JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Rapid Ligations with Equimolar Reactants in Water with the Potassium Acyltrifluoroborate (KAT) Amide Formation

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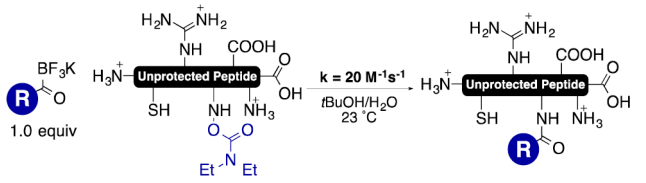
Supporting Information

ABSTRACT: The identification of fast, chemoselective bond-forming reactions is one of the major contemporary challenges in chemistry. We show that chemoselective amide-forming ligations of potassium acyltrifluoroborates (KATs) and *O*-carbamoylhydroxylamines proceed in the presence of all unprotected functional groups with a second-order rate constant of 20 M^{-1} s^{-1}. PEG chains, lipids, biotin, and dyes were introduced onto an unprotected 31-mer peptide (a GLP-1 analogue) with equimolar ratios of reactants within minutes at 1 mM and within 1 h at 100 μM, even with M_w 20 000 PEG. This conjugation reaction provides a new approach to the synthesis of molecules such as protein−protein and protein−polymer conjugates.

The ideal chemoselective conjugation reaction would allow rapid, covalent-bond formation between two unique but chemically stable moieties under aqueous conditions using equimolar amounts of the ligation partners, regardless of the size of the substrate or number and nature of the unprotected functional groups. Chemical reactions that come close to this ideal, such as the Cu-catalyzed Click reaction of azides and alkynes[1] or the addition of thiols to electrophiles, have transformed the practice of organic and bioorganic chemistry.[2] Limitations of these methods include the presence of toxic reagents, the formation of unnatural bonds, and relatively slow reaction rates necessitating the use of an excess of one of the reactants. The remaining challenge in this area is the identification of faster ligations (second-order rate constants >10 M^{-1} s^{-1}) that form natural bonds under aqueous conditions without added reagents or catalysts.[3] Such feats of bond construction, such as DNA ligation,[4] are routinely accomplished by specific biochemical enzymes and can, in certain circumstances, be co-opted for synthetic applications. Fast, selective, natural-bond forming chemical ligations are currently unknown.

Here we show that the chemoselective amide-bond forming reaction of potassium acyltrifluoroborates (KATs)[5] and *O*-carbamoylhydroxylamines tolerates all common unprotected functional groups and forms amide bonds under aqueous conditions with a second-order rate constant of 20 M^{-1} s^{-1} (Scheme 1). This reaction enables selective conjugations of large molecules using equimolar amounts of reactants, opening an avenue to the synthesis of much larger, structurally defined molecules including multidomain proteins, protein−polymer conjugates,[6] and oligomeric biomolecules.[7]

Scheme 1. Chemoselective Amide-Formation with an Equimolar Ratio of Reactants by KAT Ligation



We have previously documented chemical protein synthesis[8] using the α-ketoacid-hydroxylamine (KAHA) amide-forming ligation, which proceeds chemoselectively under aqueous conditions.[9] Although this reaction works well for the assembly of peptide segments, the comparatively slow reaction rate[10] requires higher temperatures (40−60 °C), relatively long reaction times (8−24 h) and reactant concentrations of at least 5 mM, which is not always feasible for higher molecular weight substrates. As part of our search for alternative amide-forming reactions,[11] we recently reported coupling of *O*-Bz hydroxylamines and KATs in *t*BuOH/H_2O, but our study was limited to small organic molecules lacking unprotected functional groups.[12] KATs showed rapid amide formations along with excellent stability, prompting us to consider developing this reaction as a solution to the long-standing problem of rapid, chemoselective conjugation of stoichiometric quantities of substrates under mild aqueous conditions.

