Bioconjugate Chemistry

Article

pubs.acs.org/bc

Site-Specific Conjugation of Peptides and Proteins via Rebridging of Disulfide Bonds Using the Thiol–Yne Coupling Reaction

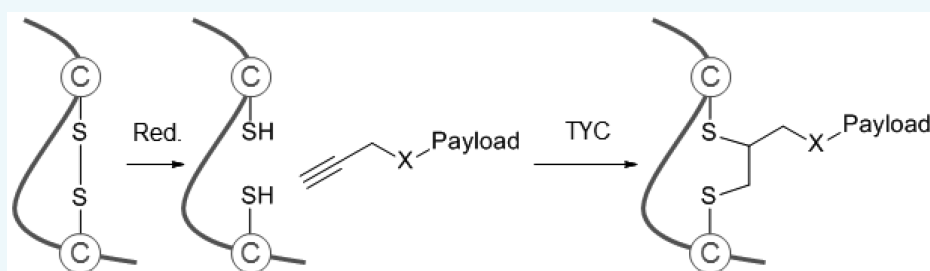
Nils Griebenow,*,[†] Alicia M. Dilmaç,[†,§] Simone Greven,[‡] and Stefan Bräse*,[§,∥]

[†]Medicinal Chemistry Wuppertal and [‡]Research Analytics, Bayer Pharma AG, Drug Discovery, Aprather Weg 18a, 42113 Wuppertal, Germany

[§]Institute of Organic Chemistry, Karlsruhe Institute of Technology (KIT), Fritz-Haber-Weg 6, D-76131 Karlsruhe, Germany

[∥]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology (KIT), Hermann-von-Helmholtz-Platz 1, D-76344 Eggenstein-Leopoldshafen, Germany

Supporting Information



ABSTRACT: Herein, we describe an extension of our previously reported photomediated disulfide rebridging methodology to the conjugation of peptides and proteins. The methodology proved to be reproducible with various alkynes and different peptides. This study includes the first rebridging of the disulfide bond of a peptide through a thiol–yne reaction with a cyclooctyne. In all cases, the rebridging was proven by MS analyses and confirmed by the absence of olefinic protons on ^{1}H NMR spectra of the resulting products. Finally, this one-pot reduction thiol–yne conjugation was successfully applied to an antibody Fab fragment with a promising conversion, which set a good ground for the future syntheses of new protein and antibody conjugates.

INTRODUCTION

In recent years, the synthesis of peptide and protein conjugates as therapeutics, as diagnostics, and as tools to elucidate biological mechanisms has become increasingly important.[1] In detail, such a conjugation enables modulation of the properties of peptides and proteins in a desired direction. For example, the conjugation of peptides with biocompatible polymers such as PEG serves for a prolonged half-life in the plasma circulation accompanied by a longer biological effect or diminishes the immunogenicity of the peptide precursor.[2] Furthermore, peptides and proteins can be conjugated with biochemical markers, dyes, or reactive functional groups to enable the exploration of binding events in certain organs or cell regions.[3] In addition, drug targeting with conjugated peptides and proteins has evolved into a rapidly growing area of research. Recent examples include antibody drug conjugates (ADCs), of which the first drugs have reached the market.[4] Today, a plethora of methods for the conjugation of peptides and proteins are available. Biological molecules can be functionalized with reactive groups, which serve as attachment points for certain payloads.[5] Furthermore, peptides and proteins can also be conjugated by using the functional groups of their native amino acids.[6] The major challenge herein is to find methods which allow for the selective transformation of the desired group in the presence of other functional groups of unprotected amino acid side chains. Further methods include chemical or biochemical modification of peptides and proteins, which generate an attachment point for conjugation. A well-known example is the reduction of native disulfide bonds, followed by selective conjugation of the liberated cysteine thiol groups, which is frequently accomplished by the reaction with maleimides.[7] Other sophisticated methods make use of bifunctional reagents, which allow for bridging two thiols generated from the reduction of a disulfide bond. On one hand, such rebridging of disulfide bonds was accomplished with double-reactive Michael acceptors to give a C1- or C3-bridge.[8] On the other hand, bis-reactive electrophilic maleimides were used, to provide a C2-bridge.[9] Herein, we describe a conjugation method which makes use of the thiol–yne coupling (TYC) for the rebridging of disulfide bonds by two carbon atoms (Scheme 1).

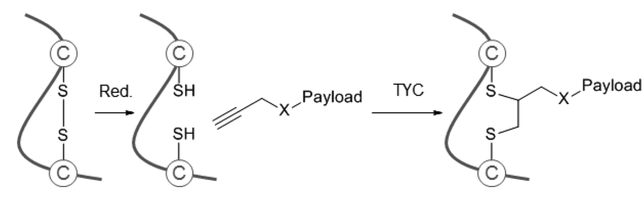
Previously, we described the reaction sequence with an amino acid derivative that allows for the direct insertion of an alkyne into a disulfide bond (Scheme 2).[10] After reduction of

Received: December 22, 2015
Revised: February 23, 2016

ACS Publications © XXXX American Chemical Society

DOI: 10.1021/acs.bioconjchem.5b00682
Bioconjugate Chem. XXXX, XXX, XXX–XXX

Scheme 1. Reduction and Thiol−Yne Coupling (TYC) for the Rebridging of Disulfide Bonds in Peptides and Proteins



the disulfide bond with TCEP, thiol−yne reaction in a photochemical batch reactor yielded the desired dithioether **3** with quantitative conversion and 49% isolated yield as a mixture of diastereomers in about a 1:1 ratio.

