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Unexpected Cyclization Product Discovery from the Photoinduced Bioconjugation Chemistry between Tetrazole and Amine

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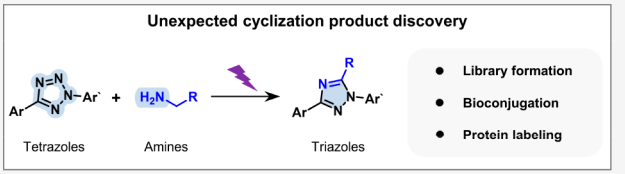
ABSTRACT: Bioconjugation chemistry has emerged as a powerful tool for the modification of diverse biomolecules under mild conditions. Tetrazole, initially proposed as a bioorthogonal photoclick handle for 1,3-dipolar cyclization with alkenes, was later demonstrated to possess broader photoreactivity with carboxylic acids, serving as a versatile bioconjugation and photoaffinity labeling probe. In this study, we unexpectedly discovered and validated the photoreactivity between tetrazole and primary amine to afford a new 1,2,4-triazole cyclization product. Given the significance of functionalized *N*-heterocycles in medicinal chemistry, we successfully harnessed the serendipitously discovered reaction to synthesize both pharmacologically relevant DNA-encoded chemical libraries (DELs) and small molecule compounds bearing 1,2,4-triazole scaffolds. Furthermore, the mild reaction conditions and stable 1,2,4-triazole linkage found broad application in photoinduced bioconjugation scenarios, spanning from intramolecular peptide macrocyclization and templated DNA reaction cross-linking to intermolecular photoaffinity labeling of proteins. Triazole cross-linking products on lysine side chains were identified in tetrazole-labeled proteins, refining the comprehensive understanding of the photo-cross-linking profiles of tetrazole-based probes. Altogether, this tetrazole-amine bioconjugation expands the current bioconjugation toolbox and creates new possibilities at the interface of medicinal chemistry and chemical biology.



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INTRODUCTION

The modification of biomolecules is a central endeavor for the advancement of drug discovery, chemical biology, and molecular biology.[1−5] Bioconjugation chemistry has been extensively utilized for the modification of diverse biomolecules, ranging its application from nucleotide cross-linking, peptide cyclization, protein modification, and covalent drug discovery. Hence, novel chemistry shall be constantly explored and investigated to broaden the current bioconjugation toolbox for diverse aims.

Among the bioconjugation functionalities, tetrazole ranks as a versatile moiety due to its broad reactivity as a photoinducible nitrile imine precursor.[6] Although initially proposed to be a bioorthogonal handle that undergoes phototriggered 1,3-dipolar cycloaddition with exogenously introduced alkenes, tetrazole was later reported to possess photoreactivity with native carboxylic acids on proteins.[7−10] This biocompatible and photocontrollable reactivity conferred tetrazole a panel of applications in biomolecule conjugation, affinity-based protein labeling, and covalent drug discovery.[11−14] Nevertheless, exploring new chemical reactivity and investigating the chemoselectivity of tetrazole photoreaction is still in urgent demand.

Crucial to bioconjugation chemistry are the mild reaction conditions, which are compatible with biomolecules. These bioconjugation reactions should also demonstrate efficient conversion and good selectivity and ideally generate stable linkage or products, which may be highly desired in the manufacturing of bioconjugates (Figure 1a). Besides protein modification, such as antibody−drug conjugates, bioconjugation chemistry is highly demanded in the modification of oligonucleotides, although the criteria for bioconjugation chemistry applied to different biomolecules (proteins, nucleotides, sugars, etc.) may slightly vary. Recently, bioconjugation chemistry that complies with the standards of DNA compatibility and creates drug-like scaffolds is valuable for DNA-encoded chemical library (DEL) construction and further selection to isolate bioactive molecules.[15−21] Considering the diverse transformation of the tetrazole moiety, our group has previously explored DNA-compatible chemical reactions focused on tetrazoles. We reported the application of 1,3-dipolar cycloaddition reaction between tetrazoles and alkenes to assemble pyrazoline scaffold meaningful DELs.[22] Following this vein, we explored the broader reactivity of tetrazole from the aspect of bioconjugation reactions and unexpectedly discovered a new reactivity between tetrazole and primary amine to form a stable 1,2,4-triazole cyclization

