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Supplementary Information

1) Active site model and computational enzyme design

Distances, angles and dihedrals of the corresponding active sites were measured for all interacting inhibitor-residue atom pairs as shown in Table S1: Zn to $O\epsilon^1$ or $O\epsilon^2$ atoms for Glu_{cat} and Zn to $N\epsilon^2$ atoms for His₁₋₃; periodicity of 360° was set for all geometrical parameters except for χ_{AB} in His₁₋₃ where it was set to 11.25°, corresponding to discrete rotation of the imidazole ring around the C β -C γ bond. Interactions were modelled as pseudocovalent for His1-3-Zn and as non-bonded for Glucat-Zn, with higher k values for distances than for angles and dihedrals to penalize designs with distorted coordination geometries. Two parameter sets were used to sample the x0±xtol value range at regular intervals: the first with n=1 to sample 2n+1=5 discrete values for all 6 geometrical parameters; the second with the "rule-of-thumb" n=xtol/0.1 for d_{AB} and n=xtol/5 for remaining geometrical parameters (n<4, maximum 9 discrete values). The first set was used to screen and design NMR structures; the second set was used to screen and design X-ray structures since it allows increased conformational sampling of side chain orientations. The 2010 Dunbrack rotamer library was used, with $\chi'_{1,2} \pm 2\sigma$ side chain sampling for His1-3. The model substrate diAla was based on atomic coordinates of the highresolution structure of astacin with bound inhibitor molecule (PDB ID: 1QJI).¹ The Inhibitor was trimmed and the phosphate atom replaced by a carbon atom. This allowed for reconstruction of alanine side chains in extended conformation. Conformers were generated with the Open Babel software ²: 6 rotatable bonds identified, 7776 conformers tested and final 306 conformers produced, including the original extended conformer. The corresponding files and options used as input for Rosetta are described in Table S2. The resulting Rosetta cst file of the model active site is shown in Table S3.

The options used for both the matcher and enzyme design executables are given along active site definition in Table S3. The complete list of obtained designs is shown in Table S4. The final step of design did not evaluate repacking in the absence of diAla, as reference for REU calculation, sincezinc was modelled as part of the substrate (charge repulsion in the absence of zinc render the preorganization of His1-3 unfavourable). The parameters used to build the correlation matrix were: total_score (Scoretotal), fa_rep (repulsive LJ), hbond_sc (H-bond energy), all_cst (constraint, k), tot_pstat_pm (Packing), tot_nlsurfaceE_pm (surface energy), SR_1_total_score (Glu_{cat}), SR_2_total_score (His1), SR_3_total_score (His2), SR_4_total_score (His3), SR_5_total_score (diAlascore), SR_5_fa_rep (diAla repulsive LJ), SR_5_hbond_sc (diAla H-bond energy), SR_5_all_cst (diAla constraint, k), SR 5 interf E 1 2 (diAla interface energy), and SR 5 dsasa 1 2 (diAla solvent accessible surface area, SASA). Sequence length (L) was included as an additional parameter to account for the system size variability. This set does not contain scaffold-specific parameters, such as pose metric calculators. For enhanced discrimination of results, the parameters related with constraint penalty k, all_cst and SR5_all_cst, were represented in k logarithm scale. Results of PCA analysis are given in Figure S1.

2) List of reagents and equipment

Synthesis of peptides was done in an Initiator+ Alstra Automated Microwave Peptide Synthesizer (Biotage) or in a Liberty Microwave-Assisted Automated Peptide Synthesizer (CEM GmbH) using the supplier's protocols and optimized accordingly to the methods developed in our laboratory. Fmoc-amino acids were purchased from CEM, Novabiochem (now Merk GmBH) and Iris Biotech GmbH. Rink Amide 4-methylbenzhydrylamine (100-200 mesh, loading 0.59 mmol/g or 100-200 mesh, loading 0.36mmol/g) resin and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate were obtained from Novabiochem. Trifluoroacetic acid, anisole, thioanisole, 1,2-ethanedithiol, triisopropylsilane, 1hydroxybenzotriazole and piperidine were acquired from Merck. Acetonitrile, dimethylformamide, diethyl ether, dichloromethane, N-methyl-2-pyrrolidone, N,N-diisopropylethylamine, acetic anhydride and triethylamine were obtained from different commercial suppliers. All reagents used were the highest grade available. DiAla was purchased from POP-UP (Peptide Synthesis Facility at University of Porto, Portugal) with a purity of 92% and used without further purification. Preparative HPLC was made using linear gradient methods with mobile phase of solvent A (H₂O/TFA 99.9:0.1 v/v) and solvent B (ACN/H₂O/TFA 90:9.9:0.1 v/v), using a C18 Phenomenex Jupiter 250 mm x 21.2 mm, 15 μm 300 Å column. Analytical HPLC was made with the same methods adapted accordingly in either a Phenomenex C12 Jupiter Proteo, 250 mm x 4.6 mm, 4 µm, 300 Å or C18 Phenomenex Jupiter 250 mm x 4.6 mm, 15 µm, 300 Å and Discovery HS 250 mm x 4.6 mm, 5 µm. CD spectroscopy was performed in a Jasco J-815 CD spectropolarimeter (Integration time 1 or 2 seg, bandwidth 1 or 2 nm, 8 accumulations, scan speed 100 nm/min) with external temperature controller Jasco CDF-426S/15 or Applied Photophysics Chirascan[™] qCD spectrometer (Integration time 3 s, bandwidth 1 nm, 3 accumulations, scan speed 20 nm/min). UV-Vis spectroscopy was performed in a Cary 100 Bio spectrophotometer with a Peltier temperature controller or in 96-well plates at room temperature in a Tecan Infinite F200 microplate reader. NMR spectroscopy was performed in a Bruker Avance II+ 800 MHz or Bruker Avance II+ 500 MHz. Assays done at different pH values used compositions shown in Table S5.

