Supporting Information

Potent inhibitors targeting Cyclin-dependent kinase 9 discovered via high-throughput virtual screening and absolute binding free energy calculations

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Table S1. Setup of MD Simulations.

Simulation type	/	/
Conventional	Ensemble	NPT
	Simulation time	100 ns
	Time step	2 fs
	total atomic number	~75500
Alchemical	λ-windows	Restraint: 0.0, 0.01, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2, 0.35, 0.5, 0.75, 1.0 Ligand in complex: 0.0, 0.01, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0
	Ensemble	NPT
	Simulation time	1 ns * 32
	Time step	1 fs
	Restraint type	Boresch-restraint

Table S2. Top 200 docking molecules from Zinc15 database. The red-highlighted Rank ID indicates the existence of hydrogen bonding in the docking conformation with the hinge region of CDK9.

Rank	ZINC ID	Vina docking score (kcal/mol)
001	ZINC000095869672	-14.642
002	ZINC000012377403	-14.608
003	ZINC000218224996	-13.017
004	ZINC000020477868	-12.726
005	ZINC000253534673	-12.644
006	ZINC000020477880	-12.414
007	ZINC000006763566	-12.296
008	ZINC000095916638	-12.285
009	ZINC000100643778	-12.251
010	ZINC000015218322	-12.146
011	ZINC000005853248	-12.114
012	ZINC000085776324	-11.864
013	ZINC000095916633	-11.844
014	ZINC000057497264	-11.842
015	ZINC000085776320	-11.834
016	ZINC000217379534	-11.834
017	ZINC000253629646	-11.829
018	ZINC000226860140	-11.767
019	ZINC000095916637	-11.766
020	ZINC000409402836	-11.751
021	ZINC000409352511	-11.742
022	ZINC000085776323	-11.726
023	ZINC000004027396	-11.703
024	ZINC000057497261	-11.681
025	ZINC000408721756	-11.663
026	ZINC000095916634	-11.625
027	ZINC000094156261	-11.619
028	ZINC000033784438	-11.615
029	ZINC000022001476	-11.614
030	ZINC000004416776	-11.610
031	ZINC000009357335	-11.596
032	ZINC000095916626	-11.592
033	ZINC000104014035	-11.549
034	ZINC000019332604	-11.536
035	ZINC000100511559	-11.525
036	ZINC000138162479	-11.524
037	ZINC000100162583	-11.519
038	ZINC000104301950	-11.518
039	ZINC000004706349	-11.507

040	ZINC000044138285	-11.507
041	ZINC000085776327	-11.506
042	ZINC000004611090	-11.489
043	ZINC000005753111	-11.485
044	ZINC000020673951	-11.482
045	ZINC000006417103	-11.477
046	ZINC000409136801	-11.477
047	ZINC000020477910	-11.473
048	ZINC000020673948	-11.468
049	ZINC000013549435	-11.460
050	ZINC000004006341	-11.458
051	ZINC000100643779	-11.456
052	ZINC000409359730	-11.435
053	ZINC000588225119	-11.430
054	ZINC000016952802	-11.430
055	ZINC000001027893	-11.419
056	ZINC000094156260	-11.415
057	ZINC000409348728	-11.407
058	ZINC000005554236	-11.385
059	ZINC000409363068	-11.370
060	ZINC000225683042	-11.366
061	ZINC001554504429	-11.356
062	ZINC000096269095	-11.344
063	ZINC000409358646	-11.344
064	ZINC000409353839	-11.338
065	ZINC000409354366	-11.337
066	ZINC000409351662	-11.334
067	ZINC000005853513	-11.331
068	ZINC000001636607	-11.331
069	ZINC000008653206	-11.329
070	ZINC000008653207	-11.329
071	ZINC000409357147	-11.327
072	ZINC000003843479	-11.325
073	ZINC000020673341	-11.320
074	ZINC000020673338	-11.320
075	ZINC000012551619	-11.319
076	ZINC000032506883	-11.313
077	ZINC000004721192	-11.310
078	ZINC000061999358	-11.301
079	ZINC000104278237	-11.299
080	ZINC000100133448	-11.292
081	ZINC000408902610	-11.291
082	ZINC000015133646	-11.290

