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Investigation of mechanism-based thrombin inhibitors: Implications of a highly conserved water molecule for the binding of coumarins within the S pocket

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Abstract—The synthesis of novel coumarins bearing on the lateral side chain in the 3-position an amine or a guanidine group is described. In vitro evaluation highlighted **14d** which possesses a *meta* aniline side chain as a very potent THR inhibitor. Surprisingly, the introduction of a guanidine moiety always led to a decrease in THR inhibiting properties. We, thus, used docking experiments to rationalize the SAR in the series. This study showed the crucial role of a conserved water molecule in the specificity pocket of THR during docking simulation in order to explain the inactivity of guanidine derivatives.

Thrombin (THR) is a trypsin-like serine protease which plays a pivotal role in the process of haemostasis and thrombosis. Besides exerting multifunctional activities in the coagulation cascade, it is also one of the main activators of platelet secretion and aggregation.[1,2] So, THR has earlier been recognized as a key target for the development of new antithrombotics.

As part of a project aiming at the development of coumarins as selective serine protease inhibitors, we have recently described a series of coumarins as THR and factor Xa (FXa) inhibitors.[3] They are characterized by a chloromethyl moiety in the 6-position and a hydrophobic alkyl-, aryl-, heteroaryl-ester, amide or thioster in the 3-position. These compounds were found to act as mechanism-based inhibitors. The first step of their inhibition mechanism consists in the nucleophilic attack by the activated hydroxyl group of Ser195 on the lactone moiety, leading to the acyl-enzyme. Then, the departure of the chlorine atom promotes the formation of a highly reactive quinone methide which could be alkylated and thus leads to the irreversible inactivation of the enzyme.

In the present report, we investigated the synthesis and structure–activity relationships (SAR) of novel coumarins bearing an amine or a guanidine on the ester lateral side chain in the 3-position. These basic moieties could advantageously interact with the negatively charged Asp189 in the specificity (S) pocket. This strategy should therefore lead to new potent THR inhibitors. Pyridinic derivatives have been also investigated.

The synthetic routes of the newly designed coumarins are depicted on Schemes 1 and 2. The introduction of the ester lateral side chains on the 3-position needs the preparation of the protected guanidino (**5a**–**d**) and amino alcohols (**6a**–**f**). Among the various methods developed to prepare Boc-protected guanidines,[4,5] we chose the *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-triflylguanidine **3** which allows to access the alcohols (**5a**–**d**) in one step, starting from the commercially available amino derivatives **4**.[6,7] The intermediate **3** was easily obtained from the guanidinium hydrochloride **1**, by reaction with di-

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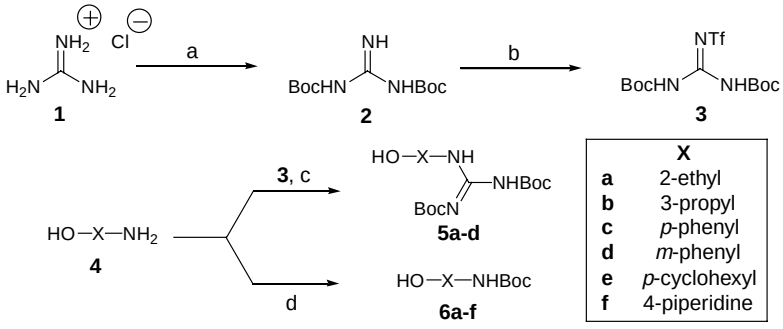
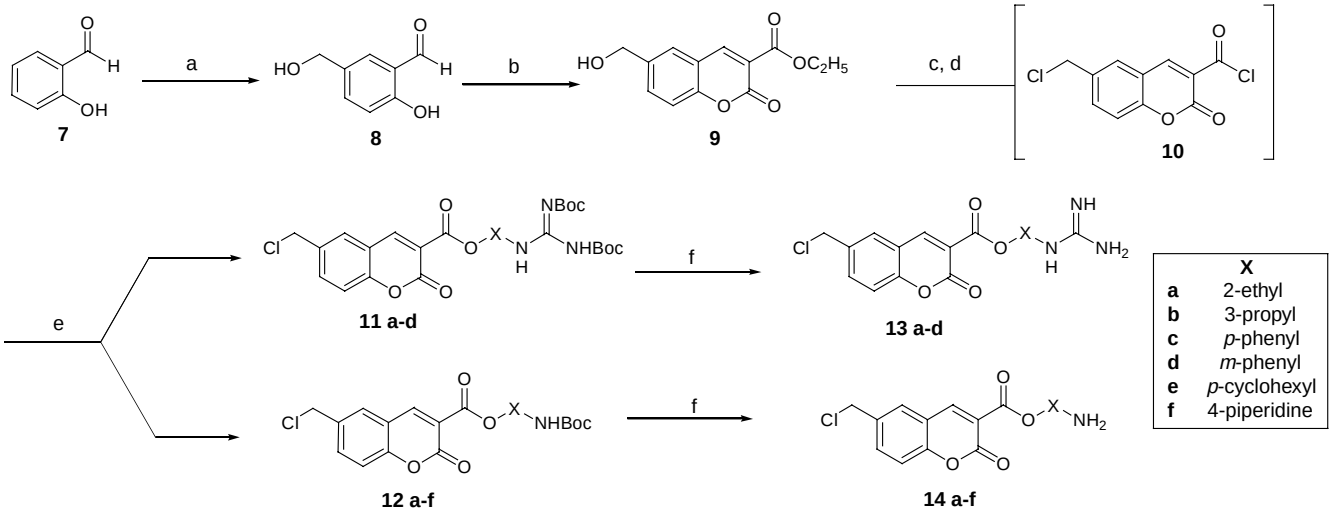


