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Synthesis of a novel cyclic prodrug of RGD peptidomimetic to improve its cell membrane permeation

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Abstract

The objective of this work was to synthesize cyclic prodrug **2** derived from the parent RGD peptidomimetic **1** and to evaluate its chemical and enzymatic stabilities and antithrombic activity. Cyclic prodrug **2** was formed to improve the cell membrane permeation of RGD peptidomimetic **1** by transiently masking the unfavorable physicochemical properties of compound **1**. Cyclic prodrug **2** was synthesized by linking the amino and carboxylic acid groups of parent **1** via the (acyloxy)alkoxy promoiety. The prodrug-to-drug conversion of cyclic prodrug **2** was evaluated in isolated esterase and human plasma in the absence and presence of the esterase inhibitor paraoxon. The rate of hydrolysis of cyclic prodrug **2** was significantly faster in plasma ($t_{1/2} = 33.5 \pm 0.6$ min) than in PBS ($t_{1/2} = 314 \pm 11$ min). Cyclic prodrug **2** was converted by esterase to the parent compound **1** and this conversion was inhibited by an esterase inhibitor, paraoxon. The IC_{50} (4 μM) of cyclic prodrug **2** was higher than the IC_{50} (1.9 μM) of parent drug **1**. The antithrombic activity of cyclic prodrug **2** depends on the incubation time in platelet-rich plasma; the activity increases with incubation time, suggesting that the prodrug-to-drug conversion is time-dependent and mediated by esterase. Cyclic prodrug **2** was more stable under acidic

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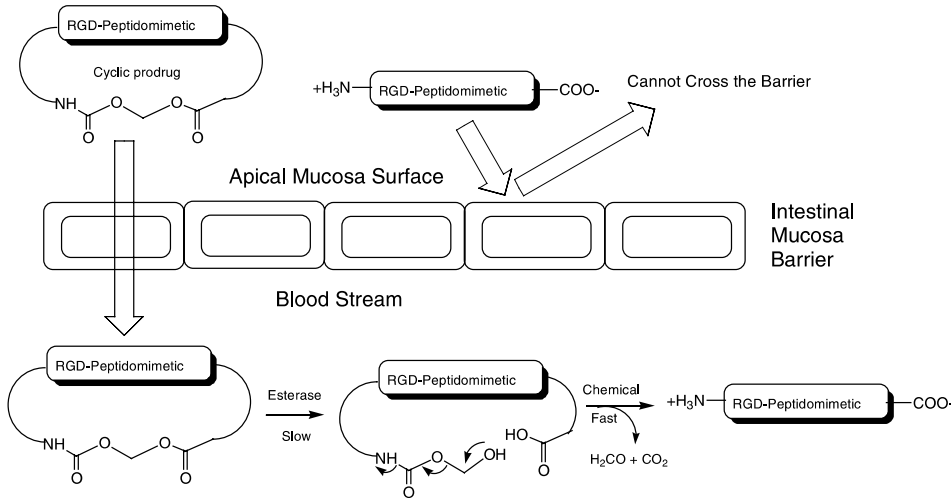
and neutral conditions than under basic conditions, suggesting that handling and formulation of this prodrug should be undertaken under acidic conditions.

Keywords: Cyclic prodrug; RGD peptidomimetic; (acyloxy)alkoxy linker; Antithrombic agents

1. Introduction

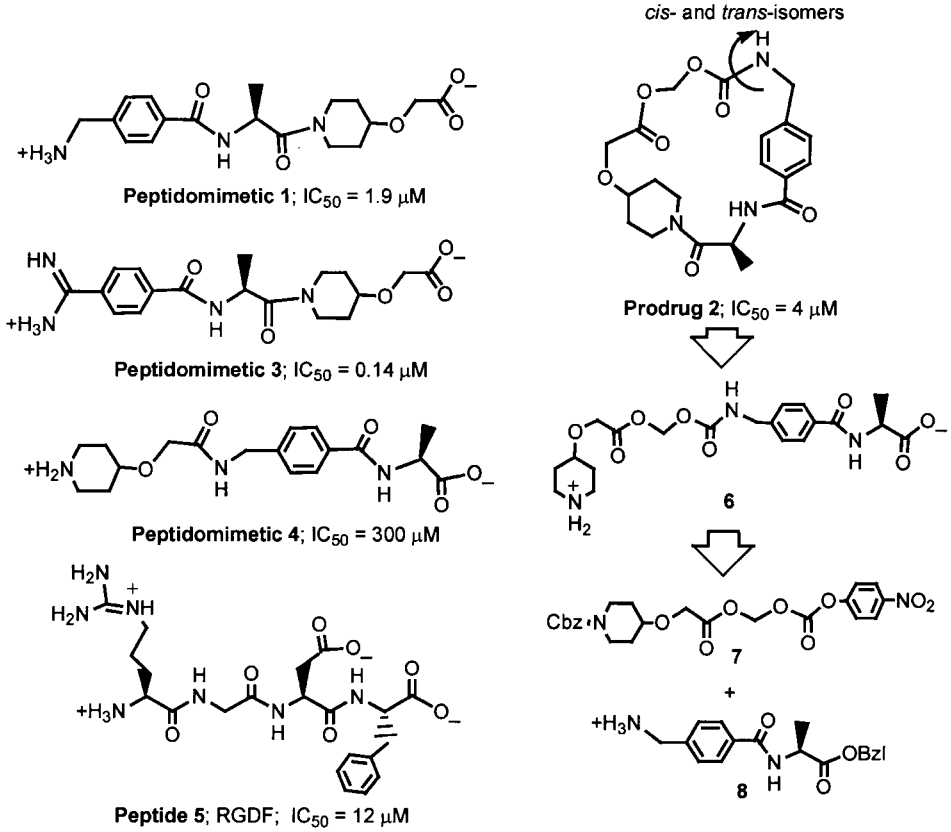
Thrombotic disease is one of the major causes of death in the United States; in addition, more than one-and-one-half million people are hospitalized with myocardial infarctions each year [1,2]. The basic cause of this group of diseases is the formation of a thrombus that attaches to blood vessels, causing disruption of normal blood flow as well as tissue damage. There is a need to develop inhibitors for thrombosis that can be delivered orally because oral drug delivery is convenient and cost-effective [3]. In the process of thrombosis, activated platelets aggregate to form blood clots via multivalent interactions between GPIIb/IIIa receptors on the surface of platelets and fibrinogen (Fg) [4–7]. Binding between Fg and GPIIb/IIIa receptors is mediated by the Arg–Gly–Asp (RGD) sequence on fibrinogen α- and γ-chains [8–12]. Therefore, RGD peptides and peptidomimetics have been developed as antithrombic agents to inhibit platelet aggregation in vitro and in vivo [2,13–17].

