Articles

Toward an Understanding of the Chemiluminescence Accompanying the Reaction of 9-Carboxy-10-methylacridinium Phenyl Ester with Hydrogen Peroxide

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Received March 26, 1998

The reaction between 9-carboxy-10-methylacridinium phenyl ester (CMAPE) and hydrogen peroxide leading to the formation of electronically excited 10-methyl-9-acridinone has been examined at the semiempirical PM3 level of theory. The reaction mechanisms proposed in the literature were verified and the details of the rather complicated processes leading to the emission of light (chemiluminescence) explained by these theoretical studies. Particular reaction steps were characterized at the thermodynamic and kinetic levels. The influence of the solvent was studied by means of the PM3-COSMO method (aqueous medium). The bottleneck steps determining the kinetics of the overall process were identified. Structural modifications of CMAPE that could improve its analytical usefulness were indicated on the basis of theoretical considerations.

Introduction

Chemiluminescence is a very interesting phenomenon, both from the cognitive and practical point of view.[1−8] As regards the latter aspect, the analytical applications of chemiluminescence are highly significant. Consequently, reactions giving rise to luminescence are widely used in acid−base and redox titrations, as well as in determinations of metal ions, molecular oxygen, and various organic compounds.[5] Attempts have been made to employ chemiluminescence to determine ATP in biological systems.[9] As the latter entity is involved in many essential biochemical processes, this finding has opened the way to the application of chemi- or bioluminescence in such disciplines as clinical chemistry, marine biology, and soil science.[9,10] The growing importance of chemiluminescence in immunoassays should also be mentioned:[1,10−20] the high specificity and sensitivity of antigen−antibody reactions, combined with the equally high sensitivity of chemi- and bioluminescence processes, are used here. The method was introduced as an alternative to the standard radioimmunoassays.[1,10−20] The growing interest in chemiluminescent labels is due to their considerable advantages over radioactive tracers: the former entities can be assayed with greater speed and sensitivity, and radioactive waste disposal is no longer a problem. Furthermore, proteins cannot be heavily labeled with ^{125}I because of the radiolysis effect, whereas, at least in principle, there is no limit to the number of chemiluminescent molecules incorporated.[5,11]

Connected through a spacer to an active group capable of interacting with macromolecules in living matter, 9-carboxyacridinium phenyl esters are one of the most efficient chemiluminescent labels used in immunoassays since 1980.[1,10,12−20] A chemiluminescent section of these labels, 9-carboxy-10-methylacridinum phenyl ester (CMAPE), has thus attracted much attention. These are the reasons why we chose CMAPE for our studies.

From the analytical point of view the sensitivity of a given method is one of the most important factors determining its usefulness. In the case of immunoassays, better sensitivity can be obtained by more heavily labeling macromolecules with chemiluminescent substances.[13] Another approach is to synthesize more efficient labels. However, to accomplish this task in a rational manner, an understanding of the mechanism of the processes

(1) Rauhut, M. M.; Sheehan, D.; Clarke, R. A.; Roberts, B. G.; Semsel, A. M. *J. Org. Chem.* **1965**, *30*, 3587.
(2) McCapra, F. *Pure Appl. Chem.* **1970**, *24*, 611.
(3) White, E. H.; Roswell, D. F. *Acc. Chem. Res.* **1970**, *3*, 541.
(4) McCapra, F. *Acc. Chem. Res.* **1976**, *9*, 201.
(5) Gundermann, K. D. *Proc.-Int. Symp. Anal. Appl. Biolumin. Chemilumin.* 1978 (Pub. **1979**), 37.
(6) Lee, C.; Singer, L. A. *J. Am. Chem. Soc.* **1980**, *102*, 3823.
(7) White, E. H.; Roswell, D. F.; Dupont, A. C.; Wilson, A. A. *J. Am. Chem. Soc.* **1987**, *109*, 5189.
(8) Abdel-Lalif, M. S.; Guilbault, G. G. *Anal. Chem.* **1988**, *60*, 2671.
(9) Gundermann, K. D.; McCapra, F. In *Reactivity and Structure*, Vol. 23 (Chemiluminescence in Organic Chemistry); Springer-Verlag: Berlin, Heidelberg, 1987.
(10) Zomer, G.; Stavenuiter, J. F. C.; Van Den Berg, R. H.; Jansen, E. H. J. M. *Pract. Spectrosc.* **1991**, *12*, 505.
(11) Simpson, J. S. A.; Campbell, A. K.; Ryall, M. E. T.; Woodhead, J. S. *Nature* **1979**, *279*, 646.
(12) Weeks, I.; Beheshti, I.; McCapra, F.; Campbell, A. K.; Woodhead, J. S. *Clin. Chem.* **1983**, *29*, 1474.
(13) Zomer, G.; Van Den Berg, R. H.; Jansen, E. H. J. M. *Anal. Chim. Acta* **1988**, *205*, 267.
(14) Zomer, G.; Stavenuiter, J. F. C. *Anal. Chim. Acta* **1989**, *227*, 11.
(15) Shellum, C.; Gubitz, G. *Anal. Chim. Acta* **1989**, *227*, 97.
(16) Batmanghelich, S.; Woodhead, J. S.; Smith, K.; Weeks, I. *J. Photochem. Photobiol. A: Chem.* **1991**, *56*, 249.
(17) Kaltenbach, M. S.; Arnold, A. M. *Mikrochim. Acta* **1992**, *108*, 205.
(18) Papadopoulos, K.; Hadjianestis, J.; Nikokavouras, J. *J. Photochem. Photobiol. A: Chem.* **1993**, *75*, 91.
(19) Sakanishi, K.; Kato, Y.; Mizukoshi, E.; Shimizu, K. *Tetrahedron Lett.* **1994**, *35*, 4789.
(20) Sato, H.; Mochizuki, H.; Tomita, Y.; Izako, T.; Sato, N.; Kanamori, T. *J. Biolumin. Chemilumin.* **1996**, *11*, 23.

