Carotenoids

DOI: 10.1002/anie.200803668

3,3′-Dihydroxyisorenieratene, a Natural Carotenoid with Superior Antioxidant and Photoprotective Properties**

Hans-Dieter Martin, Sebastian Kock, Roger Scherrers, Kaya Lutter, Tanja Wagener, Claas Hundsdörfer, Susanne Frixel, Klaus Schaper, Hansgeorg Ernst, Wolfgang Schrader, Helmut Görner, and Wilhelm Stahl**

Carotenoids comprise a group of yellow to purple dyes widely distributed in the plant and animal kingdoms. To date, more than 750 carotenoids have been isolated. Not only do they serve as colorants, they are also efficient antioxidants and photoprotective agents that scavenge reactive oxygen species such as singlet oxygen and free radicals. Thus, they protect biologically important molecules from oxidative degradation. In vitro and in vivo studies demonstrate that natural carotenoids prevent UV-induced DNA damage and inflammation.

3,3′-Dihydroxyisorenieratene (DHIR, **1**), a carotenoid with an unusual structure bearing methylated phenolic end groups, was first isolated from the bacterium *Streptomyces mediolani*. It is also present in the membrane of *Brevibacterium linens*,[1] which is used in dairy industry for the production of various red smear cheeses such as Munster, Limburger, and Romadur cheeses.

We studied various carotenoids known to be excellent antioxidants with four different model systems. DHIR (**1**) proved to be superior to other carotenoids including astaxanthin, cryptoxanthin, and the macula lutea pigments zeaxanthin and lutein (for structural formulas see the Supporting Information).[2] The data suggest that **1** acts as a bifunctional radical scavenger owing to its polyenic and phenolic substructures. Its activity in singlet oxygen quenching is comparable to that of lutein and other polyenic carotenoids; **1** acts as a fast-quenching polyene and not like a slower-reacting phenol. Studies in more complex systems such as liposomes and human fibroblasts reveal that **1** prevents photo- and photooxidative damage. The compound inhibits UV-induced lipid oxidation, suppresses heme oxygenase-1 (HO-1) expresssion, and prevents the formation of thymidine dimers (eight assays are described in the Supporting Information).

Compound **1** was synthesized previously,[1a] but this approach did not provide sufficient amounts for extended analytical, antioxidant, and biochemical studies. Scheme 1 describes a new total synthesis of **1** and the corresponding quinone **2**,[3] which had not been fully characterized.[3] In the present synthesis protecting groups are not needed for the final Wittig reaction (**7** + **8** → **1**), and the product is obtained in 62% yield (for details and characterization see the Experimental Section and the Supporting Information).

Antioxidant capacities can be compared easily by using inhibition times determined by the cumene hydroperoxide inhibition assay introduced by Terao[4] and later modified.[2b,d,5] For lutein (Figure 1) an inhibition time of 20 min (2×10^{-4} M) was determined, whereas **1** is an outstanding antioxidant with an inhibition time of 107 min at the same concentration (Figure 1). Compound **1** can be considered an efficient hybrid or bifunctional antioxidant with characteristics between those of polyphenols and carotenoids. The special character of **1** becomes even more distinct when the inhibition times of other important carotenoids are plotted against their concentrations (see Figure 1 and also Figure 2 in the Supporting Information). The intermediary formation of quinone **2** is evident in this assay from the change in color from red to blue to yellow.

Compound **1** proved to be the best antioxidant in this series. Blocking of the phenolic hydroxy groups by methylation (dimethoxyisorenieratene in Figure 1) leads to distinct loss of antioxidant capacity. Thus, it is likely that oxidation of

[*] Prof. H.-D. Martin, Dr. S. Kock, Dr. R. Scherrers, C. Hundsdörfer, Dr. S. Frixel
Institute of Organic Chemistry and Macromolecular Chemistry, Natural Compounds, and Photoprotection
Heinrich-Heine-University Düsseldorf
Universitätsstrasse 1, 40225 Düsseldorf (Germany)
Fax: (+49) 211-811-4324
E-mail: martin@uni-duesseldorf.de
wilhelm.stahl@uni-duesseldorf.de

