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A WATER-SOLUBLE QUATERNARY AMMONIUM ANALOG OF α-TOCOPHEROL, THAT SCAVENGES LIPOPEROXYL, SUPEROXYL AND HYDROXYL RADICALS

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The new water-soluble ammonium-analog of α-tocopherol (vitamin E) (compound **1**: 3,4-dihydro-6-hydroxy-N,N,N-2,5,7,8-heptamethyl-2H-1-benzopyran-2-ethanaminium 4-methylbenzenesulfonate) and its tertiary amine derivative (compound **2**: 3,4-dihydro-2-(2-dimethylaminoethyl)-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol hydrochloride) were investigated as scavengers of oxygen-derived free radicals. Compounds **1** and **2** were at least 40 times more potent inhibitors of Fe-driven heart microsomal lipid peroxidation than Trolox. While the α-tocopherol analogs had the same potency as scavengers of xanthine/xanthine oxidase-generated superoxyl radicals, the thiol compounds D, L-penicillamine and N-2-mercaptopropionyl glycine reacted at a much slower rate. The O-acetyl derivatives of compounds **1** and **2** were not scavengers of superoxyl radicals. Considerable differences between the α-tocopherol analogs were observed in their competition with 2-deoxyribose for hydroxyl radicals (OH·). Compound **2** was equipotent with Trolox and thiourea, whereas the reactivity of these substances was diminished by more than 30% as compared to compound **1**. Although showing lower reactivity, the O-acetyl derivatives of compounds **1** and **2** were active nevertheless as OH·-scavengers.

The previously reported high potency of compound **1** in reducing infarct size during myocardial ischemia/reperfusion appears to be due to its radical-scavenging properties, likely to be enhanced by its previously described cardioselectivity.

KEY WORDS: α-tocopherol analog, lipid peroxidation, hydroxyl radical, superoxyl radical.

INTRODUCTION

Superoxyl radical anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·) are formed in aerobic organisms in the course of different cellular functions.[1] In a number of pathologic situations, as for example ischemia/reperfusion and inflammation,[2,3] release of such reduced oxygen species can initiate autoxidation of polyunsaturated fatty acids and exert detrimental effects on enzymes, membrane proteins or nucleic acids.[4–6] Of the numerous defense systems available in mammalian organisms, α-tocopherol (vitamin E) is especially effective as lipoperoxyl radical scavenger[7,8] by intercalation into cellular or subcellular membranes. α-Tocopherol is also a scavenger of O_2^-,[9] singlet oxygen[10,11] and OH·.[12] However, these species are expected to react primarily with the phospholipids surrounding the α-tocopherol molecule, which then neutralizes the secondary radicals formed by chain reactions within the membrane.[12] The α-tocopherol content is known to decrease during

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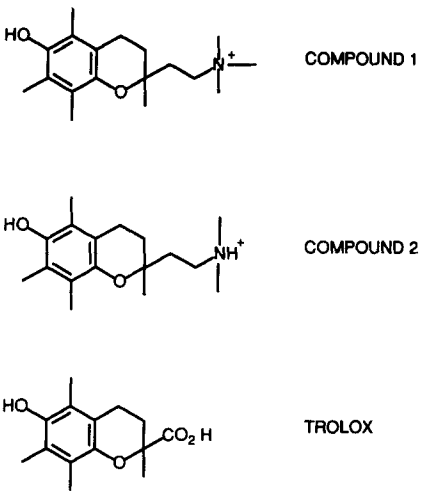

FIGURE 1 Structures of the α-tocopherol analogs.

enhanced microsomal lipid peroxidation[13] or in heart tissue following cardioplegia.[14] Treatment with vitamin E[15] or Trolox,[16] a water-soluble α-tocopherol analog, in combination with vitamin C has been reported to decrease infarct size after myocardial ischemia/reperfusion.

Utilizing the chromanol structure of α-tocopherol, a quartenary ammonium analog was synthesized (compound **1**) (Figure 1).[17] This analog was hoped to be cardioselective[17] while retaining the radical scavenging properties of the parent compound. It could thus react with oxygen-derived free radicals fast enough to interfere with the initiation of radical chain reactions. Indeed, compound **1** was shown to accumulate in rat heart tissue over 10 times more efficiently than compound **2** and to decrease infarct size in a rat model of myocardial ischemia/reperfusion at lower doses.[18] The effects of the structural modifications on the free radical scavenging properties of these new α-tocopherol analogs, in comparison with Trolox and thiol antioxidants, are reported and evaluated in this communication.

