Novel Antiplatelet Constituents from Formosan Moraceous Plants

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Sixteen constituents from Formosan Moraceous plants were tested for their antiplatelet activities in rabbit platelet suspension and human platelet-rich plasma. Cycloartocarpin A, cycloheterophyllin, broussochalcone A, kazinol A, broussoaurone A, and broussoflavonol F showed strong inhibition of arachidonic acid (AA)-induced platelet aggregation. Of the compounds tested, broussochalcone A exhibited the most potent inhibition of platelet aggregation induced by AA (IC_{50} = 6.8 μM). The antiplatelet effects of cycloheterophyllin, broussochalcone A, kazinol B, broussoaurone A, and broussoflavonol F are partially due to an inhibitory effect on cyclooxygenase.

Introduction

In the course of continued screening work on antithrombotic Chinese herbs, especially those having antiplatelet effect, 16 flavonoids from Formosan *Artocarpus heterophyllus* Lam. and *Broussonetia papyrifera* Vent. have been isolated and reported.[1−7] In a previous paper,[8] the antiplatelet activity of prenylflavonoids isolated from Formosan *Artocarpus communis* J. R. Forst was reported. In the present paper the antiplatelet effects and structure−activity relationships of flavonoids isolated from Formosan Moraceous plants, *A. heterophyllus* and *B. papyrifera*, are reported, and mechanisms of action are investigated.

Results and Discussion

The antiplatelet effects of flavonoids (Figure 1) were studied on the aggregation of washed rabbit platelets induced by AA (100 μM), collagen (10 μg/mL), PAF (2 ng/mL), and thrombin (0.1 U/mL). As shown in Tables 1 and 2, compounds **4**, **5**, **10**, **11**, **14**, and **16** showed strong inhibition of AA-induced platelet aggregation. Compounds **4**, **10**, and **11** showed strong inhibition of collagen-induced platelet aggregation. Compound **11** inhibited markedly PAF-induced platelet aggregation. The acetylated product of **5** (**5** diacetate, <100 μM) showed enhancement of the inhibitory effect on collagen-induced platelet aggregation while the other acetylated product of **5** (**5** peracetate, <100 μM) did not potentiate the antiplatelet effect on collagen-induced platelet aggregation.

Considering Tables 1 and 2, it is clearly indicated that the C-3 prenyl chain of **6** oxidatively cyclized with 2′-hydroxy group of B ring (i.e., **5**) showed enhancement of the inhibitory effect on AA-induced platelet aggregation. Although compounds **1** and **3** possess the same A−C ring moiety, **3** exhibited much stronger inhibitory effects on AA-, collagen-, and PAF-induced platelet aggregation than **1**. Prenylation at C-8, C-5′, and C-6′ of quercetin (i.e., **15**) or C-8 and C-3′ of kaempferol (i.e., **16**) markedly enhanced the inhibitory effects on platelet aggregation induced by AA and collagen, while compared with those of quercetin and kaempferol, respectively.[9]

Aspirin was used in this study as a positive control. It was found that aspirin (50 μM) completely inhibited the platelet aggregation induced by AA but not that induced by collagen or PAF or thrombin (Table 1).

The antiplatelet effects of **5**, **10**, and **12** were also studied on the platelet aggregation of human PRP induced by adrenaline (5 μM). As shown in Table 3, **5**, **10**, and **12** showed significant antiplatelet effect on adrenaline-induced platelet aggregation. This effect appeared to be concentration dependent. In adrenaline-induced platelet aggregation, **5** and **12** prevented secondary aggregation at low concentration and completely abolished the aggregation at high concentration, but **10**