K. Lutter, T. Wagener, Prof. W. Stahl
Institute of Biochemistry and Molecular Biology I
Heinrich-Heine-University Düsseldorf
Universitätsstrasse 1, 40225 Düsseldorf (Germany)

Dr. K. Schaper
Institute of Organic Chemistry and Macromolecular Chemistry, Organic Photochemistry, Heinrich-Heine-University Düsseldorf

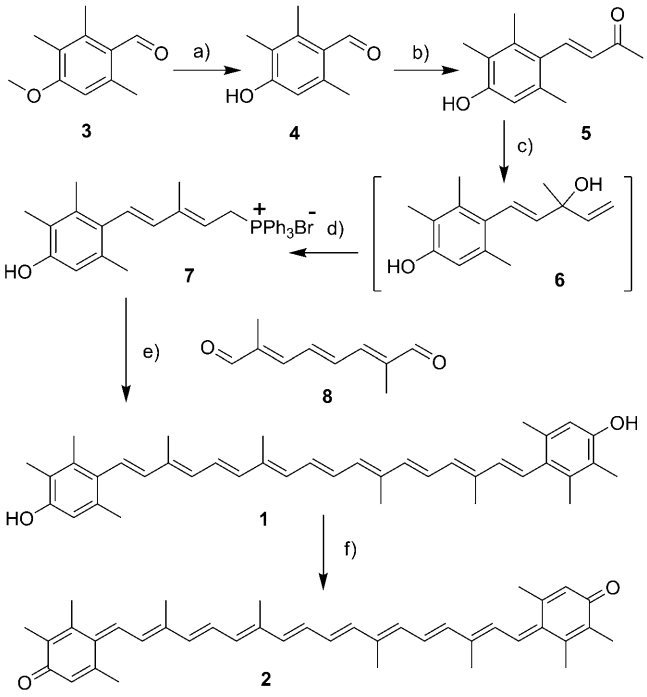
Dr. H. Ernst
GVF/A, BASF SE
67056 Ludwigshafen (Germany)

Dr. W. Schrader
Max-Planck-Institut für Kohlenforschung
45470 Mülheim an der Ruhr (Germany)

Dr. H. Görner
Biophysicalische Chemie
Max-Planck-Institut für Bioanorganische Chemie
45413 Mülheim an der Ruhr (Germany)

[**] The present work has been performed as a project of the SFB 663 (B1) at the Heinrich-Heine-University Düsseldorf and is printed at its instigation with financial support provided by the Deutsche Forschungsgemeinschaft.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200803668.



Scheme 1. a) BBr_3, 0 °C, CH_2Cl_2; b) (triphenylphosphaniumyl)propan-2-on-1-ide, 110 °C, toluene; c) vinylmagnesium bromide, −5 °C, THF; d) triphenylphosphane hydrobromide, 0 °C, CH_2Cl_2; e) 1,2-epoxybutane, reflux, ethanol; f) Ag_2CO_3, acetone. (The full synthesis is described in the Supporting Information.)

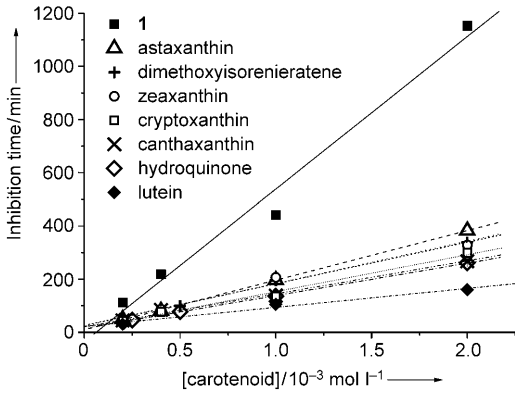


Figure 1. Plot of inhibition time of cumune hydroperoxide formation against concentration. The antioxidant capacity decreases from the top (**1**, ■) to the bottom (lutein, ◆) of the sequence shown. The concept of induction period, inhibition times, and chain-breaking antioxidants is further explained in the Supporting Information.[5]

the phenolic groups to give the conjugated quinoid system **2** is an additional option in **1** not feasable in typical nonphenolic carotenoids.

