Bioactive Constituents of *Morus australis* and *Broussonetia papyrifera*

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The biological activities of the active principles of two plants in the Moraceae have been investigated. A new prenylflavonoid, australone A (**1**), and a new triterpenoid, 3β-[(*m*-methoxybenzoyl)oxy]urs-12-en-28-oic acid (**2**) were isolated from the root bark of *Morus australis*, and their structures determined by spectroscopic methods. Also isolated from this plant were seven known compounds, morusin (**3**), kuwanon C (**4**), betulinic acid, β-amyrin, quercetin, ursolic acid, and compound A. Morusin (**3**) showed significant effects on arachidonic acid-, collagen-, and PAF-induced platelet aggregation, while kuwanon C (**4**) was active in the arachidonic acid- and PAF-induced platelet aggregation assays. In biological work on a second plant, *Broussonetia papyrifera*, broussoflavonols F (**5**) and G (**6**), broussoflavan A (**7**), and broussoaurone A (**8**) potently inhibited Fe^{2+}-induced lipid oxidation in rat-brain homogenate. Compounds **5**−**7** also significantly inhibited the proliferation of rat vascular smooth muscle cells.

In previous papers, the antiplatelet activity of prenylflavonoids isolated from Formosan Moraceous plants were reported.[1,2] In a continued search for bioactive constituents from plants in this family, two new compounds, australone A (**1**) and an ursolic acid ester (**2**), and seven known compounds, morusin (**3**), kuwanon C (**4**), betulinic acid, β-amyrin, quercetin, ursolic acid, and compound A, were isolated from *Morus* australis Poir. In the present paper the characterization of **1** and **2**, and the antiplatelet effects of **3** and **4** are reported.

Cardiovascular disease remains the chief cause of death in the United States and Western Europe.[3] In the course of a continued screening investigation on active cardiovascular agents from Chinese herbs, the antioxidant properties and effects on the proliferation of rat vascular smooth muscle cells of four compounds, broussoflavonols F (**5**) and G (**6**), broussoflavan A (**7**), and broussoaurone A (**8**), whose structures were recently reported[2] and which were isolated from *Broussonetia papyrifera*, also are described in this paper.

Results and Discussion

The HRMS of **1** revealed a $[M]^+$ at *m*/*z* 420.1557, which corresponded to the molecular formula $C_{25}H_{24}O_6$. The IR spectrum of **1** showed hydroxyl and chelated carbonyl absorption bands at 3300 and 1655 cm^{-1}, respectively. The UV spectrum of **1** exhibited similar absorption maxima to those of artocarpetin and artocarpetins A and B.[4] The ^{1}H-NMR (acetone-d_6) spectrum of **1** showed signals of a 2-methyl-2-(4-methylpent-3-enyl)chromene ring at δ 1.45, 1.57, and 1.64 (9H, 3s, $3CH_3$), 1.7−1.9 (2H, m, H_2-13), 2.1−2.2 (2H, m, H_2-14), 5.13 (1H, t, J = 7.2 Hz, H-15), 5.72 (1H, d, J = 10 Hz, H-10), and 6.71 (1H, d, J = 10 Hz, H-9); a 2′,4′-dihydroxy-substituted B ring at δ 6.57 (1H, dd, J = 8.8, 2.4 Hz, H-5′), 6.61 (1H, d, J = 2.4 Hz, H-3′), and 7.87 (1H, d, J = 8.8 Hz, H-6′), and two singlets at δ 6.46 (1H, s, H-6 or H-8) and δ 7.09 (1H, s, H-3), indicating that it was a prenylflavonoid.[5] In addition, the UV spectrum of **1** showed no bathochromic shift upon addition of aluminum chloride,[6] the signals observed at δ 6.46 and 6.71 in the ^{1}H-NMR spectrum of **1** were closely comparable to those of the H-8 and H-9 (olefinic proton on pyran ring) signals of a linear type of pyranoflavone.[7] Therefore, the signal appearing at 6.46 was assigned to H-8 of **1**. On the basis of the above evidence, australone A was characterized as 5,2′,4′-trihydroxy-6,7-[2-methyl-2-(4-methylpent-3-enyl)chromeno] flavone (**1**). The ^{13}C-NMR (pyridine-d_5) assignments (Table 1) of **1**

