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Facile Synthesis of Aminomethyl Phosphinate Esters as Serine Protease Inhibitors with Primed Site Interaction

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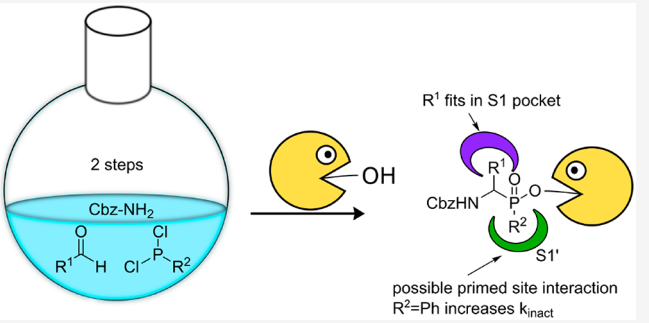
ABSTRACT: Serine proteases comprise about one-third of all proteases, and defective regulation of serine proteases is involved in numerous diseases. Therefore, serine protease inhibitors are promising drug candidates. Aminomethyl diphenyl phosphonates have been regularly used as scaffolds for covalent serine protease inhibition and the design of activity-based probes. However, they cannot make use of a protease's primed site. Therefore, we developed a facile two-step synthesis toward a set of phenyl phosphinates, which is a related scaffold but can interact with the primed site. We tested their inhibitory activity on five different serine proteases and found that a phenyl group directly attached to the phosphorus atom leads to superior activity compared with phosphonates.



KEYWORDS: *Covalent inhibitors, phosphinates, protease inhibitor, serine proteases*

Serine proteases comprise about one-third of all proteases. A multitude of them are implicated in human diseases. Neutrophil elastase, for instance, plays a role in inflammatory diseases such as inflammatory bowel disease,[1] chronic obstructive pulmonary disease,[2] and cystic fibrosis.[3] Furthermore, it is a component of neutrophil extracellular traps (NETs), a network of chromatin and granular proteins that is secreted by neutrophils to trap and kill pathogens.[4] Chymase is linked to atherosclerosis and gastric cancer, and numerous other serine proteases are involved in carcinogenesis, tumor progression, and neurodegeneration.[5] Hence, serine proteases are attractive drug targets and protease inhibitors represent promising drug candidates. For example, *camostat* is an approved drug for treatment of chronic pancreatitis.

The development of covalent inhibitors is a re-emerging topic in drug development.[6,7] Various recently approved drugs, such as ibrutinib for the treatment of leukemia, have a covalent inhibitory mechanism.[7] In the past, several covalent inhibitor scaffolds for serine proteases have been developed, such as isocoumarins,[8] benzoxazinones,[9] *N*-(sulfonyloxy) and *N*-(acyloxy) phtalimides,[10] sulfonyl[11] and phosphonyl fluorides,[12] 1,2,5-thiadiazolidin-3-one 1,1-dioxides,[13] diphenyl phosphonates,[14] β-lactams, and more recently oxolactams.[15,16] Interestingly, these can not only be used as inhibitors but also as warheads for activity-based probes (ABPs).

α-Aminomethyl diphenyl phosphonates are of particular interest due to their ease of synthesis by a one-step three-component reaction.[17] Although usually synthesized as an enantiomeric mixture, the *R*-configuration, which corresponds to the natural *L*-configuration of amino acids, is usually the active species.[18,19] Because of their close resemblance to amino acid residues, peptidyl diphenyl phosphonates allow for a rational inhibitor design, using knowledge of the protease substrate specificity, which is determined by subsites neighboring the active site. The subsites interact with side chains of individual amino acid residues around the cleaved peptide bond (scissile bond).[20] For S1 family proteases, the biggest family of serine proteases, the S1 pocket is the primary recognition site, but other subsites also contribute to selectivity. Therefore, extending phosphonate inhibitors beyond the P1 position can improve selectivity. For example, inclusion of non-natural amino acids in the P2−P4 position has led to highly active and selective inhibitors for the serine protease human neutrophil elastase (HNE).[21,22]

