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ORIGINAL ARTICLE

The development of 1,3-diphenylisobenzofuran as a highly selective probe for the detection and quantitative determination of hydrogen peroxide

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ABSTRACT

1,3-Diphenylisobenzofuran (DPBF) has been developed as a selective probe for the detection and quantitative determination of hydrogen peroxide in samples containing different reactive nitrogen and oxygen species (RNOS). DPBF is a fluorescent probe which, for almost 20 years, was believed to react in a highly specific manner toward some reactive oxygen species (ROS) such as singlet oxygen and hydroxy, alkyloxy or alkylperoxy radicals. Under the action of these individuals DPBF has been rapidly transformed to 1,2-dibenzoylbenzene (DBB). In order to check if DPBF can act as a unique indicator of the total amount of different RNOS, as well as oxidative stress caused by an overproduction of these individuals, a series of experiments was carried out, in which DPBF reacted with peroxynitrite anion, superoxide anion, hydrogen peroxide, hypochlorite anion, and anions commonly present under biological conditions, namely nitrite and nitrate. In all cases, except for hydrogen peroxide, the product of the reaction is DBB. Only under the action of H_2O_2 9-hydroxyanthracen-10(9H)-one (oxanthrone) is formed. This product has been identified with the use of fluorescence spectroscopy, NMR spectroscopy, high performance liquid chromatography coupled with mass spectrometry, infrared spectroscopy, elemental analysis, and cyclic voltammetry (CV). A linear relationship was found between a decrease in the fluorescence intensity of DPBF and the concentration of hydrogen peroxide in the range of concentrations of 0.196–3.941 mM. DPBF responds to hydrogen peroxide in a very specific way with the limits of detection and quantitation of 88 and 122.8 μM, respectively. The kinetics of the reaction between DBBF and H_2O_2 was also studied.

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1,3-diphenylisobenzofuran; hydrogen peroxide; fluorescence spectroscopy; high performance liquid chromatography coupled with mass spectrometry; selectivity; limit of detection and quantitation

Introduction

Hydrogen peroxide belongs to reactive oxygen species (ROS), which next to reactive nitrogen species (RNS) play a positive and important role in the processes of cell communication. The maintenance of their certain levels in cells is necessary for the information flow between cells and inside cells [1]. It is known that ROS, especially superoxide anion and hydrogen peroxide, are important signaling molecules in cardiovascular cells [2–4]. Although reactive nitrogen and oxygen species (RNOS) are essential for a signalization, they also contribute (in excess) to the pathogenesis of many diseases, such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis. Even slight fluctuations in the basic level of RNOS significantly affect the change in the cells' metabolism, an expression of genes and modifications of proteins. All reactive oxygen (i.e. superoxide anion, hydroxyl radical, singlet oxygen, hydrogen peroxide, hypochlorite anion, alkyloxyl radical, and alkylperoxyl radical) and nitrogen (i.e. nitric oxide, nitrogen dioxide, and peroxynitrite ion) species react with biological structures. As a result, proteins can be altered with a loss of function, DNA can be cleaved, and lipid components can be oxidized to disrupt membranes [5–7]. Hydrogen peroxide itself in the excess is an important mediator of pathological processes in various diseases. It is implicated in neurodegenerative disorders and appears to act as a neuromodulator in the brain [8]. Increased rates of its vascular production contribute to an initiation of pro-inflammatory events [9]. Hydrogen peroxide is generated as a by-product in a wide range of biological processes. It has a diverse array of physiological and pathological effects within living cells, depending on the extent, timing, and location of its production. As the cell membranes are highly permeable to H_2O_2, it can diffuse between compartments and mediate the cell-to-cell communication [10].

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Supplemental data for this article can be accessed here.

Fluorescent probes, designed to detect and determine individual RNOS with a high selectivity and sensitivity, seem to be the most promising tools in signaling H_2O_2 as they provide spatial and temporal information about the species in biological systems. The development of the probes suitable for a fluorescence imaging of H_2O_2 have become an active area of research in recent years [11]. As each RNOS possess their own unique physiological activity, the need for novel fluorescent sensors for detecting such an individual species with a high selectivity and specificity is still increasing.

1,3-Diphenylisobenzofuran (DPBF) is a fluorescent compound, which when excited at 410 nm expresses a fluorescence emission centered at *ca.* 455 nm [12]. As DPBF is compatible with the biological conditions, it can be utilized in an investigation of cell membranes where the presence of proteins does not interfere with the fluorescence properties of the probe. This is the main reason why the use of DPBF as an indicator of RNOS is of great scientific and practical importance [13,14]. For almost 20 years DPBF was believed to react in a highly specific way toward some ROS such as singlet oxygen ($^1O_2$), hydroxy ($HO^\bullet$), alkyloxy ($RO^\bullet$), and alkylperoxy ($ROO^\bullet$) radicals [15,16]. In all cases, studied so far, the reaction product is 1,2-dibenzoylbenzene (DBB). Recently, we have demonstrated that DPBF can react with nitrogen dioxide (NO_2) – an example of RNS – also yielding DBB as the final product [17]. The fact that the reaction of DPBF with NO_2 and different ROS gives the same product has an immense influence on the detection and quantitative determination of RNOS. If it is confirmed that DPBF forms the same product (DBB) in the reaction with all RNOS present in matrices studied, it may turn out to be a unique indicator of a cellular stress caused by such chemical individuals. According to this, the main goal of the work was comprehensive characteristics of the interactions between DPBF and the most important (and not yet studied) RNOS, which are hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), hypochlorite anion (OCl^-), peroxynitrite anion ($ONOO^-$) as well as nitrite (NO_2^-) and nitrate (NO_3^-) – the ions commonly present in biological matrices.