Table 1. ^{13}C-NMR Chemical Shift Assignment of **1** and **2**[a]

carbon	**1** (pyridine-d_5)[b]	carbon	**2** ($CDCl_3$)[c]	carbon	**2**
1		1	38.1	27	23.6
2	163.0	2	23.3	28	184.1
3	108.2	3	81.7	29	17.1
4	183.2	4	38.3	30	21.2
4a	105.3	5	55.4	1′	132.3
5	157.0	6	18.2	2′	114.2
6	105.7	7	32.8	3′	159.5
7	159.8	8	39.5	4′	119.0
8	95.2	9	47.9	5′	129.3
8a	157.6	10	36.7	6′	121.9
9	116.4	11	18.2	OMe	55.3
10	129.2	12	125.7	CO	166.1
11	80.6	13	137.9		
12	27.0	14	41.0		
13	41.8	15	28.0		
14	23.1	16	24.0		
15	124.5	17	47.4		
16	131.8	18	52.5		
17	17.6	19	39.0		
18	25.7	20	38.8		
1′	110.2	21	30.6		
2′	160.7	22	36.9		
3′	104.4	23	28.2		
4′	163.6	24	17.0		
5′	108.9	25	15.5		
6′	130.7	26	17.0		

[a] The number of protons directly attached to each carbon was verified by a DEPT experiment. [b] Signals obtained by ^{1}H−^{1}H COSY, HMQC, and NOESY techniques. [c] Signals obtained by ^{1}H−^{1}H, ^{1}H−^{13}C COSY, and ^{1}H-^{13}C long-range coupling techniques.

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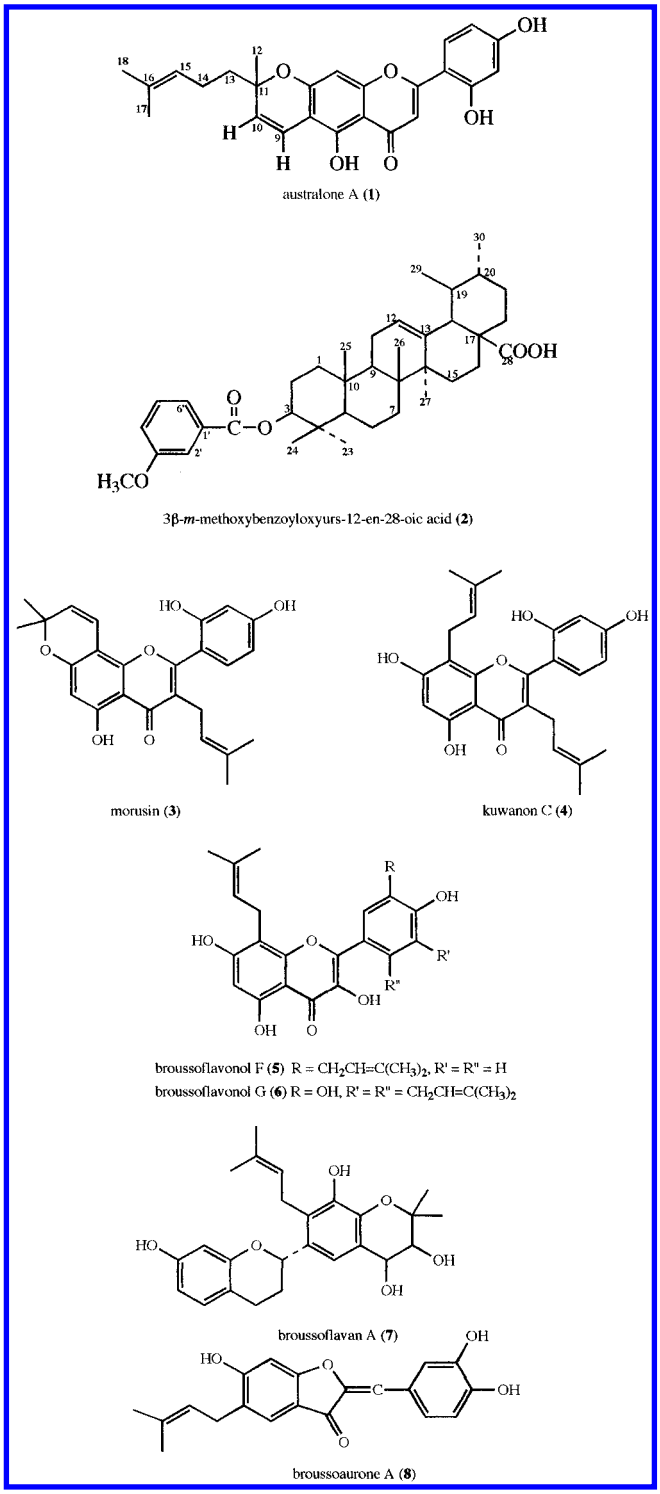

were made by performing ^{1}H-decoupled, DEPT, and 2D ^{1}H−^{13}C correlation experiments (Figure 1) and from related data in the literature.[5,8] The data obtained from the MS, and from the ^{13}C-NMR and HMBC (Figure 1) spectra also supported the structural assignment of **1**.

