Bioorganic & Medicinal Chemistry Letters, Vol 2, No.12, pp. 1607-1612, 1992
Printed in Great Britain

0960-894X/92 $5 00 + .00

α–HYDROXY- AND α-KETOESTER FUNCTIONALIZED THROMBIN INHIBITORS

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(*Received* 31 *August* 1992)

Abstract

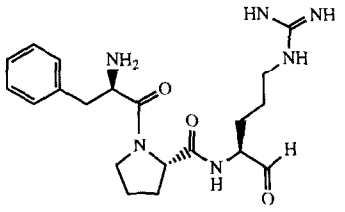
α-Hydroxy- and α-ketoester functionalized D-Phe-Pro-Lys tripeptides were found to be potent thrombin active site inhibitors. The ketoester derivatives were characterized by slow binding kinetics. The most potent of the series was **9** (**BMS 181,412**) with an overall inhibition constant K_i^* of 0.0017 μM.

Introduction

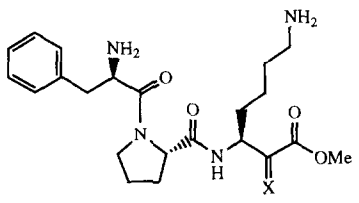
Thrombin, the serine protease product of the blood coagulation cascade, catalyzes the cleavage of fibrinogen to form fibrin and potently stimulates blood platelets, vascular smooth muscle and endothelium.[1] Most or all of these actions require serine protease catalytic activity. As a result, inhibitors of thrombin catalytic activity have been considered as potential targets for drug design as antithrombotic and anticoagulant agents. A number of compounds have been synthesized to address the current need for such agents.[2]

Recent studies have examined the anticoagulant and antiplatelet properties *in vivo* and *in vitro* of peptidic inhibitors of thrombin in which the scissile bond is replaced with an aldehyde moiety.[3] With related serine proteases, other electrophilic residues susceptible to the formation of (hemi-) acetals or ketals have been employed to form a covalent bond between inhibitors and the active site serine hydroxyl.[4,5,6] In the case of α-chymotrypsin, the replacement of an aldehyde by an α-ketoester on a peptidic inhibitor provides a significant increase in potency.[4b]

Thrombin is selective for substrates containing a basic group at P1, especially arginine. Reported here are compounds in which the arginine residue is replaced by lysine, and the electrophilic aldehyde has been replaced by a ketoester. These inhibitors were modeled after the D-Phe-Pro-Arg sequence exemplified by **RGH 2958**.[7] In addition to the ketoester **1a**, the corresponding diastereomeric hydroxyesters **1b** were also of interest as active site inhibitors.



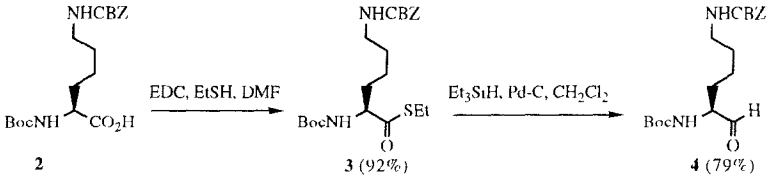
RGH 2958



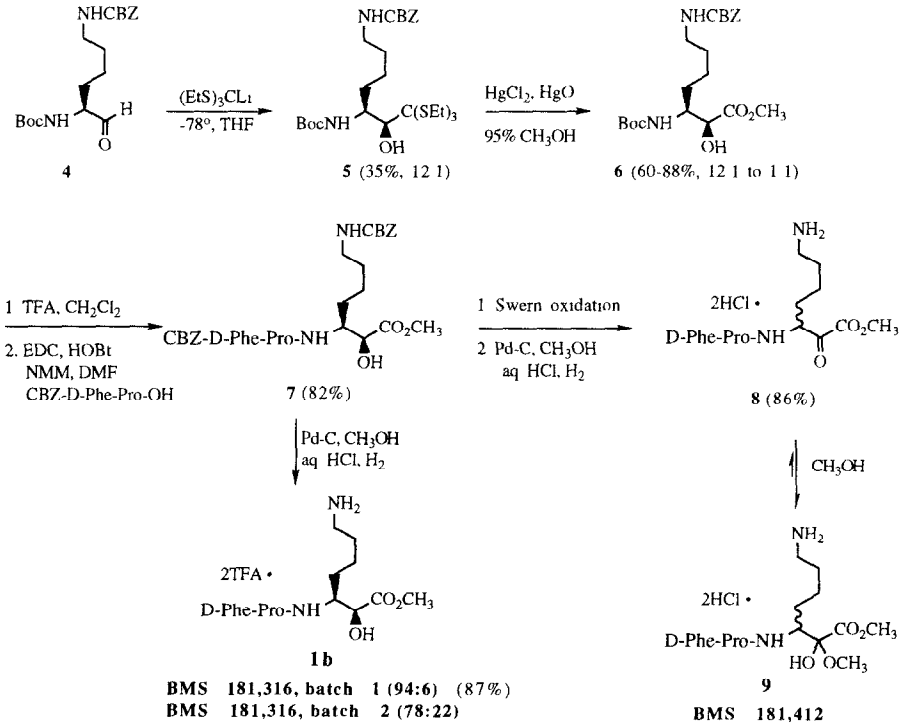
1a (X= O)
1b (X= HO, H)