10.1021/jo980566u CCC: $18.00
Published on Web 04/03/1999

giving rise to chemiluminescence seems to be indispensable. As some insight into this mechanism might be achieved by a theoretical examination of the elementary reactions between CMAPE and hydrogen peroxide, this became the principal aim of the present work. Although chemiluminescence occurs from electronically excited states, we focused our studies on ground state reaction profiles. (To describe the steps related directly to the conversion of chemical energy into electronic excitation, computational tools at present inaccessible would be required.) Despite this simplification, we identified elementary reactions that are sufficiently exothermic to populate electronically excited 10-methyl-9-acridinone. Thus, our calculations indirectly proved the possibility of an electronically excited molecule being formed during the processes studied. On the other hand, they also enabled a number of points concerning the chemiluminescence mechanisms proposed in the literature to be explained and clarified.[1,2,5,7,9,10,21−23]

Computational Methods

The semiempirical PM3 method,[24] implemented in the MOPAC93 molecular orbital package,[25] was used to examine the reaction mechanism of CMAPE with hydrogen peroxide in the gaseous phase and in aqueous solution. This method was chosen since it performs better than other semiempirical methods with respect to prediction geometries, dipole moments, heats of formation, and other molecular properties.[26] The starting geometries for quantum mechanical calculations were generated by molecular mechanics computations in the force field incorporated in the SPARTAN v. 4.0 program package.[27] Unconstrained geometry optimizations of entities in the ground electronic state were carried out using standard PM3 orbitals[24,26] and following the EF procedure.[28] In the CI calculations a total of 36 configurations generated in the active space of HOMO-1, HOMO, LUMO, and LUMO+1 were used.[29] The final values of the energy gradient were always lower than 0.1 kcal/mol, and the eigenvalues of the Hessian matrix were all positive. Three consecutive steps were considered for the location of the transition states on the reaction path. First, "saddle" calculations[30,31] led to the approximate saddle point structure. Next, the eigenmode method[28] was used to determine the transition state configurations. Finally, vibrational analysis was performed to demonstrate that only one negative eigenvalue appears in the Hessian matrix.

Molecular entropies and thermal energy contributions were calculated using vibrational frequencies derived from the Hessian energy and standard statistical thermodynamics formulas.[32] Values of the heats of formation ($\Delta_{f,298}H°$) and entropies ($_{298}S°$) derived in accordance with the MOPAC93 routines were used to calculate the enthalpy ($\Delta_{r,298}H°$), entropy ($\Delta_{r,298}S°$), and free enthalpy ($\Delta_{r,298}G°$) of the reaction at a temperature of 298.15 K and standard pressure of 1 atm.[33] Similarly, changes in the thermodynamic functions relevant to the activation steps were determined.

To determine solvent effects, the conductor-like screening model (COSMO),[34] implemented in the MOPAC93 program package, was employed. The dielectric constant of water was taken to be 78.4 (at 298 K). Such a formalism enables only the energy of electrostatic interactions between solute and solvent, and hence the relevant contributions to the solvation energy (free energy), to be evaluated.

To gain insight into the mechanism of the decarboxylation step, regarded as the rate-determining step,[1,2,5,7,10] the calculations were carried out at a more advanced level of theory, i.e., by using the density functional (DFT) method.[35] They were performed employing the B3LYP functional, which combines Becke's three-parameter exchange functional[36] and the no-local correlation functional of Lee, Yang, and Parr,[37] together with the split valence 6-31G basis set[38] augmented with d-type polarization functions on heavy atoms and p-type polarization functions on the hydrogen atoms (6-31G** [39]). Relative atomic partial charges were determined by fitting the Coulomb equation to the molecular electrostatic potential at points selected according to the Besler−Merz−Kollman scheme.[40] It has been found for a wide variety of systems that the DFT method, and the hybrid B3LYP method in particular, provides a relatively accurate approach in the determination of molecular structures and energetics, including reaction activation barriers.[41] The DFT calculations were performed using the GAUSSIAN 94 program.[42] The entropy and thermal energy contributions were calculated in the same way as those described above and in our other papers.[43,44]

