Isolation of Translactone-containing Triterpenes with Thrombin Inhibitory Activities from the Leaves of *Lantana camara*

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Methanolic extracts prepared from the leaves of *Lantana camara* have been found to inhibit human thrombin. An assay, in which thrombin activity is measured as a function of clot formation from fibrinogen, was used to guide the fractionation and purification of five principal active constituents (**1**−**5**), which were all characterized as 5,5-*trans*-fused cyclic lactone-containing euphane triterpenes.

Lantana camara L. (Verbenaceae), commonly known as wild sage, is a flowering shrub indigenous to tropical America and Africa and is now widely cultivated throughout the world as an ornamental.[1] The species is known to be toxic to grazing animals, which, on ingestion of the leaves, develop hepatotoxicity and photosensitization.[1−3]

The molecular mechanism of action of *Lantana* toxicity to hepatocytes has not been fully elucidated. A study of hematological changes in sheep after *Lantana* poisoning indicated a significant increase in coagulation time and prothrombin time, with an associated decrease in blood sedimentation rate, total plasma protein, and fibrinogen.[2]

Extensive phytochemical studies on *Lantana* have led to the isolation of triterpenoids, flavonoids, iridoids, and the phenylpropanoids glycosine and verbascoside.[1,3,4] The toxic effects of the leaves of the species have been attributed to a series of pentacyclic triterpenes, of which lantadenes A and B are typical members.[3]

During a screening program to look for inhibitors of human thrombin, methanolic extracts of *L. camara* leaves obtained from a garden in England were found to show highly potent activity. The present report describes the bioassay-guided purification, structure elucidation, and antithrombin activity of the principal active components (**1**−**5**).

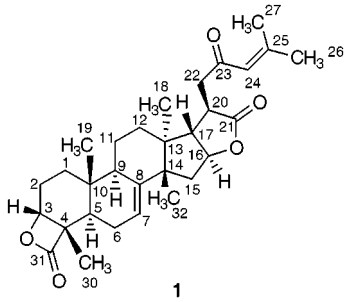
Results and Discussion

After the initial observation of the thrombin-inhibitory activity of a methanolic extract prepared from leaves of *L. camara,* large-scale extraction and fractionation was undertaken according to the protocols described in the Experimental Section. Thrombin-inhibitory activity was found to be associated with a series of novel 5,5-*trans*-fused cyclic lactone-containing euphane triterpenes (**1**−**5**).

A combination of HRMS data (Table 1) and ^{1}H and ^{13}C NMR data (Tables 2 and 3, respectively) showed that the molecular formula of compound **1** was $C_{30}H_{40}O_5$. In addition to the four singlet methyls, three carbonyls, and two sp^2 and four sp^3 quaternary carbons, analysis of the ^{1}H−^{1}H and one bond ^{1}H−^{13}C correlation data (PSDQFCOSY and HMQC) enabled a number of other fragments to be identified. The connections between these (Figure 1) were derived from long-range ^{1}H−^{13}C correlation data (HMBC). The presence of both a β- and a γ-lactone was postulated

Table 1. HRMS Data of Compounds 1−5

compound	molecular formula	molecular weight	measured (MH^+)	calculated	error (ppm)
1	$C_{30}H_{40}O_5$	480	481.2952	481.2954	0.5
2	$C_{32}H_{44}O_7$	540	541.3159	541.3165	1.2
3	$C_{32}H_{44}O_8$	556	557.3127	557.3114	2.2
4	$C_{38}H_{54}O_{14}$	734	735.3616	735.3592	3.3
5	$C_{40}H_{56}O_{15}$	776	777.3685	777.3698	1.6



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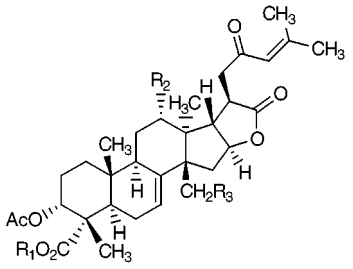

	R_1	R_2	R_3
2,	H	H	H
3,	H	OH	H
4,	β-D-Glucose	OH	OH
5,	β-D-Glucose	OH	OAc

to account for the observed chemical shifts and to fit the molecular formula. This was confirmed by bands at 1811 and 1775 cm^{-1}, respectively, in the IR spectrum. The relative stereochemistry was derived from NOE difference data (Table 4). A subsequent X-ray crystal structure (Figure 2, Table 5) confirmed all of these conclusions.