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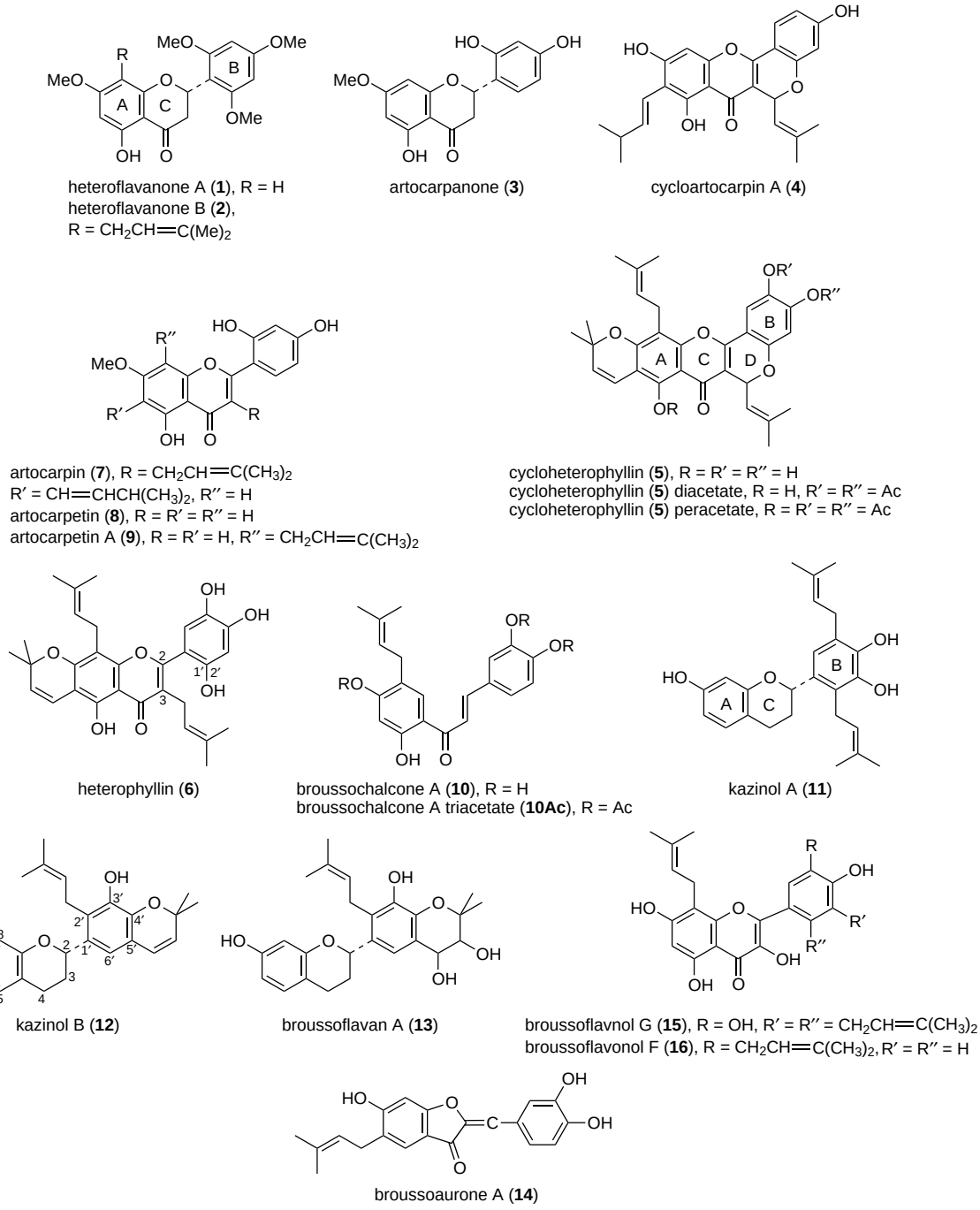

Figure 1. Structures of flavonoids.

prevented secondary aggregation even at high concentration (Figure 2).

Compounds **5**, **10**, **12**, **14**, and **16** were selected to study the mechanisms of the antiplatelet actions of flavonoids. When the activity of fatty acid cyclooxygenase from ram vesicular glands was measured in the presence of each of the compounds, all the compounds inhibited the enzyme in a dose-dependent manner. As shown in Table 4, the IC_{50} values depended on the chemical skeleton of the compound. Compounds with the flavan skeleton (i.e., **12**) showed higher IC_{50} values than **5**, **10**, **14**, and **16**.

