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# Synthesis and comparison of physicochemical, transport, and antithrombic properties of a cyclic prodrug and the parent RGD peptidomimetic

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**Abstract**—Cyclic prodrug **1** was derived from RGD peptidomimetic **2** by linking the amino and carboxylic acid groups via an (acyloxy)alkoxy linker. The formation of a cyclic prodrug can transiently alter the physicochemical properties of the RGD peptidomimetic. Cyclic prodrug **1** was synthesized via the key intermediate **8**, and the synthesis of this key intermediate was accomplished using two different routes. Cyclic prodrug **1** has a smaller hydrodynamic radius and higher membrane interaction potential than those of the parent RGD peptidomimetic **2**. The cell membrane permeation of cyclic prodrug **1** is twice that of the parent peptidomimetic **2**. The prodrug-to-drug conversion can be carried out in isolated porcine esterase as well as human plasma. The cyclic prodrug is more stable at pH 4 than pH 7, and is very unstable at pH 10. The cyclic prodrug has antithrombic activity, suggesting that it can be converted to the RGD peptidomimetic in platelet-rich plasma (PRP). 

## 1. Introduction

Arg-Gly-Asp (RGD) peptides and peptidomimetics have been developed as antithrombic[1–3] and antitumor agents.[4,5] The biological activities of these molecules are due to their ability to bind integrin receptors on the cell surface and inhibit cell adhesion to extracellular matrix (ECM) proteins such as fibronectin (FN), fibrinogen (FG), and vitronectin (VN).[6–8] Aggrastat is one example of an RGD peptidomimetic that has been used clinically to treat thrombosis by binding to GPIIb/IIIa receptors and inhibiting platelet aggregation.[9,10] Aggrastat is delivered clinically via intravenous (IV) injection because it is not orally bioavailable. Therefore, any method to improve the oral bioavailability of RGD peptidomimetics will be beneficial for patients. Aggrastat and other RGD peptidomimetics have charged amino and carboxylic acid groups that are necessary for their biological activity. Unfortunately, these charges make the RGD peptidomimetics very hydrophilic and prevent their partitioning into cell membranes of the intestinal mucosa for oral bioavailability. Therefore, it is proposed that the membrane permeation of the RGD peptidomimetic can be improved by modifying its physicochemical properties.

The physicochemical properties of RGD peptidomimetics can be transiently altered with the formation of cyclic prodrugs (Scheme 1). Cyclic prodrugs can be synthesized by linking the amino and carboxylic acid groups using different linkers such as (acyloxy)alkoxy,[11,12] phenylpropionic acid,[13,14] modified phenylpropionic acid,[15] coumarinic acid,[16–17] and modified coumarinic acid.[20] Previously, cyclic prodrugs have been shown to have better membrane permeation than their respective parent peptides or peptidomimetics.[20–23] The improved membrane permeation is due to the increased hydrophobicity, conformation stability, and enzymatic stability.[24–27] In addition, cyclic prodrugs have lower hydrodynamic radii and hydrogen-bonding potentials than their parent compounds.[24,28,29]

In this work, we developed two methods to synthesize cyclic prodrug **1** using an (acyloxy)alkoxy linker. The first method follows the previously described method;[11,12] the second method is a more efficient way to make the (acyloxy)alkoxy cyclic prodrug. The membrane permeation of cyclic prodrug **1** was compared with that of parent RGD peptidomimetic **2**. The physicochemical properties (i.e. hydrophobicity, size)

*Keywords*: RGD peptidomimetic; (acyloxyl)alkoxy linker; cyclic prodrug; antithrombic activity; membrane permeation.

*Abbreviations:* ADP, adenosine diphosphate; Boc, *t*-butyloxycarbonyl; DIEA, diisopropylethyl amine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; FAB-MS, fast atom bombardment mass spectrometry; HBSS, Hanks balanced salt solution; HBTU, *O*-benzotriazol-1yl-*N*,*N*,*N*′,*N*′-tetramethyl uronium hexaflourophosphate; HOBT, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectra; IAM, immobilized artificial membrane; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; PBS, phosphate buffer solution; PRP, platelet-rich plasma; Tce, trichloroethanol; TFA, trifluoroacetic acid; TMS, tetramethylsilane.

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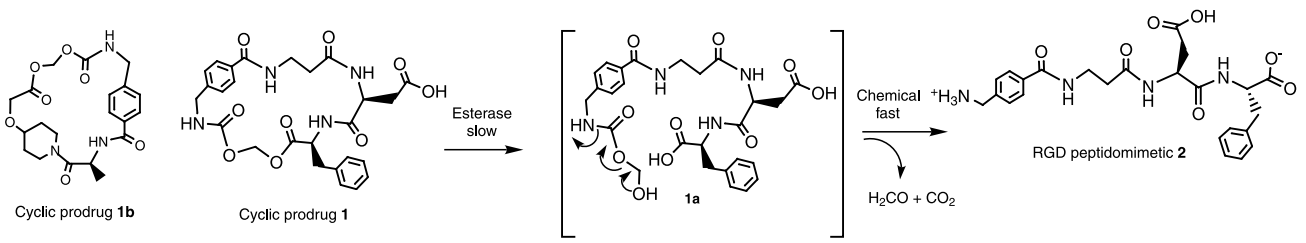



**Scheme 1.**

of both compounds were studied for their correlation to the transport properties. The antithrombic activity of cyclic prodrug **1** was compared to that of the parent drug **2**. The rate of prodrug-to-drug conversion was determined using isolated esterase and human plasma to prove that RGD peptidomimetic **2** can be released from cyclic prodrug **1**. Finally, the chemical stability of this prodrug was evaluated at pH 4, 7, and 10.

## 2. Experimental procedures

### 2.1. General methods

$^1$H NMR spectra were obtained using a Bruker DRX-400 (400 MHz) or Varian XL-300 (300 MHz) instrument. Chemical shifts are expressed in parts per million ($\delta$) relative to tetramethylsilane (TMS) or residual solvent as an internal reference. Abbreviations are reported as (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet and (br) broad. FAB-MS and HRMS were obtained using a VG Analytical ZAB double-focusing spectrometer; several matrixes were used, including nitrobenzyl alcohol (NBA) and thioglycerol/glycerol (TG/G). All starting materials were purchased from Aldrich Chemical Co., Sigma Chemical Co., Fluka Chemicals, and Bachem Bioscience Inc., and were 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 with a semi-preparative reversed-phase HPLC system using a C-18 column (12 μm, 300 Å, 25 cm×21.4 mm i.d., flow rate 10 mL/min) eluting with a gradient of solvents A (0.1% TFA/$H_2O$:5% MeCN) and B (100% MeCN). The gradient method used for the semi-preparative HPLC started from 0 to 35% of solvent B in 23 min, followed by an increase to 100% of solvent B in 10 min. Elution at 100% of solvent B was maintained for 2 min, and ended with gradient elution to 0% of solvent B in 2 min. The desired peptide was evaluated by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm×4.6 mm i.d., flow rate 1 mL/min) eluting with a gradient of solvents A and B. The total HPLC analysis run time was 18 min. The gradient started with solvent B from 0 to 50% over 12 min; then solvent B was increased to 100% in 2 min and kept constant at 100% of solvent B for 2 min. Finally, the gradient was changed back to 0% solvent B over 2 min.

