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Chemiluminogenic Properties of 10-Methyl-9-(phenoxycarbonyl)acridinium Cations in Organic Environments

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The chemiluminogenic (CL) properties of aryl esters of 9-carboxy-10-methylacridinium acid and 9-carboxy-2-methoxy-10-methylacridinium acid (AE), variously substituted in the benzene ring (2-H, 2-CH₃, 2-Cl) were investigated in aliphatic alcohols, acetonitrile, and dimethyl sulfoxide in the presence of hydrogen peroxide and different bases—potassium hydroxide, tetra-*n*-butylammonium hydroxide, and 1,8-diazabicyclo[5.4.0]undec-7-ene. The dependence of their CL properties (decay rate constants (k_{CL}) and relative efficiencies (RE)) on solvent parameters, the nature and concentration of base, as well as H₂O₂ concentration were investigated. Comparison of the various AE revealed that substituents at the benzene ring strongly influence the reaction kinetics, while 2-OCH₃ substitution of the acridine nucleus is manifested, in general, by a red shift in the emission spectrum and slight increase in CL efficiency. The values of k_{CL} depend linearly on polarity and acid–base properties of solvents as well as on concentration of bases (over certain concentration ranges) and demonstrate a nonlinear dependence on H₂O₂ concentration. RE values depend on solvent polarity and nucleophilicity but are rather weakly dependent on base and oxidant concentrations. The CL properties of the above systems are discussed in the context of their physicochemical features gained from fluorescence spectroscopy, spectrophotometric titration, MS, and HPLC. Electronically excited 10-methyl-9-acridinones are the light-emitting entities in both organic and aqueous environments. It was also found that the tendency for an unwanted side-process, the production of a pseudobase form of AE, to take place was similar in alcoholic and aqueous media, although 2-methoxy ring-substituted derivatives seemed to be less susceptible to this dark-type conversion. On the basis of these results new CL systems are postulated that are more efficient than their aqueous counterparts.

Introduction

Derivatives of 10-methylacridinium-9-carboxylic acid play an important role in the systems employed today for the chemiluminogenic assay of biological molecules such as antigens, antibodies, hormones, and others.^{1–6} They are also used as tracers in assays of oxidative entities (e.g., hydrogen peroxide, peroxide anion) and other nucleophilic entities in environmental, biological, or pharmaceutical samples.^{7–11} Quantitative analyses utilizing aromatic esters of 10-methylacridinium-9-carboxylic acid (acridinium esters) are regarded as some of the most sensitive analytical methodologies available nowadays, enabling analytes to be detected even at the sub-attomole level.^{12,13} Recently, new systems based on AE have been developed for high-sensitivity ligand binding investigations in homogeneous nucleic acid hybridization assays utilizing luminescence quenching.^{14,15}

Typically, the oxidation of AE in an aqueous environment is initiated by the action of a peroxide anion (OOH[−]) generated in situ in basic hydrogen peroxide solution; the process is accompanied by the relatively effective emission of light (chemiluminescence), reaching quantum yields of ca. 2% and lying within the blue range of spectrum.^{16–19} We investigated the mechanism of this rather complicated process in detail with respect to the simplest AE (10-methyl-9-(phenoxycarbonyl)acri-

dinium cation) using modern quantum chemistry methods, including the DFT level of theory.²⁰ The results revealed the multistep nature of the oxidation of AE and of the intermediates that may be produced along the reaction pathway. Even so, there is still not enough experimental support for theoretical findings.

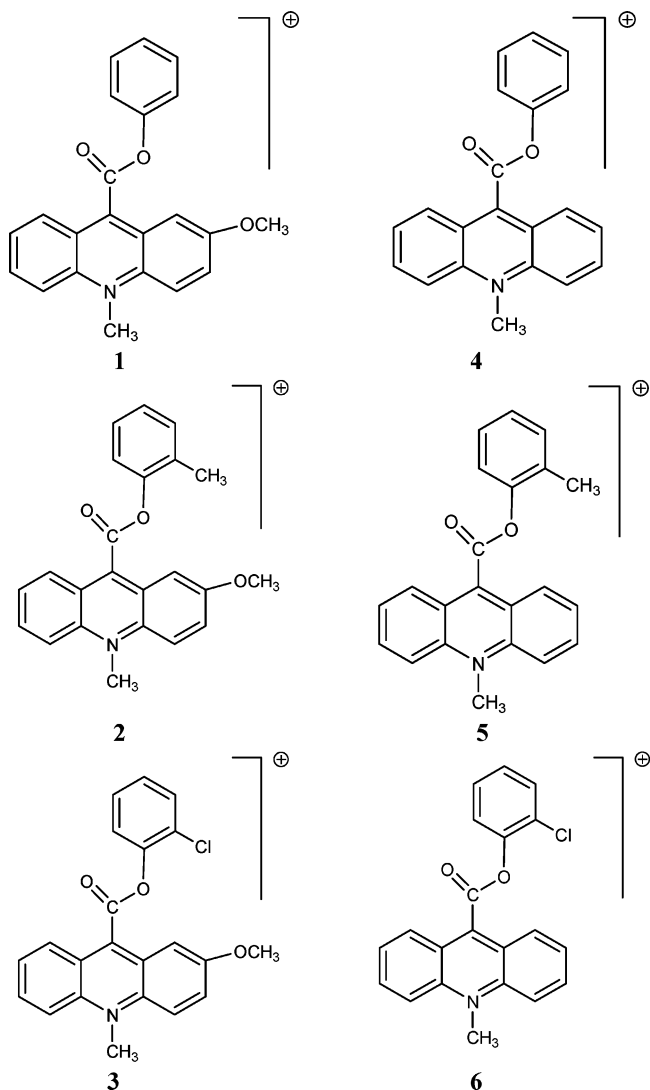
Despite the numerous papers and patents describing the application of acridinium chemiluminogenic systems in modern analytical procedures^{5,12,21} not much research has been directed at determining the physicochemical properties of AE bearing various substituents in the benzene or acridine rings as well as their CL properties in nonaqueous media. It was observed that the kinetics of CL and the efficiency of light emission of AE depend more or less on the structure of the leaving group (the phenoxy or phenoxycarbonyl fragment).^{22–24} In the group of acridinium tracers bearing an electron-donating (CH₃, OCH₃) and electron-accepting (Br) substituents, CL efficiency depends not only on the type of the latter but also on their location, which suggests that ortho- or para-substituted systems perform better than meta-substituted ones. However, no simple explanation has been given for the differences between the luminogenic ability of variously phenyl-substituted derivatives; the results are not always predictable.²⁴

Up to date no systematic investigations have been undertaken to quantitate the influence of substituents on the above-mentioned and other luminogenic features of 10-methyl-9-(phenoxycarbonyl)acridinium cations (e.g., the wavelength of emitted light). Moreover, very few investigations have been

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SCHEME 1: Structures of the Chemiluminogenic Cations Investigated

undertaken on derivatives containing substituents in the acridine nucleus.^{25–27} The latter aspect seems to be important, as the substitution of the acridine ring alters the electronic properties of the parent chemiluminogenic cation and the resulting emitter, the excited derivative of 10-methyl-9-acridinone.²⁸

Another problem we considered in the work is the influence of the physicochemical properties of the liquid phase on a system's chemiluminogenic performance, i.e., the type of solvent and base as well as concentrations of triggering reagents. We carried out these investigations with the aim of postulating new AE-based chemiluminogenic systems that may perform better in terms of efficiency of light emission and stability in comparison with a typical aqueous CL system based on simple AE. We also attempted to disclose the multiparametric description of the CL systems investigated in this work.

We believe the physicochemical investigations of simple acridinium cations presented in this work may occur helpful in rational designing of chemical labels or tracers utilizing the phenomenon of chemiluminescence.

Experimental Section

Syntheses and Analyses of AE. Acridinium salts investigated (Scheme 1) were synthesized starting from 2-methoxyacridine-

9-carboxylic acid (**1–3**: 2-methoxy-10-methyl-9-(phenoxycarbonyl)acridinium (**1**), 2-methoxy-10-methyl-9-[(2-methylphenoxy)carbonyl]acridinium (**2**), and 9-[(2-chlorophenoxy)carbonyl]-2-methoxy-10-methylacridinium (**3**)) or acridine-9-carboxylic acid (Aldrich) (**4–6**: 10-methyl-9-(phenoxycarbonyl)acridinium (**4**), 10-methyl-9-[(2-methylphenoxy)carbonyl]acridinium (**5**), and 9-[(2-chlorophenoxy)carbonyl]-10-methylacridinium (**6**)) and the appropriate hydroxybenzene (Aldrich): phenol, 2-methylphenol, or 2-chlorophenol.^{2,29,30} Detailed information concerning synthesis of the compounds and their analyses are given in the Supporting Information. Physicochemical properties concerning derivatives **4–6** are reported in some of our other papers: spectral,³⁰ thermal^{31,32} and NMR (¹H + ¹³C).³³ Crystallographic data relevant to some of the derivatives discussed here are given elsewhere.^{29–32,34–36}

The chromatographic parameters of AE were established using a chromatographic set consisting of a Waters 600E Multisolute Delivery System, a W2487 Dual λ Absorbance Detector ($\lambda(\text{obsd}) = 268, 254 \text{ nm}$), a Gemini 5 μm C6 phenyl 110 column (Phenomenex), and a 60/40 v/v % acetonitrile/water mixture containing 2 mM phosphates ($\text{Na}_2\text{HPO}_4 + \text{NaHPO}_4$, pH = 3.5) as stationary and mobile phases, respectively. Retention times (R_T) and % areas under signals are presented as the average values of at least three runs. To assess the composition of post-CL reaction mixtures, 20 μL of the stock solution of **1–6** in acetonitrile (5 mM) was mixed with 50 μL of 18 mM H_2O_2 in methanol or acetonitrile, followed by the addition of 50 μL of 0.2 M solution of base (KOH, DBU) in appropriate solvent. After completion of CL, the mixtures were acidified with a drop of trifluoroacetic acid (TFA), followed by addition 125 μL of mobile phase and subjected to HPLC analysis. The signals were ascribed by comparison their retention times (R_t) with the ones obtained for standard substances. R_t values for standard substances (commercial or synthesized previously according to described methods), namely, 10-methyl-9-acridinone,²⁸ 2-methoxy-10-methyl-9-acridinone,³⁷ 10-methyl-acridinium-9-carboxylic acid, and 2-methoxy-10-methylacridinium-9-carboxylic acid,^{38,39} phenol, 2-methylphenol, and 2-chlorophenol (Aldrich), were estimated as the mean values of at least three independent runs. Population of products in the post-CL reaction mixtures was estimated by comparison of the areas under each signal with the respective area of signal found for an appropriate standard having known concentration.