3) Zinc binding, thermal stability and hydrolytic activity

For CD spectroscopy, the signal (θ_{obs}) was converted from ellipticity to mean residual weight ([θ]_{MRW}) in units of deg.dmol.cm² by the equation 1:

$$[\theta]_{MRW} = \frac{\theta_{obs}}{10lcm} \tag{1}$$

where *I* is the path-length of cell, 10 is the conversion factor from mol to dmol, *c* is the molar concentration of peptide (mol/L) and *m* is the number of peptide bonds in case of Sp1f2/RD01 (m= 30) and HP35/RD02 (m= 34).

For esterase activity determination using 4-nitrophenyl acetate, the rates of product formation k_{obs} were calculated by equation 2:

$$k_{obs}(M.s^{-1}) = \frac{A_{400}}{l\varepsilon} \times \frac{1}{s} = \frac{[4 \cdot nP]}{s}$$
 (2)

where A₄₀₀ was converted to [4-nP] using ϵ_{400} according to Table S5. Linearity considered when R² > 0.99 over recorded time. The ϵ_{400} values were determined under the same experimental conditions for

each pH (including 5% ACN) by measuring A₄₀₀ values of product 4-nitrophenol. Rates of product formation obtained for control assays were subtracted in the corresponding catalyst assays (free peptide and zinc complex). The [Zn]/[RD] used for each pH was chosen according to Figure S4. The first order rate constant V_{cat} was calculated accordingly for assays with different concentrations of catalyst by equation 3:

$$V_{cat}(s^{-1}) = (k_{obs,catalyst} - k_{obs,background}) \times \frac{1}{[catalyst]}$$
(3)

Second-order rate constant k_2 was calculated according to equation 4:

$$k_2 (M^{-1}s^{-1}) = \frac{V_{cat}}{[4-nPA]}$$
(4)

Single amino acid p-nitroanilides (X-pNA, where X= Ala, Gly, Glu, Arg, Met, Leu) were purchased from Sigma-Aldrich and 100 mmol/L stock solutions were prepared in corresponding solvents (acetone, acetonitrile or water). The formation of the product p-nitroaniline (pNA) was monitored by following A₄₀₅ increase under the experimental conditions described for esterase assays over a period of at least 2 days. The extinction coefficient of product aniline, ε_{405} =5329 M⁻¹cm⁻¹ from a nitroaniline solution. The ab112146 MMP Activity Assay Kit (fluorometric – green) was purchased from Abcam and assays were performed according to the provided protocol. Fluorescence signal was monitored in microplate format at room temperature (V_T= 100 µL) with an Ex/Em = 490/525 nm for variable time intervals up to a total duration of 2 days. A 1 mM diAla stock solution was prepared in D₂O 50mM NaCl (molecular weight 201.11 g/mol, 0.2 mg/mL) and its pH adjusted to 7.47 with additions of concentrated NaOH and HCl solutions. Assays were done by direct addition of both 25 µM peptide and 75 µM ZnCl₂ to diAla 1 mM solutions (V_T= 600 µL). ¹H NMR spectra were recorded immediately after the addition of at least 2 days.