Scheme 1. Reagents and conditions: (a) Boc_2O, NaOH, 0 °C; (b) Tf_2O, Et_3N, −78 °C; (c) CH_2Cl_2, rt; (d) Boc_2O, dioxane, rt, 2 h.



Scheme 2. Reagents and conditions: (a) HCOH, HCl, 80 °C, 20 min; (b) diethyl malonate, piperidine, AcOH, EtOH, reflux, 17 h; (c) HCl (3 N), EtOH, reflux, 3 h; (d) $SOCl_2$, reflux, 3 h; (e) **5a–d** or **6a–f**, dioxane, pyridine, rt, 2 h; (f) TFA, CH_2Cl_2 (1:1), rt, 10 min.

tert-butyl-dicarbonate and sodium hydroxide at 0 °C followed by reaction at −78 °C with triflic anhydride and Et_3N (Scheme 1).[8] The Boc-protected amino alcohols (**6a–f**) were synthesized by reaction of the hydroxyl-amino (**4a–f**) with di-*tert*-butyl-dicarbonate.

Then, the suitable *N*-protected alcohol was reacted with the acyl halide (**10**), obtained by a previously described procedure,[9] to afford (**11a–d**) and (**12a–f**) (Scheme 2).[8] Finally, deprotection of derivatives (**11a–d**) and (**12a–d**) led to the targeted guanidines (**13a–d**) and amines (**14a–f**).[8]

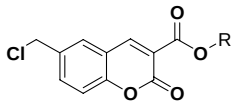
Pyridinic compounds **15a–e** were obtained according to our previously published procedure.[9,10]

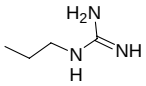
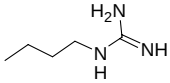
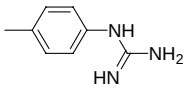
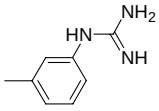
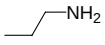
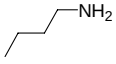
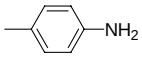
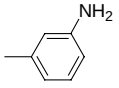
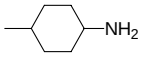
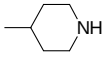
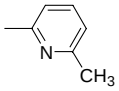
Table 1 summarizes the THR inhibitory potency of the newly synthesized compounds.[11] Surprisingly, guanidine derivatives (**13a–d**) are found to be almost inactive. Only **13a** and **13c**, bearing, respectively, a 2-guanidino-ethyl and a 4-guanidino-phenyl as side chain in the 3-position, possess a weak THR inhibitory potency. On the contrary, in the amino series, potent THR inactivators are obtained when the amine group is introduced on a phenyl ring (**14c–d**), particularly in the *meta*-position (**14d**). However, the introduction of an amine moiety on linear (**14a–b**) or cyclic (**14e–f**) aliphatic side chains always leads to a decrease in THR inhibitory potency. Compound **14d**, which possesses a *meta*-aminophenyl as side chain in the 3-position, is the best compound in this series with an IC_{50} value of 1.98 μM. For comparison purpose, we determined their kinetic parameters k_i and K_I (Table 2).[12] **14d** possesses a k_i/K_I ratio equal to 3570 M^{-1} s^{-1} and thus appears to be about 10-fold less potent than **16**, the best THR inhibitor of our previous study.[3] Moreover, the introduction of basic moieties such as guanidine, which proved to be very efficient on other series,[13] leads to an important decrease in THR inhibitory potency.

