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Enantiomeric separation of 2-aryloxyalkyl- and 2-arylalkyl-2-aryloxyacetic acids on a Penicillin G Acylase-based chiral stationary phase: Influence of the chemical structure on retention and enantioselectivity

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Abstract

The chiral recognition mechanism of Penicillin G Acylase (PGA) was investigated with a set of 18 new chiral acidic compounds. A series of 2-aryloxyalkyl- and 2-arylalkyl-2-aryloxyacetic acids in which the absolute configuration has been reported to exert a strong influence on pharmacological activity, were synthesized and analysed on PGA-based chiral stationary phase (CSP) and 11 racemates were completely resolved with a mobile phase composed of 50 mM phosphate buffer (pH 7.0). The influence of structural variations of analytes on retention and enantioselectivity was investigated by application of molecular modelling studies. Docking experiments were also carried out to rationalize the observed enantioselective behaviour. The computation approach revealed to be helpful in elucidating the molecular basis of the enantioselectivity observed on PGA-CSP.

Keywords: Chiral HPLC; Monolithic PGA column; Enantiomeric discrimination; Molecular modelling

1. Introduction

Chiral chromatographic techniques have been used for the analytical and preparative separation of enantiomers for many years, and it can be assert that chiral HPLC is today the most widely used methodology for the separation of optical isomers both in academia and industry [1]. For this reason we assisted in the last few decades to the development of a great number of chiral stationary phases (CSP) and a lot of efforts have been spent to explain and to investigate the chiral recognition processes [2–4]. In this wide scenario, proteins, as chiral selectors, are of special interest because of their peculiar enantioselective properties and because they can separate a wide spectrum of chiral compounds [5]. A single protein may contain several sites that can contribute to the enantioseparation, therefore, the complex process of chiral recognition mechanism of protein-based CSP is difficult to be investigated [6].

Penicillin G Acylase (PGA) of *Escherichia coli* ATCC 11105 (EC 3.5.1.11) is mainly used in the pharmaceutical industry for the hydrolytic cleavage of Penicillin G into 6-aminopenicillanic acid (6-APA) and phenylacetic acid (PAA) [7]. Analysis of the crystal structure of *E. coli* PGA [8] identified hydrophilic and hydrophobic parts in the substrate binding site. In particular, the binding site has been found to consist of three major regions: the catalytic residue SerB1, the oxyanion hole (stabilizing the negative charge present on the ligand carboxylate group) formed by GlnB23, AlaB69, AsnB241 and a hydrophobic pocket which is able to accommodate lipophilic groups.

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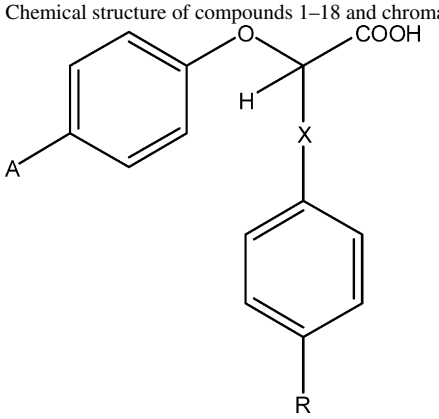
Table 1
Chemical structure of compounds 1–18 and chromatographic parameters obtained for racemates analysed on PGA-CSP



Compounds	A	X	R	k_1[a]	α	Rs
1	Cl	CH_2	H	1.087	1.00	–
2	Cl	$(CH_2)_2$	H	2.16 (R)	2.90	1.10
3	Cl	$(CH_2)_3$	H	9.58	1.00	–
4	Cl	$(CH_2)_4$	H	10.00	1.92	2.50
5	Cl	CH_2	Cl	5.81	2.30	2.50
6	Cl	$(CH_2)_2$	Cl	13.35	3.30	4.87
7	Cl	$(CH_2)_3$	Cl	56.01	1.00	–
8	Cl	$(CH_2)_4$	Cl	78.44	1.48	1.30
9	Cl	CH_2O	Cl	7.40	1.00	–
10	Cl	$(CH_2)_2O$	Cl	18.65 (R)	2.04	2.06
11	Cl	$(CH_2)_3O$	Cl	45.11	1.00	–
12	Cl	$(CH_2)_4O$	Cl	112.73	1.00	–
13	Cl	$(CH_2)_5O$	Cl	66.70	1.00	–
14	Cl	$(CH_2)_3O$	H	22.74	1.24	0.77
15	Br	$(CH_2)_3O$	H	14.06	1.16	0.68
16	$COCH_3$	$(CH_2)_3O$	H	7.02	1.40	1.23
17	CF_3	$(CH_2)_3O$	H	11.04	1.20	0.81
18	C_6H_5	$(CH_2)_3O$	H	25.07	1.27	1.10

[a]Absolute configuration of the less retained enantiomer.