MATERIALS AND METHODS

Reagents: Malondialdehyde bis-dimethylacetal, 2-thiobarbituric acid, D,L-penicillamine, thiourea, Trolox (3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyrane-2-carboxylic acid) and trimethylamine and tetramethylammonium hydrochlorides were from Aldrich (Strasbourg, France), $FeSO_4 \times 7H_2O$ from Janssen (Beerse, Belgium) and 2-deoxy-D-ribose from Serva (Heidelberg, FRG). Xanthine sodium salt, D-mannitol, phenylmethylsulfonyl fluoride, N-2-mercaptopropionyl glycine, nitro-blue tetrazolium, adenosine 5′-diphosphate (sodium salt, grade III), ascorbic acid, bovine serum albumin and superoxide dismutase (from bovine erthrocytes, E.C. 1.15.1.1., 3,200 units/mg), catalase (from bovine liver, E.C. 1.11.1.6., 13,000 units/mg), xanthine oxidase (from buttermilk, grade 1, E.C. 1.2.3.2., 0.59 units/mg) and glucose oxidase (from aspergillus niger, E.C. 1.1.3.4., 150 units/mg) were from Sigma Chimie (La Verpillière, France). Compound **1**

(MDL 73404: 3,4-dihydro-6-hydroxy-N,N,N-2,5,7,8-heptamethyl-2H-1-benzopyran-2-ethanaminium 4-methylbenzenesulfonate) and compound **2** (MDL 73335; 3,4-dihydro-2-(2-dimethylaminoethyl)-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol hydrochloride) (Figure 1) as well as their O-acetates were prepared as described.[17] Instrumentation: For the photometric measurements a Beckman DU-7 spectrophotometer was used.

Lipid Autoxidation in Rat Brain Homogenate

The method originally described by Stocks *et al.*[19] was adapted. Fresh brains from adult Sprague Dawley rats were used instead of bovine brains. The tissue was homogenized at 1,000 RPM in a glass/teflon-homogenizer 1 + 4 (w/v) in ice-cold 40 mM potassium phosphate buffer (pH 7.4, 0.142 M NaCl) and centrifuged for 15 min at 1,000 × g in a cooled centrifuge and the supernatants were frozen at −80°C.

For the assay, the homogenate was diluted 2 : 5 (v/v) with the same buffer. Duplicate samples of total volume 1 ml containing 800 μl of diluted brain homogenate and with the scavenger at an appropriate dilution were incubated. Nonincubated samples were taken as background. Controls were run without scavenger and a sample containing only buffer was taken as blank. After incubation at 37°C for 30 min, 200 μl of 35% perchloric acid was added, the samples centrifuged and 800 μl of the supernatants mixed with 200 μl of 1% thiobarbituric acid. The pink condensation product of thiobarbituric acid-reactive material was developed at 100°C in a boiling water bath for 15 min, and absorbance read at 532 nm. Including 1 mM Trolox as an antioxidant at this step did not affect the final reading. A straight calibration line was obtained between 4 and 20 nmol with malondialdehyde dimethylacetal as standard, independent of the scavengers at the concentrations utilized.

Lipid Peroxidation in Rat Heart Microsomes

Microsomes were isolated from the hearts of adult male rats starved for 24 h prior to being killed by decapitation, as has been described[20] and frozen at 2 mg protein/100 μl 1.15% KCl at −80°C in 0.5 ml portions.