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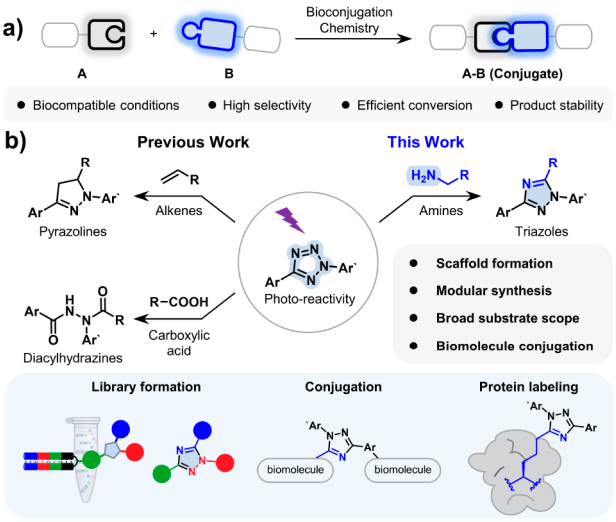

Figure 1. Discovery and application of the new reactivity between tetrazole-amine bioconjugation. (a) The general scheme and criteria of bioconjugation chemistry between two biomolecules. (b) Previous works mainly investigated photoinduced tetrazole reactions with alkenes and carboxylic acids. This work discovered a unique 1,2,4-triazole cyclization product of tetrazole-amine bioconjugation chemistry and showed its diverse applications.

product via photomediated reaction, which was not reported previously.

With the discovery and validation of the unexpected cyclization product generated from the tetrazole-amine bioconjugation, we showcased its capability as a biomolecule-compatible reaction. Applying the bioconjugation reaction to DEL and combinatorial chemistry efficiently generated pharmacologically meaningful scaffolds and synthesized compound libraries. Further experiments in intramolecular peptide macrocyclization, DNA-templated nucleotide cross-linking, and photoaffinity protein labeling demonstrated its universal applicability in the modification of diverse biomolecules, expanding the bioconjugation toolbox and revealing hidden insights of tetrazole probe's photo-cross-linking profiles (Figure 1b).

RESULTS AND DISCUSSION

1. Unexpected Discovery of a New 1,2,4-Triazole Cyclization Product from DNA-Compatible Tetrazole-Amine Conjugation. Due to its diverse reactivity as a synthetic intermediate, tetrazole has been widely explored in the construction of diverse chemical structures in a biomolecule-compatible manner. Recently, by harnessing the classical photomediated cycloaddition between tetrazole and alkene, we achieved DNA-compatible synthesis of polysubstituted pyrazoline scaffolds for DEL construction purposes.[22] During the process of expanding its substrate scope to accommodate more chemically diverse alkene-bearing building blocks, we were intrigued by some results beyond our expectations. As shown in Figure 2a, when we examined the reactivity of the DNA-tetrazole conjugate (**a1**) with an alkene bearing an amine group (**b1**), instead of the proposed 1,3-dipolar cycloaddition, an unexpectedly dominant product was observed. The molecular weight (5238 Da) deconvoluted from the MS data was not consistent with the expected pyrazoline product **c1** generated from alkene cycloaddition or with the previously reported product of nucleophilic addition to amine

