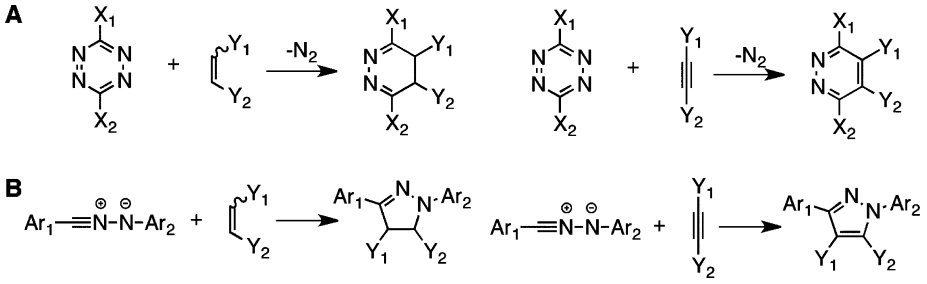
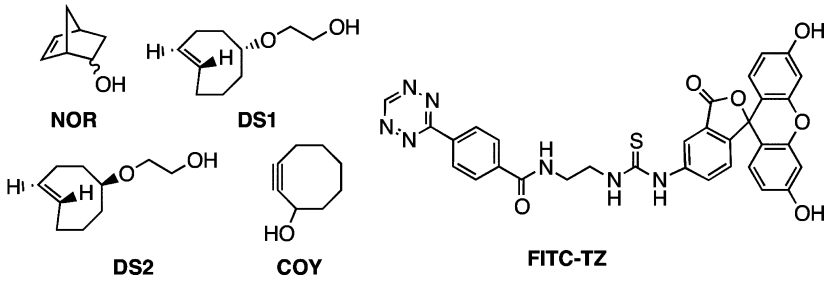
Scheme 1. (A) Tetrazine-Alkene/Alkyne IEDDAC and (B) NADC Reactions



Scheme 2. Structures of Four Strained Alkene/Alkyne Molecules and a Fluorescein Tetrazine Dye



