Structure–Activity Relationships of New 2-Acylamino-3-thiophenecarboxylic Acid Dimers as Plasminogen Activator Inhibitor-1 Inhibitors

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Small molecule inhibitors of plasminogen activator inhibitor (PAI)-1 have been reported to date but their clinical effects still remain unknown. The present study was undertaken to investigate the structure–activity relationships (SAR) of newly synthesized 2-acylamino-3-thiophenecarboxylic acid dimers based upon a core structure of TM5001 (1) and TM5007 (2) that we have previously identified as orally effective PAI-1 inhibitors. In general, compounds possessing bulky or/and hydrophobic substituents (*e.g.* phenyl, isobutyl group) on the both thiophene rings showed potent PAI-1 inhibitory activities irrespective of the positions of the substitution. The monocarboxyl derivative (10) exhibited PAI-1 inhibition comparable to the corresponding dicarboxyl compound (9f).

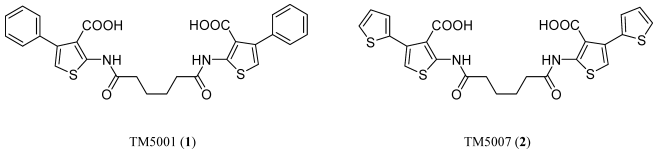
Key words plasminogen activator inhibitor-1; inhibitor; structure–activity relationship; tissue-type plasminogen activator; aminothiophenecarboxylic acid derivative

Plasminogen activator inhibitor (PAI)-1, a serine protease inhibitor, inhibits tissue- and urokinase-type plasminogen activators (tPA and uPA) and plays an important role in the clotting-fibrinolytic system. PAI-1 is implicated in a variety of pathological conditions, including thrombosis, fibrosis, atherosclerosis, radiation damage, obesity, cancer.[1—4] PAI-1 inhibition is therefore expected to have therapeutic potential for the treatment of these diseases. Furthermore, disruption of the PAI-1 gene does not result in any serious pathological defects and induces little sign of bleeding, a most common and critical adverse effect associated with various types of current anti-thrombotic drugs.[3—5] Although small molecule PAI-1 inhibitors such as PAI-039 (tiplaxtinin) and PAI-749 (diaplasinin)[4,6,7] have been reported to be effective in animal experiments, their clinical efficacy still remains unknown.

We have previously used structure-based drug design (SBDD) and *in silico* screening based on the human PAI-1 tertiary structure, and identified novel dimeric 2-acylamino-3-thiophenecarboxylic acid derivatives, TM5001 (**1**) and TM5007 (**2**) (Fig. 1),[8] with *in vitro* effects comparable to PAI-039 and PAI-749. They also exhibited significant anti-thrombotic effects when administered orally in rodents.[8]

The present study was undertaken to investigate the structure–activity relationships (SAR) of this series of compounds in more detail in order to develop a novel and more effective PAI-1 inhibitor than **1** and **2**.

Compounds having various substituents (R_1 and R_2) on the thiophene ring were synthesized as shown in Chart 1. The linker L which combines two aminothiophenecarboxylic acid moieties symmetrically through two amide bonds was also varied.



TM5001 (**1**) TM5007 (**2**)

Fig. 1. TM5001 and TM5007

Ketones or aldehydes (**3**) were subjected to the Gewald reaction[9] to prepare the starting 2-amino-3-thiophenecarboxylates (**6**) in one pot reaction or two step reactions. Then **6** was acylated with 0.5 eq of dicarbonyl chlorides (**7**) to give the diesters (**8**) in good yields. Diacids (**9**) were obtained from **8** by alkaline hydrolysis (when R_3 is methyl) or by treatment with trifluoroacetic acid (TFA) (when R_3 is *tert*-butyl). An ester-carboxylic acid (**10**) was able to be isolated on treatment of the corresponding **8** with TFA under mild conditions and subsequent chromatography.

Results and Discussion

Structures of newly synthesized compounds as described above and their *in vitro* biological activities are listed in Table 1, together with those of tiplaxtinin and diaplasinin. The *in vitro* PAI-1 activity is expressed as the remaining activity in percent (%) after incubation of PAI-1 with test compounds as described in the experimental section. Method A and Method B differ with the relative molar ratio of PAI-1 and tPA added to the medium (Method A; tPA : PAI-1 = 1 : 6.9, Method B; tPA : PAI-1 = 1 : 2.5). Method B provides a more sensitive assay than Method A to evaluate the PAI-1 activity at lower concentrations.

