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Solid-Phase Synthesis of Thrombin Inhibitors

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Abstract—A newly developed convergent solid-phase synthesis provides efficient access to thrombin inhibitors of the D-Phe-Pro-Arg type. Members of the synthesized libraries inhibited thrombin with IC_{50}s in the nanomolar range.

Thrombin is a trypsin-like serine protease which plays a crucial role in blood coagulation. It cleaves soluble fibrinogen into fibrin. Subsequent polymerization of fibrin stabilizes the initially formed thrombozyte clots at the site of a blood vessel damage.

Undesired activation of the blood coagulation cascade, however, can result in cardiovascular disorders such as deep vein thrombosis, myocardial infarction, unstable angina, pulmonary embolism, and ischaemic stroke. Thrombin has therefore become an important target for the treatment of thromboembolic diseases.[1]

Many approaches to thrombin inhibitors are centred around the D-Phe-Pro-Arg motif that mimics the natural substrate.[1] D-Phe replaces the Phe residue in fibrinogen which normally occupies the lipophilic D pocket. The guanidine moiety of Arg which is also present in the natural substrate forms a salt bridge with Asp189 at the bottom of the specificity pocket of thrombin. Interactions with Ser195 in the active center of thrombin were shown not to be essential for high affinity to the enzyme.[1]

Figure 1 shows a typical representative of this class of thrombin inhibitors. Arg in P1 has been replaced by a heteroaryl amidine, Pro in P2 by dehydroproline, and D-Phe in P3 by D-Cha. Inhibitors of this type, although very potent, tend to lack selectivity towards related serine proteases. Optimization of each moiety's fit to its binding pocket should lead to improved selectivity.

In our own ongoing efforts towards potent and selective thrombin inhibitors[2] we felt that this optimization could best be achieved by means of combinatorial chemistry.[3]

We therefore designed a convergent solid-phase synthesis which permits independent variation of the P1-, P2-, P3- and P4-positions.

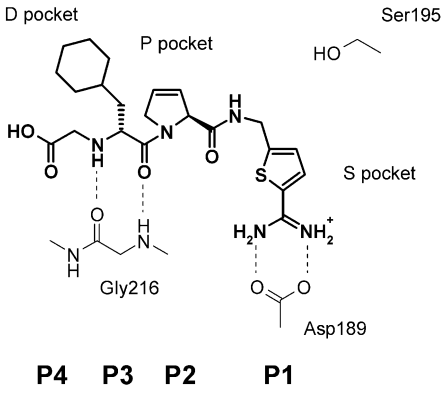

Figure 1. Binding of an inhibitor to the active site of thrombin.

Chemistry

The desired compounds can in principle be assembled from either end, that is with the P1- or the P4-moiety bound to the solid support. Both strategies were evaluated, the latter was abandoned because of unsatisfactory coupling yields and purities.

We decided to adopt a convergent approach in which a protected P2–P4-fragment was synthesized on solid phase separately and then coupled to a resin bound P1-unit.

