shaker at 0–4 °C. The procedure was repeated with a fresh pellet. After each incubation, the charcoal was removed by centrifugation. The serum was sterilized through a 0.20-μm filter (Sartorius, Göttingen, FRG) and stored at −20 °C. All of the experiments were performed in the presence of phenol red.

Cells were grown in a humidified incubator in 5% CO_2 at 37 °C. Two weeks before start of the experiment, cells were switched from NCS to CCS and received two additional media changes before they were harvested with 0.05% trypsin/0.2% EDTA in 0.15 M NaCl. They were syringed gently to prevent clumping, and approximately 2×10^4 cells in 2 mL were plated replicately in six-well dishes (Costar). Cells were switched 2 days later to a medium containing the substances and 0.1% DMF in which the compounds had been dissolved. The medium of control wells contained an equal volume of DMF. On the 4th day, media were changed and substances added again. Cells were labeled 3 days later with 1 μCi [^{3}H]thymidine per well for 2 h. Cells were washed with cold PBS and harvested in PBS containing 0.02% EDTA. After centrifugation, the cell pellet was resuspended in 1 mL of PBS and divided in two equal parts. One part was counted in a Z I Coulter Counter the other one was sonicated. After addition of 4 mL of 10% trichloroacetic acid (TCA), the acid-insoluble fraction was collected on a 0.45-μm filter (Metricel, Gelman) and counted after addition of 10 mL of scintillation liquid (Quickszint 212, Zinsser) in a LS 1801 Beckman scintillation counter.

MDA-MB 231 Human Breast Cancer Cells. The MDA-MB 231 cell line was also obtained from ATCC. Cells were grown in McCoy 5a medium (Boehringer Mannheim, FRG) supplemented with 10% NCS and gentamycin (40 μg/mL). The experiments were performed as described for the MCF-7 cells with one exception: the incubation period was reduced from 5 to 2 days because the cell population doubling time is less than half of that of MCF-7 cells (16 vs 36 h[16]).

Transplanted MXT-Mammary Tumors of the Mouse. The MXT-mammary tumors were generously provided by Dr. A. E. Bogden, EG & G Mason Research Institute, Worcester, MA and Dr. G. Leclercq, Institute Jules Bordet, Brussels, Belgium. Hormone-sensitive tumors grew for 4–5 weeks in the host animals before transplantation. Tumor pieces of 1 mm^2 were serially transplanted into 8–9-week-old female B6D2F1 mice, obtained from Charles-River-Wiga, Sulzfeld, FRG. Animals were assigned randomly in groups of 10 and treatment was started 24 h after transplantation. Drugs were dissolved or suspended in polyethylene glycol 400/1.8% saline (1:1) and administered subcutaneously on Monday, Wednesday, and Friday. After a 6-week period of treatment, animals were killed and autopsied. Tumors were removed and weighed. The uterine dry weight was determined as described above. The change of body weight between start and end of therapy was recorded in order to detect obvious toxicity.

Hormone-resistant tumors were kept in ovariectomized B2D2F1 mice. Treatment was started 24 h after transplantation and lasted 2 weeks. The administration scheme was the same one as outlined for hormone-sensitive tumors. Since the hormone-resistant tumors can not be dissected free of connective tissue, the tumor area was determined instead of tumor weight. The tumor area was obtained by transdermal caliper measurements of two perpendicular axes, one across the largest diameter.

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Registry No. **1b**, 91444-18-7; **2a**, 115119-04-5; **2b**, 115119-05-6; **2c**, 115119-06-7; **2d**, 115084-37-2; **3a**, 132751-72-5; **3b**, 132751-78-1; **3c**, 132751-79-2; **3d**, 116041-09-9; **4**, 132751-73-6; **5a**, 132751-74-7; **5b**, 132751-80-5; **5c**, 132751-81-6; **5d**, 115084-42-9; **5e**, 132751-82-7; **6a**, 1121-60-4; **6b**, 1122-62-9; **6c**, 3238-55-9; **7a**, 132751-75-8; **7b**, 132751-83-8; **7c**, 132751-84-9; **7d**, 116041-11-3; **7e**, 132751-85-0; **7f**, 132751-86-1; **7g**, 132751-87-2; **8a**, 132751-76-9; **8b**, 132751-88-3; **8c**, 132751-89-4; **8d**, 116041-10-2; **8e**, 132751-90-7; **8f**, 132751-91-8; **8g**, 132751-92-9; **9a**, 132751-77-0; **9b**, 132751-93-0; **9c**, 132751-94-1; **9d**, 116041-12-4; **9e**, 132751-95-2; **9f**, 132751-96-3; **9g**, 132751-97-4; **10a**, 115357-87-4; **10b**, 115357-86-3; **10c**, 132751-98-5; **10d**, 115357-85-2; **10e**, 115357-88-5; **10f**, 115362-88-4; **10g**, 115378-88-6; K_2PtCl_4, 10025-99-7; phthalimide-K, 1074-82-4; 2-cyanopyridine, 100-70-9; 7-bromoheptanonitrile, 20965-27-9.

Supplementary Material Available: ^{1}H NMR data of 1-(phthalimidoalkyl)-2-phenylindoles (**3a–d**), 1-(aminoalkyl)-2-phenylindoles (**5a–e**), 1-[ω-[(2-pyridylmethylene)amino]alkyl]-2-phenylindoles (**7a–g**), 5-methoxy-2-(4-methoxyphenyl)-1-[ω-[(2-pyridylmethyl)amino]alkyl]indoles (**8a–g**), 5-hydroxy-2-(4-hydroxyphenyl)-1-[ω-[(2-pyridylmethyl)amino]alkyl]indoles (**9a–g**), and [5-hydroxy-2-(4-hydroxyphenyl)-1-[ω-[(2-pyridylmethyl)amino]alkyl]indole]dichloroplatinum(II) complexes (**10a–g**) (9 pages). Ordering information is given on any current masthead page.

