2,3-Diarylxanthones as strong scavengers of reactive oxygen and nitrogen species: A structure–activity relationship study

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A B S T R A C T

Xanthones are a class of oxygen-containing heterocyclic compounds widely distributed in nature. The natural derivatives can present different substitutions in the xanthone core that include hydroxyl, methoxyl, prenyl and glycosyl groups. The inclusion of aryl groups has only been reported for a few synthetic derivatives, the 2,3-diaryl moiety being recently introduced by our group. Xanthones are endowed with a broad spectrum of biological activities, many of them related to their antioxidant ability, including the scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as metal chelating effects. Considering the interesting and promising antioxidant activities present in compounds derived from the xanthone core, the main goal of this work was to evaluate the scavenging activity of the new 2,3-diarylxanthones for ROS, including superoxide radical (O2•−), hydrogen peroxide (H2O2), singlet oxygen (1O2), peroxyl radical (ROO•) and hypochlorous acid (HOCl), and RNS, including nitric oxide (NO) and peroxynitrite anion (ONOO−). The obtained results revealed that the tested 2,3-diarylxanthones are endowed with outstanding ROS and RNS scavenging properties, considering the nanomolar to micromolar range of the IC50 values found. The xanthones with two catechol rings were the most potent scavengers of all tested ROS and RNS. In conclusion, the new 2,3-diarylxanthones are promising molecules to be used for their potential antioxidant properties.

1. Introduction

Xanthones are a class of oxygen-containing heterocyclic compounds widely distributed in nature. They occur in two major plant families, Guttiferae and Gentianaceae and in some families of fungi and lichens. These natural derivatives can present different substitutions in the xanthone core that include hydroxyl, methoxyl, prenyl and glycosyl groups. The inclusion of aryl groups has only been reported for a few synthetic derivatives and the 2,3-diaryl moiety has never been presented before our synthetic work.

The pharmacological properties of xanthones published in the last decades reveal the growing interest in this type of compounds. The pioneering work of Finnegan et al., in 1968, reported the diuretic action of mangiferin, a natural xanthone glycoside. Later on, Da Re et al. reported the analeptic activity of synthetic aminooalkylxanthone derivatives. Since then, the biological profiles of natural and even synthetic analogues have been extensively reported in the literature, namely anti-allergic, antifungal, anti-inflammatory, antimalarial, hepatoprotective activity, inhibition of monoamine oxidase, and other enzymes, like cholinesterase and angiotensin I converting-enzyme. In this broad spectrum of biological activities special mention deserves also the antioxidant activity demonstrated by several xanthones. The antioxidant properties have been exemplified by their action as free radical scavengers, inhibitors of lipid peroxidation and as metal chelators.

The recognized participation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in several pathologies and in cellular signalling systems has attracted growing interest from the health sector over the last few decades. In addition, the quest for effective antioxidants for food, cosmetic and pharmaceutical purposes has become a major industrial and scientific research challenge. Considering the interesting and promising studies already performed in xanthones, concerning their antioxidant activities, the development of new and effective scavengers of ROS and RNS using a xanthone scaffold seems therefore to be an interesting approach in this area. Thus, the main goal of this work was to evaluate the scavenging activity of the new synthetic 2,3-diarylxanthones derivatives for ROS, such as superoxide radical...
(O$_2^*$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (1O$_2$), peroxyl radical (ROO$^*$) and hypochlorous acid (HOCl), and RNS, such as nitric oxide (NO) and peroxynitrite anion (ONOO$^-$) (Fig. 1).

2. Results

2.1. Superoxide radical scavenging activity

Only 2,3-diarylxanthones 3a–c could prevent the O$_2^*$-dependent reduction of NBT in a concentration-dependent manner (Table 1). Compound 3c was considerably the most efficient, of this group and of all the tested compounds, presenting an IC$_{50}$ of 10.4 ± 0.8 μM. Compounds 2c, 1c and 3b are also potent scavengers, providing IC$_{50}$s of 20.3 ± 2.5, 28.1 ± 2.3 and 31.3 ± 3.2 μM, respectively.

