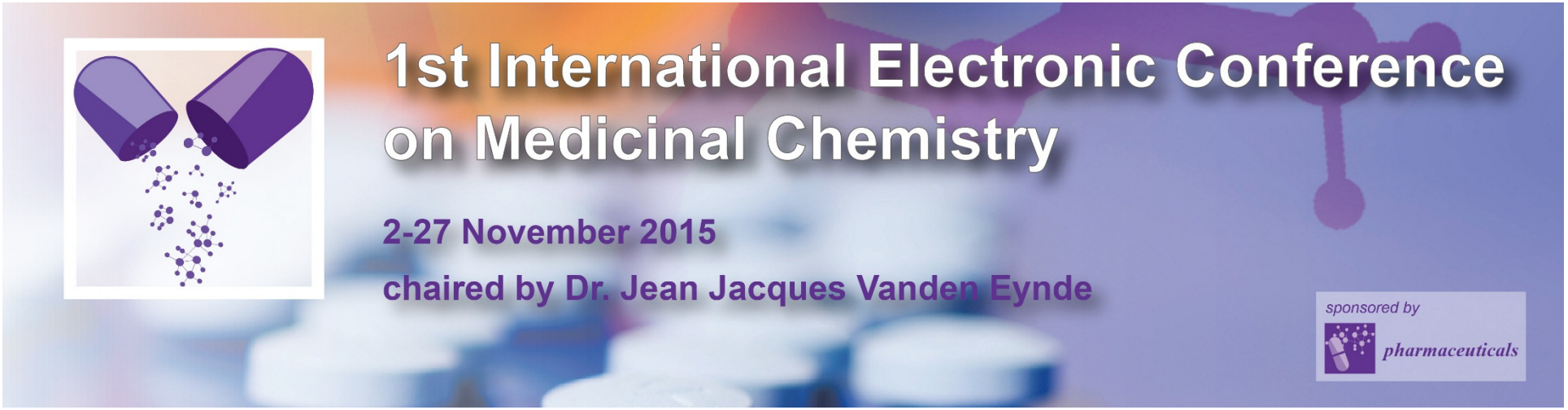

Aminophosphonates as Novel Activity-Based Probes for Matriptase-2

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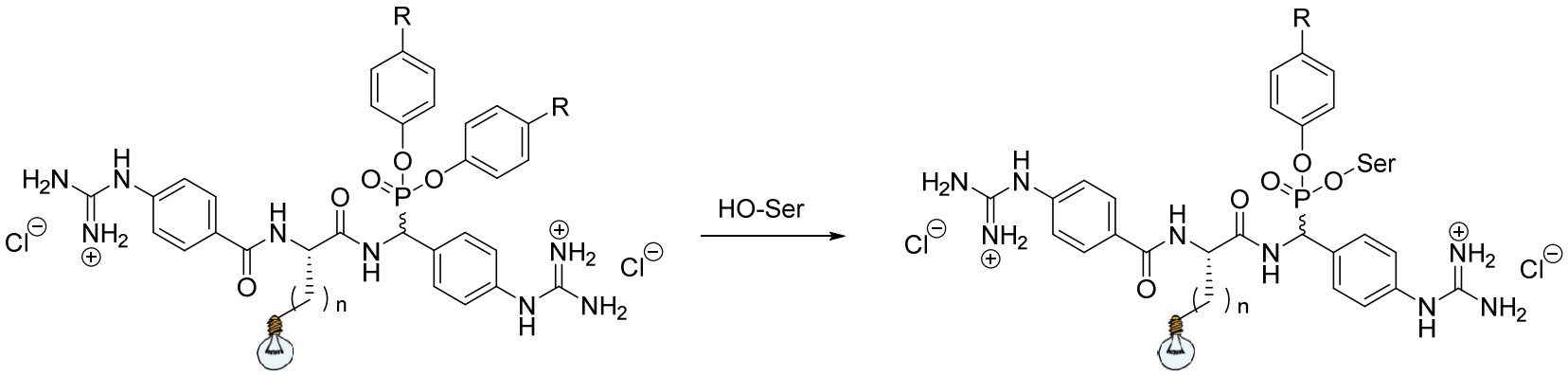


