Tetrahedron 64 (2008) 5784–5793

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Total synthesis of aeruginosin 298-A analogs containing ring oxygenated variants of 2-carboxy-6-hydroxyoctahydroindole

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ARTICLE INFO

Article history:
Received 21 November 2007
Received in revised form 11 March 2008
Accepted 31 March 2008
Available online 8 April 2008

ABSTRACT

Aeruginosins are a family of naturally occurring oligopeptides sharing a common perhydroindole-2-carboxylic acid (L-Choi) core structure. Many aeruginosins exhibit inhibitory activity against serine proteases including thrombin. Aeruginosin 298-A is a tetrapeptide containing the L-Choi core structure and three other unusual amino acids or their derivatives; it is a thrombin and trypsin inhibitor. As part of our effort in finding effective thrombin inhibitors from natural product analogs and to understand the influence of the rigid bicyclic amino acid to the thrombin inhibition activities, we synthesized two aeruginosin 298-A analogs in which the L-Choi is replaced with ring oxygenated perhydroindole-2-carboxylic acids. The Choi variants are enantiomers synthesized from D- and L-glucose, respectively. The preparation of the aeruginosin 298-A analogs containing the ring oxygenated Choi variants and their inhibition activities toward thrombin and trypsin are reported.

1. Introduction

Aeruginosins are a class of naturally occurring oligopeptides containing a common bicyclic core structure, 2-carboxy-6-hydroxyoctahydroindole (Choi), and other unusual amino acids or their derivatives.[1–10] More than 20 compounds in this family have been isolated and identified so far.[10] Typically, these compounds are tetrapeptides containing the 2-carboxy-6-hydroxyoctahydroindole core structure (L-Choi **1**, Fig. 1), which has the configuration of 2*S*,3a*S*,6*R*,7a*S*.[11–13] While a majority of aeruginosins share

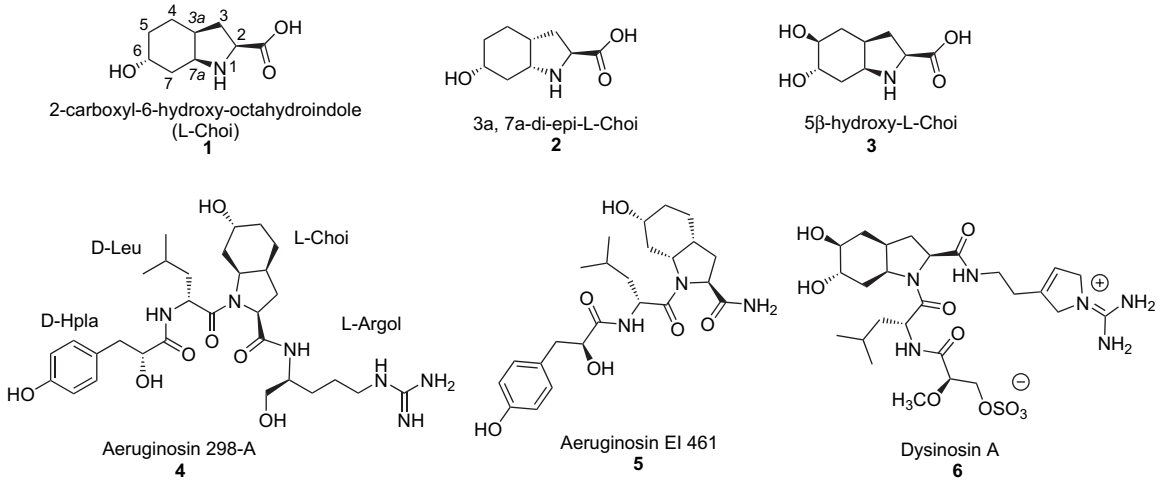

Figure 1. Structures of several octahydroindole-2-carboxylic acids and aeruginosins.

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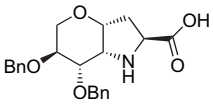
doi:10.1016/j.tet.2008.03.107

the same Choi structure as in aeruginosin 298-A (**4**), several other Choi structures with different stereochemistry or substituents have also been reported. These include the 3a,7a-*diepi*-L-Choi **2** in aeruginosin EI 461 (**5**)[15] and the 5,6-dihydroxyl octahydroindole-2-carboxylic acid **3** in dysinosins.[9,10]

Many aeruginosins have shown activities against serine proteases such as trypsin, thrombin, and factor VIIa. Thrombin (factor IIa) is the key enzyme in blood coagulation cascade; direct inhibition of thrombin has been shown to be an important method to treat thrombosis and other blood clotting disorders.[16–21] Aeruginosin 298-A (**4**) was the first compound in the family exhibiting serine protease inhibitory activities. It contains the sequence of D-*p*-hydroxylphenyllactic acid (D-Hpla), D-leucine, L-2-carboxy-6-hydroxyotahydroinhole (L-Choi), and L-argininol (L-Argol) through peptidic linkages. Aeruginosin 298-A inhibits thrombin at IC_{50}=0.5 μM and trypsin at IC_{50}=1.7 μM. The X-ray crystal structure[11] of the ternary complex of 298-A bound to thrombin–hirugen indicated that its binding mode at the active site is similar to that of D-Phe-Pro-Arg chloromethyl ketone and other serine protease inhibitors.[21] The P_1 residue Argol occupies the S_1 binding site of thrombin, and the D-Leu and D-Hpla bind to the S_3 subsite.[11] The five-membered ring of the Choi residue occupies the hydrophobic S_2 binding site, while its six-membered ring projects out and loosely interacts with Tyr60A and Trp60D from thrombin. Dysinosin A (**6**) is a factor VIIa inhibitor (K_i=0.11 μM) and thrombin inhibitor (K_i=0.45 μM).[9] The X-ray crystal structure of **6** with thrombin reveals that it occupies the S_1–S_2–S_3 binding sites. The Choi is underneath tryptophan 86 and it appears not to be hydrogen bonded with the S_2 binding site.[9]

The unusual structure and interesting biological activities of this class of molecules have attracted several research groups to carry out their total syntheses.[10,12,13,22–24] Several methods are also available for synthesizing the Choi core structure and its analogs.[25–29] Moreover, there also have been some confusions about the structure assignment for aeruginosins, several proposed structures of aeruginosins including aeruginosin 298-A, EI 461 have been revised or reassigned,[12–14,15,24] there is also a proposed structure revision for aeruginosin 205 based on synthesis.[14] Besides the synthesis of the aeruginosins, there is also a great interest in preparing analogs of the aeruginosin natural products and understanding the structure–activity relationship (SAR) pattern for these serine protease inhibitors. While several aeruginosin 298-A analogs have been synthesized, most of the modifications or variations of structures are on the units other than the Choi.[25,26,30,31] These include the preparation of a combinatorial library and the study of stereochemical influences of the amino acid residues at P_3, P_4 or P_1 to the trypsin inhibition activity[30] and several other analogs of aeruginosins with different amino acid residues.[31] Many aeruginosin analogs have exhibited trypsin inhibition potency and are not very selective toward thrombin. Gaining selectivity among serine proteases is an important issue in developing effective thrombin inhibitor based anticoagulants.

In order to understand the function of the Choi core structures and stereochemical effect of the P_2 unit toward thrombin inhibition, we synthesized novel aeruginosin 298-A analogs (**I** and **II**) in which the P_2 Choi structure is replaced with a ring oxygenated variant. Previously we had reported a general method for converting D-glucose to the ring oxygenated variants (**7**, **8**) of the Choi core structures in which the methylene at C-4 position is replaced with an oxygen atom and with an additional hydroxyl group at the C-7 position.[27]



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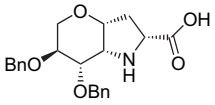

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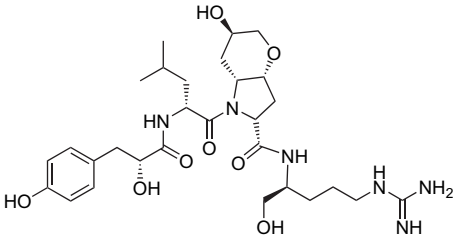
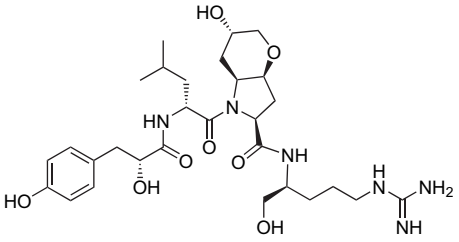
Here we report the synthesis of the ring oxygenated variants of Choi without the additional hydroxyl group, and the aeruginosin 298-A analogs **I** and **II** containing the corresponding *O*-Choi variants and their activities toward thrombin and trypsin. These tetrapeptides (**I**, **II**) have the same amino acid sequence as in aeruginosin 298-A except that the Choi is replaced by a pair of enantiomeric oxygenated Choi variants.



