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Natural and newly synthesized hydroxy-1-aryl-isochromans: A class of potential antioxidants and radical scavengers

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Abstract

We investigated the antioxidant and radical scavenging activity of polyphenolic isochromans. To assess the relation between structure and scavenging properties the natural occurring 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochroman (ISO-3, three OH groups) was compared with three newly synthesized derivatives that differ in their degree of hydroxylation by substitution with methoxy-groups (ISO-4: four OH groups; ISO-2: two OH groups and ISO-0: fully methoxylated). We found that ISO-4 is a 2-fold better scavenger for the artificial radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, 100 μM) with an EC_{50} = 10.3 μM compared to the natural ISO-3 (EC_{50} = 22.4 μM) and to ISO-2 (EC_{50} = 25.1 μM), while ISO-0 did not react with DPPH. The scavenging capacity for superoxide ($O_2^{\cdot-}$) enzymatically generated in a hypoxanthin-xanthinoxidase reaction was the highest for ISO-4 (EC_{50} = 34.3 μM) compared to those of ISO-3 (EC_{50} = 84.0 μM) and ISO-2 (EC_{50} = 91.8 μM), while ISO-0 was inactive. In analogy, ISO-4 scavenged peroxynitrite ($ONOO^-$, EC_{25} = 23.0 μM) more effective than ISO-3, ISO-2 and ISO-0.

When C6 rat glioma cells loaded with the reactive oxygen/nitrogen (ROS/RNS)-sensitive fluorochrome 2,7-dichlorodihydrofluorescein, were exposed to hydrogen peroxide, the lowest stress level as indicated by the fluorescence signal was detected when the cells were pretreated with ISO-4 or ISO-2 but to a much lesser extent with ISO-3, while ISO-0 did not show any effect. All tested hydroxyisochromans superceded the scavenging effect of trolox.

The excellent radical and ROS/RNS scavenging features of the hydroxy-1-aryl isochromans and their simple synthesis let these compounds appear to be interesting candidates for pharmaceutical interventions that protect against the deleterious action of ROS/RNS.

Keywords: *Hydroxyisochromans, reactive oxygen species, hydrogen peroxide, reactive nitrogen species, peroxynitrite, astrocytes*

Abbreviations: *DCF, 2,7-dichlorofluorescein; DMSO, dimethyl sulfoxide; DMSO-d_6, hexadeutero dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; H_2DCF, 2,7-dichlorodihydrofluorescein; H_2DCF-DA, 2,7-dichlorodihydrofluorescein diacetate; H_2O_2, hydrogen peroxide; HT, hydroxytyrosol; ISO-0, 1-(3′,4′-dimethoxy)phenyl-6,7-dimethoxy-isochroman; ISO-2, 1-(3′4′-dimethoxyphenyl)-6,7-dihydroxy-isochroman; ISO-3, 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochroman; ISO-4, 1-(3′,4′-dihydroxy)phenyl-6,7-dihydroxy-isochroman; MeOH, methanol; NaOONO, sodium peroxynitrite; NBT, nitroblue tetrazolium; $O_2^{\cdot-}$, superoxide anion radical; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species*

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Introduction

A new class of isochromans (ISO), the so-called hydroxy-1-aryl-isochromans, was recently identified by Bianco et al. [1] in extra-virgin olive oil. In particular, the presence of two representatives of this class namely 1-phenyl-6,7-dihydroxy-isochroman and 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochroman (ISO-3, see Figure 1) was found in olive oil as demonstrated by high performance liquid chromatography—mass/mass spectroscopy (HPLC-MS/MS). Evidence is mounting that such polyphenols when consumed with olive oil may play a particular role in the regional low incidence of coronary heart disease (CHD) [2,3]. Only a few other plant and fungal species have, so far, been described to contain isochromans [4,8]. These substances are also synthetically accessible since Guiso et al. reported a facile high-yield synthesis of isochroman derivatives by the acid-catalyzed *oxa*-Picted-Spengler reaction starting from hydroxytyrosol (HT, see Figure 2) [9,10].

Based on the commonly accepted beneficial antioxidant effects of polyphenols and their proposed role in the mediterranean diet, these natural substances or their synthetic derivatives are of interest as potential protectants against oxidative/nitrosative stress caused by free radicals and other reactive oxygen/nitrogen species (ROS/RNS).

Excessive ROS production, like hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$) or their metabolites is known to contribute to many cardiovascular and neurological pathologies that range from aging to heart infarctation and stroke [11,12]. Potentially hazardous levels of ROS may arise either from overstimulation of enzymes like NAD(P)H oxidases or from a deregulation of the mitochondrial electron transport resulting in free radical leakage from mitochondria [13,14]. ROS-generating pathways involve also enzyme-mediated lipid auto-oxidation, prooxidant activities of toxins and exposure to ionizing radiation [15]. Another form of cellular stress, the so-called nitrosative stress, is based on the RNS production such as the radical nitric oxide (NO) and its metabolite peroxynitrite ($ONOO^-$) that is formed by the reaction of NO with $O_2^{\cdot -}$ [16]. $ONOO^-$ is a molecule that can cause damage to proteins and DNA, an effect which is involved in the pathogenesis of many human diseases, including neurodegeneration and inflammation, as well as cancer, diabetes, arthritis, and asthma (for review see Ref. [17,18]).

Therefore the quest for potent antioxidative/antinitrosative drugs is of great interest to the pharmaceutical, cosmetic and nutritional supplement industry [19,20].

The aim of this study is to assess the potential antioxidative activities of different isochroman derivatives. For this purpose, we used the synthesis described by Guiso et al. [9] to generate the naturally occurring isochroman ISO-3 that is contained in olive oil. It was our goal to investigate the dependence of the radical scavenging capacity on the aromatic backbone structure activation by the hydroxylation degree of the isochromans.

Based on theoretical considerations that aromatic structures are activated by phenoxyl radical forming phenolic OH groups [21], we hypothesized that the tetrahydroxy substituted derivative ISO-4 (1-(3′,4′-dihydroxy)phenyl-6,7-dihydroxyisochroman, Figure 1) possesses the highest scavenging activity and that a successive blocking of the OH groups by methoxylation leads to a step-wise loss in activity. Therefore, we additionally synthesized the new derivatives ISO-4, ISO-2 and ISO-0 (Figure 1) that differ in the number of OH groups substituted by methoxylation and we included these compounds in our scavenging assays.

