

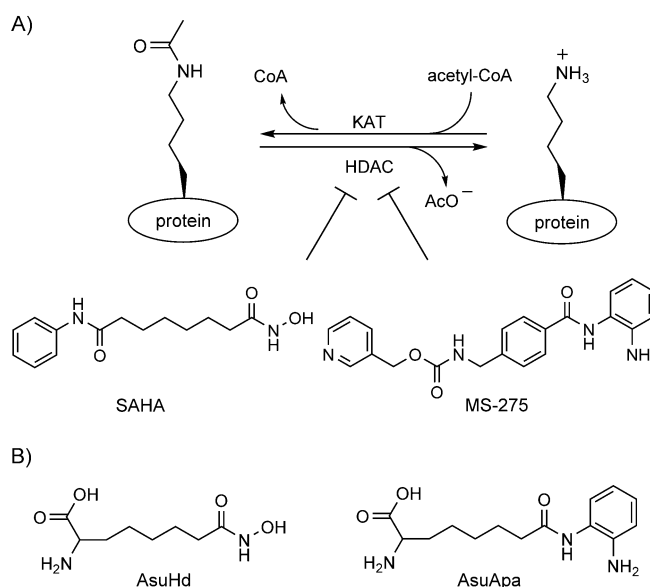
Peptide-Based 2-Aminophenylamide Probes for Targeting Endogenous Class I Histone Deacetylase Complexes

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Lysine deacetylases or histone deacetylases (HDACs) remove acetylation markers from numerous cellular proteins, thereby regulating their function and activity. Recently established peptide probes containing the HDAC-trapping amino acid α -aminosuberic acid ω -hydroxamate (AsuHd) have been used to investigate the compositions of HDAC complexes in a site-specific manner. Here we report the new HDAC-trapping amino acid 2-amino-8-[(2-aminophenyl)amino]-8-oxooctanoic acid (AsuApa) and the utility of AsuApa-containing probes for HDAC complex profiling on a proteome-wide scale. Unlike AsuHd-containing probes, AsuApa enriched only HDACs 1, 2, and 3 efficiently and was the most potent probe tested for capturing the last of these. These findings indicate that the inherent specificity of reported small-molecule pimelic diphenylamide HDAC inhibitors is preserved in AsuApa and that this HDAC-trapping amino acid represents a potent tool for investigating class I HDAC complexes.

Lysine acetylation is a frequent and reversible posttranslational modification (PTM) of eukaryotic proteomes.^[1] This PTM was first discovered on histones, where it plays a central role in regulating transcription and chromatin condensation states. More recent investigations have shown that lysine acetylation also features in a multitude of non-histone proteins and represents one of the most abundant PTMs in nature.^[2] Lysine acetylation is installed through the action of lysine acetyltransferases (KATs) and removed through that of lysine deacetylases or histone deacetylases (HDACs, Scheme 1 A).

The Zn²⁺-dependent HDACs (hereafter referred to simply as HDACs) form a major family among the deacetylases and cleave the *N* ϵ -amides by direct hydrolysis of the amide bond.^[3] This mechanism is distinct from that of NAD⁺-dependent sirtuins, which form a further major deacetylase family.^[4] HDACs



Scheme 1. A) Histone deacetylases (HDACs) remove acetyl groups of modified lysine residues and can be inhibited by small-molecule HDAC inhibitors such as SAHA and MS-275. B) HDAC-trapping amino acids AsuHd and the newly established AsuApa.

can be further subdivided into classes I, IIa, IIb, and IV by homology to yeast HDACs Rpd3 and HD1.

