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Supporting Information

Recognition and Conformational Properties of an Alternative Antithrombin Binding Sequence Obtained by Chemoenzymatic Synthesis

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	Glycosidic dihedral angles of Hexa-4/AT complex					
tr-NOE select. Struct. (ns)	**GlcNS6S-GlcA**	**GlcA-GlcNS3S6S**	**GlcNS3S6S-IdoA2S**	**IdoA2S-GlcNS6S**	**GlcNS6S''-GlcA''**	**Rave**
209	-50/-37	47/24	-69/-54	53/19	-24/-18	0.13
227	-39/-33	53/22	-47/-58	51/19	-23/-30	0.09
240	-59/-38	45/27	-51/-56	39/21	-16/-17	0.22
257	-45/-40	50/25	-62/-39	48/24	-14/-28	0.17
270	-43/-31	39/44	-59/-52	70/12	-26/-25	0.19
AVERAGE	**-47(3)/-36(2)**	**47(2)/28(4)**	**-58(4)/-52(4)**	**52(5)/19(2)**	**-21(2)/-24(4)**	**-**
Hexa-4	**-41(2)/-27(1)**	**53(1)/5(1)**	**-41(1)/-45(1)**	**45(1)/1(1)**	**-42(1)/-20(1)**	**-**
	Glycosidic dihedral angles of Hexa-8/AT complex					
tr-NOE select. Struct. (ns)	**GlcNS6S-GlcA**	**GlcA-GlcNS3S6S**	**GlcNS3S6S-IdoA2S**	**IdoA2S-GlcNS6S**	**GlcNS6S''-GlcA''**	**Rave**
214	-41/-33	50/8	-44/-40	45/11	-69/-46	0.20
235	-16/-28	44/6	-57/-40	45/23	-63/-37	0.18
243	-23/-24	47/4	-56/-47	54/21	-70/-48	0.21
258	-37/-1	38/8	-62/-50	56/9	-85/-34	0.18
AVERAGE	**-29(6)/-22(7)**	**45(3)/7(1)**	**-55(4)/-44(3)**	**50(3)/16(4)**	**-72(5)/-41(3)**	**-**
Hexa-8	**-42(1)/-23(1)**	**59(1)/8(1)**	**-46(1)/-41(1)**	**33(1)/11(2)**	**-47(2)/-28(2)**	**-**
	(Hricovini et al 2001)					
AGA*IA/AT	**-56/-60**	**40/12**	**-32/-43**	**45/16**	**-**	**-**
AGA*IA	**-34/-27**	**47/6**	**-35/-42**	**43/6**	**-**	**-**

Table S1 Glycosidic dihedral angles (ϕ/ψ) of Hexa/AT complexes generated by MD simulation and validated by selected Tr-NOEs, corresponding to geometries at instant time: 209, 227, 240, 257, 270 ns for Hexa-4/AT and 214, 235, 243, 258 ns for Hexa-8/AT. The last column (Rave) report the R factor averaged on the selected set of proton-proton inter-glycosidic Tr-NOEs used as constraints. The label "AVERAGE" (light blue background) indicate the average and estimated error calculated on the respective set of glycosidic dihedral angles. Glycosidic dihedral angles of Hexa-4 and Hexa-8 in free state are reported as averages and errors calculated on the MD simulation trajectories after 40ns of relaxation time (Hexa-4 and Hexa-8 labels in orange background). The corresponding dihedral angles ϕ/ψ of the AGA*IA and AGA*IA/AT complexes as determined by Hricovini et al. 2001 are reported in the last two rows in orange and light blue background .

	Hexa-4 and Hexa-4/AT		Hexa-8 and Hexa-8/AT	
	H2-H5/H4-H5	H2-H5/H4-H5	H2-H5/H4-H5	H2-H5/H4-H5
Mixing time (ms)	NOE	tr-NOE	NOE	tr-NOE
200	2.2/4.9 (=0.45)	19.9/15.6 (=1.28)	0.9/3.4 (=0.26)	5.1/5.7 (=0.89)
300	2.8/7.8 (=0.36)	29.8/24.2 (=1.23)	1.7/5.6 (=0.30)	7.4/9.4 (=0.79)
500	5.2/12.4 (=0.42)	51.8/44.4 (=1.17)	2.2/10.0 (=0.22)	15.5/18.5 (=0.84)

Table S2 Selected H2-H5 and H4-H5 intra-residue NOEs and Tr-NOEs for IdoA in Hexa-4 and Hexa-8 in free and in bound state with AT, respectively. The ratios between the two signals are reported in red in brackets.