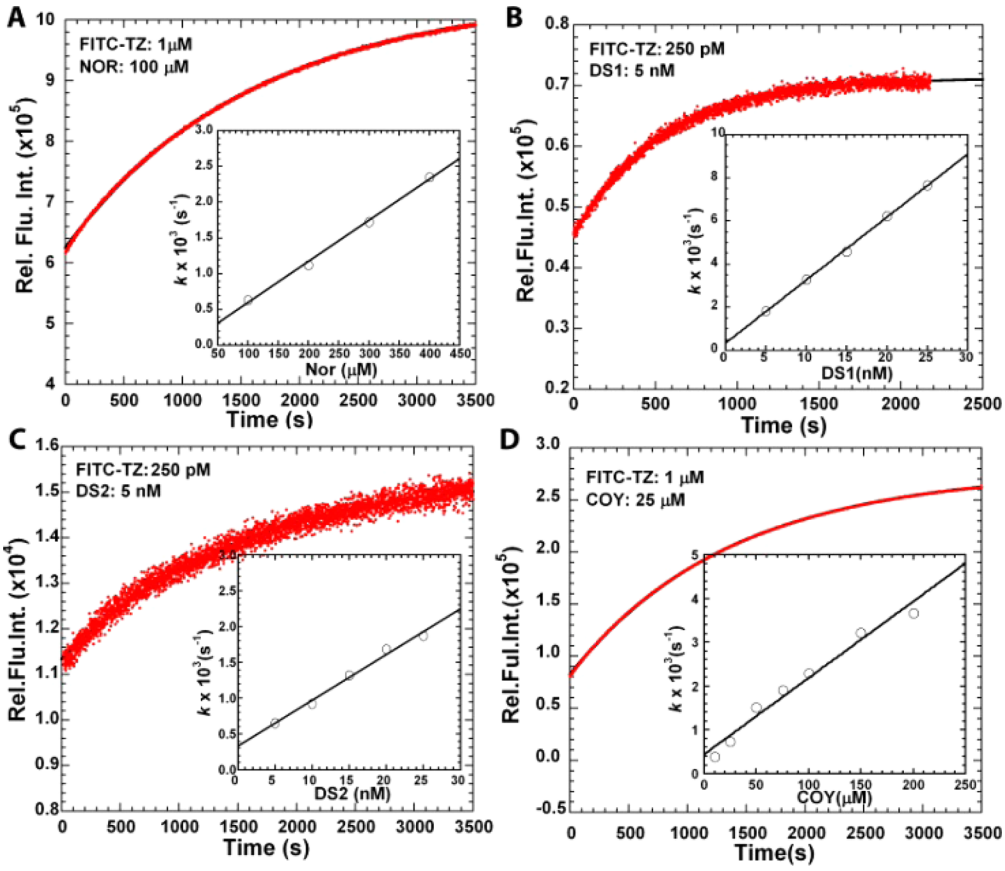


Figure 1. Characterization of FITC-TZ reactions with (A) NOR, (B) DS1, (C) DS2, and (D) COY. All reactions were carried out in PBS buffer at pH 7.4. The fluorescence emission was detected at 515 nm with excitation at 493 nm. For A–D, each presents the fluorescence change as a function of time at a given concentration shown in the top left corner. The insets show the linear dependence of the pseudo-first-order rate constants of a reaction on dienophile concentrations.

COY is a racemic mixture. Its two stereoisomers are enantiomers that are expected to display the same reactivities toward FITC-TZ. Therefore, the two stereoisomers were not separated for undertaking kinetic analysis. FITC-TZ has a tetrazine entity that efficiently quenches the fluorescence emission of fluorescein until reaction with a dienophile, enabling easy tracking of the reaction progress. The reactions of all four dienophiles with FITC-TZ were carried out in

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Table 1. Second-Order Rate Constants of FITC-TZ Reactions with Strained Alkene/Alkyne Dienophiles

Dienophile	NOR	DS1	DS2	COY
$k/M^{-1}\ s^{-1}$	5.7 ± 0.2	292 000 ± 6000	64 000 ± 4000	17 ± 1

pseudo-first-order conditions, in which the dienophile was present in a 20-fold excess compared to FITC-TZ (Figure 1). For NOR and COY, FITC-TZ was used at a 1 μM concentration for an optimal dynamic range of the signal, while for DS1 and DS2, the reactions with FITC-TZ are so fast that the concentration of FITC-TZ had to be significantly reduced to 250 pM in order to detect the reaction progress. Data collected at all conditions was fit to a single exponential increase equation $F = F_1 - F_2 \times e^{(-k' \times t)}$, where F is the detected fluorescent signal at a given time t, F_1 is the final fluorescence, $F_1 - F_2$ is the background fluorescent signal, and k' is the apparent pseudo-first-order rate constant. The determined values for k' were plotted against dienophile concentrations and fitted to the equation $k' = k \times [\text{dienophile}] + C$, where k is the second-order rate constant of a dienophile reaction with FITC-TZ. The calculated second-order rate constants for all four dienophiles are shown in Table 1.

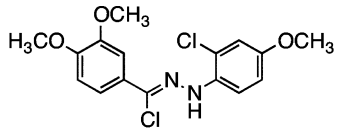
Among all four dienophiles, NOR reacts most slowly with FITC-TZ. The determined rate constant is similar to what has been reported for reactions between norbornenes and tetrazines.[26−28] The determined rate constant for COY is 20-fold lower than what was reported previously,[29] which may be attributed to structural differences in the tetrazine dyes used in the two analyses. Both DS1 and DS2 react exceedingly fast with FITC-TZ, several orders of magnitude faster than the reactions of the NOR and COY dienophiles. For DS2, its reaction with FITC-TZ has a rate constant in the same range as of those of reported rapid *trans*-cyclooctene reactions with tetrazines.[25,26,30,31] Interestingly, the reaction of DS1 with FITC-TZ is almost 5-fold higher than that of DS2 demonstrating that small stereochemical differences can have significant effects on IEDDAC reactions. With this fast rate constant, a reaction with both reactants at 1 μM reaches 97% conversion in just 100 s. Thus, DS1 is a better choice than DS2 for applications that require labeling molecules at very dilute concentrations such as in living systems.

To further analyze this reactivity difference between DS1 and DS2, we carried out a detailed computational study at the CPCM-M06-2X/6-311+G(d,p) level of theory[32−34] (see Supporting Information for details). This level of ab inito calculations provides accurate reaction barriers for Diels−Alder cycloaddition reactions and has been employed in similar studies of strain-promoted click reactions.[35] We predict free energy barriers of 14.0 and 15.5 kcal/mol for the IEDDAC reaction of slightly simplified models of DS1 and DS2 with 3-phenyl-1,2,4,5-tetrazine, respectively. This corresponds to a predicted rate for DS1 that is 12-times that of DS2, in agreement with the experimentally observed 5-fold difference. The 1.5 kcal/mol difference in free energy barrier can be explained in part by distortion-interaction analyses, in which the energy barrier for a bimolecular reaction is deconstructed into the energy required to distort the reactants into the transition state geometry (E_{dist}) and the stabilization provided by the interaction of these two distorted reactants (E_{int}). In particular, we find that both the interaction energy and the distortion energy slightly favor DS1 over DS2. This net 0.8 kcal/mol difference, combined with a 0.9 kcal/mol contribution from vibrational and entropy effects, leads to the final free energy difference of 1.5 kcal/mol.

