Supporting Information

Protein-induced Change in Ligand Protonation during Trypsin and Thrombin Binding: Hint on Differences in Selectivity Determinants of both Proteins?

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1. mF_o-DF_c omit maps and numbering of water molecules

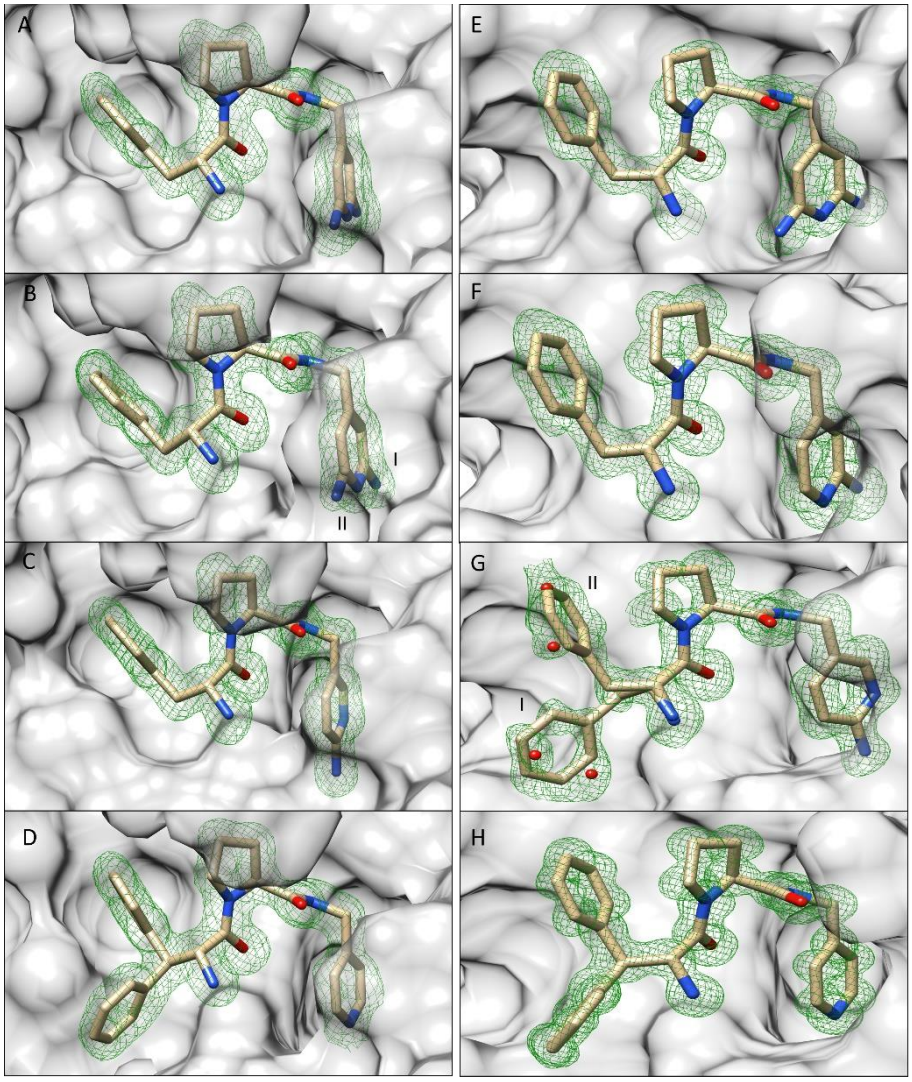


Figure S1: Coordinates and structure factors have been deposited in the Protein Data Bank and will be released upon article publication. mF_o-DF_c omit maps of ligands 6HSX (**1**, A), 6T3Q (**2**, B), 6T4A (**3**, C) and 6TDT (**4**, D) in the S1 pocket of thrombin (left) compared to the structures of trypsin (right) by ligands 6T0M (**1**, E), 6T0P (**2**, F), 6T5W (**3**, G) and 6SY3 (**4**, H) are displayed in green at 3σ. Figure S1B shows ligand **2** with an alternative conformation of the P1 head group in thrombin (occupancy 76% for I and 24% for II). Figure S1G represents ligand **3** with an alternative conformation of the P3 group in trypsin (group occupancies each to two water molecules for 51% for I and 49% for II). In the other complex structures, all ligand atoms were refined to 100% occupancy. mF_o-DF_c map of ligand **1** in complex E indicates some additional electron density than expected at the *N*-terminal amino group of the P3 group which we cannot explain.

