Synthesis and Evaluation of Novel Coumarin-Based Esterase-Sensitive Cyclic Prodrugs of Peptidomimetic RGD Analogs with Improved Membrane Permeability

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Earlier, we reported the development of a coumarin-based prodrug system that could be used for the preparation of cyclic prodrugs of opioid peptides. These cyclic prodrugs exhibited excellent membrane permeability characteristics. Therefore, it was of interest to determine the effects of this prodrug strategy on the membrane permeabilities of peptidomimetics which also have low membrane permeabilities. For this study, we have chosen two RGD (Arg–Gly–Asp) peptidomimetics, which have the potentials to be developed clinically as orally active antithrombotic agents. However, the clinical development of oral dosage forms of these RGD analogs has been hindered by their low intestinal mucosal permeability. Therefore, we have synthesized the corresponding coumarin-based cyclic prodrugs of these RGD peptidomimetics, which have the two most polar functional groups, a carboxyl and an amino group, masked as an ester and an amide, respectively. These cyclic prodrugs were shown to have higher membrane interaction potentials, as estimated by their partitioning between aqueous buffer and an immobilized artificial membrane, than the corresponding RGD analogs suggesting that they should exhibit good membrane permeation characteristics. Subsequently, in a separate study these cyclic prodrugs were shown to be 5 to 6-fold more able to permeate monolayers of Caco-2 cells, an *in vitro* cell culture model of the intestinal mucosa barrier, than the corresponding RGD peptidomimetics.

Key words prodrug; membrane permeability; RGD; fibrinogen antagonist; coumarin-based cyclic prodrug; peptidomimetic

For many potent biologically active peptides, undesirable physicochemical properties (*e.g.*, charge, size, hydrophilicity, high hydrogen bonding potential) cause these molecules to have low permeabilities across intestinal mucosal cells, which ultimately result in low bioavailability when administered *via* the oral route. This low oral bioavailability often hinders the clinical development of these pharmacologically interesting molecules.[1—3] Recently, our laboratories described three linker technologies that can be used to prepare cyclic prodrugs of peptides.[4,5] These cyclic prodrugs were shown to exhibit much improved membrane permeability characteristics compared to the parent peptides.[5] The linkers used to prepare these cyclic prodrugs include a coumarin-based prodrug moiety (Chart 1),[6—8] a phenylpropionic acid-based prodrug moiety,[9,10] and an acyloxyalkoxy-based prodrug moiety.[11,12]

Like peptides, many peptidomimetics also exhibit undesirable physicochemical properties which limits their intestinal mucosal permeabilities; thus, their oral bioavailabilities. Therefore, it was of interest in this study to determine whether the coumarin-based cyclic prodrug strategy described previously for peptides could be used with peptidomimetics to increase their cell membrane permeation characteristics. With both peptides and peptidomimetics, this cyclic prodrug strategy could mask the charges (*e.g.*, *N*-terminal amino and *C*-terminal carboxyl groups) which would have a favorable effect on their abilities to interact with biological membranes. Based on our earlier results, we concluded that coumarin-based cyclic prodrugs of peptides also have unique solution structural features that include intramolecular hydrogen bonds.[7,13—15] Hydrogen bond potentials are known to affect the membrane permeability of peptides.[16—20] However, the situation with peptidomimetics is very different from peptides. Peptidomimetics lack the regularly spaced amide bonds which can form these intramolecular hydrogen bonds. Therefore, it was of interest to determine how these structural differences between peptides and peptide mimetics could affect the membrane permeability enhancing effects of these cyclic prodrug strategies.

For the studies described in this manuscript, we have chosen to evaluate the coumarin-based cyclic prodrug strategy on two RGD (Arg–Gly–Asp) peptidomimetics. The RGD peptidomimetics chosen for this study are known glycoprotein (Gp) IIb/IIIa receptor antagonists, which inhibit the binding of fibrinogen to activated platelets.[21] Because fibrinogen binding to the platelet Gp IIb/IIIa receptor mediates the platelet aggregation process, such antagonists have the potential as antithrombotic agents clinically.[22—30] One problem that has hindered the clinical development of some RGD peptidomimetics is their low oral bioavailability.[22,27,29] Although many factors, including dissolution, metabolism, absorption, and clearance could affect the oral bioavailability of a drug, the unfavorable physicochemical properties (*e.g.*, charge, polarity, hydrogen bonding potential and size) of these RGD analogs have been thought to play an important role in limiting their oral bioavailability by limiting their permeation through the intestinal mucosa.[22,31,32] In addition to *de novo* drug design approaches,[32—36] many efforts to improve the oral bioavailability of RGD analogs have also been focused on the modification of their unfavorable physicochemical properties.[32,33,37—39] Our coumarin-based cyclic prodrug strategy allows for the simultaneous masking of the two most polar functional groups of an RGD analog, the carboxyl and the amino functional groups. Consequently, the cyclic prodrugs should have significantly decreased charge and polarities, which in turn should help to increase the

