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COMMUNICATION

Triazolecarbaldehyde Reagents for One-step N-Terminal Protein Modification**

Akira Onoda, Nozomu Inoue, Eigo Sumiyoshi, and Takashi Hayashi**

Abstract: Site-specific modification of peptides and proteins is a key aspect of protein engineering. We developed a method for modification of the N-terminus of proteins using 1*H*-1,2,3-triazole-4-carbaldehyde (TA4C) derivatives, which can be prepared in one step. The N-terminal specific labeling of bioactive peptides and proteins with the TA4C derivatives proceeds under mild reaction conditions in excellent conversion (angiotensin I: 92%, ribonuclease A: 90%). This method enables site-specific conjugation of various functional molecules such as fluorophores, biotin, and polyethylene glycol attached to the triazole ring to the N-terminus. Furthermore, a functional molecule modified with a primary amine moiety can be directly converted to a TA4C derivative through a Dimroth rearrangement reaction with 1-(4-nitrophenyl)-1*H*-1,2,3-triazole-4-carbaldehyde. This method can be used to obtain N-terminal modified proteins via only two steps; (i) convenient preparation of a TA4C derivative with a functional group and (ii) modification of the N-terminus of the protein with the TA4C derivative.

Site-specific modification of peptides and proteins is required for the development of pharmaceutical conjugates, bioimaging and medical diagnostics reagents, and protein-based materials.[1-9] Chemical approaches have been important tools for modification of reactive side chains in canonical amino acid residues including Lys, Cys, Tyr, Trp, Arg, and Met.[7-13] The N-terminus is an attractive accessible site for specific chemical modification because it represents a unique, yet ubiquitous position in proteins. Advantageously, the N-terminus is typically not included in a folded structure. It is therefore likely that N-terminal modification will not affect the inherent function of wild-type proteins or enzymes in most cases.[14] Since an N-terminal α-amino group has a lower pK_a value than the ε-amino group of the Lys side chain,[15] a reagent targeting the N-terminal amino acid is expected to be particularly effective for modification of this site.[16-35] For example, Francis and co-workers, and other researchers have reported a site-specific and one-step N-terminal α-amino group modification scheme using 2-pyridinecarbaldehyde derivatives.[28-35]

A more simple and versatile method for preparing N-terminal modified proteins via minimum reaction steps would be expected to accelerate efforts to prepare proteins with various functional moieties for many different applications. Herein, we report a new one-step N-terminal specific protein modification with 1*H*-1,2,3-triazole-4-carbaldehyde (TA4C) derivatives which can form a 4-imidazolidinone ring (Figure 1). The reagent is easily prepared from a variety of synthetically accessible azide compounds and 3,3-diethoxyprop-1-yne via copper-catalyzed azide-alkyne cycloaddition (CuAAC).[36] Furthermore, it is found that a primary amine compound containing a functional molecule can be converted to a TA4C reagent through a unique Dimroth rearrangement.[37-38] This reaction scheme represents the simplest process developed thus far for preparing N-terminal modified proteins using a TA4C derivative (Figure 1b).

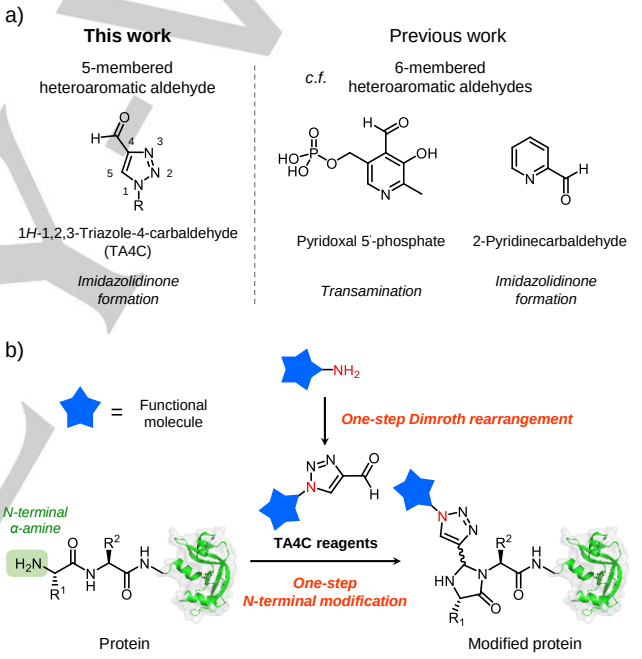

Figure 1. (a) Molecular structure of N-terminal specific modification reagent 1*H*-1,2,3-triazole-4-carbaldehyde (TA4C) and related compounds. (b) Scheme showing a one-step preparation of TA4C and a one-step bioconjugation through the formation of a 4-imidazolidinone ring with two nitrogen atoms of the N-terminus and adjacent residues. The blue star represents a functional molecule for N-terminal modification.

