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TRIPEPTIDYL PYRIDINIUM METHYL KETONES AS POTENT ACTIVE SITE INHIBITORS OF THROMBIN

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Abstract

Several substituted methyl ketone derivatives of tripeptides with a C-terminal arginyl residue were synthesized as active site inhibitors of human α-thrombin. The most active compound among this series was the pyridinium methyl ketone D-Cha-Pro-Arg-PMK, which was characterized as a slow-binding inhibitor with a K_i of 0.19 nM. Copyright © 1996 Elsevier Science Ltd

Introduction

The multifunctional trypsin-like serine protease α-thrombin is the key enzyme in the blood coagulation cascade. It is responsible for the conversion of fibrinogen into polymerizable fibrin and amplifies its own generation through the activation of factor V and VIII. In addition, thrombin activates factor XIII, protein C and stimulates platelet aggregation, endothelial and smooth muscle cells. Therefore, thrombin is a primary target for the development of antithrombotic and anticoagulant agents.[1]

A well established method for the development of highly potent protease inhibitors is the design of substrate analog structures, which can mimic the transition state of the substrate hydrolysis. These compounds lack the cleavable carbonamide linkage but have groups, without cleavable residues, prone to the addition of nucleophiles. Different types of such transition state analogues derived from the tripeptide D-Phe-Pro-Arg were synthesized as thrombin inhibitors, including peptidyl aldehydes[2] and a series of ketone derivatives[3]. Recently a new type of substrate analog inhibitors, the peptidyl ammonium methyl ketones, was introduced as potent inhibitors for prolyl endopeptidase[4]. The characteristic feature of these compounds is their permanent positively charged ammonium (or pyridinium) methyl ketone moiety. This group has a strong electron withdrawing effect in the close neighbourhood to the P_1-carbonyl group. In addition, these compounds were characterized as slow binding inhibitors, therefore it was assumed, that they act also in a transition state manner with their target proteases.

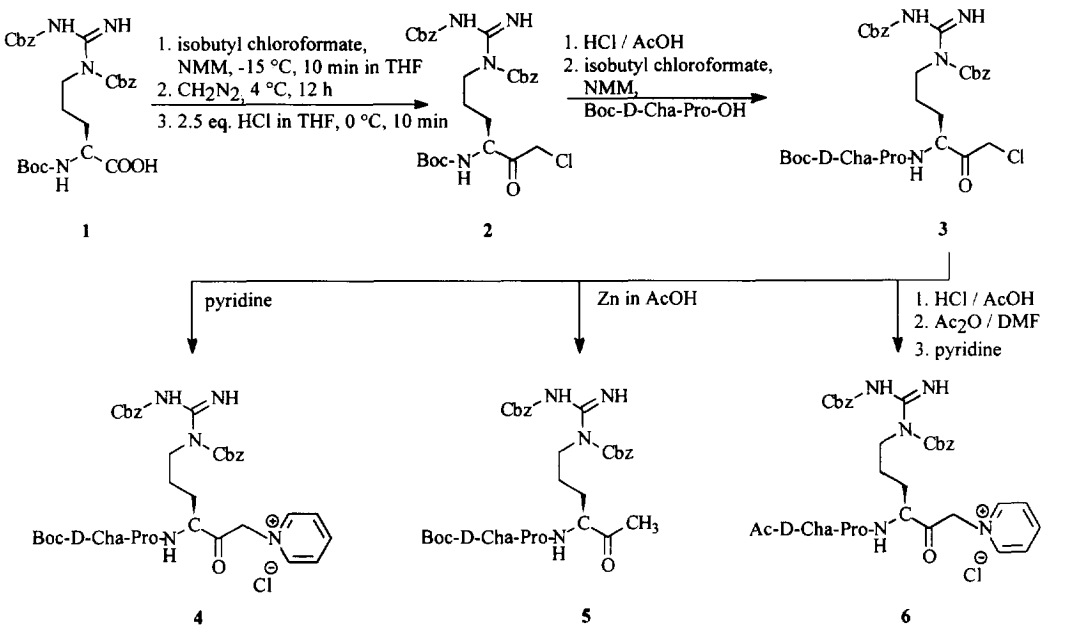
Reported here are potent substrate analog thrombin inhibitors of the pyridinium methyl ketone type, their activity is compared to sequence analog compounds lacking the permanent positively charged moiety.