MS analyses were performed using a Fennigan LCQ Deca Surveyor LC/MS/MS spectrometer (electrospray ionization).³¹ Mass spectra were acquired in the positive polarity mode in the m/z scan range of 100–600 (N_2 sheath gas), with the source, capillary, and trap voltages set at 5 kV, 17 V, and 10 V, respectively. Exemplary mass spectra are given in Supporting Information (Figure 1S). To examine the reaction of AE with a base, ethanolic solutions (100 μL , 0.5 mM) of **2** or **5** (Scheme 1) were stirred with 10 μL of a 1 mM solution of NaOH in ethanol and the resulting mixtures immediately introduced into the apparatus described above (50 μL of each sample). Spectra were recorded over several time spans ranging from 0.4 to 40 min. The signals found in m/z (relative abundance) were for **2** 223 (58%), 239 (6%), 251 (7%), and 358 (100%) and for **5** 193 (88%), 210 (26%), 221 (8%), and 328 (100%).

Chemiluminescence Kinetics and Efficiency. CL profiles (temporary intensities (I_{CL}) versus time) were recorded using a Fluoroskan Ascent FL plate luminometer (Labsystems, Finland) with the photomultiplier tube voltage operating at 300 mV.

Stock solutions of **1–6** (5 mM) were diluted to a final concentration of AE of $4 \times 10^{-5} \text{ M}$ with one of the following

solvents (spectroscopic or anhydrous grade (>99.5%), all from Aldrich): methanol (MeOH), ethanol (EtOH), 2-propanol (2-PrOH), 2-butanol (2-BuOH), acetonitrile (AN), dimethyl sulfoxide (DMSO), and water (HPLC grade, Millipore); all solvents were acidified with traces of TFA prior to use. These solutions were placed on a white 96-well Cliniplate plate (200 μ L in each well, $n = 5$), and 100 μ L portions of a 18 mM solution of hydrogen peroxide (Aldrich) in appropriate solvent were added to each well, after which the plate was shaken for 10 s. CL profiles (temporary CL intensity (I_{CL}) versus time) were recorded at 298 K, following the addition of 100 μ L of a 0.20 M solution of base (potassium hydroxide (KOH), tetra-*n*-butylammonium hydroxide (TBAOH) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), p.a. grade, Aldrich) in the relevant solvent. The pseudo-first-order CL decay constants (k_{CL}) were obtained by linearizing ($\ln I_{CL}$) the descending section of the averaged CL profiles (at least 95% of the height of each profile) and taking the reciprocal of each slope.

CL intensities were recorded in that the areas under the temporary CL profiles were calculated using an integration algorithm implemented in the Ascent FL software (LabSystems, Finland). The areas under the CL profiles (in RLU) were normalized to the value obtained for the unsubstituted derivative (**4**) in the H₂O/H₂O₂/KOH system (regarded here as the reference system) under the above conditions. The experiments involving various concentrations of reagents (AE, base, or H₂O₂) were performed with all the other settings kept constant (see above). Second-order kinetic constants (k_{2CL}) were obtained as slopes of linear relationships between k_{CL} values and base concentrations.

Absorption and Emission Measurements. A Varian Cary spectrofluorimeter was employed to record the stationary spectra of CL. One milliliter of 4×10^{-5} M solutions of AE (**1**, **3**, **4**, **5**) in methanol, acetonitrile, or H₂O were stirred with 0.5 mL of 18 mM solution of H₂O₂ in an appropriate solvent and acidified with traces of TFA. A quartz cuvette containing the mixture was then placed in the apparatus chamber equipped with a small stirring unit, and 0.5 mL of 0.20 M base (KOH, TBAOH or DBU) was added in the dark.

Stationary CL spectra were measured in two steps. First, emission spectra were measured over the 400–600 nm range of the luminescing solution; recording of the spectra was carried out simultaneously with the addition of base. Then, the fluorescence spectrum of 2-methoxy-10-methyl-9-acridinone was recorded at the same settings using an excitation wavelength of 360 nm.

Time-resolved fluorescence experiments were performed using the apparatus described in ref 40. The experimental resolution was 0.1 ns, the standard error being $\pm 0.05\%$ of the measured value. The excitation wavelength was set at 360 nm and the observation wavelengths at 470 and 450 nm for **1** and **4**, respectively. Fluorescence decays were measured in post-CL reaction solutions of AE in organic solvent (methanol, acetonitrile) or water on completion of light emission. The proportions of reagents were kept the same as in the lumino-metric experiments (Tables 1 and 2); the reaction mixtures were diluted 80 times with an appropriate solvent prior to the measurements.

To perform the spectrophotometric titrations, **1–6** were dissolved in acidified (TFA) solvent to obtain a final AE concentration of 0.4 mM. Aliquots of 1.9 mL were titrated against KOH solution (9.0 mM) in an appropriate solvent in steps of 4×10^{-3} mL to a final pH of 10. A Cerko Lab System microtitration unit, consisting of a calibrated 0.5 mL Hamilton syringe, a precision 18-bit pH-meter, a Mettler-Toledo InLab

TABLE 1: Pseudo-First-Order Kinetic Constants of CL Decay (k_{CL} , in s^{-1}) Obtained for Compounds **1–6 in Various Systems (RSD = 2–8%)^a**

compd no.	base	k_{CL}						
		MeOH	EtOH	2-PrOH	2-BuOH	AN	DMSO	H ₂ O
1	KOH	1.58	1.99	2.32	2.43		3.17	2.96
	TBAOH	1.90	2.20	2.62	2.77	13.4	3.53	3.45
	DBU	1.07	0.76	0.32	0.25	8.95	0.95	
2	KOH	0.07	0.37	1.46	1.57		1.74	0.13
	TBAOH	0.10	0.52	2.42	2.66	12.8	4.63	0.20
	DBU	0.05	0.03	0.02	0.02	3.42	0.42	
3	KOH	2.86	2.06	2.42	3.23		3.39	5.66
	TBAOH	3.04	2.63	2.94	5.15	13.8	4.57	3.81
	DBU	3.37	2.56	2.41	2.22	15.1	1.52	
4	KOH	1.27	1.99	2.03	3.08		3.16	3.01
	TBAOH	1.61	2.23	2.08	4.47	10.7	4.51	3.29
	DBU	0.84	1.08	0.29	0.24	10.4	1.14	
5	KOH	0.06	0.39	1.27	1.44		1.89	0.14
	TBAOH	0.09	0.42	3.13	2.70	12.2	3.37	0.20
	DBU	0.04	0.02	0.02	0.02	3.35	0.40	
6	KOH	2.91	2.19	3.28	3.82		3.43	6.77
	TBAOH	2.80	2.88	3.20	5.32	13.7	4.28	4.05
	DBU	3.69	2.67	2.07	1.94	10.4	2.12	

^a Concentrations of the reagents were kept constant in all experiments ($c(\text{AE}) = 2.0 \times 10^{-5}$ M, $c(\text{H}_2\text{O}_2) = 4.5 \times 10^{-3}$ M, and $c(\text{base}) = 5 \times 10^{-2}$ M; final values).

TABLE 2: Relative Efficiencies of CL (RE) of Compounds **1–6 in Various Systems (RSD = 2–8%)^a**

compd no.	base	relative efficiencies						
		MeOH	EtOH	2-PrOH	2-BuOH	AN	DMSO	H ₂ O
1	KOH	0.76	1.37	1.18	1.10		0.70	0.64
	TBAOH	0.81	1.57	0.95	0.71	0.89	0.53	0.57
	DBU	0.74	1.03	1.22	0.71	1.95	0.23	
2	KOH	0.77	1.87	1.53	0.95		0.67	0.59
	TBAOH	0.69	1.68	0.95	0.69	0.84	0.55	0.58
	DBU	0.59	1.19	0.93	0.69	2.13	0.32	
3	KOH	0.80	1.31	1.49	1.12		0.73	0.75
	TBAOH	0.93	1.75	0.92	0.67	0.90	0.60	0.55
	DBU	0.76	1.79	1.55	1.17	1.81	0.39	
4	KOH	0.81	1.28	1.20	0.80		0.19	1.00 ^b
	TBAOH	0.81	1.59	0.68	0.53	0.67	0.18	0.92
	DBU	0.69	1.22	0.83	0.53	1.37	0.09	
5	KOH	0.70	1.40	1.22	0.63		0.16	0.94
	TBAOH	0.61	1.15	0.62	0.51	0.66	0.16	0.84
	DBU	0.54	0.98	0.75	0.47	1.41	0.10	
6	KOH	0.74	1.33	1.06	0.68		0.16	1.02
	TBAOH	0.79	1.24	0.50	0.47	0.62	0.16	0.86
	DBU	0.68	1.57	1.09	0.98	1.32	0.10	

