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Preparation of L-proline based aeruginosin 298-A analogs: Optimization of the P_1-moiety

Guijun Wang *, Navneet Goyal, Branden Hopkinson

Department of Chemistry, University of New Orleans, New Orleans, LA 70148, United States

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ABSTRACT

Aeruginosins are a family of naturally occurring oligopeptides that share a common bicyclic amino acid core structure. Many compounds in the family are inhibitors of serine proteases, such as thrombin and trypsin. Thrombin is an important enzyme in the blood coagulation cascade, and is a promising target for anticoagulant drug development. In order to understand the structure–activity relationship (SAR) and to find selective thrombin inhibitors, we synthesized a series of aeruginosin 298-A analogs, in which the P_2 bicyclic amino acid was replaced by a L-proline residue. The structure optimization was focused on modification of the P_1 position. In choosing the P_1 group, an effort was made to avoid using the highly basic guanidine groups present in nearly all naturally occurring aeruginosins. The synthesis and enzyme assays of these aeruginosin analogs against thrombin and trypsin are reported. We found that several compounds with neutral P_1 groups exhibit excellent selectivity over trypsin and good potency against thrombin. The SAR data of the P_1 groups obtained here can be used in preparing other thrombin inhibitors with better selectivity against trypsin.

Blood coagulation disorders can lead to serious diseases, including deep vein thrombosis and pulmonary embolism. The two main drugs currently used to treat such disorders are heparin and warfarin, but both suffer certain limitations. There have been intense efforts over the past few decades to develop safer and more effective anticoagulants.[1–5] Because thrombin is an important enzyme in the blood coagulation cascade, it has become a promising target. Direct inhibition of thrombin has led to the development of several effective anticoagulants, such as argatroban and hirudin.[6,7] However, the discovery of new, orally available, safe drugs to treat thromboembolism is still very challenging.[8,9] The blood coagulation factors, including thrombin, are trypsin-like serine proteases. These serine proteases have similar binding sites especially at their S_1 binding sites. While thrombin affects coagulation of the blood, trypsin is an important digestive enzyme. Because of the similar active sites, gaining selectivity between these serine proteases has been a challenge.

Aeruginosins are small linear peptides that contain a common, fused bicyclic amino acid core structure, 2-carboxy-6-hydroxyoctahydroindole (Choi). More than 20 compounds in the family have been identified so far, and many of them exhibit serine protease inhibition activities.[10–18] Typically, aeruginosins contain 4 amino acid residues; the structures of several aeruginosins are shown in Figure 1. The majority of the family contains a Choi core with the configuration of 2*S*, 3a*S*, 6*R*, 7a*S*, as shown in aeruginosin 298-A.

Aeruginosin 298-A (**1**) contains the structure D-Hpla-D-Leu-L-Choi-L-Argol, and it inhibits thrombin and trypsin with IC_{50} values of 0.5 and 1.7 μM, respectively.[18a] Aeruginosins 89-A, 89-B, and 102 contain an argininal (Argal) at the P_1 unit; they also exhibit good potency against thrombin and trypsin. Aeruginosin 89-A (**2a**) inhibits thrombin and trypsin at 0.03 and 0.4 μg/mL, respectively, while aeruginosin 89-B (**2b**) inhibits thrombin and trypsin at IC_{50} = 0.05 μg/mL and 6.6 μg/mL, respectively. Although the Argal group can provide good potency, it has been shown that Argal containing compounds are not suitable drug candidates due to the reactivity of the aldehyde functional group. Oscillarin (**4**) has the common Choi unit, but contains different P_1 and P_3 residues.[19] It contains a cyclic 3-aminoethyl 1-guanidylpyrroline group at the P_1 position. Oscillarin is one of the most potent thrombin inhibitors in the aeruginosin family, with an IC_{50} = 28 nM. Dysinosins have the 5,6 dihydroxyl-octahydroindole 2-carboxylic acid core structure instead.[16,17,20] Dysinosin A (**5**) is both a factor VIIa (K_i = 0.11 μM) and a thrombin inhibitor (K_i = 0.45 μM).[16a] Aeruginosin 98-B (**6**) has a sulfated Choi unit, and it is more selective for trypsin, with IC_{50} values of 0.6 μg/mL and 10 μg/mL against trypsin and thrombin, respectively.[18b] Aeruginosin EI461 does not have a P_1 subunit, and it only weakly inhibited 15% of trypsin activity at 45.5 μg/mL concentration.[15a]

