BOPC1 enantiomers preparation and HuR interaction study. From molecular modeling to a curious DEEP-STD NMR application.

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SUPPORTING INFORMATION

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S1. Synthesis, enantiomeric resolution by chiral HPLC and compound characterization

S1.1 General procedures

Reagents and solvents for synthesis were purchased from Sigma-Aldrich (Italy). Unless otherwise specified, commercially available reagents were used as received from the supplier. Solvents were purified according to the guidelines in Purification of Laboratory Chemicals. Analytical thin-layer-chromatography (TLC) was carried out on silica gel precoated glass-backed plates (Fluka Kieselgel 60 F254, Merck) and visualized by ultra-violet (UV) radiation and potassium permanganate. Flash chromatography (FC) was performed on Silica Gel 60 (particle size 230–400 mesh, purchased from Nova Chimica). IR spectra were recorded on a Jasco FT/IR-4100 spectrophotometer with ATR module; only noteworthy absorptions are reported. Unless otherwise specified, ^{1}H, COSY, HSQC, NOESY and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer at 298K. A Laborota 4000 Efficient (Heidolph) rotary evaporator was exploited for solvent evaporation of each fraction.

Specific rotations were determined on a DIP 100 JASCO polarimeter, equipped with sodium ($\lambda = 589$ nm) and mercury ($\lambda = 405$ nm) lamps. HPLC analyses were performed on a JASCO system: pump (PU-1580), degasser (DG-2080-53) and mixer (LG-1580-02); PDA (photo array detector, MD-1510), injection system Rheodyne valve 7125 equipped with a 10 µL loop. Semi-preparative methods were conducted on the same system equipped with a 1 mL loop.

HPLC solvents (*n*-hexane, isopropanol, ethanol, methanol), were acquired from Sigma Aldrich and VWR.

S1.2 Preparation of (±)-trans-4, (+)-trans-4, (–)-trans-4

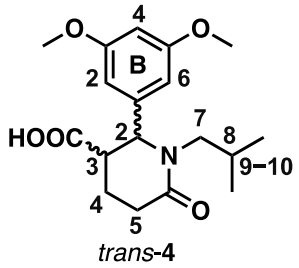
2-(3,5-Dimethoxyphenyl)-1-isobutyl-6-oxopiperidine-3-carboxylic acid

To a solution of aldehyde 1 (100 mg, 0.60 mmol) in toluene (2 mL) at room temperature (rt), isobutylamine 2 (60 µL, 0.60 mmol) and molecular sieves 4Å (MS 4Å, 75.5 mg) were sequentially added. The reaction mixture was stirred for 4 hours after which, the MS was filtered and amberlyst resin IRC50 added for 20 minutes. The yellow solution obtained was then evaporated to dryness, dissolved in p-xylene (2 mL) and added glutaric anhydride 3 (54 mg, 0.60 mmol). The stirred suspension was refluxed overnight. The solvent was then decanted and the remaining white solid washed with ether giving (±)-trans-4 as a white solid (125 mg, 62%); identity was confirmed by NMR analysis. mp=176−179°C.The enantiomers of **(±)-*trans*-4** were separated by chiral HPLC using a Daicel Chiralpak IA column (25 × 1.0 cm i.d.), eluting with isopropyl alcohol (IPA) added with 0.1% diethyl amine (DEA) and 0.3% trifluoroacetic acid (TFA) at a flow rate of 2 mL/min. After solvent evaporation, the first

fraction eluted at $t_1 = 6.9$ min gave (+)-*trans*-**4** ($[\alpha]_D^{20} = +51.0$, c = 0.3 in $CHCl_3$, *ee* = 98.2%) and the second at $t_2 = 8.1$ min gave (–)-*trans*-**4** ($[\alpha]_D^{20} = -49.0$, c = 0.3 in $CHCl_3$, *ee* = 97.5%), were isolated as white solids. The enantiomeric excess of each enantiomer was determined using Chiralpak IA (4.6 mm I.D. x 250 mm), eluting with IPA:DEA:TFA 100:0.1:0.3 at flow rate of 0.5 mL/min. The ^{1}H NMR spectra were identical to the spectrum of (±)-*trans*-**4**.

