Antioxidant and Neutrophil-Inhibiting Properties of New 2-O-Methyl-6-(alkylthio)ascorbic Acid Derivatives†

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A series of new 6-(alkylthio)ascorbic acids was synthesized, and their inhibitory effects on lipid peroxidation and the oxidative burst of human neutrophils were tested. Of 12 structurally different lipophilic ascorbic acid derivatives 6-*S*-*n*-hexadecyl-2-*O*-methyl-6-deoxy-6-thio-L-ascorbic acid (**7b**; B-003) inhibited the Fe^{2+}/ADP-induced lipid peroxidation of rat liver microsomes with an IC_{50} value of 2 μM. In human neutrophils, **7b** most potently inhibited the fMLP-induced oxidative burst in a cell density-dependent manner with an IC_{50} value of 0.6 μM at 5×10^5 cells/mL. Shorter alkyl chain lengths decreased the inhibitory potency for both lipid peroxidation and oxidative burst, but in general no correlation was found between the two parameters. Likewise, 6-*S*-*n*-hexadecyl-3-*O*-methyl-6-thio-L-ascorbic acid (**7c**; B-015), the regioisomer of **7b**, was a potent antioxidant but did not affect the oxidative burst. Since superoxide anions generated by xanthine/xanthine oxidase were not quenched by **7b**, it became evident that its target was somewhere between receptor stimulation and NADPH-oxidase activation. By measuring the cellular concentrations of **7b** and **7c**, an accumulation of the first was found explaining its potency and the dependence on cell density. Expecting a pK_a value of 3.3 for **7b** and 7.7 for **7c** a protonophore action of **7b** was likely and could be verified by the drop in intracellular pH (pH_i) which did not occur with **7c**. Ionophores such as nigericin, CCCP, or propionic acid also lowered the pH_i but did not inhibit the oxidative burst, indicating that the pH_i drop was not the cause for this inhibition. **7b** also strongly inhibited the fMLP-induced secretion of azurophilic (IC_{50} = 7 μM) and specific (IC_{50} = 2.5 μM) granules.

Introduction

Human neutrophils play a pivotal role in the body's cellular defense against invading microorganisms. After recruitment to the inflamed area and stimulation by complement or bacterial degradation products, neutrophils answer with massive production of superoxide anion radicals known as oxidative burst and with secretion of a number of degrading enzymes from azurophilic or specific granules.[1,2] After activation of the oxidative burst, other reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^·$), and hypohalites (e.g. OCl^-) are produced in a spontaneous or an enzyme (myeloperoxidase)-catalyzed reaction.[1] Under physiological conditions, neutrophils use ROS for the site-directed intraphagosomal killing as well as for the extracellular killing of tumor cells or virus-infected cells (cytotoxicity). Under certain pathological conditions like heart attack, septic shock, rheumatoid arthritis, or in reperfused ischemic organs the uncontrolled release of ROS or granule contents from invading neutrophils might lead to massive cell death and necrosis.[3-5] There are several reports that myeloperoxidase activity and leukocytes accumulate in postischemic tissue.[6,7] In support of neutrophil damage during reperfusion of ischemic organs, neutrophil-mediated depletion or administration of agents that decrease neutrophil accumulation reduce infarct size.[8]

The use of scavengers like superoxide dismutase (SOD) or catalase have been shown protective in several ROS-mediated inflammatory models in vivo, although the pharmacological half-life of these enzymatic scavengers is disadvantagely short.[9,10] Lipid-soluble antioxidants, including vitamine E and its derivatives, were also found useful and reduced myocardial injury during ischemia and reperfusion, although high doses were required.[11,12]

Following a strategy of fortifying the antioxidant potential of the activated neutrophil in the aqueous as well as the lipid phase, we had studied the effect of the known lipophilic antioxidant L-ascorbic acid 6-palmitoate on neutrophils and found an effective reduction of the oxidative burst.[13] More recently, the 2-*O*-octadecylascorbic acid CV-3611 was also shown to inhibit the superoxide production of activated neutrophils and to have beneficial effects in different ischemia-reperfusion models[14-16] where ROS are thought to be involved.[17] There seemed to be a clear-cut correlation of the protective effects with the ROS-scavenging property of this compound. In the course of our further studies, however, we confirmed our previous findings[13] that degranulation of neutrophils was also inhibited by L-ascorbic acid 6-palmitoate. This suggested an additional, scavenging-independent mechanism of action of this class of compounds, which we tried to elucidate by comparing a series of 6-deoxy-6-(*n*-alkylthio)-ascorbic acid derivatives in their structure–activity relationships.

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† Abbreviations: fMLP, *N*-formylmethionylleucylphenylalanine; PBS, phosphate buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; LECL, luminol-enhanced chemiluminescence; DMSO, dimethyl sulfoxide; NADPH, nicotinamide adenine dinucleotide phosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; BCECF, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; bisoxonol, bis-(1,3-dibutylbarbituric acid)trimethine oxonol; DTT, dithiothreitol; SOD, superoxide dismutase; CAT, catalase; HPLC, high-pressure liquid chromatography; PMN, polymorphonuclear leukocytes. Enzymes: β-D-glucuronidase (EC 3.2.1.31); lysozyme (EC 3.2.1.17).

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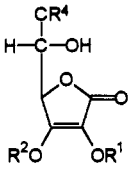
Table I. Chemical and Physical Data of the 6-S-n-Alkyl-6-deoxy-6-thio-L-ascorbic Acids and Related Compounds



compd	R^1	R^2	R^4	% yield[a]	mp (°C)	recryst[b] solvent	formula[c]
3b	H	CH_3	Br	83	oil		$C_7H_9BrO_5$[d]
3c	CH_3	H	Br	23	155–158	AT	$C_7H_9BrO_5$
4b	CH_3	H	SPh	79	174.5–177	T	$C_{13}H_{14}O_5S$
5a	H	H	$S(CH_2)_7CH_3$	45	95.5–101.5	DC	$C_{14}H_{24}O_5S$
5b	CH_3	H	$S(CH_2)_7CH_3$	38	130–132.5	I	$C_{15}H_{24}O_5S$
6a	H	H	$S(CH_2)_{11}CH_3$	33	101.5–106	I	$C_{18}H_{32}O_5S$
6b	CH_3	H	$S(CH_2)_{11}CH_3$	52	130–132.5	I	$C_{18}H_{34}O_5S$
6v	H	CH_3	$S(CH_2)_{11}CH_3$	21	84–85	P	$C_{19}H_{34}O_5S$
7a	H	H	$S(CH_2)_{15}CH_3$	45	111.5–115	CA	$C_{22}H_{40}O_5S$
7b	CH_3	H	$S(CH_2)_{15}CH_3$	70	130–131	H	$C_{23}H_{42}O_5S$
7c	H	CH_3	$S(CH_2)_{15}CH_3$		89.5–91.3	P	$C_{23}H_{42}O_5S$
7d	CH_3	CH_3	$S(CH_2)_{15}CH_3$	39	58–60	C	$C_{24}H_{44}O_5S$
8b	CH_3	H	$S(CH_2)_{17}CH_3$	28	125–130	P[d]	$C_{25}H_{46}O_5S$

[a] Yield reported represents yield after recrystallization. [b] D = diethyl ether, C = cyclohexane, I = diisopropyl ether, A = EtOAc, M = MeOH, H = H_2O, T = toluene, E = ethanol, P = petroleum ether. [c] Analyses for the elements C, H, N, S were within ±0.3% of the theoretical values. [d] The compound was resuspended and washed. [e] The raw material was used without purification, no analytical data available.