### 2.2. Synthesis

**2.2.1. Synthesis of cyclic prodrug 1.** 4-*N*-Boc-aminomethylbenzoic acid (**3**), TFA·H-Asp(OBzl)-OTce (**16**), Boc-[(phenylalanyloxyl)methyl]-*p*-nitrophenyl carbonate (**17**) and Boc-PheO$^-$Cs$^+$ (**18**), were prepared according to published methods.[11,12] Due to the side reaction, compound **17** was obtained at 37% yield.

**2.2.1.1. 4-(*N*-Boc-aminomethyl)benzoyl-β-Ala-OBzl (4).** Acid **3** (2.52 g, 10 mmol), EDC (1.92 g, 10 mmol), HOBT (1.35 g, 10 mmol) and NMM (1.0 g, 10 mmol) were dissolved in 150 mL of $CH_2Cl_2$ in an ice bath. After 1 min, β-Ala-OBzl (3.57 g, 10 mmol) was added to the mixture and stirred in the ice bath for 4 h. Following this, it was stirred for 24 h at ambient temperature. The mixture was concentrated under reduced pressure. The residue was dissolved in 150 mL of EtOAc and was successively washed 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 to yield a white solid. The white solid was recrystallized from EtOAc–petroleum ether to give a pure compound **4** (3.85 g, 94%). $^1$H NMR ($CDCl_3$, $\delta$): 1.48 (9H, s), 2.72 (2H, t, $J$=5.6 Hz), 3.75 (2H, q, $J$=5.6 Hz), 4.36 (2H, s), 4.97 (1H, s), 5.17 (2H, s), 6.81 (1H, s), 7.33 (7H, m), 7.68 (2H, d, $J$=7.8 Hz). MS (FAB) $m/z$: 413 ($M^+$+1).

**2.2.1.2. 4-(*N*-Boc-aminomethyl)benzoyl-β-Ala-OH (5).** 10% Pd/C catalyst (0.38 g) was added to a solution of **4** (3.85 g, 9.4 mmol) in 50 mL of MeOH, and the reaction mixture was stirred for 24 h under $H_2$ atmosphere using a balloon. The reaction mixture was filtered by celite to remove the catalyst; the filtrate was concentrated under vacuum to yield a crude product. The crude product was recrystallized from EtOAc–hexane to give a pure white solid, compound **5** (2.85 g, 95%). $^1$H NMR (DMSO-$d_6$, $\delta$): 1.39 (9H, s), 3.46 (2H, m), 4.15 (2H, d, $J$=6.0 Hz), 7.28 (2H, d, $J$=7.9 Hz), 7.46 (1H, t, $J$=6.0 Hz), 7.77 (2H, d, $J$=7.9 Hz), 8.48 (1H, t, $J$=5.0 Hz). MS (FAB) $m/z$: 323 ($M^+$+1).

**2.2.1.3. 4-(*N*-Boc-aminomethyl)benzoyl-β-Ala-Asp-(OBzl)-OTce (6).** Compound **5** (1.61 g, 5.0 mmol) in 100 mL of $CH_2Cl_2$ was activated by adding EDC (0.96 g, 5.0 mmol), HOBT (0.68 g, 5.0 mmol), and NMM (2.02 g, 20 mmol). The mixture was stirred at 0°C for 30 min before the addition of Asp(OBz)-OTce (**16**; 2.34 g; 5.0 mmol). This reaction mixture was stirred at 0°C for 4 h and then at ambient temperature for 24 h. The solvent was then evaporated using a rotary evaporator under reduced pressure. The residue was dissolved in 200 mL of EtOAc and washed with 10% aqueous citric acid (2×20 mL), $H_2O$ (2×50 mL), saturated aqueous $NaHCO_3$ (2×20 mL), saturated aqueous NaCl (20 mL), and $H_2O$ (2×50 mL). The organic layer was dried over anhydrous $Na_2SO_4$, filtered, and concentrated to give compound **6** as a pale yellow oil in 91% yield. $^1$H NMR ($CDCl_3$, $\delta$): 1.47 (9H, s), 2.57 (2H, t,

$J$=5.2 Hz), 2.29–2.97 (1H, dd, $J$=4.3 Hz), 3.15–3.21 (1H, dd, $J$=4.7 Hz), 3.69–3.81 (2H, m), 4.35 (2H, s), 4.72 (2H, q, $J_1$=21.1 Hz, $J_2$=11.9 Hz), 4.91 (1H, s), 5.04–5.14 (3H, m), 6.70 (1H, d, $J$=8.4 Hz), 7.11 (1H, s), 7.31–7.40 (7H, d, $J$=8.1 Hz). MS (FAB) $m/z$: 658 ($M^+$+1), 602 ($M^+-C_4H_9$+1).

**2.2.1.4. TFA·(4-Aminomethyl)benzoyl-β-Ala-Asp-(OBzl)-OTce (7).** TFA (10 mL) was added to a stirred solution of compound **6** (3.0 g, 4.5 mmol) in 10 mL of $CH_2Cl_2$ at 0°C followed by stirring for 1 h at room temperature. The reaction mixture was concentrated under vacuum to give a crude product, which was triturated and washed with anhydrous $Et_2O$ to give a white solid. The white solid was isolated by decantation and dried under vacuum to give pure compound **7** (2.93 g, 96%). $^1$H NMR ($CD_3OD$, $\delta$): 2.58 (2H, t, $J$=6.6 Hz), 2.93–3.07 (2H, m), 3.65 (2H, m), 4.18 (2H, s), 4.80 (2H, dd, $J_1$=8.2 Hz, $J_2$=12.0 Hz), 5.00 (2H, m), 5.13 (2H, s), 7.35 (5H, m), 7.55 (2H, d, $J$=8.1 Hz), 7.90 (2H, d, $J$=8.1 Hz). MS (FAB) $m/z$: 558 ($M^+$+1).

**2.2.1.5. 4-Aminomethylbenzoyl-β-Ala-OBzl (12).** Compound **4** (2.60 g, 6.31 mmol) was stirred in 25 mL of 50% TFA in $CH_2Cl_2$. The mixture was concentrated under reduced pressure to give a white solid upon addition of cold ether ($Et_2O$). The solid was filtered and washed with ether. The residual ether was removed under vacuum to give compound **12** (1.70 g, 87%). $^1$H NMR ($CD_3OD$, $\delta$): 2.73 (2H, t, $J$=6.7 Hz), 3.68 (2H, t, $J$=6.7 Hz), 4.20 (2H, s), 5.16 (2H, s), 7.29–7.37 (5H, m), 7.55 (2H, d, $J$=8.2 Hz). 7.85 (2H, d, $J$=8.2 Hz). MS (FAB) $m/z$: 313 ($M^+$+1).

