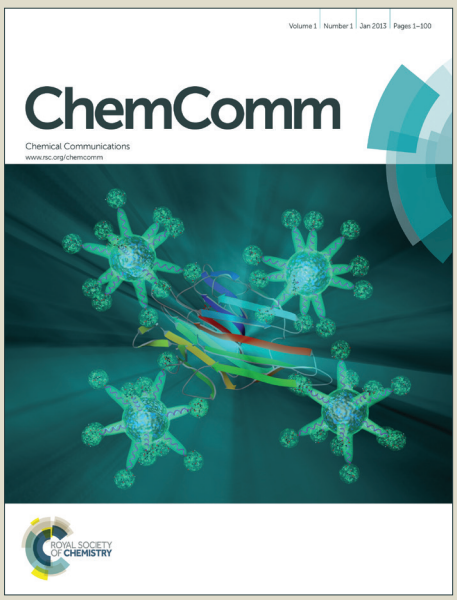
# ChemComm

Accepted Manuscript





ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

# Native Chemical Ubiquitination Using a Genetically Incorporated Azidonorleucine†

Cite this: DOI: 10.1039/x0xx00000x

Renliang Yang[a], Xiaobao Bi[a], Fupeng Li, Yuan Cao, and Chuan-Fa Liu*

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/ChemComm

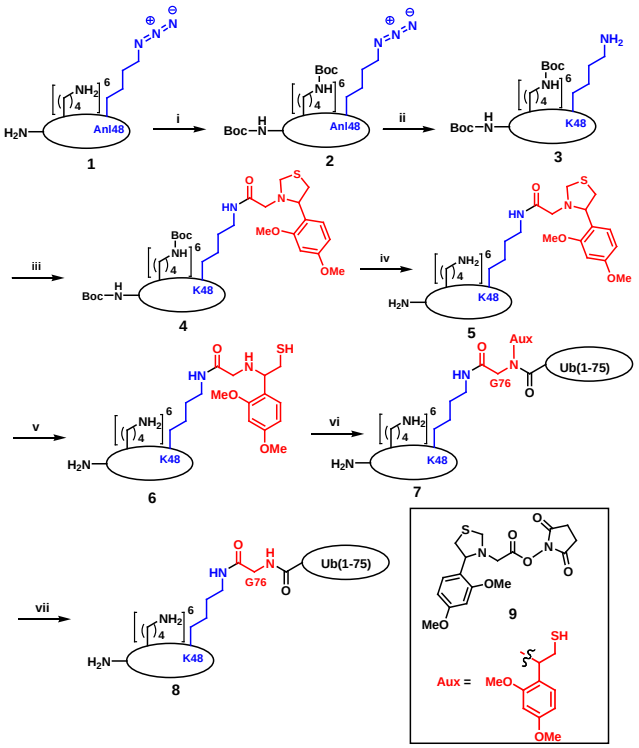
**A robust chemical ubiquitination method was developed. The method employed a genetically incorporated azidonorleucine as an orthogonal lysine precursor for the installation of a Gly residue bearing an Nα-auxiliary which mediated the ligation between ubiquitin(1-75)-thioester and the target protein. To demonstrate our methodology, a model protein, K48-linked diubiquitin, was synthesized with an overall yield of 35%.**

