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A Bisbenzamidine Phosphonate as a Janus-faced Inhibitor for Trypsin-like Serine Proteases

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A hybrid approach was applied for the design of an inhibitor of trypsin-like serine proteases. Compound **16** [(*R*,*R*)- and (*R*,*S*)-diphenyl (4-(1-(4-amidinobenzylamino)-1-oxo-3-phenylpropan-2-ylcarbamoyl)phenylamino)(4-amidinophenyl)methylphosphonate hydrochloride], prepared in a convergent synthetic procedure, possesses a phosphonate warhead prone to react with the active site serine residue in a covalent, irreversible manner. Each of the two benzamidine moieties of **16** can potentially be accommodated in the S1 pocket of the target enzyme, but only the benzamidine close to the phosphonate group would then promote an irreversible interaction. The Janus-faced inhibitor **16** was evaluated against several serine proteases and caused a pronounced inactivation of human thrombin with a second-order rate constant (k_{inac}/K_i) of 59500 $M^{-1}s^{-1}$. With human matriptase, **16** showed preference for a reversible mode of inhibition ($IC_{50} = 2.6$ μM) as indicated by linear progress curves and enzyme reactivation.

Trypsin-like serine proteases possess primary substrate specificity for basic amino acids. Several members of this family are promising drug targets, and the first orally available inhibitors of trypsin-like serine proteases have been approved for clinical use.[1,2] For example, inhibitors of the coagulation proteases thrombin and factor Xa are used as anticoagulant agents to prevent deep-vein thrombosis and as therapeutics against angina pectoris, stroke, and heart attack, which are major causes of death and morbidity. Thrombin cleaves fibrinogen to form fibrin and activates factors V, VIII, XI, XIII, as well as TAFI and protein C.[2,3] Moreover, thrombin stimulates platelet secretion and aggregation. Different types of antithrombotic drugs have been designed.[2,4] Factor Xa is a much more specific protease than thrombin. Together with factor Va and calcium ions, it forms the prothrombinase complex on a phospholipid surface, which is responsible for the conversion of prothrombin into thrombin. Thus, inhibitors of factor Xa attenuate the generation of thrombin without affecting existing thrombin levels, which are thought to be sufficient to ensure primary hemostasis via weak platelet activation.[5,6]

Matriptase, the eponymous enzyme of the matriptase subfamily, is a member of the type II transmembrane serine proteases (TTSPs). This enzyme processes and activates several substrates, which themselves play critical roles in tumorigenesis, such as hepatocyte growth factor/scatter factor, urokinase plasminogen activator, and protease-activated receptor 2. Inhibition of matriptase is therefore thought to be a possible strategy for cancer treatment.[7,8]

Matriptase-2, another member of the TTSPs, has attracted considerable attention because of its critical role in iron homeostasis.[9] Hepcidin, a hepatic peptide hormone that binds to and down-regulates the iron transporter ferroportin, is affected by matriptase-2, which cleaves hemojuvelin, the bone morphogenetic protein (BMP) co-receptor, at the cell surface of hepatocytes.[10] This leads to modulation of the BMP/SMAD signaling pathway and to suppression of the expression of the hepcidin-encoding gene. Accordingly, by applying matriptase-2 inhibitors, hepcidin expression will be promoted, and therefore, this enzyme has been considered a potential target for the treatment of iron-overload diseases.[9,11,12]

The benzamidine moiety represents an arginine mimetic which has been used extensively as a pharmacophore prone to interact with protein regions bearing anionic and hydrogen bond acceptor groups. In particular, benzamidines have been frequently incorporated as P1 fragments of inhibitors for trypsin-like serine proteases.[13] In the protease–inhibitor complexes, the benzamidine groups were shown to be deeply buried in the S1 specificity pocket.[14,15] Substrate analogue inhibitors can be derived from the particular substrate structure, for example, from P3 to P1, by incorporating the benzamidine moiety into the P1 side chain and removing the carboxyl group of the P1 amino acid. Hence, the altered structure of the N-terminal product of proteolytic cleavage leads to an improved binding affinity for the active site. Several so-designed peptidomimetics have been reported as inhibitors for trypsin-like serine proteases, such as thrombin, factor Xa, matriptase, and matriptase-2.[12,16,17] The structure of the reversible direct thrombin inhibitor melagatran (**1**), a major milestone to this structural class of antithrombotics, is shown in Figure 1.

