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Bovine Serum Amine Oxidase and Polyamine Analogues: Chemical Synthesis and Biological Evaluation Integrated with Molecular Docking and 3-D QSAR Studies

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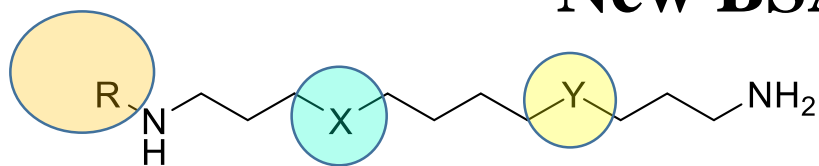
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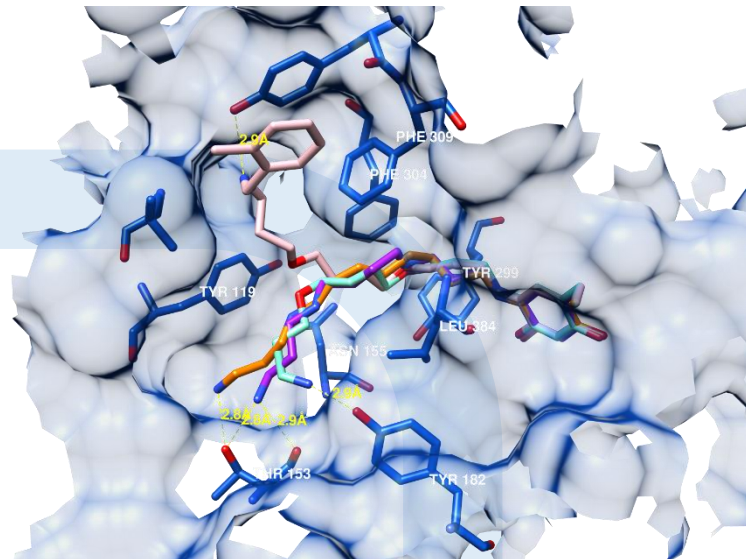
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Journal of Chemical Information and Modeling

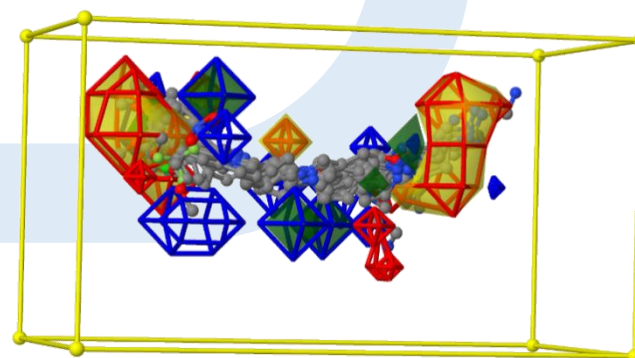
New BSAO Substrates



#	R	X	Y	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)
10		N	N	7.2	0.109
13		N	N	6.0	0.098
21		O	O	3.8	0.124



Molecular Docking

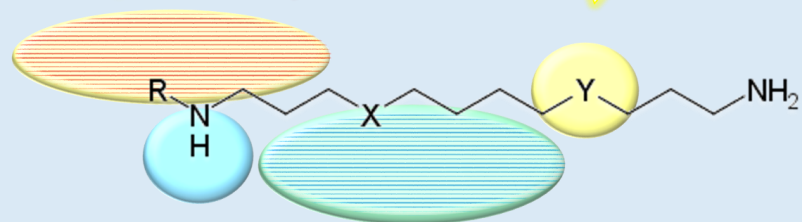


3-D QSAR

Avoid flexible and hydrophobic substituents

Insert HB donor groups

Reduce steric interactions
Y=N,O preferred than C



Maintain HB donor group and prefer electrostatic rather than steric interactions

Branching and inserting constraints to increase steric hindrance and reduce flexibility
X=N~O

Bovine Serum Amine Oxidase and polyamine analogues: chemical synthesis and biological evaluation integrated with molecular docking and 3-D QSAR studies.

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13 ‡ these authors contributed equally to this work
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16
17 This work is a tribute to Giampiero Tempera Ph.D.
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22 **Keywords:** Bovine Serum Amine Oxidase; Polyamine Analogues; Covalent-Docking; 3-D
23 QSAR; Py-CoMFA; Py-ComBinE
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27 **Abbreviations** (footnote 1st page): BSAO: bovine serum amine oxidase; PA: polyamine; SB:
28 structure-based; LB ligand-based; 3-D QSAR: three-dimensional quantitative structure-activity
29 relationships; ComBinE: comparative binding energy; MD: molecular docking; CMD: covalent
30 molecular docking; RMD: reversible molecular docking; Spm: spermine.
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Abstract

Natural polyamines (PAs) are key players in cellular homeostasis by regulating cell growth and proliferation. Several observations highlight that polyamines (PAs) are also implicated in pathways regulating cell death. Indeed, the PAs accumulation cytotoxic effect, maximized with the use of Bovine Serum Amine Oxidase (BSAO) enzyme, represents a valuable strategy against tumor progression. In the present study, along with the design, synthesis and biological evaluation of a series of new spermine (Spm) analogs (**1-23**), a mixed Structure-Based (SB) and Ligand-Based (LB) protocol was applied. Binding modes of BSAO-PA modeled complexes led to clarify electrostatic and sterical features likely affecting the BSAO-PA biochemical kinetics. LB and SB Three-Dimensional Quantitative Structure-Activity Relationship (Py-CoMFA and Py-ComBinE) models were developed by means of the 3d-qsar.com portal and their analysis represent a strong basis for future design and synthesis of PAs BSAO substrates for potential application in oxidative stress-induced chemotherapy.

Introduction

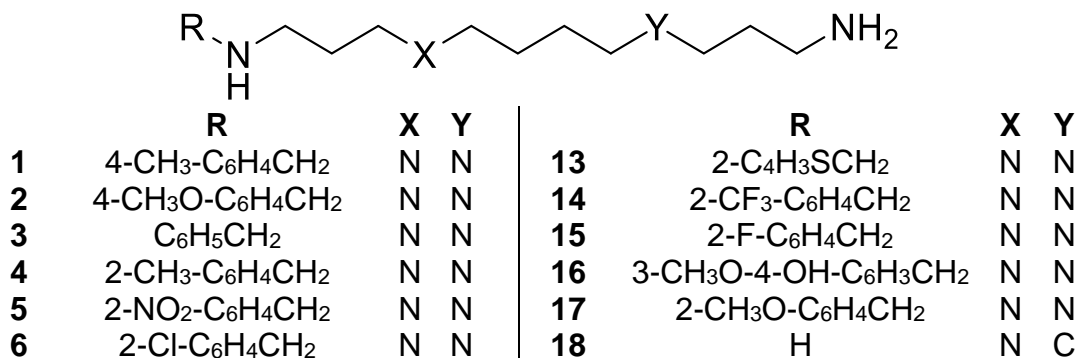
The natural polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm), are ubiquitous polycationic molecules present in all organism cells, where they support a wide variety of cellular functions.¹ Due to their polycationic structures, PAs are able to interact with various biological macromolecules, such as nucleic acids, phospholipids and proteins (receptors, ionic channels and enzymes), thus modulating various metabolic and signaling pathways. Indeed, PAs are involved in cellular homeostasis, cell growth, proliferation, and cell death.¹ The dysregulation of PA metabolism is associated with various pathological conditions. In particular, the PA biosynthetic pathway is very active during cancer cell growth, and PA content is increased in tumor cells and tissues, such as breast, colon, skin and prostate cancers.^{2, 3} Given the role of natural polyamines in cancer, efforts have been made in search for strategies that target the PA metabolism, which might be useful for tumor treatment.^{4, 5} In this context, the main investigated strategies have been pointed to reduce intracellular PA concentration by inhibition of their synthesis or transport across cell membranes, as well as through the use of analogues that interfere with PA metabolism. More recently, the strategy of exploiting PA-degrading enzymes to induce cancer cells death has been applied.^{5, 6} Indeed, the oxidative deamination of PAs catalyzed by some amine oxidases causes an over-production of aldehydes and hydrogen peroxide, that are cytotoxic and can damage cancer cells causing apoptosis/necrosis, and ultimately cell death.⁷⁻¹¹ The induction of oxidative stress by increasing ROS has recently received great attention in anticancer therapies and the effect of various anticancer drugs is based on the principle of oxidative stress-induced chemotherapy,¹² for example, 2-methoxyestradiol induces mitochondrial production of hydrogen peroxide and subsequently activates c-Jun N-terminal kinase (JNK), resulting in apoptosis initiation.¹³

Therefore, the induction of Spm oxidase by PA analogues^{13, 14} or the simultaneous delivery of bovine serum amine oxidase (BSAO) with Spm to produce polyamine metabolites are currently explored as a promising strategy in tumor therapy.¹⁵⁻¹⁹

In particular, the possibility of assembling both BSAO and/or PA analogues in nanoparticles to target cancer cells is recently been explored and seems very promising in this field.^{15, 17, 20} BSAO is a copper and semicarbazide-sensitive-amine oxidase, whose crystal 3D-structure has been resolved, either alone (PDB entry code 1TU5) or complexed with clonidine^{21, 22} (PDB entry code

2PNC). Currently, no BSAO crystal structures complexed with a PA derivative are yet available. The structural studies on BSAO together with the functional studies performed with different substrates, gave information about the most important molecular properties (aliphatic chain length, charge distribution, steric hindrance) for an efficient BSAO substrate.²³⁻²⁶ In particular, in physiological conditions, the electrostatic interaction between the positively charged polyamine skeleton and active site of the enzyme play a crucial role, together with the flexibility of the BSAO secondary structure, which allows the enzyme to accommodate compound/substrate sterically hindered.^{21, 23} Further studies are needed to perform structural optimization of BSAO substrate(s) useful for this specific application in anticancer therapies: substrate(s) for which BSAO should demonstrate high affinity and catalytic efficiency, easy to deliver to cells, soluble in physiological conditions. Molecular docking (MD),²⁷ ligand-based (LB) and structure-based (SB) three-dimensional quantitative structure-activity relationships²⁸ (3-D QSAR) and comparative molecular binding analysis²⁹⁻³¹ (COMBINE) are well- recognized tools to develop quantitative models with the aim to investigate on both the binding mode and the main chemical features taking part to the ligand-macromolecule interaction.

Herein a series of new Spm analogues (**1-23**, Figure 1 and Table 1), characterized by different aryl methyl substituents on one primary amino group of Spm, was designed, synthesized and used as BSAO active site probes through extensive biological kinetic analysis. The experimental data were then integrated with a molecular modeling protocol including reversible and covalent MD simulations and structure-based (SB) and ligand-based (LB) quantitative methods to shed light on the main features guiding the BSAO/PA interactions for future drug design of new and more effective PA analogues.



7	2,3,4-CH ₃ O-C ₆ H ₄ CH ₂	N	N	19	2-CH ₃ -C ₆ H ₄ CH ₂	N	C
8	3,4,5-CH ₃ O-C ₆ H ₄ CH ₂	N	N	20	2-C ₄ H ₃ SCH ₂	N	C
9	2-C ₅ H ₅ N	N	N	21	2-CH ₃ -C ₆ H ₄ CH ₂	O	O
10	4-C ₅ H ₅ N	N	N	22	2-C ₄ H ₃ SCH ₂	O	O
11	3-C ₅ H ₅ N	N	N	23	H	O	O
12	2-C ₁₀ H ₇ CH ₂	N	N	Spm	H	N	N

Figure 1. General structure of the newly synthesized Spm analogues **1-23** as BSAO substrates

Results and discussion

Structural design of PA analogues

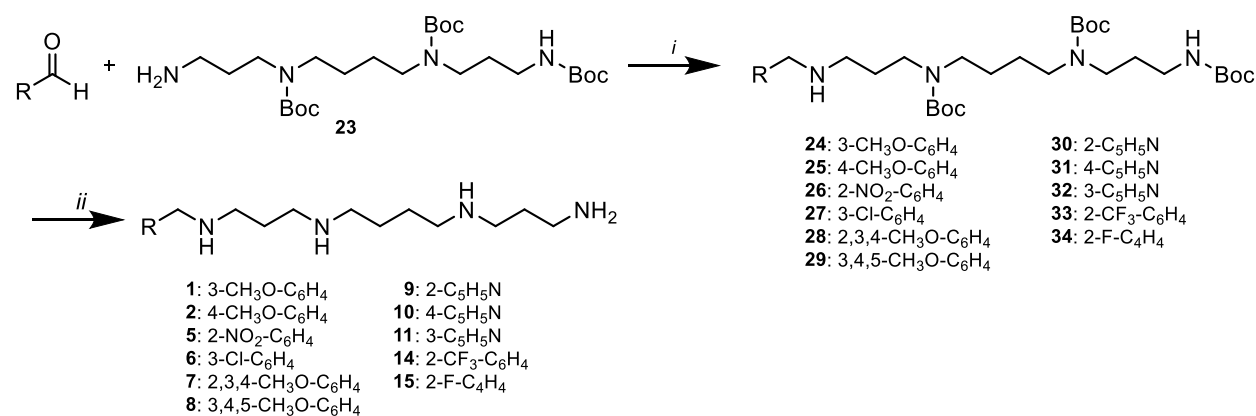
The structure modifications applied on the Spm scaffold, concerned the insertion of unsubstituted and substituted (hetero)aromatic rings on one of the primary amine functions, and the replacement of one or both the inner secondary amine groups in order to explore the activity towards BSAO.

In particular, compounds **1-8** and **14-17** are direct Spm derivatives characterized by a benzyl ring, carrying substituents with different steric and electronic properties. Spm was also substituted with a larger aromatic ring, such as naphthalene (**12**), and heteroaromatic ring, such as pyridine (**9-11**) and thiophene (**13**). To evaluate the importance of a polycationic chain in favor of BSAO activity, triamine derivatives **18-20** and diamines **21** and **22**, the commercially available Spm dioxo analogues (**23**) and Spm were also included in the study. In particular, one of the inner nitrogen atoms of Spm of compounds **4** and **13** was replaced with a methylene group, affording compounds **18, 19** and **20**, respectively, while the replacement of both inner nitrogen atoms of compounds **4** and **13** with oxygen atoms led to diamino-ether derivatives **21** and **22**, respectively.

