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Synthesis and physicochemical properties of novel lophine derivatives as chemiluminescent in vitro activators for detection of free radicals

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Abstract The overproduction of free radicals and reactive oxygen species (ROS) has been proved as a basic damage mechanism and cause for oxidative stress. Their measurement is often hindered by the low signal. This could be resolved with the application of luminescent probes (lophines, luminol, lucigenin, etc.). The focus of this study is to synthesize and describe the spectral properties and physicochemical characteristics of lophine and its derivatives as new chemiluminescent in vitro activators. The prepared luminophores are analogues of lophine. Their absorption maxima are in the range 329–340 nm, with good-to-high extinction coefficients. Their spectral properties are measured in methanol and buffer solutions with pH 3.5, 7.4 and 8.5. Same conditions were applied in the systems for chemiluminescent assay in vitro: (1) Fenton's (Fe^{2+}+H_2O_2) for the generation of ·OH and –OH species, (2) Hydrogen peroxide (H_2O_2), (3) Iron (II) sulfate ($FeSO_4$), (4) Glutathione-peroxidase, monitoring the deactivation of H_2O_2, (5) Ascorbic acid-Fenton's reagent: Vit.C appears a strong oxidant, generating free-radical products when applied in higher than physiological concentrations, (6) Reduced α-nicotinamide adenine dinucleotide (NADH)-phenazine methosulfate—for the generation of superoxide radicals ($O_2^{\cdot-}$). Lophine and all novel compounds do not alter the kinetics, except of the dimethyl amino substituted derivative (4-(3a,11b-dihydro-1H-imidazo[4,5-f][1,10]phenanthrolin-2-yl)-N,N-dimethylaniline) in the glutathione-peroxidase system, at pH 8.5. Same derivative showed a comparable or higher activity than Lucigenin and Rhodamine 6G. In neutral and acidic medium, in the Fenton's system, Rhodamine 6G was the most appropriate probe. In alkaline pH and oxidant H_2O_2, Lucigenin induced a signal twice as strong as the signal compared to all other activators.

Keywords Lophine · ROS · Chemiluminescent probes

Introduction

Free radical reactions and the generation of reactive oxygen species (ROS) are vital in metabolism. These processes support homeostasis, functional activity, and the adaptation of the organism. The rate of these reactions is controlled by complex regulatory systems and any imbalances are proven basic damage mechanism for oxidative stress in many diseases. Free radicals such as ROS, reactive nitrogen species (RNS) and lipid peroxidation products are short-living and unstable structures. They chain in metabolism and their evaluation needs high sensitivity methods (Siess 1985; Halliwell 1994; Bartosz 2006). ROS/RNS include hydrogen peroxide; hydroxyl, superoxide and thiyl radicals; carbonate radical-anion; nitric oxide, nitrogen dioxide and peroxynitrite. Their direct detection in biological or other systems is hindered by the low signal and differential distinction between samples. This could be resolved with the application of luminescent probes such as lophines, luminol, lucigenin, etc. (Wardman 2007). However, probes based on reduced dyes lack selectivity and may require an additional catalyst for the reaction. On other hand, Lophine (2,4,5-triphenyl-1*H*-imidazole) is a traditional chemiluminescent and fluorescent probe with various practical applications. Its derivatives are also widely applied for ROS detection (Kenichiro 2003) (http://en.wikipedia.org/wiki/Lucigenin).

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The use of the chemiluminescent methods for practical applications, for instance cellular systems, requires probes to be loaded into cells. ROS can compete with antioxidants and that could negatively affect the intensity of the signal. The products that are being measured can react further with radicals, and intermediate probe radical's products are often reactive towards additional antioxidants (i.e., oxygen, to generate superoxide, etc.). The most optimal results of use of such probes could only be achieved with proper understanding of the limitations and quantitation of all the mechanistic pathways involved (Wardman 2007).

The major goal of this study was to synthesize and describe the spectral properties and physico-chemical characteristics of lophine and its derivatives as new chemiluminescent in vitro activators.

Materials and methods

All materials and solvents were purchased from commercial suppliers (Sigma-Aldrich, etc.) and used without further purification unless otherwise specified. The general procedure for the structures 4-morpholinobenzaldehyde **1a**, 4-(piperidin-1-yl)benzaldehyde **1b**, 4-(pyrrolidin-1-yl)benzaldehyde **1c**, 1,10-phenanthroline-5,6-dione **2** and final products **3a**–**3e** are described and are well known from the literature (Gale and Wilshire 1970; Che et al. 2006; Khosropour 2008; Xie and Chen 2007). The final compounds, except for **3d**, are all novel (never previously described or used as probes). The reactions were carried out in a Diamondback-UD50SH ultrasonic processor operating at frequency of 40 kHz. The progress of the reactions was monitored by TLC (Macherey-Nagel F 254 silica gel; dichloromethane). Analytical samples of the dyes were obtained by recrystallization from ethanol. ^{1}H-NMR spectra was recorded on a Bruker Avance AV 600-MHz instrument in DMSO-d6. Absorption spectra for determining extinction coefficients were detected by a Cecil Aurius CE3021 UV–VIS spectrophotometer in CH_3OH und phosphate buffer). The molar extinction coefficients were determined in methanol with concentration 1×10^{-5} M. Fluorescence maxima of the solutions were determined by a Perkin-Elmer LS45 fluorescence spectrometer in phosphate buffer (pH 3.5, 7.4 and 8.5) at room temperature (22 °C). Stock solutions were prepared in DMSO with concentrations 1×10^{-3} M and further diluted with appropriate buffer to a final concentration of 1×10^{-5} M.

General procedure for synthesis of the compounds

4-(4-(3a,11b-dihydro-1H-imidazo[4,5-f] (Siess 1985; Xie and Chen 2007) phenanthrolin-2-yl)phenyl) morpholine (3a)

The starting compound **1a** (0.0052 mol), 1,10-phenanthroline-5,6-dione 2 (0.005 mol) and fresh ammonium acetate (0.0052 mol) were mixed in 10 ml of ethanol + zirconium (IV) containing catalyst; acetylacetonate (0.001 mol) was added. The mixture was sonicated at room temperature for 30 min. The progress of the reaction was monitored by TLC. The product was precipitated by dilution with water (50–100 ml), filtered off, and purified by column chromatography (dichloromethane–ethyl acetate 5:1), yield 68 %.

^{1}H-NMR (600 MHz, DMSO-d6, δ ppm): 3.27 t 4H (CH_2OCH_2), 3.78 t 4H (CH_2NCH_2), 7.83 d 2H (ArH), 8.16 d 2H (ArH), 7.17 d 2H (ArH), 8.91 d 2H (ArH), 9.02 d 2H (ArH), 13.52 s 1H (NH).