Potent RGD peptidomimetics contain a positively charged guanidino or amino group and a negatively charged carboxylic acid group, separated by a certain distance for their binding selectivity to GPIIb/IIIa [16,18–20]. Unfortunately, these charges and other physicochemical properties (i.e., hydrogen-bonding potential, conformation) hinder the permeation of RGD peptidomimetics through cell



Scheme 1.

membranes of the intestinal mucosa for oral bioavailability (Scheme 1); for this reason, RGD peptidomimetics have been developed only for intravenous (IV) applications [2]. Many attempts have been made to develop orally active RGD peptidomimetics; however, some of these failed in clinical trials. Thus, a new method to improve cell membrane permeation of RGD peptidomimetics is necessary to increase their oral bioavailability. One approach to increase membrane permeation of RGD peptidomimetics is in transiently altering the physicochemical properties (e.g., charge, hydrogen-bonding potential, conformation, hydrodynamic radii, and lipophilicity) of the RGD peptidomimetics. This can be done by linking the amino and carboxylic acid groups via the acyloxyalkoxy promoiety to form cyclic prodrugs (Scheme 1) [21–23]. Previously, we have shown that the formation of cyclic prodrugs improves the permeation of peptides via the Caco-2 cell monolayer, a model for the intestinal mucosa [23–25]. The cyclic prodrugs permeate through the cell membranes more significantly than do the parent linear peptides [23,24,26,27]. The improvement in membrane permeation of the cyclic prodrugs is due to: (a) increased conformational stability and intramolecular hydrogen-bonding potential, (b) an increase in hydrophobicity produced by masking the charges in the N- and C-termini, (c)



Scheme 2.

improvement of enzymatic stability against exopeptidase and endopeptidases, and (d) reduction of hydrodynamic radii [25,28,29]. After crossing the cell membranes, the prodrug can be converted to the parent drug by esterase in the bloodstream.

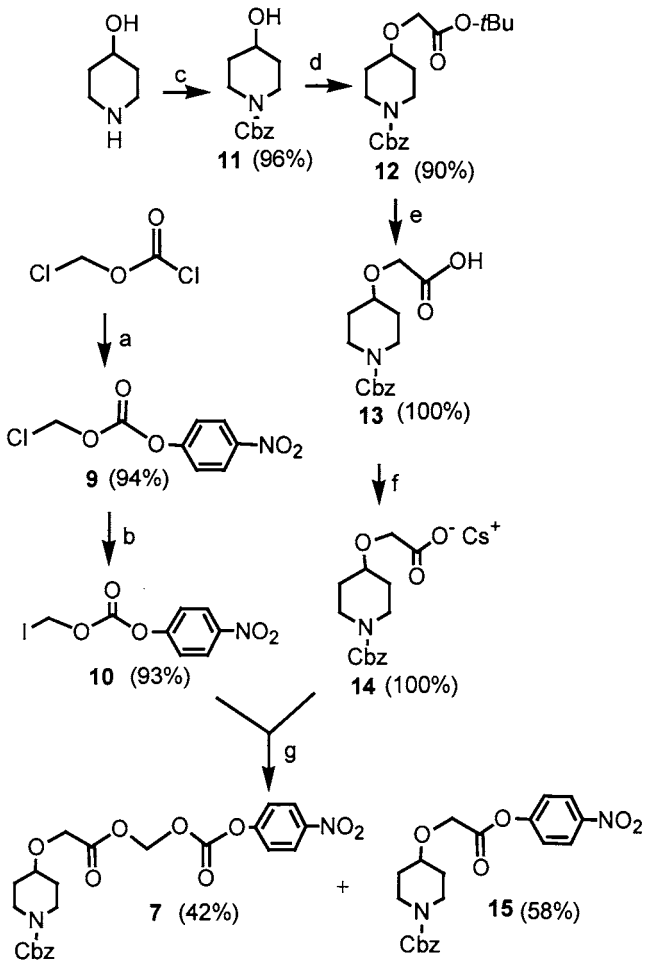
Herein, we describe the synthesis of cyclic prodrug **2** from the parent RGD peptidomimetic **1** (Scheme 2) to improve its membrane permeation properties. The parent peptidomimetic **1** is the derivative of a known potent RGD peptidomimetic **3** [30]; the amidine group in compound **3** was replaced with an amino group in compound **1**. The antithrombic activity of cyclic prodrug **2** was compared to that of the parent compound **1**, RGD peptidomimetics **3** and **4**, and RGD peptide **5**. The synthesis of cyclic prodrug **2** was accomplished by linking the amino and carboxylic acid groups via the acyloxyalkoxy promoiety. Because RGD peptidomimetic **1** has aromatic and piperidine rings, cyclic prodrug **2** has high rigidity, which contributes to the physicochemical and biopharmaceutical properties of the prodrug. The enzymatic conversion of cyclic prodrug **2** was evaluated to determine the ability of the prodrug to release the RGD peptidomimetic **1**.

2. Results and discussion

2.1. Synthesis of cyclic prodrug 2

The synthesis of cyclic prodrug **2** was accomplished by forming an amide bond between the amino functional group of the piperidine ring and the carboxylic acid of the alanine in linear precursor **6**, as shown in Scheme 2. The linear precursor **6** was synthesized from intermediate **18**, which can be formed via two different paths (Scheme 4). The first path is conjugation of the two key intermediates **7** and **8**. Unfortunately, intermediate **7** contains an (acyloxy)alkoxy promoiety linked to the piperidine acetic acid group and has a side product **15**. Intermediate **8** is the 4-aminomethylbenzoylalanine peptide. The second path to make intermediate **18** is the formation of intermediate **21** from compound **8**. Intermediate **21** was reacted with compound **14** to give intermediate **18** without a side product.

The synthesis of intermediate **7** (Scheme 3) was completed by conjugating compounds **10** and **14**. The synthesis of compound **10** was initiated using commercially available 1-chloromethyl chloroformate. Treatment of 1-chloromethyl chloroformate with *p*-nitrophenol in the presence of *N*-methylmorpholine (NMM) produced compound **9** in 94% yield. The chloride atom in **9** was replaced with iodine by reacting it with sodium iodide in acetone to produce compound **10** in 93% yield. Synthesis of compound **14** was started from the commercially available 4-hydroxypiperidine [30]. The secondary amine of 4-hydroxypiperidine was protected with a benzyloxycarbonyl (Cbz) group; this was carried out by reacting 4-hydroxypiperidine with benzylchloroformate to obtain compound **11** in 96% yield. The hydroxyl group in compound **11** was alkylated with 2-bromoacetate-*t*-butyl ester to yield 90% of compound **12**. The *t*-butyl ester-protecting group in **12** was removed with trifluoroacetic acid (TFA) in CH_2Cl_2 to produce acid **13** in 100% yield. Acid **13** was converted to cesium salt by treating it with Cs_2CO_3 in methanol to give a quantitative yield of salt **14**. Addition of cesium salt **14** into iodo intermediate **10** in



a) *p*-Nitrophenol, NMM/CH_2Cl_2, 0-25°C; b) NaI/Acetone, 40°C, 6 h; c) CBz-Cl, Et_3N/CH_2Cl_2, 0-25°C, 17 h; d) $BrCH_2COOt$-Bu, $(n\text{-Bu})_4NHSO_4$, 50% NaOH:$PhCH_3$ (1:1), 20 h; e) TFA/CH_2Cl_2; f) Cs_2CO_3/MeOH; g) DMF, 0-25°C