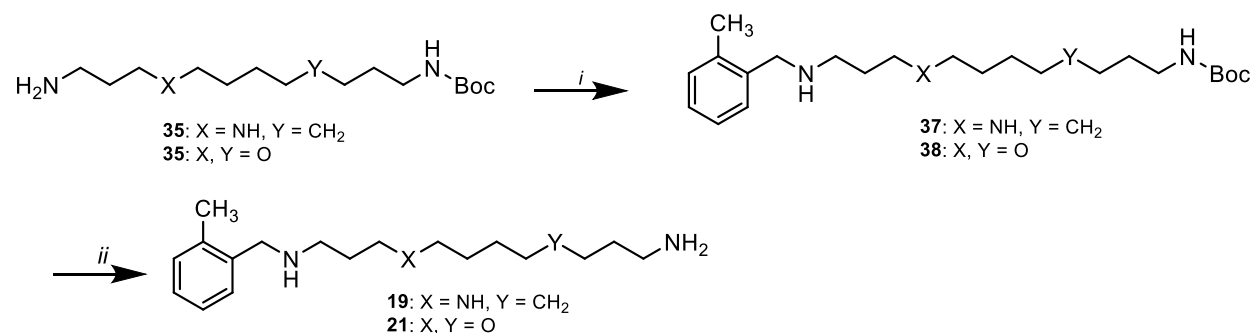
Chemistry

The syntheses of the PA analogues are reported in Schemes 1 and 2. Tetraamines **3, 4**,³² **17**,³³ **16**,³⁴ **12, 13, 20** and **22**³⁵ were synthesized as previously reported. Tetraamines **1, 2, 5-11, 14** and **15** were synthesized as depicted in Scheme 1: the appropriate aldehydes were reacted with tri-Boc-spermine³⁶ to afford the corresponding Schiff bases which were reduced in situ to give compounds **24-34**; acidic deprotection of such compounds led to the corresponding tetraamines **1, 2, 5-11, 14** and **15** as tetrahydrochloride salts.

Synthesis of **19** and **21** was performed as shown in Scheme 2. Briefly, 2-methylbenzaldehyde was reacted with the appropriate mono-Boc-protected diamines **35** and **36**³⁵ to afford the corresponding Schiff bases which were reduced in situ to give compounds **37** and **38**, respectively; acidic deprotection of such compounds led to the corresponding target compounds **19** and **21** as dihydrochloride salts, respectively.



Scheme 1. (i) (a) toluene/ Δ /3 h, (b) NaBH₄/EtOH/rt/4 h, (ii) HCl 3M/rt/overnight; Boc = (CH₃)₃COCO-



Scheme 2. (i) (a) 2-CH₃-C₆H₄CHO, toluene, reflux, 3 h, (b) NaBH₄, EtOH, rt, 4h; (ii) HCl 3M, rt, overnight quantitative yields; Boc = (CH₃)₃COCO-

Biological assay. Kinetic parameters of BSAO for the various polyamine analogues

The PA derivatives **1-23** were evaluated through biological assays aimed to determine their kinetic parameters. The K_M values showed that the affinity of BSAO for the various analogs spanned in the range 2-100 μ M. Even if BSAO showed the best affinity (lowest K_M values), for either the

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3 physiological Spm or the diamine Dioxa (**23**), some analogues were also found to be adequate
4 BSAO substrates. In particular, **4**, **10**, **12** and **13**, among the Spm derivatives, and **20** (triamines)
5 and **21**, **22** (diamines) showed K_M values in the single digit micromolar range. Low- affinity, that
6 is high K_M values, were obtained for **3**, **6**, **7**, **15**, and **19**, being the last one with the lowest affinity
7 among all the tested compounds ($K_M = 97 \mu\text{M}$). This strong effect on K_M values depending on the
8 substituents (R) on the primary amine of Spm skeleton (“N-C₍₃₎-N-C₍₄₎-N-C₍₃₎-N”), likely indicates
9 the important role of this moiety in substrate docking /anchoring into BSAO active site. The
10 catalytic constant (k_{cat}) values spanned in the range 0.15-1.5 s⁻¹, that is in a narrower range than
11 that of K_M . In addition to Spm, ($k_{\text{cat}} = 1.5 \text{ s}^{-1}$), the highest k_{cat} values (1.29-0.91s⁻¹) were found for
12 **8**, **9**, **11** and **23**. Most of the other k_{cat} values spanned in the range 0.46-0.80 s⁻¹; k_{cat} values lower
13 than 0.45 were found only for **4**, **12**, **18** and **19**, being this last one the worst substrate ($k_{\text{cat}} = 0.04$
14 s⁻¹). These data suggest that the different type of moiety bonded at the SPM tail could affect the
15 chemical step of the oxidative deamination of the substrate (H₂O₂ and aldehyde production and
16 release).

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27 The BSAO catalytic efficiency values (k_{cat}/K_M) for these compounds, a kinetic parameter that
28 controls the reaction rate under the non-saturating concentration of substrate ($[S] \ll K_M$), were
29 found to be lower than that for the physiological Spm substrate, with the exception of **23**, for which
30 a catalytic efficiency 1,2 times that for SPM was found. For the other compounds, if compared to
31 Spm, the resulting relative catalytic efficiency values, $(k_{\text{cat}}/K_M)/(k_{\text{cat}}/K_{M_Spm})$, were higher than 0.2
32 ($k_{\text{cat}}/K_M > 0.1 \mu\text{M}^{-1} \text{ s}^{-1}$) for **8**, **10**, **13** and **21** only. The other relative catalytic efficiency values were
33 in the medium range 0.2-0.1 for **9**, **11**, **12**, **20** and **22** (k_{cat}/K_M values in the range 0.1-0.05 $\mu\text{M}^{-1} \text{ s}^{-1}$
34), and lower of more than one order of magnitude for the other compounds (relative catalytic
35 efficiency < 0.1), being **19** the compound with the lowest catalytic efficiency ($k_{\text{cat}}/K_M = 0.0004$
36 $\mu\text{M}^{-1} \text{ s}^{-1}$).

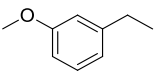
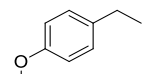
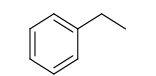
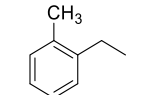
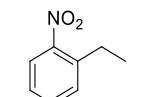
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45 The lack of one of the secondary amine groups in the PA skeleton as in triamine **18**, (N-C₍₃₎-N-
46 C₍₄₎-C-C₍₃₎-N vs Spm) led to a decrease in the k_{cat} value (0.31 vs 1.5 s⁻¹), even more relevant in
47 compound **19**, bearing a toluene ring with an associated lowest affinity (the highest K_M value), k_{cat}
48 and k_{cat}/K_M . Differently, the substitution of both of the secondary amine groups with oxygen atoms
49 in the Spm skeleton as in **23**, determined a decrease in k_{cat} value (0.9 vs 1.5 s⁻¹ of Spm) and
50 improved the affinity and catalytic efficiency respect to Spm. Also, in this case, the addition of a
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toluene ring on the tail (compound **21**) induced a decrease in the affinity and catalytic constant respect to **23**, even if not so strong of that observed as in the case of the triamine (**18** vs **19**).


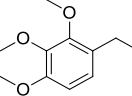
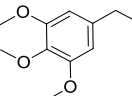



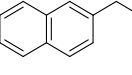



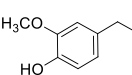





Among the tested analogues, the non-physiological substrates with the best profile in terms of low K_M and high k_{cat} and k_{cat}/K_M values among the tested analogues were the tetramines **8** and **10** and the diamine **23**, bearing a trimethoxy benzyl and a pyridinyl moiety, suggesting the importance of both the role of the Spm skeleton and the substituents in the recognition with BSAO active site and therefore on the associated catalytic process. Additionally, **13** and **21**, endowed with high affinity (K_M) and significative catalytic efficiency (k_{cat}/K_M), even though with a middling k_{cat} value, can be considered as interesting BSAO substrates.

Furthermore, as **3**, **11**, **13** and **20**, were previously shown to be substrates both of spermine oxidase^{37, 38} (SMOX) and acetylpolymine oxidase³⁹ (APAOX), it cannot be excluded that **8** and **10**, and/or other of BSAO analogues might be substrate/s of these enzymes, that, producing cytotoxic reaction products, could potentiate the effect of BSAO and to be useful/synergic in the anticancer therapy.

Table 1. Molecular structure of PA substrates **1-23** and associated experimental kinetic parameters of BSAO

# PA	R	X	Y	K_M (μM) ^a	k_{cat} (s^{-1}) ^a	k_{cat}/K_M ($\text{s}^{-1} \times \mu\text{M}^{-1}$)	Relative k_{cat}/K_M ^b
1		N	N	18.6±2.8	0.52±0.08	0.0280	0.06
2		N	N	40.7±4.1	0.66±0.07	0.0162	0.03
3^{c,e}		N	N	33.9±4.1	0.51±0.06	0.0150	0.03
4		N	N	7.2±0.9	0.25±0.03	0.0347	0.07
5		N	N	16.8±2.5	0.73±0.11	0.0435	0.09

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6		N	N	29.7±4.6	0.80±0.12	0.0269	0.06
7		N	N	35.0±4.6	0.67±0.09	0.0191	0.04
8		N	N	11.0±1.4	1.29±0.16	0.1173	0.25
9		N	N	12±1.8	0.91±0.14	0.0758	0.16
10		N	N	7.2±0.9	0.79±0.10	0.1097	0.23
11^{d,e}		N	N	23.8±3.6	1.25±0.19	0.0525	0.11
12^{d,e}		N	N	7.8±1.2	0.40±0.06	0.0513	0.11
13^{d,e}		N	N	6.0±0.8	0.59±0.08	0.0983	0.21
14		N	N	17.0±2.6	0.61±0.09	0.0359	0.08
15		N	N	38.0±4.9	0.52±0.07	0.0137	0.03
16		N	N	16.2±2.4	0.57±0.09	0.0352	0.08
17		N	N	19.8±2.6	0.47±0.06	0.0237	0.05
18	H	N	C	10±1.3	0.31±0.04	0.0310	0.07
19		N	C	97.0±14.6	0.04±0.01	0.0004	0.00
20^e		N	C	8.4±1.1	0.46±0.06	0.0548	0.12
21		O	O	3.8±0.5	0.47±0.06	0.1237	0.26
22^c		O	O	7.0±1.0	0.46±0.07	0.0657	0.14

23	H	O	O	1.6±0.2	0.90±0.12	0.5625	1.20
Spm	H	N	N	3.2±0.46	1.5±0.2	0.4688	1.00

^a Kinetic parameters determined in buffer potassium phosphate 0.01 M at pH 7.6 (T=37°C)

^b: k_{cat}/K_M of BSAO for Spm, taken as reference substrate, was: $0.469 \pm 0.050 \text{ s}^{-1} \mu\text{M}^{-1}$.

^c **3** = BnSPM.^{32, 34}

^d Compounds previously tested as amine oxidase substrate/inhibitor.³²

^e Compounds previously tested as a substrate of amine oxidase.³⁵

Molecular Modeling

Methodology overview.

At the best of the authors knowledge, only two experimental BSAO structures were actually available from the protein databank⁴⁰ (Figure 2): an inhibited one co-crystallized with the inhibitor clonidine²¹ (CLO) (PDB entry code 2PNC) and the free unbound BSAO protein²² (PDB entry code 1TU5).

The 2PNC BSAO/CLO was used to select the docking software through a reversible docking assessment procedure. Furthermore, cross-docking experiments^{41, 42} were also performed as the BSAO bound topaquinone cofactor^{43, 44} (TPQ, 2,4,5-trihydroxyphenylalanine quinone) was observed to have two distinct sidechain conformations in either the CLO bound (2PNC) or the free structure (1TU5). Slightly different TPQ conformations were also visible within the two copies for each BSAO crystal structure (Figure 2).

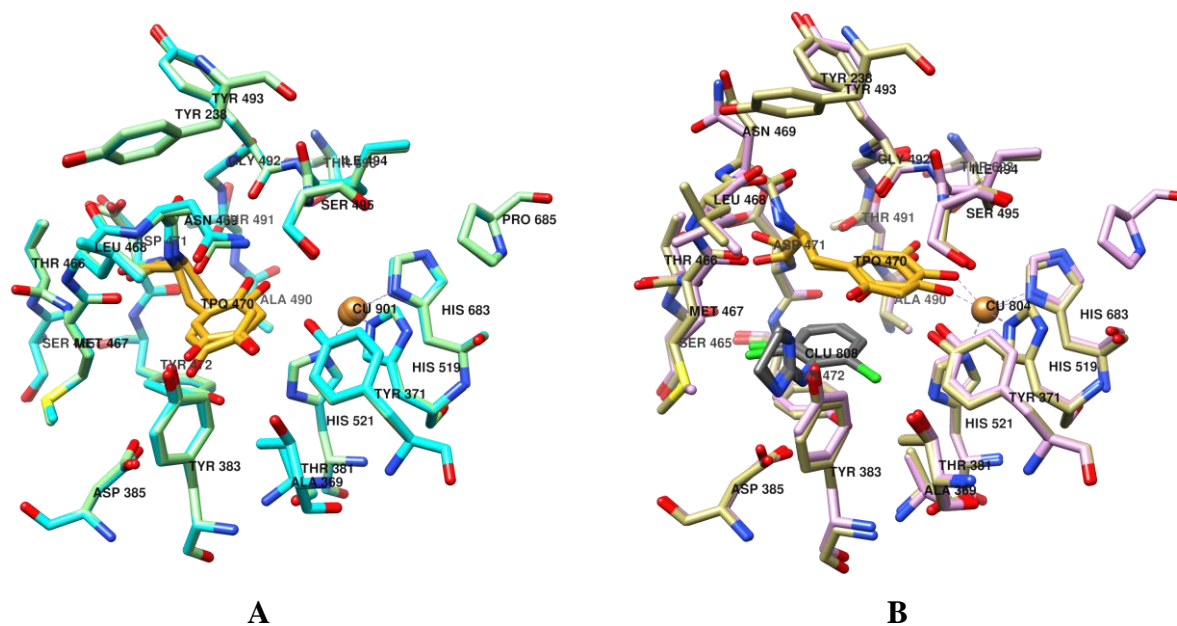


Figure 2. BSAO binding site as experimentally observed in 1TU5 (A) and 2PNC (B) crystal structures. As for each deposited PDB code two copies (chains A and B) of BSAO were available in the crystal unit cell both chains are depicted. 1TU5A and 1TU5B are reported in cyan and light green colored carbon atoms, respectively. While 2PCNA and 2PCNB are depicted in dark khaki and orchid colored carbon atoms, respectively. Gold and light gray colored carbon atoms indicate TPQ and clonidine (only for 2PNC), respectively. Color codes are referred to those indicated in the UCSF Chimera program.