Synthesis

A synthetic scheme reported by Burkhart was used to construct the carbon framework of the α-hydroxy- and the α-ketoester residues.[8] Bis-protected lysine **2** was readily converted to orthothioester **3**, which was then transformed to aldehyde **4** using Fukuyama's conditions.[9]

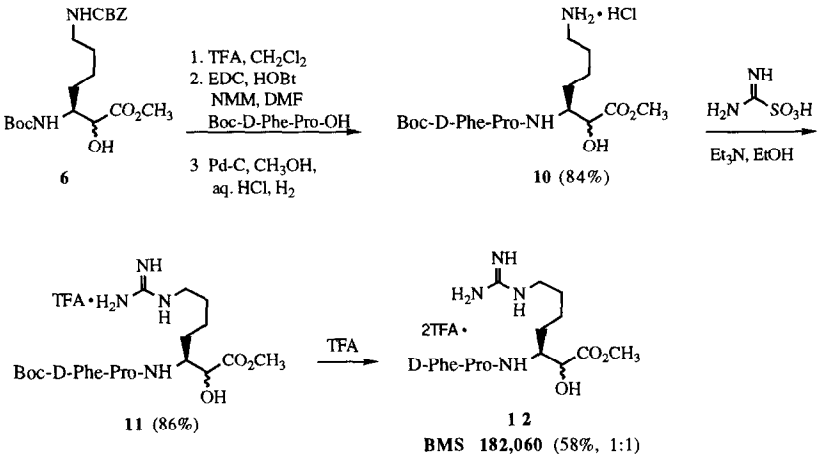


Treatment of **4** with lithiated tris(ethylthio)methane provided a mixture of diasteromeric alcohols **5** that was enriched in the *syn* isomer as shown in the scheme.[10] Transformation of **5** into the corresponding esters **6** proved to be sensitive to reaction conditions, giving variable yields and diastereomeric ratios of the methyl esters.[11] The *t*-butyl carbamate (Boc) group was removed, and the free amine was reacted with CBZ-D-Phe-Pro-OH to give the coupled adduct **7**.[12] Both benzyl carbamate (CBZ) groups were then removed by hydrogenation, and the targets were purified using preparative HPLC to give **1b [BMS 181,316**, batch 1 (94% isomer A, 6% isomer B) and batch 2 (78% isomer A, 22% isomer B)].



The penultimate compound **7** was subjected to Swern oxidation conditions to provide the α-keto ester; in the process the stereochemical integrity of the β-carbon was lost. Catalytic hydrogenation provided diamine **8** contaminated with the corresponding hydrate and hemiketal **9**.[13] The highly electrophilic nature of the α-keto group was evident by ^1H and ^{13}C NMR (CD_3OD) where the diastereomeric hemiketals were observed.[14] Efforts in purification led to imine formation and hydrolysis of the methyl ester.[15] Treatment of **8** with methanol a gave mixture of the diastereomeric hemiketals **9**, which was tested without further purification.

The synthesis of homoarginine **12**, unlike the lysine analogs, required different protecting groups that could be selectively removed. Thus, the corresponding salt of **6** was coupled with Boc-D-Phe-Pro-OH to give the protected tripeptide. Hydrogenation selectively removed the benzyl carbamate to give **10**. Reacting the amine with aminoiminomethanesulfonic acid provided guanidine **11**, which was purified by preparative HPLC to give 86% of **11** and 13% of **12**.[16] The final deprotection step provided **12** (**BMS 182,060**).