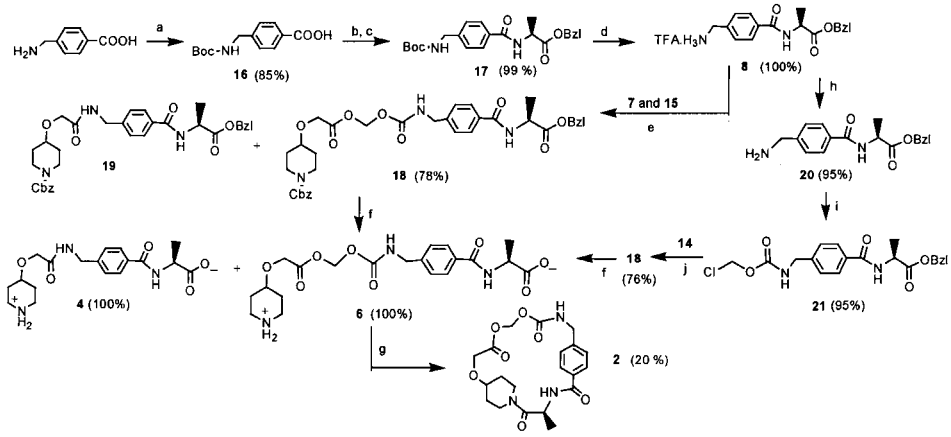
Scheme 3.

dimethylformamide (DMF) gave the key intermediate **7** in 42% yield; the low yield was due to the formation of side product **15** in 58% yield. Previously, we have shown that this is one of the most challenging steps in the synthesis of this type of cyclic prodrug; the yield of this intermediate depends on the structure of the acid [21]. The mixture of compounds **7** and **15** was used directly in the next reaction due to the difficulty in separating them.

The key intermediate **8** was produced by coupling the Boc-*p*-aminomethyl benzoic acid (**16**) and the alanine benzyl ester as shown in Scheme 4. Protection of the amino group in *p*-aminomethylbenzoic acid with the *t*-Boc group gave compound **16** in 85% yield. Activation of the carboxylic acid group of **16** was accomplished using EDC and HOBT; the activated acid was subsequently reacted with alanine benzyl ester to give compound **17** in 99% yield. Deprotection of the Boc group with TFA/CH_2Cl_2 produced the desired intermediate **8** in a quantitative yield.

The final process in the synthesis of cyclic prodrug **2** was the formation of key intermediate **6**, which can be formed by hydrogenation of compound **18**. Early in our study, compound **18** was made by conjugation of intermediate **8** with a mixture of compounds **7** and **15** in DMF in the presence of HOBT and NMM. This reaction gave a side product **19**, which did not contain the (acyloxy)alkoxy promoiety. Compound **18** (78%) can be separated with difficulty from the side product **19** using silica gel column chromatography. In a separate attempt, the mixture was also subjected to the next reaction without purification; the Cbz- and OBzl-protecting groups in compounds **18** and **19** were removed simultaneously by hydrogenation to give compounds **6** and **4**. Compound **6** was purified by semi-preparative HPLC on a C-18 reversed-phase column. Compound **4** was also isolated and was tested for antithrombic activity. Because this synthetic route is extremely time-consuming and the yield is low, we developed a new route to prepare **18** in fewer steps with higher yield and easier purification (Scheme 4).

When the TFA salt **8** was initially converted to free amine **20**, it reacted readily with 1-chloromethyl chloroformate in the presence of a base (TEA) to produce compound **21** in 95% yield. Unlike the formation of compound **10** in Scheme 3, metathesis of chloride to iodide atom in **21** was unsuccessful. However, the coupling reaction between **21** and cesium salt **14** proceeded smoothly to give the key intermediate **18** in 76% yield after silica gel column chromatography. Because no side products were formed, the subsequent hydrogenation compound **6** could be used



a) $(Me_3COCO)_2O$, 1,4-Dioxane/H_2O (1:1), 1.0 M NaOH; b) EDC, HOBT, NMM/CH_2Cl_2, 0-25°C; c) H-Ala-OBzl; d) TFA/CH_2Cl_2; e) HOBT, NMM/DMF; f) H_2, 10% Pd-C/MeOH; g) HBTU, NMM/DMF; h) 2 M NaOH/CH_2Cl_2; i) $ClCH_2OCOCl$, TEA/CH_2Cl_2, 0°C; j) DMF

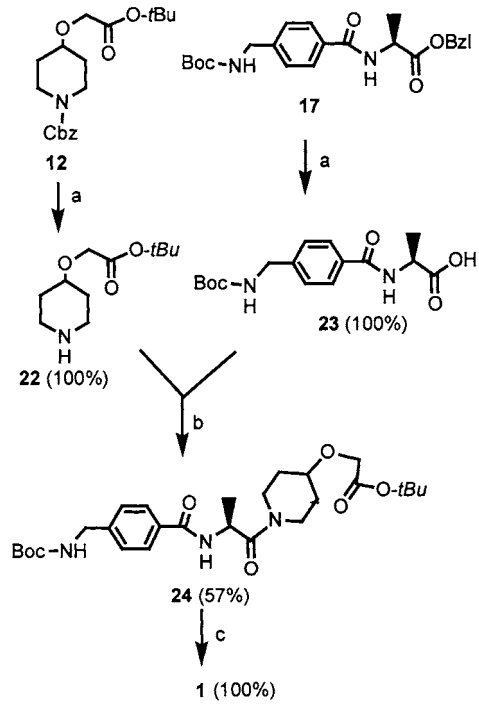
Scheme 4.

without purification. This optimal method can provide large amounts of cyclic prodrug for in vitro and in vivo studies. Cyclization of linear precursor **6** was accomplished under high-dilution conditions in DMF using HBTU as an activating reagent in the presence of *N*,*N*-diisopropylethyl amine (DIEA); the desired cyclic prodrug **2** was isolated in 20% yield after HPLC purification. The low yield obtained in this cyclization step may be due to (a) the difficulty in the formation of a rigid macrocyclic ring, (b) the possibility of forming oligomers, even under high-dilution conditions, or (c) the low recovery during purification by preparative HPLC.

The proton NMR spectrum of cyclic prodrug **2** showed two conformers; these conformers were derived from the *cis*- and *trans*-isomerization at the peptide bond between the (acyloxy)alkoxy linker and the aminobenzoic acid group as shown in Scheme 2. A similar isomerization phenomenon was also found in other cyclic prodrugs that contain the (acyloxy)alkoxy linker [29,31].