Rate constants ($_{298}k°$) were obtained using the equation

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

resulting from the transition state theory,[33] and the reaction completion times ($_{298}\tau°_{99}$) from the formula

$$_{298}\tau°_{99} = \ln 100/_{298}k°$$

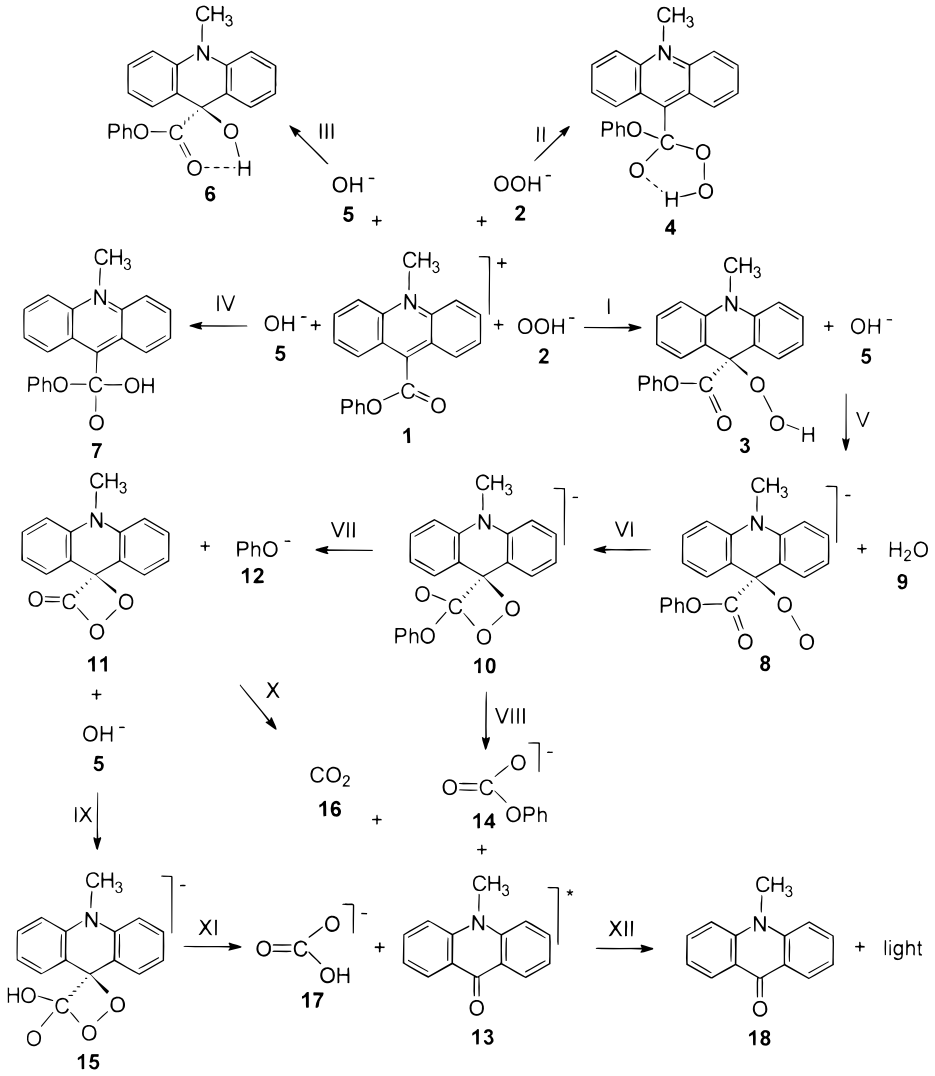
where R, T, N, and h denote the gas constant, temperature (298.15 K), Avogadro constant, and Planck constant, respectively.

Results and Discussion

The mechanism of formation of electronically excited 10-methyl-9-acridinone is depicted in Scheme 1. The process is initiated by the nucleophilic attack of OOH^- (**2**) on the carbon atom of CMAPE (**1**) in position 9, which leads to the formation of an addition product (**3**) (Figure

