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Amino-Acid-Catalyzed Direct Aldol Bioconjugation

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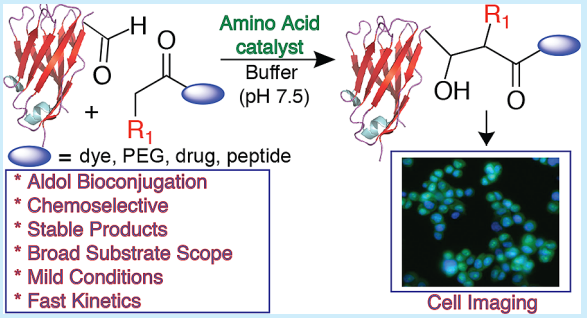
Supporting Information

ABSTRACT: A site-specific bioconjugation was developed based on direct aldol coupling using amino-acid-derived organocatalysts. The functionalization exhibits fast kinetics and occurs under mild, biocompatible conditions (viz., aqueous media, moderate temperature, and neutral pH). The resulting bioconjugates were found to be stable toward abundant aldolase enzymes, as well as acidic and basic pH. The methodology was demonstrated through conjugation of a variety of small molecules, dyes, and peptides to proteins, including a single-domain antibody, which was then used for cellular imaging.



Post-translational modification of proteins to install new functionality is widely known in nature, yet similar selectivity and efficiency is currently unattainable from the available chemical methods.[1,2] Single-site biomolecule functionalization to generate homogeneous constructs in the presence of amino acids with diverse reactivity in both a chemo- and regioselective manner is an exceptional challenge, which is further exacerbated by the requirement for nondenaturing conditions.[3] Nevertheless, bioorthogonal chemical reactions have emerged for modification of proteins with both natural and unnatural amino acids. For example, aldehydes and ketones can be readily installed onto a protein's N-terminus by well-established methods.[4] They then become key handles for further transformation, such as the commonly employed conjugations with hydrazines and hydroxylamines to generate hydrazones and oximes, respectively.[5,6] However, this approach has limitations, such as slow kinetics, acidic (pH 5−6) conditions, high concentrations of harmful catalysts, cross-reactivity with metabolites, and poor stability of the resulting conjugates due to reversibility.[6,7] This leads to the development of more advanced aldehyde bioconjugations, which exhibit improved stability of conjugates, such as Pictet−Spengler,[8] indium-mediated allylation,[9] and Wittig reactions.[10]

We considered that amino acids and/or peptides could be used for the conjugation of proteins by catalyzing the aldol reaction, which would lead to carbon−carbon bond formation at the site of conjugation (Figure 1).[11,12] Existing bioconjugation methods utilizing the aldol reaction are limited by poor substrate scope and slow kinetics.[13] While the efficiency and selectivity of the proposed amino-acid-catalyzed reaction may be impeded by the presence of a variety of functional groups

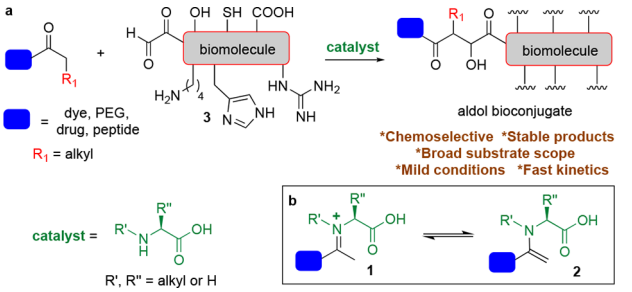


Figure 1. (a) Proposed Aldol bioconjugation reaction using amino-acid-derived catalysts. (b) Key reactive intermediates.

within complex biomolecules, we envisioned that careful use of particular amino acids or peptides would serve as highly reactive catalysts for the desired transformations under physiologically relevant conditions. The interaction between the amino group of the catalyst and the aldehyde/ketone would guide its selectivity by formation of an iminium ion-enamine complex. The ability to achieve high levels of chemo- and regioselectivity and fast reaction kinetics with aldehydes and ketones under aqueous conditions by using well-designed amino-acid-derived organocatalysts suggests that they could be very attractive for the development of a new site-selective bioconjugation method leading to the modification at the N-terminus of proteins. In addition, this method has the potential to generate a chiral center at the site of bioconjugation with high stereoselectivity through judicious organocatalyst selec-

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tion, which may be of therapeutic significance (see Supporting Information).

