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Combinatorial approaches towards the discovery of new tryptase inhibitors

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Abstract—The synthesis and evaluation as tryptase inhibitors of a library of 2,5-diketopiperazine derivatives containing guanidine or amidine functional groups is reported. Among the compounds evaluated, derivatives **6**{CG4-CG8} and **6**{CG4-CG9} are the most active compounds and have marked selectivity towards tryptase in front of trypsine.

Most of the allergic inflammation diseases including asthma,[1] allergic conjunctivitis,[2] allergic rhinitis[3] and others have been related to the presence of elevated levels of human β-tryptase,[4] a structurally unique serine protease with trypsin-like activity released from mast cell secretory granules.

Studies carried out both in vitro[5] and with animal models[6] with potent and selective tryptase inhibitors has facilitated the validation of this protease as an important therapeutic target. Furthermore, the presence of two tryptase inhibitors in clinical trials, RWJ-58643[7] and APC-366,[8] in phase I and II, respectively, have provided support for the involvement of tryptase in asthma pathology.

In the literature,[9] four classes of selective tryptase inhibitors have been described, of which the first three types are synthetic and the fourth one has natural origin: (i) peptidic inhibitors (APC-366), (ii) dibasic inhibitors, (iii) Zn^{2+}-mediated inhibitors (BABIM-like) and (iv) heparin antagonist (e.g., lactoferrin). In particular, the most studied class in the last years is that based in dibasic inhibitors. This tendency is related to the publication of its resolved crystal structure, which has revealed that the active enzyme consists of four identical subunits (A, B, C and D) assembled with the four active sites pointing towards an oval central pore.[10] This array of the four negatively charged S1 binding pockets represents an ideal basis for the rational design of divalent/dibasic inhibitors.[11] Dibasic ligands of appropriate length could interact simultaneously with two neighbouring active sites giving compounds with high affinity and selectivity.[5b,12]

In the present work, we have systematically explored by a combinatorial approach the structural requirements (length, spatial distribution, hydrophobicity, basic group type) of a dibasic inhibitor based on a 2,5-diketopiperazine scaffold (DKP). Besides its scaffold role the DKP moiety can also contribute to the desired tryptase inhibition activity specially exploiting its capacity to form H-bonds.[13] Due to the fact that 2,5-diketopiperazines with a *trans*-geometry have been previously explored as scaffold on the synthesis of dibasic inhibitors with good results,[14] herein the structural features for the *cis*-geometry has been explored.

Interpreted through our lens, the structures of some examples of tryptase inhibitors in preclinical and clinical studies (Fig. 1), it is feasible to observe the highly frequent presence of the guanidine functional group in their structures. This motif has extensively been explored due to its particular features such as its extremely high basicity and its consequent participation in specific interactions ligand–receptor or substrate–enzyme through hydrogen bonds and/or electrostatic

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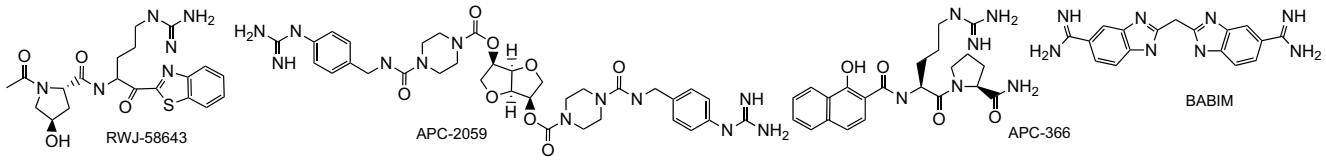


Figure 1. Some examples of tryptase inhibitors.

interactions.[15] In the present work the influence between guanidine/amidine as binding heads has been studied.

The diversity aspects of the library designed are generally introduced by a symmetric or asymmetric spatial arrangements and by different relative distributions of the guanidine groups. To achieve these diversity considerations two aspects have been considered: on one side the *nature* of the scaffold (the asymmetric DKPs: *cyclo*[L-Arg-L-Lys] and *cyclo*[L-Lys-L-Asp]; and the symmetric DKP, *cyclo*[L-Lys-L-Lys]) and on the other side the binding onto the DKPs of different guanidine-containing molecules (Fig. 2).