Synthesis

A slightly modified procedure reported previously by Aplin et al.[5] was used to construct the tripeptidyl chloromethyl ketones as starting materials. Commercial available Boc-Arg(Cbz_2)-OH **1** was readily converted by the mixed anhydride activation and addition of diazomethane to the diazomethyl ketone, treatment with HCl in THF gave the chloromethyl ketone **2**. The Boc group was removed and the free amine was reacted with Boc-D-Cha-Pro-OH (or Boc-D-Phe-Pro-OH) to give the coupled adducts **3**.

Treatment of **3** with pyridine gave the fully protected tripeptidyl pyridinium methyl ketone **4**. In contrast, reduction of **3** with zinc dust in glacial acetic acid gave the analog methyl ketone **5** according to procedures described by Fittkau[6]. For N-terminal acetylation compound **3** was deprotected and treated with acetic anhydride. The intermediate was solved in pyridine to give **6** (Scheme 1).

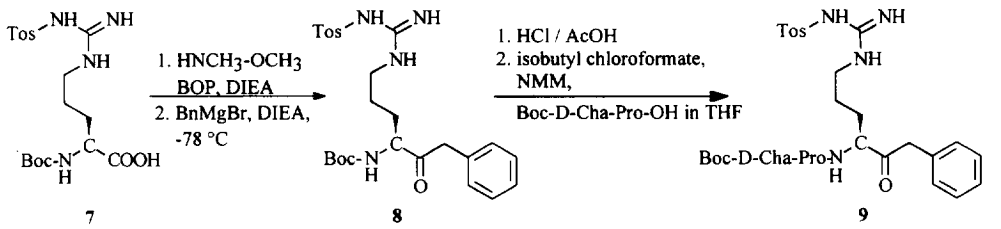
Scheme 1:



The tripeptidyl benzyl ketone was synthesized from Boc-Arg(Tos)-OH **7**, which was converted in the Boc-Arg(Tos)-N-methoxy-N-methyl-amide, followed by treatment with benzyl magnesium bromide to give the appropriate Boc-Arg(Tos)-Benzylketone **8**. According to the procedure described above, the N-terminal amino acids were coupled as a dipeptide to give the protected tripeptidyl benzyl ketone **9** (Scheme 2).

All compounds were deprotected finally by liquid hydrogen fluoride containing anisole and dimethyl sulfide (10% by volume) at -5 °C.

Scheme 2:



7 8 9

Methods

The determination of the inhibition constants with Tos-Gly-Pro-Arg-AMC and the clotting assays with fibrinogen as substrate were carried out as described by Szewczuk[7]. The peptidyl pyridinium methyl ketones were preincubated in the assay buffer at pH 7.8 for 15 hours before kinetic measurements[8].

Results

The inhibition constants for all inhibitors were determined from Dixon-Plots. Table 1 shows the results of the amidolytic assays and clotting tests.