Methods

Thrombin (obtained from J. Fenton II, Albany, NY) cleavage of the synthetic substrate S-2238 (D-Phe-Pip-Arg-pNA) was measured at room temperature (23-25 °C) using a kinetic microplate reader (UV_{max} microtiter plate reader, Molecular Devices Corporation). Enzyme reactions took place in 300 μL final volume containing 145 mM NaCl, 5 mM KCl, 1 mg/ml Polyethylene glycol (PEG-8000), 30 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid at pH 7.4. The K_m for the substrate (S-2238) was determined by curve fitting the enzyme velocity versus substrate concentration curves and was 2.45 μM under the conditions used in this assay. The inhibitors were dissolved in water or dimethyl sulfoxide, when required. The concentration of dimethyl sulfoxide (final concentration 3.3 %) used had no effect on the enzyme activity when the synthetic substrate cleavage was measured. Data acquisition (optical density at 405 nM was monitored) and computer control of the plate reader were performed using an Apple Macintosh IIci computer running Softmax (version 2.02). Clotting time measurements were determined optically at 37 °C using an MLA 700 coagulation timer. Thrombin clotting time was determined with 0.1 mL of citrated human plasma (1/10th final volume of 0.129 M buffered citrate) added to 0.1 mL of Owren's buffer and stimulated with 0.1 mL of 10 U/mL human α-thrombin (Sigma). The concentration of inhibitor that caused a doubling of thrombin clotting time was determined.

Results and Discussion

In initial testing, thrombin was incubated with the inhibitor for three minutes prior to starting the reaction with 10 μM S-2238. **RGH 2958**, **1b** (Batches 1 and 2), **9** and **12** were found to inhibit the cleavage of the synthetic substrate (Table 1). The hemiketal **9** was found to be a potent inhibitor of thrombin, comparable to **RGH 2958**. The diastereomeric hydroxyesters **1b** (Batches 1 and 2) were also found to be active site inhibitors of thrombin. When the inhibitors were added directly to plasma (no preincubation with thrombin), **RGH 2958** and **9** prolonged clotting times to comparable degrees (Table 1).

Table 1

Compound	Thrombin IC_{50} (μM, 3 min)	Clotting Time* (μM)
RGH 2958	**0.049**	**0.40**
1b (BMS 181316, Batch #1)	**30.5**	**>300**
1b (BMS 181316, Batch #2)	**20.2**	**>300**
9 (BMS 181412)	**0.032**	**0.39**
12 (BMS 182060)	**48.0**	**----**

* Inhibitor concentration necessary to double thrombin clotting time

Compound **12** was found to be a simple competitive inhibitor of thrombin catalytic activity (no time dependence of thrombin inhibition), with a K_i determined from Dixon plots (using three different concentrations of substrate) of 28.2 ± 10.0 (m ± SD, N=5) μM.

Compounds **1b** and **9** (as well as the **RGH 2958**) were found to inhibit thrombin catalytic activity in a time-dependent manner, typical of slow-binding enzyme inhibitors (Fig. 1).

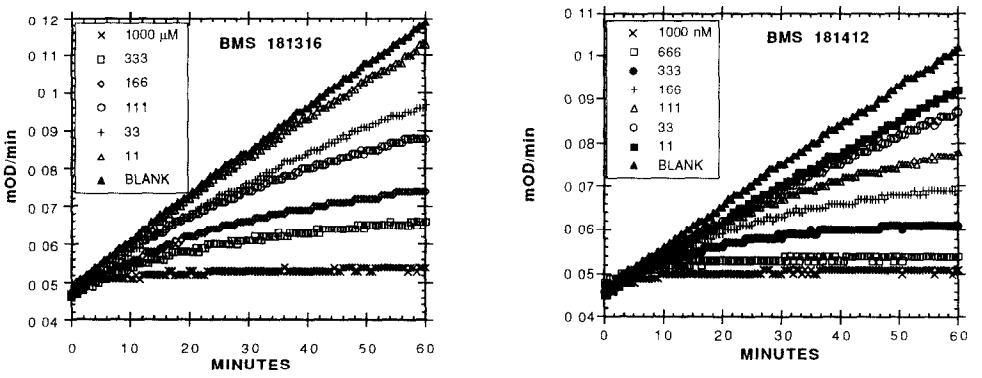


Figure 1. Progress curves showing the time-dependent inhibition of thrombin with increasing concentrations of inhibitors. The panel on the left depicts data obtained with **1b** (**BMS 181,316**) and the right panel **9** (**BMS 181,412**). The concentration of substrate (S-2238) was 100 μM and the reaction was started with the addition of 0.3 U/mL α-thrombin. The concentration of the enzyme was chosen such that less than 10% of the substrate was consumed during the time course of enzymatic determination. These curves are representative of four determinations.

