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Synthesis and Antiplatelet, Antiinflammatory, and Antiallergic Activities of Substituted 3-Chloro-5,8-dimethoxy-1,4-naphthoquinone and Related Compounds

Li-Jiau Huang,[a] Fu-Chaio Chang,[a] Kuo-Hsiung Lee,[b] Jih-Pyang Wang,[c] Che-Ming Teng[d] and Sheng-Chu Kuo[a,*]

[a]*Graduate Institute of Pharmaceutical Chemistry, China Medical College, Taichung, Taiwan*
[b]*Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, U.S.A.*
[c]*Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan*
[d]*Pharmacological Institute, College of Medicine, National Taiwan University, Taiwan*

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Abstract—2-Amino (**6**), 2-alkylamino (**7–8**), 2-methoxy (**9**), 2-acetamido (**10**), and 5,8-diacetoxy (**11**) derivatives of the lead compound 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (**4**) were synthesized, together with 6,7-dichloro-5,8-dimethoxy-1,4-naphthoquinone (**5**), a positional isomer of **4**. Antiplatelet, antiinflammatory, and antiallergic activities were evaluated, and most compounds were quite potent in all assays. Compounds **5** and **9–11** were especially active; however, **5** was ineffective against neutrophil superoxide formation, and **10** was ineffective against mast cell degranulation.

Introduction

In previous papers,[1,2] we reported the synthesis and antiinflammatory and antiallergic activities of 2,3-disubstituted derivatives of 2,3-dichloro-1,4-naphthoquinone (A). The substituent group at the 2-position played an important role in the biological activities. For instance, when the acetamido substituent in active compound B was replaced by an alkylamino group (compound C), the activity decreased dramatically. However, if the acetamido group was replaced by an alkoxy group as in compound D, the potency remained high. In the present work, we selected 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (**4**) as a lead compound, after discovering its outstanding biological activities. We have now synthesized 2-substituted derivatives of **4**, together with related compounds, and describe herein their synthesis and preliminary biological evaluation (Figure 1).

Chemistry

Lead compound **4** was prepared according to the method of P. Brassard.[3] As shown in Scheme 1, *p*-dimethoxy benzene (**1**) and excess dichoromaleic anhydride (**2**) were condensed in a molten mass of $AlCl_3$ and NaCl to yield intermediate **3**. This compound probably exists as a mixture of **3a** and **3b**, since methylation in the presence of Ag_2O afforded both 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (**4**)[3] and its positional isomer 6,7-dichloro-5,8-dimethoxy-1,4-naphthoquinone (**5**).[3] Their structures were confirmed by ^1H NMR data.

As illustrated in Scheme 2, treating **4** with ammonia water or alkylamines produced the corresponding 2-amino-3-chloro-5,8-dimethoxy-1,4-naphthoquinone (**6**) or 2-alkylamino-3-chloro-5,8-dimethoxy-1,4-naphthoquinones (**7–8**). 2-Chloro-3,5,8-trimethoxy-1,4-naphthoquinone (**9**) was formed by reacting **4** with sodium methoxide.

Acetylation of **6** with Ac_2O led to 2-acetamido-3-chloro-5,8-dimethoxy-1,4-naphthoquinone (**10**) together with

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*Corresponding author. Tel: 919 966 1121; Fax: 919 966 6919.

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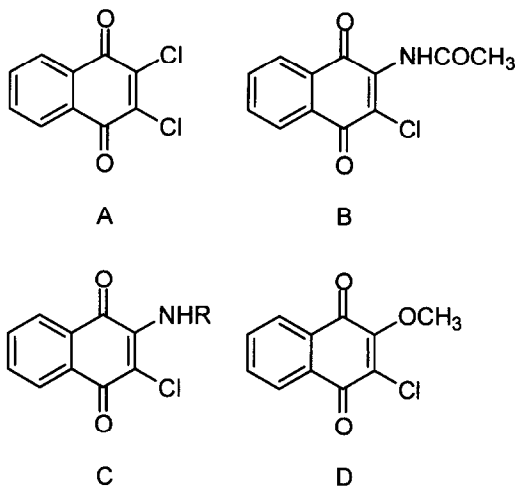


Figure 1.

starting material **6**. In addition to the desired target compound 5,8-diacetoxy-2,3-dichloro-1,4-naphthoquinone (**11**), acetylation of **3** gave a small amount of the isomeric 6,7-dichloro-5,8-diacetoxy-1,4-naphthoquinone and starting material **3** (Scheme 3).

Results and Discussion

Antiplatelet activity

Table 1 lists the antiplatelet activities of all target compounds. At 20 μg/mL, the lead compound **4** completely inhibited the platelet aggregation induced by thrombin (0.1 unit/mL), arachidonic acid (AA) (100 μM), collagen (10 μg/mL), and platelet activating factor (PAF) (2 ng/mL). Even at a lower concentration of 2 μg/mL, **4** inhibited AA- and collagen-induced aggregation completely, but was less active against PAF- and thrombin-induced aggregation.

Compound **5** was slightly less potent than **4**, as were the 2-methoxy (**9**), 2-acetamido (**10**), and the 5,8-diacetoxy (**11**) derivatives. However, when the 2-chloro group of **4** was replaced with amino groups (**6–8**), the antiplatelet potency dropped dramatically.