(21) McCapra, F. *J. Chem. Soc., Chem. Commun.* **1977**, 946.
(22) Koo, J.-Y.; Schuster, G. B. *J. Am. Chem. Soc.* **1978**, *100*, 4496.
(23) Adam, W.; Cueto, O. *J. Am. Chem. Soc.* **1979**, *101*, 6511.
(24) Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209, 221.
(25) Stewart, J. J. P. MOPAC 93; Copyright Fujitsu: Tokyo, Japan, 1993.
(26) Stewart, J. J. P. *J. Comput.-Aided Mol. Design* **1990**, *4*, 1.
(27) Available from Wavefunction, Inc., 18401 Von Karman, Suite 370, Irvine, CA 92715.
(28) Baker, J. *J. Comput. Chem.* **1986**, *7*, 385; **1988**, *9*, 465.
(29) Amstrong, D. R.; Perkins, P. G.; Stewart, J. J. P *J. Chem. Soc., Faraday Trans. 2* **1972**, *68*, 1839.
(30) Dewar, M. J. S.; Healy, E. F.; Stewart, J. J. P. *J. Chem. Soc., Faraday Trans. 2* **1984**, *80*, 227.
(31) Dewar, M. J. S. *Int. J. Quantum Chem., Quantum Chem. Symp.* **1988**, *22*, 557.
(32) Dewar, M. J. S.; Ford, G. P. *J. Am. Chem. Soc.* **1977**, *99*, 7822.
(33) Atkins, P. W. *Physical Chemistry*, 5th ed.; Oxford University Press: Oxford, 1994.
(34) Klamt, A.; Schuurmann, G. Z. *J. Chem. Soc., Perkin Trans. 2* **1993**, 799.
(35) *Density Functional Methods in Chemistry*; Labanowski, K. J., Andzelm, J. W., Eds.; Springer-Verlag: New York, 1991.
(36) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 1372, 5648.
(37) Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785.
(38) Hariharan, P. C.; Pople, J. A. *Theor. Chim. Acta* **1973**, *28*, 213.
(39) Francl, M. M.; Pietro, W. J.; Hehre, W. J.; Binkley, J. S.; Gordon, M. S.; DeFrees, D. J.; Pople, J. A. *J. Chem. Phys.* **1982**, *77*, 3654.
(40) Besler, B. H.; Merz, K. M.; Kollman, P. A. *J. Comput. Chem.* **1990**, *11*, 431.
(41) Kim, K.; Jordan, K. D. *J. Phys. Chem.* **1994**, *98*, 10089.
(42) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; DeFrees, D. J.; Baker, J.; Stewart, J. J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. *GAUSSIAN 94*, Revision C.3; Gaussian, Inc: Pittsburgh, PA, 1995.
(43) Rak, J.; Skurski, P.; Liwo, A.; Blazejowski, J. *J. Am. Chem. Soc.* **1995**, *117*, 2638.
(44) Rak, J.; Skurski, P.; Gutowski, M.; Jozwiak, L.; Blazejowski, J. *J. Phys. Chem. A* **1997**, *101*, 283.

Scheme 1. Pathways of the Reaction of CMAPE with Hydrogen Peroxide (H-bonding is indicated by the dotted line)



1). Because ions of opposite signs are involved in the reaction, the process is highly exothermic and spontaneous in the gaseous phase (Table 1). The negative (unfavorable) entropy change in step I only slightly diminishes the tendency toward the formation of **3**. In aqueous solution, the enthalpy change for step I is markedly reduced relative to that in the gaseous phase, even though it remains negative. Despite the unfavorable contribution of solvation, the process should proceed spontaneously, at least from the point of view of the energetics (the PM3-COSMO method does not evaluate the entropy changes accompanying solvation). The addition of OOH^- to CMAPE does not require any activation barrier to be overcome (we were unable to locate any transition state for step I), hence the kinetics of the process are controlled exclusively by thermodynamics.

The nucleophilic attack of OOH^- on the carbon atom of the carboxy group (step II) rather than on the carbon in position 9 of the acridine nucleus had been proposed earlier as the first step of the reaction between CMAPE and hydrogen peroxide.[10] This assumption was based on the fact that experimentally the methoxy groups in positions 2 and 7 did not affect the chemiluminescence efficiency. This inference might be valid if the attack of OOH^- on CMAPE were the rate-determining step.[1,10] However, our calculations indicate that steps I and II are not in fact the rate-determining ones. They proceed without an activation barrier having to be surmounted and therefore should be completed rapidly (ion−ion reactions are among the fastest processes). On the other hand, comparison of the data in Table 1 shows that the thermodynamic driving force in step I is considerably greater than that in step II. The inclusion of the water causes step II to become slightly endothermic, whereas step I is always exothermic. Therefore, thermodynamics favors the formation of **3** rather than **4** in both gaseous and aqueous phases. A further point in favor of the inference that OOH^- attacks the carbon atom in position 9 emerges from the analysis of the LUMO orbital distribution on the electron isodensity surface.[45] In CMAPE the density of LUMO is highest at carbon atom 9, whereas it is almost 10 times lower at the carboxy group carbon atom (i.e. 0.026 and 0.0015, respectively). This implies that it is the carbon atom 9 rather than the carbon atom of the carboxy group that should be the site of the nucleophilic attack of OOH^-.

In step V, proton abstraction from **3** by OH^- takes place (Scheme 1).[10,17] The geometry of the anion (**8**) formed in this step is shown in Figure 1, and its thermodynamic

(45) Fleming, I. *Frontier Orbitals and Organic Chemical Reactions*; Wiley: London, New York, 1976.