2.2. Synthesis of compounds *1* and *3*

Compound 1: The synthesis of parent compound **1** is shown in Scheme 5; it is started from compounds **12** (Scheme 3) and **17** (Scheme 4). The Cbz-protecting



a) H_2, 10% Pd-C, MeOH; b) HBTU, NMM, DMF, 0-25°C;
c) TFA/CH_2Cl_2

Scheme 5.

group in compound **12** was removed by hydrogenation to give amine **22** in a quantitative yield. Similarly, the benzyl ester-protecting group in compound **17** was also removed by hydrogenation to give acid **23** in a quantitative yield. Acid **23** was activated with HBTU and NMM followed by reaction with amine **22** to give compound **24** in 57% yield. Boc- and *t*-Bu-protecting groups in compound **24** were removed with TFA in dichloromethane to give the desired parent compound **1** in 100% yield.

Compound 3: The synthesis of compound **3** was accomplished using the method described by Alig et al. [30].

2.3. Biological study

Antithrombic activity: The antithrombic activities of compounds **1**–**5** were evaluated using an ADP-activated platelet aggregation assay and their IC_{50} results are given in Scheme 2 [32]. RGDF peptide **5**, with an IC_{50} of 12 μM, was used as a positive control. RGD peptidomimetic **1** has an IC_{50} of 1.9 μM, about tenfold higher than that of parent peptidomimetic **3** (IC_{50} of 0.14 μM). Replacement of the amidinine group with an amino group in **1** lowers the selectivity of RGD peptidomimetics for GPIIb/IIIa. This may be due to the preference of the GPIIb/IIIa receptor for an amidinine over an amino group. The side product **4** was also tested in the platelet aggregation assay; this compound has an IC_{50} of 300 μM. Although the charge separation in **4** is the same as that in **1**, compound **4** is less active than compound **1**; this result confirms the hypothesis that not only the charge separation but also the structure of RGD peptidomimetic is important. To prove that cyclic prodrug **2** can be converted to parent drug **1**, the anti-platelet aggregation activity of cyclic prodrug **2** was investigated. The inhibitory activity of cyclic prodrug **2** was evaluated under different conditions. Cyclic prodrug **2** has an IC_{50} of 10 μM when platelet aggregation is induced immediately after the addition of the prodrug; however, it has an optimal IC_{50} of 4 μM when incubated for 10–20 min PRP, prior to induction of aggregation. This result suggests that the effective conversion of prodrug to drug by esterase in PRP takes about 10 min.

Chemical and enzymatic stability: The chemical stability of cyclic prodrug **2** was evaluated at pH 4, 7, and 10 in PBS (Table 1). Prodrug **2** is more stable at pH 4.0 ($t_{1/2} = 985 \pm 37$ min) than at pH 7.0 ($t_{1/2} = 314 \pm 11$ min) or pH 10.0 ($t_{1/2} = 1.46 \pm 0.07$ min). As we observed previously, the (acyloxy)alkoxy promoiety is chemically

Table 1
Half-life ($t_{1/2}$, min) for the stability in aqueous buffer of various pH values (37 °C, $\mu = 0.15$) and enzymatic stability of cyclic prodrug **2**

Enzymatic stability	No paraoxon	With paraoxon	
Isolated esterase	16.5 ± 0.9	567 ± 14	
Plasma	33.5 ± 0.6	75.1 ± 2.1	
Chemical stability	pH = 4	pH = 7	pH = 10
PBS	985 ± 37	314 ± 11	1.46 ± 0.07

unstable at basic pH and stable at acidic pH [21,24,33]. This result suggests that cyclic prodrug **2** will be stable at the pH of the stomach.

The ability of esterase to hydrolyze the ester bond of the promoiety is necessary for releasing the RGD peptidomimetic from the cyclic prodrug into the bloodstream. The target for RGD peptidomimetics is the platelets in the bloodstream. The conversion of prodrug **2** to parent drug **1** by esterase was confirmed by comparing the stability of the prodrug in platelet-rich plasma or isolated esterase in the presence and absence of the esterase inhibitor paraoxon [24,26]. As shown in Table 1, the prodrug can be converted to the parent drug by isolated porcine and plasma esterases as determined by HPLC. In general, the half-lives of the prodrugs in isolated esterase (Table 1) were shorter than those in PBS at pH 7.0, suggesting that esterase can hydrolyze the prodrugs. The rate of hydrolysis of cyclic prodrug **2** was significantly faster in human plasma in the absence of paraoxon (33.5 ± 0.6 min) than in the presence of paraoxon ($t_{1/2} = 75.1 \pm 2.1$ min) (Table 1). This enzymatic reaction was also conducted in isolated esterase in the presence and the absence of esterase inhibitor. The rate of conversion of prodrug **2** in isolated esterase solution showed a half-life of 16.5 ± 0.9 min in the absence of paraoxon and 567 ± 14 min in the presence of paraoxon. These results suggest that prodrug **2** is converted to parent compound **1** by esterase. The production of parent compound **1** in the enzymatic reaction was observed by HPLC. Paraoxon was less effective in inhibiting the prodrug-to-drug conversion in plasma than in isolated esterase; this may be due to the presence of many different types of esterases in human plasma. Thus, paraoxon may not effectively inhibit all of the different esterases in the plasma.

In another study, the intrinsic membrane permeation of cyclic prodrug **2** was compared to that of the parent compound **1** using Caco-2 cell monolayers, an in vitro model for the intestinal mucosa. The cyclic prodrug **2** ($P_{app} = 10.3 \pm 0.74 \times 10^{-7}$ cm/s) can permeate the cell membrane about 13 times better than the parent RGD peptidomimetic **1** ($P_{app} = 0.77 \pm 0.10 \times 10^{-7}$ cm/s). These results are congruent with previous studies on cyclic prodrugs of RGD peptidomimetics using coumaric acid linkers [34,35]. The improvement in the membrane permeation of cyclic prodrug **2** is due to the alteration of its physicochemical properties (i.e., charges, hydrogen-bonding potential, and conformation) compared to those of parent RGD peptidomimetic **1**. Although the in vitro results suggest that cyclic prodrug formation may improve the oral bioavailability of RGD peptidomimetics, in vivo studies of the prodrugs will be essential for determining the utility of this method.

3. Conclusions

We have developed a route to synthesize a new rigid cyclic peptide prodrug **2** by using an (acyloxy)alkoxy promoiety to link the amino and carboxylic acid groups of the parent peptidomimetic **1**. Cyclic prodrug **2** is susceptible to esterase metabolism (slow step), leading to a cascade of chemical reactions and resulting in the release of the linear parent peptidomimetic **1**. The antithrombic activity of cyclic prodrug **2** is due to prodrug-to-drug conversion by esterase in the plasma. The chemical stability

of the cyclic prodrug **2** is higher at acidic pH than at neutral and basic pH. Finally, prodrug **2** can permeate the cell membrane better than parent compound **1**.