The *O*-Bz hydroxylamines used in our first report were not sufficiently stable toward standard synthetic protocols[13] and could not be easily incorporated into peptides or other synthetic targets. We therefore evaluated a panel of hydroxylamine derivatives for stability and reactivity in the amide-forming reaction (Table 1). This led to our selection of *N*,*N*-diethylcarbamate **1i**, a derivative that was almost as reactive as the *O*-Bz substrates. Importantly, it was stable toward the ligation conditions and unprotected primary amines, tolerant of acidic (TFA) deprotection of Boc and related protecting groups, and compatible to Fmoc-cleavage with piperidine in its Boc-protected form.

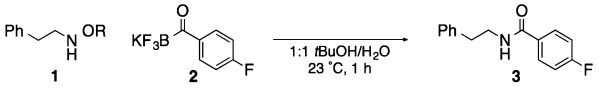
In order to properly assess the kinetics and chemoselectivity of this ligation, we prepared a β-alanine derived monomer containing the *O*-diethylcarbamoyl-hydroxylamine and attached this to the N-terminus of a model peptide. The sequence was chosen to include unprotected amines (Lys), thiols (Cys), and carboxylic acids (Glu/Asp), which are the functional groups

Received: February 26, 2014
Published: March 31, 2014

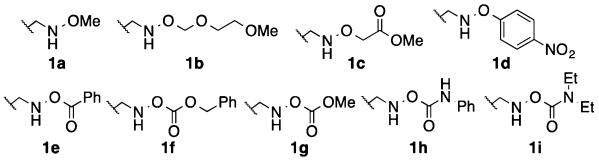
dx.doi.org/10.1021/ja5018442 | *J. Am. Chem. Soc.* 2014, 136, 5611−5614

Table 1. Determination of Suitable Ligation Partners

A) Reactions of hydroxylamine 1 and KAT 2.



B) *O*-Substituted hydroxylamines evaluated.



C) A chart of reactivity and stability of 1.

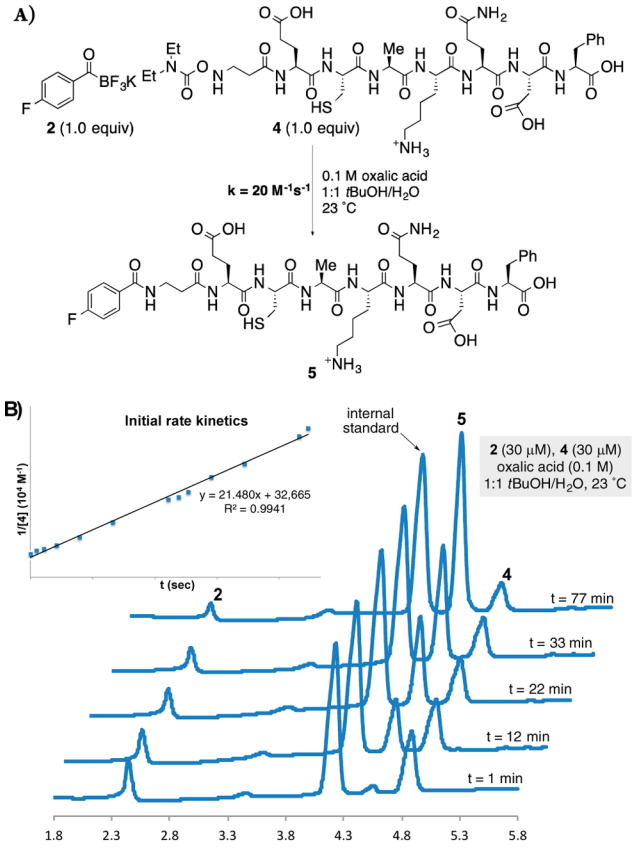
hydroxyl-amine	relative reactivity[a]	stability to 2° amine[b]	stability to 1° amine[c]	stability to acid[d]
1a	NR	–	–	–
1b	NR	–	–	–
1c	NR	–	–	–
1d	14	–	–	–
1e	100	no	no	yes
1f	117	no	no	no
1g	124	no	no	no
1h	78	no	no	no
1i	87	yes	yes	yes

[a]1:1 *t*BuOH/H_2O, 23 °C, 1h. [b]*N*-Boc derivatives of **1** were used as substrates. 20% piperidine in DMF, 23 °C, 24 h. [c]2-Phenylethylamine (10 equiv), DMF, 23 °C, 1h. [d]50% TFA in CH_2Cl_2, 23 °C, 3 h. NR; no reaction.

