Synthesis and purification of photoreactive peptides targeting epigenetic enzymes

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Chromatography Application Note AN127

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Abstract

DNA and histone methylation/demethylation influences the regulation of gene expression at the transcriptional level. Precise regulation of methylation patterns provides fundamental protection against cellular transformation; consequently, the disruption of epigenetic patterns is a trademark for cellular abnormalities which can lead to cancer.[1] Despite the therapeutic significance of epigenetic disruptions, the development of potent, selective chemical biology tools remains challenging. Peptides are an emerging class of pharmacological molecules that are attractive in a therapeutic setting due to high affinities and high specificities that they can achieve against a variety of targets. This project discusses the synthesis, purification and characterization of histone peptides containing azide handles and photosensitive groups, and the use of these peptides to study the biological significance of histone modifying enzymes. The sequence of the histone peptides was chosen based on David Li et al. 2021[2]

Background

Solid-phase peptide synthesis (SPPS) is a popular and rapid method of peptide synthesis preferred over solution synthesis, as during SPPS all reactions can be carried out in the same vessel, and excess amino acids and reagents act as a driving force for the completion of the reaction. The excess amino acids and reagents can be filtered from the system, thus eliminating the need to purify the intermediates after each step. The solid-phase method consists of many repetitive steps, which makes automation possible. One disadvantage of SPPS is that the removal of peptide byproducts during synthesis is impossible.[3]

Results and discussion

Peptide synthesis

Peptides were synthesized using fluorenylmethyloxycarbonyl (Fmoc) Solid Phase Peptide Synthesis (SPPS) using N,N'-Diisopropylcarbodiimide/Oxyma as peptide coupling reagents and Rink-amide resin. Preparative high pressure liquid chromatography (HPLC) was needed to separate the peptide of interest from impurities.

Table 1: Sequence, molecular weight and exact mass of the peptides synthesized.
Legend: KN_3- azidolysine, KMe_3 – trimethyl lysine, Lp – photo-leucine.

Peptide ID	Sequence	Molecular formula	Exact mass/ Da
H3K4Me_3 C-KN_3	ARTKMe_3QTARKSTGGKAKN_3	$C_{75}H_{138}N_{29}O_{23}$	1812.06802
H3K4Me_3 A7Lp C-KN_3	ARTKMe_3QTLpRKSTGGKAKN_3	$C_{77}H_{141}N_{32}O_{22}$	1866.08982
H3K9Me_3 C-KN_3	ARTKQTARKMe_3STGGKAKN_3	$C_{75}H_{138}N_{29}O_{23}$	1812.06802
H3K9Me_3 A7Lp C-KN_3	ARTKQTLpRKMe_3STGGKAKN_3	$C_{77}H_{141}N_{32}O_{22}$	1866.08982

Peptide purification

The peptides were purified using a Teledyne ISCO ACCQ*Prep* HPLC system with a Phenomenex Gemini-NX 5 µm C18 110 Å 250x30 mm column with UV Detection at 220 nm. The histone peptides were purified using a 2-25% acetonitrile (MeCN) gradient, 0.1% TFA, for 30-45 min. Pure fractions were detected using MALDI-TOF mass spectrometry, and methods were adjusted to collect all samples within the time window in which the peptide was expected after an initial 'scouting' run for each peptide (Fig.1).

