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NOVEL ANTIPLATELET NAPHTHALENE FROM *RHAMNUS NAKAHARAI*[1]

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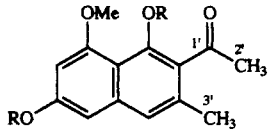
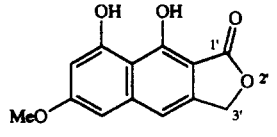
ABSTRACT.—A new naphthalene derivative, isotorachrysone [**1**], was isolated from the stem bark of *Rhamnus nakaharai* along with several known compounds. The antiplatelet effects of isotorachrysone [**1**], isotorachrysone peracetate [**2**], 6-methoxysorigenin [**3**], quercetin 3-*O*-methyl ether [**4**], and quercetin 3-*O*-methyl ether peracetate [**5**] were studied using washed rabbit platelets. Of the compounds tested, **1**, **2**, **4**, and **5** showed potent antiplatelet effects on arachidonic acid (AA-) and collagen-induced platelet aggregation. Compound **5** also showed potent antiplatelet effects on platelet-activating factor-(PAF-) induced platelet aggregation. Isotorachrysone [**1**] and its peracetate [**2**] were also studied for antiplatelet activity in human platelet-rich plasma (PRP) and both showed potent inhibition of the secondary aggregation induced by epinephrine. The antiplatelet effects of **1** and **2** are due partially to an inhibitory effect on thromboxane formation.

Several new anthraquinone, naphthalene, and flavonol glycosides from the roots of *Rhamnus nakaharai* Hayata (Rhamnaceae) have been reported previously by our group (1,2). In a continued study of this plant, a new naphthalene derivative, isotorachrysone [**1**], and the known compounds 6-methoxysorigenin [**3**] and quercetin 3-*O*-methyl ether [**4**] were isolated. Recently, we demonstrated that some flavonol derivatives of Formosan *Rhamnus* species inhibited the aggregation of rabbit platelets elicited by various inducers (3). From a search for antithrombotic agents from Formosan *Rhamnus* species, we report herein the characterization and antiplatelet effects of **1**, **1** peracetate [**2**], **3**, **4**, and **4** peracetate [**5**].

Compound **1** exhibited a uv spectrum similar to that of torachrysone (4), and the ^{1}H-nmr spectrum showed aromatic methyl, acetyl methyl, and methoxy



1 R=H
2 R=COMe



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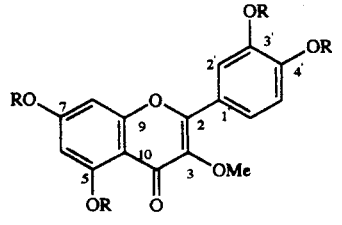

4 R=H
5 R=COMe

[1]Part 9 in the series "The Constituents of Formosan *Rhamnus* Species." For part 8, see Lin *et al.* (1).

signals at δ 2.52, 2.76, and 3.78, respectively. The aromatic protons appeared at δ 6.71 and 6.78 as a pair of meta-coupled signals (d, J=2.2 Hz), and a singlet at δ 7.06 was due to H-4. In the ^{1}H-nmr spectrum of **2**, the meta-coupled aromatic proton signals did not show apparent downfield shifts when compared to those of **1**. This did not correlate with an acetylation-induced shift (5). In the nOeds spectrum of **1** (Figure 1, in DMSO-d_6), irradiation of the methoxy signal showed enhancements of 5.64% and 5.00% for the doublet signal at δ 6.71 and the aromatic acetyl signal at δ 2.76. Thus, the nOeds spectrum indicated that the methoxyl group must be located at C-8 of **1**. In this manner, isotorachrysone [**1**] was characterized as 2-acetyl-8-methoxy-3-methylnaphthalene-1,6-diol.

The ^{13}C-nmr assignments of **1**, **3**, and **4** (Table 1) were obtained by ^{1}H-decoupled and DEPT pulse sequence experiments and by comparison with published data (6–8). The ^{13}C-nmr spectrum of **1** also supported structure **1**.