The substituents R_1 and R_2 on the two thiophene rings were first evaluated for adipoyl diamide-type compounds [L = $(CH_2)_4$]. Although replacement of the phenyl or 2-thienyl group of **1** or **2** with isopropyl group retained PAI-1 inhibitory activity, its potency was significantly decreased (**1**, **2** *vs.* **9a**), suggesting the importance of these substituents. Thus, compounds with other substituents were prepared in order to identify additional structural requirements concerning R_1 and R_2. Introduction of isobutyl group as R_2 (**9b-4**) largely improved the *in vitro* activity compared to **9a**. It is of interest that the activity of **9b-4** (R_2 = isobutyl) is more potent than **9a** (R_2 = isopropyl), and is comparable or superior to

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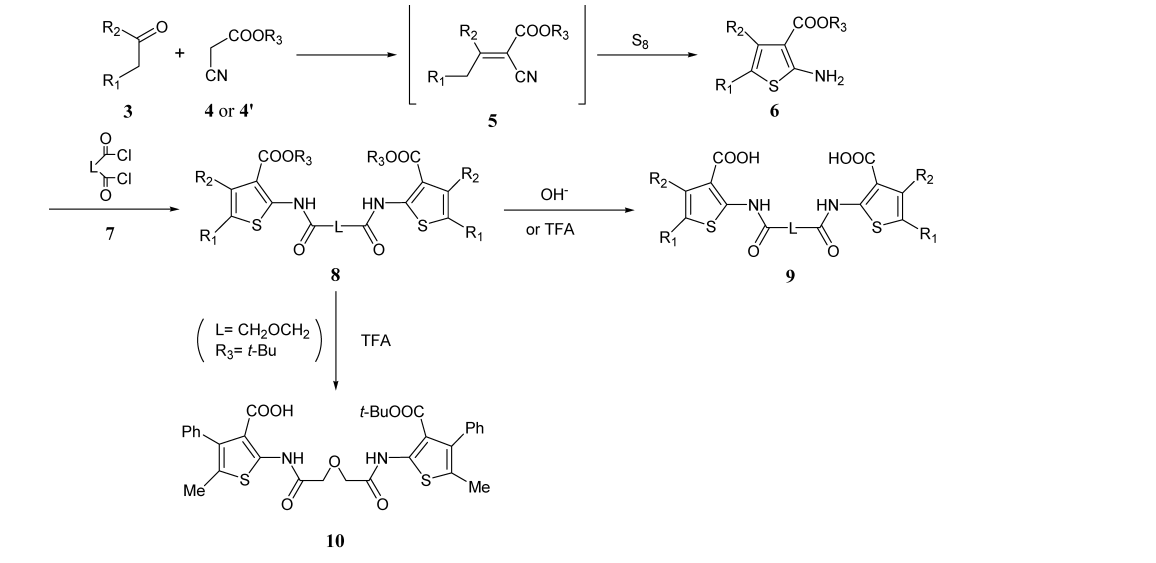


3a R_1 = H, R_2 = *i*-Pr
3b R_1 = H, R_2 = *i*-Bu
3c R_1 = H, R_2 = Ph
3d R_1 = Me, R_2 = H
3e R_1 = Ph, R_2 = H
3f R_1 = Me, R_2 = Ph

4 R_3 = *t*-Bu
4' R_3 = Me

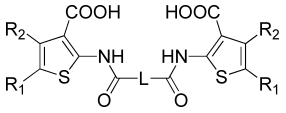
6a R_1 = H, R_2 = *i*-Pr, R_3 = *t*-Bu
6b R_1 = H, R_2 = *i*-Bu, R_3 = Me
6c R_1 = H, R_2 = Ph, R_3 = *t*-Bu
6d R_1 = Me, R_2 = H, R_3 = *t*-Bu
6e R_1 = Ph, R_2 = H, R_3 = *t*-Bu
6f R_1 = Me, R_2 = Ph, R_3 = *t*-Bu

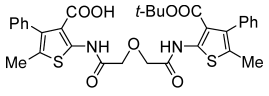
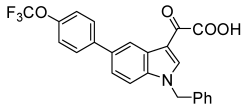
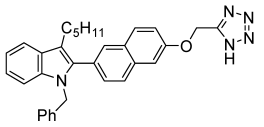
8a R_1 = H, R_2 = *i*-Pr, R_3 = *t*-Bu, L = $-(CH_2)_4-$
8b-1 R_1 = H, R_2 = *i*-Bu, R_3 = Me, L = $-(CH_2)_2-$
8b-2 R_1 = H, R_2 = *i*-Bu, R_3 = Me, L = $-(CH_2)_3-$
8b-3 R_1 = H, R_2 = *i*-Bu, R_3 = Me, L = $-CH_2C(CH_3)_2CH_2-$
8b-4 R_1 = H, R_2 = *i*-Bu, R_3 = Me, L = $-(CH_2)_4-$
8c-1 R_1 = H, R_2 = Ph, R_3 = *t*-Bu, L = $-(CH_2)_5-$
8c-2 R_1 = H, R_2 = Ph, R_3 = *t*-Bu, L = $-CH_2OCH_2-$
8d R_1 = Me, R_2 = H, R_3 = *t*-Bu, L = $-(CH_2)_4-$
8e-1 R_1 = Ph, R_2 = H, R_3 = *t*-Bu, L = $-(CH_2)_4-$
8e-2 R_1 = Ph, R_2 = H, R_3 = *t*-Bu, L = $-CH_2OCH_2-$
8f R_1 = Me, R_2 = Ph, R_3 = *t*-Bu, L = $-CH_2OCH_2-$