Elemental analysis calcd (%) for $C_{23}H_{21}N_5O$: C 72.42, H 5.02, N 18.36, O 4.19; found: C 72.45, H 5.05, N 18.33, O 4.21.

2-(4-(piperidin-1-yl) phenyl)-3a,11b-dihydro-1H-imidazo[4,5-f] phenanthroline (3b): yield 71 %

^{1}H-NMR (600 MHz, DMSO-d6, δ ppm): 2.05–2.09 m 6H ($CH_2CH_2CH_2$), 4.07 t 4H (CH_2NCH_2), 7.67–7.74 m 2H (ArH), 8.19 d 2H (ArH), 8.80 d 2H (ArH), 8.93 d 2H (ArH), 9.05 d 2H (ArH), 9.15 d 2H (ArH), 13.81 s 1H (NH) (Siess 1985; Xie and Chen 2007).

Elemental analysis calcd (%) for $C_{23}H_{21}N_5O$: C 75.97, H 5.58, N 18.46; found: C 75.95, H 5.53, N 18.49.

2-(4-(pyrrolidin-1-yl) phenyl)-3a,11b-dihydro-1H-imidazo[4,5-f] (Siess 1985; Xie and Chen 2007) phenanthroline (3c): yield 77 %

^{1}H-NMR (600 MHz, DMSO-d6, δ ppm): 1.99 t 4H (CH_2CH_2), 3.33 t 4H (CH_2NCH_2), 6.71 d 2H (ArH), 7.79–7.84 m 2H (ArH), 8.10 d 2H (ArH), 8.90 d 2H (ArH), 9.01 d 2H (ArH), 13.37 s 1H (NH).

Elemental analysis calcd (%) for $C_{23}H_{21}N_5O$: C 75.59, H 5.24, N 19.16; found: C 75.61, H 5.59, N 19.12.

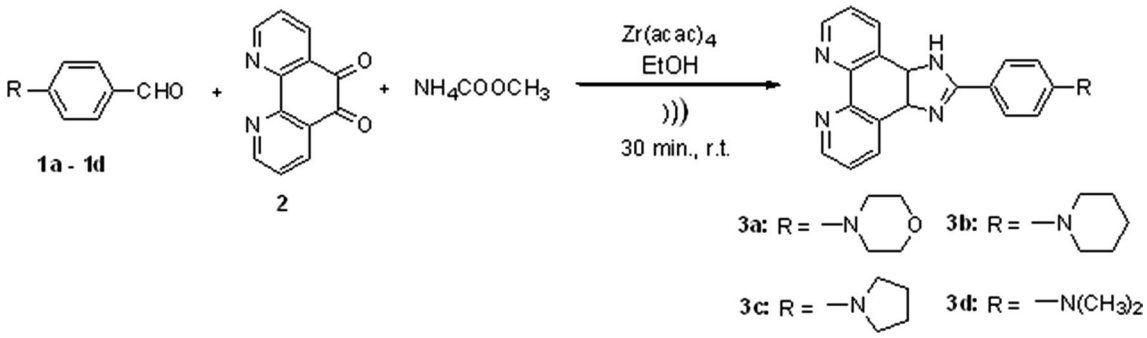
4-(3a,11b-dihydro-1H-imidazo[4,5-f](Siess 1985; Xie and Chen 2007) phenanthrolin-2-yl)-N,N-dimethylaniline (3d) (Parveen 2013): yield 78 %

^{1}H-NMR (600 MHz, DMSO-d6, δ ppm): 3.52 s 6H ($2CH_3$), 7.87–7.94 m 2H (ArH), 8.21 d 2H (ArH), 8.90 d 2H (ArH), 8.95 d 2H (ArH), 9.15 d 2H (ArH), 13.79 s 1H (NH).

Elemental analysis calcd (%) for $C_{23}H_{21}N_5O$: C 74.32, H 5.05, N 20.63; found: C 74.28, H 5.09, N 20.66.

1,4-bis(1H-imidazo[4,5-f] (Siess 1985; Xie and Chen 2007) phenanthrolin-2-yl) benzene (3e): yield: 74 % (Krieg and Manecke 1967; Nakashima et al. 1995)

^{1}H-NMR (600 MHz, DMSO-d6, δ ppm): 7.83 d 4H (ArH), 8.12 d 4H (ArH), 8.65 d 4H (ArH), 9.03 s 4H (ArH), 13.96 s 2H (NH).



Scheme 1 General scheme for the synthesis of the target structures **3a–3d**

Elemental analysis calcd (%) for $C_{23}H_{21}N_5O$: C 74.70, H 3.53, N 21.78; found: C 74.74, H 5.59, N 21.75.

Results and discussion

Conventional procedures for obtaining 2,4,5-trisubstituted imidazoles are based on the interaction between aldehyde, benzyl and ammonium acetate by refluxing conditions, usually in acetic acid.

Recently, the use of zirconium (IV) containing catalysts for synthesis of imidazole heterocycle was described in several publications, emphasizing its advantages such as non-toxicity, low cost and high catalytic activity (Khosropour 2008; Babu and Surendhar 2014). A simple, efficient, and environmentally acceptable procedure for the synthesis of 2,4,5-trisubstituted imidazoles, catalyzed by $Zr(acac)_4$ under ultrasound irradiation was used for the preparation of the final lophine structures **3a**–**3d** (Scheme 1). Such an improved approach offers considerable advantages in comparison to the classical synthetic methods for the preparation of similar compounds—significantly higher yield of the target products, simplified synthetic procedure, and shorter reaction times at mild conditions, preventing the use of toxic and high boiling solvents. The key intermediates required for the synthesis of the target dyes were aryl and substituted aryl aldehydes, which were prepared according to the conventional described in the literature method by the reaction of 4-fluorobenzaldehyde with morpholine, pyrrolidine, or piperidine in dimethyl sulfoxide, at the presence of calcium carbonate (Gale and Wilshire 1970).

It was synthesized 1,4-bis(4,5-diarylimidazolo) (as a benzene derivative of lophine) in order to obtain new structure with higher fluorescent intensity (**3e**, Fig. 1) (Xie and Chen 2007). The properties of the new compound as a chemiluminescent activator were compared with the non-symmetrical compounds.

The prepared luminophores can be described as analogues of lophine, possessing more rigid structure, due to the introduction of a phenanthroline residue, which also influenced their luminescent properties. The final structures of the lophine derivatives were proved by ^{1}H-NMR and elemental analyses.