α-Aminoalkylphosphonate diphenyl esters are phosphonic analogues of naturally occurring amino acids. Their peptidyl derivatives, such as the thrombin inhibitor **2** (Figure 1),[18] have been reported as inactivators of serine proteases,[19,20] or activity-based probes.[21–23] The irreversible mode of action of such

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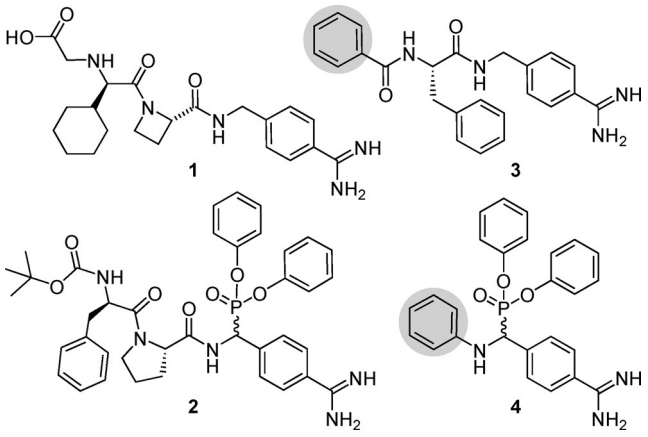
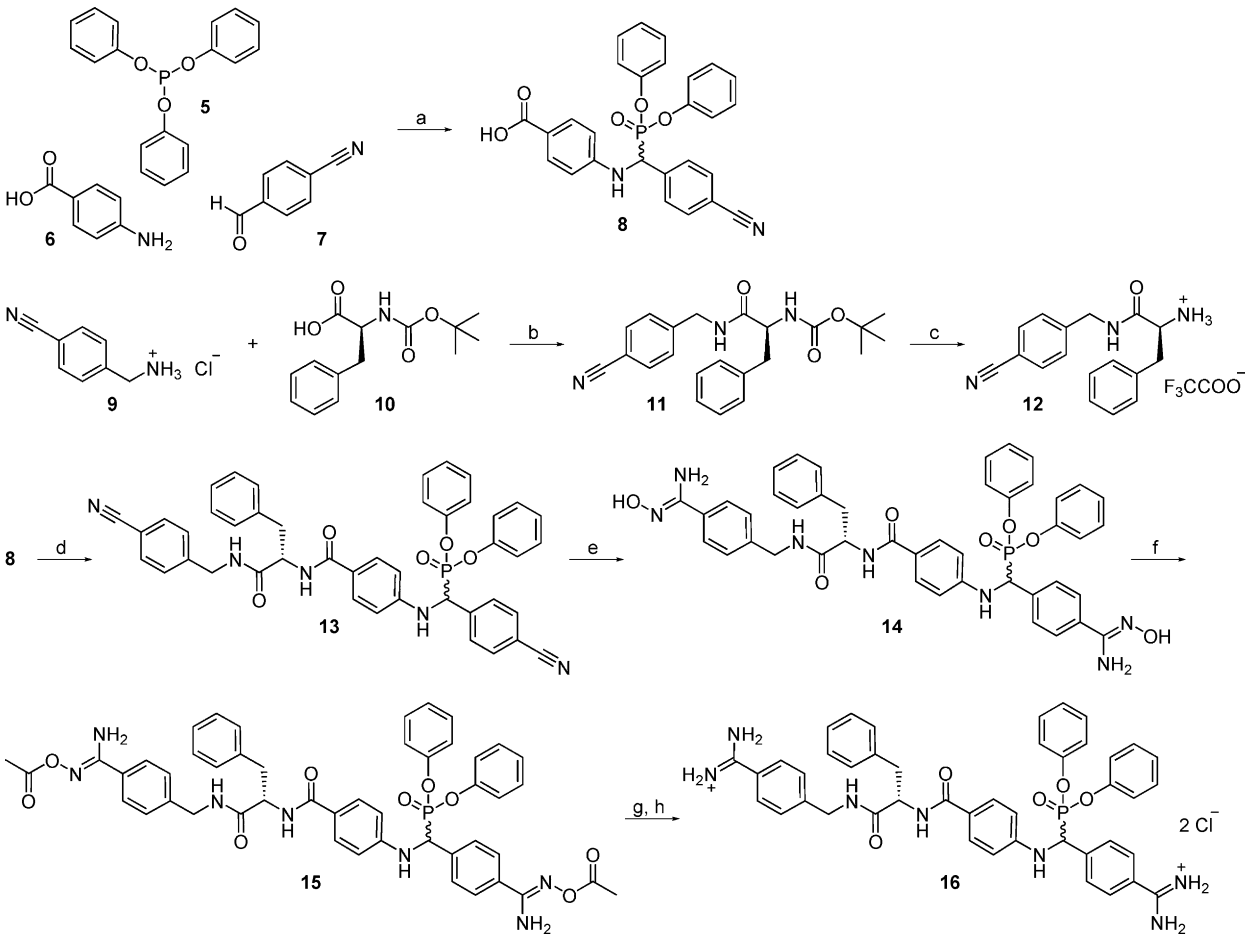


