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Synthesis and matched molecular pair analysis of covalent reversible inhibitors of the cysteine protease CPB

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ABSTRACT

Cysteine protease B (CPB) can be targeted by reversible covalent inhibitors that could serve as antileishmanial compounds. Here, sixteen dipeptidyl nitrile derivatives were synthesized, tested against CPB, and analyzed using matched molecular pairs to determine the effects of stereochemistry and *p*-phenyl substitution on enzyme inhibition. The compound (S)-2-(((S)-1-(4-bromophenyl)-2,2,2-trifluoroethyl)amino)-N-(1-cyanocyclopropyl)-3-phenylpropanamide (**5**) was the most potent CPB inhibitor (pKi = 6.82), which was also selective for human cathepsin B (pKi < 5). The inversion of the stereochemistry from *S* to *R* was more detrimental to potency when placed at the P2 position than at P3. The *p*-Br derivatives were more potent than the *p*-CH_3 and *p*-OCH_3 derivatives, probably due to intermolecular interactions with the S3 subsite.

Leishmaniasis is a neglected disease endemic in many countries, including Brazil. It is considered an emergent and uncontrolled disease, caused by more than 20 species of *Leishmania*.[1] Leishmaniasis can be classified into three forms, the two most common being tegumentary and visceral. The most frequent types in Brazil are cutaneous and mucocutaneous leishmaniasis, caused by *Leishmania* (*Viannia*) *braziliensis*[2]; however, the visceral form is the deadliest. According to the World Health Organization (WHO), visceral leishmaniasis affected 500,000 people in 88 countries worldwide in 2017; 556 million people live in endemic areas. Six countries, including Brazil, account for 94% of cases.[3]

The disease is transmitted to humans by the bite of infected female sandflies. These parasites are present inside the vector gut as extracellular promastigotes; then, they transform into intracellular amastigotes in mammalian host cells.[4] Currently, leishmaniasis treatment includes pentavalent antimonials, amphotericin B and miltefosine. Pentavalent antimonials are the first-line treatment; however, these are associated with side effects such as hepatotoxicity and cardiotoxicity.[5] Amphotericin B, another common antileishmanial drug, causes renal toxicity.[6] For these reasons, it is crucial to develop new drugs, and cysteine proteases are attractive targets; these are associated with physio-pathological processes such as cancer (for cathepsins),[7] Chagas disease that is caused by *Trypanosoma cruzi* (where cruzipain is expressed throughout the whole life cycle), and leishmaniasis, caused by *Leishmania* spp. (for the enzymes CPA, CPB, and CPC).[8] These enzymes irreversibly hydrolyze peptide bonds by putative mechanism involving nucleophilic attack provided by the thiolate, resulting in formation of a tetrahedral intermediate that is followed by formation of a thioester intermediate and its hydrolysis. The active site of the cysteine proteases from the papain family recognizes the protein sequence to be cleaved (or the biomimetic inhibitor) in which the substituents in the α-carbon of the amino acids (or the inhibitors) bind to the subsites.[9]

Many research groups been working with derivatives of dipeptidyl nitrile that bind reversibly with cysteine proteases.[10,11] The covalent bond is formed between the carbon from the nitrile warhead and the thiolate of the catalytic cysteine forming the thioimidate; nevertheless, this is unstable and degrades to return the system to the initial state. Therefore, these compounds are classified as covalent reversible inhibitors.[12]

There are many reports in the literature of various cysteine proteases inhibitors, especially reversible and irreversible covalent inhibitors.[13,14] Moreover, inhibitors that do not form a covalent bond with the active site usually show lower potency. By contrast, those that form irreversible covalent bonds may have toxicity due to off-target

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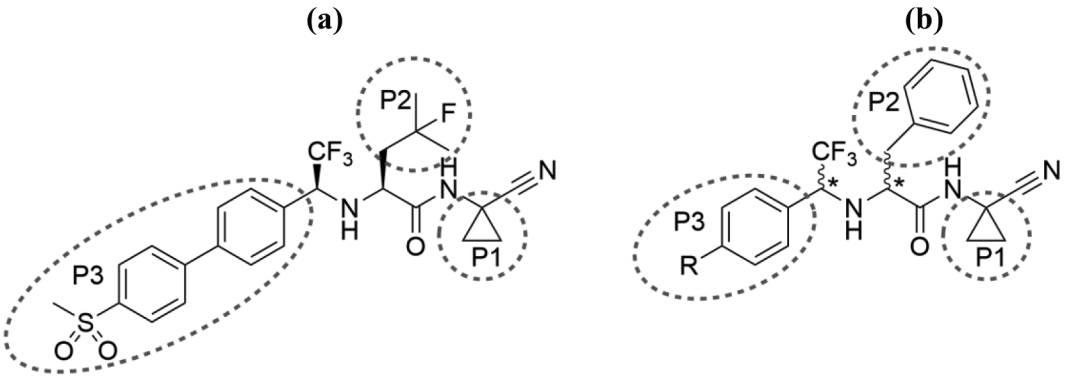