Results

Antioxidative Properties. The series of 12 ascorbic acid derivatives was designed to combine the radical scavenging properties of ascorbic acid with the lipophilicity of long-chain alkyl groups. Since the unfavorable autoxidation of ascorbate originates from its enediol structure, a methylation of the 2- or 3-hydroxy group would leave the enol which still would possess antioxidative but no autoxidative potential. The influence of the alkyl group chain length could be studied by substitution at the 6-position in which oxygen was replaced by sulfur. These compounds are readily accessible by reaction of the nucleophilic mercaptanes with 6-bromo-6-deoxyascorbic acids. For control purposes the 2,3-dimethylated compound 7d was synthesized to eliminate the antioxidative properties completely.

Lipid peroxidation is a radical-initiated reaction which can serve as a suitable test system for evaluating the antioxidative properties of the synthesized derivatives. The autoxidation of Fe^{2+}/ADP causes a massive lipid peroxidation in liver microsomes which can be followed by the generation of malondialdehyde. When investigating the effects of the new ascorbic acid derivatives (Table I) on the Fe^{2+}/ADP-induced lipid peroxidation of rat liver microsomes, we observed potent antioxidative activities by long chain (C_{12}, C_{16}, C_{18}) alkylascorbic acid derivatives which strongly reduced malondialdehyde production to basal levels (Figure 1a). The enediol compound 4a (not shown in Figure 1a) like **4b** contains an *S*-phenyl group and only inhibited the lipid peroxidation by 30%, suggesting that a straight-chain alkyl group with more than eight carbon atoms is essential. The long-chain 6-*S*-alkyl derivatives 7a and 6a which possess free enediol groups, thus having a 2-electron donating capacity, did not show stronger inhibitory effects than the 2-methoxylated compounds (data not shown). In the same way 7c (6-*S*-hexadecyl-3-*O*-methyl-6-thio-6-deoxy-L-ascorbic acid), the regioisomer of **7c**, did not differ significantly in its inhibitory action toward the lipidperoxidative processes (Figure 1a). The redox-inactive 2,3-*O*-dimethylated derivative **7d** was used as a control and did not lead to any reduction of malondialdehyde formation.

Table II. ^1H NMR Analysis Data of the Synthesized 6-S-n-Alkyl-6-deoxy-6-thio-L-ascorbic Acids and Related Compounds

compd	^1H NMR spectral data, δ (DMSO-d_6)
3b	3.55 (2 H, m), 3.68 (3 H, s), 4.02 (1 H, m), 4.82 (1 H, s)
3c	3.49 (2 H, m), 3.86 (1 H, m), 4.06 (3 H, 2), 4.89 (1 H, s)
4b	3.16 (2 H, m), 3.68 (3 H, s), 3.91 (1 H, m), 4.88 (1 H, d, *J* = 1.3 Hz), 7.37 (5 H, m)
5a	0.85 (3 H, t, *J* = 6.3 Hz), 1.27 (10 H, m), 1.55 (2 H, m), 2.55 (2 H, d, *J* = 7.1 Hz), 2.63 (2 H, d, *J* = 7.2 Hz), 3.82 (1 H, m), 4.77 (1 H, d, *J* = 1 Hz), 5.2 (1 H, ba), 8.3 (1 H, bs), 11.1 (1 H, bs)
5b	0.86 (3 H, t, *J* = 6.1 Hz), 1.25 (12 H, m), 1.53 (2 H, m), 2.56 (2 H, d, *J* = 7 Hz), 2.63 (2 H, d, *J* = 7 Hz), 3.66 (3 H, s), 3.85 (1 H, t, *j* = 6.8 Hz), 4.83 (1 H, s),5.22 (1 H, bs), 11.80 (1 H, bs)
6a	0.85 (3 H, t, *J* = 6.1 Hz), 1.24 (20 H, m), 1.52 (2 H, m), 2.55 (2 H, d, *J* = 7 Hz), 2.64 (2 H, d, *J* = 7 Hz), 3.4 (1 H, bs), 3.82 (1 H, t, *J* = 7 Hz), 4.75 (1 H, s)
6b	0.86 (3 H, t, *j* = 6.1 Hz), 1.25 (20 H, m), 1.51 (2 H, m), 2.55 (2 H, d, *J* = 6.9 Hz), 2.64 (2 H, d, *J* = 6.9 Hz), 3.67 (2 H, s), 3.86 (1 H, m), 4.83 (1 H, s), 5.23 (1 H, bs), 11.79 (1 H, bs)
6c	0.85 (2 H, t, *J* = 6.1 Hz), 1.24 (20 H, m), 1.51 (2 H, m), 2.54 (2 H, d, *J* = 7.2 Hz), 2.62 (2 H, d, *J* = 7.1 Hz), 3.69 (1 H, m), 4.04 (3 H, s), 4.80 (1 H, s), 5.25 (1 H, d, *J* = 6.4 Hz exchange), 8.76 (1 H, s)
7a	0.85 (3 H, t, *J* = 4.8 Hz), 1.24 (28 H, m), 1.52 (2 H, m), 2.55 (2 H, d, *J* = 7.2 Hz), 2.67 (2 H, d, *J* = 7.1 Hz), 3.81 (1 H, m), 4.77 (1 H, s), 5.16 (1 H, d, *J* = 6.8 Hz), 8.36 (1 H, bs), 11.06 (1 H, bs)
7b	0.85 (3 H, t, *J* = 6.2 Hz), 1.24 (28 H, m), 1.52 (2 H, m), 2.55 (2 H, d, *J* = 7.0 Hz), 2.63 (2 H, d, *J* = 7.0 Hz), 3.35 (1 H, s, br), 3.66 (3 H, s), 3.89 (1 H, m), 4.83 (1 H, s), 5.25 (1 H, bs)
7c	0.85 (2 H, m), 1.24 (28 H, m), 1.51 (2 H, m), 2.55 (2 H, d, *j* = 7 Hz), 2.62 (2 H, d, *J* = 6.9 Hz), 3.80 (1 H, m), 4.04 (23 H, s), 4.81 (1 H, s), 5.25 (1 H, d, *J* = 6.3 Hz), 8.77 (1 H, s)
7d	0.85 (3 H, t, *J* = 6.4 Hz), 1.24 (28 H, m), 1.50 (2 H, m), 2.63 (2 H, d, *J* = 7.4 Hz), 3.71 (3 H, s), 7.53 (1 H, m), 4.07 (3 H, s), 4.86 (1 H, d, *J* = 1.5 Hz), 5.29 (1 H, d, *J* = 6.4 Hz, OH)
8b	0.85 (2 H, t, *J* = 6.8 Hz), 1.23 (32 H, m), 1.50 (2 H, m), 2.55 (2 H, d, *J* = 7.0 Hz), 2.64 (2 H, d, *J* = 7.1 Hz), 3.66 (3 H, s), 3.85 (1 H, m), 4.82 (1 H, s)

The 6-*S*-hexadecyl compound **7b** inhibits lipid peroxidation with an IC_{50} value of 2 μM, whereas the 6-*S*-octyl derivative **5b** was almost inactive even at the high

