Affinity Probes

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A Selective Chemical Probe for Coenzyme A-Requiring Enzymes**

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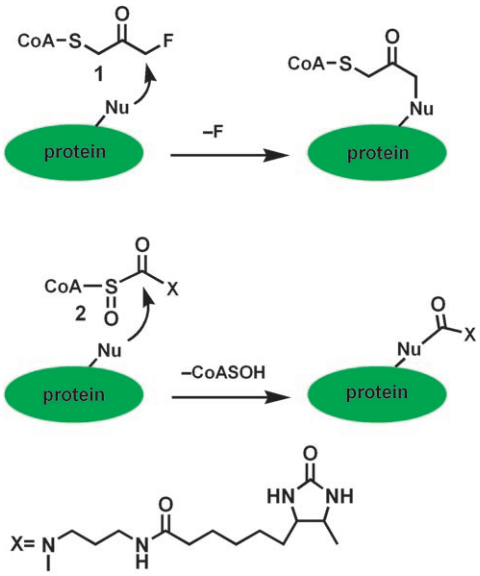
Structural and functional annotation of the human proteome is one of the critical challenges in biomedical research. Recent chemical approaches have been brought to bear on systematically interrogating enzymes involved in protein phosphorylation, proteolysis, lipid modification, glycosylation, deimination, methyl transfer, and acetylation.[1] An elegant strategy for analyzing substrates of acetyltransferases by using chloroacetyl coenzyme A (CoA) has been reported.[1q] However, to our knowledge, no chemical probes have yet been established to specifically tag CoA-dependent proteins by covalent cross-linking. These probes could be especially useful in the identification and analysis of histone and protein acetyltransferases, which contain many families with limited homology.[2] Herein, we describe the synthesis and functional analysis of a new CoA chemical probe that shows selectivity in tagging acetyltransferases.

There are no absolutely conserved catalytic residues throughout the different groups of acetyltransferases, although there are elements of phosphopantetheine binding that appear to be structurally conserved across many of these enzymes.[2] Consequently, we designed two probes to interrogate acetyltransferases which exploit the CoA binding pocket. The fluoroacetonyl CoA **1** (Scheme 1) was envisaged to be a mimic of acetyl CoA and take advantage of the known reactivity of α-halocarbonyl compounds toward nucleophilic attack. ^{32}P-labeled **1** produced enzymatically[3] (Scheme S1 in the Supporting Information) was examined as an affinity agent against a series of acetyltransferases and control proteins.

As can be seen by autoradiography (Figure S1 in the Supporting Information), compound **1** could selectively label two acetyltransferases (p300[4] and arylalkylamine *N*-acetyltransferase (AANAT)[2]) but not a third (p300/CBP-associated factor (PCAF)[2]). Although **1** did not label two non-acetyltransferases, 14-3-3[5] or low-molecular-weight phosphotyrosine phosphatase (LMW-PTP)[6], **1** did label two protein kinases, Csk[7] and Src.[7] Compound **1** did not perform well in cellular extracts (Figure S1 in the Supporting Information) and in competition experiments with acetonyl CoA (actCoA; Figure S1 in the Supporting Information).



Scheme 1. Strategy for labeling proteins based on CoA affinity. Nu = nucleophile.

Given the limitations of **1**, we turned to a different electrophilic probe **2** (Scheme 1), which we hoped could be more useful. Compound **2** was designed to have the potential to introduce a desthiobiotin moiety at the protein modification site, and the covalent desthiobiotin could then be used as an affinity handle (Scheme 1). Although to our knowledge a thiocarbamate sulfoxide functionality has not previously been used in protein modification, it was envisaged that this group could be attacked by enzyme nucleophiles, especially cysteines, at the carbonyl carbon atom,[8] thus generating carbamylated targets and liberating CoA sulfenic acid.

