Organic LETTERS

Letter

pubs.acs.org/OrgLett

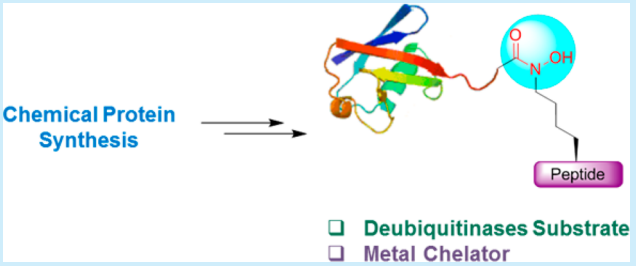
Synthesis of *N*-Hydroxy Isopeptide Containing Proteins

Suman Kumar Maity, Shimrit Ohayon, and Ashraf Brik*

Schulich Faculty of Chemistry Technion—Israel Institute of Technology, 3200008 Haifa, Israel

Supporting Information

ABSTRACT: Chemical synthesis of a peptide−ubiquitin conjugate linked by an *N*-hydroxy isopeptide bond to determine what effect the *N*-hydroxy group has on the enzymatic hydrolysis of the isopeptide linkage by deubiquitinases is reported. This conjugate was subjected to proteolysis by UCH-L3 in the presence and absence of various metal ions, and no substantive difference in hydrolysis was seen compared to a control lacking the *N*-hydroxy group. The accessibility of *N*-hydroxy ubiquitinated substrates may find uses to study other deubiquitinases in particular those which use a zinc ion as a part of their catalytic mechanism.



The ability to fine-tune the atomic structure of peptides and proteins enables researchers to introduce new properties into these macromolecules that are important in various research areas. Compared to proteins, peptides and peptidomimetics are more amenable for such modifications because of their smaller size and relatively straightforward synthesis. For example, peptidomimetics such as siderophores, which contain *N*-hydroxy amide bonds as chelators of Fe(III) ions to assist cell membrane transport in microorganisms,[1] have been synthesized and extensively studied. *N*-Hydroxy amide containing peptides and peptidomimetics, which exhibit excellent inhibition properties for metalloproteases such as thermolysin,[2] methionine aminopeptidase,[3] leukotriene A4 hydrolase,[4] encephalin degrading enzyme,[5] and peptide deformylase,[6] have also been synthesized and studied. However, to the best of our knowledge, the synthesis of proteins containing *N*-hydroxy amide has not been reported so far. Here we report on the synthesis of ubiquitinated peptides bearing a *N*-hydroxy isopeptide bond to examine the effect of this modification on the cleavage of this bond by known proteases.

It has been reported that the *N*-hydroxy amide inserted in short peptides exhibits different stability to enzymatic hydrolysis compared to the native amide.[7] We reasoned that the introduction of this moiety into the isopeptide bond linking peptides or proteins through the Lys side chain to the C-terminal of ubiquitin or ubiquitin-like modifiers (e.g., SUMO) could in principle influence the electronic and conformational[8] properties of the isopeptide bond. As a result this might affect the enzymatic hydrolysis by deubiquitinases (DUBs), which are known to cleave the isopeptide bond.[9] To test this hypothesis we set the goal of preparing a short peptide linked to ubiquitin (Figure 1), applying state-of-the-art chemical protein synthesis approaches.

Several methods have been reported for the synthesis of *N*-hydroxy amide containing peptides (Supporting Information, Scheme S1). For example, acylation of free amine of a tripeptide by *N*-benzyloxy-*N*-carboxy α-amino acid anhydrides afforded the corresponding *N*-benzyloxytetrapeptide.[10] *N*-Hydroxy glycine residue was incorporated in the peptide backbone by coupling of bromoacetic acid on a solid support followed by nucleophilic substitution by *O*-benzylhydroxylamine and subsequent peptide elongation.[7a] In another report, it was found that acylation of *N*-(benzoyloxy) phenethylamine with the acid chloride of N^{α}-Fmoc-L-leucine provided an N^{α}-Fmoc-*N*-(benzoyloxy)-L-leucinamide, where the free hydroxyl can be achieved by benzoyl group deprotection.[11] Coupling of α-amino acids with α-keto acyl chlorides was found to produce the corresponding α-keto amides, which upon condensation with hydroxylamine gave the oxime bond. It has also been reported that reduction of oxime followed by selective *N*-acylation with Fmoc-protected amino acid chlorides afforded the *N*-hydroxy peptide linkage.[12] Bode and co-workers reported the synthesis of enantiopure 5-oxaproline and *N*-benzylidene nitrone protected *N*-hydroxyamino acids, which were readily incorporated in peptides by solid phase peptide synthesis (SPPS) and used in chemical protein synthesis.[13] In addition, the ε-amine of lysine has been hydroxylated by oxidation with dibenzoyl peroxide followed by acylation with formic acid.[14]