We conducted a second assay, in which the dependence of the rate of oxygen consumption on the carotenoid concentration and partial pressure of oxygen is determined.[2b,d] This assay has its origin in studies of Burton and Ingold,[6] and allows a deeper insight into the oxygen consumption, the influence of concentration, and the possible appearance of prooxidative effects (Figure 2).

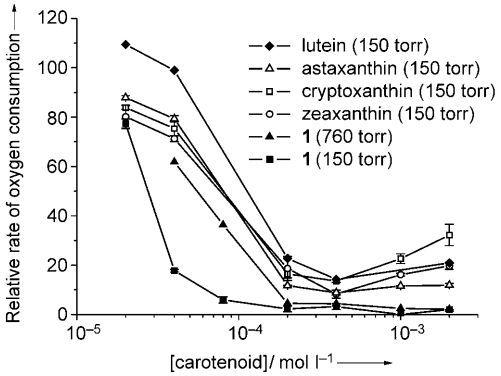


Figure 2. Dependence of the relative rate of oxygen consumption on carotenoid concentration and partial pressure of oxygen at 200 hPa (150 torr) and 1013 hPa (760 torr) for **1**.[2b,d,6] No prooxidation is seen for **1** even at 1013 hPa (760 torr) partial pressure of oxygen.

Comparing the data of the established antioxidants cryptoxanthin, zeaxanthin, lutein, and particularly astaxanthin, with that of **1** several features are apparent: Compound **1** gives rise to a much steeper decline in the rate of oxygen consumption and a deeper minimum around zero, and the rate does not recover (even at 760 Torr (1013 hPa), see Figure 2) as with the three other carotenoids even at 150 Torr (200 hPa). There is no evidence that **1** has prooxidant behavior.[6]

A third assay, which was developed by Re et al.,[7] measures the activity against non-oxygen-free radicals. The ABTS assay (see the Supporting Information for details) records the dependence on concentration and reaction time of the antioxidant on the reduction of $ABTS^{\bullet+}$ radical cations.[8] The results of this assay are given as TEAC (trolox equivalent antioxidant capacity for specific times) and/or RAA(AUC) (relative antioxidant activity as area under curve for the whole time range).[7] Results for **1** are: $TEAC_{1\,min} = 1.46$, $TEAC_{5\,min} = 2.48$, $RAA(AUC)_{5\,min} = 1.86$ (cf. RAA(AUC) of the phenol derivatives α-tocopherol (0.90)[7] and luteolin (1.49),[7] and of the polyenes β-carotene (2.50),[7] lycopene (3.04),[7] and lutein (1.35); mean ≈1.9 Figure 3). Phenols yield lower values which may increase somewhat over time, and carotenoids show higher values from the start. Compound **1** behaves as a phenol at the start and then as a polyene after minutes as a result of its bifunctionality. For comparison, the mean of approximately 1.9 of the TEAC/RAA(AUC) of the five above-mentioned compounds is similar to the RAA(AUC) of DHIR **1.** Lutein is less active, possibly because it has one conjugated double bond less (Figure 3).

The fourth assay probes the activity against damaging species in excited states, that is, $O_2(^1\Delta_g)$ singlet oxygen.[9,2a,c,d] Most carotenoids quench singlet oxygen by a mechanism that involves electronic energy transfer from a $^1(^1\Delta{\cdot}S_0)$ encounter complex that deactivates irreversibly by internal conversion to an $^1(^3\Sigma{\cdot}T_1)$ encounter complex.[9c,d] It was of interest to check whether **1** uses predominantly phenolic or polyenic quenching behavior. The rate constant (k_q) of quenching of singlet molecular oxygen was determined as follows: $O_2(^1\Delta_g)$ is formed upon generation of the triplet state of acridine as