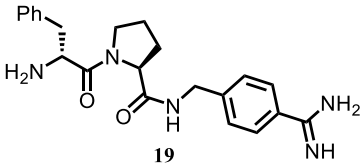
Table S1. Corresponding water molecule numbering from Figure 1.

Inhibitor	**Thrombin**	**Trypsin**
1	W1 = HOH413, W2 = HOH501, W3 = HOH499, W4 = HOH424	W1 = HOH428, W2 = HOH472
2	W1 = HOH404, W2 = HOH538, W3 = HOH537, W4 = HOH421	W1 = HOH424, W2 = HOH481
3	W2 = HOH416, W3 = HOH542, W4 = HOH412	W1 = HOH503, W2 = HOH549
4	W1 = HOH406, W2 = HOH550, W4 = HOH461, W5 = HOH423, W6 = HOH515	W1 = HOH413, W2 = HOH458

The numbering of W1-W6 in Figure 1 corresponds to the above given numbers in the deposited PDB structures. Water molecules are listed in chain H for thrombin and chain A for trypsin.

2. ITC results from displacement titrations

Table S2. Thermodynamic data of $\Delta H°$, $\Delta G°$ and $-T\Delta S°$[a] by displacement titration in trypsin.

Method	Inhibitor	$\Delta H°$ / kJ mol^{-1}	$\Delta G°$ / kJ mol^{-1}	$-T\Delta S°$ / kJ mol^{-1}	K_d / µM
Displacement titration	**1**	-21.1 ± 0.2	-23.5 ± 0.4	-2.1 ± 0.6	88 ± 28
	2	-18.5 ± 0.3	-24.2 ± 0.5	-5.7 ± 0.2	70 ± 22
	3	-21.5 ± 1.0	-23.6 ± 0.8	-2.1 ± 1.7	64 ± 27
 **19**		-35.1 ± 0.5	-43.4 ± 0.7	-8.3 ± 1.2	0.026 ± 0.006

Displacement ligand **19** was synthesized according to a procedure of L. Muley et al.[1] and was performed by direct titration in Tris buffer. The values for the inhibitors **1-3** were determined by displacement titration in the same buffer. Standard deviations were obtained from three measurements. [a]$-T\Delta S°$ was calculated as the difference between $\Delta G°$ and $\Delta H°$.