The efficacy of the ISO test compounds to scavenge pathophysiologically-relevant free radicals and reactive oxygen or nitrogen species (ROS/RNS) like the highly reactive molecules $O_2^{\cdot -}$, H_2O_2, and $ONOO^-$ was assessed in comparison to classical antioxidants like trolox, a water-soluble vitamin E derivative, and ascorbic acid.

Materials and methods

Reagents

Hydroxytyrosol (HT, 2-(3′,4′-dihydroxy)phenylethanol) was prepared by reducing 3,4-dihydroxyphenylacetic acid methylester with sodium borohydride ($NaBH_4$) according to a protocol of Bianco et al. [22].

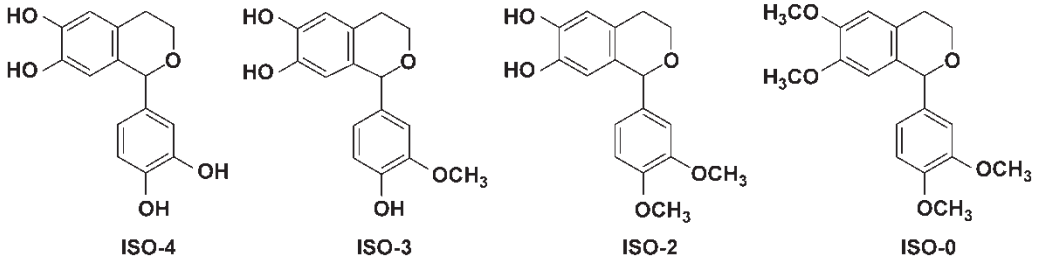


Figure 1. Chemical structures of the investigated isochromans.

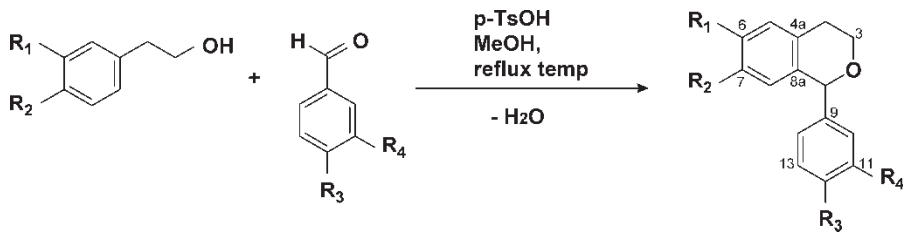


Figure 2. Synthesis of the isochroman derivatives by condensation of hydroxytyrosol ($R_1 = R_2 =$ OH) and an aromatic aldehyde via an acid-catalyzed *oxa*-Picted-Spengler reaction (*p*-TsOH = *p*-toluene sulfonic acid). The numbering inside the isochroman formula corresponds with the C-atom numbering of the NMR data (see: results; analytical and spectral data).

Hypoxanthin was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Xanthinoxidase (XOD, grade I from buttermilk), pyrogallol red (pyrogallolsulfonephthalein), and nitroblue tetrazolium (NBT) were purchased from Sigma (Steinheim, Germany). Manganese (IV) oxide (activated, <5 μm, ca. 85%) and trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carbonic acid) were obtained from Sigma–Aldrich (Steinheim, Germany), 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) from Molecular Probes Europe BV (Leiden, Holland). The other chemicals were obtained at analytical grade from Merck (Darmstadt, Germany) or Sigma–Aldrich (Taufkirchen, Germany).

Analytical and spectral analysis of the substances

The purity of the analytes, their mass spectrometric identification and UV–vis spectra were assessed by a HPLC–UV–MS/MS system. From a solution of the compounds (0.1 mg/ml) in methanol (MeOH)/water (3/6 v/v) were 10 μl samples injected into the HPLC–UV–MS/MS system. The HPLC system consisted of a Agilent 1100 system (Waldbronn, Germany), equipped with a binary gradient pump and an diode-array UV–vis detector. The chromatographic separation was achieved by a Merck Superspher 100 RP18e 125 × 4 mm column. The two-stage mass spectrometric (MS/MS) analysis was accomplished by a Finnigan LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, USA) equipped with an atmospheric pressure chemical ionization ion source. The substances were eluted from the HPLC-column with a formic acid buffer–MeOH gradient starting with 40% MeOH/water (v/v). The MeOH fraction increased to 70% within 10 min. The total runtime was 12 min. Full scan mass spectra of the HPLC-eluate were recorded during the runtime, resulting in the signals of the $[M + H]^+$ ions of the analytes. To gain structural information, these ions were trapped and fragmented to yield the precursor-product pattern of the analytes. The nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) on a VARIAN-INOVA-500 spectrometer. Chemical shifts are reported in δ (ppm), and the solvent was used as an internal reference; DMSO-d_6 (^{1}H: $\delta = 2.50$; ^{13}C = 39.43).

Synthesis of the hydroxy-1-aryl-isochromans (ISO-4, ISO-3, ISO-2)

The hydroxy-1-aryl-isochromans were synthesized via a reaction of hydroxytyrosol (HT) with the selected aromatic aldehyde (Figure 2) [9]. In brief, 1.45 g (9.4 mmol) HT was dissolved in MeOH (70 ml) together with the appropriate aromatic aldehyde (9.4 mmol) in presence of a catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH, 200 mg). The reaction mixture was kept at reflux temperature (64°C) for 3–6 h. After chromatographic control by thin layer chromatography (TLC, solid phase: Merck silica 60 plates; liquid phase: chloroform/MeOH (9/1 v/v) the solvent was removed by rotaevaporation and the residue purified over vacuum liquid chromatography (VLC) on a silica column (78 g TLC grade Merck silica 60, preconditioned with chloroform), by eluting with chloroform/MeOH (9/1 v/v) under vacuum suction. The fractions which contained the pure compounds were unified, the solvent was distilled off and the residue dried under vacuum. The isolated compounds were found to be = 95% pure by HPLC and NMR.

Synthesis of the 1-(3′,4′-dimethoxyphenyl) -6,7-dimethoxyisochroman (ISO-0)

A mixture of 3.00 g (16.46 mmol) 2-(3,4-dimethoxyphenyl)ethanol and 2.73 g (16.46 mmol) 3,4-dimethoxybenzaldehyde, 146 ml MeOH and *p*-TsOH (400 mg) was refluxed for 5 days. The reaction product was purified as described above by VLC (TLC grade Merck silica 60, preconditioned with 2-methylbutane, gradient 2-methylbutane/ethylacetate = 5/1 to 1/1.5, v/v). The yielded raw material was further purified by recrystallization from ethylacetate.