Class I HDACs consist of HDACs 1, 2, 3, and 8. With the exception of HDAC8 these enzymes are confined to the nucleus, where they regulate transcription through deacetylation of histones and also of non-histone proteins.^[5]

HDACs of class II shuttle between nucleus and cytoplasm and are further divided into the poorly active class IIa enzymes (HDACs 4, 5, 7, and 9) and the active class IIb HDACs (HDAC6 and HDAC10).^[5] Whereas HDAC6 is an abundant lysine deacetylase with many cytosolic substrates, HDAC10 was reported to possess polyamine deacetylase activity.^[6]

Finally, little information is available about the physiological role of HDAC11, the only known member of the class IV HDACs. However, recent publications have shown that this enzyme is capable of removing long-chain *N*-acyl modifications from lysine residues.^[7]

HDACs represent promising drug targets, due to their regulatory function in chromatin, signaling, and metabolism, and several HDAC inhibitors, including the FDA-approved suberoylanilide hydroxamic acid (SAHA), have been developed (Scheme 1 A).^[3,5] However, HDACs are known to form multiprotein complexes, and many components of HDAC complexes

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potentially modulate activity and specificity of the imbedded deacetylases, thereby complicating biochemical research.^[8] Strategies for profiling HDAC complexes in a substrate-site-dependent manner have been established.^[9] HDAC-trapping amino acids, such as AsuHd, the hydroxamic acid derivative of 2-aminosuberic acid, were developed for replacing acetyllysine residues in peptide probes derived from known acetylation sites (Scheme 1 B).^[10]

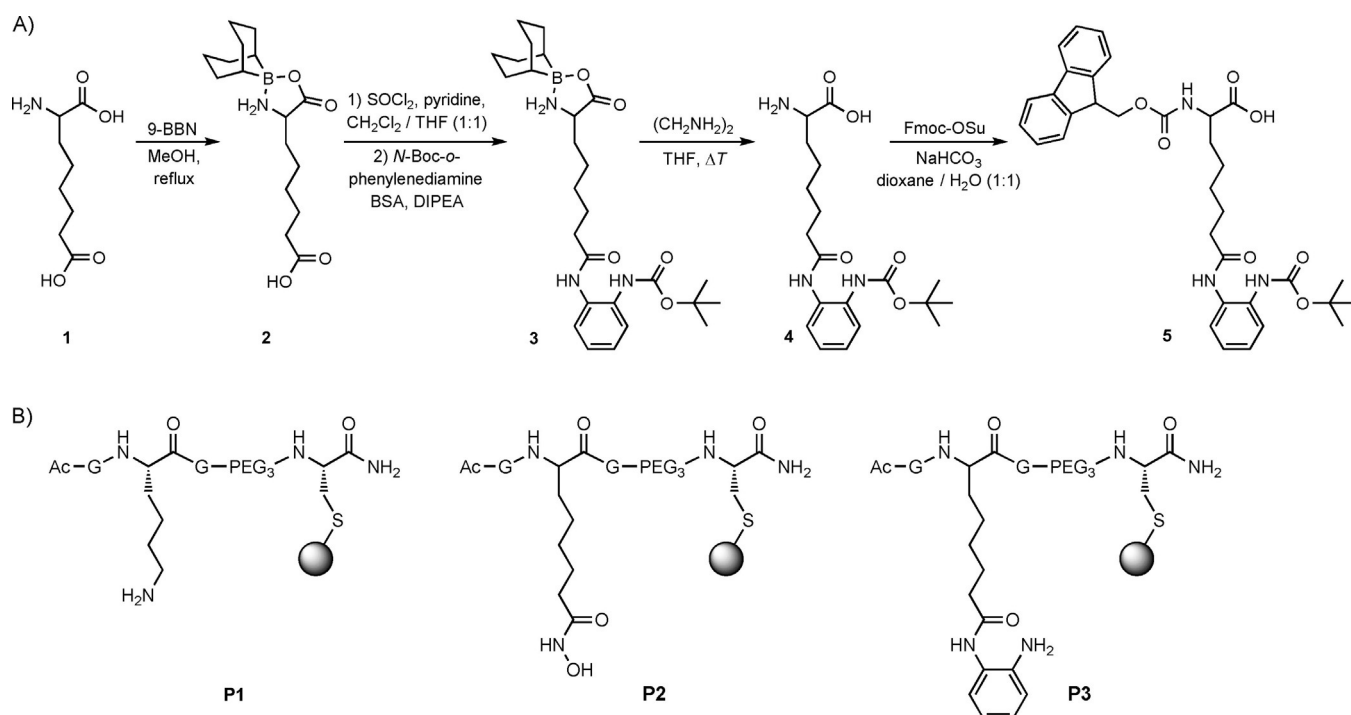
When combined with MS-based methods of interactome profiling, these peptide-based probes can uncover the compositions of HDAC complexes in their associated peptide substrate contexts. However, HDAC-trapping amino acids likely possess an inherent selectivity for HDACs themselves, so a broad set of HDAC-trapping amino acids is desirable for optimal targeting of individual HDAC complexes.