Ubiquitination is one of the most important protein posttranslational modifications in eukaryotic cells and is involved in almost all of the cellular processes, including protein degradation and the regulation of gene expression.[1] It refers to the linking of the C-terminus of ubiquitin protein (76 amino acids) to the lysine side chain of the target protein through an isopeptide bond. To study the physiological roles of ubiquitination, it is crucial to generate homogeneously ubiquitinated proteins. Biologically, ubiquitination is achieved through the consecutive action of three enzymes, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3).[1] Due to the difficulties in identifying or isolating the substrate specific ligases, enzymatic ubiquitination *in vitro* often has limited practical value. Chemical ubiquitination offers a better solution. Since the first report of Nα-auxiliary mediated ubiquitination by Muir et al.,[2] several different chemical ubiquitination approaches have been developed. Our group, Brik's group and Ovaa's group reported the chemical synthesis of ubiquitinated peptides and proteins through γ- or δ-thiolysine mediated chemical ligations.[3-5] Later, Chin et al. reported the genetic incorporation of δ-thiolysine derivatives into recombinant proteins, which avoided the laborious work of installing thiolysine into proteins through peptide synthesis and chemical ligation.[6] The same group also reported a genetically encoded orthogonal protection and activated ligation (GOPAL) approach to synthesize K6- and K29-linked diubiquitins.[7] More recently, Cropp and Fushman modified the approach and applied it to the synthesis of more complicated oligo-ubiquitins.[8] Recently, Brik et al. also reported the expeditious synthesis of ubiquitinated peptides employing orthogonal protection and chemical ligation.[9] All these methods generated ubiquitin conjugates with the native linkage. Methods for preparing ubiquitin conjugates with non-native linkages including a disulfide bond[10], oxime[11], triazole[12], thioether[13], as well as isopeptide bond with the C-terminal Gly76 mutated to Cys[14] or Ala[15] and isopeptide bond with Nε-methylation[14b,16] have also been reported. While these non-native ubiquitin conjugates are useful for many studies, they do not reflect all the genius aspects of the physiological properties of their native counterparts.

Among all these currently developed methods for synthesizing native Ub-proteins, native chemical ligation[17] as well as its extended version – Nα-auxiliary-mediated ligation[18] – remain the best choice due to their chemoselective and protection-free nature. Various ubiquitin conjugates have been created through chemoselective ligation between a ubiquitin thioester and the target protein of which the lysine side chain bears a ligatable functionality, such as a γ- or δ-thiol group,[3-6] or a Gly with an Nα-auxiliary[2] or Cys[14, 15] on the ε-N. These existing ligation-based ubiquitination methods shared a common feature that all these ligation functionalities are pre-installed at the desired position during the production of peptide or protein substrates through solid-phase peptide synthesis (SPPS) or genetic incorporation. The pre-installation synthetic steps are usually laborious and limit the overall yield. Therefore, we have been actively seeking an alternative chemical ligation-based ubiquitination method in which the ligatable functionality is post-synthetically or posttranslationally installed through manipulating the full-length target protein.

The successful site-specific and post-synthetic installation of the ligatable functionality relies on distinguishing the lysine involved in ubiquitination from all the others which are not involved. We realized that azidonorleucine (Anl) can serve this purpose. Recently, azide has been recognized by peptide chemists as a lysine side chain protection group for peptide condensation.[19] However, this application has not been performed on recombinant proteins despite the successful genetic incorporation of Anl reported long time ago.[20] Previously, the genetically incorporated Anl was solely used as a source of azide functionality for protein labeling via click chemistry.[20] Here, we propose that the genetically incorporated Anl can also serve as a temporary and orthogonal lysine precursor in recombinant proteins for chemical ubiquitination (Scheme 1). To do this, all the other amines in the substrate protein which are not involved in ubiquitination are first protected with Boc. A reduction reaction then converts the Anl residue to Lys onto which a ligatable functionality (**9**, Scheme 1) can then be installed. Subsequently, site-specific ubiquitination can be achieved through auxiliary-mediated

ligation (Scheme 1). To illustrate our methodology, a model protein, K48-linked diubiquitin, was synthesized.




**Scheme 1** Synthesis of K48-linked diubiquitin through the combination of genetic Anl incorporation and Nα-auxiliary-mediated native chemical ligation. Reagents and conditions: i) Boc anhydride, DIEA, DMSO; ii) 1 M TCEP in $H_2O$, DMSO; iii) **9**, DIEA, DMSO; iv) TFA/TIS/ $H_2O$ (95/2.5/2.5), 56% (four steps); v) 6 M Gdn•HCl, 0.2 M phosphate, 0.4 M $MeONH_2$, pH 4.0, 85%; vi) Ub(1-75)-MES, 6 M Gdn•HCl, 0.2 M phosphate, 25 mM TCEP, 25 mM MPAA, pH 8.0, 85%; vii) TFA/TIS/ $H_2O$ (95/2.5/2.5), 86%.