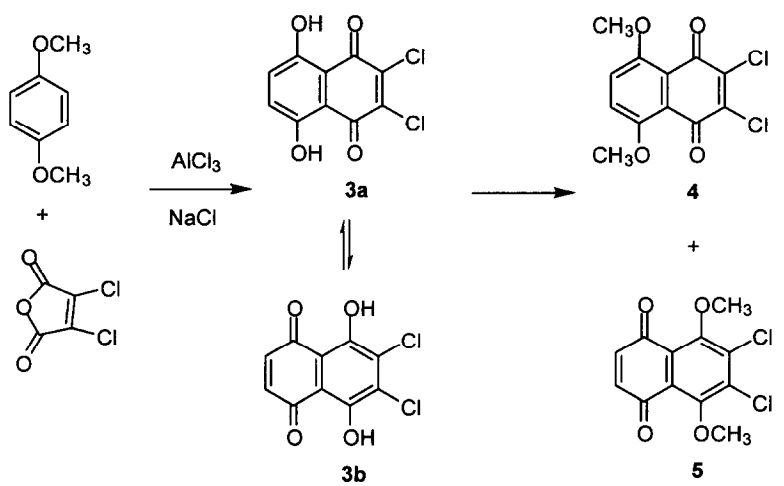
Antiinflammatory activity

Effect on neutrophil degranulation. The activities of **4–11** against neutrophil degranulation were also examined. As shown in Table 2, lead compound **4** was extremely potent ($IC_{50} = 0.19 \pm 0.08$ μg/mL) against neutrophil degranulation induced by FMLP (1 μM). The positional isomer **5** was also considered potent, but about one-half as potent as **4**.

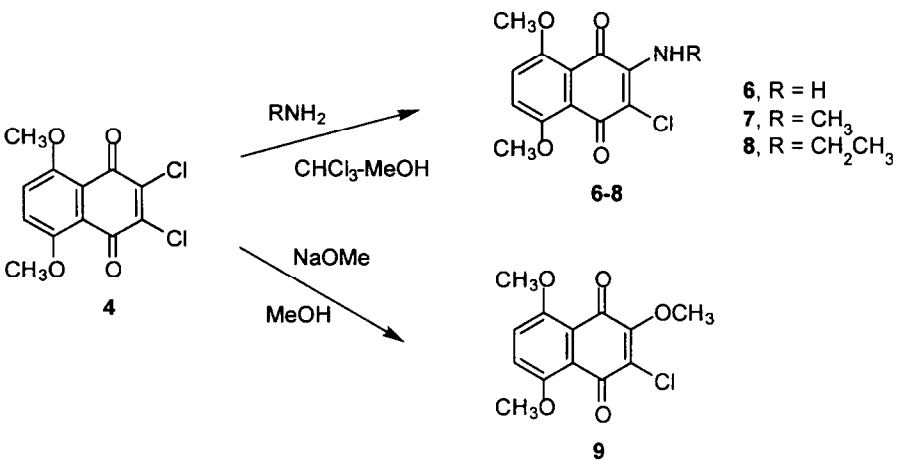
The activity of the 2-amino derivatives (**6–8**) was low, but the 2-methoxy derivative (**9**) was quite potent ($IC_{50} = 0.3 \pm 0.11$ μg/mL). The 3-acetamido derivative (**10**) also displayed significant potency ($IC_{50} = 1.6 \pm 0.4$ μg/mL).

Replacing the 5,8-dimethoxy groups of **4** with diacetoxy groups (**11**) increased activity two-fold ($IC_{50} = 0.07 \pm 0.02$ μg/mL) compared with that of the lead compound **4**. Compound **11** was ca. 120 times more active than the positive control ($IC_{50} = 8.5 \pm 0.4$ μg/mL).

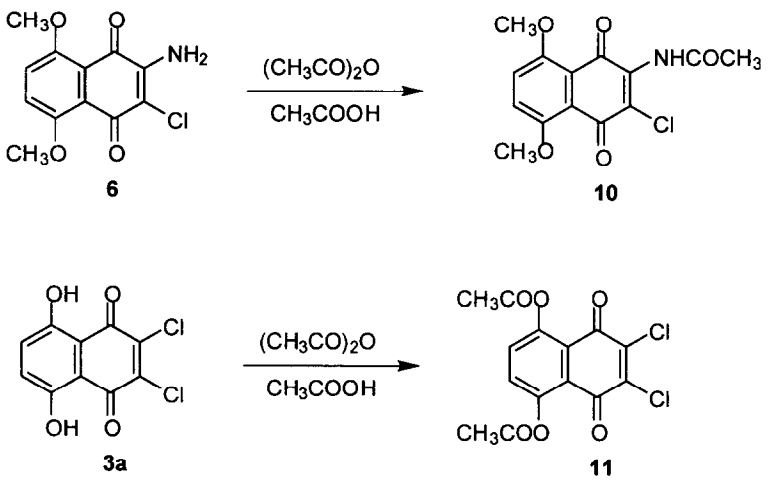
Effect on neutrophil superoxide formation. As shown in Table 3, lead compound **4** showed significant inhibition ($IC_{50} = 0.6 \pm 0.2$ μg/mL) of the neutrophil superoxide formation induced by FMLP (1 μM). Surprisingly, the positional isomer **5** was not significantly active in this assay, in contrast to its potent effect against neutrophil degranulation.



Scheme 1.



Scheme 2.



Scheme 3.

Again, the 2-amino derivatives (**6–8**) were not significantly active, while both the 2-methoxy (**9**, $IC_{50} = 0.9 \pm 0.2\,\mu g/mL$) and 3-acetamido (**10**, $IC_{50} = 1.6 \pm 0.3\,\mu g/mL$) derivative did have potent inhibitory effects. The 5,8-diacetoxy derivative (**11**) showed some interference in the assay system and could not be adequately quantified. Except for compound **5**, the relative inhibitory potencies of compounds **4** and **6–10** followed by same pattern in the neutrophil superoxide formation and degranulation assays.

Antiallergic activity

Effect on mast cell degranulation. As listed in Table 4, compound **4** was quite potent ($IC_{50} = 0.12 \pm 0.02\,\mu g/mL$) against mast cell degranulation induced by compound 48/80. Compounds **5**, **9** and **11** were also significantly active. However, although the 3-acetamido derivative (**10**) was active in the neutrophil degranulation assay, it had negligible activity against mast cell degranulation at a concentration of 10 μg/mL. The 2-amino derivatives **6–8** again were inactive.

Therefore, except for compound **10**, the relative inhibitory potencies in this series of compounds followed the same trend in the mast cell and neutrophil degranulation assays.