Kinetic Analysis of NADC Reactions of Norbornene, *trans*-Cyclooctene, and Cyclooctyne. Previously, it was shown that olefins, serving as dipolarophiles, react with diaryl nitrilimines to form fluorescent pyrazoline products with appreciable reaction rates under physiological conditions.[20] Due to the labile nature of nitrilimines, currently two methods are used to generate them transiently in aqueous conditions. One is the photolysis of tetrazoles, while the other is the dissociation of hydrazonyl chlorides in water.[36,37] The first method has been significantly expanded by Lin and co-workers for photoclick labeling of biomolecules.[38−41] The second method has been recently applied to label biomolecules that contain norbornene and acrylamide functionalities and used to understand the NADC reaction mechanism in water.[42−44]

Recently, we reported that NOR reacts readily with a hydrazonyl chloride (HZCL in Scheme 3) with a rate constant

Scheme 3. Structure of a Hydrozonoyl Chloride, HZCL



HZCL

of 0.75 $M^{-1}\ s^{-1}$.[44] Similar kinetic characterizations were also carried out for DS1, DS2, and COY. Like NOR, both DS1 and DS2 also reacted readily with HZCL, as detected by following the fluorescence emission of the cycloaddition products (Figure 2). To determine second-order rate constants of the reactions of DS1 and DS2 with HZCL, reactions were set up under pseudo-first-order conditions in PBS/acetonitrile (1:1) at pH 7.4. Acetonitrile was provided to increase the solubility of all tested reactants and corresponding fluorescent products. At a 1:1 ratio, PBS and acetonitrile forms a homogeneous solvent system at pH 7.4 that can still be considered mimic to physiological conditions. The determined k' values were plotted against dipolarophile concentrations and fitted to the equation $k' = k_1 \times [\text{dipolarophile}] + k_2$, where k_1 is the second-order rate constant of a dipolarophile reaction with HZCL and k_2 is the rate constant of HZCL hydrolysis. The determined rate constants for DS1 and DS2 are shown in Table 2. Unlike NOR, DS1, and DS2, COY does not yield a fluorescent product when it reacts with HZCL. In order to kinetically characterize the reaction of COY with HZCL using fluorescence spectroscopy, acrylamide, which competes against COY to react with HZCL to form a fluorescent product, was provided at 50 μM. Reactions at all conditions showed single exponential fluorescent increase over time. The determined pseudo-first-order rate constants were plotted against COY concentrations and fitted to the equation $k' = k_1 \times [\text{COY}] + k_2'$, where k_1 is the second-order rate constant of the COY reaction with HZCL and k_2' is the sum of the rate constant of HZCL hydrolysis and the pseudo-first-order rate constant of the