The synthesis of K48-linked diubiquitin starts with the expression of the base ubiquitin with K48 replaced by Anl (ub **1**). Anl has been successfully incorporated into proteins through an engineered methioninyl-tRNA synthetase (MetRS) in Met-auxotrophic *E. coli* cells.[20] It has been reported that MetRS with a single mutation (L13G) is sufficient for the efficient Anl incorporation. In our study, we found that MetRS with L13A mutation (MetRSL13A) could also catalyze the incorporation of Anl efficiently. There are no other Met residues except the initiator Met present in the ubiquitin sequence. To express ub **1** (Scheme 1), the codon coding for K48 was mutated to that for Met. To ensure that the initiating Anl can be removed after protein expression within *E. coli* cells, two extra residues Ala and Ser were introduced after the initiating residue. The expression of ub **1** was done with MetRSL13A in the presence of 1 mM Anl. After purification, homogeneous ub **1** was obtained with a yield of 10 mg/L. Electrospray ionization mass spectrometry (ESI-MS) analysis of the protein confirmed that the initiating Anl had been completely removed (Fig. 1).

To install the ligation auxiliary at the K48 side chain of ub, all the free amines of ub **1** were first protected using $(Boc)_2O$. Under the conditions used (ESI†), the reaction was completed in 1 h at room temperature and the crude ub **2** product was obtained using diethyl ether precipitation. MS data (Fig. 1B trace b) showed the presence of 7 or 8 Boc groups on the product. There are seven free amines in **1** and the eighth Boc group might have been added on the His residue in ub. It is worth mentioning that, when an over-excessive amount of DIEA was used, a significant side product with 17-18 Da less than the expected product was observed (data not shown). This was possibly due to deamination or dehydration under the over-alkaline conditions. After Boc protection, the azide group of Anl in ub **2** was reduced to amine by tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in DMSO. The reduction reaction was finished in 3 h at room temperature as confirmed by ESI-MS (Fig. 1 B trace c). The reduced product ub **3** was ether-precipitated and used directly for introducing the ligation auxiliary using compound **9** (Scheme 1). After 45 min, crude ub **4** product was obtained by ether precipitation (Fig. 1B trace d). Finally, global Boc deprotection was done on the crude ub **4** using TFA for 20 min. After ether precipitation, the crude deprotection product was analyzed by C18 analytical HPLC (Fig. 1A, trace b) which showed an excellent purity profile of the desired product **5** with only a small amount of the amine form of ub **1** as a noticeable side product. After HPLC purification, 4.9 mg of **5** was obtained. The overall yield for the first four steps was 56%. This good yield is mostly due to that only one HPLC purification step was employed in the 4-step process of synthesizing **5**. The analytical HPLC and deconvoluted ESI-MS of **5** is shown in Fig. 1 (panel A trace c and panel B trace e).