The general synthetic strategy followed to obtain the library members is based on the union by an amide linkage between the DKP scaffold derived from the cyclization of a dipeptide- and a molecule bearing either a guanidine or an amidine group. In the sublibraries in which Lys is one of the DKP constitutive amino acids, the union is performed between the ε-amine group of this amino acid and a carboxylic group of a guanidine/amidine-containing molecule (*CGx*/*CAy*).[16] On the other side, when Asp forms part of the DKP, the linkage occurs between the β-carboxylic group of the amino acid and an amine group of the guanidine/amidine-containing molecule (*AGz*/*AAw*). The general structure of the resulting molecules is represented in Figure 3.

The DKP scaffold was generated following a previously published methodology,[17] by cyclization on solid-phase of orthogonally side chain protected dipeptides attached through the peptide bond nitrogen to a backbone amide linker (BAL). The different sublibraries were obtained starting from the corresponding protected DKPs following the deprotection/coupling protocol illustrated in Scheme 1.

The inhibitory effect of tryptase/trypsine catalytic activity was assessed at 10 μM of test substance by determination of the residual tryptase/trypsine activity to cleave the chromogenic substrate (Tosyl-Gly-Pro-Lys-*p*-nitroanilide, Tosyl-GPL-pNa).[18] No effect was detected in none of these compounds when the trypsine

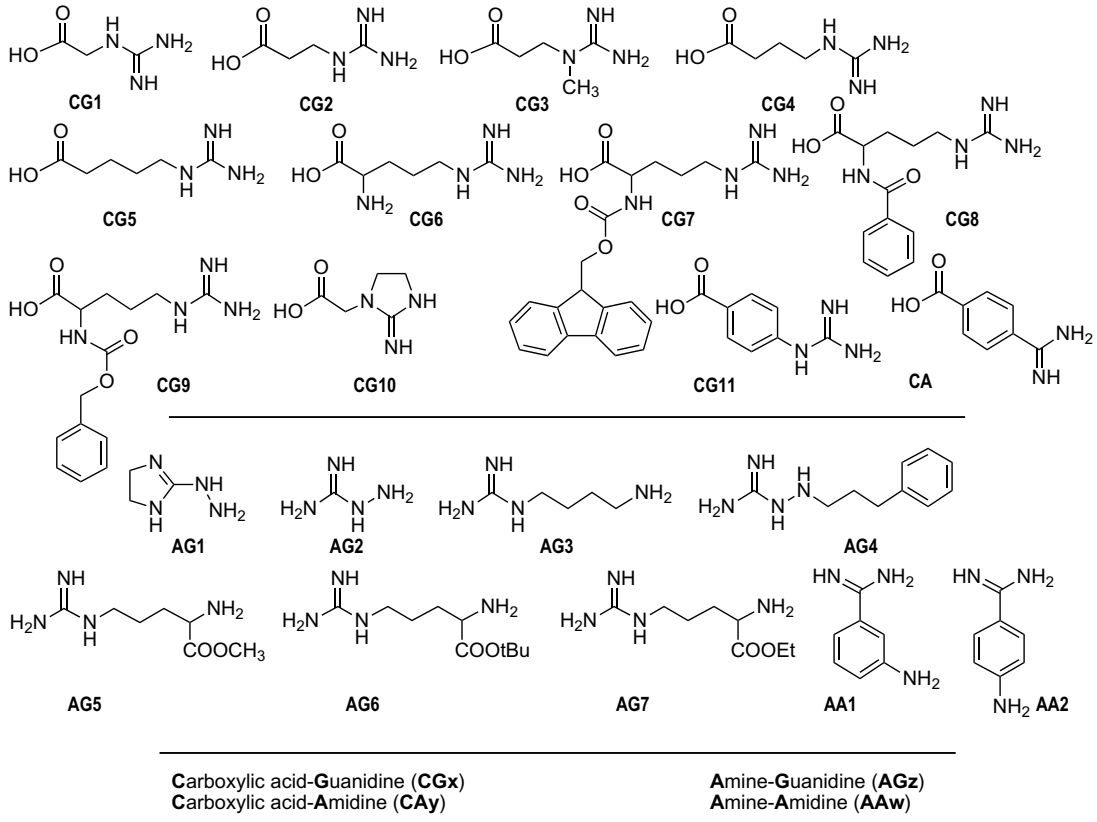


Figure 2. Guanidine-containing molecules introducing diversity on the DKP scaffold.