	Hexa-4 Experimental NOE (Simulated NOE)			
Tmix	**GlcNS6S-GlcA**	**GlcNS3S6S-IdoA2S**	**IdoA2S-GlcNS6S''**	**GlcNS6S''-GlcA''**
(ms)	**H1-H4**	**H1-H4/H1-H3**	**H1-H4/H1-H6**	**H1-H4/H1-H3**
200	2.9 (2.8)	(4.1) 4.3/4.0 (3.4)	(8.8) 3.2/3.4 (2.5)	(6.1) 2.3/0.7 (0.50)
300	4.5 (5.1)	(6.2) 6.2/6.1 (5.2)	(13.2) 4.9/5.2 (4.0)	(9.2) 3.7/1.1 (0.81)
500	7.9 (8.6)	(10.3) 10.5/10.2 (8.7)	(22.0) 8.0/7.8 (7.8)	(15.3) 6.0/1.9 (1.42)
	Hexa-8 Experimental NOE (Simulated NOE)			
Tmix	**GlcNS6S-GlcA**	**GlcNS3S6S-IdoA**	**IdoA-GlcNS6S''**	**GlcNS6S''-GlcA''**
(ms)	**H1-H4**	**H1-H4/H1-H3**	**H1-H4/H1-H6**	**H1-H4/H1-H3**
200	2.1 (6.3)	(3.9) 5.4/6.7 (9.5)	(11.4) 4.0/1.6 (2.1)	(4.3) 1.4/0.1 (0.9)
300	3.5 (9.4)	(5.9) 8.0/10.2 (14.3)	(17.1) 6.4/2.2 (3.5)	(6.4) 2.2/0.4 (1.3)
500	5.8 (15.7)	(10.1) 13.2/16.7 (23.9)	(28.4) 10.5/3.6 (6.9)	(10.8) 4.0/0.7 (2.2)

Table S3. Experimental NOEs of Hexa-4 and Hexa-8 in solution for selected Inter-glycosidic proton-proton correlation. The corresponding simulated NOEs are reported in brackets (red color), and are generated using NOEPROM, with an isotropic model of motion (Tc = 700 ps). The Hexa-4 and Hexa-8 structure and conformations are obtained from MD simulation, adjusting each ϕ_i/ψ_i glycosidic dihedral angle pair with the average values calculated as reported in materials and methods.

