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Using peptidyl aldehydes in activity-based proteomics

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ABSTRACT

The broad inhibitory spectrum of aldehydes and the possibility that amino acid residues modulate their specificity point to the potential of using peptidyl aldehydes as activity-based probes. Here, we establish the potential of peptidyl aldehydes in activity-based proteomics by synthesizing different probes and using them to specifically label a well-known serine protease in an activity-dependent manner. From our results, peptidyl aldehydes emerge as promising activity-based probes that enable multiple enzymatic-class detection by substrate recognition and can be used in diverse activity-based proteomics applications like protein identification and activity profiling.

Activity-based proteomics (ABP) is a chemical strategy that uses probes that covalently bind an enzyme active site for protein activity profiling and for discovering new therapeutic targets and enzyme inhibitors. Natural substrates and covalent inhibitors of diverse enzymatic classes (e.g., proteases,[1–6] glycosidases,[7,8] and phosphatases[9]) have been used for designing several activity-based probes. In most cases, the reactive moiety of these probes consists in electrophilic functional groups such as fluorophosphonates,[1,2] epoxides,[3,5] and acyloxymethylketones.[10,11]

Aldehydes are electrophilic functional groups that have interesting inhibitory properties toward different kinds of proteolytic enzymes. Many compounds with an aldehyde moiety have been recently described as covalent reversible inhibitors of serine and cysteine proteases like trypsin, thrombin,[12] and cathepsins[13–15] among others.[16–19] Apart from these proteases, aldehydes also inhibit metalloproteases[20] and aspartic proteases[21,22] like γ-secretase, HIV protease, and renin, and they can also interact with some non-proteolytic proteins like tyrosine phosphatases[23,24] and SH2 domains.[25]

Although aldehydes can inhibit a wide number of enzyme types due to their broad reactivity, several studies succeeded in limiting their interaction to certain enzymatic subclasses.[13,18,26,27] Obtaining specific aldehyde inhibitors has been possible with the synthesis of peptidyl aldehydes and the subsequent modification of the amino acid residues placed in positions P1 and P2 according to the natural substrates of each enzyme.[26,27]

The broad inhibitory spectrum of aldehydes and the possibility that amino acid residues modulate their specificity point to the potential of using peptidyl aldehydes as activity-based probes. Here, we explore for the first time the potential of peptidyl aldehydes in ABP. For this purpose, we synthesized different probes and, as a proof of principal, we used them to specifically label a well-known serine protease in an activity-dependent manner.

Prolyl oligopeptidase (POP; EC 3.4.21.26) is a post-proline serine protease that hydrolyses small proline-containing peptides.[28] POP is involved in the regulation of many bioactive peptides in vivo like substance P and thyrotropin-releasing hormone, among others[29–32] and has been associated to several neuropsychiatric disorders like schizophrenia and bipolar affective disorder.[33] Although the mechanism of action of this protease remains unknown, several studies suggested that POP may produce its effect through the metabolism of inositol-1,4,5-triphosphate, a key molecule in the transduction cascade of neuropeptide signaling.[34,35] In our laboratory, POP was recently cloned from human brain RNA, expressed in *Escherichia coli*, and an homologous model based on the X-ray structure of porcine POP was obtained.[36]

To evaluate the use of peptidyl aldehydes in ABP, three peptidyl aldehyde activity-based probes, Aha-Bpa-Pro-Pro-H (**1**), Aha-Bpa-Ahx-Pro-Pro-H (**2**), Aha-Bpa-Peg-Pro-Pro-H (**3**) (Aha: hexynoic acid; Ahx: ε-aminohexanoic acid; Bpa: benzoylphenylalanine; Peg: 15-amino-4,7,10,13-tetraoxapentadecanoic acid) were synthesized and used to specifically label active POP (Fig. 1a). It is worth mentioning that unlike phosphonates, sulfonates and other reactive groups traditionally used in activity-based probes, the use of aldehydes facilitates probe synthesis through easy and rapid solid-phase peptide synthesis (SPPS) strategies.