In biological systems, pyridoxal phosphate is an important cofactor which is involved in post-translational modification of an amino group in peptides and proteins.[39-41] Specific N-terminal modification of a protein with 2-pyridylcarbaldehyde, a nitrogen-containing heteroaromatic compound with a formyl group which is similar to the pyridoxal phosphate cofactor, was reported to proceed via formation of a 4-imidazolidinone ring involving two nitrogen atoms of the N-terminus and adjacent residues.[28-33,35] On the basis of this knowledge, we began our investigation by examining a series of five-membered nitrogen-containing heteroaromatic aldehydes as N-terminal specific modification reagents. The reagents **1-5** including a triazole, imidazole, or pyrazole ring bearing a benzyl group were tested for N-terminal

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modification of a bio-active peptide, angiotensin I (DRVYIHPFHL) (Figures 2 and S1). The conversions of the peptide after treatment with the reagents were analyzed by LC-MS (Figure S1). Interestingly, both 1-benzyl-1*H*-1,2,3-triazole-4-carbaldehyde (**1**) (90%) and 2-benzyl-2*H*-1,2,3-triazole-4-carbaldehyde (**2**) (86%) are found to provide excellent conversions in N-terminal modification. In contrast, 1-benzyl-1*H*-1,2,3-triazole-5-carbaldehyde (**3**) containing a benzyl group at the 1-position did not produce the modified product, indicating that the positioning of an aldehyde group adjacent to the nitrogen atom is an important feature. Imidazole **4** (49%) and pyrazole **5** (16%) produce moderate to low conversions. Considering the two triazole reagents that gave excellent conversions, carbaldehyde **1** can be easily synthesized from benzyl azide and 3,3-diethoxyprop-1-yne via CuAAC in a one-pot synthesis (Scheme S1).[36] In addition, the azide precursors are also prepared from organobromine or borate compounds in one-pot syntheses.[36,42] On the basis of these encouraging results, we set out to investigate the potential of TA4C derivatives as N-terminal modification reagents.

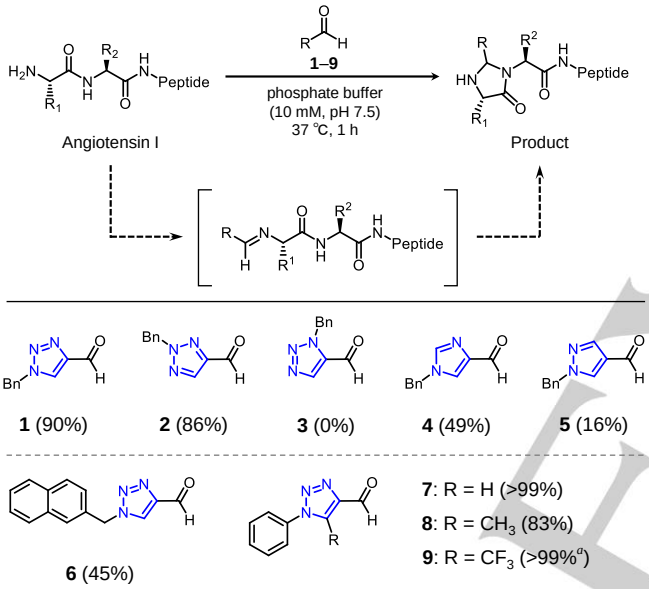

Figure 2. N-terminal modification of angiotensin I with heteroaromatic aldehydes (**1**–**9**). The conversion of the peptide treated with each reagent is determined by LC-MS. Conditions: angiotensin I (100 μM), aldehyde (10 mM), phosphate buffer (10 mM, pH 7.5) at 37 °C for 1 h. [a]The conversion efficiency was determined by the relative ratio of modification after 24 h.