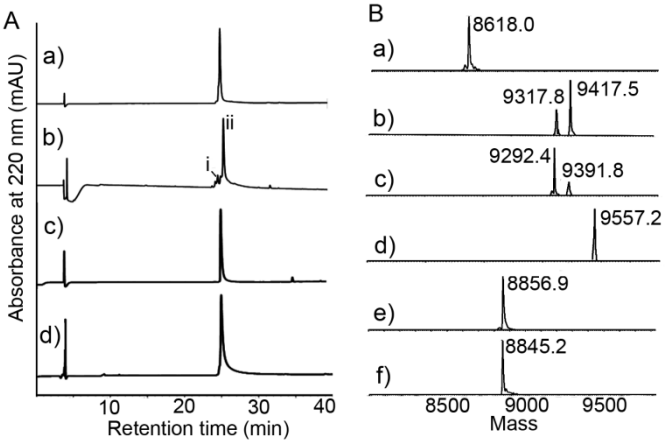



**Fig 1** C18 analytical HPLC (A) and deconvoluted ESI-MS (B) of the key intermediates during the installation of auxiliary to the side chain of ubiquitin K48. (A): a) purified ub **1**; b) crude ub **5 (**peak **ii**: ub **5**; peak **i**: amine-form ub **1)**; c) purified ub **5**; d) purified ub **6**. Gradient: 0-80% buffer B (90% acetonitrile, 10% $H_2O$ containing 0.045% TFA) in buffer A ($H_2O$ containing 0.045% TFA) for 40 min. B) a-f: Deconvoluted ESI-MS of ub **1-6**. Average mass of **1**, calculated 8617.70, observed 8618.0; average mass of **2**: $\mathbf{Boc_8}$, calculated 9418.62, found 9417.5, $\mathbf{Boc_7}$, calculated 9318.50, found 9317.8; average mass of **3**: $\mathbf{Boc_8}$, calculated 9392.63, found 9391.8, $\mathbf{Boc_7}$, calculated 9292.51, found 9292.4; average mass of **4**: calculated 9557.84; found 9557.2 (Only $Boc_7$ product was observed); average mass of **5**: calculated 8857.03; found 8856.9; average mass of **6**: calculated 8845.02; found 8845.2.

After the installation of the auxiliary group, the thiazolidine protection group of **5** was removed by methoxylamine. So 2.9 mg of **5** was dissolved in 750 µL of the reaction solution (6 M Gdn•HCl, 0.2 M phosphate, 0.4 M $MeONH_2$, pH 4). After 5 h at 37°C, 2.5 mg of deprotected product **6** was obtained after HPLC purification (yield 85%) (Fig. 1A trace d and B trace f).

To form diubiquitin **7**, ub **6** was ligated with the 75-aa ubiquitin thioester ub(1-75)-MES through auxiliary-mediated ligation. The thioester was generated through the thiolysis of a ubiquitin-intein fusion protein[21] with sodium mercaptoethanesulfonate (MESNa). For the auxiliary-mediated ligation, initially we chose MESNa as the thiol additive. 2.0 mg of ub(1-75)-MES and 1.4 mg of ub **6** were dissolved in 200 µL of ligation buffer (6 M Gdn•HCl, 0.2 M phosphate, 0.1 M MESNa, 20 mM TCEP, pH 8). At room

temperature, the ligation reaction was extremely sluggish. After 24 h, less than 20% of the ligation product was formed. The majority of the ubiquitin thioester was hydrolyzed. After incubation for another 24 h, there was no significant improvement in ligation yield (ESI†). Based on these observations, we realized that MESNa was not a good thiol additive for our auxiliary-mediated ligation. The use of thiol additives in NCL has long been practiced[22] and more recently it has been shown that aromatic thiols are more effective additives than alkyl thiols.[23] So next, we tested the aromatic thiol 4-mercaptophenylacetic acid (MPAA) which is a very efficient thiol additive.[21] For a typical ligation reaction, 2.8 mg of ub(1-75)-MES and 1.7 mg of ub **6** were dissolved in 200 μL of ligation buffer (6 M Gdn•HCl, 0.2 M phosphate, 25 mM MPAA, 25 mM TCEP, pH 8). Here we used a minimum concentration of MPAA to avoid the possible overlap of the MPAA peak and ubiquitin peaks on HPLC profiles. To our delight, the ligation underwent efficiently in the presence of MPAA at such a concentration. After 7 h at room temperature, most ub 6 was consumed to form the ligation product. After another 5 h, almost all of the **6** was consumed (Fig. 2A). After HPLC purification, 2.9 mg ligation product **7** was obtained with an isolated yield of 85%. Based on these observations, we concluded that our auxiliary-mediated ligation was very efficient with MPAA as the thiol additive even though the ligation was at a sterically hindered secondary amine.

