Electronic Supplementary Material (ESI) for Chemical Science.

Small Molecule Probes That Perturb A Protein-protein Interface In Antithrombin

Dongyue Xin,[1] Andreas Holzenburg[2], and Kevin Burgess[1*]

[1] Department of Chemistry, Texas A & M University, Box 30012, College Station, TX 77842

[2] Microscopy and Imaging Center, Department of Biology, and Department of Biochemistry & Biophysics TAMU, College Station, TX 77843-2257

E-mail: burgess@tamu.edu

A. Matching With EKO

QMD (quenched molecular dynamics)

QMD was used to generate the simulated solution conformations for all the diastereomers of **1aaa**. [1]

NAMD[2] was used for the molecular simulations performed in this work. Explicit atom representations were used throughout the study. The protein structure files (PSF) for all the peptidomimetics were built using Discovery Studio 2.5 (Accelrys Inc) using the CHARMm force field.

Quenched molecular dynamics simulations were performed using the CHARMm force field as implemented in Discovery Studio 2.5. All four molecules were modeled as neutral compounds in a dielectric continuum of 80 (simulating H_2O). Thus, the starting conformers were minimized using 3000 steps of conjugate gradient. The minimized structures were then subjected to heating, equilibration, and dynamics simulation. Throughout, the equations of motions were integrated using the Verlet algorithm with a time step 1 fs. Each peptidomimetic was heated to 1000 K over 10 ps and equilibrated for another 10 ps at 1000 K, then molecular dynamics runs were performed for a total time of 600 ps with trajectories saved every 1 ps. The resulting 600 structures were thoroughly minimized using 1000 steps of SD followed by 3000 steps of conjugate gradient. Structures with energies less than 3.0 kcal mol^{-1} relative to the global minimum were selected for further analysis.

The VMD[3] package was used to display, overlay, and classify the selected structures into conformational groups. The best clustering was obtained using a grouping method based on calculation of RMS deviation of a subset of atoms, in this study these were the Cα - and Cβ- atoms. Thus, threshold cutoff values 0.5 Å were selected to obtain families with reasonable homogeneity.

Data Mining Of 3D Complex Database With EKO

The data mining of 3D complex database was performed for unique conformers of all diastereomers of **1aaa** according to the procedure described previously.[4] PPI interface of dimeric α-Antithrombin (PDBID: 2ZNH)[5] was found in the mining of LLD-**1aaa** (RMSD = 0.42 Å, S385-A383-E374).

Overlaying All Preferred Simulated Conformers On Dimeric α-Antithrombin PPI Interface

All the conformers within 3.0 kcal/mol were considered to be "preferred". Each of these was overlaid on α-antithrombin dimeric interface (PDBID: 2ZNH) using an in house generated algorithm that compared Cα - Cβ coordinates of the side chains which generates a list of structures ranked in terms of the RMSD for the overlay process. All the unique hits within 0.37 Å RMSD are listed in Table S1.

Table S1. Unique hits from EKO for dimeric α-antithrombin

isomer	RMSD(Å)	residues	polarity
LDD	0.26	V389-T386-A384	antiparallel
LLL	0.29	S385-A383-E374	antiparallel
LLL	0.33	K370-A383-E381	antiparallel
LLD	0.33	L373-A371-T386	antiparallel
LDL	0.35	D366-A387-K370	parallel
DDD	0.35	A383-S385-F368	parallel
LLD	0.36	F372-K370-T386	antiparallel
LDD	0.37	A382-A371-H369	antiparallel

DLL	0.37	L373-A384-T386	parallel

Overlaying All Preferred Simulated Conformers On *pseudo*-Dimeric α-Antithrombin PPI Interface

The strand 361 – 393 in the monomeric α-Antithrombin(PDBID: 2ANT), which corresponds to one strand of the β-hairpin at the dimeric interface, was artificially disconnected from the monomeric protein and considered as an independent chain. This artificial chain and the rest of the protein formed the *pseudo*-dimeric α-antithrombin PPI interface. Each of the preferred conformers was overlaid on *pseudo*-dimeric α-antithrombin PPI interface using an in house generated algorithm that compared $C\alpha$ - $C\beta$ coordinates of the side chains which generates a list of structures ranked in terms of the RMSD for the overlay process. The best hit was shown in Figure S1.