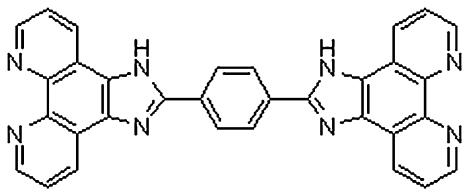


Fig. 1 Structure of the symmetrical bis-derivative **3e**

Spectral properties: influence of the structure rigidity

Absorption and emission spectra of the novel compounds were measured in methanol and buffer solutions with different pH—3.5, 7.4, 8.5.

The absorption maxima of derivatives **3a**–**3d** are in the range 329–340 nm with hypsochromic shifted shoulder around 266–290 nm (Fig. 2), probably due to the aggregation of molecules. The solution of bis-derivative **3e** showed absorption maxima at 275 nm and a shoulder at 334 nm. The absorption maxima of the other derivatives were also observed. They were characterized with low solubility of the structures under these conditions and domination of aggregates. The extinction coefficients determined in methanol solutions were in the range 36,830–53,780 M^{-1} cm^{-1}. In aqueous buffer solutions the absorption maxima of structures **3a**–**3d** exhibit hypochromic shift. Higher pH induced increase of the hypsochromic shifted band. Lophine exhibited higher molar absorptivity in methanol solution, in comparison to the buffer solutions.

All derivatives, with the exception of **3a** and **3c**, possess fluorescence with high intensity in methanol solutions at concentration 1×10^{-5} M (Fig. 3). Structures **3b** and **3e** showed high fluorescent intensity in all three buffer solutions. Compound

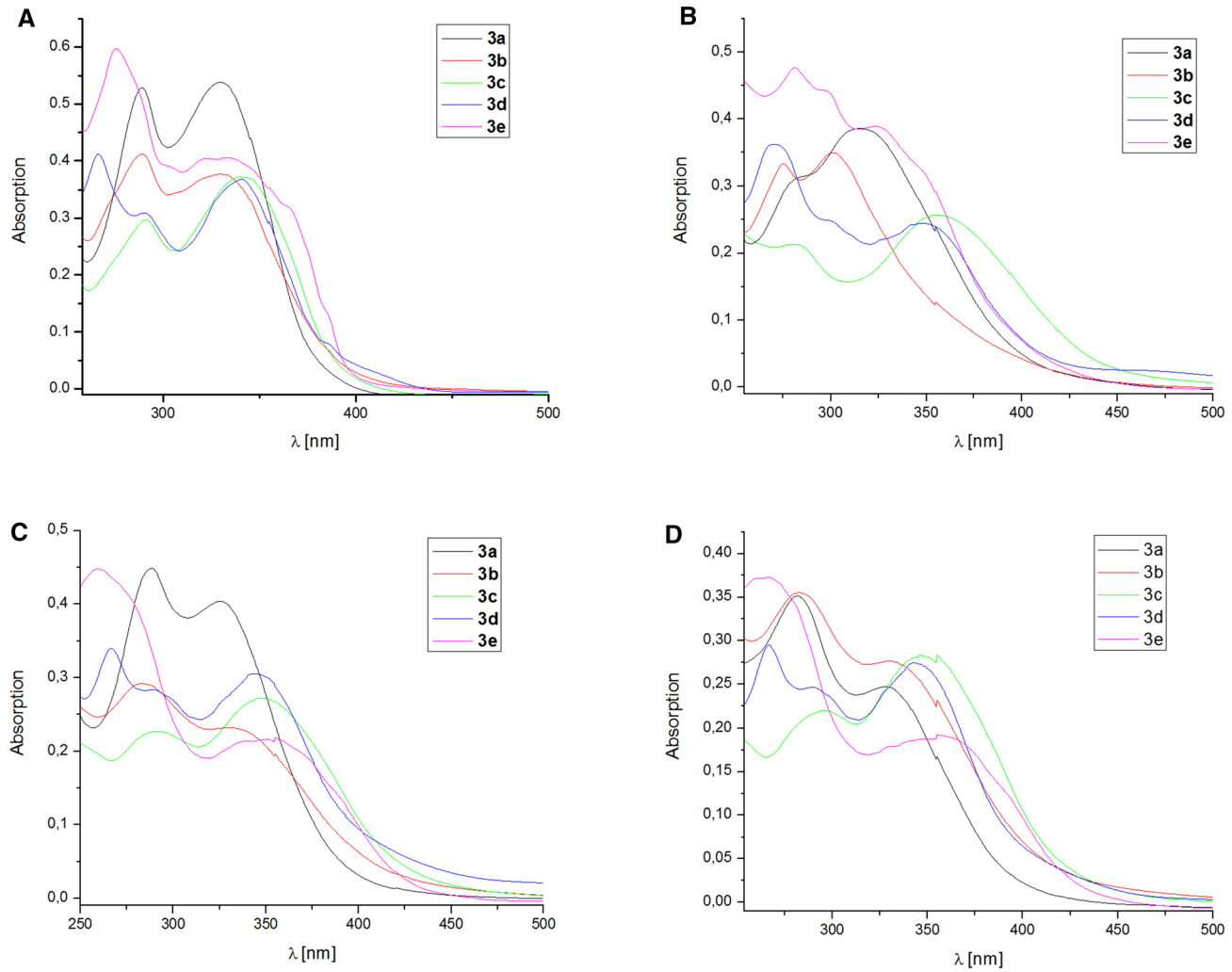


Fig. 2 Absorption spectra of **3a–3f** dyes in methanol (A) and phosphate buffer–pH 3.5 (B) pH 7.4 (C) and pH 8.5 (D) with concentration 1×10^{-5} M

3d exhibited lower fluorescence intensity in methanol in comparison to the other probes, which could be explained with the free rotation around the single C–C bonds of the dimethyl-amino group, which leads to non-radiative transition and decreases the fluorescence intensity. In compound **3a–3d**, such rotation was limited due to structure rigidity and therefore non-radiative transitions were with lower intensity.

Fluorescence with higher intensity was observed in pH 3.5 buffer solutions, which could be explained by the protonation process. The intensity of fluorescence at pH 7.4 and 8.5 is lower than that in pH 3.5 (Fig. 3).

In vitro chemiluminescent comparison for the application of lophine and its derivatives as pro-oxidant and anti-oxidant probes

The chemical activators of chemiluminescence are compounds reacting with ROS or organic free radicals, generating products at excited electronic state. The observed chemiluminescence is usually associated with the transition of the molecules to the ground state, through emission of photons. This type of chemiluminescence is based on the reaction between luminol (5-amino-2,3-dihydro-1,4-ftalazindionom) and hydrogen peroxides, similar to the reactions of H_2O_2 with luminol isoforms (isoluminol (6-amino-2,3-dihydro-1,4-ftalazindionom)), lucigenin (10.10-dimethyl-9,9 biakardnium dinitrate), etc. Hydrogen peroxide, hypochlorite radicals ($ClO^{\cdot -}$), and other oxygen radicals such as superoxide ($O_2^{\cdot -}$) and hydroxyl ($HO^{\cdot}$) are vital in metabolism (Yamaguchi et al. 2010; Lu et al. 2006).