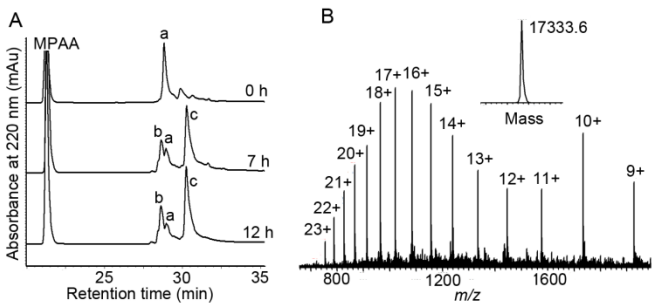



**Fig 2** (A) C8 analytical HPLC monitored ligation reaction between ub **6** and ub(1-75)-MES with MPAA as the thiol additive at 0 h, 7 h and 12 h. Peak a: at 0 h and 7h: mixture of **6** and ub(1-75)-MES; at 12 h, only ub(1-75)-MES remaining; peak b: ub(1-75)-OH; peak c: ligation product **7**. (B) The raw and deconvoluted ESI-MS of **7**. Average mass calculated 17334.7; found 17333.6.

The final step for the synthesis of K48-linked diubiquitin **8** is to remove the auxiliary. To do this, 2.9 mg of 7 was treated with a cocktail containing TFA/TIS/$H_2O$ (95/2.5/2.5) for 20 min on ice. After purification, 2.5 mg of **8** was obtained with an isolated yield of 86%. The SDS-PAGE, ESI-MS and C8 analytical HPLC analysis of **8** were shown in Fig. 3A (lane 2), B and C (trace a). The overall yield for our seven-step diubiquitin synthesis was about 35%, which makes our approach one of most efficient methods for chemical diubiquitin synthesis.

To generate native-folded K48-linked diubiquitin, the chemically synthesized diubiquitin **8** was refolded through dialysis (ESI†). To test whether the refolded diubiquitin was biologically active, the diubiquitin was analyzed by western blot using ubiquitin monoclonal antibody P4D1. A single band corresponding to the diubiquitin was detected in western blot (Fig. 3A). Deubiquitinase assays with two ubiquitin deconjugating enzymes, isopeptidase T (IsoT) and A20 catalytic domain ($A20_{CD}$), were also performed (Fig. 3C; ESI†). It was observed that with a 350:1 substrate-to-enzyme ratio, IsoT could effectively hydrolyze almost all the K48 diubiquitin in 2 h at 37°C. Compared to IsoT, $A20_{CD}$ was found to be less efficient in hydrolyzing K48 diubiquitin. With a substrate enzyme ratio of 24:1, only about 60% of the diubiquitin was hydrolyzed by $A20_{CD}$ after 2 h at 37°C. After incubation for another 2 h, about 85% of the diubiquitin was hydrolyzed by $A20_{CD}$.