3-*O*-Alkylascorbic Acids as Free-Radical Quenchers: Synthesis and Inhibitory Effect on Lipid Peroxidation

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A novel series of 3-*O*-alkylascorbic acids (3-RASA, **3a–n**) was synthesized to act as radical scavengers for active oxygen species and free radicals, and their redox potentials and inhibitory effects on lipid peroxidation in rat liver microsomes were evaluated. The redox potentials of the 3-RASA compounds were increased by the substituent group to 90–190 mV above the potential for ascorbic acid (i.e., 3-RASA compounds were harder to oxidize). Although 3-*O*-dodecylascorbic acid (**3c**) and 3-*O*-(decylcarbomethyl)ascorbic acid (**3i**) differed in their redox potentials, they both markedly inhibited lipid peroxidation in rat liver microsomes to a similar extent (IC_{50} = 3.1 and 3.3×10^{-6} M, respectively). Structure–activity relationship studies demonstrated that the anti lipid peroxidation activity of the 3-RASA compounds was markedly dependent upon their hydrophobicity.

Introduction

It is clear that active oxygen species (AOS, such as superoxide, •OH, •OOH, etc.), as well as the free radicals derived from the biochemical utilization of O_2 or the prooxidant stimulation of O_2 metabolism, participate in the development or exacerbation of various diseases: e.g., ischemia–reperfusion disturbances in the brain and heart, rheumatism, inflammatory disorders, gastric ulcer, and cancer.[1]

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Table I. Chemical and Physical Data and Inhibitory Effect on Lipid Peroxidation of 3-RASA and 2-RASA Compounds

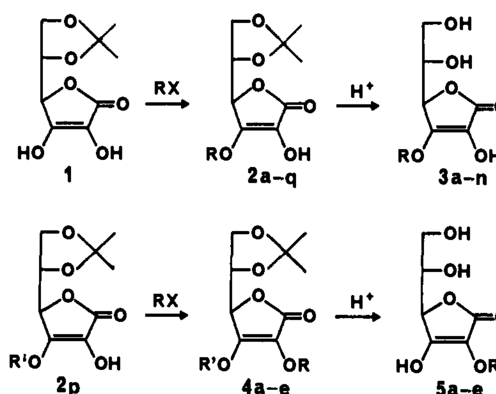
no.	R	mp, °C	formula	yield, %: alkyl.[a] $1 \rightarrow$ 2a–n	yield, %: deprotect.[b] 2a–n $\rightarrow$ 3a–n	% LO inhibn[c]: Fe^{3+}–ADP[d]	% LO inhibn[c]: Fe^{2+}[d]	k′[j]
	H (ascorbic acid)		$C_6H_8O_6$			NT[e]	(72)[f]	<0.10
3a	$(CH_2)_7CH_3$	58–60	$C_{14}H_{24}O_6$	71	70	40[g]	9[i]	0.62
3b	$(CH_2)_9CH_3$	73–75	$C_{16}H_{28}O_6$	70	90	91[g]	44[g]	1.27
3c	$(CH_2)_{11}CH_3$	86–88	$C_{18}H_{32}O_6$	90	71	88[g]	34[g]	2.49
3d	$(CH_2)_{13}CH_3$	68–69	$C_{20}H_{36}O_6$	60	90	81[g]	30[g]	4.78
3e	$(CH_2)_{15}CH_3$	73–74	$C_{22}H_{40}O_6$	55	90	74[g]	21[g]	9.09
3f	$(CH_2)_{17}CH_3$	102–103	$C_{24}H_{44}O_6$	60	90	45[g]	7[i]	17.34
3g	$CH_2CO(CH_2)_5CH_3$	99–100	$C_{14}H_{22}O_7$	37	90	27[h]	20[g]	0.24
3h	$CH_2CO(CH_2)_7CH_3$	109–110	$C_{16}H_{26}O_7$	40	55	64[g]	33[g]	0.55
3i	$CH_2CO(CH_2)_9CH_3$	105–106	$C_{18}H_{30}O_7$	28	84	88[g]	44[g]	1.14
3j	$CH_2CO(CH_2)_{11}CH_3$	113–114	$C_{20}H_{34}O_7$	33	83	81[g]	30[g]	2.25
3k	$CH_2CO(CH_2)_{13}CH_3$	116–117	$C_{22}H_{38}O_7$	33	83	65[g]	33[g]	4.33
3l	$CH_2CO(CH_2)_{15}CH_3$	113–116	$C_{24}H_{42}O_7$	74	56	43[g]	28[g]	8.26
3m	$CH_2COO(CH_2)_9CH_3$	110–111	$C_{18}H_{30}O_8$	55	52	NT[e]	31[g]	NT[e]
3n	β-picolyl	159–160	$C_{12}H_{13}NO_6$	23[a,*]	60	02[i]	NT[e]	<0.10
5a	$(CH_2)_7CH_3$	118–119	$C_{14}H_{24}O_6$	(65)[a,**]	(70)	20[g]	NT[e]	<0.20
5b	$(CH_2)_{11}CH_3$	127–128	$C_{18}H_{32}O_6$	(63)[a,**]	(79)[b,*]	83[g]	NT[e]	0.63
5c	$(CH_2)_{17}CH_3$	128–129	$C_{24}H_{44}O_6$	(70)[a,**]	(60)[b,*]	88[g]	29[g]	1.13
5d	$CH_2CO(CH_2)_{15}CH_3$	117–119	$C_{24}H_{42}O_7$	(24)[a,**]	(75)[b,*]	55[g]	NT[e]	0.45
5e	$CH_2COO(CH_2)_3CH_3$	34–35	$C_{12}H_{18}O_8$	(91)[a,**]	(20)[b,*]	10[i]	NT[e]	NT[e]
6	CH_2COCH_3	89	$C_{12}H_{16}O_7$	*l*	*l*	02[i]	NT[e]	<0.10
7	2,3-*O*-$[(CH_2)_9CH_3]_2$	65	$C_{26}H_{48}O_6$	*l*	*l*	02[i]	NT[e]	NT[e]