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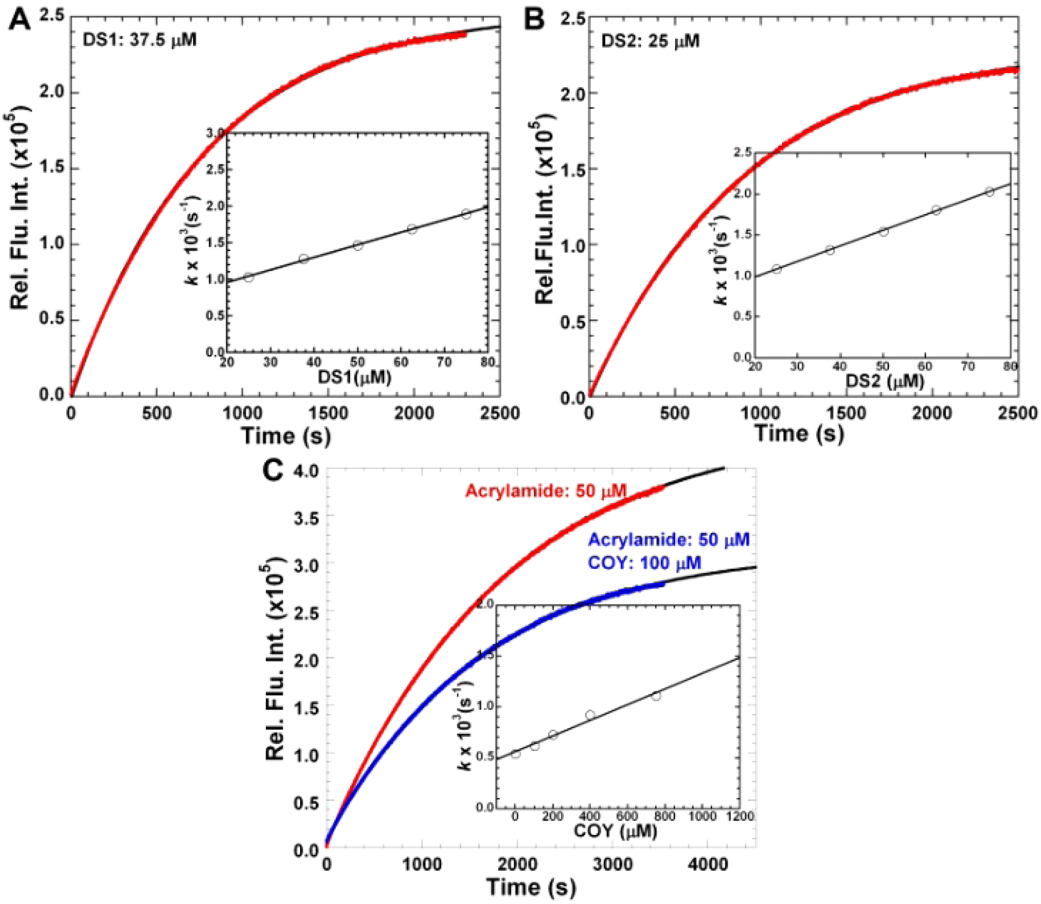


Figure 2. Characterization of HZCL reactions with (A) DS1, (B) DS2, and (C) COY. All reactions were carried out in PBS/acetonitrile (1:1) at pH 7.4. HZCL was provided at 1 μM. The fluorescence emission was detected at 480 nm with excitation at 318 nm. The detected fluorescence data for 37.5 μM DS1 and 25 μM DS2 are presented in A and B. C shows data at two conditions, one with only acrylamide and the other with both acrylamide and COY. The insets show the linear dependence of the pseudo-first-order rate constants on the dipolarophile concentrations.

Table 2. Second-Order Rate Constants of HZCL Reactions with Strained Alkene/Alkyne Dipolarophiles

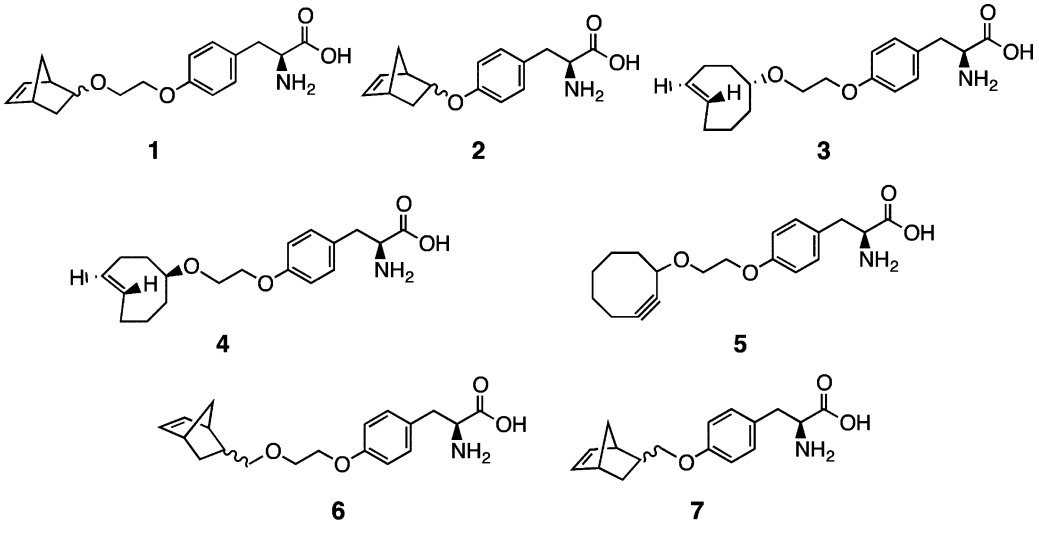
Dipolarophile	NOR[a]	DS1	DS2	COY
$k/M^{-1}\ s^{-1}$	0.75 ± 0.03	17.2 ± 0.4	19.0 ± 0.3	0.77 ± 0.07

[a]This rate constant was determined in ref 44.