^a Concentrations of the reagents were kept constant in all experiments ($c(\text{AE}) = 2.0 \times 10^{-5}$ M, $c(\text{H}_2\text{O}_2) = 4.5 \times 10^{-3}$ M, and $c(\text{base}) = 5 \times 10^{-2}$ M; final values). ^b Chemiluminescence quantum yield for this system is equal to 1.88 (± 0.02)%.³⁸

423 pH microelectrode, and a host PC, was used in all the experiments. The pH electrode was calibrated before use against at least five buffers.⁴¹ After the addition of each aliquot of base, UV–vis spectra in the 250–500 nm range were recorded at 298 K using a Perkin-Elmer Lambda 40 spectrophotometer and a 1 cm quartz cuvette. Changes in absorbance were monitored and plotted on one graph. The absorption at selected wavelengths plotted against the actual pH value gave sigmoid curves with one inflection point, from which the constants of pseudobase formation (pK_{OH}) were calculated using the Henderson–Hasselbach algorithm⁴² implemented in Spectral Data Lab software.⁴³

Quantum Chemical and Statistical Calculations. Multi-parametric correlation analysis was applied according to the standard procedure described in ref 44 using the Statistica 8.0 software.⁴⁵ The quality of correlation was evaluated and the

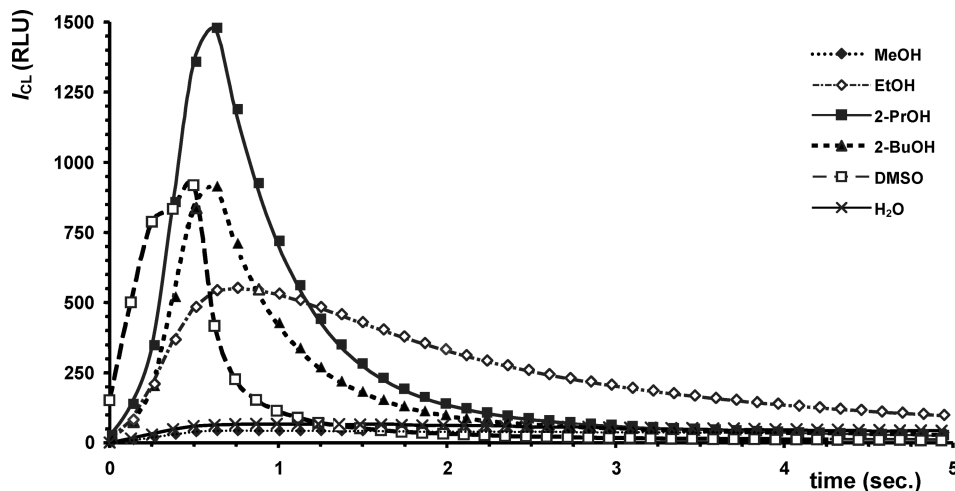


Figure 1. Chemiluminescence profiles for **2** in various solvents using the $\text{H}_2\text{O}_2/\text{KOH}$ triggering system.

optimal equation chosen on the basis of the optimal values of two parameters—squared correlation coefficients and standard errors.

Values of Mulliken charges and LCAO coefficients of the p_z LUMO orbital⁴⁶ at selected atoms were obtained at the DFT level of theory (B3LYP/6-31G**) in exactly the same way as described in ref 47. The calculations were carried out on computers of the Tri-City Academic Network Computer Centre in Gdansk (TASK).

Results and Discussion

Chemiluminescence and Solvent Properties. The structures of acridinium salts investigated are presented in Scheme 1. The chemiluminescence profiles of **2** in various solvents (methanol, ethanol, 2-propanol, 2-butanol, dimethyl sulfoxide, and water) using a $\text{H}_2\text{O}_2/\text{KOH}$ triggering system are presented in Figure 1. Linearization of the kinetic profiles ($\ln(I_{\text{CL}})$ versus time) in the decay range of the curves yielded relationships characterized by correlation coefficients in the 0.92–0.99 range. The pseudo-first-order kinetic constants of CL decay (k_{CL}), derived from the above graphs, are summarized in Table 1. Integration of the areas under the profiles and their normalization to the area obtained for unsubstituted derivative **4** in the aqueous environment (the reference system) disclosed the relative efficiencies of light emission (RE), summarized in Table 2.

A brief look at the profiles presented in Figure 1 enables the CL kinetics of **2** to be compared in various environments differing in terms of solvent properties. The profiles differ substantially even in the group of neutral protic solvents. Glow-type emissions occur in methanol and water, although flash-type emissions are the rule (emission is complete in ca. 2 s) in higher alcohols (2-propanol, 2-butanol). The fastest emission was observed in dimethyl sulfoxide; however the latter environment displayed only limited stability during the experiments.

There are certain regularities in the values of k_{CL} listed in Table 1 (the values for an aqueous environment are presented in the last column for comparison). In all systems the pairs of compounds **1/4**, **2/5**, and **3/6** behaved similarly in terms of CL kinetics. Thus, the presence of an electron-donating substituent in the acridine ring system appears to have had only a minor influence on the reaction kinetics. In contrast, the type of substituent introduced in the ortho position of the benzene ring (the ester fragment of AE) strongly influenced the values of k_{CL} . Accordingly, CL decay constants were highest for 2-chloro derivatives (**3** and **6**) (flash-type CL) and lowest (glow-type CL)

for 2-methyl derivatives (**2** and **5**). A strongly electronegative substituent, such as a chlorine atom, obviously facilitates the nucleophilic attack of an oxidizing ion at the C9 atom of the acridine nucleus, whereas the presence of a methyl group—weakly electron donating—slows the reaction down substantially.

The DFT-calculated Mulliken charges at C9 (where the attack of OOH^- takes place²⁰), are slightly higher (3–6%) in the case of **3/6** than in **2/5** (Table 1S in the Supporting Information).

However, this trend is reversed if one considers the LCAO coefficients of the p_z LUMO orbital at C9, although the differences are small. It must be pointed out here that Mulliken population analysis predict rather small negative charge deficiency at C9 but substantial negative charge deficiency at C15 atom, suggesting that carbonyl carbon is the favored site for primary attack of nucleophile (OOH^- or OH^-). However, when LCAO coefficients of the p_z LUMO orbital at C9 (which might also serve as a measure of susceptibility to nucleophile substitution) are considered, the values at the latter atom are more than 10 times higher than those at C15, suggesting C9 as a center for the primary attack of anion. Thus, a simple theoretical approach applied to this problem does not reveal conclusive results in the above-discussed context.

The relative CL efficiencies (RE) of **1–6**, i.e., the ratios of the averaged areas under the actual CL profile to the area found for the reference system, are summarized in Table 2. Generally, there are not such big differences among the RE values from investigated salts as compared to the values of k_{CL} . In the organic systems 2-methoxy ring-substituted derivatives (**1–3**) are generally slightly more efficient than unsubstituted ones (**4–6**). This trend is most pronounced in aprotic solvents (DMSO, AN), while in lower alcohols only minor differences were recorded.

Another trend that emerges from the data in Table 2 is that in alcoholic media the maximum efficiency was always attained in ethanol or 2-propanol, regardless of the type of base used; in some cases RE values in alcohols were as high as 1.8–1.9 (**2** with KOH as a base or **3** with DBU).

The highest values of RE, which in some cases are more than double the efficiency of the reference system, were in most cases recorded in the AN/DBU system, whereas RE was generally low in DMSO and 2-BuOH, especially when derivatives contained no substituent in the acridine ring (**4–6**). One cannot rule out the possibility that in these relatively viscous environments (2-BuOH, $\eta = 2.30$ cP; DMSO, $\eta = 1.99$ cP)⁴⁸ effective mixing of reagents might be, to some extent, rendered by diffusion upon the experiment conditions. It should be pointed

TABLE 3: Multiparametric Relationships between Pseudo-First-Order CL Decay Constants (k_{CL}) in Organic Systems and the Relative CL Efficiencies (RE) of Organic CL Systems

(A) Multiparametric Relationships between Pseudo-First-Order CL Decay Constants (k_{CL}) in Organic Systems and Parameters of Solvents: Kirkwood Polarity Constant (Y), Nucleophilicity (B), and Electrophilicity (E)										
compd no.	base/equation for k_{CL}									
	KOH	R^2	s	TBAOH	R^2	s	DBU	R^2	s	
1	$24.0Y - 0.041B - 0.255E + 3.20$	0.99	0.07	$-0.112B - 0.629E + 34.4$	0.97	0.89	$-0.089B - 0.409E + 25.2$	0.97	0.70	
2	$-11.8Y - 0.049B - 0.363E + 14.0$	0.98	0.21	$-0.103B - 0.814E + 33.5$	0.95	1.25	$-0.034B - 0.202E + 9.85$	0.97	0.39	
3	$29.9Y - 0.121B - 0.583E + 22.7$	0.99	0.30	$-0.104B - 0.643E + 33.8$	0.97	0.86	$-0.143B - 0.464E + 40.3$	1.00	0.50	
4	$13.9Y - 0.064B - 0.380E + 14.5$	0.80	0.52	$-0.078B - 0.504E + 25.8$	0.89	1.25	$-0.104B - 0.200E + 29.2$	0.96	1.01	
5	$-0.768Y - 0.043B - 0.338E + 14.6$	0.99	0.14	$-0.100B - 0.756E + 31.9$	0.94	1.25	$-0.033B - 0.198E + 9.66$	0.97	0.28	
6	$10.4Y - 0.162B - 0.748E + 43.3$	1.00	0.03	$-0.103B - 0.617E + 33.3$	0.97	1.00	$-0.090B - 0.282E + 26.3$	0.98	0.57	