Figure 1. Structures (**1**, **2**) or fragments (**3**, **4**) of substrate analogue inhibitors (**1**, **3**) or irreversible inhibitors (**2**, **4**) for trypsin-like serine proteases.

phosphonates relies on the nucleophilic attack of the active serine at the phosphorus atom and simultaneous release of one phenolic residue. The resulting serine phosphono diester is known to undergo a slow 'aging' upon loss of the second phenoxy group to form a phosphono monoester. A complete lack of activity against cysteine or threonine proteases is an advantage over other inhibitor classes such as chloromethyl ketones.[19] The selectivity of the phosphonate esters toward trypsin-like serine proteases can be enhanced by introducing residues of basic amino acids such as arginine, lysine, homolysine, and ornithine in the P1 position, or likewise, the benzamidine pharmacophore.[18,19,23,24]

Fragments **3** and **4** represent hypothetical prototypes of either substrate analogue inhibitors (**3**) or irreversible inhibitors (**4**) for trypsin-like proteases. In both cases, the benzamidine moiety would be expected to be accommodated in the S1 specificity pocket of the protease. We performed a hybrid design approach by combining both fragments to a single inhibitor structure.[25,26] One phenyl ring present in both **3** and **4** (highlighted) was merged, and the resulting bisbenzamidine phosphonate was synthesized and evaluated at the aforementioned proteases, i.e., thrombin, factor Xa, matriptase, and matriptase-2. Inhibition of the reference enzyme trypsin as well as leukocyte elastase and chymotrypsin was also examined. This study is aimed at investigating the biological activity and selectivity profile of such a Janus-faced molecule.

The inhibitor **16** was produced in a convergent seven-step synthetic route outlined in Scheme 1. To generate the



Scheme 1. Synthesis of the bisbenzamidine phosphonate **16**. *Reagents and conditions*: a) $Mg(ClO_4)_2$, THF, reflux, 80 h, 55%; b) NMM, $ClCO_2iBu$, TEA, THF, −25 °C→RT, overnight, 98%; c) TFA, CH_2Cl_2, 1 h, RT; d) HATU, DIPEA, DMF, RT, 16 h, 89%; e) H_2N-OH·HCl, DIPEA, EtOH, reflux, 2 h, 61%; f) Ac_2O, AcOH, RT; g) Pd/C/H_2, AcOH, 275 kPa, RT, 2 h; h) preparative HPLC, lyophilization, 9%.

α-aminophosphonate **8**, a Kabachnik–Fields reaction was chosen. This involves a three-component reaction between 4-cyanobenzaldehyde (**7**), 4-aminobenzoic acid (**6**), and triphenylphosphite (**5**). The conversion is usually promoted by Lewis or Brønsted acids.[27] In our case, $Mg(ClO_4)_2$ was used, which was reported to be an efficient catalyst for the formation of α-aminophosphonates.[28] Compound **8**, a diphenyl phosphonate containing a nitrile functionality on the one hand and a carboxylic acid moiety on the other hand, was obtained in good yield. This building block represents the *irreversible inhibitor part* of the desired final product.

The *substrate analogue inhibitor segment* of the target molecule **16** was synthesized as follows: 4-(Aminomethyl)benzonitrile (**9**) and Boc-protected phenylalanine (**10**) were coupled by the mixed-anhydride method using isobutyl chloroformate and *N*-methylmorpholine to yield the amino acid amide **11**. To remove the N-protecting group, a mixture of dichloromethane and trifluoroacetic acid was used. The salt **12** was directly subjected to a coupling reaction with the α-aminophosphonate **8** produced before. By applying HATU as reagent and DIPEA as base,[22,29] the bisnitrile phosphonate **13** was obtained.