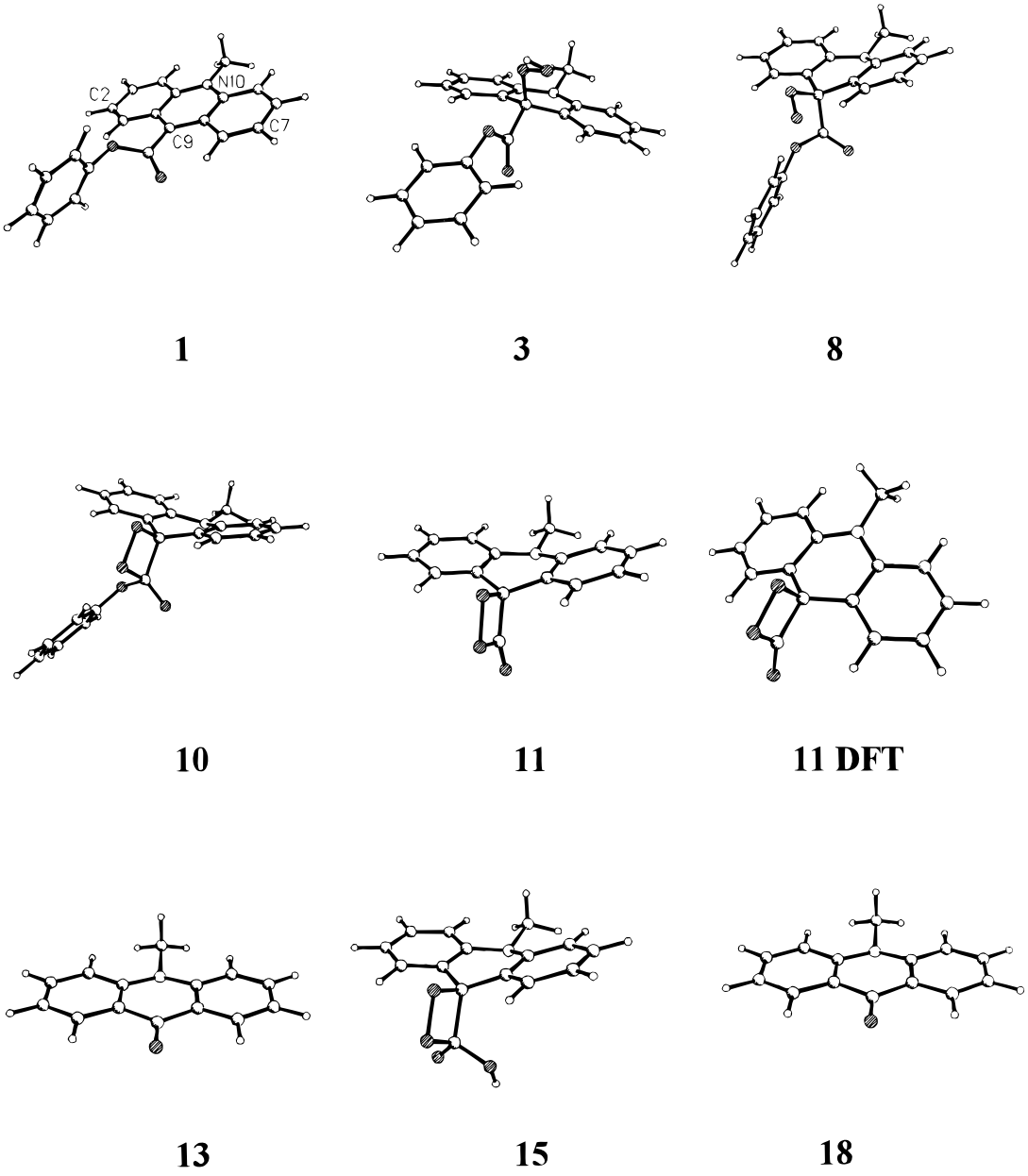


Figure 1. PM3 (and DFT) optimized geometries of the entities occurring on the pathway of the reaction of CMAPE with hydrogen peroxide.

characteristics are given in Table 1. In the gaseous phase, the process is spontaneous ($\Delta_{r,298}G°$ is negative) and exothermic ($\Delta_{r,298}H°$ is also negative) but only slightly endothermic in water (with negligible entropy changes, since the reaction on both sides is bimolecular). Two weak molecular complexes of **3**···**5** and **8**···**9**, as well as the transition state between them, were formed in this elementary reaction. However, their energies are all much lower than the sum of substrate energies (**3** and **5**); this is practically tantamount to the lack of a kinetic energy barrier in step V. Our previous studies showed that the activation barrier for this type of reaction, if it exists at all, is not very high and that the behavior of the reactants is governed by thermodynamics.[46,47]

It is well-known that an alkaline medium is required to produce chemiluminescence in the reaction of CMAPE with hydrogen peroxide.[10,17] If in step V it was the water molecule that reacted rather than the OH^-, the enthalpy of the reaction leading to **8** and H_3O^+ would increase to 198 and 48 kcal/mol in the gaseous and aqueous phases, respectively. Therefore, in both phases the reaction would be highly endothermic and thus not very likely, which confirms the necessity for an alkaline environment.

In step VI the intramolecular rearrangement of **8** to the cyclic product **10** (Scheme 1 and Figure 1) takes place. The free energy of the reaction is slightly negative, with $\Delta_{r,298}S°$ being small and slightly positive (Table 1), which indicates that the equilibrium between **8** and **10** shifts in favor of **10**. This elementary step proceeds via transition state **8** → **10** (Figure 2); however, the free energy of

(46) Madaj, J.; Rak, J.; Skorupowa, E.; Lopacinska, A.; Sokolowski, J.; Wisniewski, A. *J. Chem. Soc., Perkin Trans. 2* **1995**, 569.