Summary

5,8-Dimethoxy-2,3-dichloro-1,4-naphthoquinone (**4**), derived from compound A, was selected as a new lead compound. 2-Amino (**6**), 2-alkylamino (**7,8**), 2-methoxy

Table 1. Inhibitory effects of **4–11** on platelet aggregation induced by thrombin, AA, collagen, and PAF

Compd	Conc (μg/mL)	Thrombin	AA	Collagen	PAF
Control		92.3 ± 0.7(3)	89.5 ± 1.1(3)	90.0 ± 1.4(3)	91.9 ± 0.8(3)
Indomethacin	7.2	91.8 ± 1.3	0.0 ± 0.0***	70.0 ± 8.0	89.7 ± 1.4
4	100	0.0 ± 0.0(3)***	0.0 ± 0.0(3)	0.0 ± 0.0(3)	0.0 ± 0.0(3)
	20	0.0 ± 0.0(3)***	0.0 ± 0.0(3)	0.0 ± 0.0(3)	0.0 ± 0.0(3)
	10	17.9 ± 4.4(3)	0.0 ± 0.0(3)	0.0 ± 0.0(3)	0.0 ± 0.0(3)
	5	31.7 ± 4.3(3)***	0.0 ± 0.0(3)	0.0 ± 0.0(3)	2.8 ± 2.3(3)
	2	68.5 ± 2.6(3)**	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***	30.9 ± 11.8(4)**
	1	89.6 ± 0.8(3)	71.2 ± 2.0(3)	63.2 ± 6.7(3)	69.1 ± 6.4(3)
5	100	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***
	10	2.8 ± 2.3(3)	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***
	5	74.9 ± 9.9(3)*	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***	17.4 ± 8.0(3)
	2	87.1 ± 3.4(3)	0.0 ± 0.0(3)***	31.5 ± 11.3(3)***	73.1 ± 1.5(4)***
	1	—	59.8 ± 2.3(3)	—	79.4 ± 1.1(3)
	0.5	—	69.7 ± 1.0(3)***	—	—
	0.2	—	79.4 ± 0.9(3)***	—	—
6	100	84.7 ± 2.0(5)	31.4 ± 3.8(4)	35.8 ± 5.9(3)	66.6 ± 2.9(5)
	50	—	38.4 ± 13.7(4)	74.7 ± 1.2(3)	—
	20	—	57.3 ± 1.0(4)	—	—
	10	—	68.1 ± 1.0(3)	—	—
		91.7 ± 0.1(6)	85.1 ± 1.6(5)	87.5 ± 0.5(4)	86.0 ± 0.6(5)
7	50	75.7 ± 7.1(3)**	59.0 ± 10.9(4)**	65.5 ± 5.2(3)**	75.2 ± 2.2(4)***
	20	—	76.8 ± 3.7(3)**	—	79.1 ± 0.9(4)***
	10	—	77.7 ± 3.3(3)**	—	78.4 ± 3.9(4)*
8	100	83.7 ± 1.3(3)***	18.9 ± 12.1***	—	42.3 ± 9.6(4)***
	50	—	30.8 ± 15.5(4)**	—	62.3 ± 4.6(3)***
	20	—	41.3 ± 15.3(4)**	—	75.2 ± 3.3(3)***
	10	—	64.5 ± 5.5(4)***	—	79.3 ± 2.1(3)***
	5	—	75.2 ± 3.0(4)**	—	—
9	100	12.1 ± 5.1(3)***	0.0 ± 0.0(3)***	0.0 ± 0.0(3)**	0.0 ± 0.0(3)***
	10	—	—	—	0.0 ± 0.0(3)***
	5	—	0.0 ± 0.0(3)***	—	63.4 ± 6.0(3)***
	2	—	67.2 ± 2.8(3)***	—	83.0 ± 1.0(3)***
10	100	19.3 ± 2.3(3)***	0.0 ± 0.0(3)***	0.0 ± 0.0(3)***	0.0 ± 0.0(4)***
	20	—	0.0 ± 0.0(3)***	—	0.0 ± 0.0(3)***
	10	—	0.0 ± 0.0(3)***	—	15.4 ± 12.5(3)***
	5	—	0.0 ± 0.0(3)***	—	70.8 ± 2.1(3)***
	2	—	80.0 ± 3.4(3)	83.3 ± 2.7(3)*	—
11	100	25.5 ± 10.6(3)***	0.0 ± 0.0(3)	8.9 ± 7.3(3)***	0.0 ± 0.0(3)***
	10	—	—	—	0.0 ± 0.0(3)***
	5	—	—	—	34.5 ± 1.9(3)***
	2	—	0.0 ± 0.0(3)***	—	37.3 ± 6.8(3)***
	1	—	6.2 ± 5.0(3)***	—	56.6 ± 5.9(3)***
	0.5	—	66.5 ± 4.8(3)***	—	84.5 ± 0.3(3)
	0.2	—	90.7 ± 2.4(3)	—	—

Platelets were incubated with a test sample or 0.5% DMSO at 37 °C for 1 min, then thrombin (0.1 unit/mL), AA (100 μM), collagen (10 μg/mL), or PAF (2 ng/mL) was added to trigger the aggregation. Indomethacin is a positive control. Values are the percentage of platelet aggregation and are expressed as mean ± S.D. Numbers in parentheses are number of separate experiments.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; — not determined. All values are averages of three to five experiments.

(**9**), and 2-acetamido (**10**) derivatives were prepared, as well as a 5,8-diacetoxy derivative (**11**) and a positional isomer (**5**).