Table 1: *Results of the amidolytic assays and clotting tests for the inhibition of human α-thrombin by the synthesized peptidyl methyl ketones.*

compound	IC_{50} (nM)	K_i (nM)
P577: Ac-D-Phe-Pro-Arg-CH_2-$^+NC_5H_5$	2700 ± 200	310 ± 60
P578: Ac-D-Cha-Pro-Arg-CH_2-$^+NC_5H_5$	180 ± 40	21 ± 6
P598: D-Cha-Pro-Arg-CH_2-$^+NC_5H_5$	1.7 ± 0.2	0.19 ± 0.06
P601: D-Cha-Pro-Arg-CH_2-C_6H_5	9.9 ± 1.5	1.25 ± 0.1
P607: D-Cha-Pro-Arg-CH_3	410 ± 22	60 ± 16
P608: Ac-D-Cha-Pro-Arg-CH_2-C_6H_5	630 ± 60	185 ± 40

The inhibition experiments usually showed linear progress curves (simple competitive inhibitors). Biphasic reaction progress curves were only observed for compound P598, as showed in Figure 1, indicating that this compound is a slow-binding inhibitor of thrombin, according mechanism A[9,10]. These progress curves were fitted to the integrated rate equation for slow-binding inhibitors (eq 1), using the data analysis application UltraFit (Biosoft) for the Macintosh PC:

$$[P] = v_S t + (v_0 - v_S)(1 - exp(-k_{obsd} t))/k_{obsd} + d \qquad \text{(eq 1)}$$

where $[P]$ is the 7-amino-4-methylcoumarin product, v_0, v_S, k and d represent the initial velocity, steady-state

velocity, apparent first-order rate constant and the displacement of *[P]* from zero at $t = 0$ respectively. In the case of P598, v_0 was constant at constant substrate concentration and different inhibitor concentrations and there was a linear dependence of k_{obsd} from the inhibitor concentration as shown in Figure 2.

Figure 1: *Progress curves for the generation of amino-methyl-coumarin by α-thrombin (60 pM) catalyzed hydrolysis of Tos-Gly-Pro-Arg-AMC (40 μM) in the presence of different concentrations of P598. Reactions were initiated by the addition of the enzyme. The progress curves were fitted to eq 1.*

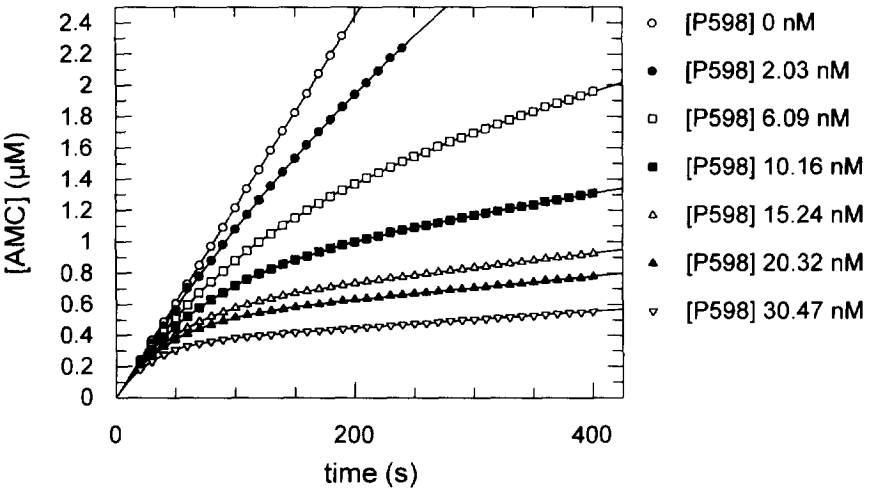
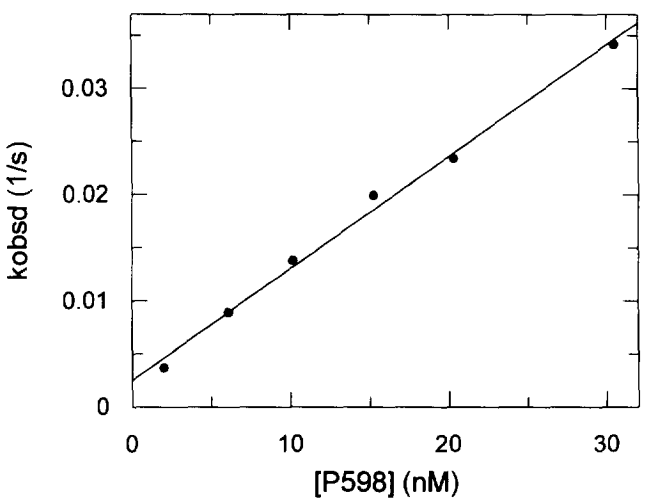