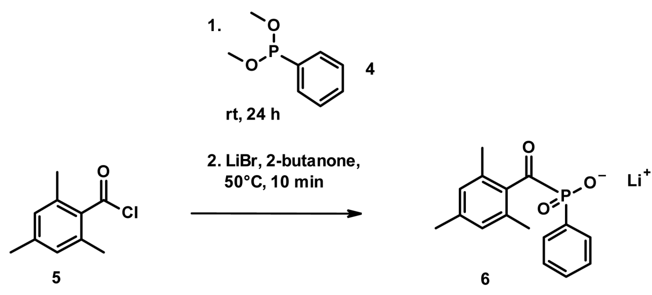
Herein, we describe the application of this protocol for the conjugation of peptides and proteins.

■ RESULTS AND DISCUSSION

Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) **6** is a water-soluble radical initiator that already showed efficiency in the thiol−yne reaction involving peptides.[11] Given this appropriate solubility, LAP appeared to be more suitable than XBPO for an application of our methodology to peptides and proteins, and was thus synthesized from 2,4,6-trimethylbenzoyl chloride **5** and dimethyl phenylphosphonite **4**, according to a procedure from the literature (Scheme 3).[12]

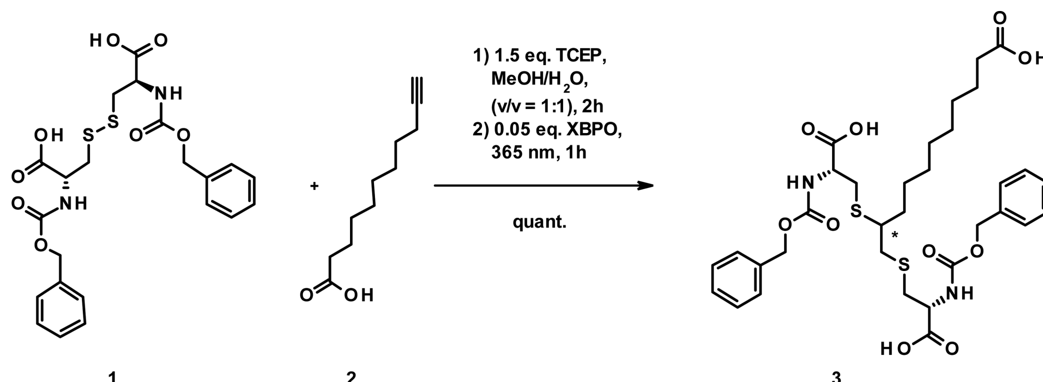
Terlipressin **7** (oxidized form) is a polypeptide bearing 12 amino acids and a single disulfide bond. Therefore, its commercial acetate salt was chosen as our first template to test our aforementioned disulfide rebridging methodology on peptides. 6-Heptynoic acid **8** was the first alkyne to be investigated in this thiol−yne reaction (Scheme 4). Terlipressin acetate (37.23 μmol) was first dissolved in water and its disulfide bond was reduced with 1.5 equiv of *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl) under an inert atmosphere. The reduction of the disulfide bond was confirmed by LC/MS analysis. 6-Heptynoic acid **8** was then dissolved in methanol (amount of methanol less than 10% of the total volume), and added to the reduced peptide. The resulting mixture was then irradiated with UV-light (365 nm) of an LED UV-pen, in the presence of LAP as radical initiator. The portion-wise addition of the latter (portions of 5 mol % each) at the beginning of every hour of irradiation appeared to be a key element to achieve satisfactory conversion rates. At this

Scheme 3. Synthesis of the Radical Initiator LAP[12]



stage, it is worth noting that several reaction products could be expected from this reaction: the vinyl sulfide products of type **10a**/**b**, resulting from a monoaddition of a thiyl radical on the alkyne, or the *bis*-addition products of type **9a**/**b**, resulting from an intramolecular thiol−ene coupling (TEC) between the alkene of **10a**/**b** and the second cysteine residue. Products **9** and **10** can also be present as two regioisomers (**a** and **b**) and an additional two stereoisomers for each regioisomer; all these compounds exhibit the same molecular weight. In our case, the monitoring of the reaction using LC/MS analyses showed the appearance of four peaks with the same m/z value of 1355 ($[M + H]^+$) over the first hour of reaction. Longer reaction times resulted in a progressive disappearance of the two having the longer retention times and of the starting material, along with an increase of the two other new peaks. If products **10a** and/or **10b** were never isolated, this behavior suggested that these intermediates were effectively formed, and progressively ring-closed through a TEC step to yield products of type **9a**/**b**. The presence of two peaks instead of four suggested that the different stereoisomers of **9a** and **9b** probably had the same or close retention times. It is worth noting that in the case of thiol−yne coupling reactions with terminal aliphatic alkynes, the second addition onto vinyl sulfides is generally expected to be three times faster than the first addition.[13] Thus, the accumulation of vinyl sulfide is normally unlikely to be observed. Fairbanks et al.[13] also showed that a second addition should not occur when the alkyne shows a higher reactivity than the vinyl sulfide product. In our case, however, the location of the two thiols on the peptide and the necessity for the latter to adopt a certain conformation to let the second addition occur might slightly delay the ring closure. Thus, the distance between the two cysteines, as well as sterical

