JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Transition Metal-Free Tryptophan-Selective Bioconjugation of Proteins

Yohei Seki,[†] Takashi Ishiyama,[†] Daisuke Sasaki,[†,‡] Junpei Abe,[†] Youhei Sohma,[†,‡] Kounosuke Oisaki,*[,†] and Motomu Kanai*[,†,‡]

[†]Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[‡]ERATO, Kanai Life Science Catalysis Project, Japan Science and Technology Agency (JST), 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Supporting Information

ABSTRACT: Chemical modifications of native proteins can facilitate production of supernatural protein functions that are not easily accessible by complementary methods relying on genetic manipulations. However, accomplishing precise control over selectivity while maintaining structural integrity and homogeneity still represents a formidable challenge. Herein, we report a transition metal-free method for tryptophan-selective bioconjugation of proteins that is based on an organoradical and operates under ambient conditions. This method exhibits low levels of cross-reactivity and leaves higher-order structures of the protein and various functional groups therein unaffected. The strategy to target less abundant amino acids contributes to the formation of structurally homogeneous conjugates, which may even be suitable for protein crystallography. The absence of toxic metals and biochemically incompatible conditions allows a rapid functional modulation of native proteins such as antibodies and pathogenic aggregative proteins, and this method may thus easily find therapeutic applications.

Engineering native proteins can vastly expand their functional diversity, enabling various applications, such as, biochemical tools, catalysts, and therapeutic agents.[1] Chemical modifications of native proteins that target proteinogenic amino acid residues[2] complement the methods relying on genetic manipulations and allow facile access to engineered proteins. In general, various structural modifications, including direct conjugation with drug or probe molecules, are possible with chemical protein modifications. Moreover, the concept mimics regulation of protein functions by post-transcriptional modifications.[3]

The functionality of engineered proteins is intimately correlated to their chemical structure.[4] Uncontrolled modifications that produce heterogenic conjugates may not only result in detrimental properties such as thermal instability, aggregation, and unfavorable pharmacokinetics but also hamper detailed analyses regarding structure−function relationships and modes of action. Even though precise control over the chemo-, site, and modification-number selectivity in protein chemical conjugates with maintained structural integrity and homogeneity is highly important, it still represents a major challenge.[5] The available toolbox of native protein conjugations does not contain satisfactory solutions to this challenge at present. In many cases, the conjugation sites are located at the side chains of Lys and Cys residues.[2,6] Despite recent improvements,[7] these methods often afford unsatisfactory levels of selectivity with disintegrated higher-order structures.[8]

Targeting less abundant amino acid residues may represent a potential solution to achieving high control over protein conjugates. Among all proteinogenic amino acids, Trp is the least abundant (∼1%),[9] exhibits little surface exposure,[10] and is included in the primary sequences of 90% of native proteins.[11] Targeting Trp should thus be a promising strategy to accomplish selectivity while maintaining structural integrity. However, methods applicable to the synthesis of protein therapeutics targeting Trp still remain elusive. Previously reported methods for the conjugation of Trp[12] require toxic heavy metals or biochemically incompatible conditions. Some of these methods also exhibit cross reactivity to other amino acids (particularly to tyrosine), thus limiting the range of applications. Herein, we report a mild, transition metal-free, Trp-selective method for the bioconjugation of peptides and proteins, which is applicable to a wide range of substrates. This method tolerates a variety of functional groups, while retaining the higher-order structure of the proteins.

We initially found that Trp-containing short peptides (**1a**) produced significant amounts of 9-azabicyclo[3.3.1]nonane-3-one-*N*-oxyl (keto-ABNO, **3a**)[13]-adducts under the previously reported conditions for the copper/**3a**/NO_x-mediated serine-selective oxidative cleavage of peptides.[14] The adducts (**2** and dehydrated **2**) resulted from the formation of a C−O bond at the 3-position of the indole moiety of a Trp residue.[15] This preliminary finding prompted us to investigate the application of **3a** to a novel Trp-selective bioconjugation reaction. After surveying several reaction parameters,[15] optimal reaction conditions could be established: treatment of 1 equiv of **3a** with 0.6 equiv of $NaNO_2$ in an aqueous medium containing 0.1% acetic acid for 30 min in the absence of transition metal salts (Figure 1).