(B) Multiparametric Relationships between the Relative CL Efficiencies (RE) of Organic CL Systems and Parameters of Solvents: Polarizability (P) and Nucleophilicity (B) ^{a,b}										
compd no.	base/equation for RE									
	KOH	R^2	s	TBAOH	R^2	s	DBU	R^2	s	
1	$-19.8P + 0.010B + 2.45$	0.99	0.05	$-19.8P + 0.010B + 2.45$	0.99	0.05	$-25.3P + 5.81$	0.72	0.24	
2	$-35.0P + 0.017B + 3.95$	0.92	0.22	$-32.4P + 0.011B + 4.50$	0.80	0.28	$-26.6P + 6.07$	0.70	0.26	
3	$-20.5P + 0.010B + 2.73$	0.81	0.19	$-26.9P + 0.010B + 3.82$	0.77	0.22	$-29.3P + 6.87$	0.97	0.11	
4	$-25.0P + 5.52$	0.92	0.17	$-19.5P + 4.38$	0.87	0.21	$-25.0P + 5.52$	0.92	0.17	
5	$-22.3P + 4.95$	0.90	0.26	$-15.5P + 3.54$	0.82	0.16	$-22.3P + 4.95$	0.90	0.26	
6	$-20.6P + 4.72$	0.90	0.16	$-16.4P + 3.68$	0.73	0.22	$-27.8P + 6.26$	0.99	0.07	

^a Experimental data were taken from Table 1, tabular data are summarized in Table 2S (Supporting Information). Key: R^2 , squared correlation coefficient; s , standard error of estimation. ^b Experimental data were taken from Table 2.

out that more pronounced deflections from linearity of $\ln I_{CL}$ versus time relationships were observed in the case of systems containing the two latter solvents.

The multiparametric analysis of the relationship between pseudo-first-order CL decay constants and solvent parameters was carried out applying the Palm equation⁴⁹

$$k_{CL} = k_{CL}^0 + yY + pP + bB + eE$$

where k_{CL} and k_{CL}^0 are the respective CL decay constants in solvent and in a vacuum, respectively, Y is the Kirkwood constant characterizing solvent polarity ($Y = (\epsilon - 1)/(2\epsilon + 1)$),⁵⁰ ϵ is the dielectric constant, P is the solvent polarizability ($P = (n^2 - 1)/(2n^2 + 1)$),⁵⁰ n is the refractive index, B is the empirical basicity index,^{51,52} E is the electrophilicity index⁵³, and y , p , b , and e denote respective coefficients of the equations. The values of each of the above parameters are collected in Supporting Information (Table 2S).

The results of the multiparametric analysis are given in Table 3 and illustrated in Figure 2(i). These data suggest that k_{CL} values correlate with the nucleophilicity and electrophilicity indices of the solvent, so that the CL decay rate depends on the acid–base properties of solvents. It follows from Table 3 that in all cases, the increase in solvent nucleophilicity and electrophilicity was manifested by a slowing down of CL decay. In systems containing TBAOH, the equations for k_{CL} were similar for all of the compounds investigated. In the case of the ones containing DBU, the influence of solvent basicity on reaction kinetics is comparable to the ones containing TBAOH, as it is indicated by respective values of b parameters; however, in the case of 2-methyl derivatives (2 and 5) this dependence seems to be much weaker. The influence of solvent electrophilicity on reaction kinetics in systems with DBU is generally lower in comparison to the ones containing base TBAOH. This may have been due to the different mechanism of AE interaction with the various bases. In the presence of KOH, the CL rate depended not only on E and B but also on solvent polarity (Y), whose

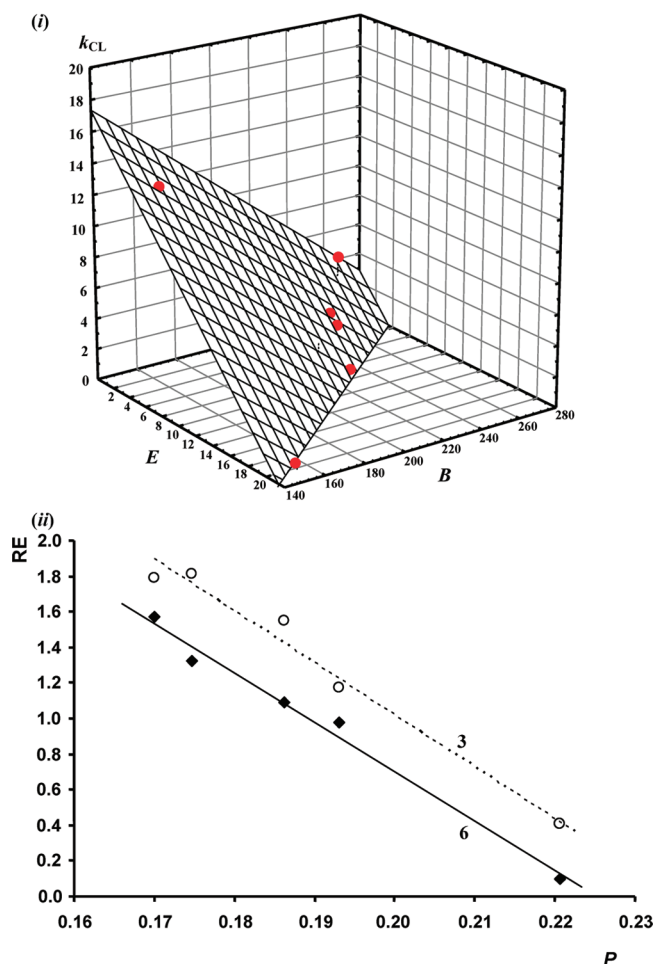


Figure 2. Upper graph (i) relationships among k_{CL} values and solvent nucleophilicity (B) and electrophilicity (E) for 2 in the presence of TBAOH. Lower graph (ii): relationships between values of RE and solvent polarizability indices (P) for 3 and 6 in the presence of DBU. The equations are presented in Table 3.

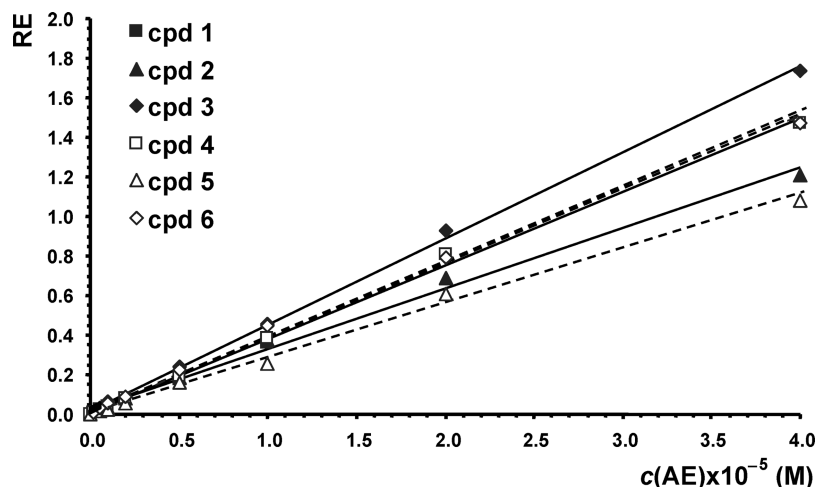


Figure 3. Dependences of relative efficiency of CL (RE) of 1–6 on AE concentration in the MeOH/TBAOH system. The equations are $RE = (3.732 \times 10^9)c(AE) + 0.005$ (1), $RE = (3.068 \times 10^9)c(AE) + 0.022$ (2), $RE = (4.367 \times 10^9)c(AE) + 0.013$ (3), $RE = (3.734 \times 10^9)c(AE) + 0.008$ (4), $RE = (2.736 \times 10^9)c(AE) + 0.007$ (5), $RE = (3.685 \times 10^9)c(AE) + 0.027$ (6).

influence seemed to be multidirectional. Increasing the solvent polarity accelerated CL decay in the case of 1/4 and 3/6 but retarded it in the case of compounds 2/5.

We also took into consideration the possible correlations between the relative efficiency of chemiluminescence (RE) and physicochemical properties of solvent (Palm's parameters, mentioned above) as well as the type of base used for triggering CL. The main factors affecting the intensity of emitted light seem to be the polarizability and nucleophilicity of the solvents. It follows from the equations presented in Table 3B that higher RE values should be obtained in media with a lower polarizability and higher nucleophilicity, although the latter parameter is often negligible. The linear dependences between the RE value and polarizability indices of solvents are clearly visible in Figure 2(ii).

It should be added that the multiparametric analysis of RE values in the context of solvent parameters was carried out with respect not only to Palm's parameters but also to solvent viscosities.⁴⁸ Nevertheless, the results showed that the influence of viscosity on RE was statistically not significant.

Chemiluminogenic Properties and Reagent Concentrations. Concentration of AE. The dependences of CL efficiency on the concentration of 1–6 in MeOH/TBAOH system are illustrated in Figure 3. The values of RE of 1–6 at various salt concentrations in MeOH/TBAOH and AN/DBU are disclosed in the Supporting Information (Table 3S).