3. Thermograms and binding isotherms from ITC measurements

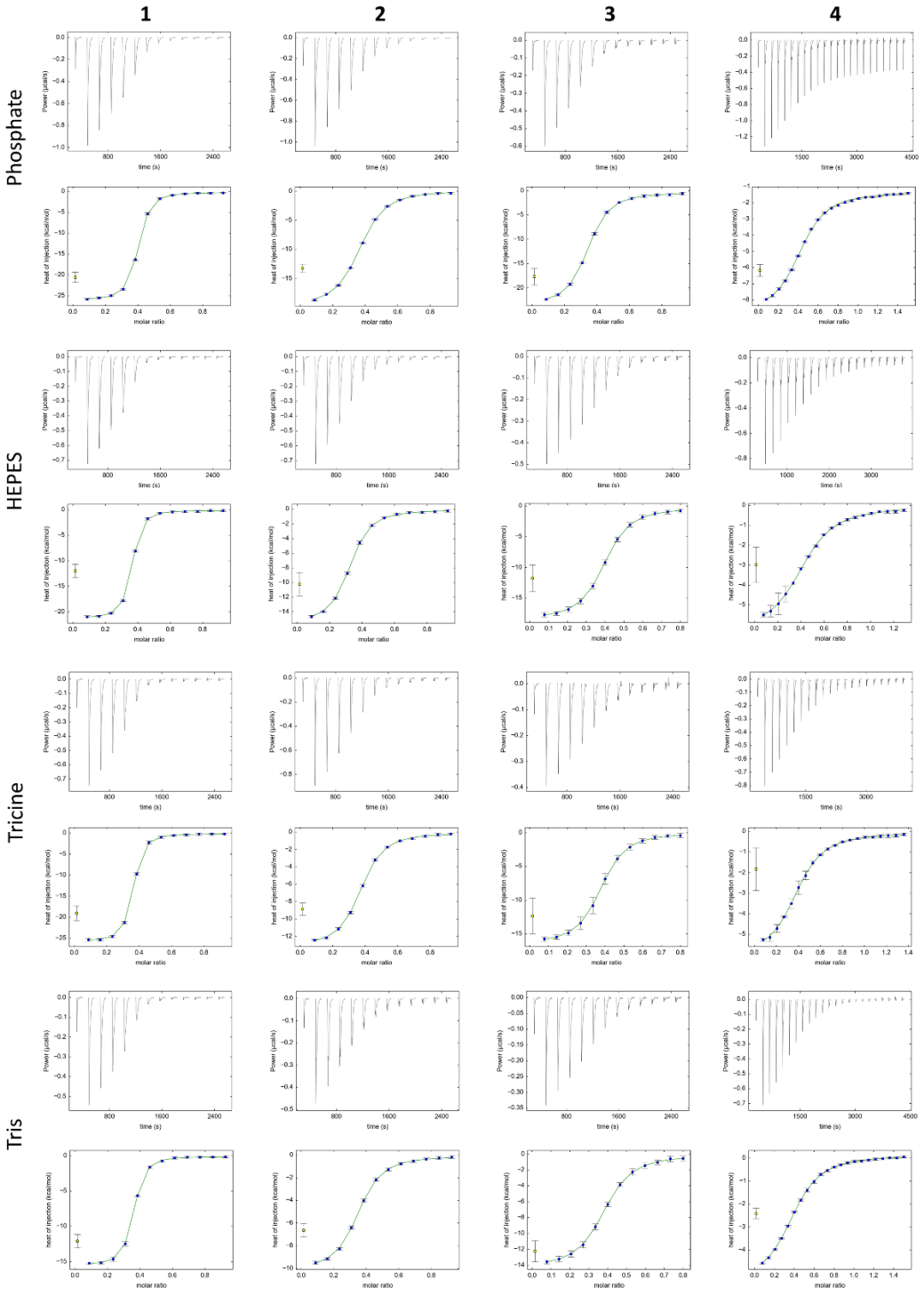


Figure S2. Representative diagrams and isotherms of inhibitor **1-4** from direct titrations in four different buffers for thrombin.