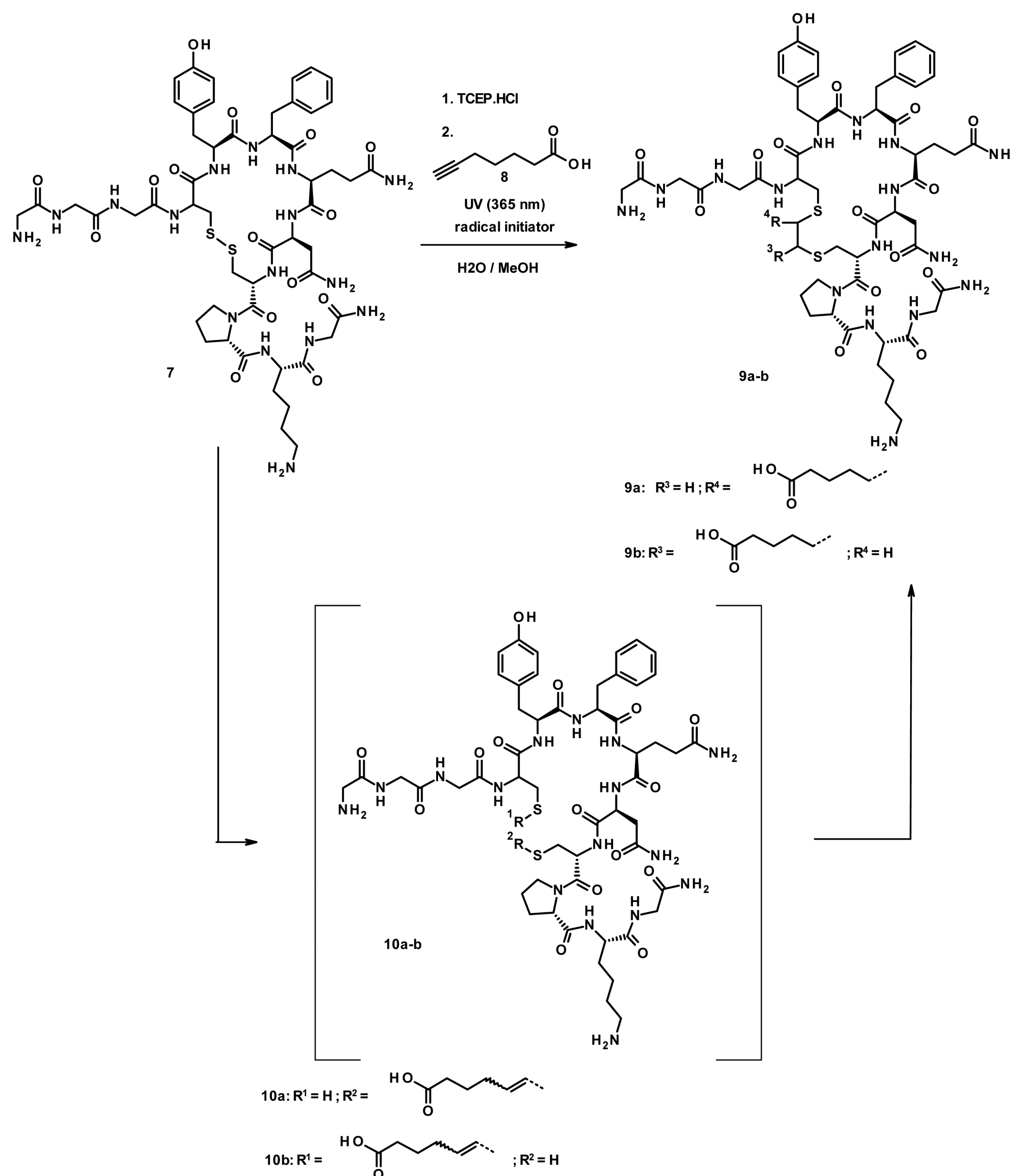
Scheme 2. One Pot Alkyne Insertion into a Disulfide Bond[a]



[a]Quantitative conversion, 49% isolated yield as a mixture of diastereomers in about a 1:1 ratio; XBPO = phenylbis(2,4,6-trimethyl benzoyl)-phosphine oxide, Irgacure 819.

DOI: 10.1021/acs.bioconjchem.5b00682
Bioconjugate Chem. XXXX, XXX, XXX−XXX

Scheme 4. Rebridging of the Disulfide Bond in Terlipressin through Reduction and TYC with 6-Heptynoic Acid



hindrance, seem to be a plausible explanation for this slight accumulation of **10a**/**b** during the first hour of reaction, and yet formation of **9a**/**b** as major product after a longer time.

A purification step via preparative HPLC allowed us to isolate a total amount of 1.3 mg (3%) of compound **9a**/**b**. The absence of olefinic protons in the ^{1}H NMR spectrum of the isolated fractions confirmed the absence of intermediates of type **10**. If the isolated fractions all contained different products of the same expected m/z value, these were mostly mixtures, which did not allow a differentiation between **9a** and **9b** to be made. However, the presence of several reaction products of the same expected mass with no olefinic protons correlates with the formation of the different regio- and stereoisomers of **9a**/**b**.

