Organic & Biomolecular Chemistry

PAPER

View Article Online
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Cite this: *Org. Biomol. Chem.*, 2014, **12**, 5435

# One-pot native chemical ligation of peptide hydrazides enables total synthesis of modified histones†

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Received 4th April 2014,
Accepted 9th May 2014

DOI: 10.1039/c4ob00715h

www.rsc.org/obc

One of the rising demands in the field of protein chemical synthesis is the development of facile strategies that yield the protein in workable quantities and homogeneity, with fewer handling steps. Although the native chemical ligation of peptide hydrazides has recently been shown to be useful for the chemical synthesis of proteins carrying acid-sensitive modification groups, previous hydrazide-based protein synthesis studies have used sequential ligation strategies. Here, we report a practical method for a "one-pot" native chemical ligation of peptide hydrazides that would circumvent the need for the isolation of the intermediate products. This method employed a fast and selective arylboronate oxidation reaction mediated by $H_2O_2$, which draws attention to the potential applications of the thus far under-exploited boron-based functionalities in protein chemical synthesis. To demonstrate the practicality and efficiency of the new one-pot method, we report its application to a scalable total synthesis of modified histones (with five analogues of H3 and H4 as examples) on a multi-milligram scale, with good homogeneity.

## Introduction

Histone post-translational modifications (PTMs, *e.g.* acetylation, methylation and phosphorylation) play a central role in regulating the structure and dynamics of chromatin, as well as DNA-driven cellular processes.[1] An understanding of how histone PTMs translate into diverse cellular events is important from both the fundamental and therapeutic perspectives. Studies for this purpose need access to homogenously modified histones that unfortunately cannot be easily isolated from natural sources, or obtained by classical biochemical methods.[2] To solve this problem four strategies have been used to make modified histones: (1) amber suppression mutagenesis,[3] (2) Cys-directed protein modification,[4] (3) expressed protein ligation[5] and (4) total chemical protein synthesis.[6] In the first three approaches the main structures of histones are produced recombinantly, whereas the modification is introduced later by chemoselective reactions with small molecules or peptides. These semi-synthetic strategies have been successfully used to produce many modified histones, enabling elegant structural and functional studies.[3–5] Nonetheless, the total chemical synthesis of histones is needed for the generation of histones with multiple, different modifications at both the terminal and middle regions.

In this context, Ottesen and coworkers described the first total synthesis of histone H3, through sequential native chemical ligations.[7] This important, pioneering work enabled the production of a fully synthetic H3 with 2–7% overall yields. A potential limitation is that the peptide segments were prepared using Boc SPPS (solid-phase peptide synthesis), whose HF cleavage conditions were unsuitable for some PTMs, such as phosphorylation. To overcome this problem Ottesen *et al.* tested the *N*-acylurea approach for the Fmoc SPPS of histone peptide thioesters.[8] Meanwhile, Aimoto and coworkers accomplished the first Fmoc-based total synthesis of H3 by using the Cys-Pro ester autoactivating unit as a thioester precursor.[9] Notwithstanding these key advances, to meet the escalating needs in the studies of histone PTMs it is helpful to develop a more scalable and cost-effective method for the total synthesis of modified histones on a multi-milligram scale.

We recently described the native chemical ligation of peptide hydrazides,[10] which can be readily converted to thioesters *via* $NaNO_2$ activation and subsequent thiolysis. We expected that this approach may allow an expedient synthesis of modified histones, as peptide hydrazides are easily synthesizable through automated Fmoc SPPS. Indeed, with the help

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†Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/c4ob00715h

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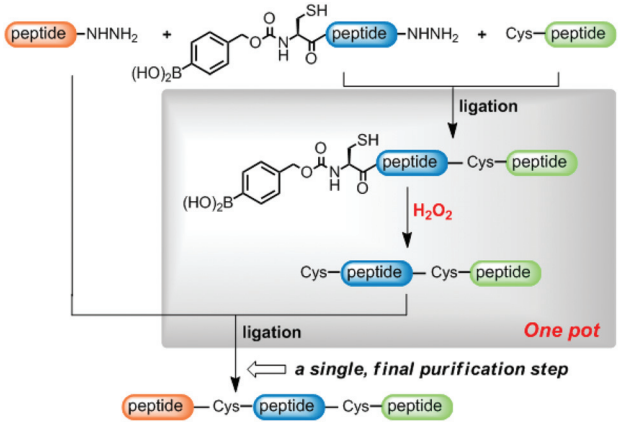


Fig. 1 One-pot ligation of peptide hydrazides.