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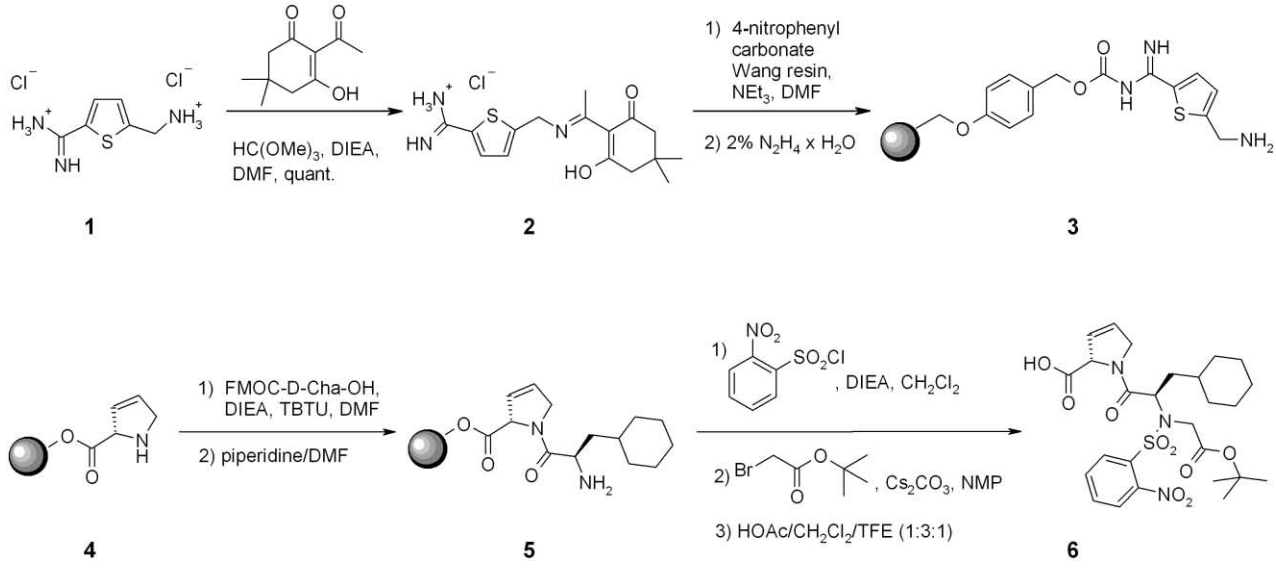
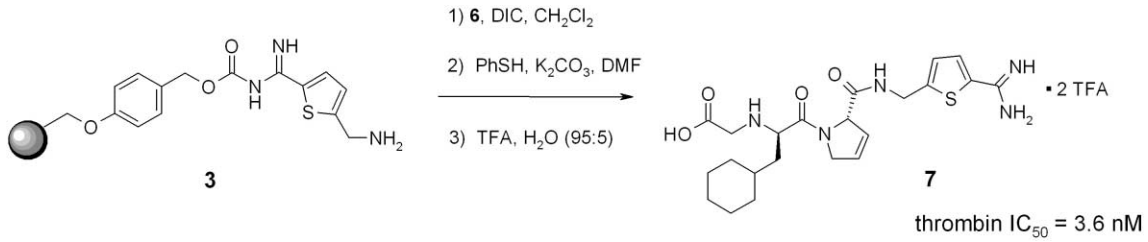


Scheme 1. Syntheses of resin bound amidine derivative **3** (P1-fragment) and P2–P4-fragment **6**.



Scheme 2. Synthesis of thrombin inhibitor **7** by coupling P2–P4-fragment **6** to support bound P1-fragment **3**.

In this way, those impurities of the P2–P4-fragment, which did not couple to P1-resin could simply be washed away and did thus not appear in the final product. In addition it would be possible to purify the P2–P4-fragment before the coupling reaction, if necessary.

To prepare the resin bound P1-fragment the Dde-protected[4] amidine derivative **2** was synthesized from acetyldimedone and **1**[5] (Scheme 1) and reacted with 4-nitrophenyl carbonate-Wang resin.[6] Subsequent deprotection with 2% hydrazine hydrate in dimethylformamide gave **3**. The partly protected P2–P4-fragment **6** was assembled starting from dehydroproline 2-chlorotrityl resin **4**[7] (Scheme 1). Subsequently dipeptide **5** was prepared using standard deprotection and coupling methods. The desired monoalkylation of **5** was achieved by conversion of the N-terminal amine into the 2-nitrobenzenesulfonamide which could be alkylated under mild conditions.[8] Cleavage from the support afforded the P2–P4-fragment **6**, which was subsequently coupled to the polymer bound amidine derivative **3** (Scheme 2). After removal of the sulfonamide protecting group with potassium thiophenolate in DMF and cleavage from the support **7** was obtained in 90% purity (RP-HPLC, UV detection at 214 nm).

A library of inhibitors with variations in the P1-, P2- and P3-positions was synthesized[9] and tested for inhibition of thrombin and other serine proteases like trypsin. Several compounds inhibited thrombin at nanomolar concentration as determined in a chromogenic assay.