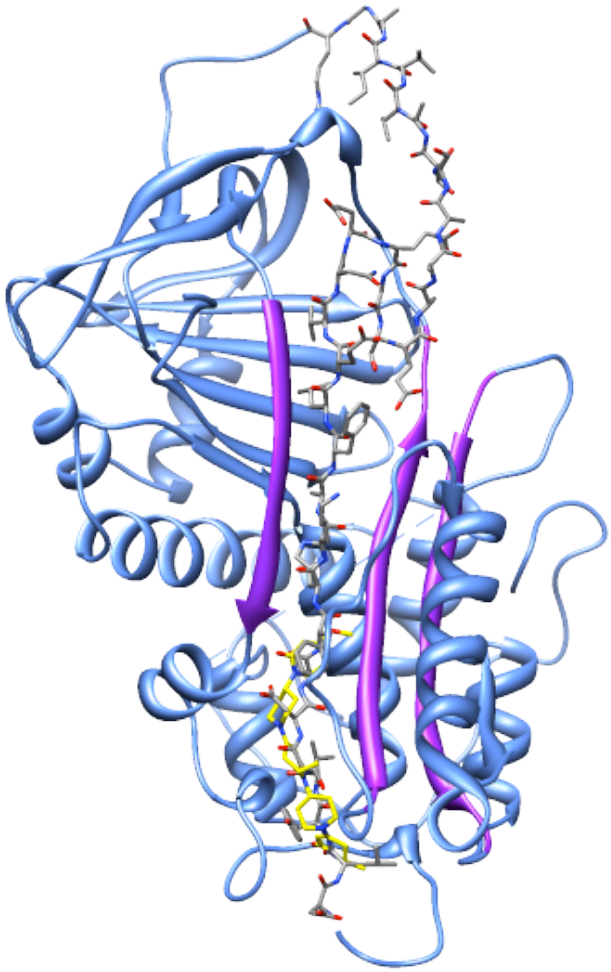


Figure S1 Conformers of scaffolds **1**(yellow) can overlay side-chains on the β-strand (gray) in the *pseudo*-dimeric α-antithrombin PPI interface.

B. Nondenaturing PAGE

General procedure: α-Antithrombin was purchased from Haematologic Technologies Inc. and diluted with pH 7.4 Tris-HCl buffer containing 50 mM tris, 50 mM NaCl, 1 mM EDTA. 20 mM stock solutions of the target compounds in DMSO were used in all the gel experiments.

0.25 mg / mL α-Antithrombin was incubated with 200 fold of the target compounds or controls at 50 °C for 1 h. The solutions were put on ice to quench the oligomerization after incubation. Aliquots of the solutions were

taken and analyzed by 8 % (w/v) acrylamide nondenaturing PAGE to evaluate the extent of oligomerization.[5] Silver stain was performed according to a literature procedure to visualize the results.[6]

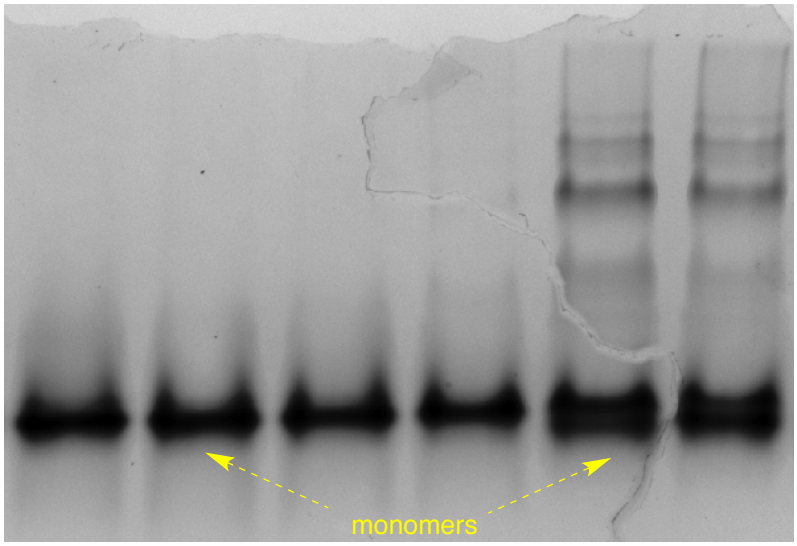


Figure S2. α-Antithrombin was incubated at 50 °C for 0 h (lane1), 1 h (lane2), 4 h (lane3), 12 h (lane4), 36 h (lane5) and 60 h (lane6).

In the absence of mimics, monomeric α-antithrombin was stable against oligomerization under the standard experimental condition for up to 12 hours. Only a small degree of oligomerization was observed even after 60 hours of incubation.