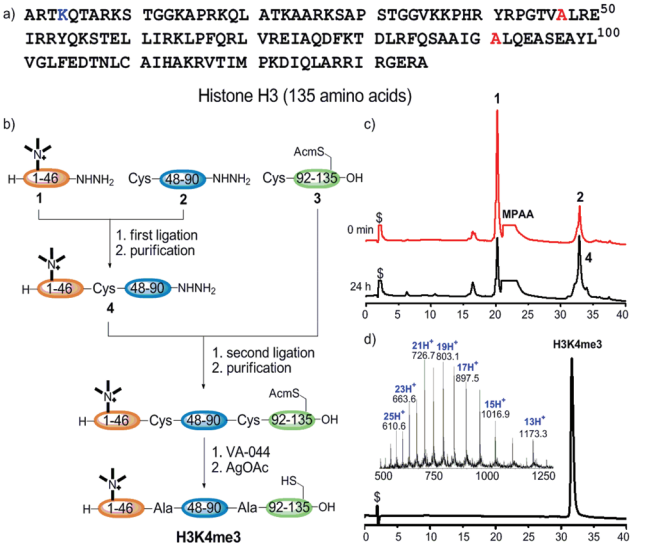


Fig. 2 Synthesis of H3K4me3 by using N-to-C sequential ligations. (a) Sequence of H3. (b) Procedure for the N-to-C sequential ligations. (c) Analytical HPLC traces ($\lambda$ = 214 nm) for the ligation between **1** and **2** after 0 min and 24 h. "$" denotes solvent fronts. (d) Analytical HPLC trace ($\lambda$ = 214 nm) for the final, purified H3K4me3 and its ESI-MS: observed mass 15 240.2 ± 0.7 Da, calc. 15 239.62 Da (average isotopes).

of peptide hydrazides, Brik *et al.* accomplished the first total synthesis of Lys34-ubiquitinated H2B.[11] However, in our synthesis of H3 and H4 using the previous sequential ligation/purification method,[10b] we encountered difficulty in separating the intermediate products, which not only complicated the experiments, but also reduced the yield. To overcome this challenge we sought to use a "one-pot" strategy that would need a single final purification step.[12] However, our own tests revealed that the previously developed one-pot procedures were not readily applicable to peptide hydrazides.[13]

In the present study, we describe a one-pot method for the ligation of peptide hydrazides (Fig. 1). We also explore the possible use of the thus far under-exploited boron-based functionalities in the chemical synthesis of proteins. The practicality and efficiency of the method was demonstrated by the multi-milligram-scale synthesis of modified histone H3 and H4. Thus, in terms of the concept as well as the technological advances, this work extends the hydrazide-based approach of native chemical ligation.

## Results and discussion

### Problem of sequential ligations

Before we describe the "one-pot" method, we wish to show, with experimental data, why a one-pot method would sometimes be practically advantageous, if not obligatory for the native chemical ligation of peptide hydrazides. For the synthesis of histone H3 trimethyl Lys4 (H3K4me3) we started with three synthetic peptide segments (Fig. 2), *i.e.* H3K4me3[Ala1-Val46]-$NHNH_2$ (**1**, 46-mer), H3K4me3[Cys47-Gly90]-$NHNH_2$ (**2**, 44-mer), and H3K4me3[Cys91-Ala135] (**3**, 45-mer). Both the Ala47 and Ala91 residues were replaced by Cys to facilitate the ligations, which would be converted back to Ala after the full-length peptide was produced.[14] Furthermore, the side chain thiol of Cys110 was protected by the Acm (acetamidomethyl) group to enable the selective desulfurization of Ala47 and Ala91 in the full-length polypeptide product.[15]

With **1**–**3** in hand, we first condensed **1** and **2** with the help of MPAA (4-mercaptophenylacetic acid) to produce an intermediate, **4**. After the purification of **4**, we conducted the second ligation between **4** and **3** to obtain the full-length peptide. Finally, the desired protein (*i.e.* H3K4me3) was generated through a free radical desulfurization reaction initiated, by VA-044 (2,2′-azobis[2-(2-imidazolin-2-yl)-propane] dihydrochloride)[16] followed by Acm deprotection. As shown in Fig. 2c, a tricky problem encountered in the above synthesis was that the retention times of **2** and **4** were almost identical in the reverse-phase HPLC under the conditions used. This rendered the monitoring of the first ligation reaction difficult. The purification of **4** was also challenging, which seriously increased the time cost of the synthesis while reducing the yield.