4) Molecular Dynamics Simulations

Residue protonation state was attributed according to the respective *pKa* values; Lys and Arg residues were modelled in protonated charged state (+1), aspartate and glutamate residues in deprotonated state with a point charge of (-1), Zn-coordinating cysteines in deprotonated state (CYM) and non-coordinating histidines (astacin) in uncharged monoprotonated state at the N ϵ^2 atom. Molecules were placed in a cubic box with edges at least 12 Å from the solute and solvated with the explicit TIP3P water model ³ (~15900 molecules for astacin, ~5500 for Sp1f2 and RD01, ~4900 for HP35 and RD02). Chloride or sodium counter-ions were added first to neutralize the total charge of the system and then added in equal proportion to achieve a 50 mM NaCl concentration. Energy minimization was done in two steps to remove eventual atom clashes in crystallographic, NMR structures or Rosetta outputted files: steepest descent minimization algorithm followed by a conjugated gradient algorithm (< 100 kJ/mol/nm). Short equilibration in a NPT ensemble was done next (Nosé-Hoover thermostat at 300 K with time-constant of 1.6 ps ^{4,5}; isotropic Parrinello-Rahman barostat at 1 bar with time-constant 5 ps ^{6,7}) with positional restrains for all hydrogen bonds in 3 consecutive steps of 100 ps each with the LINCS algorithm (order parameter 8, iteration level 2) and a force constant of 1000, 100 and 10 kJ/mol, respectively. Integration step was 2 fs and coordinates were saved each 25

ps, with long-range electrostatics being treated with the Particle-Mesh Ewald algorithm ⁸. The "gromos" clustering method described by Piana *et al.* was employed to the concatenated trajectories, with a minimum of 10 structures per cluster and a cut-off of 3 Å between backbone atoms.⁹ The 2D RMSD matrix of 40001x40001 (peptides) or 14000x14000 (astacin) elements was calculated, corresponding to equally spaced 50 ps frames. Each cluster was represented by the centroid structure, *i.e.* the one with smallest average distance to the remaining neighbours. Analysis of designed active site (d_{AB} , θ_B) done at each 50 ps frames. Production of all images was done using VMD 1.9.2 ¹⁰ and rendering of image files done with Tachyon.¹¹





Corresponding description of each one of the parameters given in 1). Selected parameters used for ranking of designs in green. Astacin designs in open circles, remaining scaffolds in closed circles



Figure S2 - – Comparison of CD spectra between native Sp1f2 and RD01. Far-UV CD spectra of 25 μ M free peptide (dashed lines) and zinc complex forms (solid lines) in 10 mM TRIS 50 mM NaCl at 25 °C, pH 8.0. Additions of ZnCl₂ were 37.5 μ M for Sp1f2 an 100 μ M for RD01. Spectra correspond to two replicates.





Left: Far-UV CD spectra of 25 µM HP35 (black line) and upon addition of 150 µM ZnCl₂ (red line) in 10 mM HEPES 50 mM NaCl at 25 °C, pH 7.5. Right: Corresponding spectra of free peptide (dashed lines) and zinc complex forms (solid lines) of HP35 and RD02, 25 µM peptide and 150 µM ZnCl₂. Spectra correspond to two replicates.



Figure S4 - CD spectra of RD01 and RD02 in free peptide and zinc complex forms for different pH values.

Free peptide and zinc-complex forms of (a) RD01 and (b) RD02, 25 µM concentration of peptide and corresponding peptide-Zn ratios at 25 °C. (c) Corresponding ratios at which the maximum ellipticity values for the zinc-complex forms were obtained.



Figure S5 - Thermal unfolding of native HP35.

Left: Far-UV CD spectra of 25 μ M HP35 in 10 mM HEPES 50 mM NaCl, pH 7.5 at 25 °C, 75 °C and after refolding at 25 °C. Right: Corresponding [θ]₂₂₂ values as a function of temperature, solid lines correspond to the fit to the two-state transition model. Data corresponds to two replicates.



Figure S6 – ¹H NMR of RD01 signal at 1.05 ppm.

Data was obtained for 150 µM peptide in 50 mM NaCl, pH 7.5 with addition of 0-534 µM of ZnCl₂ at 25 °C.



Figure S 7 - Cluster analysis of astacin MD simulations, (a) full structure and (b) detail of active site. Backbone in cartoon representation and colored based on residue index. Residues involved in metal interactions, atoms from the CaDA model shown in spheres, green for zinc ion and orange for dummy atoms. Cluster centroid and initial conformation in transparent and solid representations, respectively.