The docking into free BSAO (1TU5) was performed to confirm the software inability to find low energy poses comparable to those obtained into the inhibited protein (2PNC).

As previously reported,²² TPQ catalyzes the primary amine substrates oxidation to aldehyde and the concomitant production of hydrogen peroxide through a ping pong mechanism.^{4, 45} This reaction mechanism, is likely dependent on the TPQ conformation. Indeed, the two experimental different BSAO sidechain TPQ orientations indicate the *off copper* and *on copper* states⁴⁶ as found in 1TU5 and 2PNC, respectively. Regardless of the TPQ orientation, MD simulations were carried out on PAs analogues either with classical reversible molecular docking (RMD) procedures or with a covalent molecular docking (CMD) method through covalently binding of Spm and derivatives **1-23** to the TPQ oxygen in position 5. The two docking procedures led to define a series of PAs' aligned datasets that were separated from the proteins and used to build a LB/SB

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3 combined CoMFA-like (SB-LB 3-D QSAR) and COMBINE-like models (SB 3-D QSAR) by
4 means of the Py-CoMFA and Py-ComBinE web apps, respectively, both available from the 3d-
5 qsar.com portal.^{28, 47} LB 3-D QSAR models were also built from scratch using the PAs' SMILES
6 structures (Figure 3)
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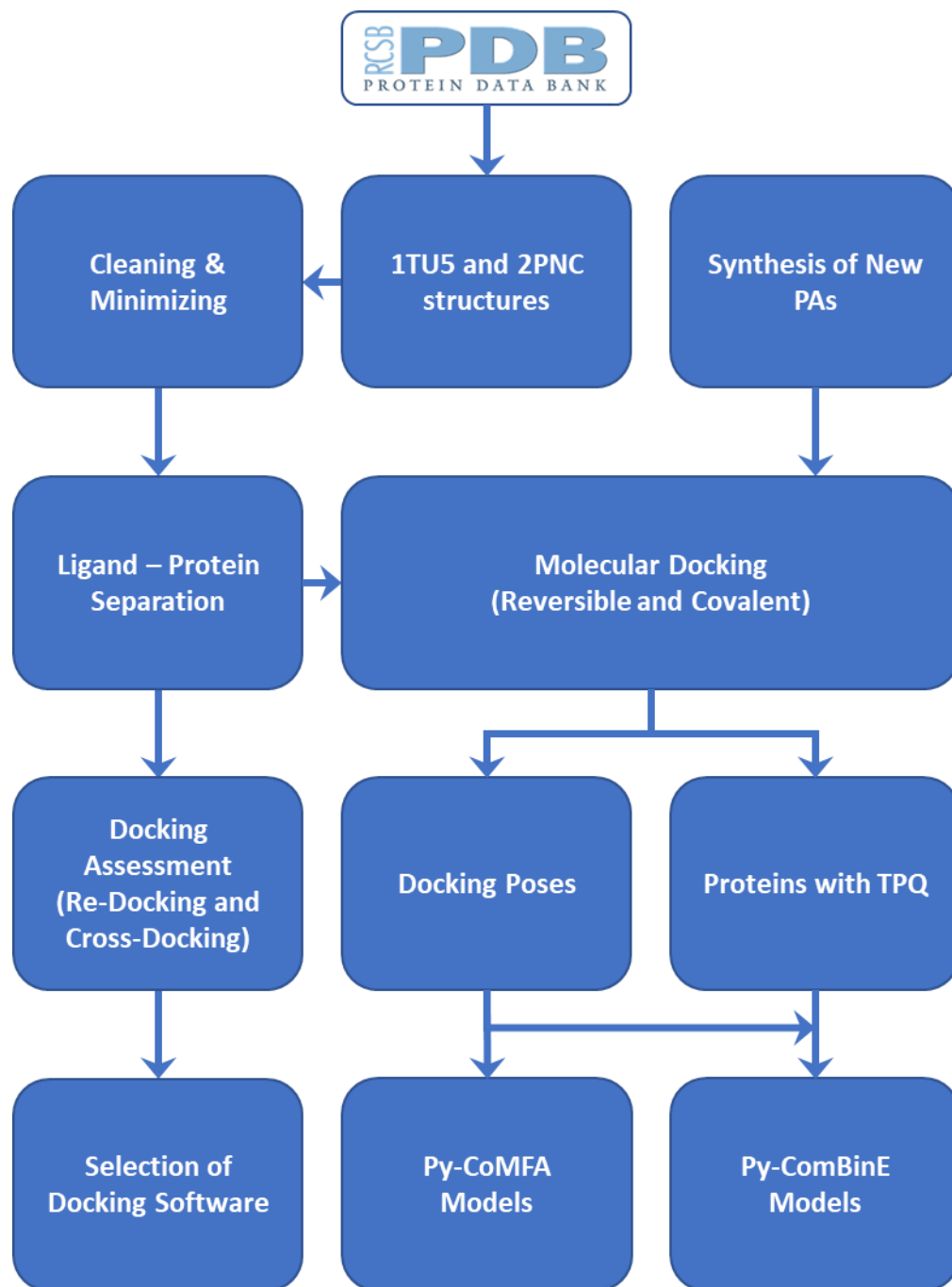


Figure 3. General procedure applied in the molecular modeling study.

RMD Assessment

As available within the 3d-qsar.com portal, the programs Smina⁴⁸ and Plants⁴⁹ were used to assess which software/scoring function combination would result in a lower average root mean square deviation (RMSD) value. As a matter of fact, the cross-docking of clonidine into the free BSAO showed a high RMSD value indicating the drug was not able to correctly bind to the *off copper* BSAO conformation (Table 2 and Supporting Information Tables SI1 and SI2). On the other hand, the RMSD values of redocked clonidine into the *on copper* BSAO conformation confirmed the programs to be able to reproduce, to some extent, the binding conformation of clonidine. In particular, the Plants/PLP program/scoring function combination revealed to be the most effective one with average RMSD values of 1.56 and 0.62 (Table 2). From re-docking experiments was also evident that clonidine was much better repositioned in the chain A of 2PCN than in chain B.

Table 2. RMSD and docking scores values for the docking assessment from clonidine experimental random conformations. Only values for the Plants/PLP combination are shown as was the best performing one. For the other results see Supporting Information Tables SI1 and SI2.

Ligand ^a	Conformation ^b	Protein ^c								Best Docked Lock ^c	Best Docked ^f		Average ^e RMSD
		1TU5B		1TU5A		2PNCA		2PNCB			RMSD	Score	
		RMSD ^d	Score ^e	RMSD	Score	RMSD	Score	RMSD	Score				
2PNCA	EC	15.34	-58.72	15.53	-59.99	2.69	-77.13	2.74	-66.52	2PNCA	2.69	-77.13	1.56
		7.86	-59.58	15.12	-59.19	0.42	-77.04	2.48	-65.91		2PNCA	0.42	
2PNCA	RC	12.22	-61.60	11.80	-62.69	0.56	-79.83	2.57	-67.62	2PNCA	0.56	-79.83	0.62
		13.54	-61.03	11.28	-61.80	0.67	-79.83	1.15	-67.06		2PNCA	0.67	

^a: PDB code from which the CLO ligand was extracted, the last letter indicates the chain.

^b: EC = experimental conformation; RC = random conformation

^c: PDB code of the protein used as a receptor, the last letter indicates the chain.

^d: Root Means Squared Deviation

^e: PLP score

^f: RMSD and Score for the best scored docked conformation (low is better)

^g: Average RMSD value for CLO docking into the 2PNC chains

RMD Docking of the tested PAs

As the Plants/PLP program/scoring function combination was the most effective it was thus applied as a tool to investigate the unbound conformation of the titled PAs into the four BSAO proteins chains extracted from 1TU5 and 2PNC. Although the new PAs should be considered covalent ligands the RMD could represent a tool to investigate the pre-covalently bound conformations. As expected, and considering that any classical docking program is normally developed to simulate binding for compounds acting as inhibitors the scores for the PAs, working as substrates, were mainly found higher (higher docking energies) for poses docked into the inhibited BSAO *on copper* protein conformation (2PNC) (Supporting Information Tables SI3-SI5). The predicted binding energies therefore indicated the PAs were not able to tightly bind BSAO. At a deeper inspection all of the tested PAs bearing one of the primary amino groups substituted by an aryl methyl group were found docked with the aryl methyl head overlapped to the bound clonidine inhibitor (Supporting Information Figure SI1). To seek for alternate and more homogeneous binding modes, further RMD runs were performed into either only 1TU5 chains or only 2PNC chains. Still, many of the PAs docked conformations were found showing the aryl methyl head in the proximity of TPQ in either docking simulations (Supporting Information Figures SI2 and SI3). Among them **7, 8, 14, 16, 17** docked into 1TU5 protein chains showed a conformation with the primary amino group in the proximity of TPQ residue. These results confirmed the RMD is not efficient for docking of BSAO substrates. Regarding the docking into only 2PCN chains only **7** and **8** were found to dock with the aryl methyl groups away from the TPQ residues, thus indicating that still the RMD would not have proposed homogenous docked poses among all the PA derivatives. Regardless the PAs' poses, a direct comparison of the

experimental kinetic data (K_M and k_{cat}/K_M) with the docking scores revealed the existence of some correlation (Table 3). In particular among K_M and docking scores a weak negative correlation was observed, while a strong direct correlation was calculated among k_{cat}/K_M and the docking scores (Table 3).

Table 3. RMD energies in comparison with the experimental kinetic data K_M and k_{cat}/K_M

PA^a	K_M	k_{cat}/K_M	Best Docked Score^b	2PNC Best Docked Score^c	1TU5 Best Docked Score^d
1	18.60	0.0280	-113.23	-113.34	-101.73
2	40.70	0.0162	-109.81	-113.16	-108.63
3	33.90	0.0150	-113.26	-111.05	-111.44
4	7.20	0.0347	-114.93	-115.17	-100.43
5	16.80	0.0435	-116.98	-114.84	-99.72
6	29.70	0.0269	-112.10	-114.86	-102.90
7	35.00	0.0191	-106.21	-104.75	-100.81
8	11.00	0.1173	-110.91	-109.25	-102.73
9	12.00	0.0758	-104.21	-105.57	-104.83
10	7.20	0.1097	-106.64	-109.14	-102.96
11	23.80	0.0525	-106.13	-107.63	-103.46
12	7.80	0.0513	-118.46	-115.55	-112.28
13	6.00	0.0983	-103.97	-103.66	-103.59
14	17.00	0.0359	-115.50	-114.41	-103.28
15	38.00	0.0137	-112.71	-115.61	-113.15
16	16.20	0.0352	-119.50	-115.56	-102.43
17	19.80	0.0237	-113.34	-113.17	-101.79
18	10.00	0.0310	-87.63	-83.03	-87.67
19	97.00	0.0004	-117.29	-116.03	-108.35
20^e	8.40	0.0548	-109.92	-107.70	-103.75
21	3.80	0.1237	-114.66	-112.71	-107.13
22	7.00	0.0657	-105.63	-105.24	-104.12

23	1.60	0.5625	-81.44	-76.55	-81.12
Spm	3.20	0.4688	-82.78	-77.44	-83.07
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Pearson correlation			-0.35	-0.37	-0.41
coefficient with K_M					
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Pearson correlation			0.77	0.80	0.76
coefficient with k_{cat}/K_M					
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^a: Polyamine number

^b: the best score from cross-docking experiments into the four chains

^c: the best score from cross-docking experiments into only 2PNC chains

^d: the best score from cross-docking experiments into only 1TU5 chains

CMD Docking of the tested PAs

Since the PAs were proved to act as BSAO substrates, they would undergo a covalent interaction with the TPQ, making a Schiff intermediate before the oxygen activation and the subsequent oxidation of the imine (Supporting Information Figure SI5).^{45, 50, 51} This mechanism was observed for the imidazole and 2-hydrazinopyridine bound to TPQ in the human semicarbazide-sensitive amine oxidase (SSAO)/vascular adhesion protein-1 (VAP1) available crystal structures 2Y74 and 2C11, respectively.^{52, 53} Following the PA oxidation mechanism (Supporting Information Figure SI5) a CMD protocol was set up to investigate the possible mechanism of interaction between BSAO and the titled PAs (Figure 4).

In the CMD procedure an overall mimicking of the reversible PA-TPQ adduct formation was simulated in discrete steps, thus the PAs' covalent docked conformations were subsequently dissociated in two steps: a first local minimization of the unbound PAs and a second one using a reconstituted TPQ structure.

