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Development of activity-based probes for trypsin-family serine proteases

Zhengying Pan,* Douglas A. Jeffery,† Kareem Chehade, Jerlyn Beltman,‡ James M. Clark, Paul Grothaus, Matthew Bogyo*,§ and Amos Baruch*

Celera Genomics, 180 Kimball Way, South San Francisco, CA 94080, USA

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Abstract—A series of diphenylphosphonate-based probes were developed for the trypsin-like serine proteases. These probes selectively target serine proteases rather than general serine hydrolases that are targets for fluorophosphonate-based probes. This increased selectivity allows detection of low abundance serine proteases in complex proteomes using simple SDS–PAGE methods. We present here the application of multiple probes in enzyme activity profiling of intact mast cells, a type of inflammatory cell implicated in allergy and autoimmune diseases.

The primary goal of proteomics is to assign functions to all proteins in a given cell, tissue or organism.[1] The challenges implicit in this field have generated novel approaches to functionally dissect the proteome. Chemical proteomics or activity-based protein profiling (ABPP) makes use of small molecule probes that form covalent complexes with their targets using an activity-dependent chemical reaction. These activity-based probes (ABPs) can be used to profile enzymatic activities in complex proteomes and provide information on protein targets at the functional, rather than the expression level.[2] Moreover, due to its use of small molecules, this approach naturally focuses on 'druggable' enzymes that show specific ligand binding.

A number of classes of ABPs have successfully been designed and applied to serine hydrolases[3] and cysteine proteases.[4] Recently the scope of this approach has expanded with the development of chemical probes that target additional important enzyme families including phosphatases[5] and kinases.[6] Many approaches have focused on broad-spectrum probes that target multiple related enzyme family members.[7] Thus, there is a great need to develop new chemical probes to selectively target sub-classes of enzymes to study their roles in biological processes.

Serine hydrolases represent a large family of enzymes, members of which participate in many crucial biological processes. One of the most intriguing sub-classes of this family is the trypsin-like serine proteases. This subgroup is comprised of 65 enzymes in humans with unique cellular and physiological regulatory roles in health and disease.[8] Several enzymes within this group, such as thrombin, factor VIIa, factor Xa, and tryptase, are being extensively pursued as drug targets in cardiovascular and inflammatory indications. A fluorophosphonate (FP) probe, similar to probe **1** (Bio-FP), has been previously reported to target the serine hydrolase family (Ref. 3). The broad reactivity of probe **1** enables simultaneous labeling of a large number of enzymes including esterases, proteases, lipases, and amidases. In many cases, this probe generates a highly complex activity profile that prevents its usage for studying low-abundance, specific enzymes using simple analytical methods such as SDS–PAGE. To

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* Corresponding authors; e-mail addresses: zypan@yahoo.com; zhengying.pan@celera.com; mbogyo@stanford.edu; amos.baruch@celera.com
† Present address: Novartis Institute of Biomedical Research, Boston, MA 02139, USA.
‡ Present address: Chiron Corporation, Emeryville, CA 94608, USA.
§ Present address: Department of Pathology, School of Medicine, Stanford University, 300 Pasteur Drive, Stanford, CA 94305, USA.

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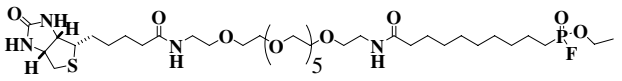


Figure 1. A general serine hydrolase probe 1.

circumvent this issue, we developed a series of selective probes that specifically target the trypsin-like serine proteases (see Fig. 1).

To achieve selectivity toward serine proteases, we focused our efforts on phosphonate-based probes. Initial attempts to use an α-amino fluorophosphonate reactive group failed due to its short half-life in an aqueous environment.[9] The more stable reactive group, diphenylphosphonate, has previously been used to generate potent irreversible serine protease inhibitors[10]. Here, we describe our efforts in developing diphenylphosphonate (DPP)-derived probes for activity profiling of trypsin-like serine proteases[11] (see Scheme 1).