Among the family of natural products, the four types of P_1 residues discovered so far are argamatine (**7**, Agma), argininol (**8**, Argol), argininal (**9**, Argal), and the aminoethyl 1-*N*-amidino-3-Δ-pyrroline (**10**, Adc) (Fig. 2). They all contain a basic guanidine group that is presumably hydrogen bonded to various aspartic

* Corresponding author. Tel.: +1 504 280 1258; fax: +1 504 280 6860.
E-mail address: gwang2@uno.edu (G. Wang).

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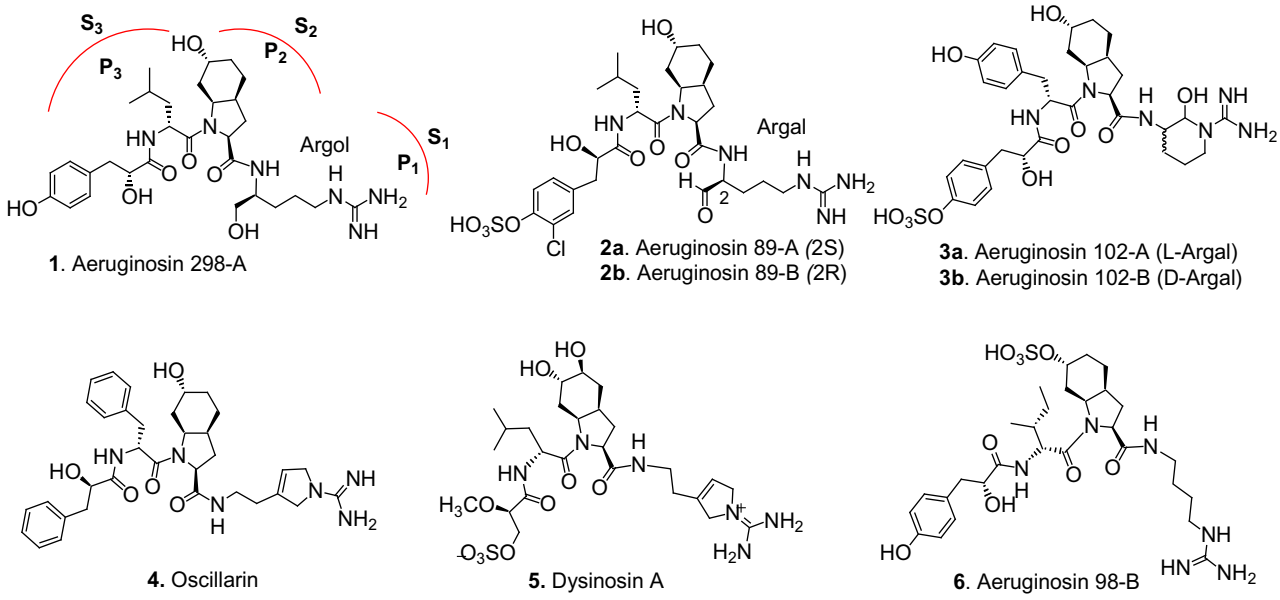


Figure 1. Structures of several aeruginosins (**1**–**6**).

residues of the proteases. The highly basic guanidine group is important for the observed biological activities, but it has been shown that highly basic groups generally lead to poor bioavailability and in vivo activity.[21,22] Considerable efforts have been devoted to finding neutral P_1 groups which can lead to good oral anticoagulants.[23–26]

