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Effective chemiluminogenic systems based on acridinium esters bearing substituents of various electronic and steric properties†

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A series of 10-methyl-9-(phenoxyacetyl)acridinium trifluoromethanesulfonates (XAEs), bearing substituents of various characteristics in the lateral benzene ring (2-halogen, 2,6-dihalogen, 2-trifluoromethyl, 2-nitro, 2-methoxy, 3-halogen and 4-halogen) were synthesized with high yields, identified and subjected to a physicochemical and theoretical investigation. The main task of the work was to assess the mechanism and optimal conditions of light emission in various liquid systems based on the above salts in order to evaluate their potential usefulness as chemiluminescence (CL) labels and indicators in ultra-sensitive analyses. Density functional theory (DFT) calculations were performed to investigate the detailed mechanism of the oxidation of 9-substituted 10-methylacridinium cations involved in XAEs by hydrogen peroxide in alkaline media. Three general pathways were drawn, which are termed the "light path" (chemiluminogenic) and there were two "dark paths" (non-chemiluminogenic): hydrolytic and "pseudobase". The CL time profiles, triggered in alkaline solutions containing hydrogen peroxide, enabled us to establish crucial physicochemical parameters, including pseudo-first order kinetic constants of CL decay and relative efficiencies of emission. In order to optimize the systems' luminogenic performance, different bases, such as sodium hydroxide, tetrabutylammonium hydroxide (TBAOH) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), as well as enhancers, such as cationic, zwitterionic and neutral surfactants (cetyltrimethylammonium chloride (CTAC), *N,N*-dimethyldodecylammonio-1,3-propane sulfonate (DDAPS) and Triton X-100, respectively) were tested. The results revealed the optimal CL systems, which enabled us to obtain substantially higher emissions than typical ones, based on acridinium esters or luminol. The derived parameters, characterizing the potential utility of the acridinium esters, such as stability in aqueous environments and usefulness (the product of emission efficiency and stability at a given pH), enabled us to reveal the best candidates and their practical applications. The post-reaction mixtures, analyzed by means of chromatography (RP-HPLC) and mass spectrometry (ESI-MS), allowed us to verify the occurrence and population of the products that were theoretically predicted, *i.e.* 10-methyl-9-acridinone (NMAON), 10-methylacridinium-9-carboxylic acid (NMACA) and substituted phenols (RPHOHs).

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Introduction

Chemiluminogenic acridinium esters (AE), first described by F. McCapra in the 1960s, were introduced in medical diagnostics about twenty years later.^{1,2} In contrast to the commonly employed phthalic acid hydrazides, they do not require the use of a catalyst to trigger CL and, in general, are characterized by

a high temporary intensity of emission – up to 100-fold higher than luminol.³ The quantum yields of most AEs are at the level of 2% in aqueous environments, which can be considered a high value compared to luminol ($\phi_{CL} = 1.23\%$, the standard used in luminometric assays⁴) enabling the direct quantification of the analyte in a given system at the femtomolar or even lower levels of concentrations.^{5,6} The carriers of AEs and some other quaternary acridinium salts (9-sulphonamides, 9-cyanides) in modern analysis are also determined by the possibility of their relatively easy chemical modifications and the simplicity of the CL assays.^{7,8} Today, applications of AEs cover a wide area of immunodiagnostics, where they are used in a chemically bound form (CL labels),⁸ as well as in environmental analyses – where they are utilized in the free form

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(CL indicators).^{6,9} Acridinium labels and indicators have been employed, among others, for assaying human α -fetoprotein, gonadotropins, thyroid stimulating hormone (TSH), viruses, markers of cancer cells, nucleic acids, oxidative enzymes (e.g. glucose oxidase, horseradish peroxidase) and other important bioanalytes.^{1–3,8,10,11} AEs were also utilized to determine trace amounts of environmental pollutants, such as hydrogen peroxide or substituted phenols.^{3,12} Recently, acridinium salts were employed in serial clinical assays performed with the aid of automated clinical analyzers – such as Abbott Prism® or Siemens Advia Centaur® – in the routine monitoring of blood quality for the presence of HBC, HCV, HIV-1, HIV-2 and other viruses.¹⁰ Other examples of diagnostic methods developed with the participation of AEs are affinity tests, applied in the investigation of nucleic acids (also in automated variants).^{13,14} The latter approach makes an alternative for DNA sequencing, gel electrophoresis, mass spectrometry and other techniques employed in nucleic acid chemistry. Recently, new variants of nucleic acid hybridization AE-based tests, with no need for their amplification, were proposed.^{15,16} The introduction of the electron-donating alkoxy substituents (e.g. OCH₃) into the conjugated ring system of AE resulted in a shift of the emission range towards longer wavelengths, and a slight enhancement of its efficiency.^{16,17} The authors also stated that the introduction into the benzene ring electron-withdrawing Br atoms (*ortho* positions) of AE enables the triggering of the ring efficient emission at relatively low pH values (pH = 9). The proposed design of new AE labels should guarantee their increased resistance to hydrolysis and good dynamics of light emission (which finally enables lower limits of detection). Other studies on variously substituted acridinium esters indicated that the introduction of oxygen-rich groups (such as polyethylene glycol (PEG) residues or alkylsulfopropyl groups) and bulky polar substituents (such as Br) into specific locations of the aromatic ring systems results in a substantial increase in the luminogenic capability of the respective compounds (up to four times).^{18–21} The polar groups improve the solubility of the related derivatives and, presumably, help to reduce their hydrophobic aggregation after conjugation with the bioanalyte accompanied with low non-specific binding.

Our previous investigations on the CL of AEs were focused on the family of alkylphenyl esters of 10-methylacridinium-9-carboxylic acids – molecular systems frequently incorporated in commercially available CL labels.^{22,23} Experimentally established values – CL decay kinetic constants and integral efficiency of emission – showed correlations with the theoretically predicted parameters, such as activation barriers and specified geometrical parameters of the molecular systems involved in the light-emitting step. The presence of bulky alkyl substituents at the *ortho*-positions of the benzene ring drastically decreased the reaction course; their slight electron-donating character is manifested by the same effect, but more subtle. Among the interdependences revealed within this family of CL systems was the correlation between the AE's emission intensity and the volume of the hydration layer of the leaving group²² during the light-generating step. The latter is

an important observation for the rational design of new acridine-based labels and indicators.

Natrajan's group determined the influence of various types of surfactants on the behavior of zwitterionic and alkyl-substituted AE labels and their protein conjugates.²⁴

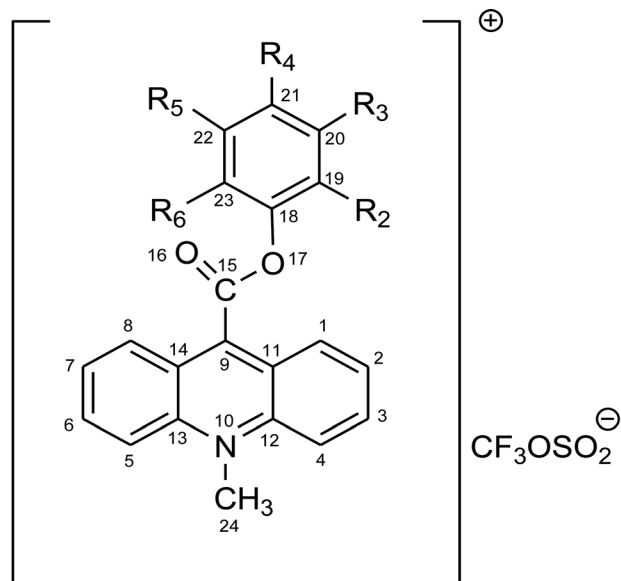
To take a deeper look at the physicochemistry and lumino-genic properties of the AEs, we have synthesized a series of chemiluminogenic salts, differing in terms of the location and the electronic and steric properties of the substituent(s) introduced into the lateral benzene ring (XAEs). In the past, we performed studies on the crystalline structures of XAEs, applying X-ray crystallography methods, as they demonstrate interesting features in the solid phase.^{25–31} On the other hand, applying differential scanning calorimetry methods to study their thermal behavior enabled us to assess important physico-chemical parameters – such as the crystal lattice energy, enthalpies of volatilization and formation and others.³²

In this work, we present investigations of the luminogenic properties of XAEs, including theoretical, structural and physicochemical studies on the liquid phase. Since most of the compounds presented here express higher emission efficiencies than their alkyl analogues,²² we attempt to reveal the possible reason(s) for this phenomenon, including analyses of the post-CL reaction mixtures. Within this work we also took into consideration the emission properties and crucial analytical parameters of XAEs in systems containing cationic, zwitterionic or neutral surfactants, being employed as CL enhancers in this context.²⁴ In order to understand the observed differences in physicochemical features, we applied computational methods based on Density Functional Theory (DFT), which enabled us to reveal the thermodynamic and kinetics parameters and to propose possible reaction pathways for chemiluminogenic oxidation of XAEs in various alkaline environments.

Results and discussion

Origin of chemiluminescence

Previous investigations by us²² and others^{8,33} on the mechanism of the oxidation of 9-substituted 10-methylacridinium cations by H₂O₂ in alkaline media indicated that the primary steps of the reaction of XAEs with either OOH[–] or OH[–] are mainly determined by the location of the electrophilic center in the cation. According to the frontier orbital theory,³⁴ the Lowest Unoccupied Molecular Orbital (LUMO) distribution of an electrophilic species determines the molecular center sensitive to nucleophilic attack. The value of the LCAO coefficient of the p_z atomic orbital in the XAEs' LUMO is *ca.* 10 times higher at the endocyclic C(9) than at the C(15) carbonyl atom (0.30 and 0.03, respectively; average for 2-F, 2-CF₃, 2-NO₂, 2-OCH₃ and 2,6-diBr) (Scheme 1). This implies that C(9) rather than C(15) marks the site of the primary nucleophilic attack of the above-mentioned anions, despite the fact that the Mulliken partial charge at C(9) is lower than at the C(15) atom of the carbonyl group (0.09 and 0.52, respectively; average for 2-F, 2-CF₃,