The antiplatelet effects of **1–5** were studied using procedures to evaluate the aggregation of washed rabbit platelets induced by thrombin (0.1 U/ml), arachidonic acid (AA) (100 μM), collagen (10 μg/ml), and platelet-activating factor (PAF) (2 ng/ml), and the results are shown in Table 2. Compounds **1–5** strongly inhibited AA- and collagen-induced platelet aggregation. Compound **5** also showed potent antiplatelet effects on PAF-induced aggregation. Esterified **4** [**5**]

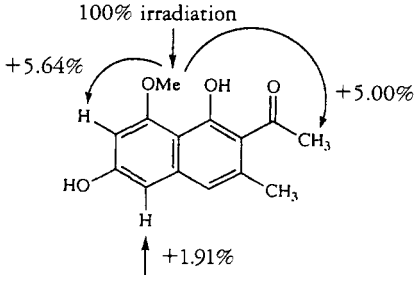

FIGURE 1. NOeds spectrum of **1**.

TABLE 1. ^{13}C-Nmr Chemical Shift Assignments of **1**, **3**, and **4**.[a]

Carbon	Compound		
	1[b]	**3**[b]	**4**[c]
1	158.4	172.7	
2	120.4	143.4	149.5
3	139.0	144.0	139.6
4	119.7	103.1	179.9
5	101.2	98.3	163.6
6	158.0	164.5	99.7
7	98.6	98.1	165.2
8	160.9	162.5	94.8
9	109.0	101.8	158.2
10	116.2	113.7	106.2
1′	204.2	176.6	123.4
2′	32.4		116.6
3′	21.6	70.2	145.2
4′			157.1
5′			116.7
6′			122.5
OMe	55.3	55.0	60.5

[a]The number of protons directly attached to each carbon was verified with the DEPT pulse sequence.
[b]Spectrum measured in pyridine-d_5.
[c]Spectrum measured in Me_2CO-d_6.

showed an enhancement of the antiplatelet effect on PAF-induced platelet aggregation. Aspirin was used as a positive control. It was also found (see Table 2) that aspirin (50 μM) completely inhibited AA-induced platelet aggregation but not aggregation induced by thrombin, collagen, or PAF. Further experiments were performed to study the effects of **1**, **2**, **4**, and **5** on AA- (**1**, **2**, **4**, and **5**), **4** and **5** on collagen-, and **5** on PAF-induced platelet aggregation at various concentrations. Of the compounds tested using AA- induced platelet aggregation, **2** was the most potent, and **1** was less potent, while **5** did not show enhancement of the antiplatelet effect of **4** (Figure 2). In collagen-induced platelet aggregation, however, **5** was slightly more potent than **4** (Figure 3). Compound **5** also inhibited PAF-induced platelet aggregation in a concentration-dependent manner (Figure 4). Of the three inducers tested (Figures 2–4), AA-induced platelet aggregation was most easily inhibited by **2**, which

TABLE 2. Effects of **1–5** and Aspirin on Platelet Aggregation Induced by Thrombin, Arachidonic Acid (AA), Collagen, and Platelet-Activating Factor (PAF) in Washed Rabbit Platelets.[a]

Agent (μM)	Aggregation (%)			
	Thrombin (0.1 U/ml)	AA (100 μM)	Collagen (10 μg/ml)	PAF (2 ng/ml)
Control	94.6 ±0.7 (4)	89.8 ±1.5 (4)	90.2 ±2.5 (3)	94.0 ±1.3 (4)
1 (400)	92.2 ±3.3 (3)	0.0 ±0.0 (3)[b]	39.1 ±4.6 (3)[b]	94.2 ±0.8 (3)
2 (300)	82.7 ±1.4 (3)[b]	0.0 ±0.0 (3)[b]	16.8 ±8.7 (3)[b]	86.7 ±0.9 (3)[b]
3 (300)	88.1 ±1.0 (4)[c]	22.1 ±18.1 (3)[c]	41.7 ±12.1 (3)[c]	80.0 ±2.4 (3)[b]
4 (300)	81.2 ±3.0 (3)[c]	0.0 ±0.0 (3)[b]	5.4 ±4.6 (4)[b]	50.1 ±8.2 (4)[b]
5 (300)	64.9 ±6.8 (3)[c]	0.0 ±0.0 (3)[b]	0.0 ±0.0 (3)[b]	0.0 ±0.0 (3)[b]
Aspirin (50)	91.9 ±2.5 (3)	0.0 ±0.0 (3)[b]	85.4 ±3.9 (3)	90.5 ±1.2 (3)

[a]Platelets were preincubated with various agents, aspirin or DMSO (0.5%, control) at 37° for 3 min, and the inducer was then added. Percentages of aggregation are presented as mean±SEM (*n*).