### One-pot ligation of peptide hydrazides: model test

To overcome the problem of the intermediate separation, we decided to use a one-pot ligation approach[12] to the native chemical ligation of peptide hydrazides. This strategy has been successfully used in many previous studies to reduce the handling time and cost and increase the yields in protein chemical synthesis.[12] For the ligation of peptide hydrazides, the one-pot method must be compatible with the $NaNO_2$ activation step.[13] Although we previously developed the sequential procedure for the ligation of peptide hydrazides,[10b] the one-pot ligation of peptide hydrazides has not been accomplished. A critical requirement is the development of a readily reversible protection of the N-terminal Cys residue. To compromise the operational ease and reaction efficiency, we were interested in the use of the *p*-boronobenzyloxycarbonyl (Dobz) group developed by Kemp and Roberts.[18] We anticipated that the Dobz-

 

protected Cys should remain un-reactive under native chemical ligation conditions, unless it was activated by the addition of $H_2O_2$.[17] In the previous studies on Dobz,[18] only small peptides (up to a pentamer, synthesized by the solution-phase coupling method) were tested. Thus, for the use of Dobz to accomplish the one-pot native ligation of peptide hydrazides in protein total synthesis, we needed to examine a number of important issues regarding the synthesis, stability and handling of the Dobz peptides.

First, we synthesized the pDobz protected cysteine (*i.e.* pDobz-Cys(Trt)-OH) in which the boronic acid was protected as a pinacol ester (Fig. 3b). We then tested the use of pDobz-Cys(Trt)-OH in standard Fmoc SPPS followed by regular trifluoroacetic acid (TFA) cleavage (Fig. 3c). The desired peptide (**5**) was obtained smoothly, in which the free boronic acid group was generated. The next test was the native chemical ligation of the Dobz-Cys-peptide-$NHNH_2$ (**5**) under the previously described $NaNO_2$ activation and MPAA thiolysis conditions (Fig. 3d).[10] The expected peptide thioester intermediate (**6**) was formed cleanly, which reacted *in situ* with the subsequently added Cys to produce the ligation product **7**, in an almost quantitative yield after 60 min (Fig. 3e). Some minor products, due to formation of a thiolactone with the internal Cys and lactam with the C-terminal Lys,[11] were observed. To further confirm the stability of the Dobz-amino protecting group to the $NaNO_2$ oxidation conditions, another test of the Dobz-Cys-peptide-$NHNH_2$ was performed (Fig. 3f) in which Ala was the ligation site and the middle Cys was eliminated. In this test, the ligation proceeded more cleanly without the formation of any thiolactone or lactam by-products (Fig. 3g). Collectively, these results showed that the Dobz group was compatible with the modern Fmoc SPPS and the native chemical ligation of peptide hydrazides.

A critical test for the Dobz group was its selective removal by $H_2O_2$ oxidation, followed by a spontaneous hydrolysis. For this purpose, we dissolved **5** in a ligation buffer containing 65 equivalents of MPAA (pH 6.5–7.0) to simulate the conditions needed by the one-pot ligation (Fig. 3h). To test whether or not the $H_2O_2$ treatment may cause any damage to the peptide, we incorporated all the redox sensitive amino acids (*i.e.* Cys, Met, Trp, Tyr, His) into **5**. When 1 equivalent of $H_2O_2$ (as 1 M aqueous solution) was added to **5** in the ligation buffer, we observed no change in **5** after 50 min, indicating that MPAA was the most susceptible to $H_2O_2$ oxidation in the system (Fig. 3i). To fully consume the MPAA and oxidize **5**, we tested the addition of 33.5 equivalents of $H_2O_2$. Gratifyingly, under this condition we observed a complete and clean conversion of the Dobz-protected **5** to the deprotected peptide product **11**, in 10 min. A further increase of $H_2O_2$ to 50 equivalents (data not shown) and 66 equivalents was found to generate a by-product, whose mass was 16 Da higher than that of **11**. According to the previous study by Schultz *et al.* on BoPhe-incorporated proteins, this by-product was assigned as peptide **12**, with the Met oxidized.[17] Nonetheless, even 66 equivalents of $H_2O_2$ did not fully oxidize **11** to **12**. Collectively, the above results suggested that $H_2O_2$ should oxidize MPAA first and then oxidize and remove the Dobz group, selectively. The Met would be oxidized if excessive $H_2O_2$ was added, but this side reaction could be prevented by using an accurately calculated quantity of $H_2O_2$.[19] Furthermore, use of norleucine to replace Met,[20] which has been a frequently employed tactic in protein chemical