The full NMR characterization of racemic and enantiomeric *trans*-4 in $CDCl_3$ at 298K is reported in S1.2.1, and the chiral chromatographic profiles in S1.2.2.

S1.2.1 Full NMR characterization of compounds (±)-*trans*-4, (+)-*trans*-4, (–)-*trans*-4 in $CDCl_3$ at 298K



trans-**4**

	^{1}H (ppm)	Mult, J (Hz)	^{13}C (ppm)
B 4	6.42	tr, 1.78	99.5
B 2,6	6.33	d, 1.78	104.5
2	5.10	d, 1.77	61.8
7a	3.98	q, 8.96; 4.53	53.3*
OCH_3	3.81	s	55.5
3	2.94	q, 2.83; 3.98	45.6
5a	2.76	m	28.9§
5b	2.64	m	28.9§
7b	2.31	q, 5.68; 7.41	53.3*
4a	2.10	m	18.4°
8	2.07	m	26.6
4b	1.98	m	18.4°
9–10	0.90	s	20.1

S1.2.2 HPLC chiral analysis of compound (±)-*trans*-4, (+)-*trans*-4, (–)-*trans*-4

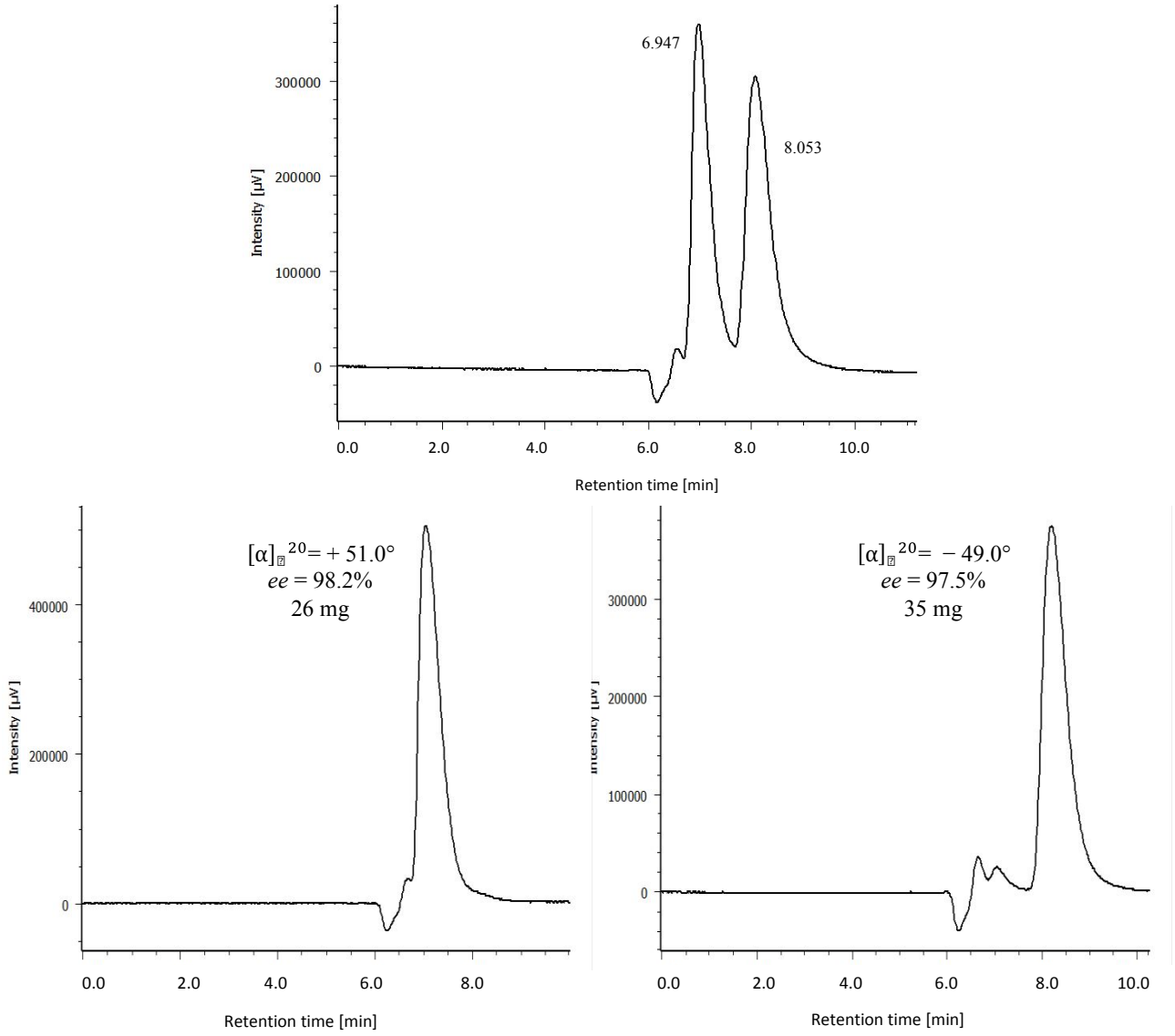


Figure S1.2.2. Top: Analysis performed on (±)-*trans*-**4**; bottom: Chromatographic profile of the two enantiomers: (+)-*trans*-**4** (*ee* 98.2%), and (–)-*trans*-**4** (*ee* 97.5%) after separation.
All analyses were performed using Chiralpak IA (4.6 mm I.D. x 250 mm), IPA:DEA:TFA 100:0.1:0.3, flow: 0.5 mL/min, 25°C, λ = 220 nm. Injection volume 10 µL (1 mg/mL in IPA).

S1.3 Preparation of (±)-*trans*-BOPC1

N-(2-(Benzylamino)ethyl)-2-(3,5-dimethoxyphenyl)-1-isobutyl-6-oxopiperidine-3-carboxamide