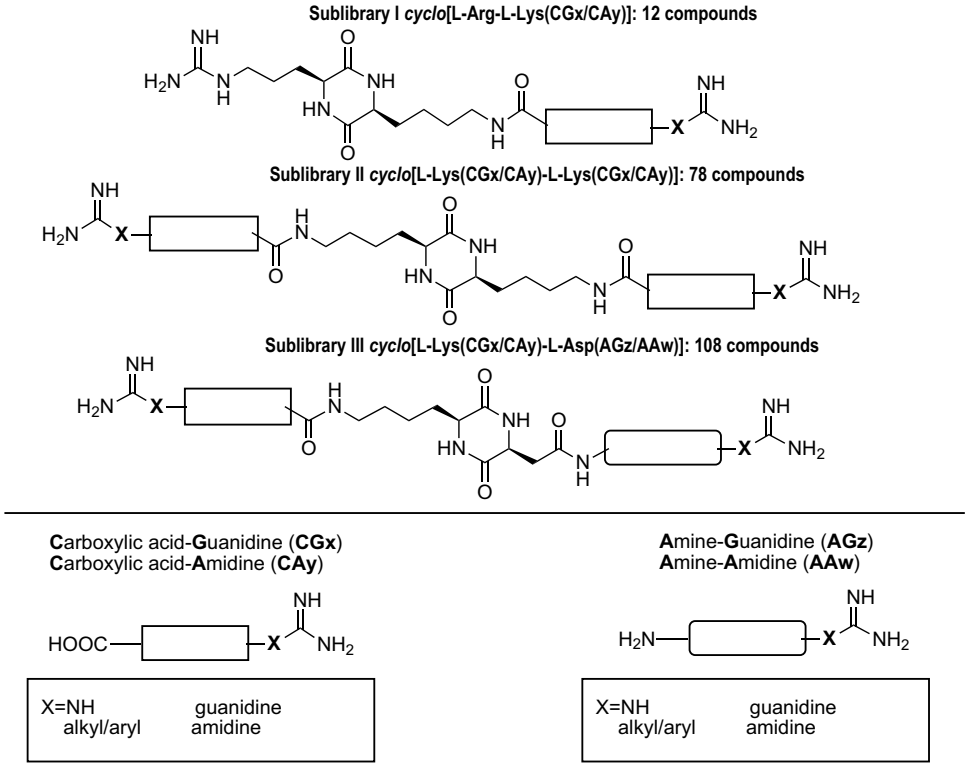
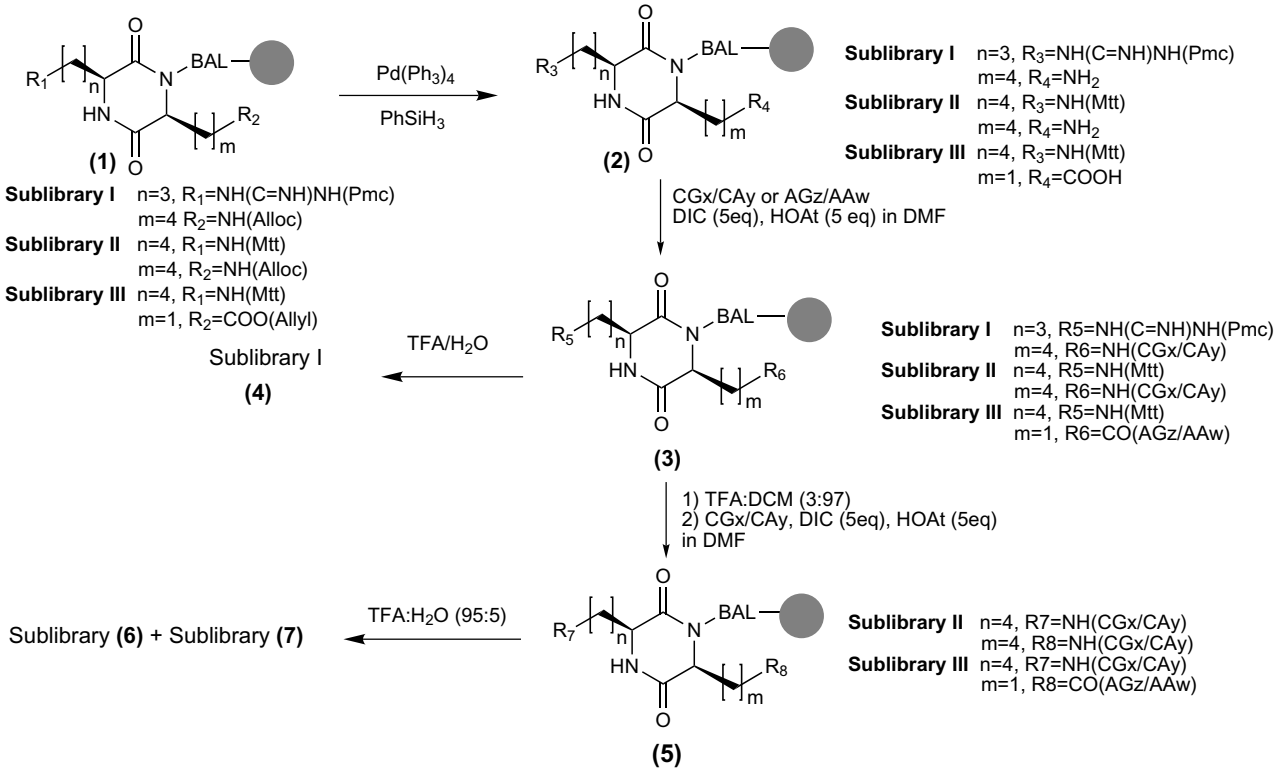