		M10					M	12				
Inte	raction	1176	1 MMP	1CIZ	1CAQ	1B8Y	1HFS	10JI	4AIG	1ATL	3G42	$\overline{x} \pm \frac{\sigma}{\sqrt{n}}$
dав	His₁	2	2.1	2.2	2.2	2.2	1.9	2.2	2.1	2	2	2.1±0.1
	His ₂	2	2.2	2.2	2.2	2.2	1.9	2.1	2.2	2.1	2	2.1±0.1
	His₃	2	2.1	2.2	2.2	2.2	1.8	1.9	2.3	2	2	2.1±0.2
	Glucat	5.1	4.9	5	5	5.2	5.5	4.7	5.2	4.6	5.1	5.0±0.3
θΑ	His₁	100.5	91.8	93.6	91.8	94.2	93.3	105	96	112.4	89.6	96.8±7.1
	His₂	88.4	94.9	88.4	96.6	93.3	88.2	108.8	83.9	106.5	92.6	94.2±8.1
	His₃	142.2	150.6	151.4	147.2	144.2	154.4	130.6	144.7	131.9	143	144.0±7.8
	Glucat	33	31	30.4	36.1	36.5	19.8	48.2	31.5	51.1	33.4	35.1±9.0
θΒ	His₁	126.7	126.1	129.4	128.2	132.5	129.3	133.1	128.8	131.3	118.9	128.4±4.1
	His₂	123.9	126.1	121.2	121.5	114.9	131.5	126.8	121	126.5	124.9	123.8±4.5
	His₃	126.6	117.3	119.1	122.6	121.6	124.7	126.2	131.4	122.9	130.5	124.3±4.5
	Glucat	88.5	96.1	93.1	94.7	90.8	83.3	85.3	94	98.8	93.3	91.8±4.8
XA	His₁	248.4	261.1	247.7	240.4	237.4	249	243.3	246.2	281.4	252.7	250.8±12.6
	His ₂	143.9	158.3	142.1	134.4	131.9	145.7	134.2	143.5	172.3	153.2	146.0±12.4
	His₃	36.2	34.2	37.4	17.5	1.7	37	10.7	37.7	53.6	38.3	30.4±15.5
	Glucat	184.7	188.5	173.8	160.6	156.3	187.2	166.1	186	220	190.3	181.4±18.4
Хав	His₁	165.5	149.8	167.8	169.1	170.4	156.5	148.9	167.3	129.1	157.6	158.2±12.9
	His ₂	208.6	197.9	191.3	198	198.3	198.3	197.4	201.9	198.9	191.2	198.2±4.9
	His₃	4.6	22.9	2.8	16.2	29.6	8.7	39.2	4.3	21.1	19.8	16.9±12.0
	Glucat	79.6	74.9	67.2	67.3	70.1	95.6	72.4	82	79.1	66.1	75.4±9.1
Хв	His₁	168	174.8	160.6	155.6	154.7	176.9	164.6	161.9	182.3	171.2	167.1±9.2
	His ₂	186	190.5	190.2	184.4	185.2	186.9	195	193	198.3	194.9	190.4±4.8
	His₃	162.6	163.4	173	176.8	163.9	167.5	164.9	171.3	165.1	158.1	166.7±5.6
	Glucat	138.7	145.7	140.3	141.3	143.8	148.4	151.6	148	151.5	141.2	145.1±4.7

 Table S1 - Geometrical parameters of AS from selected MA(M) structures. Subclan families and

 PDB identifiers on top.

# 1QJI_diala_conf.params							
NAME	dA1		-				
IO_ST	TRING	dAl Z					
TYPE	LIGA	ND					
AA UN	JΚ						
ATOM	ZN1	Zn2p	Х	1.96			
ATOM	02	OH	Х	-0.70			
ATOM	C1	CH1	Х	-0.13			
ATOM	03	00C	Х	-0.80			
ATOM	NЗ	Ntrp	Х	-0.65			
ATOM	C8	CH1	Х	-0.13			
ATOM	C2	CH3	Х	-0.31			
ATOM	H12	Наро	Х	0.06			
ATOM	H13	Наро	Х	0.06			
ATOM	H14	Наро	Х	0.06			
ATOM	С5	COO	Х	0.58			
ATOM	N2	NH2O	Х	-0.51			
ATOM	H2	Hpol	Х	0.39			
ATOM	ΗЗ	Hpol	Х	0.39			