Here we report an organocatalyzed aldol bioconjugation reaction that is applicable to a wide range of peptides and proteins. The organocatalyzed aldol bioconjugation reaction is chemoselective, site-specific, and rapid and occurs under mild reaction conditions. We illustrate the diversity of this reaction for a series of expressed and commercially available proteins and antibodies.

We started our investigation with proline as catalyst because a multitude of reports have shown that proline can form an iminium ion **1** with the ketone followed by rearrangement to an enamine **2**, as needed for carrying out nucleophilic attack on the electrophilic aldehyde **3** (Figure 1).[14] A simple amino acid aldehyde, phenylalanine aldehyde **4**, was used to optimize reaction conditions and to explore substrate scope with different ketones (**5**–**7**, Figure 2). First, we compared the

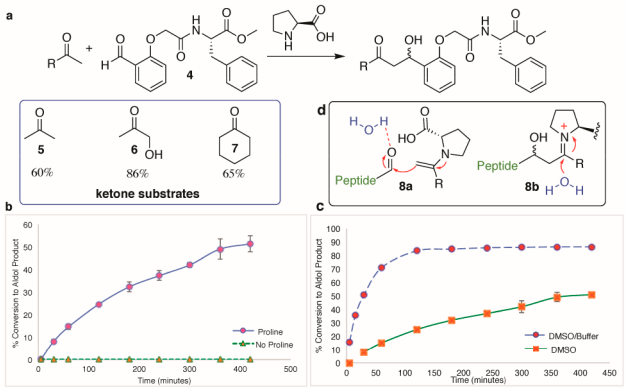

Figure 2. Model aldol bioconjugation between (a) phenylalanine aldehyde **4** and ketones (**5**–**7**) catalyzed by proline. (b) Comparison of uncatalyzed and proline-catalyzed (100 mM) reaction rates in DMSO at 37 °C between **4** (1 equiv., 7 mM) and hydroxyacetone **6** (12 equiv., 84 mM). (c) Comparison of reaction in 100% DMSO versus 1:1 sodium phosphate buffer (pH 7.4):DMSO. (d) Proposed role of water in increasing reaction kinetics.

rates of catalyzed versus uncatalyzed reactions between **4** and hydroxyacetone **6** (Figure 2b). No product was detected in the absence of an organocatalyst. While most aldehyde bioconjugations operate at slightly acidic pH (5.5 to 6.5),[5,6,8] 86% conversion occurred for the pH 7.4 phosphate-buffered reaction. Furthermore, the rate was considerably faster with aqueous buffer than in neat DMSO (Figure 2c).

The increase in the rate of the organocatalyzed reaction in buffer might be due to the dual role of water in activating the aldehyde for nucleophilic attack through hydrogen bonding **8a** and by accelerating the hydrolysis of the iminium ion intermediate **8b** formed between the aldol bioconjugate and the organocatalyst, resulting in the release of the desired bioconjugate (Figure 2d).[14] Different amino-acid-based organocatalysts were then screened for the reaction of either **4** or peptide aldehyde, VF **9**, with **6**, and the highest conversion was observed with proline (see Supporting Information). After optimizing catalyst loading and temperature, 96% product formed within 15 min using 100 mM proline at 60 °C and pH 7.5. The optimized reaction conditions (100 mM proline, 5% DMSO, 25 mM phosphate buffer, pH 7.5 at 60 °C) were then used for evaluating substrate scope (Figure 3). Unprotected peptide aldehydes **9**–