In the optimization process, the following observations were noteworthy (Figure 1). The use of sterically hindered 2,2,2′,2′-tetramethylpiperidine *N*-oxyls (TEMPOs, **7** and **8**), instead of **3a**, did not afford the conjugation product. 9-Azabicyclo[3.3.1]-nonane *N*-oxyl (ABNO, **4**), 2-azaadamantane *N*-oxyl (AZADO,

Received: June 29, 2016
Published: August 17, 2016

DOI: 10.1021/jacs.6b06692
J. Am. Chem. Soc. 2016, 138, 10798−10801

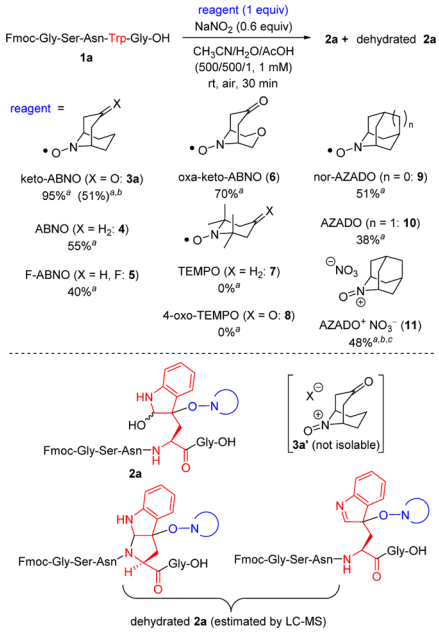
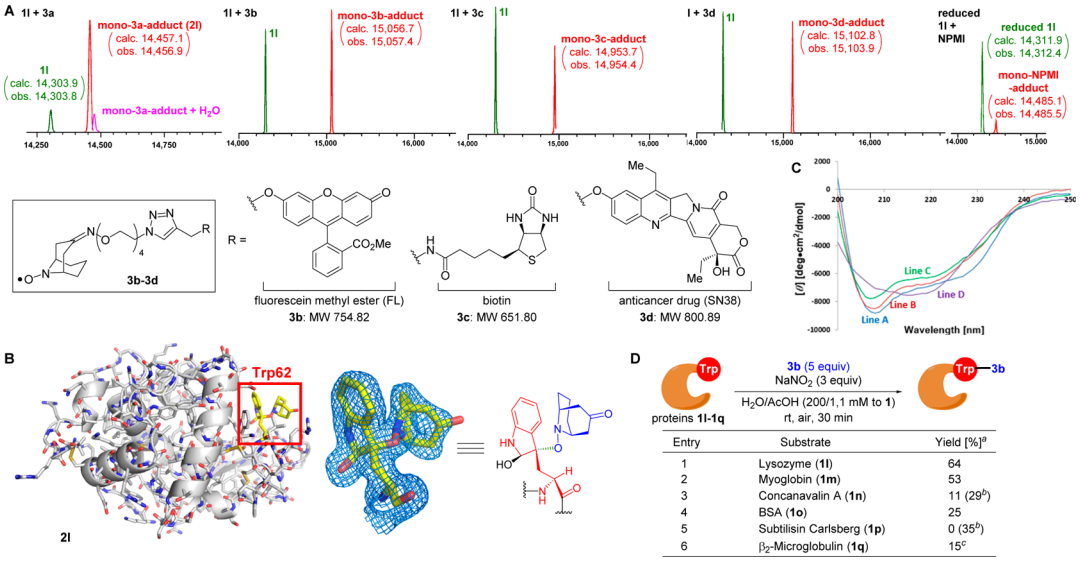


Figure 1. Effects of radicals and reagents on Trp-selective bioconjugation to Fmoc-pentapeptide **1a**. [a]Yields were calculated as combined yield of **2a** and dehydrated form(s) of **2a** based on peptide **1a** from the absorbance at 301 nm (the maximum absorbance of Fmoc group) using LC-MS analysis. [b]Reaction was conducted in the absence of $NaNO_2$. [c]Reaction was conducted in the absence of acetic acid.