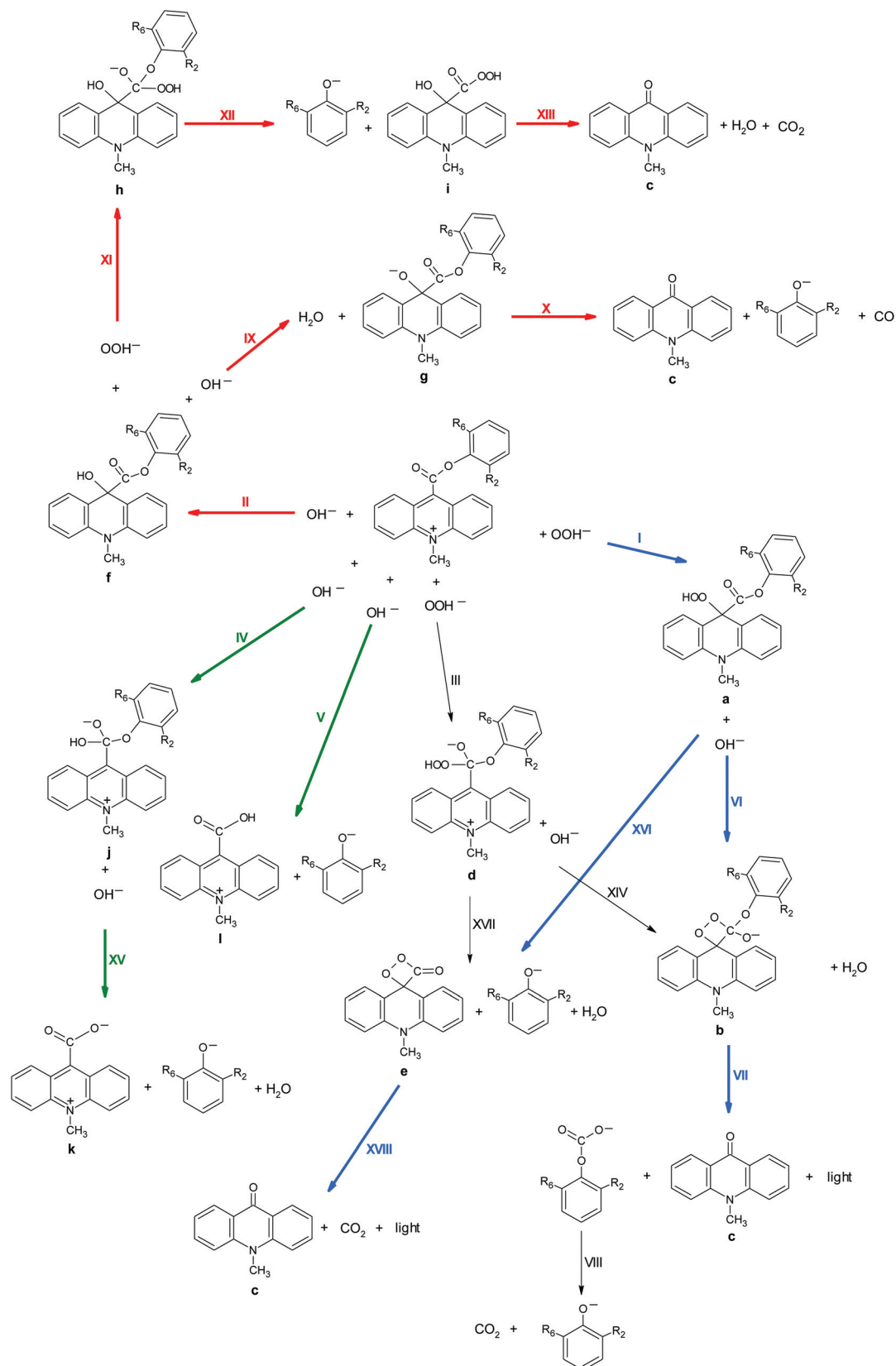


compound code	substituents in the benzene ring					name of the cation
	R ₂	R ₃	R ₄	R ₅	R ₆	
2-F	F	H	H	H	H	9-[(2-fluorophenoxy)carbonyl]-10-methylacridinium
2-Cl	Cl	H	H	H	H	9-[(2-chlorophenoxy)carbonyl]-10-methylacridinium
2-Br	Br	H	H	H	H	9-[(2-bromophenoxy)carbonyl]-10-methylacridinium
2-I	I	H	H	H	H	9-[(2-iodophenoxy)carbonyl]-10-methylacridinium
2-CF₃	CF ₃	H	H	H	H	10-methyl-9-[[2-(trifluoromethyl)phenoxy]carbonyl]acridinium
2-NO₂	NO ₂	H	H	H	H	10-methyl-9-[(2-nitrophenoxy)carbonyl]acridinium
2-OCH₃	OCH ₃	H	H	H	H	9-[(2-methoxyphenoxy)carbonyl]-10-methylacridinium
2,6-diF	F	H	H	H	F	9-[(2,6-difluorophenoxy)carbonyl]-10-methylacridinium
2,6-diCl	Cl	H	H	H	Cl	9-[(2,6-dichlorophenoxy)carbonyl]-10-methylacridinium
2,6-diBr	Br	H	H	H	Br	9-[(2,6-dibromophenoxy)carbonyl]-10-methylacridinium
2,6-diI	I	H	H	H	I	9-[(2,6-diiodophenoxy)carbonyl]-10-methylacridinium
3-F	H	F	H	H	H	9-[(3-fluorophenoxy)carbonyl]-10-methylacridinium
4-F	H	H	F	H	H	9-[(4-fluorophenoxy)carbonyl]-10-methylacridinium
4-Cl	H	H	Cl	H	H	9-[(4-chlorophenoxy)carbonyl]-10-methylacridinium
4-Br	H	H	Br	H	H	9-[(4-bromophenoxy)carbonyl]-10-methylacridinium

Scheme 1 Structural formula and systematic names of the investigated 10-methyl-9-(phenoxycarbonyl)acridinium trifluoromethanesulfonates (triflates) (XAEs) with the numbering system of C, N and O atoms given for the cations. The number of H atoms is the same as the C atoms to which the former are attached.

2-NO₂, **2-OCH₃** and **2,6-diBr**). Another premise that must be taken into account is the fact that H₂O₂ reacts spontaneously with OH[−] to yield OOH[−] (the thermodynamic data for the reaction H₂O₂ + OH[−] = H₂O + OOH[−] are: −26.8, −27.9 and −20.6 kcal mol^{−1} (enthalpy (gaseous phase), Gibbs' free energy (gaseous phase) and Gibbs' free energy (aqueous phase) of the reaction at temperature 298.15 K and standard pressure, respectively).

Taking into account the reaction pathways proposed by other authors^{8,33} and our previous investigations,²² as well as the information mentioned above, we considered the processes in accordance with general chemical knowledge that may explain the experimental findings. The pathways of the possible reactions of XAEs with OOH[−] and OH[−], outlined in Scheme 2, emerge from a thorough analysis of the results of theoretical calculations. Scheme 2 presents the processes that



Scheme 2 Pathways of the reactions of MPCA^+ with OOH^- and OH^- (pathway one (blue arrows), pathway two (red arrows) and pathway three (green arrows)).

account for CL (pathway one; steps I, VI, and VII; or I, XVI and XVIII), the formation of the so-called “pseudobase” (step II), and the following non-CL processes (pathway two; steps IX and X; or XI, XII and XIII), the appearance of 10-methyl-acridinium-9-carboxylate anions or 10-methylacridinium carboxylic acid (pathway three; steps IV and XV, and step V, respectively) among the reaction products. The theoretically predicted thermodynamic data for all the reaction steps are given in Table 1, while Table 2 contains kinetic data for the steps involving molecules in the ground electronic state. Additionally, the DFT-optimized geometries originating from the acridine molecules produced by the reaction of XAEs with OOH^- or OH^- are shown in Fig. S1 (lowest energy and transition state structures) in the ESI.†

Pathway one (“light pathway”) represents the attack of OOH^- on the XAE acridinium carbon atom (9) (step I), followed by the reaction of the addition product **a** with OH^- (step VI), the cyclization of the anion to a cyclic intermediate (**b**) (step VI), and the elimination of the phenyl carbonate anion

leading to the energy-rich 10-methyl-9-acridinone (**c**) (step VII). Interestingly, in the case of cations involved in **2-NO₂** and **2,6-diBr** (Scheme 1), we did not observe steps VI and VII. After the attack of OOH^- on the carbon atom C(9) of **2-NO₂** or **2,6-diBr** cation (step I), followed by the reaction of the addition product **a** with OH^- (step VI), the elimination of an adequate number of phenoxy anions and the resulting cyclic intermediate (**e**) (step XVI) with the subsequent elimination of carbon dioxide leads to the energy-rich 10-methyl-9-acridinone (**c**) (step XVIII). We examined the two pathways (steps I, VI and VII; and I, XVI and XVIII) for compounds **2-F**, **2-CF₃** and **2-OCH₃**. The theoretically predicted thermodynamic data indicated that these two pathways are possible for compounds **2-F**, **2-CF₃** and **2-OCH₃**. On the other hand, the enthalpy (gaseous phase), Gibbs’ free energy (gaseous phase) and Gibbs’ free energy (aqueous phase) of step XVI (298.15 K, standard pressure) are thermodynamically more preferred in the case of compounds **2-NO₂** and **2,6-diBr** than **2-F**, **2-CF₃** and **2-OCH₃**. The barriers predicted for step XVIII are similar in the aqueous phase to the

Table 1 Thermodynamic data of the elementary steps of the reactions of selected compounds with OOH^- and OH^- ^a