First, each PA was modeled from its SMILES structure and covalently linked to the TPQ, deprived of the oxygen in position 5, to form a TPQ-PA adduct residue, taking care of the proper protonation state in agreement with the experimental assay conditions (covalent docking step 1 in Figure 4). Then, by means of the latest version of Vina software⁵⁴ a water molecule was docked while letting the TPQ-PA adduct to flexibly move. Either the vina⁵⁴ or vinarado⁴⁸ scoring functions were used during the CMD and were also used to re-score each separated PAs docked conformations leading

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3 to furnish two covalently docked molecule datasets ($PA_{Cov-Vinardo}$ and $PA_{Cov-Vina}$) to be used as
4 training set in the subsequent 3-D QSAR modeling (covalent docking step 2 in Figure 4).
5 Subsequently, each PA was unbound from the respective TPQ adduct and the complex minimized,
6 through the Smina docking software minimization feature, while keeping the TPQ deoxygenated
7 in position 5, to avoid atoms overlapping. This process mimicked the formation of the C=N Schiff
8 base double bond (dissociation step 1 in Figure 4) in agreement with the experimental covalently
9 bound 2-hydrazinopyridine as found in the SSAO.⁵² The best energy scored minimized PAs
10 furnished a further couple of datasets ($PA_{Min1-Vinardo}$ and $PA_{Min1-Vina}$) to be used as a training set to
11 build either 3-D QSAR or COMBINE models (with the associated protein counterpart). Then a
12 second Smina minimization round was performed after the reconstitution of the full TPQ structure
13 leading to the $PA_{Min2-Vinardo}$ and $PA_{Min2-Vina}$ datasets as 3-D QSAR and COMBINE training sets
14 (dissociation step 2 in Figure 4). Two further COMBINE training sets were also compiled using
15 the unminimized full TPQ containing BSAOs and the $PA_{Min1-Vinardo}$ and $PA_{Min1-Vina}$ mimicking the
16 reacting PA with the TPQ oxygen in position 5 ($PA_{Min3-Vinardo}$ and $PA_{Min3-Vina}$) thus mimicking the
17 close detachment/attachment of the PA from the deoxygenated TPQ. The procedure was repeated
18 for each of the four chains available from the 1TU5 and 2PNC crystal structures.
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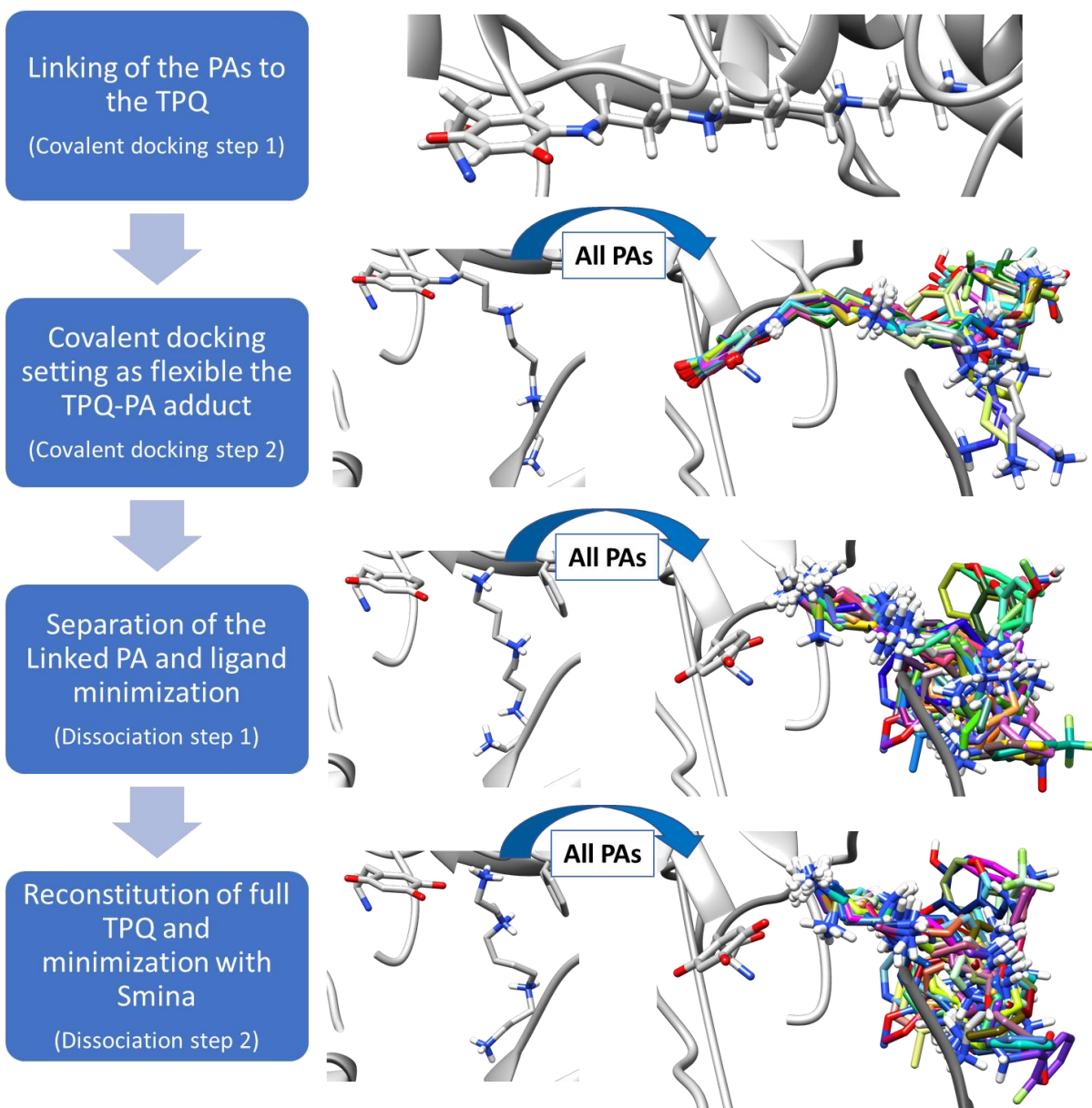
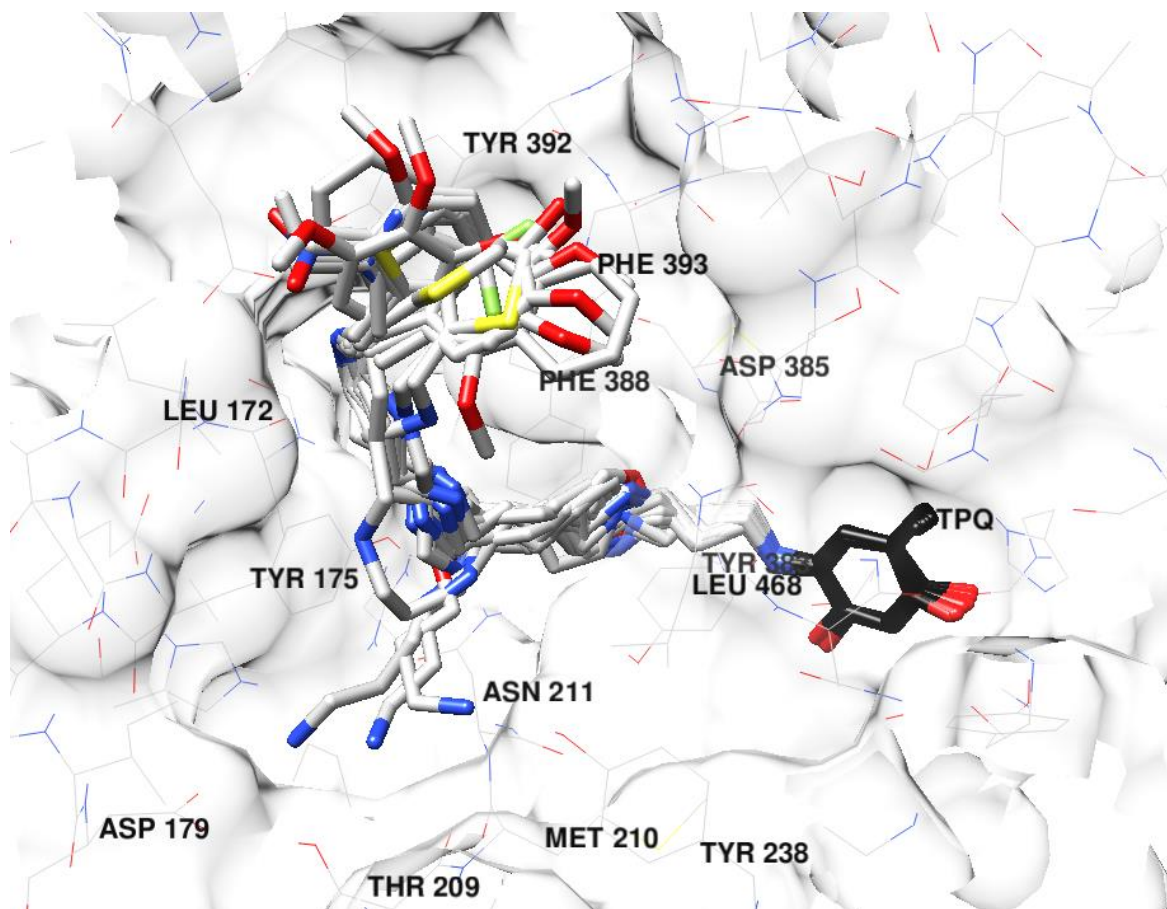


Figure 4. Workflow of the CMD procedure and the subsequent minimizations of the covalently docked PAs. The Spm is shown as an example for a given PA. Conformations of Dissociation step 1 were merged into BSAO with fully reconstituted TPQ (see main text)

Inspection of proposed docked poses obtained applying two scoring functions (vina and vinardo) in the four available chains displayed eight different scenarios. Only the CMD with the vinardo scoring function in the chain A of 1TU5 led to poses all docked in the same area as well as all the TPQ oriented in the same direction (Figure 5 and Supporting Information Figure SI4). The

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3 orientation of the TPQ was consistent with the *off copper* state (1TU5) observed in the free BSAO
4 crystal structure and overlapped to that of the 2-hydrazinopyridine/TPQ adduct as found in the
5 SSAO complex⁵² (2C11).
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42 **Figure 5.** PAs covalent binding mode proposed by the Vina program and selected by a rescore
43 with the vinardo scoring function using the CMD procedure. In black the TPQ and light gray the
44 chain A of 1TU5 residues and its 80% transparent surface.
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49 All covalently docked rescored arylmethyl derivatives **1-17** and **19-22** were placed the aryl heads
50 in a mostly hydrophobic pocket formed by Leu172, Tyr175, Glu417, Tyr392 and Phe393
51 sidechains, while compounds **18**, **23** and Spm without any arylmethyl moiety at the terminal
52 primary amino groups were all found in a different pocket mainly formed by Asp179, Thr209,
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3 Met210, Asn211, Phe226 and Tyr238. Clearly the primary amino group capped with the aryl
4 methyl somehow has a definite influence in the binding mode of PAs. For compounds **18**, **23** and
5 Spm the main driving force are the hydrogen bonds taking place between their terminal amino
6 groups and Thr209 and Tyr238 hydroxyls (Figure 6A).
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10 Notably, compound **21** showed a BSAO affinity comparable to that of Spm, nevertheless from a
11 simple analysis of steric contacts no particular feature or interaction could be associated with its
12 potency. The only difference with all the other arylmethyl derivatives is the 2-methyl group and
13 the oxygen bridge that being an ether is better hosted in the small hydrophobic tube formed by
14 Tyr175, Tyr383, Phe388, Phe393 and Leu468 (Figure 6A). A slightly different scenario was
15 observed after the minimization with Smina in which the role of Tyr238 was of greater importance
16 for PA/BSAO molecular recognition. From the two sequential minimizations of dissociated PAs,
17 it seems that Tyr238 would represent the main anchor point to drive the substrates to the
18 subsequent covalent binding to TPQ (Figure 6B). This observation was particularly constant for
19 nine out of ten compounds (Spm, **4**, **10**, **12**, **13**, **18**, **20**, **21** and **23**) characterized by K_M values up
20 to 10 μM (not shown).
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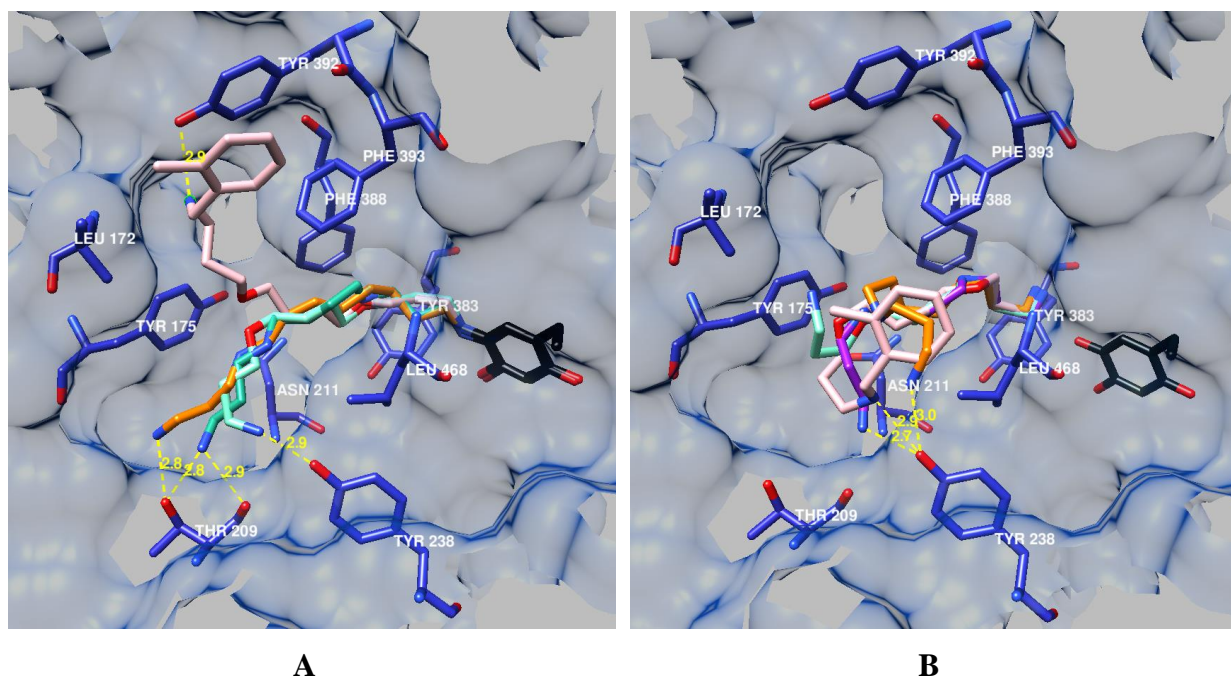


Figure 6. Spm (green in A and purple in B), **18** (orange), **21** (pink) and **23** (aquamarine) binding mode proposed by the Vina program and selected by a rescore with the vinardo scoring function using the CMD procedure. In panel A, the conformations bonded to the TPQ. In panel B the conformations after two local minimizations with Smina (last step in Figure 4). The chain A of 1TU5 residues and its 80% transparent surface were depicted in dodger blue. In black the conformation of TPQ as found during the CMD procedure.

3-D QSAR Modeling

General procedure.

3-D QSAR models were built by means of the Py-CoMFA web app available from the 3d-qsar.com freely accessible portal either starting from the PAs' SMILES structures (Full LB models) or from their docked conformations obtained from either the RMD or the CMD procedure above described (Mixed LB-SB models) (Figure 7).