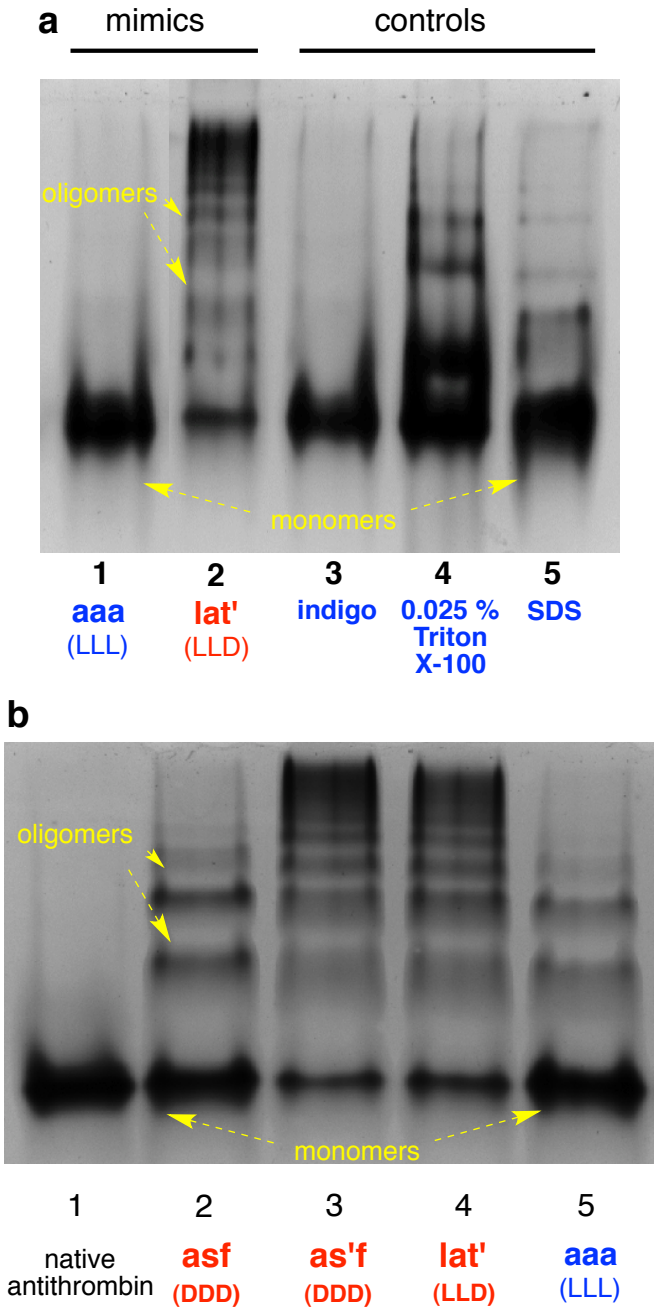


Figure S3. Throughout, controls are delineated in blue, and assays of target compounds are shown in red. **a** α-Antithrombin (0.25 mg / mL) was incubated with 200 fold compound LLL-**1aaa** (negative control in this series, lane 1), 200 fold LLD-**1lat'** (lane 2), 200 fold indigo (control for non-specific hydrophobic interactions, lane 3), 0.025 % Triton-X 100 (control for surfactant, lane 4), 200 fold sodium dodecyl sulfate (SDS, control for amphiphilic compounds, lane 5) at 50 °C for 1 h; **b** induction of oligomerization by mimics in the presence of surfactant: α-Antithrombin (0.25 mg / mL) was incubated with 200 fold compound DDD-**1asf** (lane2), DDD-**1as'f** (lane3), LLD-**1lat'** (lane4) and LLL-**1aaa** (negative control in this series, lane 5) in the presence of 0.02 % (v/v) Tween 80 at 50 °C for 1 h.

C. Selectivity Issues

Nondenaturing PAGE was used to follow the oligomerization of α-antitrypsin according to the procedure in section B:

α-Antitrypsin was dissolved in pH 7.4 Tris-HCl buffer containing 50 mM tris, 50 mM NaCl, 1 mM EDTA. 20 mM stock solutions of the target compounds in DMSO were used in all the gel experiments.

0.25 mg / mL α-Antitrypsin was incubated with 200 fold of DDD-**1as'f** or controls at various temperature for 1 h. The solutions were put on ice to quench the oligomerization after incubation. Aliquots of the solutions were taken and analyzed by 8 % (w/v) acrylamide nondenaturing PAGE to evaluate the extent of oligomerization.[5] Silver stain was performed according to a literature procedure to visualize the results.[6]

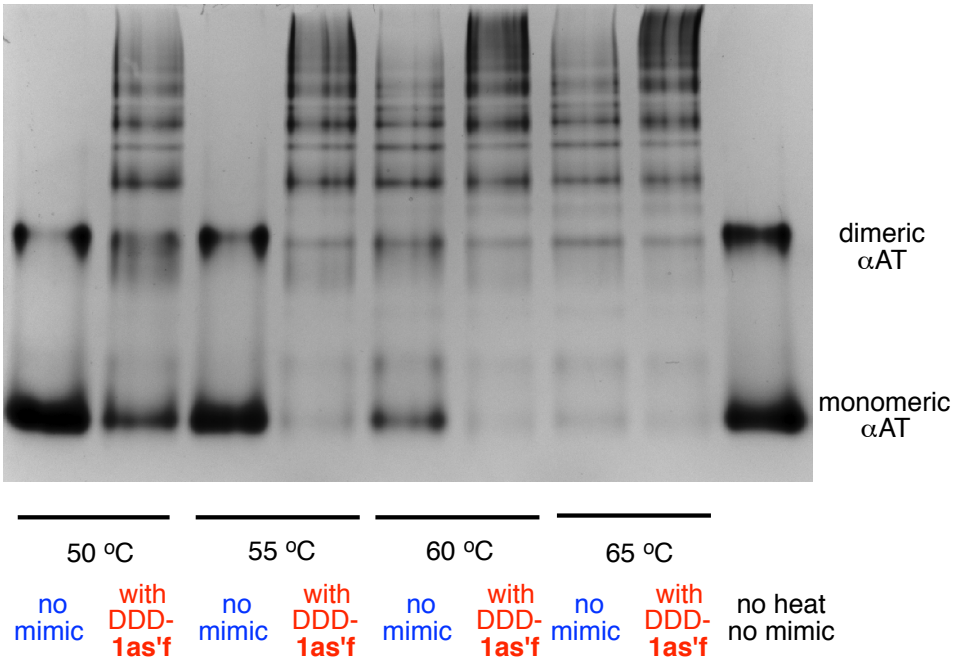
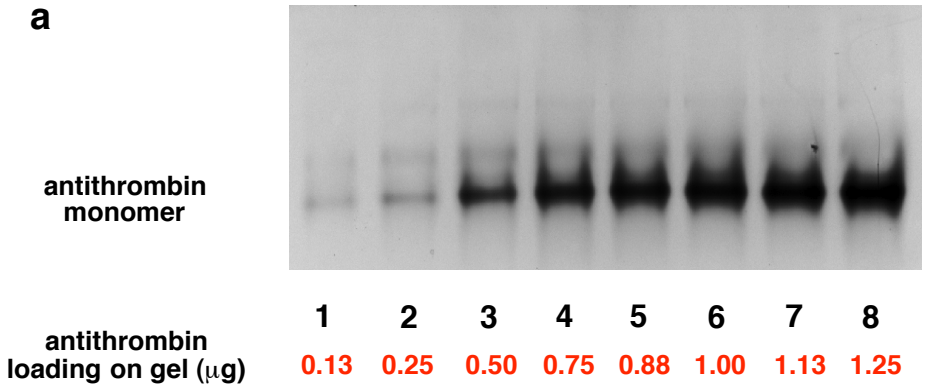