Step no. (Scheme 2)	Substituent	Gaseous phase		Aqueous phase	Step no. (Scheme 1)	Substituent	Gaseous phase		Aqueous phase
		$\Delta_{r,298}H^\circ$	$\Delta_{r,298}G^\circ$				$\Delta_{r,298}H^\circ$	$\Delta_{r,298}G^\circ$	
I	2-F	−164.2	−151.3	−43.3	X	2-F	−0.1	−24.4	−34.4
	2-CF₃	−167.1	−153.2	−44.1		2-CF₃	−6.6	−30.9	−39.0
	2-NO₂	−166.0	−153.5	−44.7		2-NO₂	−13.9	−38.5	−47.6
	2-OCH₃	−162.8	−150.5	−43.0		2-OCH₃	2.3	−21.8	−30.9
	2,6-diBr	−165.4	−152.8	−44.2		2,6-diBr	−20.2	−44.2	−45.8
II	2-F	−190.2	−180.2	−63.1	XI	2-F	−70.3	−56.0	−15.7
	2-CF₃	−192.9	−182.0	−64.4		2-CF₃	−72.6	−58.0	−16.8
	2-NO₂	−191.4	−181.1	−64.9		2-NO₂	−75.3	−61.7	−21.1
	2-OCH₃	−188.9	−178.4	−62.7		2-OCH₃	−64.4	−50.9	−12.2
	2,6-diBr	−188.0	−178.5	−62.6		2,6-diBr	−66.7	−51.0	−14.2
III	2-F	−138.4	−125.5	−25.0	XII	2-F	24.7	10.2	−3.7
	2-CF₃	−142.0	−128.2	−26.0		2-CF₃	24.4	9.8	−8.4
	2-NO₂	−135.4	−123.3	−23.0		2-NO₂	15.8	1.9	−13.1
	2-OCH₃	−133.9	−121.0	−22.4		2-OCH₃	31.5	18.6	−2.7
	2,6-diBr	−138.3	−124.9	−25.0		2,6-diBr	8.4	−6.3	−18.7
IV	2-F	−156.6	−146.6	−36.7	XIII		−94.2	−116.3	−118.6
	2-OCH₃	−152.3	−142.1	−34.9		2-F	−103.1	−104.1	−58.7
V	2-F	−75.4	−78.0	−51.7	XIV	2-CF₃	−105.3	−106.9	−60.8
	2-CF₃	−86.4	−89.1	−58.8		2-OCH₃	−100.9	−102.2	−58.2
	2-NO₂	−96.3	−99.8	−68.4		2-F	−88.4	−103.0	−82.9
	2-OCH₃	−67.2	−69.7	−46.9		2-OCH₃	−84.5	−99.1	−80.0
	2,6-diBr	−91.7	−94.8	−64.7		2-F	−66.3	−80.1	−51.6
VI	2-F	−77.2	−78.3	−40.4	XVI	2-CF₃	−74.5	−89.2	−57.9
	2-CF₃	−80.1	−81.9	−42.7		2-NO₂	−85.4	−99.6	−66.8
	2-OCH₃	−72.0	−72.6	−37.6		2-OCH₃	−59.5	−72.5	−47.1
VII	2-F	−93.6	−108.0	−117.7	XVII	2,6-diBr	−81.5	−95.4	−63.7
	2-CF₃	−95.7	−111.0	−117.8		2-F	−92.2	−105.8	−69.9
	2-OCH₃	−97.6	−112.0	−118.0		2-CF₃	−99.6	−114.2	−76.0
VIII	2-F	10.8	2.2	0.4	XVIII	2-NO₂	−116.0	−129.7	−88.5
	2-CF₃	7.7	−0.3	−3.6		2-OCH₃	−88.4	−102.0	−67.6
	2-OCH₃	16.4	8.1	2.4		2,6-diBr	−108.6	−123.2	−82.8
IX	2-F	−68.0	−68.7	−33.2			−93.7	−104.1	−106.2
	2-CF₃	−69.8	−71.4	−34.5					
	2-NO₂	−73.9	−75.4	−34.9					
	2-OCH₃	−63.5	−64.7	−32.3					
	2,6-diBr	−66.5	−67.4	−35.4					

^a $\Delta_{r,298}H^\circ$ and $\Delta_{r,298}G^\circ$ respectively represent the enthalpy and Gibbs’ free energy (gaseous phase) or free energy (aqueous phase) of the reaction corresponding to a given step number at temperature 298.15 K and standard pressure. All thermodynamic data are in kcal mol^{−1}.

Table 2 Kinetic characteristics of the elementary steps of the reaction of selected compounds with OOH^- and OH^- ^a

Step no. (Scheme 2)	Substituent	Gaseous phase			Aqueous phase
		$\Delta_{a,298}H^\circ$	$\Delta_{a,298}G^\circ$	${}_{298}k^\circ$ (${}_{298}\tau^{0.99}$)	$\Delta_{a,298}G^\circ$
VII	2-F	9.3	8.5	3.4×10^6 (1.4×10^{-6})	14.1
	2-CF ₃	10.8	10.7	9.5×10^4 (4.8×10^{-5})	14.6
	2-OCH ₃	8.2	8.6	3.3×10^6 (1.4×10^{-6})	12.6
X	2-F	8.5	7.7	3.4×10^6 (1.4×10^{-6})	14.1
	2-CF ₃	8.2	7.1	4.1×10^7 (1.1×10^{-7})	8.2
	2-NO ₂	7.7	6.5	9.8×10^7 (4.7×10^{-8})	6.6
	2-OCH ₃	8.8	7.9	1.1×10^7 (4.3×10^{-7})	8.0
	2,6-diBr	2.0	2.8	5.4×10^{10} (8.5×10^{-11})	5.2
XIII		32.6	33.0	4.0×10^{-12} (1.1×10^{12})	26.2
XVIII		10.3	10.1	2.6×10^5 (1.8×10^{-5})	15.4

^a $\Delta_{a,298}H^\circ$ and $\Delta_{a,298}G^\circ$ (both in kcal mol^{-1}) respectively represent the enthalpy and Gibbs' free energy (gaseous phase) or free energy (aqueous phase) of activation at temperature 298.15 K and standard pressure. ${}_{298}k^\circ$ (in s^{-1}) and ${}_{298}\tau^{0.99}$ (in s) (eqn. (5) and (6)) respectively denote the rate constant and the time after which the reaction is 99% complete.

barriers predicted for step VII (*e.g.* for compound 2-F $\Delta_{a,298}G^\circ$ in the aqueous phase (in kcal mol^{-1}) are 15.4 and 14.1, respectively). The computationally predicted pathways of CL generation do not clearly present which pathway operates for the cations involved in 2-F, 2-CF₃ and 2-OCH₃. Just one thermodynamically possible pathway was observed in the case of XAEs, where the elimination of phenoxy anions after the reaction of the addition product **a** with OH^- (step VI) and formation of the cyclic intermediate **e** occur. The energy released in step VII or XVIII (Table 1) exceeds that which is necessary to cause the electronic excitation of 10-methyl-9-acridinone, which is $88.2 \text{ kcal mol}^{-1}$.³⁵

Pathway two ("dark pathway") assumes the attack of OH^- on the XAE carbon atom (9) (step II), followed by the reaction of the addition product **f** ('pseudobase') with OH^- (step IX) and the elimination of an adequate number of phenoxy anions and carbon monoxide, leading to 10-methyl-9-acridinone (**c**) (step X). Alternatively, the reaction of the addition product **f** ('pseudobase') through the attack of OOH^- on the carbon atom C(15) of the carbonyl group of the XAE (step XI), followed by elimination of a phenoxy anion leads to intermediate **i** (step XII), and the further elimination of carbon dioxide and water leads to 10-methyl-9-acridinone (**c**) (step XIII). The theoretically predicted thermodynamic data indicated that these pathways are possible for all of the investigated compounds. The enthalpy (gaseous phase), Gibbs' free energy (gaseous phase) and Gibbs' free energy (aqueous phase) computed at temperature 298.15 K and standard pressure suggest that the addition of OH^- to C(9) (step II) is thermodynamically preferred over the addition of OOH^- to the same carbon atom. In this case, the possible pathway of the reaction seems to be exclusively dependent on the kinetic factors. We noted earlier,²² and have confirmed in our present calculations, that the activation barriers characterizing the addition of OOH^- or OH^- to C(9) of the acridinium esters (steps I and II, respectively) and the substitution at OOH^- of the carbonyl C(15) atom of the 'pseudobase', followed by the release of the phenoxy anion (steps XI and XII, respectively) are negligible. Activation barriers do,

however, exist for the final steps (X and XIII), characterizing the transformation of the XAE pseudobase form to 10-methyl-9-acridinone (Table 2). In the case of step X, the activation barriers fall in the range of 5–14 kcal mol^{-1} , which is comparable to the activation barriers characterizing step VII. Nevertheless, the energy released is too small to electronically excite the above product (Gibbs' free energy in the aqueous phase is between -30 and $-47 \text{ kcal mol}^{-1}$) (Table 1). The overall values of the thermodynamic functions for step XIII (Table 1) are high enough to ensure electronic excitation of the produced 10-methyl-9-acridinone, but the activation barrier characterizing this process is significantly higher than that of step VII or X (Table 2). For the above reasons, pathway two, assuming the attack of OH^- on XAEs' C(9) cannot lead to CL, represents the dark transformation of XAE cations, producing 10-methyl-9-acridinone in the ground electronic state.

Pathway three explains the occurrence of 10-methylacridinium-9-carboxylate anions or 10-methylacridinium-9-carboxylic acid (steps IV and XV, and step V, respectively) among the reaction products, as OH^- – like OOH^- – can attack the acridinic carbon atom (9) or the carbonyl carbon atom (15). The addition of OH^- to C(9) (pathway two) is more probable for the reasons mentioned above. As we noted earlier,²² the identified entities were 10-methyl-9-acridinone (NMAON), 10-methylacridinium-9-carboxylic acid (NMACA) and substituted phenols (RPhOHs). The latter acid or its zwitterionic form, 10-methylacridinium-9-carboxylate, is likely formed as a result of XAEs' hydrolysis, which assumes the attack of OH^- on the carbonyl carbon C(15) (step IV), followed by the reaction of the addition product **j** with OH^- (step XV), and the elimination of an adequate number of phenoxy anions and water molecules, leading to product (**k**). These two steps are possible only for the two species – 9-[(2-fluorophenoxy)carbonyl]-10-methylacridinium (2-F) and 9-[(2-methoxyphenoxy)carbonyl]-10-methylacridinium (2-OCH₃) cations. In other cases (2-CF₃, 2-NO₂ and 2,6-diBr), these steps presumably do not occur and NMACA is produced upon the attack of OH^- on the carbonyl carbon atom C(15) (step V), followed by simultaneous

elimination of the substituted phenoxy anion leading to product (**1**). The thermodynamic data (Table 1) indicate that this step is possible for all the theoretically investigated compounds that are **2-F**, **2-CF₃**, **2-NO₂**, **2-OCH₃** and **2,6-diBr**. In the case of step V the alkaline hydrolysis of acridinium esters occurs with the formation of a tetrahedral intermediate, which immediately decays into products (**1**) and the substituted phenoxy anion. The activation barriers characterizing the addition of OOH[−] to the C(15) atom and formation of a tetrahedral transition state are negligible (0.6 kcal mol^{−1}; average value for **2-CF₃**, **2-NO₂** and **2,6-diBr**). In our opinion the more preferred pathway is *via* the steps IV and XV, but the theoretically predicted data indicated that the latter pathway is not possible in the case of **2-CF₃**, **2-NO₂** and **2,6-diBr**.