Activity-based probes are compounds that exclusively form covalent bonds with active enzymes. They can be utilized to profile enzyme activities in vivo, to identify target enzymes and to characterize their function. Usually, such probes share three elements, a reactive head group that covalently binds to the target, a tag that allows detection (e.g. a fluorophore) and a linker to connect both.
Matriptase-2 is a transmembrane serine protease, which plays a key role in the human iron homeostasis. This enzyme exhibits a primary substrate specificity for arginine in P1 position. Our design of activity-based probes for matriptase-2 is based on linker-connected *bis*-benzguanidines. The two benzguanidine units interact as arginine mimetics with the S1 and the upper part of the S3/S4 pocket, respectively. An amino acid was introduced as a linker, which bears the coumarin fluorophore. Moreover, an incorporated phosphonate allows for a covalent interaction with the active-site serine. The resulting irreversible mode of action was demonstrated, leading to an enzyme inactivation and to a fluorescence labeling of matriptase-2. Herein, we present the synthetic approach to coumarin-labeled *bis*-benzguanidines as activity-based probes for matriptase-2.

Keywords: activity-based probe; guanidine; matriptase-2; phosphonate

Introduction

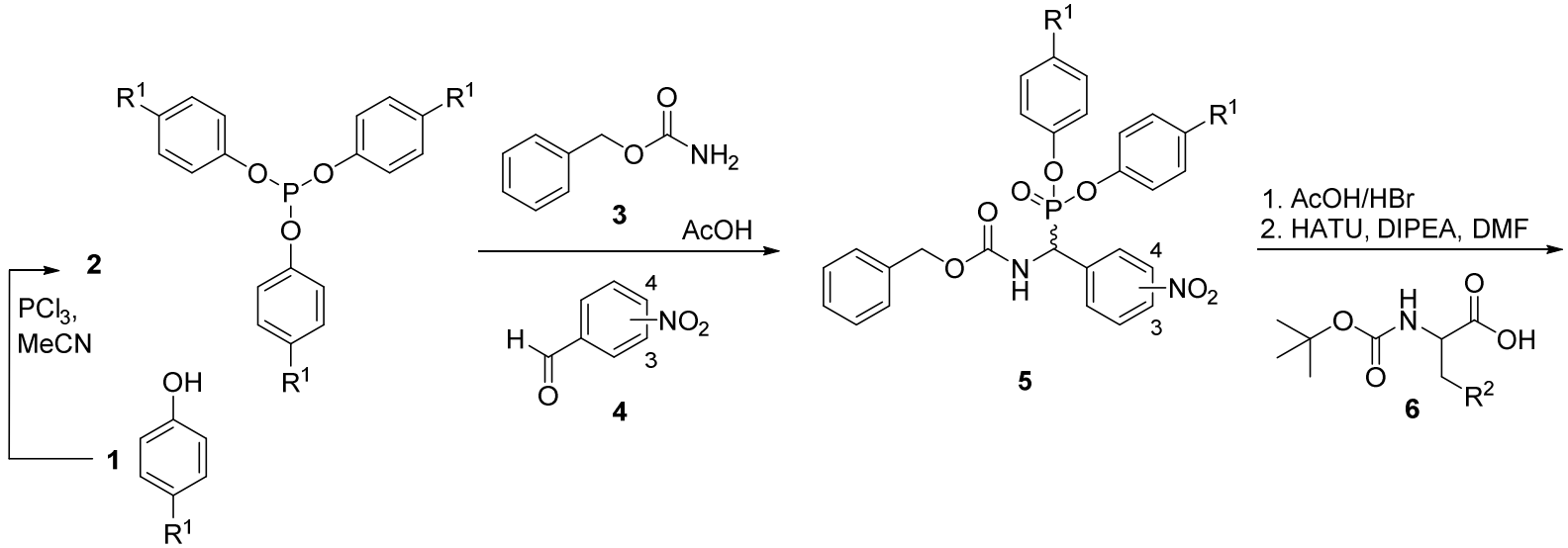
Activity-based probes form covalent bonds with active enzymes and can be utilized to profile enzyme activities *in vivo*, to identify target enzymes and to characterize their function. They exhibit (i) a reactive head group that binds to the target, (ii) a tag that allows detection (*e.g.* a fluorophore) and (iii) a linker to connect both.