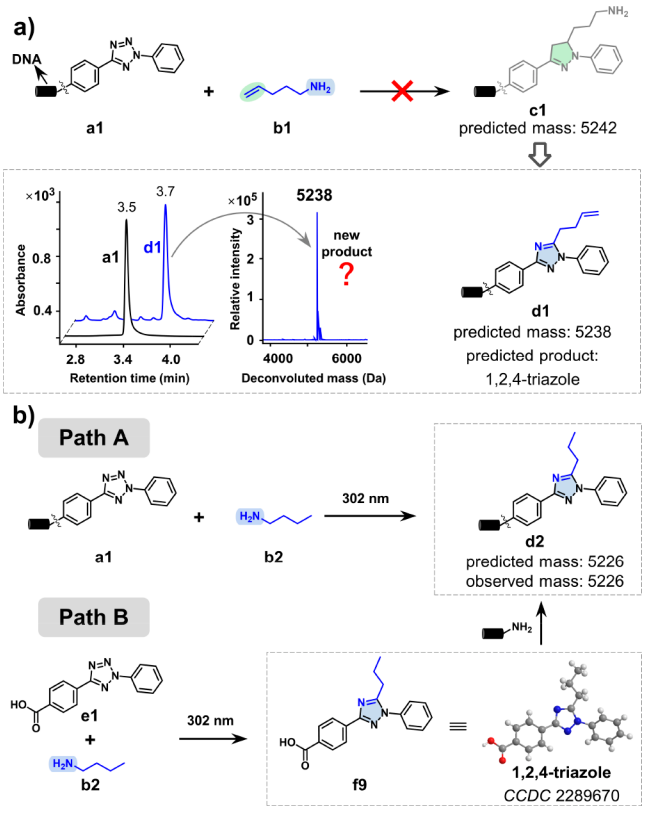

Figure 2. Discovery and validation of a new tetrazole-amine reactivity to afford the 1,2,4-triazole cyclization product in the DNA-compatible chemistry study. (a) The reaction between DNA-conjugated tetrazole probe a1 and bifunctional building block b1 yielded a product with unexpected molecular weight. Conditions: DNA-conjugated tetrazole a1 (4 μL, 50 μM in H_2O), Cs_2CO_3 (5 μL, 250 mM in H_2O), amine b1 (10 μL, 500 mM in DMF), solvent (11 μL, ACN/DMF = 7/4, v/v), 302 nm hand-held UV lamp, 25 °C, 10 min. Conversions were determined by UPLC-MS. (b) Validation of the 1,2,4-triazole product structure from both the on-DNA and off-DNA synthetic paths. Path A condition: DNA-conjugated tetrazole a1 (4 μL, 50 μM in H_2O), Cs_2CO_3 (5 μL, 250 mM in H_2O), amine b2 (10 μL, 500 mM in DMF), cosolvent (11 μL, ACN/DMF = 7/4, v/v), 302 nm hand-held UV lamp, 25 °C, 10 min. Conversions were determined by UPLC-MS. Path B condition: See the Supporting Information (SI) for details.

(5242 Da).[23,24] Hence, we speculated that a different structure was generated possibly via a cyclization reaction with amine, of which the molecular weight corresponded to the 1,2,4-triazole structure. Subsequently, to verify our speculation of the new structure, monofunctional amine substrate **b2** was tested with DNA-tetrazole conjugate **a1**. A similar unexpected product, **d2**, was observed. This indicated a unified yet undiscovered phototriggered reaction mode between tetrazole and amine. To fully investigate the product's chemical structure, we next carried out the reaction between tetrazole and amine without the DNA tags and yielded the product **f9** with the expected molecular weight. Furthermore, the structure of **f9** was validated by nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HRMS), and crystallography (see SI 3.4 and section 8). All the evidence indicated that the unexpected product formed between the tetrazole-amine reaction was the cyclized *N*-containing heterocycle 1,2,4-triazole. With the organic molecule again conjugated to the DNA as a standard, the structure of the on-DNA formed

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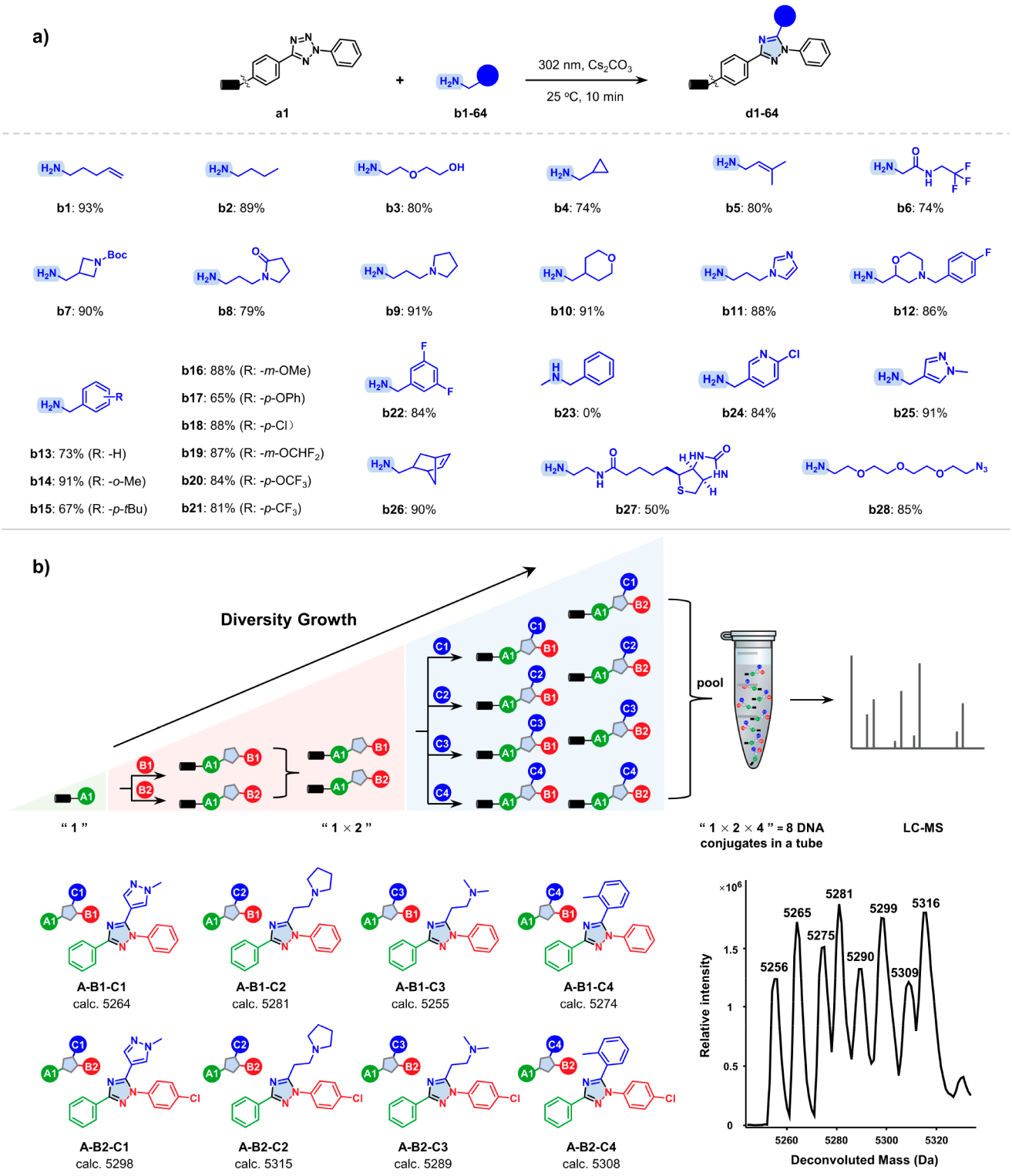