Synthetic considerations

Although the methods for the preparation of 10-substituted 9-(phenoxyacetyl)acridinium salts have been described in the literature,^{22,25–32,36} a few remarks concerning the compounds investigated here should be made (their synthesis and spectroscopic analyses are reported in the ESI†). To ensure a clean and effective synthesis of XAEs (Scheme 1), their immediate precursors, substituted 9-[(phenoxy)carbonyl]acridines, must be obtained in high yield and purity. According to this, we promptly chromatographed the reaction mixtures after the completion of the reaction, omitting numerous washings of the organic post-reaction mixture with aqueous solutions that lower the yield of reaction.²² The procedure is especially important if the sterically hindered phenols are used for esterification or the product contains hydrophilic atoms or groups of atoms. TLC tests of the chromatographically (LC) purified (9-[(phenoxy)carbonyl]acridines showed no other residual acridines by fluorescence detection; however, the elemental analyses are not always satisfactory. Further investigation revealed that the residual impurities in the chromatographed materials arose from residual phenols, characterized by a similar polarity as the uncharged esters. However, such impurities do not affect the quality of the final products (XAEs) – as phenols are washed away during the last step of the reaction (quaternization). If needed, the residual phenols can be easily removed from the final products by heating the products in a vacuum to about 100 °C – under such conditions their sublimation/evaporation takes place.

The other important synthetic issue that must be remarked upon in the reaction of the above-described esters with methyl triflate is the special treatment of the solvent before the reaction (chloroform should be passed through a dried, basic Al₂O₃ column), as well as the need for the use of a “proton sponge” (such as immobilized 2,6-di-*tert*-butylpyridine) – in order to remove the acidic impurities from the reaction mixtures, greatly reducing the yield and quality of the obtained salts. It must also be noted that, among other XAEs, the compound encoded **2,6-diI** (Scheme 1) caused experimental difficulties as it expressed limited photochemical stability, which was observed during the experiments.

Spectroscopic properties of XAEs

According to ¹H and ¹³C NMR techniques that were employed for the identification of cations involved in XAEs, the compatibility of the experimental and computational chemical shifts was satisfactory as denoted by the data collected in Tables S1–S3 and illustrated in Fig. S2 (ESI†).

In general, the ¹H NMR spectra of XAEs can be subdivided into three groups of signals³⁷ – those arising from the hydrogen atoms bonded to the acridine/acridinium nuclei (maximum range of chemical shifts, σ), the hydrogens present in the benzene ring (an intermediate range of σ), and – if present – the signals rising from the aliphatic hydrogen atoms (the lowest range of σ). In the group of 9-[(phenoxy)carbonyl]acridines (the unmethylated precursors of XAEs), the ¹H signals from the acridine nucleus H1/H8 and H4/H5 always occur at higher values of chemical shifts than H2/H7 and H3/H6 (Fig. S2, ESI†). The latter observation is likely related to the “uncover” effect, applied by the carbonyl oxygen atom (in the case of H1/H8) or the endocyclic nitrogen atom (in the case of H4/H5). Due to their spatial proximity, the H1/H8 signals also seem to be most sensitive to the substitution type in the benzene ring. The ¹H NMR spectra of XAEs are similar to the spectra of their precursors (the synthesis of XAEs, ESI†). In these cases, the signals rising from the acridinium nucleus appear at higher value chemical shifts than the respective signals for the bases – due to the lowering of the electron density, especially in a spatial area close to the endocyclic nitrogen atom. The coupling constants, characterizing the direct vicinal protons in the XAEs, appear in a range of values characteristic for aromatic molecular systems (7.0–10.0 Hz). In the case of couplings of higher orders, the values are located in the range of 0.5–2.5 Hz and reflect the individual features of the analyzed molecular systems. The ¹³C NMR spectra of XAEs (Fig. S2, ESI†) express a typical pattern, characteristic for phenyl-substituted acridinium esters.³⁷ Each of the observed signal was attributed to the respective carbon atom (Table S2, ESI†) with the aid of HETCOR ¹H–¹³C HSQC two-dimensional NMR spectra and the ACD/CNMR database.⁵⁵

The data concerning cations involved in XAEs disclosed by mass spectrometry are summarized in Table S1 (columns 1–3 from the right, ESI†) and the representative MALDI-TOF spectra are presented in Fig. S2 (ESI†). In all cases, the mass spectra present a simple and clear pattern, underlining the chemical identity of the obtained acridinium salts. The parent ions M⁺ or (M + 1)⁺/(M – 1)⁺ are observed with the relative abundances of 18–100% and 8–76%, respectively and the characteristic signal for all spectra – 10-methylacridinium cation (MeAcr⁺, $m/z \approx 193$, or (MeAcr⁺ + 1)⁺, (MeAcr⁺ – 1)⁺ signals) – are observed within relative abundances of 21–100%.

Chemiluminogenic properties of XAEs

Kinetics of CL decay in various environments. The group of the presently studied acridinium esters (XAEs) contains substituents of various electronic and steric characters in the ester

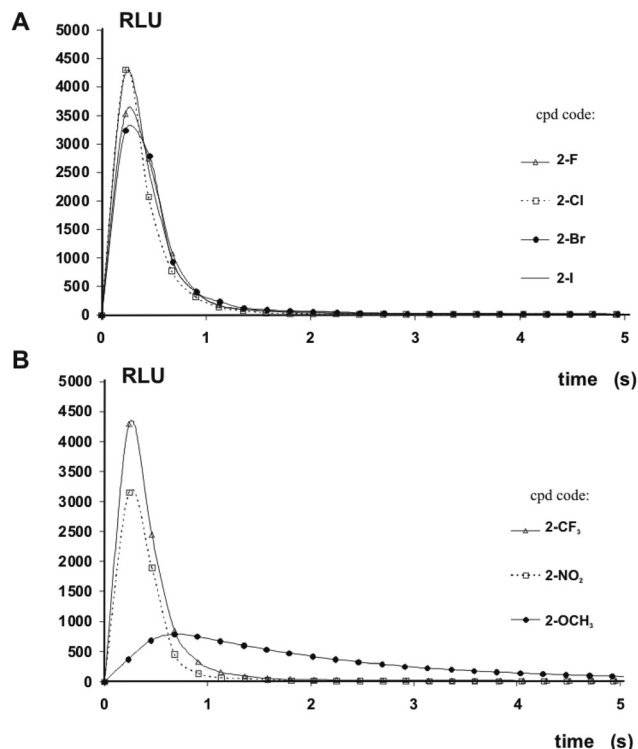


Fig. 1 Chemiluminescence profiles recorded for 2-substituted XAEs (Scheme 1) using a standard triggering system. (A) CL profiles of 2-halo-substituted acridinium salts; (B) CL profiles of other 2-substituted acridinium salts investigated. For details, see the Experimental section and note 1 to Table S4 (ESI†).

moiety (the leaving group during their chemiluminogenic oxidation) located at the *ortho*, *para* or *meta* positions of the benzene ring (Scheme 1). These substituents are electron-acceptor halogen atoms, nitro (NO_2) and trifluoromethyl (CF_3) and methoxy groups (OCH_3), expressing an electron-donor character.^{17,32}

As shown by the data presented in Fig. 1 and 2A and summarized in Table S4, column 2, ESI†, emission decays for most haloester salts, initiated by standard triggering reagents ($\text{H}_2\text{O}_2/\text{NaOH}$), represent high-speed (flash type) kinetics, with k_{CL} values falling in the range of *ca.* $3\text{--}5\text{ s}^{-1}$. Thus, the investigated systems are characterized by emissions occurring in a time order of seconds as shown in an exemplary CL time profile for a series of 2-substituted XAEs (Fig. 1). The behavior of the salts **2-NO₂** and **2-CF₃** is similar to the other 2-substituted haloesters. However, the compound bearing an electron-donor substituent (**2-OCH₃**) behaves differently in this context, exhibiting a glow-type emission ($k_{\text{CL}} \approx 0.4\text{ s}^{-1}$), typical for the majority of previously studied acridinium alkylesters.^{17,32,38} Interestingly, the influence of the steric hindrance of substituents on k_{CL} is not clearly observed, if one compares the k_{CL} values, *e.g.* for compounds **2-F/2,6-diF** or **2-Br/2,6-diBr** (Fig. 2A, Table S4, ESI†). The latter behavior differentiates XAEs (instead of **2-OCH₃**) from the above-mentioned alkylesters, where the spatial effect caused by the *ortho*-substituents is dis-

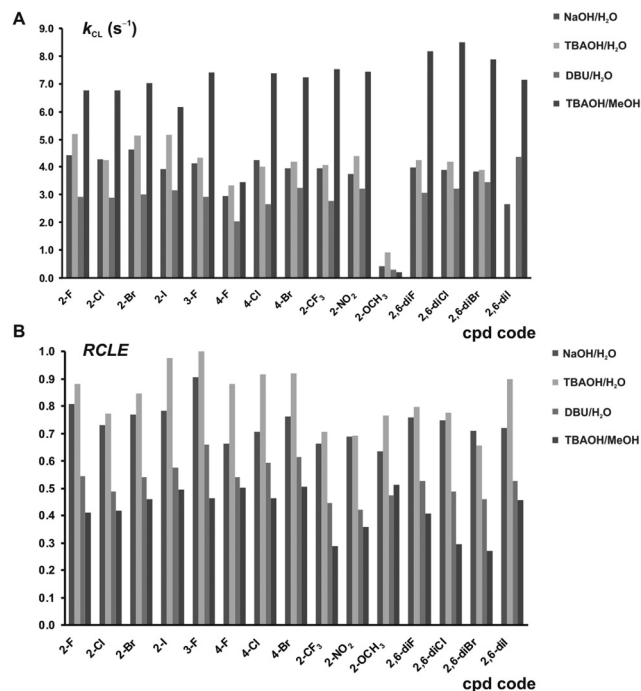


Fig. 2 CL parameters of XAEs (Scheme 1) in various environments. (A) Pseudo-first order emission decay kinetic constants (k_{CL}); (B) relative CL efficiencies (RCLE, normalized) in various environments. The coefficients of variability (CV) fall in the range of 2–8%. Abbreviations: TBAOH – tetra-*n*-butylammonium hydroxide, DBU – 1,8-diazabicyclo[5.4.0]undec-7-ene; MeOH – methanol. For details, see the Experimental section and notes to Table S4 (ESI†).

tinct.^{21,39} The use of organic bases instead of NaOH – such as sterically hindered tetrabutylammonium hydroxide (TBAOH) or a strong Lewis base, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in aqueous or methanolic environments was tested in order to examine how it affects the kinetics and efficiency of CL. We assumed that, with the use of other bases, the susceptibility of XAEs to the production of undesirable “pseudobases”, as well as the mobility of the ions engaged in the reaction, might be altered. When using TBAOH, a clear acceleration of the emission dynamics was observed if compared to the situation when typical triggers were used – by 20–50% in aqueous and by 50–80% in methanolic environments (Fig. 2A; Table S4, columns 3 and 5†). In turn, the use of DBU, in general, manifests a decrease of k_{CL} values by about 10–40% (Fig. 2A; Table S4, column 4, ESI†). Such observations may be associated with a lower degree of H_2O_2 ionization in the environment when DBU was used instead of the typical Brønsted bases. In addition, OH^- ions also act as a catalyst in stage VI of light-generating transformations (Scheme 2). Another observation, which might be annotated, is that the properties of the environment (the base, solvent) express a more pronounced influence on the emission kinetics in the group of 2,6-disubstituted than 2-substituted XAEs (Fig. 2A).