9a R_1 = H, R_2 =*i*-Pr, L = $-(CH_2)_4-$
9b-1 R_1 = H, R_2 = *i*-Bu, L = $-(CH_2)_2-$
9b-2 R_1 = H, R_2 = *i*-Bu, L = $-(CH_2)_3-$
9b-3 R_1 = H, R_2 = *i*-Bu, L = $-CH_2C(CH_3)_2CH_2-$
9b-4 R_1 = H, R_2 = *i*-Bu, L = $-(CH_2)_4-$
9c-1 R_1 = H, R_2 = Ph, L = $-(CH_2)_5-$
9c-2 R_1 = H, R_2 = Ph, L = $-CH_2OCH_2-$
9d R_1 = Me, R_2 = H, L = $-(CH_2)_4-$
9e-1 R_1 = Ph, R_2 = H, L = $-(CH_2)_4-$
9e-2 R_1 = Ph, R_2 = H, L = $-CH_2OCH_2-$
9f R_1 = Me, R_2 = Ph, L = $-CH_2OCH_2-$

Chart 1. Synthesis of 2-Acylamino-3-thiophenecarboxylic Acid Dimers

Table 1. PAI-1 Inhibitory Activity of 2-Acylamino-3-thiophenecarboxylic Acid Dimer Derivatives



Compound	R_1	R_2	L	PAI-1 activity (%) (method A)[a]	
				100 μM	50 μM
1	H	Ph	$-(CH_2)_4-$	15.9±8.3	51.4±5.9
2[b]	H	2-thienyl	$-(CH_2)_4-$	—	32.1±2.4
9a[c]	H	*i*-Pr	$-(CH_2)_4-$	14.3±0.8[d]	
9b-1	H	*i*-Bu	$-(CH_2)_2-$	45.3±9.6	98.5±0.9
9b-2	H	*i*-Bu	$-(CH_3)_3-$	20.1±7.4	85.6±1.2
9b-3	H	*i*-Bu	$-CH_2C(CH_3)_2CH_2-$	17.9±5.0	54.5±9.7
9b-4	H	*i*-Bu	$-(CH_2)_4-$	1.8±3.1[e]	49.8±15.3
9c-1	H	Ph	$-(CH_2)_5-$	10.3±3.8[e]	22.4±4.4
9c-2	H	Ph	$-CH_2OCH_2-$	19.8±4.4	94.2±7.3
9d[c]	Me	H	$-(CH_2)_4-$	70.6±6.6[f]	
9e-1	Ph	H	$-(CH_2)_4-$	12.4±5.5	16.4±4.8
9e-2	Ph	H	$-CH_2OCH_2-$	9.3±1.3	12.9±3.1
9f	Me	Ph	$-CH_2OCH_2-$	7.9±3.9[e]	15.5±3.6
10[c]				—	12.7±4.0
Tiplaxinin[c] (PAI-039)				—	34.8±3.0
Diaplasinin[c] (PAI-749)				—	30.5±3.6

Data are expressed as mean±S.D. *a*) See Experimental, *b*) PAI-1 activity (%) of **2** tested by method B is 14.1±6.1% at 50 μM, *c*) tested by method B (see Experimental), *d*) PAI-1 activity (%) at 200 μM is shown, *e*) tPA is slightly inhibited in the test, *f*) PAI-1 activity (%) at 300 μM is shown.

those of **1** and **2** with aromatic-ring substituents, or those of the reference compounds tiplaxtinin and diaplasinin as shown in the Table 1. Since there was no distinct difference in activity between isomeric **9e-1** and **1** which have a phenyl moiety at different positions (R_1 and R_2, respectively), hydrophobicity or/and sterical bulkiness adjacent to the thiophene rings appears to be more important than the position of the substitution. This observation was supported by the activity of compound **9d** which showed much reduced potency indicating that the methyl group at R_1 is too small or/and not sufficiently hydrophobic. The linker L which connects the two thiophene rings *via* a diamide structure was varied next to investigate its potential for enhancing potency. However, PAI-1 inhibitory activity was not found to be significantly influenced by either the distance (2 to 5 atoms) between the two amide linkages (see **9b-1**, **9b-2** and **9b-4**), nor by introducing a dimethyl branch to increase bulkiness or hydrophobicity (see **9b-3**). Introduction of an oxygen atom into the linker L also retained the activity observed with the original alkylene-type derivatives (see **9c-2** and **9e-2**). Compound **9f** which has both phenyl and methyl groups on the same ring also showed potent activity.

The above-mentioned observations suggest that it is worthy of investigating further SAR both on the substituent and the linker of the molecule in future modifications.

Of note, a compound (**10**) with a carboxyl group on a thiophene ring and an ester moiety on the other ring, provides potent inhibitory activity almost comparable to the corresponding dicarboxylic acid (**9f**). This suggests that one carboxylic acid group is sufficient to elicit potent PAI-1 inhibition, and a symmetrically substituted, dimeric structure may not be necessary.