When chemiluminescent probes are used for detection of clinically important metabolites, it is of crucial importance that the luminescence activator does not affect the progress of the reaction. Chemiluminescent probes should only increase the luminous intensity—the quantum yield of the reaction, but not interfere with the kinetics of the reaction.

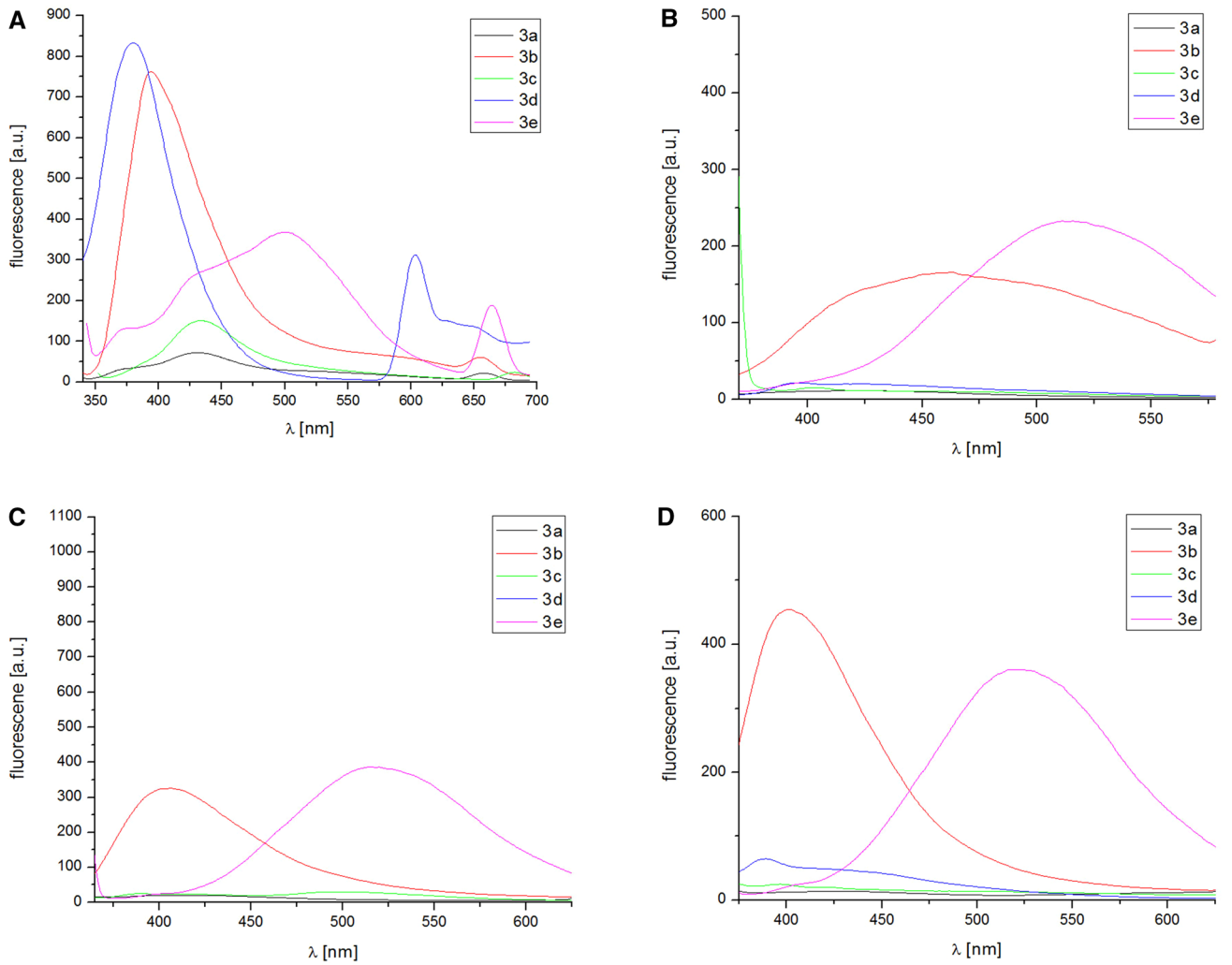


Fig. 3 Fluorescent spectra of structures **3a**–**3e** in methanol (A) and phosphate buffer pH 3.5 (B) pH 7.4 (C) and pH 8.5 (D) at concentration 1×10^{-5} M

The basis of this specific mechanism of action is the physical process of interaction and activation between the compounds—the energy transfer from the molecules of the product (for example, a ketone) to the molecules of the activator.

Basic chemiluminescent probes

Rhodamine 6G ($C_{28}H_{31}N_2O_3Cl$, Mr 479.02)[1]

Rhodamine 6G is a fluorescent molecule, emitting light with a peak at 566 nm. It is traditionally used as a dye, but recently is more frequently applied as a tool for the detection of biological substances in molecular biology.

Luminol ($C_8H_7N_3O_2$, Mr 177.16)[2]

Luminol emits bright blue glow, when mixed with a suitable oxidant (Fig. 4). Luminol is frequently used to detect traces of blood (since it contains iron in hemoglobin). Luminol rapidly reacts to other strong oxidizing compounds and could detect copper, iron, cyanide and others[3]

[1] http://en.wikipedia.org/wiki/Rhodamine, http://chemicalland21.com/specialtychem/finechem/VICTORIA%20BLUE%20B.htm.

[2] http://en.wikipedia.org/wiki/Luminol.

[3] http://chemicalland21.com/specialtychem/finechem/VICTORIA%20BLUE%20B.htm.