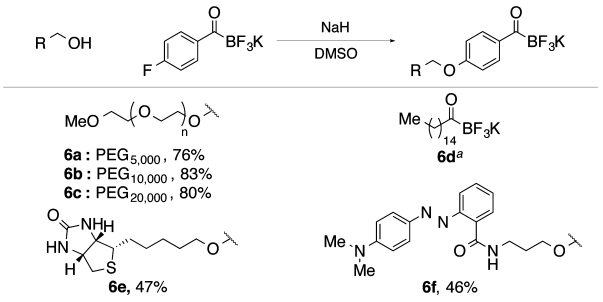
most likely to be encountered in applications of a chemoselective ligation (Scheme 2A). Due to the fast reaction rates, we performed studies on the second-order kinetics using a 1:1 mixture of KAT **2** and peptide **4** at 30 μM at 23 °C in 1:1 *t*BuOH/H_2O. Under these dilute conditions, we could accurately monitor the conversion to product and the disappearance of the starting materials by HPLC (Scheme 2B). At higher concentrations (100 μM) the ligations were complete within 1 h, but the reaction was too fast for accurate monitoring of the initial rate. We also found that the ligations are faster at lower pH and added oxalic acid for these studies; other acids such as phosphoric acid are also suitable. We determined a second-order rate constant of 20 M^{-1} s^{-1}, considerably faster than the majority of ligation reactions in common use.[14] It does not approach the reported rates of the tetrazine–*trans*-cyclooctene ligation[15] but has the distinct advantage of using chemically stable, easily handled functional groups and forming an amide bond.[16] We anticipate that further development of the reaction partners will lead to faster rates and ligations under neutral conditions, but even at the current stage of development the reaction is fast enough for stoichiometric ligations at <50 μM with reasonable times.

In order to easily prepare KATs for this ligation, we took advantage of the strong electron-withdrawing nature of the potassium acyltrifluoroborate moiety and devised a convenient approach for attaching PEG chains of various molecular weights,[17] biotin,[18] and dyes[19] by nucleophilic aromatic substitution (Scheme 3). This proved particularly convenient for the preparation of PEG KATs **6a**–**6c**; the desired product could be separated from excess **2** by dialysis to give pure PEGylated reagents without any additional purification. Lipid-derived KAT **6d** was prepared by a modification of our previous method.[20] Ongoing work in our laboratories and others will