Peptidyl aldehyde probes were initially designed from the well-known covalent POP inhibitor, *Z*-prolyl-prolinal (ZPP, Fig. 1b),[37]

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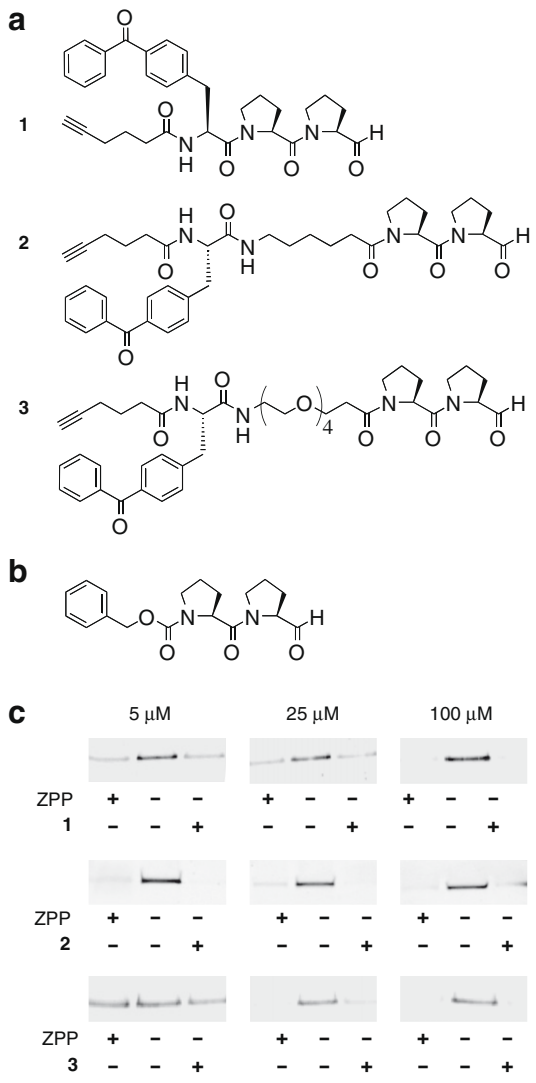


Figure 1. (a) Structure of peptidyl aldehyde probes Aha-Bpa-Pro-Pro-H (**1**), Aha-Bpa-Ahx-Pro-Pro-H (**2**), Aha-Bpa-Peg-Pro-Pro-H (**3**). Probes were synthesized by SPPS using a *t*-Bu/Fmoc standard strategy except for the first amino acid residue, which was coupled in the semicarbazide resin as Fmoc-Pro-H in THF and acetic acid (1%). (b) Structure of *Z*-prolyl-prolinal. (c) Competitive inhibitory assays to evaluate the recognition of POP active site by the new synthesized probes. In these assays POP (20 ng) was incubated for 15 min at 25 °C with increasing amounts (5 μM, 25 μM and 100 μM) of either probe or ZPP. Afterwards, FP-Rh (1 μM) was added and the incubation was maintained for an additional 60 min at 25 °C. The assay was resolved on an SDS–PAGE and the FP-Rh-labeled POP was visualized using a fluorescence scanner.

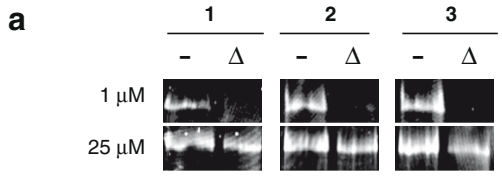
and subsequently modified to fulfill the requirements previously described for ABP probes.[38,39] One of these requirements is the establishment of covalent irreversible bonds between the probe and the target protein. However, aldehydes establish covalent reversible bonds with the active site of serine and cysteine proteases and non-covalent bonds when interacting with aspartic proteases and metalloproteases. This aldehyde reversibility could constitute a certain restriction in the use of peptidyl aldehydes in ABP applications. Therefore, to ensure covalent irreversible binding between the synthesized probes and the target protein a photoreactive benzophenone residue was introduced at different positions to induce photocrosslinking by UV light irradiation. This strategy is similar to that previously used in the characterization of aspartic proteases and metalloproteases with hydroxyethylenes[40,41] and hydroxamates.[42] Finally, synthesized peptidyl aldehyde probes included an alkyne functional group in the N-terminus to allow fluorescent and biotin label conjugation by Cu(I)-catalyzed Huisgen [3+2] cycloaddition.[43,44]