Figure S4. α-Antitrypsin (0.25 mg / mL) was incubated with or without 200 fold compound DDD-**1as'f** at 50 °C, 55 °C, 60 °C or 65 °C for 1 h.

D. α-Antithrombin Oligomerizartion Kinetics Determined By Nondenaturing PAGE

Kinetics of α-antithrombin oligomerization was followed by nondenaturing PAGE according to the literature procedure except that silver staining was used to visualize the protein.[7,8] Since silver staining method generally has a limited linear dynamic range, silver staining for α-antithrombin at various sample loading was performed to calibrate the band intensity relative to the amount of protein (**Figure S5**). The linear range was determined to be between 0 μg and 0.75 μg for protein loading on gel. The protein loading in the kinetics studies was kept in this range to make sure the linear correlation between the amount of monomer and band intensity after silver staining.



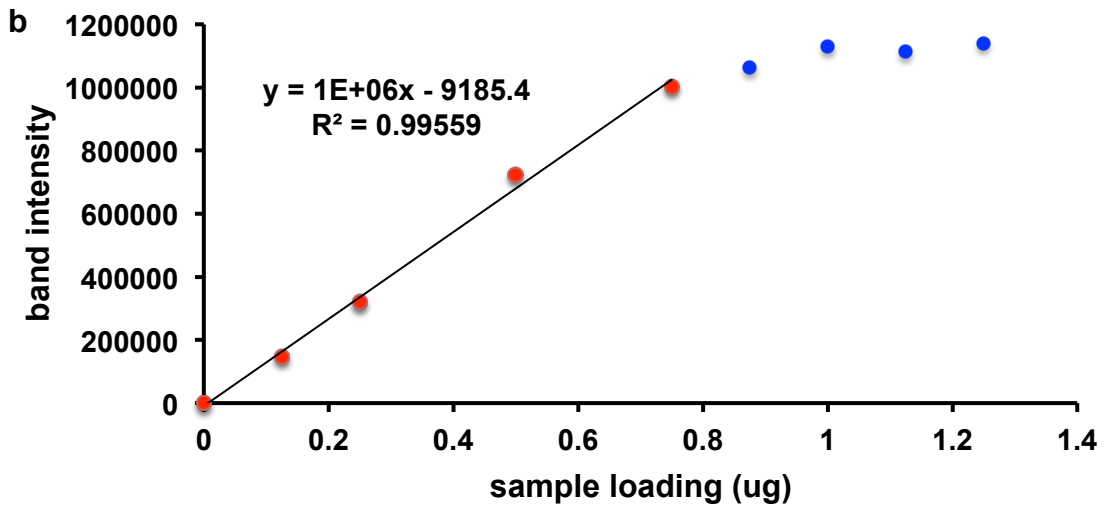
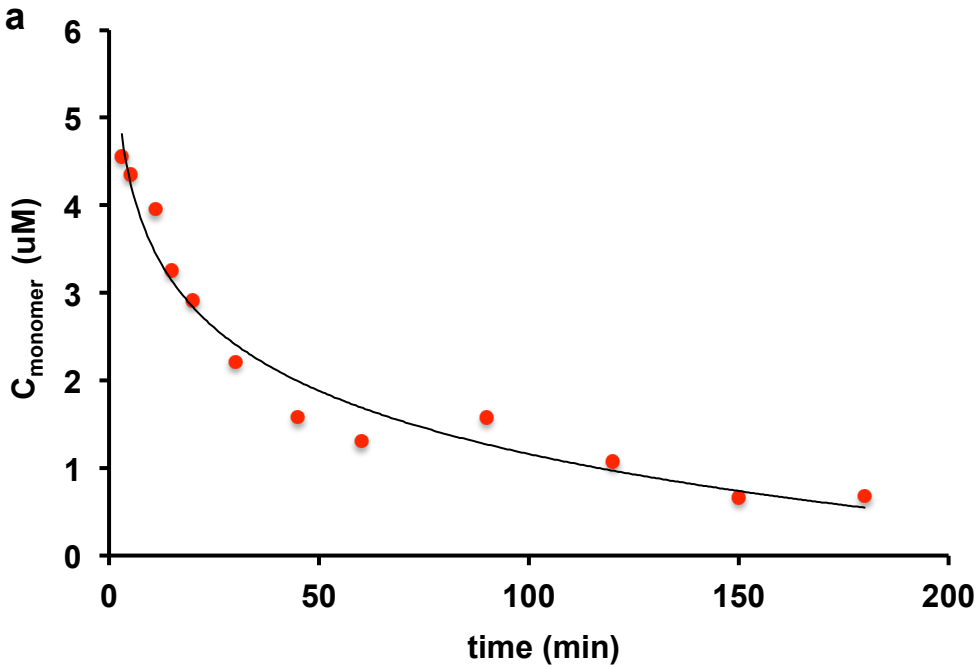


Figure S5. a Band intensity of α-antithrombin monomer at different protein loading on nondenaturing PAGE; b Determination of the linear dynamic range for silver staining.