Preparation of isochroman solutions for the antioxidant/radical scavenging assays

Stock solutions of all synthesized isochromans (25, 50, 100, 200, 300 mM), herein called test compounds, were prepared in dimethyl sulfoxide (DMSO). For cell culture experiments the solutions were sterilized by passing them through a 0.22 μm Nalgene nylon filter. Stock solutions were kept as aliquots at − 20°C until use.

DPPH radical scavenging capacity

The DPPH assay was performed as described earlier [23]. In brief, the test compounds or vehicle (DMSO) were added to 2.0 ml of methanolic 1,1-diphenyl-2-picrylhydrazyl (DPPH, 100 μM) and incubated for 30 min at 37°C. The absorbance at $\lambda_{max} = 516$ nm (A_{516}) was measured using a Perkin-Elmer UV–vis spectrophotometer (λ_2). The EC_{50} values were calculated from the resulting absorbance curves for each test compound by regression analysis. Scavenging activities were expressed as 50% of inhibitory concentration (EC_{50}) value, which denotes the concentration of the test compound (μM) required to give a 50% reduction in A_{516} relative to that of the control, whereby a high scavenging capacity is indicated by a low EC_{50} value. The efficacy of common radical scavengers like ascorbic acid or trolox were also tested in our assays to enable a comparison with that of the isochromans.

$O_2^{\cdot-}$ *scavenging capacity*

The $O_2^{\cdot-}$ scavenging activity was determined by a modified procedure that was described by Furuno et al. using an enzymatic $O_2^{\cdot-}$ generation that was visualized via a reaction with NBT yielding a colored formazan product [24]. The assay is based on a competition for $O_2^{\cdot-}$ that leads to the effect that the stronger the scavenging capacity of the test compound is, the lower is the amount of NBT that is converted into the blue formazan product. In brief, 3 μl of the various test compounds in DMSO solution was added to 1.0 ml of 50 mM Tris buffer at pH 7.4 containing 54 μM hypoxanthin and 45.1 μM NBT. One micro liter of xanthinoxidase (XOD) at a final concentration of 34.2 mU/ml was added to the mixture to initiate the $O_2^{\cdot-}$ generation. The mixture was incubated at 37°C for 10 min and the formazan absorption was measured at $\lambda_{max} = 560$ nm (A_{560}) against blank samples which did not contain the enzyme. DMSO did not interfere with the assay system in the concentration range that was used, up to a final concentration of 0.3%. The formazan formation was measured 10 min after addition of XOD. The EC_{50} values of the different isochroman derivatives were determined by absorption analysis at five different concentrations of each test compound to obtain the concentration-inhibition curves. A minimum of 3 independent sets of experiments were measured in duplicates ($n = 6$).

Preparation of sodium peroxynitrite (NaOONO) solution

Sodium peroxynitrite solution (NaOONO) has been prepared according to Hughes et al. [25] To an ice-cooled solution of 0.6 M $NaNO_2$ (20 ml) and 1.2 M NaOH (20 ml) a hydrochloric acid solution of hydrogen peroxide (0.6 M H_2O_2 in 0.7 M HCl, 20 ml) was added drop-wise under stirring. The yellow-colored NaOONO solution was then treated with manganese(IV) oxide (MnO_2, activated, 200 mg) to remove the residual H_2O_2 and filtered through a 0.22 μm Nalgene nylon filter. $ONOO^-$ concentration was determined spectrophotometrically at $\lambda_{max} = 302$ nm (A_{302}; $\varepsilon_{max} = 1670\ mol^{-1}\,l\,cm^{-1}$) [26]. The desired $ONOO^-$ concentration (10 mM) was prepared fresh by dilution of the stock solution with ice-cooled aqueous 1.0% NaOH. The $ONOO^-$ solution was kept in an ice bath until use.

$ONOO^-$ scavenging assay using pyrogallol red bleaching

The $ONOO^-$ scavenging capacity of the isochromans was determined using a modified protocol described by Balavoine et al. [26].

A test cuvette was charged with 2.997 ml of pyrogallol red solution (32 μM) in phosphate buffered saline (27.55 mM K_2HPO_4, 40.99 mM K_2HPO_4, pH 7.4) and 3 μl of the test compound (final concentrations: 25, 50, 100 or 200 μM) in DMSO solution. Subsequently, 5 aliquots (5 μl each) of 10 mM NaOONO solution (final concentration 66.2 μM) were added to the mixture. The cuvette was sealed by Parafilm, turned over 5 times, and the absorbance at $\lambda_{max} = 541$ nm (A_{541}; $\varepsilon_{max} = 32,467\ l.mol^{-1}.cm^{-1}$) was measured against the blank after each NaOONO addition. The total shift of pH, due to an addition of NaOONO solution was within 0.1 units, while the change of the volume was within 1.0%. The addition of the vehicle without NaOONO treatment was set to 0% bleaching. The pyrogallol red absorption after the addition of five NaOONO aliquots without the addition of the test compound was set to 100% bleaching.

EC_{25} values were calculated from the portion of the dose/effect graph with linear slope to enable the drug effectiveness.

Intracellular oxidative stress assay

Rat glioma cell cultures (C6) were chosen to test the efficiency of the isochromans to reduce intracellular oxidative stress. The cells were seeded at 1×10^6 cells/ml in 35-mm petridishes with coverslips. The cultures were maintained for 1 day *in vitro* (DIV) as described previously [23] in RPMI 1640 medium (Gibco, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% filtered fetal calf serum, penicillin (5 IU/ml), streptomycin (5 μg/ml) and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 atmosphere.