9), and 9-azanoradamantane *N*-oxyl (nor-AZADO, **10**),[16a] which exhibit a steric demand similar to that of **3a** even though they lack the electron-withdrawing carbonyl group, produced the corresponding conjugates in lower yields than **3a**. Moreover, 3-fluor-9-azabicyclo[3.3.1]nonane *N*-oxyl (F-ABNO, **5**) and 7-oxa-9-azabicyclo[3.3.1]nonan-3-one *N*-oxyl (oxa-keto-ABNO, **6**)[16b] containing electron-withdrawing group(s) produced less satisfactory results than **3a**. The higher reactivity of **3a** is most likely due to both its small steric demand and the high oxidation potential, which arises from the presence of an electron-withdrawing keto group.[13,16b] The keto group is moreover able to facilitate the covalent attachment of various functional molecules to the radical, such as fluorescent probes and anticancer drugs (see Figure 2). Therefore, **3a** is the best choice in view of both reactivity and applicability. The result using $AZADO^+NO_3^-$ (**11**) suggested that the actual reactive species should be oxoammonium **3a′**, which is derived from **3a** via acid-promoted disproportionation and oxidation by NO_x, which in turn is generated from $NaNO_2$ and acetic acid.[17,18] The conjugation reaction should proceed via a nucleophilic attack of the indole ring of a Trp residue to **3a′**, followed by the attack of water toward the resulting imine.[15]

Subsequently, we studied the scope of the Trp-selective conjugation to peptide substrates under the optimized conditions (Table 1). Peptides **1a**−**1f** comprise oxidation-sensitive Ser (aliphatic alcohol), Lys (aliphatic primary amine), His (imidazole), Tyr (phenol), Met (sulfide), or Cys-Cys (cyclic disulfide) moieties, which have often exhibited cross reactivity in previously reported Trp-selective modification methods. However, using the present method, the expected conjugates were generated in high yield without affecting these amino acid residues (entries 1−6). The reaction proceeded in the absence of organic cosolvents (entries 7−10), a feature that is especially desirable in the context of protein bioconjugation (*vide infra*). Biologically relevant oligopeptides such as neuromedin B (**1g**), the luteinizing hormone-releasing hormone (LH-RH; **1h**), the δ sleep-inducing peptide (DSIP; **1i**), and the kisspeptin-10 (**1j**) could also be used in this reaction (entries 7−10). Furthermore, the pH-sensitive Alzheimer disease-related 26-*O*-acyl isoamyloid β $(A\beta)_{1-42}$,[19] which is capped with an N-terminal Trp (**1k**; entry 11), could also be transformed. We also carried out the

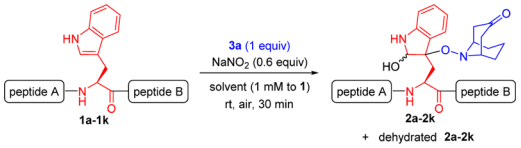


Entry	Substrate	Yield [%][a]
1	Lysozyme (**1l**)	64
2	Myoglobin (**1m**)	53
3	Concanavalin A (**1n**)	11 (29[b])
4	BSA (**1o**)	25
5	Subtilisin Carlsberg (**1p**)	0 (35[b])
6	β_2-Microglobulin (**1q**)	15[c]

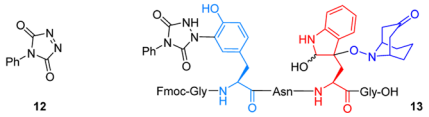
Figure 2. Trp-selective protein bioconjugation of proteins. (A) Deconvoluted ESI-MS charts after the Trp-selective bioconjugation of **1l** and **3a**−**3d**, as well as the Cys-selective bioconjugation[6] of **1l** and NPMI (MW: 173.17). (B) The crystal structure of **2l** and the $2F_0 - F_c$ electron density map (contoured at the 0.7 σ level) of **3a**-conjugated Trp62 in **2l**. (C) Circular dichroism of the **1l**−**3a** and **1l**−NPMI conjugates. Line A, products after the conjugation of **1l** with **3a**; line B, products after the reaction of **1l** and $NaNO_2$ in the absence of **3a**; line C, **1l**; line D, products after Cys-selective conjugation of reduced **1l** with NPMI. (D) Scope of proteins. [a]Assuming that the product was the mono-**3b**-adduct, the yield was calculated from the fluorescence intensity of SDS-PAGE bands, based on a calibration curve. [b]0.1 M aqueous HCl was used as the solvent. [c]H_2O/AcOH (2000/1; 0.1 mM with respect to 1) was used as the solvent.