Materials and methods

Materials

DPBF, hydrogen peroxide, potassium dioxide, sodium hypochlorite solution, sodium nitrite, sodium nitrate, and acetonitrile were purchased from Sigma-Aldrich (Poznan, Poland). The solution of sodium peroxynitrite in 0.3 M sodium hydroxide was purchased from Cayman Chemical (Ann Arbor, MI). Formic acid was purchased from POCH S.A. (Gliwice, Poland). The stock solution of DPBF ($c = 10^{-3}$ M) was prepared in acetonitrile. The solution of potassium dioxide was prepared by dissolving a powder in dimethylsulfoxide, while other solutions were prepared in DI water. All solutions were prepared immediately prior to use.

Fluorescence measurements

Fluorescence emission spectra and kinetic measurements were performed using the Cary Eclipse Varian stationary spectrofluorimeter (Agilent, Santa Clara, CA), equipped with a temperature controller set at 20 °C (± 0.01 °C). Fluorescence emission spectra were recorded at a scanning rate of 600 nm min^{-1} and excitation and emission slits set to 10 and 5 nm, respectively. The samples were excited at 410 nm and the fluorescence emission was recorded in the range of 420–600 nm. During kinetic measurements excitation and emission slits were set to 5 nm. Before each analysis the solution of DPBF (500 μL, 10^{-4} M) was deoxidized by flushing with argon for 15 min. After transferring the above solution to a quartz cuvette (1.00 cm), different amounts of H_2O_2 solution (300–700 μL) and water to attain the total volume of 2.5 mL were added. Immediately after rapidly mixing the reagents, the fluorescence emission was monitored at 455 nm (the maximum of DPBF emission intensity). The rate constants of the reaction were determined by monitoring of the decrease in fluorescence intensities under the action of hydrogen peroxide. All kinetic measurements were carried out until the fluorescence intensity decreased near zero. The observable rate constants (*k*) were computed using the Origin 8.5 software (OriginLab, Northampton, MA), assuming pseudo-first order kinetic pattern of the reaction course. Each value of the rate constants was assessed as an average value of the three independent measurements.

The analytical characteristic of the presented method was assessed employing EnSpire Multimode Plate Reader (Perkin-Elmer, Hamburg, Germany) with the integrated software, using Cliniplate 96-well white microplates (Thermo Scientific Inc., Waltham, MA). Excitation and emission slits were set to 5 nm. The spectra were recorded in the range of 300–600 nm. The stock solutions of DPBF in spectral-grade acetonitrile (20 μM) and H_2O_2 in distilled water (0.9790 M) were prepared from the chemically pure products (>99%) and stored in the dark at a lowered temperature (3–5 °C). The working solutions were prepared from stock solutions just before use. Accordingly, the mixture of 100 μL of DPBF stock solution and 100 μL of H_2O_2 solution was placed in the dark for 15 h (3–5 °C). The intensity of the fluorescence emission was recorded at 455 nm with reference

to DPBF solution, in order to take into account the effect of the oxidation of this reagent by air.

High performance liquid chromatography

To monitor the reaction between DPBF and H_2O_2, reversed-phase chromatography (RP HPLC) was applied using Beckman Gold System (Brea, CA) equipped with the UV DAD detector. The chromatograms were recorded at different wavelengths (220, 260, 280, and 300 nm) for monitoring the effluents. The Wakopak reverse-phase C18 column (4.6 mm × 150 mm; particle size = 5 μm) and deionized water/acetonitrile mixture (90/10, v/v; flow rate = 1 mL min^{-1}) were used as a stationary and a mobile phase, respectively. The chromatographic conditions were optimized and established as follows: an isocratic elution for 10 min with the use of deionized water, acetonitrile and 1% formic acid (pH = 2.55; 87.7/2.0/10.3, v/v/v), then linear gradient elution for 30 min to attain 40% acetonitrile in the mobile phase.

High performance liquid chromatography coupled with mass spectrometry

Agilent 1200 Technologies HPLC System (Santa Clara, CA) was employed for LC-MS/MS experiments. RP HPLC analytical conditions are described in the above paragraph (High Performance Liquid Chromatography). The effluent was coupled to the HCT Ultra ion-trap mass spectrometer, with electrospray (ESI) ion source, which was operated in the positive and negative ion modes. The mass spectrometer was set up to MS or MS/MS mode. A 20 μL of sample solution was injected in each run. The ion source parameters were set as follows: the source temperature 360 °C, spray voltage ±4.0 kV, the drying gas (N_2) pressure 50 psi and the drying gas flow rate 11 L min^{-1}. Each spectrum was obtained by averaging three scans, and the time for each scan was 0.1 s.

Elemental analysis and IR spectroscopy

Elemental analysis of the reaction product between DPBF and H_2O_2 was performed by using a Vario El Cube CHNS Element Analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany). The IR spectrum of the above was obtained by employing a Bruker IFS66 FT-IR spectrometer (Bruker Optics Inc., Billerica, MA).