The HRMS of **2** gave a molecular ion at *m/z* 590.3949, which corresponded to the molecular formula $C_{38}H_{54}O_5$. It showed a positive Libermann−Burchard reaction and IR absorption bands at 1700 and 1690 cm^{-1} for ester carbonyl and carboxyl groups, respectively. The EIMS displayed two characteristic fragment ion peaks (*m/z* 248 and 203) due to *retro*-Diels−Alder-type fragmentation of an olean-12-en- or an urs-12-en-28-oic acid derivative bearing no substituent in either the C, D, or E rings.[9] The ^{1}H-NMR spectrum of **2** showed five

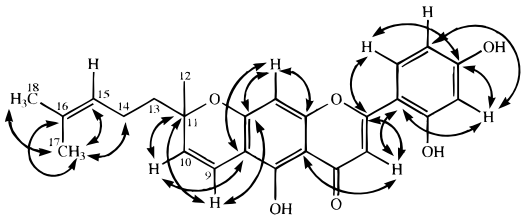

Figure 1. C/H long-range correlations of **1** obtained from HMBC spectra.

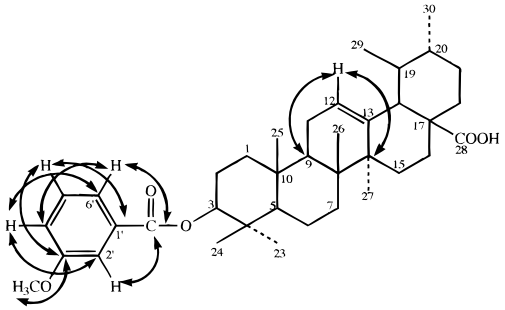

Figure 2. C/H long-range correlations of **2** obtained from HMBC spectra.

tertiary methyls, two secondary methyls, one deshielded methine proton on C-3 bearing an OCOR group, a methoxy, an olefinic proton, and a 1,3-disubstituted phenyl group. On the basis of the data obtained and the appearance of two characteristic fragment ion peaks at *m/z* 438 [M − methoxybenzoic acid]$^+$ and 544 [M − COOH − H]$^+$ in the mass spectrum, **2** was characterized as 3*β*-[(*m*-methoxybenzoyl)oxy]urs- or -olean-12-en-28-oic acid. The ^{13}C-NMR spectrum of **2** (Table 1) was assigned by conducting ^{1}H-decoupled, DEPT, ^{1}H−^{13}C COSY, and ^{1}H-^{13}C long-range correlation experiments (Figure 2) and by comparing the chemical shift values with those of corresponding data for methyl 3*β*-acetylursolate.[10] The ^{13}C-NMR data therefore permitted the characterization of **2** as 3*β*-[(*m*-methoxybenzoyl)oxy]urs-12-en-28-oic acid (**2**).

The antiplatelet effects of the constituents isolated from *Morus australis* (**1**−**4**) were studied on the aggregation of washed rabbit platelets induced by arachidonic acid (AA, 100 *μ*M), collagen (10 *μ*g/mL), PAF (platelet-aggregating factor) (2 ng/mL), and thrombin (0.1 U/mL). As shown in Table 2, compounds **3** and **4** inhibited the aggregation of washed rabbit platelets. The degree of the inhibition was shown to be different depending upon the type of aggregation inducer. AA-, collagen-, and PAF-induced aggregations were almost completely inhibited by **3** and **4**, while that induced by thrombin was slightly affected by **3** but markedly inhibited by **4**. Prenylflavonoids **3** and **4** with a different chemical moiety only at the C-8 substituent showed different antiplatelet effects. Because AA- and collagen-induced aggregations were markedly inhibited by **3** and **4**, part of the antiaggregatory effects mediated by these compounds may be due to inhibition of thromboxane A_2 formation. Compounds **3** and **4** also inhibited platelet aggregation induced by PAF, which did not cause thromboxane formation in rabbit platelet.[11] Further experiments are required to elucidate their inhibitory mechanism.