Our newly synthesized compounds will provide useful tools for a better understanding of the pathophysiological functions of PAI-1. However, in general they possess somewhat high molecular weights and poor solubility in both aqueous and organic solvents, and may not be sufficiently bioavailable and bioeffective upon oral administration in animals. Further modifications to find more elaborated compounds with more drug-like properties, *i.e.*, lower molecular weights, better solubility, better oral bioavailability, *etc.* are to be required.

Conclusion

On the basis of structure of **1** and **2**, we synthesized and evaluated the SAR of several novel derivatives of this series. Compounds possessing bulky or/and hydrophobic substituents on the thiophene rings, exhibited significant PAI-1 inhibitory activities *in vitro*. Further studies suggested that the linker L was tolerant to variation and only one carboxyl moiety was sufficient to elicit potent PAI-1 inhibitory activity. Therefore, we have now started to synthesize simpler derivatives, particularly those with an unsymmetrical structure and will report our results in due course. These studies will not only be useful for a better understanding of the pathophysiology of PAI-1 but also may help to identify potential new treatments for diseases where PAI-1 has been implicated.

Experimental

General Melting points (mp) were determined on Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer, with tetramethylsilane as an internal standard. TLC analyses were carried out on MERCK Silica gel 60 F_{254} plates. Elemental analyses were carried out by Takeda Analytical Laboratories, Ltd., and are within $\pm$0.4% of the theoretical values unless otherwise noted. Chromatographic purification was carried out on silica gel columns (MERCK Silica gel 60, 0.040—0.063 mm) unless otherwise noted. The yields reported are not optimized. Analytical HPLC was conducted on a Puresil C_{18} column (5 μm, 4.4×150 mm i.d.) eluted with 0.1% (v/v) TFA in water (solvent A) and 0.08% (v/v) TFA in acetonitrile (MeCN)/water (80%/20% (v/v)) (solvent B), according to the following elution gradient: 0—20 min, 50—80% B; 20—45 min, 80% B at a flow rate of 0.8 ml/min. LC/MS spectra were recorded on an Alliance HPLC system using a ULTORON VX-ODS C_{18} column (5 μm, 4.6×150 mm i.d.) coupled with an micromass ZQ as MS detector and a gradient of 5—100% B over 20 min was used with a flow rate of 1.0 ml/min. Formic acid 0.1% was added to solvents A and B (Water (A) and MeCN (B)).

***tert*-Butyl 2-Amino-4-phenylthiophene-3-carboxylate (6c)** To a solution of acetophenone (**3c**) (10.6 g, 88.2 mmol) in toluene (50 ml) were added *tert*-butyl cyanoacetate (**4**) (19.9 g, 141.7 mmol), acetic acid (AcOH) (5.3 g, 88.2 mmol) and morpholine (6.2 g, 70.6 mmol). The reaction mixture was refluxed with stirring for 12.5 h under azeotropic removal of water using a Dean–Stark apparatus. After cooling, water was added to the reaction mixture and the whole mixture was extracted with toluene three times. The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated *in vacuo*. To a solution of this residue in dimethylformamide (DMF) (100 ml) were added sulfur (2.83 g, 88.2 mmol) and morpholine (7.68 g, 88.2 mmol) and the whole mixture was stirred at room temperature for 16 h. Water was added to the reaction mixture and the resulting solution was extracted with EtOAc three times. The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (acetone/hexane 1/10) to afford 14.7 g (yield 61%) of **6c** as a solid, ^{1}H-NMR ($CDCl_3$) δ: 1.19 (9H, s), 6.01 (2H, br), 6.03 (1H, s), 7.20—7.36 (5H, m).

***tert*-Butyl 2-Amino-5-methyl-4-phenylthiophene-3-carboxylate (6f)** This compound was prepared in 33% yield as a solid from propiophenone (**3f**) following a procedure similar to that described for the preparation of **6c**, ^{1}H-NMR ($CDCl_3$) δ: 1.10 (9H, s), 2.02 (3H, s), 5.89 (2H, br), 7.09—7.17 (2H, m), 7.20—7.38 (3H, m).

Di-*tert*-butyl 2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis[4-(propan-2-yl)thiophene-3-carboxylate] (8a) Adipoyl chloride (1.37 g, 7.5 mmol) was added to a solution of *tert*-butyl 2-amino-4-(propan-2-yl)thiophene-3-carboxylate (**6a**)[10] (3.62 g, 15.0 mmol) in *N*,*N*-dimethylacetamide (DMA) (20 ml) at 0 °C, and the resulting mixture was stirred at room temperature for 15 h. Aqueous $NaHCO_3$ solution was added to the reaction mixture and the resulting precipitate was collected by suction filtration, washed with water and ethyl acetate (EtOAc), and dried *in vacuo* to afford 4.19 g (yield 93%) of **8a** as a solid, ^{1}H-NMR ($CDCl_3$) δ: 1.21 (12H, d, J=6.8 Hz), 1.60 (18H, s), 1.75—1.95 (4H, m), 2.45—2.65 (4H, m), 3.34—3.58 (2H, m), 6.42 (2H, s), 11.54 (2H, br).