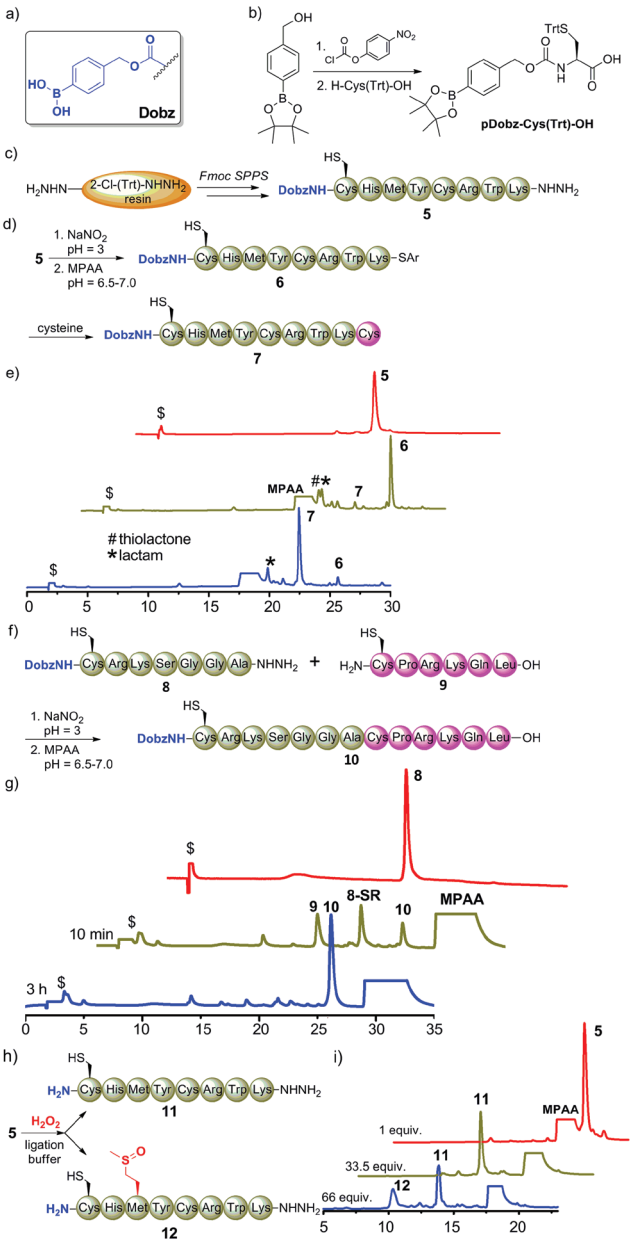


**Fig. 3** Tests for the use of Dobz-peptides. (a) Structure of Dobz. (b) The synthesis of pDobz-Cys(Trt)-OH. (c) Fmoc SPPS of Dobz-Cys-peptide-$NHNH_2$. (d) Conversion of Dobz-Cys-peptide-$NHNH_2$ to a thioester and its ligation with Cys. (e) Analytical HPLC traces ($\lambda$ = 214 nm) for purified **5**, crude reaction mixture of **5** with $NaNO_2$ and MPAA, and crude ligation product with Cys. "$" denotes solvent fronts. (f) Ligation of **8** with **9**. (g) Analytical HPLC traces ($\lambda$ = 214 nm) for **8**, ligation mixture of **8** and **9** after 10 min and 3 h. (h) Treatment of **5** with $H_2O_2$ in the ligation buffer. (i) Analytical HPLC traces ($\lambda$ = 214 nm) after treatment of **5** (in the presence of 65 equiv. MPAA) with 1 equiv. $H_2O_2$ (50 min), 33.5 equiv. $H_2O_2$ (10 min), and 66 equiv. $H_2O_2$ (10 min).

synthesis, would completely circumvent the Met oxidation problem. It is important to emphasize that except for Met (and Cys that forms disulfides), we did not observe the reaction of $H_2O_2$ with any other amino acid residues (in particular, Tyr or Trp) under the conditions used to remove the Dobz group.[19]

### Total synthesis of modified histones

The efficient and selective oxidation of the Dobz group by $H_2O_2$ and its removal from the N-terminal Cys allowed for the design of a $H_2O_2$-controlled one-pot ligation method suitable for peptide hydrazides (Fig. 1). From an operational perspective, the $H_2O_2$ treatment did not need any pH change and it released water as the only by-product. With this key chemistry for the one-pot native chemical ligation of peptide hydrazides in hand, we carried out a new synthesis of H3K4me3.