Emission efficiency in various environments. The normalized values of the relative efficiencies of XAE emission (RCLE)

are compared in Fig. 2B and summarized in Table S4 (columns 6–9, ESI†). One can observe that, among the various compounds investigated, the differences in *RCLE* are not substantial, as was observed in the case of the above-discussed values of k_{CL} . However, if one takes into consideration the values of this parameter in various environments, the differences are more distinct.

Generally, the highest values of *RCLE* are obtained in TBAOH/H₂O, while the lowest values are obtained in the TBAOH/CH₃OH environment; the typical aqueous environment (NaOH/H₂O) is characterized by moderate efficiency – located in between the two former ones. Comparing the two graphs presented in Fig. 2(A and B), it can be concluded that, in aqueous systems, a faster emission is generally accompanied by a higher efficiency. To some extent, this observation might be explained in terms of various amounts of 10-methylacridinium-9-carboxylic acid (NMACA), detected among CL reaction products.²² The latter finding indicates various levels of participation of the hydrolytic dark paths IV and V (Scheme 2) among the reaction pathways. This problem will be further discussed below (the products secreted upon XAEs' chemiluminescence). On the other hand – faster kinetics in methanol is not accompanied by a higher efficiency emission of XAEs – as *RCLEs* characterizing the TBAOH/MeOH system take the lowest values (instead of 2-OCH₃, Fig. 2B, Table S4 (ESI†)). The circumstances, whose importance is hard to estimate just on the basis of CL measurements – such as an altered mechanism of light generation in an organic environment or different excitation efficiency of the oxidation product – might be considered to explain that observation.

The values of quantum yields (QY) determined for XAEs, using luminol as a reference,^{22,40} are collected in column 10 of Table S4 (ESI†). Generally, slightly higher emission yields for XAEs than for the previously investigated acridinium alkyls in an alkaline aqueous environment (NaOH/H₂O) can be noted.²² In this context, the fluorinated derivatives are characterized by maximal QY values in the group of investigated salts, e.g. 2.3–2.4% in the case of 2-F and 3-F – which is more than 25% higher than those obtained for the unsubstituted AE in the same environment.

Alkalinity of environment vs. CL efficiency. As regards the influence of the environment's pH on the emission efficiency (*RCLE*) in the group of XAEs, the information obtained from CL measurements is compared in Fig. 3A and summarized in Table S4 (columns 11–13, ESI†). In general, a lower pH of the CL environment is manifested by a substantial reduction in *RCLE* values. This behavior is likely accompanied by a lower population of OOH[−] ions (pK_a of H₂O₂ = 11.75)⁴¹ in solutions of lower alkalinity. The decrease in efficiency is irregular – a change of pH from 12 to 10 leads to a slight (few percent) reduction in the *RCLE* (as it takes place in the case of 2- and 4-substituted XAEs), whereas a further lowering of the pH value (pH = 8) is manifested in a substantial reduction of emission efficiency (up to several dozen percent). In the case of 2-OCH₃ the emission practically disappears at pH = 8. However, some XAEs retain their emission ability at pH = 8

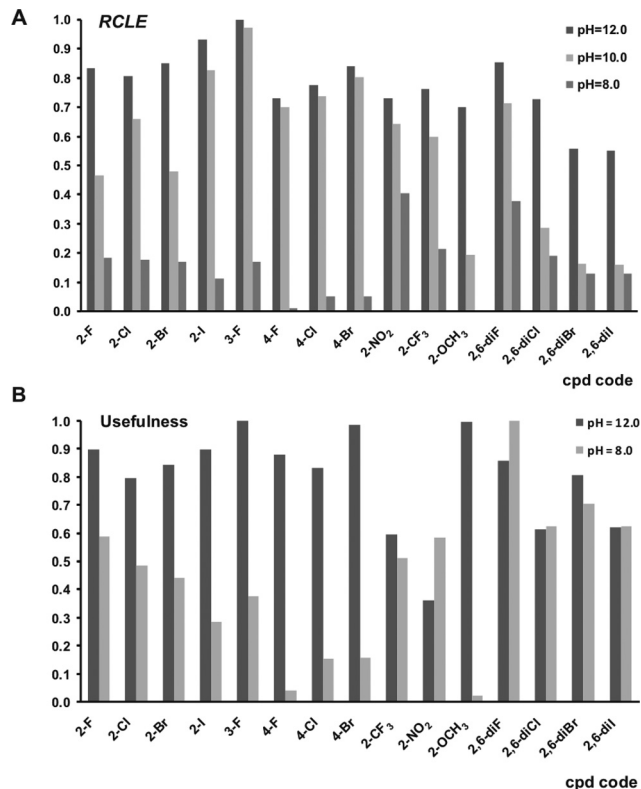


Fig. 3 CL parameters of XAEs (Scheme 1) in aqueous environments of different alkalinity. (A) The dependence of *RCLE* (normalized to the highest value) on the pH. (B) Usefulness (the product of stability at pH = 8.0 and the emission efficiency at given pH, normalized) in aqueous environments of different alkalinity. The coefficients of variability (CV) fall in the range of 2–8%. For details, see the Experimental section and notes to Table S4 (ESI†).

reasonably well (about 50% of the original value) – such as compounds 2,6-diF and 2-NO₂. The latter effect is likely associated with their substituent-driven activation towards nucleophile attack (OOH[−]) onto the C(9) atom, starting the “light pathway” of the oxidation of XAEs (Scheme 2, pathway I).

Other parameters characterizing XAEs' emission ability. The last column in Table S4 (ESI†) contains parameters named stability, reflecting the degree of the retained XAE emission activity after incubation in alkaline phosphate buffer (pH = 8.0, t = 30 min, T = 298 K). This parameter seems to be important in the context of the utilization of XAEs as fragments of CL labels – where the covalent attachment of a label to a biomolecule requires the incubation of the ester under the above-mentioned conditions.⁸ As revealed from the data presented in Table S4,† the stability of most XAEs fluctuates around 50%, although some compounds (2-OCH₃, 2,6-diBr) retain more than 70% of their emission ability under experimental conditions. The possible reasons for this might be considered in the context of the electronic and steric influence of the substituents that are present in the benzene ring. In the group of

2,6-dihaloesters, the trend is towards strengthening the compounds' stability in the order: 2,6-diBr > 2,6-diCl > 2,6-diF. The least stable compound, that is 2-NO₂, loses ca. 75% of its emission ability under the same conditions – apparently because of its strong susceptibility to alkaline hydrolysis.

Interesting conclusions can be drawn from the chart, presenting the “usefulness” of XAEs (Fig. 3B). Usefulness is the original parameter, which we had proposed previously,²² as the product of emission efficiency at a given pH (12 or 8) and the stability in an alkaline environment (pH = 8). It can be employed – among other physicochemical characteristics, such as kinetics of emission, thermodynamic stability – as a measure of potential utility of a given compound as a CL label or an indicator. According to this, the compounds that are intended to be used as CL labels should be characterized by high usefulness at high pH values – under which the emission of the labeled conjugates is triggered. Accordingly, mono-fluorinated compounds, such as 3-F, 2-F, 4-F or 4-Br and 2-OCH₃, seem to be particularly useful in this context (Fig. 3B, dark bars). On the other hand, if relatively low alkalinity, at which the emission is triggered is important – which, for example, takes place in nucleic acid diagnostics or CL ELISA applications (where AEs are used as CL indicators) – obtaining a high usefulness of CL reagents at pH = 8 would be beneficial. In this context, the XAEs containing two halogen atoms in both *ortho*-positions – in particular the compound 2,6-diF (Fig. 3B, light bars) – would be beneficial.

The previously reported trend among acridinium chemiluminogenic salts indicated that, in general, the dynamics of light emission increases as the pK_a of the leaving group decreases, *i.e.* the moiety detached upon CL becomes a “better” leaving group.⁴² In the present work we were unable to observe a clear relationship between kinetics or efficiency and pK_a of the respective phenol among all the investigated XAEs. However, in the sub-group of the salts with no steric hindrance, *i.e.* in the series of 2- or 4-halosubstituted esters, an interdependence between CL decay kinetics constants (k_{CL}) and pK_a values of the respective phenols was observed (Fig. 4).

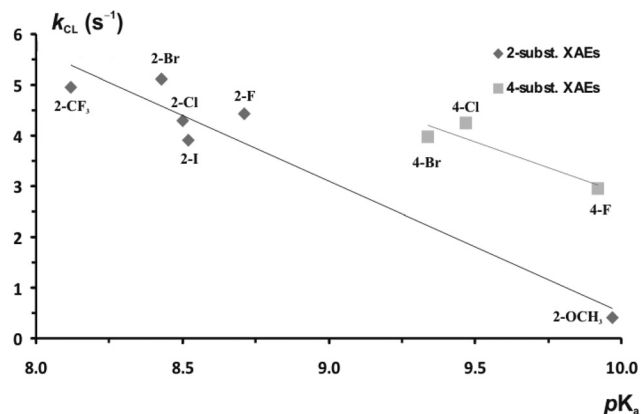


Fig. 4 The interdependencies between estimated ionization constants for respective phenols (pK_a) [ACD⁵⁵] and the pseudo-first order constants of emission decay (k_{CL}) (Table S4, column 2, ESI†) for 2- or 4-substituted XAEs (Scheme 1). The lines correspond to the following equations: $k_{CL} = -2.593pK_a + 26.44$ (2-subst. XAEs), $k_{CL} = -2.045pK_a + 23.31$ (4-subst. XAEs). For details, see the Experimental section and note 1 to Table S4 (ESI†).