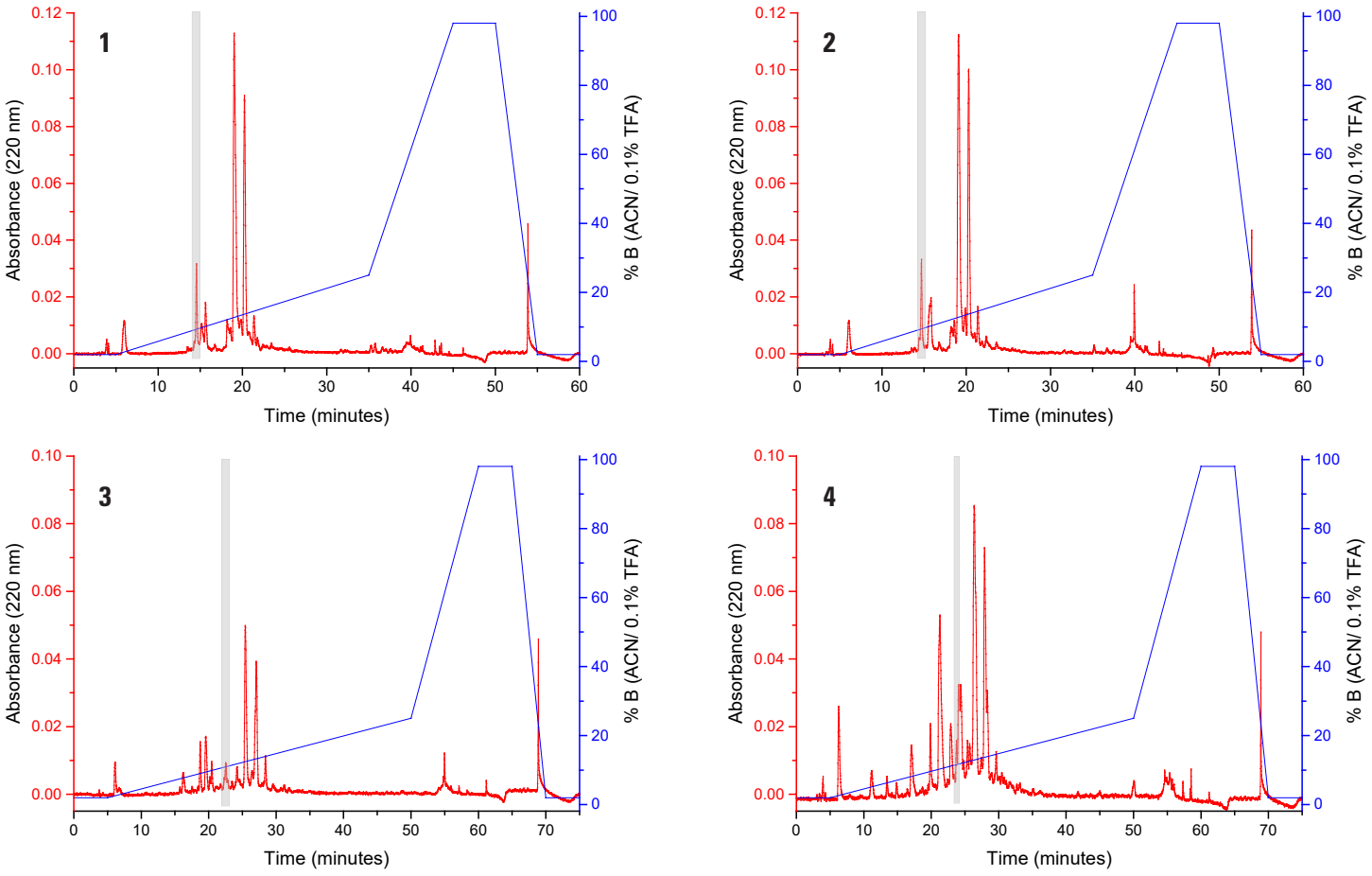

Figure 1: Preparative HPLC chromatogram of the H3 peptide series using optimised conditions.
(Phenomenex Gemini-NX 5 µm C18 110 Å 250x30 mm column with UV Detection at 220 nm, 2-25% MeCN gradient, 0.1% TFA.)
Legend:
1 - H3K4Me$_3$ C-KN$_3$
2 - H3K9Me$_3$ C-KN$_3$
3 - H3K4Me$_3$ A7Lp C-KN$_3$
4 - H3K9Me$_3$ A7Lp C-KN$_3$