In the last years, we have published a series of papers [9–12] regarding the development and the applications of a new CSP based on Penicillin G Acylase immobilized on silica columns. We have demonstrated that PGA-CSP is an excellent chiral selector for the enantiomeric separation of acidic aromatic compounds; this result was not surprising taking into account the reactions catalyzed by PGA. We have also shown that the catalytic site of PGA is involved in the chiral discrimination process, therefore, our attention was focused on the study of the enantioselective mechanism.

Along this line, a large number of closely related chiral 2-aryloxy-2-aryl-acetic acids, 2-aryloxyalkanoic acids and isosteric analogs were analyzed on PGA-CSP. Docking and molecular dynamic studies were also carried out [13,14]. In these works we have found that electron-withdrawing substituents on the aromatic rings are important for the stabilization of the complex between the analytes and the chiral selector leading to more effective charge-transfer interactions which, in addition to polar interactions, play a crucial role in determining the retention factors and the enantioselectivities observed. Moreover, it was observed that steric hindrance prevents the interaction of the analyte with the stereoselective binding site resulting in a loss of enantioselectivity.

The main goal of this paper is to extend the number of enantiomeric pairs analysed on PGA-CSP and to further clarify the structural features that may be responsible for chiral recognition and enantioselectivity values observed during chiral HPLC separation. Therefore, a group of structurally related compounds (1–18), having pharmacological importance as blockers of the skeletal muscle membrane chloride conductance [15], hypolipidemic agents [16] and peroxisome proliferator-activated receptor (PPAR) agonists [17] (Table 1) were synthesized and analysed on PGA-CSP in a HPLC system and a computational study was carried out.

The new series of compounds was designed to substantiate the results obtained in previous works and to derive information that can be used for faster and efficient development of enantioselective chromatographic methods.

2. Experimental

2.1. Chemicals

Epoxy-modified silica Chromolith® SpeedRod monolithic column (50 mm × 4.6 mm i.d.) cladded with PEEK was prepared

as research samples at Merck KGaA (Darmstadt, Germany) following a previously reported procedure [10]. Potassium dihydrogenphosphate, dipotassium hydrogenphosphate for the preparation of the mobile phase were purchased from Merck (Darmstadt, Germany).

Penicillin G Acylase crude extract from *E. coli* ATCC 11105 (EC 3.5.1.11) was kindly donated by Recordati (Milan, Italy) and used as received.

All solvents were of analytical grade and purchased by Carlo Erba (Milan, Italy).

2.2. Preparation of the analytes

The chemical structures of the racemic analytes used for the study are given in Table 1.

The synthesis of the compounds is briefly described. Racemates 1–8 were prepared by condensation of diethyl 4-chlorophenoxy-malonate with the suitable aryl-alkyl-bromides in the presence of NaH followed by alkaline hydrolysis and thermal decarboxylation at 160 °C. Stereoisomers of compound 2 were obtained by Mitsunobu condensation of 4-chlorophenol with *R*- or *S*-ethyl 2-hydroxy-4-phenylbutanoate. This reaction, that is known to occur with inversion of configuration, afforded, after hydrolysis, *S*-2 and *R*-2, respectively. Racemates 9–13 were prepared as previously reported [15]. A similar synthetic pathway was carried out for the preparation of acids 14–18; the condensation products, derived from the reaction between phenoxypropylbromide and the appropriate diethyl aryloxy-malonate, gave rise, after hydrolysis and decarboxylation to the desired compounds The enantiomers of 10 were obtained following an alternative procedure. The acid-catalyzed hydrolysis in CH_3OH of the product achieved by the Mitsunobu condensation between 4-chlorophenol and *R*- or *S*-2-hydroxy-butyrolactone gave a hydroxy-ester intermediate which underwent a second condensation with 4-chlorophenol under Mitsunobu conditions and a final hydrolysis to give *R*- and *S*-10. The desired acids had the opposite absolute configuration of the starting *R*- or *S*-2-hydroxy-butyrolactone as assigned on the basis of the known stereochemical course of the Mitsunobu reaction. All the synthetic details as well as the characterization of the compounds used for this study have been reported elsewhere [17].