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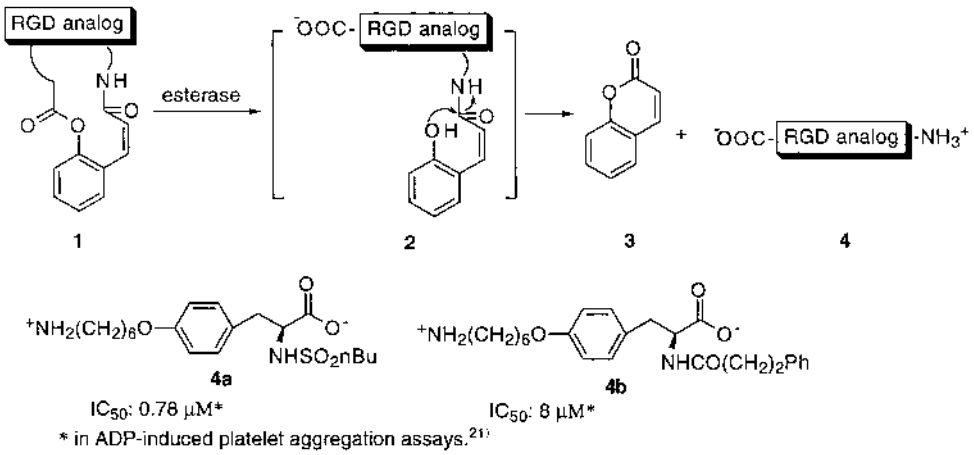

Chart 1. Esterase-Sensitive Cyclic Prodrugs of Two RGD Analogs

membrane permeabilities of these RGD analogs. We, therefore, synthesized the coumarin-based esterase-sensitive cyclic prodrugs of two RGD analogs (Chart 1). To achieve a better understanding of the factors controlling transport of peptidomimetic RGD analogs and their prodrugs, the effect of the prodrug cyclization on the physicochemical properties was quantified through the determination of their membrane interaction potentials and molecular sizes. In a separate study, the membrane permeability of these RGD analogs (**4a**, **b**) and their prodrug drugs (**1a**, **b**) was evaluated using monolayers of Caco-2 cells, a cell culture model of the intestinal mucosa.[40] The correlation of the physicochemical properties of the prodrugs (**1a**, **b**) and their improved membrane permeability was discussed.

Results and Discussion

Synthesis The synthesis started with coumarin (**3**). By following procedures published earlier,[6] the TBDMS (*tert*-butyldimethylsilyl) protected alcohol **6** was prepared through $LiAlH_4$ reduction and TBDMS protection. The two Boc-protected RGD analogs **5a**, **b** were prepared according to literature procedures.[21] Coupling of the protected RGD analogs **5a**, **b** to the free phenol hydroxyl group of the TBDMS protected alcohol **6** was accomplished by using DCC (dicyclohexylcarbodiimide) as the activating reagent in the presence of DMAP (4-dimethylaminopyridine) to give **7a**, **b**, respectively (Chart 2). Then the TBDMS protecting group of **7a**, **b** was cleaved using a mixture of HOAc, water, and THF to give the alcohols **8a**, **b**, respectively. The free allylic hydroxyl group of **8a** and **8b** was converted to a carboxyl group in a two-step oxidation to give the corresponding free acids **10a**, **b**, respectively. The oxidation of **8a**, **b** to the corresponding aldehydes **9a**, **b** was accomplished using manganese dioxide (MnO_2) in about 75—82% yields and the conversion of the aldehydes **9a**, **b** to the carboxylic acids **10a**, **b** was accomplished using hydrogen peroxide in the presence of sodium chlorite ($NaClO_2$) under weakly acidic conditions (pH 4) in 67—75% yields. Deprotection of the *N*-Boc group of **10a**, **b** was followed by cyclization in dilute solution using bis (2-oxo-3-oxazolidinyl) phosphinic chloride (Bop-Cl)[41] as the activating reagent in the presence of triethyl amine (TEA) to afford the respective cyclic prodrugs **1a**, **b** in 5—33% yields. It should be noted that the low cyclization yield (5%) for **1a** seems to be an exception since we have synthesized a number of other coumarin-based cyclic prodrugs of peptides and peptidomimetics and the cyclization yields were approximately 30—40%. For example, the cyclization reaction leading to the formation of **1b** gave a 33% yield and similar cyclizations for the preparation of cyclic prodrugs of two opioid pentapeptides also gave about 30—40% yields.[7,15] It is not readily clear to us why the cyclization yield for **1a** was so low. The major product(s) of the reaction for the preparation of **1a** seems to be oligomers as they are more polar than the starting material and stayed at the origin of the TLC [silica gel, ethyl acetate : hexanes (1 : 1)] Further treatment of the side product(s) with Bop-Cl did not give anything that would move beyond the origin on TLC under identical conditions. However, no attempt was made to fully characterize the side product(s). It is understood that in such a cyclization reaction, there is always the possible competition between the intramolecular and the intermolecular reactions. The high dilution conditions used for the preparation of **1a** was designed to favor the desirable intramolecular reaction. However, if there are other factors that disfavor the cyclization reaction, the competing intermolecular reaction could predominate leading to the formation of oligomers. Conformational effects are known to affect cyclization reactions.[42] One possible explanation is that the sulfonamide side chain of **1a** could have caused an unusual conformation that was unfavorable for the cyclization reaction. However, detailed studies of this aspect is beyond the scope of this paper.