(47) Madaj, J.; Rak, J.; Sokolowski, J.; Wisniewski, A. *J. Org. Chem.* **1996**, *61*, 2988.

Table 1. Thermodynamic Data for the Elementary Steps of the Reaction of CMAPE with Hydrogen Peroxide[a]

	$\Delta_{r,298}H°$		$\Delta_{r,298}S°$	$\Delta_{r,298}G°$
step no.	PM3	PM3-COSMO	PM3	PM3
I	−163.7	−13.6	−47.1	−149.7
II	−124.1	3.5	−48.5	−109.7
III	−193.0	−28.0	−42.4	−180.3
IV	−149.3	−3.9	−36.5	−138.4
V	−50.3	6.9	−7.0	−48.2
VI	−6.8	−4.9	2.1	−7.4
VII	5.8	−8.3	43.8	−7.2
VIII	−14.4	−52.6	56.7	−31.3
IX	−70.2	−18.2	−32.8	−60.4
X	−5.8	−38.3	50.3	−20.8
XI	−10.5	−53.0	53.9	−26.6
XII	−83.4	−62.7	−7.5	−81.2

[a] $\Delta_{r,298}H°$, $\Delta_{r,298}G°$ (both in kcal/mol), and $\Delta_{r,298}S°$ (in cal/(mol K)) represent the enthalpy, free energy, and entropy of reaction (Scheme 1), respectively, at standard temperature 298.15 K and pressure (1 atm). The values in the PM3 columns refer to the gaseous phase and those in the PM3-COSMO column to the aqueous phase.

activation is only 6.6 kcal/mol (Table 2), suggesting that the equilibrium state is achieved in nanoseconds (see $_{298}\tau°_{99}$).

In the next step (VII), the unimolecular detachment of the phenoxy anion (PhO^-, **12**) from **10** yields product **11**. As one molecule of the substrate decomposes to two molecules of products, the entropy term contributes substantially to the thermodynamic driving force (Table 1). This is because even though $\Delta_{r,298}H°$ is slightly positive, $\Delta_{r,298}G°$ becomes negative in the gaseous phase, so the process is thermodynamically spontaneous. On the other hand, the negative value of $\Delta_{r,298}H°$ in water indicates that the process is more likely to occur there than in the gaseous phase. The transfer from **10** to **11** accompanied by the elimination of PhO^- proceeds through the transition state **10** → **11** (Figure 2). The enthalpy and free energy barriers in step VII are both positive in the gaseous phase and almost equal, since the activation entropy is small (Table 2). The rate constant indicates that the process ought to reach completion in microseconds rather than nanoseconds. $\Delta_{a,298}H°$ is somewhat higher in water than in the gaseous phase, which is due to the better solvation of **10** than of the transition state **10** → **11**. Analysis of the electrostatic potential around **10** and **10** → **11** shows that at comparable distances from both molecules it is much higher in the former case. Thus, **10** should be more susceptible to hydration than **10** → **11**, and this is indeed manifested in the increase in the energy gap between the relevant states of the molecules.

If **10** eliminated PhO^- in step VII, **11** could undergo decarboxylation, which would also lead to the electronically excited 10-methyl-9-acridinone (step X). Step VII should be spontaneous in the gaseous phase (negative $\Delta_{r,298}G°$) despite the slightly positive value of $\Delta_{r,298}H°$, since the entropy term is highly positive (Table 1).

Transfer from **11** to **13** (Figure 1) can proceed via concerted or nonconcerted CO_2 elimination from the former molecule.[4,48] Analysis of the activation parameters indicates that concerted CO_2 elimination (for the transition state structure, see Figure 2) needs to overcome a relatively high enthalpy activation barrier, both in the gaseous and aqueous phases, and so the process would take hours to complete (Table 2). Another possibility is nonconcerted CO_2 elimination from **11**. Such a pathway could reflect the CIEEL mechanism, which in the past has been considered an important step in the formation of electronically excited 10-methyl-9-acridinone.[21,22] However, the activation barriers predicted for this pathway (53.8 and 41.4 kcal/mol at the PM3 and PM3-COSMO levels, respectively) are much higher than those for concerted CO_2 elimination. As other researchers have regarded step X as the main route by which electronically excited 10-methyl-9-acridinone[1,2,5,7,10] is formed, we examined it in greater detail. We used the DFT method, which, though numerically much more demanding, is much more advanced than the PM3 one. While the enthalpy activation barrier for concerted CO_2 elimination predicted at this level is somewhat lower than that obtained at the PM3 level, it is still too high to ensure rapid completion of the process. We thus believe that decarboxylation via steps VII and X is not essential to the formation of electronically exited 10-methyl-9-acridinone.

Calculated at the DFT level (Table 2), the rate constant for step X compares very well with that reported for the decomposition of 4,4-dimethyl-1,2-dioxetan-3-one (at 300 K) (10.2×10^{-4} s^{-1}).[23] This finding is somewhat accidental, however. The enthalpy activation barrier for the concerted elimination of CO_2 from this compound, calculated at the DFT level, is 26.7 kcal/mol, and the corresponding rate constant $_{298}k°$ is 3.9×10^{-7} s^{-1}. The calculated activation barrier for CO_2 elimination from 4,4-dimethyl-1,2-dioxetan-3-one is thus higher and the rate constant lower than that for step X. The discrepancies may be due to the fact that predicted kinetic data refer to the gaseous phase and the experimental ones to the liquid phase (CH_2Cl_2[23]).