[b]$p<0.001$ as compared with control values.

[c]$p<0.01$ as compared with control values.

had an IC_{50} value of about 5.1 μM (Figure 2).

Recently, we have demonstrated that quercetin-related compounds inhibit the aggregation and release reaction of rabbit platelets caused by various inducers and that glycosylation of quercetin-related compounds at OH-3, with one sugar molecule, produces enhancement of antiplatelet effects (3,9). In this study and previous reports (3,9), we have further demonstrated that among the quer-

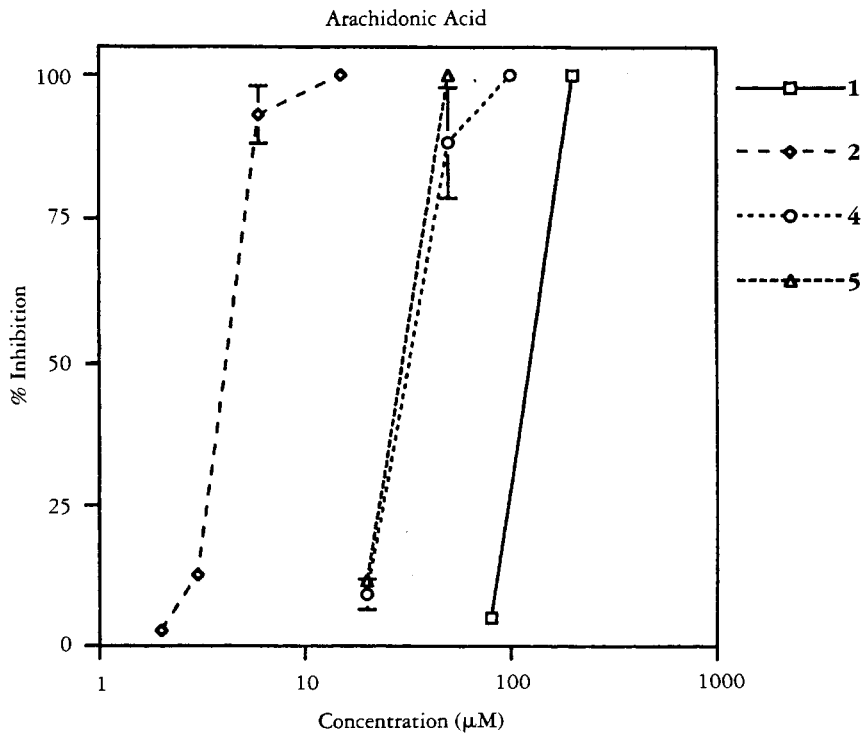

FIGURE 2. The effects of **1**, **2**, **4**, and **5** on AA-induced platelet aggregation. Washed rabbit platelets were incubated with various concentrations of these agents and AA (100 μm) was added.