Figure 2: *Linear dependence of the apparent first-order rate constant k_{obsd}, obtained from fitting the progress curves to eq 1, from the inhibitor concentration for the inhibition of α-thrombin by P598.*



The $\{k_{obsd}$; $[I]\}$ data pairs were fitted to eq 2[9]:

$k_{obsd} = k_{on}[I] / (1 + [S] / K_m) + k_{off}$ (eq 2)

When K_m and $[S]$ are set to 6.09 μM and 40 μM respectively, linear least-squares analysis yielded a value for

$k_{on} = 7.97 \cdot 10^6 \pm 3.1 \cdot 10^5\ M^{-1} s^{-1}$

For slow-binding mechanism A K_i depends from k_{on} and k_{off} according to eq 3[9]:

$K_i = k_{off} / k_{on}$ (eq 3)

Therefore the dissociation rate constant was calculated to be $k_{off} = 1.1 \cdot 10^{-3}\ s^{-1}$.

Discussion

The substrate analog peptidyl pyridinium methyl ketones are very potent inhibitors of human α-thrombin. However, it was still unclear, if the reason for the high activity was based on the electron withdrawing effect of the permanent positive charge on the pyridinium nitrogen, which possibly could lead to the formation of a transition state analog enzyme/inhibitor complex, or if the increase in affinity was based only on enhanced hydrophobic interactions induced by the aromatic pyridinium ring in the P_1'-region. Therefore, the peptidyl benzyl ketone P601 and the peptidyl methyl ketone P607 were synthesized as analogs to P598. The first compound lacks the permanent positive charge, but still has an aromatic ring in the P_1'-position, the second analog P607 has neither the positive charge nor the aromatic and hydrophobic P_1'-residue. Interestingly, the benzyl ketone P601 was around one order of magnitude less active than P598, but on the other side ca. 50 times more potent than P607. In contrast to the slow-binding inhibitor P598 both compounds were characterized as classical competitive inhibitors, they did not show any time-dependent inhibition. These kinetic results lead to the conclusion that the high activity of the peptidyl pyridinium methyl ketones is based on both the electron withdrawing and the hydrophobic effects of the aromatic pyridinium moiety. Possibly, independent from the design of transition state inhibitors, the incorporation of hydrophobic residues in the P_1'-region could also enhance the activity of other types of thrombin inhibitors. A recently published crystal structure of the complex between thrombin and a bivalent thrombin inhibitor containing an arginyl pyridinium acetic acid methyl ketone in the P_1-P_1' region could prove the existence of a covalent tetrahedral intermediate between Ser^{195} of thrombin and the P_1 carbonyl group of the inhibitor[12].

The acetylation of the amino group in P_3-position results in a very strong decrease in potency of these substrate analog inhibitors. This is clearly demonstrated by the comparison of compound P578 with P598 and P608 with P601. This corresponds well with results published previously by Stone[11], where the reaction rate of thrombin (k_{cat}/K_m) with chromogenic substrates is much faster, if the N-terminal D- amino acid in P_3-position is unprotected. Probably, the reason is the formation of a stronger hydrogen bond between the carbonyl oxygen of Gly^{216} from thrombin with the protonated amino group of the P_3 amino acid compared to the acetylated amino group[11].

The peptidyl pyridinium methyl ketones may also have potential value for the design of transition state inhibitors for other types of serine proteases.

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$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} E:S \xrightarrow{k_{cat}} E + P$$

$$k_{off} \uparrow\downarrow k_{on}[I]$$

$$E:I$$

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