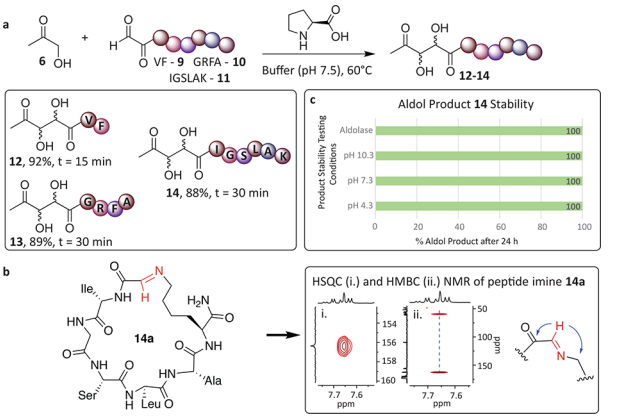

Figure 3. Chemoselective nature of aldol bioconjugation. (a) Reaction conditions: peptide aldehydes **9**–**11** (7 mM), hydroxyacetone **6** (84 mM), and proline (100 mM) in 25 mM phosphate buffer (pH 7.5) and up to 15% DMSO at 60 °C. (b) Peptide **11** containing Lys can form macrocyclic imine **14a**, but this did not impede the formation of aldol bioconjugate **14**. (c) Stability of **14** with acid, base, or aldolase at 37 °C.

11, which were generated from N-terminal serine-containing peptides by sodium periodate ($NaIO_4$) oxidation,[4a] were reacted with hydroxyacetone **6** resulting in high conversions to the desired conjugated products **12**–**14** within 15 to 30 min. No interference was observed from the presence of other reactive amino acid side chains. In the case of lysine, self-condensation of **11** resulted in the formation of a macrocyclic imine **14a** (Figure 3b). However, reaction of **14a** with **6** ultimately leads to the desired aldol bioconjugate **14**, presumably due to the high rate of transamination reaction of the imine **14a** with proline catalyst.[15]

The stability of the aldol bioconjugation product was then evaluated against acidic and basic conditions, as well as aldolase enzymes (Figure 3c). As expected, incubation of **14** for 24 h at pH 4.3, 7.3, and 10.3 at both room temperature and 37 °C leads to no detectable degradation. Notably, **14** was also found to be stable toward the retro-aldol reaction catalyzed by aldolases, which are highly abundant in nature. The stability of the aldol bioconjugate toward aldolases is presumably due to the enzyme's high substrate specificity.[16]

We next explored the aldol bioconjugation reaction with horse heart myoglobin (Figure 4). The N-terminal glycine residue was converted to an aldehyde using the well-known pyridoxal 5′-phosphate (PLP) reagent.[4b] Hydroxyacetone **6** was then reacted with myoglobin aldehyde **15** at 37 °C in buffer using proline resulting in bioconjugated myoglobin **16** with 80% conversion within 30 min as determined by LC-MS. The analysis of the resulting myoglobin bioconjugate **16** using CD and UV showed that the structure of modified protein **16** remained intact after the bioconjugation (see Supporting Information). Two control reactions were then performed using (1) unmodified myoglobin (i.e., no aldehyde handle) and (2) no organocatalyst. In both cases, no bioconjugation product was observed after 24 h.

Peptide ketones, GIRVF **17** and ACF **18**, were generated from reaction of PLP with peptides, AGIRVF and AACF, respectively. These were then reacted with myoglobin aldehyde **15** resulting in desired protein bioconjugation products, **19** (66%) and **20** (83%), respectively, when using O-tBu-Thr as organocatalyst in place of proline. Peptide ketones gave high conversion to bioconjugated products with

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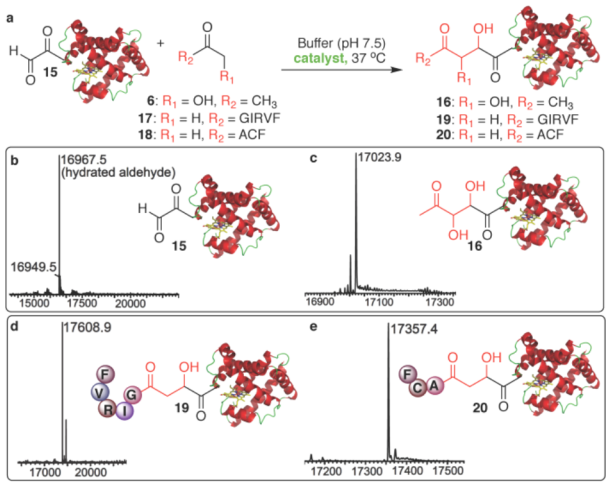

