3-*O*-Alkylascorbic Acids as Free Radical Quenchers. 3. Protective Effect on Coronary Occlusion–Reperfusion Induced Arrhythmias in Anesthetized Rats

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Structural modification of ascorbic acid by substitution of the 3-hydroxy group with lipophilic moieties has allowed the development of agents for treating reperfusion injury. These ascorbic acid derivatives inhibited lipid peroxidation, and some of them also reduced coronary reperfusion-induced arrhythmias in anesthetized rats. We found that 3-*O*-[(dodecylcarbonyl)methyl]ascorbic acid (8) was protective against reperfusion injury without directly influencing hemodynamics. 2-*O*-Octadecylascorbic acid (19) and 5,6-*O*-dodecylideneascorbic acid (15) also exhibited a marked effect on reperfusion injury, but significantly reduced the arterial blood pressure and heart rate in rats.

Introduction

Better treatments for reperfusion disturbances need to be developed for use after angioplasty, thrombolytic therapy, or coronary bypass graft surgery in patients with myocardial infarction.[1] Many investigators have detailed the possible mechanisms of reperfusion injury, and Bolli stated that among the numerous mechanisms proposed for myocardial stunning (which is a manifestation of reperfusion injury), there appeared to be plausible (1) generation of oxygen radicals, (2) calcium overload, and (3) excitation–contraction uncoupling.[2]

It has been reported that calcium antagonists,[3] adrenoceptor antagonists,[3] prostaglandin E_1,[4] prostacyclin,[3a] active oxygen species scavengers,[5] antioxidants,[6] adenine nucleotide translocase modulators,[7] and Na^+–H^+ exchange inhibitors[8] are effective in experimental modes of reperfusion injury. It has been clear that active oxygen species production and the generation of free radicals participate in the development or exacerbation of reperfusion injury in the heart, kidneys, liver, and brain,[9] since Granger et al. proposed that the reperfusion disturbances were amplified by oxidative stress.[10]

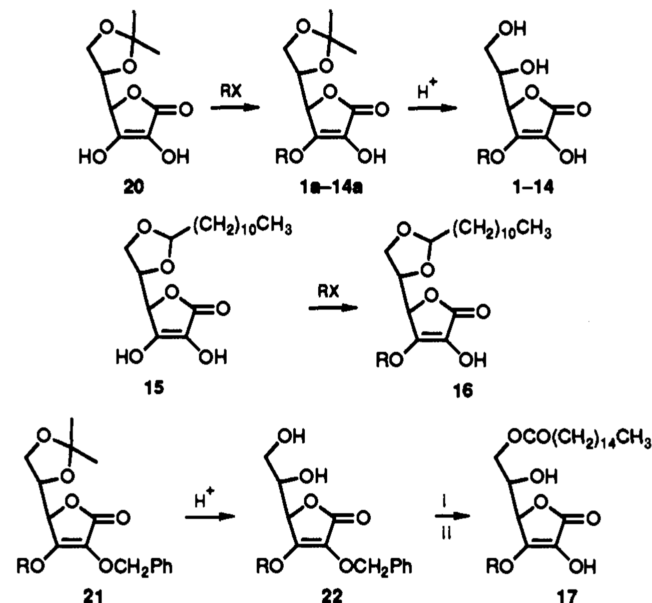
In fact, combined treatment with superoxide dismutase and urokinase-like plasminogen activator have enhanced myocardial salvage in a canine coronary thrombosis model.[11] Kato et al. have reported that 2-*O*-octadecylascorbic acid (19; CV-3611) reduced the myocardial lesions induced by ischemia and reperfusion in rats.[12] On that basis, we prepared several 3-*O*-alkylascorbic acids and then evaluated their antioxidant activities. These compounds, typified by 3-*O*-[(decylcarbonyl)methyl]ascorbic acid (8; HX-0112) were found to be strong chain-breaking antioxidants with a high affinity for biomembranes,[13] suggesting that some lipophilic ascorbic acid derivatives could be beneficial for protection against reperfusion injury.

In this report, we describe the effect of 3-*O*-alkylascorbic acids and the known lipophilic ascorbic acid derivatives (15,[14] 18,[15] 19[12]) on arrhythmias induced by coronary occlusion–reperfusion in anesthetized rats, as well as the structure–activity relationships and the related pharmacological effects of these lipophilic ascorbic acids.

Results

Chemistry. The routes of synthesis of the ascorbic acid derivatives listed in Table I are summarized in Scheme I. The compounds 1–14 were prepared as described in our previous report.[13a] Initially, 5,6-*O*-isopropylideneascorbic acid (20) was reacted with alkyl halides in $NaHCO_3$/DMSO, yielding 3-*O*-alkyl-5,6-*O*-isopropylideneascorbic acids 1a–14a that gave compounds 1–14 after acid hydrolysis.