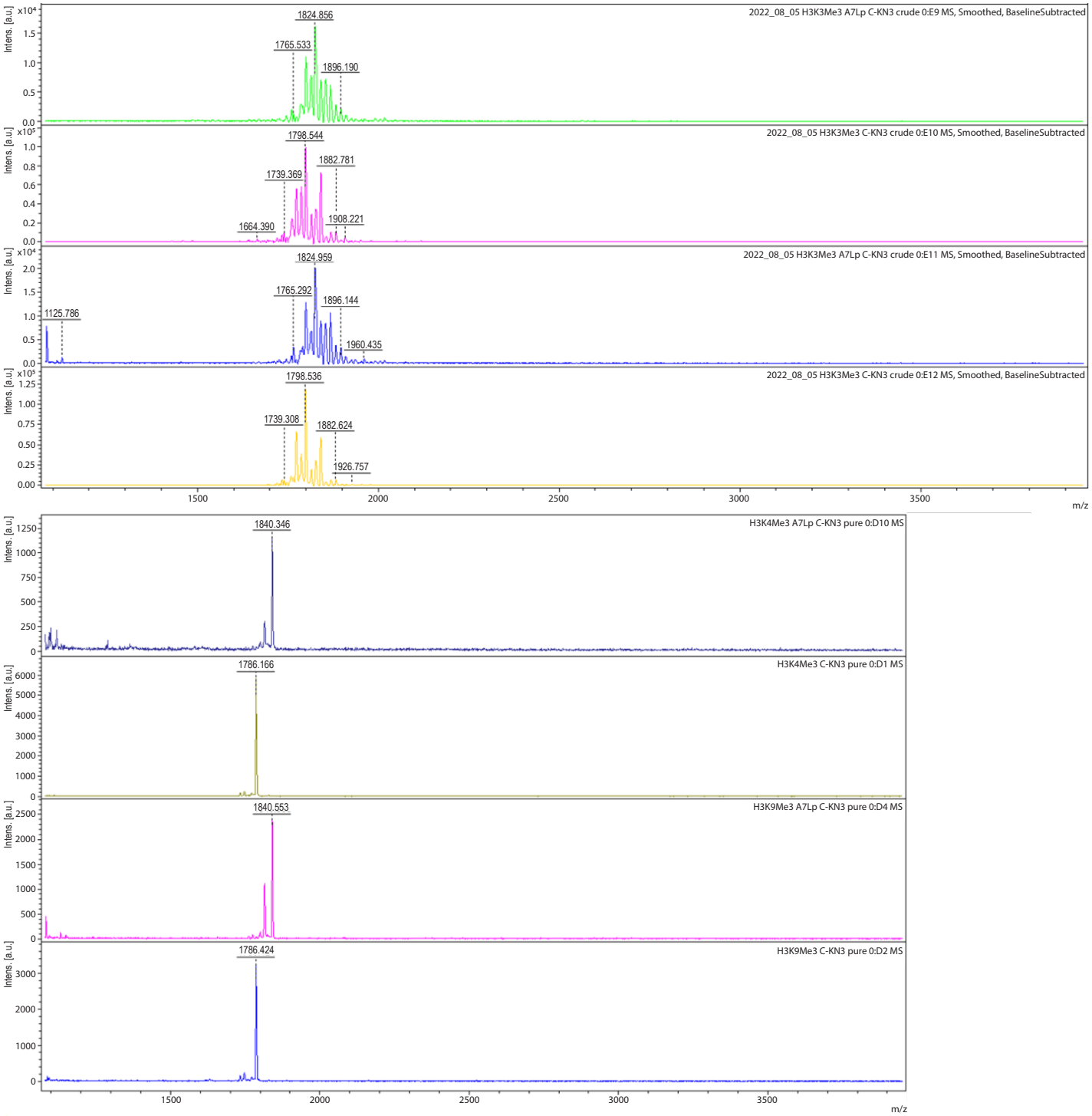

Figure 2: MALDI-TOF MS spectra of crude (a-d) and purified H3 peptide series (e-h).
Legend:
a - H3K4Me$_3$ A7Lp C-KN$_3$
b - H3K4Me$_3$ C-KN$_3$
c - H3K9Me$_3$ A7Lp C-KN$_3$
d - H3K4Me$_3$ C-KN$_3$
e - H3K4Me$_3$ A7Lp C-KN$_3$
f - H3K4Me$_3$ C-KN$_3$
g - H3K9Me$_3$ A7Lp C-KN$_3$
h - H3K4Me$_3$ C-KN$_3$