In the investigated systems CL efficiency is expressed by a linear dependences ($R^2 > 0.99$) on AE concentration. In contrast, within the studied range of $c(AE)$, the changes in k_{CL} values were not significant, varying ca. 2–10% around the values listed in Table 1 (data not shown). The region of RE linearity involves 3 orders of magnitude of AE concentrations (1×10^{-8} to 4×10^{-5} M) at constant detector sensitivity (PMT = 300 mV). Other concentrations were not investigated within the above-mentioned settings due to the detection limit (regarded here as 10 intensities of blank) or detector saturation. However, by adjusting the detector sensitivity to the maximum value (PMT voltage = 1000 mV), limits of detection in the range of 10^{-14} – 10^{-12} M (for 2-Cl and 2-Me substituted derivatives, respectively) were attainable.

The above calibration graphs enabled a comparison of AE in terms of their relative CL efficiencies. Thus, the order of RE in MeOH/TBAOH is $3 > 6 > 1 \approx 4 > 2 > 5$. Clearly, flashing systems (containing 2-Cl substituted derivative) emitted light

more efficiently than glowing systems (containing 2-CH₃ substituted derivative). Another finding is that acridinium salts bearing a CH₃O substituent in the acridine nucleus (1–3) were generally better emitters than their ring-unsubstituted analogues (4–6). Similar trends were observed in the AN/DBU system. Experiments suggest that altering the electronic properties of the acridine ring—by introducing an electron-donating substituent—is manifested by slight increase in the CL efficiency of the respective AE in organic environment. The introduction into the ring of more substituents with diverse electronic properties may well produce a conspicuous improvement in the performance of luminescing systems based on acridinium esters. We plan to explore this aspect in the future.

Concentration of Base. The influence of base concentration on the parameters of AE chemiluminescence (pseudo-first-order kinetic constants of CL decay and relative efficiencies) for representative systems is outlined in Figures 4 and 5 and the data are summarized in the Supporting Information (Table 4S).

The relationships between k_{CL} and base concentrations for 1–6 in MeOH/TBAOH are illustrated in Figure 4(i). They are linear in the region of low concentrations of base; at higher concentrations k_{CL} values diminish gradually. Interestingly, the region of linearity is different for various derivatives: it may extend up to 0.15 M TBAOH for 2 and 5 (slow-decaying systems) but is much narrower (up to ca. 0.04 M TBAOH) for 3 and 6 (fast-decaying systems). Other observations are that the order of k_{CL} is $3, 6 > 1, 4 > 2, 5$ (2-Cl > 2-H > 2-CH₃ derivatives) over the whole base concentration range; 2-OCH₃ derivatives (1–3) are always characterized by slightly higher values of k_{CL} than their ring-unsubstituted analogues (4–6) for the reactions performed in MeOH/TBAOH. Similar trends have been observed in other systems investigated (Table 4S, Supporting Information).

Figure 5 presents relationships between the base concentrations and constants of CL decay for 2 in methanol. Such dependences enabled us to estimate, within the range of acceptable linearity ($R^2 > 0.95$), second-order kinetic constants for the CL process (k_{2CL}); these are summarized in Table 4. Comparison of the data for ring-substituted and unsubstituted analogues (1–3 and 4–6) shows that they are characterized by similar k_{2CL} values in a given environment. Generally, the faster the system decays, the higher the dependence of reaction kinetics on the base concentration, as it is indicated by the order of k_{2CL} values (2-Cl > 2-H > 2-CH₃-substituted derivatives).

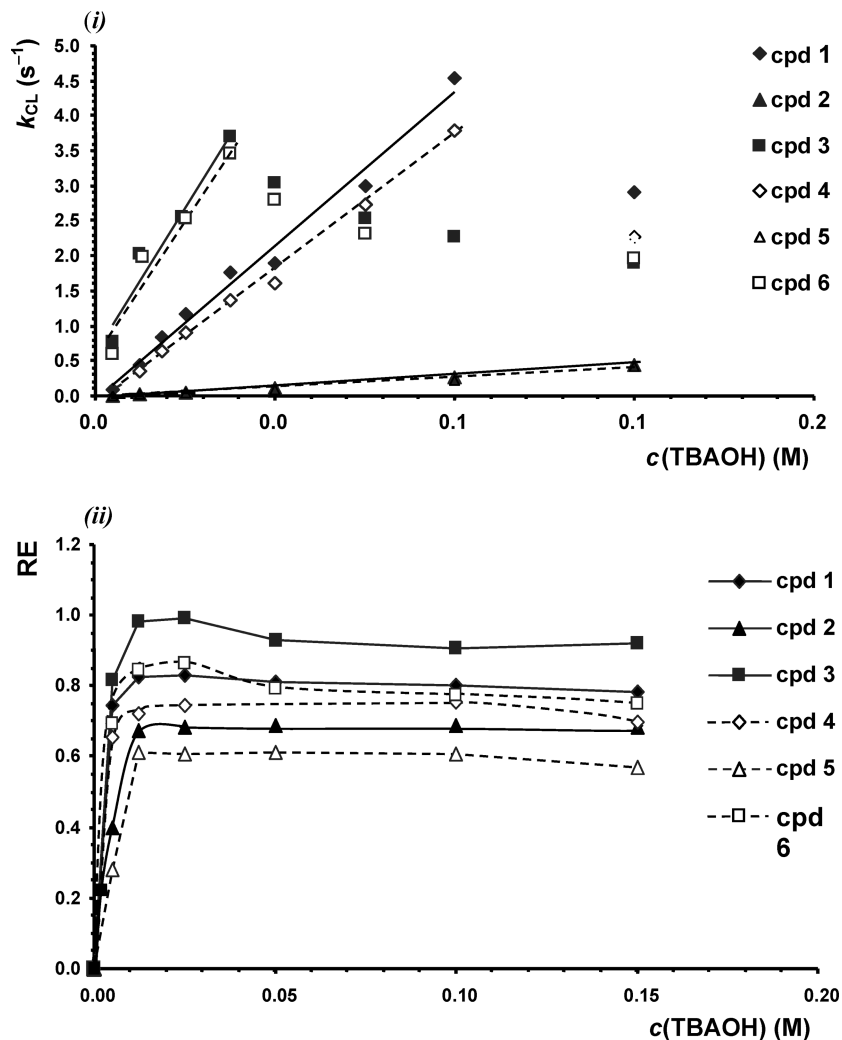


Figure 4. Upper graph (i): relationships for 1–6 in the MeOH/TBAOH system between the pseudo-first-order kinetic constants of CL decay (k_{CL}). Lower graph (ii): relative CL efficiencies (RE) and base concentrations.

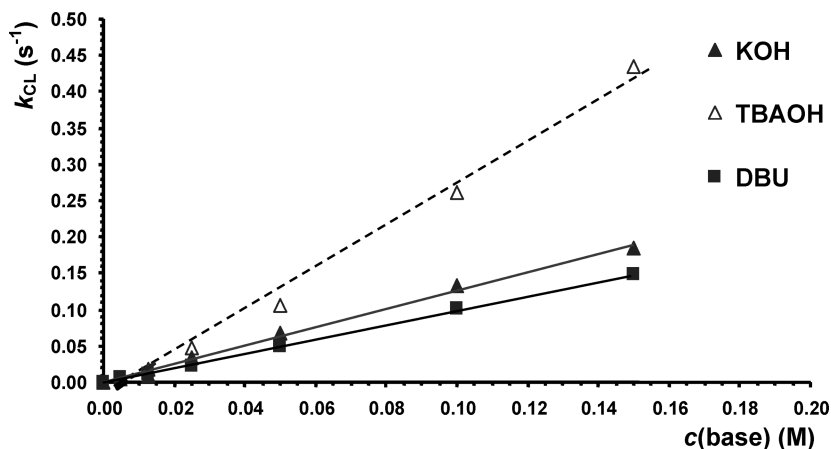


Figure 5. Dependencies between base concentrations and pseudo-first-order kinetic constants of CL decay (k_{CL}) of 2 in methanol. The equations are $k_{CL} = 1.264c(\text{KOH})$, $k_{CL} = 2.912 c(\text{TBAOH}) - 0.018$, $k_{CL} = 0.999c(\text{DBU})$.

The relative efficiencies (RE) of 1–6 in MeOH/TBAOH as a function of base concentration are presented in Figure 4 (ii). In all cases a fast increase in RE was observed in the low base-concentration region (up to ca. 15 mM). Above that point RE values stabilized (2, 5) or reached a plateau (3, 6). At concentrations above of 50 mM (the value used in most experiments), a slight reduction of CL efficiency was recorded

in all cases. Similar relationships have been found for other systems investigated (Supporting Information, Table 4S).

Concentration of the Oxidant. The graphs showing the dependence of CL decay constants and efficiency of 1–6 on hydrogen peroxide concentration in the AN/DBU system are illustrated in Figure 6. The data for these systems are summarized in Supporting Information (Table 5S).

TABLE 4: Second-Order Kinetic Constants of CL Decay ($k_{2\text{CL}}$, in $\text{M}^{-1} \text{s}^{-1}$) for 1–6 in Various Organic Systems

compd no.	$k_{2\text{CL}}$			
	MeOH + KOH	MeOH + TBAOH	MeOH + DBU	AN + DBU
1	29.8	44.3	19.6	50.6
2	1.26	2.98	1.00	49.2
3	90.4	83.2		166
4	25.5	38.3	15.6	146
5	1.15	2.77	0.85	46.3
6	129	80.7		

The relationships between k_{CL} and H_2O_2 concentration were not linear in any of the investigated concentration ranges (Figure 6(i)). However, all the relationships expressed a maximum, the position of which differed slightly for various groups of compounds. Hence k_{CL} for **1** and **4** reached the maximum value at ca. 50 mM of H_2O_2 , while that of **2** and **5** was ca. 5 mM and that of **3** and **6** was ca. 90 mM. The kinetic constants attained comparable values for the above-mentioned pairs of AE within the entire range of oxidant concentrations; within the pairs of analogues the mutual relationships among k_{CL} exhibited quite minor differences (**1** \approx **4**, **3** > **6**, **5** > **2**).

