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ARTICLE *in* BIOCONJUGATE CHEMISTRY · OCTOBER 2009

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Expedited Solid-Phase Synthesis of Fluorescently Labeled and Biotinylated Aminoalkane Diphenyl Phosphonate Affinity Probes for Chymotrypsin- and Elastase-Like Serine Proteases

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Received May 14, 2009; Revised Manuscript Received August 24, 2009

In this study, we report on a novel, expedited solid-phase approach for the synthesis of biotinylated and fluorescently tagged irreversible affinity based probes for the chymotrypsin and elastase-like serine proteases. The novel solid-phase biotinylation or fluorescent labeling of the aminoalkane diphenyl phosphonate warhead using commercially available Biotin-PEG-NovaTag or EDANS NovaTag resin permits rapid, facile synthesis of these reagents. We demonstrate the kinetic evaluation and utilization of a number of these irreversible inactivators for chymotrypsin-like (chymotrypsin/human cathepsin G) and elastase-like serine proteases. Encouragingly, these compounds display comparable potency against their target proteases as their *N*-benzyloxycarbonyl (Cbz)-protected parent compounds, from which they were derived, and function as efficient active site-directed inactivators of their target proteases. We subsequently applied the biotinylated reagents for the sensitive detection of protease species via Western blot, showing that the inactivation of the protease was specifically mediated through the active site serine. Furthermore, we also demonstrate the successful detection of serine protease species with the fluorescently labeled derivatives "in-gel", thus avoiding the need for downstream Western blotting. Finally, we also show the utility of biotinylated and pegylated affinity probes for the isolation/enrichment of serine protease species, via capture with immobilized streptavidin, and their subsequent identification via de novo sequencing. Given their selectivity of action against the serine proteases, we believe that these reagents can be exploited for the direct, rapid, and selective identification of these enzymes from biological milieu containing multiple protease subclasses.

INTRODUCTION

The completion of the human genome sequencing project (*1*) has facilitated a comprehensive analysis of known and potential gene products which may represent novel targets for pharmacological intervention/modulation. Original estimates of the "druggable genome" (i.e., those genes that encode protein products predicted to possess structural domains or motifs which are likely to favor interactions with drug-like molecules) indicated that approximately 3000 genes belonged to this unique group (*2*). More recent functional genomic analysis of the druggable genome has been in close agreement with these earlier estimates, although, significantly, the proportion of the predicted major target families of proteins appears to have changed somewhat. For example, these latter studies indicate a reduction in the numbers of putative protein kinases and rhodopsin-like G-protein coupled receptors (GPCRs) and an increased number of putative protease targets which are potentially "druggable" (*3*). Proteases have long been regarded as important therapeutic targets, in recognition of their multifarious roles in numerous diseases, and much focus has been directed toward protease inhibition in pathological conditions. However, it is significant that, although many proteases have recently been identified through genomic analysis, only a small proportion (∼15%) have been characterized through functional studies (*4*). Thus, the application of specific and sensitive protease detection methodologies that can be utilized in high-throughput proteomic formats to facilitate the functional identification of poorly characterized species is urgently required. A number of substrate approaches have previously been developed for the detection of protease activities, following electrophoresis from a range of biological samples (*5, 6*).

We have previously reported on the synthesis and methodologies for the profiling of serine proteases by utilizing biotinylated aminoacylchloromethanes (*7*) and cysteine proteases, using biotinylated peptidyldiazomethanes (*8*−*10*). Powers and coworkers (*11*) pioneered the development of potent and exquisitely selective peptide derivatives of diphenyl [1-(*N*-peptidylamino)alkyl] phosphonate esters as irreversible inactivators of the serine proteases and utilized biotinylated and fluorescently labeled derivatives to detect a range of serine proteolytic activities including lymphocyte serine proteases (granzymes) (*12, 13*). We have also reported on the application of such phosphonate-based inhibitors for the detection of trypsin-like serine proteases (*14*) and, more recently, on the development of an active site directed probe for dipeptidyl peptidase IV-like serine proteases, based on a biotinylated dipeptide proline diphenylphosphonate (*15, 16*). In a related approach, Cravatt and co-workers have employed fluorophosphonate derivatives of long-chain fatty acids for visualization and characterization of catalytically active serine hydrolases including fatty acid esterases and serine proteases (*6, 17*). Development of such "activity profiling" approaches has been particularly useful in assigning function to proteolytic enzymes from diverse biological milieu. Indeed, such approaches may be of particular utility in validating protease targets in disease processes where analysis of levels of transcription and translation of a protease do not necessarily correlate well with enzyme activity (*18*).