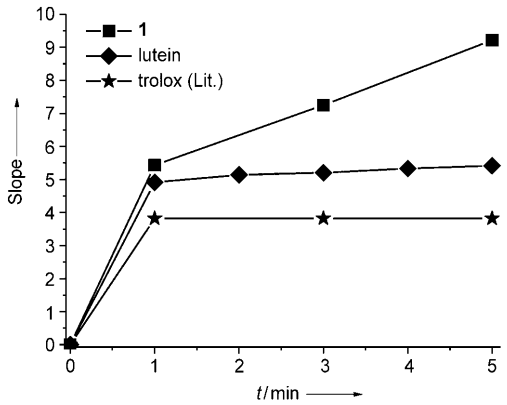


Figure 3. Plotting the percentage inhibition of absorbance of $ABTS^{\bullet+}$ at 734 nm[7,8] versus concentration yields a slope, which when plotted as a function of time of reaction allows the calculation of the area under the curve (AUC). The ratio between the area under the curve (AUC) for the reaction of the specific antioxidant and that for trolox gives the relative antioxidant activity RAA(AUC),[7] trolox $RAA(AUC)_{[5min]}$: 1.00 (by definition), lutein: $RAA(AUC)_{[5min]}$: 1.35, $\mathbf{1}_{[5min]}$: 1.86.

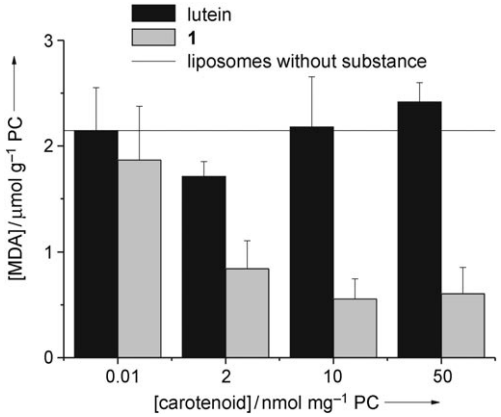


Figure 4. MDA (malondialdehyde) levels in liposomes loaded with **1** or lutein after irradiation with UV-A light (27.5 J cm^{-2}).

the sensitizer ($\lambda_{exc} = 308$ nm) and quenching of the triplet by oxygen. The lifetime of singlet oxygen ($\tau_s = 1/k_S$) is solvent dependent, and in dichloromethane τ_S is 0.03 ms. On addition of a quencher, the rate constant (k_S) increases with quencher concentration. The molar absorption coefficient of **1** in dichloromethane is $\varepsilon_{466} = 1.3 \times 10^5\,M^{-1}\,cm^{-1}$. Analysis of the data with the Stern–Volmer relation (Figure 4 in the Supporting Information) yields $k_q(DHIR) = 9.7 \times 10^9\,M^{-1}\,cm^{-1}$. Compared with other carotenoids (usual range $k_q \approx 1 \times 10^9$–$10^{10}\,M^{-1}\,cm^{-1}$; $k_q(lutein) = (12 \pm 4) \times 10^9\,M^{-1}\,cm^{-1}$)[9,2a,c,d] and phenols ($k_q \approx 1 \times 10^6\,M^{-1}\,cm^{-1}$)[10] this is evidence that **1** uses its polyene chain for efficient quenching and not the slower-quenching phenolic subunits.[10] The appearance of quinone **2**[3] in the first assay (see above) can be followed visually, but whether **2** plays a role in other assays remains unclear.

To determine the effects of **1** against photooxidation a liposomal model was applied (for details see the Supporting Information). Compound **1** and, for comparison, lutein were incorporated into multilamellar liposomes composed of egg yolk phospholipids. Photooxidation was initiated with UV light (UV-A or UV-B light), and the decomposition product malondialdehyde (MDA) was determined as a measure of oxidative lipid damage. The result of a typical experiment is shown in Figure 4. Upon irradiation of control liposomes with UV-A light at 27.5 J cm^{-2}, about 2.1 µmol MDA per mg phospholipids forms (black line). When liposomes are loaded with increasing amounts of **1** (0.01–50 nmol mg^{-1} phospholipids) MDA formation decreases significantly to about 0.5 µmol MDA per mg phospholipids, indicating antioxidant activity. With lutein, only a moderate inhibition of lipid oxidation was found. Protective effects of **1** were also observed at doses of 10 J cm^{-2} UV-A light and less pronounced at 0.5 and 1.5 J cm^{-2} UV-B light. The bifunctional structure of **1**, providing UV absorption and radical-scavenging properties, is also in this system likely responsible for the superior activity of the compound. HPLC analyses revealed that **1** was more stable than lutein under UV irradiation. UV-protective properties of **1** were further investigated in cell culture with human dermal fibroblasts (ATCC-CRL-2076); again lutein was applied for comparison. None of the carotenoids was cell toxic (with and without UV irradiation) as proven with the sulforhodamine B assay.[11] Cellular UV-A response was determined by analyzing the expression of the enzyme heme oxygenase 1 (HO-1) at the protein level by Western blot analyses. Following UV-A-induced photooxidation, HO-1 expression is increased. Compared to the irradiated control (20 J cm^{-2}), HO-1 levels were significantly lowered by about 30% when the cells were preincubated with 1.5 µM **1** for 24 h prior to irradiation. In contrast, no photoprotection was found when the cells were pretreated with similar amounts of lutein.