Most compounds showed similar trends with respect to antiplatelet, antiinflammatory, and antiallergic activities. In general, if a compound was potent in one assay, it was also potent in the others. Interestingly, the structure–activity relationships (SAR) resembled those found in our previous report for derivatives of compound A.[1,2] Two exceptions, however, were noted. The 3-acetamido derivative (**10**) of **4** had considerable inhibitory effects

Table 2. Inhibitory effects of **4**–**11** on the release of β-glucuronidase and lysozyme from neutrophils

Compd	Conc (μg/mL)	β-Glucuronidase		Lysozyme	
		% Release	% Inhibition	% Release	% Inhibition
Control		21.6 ± 2.1		39.1 ± 3.5	
Trifluoperazine	IC_{50}	8.5 ± 0.4		7.8 ± 0.3	
4	0.3	1.4 ± 4.2**	97.6 ± 14.1	13.7 ± 0.3**	63.5 ± 1.9
	0.2	7.6 ± 3.7**	64.1 ± 7.5	21.5 ± 2.3*	48.3 ± 7.6
	0.1	24.3 ± 5.5	−8.9 ± 15.3	29.7 ± 1.8	17.9 ± 7.2
	IC_{50}	0.19 ± 0.08		0.22 ± 0.22	
5	1	−1.9 ± 2.9**	109.6 ± 11.0	3.7 ± 3.1**	90.7 ± 8.3
	0.5	6.2 ± 3.1**	70.5 ± 8.3	12.3 ± 2.6**	63.4 ± 7.5
	0.3	15.6 ± 2.5	27.9 ± 5.3	26.9 ± 6.5	30.9 ± 14.4
	IC_{50}	0.43 ± 0.12		0.46 ± 0.16	
6	10	18.0 ± 2.4	17.3 ± 4.3	24.1 ± 7.0	40.5 ± 14.4
7	10	12.9 ± 2.6*	41.2 ± 8.4	28.2 ± 2.9	33.6 ± 8.6
	3	22.1 ± 0.1	6.1 ± 4.7	35.0 ± 7.6	12.7 ± 15.4
8	10	14.1 ± 3.2*	36.0 ± 11.2	23.2 ± 5.5*	41.7 ± 11.0
	3	16.3 ± 3.9	27.0 ± 11.7	34.6 ± 5.2	12.3 ± 7.2
9	1	−0.5 ± 1.9**	104.7 ± 8.6	11.2 ± 3.3**	62.5 ± 6.7
	0.5	5.7 ± 2.1**	74.7 ± 7.8	21.2 ± 3.2**	40.2 ± 6.4
	0.3	11.7 ± 1.4**	44.9 ± 1.1	26.7 ± 5.3	8.1 ± 10.6
	IC_{50}	0.30 ± 0.11		0.76 ± 0.23	
10	10	−5.2 ± 1.3**	127.9 ± 6.7	−7.9 ± 1.4**	112.8 ± 2.2
	3	4.0 ± 1.4**	80.3 ± 6.5	12.5 ± 3.4**	68.5 ± 4.8
	1	8.7 ± 3.5**	58.7 ± 10.0	23.6 ± 4.1	19.6 ± 3.8
	IC_{50}	1.6 ± 0.4		3.0 ± 0.5	
11	0.1	3.9 ± 2.8**	77.9 ± 6.5	18.5 ± 5.7*	46.5 ± 11.4
	0.05	14.5 ± 2.3*	36.3 ± 6.4	29.3 ± 3.3	26.1 ± 4.5
	0.03	26.2 ± 4.2	−31.4 ± 14.2	38.8 ± 3.8	2.9 ± 2.0
	IC_{50}	0.07 ± 0.02			

The neutrophil suspension was preincubated with a test sample or 0.5% DMSO at 37 °C for 3 min in the presence of cytochalasin B (5 μg/mL). Forty-five min after the addition of FMLP (1 μM), β-glucuronidase and lysozyme in the supernatant were determined. Trifluoperazine acts as a positive control. Values are expressed as mean ± S.D.
*$p < 0.05$, **$p < 0.01$. All values are the averages of three to five separate experiments.

on platelet aggregation, neutrophil degranulation, and neutrophil superoxide formation, but a very weak effect on mast cell degranulation. This behavior was not seen previously in derivatives of A. Second, although compound **5** had similar inhibitory effects to those of **4** on platelet aggregation, neutrophil degranulation, and mast cell degranulation, it was ineffective against neutrophil superoxide formation. This is in strong contrast to the overall potency of compound **4**, and appears to indirectly emphasize the significance of the 2,3-dichloro, 2- or 3-methoxy, and 2- or 3-acetamido groups to the activity against neutrophil superoxide formation.

Overall, the lead compound **4** and its 3-methoxy derivative (**9**) both had significant potency in all four screening assays. Compounds **5** and **11** both demonstrated impressive effects on platelet aggregation, neutrophil degranulation, and mast cell degranulation, and **10** had significant effects on platelet aggregation, neutrophil degranulation, and neutrophil superoxide formation. We have selected these five compounds for further pharmacological investigation. The results will be presented later.

Experimental

General

All melting points are uncorrected. IR spectra were recorded on Shimadzu IR-440 and Nicolet Impact 400 FT-IR spectrophotometers as KBr pellets. NMR spectra were obtained on Bruker ARX-300 and Varian VXR-300 FT-NMR spectrometers in $CDCl_3$. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. MS were

Table 3. Inhibitory effects of **4–11** on superoxide formation in neutrophils

Compound	Conc (μg/mL)	Superoxide anion formation (nmol/10^6 cells)	% Inhibition
Control		1.82 ± 0.16	
Trifluoperazine	IC_{50}	7.4 ± 0.5 μg/mL	
4	1	0.43 ± 0.17**	70.1 ± 11.5
	0.3	1.08 ± 0.12	23.9 ± 10.7
	0.1	1.63 ± 0.13	11.5 ± 6.2
	IC_{50}	0.6 ± 0.2 μg/mL	
5	10	1.82 ± 0.18	2.3 ± 6.2
6	10	1.85 ± 0.22	−3.4 ± 9.9
7	10	1.02 ± 0.14*	42.3 ± 8.5
	3	1.92 ± 0.44	−9.8 ± 14.9
8	10	1.55 ± 0.25	−3.3 ± 13.9
	3	1.41 ± 0.21	17.5 ± 9.5
9	3	0.36 ± 0.06**	80.0 ± 1.5
	1	0.64 ± 0.14**	64.4 ± 1.7
	0.3	1.20 ± 0.15	33.6 ± 4.3
	IC_{50}	0.9 ± 0.2 μg/mL	
10	3	0.41 ± 0.08**	77.6 ± 3.7
	1	0.99 ± 0.13*	46.2 ± 3.8
	0.3	1.56 ± 0.16	14.4 ± 7.9
	IC_{50}	1.6 ± 0.3 μg/mL	
11		ND	