Figure 4. Organocatalyzed protein modification. (a) Ligation of N-terminal myoglobin aldehyde **15** (0.15 mM) with different ketones (**6**, **17**, and **18** (10 mM)) using either proline (b, c) or O-tBu-Thr (d, e) (100 mM) as catalysts in 25 mM phosphate buffer (pH 7.5) and up to 15% DMSO at 37 °C. Deconvoluted ESI-MS spectra of (b) unconjugated myoglobin aldehyde **15**, (c) myoglobin hydroxyacetone bioconjugate **16** generated by reaction with **6**, and (d and e) myoglobin–peptide bioconjugates **19** (60%) and **20** (40%) generated by reaction with peptide ketones GIRVF **17** and ACF **18**.

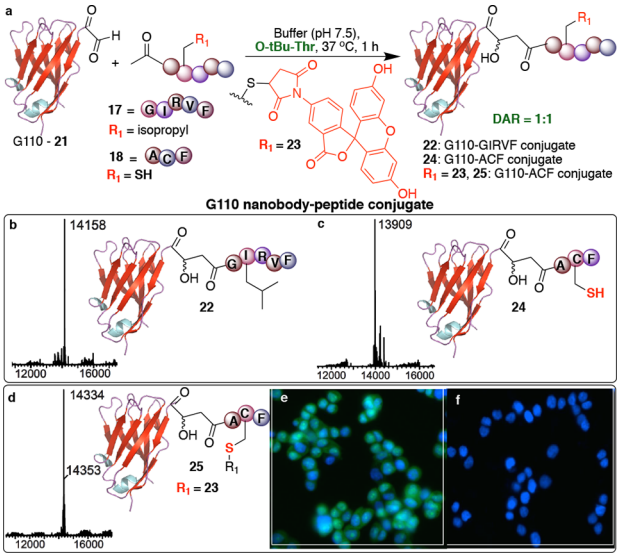

Figure 5. Nanobody–drug conjugates. (a) Synthesis of nanobody–drug conjugates (NDCs) at 1:1 drug-to-nanobody ratio (DNR). Reaction conditions: G110 **21** (0.15 mM), ketones **17** or **18** (10 mM), O-tBu-Thr (100 mM) in 25 mM phosphate buffer, pH 7.5, and up to 15% DMSO at 37 °C. Deconvoluted ESI-MS spectra of (b) NDC **22** (66%), (c) NDC **24** (83%), and (d) dual labeled NDC **25** (99%). (e) Fluorescent imaging with **25** indicates that these conjugates bind to cell surface receptors. (f) Control reaction: cells treated with dye but no nanobody or nanobody lacking dye do not fluoresce.

O-tBu-Thr as compared to proline (see Supporting Information). Further, reaction of myoglobin aldehyde with drug, FITC, and biotin ketones (see Supporting Information) demonstrated the flexibility of this approach with various ketones which is in contrast to known bioconjugation techniques, where reaction works with only one type of ketone.[13]

Antibody–drug conjugates (ADCs) are utilized for targeted drug delivery, and the synthesis of homogeneous conjugates is critical for both the safety and efficacy of this therapy.[17] We aimed to conjugate a peptide ketone, GIRVF **17**, to a single domain antibody G110. Single domain antibodies, also referred to as nanobodies, have been shown to be equally specific as normal antibodies and in some cases more bioactive.[18,19] We chose a nanobody for conjugation because of their small size, robust stability, and simpler expression and purification in *E. coli* cells. The bioconjugation of **17** to nanobody G110 containing an N-terminal aldehyde **21** was catalyzed by O-tBu-Thr and produced homogeneous nanobody-drug conjugate NDC **22** (1:1) with 66% conversion (Figure 5).