Matriptase-2, a type II transmembrane serine protease, plays a key role in the human iron homeostasis. By negatively regulating hepcidin, the systemic iron regulatory hormone, active matriptase-2 provokes increased plasma iron levels. Therefore, inhibition of matriptase-2 is considered as an attractive strategy for the treatment of iron overload diseases.

We aimed at synthesizing dipeptidomimetic inhibitors with the following structural features, (i) an aminophosphonate warhead, to enable irreversible inhibition, (ii) two arginine-mimetic substructures for interaction with the S1 and S3/S4 subsites, (iii) an optional coumarin-labeled amino acid for activity-based probing.

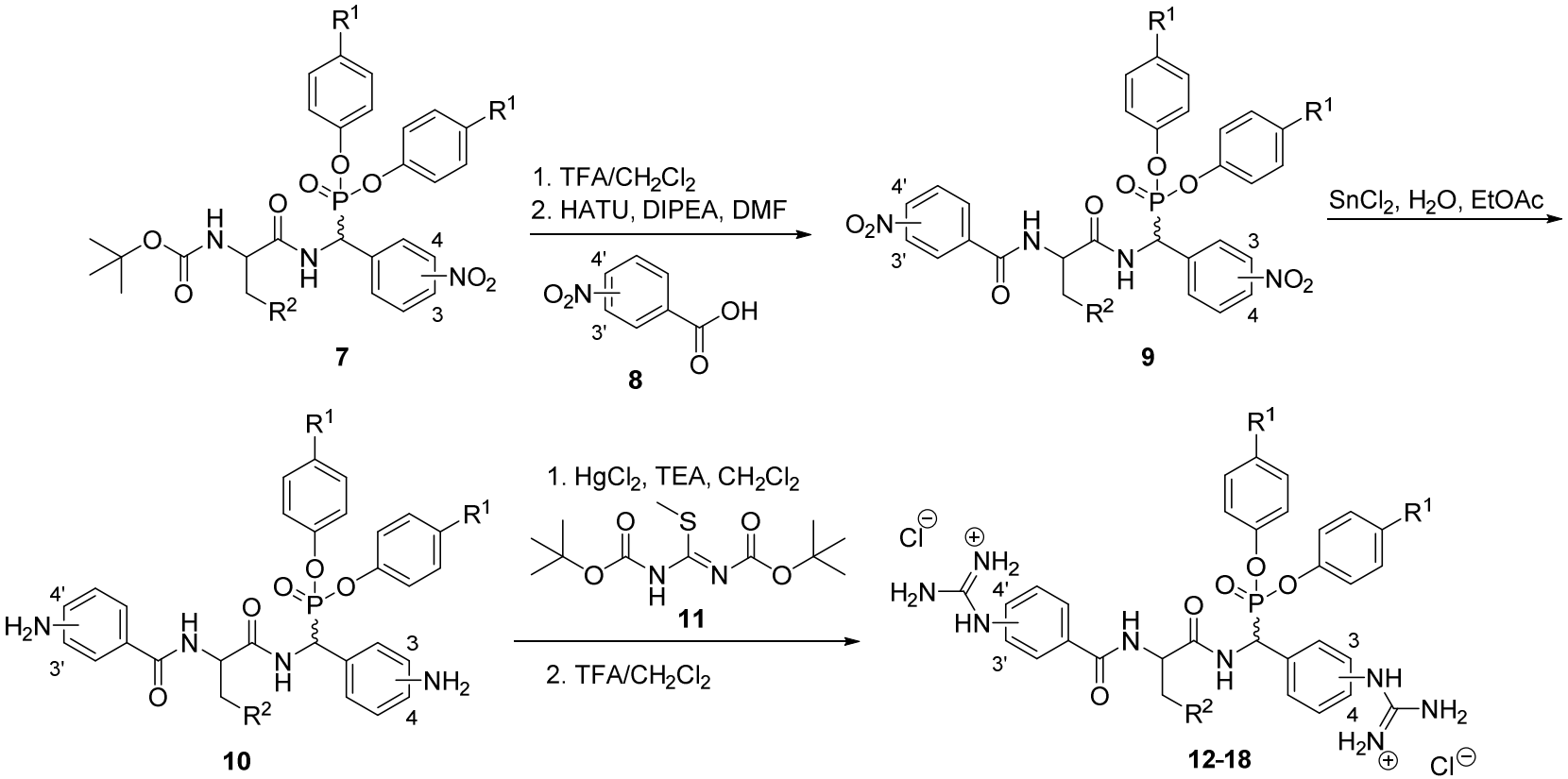
Synthesis

Seven dipeptidomimetic inhibitors were synthesized. Five points of diversity were introduced, (i) and (ii) the position of the guanidine group at both benzguanidines, (iii) and (iv) the nature and stereochemistry of the N-terminal amino acid and (v) the O-substitutents at the phosphonate. Finally, five active compounds were identified and kinetically characterized as inactivators of matriptase-2.



Scheme 1a. Synthesis of irreversible inhibitors of matriptase-2.

Synthesis



Scheme 1b. Synthesis of irreversible inhibitors of matriptase-2.

Molecular Docking

Each product was obtained as a mixture of two diastereomers and the biologically active products (**14**-**18**) were separated into their single epimers. They clearly differed in their matriptase-2 inhibitory activity.

The covalently bound inhibitor **15** was energy minimized within the matriptase-2 complex and subjected to covalent docking using AutoDock 4.2, yielding a reasonable orientation of the ligand with a (*R*)-phosphonate. However, docking of the (*S*)-phosphonate did result in a less plausible binding mode.