Dimethyl 2,2′-[(1,4-Dioxobutane-1,4-diyl)diimino]bis[4-(2-methylpropyl)thiophene-3-carboxylate] (8b-1) This compound was prepared in 84% yield as a solid from methyl 2-amino-4-(2-methylpropyl)thiophene-3-carboxylate (**6b**)[11] and succinyl chloride following a procedure similar to that described for the preparation of **8a**, ^{1}H-NMR ($CDCl_3$) δ: 0.89 (12H, d, J=6.6 Hz), 1.67—1.91 (2H, m), 2.61 (4H, d, J=6.6 Hz), 2.95 (4H, s), 3.90 (6H, s), 6.35 (2H, s), 11.42 (2H, br).

Dimethyl 2,2′-[(1,5-Dioxopentane-1,5-diyl)diimino]bis[4-(2-methylpropyl)thiophene- 3-carboxylate] (8b-2) Glutaryl chloride (254 mg, 1.5 mmol) was added to a solution of **6b** (0.64 g, 3 mmol) in DMA (6 ml) at 0 °C, and the resulting mixture was stirred at room temperature for 16 h. Aqueous $NaHCO_3$ solution was added to the reaction mixture and the whole mixture was extracted with EtOAc three times. The combined organic layers were washed with water, dried (Na_2SO_4), and concentrated *in vacuo* to afford 0.73 g (yield 94%) of **8b-2** as a solid, ^{1}H-NMR ($CDCl_3$) δ: 0.89 (12H, d, J=6.8 Hz), 1.70—1.90 (2H, m), 2.10—2.30 (2H, m), 2.62 (4H, d, J=6.7 Hz), 2.63 (4H, t, J=7.1 Hz), 3.88 (6H, s), 6.36 (2H, s), 11.35 (2H, br).

Dimethyl 2,2′-[(3,3-Dimethyl-1,5-dioxopentane-1,5-diyl)diimino]bis[4-(2-methylpropyl)thiophene-3-carboxylate] (8b-3) (i) To a solution of 3,3-dimethylpentanedioic acid (20 g, 124 mmol) in tetrahydrofuran (THF) (40 ml) were added oxalyl chloride (80 g, 374 mmol) and DMF (cat. amount) under 0 °C. After stirring at room temperature for 0.5 h, the mixture was concentrated *in vacuo*. The residue was purified by distillation under reduced pressure (18 mmHg, 80 °C) to afford 13.5 g (yield 54%) of 3,3-dimethylpentanedioyl dichloride as an oil, ^{1}H-NMR ($CDCl_3$) δ: 1.18 (6H, s),

3.13 (4H, s).

(ii) 3,3-Dimethylpentanedioyl dichloride (0.46 g, 2.34 mmol) was added to a solution of **6b** (1.0 g, 4.69 mmol) in DMA (3 ml) at 0 °C, and the resulting mixture was stirred at room temperature for 16 h. Aqueous $NaHCO_3$ solution was added to the reaction mixture and the whole mixture was extracted with EtOAc three times. The combined organic layers were washed with brine and water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (EtOAc/hexane 1/4) to afford 1.19 g (yield 92%) of **8b-3** as a solid, ^{1}H-NMR ($CDCl_3$) δ: 0.90 (12H, d, J=6.6 Hz), 1.23 (6H, s), 1.69—1.92 (2H, m), 2.61 (4H, s), 2.63 (4H, d, J=6.0 Hz), 3.91 (6H, s), 6.38 (2H, s), 11.35 (2H, br).

Dimethyl 2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis[4-(2-methylpropyl)thiophene-3-carboxylate] (8b-4) This compound was prepared in 94% yield as a solid from **6b** and adipoyl chloride following a procedure similar to that described for the preparation of **8b-2**, ^{1}H-NMR ($CDCl_3$) δ: 0.89 (12H, d, J=6.6 Hz), 1.70—1.95 (6H, m), 2.40—2.60 (4H, m), 2.61 (4H, d, J=6.8 Hz), 3.89 (6H, s), 6.36 (2H, s), 11.36 (2H, br).

Di-*tert*-butyl 2,2′-[(1,7-Dioxoheptane-1,7-diyl)diimino]bis(4-phenylthiophene-3-carboxylate) (8c-1) This compound was prepared in 55% yield as a solid from **6c** and pimeloyl chloride following a procedure similar to that described for the preparation of **8a**, ^{1}H-NMR ($CDCl_3$) δ: 1.19 (18H, s), 1.43—1.64 (2H, m), 1.76—1.94 (4H, m), 2.56 (4H, t, J=7.4 Hz), 6.55 (2H, s), 7.20—7.40 (10H, m), 11.33 (2H, br).

Di-*tert*-butyl 2,2′-{Oxybis[(1-oxoethane-2,1-diyl)imino]}bis(4-phenylthiophene-3-carboxylate) (8c-2) This compound was prepared in 70% yield as a solid from **6c** and diglycolyl chloride following a procedure similar to that described for the preparation of **8b-2**, ^{1}H-NMR ($CDCl_3$) δ: 1.11 (18H, s), 4.46 (4H, s), 6.60 (2H, s), 7.18—7.39 (10H, m), 12.16 (2H, br).