The influence of surfactants on chemiluminescence. The cationic, zwitterionic and neutral surfactants (CTAC, DDAPS and Triton X-100, respectively) added to aqueous CL systems, containing XAEs, generally increase both the dynamics as well as the efficiency of emission of light. The parameters quantifying this behavior are Δk_{CL} and $\Delta RCLE$, respectively and the relevant data are summarized in Table 3. The acceleration of CL kinetics decay for the investigated XAEs obeys the order (average values of Δk_{CL} are given in parentheses): DDAPS (1.5) > CTAC (1.1) > Triton X-100 (0.9). The general trend observed in all cases (CTAC, DDAPS, Triton X-100) is that the values of Δk_{CL} increase with the increase of the inductive constants of substituents present in the benzene ring ($\sigma_I = 0.76, 0.52, 0.47, 0.50$ and 0.39 for NO₂, F, Cl, Br and I, respectively).⁴⁵ On the other hand, the enhancements of CL efficiency for the investigated XAEs are arranged in the following order (average values of $\Delta RCLE$ are given in parentheses): DDAPS (2.2) $\approx$ Triton

Table 3 Effects of surfactants on CL parameters for selected XAEs in alkaline aqueous environments. For details see the Experimental section

Compound code	CTAC			DDAPS			Triton X-100		
	Δk_{CL}^a	$\Delta(RCLE)^b$	$LOD/LOQ \times 10^{-11}$ M	Δk_{CL}	$\Delta(RCLE)$	$LOD/LOQ \times 10^{-11}$ M	Δk_{CL}	$\Delta(RCLE)$	$LOD/LOQ \times 10^{-11}$ M
2-F	1.14	2.42	4.60/7.47	1.21	2.72	9.10/14.8	0.81	3.19	10.7/17.4
2-I	1.15	1.89	7.85/12.7	1.23	2.35	11.0/17.9	0.80	2.02	19.6/31.5
2-NO ₂	1.24	2.12	2.93/4.78	1.37	2.36	7.24/11.8	1.00	2.45	6.36/10.2
2,6-diF	1.18	1.84	1.29/2.13	2.02	3.15	3.05/5.14	1.24	2.40	7.28/11.8
2,6-diCl	1.09	1.37	7.16/11.3	1.62	2.07	27.7/44.3	1.20	2.47	17.1/27.2
2,6-diBr	1.04	1.22	6.73/10.7	1.43	1.85	16.9/26.9	0.77	1.80	14.5/23.1
2,6-diI	1.10	1.26	15.0/24.0	1.79	1.16	14.3/22.9	0.49	0.93	17.3/27.5

Abbreviations: ^a Δk_{CL} – denotes the change of CL decay kinetic constant value relative to the value obtained with no surfactant using the standard triggering system (H₂O₂/NaOH) (see footnote 1 under Table S4 (ESI)); ^b $\Delta(RCLE)$ – denotes the change of integral CL intensity relative to the value obtained with no surfactant using the standard triggering system (H₂O₂/NaOH) (also see Table S3 (ESI)); ^c LOD/LOQ – denotes the limit of detection and the limit of quantification, respectively.

X-100 (2.2) > CTAC (1.7). The most efficient CL systems among the studied ones such as 2,6-diF/DDAPS or 2-F/Triton X-100, express more than three-fold enhancement of CL (Table 3, columns 6, 9) in comparison with the typical environment, in which the emission of XAEs is triggered (Table S4, column 6, ESI†).

As it was observed for similar systems, the dynamics and efficiency of CL are distinctly accelerated in the presence of cationic or zwitterionic surfactants, such as CTAC and DDAPS.^{24,43} Such a bimolecular reaction can undergo micellar catalysis, based on the ability of the surfactants to bind various substrates and attract oppositely charged entities towards their surfaces.⁴⁴ In the context discussed here, the latter phenomenon is presumably manifested by the local increase in the concentration of the hydroperoxide ions at the oppositely charged micellar surfaces and, resultantly the more efficient formation of the adequate hydroperoxide adducts (Scheme 2, a) that initiate the "light path" of the reaction (Scheme 2, pathway I). In contrast to the above, when using a neutral surfactant such as Triton X-100, no impact (or even a slight decrease) of the rate of CL was detected – which is consistent with the previous observations made on similar CL systems.^{24,43} However, a clear influence of the above neutral detergent on the efficiency of emission is observed in the case of the herein considered group of compounds, which differentiates the behavior of XAEs from other acridinium esters (labels and their conjugates) investigated in this context.²⁴ It should be noted that the chemiluminogenic adduct, from which the transformations start, is neutral in the case of the molecular systems studied here (Scheme 2, a). Taking into account the structure of Triton X-100, which is similar to the polyethylene glycols (PEGs), one can expect the occurrence of specific intermolecular interactions of the latter intermediate (a) with this neutral detergent – such as hydrogen bondings and the hydrophobic interactions of the XAEs' aromatic ring systems with the micelles. The latter phenomenon can lead to the increased local concentration of the chemiluminogenic adduct, and – resultantly – an increase in emission efficiency (ΔRCLE) and, at the same time, diminution of the reaction rate due to the involvement of a in the above mentioned interactions. Our further investigations of this interesting problem are in progress.

For the above discussed enhanced CL systems, we also established the limits of detection (*LOD*) and limits of quantification (*LOQ*) at the maximal detector's sensitivity (Table 3, columns 4, 7, and 10). The above parameters, depending basically on the slope of the calibration graphs, the deviation of the measured points from the linear regression, and the number of measured points (eqn (S1) and (S2), ESI†) are of great analytical importance.⁴⁶ The relationships used for the calculation of *LOD*/*LOQ* and the exemplary calibration graphs are presented in Fig. S3B (ESI†). Among the studied CL systems, the lowest values of *LOD*/*LOQ* – falling at the level of $1.3\text{--}2.9 \times 10^{-11}$ M for *LOD* and $2.1\text{--}4.8 \times 10^{-11}$ M for *LOQ* – were obtained with the participation of 2,6-diF and 2-NO₂ and a cationic surfactant CTAC.

The products secreted upon XAEs' chemiluminescence

The mixtures obtained after the completion of CL were immediately subjected to RP-HPLC analyses in order to reveal the type and population of the products. An exemplary chromatogram for the reaction mixtures is presented in Fig. S4 (ESI†). The products found after CL of XAEs were always 10-methyl-9-acridinone (NMAON) (the most intense signals were at 254 nm and 268 nm), 10-methylacridinium-9-carboxylic acid (NMACA) and the substituted phenol (RPhOH). The above signals were attributed by a comparison of their retention times (*R_T*) with the ones found for previously prepared standard substances (NMAON, NMACA)¹⁷ or those purchased (RPhOHs) as reference compounds. The other trace signals observed in the chromatograms were analytically insignificant and they were therefore not quantified. The data concerning the content of NMACA in post-CL mixtures, assayed for compounds 2,6-diF, 2,6-diCl, 2,6-diBr and 2,6-diI under standard CL conditions are summarized in Table 4 (column 2). As the emissions from the above haloesters represent flash-type decays, the content of the acid, assessed with the use of calibration graphs (Fig. S4B, ESI†), is generally low and falls in the range of *ca.* 0.2–2.7 mol%. The results indicate that, in the group of investigated compounds, the content of acid in the reaction mixtures does not clearly depend on the type of substituent. Interdependences were observed among the pseudo-first order kinetic constants of the alkaline hydrolysis of 2,6-disubstituted XAEs at pH = 12 (*k_{OH}*) and the size parameters, characterizing the substituents (halogen atoms) (Table 4, columns 4 and 5). According to this, *k_{OH}* takes the values which are approximately 1.0, 0.2 and 0.06 for 2,6-diF, 2,6-diCl and 2,6-diBr, respectively.

The ESI-MS mass spectra obtained for the mixtures under CL or just after the completion of CL reaction with the participation of selected XAEs in an alkaline aqueous environment are given in Fig. 5. It can be seen that, instead of the cations

Table 4 Chromatographically (RP-HPLC) assessed content of XAEs' alkaline hydrolysis product, 10-methylacridinium-9-carboxylic acid (NMACA) in the post-CL reaction mixtures. Pseudo-first order kinetics constants of XAEs' alkaline hydrolysis at pH = 12 (*k_{OH}*). For details see the Experimental section

Compound code	NMACA content ^a (mol%)	CV (%)	<i>k_{OH}</i> ^b ($\times 10^{-2}$ s ⁻¹)	Charton's size values (σ_v)/Taft size parameter (<i>E_s</i>) for adequate halogen atom [H]
2,6-diF	0.18	0.0	4.69	0.27/−0.46
2,6-diCl	1.66	3.8	0.89	0.55/−0.97
2,6-diBr	2.73	5.9	0.31	0.65/−1.16
2,6-diI	1.07	4.9	—	0.78/−1.40

^a Signals were identified by the comparison of their retention times with the ones assessed for the reference material. Values were established based on the areas under the signal of NMACA in relationship to the calibration plots (Fig. S4 (ESI)). ^b Values were obtained upon the linearization of $\ln C_t/C_0 = f(\text{time})$ interdependences ($n = 6\text{--}8$, $R^2 = 0.97\text{--}0.99$) (Fig. S5 (ESI)).