Sample preparation was carried out by dissolving known amounts of the chiral drug in 1-propanol (analytical grade) and each solution was diluted with the mobile phase buffer to a concentration of 0.1 mM.

2.3. Preparation of HPLC PGA-CSP

The in situ modification was used for the immobilization of PGA on epoxy monolithic support, and the characterization of the CSP was carried out as previously reported [13].

The amount of immobilized units was 2178.87 U/column.

When not in use, the columns were stored at 4 °C in a 0.01% (w/v) solution of sodium azide.

2.4. Apparatus and chromatographic conditions

Chromatographic experiments were performed with an Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) with a Rheodyne sample valve (20 μl loop) equipped with an Agilent 1100 variable-wavelength detector and an 1100 thermostat. The system was connected to a HPLC ChemStation, (Revision A.04.01). Mobile phase used for this study was 50 mM phosphate buffer (pH 7.0). Water was deionized by passing through a Direct-Q^{TM} (Millipore) system (Millipore, Bedford, MA, USA).

All the chromatographic experiments were carried out at a constant oven temperature of 25 °C and the column flow-rate was set at 1.5 ml/min. The UV signal was followed at 225 nm.

The retention factor (k) was calculated using the equation $k = (t_r/t_o) - 1$, where t_r is the retention time of the analyte and t_o is the retention time of an unretained compound. The void time (t_o) was determined at the first disturbance of the baseline after injection. The separation factor (α) was calculated using the equation, $\alpha = k_2/k_1$ where k_1 and k_2 are the retention factors for the first and last eluted enantiomers, respectively. The determination of the enantiomeric elution order was carried out injecting solutions of (*R*) and (*S*) isomers with a concentration ratio of 2.

2.5. Computational methods

2.5.1. Molecular modelling

Molecular modelling and graphics manipulations were performed using the SYBYL [SYBYL Molecular Modelling System (version 7.1), TRIPOS Assoc., St. Louis, MO] and UCSF CHIMERA software packages [18], running it on a Silicon Graphics Tezro R16000 workstation. Model building and geometry optimizations of compounds 6, 7, 10 and 13 were accomplished with the TRIPOS force field [19] available within SYBYL.

2.5.2. Docking simulations

Docking of both *R*- and *S*-enantiomers of compounds 6, 7, 10 and 13 to PGA was carried out using GOLD 3.1 version [20], a genetic algorithm-based software, selecting GOLDScore as a fitness function. GOLDScore is made up of four components that account for protein-ligand binding energy: protein-ligand hydrogen bond energy (external H-bond), protein-ligand van der Waals energy (external vdw), ligand internal vdw energy (internal vdw) and ligand torsional strain energy (internal torsion). Parameters used in the fitness function (hydrogen bond energies, atom radii and polarizabilities, torsion potentials, hydrogen bond directionalities, and so forth) are taken from the GOLD parameter file. The fitness score is taken as the negative of the sum of the energy terms, so larger fitness scores indicated better bindings. The fitness function has been optimized for the prediction of ligand binding positions rather than the prediction of binding affinities, although some correlation with the latter can be also found [21]. The protein input file may be the entire protein structure or a part of it comprising only the residues that are in the region of the ligand binding site. In the present study, GOLD was allowed to calculate interaction energies within a sphere of

a 10 Å radius centered on the HZ hydrogen atom of Phe71 in PGA.

2.5.3. Ligand setup

The structures of the ligands 6, 7, 10 and 13 were constructed using standard bond lengths and bond angles of the SYBYL fragment library. Geometry optimizations were carried out with the SYBYL/MAXIMIN2 minimizer by applying the BFGS (Broyden, Fletcher, Goldfarb, and Shannon) algorithm [22] and setting a rms gradient of the forces acting on each atom of 0.001 kcal mol^{-1} $Å^{-1}$ as the convergence criterion.

