Bioconjugate Chemistry

Communication

pubs.acs.org/bc

MAPN: First-in-Class Reagent for Kinetically Resolved Thiol-to-Thiol Conjugation

Oleksandr Koniev,[#,†,§] Sergii Kolodych,[#,†,§] Zoljargal Baatarkhuu,[†] Johann Stojko,[‡,⊥] Jitka Eberova,[†] Jean-Yves Bonnefoy,[§] Sarah Cianférani,[‡,⊥] Alain Van Dorsselaer,[‡,⊥] and Alain Wagner*[,†]

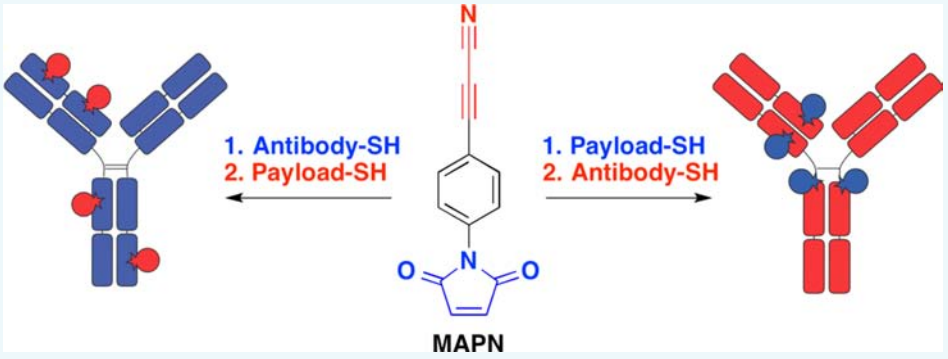
[†]Laboratory of Functional ChemoSystems (UMR 7199), Labex Medalis, University of Strasbourg, 74 route du Rhin, 67401 Illkirch-Graffenstaden, France

[§]Syndivia SAS, 4 Rue Boussingault, 67000 Strasbourg, France

[‡]BioOrganic Mass Spectrometry Laboratory (LSMBO), IPHC, University of Strasbourg, 25 rue Becquerel, 67087 Strasbourg, France

[⊥]IPHC, CNRS, UMR7178, 25 rue Becquerel, 67087 Strasbourg, France

Supporting Information



ABSTRACT: Thiols are among the most frequently used functional groups in the field of bioconjugation. While there exists a variety of heterobifunctional reagents that allow for coupling thiols to other functions (e.g., amines, carboxylic acids), there is no specific reagent for creating heteroconjugates using two different thiols. In response to the ever-increasing demand for bioconjugation tools, we have developed *p*-(maleimide)-phenylpropionitrile (MAPN)—an efficient reagent for kinetically resolved thiol-to-thiol coupling. In a comparative study with its closest commercially available analogue, *p*-phenylenedimaleimide, MAPN has shown substantial advantages for the preparation of thiol−thiol heteroconjugates. Namely, an antibody−drug conjugate (ADC) with mertansine (DM1), conjugated to the cysteine residues of Trastuzumab, was prepared for the first time.

INTRODUCTION

Heterobifunctional reagents are comprehensively applied in biological research for linking biological molecules to the functional entities of interest.[1,2] Among the functional groups used for coupling, sulfhydryl groups are of paramount importance[3] for three primary reasons. First, their high nucleophilicity allows for specific reactions with many reactive electrophiles.[4] Second, these groups have low abundance[5] but omnipresence in biological molecules, which allows for the preparation of well-defined bioconjugates. Free sulfhydryl functions on proteins can for instance be obtained through the reduction of disulfide bonds,[6] naturally present in most proteins, or introduced via lysine modification with SATA[7] or Traut's reagent.[8] Third, many molecular tags (dyes, linkers, drugs, DNA, RNA, tracers, lipids) are available as sulfhydryl derivatives. Therefore, thiol-to-thiol coupling appears as a highway for the preparation of bioconjugates.