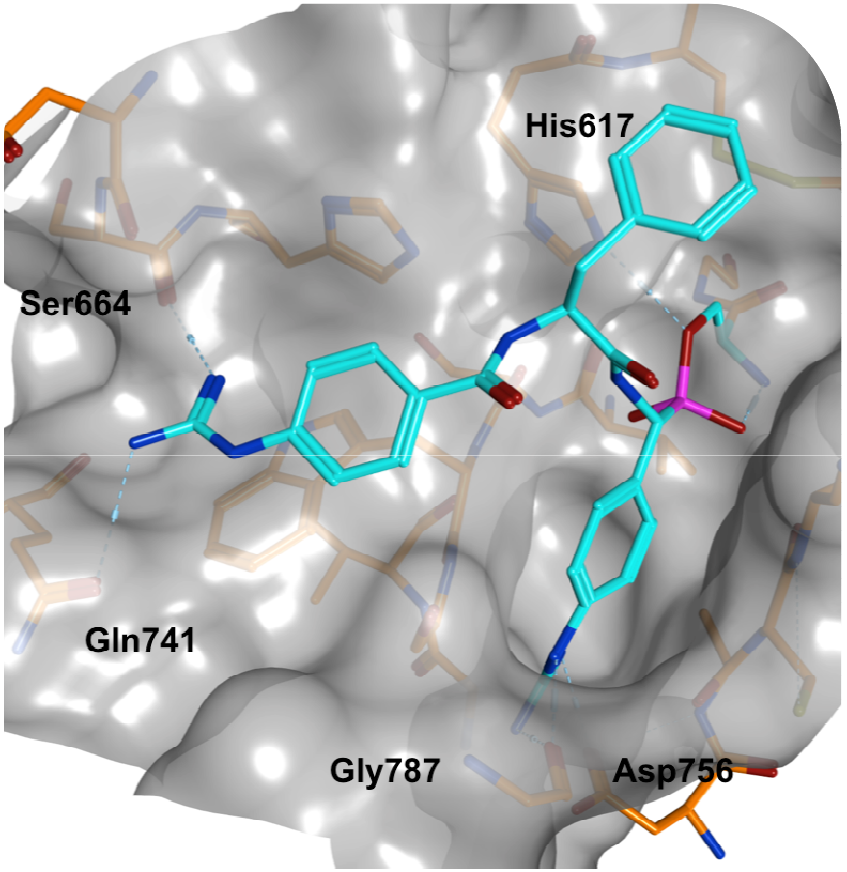


Figure 1. Covalent docking of **15** bound to Ser762 in the binding site of a matriptase-2 homology model.

Biochemical Evaluation

The phosphono dipeptides **12**-**18** were evaluated at matriptase-2 and related serine proteases, *i.e.* human thrombin and bovine factor Xa. These enzymes where assayed with fluorogenic peptide substrates. In most cases, active compounds exhibited a time-dependent inhibition, and linear regression of the progress curves provided pseudo-first order rate constants of irreversible inhibition, k_{obs}. These k_{obs} values were then plotted versus the inhibitor concentrations, and from the second-order rate constants, $k_{obs}/[I]$, obtained by linear regression, the parameters k_{inac}/K_i were calculated. The results of the kinetic experiments are summarized in Table 1.

Compound **18A** with (*S*)-configuration at the N-terminal amino acid, (*R*)-configuration at the phosphonate carbon, both guanidine residues in *para* position and methylthiophenoxy groups was the most potent matriptase-2 inactivator with a k_{inac}/K_i value of 2790 $M^{-1}s^{-1}$.