DOI: 10.1021/jacs.6b06692
J. Am. Chem. Soc. 2016, 138, 10798−10801

Table 1. Transition Metal-Free Trp-Selective Bioconjugation of Peptides



Entry	Substrate	Yield [%]
1[a]	Fmoc-Gly-Ser-Asn-Trp-Gly-OH (**1a**)	95
2[a]	Fmoc-Gly-Lys-Asn-Trp-Gly-OH (**1b**)	85
3[a]	Fmoc-Gly-His-Asn-Trp-Gly-OH (**1c**)	90
4[a]	Fmoc-Gly-Tyr-Asn-Trp-Gly-OH (**1d**)	95
5[a]	Fmoc-Gly-Met-Asn-Trp-Gly-OH (**1e**)	96
6[a]	Fmoc-Cys-Gly-Trp-Arg-Ala-Cys-Gly-OH (**1f**)	84
7[b]	Neuromedin B: Ala-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2 (**1g**)	84
8[b]	LH-RH: Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (**1h**)	91
9[b]	DSIP: Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH (**1i**)	84
10[b]	Kisspeptin-10: Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2 (**1j**)	83
11[c]	*N*-Ac-Trp-(26-*O*-acyl-isoAβ_{1-42})-OH (**1k**)	ND[f]
12[d]	Fmoc-Gly-Tyr-Asn-Trp-Gly-OH (**1d**)	54[g]
13[e]	Fmoc-Gly-Tyr-Asn-Trp-Gly-OH (**1d**)	52[g]



[a]CH_3CN/H_2O/AcOH (500/500/1) was used as the solvent. HPLC yield was combined yield of **2** and dehydrated **2** based on the starting peptide, determined at 301 nm absorbance derived from the Fmoc group. [b]H_2O/AcOH (1000/1) was used as the solvent. HPLC yield was determined at 250 nm absorbance. [c]**1** in CH_3CN/H_2O/AcOH (333/333/1), **3a** (1.5 equiv), and $NaNO_2$ (0.9 equiv) were used. [d]A one-pot sequential double modification protocol for Trp and Tyr[20] was applied to generate **13**. [e]The modification sequence was reversed from entry 12 to generate **13**. [f]ESI-MS and HPLC analyses suggested almost quantitative conversion from **1k** to **2k**. The yield was not determined (ND) on account of the aggregation of **2k**. [g]HPLC yield of **13** after two steps.

Trp-modification orthogonally to another amino acid-targeting bioconjugation, a Tyr-modification.[20] The Trp-selective conjugation of **1d** with **3a**, followed by the Tyr-selective conjugation with **12** afforded the doubly modified peptide **13** in 54% overall yield (entry 12). Applying the corresponding reversed double-modification sequence furnished the same product in similar yield (52%, entry 13).