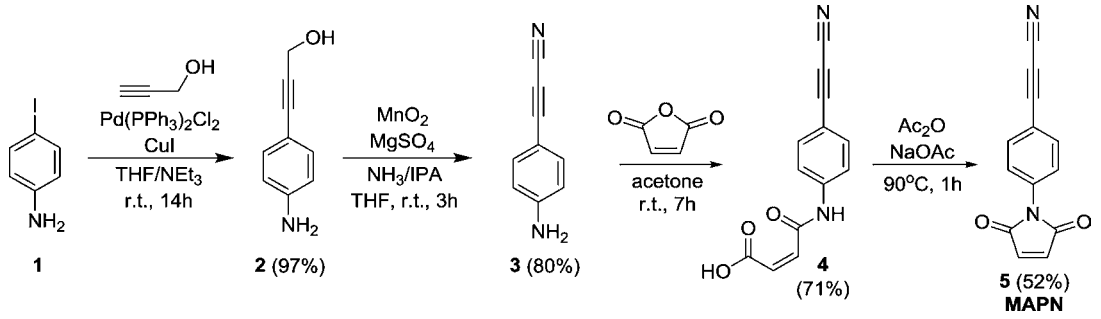
Most commonly, two thiols are coupled through the formation of disulfide bonds.[9] While this strategy is widely applied, it has some significant drawbacks, which limits the scope of its application. The drawbacks include the instability of the conjugate in reducing media and in exchange with other thiols.[10] The stability issue is, for instance, of crucial importance in the development of antibody−drug conjugates (ADC), since the extremely potent cytotoxic payload conjugated to the antibody should not be lost before the ADC reaches the target cell. In order to create a stable bond, thiol-containing drugs such as mertansine are usually conjugated to the lysine residues of the antibody, using amine-to-thiol heterobifunctional coupling reagents such as SMCC[11] or CBTF.[12] However, these conjugation strategies provide highly heterogeneous ADCs, due to the presence of more than 60 lysine residues in the antibody backbone.

One successful way to decrease heterogeneity is through the cysteine modification, using the reduction-alkylation strategy. Indeed, IgG1 antibodies contain only four solvent-exposed