Figure 3. Construction of 1,2,4-triazole-focused DNA-encoded libraries. (a) Substrate scope of amines for on-DNA 1,2,4-triazole scaffold assembly. Standard conditions: DNA-conjugated tetrazoles (4 μL, 50 μM in H_2O, 0.2 nmol), Cs_2CO_3 (5 μL, 250 mM in H_2O), amines (10 μL, 500 mM in DMF), ACN/DMF (11 μL, v/v = 7/4), 302 nm hand-held UV lamp, 25 °C, 10 min. Conversions were determined by UPLC-MS. (b) Synthesis of a DNA-encoded mock library featuring polysubstituted 1,2,4-triazole derivatives. See the Supporting Information (SI) for details.

product was further validated to be 1,2,4-triazole via a coinjection experiment (Figure 2b).

Tetrazole was known to transform into the 1,3-dipolar nitrile imine intermediate amenable to both cycloaddition and nucleophilic addition upon photoirradiation. However, further cyclization following nucleophilic addition of primary amine was not reported before. Our results demonstrated that a unique 1,2,4-triazole cyclization product was generated via the photomediated reaction between tetrazole and amine. This process was probably triggered by radical-mediated cyclization and further oxidation (see SI section 3.6). Given that the phototriggered tetrazole-amine reaction was biomolecule-

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compatible and the cyclized 1,2,4-triazole scaffold was pharmacologically important, we next explored the potential of this reaction in DNA-encoded library synthesis.

2. Utilizing the Tetrazole-Amine Bioconjugation for the Synthesis of 1,2,4-Triazole-Focused Combinatorial Libraries. The DEL technology, based on DNA-small molecule bioconjugates, has emerged as a promising high-throughput drug discovery platform to obtain bioactive compounds of target proteins and a wide range of other biomacromolecules in both industrial and academic realms, evidenced by the generation of several clinical candidates.[25−35] By combining the advantage of combinatorial chemical synthesis with the convenience of genetic barcoding, DEL allows the systematic and high-throughput implementation of hundreds of reactions in parallel. A structurally diverse DEL is crucial for the advancement of hit identification, which calls for the development of new DNA-compatible reactions.[36−52] Logically, the DEL demonstrates mutual reciprocity with biocompatible conjugation reaction discovery. On one side, harnessing DNA-compatible synthetic methodologies for DEL synthesis significantly facilitates the creation of chemically diverse combinatorial libraries. On the other side, earlier studies have documented DNA-templated reaction discovery systems, unveiling a range of biocompatible ligation and cleavage reactions.[53−56]

Polysubstituted 1,2,4-triazole derivatives, the cyclized product discovered above, have been extensively employed in chemistry, material science, and coordination chemistry.[57−62] Due to the widespread application of 1,2,4-triazole derivatives, several synthetic strategies have been previously reported.[63−67] However, some harsh conditions involving heating (>100 °C)[66] and oxidant (TBHP/I_2)[63] impeded their use in DNA-compatible reactions. In contrast, the synthetic route to 1,2,4-triazole heterocycles we reported is mild in condition and DNA-compatible, making it ideal for DEL synthesis. Therefore, we applied this phototriggered tetrazole-amine bioconjugation chemistry to DNA-compatible chemical transformations.