4. Experimental methods

4.1. General methods

^{1}H-NMR spectra were recorded on 500 and 400 MHz Bruker NMR instruments. Chemical shifts are expressed in parts per million (δ), relative to the internal standard tetramethylsilane (TMS) or residual proton from solvent. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. High-resolution mass spectra (HRMS) were obtained using a VG Analytical ZAB double-focusing spectrometer. Elemental analyses were performed by Quantitative Technologies, New Jersey. All starting materials were purchased from Aldrich Chemical, Sigma Chemical, Fluka Chemical, or Bachem Bioscience and used as received.

HPLC purification and analysis were conducted using a Rainin HPXL gradient system with a Dynamax UV detector. The desired product was purified by semi-preparative reversed-phase HPLC using a C-18 column (12 μm, 300 Å, 25 cm × 21.4 mm i.d., flowrate 10 mL/min) and eluting with a gradient of solvent A (0.1% TFA/H_2O:5% MeCN) and solvent B (100% MeCN). The gradient method used for the preparative HPLC started from 0% to 35% of solvent B for 23 min, followed by an increase to 100% of solvent B in 10 min. The final elution was at 100% solvent B for 2 min, followed by gradient change to 100% solvent A in 2 min. The desired peptide was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm i.d., flowrate 1 mL/min) eluting with a gradient of solvents A and B. The total HPLC analysis run time was 18 min. The solvent gradient used for the analytical HPLC was 0–50% of solvent B over 12 min. The composition of solvent B was increased to 100% in 2 min, followed by elution at 100% of solvent B for 2 min. Finally, the solvent was changed to 0% solvent B over 2 min for the final equilibration.

1-Chloromethyl-p-nitrophenyl carbonate (***9***). Compound **9** was prepared and obtained (94%) in the same way, as reported in the literature [21].

1-Iodomethyl-p-nitrophenyl carbonate (***10***). Compound **10** was synthesized (93%) from compound **9** according to reported procedures [21].

N-Benzyloxycarbamate-4-hydroxypiperidine (***11***). Triethylamine (TEA) (5.06 g, 50 mmol) and benzyl chloroformate (8.53 g, 50 mmol) were added to a solution of 4-hydroxypiperidine (5.06 g, 50 mmol) in CH_2Cl_2 at 0 °C. The reaction mixture was then stirred at room temperature for 16 h. The resulting suspension was filtered and the residue after solvent evaporation was taken up in EtOAc (50 mL), which was washed with H_2O (2 × 20 mL) and 10% citric acid (40 mL) and dried over anhydrous Na_2SO_4. The solvent was removed to give the desired compound **11** (11.32 g, 96%) as a pale yellow oil. This compound was used without further purification. ^{1}H-NMR ($CDCl_3$, δ): 1.48 (2H, m), 1.85 (2H, m), 3.15 (2H, m), 3.89 (3H, m), 5.12 (2H, s), 7.35 (5H, m). The NMR spectrum of **11** was identical to that reported in the literature [30].

2-(N-Benzyloxycarbamate-4-piperidinyloxy)-t-butyl acetate (***12***). *Tert*-butyl bromoacetate (8.8 g, 45 mmol) and *tetra-n*-butylammonium hydrogen sulfate (0.339 g, 1 mmol) dissolved in H_2O (5 mL) were added to toluene solution (50 mL) of compound **11** (7.058 g, 30 mmol). A solution of NaOH (0.625 M, 25 mL) was added dropwise to the reaction mixture followed by vigorous stirring for 20 h. The organic layer was then separated and dried over anhydrous Na_2SO_4. The solvent was removed to give compound **12** (9.46 g, 90%) as light yellow oil. ^{1}H-NMR ($CDCl_3$, δ): 1.48 (9H, s), 1.61 (2H, m), 1.85 (2H, m), 3.15 (2H, m), 3.58 (1H, m), 3.83 (2H, m), 4.0 (2H, s), 5.16 (2H, s), 7.34 (5H, m).

2-(N-Benzyloxycarbamate-4-piperidinyloxy)-acetic acid (***13***). TFA (25 mL) was added to a stirred solution of compound **12** (9 g, 25.8 mmol) in CH_2Cl_2 at 0 °C. The reaction mixture was stirred at room temperature for 1 h. Volatile compounds in the reaction mixture were removed by rotary evaporation under vacuum. The residue was triturated and washed with Et_2O/petroleum ether (1:1) to give a white solid. The residual solvent was removed under vacuum to give compound **13** as a white solid (7.56 g, 100%); this compound was used in the next step without further purification.

2-(N-Benzyloxycarbamate-4-piperidinyloxy)-acetyl-oxymethyl-p-nitro-phenyl carbonate (*7*). The cesium salt of compound **13** (2.93 g, 10 mmol) was prepared by reacting it with Cs_2CO_3 (1.7 g, 5.2 mmol) in MeOH (35 mL). After stirring for 1 h, the solvent was removed under reduced pressure to afford a white powder of cesium salt **14**. Cesium salt **14** (4.25 g, 10 mmol) in DMF (150 mL) was added slowly to an ice-cold stirred solution of iodomethyl-*p*-nitrophenyl carbonate **10** (3.23 g, 10 mmol) in DMF (50 mL) over a period of 2 h. After stirring for 29 h at room temperature, DMF was removed under reduced pressure to give an oily residue. This residue was dissolved in ethyl acetate (150 mL), followed by washing with 10% $NaHCO_3$ (2×40 mL), H_2O (2×50 mL), and brine (40 mL). The ethyl acetate layer was separated and dried over anhydrous Na_2SO_4; the solvent was then removed to give the desired compound **7** (42%) and the side product **15** (58% yield) as determined by proton NMR. Due to the difficulty of separating these compounds, this mixture was used in the next step. Compound **7** ^{1}H-NMR ($CDCl_3$, δ): 1.65 (2H, m), 1.90 (2H, m), 3.23 (2H, m), 3.68 (1H, m), 3.84 (2H, m), 4.24 (2H, s), 5.13 (2H, s), 5.96 (2H, s), 7.36 (7H, m), 8.29 (2H, d, $J = 9.0$ Hz). MS (FAB) *m/z*: 489 ($M^+ + 1$).