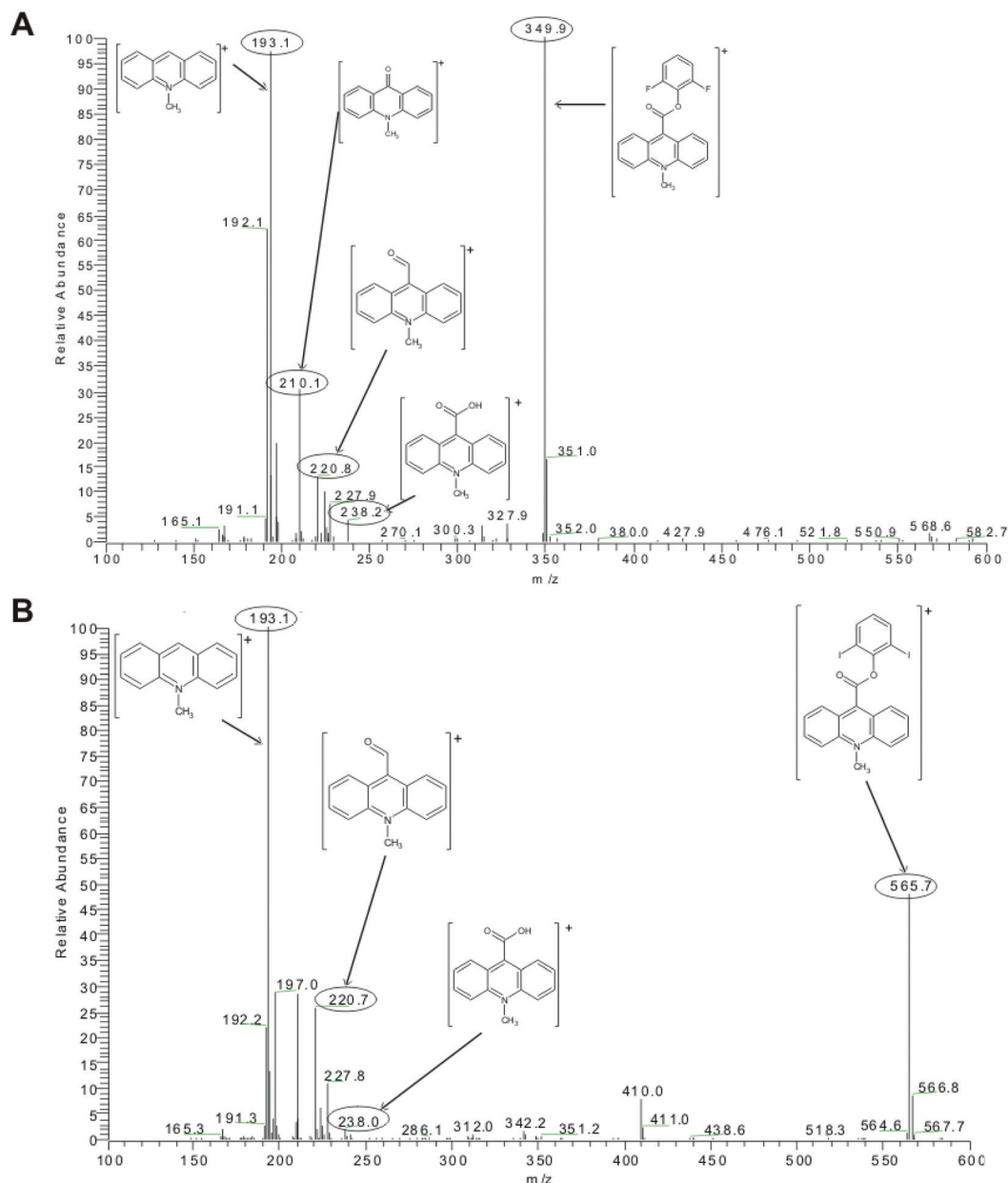


Fig. 5 ESI-MS spectra recorded in the positive ion regime for exemplary XAEs (Scheme 1). (A) 9-[(2,6-difluorophenoxy)carbonyl]-10-methylacridinium triflate (**2,6-diF**); (B) 9-[(2,6-diiodophenoxy)carbonyl]-10-methylacridinium triflate (**2,6-diI**). For details, see the Experimental section.

observed in the MALDI-TOF experiments (Fig. S2 (ESI[†])), signals arise from the charged products of the oxidative decomposition of the acridinium salts, *i.e.* $m/z \approx 210$ (NMAON) and $m/z \approx 238$ (NMACA). The relative abundance of the latter cation is traceable in the case of the investigated 2,6-disubstituted salts (**2,6-diF** and **2,6-diI**) – which underlines the statements drawn from the above-discussed RP-HPLC experiments. It should also be noted that, among the cations detected, an acridinium fragment corresponding to the detachment of the substituted phenoxy fragment from the parent acridinium cation ($m/z \approx 221$ ($M - 1$)) has a relative abundance at the level

of several percents (*ca.* 15 and 27% for **2,6-diF** and **2,6-diI**, respectively).

Conclusions

Newly synthesized acridinium esters containing substituents of various electronic and steric characters such as halogens, CF_3 , NO_2 , and OCH_3 located at the *ortho*-, *para*- or *meta*-positions of the benzene ring (XAEs, Scheme 1) were

subjected to a comprehensive theoretical and physico-chemical investigation. The main tasks of this work included the assessment of the mechanism of their chemiluminogenic oxidation in basic protic solvents, as well as a thorough evaluation of their emission abilities in various environments.

By applying computational methods, based on Density Functional Theory (DFT), we have proposed a number of possible steps concerning the transformations of XAEs in an aqueous alkaline environment, with the participation of ions such as OH^- and OOH^- . The latter may compete for the electron-deficient acridinium C(9) atom in the initial steps of the XAE transformations. In general, three concurrent reaction pathways have been proposed. Pathway one (blue arrows, Scheme 2) comprises the “light way” of XAE oxidation, involving 1.7–2.4% of the reaction energy released upon the transformation with regard to experimentally obtained values of quantum yields (QY, Table S4 (ESI†)). As stated before,^{22,33} CL of the acridinium cations starts from the attack of OOH^- on the electron-deficient C(9).^{22,33} Interestingly, the acridane-type adduct thus formed undergoes further transformations *via* two slightly different channels – suggesting thermodynamic features of the investigated systems and the values of the p_z LUMO orbital at the acridinium C(9) atom, reflecting susceptibility of XAEs for the attack of a nucleophile. The two alternative pathways seem to be dependent on the character of the “leaving group”, being detached upon the reaction. The fluorinated esters such as **2-F**, **2- CF_3** and 2-methoxy (**2-OCH₃**) are characterized by the occurrence of the cyclic dioxethane anionic form (**b**, Scheme 2) at the “bottleneck” stage of the pathway and behave similarly to the previously investigated alkyl-acridinium esters.²² The second pathway includes the formation of the cyclic dioxethanone – the structure suggested in the literature as the crucial intermediate of the acridinium ester CL transformations – although till now there is no clear experimental evidence demonstrating its occurrence. Both cyclic intermediates make an immediate, high-energetic precursor of the electronically excited molecules of 10-methyl-9-acridinone (NMAON), the final emitter in all the CL systems based on acridinium salts.^{17,47}

The other two general pathways are considered as “dark” – making concurrent reaction channels to the above-discussed “light way”. The latter two pathways denote a “pseudobase way” (pathway two, red arrows, Scheme 2) – ending with the formation of NMAON in an electronic ground state and a “hydrolytic way” (pathway three, green arrows, Scheme 2) – ending with the formation of 10-methylacridine-9-carboxylic acid (NMACA) in the ground electronic state. Each of the three pathways ends up with chemically stable products, which were observed experimentally using chromatographic (RP-HPLC) and mass spectrometry (ESI-MS) techniques. Their population in the reaction mixtures upon the completion of CL reflects the character of the substituents present in the benzene ring of the XAEs. However, at this stage of the research, we were unable to observe any of the high-energetic reaction intermediates revealed by computations.

The measurements of the XAE time profiles enabled us to assess the CL decay constants (k_{CL}) and the Relative Chemiluminescence Efficiencies ($RCLE$) in various environments (Table S4, ESI†). To do so, we tested XAEs in aqueous or methanolic solutions, containing various bases and additives such as sodium hydroxide, tetrabutylammonium hydroxide and 1,8-diazabicyclo[5.4.0]undec-7-ene, as well as cationic, zwitterionic or neutral surfactants such as cetyltriethylammonium chloride, dimethyldodecylammonio-1,3-propane sulfonate and Triton X-100. All the XAEs, except **2-OCH₃** (the only chemiluminogenic salt in the studied group, bearing an electron-donor substituent) emit a flash-type blue radiation (*ca.* 450–455 nm) in aqueous or methanolic solutions, completed within 1–2 seconds, regardless of the steric hindrance caused by spatial atoms (Br, I) attached at the *ortho* positions of the lateral benzene ring of XAEs. In all cases the aqueous triggering reagents, including quaternary ammonium base (H_2O_2 + TBAOH) allowed obtaining 2–20% higher $RCLE$ if compared to standard (H_2O_2 + NaOH) or other triggers (H_2O_2 + DBU; H_2O_2 + TBAOH in CH_3OH) (Fig. 2B). A distinct decrease in the XAEs’ $RCLE$ values upon lowering of the aqueous environment’s pH was observed (Fig. 3A). The effect was most pronounced in the case of the **2-OCH₃** (deactivated by the substituent for the nucleophilic attack) while it is less pronounced in the cases where the esters bear substituents of a distinct negative inductive effect, such as **2-NO₂** or **2,6-diF** (retaining *ca.* 50% of emission ability at pH = 8). Among the compounds investigated, **2-OCH₃**, **4-Br** and **3-F** can be recommended as fragments of CL labels due to their high usefulness at pH = 12 (the product of relative efficiency and stability at given pH), while compounds **2,6-diF**, **2-NO₂**, expressing high usefulness at pH = 8, are interesting reagents in applications where high alkalinity of the environment makes a limitation (Fig. 3B).

The screening of the influence of surfactants on the emission ability and kinetics enabled us to select the CL systems of optimal performance – some of them, containing pairs of reagents such as **2,6-diF/DDAPS** or **2-F/Triton X-100**, express more than 3 times higher efficiency compared to the emission in typical aqueous environments with no enhancers ($\text{H}_2\text{O}_2/\text{NaOH}$).

The amounts of the acid (NMACA), the product of XAEs’ alkaline hydrolysis, detected in the post-CL reaction mixtures, are traceable (below 2.5 mol%). The latter presumably arise from the fact that the values of alkaline hydrolysis constants (k_{OH}) are 2–3 orders of magnitude lower than the respective constants of CL decay (k_{CL}), as revealed among acridinium 2,6-dihaloesters (Table 3 and S3 (ESI†)). Other experiments revealed that the alkaline hydrolysis at pH = 12 of sterically hindered compounds, such as **2,6-diBr**, occurs *ca.* 17 times slower than the hydrolysis of the compound **2,6-diF**, as indicated by values of k_{OH} (Table 4).

We hope the present work will enhance the knowledge on the mechanism of oxidative transformations and physico-chemistry of the acridinium esters, bearing various atoms or groups of atoms in their phenoxy moieties, involved in the generation of light. Knowledge on their CL properties in

various liquid environments is useful in the rational design of new luminogenic labels and indicators to be employed in ultrasensitive biomedical and/or environmental analytics.