2.5.4. Protein setup

The crystal structure of PGA in complex with *p*-nitrophenylacetic acid (entry code: 1AJN) [23], recovered from Brookhaven Protein Database [24], was used. The structure was set up for docking as follows: polar hydrogens were added by using the BIOPOLYMERS module within the SYBYL program (residues Arg, Lys, Glu and Asp were considered ionized, while all His were considered to be neutral by default), the *p*-nitrophenylacetic acid and all waters were removed.

2.5.5. GOLD docking

Fifty independent docking runs were performed for each docking experiment. All docking runs were carried out using standard default settings with a population size of 100, a maximum number of 100,000 operations, and a mutation and crossover rate of 95. The best generated 10 solutions of each ligand were ranked according to their fitness scores calculated by the GOLDScore function.

3. Results and discussion

Over the last years we have demonstrated that immobilized PGA can be used as chiral selector for acidic compounds [9–12]. In particular, complete chiral resolution of many 2-aryloxyalkanoic acids, isosteric analogs and 2-arylpropionic acids was achieved with PGA-CSP which shows a preferential enantioselective binding for the *S* enantiomers. These experimental observations provided evidence that the catalytic site is involved in the chiral mechanism.

On the basis of our previous results, in the present work we investigated the chiral properties of a PGA column towards a new series of 2-aryloxyacetic acids (Table 1) characterized by the presence into the side chain of a phenyl ring (compounds 1–8) or a further phenoxy group (compounds 9–18) at variable distance from the chiral center. Half of these compounds, moreover, had an additional chlorine atom in the *para*-position of the side chain benzene ring, whereas different atoms or groups with different size, shape and electronic properties were considered for the *para*-position of the phenoxy group directly linked to the chiral center.

One goal of this study was also to enhance the knowledge of the chiral recognition mechanism and process on PGA-CSP. A molecular modelling study was, therefore, undertaken, allowing to describe the relationships between the chemical structure of the analytes and the chromatographic results.

3.1. Chromatographic studies

Compounds 1–18 were chromatographed on PGA-CSP using a mobile phase of 50 mM phosphate buffer pH 7.0. The chromatographic results are summarized in Table 1: PGA showed enantiorecognition for 11 racemates (α greater than 1.0) but only compounds 4, 5, 6 and 10 present an Rs value better than 1.5. It could be expected that further optimization of the chromatographic conditions can improve the chiral separations.

Some representative chromatograms are reported in Fig. 1.

For compounds 1–8 an increase in retention time was achieved increasing the length of the alkyl chain between the phenyl ring and the chiral center. The presence of a second chlorine atom on the side chain aromatic ring (Ar′) (compounds 5–8) led to a large increase in retention times. The analogs with two carbon atoms in the alkyl chains, present the better enantioresolutions ($\alpha = 2.90$ and 3.30 for compounds 2 and 6, respectively). In this sub-group of analytes only the racemic mixtures of compounds 1 and 7 were not resolved.

From these data seemed that the length of the chain is not the most significant structural parameter for the interaction with the enantioselective site, while the substituent on Ar′ plays a more important role.

In the subset of compounds 9–13, an oxygen atom was introduced between the aliphatic chain up to five methylenes and Ar′ while maintaining the chlorine substituents on both the aromatic rings. As expected, the retention was increased by the lengthening of the carbon chain up to four C atoms, while for compound 13 (5 C) a surprising reduction of the *k* was obtained. A possible explanation of this behaviour might be the restricted dimensions of the binding pocket or the necessity of an optimal distance between two areas of the binding site that can interact with the analyte. Enantioselectivity was observed only for compound 10 with a carbon chain of two atoms: $\alpha = 2.04$. This compound was the only oxygenated derivative to show a better enantioresolution in comparison to the corresponding methylenic isoster (10 versus 7).

Finally, we prepared a third group of derivatives (14–18) in which the phenoxypropyl side chain was maintained constant while varying the 4-substituent on the aromatic ring of the phenoxy group (Ar) directly linked to the chiral center. These compounds were selected for their pharmaceutical interest being analogs of 11 which is the most active blocker of the skeletal muscle membrane chloride conductance [15].

The introduction of a phenyl ring in position 4 of Ar led to a substantial increase in retention, while the acetyl group determined a significant reduction in retention time. The elution order of compounds 14, 15 and 17, with Cl, Br and CF_3 as substituents, is in agreement with the electron-withdrawing power of the considered substituents.