Received: January 29, 2015
Revised: August 29, 2015
Published: September 3, 2015

DOI: 10.1021/acs.bioconjchem.5b00440
Bioconjugate Chem. 2015, 26, 1863−1867

Scheme 1. Synthesis of MAPN



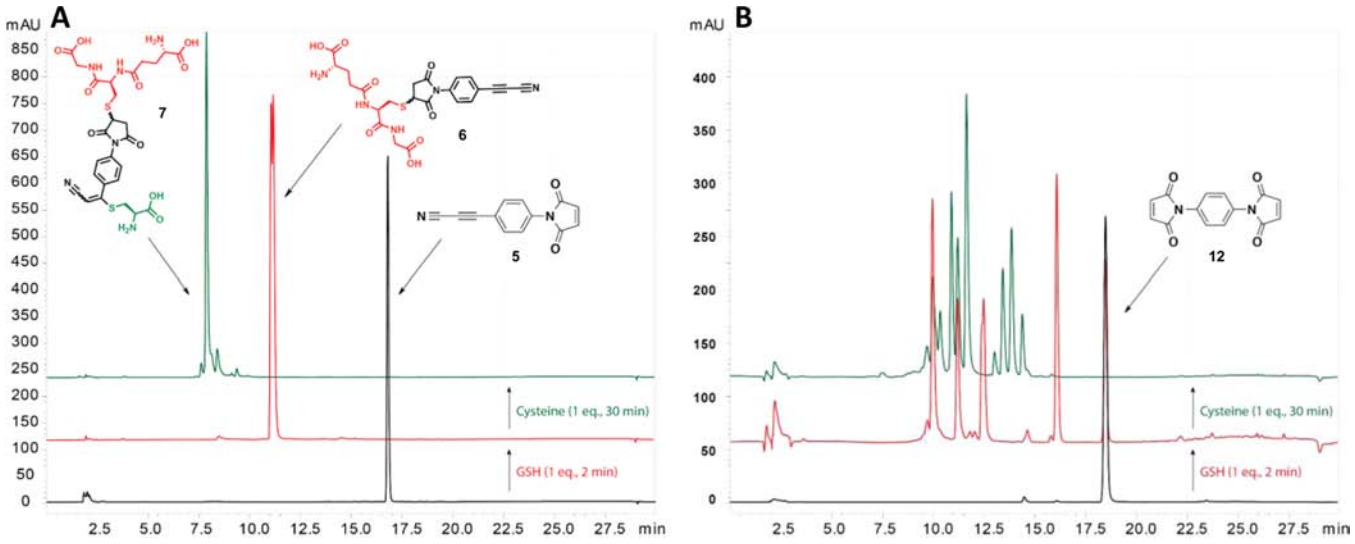


Figure 1. Heteroconjugation of glutathione and cysteine using MAPN (A) and *p*-phenylenedimaleimide (B). HPLC traces of MAPN and *p*-phenylenedimaleimide prior to conjugation are indicated in black; HPLC traces after 2 min of incubation of the reagents with glutathione are indicated in red; HPLC traces after a subsequent 30 min of incubation with cysteine are shown in green.

interchain disulfide bonds that can be reduced in order to yield free thiol residues. Selective alkylation of these nucleophilic residues was shown to yield a much cleaner mixture of conjugate. It therefore seems appealing to combine this reduction-alkylation strategy with a thiol-to-thiol heterocoupling reagent in order to access well-defined conjugates using readily available sulfhydryl molecules.

Herein, we describe the development and application of *p*-(maleimide)-phenylpropionitrile (MAPN, **5**), a ready-to-use reagent that allows for the covalent heterocoupling of two thiol-containing entities. MAPN contains two thiol-specific reactive groups, an APN[13] and a maleimide, exhibiting distinctive reaction rates. This feature allows for the selective formation of monoadduct upon coupling of the more reactive group with the first thiol, leaving the second reactive group intact for the subsequent reaction with the second thiol.

RESULTS AND DISCUSSION

Recently, we described a 3-arylpropyonitrile (APN) group as an efficient reactive function for the selective and irreversible labeling of thiols.[13] Given the at least 10-fold higher reaction rate of maleimide ($k > 50$ M^{-1} s^{-1}) compared to APN ($k = 3.1$ M^{-1} s^{-1}), we hypothesized that combining these two functions into a heterobifunctional reagent will allow for kinetically resolved thiol-to-thiol coupling.

We synthesized MAPN **5** with a 29% overall yield from 4-iodoaniline **1** in a four-step reaction sequence (Scheme 1).

Briefly, 4-iodoaniline reacted with propargylic alcohol under standard Sonogashira coupling conditions to yield intermediate **2**, which was oxidized with MnO_2 in the presence of ammonia to form 3-(4-aminophenyl)propionitrile **3**.[14] This intermediate reacted with maleic anhydride to yield product **4**. Finally, MAPN was obtained by dehydrative cyclization of **5** in the presence of acetic anhydride and sodium acetate.

In order to confirm the applicability of MAPN for thiol-to-thiol heterocoupling, we carried out a reaction between two model substrates: glutathione and cysteine. The reaction was conducted in PBS/DMSO (9:1, pH 7.4) in a sequential manner (Figure 1A). First, 1 equiv of MAPN **5** was added to 1 equiv of the reduced glutathione (1 mM solution). After 2 min of incubation at 25 °C, HPLC analysis showed full conversion into the single adduct **6**. To the resulting solution, we added 1 equiv of cysteine and incubated the mixture for 30 min to allow for clean formation of the glutathione-cysteine adduct **7**. Compounds **6** and **7** were isolated and characterized in order to confirm the order of thiol addition to the maleimide and APN functions. A side-by-side comparison with the closest commercially available thiol-to-thiol coupling reagent, *p*-phenylenedimaleimide, was carried out under the same conditions (Figure 1B). As expected, following the addition of 1 equiv of glutathione, *p*-phenylenedimaleimide yielded a mixture with four main products: the adduct, the homocoupling product, the hydrolysis product, and the starting compound.