[a] Yield of 3-*O*-alkylation step (method A); *2 $\rightarrow$ **3n** (method B); ****3p** $\rightarrow$ **5a–c**, 2-*O*-alkylation step.[11] [b] **4a–n** (**3a–n** $\rightarrow$ **4a–n**), ***6a–c**, (**5a–c** $\rightarrow$ **6a–c**); yield of deprotection step. [c] LO (lipid peroxidation), compound concentration 1×10^{-5} M. [d] Producing malondialdehyde at control, 11.9 ± 0.4 nM/mg of protein by Fe^{3+}–ADP, 9.5 ± 0.6 nM/mg of protein by Fe^{2+}. [e] Not tested. [f] Percent stimulation. [g] Percent inhibition; statistically significant compared to control, $p < 0.01$. [h] $p < 0.05$. [i] Percent inhibition; not statistically different from control. [j] k' = partition ratio obtained from HPLC analysis. [l] See the Experimental Section.

Although the exact mechanisms of action and the roles of AOS in these diseases are not entirely clear, a vast number of studies have been undertaken on the systems for the generation and elimination of AOS and free radicals both in vitro and in vivo.[2] In particular, much work has been devoted to AOS generation in ischemic organs and the damaging effect of AOS on cell membranes and DNA synthesis.[3] Eventually, it is hoped that the development of quenchers for AOS and free radicals will provide a new method of treatment for many diseases.

Some AOS, hydroxyl radicals, and complexes of oxygen radicals and Fe ions, may attack proteins, DNA, and lipid membranes, leading to a decrease in cellular activity and the deterioration of function.[4] Such active species are produced under conditions where the oxygen tension is increased within cells as well as during the lipid peroxidation of biological membranes. Unsaturated fatty acids, a constituent of cell membranes, are particularly susceptible to lipid peroxidation. It is not yet clear whether or not lipid peroxidation is the cause of any of the diseases mentioned above, but increased generation of lipid peroxides has been clearly shown in these conditions.[5] Idebenone, a benzoquinone derivative, is used in Japan for the treatment of senile dementia, and its mode of action is thought to be as an inhibitor of the lipid peroxidation of biological membranes.[6]

Scheme I



Ascorbic acid (ASA) is one of the most potent reducing agents in the biological millieu, but since it reacts with trace metal ions to yield AOS,[7] it may also act as a prooxidant. ASA is unstable in aqueous solution, and its autooxidation is also stimulated by trace metals. Furthermore, the cytotoxicity of ASA was reported to be oxygen-dependent and linked to its autooxidation.[8] Many attempts have made to synthesize ASA derivatives with an improved resistance to autooxidation,[9] but no satisfactory compounds have been obtained yet. We have reported elsewhere[10] that 3-*O*-alkylascorbic acids were found

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Table II. Catalytic Effects of PTC on the Yield of 5,6-*O*-Isopropylidene-3-*O*-acetonylascorbic Acid (2o)

PTC	solvent	base	temp	h	% yield
	DMSO	$NaHCO_3$	RT	20	35
	DMSO	$NaHCO_3$	50	16	72
	DMF	K_2CO_3	50	16	58
$(Me)_3PhN\cdot I$	DMSO	$NaHCO_3$	RT	20	52
$(Me)_3PhN\cdot I$	DMSO	K_2CO_3	RT	20	75
$(n\text{-}Bu)_4N\cdot Br$	MEK/H_2O	$NaHCO_3$	70	16	98

to be stable in ointments and to suppress intracellular melanin accumulation in the skin.

Recently, Kato et al. reported that 2-*O*-octadecylascorbic acid (**5c**) markedly inhibited the lipid peroxidation of biological membranes and alleviated myocardial lesions induced by ischemia–reperfusion treatment in rats.[11] They showed that 3-*O*-octadecylascorbic acid (**3f**), the regioisomer of **5c**, was less effective in its inhibition of lipid peroxidation in brain homogenate than was **5c**. There has not been any previous detailed report on the biochemical antioxidant effect of the other 3-*O*-alkylascorbic acid. We designed and synthesized various 3-*O*-alkylascorbic acids (3-RASA) and examined their structure–activity relationships to determine their electron-donating potency and effects on lipid peroxidation. Additionally, we studied the role of the 3-*O*-alkyl moiety of these 3-RASA derivatives in relation to their antioxidant activity in rat liver microsomes and their stability against autooxidation.

Chemistry

The 2-RASA and 3-RASA compounds listed in Table I were synthesized as shown in Scheme I. The regioselective alkylation of the 3-hydroxyl group on 5,6-*O*-isopropylideneascorbic acid (**1**) was achieved by use of sodium bicarbonate as a suitable base. The starting material (**1**) was alkylated with alkyl halides in DMSO in the presence of sodium bicarbonate to give the corresponding intermediates **2a–n**, with the yields shown in Table I. We found that 2,3-dialkylascorbic acid was formed as a byproduct in the presence of potassium carbonate in lieu of sodium bicarbonate in the alkylating reaction.

Next, 5,6-*O*-isopropylidene-3-*O*-acetonylascorbic acid (**2o**) was quantitatively obtained by using *tert*-butylammonium bromide as a phase-transfer catalyst (PTC) in MEK/H_2O (Table II), and 5,6-*O*-isopropylidene-3-*O*-β-picolylascorbic acid (**2n**) was only formed in the presence of this catalyst.