The fluorescence intensity of the fluorochrome 2,7-dichlorofluorescein (DCF) was measured as an indicator of the intracellular oxidative stress level [23,27]. The cultures were co-incubated for 30 min with 50 μM H_2DCF-DA with either one of the test

compounds (100 μM) in Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 15 mM Hepes, and 10 mM D-glucose, pH 7.3). The cultures were washed with Locke's solution, mounted in a steel chamber (Attoflour) on an inverted microscope, and covered with 1 ml of Locke's solution. Experiments were conducted using a Zeiss (Jena, Germany) laser scanning microscope (LSM 410 Axiovert, Zeiss 40 × oil lense; excitation 488-nm argon laser, emission, 515-nm long pass filter). Laser attenuation, pinhole diameter, photomultiplier sensitivity, and offset were kept constant for every set of experiments. For continuous monitoring of DCF fluorescence, a time series of images was started and H_2O_2 (200 μM) was added to the cells after 3 min of baseline fluorescence collection. Fluorescence intensity was monitored at an interval of 30 s for a total period of 14.5 min. The fluorescence intensity data, obtained as average intensity within boxes drawn over the somata of individual cells, were quantitatively analyzed using the Zeiss LSM software. Fluorescence values were normalized to the H_2O_2 pretreatment levels. The DCF fluorescence increase was calculated as area % of control (DMSO and H_2O_2 treated cultures) over baseline.

Statistical analysis

All quantitative data are shown as mean ± SEM. Gaussian distribution of the data was determined by a completely randomized one-way ANOVA (drug × dosage) that was followed by a Tukey/Kramer *t* test if appropriate. Probability (*p*) values less than 0.05 were considered to be significant. EC_{50} and EC_{25} values were obtained by regression analysis using the Microcal Origin program, version 4.10 (Microcal Software Inc., Northampton, MA, USA).

Results

Chemistry

For the preparation of the known natural isochroman ISO-3 we successfully applied the synthesis reported by Guiso et al. [9]. Here we also report for the first time the synthesis of three novel isochromans ISO-4, ISO-2 and ISO-0. The prepared isochromans did not show optical activity when measured ($[\alpha]_D^{25}$ 0° (c 0.98, MeOH) indicating that the compounds are racemates.

Analytical and spectral data

The assignment of the following NMR spectral data of the novel synthesized isochroman derivatives (Figure 2) was based on comparison with already published data of similar compounds [9,10]. Correlation of the NMR chemical shifts based also on ^{1}H, ^{13}C-COSY NMR experiments (data not shown). Isochromans contain carbon atoms that are called *ipso* carbon atoms (positions 4a, 8a and 9, see Figure 2).

Because *ipso* carbon atom shift correlations are usually uncertain to assign, the 'ACD/NMR Predictor' program, version 7.0 (ScienceServe GmbH, Pregnitz, Germany) was used for assignment of these shifts.

1-(3′,4′-Dihydroxy)phenyl-6,7-dihydroxy-isochroman (ISO-4). Yield: 85.3%; TLC (Rf): 0.21; Mp: 177–179°C; UV–vis (λ_{max}, MeOH): 285 nm (log $\varepsilon_{3.822}$); ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.80 (s, 1H, OH), 8.77, (s, 1H, OH), 8.64 (s, 1H, OH), 8.59 (s, 1H, OH), 6.67 (d, 1H, $\mathcal{J}$ = 7.9 Hz, H-13), 6.57 (s, 1H, H-10), 6.54 (d, 1H, $\mathcal{J}$ = 8.2 Hz, H-14), 6.48 (s, 1H, H-5), 6.06 (s, 1H, H-8), 5.33 (s, 1H, H-1), 3.95 and 3.67 (2H, m, 2H-3), 2.78 and 2.52 (2H, m, 2H-4); ^{13}C NMR (DMSO-d_6, 126 MHz): δ 144.76, 143.83, 143.09 (C6, C7, C11, C12, one signal not observed), 133.87 (C8a), 128.31 (C4a), 123.82 (C9), 119.73 (C14), 115.78 (C10), 114.85 (C13), 114.77 (C5), 113.33 (C8), 78.06 (C1), 62.84 (C3), 27.52 (C4). $[M + H]^+ = 275.0$; fragmentation ions: 256.9 $[M + H - H_2O]^+$, 164.7, 137.1 and 123.0.

1-(3′-Methoxy-4′-hydroxy)phenyl-6,7-dihydroxyisochroman (ISO-3). Yield: 66.2%; TLC (Rf): 0.42; Mp.: 154–156°C; UV–vis (λ_{max}, MeOH): 285 nm (log ε 3.728). The ^{1}H- and ^{13}C-NMR data were consistent with those of the literature [9]. $[M + H]^+ = 289.0$; fragmentation ions: 271.2 $[M + H - H_2O]^+$, 165.0 and 137.2.

1-(3′,4′-Dimethoxy)phenyl-6,7-dihydroxyisochroman (ISO-2). Yield: 45.8%; TLC (Rf): 0.58; Mp.: 156–158°C; UV–vis (λ_{max}, MeOH): 283 nm (log ε 3.689), ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.68 (s, 1H, OH), 8.59 (s, 1H, OH), 6.90 (d, 1H, $\mathcal{J}$ = 8.3 Hz, H-13), 6.83 (s, 1H, H-10), 6.78 (d, 1H, J = 7.8 Hz, H-14), 6.51 (s, 1H, H-5), 6.05 (s, 1H, H-8), 5.45 (s, 1H, H-1), 4.00 and 3.71 (m, 2H, 2H-3), 3.74 (3H, s, OCH_3), 3.70 (3H, s, OCH_3, overlapped with H-3 signal), 2.84 and 2.53 (2H, m, 2H-4). ^{13}C NMR (DMSO-d_6, 126 MHz): 148.41, 148.24 (C12, C11), 143.90, 143.13 (C6, C7), 135.30 (C8a), 127.99 (C4a), 123.81 (C9), 120.78 (C14), 114.98 (C5), 113.20 (C8), 111.99 (C10), 111.12 (C13), 78.13 (C1), 63.18 (C3), 55.39 (OCH_3), 55.36 (OCH_3), 27.45 (C4). $[M + H]^+ = 303.3$; fragmentation ions: 288.0 $[M + H - CH_3]^+$, 285.0 $[M + H - H_2O]^+$, 164.9, 151.0 and 137.0.