The three peptide segments (*i.e.* **1**, **13**, and **3**, Fig. 4a) were prepared by using automated Fmoc SPPS and sufficient purified materials were obtained. However, the key segment **13** was synthesized with a low yield. After analysis of the crude **2** and **13** (Fig. S2 and S11†), we concluded that the low yield of **13** was due to the nature of the peptide sequence, but not due to the introduction of the Dobz group. The segments were condensed in a one-pot fashion as follows: firstly, **13** (21 mg) was ligated with **3** (20 mg) at pH 6.8 for 1 hour; secondly, the reaction mixture was treated with 115 μL of 1 M aqueous $H_2O_2$ for 10 minutes and finally, **1** (37 mg, pre-activated by $NaNO_2$ and thiolyzed with MPAA) was added to the reaction mixture. Because the second ligation occurred at a Val-Cys junction, it was allowed to proceed for 48 hours. The full-length peptide was obtained through an HPLC purification process in 12 mg, corresponding to an isolated yield of 21% for the one-pot reactions (Fig. 4b). The HPLC traces of this one-pot transformation were relatively clean, indicating that the intended reactions proceeded smoothly (For an explanation of every peak in Fig. 4b, please refer to Fig. S12†). Subsequently we carried out a desulfurization and Acm deprotection on the HPLC purified materials, to generate 8.3 mg of the target product, H3K4me3, corresponding to a 14.4% overall isolated yield. A repeated experiment using 14 mg **13**, 13 mg **3**, and 25 mg **1** afforded 7.7 mg of H3K4me3, corresponding to a 19.8% overall isolated yield. Through analysis of the MALDI-TOF spectra (Fig. S14†) and every peak in the ESI-MS (data are not shown), we concluded that the $Met_{120}$ in H3K4me3 was not oxidized in the $H_2O_2$ treatment. Thus, our experiments showed that modified H3 could be practically synthesized on a multi-milligram scale.

To further characterize the final synthetic H3K4me3, we conducted an isotope envelope analysis (Fig. 5a and Fig. S15, Table S1†). The results showed that the observed isotope envelope was fully consistent with the theoretically predicted pattern of the target H3K4me3, but not the oxidized side product (+16 Da). Although there were peaks in the region of the predicted oxidized product (Fig. S15b†), we could not

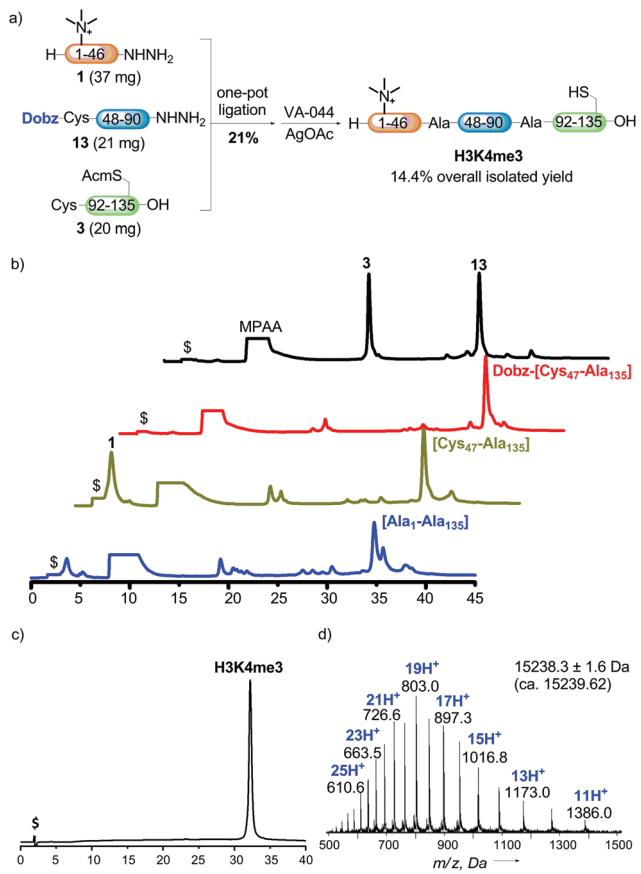


**Fig. 4** Synthesis of H3K4me3 by using $H_2O_2$-controlled one-pot native chemical ligation of peptide hydrazides. (a) The overall procedure. (b) Analytical HPLC traces ($\lambda$ = 214 nm) for the different steps in the one-pot ligation. Black: beginning of the whole process; Red: ligation mixture of **3** and **13** after 1 h; Olive: ligation mixture after the $H_2O_2$ treatment; Blue: the final ligation mixture. "$" denotes solvent fronts. For more explanation of each peak, please refer to Fig. S12.† (c) Analytical HPLC trace ($\lambda$ = 214 nm) for the final, purified H3K4me3. (d) its ESI-MS: observed mass 15 238.3 ± 1.6 Da, calc. 15 239.62 Da (average isotopes).