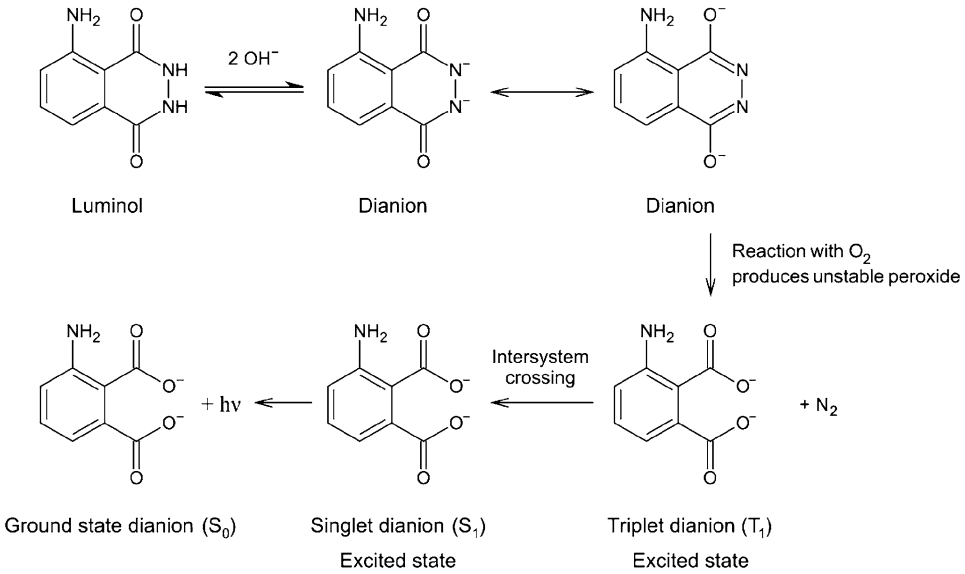


Fig. 4 Mechanism of the chemiluminescence of luminol: the reaction between luminol and hydroxide anion generates a dianion. The oxygen produced by hydrogenperoxide reacts to the dianion of luminol. The product of this reaction is an organic peroxide, which is very unstable and immediately decomposes with the loss of nitrogen in the production of 3-aminopthalic acid, with electrons in the excited electronic state. The energy excess is emitted as light (http://en.wikipedia.org/wiki/Luminol)

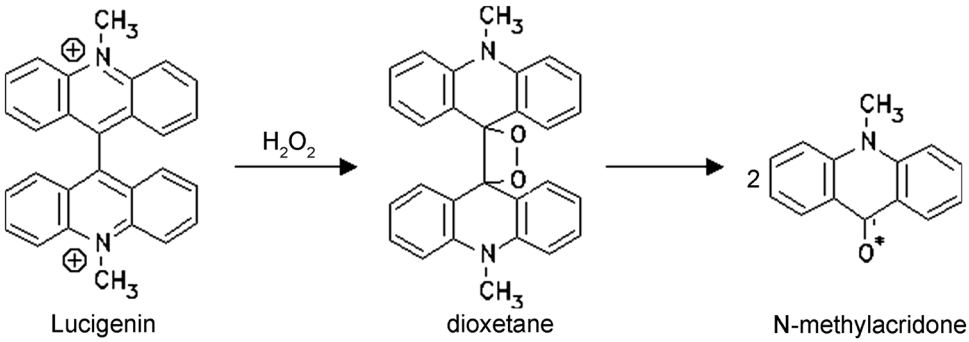


Fig. 5 Mechanism of the chemiluminescence of lucigenin: lucigenin undergoes reaction with hydrogen peroxide and forms unstable dioxetan lucigenin forms. When they disslolve, it is produced N-methylacridon in electronic excited state

Lucigenin ($C_{28}H_{22}N_4O_6$, Mr 510.5)[4]

Lucigenin is a specific chemiluminescence probe used for detection and measurements of superoxide radicals.[5] Lucigenin and luminol could be successfully used for non-specific detection of hydrogen peroxide (www.google.bg/imgres?imgurl=http://www.demochem.de/Grafik/D-Luminol-e.gif&imgrefurl=http://www.chemie.uni-regensburg.de/Organische_Chemie/Didaktik/Keusch/D-Luminol-e.htm&usg=__2PZXQYHS0BtMnEk7NLZffuG mDI=&h=320&w=617&sz=7&hl=bg&start=4&zoom=1&tbnid=jOKrOiB3fMB6bM:&tbnh=71&tbnw=136&ei=0mVpTafgAoGEswbc1ez0DA&prev=/images%3Fq%3DLucigenin%26um%3D1%26hl%3Dbg%26client%3Dfirefox-a%26sa%3DN%26rls%3Dcom.mozilla.campus:en-US:official%26tbs%3Disch:1&um=1&itbs=1). The mechanism of lucigenin chemiluminescence is presented at Fig. 5.

Lophine ($C_{21}H_{16}N_2$, Mr 293.36)[6]

Lophine is a fluorescent probes used in neutralization assays. The chemiluminescent reaction of lophine emits yellow light when reacting with oxygen in the presence of a strong base. For maximum quantum yield, lophine should be dissolved in DMSO. The mechanism of chemiluminescence is shown at Fig. 6.

[4] http://en.wikipedia.org/wiki/Lucigenin.

[5] http://sciencelinks.jp/j-east/article/200324/000020032403A0643390.php.

[6] http://journals.iucr.org/e/issues/2009/04/00/hb2915/hb2915bdy.html#BB16.

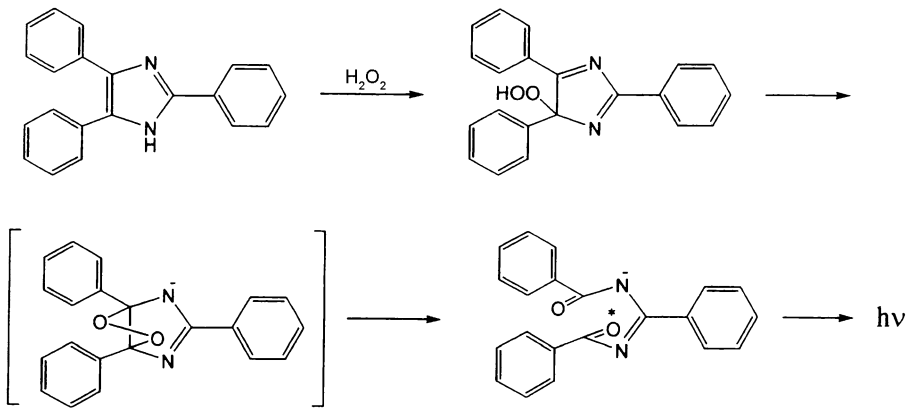


Fig. 6 Mechanism of the chemiluminescence of lophine

In vitro systems for induced chemiluminescence

The selection of a suitable activator that enhances the chemiluminescent signal with distinction in systems for generation of free radicals and ROS is crucial for practical medical and biological applications. The intensity of the signal depends proportionally on factors such as temperature and acidity of the medium. In routine analysis is usually applied to alkaline pH, which enhances much higher quantum yield. However, for a thorough description of the probe's activity, it should be tested in acidic, neutral, and alkaline media, in standard systems for the generation of ROS. Since the chemiluminescent kinetics curves vary, due to the oxidation reaction response, it is needed longer period monitoring of the reactions than the instantaneous flash registered in the first seconds of the interaction.