An analysis of the HRMS and 1D and 2D NMR data for compound **4** similar to that described above for **1** led to the structure proposed. Once again NOE difference studies served to establish the relative stereochemistry (Table 4). The absolute stereochemistry, including that of the glucose

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Table 2. ^{1}H NMR Data of Compounds 1−5

	compound				
position	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]
1	1.60 (m), 1.34 (m)	1.40 (m)	1.47−1.37 (m)	1.39 (m)	1.38 (m)
2	2.08 (m)	1.90 (m)	1.91 (m)	1.91 (m)	1.91 (m)
	1.90 (m)	1.76 (dq, 15, 3.5)	1.76 (dq, 15, 3.5)	1.77 (dq, 15, 3)	1.77 (dq, 15, 3.5)
3	4.43 (dd, 3, 1)	5.02 (dd, 3.5, 2.5)	5.03 (dd, 3.5, 2.5)	5.09 (dd, 3, 2)	5.09 (dd, 3.5, 2)
5	2.18 (m)	2.48 (dd, 12, 6)	2.48 (dd, 12, 5)	2.48 (dd, 12, 5)	2.53−2.45 (m)
6	2.18 (m)	2.32 (m)	2.35 (m)	2.45 (m)	2.53−2.45 (m)
	2.02 (m)	1.99 (m)	1.99 (m)	2.04 (m)	obscured
7	5.31 (m)	5.30 (m)	5.35 (m)	5.34 (m)	5.47 (m)
9	2.59 (m)	2.69 (m)	2.82 (m)	2.85 (m)	2.86 (m)
11	1.76 (m)	1.67 (m)	2.13 (m)	2.13 (m)	2.13 (m)
	1.45 (m)	1.58 (m)	1.51 (ddd, 15, 8, 2)	1.56 (ddd, 15.5, 8.5, 2.5)	1.59 (ddd, 15.5, 8, 2.5)
12	1.56 (m), 1.47 (m)	1.62−1.48 (m)	3.92 (dd, 9, 2)	3.97 (dd, 9, 2.5)	3.89 (dd, 9, 2.5)
15	2.28 (dd, 13, 10)	2.33 (dd, 13, 10)	2.41 (dd, 13.5, 10)	2.21 (dd, 14, 8)	2.30 (dd, 14, 9.5)
	1.73 (dd, 13, 7.5)	1.67 (dd, 13, 7.5)	1.70 (dd, 13.5, 7.5)	2.17 (dd, 14, 9.5)	2.09 (dd, 14, 8)
16	4.28 (ddd, 11, 10, 7.5)	4.35 (ddd, 11.5, 10, 7.5)	4.33 (ddd, 11.5, 10, 7.5)	4.37 (ddd, 11, 9.5, 8)	4.37 (ddd, 11.5, 9.5, 8)
17	2.06 (dd, 12, 11)	2.22 (t, 11.5)	2.58 (t, 12)	2.91 (dd, 13, 11)	2.84 (t, 11.5)
18	1.09 (s)	1.10 (s)	1.01 (s)	1.05 (s)	1.07 (s)
19	0.67 (s)	0.88 (s)	0.87 (s)	0.85 (s)	0.79 (s)
20	3.02 (ddd, 12, 9, 2.5)	3.06 (m)	3.01 (m)	3.05 (dt,13,5)	3.02−2.92 (m)
22	3.15 (dd, 17.5, 2.5)	3.02 (m)	2.97 (m)	2.88 (m)	3.02−2.92 (m)
	2.51 (dd, 17.5, 9)	2.64 (dd, 17.5, 8.5)	2.90 (m)		
23					
24	6.07 (m)	6.20 (m)	6.19 (m)	6.19 (m)	6.18 (m)
26	1.91 (d, 1)	1.93 (d, 1)	1.92 (d, 1)	1.91 (d, 1)	1.91 (d, 1)
27	2.17 (d, 1)	2.16 (d, 1)	2.13 (d, 1)	2.13 (d, 1)	2.13 (d, 1)
30	1.35 (s)	1.31 (s)	1.30 (s)	1.36 (s)	1.36 (s)
32	1.20 (s)	1.23 (s)	1.19 (s)	3.58 (d, 11)	4.06 (d, 11)
				3.14 (d, 11)	3.83 (d, 11)
Ac-3		2.01 (s)	2.00 (s)	2.03 (s)	2.03 (s)
1′				5.40 (d, 8)	5.40 (d, 8)
2′				3.28 (dd, 9, 8)	3.28 (dd, 9, 8)
3′				3.38 (t, 9)	3.38 (t, 9)
4′				3.30[c]	obscured
5′				3.35 (m)	3.35 (m)
6′				3.82 (dd, 12, 2.5)	3.82 (dd, 12, 2.5)
				3.65 (dd, 12, 5.5)	3.65 (dd, 12, 5)
Ac-32					2.08 (s)