The consistent nanobody–peptide conjugate ratio is in contrast to some other bioconjugation techniques generating heterogeneous mixtures of ADCs with variable drug to antibody ratio (DAR).[20] The mild conditions required for this bioconjugation method maintained the structurally critical disulfide bond in G110, which was confirmed by reaction with a maleimide dye **23** that resulted in no cysteine modification. While traditional ADCs use a variety of linkers to attach drug molecules to antibodies, our method has the capability of being linker-free.

Modification of proteins in the presence of free cysteines is particularly challenging due to the strong nucleophilicity of the thiol group. To assess the compatibility of our approach with free cysteines, we synthesized peptide ketone ACF **18** and conjugated it to **21**, which formed nanobody–peptide conjugate **24** (83% conversion, Figure 5c). Cys remained unreacted and allowed for further ligation with a maleimide dye **23** generating multiply labeled G110 **25** (Figure 5d). Fluorescence microscopy showed that this labeled G110 nanobody **25** can bind to an EGF receptor-expressing breast cancer cell line, MDA-MB468, which confirms the stability of aldol bioconjugates in cellular assays (Figure 5e). Thus, this method can be used when the chemistry for Cys modification is not compatible or for applications that require specific labeling at multiple locations through orthogonal methods.

In all the above experiments, a peptide or protein with an electrophilic N-terminal aldehyde was conjugated to a small molecule containing a nucleophilic ketone, also reported by Spears and colleagues while our manuscript was under review.[21] We report that these roles can be reversed. A ketone was generated on protein α-lactalbumin **26** containing glutamic acid at the N-terminus, followed by reaction with 2-pyridine carboxyaldehyde (2-PCA) **27** and peptide aldehyde VF **9** using O-tBu-Thr as organocatalyst, yielding aldol bioconjugates **28** and **29** (57%), respectively (Figure 6). Notably, the resulting aldol bioconjugate **28** further underwent dehydration to the more stable α,β-unsaturated enone **30** (50%) in 1 h. The condensation product was only observed with 2-PCA **27** presumably due to stabilization of the resulting double bond by conjugation with the aromatic ring of 2-PCA. Further, reaction with other peptide ketones and aldehydes (see Supporting Information) demonstrated the broad applicability of this approach for modification of different proteins under mild conditions.

We note that the ease of utilizing amino acids as catalysts makes them particularly attractive for routine modification of biomolecules for application in chemical biology, in medicine, and for generating peptide–polymer conjugates in material science. Here, we demonstrated an organocatalyzed bioconju-

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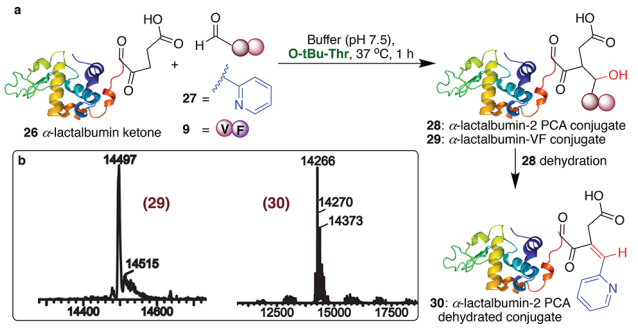

Figure 6. Protein ketone with aldehydes 2-PCA **27** and VF **9**. (a) Formation of α-lactalbumin conjugates **29** and **30**. (b) ESI-MS of α-lactalbumin conjugates **29** and **30**.

gation strategy that proceeds in aqueous media under mild temperature and pH conditions while being able to tolerate a wide variety of reactive functional groups and leads to formation of a stable C−C bond at the site of conjugation. Additionally, this method demonstrates excellent selectivity and broad scope for modification of a vast majority of proteins with different amino acids at the N-terminus by converting them into either aldehyde or ketone handles. Importantly, these amino-acid-based organocatalysts catalyze the bioconjugation reaction efficiently, independent of the nature of proteins as an electrophile or nucleophile. No such role reversal was reported for the existing bioconjugation approaches.[21] Furthermore, the compatibility of this reaction with free cysteine groups was utilized for dual labeling of proteins for cellular imaging. Thus, this method has the potential for synthesizing multiple conjugated biomolecules for various biophysical studies and drug delivery.