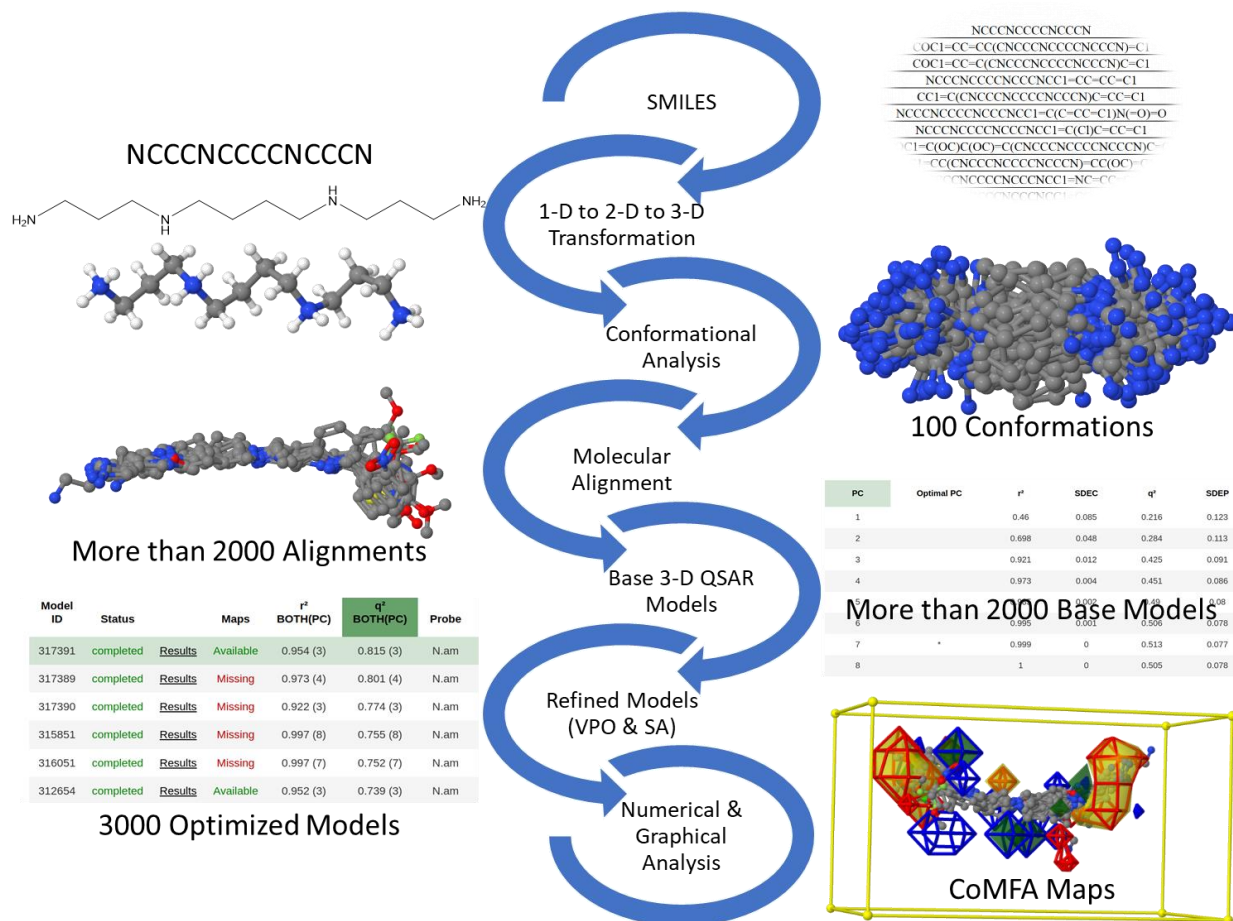


Figure 7. Workflow to build the 3-D QSAR models. LB models started from SMILES structures, while SB started directly from the “Default 3-D QSAR Models” step.

Full Ligand-Based Models

The Full LB models were built by first seeking for the best alignment rule through a series of eleven conformational analyses run on all PAs.⁵⁵ Then for each conformational analysis 32 different molecule templates and 6 combinations of alignment software settings led to 2112 molecular alignments used to build 2112 Py-CoMFA models using the default settings (see experimental section). The procedure was applied to either pK_M ($-\log_{10}[K_M]$) or $p(k_{cat}/K_M)$ ($-\log_{10}[k_{cat}/K_M]$) values as dependent variable (Table 1). The model (LB1, Table 4) endowed with the highest q^2 value (0.47 at 5 principal components, Supporting Information Table SI6) was obtained using the pK_M values, conformational analysis calculated with the balloon software⁵⁵ using the MMFF94 force field and aligned by means of RDKit library (<http://www.rdkit.org>) using

the “Best Score” alignment scoring function. As template molecules the one with highest LogP value (**19**, LogP = 3.364) in its longest conformation (Supporting Information Table SI7 and Figure SI6) was used. Using the pK_{cat}/K_M activity value a maximum q^2 value of 0.41 was reached.

Model LB1 was optimized by running 3000 random variable pretreatment optimizations (VPO, Supporting Information Figure SI7) to furnish model LB2, selected based on highest q^2 (0.74) and lowest number of optimal PCs (3) (Table 4, Supporting Information Table SI8) obtained with the amidic probe atom (N.am) and charge model, minimum sigma, grid spacing, dielectric constant and cutoff of MMFF94, 0.8, 2.7, 1.0, 3 and 2.0, respectively (Supporting Information Table SI9). The final LB3 model (Table 4, Supporting Information Tables SI10 and SI11) was obtained by means of a simulated annealing (SA) feature selection and characterized by a q^2 value of 0.82 with significant increments of 74% and 11% with respect to LB1 and LB2 models.

Table 4. LB1, LB2 and LB3 Py-CoMFA models’ statistical coefficients.

# ^a	Probe Atom ^b	# PCs ^c	r^2 ^d	SDEC ^e	q^2 ^f	SDEP ^g
LB1	N.am	5	0.99	0.01	0.47	0.08
LB2	N.am	3	0.95	0.01	0.74	0.04
LB3	N.am	3	0.95	0.01	0.82	0.03

^a: model ID

^b: atom to calculate the molecular interaction fields

^c: optimal number of PLS principal components

^d: squared correlation coefficient

^e: Standard error of calculation by the model

^f: cross-validated squared correlation coefficient

^g: Standard error of prediction during cross-validation

Model LB3 was analyzed by inspecting the CoMFA-like maps obtained by the product of the PLS coefficients (Coeffs) by the average molecular interaction field (MIF) values to give the average activity contribution (AAC) maps for either steric (STE) or electrostatic (ELE) MIFs (Supporting Information Figure SI9). Although model LB3 was of outstanding statistical levels being the model built on highly closely related compounds the associate AAC maps were of low utility. The most

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3 important characteristic that LB3 model highlighted relates to the length of the molecules. In fact,
4 arylmethyl head bearing PAs' are longer than those with unsubstituted two primary amine groups.
5 This was clearly underlined by the Py-CoMFA yellow polyhedrons (STE negative AAC, panel A
6 and C of Supporting Information Figure SI9) that resided on top of the molecules' ends. While
7 compound **23** did not overlap the above polyhedrons, the lowest active derivative (**19**) being longer
8 than **23** extended both ends over the two yellow polyhedrons. This would suggest that the aryl
9 methyl heads should not be used as substituents leading to longer molecule, but instead as
10 replacements of the propylamino groups of both Spm and **23** so to keep as much as possible their
11 molecule length. On the other hand, a few small green polyhedrons (positive AAC_{STE}) are around
12 the overlapped molecules (not shown) without a clear indication on which possible substitution
13 could exert some influence on pK_M. These observations thus suggested that to expand the model
14 applicability some branched PA should also be investigated.

15 Regarding AAC_{ELE} maps, similarly to AAC_{STE}, the most important polyhedrons were the two red
16 on the extremities of the molecules indicating the importance of a hydrogen bond donating group
17 in those regions (panel B and D of Supporting Information Figure SI9). While one end largely
18 satisfied these requirements, the ends corresponding to the aryl methyl head were mostly highly
19 hydrophobic and lacked of hydrogen bond donor groups. Some compounds partially filled this
20 lack with the presence of heteroatoms or hydroxyl groups (i.e. **10** or **16**), therefore it could be
21 speculated that the insertion of more than one hydroxyl or even an aminomethyl group would
22 likely lead to a better biochemical kinetic profile.

39 **Ligand-Based and Structure-Based Mixed Models**

40 From the PA analogues docking results, a series of SB-aligned datasets were obtained and promptly
41 uploaded into the 3d-qsar.com portal to build 3-D QSAR model with the same protocol applied to
42 already aligned molecules.²⁸ Default Py-CoMFA models were built for the RMD datasets, using
43 either pK_M or pK_{cat}/K_M, but none of them showed satisfying q² values and therefore were not further
44 investigated. A different scenario was observed for the six datasets (PA_{Cov-Vinardo}, and PA_{Cov-Vina},
45 PA_{Min1-Vinardo}, PA_{Min1-Vina}, PA_{Min2-Vinardo} and PA_{Min2-Vina}) obtained with the CMD procedure (Figure
46 4). In agreement with both the LB models and docking results, acceptable models were only
47 obtained (data not shown) using pK_M and the PA conformations docked using the vinardo scoring
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3 function (Figure 5) obtained within the above described dissociation step 1 (Figure 4). Therefore,
4 the $PA_{\text{Min1-Vinardo}}/pK_M$ dataset was subjected to the same Py-CoMFA procedure described above
5 (Figure 7) by skipping the alignment steps. Therefore after 3000 random VPO optimizations
6 (Supporting Information Table SI12) the top three different models were further investigated by
7 SA feature elimination to select the model characterized by the amidic atom probe (N.am) and
8 charge model, minimum sigma, grid spacing, grid extension, dielectric constant and cutoff of q_{eq} ,
9 0.4, 2.9, 5, 59 and 28, respectively (Supporting Information Tables SI12-SI14 and Figure SI10).
10 Being aligned by a SB method the Py-CoMFA model associated maps can be directly interpreted
11 overlapped to the BSAO binding site to combine the docking results with the 3-D QSAR data
12 (Figure 8). This kind of overlapping clearly shows the convergence of the two techniques (CMD
13 and 3-D QSAR). Taking as references Spm and **19** as the highest affinity and lowest processed
14 substrates, respectively, the AAC_{STE} (Figure 8A) and the AAC_{ELE} (Figure 8B) polyhedrons were
15 found in agreement with the above discussed binding mode. In particular, Spm was mainly
16 surrounded by green polyhedron (positive AAC_{STE}) and the largest one was actually indicating as
17 important for the Spm-Tyr238 interaction. On the other hand, a big yellow (negative AAC_{STE})
18 polyhedron greatly overlapped to the **19** benzylaminopropyl portion while fulfilling the
19 hydrophobic pocket formed mainly by Leu172, Phe388, Tyr392 and Phe393 residues. Regarding
20 the AAC_{ELE} plot, a small blue polyhedron highlighted the hydrogen bond between the Spm primary
21 amino group and the Tyr238 hydroxyl moiety, other blue spots were also visible, but no specific
22 association can be done. Differently, a big red polyhedron run over the big yellow one, thus
23 indicating that not only the occupation of that portion was negatively associated with the pK_M but
24 also that hydrogen donating groups could enhance the biological activity, in agreement with the
25 full LB models.
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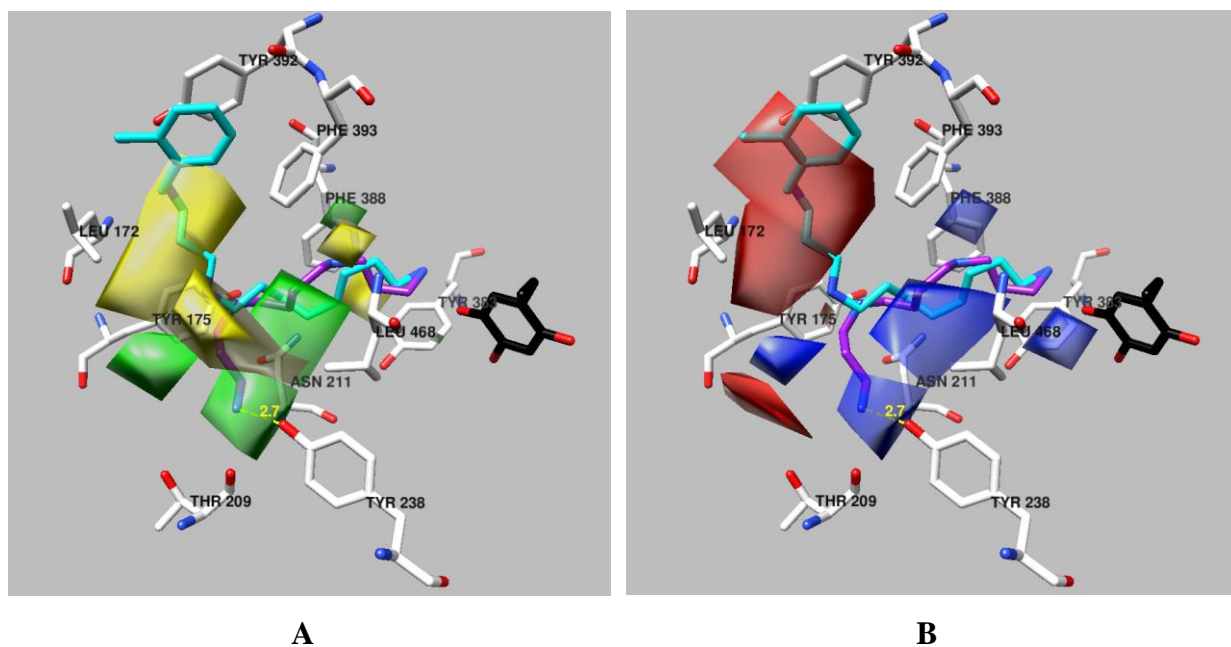


Figure 8. SB 3-D QSAR model AAC maps overlapped to the 1TU5 chain A (white). In panel A the AAC_{STE} plot overlapped to Spm (purple) and **19** (cyan). In panel B AAC_{ELE} plot. In black is also represented the reconstituted TPQ after the separation of the covalently docked PA. The polyhedrons represent the AAC obtained by the product of the PLS coefficients with the average MIF values.