The neutrophil suspension in the presence of ferricytochrome *c* was preincubated at 37 °C with 0.5% DMSO or test compound for 3 min. Fifteen minutes after the addition of FMLP (0.3 μM), the absorbance we determined at 550 nm. Trifluoperazine acts as a positive control. Values are presented as mean ± S.D.
*$p < 0.05$, **$p < 0.01$. All values are the averages of three to five separate experiments. ND: Not determined.

measured with a HP 5995 GC-MS instrument. The UV spectra were recorded on a Shimadzu UV-160A UV-Vis recording spectrophotometer as alcoholic solutions. Elemental analyses (C,H,N) were performed by National Cheng Kung University and National Chung Hsing University, Taiwan.

2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone (3a, 3b).[3] A mixture of $AlCl_3$ (142 g, 1 mol) and NaCl (28.3 g, 0.5 mol) was stirred and melted by heating to 140–150 °C. Then, *p*-dimethoxy benzene (**1**, 16.6 g, 0.11 mol) and dichloromaleic anhydride (**2**, 40.1 g, 0.24 mol) were added in portions. The mixture was heated to 170–175 °C, stirred for 1–2 min, and allowed to cool to 25 °C. After adding water (1.5 L) and conc. HCl (100 mL), the mixture stood for 12 h and was extracted with EtOAc, and concentrated to give crude product. Recrystallization from petroleum ether gave yellow needles of the mixture of **3a** and **3b** (23 g, 81% yield). mp 198–199 °C; MS (*m/z*): 259 [M^+], 261 [$M^+ + 2$]; UV (EtOH): λ_{max} 293 nm (log ε = 3.7); IR (KBr): ν_{max} 3422 (OH), 1628, 1591 (C=O) cm^{-1}; ^1H NMR: δ 7.30 (2H, s, H-6, H-7), 12.31 (2H, s, 5,8-OH); ^{13}C NMR: δ 110.4 (C-4a, C-8a), 131.1 (C-6, C-7), 142.8 (C-2, C-3), 161.2 (C-5, C-8), 177.1 (C-1, C-4); Anal. calcd For $C_{10}H_4Cl_2O_4$: C, 46.37; H, 1.56. Found: C, 46.09; H, 1.23.

2,3-Dichloro-5,8-dimethoxy-1,4-naphthoquinone (4) and 6,7-Dichloro-5,8-dimethoxy-1,4-naphthoquinone (5). CH_3I (8 mL) and Ag_2O (8 g) were added to a solution of **3** (5.2 g. 0.02 mol) in $CHCl_3$. The mixture was stirred at 25 °C and the same quantities of CH_3I and Ag_2O were added again at 6 and 16 h. The total reaction time was 22 h. The mixture was then filtered and concentrated in vacuo. The residue was purified by column chromatography (silica gel, $CHCl_3$/EtOAc) to give **4** (2.6 g, 45%) as a reddish brown solid and **5** (1.7 g, 30%) as a yellow solid.

Compound **4**: mp 237–239 °C; MS (*m/z*): 286 [M^+], 288 [$M^+ + 2$]; UV (EtOH): λ_{max} 278 nm (log ε = 3.9); IR (KBr): ν_{max} 1671 (C=O) cm^{-1}; ^1H NMR: δ 3.94 (6H, s, 5-OCH_3, 8-OCH_3), 7.34 (2H, s, H-6, H-7); ^{13}C NMR: δ 56.9 (5-OCH_3, 8-OCH_3), 119.6 (C-4a, C-8a), 120.9 (C-6, C-7), 142.5 (C-2, C-3), 154.5 (C-5, C-8), 174.8 (C-1, C-4); Anal. calcd For $C_{12}H_8Cl_2O_4$: C, 50.20; H, 2.81. Found: C, 50.46; H, 2.69.

Compound **5**: mp 203–204 °C; MS (*m/z*): 286 [M^+], 288 [$M^+ + 2$]; UV (EtOH): λ_{max} 251 nm (log ε = 3.5); IR (KBr): ν_{max} 1669 (C=O) cm^{-1}; ^1H NMR: δ 3.92 (6H, s, 5-OCH_3, 8-OCH_3), 6.82 (2H, s, H-2, H-3); ^{13}C NMR: δ 61.9 (5-OCH_3, 8-OCH_3), 124.3 (C-2, C-3), 137.0 (C-4a, C-8a), 138.4 (C-6, C-7), 153.5 (C-1, C-4), 182.8 (C-5, C-8); Anal. calcd For $C_{12}H_8Cl_2O_4$: C, 50.20; H, 2.81. Found: C, 50.10; H, 2.61.

2-Amino-3-chloro-5,8-dimethoxy-1,4-naphthoquinone (6). To a solution of **4** (1.5 g, 0.005 mol) in CH_2Cl_2 (15 mL) and EtOH (15 mL) was added conc. ammonia water. The reaction mixture was refluxed for 6 h during which a slow stream of ammonia gas was introduced. The reaction mixture was evaporated, and the residue was purified by column chromatography (silica gel, $CHCl_3$/EtOAc) to yield **6** (0.5 g, 37%) as an orange-yellow powder. mp 235–237 °C; MS (*m/z*): 267 [M^+], 269 [$M^+ + 2$]; UV (EtOH): λ_{max} 244 nm (log ε = 4.2); IR (KBr): ν_{max} 3398, 3295 (NH_2), 1624 (C=O) cm^{-1}; ^1H NMR: δ 3.92 (3H, s, 5-OCH_3), 3.96 (3H, s, 8-OCH_3), 5.32 (2H, br, NH_2), 7.24 (1H, d, J = 9.3 Hz, H-7), 7.26 (1H, d, J = 9.3 Hz, H-6); ^{13}C NMR: δ 56.7 (8-OCH_3), 57.4 (5-OCH_3), 112.2 (C-2), 118.8 (C-6, C-4a), 123.1 (C-7, C-8a), 144.3 (C-3), 153.9 (C-5), 154.8 (C-8), 177.9 (C-1, C-4); Anal. calcd For $C_{12}H_{10}ClNO_4$: C, 53.85; H, 3.77; N, 5.23. Found: C, 53.96; H, 3.97; N, 5.39.