Interestingly, all the racemic mixtures of this subset of derivatives were resolved and the enantioselectivity was almost constant for all the compounds. Therefore, the nature of the substituents on this aromatic ring seems to have a very little

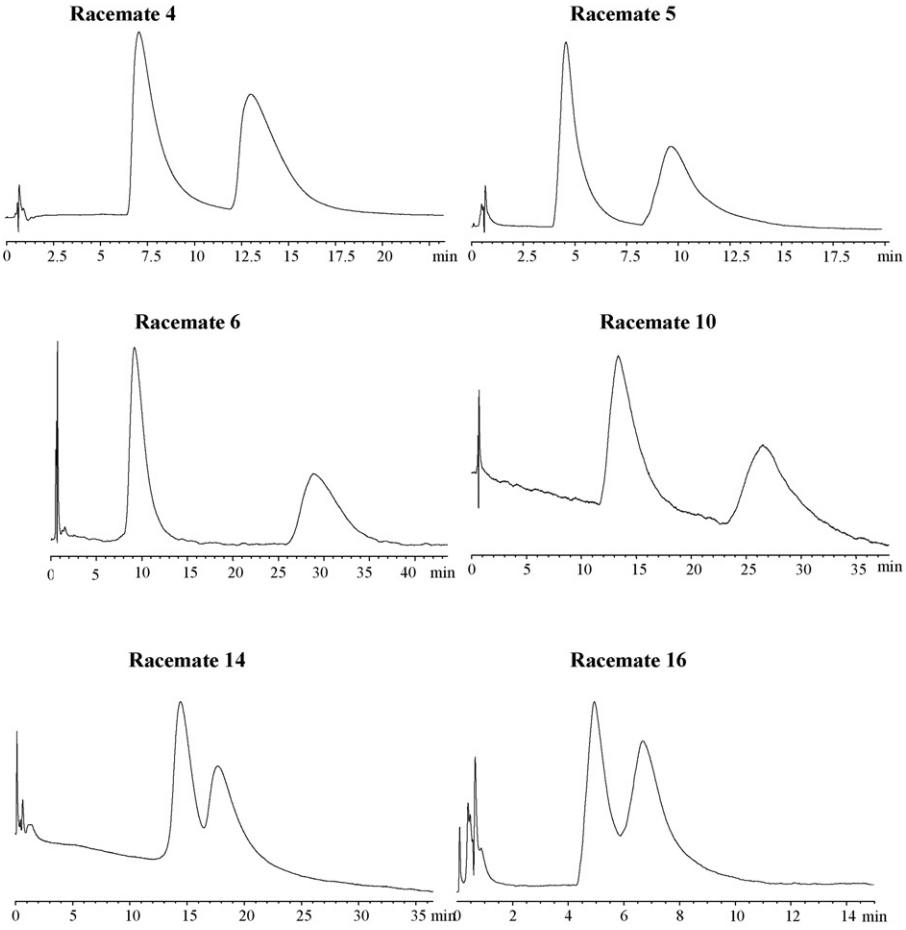


Fig. 1. Chromatograms of racemates 4, 5, 6, 10, 14 and 16 on PGA column. Mobile phase: 50 mM phosphate buffer (pH 7.0). Flow: 1.5 ml/min.

influence on enantioselectivity. On the other hand, the lacking of the chlorine on Ar′ seems to favour the chiral discrimination in compounds 14 and 11.

A complete overview of the data led us to derive more complete considerations regarding cross-correlation between the effect of the chlorine atom on Ar′ and the length of the carbon chain (X). In fact, the presence of chlorine atom on Ar′ seems to determine (compounds 5 and 1) or to enhance (compounds 6 and 2) the enantioselectivity, but an opposite trend was observed when comparing compounds 8 with 4, and compounds 11 with 14. One explanation of the contradictory behaviour observed could be the crucial role played by length of the carbon chain (X). The presence of more than 3 C does not allow the Ar′ to fit in the hydrophobic pocket of the active site when carrying the chlorine atom, due to steric hindrance of this substituent. This behaviour was not observed for compounds 5 in comparison to 1 and 6 in comparison to 2 where the carbon chain is composed by one or two C, respectively. We can suppose that in this case, the length of the chain allows the Ar′ ring to fit in the hydrophobic pocket, and Cl substituent to positively affect the chiral discrimination.

Of the whole series, only analogs 2 and 10 were prepared, also, as optically active isomers with assigned absolute configuration. The elution order of these enantiomers was found to be *R*/*S* and these data are consistent with the previously observed chiral interactions [9,12].