**2.2.1.6. 1-Chloromethyleneoxycarbonyl-4-aminomethyl-benzoyl-β-Ala-OBzl (13).** A solution of 1-chloromethyl chloroformate (0.85 g, 6.59 mmol) in dry $CH_2Cl_2$ (20 mL) was added dropwise to a mixture of **12** (1.70 g, 5.47 mmol) and TEA (0.66 g, 6.52 mmol) in ice-cold dry $CH_2Cl_2$ (50 mL). After stirring overnight at room temperature, the mixture was concentrated to give a white residue. This residue was dissolved in 150 mL of EtOAc and extracted with 5% $KHSO_4$ (2×50 mL), brine (2×50 mL), and $H_2O$ (2×50 mL); the organic layer was dried over anhydrous $MgSO_4$. After filtering the $MgSO_4$, the filtrate was concentrated and the crude product was purified on a silica gel column using EtOAc–hexane (2:1) as eluent to give compound **13** (1.70 g, 77%). $^1$H NMR ($CDCl_3$, $\delta$): 2.70 (2H, m), 3.72 (2H, m), 4.43 (2H, m), 5.16 (2H, m), 5.78 (2H, m), 6.89 (1H, s), 7.24–7.40 (6H, m), 7.64 (2H, d, $J$=8.0 Hz). MS (FAB) $m/z$: 405 ($M^+$+1).

**2.2.1.7. Boc-Phe-methyleneoxycarbonyl-4-aminomethyl-benzoyl-β-Ala-OBzl (14).** Compound **13** (1.70 g, 4.21 mmol) in 20 mL DMF was added dropwise to a stirred solution of cesium salt **18** (1.84 g, 4.64 mmol) in ice-cold DMF (30 mL). The mixture was stirred overnight and the solvent was removed under reduced pressure. This residue was dissolved in 150 mL of EtOAc and extracted with 5% $KHSO_4$ (2×50 mL), brine (2×50 mL), and $H_2O$ (2×50 mL). The organic layer was dried over anhydrous $MgSO_4$. The mixture was filtered, and the filtrate was concentrated. The crude product was purified on a silica gel column using EtOAc–hexane (2:1 to 3:1) as eluent to give compound **14** (1.91 g, 72%). $^1$H NMR ($CDCl_3$, $\delta$): 1.42 (9H, s), 2.71 (2H, t, $J$=5.8 Hz), 3.06–3.15 (2H, m), 3.74 (2H, q, $J$=5.7 Hz), 4.45 (2H, d, $J$=5.9 Hz), 4.98 (1H, d, $J$=5.8 Hz), 5.16 (2H, s), 5.51 (1H, s), 5.80 (2H, dd, $J$=5.8 Hz), 6.85 (1H, s), 7.15 (2H, d, $J$=6.8 Hz), 7.20–7.37 (9H, m), 7.68 (2H, d, $J$=8.0 Hz). MS (FAB) $m/z$: 634 ($M^+$+1), 534 ($M^+-$Boc+1).

**2.2.1.8. Boc-Phe-methyleneoxycarbonyl-4-aminomethyl-benzoyl-β-Ala-OH (15).** 10% Pd/C catalyst (0.20 g) was added to a solution of **14** (1.91 g, 3.01 mmol) in 50 mL of MeOH, and the reaction mixture was stirred for 24 h under $H_2$ atmosphere using a balloon. The reaction mixture was filtered by celite to remove the catalyst; the filtrate was concentrated under vacuum to yield a pure product, **15** (1.40 g, 85%). $^1$H NMR (DMSO-$d_6$, $\delta$): 1.32 (9H, s), 2.75–3.00 (2H, m), 3.40–3.50 (5H, m), 4.20 (1H, m), 4.26 (2H, d, $J$=6.0 Hz), 5.70 (2H, dd, $J_1$=5.9 Hz, $J_2$=6.1 Hz), 7.20–7.38 (8H, m), 7.77 (2H, d, $J$=8.0 Hz), 8.20 (1H, t, $J$= 5.8 Hz), 8.48 (1H, t, $J$=5.2 Hz). MS (FAB) $m/z$: 544 ($M^+$+1), 488 ($M^+-C_4H_9$+1), 444 ($M^+-$Boc+1).

**2.2.1.9. Boc-Phe-methyleneoxycarbonyl-4-aminomethylbenzoyl-β-Ala-Asp(OBzl)-OTce (8).** *Method 1*. A solution of compound **17** (2.02 g, 3.0 mmol) and NMM (1.21 g, 12 mmol) in 10 mL of DMF was added to a mixture of compound **7** (1.39 g, 3.0 mmol) and HOBT (0.41 g, 3.0 mmol) in 50 mL of DMF at 0°C. The mixture was stirred for 24 h at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in 100 mL of $CH_2Cl_2$. The organic solution was washed with saturated aqueous $NaHCO_3$ (2×20 mL), saturated aqueous NaCl (20 mL), and $H_2O$ (2×50 mL). The $CH_2Cl_2$ layer was dried over anhydrous $Na_2SO_4$, filtered, and evaporated to give a crude product. The crude product was purified using column chromatography (63–200 μm silica gel with EtOAc–hexane–MeOH (300:100:2)) to give compound **8** (1.88 g, 71%).

*Method 2*. EDC (0.37 g, 1.93 mmol), HOBT (0.22 g, 1.63 mmol), and NMM (0.20 g, 1.98 mmol) were added to a stirred ice-cold solution of **15** (0.72 g, 1.63 mmol) in 30 mL of $CH_2Cl_2$. After 1 min stirring, compound **16** (0.80 g, 1.71 mmol) in 10 mL of $CH_2Cl_2$ was added and the mixture was stirred overnight. The mixture was concentrated under reduced pressure, and the residue was dissolved in 100 mL of $CH_2Cl_2$. The organic solution was washed with saturated aqueous $NaHCO_3$ (2×20 mL), saturated aqueous NaCl (20 mL), and $H_2O$ (2×50 mL). The $CH_2Cl_2$ layer was dried over anhydrous $Na_2SO_4$, filtered, and evaporated to give a crude product. The crude product was purified using column chromatography (63–200 μm silica gel with EtOAc–hexane–MeOH (300:100:2)) to give compound **8** (1.09 g, 94%). $^1$H NMR ($CDCl_3$, $\delta$): 1.42 (9H, s), 2.50–2.58 (2H, m), 2.69 (2H, t, $J$=5.6 Hz), 2.90–3.20 (4H, m), 3.73 (2H, m), 4.43 (2H, m), 4.58–4.77 (5H, m), 4.07–5.13 (4H, m), 5.41 (1H, br s), 5.80 (2H, dd, $J_1$=19.0 Hz, $J_2$=5.4 Hz), 6.85 (1H, d, $J$=8.2 Hz), 7.14 (2H, $J$=6.7 Hz), 7.24–7.40 (10H, m), 7.55–7.76 (4H, m), 7.92 (2H, d, $J$=8.1 Hz). MS (FAB) $m/z$: 881 ($M^+$+1), 781 ($M^+-$Boc+1).