We then examined the Trp-selective bioconjugation to a native protein, lysozyme (**1l**) (Figure 2A–C), which comprises 129 amino acid residues including six Trp residues (Trp28, Trp62, Trp63, Trp109, Trp112, and Trp124). When **1l** was treated with **3a** under standard conditions, the monoconjugated species (**2l**) was obtained as the major product (Figure 2A, **1l** + **3a**). Crystallization of **2l** was possible by storing the reaction mixture in an aqueous solution containing 0.8 M NaCl and 0.05 M AcOH (pH 4.5) at room temperature for 3 days. The X-ray diffraction analysis revealed that the major modification site was Trp62 (Figure 2B). The other five Trp residues, other oxidation-sensitive amino acid residues such as Tyr20, Tyr23, and Tyr53, and the S–S bonds of Cys6-Cys127, Cys30-Cys115, Cys64-Cys80, and Cys74-Cys76 remained intact.[15] The selectivity of the conjugation toward Trp62 is probably due to its location, which should be the least sterically hindered among the six Trp residues present in the native conformation of **1l**.[21] The chemical conjugation did not affect the conformation of the main chain of the protein, and a significant difference between the three-dimensional structures of modified **1l** (i.e., **2l**) and native **1l** could not be observed by crystallographic analysis.[15] The structural integrity of **2l** was confirmed by circular dichroism (CD) spectroscopy (Figure 2C). The spectral profile of the sample after Trp-modification (line A) was almost identical to those of a control sample under reaction conditions without **3a** (line B) and pure **1l** (line C). Conversely, the Cys-conjugation with *N*-phenylmaleimide (NPMI) after reductive cleavage of the disulfide bonds[6] with tris(2-carboxyethyl)phosphine (TCEP) afforded a markedly altered CD spectrum (line D), indicating that the products contain non-native conformations. Because the current Trp-selective bioconjugation proceeds under mild conditions reflecting the local steric environment around each Trp residue, the method will be a unique tool to gain information about the tertiary structure of proteins in a solution phase.

Using lysozyme (**1l**) as a model protein substrate, we then investigated the scope of this conjugation with derivatives of **3** (1 equiv) that were functionalized with a fluorescein methyl ester (**3b**), biotin (**3c**), or the anticancer drug SN38 (**3d**) (Figure 2A). In all cases, single modification products were obtained, which corroborates the precise selectivity of the present method and its high tolerance toward functional groups in the small molecule side. In sharp contrast, a conventional Cys-selective modification of **1l** with NPMI produced the adduct in low conversion due to the inevitable aggregation after S–S cleavage (Figure 2A, reduced **1l** + NPMI). Moreover, the Trp-selective bioconjugation products were chemically and thermally more stable than the products of the Cys-bioconjugation.[15]

Subsequently, we investigated the substrate scope with respect to native proteins (Figure 2D). Lysozyme (**1l**), myoglobin (**1m**; 153 amino acid residues including the two tryptophan residues; Trp7 and Trp14, and containing an iron-heme complex), concanavalin A (**1n**; 235 amino acid residues; Trp40, Trp88, Trp109, and Trp188), BSA (**1o**; 583 amino acid residues; Trp215), subtilisin Carlsberg (**1p**; 274 amino acid residues; Trp113), and β_2-microglobulin (**1q**; 99 amino acid residues; Trp60 and Trp95) afforded the expected **3b**-conjugates in 11–64% yield with use of increased amount of reagents (5 equiv of **3b** and 3 equiv of $NaNO_2$). When an organic cosolvent was used for **1m**, leaching of the heme complex was observed.[15] However, conditions in the absence of an organic cosolvent suppressed the leaching and furnished the **3b**-conjugate in high yield (53%, Figure 2D, entry 2). The use of acidic media with hydrochloric acid improved the product yield for **1n** and **1p**, which might be attributed to the exposure of more Trp residues on the protein surface through partial denaturation (entries 3 and 5).

This method was also applicable to the synthesis of an antibody–small molecule conjugate, whereby the binding ability of the protein was not diminished. A commercially available anti-Aβ_{1-16} antibody (6E10) was conjugated with fluorescein-containing **3b**. The fluorescence of this **3b**-conjugate suggested that conjugation occurred both at the light and heavy chains of the antibody (Figure 3A). In order to confirm that the functionality of the antibody remained intact, dot blot assays targeting Aβ were carried out (Figure 3B). The observed fluorescence intensity was consistent with the concentration of Aβ adsorbed on the blot. Relative to a conventional Aβ dot blot assay, which comprises an enzyme-linked immunosorbent assay,[22] the present method using the 6E10–**3b** conjugate does not require a secondary antibody. This is a significant advantage with respect to operational ease for the detection of Aβ, which is a potential cause of Alzheimer's disease after amyloid formation.