The potential of this method to generate chiral centers at the site of conjugation is of high importance in drug discovery since stereoselectivity is crucial for the active binding conformation.[12] A full investigation in this regard is currently underway in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b02265.

Supporting figures, experimental procedures, and analytical data for new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) McKay, C. S.; Finn, M. G. *Chem. Biol.* **2014**, *21*, 1075−1101.
(2) Zheng, M.; Zheng, L.; Zhang, P.; Li, J.; Zhang, Y. *Molecules* **2015**, *20*, 3190−3205.
(3) Shih, H.-W.; Prescher, J. A. *J. Am. Chem. Soc.* **2015**, *137*, 10036−10039.
(4) (a) Geoghegan, K. F.; Stroh, J. G. *Bioconjugate Chem.* **1992**, *3*, 138−146. (b) Witus, L. S.; Francis, M. *Curr. Protoc. Chem. Biol.* **2010**, *2*, 125−134. (c) Witus, L. S.; Netirojjanakul, C.; Palla, K. S.; Muehl, E. M.; Weng, C. H.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2013**, *135*, 17223−17229.
(5) Dirksen, A.; Dirksen, S.; Hackeng, T. M.; Dawson, P. E. *J. Am. Chem. Soc.* **2006**, *128*, 15602−15603.
(6) Dirksen, A.; Hackeng, T. M.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 7581−7584.
(7) (a) Wendeler, M.; Grinberg, L.; Wang, X.; Dawson, P. E.; Baca, M. *Bioconjugate Chem.* **2014**, *25*, 93−101. (b) Kalia, J.; Raines, R. T. *Angew. Chem., Int. Ed.* **2008**, *47*, 7523−7526.
(8) Sasaki, T.; Kodama, K.; Suzuki, H.; Fukuzawa, S.; Tachibana, K. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4550−4553.
(9) Alam, J.; Keller, T. H.; Loh, T.-P. *Chem. Commun.* **2011**, *47*, 9066−9068.
(10) Han, M. J.; Xiong, D. C.; Ye, X. S. *Chem. Commun.* **2012**, *48*, 11079−11081.
(11) Tang, Z.; Yang, Z.-H.; Cun, L.-F.; Gong, L.-Z.; Mi, A.-Q.; Jiang, Y.-Z. *Org. Lett.* **2004**, *6*, 2285−2287.
(12) Nguyen, L. A.; He, H.; Pham-Huy, C. *Int. J. Biomed. Sci.* **2006**, *2*, 85−100.
(13) Alam, J.; Keller, T. H.; Loh, T. *J. Am. Chem. Soc.* **2010**, *132*, 9546−9548. (b) Wang, P.; Zhang, S.; Meng, Q.; Liu, Y.; Shang, L.; Yin, Z. *Org. Lett.* **2015**, *17*, 1361−1364.
(14) Raj, M.; Singh, V. K. *Chem. Commun.* **2009**, 6687−6703.
(15) Byeon, J.-Y.; Limpoco, F. T.; Bailey, R. C. *Langmuir* **2010**, *26*, 15430−15435.
(16) Galkin, A.; Li, Z.; Li, L.; Kulakova, L.; Pal, L. R.; Dunaway-Mariano, D.; Herzberg, O. *Biochemistry* **2009**, *48*, 3186−3196.
(17) Agarwal, P.; Bertozzi, C. R. *Bioconjugate Chem.* **2015**, *26*, 176−192.
(18) Harmsen, M. M.; De Haard, H. *Appl. Microbiol. Biotechnol.* **2007**, *77*, 13−22.
(19) Siontorou, C. G. *Int. J. Nanomed.* **2013**, *8*, 4215−4227.
(20) Kim, M. T.; Chen, Y.; Marhoul, J.; Jacobson, F. *Bioconjugate Chem.* **2014**, *25*, 1223−1232.
(21) Spears, R. J.; Brabham, R. L.; Budhadev, D.; Keenan, T.; McKenna, S.; Walton, J.; Brannigan, J. A.; Brzozowski, A. M.; Wilkinson, A. J.; Plevin, M.; Fascione, M. A. *Chem. Sci.* **2018**, *9*, 5585−5593.

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