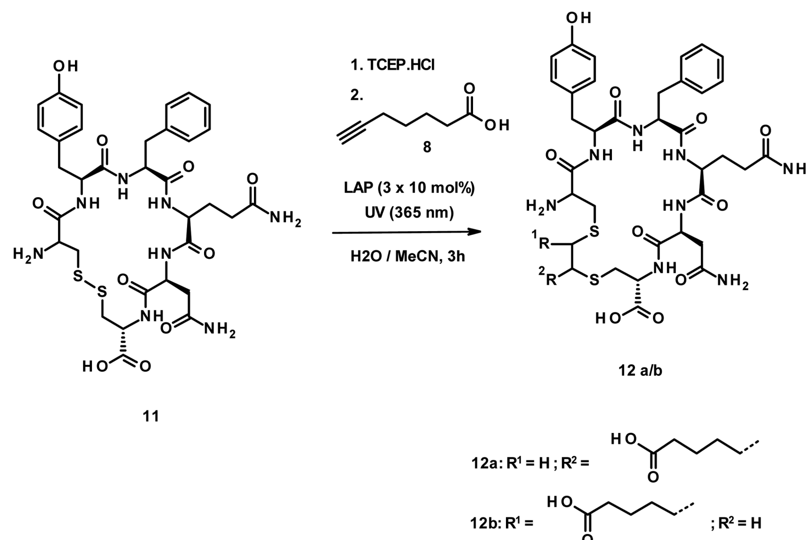
Pressinoic acid **11** is a hexapeptide corresponding to the cyclic moiety that terlipressin and vasopressin have in common. Using a procedure similar to the one mentioned before, the disulfide bridge of pressinoic acid was successfully reacted with the same alkyne **8**, through successive reduction and TYC reaction and in a one-pot manner. In this case, however, the use of a 1:1 mixture of acetonitrile/0.1% acetic acid aqueous solution as the reaction solvent was necessary to avoid the precipitation of the reactants at either steps of the sequence (Scheme 5). The conversion of pressinoic acid into the desired reaction products **12a**/**b** was followed by successive LC/MS analyses. Purification via preparative HPLC yielded three different fractions containing mixtures of isomers of **12a**/**b**, with a total isolated yield of 15%.

The next step of this study consisted of exploring the possible extent of our methodology to the use of different type of alkynes. While alkyne **19** (Scheme 6) was commercially available, alkynes **15** and **18** were synthesized as depicted in Scheme 6, before being conjugated to terlipressin. These alkynes were selected not only for their structural diversity, but also for their water solubility, which might become important when applying this methodology on larger biomolecules.

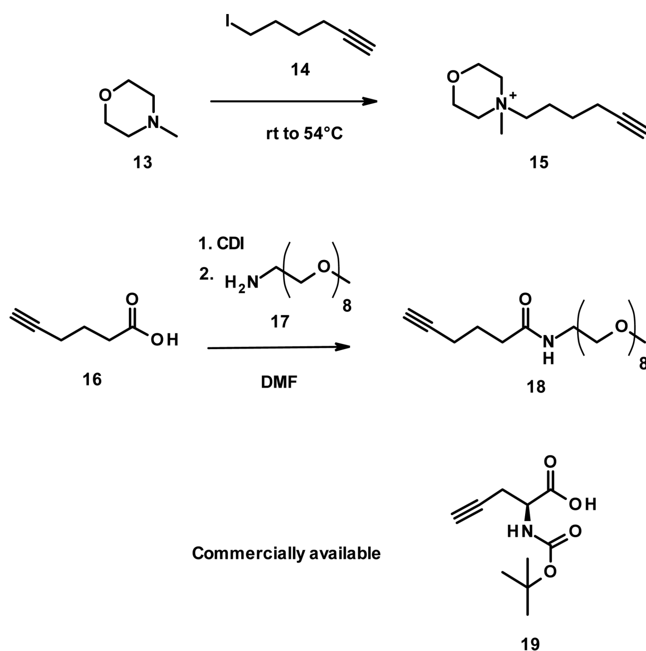
After reduction of terlipressin acetate with TCEP·HCl in a DPBS buffered solution, alkyne **15** and LAP (10 mol % at each hour of UV-irradiation) were also dissolved in DPBS buffer and

DOI: 10.1021/acs.bioconjchem.5b00682
Bioconjugate Chem. XXXX, XXX, XXX−XXX

Scheme 5. Rebridging of the Disulfide Bond in Pressinoic Acid with 6-Heptynoic Acid



Scheme 6. Alkynes That Were Investigated in the Rebridging of Disulfide Bond in Peptides and Their Syntheses



added to the reaction solution, which was then photoirradiated (365 nm) for 3 h (Scheme 7). Analytical LC/MS analyses after each hour of photoreaction clearly showed the disappearance of the peak of reduced terlipressin and the appearance of two very close peaks of m/z = 705.8, which correspond to the $[M+2H]^{2+}$ mass of the expected product **20**. Separation via preparative HPLC yielded two fractions containing **20a/b**, with a total isolated yield of 12%. Unfortunately, the closeness in the retention times of the reaction product and the side impurities did not allow a better separation to be obtained. Nevertheless, ^{1}H NMR analyses of the resulting fractions did not show the presence of olefinic protons, proving again the absence of the olefinic intermediates and their effective ring closure to yield **20a/b**.