[a] In $CDCl_3$; referenced to residual $CHCl_3$ at δ 7.26. [b] In CD_3OD; referenced to residual CHD_2OD at δ 3.31. [c] From $^1H-^{13}C$ correlation.

residue was established by X-ray crystallographic studies carried out on the complex formed between **4** and α-thrombin.[5]

The remaining compounds (**2**, **3**, and **5**) were identified from their MS (Table 1) and 1D ^{1}H NMR spectral (Table 2) data. Thus, comparison of the data for **5** with those for **4** showed that **5** contained an extra acetate group (2.08 ppm). The characteristic acylation shift observed for the protons of the CH_2 at the C-32 position served to indicate the point of attachment. Compound **3** clearly lacked the sugar residue, and the absence of the hydroxyl group at C-32 followed from the presence of an additional methyl singlet (1.19 ppm). Comparison of the data for compound **2** with those for **3** showed that they differed only in that the former lacked the hydroxyl group at the C-12 position.

Compounds **1**−**5** are all potent inhibitors of thrombin as shown in Table 6. The biological activity of this series of 5,5-*trans*-fused cyclic lactones has been studied in detail and is described elsewhere.[5] The mechanism of action as inhibitors of the blood-clotting cascade has been shown to be via acylation of the active-site Ser195 residue of thrombin. This acylating activity has been found to be generic against other serine protease enzymes, and this finding forms a basis for exploitation in drug discovery.

Experimental Section

General Experimental Procedures. UV spectra were obtained in MeOH using a Hewlett-Packard 8452-A diode array spectrophotometer. IR spectra were recorded in $CDCl_3$ on a Nicolet 20SXB instrument. NMR spectra were recorded on a Bruker AM500 instrument using standard pulse sequences. PSDQFCOSY and HMBC spectra were acquired in 512 increments, each with 4 K data points. HMQC spectra were acquired in 256 increments, each with 1 K data points. Delays were set using $^1J_{CH} = 135$ Hz and $^{LR}J_{CH} = 10$ Hz. 1D NOE difference experiments were recorded using a 5 s irradiation period and an acquisition time between 1.2 and 1.7 s. High-resolution liquid secondary-ion mass spectrometry measurements were made using a Micromass Autospec Q instrument (Micromass, Altrincham, Cheshire, UK). The magnet was scanned between *m*/*z* 140 and 1000 in 10 s at a resolution of 10 000. Ionization was via a cesium beam, 3 μA at 30 kV. The reference compound was PEG, and the matrix was *m*-nitrobenzyl alcohol. HPLC was performed using Perkin−Elmer, Gilsen, or Waters chromatographic equipment using the conditions described below.