1-(3′,4′-Dimethoxy)phenyl-6,7-dimethoxyisochroman (ISO-0). Yield: 49.6%; TLC (Rf): 0.94; Mp.: 80–81°C; UV–vis (λ_{max}, MeOH): 282 nm (log ε 3.806);

^{1}H NMR (DMSO-d_6, 500 MHz): δ 6.90 (1H, d, $\mathcal{J}$ = 8.3 Hz, H-13), 6.85 (1H, d, $\mathcal{J}$ = 1.34 Hz, H-10), 6.76 (1H, s, H-5), 6.74 (1H, dd, $\mathcal{J}$ = 1.7, 8.3 Hz, H-14), 6.25 (1H, s, H-8), 5.58 (1H, s, H-1), 3.98 and 3.74 (2H, m, 2H-3), 3.74 (6H, s, 2 OCH_3, overlapped with H-3 signal), 3.70 (3H, s, OCH_3), 3.52 (3H, s, OCH_3), 2.89 and 2.68 (2H, m, H-4); ^{13}C NMR (DMSO-d_6, 126 MHz): 148.40, 148.30, 147.50, 146.71 (C11, C6, C12, C7), 134.76 (C9), 128.80 (C4a), 125.75 (C8a), 120.88 (C14), 112.08 (C10), 111.68 (C5), 111.03 (C13), 109.99 (C8), 77.62 (C1), 62.26 (C3), 55.43 (OCH_3), 55.35 (OCH_3), 55.14 (2OCH_3), 27.66 (C4). $[M + H]^+ = 331.1$; fragmentation ions: 313.2 $[M + HH_2O]^+$, 192.9, 165.2 and 151.2.

Hydroxy-1-aryl-isochromans are effective radical scavengers

To assess the general radical scavenging capacity of the isochromans we used an assay that monitors their reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH), a moderately stable artificial radical. The investigated isochroman derivatives demonstrated to be effective scavengers for DPPH as indicated by the concentration dependent decrease in the absorbance of the radical in a cell free assay (Figure 3). There was a linear inverse proportional relationship between the decrease in absorbance of the DPPH and the concentrations of the drugs ($p < 0.01$). ISO-4 was found to be most effective compared to the other tested isochromans ($EC_{50} = 10.37\ \mu M$). ISO-3 displayed a lesser activity than ISO-4 and was slightly more effective than ISO-2 ($EC_{50} = 22.45$ and $25.17\ \mu M$, $p < 0.01$). When the phenolic OH groups were completely substituted by methoxylation ($-OCH_3$), i.e. in the test compound ISO-0, no scavenging activity was detected. In comparison, known strong radical scavengers, such as ascorbic acid or trolox, displayed EC_{50} values of 23.60 and $24.34\ \mu M$ (Table 1). Also resveratrol (*trans*-3,4′,5-trihydroxystilbene), another polyphenolic radical scavenger, [23] have shown a moderate reactivity with DPPH in our assay ($EC_{50} = 29.31\ \mu M$, data not shown). However, resveratrol was excluded from the other assays because of precipitation problems in aqueous solution at higher concentrations. Interestingly, ISO-4 was revealed in this test as an even better scavenger (2.4-fold stronger, $p < 0.01$) for DPPH than ascorbic acid and trolox.

ISO-4 is an effective $O_2^{\cdot-}$ scavenger

To investigate the ability of the isochromans to scavenge $O_2^{\cdot-}$ we applied an NBT reduction assay. Our results show that ISO-4 most strongly inhibited the NBT formazan production (see Figure 4, Table 1). At an ISO-4 concentration of 100 μM more than 90% of the $O_2^{\cdot-}$ was scavenged ($EC_{50} = 34.37\ \mu M$, $p < 0.01$), whereas the isochromans ISO-3 and ISO-2 were less effective ($EC_{50} = 84.02$ and $91.84\ \mu M$). ISO-0 and trolox were little or not effective in reducing the NBT formazan absorption. Ascorbic acid was not used for comparison in this assay because it is considered to be a reducing agent for NBT [28].

Hydroxy-1-aryl-isochromans prevent pyrogallol red bleaching caused by $ONOO^-$

When NaOONO (66.2 μM) was added to pyrogallol red solution (32 μM), without the test compounds

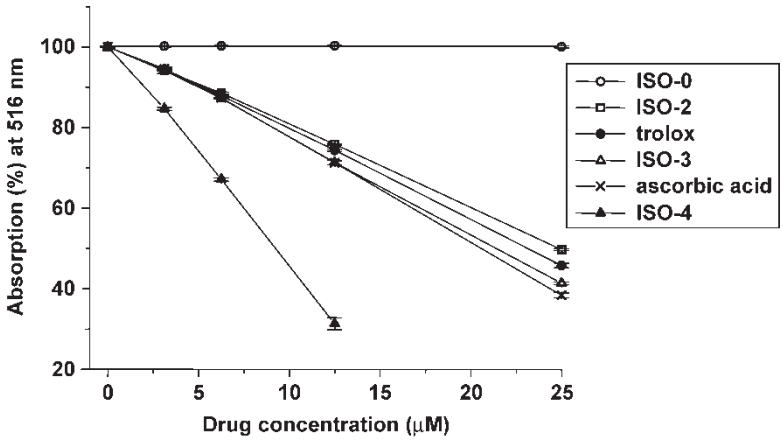


Figure 3. ISO-4 scavenges the model radical DPPH at a higher rate than ascorbic acid, ISO-3, trolox and ISO-2. DPPH spectrophotometric assay is based on the quenching effect of radical scavengers on the absorbance of DPPH. The tested compounds (3.125, 6.25, 12.5 and 25 μM) were incubated with DPPH (100 μM) for 30 min at 37°C in a MeOH solution. ISO-4 ($EC_{50} = 10.37 \pm 0.27\ \mu M$) shows the strongest scavenging effect against DPPH free radicals in comparison to ISO-3 and ISO-2 ($EC_{50} = 22.45 \pm 0.77$ and $25.17 \pm 0.47\ \mu M$). The proportional decrease in absorbance was compared with that of the blank and the data normalized to that of the control (vehicle = 100%). The vehicle (DMSO) only slightly altered the DPPH absorbance. Data represent means ± SEM of three sets of experiments in duplicates ($n = 6$). The figure displays only the linear portion of the graphs. A one-way ANOVA shows that the addition of the drugs, except that of ISO-0, causes a significant decrease in absorbance at all concentrations measured ($p < 0.01$).

Table 1. Radical (DPPH), superoxide ($O_2^{\cdot-}$) and peroxynitrite ($OONO^-$) scavenging activity of the isochroman derivatives.