Di-*tert*-butyl 2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis(5-methylthiophene-3-carboxylate) (8d) Adipoyl chloride (472 mg, 2.58 mmol) was added to a solution of *tert*-butyl 2-amino-5-methylthiophene-3-carboxylate (**6d**)[12] (1.0 g, 4.69 mmol) in DMA (10 ml) at 0 °C, and the resulting mixture was stirred at room temperature for 3 h. Aqueous $NaHCO_3$ solution was added to the reaction mixture and the resulting precipitate was collected by suction filtration and washed with water and hexane to give a crude product, which was recrystallized from a mixed solvent of EtOAc, THF and hexane to afford 965 mg (yield 77%) of **8d** as a solid, ^{1}H-NMR ($CDCl_3$) δ: 1.56 (18H, s), 1.80—1.90 (4H, m), 2.36 (6H, d, J=1.1 Hz), 2.48—2.47 (4H, m), 6.77 (2H, q, J=1.1 Hz), 10.95 (2H, br).

Di-*tert*-butyl 2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis(5-phenylthiophene-3-carboxylate) (8e-1) This compound was prepared in 31% yield as a solid from *tert*-butyl 2-amino-5-phenylthiophene-3-carboxylate (**6e**)[13] and adipoyl chloride following a procedure similar to that described for the preparation of **8b-3**, ^{1}H-NMR ($CDCl_3$) δ: 1.60 (18H, s), 1.85—1.95 (4H, m), 2.55—2.65 (4H, m), 7.20—7.42 (6H, m), 7.32 (2H, s), 7.54—7.62 (4H, m), 11.10 (2H, br).

Di-*tert*-butyl 2,2′-{Oxybis[(1-oxoethane-2,1-diyl)imino]}bis(5-phenylthiophene-3-carboxylate) (8e-2) This compound was prepared in 65% yield as a solid from **6e** and diglycolyl chloride following a procedure similar to that described for the preparation of **8a**, ^{1}H-NMR ($CDCl_3$) δ: 1.49 (18H, s), 4.46 (4H, s), 7.22—7.44 (6H, m), 7.36 (2H, s), 7.55—7.64 (4H, m), 11.92 (2H, br).

Di-*tert*-butyl 2,2′-{Oxybis[(1-oxoethane-2,1-diyl)imino]}bis(5-methyl-4-phenylthiophene-3-carboxylate) (8f) This compound was prepared in 80% yield as a solid from **6f** and diglycolyl chloride following a procedure similar to that described for the preparation of **8b-3**, ^{1}H-NMR ($CDCl_3$) δ: 1.04 (18H, s), 2.12 (6H, s), 4.43 (4H, s), 7.05—7.15 (4H, m), 7.20—7.40 (6H, m), 12.10 (2H, s).

2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis[4-(propan-2-yl)thiophene-3-carboxylic Acid] (9a) To a solution of **8a** (1.19 g, 2.0 mmol) in $CHCl_3$ (10 ml) was added TFA (2 ml) at 0 °C. After stirring at room temperature for 16 h, the mixture was concentrated *in vacuo*, and water was added to the residue. The precipitate was collected by suction filtration, washed with water and diethyl ether (Et_2O), dried *in vacuo* to afford 730 mg (yield 75%) of **9a** as a solid, mp 255—257 °C, ^{1}H-NMR (DMSO-d_6) δ: 1.16 (12H, d, J=6.8 Hz), 1.60—1.75 (4H, m), 2.50—2.65 (4H, s), 3.35—3.62 (2H, m), 6.65 (2H, s), 11.32 (2H, s), HPLC (250 nm): 99.1% (t_R=37.4 min), LC/MS (ESI) m/z: 481 $(M+H)^+$, 479 $(M-H)^-$.

2,2′-[(1,4-Dioxobutane-1,4-diyl)diimino]bis[4-(2-methylpropyl)thiophene-3-carboxylic Acid] (9b-1) To a solution of **8b-1** (610 mg, 1.2 mmol) in THF (10 ml) was added 1 N aqueous NaOH solution (4.8 ml) and the mixture was stirred for 6 h at 60 °C. After cooling, THF was removed *in vacuo*. The resulting precipitate was collected by filtration and washed with water and EtOAc. The crude product was suspended in 1 N aqueous HCl solution and extracted with a mixed solvent of EtOAc and THF. The organic layer was washed with brine, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by recrystallization from EtOH to afford 152 mg (yield 26%) of **9b-1** as a solid, mp 278—280 °C (EtOH), ^{1}H-NMR (DMSO-d_6) δ: 0.84 (12H, d, J=6.6 Hz), 1.81 (2H, septet, J=6.8 Hz), 2.61 (4H, d, J=6.8 Hz), 2.86 (4H, s), 6.60 (2H, s), 11.35 (2H, br), HPLC (250 nm): 97.7% (t_R=21.1 min), LC/MS (ESI) m/z: 481 $(M+H)^+$, 479 $(M-H)^-$.