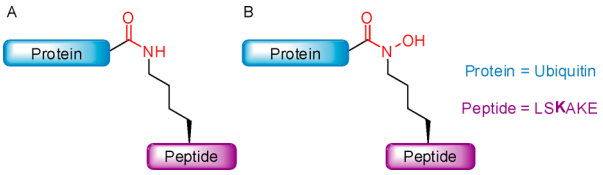


Figure 1. Schematic presentation of a short peptide linked to protein (e.g., ubiquitin) through (A) native and (B) *N*-hydroxy isopeptide bonds.

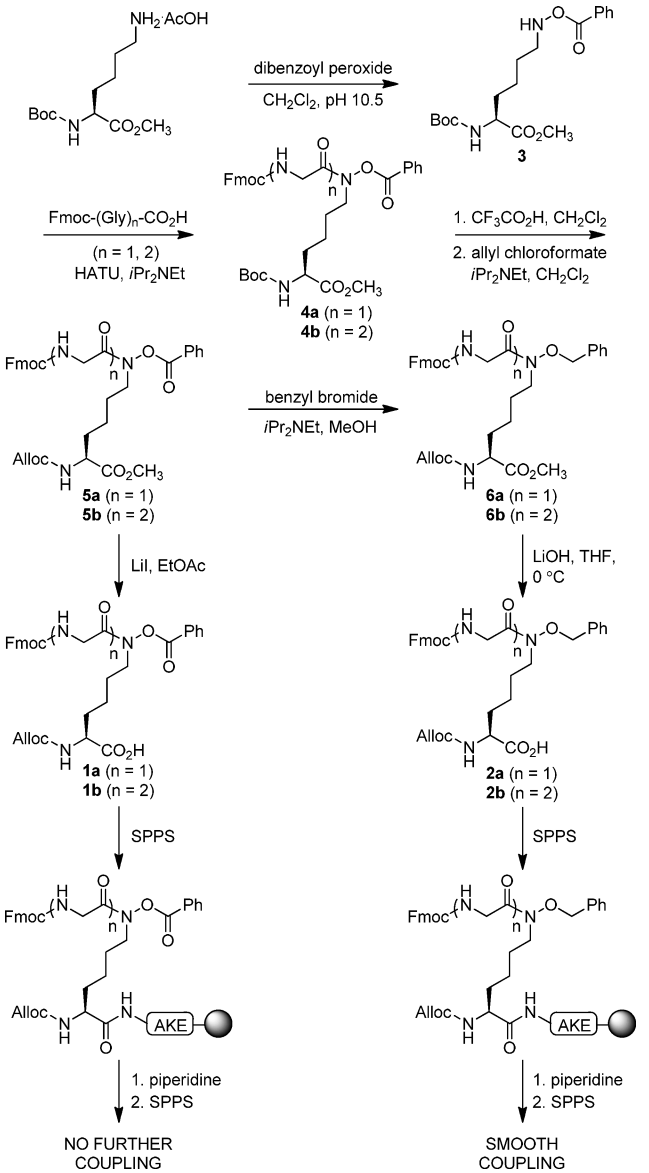
Received: May 17, 2015

DOI: 10.1021/acs.orglett.5b01443
Org. Lett. XXXX, XXX, XXX−XXX

Despite the fact that these methods have been successfully exercised on peptides, their extension to proteins is not trivial.