We previously reported that prenylflavonoids, cyclomulberrin, dihydroisocycloartomunin, cyclocommunol, and cyclocommunin had potent antiplatelet actions on collagen- and AA-induced platelet aggregation with little or no effect on PAF-induced platelet aggregation.[8] In the present study, we found that prenylflavonoids in Table 1 had the same antiplatelet actions as those of prenylflavonoids reported previously.[8] Prenylflavonoids with a D ring (Figure 1) such as **4** and **5** always showed stronger antiplatelet effect than those of prenylflavonoids without a D ring. These indicate that the D ring of prenylflavonoid may modulate the antiplatelet effect. The antiplatelet effects of **1**, **2**, **8**, and **9** further support that a prenyl group substituted at C-6 or C-8 of flavonoids may modulate antiplatelet effects.[8] Flavanones **1** and **3** with the same chemical structure, except for the B ring, flavans **11**, **12**, and **13** with a different chemical moiety only at the B ring, and quercetin-related flavonoids **15** and **16** showed different antiplatelet potencies, respectively. Variation in the B ring showed an interesting structure−activity correla-

Table 1. Effects of Constituents on the Platelet Aggregation Induced by Arachidonic Acid, Collagen, PAF, and Thrombin in Washed Rabbit Platelets[a]

compound	aggregation (%)			
	AA (100 μM)	collagen (10 μg/mL)	PAF (2 ng/mL)	thrombin (0.1 U/mL)
control	87.7 ± 0.7	89.0 ± 0.3	89.1 ± 1.0	92.0 ± 0.9
heteroflavanone A (**1**) (300 μM)	27.9 ± 4.0[d]	66.1 ± 6.6[c]	81.0 ± 7.3	83.1 ± 2.9
heteroflavanone B (**2**) (300 μM)	81.5 ± 2.4[d]	68.9 ± 4.0[d]	84.5 ± 1.2	85.7 ± 3.7
artocarpanone (**3**) (300 μM)	0.0 ± 0.0[d]	1.1 ± 0.9[d]	38.6 ± 19.7[b]	90.2 ± 1.2
cycloartocarpin A (**4**) (20 μM)	39.5 ± 20.1[b]	60.9 ± 18.8[b]	86.5 ± 1.6	89.9 ± 2.2
cycloheterophyllin (**5**) (300 μM)	0.0 ± 0.0[d]	33.8 ± 16.6[d]	4.5 ± 3.2[d]	82.4 ± 3.5[b]
cycloheterophyllin (**5**) diacetate (<100 μM)	83.4 ± 1.8[b]	4.9 ± 1.5[c]	86.0 ± 1.9	91.3 ± 1.7
cycloheterophyllin (**5**) peracetate (<100 μM)	74.0 ± 2.1[d]	73.0 ± 6.7[c]	78.3 ± 5.0[b]	91.0 ± 2.9
heterophyllin (**6**) (100 μM)	0.0 ± 0.0[d]	0.0 ± 0.0[d]	32.0 ± 8.6[d]	80.2 ± 4.2[b]
artocarpin (**7**) (20 μM)	54.8 ± 23.8	84.0 ± 2.4[b]	76.0 ± 3.9[c]	87.3 ± 2.4
artocarpetin (**8**) (300 μM)	0.0 ± 0.0[d]	73.8 ± 4.9[b]	85.7 ± 2.4	88.2 ± 2.2
artocarpetin A (**9**) (300 μM)	61.8 ± 5.2[d]	46.8 ± 8.1[d]	59.8 ± 4.2[d]	80.4 ± 1.8[d]
control	87.4 ± 2.3	91.3 ± 0.7	86.3 ± 0.9	93.1 ± 1.2
broussochalcone A (**1**) (60 μM)	ND	0.0 ± 0.0[d]	67.8 ± 0.9[d]	ND
broussochalcone A (**10**) (15 μM)	0.0 ± 0.0[d]	55.1 ± 10.8[c]	ND	ND
broussochalcone A triacetate (**10Ac**) (300 μM)	9.3 ± 4.6[d]	23.1 ± 2.7[d]	0.0 ± 0.0[d]	88.4 ± 1.3
broussochalcone A triacetate (**10Ac**) (100 μM)	9.6 ± 1.0[d]	ND	82.1 ± 3.7[b]	ND
kazinol A (**11**) (30 μM)	0.0 ± 0.0[d]	0.0 ± 0.0[d]	65.8 ± 3.1[d]	ND
kazinol B (**12**) (50 μM)	0.0 ± 0.0[d]	4.0 ± 3.2[c]	42.6 ± 6.6[d]	79.8 ± 2.6[d]
broussoflavan A (**13**) (300 μM)	0.0 ± 0.0[d]	0.0 ± 0.0[d]	87.9 ± 2.3	92.0 ± 0.8
broussoaurone A (**14**) (300 μM)	0.0 ± 0.0[d]	0.0 ± 0.0[d]	89.5 ± 2.7	93.7 ± 2.3
broussoflavonol E (**15**) (100 μM)	0.0 ± 0.0[d]	0.0 ± 0.0[d]	86.4 ± 1.3[c]	91.8 ± 1.2
broussoflavonol F (**16**) (100 μM)	0.0 ± 0.0[d]	0.0 ± 0.0[d]	84.8 ± 1.1[d]	93.1 ± 0.9
aspirin (50 μM)	0.0 ± 0.0[d]	85.4 ± 3.9	90.5 ± 1.2	91.9 ± 2.5