2,2′-[(1,5-Dioxopentane-1,5-diyl)diimino]bis[4-(2-methylpropyl)thiophene-3-carboxylic Acid] (9b-2) To a solution of **8b-2** (730 mg, 1.4 mmol) in THF (12 ml) was added 1 N aqueous NaOH solution (8 ml) and the mixture was stirred for 16 h at 60 °C. After cooling and evaporation of THF *in vacuo*, the resulting precipitate was collected by suction filtration and washed with water and EtOAc. The crude product was suspended in 1 N aqueous HCl solution and extracted with EtOAc. The extract was washed with brine, dried ($MgSO_4$), and concentrated *in vacuo*. Et_2O was added to the residue and the resulting crystal thus formed was collected by suction filtration, washed with Et_2O, and dried *in vacuo* to afford 119 mg (yield 17%) of **9b-2** as a solid, mp 217—218 °C, ^{1}H-NMR (DMSO-d_6) δ: 0.84 (12H, d, J=6.6 Hz), 1.70—2.10 (4H, m), 2.50—2.70 (4H, m), 2.61 (4H, d, J=6.8 Hz), 6.60 (2H, s), 11.30 (2H, br), HPLC (250 nm): 99.6% (t_R=21.7 min), LC/MS (ESI) m/z: 495 $(M+H)^+$, 493 $(M-H)^-$.

2,2′-[(3,3-Dimethyl-1,5-dioxopentane-1,5-diyl)diimino]bis[4-(2-methylpropyl)thiophene-3-carboxylic Acid] (9b-3) This compound was prepared in 22% yield as a solid from **8b-3** following a procedure similar to that described for the preparation of **9b-1**, mp 198—201 °C (EtOAc/hexane), ^{1}H-NMR (DMSO-d_6) δ: 0.84 (12H, d, J=6.6 Hz), 1.12 (6H, s), 1.70—1.93 (2H, m), 2.60 (4H, s), 2.61 (4H, d, J=5.6 Hz), 6.60 (2H, s), 11.37 (2H, br), HPLC (250 nm): 97.6% (t_R=29.7 min), LC/MS (ESI) m/z: 523 $(M+H)^+$, 521 $(M-H)^-$.

2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis[4-(2-methylpropyl)thiophene-3-carboxylic Acid] (9b-4) This compound was prepared in 61% yield as a solid from **8b-4** following a procedure similar to that described for the preparation of **9b-2**, mp 224—225 °C, ^{1}H-NMR (DMSO-d_6) δ: 0.84 (12H, d, J=6.8 Hz), 1.50—1.75 (4H, m), 1.70—1.90 (2H, m), 2.50—2.70 (8H, m), 6.59 (2H, s), 11.29 (2H, br), HPLC (250 nm): 98.7% (t_R=23.2 min), LC/MS (ESI) m/z: 509 $(M+H)^+$, 507 $(M-H)^-$.

2,2′-[(1,7-Dioxoheptane-1,7-diyl)diimino]bis(4-phenylthiophene-3-carboxylic Acid) (9c-1) To a solution of **8c-1** (0.66 g, 0.98 mmol) in CH_2Cl_2 (3 ml) was added TFA (2 ml) at 0 °C. After stirring at room temperature for 16 h, the mixture was concentrated *in vacuo*, and diisopropyl ether (IPE) was added to the residue. The precipitate thus formed was collected by suction filtration, washed with EtOAc, and dried *in vacuo* to afford 467 mg (yield 85%) of **9c-1** as a solid, mp 204—206 °C, ^{1}H-NMR (DMSO-d_6) δ: 1.30—1.50 (2H, m), 1.55—1.80 (4H, m), 2.57 (4H, t, J=7.3 Hz), 6.84 (2H, s), 7.20—7.40 (10H, m), 11.26 (2H, s), 12.96 (2H, br), HPLC (250 nm): 96.3% (t_R=21.6 min), LC/MS (ESI) m/z: 563 $(M+H)^+$, 561 $(M-H)^-$.

2,2′-{Oxybis[(1-oxoethane-2,1-diyl)imino]}bis(4-phenylthiophene-3-carboxylic Acid) (9c-2) This compound was prepared in 95% yield as a solid from **8c-2** following a procedure similar to that described for the preparation of **9c-1**, mp 250—252 °C, ^{1}H-NMR (DMSO-d_6) δ: 4.52 (4H, s), 6.92 (2H, s), 7.20—7.40 (10H, m), 12.02 (2H, s), 13.06 (2H, br), HPLC (250 nm): 99.2% (t_R=17.6 min), LC/MS (ESI) m/z: 537 $(M+H)^+$, 535 $(M-H)^-$.

2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis(5-methylthiophene-3-carboxylic Acid) (9d) This compound was prepared in 55% yield as a solid from **8d** following a procedure similar to that described for the preparation of **9c-1**, mp 273—275 °C (THF/hexane), ^{1}H-NMR (DMSO-d_6) δ: 1.55—1.75 (4H, m), 2.32 (6H, d, J=1.1 Hz), 2.50—2.65 (4H, m), 6.82 (2H, d, J=1.1 Hz), 10.89 (2H, s), 12.98 (2H, br), HPLC (250 nm): 95.2% (t_R=10.1 min), LC/MS (ESI) m/z: 425 $(M+H)^+$, 423 $(M-H)^-$.