Scheme 2. KAT ligation of 2 with model peptide 4



Scheme 3. Syntheses of KAT 6 via S_NAr of 2



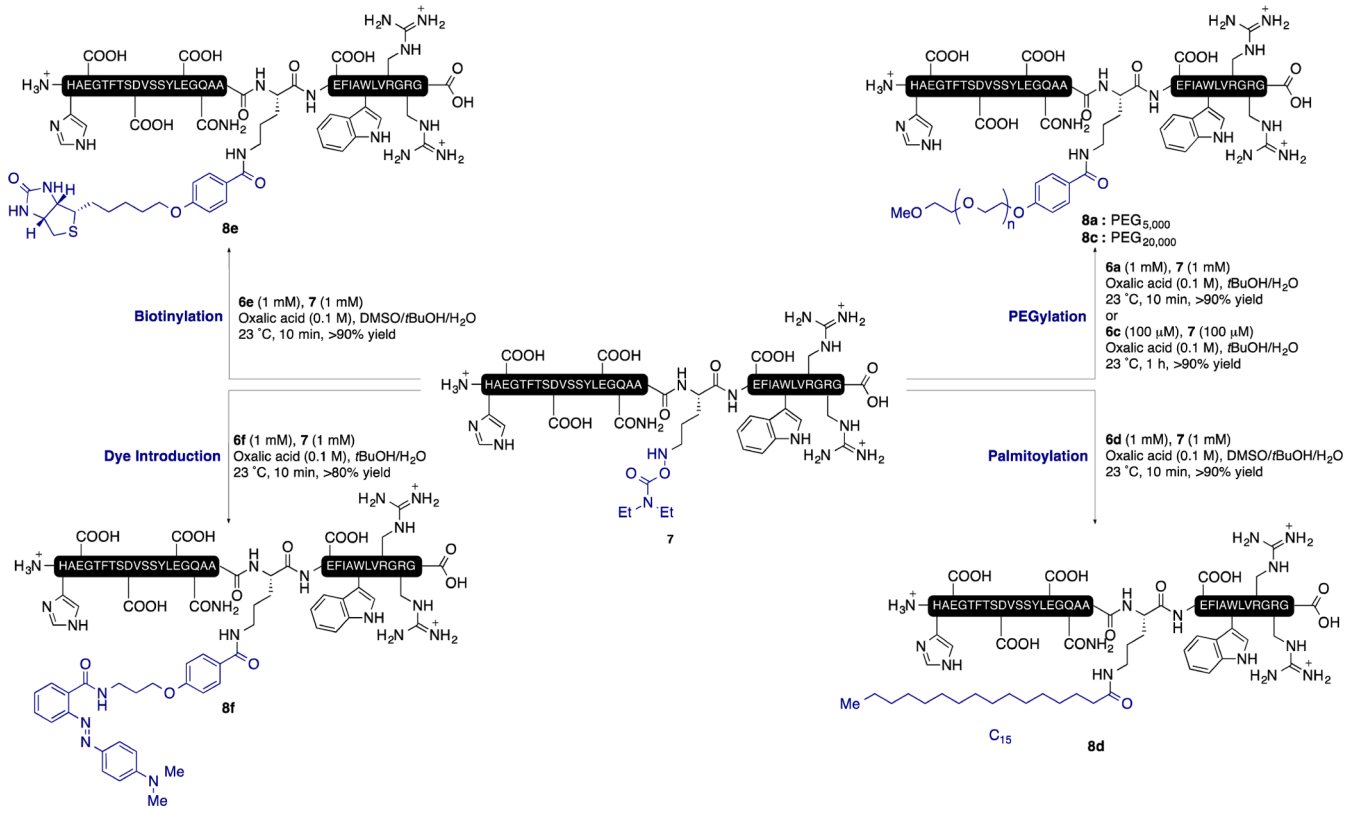
[a]**6d** was prepared by a modification of the reported procedure. See Supporting Information for details.

further simplify the preparation of other KATs in the near future.

The use of this ligation for synthetic conjugations also requires a means to prepare complex molecules containing the requisite hydroxylamine functionality. Given our interest in fully synthetic constructs such as PEGylated peptides[21] and synthetic protein–protein conjugates, we first targeted the preparation of peptides containing the hydroxylamine as a side-chain functional group. We selected a 31-amino acid residue analogue of the antidiabetic peptide GLP-1 as a first target, as conjugations to these peptides are a critical and troublesome step in the manufacture and development of new treatments.[22] Several PEGylated variants are in development and a lipidated form, liraglutide, is an approved and marketed drug.[23] Improved methods for conjugating molecules to fully unprotected peptides or proteins are in great demand for

dx.doi.org/10.1021/ja5018442 | J. Am. Chem. Soc. 2014, 136, 5611–5614

Scheme 4. Chemoselective Ligations of Unprotected GLP-1 Analogue with Equimolar Reactants



both synthetic and semisynthetic therapeutics, particularly as some of the PEGs and other conjugates can be even more expensive than the peptide or protein they are meant to modify. The ideal conjugation reaction would allow the use of 1:1 stoichiometry of the two reagents, proceed rapidly under aqueous conditions, tolerate all unprotected functional groups, and operate without toxic reagents or byproducts. We were pleased to find that the KAT ligation fulfills all of these criteria and offers the further advantage of forming a stable, biocompatible amide bond.

Synthetic GLP-1 analogue **7**[24] (1.0 equiv) was allowed to react with KATs **6a**-**6f** (1.0 equiv) at either 1 mM or 100 μM in H_2O (Scheme 4). In all cases, clean conversion to the desired product was observed in <1 h, even when M_w 20 000 PEG reagent **6c** was used. KAT ligations are accelerated in aqueous solvents and are not affected by the nature or presence of organic cosolvents. We added DMSO as a cosolvent in the reactions with **6d** and **6e** due to the low solubilities of these KATs. Both ligations proceeded smoothly with essentially the same rate as other cases. The presence of water, however, is essential; under strictly anhydrous conditions the rate of the ligation slows dramatically.