Stability Studies The cyclic prodrugs **1a**, **b** were designed to undergo esterase-catalyzed release of the RGD peptidomimetics (Chart 1). Therefore, we studied the stability of these cyclic prodrugs in the presence of porcine liver esterase (PLE) (Sigma, EC 3.1.1.1) in a phosphate buffer (pH 7.4, 0.05 M, 37 °C) following procedures reported before.[6,7] Specifically, for the esterase stability studies the UV absorbency change at 276 nm was monitored during the reaction and used for the calculation of the pseudo first-order rate constants. The experiments were conducted in triplicates. As can be seen from Table 1, PLE was able to catalyze the facile release of the RGD peptidomimetics **4a** and **4b** from their corresponding cyclic prodrug **1a** and **1b**, respectively. The hydrolysis rates were about the same for both prodrugs ($t_{1/2}$

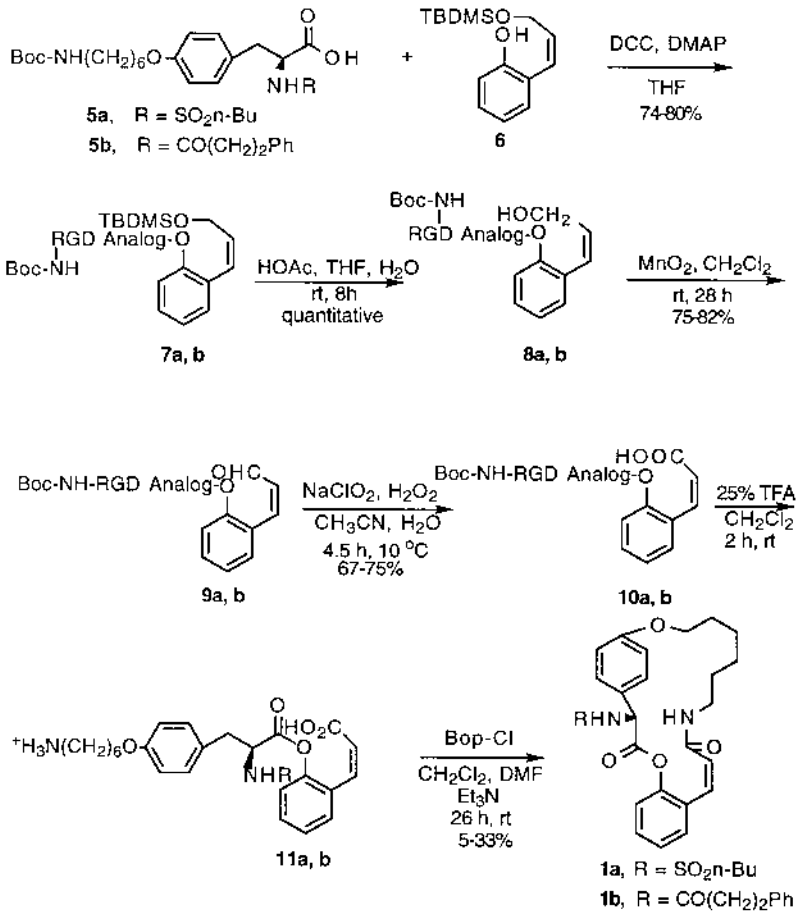

Chart 2. Synthesis of Coumarin-Based Prodrugs of RGD Analogs

Table 1. Stability of the Cyclic Prodrugs **1a**, **b** in the Presence of PLE

	Esterase activity (U/ml)	$t_{1/2}$ (min)	k_{obs} ($\times 10^4$) (s^{-1})
1a	1.02	84± 3	1.37±0.04
1b	1.02	95±10	1.23±0.14

Table 2. Physicochemical Properties of the RGD Analogs and Their Cyclic Prodrugs

	r (Å)	M.W.	$\log k_w$ [a)]
Prodrug **1a**	4.96	529	3.60
RGD analog **4a**	4.75	401	1.22
Prodrug **1b**	4.55	541	3.71
RGD analog **4b**	4.37	413	1.23

a) Capacity factors were determined from the partitioning of the solute between phosphate buffer and an immobilized artificial membrane (see Experimental section for details).[47,48]

84—95 min). In contrast, minimal hydrolysis (<1%) of the prodrugs were observed within the first 10 h of reaction in the absence of PLE. Using HPLC, the conversion of the cyclic prodrugs **1a** and **1b** to the RGD peptidomimetics **4a** and **4b**, respectively, as well as coumarin, were confirmed.

Physicochemical Characteristic Determinations Because the coumarin-based cyclic prodrug strategy was designed to modify the physicochemical properties of these RGD analogs, it was important to quantify these changes. In general, permeation of hydrophilic peptidomimetics across cell membranes is restricted to the paracellular pathway, which consists of aqueous pores created by the cellular tight junctions.[43] The average size of these aqueous pores in the small intestine is approximately 7—9 Å.[43] These aqueous pores restrict permeation of compounds based on the size and charge of the molecule.[43,44] In contrast to hydrophilic compounds, hydrophobic compounds that lack charge and exhibit a low hydrogen bonding potential, can traverse the intestinal mucosa by passive diffusion *via* the transcellular pathway.[16,18,45] Therefore, optimal permeation of these RGD peptidomimetics through the biological membranes may be achieved by shifting the mechanism of transport from the paracellular to the transcellular route.

To quantify the changes in physicochemical properties and to correlate these changes with their membrane permeability, we determined the molecular sizes and interaction potentials of these cyclic prodrugs, as well as their corresponding RGD peptidomimetics, with immobilized lipophilic membrane.