3.2. Docking studies

In order to get a better comprehension of the molecular recognition process between PGA and its analytes, docking experiments were performed using the crystal structure of PGA in complex with the 2-(4-nitrophenyl)acetic acid (PDB entry code: 1AJN) [23]. The GOLD 3.1 program was used to carry out docking simulations, [20] since in several studies it yielded better performances compared to other similar programs [25–27]. An analysis of principles and methods adopted by GOLD for energy calculations, conformational search and clustering, and energy ranking is briefly presented in the Section 2, whereas a fully detailed description may be found elsewhere [28].

Docking investigations were focused on both *R*- and *S*-isomers of 6 and 10, whose racemate were efficiently separated by PGA. An in-depth look at the conformer population of *S*-6 and *S*-10 generated during the docking simulations into the PGA active site revealed that a convergent binding mode was largely adopted with a high fitness score (64 kJ/mol for *S*-6 and 61 kJ/mol for *S*-10). As depicted in Fig. 2a and c, the associated binding mode is characterized by the presence of H-bonds involving the carboxylate group and the OH groups of both

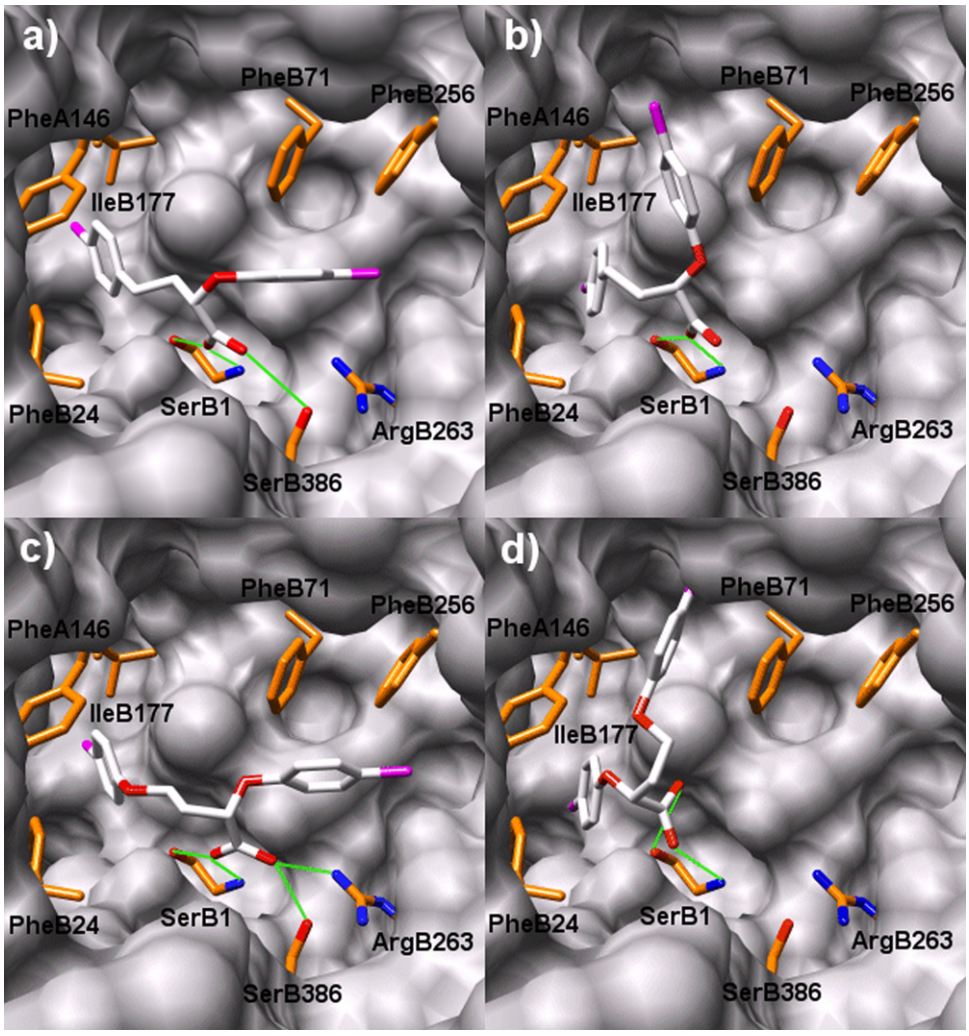


Fig. 2. Binding mode of *S*-6 (a), *R*-6 (b), *S*-10 (c) and *R*-10 (d) within PGA. For clarity reasons only interacting residues are displayed and labeled. Hydrogen bonds between ligand and protein are shown as green lines. Ligand (white) and interacting key residues (orange) are represented as stick models, while the protein as a light grey Connolly surface.