In this report, we present the first synthesis of a protein containing a *N*-hydroxy isopeptide bond. We chose ubiquitin as a protein model since the preparation of ubiquitinated peptides with a more labile isopeptide bond might enable the synthesis of more efficient substrates for high throughput screening of inhibitors against DUBs.[15] Moreover, since some DUBs employ a zinc ion as a part of their catalytic apparatus, such conjugates could also be used to trap the catalytic zinc and generate inactive DUB bound to its substrate for a variety of studies. To achieve the preparation of such a conjugate, we synthesized Lys derivative **1a** starting from N^{α}-Boc-Lys-OMe by oxidation of the ε-amine with dibenzoyl peroxide to *N*-(benzoyloxy) amine followed by acylation with Fmoc-Gly-OH using 1-[Bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate (HATU) as a coupling agent (Scheme 1). This derivative was incorporated on a Rink amide resin via conventional Fmoc-SPPS (Scheme 1).

Scheme 1. Synthesis of Peptide Fragment Containing *N*-Hydroxy Isopeptide from Lysine Derivatives



However, in the case of **1a**, we did not observe further coupling on the free amine (Supporting Information, Figure S1).

We then wondered if SPPS would proceed without particular problems subsequent to the incorporation of **1b**, which has two glycine residues at the ε-amine of the Lys residue. In this case, despite the fact that we were able to couple the next amino acids, several unidentified side products were observed as we progressed with the synthesis (Supporting Information, Figures S2 and S3). Moreover, when coupling was performed after 48 h of the Fmoc removal, the majority of the starting material remained unreacted (Supporting Information, Figure S4). We reasoned that the amine was capped by the transfer of the benzoyl protecting group from the *N*-benzoyloxy group to the glycine amine, after the piperidine treatment step (Supporting Information, Scheme S2).[16]

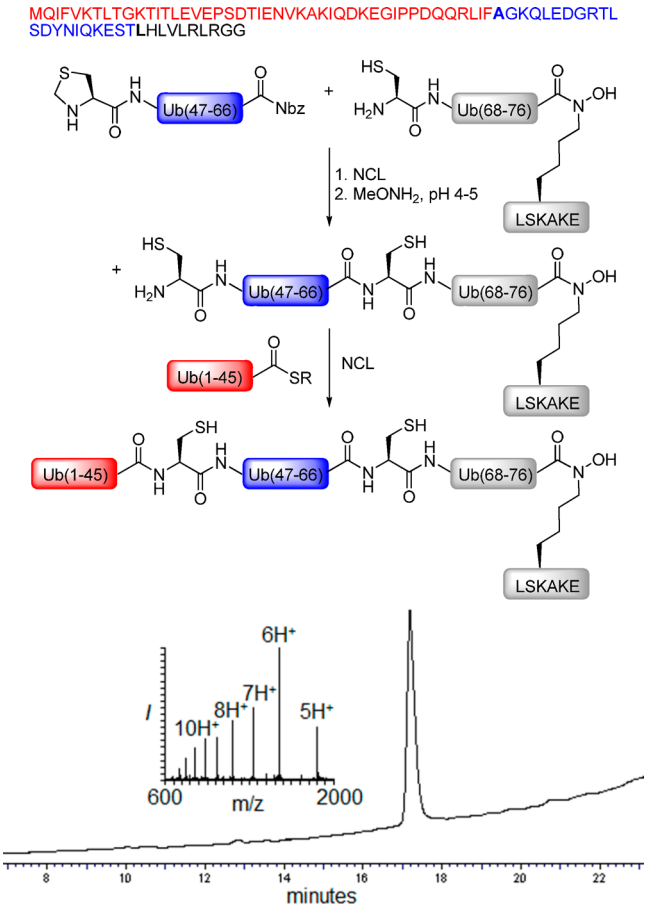
We then changed the benzoyl-protecting group to the benzyl derivative and synthesized the modified Lys with two glycine residues (Scheme 1). The incorporation of a benzyl protecting group was carried out through solvolysis in methanol and *N,N*-diisopropylethylamine (DIEA) in the presence of benzyl bromide (Scheme 1). Compound **2a** was successfully incorporated on the resin and the couplings of the next amino acids were performed smoothly, supporting that the free amine was capped in the case of benzoyl protection. With this approach, we synthesized the Cys-Ub(47−76) fragment with a mutation of Ala46Cys to enable native chemical ligation (NCL)[17] with the complementary Ub(1−45)-thioester. The N^{α}-Alloc protecting group on the backbone peptide was removed followed by coupling of Ser and Leu. Unfortunately, the removal of the benzyl-protecting group using either hydrogenation over Pd/C or trifluoromethanesulfonic acid (TFMSA) was problematic and a major product with a mass that was 16 Da less than that of the desired product was obtained (Supporting Information, Figure S5). This probably occurred due to the removal of an oxygen atom from the *N*-benzyloxy functionality during the deprotection step.