Dependences of RE on concentration of H_2O_2 for **1**–**6** in AN/DBU are presented in Figure 6(ii). Generally, the values of RE increased strongly in the region of low oxidant concentrations (2–10 mM); in the cases of flashing derivatives (**1**, **3**, **4**, **6**) this trend continued—but was less pronounced—up to 50 mM. Beyond that value, the efficiency of CL decreased slowly for all the systems investigated. As in the other experiments (e.g., Figure 3), ring-substituted derivatives (**1**–**3**) were generally more efficient (by average 1.5 times) than their unsubstituted analogues (**4**–**6**), especially in the region of low concentration of H_2O_2 ($<10^{-1}$ M). It should be pointed out that, over the whole range of H_2O_2 concentrations, all the acridinium salts attained higher values of RE in AN/DBU in comparison to the reference system.

Products of Transformations of AE in Organic Media.

To acquire further insight into AE transformations in organic environments, we analyzed the spectral parameters of emitting forms, estimated the equilibrium between cationic and nonluminescing pseudobase forms of AE in solutions, and attempted to estimate the population of products that appear in the postreaction mixtures on completion of light emission.

Origin of Emitting Forms. According to the all of reported mechanisms of AE CL, light emission is due to the relaxation of excited molecules of 10-methyl-9-acridinones, which are the main final products of AE oxidation.^{16,17,20} Hence, the emission spectra recorded during CL (stationary CL spectra) should correspond to the fluorescence spectra of the respective derivative of 9-acridinone present in the postreaction mixture and, if it is the only emitter, to the fluorescent spectra of the latter.

The stationary CL spectra of **3** in representative systems (MeOH/KOH, AN/DBU) together with the spectrum obtained for the aqueous system are illustrated in Figure 7. Additionally, fluorescence spectra of 2-methoxy-10-methyl-9-acridinone were plotted in the above systems over the same range of wavelengths. Generally speaking, the spectrum of this derivative resembled the stationary spectra obtained for ring-unsubstituted AE in aqueous systems.³⁸ However, the position of the emission maximum of **3** was shifted by ca. 35–40 nm toward higher wavelengths in comparison with the ring-unsubstituted analogues.

In view of the similarity between the CL spectra of the emitting mixture and the fluorescence spectra of relative

acridinone, it can be concluded that the latter compound is the main (or even the only) emitting entity during light emission. Similar observations have been made in the past with other CL systems based on AE.⁵⁴ Evidently, the above-mentioned red shift is due to the change in electronic properties of the emitting entity bearing, in the case of **3**, an electron-donating group (CH_3O) at position 2 of the acridine ring system. Additionally, the position of the maximum depends somewhat on the properties of the liquid phase, centring around 486 nm in water, 470 nm in methanol, and 458 nm in acetonitrile. In comparison with the latter solvent, the red shift in H_2O or MeOH might be due to the formation of hydrogen-bonded structures involving the carbonyl O atom of 2-methoxy-10-methyl-9-acridinone in the protic environments.^{28,55} Similar results were obtained for other AE (data not shown); the spectra for **1**–**3** and **4**–**6** under the same conditions were comparable, suggesting that the range of emission depended primarily on the structure of the acridine nucleus and to a lesser extent on the properties of the liquid phase (solvatochromism).⁵⁶ To shed more light on the above statements, we subjected the postreaction mixtures of the representative compounds (**1**, **4**) to time-resolved fluorescence spectroscopy experiments.^{39,56} Analysis of the data revealed the presence of two-exponential fluorescence decays involving short-lived and long-lived components. The fluorescence lifetime (τ_{F}) of the short-lived component fell within the range of the apparatus's resolution (0.1 ns or less). The latter emission could be associated with 10-methylacridinium-9-carboxylic acid (the product of alkaline hydrolysis of AE), whose lifetime recorded at the same settings was $\tau_{\text{F}} = 0.16$ ns (water). Both values represent the same order; however, the differences could have arisen from the various chemical environments of the cation in the two cases (solvent + other products of CL reaction in the first case versus pure solvent in the second case). The above suggestion was supported by other experiments (see below). The mean fluorescence lifetime of a long-lived component was equal to 15.6, 12.0, and 9.75 ns for **1** and to 13.3, 10.3, and 7.34 ns for **4** in water, methanol, and acetonitrile, respectively, and could be attributed to the fluorescence decay of the corresponding 9-acridinone derivative. These data reveal that CL systems emitting at higher wavelengths (e.g., those containing **1**) express longer fluorescence decay lifetimes than systems emitting at shorter wavelengths (containing **4**).

The above results demonstrate that the light emission recorded during AE chemiluminescence in organic media is caused by the production of excited molecules of 2-methoxy-10-methyl-9-acridinone or 10-methyl-9-acridinone; this suggests that the mechanisms of CL in organic solutions and aqueous environments may well be similar. Minor changes in the spectral properties of CL in various organic solvents probably reflect different interactions of the excited fluorophore with organic media.

Composition of Post-CL Reaction Mixtures. The composition of post-CL reaction mixtures of **1**–**6** in the case of the MeOH/KOH and AN/DBU systems was evaluated using high-performance liquid chromatography. The results are compiled in Table 5, and the exemplary chromatogram is presented in the Supporting Information (Figure 2S).

In general, the chromatograms exhibited three main signals: in the case of 2-methoxy derivatives (**1**–**3**) at 3.75–3.88, 6.37–6.45, and 4.90–5.52 min and in the group of ring-unsubstituted derivatives (**4**–**6**) at 3.18–3.24, 6.33–6.34, and 4.90–5.52 min. We ascribed those signals by comparing them with retention times (R_t) of standard compounds, recorded at the same settings. Thus, the signals with the lowest R_t values

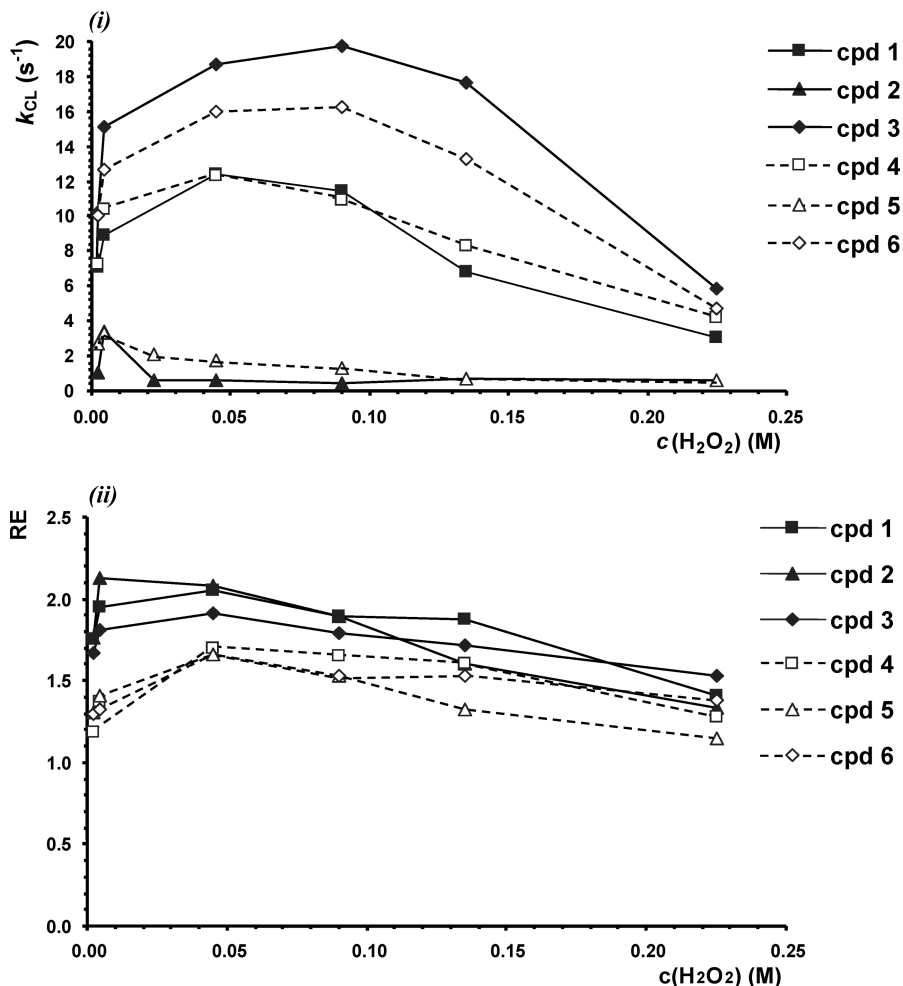


Figure 6. Upper graph (i): relationships for 1–6 between H_2O_2 concentration and pseudo-first-order kinetic constants of CL decay (k_{CL}). Lower graph (ii): the relative efficiencies of light emission (RE) versus concentration of H_2O_2 for 1–6 in the AN/DBU system.