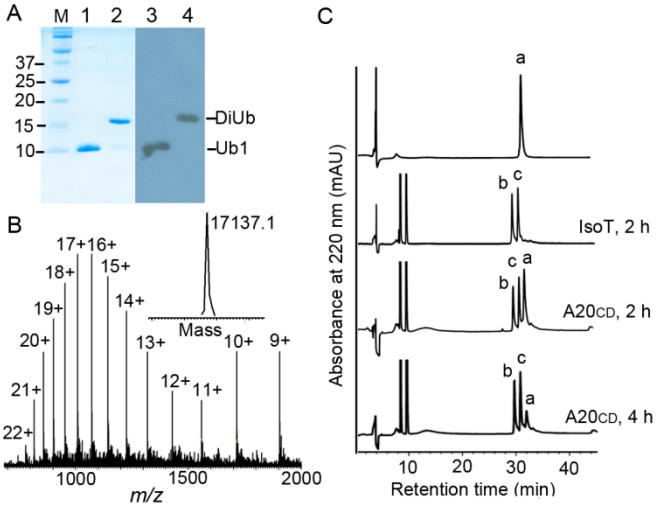



**Fig. 3** (A) SDS-PAGE with coomassie blue staining (lane 1 and 2) and western blot (lane 3 and 4) of the synthesized K48-linked diubiquitin **8**. Lane 1 and 3: ub**1**; Lane 2 and 4: diub**8**. (B) The raw and deconvoluted ESI-MS of **8**. Average mass calculated 17138.43; found 17137.1. (C) Deubiquitinase assays of **8** performed with IsoT and $A20_{CD}$ monitored by C8 analytical HPLC. Peak a: **8**; peak b: ub **1**; peak c: wild type ub.

## Conclusions

In conclusion, we have developed a novel and efficient native chemical ligation-based method for protein ubiquitination. Different from all the previously reported methods, ubiquitin ligation is facilitated by an auxiliary which is installed on a recombinant protein containing a genetically incorporated Anl. The method combines the advantageous features of Muir's Nα-auxiliary-mediated site-specific ubiquitination approach and Chin's GOPAL approach. The key ubiquitination step in our approach is based on the highly efficient and selective NCL which allows the use of completely unprotected reaction partners for ligation in aqueous buffer. The only step that requires protection in our scheme is the installation of the auxiliary group on the substrate protein. This is different from the GOPAL approach in which the key ubiquitination reaction step involves $Ag^+$-mediated activation of a C-terminal thioester[24] in the acyl ubiquitin for condensation with the amine partner in DMSO and requires protection of all the uninvolved amines in both reaction partners, and the large size of the reaction components also limits the efficiency of condensation. Our work shows an interesting new application of the Anl genetic incorporation method. A limitation of our approach lies with that Anl would substitute for all Met residues in the target protein. Nevertheless, Met is a rare amino acid and can often be replaced by other amino acids such as Leu without affecting protein function.[25] The synthesis of K48-linked diubiquitin with native isopeptide linkage at an overall yield of 35% demonstrates the robustness of our approach. This, coupled with the relative easiness to express Anl-containing proteins, makes our method an excellent addition to the list of chemical

ubiquitination methods which together have made the difficult-to-make ubiquitinated proteins more accessible for the concerned research community.

This work is supported by the Agency for Science, Technology and Research (A*STAR) of Singapore (grant No. SERC 112 120 2017) and Nanyang Technological University.

## Notes and references

Division of Structural Biology and Biochemistry, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551. E-mail: cfliu@ntu.edu.sg; Fax: (+)65 67953021

[a]These two authors contributed equally to this work.

† Electronic Supplementary Information (ESI) available: Detailed experimental procedures; NMR, HPLC and MS data. See DOI: 10.1039/c000000x/

1 (a) M. Hochstrasser, *Nat. Cell Biol.*, 2000, **2**, E153-E157; (b) C. M. Pickart, *Annu. Rev. Biochem.*, 2001, **70**, 503-533; (c) O. Kerscher, R. Felberbaum and M. Hochstrasser, *Annu. Rev. Cell Dev. Biol.*, 2006, **22**, 159-180; (d) M. Hochstrasser, *Nature*, 2009, **458**, 422-429; (e) Z. J. Chen and L. J. Sun, *Mol. Cell*, 2009, **33**, 275-286; (f) F. Ikeda, N. Crosetto and I. Dikic, *Cell*, 2010, **143**, 677-681.

2 (a) C. Chatterjee, R. K. McGinty, J.-P. Pellois and T. W. Muir, *Angew. Chem., Int. Ed.*, 2007, **46**, 2814-2818; (b) R. K. McGinty, J. Kim, C. Chatterjee, R. G. Roeder and T. W. Muir, *Nature*, 2008, **453**, 812-816.