Plant Material. *Lantana camara* leaves were obtained initially from Worldwide Butterflies, Sherborne, Dorset, and subsequently from Costa Rica and Guatemala. Herbarium specimens have been deposited in the Field Museum of Natural History, Chicago, Illinois.

Extraction and Isolation. Protocol 1: Dried leaves (0.5 g) were extracted overnight with 1.5 mL of MeOH to provide a sample for screening. The supernatant was removed and added to 100 mg of insoluble poly(vinylpyrrolidone) (PVP, Sigma) to adsorb polyphenols. The material was centrifuged for 5 min at 12 000 rpm, and the supernatant (10 μL) was used for screening in the fibrinogen clot assay.

Protocol 2: Dried leaves (46 kg) were extracted at room temperature with MeCN (400 L, 200 L). The filtrate was

Table 3. ^{13}C NMR Data for Compounds **1** and **4**

	compound	
position	**1**[a]	**4**[b]
1	30.0	31.5
2	22.6	22.8
3	78.8	76.8
4	52.5	50.1
5	41.9	42.3
6	25.4	26.3
7	118.0	123.2
8	143.5	138.9
9	45.0	50.2
10	33.4	36.1
11	16.9	31.4
12	28.1	71.0
13	39.2	47.0
14	55.2	62.1
15	35.6	30.8
16	82.8	84.7
17	58.9	57.0
18	21.3	16.0
19	11.9	13.2
20	40.8	42.0
21	180.2	182.7
22	43.6	43.2
23	197.0	200.4
24	123.1	124.7
25	156.1	157.3
26	27.5	27.6
27	20.8	20.9
30	17.3	17.5
31	175.6	174.9
32	32.5	69.4
Ac-3		172.3, 21.2
1′		95.6
2′		73.7
3′		78.1
4′		71.1
5′		78.7
6′		62.2

[a] In $CDCl_3$; referenced to solvent at δ 76.8. [b] In CD_3OD; referenced to solvent at δ 48.9.

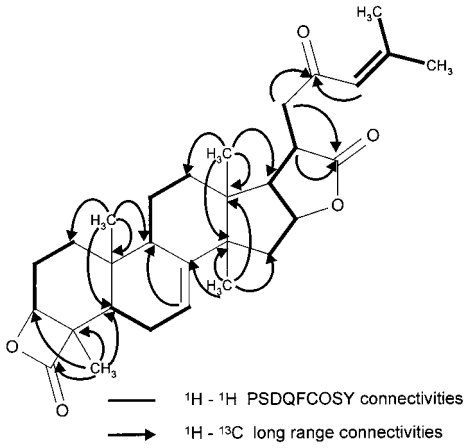

Figure 1. Summary of important $^1H-^1H$ and long-range $^1H-^{13}C$ NMR correlations observed for compound **1**.