Here we report the synthesis and characterization of the HDAC-trapping amino acid Δ L-2-amino-8-[(2-aminophenyl)amino]-8-oxooctanoic acid (AsuApa), designed on the basis of the HDAC inhibitor MS-275 (Scheme 1).^[11] AsuApa has previously been installed into a cyclic peptide HDAC inhibitor by an on-resin strategy.^[12] We have followed a different approach, by synthesizing a protected AsuApa building block for solid-phase peptide synthesis (SPPS). This appeared more flexible for the design of peptide-based HDAC probes. The 2-aminophenylamide-containing Δ L-AsuApa was synthesized from Δ L-2-aminosuberic acid (**1**, Scheme 2 A). The α -amino and -carboxylic acid groups were first protected by complexation with 9-borabicyclo[3.3.1]nonane (9-BBN) to form **2**, followed by acyl chloride formation at the side chain with thionyl chloride.^[13] The freshly formed acyl chloride was coupled with *N*-Boc-1,2-phenylenediamine, resulting in 9-BBN-protected AsuApa(Boc) (**3**). Treatment of **3** with ethylenediamine to remove 9-BBN resulted in

4, and subsequent installation of the fluorenylmethoxycarbonyl (Fmoc) protecting group with Fmoc *N*-hydroxysuccinimide ester (Fmoc-OSu) furnished the Fmoc-AsuApa(Boc)-OH (**5**) building block (Scheme 2 A).

With Fmoc-AsuApa(Boc)-OH to hand we set out to synthesize the corresponding AsuApa-containing peptide probe mini-AsuApa (**P3**, Scheme 2 B). Previous reports have shown that probes containing α -aminosuberic acid ω -hydroxamate (AsuHd) efficiently recruit HDAC complexes of class I and IIb from lysates of mammalian cells. The general design of this mini-AsuHd probe (**P2**) consisted of the AsuHd building block flanked by glycine residues and a C-terminal poly(ethylene glycol) (PEG) and cysteine extension used for immobilization on a solid support (Scheme 2 B). Mini-Lys probe **P1**, with AsuHd replaced by a lysine residue, did not recruit HDACs and served as control. Mini-AsuApa (**P3**) was designed in the same way with AsuApa as the central amino acid. The peptide was assembled on the solid support by standard SPPS methods, Fmoc-AsuApa(Boc)-OH being fully compatible with these conditions. After synthesis the mini-AsuApa peptide was purified by HPLC and immobilized on iodoacetyl-modified agarose to form the mini-AsuApa probe. Mini-AsuHd and mini-Lys peptides and probes were synthesized as reported previously.

We analyzed the ability of mini-AsuApa, in comparison with mini-AsuHd and mini-Lys probes, to recruit endogenous HDAC complexes from cellular lysates. Native lysate of HeLa cells was used as input at a fixed concentration of 1 mg mL⁻¹ (200 μ g total protein). Upon pull-down, the resins were washed, and proteins recruited to either of these probes were eluted, resolved on SDS-PAGE, and analyzed by western blot. The blots were probed with antibodies against all HDACs of class I and class IIb and against HDAC4 as a representative member of the



Scheme 2. A) Synthesis of Fmoc-AsuApa(Boc)-OH (**5**). B) Peptide-based probes mini-Lys (**P1**), mini-AsuHd (**P2**), and mini-AsuApa (**P3**).