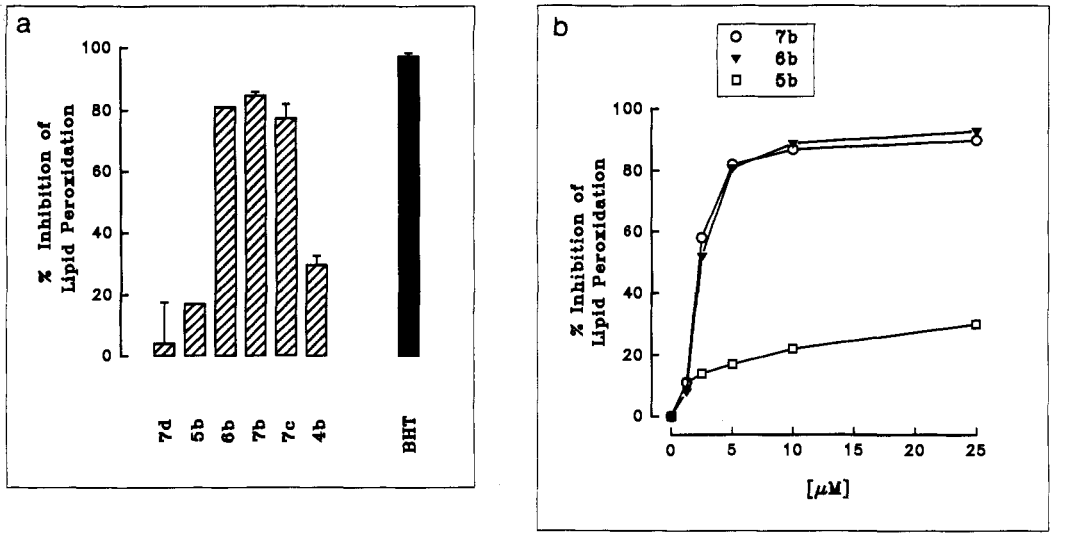


Figure 1. Influence of ascorbic acid derivatives on lipid peroxidation of rat liver microsomes. (a) Structure–activity relationship. After a 3-min preincubation of rat liver microsomes (1 mg/mL) with 5 μM of the indicated substances, lipid peroxidation was induced as described under Materials and Methods. After 15 min, malondialdehyde formation was determined at 532 nm by the thiobarbituric acid method. Values are given as mean ± SEM of $n = 3$ experiments. (b) Concentration-dependent inhibition of lipid peroxidation. Incubation conditions as described under part a.

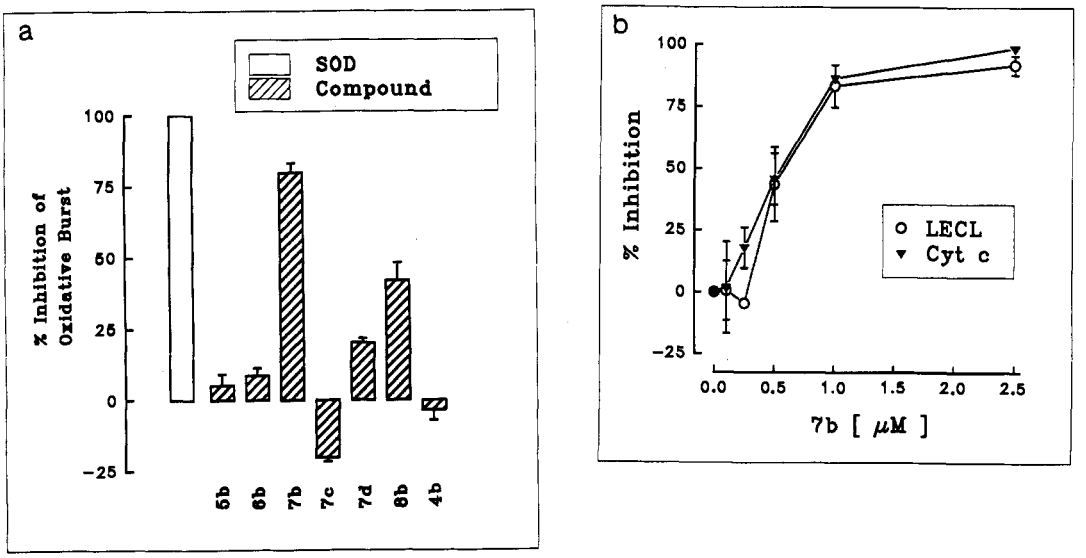


Figure 2. Effects of ascorbic acid derivatives on the fMLP-stimulated oxidative burst of human PMN. (a) Structure–activity relationship. Cells (5×10^5/mL) were preincubated with 1 μM of the indicated ascorbic acid derivatives for 3 min at 37 °C. After stimulation with 100 nM fMLP, $O_2^{\cdot}$ release was detected by SOD-sensitive cytochrome *c* reduction as described under Materials and Methods. Values are given as mean ± SEM from three to six independent experiments. (b) Concentration-dependent effect of **7b**. Cells (5×10^5/mL) were preincubated with the indicated concentrations of B-003 (0.1% DMSO) for 3 min at 37 °C and stimulated with 100 nM fMLP. LECL (open circles) or cytochrome *c* reduction (filled triangles) was monitored continuously as described under Materials and Methods.

concentration of 25 μM (Figure 1b). The inhibitory effect of the 6-*S*-dodecyl derivative **6b** did not differ significantly from **7b** and showed an IC_{50} value of 2.5 μM (Figure 1b). These results indicate that **7b**, although weakened in its electron-donating property due to the methylation at position 2, still possesses a strong antioxidant capacity. As a positive control for the assay system we used 5 μM of the known antioxidant *tert*-butylhydroxytoluene (BHT) which strongly suppressed MDA formation, whereas 25 μM ascorbic acid in agreement with its known prooxidative effects was found to enhance lipid peroxidation severalfold (not shown).

Effect on Superoxide Radical Generation of Human Neutrophils. We anticipated that the antioxidant properties of the different ascorbic acid derivatives should correlate with their abilities to quench superoxide anions produced in stimulated neutrophils. This oxidative burst can be measured by the SOD-sensitive cytochrome *c* reduction, but this test is not valid for all enediol derivatives which by themselves reduce cytochrome *c* due to their low potential one-electron-reducing capacities. Oxygen consumption as a parameter would not be applicable if the inhibition would occur through a reduction of O_2^-, and the commonly used inhibition of luminol-enhanced chemiluminescence is a complex series of oxidative events where antioxidants could already interfere with the peroxidase-dependent assay. Hence we chose the cytochrome *c* assay and had to omit the enediol derivatives [**5a–7a**] from the screening. Figure 2a contains the most relevant derivatives and shows their effects on superoxide anion formation of fMLP-stimulated neutrophils.

At 1 μM, **7b** resulted in about 80% inhibition, and **8b** with a two-carbon longer side chain inhibited about 40%.

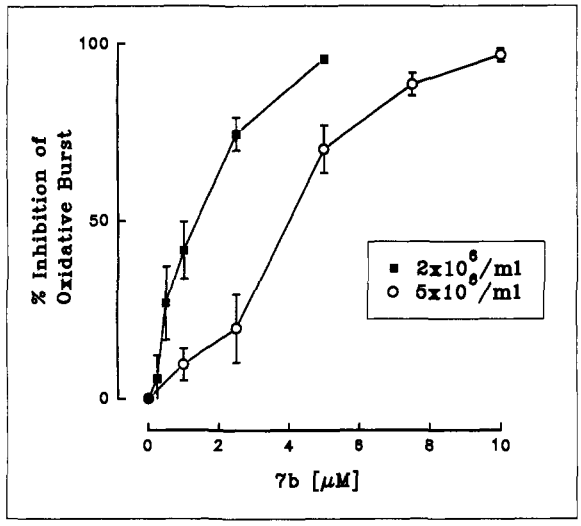


Figure 3. Inhibition of the fMLP-stimulated oxidative burst by **7b** dependent on cell density. Samples, 2×10^6 PMN/mL (filled squares) or 5×10^6 PMN/mL (open circles), were preincubated for 3 min at 37 °C with the indicated concentrations of **7b**. After stimulation of the cells with 100 nM fMLP, superoxide radical generation was monitored by SOD-sensitive cytochrome *c* reduction as described under Materials and Methods. Values are given as mean ± SEM of three to five independent experiments.