Detailed tests comparing lophine and the first synthesized derivative–lophine 5/**3d** with standard luminescent activators (luminol, lucigenin, and Rhodamine 6G) showed the following results: the kinetic records for all chemiluminescent probes showed fast flash up to the tenth second after the start of the interaction. Different activators (all dissolved in DMSO) show various intensities of emitted luminescence, depending on the reaction conditions and type of chemical system used for the generation of ROS.

- Lophine manifests as an activator of chemiluminescence at alkaline pH and oxidant Fe^{2+}. It induces the signal about 50 % in comparison to Rhodamine 6G, which is a typical physical activator of luminescence and in comparison to the other chemical activators.
- At alkaline and acidic pH in the Fenton's system (ferrous sulfate-hydrogen peroxide), the strongest signal demonstrates Rhodamine 6G—60 % stronger than luminol and the other selected activators. The kinetics curve of this probe presents a quick and sharp initial flash in the first 15 s of the interaction. Rhodamine 6G is most suitable for the Fenton system in neutral medium because it is a physical activator and does not react to the reagents but takes part in the process of migration of energy from the products of the reaction to the activator.
- Lucigenin extremely intensifies the chemiluminescent emission—three times stronger than any other probe, at alkaline pH in the enzymatic oxidase system for generation of superoxide radicals. It is the only suitable probe for this enzyme system and medium.
- Surprisingly, at acidic pH in an oxidase enzyme system, lophine exhibits the strongest signal—30 % more in comparison to all the other activators. Lucigenin, luminol and Rhodamine 6G induce equal signals, slightly brighter. Further studies are needed to study the interaction between lophine and the generated in the system superoxide radicals.
- Lucigenin induces a signal more than twice as strong in comparison to the other activators at alkaline pH and oxidant H_2O_2. Lophine, Rhodamine 6G and luminol exhibit the lowest chemiluminescent signal in that model in vitro system.

In conclusion, we found that at alkaline pH and an oxidant iron, lophine induces highest quantum yield and is the best application choice for this system. It behaves similarly in the oxidase enzyme system at pH 3.5.

- The synthesized chemiluminescence activator—a derivative of lophine–lophine 5/**3d**, ($C_{21}H_{19}N_5$, M_R 339.39) shows a comparable or higher activity than the commonly applied chemiluminescent probes lucigenin and Rhodamine 6G.
- In a system with pH 3.5 and oxidant hydrogen peroxide, the derivative of lophine (5/**3d**) and luminol demonstrate a significant and reliable more intensive signal in comparison to Rhodamine 6G and lucigenin. The kinetics of all four probes is identical, following a common curve, but varying intensity.

- At neutral and acidic pH and an oxidant iron, the derivative of lophine (5/**3d**) demonstrates the strongest signal–twice higher than the other tested probes.
- In neutral and acidic medium in Fenton's system, Rhodamine 6G emits much more light, compared to the other probes.
- At alkaline pH and oxidant H_2O_2, lucigenin induces a twice stronger signal in comparison to all other activators.

The obtained pilot results show that the application of the enzyme xanthine–xanthine oxidase system for the generation of reactive species is not suitable for assay of the researched effects.

After the discussion of the obtained initial and detailed results, there are also other synthesized derivatives of lophine, whose activity is tested and compared in the following selected for this purpose in vitro model chemiluminescent systems (the given concentrations are final, within total volume 2 ml; all activators are dissolved in DMSO, dripped as 20 μl, in order to minimize the suppression of the luminescence with the addition of the organic solvent):

System 1

Each sample contains 0.2 M sodium hydrogen phosphate buffer with a chosen pH, Fenton's reagent: $FeSO_4$ (5.10^{-4} M)—H_2O_2 (1.5 %) and a chemiluminescent probe (10^{-4} M). Free radicals and ROS are generated, according to the reaction by the scheme:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^- \quad (1)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+. \quad (2)$$

System 2

Each sample contains 0.2 M sodium hydrogen phosphate buffer, with a chosen pH, H_2O_2 (1.5 %) and a chemiluminescent probe (10^{-4} M). In this system, hydrogen peroxide reacts as an oxidizing agent and a reactive oxygen species.

System 3

Each sample contains 0.2 M sodium hydrogen phosphate buffer with a chosen pH, $FeSO_4$

(5.10^{-4} M) and a chemiluminescent probe (10^{-4} M). The oxidizer in this system is iron sulfate. It also appears to be a source of iron ions, which could affect the activity of the tested activators.

System 4

Each sample contains 0.2 M sodium hydrogen phosphate buffer with a chosen pH, glutathione (5.10^{-4} M), H_2O_2 (1.5 %), peroxidase (9459U) and a chemiluminescent probe (10^{-4} M). Glutathione peroxidase is an Se-containing enzyme that catalyzes the following physiologically important reactions:

$$H_2O_2 + 2G\text{-}SH \rightarrow G\text{-}SS\text{-}G + 2H_2O$$

$$ROOH + 2G\text{-}SH \rightarrow G\text{-}SS\text{-}G + 2H_2O + ROH.$$

System 5

Each sample contains 0.2 M sodium hydrogen phosphate buffer, with a chosen pH, ascorbic acid (5×10^{-4} M), $FeSO_4$ (5×10^{-4} M), H_2O_2 (1×5 %) and a chemiluminescent probe (10^{-4} M).

System 6

Each sample contains 0.2 M sodium hydrogen phosphate buffer, with a chosen pH, NAD.H (10^{-4} M), phenazine-meta-sulfate (FMS), (10^{-6} M) and a chemiluminescence probe (10^{-4} M). The scheme for generation of superoxide radicals in this chemical system is presented below (Nishikimi et al. 1972; Faulkner and Fridovich 1993):

$$FMS + NAD.H + H^+ \rightarrow FMS.H_2 + NAD^+$$

$$FMS.H_2 + FMS \rightarrow 2FMS.H^{\cdot}$$

$$FMS.H^{\cdot} + O_2 \rightarrow FMS + O_2^{-\cdot} + H^+$$

Lucigenin-enhanced chemiluminescence registers the concentration of $O_2^{\cdot -}$radicals at 470 nm (Scheme 2):

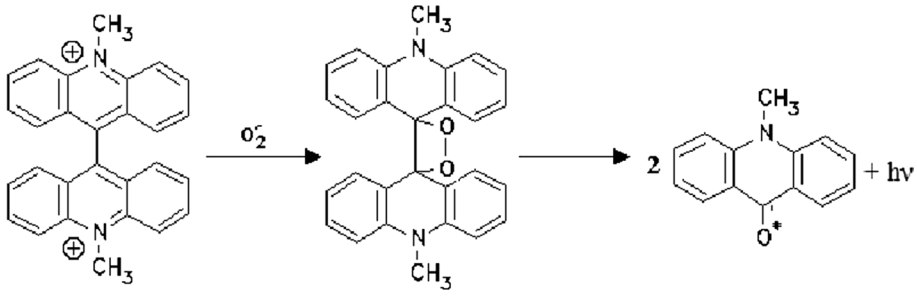
All measurements are performed in triplicate at constant temperature (25 °C) and acidity of the medium at three chosen pH levels—3.5, 7.4, and 8.5, by chemiluminometer LKB1251, Sweden. All studied kinetics were monitored for 3-min periods at 3-s intervals.