To simultaneously convert the two nitrile moieties of compound **13** into amidines, the method of Judkins et al. was used.[30] Hydroxylamine was reacted to form amidoxime **14**. The following treatment with acetic anhydride furnished acetamidoxime **15**. After a palladium/carbon-catalyzed reduction, the obtained crude product was purified by preparative HPLC to give the final bisbenzamidine phosphonate **16**.

Compound **16** was evaluated at selected proteases; the kinetic parameters are listed in Table 1. We used fluorogenic and chromogenic peptide substrates for these assays. Because different shapes of the progress curves were observed, an estimation of the respective inhibitory capacity was based on product formation after 15 min in the presence of five different inhibitor concentrations, and the IC_{50} values were obtained on the basis of these end-point determinations. The IC_{50} values were considered to be meaningful for a comparative assessment of the inhibitory activity toward the seven proteases, as: 1) an active-site-directed mode of interaction was supposed, and 2) the substrate concentrations used were in the range of the corresponding K_M values (Table 1). Weak inhibition was found for factor Xa, elastase, and trypsin. In contrast, we observed a relatively strong inhibition of thrombin, and a moderate decrease in the activity of matriptase, matriptase-2, and chymotrypsin. These differences were unexpected and might result from different binding modes. The bisbenzamidine **16** might either occupy the S1 pocket with the benzamidine from its *irreversible inhibitor part* or its *substrate analogue inhibitor segment*. In the first binding mode, inactivation would be facilitated, because the aforementioned benzamidine would direct the electrophilic phosphonate close to the nucleophilic serine residue. In the alternative binding mode, noncovalent inhibition would occur. As a matter of course, each interaction might lead to both situations and hence to a mixture of two different enzyme–inhibitor complexes. Nevertheless, the different shapes of the progress curves indicate a preference for either irreversible or substrate analogue inhibition. For example, as depicted in Figure 2, the reaction of **16** with thrombin resulted in time-dependent inhibition, and the second-order rate constant of inactivation, k_{inac}/K_i, could be determined. This was also the case for matriptase-2 and trypsin (Table 1). For the other enzymes, the progress curves did not follow an exponential equation and were instead rather linear, as shown for the inhibition of matriptase in Figure 3. Such a behavior was also observed for factor Xa, elastase, and chymotrypsin.[31] In a control experiment, the established inhibitor melagatran **1** (Figure 1) was inspected toward thrombin. Linear time-independent inhibition was observed, in agreement with the known noncovalent binding mode, and a K_i value of 2.4 nM was determined (lit.: $K_i = 4$ nM).[15] The data are shown in Figure S1 (Supporting Information).

From these findings, it was concluded that thrombin preferentially forms a covalent enzyme–inhibitor complex with **16**. To illustrate the resulting irreversible mode of interaction,

Table 1. Protease inhibition by bisbenzamidine phosphonate **16**: assay conditions and parameters of inhibition.

Enzyme	Substrate	[S] [μM][a]	K_M [μM]	IC_{50} [μM][b]	k_{inac}/K_i [$M^{-1}s^{-1}$][c]
Human thrombin	Cbz-Gly-Gly-Arg-AMC	40	40[d]	0.075 ± 0.019	59500 ± 3800
Human matriptase	Mes-D-Arg-Pro-Arg-AMC	10	8.00 ± 0.65[e]	2.6 ± 0.1	ND[f]
Human matriptase-2	Boc-Gln-Ala-Arg-AMC	40	32[d]	1.5 ± 0.5	1060 ± 10
Bovine factor Xa	Boc-Ile-Glu-Gly-Arg-AMC	100	59.0 ± 3.2[g]	120 ± 10	ND[f]
Human neutrophil elastase	MeO-Suc-Ala-Ala-Pro-Val-pNA	100	54[h]	23 ± 7	ND[f]
Bovine trypsin	Boc-Gln-Ala-Arg-AMC	40	22[d]	9.8 ± 1.1	121 ± 11
Human chymotrypsin	Suc-Ala-Ala-Pro-Phe-AMC	40	15.9 ± 1.7[i]	0.85 ± 0.08	ND[f]