083	ZINC000005376013	-11.285
084	ZINC000014921305	-11.283
085	ZINC000021981448	-11.271
086	ZINC000001705708	-11.271
087	ZINC000104207302	-11.268
088	ZINC000104207309	-11.268
089	ZINC000003057249	-11.261
090	ZINC000059514393	-11.260
091	ZINC000409357936	-11.256
092	ZINC000409356947	-11.255
093	ZINC000022608226	-11.251
094	ZINC000006651549	-11.245
095	ZINC000068568685	-11.241
096	ZINC000036589708	-11.237
097	ZINC000017311037	-11.230
098	ZINC000104207323	-11.229
099	ZINC000104207316	-11.229
100	ZINC000004166422	-11.227
101	ZINC000095869672	-10.935
102	ZINC000012377403	-10.922
103	ZINC000218224996	-10.909
104	ZINC000020477868	-10.896
105	ZINC000253534673	-10.883
106	ZINC000020477880	-10.869
107	ZINC000006763566	-10.856
108	ZINC000095916638	-10.843
109	ZINC000100643778	-10.830
110	ZINC000015218322	-10.817
111	ZINC000005853248	-10.804
112	ZINC000085776324	-10.791
113	ZINC000095916633	-10.777
114	ZINC000057497264	-10.764
115	ZINC000085776320	-10.751
116	ZINC000217379534	-10.738
117	ZINC000253629646	-10.725
118	ZINC000118925436	-11.161
119	ZINC000032508293	-11.160
120	ZINC000045789132	-11.154
121	ZINC000100006925	-11.154
122	ZINC000012559276	-11.154
123	ZINC000408718240	-11.153
124	ZINC000032508299	-11.148
125	ZINC000016990249	-11.147

126	ZINC000016033727	-11.142
127	ZINC000005024038	-11.141
128	ZINC000100162630	-11.124
129	ZINC000409353161	-11.123
130	ZINC000253618165	-11.123
131	ZINC000019237438	-11.122
132	ZINC000100006436	-11.120
133	ZINC000409346250	-11.118
134	ZINC000007066199	-11.114
135	ZINC000408731495	-11.113
136	ZINC000001087348	-11.112
137	ZINC000005725191	-11.109
138	ZINC000004742460	-11.109
139	ZINC000104301937	-11.106
140	ZINC000001697065	-11.105
141	ZINC000409351421	-11.102
142	ZINC000101232459	-11.098
143	ZINC000102629257	-11.096
144	ZINC000006415633	-11.093
145	ZINC000004705939	-11.092
146	ZINC000409340652	-11.091
147	ZINC000006476384	-11.090
148	ZINC000059514550	-11.084
149	ZINC000094156263	-11.083
150	ZINC000001050326	-11.082
151	ZINC000247646417	-11.082
152	ZINC000408722162	-11.081
153	ZINC000034074220	-11.079
154	ZINC000118929793	-11.071
155	ZINC000032505477	-11.064
156	ZINC000032506879	-11.061
157	ZINC000409356050	-11.060
158	ZINC000408730299	-11.057
159	ZINC000004690292	-11.056
160	ZINC000101654670	-11.053
161	ZINC000096306652	-11.052
162	ZINC000032507412	-11.052
163	ZINC000096161427	-11.051
164	ZINC000118917616	-11.049
165	ZINC000096306678	-11.048
166	ZINC000409359534	-11.046
167	ZINC000409354090	-11.046
168	ZINC000101139397	-11.043

169	ZINC000079096590	-11.041
170	ZINC000032508224	-11.038
171	ZINC000026507889	-11.038
172	ZINC000072351420	-11.034
173	ZINC000101318196	-11.032
174	ZINC000009287909	-11.031
175	ZINC000004933922	-11.029
176	ZINC000017054917	-11.024
177	ZINC000248332081	-11.018
178	ZINC000068601390	-11.016
179	ZINC000032508222	-11.016
180	ZINC000409353566	-11.012
181	ZINC000252515700	-11.009
182	ZINC000118926369	-11.009
183	ZINC000104002935	-11.007
184	ZINC000003080766	-11.005
185	ZINC000105492283	-11.003
186	ZINC000118929318	-11.000
187	ZINC000012558757	-11.000
188	ZINC000409288042	-10.998
189	ZINC000037263681	-10.997
190	ZINC000012558795	-10.996
191	ZINC000012284765	-10.996
192	ZINC000253617332	-10.994
193	ZINC000004897164	-10.991
194	ZINC000017032133	-10.990
195	ZINC000041589287	-10.988
196	ZINC000118915169	-10.987
197	ZINC000409353866	-10.983
198	ZINC000408720985	-10.983
199	ZINC000253476472	-10.981
200	ZINC000118929733	-10.978

Table S3. Hydrogen bonding analysis of molecules obtained from virtual screening with CDK9. The hydrogen bonding interaction was judged by the distance between hydrogen bond donor and acceptor within 3.5 Å and the hydrogen bonding angle greater than 135°.