Conclusions

The summarized results are presented in the figures below. The obtained data are processed statistically. We calculated the mean and standard variation of the mean for each point on the kinetic curve; it is defined the maximum point for each curve.

The obtained results for the activity of the newly synthesized chemiluminescent probes, as compared with conventional activators lucigenin and luminol, show the following trends (Fig. 7):

- System 1 (Fig. 7a1, a2, a3): at neutral and alkaline pH (Fig. 7a2, a3), lucigenin shows an expected very strong



Scheme 2 The interaction between Lucigenin and a superoxide radical (Faulkner and Fridovich 1993)

effect comparable to earlier data obtained by our team and other authors (Wardman 2007; Kenichiro 2003; Krieg and Manecke 1967; Babu and Surendhar 2014; Владимиров 1997). Lophine 3/**3b** exhibits comparable to lucigenin activity at pH 7.4 (Fig. 7a2); at pH 8.5 lophine 3 retains its absolute activity, demonstrating an increase of the signal by approximately 60 % compared to the control, but 40 times lower when compared to lucigenin (Fig. 7a3). Luminol, lophine, and its newly synthesized other derivatives have no luminescent activity in the studied in vitro model systems.

- System 2 (Fig. 7b1, b2, b3): An oxidizing agent and a reactive oxygen species is hydrogen peroxide. This system could be a reference system to system 1. Lucigenin exhibits expected strong luminescent activity at neutral and alkaline pH (Fig. 7b2, b3). Luminol, lophine, and its newly synthesized derivatives show prominent luminescent activity at acid and neutral pH and at alkaline pH suppress the light emission.
- System 3 (Fig. 7c1, c2, c3): The oxidant is Fe^{2+} ions. This system could also be a reference system to system 1. We registered no significant effects of all the studied compounds. Iron ions do not affect the activity of the tested probes.
- System 4 (Fig. 7d1, d2, d3) is a typical model enzyme system that monitors the deactivation of the hydrogen peroxide, respectively, the activity of the enzyme. However, in this case, we test the capacity of the chemiluminescent probe to faithfully amplify the signal of this reaction. At acidic conditions, we observed no significant effect, which can be explained by the deactivation of the enzyme and consequent suppression of the luminescent response (Fig. 7d1). At physiological pH (7.4), the most efficient probes are lucigenin and lophine 3/ 3b (80 % higher activity than lucigenin) (Fig. 7d2); luminol and the other lophine derivatives have poor luminescent activity. At alkaline pH, an outstanding effect have lucigenin, lophine 3 (3b) and lophine 5 (3d)—respectively 50 and 160 % higher activity than the control (Fig. 7d3). It should be noticed that lophine 5 (3d) is very unstable during the reaction, although its high intensifying ability. Luminol and the novel synthesized derivatives of lophine inhibit the light emission to different extent.
- System 5 (Fig. 7e1, e2, e3): At the given concentration, when combined with the Fenton's reagent, ascorbic acid (vitamin C) exhibits strong oxidant effect, generating free-radical products. At acidic pH lophine 5 (3d) has low luminescent activity (3 times amplification compared to controls, Fig. 7e1). At physiological pH (7.4), lucigenin, lophine 1 (3a), and lophine 3 (3b) exhibit a significant and comparable between each other enhancing effect (19 times amplification compared to controls, (Fig. 7e2). At pH 8.5, lucigenin is most active; lophine 3 (3b) amplifies the signal 13 times when compared to the control, but 55 times lower in comparison to lucigenin (Fig. 7e3).
- System 6 (Fig. 7f1, f2, f3) is a typical model system for in vitro chemical generation of superoxide radicals. A standard applied probe is lucigenin. It exhibits expected high activity at alkaline pH (about 25 times induction, compared to the control) and about three times at pH 7.4 (Fig. 7f2, f3). Luminol, lophine, and the newly synthesized derivatives of lophine show no trap activity towards the generated superoxide radicals in the various model conditions.
- The registered tracked curves in varying pH oxidation reactions and generation of free radicals show that lophine and all its newly synthesized derivatives do not alter the reaction kinetics, except of lophine 5/3d in system 4, at pH 8.5 (Fig. 7). All interactions follow the kinetic curve of the control system. There are usually registered three main phases of the interaction: (1) spontaneously flash (0–10 s) (2) fast chemiluminescence (10–50 s) and (3) latent period (50–300 s). With lophine 5/3d, the curve has the same form, but the phases are shifted and delayed in time in system 4 at pH 8.5. For comparison, lucigenin in system 1, 2, and 4 at pH 8.5, amplify the signal with time, due to the accumulation and registration of secondary free radicals and reactive oxygen species.

Further research is needed on lucigenin and lophine 3 (3b) in systems 1 and 4, and lophine 1 (3a) and lophine 3