Figure 3. General structure of the library members.



Scheme 1. General synthetic pathway for the syntheses of the members of the library.

inhibitory activity was tested, indicating therefore a marked selectivity towards tryptase.

None of the compounds belonging to Sublibrary I (with DKP *cyclo*[L-Arg-L-Lys] as scaffold) have shown

Table 1. Tryptase inhibitory activity of some members of Sublibraries I, II[a] and III at 10 μM

Sublibrary I	% Tryptase inhibition	Sublibrary II	% Tryptase inhibition	Sublibrary III	% Tryptase inhibition
4{CG1}	−2.55	**6**{CG2,CG4}	**51.68**	**7**{CG11,GA2}	42.54
4{CG2}	−6.03	**6**{CG2,CG8}	31.17	**7**{CG7,GA4}	49.26
4{CG3}	−2.55	**6**{CG4,CG3}	36.73	**7**{CG1,AA1}	**58.63**
4{CG4}	8.92	**6**{CG4,CG4}	**59.33**	**7**{CG2,AA1}	**76.45**
4{CG5}	0.23	**6**{CG4,CG5}	44.38	**7**{CG3,AA1}	**62.65**
4{CG6}	3.71	**6**{CG4,CG8}	**80.53**	**7**{CG4,AA1}	**54.43**
4{CG8}	7.53	**6**{CG4,CG9}	**82.97**	**7**{CG7,AA1}	**62.61**
4{CG9}	1.97	**6**{CG5,CG8}	**47.51**	**7**{CG8,AA1}	**70.56**
4{CG10}	−3.24	**6**{CG5,CG9}	**68.02**	**7**{CG9,AA1}	**59.03**
4{CG11}	38.82	**6**{CG8,CG9}	**43.34**	**7**{CG10,AA1}	47.71
4{CA}	−2.90	**6**{CG9,CG9}	**48.55**	**7**{CG11,AA1}	47.27
		6{CG10,CG11}	**61.07**	**7**{CG7,AA2}	**64.49**
				7{CG8,AA2}	45.01
				7{CG9,AA2}	41.60
				7{CG11,AA2}	**73.92**

[a] Due to problems of high insolubility of CG1 in the reaction media it has not been possible its incorporation onto the corresponding batch of DKP *cyclo*[Lys-Lys (Mtt)]-®. Instead, it has been possible to introduce CG1 onto the corresponding small batches of DKP *cyclo*[Lys(CG#)-Lys]-® because performing the couplings with small amounts of either resin and CG1 has facilitated the solubility of the latter.