ATOM 04 ONH2 X -0.59 ATOM H1 Hapo X 0.06 ATOM H7 Hpol X 0.39	
ATOM C6 CH1 X -0.13 ATOM N1 Ntrp X -0.65	
ATOM C4 COO X 0.58 ATOM O1 ONH2 X -0.59	
ATOM C3 CH3 X -0.31	
ATOM H10 Hapo X 0.06	
ATOM H11 Hapo X 0.06 ATOM H16 Hpol X 0.39	
ATOM C7 CH3 X -0.31 ATOM H4 Hapo X 0.06	
ATOM H5 Hapo X 0.06	
ATOM H6 HADO X 0.06 ATOM H9 HADO X 0.06	
ATOM H15 Hpol X 0.39 BOND C1 O2	
BOND C1 03 BOND C1 N3	
BOND C1 C6	
BOND C2 C8 BOND C2 H12	
BOND C2 H13 BOND C2 H14	
BOND N1 C4 BOND N1 C6	
BOND NI H16	
BOND N2 C5	
BOND N2 H2 BOND N2 H3	
BOND 02 H15 BOND 02 ZN1	
BOND O4 C5	
BOND C3 H8	
BOND C3 H10 BOND C3 H11	
BOND N3 C8 BOND N3 H7	
BOND C5 C8	
BOND C6 H9	
BOND C7 H4 BOND C7 H5	
BOND C7 H6 BOND C8 H1	
CHI 1 ZN1 02 C1 03 CHI 2 02 C1 N3 C8	
CHI 2 02 CI N3 C8 CHI 3 02 CI C6 N1	
CHI 4 C6 N1 C4 O1 CHI 5 C1 C6 N1 C4	
CHI 6 C1 N3 C8 C2 CHI 7 N3 C8 C5 N2	
NBR_ATOM 02	
NBR_RADIUS 8.363764 ICCOR_INTERNAL ZN1 0.000000 0.000000 ZN1 O2 C1	
ICOOR_INTERNAL 02 0.000000 180.000000 1.849189 ZN1 02 C1 ICOOR_INTERNAL C1 0.000001 56.729696 1.595460 02 ZN1 C1	
ICOOR_INTERNAL 03 5.414501 78.336182 1.568874 C1 02 ZN1	
ICOOR_INTERNAL NS II9.370020 65.773055 I.655588 CI O2 O3 ICOOR_INTERNAL C8 -76.489396 61.101004 1.535813 N3 C1 O2	
ICCOR_INTERNAL C2 77.096851 69.172846 1.539788 C8 N3 C1 ICCOR INTERNAL H12 -116.166899 70.478464 1.089474 C2 C8 N3	
ICOOR_INTERNAL H13 -120.051541 70.526968 1.090604 C2 C8 H12	

ICOOR INTERNAL	C 5	121 054923	70 835105	1 524968	C8	N3	C2
ICOOR INTERNAL	N2	132 297922	63 393354	1 341865	C5	C8	N3
ICOOD INTERNAL	112	2 246025	50 110010	1 000561	NT O	CE	C 9
ICOOR_INTERNAL	п∠	=3.240955	59.110010	1.009561	IN Z	05	
ICOOR_INTERNAL	HЗ	-179.985254	60.445997	1.009692	N2	C5	H2
ICOOR_INTERNAL	04	179.726837	58.151996	1.223927	С5	C8	N2
ICOOR_INTERNAL	H1	119.995376	71.118126	1.089578	C8	NЗ	C5
ICOOR_INTERNAL	H7	-121.397122	72.908834	1.009422	NЗ	C1	C8
ICOOR INTERNAL	C6	126.918397	69.610456	1.707063	C1	02	N3
ICOOR INTERNAL	N1	53.919212	64.020083	1.446771	C6	C1	02
ICOOR INTERNAL	C4	-101.124052	59.540397	1.324118	N1	C6	C1
ICOOR_INTERNAL	01	-0.031823	56.993050	1.232007	C4	N1	C6
ICOOR_INTERNAL	C3	-178.207807	63.265011	1.513557	C4	N1	01
ICOOR INTERNAL	H8	-97.218306	69.813070	1.090471	C3	C4	Nl
ICOOR INTERNAL	H10	119.199821	68.909778	1.089870	C3	C4	Н8
ICOOR INTERNAL	H11	120.098134	72.723062	1.089461	C3	C4	H10
ICOOR_INTERNAL	H16	-179.110947	63.140534	0.980142	N1	C6	C4
ICOOR_INTERNAL	C7	127.125575	66.295147	1.525871	C6	C1	Nl
ICOOR INTERNAL	H4	-108.636956	70.497439	1.090028	С7	C6	C1
ICOOR INTERNAL	H5	-120.078698	70.505253	1.090535	С7	C6	H4
ICOOR INTERNAL	НG	-119.955610	70.572417	1.090338	С7	C6	Н5
ICOOR_INTERNAL	Н9	117.493922	78.793685	1.089756	C6	C1	C7
ICOOR_INTERNAL	H15	125.262060	73.810349	0.956392	02	ZN1	C1
PDB_ROTAMERS 1QJI	_diA	la_conf_Zn_cor	.pdb				