Ligand	Acceptor	Donor	Fraction	Distance(Å)	Angle(°)
006	006@N6	CYS_106@N	0.81	3.21	158.28
	ASP_109@OD2	006@N4	0.56	3.02	147.13
	ASP_109@OD1	006@N4	0.53	3.02	146.66
	ASP_109@OD2	006@N3	0.45	2.90	153.01
	006@N7	CYS_106@N	0.44	3.29	151.77
	ASP_109@OD1	006@N3	0.43	2.90	153.14
008	008@O2	CYS_106@N	0.87	3.08	159.08
	008@F1	ASP_167@N	0.05	3.14	141.21
149	149@O3	CYS_106@N	0.88	3.03	158.61
	149@O4	ASP_167@N	0.76	3.08	157.04
	149@N1	ASP_109@N	0.66	3.26	154.52
	149@O5	ASN_116@ND2	0.43	3.02	157.51
052	CYS_106@O	052@N3	0.97	2.80	160.62
	052@O1	CYS_106@N	0.89	3.06	157.27
	GLY_31@O	052@N4	0.11	2.94	146.54
124	124@O2	GLN_27@N	0.40	2.97	160.64
	124@O2	GLN_27@NE2	0.02	2.97	155.54
	124@O3	CYS_106@N	0.01	2.99	157.67