**2.2.1.10. Boc-Phe-methyleneoxycarbonyl-4-aminomethylbenzoyl-β-Ala-Asp(OBzl)-OH (9).** Compound **8** (1.09 g, 1.24 mmol) was dissolved in acetic acid (30 mL) followed by addition of Zn dust (1 g) over a 1 h time period. The reaction mixture was stirred for 18 h at room temperature to remove the Tce-protecting group, and filtered to remove the insoluble material. The filtrate was concentrated under vacuum to give an oily residue. The crude product was purified using column chromatography

(63–200 μm silica gel with EtOAc–hexane–MeOH (300:100:2)) to give compound **9** (0.75 g, 80%). $^1$H NMR ($CDCl_3$, $\delta$): 1.42 (9H, s), 2.65–3.13 (6H, m), 3.50–3.70 (3H, m), 4.35 (3H, m), 4.60 (1H, s), 4.80 (1H, m), 5.06 (2H, s), 5.74–6.00 (3H, m), 7.12–7.34 (13H, m), 7.67 (2H, d, $J$=8.0 Hz). MS (FAB) $m/z$: 749 ($M^+$+1), 649 ($M^+$− Boc+1).

**2.2.1.11. TFA·H-Phe-methyleneoxycarbonyl-4-aminomethylbenzoyl-β-Ala-Asp(OBzl)-OH (10).** Compound **9** was dissolved in 25 mL of 50% TFA in $CH_2Cl_2$ and stirred for 2 h. The mixture was concentrated under reduced pressure. The reaction mixture was concentrated under vacuum to give a crude product, which was triturated and washed with anhydrous $Et_2O$ to give a white solid. The white solid was isolated by decantation and dried under vacuum to give pure compound **10** in 95% yield. The peptide was analyzed using analytical reversed-phase HPLC with a C-18 column (5 μm, 300 Å, 25 cm×4.6 mm i.d., flow rate 1 mL/min) to give a retention time of 13.14 min. $^1$H NMR (DMSO-$d_6$, $\delta$): 2.70 (2H, t, $J$=7.0 Hz), 2.76 (2H, m), 3.00 (2H, m), 3.46 (2H, m), 3.87 (1H, q, $J$=6.0 Hz), 4.13 (2H, m), 4.91 (1H, m), 5.13 (2H, s), 5.96 (2H, m), 7.28 (12H, m), 7.88 (2H, d, $J$=9.0 Hz). MS (FAB) $m/z$: 649 ($M^+$+1), 633 ($M^+-NH_2$+1). HRMS: calcd for $C_{33}H_{36}N_4O_{10}$ 649.2509. Found 649.2501.

**2.2.1.12. Cyclic prodrug (1).** Compound **10** (0.129 g, 0.2 mmol) and HBTU (0.379 g, 1.0 mmol) were dissolved in DMF (300 mL) and stirred at room temperature for 0.5 h under $N_2$. DIEA (0.258 g, 2.0 mmol) in DMF (25 mL) was added dropwise over 1 h and the reaction mixture was stirred at room temperature for 8 h under $N_2$. The mixture was concentrated under vacuum to yield a light brown residue, which was purified using preparative reversed-phase HPLC with a C-18 column (12 μm, 300 Å, 25 cm×21.4 mm i.d., flow rate 10 mL/min). The desired fractions were analyzed by analytical HPLC with a C-18 column (5 μm, 300 Å, 25 cm×4.6 mm i.d., flow rate 1 mL/min) to give a retention time of 15.47 min. The desired fractions were combined, concentrated, and lyophilized to give pure compound **11** (0.033 g, 26%). MS (FAB) $m/z$: 631 ($M^+$+1). HRMS: calcd for $C_{33}H_{34}N_4O_9$ 631.2404. Found 631.2379. A mixture of compound **11** (0.033 g, 0.052 mmol) and 10% Pd/C (0.020 g) in MeOH (10 mL) was stirred for 24 h under a balloon $H_2$ atmosphere. The reaction mixture was filtered through celite to remove Pd/C, and the filtrate was concentrated to give a crude product, which was purified using preparative reversed-phase HPLC with a C-18 column (12 μm, 300 Å, 25 cm×21.4 mm i.d., flow rate 10 mL/min) to give pure cyclic prodrug **1** (0.027 g, 96%). Cyclic prodrug **1** was analyzed using analytical reversed-phase HPLC with a C-18 column (5 μm, 300 Å, 25 cm×4.6 mm i.d., flow rate 1 mL/min) to give a retention time of 11.41 min. $^1$H NMR (DMSO-$d_6$, $\delta$): 2.17 (2H, m), 2.86 (2H, m), 3.04 (2H, m), 3.46 (2H, q, $J$=5.8 Hz), 3.97 (1H, m), 4.26 (2H, m), 4.55 (1H, m), 5.46 (2H, m), 7.25 (7H, m), 7.68 (2H, d, $J$=9.0 Hz). MS (FAB) $m/z$: ($M^+$+1). HRMS: calcd for $C_{26}H_{28}N_4O_9$ 541.1935. Found 541.1957.

### 2.2.2. Synthesis of parent compound 2

**2.2.2.1. Boc-Asp(OBzl)-Phe-OBzl (19).** The procedure to make compound **19** was similar to that for compound **4**. Yield: 95%. $^1$H NMR ($CDCl_3$, $\delta$): 1.43 (9H, s), 2.67–2.73 (1H, m), 3.04 (1H, s), 3.11 (2H, d, $J$=5.7 Hz), 4.54 (1H, s), 4.86 (d, $J$=6.5 Hz), 5.09–5.17 (4H, m), 5.64 (1H, d, $J$=7.5 Hz), 6.98 (1H, d, $J$=5.9 Hz), 7.06 (2H, d, $J$=3.7 Hz), 7.24–7.36 (12H, m).

**2.2.2.2. TFA·H-Asp(OBzl)-Phe-OBzl (20).** The procedure to prepare compound **20** was similar to that for compound **7**. Yield: 98%. $^1$H NMR ($CD_3OD$, $\delta$): 2.73–3.03 (3H, m), 3.20 (1H, m), 4.20 (1H, m), 4.78 (1H, t, $J$=3.0 Hz), 5.12–5.20 (4H, m), 7.18–7.88 (15H, m).