We now report on a novel solid-phase synthesis approach for the generation of active site directed affinity probes based upon aminoalkyl diphenyl phosphonates and their application for the profiling and characterization of serine proteases obtained

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10.1021/bc9002162 CCC: $40.75
Published on Web 10/07/2009

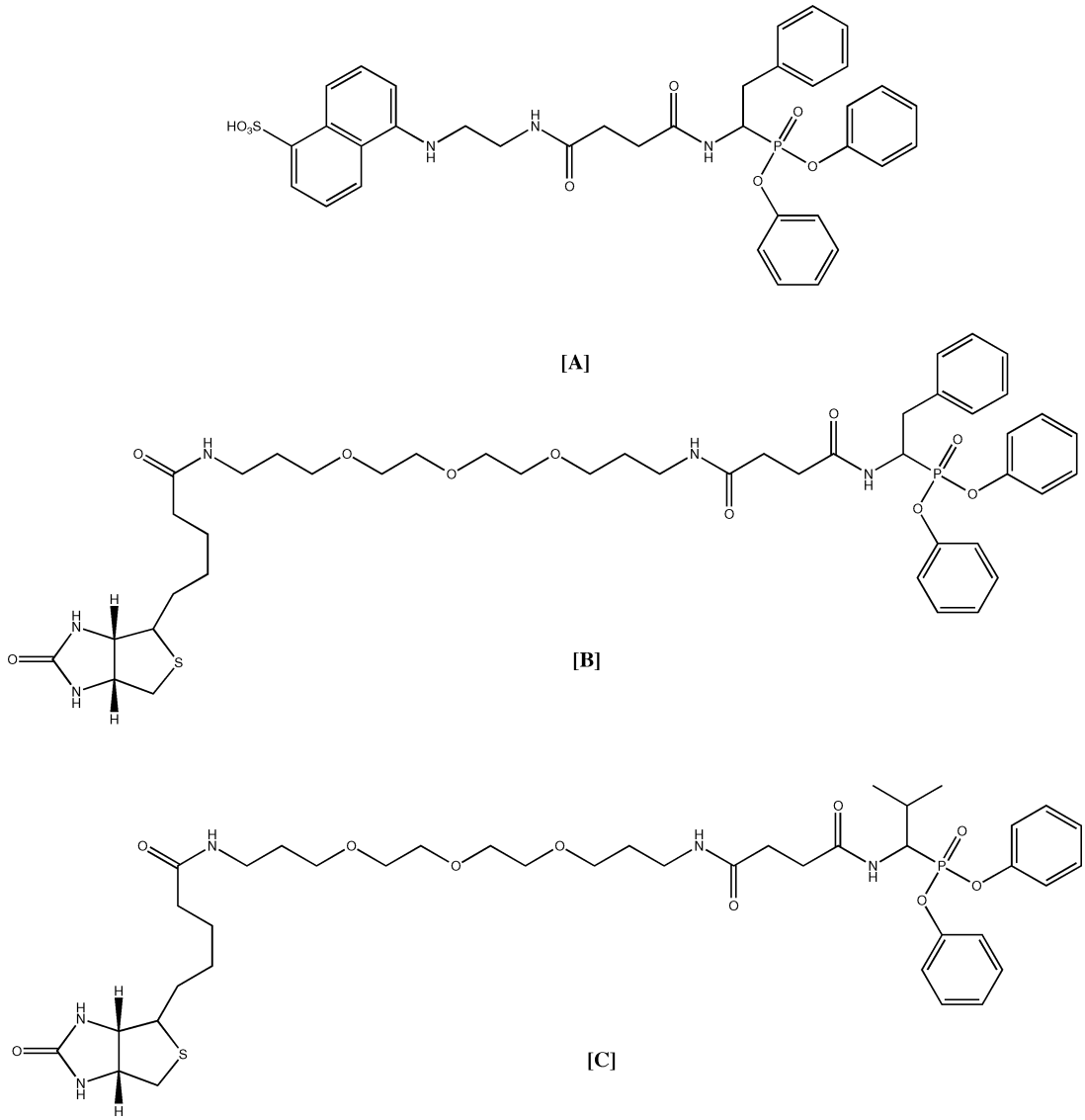


Figure 1. Affinity based probes synthesized for this study, according to Scheme 1. [A] EDANS-succinyl-Phe^{P}(OPh)$_2$. [B] Biotinyl-PEG-succinyl-Phe^{P}(OPh)$_2$. [C] Biotinyl-PEG-succinyl-Val^{P}(OPh)$_2$.

from a broad variety of biological sources. The synthetic scheme employs recently available NovaTag resin technology that enables the facile synthesis of activity probes bearing biotin or fluorescent tags and which can also incorporate pegylated spacer units, if desired. Using this approach, we have synthesized a number of affinity probes that target serine proteases with differing P1 specificities and demonstrate their detection by Western blot. Furthermore, the application of fluorescently tagged active site directed probes for "in-gel" detection is examined. We also demonstrate the utility of biotinylated and pegylated affinity probes for the isolation/enrichment of serine protease species, in this case, the *Pseudomonas aeruginosa* MucD peptidase (*19*), via exploitation of their interaction with immobilized streptavidin, and their subsequent *de novo* sequencing, using mass spectrometry.