We have previously reported that the constituents of *Broussonetia papyrifera*, broussoflavan A (**5**), broussoflavonol F (**6**), broussoflavonol G (**7**), and broussoau-

Table 2. Effects of Compounds **3** and **4** and Aspirin on Platelet Aggregation Induced by Thrombin, AA, Collagen, and PAF in Washed Rabbit Platets[a]

	aggregation (%)			
agent (μM)	thrombin (0.1 unit/mL)	AA (100 μM)	collagen (10 μg/mL)	PAF (2 ng/mL)
control	91.1 ± 1.9 (4)	87.0 ± 1.7 (5)	90.4 ± 2.4 (4)	90.0 ± 1.2 (5)
3 (100)	81.8 ± 3.5 (3)[d]	0.0 ± 0.0 (3)[b]	5.3 ± 4.6 (4)[b]	0.0 ± 0.0 (3)[b]
(50)		74.2 ± 2.7(3)[b]		62.5 ± 8.9 (3)[c]
(20)		82.8 ± 2.8 (3)		81.3 ± 2.3 (3)[c]
4 (300)	54.8 ± 10.6 (4)[c]	0.0 ± 0.0 (5)[b]	5.5 ± 2.3 (3)[b]	0.0 ± 0.0 (5)[b]
(100)		5.9 ± 3.7 (5)[b]		26.9 ± 1.5 (5)[b]
(50)		60.2 ± 13.7 (5)		67.6 ± 6.3 (5)[c]
(20)		83.9 ± 2.5 (5)		78.8 ± 4.0 (5)[d]
aspirin (50)	91.9 ± 2.5 (3)	0.0 ± 0.0 (3)[b]	85.4 ± 3.9 (3)	90.5 ± 1.2(3)

[a] Washed rabbit platelets were preincubated with various agents, aspirin, or DMSO (0.5%, control) at 37 °C for 3 min, and the inducer was then added. Percentages of aggregation are presented as means ± S.E.M. (n). [b] $P < 0.001$. [c] $P < 0.01$. [d] $P < 0.05$ as compared with control values.

Table 3. Effects of Constituents of *Broussonetia papyrifera* on Thiobarbituric Acid-Reactive Substance Formation Caused by Fe^{2+} in Rat-Brain Homogenate

compound	IC_{50} (μM)[a]
broussoflavan A (**5**)	2.1
broussoflavonol F (**6**)	2.7
broussoflavonol G (**7**)	1.0
broussoaurone A (**8**)	1.2
butylated hydroxytoluene (100 μM)	14.5

[a] Data are mean values from two independent experiments.

Table 4. Effects of Constituents of *Broussonetia papyrifera* on [^{3}H]Thymidine Incorporation into DNA in the Proliferation of Rat VSMCs[a]

compound	FCS (10%)	PDGF (20 ng/mL)	ADP (10 μM)	5-HT (10 μM)
broussoflavan A (**5**)	39.5	258	67	19
broussoflavonol F (**6**)	0	127	7.8	0
broussoflavonol G (**7**)	0	127	0.4	0

[a] Inhibition by compounds **5**−**7** of [^{3}H]-thymidine incorporation in rat VSMCs stimulated with fetal calf serum (FCS), PDGF, ADP, or 5-HT. Data (expressed as % of control) are mean values from two independent experiments.

rone A (**8**) have antiplatelet effects.[2] In the course of a screening investigation on antioxidant constituents with antiproliferative effects in rat vascular smooth muscle cells (VSMC), the effects of compounds **5**−**8** on thiobarbituric acid-reactive substance (TBARS) formation caused by Fe^{2+} in rat-brain homogenate, and the effects of **5**−**7** on fetal calf serum (FCS)-, platelet-derived growth factor (PDGF)-, 5-hydroxytryptamine (5-HT)-, or ADP-induced [^{3}H]-thymidine incorporation into DNA as an indicator of antiproliferation were examined. Compounds **5**−**8** inhibited the Fe^{2+}-induced formation of TBARS in a concentration-dependent manner (Table 3). Butylated hydroxytoluene was used as positive control. It (100 μM) indicated a 91.3% inhibition of the Fe^{2+}-induced formation of TBARS. Compounds **6** and **7** inhibited FCS-, ADP-, and 5-HT-induced [^{3}H]thymidine incorporation into rat VSMC in a concentration-dependent manner, in each case. This indicates that compounds **6** and **7**, which have potent antiplatelet agents with antioxidant properties and inhibitory effects on proliferation of rat VSMC, may have therapeutic implications for atherosclerosis and vascular diseases.