General procedure: 0.25 mg / mL α-Antithrombin was incubated with 200 fold DDD-**1as'f** at 50 °C for 3 h. Aliquots of the solutions were taken and cold quenched on ice in every 10 mins in the first hour and then in every 30 min afterwards. The relative amount of residual monomer was analyzed by 8 % (w/v) acrylamide nondenaturing PAGE. The intensities of the α-antithrombin monomer bands were measured and fitted to a second order kinetics model to determine the rate constant in terms of the loss of monomer. A representative example for DDD-**1as'f** was shown in Figure S6.



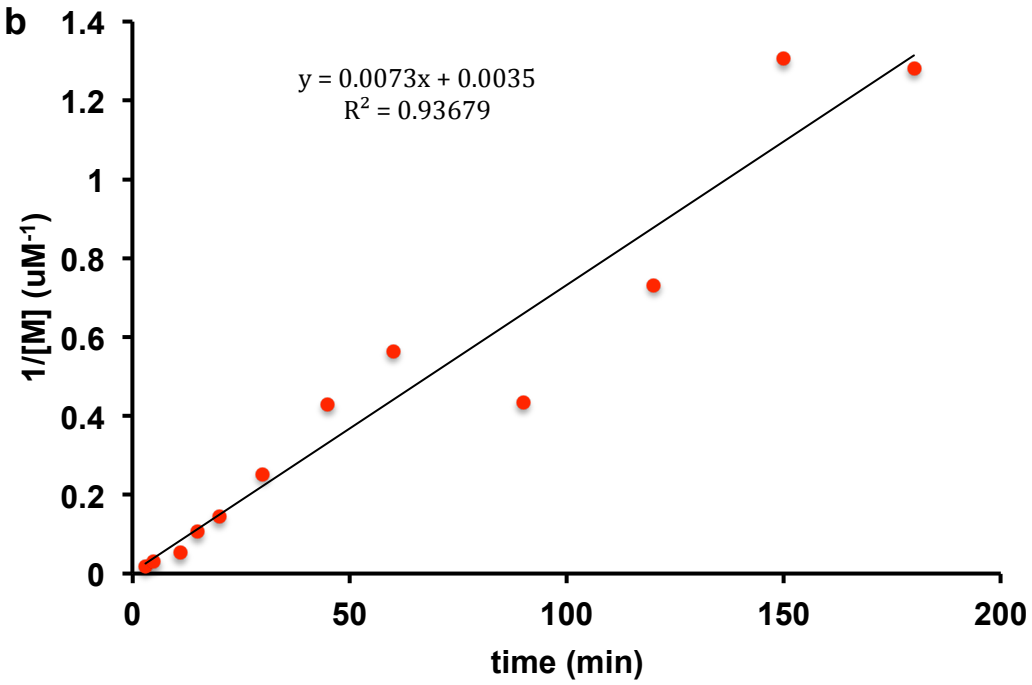


Figure S6. a Loss of monomeric α-antithrombin measured by nondenaturing PAGE; b Fitting into a second order kinetics model.

E. Thrombin Activity Assay

0.25 mg / mL a-Antithrombin was incubated with 200 fold DDD-**1as'f** or DDD-**1asf** at 50 °C for 3 -5 h. Aliquots of the solutions were taken at indicated time and quenched on ice to stop oligomerization. The antithrombin solutions at different degrees of oligomerization were then incubated with 4 nM thrombin (purchased from Haematologic Technologies Inc.) in 20 mM Tris-HCl buffer containing 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1 % PEG8000, 0.02 % Tween80 at pH = 7.4 in the presence of 10 nM heparin. After incubation of 5 min at room temperature, chromogenic substrate Spectrozyme TH (purchased from American diagnostic inc.) was added. The residual enzyme activity was determined from the initial rate of increase in absorbance at 405 nm with a plate reader. [9]

F. α-Antithrombin Conformation Change By CD

Circular dichroism (CD) was performed in Tris buffer (50 mM tris, 50 mM NaCl, 1mM EDTA, pH = 7.4), at 50 °C. Spectra in the range of 200 - 250 nm) were determined with a 0.1 cm path length cell. Conformation changes of α-antithrombin in the presence of 10 x DDD-**1as'f** was determined by measuring the relative change in ellipticity at 222 nm of 0.25 mg/mL α-antithrombin at 50 °C.[10] A control experiment at the same temperature but without DDD-**1as'f** was also run (Figure S7).