[a] Substrate concentration. [b] IC_{50} values were obtained from duplicate measurements in the presence of five different inhibitor concentrations. Reactions were followed over 15 min, and the formation of the product was plotted versus inhibitor concentration, [I]. IC_{50} values were determined by nonlinear regression using equation $[P] = [P_0]/(1 + [I]/IC_{50})$, in which [P] is the product concentration after 15 min in the presence of various inhibitor concentrations, and $[P_0]$ is the product concentration after 15 min in the absence of inhibitor. [c] The reactions were followed over 45 min. The second-order rate constant of inactivation (k_{inac}/K_i) was obtained from linear regression of plots of the k_{obs} values versus [I]. Under conditions of linear dependency of k_{obs} on [I] (i.e., $[I] \ll K_i\ (1 + [S]/K_M)$), the equation $k_{obs} = k_{inac}[I]/(K_i(1 + [S]/K_M) + [I])$ simplifies to $k_{inac}/K_i = k_{obs}/[I](1 + [S]/K_M)$, and k_{inac}/K_i values were calculated accordingly. [d] Data from ref. [16]. [e] The K_M value was obtained by triplicate measurements at nine different substrate concentrations (2, 4, 6, 8, 10, 15, 20, 25, and 30 μM). [f] Not determined; time-dependent inhibition was not observed. [g] The K_M value was obtained by triplicate measurements at 12 different substrate concentrations (10, 20, 40, 60, 80, 100, 150, 200, 250, 300, 400, and 500 μM). [h] Data from refs. [12,32]. [i] The K_M value was obtained by duplicate measurements at 14 different substrate concentrations (4, 6, 8, 12, 16, 20, 40, 60, 80, 100, 120, 140, 160, and 180 μM).

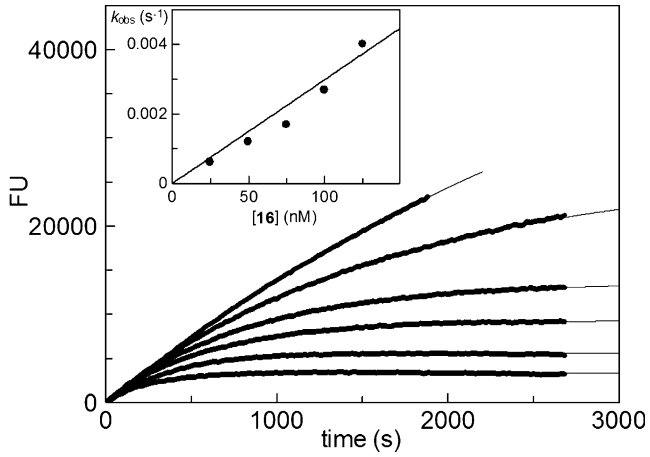


Figure 2. Inhibition of human thrombin by bisbenzamidine phosphonate **16**. Measurements were performed with a plate reader. From top to bottom: uninhibited reaction, [I] = 25 nM, [I] = 50 nM, [I] = 75 nM, [I] = 100 nM, [I] = 125 nM. The first-order rate constants (k_{obs}) were obtained by nonlinear regression of the progress curves using the equation $[P] = [P_\infty](1\text{-}\exp(-k_{obs}t)$, for which [P] is the product concentration, and $[P_\infty]$ is the product concentration at infinite time. Time-dependent inhibition was analyzed as noted in Table 1. The inset shows a plot of k_{obs} values of duplicate measurements versus inhibitor concentrations, [I]. Linear regression gave an apparent second-order rate constant ($k_{obs}/[I]$) of $29\,800 \pm 1900\ M^{-1}s^{-1}$.

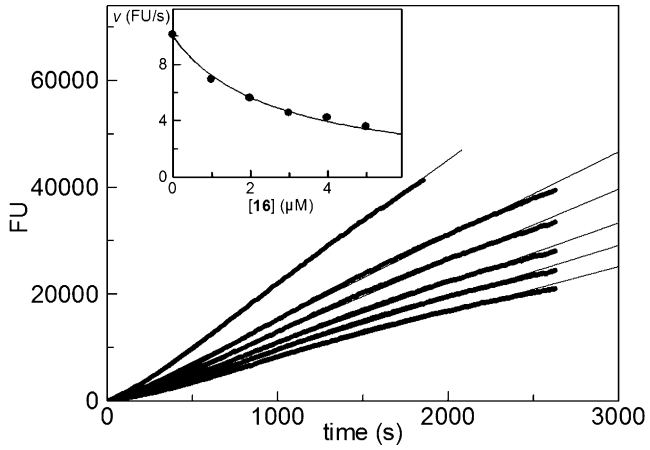


Figure 3. Inhibition of human matriptase by bisbenzamidine phosphonate **16**. The measurements were performed with a plate reader. From top to bottom: uninhibited reaction, [I] = 1 μM, [I] = 2 μM, [I] = 3 μM, [I] = 4 μM, [I] = 5 μM. Rates (v) were obtained by linear regression of the progress curves. Assay conditions are noted in Table 1. The inset shows a plot of the rates of duplicate measurements versus inhibitor concentrations, [I]. Nonlinear regression using the equation $v = v_0/(1 + ([I]/IC_{50}))$ gave an IC_{50} value of 2.6 ± 0.1 μM.