The intermediates **2a–n**, protected with the isoproplidene group, were then subjected to acid hydrolysis to give the corresponding 3-RASA compounds, **3a–n**, in a good yield (Table I).

In addition, various 2-*O*-alkylascorbic acids (2-RASA: **5a–e** and **6**) were synthesized according to the method described elsewhere.[11] The structures of these derivatives were determined by NMR and UV spectrometry. At pH 7.4 the maximal absorption in the UV spectrum of **3c** and its regioisomer **5b** was 247 and 263 nm, respectively. When the pH of the solution was changed from 7.4 to 4.0, the maximal absorption of **5b** (a 2-RASA) was shifted from 263 to 237 nm, but that of **3c** (a 3-RASA) remained unchanged in the pH range of 4.0–7.4. At pH 10.0 the maximal absorption of **3c** and **7** (a 2,3-dialkyl-ASA) was 276 and 246 nm; respectively. In the ^{13}C NMR spectrum of **3c**, the signal of the 2-carbon was most largely shifted downfield (10.8 ppm) and that of the 1-carbon was also shifted downfield (6.8 ppm) while that of the 3-carbon was shifted high field (5.7 ppm) when the pH of the solution was altered from 7 to 10. Under the condition the signal of the 4-, 5-, and 6-carbon was least shifted. In the spectrum of **5b**, the signal of the 3-carbon was most largely shifted downfield (8.4 ppm), that of the 1- and 4-carbon was also shifted downfield (2.0 ppm) and that of the 2-carbon was shifted high field (2.0 ppm) when the pH of the solution was altered from 4 to 8. Under the latter condition the signal of the 5- and 6-carbon was little shifted. These findings were due to a difference in the pK_a values of the regioisomers[11] and are consistent with the observation that compound **3c** was not ionized at its 2-hydroxyl group while **5b** was ionized at its 3-hydroxyl group at pH 7.4.

In the stability experiments, compound **3c** and its regioisomer **5b** were dissolved in DMF and 0.05 M Tris-HCl buffer (pH 7.0 and 9.0). Irrespective of the pH, **3c** was far more stable than **5b**; it was found that **5b** underwent 50%

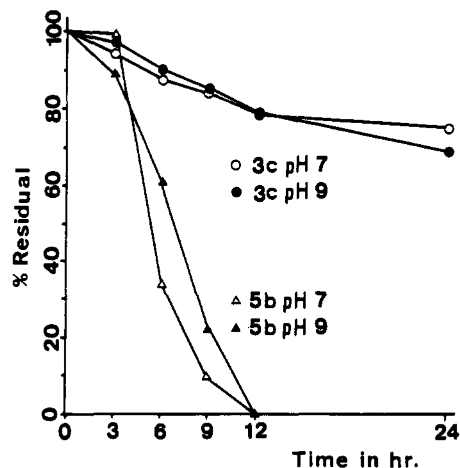

Figure 1. Stability of compounds **3c** and **5b** at pH 7 and 9.

Table III. Redox Potentials and Reducing Activities of 3-RASA and 2-RASA Compounds and of ASA Itself

compound	peak potential, mV at pH 7.4[a]	reducing % in EtOH[b]
2o, CH_2COCH_3		37
2p,[c] CH_2OCH_3		74
2q,[d] benzyl		68
3a, $(CH_2)_7CH_3$		58
3c, $(CH_2)_{11}CH_3$	300	61
3g, $CH_2CO(CH_2)_5CH_3$	375	30
3i, $CH_2CO(CH_2)_9CH_3$	380	39
3m, $CH_2COO(CH_2)_9CH_3$	340	51
3n, β-picolyl	420	40
5a, $(CH_2)_7CH_3$		>90
5b, $(CH_2)_{11}CH_3$	245	>90
5c, $(CH_2)_{17}CH_3$	240	>90
5d, $CH_2CO(CH_2)_9CH_3$		85
5e, $CH_2COO(CH_2)_3CH_3$		>90
6,[e] CH_2COCH_3		>90
7,[f] 2,3-*O*-[$(CH_2)_9CH_3$]		<5
ASA	210	>90

[a] The redox potentials were determined by differential pulse voltammetry. The reference electrode was Ag/AgCl. [b] Reducing activity against α,α-diphenyl-β-picrylhydrazyl of equimolar amounts of the test compounds for 15 min. [c] 5,6-*O*-Isopropylidene-3-*O*-methoxymethylascorbic acid. [d] 5,6-*O*-Isopropylidene-3-*O*-benzylascorbic acid. [e] 5,6-*O*-Isopropylidene-2-*O*-acetonylascorbic acid. [f] 2,3-*O*-Di-*n*-decylascorbic acid.

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degradation within 6–7 h and was completely degraded after 12 h, while more than 70% of **3c** remained intact after 24 h (Figure 1).

The redox potentials of the 3-RASA compounds were measured by differential pulse voltammetry to evaluate their electrochemical characteristics as electron donors (a detailed account is given in the Experimental Section). ASA, **5c**, and the 3-RASA derivatives were irreversibly oxidized in 0.01 M phosphate buffer at pH 7.4. Table III shows the redox potentials of ASA, **5c**, and the 3-RASA compounds obtained by differential pulse voltammetry. ASA had the lowest potential among the compounds tested, and the 3-RASA compounds had significantly higher E_1 values than ASA (increased by 90–190 mV). Compound **5c** had an E_1 at least 30 mV higher than that of ASA. Among the 3-RASA compounds synthesized, compound **3i** that was substituted with a β-ketoalkyl residue required much higher potentials than did **3c** that was substituted with a saturated alkyl group.