With these highly efficient reagents for peptide modification in hand, we investigated 1*H*-1,2,3-triazole-4-carbaldehydes for effectiveness in the N-terminal modification of angiotensin I. TA4C derivatives with substituents at the N1- or C5-position of the triazole ring were tested (Figures 2 and S2). TA4C derivative **6,** which has a naphthylmethyl group at the N1 position, provides a moderate conversion (45%) because of low solubility in buffer. TA4C derivative **7**, which has a phenyl group at the N1 position provides excellent conversion (99%). Another derivative **8** which has a methyl group at the C5-position also provides good conversion (83%). TA4C derivative **9**, which has an electron-withdrawing trifluoromethyl group at the same position, provided a full conversion over a longer reaction time.

Next, we investigated modification of other bio-active peptides with reagent **1** (Table 1, Figure S3). Peptides with an α-amino group at the N-terminus were modified by **1** in excellent conversions (Table 1, entries 1−4). In addition, MS/MS analysis indicates that the modification proceeds only at the N-terminus (Figures S4 and S5). In sharp contrast, the modification does not occur when the α-amino group of at the N-terminus is blocked by acylation or pyroglutamylation (Table 1, entries 5 and 6). Bradykinin, RPPGFSPFR, in which the second amino acid residue is proline lacking a secondary amide group, does not provide the modified product (Table 1, entry 7). These results are explained by the reaction mechanism which appears to proceed through formation of a 4-imidazolidinone ring at the N-terminus (Figure 2). Furthermore, the formation of the 4-imidazolidinone ring after treatment of tripeptide Leu-Gly-Gly with TA4C derivatives was confirmed by ^{1}H NMR measurements (Figure S6). The ^{1}H NMR spectrum has two peaks at 5.64 and 5.67 ppm assignable to protons at the 2-position of the 4-imidazolidinone diastereomer. The high N-terminal selectivity originates from formation of the 4-imidazolidinone ring via nucleophilic attack of the amide nitrogen of the backbone followed by formation of an imine with TA4C.

Table 1. Modification of Peptides with Reagent **1**.[a]

Entry	Peptide	Modification[b] (%)
1	DRVYIHPFHL (Angiotensin I)	92
2	WAGGDASGE (Delta-sleep inducing peptide)	91
3	YGGFMRRVGRPE (BAM-12P)	96
4	LRQFLQKSLAAAA-NH_2 (Neuronostatin-13)	98
5	**Ac**-YVG	–
6	**pE**AKSQGGSN (Serum thymic factor)	–
7	R**P**PGFSPFR (Bradykinin)	–

[a]Conditions: peptide (100 μM), **1** (10 mM), phosphate buffer (10 mM, pH 7.5) at 37 °C for 4 h. [b]The relative ratio of modification was analyzed by LC-MS measurements and used to determine the conversion.

Encouraged by the high efficiency observed in the N-terminal modification of peptides by **1**, we next examined examples of site-specific modification at the N-terminus of proteins. Under optimized reaction conditions (pH 7.5, 37 °C, 16 h) (Figure S7), a series of proteins were treated (Figures 3a and S8). RNase A (RNase, N-terminal: Lys, 10 Lys residues), aldolase (N-terminal: Pro, 25 Lys residues), and bovine serum albumin (BSA, N-terminal: Asp, 59 Lys residues) were found to be specifically modified at the N-terminus in high conversion. Cytochrome b_{562} (N-terminal: Ala, 13 Lys residues), which has a hindered N-terminus, results in low modification efficiency. Cytochrome *c* (N-terminal: Ac-Gly, 19 Lys residues) with an acetylated N-terminal α-amino group was not modified. These results support the previous observation that the modification proceeds specifically at the N-terminal α-amino group without any ε-amino groups of Lys residues being modified under the present conditions.[28] We also confirmed that RNase with the N-terminal modification retains the native structure and activity (Figure S9 and Figure S10).