COMBINE Models

Differently from the above CoMFA-like 3-D QSAR approaches (Py-CoMFA), COMBINE represents a full SB 3-D QSAR where the descriptive variables (regression parameters) are represented by the ligand/protein interactions on a per residue basis energy deconvolution. Therefore, as the $PA_{\text{Min1-Vinardo}}/pK_M$ dataset showed some agreement among the docked conformations and the built over SB/LB 3-D QSAR model a COMBINE approach was therefore applied utilizing the Py-ComBinE web app available on the www.3d-qsar.com website. The COMBINE model was built by combining the **1-23** and **Spm** docked conformations with the BSAO containing the reconstituted TPQ as above described in the covalent docking procedure before running the second Smina minimization to produce the $PA_{\text{Min2-Vinardo}}$ dataset. For a complete description of the ligand/BSAO recognition playing forces, four types of ligand/protein interactions were calculated: van der Waals (STE), coulombic (ELE), desolvation (DRY) and

hydrogen bonding (HB) and correlated through the partial least square (PLS) to the pK_M values' vector. To reduce data redundancy, the optimal PLS model was carried out through a SA feature selection leading to a COMBINE model characterized by r^2 , SDEC, q^2 and SDEP values of 0.87, 0.14, 0.62 and 0.25, respectively (Table 5 and Supporting Information Table SI15 and Figure SI11).

Table 5. Py-ComBinE model statistical coefficients.

# PC	r^2 ^b	SDEC ^c	q^2 ^d	SDEP ^e	SDEP Decrement
1	0.37	0.31	0.17	0.36	
2	0.65	0.24	0.36	0.32	11.91%
3	0.83	0.17	0.46	0.29	8.49%
4* ^a	0.87	0.14	0.62	0.25	15.46%
5	0.88	0.14	0.59	0.25	-2.85%

^a: optimal number of PLS principal components

^b: squared correlation coefficient

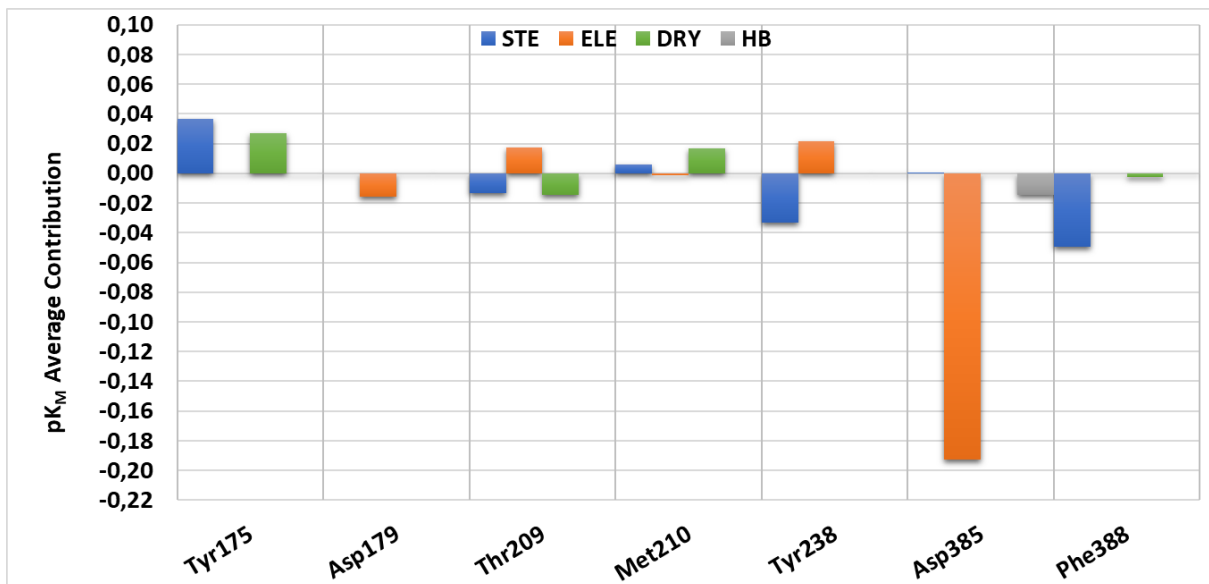
^c: Standard error of calculation by the model

^d: cross-validated squared correlation coefficient

^e: Standard error of prediction during cross-validation

Analogously as for the above CoMFA models, per residue AAC were generated by multiplying each interaction energy by the associated PLS coefficients (AAC_{STE} , AAC_{ELE} , AAC_{DRY} , AAC_{HB} , Figure 9). A strong negative AAC_{ELE} was found to be associated with Asp385 indicating that the lack of interaction with that residue reduces the affinity of about 0.2 pK_M units. This interaction is connected to the TPQ interacting amino group, therefore an optimal distance would ensure the correct formation of the covalent bond. Effective substrates such as compound **23** and Spm showed a distance of 2.7 Å between the primary amino group nitrogen and the Asp385 delta oxygen atom. Longer distances were observed for less efficient substrates such as **2** (2.8 Å), **5** (2.8 Å), **15** (2.8 Å), **19** (2.8 Å) and **20** (2.9 Å) (not shown). Negative AAC_{STE} of about 0.01-0.05 pK_M units were associated with Thr209, Tyr238 and Phe388. As above discussed Thr209 and Tyr238 were already recognized as important residues for hydrogen bonding (Figure 10), therefore any interaction not

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3 electrostatic-like would not have been well accepted. In fact, a positive AAC_{ELE} of 0.02 pK_M units
4 was also associated with Tyr238. Phe388 was already depicted to participate in the shape fitting
5 of the docked PAs and the rigid nature of the COMBINE method cannot account exactly for its
6 role. A positive AAC_{STE} of almost 0.04 pK_M units was associated with Tyr175 which was above
7 recognized as an important residue in hosting the PAs' heads. Regarding AAC_{DRY} , a notably
8 positive value was observed for Tyr175 that summed to the AAC_{STE} further highlights its role for
9 non-electrostatic type interactions, also supported by the lack of any AAC_{ELE} or AAC_{HB} . Met210
10 follows the same tendency as Tyr175 and its hydrophobic contributions are congruent with the full
11 LB and mixed LB/SB studies (Figure 10). A different profile was instead found for Thr209 whose
12 low AAC_{STE} and AAC_{DRY} values supported its role for ELE or HB type interactions.
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41 **Figure 9.** AAC plot for the COMBINE model. To reduce redundancy values were filtered at 0.01
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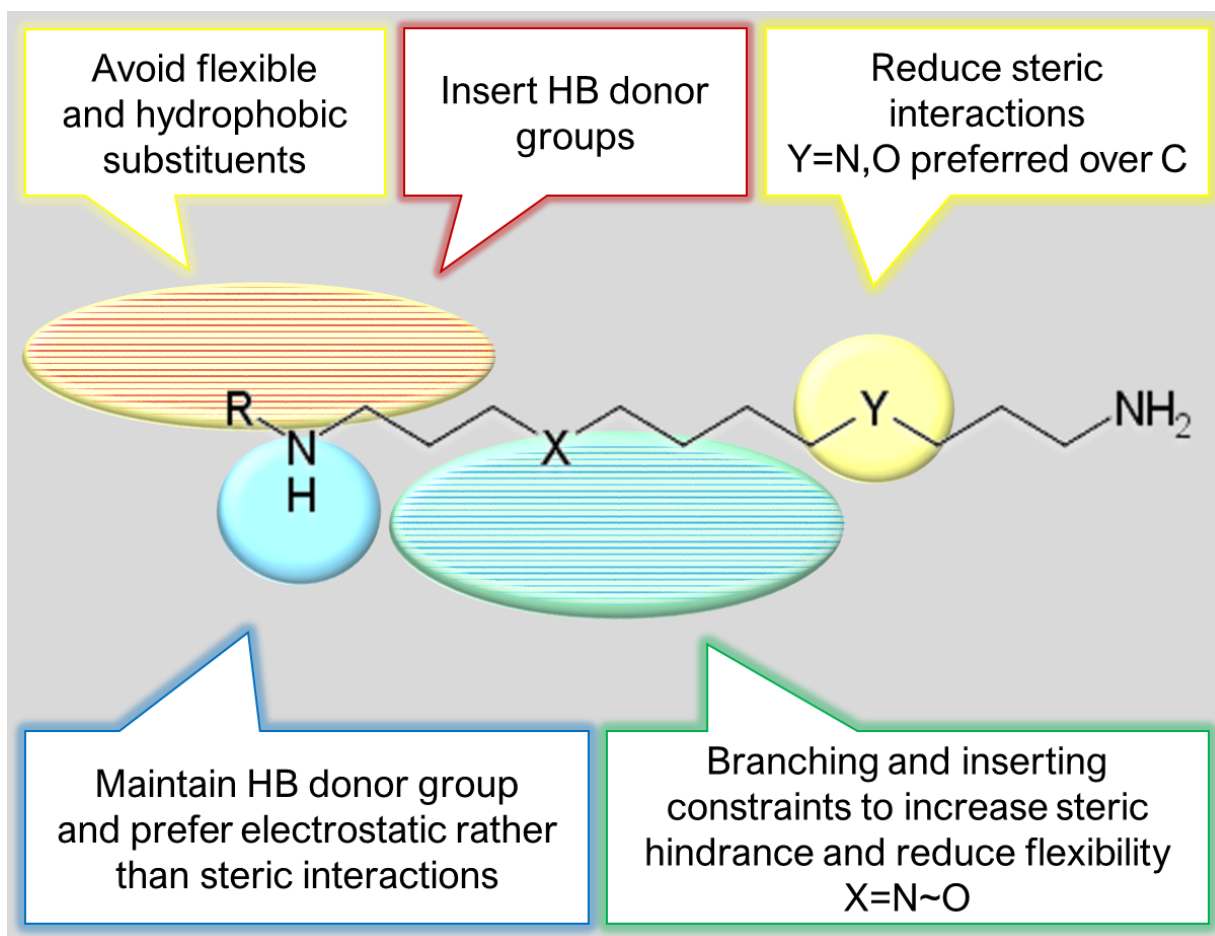


Figure 10. Overall 3-D SAR of Full LB, LB/SB 3-D QSAR and COMBINE models.

Summary of the main conclusions from the molecular modeling studies

Reversible docking (RMD protocol) showed the PA uniformly binding into BSAO with conformations resembling an inhibitor, notwithstanding their energies of docking as predicted by the vina scoring function were found in good agreement with the K_M values, indicating that this tool could be used to fast screen other substrates to subject to deeper molecular modeling investigations. Interestingly the CMD procedure was applied in a fashion to simulate the formation/dissociation of the Schiff base, the first step of the BSAO catalytic mechanism.⁴⁵ The Vina program with the vina scoring function was the selected combination as during the covalent docking was the only one proposing a uniform series of docked conformations. Inspection of the covalently docked PAs indicated that substrates bearing a second free primary amino group preferred bindings slightly different than those bearing an aryl methyl substituent on the amino

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3 termini. Short branched derivatives could couple the two binding modes likely leading to better
4 substrates or BSAO ligands.

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6 The pK_M derived LB 3-D QSAR was useful to state that the optimal substrate should not be too
7 longer than that of the natural substrate Spm. Furthermore, in agreement with the binding mode
8 analysis, the model also suggested that branched derivatives should be designed to expand the
9 model applicability. The LB model could represent a second filter in a virtual screening campaign.
10 A different scenario was observed with the LB-SB mixed 3-D QSAR, obtained with the pK_M
11 values and ligand conformations through the covalent docking procedure. In particular the
12 conformations related to the first minimized dissociated covalently docked PAs furnished the best
13 model. The statistical coefficients goodness suggests that the model could be used as a third filter
14 to select compounds likely able to covalently bind the TPQ similarly as previously reported.⁵⁶ As
15 last, modeling investigation a COMBINE model was developed as a final selection tool for new
16 potential BSAO substrates. As the method associated docking and SB 3-D QSAR^{57, 58} it implies
17 the highest level of investigation that could lead to the selection/design of new potential BSAO
18 substrates. Inspection of the Py-ComBinE model derived on the $PA_{Min3-Vinardo}$ dataset gave insights
19 on the substrate/BSAO residue interactions to be avoided or maintained for future designed PA
20 derivatives.
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34 **Conclusions and perspectives**

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36 A series of new Spm derivatives along with recently reported analogues was herein investigated
37 as BSAO substrates to derive a tool to be applied in the design of compounds to potentially be
38 used in a future anticancer therapy. BSAO as an enzyme that oxidatively deaminates natural PAs
39 while freeing *in situ* cytotoxic compounds (H_2O_2 and aldehydes) known to induce cancer cell
40 death.
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44 From the experimental biological assays results, new effective BSAO substrates emerged among
45 the tested analogues. In particular, compared to the physiological Spm, BSAO showed relevant
46 levels of catalytic constant, efficiency and affinity values for **8** and **10**, **13** (with a Spm skeleton),
47 **21** and **23** (a diamine derivative). As **3**, **11**, **13** and **20**, were previously found to be also substrates
48 for either SMOX or APAOX,³⁹ it cannot be excluded that also **8** and **9**, and/or other analogues
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3 might be substrates for APAOX and/or SMOX and consequently could potentiate the effects in an
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5 eventual anticancer therapy.

6 The experimentally kinetic parameters (K_M and k_{cat}) of the studied compounds were used in LB
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8 and SB applications (CMD, 3-D QSAR and COMBINE) as tools to define 3-D structure-activity
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10 relationships for the future design of new BSAO substrates.

11 Molecular docking combined with LB and SB 3-D QSARs suggested that short branched
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13 derivatives could leading to better substrates or BSAO ligands.

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15 In conclusion, this study represents the first application of a combination of molecular modeling
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17 procedures that could be used for the design of novel BSAO substrates with potential future
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19 pharmacological application.