The actual quenching potencies of the 3-RASA compounds were determined in comparison with 2-RASA compounds and ASA itself using the stable radical α,α-diphenyl-β-picrylhydrazyl (DPPH),[12] and the results are shown in Table III. The radical-scavenging activity of the 3-RASA compounds was also lower than that of the 2-RASA compounds and ASA. The chemical reactivity of the 3-RASA compounds was apparently influenced by the electron-attracting effect of the substituted moiety. In this assay, the 2-RASA compounds exhibited almost the same reducing activity as ASA itself, while 2,3-didecylascorbic acid (**7**) completely lost its reducing activity.

Pharmacology

The effects of the 2-RASA and 3-RASA compounds on lipid peroxidation induced in rat microsomes by ferrous ion (Fe^{2+}) and ferric ion-ADP (Fe^{3+}–ADP) are shown in Table I. Microsomal lipid peroxidation was propagated by Fe^{2+} as a nonenzymatic inducer and by Fe^{3+}–ADP in the presence of NADPH as an enzymatic inducing system and was monitored by the formation of thiobarbituric acid reactive substances. The 3-RASA compounds (**3b–d,i,j**) with long alkyl chains exhibited a potent inhibitory effect on lipid peroxidation in both systems, while ASA enhanced lipid peroxidation. We found that compound (**3f**) was less effective in preventing liver microsomal lipid peroxidation than its regioisomer (**5c**), the same relationship as reported previously for the autooxidation of rat brain homogenates.[11] The most potent 3-RASA compounds were **3c** and **3i**, which exhibited the same or a slightly stronger potency than **5c**. The IC_{50} values of compounds **3c** and **3i** were 3.1 and 3.3 × 10^{-6} M, respectively, against Fe^{3+}–ADP-induced lipid peroxidation and 40 and 45 × 10^{-6} M against Fe^{2+}-induced lipid peroxidation. The numerical value of hydrophobicity was expressed as the respective capacity ratio (k') as obtained by HPLC using a C8 reversed-phase column (Table I). The k' value increased in proportion to the length of the substituted alkyl group. Among the 3-RASA compounds substituted with a saturated alkyl moiety (**3a–f**) or a β-ketoalkyl moiety (**3g–l**), compounds **3b**, **3c**, **3i**, and **3j** were potent lipid peroxidation inhibitors (Figure 2) irrespective of their redox potentials and radical-scavenging activities. Their hydrophobicities were scattered in the range of k' values 1–3. Compound **5c** was also active in inhibiting lipid peroxidation (Table I) and was located in the same k' range as **3b**, **3c**, **3i**, and **3j**. It therefore appears that the inhibitory effect of 3-RASA compounds on lipid peroxidation is increased by the acquisition of an optimum hydrophobicity and decreased by an insufficient or excessive hydrophobicity.

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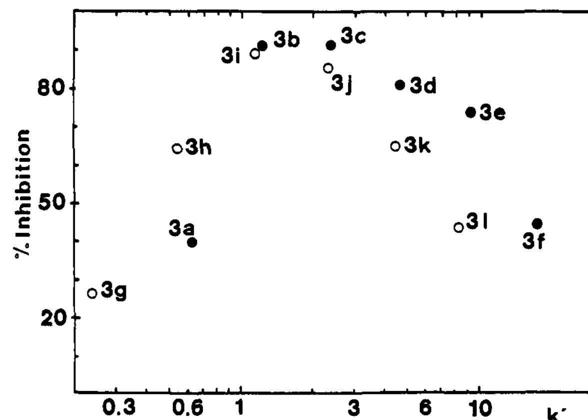

Figure 2. Relationship between the hydrophobicity (k') of 3-RASA compounds (**3a–l**) and their antioxidant activity (percent inhibition at 10^{-5} M) against Fe^{3+}–ADP-induced lipid peroxidation. Closed circles (**3a–f**): 3-RASA compounds substituted with a saturated alkyl moiety. Open circles (**3g–l**): 3-RASA compounds substituted with a β-ketoalkyl moiety.

Discussion

We regioselectively synthesized various 3-RASA compounds in which the 3-hydroxyl group of ASA was substituted by various alkyl groups. Regioselective alkylation was achieved in sodium bicarbonate, by the utilization of the differences between the pK_a values of the 2-hydroxyl and 3-hydroxyl groups. Differences in the pK_a may exert a great influence on the stability and electrochemical characteristics of these 2-RASA and 3-RASA compounds.[11] Compound **3c**, which was unionized at the 2-hydroxyl group, was more stable against autooxidation in buffer solutions at pH 7 and 9 than regioisomer **5b**, which was ionized. This observation supports the proposition that ionization of the 3-hydroxyl group is the rate-limiting step for the subsequent autooxidation of ASA.[13]

It has been reported that when ASA is oxidized by superoxide, its 2- and/or 3-hydroxyl group acts as an essential group in electron donation.[14] Compound **5c** and the 3-RASA compounds, which were respectively alkylated on the 2-hydroxyl and 3-hydroxyl groups of ASA, had much higher redox potentials at pH 7.4 than ASA itself, and the 3-RASA compounds had much higher potentials than **5c** in electrode oxidation. In addition, the reducing activity of the 3-RASA compounds tested against the free radical was chemically lower than that of either the 2-RASA compounds or ASA itself. It appears that the electron-donating activity of ASA is reduced by monoalkylation at an enolic 2- or 3-hydroxyl group, and the redox potentials of 3-RASA compounds are influenced by the electron-attracting effect of the substituent group. However, the 3-RASA compound **3n** had a higher potential of 420 mV (i.e., it was more resistant to electrical oxidation), which is thought to be sufficient for reducing superoxide anion. We also found that compounds **3c**, **3i**, and **5c** exhibited almost the same inhibitory effects on the lipid peroxidation of rat microsomes, although their redox potentials varied, being 300, 380, and 240 mV, respectively.

However, the anti lipid peroxidation effects of the 3-RASA compounds were shown to be dependent upon the

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hydrophobicity values obtained from HPLC analysis; i.e., **3c**, **3i**, and **5c** were nearly equal in hydrophobicity and in anti lipid peroxidation activity.