N-Boc-4-aminomethyl-benzoic acid (***16***). 4-Aminomethyl benzoic acid (5.36 g, 20 mmol) and di-*t*-butyl dicarbonate were dissolved in a mixture of water (67 mL) and dioxane (100 mL). NaOH (1.0 N) was added dropwise to this solution at 0 °C to adjust the pH to 9.0. The reaction mixture was stirred at room temperature for 1.5 h and during this period some white precipitate was formed. The reaction mixture was concentrated to a volume of 60 mL, followed by addition of EtOAc (70 mL). The mixture was cooled in an ice bath, followed by addition of 15% citric acid to adjust the pH to 3.0. The aqueous layer was extracted with EtOAc (4×40 mL) and the combined organic layer was dried over anhydrous Na_2SO_4. A white solid after solvent evaporation was purified by recrystallization in EtOAc to yield pure compound **16** (5.02 g, 85%). ^{1}H-NMR (DMSO-d_6, δ): 1.38 (9H, s), 4.16 (2H, d, $J = 6.0$ Hz), 7.31 (2H, d, $J = 8.0$ Hz), 7.46 (1H, t, $J = 6.0$ Hz), 7.86 (2H, d, $J = 8.0$ Hz).

4-(N-Boc-aminomethyl)-benzoyl-Ala-OBzl (***17***). Acid **16** (3.765 g, 15 mmol), HOBT (2.027 g, 15 mmol), and EDC (2.876 g, 15 mmol) were dissolved in CH_2Cl_2

(120 mL) at 0 °C. NMM (1.52 g, 15 mmol) and H-Ala-OBzl (2.685 g, 15 mmol) were added to the reaction mixture, followed by stirring at 0 °C for 4 h and at ambient temperature for 24 h. A residue remaining after solvent evaporation was dissolved in EtOAc (150 mL); it was successively washed with 10% aqueous citric acid (2×40 mL), H_2O (60 mL), saturated $NaHCO_3$ (2×40 mL), H_2O (60 mL), and brine (50 mL), and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure to give a white solid, which was purified by recrystallization in EtOAc to yield compound **17** (6.1 g, 99%). ^{1}H-NMR ($CDCl_3$, δ): 1.45 (9H, s), 1.53 (3H, d, $J = 7.3$ Hz), 4.36 (2H, d, $J = 6.1$ Hz), 4.82 (1H, m), 5.21 (2H, dd, $J_1 = 5.0$ Hz, $J_2 = 13.0$ Hz), 6.8 (1H, br), 7.38 (7H, m), 7.76 (2H, d, $J = 8.0$ Hz). MS (FAB) *m/z*: 413 ($M^+ + 1$). HRMS: Calcd for $C_{23}H_{28}N_2O_5$, 413.2076. Found: 413.2079.

TFA · 4-Aminomethyl-benzoyl-Ala-OBzl (***8***). Compound **17** (4.12 g, 10 mmol) in CH_2Cl_2 (20 mL) was added to TFA (20 mL) at 0 °C. After stirring at room temperature for 1 h, the reaction mixture was concentrated by a rotary evaporator under reduced pressure. The remaining residue was triturated with Et_2O/petroleum ether (1:1) to give a white solid. The solid was isolated by decantation and the residual ether was removed under vacuum to give compound **8** (4.26 g, 100%). This compound was used in the next step without further purification. ^{1}H-NMR (DMSO-d_6, δ): 1.42 (3H, d, $J = 7.3$ Hz), 4.11 (2H, t, $J = 5.4$ Hz), 4.52 (1H, quintet, $J = 7.2$ Hz), 5.13 (2H, dd, $J_1 = 2.6$ Hz, $J_2 = 12.7$ Hz), 7.29–7.38 (5H, m), 7.54 (2H, d, $J = 8.2$ Hz), 7.92 (2H, d, $J = 8.2$ Hz), 8.28 (3H, br), 8.87 (2H, d, $J = 6.9$ Hz). MS (FAB) *m/z*: 313 ($M^+ + 1$). HRMS: Calcd for $C_{18}H_{21}N_2O_3$, 313.1552. Found: 313.1541.

4-Aminomethyl-benzoyl-Ala-OBzl (***20***). Compound **8** (2.30 g, 5.40 mmol) was dissolved in a mixture of CH_2Cl_2 (100 mL) and NaOH (2 M, 50 mL). The organic phase was separated, washed thoroughly with NaOH (2 M, 50 mL), and dried over anhydrous $MgSO_4$. Solvent was removed to yield a white solid, which was dried under vacuum to give compound **20** (1.60 g, 95%). ^{1}H-NMR (CD_3OD, δ): 1.53 (3H, d, $J = 7.3$ Hz), 3.86 (2H, s), 4.61–4.69 (1H, m), 5.21 (2H, dd, $J_1 = 6.0$ Hz, $J_2 = 12.4$ Hz), 7.40–7.38 (5H, m), 7.45 (2H, d, $J = 8.2$ Hz), 7.84 (2H, d, $J = 8.2$ Hz).

1-Chloromethyleneoxycarbonyl-4-aminomethyl-benzoyl-Ala-OBzl (***21***). A solution of 1-chloromethyl chloroformate (0.79 g, 6.12 mmol) in dry CH_2Cl_2 (20 mL) was added dropwise to compound **20** (1.60 g, 5.13 mmol) in dry CH_2Cl_2 (40 mL) and TEA (0.62 g, 6.12 mmol) in an ice bath. After stirring overnight at room temperature, the white residue remaining after solvent evaporation was taken up in EtOAc (150 mL); this was followed by washing with 5% $KHSO_4$ (2×50 mL), H_2O (2×50 mL), and brine (2×50 mL). The organic layer was separated, dried over anhydrous $MgSO_4$, and concentrated until a precipitate formed; petroleum ether (150 mL) was added to complete the precipitation. The precipitate was filtered, washed with petroleum ether, and dried under vacuum to give compound **21** (2.0 g, 95%). ^{1}H-NMR ($CDCl_3$, δ): 1.55 (3H, d, $J = 7.2$ Hz), 4.47 (2H, d, $J = 6.1$ Hz), 4.78–4.89 (1H, m), 5.24 (2H, dd, $J_1 = 5.0$ Hz, $J_2 = 12.3$ Hz), 5.50 (1H, s), 5.80 (2H, s), 6.79 (1H, d, $J = 7.0$ Hz), 7.34–7.42 (7H, m), 7.77 (2H, d, $J = 8.1$ Hz). MS (FAB) *m/z*: 405 ($M^+ + 1$).