2,3-Diarylxanthones 1a, 1b and 2a were completely ineffective up to the highest tested concentration (compound 1a, 25 μM and compounds 1b and 2a, 200 μM).

The IC$_{50}$ values presented in Table 1 for O$_2^*$ scavenging activity vary from 10.4 to 166 μM (compound 2b) and are significantly lower than the IC$_{50}$ value found for the positive control, tiron (IC$_{50}$ = 273 ± 32 μM).

2.2. Hydrogen peroxide scavenging activity

The results presented in Table 1 refer only to the H$_2$O$_2$ scavenging activity of the tested 2,3-diarylxanthones in a range of 125–250 μM, due to the precipitation that occurred in the sample wells at higher concentrations.

Vestigial scavenging activity was observed for xanthones 3a–c. Compounds 3b and 3c caused 17% and 12% inhibition of chemiluminescence, respectively, at their maximum concentration (250 μM). From 2a–c, only 2c presented a slight effect of 14% at the highest tested concentration (250 μM). The derivatives 3a and 1c presented similar effects and did not reach 10% effect at the maximum tested concentration (125 μM).

No scavenging activity was found for 2,3-diarylxanthones 1a,b and 2a,b, at concentrations up to 125 μM.

The positive control, ascorbic acid, provided an IC$_{50}$ of 602 ± 80 μM.

2.3. Hypochlorous acid scavenging activity

All the tested compounds were able to scavenge HOCl in a concentration-dependent manner. The HOCl-induced oxidation of dihydrolipoic acid 123 (DHR) was efficiently prevented by hydroxy-2,3-diarylxanthones 3a–c, being 3c and 3b the most effective derivatives, providing IC$_{50}$ values of 1.2 ± 0.02 and 7.5 ± 0.7 μM, respectively (Table 1).

From compounds 1a–c and 2a–c, 1c and 2c proved to be the most active ones (IC$_{50}$ = 15.7 ± 1.1 and 14.7 ± 1.3 μM, respectively). However, the IC$_{50}$ values were slightly higher than the obtained for the compound 3a (IC$_{50}$ = 10.8 ± 0.4 μM).

It was possible to determine the IC$_{50}$ of 2,3-diarylxanthone 1a, along the studied concentration range (25–200 μM) at 155 ± 25 μM.

The IC$_{50}$ of the positive control dihydrolipoic acid was 2.3 ± 0.3 μM.

2.4. Singlet oxygen scavenging activity

The 2,3-diarylxanthones proved to be effective scavengers of 1O$_2$ in a concentration-dependent manner, except for compound 1a, which only reached 27% effect at the highest tested concentration (100 μM) (Table 1). For compounds 1a–c, 1c (IC$_{50}$ = 6.0 ± 1.0 μM) was noticeably more active than compound 1b (IC$_{50}$ = 80 ± 11 μM).

For compounds 2a–c, 2c was the most potent (IC$_{50}$ = 3.3 ± 0.7 μM) and the obtained IC$_{50}$ for 2b (IC$_{50}$ = 58.4 ± 4.9 μM) was slightly lower than that of 2a (IC$_{50}$ = 68.8 ± 6.2 μM).

2,3-Diarylxanthones 3a–c revealed to be very active compounds, the scavenging activity order being 3c > 3a > 3b, with IC$_{50}$s of 2.5 ± 0.2, 4.5 ± 0.6 and 6.8 ± 0.5 μM, respectively. Quercetin (positive control) efficiently scavenge 1O$_2$ (IC$_{50}$ = 1.8 ± 0.1 μM).

2.5. Peroxy radical scavenging activity

As seen in Table 1, all the assayed compounds, except 1a, were able to delay the loss of fluorescence, due to ROO$^*$-dependent fluorescein oxidation, in a concentration-dependent way. Compound 1a did not show any scavenging activity along the studied concen-

![Figure 1](image-url)
2.6. Nitric oxide scavenging activity

All the tested compounds were able to scavenge NO-oxidation of 4,5-diaminofluorescein (DAF-2) in a concentration-dependent manner (Table 2).

Compounds 2c and 3c were the most effective ones, presenting a very similar activity with IC50 ranging 0.4 µM, followed by 1c, 3a and 2b. The order of potencies found for these derivatives was: 3c > 2c > 3b > 3a > 1c.

2.3-Diarylxanthones 1b, 2a and 2b were the most powerful scavengers of ROO\(^·\)/C5\(^·\), providing ORAC values of 1.05 ± 0.09, 2.08 ± 0.16 and 2.88 ± 0.09 µM, respectively.

In contrast, compounds 3a-c along with compound 1c were the less active ones, presenting ORAC values from 0.28 to 0.84 µM.

2.7. Peroxynitrite scavenging activity

The 2.3-diarylxanthones tested were shown to efficiently inhibit ONOO\(^−\)-induced oxidation of DHR, in a concentration-dependent manner. However, the concentration-effect relationship on scavenging activity was different in the absence or in the presence of 25 mM NaHCO3. Generally, the compounds were shown to be more effective in the absence of NaHCO3 than in its presence, with the exception of compounds 2a and 2b.

Compound 3c proved to be the most active derivative, with IC50 of 0.17 ± 0.01 and 0.33 ± 0.06 µM, in the absence and in the presence of NaHCO3, respectively (Table 2).

Concerning other results in the absence of NaHCO3, compounds 2c and 3b (IC50 = 0.26 ± 0.05 and 0.22 ± 0.03 µM, respectively) were visibly more efficient than compounds 1c and 3a (IC50 = 0.40 ± 0.03 and 0.37 ± 0.09 µM, respectively). However, in the presence of NaHCO3, the relative order of potencies for these compounds is the opposite. The scavenging activity order was: 1c > 3a > 2c > 3b, with IC50 values of 0.54 ± 0.08, 0.67 ± 0.09, 0.78 ± 0.26 and 0.89 ± 0.18 µM, respectively.

Similar results were obtained for compounds 1b and 2b, being the former more efficient in the absence of NaHCO3 and the latest more effective in the presence of this salt.

Compound 1a was also able to scavenge ONOO\(^−\) although its effect could only reach 29% and 48%, at the maximum concentration tested (50 µM), in the absence or in the presence of NaHCO3, respectively.

Ebselen, a well known ONOO\(^−\) scavenger, presented an IC50 of 0.50 ± 0.03 µM in the absence of NaHCO3 and 2.01 ± 0.22 µM in its presence.

3. Discussion

The present study indicates that the tested 2,3-diarylxanthones 1–3 are promising molecules to be used for their potential antioxidant properties. The scavenging activity for ROS/RNS of these xanthone derivatives is reported for the first time, with outstanding results, considering the nanomolar to micromolar range of the IC50 values found. In our first approach, O2\(^·\)– scavenging activity was assayed using a non enzymatic (NADH/phenazine methosulfate) generating system. The results seem to be largely affected by the number and position of hydroxyl groups in D and E rings of the xanthone core. Several authors had already referred the importance of structure–activity relationship for the ability of several hydroxxyanthones to scavenge O2\(^·\)–, using different methodologies.50–52 Compound 3c, bearing 4 hydroxyl groups, is the most effective scavenger of O2\(^·\)– (Fig. 2) followed by compounds 3b and 2c, which possess 3 hydroxyl groups. In this case, the scavenging activity of compound 2c is slightly higher than compound 3b, suggesting that the presence of a catechol group in D-ring is more relevant than the presence of a catechol group in E-ring. The same effect was observed in compounds 1c and 3a, possessing an ortho-dihydroxy substitution in D-ring and E-ring, respectively. Compound 2b that contains two hydroxyl groups, but no catechol moieties, was noticeably less potent than the compounds described before; the remaining phenol derivatives showed no activity.

Poor H2O2 scavenging activity was observed for all the tested compounds. However, it was not possible to test beyond the concentration of 250 µM due to the low solubility of 2,3-diarylxanthones in the tested system. Nevertheless, the scavenging values of derivatives 2c, 3b and 3c (12–17% at a 250 µM concentration) also suggest that the number and the position of hydroxyl groups are responsible for the H2O2-scavenging effects (Table 1). This hypothesis is only partially confirmed in the literature. Sun et al.51 monitored the H2O2-induced oxidation of luminol (a similar methodology to the described in this paper) and showed that α-mangostin (a trihydroxyxanthone) presented evident inhibitory effect on H2O2 when compared with γ-mangostin (a dihydroxyxanthone). On the other hand, Pedraza-Chaverri et al.,53 using a different procedure, showed that α-mangostin was unable to scavenge H2O2. From the analysis of our results, the tetrahydroxy-2,3-diarylxanthone 3c is more active than the trihydroxyxanthones, 2c and 3b. Therefore comparing the results of both trihydroxyxanthones one can conclude that the presence of a catechol unit in the D-ring of compound 2c can contribute for their slight higher effect relatively to the corresponding isomer 3b.

The HOCl-induced oxidation of DHR was efficiently prevented by all the tested 2,3-diarylxanthones. The results from the HOCl-scavenging assay show, once again, the importance of the ortho-dihydroxyl substitution for the antioxidant activity of 2,3-diarylxanthones (Fig. 3). In addition, the number of the hydroxyl groups also seems to contribute to the scavenging effect of these compounds. Indeed, compound 2b, possessing two hydroxyl groups but not a catechol moiety, presents almost 100% inhibition of HOCl-induced oxidation at a concentration of 100 µM. For lower concentrations, the scavenging activity of the tested xanthones...
was visibly more affected by the presence of the catechol group rather than the number of hydroxyl substituents. This is clear by the analysis of the results, which shows that derivatives 3a–c in addition of compounds 1c and 2c were all more effective scavengers than compound 2b, at 6.25 and 3.125 \( \mu \)M concentration (Fig. 3).

In agreement with the results from other ROS, the \( ^1 \)O\(_2\) scavenging activity of 2,3-diarylxanthones depends particularly on the OH-substitution pattern. Compounds with a catechol unit presented a considerably higher effect than those lacking this feature as it can be confirmed by the IC\(_{50}\) values. 2,3-Diarylxanthones 1c, 2c, 3a–c presented IC\(_{50}\) values in a range of 2.5–6.8 \( \mu \)M and compounds 1b, 2a, 2b, having a phenol instead of a catechol ring, were noticeably less potent, with IC\(_{50}\)s rounding 58.4–80 \( \mu \)M (Table 1).

Other interesting feature could be suggested by the analysis of Figure 4, which presents the results of the \( ^1 \)O\(_2\) scavenging activity of 2,3-diarylxanthones possessing at least a catechol unit. We can observe that, for concentrations of 10–20 \( \mu \)M, the presence of two catechol groups in compound 3c seems to be essential for the high scavenging potential of this derivative. In contrast, for the lower concentrations, the number of catechol units does not seem to bring any advantage to the scavenging activity, all the derivatives presenting similar effects.