To a solution of (±)-*trans*-**4** (100 mg, 0.30 mmol) dissolved in THF (3 mL), TBTU (115,3 mg, 0.36 mmol) and DIPEA (0.109 mL, 0.62 mmol) were sequentially added.[5] The reaction mixture was stirred at rt for 30 minutes. *N*-benzylethylendiamine (**5**) (0.045 mL, 0.30 mmol) was added and the solution was stirred overnight at room temperature. The reaction mixture was concentrated *in vacuo*, dissolved in DCM (10 mL) and washed with water (20 mL). The organic layers were dried over Na_2SO_4, filtered and the solvent evaporated *in vacuo*. The crude was purified by FC eluting with DCM/MeOH (8:2, v/v), giving **(±)-*trans*-BOPC1** as yellow oil (99.6 mg, 71%)

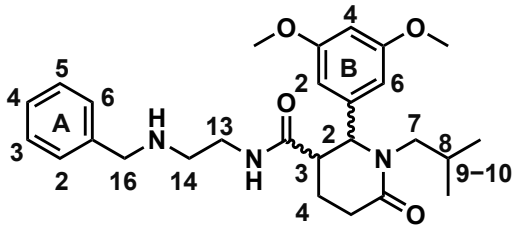
^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.25 (ddd, J = 7.4, 4.5, 1.5 Hz, 2H), 7.22-7.15 (m, 3H), 6.30–6.27 (m, 1H), 6.25 (d, J = 2.2 Hz, 2H), 5.96 (s, 1H), 4.75 (d, J = 7.5 Hz, 1H), 3.87-3.80 (m, 1H), 3.67 (d, J = 3.0 Hz, 6H), 3.62 (d, J = 4.5 Hz, 2H), 3.23 (ddd, J = 16.9, 11.9, 5.1 Hz, 1H), 3.18-3.08 (m, 1H), 2.61 (dq, J = 8.7, 4.9 Hz, 1H), 2.57-2.49 (m, 2H), 2.46−2.34 (m, 2H), 2.15 (dd, J = 13.6, 6.2 Hz, 1H), 2.02–1.83 (m, 4H), 0.78 (d, J = 5.4 Hz, 3H), 0.76 (s, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 175.26, 171.13, 161.21, 142.42, 104.53, 99.23, 61.68, 55.31, 52.91, 45.75, 28.78, 26.41, 20.18, 19.97, 18.50. IR (νmax/cm^{-1}): 836.9, 910.4, 991, 1061.62, 1156.12, 1203.36, 1607.38, 1746.23, 3002.62, 3027.69, 3381.57. UHPLC-ESI-MS: ABS tR= 1.55 min, 96% pure (λ = 210 nm), calculated for $C_{27}H_{38}N_3O_4$ $[M+H]^+$ 468.28623, found 468.28601.