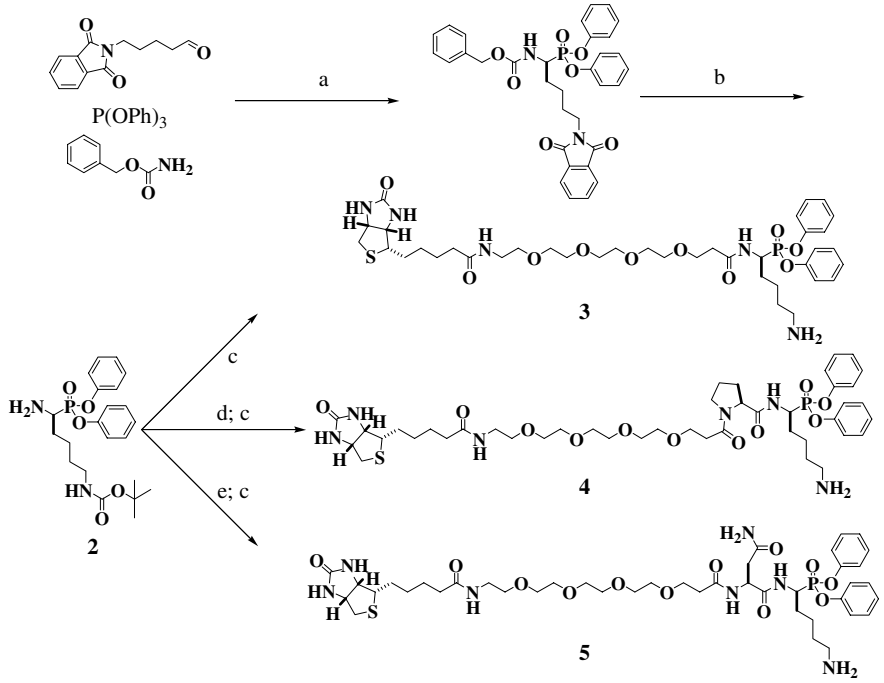
Based on previous studies of substrate specificity,[12] a lysine residue was incorporated at the P_1 site with either a proline or asparagine at the P_2 position to generate a general trypsin-family protease probe **4** (Bio-PK-DPP) and a β-tryptase-selective probe **5** (Bio-NK-DPP), respectively. Lysine-based DPPs can be made through the Oleksyszyn 3-component reaction using either conventional heating or microwave-assisted agitation. Intermediate **2** was obtained through three steps of protecting group manipulations. A biotinylated probe **3** (Bio-K-DPP) was made by coupling **2** with a commercially available biotinylation reagent NHS-$(PEG)_4$-biotin. Following addition of an amino acid residue at the P_2 position, similar procedures produced biotinylated probes **4** and **5**.

Apparent inhibition constants [$K_{i(app)}$] were obtained for these probes against four trypsin-like serine proteases (Table 1). The addition of a P_2 proline residue increased the overall reactivity of probe **4** toward trypsin-like serine proteases. Incorporation of an asparagine residue at P_2 yielded a 23-fold selectivity increase for probe **5** favoring tryptase over its closely related family member trypsin.

To confirm that the potency and selectivity observed in kinetic assays were reflected in enzyme labeling profiles, probes **1**, **4**, and **5** were used for activity-based labeling of a panel of recombinant enzymes from different serine hydrolase families (Fig. 2). The extent of covalent labeling was determined by Western blotting with streptavidin detection of the biotin reporter group. Probe **1** labeled all recombinant enzymes tested including butyrylcholine esterase (BCE), a hydrolase enzyme. In contrast, the lysine-DPP-based probes were completely inactive against both chymotrypsin and BCE. The general trypsin-family probe **4** efficiently labeled all trypsin-

Table 1. Apparent inhibition constants [$K_{i(app)}$ μM] of probes **1**, **4**, and **5** for trypsin-like serine proteases following 30 min of incubation[13]

Enzymes	Bio-FP **1**	Bio-PK-DPP **4**	Bio-NK-DPP **5**
β-Tryptase	30	6.2	2.5
Trypsin	16.5	0.57	57.3
Thrombin	7.9	1.07	>100
Plasmin	>100	2.9	4.03



Scheme 1. Synthesis of ABPs with the diphenylphosphonate reactive group. Reagents and conditions: (a) HOAC as solvent, microwave, 150 °C, 5–10 min or oil bath, 70 °C, 1–3 h; (b) hydrazine; Boc_2O; H_2, Pd–C; (c) NHS-$(PEG)_4$-biotin, triethylamine; then TFA; (d) Cbz-Pro-OH, EDCI, HOBt; H_2, Pd–C; (e) Cbz-Asn(Trt)-OH, EDCI, HOBt; H_2, Pd–C.