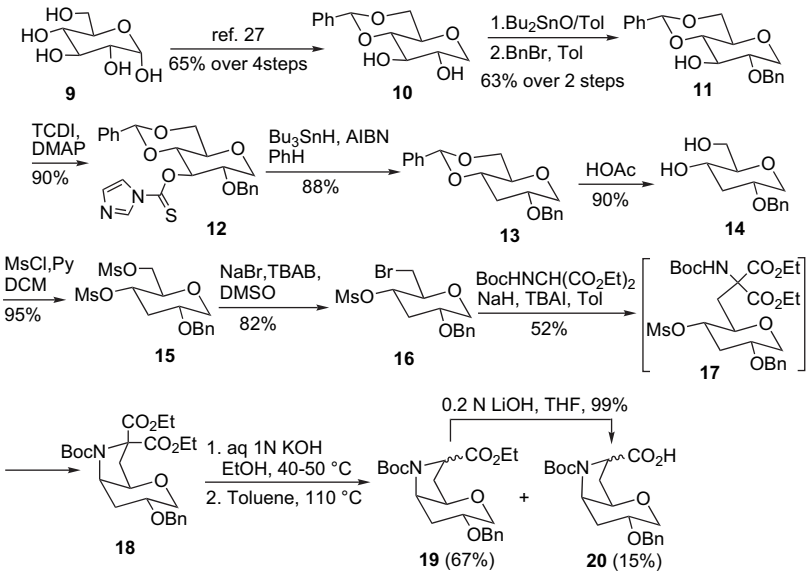
I



II

2. Results and discussion

The Choi analogs in **I** and **II** can be prepared by a modified method for the synthesis of **7** and **8**. The preparation of the protected Choi analog in compound **I** is shown in Scheme 1. D-Glucose was converted to the diol **10** in 65% yield over four steps.[27] Subsequent mono-benzylation of the 2-hydroxyl group of **10** using dibutyltin oxide and benzyl bromide gave **11** in 63% yield along with 13% of 3-hydroxyl benzylated byproduct. The 3-benzyloxy byproduct can be converted back to **10** by catalytic hydrogenolysis and dimethoxyl benzylidene acetal protection and reused again. Compound **11** was treated with TCDI to give the thiocarbamate **12**, which was then subjected to radical reduction using tributyltin hydride, producing 3-deoxy acetal **13** in high yield. Hydrolysis of acetal **13** with 80% acetic acid followed by dimesylation and selective bromo displacement of the primary mesylate afforded bromide **16** in good overall yield. The alkylation reaction of *N*-Boc diethyl amino malonate with bromide **16** led to the cyclized product **18** directly presumably via intermediate **17** by displacement of mesylate. In an analog with an additional 3-hydroxy substituent, the open-chained alkylation product was obtained instead and subsequent cyclization was accomplished by deprotecting the Boc group to the free amine.[27] The mono hydrolysis of **18**, however, proved not so straightforward. The exact condition applied successfully to a similar but uncyclized acyclic amino acid diester (1 N aq NaOH, EtOH, room temperature, 5 h)[27] was not working in



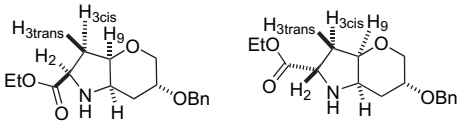
Scheme 1. Synthesis of protected Choi analog **20** from D-glucose.

this case. Unchanged starting material was found even after stirring at room temperature for almost two days. The difference in hydrolysis was understandable since the cis-fused ring system would be more hindered for saponification than the acyclic chain system. After several trials, we found that under conditions of stronger base (1 N KOH) and elevated temperature (40–50 °C), compound **18** was hydrolyzed completely in less than 12 h. Subsequent decarboxylation in refluxing toluene afforded both ester **19** and acid **20** in 67 and 15% yield, respectively, and the presence of acid **20** is due to over hydrolysis of **18** to a diacid under those fairly harsh conditions. Since compound **19** can be hydrolyzed to **20** almost quantitatively, the separation of the acid with ester is unnecessary; the crude mixture of **19** and **20** obtained in the decarboxylation reaction was subjected to hydrolysis directly without separation, producing the acid **20** with 7:1 diastereomeric selectivity in 85% yield from **18** over 3 steps.

The decarboxylation products **19** and **20** were obtained as mixtures of diastereomers. The major isomers **19a** and **20a** are assigned as *R*-amino acids based on the decarboxylation–tautomerization mechanism shown in Figure 2. The protonation of the intermediate enediol would be on the more accessible convex face (*Re* face) of the cis-fused ring system giving the *R*-isomer as the major product. This assignment is confirmed by 2D NOESY NMR study as well.

The diastereomeric mixture, either **19a** and **19b**, or **20a** and **20b**, could not be separated by chromatography efficiently, however, the derivatives **21a** and **21b** from the mixture of **19a** and **19b** can be separated from each other cleanly via flash chromatography. The NMR studies of amines **21a** and **21b** further confirmed the above structural assignment (Fig. 3).

For compound **21a**, the signals corresponding to protons H-2 (3.74 ppm), H-9 (3.78 ppm), H-3cis (2.27 ppm), and H-3trans (2.13 ppm), can be assigned unambiguously based on coupling



21a: δ (ppm): H_2 3.74, H_{3trans} 2.13, H_{3cis} 2.27, H_9 3.78.
J (Hz): $J_{2\text{-}3cis}$= 10.2, $J_{2\text{-}3\text{-}trans}$= 3.3, $J_{9\text{-}3cis}$= 4.1, $J_{9\text{-}3trans}$= 0

21b: δ (ppm): H_2 4.00, H_{3trans} 2.26, H_{3cis} 2.04, H_9 3.93.
J (Hz): $J_{2\text{-}3cis}$= 7.7, $J_{2\text{-}3trans}$= 8.5, $J_{9\text{-}3cis}$= 4.7, $J_{9\text{-}3trans}$= 0

Figure 3. Configurations and ^{1}H NMR chemical shifts of the diastereomers **21a** and **21b**.

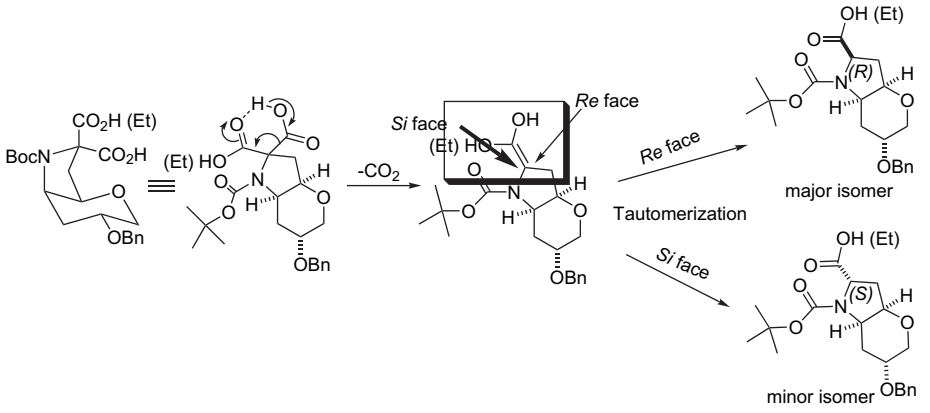
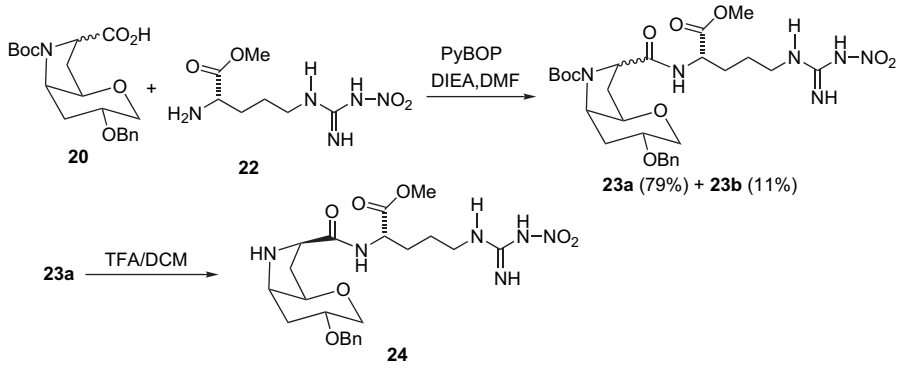


Figure 2. Stereochemistry outcome of the decarboxylation step.