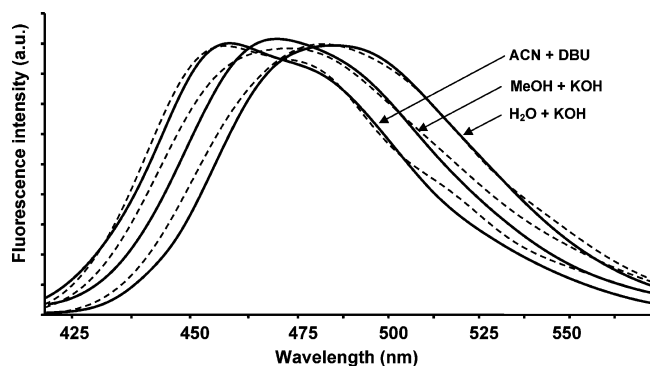


Figure 7. Stationary CL spectra of compound 3 recorded in various systems (solid lines). Dashed lines show fluorescence emission spectra of 2-methoxy-10-methyl-9-acridinone recorded in the same medium.

were attributed to 2-methoxy-10-methylacridinium-9-carboxylic acid ($R_t = 3.50$ min) or 10-methylacridinium-9-carboxylic acid ($R_t = 3.19$ min), the signals appearing at around 5 min to the respective hydroxybenzenes, phenol ($R_t = 4.93$ min), 2-methylphenol ($R_t = 5.50$ min), or 2-chlorophenol ($R_t = 5.55$ min), and the most intense signals to 2-methoxy-10-methyl-9-acridinone ($R_t = 6.48$ min) or 10-methyl-9-acridinone ($R_t = 6.37$ min). Analysis of the areas under the signals and their comparison with the areas obtained for standard solutions of known concentrations enabled us to estimate the molar fractions of the products appearing in the postreaction mixtures (Table 5).

TABLE 5: Chromatographic (HPLC) Data Obtained for Post-CL Mixtures of Compounds 1–6 in Representative Systems (RSD = 2–10%)

compd no.	acid ^a		acridone ^b		LG ^c	
	mf ^d	R_t^e	mf	R_t	mf	R_t
MeOH + KOH						
1	1.99	3.23	98.0	6.45	71.1	4.92
2	3.81	3.88	92.2	6.39	31.3	5.42
3	0.69	3.80	99.3	6.43	86.4	5.52
4	2.55	3.22	97.4	6.34	41.4	4.91
5	7.81	3.68	96.2	6.34	67.6	5.45
6	2.59	3.24	97.4	6.33	87.6	5.50
AN + DBU						
1	1.67	3.28	98.3	6.45	81.7	4.92
2	0.46	3.75	99.5	6.37	88.7	5.40
3	0.52	3.83	99.5	6.43	97.4	5.52
4	5.68	3.19	94.3	6.34	52.8	4.90
5	1.93	3.18	98.1	6.33	85.3	5.45
6	9.25	3.19	90.7	6.34	100	5.51

^a 2-Methoxy-10-methylacridinium-9-carboxylic acid (for 1–3) or 10-methylacridinium-9-carboxylic acid (for 4–6). ^b 2-Methoxy-10-methyl-9-acridinone (for 1–3) or 10-methyl-9-acridinone (for 4–6). ^c Leaving group. The numbers were calculated using standard solutions of respective phenol derivatives. ^d Molar fraction in the post-CL reaction mixture (in mol %). ^e Retention time (in minutes).

In most cases, there were larger quantities of respective acids—side products resulting from the alkaline hydrolysis of AE—in the group of ring-unsubstituted compounds (4–6) than

in the case of **1–3**. The following trend was observed in a protic environment: the slower the CL decay (Table 1), the greater the quantity of acid appearing in the postreaction mixture. Thus, in MeOH/KOH, the respective amounts of acids for **2/5** and **3/6** were at the levels of 3.8/7.8 mol % and 0.7/2.6 mol %. Presumably, side processes such as alkaline hydrolysis increase in importance when emission is longer. The occurrence of the above-mentioned acids in reaction mixtures could be one of the factors accounting for the diminution of CL efficiency of some longer-decaying AE.

However, the analyses of post-CL reaction mixtures of AE in AN/DBU gave slightly different results in the context under discussion here. There were relatively smaller amounts of alkaline hydrolysis products in mixtures containing 2-methyl derivatives (ca. 0.5 and 1.9 mol % for **2** and **5**, respectively) than in those containing 2-chloro derivatives (ca. 0.5 and 9.2 mol % for **3** and **6**, respectively). At the present moment, it is difficult to explain these findings; as already hinted, however, the oxidation of AE in the aprotic solvent may express some differences with regard to reaction mechanism.

The chromatograms of all the reaction mixtures also revealed the presence of signals attributed to the ester fragment of AE (the leaving groups, LG). We calculated the amounts of these substances, assuming that in an acidic environment they represent the various phenols that may dissociate from the substrate upon oxidation.^{2,16,17} However, the results are not fully conclusive, as in most cases we obtained values much lower than the expected 100 mol % (we obtained values close to 100% only for **3** and **6** in AN/DBU). One cannot exclude the possibility that the signals occur accidentally in the range characteristic of phenols, but they were in fact due to different substances, e.g., phenyl carbonates. The occurrence of such products was suggested by a theoretical analysis of the oxidation mechanism of the cation involved in **4** with H₂O₂ in a basic aqueous environment.²⁰

We shall give due attention to the above problems in our future research.

Dark Processes Attenuating CL. The occurrence of pseudobase forms of AE in alkaline solutions, detected experimentally,⁵⁸ was expected to be one of the main reasons for the diminution of their CL efficiency; this is their most important disadvantage, limiting their stability in aqueous environments and hence restricting their practical applications.^{2,58}

Products Occurring in an Alkaline Environment. To acquire further insight into the nature of the products occurring in an alkaline environment containing AE, ethanolic solutions of representative compounds **2** and **5** were mixed with a solution of NaOH, and ESI-MS spectra were recorded at regular time intervals. Spectra were analyzed together with the results obtained for the parent salts. The patterns obtained are generally similar in both cases. The most intense signals were attributed to the 10-methylacridinium cations ($m/z = 223$ and 193 for **2** and **5**, respectively) and to the parent cations, characterized by m/z of 358 and 328, respectively. In both cases we detected weak signals attributed to the $(M + 1)^+$ or $(M - 1)^+$ ions of 2-methoxy-10-methyl-9-acridinone or 10-methyl-9-acridinone at m/z at 239 and 210, respectively, as well as signals ascribed to 10-methylacridin-9(10*H*)-ylidene methanones (at m/z of 251 and 221 in the case of **2** and **5**, respectively).

The spectra obtained for the AE–NaOH mixtures show that both systems underwent dynamic changes in time (Figure 8). At about 3 min two distinct signals were recorded at m/z ca. 290/260 and at m/z ca. 398/368 for **2/5** respectively. We ascribed the first two signals to the sodium salts of 2-methoxy-10-

methylacridinium-9-carboxylic acid ($m/z = 290$) or 10-methylacridinium-9-carboxylic acid ($m/z = 260$), and the other two to the AE–NaOH adducts, which could be regarded as pseudobase forms of AE.^{2,20,58} The above signals tended to intensify with time; however, during the same time interval, the relative intensity of the signal due to the AE–NaOH adduct was higher for **5** than for **2**. This result suggests that the 2-OCH₃ derivative (**2**) is less likely than its analogue unsubstituted in the acridine ring (**5**) to form the pseudobase.

The latter observation concurs with the mentioned before theoretical values of both Mulliken charges and LCAO coefficients of the p_z LUMO orbital at C9 of the acridinium nucleus (Table 1S in the Supporting Information), where the nucleophilic attack takes place.²⁰ These parameters attain respective values of 0.082/0.295 and 0.093/0.310 for **2** and **5**. The higher values in the case of **5** as compared to **2** suggest that the former cation displays a greater tendency to accept the nucleophile, which is thus more likely to form a pseudobase-type adduct in an alkaline environment.

Constants of Pseudobase Formation. To evaluate the constants of pseudobase formation, spectrophotometric titrations were conducted for **1–6** in protic environments (MeOH/KOH, H₂O/KOH). Titrations in acetonitrile were not performed because of problems with electrode calibration. Titration curves illustrating acid–base transformations of **1** are illustrated in Figure 9. In another paper, we reported on spectrophotometric titrations of **4** and **5** in an aqueous environment.³⁸ Those results showed that the introduction of an alkyl substituent into the benzene ring of AE had practically no influence on the position and shape of the resulting titration curves.

The titration curves of **1** with KOH in methanol exhibit two maxima—a short-wavelength one at ca. 270 nm and a long-wavelength one at ca. 375 nm. These maxima, characteristic for the cationic form of AE,³⁰ gradually disappear with increasing pH and, simultaneously, a new band comes into existence at ca. 290 nm. Two isosbestic points characterize the titration curves of **1**: one at short wavelengths (ca. 282 nm) and one at longer wavelengths (ca. 350 nm). These results indicate that—as was the case for ring unsubstituted AE—two reversible forms are present in protic solvent, 2-methoxy-10-methyl-9-(phenoxycarbonyl)acridinium cation (capable of CL) and 9-hydroxy-2-methoxy-10-methyl-9-(phenoxycarbonyl)acridane (a pseudobase, incapable of CL).

Comparison of the absorption spectra obtained for **1** in alcoholic and aqueous environments revealed no significant changes (Figure 3S in the Supporting Information). However, a small red shift (up to 5 nm) was observed for all the characteristic points of the spectra; this could have been due to minor solvatochromic effects.⁵⁷ Comparison of the spectra of the two analogues **1** and **4** in an aqueous environment showed the differences to be more pronounced.³⁸ All the above-mentioned characteristic points for the 2-methoxy derivative were shifted by ca. 8–20 nm toward longer wavelengths as compared to the unsubstituted derivative.