The *trans*-configured enantiomers of **BOPC1** were separated on a semi-preparative scale on Chiralpak IC by eluting with n-Hexane:IPA:DEA 75:25:0.1 (v/v/v), flow rate 2 mL/min.

After solvent evaporation, the first fraction eluted at t_1 = 35.3 min gave (+)-*trans*-**BOPC1** (+ 74.0, c = 0.3 in MeOH, *ee* = 99.0%) and the second eluted at t_2 = 43.1 min gave (–)-*trans*-**BOPC1** ($[\alpha]_D^{20}$ = – 74.6 *ee* = 95.6%), as colorless oils. The enantiomeric excess of both enantiomers was determined using using Chiralpak IC (4.6 mm I.D. x 250 mm) and eluting with n-Hex:IPA:DEA 75:25:0.1 (v/v/v), flow rate 0.5 mL/min. The ^{1}H-NMR spectra were identical with the spectrum of (±)-*trans*-**BOPC1.**

Compounds (+)-*trans* and (–)-*trans*-**BOPC1** were then converted into the corresponding hydrochloride by addition of 37% HCl (4 µL, 0.128 mmol) to a stirred solution of (+)-*trans* and (–)-*trans*-**BOPC1** (30 mg, 0.064 mmol) in methanol (500 µL) and successive evaporation of the solvent *in vacuo* obtaining (+)-(2S,3S)- and (–)-(2R,3R)-**BOPC1** HCl as white solids. Full NMR characterization in phosphate buffer is reported in S1.3.1, and the chiral chromatographic profiles in S1.3.2.

S1.3.1 Full NMR characterization of compound (±)-*trans*-BOPC1, (+)-*trans*-BOPC1 and (–)-*trans*-BOPC1 in a 20 mM phosphate buffer at pH = 7.4 and 283K.



trans-**BOPC1**

	^{1}H (ppm)	Mult., J (Hz)	^{13}C (ppm)
A 3,4,5	7.21	bs	129.28
A 2,6	7.12	bs	129.42
B 4	6.19	d, J = 1.8	99.93
B 2,6	6.17	d, J = 1.8	105.83
2	4.59	-	63.50
16	3.69	s	51.10
OCH_3	3.47	s	55.50
7b	3.36	m	51.40
14	3.05	m	36.20
15	2.74−2.63	m	45.80
3	2.56	m	49.30
5	2.35	t, J = 6.8	30.60
7a	2.05	dd, J = 6.52; 14.40	51.40
4	1.72	m	22.00
8	1.63	m	25.40
9–10	0.50	d, J = 3.35	19.11

S1.3.2 HPLC chiral analysis of compound (±)-trans-BOPC1, (+)-trans-BOPC1, (–)-trans-BOPC1