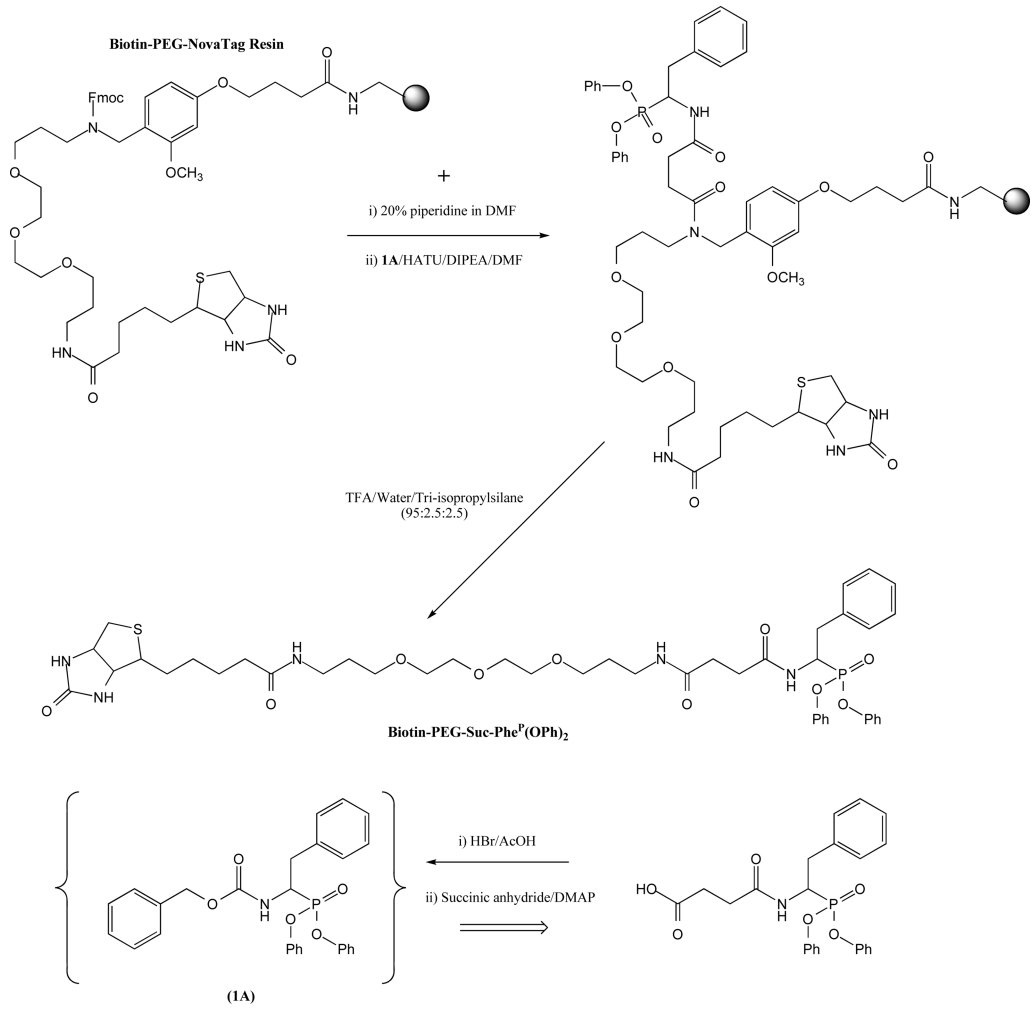
EXPERIMENTAL PROCEDURES

Biotin-PEG NovaTag resin, EDANS NovaTag resin, and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) were purchased from Novabiochem, Nottingham, U.K. *N*,*N*-Dimethylformamide (DMF) was supplied by BDH Chemicals Ltd. (Poole, England). *N*,*N*-Diisopropylethylamine (DIPEA) and piperidine/DMF (20% v/v) were supplied by PerSeptive Biosystems Ltd. (Warrington, England). Succinic anhydride and dimethylamino pyridine were purchased from Aldrich Chemical Co., Poole, Dorset (UK). All chemicals and solvents were of the highest purity and were subjected to no further purifications prior to use. Horseradish peroxidase streptavidin was obtained from Vector Laboratories Ltd., Burlingame, CA, USA. SuperSignal West Pico Chemiluminescent Substrate was supplied by Pierce Biotechnology Inc., Rockford, IL, USA.

Synthesis of Active Site Probes. The protected aminoalkane diphenylphosphonate derivatives diphenyl-1-[*N*-benzyloxycarbonyl]-amino phenylethane phosphonate (Cbz.Phe^{P}(OPh)$_2$)[1] and diphenyl-1-[*N*-benzyloxycarbonyl]-amino isobutane phosphonate (Cbz.Val^{P}(OPh)$_2$) were prepared by reacting triphenylphosphite, benzyl carbamate and the appropriate aldehyde corresponding to the desired amino acid side chain according to the method of Oleksyszen et al. (*11, 20*). Each of the

[1]Abbreviations: Bio; Biotin, Cbz; benzyloxycarbonyl, EDANS; 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid, HNE; Human Neutrophil Elastase, ITPG; isopropyl-β-D-1-thiogalactopyranoside, PEG; poly(ethylene glycol), PMSF; phenylmethanesulphonylfluoride, SDS; sodium dodecyl sulfate, Suc-; succinyl, TFA; trifluoroacetic acid, TIPS; tri-isopropylsilane, (Xaa)^{P}(OPh)$_2$; Xaa-diphenyl phosphonate.

Scheme 1. General Solid-Phase Synthetic Scheme for Biotinylated P1-Aminoalkane Diphenylphosphonate Affinity Probes for the Serine Proteases Utilizing Biotin PEG NovaTag Resin



compounds was found to be correct by elemental analysis and ^{1}H NMR, and had melting points in close agreement with the literature values (*11, 20*). Each derivative was obtained as a 50/50 mix of the L and D enantiomers.