NMR spectroscopy

^1H NMR spectra for the products of reaction between DPBF and RNOS studied as well as for 9,10-anthraquinone (AQ) were recorded at 500 MHz, employing Varian Unity 500 Plus spectrometer (Oxford Instruments, Abingdon, UK) with 5 mm PFG probe attached. Dried samples were dissolved in $CDCl_3$ or CD_2Cl_2 and subjected to series of experiments at various time spans at 298 – 305 K. The ^1H nuclei signals were attributed by applying standard analysis basing on integration, multiplicity, and chemical shifts with the assistance of ACD HNMR and CNMR software (Cambridge, UK).

Cyclic voltammetry

Electrochemical measurements were performed using a PGSTAT 128N potentiostat (Metrohm Autolab B.V., Utrecht, The Netherlands), controlled with GPS 4.9 software. The cyclic voltammetry (CV) experiments were carried out in a standard three-electrode cell at the scan rate of 100 mV s^{-1} (2 mM acetonitrile solutions containing 0.1 M nBu_4NPF_6 as the supporting electrolyte, 298 K). The working electrode was a 3 mm diameter glassy carbon; a platinum wire and Ag/AgCl system were employed as a counter and reference electrode, respectively.

Quantum chemistry calculations

To calculate standard-state free energies of reactions leading to the formation of oxanthrone from DPBF a Hartree-Fock method with STO-3G basis set was used from Gaussian 09 program (Wallingford, CT) [18–20]. The molecular geometries for all molecular species in water as a solvent at 298.15 K have been fully optimized and vibrational frequencies have been obtained at corresponding equilibrium geometries.

Results and discussion

The main aim of the studies was to determine the products of the reactions between DPBF and different RNOS as well as nitrite and nitrate ions with the use of steady-state fluorescence spectroscopy, high performance liquid chromatography coupled with mass spectrometry, and NMR spectroscopy. In case of the reaction between DPBF and hydrogen peroxide the structure of the main product was additionally confirmed with the use IR spectroscopy, elemental analysis, and CV. In case of the reaction between DPBF and hydrogen peroxide, the additional aim of the studies was to determine its kinetics as well as the limits of detection and quantitation of H_2O_2.

Firstly, to assure that DPBF indeed reacts with peroxynitrite anion, superoxide anion, hydrogen peroxide,

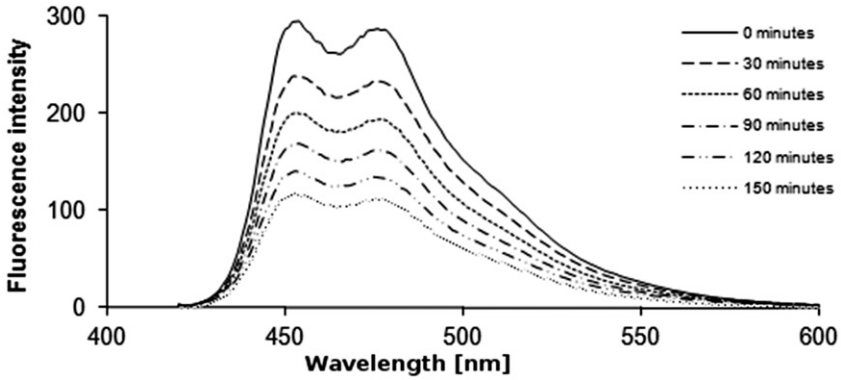

Figure 1. The decrease in the fluorescence intensity upon interaction of DPBF with H_2O_2. An excitation wavelength – 410 nm, spectra recorded every 30 min. Reaction conditions: $c_{DPBF} = 2 \times 10^{-5}$ M; $c_{RNOS/NO_3^-/NO_2^-}$=1.75 M; T = 20 °C.

hypochlorite anion, nitrite, and nitrate ions and to reject the possibility of a fluorescence quenching of DPBF by these species, several fluorescence emission spectra of the fluorophore were recorded every 30 min after the addition of a fixed amount of each reactive oxygen or nitrogen species solution. Examples of such spectra, registered under the action of hydrogen peroxide solution on DPBF, are presented in Figure 1. As it can be observed, the spectrum characterized by the highest intensity of fluorescence emission was obtained in the absence of hydrogen peroxide. On the other side, the spectrum of the lowest fluorescence intensity was registered after 150 min after the addition of H_2O_2. A permanent and time-dependent decrease in fluorescence intensity indicates the occurrence of a chemical transformation that obviously takes place under the experimental conditions. It has to be mentioned that similar spectra and observations were registered for all RNOS studied, as well as for nitrite and nitrate ions.