Compound[a]						Enzyme inactivation, k_{inac}/K_i ($M^{-1}s^{-1}$)		
	No	Position of the guanidine residues		R^1	R^2	matriptase-2	thrombin	factor Xa
12	((*S*)-, *rac*)	3´	3	H	Ph	n.i.[b]	IC_{50} = 1.66 ± 0.12 µM[c]	n.i.
13	((*S*)-, *rac*)	4´	3	H	Ph	n.i.	IC_{50} = 2.72 ± 0.20 µM[c]	n.i.
14	((*S*)-, *rac*)					59.1 ± 4.7	n.i..	n.i.
	((*S*)-, (*R*)-)	3´	4	H	Ph	197 ± 4	n.i..	n.i.
	((*S*)-, (*S*)-)					n.i.	n.i.	n.i.
15	((*S*)-, *rac*)					120 ± 10	n.i.	n.i.
	((*S*)-, (*R*)-)	4´	4	H	Ph	283 ± 14	n.i.	n.i.
	((*S*)-, (*S*)-)					n.i.	n.i.	n.i.
16	((*R*)-, *rac*)					58.7 ± 4.6	n.i.	n.i.
	((*R*)-, (*S*)-)	4´	4	H	Ph	n.i.	n.i.	n.i.
	((*R*)-, (*R*)-)					154 ± 20	n.i.	n.i.
17	((*S*)-, *rac*)					65.0 ± 2.3	n.i.	n.i.
	((*S*)-, (*R*)-)	4´	4	H	$(CH_2)_3NH$-Cbz	73.6 ± 5.3	n.i.	n.i.
	((*S*)-, (*S*)-)					n.i.	n.i.	n.i.
18	((*S*)-, *rac*)					1750 ± 40	n.i.	n.i.
	((*S*)-, (*R*)-)	4´	4	SMe	Ph	2790 ± 60	n.i.	n.i.
	((*S*)-, (*S*)-)					1280 ± 50	n.i.	n.i.

Table 1. [a] The stereochemistry is given in parentheses. The first entry refers to the configuration at the amino acid, the second to the configuration at the aminophosphonate, [b] n.i.: no inhibition, [c] No time-dependent inhibition was observed.