SerB1 and SerB386. Moreover, an ionic interaction is formed between the negatively charged carboxylate group and the positively charged N-terminal SerB1. It is to note that an additional H-bond is established between the carboxylate of *S*-10 and the ArgB263 side chain. The side chain *p*-Cl-phenyl ring occupies a hydrophobic pocket made up by residues PheB24, PheB57, PheA146, MetA142, TrpB154 and IleB177, where it establishes a π-stacking charge-transfer interaction with PheB24 aromatic ring. The *p*-Cl-phenoxy ring directly linked to the chiral center projects towards a large pocket and is well oriented to form T-shaped charge-transfer interactions with both PheB71 and PheB256 aromatic rings. The importance of charge-transfer interactions in the predicted binding poses is confirmed by the experimental data indicating an increase in retention of both *R*- and *S*-enantiomers for those compounds bearing electron-withdrawing substituents on Ar and Ar′. In Fig. 3 is depicted the 3D structure of PGA in complex with isomer *S*-6, the enantiomer showing the best interaction with the catalytic pocket.

As regards *R*-6 and *R*-10, results from GOLD runs suggested multiple docking poses characterized by quite diverse binding modes but comparable fitness scores (in the range 63–57 kJ/mol for *R*-6 and 60–55 kJ/mol for *R*-10). Analysis of the top-ranking docking solutions (Fig. 2b and d) revealed that the carboxylate group H-bonds both OH and N-terminal groups of SerB1, but it does not contact neither the SerB386 OH group nor the ArgB263 side chain. Moreover, the side chain *p*-Cl-phenyl ring is hosted in the same hydrophobic pocket occupied by the *p*-Cl phenyl ring in the *S*-isomer, whereas the *p*-Cl-phenoxy ring directly linked to the chiral center points in a different region of the enzyme, where it contacts the PheB146 and PheB71 aromatic rings. The poor docking run convergence towards a single binding mode, the lower estimated GOLD fitness score and the absence of H-bonds with SerB386 and ArgB263 would suggest that the *R*-isomers of 6 and 10 form a less stable complex with PGA. In fact, compound 10 was synthesized in enantiomeric form confirming that the elution order was *R*:*S*.

As shown in Table 1, racemate 7 is not separated by PGA. Remarkably, our docking study confirmed these experimental results. In fact, for both enantiomers of 7, GOLD calcu-

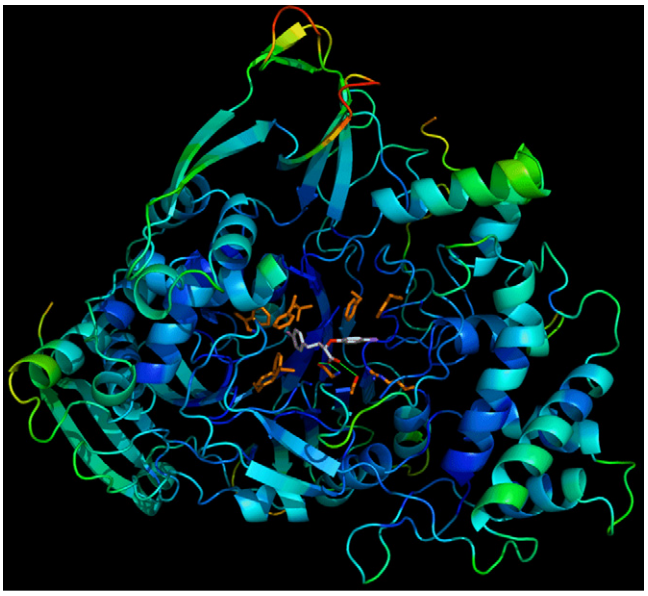


Fig. 3. 3D structure of PGA in complex with isomer *S*-6 (white) displayed as stick model. The protein is coloured by B-factor: blue regions correspond to low temperature factors, whereas green, yellow and red colours characterize regions of subsequently increasing B-factor. Residues lining the ligand position are highlighted in orange, while the H-bonds are represented by dashed lines.