Table 4. Inhibitory effects of **4–11** on the release of β-glucuronidase and histamine from mast cells

		β-Glucuronidase		Histamine	
Compd	Conc (μg/mL)	% Release	% Inhibition	% Release	% Inhibition
Control		38.8 ± 3.2		41.2 ± 5.3	
Mepacrine	IC_{50}	6.2 ± 2.1		7.0 ± 2.6	
4	1	−2.4 ± 4.3**	112.2 ± 15.0	0.3 ± 2.5**	100.3 ± 5.1
	0.3	6.8 ± 3.5**	82.3 ± 7.6	7.9 ± 3.2**	79.7 ± 7.6
	0.1	23.5 ± 4.1*	40.2 ± 8.6	26.5 ± 3.3	37.2 ± 8.4
	IC_{50}	0.12 ± 0.02		0.17 ± 0.02	
5	3	10.0 ± 3.0**	70.2 ± 12.8	12.4 ± 4.6**	69.9 ± 9.8
	1	18.3 ± 3.2**	51.4 ± 9.7	21.5 ± 3.8**	43.1 ± 8.5
	0.3	26.8 ± 4.2	30.1 ± 3.5	33.6 ± 3.5	21.0 ± 8.2
	IC_{50}	1.3 ± 0.2		1.7 ± 0.3	
6	10	30.3 ± 6.3	21.5 ± 13.8	36.4 ± 4.0	10.9 ± 7.5
7	10	36.7 ± 3.2	5.4 ± 1.2	33.4 ± 4.7	14.2 ± 0.4
	3	22.1 ± 0.1	6.1 ± 4.7	35.0 ± 7.6	12.7 ± 15.4
8	10	42.4 ± 5.8	−8.4 ± 15.9	37.5 ± 3.9	8.1 ± 5.5
	3	37.2 ± 3.1	4.1 ± 1.5	36.3 ± 4.1	11.5 ± 3.1
9	1	18.1 ± 4.7**	68.7 ± 17.5	19.0 ± 4.5**	86.2 ± 8.7
	0.3	29.1 ± 2.9	25.7 ± 9.2	23.8 ± 4.5	33.0 ± 9.1
	0.1	34.5 ± 2.8	11.9 ± 6.5	33.1 ± 3.6	14.7 ± 8.1
	IC_{50}	0.6 ± 0.2		0.5 ± 0.2	
10	10	37.1 ± 3.4	15.3 ± 7.7	34.0 ± 3.8	20.1 ± 6.0
11	0.3	10.1 ± 2.9**	74.2 ± 11.1	2.6 ± 1.9**	96.6 ± 5.6
	0.2	19.2 ± 4.8*	50.1 ± 12.1	20.9 ± 6.7*	57.5 ± 12.3
	0.1	30.9 ± 4.2	11.5 ± 12.4	31.2 ± 7.0	37.2 ± 14.1
	IC_{50}	0.21 ± 0.02		0.15 ± 0.03	

The mast cell suspension was preincubated with a test sample or 0.5% DMSO at 37 °C for 3 min. Fifteen minutes after the addition of compound 48/80 (10 μg/mL, β-glucuronidase and histamine in the supernatant were determined. Mepacrine acts as a positive control. Values are expressed as mean ± S.D.

*$p < 0.05$, **$p < 0.01$. All values are the averages of three to five separate experiments.

2-Chloro-3-methylamino-5,8-dimethoxy-1,4-naphthoquinone (7). To a solution of **4** (0.2 g, 0.68 mmol) in EtOH (15 mL) was added CH_3NH_2 (2 mL). The reaction mixture was refluxed for 20 min, then poured into ice water (150 mL) and extracted with $CHCl_3$. The organic phase was concentrated in vacuo and the residue purified by column chromatography (silica gel, $CHCl_3$/EtOAc) to give **7** (0.12 g, 63%) as a yellow solid. mp 202–205 °C; MS (*m/z*): 281 [M^+], 283 [$M^+ + 2$]; UV (EtOH): λ_{max} 249 nm (log ε = 4.2); IR (KBr): ν_{max} 3340 (NH), 1616 (C=O) cm^{-1}; ^1H NMR: δ 3.35 (3H, d, J = 5.7 Hz, $NHCH_3$), 3.91 (3H, s, 8-OCH_3), 3.93 (3H, s, 5-OCH_3), 5.87 (1H, br, NH), 7.23 and 7.24 (each 1H, d, J = 9.3 Hz, H-6, H-7); ^{13}C NMR: δ 32.3 ($NHCH_3$), 56.7 (5-OCH_3), 57.4 (8-OCH_3), 110.4 (C-3), 118.2 (C-6), 118.8 (C-4a), 121.5 (C-8a), 123.1 (C-7), 144.1 (C-2), 153.4 (C-8), 154.5 (C-5), 176.5 (C-1), 179.3 (C-4); Anal. calcd For $C_{13}H_{12}ClNO_4$: C, 55.40; H, 4.29; N, 4.97. Found: C, 55.21; H, 4.50; N, 4.76.