The formation of cyclobutane pyrimidine dimers is a major mechanism of DNA damage in tissues exposed to UV-B light. When human dermal fibroblasts are exposed to UV-B light (300 mJ cm^{-2}) thymidine dimers are formed and can be visualized with specific antibodies (Figure 5 A). If the cells are preincubated for 24 h with **1** less dimer formation is observed (Figure 5 B) than in solvent controls. Quantitative evaluation showed that the formation of thymidine dimers in the presence of **1** is 25% lower than in the control; lutein had no effect (Figure 6). Since the generation of cyclobutane

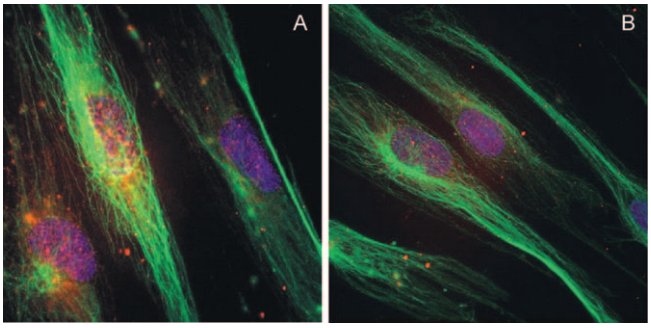


Figure 5. Thymidine dimer (immunostaining, red) formation in human skin fibroblasts; control (A), preincubated with **1** (B). DNA (DAPI staining, blue), cytoskeleton-β-tubulin (immunostaining, green). DAPI = 4′,6-diamidino-2-phenylindole.

pyrimidine dimers is a photochemical reaction of DNA bases and not related to photooxidation it is likely that UV-B-absorbing properties of **1** are responsible for the effect.

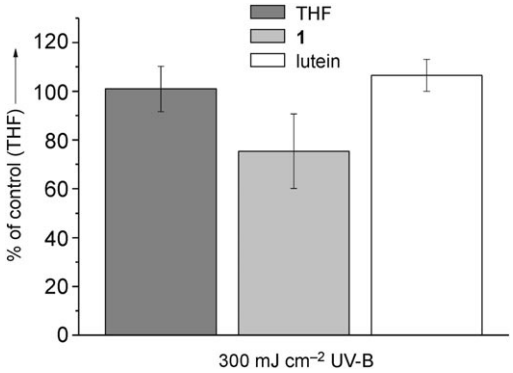


Figure 6. The formation of thymidine dimers in human skin fibroblasts preincubated with **1** or lutein; control: THF.

In summary, the natural phenolic carotenoid **1** is distinguished by its outstanding antioxidative and photoprotection properties. In eight independent assays we found **1** superior to other carotenoids, for example, lutein, the main carotenoid in the human macula lutea. Use and application of **1** and its oxidation product, quinone **2**, as natural colorants for food and feed, in cosmetics, and as antioxidants or photoprotecting agents are under investigation. These compounds may also be useful in the prevention of degenerative diseases such as age-related degeneration of the macula lutea.

Experimental Section

Full procedures, general methods, characterization data and structural formulas of all compounds are given in the Supporting Information.

Important data of **1** and **2**: **1**: Yield: 62%; m.p. 228–230°C; UV/Vis (THF): λ_{max} (ε) = 280 (25970), 466 nm (130000); HRMS (Finnigan MAT95/70 eV, EI): calcd for $C_{40}H_{48}O_2$: 560.365057; found: 560.365431. **2**: Yield: 61%; m.p. > 250°C; Vis (*n*-hexane): λ_{max} (ε) = 548 nm (151000); HRMS (ESIpos): calcd for $C_{40}H_{46}O_2Na$: 581.339270; found: 581.338995.