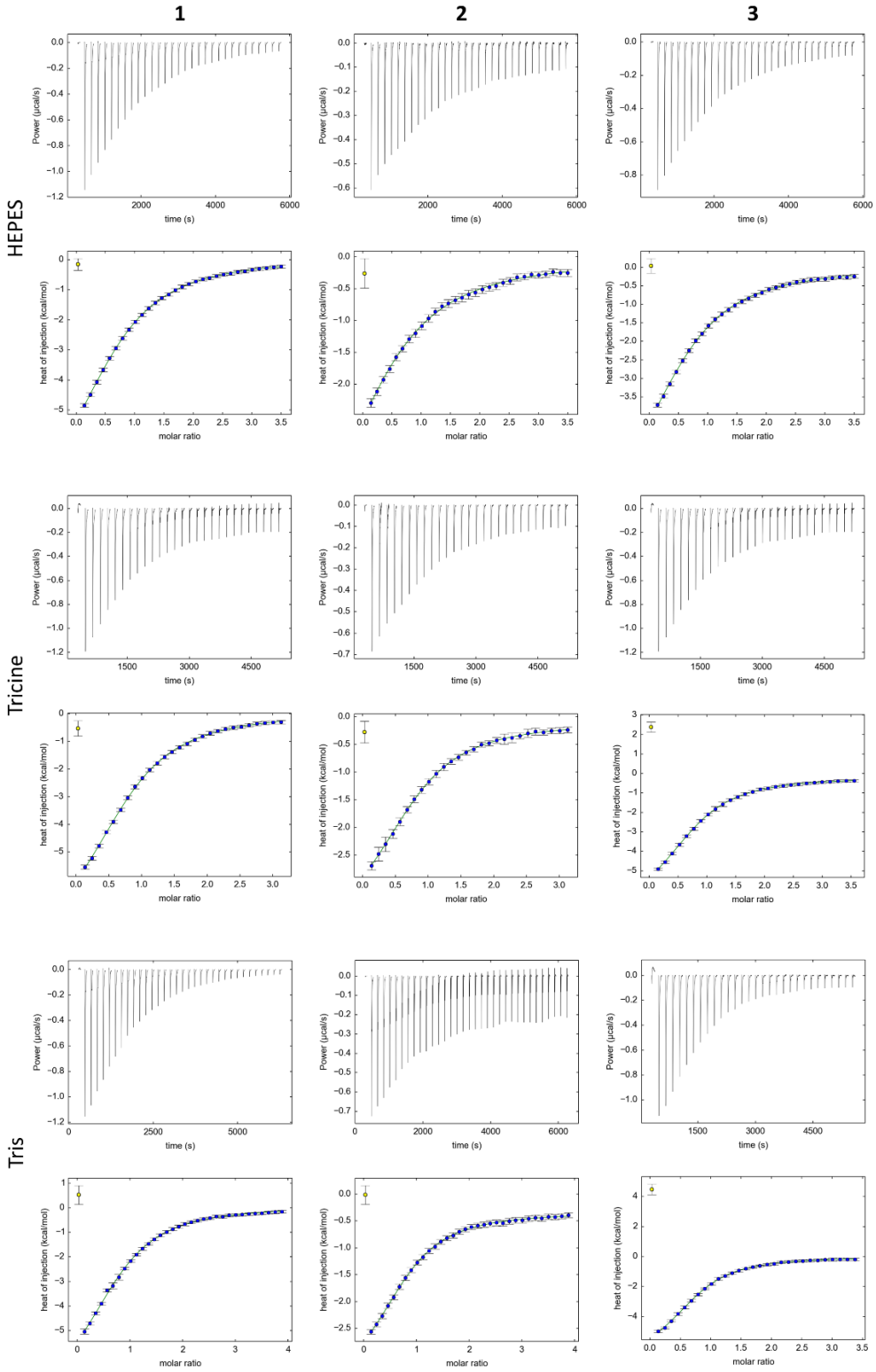
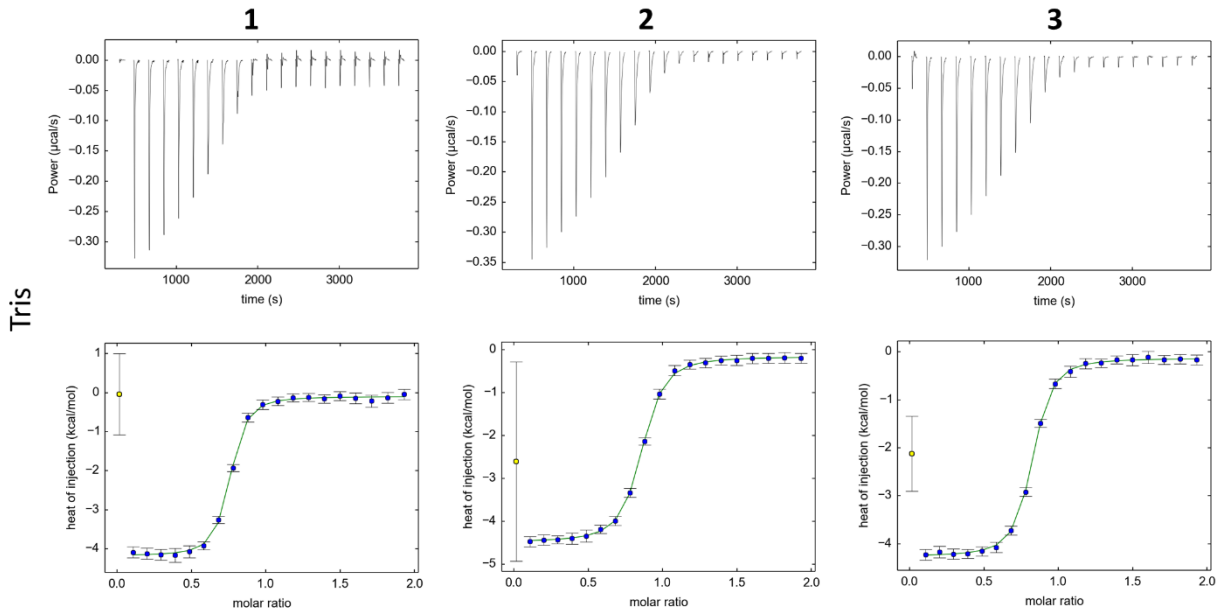