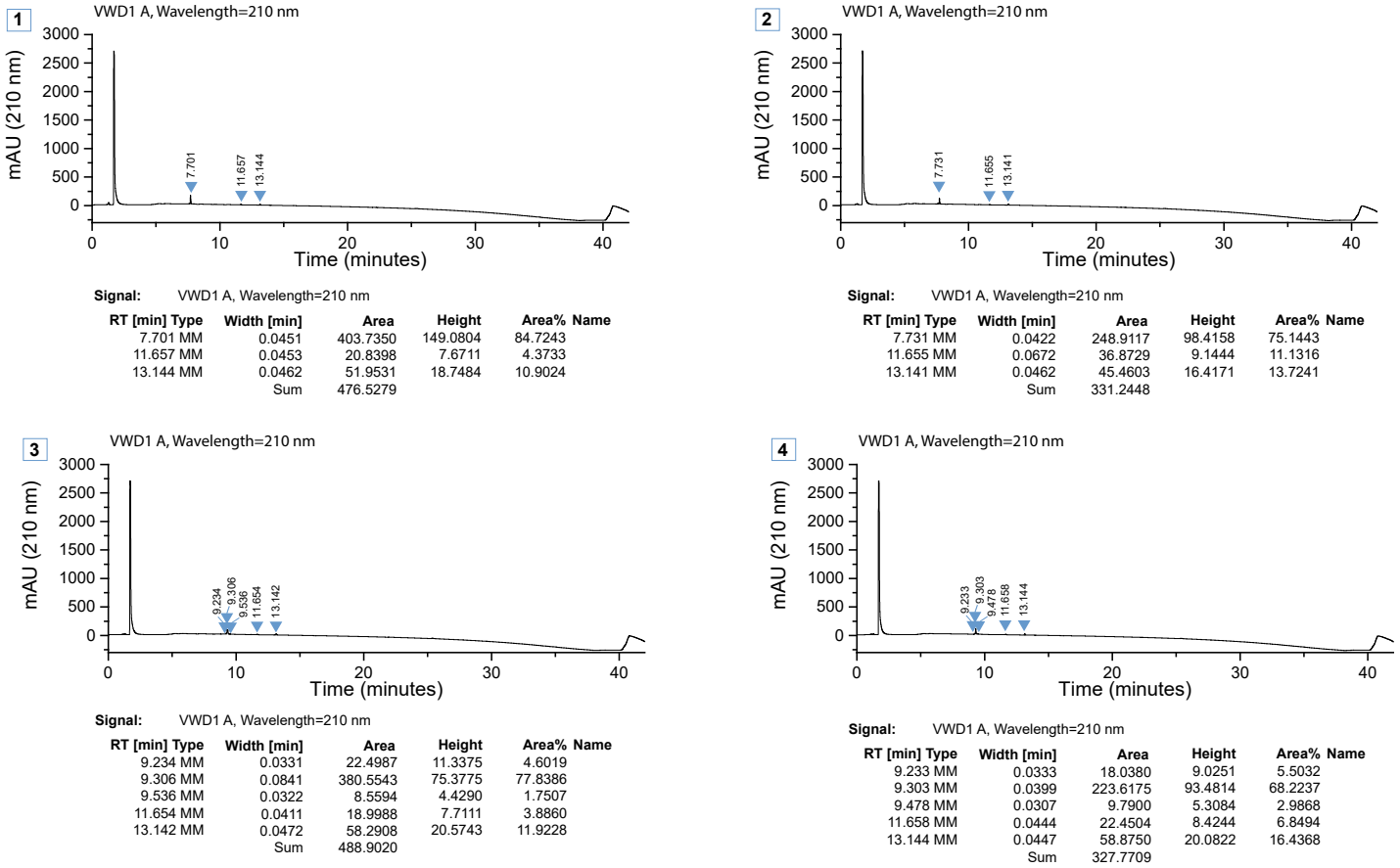

Figure 3: Analytical HPLC chromatogram of the H3 peptide series. The first peak in each spectrum is DMSO.
Legend:
1 - H3K4Me$_3$ C-KN$_3$
2 - H3K9Me$_3$ C-KN$_3$
3 - H3K4Me$_3$ A7Lp C-KN$_3$
4 - H3K9Me$_3$ A7Lp C-KN$_3$

Figure 2 shows MALDI-TOF MS spectra of the crude peptides and the purified product, highlighting the importance of HPLC purification to work with these peptides. Many mass spectra contain –26 Da and –52 Da peaks due to the presence of azide and diazirine groups which can lose N_2 during ionisation using MALDI-TOF MS, as a high voltage laser is used for this process. Analytical HPLC chromatograms of the purified peptides (Fig. 3), in combination with the mass spectra obtained in Fig. 2 and using high resolution mass spectrometry, support that the major peaks in analytical HPLC chromatograms correspond to the peptide of interest. The purification method chosen resulted in peptide purities ranging from 68-85% (Fig. 3). Given the challenging nature of peptide purification, this is a great result which enables further characterization of the peptides in photocross-linking experiments.