class IIa enzymes. Consistent with previous reports, we observed that all endogenous HDACs of classes I and IIb bound mini-AsuHd, but not mini-Lys (Figure 1). Recruitment of HDAC4 to mini-AsuHd was weak and barely detectable. Mini-AsuApa showed a different binding pattern from mini-AsuHd and did not recruit class IIb enzymes, HDAC8, or HDAC4. However, efficient binding of the other class I enzymes—HDAC1, HDAC2, and HDAC3—was observed (Figure 1). This binding pattern is reminiscent of the reported specificity of benzamide HDAC inhibitor MS-275 (Entinostat), which inhibits HDAC1 and HDAC3 strongly, but not HDAC2 and class II HDACs. The poor recruitment of HDAC4 to mini-AsuApa also fits the specificity profile of MS-275 (Figure 1).^[14]

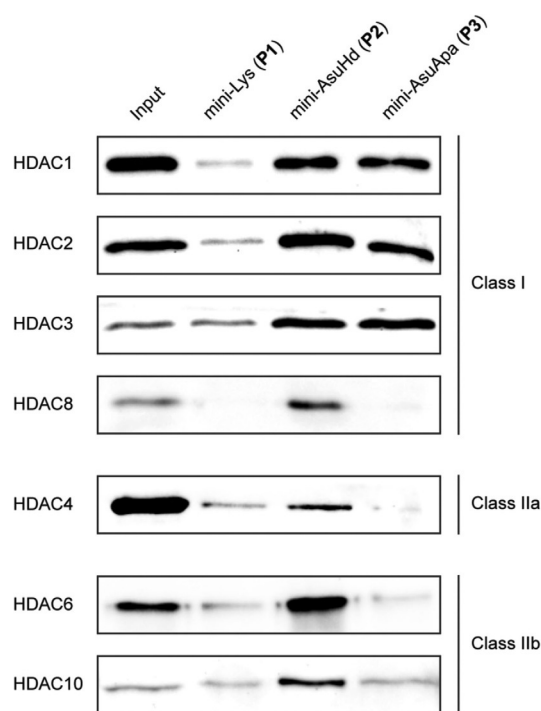


Figure 1. Western blot analysis of HDAC pull-down experiments from HeLa whole cell lysates. Proteins were eluted in a volume of 20 μ L and quantitatively loaded into the SDS-PAGE; 16 μ g HeLa lysate was used for input samples. The blots were probed with antibodies against HDACs 1, 2, 3, 4, 6, 8, and 10.

To obtain quantitative information about the HDAC binding properties of mini-AsuApa and the compositions of recruited HDAC complexes, we performed a chemical proteomics profiling of mini-AsuApa, in comparison with mini-AsuHd and mini-Lys, by label-free LC-MS/MS analysis. Pull-downs from HeLa whole cell lysates were performed as before, but eluted proteins were alkylated at cysteine residues, subjected to tryptic digestion, and identified and relatively quantified by LC-MS/MS with use of the MaxLFQ algorithm of the MaxQuant software package (Tables S1–S3 in the Supporting Information).^[15] Proteins enriched on mini-AsuHd and mini-AsuApa, in comparison with mini-Lys, from three biological replicates were visualized by use of volcano plots (Figure 2A and B). The \log_2 -fold enrichment ratios of mini-AsuHd and mini-AsuApa versus mini-Lys