Secondly, in order to determine the products of the reactions among DPBF and RNOS as well as nitrite and nitrate ions, the appropriate RP-HPLC chromatograms were registered and presented in Figure 2. The results suggest a high probability that in the all above cases – except for H_2O_2 – the product of the reaction with DPBF makes the same compound. That hypothesis was confirmed by the registration of mass spectra of these compounds (the product of each reaction) after the addition of appropriate RNOS as well as nitrite and nitrate ions, followed by the isolation of the product with the aim of RP-HPLC. Registration of these spectra enabled us to determine the molecular masses of the products of the reactions studied. For hypochlorite anion, superoxide anion, peroxynitrite anion, nitrogen dioxide, nitrate, and nitrite ions the mass spectra of the products of their reaction with DPBF were almost identical, disclosing the same *m/z* value of the molecular cations ($[M + H]^+$) around 287. Figure 3(a) presents an exemplary MS spectrum of the product of the reaction between DPBF and peroxynitrite anion. The ion of *m/z* 309 is attributed to $[M + Na]^+$ ion. The presented product-ion spectrum of the $[M + H]^+$ ion (*m/z* 287) reveals that the product of the reaction is characterized by a molecular mass of about 286. In order to determine its structure, a further fragmentation of the above parent ion was performed with the aim of MS/MS analysis. The fragmentation of that ion is shown in Figure 3(b). The ion of *m/z* 209 is attributed to the loss of a phenyl moiety from a parent molecule. The $[M + H]^+$ ion of the product can undergo the facile loss of a water molecule to give the ion of *m/z* 269. Furthermore, it may eliminate two molecules of water to afford the ion of *m/z* 251. Further fragmentation of the *m/z* 209 ion leads to the formation of the ion *m/z* 181, which can be attributed to the benzophenone cation (Table 1). ESI QTOF MS spectra reveal that the product of the reaction between DPBF and the following compounds – peroxynitrite anion, nitrogen dioxide, hypochlorite anion, superoxide anion, nitrite, and nitrate is DBB – seems to be identical as that obtained upon the reaction among DPBF and $^1O_2$, $HO^\bullet$, $RO^\bullet$, and $ROO^\bullet$ [15–17].

In order to determine the molecular mass of the product of the reaction between DPBF and hydrogen peroxide, the chromatographic runs in altered conditions as well as the mass spectra under both positive and negative modes of ionization were registered. Representative results are disclosed in Figure 4. In Figure 4(b) the product-ion spectrum of the $[M + H]^+$ ion in the positive mode reveals the parent ion of *m/z* 211, while the product-ion spectrum of the $[M–H]^-$ ion in the negative mode gives an ion of *m/z* 209. This reveals that the product of the reaction between DPBF and H_2O_2 is characterized by a molecular mass of 210. In order to determine the structure of the latter ion, a fragmentation was performed with the use of MS/MS-type of mass analysis and the results are presented

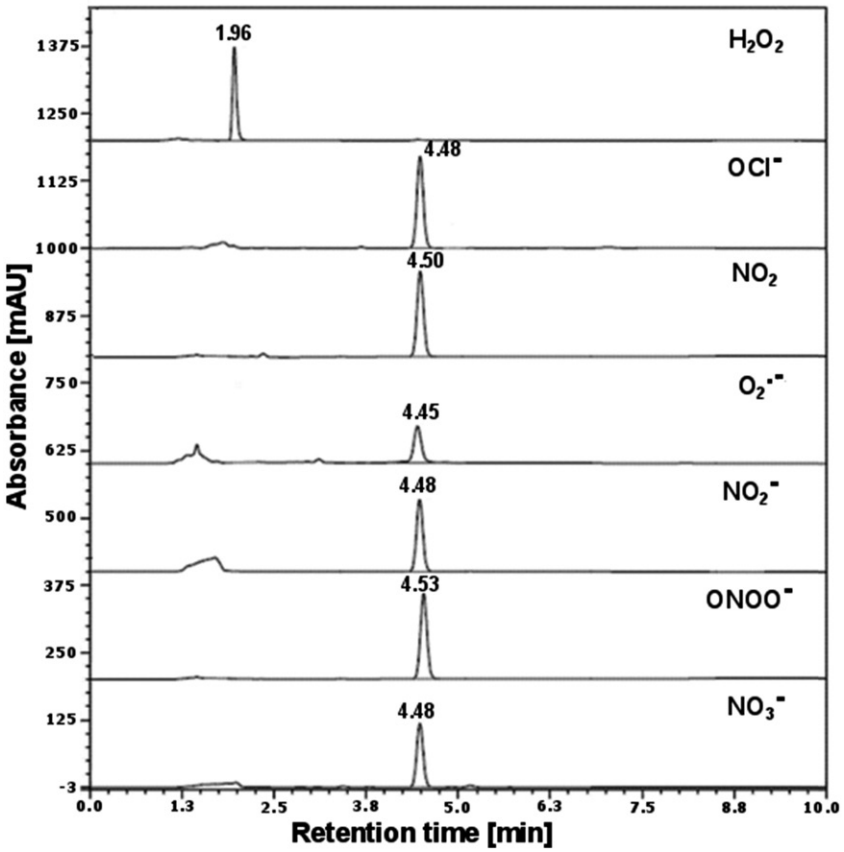

Figure 2. RP-HPLC chromatograms of the products of the reactions among DPBF and various reactive nitrogen and oxygen species, nitrate and nitrite ions (absorbance was measured at 260 nm, flow rate =1 mL min^{-1}; mobile phase: DI water/acetonitrile =90/10, v/v). Reaction conditions: $c_{DPBF} = 2 \times 10^{-5}$ M; $c_{RNOS/NO_3^-/NO_2^-} = 2 \times 10^{-5}$ M; T = 20 °C; each chromatogram was registered upon the reaction completion, i.e. when the mixture exhibited no fluorescence ($\lambda_{ex} = 410$ nm; $\lambda_{em} = 455$ nm).

in Figure 5. The ion of *m/z* 182 is attributed to the loss of a CHO fragment from the parent ion. The latter can also eliminate two CHO fragments to afford the ion of *m/z* 153 (Table 1). Generally, such a fragmentation makes a characteristic pattern for different derivatives of 9,10-anthraquinone [21]. The structure of the product obtained was additionally confirmed by elemental analysis and IR spectroscopy.