Aminomethyl diphenyl phosphonates make no efficient use of the primed site of the protease. Although adding substituents to the phenyl group has been shown to influence the activity,[23,24] this may be a result of the leaving group capacity, possible primed site interaction, or a combination thereof. However, the leaving groups are not observed in crystal structures of the compounds due to "aging" (Figure 1, upper part). Knowledge of the interaction with the primed site,

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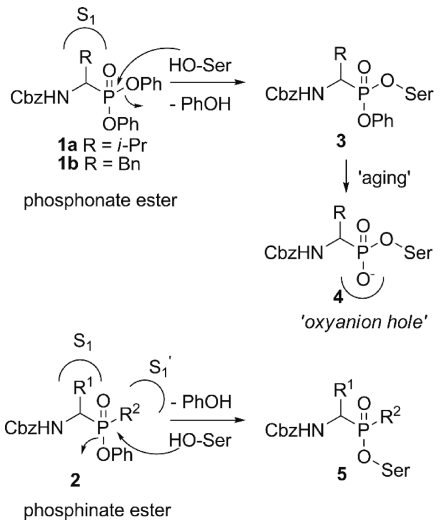


Figure 1. Phosphonate esters (**1**) and phosphinate esters (**2**) bind with their side chain to the protease S1 subsite. Phosphinates can potentially make use of the primed sites with their R^2 group. Diphenyl phosphonates (upper) react with the nucleophilic active site serine of the protease, thereby expelling a phenoxy leaving group. The second phenoxy group is later lost in an "aging" process, and the negatively charged oxygen occupies the oxyanion hole. Phosphinates react in the same manner but do not undergo the aging process.

however, could lead to a potential gain in selectivity. Phosphinates are structurally similar to phosphonates but have one of the ester bonds replaced by a carbon substituent. As a result, phenyl phosphinate esters only possess one leaving group (Figure 1, lower part) but have an additional stereocenter at the phosphorus atom. With their carbon substituent, phosphinates may have the ability to interact with the primed site, thereby potentially increasing selectivity.

A small number of studies have reported phosphinate serine protease inhibitors,[25−27] but these studies only incorporated one carbon substituent, preventing a thorough analysis of their influence on activity. Additionally, the synthesis of most of these compounds requires three to five synthetic steps from commercially available starting materials.[26]

Here we report a facile one-pot (for benzaldehydes) and two-pot (for other aldehydes) two-step synthesis of α-amino phenyl phosphinate esters as covalent, irreversible serine protease inhibitors. We show that they display better inhibition of various S1 family serine proteases compared with diphenyl phosphonates and we present a possible binding mode of the different carbon substituents in the active site of several serine proteases.

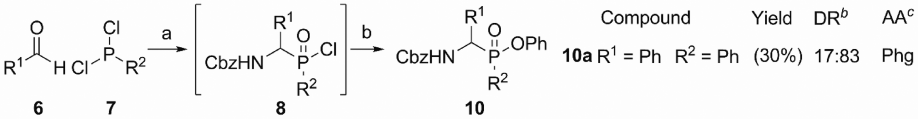
Inspired by work on the synthesis of phosphinodepsipeptides by Meng and Xu,[28] we explored a two-step one-pot synthesis of the phosphinate scaffold in a pseudo-four-component condensation reaction (Scheme 1). The procedure starts with a three-component Mannich-type reaction between commercially available aldehydes **6**, dichlorophosphines **7**, and benzyl carbamate (Scheme 1). From these materials, the aldehyde R^1 substituent forms the eventual amino acid side chain mimic, whereas the R^2 substituent on the phosphine constitutes the potential primed site binding element. The formed phosphinyl chloride intermediate **8** is then reacted with phenol and triethylamine to yield the final phosphinate ester **10**. Although successful for benzaldehyde as a starting material, the one-pot procedure did not succeed with aliphatic aldehydes. As an alternative, we directed our efforts toward a two-step synthesis, using the same starting materials (Scheme 2). Here, we isolated intermediate phosphinic acids **9**, which were then reacted with phenol in a separate, Steglich-type esterification using DMAP and DIC (Scheme 2). Satisfyingly, this yielded the desired compounds **10**−**13**, as a mixture of diastereomers. Since no purification of the intermediate is necessary, this two-step synthesis is a facile way to access α-amino phosphinate esters with aliphatic as well as aromatic amino acid side chains.