At this point we thought to divide the Cys-Ub(47−76) fragment into two fragments, which could lead to a more facile deprotection. Accordingly, the Cys-Ub(47−76) peptide was divided into two fragments, one containing C-terminal *N*-acyl-benzimidazolinone (Nbz) as well as N-terminal thiazolidine (Thz), Thz-Ub(47−66)-Nbz (Supporting Information, Figure S7), while the other contains the *N*-hydroxy isopeptide bond with N-terminal Cys, Cys-Ub-(68−76), to enable NCL. Fortunately, by using this approach the benzyl removal was successfully achieved employing a mixture of TFMSA/TFA/*m*-cresol (Supporting Information, Figure S6 and Scheme S3). Subsequently, the short peptides were ligated and the N-terminal Thz was unmasked by methoxylamine to produce Cys-Ub(47−76), ready for a second ligation (Scheme 2). This fragment was then ligated with Ub(1−45)-thioester to produce the full ubiquitin with the *N*-hydroxy isopeptide bond (Supporting Information, Figures S8 and S9). The ubiquitinated peptide with the native isopeptide bond, as a control, was also prepared from similar fragments (Supporting Information, Figures S10 and S11).

To check the effect of *N*-hydroxy functionality on the electronic environment of the isopeptide carbonyl bond, we synthesized the LSKAKE peptide linked to the Ub(72−76) fragment with and without the *N*-hydroxy functionality, where the Gly76 residue contained ^{13}C enriched carbonyl (Supporting Information, Figures S12 and S13). The carbonyl from the *N*-

DOI: 10.1021/acs.orglett.5b01443
Org. Lett. XXXX, XXX, XXX−XXX

Scheme 2. Synthesis of *N*-Hydroxy Ubiquitinated Peptide from Three Fragments; HPLC and Mass Spectra of the Pure *N*-Hydroxy Ubiquitinated Peptide with the Observed Average Mass 9298.8 ± 0.6 Da, Calcd 9297.7 Da (Average Isotopes)



hydroxy isopeptide exhibited a δ value of 170.54 ppm, whereas that of the native isopeptide exhibited 172.06 ppm (Figure 2), indicating that the carbon is slightly electron rich compared to the native amide carbon. Next, we started to examine their behavior with known DUBs, and we initially chose to work with UCH-L3 since it is known to cleave small peptides from the C-terminal of ubiquitin. The cleavage reactions were monitored by HPLC following the ubiquitin formation. Testing both substrates revealed that the extent of hydrolysis of the ubiquitinated peptide bearing an *N*-hydroxy isopeptide bond was lower by only 8% compared to the native isopeptide bond (Figure 2C).

Next, we wondered about effect of metal ions binding on the electronic environment of the amide carbonyl and the enzymatic hydrolysis. The ^{13}C NMR of the *N*-hydroxy in the presence of 1 equiv of Zn(II) ion in HEPES buffer showed a large upfield shift in the δ value, from 170.54 to 161.78 ppm, whereas the native isopeptide bond did not show any shift (Figure 2a and 2b).[18] This indicates that due to metal ion binding the carbonyl group of the *N*-hydroxy isopeptide bond becomes more electron rich.

We next studied the effect of Zn(II) ions on the hydrolysis of ubiquitinated peptides containing *N*-hydroxy and native isopeptide bonds by UCH-L3. Surprisingly, with UCH-L3, no difference was observed in the extent of hydrolysis with the