Molecular sizes of these RGD analogs **4a**, **b** and their respective cyclic prodrugs **1a**, **b** were determined by measuring their diffusion coefficients using NMR spectroscopy. The molecular radii were then calculated from these diffusion coefficients according to the Stokes–Einstein equation[46] and the values are given in Table 2. Overall, the molecular radii of these RGD analogs and their cyclic prodrugs range from

4.37 to 4.96 Å. These minor changes in molecular sizes are not expected to have any significant effect on their permeation across a cell monolayer *via* the paracellular route, considering the typical size of the tight junction pore being approximately 7—9 Å.[43]

The abilities of the RGD analogs **4a**, **b** and their prodrugs **1a**, **b** to interact with membranes, the membrane interaction potentials, were estimated by determining their partitioning between 10 mM phosphate buffer, pH 7.4/acetonitrile at various concentrations and an immobilized artificial membrane as described by El Tayar and colleagues.[47,48] The results given in Table 2 show that the cyclic prodrugs **1a**, **b** have much improved membrane interaction potentials compared with the RGD analogs **4a**, **b** themselves. For example, the membrane interaction potentials for prodrugs **1a**, **b** were determined to be 3.60 and 3.71, respectively. Whereas, the membrane interaction potentials of the corresponding linear RGD analogs **4a**, **b** were determined to be 1.22 and 1.23, respectively. These data suggest that these cyclic prodrugs **1a**, **b** may be more able to transverse a cell monolayer *via* the transcellular route and, therefore, allow the cyclic prodrugs to permeate the cell monolayer at a higher rate than the linear RGD analogs **4a**, **b**.

In a separate study, we have measured the intrinsic membrane permeabilities of these RGD analogs **4a**, **b** and their prodrugs **1a**, **b** using monolayers of Caco-2 cells, an *in vitro* cell culture model of the intestinal mucosa.[40] It should be noted that monolayers of Caco-2 cells have been widely used in drug discovery research as a model to screen for the intestinal permeability of potential drug candidates.[49,50] The apparent membrane permeability of RGD analogs **4a** and **4b** were determined to be $3.94\pm0.05\times10^{-7}$ and $3.88\pm0.10\times10^{-7}$ cm/s, respectively.[40] The coumarin-based prodrugs **1a** and **1b** exhibited apparent membrane permeabilities that were approximately 6- ($2.42\pm0.29\times10^{-6}$ cm/s) and 5-fold ($1.90\pm0.21\times10^{-6}$ cm/s) higher than their corresponding RGD analogs, respectively. These data indicate that the coumarin-based prodrug strategy can indeed be used to improve the membrane permeabilities of such RGD analogs. However, the magnitude of the improvement is less than what we observed with a cyclic prodrug of a metabolically stable opioid peptides (DADLE) which was 31 times more permeable than the peptide itself.[14] One possible explanation for this difference is that intramolecular hydrogen bond formation in the cyclic prodrug of DADLE could help to further improve their membrane permeabilities.[13] Whereas, similar intramolecular hydrogen bonds cannot be formed with the cyclic prodrugs **1a**, **b** of these RGD peptidomimetics because of the lack of the regularly spaced amide bonds.

Conclusion

In conclusion, we have described the evaluation of the coumarin-based prodrug strategy through the synthesis and evaluation of two coumarin-based cyclic prodrugs of peptidomimetic RGD analogs by linking the *N*- and *C*-terminal ends. These prodrugs have enhanced membrane interaction potentials and, therefore, higher membrane permeabilities. Such a strategy also has the potential to be applied for the preparation of cyclic prodrugs of other peptidomimetics for enhanced membrane permeability.

Experimental

General Methods All ^{1}H-NMR spectra were recorded on a Varian 300 MHz with tetramethylsilane (TMS) as the internal standard. Column chromatography was performed using silica gel (200—400 mesh size) from Aldrich. Elemental analyses were performed by Midwest Microlab, Indianapolis, Indiana and Atlantic Microlab Inc., Norcross, GA. Mass spectral analyses were conducted by North Carolina State University Mass Spectrum Laboratory. Commercially available starting materials and reagents were purchased from Aldrich. Tetrahydrofuran (THF) was distilled from Na and benzophenone. Dichloromethane (CH_2Cl_2) was distilled from CaH_2. A Shimadzu 1601 UV-visible spectrophotometer was used for the esterase kinetics study. All pH values were determined with an Accumet 1003 Handhold pH/mV/Ion Meter (Fisher Scientific).

2-[(*Z*)-3-{[1-*tert*-Butyl)-1,1-dimethylsilyl]oxy}-1-propenyl]phenyl Boc-RGD Ester 7a To a solution of **5a** (1.08 g, 2.16 mmol) in 10 ml of dry THF at 0 °C under nitrogen atmosphere with stirring was added DCC (534 mg, 2.59 mmol). After about 3 min, alcohol **6** (570 mg, 2.16 mmol) and DMAP (316 mg, 2.59 mmol) were added. The resulting mixture was stirred at 0 °C for 2 h, then room temperature (RT) for 7 h. The white precipitate was filtered off. Then solvent was evaporated to yield an oil. The oil was dissolved in 150 ml of ethyl acetate, which was then washed with 5% $NaHCO_3$ (2×50 ml), 10% citric acid (30 ml) and brine (50 ml), and dried ($MgSO_4$). Solvent evaporation gave a slightly yellow oily product, which was purified on a silica gel column (30 g, eluent: EtOAc–hexanes: from 1 : 5 to 1 : 4) to afford an oily product (1.19 g, 74%). ^{1}H-NMR ($CDCl_3$) δ: 7.23—7.31 (m, 3H), 7.19 (1H, d, J=8.4 Hz), 7.02 (1H, d, J=7.7 Hz), 6.87 (2H, d, J=8.4 Hz), 6.32 (1H, d, J=11.7 Hz), 5.89 (1H, m), 4.82 (1H, d, J=9.3 Hz), 4.52 (1H, m), 4.28 (2H, d, J=6.3 Hz), 3.93 (2H, d, J=6.3 Hz), 3.27 (1H, dd, J_1=14.0 Hz, J_2=5.4 Hz), 3.03—3.14 (3H, m), 2.79 (2H, t, J=8.0 Hz), 1.74—1.80 (2H, m), 1.58—1.66 (2H, m), 1.44 (9H, s), 1.38—1.55 (6H, m), 1.30 (2H, q, J=7.5 Hz), 0.89 (9H, s), 0.86 (3H, t, J=7.5 Hz), 0.04 (6H, s). MS (FAB) *m*/*z*: 647.5 (M+1). *Anal*. Calcd for $C_{39}H_{62}N_2O_8SSi$: C, 62.70; H, 8.36; N, 3.75. Found: C, 62.49; H, 8.36; N, 3.69.