Since one of our aims was to identify the influence of R^2, we used different dichlorophosphines to vary this substituent. Unfortunately, our choice was limited by the small amount of commercially available dichlorophosphines. Eventually, we selected three different ones, each with a distinct carbon substituent: **7a** with a phenyl substituent would most closely resemble the diphenyl phosphonate counterparts and was the only commercially available dichlorophosphine with an aromatic substituent (**1**, see Figure 1B). Besides this, **7b** with *n*-propyl as a sterically small and **7c** with *t*-butyl as a bulky, sterically demanding substituent were chosen. The aldehydes were selected according to the proteases to be targeted. We decided for phenylacetaldehyde, benzaldehyde, isovaleraldehyde, and isobutyraldehyde leading to analogues of Phe, Phg, Leu, and Val, respectively.

The free acid intermediates **9** were generally obtained as pure compounds after a simple aqueous workup. Unfortunately, despite several efforts, the reaction with **7b** or **7c** and phenylacetaldehyde did not yield the desired product. Overall, the obtained yields are generally higher for reactions with *n*-propyldichlorophosphine and lower for the sterically more demanding *t*-butyldichlorophosphine, whereas the ones for phenyldichlorophosphine are found in between.

Having the small series of phosphinates in hand, we tested their inhibitory activity on different serine proteases from the S1 family of serine proteases. We decided for neutrophil elastase (NE), porcine pancreas elastase (PPE), and proteinase 3 (PR3) which possess a preference for small hydrophobic amino acids in their S1 site as well as for chymotrypsin (ChT) and cathepsin G (CatG) which in turn prefer cleavage after large hydrophobic amino acids. In order to select active compounds, we first performed a competitive activity-based protein profiling experiment (competitive ABPP) at high

Scheme 1. Two-Step, One-Pot Synthesis of Phosphinate Esters[a]



[a]Reagents and conditions: (a) $CbzNH_2$, acetonitrile, reflux; (b) TEA, phenol. [b]Diastereomeric ratio as determined after silica chromatography. [c]Mimic of amino acid: Phg = phenylglycine.

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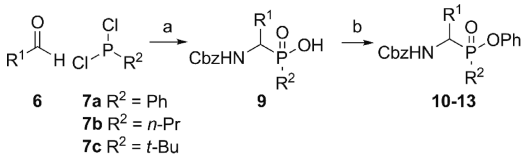
Scheme 2. Two-Step, Two-Pot Synthesis of Phosphinate Esters[a]



Compound	Yield	DR[b]	AA[c]
10a R1 = Ph R2 = Ph	(22%)	26:74	Phg
10b R1 = Ph R2 = *n*-Pr	(28%)	35:65	Phg
10c R1 = Ph R2 = *t*-Bu	(10%)	32:68	Phg
11a R1 = *i*-Bu R2 = Ph	(28%)	7:93	Leu
11b R1 = *i*-Bu R2 = *n*-Pr	(27%)	28:72	Leu
11c R1 = *i*-Bu R2 = *t*-Bu	(9%)	36:64	Leu
12a R1 = *i*-Pr R2 = Ph	(14%)	14:86	Val
12b R1 = *i*-Pr R2 = *n*-Pr	(26%)	19:81	Val
12c R1 = *i*-Pr R2 = *t*-Bu	(4%)	0:100	Val
13a R1 = Bn R2 = Ph	(8%)	39:61	Phe

[a]Reagents and conditions: (a) $CbzNH_2$, acetonitrile, reflux, aqueous workup; (b) phenol, DIC, DMAP, toluene, 80 °C, 4 h. [b]Diastereomeric ratio as determined after silica chromatography. [c]Mimic of amino acid: Phg = phenylglycine.