Figure S3. Representative diagrams and isotherms of inhibitor **1**-**3** from direct titrations in three different buffers for trypsin.



Displacement titration of inhibitor **4**:

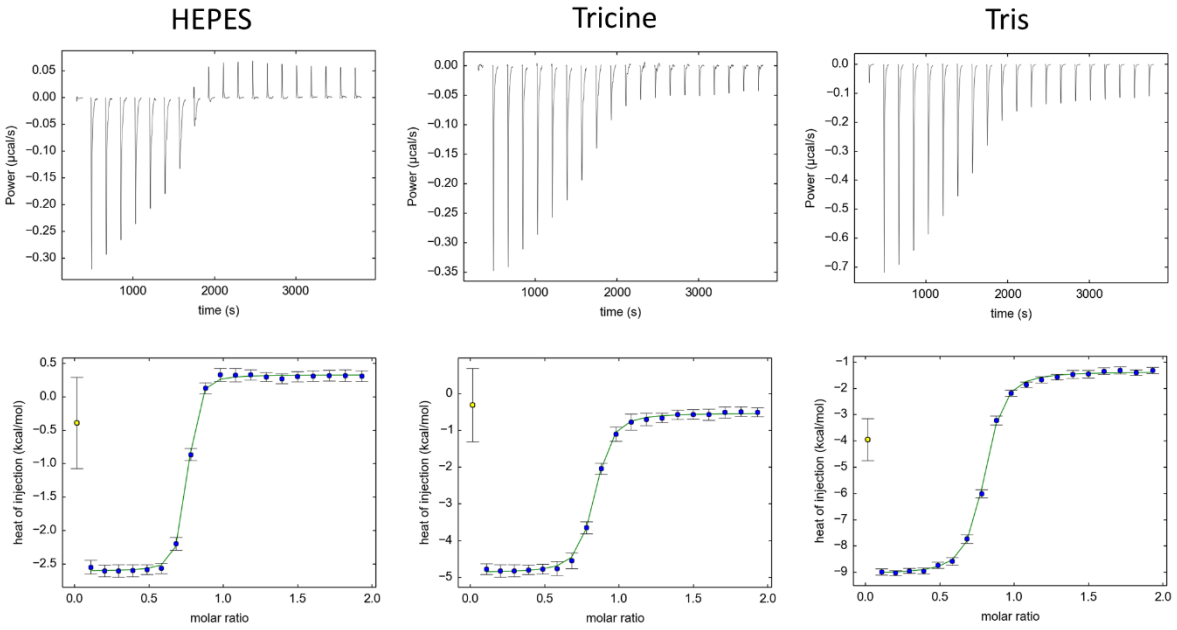


Figure S4. Representative diagrams and isotherms of inhibitor **1-3** from displacement titrations in Tris buffer for trypsin. The displacement titration of **4** was performed in three different buffers to obtain the protonation state.