The progress curves (four sets of data for each compound) were fit to the integrated rate equation for slow binding inhibitors[17] (using KaleidaGraph data analysis/graphics application for the Macintosh PC):

$$P = v_S t + (v_O - v_S)(1 - e^{-kt})/k$$

where P is the para-nitroaniline product. v_O, v_S, and k represent the initial velocity, steady-state velocity and apparent first-order rate constant respectively. The data were fit to the above equation to derive v_O, v_S, and k. The initial enzyme velocity did not change as a function of the inhibitor concentration indicating a type A mechanism.[17] This designation was confirmed by linear plots of k vs [I] (not shown). The overall inhibition constant, designated K_i^*, was determined using the steady state enzyme velocity (v_S) for competitive inhibition fit to the following equation.

$$v_S = v_{max} [S] / K_m(1 + [I] / K_i^*)+[S]$$

The K_i^* values for **1b** (**BMS 181,316**) and **9** (**BMS 181,412**), determined by curve fitting four sets of data, were 1.1 μM and 0.0017 μM, respectively.

Conclusion

Many efforts to develop potent peptidic inhibitors of proteases have focused on the introduction of residues which mimic tetrahedral species found in the normal proteolytic mechanism. We have described α-hydroxyester and derivatized α-ketoester functionalized D-Phe-Pro-Lys inhibitors of thrombin catalytic activity.

Both **1b** and **9** may be described as slow binding inhibitors, a characteristic commonly associated with analogs of intermediates of enzymatic reactions. In both cases the initial enzyme velocity did not change as a function of inhibitor concentration suggesting a type A mechanism.[18] A slow on-rate is characteristic of an A type mechanism, owing to barriers the inhibitor and/or enzyme may encounter in the process of binding at the active site.[17b]

Unlike **1b**, the stability of **9** in the enzyme assay is in question. Compound **10** may hydrolyze to ketoester **1a** which would be in equilibrium with the corresponding hydrate **13**.[19] This additional complexity prevents us from assigning the individual contribution of each of the three species at maximal inhibition.

By suitable replacement of the aldehyde and arginyl residues of **RGH 2958**, we have fashioned a potent active site inhibitor of thrombin: **9** (**BMS 181,412**). In addition to potent inhibition of thrombin *in vitro*, we also observed marked activity in plasma on clotting times.

Acknowledgment

We acknowledge Mr. Mark Bolgar's contributions in identification of materials by mass spectral analysis.

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10. The major isomer was assigned by comparing products of similar addition reactions to α-aminoaldehydes.
11. Elevated temperatures gave increased yields but with a loss in diastereomeric purity.
12. Dipeptides for the C terminus (CBZ-D-Phe-Pro-OH and Boc-D-Phe-Pro-OH) were prepared under standard peptide coupling conditions.
13. Mass spectral and infrared analysis suggested the ketoester as well as the corresponding hydrate and hemiketal were present.
14. The NMR spectra were complex, however, four diastereomers were observed. ^{13}C NMR signals at δ 99.91 and 99.85 ppm (CD_3OD) were assigned as the hemiketal carbons of the two major diastereomers.
15. Mass spectral analysis of preparative HPLC fractions suggested the carboxylic acid and the cyclic imine formed during the attempted purification.
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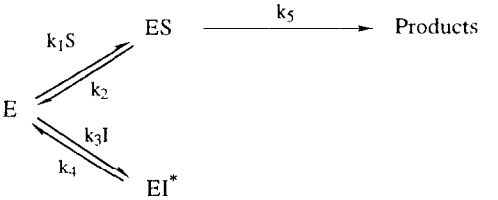
18. The type A mechanism as described by Morrison and Walsh is given below.

Mechanism A



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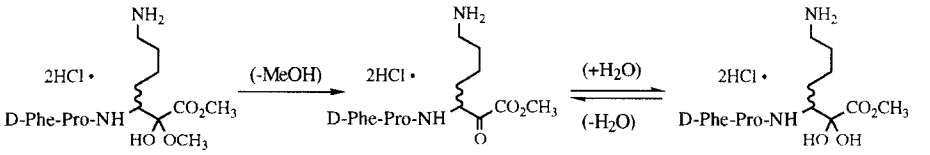

BMS 181,412-01 **1a** **13**