**2.2.2.3. Boc-(4-aminomethylbenzoyl)-β-Ala-Asp(OBzl)-Phe-OBzl (21).** The procedure to prepare compound **21** was similar to that for compound **4**. Yield: 90%. $^1$H NMR ($CDCl_3$, $\delta$): 1.48 (9H, s), 2.47 (2H, m), 2.70 (1H, dd, $J$=6.6 Hz). 2.93–3.11 (3H, m), 3.70 (2H, m), 4.30 (2H, s), 4.82 (2H, t, $J$=6.2 Hz), 4.90 (1H, s), 5.06–5.17 (7H, m), 7.01 (4H, m), 7.30–7.53 (15H, m), 7.73 (2H, d, $J$=7.7 Hz), 7.86 (2H, d, $J$=7.7 Hz). MS (FAB) $m/z$: 765 ($M^+$+1), 709 ($M^+-C_4H_9$+1).

**2.2.2.4. Boc-(4-aminomethylbenzoyl)-β-Ala-Asp-Phe-OH (22).** Compound **22** was prepared using the same procedure as that to make compound **5**. Yield: 65%. $^1$H NMR (DMSO-$d_6$, $\delta$): 1.39 (9H, s), 2.35–2.43 (3H, m), 2.64 (2H, dd, $J_1$=11.6 Hz, $J_2$=5.0 Hz), 2.92–3.02 (2H, m), 3.42–3.48 (4H, m), 4.15 (2H, d, $J$=5.7 Hz), 4.37 (1H, d, $J$=5.9 Hz), 4.61 (1H, d, $J$=6.3 Hz), 7.18–7.45 (7H, m), 7.76 (2H, d, $J$=7.7 Hz), 7.98 (1H, d, $J$=7.3 Hz), 8.22 (1H, d, $J$=7.7 Hz), 8.41 (1H, s). MS (FAB) $m/z$: 585 ($M^+$+1), 529 ($M^+-C_4H_9$+1).

**2.2.2.5. TFA·(4-aminomethylbenzoyl)-β-Ala-Asp-Phe-OH (2).** The removal of the Boc-protecting group in compound **2** was accomplished using a procedure similar to that used for making compound **7**. Yield: 98%. $^1$H NMR ($CD_3OD$, $\delta$): 2.53 (2H, t, $J$=6.6 Hz), 2.62 (1H, dd, $J_1$=8.7 Hz, $J_2$=8.2 Hz), 2.81 (1H, dd, $J_1$=11.3 Hz, $J_2$=5.5 Hz), 3.05 (1H, dd, $J_1$=5.9 Hz, $J_2$=7.9 Hz), 3.20 (1H, dd, $J_1$=8.5 Hz, $J_2$=5.2 Hz), 3.60–3.68 (2H, m), 4.18 (2H, s), 4.64 (1H, q, $J_1$=2.6 Hz, $J_2$=5.3 Hz), 4.77 (1H, q, $J_1$=2.7 Hz, $J_2$=5.5 Hz), 7.18–7.30 (5H, m), 7.54 (2H, d, $J$=8.4 Hz), 7.91 (2H, d, $J$=8.4 Hz). MS (FAB) $m/z$: 485 ($M^+$+1).

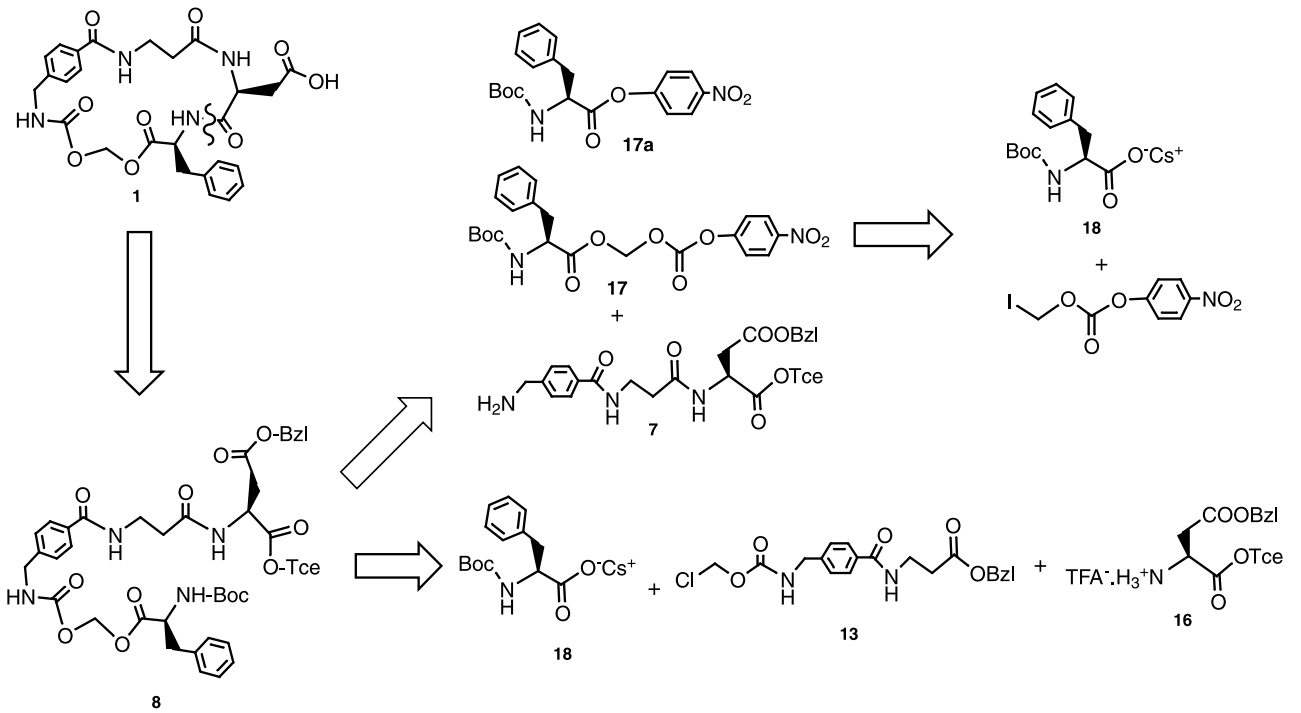
## 2.3. Platelet aggregation

The platelet aggregation assay was carried out using a previously described protocol.[3,30] The assay was done using fresh platelet-rich plasma (PRP) in Tyrodes buffer.