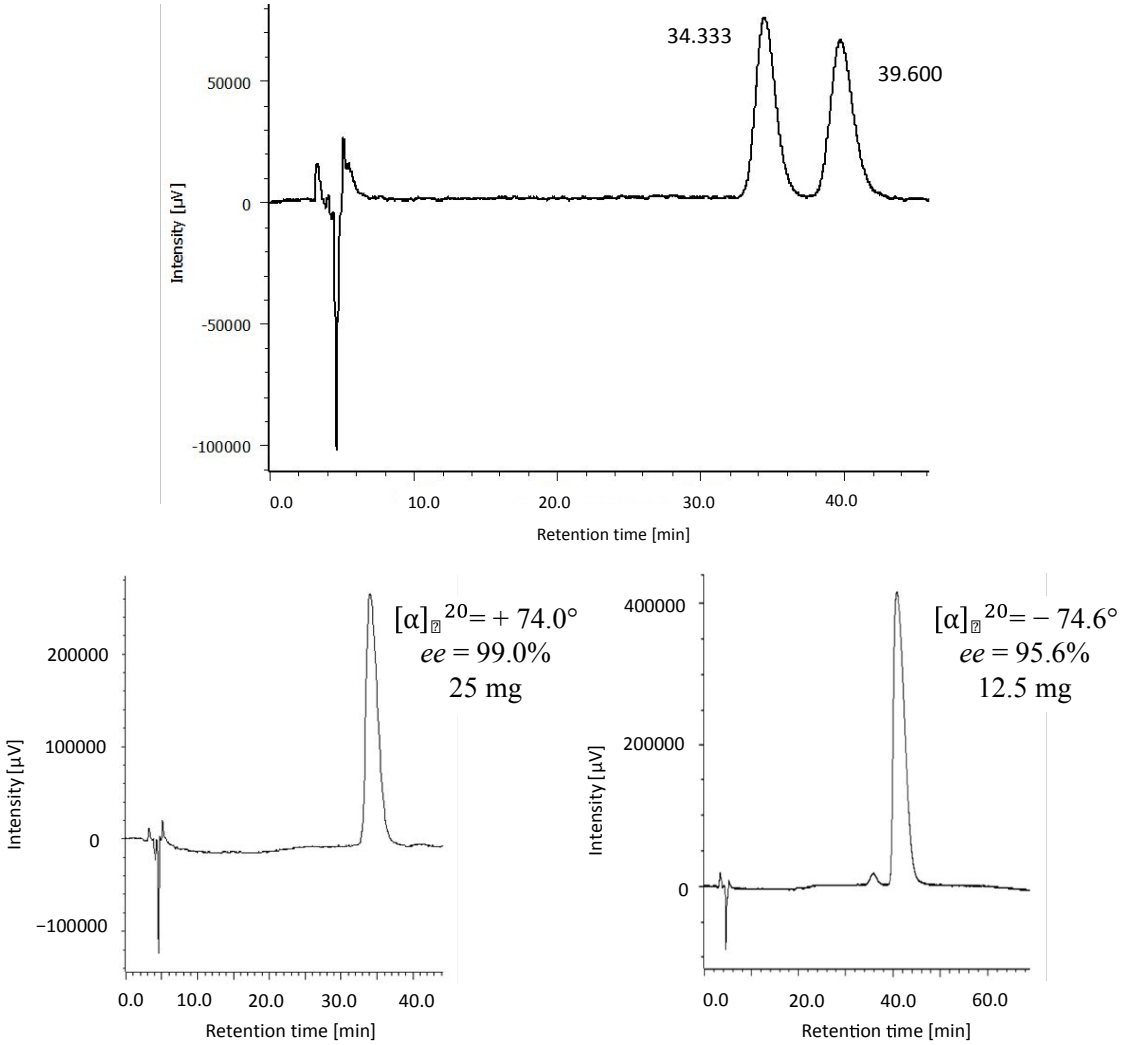


Figure S2.3.1. Top: Analysis performed on (±)-*trans*-**BOPC1**; bottom: Chromatographic profile of the two enantiomers: first, (+)-*trans*-**BOPC1** (ee 99%), and second, (–)-trans-BOPC1 (ee 95.66%) after separation. All analyses were performed using Chiralpak IC (4.6 mm I.D. x 250 mm), n-Hex:IPA:DEA 80:20:0.1 (v/v/v), flow: 0.5 mL/min, 25°C, λ = 220 nm. Injection volume 10 µL (1 mg/mL in n-Hex:IPA 75:25).