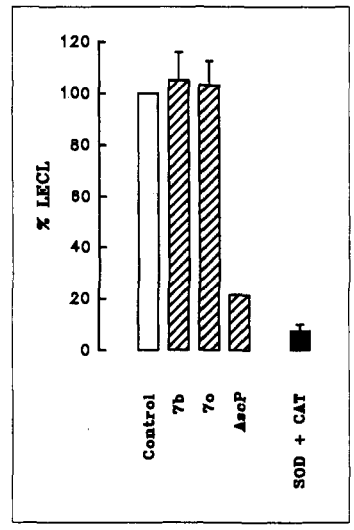


Figure 4. Examination of the superoxide-scavenging activity. The influence of 20 μM of the indicated substances (0.1% DMSO) on the superoxide radical-generated LECL by a cell-free xanthine/xanthine oxidase system was examined as described under Materials and Methods. Values are given as mean ± SEM of three experiments.

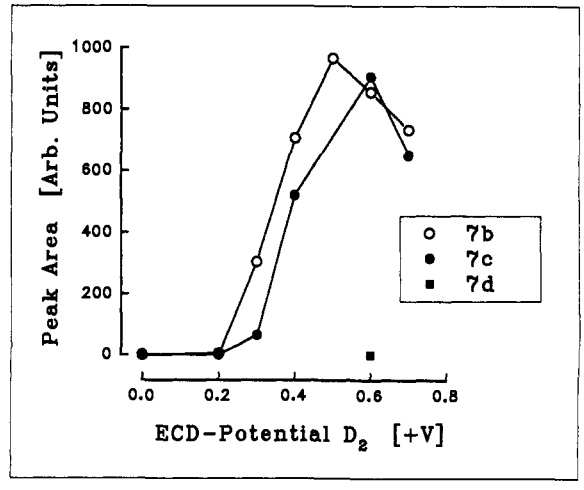


Figure 5. Voltammogram of **7b** and **7c**. Samples (100 ng) of the indicated substances were injected on the HPLC/ECD system, and the individual electrochemical signals were monitored at varying detector potentials at a gain of 200 as described under Materials and Methods. Mean of the values from three experiments for each compound are shown.

The compound **4b** which carries a phenyl group at the sulfur as well as the derivatives **5b** and **6b** having a C_8 or C_{12} alkyl side chain did not inhibit or only weakly inhibited. In the same way, **7d**, the nonreducing control compound, only weakly inhibited, whereas **7c** the regioisomer of **7b** at 1 μM even had a stimulatory effect. This was a clear indication that the antioxidant properties seem to be unrelated to the inhibition of the oxidative burst.

Since **7b** proved to be the most efficient inhibitor, we carried out more detailed studies with this compound. A concentration dependency resulted in an IC_{50} value of 0.6 μM, and the same value was obtained when measuring the inhibition of the luminol-enhanced chemiluminescence after stimulation of the cells with fMLP (Figure 2b).

We noticed that the inhibitory effect of **7b** on the fMLP-induced oxidative burst of human neutrophils was strongly dependent on cell density. As shown in Figure 2b, at a cell density of 5×10^5 PMN/mL, **7b** half-maximally inhibits at 0.6 μM. Increasing the cell density to 2×10^6/mL, the IC_{50} value shifted to 1.25 μM, whereas maximal inhibition was observed at 5 μM **7b** (Figure 3). At a cell density of 5×10^6/mL, **7b** inhibited the fMLP-stimulated superoxide production with an IC_{50} value of 3.7 μM, and maximal inhibition was achieved by 10 μM. Again, the regioisomer **7c** was inactive under these conditions (not shown).

Differences in the Action of 7b and the Regioisomer 7c. It was surprising to find extreme differences in the inhibition of the oxidative burst between 7b and its regioisomeric compound **7c**. This allowed us to further investigate the possible mechanism of action of **7b**. Both compounds were comparably active in the inhibition of lipid peroxidation which already indicated that the antioxidant properties seem to be not essential for the inhibition of the oxidative burst. Furthermore, no interaction of **7b** with superoxide anions occurs as shown in Figure 4.

Superoxide radicals are formed in the system xanthine/xanthine oxidase and can be registered by LECL. No interference of **7b** and **7c** could be observed, although L-ascorbic acid 6-palmitoate was an efficient quencher, and SOD/catalase almost completely prevented chemiluminescence.

As a basic requirement for an inhibition of the fMLP-induced oxidative burst, the compounds have to enter the cells, and this can be measured by following the cell-bound concentrations of **7b** and **7c**. Since the expected amounts would be small, we employed the electrochemical detection after HPLC separation and first established the voltammograms for both compounds (Figure 5). **7b** showed a full oxidation at about 0.5 V whereas **7c** was maximally oxidized at a potential of about 0.6 V. Such differences could be expected since for both compounds its enolic structures should be different in their pK_a values. Due to the hydrophobic character of the alkylated compounds, no direct titration of the compounds in water was possible, but the dealkylated precursors yielded pK_a values of 3.32 ± 0.02 and 7.70 ± 0.09 which then were assumed also for **7b** and **7c**, respectively (Table III).

Table III. Determination of the pK_a of 2- and 3-O-Methylascorbic Acid[a]

	$pK_a \pm$ SEM
2-O-methylascorbic acid	3.32 ± 0.02
3-O-methylascorbic acid	7.70 ± 0.09

[a] Since the hydrophobicity of the compounds **7b** and **7c** led to a poor solubility in aqueous systems and hampered the potentiometric estimation of the pK_a, analogues in which the long chain alkyl groups were replaced by methyl groups were used to determine the pK_a values by nonlinear regression from titrations curves obtained potentiometrically. Mean ± SEM from three titrations were shown.

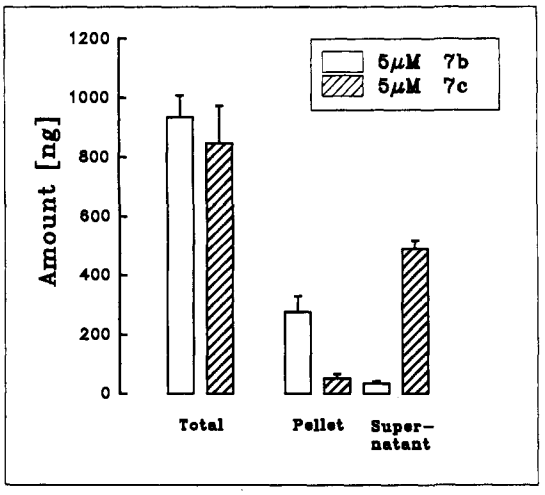


Figure 6. Differences in the permeation of **7b** and **7c** into human PMN. Cells (2×10^6/mL) were preincubated with 5 μM **7b** or 5 μM **7c** (0.1% DMSO) at 37 °C. After 3 min, cells were pelleted and washed once as described under Materials and Methods. After extraction with ethyl acetate the amount of both substances in the cell pellets and supernatants was detected by the established HPLC/ECD system (see Materials and Methods) and calculated by standard curves set up for each (not shown). Values are given as mean ± SEM of four independent experiments.