The unimolecular decomposition of **10** via step VIII is most likely the route by which electronically excited 10-methyl-9-acridinone is formed (**13**). The process is predicted to be sufficiently exothermic to populate **13**, both in the gaseous and liquid phases, and spontaneous (negative $\Delta_{r,298}G°$) (Table 1). Moreover, the enthalpy (in both phases) and the free energy barriers in the process **10** → **13** (Figure 1) are small, which implies that decomposition of **10** via step VIII should be much faster than dissociation through steps VII and X (Table 2).

As OH^- are present in the medium we checked whether they could participate by chance in the decarboxylation of **11**. Indeed, these ions would spontaneously undergo addition to **11** in step IX (Table 1) without the necessity of overcoming any activation barrier. The addition product (**15**; Figure 1) would subsequently decompose very rapidly to electronically excited 10-methyl-9-acridinone in step XI. This emerges from the negative values of both $\Delta_{r,298}H°$ and $\Delta_{r,298}G°$ (Table 1) and the negligible activation barrier in this step (Table 2). Steps IX and XI would be important if the contribution of step VII to the overall process were substantial. However, as step VIII competes effectively with step VII, electronically excited 10-methyl-9-acridinone should largely be produced via the former.

Thus far, it is the main reaction path that has been scrutinized. Now the initial addition of the hydrogen peroxide anion to CMAPE can compete with side reactions—nucleophilic attacks of OH^- on carbon atom 9 (step III) or the carboxy group carbon atom (step IV). Both the latter are ion−ion processes and, like steps I

(48) O'Neal, H. E.; Richardson, W. H. *J. Am. Chem. Soc.* **1970**, *92*, 6553.

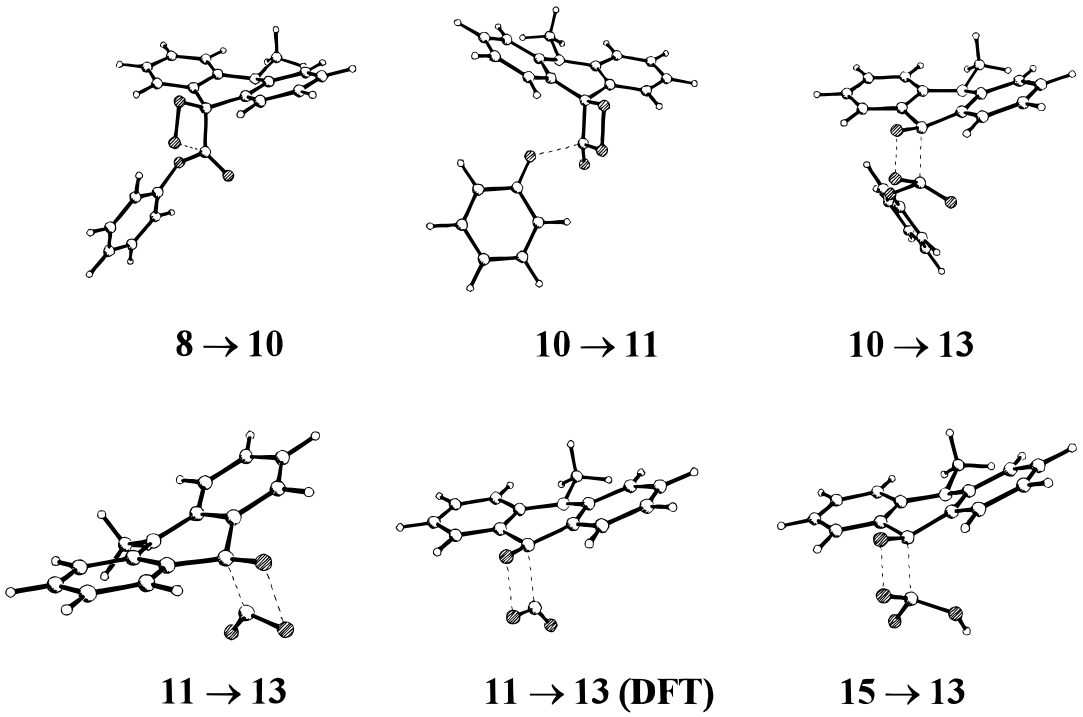


Figure 2. PM3 (and DFT) optimized geometries of the entities relevant to the transition states. Dotted lines represent the bonds that are formed and broken during the course of the reaction.