reactivation experiments were performed with thrombin and compared with matriptase, for which a preferred noncovalent interaction was assumed. Both enzymes were incubated with **16** at a concentration of $\sim 2 \times IC_{50}$. After 30 min, the samples were centrifuged to remove unbound inhibitor. The appropriate substrate was added to each enzyme concentrate and product formation was monitored.

Whereas matriptase recovered activity (Figure 4), thrombin was shown to remain inactivated by **16** (Figure 5). A further

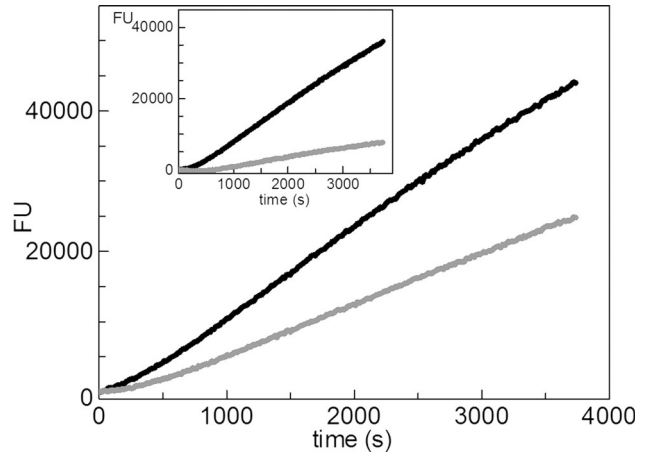


Figure 4. Reactivation of human matriptase, inhibited by bisbenzamidine phosphonate **16**. Reactions were performed with a plate reader. Dark grey: the enzyme was incubated with **16** (4 μM) for 30 min in a volume of 200 μL. Assay buffer was added to a total volume of 4 mL, which then was decreased to ~150 μL by centrifugation to remove unbound inhibitor. The sample was filled to 1 mL, and the centrifugation was repeated. The obtained volume of ~160 μL was filled to a volume of 180 μL. Finally, substrate (20 μL, 100 μM) was added, and the product formation was assayed. The concentration of matriptase was 0.0005 μg mL^{-1}. Black: reaction in the absence of **16**. Inset: reactions were performed using 162 μL assay buffer and 8 μL DMSO (black) or inhibitor solution in DMSO (dark grey). Matriptase was added and was incubated at 37 °C for 30 min, without subsequent filtration. The reaction was initiated by the addition of substrate. Final concentrations: [matriptase] = 0.0005 μg mL^{-1}, [S] = 10 μM, [I] = 4 μM.