DOI: 10.1021/acs.bioconjchem.5b00440
Bioconjugate Chem. 2015, 26, 1863−1867

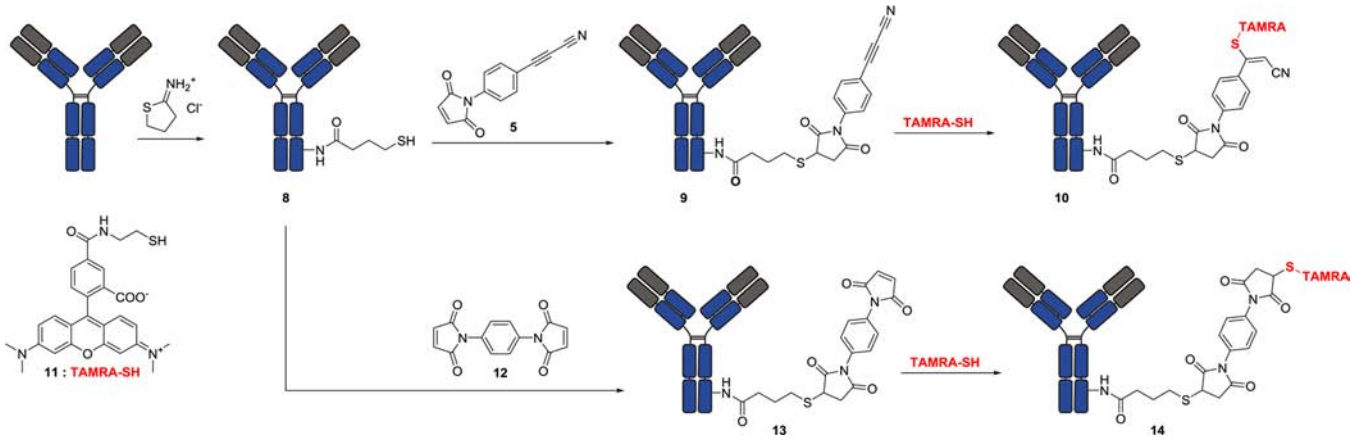


Figure 2. Conjugation of the antibody with TAMRA-SH using MAPN and *p*-phenylenedimaleimide.

Subsequent addition of 1 equiv of cysteine resulted in a highly complex mixture.

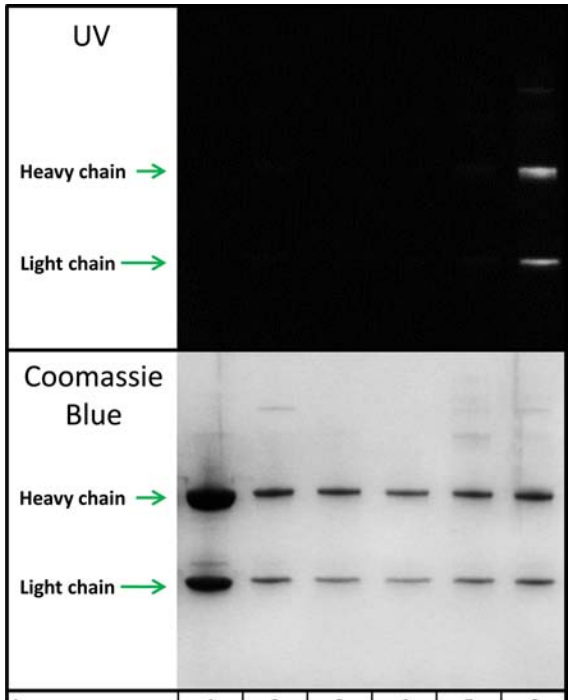
Encouraged by these results, we decided to test the MAPN reagent in the heterocoupling of a biomolecule and a fluorescent tag (Figure 2). Again, a side-by-side comparison with *p*-phenylenedimaleimide was carried out in the coupling of the Trastuzumab with a thiol-containing fluorophore **11**.