With the optimal conditions in hand, a number of primary amines bearing a variety of substituents were investigated (Figure 3a and SI section 4.1). First, primary amines (**b1**−**b6**) containing alkenyl, cyclopropyl, or alcohol groups were well tolerated to give the corresponding 1,2,4-triazoles with high conversion. It was also found to be highly reactive toward primary amines containing heterocycles (**b7**−**b12**), including imidazole and several saturated *O*- and *N*-heterocycles. Intriguingly, amines containing bioorthogonal handles and functionalities, such as alkyne (**b1**, **b26**), biotin (**b27**), and azide (**b28**), reacted successfully and therefore permitted further diversifications. It is worth noting that when both norbornene and amine groups are present simultaneously (**b26**), tetrazole reacts preferentially with the amine group under our reaction conditions.

In order to construct triaryl-substituted 1,2,4-triazole derivatives, various benzylamine derivatives (**b13**−**b22**) were tested. The results showed that substrates with both electron-rich (**b14**−**b17**) and electron-deficient (**b18**−**b22**) substituted benzylamine could be converted into their 1,2,4-triazoles in good conversion (65%−91%). Notably, it was also demonstrated that the secondary amine (**b23**) was inert under this reaction condition. In addition, amines containing medicinally important unsaturated heterocycles, such as pyridine and pyrazole, could afford excellent conversion (**b24**−**b25**). Satisfyingly, among 59 amines, 48 afforded the corresponding products in >70% conversion.

Furthermore, we prepared 16 DNA-tetrazole conjugates (green, representative of aldehydes; red, representative of aryl amines) with DNA-compatible synthetic routes and then investigated the substrate scope of these conjugates (see SI section 4.2).[22] The resulting DNA-tetrazole conjugates achieved efficient conversion toward triazoles (15 substrates afforded >70% conversion and 1 achieved >50% conversion). The abundant scope of structurally diverse, commercially available arylamines, aldehydes, and primary amines makes it a particularly attractive strategy for utilization in DEL.

To demonstrate the synthetic compatibility of this new chemistry for DEL synthesis, we employed the "split-and-pool" procedure to prepare a mock library of 1,2,4-triazole in three cycles (Figure 3b). Using the DNA-tagged benzaldehyde as the starting species and two dimensions of building blocks, we successfully obtained a three-dimensional 1,2,4-triazole mock pool library containing 1 × 2 × 4 members, which was clearly characterized by LC-MS. These data revealed the combinatorial synthetic diversity of this tetrazole-amine reaction. Meanwhile, we wondered whether this reaction would preserve the DNA integrity and be compatible with enzymatic ligation. Toward this goal, we prepared a 1 × 1 × 1 model 1,2,4-triazole compound via three cycles of synthesis together with encoding. To our delight, all three ligation products were confirmed by polyacrylamide gel electrophoresis (PAGE) analysis (see SI section 4.3 and 4.4). Taken together, the study of the tetrazole-amine reaction provided a foundation for the subsequent synthesis of a DNA-encoded 1,2,4-triazole library for high-throughput drug discovery.

Besides its role in DEL construction, we further explored the potential of this new chemistry for conventional organic compound synthesis. A series of primary amines and tetrazoles were investigated to broadly assess the practicability of the reaction.

First, we chose tetrazole **e4** as the model substrate to evaluate the reactivity of the different amines. As shown in Figure 4, desired products were obtained with several amines (**b4**, **b8**, **b10**, **b13**, **b25**, **b26**, **b48**, and **b54**) in 46%−84% isolated yields. We further examined the generality of tetrazoles reacting with amine **b2**. A total of six tetrazoles with different substituents (**e1**−**e6**) proceeded smoothly under the standard reaction conditions to give the expected products, all of which exhibited favorable isolated yields (55%−82%).

All of the above experimental results demonstrated that the tetrazole-amine reaction could be successfully performed not only in on-DNA library construction but also in off-DNA synthesis. Besides the mild conditions and good yield, the strategy also has the potential to be used not only for diverse combinatorial chemical library construction but also in off-DNA synthesis for DEL hit validation. Besides the mild condition and good yield, the strategy also has the potential to be used for diverse combinatorial chemical library construction. Integrating the DNA-compatible synthesis with parallel off-DNA synthesis, this synthetic approach would greatly accelerate the synthesis and validation process of the hit compound selected from DNA-encoded libraries.