When human neutrophils were incubated with 5 μM of each compound, washed, and then extracted for HPLC, it turned out that the total amount of **7b** was about 6 times higher in the pellet than in the supernatant, whereas with **7c** it was reversed (Figure 6). On the basis of the volume of the PMN, this means a considerable accumulation of **7b** above the extracellular concentration. This was difficult to understand in view of the charged molecule **7b** and the predominantly uncharged compound **7c** at a physiological pH. The efficiencies of extraction into hexane which was much higher for **7c** than for **7b** confirmed the charged character of **7b**. An unspecific absorption of **7b** at the neutrophils could be excluded since the accumulation was not observable after short time exposures (<10 s). Therefore it seemed that a time-dependent active process was involved which might determine part of the action of **7b** compared to **7c**.

Intracellular pH and Membrane Potential. Considering **7b** as a charged species being transported into the cell, we investigated its effect on intracellular pH (pH_i) by following the pH-dependent fluorescence of the pH-sensitive dye BCECF. In agreement with literature data, the stimulation of neutrophils with 100 nM fMLP caused a rapid acidification indicated by a decrease in fluorescence followed by a delayed increase which could be partially blocked by amiloride (Figure 7a). This would be consistent with an inhibition of the postulated Na^+/H^+-antiporter.[18] After addition of 5 μM **7b** to resting neutrophils, the pH_i dropped in a monophasic reaction to about the same level as observed after stimulation with 100 nM fMLP (Figure 7a). With 10 μM **7b** the decrease in fluorescence was about doubled and the subsequent increase after stimulation with fMLP was slowed down. The compound **7c** was completely inactive, and L-ascorbic acid 6-palmitoate as well as the 2-O-alkylascorbic acid CV-3611 reached about half of the initial velocity and extent of acidification (Figure 7b).

We then compared these effects with those of known protonophores like nigericin, CCCP, and propionic acid (Figure 8). The acidification occurred as expected, but the effects on the oxidative burst were negligible. Nigericin (Figure 8) as well as 10 mM propionic acid (data not shown) did not give any inhibition, whereas CCCP caused about 10%. The latter had about the same pH_i-lowering potential as **7b** which however caused an about 60% inhibition at this cell density of 5×10^6/mL.

By a similar fluorescence assay the resting membrane potential of granulocytes can be determined. In Figure 9 the potential-sensitive dye bisoxonol was preincubated with 2×10^6 PMN as described under Materials and Methods, and then nigericin, CCCP, and **7b** were added. Clearly the two protonophores showed an increase in fluorescence, thus proving the expected change of the membrane potential whereas **7b** had no effect. As a control we used the cell-permeabilizing detergent digitonin, which, at 5 μM, led to a pronounced change in membrane potential similar to CCCP (data not shown).

Degranulation. Due to the described inhibitory effects of L-ascorbic acid 6-palmitoate on neutrophil secretion, we investigated the influence of **7b** on other parameters of neutrophil activation. As shown in Figure 10, we observed a comparably strong inhibition of the fMLP-induced degranulation from azurophilic as well as specific granules. At a cell density of 5×10^6/mL, the IC_{50} values for **7b** were 7 μM for the secretion of β-glucuronidase (azurophilic granules) and 2.5 μM for lysozyme secretion (azurophilic and specific granules). Preincubation of the cells with 10 μM **7c** did not influence the fMLP-triggered secretion of lysozyme (data not shown).

Discussion

The synthesis of lipophilic ascorbic acid derivatives resulted in the discovery of **7b** (B-003) as a very potent inhibitor of the oxidative burst in human neutrophils. At variance with the starting strategy of synthesizing lipophilic antioxidants it turned out that the antioxidant properties of this compound were unrelated to its mechanism of action. The structure–activity relationship revealed the requirement of an alkyl side chain of at least 16–18 carbons. This chemical feature was also present in the earlier investigated L-ascorbic 6-palmitoate and can be found in the 2-O-octadecylascorbic acid CV-3611 which both are inhibitors of the oxidative burst. It may be speculated that this dependency on a longer alkyl chain may be linked to a binding site for fatty acids in membranes or proteins. It was surprising to find a similar dependence on a C_{16}–C_{18} alkyl chain in the inhibition of lipid peroxidation although this mechanism turned out to be independent of the inhibition of the oxidative burst since the 2-O-methylated-6-S-dodecyl derivative was almost inactive. The results on lipid peroxidation are in agreement with earlier findings by Kato et al.[14] who observed comparably strong antilipid-peroxidative properties and