Removal of the Cbz-protecting group was achieved by treatment with HBr/acetic acid (~33% v/v) for 90 min at room temperature. The succinyl derivatives of each of the deprotected aminoalkyl diphenyl phosphonate were then prepared by reaction with succinic anhydride, in dry DMF, at room temperature, overnight, in the presence of 2.0 equiv of DIPEA. These succinyl derivatives were then used to acylate samples of Biotin-PEG NovaTag (Novabiochem: cat no 04−12−3908) or EDANS-NovaTag (Novabiochem: cat no 04−12−3904) resin. This was achieved by first removing the Fmoc-protecting group from the benzyl-linked amine of the resin, by treatment with piperidine/DMF (20%, v/v), for 15 min at room temperature. After standard washing procedures (*8*), the succinylated aminoalkyl diphenylphosophonates were then coupled to the newly exposed secondary amine grouping on the resin via HATU-mediated condensation. The labeled diphenyl phosphonate derivatives of phenylalanine and valine were then cleaved from the resin by treatment with TFA/H_2O/TIPS (95/2.5/2.5; v/v/v) for 90 min at room temperature.

Kinetic Evaluation of Affinity Based Probes. Stock solutions (10 mM) of each affinity based probe were diluted in the appropriate assay buffer to give final concentrations of 5 μM, 10 μM, 25 μM, 50 μM, and 100 μM, which are evaluated for inhibitory activity against each target proteinase using a standard microtiter plate assay. Fluorogenic substrate was added such that a final concentration of 50 μM in each well initiates the reaction. The inhibitory potency of each inhibitor was determined at five individual concentrations using a standard microtiter plate assay where the rate of substrate hydrolysis at 37 °C, in each instance, was monitored every 60 s over a period of 90 min by measuring the rate of increase of fluorescence at (excitation λ_{nm} 395 ± 45, emission λ_{nm} 460 ± 40) in a CYTOFLUOR Multiwell Plate Reader Series 4000 spectrofluorimeter. Each experiment is performed in triplicate. Typical progress curves are shown in Figure 2.

Labeling and Detection of Peptidases. *Electrophoresis.* Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed by a modification of the method of Laemmli (*21*). Initially, SDS-PAGE was performed on NuPage Novex 4−12% Bis-Tris gels. Labeled proteins were denatured by boiling for 10 min in Laemmli treatment buffer. SeeBlue Plus2 prestained molecular weight standards were included in each gel.

Western Blotting. Electroblotting of the separated proteins was carried out using a modification of the methodology described first by Towbin et al. (*22*). Separated proteins were electroblotted onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Milipore) in NuPage Transfer Buffer (Invitrogen Ltd.). The running time was 2 h at 200 mA, at room temperature.

Detection of Labeled Peptidases. Labeled peptidases were detected using the methods previously described (*9, 16*).

Table 1. Mass Spectrum Analysis

reagent	predicted	observed
Bio-PEG-Suc-Val^P(OPh)$_2$	833.99	834.2/835.2
Bio-PEG-Suc-Phe^P(OPh)$_2$	882	882.2/883.2
EDANS-Suc-Phe^P(OPh)$_2$	635	636.3

Essentially, following protein transfer, unbound sites on the Immobilon-P PVDF membrane were blocked by incubation of the blot membrane in a 3% (w/v) solution of bovine serum albumin (BSA) in Tris-buffered saline (TBS; 0.05 M Tris/HCl, 0.1 M NaCl, pH 7.4) for at least 1 h at room temperature. After washing with TBS, the solution was discarded and replaced with streptavidin/horseradish peroxidase (Streptavidin-HRP) (Vector Laboratories) prepared at a dilution of 1 in 10 000 in blocking solution containing 0.3% BSA at room temperature for 45 min. The membrane was washed at least six times with TBS containing 0.1% Tween 20 and excess liquid removed. Horseradish peroxidase activity was detected by overlay with SuperSignal West Pico chemiluminescent substrate. After removal of the substrate, the blot was placed between acetate sheets and overlaid with photographic film in a film cassette. Films were exposed for ~30 s developed and fixed.

Direct Detection. For labeling studies employing EDANS-labeled probe, the method above was followed except that the electrophoresis unit was protected from light during each run. The gel was removed from the cassette, and labeled peptidases were viewed directly on a high-performance ultraviolet transilluminator (Ultraviolet Products Ltd., Cambridge, UK). Images were captured using a Syngene Gene-Genius Bioimaging system with on-board UV transilluminator or using standard photographic film.

Labeling of Recombinant MucD in Crude Cell Lysates. The MucD gene of *Pseudomonas aeruginosa* PA01 was amplified from genomic DNA by PCR and subsequently cloned into the *E. coli* expression vector pQE30 (Qiagen). Site-directed mutagenesis of the putative active site serine to an alanine at position 217 was carried out using site-directed mutagenesis kit (Stratagene) as per the manufacturers instructions. TOP10F' *E. coli* cells (Invitrogen) harboring empty pQE30 plasmid, wild-type MucD, and Ser-Ala active site mutant grown to early log phase (37 °C) were induced using isopropyl-β-D-1-thiogalactopyranoside (ITPG, 1 mM final concentration), prior to a further 3 h incubation. Cells were harvested and lysed by sonication in assay buffer (25 mM Hepes, 5 mM $CaCl_2$, pH 7.5). Biotinylated DPP probes (final concentration, 50 uM) were incubated with the freshly prepared lysates for 90 min at either 37 or 45 °C. Labeled peptidases were detected as described previously.