Elemental analysis calculated (%) for $C_{14}H_{10}O_2$ (210.2): C 79.98, H 4.79; found C 80.18, H 4.98.

IR (KBr): ν(O–H) = 3332 cm^{-1}, ν(C = O) = 1660 cm^{-1}, ν(C–H) = 3067 cm^{-1}, δ(C–H) = 1279 cm^{-1}.

The results of analyses indicate, that main product of reaction between DPBF and H_2O_2 under described conditions seems to express an unexpected structure, different than the typical product, obtained upon the reaction between DPBF and other RNOS (1,2-dibenzoylbenzene, DBB). The unique product appears in the reaction mixtures in varying amounts, depending on the reaction conditions. To get deeper insight into its structure, we employed additional methods, such as nuclear magnetic resonance spectroscopy, cyclic voltammetry (CV), and quantum chemistry calculations. The results are presented in Supplemental data file. They reveal that the substance under study expresses similar polarity to AQ (Figure S2, ESI), but differs markedly in terms of spectroscopic features (Figure S5, ESI), both from AQ (Figure S6, ESI) as well as from the typical product of DPBF oxidation, namely DBB (Figure S3, S4). The molecule under study expresses the bands, that are characteristic for carbonyl group in quinones as well as for hydrogen-bonded hydroxyl groups – as it is manifested in FT-IR spectrum (Figure S7).

The CV was employed to characterize electrochemical behavior of the new product; its activity was then compared to the behavior of the AQ at analogous conditions (Figure S8). The latter (reference) compound undergoes characteristic two one-electron reduction steps to give semiquinone ($AQ^{\bullet-}$) and then – quinone dianion (AQ^{2-}), expressed by the presence of two reversible peaks (blue dotted line). The change in environment's acidity (low pH values) leads to the appearance of single reduction peak, shifted towards positive values of potential, thus indicating the transfer of two electrons (red dashed line). The position and shape of the peak of the product of reaction between DPBF and

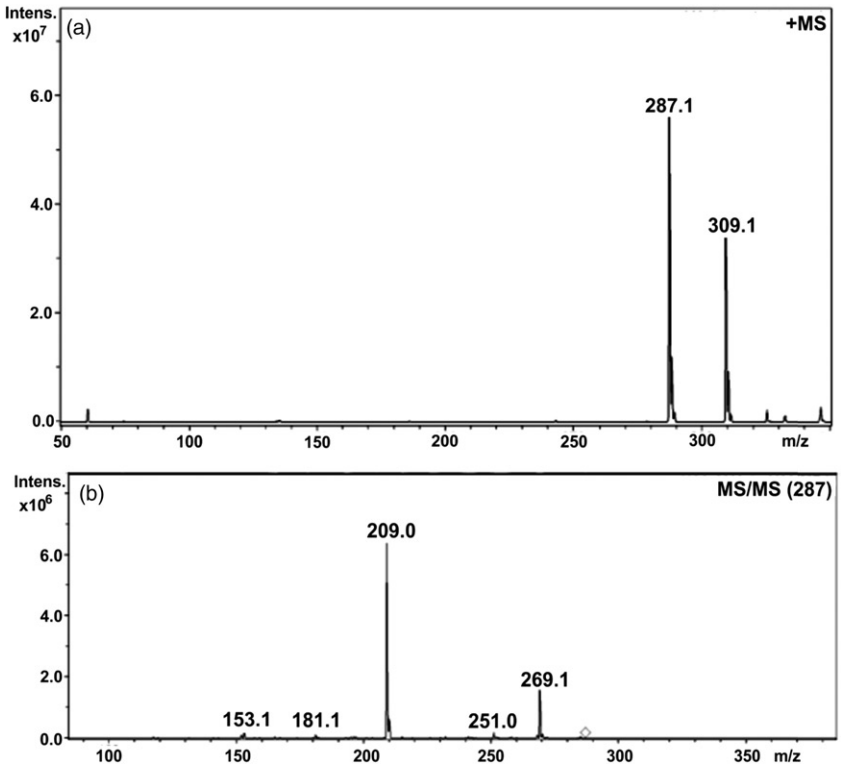

Figure 3. (a) The product-ion ESI-QTOF MS spectrum of the $[M+H]^+$ ion of the product of the reaction between DPBF and peroxynitrite anion; (b) ESI-QTOF MS/MS spectrum of the $[M+H]^+$ ion of the product of the reaction between DPBF and peroxynitrite anion.

Table 1. Mass measurements of the $[M+H]^+$ ions and product ions observed in the MS/MS of the $[M+H]^+$ ions of the two identified products.

1,2-dibenzoylbenzene		9-hydroxyanthracen-10(*9H*)-one	
m/z	Ion identities	*m/z*	Ion identities
287.1	$[M+H]^+$	211.0	$[M+H]^+$
269.1	$-H_2O$	210.0	$[M]^+$
251.0	$-H_2O$, $-H_2O$	182.0	–CHO
209.0	$-C_6H_6$	153.0	–CHO, –CHO
181.1	$-C_6H_6$, –CO		

H_2O_2 clearly indicate its different red-ox behavior in comparison with AQ and suggest the one-electron reversible reduction step in the case of the original product, (black solid line).