Scheme I[a]



[a] (i) Palmitoyl chloride; (ii) Pd/C + H_2.

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Table I. Effects of Ascorbic Acid Derivatives and Verapamil on Lipid Peroxidation in Rat Liver Microsomes and Coronary Reperfusion-Induced Arrythmias in Anesthetized Rats

compd no.	R	lipid peroxidation: % inhibn[a]	dose,[b] mg/kg (iv)	arrhythmias times (VF + VT)[b]
	3-O-Alkylascorbic Acid			
	(i) n-alkyl group			
1	3-O-CH_2CH_3	2 ± 2 (78 ± 3)[c]	30	591 ± 5
2[d]	3-O-$(CH_2)_9CH_3$	93 ± 3[d]	1	291 ± 88*
3[d]	3-O-$(CH_2)_{11}CH_3$	91 ± 2[d]	10	49 ± 20**
4[d]	3-O-$(CH_2)_{13}CH_3$	78 ± 3[d]	10	352 ± 88
5[d]	3-O-$(CH_2)_{17}CH_3$	45 ± 2[d]	30	447 ± 96
	(ii) β-ketoalkyl group			
6[d]	3-O-$CH_2CO(CH_2)_7CH_3$	64 ± 2[d]	10	433 ± 157
7[d]	3-O-$CH_2CO(CH_2)_9CH_3$	88 ± 4[d]	1	205 ± 91*
8[d]	3-O-$CH_2CO(CH_2)_{11}CH_3$	85 ± 3[d]	1	185 ± 36**
9[d]	3-O-$CH_2CO(CH_2)_{13}CH_3$	65 ± 3[d]	30	445 ± 137
10[d]	3-O-$CH_2CO(CH_2)_{15}CH_3$	43 ± 2[d]	10	521 ± 71
	(iii) (n-alkoxycarbonyl)methyl group			
11	3-O-$CH_2COO(CH_2)_{17}CH_3$	30 ± 3	30	413 ± 89
	(iv) phenacyl group			
12	3-O-phenacyl	1 ± 2	30	429 ± 60
13	3-O-(4'-butylphenacyl)	82 ± 3	30	325 ± 90
14	3-O-(4'-octylphenacyl)	88 ± 2	10	461 ± 82
	Miscellaneous Lipophilic Ascorbic Acid Derivatives			
15[e]	5,6-O-dodecylidene	89 ± 3	1	186 ± 31**
16	3-O-CH_2COCH_3, 5,6-O-dodecylidene	44 ± 2	10	478 ± 50
17	3-O-CH_2COCH_3, 6-O-palmitoyl	53 ± 3	10	353 ± 88
18[f]	6-O-palmitoyl	80 ± 2	3	414 ± 89
19[g]	2-O-$(CH_2)_{17}CH_3$	88 ± 3[d]	0.1	238 ± 119*
verapamil		(37 ± 3)[c]	0.3	121 ± 57**

[a] Malondialdehyde production under control conditions was 11.9 ± 0.4 nmol/mg protein ($n = 10$), being induced by ADP/Fe^{3+} including NADPH. All mean ± SD inhibition % values ($n = 3$–6) were determined, at 10^{-5} M, except for verapamil (10^{-4} M, see footnote c). [b] The intervals of ventricular tachycardia (VT) and ventricular fibrillation (VF) were measured and summed to give the total duration of arrhythmia within 600 s, mean ± SE. [c] In the control group (vehicle), the range of times from each experiment ($n = 8$) was 565 ± 21 s. Each rat ($n = 3$–6) was treated iv with the compound 2 min before coronary occlusion. Significantly different from vehicle at $p < 0.01$ (**), $p < 0.05$ (*). [d] See ref 13a. [e] See ref 14. [f] See ref 15. [g] See ref 12.

5,6-O-Dodecylideneascorbic acid (15) was prepared from ascorbic acid and reacted with dodecanal in the presence of p-toluenesulfonic acid according to the method of Bharucha.[14] The resulting compound 15 was alkylated as described above to give 3-O-acetonyl-5,6-O-dodecylidene-ascorbic acid (16).

3-O-Acetonyl-5,6-O-isopropylideneascorbic acid[13a] was protected with benzyl bromide, and then the acetal group

Table II. Effects of Ascorbic Acid Derivatives and Verapamil on Coronary Reperfusion-Induced Arrythmias in Anesthetized Rats[a]

compound	dose (po), mg/kg	VF + VT, n = 5–10, mean ± SE)
vehicle		518.6 ± 41.3
2	30	283.6 ± 129.3
8	10	378.2 ± 61.4
	30	313.3 ± 81.7*
	100	85.4 ± 28.9**
15	30	420.8 ± 109.9
19	10	413.2 ± 56.9
	100	39.4 ± 12.3**
verapamil	10	56.7 ± 50.8*[b]

[a] Each rat was treated po with the compound at 1 h before coronary occlusion. Significant difference from the vehicle control at $p < 0.05$ (*), $p < 0.01$ (**). [b] $n = 3$.

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Table III. Antirrhythmic and Hemodynamic Effects of Compounds 8, 15, 19, and Verapamil in Anesthetized Rats[a]

subject (route)	8	15	19	verapamil
1. efficacy against:				
reperfusion (iv)	>1 mg/kg	>1 mg/kg	>0.1 mg/kg	>0.3 mg/kg
arrhythmia (po)	>30 mg/kg	30 mg/kg 19%[c]	10 mg/kg 20%[c]	10 mg/kg 87%[c]
effective dose[b]			100 mg/kg 92%**[c]	
2. effect on hemodynamics:				
(a) changes in arterial blood pressure (iv)	dose,[d] Δ mmHg[e]	dose,[d] Δ mmHg[e]	dose,[d] Δ mmHg[e]	dose,[d] Δ mmHg[e]
	10, −5.0 ± 1.7	1, −7.0 ± 1.5	0.1, −10.0 ± 2.9	0.3, −46.7 ± 3.3**
	30, −17.7 ± 3.5*	3, −30.3 ± 2.9**	0.3, −35.3 ± 2.3**	
(b) changes in heart rate (iv)	dose,[d] Δ beats/min[e]	dose,[d] Δ beats/min[e]	dose,[d] Δ beats/min[e]	dose,[d] Δ beats/min[e]
	10, −8.3 ± 8.3	1, −10.3 ± 3.2	0.1, −10.0 ± 5.8	0.3, −70.0 ± 17.3**
	30, −35.0 ± 8.7*	3, −75.0 ± 13.2**	0.3, −55.0 ± 11.5**	
effective dose ratio:				
antiarrhythmic dose/hemodynamic effect dose	30	3	3	1

[a] Statistically significant when compared with vehicle, (*) $p < 0.05$, (**) $p < 0.01$. [b] Minimum effective dose. See Tables I and II. [c] Percent inhibition. [d] Dose iv mg/kg. [e] Mean ±SE, $n = 3$–4.

Table IV. Effect of Ascorbic Acid Derivatives and Verapamil on Atrial Rate and Contractility in Isolated Guinea Pig Right Atria[a]

	% inhibition (mean ± SE)					
	atrial rate			atrial contractility		
vehicle	0.5 ± 0.2	0.7 ± 0.3	0.9[b]	8.9 ± 1.6	12.2 ± 1.8	12.2[b]
concn (mg/mL)	0.03	0.1	0.3	0.03	0.1	0.3
8	12.9 ± 2.5*	14.6 ± 1.8*	13.0[b]	25.0 ± 2.3**	30.2 ± 2.9**	41.3[b]
13	8.8 ± 1.2*	17.0 ± 2.0*	25.6[b]	36.2 ± 2.0**	52.8 ± 2.6**	67.1[b]
14	−0.4 ± 0.4	−0.9 ± 0.4	0.3[b]	15.8 ± 1.7*	33.5 ± 2.9**	45.0[b]
18	−2.6 ± 1.4	−6.2 ± 1.6*	−10.9[b]	3.7 ± 1.2	3.7 ± 1.1	19.2[b]
19	−1.8 ± 1.2	−6.0 ± 1.7*	−13.6[b]	−10.7 ± 1.3**	−16.7 ± 1.0*	−39.9[b]
concn (mg/mL)	0.003	0.01	0.03	0.003	0.01	0.03
verapamil	37.9 ± 3.0**	63.0[b]	100[b]	49.9 ± 3.2**	77.8[b]	100[b]

[a] Significantly different from vehicle at (*) $p < 0.05$, (**) $p < 0.01$. Each test $n = 2$–4. [b] $n = 2$.

was removed by acid hydrolysis. The resulting 2-*O*-benzyl-3-*O*-acetonylascorbic acid (**22**) was acylated with palmitoyl chloride and then catalytically reduced to yield compound **17**.

Pharmacology. The effects of 19 ascorbic acid derivatives (**1**–**19**) and verapamil on lipid peroxidation of rat liver microsomes by $FeCl_3$–ADP in the presence of NADPH were studied. Peroxides were determined as malondialdehyde by Ohkawa's method.[16] As shown in Table I, compounds **2**, **3**, **7**, **8**, **13**–**15**, **18**, and **19** exhibited strong inhibitory effects on lipid peroxidation. Verapamil also demonstrated significant inhibition, although its activity was lower than that of the ascorbic acid derivatives.

The effects of these compounds on coronary reperfusion arrhythmias (ventricular tachycardia (VT) + ventricular fibrillation (VF)) were studied in anesthetized rats.[17] The left anterior descending artery was ligated for 5 min, and the duration of VF and VT was determined following reperfusion for 10 min. Each rat was treated with the test compounds before arterial occlusion (2 min before for iv and 1 h before for po administration). Compounds **2**, **3**, **7**, **8**, **15**, and **19**, which exhibited a potent effect against lipid peroxidation, as well as verapamil, greatly reduced the VF + VT intervals as compared with vehicle alone (control) as shown in Table I. Compounds **13**, **14**, and **18**, however, did not affect postreperfusion arrhythmias although they were also strong inhibitors of lipid peroxidation. All weak inhibitors of lipid peroxidation (except verapamil) were also ineffective. Compound **8** exhibited the strongest protective effect of the 3-*O*-alkylascorbic acid analogues on reperfusion injury, the lowest effective dose being 1 mg/kg iv or 30 mg/kg po (Tables I and II). For compounds **2**, **15**, **19**, and verapamil, the iv dose was 1, 1, 0.1, and 0.3 mg/kg, respectively, but compound **2** and **15** had no effect at 30 mg/kg po.

The effects of these compounds on hemodynamics were investigated in anesthetized rats. Electrocardiograms and arterial blood pressure (ABP) were monitored in the anesthetized rats given compound **19**, verapamil (both >0.3 mg/kg iv), or compound **15** (>3 mg/kg iv). Compound **15**, **19**, and verapamil strongly influenced the hemodynamics to reduce heart rate and ABP in rats, but compound **8** had no effect up to 30 mg/kg iv, as shown in Table III. The other compounds **2**, **3**, and **7**, effective against reperfusion arrhythmias, also significantly reduced ABP in anesthetized rats when administered at doses beyond the range of 3–10 mg/kg iv (data not shown).

The effects of compounds **8**, **13**, **14**, **18**, **19**, and verapamil on the atrial rate and atrial contraction were investigated in isolated guinea pig right atria. Verapamil inhibited the atrial rate and atrial contractility at 0.003 mg/mL while the ascorbic acid derivatives had no influence at higher concentrations (Table IV).

The effects of compounds **2**–**9** and **19** on the production of active oxygen species were investigated in peritoneal exudate macrophages obtained from male Wistar rates by the intraperitoneal injection of liquid paraffin. The active oxygen species production, most of which was actually superoxide anion because the consumption of NADH was almost completely inhibited (>95%) by adding superoxide dismutase to the exudate macrophages, was determined by the NADH–LDH method.[18] Compound **8** had no effect on active oxygen species production, although compound **19** and its regioisomer compound **5** significantly inhibited

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Table V. Effect of Various Compounds 2–9 and 19 on the Production of Active Oxygen Species by Rat Peritoneal Exudate Macrophages

compound no.	R	inhibition (%)[a]
2	3-O-$(CH_2)_9CH_3$	15.9 ± 4.2
3	3-O-$(CH_2)_{11}CH_3$	38.7 ± 6.7*
4	3-O-$(CH_2)_{13}CH_3$	40.7 ± 7.4*
5	3-O-$(CH_2)_{17}CH_3$	53.3 ± 6.3**
6	3-O-$CH_2CO(CH_2)_7CH_3$	4.9 ± 2.5
7	3-O-$CH_2CO(CH_2)_9CH_3$	11.1 ± 4.5
8	3-O-$CH_2CO(CH_2)_{11}CH_3$	6.3 ± 3.9
9	3-O-$CH_2CO(CH_2)_{13}CH_3$	36.8 ± 6.2*
19	2-O-$(CH_2)_{17}CH_3$	87.0 ± 7.1**

[a] Mean ± SE value (n = 4–6) at 1×10^{-4} M. Final cell number was $(2-3) \times 10^6$ cells/mL. Control group (n = 12), NADH 32.3 ± 3.4 nmol/mL was oxidized for 10 min; blank group (n = 12) without cells, NADH 7.5 ± 1.8 nmol/mL, respectively. Significantly different from control, (*) $p < 0.05$, (**) $p < 0.01$.

the production of active oxygen species, as did compounds 3 and 4 (Table V).

Discussion

We previously reported that lipophilic ascorbic acids 2, 3, 7, and 8, as well as compound 19 with nearly equal hydrophobicity, strongly inhibited microsomal lipid peroxidation to a similar extent, whereas compounds 5 and 10 with higher hydrophobicity had decreased activity against lipid peroxidation.[13]

In the present study, lipophilic ascorbic acid derivatives 13, 14, 15, and 18 exhibited strong inhibitory effects, equal to those of compounds 2, 3, 7, 8, and 19, on lipid peroxidation. However compounds 13 and 14 (bearing a 4'-alkylphenacyl group at the 3-position) and compound 18 (acylated with a palmitoyl group at the 6-position) did not reduce reperfusion arrhythmias. The latter compound would have been promptly metabolized in vivo, but we do not understand the cause of the lack of effectiveness of 3-O-(4'-alkyl)phenacylascorbic acids 13 and 14 regardless of their strength as antioxidants. Verapamil inhibits the rate and strength of atrial contraction,[19] so we investigated the inhibitory activity of compounds 8, 13, 14, 18, and 19 on these parameters in isolated guinea pig right atria. However, the weak effects of the ascorbic acid derivatives on these atrial properties did not explain the unexpected results for compounds 13 and 14 in the reperfusion injury test. Thus, the phenacyl group is not a suitable substituent for compounds to protect against reperfusion injury, although the cause so far remains unclear.

The significantly active compounds 2, 3, 7, and 8 protecting against reperfusion injury were selected by screening for the optimal length of the aliphatic alkyl chain. When the compounds 1–11 underwent the lipid peroxidation test (in vitro) and the reperfusion injury test (in vivo), significant activity in vivo corresponded with the maximal activity in vitro.

It has been proposed that the production of active oxygen species by neutrophils and macrophages is concerned with reperfusion injury.[20] Compounds 3–5 and 19 bearing a long n-alkyl chain ($>C_{12}$) inhibited the production of superoxide anion by peritoneal exudate macrophages, but compounds 6–8 bearing a β-ketoalkyl chain were ineffective in preventing the production of superoxide anion. However, there was no relationship between this activity and that of the antiarrhythmic effect of these compounds.

Compounds 15 and 19 exhibited a strong protective effect against reperfusion injury, and these compounds strongly reduced ABP and heart rate in anesthetized rats at lower doses than compound 8. Compound 19 also had strong hemodynamic effects which were equal to those of verapamil, although at a low concentration it did not influence atrial rate and contractility in the isolated atrial preparation. The marked protective effect of compound 19 against reperfusion injury may have been due to inhibition of lipid peroxidation plus its potent hemodynamic actions. Since compound 8 did not influence atrial contractility, atrial rate, and superoxide production had little influence on hemodynamics, the protective effect of this compound against reperfusion injury would appear to be dependent on the potency of its antioxidant effect on lipid membranes. Additionally, compound 8 has been shown to reduce myocardial infarct size in dogs and to be effective against renal damage in rats, both of the animal models involving occlusion–reperfusion.[21] Chain-breaking antioxidants are now proposed as a new class of agents for treating reperfusion injury while not directly affecting hemodynamics.[22] Compound 8 was more specific against reperfusion-induced arrhythmias and in its effects on the cardiovascular system than the other effective ascorbic acid derivatives.

Experimental Section

Chemistry. Column chromatography was carried out on a Kieselgel 60 column (70–230 mesh, Merck). Melting points were obtained with a micro melting point apparatus (Yanagimoto) and were uncorrected. NMR spectra were recorded on a JEOL FX-90Q spectrometer (90 MHz), using Me_4Si as an internal standard. All elemental analyses were found to be within ±0.4% of the calculated values. Compounds 2–11 and 19 were prepared as previously reported.[13a]

General Procedure for Obtaining the 3-O-Alkylascorbic Acids 1–14.[13a] **3-O-Ethylascorbic Acid (1).** Sodium bicarbonate (2.52 g, 0.03 mol) was added to a solution of 5,6-O-isopropylideneascorbic acid[23] (**20**, 4.3 g, 0.02 mol) in 25 mL of DMSO. The mixture was stirred for 20 min and ethyl bromide (3.27 g, 0.03 mol) was added. This mixture was stirred for 16 h at 50 °C and then diluted with water (50 mL), neutralized with 1 M HCl, and extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue was then washed with hexane to give the crude 3-O-ethyl-5,6-O-isopropylideneascorbic acid (**1a**). The residue was recrystallized from benzene to give 3.42 g of compound **1a** (70%, mp 105–106 °C). To compound **1a** (3.0 g, 0.012 mol) in MeOH was added 2 M HCl (20 mL), and then the mixture was stirred for 3 h at 60 °C. The mixture was concentrated to $^1/_5$ volume, neutralized with 5% sodium bicarbonate, and extracted with EtOAc/IPA (9:1). The organic layer was separated, washed with water, dried, and

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evaporated in vacuo. Then the crude product was recrystallized from EtOAc to give 2.41 g of compound 1: yield 98%; mp 113–114 °C; NMR (MeOH-d_4) δ 1.36 (3 H, t, 7 Hz), 3.69 (2 H, m), 3.90 (1 H, m), 4.54 (2 H, q, 7 Hz), 4.75 (1 H, d, 1 Hz). Compounds 11–14 were prepared according to this procedure, with silica gel column chromatography to purify their ketals. For compound 11, 12, 13, and 14, the yield after alkylation was 50%, 45%, 55%, and 45%, respectively, while that after deprotection was 58%, 70%, 90%, and 93%, respectively. Compound 11 ($C_{26}H_{46}O_8$), mp 113–114 °C. Compound 12 ($C_{14}H_{14}O_7$), mp 91–92 °C. Compound 13 ($C_{18}H_{22}O_7$), mp 103 °C dec. Compound 14 ($C_{22}H_{30}O_7$), mp 128–130 °C.

5,6-*O*-Dodecylideneascorbic Acid (15).[14] Ascorbic acid (13.8 g, 0.078 mol), *p*-toluenesulfonic acid (1.2 g, 0.007 mol), and *n*-dodecylaldehyde (4.05 g, 0.022 mol) were dissolved in dimethylacetamide (60 mL) and stirred for 20 h at 60 °C. After cooling, ether and water (100 mL each) were added, and the mixture was vigorously shaken. The organic layer was separated, and the extraction procedure was repeated twice. The combined organic layers were washed with water, dried, and evaporated in vacuo. Hexane was added to the residue and reevaporated. The residue was then recrystallized from EtOAc/hexane to give 10.8 g of compound 15: yield 40%; mp 130–131 °C (lit. 128–130 °C);[14] NMR (MeOH-d_4) δ 0.89 (3 H, m), 1.28 (20 H, m), 3.90–4.27 (3 H, m), 4.64 (1 H, m), 4.69 (1 H, d, 3 Hz).

3-*O*-Acetonyl-5,6-*O*-dodecylideneascorbic Acid (16). Sodium bicarbonate (0.4 g, 0.005 mol) was added to a solution of compound 15 (1.70 g, 0.005 mol) in 20 mL of DMSO. The mixture was then stirred for 20 min, and chloroacetone (0.5 g, 0.0054 mol) was added. The mixture was subsequently stirred for 16 h at 50 °C, diluted with water (100 mL), neutralized with 0.5 M HCl, and extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. Then the residue was subjected to silica gel column chromatography and eluted with EtOAc/benzene to give 0.5 g of compound 16: yield 25% ($C_{21}H_{34}O_7$); mp 124–125 °C (recrystallized from EtOAc/hexane); NMR (MeOH-d_4) δ 0.89 (3 H, m), 1.29 (20 H, m), 2.18 (3 H, s), 3.89–4.28 (3 H, m), 4.79 (1 H, m + 1 H, d, 3 Hz), 5.07 (2 H, s).

3-*O*-Acetonyl-6-*O*-palmitoylascorbic Acid (17). Benzyl bromide (2.05 g, 0.012 mol) and potassium carbonate (1.66 g, 0.012 mol) were added to a solution of 3-*O*-acetonyl-5,6-*O*-isopropylideneascorbic acid (2.80 g, 0.012 mol)[13a] in 20 mL of DMSO. The mixture was stirred for 16 h at 50 °C, diluted with water (100 mL), and extracted with EtOAc. Then the organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue was subjected to silica gel column chromatography and eluted with EtOAc/hexane to give 2-*O*-benzyl-3-*O*-acetonyl-5,6-*O*-isopropylideneascorbic acid (21, 2.21 g, 57%). After removal of the protective group of compound 21 as described above, 2-*O*-benzyl-3-*O*-acetonylascorbic acid (22, 1.92 g, 99%) was obtained. To a solution of compound 22 (1.90 g, 0.0067 mol) in CH_2Cl_2 (40 mL) triethylamine (0.70 g, 0.0079 mol) and palmitoyl chloride (2.00 g, 0.0073 mol) were added slowly at 4 °C. The mixture was stirred at 4 °C overnight and then evaporated to $^1/_3$ of the original volume. EtOAc (100 mL) and 0.5 M HCl (50 mL) were added, and the mixture was shaken, after which it was extracted with EtOAc (100 mL × 2). The organic layer was separated, and the combined organic solution was then washed with 5% $NaHCO_3$ and water, dried, and evaporated in vacuo. Then the residue was dried in vacuo to give 2-*O*-benzyl-3-*O*-acetonyl-6-*O*-palmitoylascorbic acid (2.20 g, 60%). To a solution of the benzylated compound (2.20 g, 0.0042 mol) in 40 mL of EtOAc was added 10% palladium charcoal (0.30 g), and the mixture was hydrogenated for 12 h at atmospheric pressure. The catalyst was removed by filtration, and the filtrate was concentrated. Then the residue was recrystallized from EtOAc/hexane to give 1.40 g of compound 17: yield 77% ($C_{25}H_{42}O_8$); mp 97–98 °C; NMR (DMSO-d_6) δ 0.86 (3 H, m), 1.24 (24 H, m), 2.11 (3 H, s), 2.32 (2 H, m), 4.06 (3 H, m), 4.82 (1 H, b s), 5.03 (2 H, m).