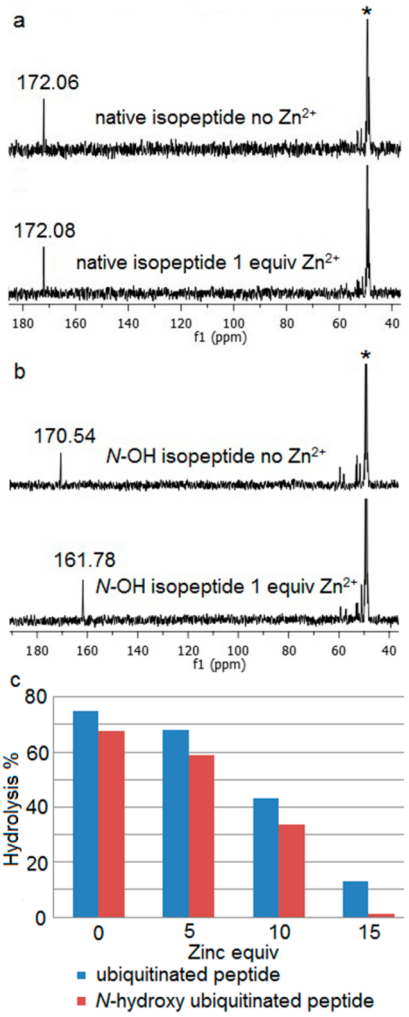


Figure 2. ^{13}C NMR spectra of (a) Ub(72−76)-peptide (b) *N*-hydroxy-Ub(72−76)-peptide with ^{13}C labeled isopeptide bond with and without Zn(II) ions. (*) is the reference peak from methanol-d_4. (c) The extent of hydrolysis of ubiquitinated and *N*-hydroxy ubiquitinated peptides with respect to different equivalents of Zn(II) ions by UCH-L3.

addition of 5 equiv of Zn(II), and only with an increasing amount of Zn(II) ions we observed an additional decrease in the hydrolysis in the case of the *N*-hydroxy ubiquitinated peptide (Figure 2c). This may suggest that Zn(II) ions also bind to ubiquitin (as it contains cysteine and histidine residues) and/or to the enzyme. The excess of Zn(II) ions may harm the activity of the enzyme, as we observed in the case of the ubiquitinated peptide where the extent of hydrolysis was decreased with an increasing amount of zinc. In the case of the *N*-hydroxy ubiquitinted peptide despite the relatively large chemical shift in the NMR upon addition of Zn(II) ions, the extent of hydrolysis was lower only by 13%.[19]

We also studied the effect of Fe(III) ions on the hydrolysis of ubiquitinated and *N*-hydroxy ubiquitinated peptides by UCH-L3, where we observed that 15 equiv of Fe(III) ions had no effect on the hydrolysis (Supporting Information, Figure S16).

The ^{13}C NMR of the labeled peptides in the presence of Ga(III) ions which show similar coordination properties such as Fe(III) ions[20] exhibited no shift in the ^{13}C spectra of the *N*-hydroxy model peptide suggesting that Ga(III) ions were not coordinating with the *N*-hydroxy functionality (Supporting

DOI: 10.1021/acs.orglett.5b01443
Org. Lett. XXXX, XXX, XXX−XXX

Information, Figure S17). Finally, we examined the effect of hydrolysis by UCH-L3 in the presence of Cu(II) ions.[21] In presence of 15 equiv of Cu(II) ions, the ubiquitinated peptide was hydrolyzed to 58% and the *N*-hydroxy ubiquitinated peptide was hydrolyzed to 45% (Supporting Information, Figure S18). This indicated that Cu(II) ions had less effect on ubiquitin and/or enzyme compared to Zn(II) ions, as the extent of hydrolysis with Cu(II) ions was significantly more, compared to the presence of 15 equiv of Zn(II) ions (Supporting Information, Table S1).

In summary, for the first time the synthesis of a *N*-hydroxy isopeptide bond-containing protein was achieved by applying small molecule, peptide, and protein synthesis. The presence of such functionality in the free or metal bound forms did not significantly affect the UCH-L3 activity. Expanding this study to other DUBs is currently underway in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

All experimental procedures, analytical data of synthetic compounds, enzymatic hydrolysis studies of ubiquitinated peptides. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01443.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: abrik@technion.ac.il.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Israel Science Foundation for financial support (A.B.). S.K.M. thanks the Israel Council of Higher Education for a fellowship under the PBC program. A.B. is a Neubauer Professor and a Taub Fellow-Supported by the Taub Foundations.