separated and concentrated *in vacuo* to give an inactive solid material (450 g) and an active concentrated liquid extract (30 L). The liquid extract was stirred with Partisil Prep 40 (Whatman, 3 L) and H_2O (90 L), and then filtered. The slurry was loaded onto a column of Partisil Prep 40 (16 L) in MeCN−H_2O (2:3) and was chromatographed using a stepwise MeCN−H_2O gradient [2:3 (22 L), 1:1 (32 L), 3:2 (38 L), 7:3 (38 L), 4:1 (33 L)]. After 66 L, two active fractions eluted: fraction 1, with 42 L of eluent and fraction 2, with a further 54 L of eluent. Fraction 1 yielded 10.5 g of solid after removal of solvent. This was subjected to sequential chromatography in portions first on Kromasil C_8 7 μM, 25 × 15 cm i.d. in MeCN−0.05 M $NH_4H_2PO_4$, 3:2, 500 mL/min, detection λ 234 nm and then, after removal of salt and solvent, on Spherisorb S5 ODS2, 25 × 2.1 cm i.d. in MeCN−0.05 M $NH_4H_2PO_4$ 2:3, 25 mL/min, detection at λ 234 nm. Removal of salt and solvent from combined fractions with biological activity gave **3** (25 mg). Fraction 2, after removal of solvent, yielded 42.3 g of solid. This was subjected in portions to chromatograhy on Kromasil C_8 7 μM, 25 × 15 cm i.d. in MeCN−0.05 M $NH_4H_2PO_4$, 7:3, 580 mL/min, detection at λ 234 nm to yield, after bulking, two fractions with biological activity. Solvent and salt were removed from the earlier-eluted fraction, and the residue was subjected to chromatography on Spherisorb S5 ODS2, 25 × 2.1 cm i.d. in MeCN−0.1 M $NH_4H_2PO_4$, 52.5:47.5, 25 mL/min, detection at λ 234 nm. After removal of salt and solvent from the combined fractions with biological activity, minor impurities were removed from the residue by further chromatography on Sephadex LH-20 800 × 5 cm i.d. in MeCN. Evaporation of solvent from the fraction with biological activity yielded **2** (67 mg). Solvent and salt were removed from the later-eluted fractions to yield **1** (28 mg from half of combined fractions), which crystallized from MeOH.

Table 4. NOE Data for Compounds **1** and **4**

compound **1**		compound **4**	
signal irradiated	NOE seen at	signal irradiated	NOE seen at
18	9, 16, 20	18	9, 16, 20
19	3, 30, 32	19	
30	3, 19,	30	3, 19
32	17, 19,	32	12, 17

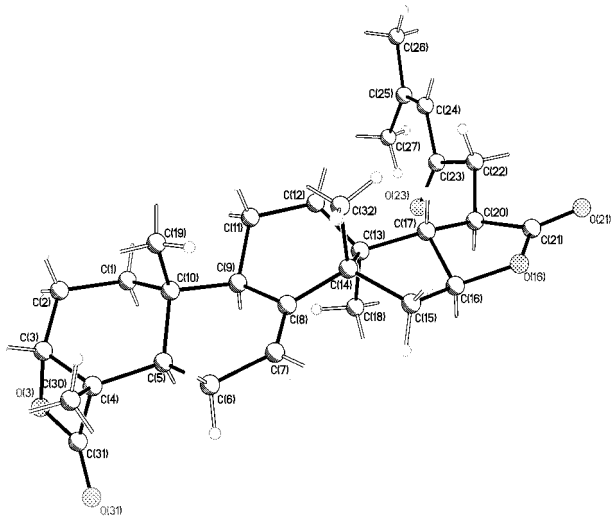

Figure 2. X-ray structure of compound **1**.

Protocol 3: Dried leaves (28 kg), initially treated with MeCN (2 × 200 L) and filtered, were subsequently extracted at room temperature in MeCN−H_2O−HCOOH (80:20:0.1). The acidic aqueous extract was separated from the plant material and treated with insoluble poly(vinylpyrrolidone) (PVP, 500 g, Sigma) to remove polyphenols and filtered. The filtrate was chromatographed over XAD16, eluting with 50% MeCN containing 0.01% HCOOH, and the fractions were screened for their ability to inhibit thrombin activity. Active fractions were rechromatographed on XAD16 using a stepwise gradient of MeCN−H_2O from 1:4 to 1:1 to give two pools of active fractions. Pool 1, eluted with MeCN−H_2O (2:3), was evaporated to dryness and chromatographed by sequential isocratic preparative HPLC using PRP-1 (MeCN−H_2O, 2:5), PRP-1 (MeCN−H_2O, 3:10), and Spherisorb ODS-2 (MeCN−H_2O, 3:10) to give **4** (47 mg). Pool 2 was fractionated by preparative HPLC on PRP-1 (MeCN−H_2O, 2:3) to give pools 3 and 4, which were subsequently chromatographed by HPLC on ODS-2 (MeCN−H_2O, 2:3) to give **5** (12 mg).