20 Future perspective will be a further investigation on the novel BSAO substrates here found (**8**, **10**,
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22 **13**, **21** and **23**) and on new designed substrates on the basis of the MD combination with 3-D
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24 QSARs studies to evaluate their cytotoxic effect also in combination with nanoparticles as drug-
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26 delivery system in anti-cancer therapies. In particular, the conjugation of core-shell nanoparticles
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28 with BSAO is a new approach to immobilize this copper-enzyme on gold-polymer nanosystems
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30 stabilized by a hydrophilic macromolecule. The conjugated BSAO-nanoparticles coupled with
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32 novel substrates could have several applications in pharmaceutical fields, as a biosensor, and in
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34 medicine as anti-cancer therapy.^{16, 19}

35 36 **Experimental section**

37 **Chemistry**

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39 The chemicals were analytical grade. Purified bovine serum amine oxidase was prepared as
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41 previously reported.^{17, 18, 44} All the commercially available reagents and solvents were purchased
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43 from Sigma-Aldrich (St. Louis, MO, USA), Alpha Aesar (Haverhill, MA, USA), VWR (Radnor,
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45 PA, USA), and TCI, which were used without further purification. Reactions were followed by
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47 analytical thin layer chromatography (TLC) on precoated TLC plates (layer 0.20 mm silica gel 60
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49 with a fluorescent indicator UV254, from Sigma-Aldrich). Developed plates were air-dried and
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51 analyzed under a UV lamp (UV 254/365 nm), KMnO₄stain. Nuclear magnetic resonance (NMR)
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53 experiments were run on Varian VXR 400(400 MHz for¹H, 100 MHz for¹³C). Chemical shifts
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55 (d) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal

reference and coupling constants (J) are reported in hertz (Hz). The spin multiplicities are reported as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass-spectra were recorded on a VG707EH-F apparatus, and electrospray ionization (ESI) both in positive and negative mode was applied. Compounds were named following IUPAC rules as applied by ChemBioDraw Ultra (version 17.0). All of the final compounds showed 95% purity by elemental analysis.

General procedure for the synthesis of 24-31 and 33-34.

A solution of tri-Boc-spermine (0.502 g, 1 mmol) and the appropriate aldehyde (1.1 mmol) in toluene (25 ml) was refluxed in a Dean-Stark apparatus for 3 h. After cooling, the solvent was removed in vacuo, the residue was taken up in EtOH (30 ml) and NaBH₄ (0.151 g, 4 mmol) was added to a cooled (0 °C) solution and stirring was continued at room temperature for 4 h. The solvent was removed, and the residue was dissolved in CH₂Cl₂(30 ml) and washed with H₂O (3x20 ml). Removal of the dried solvent in vacuo gave a residue that was purified by flash chromatography eluting with CH₂Cl₂/MeOH/33% aq. NH₄OH (9:1:0.05).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((3-methoxybenzyl)amino)propyl)carbamate (24).** Synthesized from 3-methoxybenzaldehyde (0.151 g). Obtained 0.430 g of a yellow oil, 69 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.45 (s, 27H), 1.63-1.76 (m, 6H), 2.64 (t, J = 6.6 Hz, 2H), 3.98-3.25 (m, 12H), 3.78 (s, 2H), 3.82 (s, 3H), 6.81 (d, J = 8.2 Hz, 1H), 6.92 (m, 2H), 7.25 (t, J = 7.2 Hz, 1H).

***tert*-butyl4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((4-methoxybenzyl)amino)propyl)carbamate (25).** Synthesized from 4-methoxybenzaldehyde (0.151 g). Obtained 0.450 g of a yellow oil, 72 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.45 (s, 27H), 1.59-1.70 (m, 4H), 2.63-2.77 (m, 4H), 3.09-3.24 (m, 12H), 3.48 (s, 2H), 3.80 (s, 3h), 6.86 (d, J = 6.3 Hz, 2H), 7.27 (d, J = 6.6 Hz, 2H).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((2-nitrobenzyl)amino)propyl)carbamate (26).** Synthesized from 2-nitrobenzaldehyde (0.166 g). Obtained 0.490 g of a yellow oil, 77 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.46 (s, 27H), 1.69-1.75 (m, 4H), 2.60-2.66 (m, 4H), 4.05 (s, 2H), 7.28-7.30 (m, 1H), 7.42-7.44 (m, 1H), 7.58-7.61 (m, 1 H), 7.93-7.97d (m, 1H).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((2-chlorobenzyl)amino)propyl)carbamate (27).** Synthesized from 2-chlorobenzaldehyde (0.154 g). Obtained 0.530 g of a yellow oil, 84 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.45 (s, 27H), 1.63-1.75 (m, 6H), 2.64 (t, J = 6.6 Hz, 2H), 3.09-3.25 (m, 12H), 3.90 (s, 2H), 7.17-7.39 (m, 4H).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((2,3,4-trimethoxybenzyl)amino)propyl)carbamate (28).** Synthesized from 2,3,4-trimethoxybenzaldehyde (0.216 g). Obtained 0.530 g of a yellow oil, 84 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.39 (s, 27H), 1.60-1.67 (m, 6H), 2.56 (t, J = 6.2 Hz, 2H), 3.04-3.19 (m, 12H), 3.78 (s, 2H), 3.92 (s, 9H), 6.94-7.12 (m, 2H).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((3,4,5-trimethoxybenzyl)amino)propyl)carbamate (29).** Synthesized from 3,4,5-trimethoxybenzaldehyde (0.216 g). Obtained 0.470 g of a yellow oil, 69 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.44 (s, 27H), 1.63-1.76 (m, 6H), 2.64 (t, J = 6.2 Hz, 2H), 3.07-3.22 (m, 12H), 3.72 (s, 2H), 3.81 (s, 3H), 3.85 (s, 6H), 6.60 (s, 2H).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((pyridin-2-ylmethyl)amino)propyl)carbamate (30).** Synthesized from 2-pyridylcarboxaldehyde (0.118 g). Obtained 0.570 g of a yellow oil, 96 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.46 (s, 27H), 1.62-1.76 (m, 6H), 2.65 (s, J = 6.2 Hz, 2H), 3.90 (s, 2H), 7.14-7.33 (m, 2H), 7.61-7.69 (td, J = 7.6, 1.8 Hz, 1H), 8.55 (d, J = 4.6 Hz, 1H).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((pyridin-4-ylmethyl)amino)propyl)carbamate (31).** Synthesized from 4-pyridylcarboxaldehyde (0.118 g). Obtained 0.460 g of a yellow oil, 96 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.457(s, 27H), 1.65-1.72 (m, 4H), 1.91-2.03 (m, 2H), 2.15-2.18 (m, 2H), 3.01-3.12 (m, 10H), 3.16-3.21 (m, 2H), 4.59 (s, 2H), 7.99 (d, 2H, J = 5.1 Hz), 8.65 (d, 2H, J = 4.8 Hz).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((2-(trifluoromethyl)benzyl)amino)propyl)carbamate (32).** Synthesized from 2-trifluoromethylaldehyde (0.191 g). Obtained 0.350 g of a yellow oil, 89 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.45 (s, 27H), 1.47-1.73 (m, 4H), 2.64 (t, 2H, J = 6.0 Hz), 3.08-3.24 (m, 12H), 3.94 (s, 2H), 7.18-7.29 (m, 2H), 7.75-7.92 (m, 2H).

***tert*-butyl(4-((*tert*-butoxycarbonyl)(3-((*tert*-butoxycarbonyl)amino)propyl)amino)butyl)(3-((3-chlorobenzyl)amino)propyl)carbamate (33).** Synthesized from 2-trifluoromethylaldehyde (0.191 g). Obtained 0.300 g of a yellow oil, 83 % yield. ¹H-NMR (300 MHz, CD₃Cl) δ 1.47 (s, 27H), 1.62-1.75 (m, 4H), 2.64 (t, 2H, J = 6.2 Hz), 3.09-3.25 (m, 12H), 3.90 (s, 2H), 7.17-7.42 (m, 4H).

General procedure for the synthesis of the target compounds (1, 2, 5-10, 14 and 15).

A solution of the appropriate compounds (0.5 mmol) in 30 ml of MeOH and 20 ml of aq. HCl 3 M (10 ml) was stirred overnight at room temperature. Following solvent removal, the residue was washed with Et₂O. The resulting solid was filtered and dried to afford the corresponding tetrahydrochloride salt as a white solid. All the compounds were obtained in quantitative yields.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((3-methoxybenzyl)amino)propyl)butane-1,4-diamine (1).** ¹H-NMR (300 MHz, D₂O) δ 1.88–1.62 (m, 4H), 2.91-2.97 (m, 12H), 3.72 (s, 3H), 3.92-4.00 (m, 2H), 6.91-6.94 (m, 3H), 7.26-7.31 (m, 1H); ¹³C-NMR (75 MHz, D₂O) δ 22.67, 22.72, 22.69, 36.50, 43.90, 44.48, 46.93, 51.02, 55.41, 115.08, 115.31, 122.29, 130.53, 131.18, 159.20; MS (ES⁺) 323 (M+H)⁺. ¹H-NMR (300 MHz, CD₃Cl) δ 22.67, 22.72, 23.70, 36.51, 43.90, 44.,159.20. MS (ESI⁺) 162 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((4-methoxybenzyl)amino)propyl)butane-1,4-diamine (2).** ¹H-NMR (300 MHz, D₂O) δ 1.60-1.64 (m, 4H), 1.90-1.99 (m, 4H), 2.93-3.01 (m, 12H), 3.72 (s, 3H), 4.06 (s, 2H), 6.91-6.94 (m, 2H), 7.27-7.31 (m, 2H); ¹³C-NMR (75 MHz, D₂O) δ 25.20, 25.28, 26.27, 39.08, 46.16, 47.05, 49.50, 53.21, 57.95, 117.14, 125.50, 134.06, 162.31; MS (ES⁺) 323 (M+H)⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((2-nitrobenzyl)amino)propyl)butane-1,4-diamine (5).** ¹H-NMR (300 MHz, D₂O) δ 1.65-1.68 (m, 4H), 1.93-2.00 (m, 4H), 2.95-3.09 (m, 10H), 3.21-3.26 (m, 2H), 4.42 (s, 2H), 7.57-7.65 (m, 2H), 7.71-7.75 (m, 1H), 8.15-8.17 (m, 1H); MS (ESI⁺) 169 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((2-chlorobenzyl)amino)propyl)butane-1,4-diamine (6).** ¹H-NMR (300 MHz, D₂O) δ 2.09-2.13 (m, 4H), 2.42-2.50 (m, 4H), 3.42-3.51 (m, 10H), 3.57-3.61 (m, 2H), 4.77 (s, 2H), 7.76-7.82 (m, 2H), 7.87-7.90 (m, 2H); MS (ESI⁺) 164 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((2,3,4-trimethoxybenzyl)amino)propyl)butane-1,4-diamine (7).** ¹H-NMR (300 MHz, D₂O) δ 1.79-1.83 (m, 4H), 2.10-2.18 (m, 4H), 3.12-3.21 (m, 12H), 3.91 (s,

3H), 3.92 (s, 3H), 3.98 (s, 3H) 4.25 (s, 2H), 6.94 (d, 1H, J = 8.8 Hz), 7.20 (d, 1H, J = 8.8 Hz); ¹³C-NMR (75 MHz, D₂O) δ 22.46, 22.68, 23.67, 36.48, 43.64, 44.44, 44.47, 46.94, 56.02, 60.95, 61.38, 108.27, 116.38, 126.66, 140.97, 151.70, 154.67; MS (ESI⁺) 192 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((3,4,5-trimethoxybenzyl)amino)propyl)butane-1,4-diamine (8).**

¹H-NMR (300 MHz, D₂O) δ 1.79-1.83 (m, 4H), 2.12-2.19 (m, 4H), 3.12-3.22 (m, 12H), 3.83 (s, 3H), 3.91 (s, 6H), 4.24 (s, 2H), 6.89 (s, 2H); ¹³C-NMR (75 MHz, D₂O) δ 22.60, 22.68, 23.67, 36.48, 43.77, 44.43, 44.47, 46.93, 51.25, 56.15, 60.85, 107.42, 126.93, 137.45, 152.77; MS (ESI⁺) 192 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((pyridin-2-ylmethyl)amino)propyl)butane-1,4-diamine (9).**

¹H-NMR (300 MHz, D₂O) δ 1.76-1.80 (m, 4H), 2.04-2.12 (m, 2H), 2.16-2.24 (m, 2H), 3.07-3.21 (m, 10H), 3.33-3.36 (m, 2H), 4.66 (s, 2H), 7.98-8.01 (m, 1H), 8.07-8.09 (m, 1H), 8.49-8.54 (m, 1H), 8.82-8.83 (m, 1H); ¹³C-NMR (75 MHz, D₂O) δ 22.58, 22.68, 23.66, 36.45, 44.29, 44.47, 44.97, 46.91, 46.96, 48.11, 127.26, 127.41, 144.54, 145.49, 145.73; MS (ESI⁺) 147 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((pyridin-4-ylmethyl)amino)propyl)butane-1,4-diamine (10).**

¹H-NMR (300 MHz, D₂O) δ 1.64-1.68 (m, 4H), 1.94-2.00 (m, 2H), 2.06-2.11 (m, 2H), 2.95-3.09 (m, 10H), 3.20-3.24 (m, 2H), 4.52 (s, 2H), 8.05 (d, 2H, J = 5.1 Hz), 8.76 (d, 2H, J = 4.8 Hz), 8.49-8.54 (m, 1H), 8.82-8.83 (m, 1H); ¹³C-NMR (75 MHz, D₂O) δ 22.58, 22.69, 22.67, 36.49, 44.33, 44.48, 45.14, 46.98, 49.36, 127.34, 142.16, 150.96; MS (ESI⁺) 147 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((2-(trifluoromethyl)benzyl)amino)propyl)butane-1,4-diamine**

(14). ¹H-NMR (300 MHz, D₂O) δ 1.64-1.68 (m, 4H), 1.93-2.09 (m, 4H), 2.76-3.06 (m, 10H), 3.16-3.18 (m, 2H), 4.34 (s, 2H), 7.51-7.55 (m, 2H), 7.56-7.64 (m, 1H), 7.72-7.74 (m, 1H); MS (ESI⁺) 181 (M+2H)²⁺.

***N*¹-(3-aminopropyl)-*N*⁴-(3-((2-fluorobenzyl)amino)propyl)butane-1,4-diamine (15).**

¹H-NMR (300 MHz, D₂O) δ 1.61-1.68 (m, 4H), 1.93-2.03 (m, 4H), 2.95-3.10 (m, 12H), 4.22 (s, 2H), 7.11-7.8 (m, 2H), 7.35-7.43 (m, 2H); MS (ESI⁺) 156 (M+2H)²⁺.