In the pyridine series (**15a–e**), only derivatives possessing the pyridine nitrogen in the 2′-position (**15a–b**) inhibit THR and particularly when a chlorine atom is located in the 3′-position.

With a view to helping in the understanding of these surprising results, we explored the binding mode of representative compounds within the THR active site by means of molecular modelling. Derivatives **13c**, **13d**, **14c** and **14d** were docked into the THR cavity (from

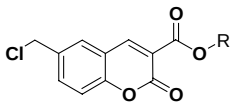
Table 1. Inhibitory effect on THR and physical and synthetic data for compounds **13a–d**, **14a–f** and **15**



Compound	R	Yield[a] (%)	mp (°C)	IC_{50}[b] (μM)
13a		67	141–143	107 (23–495)
13b		78	144–145	>300
13c		15	157–161	151 (69–330)
13d		11	137–140	>300
14a		72	149–151	284 (137–589)
14b		86	127–129	>300
14c		22	280–288	48 (25–93)
14d		15	335[c]	1.98 (0.76–5.17)
14e		76	215–218	>300
14f		45	210–213	>300
15a		—	—	0.87 (0.30–2.01)
15b		—	—	1.39 (0.64–3.01)
15c		—	—	>300
15d		—	—	ni[d]
15e		—	—	ni[d]

[a] Overall yield on steps e, f (Scheme 2).
[b] Calculated by nonlinear regression from dose–response curves. Values in parentheses are 95% confidence intervals.
[c] Decomposition temperature.
[d] ni, no inactivator at maximum solubility.

Table 2. Comparison of the kinetic parameters k_i and K_I for derivatives **14d** and **16**



Compound	R	THR k_i/K_I (M^{-1} s^{-1})
14d	3′-Aminophenyl	3570 ± 655 (0.0020 s^{-1}, 0.548 μM)
16[a]	2′,5′-Dichlorophenyl	37,000 ± 5150 (0.016 s^{-1}, 0.424 μM)

[a] Compound **30** in Ref. 3.

PDB entry 1A4W) using the automated *GOLD* program.[14] To take protein flexibility into account, the selected enzyme–inhibitor complexes were further refined by molecular mechanics using the *DISCOVER3* module[15] (CVFF forcefield) from *INSIGHTII*.[16]

Initially, the docking study was conducted on the THR active site, in the absence of water molecules. This unfortunately did not allow to explain the observed biological trends, that is, the inactivity of guanidine derivatives (**13c**, **d**) versus the activity of amine compounds (**14c**, **d**). According to a recent paper[17] that highlighted the importance of water in the binding properties of inhibitors, especially in the case of FXa, another trypsin-like serine protease structurally related to THR, we thought about the possible role played by water in the binding of these coumarins to THR. Therefore, we compared the X-ray structures of several THR-inhibitor complexes (PDB entries: 1A4W, 1BA8, 1OYT, 1QHR, 1SB1, 1EB1, 8KME and 1D9I). Their superimposition revealed a highly conserved water molecule (H_2O395), located just above the aromatic ring of Tyr228 (distance between the O atom and the phenyl centroid of ~3.5 Å) in the S pocket. New docking studies were thus performed in the presence of H_2O395. This latter was allowed to switch on and off during the simulation, that is, *GOLD* predicted if the molecule was displaced or not by the inhibitors.

As can be seen in Figure 1, all the complexes involve the conserved water molecule, underlining the importance to consider it in the docking of coumarins. In the amino series (Fig. 1a), both compounds (**14c**, **d**) adopt a similar orientation within the THR active site. The phenyl ring of coumarin favourably interacts with the hydrophobic proximal (P) pocket, composed of Tyr60A and Trp60D. The chloromethyl moiety fills in part the distal binding (D) pocket, interacting through hydrophobic contact with Trp215. The main difference in the binding of the two compounds arises from the specificity (S) pocket. While both aniline groups are stabilized through H-bonding with Asp189, an additional H-bond with Gly219 or with H_2O395 (Fig. 1a; represented in cyan) is observed for the *meta* (**14d**) and *para* (**14c**) derivatives, respectively. In both complexes, the lactone carbonyl moiety is located next to the catalytic Ser195, allowing the formation of the acyl-enzyme. Indeed, the distance of 4.2 and 4.0 Å between the lactone carbonyl C atom of **14c** and **14d**, respectively, and the hydroxyl O atom of Ser195, activated via H-bonding with the carbonyl O atom of exocyclic ester, is consistent with the nucleophilic attack of this residue onto the lactone, as described in the inhibition mechanism.[3,18] As both compounds are equally stabilized within the THR active site, and exhibit a proper orientation with regard to Ser195 (for acyl-enzyme formation), no direct conclusion can be derived from these results that would explain the small difference in potency of **14c** and **14d**.