In order to estimate the tendency for the pseudobases of **1–6** to form, we calculated the constants of their dissociation (pK_{OH}) from a numerical analysis of the titration curves. Thus, the respective pK_{OH} values in water of 2-methoxy substituted derivatives were 4.46, 4.38, and 4.44 for **1**, **2**, and **3** (the variability factor (RSD) ranged from 1.3% to 2.0%), while those of the ring-unsubstituted derivatives (**4**, **5**, **6**) were 4.73, 4.74, and 4.76 (RSD = 0.63–1.1%). In methanolic solutions, pK_{OH} values of **1**, **2**, and **3** were 4.58, 4.39, and 4.65 (RSD = 0.87–1.7%), respectively. The results show that a sub-

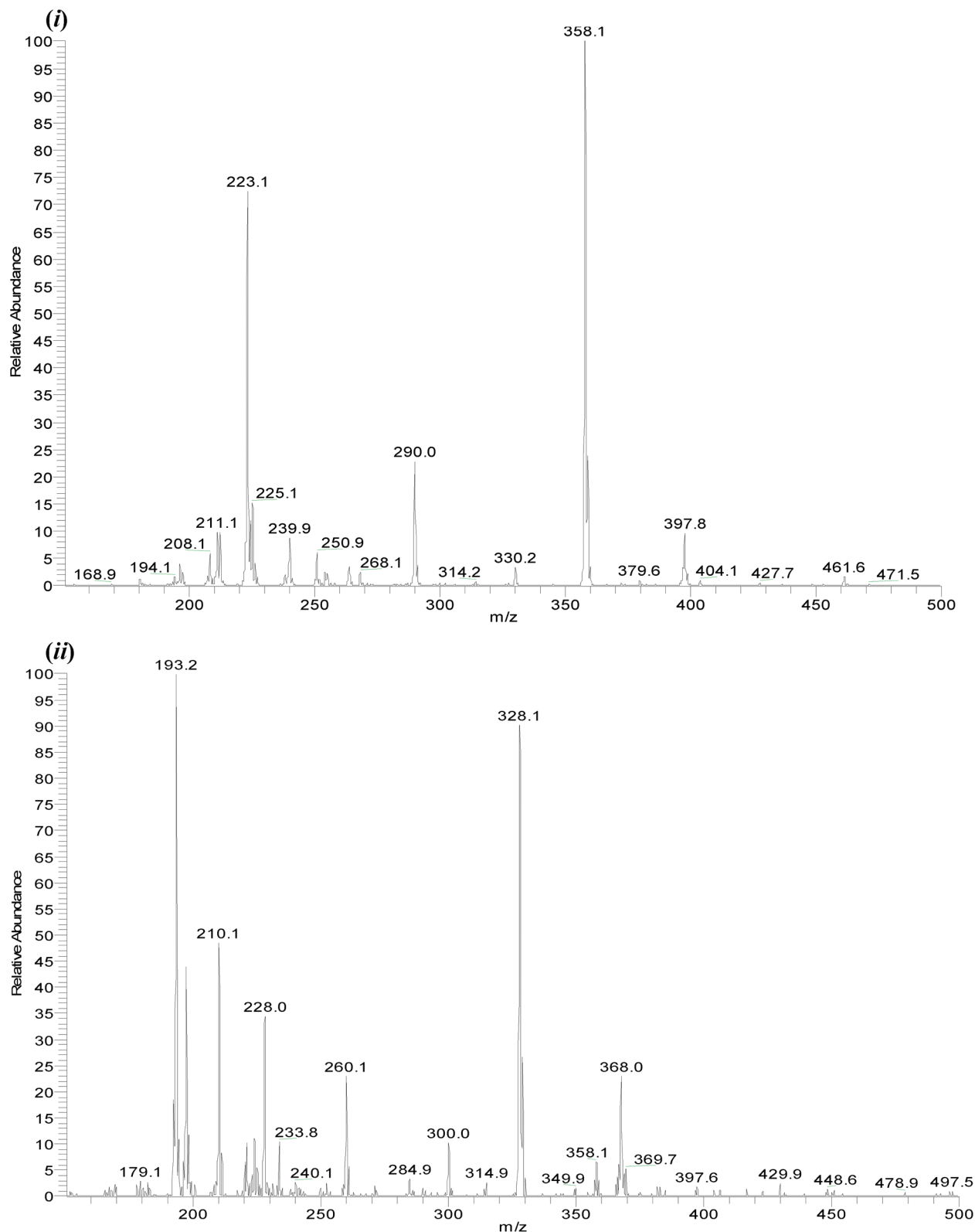


Figure 8. Mass spectra of **2** (i, upper graph) and **5** (ii, lower graph) after 3 min of incubation in an ethanolic solution of NaOH.

stituent present in the ester fragment of AE (H, CH₃, and Cl) hardly affected the ability of the pseudobase to form (**1–3** and **4–6** displayed only minor differences in their pK_{OH} values; in most cases they fell within the limits of experimental error). The differences in methanol were somewhat more pronounced, giving a pK_{OH} order of **3** > **1** > **2**, analogous to the order of k_{CL} (Table 1), expressing the susceptibility of

the cations to attack by OOH^- . In contrast, the introduction of an electron-donating substituent into the acridinium nucleus was manifested by a lowering of the relative pK_{OH} values (by 7% on average). These results lead to the conclusion that 2-methoxy derivatives are less likely than their unsubstituted analogues to form nonchemiluminogenic pseudobases in an alkaline environment.

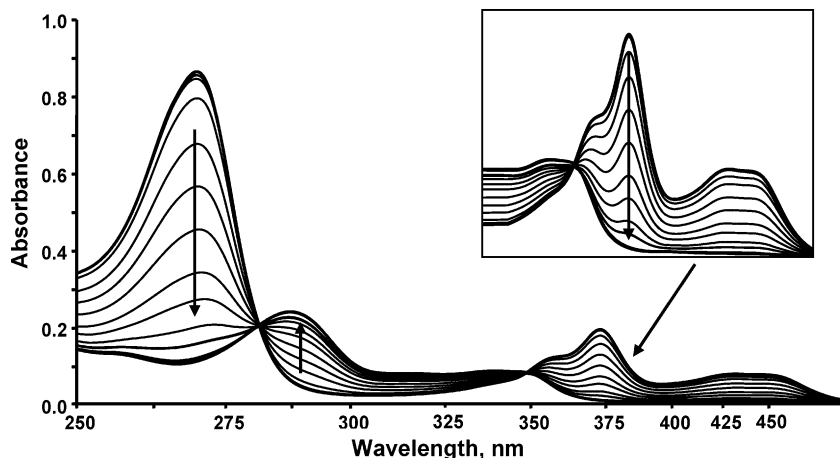


Figure 9. Spectrophotometric titration curves of **1** with KOH in methanol (pH range = 3.5–10.0).

Conclusions

10-Methyl-9-(phenoxy-carbonyl)acridinium salts differing in the type of substituent introduced in the position 2 of the benzene ring (H, CH₃, Cl) or in position 2 of the acridine nucleus (H, CH₃O) exhibit various trends in chemiluminogenic properties when oxidized with H₂O₂ in organic environments (aliphatic alcohols, acetonitrile, and dimethyl sulfoxide) in conjunction with the bases KOH, TBAOH, or DBU.

The kinetics of CL is controlled almost exclusively by the structure of the ester fragment of AE—in all cases the order of the kinetic constants of CL decay is 2-Cl > 2-H > 2-CH₃ substituted derivatives. In most cases AE substituted in the acridine ring displayed a somewhat better emission efficiency in organic environments than their unsubstituted analogues. The properties of the organic environment also influenced the efficiency of light emission; the highest values being attained with the AN/DBU and EtOH/TBAOH systems.

Statistical analysis indicated that emission decay constants depended primarily on the acid–base properties of solvents (i.e., their nucleophilicity and electrophilicity). The relative efficiencies of CL depended primarily on the polarizability of solvents and, to a lesser extent, on their nucleophilicity.

The dependence of RE on AE concentration was clearly linear over several orders of magnitude; the lowest detection limits of AE were at the level of 10^{−14} M.

The dependence of CL kinetics on base concentration was linear in the range of low concentrations of base. The interdependences between CL parameters and oxidant concentration were not linear.

The CL properties of AE are discussed in the context of stationary and time-resolved fluorescence, high-performance liquid chromatography, mass spectrometry (ESI), and spectrophotometric titration. Introduction of the 2-OCH₃ group into the acridine ring system was manifested primarily by an emission shift toward higher wavelengths as compared to the ring-unsubstituted cations; the position of the maximum varied slightly with the properties of the liquid phase. The emitting entities in all of the investigated systems seem to be excited molecules of 10-methyl-9-acridinones. HPLC analyses of post-CL reaction mixtures showed that 10-methylacridinium-9-carboxylic acids, the products of alkaline hydrolysis of AE, also occurred among the AE oxidation products, 2-methoxy ring-substituted amounts of these acids were generally lower. Analysis of mass spectra suggested that ring-unsubstituted compounds displayed a greater tendency than their 2-methoxy analogues to form nonchemiluminogenic pseudobases in basic

environment. This was also reflected by the constants of pseudobase formation, which were somewhat higher in the group of ring unsubstituted derivatives.

The experimental findings generally concurred with the values of Mulliken charges and the LCAO coefficient of the p_z LUMO orbital. Both parameters attained higher values in the group of ring-unsubstituted derivatives as compared to 2-methoxy derivatives, indicating the greater susceptibility of the former to attack by a nucleophile.

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Supporting Information Available: Detailed description of syntheses and analyses, Mulliken charges and LCAO coefficients of the p_z LUMO orbital obtained at the DFT level of theory (B3LYP/6-31G**), solvent parameters used for multi-parametric regression analysis, parameters of CL (pseudo-first-order of CL decay (*k*_{CL})) and relative efficiency of light emission (RE) in organic systems containing various concentrations of AE, bases, and hydrogen peroxide, selected mass spectra, HPLC chromatograms, and spectrophotometric titration curves. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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