The high specificity of the N-terminal modification by the TA4C reagent was also revealed by control experiments using an *N*-hydroxysuccinimide ester (NHS-ester) reagent (Figure S11). When RNase was treated with benzoic acid NHS-ester (1 equiv. to protein) at pH 7.0 and 8.0 at room temperature for 12 h, multiple modifications at the N-terminal amino group and other residues were observed (Figure S11a). In addition, the modification efficiency is relatively low and even lower at pH 6.0. In contrast, the site-specific modification at N-terminus by **1** proceeds in excellent efficiency within the pH range from 6.0 to 8.0 (Figure S11b). Bioconjugation of RNase with a TA4C derivative having a range of functional molecules was performed (Figures 3b and S12). Fluorescein and biotin-attached TA4C derivatives **10** and **11** were conjugated to

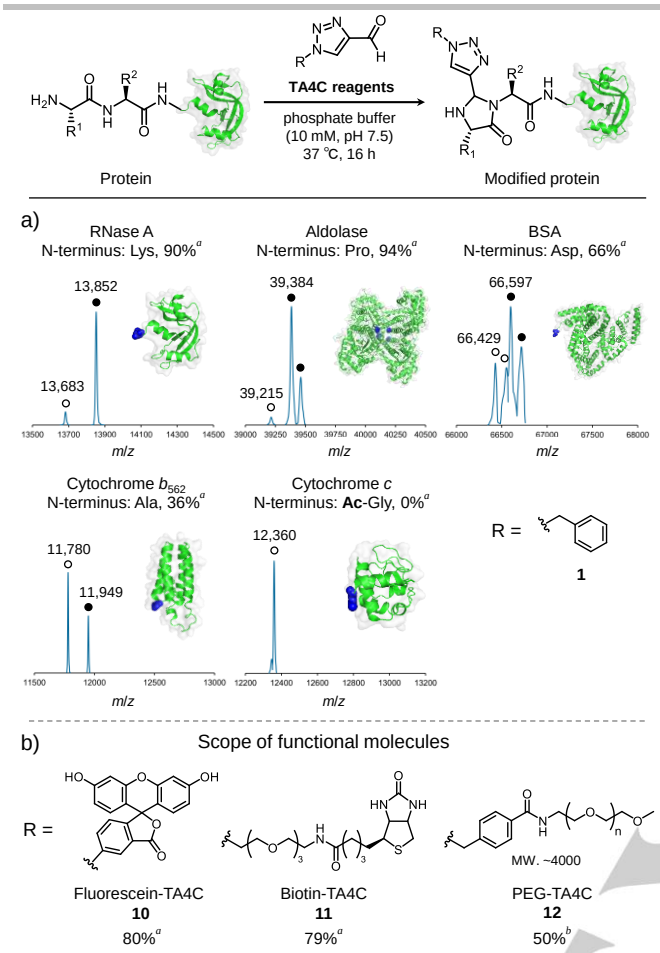

Figure 3. (a) N-terminal modification of proteins using TA4C derivatives. Deconvoluted ESI-MS spectra of modified proteins, RNase, aldolase, bovine serum albumin (BSA), cytochrome b_{562}, and cytochrome *c*, are shown. Unmodified proteins are labeled with open circles, and species corresponding to the correctly modified protein are labeled with filled circles. (b) Chemical structures of functional molecules attached to a TA4C moiety and relative ratio of modification of RNase. Conditions: protein (50 μM), **1**, **10**, **11** (10 mM) or **12** (20 mM), phosphate buffer (10 mM, pH 7.5) at 37 °C for 16 h. [a]The relative ratio of modification was analyzed by LC-MS measurements (Figure S12). [b]The relative ratio of modification was analyzed by SDS-PAGE analysis (Figure S14).

RNase in 80% and 79% conversions, respectively. The relative ratio of modification was also confirmed by UV-vis absorption of RNase having fluorecein, RNase-**10** (79%). The result is in a good accordance with the LS-MS result (Figure S13) We further applied the reaction to prepare a protein−polymer conjugate using polyethylene glycol (PEG) with 50% conversion determined by SDS-PAGE (Figure S14).