2-Chloro-3-ethylamino-5,8-dimethoxy-1,4-naphthoquinone (8). Compound **8** (0.11 g, 52%) was prepared from **4** (0.2 g, 0.68 mmol) and $EtNH_2$ (2 mL) in the same manner as described above for **7**. mp 129–131 °C; MS (*m/z*): 295 [M^+], 297 [$M^+ + 2$]; UV (EtOH): λ_{max} 366 nm (log ε = 3.7); IR (KBr): ν_{max} 3340 (NH), 1620 (C=O) cm^{-1}; ^1H NMR: δ 1.26 (3H, t, J = 7.2 Hz, $NHCH_2CH_3$), 3.80 (2H, m, $NHCH_2$), 3.89 (3H, s, 8-OCH_3), 3.92 (3H, s, 5-OCH_3), 5.75 (1H, br, NH), 7.16 (1H, d, J = 9.5 Hz, H-6), 7.16 (1H, d, J = 9.5 Hz, H-7); Anal. calcd For $C_{14}H_{14}ClNO_4$: C, 56.86; H, 4.77; N, 4.74. Found: C, 56.63; H, 4.60; N, 4.62.

2-Chloro-3,5,8-trimethoxy-1,4-naphthoquinone (9). NaOMe (2.2 g, 41 mmol) was prepared by adding Na (1 g, 43 mmol) to MeOH (30 mL), refluxing the resulting mixture until completely dissolved, and evaporating the solvent. The resulting white powder (0.1 g) was added to compound **4** (0.1 g, 0.35 mmol) dissolved in MeOH

(20 mL). After stirring for 30 min, the solution was poured into water (100 mL). The precipitate was filtered and purified by column chromatography (silica gel, $CHCl_3$/EtOAc) to afford **9** as a yellow solid (25 mg, 25%). mp 146–148 °C; MS (*m/z*): 282.5 [M^+], 284.5 [$M^+ + 2$]; UV (EtOH): λ_{max} 280 nm (log $\varepsilon = 4.0$); IR (KBr): ν_{max} 1670, 1656 (C=O) cm^{-1}; ^1H NMR: δ 3.88 (3H, s, 8-OCH_3), 3.90 (3H, s, 5-OCH_3), 4.16 (3H, s, 3-OCH_3), 7.22 (1H, d, $J = 9.5$ Hz, H-6), 7.26 (1H, d, $J = 9.5$ Hz, H-7); Anal. calcd For $C_{13}H_{11}ClO_5$: C, 55.24; H, 3.92. Found: C, 55.41; H, 3.70.

2-Acetamido-3-chloro-5,8-dimethoxy-1,4-naphthoquinone (10). To a suspension of **6** (0.2 g, 0.75 mmol) in Ac_2O (10 mL) was added 1 mL of AcOH. The reaction mixture was refluxed for 24 h, then evaporated in vacuo. The residue was purified by column chromatography (neutral alumina, benzene) to give **10** (24 mg, 10%) as a brown powder. mp 246–248 °C; MS (*m/z*): 309 [M^+], 311 [$M^+ + 2$]; UV (EtOH): λ_{max} 280 nm (log $\varepsilon = 3.9$); IR (KBr): ν_{max} 3255 (NH), 1667, 1655 (C=O) cm^{-1}; ^1H NMR: δ 2.23 (3H, s, $COCH_3$), 3.93 (3H, s, 8-OCH_3), 3.94 (3H, s, 5-OCH_3), 7.29 (1H, d, $J = 9.3$ Hz, H-7), 7.33 (1H, d, $J = 9.3$ Hz, H-6), 7.63 (1H, br, NH),; ^{13}C NMR: δ 23.9 ($COCH_3$), 56.9 (8-OCH_3), 57.0 (5-OCH_3), 119.3 (C-8a), 120.2 (C-7), 121.5 (C-6), 132.1 (C-4a), 128.2 (C-2), 139.0 (C-3). 154.1 (C-5), 154.2 (C-8), 166.8 (CO), 176.4 (C-4), 178.5 (C-1); Anal. calcd For $C_{14}H_{12}ClNO_5$: C, 54.30; H, 3.91; N, 4.52. Found: C, 54.62; H, 3.69; N, 4.31.

2,3-Dichloro-5,8-diacetoxy-1,4-naphthoquinone (11). To a suspension of **3** (1 g, 4 mmol) in Ac_2O (10 mL) was added 1 mL of AcOH. The reaction mixture was refluxed for 1 h, then poured into water (50 mL). The mixture was extracted with benzene, and the organic layer dried over $MgSO_4$ and evaporated in vacuo. The residue was purified by column chromatography (neutral aluminum oxide, benzene) to give 11 (75 mg, 5.6%). mp 204–206 °C; MS (*m/z*): 243 [M^+], 245 [$M^+ + 2$]; UV (EtOH): λ_{max} 279 nm (log $\varepsilon = 3.8$); IR (KBr): ν_{max} 1781, 1694 (C=O) cm^{-1}; ^1H NMR: δ 2.44 (6H, s, $COCH_3$) 7.42 (2H, s, H-6, H-7); ^{13}C NMR: δ 20.9 ($COCH_3$), 123.5 (C-4a, C-8a), 131.8 (C-6, C-7), 143.1 (C-2,C-3). 148.4 (C-5, C-8), 168.9 (CO), 173.9 (C-1, C-4); Anal. calcd For $C_{14}H_8Cl_2O_6$: C, 49.01; H, 2.35. Found: C, 49.16; H, 2.60.

Evaluation of antiplatelet aggregation activity

Materials. Collagen (type 1, bovine Achilles tendon), obtained from Sigma Chemical Co., was homogenized in 25 mL HOAc and stored at −70 °C. Arachidonic acid, bovine serum albumin (BSA), EDTA (disodium salt), sodium citrate, dimethyl sulfoxide (DMSO), and platelet-activating factor (PAF) were purchased from Sigma Chemical Co. Thrombin (bovine) was obtained from Park Davis Co. and dissolved in 50% (v/v) glycerol to give a stock solution of 100 NIH units/mL.