3. Tetrazole-Amine Reaction for Biocompatible Conjugation and Labeling of Diverse Biomolecules. Encouraged by the successful studies in DNA-encoded library synthesis and combinatorial chemistry, we envisioned the capability of tetrazole-amine conjugation chemistry in the

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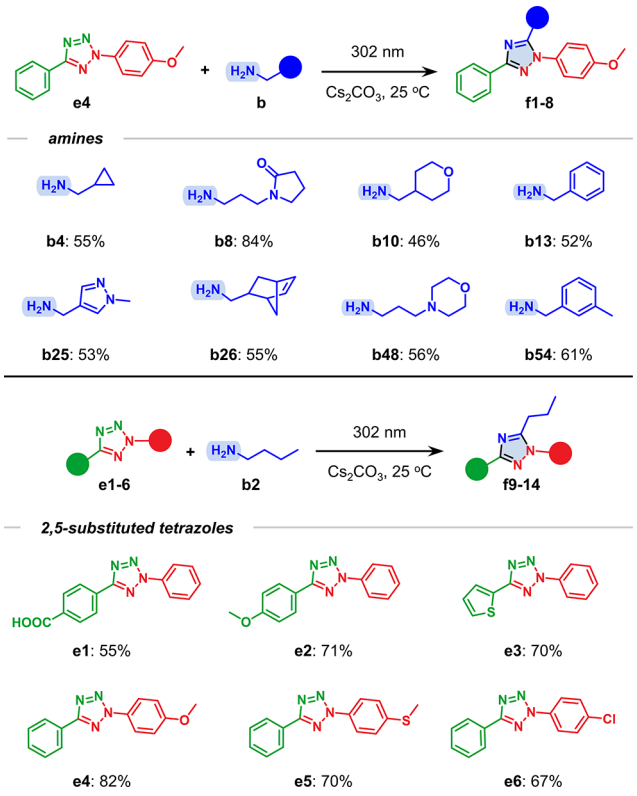

Figure 4. Substrate scope study of organic synthesis of 1,2,4-triazole-centered molecules. In the off-DNA organic synthesis, both the scopes of primary amines and tetrazoles were investigated. See the Surpporting Information (SI) for details. Isolated yields were obtained after flash chromatography.

modification of biomolecules in diverse scenarios, from the intramolecular peptide macrocyclization to templated nucleotide-nucleotide cross-linking and then photoaffinity protein labeling. To this end, we first explored whether the reaction could be used in intramolecular peptide macrocyclization. For this study, we chose the peptide LTFEHYWAQLTS, which was discovered via phage display as a potent peptide dual inhibitor of p53-Mdm2/Mdmx.[68] However, this peptide inhibitor was prevented from further development as a therapeutic agent due to cell permeability limitations. Stapled peptides and macrocyclization of peptides are practicable strategies for overcoming this problem. To improve the cell permeability of peptide inhibitors, Lin and co-workers previously reported a strategy for stapling the modified peptide with tetrazole and alkene via photomediated 1,3-dipolar cycloaddition.[69,70] As compared to the previous studies requiring dual side chain modifications to make the stapled peptide, we considered that the cyclization between tetrazole and the natural Lys side chain in peptides may be advantageous. To demonstrate the peptide cyclization, the modified peptide inhibitor **S1** with an (i, i+4) reaction pair of lysine and tetrazole was prepared (Figure 5a). As expected, the cyclized product **S2** was obtained with 75% conversion after the linear peptide inhibitor was subjected to 302 nm UV irradiation in a mixed ACN/PBS solution for 20 min (see SI section 6.1). These results indicated that our method could serve as a powerful tool for constructing novel stapled and cyclized peptides in a modular manner, and the functionalities involved in this bioconjugation reaction were readily compatible with solid-phase peptide synthesis (SPSS).

Similar to the intramolecular reaction, template-controlled reactions also provide a higher effective molarity to accelerate chemical reactions. Accordingly, we next explored the application of tetrazole-amine reaction in DNA-templated nucleotide-nucleotide cross-linking. Currently, various DNA-templated chemical reactions are widely used for purposes ranging from drug discovery,[71,72] to nucleic acid diagnostics[23,73−75] and drug release.[76,77] Successful DNA-templated chemistry requires milder conditions that facilitate DNA hybridization, such as an aqueous buffer, high salt, and low temperature. In this regard, we attempted to examine whether the tetrazole-amine reaction could occur via DNA-templated synthesis to generate the 1,2,4-triazole scaffold. As shown in Figure 5b, the optimized conditions were applied (lane 2) to form 1,2,4-triazole, with the hybridization between the reagent DNA strands (**R1**, **R2**) and the template strand (**T**). In contrast, no irradiation or mismatched template DNA or no template DNA resulted in the absence of product **R1**+**R2** formation (lanes 1, 3, 4). These control experiments demonstrated that the bioconjugation reaction relied on hybridization between the reagent DNA strands (**R1**, **R2**) and template (**T**) as well as 302 nm irradiation. Furthermore, we verified the cross-linking results by LC-MS and observed the expected molecular weight of the 1,2,4-triazole cyclization product (see SI section 6.2). These results suggested that DNA-templated synthesis was capable of directing the tetrazole-amine reaction to form the cyclized product linking two oligonucleotide backbones. Compared with gel electrophoresis, the characterization of DNA-templated chemical reactions by LC-MS provided more accurate information, which not only characterized conversion yields but also revealed structural information on the generated products.