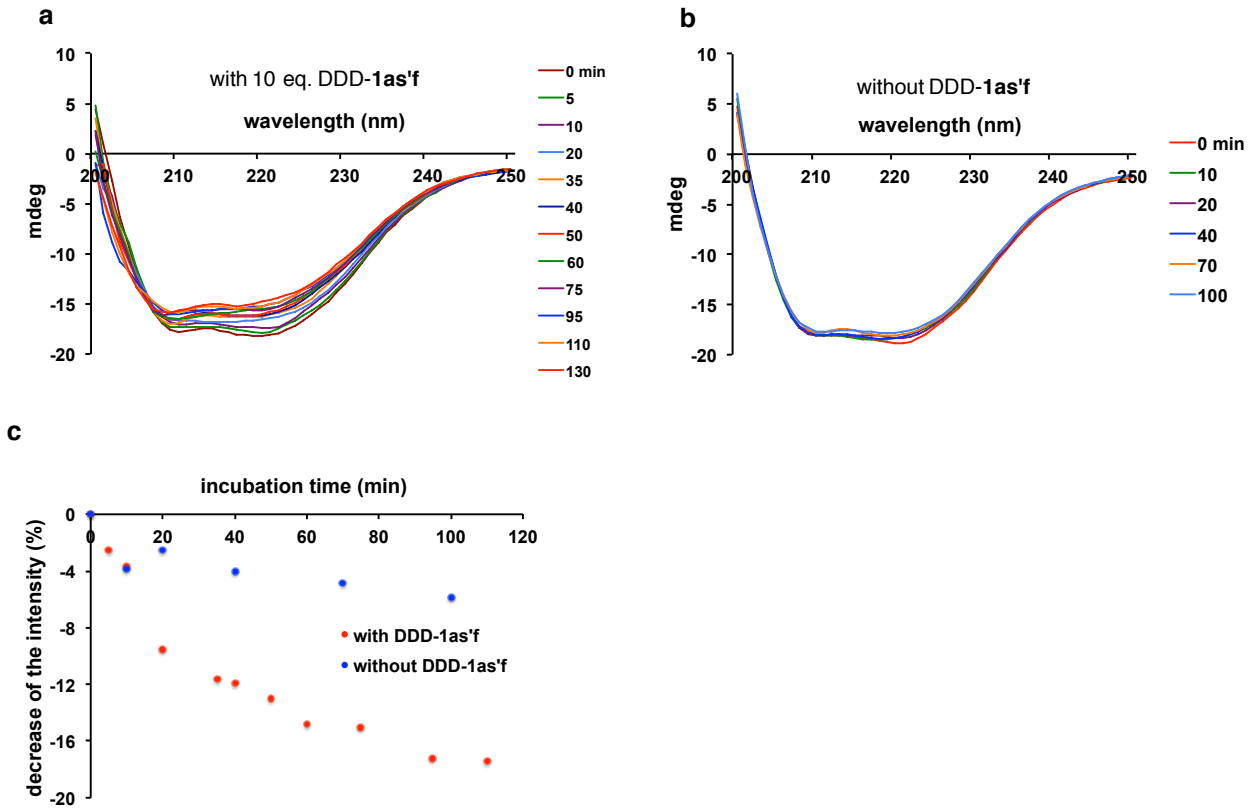


Figure S7. Change in CD spectrum of α-antithrombin with DDD-**1as'f** (a) and without DDD-**1as'f** (b) at 50 °C for the indicated time. c. Reduced CD signal at 222 nm with DDD-**1as'f** (red) and without DDD-**1as'f** (blue).

G. Electron Microscopy

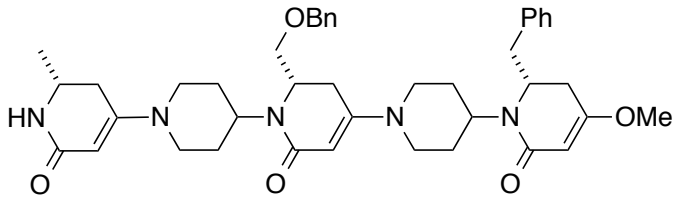
0.25 mg / mL α-Antithrombin was incubated with or without 200 fold DDD-**1as'f** at 50 °C for 1 h. The solutions were put on ice to quench the oligomerization after incubation. The solution was diluted to a concentration of 0.05 mg / mL in pH 7.4 Tris-HCl buffer containing 50 mM tris, 50 mM NaCl, 1 mM EDTA for use in the EM experiments. Specimens were prepared using an aqueous solution of 1% wt/vol uranyl acetate (pH 4.25). Uranyl acetate has been shown to fix the structure of protein molecules within 10 ms and prior to blotting the buffer off.[11] Therefore it can be reasonably assumed that the preparation reflects the situation in solution. Specimens were observed in a Jeol 1200 EX TEM operated at an accelerating voltage of 100 kV. Images were captured at calibrated magnifications using an optically coupled 3 k slow scanCCD camera (model 15C, SIA, Duluth, GA) and Maxim DL imaging software.

H. Syntheses And Characterization Of Key Compounds

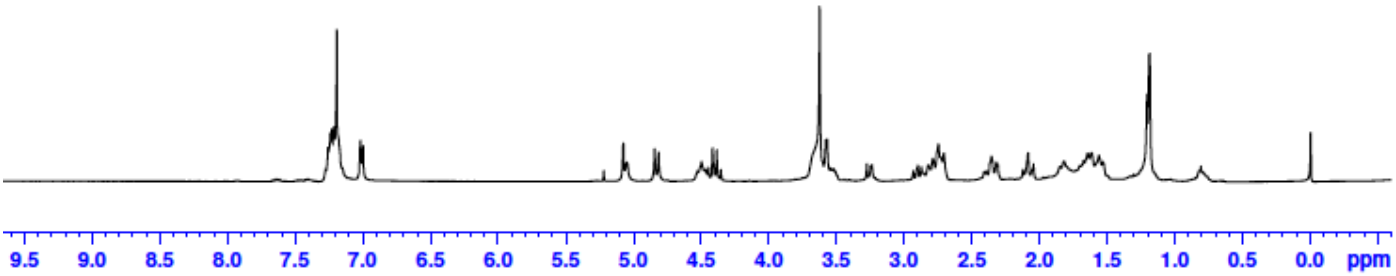
Syntheses and structural characterization of most of the compounds used in this study have been published in our previous paper.[12] The new compounds DDD-**1as'f** and DDD-**1asf** were synthesized via the same procedures.