Table S3 – MA(M):diAla cst file (top) and matcher and enzyme design options (bottom).

```
# MAM:diAla cst file
# 02 corresponds to Ow atom. C1 to the tetrahedral carbon bound to Ow and Op.
# diAla has 3-letter code dA1.
# 6th column of distanceAB value set to 0 for non-bonded interaction, set to 1
for pseudocovalent interaction.
# When secondary algorithm is used, angle A angle B torsion A torsion AB
torsion B commented out.
#Glu cat - catalytic interaction
CST::BEGIN
 TEMPLATE::
              ATOM MAP: 1 atom name: ZN1 02 C1
 TEMPLATE::
              ATOM MAP: 1 residue3: dA1
             ATOM MAP: 2 atom_type: OOC ,
 TEMPLATE::
                                               #either OE1 or OE2
 TEMPLATE:: ATOM MAP: 2 residue1: E
 CONSTRAINT:: distanceAB: 5.0 0.3 100 0
                                                 1
                                                    #2
 CONSTRAINT:: angle_A: 35.1 9.0
CONSTRAINT:: angle_B: 91.8 4.8
                                       30 360 1
                                                    #2
                                        30
                                            360
                                                 1
                                                    #1
 CONSTRAINT:: torsion A: 181.4 18.4
                                        30 360 1
                                                    #4
 CONSTRAINT:: torsion AB: 75.4 9.1
                                        30
                                           360 1 #2
 CONSTRAINT:: torsion B: 145.1 4.7
                                       30 360 1 #1
CST::END
# His3 - Zn(II) 1st coord. sphere
CST::BEGIN
 TEMPLATE::
              ATOM_MAP: 1 atom_name: ZN1 O2 C1
             ATOM MAP: 1 residue3: dA1
 TEMPLATE::
 TEMPLATE:: ATOM MAP: 2 atom type: Ntrp , #either ND1 or NE2
 TEMPLATE:: ATOM MAP: 2 residue1: H
 CONSTRAINT:: distanceAB:
                           2.1 0.2 100 1
                                                  1
                                                     #2
               angle_A: 144.0 7.8
                                        30 360
 CONSTRAINT::
                                                  1
                                                     #2
                 angle B: 124.3 4.5
 CONSTRAINT::
                                        30 360
                                                  1
                                                     #1
 CONSTRAINT:: torsion_A: 30.4 15.5
CONSTRAINT:: torsion_AB: 16.9 12.0
                                        30 360
                                                     #З
                                                  1
                                        30 11.25 0
                                                     #1
 CONSTRAINT:: torsion B: 166.7 5.6
                                        30
                                           360
                                                  1
                                                     #2
#ALGORITHM INFO:: match ;not commented out when secondary algorithm is used
#SECONDARY MATCH: DOWNSTREAM ; not commented out when secondary algorithm is used
#ALGORITHM_INFO::END ;not commented out when secondary algorithm is used
CST::END
# His1 - Zn(II) 1st coord. sphere
CST::BEGIN
              ATOM MAP: 1 atom name: ZN1 02 C1
 TEMPLATE::
 TEMPLATE::
             ATOM MAP: 1 residue3: dA1
 TEMPLATE::
              ATOM_MAP: 2 atom_type: Ntrp, #either ND1 or NE2
```

```
TEMPLATE:: ATOM MAP: 2 residue1:
                                    Н
 CONSTRAINT:: distanceAB: 2.1 0.1 100 1
                                                1 #1
 CONSTRAINT:: angle A: 96.8 7.1 30 360
                                               1 #2
                angle_B: 128.4 4.1 30 360
 CONSTRAINT::
                                                1
                                                   #1
 CONSTRAINT:: torsion_A: 250.8 12.6
                                      30
                                          360
                                                1
                                                   #3
                                      30 11.25 0
 CONSTRAINT:: torsion AB: 158.2 12.9
                                                   #1
 CONSTRAINT:: torsion B: 167.1 9.2 30 360 1 #2
#ALGORITHM INFO:: match
                           ;not commented out when secondary algorithm is used
#SECONDARY_MATCH: DOWNSTREAM ;not commented out when secondary algorithm is used
#ALGORITHM INFO::END
                     ;not commented out when secondary algorithm is used
CST::END
# His2 - Zn(II) 1st coord. sphere
CST::BEGIN
 TEMPLATE::
             ATOM MAP: 1 atom name: ZN1 02 C1
             ATOM_MAP: 1 residue3: dA1
 TEMPLATE::
            ATOM_MAP: 2 atom_type: Ntrp , #either ND1 or NE2
ATOM_MAP: 2 residue1: H
 TEMPLATE::
 TEMPLATE::
 CONSTRAINT:: distanceAB: 2.1 0.1 100 1
                                               1 #1
 CONSTRAINT:: angle A: 94.2 8.1 30 360 1 #2
                angle_B: 123.8 4.5 30 360
                                               1 #1
 CONSTRAINT::
                                      30 360 1
30 11.25 0
 CONSTRAINT:: torsion A: 146.0 12.4
                                                   #3
 CONSTRAINT:: torsion_AB: 198.2 4.9
                                                   #1
 CONSTRAINT:: torsion B: 190.4 4.8 30 360 1 #1
#ALGORITHM INFO:: match
                           ;not commented out when secondary algorithm is used
#SECONDARY MATCH: DOWNSTREAM ; not commented out when secondary algorithm is used
                             ;not commented out when secondary algorithm is used
#ALGORITHM_INFO::END
CST::END
# options_matcher.flags
                                      # options_enzdes.flags
-packing
                                      -dun10
-ex1:level 3 #Chi'1 sampling level
                                      -packing
 -ex2:level 3 #Chi'2 sampling level
                                       -use input sc
                                       -ex1:level 6 #Chi'1 sampling level
-ex2aro
                                       -ex2:level 6 #Chi'2 sampling level
-exlaro
-use_input_sc
                                       -exlaro:level 6
-linmem ig 10
                                       -ex2aro:level 6
                                       -soft rep design
-match
-bump tolerance 0.2
                                       -linmem ig 10
-filter colliding upstream residues
                                      -enzdes
                                      -parser read cloud pdb true
filter upstream downstream collisions
                                       -cst opt
-output_format CloudPDB
                                       -chi min
-enumerate_ligand_rotamers
                                       -bb min
-dun10
                                       -cst design
-mute protocols.idealize
                                       -cst_min
                                       -design min cycles 4
                                       -detect design interface
                                       -cut1 6.0
                                       -cut2 8.0
                                       -cut3 10.0
                                       -cut4 12.0
                                      -score
                                         -weights enzdes.wts
                                      -fix catalytic aa
                                      -nstruct 1 #for design or -nstruct 10 for
                                      full sequence design
```