Experimental Section

General Experimental Procedures. Melting points are reported uncorrected. Optical rotations were obtained on JASCO model DIP-370 digital polarimeter; UV spectra were obtained on a JASCO model 7800 UV-vis spectrophotometer; IR spectra were recorded on a Hitachi model 260-30 spectrophotometer; ^{1}H- and ^{13}C-NMR (400 MHz) spectra were recorded on a Varian Unity-400 spectrometer; and MS were obtained on a JMS-HX 100 mass spectrometer.

Plant Material. The roots of *Morus australis* (20 kg) were collected at Kaohsiung Hsien, Taiwan, during May 1995, and a voucher specimen has been deposited in the authors' laboratory (voucher no. M-03).

Extraction and Isolation. The cortex (0.85 kg) and decorticated root bark (2 kg) of *M. australis* were chipped and extracted with $CHCl_3$ at room temperature sequentially. The $CHCl_3$ extract of the cortex was chromatographed over Si gel. Elution with cyclohexane−EtOAc (4:1) yielded betulinic acid (1.2 g); cyclohexane−EtOAc (3:1) yielded **3** (3.5 g); cyclohexane−EtOAc (2:1) yielded **4** (30 mg); cyclohexane−EtOAc (1:1) yielded **1** (10 mg); EtOAc−MeOH (9:1) yielded quercetin (50 mg); and CH_2Cl_2−MeOH (9.5:0.5) yielded ursolic acid (0.1 g) and compound A (35 mg). The $CHCl_3$ extract of the decorticated root bark also was chromatographed on Si gel. Elution with cyclohexane−Me_2CO (19:1) yielded **2** (20 mg) and benzene−cyclohexane−EtOAc (5:4:1) yielded β-amyrin (10 mg). Betulinic acid, **3**, **4**, quercetin, ursolic acid, compound A, and β-amyrin were identified by spectroscopic methods and comparison with authentic data.[12−16]

Australone A (1): obtained as yellowish needles (C_6H_{12}−Me_2CO); mp 195−197 °C; $[\alpha]^{25}_D$ −36° (*c* 0.05, Me_2CO); UV (MeOH) λ_{max} (log ϵ) 230 (4.40), 2.90 (4.44), 310 (sh) (4.24), 358 (4.42) nm, (MeOH−$AlCl_3$) 235, 295, 325 (sh), 385 nm, (MeOH−NaOAc) unchanged, (MeOH−NaOAc−H_3BO_3) unchanged, (MeOH−NaOMe) 270, 285 (sh), 307 (sh), 425 nm; IR (KBr) ν_{max} 3300 (OH), 1655 (chelated CO); ^{1}H NMR (acetone-d_6), see text; ^{13}C NMR (pyridine-d_5), see Table 1; EIMS (70 eV) *m/z* $[M]^+$ 420 (24), 405 (6), 377 (3), 351 (2), 337 (100), 323 (1), 203 (40), 176 (5), 147 (7), 136 (16); HRMS, *m/z* found 420.1557, calcd for $C_{25}H_{24}O_6$, 420.1572.

3β-[(*m*-Methoxybenzoyl)oxy]urs-12-en-28-oic acid (2): obtained as a colorless powder ($CHCl_3$); mp 174−176 °C; $[\alpha]^{27}_D$ 4° (*c* 0.1, MeOH); IR (KBr) ν_{max} 1700 (CO), 1690 (COOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.79 (3H, s, Me-26), 0.87 (3H, d, J = 6.4 Hz, Me-29), 0.94 (3H, s, Me-23), 0.95 (3H, d, J = 6.4 Hz, Me-30), 1.01 (6H, s, Me-24 and Me-25), 1.10 (3H, s, Me-27), 3.85 (3H, s, MeO), 4.74 (1H, q, J = 5.6, 5.2 Hz, H-3α), 5.25 (1H, t, J = 3.2 Hz, H-12), 7.10 (1H, m, H-4′), 7.34 (1H, t, J = 8.0 Hz, H-5′), 7.57 (1H, m, H-2′), 7.64 (1H, m, H-6′); ^{13}C NMR ($CDCl_3$), see Table 1; EIMS (70 eV) *m/z* $[M]^+$ 590

(3), $[M - COOH - H]^+$ 544 (13), [M − methoxybenzoic acid]$^+$ 438 (19), 248 (100), 203 (63), 190 (67), 189 (43), 135 (75), 133 (46); HRMS, *m/z* found 590.3949, calcd for $C_{38}H_{54}O_5$, 590.3971.