[a] Platelets were preincubated with DMSO (0.5%, control) or constituents at 37 °C for 3 min, and the inducer was then added. Values are presented as means ± SEM (N = 3−5). ND: no determined. [b] $p < 0.05$. [c] $p < 0.01$. [d] $p < 0.001$ as compared with the respective control.

Table 2. IC_{50} Values of Flavonoids on the Platelet Aggregation-Induced Arachidonic Acid, Collagen, and PAF

reagent	IC_{50} (μM)		
	AA	collagen	PAF
artocarpanone (**3**)	41.0	90.7	
cycloartocarpin A (**4**)	18.5	23.7	
cycloheterophyllin (**5**)	10.9		
heterophyllin (**6**)	49.8	48.7	
artocarpetin (**8**)	95.9		
broussochalcone A (**10**)	6.8	22.4	118.8
broussochalcone A triacetate (**10Ac**)	91.7		
kazinol A (**11**)	11.4	20.7	54.6
kazinol B (**12**)	32.6		
broussoflavan A (**13**)	86.7		
broussoaurone A (**14**)	15.4		
broussoflavonol E (**15**)	39.9		
broussoflavonol F (**16**)	16.9		

Table 3. Effects of Cycloheterophyllin, Broussochalcone A, and Kazinol B on the Platelet Aggregation Induced by Adrenaline in Human Platelet-Rich Plasma (PRP)[a]

compound	treatment (μM)	aggregation (%)
control		96.9 ± 1.2
cycloheterophyllin (**5**)	300	35.5 ± 13.8[c]
	150	55.1 ± 8.5[c]
	100	61.6 ± 5.5[d]
broussochalcone A (**10**)	450	40.1 ± 2.0[d]
	300	74.0 ± 13.6
kazinol B (**12**)	300	23.1 ± 2.0
	150	27.6 ± 2.0[d]
	100	27.9 ± 1.4[d]
aspirin	50	39.6 ± 15.4[b]

[a] PRP was preincubated with DMSO (0.5%, control), **5**, **10**, **12**, or aspirin at 37 °C for 3 min, and the inducer, adrenaline (5 μM), was then added. Values are presented as means ± SEM (N = 3−5). [b] $p < 0.05$. [b] $p < 0.01$. [c] $p < 0.001$ as compared with the respective control.

tion. Appropriate substitution in the B ring appeared to be required for activity.