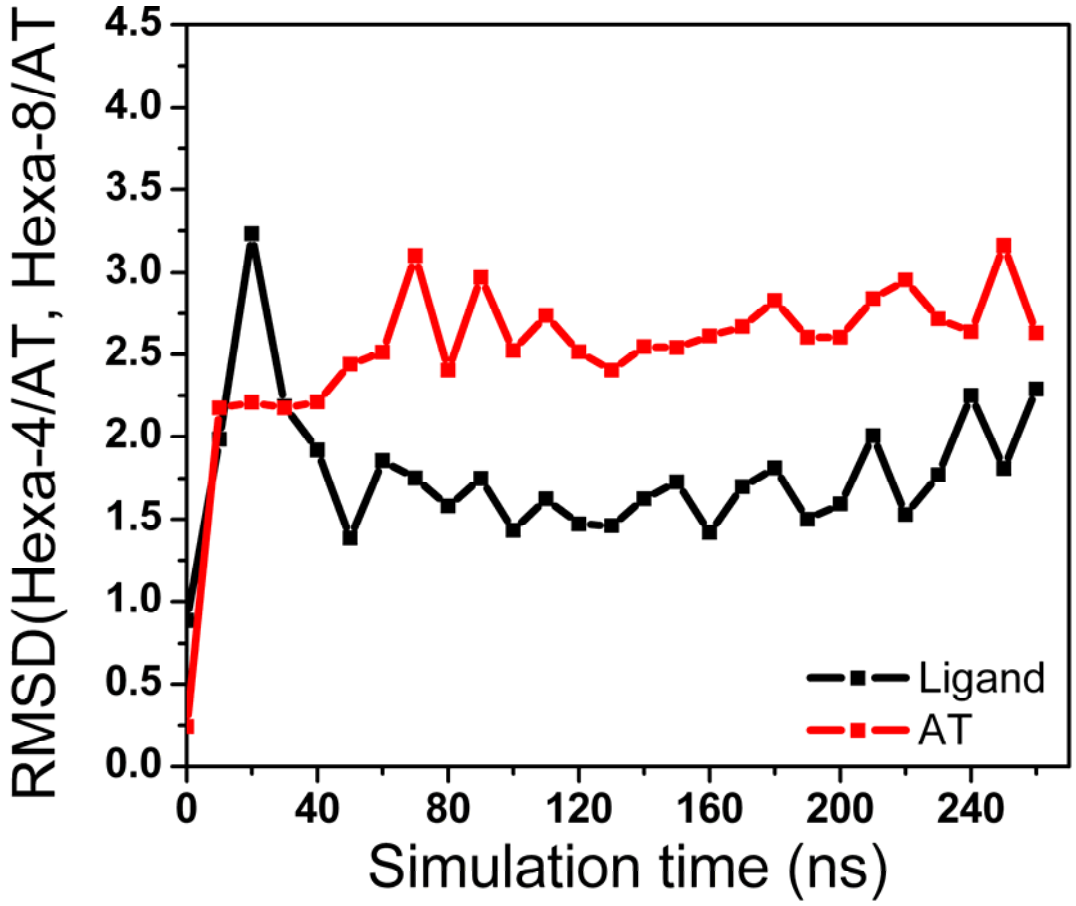

Figure S1 The black line show the RMSD distance calculated between the two ligands (C1, C2, C3, C4, C5, O5 for each residue) after superposition of the A-helix (Cα backbone) in glycan-AT structures, for simulation time 0 to 260 ns. The red line correspond to the RMDS distance between AT of the two complexes calculated on the Cα backbone. Plotting the RMSD between Hexa-4 and Hexa-8 at different simulation time, it is possible to draw a time scale in which the ligand relative position in AT RBS change, giving an estimation of the MD relaxation time. All the distances are expressed in Å

	H1-H2 Hexa-4/AT time = 209ns			H1-H2 Hexa-4/AT time = 227ns			H1-H2 Hexa-4/AT time = 240ns		
Tmix ms	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**
200	15.4 (11.5)	12.3 (17.7)	9.4 (14.6)	15.4 (10.9)	12.3 (10.6)	9.4 (10.0)	15.4 (6.1)	12.3 (10.1)	9.4 (5.7)
300	22.4 (17.3)	17.2 (25.8)	21.3 (14.1)	22.4 (16.1)	17.2 (15.6)	21.3 (14.5)	22.4 (8.7)	17.2 (14.5)	21.3 (8.1)
500	38.0 (30.3)	34.9 (42.1)	33.1 (24.3)	38.0 (27.1)	34.9 (26.5)	33.1 (23.4)	38.0 (14.1)	34.9 (23.4)	33.1 (12.8)
R	**0.05**	**0.09**	**0.09**	**0.08**	**0.05**	**0.09**	**0.39**	**0.09**	**0.38**

	H1-H2 Hexa-4/AT time = 257ns			H1-H2 Hexa-4/AT time = 270ns		
Tmix ms	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**
200	15.4 (11.6)	12.3 (9.8)	9.4 (6.7)	15.4 (13.2)	12.3 (10.3)	9.4 (6.3)
300	22.4 (16.1)	17.2 (14.3)	21.3 (9.6)	22.4 (19.1)	17.2 (15.1)	21.3 (9.4)
500	38.0 (25.1)	34.9 (23.8)	33.1 (15.9)	38.0 (31.2)	34.9 (25.2)	33.1 (16.0)
R	**0.10**	**0.08**	**0.28**	**0.03**	**0.06**	**0.28**

	H1-H2 Hexa-8/AT time = 214ns			H1-H2 Hexa-4/AT time = 235ns			H1-H2 Hexa-4/AT time = 243ns		
Tmix ms	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**
200	9.2 (10.0)	8.2 (9.0)	8.5 (9.3)	9.2 (5.7)	8.2 (6.8)	8.5 (6.1)	9.2 (7.2)	8.2 (8.1)	8.5 (8.1)
300	15.7 (13.2)	12.0 (12.6)	13.1 (12.7)	15.7 (7.9)	12.0 (9.6)	13.1 (8.8)	15.7 (9.9)	12.0 (11.5)	13.1 (11.5)
500	31.5 (18.6)	22.8 (19.8)	25.0 (18.6)	31.5 (12.5)	22.8 (15.4)	25.0 (14.1)	31.5 (15.0)	22.8 (18.4)	25.0 (17.9)
R	**0.13**	**0.01**	**0.05**	**0.33**	**0.09**	**0.16**	**0.23**	**0.03**	**0.06**