Fig. 1. Representation of odanacatib (a) and the scaffold for the set of compounds (b) with the numbering of the respective substituents that interact with the subsites of the cysteine protease. The chiral carbons marked in (b) were studied to identify the stereogenic recognition by the CPB enzyme. R: H, Br, CH_3, or OCH_3.

effects.[15]

Here we describe a series of cysteine protease inhibitors based on derivatives of the dipeptidyl nitrile scaffold. These compounds were designed based on a simplification of the odanacatib structure. Odanacatib is a potent cathepsin K inhibitor that is being tested in phase 3 clinical trials.[16] The mode of binding is well-described with substituents interacting at various subsites of the active site of the cathepsin K (Fig. 1a).[17] All compounds maintain the 2,2,2-trifluoroethylamine moiety from odanacatib as an amide isostere, which is often used to improve the biological stability with respect to the amide itself. However, two alterations of the chemical structure of odanacatib were made:

(i) The phenylalanine was placed in position P2 (Fig. 1b) instead of the unnatural amino acid 4-fluoroleucine. Our previous study with another parasitic cysteine protease (cruzain) demonstrated that phenylalanine is also recognized by enzymes from the papain family,[10] in which the homology model of the CPB structure shares structural features and ligand recognition in the active site.[18] The stereochemistry is an important feature for these compounds, and *R* and *S* derivatives were studied for the α-carbon of the phenylalanine amino acid in position P2 (Fig. 1b). The S2 subsite of these cysteine proteases is deemed as a selective point,[15] and it was then exploited in this study to identify the best stereochemistry.

(ii) A simplification of the chemical structure was made in the position P3, where the phenyl derivatives substituted in the *para* position were tested (Fig. 1). The intermolecular interactions in the S3 subsite could improve the potency; hence, two distinct modifications were devised: (i) the *p*-phenyl substitution, and (ii) the stereochemistry of the CF_3 group.

There are few reports of CPB inhibition by reversible covalent dipeptidyl nitrile derivatives and their effects on leishmania parasites.[19,20] Thus, this work demonstrates the hypothesis-driven asymmetrical synthesis of a series with 16 derivatives of dipeptidyl nitriles and their evaluation as inhibitors of the leishmanial cysteine protease CPB. As seen in Fig. 1b, two stereocenters are found for each compound. All stereoisomers were synthesized and evaluated in biochemical assays to describe their bioactivity profiles and the influence of their stereochemistry on CPB inhibition.

The synthesis of the amino acid intermediates used in the peptide coupling is shown in Scheme 1. *D* and *L*-Amino esters were purchased, and the synthetic approach from Chen *et al.* was used in this step.[21] Amino esters reacted with the opportune 2,2,2-trifluoroacetophenone in a reflux system in the presence of potassium carbonate and anhydrous methanol at 75 °C for 18 h, to produce the respective imines (Scheme 1). The basic reaction condition at elevated temperature promoted the hydrolysis of the methyl ester group, providing the potassium salts of the 2,2,2-trifluorophenyl imines. Due to the imine instability, all intermediates were used in the next step without purification.