Compound **5** inhibited the activity of cyclooxygenase and prevented the secondary aggregation induced by adrenaline in human PRP. Compound **10** inhibited cyclooxygenase markedly (Table 4) but significantly prevented secondary aggregation induced by adrenaline in human PRP at high concentration (Table 3 and Figure 2). This could be due to the higher binding capacity of plasma for this inhibitor. Compounds **14** and **16** also inhibited cyclooxygenase markedly (Table 4). Therefore, the antiplatelet actions of **5**, **10**, **14**, and **16** are partially due to an inhibitory effect on cyclooxygenase activity and diminishing thromboxane formation.[10−12] However, the exact mechanism of action of these flavonoids may be different from that of aspirin, a cyclooxygenase inhibitor, because the latter inhibites AA but not collagen- and PAF-induced platelet aggregation.

Compound **12** inhibited the activity of cyclooxygenase at a relatively high IC_{50} value and suppressed secondary platelet aggregation induced by adrenaline in human PRP. This indicated that the antiplatelet effects of kazinol B (**12**) is partially due to an inhibitory effect on cyclooxygenase and possibly also on thromboxane synthase leading to diminished thromboxane formation.[10−12] Further experiments are needed to elucidate these actions.

Experimental Section

Materials. Flavonoids were isolated from Formosan *A. heterophyllus* and *B. papyrifera*[1−7] and dissolved in dimethyl sulfoxide (DMSO). In order to eliminate the effect of the 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 acetic acid 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 chloroform and diluted into 0.1% bovine serum albumin−saline solution immediatly prior