The data presented in Table 1 show that all the hydroxyxanthones tested provided higher ORAC values than the endogenous antioxidant ascorbic acid. The ROO\(^{\bullet}\) scavenging activity of these compounds seems to mostly depend on the hydroxylation pattern.
in the aryl groups. However, in this assay, the presence of phenol groups seems to be extremely important for the scavenging effect. The highest ORAC value was obtained for compound 2b, a xanthone bearing two phenol groups as substituents. The introduction of an additional OH substituent drastically decreases the scavenging activity as it is evident by the results of compounds 3a–c. 2,3-Diarylxanthone 3c, a derivative with two ortho-dihydroxyl groups, provided the lowest ORAC value from all the tested hydroxyxanthone. These observations are not in accordance to the knowledge about structure–activity relationship of flavonoids and phenolic compounds. The ortho-dihydroxyl substitution is particularly important to the ROO\(^*/C_5\) absorbing activity of those classes of compounds.\textsuperscript{34–36} The mechanism involves the H-atom donating ability of the antioxidant to the ROO\(^*\), thus acting as chain radical terminator.

The 'NO scavenging activity indicates that the OH-substitution pattern in the aryl groups is the main factor responsible for the scavenging effect of this RNS. The compounds with a catechol group play an important role as protectors of the 'NO-dependent oxidation of DAF-2 (Fig. 5). Yet, this group of compounds is also much more effective than rutin, a flavonoid, which is a well-known 'NO scavenger. The other 2,3-diarylxanthones provided a much weaker effect, as can be confirmed by the analysis of the results presented in Table 2.

Some of us had already studied the 'NO scavenging activity of some flavones and 2-styrylchromones.\textsuperscript{37,38} In general, the

![Figure 4](image-url) `\( ^1\text{O}_2 \) scavenging activity of 2,3-diarylxanthones 1c, 2c, 3a–c. Each column represents the values obtained from five experiments, performed in triplicate (mean ± SEM).

![Figure 5](image-url) 'NO scavenging activity of 2,3-diarylxanthones 1c, 2c, 3a–c. Each column represents the values obtained from five experiments, performed in triplicate (mean ± SEM).
compounds presenting a catechol moiety, have shown to be more potent than those lacking this unit. The same behaviour was observed in the present study and the tested 2,3-diarylxanthones presented similar effects on the NO scavenging activity to those presented by the structural similar compounds. Other authors also reported that some flavonoids were good NO scavengers. Thus, considering the structural similarity between the above-referred compounds, it is very likely that the studied hydroxyxanthones might present some therapeutic value.

Concerning $\text{ONOO}^-$, all the studied hydroxy-2,3-diarylxanthones were able to scavenge this RNS in a very efficient way. Apparently, the scavenging effect is related to the number and position of the hydroxyl substituents on the D and E-rings. However, these features have a different contribution when the assays are performed in the absence or in the presence of NaHCO$_3$. Figure 6 describes the ONOO$^-$ scavenging activity of the catecholic-2,3-diarylxanthones, in the absence of bicarbonate. We can observe that by increasing the number of hydroxyl substituents, higher is the scavenging effect of these compounds. Derivatives 2c, 3b and 3c were visibly more active than the compounds 1c and 3a, which possess only two hydroxyl groups. Heinjen et al. studied the ONOO$^-$ scavenging of substituted phenols and several flavonoids and concluded that the activity is positively influenced by the number of OH groups and by the presence of catechol units in the above-referred molecules. When we performed this assay in the presence of bicarbonate the results seemed to be dependent on the position of the ortho-di-hydroxyxanthone moiety, which were sometimes favourable and others unfavourable to the scavenging activity, depending on the concentration of the antioxidant species (Fig. 7). The reaction between ONOO$^-$ and CO$_2$ is very fast ($K = 3–5.8 \times 10^5 \text{M}^{-1} \text{s}^{-1}$) and results in the formation of the nitroperoxycarbonate anion (ONOOCO$_3^-$), whose decomposition leads to the formation of different species including the highly reactive $\text{NO}_2$ and $\text{CO}_3^-$ radicals. Thus, the observed variation in the scavenging activities is probably due to the different ability to scavenge the resulting radicals.