Both 3-RASA and 2-RASA compounds require long alkyl moieties and the electron-donating activity of their enolic hydroxyl group in the suppression of lipid peroxidation. However, an optimum range of k' values was observed for the antioxidant activity of the 3-RASA compounds. Furthermore, it has been reported that lipid peroxidation is closely related to membrane fluidity.[15]

These findings taken together suggest that the long alkyl chain acts as an essential anchor to the lipid bilayer and that too long an alkyl chain or too high a hydrophobicity of the compound results in the decreased mobility of the radical scavenger in the lipid bilayer.

Another possibility is that the cutoff phenomenon of 3-RASA anti lipid peroxidative activity that we noted may be explained by the solubility of these 3-RASA analogues in the lipid membrane. Although the mechanism producing the parabolic relationship between k' and the inhibition of lipid peroxidation by 3-RASA compounds is still unclear, we showed that the chemical antioxidant activity of **3i** was lower than that of **3c**. Howecver, the anti lipid peroxidative potency of **3i** in liver microsomes was similar to that of **3c**.

Therefore, the results obtained in this study indicate that 3-RASA analogues with an appropriate hydrophobicity can easily penetrate and/or diffuse into lipid bilayers and act as free-radical quenchers that potently protect against the lipid peroxidation of the biomembrane.

Experimental Section

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. HPLC analysis was performed with a JASCO Model 801 system, and separation was performed with a Cosmosil C8 column (10 μm, 4.6 × 150 mm) at a flow rate of 1 mL/min. The mobile phase consisted of H_2O/MeOH (20/80) and detection was performed at 252 nm. Reproducibility of the retention volume as expressed by the coefficient of variance was 0.09%. NMR spectra were recorded on a JEOL FX-90Q spectrometer (90 MHz), using Me_4Si as an internal standard. UV spectra were recorded on a Hitachi UV spectrometer. All elemental analyses were found to be within ±0.4% of the calculated values.

5,6-*O*-Isopropylideneascorbic Acid (1).[16] Acetyl chloride (15 mL, 0.06 mol) was added to a stirred solution of ASA (180 g, 1 mol) in acetone (2 L). The reaction mixture was stirred at 40 °C for 4 h, the precipitate was separated by filtration, washed with *n*-hexane and 20% acetone in *n*-hexane, and then dried in vacuo. Recrystallization from acetone gave 1 (180 g, 83%): mp 201–203 °C; NMR (DMSO-d_6) δ 1.33 (6 H, s), 3.90–4.46 (4 H, m), 4.62 (1 H, d, J = 3 Hz). Anal. ($C_9H_{12}O_6$) C, H.

General Procedure for Obtaining β-Ketoalkyl Bromides.[17] **1-Bromooctadecan-2-one (8g).** To a solution of 1-octadecene (7.6 g, 0.03 mol) in dioxane (50 mL) was added AcOH (10 mL). $NaBrO_2$ solution (150 mL, 0.18 mol in water) was then added dropwise over 60 min to the solution, which was kept at 10 °C. The reaction mixture was then stirred at room temperature for 12 h and neutralized with 5% $NaHCO_3$. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue was subjected to column chromatography on a silica gel column (CH_2Cl_2/petroleum ether 1:10) to give 2.3 g (24%) of crude **8g**. The typical NMR spectrum for **8g**: ($CDCl_3$) δ 0.89 (3 H, m), 1.29 (8 H, m), 2.64 (2 H, t), 3.88 (2 H, s). The crude alkylbromides obtained were used in the next reaction without further purification. The other β-ketoalkyl bromides **8h–k**, corresponding to **3h–k** in Table I, were also obtained in the same manner.

General Procedure for the Alkylation of the 3-Hydroxyl Group. Method A (5,6-*O*-Isopropylidene-3-*O*-alkylascorbic Acids, 2a–q except 2n). To a solution of 1 (4.3 g, 0.02 mol) in DMSO (25 mL) was added sodium bicarbonate (2.52 g, 0.03 mol). The mixture was then stirred for 20 min, and alkyl halide (0.03 mol) was added. The mixture was next stirred for 8–16 h at 50 °C and was 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 purified on a silica gel column (EtOAc/benzene or EtOAc/hexane) to give **2a–m** and **2o–q**. For **2b**: NMR (acetone-d_6) δ 0.89 (3 H, m), 1.29 (22 H, m), 4.16 (3 H, m), 4.49 (2 H, t), 4.66 (H, d, J = 3 Hz). For **3h**: NMR ($CDCl_3$) δ 0.88 (3 H, m), 1.26 (12 H, m), 1.38 (3 H, s), 1.40 (3 H, s), 2.45 (2 H, t), 4.12 (3 H, m), 4.69 (H, d, J = 4 Hz), 5.00 (2 H, s).

Method B (2n and 2o). To a solution of 1 (10.8 g, 0.05 mol) in MEK (200 mL) was added 4.15% sodium bicarbonate solution (100 mL) and the mixture was allowed to stand for 5 min. This was followed by the addition of alkyl halide (0.055 mol) and tetra-*n*-butylammonium bromide (0.8 g, 2.5 mmol). The resultant mixture was stirred at 70 °C for 16 h. Then the organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue was subjected to silica gel column chromatography (EtOAc/benzene) to give **2n** and **2o**. For **2n**: NMR (MeOH-d_4) δ 1.29 (6 H, s), 4.14 (3 H, m), 4.72 (H, d, J = 2 Hz), 5.59 (2 H, s), 7.47 (H, m), 7.97 (H, m), 8.53 (H, m), 8.63 (H, bs).