Although many aeruginosins exhibit promising biological activities, their structures need to be optimized to obtain useful therapeutic agents. One challenge is to obtain selectivity among the different serine proteases; other problems may include their peptide like structures and structural complexity. In recent years, there has been growing interest in synthesizing analogs of aeruginosins.[10,11,27–31] L-Proline has been a commonly used P_2 group in thrombin inhibitor designs including orally active thrombin inhibitors[32–34] and aeruginosin analogs.[27] With the seminal works reported by Hanessian's group and several other research teams, a significant amount of knowledge has been obtained regarding the SAR of the aeruginosin families. Among the various modified aeruginosin analogs, many showed improved potency compared to their original natural products; however, many of these compounds are also potent inhibitors for trypsin. The blood coagulation factors, such as thrombin, and factors Xa and VIIa, are all trypsin-like enzymes which share similar active sites and can thus bind similar compounds. Therefore, it is important to find molecules that can exhibit selectivity for thrombin and/or other blood coagulation factors over trypsin in order to avoid possible side effects. Previously, we had prepared aeruginosin analogs **11** and **12** (Fig. 3), in which the L-Choi is replaced with a pair of enantiomers.[31] The compound **11**, with the opposite stereochemistry to that of Choi, showed remarkably improved selectivity over trypsin with slightly diminished activity against thrombin.

In order to optimize the structures of the naturally occurring aeruginosins we synthesized several novel analogs of aeruginosin 298-A. In these analogs, the L-Choi is replaced with L-proline, and the Argol group is replaced with a series of functional groups including less basic or neutral P_1 groups. Although L-proline based thrombin inhibitors have been well studied, there have been no SAR studies of the P_1 position in which the P_2 unit is replaced by L-proline but the P_3 and P_4 units are the same as those found in aeruginosin 298-A. We hope to find compounds with good selectivity toward thrombin and potentially improved pharmacokinetic properties. The preparation of these compounds and their preliminary activities against thrombin and trypsin are shown below.

The synthesis of the aeruginosin analogs is shown in Schemes 1 and 2. L-Proline was converted to the ester **14**, which was then coupled with *N*-Boc-D-Leucine to give the dipeptide **15**. Next, the Boc group was removed to give the amine **16**, which was subsequently coupled with the protected form of D-Hpla **17** to afford the tripeptide **18**. With **18** in hand, ester groups were hydrolyzed to give the free acid **19**, which was then coupled to various P_1 groups.

One example of the coupling reaction used to prepare the tetrapeptide is shown in Scheme 2. The diamine **20** was treated with *N*-Boc protected thiomethyl pseudourea to give the intermediate **21**. Compound **21** was coupled with the tripeptide **19** using typical coupling reagents such as HATU, PyBOP, or EDCI and HOBt, depending on which amine was used. Compound **22** was then converted to the tetrapeptide **23** after removal of benzyl and Boc protecting groups.

Several compounds with the general structure **24** were synthesized, and enzyme assays of the compounds were conducted.[31] The structures of the various P_1 groups and their 50% inhibition concentrations are shown in Table 1. Commercial thrombin inhibitor PPACK (520222, Calibiochem) was used as the standard for the

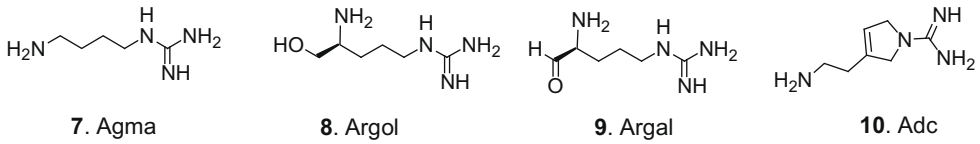


Figure 2. The various P_1 groups in the natural products.