To each sample of 0.5 mg microsomal protein in 400 μl 15 mM potassium phosphate buffer pH 7.4 were added (50 μl each): The scavenger at appropriate concentration (duplicate), adenosine 5′-diphosphate (1.7 mM final concentration) $FeSO_4 \times 7H_2O$ (in 0.1 mM HCl; final concentration 10 μM) and ascorbic acid (0.5 mM final concentration) in a total volume of 500 μl. Incubation mixtures not containing iron and ascorbic acid were taken as blanks. Controls were run without scavenger. After 30 min at 37°C incubations were stopped by adding 233 μl 35% trichloroacetic acid. An aqueous solution of 1.5% thiobarbituric acid (233 μl) was added and the color was developed as before. Then 233 μl of 70% trichloroacetic acid was added and after cooling to room temperature the samples were extracted with 500 μl of chloroform and centrifuged. The absorbance of the aqueous phase was read at 532 nm. The microsomal protein content was determined by using the Lowry method as modified by Hartree.[21]

Formation and Detection of Superoxyl Radicals

The procedure of Beauchamp and Fridovich[22] was adapted. Incubation was carried out at 25°C in 1 ml *tris*/HCl buffer (50 mM; pH 7.5 containing 0.15 M KCl and

0.1 mM EDTA) containing xanthine at 0.1 mM, nitro-blue tetrazolium at 40 μM, scavenger at varying concentrations and 100 μl of diluted xanthine oxidase (7 μg). The rate of formazan formation, measured by the absorbance change at 560 nm between 5 and 10 min after addition of the enzyme, was determined in duplicate. From the control rate, in the absence of scavengers, a residual activity of 10% was subtracted, that was not inhibited by 10 μg (30 units) of superoxide dismutase. The presence of the scavengers up to 200 μM did not impair formation of urate from xanthine as determined by the increase in absorbance at 290 nm ($\Delta A_{290} = 0.0525 \pm 0.0009\,min^{-1}$ (mean $\pm$ S.D.; n = 10)). Nitro-blue tetrazolium was not reduced directly by the scavengers, which occurred with cytochrome c if this was used as an indicator.

Hydroxyl Radical-Dependent Formation of Malondialdehyde from 2-Deoxyribose

A modified version of the method described by Gutteridge[23] was used. The reaction was performed in ice-cold potassium phosphate buffer (pH 7.4, containing 40 mM sodium chloride and 0.1 mM EDTA). The solution contained in a total volume of 500 μl 0.5 mM 2-deoxyribose and scavenger at varying concentrations (0.05–0.5 mM). A solution of $FeSO_4$ in water was added rapidly to give a final Fe^{2+}-concentration of 0.1 mM. The samples (in duplicate) were left at 0°C for 15 min, and then 250 μl of each, 3% trichloroacetic acid and 1.5% thiobarbituric acid in water, were added and the colour developed (see lipid peroxidation). The absorbance was read at 532 nm (control: $A_{532\,nm} = 0.18 \pm 0.02$ (mean $\pm$ S.D.; n = 17)).

Reaction of Compound 1 or Trolox with O_2^- Generated by Xanthine/Xanthine Oxidase

In a total volume of 1 ml Krebs–Ringer phosphate buffer pH 7.4, prepared as described before,[24] 0.1 mM xanthine, 0.25 mM **1** or Trolox and 1.3 to 13 μg xanthine oxidase were incubated at 37°C in the absence or presence of 0.3 μg superoxide dismutase. For determining the amount of the 8a-hydroxychroman-6-one derivative formed the absorbance change at 250 nm (0–3 min; $\varepsilon = 12{,}000\,M^{-1}cm^{-1}$)[25] and for urate formation the absorbance change at 295 nm (0–10 min; $\varepsilon = 11{,}000\,M^{-1}cm^{-1}$)[26] were continuously recorded. At 250 nm the spectral change due to the conversion of xanthine to uric acid showed an isosbestic point. The $\Delta A_{250\,nm}$/min was linear only during the initial 3 min because the 8a-hydroxychroman-6-one derivative formed is hydrolysed under this condition.[25] In exchange of xanthine/xanthine oxidase, 1 to 10 μg glucose oxidase in the presence of 1 mM D-glucose was also used.

Reaction with hydrogen peroxide

The absorbance at 240 nm of a 13 mM solution of H_2O_2 in 50 mM sodium phosphate buffer pH 7.0 at 25°C was followed. 25 U of catalase were used as a positive control.