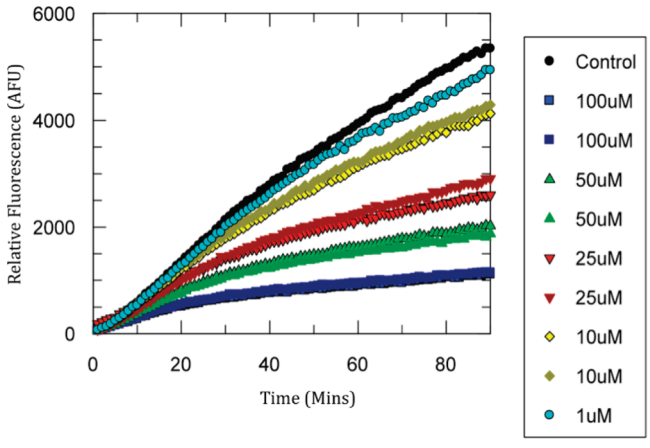


Figure 2. Typical progress curves for the generation of 7-amino-4-methylcoumarin from the chymotrypsin catalyzed hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-NHMec in the presence of EDANS-succinyl-Phe^P(OPh)$_2$ The figures represent the best fit of data to the equation for irreversible inhibition (Walker and Elmore, 1984) of chymotrypsin by EDANS-succinyl-Phe^P(OPh)$_2$ in the presence of N-succinyl-Ala-Ala-Pro-Phe-NHMec. The inactivation studies were carried out at a fixed substrate concentration (50 μM) and varying concentrations of inactivator as indicated above.

Mechanism Based Affinity Capture of Recombinant MucD in Crude Cell Lysates. *Escherichia coli* TOP10F' cells harboring pQE30 plasmid expressing wild-type MucD and Ser-Ala active site mutant were grown to early log phase (37 °C) and induced using ITPG (1 mM final concentration), prior to a further 3 h incubation. Cells were harvested and lysed by sonication in MucD assay buffer (25 mM HEPES, 5 mM $CaCl_2$, pH 7.5). The Biotin-PEG-Suc-Val^P(OPh)$_2$ probe (final concentration, 50 μM) was incubated with the freshly prepared lysates for 90 min at 37 °C. Samples were subjected to affinity capture with strepavadin magnetic Dynabeads (dynal Biotech) and washed 6 times in PBS Tween 20 (0.1%). Samples were subjected to SDS-PAGE electrophoresis and stained with Brilliant Blue G - Colloidal Coomassie. Protein bands were excised from the Coomassie stained gel, treated with acetonitrile, and dried. Sequencing grade trypsin (Promega) was added, and samples were incubated at 30 °C overnight. Digested peptides were extracted from the gel pieces using 5% v/v trifluoroacetic acid and 50% v/v acetonitrile and dried. The samples were then rehydrated, desalted, and concentrated using in-tip reverse-phase resin (Zip Tip C18, Millipore). Using a Thermoquest LCQ Deca mass spectrometer, in nanospray mode, the samples were analyzed and MS/MS data collected by Xcalibur. Finally, MS/MS ion search was performed by Mascot (*23*), using the database NCBInr.

RESULTS

Synthesis of Affinity Probes. Our primary aim in this study was the development of a robust and broadly applicable solid-phase approach for the synthesis of diphenyl phosphonate activity probes for the serine proteinases. As an example, the synthetic scheme for the synthesis of the P_1−Phe containing inhibitor (Biotin-PEG-Phe^P(OPh)$_2$) is shown in Scheme 1. In essence, this inhibitor sequence was constructed from the succinyl-derivatized diphenyl phosphonate analogue of phenylalanine (**1A**), which in turn was prepared from the benzloxycarbonyl protected analogue (Cbz-Phe^P(OPh)$_2$). The synthesis of Biotin-PEG-Phe^P(OPh)$_2$ was expedited, using standard Fmoc solid-phase protocols, employing a CEM Liberty instrument, and using the recently developed Biotin-PEG NovaTag. We have found that the use of this solid-phase approach has eliminated the problems typically encountered with the solution-phase synthesis of biotin-containing derivatives, namely, poor solubility and sluggish coupling kinetics of biotin and its derivatives. We have incorporated the "PEG unit" into the inhibitor sequence to minimize steric hindrance limitations in the interaction between the biotin reporter group and streptavidin. This reporter moiety facilitates both the detection of labeled species using steptavidin horseradish peroxidase/alkaline phosphatase conjugated by Western blot and also the capture and purification inhibitor/protease complex for the purposes of sequencing by mass spectrometry (described below). The synthesis of EDANS-derived affinity probes follows exactly the same synthetic pathway as in Scheme 1, using the EDANS-NovaTag resin. The structures of the affinity-based probes synthesized in this study, according to Scheme 1, are shown in Figure 1. The identity of each compound was confirmed by electrospray mass spectroscopy, and the results shown in Table 1.