Summarizing the results of physicochemical investigations carried on the product of the reaction between DPBF and hydrogen peroxide (elemental analysis, RP-HPLC, ESI-QTOF MS, ^{1}H NMR, FT-IR, and CV) it can be concluded, that the new substance is presumably 9-hydroxyanthracen-10(*9H*)-one (oxanthrone) – the derivative of AQ which, to our knowledge, has not yet been isolated in pure form.

Based on the obtained results a proposed scheme of the reaction between DPBF and hydrogen peroxide is presented in Figure 6. In the first step, DPBF reacts with H_2O_2 to form DBB. The final product – 9-hydroxyanthracen-10(*9H*)-one (oxanthrone) – is formed beside the phenol and oxygen in the second step, as a result of the reaction of two molecules of DBB with two molecules of H_2O_2. The proposed scheme of the reaction remains in a good agreement with quantum chemistry calculations (HF/STO-3G level of theory), indicating the thermodynamic possibility of the formation of both types of products, that are DBB and 9-hydroxyanthracen-10(*9H*)-one under the experimental conditions (standard-state free Gibbs energies ΔG^0 are negative for both reactions and are equal to -8.3 kcal/mol for the formation of DBB from DPBF and -60.9 kcal/mol for the formation of oxanthrone from DBB, respectively).

While carrying out the kinetic measurements on the interactions between DPBF and hydrogen peroxide it was noted that for the temperature and concentrations studied the approximated curves changed mono-exponentially. All curves studied are presented in Figure 7. This shows that the DPBF fluorescence intensity greatly depends on the hydrogen peroxide concentration and consistently decreases. The rate constants for all decays presented were obtained by fitting the rate data to the pseudo-first order kinetic Equation (1):

$$-dF/dT = F_0 \cdot e^{kt} \quad (1)$$

In this equation, the symbol F means fluorescence intensity in time *t*, F_0 refers to maximal fluorescence intensity and *k* is a rate constant. The appropriate plots

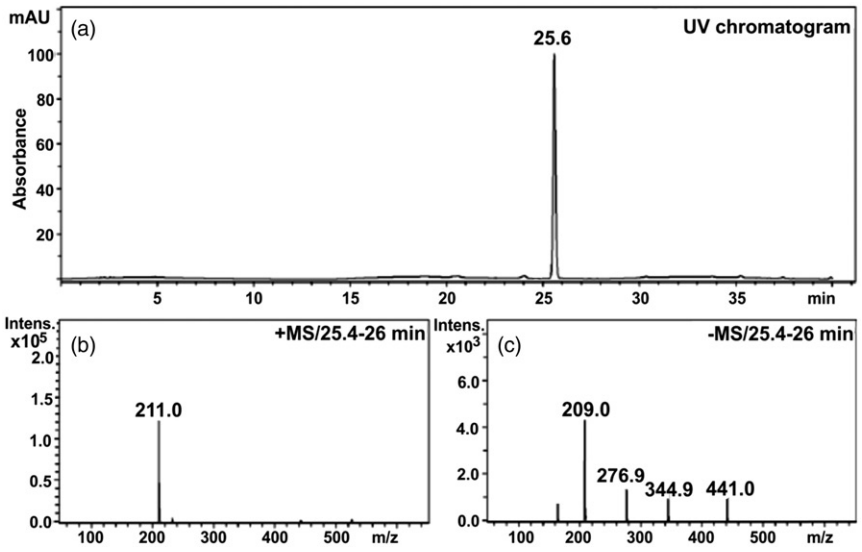

Figure 4. (a) RP-HPLC chromatogram of the product of the reaction between DPBF and H_2O_2 (absorbance measured at 260 nm, the flow rate – 1 mL min^{-1}, a mobile phase – isocratic elution for 10 minutes [DI water/acetonitrile/1% formic acid in water =87.7/2.0/10.3, v/v/v; pH 2.55], followed by 30 min linear gradient to attain 40% acetonitrile); (b) the mass spectrum of the product of the reaction between DPBF with H_2O_2, recorded in the positive ions mode; and (c) the mass spectrum of the product of the reaction of DPBF with H_2O_2, recorded in the negative ions mode.

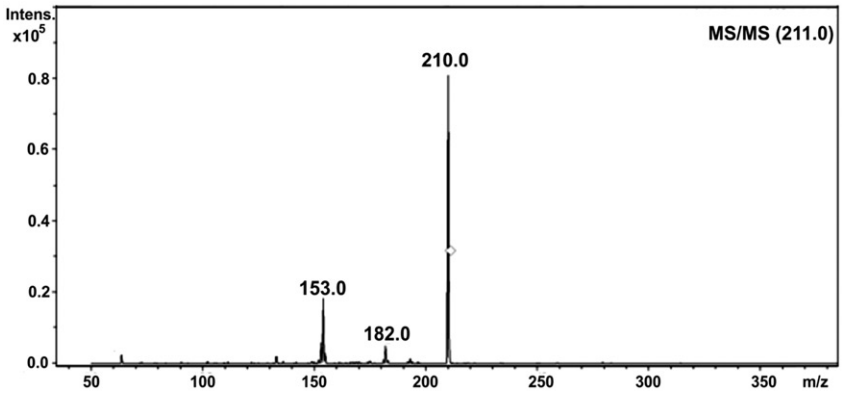

Figure 5. The MS/MS spectrum of $[M + H]^+$ ion of the product of the reaction between DPBF and H_2O_2.