HZCL reaction with 50 μM acrylamide. The determined rate constant for COY is also presented in Table 2.

As shown in Table 2, COY and NOR have similar reactivity toward HZCL. Both DS1 and DS2 react rapidly with HZCL with rate constants more than 20-fold higher than those for NOR and COY. Although elevated energy states of DS1 and

Scheme 4. Structures of *O*-Alkylated Tyrosine Derivatives That Contain Strained Alkene/Alkyne Functionalities



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DS2 lead to higher reactivities toward HZCL than NOR and COY, the rate enhancement is not as significant as observed in the IEDDAC reactions. In addition, DS1 and DS2 have similar reactivities toward HZCL, agreeing well with small stereochemistry differences between DS1 and DS2.

Genetic Incorporation of Tyrosine Derivatives Bearing Strained Alkene/Alkyne Functionalities into Proteins in *E. coli*. Lysine derivatives that contain strained alkene/alkyne functionalities were previously incorporated into proteins in live cells using engineered pyrrolysyl-tRNA synthetase (PylRS)-$tRNA^{Pyl}_{CUA}$ pairs.[26,28,29,45,46] Here, we took a different route to install strained alkene/alkyne functionalities into proteins. Previously we engineered a N346A/C348A mutant of PylRS (PylRS(N346A/C348A)) and demonstrated that this mutant enzyme in conjunction with $tRNA^{Pyl}_{CUA}$ was able to incorporate a wide range of tyrosine and phenylalanine derivatives into proteins in response to the amber codon in *E. coli*.[47−52] Since one of the successfully tested tyrosine derivatives, *O*-benzyl-tyrosine, is bulky and hydrophopic, we hypothesized that PylRS(N346A/C348A) might be applicable for the genetic incorporation of noncanonical amino acids (NCAAs) bearing functionalities with similar bulky and hydrophobic characteristics such as norbornene, *trans*-cyclooctene, and cyclooctyne. Seven *O*-alkylated tyrosine derivatives with strained alkene/alkyne functionalities were synthesized (structures **1**−**7** in Scheme 4). Their genetic incorporation into protein was tested in an *E. coli* BL21 system that contained two plasmids, pEVOL-pylT-N346A/C348A and pET-pylT-sfGFP2TAG, with genes coding PylRS(N346A/C348A), $tRNA^{Pyl}_{CUA}$, and superfolder green fluorescent protein (sfGFP) with an amber mutation at its 2 position. However, this *E. coli* system was not able to express sfGFP in GMML (a minimal medium supplemented with 1% glycerol and 0.3 mM leucine) in the absence or presence of **1**−**7** (2 mM). Apparently, the active site pocket of PylRS(N346A/C348A) could not accommodate the large side chains of **1**−**7**. To expand the active site pocket, we introduced two more mutations, Y306A and Y384F, to PylRS(N346A/C348A), generating PylRS(Y306A/N346A/C348A/Y384F). The Y384F mutation has been shown to improve the genetic incorporation of several NCAAs and the Y306A mutation has been introduced to the wild type enzyme to accommodate lysine derivatives with bulky side chains.[29,42,46,53−55] The crystal structure of PylRS(Y306A/Y384F) complexed with a furan-containing NCAA was recently published and indicated a very deep and large hydrophobic pocket for favorable interactions with bulky and hydrophobic chemical moieties.[56,57] *E. coli* BL21 cells transformed with pEVOL-pylT-Y306A/N346A/C348A/Y384F and pET-pylT-sfGFP2TAG were not able to express sfGFP in LB medium in the absence of **1**−**7**. The Y306A mutation apparently decreased the activity of the original mutant enzyme PylRS(N346A/C348A) and reduced the mis-incorporation of phenylalanine in a rich medium.[47] However, supplementing LB with 1 mM of one of five NCAAs (**1**, **2**, **4**, **5**, or **6**) induced sfGFP expression (Figure 3). The addition of **3** or **7**, even at 2 mM, led to no sfGFP expression. All five purified sfGFP variants were confirmed by ESI-MS (Table 3).