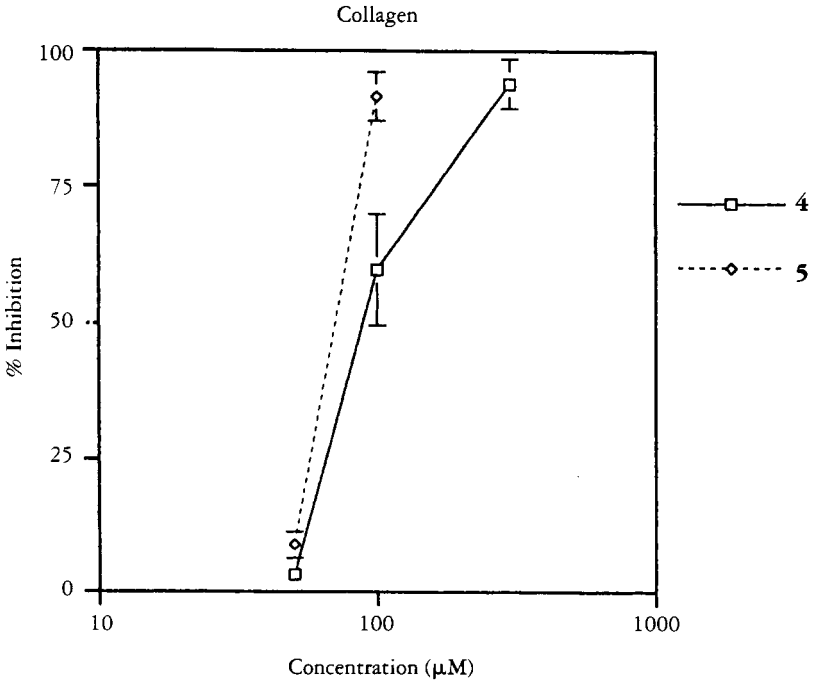

FIGURE 3. The effects of **4** and **5** on collagen-induced platelet aggregation. Washed rabbit platelets were incubated with various concentrations of these agents and collagen (10 μg/ml) was added.

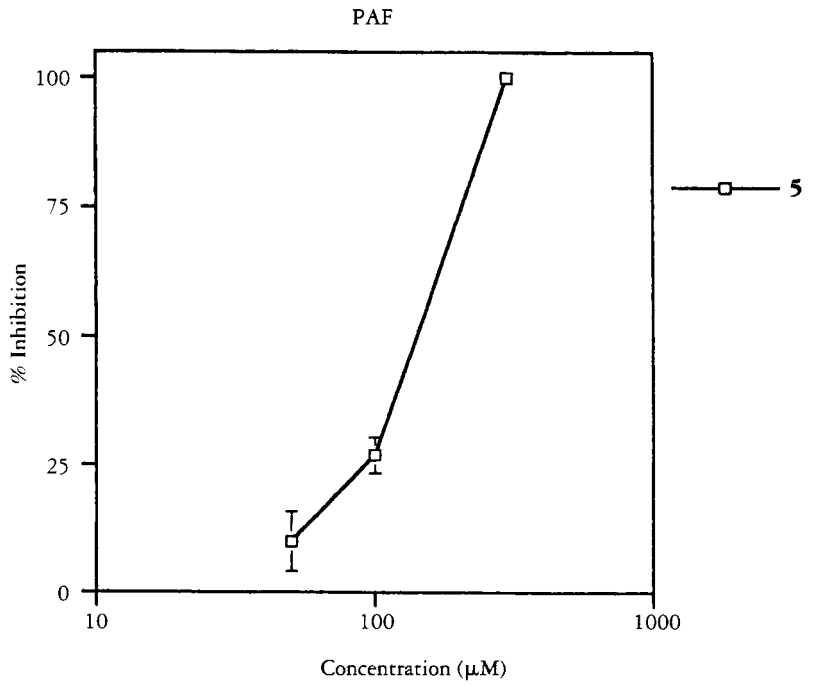

FIGURE 4. The effects of **5** on PAF-induced platelet aggregation. Washed rabbit platelets were incubated with various concentrations of **5** and PAF (2 ng/ml) was added.

cetin-related compounds studied, **4** and **5** were the most potent inhibitors of AA-induced platelet aggregation (Figure 2). Thus, the OH-3 of quercetin-related compounds appears to be the functional group related to antiplatelet effect on AA-induced platelet aggregation.

Antiplatelet effects of **1** and **2** were also studied on the aggregation of human platelet-rich plasma (PRP) induced by epinephrine (5 μM). As shown in Table 3, **1** and **2** had potent antiplatelet effects. In human PRP, these two compounds prevented secondary aggregation induced by epinephrine (Figure 5). We suggest that their mechanism of action is par-

TABLE 3. Antiplatelet Effects of **1** and **2** on Epinephrine-induced Human PRP.[a]

Treatment (μM)	Aggregation (%)
	Epinephrine (5 μM)
Control	93.7±2.9 (3)
1 (100)	20.2±1.3 (4)[b]
2 (50)	13.0±5.1 (4)[b]
2 (20)	83.0±0.7 (5)[c]
Aspirin	39.6±15.4

[a]PRP was preincubated with DMSO (0.5%, control), **1**, **2**, or aspirin at 37° for 3 min, and epinephrine was then added. Values are presented as mean±SEM (n).

[b]$p<0.001$.