RESULTS

Prevention of Lipid Peroxidation

With rat heart microsomes (Fe-dependent lipid peroxidation) the new α-tocopherol analogs **1** ($IC_{50} = 0.17\,\mu M$) and **2** ($IC_{50} = 0.07\,\mu M$) were potent scavengers of

lipoperoxyl radicals (IC_{50} means concentration at which 50% inhibition is obtained). As for spontaneous lipid peroxidation of rat brain homogenate,[17] **2** was slightly more effective than **1**, may be due to its more pronounced lipophilic character.[17] Though qualitatively in agreement, the inhibitory concentrations of **1** and **2** determined with heart microsomes were about 10 times lower than in brain homogenate (**1**: IC_{50} = 1.7 μM; **2**: IC_{50} = 0.5 μM).[17] On the other hand, Trolox and D,L-penicillamine (rat brain homogenate IC_{50} = 12 ± 1 and 67 ± 7 μM, respectively; mean ± S.D., n = 3) were even less active in the microsomal preparation (IC_{50} = 75 μM and 270 μM, respectively). Ascorbic acid at 200 and 20 μM enhanced lipid peroxidation in the rat brain homogenate by about 10%.

Acetylation of the phenolic hydroxyl in **1** reduced the antioxidative potency (Fig. 2; IC_{50} = 15 μM). Part of the still-considerable activity was the consequence of a 1% impurity by the free phenol. For the other part, enzymatic cleavage of the acetate by an arylesterase present in the brain homogenate[27] may account, since in the presence of 1 mM of the esterase inhibitor phenylmethylsulfonyl fluoride the scavenging activity of O-acetylated **1** was further reduced by 44% (Figure 2). The inhibitor did not influence the IC_{50} obtained with **1**. Although **1** and **2** were at least 10 times more potent lipoperoxyl radical scavengers than vitamin E in this assay,[17] the free phenolic hydroxyl can be nevertheless assumed to be essential, as has been previously shown with α-tocopherol.[28]

Hydroxyl Radical Scavenging

It has been demonstrated that iron-dependent formation of malondialdehyde from 2-deoxyribose is mediated by OH·.[29] Relative rate constants of the scavengers (Table I) were based on the known bimolecular rate constant for the reaction of 2-deoxyribose with OH·: $k_{DR} = 1.9 \times 10^9\,M^{-1}\,sec^{-1}$.[30] The linear relationship $A_0/A = 1 + k_S/k_{DR}$ [(competitor]/[2-deoxyribose]) was used (A_0 and A are the absorbances in the absence and presence of competitor, respectively; k_S = rate constant for reaction of competitor with OH·)).

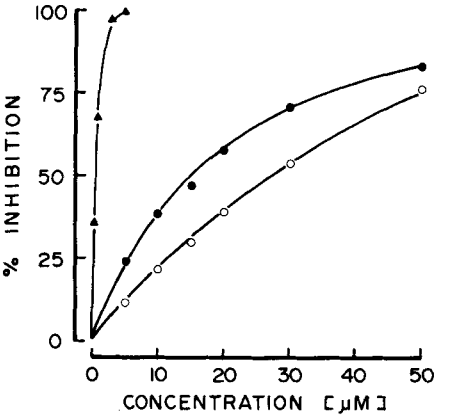

FIGURE 2 Prevention of spontaneous lipid peroxidation in rat brain homogenate by compound **1** and its O-acetyl derivative. Lipid peroxidation was inhibited by the free phenol (▲) and the O-acetyl form in the absence (●) or presence (○) of 1 mM of the esterase inhibitor phenylmethylsulfonyl flouride. Points represent means of duplicate determinations of thiobarbituric acid reactive material. The absorbance of the control samples was $A_{532\,nm}$ = 0.7.

TABLE I
Relative rate constants for reaction of hydroxyl radicals with different scavengers

Compound	$k_s[M^{-1}sec^{-1}] \times 10^{-9}$
1	9.5 ± 1.4 (5)*
1 (O-acetylated)	6.5 ± 0.8 (3)
2	5.3 ± 1.1 (5)
2 (O-acetylated)	2.4 ± 0.3 (3)*
Trolox	5.3 ± 1.1 (4)
N-2-mercaptopropionyl -glycine	3.6 ± 1.0 (4)*
D, L-Penicillamine	2.5 ± 0.2 (3)*
Thiourea	6.1 ± 0.5 (4)
D-Mannitol	1.1 ± 0.1 (3)*
L-Ascorbic acid	1.2 ± 0.1 (3)*

Values are means ± S.D.; number of independent determinations in parentheses.

*Significantly different from **1** (O-acetylated) as determined by ANOVA with multiple comparisons ($p \leq 0.05$).