2-[(*Z*)-3-Hydroxy-1-propenyl]phenyl Boc-RGD Ester 8a Compound **7a** (1100 mg) was treated with 25 ml of a mixture of THF–HOAc–H_2O (1 : 3 : 1) at RT for 8 h. The solvents were removed *in vacuo* to afford an oil. The residue was dissolved in 150 ml of EtOAc, then washed with 5% sodium bicarbonate (2×35 ml) and brine (45 ml), and dried ($MgSO_4$). Solvent evaporation gave an oil (995 mg, 100%). ^{1}H-NMR ($CDCl_3$) δ: 7.24 (3H, m), 7.19 (2H, d, J=8.4 Hz), 7.02 (1H, d, J=7.7 Hz), 6.86 (2H, d, J=8.4 Hz), 6.32 (1H, d, J=11.7 Hz), 5.89 (1H, m), 5.14 (1H, d, J=9.3 Hz), 4.52 (1H, m), 4.29 (2H, d, J=6.3 Hz), 3.93 (2H, t, J=6.3 Hz), 3.25 (1H, dd, J_1=14.0 Hz, J_2=7.7 Hz), 3.12 (2H, br d, J=6.2 Hz), 3.05 (1H, dd, J_1=14.0 Hz, J_2=8.0 Hz), 2.79 (2H, t, J=7.9 Hz), 1.78 (2H, m), 1.45 (9H, s), 1.29—1.63 (10H, m), 1.27 (2H, q, J=7.2 Hz), 0.86 (3H, t, J=7.2 Hz). MS (FAB) *m*/*z*: 633.4 (M+1). *Anal*. Calcd for $C_{33}H_{48}N_2O_8S$: C, 62.63; H, 7.65; N, 4.43. Found: C, 62.64; H, 7.69; N, 4.35.

2-[(*Z*)-3-Oxo-1-propenyl]phenyl Boc-RGD Ester 9a To a solution of 895 mg (1.42 mmol) of **8a** in 22 ml of methylene chloride was added in one portion 85% activated MnO_2 (290 mg, 2.84 mmol). The reaction solution was kept stirring and more MnO_2 was added at various intervals (2, 5, 8, 21, 27, 30 h) in one 290 mg-portion during a period of 34 h. The reaction mixture was filtered through Celite and washed with methylene chloride. Solvent evaporation gave a yellow oil (658 mg, 75%). The crude product was used for the next step reaction without purification. ^{1}H-NMR ($CDCl_3$) δ: 9.81 (1H, d, J=8.0 Hz), 7.29—7.49 (4H, m), 7.17 (2H, d, J=8.4 Hz), 7.12 (1H, d, J=8.3 Hz), 6.87 (2H, d, J=8.4 Hz), 6.18 (1H, dd, J_1=11.4 Hz, J_2=8.0 Hz), 4.74 (1H, d, J=9.3 Hz), 4.49 (1H, m), 3.93 (2H, t, J=6.3 Hz), 3.22 (1H, dd, J_1=14.0 Hz, J_2=5.3 Hz), 3.13 (2H, br d, J=6.0 Hz), 3.02 (1H, dd, J_1=14.0 Hz, J_2=8.0 Hz), 2.79 (2H, t, J=7.8 Hz), 1.78 (2H, m), 1.44 (9H, s), 1.35—1.58 (8H, m), 1.29 (2H, q, J=7.3 Hz), 0.86 (3H, t, J=7.3 Hz). MS (FAB) *m*/*z*: 631.5 (M+1). *Anal*. Calcd for $C_{33}H_{46}N_2O_8S$: C, 62.83; H, 7.35; N, 4.44. Found: C, 62.82; H, 7.34; N, 4.51.