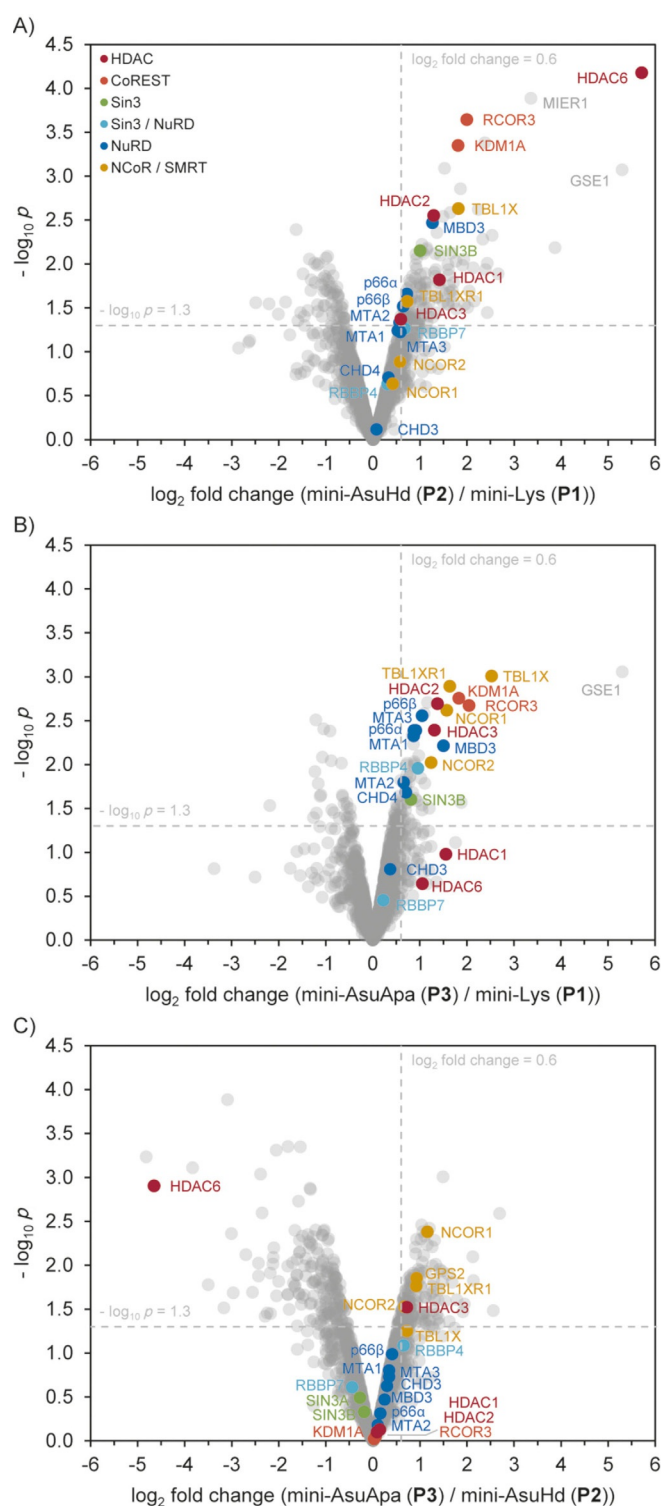


Figure 2. A) Volcano plot of proteomic pull-downs of mini-AsuHd (P2) versus mini-Lys (P1). B) Volcano plot of pull-downs of mini-AsuApa (P3) versus mini-Lys (P1; out of scale: MIER1 \log_2 -fold change = 3.7, $-\log_{10} p = 5.1$) C) Volcano plot of pull-downs of mini-AsuApa (P3) versus mini-AsuHd (P2). The p value of the statistical analysis (limma) is plotted in negative logarithmic scale against the average \log_2 -fold change of enrichment. Cut-offs for statistical significance and enrichment were set at $p \leq 0.05$ and \log_2 -fold enrichment ≥ 0.6 . All experiments were performed as biological triplicates. Gray-labeled proteins have been proposed as HDAC binding proteins that do not belong to the CoREST (orange), Sin3 (green), NuRD (blue), or NCoR/SMART (yellow) complexes.

were plotted against the negative $\log_{10} p$ value, showing significantly enriched proteins in the upper right-hand sections of the volcano plots. Statistical analysis was performed with proteins identified and quantified in all three biological replicates—including HDAC1, HDAC2, HDAC3, and HDAC6—that fulfilled these criteria. Consistently with previous reports we observed that all of the above HDACs were significantly enriched on mini-AsuHd in comparison with mini-Lys.^[10] HDAC6 shows the strongest enrichment of all detected proteins. Furthermore, proteins of known complexes of HDAC1 and HDAC2, including RBBP7 and p66 of NuRD and Sin3A complexes, as well as KDM1A and RCOR3 of the CoREST complex, were enriched on mini-AsuHd.^[16] Components of the latter complex showed stronger enrichment than proteins of the former. HDAC3 was also enriched on mini-AsuHd, but not to the same extent as HDACs 1, 2, and 6. This pattern is also reflected in the enrichment of components of the NCoR/SMRT complex, such as NCOR1, NCOR2, and TBL1X, which contains HDAC3 as catalytic subunit.^[17]

The volcano plot of the interactome of mini-AsuApa versus mini-Lys showed a different binding profile from that of mini-AsuHd (Figure 2B): HDAC6 was only marginally enriched on mini-AsuApa; this is consistent with the western blot analysis (Figure 1). A major fraction of proteins enriched on mini-AsuApa belongs to the NuRD/Sin3A and CoREST complexes; this further supports the notion that mini-AsuApa serves as a probe for HDACs 1 and 2. Furthermore, HDAC3 and proteins of the corresponding NCoR/SMRT complex bound strongly to mini-AsuApa, and this interaction appeared more pronounced than the corresponding enrichment on mini-AsuHd. NCoR/SMRT proteins TBL1X and NCOR1, for example, rank among the most highly enriched proteins identified with mini-AsuApa. Plotting of the enrichment of proteins on mini-AsuApa against mini-AsuHd (Figure 2C) allowed a more direct comparison of the binding properties of both probes. Proteins enriched on mini-AsuApa are located in the upper right-hand section of the plot, whereas proteins enriched on mini-AsuHd are found in the upper left-hand section. Proteins binding evenly to both probes are confined to the center (Figure 2C). HDAC6 is located in the upper left-hand segment of the plot, thus confirming that mini-AsuApa is not capable of efficient HDAC6 recruitment. Proteins of the NuRD, Sin3A, and CoREST complexes including HDACs 1 and 2 are located in the center, thus indicating comparable recruitment to mini-AsuHd and mini-AsuApa. Proteins of the NCoR/SMRT complex are primarily located in the upper right-hand section of the plot, thus showing that mini-AsuApa is a superior probe recruiting HDAC3 and complexes imbedding HDAC3.

Collectively, the interactome studies and western blot analysis showed that AsuApa serves as a probe for complexes of HDACs 1, 2, and 3 when imbedded in a peptide scaffold. No significant interactions with other HDACs were observed. Furthermore, mini-AsuApa was more potent than mini-AsuHd in enriching HDAC3 and NCoR/SMRT complex proteins. The binding properties of mini-AsuApa differ from the inhibitory properties of the parent MS-275, which inhibits both HDAC1 and HDAC3 with similar efficiency.^[14] Furthermore, activity-based

probes derived from MS-275 interacted only weakly with endogenous HDACs of HeLa nuclei lysates.^[18] The *o*-phenylenediamine moiety is linked to an aliphatic carboxylic acid in AsuApa, unlike in the MS-275 scaffold, in which this moiety is coupled to an aromatic benzoic acid derivative. This observation prompts the conclusion that the aliphatic side chain of AsuApa is important for HDAC3 selectivity; this is also supported by pimelic diphenylamide inhibitor 106, which displays HDAC binding properties similar to those of mini-AsuApa.^[19] Furthermore, an activity-based probe derived from compound 106 was shown to interact with HDAC3 most efficiently, thus indicating that the inherent selectivity of this small-molecule compound is also preserved in mini-AsuApa.^[19]

Peptide-based probes represent important tools for investigating the compositions of HDAC complexes in a substrate-site-dependent manner. HDAC-trapping amino acids commonly possess an inherent HDAC specificity, so the establishment of a set of such amino acids is important for covering all 11 mammalian HDACs. The initially introduced AsuHd binds all HDACs of class I and IIb.^[10] Further reported HDAC-binding amino acids include *N* ϵ -acetyl-*N* ϵ -hydroxy-L-lysine [Lys[N(O-H)Ac]], which interacts with HDACs 1, 2, and 6, and the recently reported trifluoromethyl ketone amino acid Atona, which targets HDACs of class IIa.^[20] With AsuApa, we now introduce a new HDAC-trapping amino acid that allows HDACs 1 and 2 and, in particular, HDAC3 and its binding proteins to be addressed. The abundance of HDACs and HDAC complexes can vary strongly between cells, thus imposing a major obstacle to targeting of low-abundance endogenous deacetylases. The enrichment of HDACs on peptide-based probes is determined by affinity and abundance of the enzymes, and low-abundance HDACs might be driven off pan-specific probes by high-abundance HDACs. Use of HDAC-specific trapping residues in peptide probes seems an attractive strategy for addressing this problem. This broad set of HDAC-trapping amino acids now available can be used to investigate the impact of acetylation sites on the composition of the HDAC complex by combining HDAC-trapping amino acids with peptide contexts derived from acetylation sites in a mix-and-match type of fashion.