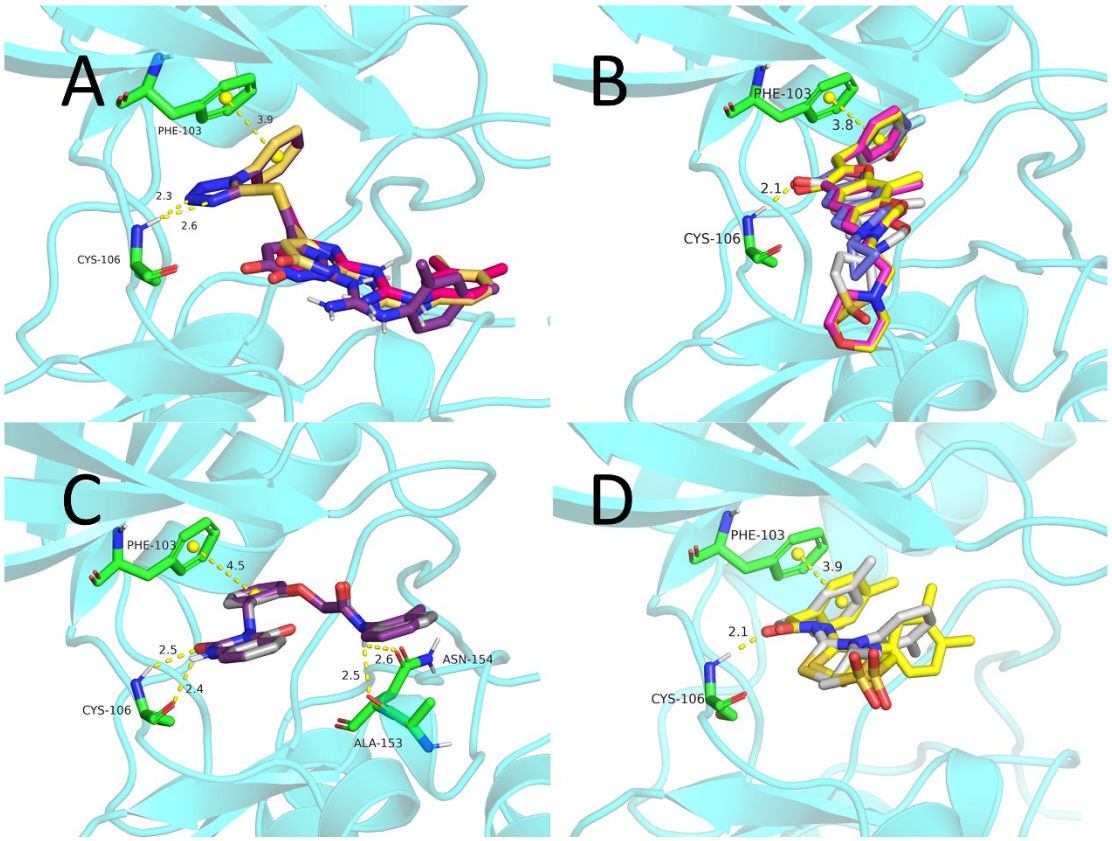

Fig. S1 Binding modes of the four types of molecular structures at the ATP-binding site, the backbone structures of the same class of molecules basically overlap. hydrogen bonding and π – π stacking are indicated by the yellow dashed line.

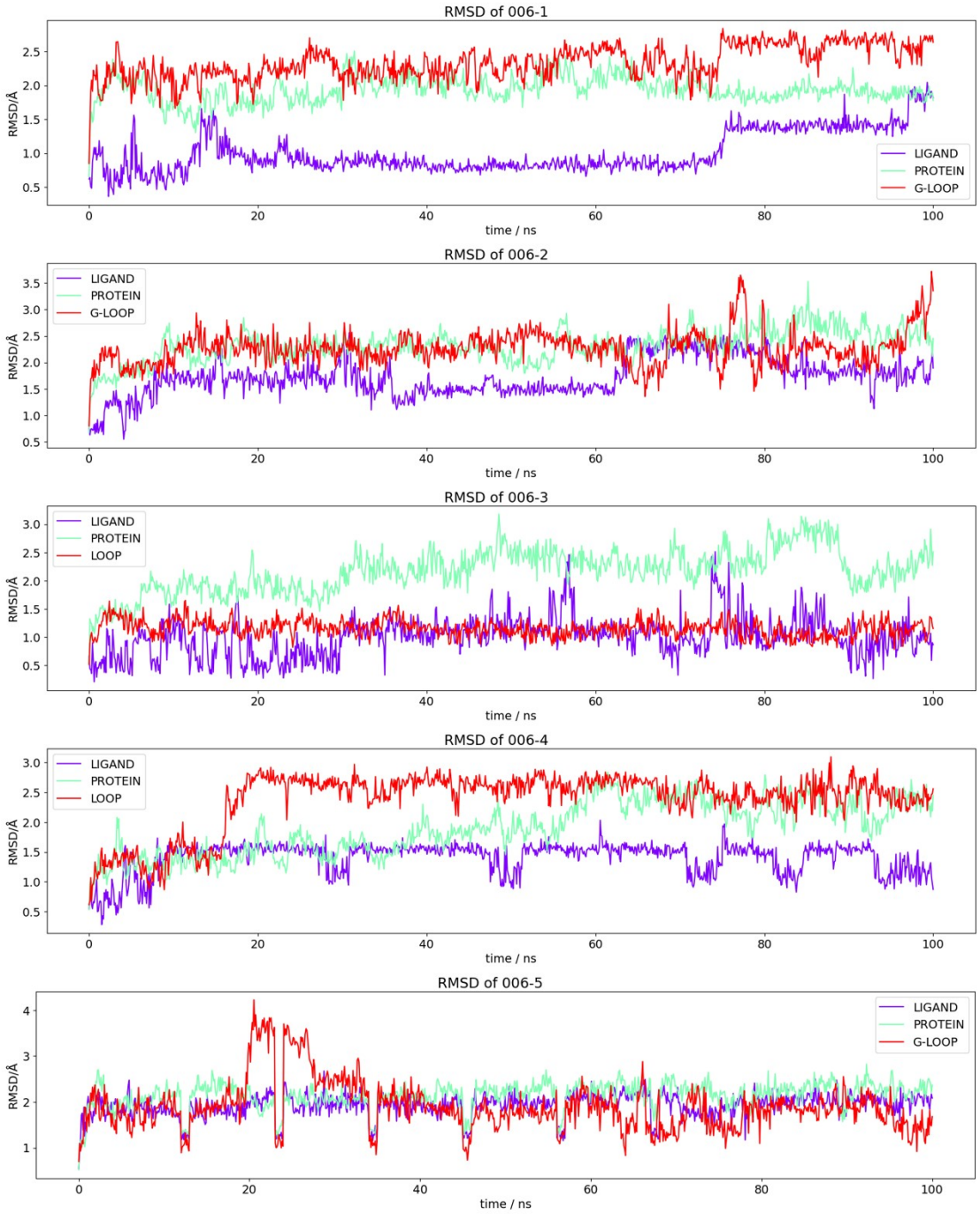

Fig. S2 RMSD of molecules in complex with CDK9, ligands, protein backbone atoms and G-loop non-hydrogen heavy atoms indicated in purple, green and red, respectively.