S1.4 Preparation of (+)-(2S,2S)-BOPC1

(+)-(2S,3S)-N-(2-(Benzylamino)ethyl)-2-(3,5-dimethoxyphenyl)-1-isobutyl-6-oxopiperidine-3-carboxamide

To a solution of (–)-*(2S,3S)*-**4** (10 mg, 0.03 mmol) dissolved in THF (1 mL), TBTU (11.5 mg, 0.04 mmol) and DIPEA (10 μL, 0.06 mmol) were added. After stirring at rt for 30 minutes, *N*-benzylethylendiamine **5** (0.045 mL, 0.30 mmol) was added and the solution stirred overnight at rt. The reaction mixture was concentrated *in vacuo*, dissolved in DCM (2 mL) and washed with water (2 mL). The organic layers were dried over Na_2SO_4, filtered and the solvent evaporated *in vacuo*. The crude was purified by FC eluting with DCM/MeOH (8:2, v/v), giving (+)-**(***2S,3S)***-BOPC1** as a yellow oil. UHPLC-ESI-MS: ABS tR= 1.56 min, 96% pure (λ = 210 nm), calculated for $C_{27}H_{38}N_3O_4$ $[M+H]^+$ 468.28623, found 468.28612.

S2. STD NMR and DEEP-STD NMR experiments

S2.1 General Procedures

All protein−ligand samples were prepared in a 1000:1 ligand/protein ratio. Typically, the final concentration of the samples was 400 μM of ligand and 0.4 μM of HuR, and the final volume was 200 μL. The buffer used is a 20 μM deuterated phosphate buffer pH 7.4.

^{1}H-STD NMR experiments were performed on a 600 MHz Bruker Avance spectrometer. The probe temperature was maintained at 283K. In the STD experiments, water suppression was achieved by the WATERGATE 3-9-19 pulse sequence. The on-resonance irradiation of the protein was performed at −0.05 ppm. Off-resonance irradiation was applied at 200 ppm, where no protein signals are visible. Selective presaturation of the protein was achieved by a train of Gauss-shaped pulses of 49 ms length each. The STD NMR spectra were acquired with an optimized total length of saturation train of 2.94 s. Blank experiments were conducted in absence of protein in order to avoid artefacts.

For applying the DEEP-STD NMR strategy, we measured the samples in differential solvent conditions, namely in a 100% D_2O-buffer and a H_2O:D_2O 90:10-buffer (indicated with H_2O).[1]

Intensities of all STD effects (absolute STD) were calculated by division through integrals over the respective signals in STD NMR reference spectra. The different signal intensities of the individual protons are best analyzed from the integral values in the reference and STD spectra, respectively. $(I_0-I_{sat})/I_0$ is the fractional STD effect, expressing the signal intensity in the STD spectrum as a fraction of the intensity of an unsaturated reference spectrum. In this equation, I_0 is the intensity of one signal in the off-resonance or reference NMR spectrum, I_{sat} is the intensity of a signal in the on-resonance NMR spectrum, and $I_0–I_{sat}$ represents the intensity of the STD NMR spectrum.[2,3] Further processing for building epitope maps within single ligands involved the calculation of two related values, absolute and relative STD intensities (both values are given as percentages). To facilitate comparison of protons within a single molecule, relative STD % was subsequently calculated: the proton with the highest absolute STD % was given the arbitrary value of 100%; the values of the other protons are then calculated relative to this proton. In case of multiple protons under the same chemical shift, this number was normalized. While within the same spectrum, higher absolute STD values are symptomatic of a higher affinity for the macromolecular target, it is not commonly accepted to compare absolute STD values of different spectra or of different compounds to rank their affinity to the macromolecule. This comparison can instead be afforded by processing different spectra according to the DEEP-STD NMR equation:[1]

$$\Delta\mathrm{STD}_i = \frac{STD_{\ exp1,i}}{STD_{\ exp2,i}} - \frac{1}{n}\sum_{i}^{n}\left(\frac{STD_{\ exp1,i}}{STD_{\ exp2,i}}\right)$$

ΔSTDi = differential STD value
*exp*1 and 2 = two different experimental conditions
i= each proton

To obtain a consistent scale of $\Delta\mathrm{STD}_i$ factors, *exp*1 should always be the experiment which shows larger total ligand saturation. The highlighted section of the equation represents the intrinsic differences in saturation levels under different conditions. For this reason, contrary to STD intensities which are always positive numbers or 0, in DEEP STD processing, values can be positive and negative, as well as numbers close to 0. Positive values indicate higher influence of the *exp*1 conditions, while negative values imply higher impact of the *exp*2 conditions on a determined proton. Finally, the protons showing low variation due to exp conditions will give values in a range around zero; this cut-off of statistical significance was defined for the system under study as 0.4. We thus built DEEP epitope maps similarly to STD epitope maps with color codes identifying the different type of amino acid residue interacting with each interacting proton and thus improving the predicted placement of the ligand inside the binding site.