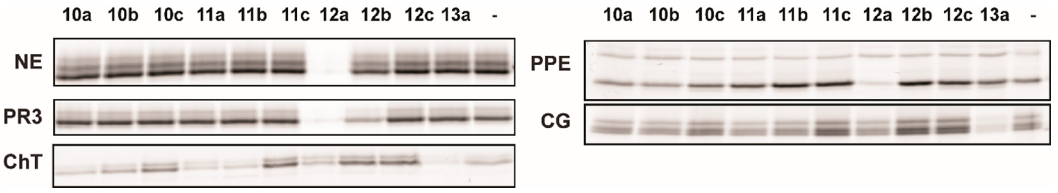


Figure 2. Initial testing of compounds by competitive activity-based protein profiling. Proteases were treated with the indicated compound at 100 μM or DMSO control (−), after which residually active enzyme was fluorescently labeled with the activity-based probe FP-Rh. Hence, a disappearance of a band corresponds to an active compound. NE = human neutrophil elastase, PR3 = human proteinase 3, ChT = bovine chymotrypsin, PPE = porcine pancreatic elastase, CG = human cathepsin G.

Table 1. Kinetic Values of the Synthesized Inhibitors

entry	enzyme	compound	AA[a]	k_{inact} (10^{-3} s^{-1})	K_I (μM)	k_{inact}/K_I (M^{-1} s^{-1})	DR[b]
1	NE	**12a**	Val	13.0 ± 3.0	51.0 ± 15.4	255 ± 96	11:89
2		**1a** (Val^P)	Val	0.59 ± 0.04	2.96 ± 0.85	200 ± 59	
3	PR3	**12a**	Val	11.0 ± 1.6	26.5 ± 6.2	415 ± 115	11:89
4		**12b**	Val	1.75 ± 0.20	83.8 ± 18.3	21 ± 5	54:46
5		**1a** (Val^P)	Val	n.d.[c]	n.d.[c]	n.i.[d]	
6	PPE	**12a**	Val	7.80 ± 0.79	132 ± 19	59 ± 10	11:89
7		**1a** (Val^P)	Val	n.d.[c]	n.d.[c]	n.i.[d]	
8	ChT	**12a**	Val	3.04 ± 0.15	7.42 ± 0.83	409 ± 50	11:89
9		**10a**	Phg	1.57 ± 0.07	0.46 ± 0.076	(3.4 ± 0.6) × 10^3	20:80
10		**10b**	Phg	5.84 ± 0.9	14.5 ± 3.8	404 ± 122	55:45
11		**11a**	Leu	21.1 ± 2.0	21.6 ± 3.3	978 ± 176	44:56
12		**11b**	Leu	2.44 ± 0.31	13.0 ± 2.9	188 ± 48	53:47
13		**13a**	Phe	23.0 ± 1.7	1.40 ± 0.28	(16.4 ± 3.6) × 10^3	39:61
14		**1b** (Phe^P)	Phe	5.63 ± 0.24	0.69 ± 0.07	(8.1 ± 0.9) × 10^3	

[a]The tested compound mimics the indicated amino acid. [b]Compounds were tested as indicated mixtures of diastereomers (either as obtained in the reaction or as a mixture with a DR of approximately 50:50). [c]n.d.: not determined; since no inhibition was seen, these values were not determined. [d]n.i.: no inhibition; no good inhibition was seen in a dilution series between 200 and 26.7 μM.