X-ray Crystallography on 1. Three-dimensional, room-temperature (295 K) X-ray data were collected on a crystal of

Table 5. Atomic Coordinates ($\times 10^4$) and Equivalent Isotropic Displacement Parameters (Å^2 $\times$ 10^3) for **1**[a]

	x	*y*	*z*	*U*(*eq*)
C(1)	−8940(3)	−475(8)	−117(3)	67(1)
C(2)	−9263(3)	125(10)	792(3)	87(2)
C(3)	−8751(3)	−994(8)	1543(3)	72(1)
O(3)	−9149(3)	−3105(8)	1716(2)	104(1)
C(4)	−7757(3)	−1956(7)	1441(2)	56(1)
C(5)	−7447(2)	−2202(6)	461(2)	47(1)
C(6)	−6364(3)	−2555(8)	418(2)	65(1)
C(7)	−6019(3)	−2402(7)	−503(2)	58(1)
C(8)	−6540(2)	−1759(5)	−1203(2)	43(1)
C(9)	−7609(2)	−1284(6)	−1133(2)	43(1)
C(10)	−7841(3)	−517(6)	−185(2)	46(1)
C(11)	−8040(2)	145(7)	−1890(2)	51(1)
C(12)	−7704(2)	−279(6)	−2836(2)	45(1)
C(13)	−6902(2)	−1858(5)	−2856(2)	40(1)
C(14)	−6122(2)	−1332(5)	−2086(2)	43(1)
C(15)	−5189(2)	−2543(7)	−2335(2)	53(1)
C(16)	−5410(2)	−3139(6)	−3295(2)	50(1)
O(16)	−4703(2)	−2865(4)	−3968(2)	59(1)
C(17)	−6234(2)	−1782(6)	−3628(2)	42(1)
C(18)	−7324(3)	−4074(6)	−2766(3)	51(1)
C(19)	−7378(3)	1605(6)	17(3)	70(1)
C(20)	−6315(2)	−2517(6)	−4589(2)	50(1)
O(21)	−4844(2)	−2537(5)	−5439(2)	67(1)
C(21)	−5230(3)	−2641(6)	−4746(3)	54(1)
C(22)	−6850(3)	−1140(7)	−5258(3)	59(1)
O(23)	−8272(2)	−3064(6)	−5020(2)	94(1)
C(23)	−7919(3)	−1511(7)	−5332(3)	55(1)
C(24)	−8476(3)	142(8)	−5794(3)	64(1)
C(25)	−9402(3)	89(8)	−6098(3)	68(1)
C(26)	−9830(4)	1959(10)	−6557(4)	103(2)
C(27)	−10 054(3)	−1713(10)	−6026(4)	98(2)
C(30)	−6999(3)	−1186(9)	2119(3)	85(2)
C(31)	−8256(5)	−3928(9)	1721(3)	82(2)
O(31)	−8026(4)	−5669(7)	1907(3)	136(2)
C(32)	−5844(3)	1015(6)	−2065(3)	55(1)

[a] U(*eq*) is defined as one-third of the trace of the orthogonalized *Uij* tensor.

Table 6. Activities of Triterpenes Isolated from *L. camara* Leaf Extract in a Fibrinogen Clot Assay

sample	IC_{50} nM
1	50
2	18
3	90
4	130
5	70
Hirudin	12

1 with approximate dimensions 0.16 × 0.16 × 0.08 mm, using a Siemens *R*3m/V diffractometer with monochromatized Cu Kα X-radiation. The $2\theta/\omega$ mode with a scan range (ω) of 1.20° plus Kα and a variable scan speed (1.95−14.65° min^{-1}) were used. Altogether, 3830 reflections were measured ($3 < 2\theta < 115°$, minimum *hkl* −15 −7 0, maximum *hkl* 15 7 17) of which 3663 were unique [$R(\sigma) = 0.0551$, Friedel opposites merged] and 2827 had $I > 2\,\sigma(I)$. Two control data, monitored every 98 reflections, showed no appreciable decay during 54.9 h of exposure of the crystal to X-rays.