■ REFERENCES

(1) Drechsel, H.; Jung, G. *J. Pept. Sci.* **1998**, *4*, 147.
(2) Nishino, N.; Powers, J. C. *Biochemistry* **1978**, *17*, 2846.
(3) Hu, X. B.; Zhu, J. G.; Srivathsan, S.; Pei, D. H. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 77.
(4) Hogg, J. H.; Ollmann, I. R.; Wetterholm, A.; Andberg, M. B.; Haeggstrom, J.; Samuelsson, B.; Wong, C. H. *Chem.—Eur. J.* **1998**, *4*, 1698.
(5) Bouboutou, R.; Waksman, G.; Devin, J.; Fourniezaluski, M. C.; Roques, B. P. *Life Sci.* **1984**, *35*, 1023.
(6) Apfel, C.; Banner, D. W.; Bur, D.; Dietz, M.; Hubschwerlen, C.; Locher, H.; Marlin, F.; Masciadri, R.; Pirson, W.; Stalder, H. *J. Med. Chem.* **2001**, *44*, 1847.
(7) (a) Bianco, A.; Zabel, C.; Walden, P.; Jung, G. *J. Pept. Sci.* **1998**, *4*, 471. (b) Bianco, A.; Kaiser, D.; Jung, G. *J. Pept. Res.* **1999**, *54*, 544.
(8) Marraud, M.; Dupont, V.; Grand, V.; Zerkout, S.; Lecoq, A.; Boussard, G.; Vidal, J.; Collet, A.; Aubry, A. *Biopolymers* **1993**, *33*, 1135.
(9) (a) Komander, D.; Clague, M. J.; Urbe, S. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 550. (b) Haj-Yahya, M.; Eltarteer, N.; Ohayon, S.; Shema, E.; Kotler, E.; Oren, M.; Brik, A. *Angew. Chem., Int. Ed.* **2012**, *51*, 11535. (c) Haj-Yahya, N.; Haj-Yahya, M.; Castaneda, C. A.; Spasser, L.; Hemantha, H. P.; Jbara, M.; Penner, M.; Ciechanover, A.; Fushman, D.; Brik, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 11149.
(10) Akiyama, M.; Iesaki, K.; Katoh, A.; Shimizu, K. *J. Chem. Soc., Perkin Trans. 1* **1986**, 851.
(11) Wang, L.; Phanstiel, O. *J. Org. Chem.* **2000**, *65*, 1442.
(12) Lawrence, J.; Cointeaux, L.; Maire, P.; Vallee, Y.; Blandin, V. *Org. Biomol. Chem.* **2006**, *4*, 3125.
(13) (a) Pattabiraman, V. R.; Ogunkoya, A. O.; Bode, J. W. *Angew. Chem., Int. Ed.* **2012**, *51*, 5114. (b) Medina, S. I.; Wu, J. A.; Bode, J. W. *Org. Biomol. Chem.* **2010**, *8*, 3405.
(14) Fennell, K. A.; Miller, M. J. *Org. Lett.* **2007**, *9*, 1683.
(15) (a) Ohayon, S.; Spasser, L.; Aharoni, A.; Brik, A. *J. Am. Chem. Soc.* **2012**, *134*, 3281. (b) Ohayon, S.; Refua, M.; Hendler, A.; Aharoni, A.; Brik, A. *Angew. Chem., Int. Ed.* **2014**, *54*, 599.
(16) Braslau, R.; Axon, J. R.; Lee, B. *Org. Lett.* **2000**, *2*, 1399.
(17) Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.
(18) Zoroddu, M. A.; Medici, S.; Peana, M.; Anedda, R. *Dalton Trans.* **2010**, *39*, 1282.
(19) Bozza, W. P.; Liang, Q.; Gong, P.; Zhuang, Z. *Biochemistry* **2012**, *51*, 10075. Note: See this reference where the attack of the catalytic cysteine on the isopeptide carbonyl is not shown to be the rate-determining step in the DUB mechanism.
(20) Hara, Y.; Shen, L. T.; Tsubouchi, A.; Akiyama, M.; Umemoto, K. *Inorg. Chem.* **2000**, *39*, 5074.
(21) Farkas, E.; Kiss, I. *J. Chem. Soc., Dalton Trans.* **1990**, 749.

DOI: 10.1021/acs.orglett.5b01443
Org. Lett. XXXX, XXX, XXX−XXX