Method C (2o). To a solution of 1 (4.3 g, 0.02 mol) in DMSO (20 mL) were added sodium bicarbonate (1.68 g, 0.02 mol) and phenyltrimethylammonium iodide (0.1 g, 0.4 mmol), and the mixture was stirred for 30 min. Chloroacetone (2.0 g, 0.025 mol) was then added slowly and stirring was continued at room temperature for 20 h. The reaction mixture was next treated as described above to give **2o** (2.9 g, 52%): mp 76–77 °C; NMR ($CDCl_3$) δ 1.29 (6 H, s), 2.09 (3 H, s), 4.16 (3 H, m), 4.86 (H, d, J = 3 Hz), 5.03 (2 H, s). Anal. ($C_{12}H_{16}O_7$) C, H (±0.03%).

General Procedure for Removal of the Protective Group. To a solution of 5,6-*O*-isopropylidene-3-*O*-alkylascorbic acid (0.01 mol of **2a–n**) in MeOH (40 mL) was added 2 M HCl (20 mL) and the resultant mixture was stirred at 50 °C for 1–2 h. The reaction mixture was concentrated with an evaporator and the residue was extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The crude product was then recrystallized from EtOAc or CH_2Cl_2/*n*-hexane to give **3a–n** in Table I. For **3h**: ^{13}C NMR (MeOH-d_4) δ 63.22 (C_6), 70.81 (C_5), 76.71 (C_4), 121.61 (C_2), 150.50 (C_3) and 172.11 (C_1) for the ASA moiety, 75.19 (α-CH_2), 207.71 (β-CO) for the substituted group. For **3b**: NMR (acetone-d_6 + D_2O) δ 0.88 (3 H, m), 1.30 (16 H, m), 3.69 (2 H, m), 3.90 (H, m), 4.48 (2 H, t), 4.79 (H, d, J = 1.5 Hz).

2-*O*-Alkylascorbic Acids (5a–e). To a solution of 5,6-*O*-isopropylidene-3-*O*-(methoxymethyl)ascorbic acid (**2p**, 0.01 mol) in DMSO (20 mL) were added potassium carbonate (0.01 mol) and an alkyl halide (0.011 mol). The reaction mixture was stirred at room temperature for 15 h, diluted with water (100 mL), neutralized with 2 M HCl, and extracted with EtOAc. The organic layer was then separated, washed with water, dried, and evaporated in vacuo. The residue was next subjected to silica gel column chromatography (EtOAc/benzene) to give **4a–e**. After removal of the protective group as described above, compounds **4a–e** were subjected to acid hydrolysis to yield compounds **5a–e**, respectively. For **5c**: NMR (DMSO-d_6) δ 0.87 (3 H, m), 1.26 (32 H, m), 3.38–3.93 (5 H, m), 4.74 (H, bs).

5,6-*O*-Isopropylidene-2-*O*-acetonylascorbic Acid (6). To a solution 5,6-*O*-isopropylidene-3-*O*-benzylascorbic acid (**2q**, 3.1 g, 0.01 mol) in DMSO (20 mL) were added potassium carbonate (1.4 g, 0.01 mol) and chloroacetone (1.03 g, 0.011 mol). The reaction mixture was stirred at 40 °C for 6 h, diluted with water (100 mL), neutralized with 1 M HCl, and extracted with EtOAc. The residue was subjected to silica gel column chromatography (EtOAc/benzene) to give 5,6-*O*-isopropylidene-2-*O*-acetonyl-3-*O*-benzylascorbic acid (2.15 g, 59%). To a solution of 5,6-*O*-isopropylidene-2-*O*-acetonyl-3-*O*-benzylascorbic acid (2.0 g, 5.6 mmol) in EtOAc (20 mL) was added 10% palladium charcoal (0.5 g) and

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the mixture hydrogenated for 14 h at atmospheric pressure. The catalyst was removed by filtration, and the filtrate was concentrated. The residue was recrystallized from EtOAc/*n*-hexane to give **6** (1.66 g, 92%): mp 89 °C; NMR ($CDCl_3$) δ 1.24 (6 H, s), 2.09 (3 H, s), 4.10 (3 H, m) 4.55 (2 H, s) 4.80 (H, d, J = 2 Hz).

2,3-*O*-Didecylascorbic Acid (7). To a solution of 1 (2, 16 g, 0.01 mol) in DMSO (20 mL) were added potassium carbonate (2.76 g, 0.02 mol) and *n*-decyl bromide (4.77 g, 0.022 mol). The reaction mixture was stirred at 40 °C for 6 h, diluted with water (100 mL), neutralized with 2 M HCl, and extracted with EtOAc. The organic layer was then separated, washed with water, dried, and evaporated in vacuo. The residue was subjected to silica gel column chromatography (EtOAc/*n*-hexane) to give 5,6-*O*-isopropylidene-2,3-*O*-didecylascorbic acid (1.74 g, 35%). After removal of the protective group as described above, 5,6-*O*-isopropylidene-2,3-*O*-didecylascorbic acid was subjected to acid hydrolysis to yield **7** (1.43 g, 90%): mp 65 °C (recrystallized from CH_2Cl_2/*n*-hexane); NMR ($CDCl_3$) δ 0.86 (6 H, m), 1.27 (32 H, m), 3.82–4.12, (6 H, t + m), 4.44 (3 H, t), 4.66 (H, d, J = 2.4 Hz).

Physicochemical and Biological Experiments. Stability of Compounds 4c and 5b at pH 7 and 9 in 0.05 M Tris-HCl Buffer. Compounds **4c** and **5b** were dissolved in DMF and 5 mL of 0.05 M Tris-HCl buffer (pH 7 and 9) was added to make a final test solution concentration of 1%. The solutions were made in triplicate and stored at 20 °C, and 0.01-mL aliquots were taken at 3-h intervals for 24 h. The test compound concentrations were estimated by HPLC. Separation was performed on a Cosmosil C18 column (10 μm, 4.6 × 150 mm, Nacalai Tesque) with a flow rate of 1.0 mL/min. The mobile phase consisted of H_2O/MeOH/AcOH (20/80/1) and detection was performed at 254 nm.