2-(N-Benzyloxycarbamate-4-oxypiperidinyl)-acetyl-methyleneoxycarbonyl-4-aminomethyl-benzoyl-Ala-OBzl (***18***). Method 1: A solution of compound **8** (2.03 g,

4.76 mmol) and NMM (0.97 g, 9.6 mmol) in DMF (50 mL) was added to a stirred solution of compound **7** (0.976 g, 2.0 mmol), side product **15** (1.11 g, 2.76 mmol), and HOBT (0.643 g, 4.76 mmol) in DMF (25 mL). The reaction mixture was stirred at room temperature for 3 h and then DMF was removed under reduced pressure to give an oily residue. The oily residue was dissolved in EtOAc (150 mL), followed by washing with 10% $NaHCO_3$ (2×50 mL), H_2O (4×50 mL), and brine (40 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to yield a pale yellow oily mixture of compound **18** and by-product **19**. The pure compound **18** (1.035 g, 78%) was obtained as a colorless solid after silica gel column purification using EtOAc:hexane:isopropanol (4:2:1) as eluent. ^{1}H-NMR ($CDCl_3$, δ): 1.53 (3H, d, $J = 7.3$ Hz), 1.61 (2H, m), 1.85 (2H, m), 3.22 (2H, m), 3.60 (1H, m), 3.73 (2H, m), 4.18 (2H, s), 4.44 (2H, d, $J = 6.1$ Hz), 4.84 (1H, m), 5.12 (2H, s), 5.21 (2H, dd, $J_1 = 5.0$ Hz, $J_2 = 13.0$ Hz), 5.82 (2H, s), 6.8 (1H, br), 7.36 (13H, m), 7.76 (2H, d, $J = 8.0$ Hz). MS (FAB) *m/z*: 662 ($M^+ + 1$). HRMS: Calcd for $C_{35}H_{40}N_3O_{10}$, 662.2713. Found: 662.2736. Method 2: Compound **21** (3.0 g, 7.43 mmol) in DMF (40 mL) was added dropwise to the stirred cesium salt **14** (3.77 g, 8.87 mmol) in DMF (60 mL). After stirring overnight at room temperature, the white residue left after solvent removal was taken up in EtOAc (200 mL), washed with 5% $KHSO_4$ (2×50 mL), H_2O (2×50 mL), and brine (2×50 mL), and dried over anhydrous $MgSO_4$. A white solid remaining after solvent evaporation was purified on a silica gel column using EtOAc:hexane (2:1 to 3:1) as eluent to give compound **18** (3.7 g, 76%).

TFA · 2-(4-oxypiperidinyl)-acetyl-methyleneoxycarbonyl-4-aminomethyl-benzoyl-Ala-OH (*6*). Compound **18** (0.661 g, 1.0 mmol) was dissolved in MeOH (40 mL) and TFA (2 mL) and to this solution was added 10% Pd/C (60 mg). The reaction mixture was stirred at room temperature under a H_2 atmosphere using a balloon on the top of a round-bottomed flask. After stirring for 5 h, the reaction mixture was filtered to remove the catalyst (Pd/C); the solvent was removed under reduced pressure to give an oily residue. This residue was washed with Et_2O (3×20 mL) to afford the TFA salt of compound **6** (0.552 g) in a quantitative yield. This compound was further purified using semi-preparative HPLC. ^{1}H-NMR (DMSO-d_6, δ): 1.38 (3H, d, $J = 7.0$ Hz), 1.66 (2H, m), 1.92 (2H, m), 2.95 (2H, m), 3.15 (2H, m), 3.65 (1H, m), 4.25 (2H, s), 4.26 (2H, d, $J = 6.0$ Hz), 4.38 (1H, m, $J = 7.0$ Hz), 7.32 (2H, d, $J = 8.0$ Hz), 7.82 (2H, d, $J = 8.0$ Hz), 8.20 (1H, t, $J = 7.0$ Hz), 8.61 (1H, d, $J = 6.0$ Hz). MS (FAB) *m/z*: 438 ($M^+ + 1$). HRMS: Calcd for $C_{20}H_{28}N_3O_8$, 438.1876. Found: 438.1891.

Cyclic prodrug (*2*). Compound **6** (0.25 g, 0.45 mmol) was dissolved in DMF (500 mL), followed by addition of HBTU (0.853 g, 2.25 mmol). The mixture was stirred at room temperature for 30 min under N_2, followed by addition of DIEA (0.581 g, 4.5 mmol). After the reaction mixture was stirred for 16 h, the DMF was removed under reduced pressure to give a light brown residue. Cyclic peptide **2** was purified from this residue by semi-preparative HPLC with a C-18 column to give 20% yield of pure product. The HPLC run used a stepped gradient of 0–35% solvent B for 23 min, 35–100% solvent B for 8 min, and 100% solvent B for 2 min. Cyclic peptide **2** was analyzed by analytical reversed-phase HPLC using a C-18 column (5.0 μm, 300 Å, 25 cm × 4.6 mm i.d., flowrate 1 mL/min) to give a retention time of

10 min. The cyclic prodrug can also be purified by recrystallization from $CHCl_3$/hexane. ^{1}H-NMR (DMSO-d_6, δ) for *major conformer*: 1.24 (3H, d, $J = 6.2$ Hz), 1.44 (2H, br), 1.74 (2H, br), 2.77 (2H, br), 2.80 (2H, br), 3.73 (1H, d, $J = 17$ Hz), 3.83 (1H, d, $J = 17$ Hz), 4.04 (1H, dd, $J_1 = 6.3$ Hz, $J_2 = 14$ Hz), 4.12 (1H, dd, $J_1 = 5.6$ Hz, $J_2 = 14$ Hz), 4.35 (1H, br), 4.97 (1H, m, $J = 7.3$ Hz), 5.52 (1H, d, $J = 6.0$ Hz), 5.63 (1H, d, $J = 6.0$ Hz), 7.35 (2H, d, $J = 8.0$ Hz), 7.85 (2H, d, $J = 8.0$ Hz), 8.12 (1H, t, $J = 6.2$ Hz), 8.90 (1H, d, $J = 9.0$ Hz). ^{1}H-NMR (DMSO-d_6, δ) for *minor conformer*: 0.55 (2H, br), 0.87 (1H, bm), 1.22 (3H, d, $J = 6.0$ Hz), 1.35 (1H, br), 2.35 (1H, br), 2.46 (1H, br), 2.67 (1H, br), 2.74 (1H, br), 3.84 (1H, d, $J = 6.0$ Hz), 3.57 (1H, d, $J = 6.0$ Hz), 4.04 (1H, dd, $J_1 = 6.3$ Hz, $J_2 = 14$ Hz), 4.12 (1H, dd, $J_1 = 5.6$ Hz, $J_2 = 14$ Hz), 4.24 (1H, br), 4.97 (1H, m, $J = 7.3$ Hz), 5.55 (1H, d, $J = 6.0$ Hz), 5.70 (1H, d, $J = 6.0$ Hz), 7.25 (2H, d, $J = 8.0$ Hz), 7.85 (2H, d, $J = 8.0$ Hz), 7.91 (1H, t, $J = 6.2$ Hz), 8.95(1H, d, $J = 9.0$ Hz). MS (FAB) *m/z*: 420 ($M^+ + 1$). HRMS: Calcd for $C_{20}H_{25}N_3O_7$, 420.1771. Found: 420.1797. *Anal.* Calcd for $C_{20}H_{25}N_3O_7 \cdot 0.8H_2O$: C, 55.37; H, 6.18; N, 9.69%. Found: C, 55.42; H, 6.07; N, 9.62%.