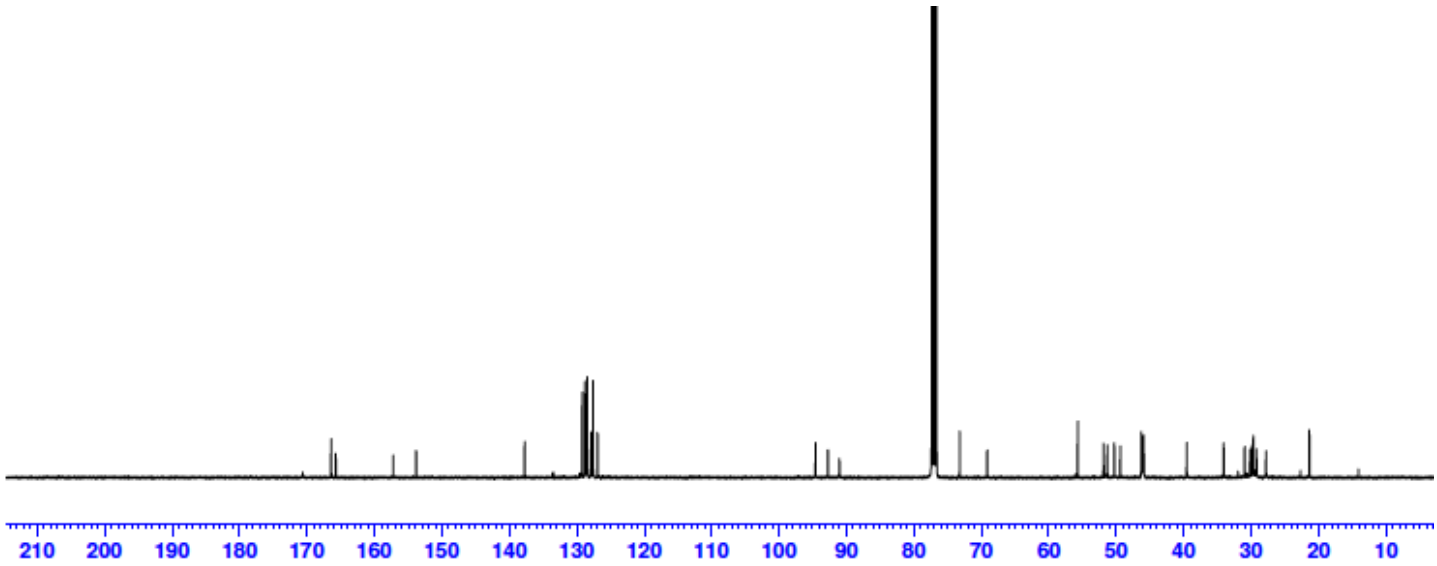
Compound DDD-**1as'f**

(*R*)-6-benzyl-1-(1-((*S*)-2-((benzyloxy)methyl)-1-(1-((*R*)-2-methyl-6-oxo-1,2,3,6-tetrahydropyridin-4-yl)piperidin-4-yl)-6-oxo-1,2,3,6-tetrahydropyridin-4-yl)piperidin-4-yl)-4-methoxy-5,6-dihydropyridin-2(1*H*)-one



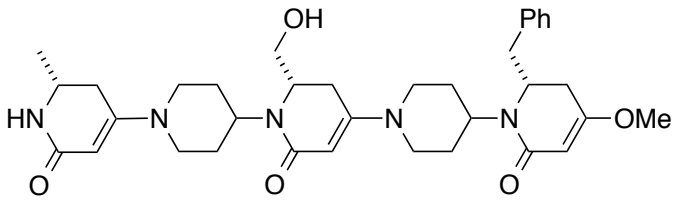
Light yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.27-7.13 (m, 8H), 6.99-6.91 (m, 2H), 5.08 (s, 1H), 5.00 (s, 1H), 4.84 (s, 1H), 4.77 (s, 1H), 4.56-4.43 (m, 4H), 3.75-3.52 (m, 12H), 3.29-3.20 (m, 1H), 2.95-2.64 (m, 7H), 2.40-2.26 (m, 2H), 2.14-2.01 (m, 2H), 1.90-1.50 (m, 8H), 1.25 (d, *J* = 7.2 Hz, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.6, 166.5, 166.4, 165.7, 157.2, 153.8, 137.8, 137.7, 129.2, 128.7, 128.4, 127.8, 127.6, 126.9, 94.5, 92.8, 91.0, 73.2, 69.1, 55.6, 51.9, 51.3, 50.2, 49.4, 46.2, 46.1, 45.9, 45.8, 39.5, 34.0, 30.9, 30.1, 29.8, 29.7, 29.6, 29.2, 27.7, 21.3; HRMS (ESI) m/z calcd for $C_{42}H_{54}N_5O_5$ $(M+H)^+$ 708.4125; found 708.4119.