In the guanidine series (Fig. 1b), **13c** and **13d** adopt two distinct binding modes within the THR active site. Both guanidinophenyls fill the S pocket and are stabilized by several H-bonds involving Gly219, Asp189 and H_2O395,

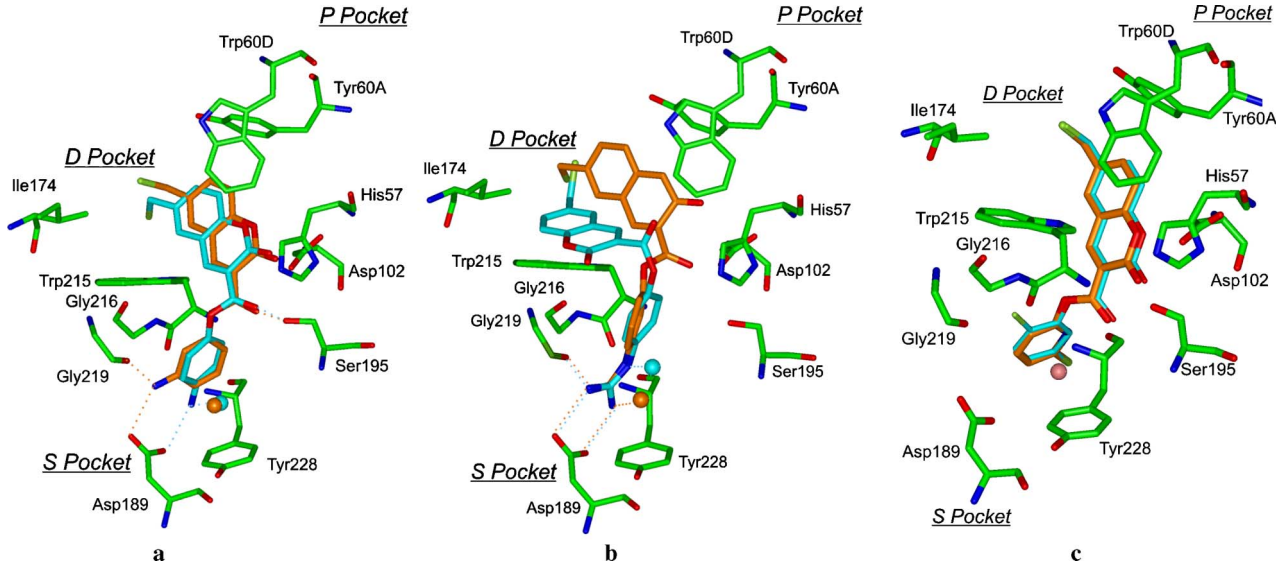


Figure 1. Superimposition of the docking simulation for (a) **14c** (cyan) and **14d** (orange), (b) **13c** (cyan) and **13d** (orange) and (c) **16** (cyan) and **15a** (orange). For (a) and (b), the conserved water molecule and H-bonds are represented in the colour of the inhibitor with which they interact. For (c) the red sphere represents the position of the conserved water molecule before its ejection of the active site during the docking simulation.

whereas the coumarinic nucleus presents different orientations. In **13d** (Fig. 1b, coloured in orange), the two carbonyl moieties are in the same direction (*syn*-conformation) and the coumarin ring and chloromethyl group interact favourably within the P and D pockets, respectively. In **13c** (Fig. 1b, coloured in cyan) in contrast, the carbonyl moieties lie in opposition (*anti*-conformation) and the coumarin ring interacts solely with the D pocket, and especially Trp215.

In comparison to amino derivatives, the bulky guanidine forces the coumarin ring to move away from the catalytic Ser195, increasing the distance between the lactone carbonyl C atom and the hydroxyl O atom of Ser195 (distance of 8.01 and 6.43 Å for **13c** and **13d**, respectively). This clearly accounts for the inactivity of guanidine versus amine derivatives.