Next, to explore a facile process for generation of TA4C reagents, we focused on a Dimroth rearrangement reaction via a ring-degenerate rearrangement of 1-substituted 1,2,3-triazole-4-carbaldehyde (Figure 4).[37-38] In this process, the primary amine was found to be incorporated into the triazole moiety when 1-(4-nitrophenyl)-1*H*-1,2,3-triazole-4-carbaldehyde (**13**) with an electron-withdrawing 4-nitrophenyl group is used.[38] We therefore designed a one-step process to generate the TA4C reagent linked with a functional moiety from precursor **13**. The reaction of **13** with benzylamine proceeds in 56% conversion at 99 °C for 30 min. To improve the conversion, we screened the reaction conditions in the presence of a series of acids in 5 mol% (Scheme S13, Table S1). Sulfonate compounds such as methanesulfonic acid and

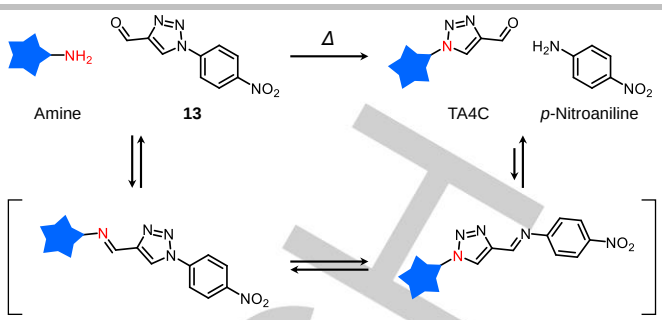

Figure 4. Dimroth rearrangement replacing 1-(4-nitrophenyl)-1*H*-1,2,3-triazole-4-carbaldehyde (**13**) with a labeling molecule having an amino group.

p-toluenesulfonic acid provide remarkable increases in conversion (up to 85%). We then investigated Good's buffer salts as an additive and found out that 2-(*N*-morpholino)ethanesulfonic acid (MES) and 3-(*N*-morpholino)propanesulfonic acid (MOPS) also accelerate the conversion (86%). The conversion was further improved with 10 mol% MOPS (91%). The use of the buffer salts at this stage is advantageous because it provides a shortcut in providing the TA4C reagent with the functional molecule and a buffered protein solution without a requirement to remove reaction additives.

After establishing conditions for the one-step generation of the TA4C reagent from benzylamine and **13** through Dimroth rearrangement, we applied the method for the preparation of TA4C reagents with other functional molecules. The reaction producing the TA4C reagents was analyzed by ^{1}H NMR (Figures S15-S18). The TA4C reagent **10** with a fluorescein molecule was obtained from commercially available 5-aminofluorescein in 81% conversion. Furthermore, the preparation of TA4C reagent with an alkyne or an azide group as a key tag for chemical modification was also investigated. The TA4C reagents **14** with an alkyne group and **15** with an azide group are also obtained from commercially available amine compounds in excellent conversion (>95%). It is noted that the Dimroth rearrangement enables us to prepare a TA4C reagent with an attached alkyne or azide group for the N-terminal protein modification, although it was not possible to directly prepare such reagents via conventional synthetic methods for triazole using the reaction of CuAAC with alkyne and azide compounds.

The method using the Dimroth rearrangement enables direct preparation of TA4C reagents with a tag, and also shows excellent conversion for N-terminal modification of RNase (Figure 5). Benzylamine was incorporated into the triazole ring to yield TA4C **1**, which was then directly used for the N-terminal modification. Interestingly, we obtained the N-terminal modified protein in excellent conversion (85%) without purification and isolation of the TA4C reagent, which is in a similar range of the conversion when the purified TA4C reagent **1** was used for the conjugation. RNase labeled with fluorescein was prepared in 65% conversion. Furthermore, preparation of TA4C reagent with an alkyne group and an azide group as key tags for chemical modification was also investigated for our two-step modification procedure from the corresponding amine compounds. The conversions to generate the modified protein using **14** and **15** are also very high (83% and 78%) in this two-step method. Moreover, we demonstrate that the azide tag **15** can be used for subsequent modification at the N-terminus of RNase to attach functional molecules such as fluorescein tethering a dibenzocyclooctyne (DBCO) moiety through a strain-promoted azide-alkyne cycloaddition reaction (SPAAC) (Figure S19).