Based on the application of the tetrazole-amine reaction in intramolecular peptide cyclization and DNA-templated cross-linking, we hypothesized that this reaction might also find applications in photoaffinity protein labeling due to its reactivity with proteinogenic residues. Previous work indicated that tetrazole was capable of labeling endogenous carboxylic acids via photomediated reactions under physiological conditions.[9−11,13,14,78,79] In addition to carboxylic acid, primary amine in the side chain of lysine residue serves as an abundant source of alternative nucleophilic reagents in native proteins,[80] and has been investigated in targeted covalent inhibitors (TCIs) and affinity-based probes (AfBPs).[81−83] With tetrazole probe **e4** in hand, we next assessed their protein labeling capabilities. As shown in Figure 5c, labeling toward lysine residues was observed in myoglobin with an Δmass of +220 Da ($C_{14}H_8N_2O$) in the pH 9.0 PBS buffer condition, indicating the formation of the expected 1,2,4-triazole product. Encouraged by this result, we repeated the experiments for tetrazole labeling of myoglobin previously reported under neutral conditions (PBS, pH 7.4).[9] Gratifyingly, we found that both endogenous carboxylic acids and amines on myoglobin were labeled with tetrazole under neutral conditions. Comparison between cross-linking samples under different pH (7.4, 9.0 and 10.0) indicated that although tetrazole majorly labeled glutamate/aspartate in the neutral pH condition, selectivity toward lysine over glutamate/aspartate was promoted by higher pH conditions (see SI section 9.1). Notably, among the labeled lysine sites, triazole was the major labeling product for tetrazole-amine conjugation (SI section 9.1). Furthermore, the results were verified in the labeling of RNase A with tetrazole probe **e4** (see SI section 9.2).

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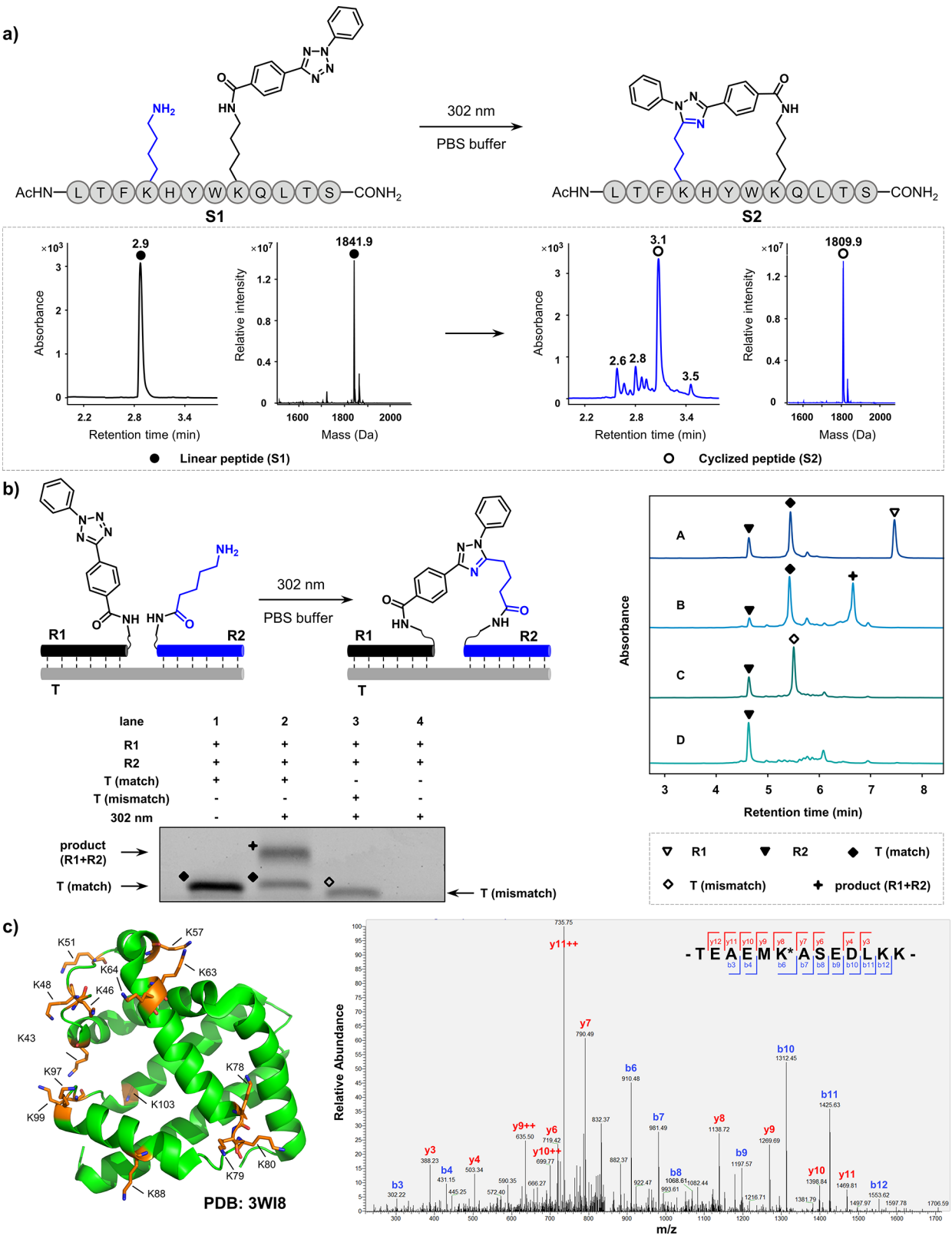