4. HPLC traces

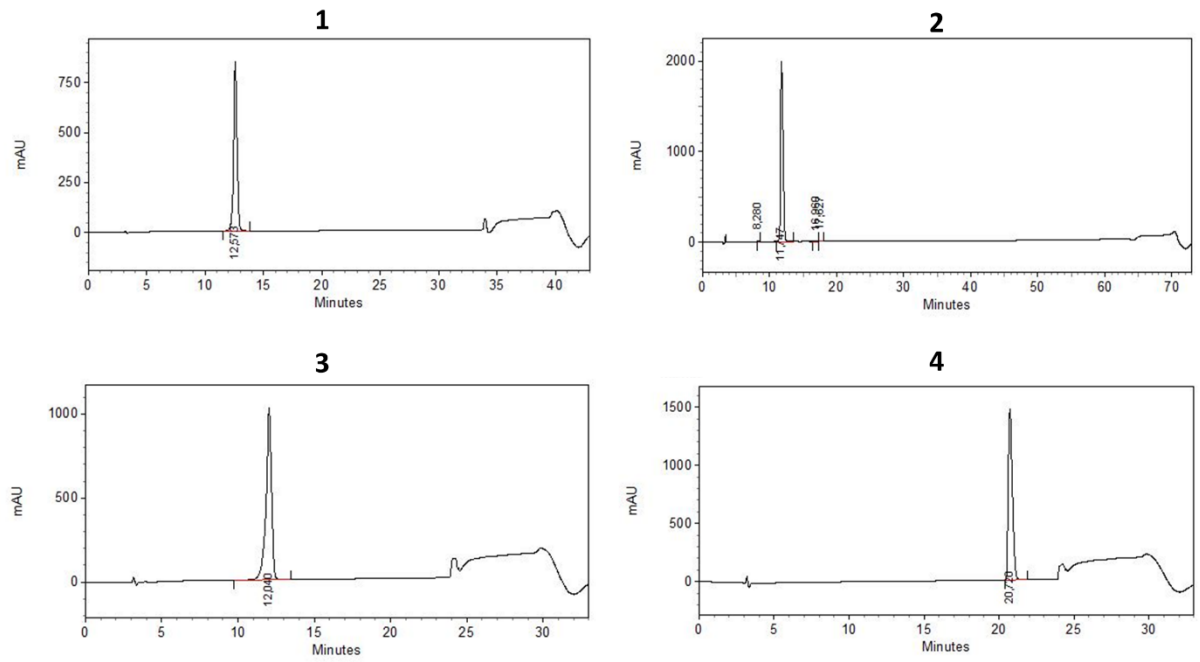


Figure S5. HPLC traces present the purity of the synthesized inhibitors **1-4** from at least 95%, start at 10% MeCN respectively. The retention time (RT) of **1** (RT = 12.57 min,), **2** (RT = 11.75 min), **3** (RT = 12.04 min) and **4** (RT = 20.72 min) is displayed in the chromatograms.

5. References

(1) Muley, L.; Baum, B.; Smolinski, M.; Freindorf, M.; Heine, A.; Klebe, G.; Hangauer, D. G. Enhancement of Hydrophobic Interactions and Hydrogen Bond Strength by Cooperativity: Synthesis, Modeling, and Molecular Dynamics Simulations of a Congeneric Series of Thrombin Inhibitors. *J. Med. Chem.* **2010**, *53* (5), 2126–2135.