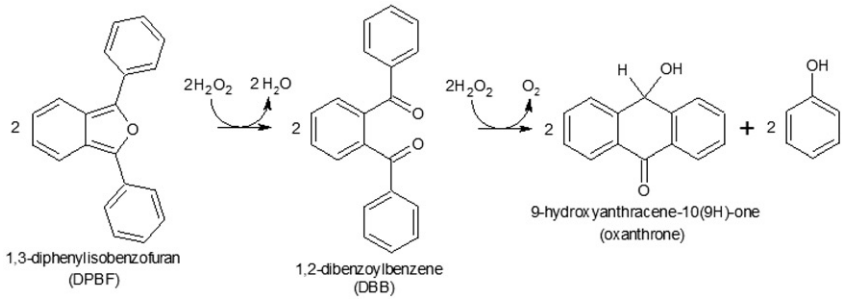

Figure 6. The proposed scheme of the reaction between 1,3-diphenylisobenzofuran and hydrogen peroxide.

are presented in Figure 8. The rate constants at different temperatures are collected in Table 2.

In order to determine limits of detection (LOD) and quantitation (LOQ) of hydrogen peroxide with the use of DPBF, the results of its relative fluorescence intensity measurements were used to prepare a calibration graph. A linear relationship was found between the decrease of the fluorescence intensity of DPBF at

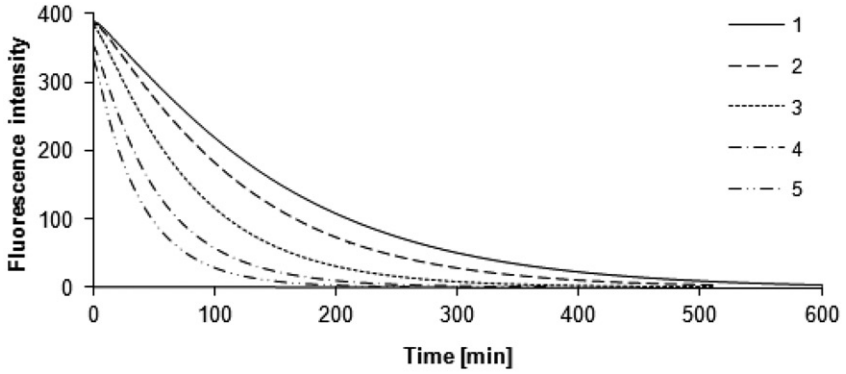

Figure 7. A mono-exponential decay of the fluorescence intensity for the reaction between 1,3-diphenylisobenzofuran and hydrogen peroxide; T = 20 °C, $c_{DPBF} = 2 \times 10^{-5}$ M, $c_{H_2O_2}$ = 1.175 M (1); 1.567 M (2); 1.959 M (3); 2.351 M (4); and 2.752 M (5).

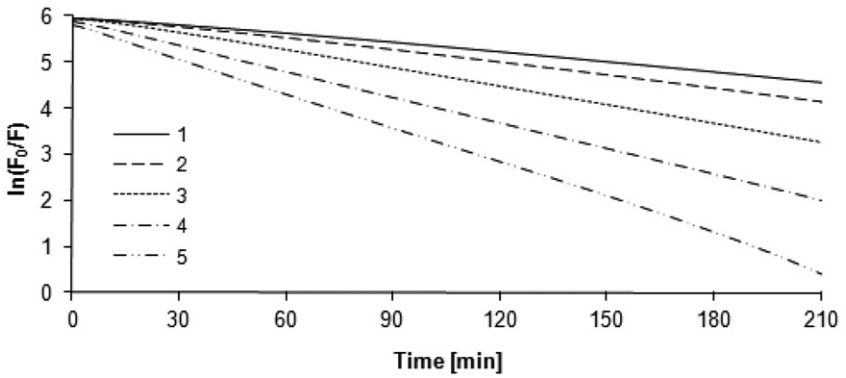

Figure 8. Plots of $\ln(F_0/F)$ versus time for the reaction between 1,3-diphenylisobenzofuran and hydrogen peroxide; T = 20 °C, $c_{DPBF} = 2 \times 10^{-5}$ M, t = 3,5 h; $c_{H_2O_2}$ = 1.175 M (1); 1.567 M (2); 1.959 M (3); 2.351 M (4); and 2.752 M (5).

Table 2. Rate constants, standard deviations of rate constants and correlation coefficients for the reaction between DPBF and H_2O_2 measured at temperature 20 °C and for different hydrogen peroxide concentrations.

	$c_{H_2O_2}$ [M]	k [s^{-1}]	SD [s^{-1}]	R^2
1	1.175	1.351×10^{-4}	0.003×10^{-4}	0.992
2	1.567	1.714×10^{-4}	0.003×10^{-4}	0.995
3	1.959	2.340×10^{-4}	0.002×10^{-4}	0.999
4	2.351	3.131×10^{-4}	0.002×10^{-4}	0.999
5	2.752	4.225×10^{-4}	0.004×10^{-4}	0.999

Table 3. Analytical characteristics of a calibration graph reflecting the relationship between DPBF's fluorescence intensity at 455 nm and H_2O_2 concentration.

Linearity range [mM]	0.196–3.941
Correlation coefficient (R^2)	0.9997
CV [%]	2.78
Limit of detection (LOD) [mM]	0.088
Limit of quantification (LOQ) [mM]	0.123

455 nm and the concentration of hydrogen peroxide in the range of 0.196–3.941 mM. An analytical characteristic of a calibration graph is presented in Table 3. The limit of detection determines the sensitivity of the method, and its value for hydrogen peroxide is satisfactory. Additionally, the coefficient of variation (CV <5%) confirms the high precision of the method.