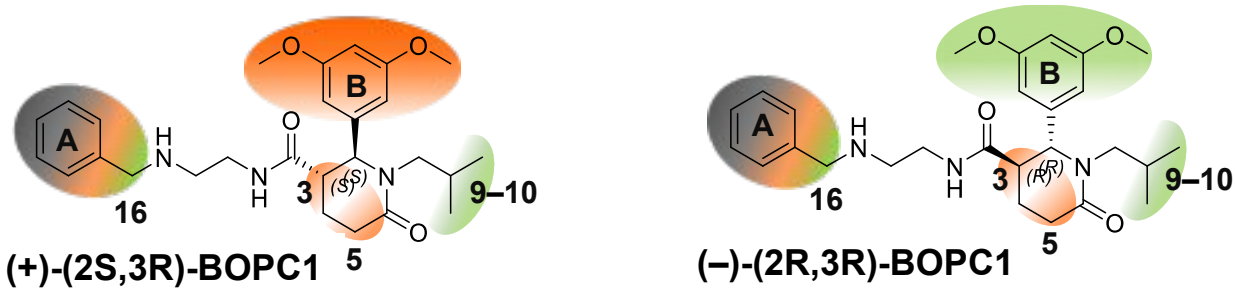
S2.2 STD NMR in D_2O for single enantiomers (+)-(2S,3S)- and (–)-(2R,3R)-BOPC1 vs HuR

(2S,3S)-BOPC1 (D_2O)

	absolute STD%	relative STD %
Ring A (3,4,5)	0.62	100
Ring A (2,6)	0.44	71
Ring B (4)	0.38	61
Ring B (2,6)	0.34	55
16	0.15	24
OCH_3	0.26	42
3	0.4	65
5	0.43	69
9–10	0.17	27

(2R,3R)-BOPC1 (D_2O)

	absolute STD%	relative STD %
Ring A (3,4,5)	0.79	100
Ring A (2,6)	0.32	41
Ring B (4)	0.21	27
Ring B (2,6)	0.22	28
16	0.28	35
OCH_3	0.15	19
3	0.56	71
5	0.34	43
9–10	0.27	34



Representation of the group epitope map for (2S,3S)-**BOPC1** and (2R,3R)-**BOPC1** binding to HuR. Relative STD % ranges are conveyed by color code: black for 100%, orange for values between 40 and 80%, and green for values below 40%. (bottom)

S2.3 Enantio DEEP-STD NMR in D_2O

	absolute STD% (2S,3S)-BOPC1	absolute STD% (2R,3R)-BOPC1	ratio STD (2S,3S)/(2R,3R)	ΔSTD
Ring A (3,4,5)	0.62	0.79	0.78	–0.37
Ring A (2,6)	0.44	0.32	1.38	0.23
Ring B (4)	0.38	0.21	1.81	0.66
Ring B (2,6)	0.34	0.22	1.55	0.40
16	0.15	0.28	0.54	–0.61
OCH_3	0.26	0.15	1.73	0.58
3	0.4	0.56	0.71	–0.44
5	0.43	0.34	1.26	0.11
9–10	0.17	0.27	0.63	–0.52
	sum	**sum**	**STD average**	
	3.19	3.14	1.15	

S2.4 STD NMR in H_2O for single enantiomers vs HuR

(2S,3S)-BOPC1 (H_2O)

	absolute STD%	relative STD %
Ring A (3,4,5)	0.38	100
Ring A (2,6)	0.12	32
Ring B (4)	0.29	76
Ring B (2,6)	0.21	55
16	0.31	82
OCH_3	0.2	53
3	-	-
5	-	-
9–10	0.32	84

(2R,3R)-BOPC1 (H_2O)

	absolute STD%	relative STD %
Ring A (3,4,5)	0.28	85
Ring A (2,6)	0.22	67
Ring B (4)	0.28	85
Ring B (2,6)	0.21	64
16	-	-
OCH_3	0.18	55
3	0.28	85
5	0.33	100
9–10	0.26	79