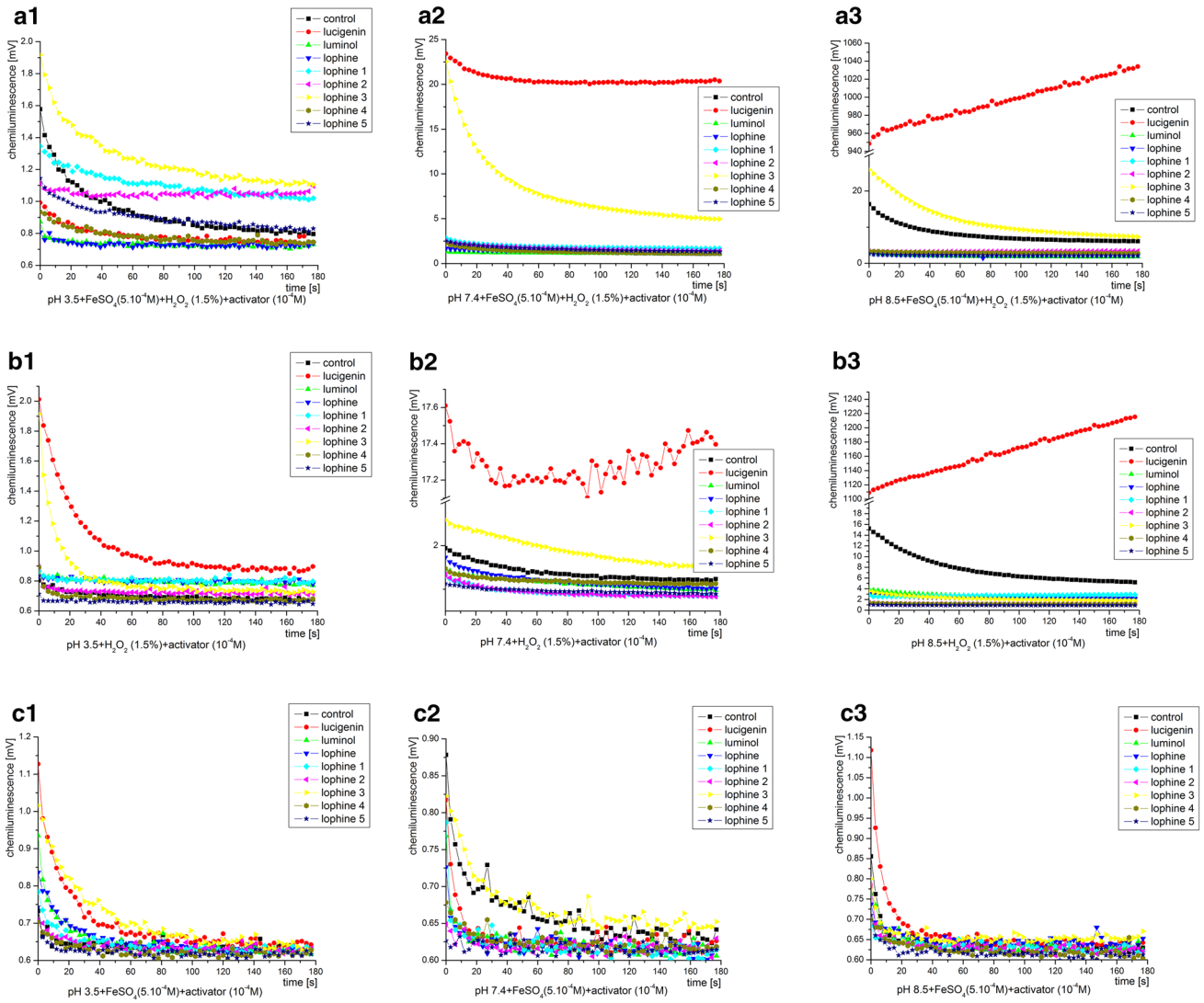


Fig. 7 Chemiluminescent kinetic curves of lophine and its newly synthesized derivatives compared to luminol and lucigenin in model systems for oxidation and generation of reactive oxygen species

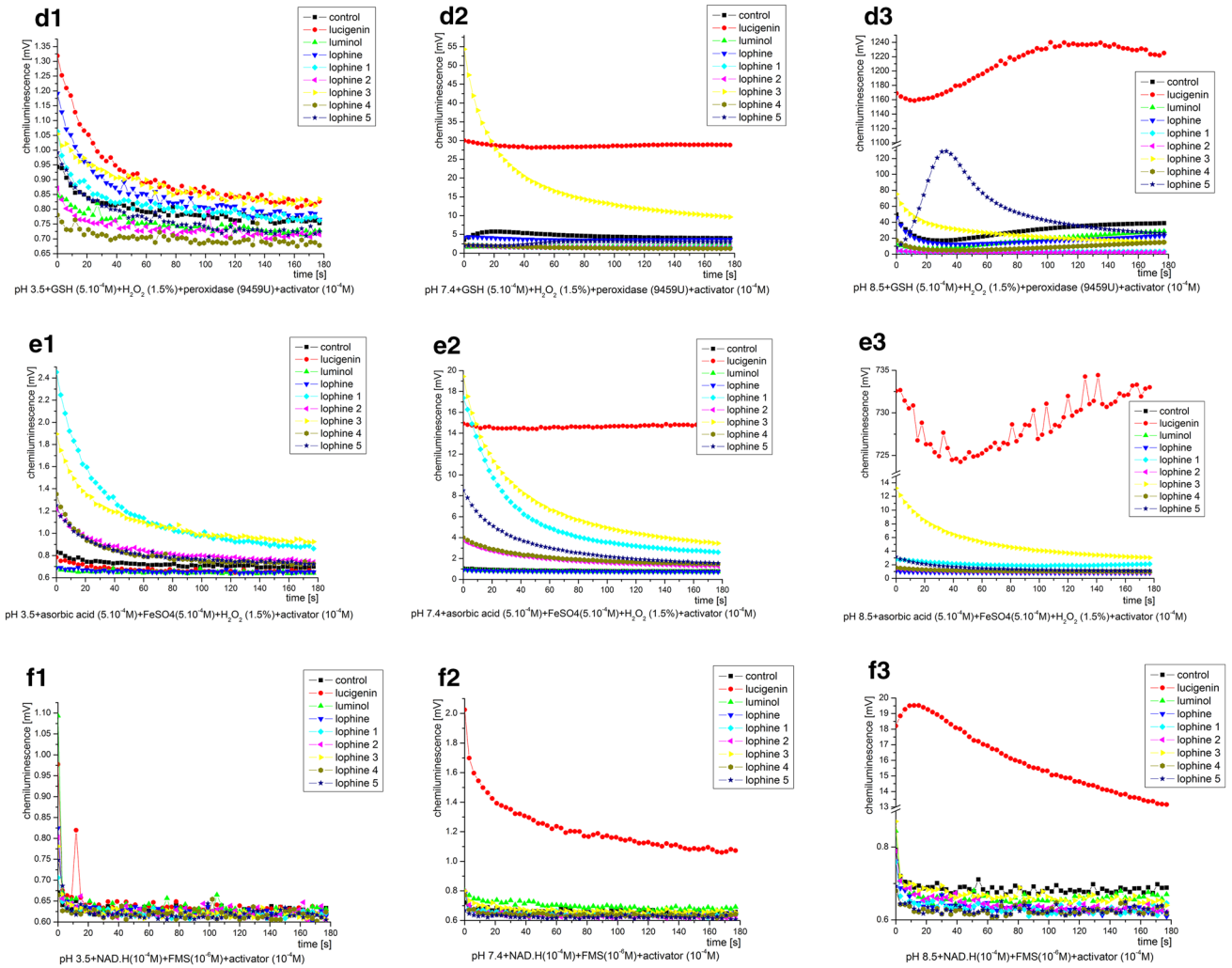


Fig. 7 continued

(3b) in system 5 for biological and medical practical applications (Vladimirov et al. 1991; Armstrong and Browne 1994; Lu et al. 1996).

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Compliance with Ethical Standards

Conflict of interest Each author declares no financial or commercial conflicts of interest.

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