moderate activity for tryptase inhibition at 10 μM, but compounds belonging to the families DKPs *cyclo*[L-Lys-L-Lys] and *cyclo*[L-Lys-L-Asp] (Sublibraries II and III, respectively), reach values of tryptase inhibition from 50% to more than 80% (Table 1). Therefore, it seems that for the interaction with the tryptase receptor it is necessary a relative long distance between the two guanidine or amidine groups, which is not encountered in those molecules obtained from *cyclo*[Arg-Lys], with a number of bonds connecting guanidine/amidine groups between 15 and 18. This fact agrees with the results already presented in the literature.[5b,12,14]

For those molecules that show higher inhibitory capacities, the corresponding IC_{50} values have been established; such values and the corresponding molecule structures are depicted below (Figs. 4 and 5).

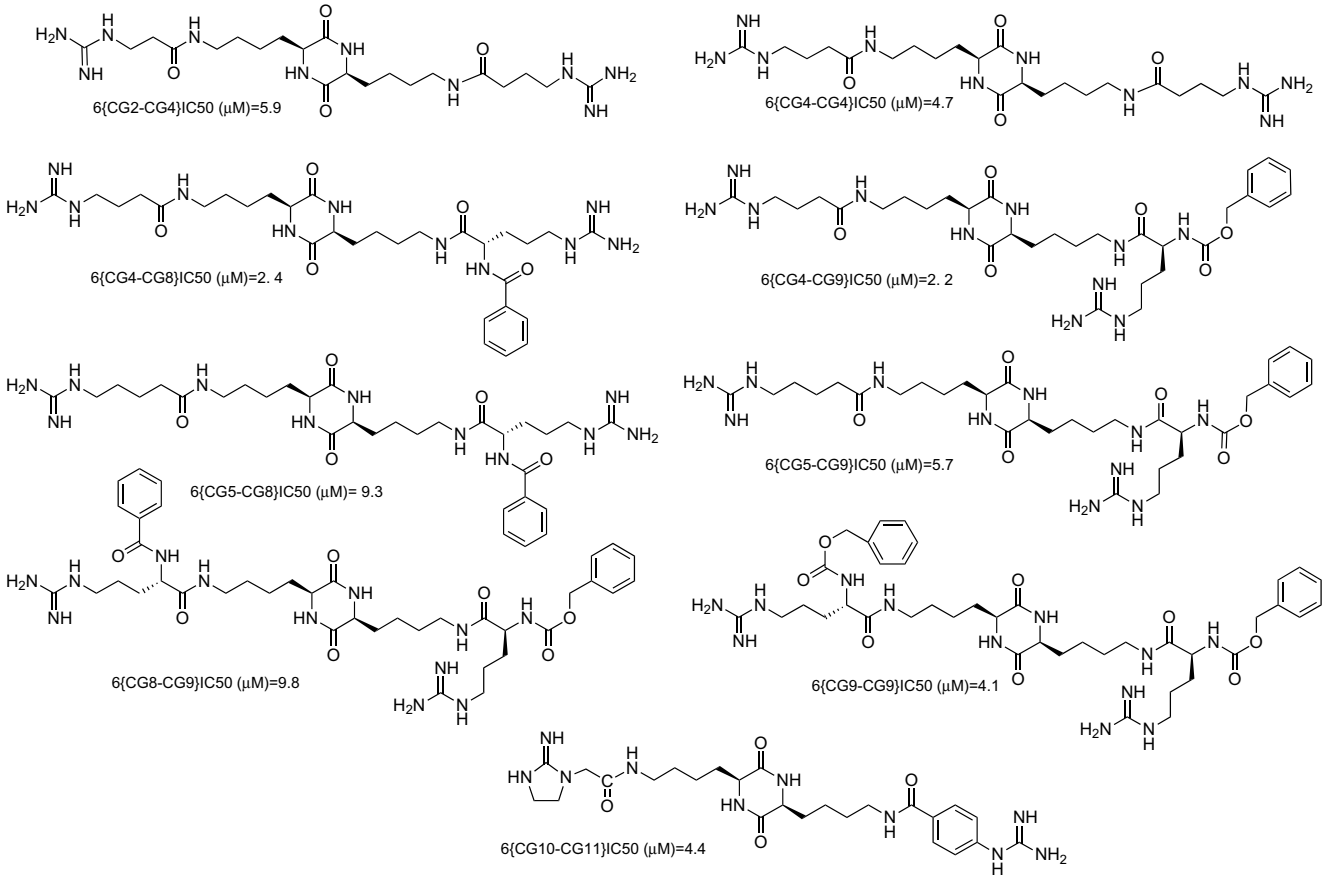


Figure 4. Members of Sublibrary II showing activity as tryptase inhibitors.

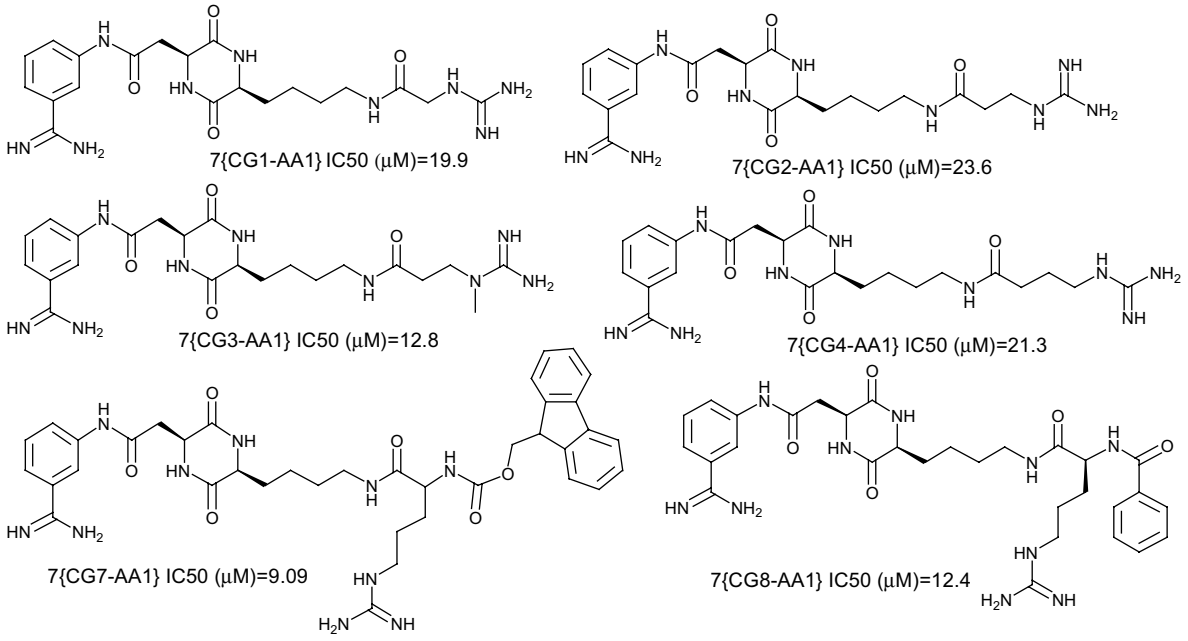


Figure 5. Members of Sublibrary III showing activity as tryptase inhibitors.

From the analysis of the most active molecules, it is possible to establish some conclusions either referring to the scaffold used as well as to the different guanidine-containing molecules attached to it. Thus, it seems that for the interaction with the tryptase receptor, it is necessary a relative long distance between the two guanidine or amidine groups, which is not encountered in those molecules obtained from *cyclo*[Arg-Lys]. On the other hand from Sublibrary II, it can be conferred that the presence of CG4, CG8 or CG9 seems crucial for the activity of the compounds. From Sublibrary III, it seems that the presence of carboxylic acids AA1 and AA2 has an important contribution to the activity of the products.

In conclusion, among the evaluated sublibrary members, **6**{CG4-CG8} and **6**{CG4-CG9} are the most active compounds (with IC_{50} values of 2.4 and 2.2 μM, respectively), and have marked selectivity towards tryptase in front of trypsine inhibitory activity. Further development of these lead compounds taking on account the importance of the information obtained from this preliminary study should lead to potent, selective tryptase inhibitors.

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