Boc-RGD-protected Coumarinic Acid 10a A solution of 80% $NaClO_2$ (353 mg, 2.08 mmol) in 2.9 ml of water was added dropwise within a 1.5 h period to a stirred solution of **9a** (658 mg, 1.04 mmol) in a mixture of CH_3CN (1.7 ml), NaH_2PO_4 (67 mg, 0.54 mmol) in 0.8 ml of water and 0.23 ml of H_2O_2 (30%) at 10 °C. Oxygen evolution was observed during the reaction. The solution was stirred for an additional 4.5 h at 10 °C. Sodium sulfite (550 mg) was added and the solution was stirred for 20 min. Then the solution was acidified with 1 N HCl to pH 1—2. The mixture was extracted with EtOAc (2×80 ml). The combined organic layers was washed with brine (2×25 ml) and dried ($MgSO_4$). The solvent was evaporated and the residue was purified on a chromatotron (2 mm silica gel plate, eluent: EtOAc–hexa-

nes 2 : 3) to give a white foam (502 mg, 75%). ^{1}H-NMR ($CDCl_3$) δ: 7.50 (1H, d, J=7.3 Hz), 7.20—7.37 (2H, m), 7.16 (2H, d, J=8.5 Hz), 7.06 (1H, d, J=7.9 Hz), 6.84 (2H, d, J=8.5 Hz), 6.83 (1H, d, J=12.4 Hz), 6.00 (1H, d, J=12.4 Hz), 5.24 (1H, d, J=9.3 Hz), 4.57 (1H, br s), 4.44 (1H, dd, J_1= 14.2 Hz, J_2=8.6 Hz), 3.92 (2H, t, J=6.4 Hz), 2.96—3.23 (4H, m), 2.78 (2H, t, J=7.6 Hz), 1.77 (2H, m), 1.44 (9H, s), 1.37—1.58 (8H, m), 1.26 (2H, m), 0.83 (3H, t, J=7.2 Hz). MS (FAB) m/z: 647.4 (M+1). *Anal*. Calcd for $C_{33}H_{46}N_2O_9S$: C, 61.28; H, 7.17; N, 4.33. Found: C, 61.10; H, 7.24; N, 4.32.

Cyclic Prodrug 1a Acid **10a** (502 mg, 0.78 mmol) was treated with 25% TFA in methylene chloride (16 ml) at RT for 2 h under N_2. Then solvent was removed and crude ^{1}H-NMR (CD_3OD) was taken to monitor the completion of the reaction. The crude product was used for the next step reaction without purification. To a solution of the above-mentioned product in 610 ml of methylene chloride and 8 ml of DMF were added 1426 mg of Bop-Cl and 1.1 ml of TEA. The resulting mixture was stirred at RT for 26 h. Then solvent was removed and the residue was dissolved in 150 ml of EtOAc, which was washed with water (25 ml), 5% citric acid (25 ml), 5% sodium bicarbonate (25 ml), and brine (25 ml) and dried ($MgSO_4$). Solvent evaporation gave a residue, which was purified on a 2 mm chromatotron silica gel plate eluting with EtOAc and hexanes (1 : 1) to give a white solid (22 mg, 5%). ^{1}H-NMR ($CDCl_3$) δ: 7.18—7.34 (5H, m), 7.07 (1H, d, J=8.0 Hz), 6.84 (2H, d, J=8.4 Hz), 6.63 (1H, d, J=12.1 Hz), 6.05 (1H, d, J=12.1 Hz), 5.44 (1H, br s), 5.19 (1H, d, J=9.5 Hz), 4.56 (1H, m), 4.12 (2H, t, J=5.4 Hz), 3.38 (1H, dd, J_1=13.7 Hz, J_2=4.4 Hz), 3.02—3.15 (3H, m), 2.94 (2H, m), 1.86 (2H, m), 1.44—1.55 (6H, m), 1.24—1.28 (4H, m), 0.97 (3H, t, J=7.3 Hz). HRMS Calcd for $C_{28}H_{36}N_2O_6S$ 529.2372. Found 529.2366.

2-[(*Z*)-3-{[1-*tert*-Butyl)-1,1-dimethylsilyl]oxy}-1-propenyl]phenyl Boc-RGD Ester 7b To a solution of **5b** (830 mg, 1.62 mmol) in 10 ml of dry methylene chloride and 5 ml of dry THF at 0 °C under nitrogen atmosphere with stirring was added DCC (400 mg, 1.94 mmol). After 3 min, alcohol **6** (428 mg, 1.62 mmol) and DMAP (237 mg, 1.94 mmol) were added. The resulting mixture was stirred at 0 °C for 2.5 h, then RT for 4 h. The white precipitate was filtered off. Then solvent was evaporated to yield an oil. The oil was dissolved in 130 ml of ethyl acetate, which was then washed with 5% $NaHCO_3$ (2×30 ml), 10% citric acid (20 ml), and brine (20 ml), and dried ($MgSO_4$). Solvent evaporation gave a slightly yellow oil, which was purified on a silica gel column (30 g, eluent: EtOAc–hexanes 1 : 4) to afford an oil (980 mg, 80%). ^{1}H-NMR ($CDCl_3$) δ: 7.18—7.30 (5H, m), 6.99 (1H, d, J=7.8 Hz), 6.98 (2H, d, J=8.4 Hz), 6.79 (2H, d, J=8.4 Hz), 6.29 (1H, d, J= 11.6 Hz), 5.86 (2H, m), 5.06 (1H, dd, J_1=13.4 Hz, J_2=5.9 Hz), 4.50 (1H, br s), 4.29 (2H, d, J=6.2 Hz), 3.92 (2H, t, J=6.4 Hz), 3.11—3.14 (4H, m), 2.96 (2H, m), 2.52 (2H, m), 1.76 (2H, m), 1.44 (9H, s), 1.24—1.51 (6H, m), 0.88 (9H, s), 0.03 (6H, s). MS (FAB) m/z: 759.5 (M+1). *Anal*. Calcd for $C_{34}H_{62}N_2O_7SSi$: C, 69.62; H, 8.23; N, 3.69. Found: C, 69.34; H, 8.20; N, 3.82.