inhibitor concentration. To this end, each of these enzymes was incubated with 100 μM of the compounds, and residual activity was measured by labeling with the general serine hydrolase ABP FP-rhodamine.[29] As can be seen in Figure 2, NE, PR3, and PPE are best inhibited by the valine derivative **12a**, in agreement with their P1 preference. The substituent on the phosphorus, however, seems crucial. Whereas the phenyl substituent results in an active compound (**12a**), the *n*-propyl substituent renders the compound inactive (NE and PPE) or dramatically reduces the inhibitory capacity (PR3) and a *t*-butyl inactivates it completely. For CatG, the Phe derivative **13a** is the most active compound. All compounds with the exception of the *t*-butyl-substituted ones and **12b** show some degree of inhibition of ChT, in agreement with the general preference of the digestive protease ChT for large hydrophobic residues in the P1 position. Overall, the compounds with the phenyl substituent on the phosphorus seem most active, followed by the *n*-propyl ones. The *t*-butyl leads to inactivity for all tested proteases. In addition to the general screening, we tested **12a** as the best compound with a small aliphatic residue in different concentrations on NE and, for comparison, on ChT to get an insight into the compound's activity and selectivity. Furthermore, we performed a titration of **13a**, the best compound with a large hydrophobic residue on ChT as well as NE. To our delight, both of these experiments reveal high selectivity of the individual compound for their respective target protease over the off-target protease (see Figure S1).

Past reports about phosphinate esters as serine protease inhibitors in comparison with phosphonate esters have been contradictory in their results. Boduszek et al. reported di- and tripeptidyl phosphinate esters to be nearly unreactive with NE and PPE, whereas similar phosphonate esters were found to be

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active.[25] In contrast, Walker et al. found that phosphinate esters with a single amino acid in the P1 position displayed consistently higher activity than their phosphonate counterparts.[26]

To shed more light on the difference in activity of phosphinate and phosphonate esters, we made a comparable assessment of the potency of the here described compounds as well as their diphenyl phosphonate ester counterparts **1a** and **1b**. To this end, we performed inhibition kinetics experiments. Because of the covalent, irreversible nature of the inhibitors, we used a two-step binding model (Figure S2) consisting of a first, reversible, non-covalent interaction and a second, irreversible reaction between enzyme and inhibitor. Applying this binding model, we determined k_{inact} and K_I. k_{inact} is the rate constant of the second, irreversible step in this process at infinite inhibitor concentration and as such an indicator of compound reactivity. The inhibitor concentration that results in an observed rate constant of $^1/_2k_{inact}$ is called K_I. It reflects the reversible binding process between inhibitor and enzyme before reaction. The overall inhibitory potency is reflected in the ratio of these two values with better inhibitors possessing higher k_{inact}/K_I values.

Valine derivatives **12a** and **1a** were tested against three enzymes with elastase-like activity (NE, PR3, and PPE). Additionally, **12b** was tested against PR3. Whereas **12a** showed activity with the highest k_{inact}/K_I against PR3, the diphenyl phosphonate was mostly inactive, in line with previous reports,[23] except against NE (Table 1, entries 1−7). Compound **12b**, which only showed activity against PR3 in the gel-based experiments (Figure 2), displayed much lower activity than **12a**.

Unfortunately, the kinetic experiments with cathepsin G gave no good progression curves and thus no good fitting was possible in that case. Hence, phosphinates with large hydrophobic P1 residues (Leu, Phe, Phg) were tested against chymotrypsin, as this protease was inhibited by the largest number of compounds (Figure 2) and may be able to give more insight into the primed site effect. Phe in the P1 position is clearly preferred with its k_{inact}/K_I value being 5−20-fold higher than the values of the corresponding Phg and Leu derivatives with the same primed site element (Table 1, entries 9−13). Additionally, it is twice as active as the corresponding diphenyl phosphonate inhibitor **1b**, which is especially due to a higher k_{inact}. Unfortunately, the Phe phosphinates could only be synthesized with one R^2 group. Therefore, comparison of the primed site binding elements can only be made with the Leu and Phg derivatives. In both cases, the inhibitors with a phenyl R^2 substituent displayed higher potency. Interestingly, for the Leu phosphinate esters, the difference was mainly caused by a higher k_{inact} for the R^2=Ph, whereas, for the Phg phosphinate esters, the underlying potency difference had its cause in a better K_I for the R^2=Ph compound.