Figure 5. Application of the tetrazole-amine chemistry for conjugation and modification of diverse biomolecules. (a) The preinstalled tetrazole handle allowed intramolecular peptide macrocyclization with a native lysine residue. (b) DNA-templated tetrazole-amine reaction for nucleotide cross-linking. 20% denaturing PAGE analysis: R1/R2/T (matched template DNA): 0.5 μM. Lane 1: R1/R2/T (match), no irradiation; lane 2: matched template DNA; lane 3: mismatched template DNA; lane 4: R1/R2, no template DNA. LC-MS analysis: A: R1/R2/T (matched template DNA), no irradiation; B: matched template DNA; C: mismatched template DNA; D: R1/R2, no template DNA. (c) LC-MS/MS analysis of tetrazole probe **e4**-labeled myoglobin identified lysine residues as photoaffinity labeling sites. The typical spectrum confirming the cyclization product on K57 were shown. See the Surpporting Information (SI) for details.

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These data demonstrated that the tetrazole probes could react with both endogenous carboxylic acids and amines on proteins in neutral or basic buffers. This new reactivity expanded the reactive residue scope for tetrazole as a photo-cross-linking chemical probe. Notably, the 1,2,4-triazole product was not detected in previous protein labeling works involving tetrazole chemistry. Therefore, this study implies the presence of untapped information for proteomics analysis, and these proteomic data may be reinvestigated to dig out extra information about cross-linking protein targets and interaction sites.

CONCLUSIONS

In summary, in pursuit of expanding the diverse bioorthogonal chemical reaction scope of tetrazoles, we have unexpectedly discovered a novel 1,2,4-triazole cyclization product generated from photomediated tetrazole-amine conjugation under biocompatible conditions. This novel phototriggered biocompatible synthetic route to this product was verified by NMR, HRMS, and crystallography and was not previously reported. From the aspects of medicinal chemistry, this methodology for constructing the drug-like 1,2,4-triazole scaffold was successfully applied in the construction of DNA-encoded libraries and small molecule organic synthesis. In addition, the biocompatible mild reaction condition of tetrazole-amine chemistry allowed for diverse bioconjugation applications, ranging from intramolecular peptide macrocyclization to DNA-templated nucleotide-nucleotide cross-linking and photoaffinity protein labeling. Given the excellent reactivity as well as the abundance of amine-containing biomolecules, the presented tetrazole-amine bioconjugation chemistry provided new opportunities for labeling and cross-linking in biocompatible conditions. Overall, we expect that this tetrazole-amine reaction will find wide applications in the modification of biomolecules, synthesis of pharmaceuticals, and other related fields.

ASSOCIATED CONTENT

Data Availability Statement

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

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c11574.

General information and experimental details, supporting figures and tables, UPLC-MS data (PDF)

Accession Codes

CCDC 2289670 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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