General procedure for the synthesis of 15 and 16

A solution of **13** or **14** (1 eq) and 2-methylbenzaldehyde (1 eq) in toluene (10 ml) was stirred at the refluxing temperature in a Dean-Stark apparatus for 3 h. After cooling, the solvent was evaporated, and the residue was dissolved in EtOH (10 ml), cooled in an ice bath, and treated with

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3 NaBH₄ (4 eq); the reaction mixture was stirred 4h at room temperature. The solvent was then
4 removed and the residue was dissolved in DCM (20 ml) and washed with H₂O (3 x 20 ml).
5
6 Removal of the dried solvent in vacuo gave a residue that was purified by flash chromatography
7
8 with a mixture of petroleum ether/CH₂Cl₂/MeOH/33% aq. NH₄OH (5:4:1:0.02) to give **15** or **16**
9
10 as a yellow oil.

11 **tert-butyl (8-((3-((2-methylbenzyl)amino)propyl)amino)octyl)carbamate (37):** compound **35**
12 (0.200 g, 0.7 mmol), 2-methylbenzaldehyde (0.7 mmol, 0.090 g, 0.08 ml), sodium borohydride
13 (2.8 mmol, 0.106 g); yield 64%. ¹H-NMR (400 MHz, CdCl₃) δ 1.28 (s, 9H), 1.44-1.51 (m, 12H).
14
15 1.75-1.79 (m, 2H), 2.23 (s, 3H), 2.61-2.63 (m, 2H), 2.75-2.79 (m, 4H+2H exch D₂O), 3.08-3.10
16
17 (m, 2H), 3.77 (s, 2H), 4.67 (brs exch D₂O, 1H), 7.14-7.16 (m, 3H), 7.28-7.29 (m, 1H).

18
19 **tert-butyl (3-(4-(3-aminopropoxy)butoxy)propyl)carbamate (38):** compound **36** (0.213 g, 0.7
20
21 mmol), 2-methylbenzaldehyde (0.7 mmol, 0.090 g, 0.08 ml), sodium borohydride (2.8 mmol,
22
23 0.106 g); yield 54%. ¹H-NMR (400 MHz, CdCl₃) δ 1.54 (s, 9H), 1.60-1.88 (m, 9H+2H exch D₂O),
24
25 2.36 (s, 3H), 2.75-2.81 (m, 2H), 3.17-3.27 (m, 2H), 3.42-3.54 (m, 8H), 3.77 (s, 2H), 5.03 (brs exch
26
27 D₂O, 2H), 7.15-7.23 (m, 3H), 7.28-7.32 (m, 1H).

28
29 **General procedure for the synthesis of 19 and 21.** A solution of **37** or **38** in MeOH (10 ml) and
30
31 HCl 3M (10 ml) was stirred overnight at room temperature. Following solvent removal, the residue
32
33 was washed with Et₂O (5 x20 ml). The resulting solid was filtered and dried to afford EB20 or
34
35 EB22 as trihydrochloride salt.

36 **N¹-(3-((2-methylbenzyl)amino)propyl)octane-1,8-diamine 19.** White solid; quantitative yield;
37
38 mp > 250 C; ¹H-NMR (400 MHz, D₂O) δ 1.14-1.25 (m, 8H), 1.50-1.56 (m, 4H), 1.97-2.03 (m,
39
40 2h), 2.26 (s, 3H), 2.82-2.93 (m, 4H), 2.98-3.02 (m, 2H), 3.09-3.15 (m, 2H), 4.18 (s, 2H), 7.17-7.29
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42 (m, 4H); ¹³C-NMR (100 MHz, D₂O) δ 18.29, 21.75, 22.58, 25.37, 25.47, 26.52, 27.81, 36.01,
43
44 36.94, 39.45, 44.26, 47.80, 48.50, 126.62, 128.93, 129.84, 130.15, 131.00, 137.71; MS (ESI+) m/z
45
46 = 153 (M + 2H)²⁺.

47 **3-(4-(3-aminopropoxy)butoxy)-N-(2-methylbenzyl)propan-1-amine 21.** White solid;
48
49 quantitative yield; mp > 250 C; ¹H-NMR (400 MHz, D₂O) δ 1.35-1.41 (m, 4H), 1.75-1.87 /m, 4H),
50
51 2.23 (s, 3H), 2.90-2.94 (m, 2H), 3.04-3.08 (m, 2H), 3.27-3.44 (m, 8H), 4.12 (s, 2H), 7.16-7.25 (m,
52
53 4H); ¹³C-NMR (100 MHz, D₂O) δ 18.38, 25.14, 26.43, 37.41, 45.40, 47.99, 67.45, 67.69, 70.28,
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55 70.34, 126.53, 129.03, 129.62, 129.96, 130.88, 137.43; MS (ESI+) m/z = 309 (M + H)⁺.

Amine oxidase activity assay and kinetic analyses

Bovine serum amine oxidase activity was determined by measuring hydrogen peroxide generation rate (one of the reaction products) by a peroxidase-coupled continuous assay previously described.⁵⁹ The H₂O₂ produced during Spm derivatives oxidation was continuously monitored by the change of absorbance at 555 nm, under continuous stirring, using an extinction coefficient, ϵ , of $2.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. Polyamine substrate concentration was varied in the range 0.5-100 μM .

All experiments were carried out in air-saturated solutions of 10 mM potassium phosphate buffer pH 7.6, at temperature $37^\circ\text{C} \pm 0.5^\circ\text{C}$. The specific activity of the BSAO preparations was $0.36 \pm 0.02 \text{ U/mg}$, where one enzyme unit is the amount of enzyme that transforms 1 μmol of benzylamine (standard substrate) per minute.⁵⁹ Protein concentration was measured according to the Bradford method,⁶⁰ which uses bovine serum albumin as a standard.

Steady state kinetic parameters (V_{max} and K_{M}) were calculated by fitting the Michaelis-Menten equation to the experimental data (initial rate of reactions vs substrate concentrations), with Sigma Plot software, version 10.0 (Jandel Scientific, San Rafael, CA, USA).

Catalytic constant (k_c) and catalytic efficiency (k_c/K_{M}) were calculated from the V_{max} , assuming a BSAO molecular mass of 170 kDa.⁴⁵

Computational studies

Modeling of compounds

Derivatives **1-23** and Spm were all modeled through the free for academic MolEdit molecular editor (<https://www.molsoft.com/moledit.html>) embedded in the www.3d-qsar.com Py-MolEdit web app.⁶¹ Canonical SMILES structures were obtained with a simple python script using the openbabel APIs⁶² (Supporting Information Table SI16).

BSAO structures preparations

The BSAO structures available from PDB, 1TU5 and 2PNC, were processed through the www.3d-qsar.com Py-PDB web app. The structures were retrieved and cleaned up of all ions and molecules not involved in the catalytic process. Together with the standard aminoacid residues the TPQ and the catalytic copper ion were retained. The cleaned structures were then added of the hydrogens considering a pH of 7.6 and subjected to a short energy minimization (100 iterations of steepest

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3 descent and 50 iterations of conjugated gradient) with the universal force field (UFF) force field⁶³
4 as implemented in the openbabel suites. The BSAO apoproteins and the CLO ligand we saved
5 separately in a dataset container.
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8 **Reversible Molecular Docking**

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10 Through the Py-Docking web app available in www.3d-qsar.com two docking programs, Smina⁴⁸
11 and Plants,⁴⁹ were applied to the 1TU5 and 2PNC BSAO complexes considering all the
12 implemented scoring functions (vina, vinardo and ad4 for Smina, and PLP, PLP95 and ChemPLP
13 for Plants). Re-docking and cross-docking assessment experiments were run directly through the
14 www.3d-qsar.com server. All derivatives **1-23** and Spm were then docked using the most
15 appropriate program/scoring function combination (see results section in the main text) saving in
16 a separate dataset container the lowest docked conformations for each derivative. The default
17 settings were used for the dockings except by setting a cubic box extending 30 Å centered to the
18 CLO center of mass. For Smina an exhaustiveness of 32 was set and the search_speed key to 1 for
19 Plants.
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27 **Covalent Molecular Docking**

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29 A procedure as previously described was applied.⁶⁴ For the covalent docking procedure, for each
30 derivative the TPQ-PA adduct was firstly prepared by means of a SMILES structure. To this the
31 TPQ SMILES structure deprived of the oxygen in position 5 was added in turn of each PA SMILES
32 structure. Then the TPQ-PA SMILES adduct was converted into a three-dimensional (3-D)
33 structure with openbabel at pH 7.6. The 3-D structure was then merged in the BSAO replacing the
34 original TPQ structure and converted into a flexible residue PDBQT file format through the
35 openbabel file format conversion utility. Then a water molecule was prepared as a fake ligand to
36 be reversibly docked. The version 1.2 of Vina program⁵⁴ was used to run the docking simulations.
37 The simulation was run by setting a 50 Å cubic box centered to the copper atom, an exhaustiveness
38 of 32, 100 docked conformations and leaving all the other settings to the defaults. The 100 docked
39 conformations of the TPQ-PA adducts were then separated and reversibly rescored with the same
40 scoring function to select the best docked conformation.⁴²
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50 Each of the separated covalently docked PA conformations was locally minimized with Smina
51 (1000 iterations) and the lowest energy complex was saved into a new series of the **1-23** PAs and
52 Spm by maintaining the TPQ without the oxygen in position 5 to avoid overlaps and also to mimic
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3 the TPQ-PA association/dissociation. A further 1000 iterations of local minimization was run on
4 each of the PA after the constitution of the full TPQ structure.

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6 Each step during the process was run with either vina or vinardo scoring functions. The whole
7 process is depicted in Figure 3 and Figure 4 and also described in the results section of the main
8 text.
9

10 11 **3-D QSAR modeling**

12 **LB models.** The **1-23** and Spm structures prepared as above described were subjected to
13 conformational search with three methods (Balloon, openbabel and RDKit) as implemented in the
14 Py-ConfSearch web app available from www.3d-qsar.com saving 100 conformations for each
15 derivative and setting a minimum value of 2 for the RMSD to prune similar conformations. The
16 conformational analysis was then subjected to automatic molecular alignments using the Shape
17 and RDKit methods (Py-Align web app in www.3d-qsar.com) and using all the implemented
18 scoring functions. 16 different templates molecules were used in either the lowest energy or the
19 longest conformation. The templates were selected based on the lowest or highest values of activity
20 (pK_M or $p[k_{cat}/K_M]$), logP, MR, TPSA, HDB, HBA, number of rotatable bonds, highest MW and
21 longest molecule.
22

23
24 The aligned conformations were automatically saved into datasets and 3-D QSAR models
25 elaborated by the Py-CoMFA web app for each dataset using the defaults settings (C.3 probe atom,
26 grid step of 2.0 Å, grid extension of 5 Å, minimum sigma of 2.0, Gasteiger charge model, dielectric
27 constant of 8 and a cutoff energy value of 30 kcal/mol). The highest q^2 endowed models were then
28 subjected to a variable pretreatment optimization (VPO) by randomly varying the settings. 3000
29 VPO models were picked up among millions of combinations. The model characterized by the
30 highest q^2 values was ultimately optimized by a feature selection with a standard implementation
31 of a SA algorithm⁶⁵ with an initial temperature of 10, a final temperature of 0.01 and a descending
32 gradient of 0.9999.
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34
35 The results of the model were graphically inspected with UCSF Chimera ver 1.16⁶⁶ with the
36 downloaded dataset molecules and the associated maps (PLS Coeff \times Average MIF values). The
37 optimal number of principal components (PCs) was determined by the SDEP decrement, selecting
38 the PCs in which at least a 5% decrement was observed.
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3 **Mixed LB-SB models.** The mixed LB_SB 3-D QSAR models were elaborated applying directly
4 the Py-CoMFA web app on the reversibly/covalently docked conformations using the same
5 procedure to define the LB models.
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10 **COMBINE models**

11 COMBINE-like⁶⁷ models were built through the Py-ComBinE web app available from www.3d-
12 qsar.com. Differently from the original Wade's method here per residue interaction energies are
13 calculated with the Autodock force field (ver 4.2) directly on the PA/BSAO complexes. Four types
14 of interaction were calculated (STE, ELE, DRY and HB) and used by block scaling each
15 interaction block.⁶⁸ Similarly, as for the 3-D QSAR models, a SA feature selection (initial
16 temperature of 10, a final temperature of 0.01 and a descending gradient of 0.9999) was run to
17 obtain the final models. The model was inspected through histogram plots of the AACs associated
18 with each residue. The optimal number of PCs was determined by the SDEP decrement, selecting
19 the PCs in which at least a 5% decrement was observed.
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29 **Data and Software Availability**

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31 All computation for the 3-D QSAR and COMBINE model generation were run on the 3d-qsar.com
32 portal (<https://www.3d-qsar.com/>) freely available to anyone for not profit usage, designed and
33 maintained by the authors. All other used stand alone or command line software was free and
34 publicly available: UCSF Chimera (<https://www.cgl.ucsf.edu/chimera/download.html>), anaconda
35 was used as python environment (<https://www.anaconda.com/products/distribution>) with the free
36 and open source available libraries (RDKit - <https://www.rdkit.org/>; OpenMM -
37 <https://openmm.org/>; sci-kit learn - <https://scikit-learn.org/stable/> and openbabel -
38 <http://openbabel.org/>).

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50 The used proteins structure data were available from PDB. All structures were computed starting
51 from SMILES structures and are available in the Supporting Information Table SI16 and as excel
52 file.
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5 **Supporting Information:** Please find enclosed Figs. from SI1 to SI11 and number 15 Tables:
6 from Table SI1 to Table SI15.
7
8
9

10 **Author Contributions:** E.A., R.R., A.M., M.L.D.P. and G.C. conceived this study and
11 coordinated the collaboration among the authors. E.A., R.R., A.M., V.T., L.A., E.P., R.F., P.F.,
12 T.T., and L.R. performed all the experiments. E.A., R.R., A.M., M.L.D.P. and G.C. optimized the
13 protocols for the analyses and techniques performed by 3-D QSAR and docking. M.F., E.P., A.G.
14 and P.F. discussed the project, focusing on the use of the more active molecules in future
15 therapeutic applications. All authors wrote the manuscript and all authors have read and approved
16 the final manuscript.
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23

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29
30
31
32
33

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35

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37 Supporting Information are also enclosed.
38
39
40

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43
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46 **Conflicts of Interest:** The authors declare no conflict of interest.
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