Synthesis of compound **2** was carried out as shown in Scheme 2. The starting Boc diamine **3** was bilaterally derivatized to install the thiocarbamate and desthiobiotin groups. Oxidation of the thiocarbamate with excess Oxone led to sulfone **5**, which was used in *trans* acylation with CoASH. Controlled oxidation of the thiocarbamyl moiety of the penultimate intermediate with Oxone resulted in successful conversion to the desired target compound **2**. As hoped, **2** proved to be reasonably stable under physiological conditions and resistant to reaction with low concentrations of thiols.

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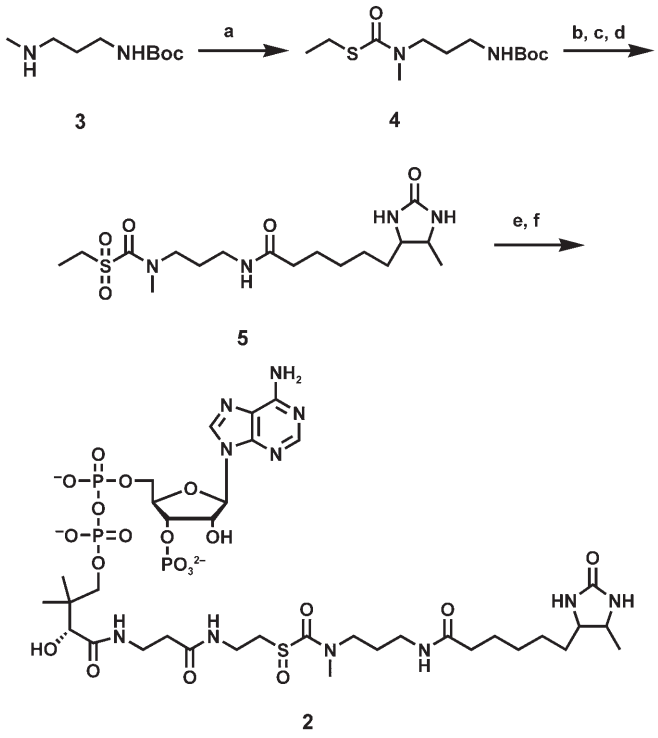

Scheme 2. Synthesis of sulfoxide **2**. a) Ethyl thiochloroformate ($ClCOSC_2H_5$), 0 °C, aqueous NaOH/diethyl ether; b) 6 N HCl, MeOH, RT; c) TSTU, desthiobiotin, DIPEA, RT, dioxane/H_2O/DMF; d) Oxone, RT, MeOH/H_2O; e) CoASH, LiOH, MeOH/H_2O; f) Oxone, 0 °C, MeOH/H_2O then reversed-phase column chromatography. Boc = *tert*-butoxycarbonyl, TSTU = *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate, DIPEA = ethyldiisopropylamine.