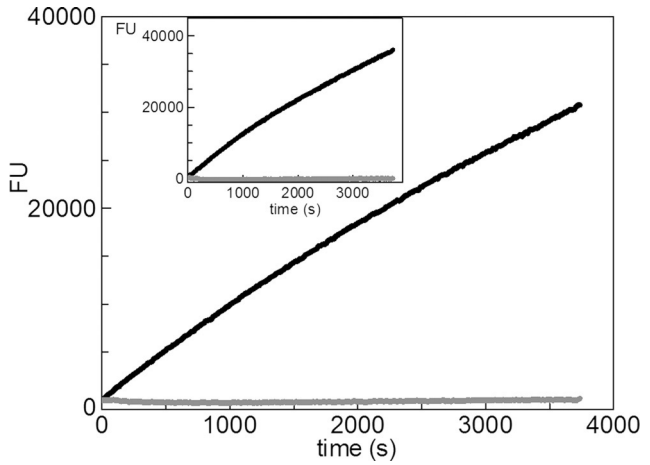


Figure 5. Reactivation of human thrombin, inhibited by bisbenzamidine phosphonate **16**. The reactions were performed with a plate reader. Dark grey: the enzyme was incubated with **16** (125 nM) for 30 min in a volume of 200 μL. Assay buffer was added to a total volume of 4 mL, which was then decreased to ~150 μL by centrifugation to remove unbound inhibitor. The sample was filled to 1 mL, and the centrifugation was repeated. The obtained volume of ~160 μL was filled to a volume of 190 μL. Finally, the substrate (10 μL, 800 μM) was added, and product formation was assayed. The concentration of thrombin was 2 U mL^{-1}. Black: reaction in the absence of **16**. Inset: reactions were performed using 160 μL assay buffer and 10 μL DMSO (black) or inhibitor solution in DMSO (dark grey). Thrombin was added and was incubated at 25 °C for 30 min, without subsequent filtration. The reaction was initiated by the addition of substrate. Final concentrations: [thrombin] = 2 U mL^{-1}, [S] = 40 μM, [I] = 125 nM.

independent experiment was carried out in which both proteases were inhibited with **16**, and after a significant increase in the substrate concentration, subsequent product formation was compared for the inhibited and non-inhibited enzyme. In this case, we observed only marginally enhanced thrombin activity (Supporting Information Figure S2), whereas matriptase was reactivated to a significant extent (Figure S3). These results indicate that the favored accommodation of **16** differs at the active sites of thrombin and matriptase.

The covalent modification of thrombin by **16** was further supported by MS peptide map fingerprinting of a tryptic digest of the excised SDS-PAGE protein band after incubation of thrombin (20 U mL^{-1}) with **16** (1.25 μM) for 30 min. Thus, compared with the activity assay, a tenfold concentration of the enzyme and the inhibitor was chosen. Two different peptide fragments of 15 or 16 amino acids containing the active site serine were obtained after tryptic digestion. In the course of the LC–MS sample preparation, methionine was oxidized to the sulfone, and cysteine was alkylated by iodoacetamide. The peptides originating from thrombin treated with compound **16** showed two additional modifications with high peak intensity. The first mass difference (685.2566) was due to the formation of the serine phosphono diester, and the second difference (609.2253) was due to the subsequent 'aging', that is, hydrolysis to the phosphono monoester (Supporting Information Table S1). Hence, in the sample resulting from incubation with **16**, the peptides were predominately modified by the bisbenzamidine phosphonate, confirming the covalent modification of thrombin by **16**.

In conclusion, we have designed the Janus-faced bisbenzamidine **16** by merging two benzamidine fragments. The fragments introduce the capacity of either irreversible or reversible inhibition into the molecule. Unexpected differences in the potency of **16** toward trypsin-like protease were found. This compound exhibited remarkable inhibitory activity against human thrombin, with a second-order rate constant of inactivation of 59 500 M^{-1} s^{-1}. Thus, our hybrid approach appears to be useful for obtaining potent and even selective inhibitors and will be extended by combining other benzamidine fragments in future studies.

Experimental Section

Synthesis: Compound **8** was synthesized in a one-pot reaction of **5** (1 equiv), **6** (1 equiv), and **7** (1 equiv) with $Mg(ClO_4)_2$ (0.05 equiv) as catalyst in THF at reflux for 80 h. Boc-Phe-OH (**10**, 1 equiv) was dissolved in THF and cooled to −25 °C. It was then coupled to 4-(aminomethyl)benzonitrile hydrochloride (**9**, 1 equiv) using NMM (1.1 equiv), $ClCO_2$*i*Bu (1.1 equiv), and TEA (2 equiv) at RT overnight. The Boc protecting group was cleaved with TFA/CH_2Cl_2 (1:1) for 1 h at RT. Coupling of compounds **8** (1 equiv) and **12** (1 equiv) occurred in DMF using HATU (1 equiv) and DIPEA (2 equiv). The mixture was stirred for 16 h at RT. Amidoxime **14** was furnished by holding **13** at reflux with H_2N-OH·HCl (6 equiv) and DIPEA (6 equiv) in EtOH for 2 h. Compound **14** (1 equiv) was dissolved in glacial acetic acid; acetic anhydride (3 equiv) was added, and the mixture was stirred at RT. After 2 h, the solution of the resulting acetylamidoxime **15** was added to 10% palladium on carbon and hydrogenated at 275 kPa for 2 h. The final product **16** was initially purified by preparative reversed-phase HPLC. A few drops of 1 N HCl were added to form the salt **16**, which was obtained after lyophilization.

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Keywords: benzamidines · enzyme inhibition · hybrid compounds · phosphonates · serine proteases

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