For further insight into the different binding modes, we performed covalent docking with AutoDock 4.2[30] of some selected compounds in crystal structures of NE, PR3, and ChT (Figure 3). In these experiments, only the *R*-configurations at the α-carbon to the phosphorus were assessed, because these reflect the natural *L*-configuration of amino acids and correspond to the active species, as previously shown in structural and kinetics studies.[27,31] However, we included both configurations at the phosphorus atom: the bound *RR*- and *RS*-diastereomers. Note that the *R*-configuration at the phosphorus bound to the enzyme originates from the *S*-configuration at the phosphorus in the inhibitor, because the stereochemistry

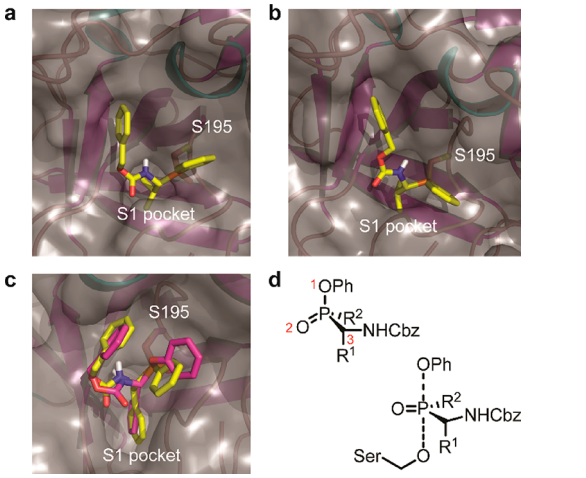

Figure 3. Proposed binding models by *in silico* covalent docking. Shown are close-ups of the active site with the protein in cartoon format (α-helices in cyan, β-sheets in magenta, loops in pink), molecular surface depicted as gray transparent, and inhibitors as stick models. All phosphinates are colored yellow. Water molecules and inhibitor molecules originally present in the structure were removed prior to docking. (a) Compound **12a** covalently bound to S195 of human NE (PDB code: 4WVP). (b) Compound **12a** covalently bound to S195 of human PR3 (PDB code: 1FUJ). (c) Compound **13a** and diphenyl phosphonate **1b** (in magenta) bound to S195 of bovine α-chymotrypsin (PDB code: 4Q2K). (d) Chirality at the phosphorus atom with priorities of the substituents (the P═O bond is treated as a normal single-bonded substituent according to IUPAC rules). Here, the *S*-enantiomer is depicted. Note that the chirality is inverted upon reaction with the enzyme in an S_N2P-type mechanism.

inverts upon reaction with the active site serine in an S_N2P-type mechanism with trigonal bipyramidal transition state (Figure 3D) Strikingly, we found the *RS*-diastereomer to be consistently superior to the *RR*-diastereomer in binding energy, with the only exception being **11a** (Table S1). Additionally, a visual inspection of the top scoring poses showed that the *RR*-derivatives generally displayed unlikely binding modes (Figure S3). Therefore, we propose that the *RS*-diastereomers are probably the (most) active species. Future efforts in synthesis toward enantiomerically pure phosphinate esters may address this further and result in compounds with higher potency.

For NE and PR3 we took a closer look at the interactions of **12a**. In both proteases the valine side chain docks well into the S1 pocket and the Cbz group occupies the S2 pocket, whereas the phenyl substituent at the phosphorus atom reaches toward the primed site (Figure 4A,B). The better K_I value seen for PR3, as measured in the kinetics experiments, may be explained by the somewhat different orientation of the Cbz and Ph groups as well as the preference of PR3 for slightly larger P1 and P2 residues, as has been shown before.[21,22,31]

For ChT, we compared the most active compound **13a** with the reference diphenyl phosphonate **1b**. For both compounds, the Phe side chain reaches deep into the S1 pocket and the carboxybenzyl substituent interacts with the S2 pocket. The phenoxy as well as the phenyl group are both oriented toward the S1′ pocket. The overall very similar binding mode may explain their comparable K_I values.