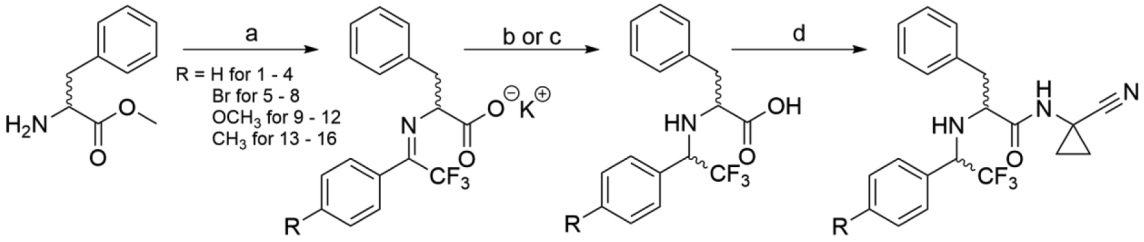
The diastereoselective reduction was described by Hughes *et al.*,[22] where the imines were reduced with $Zn(BH_4)_2$ to obtain the *S,S*-diastereoisomers (supplementary information, Figs. S1 and S2).[23,24,25] The final step of the synthesis to obtain compounds **1–16** involved the coupling of the amino acids with the respective amine and HATU. All compounds have the cyano group as a warhead (the reactive group responsible for covalent bond formation). The overall yields were good (65–70%), with high purity (> 95%), according to the chiral HPLC system applied to obtain the stereoisomers. All compounds were characterized by NMR spectroscopy, mass spectrometry, IR, and melting point, as described in the supporting information (characterization data for **1**, **2** and **5** were reported elsewhere).[10]

The stereochemistry was confirmed after the analysis of crystals of compound **12** ($C_{22}H_{22}F_3N_3O$, Fig. 2). It was crystallized by vapor diffusion of hexane into the dichloromethane solution over 72 h. A suitable crystal was selected and mounted in a fomblin film on micro mount on a SuperNova, Atlas S2 diffractometer.[26] Fig. 2 shows the absolute *R,R* configuration of compound **12**. This result confirmed that the experimental procedure described by Hughes *et al.*[22] indeed produces the *syn* isomer when the diastereoselective reduction was performed using $Zn(BH_4)_2$. Complementary data are available in the supplementary information, in which the packing of the compound **12** is shown (crystallography section), featuring the intermolecular interactions in the crystal. A network of hydrogen bonds between oxygen and nitrogen atoms of the amide groups and hydrogen-pi interactions between phenyl groups were observed in the *P*2 and *P*3 positions. Of note, compounds with the *anti*-configuration could impair the formation of the same set of interactions and did not provide a crystallographic structure despite the effort made.

All compounds were then tested against the CPB enzyme aiming to identify submicromolar inhibitors. The determination of the inhibition constant was done using an indirect assay, in which the inhibitor and substrate were present in solution alongside the enzyme. The protocol for the competitive inhibition assay was reported elsewhere[24] (further information can be found in the supplementary material).

The stereochemistry is one of the most critical aspects exploited in this series of dipeptidyl nitrile derivatives. Therefore, all compounds were tested to quantify the constant of inhibition (K_i), obtaining their respective p*K*i values (Table 1). Three of 16 compounds gave K_i values below 1 μM, and compound **5** was the most potent CPB inhibitor. Compounds that did not reach the threshold of 10 μM were assigned the percentage inhibition at this cut-off concentration.

The set of compounds was analyzed according to the concept of matched molecular pairs (MMP)[27,28] to observe how a single modification of a determined structure would affect bioactivity. The first subset of compounds proposed in this work (compounds **1–8**) was prepared to determine the influence of the phenylalanine and CF_3



Scheme 1. Reagents and conditions: (a) 2,2,2-trifluoroacetophenone, K_2CO_3, CH_3OH, 75 °C, reflux, 18 h; (b) 1 M $Zn(BH_4)_2$ in THF, CH_3CN/CH_3OH (5:1), −40/-45 °C, 4 h; (c) $NaBH_4$, THF, r.t., 6 h; (d) Hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU), N,*N*-diisopropylethylamine (DIPEA), opportune amine, r.t. 2 h.

configurations, and the effect of the *p*-bromine in the *P*3 position (Fig. 3) on enzyme recognition and inhibition using a kinetic assay. As shown in Table 1 and Fig. 3 (**1** → **5** and **4** → **8**), the *p*-bromine indeed increased the potency of the derivatives by one logarithmic unit. The *S,S* derivatives led to more potent inhibitors than the *R,R* enantiomers. Furthermore, an additive effect was observed for these compounds (Fig. 3).

Another striking result was achieved when the most promising CPB inhibitor (**5**) along with some of the derivatives were analyzed against the orthologous human enzyme cathepsin B (protocol provided in the supplementary information). According to Table 1, all compounds had pKi < 5, and were therefore considered selective inhibitors for the *Leishmania* enzyme CPB.