2-[(*Z*)-3-Hydroxy-1-propenyl]phenyl Boc-RGD Ester 8b Compound **7b** (900 mg, 1.19 mmol) was treated with a mixture of THF–HOAc–H_2O (1 : 3 : 1, 20 ml) at RT for 7 h. Then solvents were removed *in vacuo* to afford an oil. The residue was dissolved in 150 ml of EtOAc, which was then washed with 5% sodium bicarbonate (2×30 ml), brine (40 ml) and dried ($MgSO_4$). Solvent evaporation gave an oil (736 mg, 96%). ^{1}H-NMR ($CDCl_3$) δ: 7.16—7.31 (5H, m), 7.09 (2H, d, J=8.4 Hz), 6.97 (1H, d, J=7.1 Hz), 6.80 (2H, d, J=8.4 Hz), 6.32 (1H, d, J=11.5 Hz), 6.32 (1H, br s), 5.87 (2H, m), 5.04 (1H, dd, J_1=13.7 Hz, J_2= 6.1 Hz), 4.52 (1H, br s), 4.17 (2H, d, J= 6.7 Hz), 3.93 (2H, t, J=6.3 Hz), 3.11 (4H, m), 2.95 (2H, m), 2.51 (2H, m), 1.78 (2H, m), 1.44 9H, s), 1.35—1.65 (6H, m). MS (FAB) m/z: 645.4 (M+1). *Anal*. Calcd for $C_{33}H_{48}N_2O_7$: C, 70.78; H, 7.50; N, 4.34. Found: C, 70.89; H, 7.63; N, 4.44.

2-[(*Z*)-3-Oxo-1-propenyl]phenyl Boc-RGD Ester 9b To a solution of alcohol **8b** (730 mg, 1.13 mmol) in 15 ml of methylene chloride was added in one portion 85% activated MnO_2 (232 mg, 2.26 mmol). The reaction solution was kept stirring and more MnO_2 was added at various intervals (2, 5, 8, 18, 24, 27 h) in one 232 mg-portion during a period of 31 h. The reaction mixture was filtered through a Celite pad and washed with methylene chloride. Solvent evaporation gave a yellow oil (600 mg, 82%). The crude product was used for the next step reaction without purification. ^{1}H-NMR ($CDCl_3$) δ: 9.78 1H, (d, J=8.1 Hz), 7.19—7.48 (9H, m), 7.07 (1H, d, J=8.1 Hz), 6.98 (2H, d, J=8.4 Hz), 6.81 (2H, d, J=8.4 Hz), 6.13 (1H, dd, J_1=11.5 Hz, J_2=8.1 Hz), 5.76 (1H, d, J=7.1 Hz), 4.98 (1H, dd, J_1=13.4 Hz, J_2=6.6 Hz), 3.92 (2H, t, J=6.3 Hz), 3.08—3.13 (4H, m), 2.96 (2H, m), 2.52 (2H, m), 1.78 (2H, m), 1.44 (9H, s), 1.38—1.51 (6H, m). MS (FAB) m/z: 643.4 (M+1). *Anal*. Calcd for $C_{33}H_{46}N_2O_7$: C, 61.28; H, 7.17; N, 4.33. Found: C, 61.10; H, 7.24; N, 4.32.

Boc-RGD-protected Coumarinic Acid 10b A solution of 80% $NaClO_2$ (288 mg, 2.53 mmol) in 2.6 ml of water was added dropwise within a 2.5 h period to a stirred solution of **9b** (580 mg, 0.90 mmol) in a mixture of CH_3CN (1.5 ml), NaH_2PO_4 (60 mg, 0.50 mmol) in 0.7 ml of water and 0.21 ml of H_2O_2 (30%) at 10 °C. Oxygen evolution was observed during the addition. The solution was stirred for an additional 3 h at 10 °C. Sodium sulfite (500 mg) was then added and the solution was stirred for 20 min. The solution was then acidified with 1 N HCl to pH 1—2. The mixture was extracted with EtOAc (2×70 ml). The combined organic layers was washed with brine (2×30 ml) and dried ($MgSO_4$). The residue after solvent evaporation was purified on a silica gel column (18 g, eluent: EtOAc–hexanes 1 : 2) to give 378 mg (67%) of the acid **10b**. ^{1}H-NMR ($CDCl_3$) δ: 7.51 (1H, d, J= 7.5 Hz), 7.15—7.29 (7H, m), 6.97 (3H, d, J=8.4 Hz), 6.78 (2H, d, J=8.4 Hz), 6.73 (1H, d, J=12.4 Hz), 6.07 (1H, br s), 5.95 (1H, d, J=12.4 Hz), 5.01 (1H, dd, J_1=13.5 Hz, J_2=6.3 Hz), 4.60 (1H, br s), 3.91 (2H, t, J=6.3 Hz), 3.07 (4H, m), 2.90 (2H, m), 2.50 (2H, m), 1.73—1.78 (2H, m), 1.44 (9H, s), 1.35—1.48 (6H, m). MS (FAB) m/z: 659.4 (M+1). *Anal*. Calcd for $C_{33}H_{46}N_2O_8$: C, 69.28; H, 7.04; N, 4.25. Found: C, 68.93; H, 7.10; N, 4.25.