The synthesis of large molecules (M_w >5000) bearing many types of diverse functional group is a frontier of synthetic organic chemistry.[25] The limits on size, complexity, and stoichiometry arise from the inherently slow reaction rates or poor starting material stability of most organic reactions suitable for selective bond formation in the presence of unprotected functional groups.

The KAT ligation offers a natural-bond forming ligation fast enough (20 M^{-1} s^{-1}) for synthetic approaches to protein engineering, biomolecule conjugates,[26] functional polymers, and the formation of oligomers of complex molecules. Further work on the preparation of proteins bearing the hydroxylamines and KAT moieties, by both synthetic and semisynthetic approaches, is in progress and is aided by the high stability of both of these functional groups to aqueous conditions, HPLC purification, and standard reagents.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): ETH Zürich has filed a patent application for the use of this technology for PEGylation.

■ ACKNOWLEDGMENTS

G.E. was supported by a Novartis Postdoctoral Fellowship. We thank the LOC MS Service for analyses and Safwan Aroua, Jessada Mahatthananchai, and Aaron Dumas for helpful discussions. This work was supported by ETH Zürich and an ETH Research Grant (ETH-43 13−2).

■ REFERENCES

(1) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596.
(2) Finn, M. G.; Fokin, V. V. *Chem. Soc. Rev.* **2010**, *39*, 1231.
(3) Kalia, J.; Raines, R. T. *Curr. Org. Chem.* **2010**, *14*, 138.