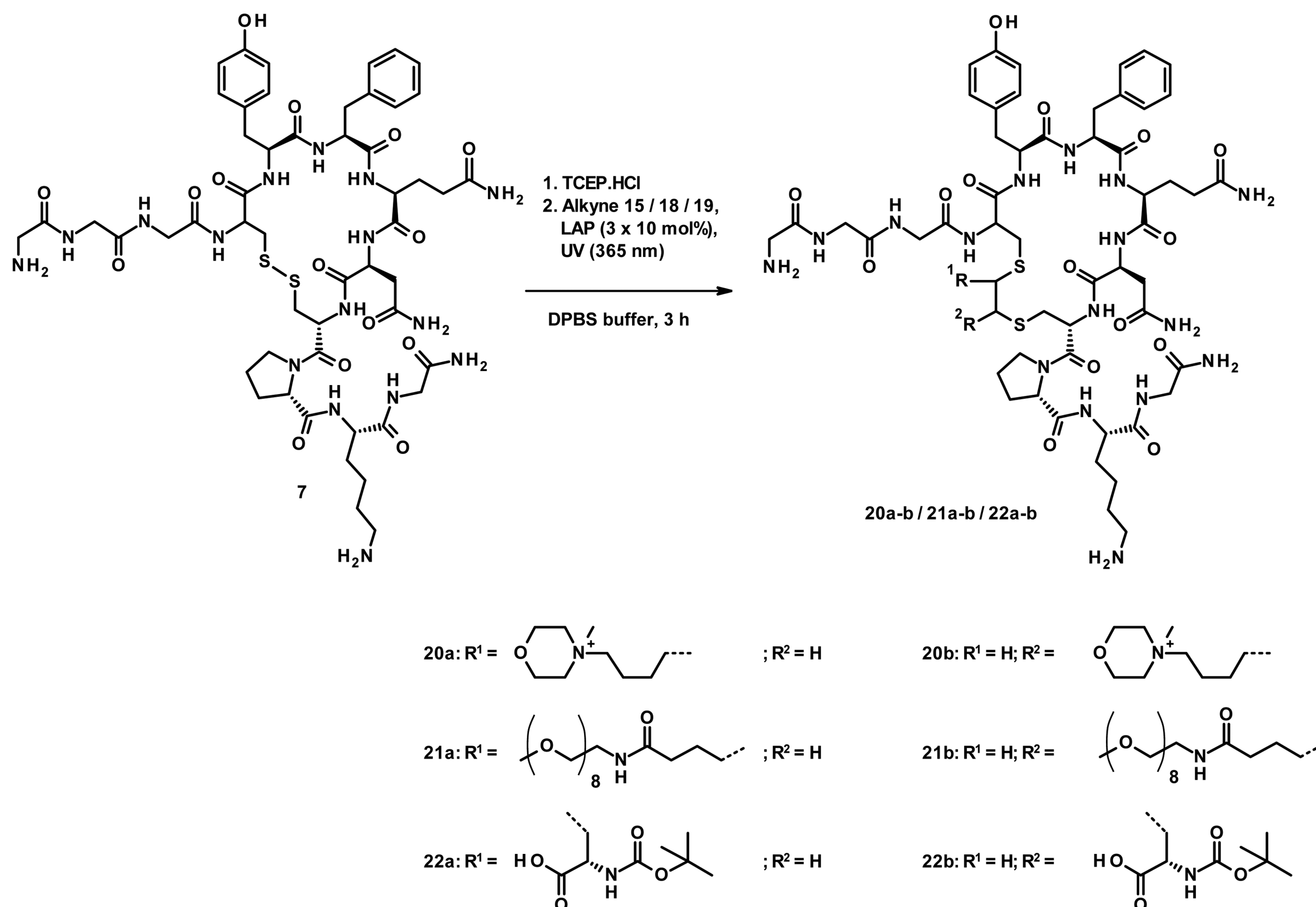
Terlipressin was also successfully PEGylated by bridging its disulfide bond with alkyne **18**, using the same methodology. Product **21a/b** was obtained here again as a mixture of isomers, with a purity reaching 99% and a total isolated yield of 30%. PEGylation is a recognized means to increase the plasma stability of peptides during circulation,[14] while cyclic peptides are known to generally exhibit higher stability than their linear counterparts. Thus, with the synthesis of **21a/b**, we show the possibility to reach these two stabilizing aspects using our new methodology.

Another alkyne that could be inserted into the disulfide bond of terlipressin was *N*-Boc-L-propargylglycine **19**. Here again, LC/MS analyses showed the formation of conjugates of m/z = 721.81, which correspond to the $[M+2H]^{2+}$ adduct of **22a/b**. ^{1}H NMR of the fractions proved the absence of olefinic protons. Although each fraction collected from the purification step over preparative HPLC showed different retention times on analytic HPLC/MS and purities of maximum 94%, ^{1}H NMR analysis showed that only a mixture of isomers was collected, which illustrates the difficulty to separate them. Product **22a/b** was obtained with a total isolated yield of 22%. The absence of olefinic protons confirmed, in this case again, the effective bridging of the disulfide bond. Just like with 6-heptynoic acid, the amino acid conjugated with terlipressin can allow further functionalization of the resulting conjugates. This successful conjugation can also be seen as the first step toward the development of a new peptide−protein conjugation methodology.