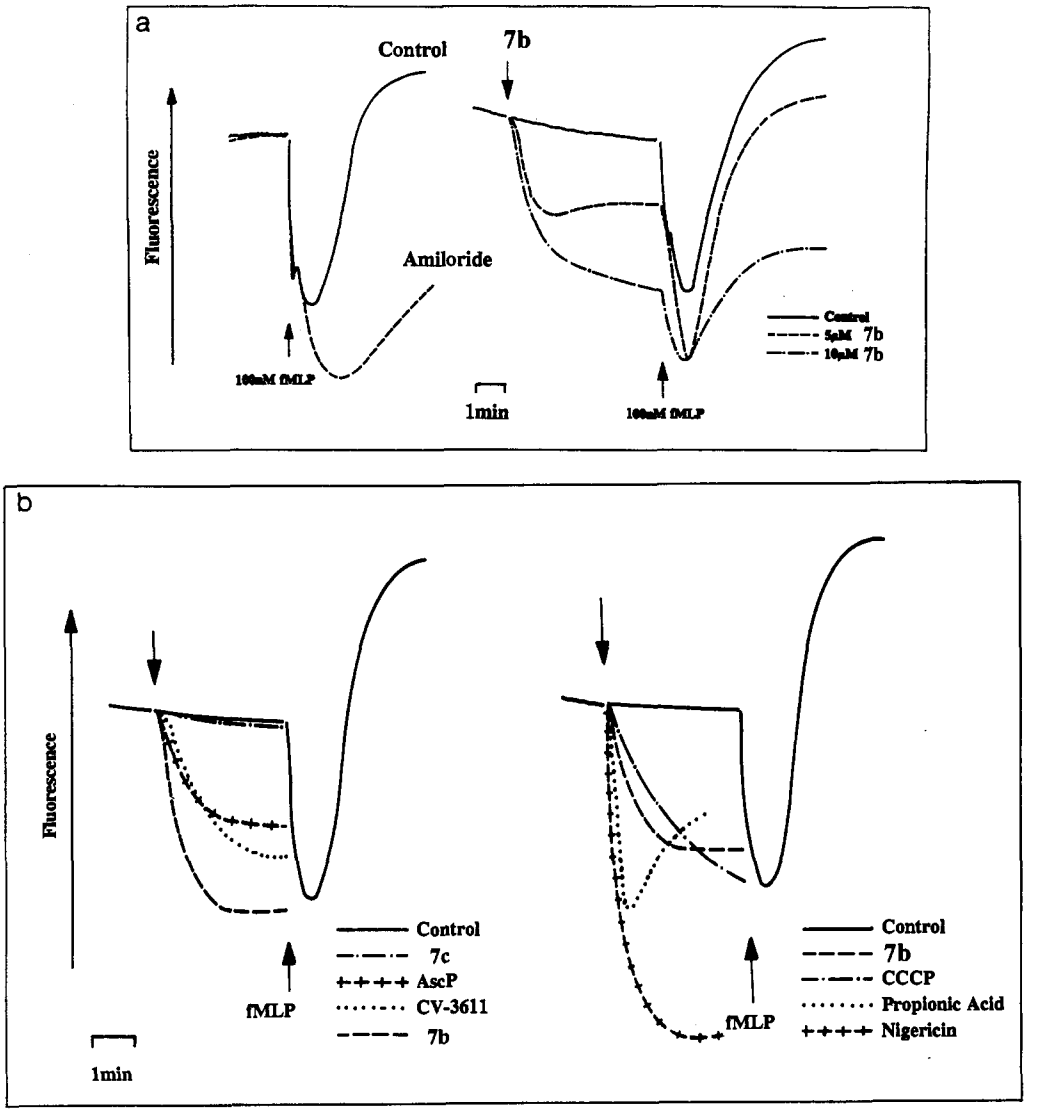


Figure 7. Effect of 7b on the intracellular pH (pH_i) of human PMN. (a) Comparison to pH_i changes triggered by fMLP. After preincubation with 200 μM amiloride for 10 min at 37 °C (left panel) or addition of **7b** (right panel, indicated by an arrow), BCECF-loaded PMN (5×10^6/mL) was stimulated with 100 nM fMLP (below arrow), and the BCECF-fluorescence was continuously monitored as described under Materials and Methods. (b) Comparison to other lipophilic ascorbic aicd derivatives and known protonophors. The indicated substances were added to BCECF-loaded PMN (5×10^6/mL) 3 min before stimulation with 100 nM fMLP and the fluorescence monitored as described under part a. The concentrations for the individual compounds were as follows: 5 μM of **7b**, **7c**, CV-3611, and AscP (L-ascorbic acid 6-palmitoate) (left panel); 0.25 μM nigericin; 10 mM propionic acid; 5 μM CCCP (right panel). Representative traces of three or four independent experiments are shown.

a similar structure–activity relationship with a series of 2-*O*-alkylated ascorbic acids. In this context one can imagine that the long chain 6-*S*-alkyl group enables the anchoring of the antioxidative ascorbyl derivatives to the membranous phospholipid layer similar to the proposed incorporation of the physiological antioxidant vitamine E.[19]

A second essential requirement for inhibition of the burst seems to be a free 3-hydroxyl at the enol group which is acidic and deprotonated at physiological pH values. This may allow an active uptake of **7b** into neutrophils by passive diffusion of the protonated form across the cell membrane and deprotonization at the inside. This would explain the drop in pHi and the accumulation of **7b** in the cell pellet. The regioisomer **7c** which has the same favorable alkyl chain but only a weakly acidic enolic group was not accumulated in neutrophils and also turned out to be inactive on neutrophil superoxide formation. Therefore, the charge in position 3 may be an essential criterion for the efficacy of **7b**. The known protonophore CCCP led to a similar decrease in intracellular pH but to no inhibition of the oxidative burst, indicating that both events are not causally linked. CCCP and nigericin showed a change in membrane potential whereas this did not occur with **7b** (Figure 8). This is surprising since even an unspecific destruction of the membrane would have depolarized the membrane as was observed with 5 or 10 μM digitonin.

In summary the compound **7b** owes its strong inhibition of the oxidative burst mainly to an uptake into neutrophils which seems to be based on the low pK_a value of the enolic 3-hydroxy group. In addition the alkyl side chain of 16–18 carbon atoms was required but not the antioxidative potential of the enolic group. The accumulation of **7b** inside the cells also explains the dependence of the IC_{50} values on the cell concentration.

Nothing is known yet about the point of attack of **7b** at the signal transduction pathway of NADPH-oxidase activation, which obviously requires a cell-free preparation of NADPH-oxidase for further investigations. This is the

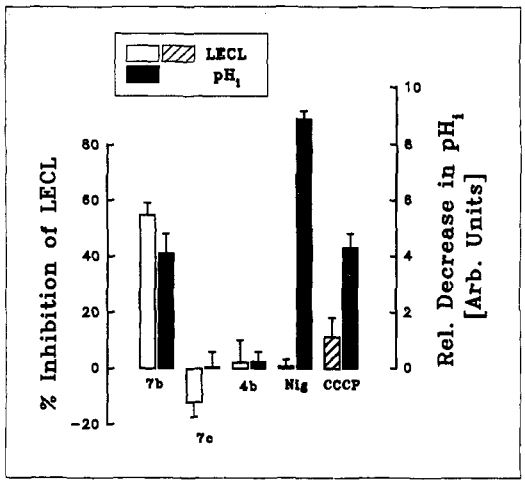


Figure 8. Effect of CCCP and nigericin on the intracellular pH and the fMLP-stimulated superoxide production in comparison to the 6-(alkylthio)ascorbic acid derivatives **7b**, **7c**, and **4b**. The relative changes in pH_i of resting BCECF-loaded PMN (5×10^6/mL) were monitored after addition of 5 μM of the indicated ascorbic acid derivatives, 5 μM CCCP or 0.25 μM nigericin, as described under Materials and Methods. The results of three to five independent experiments are calculated as relative decreases in pHi from the original traces and depicted as mean ± SEM (filled bars) in arbitrary units. After 3 min preincubation of PMN (5×10^6/mL) with the indicated substances and stimulation with 100 nM fMLP, the oxidative burst was monitored as LECL as described under Materials and Methods. Due to the much faster change in pH_i, nigericin was preincubated only for 1 min. The results of four to five independent experiments are depicted as mean ± SEM.

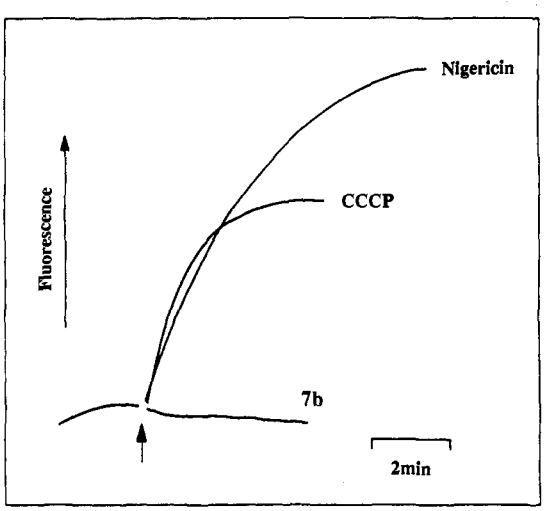


Figure 9. Effects of CCCP and nigericin on the membrane potential of resting PMN in comparison to **7b**. PMN (2×10^6/mL) was loaded with 150 nM of the potential-sensitive dye bisoxonol as described under Materials and Methods. After equilibration of the dye at 37 °C, 2.5 μM CCCP, 2.5 μM **7b**, or 1 μM nigericin were given to the cells as indicated by the arrows and the change in fluorescence was monitored at 530 nm. Representative tracings of three independent experiments are shown.

subject of ongoing studies. It must be considered that a rather general pathway of signal transduction is affected since secretion is inhibited as well.