Table S4. Hydrogen bonding analysis of molecules obtained from structure modification with CDK9. The hydrogen bonding interaction was judged by the distance between hydrogen bond donor and acceptor within 3.5 Å and the hydrogen bonding angle greater than 135°.

Ligand	Acceptor	Donor	Fraction	Distance(Å)	Angle(°)
006	006@N6	CYS_106@N	0.81	3.21	158.28
	ASP_109@OD2	006@N4	0.56	3.02	147.13
	ASP_109@OD1	006@N4	0.53	3.02	146.66
	ASP_109@OD2	006@N3	0.45	2.90	153.01
	006@N7	CYS_106@N	0.44	3.29	151.77
	ASP_109@OD1	006@N3	0.43	2.90	153.14
006-1	006-1@N6	CYS_106@N	0.65	3.14	158.91
	006-1@N7	CYS_106@N	0.33	3.30	150.54
	006-1@N8	CYS_106@N	0.12	3.26	152.69
	ALA_153@O	006-1@N5	0.09	2.99	154.91
	ALA_153@O	006-1@N5	0.05	2.95	155.30
006-2	ALA_153@O	006-2@N2	0.33	3.00	155.80
	ALA_153@O	006-2@N2	0.29	2.99	155.66
	ASP_109@OD2	006-2@N2	0.29	2.90	159.45
	ASP_109@OD2	006-2@N2	0.27	2.90	159.14
	006-2@N7	CYS_106@N	0.27	3.31	153.65
	006-2@N8	CYS_106@N	0.25	3.27	154.36
	006-2@N6	CYS_106@N	0.21	3.24	160.53
006-3	006-3@N6	CYS_106@N	0.63	3.21	155.33
	ASP_109@OD1	006-3@N3	0.56	2.78	162.55
	ASP_109@OD2	006-3@N3	0.45	2.79	162.38
	006-3@N7	CYS_106@N	0.36	3.28	153.74
	ASP_109@OD1	006-3@N1	0.30	2.95	152.13
	ASP_109@OD2	006-3@N1	0.27	2.94	152.84
006-4	006-4@N6	CYS_106@N	0.87	3.17	158.13
	ASP_109@OD1	006-4@N1	0.61	2.94	152.79
	ASP_109@OD2	006-4@N1	0.58	2.97	152.19
	ASP_109@OD2	006-4@N3	0.53	2.82	159.14
	006-4@N7	CYS_106@N	0.49	3.30	154.66
	ASP_109@OD1	006-4@N3	0.48	2.81	158.90
006-5	006-5@N6	CYS_106@N	0.80	3.21	159.93
	ILE_25@O	006-5@N3	0.78	2.91	155.99
	006-5@N7	CYS_106@N	0.23	3.36	151.12
	006-5@O1	ASP_109@N	0.03	3.22	142.81

Table S5. The results of ABFE calculations for the structures in **Fig.6**. Data were analyzed by the MBAR method , using three independent calculations to obtain the mean with standard deviation, and all data are in kcal/mol.

ligand	$\Delta G^{\text{decouple}}_{\text{solution}}$	$-\Delta G^{\text{decouple}}_{\text{site}}$	$\Delta G^{\text{restraint}}_{\text{solution}} - k_B T \ln$	$\Delta G^{\text{restraint}}_{\text{site}}$	ΔG°_{b}	ΔG°_{b}(average)
006	39.2	-58.5	11.421	-3.2	-11.0	-10.7 ± 0.7
	39.4	-58.9		-3.1	-11.3	
	39.2	-57.5		-3.1	-9.9	
006-1	35.9	-55.2	12.021	-3.9	-11.1	-10.5 ± 0.7
	36.1	-54.0		-4.0	-9.8	
	35.9	-54.8		-3.8	-10.7	
006-2	36.2	-54.5	10.962	-3.4	-10.7	-10.6 ± 3.4
	34.6	-56.1		-3.5	-14.0	
	35.7	-50.5		-3.4	-7.2	
006-3	21.3	-44.8	11.439	-3.2	-15.3	-14.4 ± 0.7
	21.7	-44.0		-3.2	-14.1	
	21.6	-43.8		-3.1	-13.9	
006-4	25.4	-45.7	11.784	-4.7	-13.2	-12.9 ± 0.4
	26.2	-46.6		-4.4	-12.9	
	25.3	-45.2		-4.4	-12.5	
006-5	22.2	-43.6	12.115	-4.5	-13.7	-12.7 ± 1.1
	22.7	-42.6		-4.5	-12.2	
	22.5	-41.7		-4.5	-11.3	