## 2.4. Chemical and enzymatic stabilities

The chemical stability of prodrug **1** was studied in aqueous phosphate buffer solutions (pH=4, 7, 10) at 37±0.5°C, and a constant ionic strength of $\mu$=0.15 was maintained using NaCl.[31] The purified prodrug **1** was dissolved in buffer at a concentration of 0.1 mM. At appropriate time intervals, aliquots were removed in triplicate, frozen immediately in dry ice and stored at −70°C until HPLC analysis. Prior to analysis the appropriate sample was fast-thawed.[31]

For the enzymatic stability studies, a purified porcine liver esterase (carboxylic-ester hydrolase; EC 3.1.1.1, Sigma) was dissolved in phosphate buffer (0.05 M, $\mu$=0.15, pH=7.4) to a concentration of 20 unit/mL.[22] For stability studies in plasma, human plasma was obtained from the Community Blood Center of Greater Kansas City, Kansas City, MO. Stock solutions of prodrug **1** were diluted with enzyme solution or human plasma to give the final




**Scheme 2.**

concentration of 0.1 mM.[22] The milieu was maintained at 37±0.5°C. At various time intervals 100 μL samples were removed and the residual enzyme activity quenched by adding 100 μL of a freshly prepared 6N guanidinium hydrochloride solution in Hanks' balanced salt solution containing 0.01% (v/v) phosphoric acid. This mixture was transferred to an Ultrafree-MC 5000 NMWL filter unit (Milipore, Bedford, MA) and centrifuged at 9000 rpm for 5 min (4°C). Aliquots (50 μL) of the filtrate were diluted with mobile phase and analyzed by HPLC. Recovery for the prodrug was found to be 96.4±1.1%. The effect of an esterase on the rate of degradation of cyclic prodrug **1** in various biological media was determined using an esterase inhibitor, paraoxon. The biological media was incubated with paraoxon (final concentration 1 mM) for 15 min at 37°C before adding the prodrug.

### 2.5. Physicochemical property characterization

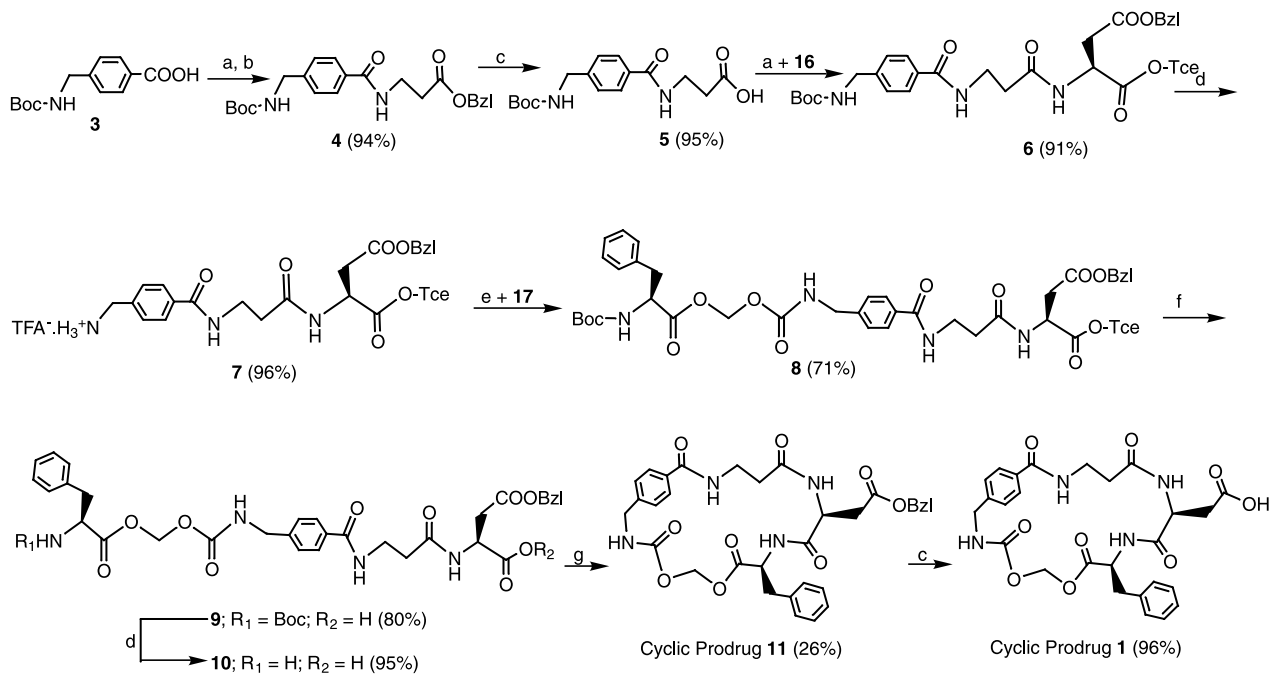
The molecular size of the cyclic prodrug and the parent peptidomimetic was determined by measuring their diffusion coefficients using NMR spectroscopy. The molecular radius was then calculated from the molecular diffusion coefficient using the Stokes–Einstein equation.[14] Lipophilicity of the cyclic prodrugs and the parent compounds was evaluated by partitioning experiments using immobilized artificial membrane (IAM) column chromatography.[32]

## 3. Results and discussion

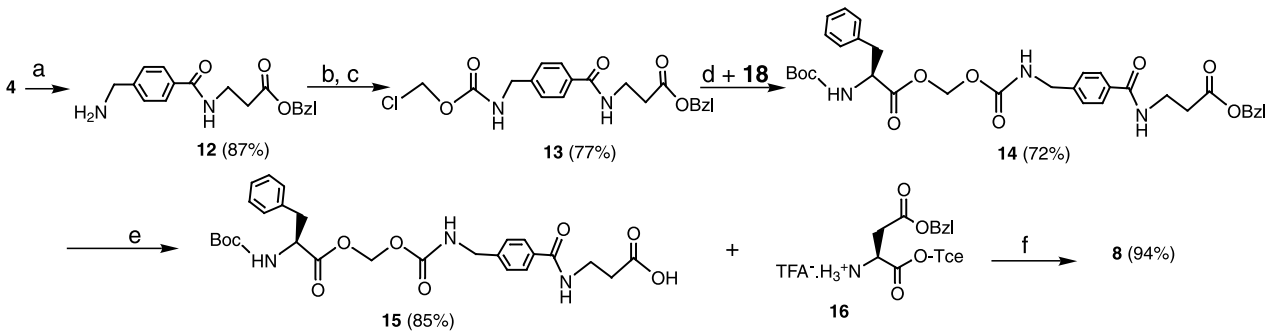
### 3.1. Synthesis of cyclic prodrug 1

Cyclic prodrug **1** was formed by linking the N-terminus of the benzylamine moiety and the C-terminus of the Phe via the (acyloxy)alkoxy linker (Scheme 1). Synthesis of cyclic prodrug **1** was accomplished by cyclization of linear intermediate **10**, which is a deprotection product of intermediate **8** (Scheme 2). We explored two ways to make intermediate **8** (Schemes 3 and 4). At first (Scheme 3), intermediate **8** was formed by conjugation of two key intermediates, **7** and **17**. Unfortunately, our previous experiment indicated that the synthesis of intermediate **17** produced side product **17a**.[11,33] It is very difficult to purify intermediate **17** from the side product intermediate **17a**, resulting in the low yield of **17**. Therefore, we investigated a second route to make intermediate **8** that is more efficient than the first. This route involves making the key intermediate **13** and reacting this intermediate with Boc-PheO$^{-}$Cs$^{+}$ (**18**) to produce the coupling product **14** (see Scheme 4) without generating any side product.