Pharmacology. Preparation of a Microsomal Suspension. Wistar rats were fasted for 24 h and killed by exanguination under light anesthesia. Their livers were removed, excised, and homogenized in cold 0.15 M KCl. The homogenate was then centrifuged at 8000*g* for 20 min, and the supernatant was recentrifuged at 10500*g* for 1 h to obtain the microsomal fraction. The pellet thus obtained was resuspended in fresh 0.15 M KCl, and the protein concentration was determined by the method of Lowry et al.[24]

Measurement of Lipid Peroxidation. The assay system (1 mL) consisted of 83.5 mM KCl and 37.2 mM Tris-HCl buffer (pH 7.4), the test compound in DMF (0.01 mL), 1 mM ADP, 10 μM $FeCl_3$, the microsomal fraction (2.0 mg of protein), and 0.02 mM NADPH. Reaction mixtures were incubated at 37 °C for 20 min and then cooled on ice to terminate the reaction. Lipid peroxides generated were determined by the method of Ohkawa et al.[16] In brief, 8.1% sodium dodecyl sulfate (0.2 mL), 20% AcOH containing 0.27 M HCl adjusted to pH 3.5 with NaOH (1.5 mL), and 0.8% thiobarbituric acid (1.5 mL) were added to the reaction mixture. The mixture was then heated at 95 °C for 20 min, and the reaction was stopped by cooling on ice. Thereafter, *n*-BuOH/pyridine (15:1, 5.0 mL) was added with vigorous mixing. After centrifugation at 800*g* for 10 min, the organic layer was separated and the absorbance was measured at 532 nm.

Effect on the Cardiovascular System of Anesthetized Rats. Experiments were performed in male Wistar rats that were anesthetized with sodium pentobarbital (60 mg/kg ip) and placed in the supine position. Blood pressure was measured at the femoral artery through a pressure transducer (Nippon Koden); the electrocardiogram was recorded from standard limb lead II, and heart rate was measured by an instantaneous beat-to-beat tachograph. Test compounds were suspended in saline containing 1% olive oil and 1% Tween 80 and injected via the femoral vein. The heart rate and blood pressure were monitored for 10 min after the injection of each compound.

Effect on Coronary Reperfusion-Induced Arrhythmias in Anesthetized Rats.[17] Experiments were performed on artificially ventilated supine male Wistar rats (350–450 g) anesthetized with sodium pentobarbital (60 mg/kg ip). A thoracostomy was performed at the 5th left intercostal space, and the heart was supported in a pericardial cradle. The left anterior descending coronary artery was ligated for 5 min, followed by reperfusion for 10 min. During this period, the durations of ventricular tachycardia (VT) and ventricular fibrillation (VF) were measured and summed to give the total duration of arrythmia within 600 s. Rats were given the test compounds 2 min (iv) or 1 h (po) before coronary occlusion.

Effect on Isolated Guinea Pig Atria. Male Hartley guinea pigs (450–550 g) were killed by a blow to the head. The hearts were removed, and the right atria were carefully dissected. The atria were suspended with a load of 0.5 g and incubated at 37 °C in Tyrode's solution gassed with a mixture of 95% O_2 + 5% CO_2. The atrial rate and contractile force were recorded on a polygraph, and the effects of the test compounds were monitored under spontaneously beating conditions after equilibration for at least 30 min (until the rate did not vary by more than 5 beats/min).

Preparation of Rat Peritoneal Exudate Macrophages.[18] Male Wistar rats (300–350 g) were injected ip with 30 mL of liquid paraffin oil and killed 3 days later. Cells were washed out from the peritoneal cavity with fresh Eagle's MEM (100 mL). Cells from three to five animals were centrifuged at 500*g* for 10 min at room temperature and then washed once with the same medium. Preparations containing numerous red cells were discarded. More than 90% of the cells thus obtained were viable macrophages.

Assay of Active Oxygen Species Production by Peritoneal Exudate Macrophages.[18] The decrease in NADH absorption at 340 nm was recorded continuously at 37 °C with a Hitachi UV spectrophotometer. The assay system (3 mL) consisted of 125 mM sodium phosphate buffer (pH 6.5), 0.08 mM EDTA, 1.2 units of LDH, 0.32 mM NADH, (2–3) × 10^6 macrophages, and the test compound in 0.03 mL of DMF. The reaction was started by the addition of NADH. Test compounds with no macrophages were used to obtain basal values for NADH oxidation, and the percent inhibition of oxidation was calculated from the following equation:

$$\{1 - [\Delta A\ (\text{macrophages + test compound}) - \Delta A\ (\text{test compound basal})] / [\Delta A\ (\text{macrophage control}) - \Delta A\ (\text{medium basal})]\} \times 100$$

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where ΔA is the difference in absorption at 340 nm before and after the reaction.