Cyclic Prodrug 1b The acid **10b** (378 mg, 0.574 mmol) was treated with 25% TFA in methylene chloride (15 ml) at RT with stirring for 2 h under N_2 atmosphere. The solvents were then removed and crude ^{1}H-NMR (CD_3OD) was taken to monitor the completion of the reaction. The crude product **11b** was used for the next step reaction without purification. To a solution of **11b** in 800 ml of methylene chloride and 8 ml of DMF were added 1023 mg (4.02 mmol) of Bop-Cl and 0.80 ml (5.74 mmol) of TEA. The resulting mixture was stirred at RT for 38 h. The residue after solvent evaporation was dissolved in 150 ml of EtOAc, which was then washed with water (30 ml), 5% citric acid (30 ml) and brine (25 ml), and dried ($MgSO_4$). Solvent evaporation gave a residue, which was purified on a silica gel column (18 g, eluent: EtOAc–hexanes 2 : 1) to afford the cyclic prodrug **1b** as a white solid (101 mg, 33%). ^{1}H-NMR ($CDCl_3$) δ: 7.12—7.32 (10H, m), 7.03 (1H, d, J=7.7 Hz), 6.83 (2H, d, J=8.4 Hz), 6.52 (1H, d, J=12.1 Hz), 5.93 (1H, d, J=12.1 Hz), 4.72 (1H, dd, J_1=10.8 Hz, J_2=5.0 Hz), 4.06 (2H, m), 3.20 (1H, dd, J_1=13.5 Hz, J_2=5.0 Hz), 3.05—3.11 (1H, m), 2.85—3.00 (2H, m), 2.64 (2H, t, J=7.6 Hz), 1.56 (2H, m), 1.30 (2H, m), 1.15 (2H, m), 0.96 (2H, m). HRMS Calcd for $C_{33}H_{36}N_2O_5$: 541.2702. Found 541.2683.

Purified Esterase Kinetics Purified PLE (carboxylic-esterase hydrolase; EC 3.1.1.1; E-2884) was obtained from Sigma as a suspension in a 3.2 M $(NH_4)_2SO_4$ solution (pH 8). Then 1.5 μl of this suspension (containing 6800 units of enzyme per ml) was diluted with 9.7 μl of phosphate buffer (0.05 M, pH 7.4) and 0.2 ml dimethylsulfoxide (DMSO). One hundred microliters of the 0.01 M stock solution of the cyclic prodrugs **1a** or **1b** in DMSO was then combined with the above-mentioned PLE/buffer solution. The mixture was shaken for 30 s, then kept in a water bath at 37±0.5 °C. Aliquots (475 μl) were taken from the reaction mixture at various times. The absorbency (abs) of the samples at 276 nm was determined using a UV spectrophotometer and used to calculate the pseudo first-order rate constants. The endpoints were obtained at about seven half-lives, at which point the reaction was over 99% complete. Then $\ln(A_\infty - A_t)$ for four half-lives was plotted *vs*. time and pseudo first-order rate constants were calculated based on the slope of the linear curve.

Chemical Stability Studies The chemical stability studies were carried out under identical conditions as for the esterase stability studies except in the absence of porcine liver esterase. The area of the coumarin peak in the HPLC chromatogram was used to calculate the percentage of the reaction. A standard curve was used for the quantitation of coumarin. A Shimadzu HPLC system consisting of a SCL-10A system controller, two LC-10AS pumps, an SPD-10AV UV-VIS detector, and an SIL-10A auto injector was used for the kinetic studies. A reversed-phase C-18 column (YMC, L=25 cm, i.d.=4.6 mm, particle size=5 μm) was used. The mobile phase consisted of HPLC grade acetonitrile (55%) (Fisher Scientific) and 0.1% TFA in distilled water (45%) filtered through a Millipore Milli-Q water purification system. A detection wavelength of 285 nm was used.

The Membrane Interaction Potential Determination The ability of the RGD analogs **4a**, **b** and their prodrugs **1a**, **b** to interact with membranes, the membrane interaction potential, were estimated by determining their partitioning between 10 mM phosphate buffer, pH 7.4/acetonitrile at various concentrations, and an immobilized artificial membrane (IAM.PC.DD column, 10 cm×4.6 cm i.d., Regis Technologies, Inc., Morton, Grove, IL) as described by El Tayar and colleagues.[47,48] Aliquots (5 μl) of RGD analog/prodrug solutions (200 mM, in running buffer) were injected on the column (flow rate 1.0 ml/min), and solutions were detected with a UV detector (254 nm).

Molecular Size Determination Diffusion coefficients of the RGD analogs **4a**, **b** and the prodrugs **1a**, **b** were experimentally measured by

NMR spectroscopy using an inverse Z-gradient probe (coil constant determined with water at 25 °C=5.2 G cm^{-1} amp^{-1}) interfaced to a PC-driven gradient generator specifically designed for diffusion studies (Digital Specialties, Chapel Hill, NC). During the experiment, the spin echo delay was held constant at 200 ms and the gradient current at 1 amp while the duration of the gradient pulses was sequentially increased (*i.e*., 0, 1, 4, 5, 6, 7 and 8 ms) as described elsewhere.[51,52] NMR spectra were processed with the FELIX software, version 950 (MSI-Biosym, San Diego, CA) on an IRIS Indigo Silicon Graphics computer. By linear regression analysis (r^2>0.99), the diffusion coefficients at 25 °C were obtained from the slope in a semilogarithmic plot of the intensity *vs*. pulse gradient. Molecular radii of the peptides were then calculated from their diffusion coefficients according to the Stokes–Einstein equation using η=2.1800 cP as the viscosity of DMSO-d_6 at 25 °C.

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