In conclusion, the results obtained in the present study revealed new promising 2,3-diarylxanthones with outstanding ROS and RNS scavenging properties. In fact, some of the studied derivatives proved to be extremely efficient scavengers, showing, in some cases, IC$_{50}$ values under 1 µM. The results obtained are largely affected by the number and position of hydroxyl groups in the xanthone core. The ROO$^-$ scavenging activity seems to depend mostly on the presence of phenolic groups while for the other tested ROS and RNS, xanthones with a catechol ring were the most potent scavengers. These findings allowed the establishment of structure–activity relationships and that 2,3-diarylxanthones can have a promising future as antioxidant pharmacophores.

4. Materials and methods

4.1. Chemicals

All the chemicals and reagents were of analytical grade. Ascorbic acid, DAF-2, dihydroliopoic acid, DHR, ebselen, 30% hydrogen peroxide, lucigenin, β-nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium chloride (NBT), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), penicillamine, phenazine methosulfate (PMS), sodium hypochlorite solution, with 4% available chlorine, rutin and tiron were obtained from Sigma–Aldrich (St. Louis, USA); α,α′-Azodiisobutyramidine dihydrochloride (AAPH), histidine, and trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt and quercetin were obtained from Aldrich (Milwaukee, USA). All the other reagents were obtained from Merck (Darmstadt, Germany).

The polyhydroxy-2,3-diarylxanthones 1a–3c were synthesized according to procedures previously described in the literature and showed to possess identical spectroscopic and analytical data. Briefly, 2,3-diarylxanthones 1–3 have been obtained by a five steps chemical sequence starting from simple starting materials (Scheme 1). The first step of this synthesis involves the esterification of 2-hydroxyacetophenone 4 with cinnamoyl chloride derivatives 5a–c (5a is commercial and 5b,c are prepared in situ from the corresponding cinnamic acids and phosphorous oxychloride in dry pyridine). Baker–Venkataraman rearrangement of esters 6a–c with potassium hydroxide in DMSO afforded β-diketones 7a–c; these were brominated and cyclodehydrated by treatment with PTT (phenyltrimethylammonium tribromide), leading to the synthesis of 2-bromo-2-styrylchromones 8a–c. The Heck reaction of bromochromones 8a–c with styrene derivatives 9a–c

Figure 6. ONOO$^-$ scavenging activity of 2,3-diarylxanthones 1c, 2c, 3a–c. Each column represents the values obtained from five experiments, performed in triplicate (mean ± SEM).
yielded 2,3-diarylxanthones 10a–c, 11a–c and 12a–c. The final step of our synthetic route consisted on the demethylation of these methoxy-2,3-diarylxanthones 10a–c, 11a–c and 12a–c by treatment with boron tribromide (2.5 equiv per methyl group) in dichloromethane.

4.2. Equipment

A microplate reader (Synergy HT, BIO-TEK), with spectrophotometric, fluorimetric, and chemiluminometric detection, plus temperature control capacity, was used for all ROS and RNS scavenging assays.

4.3. ROS and RNS scavenging assays

4.3.1. Superoxide radical scavenging assay

The O$_2^-$ was generated by the NADH/PMS system and the O$_2^-$ scavenging activity was determined by monitoring the O$_2^-$-induced reduction of NBT, according to a previously developed procedure. The effect of the tested compounds dissolved in DMSO was determined spectrophotometrically at 560 nm during 2 min. The antioxidant tiron was used as positive control. The results were expressed as the percentage inhibition of the NBT reduction to diformazan. Each assay corresponds to five experiments, conducted in triplicate.

4.3.2. Hydrogen peroxide scavenging assay

The H$_2$O$_2$ scavenging activity was measured using a previously described chemiluminescence methodology based on the H$_2$O$_2$-induced oxidation of lucigenin. The tested compounds were dissolved in DMSO and subsequently diluted in phosphate buffer (mixture 1:9). Lipoic acid was used as positive control. The results were expressed as the percentage inhibition of H$_2$O$_2$-induced oxidation of DHR. Each assay corresponds to five experiments, conducted in triplicate.