Registry No. 1, 86404-04-8; 1a, 86404-03-7; 2, 132530-91-7; 3, 88306-62-1; 4, 106396-33-2; 5, 88306-73-4; 6, 133794-55-5; 7, 133794-56-6; 8, 133794-57-7; 9, 133794-58-8; 10, 133794-59-9; 11, 132530-92-8; 12, 26633-25-0; 13, 139870-17-0; 14, 139870-18-1; 15, 69677-84-5; 16, 139870-19-2; 17, 139870-20-5; 17 (2-*O*-benzyl derivative), 139870-23-8; 18, 137-66-6; 19, 98829-12-0; 20, 15042-01-0; 21 (R = CH_2COCH_3), 139870-21-6; 22 (R = CH_2COCH_3), 139870-22-7; $ClCH_2COCH_3$, 78-95-5; $CH_3(CH_2)_{14}COCl$, 112-67-4; 3-*O*-acetonyl-5,6-*O*-isopropylideneascorbic acid, 126100-80-9.

Synthesis and Antihypertensive Activity of 3-[(Substituted-carbonyl)amino]-2*H*-1-benzopyrans

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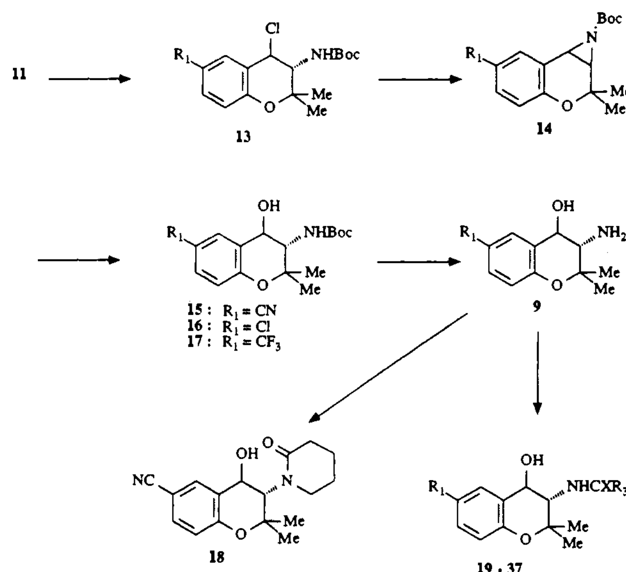
The synthesis and antihypertensive activity of a series of novel 3-[(subtituted-carbonyl)amino]-2*H*-1-benzopyran-4-ols, administered orally to spontaneously hypertensive rats, are described. Optimum activity in this series was observed for compounds with branched alkyl or branched alkylamino groups flanking the carbonyl or thiocarbonyl group (21, 31–33), which were approximately equipotent to cromakalim. Replacement of the 4-hydroxyl group by hydrogen, methoxy, or amino in this series only led to a slight reduction in potency. These observations are in marked contrast to the structure–activity relationships previously found for the 4-amidobenzopyran-3-ols. The antihypertensive activity of representative compounds 15 and 33 was attenuated by preatreatment with glibenclamide, and thus these compounds may belong to the series of drugs which have been classified as potassium channel activators.

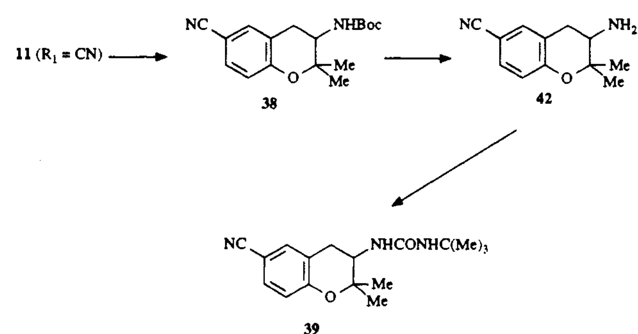
Recently we have described several series of novel antihypertensive agents based on the 4-(2-oxopyrrolidin-1-yl)-2*H*-1-benzopyran-3-ol cromakalim (1),[1-4] which has been shown to hyperpolarize the membrane potential of vascular smooth muscle cells[5] via enhanced efflux of potassium ions[6] through ATP-sensitive channels.[7] The net effect of this process is to relax blood vessels and reduce blood pressure.[8]

Since the discovery[9] of cromakalim (1) a number of other compounds such as pinacidil (2) and RP 49356 (3) have been reported[10] to be potassium channel activators. Sev-

Scheme I



Scheme II



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