(4) For a recent review on this topic: Gorska, K.; Winssinger, N. *Angew. Chem., Int. Ed.* **2013**, *52*, 6820.
(5) Molander, G. A.; Raushel, J.; Ellis, N. M. *J. Org. Chem.* **2010**, *75*, 4304.
(6) (a) Broyer, R. M.; Grover, G. N.; Maynard, H. D. *Chem. Commun.* **2011**, *47*, 2212. (b) Jung, B.; Theato, P. *Adv. Polym. Sci.* **2013**, *253*, 37. (c) Kochendoerfer, G. G.; Chen, S.-Y.; Mao, F.; Cressman, S.; Traviglia, S.; Shao, H.; Hunter, C. L.; Low, D. W.; Cagle, E. N.; Carnevali, M.; Gueriguian, V.; Keogh, P. J.; Porter, H.; Stratton, S. M.; Wiedeke, M. C.; Wilken, J.; Tang, J.; Levy, J. J.; Miranda, L. P.; Crnogorac, M. M.; Kalbag, S.; Botti, P.; Schindler-Horvat, J.; Savatski, L.; Adamson, J. W.; Kung, A.; Kent, S. B. H.; Bradburne, J. A. *Science* **2003**, *299*, 884.
(7) (a) Hutchins, B. M.; Kazane, S. A.; Staflin, K.; Forsyth, J. S.; Felding-Habermann, B.; Smider, V. V.; Schultz, P. G. *Chem. Biol.* **2011**, *18*, 299. (b) Hudak, J. E.; Barfield, R. M.; de Hart, G. W.; Grob, P.; Nogales, E.; Bertozzi, C. R.; Rabuka, D. *Angew. Chem., Int. Ed.* **2012**, *51*, 4161.
(8) (a) Dawson, P. E.; Kent, S. *Annu. Rev. Biochem.* **2000**, *69*, 923. (b) Raibaut, L.; Ollivier, N.; Melnyk, O. *Chem. Soc. Rev.* **2012**, *41*, 7001. (c) Hemantha, H. P.; Narendra, N.; Sureshbabu, V. V. *Tetrahedron* **2012**, *68*, 9491.
(9) (a) Pattabiraman, V. R.; Ogunkoya, A. O.; Bode, J. W. *Angew. Chem., Int. Ed.* **2012**, *51*, 5114. (b) Ogunkoya, A. O.; Pattabiraman, V. R.; Bode, J. W. *Angew. Chem., Int. Ed.* **2012**, *51*, 9693.
(10) Pusterla, I.; Bode, J. W. *Angew. Chem., Int. Ed.* **2012**, *51*, 513.
(11) (a) Bode, J. W.; Sohn, S. S. *J. Am. Chem. Soc.* **2007**, *129*, 13798. (b) Schäfer, G.; Matthey, C.; Bode, J. W. *Angew. Chem., Int. Ed.* **2012**, *51*, 9173.
(12) Dumas, A. M.; Molander, G. A.; Bode, J. W. *Angew. Chem., Int. Ed.* **2012**, *51*, 5683.
(13) Knowles, D. A.; Mathews, C. J.; Tomkinson, N. C. O. *Synlett* **2008**, 2769.
(14) Dirksen, A.; Dawson, P. E. *Bioconjugate Chem.* **2008**, *19*, 2543.
(15) (a) Blackman, M. L.; Royzen, M.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 13518. (b) Taylor, M. T.; Blackman, M. L.; Dmitrenko, O.; Fox, J. M. *J. Am. Chem. Soc.* **2011**, *133*, 9646.
(16) (a) Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105. (b) Kalia, J.; Raines, R. T. *Angew. Chem., Int. Ed.* **2008**, *47*, 7523. Forming an amide bond makes Staudinger ligation a popular choice for *in vivo* study in spite of its slow kinetics: Patterson, D. M.; Nazarova, L. A.; Prescher, J. A. *ACS Chem. Biol.* **2014**, *9*, 592.
(17) For reviews on PEGylation on pharmaceuticals: (a) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug. Discovery* **2003**, *2*, 214. (b) Kang, J. S.; DeLuca, P. P.; Lee, K. C. *Expert Opin. Emerg. Drugs* **2009**, *14*, 363.
(18) For a review on biotinylation: Trippier, P. C. *Chem. Med. Chem.* **2013**, *8*, 190.
(19) For a review on use of dyes in chemical biology: Gonçalves, M. S. T. *Chem. Rev.* **2009**, *109*, 190.
(20) Dumas, A. M.; Bode, J. W. *Org. Lett.* **2012**, *14*, 2138.
(21) For selected examples of site-specific PEGylation: (a) Deiters, A.; Cropp, T. A.; Summerer, D.; Mukherji, M.; Schultz, P. G. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5743. (b) Scheck, R. A.; Francis, M. B. *ACS Chem. Biol.* **2007**, *2*, 247. (c) Debets, M. F.; van Berkel, S. S.; Schoffelen, S.; Rutjes, F. P. J. T.; van Hest, J. C. M.; van Delft, F. L. *Chem. Commun.* **2010**, *46*, 97. (d) Serwa, R.; Majkut, P.; Horstmann, B.; Swiecicki, J.-M.; Gerrits, M.; Krause, E.; Hackenberger, C. P. R. *Chem. Sci.* **2010**, *1*, 596. (e) Dumas, A.; Spicer, C. D.; Gao, Z.; Takehana, T.; Lin, Y. A.; Yasukohchi, T.; Davis, B. G. *Angew. Chem., Int. Ed.* **2013**, *52*, 3916.
(22) Payne, R. W.; Murphy, B. M.; Manning, M. C. *Pharm. Dev. Technol.* **2011**, *16*, 423.
(23) Drucker, D. J.; Dritselis, A.; Kirkpatrick, P. *Nat. Rev. Drug Discovery* **2010**, *9*, 267.
(24) For the structure comparison with related peptides, see Supporting Information.
(25) (a) Hawker, C. J. *Science* **2005**, *309*, 1200. (b) Pattabiraman, V. R.; Bode, J. W. *Nature* **2011**, *480*, 471.
(26) (a) Hemantha, H. P.; Brik, A. *Bioorg. Med. Chem.* **2013**, *21*, 3411. (b) Haj-Yahya, M.; Fauvet, B.; Herman-Bachinsky, Y.; Hejjaoui, M.; Bavikar, S. N.; Karthikeyan, S. V.; Ciechanover, A.; Lashuel, H. A.; Brik, A. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 17726.