In conclusion, we have developed a new method for one-step N-terminal protein modification using 1*H*-1,2,3-triazole-4-carbaldehyde

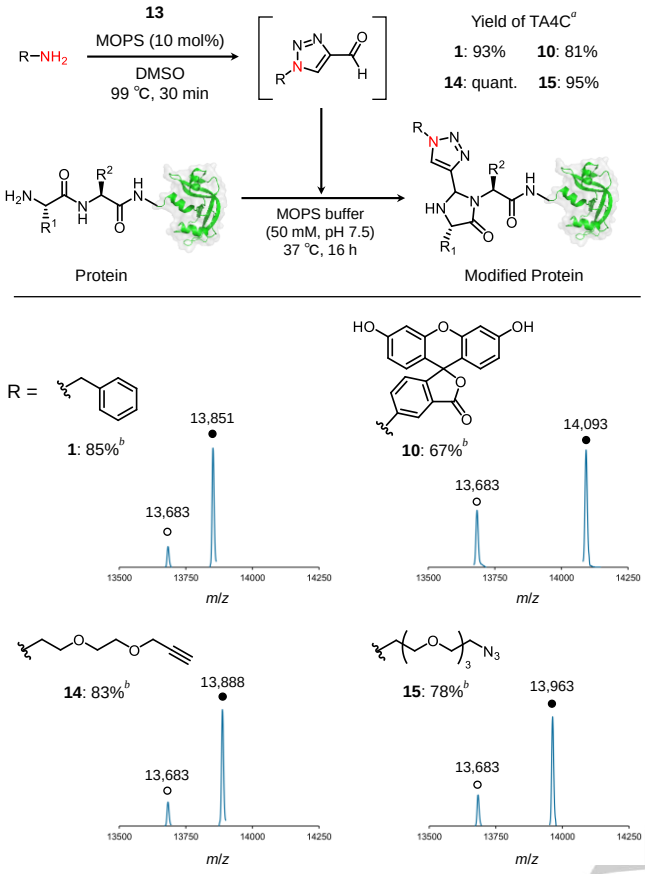

Figure 5. N-terminal modification of RNase using TA4C reagents prepared in a one-step process via a Dimroth rearrangement. Deconvoluted ESI-MS spectra of modified proteins are shown. Peaks for unmodified RNase are marked with open circles and species corresponding to the correctly modified protein are labeled with filled circles. Conditions for Dimroth rearrangement: amine precursor (100 mM), **13** (100 mM), MOPS (10 mol%), DMSO at 99 °C for 30 min. Conditions for protein modification: protein (50 μM), **1**, **10**, **14**, **15** (ca. 10 mM), MOPS buffer (50 mM, pH 7.5) at 37 °C for 16 h. [a]The conversion of Dimroth rearrangement was analyzed by ^{1}H NMR measurements. [b]The relative ratio of modification was analyzed by LC-MS measurements.

derivatives (TA4C), which are synthesized in an efficient CuAAC reaction. A series of the TA4C reagents with functional moieties such as fluorophores, biotin, and polyethylene glycol enables highly specific N-terminal modification of various proteins. Furthermore, we demonstrated direct preparation of the TA4C moiety from an amine compound having a functional group through a Dimroth rearrangement and used the product as a reagent for N-terminal protein modification. Our results indicate that protein modification using a TA4C derivative will serve as a simple and powerful method for conjugation of a wide range of molecules and biomolecules as payloads at the N-terminus to functionalize proteins in only two steps: (i) preparation of TA4C and (ii) protein conjugation. Our facile N-terminal specific modification method for proteins is expected to accelerate the generation of designed proteins loaded with multiple chemical or biomolecular entities at the N-terminus and other side chains when combined with modification methods targeting side chains. Investigations of site-specific modification technologies using our TA4C reagent for protein conjugates such as antibody-drug conjugates in medical and diagnostic applications are currently underway.

Keywords: protein modification • N-terminal modification • triazolecarbaldehyde • Dimroth rearrangement

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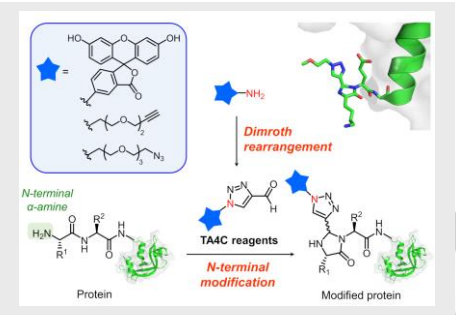
Triazolecarbaldehyde reagents for bioconjugation: A one-step N-terminal protein modification proceeds using 1*H*-1,2,3-triazole-4-carbaldehyde (TA4C) derivatives tethering a functional molecule. Furthermore, a Dimroth rearrangement enables direct preparation of TA4C derivatives from a functional group-attached amine. The modified protein with a tag at the N-terminus is easily obtained via a two-step process.



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