Trastuzumab was first reacted with 10 equiv of Traut's reagent to form the modified antibody **8** with a number of solvent-exposed thiol groups. The resulting conjugate was incubated with 10 equiv of either MAPN or *p*-phenylenedimaleimide yielding conjugates **9** and **13**, respectively. After 1 h of incubation at 25 °C, 10 equiv of TAMRA-SH **11** was added to the mixtures. Following overnight incubation, the mixtures were purified by size-exclusion chromatography.

The resulting conjugates **10** and **14** were analyzed by SDS-PAGE (Figure 3). Gel fluorescence showed that MAPN (lane 6) allowed for the fluorescent labeling of the antibody, in contrast to *p*-phenylenedimaleimide (lane 4). We hypothesized that the lack of antibody labeling in the case of *p*-phenylenedimaleimide **5** was due to the lower hydrolytic stability of maleimides, compared to APN. To verify this hypothesis, we prepared the *p*-phenylenedimaleimide-glutathione monoadduct **12a** and the MAPN-glutathione monoadduct **6** and tested their stability in PBS. Indeed, the maleimide function of **12a** underwent 50% hydrolysis in PBS (pH 7.4) within 1 h, yielding the unreactive maleic acid derivative (Supporting Information Figure S1). The APN function of **6** remained stable in the PBS solution for 24 h.

We performed a series of control experiments to confirm the absence of false positives that might be related to an unspecific conjugation process. Thus, the unmodified antibody was incubated with TAMRA-SH and showed no formation of the antibody–fluorophore conjugate (lane 1), nor was formation of the conjugate observed upon incubation of the activated antibody **8** with TAMRA-SH (lane 2). The unmodified antibody was also incubated with MAPN and *p*-phenylenedimaleimide **11** (lanes 3 and 4), followed by reaction with TAMRA-SH. In this case, gel fluorescence did not reveal any unspecific conjugation product.

To further demonstrate the utility of the MAPN reagent, we carried out the preparation of an ADC by coupling mertansine (DM1) to Trastuzumab via a reduction-alkylation strategy. In the commercial ADC (T-DM1, trade name: Kadcyla), mertansine is conjugated to lysine residues using SMCC



Lane	1	2	3	4	5	6
Trastuzumab	+	+	+	+	+	+
Traut's reagent	-	+	-	+	-	+
APN-Maleimide **5**	-	-	-	-	+	+
Dimaleimide **12**	-	-	+	+	-	-
TAMRA-SH **11**	+	+	+	+	+	+

Figure 3. SDS-PAGE analysis of antibody–dye conjugates. Control experiments were carried out on the unmodified antibody without the use of a bifunctional reagent (lane 1), with the addition of **12** (lane 3) and **5** (lane 5). The activated antibody was reacted with TAMRA-SH directly (lane 2) as a negative control. The reaction of the activated antibody with **12** and then **11** (lane 4) yielded no fluorescent labeling. Reaction of the activated antibody with MAPN and then **11** (lane 6) allowed for efficient fluorescent labeling.

reagent. Random conjugation to nearly 30 surface-exposed lysines provides a highly heterogeneous mixture of different coupling species. In contrast, the reduction-alkylation strategy offered only eight attachment points. This strategy resulted in more defined ADCs with a narrower distribution of species and with mostly even drug-to-antibody ratios (Figure 4).