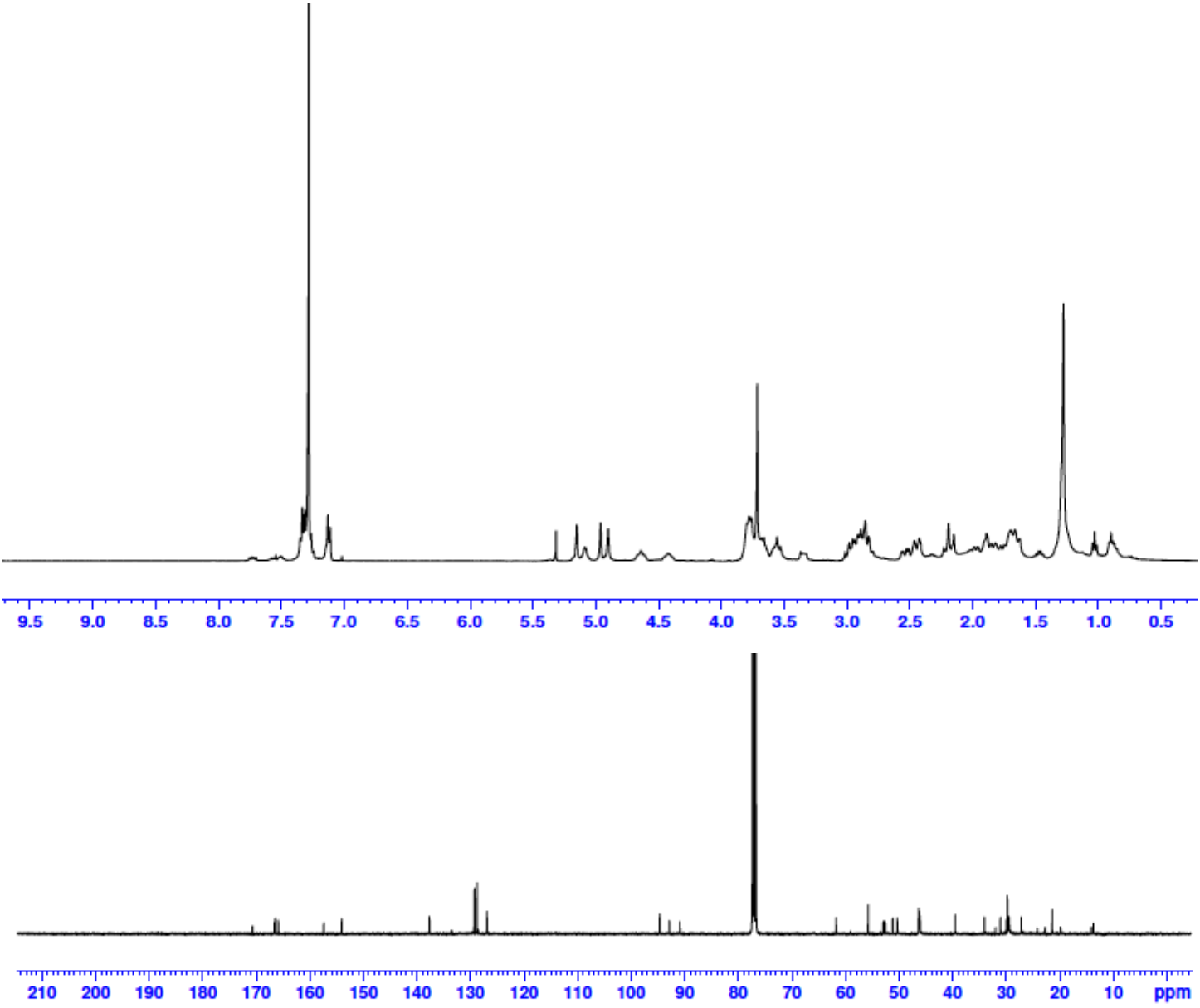


Compound DDD-**1asf**

(*R*)-6-benzyl-1-(1-((*S*)-2-(hydroxymethyl)-1-(1-((*R*)-2-methyl-6-oxo-1,2,3,6-tetrahydropyridin-4-yl)piperidin-4-yl)-6-oxo-1,2,3,6-tetrahydropyridin-4-yl)piperidin-4-yl)-4-methoxy-5,6-dihydropyridin-2(1*H*)-one



Light yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37-7.25 (m, 3H), 7.15-7.10 (m, 2H), 5.15 (s, 1H), 5.09 (s, 1H), 4.94 (s, 1H), 4.89 (s, 1H), 4.70-4.61 (m, 1H), 4.48-4.39 (m, 1H), 3.85-3.62 (m, 10H), 3.61-3.54 (m, 2H), 3.39-3.32 (m, 1H), 3.04-2.69 (m, 7H), 2.59-2.47 (m, 3H), 2.25-2.17 (m, 2H), 2.07-1.70 (m, 8H), 1.35 (d, *J* = 7.2 Hz, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.7, 166.5, 166.4, 165.8, 157.3, 154.0, 137.6, 129.2, 128.7, 126.9, 94.6, 92.8, 90.9, 61.7, 55.7, 52.9, 52.5, 51.1, 50.2, 46.3, 46.2, 46.1, 46.0, 39.4, 34.0, 31.0, 30.0, 29.7, 29.6, 29.5, 29.4, 27.1, 21.3; HRMS (ESI) m/z calcd for $C_{35}H_{48}N_5O_5$ $(M+H)^+$ 618.3655; found 618.3659.



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