Direct methods resulted in the location of all the nonhydrogen atoms. Full-matrix least-squares refinement was employed with anisotropic thermal parameters for all nonhydrogen atoms. Hydrogen atoms were refined in riding mode with common isotopic *U*s applied to some of the methyl hydrogens. Individual weights were applied according to the scheme $w = [\sigma^2(F_o^2) + (0.0727P)^2]^{-1}$, where $P = [F_o^2 + 2F_c^2]/3$ and refinement converged at *R* 0.0501, *R*w 0.1187, goodness-of-fit = 1.062. Maximum and mean shift/error in the final cycle of refinement were −0.001 and 0.000, respectively. The final electron density difference synthesis showed no peaks > 0.12 or holes < −0.12 e≈$^{-3}$. All computations were carried out using the SHELXTL for IRIX V5.03 system of programs.[6]

$C_{30}H_{40}O_5$, $M = 480.65$, monoclinic, $a = 13.940(6)$, $b = 6.434(2)$, $c = 15.151(4)$≈, $\beta = 92.79(3)°$, $V = 1357(1)$≈^{3} (by least-squares refinement on diffractometer angles for 15 automatically centered reflections, $\lambda = 1.54178$≈). Space group $P2_1$ (no. 4), $Z = 2$, $D_c = 1.18$ g cm^{-3}, $F(000) = 520$, μ (Cu Kα) = 0.59 mm^{-1}.[7]

Compound 1: obtained as colorless crystals (28 mg); UV (MeOH) λ_{max} 234 nm; IR ($CDCl_3$) ν_{max} 1811 (β-lactone C=O), 1775 (γ-lactone C=O), 1690 (α,β-unsaturated C=O) cm^{-1}; ^{1}H NMR, refer to Table 2; ^{13}C NMR, as given in Tables 3 and 4; HRMS, as given in Table 1; X-ray crystallography data, refer to Table 5.

Compound 2: obtained as an amorphous powder (67 mg); UV (MeOH) λ_{max} 234 nm; ^{1}H NMR, refer to Table 2; HRMS, refer to Table 1.

Compound 3: obtained as an amorphous powder (25 mg); UV (MeOH) λ_{max} 234 nm; ^{1}H NMR, refer to Table 2; HRMS, refer to Table 1.

Compound 4: obtained as an amorphous solid (47 mg); UV (MeOH) λ_{max} 234 nm; ^{1}H NMR, refer to Table 2; ^{13}C NMR, refer to Tables 3 and 4; HRMS, refer to Table 1.

Compound 5: obtained as an amorphous solid (12 mg); UV (MeOH) λ_{max} 234 nm; ^{1}H NMR, refer to Table 2; HRMS, refer to Table 1.

Fibrinogen-Clot Assay. Thrombin activity was measured as a function of clot formation using a turbidometric assay in microtiter plates. To each well was added 10 μL of test sample, 120 μL of fibrinogen solution (5.3 mg/mL fibrinogen in aqueous 0.16 M NaCl), 20 μL of thrombin solution (1 unit/mL at test), and 50 μL of HEPES buffer at pH 7.4. The assay plates were incubated at 37 °C, and the turbidity in the wells was measured after 60 min using a Biotek plate reader. The decreased scatter of light at 405 nm (of fibrin clots) was used quantitatively to estimate clot formation and hence thrombin activity. The observed activities for **1**−**5** are outlined in Table 6. Hirudin (Aldrich) was used as the positive control.

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