The additivity effect was observed for the comparative analyses between enantiomers and diastereomers (Fig. 3). As expected, the inversion of the stereocenter at the position P2 (Fig. 1) was less tolerated than in position P3. The S2 subsite[29,30] (in which the P2 substituent binds) does not tolerate the inversion of the phenylalanine configuration (**1** → **3** and **5** → **7**), like the subsite S3, for the CF_3 group (**1** → **2** and **5** → **6**). This is observed by the reduction of the potency in all cases where the *S*-Phe was changed by *R*-Phe (Fig. 3). The inversion of the CF_3 configuration led to mixed results, depending on the group present in the *p*-phenyl position. It did not change the potency for the non-substituted compounds (**1** → **2**), but it led to a reduction in potency for the *p*-Br derivatives (**5** → **6**). The effect is more pronounced when the enantiomers are compared (**1** → **4** and **5** → **8**).

The analysis of the bromine effect in Fig. 4 followed the additivity between the enantiomeric pairs for the *S,S* (**1** → **5**), and *R,R* (**4** → **8**) derivatives, with an increment in the potency of 10-fold when the bromine atom was in the *para* position. Intriguingly, other enantiomeric pairs provided by *S,R* (**2** → **6**), and *R,S* (**3** → **7**) did not follow the same trend (Fig. 4). The *S,R* derivatives had an increment of almost three times when the compound bears the bromine in the *para* position; however, an opposite effect was obtained for the *R,S* derivatives. These differences may be associated with the intermolecular interactions, recently described by us for the *T. cruzi* cruzipain enzyme as halogen bonding,[31] coupled with the volume of the bromine (and its steric hindrance when misplaced in the S3 subsite). The lack of a crystallographic structure of the CPB enzyme impairs further studies regarding the intermolecular interactions between the ligand and the macromolecule. However, we speculate that the effect of the *p*-bromine on incremental changes in inhibition potency might derive from halogen bonding once the *p*-methyl and *p*-methoxy derivatives had lower potencies (Table 1).

The evaluation of the *p*-methyl and the *p*-methoxy bearing compounds was also performed in relation to the non-substituted compounds (Fig. 5). However, MMP analyses were partially impaired by the loss of potency for most of the compounds. This may have resulted from steric hindrance attributable to the *para* substituents, with two out of four *p*-CH_3 derivatives considered active (**9** and **10**) and only one for the bulkier *p*-OCH_3 (**13**).

Our group is investigating a hypothesis regarding the non-additive profile to *T. cruzi* cysteine protease cruzain that may also be applicable to CPB. This refers to the hydrophobicity and the positioning of the P3 substituent.[31] The S3 subsite is more exposed to the solvent for all known cysteine protease three-dimensional structures available in the Protein Data Bank, where hydrophilic groups are usually placed in this region to interact with the amino acids. This may be the reason why the lipophilic derivatives were less potent (compounds **9–16**, Fig. 5).

Interestingly, all derivatives with the *R*-Phe were weaker inhibitors

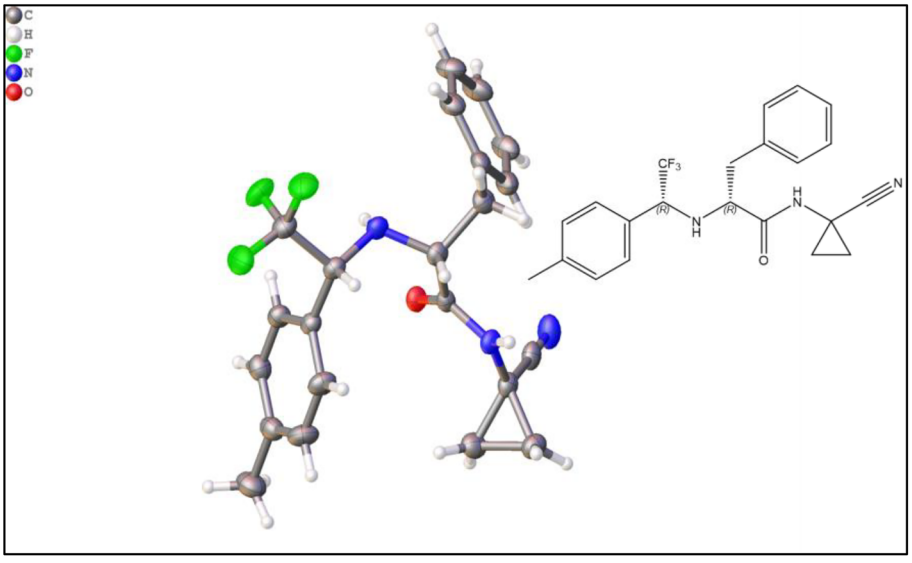
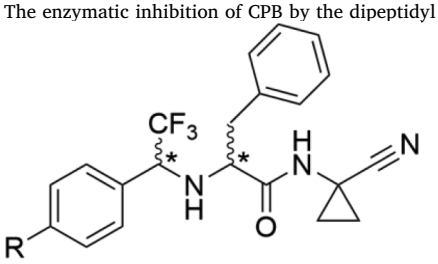