Peptidyl aldehyde probes **1**–**3** were synthesized by SPPS using a semicarbazide resin[45,46] and dipeptides Fmoc-Bpa-Pro-OH, Fmoc-Ahx-Pro-OH, and Fmoc-Peg-Pro-OH were used during peptide elongation to avoid the formation of diketopiperazines.[47] In all cases L-amino acids were used. Recognition of POP active site by probes **1**–**3** was confirmed by competitive inhibitory assays with a well-known covalent probe, fluorophosphonate rhodamine (FP-Rh), which binds to the active site of serine hydrolases.[48] In these assays POP was incubated for 15 min with increasing amounts of either probe or ZPP. Afterwards, FP-Rh (1 μM) was added and the incubation was maintained for an additional 60 min. After resolving the reaction on an SDS–PAGE, the FP-Rh-labeled POP was visualized using a fluorescence scanner. All three compounds **1**–**3** showed excellent inhibitory properties toward POP (Fig. 1c) corroborating that the introduction of additional bulky residues like benzophenone was not drastically affecting their ability to recognize the active site of the target protein.

Once peptidyl aldehyde probes were synthesized and their inhibitory properties were confirmed, next assays focused on verifying if these compounds were proper activity-based probes, that is, if they could label POP in an activity-dependent manner and distinguish its active form from heat-denatured samples. To confirm this point, active and heat-denatured POP were incubated with probes **1**–**3** at 1 and 25 μM for 15 min at rt and crosslinked by UV light irradiation for an additional 60 min at 4 °C. Afterwards, samples were labeled using a trifunctional tag that incorporates both a biotin and a rhodamine moiety[49] ($TriN_3$, Supplementary Fig. 1), and they were analyzed by SDS–PAGE and visualized with a fluorescence scanner. These assays showed that the probe specificity could be lost at high concentrations possibly due to the broad aldehyde reactivity and to the photocrosslinking step. However, peptidyl aldehydes are satisfactory activity-based probes when used at low concentrations (Fig. 2a). These results were further confirmed with pull-down experiments where the crosslinked probe–protein complex was labeled with the $TriN_3$ tag, fished out of the sample using avidin beads and detected by Western Blot using either an in-house α-POP antibody or streptavidin. This assay was performed with probe **3** (1 μM), and in both cases only the active form of POP was fished out from the sample (Fig. 2b).

Finally, activity-based probe **3** was also used for direct mass spectrometry protein identification using an nLC–MS/MS approach. In this case, probe–protein complexes were also labeled with the $TriN_3$ tag, fished out with avidin, but analyzed by reverse-phase chromatography followed by MS/MS identification. MS/MS data were analyzed with the SEQUEST software using the IPI database. The maximum false positive rate was set to 1% with PeptideProphet and ProteinProphet. This data analysis led to the identification of POP in active samples and not in heat-denatured controls (Fig. 2c). These results show that peptidyl aldehydes are adequate activity-based probes not only for specific in-gel activity profiling of proteases but also for other ABP applications like protease identification using mass spectrometry.

To verify the behavior of peptidyl aldehyde probes in complex environments, further experiments were performed mixing mouse brain homogenates and recombinant POP. First, incubation and photocrosslinking times were optimized (Supplementary Fig. 2a) and the established conditions were used to identify the most sensitive probe among the synthesized compounds (Supplementary Fig. 2b). Probe **3** was selected for subsequent experiments in which brain homogenates were mixed with increasing amounts of recombinant POP and incubated for 15 min with the probe followed by UV light irradiation during an additional 60 min. Although endogenous POP could not be detected by in-gel analysis,



c

DATABASE ID	PROTEIN
SAMPLE	
IPI00220327.3	Keratin, type II cytoskeletal 1
IPI00019359.3	Keratin, type I cytoskeletal 9
IPI00009865.2	Keratin, type I cytoskeletal 10
IPI00008164.1	**Prolyl endopeptidase**
IPI00747355.1	**Prolyl endopeptidase cDNA**
IPI00021304.1	Keratin, type II cytoskeletal 2 epidermal
IPI00217963.3	Keratin, type I cytoskeletal 16
IPI00384444.5	Keratin, type I cytoskeletal 14
IPI00290078.5	Keratin 4
CONTROL	
IPI00009865.2	Keratin, type I cytoskeletal 10
IPI00220327.3	Keratin, type II cytoskeletal 1
IPI00019359.3	Keratin, type I cytoskeletal 9
IPI00021304.1	Keratin, type II cytoskeletal 2 epidermal