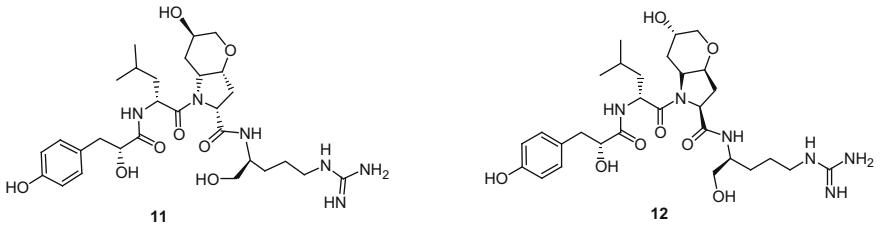
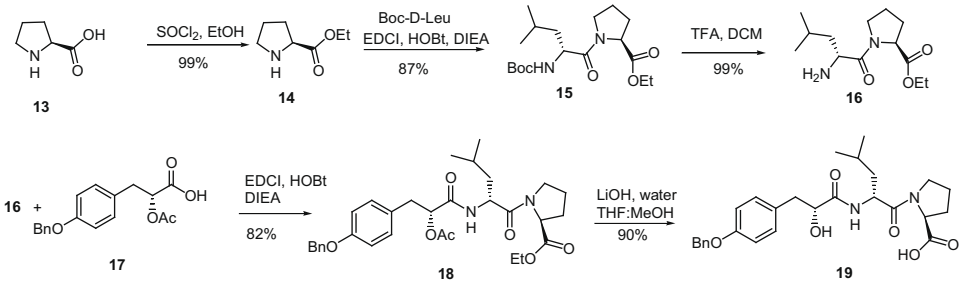
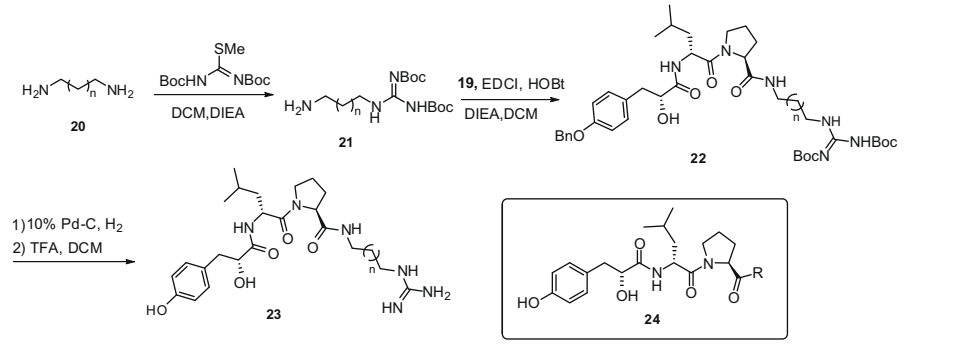


Figure 3. Structures of aeruginosin 298-A analogs containing different P_2 units.



Scheme 1. Preparation of tripeptide **19**.



Scheme 2. Preparation of the tetrapeptide **23** and the general structure of tetrapeptide **24**.

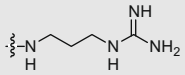
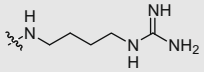
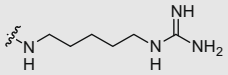
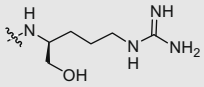
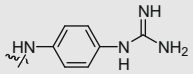
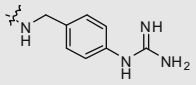
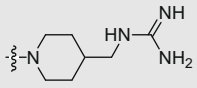
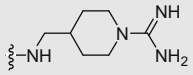
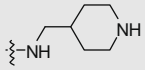
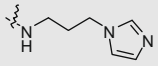
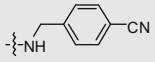
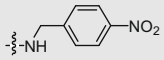
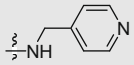
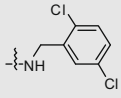
thrombin assay, and trypsin inhibitor from chicken egg white (T9523, Sigma) was used as the standard for the trypsin assay.