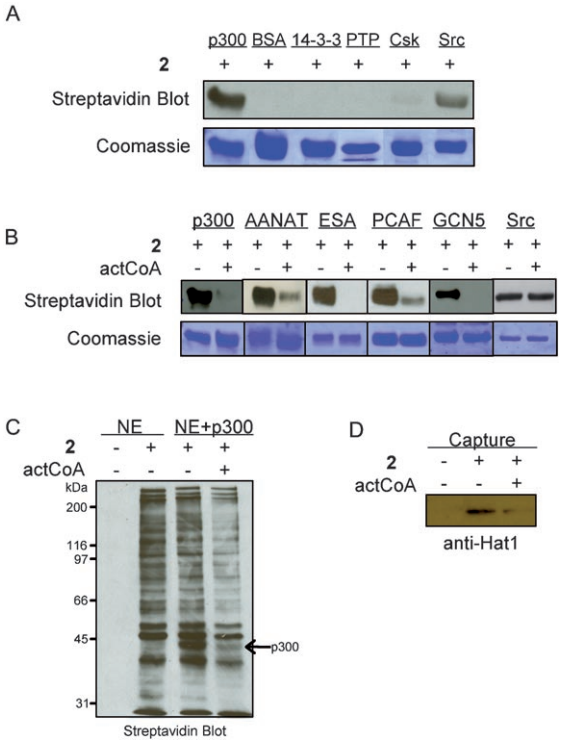

Figure 1. A) In vitro labeling experiments on non-CoA-requiring proteins with **2** (25 μM). B) In vitro labeling experiments on CoA-requiring proteins with **2** (25 μM) and competition by actCoA (3 mM). C) In vitro labeling of cell lysates with **2** (50 μM). The lysates prepared from HeLa nucleus (NE) were either spiked with recombinant p300-histone acetyltransferase (p300-HAT; approximately 1/50) or not spiked. D) Labeled cell lysates were probed by western blotting with anti-HAT1 antibody after streptavidin–agarose purification (capture). BSA = bovine serum albumin, ESA = essential SAS2-related acetyltransferase 1, GCN5 = growth-control nonrepressed 5.

We examined the reactivity of **2** with a number of protein targets and a range of acetyltransferases, as well as with other enzymes and proteins (Figure 1). Compound **2** showed efficient labeling of each of the acetyltransferases tested (p300, PCAF, AANAT, GCN5, ESA[2]) and in each of these cases, labeling was effectively competed by excess actCoA (Figure 1 B). Compound **2** did not show significant labeling of 14-3-3, PTP, or Csk (Figure 1 A). Src was also labeled by **2**, but this labeling was not significantly competed by actCoA (Figure 1 A,B), which suggests that **2** is far more selective in targeting acetyltransferases than **1**.

An advantage of **2** is that the desthiobiotin left behind can be used for pull-down experiments. This was hypothesized to be useful in the context of localizing modifications in combination with proteolytic digestion. We attempted such experiments for two different acetyltransferases, yESA1 and p300 (Figure 2). yESA1 has been characterized crystallographically and contains an active-site cysteine with the potential to attack acetyl CoA.[2] The structure of p300 has not

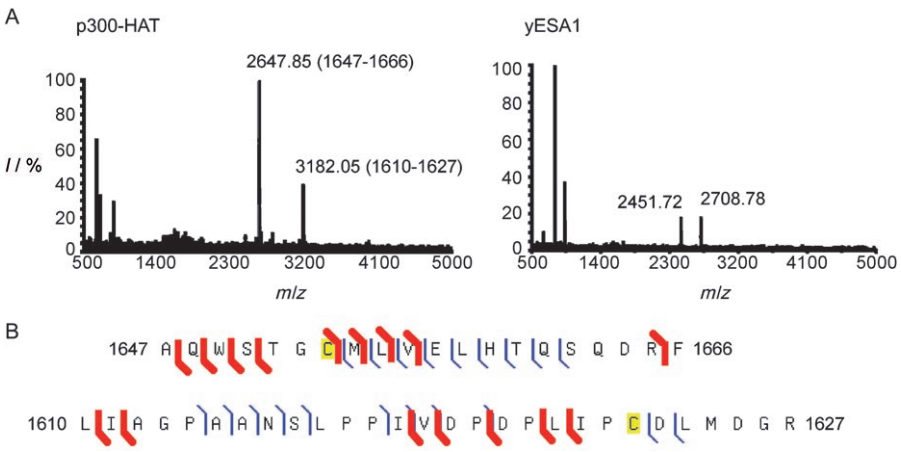

Figure 2. A) MALDI MS analysis of the streptavidin-enriched peptides from p300-HAT and yESA1 after labeling with **2** and tryptic digestion. B) Labeled peptides and confirmation of labeling site from p300-HAT by MS/MS analysis. Upward and downward slashes represent the observed *c* and *z*• ions, respectively. All the *c* or *z*• ions in red are modified while all the *c* or *z*• ions in blue are not modified, which leads to the localization of desthiobiotinylation to residues colored in yellow. The two lines in (B) correspond to yESA1 (top) and p300-HAT peptides (bottom).

been reported, and its sequence is not homologous to those of other HATs.[4]