3 (a) R. Yang, K. K. Pasunooti, F. Li, X.-W. Liu and C.-F. Liu, *J. Am. Chem. Soc.*, 2009, **131**, 13592-13593; (b) R. Yang, K. K. Pasunooti, F. Li, X.-W. Liu and C.-F. Liu, *Chem. Commun.*, 2010, **46**, 7199-7201.

4 (a) K. S. A. Kumar, M. Haj-Yahya, D. Olschewski, H. A. Lashuel and A. Brik, *Angew. Chem., Int. Ed.*, 2009, **48**, 8090-8094; (b) K. S. A. Kumar, L. Spasser, L. A. Erlich, S. N. Bavikar and A. Brik, *Angew. Chem., Int. Ed.*, 2010, **49**, 9126-9131; (c) K. S. A. Kumar, S. N. Bavikar, L. Spasser, T. Moyal, S. Ohayon and A. Brik, *Angew. Chem., Int. Ed.*, 2011, **50**, 6137-6141.

5 (a) F. El Oualid, R. Merkx, R. Ekkebus, D. S. Hameed, J. J. Smit, A. De Jong, H. Hilkmann, T. K. Sixma and H. Ovaa, *Angew. Chem., Int. Ed.*, 2010, **49**, 10149-10153; (b) R. Merkx, G. De Bruin, A. Kruithof, T. Van Den Bergh, E. Snip, M. Lutz, F. El Oualid and H. Ovaa, *Chem. Sci.*, 2013, **4**, 4494-4498.

6 S.Virdee, P. B. Kapadnis, T. Elliott, K. Lang, J. Madrzak, D. P. Nguyen, L. Riechmann and J. W. Chin, *J. Am. Chem. Soc.*, 2011, **133**, 10708-10711.

7 S. Virdee, Y. Ye, D. P. Nguyen, D. Komander and J. W. Chin, *Nat. Chem. Biol.*, 2010, **6**, 750-757.

8 C. Castañeda, J. Liu, A. Chaturvedi, U. Nowicka, T. A. Cropp and D. Fushman, *J. Am. Chem. Soc.*, 2011, **133**, 17855-17868.

9 K. S. Ajish Kumar, L. Spasser, S. Ohayon, L. A. Erlich and A. Brik, *Bioconjugate. Chem.*, 2011, **22**, 137-143.

10 (a) C. Chatterjee, R. K. McGinty, B. Fierz and T. W. Muir, *Nat. Chem. Biol.*, 2010, **6**, 267-269; (b) J. Chen, Y. Ai, J. Wang, L. Haracska and Z. Zhuang, *Nat. Chem. Biol.*, 2010, **6**, 270-272; (c) F. Meier, T. Abeywardana, A. Dhall, N. P. Marotta, J. Varkey, R. Langen, C. Chatterjee and M. R. Pratt, *J. Am. Chem. Soc.*, 2012, 134, 5468-5471; (d) B. Fierz, C. Chatterjee, R. K. McGinty, M. Bar-Dagan, D. P. Raleigh and T. W. Muir, *Nat. Chem. Biol.*, 2011, **7**, 113-119.

11 A. Shanmugham, A. Fish, M. P. A. Luna-Vargas, A. C. Faesen, F. El Oualid, T. K. Sixma and H. Ovaa, *J. Am. Chem. Soc.*, 2010, **132**, 8834-8835.

12 (a) S. Eger, M. Scheffner, A. Marx and M. Rubini, *J. Am. Chem. Soc.*, 2010, **132**, 16337-16339; (b) N. D. Weikart and H. D. Mootz, *ChemBioChem*, 2010, **11**, 774-777; (c) S. Sommer, N. D. Weikart, A. Brockmeyer, P. Janning and H. D. Mootz, *Angew. Chem., Int. Ed.*, 2011, **50**, 9888-9892.