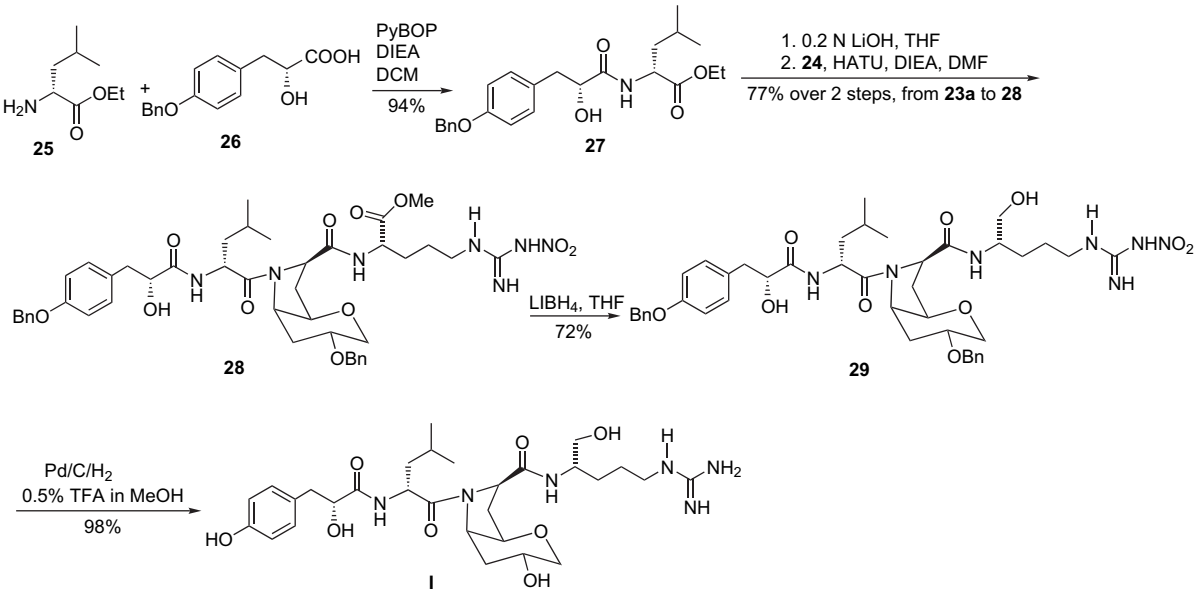
Scheme 2. Synthesis of protected *O*-Choi-Arg dipeptide **24**.

constants and COSY. The coupling constants $J_{9\text{-}3cis}$=4.1 Hz, $J_{9\text{-}3trans}$=0 Hz, suggested that the dihedral angle between H-9 and H-3trans is almost 90°, and the dihedral angle between H-9 and H-3cis is either around 44° or 133° based on Karplus equations.[32] Furthermore, strong NOE signal between H-9 and H-3cis and no NOE signal between H-9 and H-3trans clearly demonstrate that the dihedral angle between H-9 and H-3cis should be around 44° rather than 133°. Therefore, H-3cis is cis to H-9, and H-3trans is trans to H-9. For the same reason, H-2 is cis to H-3cis, and trans to H-3trans because of much larger coupling constant and stronger NOE signal between H-2 and H-3cis. Similarly, the stereochemistry of **21b** can also be elucidated based on coupling constants and NOE signal strength. Therefore, the NMR studies proved that major isomer **21a** is *R*-amino acid and minor isomer **21b** is *S*-amino acid.

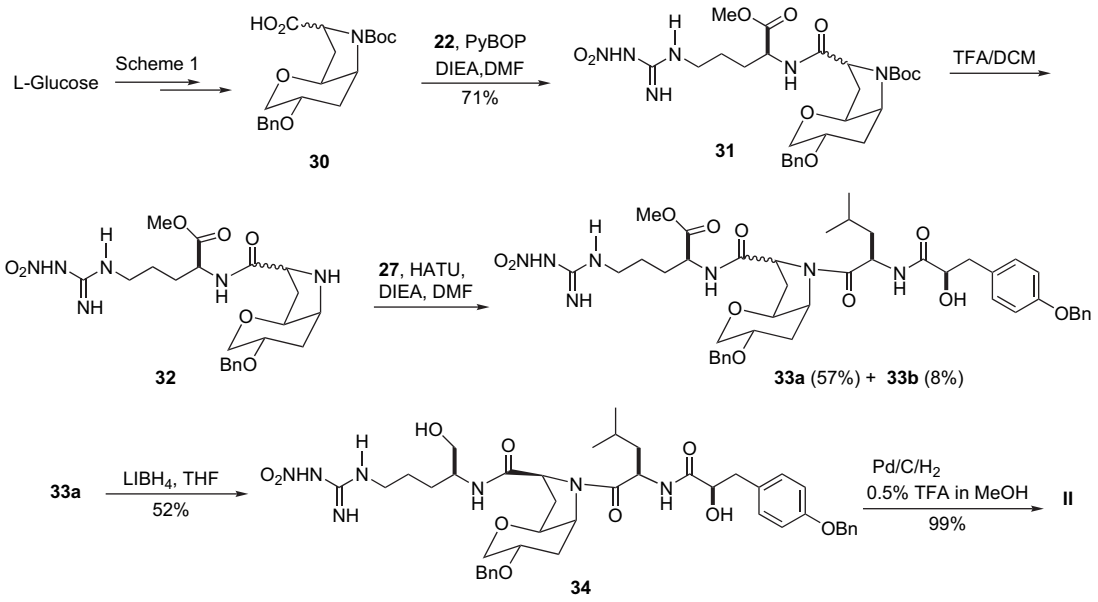
With the stereochemistry of both major and minor isomer of Choi analogs **21a** and **21b** confirmed by the NMR studies, we continued the synthesis with the peptide coupling reactions using the inseparable mixture of **20a** and **20b** with commercially available methyl ester of N^{ω}-nitro-arginine hydrochloride salt **22** (Scheme 2). The dipeptides **23a** and **23b** were separable by chromatography and were obtained in 79 and 11% yield, respectively. The major isomer compound **23a** was then deprotected to give the TFA salt **24** and is used for the coupling reaction later on.

The dipeptide Leu-HPLA **27** was prepared by coupling reaction of the commercially available D-leucine ethyl ester **25** with *O*-benzyl D-HPLA **26**, which was prepared from *O*-benzyl D-tyrosine by literature procedure.[23] It is noteworthy that the α-hydroxyl group of HPLA **26** was not protected here and this has no influence on the coupling reaction when PyBOP was used as the coupling agent. Furthermore, the racemic *O*-benzyl D,L-HPLA could also be used since the free hydroxyl group in **27** allowed easy separation of the two diastereomers by chromatography. The dipeptide **27** was hydrolyzed to the free acid and then coupled with the TFA salt **24**, affording tetrapeptide **28** in good yield. The presence of free hydroxyl group in **27** did not affect the peptide coupling reaction either. The reduction of the methyl ester group in **28** with $LiBH_4$ gave compound **29** in fairly good yield providing that the reaction was quenched immediately when only a small amount of starting material was left. Finally Pd/C catalyzed hydrogenolysis of **29** in methanol with the addition of 0.5% TFA yielded compound **I**, the analog of aeruginosin 298-A, almost quantitatively (Scheme 3).

For the preparation of analog **II**, L-glucose was used as starting material to prepare the *O*-Choi variants. As shown in Scheme 4, using exactly the same methods described in Scheme 1, the *O*-Choi variant **30** was synthesized as an inseparable mixture of diastereomers in 7:1 ratio. The diastereomers **30** can be separated by flash chromatography after removing the Boc group, similarly to their enantiomers **21a** and **21b**. The major isomer has the same stereochemistry as L-Choi in aeruginosin 298-A. To synthesize the tetrapeptide, the diastereomeric mixture **30** was used directly without separation in the coupling reaction with compound **22** to produce the dipeptide **31**. However, unlike the dipeptide **23**, the



Scheme 3. Synthesis of aeruginosin 298-A analog I.



Scheme 4. Preparation of aeruginosin analog **II**.

dipeptide **31** was an inseparable diastereomeric mixture, so the mixture was carried forward in the synthesis. The Boc group was removed to give compound **32**, which was then coupled with the dipeptide **27** in standard conditions to afford the tetrapeptide **33** as a mixture of diastereomers. The diastereomeric tetrapeptides were separated by flash chromatography to give the pure isomers **33a** (major) and **33b**. After the reduction of the methyl ester with $LiBH_4$ and global deprotection, we successfully synthesized compound **II**, the exact analog of aeruginosin 298-A with one methylene group replaced by an oxygen atom.

After completing the synthesis of the teterapeptide analogs, we carried out enzyme assays for thrombin and trypsin by standard enzymes assay conditions in the literature.[30,33,34] Commercially available thrombin inhibitor PPACK and trypsin inhibitor were used to calibrate the enzyme concentrations and as standards. The IC_{50} value for PPACK was determined as 0.018 μg/mL in this study and the standard trypsin inhibitor had IC_{50} value of 1.3 μg/mL. Compound **I** inhibited thrombin at <24 μg/mL, it showed no inhibition toward trypsin; analog **II** inhibited trypsin with IC_{50} at 2.0 μg/mL and thrombin at <22 μg/mL. The P_2 units in compounds **I** and **II** are mirror images to each other, this is the only difference of the two analogs. While compound **II** prefers trypsin over thrombin, it inhibited trypsin with IC_{50} value slightly lower than that of aeruginosin 298-A. Compound **I** has opposite configuration as aeruginosin 298-A, it inhibited thrombin similarly to compound **II**, but showed no inhibition toward trypsin. Gaining selectivity among serine proteases is an important issue in developing effective oral anticoagulants. This result indicated that compound **I** can be used as a starting point to optimize the thrombin inhibition activity. Further study modifying the P_1 and P_3 units will help us to obtain thrombin inhibitors with improved potency and selectivity.