DOI: 10.1021/jacs.6b06692
J. Am. Chem. Soc. 2016, 138, 10798–10801

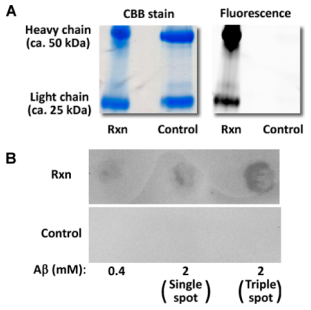


Figure 3. Trp-selective bioconjugation of the anti-Aβ_{1-16} antibody 6E10 and **3b**. (A) SDS-PAGE analysis. (B) Dot blot assay for **3b**-modified (Rxn) or unmodified (Control) 6E10. The fluorescence analysis was carried out at 473 nm (excitation) and 665 nm (detection).

In conclusion, we developed a Trp-selective bioconjugation of native proteins. Novel features of this new method are (1) no requirements for toxic transition metals, (2) facile conjugation at ambient temperature and near neutral pH for short reaction time (30 min), (3) high Trp-selectivity, (4) formation of single conjugates with high homogeneity even suitable for X-ray crystallography,[23] due to the low abundance of Trp in native proteins, (5) site-selectivity reflecting the steric demands in solution-phase tertiary structures, and (6) retention of tertiary structures and protein functions. This method should thus be highly useful for the synthesis of next-generation biologics with precisely defined chemical structures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06692. Crystallographic data for **2l** is available free of charge from the Protein Data Bank under accession numbers 5B59.

Experimental details, including optimizations, procedures, syntheses, and characterization of new products and ^{1}H NMR, ^{13}C NMR, and HPLC charts (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*oisaki@mol.f.u-tokyo.ac.jp (K.O.)
*kanai@mol.f.u-tokyo.ac.jp (M.K.)

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by ERATO from JST (M.K.), Scientific Research C (project No. 25460008) from JSPS (K.O.), and "Nanotechnology Platform" (project No. 12024046) of MEXT, Japan. Y. Seki thanks JSPS for a fellowship. We thank Dr. S. Kawashima, Dr. Y. Aoi, Dr. T. Ishiguro (The University of Tokyo), and Dr. A. Taniguchi (Tokyo University of Pharmacy and Life Sciences) for fruitful discussions and assistance with biochemical techniques.

■ REFERENCES

(1) *Protein Engineering Handbook*; Lutzu, S., Bornscheuer, U. T., Eds.; Wiley: New York, 2009, 2012; Vol. *1*−*3*.

(2) (a) *Bioconjugate Techniques*, 3rd ed.; Hermanson, G., Ed.; Adacemic Press: Cambridge, MA, 2013. (b) Stephanopoulos, N.; Francis, M. *Nat. Chem. Biol.* **2011**, *7*, 876. (c) Koniev, O.; Wagner, A. *Chem. Soc. Rev.* **2015**, *44*, 5495.

(3) Walsh, C. T.; Garneau-Tsodikova, S.; Gatto, G. J. *Angew. Chem., Int. Ed.* **2005**, *44*, 7342.

(4) *Textbook of Structural Biology*; Liljas, A., Liljas, L., Piskur, J., Lindblom, G., Nissen, P., Kjeldgaard, M., Eds.; World Scientific Publishing: Singapore, 2009.

(5) For recent reviews, see (a) Krall, N.; da Cruz, F. P.; Boutureira, O.; Bernardes, G. J. J. *Nat. Chem.* **2016**, *8*, 103. (b) Hu, W.-Y.; Berti, F.; Adamo, R. *Chem. Soc. Rev.* **2016**, *45*, 1691. (c) Akkapeddi, P.; Azizi, S.-A.; Freedy, A. M.; Cal, P. M. S. D.; Gois, P. M. P.; Bernardes, G. J. L. *Chem. Sci.* **2016**, *7*, 2954. (d) Agarwal, P.; Bertozzi, C. R. *Bioconjugate Chem.* **2015**, *26*, 176.

(6) Chalker, J. M.; Bernardes, G. J. J.; Lin, Y. A.; Davis, B. G. *Chem. - Asian J.* **2009**, *4*, 630.