[c]$p<0.01$, as compared with control values.

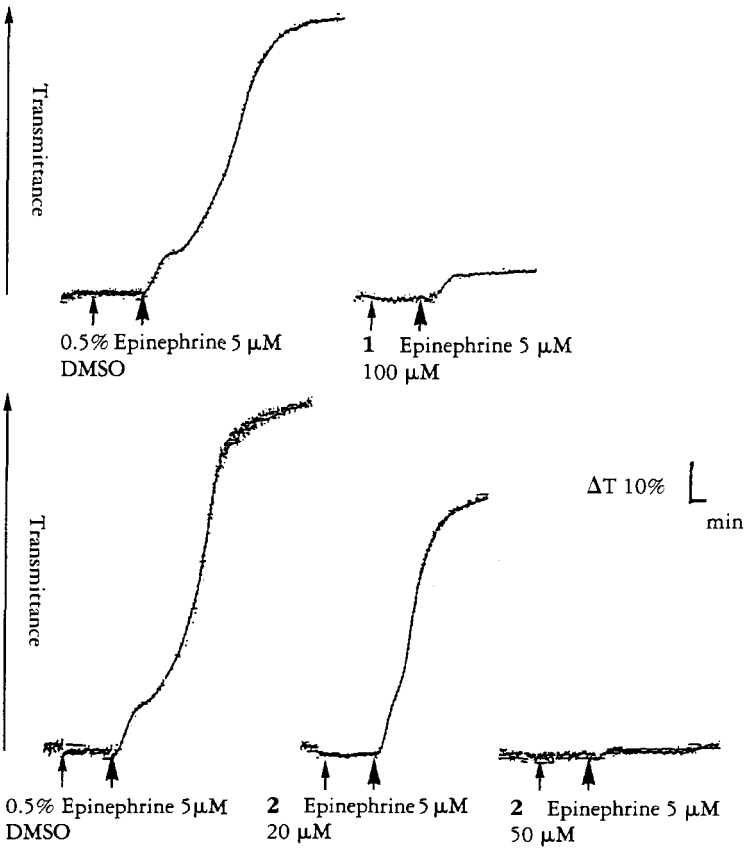

FIGURE 5. Inhibitory effects of **1** and **2** on epinephrine-induced aggregation of human platelet-rich plasma (PRP). PRP was incubated with DMSO (0.5%) and various concentrations of **1** and **2** for 3 min, then adrenaline (5 μM) was added to trigger the aggregation.

tially due to the inhibition of thromboxane formation (10–13).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All mps are reported uncorrected. Uv spectra were obtained on a Jasco uv-vis spectrophotometer; ^{1}H- and ^{13}C-nmr (200 MHz and 400 MHz, respectively) spectra were recorded on Varian Gemini-200 and Unity-400 spectrometers; ir spectra were recorded on a Hitachi Model 260-30 spectrometer; and ms were obtained on a JMS-HX 110 mass spectrometer.

PLANT MATERIAL.—Fresh stem bark (10 kg) of *Rhamnus nakaharai* Hayata (Rhamnaceae) was collected at Ali, Wu-Tai Shian, Ping-Tung Hsieng, Taiwan, during July 1990, and was chipped and extracted with $CHCl_3$ and MeOH, sequentially. A voucher specimen is deposited in our laboratory.

EXTRACTION AND ISOLATION.—The $CHCl_3$ and MeOH extracts were chromatographed on Si gel with a gradient solvent systems of cyclohexane, $CHCl_3$, and MeOH. The $CHCl_3$ extract, when eluted with $CHCl_3$-cyclohexane-MeOH (75:15:10) yielded isotorachrysone [**1**] (0.05%) and when eluted with $CHCl_3$-cyclohexane-MeOH (80:10:10) yielded 6-methoxysorigenin [**3**] (0.0005%). The MeOH extract eluted with $CHCl_3$-cyclohexane-MeOH (70:15:15) gave quercetin 3-*O*-methyl ether [**4**] (0.01%). The other fractions contained several known compounds isolated previously from the roots of this plant (1,2). The known compounds [**3**, **4**] were identified by uv, ir, nmr, and ms data (6,14). Compounds **2** and **5** were acetylated in the usual manner and **5** was also identified by uv, ir, nmr, and ms data (14).