Received: July 28, 2008
Published online: November 25, 2008

Keywords: carotenoids · photooxidation · photoprotection · quinones · singlet oxygen

[1] a) F. Arcamone, B. Camerino, E. Cotta, G. Franceschi, A. Grein, S. Penco, C. Spalla, *Experientia* **1969**, *25*, 241–242; b) W. Kohl, H. Achenbach, H. Reichenbach, *Phytochemistry* **1983**, *22*, 207–210; c) L. Dufosse, M. C. de Echanove, *Food Res. Int.* **2005**, *38*, 967–973; d) F. Guyomarćh, A. Binet, L. Dufosse, *J. Ind. Microbiol. Biotechnol.* **2000**, *24*, 64–70; e) L. Dufosse, *Food Technol. Biotechnol.* **2006**, *44*, 313–321.

[2] a) D. Baltschun, S. Beutner, K. Briviba, H. D. Martin, J. Paust, M. Peters, S. Röver, H. Sies, W. Stahl, A. Steigel, F. Stenhorst, *Liebigs Ann.* **1997**, 1887–1893; b) H. D. Martin, C. Jäger, C. Ruck, M. Schmidt, R. Walsh, J. Paust, *J. Prakt. Chem.* **1999**, *341*, 302–308; c) H. D. Martin, C. Ruck, M. Schmidt, S. Sell, S. Beutner, B. Mayer, R. Walsh, *Pure Appl. Chem.* **1999**, *71*, 2253–2262; d) S. Beutner, B. Bloedorn, S. Frixel, I. Hernández-Blanco, T. Hoffmann, H. D. Martin, B. Mayer, P. Noack, C. Ruck, M. Schmidt, I. Schülke, S. Sell, H. Ernst, S. Haremza, G. Seybold, H. Sies, W. Stahl, R. Walsh, *J. Sci. Food Agric.* **2001**, *81*, 559–568.

[3] G. Nybraaten, S. Liaaen-Jensen, *Acta Chem. Scand.* **1971**, *25*, 370–372.

[4] J. Terao, *Lipids* **1989**, *24*, 659–661.

[5] The concept of induction period, inhibition times, and chain-breaking antioxidants is described in G. W. Burton, D. O. Foster, B. Perly, T. F. Slater, I. C. P. Smith, K. U. Ingold, *Philos. Trans. R. Soc. London Ser. B* **1985**, *311*, 565–578.

[6] a) G. W. Burton, K. U. Ingold, *Science* **1984**, *224*, 569–573; b) G. W. Burton, *J. Nutr.* **1989**, *119*, 109–111.

[7] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.

[8] S. Hünig, H. Balli, H. Conrad, A. Schott, *Justus Liebigs Ann. Chem.* **1964**, *676*, 36–51.

[9] a) P. F. Conn, W. Schalch, T. G. Truscott, *J. Photochem. Photobiol. B* **1991**, *11*, 41–47; b) P. di Mascio, S. Raiser, H. Sies, *Arch. Biochem. Biophys.* **1989**, *274*, 532–538; c) R. Schmidt, *J. Phys. Chem. A* **2004**, *108*, 5509–5513; d) C. Schweitzer, R. Schmidt, *Chem. Rev.* **2003**, *103*, 1685–1757.

[10] a) C. R. Lambert, H. S. Black, T. G. Truscott, *Free Radical Biol. Med.* **1996**, *21*, 395–400; b) C. Tournaire, S. Croux, M. T. Maurette, I. Beck, M. Hocquaux, A. M. Braun, E. Oliveros, *J. Photochem. Photobiol. B* **1993**, *19*, 205–215; c) V. Avila, S. G. Bertolotti, S. Criado, N. Pappano, N. Debattista, N. A. Garcia, *Int. J. Food Sci. Technol.* **2001**, *36*, 25–33; d) S. Beutner, B. Bloedorn, T. Hoffmann, H. D. Martin, *Methods Enzymol.* **2000**, *319*, 226–241.

[11] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, M. R. Boyd, *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.