The synthetic procedure to make cyclic prodrug **1** using the first pathway is shown in Scheme 3. The synthesis was initiated by activating the carboxylic acid group of compound **3** with EDC and HOBT in the presence of NMM; the activated acid was then reacted with β-Ala-OBzl to produce dipeptide **4** in 94% yield. The benzyl ester protecting group in **4** was removed by hydrogenation with 10% Pd/C to give **5** in 95% yield. In the same way as with **3**, the carboxylic acid group in **5** was activated, followed by reaction with compound **16** to give compound **6**. Treatment of **6** with TFA in $CH_2Cl_2$ produced **7** in quantitative yield; **7** was conjugated with **17** in the presence of HOBT and NMM to give compound **8** in 71% yield after silica gel purification. The Tce- and Boc-protecting groups in **8** were removed by treatment with Zn/AcOH and TFA/$CH_2Cl_2$, respectively, to give linear peptide **10** in an overall 76% yield. The cyclization was accomplished by adding a dilute solution of **10** in DMF to a solution of HBTU in the presence of DIEA. The Bzl-protecting group in **11** was removed in the




**Scheme 3.** (a) EDC, HOBT, NMM, $CH_2Cl_2$, 0–2°C; (b) β-Ala-OBzl; (c) $H_2$, 10% Pd/C, MeOH; (d) TFA/$CH_2Cl_2$ (1:1); (e) NMM, HOBT, DMF, 0–2°C; (f) Zn, AcOH; (g) HBTU, DIEA, DMF.




**Scheme 4.** (a) TFA–$CH_2Cl_2$ (1:1); (b) 2 M NaOH–$CH_2Cl_2$; (c) $ClCH_2OCOCl$, TEA–$CH_2Cl_2$, 0°C; (d) DMF, 0–25°C; (e) $H_2$, 10% Pd/C, MeOH, (f) EDC, NMM, HOBT, $CH_2Cl_2$.

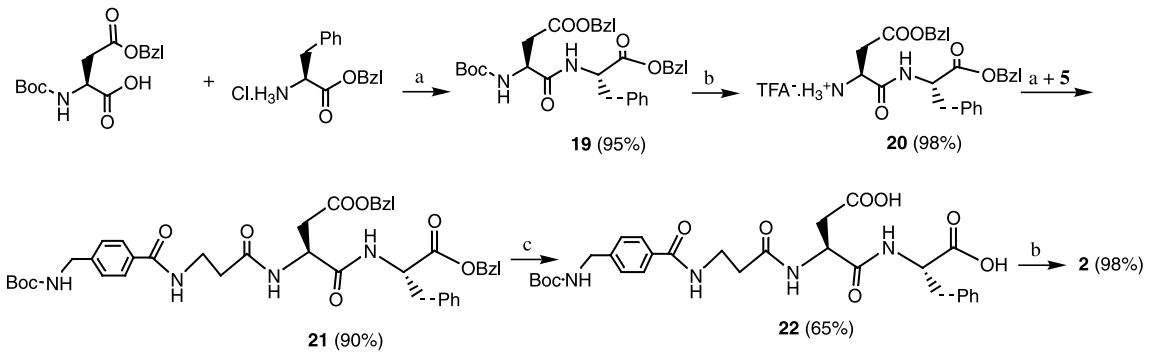
same way as with **4** to give prodrug **1** in an overall 25% yield (last two steps).

For the second pathway (Scheme 4), the TFA salt **12** was converted to free amine by using a strong base (NaOH) so that it readily reacted with 1-chloromethyl chloroformate in the presence of a weak base (TEA) to produce compound **13** in high yield. This method, however, cannot be applied to compound **7** because its Tce-protecting group could be cleaved in the presence of a strong base like NaOH. Metathesis of chloride to iodide atom in compound **13** was not necessary because the subsequent coupling reaction between **13** and cesium salt **18** proceeded smoothly to give the key intermediate **14** in 72% yield after silica gel column chromatography.[33] The Bzl-protecting group in **14** was removed in the same way as in **4** to give acid **15**, which was activated in the same way as **3** and reacted with **16** to give **8** in 94% yield.

Finally, the linear RGD peptidomimetic **2** was prepared according to Scheme 5 by typical peptide bond formation using a solution phase method. Both intermediates **19** and **21** were synthesized in the same way as **4** while the Boc-protecting group in **19** and **22** and the Bzl-protecting group in **21** were removed in the same way as with **6** and **4**, respectively, as described in Scheme 3.

### 3.2. Biological study

**3.2.1. Cell membrane permeation.** The membrane permeation properties of cyclic prodrug **1** and the parent RGD peptidomimetic **2** were evaluated using Caco-2 cell monolayers, an in vitro model of the intestinal mucosa.[34,35] The apparent membrane permeability of cyclic prodrug **1** ($0.86\pm0.11\times10^{-7}$ cm/s) was two-fold higher than that of its parent compound **2** ($0.43\pm0.14\times10^{-7}$ cm/s). This improvement was not very dramatic compared to the previously studied cyclic prodrugs.[21,22,33,36] For example, we have shown previously that a cyclic prodrug of RGD peptidomimetic (**1b**, Scheme 1) has a 13-fold increase of transport over the parent RGD peptidomimetic.[33] However, unlike cyclic prodrug **1**, this particular cyclic prodrug does not




**Scheme 5.** (a) EDC, NMM, HOBT, $CH_2Cl_2$, 0–2°C; (b) TFA/$CH_2Cl_2$ (1:1); (c) $H_2$, 10% Pd/C, MeOH.

**Table 1**. Summary of physicochemical properties of cyclic prodrug **1** and the parent drug **2**

| Compound | MW | Diffusion coefficient ($\times 10^{-6}$ cm$^2$/s) | Size (Å) | Membrane interaction potential (log $k'_{IAM}$) | |
|---|---|---|---|---|---|
| | | | | pH=4 | pH=7.4 |
| **2** | 483 | 1.69 | 5.93 | −0.230 | −0.698 |
| **1** | 539 | 1.86 | 5.38 | 0.378 | −0.193 |

have any charge.[33] On the other hand, cyclic prodrug **1** has one negative charge from the Asp residue side chain, and this negative charge may prevent the effective partitioning of the cyclic prodrug into the cell membrane. Therefore, the transcellular permeation of cyclic prodrug **1** is lower than that of the non-charged cyclic prodrug. Nonetheless, the formation of cyclic prodrug improves the transport of prodrug **1** over linear RGD peptidomimetic **2**. This suggests that there is a partial shift from a paracellular transport for linear RGD peptidomimetic **2** to an increasing contribution of transcellular transport for the cyclic prodrug **1**. Another possible explanation is that the low permeation of cyclic prodrug **1** is due to the recognition of this compound by the efflux transporter.[20,37,38]