DOI: 10.1021/acs.bioconjchem.5b00440
Bioconjugate Chem. 2015, 26, 1863–1867

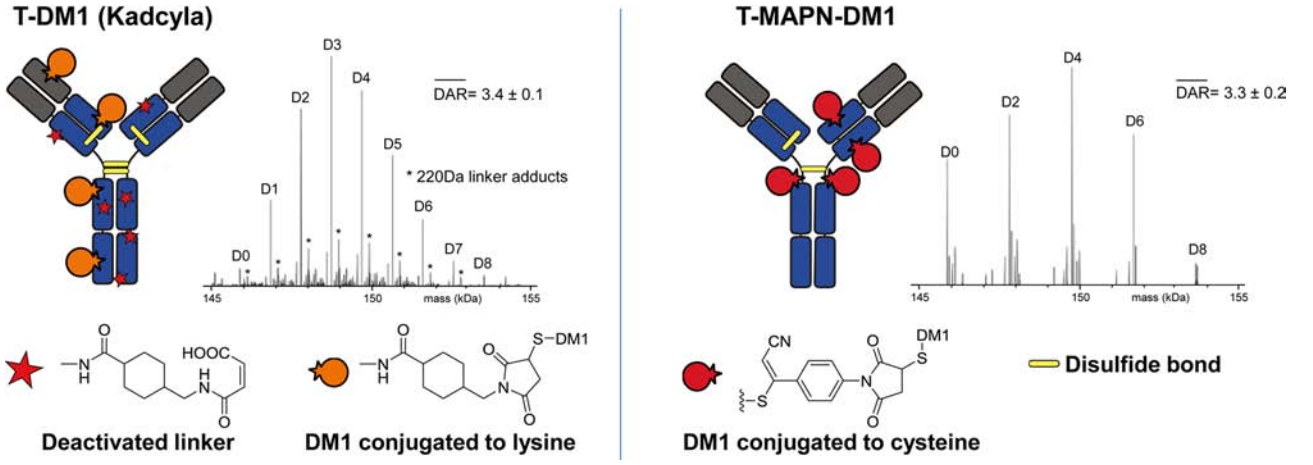


Figure 4. Representative structures and MS spectra of T-DM1 (Kadcyla) and T-MAPN-DM1.

T-MAPN-DM1 was prepared via a two-step process. First, mertansine was reacted with 1 equiv of MAPN to yield the thiol reactive payload. The payload was conjugated to the partially reduced antibody by incubating the components in the PBS buffer. The conjugate was purified by size exclusion chromatography and the average DAR was determined by native ESI-TOF-MS. T-MAPN-DM1 was shown to have the average DAR of 3.3 ± 0.2, which was comparable with the average DAR of 3.4 ± 0.1 measured for T-DM1.

Finally, in vitro cytotoxicity of the T-MAPN-DM1 conjugate was evaluated on SK-BR-3 (HER2+++) and MDA-MB-231 (HER2-) cells. Similar to T-DM1, the new conjugate showed high toxicity on HER2 positive cells and significantly lower toxicity on HER2 negative cells (Figure 5).

CONCLUSION

In conclusion, we developed MAPN—a first-in-class reagent for the efficient kinetically resolved coupling of thiols. The reagent was applied for conjugation of a thiol-activated antibody and a thiol-containing fluorophore. A side-by-side comparison with its closest commercial analog showed superior efficiency on the part of MAPN for bioconjugation applications. The MAPN reagent was further applied to conjugate mertansine and the cysteine residues of Trastuzumab, providing a more homogeneous analog of Trastuzumab emtansine (T-DM1). Considering the vast use of thiol groups in biological research, we believe that this reagent will be of great utility in the area of bioconjugation. Through an agreement with Syndivia, MAPN is now commercially available.[15]

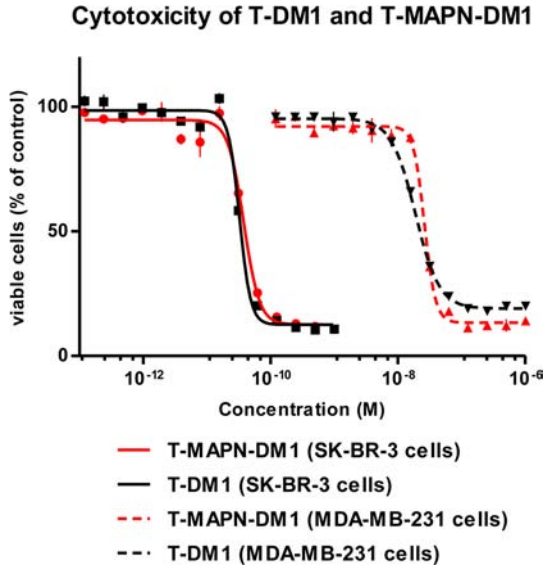


Figure 5. In vitro evaluation of T-DM1 and T-MAPN-DM1 on HER2 positive cell line (SK-BR-3) and HER2 negative cell line (MDA-MB-231).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00440.