Compounds **5**−**8** were isolated and characterized from *Broussonetia papyrifera* as previously described.[17−19]

Platelet Aggregation Assay. Washed rabbit platelets were obtained from EDTA-anticoagulated platelet-rich plasma (PRP), according to procedures described previously.[10] 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 (BSA). All glassware was siliconized. Four minutes before addition of the aggregation inducer, the platelet suspension was stirred at 1200 rpm. Aggregation was measured by a turbidimetric method.[20] The absorbance of the platelet suspension was taken as 0% aggregation and the absorbance of platelet-free Tyrode's solution as 100% aggregation. Aggregation was measured by a Lumi-aggregometer (Chrono-Log Co., Havertown, PA) 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 at HOAc and stored at −70 °C at a concentration of 1 mg/mL. Platelet-activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), purchased from Sigma, was dissolved in $CHCl_3$ and diluted into 0.1% bovine serum albumin−saline solution immediately prior to use. AA, BSA, and EDTA (disodium salt) 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.

Rat-Brain Homogenate Lipid Peroxidation Assay. Whole rat-brain homogenates were freshly prepared in Krebs' buffer containing 15 mM HEPES (pH 7.4), 10 mM glucose, 140 mM NaCl, 3.6 mM KCl, 1.5 mM $CaCl_2$, 1.4 mM KH_2PO_4, and 0.7 mM $MgCl_2$. Male Wistar rats (250−300 g) were decapitated, and the brains (minus cerebellum) were rapidly removed and homogenized in 10 volumes of ice-cold Krebs' buffer using a glass Dounce homogenizer, and centrifuged at 1000*g* for 10 min. The supernatant fraction (1 mg of protein/mL, 500 μL) was incubated with test agents or vehicle at 37 °C for 10 min, then Fe^{2+} (200 μM) was added and incubated for another 30 min. The reaction was terminated by adding 10 μL thiobarbituric acid (TBA, 5 mg/mL in 50% w/v HOAc, and boiled for 15 min. The sample was extracted with *n*-BuOH (1 mL) and centrifuged at 10000*g* for 10 min. The absorbance of the BuOH phase was determined at 532 nm in a spectrophotometer (Hitachi, Model U-3200), and the amount of TBARS present was determined by linear regression analysis of a standard curve using tetramethoxypropane as a standard.

Cell Culture. VSMC were harvested from enzymatically dissociated rat thoracic aorta according to the method of Beasley *et al*.[21] After reaching confluence, cells were passaged by harvesting with 0.1% trypsin-4 mM EDTA, placed in an equal volume of medium, and centrifuged at 600*g* for 5 min. The cells were characterized as smooth muscle cells by morphology and immunostaining with antibodies to smooth muscle α-actin.

[^{3}H]Thymidine Incorporation. VSMCs were finally grown in 24-well plates (2.5×10^4 cells/well), washed twice with 1 mL Krebs−Henseleit solution (KHS), which contained (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 5.5, HEPES 25.0, and $CaCl_2$ 2.5, then incubated in 0.5 mL DMEM/FCS-free for 48 h to induce quiescence (cell cycle stopped at G_0 phase) at 37 °C. To investigate the effect of drugs on proliferation, quiescent cells were cultured for 20 h in medium supplemented with or without 10% FCS, PDGF, (10 ng/mL, ADP (10 μM), or 5-HT (10 μM), respectively, containing or lacking experimental agents. Finally, cells were incubated for 2 h in freshly prepared medium that was additionally supplemented with [^{3}H]-thymidine (1 μCi/mL) to measure DNA synthesis by thymidine incorporation.[22,23] The experiments were terminated by washing cells with 1 mL KHS, precipitation of acid-insoluble material with 10% trichloroacetic acid and extraction of the DNA with 0.1 N NaOH. The precipitate was collected on Whatman GF/B filters, and filters were washed twice with 5 mL of ice-cold KHS. Filters were cut into pieces and shaken with 3.5 mL scintillation fluid for 24 h before liquid scintillation counting.

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