lated two prevailing binding modes in which the carboxylate group and the *p*-Cl-phenyl ring still remained anchored to the binding site interacting with SerB1, SerB386, ArgB263 and PheB24 side chains, whereas the *p*-Cl-phenoxy group projected either towards the opening of the PGA active site (Fig. 4a) or towards the large hydrophobic pocket so to contact PheB71 and PheB256 (Fig. 4b). Moreover, the associated GOLD fitness scores were comparable for each predicted binding mode in accordance with the chromatographic experiments, which display that the stationary phase is unable to discriminate between both enantiomers ($\alpha = 1$). It could be hypothesized that replacement of the oxygen atom (compound 10) with a methylenic unit (compound 7) in the aliphatic chain bearing the Ar′ decreases the gauche effect clearly present in the O–C–C–C–O chain of 10 [29] provoking the double binding orientation of the ligand. Fig. 4 illustrates a superimposition between the binding mode of *S*-10 and the two prevailing binding modes of the methylenic isoster *S*-7 into the PGA binding site.

Peculiar results were obtained when docking was performed on both *R*- and *S*-isomers of 13, bearing a carbon chain of five C atoms. No convergence towards a single binding position was observed. From a visual inspection of the ligand/PGA complex it seems clear that the increased steric hindrance of the five C chain prevents both enantiomers from adapting themselves into the stereoselective PGA binding, thus explaining the reduction of the observed k_1 value in comparison to the homologues ones (racemates 9–12).

4. Conclusions

In an effort to extend the applicability of PGA as chiral selector towards acidic compounds, a new series of 2-aryloxyalkyl- and 2-arylalkyl-2-aryloxyacetic acids were analysed on PGA-CSP and the influence of structural variations of analytes on retention and enantioselectivity was investigated.

PGA-based CSP proved resolving capability for a large number of the considered compounds. Docking experiments, based on the known 3D structure of PGA and carried out to rationalize the observed enantioselective behaviour of the immobilized enzyme, corroborate the importance of the charge-transfer interactions and steric effects on retention factors and chiral separation. Moreover, the computational studies confirmed the experimental results indicating that it would be possible to predict the enantioselectivity of PGA-CSP for the investigated analytes. The knowledge of chiral molecule-selector interactions can be useful to design a more efficient chiral selector based on mutated PGA.

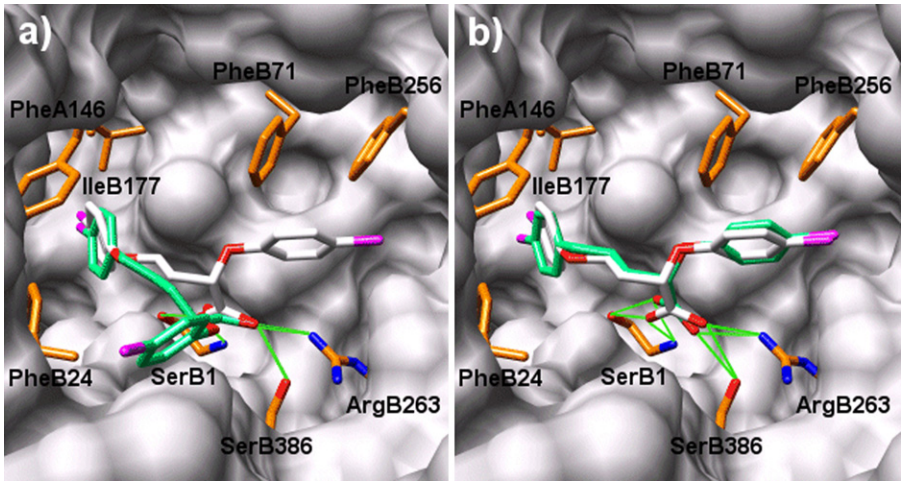


Fig. 4. Superimposition of the two prevailing binding orientations (a and b) of *S*-7 (spring green) about that of *S*-10 (white) within PGA. For clarity reasons only interacting residues are displayed and labeled. Hydrogen bonds between ligand and protein are shown as green lines. Ligand and interacting key residues (orange) are represented as stick models, while the protein as a light grey Connolly surface.

Acknowledgements

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