Scaffold	Classification (UniprotKB)	Fold	Chain length (L)	PDB	#NMR X-ray	Seconda ry	Classical (UM)
Trp-cage	De Novo Protein	α	20	1L2Y	38		
)/Dr	Viral Protein		00		40	01/	E7H14H2H17
vPg	P03300	α	22	ZBBL	10	ÜK	E20H2H17H14
HIV gp120 C5 Domain	Viral Protein P19549	α	23	1MEQ	X-ray	ОК	E9H22H5H3
Poneratoxin	Toxin P41736	α	25	1G92	10	ок	E21H6H15H17 E23H14H16H19
FBP28 WW Domain	SH3 Domain Q8CGF7	β	27	1E0L	10	ОК	E9H26H21H23
Fibritin C- terminal - Foldon	Viral Protein P10104	β	27	4NCU	X-ray		
CCK2E3	Hormone/Growth Factor Receptor P32239	α	30	1L4T	1	ок	
pGolemi	De Novo Protein	α	30	2K76	10	ок	E13H6H7H16
	Liberra e a citore de la companya de						E26H6H23H2
Parathyroid Hormone	Factor P01270	α	31	1FVY	20	ОК	
Cholecystokinin A	Hormone/Growth Factor P32238	α	31	1HZN	X-ray		
							E685H700H679H696
							E688H680H699H679
							E688H680H703H679
							E688H681H699H683
	Ubiquitin binding						E688H703H679H682
iota	domain Q9UNA4	α	32	2L0G	20	ОК	E691H680H679H683
							E700H680H699H703
							E702H682H680H699
							E706H680H703H699
							E706H682H680H699
P alamant	Nuclear Protein						E706H682H703H699
Somatic Inhibitor	Q7JPS0	α	33	2BN6	29	ОК	
			34	1UNC	25	ок	
	Actin Binding P09327						E14H29H17H25
Human Villin		a					E 10112011201122
Headpiece		~					E26H17H25H29
							E28H6H10H17
							E29H14H17H6
Advillin Human	Actin Binding 075366	α	36	1UND	25		
							E3H24H5H20
	l la mar a r a /O navy th						E7H24H5H20
nPYY	Factor	a	36	1RU5	20	ОК	E19H11H6H16
pi i i	P68005	~	00		20	ÖN	E27H36H34H24
							E30H6H27H23
							E36H20H24H5
							E5H2/H22H33
WW Domain	De Novo Protoin	ß	37	15014	10	OK	E3H27H23H33 E5H27H27H2
Prototype		р	37	1E0M	10	ОК	E5H27H24H02
							E27H5H8H24
E3-binding domain	Glycolysis P0AFG6	α	37	1BBL	1	ОК	E17H39H16H20
							E1H32H36H4
Alpha-T-alpha	De Novo Protein	α	38	1ABZ	23	ОК	E1H33H36H4
							E18H28H24H15

 Table S4 – Scaffolds screened with the MA(M):diAla model.

							E28H21H25H15
							F31H14H11H24
							E31H14H11H21
							E31U21U11U29
							E31H14H11H28
							E33H3H32H36
							E33H4H32H36
							E35H11H7H31
							E35H11H32H28
	DNA binding						E5H15H8H10
CRE-BP1	regulatory protein	α+β	38	1BHI	20	OK	E19H6H17H4
	P15336						E28H11H9H31
Phage Scaffolding Protein	Viral Protein P26748	α	40	2GP8	1	ОК	E13H28H16H32
	DNA binding						E38H53H37H41
MafG	protein	α	41	1K1V	20	ОК	E38H53H37H49
	O54790						E41H34H38H53
							F468H504H501H473
							E468H504H501H474
							E468H504H501H505
Phosphoprotein	Viral protein	~	4.4		20	OK	
XD domain	P03422	α	44	ZK9D	20	ÜK	E468H505H474H501
							E500H4/3H504H501
							E502H463H498H467
							E504H468H501H497
GA module	Albumin Binding Protein Q51911	α	45	1PRB	1	ОК	
HHR23A	C-terminal UBA domain P54725	α	45	1IFY	10	ОК	E164H171H177H168
FAF UBA domain	Apoptosis Q9UNN5	α	45	3E21	X-ray		
HIV Vpu	HIV protein P19554	α	45	1VPU	X-ray		
				2JNH	15	OK	E8H25H7H11
	Ligase Q13191	α	46				E8H21H25H15
UBA Domain						UK	E8H21H11H15
							E29H5H7H33
Sda antikinase	Signaling Protein Q7WY62	α	46	1PV0	25		
							E101H124H97H120
				2E5T			E104H97H101H120
					20		E104H124H97H120
ATP synthase	Hydrolase	α	46			OK	E104H124H101H120
ipsiion chain	Q3KUJ4						H119H99H100H116
							E127H101H124H97
							E127H101H120H97
dihydrolipoyllysin e acetyl transferase	Transferase P11961	α	47	1W4E	20	ОК	
							E4H29H7H25
							E19H47H27H37
							E19H47H27H41
							E26H47H30H27
							E27H41H37H19
Swa2n	Protein Binding	α	47	1PGY	20	OK	E27H17H37H19
Junt	Q06677	~			20		E27H17H10H41
							E27H45U41U10
							E33U7U20U40
							E44NZ/N41N3/

							E46H37H27H41
							E7H28H34H24
LysM Domain	POAE77	α+β	48	1E0G	20	OK	E7H28H24H13
	FUALZI						E48H30H26H46
							E15H52H55H14
							E27H19H17H34
							E29H52H55H14
							E31H35H33H28
Lambda-	Viral Protein		40		05	01/	E31H58H28H30
Integrase	P03700	α+β	48	1KJK	25	OK	E49H14H13H52
							E51H31H30H28
							E51H31H28H35
							E51H33H28H35
							E54H33H30H28
Translation Initiatior factor 2 N-ter	Translation P0A705	α	49	1ND9	10	ОК	
							E530H565H534H561
							E535H565H534H561
							E537H530H561H534
							E544H570H541H567
							E545H555H542H563
0075.04	Nuclear Protein P33240	α	49	2J8P	00	ОК	E546H538H551H545
CSTF-64					30		E546H538H552H545
							E546H563H551H545
							E546H563H552H545
							E551H538H555H552
							E551H563H555H552
							E560H532H536H557
РОВ	Transferase Q8ZUR6	α	51	1W4J	20	OK	E167H138H164H142
NTL9	RNA Binding Protein P02417	α+β	51	2HBB	X-ray	ОК	
Engrailed Homeodomain	DNA Binding Protein P02836	α	54	1ENH	X-ray	ок	E34H15H38H19
Protein G	Protein Binding P06654	α+β	56	2LGI	10	OK	
Protein A (Sp. aureus)	Protein Binding P38507	α	58	4NPE	X-ray	OK	
Thermolycin C	Hydrologo						E300H265H262H295
Ter	P00800	α	62	1TRL	8	OK	E302H265H262H295
							E309H284H288H267
							E6H51H55H58
Brotoin I	Immune System	a+B	64	2JZP	20	ОК	E41H34H38H47
FIOLEIIIL	Q51912	u+p	04		20		E44H18H12H10
							E52H9H57H59

Table S5 – Buffer compositions and 4-nitrophenol extinction coefficients.

рН	Buffer	NaCl	ε ₄₀₀ (M⁻¹cm⁻¹)	
7.0		10 mM		8739±261
7.5	HEFE3	(binding) 40 mM	50 mM	12754±201
8.0	TRIS/CHES			17674±455
8.6				26166±122
9.0	CHES	(catalysis)		19302±155

Scaffold	RMSD matrix	Number clusters	# top clusters
	[min, max] Å	(cut-off 3 Å)	(> 80% pop. Time)
Sp1f2	[0.3, 7.3]	5	1
RD01	[0.3, 14.0]	165	6
HP35	[0.3, 9.1]	29	1
RD02	[0.4, 12.2]	165	7
Astacin	[0.5, 4.1]	1	1

Table S6 – Cluster analysis of MD simulations.

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