Fig. 2. Crystalline structure and representation of compound **12** (CCDC 1955373).

Table 1
The enzymatic inhibition of CPB by the dipeptidyl nitrile derivatives.



Compound	Chirality	R	CPB				Cathepsin B % inh[c]
			K_i (μmol/L)	pK_i[a]	SE pK_i[b]	% inh[c]	
1	*S,S*	H	1.70	5.77	0.05		74.5
2	*S,R*	H	1.41	5.85	0.08		
3	*R,S*	H	4.40	5.37	0.02		
4	*R,R*	H	7.65	5.11	0.06		
5	*S,S*	Br	0.151	6.82	0.03		> 95
6	*S,R*	Br	0.595	6.23	0.06		
7	*R,S*	Br	> 10,000	< 5.0	–		
8	*R,R*	Br	0.795	6.10	0.07		> 95
9	*S,S*	CH_3	4.07	5.40	0.08		
10	*S,R*	CH_3	1.50	5.80	0.06		92
11	*R,S*	CH_3	> 10,000	< 5.0	–	86%	
12	*R,R*	CH_3	> 10,000	< 5.0	–	88%	
13	*S,S*	OCH_3	3.23	5.50	0.06		
14	*S,R*	OCH_3	> 10,000	< 5.0	–	70%	
15	*R,S*	OCH_3	> 10,00	< 5.0	–	95%	
16	*R,R*	OCH_3	> 10,000	< 5.0	–	95%	

[a] $pK_i = -\log_{10}(K_i/M)$.
[b] Standard Error for pK_i.
[c] % of inhibition for compounds with a K_i higher than 10.0 μmol/L.