2-Acetyl-8-methoxy-3-methylnaphthalene-1,6-diol (isotorachrysone) [**1**].—Yellow needles ($CHCl_3$/MeOH), mp 219°; ir ν max (KBr) 1642 (chelated C=O), 1595 cm^{-1}; uv λ max (MeOH) (log ϵ) 210 (4.28), 230 (4.32), 272 (4.28), 340 (sh) (3.66), 400 (3.88) nm; ^{1}H nmr (pyridine-d_5) δ 2.52 (3H, s, Ar-Me), 2.76 (3H, s, Ar-COMe), 3.78 (3H, s, OMe), 6.71 (1H, d, J=2.2 Hz, H-5), 6.78 (1H, d, J=2.2 Hz, H-7), 7.06 (1H, s, H-4); ^{13}C-nmr data (pyridine-d_5), see Table 1; eims *m/z* 246 [M]$^+$ (73), 232 (15), 231 [M−Me]$^+$ (100), 228 [M−H_2O]$^+$ (13), 203 [M−COMe]$^+$ (2), 185 (12), 175 (10), 160 (7), 157 (7), 132 (7), 128 (9), 115 (14), 81 (8), 77 (10), 69 (22), 55 (10), 43 (22); hreims, *m/z* found 246.0898; *anal.*, calcd for $C_{14}H_{14}O_3$ 246.0891.

Isotorachrysone peracetate [**2**].—Colorless mass (MeOH); mp 176°; ir ν max (KBr) 1765, 1695 (chelated C=O), 1635 cm^{-1}; ^{1}H nmr ($CDCl_3$) δ 2.31 (3H, s, Ar-Me), 2.37, 2.38 (each 3H, s, OAc), 2.50 (3H, s, Ar-COMe), 3.88 (3H, s, OMe), 6.80 (1H, d, J=2.0 Hz, H-5), 6.98 (1H, d, J=2.0 Hz, H-7), 7.46 (1H, s, H-4); eims *m/z* 330 [M]$^+$ (10), 288 [M−42]$^+$ (11), 246 [M−84]$^+$ (100), 231 (69), 228 (13), 217 (6), 43 (82).

PLATELET AGGREGATION ASSAYS.—Washed rabbit platelets were obtained from ethylene diamine tetraacetic acid (EDTA)-anticoagulated platelet-rich plasma (PRP) according to procedures described previously (15). Platelet numbers were counted by a Coulter Counter (Model ZM) and adjusted to 4.5×10^8 platelets/ml. The platelet pellets were suspended in Tyrode's solution containing (mM): 136.8 NaCl, 2.8 KCl, 11.9 $NaHCO_3$, 2.1 $MgCl_2$, 0.33 NaH_2PO_4, 1.0 $CaCl_2$, and 11.2 glucose with 0.35% bovine serum albumin. Human PRP was obtained from the supernatant after centrifugaiton of venous blood mixed with 3.8% sodium citrate (1:9 to blood). All glassware was siliconized. Four min before addition of the aggregation inducer, PRP or the platelet suspension was stirred at 1200 rev min^{-1}. Aggregation was measured by a turbidimetric method (16). The absorbance of PRP or the platelet suspension was taken as 0% aggregation and the absorbance of platelet-poor plasma or platelet-free Tyrode's solution was 100% aggregation. Aggregation was measured by a Lumi-aggregometer (Chrono-Log Co.) connected to dual channel recorders. Compounds were dissolved in DMSO. In order to eliminate the effect of solvent on platelet aggregation, the final concentration of DMSO was fixed at 0.5%. Collagen (type 1, bovine Achilles tendon), obtained from Sigma Chemical Co. (St. Louis, MO), was homogenized in 25 mM HOAc and stored at −70° at a concentration of 1 mg/ml. Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) also purchased from Sigma, was dissolved in $CHCl_3$ and diluted into 0.1% bovine serum albumin-saline solution immediately prior to use. Arachidonic acid (AA), ADP, bovine serum albumin (BSA), epinephrine, EDTA (disodium salt), and sodium citrate were also purchased from Sigma. Thrombin (bovine) was obtained from Parke Davis Co. (Detroit, MI) and dissolved in 50% glycerol to give a stock solution of 100 NIH units/ml.

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