From the enzyme assay results, we can see that the P_1 specificity group is important to the thrombin inhibition. For the compounds with guanidine groups (**25**–**32**), reasonable inhibition activity toward thrombin was typically exhibited. Compound **25** is the most potent among the three simplest compounds (**25**–**27**), which have similar structures but different chain lengths of the P_1 group. Compound **27**, with a 5-methylene linker, showed the best selectivity to thrombin over trypsin. Compound **28** has the same structure as aeruginosin 298-A, except that the Choi is replaced with L-proline; it seems to have maintained similar activities toward both enzymes. Compounds **29**–**30** contain the phenyl guanidine group, which does not seem to inhibit trypsin well, but has good activity toward thrombin. Compounds **31**–**33** have a piperidinyl group; **31**, with the secondary amine, showed good selectivity to thrombin. Without the amidine group from **32**, compound **33** showed good activity to thrombin and no inhibition to trypsin.

Several compounds without highly basic groups at the P_1 positions gave good inhibition activity against thrombin while also maintaining good selectivity over trypsin. For example, compounds with C-4-piperidinyl-methyl (**33**), propyl imidazole (**34**), cyanobenzyl (**35**), nitrobenzyl (**36**), and C-4-pyridinyl-methyl (**37**) do not contain the highly basic guanidine group. These compounds exhibited reasonably good thrombin inhibition activities, and they did not inhibit trypsin at concentrations below 100 μg/mL. Compound **35** showed about 1000-fold selectivity for thrombin over trypsin. Several others exhibited even better selectivity toward thrombin. Compound **38** has a neutral 2,5-dichlorobenzyl P_1 group, and it showed good activity against thrombin but is not as selective toward trypsin, with only about 35-fold selectivity. Several of the compounds showed diminished potency to thrombin binding, while compounds **25** and **34**–**38** showed better or comparable potencies to that of aeruginosin 298-A.

To help analyze the active binding sites of thrombin and trypsin, their X-ray crystal structures are shown in Figure 4A–C. The two proteins have very similar binding sites, especially at their S_1 binding site, as shown in the overlaid Figure 4C. Both enzymes use an Asp residue to form hydrogen bonds with the guanidine group of the aeruginosins. At the S_2 binding site, thrombin has the hydrophobic Trp86 and Tyr83 residues defining the S_2 site, which restricts it to small groups, while trypsin has a relatively more open S_2/P_2 binding site. The hydroxyl group from Hpla in **6** forms a hydrogen bond with the Ser146 residue of trypsin, while the phenyl lactic acid from **4** lacks a hydroxyl group, eliminating the possibility for such an interaction.

Table 1
The P_1 structures of compounds synthesized and their in vitro activities toward trypsin and thrombin

Compound number	P_1 group R =	IC_{50} (μg/mL) thrombin	IC_{50} (μg/mL) trypsin
25		0.092	12.5
26		1.5	6.1
27		0.62	103
28		3.0	14
29		0.50	No inhibition
30		0.43	150
31		0.57	No inhibition
32		3.5	55
33		0.65	No inhibition
34		0.084	No inhibition
35		0.20	210
36		0.28	210
37		0.43	140
38		0.41	14.2
1[12a]		0.3	1.0
PPACK		0.003	N/A
Trypsin inhibitor		N/A	1.0

No inhibition: no inhibition activity at the maximum concentration (~250 μg/mL) tested, N/A: not determined.