3. Conclusions

We have developed an efficient synthesis for aeruginosin 298-A analogs in which the P_2 Choi unit is modified by replacing a methylene group in the six-membered ring with an oxygen and different configurations. The ring oxygenated Choi variants were synthesized from D-glucose or L-glucose; the formation of the bicyclic Choi variants features alkylation and cyclization steps in a one-pot fashion and good diastereoselectivity in the decarboxylation step. Segment coupling of the dipeptide formed from Choi variants and arginine derivative with the dipeptide formed from the HPLA and D-leucine resulted in the tetrapeptides in good yields, which were then converted to the target compounds **I** and **II** efficiently. We found that compound **I** showed good selectivity to thrombin over trypsin. But compound **II**, which contains the same configuration as in the natural product showed preference for trypsin instead. Although these compounds showed some loss of activities, the SAR pattern obtained here can help us to design more selective thrombin inhibitor. Further structure optimization is necessary to obtain compounds with high potency and good selectivity among serine proteases.

4. Experimental section

4.1. General methods

Reagents, solvents, and starting materials were purchased from Aldrich, VWR, or Lancaster unless otherwise specified. Anhydrous solvents were purchased from Aldrich in sure-seal bottles and used directly without further treatment. ^1H and ^{13}C NMR spectra were acquired on a Varian 400 MHz or 300 MHz NMR machine. Melting point was measured using a Fisher-Johns melting point apparatus. Typically thin layer chromatography (60 Å pore, UV254, phosphomolybdic acid as the staining agent) was used to monitor reactions. Silica gel (230–400 meshes) was used for flash chromatography. Solvents were generally removed under reduced pressure using Buchi rotavapor R200.

4.2. Synthesis of aeruginosin analog I

*4.2.1. Preparation of 1,5-anhydro-2-O-benzyl-4,6-O-benzylidene-D-glucitol (**11**)*

A solution of 1,5-anhydro-4,6-*O*-benzylidene-D-glucitol **10**[27] (7.32 g, 29.0 mmol) and Bu_2SnO (7.95 g, 31.9 mmol) in toluene (100 mL) was heated to reflux for 3 h. Toluene was evaporated under reduced pressure. Benzyl bromide (7.6 mL, 63.8 mmol) and toluene (100 mL) were then added to the residue. The resulting solution was heated to reflux overnight and then cooled to room temperature, treated with mixture of saturated $NaHCO_3$ (100 mL) and EtOAc (100 mL), and filtered through a pad of Celite®. The organic layer was separated and the aqueous layer was extracted with

EtOAc (200 mL×3). The combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. Recrystallization of the crude with EtOAc/hexane gave 5.37 g compound **11** as pure colorless crystals. Another 0.91 g of product was obtained with column chromatography purification of the mother liquid (DCM/EtOAc/hexane, 10:9:1). Totally 6.28 g, yield 63%, mp 161–162 °C, $[\alpha]_D$ −31.9 (*c* 1.7, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$) δ 7.54–7.51 (m, 2H), 7.41–7.31 (m, 8H), 5.53 (s, 1H), 4.79 (d, 1H, *J*=11.5 Hz), 4.70 (d, 1H, *J*=11.5 Hz), 4.32 (dd, 1H, *J*=10.3, 4.8 Hz), 4.03 (dd, 1H, *J*=11.5, 5.5 Hz), 3.86 (t, 1H, *J*=9.1 Hz), 3.67 (t, 1H, *J*=10.3 Hz), 3.59 (ddd, 1H, *J*=10.3, 9.1, 5.5 Hz), 3.48 (dd, 1H, *J*=9.7, 9.1 Hz), 3.37 (dt, 1H, *J*=9.7, 4.8 Hz), 3.32 (t, 1H, *J*=10.9 Hz), 2.86 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.0, 137.0, 129.1, 128.5, 128.2, 127.9, 127.8, 126.2, 101.8, 81.0, 77.7, 74.7, 73.4, 70.9, 68.7, 68.4; HRMS calcd for $C_{20}H_{22}O_5$+H, 343.1545; found, 343.1538.

4.2.2. Preparation of 1,5-anhydro-2-O-benzyl-4,6-O-benzylidene-3-O-imidazolythiocarbonyl-D-glucitol (12)

A solution of monoalcohol **11** (5.30 g, 15.50 mmol), TCDI (thiocarbonyl diimidazole, 3.31 g, 18.60 mmol), and DMAP (2.27 g, 18.60 mmol) in chloroform (40 mL) was heated to refluxed overnight. The reaction mixture was cooled to room temperature, diluted with EtOAc (250 mL), washed with saturated aq $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated under reduced pressure. The crude material was purified by column chromatography (hexane/EtOAc, 4:1) to produce compound **12** as a light yellow solid (6.30 g, 90%), mp 96–97 °C, $[\alpha]_D$ −3.5 (*c* 0.8, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 8.21 (s, 1H), 7.58 (s, 1H), 7.40–7.38 (m, 2H), 7.33–7.32 (m, 3H), 7.26–7.19 (m, 5H), 7.04 (s, 1H), 6.18 (dd, 1H, *J*=10.0, 8.8 Hz), 5.47 (s, 1H), 4.65 (d, 1H, *J*=12.5 Hz), 4.48 (d, 1H, *J*=12.5 Hz), 4.37 (dd, 1H, *J*=10.0, 5.0 Hz), 4.20 (dd, 1H, *J*=11.3, 6.3 Hz), 3.88–3.82 (m, 1H), 3.75–3.69 (m, 2H), 3.57–3.49 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.7, 136.9, 135.3, 130.6, 129.0, 128.4, 128.3, 128.2, 127.8, 126.0, 118.2, 101.3, 82.6, 79.0, 75.0, 72.9, 71.2, 68.6, 68.5; HRMS calcd for $C_{24}H_{24}N_2O_5S$+H, 453.1484; found, 453.1476.

4.2.3. Preparation of 1,5-anhydro-4,6-O-benzylidene-3-deoxy-2-O-benzyl-D-ribo-hexitol (13)

To a magnetically stirred solution of **12** (5.76 g, 12.7 mmol) and AIBN (a few crystals) in toluene (30 mL) was added solution of Bu_3SnH (6.9 mL, 12.7 mmol) in toluene (30 mL) dropwise under N_2. The reaction mixture was immersed immediately in a pre-heated oil-bath (120 °C) once the adding of Bu_3SnH solution began. After stirring at 120 °C for 2 h, the reaction was treated dropwise with another portion of Bu_3SnH (6.9 mL, 12.7 mmol) in toluene (30 mL) and refluxed overnight. The mixture is then cooled down to room temperature, quenched with saturated $NaHCO_3$ (50 mL) and EtOAc (50 mL), filtered through a pad of Celite®, and extracted with EtOAc (100 mL×3). The combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Recrystallization of the residue with EtOAc/hexane gave 2.54 g compound **13** as pure colorless crystals. Another 1.10 g of product was obtained with column chromatography purification of the mother liquid (EtOAc/hexane, 1:20). Totally 3.64 g, yield 88%, mp 105–107 °C, $[\alpha]_D$ −40.5 (*c* 0.6, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 7.54–7.52 (m, 2H), 7.42–7.31 (m, 8H), 5.54 (s, 1H), 4.79 (d, 1H, *J*=12.5 Hz), 4.70 (d, 1H, *J*=12.5 Hz), 4.34 (dd, 1H, *J*=10.0, 5.0 Hz), 4.12 (dd, 1H, *J*=10.0, 5.0 Hz), 3.74–3.65 (m, 2H), 3.55–3.48 (m, 1H), 3.36–3.30 (m, 2H), 2.65–2.60 (m, 1H), 1.72 (q, 1H, *J*=11.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.9, 137.3, 129.0, 128.4, 128.2, 127.8, 127.6, 126.1, 101.6, 76.5, 73.3, 72.0, 71.0, 70.5, 69.2, 35.6; HRMS calcd for $C_{20}H_{22}O_4$+H, 327.1596; found, 327.1591.

4.2.4. Preparation of 1,5-anhydro-2,-O-benzyl-3-deoxy-D-ribo-hexitol (14)

A suspension of **13** (3.61 g, 11.1 mmol) in 80% HOAc (75 mL) was heated to 55 °C with stirring till all starting material was consumed. After evaporation under reduced pressure to remove most solvent, the crude was purified by recrystallization (EtOAc/hexane) to give 2.38 g of **14** as colorless crystals, 90% yield, mp 107–108 °C, $[\alpha]_D$ +7.7 (*c* 0.5, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.28 (m, 5H), 4.61 (d, 1H, *J*=12.5 Hz), 4.55 (d, 1H, *J*=12.5 Hz), 4.05 (dd, 1H, *J*=12.5, 5.0 Hz), 3.84 (dd, 1H, *J*=11.3, 5.0 Hz), 3.77 (dd, 1H, *J*=11.3, 5.0 Hz), 3.64–3.59 (m, 1H), 3.55 (dt, 1H, *J*=10.0, 5.0 Hz), 3.21 (dd, 1H, *J*=11.3, 10.0 Hz), 3.16 (dd, 1H, *J*=8.8, 5.0 Hz), 2.54–2.49 (m, 1H), 1.51 (q, 1H, *J*=11.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.9, 128.4, 127.8, 127.6, 81.2, 71.9, 71.0, 69.7, 65.7, 62.4, 38.8; HRMS calcd for $C_{13}H_{18}O_4$+H, 239.1283; found, 239.1283.