1 is significantly different from all other compounds tested ($p \leq 0.05$).

Table I shows that **1** was the most active OH· scavenger used in this test. Acetylation of the phenolic oxygen, or N-demethylation to compound **2** diminished the scavenging efficacy. Trolox, thiourea, D-mannitol, D,L-penicillamine and N-2-mercaptopropionyl glycine were all less potent than **1** by up to about one order of magnitude. To see whether the amino groups reacted with OH·, trimethylamine and tetramethylammonium were tested. Both compounds revealed only weak reactivity, with $k_S < 10^8 M^{-1} sec^{-1}$.

Catalase at 130 U inhibited the decomposition of 2-deoxyribose by 60%. Incomplete inhibition may in part be due to the low temperature employed (0°C). The involvement of O_2^- in the formation of OH· has been discussed by Gutteridge *et al.*[31], and may account for the 25% inhibition observed with 100 U superoxide dismutase.

Superoxyl Radical Scavenging

Superoxyl radicals (O_2^-) were generated by xanthine/xanthine oxidase and detected by O_2^--dependent reduction of nitro blue tetrazolium to the diformazan dye.

As judged by the competition with nitro-blue tetrazolium the α-tocopherol analogs **1**, **2** and Trolox scavenged O_2^- with equal efficacy (Fig. 3). Their second order rate constants for reaction with O_2^- were similar to that communicated for the color reagent ($k_{NBT} = 6 \times 10^4 M^{-1} sec^{-1}$).[32] The O-acetylated **1** was only a weak competitor (7% inhibition at 200 μM). Its unexpected scavenging ability may be explained by a 1% contamination with the free phenol. D,L-Penicillamine and N-2-mercaptopropionyl glycine also were only marginally active (14% inhibition at 200 μM).

Formation of 8a-Hydroxychroman-6-one Intermediate

α-Tocopherol itself[9] and α-tocopherol analogs[25,33] are known to form the 8a-hydroxychroman-6-one derivatives upon oxidation in aqueous medium. Compound **1** as well

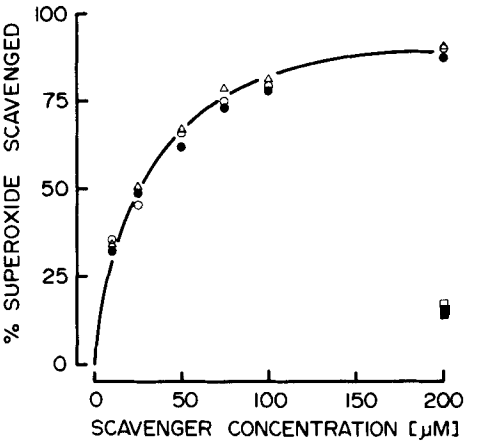

FIGURE 3 Superoxyl radical scavenging ability of α-tocopherol analogs and thiols. Superoxyl radicals generated from xanthine/xanthine oxidase were detected by reduction of nitro blue tetrazolium. Competition for superoxyl radicals was found with the α-tocopherol analogs **1** (●), **2** (○) and Trolox (△), and the thiol compounds D,L-penicillamine (□) and N-2-mercaptopropionyl glycine (■). Points represent means of duplicate determinations of rates of colour formation in the presence of scavengers. The rate in the absence of scavengers was: $\Delta A_{560\,nm}$/min = 0.0095 ± 0.0002 (mean ± S.D.; n = 6).

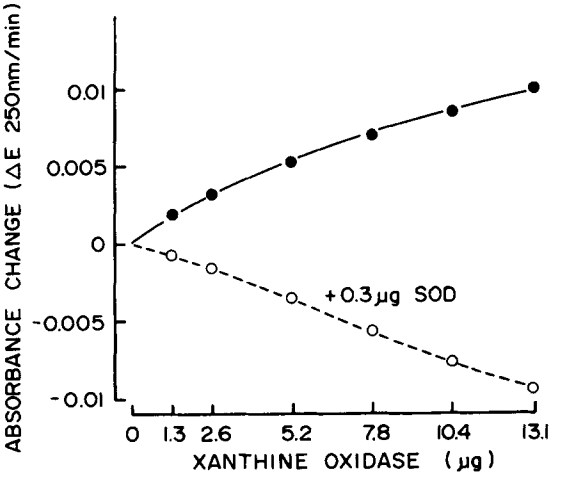

FIGURE 4 Superoxyl radical-dependent formation of the 8a-hydroxychroman-6-one form of compound **1**. An increase of absorbance in the absence of superoxide dismutase represents formation of the 8a-hydroxychroman-6-one (●). In the presence of superoxide dismutase (○) formation of a different oxidized species of compound **1** led to a decrease in absorbance. For details see Materials and Methods.