Materials and instrumentation, experimental procedures, analytical data (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: alwag@unistra.fr.

Author Contributions

#Oleksandr Koniev and Sergii Kolodych contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by SATT Conectus "BioReLiable" project, CNRS, University of Strasbourg, Région Alsace, and GIS IBiSA. The International Center for Frontier Research in Chemistry (icFRC) is also acknowledged for financial support. J.S. acknowledges the Institut de Recherche Servier for funding of his PhD fellowship.

REFERENCES

(1) Hermanson, G. *Bioconjugate Techniques*, 1996.

(2) Koniev, O., and Wagner, A. (2015) Developments and recent advancements in the field of endogenous amino acid selective bond forming reactions for bioconjugation. *Chem. Soc. Rev. 44*, 5495−5551.

(3) Chalker, J. M., Bernardes, G. J. L., Lin, Y. A., and Davis, B. G. (2009) Chemical modification of proteins at cysteine: opportunities in chemistry and biology. *Chem. - Asian J. 4*, 630−40.

DOI: 10.1021/acs.bioconjchem.5b00440
Bioconjugate Chem. 2015, 26, 1863−1867

(4) Brotzel, F., and Mayr, H. (2007) Nucleophilicities of amino acids and peptides. *Org. Biomol. Chem. 5*, 3814−20.
(5) McCaldon, P., and Argos, P. (1988) Oligopeptide biases in protein sequences and their use in predicting protein coding regions in nucleotide sequences. *Proteins: Struct., Funct., Genet. 4*, 99−122.
(6) Burns, J. A., Butler, J. C., Moran, J., and Whitesides, G. M. (1991) Selective reduction of disulfides by tris(2-carboxyethyl)phosphine. *J. Org. Chem. 56*, 2648−2650.
(7) Duncan, R. J. S., Weston, P. D., and Wrigglesworth, R. (1983) A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal. Biochem. 132*, 68−73.
(8) Traut, R. R., Bollen, A., Sun, T.-T., Hershey, J. W. B., Sundberg, J., and Pierce, L. R. (1973) Methyl 4-mercaptobutyrimidate as a cleavable crosslinking reagent and its application to the Escherichia coli 30S ribosome. *Biochemistry 12*, 3266−3273.
(9) Witt, D. (2008) Recent Developments in Disulfide Bond Formation. *Synthesis 2008*, 2491−2509.
(10) Fava, A., Iliceto, A., and Camera, E. (1957) Kinetics of the Thiol-Disulfide Exchange. *J. Am. Chem. Soc. 79*, 833−838.
(11) Yoshitake, S., Yamada, Y., Ishikawa, E., and Masseyeff, R. (1979) Conjugation of Glucose Oxidase from Aspergillus niger and Rabbit Antibodies Using N-Hydroxysuccinimide Ester of N-(4-Carboxycyclohexylmethyl)-Maleimide. *Eur. J. Biochem. 101*, 395−399.
(12) Kolodych, S., Koniev, O., Baatarkhuu, Z., Bonnefoy, J.-Y., Debaene, F., Cianférani, S., Van Dorsselaer, A., and Wagner, A. (2015) CBTF: New Amine-to-Thiol Coupling Reagent for Preparation of Antibody Conjugates with Increased Plasma Stability. *Bioconjugate Chem. 26*, 197−200.
(13) Koniev, O., Leriche, G., Nothisen, M., Remy, J.-S., Strub, J.-M., Schaeffer-Reiss, C., Van Dorsselaer, A., Baati, R., and Wagner, A. (2014) Selective irreversible chemical tagging of cysteine with 3-arylpropionitriles. *Bioconjugate Chem. 25*, 202−6.
(14) McAllister, G. D., Wilfred, C. D., and Taylor, R. J. (2002) Tandem Oxidation Processes: The Direct Conversion of Activated Alcohols into Nitriles. *Synlett*, 1291−1292.
(15) http://syndivia.com/.

DOI: 10.1021/acs.bioconjchem.5b00440
Bioconjugate Chem. 2015, 26, 1863−1867