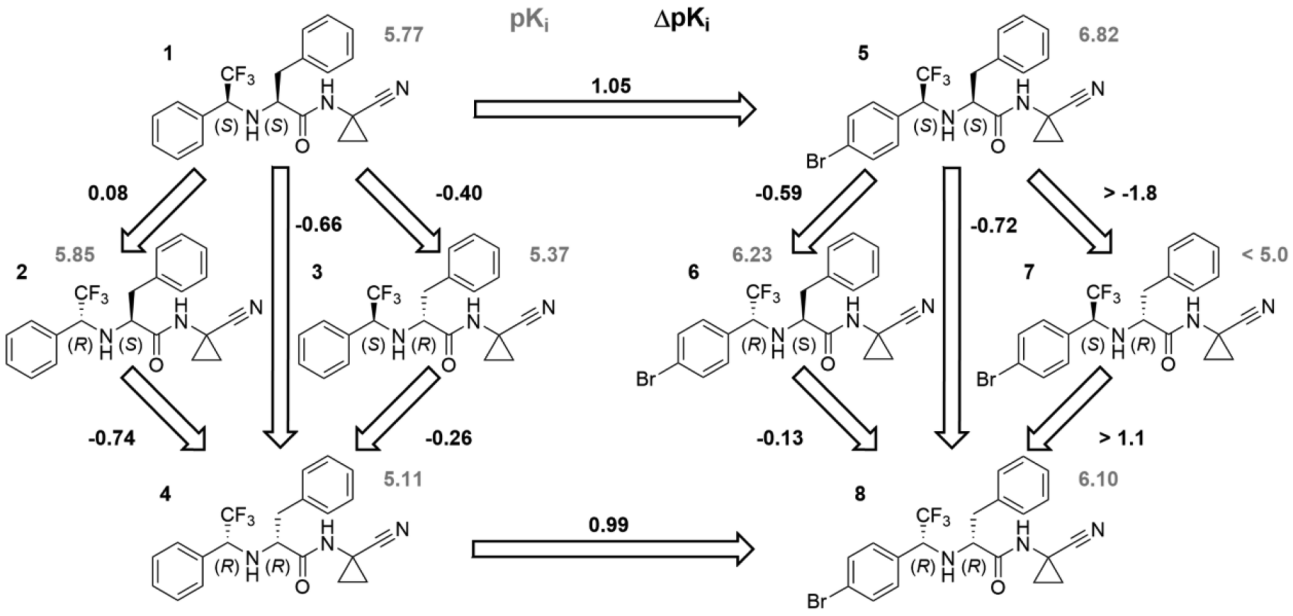


Fig. 3. Matched molecular pair analyses for the first subset (compounds **1**–**8**) focusing on the enantiomers and the additive effect of the *p*-bromine addition in P3. The additive effect was achieved for this subset.

than the *S*-Phe ones, reinforcing the notion of selectivity of the S2 subsite for *S*-Phe (Fig. 5). The lipophilic *p*-CH_3 (**9**) had almost the same potency of the hydrogen bond donor *p*-OCH_3 compound (**13**). This suggests that the presence of a hydrogen bond acceptor did not improve the potency, because the methoxy group could not interact with any hydrogen bond donor from the amino acids of the CPB enzyme. It is now necessary to increase the number of derivatives to further advance the insight described here regarding the putative formation of a halogen bonding, instead of hydrogen bonding.

This work described the synthesis and characterization of sixteen compounds against the cysteine protease CPB. SAR analysis for CPB inhibition using MMP showed that the *p*-bromine compound with (*S,S*) configuration was the most potent (**5**, pKi = 6.82). This result is probably due to a combination of (i) *S*-Phe fitting into the S2 subsite, with the change of the stereochemistry (*S* to *R*) in *P*2 leading to a loss of inhibition capacity (Fig. 1 and Table 1); and (ii) a putative halogen

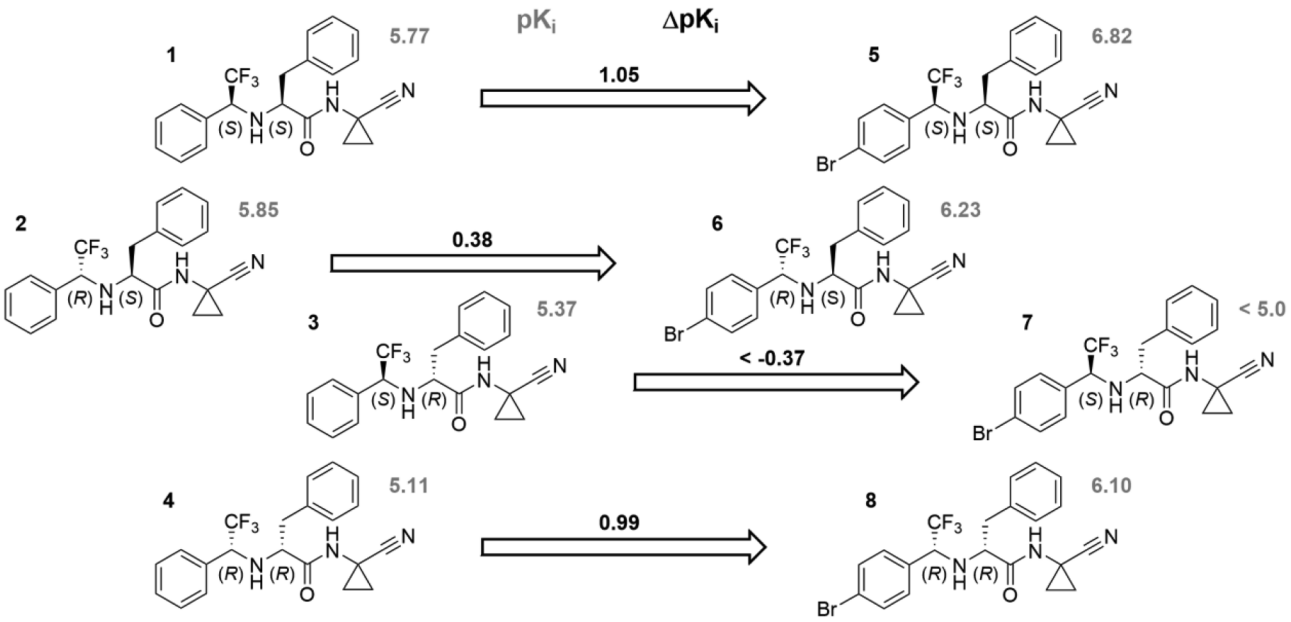


Fig. 4. Matched molecular pair analyses for the first subset (compounds **1–8**), focusing on the bromine substitution.