S2.5 Solvent DEEP-STD NMR of (2S,3S)-BOPC1 vs HuR

	absolute STD% D_2O	absolute STD% H_2O	ratio STD D_2O/H_2O	ΔSTD
Ring A (3,4,5)	0.62	0.38	1.63	0.12
Ring A (2,6)	0.44	0.12	3.67	2.16
Ring B (4)	0.38	0.29	1.31	–0.20
Ring B (2,6)	0.34	0.21	1.62	0.11
16	0.15	0.31	0.48	–1.03
OCH_3	0.26	0.2	1.30	–0.21
3	0.4	-	-	*
5	0.43	-	-	*
9–10	0.17	0.32	0.53	–0.98
	sum	**sum**	**STD average**	
	3.19	1.83	1.51	

* For protons 3 and 5, ΔSTD cannot be calculated but the contribution in D_2O is clearly prevalent, thus, the binding is mediated by hydrophobic residues.

S2.6 Solvent DEEP-STD NMR of (2R,3R)-BOPC1 vs HuR

	absolute STD% D_2O	absolute STD% H_2O	ratio STD D_2O/H_2O	ΔSTD
Ring A (3,4,5)	0.79	0.28	2.82	1.45
Ring A (2,6)	0.32	0.22	1.45	0.08
Ring B (4)	0.21	0.28	0.75	–0.62
Ring B (2,6)	0.22	0.21	1.05	–0.32
16	0.28	-	-	*
OCH_3	0.15	0.18	0.83	–0.54
3	0.56	0.28	2.00	0.63
5	0.34	0.33	1.03	–0.34
9–10	0.27	0.26	1.04	–0.33
	sum	**sum**	**STD average**	
	3.14	2.04	1.37	

* For proton 16, ΔSTD cannot be calculated but the contribution in D_2O is clearly prevalent, thus, the binding is mediated by hydrophobic residues.

S3. Molecular Modeling

Our modeling simulations were performed starting from the co-crystal structure of the two N-terminal RRM domains of HuR complexed with RNA (pdb 4ED5).[4] The complex was prepared by means of Protein Preparation Wizard implemented in Maestro using OLPS-2005 as force field allowing us to add all the missing side chains; the residual crystallographic buffer components and water molecules were removed, hydrogen atoms were added, and side chains protonation states were assigned at pH 7.4.[5–7]

The HuR–RNA complex was submitted to 10000 MacroModel minimization steps, using OPLS_2005 as force field.[7,8]

In order to consider the protein as ensembles of conformational states, we submitted the HuR–mRNA complex to 500 ns of Molecular Dynamics simulations (MDs).[9,10]

MD simulations were run using Desmond package v. 3.8 at 300 K temperature and ensemble NPT class, the system was immersed in a orthorhombic box of TIP4P water molecules, extending at least 10 Å from the protein, and counter ions were added to neutralize the system charge. The resulting trajectory was clustered with respect to RMSD (Root Mean Square Deviation), in order to explore all the collections' structures obtained, getting ten representative structures, which were submitted to 10.000 Macromodel minimization steps, using OLPS-2005 as force field.

In detail, according to Prime calculate Energy tool,[6] the *lowest* and *highest-energy* structures, were selected for the further molecular recognition studies. Furthermore, the two selected HuR conformations correspond to "open" and "closed" protein structure. For our docking studies we chose the "closed" HuR conformations according to our previous procedure.[11]

The two trans-configured isomers (2R,3R)- and (2S,3S)- BOPC1 compounds were prepared by means of LigPrep Tools, pH 7.4 and were submitted to 10000 MacroModel minimization step, using OPLS_2005 as force field.[7,8]

Docking simulations were carried out with Glide, software by using SP v. 7.8 (standard precision) algorithm, 100 poses for ligand were generated.[12] Compound poses were ranked by the Docking Scores and the best docking pose of each compound was subjected to Molecular Mechanism Generalized Born Suface Area (MM-GBSA), using VSGB as solvation model and OPLS_2005 as force field.[6,7]

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