4.2.5. Preparation of 1,5-anhydro-2-O-benzyl-3-deoxy-4,6-di-O-methylsulfonyl-D-ribo-hexitol (15)

A solution of diol **14** (1.93 g, 8.1 mmol) in DCM (10 mL) and pyridine (7.5 mL) was treated with methanesulfonyl chloride (2.5 mL, 32.4 mmol). The mixture was left stirring under anhydrous condition till completion of the reaction (usually 12 h). The reaction mixture was then poured into a beaker containing ice and saturated aq $NaHCO_3$ (20 mL). After stirring in ice bath for 1 h, the reaction mixture was extracted with DCM (50 mL×3), and washed with 1 N HCl (30 mL×3) and brine. The organic layer was dried and concentrated. Column chromatographic (EtOAc/hexane, 30:70) purification of the residue gave **15** (3.46 g, 95%) as colorless thick oil, $[\alpha]_D$ +20.4 (*c* 3.6, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 7.37–7.28 (m, 5H), 4.60 (d, 1H, *J*=11.3 Hz), 4.57 (dt, 1H, *J*=11.3, 5.0 Hz), 4.51 (d, 1H, *J*=11.3 Hz), 4.42 (d, 1H, *J*=11.3 Hz), 4.35 (dd, 1H, *J*=11.3, 5.0 Hz), 4.10 (ddd, 1H, *J*=11.3, 5.0, 2.5 Hz), 3.63 (ddd, 1H, *J*=16.3, 10.0, 5.0 Hz), 3.52–3.48 (m, 1H), 3.22 (dd, 1H, *J*=11.3, 10.0 Hz), 3.07 (s, 3H), 3.04 (s, 3H), 2.90–2.82 (m, 1H), 1.76 (q, 1H, *J*=11.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.5, 128.4, 127.9, 127.5, 76.2, 71.9, 71.2, 71.0, 70.1, 67.4, 38.6, 37.4, 37.0; HRMS calcd for $C_{15}H_{22}O_8S_2$+H, 395.0834; found, 395.0835.

4.2.6. Preparation of 1,5-anhydro-2,3-di-O-benzyl-6-bromo-6-deoxy-4-O-methylsulfonyl-D-glucitol (16)

A mixture of dimesylate **15** (3.46 g, 8.7 mmol), NaBr (5.41 g, 52.6 mmol), and TBAB (846 mg, 2.6 mmol) in DMSO (20 mL) was stirred at 60 °C for 12 h. The reaction mixture was cooled to room temperature and then poured into water (200 mL). The water phase was extracted with ethyl acetate five times, the combined organic phase was washed with water, dried (sodium sulfate), and concentrated. Flash chromatography gave 2.72 g (82% yield) colorless crystals of **16** (along with 0.29 mg of recovered starting material **15**, 8.3% recovered), mp 63–65 °C, $[\alpha]_D$ +34.0 (*c* 1.9, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.31 (m, 5H), 4.61 (d, 1H, *J*=11.3 Hz), 4.58–4.53 (m, 1H), 4.48 (d, 1H, *J*=11.3 Hz), 4.14 (dd, 1H, *J*=10.0, 3.8 Hz), 3.69 (dd, 1H, *J*=11.3, 2.5 Hz), 3.69–3.61 (m, 1H), 3.50 (dd, 1H, *J*=11.3, 5.0 Hz), 3.46–3.42 (m, 1H), 3.26 (t, 1H, *J*=11.3 Hz), 3.10 (s, 3H), 2.88–2.83 (m, 1H), 1.78 (q, 1H, *J*=11.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.4, 128.3, 127.8, 127.4, 76.5, 74.6, 71.2, 71.1, 70.1, 38.8, 36.9, 31.6; HRMS calcd for $C_{14}H_{19}BrO_5S$+H, 379.0215; found, 379.0212.

4.2.7. Preparation of the bicyclic compound 18

A stirred solution of diethyl 2-[(*tert*-butyloxycarbonyl)-amino]malonate (4.00 g, 14.5 mmol) in dry toluene (25 mL) under N_2 was treated with NaH (60% in mineral oil, 680 mg, 17.0 mmol). The reaction mixture was stirred at room temperature for 0.5 h before compound **16** (1.59 g, 4.2 mmol) in 25 mL toluene was added dropwise. The reaction mixture was stirred at room temperature for 1 h before TBAI (465 mg, 1.26 mmol) was added. The resultant reaction mixture was then heated to reflux for 17 h. The reaction mixture was cooled to 0 °C, quenched by 0.5 N HCl (25 mL), extracted with EtOAc (100 mL×3), washed with brine, dried over Na_2SO_4, filtered, and concentrated, the residue was purified by column chromatography (hexane/EtOAc, 9:1 to 7:1) to produce compound **18** as thick oil (1.03 g, 52%), $[\alpha]_D$ +30.4 (*c* 3.2, $CHCl_3$). ^1H

NMR (300 MHz, $CDCl_3$) mixture of two conformers δ 7.34–7.24 (m, 5H), 4.64–4.48 (m, 2H), 4.25–4.10 (m, 4.6H), 4.10–4.01 (m, 1.4H), 3.94–3.81 (m, 1H), 3.78–3.59 (m, 1H), 3.25–3.19 (m, 1.6H), 2.77 (d, 1.4H, J=12.9 Hz), 2.38 (d, 1H, J=12.9 Hz), 1.93–1.72 (m, 1H), 1.50 (s, 3.5H), 1.43 (s, 5.5H), 1.30–1.26 (m, 3.5H), 1.22–1.17 (m, 2.5H); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 169.3, 168.7, 168.4, 153.7, 153.2, 138.2, 128.3, 127.5, 127.4, 80.7, 80.6, 77.2, 75.6, 74.8, 72.7, 72.5, 70.6, 70.2, 69.1, 69.0, 68.3, 61.9, 61.3, 59.4, 58.6, 42.3, 41.1, 30.5, 29.3, 28.2, 27.9, 13.9, 13.8; HRMS calcd for $C_{25}H_{35}NO_8$+H, 478.2441; found, 478.2441.

*4.2.8. Synthesis of the protected O-Choi variants, ester **19** and acid **20***

A solution of **18** (400 mg, 0.84 mmol) in ethanol (10 mL) was treated with 1 N KOH (10 mL) and the reaction mixture was stirred at 40–50 °C till all starting material was consumed (about 12 h). The reaction mixture was washed with ether (10 mL×3). The combined ether solution then was washed with water (10 mL×2). The aqueous solution was combined, acidified with 1 N HCl to pH 2–3, extracted with chloroform (50 mL×5), dried over Na_2SO_4, and concentrated. The residue was subjected to decarboxylation in refluxing toluene (25 mL, about 14 h). Evaporation of the toluene under reduced pressure and chromatographic purification gave three potions, an overlapping mixture of esters **19b** and **19a** (100 mg, 29%), pure **19a** (130 mg, 38%), and mixture of acid **20a** and **20b** (50 mg, 15%), totally 82% conversion. Major isomer ester **19a** (*R*-configuration), colorless oil gradually solidified: ^{1}H NMR (300 MHz, $CDCl_3$, mixture of two conformers ratio 6:4) δ 7.37–7.24 (m, 5H), 4.68–4.43 (m, 2.4H), 4.35 (d, 0.6H, J=8.8 Hz), 4.19–4.07 (m, 2H), 4.05–3.98 (m, 1H), 3.97–3.70 (m, 3H), 3.22 (dd, 1H, J=9.3, 9.0 Hz), 3.00 (d, 0.6H, J=12.6 Hz), 2.66 (d, 0.4H, J=12.6 Hz), 2.30 (m, 0.4H), 2.25 (m, 0.6H), 2.15 (dd, 0.6H, J=9.3, 3.8 Hz), 2.10 (dd, 0.4H, J=9.3, 4.1 Hz), 1.76–1.93 (m, 1H), 1.49 (s, 3.6H), 1.44 (s, 5.4H), 1.17 (t, 3H, J=5.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 171.8, 154.7, 154.2, 138.3, 128.2, 128.1, 127.5, 127.4, 80.1, 79.8, 76.0, 75.3, 70.6, 70.2, 69.5, 69.4, 68.7, 68.6, 60.6, 59.0, 57.5, 57.2, 35.2, 34.8, 30.5, 29.4, 28.2, 13.9; HRMS calcd for $C_{22}H_{31}NO_6$+H, 406.2230; found, 406.2223.