Kinetic Evaluation. The determined values for second-order rate constant, [A], for each inhibitor concentration were used

Table 2. Inhibitor Constants[a]

protease	inhibitor	k_i (min^{-1})	K_i (M)	$(k_i)/(K_i)$ (M^{-1} min^{-1})
chymotrypsin	Bio PEG-Suc-$Phe^P(OPh)_2$	0.119 ± 0.002	1.08×10^{-6}	1.11×10^5
cathepsin G	Bio PEG-Suc-$Phe^P(OPh)_2$	0.016 ± 0.003	2.59×10^{-6}	8.41×10^4
chymotrypsin	EDANS-Suc-$Phe^P(OPh)_2$	ND	ND	0.18×10^4
cathepsin G	EDANS-Suc-$Phe^P(OPh)_2$	0.029 ± 0.002	1.39×10^{-5}	0.22×10^4
HNE	Bio PEG-Suc-$Val^P(OPh)_2$	ND	ND	1.29×10^4

[a] ND - not determined.

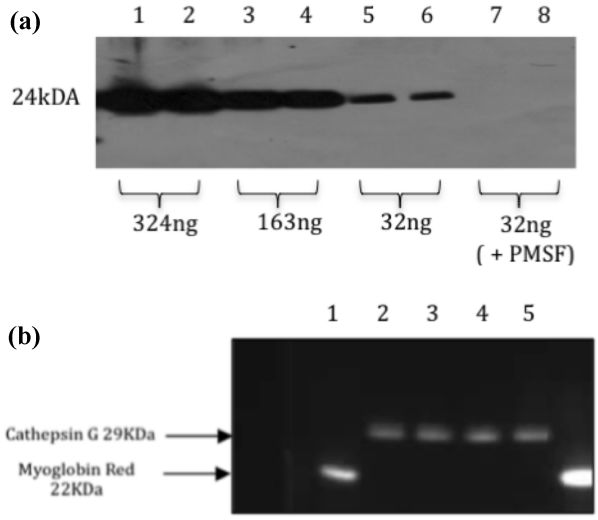


Figure 3. (a) Selective, active site directed labeling of recombinant human neutrophil elastase (HNE) by Biotin-PEG-succinyl-$Val^P(OPh)_2$ (20 μM). Lanes 1−2, 324 ng HNE; lanes 3−4, 162 ng HNE; lanes 5−6, 32 ng; lanes 7−8, 32 ng HNE preincubated with pretreated with Pefabloc (100 μM) for 15 min at 37 °C. (b) Direct visualization of purified cathepsin G protease affinity labeling using EDANS-succinyl-$Phe^P(OPh)_2$ (20 μM). Lane 1, SeeBlue prestained molecular weight markers; lanes 2−5 Cathepsin G (100 ng) incubated for 30 min with EDANS affinity based probe.

to determine the kinetic constants, K_i and k_i, for the kinetic scheme I, taken from Walker and Elmore (*25*). Progress curves were transformed using Grafit to give their linear transformations. Evaluation of the inhibitor constant K_i and the apparent first-order rate constant, k_i, for the irreversible inactivation was carried out using the methods of Tian and Tsou (*26*). A summary of determined overall second-order rate constants (k_i/K_i) for irreversible affinity-based probe inhibitors of each exemplar proteinase are shown in Table 2. In general, these compounds were found to be moderately potent irreversible inactivators of their target protease. The overall second-order rate constants are comparable to the corresponding Cbz-protected amino acid analogues Cbz-$Phe^P(OPh)_2$ (for Chymotrypsin; $k_i/K_i = (2.3 \pm 0.4) \times 10^4$ M^{-1} min^{-1}, Cathepsin G $k_i/K_i = (3.8 \pm 0.4) \times 10^3$ M^{-1} min^{-1}) and Cbz-$Val^P(OPh)_2$ (for human neutrophil elastase $k_i/K_i = (2.3 \pm 0.1) \times 10^3$ M^{-1} min^{-1}).

Proteinase Profiling. Initial profiling studies were performed on purified neutrophil elastase (HNE). Figure 3a illustrates the labeling of HNE using Bio-Peg-succinyl-$Val^P(OPh)_2$. In essence, samples of purified HNE were incubated with 20 μM biotinylated-inhibitor, for 30 min at 37 °C, prior to SDS-PAGE and Western blotting. Figure 3a shows a series of duplicates in which differing amounts of purified HNE are incubated with the fixed concentration of biotinylated inhibitor. From these results, it can be seen that, under these standard detection conditions, as little as 32 ng of protease can be detected, demonstrating the sensitivity of these irreversible probes. Crucially, the specificity of this probe was established by pre-inactivation of the protease using phenylmethanesulphonylfluoride (PMSF), which modifies the active site serine hydroxyl group, rendering the enzyme inactive. Preincubation of protease with PMSF prior to addition of the biotinylated probe completely blocked incorporation of the latter, indicating that it is active site directed.