Determination of the Redox Potentials for 3-RASA and Related Compounds. Voltammetric analysis was conducted in 0.01 M phosphate buffer (pH 7.4) which was degassed with argon before use. An Ag/AgCl reference electrode, a glassy carbon working electrode (3 mm i.e.), and a Pt counter electrode (0.05 mm i.d. × 2 cm) were used (BSA systems). The redox potentials were determined by using a BSA 100A electrochemical analyzer to obtain the peak potential in the voltammograms in the differential pulse voltammetry mode. The experimental conditions were as follows: initial E (mV) = −100, E (mV) = 500, V (mV/s) = 4, pulse amplitude (mV) = 50, pulse width (ms) = 60, and pulse ratio (ms) = 1000. Test compound solutions were added to the buffer at a final concentration of 0.05 mM (the final concentration of MeOH was 5%). Electrochemical reversibility was assessed by analyzing the reversed differential pulse voltammograms (initial E (mV) = 500, final E (mV) = −100).

Determination of the Extent of Reduction of the Stable Radical α,α-Diphenyl-β-picrylhydrazyl (DPPH).[12] To a solution of the stable radical DPPH in 5 mL of EtOH (1×10^{-4} M) was added the test compound in 25 μL of DMF (2×10^{-2} M). The reaction mixture was then vigorously stirred for 10 s and allowed to stand at room temperature for 15 min. The optical density (OD) of the solution was measured at 517 nm and the percent reduction was estimated with the following equation:

$$\% \text{ reduction} = \frac{\text{control OD (mean)} - \text{sample OD (mean)}}{\text{control OD (mean)}} \times 100$$

Each reaction was performed in triplicate. The percent reduction in control solutions from which the test compound was absent was less than 1%.

Preparation of a Microsomal Suspension. Wistar rats were fasted for 24 h and sacrificed 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. The supernatant was separated and centrifuged at 105000*g* for 1 h to obtain the microsomal fraction. The pellet thus obtained was resuspended in fresh 0.15 M KCl solution and its protein concentration was determined by the method of Lowry et al.[18]

Measurement of Lipid Peroxidation. For assaying Fe^{3+}–ADP-induced 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. For assessing Fe^{2+}-induced lipid peroxidation, the assay system (1.0 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), 10 μM $FeSO_4$, and the microsomal fraction (2 mg of protein). The reaction mixtures were incubated at 37 °C for 20 min and then cooled on ice to terminate the reaction. The lipid peroxides generated were measured by the method of Ohkawa et al.[19] In brief, to the reaction mixture was added 8.1% SDS (0.2 mL), 20% AcOH containing 0.27 M HCl adjusted with NaOH to pH 3.5 (1.5 mL), and 0.8% thiobarbituric acid (1.5 mL). The mixture was then heated at 95 °C for 20 min and the reaction was stopped by cooling on ice. Then *n*-BuOH/pyridine (15.1, 5.0 mL) was added and vigorous mixing was performed. After centrifugation at 800*g* for 10 min, the organic layer was separated and the absorbance was measured at 532 nm. The IC_{50} was calculated as the test compound concentration that reduced the amount of thiobarbituric acid positive materials to 50% of that in the vehicle control.

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Registry No. 1, 15042-01-0; **2a**, 126100-68-3; **2b**, 88307-08-8; **2c**, 126100-67-2; **2d**, 133794-46-4; **2e**, 133794-47-5; **2f**, 89202-56-2; **2g**, 133794-48-6; **2h**, 133794-49-7; **2i**, 133794-50-0; **2j**, 133794-51-1; **2k**, 133794-52-2; **2l**, 133794-53-3; **2m**, 126100-79-6; **2n**, 126100-66-1; **2o**, 126100-80-9; **2p**, 98829-07-3; **2q**, 86404-07-1; **3a**, 126100-57-0; **3b**, 132530-91-7; **3c**, 88306-62-1; **3d**, 106396-33-2; **3e**, 88306-70-1; **3f**, 88306-73-4; **3g**, 133794-54-4; **3h**, 133794-55-5; **3i**, 133794-56-6; **3j**, 133794-57-7; **3k**, 133794-58-8; **3l**, 133794-59-9; **3m**, 126100-62-7; **3n**, 126100-63-8; **4a**, 107707-25-5; **4b**, 133794-60-2; **4c**, 107347-79-5; **4d**, 107707-02-8; **4e**, 107707-04-0; **5a**, 98829-34-6; **5b**, 98829-35-7; **5c**, 98829-48-2; **5d**, 98829-49-3; **5e**, 98829-50-6; **6**, 126100-89-8; **7**, 133815-53-9; **8g**, 133794-62-4; **8h**, 39727-88-3; **8i**, 66130-89-0; **8j**, 133794-61-3; **8k**, 21436-52-2; $CH_3(CH_2)_7Br$, 111-83-1; $CH_3(CH_2)_9Br$, 112-29-8; $CH_3(CH_2)_{11}Br$, 143-15-7; $CH_3(CH_2)_{13}Br$, 112-71-0; $CH_3(CH_2)_{15}Br$, 112-82-3; $CH_3(CH_2)_{17}Br$, 112-89-0; $CH_3(CH_2)_5C(O)CH_2Br$, 26818-08-6; $CH_3(CH_2)_9OC(O)CH_2Br$, 5436-93-1; $CH_3C(O)CH_2Br$, 598-31-2; CH_3OCH_2Br, 13057-17-5; $C_6H_5CH_2Br$, 100-39-0; β-picolinyl bromide, 69966-55-8; L-ascorbic acid, 50-81-7; 1-octadecene, 112-88-9.

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