*4.2.9. Alternative method for synthesizing amino acid **20***

A solution of **18** (1.03 g, 2.16 mmol) in ethanol (20 mL) was treated with 1 N KOH (20 mL) and the reaction mixture was stirred at 40–50 °C till all starting material was consumed (about 12 h). The reaction mixture was washed with ether (10 mL×3). The combined ether solution then was washed with water (10 mL×2). The aqueous solution was combined, acidified with 1 N HCl to pH 2–3, extracted with chloroform (50 mL×5), dried over Na_2SO_4, and concentrated. The residue was subjected to decarboxylation in refluxing toluene (25 mL, about 14 h). Evaporation of the toluene under reduced pressure gave a crude mixture of ester **19** and acid **20**, which was dissolved in THF (25 mL), treated with 0.2 N LiOH (25 mL), and stirred at room temperature till all ester was consumed. The reaction mixture was acidified with 1 N HCl (pH=2–3). Extraction with chloroform (30 mL×5) followed by evaporation gave the acid **20** (diastereomers mixture ratio 7:1, 0.69 g, 85% yield for 3 steps), which was used in the peptide coupling reaction without further purification. Major isomer **20a** can be obtained by purification of a small amount of crude through column chromatography. Major isomer **20a** (*R*-configuration): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.28 (m, 5H), 4.60 (d, 1H, J=11.5 Hz), 4.52 (d, 1H, J=11.5 Hz), 4.53–4.47 (m, 1H), 4.12–4.06 (m, 1H), 4.00–3.88 (m, 2H), 3.84–3.75 (m, 1H), 3.38–3.22 (m, 1H), 2.47–2.33 (m, 1H), 2.26–2.14 (m, 1H), 1.93–1.84 (m, 1H), 1.58–1.51 (m, 1H), 1.48 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.4, 155.3, 138.2, 128.3, 127.6, 127.5, 80.7, 75.7, 70.7, 69.3, 68.6, 59.0, 57.6, 34.6, 30.1, 28.3; HRMS calcd for $C_{20}H_{27}NO_6$+H, 378.1917; found, 378.1916.

*4.2.10. Synthesis of amines **21a** and **21b***

The overlapping mixture of ester **19b** and **19a** (100 mg, 0.25 mmol) obtained from the previous step was treated with TFA/DCM (1:4, 10 mL) at 0 °C for 5 h. The solvent was evaporated under reduced pressure and saturated $NaHCO_3$ (15 mL) and EtOAc (25 mL) were added to the residue. The aqueous layer was extracted with EtOAc three times. The combined organic layers were dried over Na_2SO_4. Solvent was removed under reduced pressure. The crude was purified by column chromatography (hexane/EtOAc, 7:3) to give **21b** (12 mg, 16%) and **21a** (56 mg, 75%), totally 91% conversion. Major isomer **21a**, colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43–7.24 (m, 5H), 4.60 (d, 1H, J=11.5 Hz), 4.54 (d, 1H, J=11.5 Hz), 4.20 (q, 2H, J=7.1 Hz), 3.95 (ddd, 1H, J=10.7, 4.1, 2.2 Hz), 3.86–3.77 (m, 2H), 3.74 (dd, 1H, J=10.2, 3.3 Hz), 3.23 (m, 1H), 3.08 (dd, 1H, J=10.4, 10.2 Hz), 2.84 (br, 1H), 2.46 (dq, 1H, J=14.0, 2.2 Hz), 2.27 (ddd, 1H, J=14.3, 10.2, 4.1 Hz), 2.13 (dd, 1H, J=14.3, 3.3 Hz), 1.74 (ddd, 1H, J=14.0, 11.0, 4.9 Hz), 1.26 (t, 3H, J=7.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.5, 138.2, 128.3, 127.6, 127.5, 76.9, 71.1, 69.2, 69.1, 61.1, 59.3, 58.6, 38.0, 31.2, 14.1; HRMS calcd for $C_{17}H_{23}NO_4$+H, 306.1705; found, 306.1700. Minor isomer **21b**, colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39–7.25 (m, 5H), 4.59 (d, 1H, J=11.8 Hz), 4.55 (d, 1H, J=11.8 Hz), 4.18 (q, 2H, J=7.1 Hz), 4.05–3.97 (m, 2H), 3.93 (dd, 1H, J=4.7, 2.7 Hz), 3.82 (ddd, 1H, J=15.1, 10.2, 4.7 Hz), 3.43–3.40 (m, 1H), 3.15 (dd, 1H, J=10.7, 10.2 Hz), 2.36 (dp, 1H, J=13.7, 2.2 Hz), 2.26 (dd, 1H, J=14.0, 8.5 Hz), 2.22 (br, 1H), 2.04 (ddd, 1H, J=14.0, 7.7, 4.7 Hz), 1.68 (ddd, 1H, J=13.7, 10.7, 4.4 Hz), 1.27 (t, 3H, J=7.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.4, 138.3, 128.4, 127.6, 127.5, 77.8, 71.0, 69.3, 69.2, 61.0, 58.0, 57.9, 37.3, 32.2, 14.2; HRMS calcd for $C_{17}H_{23}NO_4$+H, 306.1705; found, 306.1703.

*4.2.11. Preparation of dipeptides **23a** and **23b***

At 0 °C, a solution of acid **20** (mixture of diastereomers, 0.69 g, 1.83 mmol) and methyl ester of N^{ω}-nitro-arginine·HCl **22** (0.49 g, 1.83 mmol) in DMF (5 mL) was treated subsequently with PyBOP (0.95 g, 1.83 mmol) and DIEA (0.64 mL, 3.66 mmol). The reaction was stirred at room temperature for 24 h before it was diluted with EtOAc (200 mL), washed with saturated $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (chloroform/hexane, 40:60 with addition of 3% MeOH) to give pure dipeptides **23a** (0.85 g, 79%) and **23b** (0.12 g, 11%). Major isomer **23a**: $[\alpha]_D$ −13.7 (*c* 2.6, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$), δ 8.82 (br, 1H), 7.80 (br, 2H), 7.35–7.26 (m, 5H), 7.12 (d, 1H, J=8.3 Hz), 4.72–4.66 (m, 1H), 4.66 (d, 1H, J=11.5 Hz), 4.58 (d, 1H, J=11.5 Hz), 4.38 (d, 1H, J=10.2 Hz), 4.10–3.84 (m, 4H), 3.76 (s, 3H), 3.62 (m, 1H), 3.36–3.25 (m, 1H), 3.21–3.13 (m, 1H), 2.85 (m, 1H), 2.39 (d, 1H, J=13.7 Hz), 2.24–2.12 (m, 1H), 2.05–1.92 (m, 1H), 1.89–1.77 (m, 1H), 1.77–1.57 (m, 3H), 1.47 (s, 9H); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 173.5, 172.4, 159.4, 156.3, 138.5, 128.4, 127.7, 127.6, 81.7, 77.2, 71.0, 69.5, 69.2, 62.1, 59.7, 52.6, 50.3, 40.2, 34.6, 31.2, 28.3, 24.3; HRMS calcd for $C_{27}H_{40}N_6O_9$+H, 593.2935; found, 593.2938. Minor isomer **23b**: ^{1}H NMR (300 MHz, $CDCl_3$), δ 8.66 (br, 1H), 7.82 (br, 2H), 7.35–7.27 (m, 5H), 6.72 (d, 1H, J=8.0 Hz), 4.66–4.60 (m, 1H), 4.59 (d, 1H, J=11.5 Hz), 4.52 (d, 1H, J=11.5 Hz), 4.31 (t, 1H, J=7.6 Hz), 4.20–4.14 (m, 1H), 4.10–4.02 (m, 1H), 4.00–3.95 (m, 1H), 3.76 (s, 3H), 3.69–3.46 (m, 2H), 3.38–3.20 (m, 2H), 2.63–2.51 (m, 1H), 2.24–2.14 (m, 1H), 2.07–1.88 (m, 3H), 1.85–1.59 (m, 3H), 1.42 (s, 9H); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 173.3, 172.2, 159.4, 154.8, 138.1, 128.5, 127.8, 127.5, 81.1, 76.2, 70.8, 69.5, 68.4, 60.8, 57.0, 52.8, 51.0, 40.4, 34.1, 30.4, 30.1, 28.4, 23.8; HRMS calcd for $C_{27}H_{40}N_6O_9$+H, 593.2935; found, 593.2930.

*4.2.12. Synthesis of dipeptide **27***

PyBOP (1.56 g, 3.0 mmol) and DIEA (1.05 mL, 6.0 mmol) were added subsequently to a stirred solution of (*R*)-β-[4-(benzyloxy) phenyl]lactic acid **26**[23] (680 mg, 2.5 mmol) and D-leucine ethyl ester hydrochloride **25** (590 mg, 3.0 mmol) in DCM (25 mL) at 0 °C. After stirring for 24 h at room temperature, the reaction mixture was diluted with EtOAc (100 mL), washed with saturated $NaHCO_3$, 1 N HCl, water, and brine, dried over Na_2SO_4, and concentrated

under reduced pressure. The crude was purified by column chromatography (hexane/EtOAc, 7:2) to get pure dipeptide **26** (970 mg, 94%), mp 85–87 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7.31 (m, 5H), 7.18 (d, 2H, J=8.5 Hz), 6.92 (d, 2H, J=8.5 Hz), 6.91 (d, 1H, J=8.2 Hz), 5.04 (s, 2H), 4.61–4.54 (m, 1H), 4.34–4.29 (m, 1H), 4.17 (q, 2H, J=7.1 Hz), 3.14 (dd, 1H, J=14.3, 4.1 Hz), 3.11 (d, 1H, J=4.1 Hz), 2.89 (dd, 1H, J=14.0, 7.4 Hz), 1.66–1.42 (m, 3H), 1.28 (t, 3H, J=7.1 Hz), 0.92 (d, 3H, J=1.7 Hz), 0.90 (d, 3H, J=1.4 Hz); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 173.0, 172.8, 157.8, 137.0, 130.7, 129.0, 128.5, 127.9, 127.4, 114.8, 72.7, 69.9, 61.3, 50.3, 41.3, 39.6, 24.6, 22.8, 21.8, 14.1; HRMS calcd for $C_{24}H_{31}N\,O_5$+H, 414.2280; found, 414.2256.