After labeling, yESA1 and p300 were treated with trypsin and the peptide digests purified with streptavidin. Mass spectrometric (MS) analysis of the purified peptides from yESA1 (Figure 2A, right) gave two major peaks (m/z 2452 and 2709) that corresponded to desthiobiotin-labeled Cys active-site peptides. MS analysis of the p300 mixture resulted in two different desthiobiotin-labeled peptides (m/z 2648 and 3182; Figure 2A, left), each of which contained a Cys unit (Figure 2B). Fourier-transform MS unequivocally demonstrated that the Cys-1621 was a site of interaction (Figure 2). Confirming the importance of Cys-1621 in p300 acetyltransferase activity, C1621A resulted in a large rise in the K_m values of acetyl CoA (Table 1), which suggests that this Cys could be important in acetyl CoA binding. The other peptide was likely labeled on Cys-1653 near the C terminus (1666). Prior studies have already implicated the C terminus of the p300-HAT domain as important.[4] These experiments illustrate the utility of **2** in providing preliminary structural analysis of poorly characterized CoA-dependent enzymes.

Table 1: Comparison of kinetic parameters of p300 [wt] and C1621A mutant.[a]

	K_m (acetyl CoA)	K_m (H4–15)	k_{cat} (acetyl CoA)
p300	6.4 μM	30 μM	0.21 s^{-1}
C1621A	100 μM	12 μM	0.24 s^{-1}

[a] For experimental details, see the Supporting Information; K_m: concentration for half-maximal rate, k_{cat}: turnover number.

Probes such as **2** could also be useful in identifying and characterizing proteins present in cellular extracts. To examine this possibility, we tested **2** in mammalian nuclear extracts spiked with a small amount of p300. As shown in Figure 1C, a range of proteins appear to be modified by **2** and the labeling of several was selectively competed by actCoA. Among these, p300 was readily identified and the modification efficiently blocked by actCoA. Compound **2** was also able to capture endogenous GCN5-related acetyltransferase (GNAT) member HAT1[2] from HeLa nuclear extracts in a manner that was competed by excess actCoA (Figure 1D). In future studies, we hope to apply **2** in proteomic studies leading to the identification of unknown CoA-binding proteins of interest in signaling or metabolism. Moreover, we anticipate that the sulfoxycarbonyl functionality may find other applications in protein labeling.

Experimental Section

Details of the synthesis of the probe **2** are provided in the Supporting Information.

Procedure for labeling recombinant proteins with sulfoxide **2**: Purified proteins (10 μL, 0.5 μg μL^{-1}) were treated with **2** (25 μM; 865 μM stock in doubly distilled (dd) H_2O) either with or without actCoA (3 mM) in assay buffer (50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 8.0, 100 mM NaCl, 0.5 mM dithiothreitol (DTT)) at 30 °C for 1 h. The assay was then quenched with a standard 5X SDS-PAGE loading buffer (reducing). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% BSA in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) at room temperature, followed by incubation for 1 h with horseradish peroxidase streptavidin in TBST. After four washes with changes every 15 min in TBST, the biotinylated proteins were visualized by enhanced chemiluminescence.

Trypsin digestion, purification, and detection of biotinylated peptides: The labeled proteins (1 μM of p300-HAT or yESA1 treated with 25 μM **2** as described above) were dialyzed against TBS to remove **2**. The dialysate was digested overnight at 37 °C with trypsin, then incubated with streptavidin–agarose beads for 1 h at room temperature. The beads were washed three times with ten volumes of low-salt wash buffer (TBS: 50 mM Tris pH 7.4, 150 mM NaCl), three times with high-salt wash buffer (50 mM Tris pH 7.4, 500 mM NaCl), and finally with ten volumes of dd H_2O (twice). After washing, the beads were eluted with 30% aqueous acetonitrile and 0.5% trifluoroacetic acid. The eluted peptides were partially dried by vacuum centrifugation and analyzed by either MALDI-TOF or liquid chromatography–MS/MS.

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