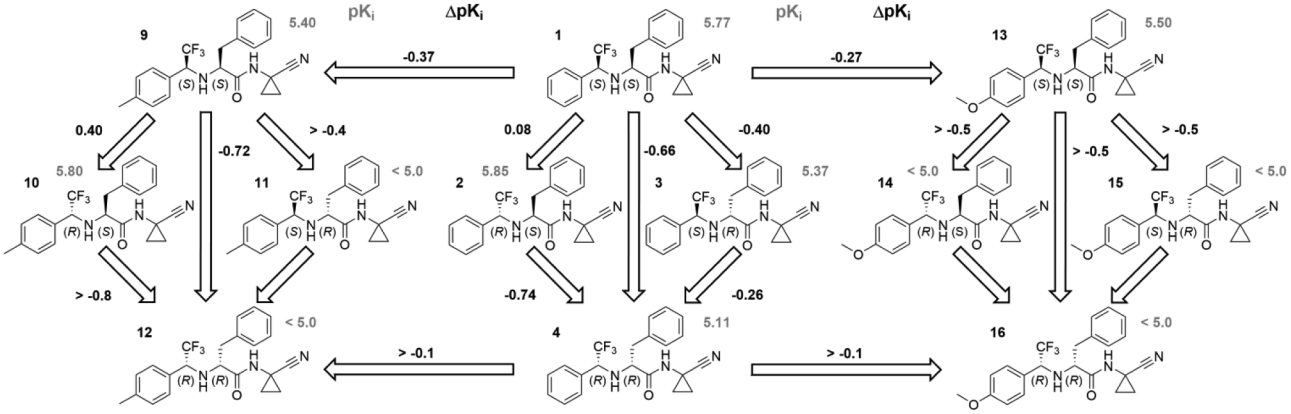


Fig. 5. Schematic analyses of compounds **1–4**, **9–12**, and **13–16**. The upper threshold for activity was 10 μmol/L.

bonding that could form between the *p*-Br derivative and an amino acid residue in the S3 subsite, which is corroborated by the other less potent *p*-CH_3 and *p*-OCH_3 compounds. Furthermore, an additive effect was observed for non-substituted compounds concerning the *p*-bromine derivatives (Fig. 3).

Finally, we reported the determination of the absolute stereochemical configuration of compound **12** using X-ray crystallography (Fig. 2). The structure corroborated the mechanism of the asymmetric synthetic route used to produce the compound with the desired stereochemistry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127439.

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23. The preference of the syn isomers over the anti comes from the formation of a complex between the Zn2+ cation and the nitrogen and oxygen atoms at the 1,3-positions of the imine. The chelated complex only allows the attack to the opposed face, once the si face is blocked. Considering that the more stable conformation of these salts puts the methine proton in the same plane of the imine face, the re face is more sterically hindered than the si face, due to the presence of bulky groups such as the benzyl group in P2 (Figure S1). To obtain the R,S-diastereoisomers (anti isomers), the reducing agent used was NaBH4 for the L-amino acid derivatives. The same effect is observed for the D-amino acid, but now the final products after the reduction with Zn(BH4)2 and NaBH4 are R,R-diastereoisomer (syn isomer) and the S,R-diastereoisomer (anti isomer), respectively.
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26. Crystal Data for C22H22F3N3O: (M = 401.42 g/mol): orthorhombic, space group P212121 (no. 19), a = 9.65500 (10) Å, b = 13.59830(10) Å, c = 15.78070(10) Å, V = 2071.87(3) Å3, Z = 4, T = 120(2) K, μ(CuKα) = 0.832 mm-1, Dcalc = 1.287 g/cm3, 19803 reflections measured (8.584° ≤ 2Θ ≤ 149.212°), 4179 unique (Rint = 0.0221, Rsigma = 0.0145) which were used in all calculations. The final R1 was 0.0256 (I > 2σ(I)) and wR2 was 0.0689 (all data). Refinement model description: Number of restraints - 0, number of constraints - unknown.
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