4.3.3. Hypochlorous acid scavenging assay

The HOCl was measured by monitoring the HOCl-induced oxidation of DHR to rhodamine 123. The fluorimetric assays were performed at 37°C and the tested compounds were dissolved in ethanol, as previously described. HOCl was daily prepared by adjusting the pH of a 1% solution of NaOCl to 6.2 with dropwise addition of 10% H$_2$SO$_4$. Lipoic acid was used as positive control. The results were expressed as the percentage inhibition of HOCl-induced oxidation of DHR. Each assay corresponds to five experiments, conducted in triplicate.

4.3.4. Singlet oxygen scavenging assay

The 1O$_2$ was generated by the thermal decomposition of a previously synthesized water-soluble endoperoxide [disodium 3,3'-(1,4-naphthalene)bispropionate (NDPO$_2$)]. The 1O$_2$ scavenging activity was measured by monitoring the oxidation of non-fluorescent DHR to fluorescent rhodamine 123, at 37°C, after a 30 min incubation period, as described in the literature. Ascorbic acid was used as positive control. The results were expressed as the percentage inhibition of 1O$_2$-induced oxidation of DHR. Each assay corresponds to five experiments, conducted in triplicate.

4.3.5. Peroxyl radical scavenging assay

The ROO· scavenging activity was measured by monitoring the fluorescence decay resulting from ROO·-induced oxidation of fluorescein and expressed as the ‘oxygen radical absorbance capacity’ (ORAC), according to a described procedure. ROO· was generated by thermal decomposition of AAPH and the tested compounds dissolved in acetone and subsequently diluted in phosphate buffer (mixture 1:9). Trolox was used as the standard control in each study. Ascorbic acid was used as positive control. The results were expressed as ORAC values. Each assay corresponds to four experiments, conducted in triplicate.

4.3.6. Nitric oxide scavenging assay

The ·NO scavenging activity was measured by monitoring the fluorescence decay resulting from ·NO-induced oxidation of non-fluorescent DAF-2 to the fluorescent triazolofluorescein (DAF-2T), using a previously described method. ·NO was generated by NOC-5, the tested compounds dissolved in DMSO and the fluorimetric signal was detected after a 30 min incubation period, at 37°C. Rutin was used as positive control. The results were expressed as the percentage inhibition of
NO-induced oxidation of DAF-2. Each assay corresponds to five experiments, conducted in triplicate.

4.3.7. Peroxynitrite scavenging assay

The ONOO$^-$ scavenging activity was measured by monitoring the ONOO$^-$-induced oxidation of non-fluorescent DHR to fluorescent rhodamine 123, as previously described. ONOO$^-$ was synthesized as reported in the literature by some of the authors. In a parallel set of experiments, the assays were performed in the presence of 25 mM NaHCO$_3$ in order to simulate the physiological CO$_2$ concentrations in vivo. This evaluation is important because, under physiological conditions, the reaction between ONOO$^-$ and bicarbonate is predominant, with a very fast rate constant ($k_2 = 3.58 \times 10^8$ M$^{-1}$ s$^{-1}$). The results were expressed as the percentage inhibition of ONOO$^-$-induced oxidation of DHR. Each assay corresponds to five experiments, conducted in triplicate.

Acknowledgements

Sincere thanks are expressed to Faculdade de Farmácia da Universidade do Porto, and also to Universidade de Aveiro, Fundação para a Ciência e a Tecnologia (Portugal) and FEDER for funding the Organic Chemistry Research Unit. M.F. and A.G. acknowledge Fundação para a Ciência e Tecnologia (FCT) and Fundo Social Europeu (FSE) their Ph.D. (SRH/BD/28502/2006) and post-doctoral (SRH/BPD/63179/2009) Grant, respectively.

References and notes