Test compound	DPPH* EC_{50} (μM)	$O_2^{\cdot-}$ † EC_{50} (μM)	$OONO^-$ ‡ EC_{25} (μM)
ISO-4	10.37 ± 0.27	34.37 ± 0.61	23.01 ± 5.24
ISO-3	22.45 ± 0.77	84.02 ± 1.86	45.26 ± 10.56
ISO-2	25.17 ± 0.47	91.84 ± 4.32	53.59 ± 22.70
ISO-0	⩾ 250	no effect	no effect
Trolox	24.34 ± 0.86	⩾ 1200	29.76 ± 5.60
Ascorbic acid	23.60 ± 1.74	–¶	3.21 ± 0.01

* Radical scavenging activity was measured by use of 100 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to Lorenz et al. [23].
† Superoxide anion ($O_2^{\cdot-}$) scavenging activity was determined using a xanthin-xanthinoxidase-NBT reduction assay modified according to a protocol of Furuno et al. [24].
‡ Peroxynitrite scavenging activity measured using a pyrogallol red bleaching assay [26].
¶ Not tested because of NBT reduction.

(vehicle only), a strong bleaching effect was observed, whereby the solution turned yellow. The absorption of this solution was set to 100% bleaching. The presence of ascorbic acid decreased the bleaching of pyrogallol red to 10.5% of control already at a concentration of 25 μM (Figure 5, $p < 0.01$).

The ranking of the tested compounds in their effectiveness to inhibit the absorbance decrease of the pyrogallol red solution after NaOONO treatment was: ascorbic acid ($EC_{25} = 3.21$ μM) > ISO-4 ($EC_{25} = 23.01$ μM) > trolox ($EC_{25} = 29.76$ μM) > ISO-2 ($EC_{25} = 53.59$ μM) > ISO-3 ($EC_{25} = 45.26$ μM; $p < 0.01$). Again, ISO-0 was unable to inhibit the bleaching reaction.

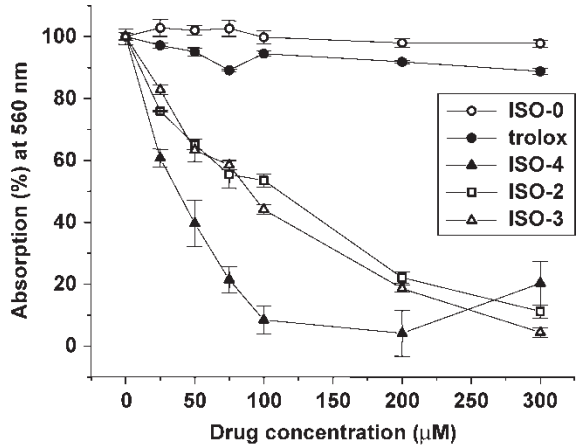


Figure 4. Analysis of the $O_2^{\cdot-}$ scavenging capacity of the isochromans. $O_2^{\cdot-}$ was generated by an enzymatic reaction of hypoxanthin and xanthinoxidase (XOD). The assay is based on the spectrophotometric detection of formazan formation due to the reaction of $O_2^{\cdot-}$ with NBT in competition with the added test compounds. The tested drugs were incubated at different concentrations (25, 50, 75, 100, 200 or 300 μM) with hypoxanthin (54 μM), NBT (45.1 μM) and XOD (34.2 mU/ml) in Tris-buffered solution (10 min, 37°C). Control samples (DMSO) showed a maximum absorption due to the reduction of NBT forming the blue formazan product. ISO-4 most strongly inhibited the NBT formation ($EC_{50} = 34.37$ μM) compared to ISO-3 and ISO-2 ($EC_{50} = 84.02$ and 91.84 μM). Trolox and ISO-0 did not inhibit the NBT reduction. Data represent means ± SEM of three sets of experiments for each treatment, measured in duplicates ($n = 6$). *The increase in the absorption observed for ISO-4 at 300 μM is due to the precipitation of the compound.

Isochromans are potent scavengers for intracellular hydrogen peroxide

To assess the general antioxidant capacity of the isochroman derivatives, C6 rat glioma cell cultures preloaded with H_2DCF-DA (50 μM for 30 min) were treated with H_2O_2 (200 μM) and the resulting increase in DCF fluorescence was measured (Figure 6). An increase to 697% over basal levels (100%) in the DCF fluorescence signal was seen in cultures that were only pretreated with vehicle (Figure 6B). Cell cultures pretreated with hydroxyisochromans (100 μM, 30 min) showed the lowest rise in the DCF fluorescence signal (ISO-4 and ISO-2 > ISO-3). Trolox, which was used for comparison, had a less pronounced effect. ISO-0 showed a somewhat weaker increase in the fluorescence, but the endpoints of the curve did not differ from that of the vehicle treated cultures.

We calculated the areas under the curves (between control or treatment graphs and the baseline) within a time interval of 0 and 720 s as a measure for the drug effectiveness. The area under the curve of the control group (H_2O_2 + vehicle) was set to 100% (see insert, Figure 6B). ISO-4 and ISO-2 reduced this area to 30 ± 0.94% and 31 ± 0.82% of control ($p < 0.01$). ISO-3 diminished the fluorescence increase to a lesser extent, to 60 ± 1.76% ($p < 0.01$) of control, similar to that pattern seen with trolox (65 ± 1.58% of control). ISO-0 reduced the area under the curve only to 81 ± 1.36% of control ($p < 0.01$).

Discussion

In the search for new antioxidants and potential radical scavengers as protectants against oxidative/nitrosative stress we focused here on the class of the hydroxy-1-aryl-isochromans. These compounds are hypothesized to have beneficial health effects. One representative, ISO-3, was recently found in natural

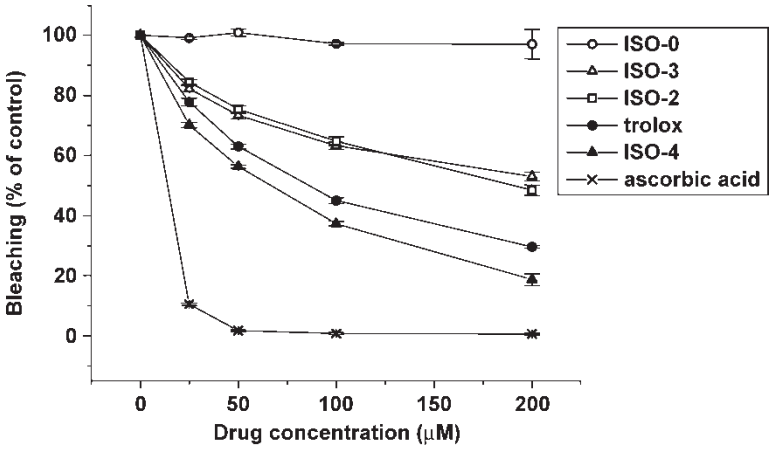


Figure 5. $ONOO^-$ scavenging assay: Bleaching of pyrogallol red (32 μM) by sodium peroxynitrite (NaOONO) was measured by absorption in the presence of the test compounds (25, 50, 100 and 200 μM) at room temperature. The absorption was recorded after each of five subsequent additions of 5 μl aliquots of NaOONO (10 mM) to the reaction mixture (final concentration: 66.2 μM). ISO-4 (EC_{25} = 23.01 μM) most effectively inhibited the pyrogallol red bleaching reaction and showed a higher activity than trolox (EC_{25} = 29.76 μM). ISO-3 and ISO-2 were less effective (EC_{50} = 45.26 and 53.59 μM). ISO-0 was ineffective to scavenge NaOONO, whereas ascorbic acid almost totally abolished the $ONOO^-$ caused bleaching at the lowest concentration (25 μM). Data represent means ± SEM of four sets of experiments for each treatment ($n = 4$).