Photocrosslinking experiments

Photoactivatable probes can be used to form a covalent linkage between the probe and protein target upon the application of UV light of a specific wavelength via generation of a highly reactive species that reacts with molecules in immediate proximity. Alkyl diazirines generate carbene reactive species by irreversible loss of N_2 upon irradiation at 302 nm. Carbenes are very reactive and can be inserted into neighbouring C-H or heteroatom-H bonds to form a covalent adduct.[4] The benefit of employing diazirines is that their reactive species have a typical half-life in the nanosecond range, which allows for a fast reaction and minimizes off-target binding. Preliminary cross-linking analysis did not show evidence of crosslinking of the peptides to the KDM4A target, as judged by SDS-PAGE and LC-MS (data not shown), so the efficacy of the probes is yet to be established.

Conclusion

MALDI-TOF MS and analytical HPLC chromatography confirmed the successful purification of the histone peptides. While further optimization of the photocrosslinking experiment conditions is required to determine the potency of the histone peptide probes against KDM4A, these probes have the potential to be used as probes for a variety of H3-binding proteins, and so have a great scope beyond the purposes of this project.

Experimental

Peptide synthesis. Peptides were synthesized using fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) on a PurePep Chorus peptide synthesiser (Gyros Protein Technologies). Rink Amide MBHA resin (100-200 mesh, AGTC Bioproducts) was used with 5 equivalents of Fmoc-protected amino acids (Sigma Aldrich, Novabiochem, Fluorochem) for most couplings on 0.05 or 0.1 mmol scale in dimethylformamide (DMF) (Peptide synthesis grade, Merck). For photo-lysine/photo-leucine (Iris Biotech), 1.5 equivalents of amino acid were used, and peptides were capped using 10% acetic anhydride/ 5% pyridine solution for 30 min at room temperature before progressing to the next coupling. Synthesis was performed with single couplings at 90 °C for 2 minutes, or double at 75 °C for arginine, and single at 50 °C for 10 minutes for cysteine/histidine using diisopropylcarbodiimide (DIC, Fluorochem) as activating agent and OxymaPure (Novabiochem) as activating base 1:1 ratio in DMF. Fmoc-deprotection cycles were performed using 20% piperidine in DMF (Peptide synthesis grade, Merck), at 50 °C for 2 minutes twice. Fmoc-deprotection was performed as the last step to leave the free amino group at the *N*-terminus. The resin was further suspended in ~5 mL of DMF and treated with 10 equivalents of chloroacetic anhydride (Sigma Aldrich) for 30 minutes at 60°C. Cleavage and removal of side-chain protection groups was performed with ~4 mL of deprotection solution: trifluoroacetic acid (TFA, 92.5% v/v), triisopropylsilane (TIPS, 2.5% v/v), 1,3-dimethoxybenzene (2.5% v/v) and H_2O (2.5% v/v) at room temperature for 3 hours using the peptide synthesizer. Peptides were precipitated by addition of 45 mL diethyl ether, followed by centrifugation at 3000 g, 4 °C for 10 minutes. Peptides were dissolved in a MeCN:H_2O solution (3mL), the ratio of which was tailored to each peptide. Peptides were frozen using liquid nitrogen and dried using a lyophilizer.

Peptide purification. Peptides were purified using a Teledyne ISCO ACCQ*Prep* HPLC system with a Phenomenex Gemini-NX 5 µm C18 110 Å 250x30 mm column, UV Detection at 220 nm. A solvent system of A = H_2O, 0.1 % (v/v) TFA and B = MeCN, 0.1 % (v/v) TFA, was used with increasing B gradient elution, customised for each peptide, over 60 minutes. The method used for the HPLC started with 2% MeCN.

Analytical High Pressure Liquid Chromatography for purity analysis. The samples (4 µL at 100 µM) were analysed using an Agilent 1220 Infinity II LC system on a Phenomenex Gemini 3 µm NX-C18, 110 Å, 250 x 4.6 mm column at a 2-98% MeCN gradient over 40 minutes.

References

1. J. Sun, J. Yang, X. Miao, H. H. Loh, D. Pei and H. Zheng, *Cell Regeneration*, 2021, **10**.

2. X. Li and X. D. Li, *Accounts of Chemical Research*, 2021, **54**, 3734–3747.

3. P. R. Hansen and A. Oddo, in *Methods in Molecular Biology*, Methods in Molecular Biology, 2015, pp. 33–50.

4. D. Murale, S. Hong, M. Haque and J. Lee, Proteome *Science*, 2016, **15**.

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