Activity-based probe

An activity-based probe **19** was obtained in an eleven-step route. The determined k_{inac}/K_i value was 122 $M^{-1} s^{-1}$. For imaging of matriptase-2 the conditioned medium of transfected HEK cells was incubated with 100 µM of **19**. The reaction mixture was resolved by SDS-PAGE and visualized by *in-gel* fluorescence detection followed by protein staining with Coomassie Brilliant Blue. In a control experiment mock cells were incubated with 100 µM of **19** and no fluorescent band for matriptase-2 appeared (right, line 3).

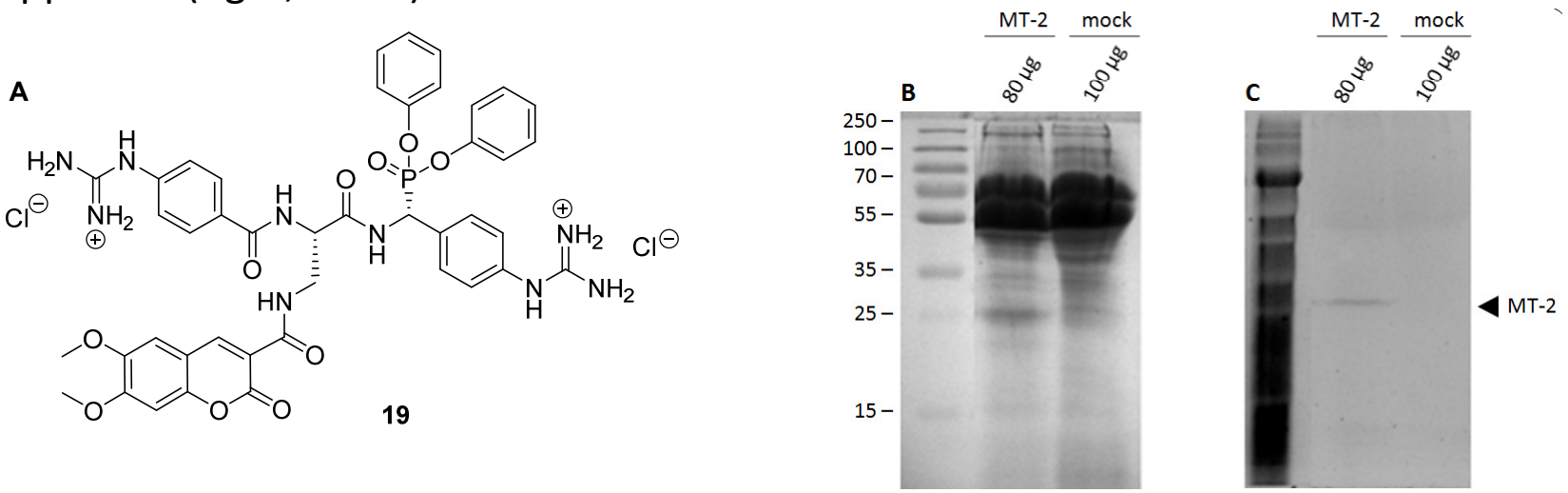


Figure 2. A, Activity-based probe **19**; **B**, Coomassie Brilliant Blue staining; **C**, *in-gel* fluorescence detection.

Conclusion

We discovered phosphono bisbenzguanidines as dipeptidomimetic inactivators for matriptase-2. Structural optimization and structure–activity relationship analyses led to the development of compound **18**. The inhibitors with (*R*)-configuration at the phosphonate carbon were found to be more potent than the (*S*)-epimers. The (*R*)-configured **18** possesses a k_{inac}/K_i value of 2790 $M^{-1}s^{-1}$ and represents the most potent irreversible inhibitor of matriptase-2 known so far. Bisbenzguanidine **15** served as a starting point for the development of an activity-based probe. This probe **19** was successfully used in an initial experiment to visualize the serine protease matriptase-2.

Acknowledgments

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