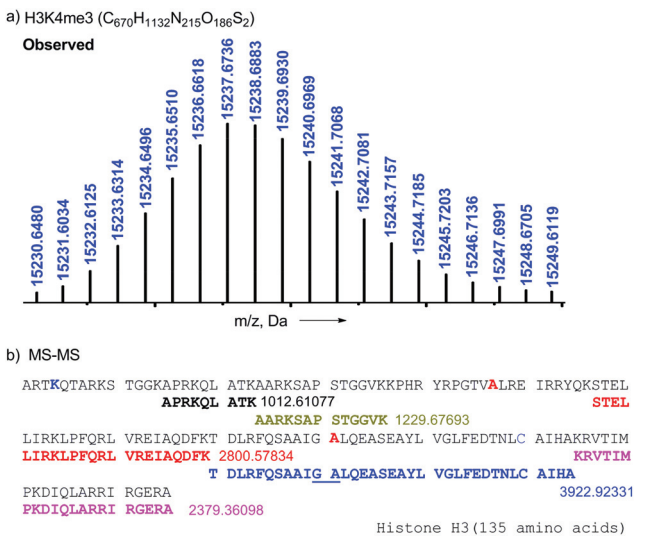


**Fig. 5** Isotope envelopes and MS-MS analysis of synthetic H3K4me3. (a) The observed isotope envelopes. (b) The MS-MS analysis data with the observed fragments marked. For more details please refer to Fig. S15 and S16.†

exclude that these were caused by baseline noise. Collectively, from the above results of the MALDI-TOF and ESI-MS, we had reason to believe that the oxidation of Met was almost prevented, albeit not completely. In the MS-MS analysis (Fig. 5b and Fig. S16†), the observed fragments closely matched the sequence of H3K4me3, especially that the ligation junction (Gly-Ala) was observed. These experimental results confirmed that the synthetic H3K4me3 was highly homogeneous.

For the application of the $H_2O_2$-controlled one-pot ligation method to the total synthesis of a modified H4 (*i.e.* H4K16ac), we also divided it into three peptide segments (Fig. 6a). They were H4K16ac[$Ser_1$-$Leu_{37}$]-$NHNH_2$ (37-mer), Dobz-H4K16ac-[$Cys_{38}$-$His_{75}$]-$NHNH_2$ (38-mer), and H4K16ac[$Cys_{76}$-$Gly_{102}$] (27-mer). Both $Ala_{38}$ and $Ala_{76}$ were changed to Cys to facilitate the ligations, which would be converted back to Ala after the full-length peptide was produced.[14] Again, these three peptide segments were prepared by using automated Fmoc SPPS (Fig. 6b). The middle segment was also difficult to synthesize, due to its hydrophobicity (Fig. S18†). Comparing the HPLC trace of the crude peptide H4K16ac[$Cys_{38}$-$His_{75}$]-$NHNH_2$ with an unprotected Cys at the N terminal (data are not shown), we confirmed that the low yield was not caused by the Dobz group.

The three segments of H4K16ac were condensed in a one-pot fashion from 28, 16, and 11 mg of the starting materials with the control of 106 μL of 1 M aqueous $H_2O_2$. The full-length peptide was then obtained through HPLC purification in 11 mg, corresponding to an isolated yield of 27.4% for the one-pot ligation (Fig. 6c). Subsequently, we carried out a desulfurization procedure to generate the target product, H4K16ac, in 7.3 mg, corresponding to an 18.3% overall isolated yield. A repeated experiment using 14 mg, 8 mg, and 5.5 mg of the starting materials afforded 4.0 mg of H4K16ac, corresponding to a 20.1% overall isolated yield. Again, the HPLC traces of this one-pot transformation (Fig. 6c) were relatively clean, indicating that the designed reactions proceeded smoothly. (For an explanation of every peak in Fig. 6c, please refer to Fig. S20†). Note that $Met_{84}$ in H4K16ac was not oxidized in the $H_2O_2$ treatment (Fig. 6e and Fig. S21†). Additionally, in the synthesis we observed that *ca.* 30% of Dobz-H4K16ac[$Cys_{38}$-$His_{75}$]-$NHNH_2$ was hydrolyzed during the ligation (Fig. 6c). The hydrolysis by-product was almost inseparable from the product of the first ligation (*i.e.* Dobz-H4K16ac[$Cys_{38}$-$Gly_{102}$]. Thus, the one-pot ligation also circumvented the intermediate separation problem that would be expected in the stepwise synthesis of H4K16ac. Furthermore,the synthetic H4K16ac was successfully characterized by the isotope envelope and MS-MS analysis (Fig. 7 and Fig. S22, S23 and Table S2†). Collectively, the above results of