For comparison purpose, we performed the docking of **15a** and **16** allowing *GOLD* to predict if H_2O395 was displaced or not (Fig. 1c). It appeared that the binding of **15a** and **16** produces the removal of water and occupation of its binding site by the chlorine atom in the *meta*-position. This observation supports our previous results which described a lipophilic contact between this chlorine atom and the aromatic ring of Tyr228 using *GOLD* with the THR active site deprived of water.[3] Moreover, water displacement allows **15a** and **16** to adopt a more favourable binding mode in which the distance between the lactone carbonyl C atom and the hydroxyl O atom of Ser195 is 3.2Å. This could explain the difference observed in the rate of THR inactivation of **16** versus **14d** ($k_i = 0.016\ s^{-1}$ and $0.0020\ s^{-1}$ for **16** and **14d**, respectively).

In conclusion, we described the synthesis of novel 3,6-disubstituted coumarins which possess an amine or a guanidine moiety in the *3*-position. The best derivative (**14d**) possesses a *meta*-aminophenyl side chain and displays a high THR inhibitory potency. Nevertheless, surprisingly the introduction of a guanidine moiety always led to a decrease in THR inhibiting properties. Rationalization of structure–activity relationships (SAR) in the series using molecular modelling revealed the need to consider a highly conserved water molecule (H_2O395) within the S cavity of THR to properly model the binding of coumarins bearing a basic moiety in the 3-position. This water molecule is of prime importance when using docking simulation to rationalize SAR, particularly in a series of mechanism-based inhibitors.

Acknowledgments

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8. Experimental details: (a) To a solution of **10** (2.27 mmols) generated in situ according to known procedure, in dry dioxane (5 mL), is added the alcohol **5a–d**, **6a–f** (1.1 equiv) and dry pyridine (1.1 equiv) and the mixture was stirred vigorously during 90 min. Then the solvents were removed, the residue redissolved in chloroform (200 mL), washed (3×) with 0.1 N HCl and brine and then dried over $MgSO_4$ and evaporated. This allowed to obtain **11a–d**, **12a–f** which were recrystallized either in hot AcOEt or acetonitrile. Deprotection was then realized by dissolving **11a–d**, **12a–f** in a 50:50 (v/v) mixture of methylene chloride/trifluoroacetic acid to generate **13a–d**, **14a–f**. Compound **14d**: overall yield: 15%; mp: 280–288 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 4.88 (2H, s) 6.40 (1H, d, $J = 8$ Hz) 6.67 (1H, s) 6.73 (1H, d, $J = 8$ Hz) 7.24 (1H, t, $J = 8$ Hz) 7.51 (1H, d, $J = 9.2$ Hz) 7.85 (dd, 1H, $J = 9.2$ Hz, $J = 2$ Hz) 8.05 (1H, d, $J = 2$ Hz) 9.02 (1H, s); ESI-MS *m/z* $(MH)^+ = 330.1$ $(MMH)^+ = 659.0$. Compound **13a**: 67%; mp: 141–143 °C ;^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.54 (m, 2H), 4.34 (t, 2H, $J = 5.6$ Hz), 4.87 (s, 2H), 7.27–7.31 (m, 4H) 7.49 (d, 1H, $J = 8.4$ Hz), 7.83 (m, 2H), 7.96 (d, 1H, $J = 2.4$ Hz), 8.81 (s, 1H); ESI-MS *m/z* $(MH)^+ = 324.1$. (b) To a solution of *N*,*N*′-bis(tert-butoxycarbonyl)-*N*″-triflylguanidine **3** (2.5 mmol) (prepared using the procedure described in Refs. 6 and 7) in dry methylene chloride (10 mL) is added Et_3N (1.1 equiv) under an argon atmosphere. To this stirred solution are then added the amino derivatives 4 at room temperature. After 2 h, the mixture is diluted with methylene chloride (10 mL), washed twice with a saturated solution of $NaHSO_4$, once with brine, dried over $MgSO_4$ and evaporated. This allowed to obtain the guanidine derivatives **5a–d** which are purified by liquid chromatography on silica gel (eluent: CH_2Cl_2 100%). Compound **5a**: yield: 85%; mp: 98–100 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50 (s, 18H), 3.24 (m, 2H), 4.40 (t, 2H), 8.50 (s, 1H), 11.27 (s, 1H).
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11. Inhibition assay was adapted from the previously described procedure[3] with an incubation time of 10 min for the enzyme/inhibitor reaction.
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