Experimental

Computations

Unconstrained geometry optimizations of isolated molecules (Scheme 1) were carried out, at the DFT level of theory,⁴⁸ using the B3LYP functional⁴⁹ and 6-31G** basis sets⁵⁰ implemented in the GAUSSIAN 09 program package.⁵¹ After completion of each optimization, the Hessian (second derivatives of the energy as a function of the nuclear coordinates) was calculated to assess whether stationary structures had been obtained.⁴⁸ The harmonic vibrational frequencies were then derived from the numerical values of these second derivatives and used to obtain the enthalpy and Gibbs' free energy contributions at 298.15 K and standard pressure with the aid of a built-in computational program of statistical thermodynamics routines.⁵² The solvent (water) effect with complete optimization of each molecule was included in the DFT calculations at the level of the Polarized Continuum Model (PCM) (UAHF radii were used to obtain the molecular cavity).⁵³

The enthalpies ($\Delta_{r,298}H^\circ$) and Gibbs' free energies (free energies in the case of DFT(PCM)) ($\Delta_{r,298}G^\circ$) of the reactions (r), as well as the enthalpies ($\Delta_{a,298}H^\circ$) and Gibbs' free energies (free energies in the case of DFT(PCM)) ($\Delta_{a,298}G^\circ$) of activation (a) indicated in Scheme 1 were calculated by following the basic rules of thermodynamics.⁵⁴ The rate constants (${}_{298}k^\circ$) for the gaseous phase reactions were obtained by applying the equation:

$${}_{298}k^\circ = \frac{RT}{Nh} \exp[-\Delta_{a,298}G^\circ/(RT)] \quad (1)$$

resulting from the transition state theory, and the reaction completion time (${}_{298}\tau_{99}$) from the formula:

$${}_{298}\tau_{99} = \ln 100/{}_{298}k^\circ \quad (2)$$

where R , T , N , and h denote the gas constant, temperature (298.15 K), Avogadro number, and Planck's constant, respectively.⁵⁴

Chemical identity of XAEs and spectroscopic analyses of XAEs

9-[(Phenoxy)carbonyl]-10-methylacridinium triflates, substituted in the benzene ring (XAEs, Scheme 1), were synthesized by adopting the procedures reported before.^{25–32} The structures and IUPAC names of the compounds obtained for this work are depicted in Scheme 1. The detailed data, concerning chemical synthesis and identity (retention times (RP-HPLC), melting points, elemental analysis) of the compounds investigated in this work are given in the ESI†

XAEs were subjected to structural analyses with the aid of nuclear magnetic resonance spectroscopy (^1H NMR, ^{13}C NMR, ^1H - ^1H COSY) and MALDI-TOF (Matrix Assisted Laser Desorption

Ionization Time of Flight) as well as ESI (Electrospray Ionization) mass spectrometry (MS) techniques. The nuclear magnetic resonance spectra (^1H NMR, ^{13}C NMR) were registered in CD_3CN , CD_3OD or $\text{DMSO}-d_6$ (300 K) using a Unity 500 Plus spectrometer (Varian) with a 5 mm PFG probe. In some cases, the two-dimensional ^1H - ^1H COSY and HETCOR techniques were employed for the unequivocal assignment of signals (compounds **2-F**, **2-Cl**, **2,6-diBr** and **4-Cl**). The experimental results were compared with the simulated spectra generated with the aid of the ^1H NMR ACD package.⁵⁵ The masses of the parent cations involved in XAEs were established using a VG Trio-3 triple quadrupole mass spectrometer (Masslab, Altrincham, UK) with 2,5-dihydroxybenzoic acid (DHB) as a matrix. Mass spectra were acquired in the positive polarity mode with the 100–700 m/z range of scan. The laser was set to 125 shots and the TOF detector was set to a linear mode with a 20 kV difference of potentials. The molecular ions recorded, together with basic fragmental ions, are summarized in Table S1 (ESI†). In the case of selected XAEs, ESI-MS spectra were registered for the post-CL reaction mixtures, using a Bruker-Esquire LC Electrospray Ionisation Mass detector (Bruker-Daltonics GmbH, Germany). The mass spectra were acquired in the positive ion mode in the scan range of m/z 100–600. Capillary exit, trap drive and skimmer voltages were set to 81.9 V, 27.6 V and 15 V, respectively.

Measurement of chemiluminescence

Investigations of XAEs' (Scheme 1) chemiluminescence were conducted in two fashions using a Fluoroskan Ascent FL plate luminometer (LabSystems-Thermo). To compare the kinetics and efficiency of the emission, the equipment was operated at moderate sensitivity (photomultiplier tube (PMT) voltage = 340 mV) and a resolution of 20 ms per point. To record the kinetic profiles, the compounds were dissolved in anhydrous acetonitrile (Aldrich) to obtain a concentration of 5.0 mM. Portions of such prepared stock solutions were diluted with 1 mM aqueous HCl to obtain a concentration of 4×10^{-5} M and 200 μl aliquots of the latter were serially dispensed ($n = 5$) over a 96-well white polystyrene plate (Cliniplate). Next, portions of H_2O_2 solution (100 μl , 0.06%) in 0.01 M HNO_3 were added to each well and the plate was shaken for 30 seconds. CL was triggered by adding 100 μl of NaOH solution (0.2 M) and collecting the light output over 2-minute periods at 298 K. The above experiments were repeated in the same manner using other bases ($c = 0.2$ M): tetrabutylammonium hydroxide (TBAOH) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Values of CL decay constants (k_{CL}) were calculated as negative values of the slopes of $\ln(I_{\text{CL}}/I_{\text{CL}}^0) = f(\text{time (s)})$ dependences.

To measure the efficiency of CL, the above acidic solutions of XAEs were serially diluted with 1 mM HCl to obtain solutions in the range of concentration 4×10^{-5} – 4×10^{-8} M and were treated as described above. The integral light outputs were collected by calculating the area under each kinetic profile, applying the implemented Ascent FL software. The calibration graphs were obtained upon linearization of the fol-

lowing relationship: Area under CL profile (A , RLU²) = $f(\text{XAE concentration (M)})$. The values of emission efficiency for each CL system were obtained as the slopes of the linear calibration graphs and normalized to the highest value (RCLE).

To compare the CL efficiencies of XAEs at various pH, 50 μL aliquots of their acidic solutions ($c = 4 \times 10^{-5}$ M) were added to the wells of the white plate ($n = 5$). Next, a 50 μL portion of 0.12% w/w H_2O_2 in 0.01 M HNO_3 was added to each well and the plate was shaken for 30 seconds. CL was triggered by adding 200 μL Britton-Robinson buffer (pH = 8.0, 10.0, 12.0) and the integral light outputs (A) were collected over 2-minute periods. The CL efficiency for XAEs was calculated from the formula:

$$\text{CL efficiency} = 100\% \times A, (\text{given pH})/A \\ \text{for the most efficient system.}$$

To assess the stability of XAEs in alkaline environments, XAE stock solutions were diluted with Britton Robinson buffer (pH = 8.0), to reach a final concentration of salt of 4×10^{-5} M. The solutions were then incubated at 298 K for 30 minutes and 200 μL aliquots of the latter mixtures were added to each well of the plate ($n = 5$), followed by the addition of 100 μL of 0.06% H_2O_2 in 0.125 M HNO_3 (to obtain pH = 3.0–3.5) and a 30-second shaking of the mixtures. To trigger the emission, 100 μL of NaOH (0.3 M) was added (final pH = 12) and the light outputs were integrated over 2 minute periods. The stability of XAEs, regarded as the preserved initial activity, was calculated from the following equation:

$$\text{Stability} = 100\% \times A (t = 30 \text{ min. incubation})/A \\ (t = 0, \text{ no incubation}).$$

A second type of CL experiment was performed at the maximal sensitivity of the apparatus (PMT voltage = 1000 mV) for selected XAEs in the presence of various surfactants: cetyltrimethylammonium chloride (CTAC), dimethyldodecylammoniumpropane sulfonate (DDAPS) and Triton X-100. The acridinium salts were serially dissolved in 1 mM HCl to obtain a series of solutions of concentrations in the range of 5.0×10^{-11} – 2.5×10^{-9} M. The solutions of surfactants (24 mM of CTAC or DDAPS and 60 mM of Triton X-100) were optimized and prepared in the oxidizing solution (0.01 M HNO_3 containing 0.06% H_2O_2). 100 μL aliquots of XAE solutions were distributed over a 96-well, white polystyrene plate ($n = 5$), followed by the addition of 50 μL of the solution of the oxidant containing each of the above surfactants. CL was triggered by the addition of 50 μL of 0.2 M KOH to each well. Temporary intensities of CL (I_{CL} , RLU) were collected every 20 ms until the reactions were complete (up to 2 minutes). For comparison, the measurements were taken under the same conditions with no surfactants. The changes in the values of CL decay kinetic constants (Δk_{CL}) and emission efficiencies (ΔRCLE) were calculated as the ratio of the value obtained under experiments carried out in the presence of a surfactant and the adequate value obtained with no surfactant (Table 3).

Products of XAEs' chemiluminescence

The products of CL were assayed directly after completion of the emission of light with the aid of a Waters chromatographic system (see the ESI†). For these experiments, the column employed was a 150×4.60 C6 Phenyl 110, 5 μm (Gemini, Phenomenex) and the mobile phase consisted of 60%/40% v/v acetonitrile/phosphate buffer (2 mM Na_2HPO_4 + NaHPO_4 , pH = 3.5). The emission of XAEs was triggered in Eppendorf vials, by mixing 20 μL of XAE (5 mM solution in anhydrous acetonitrile) with 50 μL of H_2O_2 in 0.1 M HNO_3 , followed by the addition of 0.2 M aqueous NaOH (50 μL). After completion of the emission, the mixtures were acidified with a drop of H_3PO_4 and immediately subjected to chromatographic analyses (absorption detection at 254 and 268 nm). Post-CL reaction products were identified by a comparison of their retention times (R_T) and signal intensities (A) with the analogous parameters obtained for standard substances – namely 10-methyl-9-acridinone (NMAON), 10-methylacridinium-9-carboxylic acid (NMACA) and the respective phenols (RPhOHs): 2,6-difluorophenol, 2,6-dichlorophenol, 2,6-dibromophenol and 2,6-diiodophenol. Calibration graphs prepared for NMAON and NMACA enabled the assessment of the content of the latter product in the post-CL reaction mixtures (Table 4, Fig. S4†).

To assess the pseudo-first order kinetic constants of alkaline hydrolysis for selected XAEs (k_{OH} , Table 4), the XAEs were dissolved in phosphate buffer (pH = 12, $c_{\text{XAE}} = 1.0$ – 1.5 mM) and were incubated at 298 K for up to 5 min. To stop the reaction after a certain time, 200 μL of each of the above mixtures were treated with 10 μL solution of the TFA/mobile phase (1/3 v/v) to obtain a final pH = 3. Hydrolysis constants (k_{OH}) were established, using the above-mentioned Waters HPLC system (mobile phase: acetonitrile/0.1% $\text{TFA}_{\text{aq}} = 1/1$ v/v; stationary phase: Waters Symmetry C₈, 4.6×250 mm, 5 μm) with the UV-absorption detector set at 256 nm. Chromatographic runs were recorded after specified reaction times (20–200 s, $n = 3$) and the areas under the NMAON and NMACA signals were calculated using built-in Empower™ software. The values of k_{OH} were established graphically, using a linear calibration chart, assuming c_0 values as the initial concentration of XAE in the mixture and c_t values as the temporary concentration of the hydrolysis product (NMACA), established according to the areas under the signal (Fig. S5 (ESI†)).

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