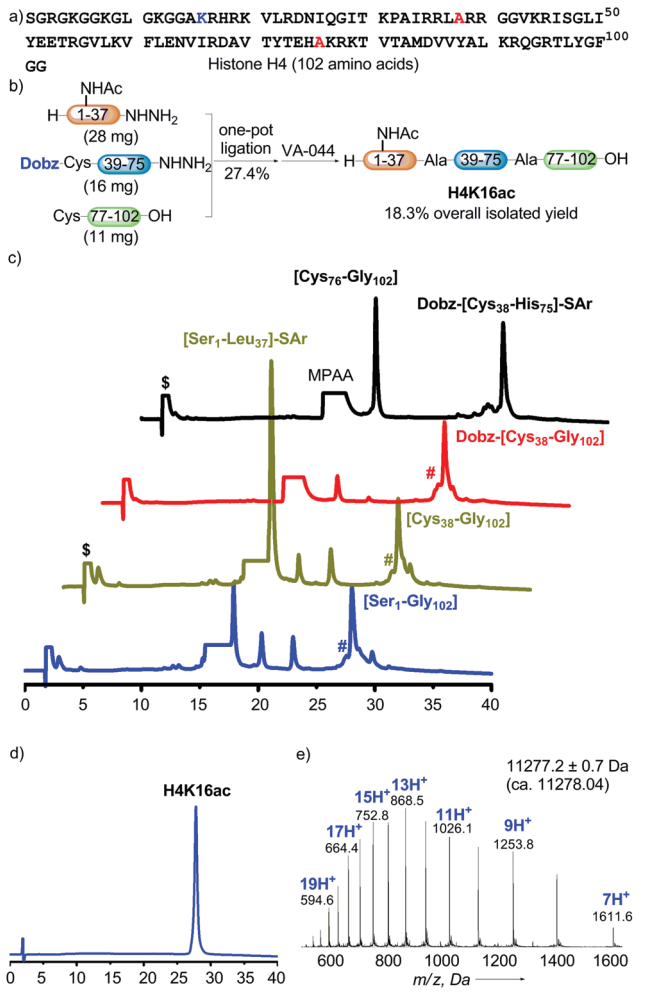


**Fig. 6** Synthesis of H4K16ac by using $H_2O_2$-controlled one-pot ligation of peptide hydrazides. (a) Sequence of H4. (b) The overall procedure. (c) Analytical HPLC traces ($\lambda$ = 214 nm) for the different steps in the one-pot ligation. Black: beginning of the first ligation; Red: reaction mixture after the first ligation; Olive: ligation mixture after the $H_2O_2$ treatment; Blue: the final ligation mixture. # correspond to the hydrolysis by-product. Red: Dobz-[$Cys_{38}$-$His_{75}$]-OH; Olive: [$Cys_{38}$-$His_{75}$]-OH; Blue: [$Ser_1$-$His_{75}$]-OH."$" denotes solvent fronts. For explanation of other peaks, please refer to Fig. S20.† (d) Analytical HPLC trace ($\lambda$ = 214 nm) for the final, purified H4K16ac. (e) ESI-MS: observed mass 11 277.2 ± 0.7 Da, calc. 11 278.04 Da (average isotopes).

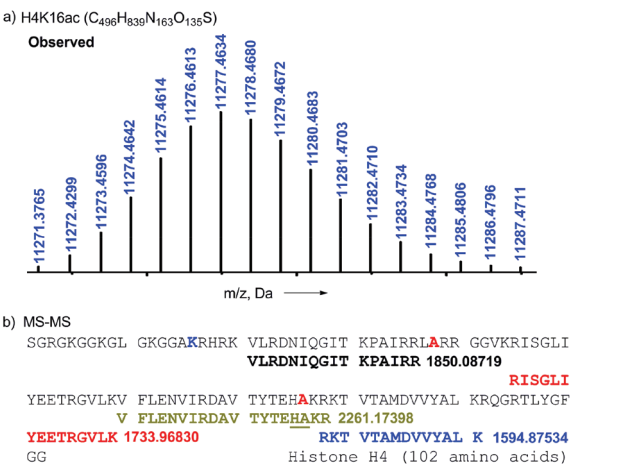


**Fig. 7** Isotope envelopes and MS-MS analysis of synthetic H4K16ac. (a) The observed isotope envelopes. (b) The MS-MS analysis with the observed fragments marked. For more details, please see Fig. S22 and S23.†

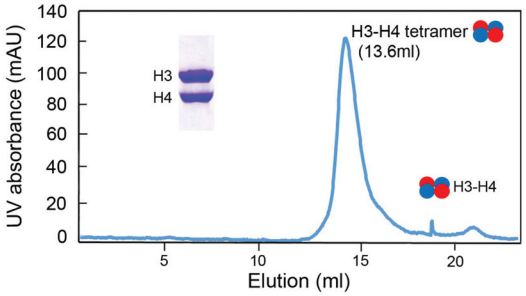


Fig. 8 Histone tetramer formation of synthetic H3K4me3 and H4K16ac. Blue curve: gel filtration profile of histone H3–H4 tetramer eluted over a Superdex 200 10/300 column. The peak of H3–H4 tetramer was eluted at 13.6 ml. Left to the peak: SDS-PAGE of the H3–H4 tetramer peak stained by coomassie brilliant blue.

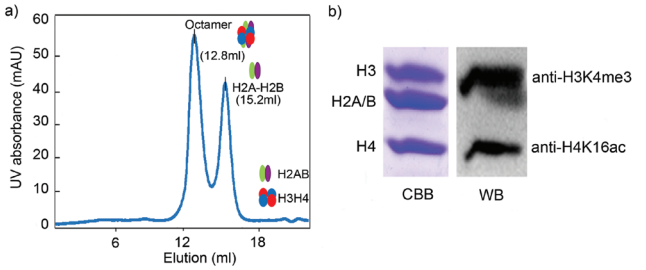


Fig. 9 Histone octamer reconstitution of synthetic H3K4me3–H4K16ac tetramer with H2A–H2B dimer. (a) Gel filtration profile of histone octamer eluted over a Superdex 200 10/300 column. Histone octamer was eluted at 12.8 ml; excess H2A–H2B dimer was eluted at 15.2 ml. (b) SDS–PAGE (left) and Western blotting (right) analysis of the reconstituted histone octamer. CBB: coomassie brilliant blue; WB: Western blotting.

the characterization confirmed that the synthetic H4K16ac was highly homogenous and the potential side reaction of oxidation was not a problem.

### Histone octamer reconstitution

The purity and identity of the synthetic H3K4me3 and H4K16ac were successfully characterized by the HPLC, MALDI-TOF and ESI-MS analyses, as well as the isotope envelope and MS-MS analyses (Fig. 4–7). To conduct biophysical characterizations, we dissolved the synthetic H3K4me3 and H4K16ac in an unfolding buffer containing 6 M guanidine hydrochloride, 5 mM dithiothreitol, and 20 mM Tris-HCl, pH 8.0. After 2 hours, the solution was subjected to four rounds of dialysis against a refolding buffer containing 2.0 M sodium chloride, 1 mM Na-EDTA, 5 mM dithiothreitol, and 20 mM Tris-HCl, pH 8.0 at 4 °C for about 48 hours. Gratifyingly, we identified and isolated the refolded H3K4me3–H4K16ac tetramer through gel filtration chromatography (Fig. 8).

With this tetramer in hand, we next mixed it with a pre-prepared recombinant histone H2A–H2B dimer at a molar ratio of 1 : 2.5. The mixture was then dialyzed against the refolding buffer over four rounds, for 48 hours at 4 °C. Through gel filtration chromatography over a Superdex 200 10/300 column (GE Healthcare), we successfully identified and isolated the histone octamer as a single mono-dispersed peak eluted at 12.8 ml (Fig. 9a), a retention volume characteristic for the histone octamer.[21] Furthermore, the identities of the H3K4me3 and H4K16ac in the reconstituted histone octamer were confirmed by Western blotting, using rabbit anti-H3K4me3 and anti-H4K16ac antibodies (Fig. 9b). Collectively, these experiments verified that our synthetic H3K4me3 and H4K16ac had the correct biophysical activities that could form the histone octamer smoothly and be recognized by antibodies.

## Conclusion

Here, we report a practical method for the one-pot native chemical ligation of peptide hydrazides. This method made use of a novel application of the Dobz protecting group, which was removable under mild conditions by a fast and selective arylboronate oxidation reaction mediated by $H_2O_2$, thus drawing attention to the interesting potential of using boron-based functionalities in protein chemical synthesis. Our experiments showed that the $H_2O_2$-based deprotection, when carefully controlled, was fast, clean and compatible with all the proteinaceous amino acids including Cys, Met, Trp, and Tyr. Thus, the present one-pot approach adds a new tool to the arsenal for the total chemical synthesis of small to medium sized proteins, which may find a complementary or unique usefulness to certain protein molecules.

By using the new one-pot method we accomplished the total chemical synthesis of the modified histone proteins H3K4me3, H4K16ac, and several others[22] on a multi-milligram scale and with good homogeneity. Our results showed that such a synthesis was fast (time cost = *ca.* 2 weeks for each protein) and scalable with regular research laboratory equipment. Given the fact that the hydrazide-based ligation method is compatible with many post-translational modifications, including phosphorylation, ubiquitination and glycosylation, we expect that the present method may find applications in the studies on histone post-translational modifications. The application of the one-pot approach to the total synthesis of histone H2A and H2B, as well as the use of fully synthetic histones to study the biology of epigenetics, are ongoing in our laboratory.

## Acknowledgements

This work was supported by the national "973" grants from the Ministry of Science and Technology (2011CB965300 for L. Liu and H. Li), NSFC (91313301 and 21225207 for L. Liu and 31270763 for H. Li), and the Specialized Research Fund for the Doctoral Program of Higher Education (grant no. 20120002130004).

 

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