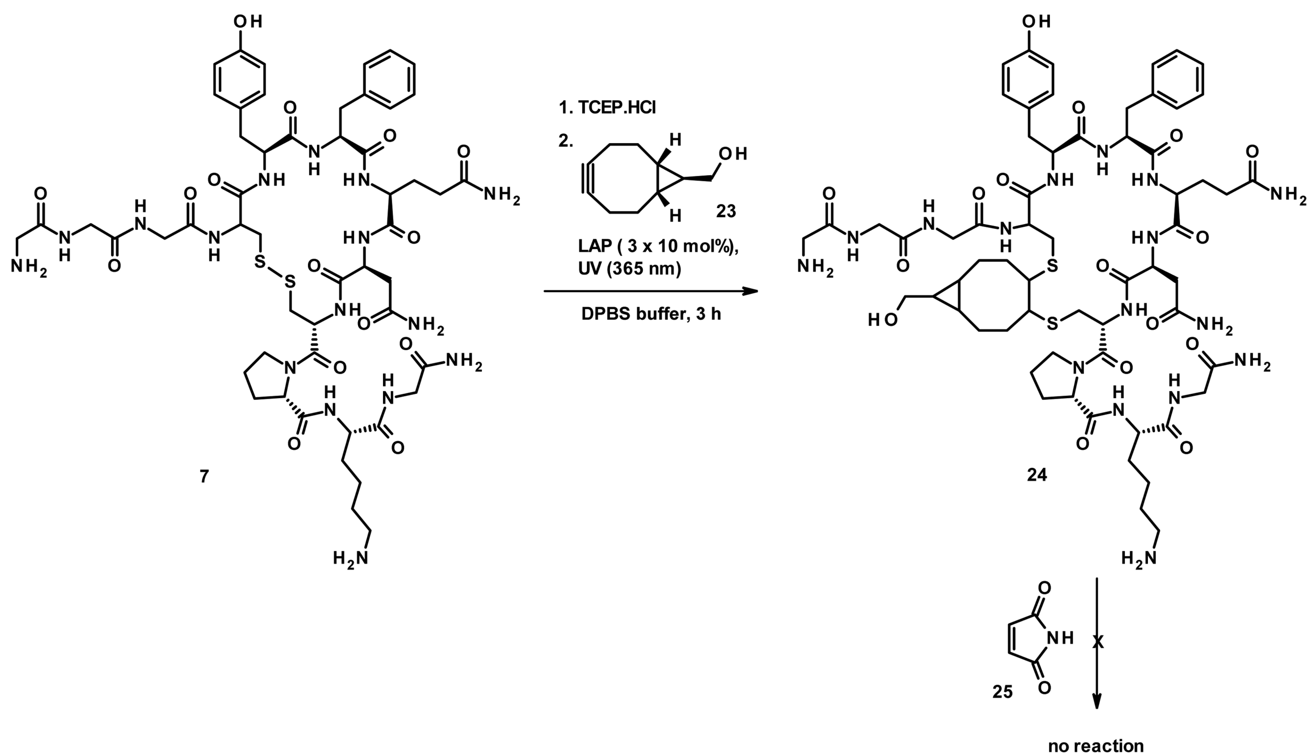
Encouraged by the reproducibility of our methodology with these various alkynes, we then question its applicability to symmetrical alkynes, which would limit the number of possible regioisomeric products. Thus, we considered investigation of symmetrical cyclooctynes. The phototriggered TYC reaction of cyclooctyne with octanethiol in the presence of a radical initiator was already investigated by B. D. Fairbanks et al.,[13] who reported the lone formation of monoaddition product and no measurable occurrence of a second addition of a thiol on the very vinyl sulfide. Yet, Geel et al.[15] reported to have observed the formation of both mono- and bis-adduct when simply incubating cysteine methyl ester with bicyclo[6,1,0]non-4-yn-9-ylmethanol (BCN alcohol). However, the provided mass spectrum seems to show the presence of remaining starting material after reaction.[15] It is noteworthy that cysteine residues of glutathione and other tested peptides also showed reactivity

DOI: 10.1021/acs.bioconjchem.5b00682
Bioconjugate Chem. XXXX, XXX, XXX−XXX

Scheme 7. Rebridging of the Disulfide Bond in Terlipressin with Various Alkynes

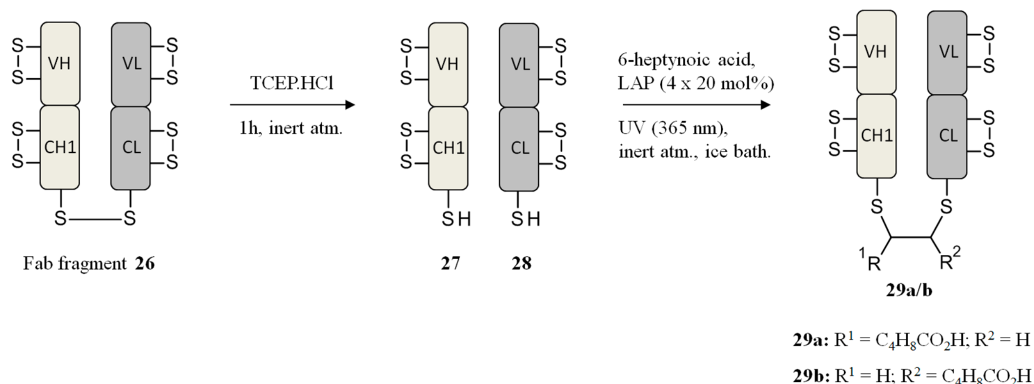


Scheme 8. TYC-Mediated Bridging of the Disulfide Bond in Terlipressin with a Cyclooctyne



DOI: 10.1021/acs.bioconjchem.5b00682
Bioconjugate Chem. XXXX, XXX, XXX−XXX

Scheme 9. Rebridging of a Disulfide Bond in a Fab Fragment



toward BCN or its derivatives, yielding vinyl sulfide products even without photoirradiation.[15] These observations, combined with the knowledge we acquired from our own experience with TYC-mediated photoconjugation, let us hypothesize that longer reaction times in the presence of a radical initiator regularly renewed and, in the presence of UV-light, might help to boost this readily occurring monoaddition, and maybe would have allowed the second addition to occur with the remaining thiol material. In this context, we decided to test our aforementioned methodology to the photoconjugation of terlipressin with the commercially available (1*R*,8*S*,9*r*)-bicyclo-[6.1.0]non-4-yn-9-ylmethanol (BCN−OH) **23** (Scheme 8). Therefore, terlipressin was submitted once again to a one-pot reduction/TYC photoreaction, in the presence of compound **23**. Due to its low solubility in DPBS buffer, the latter was dissolved in methanol before addition to the reduced terlipressin. After 3 h of UV-irradiation and a portion-wise addition of LAP at each hour of reaction, an LC/MS analysis of the reaction mixture exhibited a main product with an m/z of 903.3, corresponding to the $[M+2H]^{2+}$ adduct ion of expected product or, once again, of the corresponding vinyl sulfide. Purification via HPLC gave conjugate **24** in an isolated yield of 8%. In their report, Van Geel et al.[15] mentioned the possible reactivity of cyclooctynes toward amino acids other than cysteines. In order to confirm that both thiols were involved in our case, an aliquot was thus sampled from the reaction mixture, to which was added an excess of maleimide (tip of spatula). Indeed, maleimide is known to react readily with free thiols. A subsequent LC/MS analysis showed the persistence of our main product peaks, with no increase in the m/z value that would indicate a trapping of free thiols. This observation, combined with the observation of the correct m/z value, left no doubt for the occurrence of the double addition of the thiyl groups on the alkyne bond. To the best of our knowledge, this is the first report of the bridging of a disulfide bond using a cyclooctyne.