matrices like extra virgin olive oil [1]. Moreover, ISO-3 and some other isochroman derivatives from olive oil, were also shown to inhibit human platelet activity [29]. Although one would expect that these substances are potential antioxidants due to their polyphenolic backbone structure, little is known about their antioxidative and antinitrosative properties. We used the natural product ISO-3 as lead structure to synthesize, besides ISO-3 itself, three novel isochroman derivatives referred as ISO-4, ISO-2 and ISO-0, with varying hydroxylation degree but identical substitution pattern (Figure 1). The synthesized solid substances were soluble and stable in aqueous solutions, although ISO-0 and, quite unexpected, ISO-4 precipitated at higher concentrations (>200 μM).

Investigating the general scavenging properties by testing the isochroman derivatives with the model radical DPPH we show that ISO-4, which contains four phenolic OH groups in the molecule, is the superior scavenger among the tested substances, even more potent than the natural compound ISO-3.

The scavenging capacity of ISO-4 was 2.4-fold higher than that of the as very potent considered antioxidants trolox and ascorbic acid (Figure 3). The successive blocking of the phenolic OH groups by methoxylation resulted in a lower activity. Nevertheless, ISO-3 and ISO-2 that contain three or two phenolic OH groups, respectively, still displayed a high scavenging activity which was similar to that of trolox and ascorbic acid (Table 1 and Figure 3). As expected, the complete methoxylated isochroman ISO-0 did not react with DPPH ($EC_{50} \gg 250$ μM) at all under our assay conditions. On the other hand, it was shown before that the isochroman molecule itself is nonetheless able to react with radicals such as NO, although, this study was not performed under physiological conditions [30]. The data of our DPPH-assay indicate that at least one phenolic OH group is necessary to activate the isochroman backbone structure enabling it to react with free radicals.

The finding that the tested isochromans are good scavengers of the model radical DPPH lead us to investigate their scavenging potency for biologically relevant ROS/RNS, namely that for $O_2^{\cdot-}$, $ONOO^-$ and H_2O_2.

$O_2^{\cdot-}$ is, among other sources, a by-product of the mitochondrial respiration [31]. It is formed under conditions of oxygen deficiency such as ischemia/reperfusion [32,33] but also at particularly high oxygen concentrations and plays a keyrole, e.g. in lipid peroxidation [34,35]. Oxygen radicals like $O_2^{\cdot-}$ and also hydroxyl radicals ($^{\cdot}OH$), which are formed from $O_2^{\cdot-}$ by the Haber-Weiss reaction [36], can alter lipid, protein, and DNA structures, thereby possibly contribute to the development or exacerbation of many human diseases [34] including ischemia-reperfusion injury in heart attacks [37], stroke [38], cancer, various inflammatory-immune injuries and disorders of aging [39], as well as in several neurodegenerative disorders [40].

We used a colorimetric assay, the reaction of NBT with $O_2^{\cdot-}$ to form a stable blue formazan product, as a probe to detect the generated $O_2^{\cdot-}$ in the presence and absence of the test compounds [24]. Our results demonstrate that ISO-4 competes most strongly with NBT for reacting with $O_2^{\cdot-}$ (Figure 4). At a concentration of 50 μM ISO-4, the NBT-formazan product formation decreased to more than 60% of control, while ISO-3 and ISO-2 inhibited this reaction