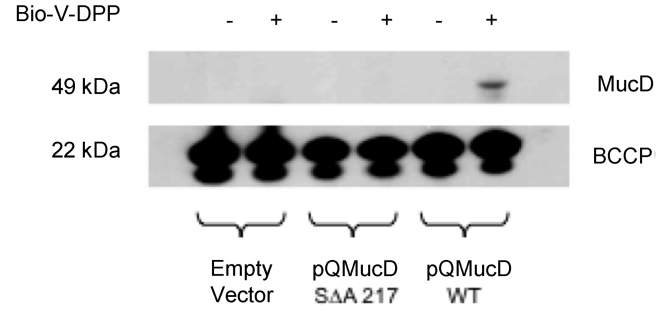


Figure 4. Bio-PEG-Succ-$Val^P(OPh)_2$ (Bio-V-DPP) specifically labels recombinant serine protease MucD in *E. coli* lysates. Bio-V-DPP (50 μM) was incubated (90 min, 37 °C) with sonicated pellets from *E. coli* cultures harboring pMucD and catalytically inactive SΔA217, with MucD expression induced with IPTG (1 mM) in all samples. Protein samples were then applied to SDS-PAGE and Western blotted; bands with incorporated biotin moieties were disclosed using streptavidin-HRP. Biotin carboxyl carrier protein (BCCP) is endogenously biotinylated and is used here as a loading control.

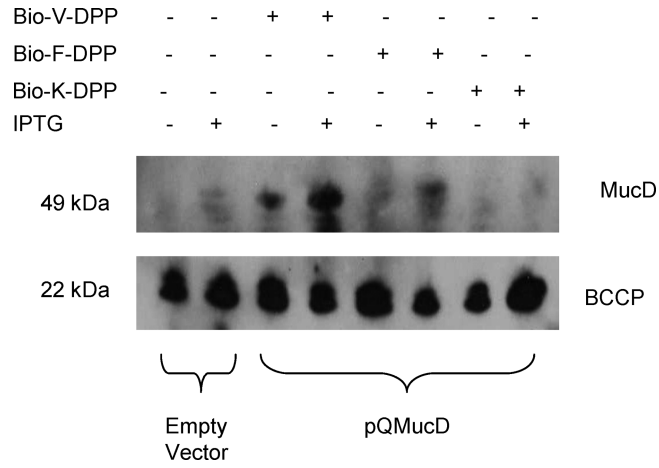


Figure 5. Biotinylated probes (50 μM) were incubated with sonicated cell pellets from cultures harboring pMucD, with MucD expression induced with IPTG (1 mM). Probes were incubated with sonicates for 90 min at 37 °C, prior to application to SDS-PAGE and Western blotting. Bands with incorporated biotin moieties are disclosed using streptavidin−HRP. Biotin carboxyl carrier protein (BCCP) is endogenously biotinylated and is used here a loading control.

The ability to detect protein species by Western blotting is a routinely used methodology, but it is not without its limitations. As it uses a number of steps beyond electrophoresis, including membrane transfer, blocking, and application of primary and/or secondary detection reagents, the process can frequently take a number of hours, if not overnight. Furthermore, the more steps involved, the more possibilities arise for human error in the visualization of the labeled proteins. Therefore, in an effort to circumvent these potential limitations, we have investigated the replacement of the biotin reporter with a fluorescent moiety to permit "in-gel" visualization. The direct labeling of cathepsin

(a)

(b)

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  1   MHTLKRCMAA MVALLALSLA MTARAELPDF TPLVEQASPA VVNISTRQKL
 51   PDRAMARGQL SIPDLEGLPP MFRDFLERSI PQVPRNPRGQ QREAQSLGSG
101   FIISNDGYIL TNNHVVADAD EILVRLSDRS EHKAKLIGAD PRSDVAVLKI
151   EAKNLPTLKL GDSNKLKVGE WVLAIGSPFG FDHSVTAGIV SAKGRSLPNE
201   SYVPFIQTDV AINPGNSGGP LLNLQGEVVG INSQIFTRSG GFMGLSFAIP
251   IDVALNVADQ LKKAGKVSRG WLGVVIQEVN KDLAESFGLD KPSGALVAQL
301   VEDGPAAKGG LQVGDVILSL NGQSINESAD LPHLVGNMKP GDKINLEVIR
351   NGQRKTLSMA VGSLPDDDEE IASMGAPGAE RSSNRLGVTV ADLTAEQRKS
401   LDIQGGVVIK EVQDGPAAVI GLRPGDVITH LDNKAVTSTK VFADVAKALP
451   KNRSVSMRVL RQGRASFITF KLAE*
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Figure 6. Capture and identification of MucD from *Pseudomonas aeruginosa* cell lysates. (a) Lysates probed with Bio-V-DPP were subsequently incubated with streptavidin agarose beads and captured proteins eluted, electrophoresed, and visualized by silver staining. A band at 50 kDa (see highlighted) was specifically captured in the presence of the probe and was subsequently excised and characterized by mass spectrometry. (b) Tryptic digestion of the protein in the band was performed and peptides subjected to MS/MS. MS/MS ion searches using MASCOT identified four peptides from *P. aeruginosa* MucD (Accession number NM_002516).

G (100 ng) using a fluorescently labeled affinity based probe (EDANS-succinyl-Phe^{P}(OPh)$_2$) is shown in Figure 3b.

Labeling, Capture, and Identification of Protease Species. Next, we wished to examine the application of these probes for the detection and identification of serine protease species in protein extracts. For these studies, we chose a model protease, MucD, from *P. aeruginosa.* MucD is an important mediator of virulence in *P. aeruginosa* infections (*26*) and is a negative regulator of alginate production (*27*). MucD mutants have been shown to exhibit reduced virulence in a number of different pathogen−host models (*26*). Furthermore, *P. aeruginosa* MucD inactivation results in decreased resistance to H_2O_2 and thermal kill (*28*). Therefore, selective inactivation of MucD may constitute a novel antivirulence approach in the treatment of *P. aeruginosa* infections.