Protein Labeling with Genetically Encoded Strained Alkene/Alkyne Functionalities Using IEDDAC and NADC Reactions. After purifying sfGFPs that contained site-specifically incorporated NCAAs with strained alkene/alkyne side chains, we next proceeded to test their bioconjugation reactions with FITC-TZ and HZCL. To label sfGFP with

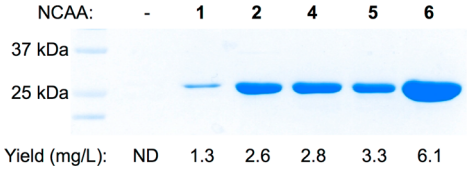

Figure 3. Site-specific incorporation of NCAAs **1**, **2**, and **4**−**6** into sfGFP at its 2 position. Proteins were expressed in *E. coli* BL21 cells transformed with pEVOL-pylT-Y306A/N346A/C348A/Y384F and pET-pylT-sfGFP2TAG. Cells were grown in LB medium supplemented with 1 mM of a NCAA for 8 h. Supplementing LB with 2 mM of **3** or **7** did not yield sfGFP expression.

Table 3. Detected and Theoretical Molecular Weights (MWs) of sfGFPs with Different NCAAs Incorporated at the Second Position

Protein	Observed MW[a]	Theoretical MW
sfGFP-**1**[b]	27 879	27 880
sfGFP-**2**[b]	27 837	27 836
sfGFP-**4**[b]	27 895	27 896
sfGFP-**5**[b]	27 894	27 894
sfGFP-**6**[b]	27 894	27 894

[a]With an error of ±1 Da. [b]sfGFP with **1**, **2**, **4**, **5**, or **6** incorporated at the S2 position.

FITC-TZ, reactions were set up in PBS buffer at pH 7.4 in the presence of 100 μM of FITC-TZ for 1 h. As expected, sfGFP containing **1**, **2**, **4**, **5**, or **6** was efficiently labeled with FITC-TZ and showed intense fluorescence in a denaturing SDS-PAGE gel under UV irradiation (Figure 4A). As a control, sfGFP with

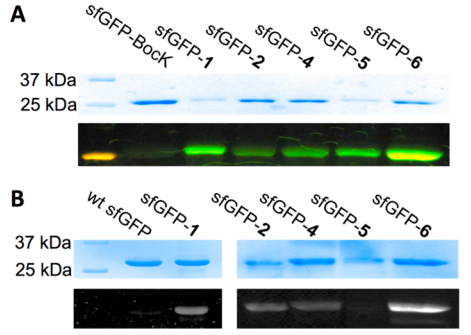

Figure 4. Selective labeling of sfGFPs that contained site-specifically incorporated NCAAs with strained alkene/alkyne functionalities with (A) FITC-TZ and (B) HZCL. In A and B, the top panels show denaturing SDS-PAGE analysis of proteins stained with Coomassie blue and the bottom panels are from fluorescent imaging of the same gels before Coomassie blue staining. In A, the fluorescent image was captured by a digital camera and displayed was real color of the emitting light. In B, the fluorescent image was captured by a Bio-Rad ChemiDoc XRS system.

N^{ε}-Boc-lysine incorporated at the second position (sfGFP-BocK) could not be labeled with FITC-TZ under the same conditions. All protein samples were treated with SDS and heated at 100 °C for 20 min to quench the intrinsic GFP fluorescence before they were analyzed by SDS-PAGE. The same treatment was also performed with proteins labeled with HZCL. For labeling with HZCL, each protein was incubated with 0.5 mM HZCL in PBS buffer at pH 7.4 for 1 h before analysis via denaturing SDS-PAGE gel and imaging under UV irradiation. SfGFP bearing **1**, **2**, **4**, or **6** was fluorescently labeled