*4.2.13. Synthesis of tetrapeptide **28***

A solution of **27** (145 mg, 0.35 mmol) in THF (7.5 mL) was treated with 0.2 N LiOH (7.5 mL) at room temperature. The reaction mixture was stirred overnight and quenched with 1 N HCl. The mixture was extracted with EtOAc, washed with brine, and dried with Na_2SO_4. After evaporation of the solvent, the crude acid **27** was dried under vacuum for 2 h.

To a solution of compound **23a** (170 mg, 0.29 mmol) in DCM (8 mL) was added TFA (2 mL) at 0 °C. The reaction mixture was warmed up to room temperature and stirred for 2 h before the solvent was evaporated under reduced pressure. The TFA salt **24** was dried under vacuum for 2 h.

HATU (135 mg, 0.35 mmol) and DIEA (0.15 mL, 0.87 mmol) were added subsequently to a stirred solution of acid **27** and TFA salt **24** in DMF (2.5 mL) at 0 °C. After stirred for 24 h at room temperature, the reaction was quenched with saturated $NaHCO_3$, extracted with EtOAc (20 mL×5), washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography (2% MeOH in chloroform) to get pure tetrapeptide **28** (192 mg, 77%), $[\alpha]_D$ +36.6 (*c* 1.4, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$, mixture of conformers) δ 8.62 (br, 1H), 8.03 (br, 1H), 7.60 (br, 2H), 7.41–7.24 (m, 10H), 7.18 (br, 1H), 7.14 (d, 2H, J=8.2 Hz), 6.91 (d, 2H, J=8.2 Hz), 5.02 (s, 2H), 4.72–4.59 (m, 2H), 4.62 (d, 1H, J=11.8 Hz), 4.50 (d, 1H, J=11.8 Hz), 4.42–4.36 (m, 1H), 4.33 (d, 1H, J=8.8 Hz), 4.30–4.13 (m, 1H), 4.10 (d, 1H, J=2.7 Hz), 4.03–3.82 (m, 2H), 3.73 (s, 3H), 3.69–3.59 (m, 1H), 3.54–3.30 (m, 2H), 3.28–3.17 (m, 1H), 3.09 (dd, 1H, J=13.7, 5.2 Hz), 2.85 (dd, 1H, J=14.0, 7.7 Hz), 2.76–2.42 (m, 2H), 2.32–2.13 (m, 2H), 2.04–1.36 (m, 6H), 1.30–1.18 (m, 1H), 0.91 (complex, 6H, J=6.6, 6.0 Hz); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 175.0, 174.6, 173.7, 173.1, 172.2, 171.7, 171.2, 159.2, 157.7, 157.6, 138.4, 138.0, 136.9, 130.6, 130.5, 130.3, 129.2, 128.8, 128.4, 127.8, 127.5, 114.8, 114.6, 114.5, 77.2, 74.4, 72.6, 72.4, 70.5, 69.8, 69.3, 60.8, 60.4, 59.6, 52.5, 52.4, 51.3, 50.3, 48.2, 40.8, 40.5, 40.0, 39.6, 39.4, 36.2, 31.0, 29.6, 29.4, 29.1, 25.0, 24.4, 23.4, 23.2, 21.4, 21.1, 20.8; HRMS calcd for $C_{44}H_{57}N_7O_{11}$+H, 860.4194; found, 860.4199.

*4.2.14. Synthesis of compound **29***

Freshly prepared 0.2 N $LiBH_4$ solution in THF (1.0 mL, 0.20 mmol) was added via syringe to a N_2 protected solution of **28** (132 mg, 0.15 mmol) in dry THF (5 mL) at 0 °C. The resulting solution was stirred at 0 °C for 30 min, another 1.0 mL 0.2 N $LiBH_4$ solution was added, and continued stirring at 0 °C for 30 min. The reaction was quenched with water followed by saturated NH_4Cl. The resulting mixture was extracted with THF/$CHCl_3$ (10:90, 20 mL×5). The extracted solution was dried over Na_2SO_4, concentrated, and the crude product was purified by chromatography (3–5% MeOH in $CHCl_3$) to give pure **29** (91 mg, 72% yield). ^{1}H NMR (300 MHz, $CDCl_3$, mixture of conformers) δ 8.63 (br, 1H), 8.07 (d, 1H, J=9.1 Hz), 7.74 (br, 2H), 7.39–7.25 (m, 10H), 7.18 (br, 1H), 7.15 (d, 2H, J=6.4 Hz), 6.89 (d, 2H, J=8.2 Hz), 4.99 (m, 2H), 4.74–4.51 (m, 2H), 4.51–4.41 (m, 1H), 4.41–4.25 (m, 3H), 4.25–4.14 (m, 1H), 4.14–4.00 (m, 2H), 3.99–3.79 (m, 1H), 3.77–3.59 (m, 2H), 3.58–3.38 (m, 2H), 3.36–2.99 (m, 3H), 2.83 (br, 2H), 2.71–2.46 (complex, 1H), 2.42–1.97 (m, 3H), 1.82–1.11 (m, 7H), 1.00–1.73 (complex, 6H, J=5.5, 5.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.9, 173.8, 172.9, 171.8, 159.2, 157.7, 138.3, 137.8, 136.8, 130.5, 130.3, 129.2, 128.8, 128.5, 128.3, 128.1, 127.9, 127.7, 127.4, 114.9, 114.7, 114.4, 77.1, 74.8, 72.7, 72.4, 70.4, 70.3, 69.8, 69.6, 69.3, 65.1, 60.7, 59.5, 50.7, 50.4, 40.7, 39.8, 39.5, 39.3, 36.3, 29.6, 29.4, 27.9, 25.1, 24.9, 24.6, 24.4, 23.4, 23.2, 21.4, 21.2, 20.7; HRMS calcd for $C_{43}H_{57}N_7O_{10}$+H, 832.4245; found, 832.4255.

*4.2.15. Preparation of aeruginosin 298-A analog **I***

Compound **29** (27 mg, 0.032 mmol) in 2.5 mL methanol containing 12.5 μL TFA was treated with Pd/C (2.0 mg). The suspension was sequentially evacuated and purged with H_2, stirred under atmosphere of H_2 (1 atm) for 24 h. The reaction mixture was filtered through a pad of Celite, which was washed with several portion of methanol. The combined solution was concentrated and dried under vacuum to afford aeruginosin 298-A analog **I** as colorless amorphous solid (30 mg, 98%), $[\alpha]_D$ +50.8 (*c* 0.4, MeOH). ^{1}H NMR (300 MHz, MeOH-d_4, mixture of conformers) δ 8.03 (d, 0.6H, J=8.5 Hz), 7.96–7.79 (br, 0.6H), 7.59 (d, 0.4H, J=8.2 Hz), 7.53–7.40 (br, 0.4H), 7.06 (d, 2H, J=8.0 Hz), 6.69 (d, 2H, J=8.2 Hz), 4.73–4.57 (m, 1H), 4.57–4.39 (m, 1H), 4.39–4.11 (m, 3H), 4.10–3.98 (m, 2H), 3.96 (s, 1H), 3.92–3.82 (m, 1H), 3.82–3.72 (m, 1H), 3.72–3.46 (m, 1H), 3.38 (d, 2.4H, J=5.2 Hz), 3.27–3.08 (m, 2H), 3.03 (t, 1.6H, J=9.6 Hz), 2.90–2.63 (m, 2H), 2.54 (dd, 1H, J=8.5, 4.4 Hz), 2.43–2.05 (m, 2H), 1.79–1.41 (m, 6H), 1.41–1.22 (m, 1H), 0.90 (d, 3H, J=6.0 Hz), 0.85 (d, 3H, J=6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$, major conformer) δ 175.0, 173.8, 171.8, 157.0, 155.5, 130.1, 127.8, 114.4, 74.5, 72.1, 70.2, 63.5, 61.4, 60.6, 59.9, 51.0, 50.4, 40.7, 39.2, 39.0, 35.4, 31.9, 27.7, 25.1, 24.1, 22.1, 19.8; HRMS calcd for $C_{29}H_{46}N_6O_8$+H, 607.3455; found, 607.3455.

4.3. Synthesis of aeruginosin 298-A analog II

The synthesis followed the same methods described in Section 4.2, only characterization data are given here.

*4.3.1. Synthesis of O-Choi variant, compound **30***

Under the same condition described for **20**, compound **30** was synthesized as an inseparable diastereomeric mixture in 85% yield. Major isomer **30a** (*S*-configuration) is the enantiomer of **20a** (*R*-configuration), thus has identical NMR spectrum as **20a**.