2,2′-[(1,6-Dioxohexane-1,6-diyl)diimino]bis(5-phenylthiophene-3-carboxylic Acid) (9e-1) This compound was prepared in 52% yield as a solid from **8e-1** following a procedure similar to that described for the preparation of **9c-1**, mp 289—291 °C (THF/hexane), ^{1}H-NMR (DMSO-d_6) δ: 1.60—1.80 (4H, m), 2.50—2.75 (4H, m), 7.20—7.45 (6H, m), 7.51 (2H, s), 7.55—7.70 (4H, m), 11.06 (2H, s), 13.30 (2H, br), HPLC (250 nm): 96.0% (t_R=23.8 min), LC/MS (ESI) m/z: 549 $(M+H)^+$, 547 $(M-H)^-$.

2,2′-{Oxybis[(1-oxoethane-2,1-diyl)imino]}bis(5-phenylthiophene-3-carboxylic Acid) (9e-2) This compound was prepared in 50% yield as a solid from **8e-2** following a procedure similar to that described for the preparation of **9c-1**, mp >280 °C, ^{1}H-NMR (DMSO-d_6) δ: 4.55 (4H, s), 7.25—7.50 (6H, m), 7.59 (2H, s), 7.60—7.75 (4H, m), 11.79 (2H, s), 13.36

(2H, br), HPLC (250 nm): 98.6% (t_R=21.6 min), LC/MS (ESI) *m/z*: 537 $(M+H)^+$, 535 $(M-H)^-$.

2,2′-{Oxybis[(1-oxoethane-2,1-diyl)imino]}bis(5-methyl-4-phenylthiophene-3-carboxylic Acid) (9f) This compound was prepared in 96% yield as a solid from **8f** following a procedure similar to that described for the preparation of **9c-1**, mp 241—243 °C, ^{1}H-NMR (DMSO-d_6) δ: 2.09 (6H, s), 4.49 (4H, s), 7.10—7.20 (4H, m), 7.20—7.45 (6H, m), 11.98 (2H, s), HPLC (250 nm): 96.9% (t_R=22.2 min), LC/MS (ESI) *m/z*: 565 $(M+H)^+$, 563 $(M-H)^-$. *Anal.* Calcd for $C_{28}H_{24}N_2O_7S_2$: C, 59.56; H, 4.28; N, 4.96. Found: C, 59.35; H, 4.24; N, 4.90.

2-{[(2-{[3-(*tert*-Butoxycarbonyl)-5-methyl-4-phenylthiophen-2-yl]amino}-2-oxoethoxy)acetyl]amino}-5-methyl-4-phenylthiophene-3-carboxylic Acid (10) To a solution of **8f** (1.5 g, 2.2 mmol) in CH_2Cl_2 (30 ml) was added TFA (3 ml) at 0 °C. After stirring at room temperature for 4 h, the mixture was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (EtOAc/hexane 1/1) to afford 262 mg (yield 19%) of **10** as a solid, mp 254—256 °C, ^{1}H-NMR (DMSO-d_6) δ: 0.98 (9H, s), 2.07 (3H, s), 2.09 (3H, s), 4.49 (4H, s), 7.05—7.20 (4H, m), 7.20—7.45 (6H, m), 11.73 (1H, s), 12.10 (1H, br), HPLC (250 nm): 99.1% (t_R=37.4 min), LC/MS (ESI) *m/z*: 621 $(M+H)^+$, 619 $(M-H)^-$. *Anal.* Calcd for $C_{32}H_{32}N_2O_7S_2$: C, 61.92; H, 5.20; N, 4.51. Found: C, 61.78; H, 5.24; N, 4.41.

Biological Procedures Method A and Method B PAI-1 activity was evaluated by two different methods. For the screening of our chemical libraries for their PAI-1 inhibitory activities, we used a biological assay utilizing a synthetic substrate for tPA. In brief, human PAI-1 (Molecular Innovations, Southfield, MI, U.S.A.) was incubated at 37 °C for 15 min in the reaction buffer containing 100 mM Tris–HCl, pH 8, 0.1% Tween 80 in the presence or absence of the tested compounds in a 96-well polystyrene plate. The mixture was subsequently incubated for 15 min with human tPA (American Diagnostica Inc., Stanford, CT, U.S.A.), and eventually fortified with a chromogenic substrate, S-2288 (Chromogenix, Milano, Italy). The final mixture contained 100 mM Tris–HCl, pH 8, 30 mM NaCl, 1% dimethyl sulfoxide (DMSO), 0.1% Tween 80, 67 nM PAI-1 (Method A) or 24.5 nM (Method B), 9.8 nM tPA, 1 mM S-2288, and tested compounds at various concentrations (20, 35, 50, 100 μM). Kinetics of *p*-nitroanilide release during peptide cleavage was monitored with a spectrophotometer at 405 nm. The residual inhibitory activity of PAI-1 was expressed as the percentage of the initial activity.

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