To study the ability to label MucD using the diphenyl phosphonate probes, the open reading frame was cloned into an *Escherichia coli* expression vector (pQE30), from which the inducible expression of MucD could be stimulated using IPTG. The insertion of the open reading frame into pQE30 also facilitates the incorporation of an N-terminal hexahistidine tag, which allowed the visualization of the expressed recombinant protein at the anticipated 49 kDa in the presence of IPTG (data not shown). Next, lysates from IPTG-induced cultures expressing MucD were incubated with Bio-PEG-Suc-Val^{P}(OPh)$_2$, where we observed clear labeling of the recombinant enzyme (Figure 4, lane 6). A catalytically inactive mutant (SerΔAla217) was also prepared, and the probe failed to label this species, further demonstrating the need for the hydroxyl side chain in the Ser residue to which the diphenyl phosphonate probes react and bind.

On the basis of these findings, we then examined the P_1 specificity in the probes on the basis of their ability to label the recombinant MucD protein. Valine, phenylalanine, and lysine derived biotinylated diphenylphosphonates where incubated with MucD overexpressing bacterial lysates, and as anticipated, the Val probe showed labeling as before (Figure 5, lanes 3 and 4). Under the same conditions, labeling by the Phe-derived probe was also visualized, albeit at lower intensities than the Val probe (lanes 5 and 6). Conversely, under these conditions no binding of the Lys probe was observed. Collectively, these data suggest that the enzyme has a clear preference for hydrophobic residues in the P_1 position (Val > Phe) over the positively charged Lys derivative. Although no comprehensive substrate specificity profiling of *P. aeruginosa* MucD has yet been published, these results are perhaps unsurprising given that MucD is orthologous to the *Escherichia coli* periplasmic protease and chaperone HtrA (DegP) (*26*), which has a demonstrated P1 preference for valine (*29*).

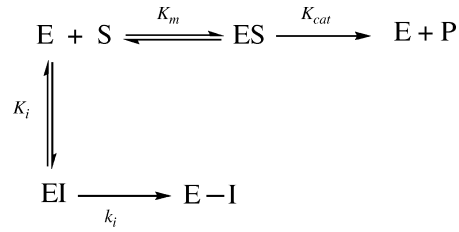
As we could identify and visualize the labeling of recombinant MucD, we then examined the application of the biotinylated Bio-PEG-Suc-V-DPP (Bio-V-DPP) probe to facilitate the capture and isolation of MucD from *P. aeruginosa* cultures. Cultures were lysed and incubated with the Bio-V-DPP probe as described and then incubated with streptavidin beads. Eluted proteins were analyzed by SDS-PAGE allowing clear visualization of a 50 kDa band specific to probe-treated samples (Figure 6a). This band was subsequently excised and protein species analyzed by mass spectrometry where it was established that MucD was successfully identified (Figure 6b). Analysis of the corresponding gel fraction from samples purified in the absence of probe did not facilitate the identification of MucD (data not shown), demonstrating that its capture and identification were dependent on the preincubation with the biotinylated probe. Collectively, these results show that these biotinylated reagents have applicability in not only the visualization of protease species, but also their activity-based capture, purification, and downstream characterization.

DISCUSSION

We have described a novel facile synthesis route for substrate-derived diphenyl phosphonate probes for the detection of serine proteases. During these studies, we have demonstrated the ability of these probes to label proteases, simply on the basis of their catalytic activity, and facilitate their downstream visualization. Using biotin reporter groups, we have demonstrated the ability to visualize bound species by Western blot. Furthermore, replacement of biotin with a fluorescent tag facilitates the direct sensitive analysis of bound species by SDS-PAGE, which has the advantage of circumventing the need for downstream Western blotting. Finally, we also demonstrate that the compounds synthesized here can be used for the affinity capture and retrieval of protease species from biological lysates.

Although the affinity-based probes developed in this study have been utilized here primarily for the detection and identification of bacterial/prokaryotic proteases, the kinetic evaluation studies were performed against eukaryotic pro-

Scheme 2. Kinetic Scheme for Irreversible Inhibitors Acting via a Complexing Mechanism, Taken from Walker and Elmore (1984)



teases, namely, bovine chymotrypsin, cathepsin G (from human leukocytes), and human neutrophil elastase demonstrate the broad applicability of these probes. Indeed, we have shown in a previous study that biotinylated proline diphenyl phosphonate derived inhibitors were capable of inactivation and labeling of dipeptidyl peptidase IV purified from porcine kidney (*16*). These observations, taken together with the extensive characterization of diphenyl phosphonate derived inhibitors against a wide range of serine protease already published in the literature (*11*−*13, 15, 16*), indicate that the affinity based probes described here will have utility in the detection, profiling, and identification of a wide range of serine proteases from both prokaryotic and eukaryotic sources. The ability to capture, purify, and characterize protease species on the basis of their catalytic activity represents a powerful discovery methodology rather than merely revealing additional members of known proteinase families through homology screening approaches. Therefore, the large-scale screening of complex protein milieu for protease species could highlight proteins of novel sequences/conformations that have yet to be demonstrated to possess proteolytic activity.

ACKNOWLEDGMENT

This work was supported by a BBSRC Project grant (BB/D005957/1).

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BC9002162