*4.3.2. Synthesis of dipeptide **33***

Under the same condition described for **22**, compound **31** was synthesized from compound **30** as an inseparable diastereomeric mixture in 71% yield. Then **31** was subjected to the same reaction condition for **28**, to give pure tetrapeptides **33b** (8%) and **33a** (57%). For major isomer **33a**: $[\alpha]_D$ +8.0 (*c* 0.6, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of conformers) δ 8.59 (br, 1H), 7.40–7.24 (m, 10H), 7.16 (d, 1.33H, J=8.4 Hz), 7.11 (d, 0.67, J=8.4 Hz), 6.90 (br, 1H), 6.87 (d, 2H, J=8.4 Hz), 4.99 (s, 0.67H), 4.97 (s, 1.33H), 4.74–4.45 (m, 6H), 4.45–4.36 (m, 2H), 4.27–4.25 (m, 0.67H), 4.19–4.13 (m, 1.33H), 4.08–4.02 (m, 1.33H), 3.93–3.84 (m, 0.67H), 3.75 (s, 1H), 3.70 (s, 2H), 3.76–3.66 (m, 2H), 3.44–3.02 (m, 4H), 2.95–2.71 (m, 2H), 2.68–2.56 (m, 1H), 2.51–2.39 (m, 1H), 2.37–2.16 (m, 2H), 2.10–1.80 (m, 1H), 1.77–1.49 (m, 3H), 1.27–1.17 (m, 2H), 0.84 (complex, 6H, J=6.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.5, 174.0, 173.0, 172.6, 172.4, 171.9, 159.6, 159.5, 158.0, 157.9, 138.3, 138.2, 137.3, 137.2, 131.0, 130.9, 129.5, 129.2, 128.8, 128.7, 128.6, 128.2, 128.0, 127.9, 127.8(2), 127.7, 114.9, 114.8, 76.0, 74.0, 72.9, 72.8, 71.1, 70.6(2), 70.1, 69.8, 69.4, 65.1, 62.3, 60.7, 59.5, 59.0, 53.0, 52.9, 52.8, 51.8, 49.6, 49.2, 41.6, 40.9, 40.7, 39.9, 39.8, 29.9, 29.7, 28.5, 24.6, 24.5, 23.6, 21.5, 21.3; HRMS calcd for $C_{44}H_{57}N_7O_{11}$+H, 860.4194; found, 860.4199.

*4.3.3. Synthesis of compound **34***

Under the same condition described for **29**, compound **33** was reduced with $LiBH_4$ to give pure compound **34** in 52% yield. ^{1}H NMR (400 MHz, $CDCl_3$, mixture of conformers) δ 8.55 (br, 1H), 7.56 (br,

2H), 7.41–7.25 (m, 10H), 7.16 (d, 2H, J=8.8 Hz), 7.15–7.05 (m, 2H), 6.89 (d, 2H, J=8.4 Hz), 4.97 (s, 2H), 4. 56 (s, 2H), 4.43–4.25 (m, 4H), 4.16–4.05 (m, 1H), 3.95–3.71 (m, 3H), 3.71–3.41 (m, 4H), 3.27–3.01 (m, 3H), 2.88–2.84 (m, 1H), 2.77–2.49 (m, 3H), 2.45–2.33 (m, 1H), 2.29–2.17 (m, 1H), 2.12–2.04 (m, 1H), 1.75–1.42 (m, 4H), 1.41–1.19 (m, 2H), 0.87 (d, 3H, J=6.2 Hz), 0.82 (d, 3H, J=6.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.8, 173.2, 172.5, 159.5, 158.0, 138.1, 137.2, 131.0, 129.4, 128.8, 128.7, 128.2, 128.0, 127.7(2), 114.8, 77.5, 74.8, 72.9, 70.9, 70.6, 70.2, 66.4, 59.9, 54.3, 50.7, 50.1, 41.2, 40.3, 39.8, 31.5, 29.4, 28.9, 24.8, 23.5, 21.5; HRMS calcd for $C_{43}H_{57}N_7O_{10}$+H, 832.4245; found, 832.4255.

4.3.4. Synthesis of aeruginosin 298-A analog ***II***

Compound **34** was subjected to the same reaction condition as for aeruginosin 298-A analog **I**, to give aeruginosin 298-A analog **II** in 99% yield, $[\alpha]_D$ +8.9 (c 0.47, MeOH). ^{1}H NMR (400 MHz, MeOH-d_4, mixture of conformers) δ 7.68–7.64 (m, 1H), 7.59–7.56 (m, 1H), 7.05 (d, 1H, J=8.4 Hz), 7.00 (d, 1H, J=8.4 Hz), 7.05 (d, 1H, J=8.4 Hz), 6.67 (d, 1H, J=8.8 Hz), 6.64 (d, 1H, J=8.1 Hz), 4.98 (d, 1H, J=8.1 Hz), 4.72–4.54 (m, 1H), 4.43–4.31 (m, 2H), 4.26–4.13 (m, 2H), 4.09–3.96 (m, 2H), 3.96–3.67 (m, 4H), 3.59–3.36 (m, 3H), 3.26–3.09 (m, 2H), 3.07–2.86 (m, 2H), 2.85–2.67 (m, 2H), 2.43–2.14 (m, 3H), 1.75–1.42 (m, 6H), 1.37–1.22 (m, 2H), 0.95–0.80 (m, 4H), 0.73 (d, 2H, J=6.2 Hz); ^{13}C NMR (100 MHz, MeOH-d_4, major conformer) δ 176.3, 176.1, 175.9, 174.3, 174.0, 173.8, 158.8, 158.7, 157.4, 157.3, 132.1, 131.0, 129.3, 129.2, 116.1, 115.6, 76.6, 75.4, 73.9, 73.7, 71.8, 68.1, 65.1, 64.8, 64.4, 62.9, 62.6, 60.9, 60.5, 54.3, 52.5, 52.4, 52.3, 50.7, 42.5, 42.4, 42.3, 41.7, 40.9, 40.8, 37.9, 33.4, 33.3, 31.0, 29.6, 29.4, 26.7, 26.3, 25.7, 25.5, 24.0, 23.9, 22.0, 21.9; HRMS calcd for $C_{29}H_{46}N_6O_8$+H, 607.3455; found, 607.3460.

4.4. General procedures for enzyme inhibition assays

Thrombin from bovine plasma, trypsin from bovine pancreas, trypsin inhibitor from chicken egg white, and trypsin substrate *N*-benzoyl-DL-arginine-4-nitroanilide hydrochloride were purchased from Sigma; chromogenic thrombin substrate, H-D-HHT-Ala-Arg-pNA·2AcOH (SPECTROZYME®), was obtained from American Diagnostica Inc. Other common chemicals and reagents were purchased from Aldrich or Lancaster. The known thrombin inhibitor, PPACK, was purchased from Calbiochem (EMD bioscience). Standard enzyme assay procedures and chromogenic substrates were used for both enzymes. UV absorbance of the cleaved *p*-nitroaniline was monitored at 405 nm on a UV spectrophotometer. Typical inhibitor concentrations are 1.0, 0.50, 0.25, 0.13, 0.063, 0.031 mg/mL, and 0 (buffer was used instead of inhibitor). Further serial dilutions were performed for compounds that showed above 90% inhibition at the lowest concentration tested. In general, test compounds were dissolved in distilled water or a small amount of DMSO and then diluted with distilled water. Thrombin buffer is composed of 10 mM Tris–HCl (pH=8), 150 mM of NaCl, 10 mM of HEPES, 0.10% of PEG6000 in distilled water. Enzyme assays were performed in 96-well microtiter plates using a microtiter plate reader (Biotek Powerwave XS Spectrophotometer). A mixture of 50 μL of inhibitor solution, 20 μL of bovine thrombin solution (0.25 NIH units/mL), and 30 μL thrombin buffer solution was incubated at 37 °C for 10 min, then 50 μL of substrate Spectrozyme (0.032 mg/mL) was added to each cell, this final mixture was then incubated for 30 min at 37 °C with slow shaking. The total volume in the cell was 150 μL. The UV absorbance at 405 nm before adding substrate was recorded as the blank. The absorbance at 405 nm was measured immediately after the incubation. The IC_{50} value was obtained from the curve of inhibitor concentration versus absorbance, the final data was obtained as an average of triplicate experiments.

Trypsin assays were run similarly; a commercial egg white trypsin inhibitor was used as the standard for calibration of the enzyme concentrations. Buffer solution is composed of 50 mM Tris–HCL (pH=8), 100 mM NaCl, 1 nM $CaCl_2$ aqueous solution. A mixture of 50 μL of inhibitor solution, 10 μL of trypsin solution (0.05 mg/mL), and 40 μL buffer solution was incubated for 5 min, then 100 μL substrate *N*-benzoyl-DL-arginine-4-nitroanilide hydrochloride (0.43 mg/mL solution in 10%DMSO and water) was added. The final volume was 200 μL, this was incubated at 37 °C for 30 min with slow shaking. The UV absorbance at 405 nm before adding substrate was recorded as the blank. The absorbance at 405 nm was recorded immediately after incubation and IC_{50} value was calculated as an average of duplicate experiment.

Acknowledgements

We are grateful to the financial support from American Heart Association scientist development grant #0430285N. We also thank Branden Hopkinson and Navneet Goyal for their help in running the enzyme assays and Sherwin Cheuk for his help in preparing some intermediates.

Supplementary data

^{1}H and ^{13}C NMR spectra for compounds **11**–**16**, **18**, **19a**, **20a**, **21a**, **21b**, **23a**, **23b**, **27**–**29**, **I**, **33a**, **34**, **II** are provided. The 2D COSY and NOESY spectra for compounds **21a** and **21b** are also provided. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.03.107.

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