Because of the differences between the P_2 binding sites of thrombin and trypsin, it should be possible to find groups that are more selective for thrombin by modifying the P_2 position. Compound **11**, with its P_2 variant configuration opposite to that of the natural product, showed no inhibition to trypsin, and moderate inhibition against thrombin.[34] The compound with the same stereochemistry (**12**) as aeruginosin 298A showed stronger inhibition to trypsin instead. Therefore, in addition to modifying the P_1 positions, it may be possible to replace the P_2 position with a D-amino acid and impart greater selectivity to trypsin. The P_3–P_4 residue used here is D-Hpla-D-Leu, as shown in Figure 4B. The Hpla is probably binding more favorably to trypsin than thrombin. In using this

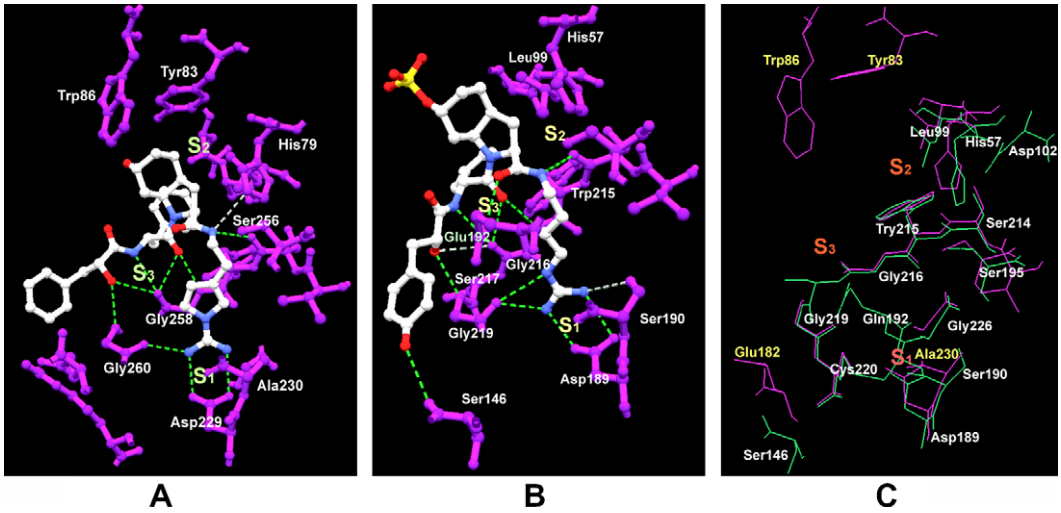


Figure 4. The X-ray crystal structures of thrombin with **4** (A, PDB code 1RIW)[19] and trypsin with **6** (B, PDB code 1AQ7).[18b] The inhibitors are shown in CPK color and protein shown in purple color. C is the overlay of the active sites of thrombin (purple) and trypsin (green). Residues from trypsin are labeled in white. Residues unique to thrombin are in yellow. The overlapping residues of thrombin are not labeled.

un-optimized P_3–P_4 residue, the selectivity obtained at the P_1 position is quite important and should allow for the design of selective and potent thrombin inhibitors with optimized P_3 and P_4 units. A simplified residue can be used to replace D-Hpla-D-Leu, such as an aryl sulfonyl group.

In conclusion, we have synthesized a series of aeruginosin 298-A analogs in which the Choi is replaced with L-proline and the P_1 Argol is replaced with various functionalities. Among these tetrapeptides, several of them exhibit excellent selectivity for thrombin inhibition over trypsin inhibition. These include cyanobenzyl, nitrobenzyl, propyl imidazole, cyanobenzyl, C-4-piperidinyl-methyl, and C-4-pyridinyl-methyl groups at the P_1 positions. These P_1 replacements are less basic or neutral, and generally have simpler or smaller groups than Argol or other guanidine containing units. The SAR data shown here is useful in finding thrombin inhibitors that are easy to synthesize and have superior selectivity toward thrombin. Further structure modifications can potentially lead to the discovery of potent and selective thrombin inhibitors that are also orally available.

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Supplementary data

Supplementary data (the synthesis and characterization of compounds **14**–**19**, **25**–**39**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.056.

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