The transport properties of cyclic prodrug **1** and its parent RGD peptidomimetic **2** are related to their physicochemical properties such as size, hydrogen bonding potential, and hydrophobicity. The size of both compounds was determined using their diffusion coefficients from the NMR studies. Prodrug **1** is only slightly smaller than its parent compound **2** (Table 1). The membrane interaction potential studies (log $k'_{IAM}$) showed that the cyclic prodrug **1** is more hydrophobic than the parent drug at both pH 4 and 7. In addition, the hydrophobicity of cyclic prodrug **1** is higher at pH 4 than at pH 7. This is due to the protonation of the carboxylic acid group of the side chain of the Asp residue. These hydrophobicity studies support the suggestion that the two-fold improvement of the cyclic prodrug transport is due to its size and hydrophobicity compared to those of the parent compound **2**.

**3.2.2. Chemical and enzymatic stability.** The precondition of prodrug strategy is that the prodrug can be converted to the parent drug in order to exert pharmacological effects (Scheme 1).[39] For this purpose, we evaluated the enzymatic and chemical stability of cyclic prodrug **1** (Table 2). The prodrug can be converted to the parent compound **2** by isolated esterase and human plasma. The rate of prodrug-to-drug conversion in both isolated esterase and human plasma was impeded by the presence of paraoxon, suggesting that the prodrug conversion was mediated by esterase. The rate of conversion in human plasma is faster than in isolated esterase, suggesting that the human plasma may have more selective esterase(s) for this conversion. The rate of enzymatic degradation was faster than the rate of chemical degradation at pH 7, suggesting that the prodrug can be converted by enzymatic degradation.

The chemical stability of cyclic prodrug **1** was also evaluated at pH 4, 7, and 10. The cyclic prodrug was more stable at acidic pH than at neutral or basic pH. As expected, the hydrolysis of the cyclic prodrug is very rapid at basic pH. The high stability of this prodrug at low pH is an advantage for oral delivery of this prodrug because it remains longer in the stomach.

**3.2.3. Antithrombic activity.** Prodrug-to-drug conversion can also be evaluated using the antithrombic activity of cyclic prodrug **1** as compared to parent compound **2** and a standard RGDF peptide. Prodrug **1** itself should not have antithrombic activity because the critical functional groups for binding to GP-IIb/IIIa receptors are masked by the linker. Thus, the biological activity of the prodrug is due to the conversion of prodrug **1** to the parent drug **2**. The

**Table 2**. The half-lives ($t_{1/2}$ in min) for cyclic prodrug **1** in biological media and aqueous buffers at various pH values (37°C, $\mu$=0.15)

| Enzymatic stability | No paraoxon ($t_{1/2}$ in min) | With paraoxon ($t_{1/2}$ in min) | |
|---|---|---|---|
| Isolated esterase | 436±13 | 877±61 | |
| Human plasma | 179±5 | 793±49 | |
| Chemical stability | pH=4 ($t_{1/2}$ in min) | pH=7 ($t_{1/2}$ in min) | pH=10 ($t_{1/2}$ in min) |
| | 4310±30 | 956±58 | 5.69±0.08 |

**Table 3**. In vitro antithrombic activity of linear peptidomimetic **2** and cyclic prodrug **1**

| Compound | $IC_{50}$±SD (μM) |
|---|---|
| RGDF | 12±0.01 |
| RGD peptidomimetic **2** | 50±4 |
| Cyclic prodrug **1** | 250±36 |

antithrombic activity of these compounds was evaluated by a platelet aggregation assay using human platelet-rich plasma (PRP) induced by adenosine diphosphate (ADP). Table 3 shows that the linear peptidomimetic **2** has an $IC_{50}$ of 50 μM, which is higher than that of the standard RGDF peptide (12 μM). Cyclic prodrug **1** has an $IC_{50}$ of 250 μM, which is five times higher than the parent peptide. In these $IC_{50}$ studies, the induction of platelet aggregation was done immediately after addition of cyclic prodrug **1**. Therefore, it is possible that the amount of drug **2** in the PRP solution is not the same as the added prodrug. This is due to the slow rate of prodrug-to-drug conversion compared to the aggregation rate of the platelets.

To prove this hypothesis, we studied the effect of prodrug incubation time on platelet aggregation inhibitory activity (Fig. 1). In this case, the prodrug was incubated in PRP at different time points prior to addition of ADP to induce the platelet aggregation. The different incubation times were used to evaluate the effect of prodrug-to-drug conversion on activity. These results show that the activity of cyclic prodrug **1** is dependent on incubation time. The inhibitory activity of the cyclic prodrug was increased from 20 to 55% when it was incubated for 10 min in PRP prior to platelet aggregation compared to no incubation prior to PRP aggregation. The activity of the prodrug plateaued when it was incubated at 10 to 20 min prior to PRP aggregation, suggesting that the prodrug can be converted to a drug that has antithrombic activity in time dependent manner.

## 4. Conclusion

We have been able to synthesize the (acyloxy)alkoxy-derived cyclic prodrug **1** to improve the synthetic methodology. The formation of cyclic prodrug **1** improves the transport properties of the RGD peptidomimetic **2** due to the increase in membrane partition potential of the cyclic prodrug compared to the parent compound. The prodrug can be converted to the parent drug by isolated esterase and human plasma. The cyclic prodrug is more stable at pH 4.0 than at pH 7.0, and is very unstable at pH 10. Finally, the prodrug can inhibit platelet aggregation, presumably because it can be converted to the parent drug in human platelet-rich plasma.

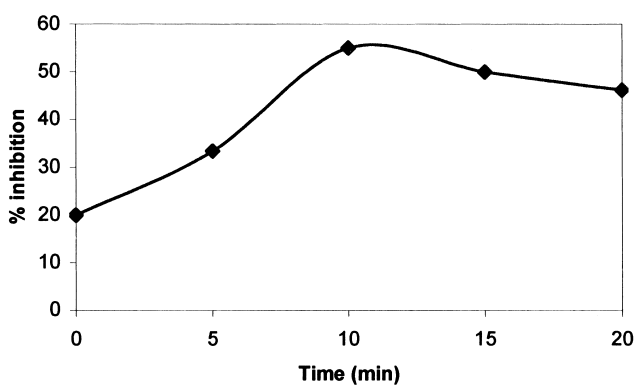



**Figure 1.** Time-dependent inhibitory activity of cyclic prodrug **1** in platelet aggregation.

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