	H1-H2 Hexa-8/AT time = 258ns		
Tmix ms	**GlcNS6S**	**GlcNS3S6S**	**GlcNS6S''**
200	9.2 (11.7)	8.2 (6.6)	8.5 (6.8)
300	15.7 (17.0)	12.0 (9.5)	13.1 (10.1)
500	31.5 (28.5)	22.8 (15.9)	25.0 (17.6)
R	**0.13**	**0.01**	**0.05**

Table S4 Experimental H1-H2 intra-residue Tr-NOEs of the glucosamines: GlcNS6S, GlcNS3S6S, and GlcNS6S belonging to Hexa-4 and Hexa-8 in bound state with AT. The corresponding simulated tr-NOEs using the selected structures of Hexa-4/AT (209, 227, 240, 257, 270 ns) and Hexa-8/AT (214, 235, 243, 258 ns) are reported in brackets (see material and methods). The agreement between each experimental and simulated proton-proton Tr-NOE pairs is expressed by the R factor calculated considering three different mixing times.

	Hexa-4/AT time = 209ns (simulated tr-NOEs are in brackets), Rave = 0.14						
Tmix	**GlcNS6S-GlcA**	**GlcNS3S6S-IdoA(2S)**		**IdoA(2S)-GlcNS6S''**		**GlcNS6S''-GlcA''**	
(ms)	H1-H4	H1-H4	H1-H3	H1-H4	H1-H6	H1-H4	H1-H3
200	14.9 (6.5)	15 (4.5)	13.9 (13.0)	9.8 (7.5)	10.4 (10.6)	16.2 (23.5)	5.7 (3.7)
300	22 (10.4)	25.5 (7.3)	23.4 (19.2)	13.3 (11.5)	15.1 (15.8)	25.1 (34.2)	10.1 (6.4)
500	41.5 (20.3)	34.9 (15.0)	32.3 (32.3)	24.1 (20.5)	29.8 (26.8)	43.3 (56.0)	24.4 (13.9)
R	**0.27**	**0.40**	**0.01**	**0.03**	**0.01**	**0.11**	**0.18**
	Hexa-4/AT time = 227ns, Rave = 0.10						
200	14.9 (10.5)	15 (5.3)	13.9 (17.3)	9.8 (10.0)	10.4 (9.2)	16.2 (20.3)	5.7 (5.1)
300	22 (15.5)	25.5 (8.6)	23.4 (25.1)	13.3 (14.5)	15.1 (13.5)	25.1 (28.8)	10.1 (8.2)
500	41.5 (26.2)	34.9 (16.9)	32.3 (41.0)	24.1 (23.6)	29.8 (22.0)	43.3 (44.7)	24.4 (16.0)
R	**0.12**	**0.34**	**0.05**	**0.00**	**0.05**	**0.01**	**0.10**
	Hexa-4/AT time = 240ns, Rave = 0.22						
200	14.9 (7.2)	15 (5.4)	13.9 (7.5)	9.8 (12.7)	10.4 (7.5)	16.2 (13.5)	5.7 (3.5)
300	22 (10.5)	25.5 (8.1)	23.4 (11.0)	13.3 (16.6)	15.1 (10.3)	25.1 (18.6)	10.1 (5.6)
500	41.5 (17.5)	34.9 (14.3)	32.3 (18.8)	24.1 (22.7)	29.8 (15.2)	43.3 (28.3)	24.4 (10.9)
R	**0.32**	**0.39**	**0.21**	**0.03**	**0.20**	**0.10**	**0.28**
	Hexa-4/AT time = 257ns, Rave = 0.18						
200	14.9 (8.6)	15 (7.1)	13.9 (13.3)	9.8 (6.8)	10.4 (10.1)	16.2 (12.2)	5.7 (2.5)
300	22 (12.7)	25.5 (10.4)	23.4 (11.4)	13.3 (9.6)	15.1 (14.1)	25.1 (17.9)	10.1 (4.2)
500	41.5 (21.9)	34.9 (18.9)	32.3 (20.4)	24.1 (15.4)	29.8 (21.7)	43.3 (29.8)	24.4 (9.1)
R	**0.21**	**0.26**	**0.16**	**0.11**	**0.05**	**0.09**	**0.38**
	Hexa-4/AT time = 270ns, Rave = 0.18						
200	14.9 (6.1)	15 (6.6)	13.9 (15.9)	9.8 (5.3)	10.4 (11.5)	16.2 (13.3)	5.7 (2.9)
300	22 (9.2)	25.5 (10.2)	23.4 (22.6)	13.3 (7.9)	15.1 (16.3)	25.1 (19.4)	10.1 (5.0)
500	41.5 (16.0)	34.9 (18.7)	32.3 (36.1)	24.1 (13.6)	29.8 (25.3)	43.3 (32.3)	24.4 (10.6)
R	**0.37**	**0.27**	**0.01**	**0.19**	**0.02**	**0.06**	**0.31**