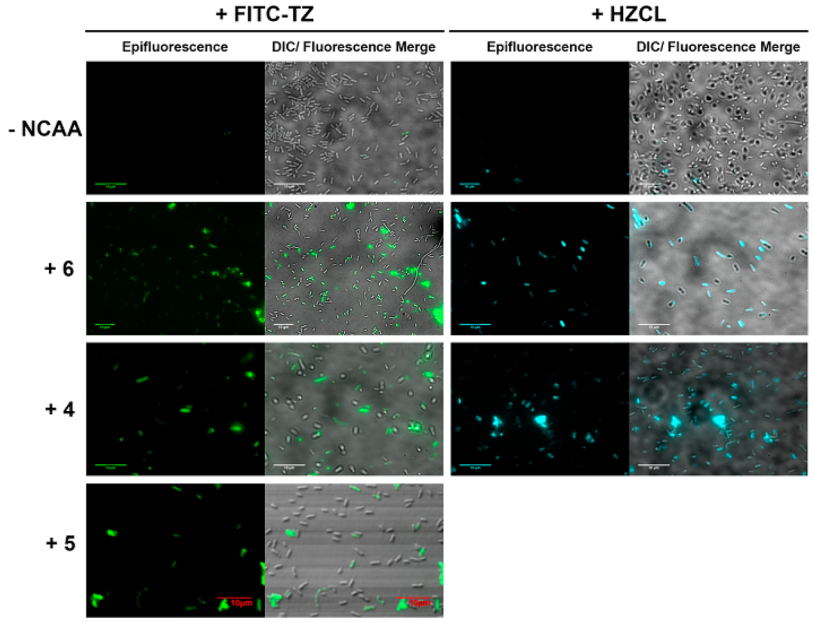

Figure 5. Selective labeling of sfGFPs that contained site-specifically incorporated NCAAs with strained alkene/alkyne functionalities with (A) FITC-TZ and (B) HZCL in *E. coli* cells. Cells were labeled with FITC-TZ and HZCL for 3 h and then washed with PBS buffer 3 times before undertaking epifluorescent and differential interface contrast (DIC) imaging.

after their reactions with HZCL. As expected, the labeling product of sfGFP-**5** could not be fluorescently visualized due to the nonfluorescent reaction product of a cyclooctyne with HZCL. As a control, wild-type sfGFP did not undergo any labeling reaction with HZCL under the same conditions.

Additionally, we tested the IEDDAC and NADC reactions in labeling proteins that bear site-specifically incorporated NCAAs with strained alkene/alkyne functionalities in living cells. pEVOL-pylT-Y306A/N346A/C348A/Y384F and a previously constructed plasmid pETDuet-OmpXTAG that contained an *E. coli* outer membrane protein OmpX gene with an AAAAXAA (A denotes alanine and X denotes the NCAA) insertion between two extracellular residues, 53 and 54, were used to transform *E. coli* BL21 cells. The transformed cells were grown in LB supplemented with 2 mM of **4**, **5**, or **6** to produce OmpX with these NCAAs site-specifically incorporated. The subsequent reactions with FITC-TZ fluorescently labeled the expressed proteins at the bacterial cell surface. Similar reactions with HZCL also fluorescently labeled the expressed proteins except for OmpX incorporated with **5**, whose labeling with HZCL led to a nonfluorescent product (Figure 5). Collectively, our assembled in vitro and cell labeling data demonstrate the high efficiency and selectivity of IEDDAC and NADC reactions in the labeling of newly developed and genetically encoded strained alkene/alkyne NCAAs.

CONCLUSIONS

In summary, we have kinetically characterized IEDDAC and NADC reactions that involve strained alkene/alkyne functionalities including norbornene, *trans*-cyclooctene, and cyclooctyne, and applied the two reactions to selectively label proteins bearing these functionalities. Being a rapid catalyst-free click reaction with a high bio-orthogonality, IEDDAC has received substantial attention recently.[17,18,23,31] Our study showed that all three strained alkene/alkyne functionalities undergo rapid IEDDAC reactions with tetrazines and rate constants close to 300 000 M^{-1} s^{-1} could be reached. Our study also revealed that all three functionalities undergo fast reactions with diaryl nitrilimines at rate constants close to 20 M^{-1} s^{-1}. To the best of our knowledge, the current study is the first report of fast reaction kinetics between *trans*-cyclooctenes and diaryl nitrilimines. To synthesize proteins with site-specifically encoded strained alkene/alkyne functionalities, a mutant PylRS-$tRNA^{Pyl}_{CUA}$ pair was developed, which has been successfully applied to incorporate five tyrosine derivatives containing norbornene, *trans*-cyclooctene, and cyclooctyne functionalities into proteins at amber mutation sites in *E. coli*. Proteins bearing these tyrosine derivatives were fluorescently labeled using IEDDAC and NADC reactions both in vitro and in living cells. Our current study has significantly expanded the tool kits for protein labeling. Potential applications of the developed methods include studies such as protein folding/dynamics, protein trafficking, and protein−protein/DNA interactions.

EXPERIMENTAL PROCEDURES

Synthesis. Synthesis of strained alkenes/alkynes, noncanonical amino acids (NCAAs), and a fluorescein tetrazine (FITC-TZ) is provided as Supporting Information due to substantial procedure descriptions and spectroscopic data.