With regard to the reported beneficial effects of 2-*O*-octadecylascorbic acid (CV-3611) in postischemic damage of the rat, we would postulate that its mode of action is related to that of **7b** rather than due to its oxygen radical scavenging properties. It is interesting that the regioisomer of CV-3611, 3-*O*-octadecylascorbic acid, which also had

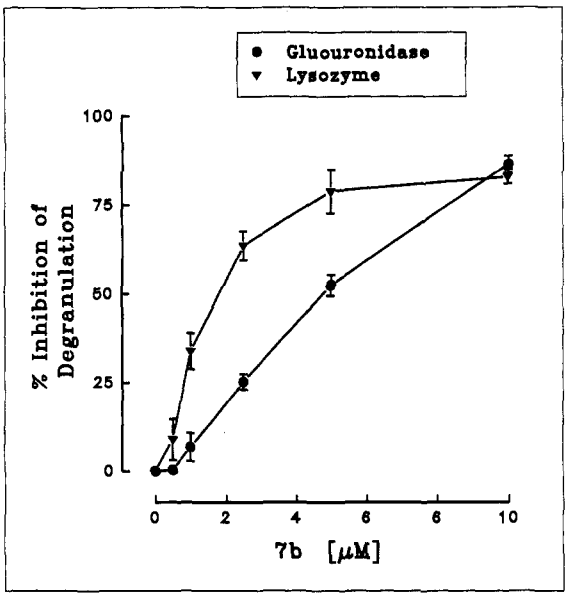


Figure 10. Concentration-dependent effects of **7b** on the secretion of azurophilic and specific granules of human PMN. After a 3-min preincubation with the indicated concentrations of **7b** (0.1% DMSO) at 37 °C, cells (5×10^6/mL) were stimulated with 100 nM fMLP. Supernatants of stimulated cells were determined for β-glucuronidase activity (azurophilic granules) (filled circles) or lysozyme activity (specific granules) (filled triangles) as described under Materials and Methods. Values are given as mean ± SEM of three independent experiments.

antiperoxidative properties, markedly differed in its cardioprotective effects.[14] Hence **7b** and CV-3611 may have in common a new mode of action with potential in neutrophil inactivation and therefore in antiinflammatory therapy.

Materials and Methods

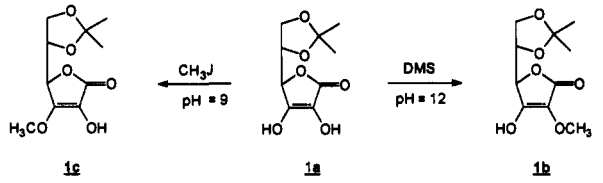
Materials. Thiobarbituric acid, xanthine, xanthine oxidase, fMLP, PMA, cytochrome *c* (type III), microperoxidase (MP-11), cytochalasin B, Na-perchlorate, digitonin, SOD, catalase, ficoll/paque, dextran, CCCP, BHT, DMSO, EDTA, 4-methylumbelliferyl-β-D-glucuronide, Triton X-100, glycine, and micrococcus lysodeikticus were obtained from Sigma Chemical Co. (Deisenhofen, FRG). ATP, luminol, ADP, ATP-bioluminescence kit (luciferin/luciferase-assay), DTT, and AMP were purchased from Boehringer-Mannheim (Mannheim, FRG). BCECF was from Calbiochem (Frankfurt, FRG), and bisoxonol was ordered from Molecular Probes (Eugene, OR). 2-*O*-octadecyl-L-ascorbic acid was a gift of Dr. S. Terao, Takeda Chemical Industries, Ltd. (Osaka, Japan).

All other reagents were commercially available and at least reagent grade. Solvents and chemicals utilized for electrochemical detection were of HPLC or analytical grade and purchased from Merck (Darmstadt, FRG).

Methods: Syntheses. The routes for the regioselective syntheses for all 6-*S*-alkyl-6-deoxy-6-thio-L-ascorbic acids started from L-ascorbic acid, which was initially converted to 5,6-*O*-isopropylidene-L-ascorbic acid (**1a**) as described by Kato et al.[14] Depending on the pH chosen for methylation, 5,6-*O*-isopropylidene-3-*O*-methyl-L-ascorbic acid (**1c**) was formed selectively at pH 9 and 5,6-*O*-isopropylidene-2-*O*-methyl-L-ascorbic acid (**1b**) at pH 12[20] (summarized in Scheme I).

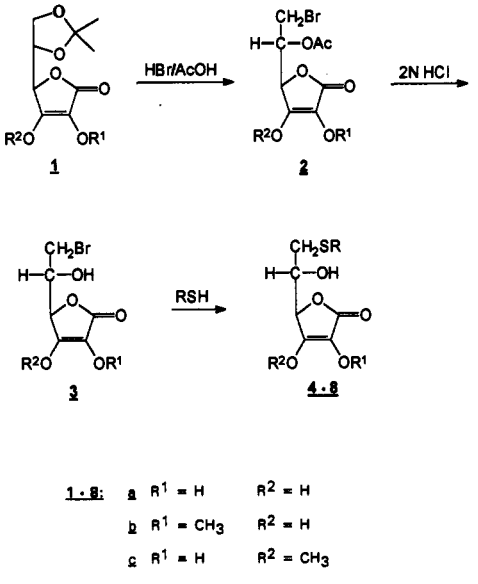
As depicted in Scheme II, 6-bromo-6-deoxy-L-ascorbic acids (**3a–c**) were obtained from 5,6-*O*-isopropylideneascorbic acids (**1a–c**) according to the procedure described by Bock et al.,[21] which initially gave the 5-acetyl-6-bromo-6-deoxy-L-ascorbic acids (**2a–c**) that without further purification were hydrolyzed to remove the 5-acetyl group. Finally the 6-bromo-6-deoxy-L-ascorbic acids were reacted with different alkyl mercaptans (RSH) to the corresponding 6-*S*-*n*-alkyl-6-deoxy-6-thio-L-ascorbic acids (**4a–8a**, **4b–7b**, and **6c–7c**) as listed in Table I.

Scheme I. Regioselective Methylation of 5,6-*O*-Isopropylidene-L-ascorbic Acid



1c 1a 1b

Scheme II. General Procedure for the Synthesis of the Different 6-*S*-*n*-alkyl-6-deoxy-6-thio-L-ascorbic acids (5a–j) from the Corresponding 5,6-*O*-Isopropylidene-L-ascorbic Acid Precursor



1 2 3 4-8

1-8: a $R^1 = H$ $R^2 = H$
b $R^1 = CH_3$ $R^2 = H$
c $R^1 = H$ $R^2 = CH_3$

6-Deoxy-6-(*n*-hexadecylthio-2,3-*O*-dimethyl-L-ascorbic acid (**7d**) was synthesized from 6-(*n*-hexadecylthio)-6-deoxy-2-*O*-methylascorbic acid (**7b**) by methylation with iodomethane in aqueous sodium carbonate.

The stereochemistry and chemical structure of each compound were confirmed by ^1H NMR analysis after isolation and purification on silica gel. The analytical data are depicted in Table II.

Antioxidative Activity. The livers of Wistar rats were removed under anesthesia with pentobarbital and homogenized in phosphate buffer, pH 7.4. Rat liver microsomes were prepared as described elsewhere.[22] After preincubation of the microsomes with the indicated ascorbic acids or DMSO as a control at 25 °C for 5 min in 50 mM potassium phosphate buffer, pH 7.2, in the presence of 50 μM ADP and 5 μM Fe_2SO_4, lipid peroxidation was initiated with 1 mM NADPH. The amount of malondialdehyde formed was determined by the thiobarbituric acid method as described by Buege et al.[23]

Isolation of Human PMN. Freshly drawn venous blood (200 ml) from healthy adult donors was supplemented with 0.38% citrate, and neutrophils were (PMN) purified by dextran sedimentation, ficoll-paque centrifugation, and hypotonic lysis of erythrocytes as described previously.[13] After isolation the cells were stored at 4 °C in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 280 mOsm) until incubated under the indicated conditions in supplemented PBS (1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose). The purity of the PMN was about 95% and the viability, as measured by trypan blue exclusion, was greater than 97%.