Next, we were intrigued to explore the potential of this new disulfide bridging technique in the synthesis of antibody drug conjugates. In order to do so, the same methodology was applied to a Fab fragment of an antibody (Scheme 9). Indeed, Fab fragments were considered to be perfect first models in our case, as they exhibit a single interchain disulfide bond, while antibodies commonly have four.

A 108.4 μL sample of 46.1 mg/mL solution of Fab-fragment M14-G07 (46 690 Da) in PBS buffer was thus reduced with TCEP·HCl over 1 h, under an argon atmosphere. The reaction vessel was then cooled down with an ice bath and the TYC was carried out over 4 h, with 6-heptynoic acid as the alkyne (1 equiv) and LAP as radical initiator (20 mol % each hour of UV-irradiation). The reaction mixture was finally purified over a Sephadex G-25 M column and centrifugation. The formation of the covalent linkage between the alkyne and the Fab fragment was then proven by MS analyses (ESI-TOF): after incubation under reductive condition, the resulting mixture showed the presence of mass peaks corresponding, respectively, to the light chain and VH−CH1 regions of the unreacted starting material, together with a mass peak of m/z 46 817.0, which was attributed to the $[M + H]^+$ adduct of the desired bridged product **29**. The absence of a vinyl sulfide type monoaddition product was proven by the absence of mass peaks corresponding to the light chain and/or VH−CH1 region with additional mass of the alkyne, which also confirms that the bridging between the heavy and light chains effectively occurred. Finally, analysis over HPLC was used to quantify the conversion of the Fab fragment into **29**. A sample of the resulting mixture was first submitted to reductive conditions with dithiothreitol (DTT) before analysis over HPLC. A comparison with the chromatogram obtained after reduction of the starting material allowed us to attribute the peaks at 6.6 and 7.7 min to the light chain and the VH−CH1 region, respectively. The new peak observed at 8.6 min was unequivocally attributed to the conjugated Fab fragment **29**. As expected, homodimer formation (LC-LC and HC-HC) did not take place during coupling, which was confirmed by mass spectrometry. Calculations from the peak areas indicated a 40% conversion of **26** to **29a/b**. A last remark is worth making on the effect of the concentration of the starting protein solution on the outcome of the reaction: Indeed, the use of more dilute solution of Fab fragments did not lead to any of the desired conjugate **29**, showing the importance of this parameter to ensure an effective thiol−yne mediated conjugation.

■ CONCLUSION

Herein, we have shown how our photomediated disulfide rebridging methodology can be successfully extended to the conjugation of peptides and proteins. In order to do, readjustments of our previously reported protocol had to be made: with peptides and proteins, the portion-wise addition of radical initiator at each hour of UV-irradiation and the concentration in the starting material was shown to be crucial to achieve satisfactory conversion rates. Our methodology was proven to be reproducible with various alkynes and different peptides. This study also allowed us to describe the first rebridging of the disulfide bond of a peptide through a thiol−

DOI: 10.1021/acs.bioconjchem.5b00682
Bioconjugate Chem. XXXX, XXX, XXX−XXX

yne reaction with a cyclooctyne. In all cases, the rebridging was proven by MS analyses and confirmed by the absence of olefinic proton on ^{1}H NMR spectra of the resulting products. Finally, this one-pot reduction thiol−yne methodology was successfully applied to a Fab fragment of an antibody with a promising conversion, which set a good ground for the future syntheses of new protein and antibody conjugates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00682.

Synthesis protocols, experimental details, and additional characterization data (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: nils.griebenow@bayer.com.
*E-mail: stefan.braese@kit.edu. Fax: +49 (0) 721 608 48581. Tel: +49 (0) 721 608 42902.

Notes

The authors declare the following competing financial interest(s): This work was funded by the Collaborative Post-Doc Program of Bayer Healthcare.

■ REFERENCES

(1) (a) Flygare, J. A., Pillow, T. H., and Aristoff, P. (2013) Antibody-drug conjugates for the treatment of cancer. *Chem. Biol. Drug Des. 81* (1), 113−21. (b) Jones, L. H., and McKnight, A. J., Eds. (2013) *Biotherapeutics: Recent Developments Using Chemical and Molecular Biology*, Royal Society of Chemistry Drug Discovery Series, Vol *36*, Royal Society of Chemistry.

(2) (a) Veronese, F. M., and Mero, A. (2008) The impact of PEGylation on biological therapies. *BioDrugs 22* (5), 315−29. (b) Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev. 54* (4), 459−76.

(3) Miller, L. W., and Cornish, V. W. (2005) Selective chemical labeling of proteins in living cells. *Curr. Opin. Chem. Biol. 9* (1), 56−61.