13 (a) E. M. Valkevich, R. G. Guenette, N. A. Sanchez, Y.-C. Chen, Y. Ge and E. R. Strieter, *J. Am. Chem. Soc.*, 2012, **134**, 6916-6919; (b) V. H. Trang, E. M. Valkevich, S. Minami, Y.-C. Chen, Y. Ge and E. R. Strieter, *Angew. Chem., Int. Ed.*, 2012, **51**, 13085-13088.

14 (a) X. Li, T. Fekner, J. J. Ottesen and M. K. Chan, *Angew. Chem., Int. Ed.*, 2009, **48**, 9184-9187; (b) N, Haj-Yahya, M. Haj-Yahya, C. A. Castañeda, L. Spasser, H. P. Hemantha, M. Jbara, M. Penner, A. Ciechanover, D. Fushman and A. Brik, *Angew. Chem., Int. Ed.*, 2013, **52**, 11149-11153.

15 (a) R. K. McGinty, M. Köhn, C. Chatterjee, K. P. Chiang, M. R. Pratt and T. W. Muir, *ACS Chem. Biol.*, 2009, **4**, 958-968; (b) B. Fierz, S. Kilic, A. R. Hieb, K. Luger and T. W. Muir, *J. Am. Chem. Soc.*, 2012, **134**, 19548-19551.

16 M. Haj-Yahya, N. Eltarteer, S. Ohayon, E. Shema, E. Kotler, M. Oren and A. Brik, *Angew. Chem., Int. Ed.*, 2012, **51**, 11535-11539.

17 P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776-779.

18 (a) P. Botti, M. R. Carrasco and S. B. H. Kent, *Tetrahedron Lett.*, 2001, **42**, 1831-1833; (b) D. W. Low, M. G. Hill, M. R. Carrasco, S. B. H. Kent and P. Botti, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 6554-6559; (c) J. Offer, C. N. C. Boddy and P. E. Dawson, *J. Am. Chem. Soc.*, 2002, **124**, 4642-4646; (d) J. Offer, *Biopolymers*, 2010, **94**, 530-541.

19 (a) H. Katayama, H. Hojo, T. Ohira and Y. Nakahara, *Tetrahedron Lett.*, 2008, **49**, 5492-5494; (b) H. Katayama, H. Hojo, I. Shimizu, Y. Nakahara and Y. Nakahara, *Org. Biomol. Chem.*, 2010, **8**, 1966-1972.

20 (a) A. J. Link, M. K. S. Vink and D. A. Tirrell, *J. Am. Chem. Soc.*, 2004, **126**, 10598-10602; (b) A. J. Link, M. K. S. Vink, N. J. Agard, J. A. Prescher, C. R. Bertozzi and D. A. Tirrell, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 10180-10185; (c) D. M. Abdeljabbar, T. J. Klein, S. Zhang and A. J. Link, *J. Am. Chem. Soc.*, 2009, **131**, 17078-17079; (d) J. T. Ngo, J. A. Champion, A. Mahdavi, I. C. Tanrikulu, K. E. Beatty, R. E. Connor, T. H. Yoo, D. C. Dieterich, E. M. Schuman and D. A. Tirrell, *Nat. Chem. Biol.*, 2009, **5**, 715-717.

21 T. W. Muir, D. Sondhi and P. A. Cole, *Proc. Natl. Acad. Sci. U.S.A.*, 1998, **95**, 6705-6710.

22 J. P. Tam, Y.-A. Lu, C.-F. Liu and J. Shao, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 12485-12489.

23 E. C. B. Johnson and S. B. H. Kent, *J. Am. Chem. Soc.*, 2006, **128**, 6640-6646.

24 S. Aimoto, *Biopolymers*, 1999, **51**, 247-265.

25. H. Yang, L. Liua, M. Wang, J. Li, N. S. Wang, G. Du and J. Chen, *Appl. Environ. Microbiol.* 2012, **78**, 7519-7526.