Superoxide Radical Generation and Determination. To assess the total ROS release of activated neutrophils we used the detection by luminol-enhanced chemiluminescence (LECL). The lipophilic chemiluminescence enhancer luminol is able to enter the cell, thus detecting intra- and extracellular processes of ROS production.[24] In brief, neutrophils at the indicated cell density were preincubated for 3 min at 37 °C with the given concentrations of the ascorbic acid derivatives or DMSO in 1.5 mL of supplemented PBS including 5 μM luminol. The cells were stimulated with 100 nM fMLP, and the LECL was continuously monitored by a LKB-Wallac 1240-chemiluminometer connected to a chromatointegrator (Merck-Hitachi, D2000). The results are given as percent inhibition and calculated from the areas under the LECL curves.

Alternatively superoxide production by stimulated neutrophils was measured as SOD-inhibitable reduction of cytochrome *c* by a modified protocol as described by Cohen and Chovaniec.[25] In brief, neutrophils at the indicated cell densities were incubated in supplemented PBS including 100 μM cytochrome *c* and stimulated with 100 nM fMLP. The reduction of cytochrome *c* was continuously recorded at 550 nm/538 nm in a dual-wavelength spectrophotometer (Aminco DW2). Superoxide radical generation was calculated by using a molar extinction coefficient of 21.5 mM^{-1} cm^{-1} at 550 nm.

The superoxide radical scavenging properties of the ascorbic acids were determined in an $O_2^{\cdot-}$-generating cell-free system consisting of xanthine and xanthine oxidase.[26] After preincubation with 20 μM of the indicated substances or DMSO in 100 mM potassium phosphate buffer, pH 7.0, in the presence of 5 μM luminol, 2 μM microperoxidase and 50 μM xanthine, the reaction was started with 0.02 units/mL xanthine oxidase and the resulting LECL signal continuously detected as given above.

Intracellular pH. The intracellular pH (pH_i) of granulocytes was measured using the membrane permeable fluorescent probe 2′,7′-bis(2-carboxyethyl)-5-carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM) according to the method described by Grinstein et al.[27] In brief, cells (10^7/mL) were loaded with the probe by incubation with the membrane-permeable precursor BCECF-AM (2 μM) for 30 min at 34 °C in HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 0.5 % BSA, 1 mM $CaCl_2$, 2 mM glucose, 10 mM HEPES, pH 7.4). After washing twice with PBS (1 mM $CaCl_2$, 2 mM glucose), cells were diluted to 5×10^6/mL in supplemented PBS (1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose) and used for fluorescence measurements in a dual wavelength spectrophotometer (Sigma ZWS II). Cell suspensions (1.5 mL) were placed in a 37 °C thermostated cuvette and the changes in BCECF-fluorescence continuously recorded under constant stirring by using alternating excitation wavelengths of 438 (isosbestic) and 500 nm. The emission wavelength was 530 nm. Substances were added after a plateau in fluorescence was reached. The nigericin/K^+ method of Thomas et al.[28] was used to control the resting level of pH_i after the labeling procedure.

Membrane Potential. Changes in the membrane potential of granulocytes were followed by using the anionic, potential-sensitive, fluorescent dye bisoxonol as described by Dho et al.[29] In brief, cells (2×10^6/mL) in supplemented PBS (1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose) were placed in a thermostated cuvette at 37 °C under constant stirring. After addition of 150 nM bisoxonol the fluorescence was continuously monitored by a Perkin-Elmer spectrofluorimeter using an excitation wavelength of 490 nm. The emission was measured at 540 nm. Before addition of substances the cells were allowed to equilibrate for approximately 10 min until a stable baseline was attained.

Degranulation Experiments. Samples (5×10^6 PMN/mL) were preincubated in supplemented PBS (1 mM Ca^{2+}, 1 mM Mg^{2+}, 5.5 mM glucose) with 5 μg/mL cytochalasin B for 4 min at 37 °C. Cells were stimulated by 100 nM fMLP, and the reaction was stopped after an additional 15 min by transferring the samples to an ice bath. After pelleting of the cells at 4 °C (2000 rpm, 5 min), aliquots of the supernatants were mixed with an equal volume of cold PBS containing 0.1% Triton X-100 and stored until assayed for enzyme activities.

For measuring β-glucuronidase activity in the supernatant, 100 μL samples were incubated with 100 μL of 4-methylumbelliferyl-β-D-glucuronide (10 mM in 100 mM sodium acetate solution pH 4.0 and 0.1% Triton X-100) for 15 min at 37 °C. The reaction was terminated by addition of 3 mL of stop-solution containing 50 mM glycine and 5 mM EDTA, pH 10.4. The reaction product, 4-methylumbelliferone, was measured fluorometrically at an excitation of 365 nm and an emission of 460 nm.

Lysozyme activity was measured as previously described.[30] In brief, 100-μL samples were added to 2.5 mL of buffer in a cuvette containing 50 mM citrate and micrococcus lysodeikticus

giving an OD_{450} = 0.3 (about 25 mg of micrococcus/100 mL of buffer). The enzyme activity was assayed photometrically at 450 nm.

Electrochemical Quantitation (ECD) of Ascorbic Acid Derivatives. The redoxactive ascorbic acid compounds were quantitatively detected by a coupled HPLC/ECD system. Samples (100 μL) of the incubates were injected in the reversed phase HPLC system consisting of a constant flow pump (LKB, Model 2150) operating at 1 mL/min, a Rheodyne injector with a fixed 100-μL sample loop, a silica-HPLC column (Nucleosil 5 C_{18} ODS, 250 × 4.6 mm, Fa. Bischoff (Leonberg, FRG)) connected to a Nucleosil 5 C_{18} precolumn. The solvent system consisted of methanol/water (93:7; v/v) including 0.2 g/L sodium perchlorate as electrolyte. The pH was adjusted to 4.8 with sodium phosphate. After passing the column, the samples were electrochemically reduced at one detector by a negative potential of −0.75 V in order to avoid loss of sensitivity by a possible oxidation of the substances during incubation and extraction. Detection of the substances was through the analytical cell (ESA, Model 5010) of the electrochemical detector (ESA Coulochem, Model 5100A) with an oxidizing potential of +0.6 V.

In order to determine the optimal working potential of the analytical cell for detection of the ascorbic acid derivatives **7b** and **7c**, separate voltammograms of each compound were established (Figure 5). Maximum oxidation for both compounds was seen at a potential between +0.5 and +0.6 V. The analysis of the peaks was done by the integration software kit Kontron DataSystem 450 run on a personal computer.

Incubations were done with 2×10^6 cells/mL in supplemented PBS at 37 °C. After addition of 5 μM of **7b** or **7c** and further incubation for 3 min the cells were immediately stopped in 4 mL of ice-cold ethyl acetate. After two extractions the organic phase was evaporated under nitrogen, and the dried samples were resuspended in 500 μL of methanol either for electrochemical detection or for storage at −20 °C for later analysis.

For examination of the permeation, the cells were centrifuged (2 min, 2200 rpm) after a 3-min incubation in the presence of 5 μM **7b** or **7c**. After washing once in PBS, the pellet was finally resuspended in 500 μL of PBS and extracted as already described. The samples were analyzed under optimum detection conditions (D_1 = −0.75 V, D_2 = +0.6 V, gain: 2000) and calculated by comparison with standard curves for each substance.

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