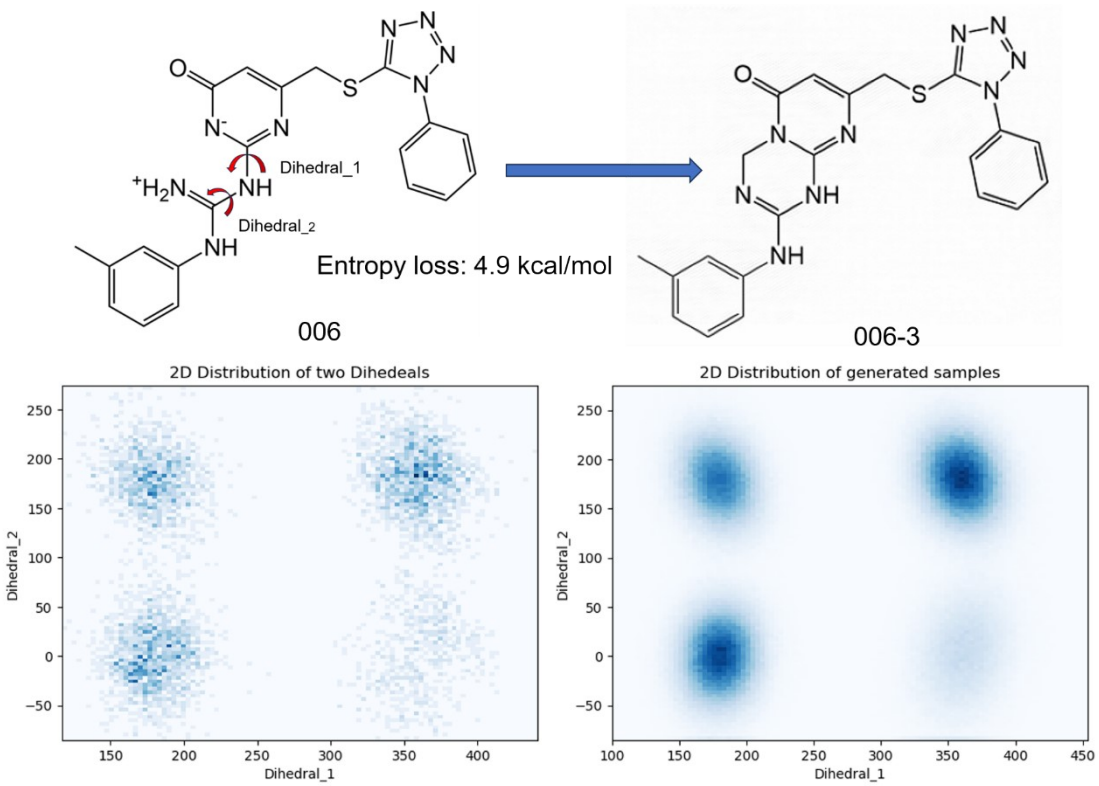

Fig. S3 Entropy loss estimation through conformational flexibility. To quantify the impact of entropy change, the distribution of two dihedral angles (highlighted with red arrows) of the 006 molecule in solution were sampled, The angles were projected onto a two-dimensional plane and underwent periodic transformations, revealing four concentrated regions. After fitting the data using a Gaussian Mixture Model (GMM) with four components from the scikit-learn library, the model-generated data distribution closely matched the original distribution, indicating a good fit. All sampled points were input into the GMM model, and the probabilities were normalized. Calculations using the formula for entropy ($S = -kT\sum_i p_i ln(p_i)$) revealed that fixing these two dihedral angles upon intramolecular cyclization results in an entropy change of 4.9 kcal/mol at room temperature. Considering that the binding affinity of 006-3 has increased by 3.7 kcal/mol compared to 006, the reduction in the entropy of these two dihedral angles has indeed contributed significantly to the enhancement of binding free energy.