Methods

Platelet suspension preparation. Blood was collected from the rabbit marginal ear vein and was mixed with EDTA to a final concentration of 6 mM. It was centrifuged at 90 *g* for 10 min at rt, and the supernatant was obtained as platelet-rich plasma. The latter was further centrifuged at 500 *g* for 10 min. The platelet pellets were washed with Tyrode's solution without EDTA. After centrifugation at the same conditions, the platelet pellets were finally suspended in Tyrode's solution of the following composition (mM): NaCl (136.8), KCl (2.8), $NaHCO_3$ (11.9), $MgCl_2$ (1.1), NaH_2PO_4 (0.33), $CaCl_2$ (1.0), and glucose (11.2). Platelet numbers were counted by Coulter Counter (Model ZM) and adjusted to 4.5×10^8 platelets/mL.

Platelet aggregation. Aggregation was measured by the turbidimetric method[4] with a dual-channel Lumiaggregometer (Model 1020, Payton, Canada). All glassware was siliconized. One minute before the addition of the aggregation inducer, the platelet suspension was stirred at 900 rpm. The percentage of aggregation was calculated as described previously.[5]

Evaluation of antiinflammatory activity

Materials. Sodium pentobarbital, bovine serum albumin (BSA), N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), ferricytochrome-*c*, superoxide dismutase (SOD), formyl-Met-Leu-Phe (FMLP), phenolphthalein-β-D-glucuronide, and Triton X-100 were purchased from Sigma Chemical Co.

Methods

Isolation of neutrophils. Rat peripheral neutrophils were isolated by a modification of the procedure described by Boyum.[6] EDTA-mixed fresh blood was obtained from the abdominal aorta of pentobarbitone (60 mg/kg, ip) anesthetized rats (Sprague-Dawley, 300–350 g). Neutrophils were separated from other blood cells by dextran sedimentation and centrifugation on a Ficoll-hypaque density gradient. Erythrocytes in the pellets were lysed by suspending the cells in 0.05% saline for 15 s followed by washing with 1.75% saline containing 0.25% BSA. Cells were resuspended in Hanks' balanced salt solution containing 4 mM Na_2CO_3 and 10 mM HEPES, pH 7.4 (HBSS) to a final concentration of 2×10^6 cells/mL. The cell preparations consisted of 90–95% neutrophils (viability approximately 95% by trypan blue exclusion).

Measurement of β-glucuronidase and lysozymes release. The neutrophil suspension was preincubated at 37 °C with DMSO or sample for 3 min, and the release reaction was triggered by the addition of 1 μM FMLP. The reaction was stopped 45 min later by the addition of ice-cold Tyrode's solution, and the mixture was centrifuged for 10 min at 1000 *g*. β-Glucuronidase activity in the supernatant was determined by spectrophotometry at 550 nm after reaction with phenolphthalein-β-glucuronide as substrate.[7] Lysozyme activity in the supernatant was measured, with *Micrococcus lysodeikticus* as substrate, by spectrophotometry at 450 nm.[8] The release of β-glucuronidase and lysozyme was expressed as percentage release = [(release elicited by secretagogue–spontaneous release)/total content] × 100. The total content was measure after treatment of the cell suspension with Triton X-100. Spontaneous release was less than 10%.

Measurement of superoxide anion production. Superoxide anion (O_2^-) production was determined by superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction as previously described[9] with modifications. Assay mixtures contained 0.2 mL cell suspension (5×10^6 cells/mL) and 0.9 mg/mL of ferricytochrome *c* in a final volume of 0.4 mL. The reference tube also received 12.5 μg/mL of SOD. Both reference and sample tubes were incubated at 37 °C for 3 min. The reactions were then started by the addition of 0.3 μM FMLP incubation at 37 °C for 30 min with occasional agitation. After centrifugation, the supernatant was transferred to a 96-well plate, and the absorbance at 550 nm was recorded with a microplate reader. The amount of O_2^- in the reaction mixture was calculated from the formula:[10] O_2^- (nmol) = 19.08 × absorbance.

Evaluation of antiallergic activity

Materials. Heparin (grade 1-A; from porcine intestinal mucosa), bovine serum albumin, compounds **48/80**, *o*-phthadialdehyde, phenolphthalein-β-D-glucuronide, and Triton X-100 were purchased from Sigma Chemical Co.

Methods

Rat peritoneal mast cell preparation. Rat peritoneal mast cells were isolated as previously described.[11] Briefly, heparinized Tyrode's solution was injected into the peritoneal cavity of exsanguinated rats (Sprague-Dawley, 250–300 g). After abdominal massage, the cells in the peritoneal fluid were harvested and separated in 38% bovine serum albumin in glucose-free Tyrode's solution. The cell pellet was washed and suspended in Tyrode's solution of the following composition (mM): NaCl (137), KCl (2.7), $NaHCO_3$ (12), $MgCl_2$ (1.0), NaH_2PO_4 (0.3), $CaCl_2$ (1.0), glucose (5.6), and bovine serum albumin (0.1%). The mast cell count was adjusted to $1–1.5 \times 10^6$ cells/mL. Cell viability was assessed with the trypan blue exclusion test.

Measurement of β-glucuronidase and histamine release. The mast cell suspension was preincubated at 37 °C with DMSO or sample for 3 min, and the release reaction was triggered by the addition of 10 μg/mL of compound **48/80**. The reaction was stopped 15 min later by the addition of ice-cold Tyrode's solution, and the mixture was centrifuged for 10 min at 1000 *g*. Histamine in the supernatant was determined by fluorescence spectrophotometry at 350/450 nm after condensation with *o*-phthaldialdehyde.[12] β-Glucuronidase activity in the supernatant was measured, with phenolphthalein-β-D-glucuronide as substrate, by spectrophotometry at 550 nm.[7] The release of histamine and β-glucuronidase was expressed as percentage release = [(release elicited by secretagogue–spontaneous release)/total content] × 100. The total content was measured after treatment of the cell suspension with Triton X-100. Spontaneous release was less than 10%.

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