Kinetic Characterizations. All kinetic measurements were carried out using a PTI QMA40 fluorescent spectrophotometer. For IEDDAC reactions between strained alkenes/alkynes and FITC-TZ, fluorescent emission was monitored at 515 nm with an excitation light at 493 nm. For NADC reactions between strained alkenes/alkynes with a hydrazonyl chloride (HZCL) that served as a nitrilimine precursor, fluorescent emission was monitored at 480 nm with an excitation light at 318 nm. Data were collected during a reaction period of 1 h.

Plasmid Construction. Plasmid pEVOL-pylT-Y306A/N346A/C348A/Y384F was derived from pEVOL-pylT-N346A/C348A[47] that contains genes coding $tRNA^{Pyl}_{CUA}$ and pyrrolysyl-tRNA synthetase (PylRS) with mutations N346A/C348A. Two primers PylRS-Y384F-Fwd: 5′-tggggataccttgatgtaatgcacg-3′ and PylRS-Y384F-Rev: 5′-aagaccatgcaggaatcgcctac-

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gat-3′ were used to run site-directed mutagenesis to introduce the Y384F mutation. Another two primers PylRS-Y306A-Fwd: 5′-aactacctgcgcaagcttgacagggc-3′ and PylRS-Y384F-Rev: 5′-cgcaaggtttggagcaagcatgggt-3′ were subsequently used to run site-directed mutagenesis to introduce the Y306A mutation.

Protein Expression. Plasmid pET-sfGFPS2TAG,[47] which contains genes coding $tRNA^{Pyl}_{CUA}$ and superfolder green fluorescent protein (sfGFP) with an amber mutation at S2, was cotransformed with plasmid pEVOL-pylT-N346A/C348A/Y384F/Y306A into *E. coli* BL21 (DE3) cells. From a single colony, a 5 mL overnight culture was prepared and inoculated into 300 mL Lysogenic Broth (LB) medium. Cells were grown to OD_{600} as 0.6 and then induced to express sfGFP site-specifically incorporated with a NCAA with the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), 0.2% arabinose, and 1 mM of the designated NCAA. After overnight expression, cells were harvested and suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and sonicated in an ice water bath. The cell lysate was clarified by centrifugation (11 000*g* at 4 °C for 60 min). The crude supernatant was decanted and allowed to bind to 5 mL of Ni-NTA superflow resin at 4 °C for 1 h. The mixture was loaded into an empty cartridge and washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The expressed sfGFP was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Eluted fractions were concentrated in a Pierce 10 KD MWCO filter unit and dialyzed against 10 mM ammonium bicarbonate. The finally purified protein was analyzed by 12% SDS-PAGE and characterized using electrospray ionization mass spectrometry (ESI-MS).

In Vitro Protein Labeling with FITC-TZ. 0.1 mM FITC-TZ was added to a solution of a NCAA-containing sfGFP in PBS (pH 7.4) buffer. The reaction was carried out at room temperature for 1 h. The protein was purified using Ni-NTA resin and analyzed by 12% SDS-PAGE. The fluorescence was detected using a regular UV transilluminator.

In Vitro Protein Labeling with HZCL. 0.5 mM HZCL was added to a solution of a NCAA-containing sfGFP in PBS (pH 7.4) buffer. The reaction was carried out at room temperature for 1 h and then directly applied to undergo analysis with 12% SDS-PAGE. The fluorescence was detected in a Bio-Rad ChemiDoc XRS+ system under UV irradiation.

Live Cell Labeling and Imaging. BL21(DE3) cells were transformed to carry plasmids pEVOL-PylT-Y306A/N346A/C348A/Y384F and pETDuet-OmpXTAG[44] that contained a gene coding *E. coli* membrane protein OmpX with an amber mutation. The transformed cells were grown to OD_{600} as 1 and then induced to express OmpX site-specifically incorporated with a NCAA with the addition of 0.5 mM IPTG, 0.2% arabinose, and 1 mM of the designated NCAA. Cells were let grown overnight, pelleted, and washed with isotonic saline three times before they were incubated with 0.1 mM FITC-TZ or 0.5 mM HZCL at room temperature. The incubation time for cell labeling was typically 3 h. After reactions, cells were pelleted, washed with PBS buffer four times, and then subjected to fluorescent imaging. Cells grown in LB without a NCAA but subjected to same labeling procedures were set up as control. Fluorescent imaging of cells was performed using an Olympus IX-81 inverted microscope.

Computational Methods. All computations were run at the M06-2X/6-311+G(d,p) level of theory, using Gaussian09.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Kinetic characterizations of strain-promoted click reactions; NCAA synthesis; plasmid construction; protein expression; protein/cell labeling; supplementary figures; ^1H and ^{13}C NMR spectra of NCAAs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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