(4-Piperidinyloxy)-t-butyl acetate (***22***). To a solution of compound **12** (3.53 g, 10.0 mmol) in MeOH (50 mL) was added 10% Pd/C (0.53 g) in one portion. The reaction mixture was stirred at room temperature for 4.5 h under a H_2 atmosphere using a balloon on the top of a round-bottomed flask. The reaction mixture was filtered to remove the catalyst (Pd/C); the solvent was removed under reduced pressure to give compound **22** (2.15 g) in quantitative yield as an oil. ^{1}H-NMR (DMSO-d_6, δ): 1.48 (2H, m, $J = 6.0$ Hz), 1.49 (9H, s), 1.81 (2H, m, $J = 6.0$ Hz), 2.5 (2H, br), 2.9 (2H, m, $J = 6.0$ Hz), 3.45 (1H, m, $J = 6.0$ Hz), 3.95 (2H, s). MS (FAB) *m/z*: 216 ($M^+ + 1$).

4-(N-Boc-aminomethyl)-benzoyl-Ala-OH (***23***). To a solution of compound **17** (1.257 g, 3.05 mmol) in MeOH (30 mL) was added 10% Pd/C (0.19 g) in one portion. The reaction mixture was stirred at room temperature for 4.5 h under a balloon H_2 atmosphere. The reaction mixture was filtered to remove the catalyst (Pd/C); the solvent was removed under reduced pressure to give compound **23** (1.0 g, 100%) as a colorless oil. ^{1}H-NMR (DMSO-d_6, δ): 1.37 (3H, d, $J = 7.0$ Hz), 1.42 (9H, s), 4.16 (2H, d, $J = 6.0$ Hz), 4.40 (1H, m, $J = 7.3$ Hz), 7.32 (2H, $J = 8.0$ Hz), 7.45 (1H, t, $J = 7.5$ Hz), 7.81 (2H, d, $J = 8.0$ Hz), 8.65 (1H, d, $J = 7.0$ Hz). MS (FAB) *m/z*: 323 ($M^+ + 1$).

2-[N-(Boc-aminomethyl-4-benzoyl-Ala)-4-piperidinyloxy]-t-butyl acetate (***24***). Compound **22** (0.645 g, 3.0 mmol) and compound **23** (0.97 g, 3.0 mmol) were dissolved in DMF (70 mL) and the reaction mixture was kept in an ice bath. HBTU (1.326 g, 3.5 mmol) was added to the stirred solution in one portion, followed by the addition of NMM (0.405 g, 4.0 mmol). The reaction mixture was stirred at 0 °C for 2 h and then at room temperature for 24 h. DMF was removed under reduced pressure to give an oily residue that was dissolved in EtOAc (100 mL), followed by washing with 10% aqueous citric acid (2×20 mL), H_2O (4×30 mL), 10% aqueous $NaHCO_3$ (2×20 mL), and brine (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to yield a colorless oil that was purified on a silica-gel column using EtOAc:hexane (2:1) as eluent. The pure compound **24** (0.89 g, 57%) was obtained as a white solid. ^{1}H-NMR ($CDCl_3$, δ): 1.42 (3H, d, $J = 7.0$ Hz), 1.48 (9H, s), 1.50 (9H, s), 1.75 (2H, m), 1.90 (2H,

m), 3.48 (1H, m), 3.70 (2H, m), 3.78 (2H, m), 4.02 (2H, s), 4.35 (2H, br), 4.90 (1H, br), 5.08 (1H, m, $J = 4.8$ Hz), 7.35 (2H, d, $J = 8.0$ Hz), 7.42 (1H, t, $J = 6.5$ Hz), 7.80 (2H, $J = 8.0$ Hz). MS (FAB) *m/z*: 520 ($M^+ + 1$).

TFA · 2[N-(Aminomethyl-4-benzoyl-Ala)-4-piperidinyloxy] acetic acid (***I***). TFA (5 mL) was added to a stirred solution of compound **24** (0.86 g, 1.65 mmol) in CH_2Cl_2 (8 mL). The reaction mixture was stirred at room temperature for 1.5 h. Volatile compounds in the reaction mixture were removed by rotary evaporation under vacuum. The residue was washed with Et_2O/petroleum ether (3:1) to afford a white solid and dried under vacuum to yield **1** (7.87 g, 100%). ^{1}H-NMR (DMSO-d_6, δ): 1.27 (3H, d, $J = 6.0$ Hz), 1.40 (2H, m, $J = 6.8$ Hz), 1.81 (2H, m, $J = 6.8$ Hz), 3.00 (1H, m, $J = 6.8$ Hz), 3.30 (1H, m, $J = 6.8$ Hz), 3.60 (1H, m, $J = 7.0$ Hz), 3.75 (1H, m, $J = 7.0$ Hz), 4.11 (2H, m, $J = 8.0$ Hz), 4.94 (1H, m, $J = 6.9$ Hz), 7.52 (2H, d, $J = 7.8$ Hz), 7.92 (1H, d, $J = 7.8$), 7.94 (1H, d, $J = 6.0$ Hz), 8.22 (3H, br), 8.71 (1H, d, $J = 7.5$ Hz). MS (FAB) *m/z*: 364 ($M^+ + 1$).

5. Platelet aggregation

Fresh platelet-rich plasma (PRP; 10 mL) was diluted with 10 mL Tyrode's buffer and centrifuged at 1000 rpm for 10 min. The PRP suspension was decanted and used for the next step. PRP (0.5 mL) in a siliconized cuvette was stirred at 1000 rpm and pre-warmed to 37 °C for 2 min. Fibrinogen solution (10 μg/mL, Sigma) was added to the cuvette and 1 min later adenosine diphosphate (ADP) (10 μM) was added. The change in light transmission was monitored with a Chrono-log Model 490 optical aggregometer. For the inhibition studies, a specific amount of peptide was added, prior to addition of ADP [32]. Each inhibitor was tested at five different concentrations for the determination of IC_{50}.

The effects of the incubation time of cyclic prodrug **2** with platelet-rich plasma were investigated as follows. Cyclic prodrug **2** was added to PRP and incubated at 37 °C for various times before aggregation was induced by adding ADP. The extent of platelet aggregation is determined by a change in light transmission through the PRP on the aggregometer.

6. Enzymatic stability

The cyclic prodrug (final concentration 24 μM) was incubated for 6 h with 1 mL of 90% human plasma in a temperature-controlled (37.0 ± 0.5 °C) shaking water bath (60 rpm). At various time points, aliquots (20 μL) were removed and the esterase activity was immediately quenched by adding 150 μL freshly prepared 6 N guanidinium hydrochloride in acidified HBSS (HBSS containing 0.01% (v/v) phosphoric acid). Aliquots (150 μL) of that acidic mixture at pH 3 were transferred to an Ultrafree-MC 5000 NMWL filter unit (Millipore, Bedford, MA) and centrifuged at 7500 rpm (5000*g*) for 60 min (4 °C). Aliquots (50 μL) of the filtrates were diluted with mobile phase and injected on the HPLC column.

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