Figure 2. (a) Activity-dependent labeling of POP at different probe concentrations. In this experiment active (–) and heat-denatured (Δ) POP (10 ng/μl) were incubated with probes **1**–**3** at 1 and 25 μM for 15 min at 25 °C and crosslinked by UV light irradiation for an additional 60 min at 4 °C. Afterwards, samples were labeled using the $TriN_3$ tag and they were analyzed in an SDS–PAGE and visualized with a fluorescence scanner. (b) Activity-dependent pull-down experiment. Probe **3** (1 μM) was used for an activity-dependent labeling of POP (10 ng/μl). After photocrosslinking and $TriN_3$ incorporation, the crosslinked complex tag–probe–protein was fished out of the sample using avidin beads and detected by Western Blot (α-POP, streptavidin). (c) List of identified proteins by mass spectrometry (LC–MS/MS) in samples with active POP and in heat-denatured controls after a pull-down experiment. A Human IPI Database was used for protein identification.

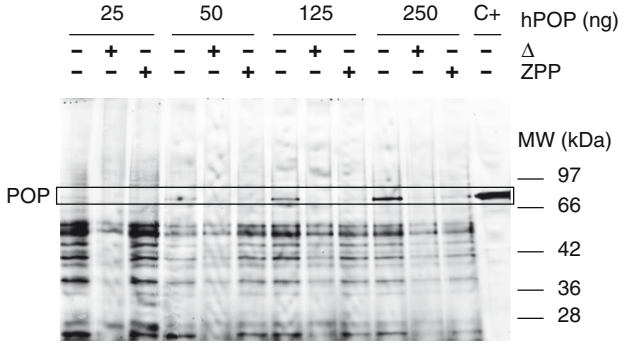


Figure 3. Activity-dependent labeling of POP in mouse brain homogenates and recombinant POP mixtures to establish the detection limit of peptidyl aldehyde probes. Brain homogenates (1 mg/ml) were mixed with increasing amounts of recombinant POP and incubated with activity-based probe **3** (1 μM). After UV light irradiation samples were labeled with $TriN_3$ and analyzed by SDS–PAGE and visualized with a fluorescence scanner. Pure recombinant POP labeled by FP-Rh (1 μM) was used as a positive control (C+). Negative controls corresponding to heat-denatured samples (Δ) and to samples with a highly specific POP inhibitor (ZPP, 100 μM) were also performed.

these experiments made possible to set the lower limit of detection of our probes around 25 ng of POP and, what is more important, they validate the use of peptidyl aldehyde probes in complex mixtures (Fig. 3). In these experiments peptidyl aldehyde probes were still able to distinguish active and non-active POP in complex homogenates despite that the aldehyde reactive group could have easily been masked or non-specifically inactivated by multiple reagent species (e.g., primary amines). Moreover, peptidyl aldehyde probes remained quite specific and, other than POP, no other proteins were clearly detected in an activity-dependent manner. These observations confirm the importance of the peptidyl region in limiting probe interaction to certain target proteases and make peptidyl aldehyde probes useful for different ABP applications like protease identification and substrate-limited activity profiling.

As shown in previous labeling assays, the photocrosslinking step reduces probe selectivity and enforces the use of probes at low concentration, which limits in-gel analysis sensitivity and prevents the detection of endogenous POP. Obviously, peptidyl aldehyde sensitivity needs to be improved for the general application of these compounds as activity-based probes. However, the sensitivity of peptidyl aldehydes reported here could still be sufficient when working with high abundance proteins.

In the light of the reported results, together with the ease of synthesis and the similarity with natural substrates, we can state that peptidyl aldehydes emerge as promising activity-based probes that enable multiple enzymatic-class detection by substrate recognition and can be used in diverse ABP applications like protein identification and activity profiling. Unlike other probes that only target one specific type at a time (e.g., serine proteases), peptidyl aldehydes can simultaneously target a variety of proteolytic enzymes. Therefore, they could become a valuable tool for identifying unknown proteases by substrate recognition (e.g., those involved in the activation of neuropeptide precursors), which could eventually lead to the establishment of new therapeutic targets. Moreover, the specificity resulting from the peptide sequence is an important feature of peptidyl aldehyde probes that could also be used to successfully monitor the activity of a particular subset of proteins.

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Supplementary data

Supplementary data (synthetic procedures, compound characterization and protocols for activity-based assays and mass spectrometry) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.148.

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