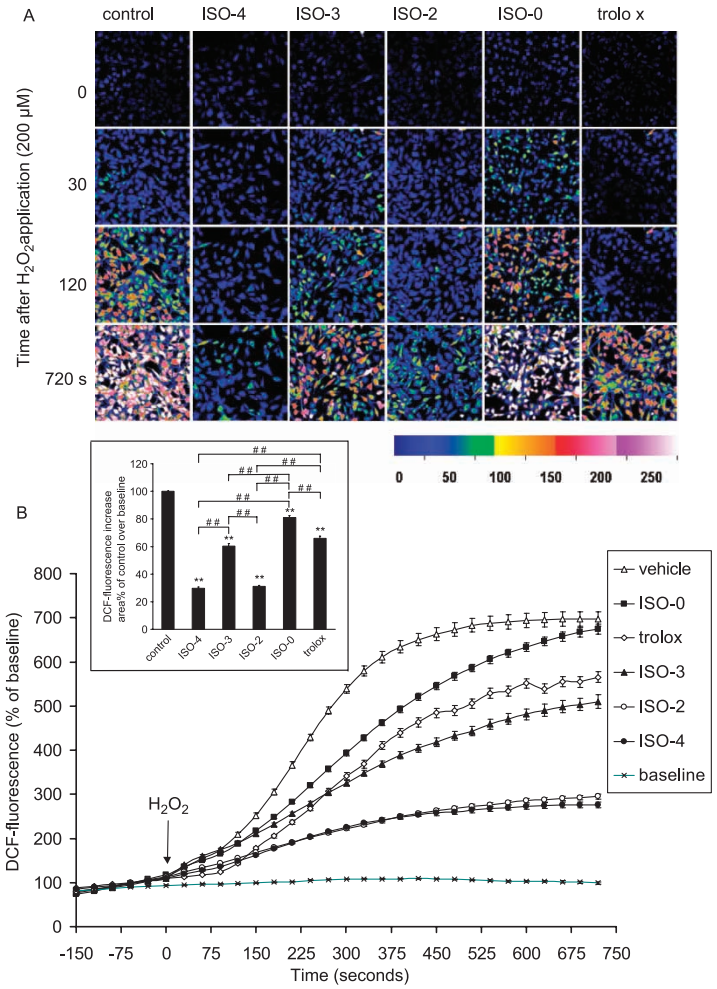


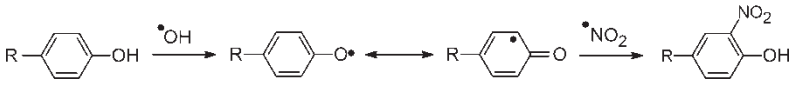
Figure 6. Fluorimetric analysis of oxidative stress induced by H_2O_2 (200 µM) application in rat C6 glioma cell cultures (1 DIV). H_2DCF-DA preloaded cells (50 µm, 30 min) showed a strong increase in DCF-fluorescence upon exposure to H_2O_2. Pretreatment with ISO-4 or ISO-2 (100 µM) almost completely abolished the increase in the fluorescence signal. The observed effect was much stronger than that of equimolar amounts of ISO-3 or trolox. ISO-0 (100 µM) displayed only a weak activity compared to control cultures. (A) Representative confocal images at different time points. (B) Quantitative analysis of averaged single cell fluorescence intensities over time. The crossed (x) line indicates the basal (pretreatment) fluorescence level that was set to 100%. Data represent means ± SEM of four sets of experiments, for each treatment averages were determined from a total of 80 cells. The insert shows the DCF-fluorescence increase calculated as the area under the curve in area% over baseline. (**$p < 0.01$, Tukey/Kramer t test corrected for number of probes compared; ##$p < 0.01$ as compared with the corresponding control (vehicle) group, one-way ANOVA).

to a lesser extent (both approx. 37%). However, extensive methoxylation of the isochroman structure resulted in a complete loss of the antioxidant properties as demonstrated by ISO-0 that was unable to react with $O_2^{\cdot-}$. Interestingly, trolox displayed only a very weak scavenging capacity for $O_2^{\cdot-}$ (8% inhibition at 50 µM) in comparison to the isochromans. This finding is contradicting to previous reports that describe vitamin E derivatives, like trolox, as excellent scavengers for $O_2^{\cdot-}$ in *in vitro* systems [41].

Furthermore, we tested if the selected isochromans react with $ONOO^-$. $ONOO^-$ is a highly reactive nitrogen species and is formed as an adduct of $O_2^{\cdot-}$ and NO. $ONOO^-$ is one of the main factors of the so-called 'nitrosative stress' and can cause deleterious effects by nitration of tyrosin residues in proteins and as a strong oxidant leading to altered or lost biological functions, that may comprise a proapoptotic signal [42]. Evidence for nitration of tyrosine as an indicator for $ONOO^-$ formation has been described e.g. in multiple sclerosis [43,44], cerebral ischaemia [32], oxygen/glucose deprivation [45] and also in the blood–brain barrier damage [46].

To investigate the potential $ONOO^-$ scavenging capacity of the isochromans we used a pyrogallol red bleaching assay [26]. We could demonstrate that isochroman ISO-4 displays a high reactivity towards $ONOO^-$ (Figure 5, Table 1). At a concentration of

Scheme 1.



Scheme 2.

100 μM it reduced the pyrogallol red bleaching to a stronger extent (to 36%) than equimolar trolox (to 45%). ISO-3 and ISO-2 inhibited the bleaching to 65% of the control level indicating a lesser potency to scavenge $ONOO^-$, while ISO-0 was completely inactive in this assay. Only in the case of the $ONOO^-$ scavenging assay, the efficacy of ISO-4 was superceded by another antioxidant, namely ascorbic acid (Figure 5, Table 1). The high reactivity of ascorbic acid with $ONOO^-$ was previously shown by others [26].

The scavenging capacity of the isochromans towards $ONOO^-$ might be explained by the reaction with the intermediate nitrogen dioxide radical ($^{\cdot}NO_2$) which is formed by the decomposition of $ONOO^-$ [47], see scheme 1.

Polyphenols like hydroxyisochromans are described to react with $^{\cdot}NO_2$ by formation of phenoxyl radicals via reaction with the simultaneously present $^{\cdot}OH$ [47,48], see scheme 2.

Hence, phenols like hydroxyisochromans, are easily nitrated even at physiological pH. This may lead to the assumption that the presence of hydroxyisochromans in a physiological environment provides a competitor for the nitration reaction that otherwise potentially alters biological molecules such as tyrosin residues.

Based on our finding that the tetra methoxylated ISO-0 does not react with $ONOO^-$ we confirm that methoxy substituents deactivate the aromatic system for nitration reactions. That was shown by Zhan et al. in analogy for the reaction of melatonin and demethoxylated melatonin with $ONOO^-$ [49].

Our results which demonstrate the excellent scavenging activity of the hydroxyisochromans for free radicals and ROS/RNS prompted us to test also the ability of the compounds to react with intracellular H_2O_2. Under physiological conditions H_2O_2 is mainly formed from $O_2^{\cdot-}$ via reaction of superoxide dismutases (SOD's) [21]. We used H_2O_2 applications in rat C6 glioma cell cultures as a biological *in vitro* model for oxidative stress. The resulting intracellular oxidative stress levels were detected by the measurement of the DCF fluorescence increase in H_2DCF-preloaded cells. Our study shows that both, ISO-4 and ISO-2 strongly reduce the H_2O_2-induced fluorescence increase to 30% of the levels observed in control cultures (Figure 6A, B). Surprisingly, the natural compound ISO-3 caused only half of the protection as measured by the DCF-fluorescence in comparison to the effect of ISO-4 and ISO-2. The unexpected difference in the effects of the test compounds may not only result from their chemical properties but may also depend on the intracellular bioavailability which possibly differs due to their methoxylation degree and their lipophilicity. The effect of ISO-3 did not significantly differ from that of trolox, indicating that the antioxidative effect of both compounds seems to be comparable. ISO-0 was much less active and reacted only weakly with the intracellular H_2O_2 in the C6 glioma cells.

Taken together, the investigated isochromans are promising compounds for antioxidant strategies. Depending on the hydroxylation degree these compounds display a high affinity to reactive molecule species like ROS/RNS, whereas methoxy substituents (OCH_3) decrease the reactivity.

Based on our data including the good water solubility of the hydroxyisochromans in pharmacologically concentrations let these compounds appear as interesting lead structures. Our results call for further studies that follow up on the antioxidative/antinitrosative properties of these substances in disease models.

Acknowledgements

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