(7) (a) Chen, X.; Muthoosamy, K.; Pfisterer, A.; Neumann, B.; Weil, T. *Bioconjugate Chem.* **2012**, *23*, 500. (b) Zhang, C.; Welborn, M.; Zhu, T.; Yang, N. J.; Santos, M. S.; Van Voorhis, T.; Pentelute, B. L. *Nat. Chem.* **2016**, *8*, 120. (c) Tamura, T.; Tsukiji, S.; Hamachi, I. *J. Am. Chem. Soc.* **2012**, *134*, 2216. (d) Shaunak, S.; Godwin, A.; Choi, J.-W.; Balan, S.; Pedone, E.; Vijayarangam, D.; Heidelberger, S.; Teo, I.; Zloh, M.; Brocchini, S. *Nat. Chem. Biol.* **2006**, *2*, 312.

(8) Hogg, P. J. *Trends Biochem. Sci.* **2003**, *28*, 210.

(9) Gilis, D.; Massar, S.; Cerf, N.; Rooman, M. *Genome Biology* **2001**, *2*, research0049.1.

(10) Moelbert, S.; Emberly, E.; Tang, C. *Protein Sci.* **2004**, *13*, 752.

(11) Gevaert, K.; Van Damme, P.; Martens, L.; Vandekerckhove, J. *Anal. Biochem.* **2005**, *345*, 18.

(12) (a) Antos, J.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 10256. (b) Ruiz-Rodríguez, J.; Albericio, F.; Lavilla, R. *Chem. - Eur. J.* **2010**, *16*, 1124. (c) Popp, B. V.; Ball, Z. T. *J. Am. Chem. Soc.* **2010**, *132*, 6660. (d) Seim, K.; Obermeyer, A.; Francis, M. B. *J. Am. Chem. Soc.* **2011**, *133*, 16970. (e) Williams, T. J.; Reay, A. J.; Whitwood, A. C.; Fairlamb, I. J. *Chem. Commun.* **2014**, *50*, 3052. (f) Hansen, M. B.; Hubálek, F.; Skrydstrup, T.; Hoeg-Jensen, T. *Chem. - Eur. J.* **2016**, *22*, 1572.

(13) Sonobe, T.; Oisaki, K.; Kanai, M. *Chem. Sci.* **2012**, *3*, 3249.

(14) Seki, Y.; Tanabe, K.; Sasaki, D.; Sohma, Y.; Oisaki, K.; Kanai, M. *Angew. Chem., Int. Ed.* **2014**, *53*, 6501.

(15) See Supporting Information for details.

(16) (a) Iwabuchi, Y. *Chem. Pharm. Bull.* **2013**, *61*, 1197. (b) Lauber, M. B.; Stahl, S. S. *ACS Catal.* **2013**, *3*, 2612.

(17) (a) Shibuya, M.; Osada, Y.; Sasano, Y.; Tomizawa, M.; Iwabuchi, Y. *J. Am. Chem. Soc.* **2011**, *133*, 6497. (b) Kishioka, S.; Ohsaka, T.; Tokuda, K. *Electrochim. Acta* **2003**, *48*, 1589.

(18) Oxoammonium **3a′** is not stable and thus could not be isolated in a pure form.

(19) Sohma, Y.; Hayashi, Y.; Kimura, M.; Chiyomori, Y.; Taniguchi, A.; Sasaki, M.; Kimura, T.; Kiso, Y. *J. Pept. Sci.* **2005**, *11*, 441.

(20) (a) Ban, H.; Gavrilyuk, J.; Barbas, C. F., III. *J. Am. Chem. Soc.* **2010**, *132*, 1523. (b) Ban, H.; Nagano, M.; Gavrilyuk, J.; Hakamata, W.; Inokuma, T.; Barbas, C. F., III. *Bioconjugate Chem.* **2013**, *24*, 520.

(21) Harata, K. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1994**, *D50*, 250.

(22) Necula, M.; Breydo, L.; Milton, S.; Kayed, R.; van der Veer, W. E.; Tone, P.; Glabe, C. G. *Biochemistry* **2007**, *46*, 8850.

(23) McPherson, A.; Nguyen, C.; Cudney, R.; Larson, S. B. *Cryst. Growth Des.* **2011**, *11*, 1469.

DOI: 10.1021/jacs.6b06692
J. Am. Chem. Soc. 2016, 138, 10798−10801