as Trolox, formed this intermediate in the presence of O_2^- generated by xanthine/xanthine oxidase (Figure 4). The simultaneous formation of urate, however, was faster than the appearance of the 8a-hydroxychroman-6-one derivative (not shown). This can be expected from the tendency of the latter to rapidly hydrolyze[25] and the fast spontaneous dismutation of the O_2^- formed. The 8a-hydroxychroman-6-one derivative, however, is not the only reaction product of O_2^- with α-tocopherol analogs.[34] The decrease in $A_{250\,nm}$ observed in the presence of superoxide dismutase (Figure 4) may, instead, reflect a more complicated situation. Oxidation of glucose by glucose oxidase in the presence of **1** did not lead to an increase in $A_{250\,nm}$. Compound **1** did not react with 13 mM H_2O_2, as evaluated by UV absorbance at 240 nm.

DISCUSSION

The water-soluble α-tocopherol analogs are potent scavengers of lipoperoxyl, hydroxyl and superoxyl radicals in vitro. While the efficacy of the lipophilic α-tocopherol depends on its intercalation into a tightly packed lipid bilayer, **1** and **2** may be able to react with free oxygen-derived radicals within an aqueous as well as a polar lipid phase, due to their amphiphilic nature.[17] Thus their effect could also be exerted via regeneration of endogenous vitamin E, as was previously shown with ascorbic acid or glutathione[35] under different circumstances. This mechanism, however, seems unlikely since ascorbic acid did not inhibit lipid peroxidation under our assay conditions. α-Tocopherol and its analogs form a resonance stabilized chromanoxyl radical.[8] As a consequence of steric hindrance by the orthomethyl groups it is less able to abstract hydrogen atoms from polyunsaturated fatty acids,[36] in addition to the OH bond-weakening effect of the para-substitution. This makes the α-tocopherol analogs effective radical chain breakers in contrast to compounds like D,L-penicillamine or N-2-mercaptopropionyl glycine, which form highly reactive thiyl radicals[37] or undergo autoxidation, forming O_2^- concomitantly.[38]

Substituents at the 2-position of the chromanol ring system influence the efficacy of the α-tocopherol analogs as lipoperoxyl radical scavengers. The weaker effect of Trolox as compared to **1** or **2** may be explained by an enhanced affinity of the amine substituted compounds for membranes which contain acidic lipids. A metal-ion chelating effect may be involved in addition. Such an effect is known to be associated with phenolic compounds containing amino groups.[39,40]

OH· reacts at high rates with many organic compounds.[30] Compound **1** revealed complete protection of 2-deoxyribose from OH·-dependent formation of malondialdehyde, with a greater relative second order rate constant for reaction with OH· than any other compound tested. Comparison of **1** with the tertiary amine and the O-acetyl derivatives demonstrates some participation of the quaternary ammonium group in the OH· scavenging activity. Hydrogen abstraction and radical formation seem to be favored by this substituent, though the higher reactivity could alternatively be ascribed to an association with the OH· precursor species. Trimethylamine and tetramethylammonium react with OH· at low rates, indicating that amino groups by themselves are not scavenging moieties. The thiol compounds were again less active in protection, probably because of secondary reactions of the thiyl radicals formed in the scavenging reaction.

Although compounds **1** and **2** *in vitro* scavenged free radicals with similar potency, the ammonium analog **1** was over 10 times more effective in reducing heart infarct size

in rats after coronary occlusion and subsequent reoxygenation.[17,18] The selective accumulation of **1** in heart tissue was thus of advantage under this condition. The data presented here support the concept that reperfusion injury following myocardial ischemia is caused by oxygen-derived reactive species. Beneficial effects of α-tocopherol analogs can also be expected in other conditions involving ischemia/reperfusion, as well as in inflammation.

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