Conclusions

In this paper, we have shown the results of our studies on the reaction between DPBF and different RNOS as well as nitrite and nitrate. The products of all reactions have been determined. In the case of peroxynitrite anion, superoxide anion, hypochlorite anion, nitrite, and nitrate the product is DBB, the same compound that is formed from DPBF under the action of singlet oxygen, nitrogen dioxide, hydroxyl, alkyloxyl, and alkylperoxyl radicals. Only in the case when hydrogen peroxide was mixed with DPBF, 9-hydroxyanthracen-10(9*H*)-one was likely the main product of the reaction. Moreover, limits of the detection and quantitation of hydrogen peroxide with the use of DPBF have been determined, being at the level of concentrations of 0.1 mM. Furthermore, the kinetics of that reaction was studied and rate constants at different concentrations of hydrogen peroxide have been determined.

The fact that the reaction of DPBF with hydrogen peroxide gives an exceptionally unique product has an

immense influence on the detection and quantitative determination of H_2O_2. As it was confirmed that DPBF reacts totally specifically only with hydrogen peroxide (among other RNOS as well as nitrite and nitrate ions commonly present under biological conditions), it seems to be a unique indicator of the presence of H_2O_2. Determined values of limits of detection and quantitation prove that DPBF may be used in the determination of trace amounts of hydrogen peroxide. Additionally, the method can be successfully applied over relatively wide range of concentrations (ca. 0.2–4.0 mM).

References

1. Lu H, Shi JX, Zhang DM, Shen J, Lin YX, Hang CH, Yin HX. Hemolysate-induced expression of intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 expression in cultured brain microvascular endothelial cells via through ROS-dependent NF-κB pathways. Cell Mol Neurobiol 2009;29:87–95.
2. Griendling KK, Sorescu D, Lassègue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscl Throm Vas 2000;20:2175–2183.
3. Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic Biol Med 2007;42:153–164.
4. del Rio LA, Sandalio LM, Corpas FJ, Palma JM, Barroso JB. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. Plant Physiol 2006;141:330–335.
5. Groves JT. Peroxynitrite: reactive, invasive and enigmatic. Curr Opin Chem Biol 1999;3:226–235.
6. Przedborski S, Ischiropoulos H. Reactive oxygen and nitrogen species: weapons of neuronal destruction in models of Parkinson's disease. Antioxid Redox Signal 2005;7:685–693.
7. Aslan M, Ozben T. Reactive oxygen and nitrogen species in Alzheimer's disease. Curr Alzheimer Res 2004;1:111–119.
8. Kulagina NV, Michael AC. Monitoring hydrogen peroxide in the extracellular space of the brain with amperometric microsensors. Anal Chem 2003;75:4875–4881.
9. Tarpey MM, Fridovich I. Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. Circ Res 2001;89:224–236.
10. Rhee SG, Chang TS, Jeong W, Kang D. Methods for detection and measurement of hydrogen peroxide inside and outside of cells. Mol Cells 2010;29:539–549.
11. Żamojć K, Zdrowowicz M, Jacewicz D, Wyrzykowski D, Chmurzyński L. Fluorescent probes used for detection of hydrogen peroxide under biological conditions. Crit Rev Anal Chem 2016;46:171–200.
12. Ohyashiki T, Nunomura M, Katoh T. Detection of superoxide anion radical in phospholipid liposomal membrane by fluorescence quenching method using 1,3-diphenylisobenzofuran. Biochim Biophys Acta 1999;1421:131–139.
13. Howard JA, Mendenhall GD. Autoxidation and photooxidation of 1,3-diphenylisobenzofuran: a kinetic and product study. Can J Chem 1975;53:2199–2201.
14. Krieg MJ. Determination of singlet oxygen quantum yields with 1,3-diphenylisobenzofuran in model membrane systems. J Biochem Biophys Meth 1993;27:143–149.
15. Carloni P, Damiani E, Greci L, Stipa P, Tanfani F, Tartaglini E, Wozniak M. On the use of 1,3-diphenylisobenzofuran (DPBF). Reactions with carbon and oxygen centered radicals in model and natural systems. Res Chem Intermed 1993;19:395–405.
16. Merkel PB, Kearns DR. Comment regarding the rate constantfor the 1,3-diphenylisobenzofuran and singlet oxygen. J Am Chem Soc 1975;97:462–463.
17. Żamojć K, Jacewicz D, Zdrowowicz M, Chmurzyński L. Kinetics of the reaction between 1,3-diphenylisobenzofuran and nitrogen dioxide studied by steady-state fluorescence. Res Chem Intermediat 2013;39:3023–3031.
18. Ochterski JW, Thermochemistry in Gaussian. Pittsburgh, PA: Gaussian Inc; 2000;1–19.
19. Cramer CJ, Essentials of computational chemistry: theories and models. Chichester, England: John Willey & Sons; 2013.
20. Sikorska C, Ignatowska D, Freza S, Skurski P. The performance of selected ab initio methods in estimating electron binding energies of superhalogen anions. J Theor Comput Chem 2011;10:93–109.
21. Spectral Database for Organic Compounds SDBS. National Institute of Advanced Industrial Science and Technology (AIST), Japan; 2001. Available from: http://sdbs.db.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi.