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Facile synthesis of colorimetric histone deacetylase substrates†

Alexander Dose,‡[a] Jan Oliver Jost,‡[a] Antje C. Spieß,[b] Petra Henklein,[c] Michael Beyermann[d] and Dirk Schwarzer*[a]

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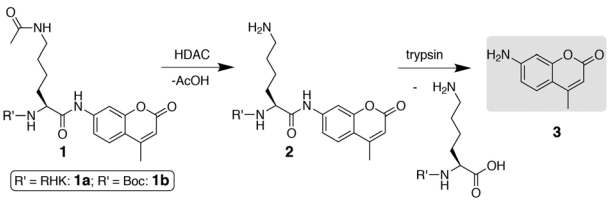
Here we report a simple procedure for generating colorimetric histone deacetylase (HDAC) substrates by solid-phase peptide synthesis based on racemization-free couplings of amino acid chlorides. We demonstrate the applicability of these substrates in HDAC assays.

Lysine acetylation constitutes one of the most prominent protein modifications found in eukaryotic organisms. Initially discovered in histone proteins, many more cellular targets of this modification are known today.[1] In the case of histones, which package DNA into chromatin, reversible lysine acetylation regulates gene activity of adjacent DNA.[2,3] Lysine acetylation is catalyzed by histone acetyl-transferases (HATs) and erased by two types of histone deacetylases (HDACs): Zn^{2+}-dependent HDACs and NAD^{+}-dependent sirtuins.[4–6] HDACs have gained considerable attention recently because they have been identified as promising drug targets for a number of human diseases including cancer.[7] At present two HDAC inhibitors (HDACis) have been approved for cancer therapy and it is likely that more of such drugs will be added to the medicinal repertoire in the near future. The development of HDACis relies on sophisticated assays for monitoring HDAC activity in a high-throughput format.[8,9] Suitable HDAC substrates should be easily accessible because recent reports indicate that residues in the vicinity of the acetylation site can influence the substrate recognition of HDACs.[10] The most frequently used type of assay couples the HDAC activity to that of the protease trypsin (Scheme 1).[11] The employed substrates contain an acetylated Lys moiety (Lys(Ac)) bound to the aromatic amine of a fluorophore (or chromophore) as in **1**. In this setting the fluorescence of the fluorophore is efficiently quenched and lysine acetylation protects the adjacent amide bond from proteolysis by trypsin. Adding HDACs converts **1** to **2** and facilitates the cleavage of the respective amide bond in **2**.



Scheme 1 Basic concept of commonly used HDAC assays.

After the addition of trypsin, the fluorescence of released **3** directly reports the prior deacetylation reaction.[11]

This type of assay is widely used and has been commercialized, but the synthesis of the required substrates is challenging. Established protocols condense Lys(Ac)-fluorophore (or chromophore) conjugates as a single building block with the C-terminus of protected peptides in solution.[11,12] This procedure is both inconvenient compared to stepwise solid-phase peptide synthesis (SPPS), and limiting with regard to the potential position of the Lys(Ac)-chromophore unit, which can only be installed at the very C-terminus of the HDAC substrates. A reported alternative employs pairs of fluorophores and quenchers installed at the N- and C-termini of the HDAC substrate and a central Lys(Ac) residue.[13] In this case alternative trypsin cleavage sites at Lys and Arg residues, which make up 20–25% of all histone amino acids, cause a fluorescence increase which is not related to the deacetylation reaction. A simple strategy for installing Lys(Ac) residues next to a reporting fluorophore or chromophore at any given position within a peptide substrate by means of SPPS would overcome the above discussed limitations.

Here we report a versatile procedure for the synthesis of colorimetric HDAC substrates by SPPS using the Fmoc/tBu strategy. We devised a colorimetric readout mechanism based on the *p*-nitroaniline derivative 5-amino-2-nitro-benzoic acid (5,2-ANB), which has been successfully applied to trypsin assays. Consistent with other reports, we observed that this moiety could be coupled to a resin-bound amino acid without protection of the 5-amino group.[14–16] This is due to the low nucleophilicity of this amino group, which is also the major obstacle to using 5,2-ANB in SPPS. Since acid halides are known to form this amide bond, we turned to amino acid chlorides, which are rarely used in SPPS due to their 'over-activated' nature.[15–17] Chlorides of 9-fluorenylmethyloxycarbonyl (Fmoc) and other urethane-protected amino acids tend to form oxazolones in the presence of a base.[18] Oxazolones are less reactive than their corresponding chlorides and their

[a] *Interfaculty Institute of Biochemistry, University of Tuebingen, Hoppe-Seyler-Str. 4, 72076 Tuebingen, Germany. E-mail: dirk.schwarzer@uni-tuebingen.de; Fax: +49-7071-29-4518; Tel: +49-7071-29-73344*
[b] *RWTH Aachen, Worringer Weg 1, 52056 Aachen, Germany*
[c] *Institut für Biochemie, Charité - Universitätsmedizin Berlin, Oudenarder Straße 16, 13347 Berlin, Germany*
[d] *Leibniz-Institut für Molekulare Pharmakologie (FMP), Robert-Roessle-Str. 10, 13125 Berlin, Germany*
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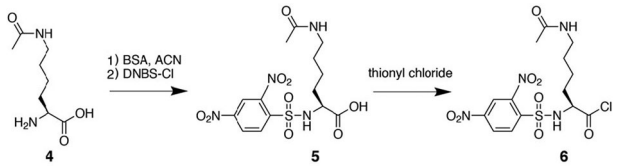
‡ These authors contributed equally to this work.



Scheme 2 Synthesis of DNBS-Lys(Ac)-Cl.

formation might even be reversible, which raises concerns about racemization of the Lys(Ac) moiety.[19,20] In contrast to urethane-based protection groups, *N*-arenesulfonyl protectants cannot form oxazolones from amino acid halides.[21] Furthermore, the greater inductive effect of the sulfonyl moiety increases the reactivity of the respective acid chlorides. Consequently, we selected the 2,4-dinitrobenzenesulfonyl (DNBS) group as a protectant for the Lys(Ac) building block.[22] This protecting group can be removed with nucleophilic thiols under mild conditions, which are fully compatible with SPPS protocols.

The synthesis of DNBS-Lys(Ac)-OH began with commercially available Lys(Ac)-OH **4**, which was first subjected to transient Nα silylation by treatment with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (Scheme 2).[23] The advantages of this course of action were two-fold: the silylation activated the Nα amino group for the subsequent installation of the DNBS group and also mitigated the otherwise poor solubility of Lys(Ac)-OH **4** in organic solvents (here acetonitrile). The addition of 2,4-dinitrobenzenesulfonyl chloride (DNBS-Cl) converted silylated **4** into the desired product DNBS-Lys(Ac)-OH **5**, which was obtained in excellent yield (ESI†). Subsequently, we established a SPPS protocol based on *in situ* formed DNBS-Lys(Ac)-Cl **6** obtained by treating **5** with thionyl chloride (Table S1, ESI†) and achieved 72% conversion when **6** was coupled to resin-bound 5,2-ANB under optimized conditions (ESI†). Deprotection of the DNBS group with thiophenolate completed the incorporation of the Lys(Ac)-5,2-ANB unit and subsequent couplings were performed under standard SPPS conditions.

Based on this scheme we synthesized the colorimetric HDAC substrate p53-5,2-ANB (**7**) covering residues 378 to 385 of the tumor suppressor protein p53. Acetylated lysine was introduced at the well-established modification site Lys382 directly upstream of 5,2-ANB (Fig. 1a).[24] To ensure that the synthesis procedure preserved the chiral integrity of the coupled Lys(Ac) residue **7** was subjected to chiral amino acid analysis, which confirmed an optical purity of 99.5% of the L-enantiomer (identical with the starting material).

In the following, we tested **7** as an HDAC substrate (Fig. 1a and Fig. S1, ESI†). Sir2.1 from *C. elegans* belongs to the NAD^+-dependent HDACs of class III, also referred to as sirtuins, which are known to deacetylate modified Lys382 of p53.[25] We treated 100 μM of **7** with recombinant Sir2.1 (1 μM) in the presence of NAD^+. The reaction was stopped after 5 minutes by adding nicotinamide, a known inhibitor of sirtuins. In a first instance we analyzed the deacetylation reaction by HPLC. After 5 minutes, 44% of **7** was deacetylated to **8**, and addition of trypsin converted **7** to **9** and deacetylated **8** to the yellow product **10** (Fig. 1b–d). Next we confirmed that the reaction could be monitored by optical readouts at 405 nm, an optimal wavelength for 5,2-ANB in **10** (Fig. S2, ESI†).

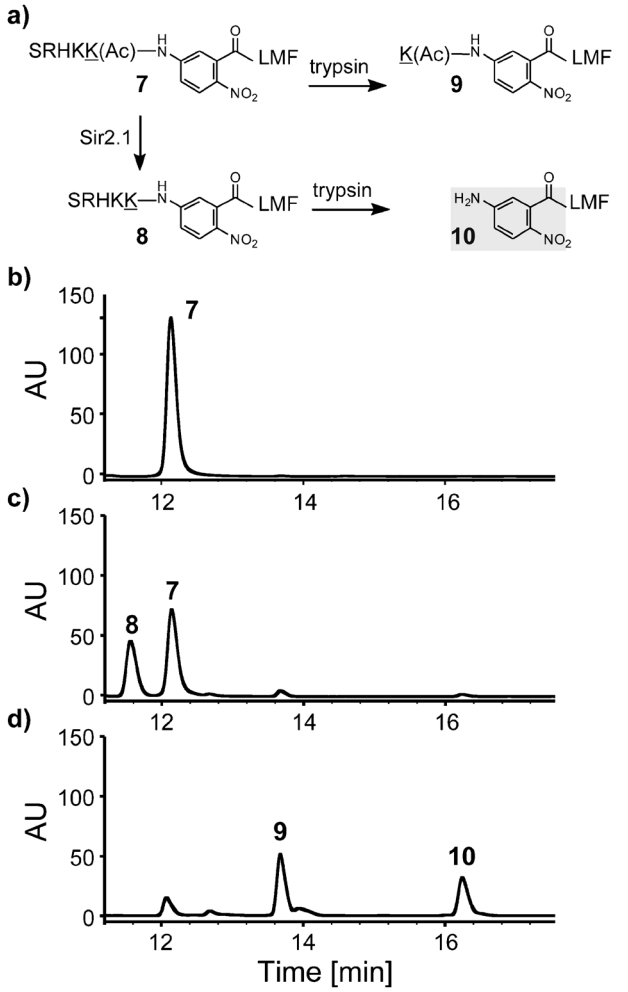


Fig. 1 Deacetylation reactions monitored at 340 nm by HPLC. (a) Products formed after Sir2.1-catalyzed deacetylation and subsequent cleavage with trypsin. (b) Chromatogram of **7**. (c) Chromatogram of the Sir2.1-catalyzed deacetylation reaction of **7** after 5 minutes of reaction time. (d) Chromatogram of the deacetylation reaction after tryptic digestion. Incubation time with trypsin was selected based on quantitative conversion of **8** to **10**.

We continued to study the properties of **7** in deacetylation assays and performed a quantitative comparison of **7** and p53-AMC (**1a**), a commercially available p53-derived HDAC substrate that uses 7-amino-4-methylcoumarin (AMC) for fluorescence readouts of deacetylase activities (Fig. 1). In these assays we used the human homolog of Sir2.1, SIRT1, which is known to deacetylate **1a** and analyzed the deacetylation activity of **7** and **1a** in parallel. Kinetic parameters were extracted from the progress curves with the incremental method taking product inhibition into account (ESI†).[26] We observed similar V_{max}/K_m values of 0.0128 min^{-1} and 0.0121 min^{-1} for **7** and **1a**, respectively, with comparable levels of product inhibition (Fig. S3 and Table S2, ESI†). Based on this, we concluded that **7** performs equally well in deacetylation assays than the commercially available **1a**.

SIRT1 and its homologs have gained considerable attention because elevated cellular levels of these enzymes have been found to induce longevity in a variety of organisms.[27] Consequently, efforts have been put forth to screen for small

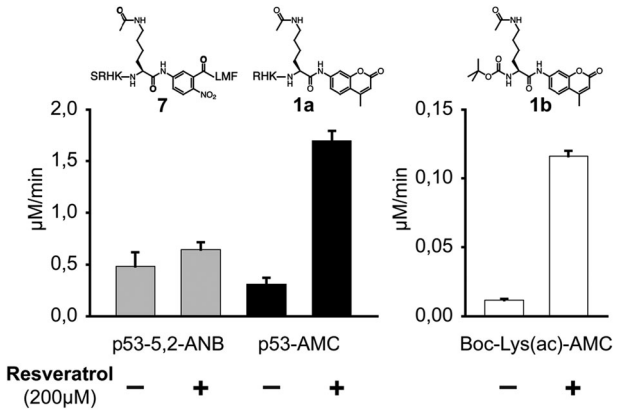


Fig. 2 Resveratrol activation of SIRT1 with 100 μM of p53-5,2-ANB (**7**), p53-AMC (**1a**) and Boc-Lys(Ac)-AMC (**1b**).

molecule activators of human SIRT1, which ultimately led to the discovery of resveratrol.[28] However, subsequent investigations indicated that this effect depends on substrates containing hydrophobic fluorophores like AMC, rendering the physiological relevance of this effect questionable.[12,29] We reasoned that **7** might be less affected by its reporter group because 5,2-ANB is less hydrophobic than AMC and fully imbedded into the peptide. We analyzed the SIRT1-catalyzed deacetylation of **7** side by side with **1a** and **1b**, both of which are conjugated to AMC. As predicted from the kinetic analysis the deacetylation rates of **7** and **1a** were similar, but the deacetylation of **1b**, which does not contain any residues flanking the acetylation site, was about 10-fold less effective (Fig. 2). In the presence of 200 μM resveratrol we detected a 5–7-fold rate acceleration for the deacetylation of **1a** and **1b**. In contrast the deacetylation of **7** showed no significant rate enhancements upon addition of resveratrol. This observation supports the notion that the direct activation of SIRT1 by resveratrol depends on AMC and may not occur with physiological substrates.

In conclusion we have established a simple and general synthesis procedure for peptide based HDAC substrates. We have demonstrated the applicability of these substrates for colorimetric readouts of HDAC activities and could show that these assays perform as well as commercially available substrates but appear to be less prone to artefacts. The convenience of the synthesis procedure allows for a greater flexibility in the designing process of HDAC substrates with respect to the amino acids flanking the acetylation site. This feature might be advantageous for future investigations into enzymatic deacetylation properties of the 3600 recently identified lysine acetylation sites of the human proteome.[1]

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