Experimental Section

Asu-BBN (2): DL-2-Aminosuberic acid (**1**, 1.89 g, 10.0 mmol, 1 equiv) was suspended in anhydrous MeOH (60 mL) and heated at reflux under nitrogen. Subsequently, 9-BBN (0.5 M in THF, 22 mL, 11.0 mmol, 1.1 equiv) was added dropwise, and the mixture was heated until the suspension had cleared (approximately 2 h). The solvent was removed under reduced pressure, and the colorless, viscous residue was purified by preparative HPLC. The desired product **2** was obtained as a white powder (2.81 g, 9.09 mmol, 91%).

AsuApa(Boc)-BBN (3): Asu-BBN (**2**, 1.55 g, 5.00 mmol, 1 equiv) was dissolved in anhydrous CH₂Cl₂/THF (1:1, 100 mL) under nitrogen. After addition of pyridine (0.40 mL, 5.00 mmol, 1 equiv) and thionyl chloride (0.36 mL, 5.00 mmol, 1 equiv) the reaction mixture was stirred at room temperature for 1 h, resulting in the acid chloride of **2**.

In parallel, *N*-Boc-1,2-phenylenediamine (1.08 g, 5.19 mmol, 1.03 equiv) was dissolved in anhydrous CH₂Cl₂ (20 mL) under nitrogen, *N,O*-bis(trimethylsilyl)acetamide (2.43 mL, 10.0 mmol, 2 equiv) was added, and the mixture was heated under reflux for 1 h. The mixture was allowed to cool down to room temperature, followed by addition of DIPEA (3.48 mL, 20.0 mmol, 4 equiv).

The solution was added dropwise to the previously prepared acid chloride of **2**. The mixture was stirred overnight at room temperature and then concentrated under reduced pressure to yield crude product **3** as a reddish brown oil, which was used in the next step without further purification.

H-AsuApa(Boc)-OH (4): Crude AsuApa(Boc)-BBN (**3**, corresponding to 5.00 mmol of **2**) was dissolved in THF (20 mL), ethylenediamine (3.34 mL, 50.0 mmol, 10 equiv) was added, and the mixture was heated for 1 min below the boiling point. Further ethylenediamine (3.34 mL, 50.0 mmol, 10 equiv) was added and the heating procedure was repeated. The solvent was removed under reduced pressure and the residue was dissolved in MeCN/H₂O. The pH was adjusted to 6 with TFA and the crude product was purified by preparative HPLC, resulting in **4**, which was obtained as a red powder (403 mg, 1.06 mmol, 21% with respect to **2**).

Fmoc-AsuApa(Boc)-OH (5): H-AsuApa(Boc)-OH (**4**, 403 mg, 1.06 mmol, 1 equiv) and NaHCO₃ (403 mg, 1.06 mmol, 1 equiv) were dissolved in dioxane/H₂O (1:1, 40 mL), and Fmoc-OSu (371 mg, 1.10 mmol, 1.04 equiv) was added. The reaction mixture was stirred for 3 h at room temperature, concentrated under reduced pressure, and lyophilized. The reddish crude product was purified by flash chromatography with silica gel as stationary and MeOH/CH₂Cl₂ (1:19, v/v) as mobile phase. The desired product **5** was obtained as a colorless solid (375 mg, 0.62 mmol, 13% with respect to **1**).

Other experimental procedures, including general methods, SPSS, pull-down assays, chemical proteomics, and data processing, can be found in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: HDACs · lysine acetylation · peptide-based probes · protein modifications · proteomics

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