Table S5 (a)

	Hexa-8/AT time = 214ns (simulated tr-NOEs are in brackets), Rave = 0.24						
Tmix	**GlcNS6S-GlcA**	**GlcNS3S6S-IdoA(2S)**		**IdoA(2S)-GlcNS6S''**		**GlcNS6S''-GlcA''**	
(ms)	H1-H4	H1-H4	H1-H3	H1-H4	H1-H6	H1-H4	H1-H3
200	5.9 (5.9)	8.6 (6.4)	7.7 (4.4)	9.8 (6.2)	4.8 (7.0)	6.1 (2.9)	1.3 (3.0)
300	9.8 (8.1)	17.3 (7.4)	14.1 (10.5)	15.3 (8.1)	6.6 (8.7)	11.0 (4.4)	2.1 (4.4)
500	21.1 (12.5)	35.7 (12.1)	28.9 (17.0)	32 (11.1)	13.5 (10.9)	24.7 (7.8)	7.7 (7.6)
R	**0.13**	**0.40**	**0.15**	**0.37**	**0.07**	**0.44**	**0.12**
	Hexa-8/AT time = 235ns, Rave = 0.24						
200	5.9 (7.0)	8.6 (6.4)	7.7 (10.4)	9.8 (7.8)	4.8 (9.7)	6.1 (4.3)	1.3 (3.1)
300	9.8 (9.8)	17.3 (9.3)	14.1 (14.7)	15.3 (10.4)	6.6 (12.6)	11.0 (6.4)	2.1 (4.7)
500	21.1 (15.2)	35.7 (15.7)	28.9 (23.6)	32 (14.9)	13.5 (17.1)	24.7 (11.2)	7.7 (8.6)
R	**0.13**	**0.40**	**0.15**	**0.37**	**0.07**	**0.44**	**0.12**
	Hexa-8/AT time = 243ns, Rave = 0.27						
200	5.9 (13.0)	8.6 (4.7)	7.7 (11.0)	9.8 (6.5)	4.8 (10.8)	6.1 (2.7)	1.3 (3.0)
300	9.8 (17.1)	17.3 (7.0)	14.1 (15.6)	15.3 (8.8)	6.6 (13.3)	11.0 (4.2)	2.1 (4.5)
500	21.1 (23.4)	35.7 (12.3)	28.9 (25.0)	32 (12.7)	13.5 (16.7)	24.7 (7.9)	7.7 (8.0)
R	**0.19**	**0.41**	**0.03**	**0.31**	**0.36**	**0.44**	**0.13**
	Hexa-8/AT time = 258ns, Rave = 0.22						
200	5.9 (11.3)	8.6 (5.3)	7.7 (15.9)	9.8 (7.3)	4.8 (8.1)	6.1 (2.9)	1.3 (2.7)
300	9.8 (16.0)	17.3 (8.1)	14.1 (22.4)	15.3 (10.5)	6.6 (11.4)	11.0 (4.6)	2.1 (4.3)
500	21.1 (25.5)	35.7 (14.9)	28.9 (35.1)	32 (17.1)	13.5 (18.1)	24.7 (9.2)	7.7 (8.5)
R	**0.15**	**0.32**	**0.16**	**0.19**	**0.22**	**0.38**	**0.11**

Table S5 (b)

Table S5 (a) and (b) Experimental selected inter-glycosdic Tr-NOEs signals measured for Hexa-4 and Hexa-8 in their interaction with AT. The corresponding simulated Tr-NOEs using the selected structures of Hexa-4/AT (209, 227, 240, 257, 270 ns) and Hexa-8/AT (214, 235, 243, 258 ns) are reported in brackets (see material and methods). The agreement between each experimental and simulated proton-proton Tr-NOE pairs is expressed by the R factor calculated at three different mixing times.

	Residues					
	A-helix type CA	**GlcNS6S**	**GlcA**	**GlcNS3S6S**	**IdoA2S/IdoA**	**GlcNS6S''**
1AZX	0	0	0	0	0	0
Hexa-4/AT, 209ns	0.53	1.63	1.92	1.62	0.99	0.44
Hexa-4/AT, 227ns	0.48	1.85	2.2	2.13	1.3	0.47
Hexa-4/AT, 240ns	0.41	1.67	2.13	1.88	1.18	0.73
Hexa-4/AT, 257ns	0.39	1.77	1.92	1.79	1.08	0.48
Hexa-4/AT, 270ns	0.47	2.28	2.58	2.46	1.61	1.18
RMSD ave (Hexa-4,1AZX)	**0.46**	**1.84**	**2.15**	**1.98**	**1.23**	**0.66**
Hexa-8/AT, 214ns	0.49	1.6	1.23	1.23	1.32	0.98
Hexa-8/AT, 235ns	0.74	1.75	1.44	0.8	1.12	1.03
Hexa-8/AT, 243ns	0.72	1.75	1.47	1.21	1.74	1.77
Hexa-8/AT, 258ns	0.71	2.13	1.07	1.07	1.42	1.69
RMSD ave (Hexa-8,1AZX)	**0.67**	**1.81**	**1.30**	**1.08**	**1.40**	**1.37**

Table S6. RMSD (Root Mean Square Distances) calculated between the common sequence of the active pentasaccharide co-crystallized with AT in PDB ID 1AZX, and the tested hexasaccharides in bound state with AT, which structures were validated by the tr-NOEs, and correspond to geometries at instant time: 209, 227, 240, 257, 270 ns for Hexa-4/AT and 214, 235, 243, 258 ns for Hexa-8/AT. In red average RMSD values are calculated for each glycan residue of Hexa-4/AT, and Hexa-8/AT complex respectively.

Interacting groups	Distances (Å)					
	Hexa-4/AT	AGA*IA/AT [1]	Hexa-8/AT	AGA*IA/AT [2]	AGA*IA/AT [3]	AGA*IA/AT [4]
GlcNS6S(**6S**)---R129(H_2N-**C**-NH_2^+)	6.4	4.3	8.3	4.9	5.2	5.0
GlcNS6S(**6S**)---K125(H_3**N**$^+$)	4.2	3.8	5.7	4.4	4.8	6.2
GlcA(**C**OO)---K125	7.2	3.5	4.4	4.1	4.1	3.7
GlcNS3S6S(**NS**)---K114	4.3	-	4.6	4.3	4.1	4.5
GlcNS3S6S(**3S**)---K114	4.4	-	4.2	4.1	4.1	4.4
GlcNS3S6S(**NS**)---R13	4.7	-	6.7	5.4	5.6	8.2
IdoA2S(**C**OO)---R47	4.4	4.5	4.1	5.6	4.5	5.1
IdoA2S(**C**OO)---R46	4.2	-	4.0	5.7	-	8.1
IdoA2S(**C**OO)---K114	3.5	3.0	3.6	3.6	3.7	3.4
IdoA2S(**S**O3$^-$)---R13	4.4	-	/	8.7	4.1	8.6
GlcNS6S''(**NS**)---R47	4.0	3.9	4.1	4.2	4.7	4.9
GlcNS6S''(**NS**)---R46	5.1	4.2	5.5	5.9	-	5.9
GlcNS6S''(**6S**)---K114	7.8	4.7	7.6	5.2	5.2	4.9
GlcNS6S''(**6S**)---R13	4.7	-	4.6	8.4	5.3	5.4

Tab. S7 Selected distances between interacting groups in Hexa-4/AT and Hexa-8/AT complexes at time 227 and 258 ns validated by tr-NOEs signals (see tr-NOEs/NOEs paragraph) are reported in 2nd and 4th column. The atoms used for distance definitions are underlined in bold face in the 1st column. For comparison the corresponding distances measured in AGA*IA/AT complex in solution by NMR techniques (Hricovini et al. 2001) [1], by x-ray diffraction '1AZX' [2] (chain I Jin et al. 1997), '3kcg' [3] (Johnson, D.J. et al. 2010), and '2gd4' (Johnson, D.J. et al. 2006) are reported in 3^{td} , 5th , 6th and 7th column respectively. Distances greater than 6 Å and smaller than 4 Å are underlined in blue and red respectively. The symbol '-' indicate distances not reported. In 3kcg structure R46 residue were not accurately resolved.