(4) (a) Garnett, M. C. (2001) Targeted drug conjugates: principles and progress. *Adv. Drug Delivery Rev. 53* (2), 171−216. (b) Wu, A. M., and Senter, P. D. (2005) Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol. 23* (9), 1137−46. (c) Sapra, P., Hooper, A. T., O'Donnell, C. J., and Gerber, H. P. (2011) Investigational antibody drug conjugates for solid tumors. *Expert Opin. Invest. Drugs 20* (8), 1131−49. (d) Chari, R. V. J., Miller, M. L., and Widdison, W. C. (2014) Antibody−Drug Conjugates: An Emerging Concept in Cancer Therapy. *Angew. Chem., Int. Ed. 53* (15), 3796−3827. (e) Klinguer-Hamour, C., Strop, P., Shah, D. K., Ducry, L., Xu, A., and Beck, A. (2014) World Antibody-Drug Conjugate Summit, October 15−16, 2013, San Francisco, CA. *mAbs 6* (1), 18−29. (f) Tian, F., Lu, Y., Manibusan, A., Sellers, A., Tran, H., Sun, Y., Phuong, T., Barnett, R., Hehli, B., Song, F., DeGuzman, M. J., Ensari, S., Pinkstaff, J. K., Sullivan, L. M., Biroc, S. L., Cho, H., Schultz, P. G., DiJoseph, J., Dougher, M., Ma, D., Dushin, R., Leal, M., Tchistiakova, L., Feyfant, E., Gerber, H.-P., and Sapra, P. (2014) A general approach to site-specific antibody drug conjugates. *Proc. Natl. Acad. Sci. U. S. A. 111* (5), 1766−1771.

(5) Ramil, C. P., and Lin, Q. (2013) Bioorthogonal chemistry: strategies and recent developments. *Chem. Commun. 49* (94), 11007−11022.

(6) Widdison, W. C., Wilhelm, S. D., Cavanagh, E. E., Whiteman, K. R., Leece, B. A., Kovtun, Y., Goldmacher, V. S., Xie, H., Steeves, R. M., Lutz, R. J., Zhao, R., Wang, L., Blattler, W. A., and Chari, R. V. (2006) Semisynthetic maytansine analogues for the targeted treatment of cancer. *J. Med. Chem. 49* (14), 4392−408.

(7) Ghosh, S. S., Kao, P. M., McCue, A. W., and Chappelle, H. L. (1990) Use of maleimide-thiol coupling chemistry for efficient syntheses of oligonucleotide-enzyme conjugate hybridization probes. *Bioconjugate Chem. 1* (1), 71−6.

(8) (a) Liberatore, F. A., Comeau, R. D., McKearin, J. M., Pearson, D. A., Belonga, B. Q., Brocchini, S. J., Kath, J., Phillips, T., Oswell, K., and Lawton, R. G. (1990) Site-directed chemical modification and crosslinking of a monoclonal antibody using equilibrium transfer alkylating crosslink reagents. *Bioconjugate Chem. 1* (1), 36−50. (b) Godwin, A. R., Pedone, E., Choi, J. W., Shaunak, S., and Brocchini, S. J. (2005) *Conjugated biological molecules and their preparation*, Int. Patent No. WO2005007197 A2. (c) Godwin, A., and Brocchini, S. J. (2010) *Conjugated proteins and peptides*, Int. Patent No. WO 2010100430 A1.

(9) Schumacher, F. F., Sanchania, V. A., Tolner, B., Wright, Z. V. F., Ryan, C. P., Smith, M. E. B., Ward, J. M., Caddick, S., Kay, C. W. M., Aeppli, G., Chester, K. A., and Baker, J. R. (2013) Homogeneous antibody fragment conjugation by disulfide bridging introduces 'spinostics'. *Sci. Rep. 3*, 1525.

(10) Griebenow, N., Braese, S., and Dilmac, A. M. (2015) Application of a novel small scale UV LED photochemical batch reactor for the thiol-yne reaction. *RSC Adv. 5*, 54301−54303.

(11) Aimetti, A. A., Feaver, K. R., and Anseth, K. S. (2010) Synthesis of cyclic, multivalent Arg-Gly-Asp using sequential thiol-ene/thiol-yne photoreactions. *Chem. Commun. (Cambridge, U. K.) 46* (31), 5781−5783.

(12) Gong, T., Adzima, B. J., and Bowman, C. N. (2013) A novel copper containing photoinitiator, copper(II) acylphosphinate, and its application in both the photomediated CuAAC reaction and in atom transfer radical polymerization. *Chem. Commun. 49* (72), 7950−7952.

(13) Fairbanks, B. D., Sims, E. A., Anseth, K. S., and Bowman, C. N. (2010) Reaction Rates and Mechanisms for Radical, Photoinitated Addition of Thiols to Alkynes, and Implications for Thiol−Yne Photopolymerizations and Click Reactions. *Macromolecules 43* (9), 4113−4119.

(14) Pelegri-O'Day, E. M., Lin, E.-W., and Maynard, H. D. (2014) Therapeutic Protein−Polymer Conjugates: Advancing Beyond PEGylation. *J. Am. Chem. Soc. 136* (41), 14323−14332.

(15) van Geel, R., Pruijn, G. J. M., van Delft, F. L., and Boelens, W. C. (2012) Preventing Thiol-Yne Addition Improves the Specificity of Strain-Promoted Azide−Alkyne Cycloaddition. *Bioconjugate Chem. 23* (3), 392−398.

DOI: 10.1021/acs.bioconjchem.5b00682
Bioconjugate Chem. XXXX, XXX, XXX−XXX