In conclusion, we report a straightforward synthetic procedure to synthesize phenyl phosphinates in one to two steps from commercially available aldehydes and dichlorophosphines. These compounds inhibit S1 family serine

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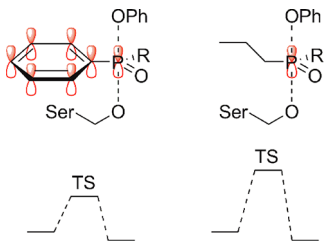

Figure 4. Proposed explanation for the higher reactivity of phosphinate esters with a phenyl ring attached to the phosphorus. In the S_N2P reaction mechanism, the orbitals on the phosphorus that form bonds to the incoming nucleophile and outgoing leaving group have overlap with the π-orbitals of the phenyl ring (left), thereby stabilizing this structure and lowering the transition state energy. This stabilization is not present with aliphatic substituents on the phosphorus (right).

proteases depending on their P1 and primed site binding elements. As expected, for the P1 position, the compounds follow the known substrate specificity preferences of the targeted enzymes: phosphinates with a P1 valine inhibit elastase-like proteases, whereas those with large, hydrophobic P1 elements are active against proteases with chymotryptic activity.

As a primed site binding element (R^2), a phenyl substituent was generally preferred. More particular, these compounds were superior to phosphonate esters, in line with a previous study by Walker et al.[26] In all compounds, the phenyl was also preferred over the *n*-propyl substituent, and all compounds with *t*-Bu substituents on the phosphorus atom were inactive. The last observation may be explained by a steric clash: the bulky *t*-Bu group could either prevent attack of the active site serine or substantially disturb the interaction with the enzyme (i.e., raising K_I). However, steric hindrance is not a logical explanation for the lower activity of the compounds with *n*-Pr substituents. Interestingly, the reason for the activity difference is mainly rooted in a better k_{inact} of the phosphinates with the Ph substituent. Hence, it is not the initial binding to the enzyme's active site but rather the reaction rate with the active site serine residue that underlies the difference in activity. Remarkably, the k_{inact} was also higher than the one for diphenyl phosphonates. This may be counterintuitive, because the electrophilicity of the phosphorus in phosphinate esters is lower than that for phosphonate esters, indicated by a lower partial Gasteiger charge as calculated during ligand preparation for *in silico* docking. As a possible explanation for the increased reactivity, we here propose that the R^2 phenyl ring stabilizes the transition state in the reaction with the enzyme (Figure 4).

We also found in compounds **12** (the Val series of phosphinates) that the *n*-Pr substituent only showed activity, albeit with low potency, against PR3 but not against NE. This result may offer future possibilities to obtain inhibitors that are selective for PR3 over the closely related NE. It also illustrates the capacity of the primed site to influence the binding to the target protease. Overall, we expect that phosphinate inhibitors with extended primed site elements as well as residues that bind the S2−S4 sites will lead to more specific inhibitors and activity-based probes. Work along these lines is currently being investigated and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00284.

Supplemental figures, supplemental tables, experimental methods, and copies of NMR spectra and LC-MS chromatograms (PDF)

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Author Contributions

S.H.L.V. and J.P.K. developed the project and designed compounds, S.L. synthesized compounds, J.P.K. performed biochemical analysis, M.A.T.v.d.P. performed covalent docking, J.P.K. wrote the manuscript, and S.H.L.V. revised the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ABPP, activity-based protein profiling; CatG, cathepsin G; Cbz, carboxybenzyl; ChT, chymotrypsin; DIC, *N,N′*-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; DR, diastereomeric ratio; HNE, human neutrophil elastase; NET, neutrophil extracellular trap; Phg, phenyl glycine; PPE, porcine pancreas elastase; PR3, proteinase 3

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