In summary this solid phase synthesis allowed us to rapidly and efficiently generate inhibitor libraries in which the P1-, P2- and P3-positions are varied and to investigate the effects of these variations on potency and selectivity. This work is in progress, results will be reported in due course.

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5. Amidine derivative **1** was prepared by analogy with procedures described in ref 2.
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9. Representative procedure: To a suspension of 1.3 g (5.7 mmol) **1** in 15 mL DMF was added 1.19 mL (6.4 mmol) DIEA, 1.25 g (6.84 mmol) acetyldimedone and 3.12 mL (28.49 mmol) trimethyl orthoformate. The mixture was stirred at rt for 2.5 h, the residue obtained after evaporation was treated with CH_2Cl_2 (5 mL) and petroleum ether (20 mL). After decanting from the solvent, **2** was obtained as a yellowish solid which was dried in vacuo. Yield: quant.

4-Nitrophenyl carbonate Wang-resin (Novabiochem, 3 g; 1.62 mmol) in 20 mL DMF was reacted with 1152 mg (3.24 mmol) Dde-protected amidine derivative **2** and 4.48 mL (32.4 mmol) triethylamine (3 d, rt). Then the resin was washed with MeOH, DMF and CH_2Cl_2 and dried in vacuo. Yield: 3.33 g.

The Dde-protecting group was cleaved by treatment with 20 mL 2% solution of hydrazine hydrate in DMF (rt, 5 min). This procedure was repeated twice. Subsequently the resin was washed with DMF, CH_2Cl_2, MeOH and CH_2Cl_2 and dried in vacuo. Yield: 2.84 g **3**.

Starting from dehydroproline-2-chlorotrityl resin **4** the dipeptide resin **5** was synthesized by standard Fmoc-deprotection (piperidine) and coupling (TBTU) methods. A solution of 261 mg (1.13 mmol) 2-nitrobenzenesulfonyl chloride in 2 mL CH_2Cl_2 was slowly added to 0.75 mmol resin **5** and 261 μL (1.5 mmol) DIEA in 5 mL CH_2Cl_2. The mixture was incubated at rt for 4 h, then the resin was filtered and washed with CH_2Cl_2, DMF, MeOH and CH_2Cl_2. The resin was suspended in 7.5 mL NMP containing 975 mg (3 mmol) Cs_2CO_3 and was shaken at rt for 15 min, followed by addition of 220 μL (1.5 mmol) *t*-butyl bromoacetate. After 20 h at rt the resin was filtered, washed with water, DMF, CH_2Cl_2, MeOH and CH_2Cl_2 and dried in vacuo. Cleavage was carried out using trifluoroethanol/acetic acid/CH_2Cl_2 (1:1:3; 1 h, rt). The residue obtained after evaporation was dissolved in glacial acetic acid and lyophilized. Yield: 226 mg (0.4 mmol) **6**.

Amidine resin **3** (81 mg; 0.04 mmol), 45 mg (0.08 mmol) **6** and 12.3 μL (0.08 mmol) diisopropylcarbodiimide in 1 mL CH_2Cl_2 were shaken at rt for 20 h (or until Kaiser ninhydrin test was negative). Then the resin was filtered and washed with CH_2Cl_2, MeOH and CH_2Cl_2.

Thiophenol solution in DMF (60 mL; 0.5 M) and 828 mg (6 mmol) K_2CO_3 were stirred under an argon atmosphere for 30 min at rt, excess K_2CO_3 was filtered off.

The resin was treated with 3 mL of the above thiophenolate solution for 20 h at rt, filtered, washed with CH_2Cl_2, MeOH and CH_2Cl_2 and dried in vacuo. Cleavage was performed with 95% aqueous TFA (1 h, rt). The residue obtained after evaporation was dissolved in water (2 mL), the aqueous phase was washed twice with CH_2Cl_2 and lyophilized. Yield: 16 mg **7** (0.023 mmol, 58%).