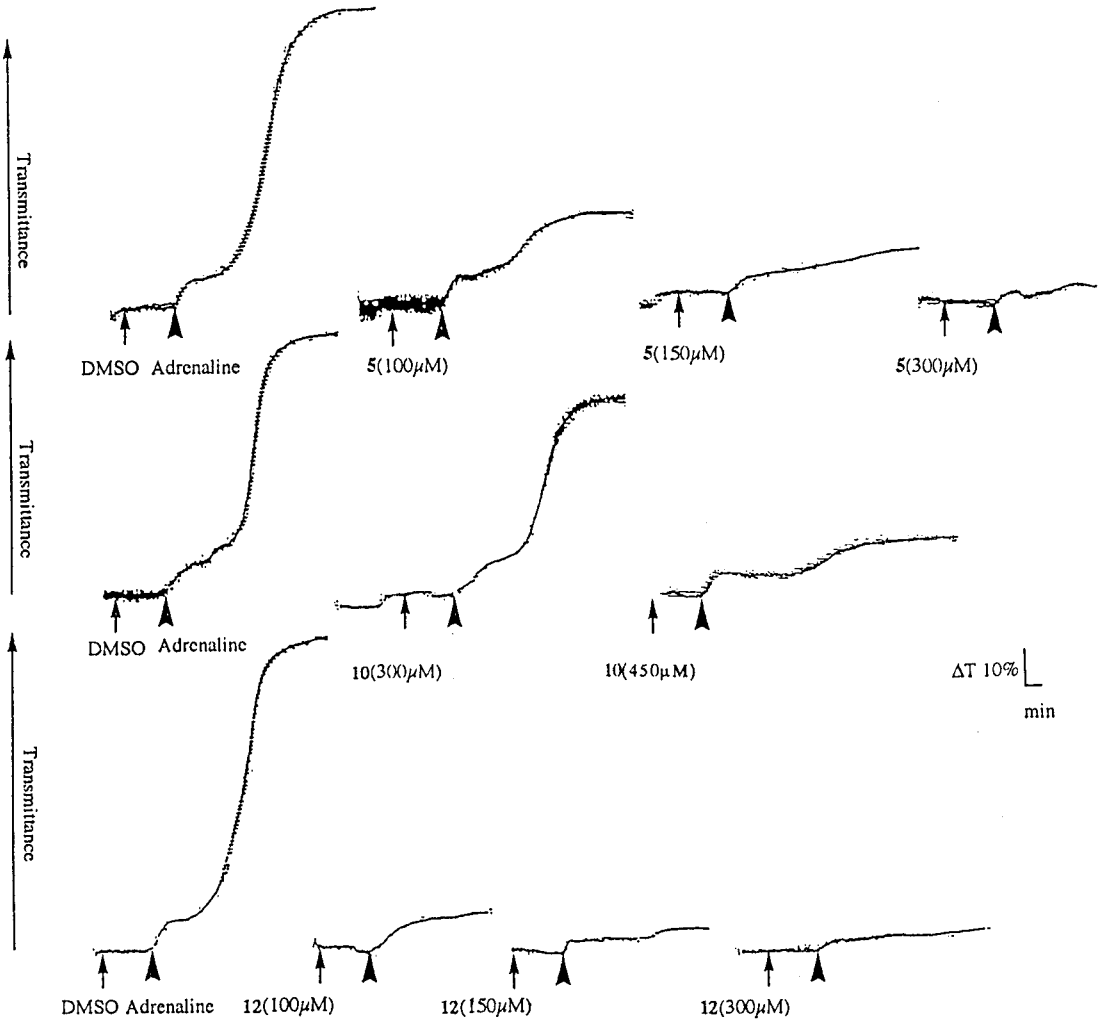

Figure 2. Inhibitory effect of **5**, **10**, and **12** on the aggregation of human platelet-rich plasma (PRP) induced by adrenaline. PRP was incubated with DMSO (0.5%) or various concentrations of **5**, **10**, or **12** for 3 min, and then adrenaline (5 μM) was added to trigger the aggregation.

Table 4. IC_{50} Values of Selected Flavonoids on Cyclooxygenase[a]

compound	IC_{50} (μg/mL)
cycloheterophyllin (**5**)	26.1 ± 1.4
broussochalcone A (**10**)	19.4 ± 1.4
kazinol B (**12**)	155.3 ± 4.1
broussoaurone A (**14**)	22.7 ± 0.8
broussoflavonol F (**16**)	17.5 ± 0.3
indomethacin	0.7 ± 0.04

[a] Tested compounds were preincubated with enzyme at 30 °C for 2 min before addition of arachidonate to start the reaction. Regression analysis was used to calculate the respective IC_{50} values. Values are presented as means ± SEM (N = 3).

to use. Arachidonic acid, ADP, bovine serum albumin (BSA), indomethacin, adrenaline, EDTA (disodium salt), sodium citrate, aspirin, hematin, and tryptophan were also purchased from Sigma. Thrombin (bovine) was obtained from the Parke Davis Co. (Detroit, MI) and dissolved in 50% (v/v) glycerol to give a stock solution of 100 NIH units/mL. Prostaglandin endoperoxide synthase, purified from ram seminal vesicles, was obtained from Biomol (Plymouth Meeting, PA).

Platelet Aggregation. Rabbit washed platelets were obtained from ethylenediaminetetraacetic acid (EDTA)-anticoagulated platelet-rich plasma (PRP) according to the washing procedures described previously.[13] Platelet numbers were counted by Coulter Counter (Model ZM) and adjusted to 4.5×10^8 platelets/mL. The platelet pellets were suspended in Tyrode's solution containing (mM) NaCl, 136.8, KCl, 2.8, $NaHCO_3$, 11.9, $MgCl_2$, 2.1, NaH_2PO_4, 0.33, $CaCl_2$, 1.0, and glucose, 11.2, with 0.35% bovine serum albumin. Human PRP was obtained from the supernatant after centrifugation of blood mixed with 3.8% sodium citrate (1:9 to blood). All glassware was siliconized. Just 1 min before the addition of the aggregation inducer, PRP or the platelet suspension was stirred at 1200 rev/min. Aggregation was measured by a turbidimetric method.[14] 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 as 100% aggregation. The aggregation was measured by a Lumi-aggregometer (Chrono-Log Co., Havertown, PA) connected to dual channel's recorders.

Measurement of Cyclooxygenase Activity. The activity of prostaglandin endoperoxide synthase purified from ram seminal vesicles was measured by a modification of a procedure described previously.[15] Briefly, tested compounds were preincubated with prostaglandin endoperoxide synthase in the reaction mixture containing 0.1 M Tris−HCl pH 8.0, 8 μM hematin, and 5 μM tryptophan at 30 °C for 2 min before addition of 50 μM arachidonate. Oxygen consumption in the reaction mixture was continuously detected with a Clark-

type oxygen electrode using a YSI oxygen monitor (Model 5300).

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