Table 2. Kinetic Characteristics of the Elementary Steps in the Reaction of CMAPE with Hydrogen Peroxide[a]

	$\Delta_{a,298}H°$			$\Delta_{a,298}S°$		$\Delta_{a,298}G°$		${}_{298}k°$ (${}_{298}\tau°_{99}$)	
step no.	PM3	PM3-COSMO	DFT	PM3	DFT	PM3	DFT	PM3	DFT
VI	6.6	5.0		0.0		6.6		9.1×10^{7} (5.0×10^{-8})	
VII	13.2	15.8		4.9		11.7		1.6×10^{4} (2.9×10^{-4})	
VIII	2.2	6.7		1.6		1.7		3.5×10^{11} (1.3×10^{-11})	
X	27.0	26.2	21.0	2.2	−1.9	26.4	21.6	3.0×10^{-7} (1.5×10^{7})	9.8×10^{-4} (4.7×10^{3})
XI	0.1	0.1		−1.7		0.7		2.0×10^{12} (2.3×10^{-12})	

[a] $\Delta_{a,298}H°$, $\Delta_{a,298}G°$ (both in kcal/mol), and $\Delta_{a,298}S°$ (in cal/(mol K)) represent the enthalpy, free energy, and entropy of activation (Scheme 1). ${}_{298}k°$ (in s^{-1}) and ${}_{298}\tau°_{99}$ (in s) respectively denote the rate constant and the time at which the reaction is 99% completed. The values in the PM3 and DFT columns refer to the gaseous phase and those in the PM3-COSMO column to the aqueous phase.

and II, proceed without activation barriers. Thermodynamics, substantially affected by the medium, thus control steps III and IV. It can be gleaned from the data in Table 1 that the thermodynamic driving force for the nucleophilic attack of OH^- on C9 is clearly greater than that for steps IV and I (Table 1). Therefore, **6** is more likely to be formed than **3**, **4**, or **7**. Indeed, a so-called pseudobase (**6**) has been detected experimentally.[16,17] The formation of such a species may partially account for the fact that only a small percentage of chemiluminogenic acridine derivatives are converted to electronically excited acridinones during their reaction with OOH^-.[1−5,7] On the other hand, even if step III competes with step I, the equilibrium can shift in favor of **3** as soon as the latter entity is consumed in the steps following step I, as indicated in Scheme 1. In this way more of **3** convertible to **13** should be available. Therefore, side reactions prevent neither the formation of electronically excited 10-methyl-9-acridinone nor their analytical applications.

Final Remarks

The theoretical approach taken in this work has enabled the mechanism of formation of electronically excited 10-methyl-9-acridinone in the reaction of the CMAPE cation with hydrogen peroxide to be analyzed, mainly at the semiempirical level of theory. The use of more advanced methods does not guarantee completion of all the necessary calculations in a reasonable length of time. Thus, only the decarboxylation step, generally regarded as the rate-determining one,[1,2,5,7,10] was analyzed at the DFT level. Nevertheless, even this relatively simple approach affords an insight into the interesting phenomenon of chemiluminescence from both the cognitive and practical point of view.

There is one problem which we did not attempt to solve in our study: how is the energy released in the chemical processes accumulated in the 10-methyl-9-acridinone molecules as electronic excitation? We intend to tackle this in the future. Even so, the results of the study enable the wavelengths of the emitted radiation to be evaluated

on the basis of the enthalpy changes in step XII (Table 1). Radiation of 343 nm should be emitted in the gaseous phase, of 456 nm in water. The latter value corresponds quite well with that found experimentally.[1]

The mechanism proposed and examined theoretically accounts well for all experimental findings. First, the catalytic role of hydroxide ions has been documented. Second, the reaction leading directly to the electronically excited 10-methyl-9-acridinone (step VIII) turned out to be exothermic and accompanied by a relatively high increase in entropy, which should result in the process proceeding spontaneously (this correlates with the literature reports[1,10]). Third, none of the kinetic barriers in any of the steps I, V, VI, and VIII are very high, which explains why the luminescence appears relatively quickly a result of the reaction of CMAPE with hydrogen peroxide. Moreover, considerations based on theoretical findings explain the role of side reactions, which could be the factors reducing the analytical utility of CMAPE (e.g. in immunoassays[10,17]).

Our studies further explained several questions related to the mechanisms of the process existing in the literature.[1,2,5,10,21−23] Thus, the primary step was in fact characterized as the nucleophilic attack of the hydrogen peroxide anion on the carbon atom 9 of the acridine nucleus. Moreover, it turns out that the subsequent rearrangement of the entity so formed (**3**) to **8** proceeds with the participation of OH^-, which acts as a catalyst (step V).

The rearrangement of **8** to **10** (step VI) appears to be the bottleneck step controlling the kinetics of formation of electronically excited 10-methyl-9-acridinone. This implies that modification of both the acridinium moiety and the phenyl fragment of the PhO should influence the rate of formation of **13** and thus the sensitivity of analytical assessment (the faster this process, the more sensitive the method).

Complementary calculations have predicted that 9-acetyl- (AMA) or 9-benzoyl-10-methylacridinium (BMA) cations react with the hydrogen peroxide anion in the same way as CMAPE in steps I and V (the thermodynamic and kinetic parameters are comparable). However, a difference appears in step VI. The theory predicts that only the phenyl ester will yield a four-membered cyclic product (**10**), whereas AMA or BMA will go directly to 10-methyl-9-acridinone and acetate or benzoate anions. The formation of a four-membered ring in the case of AMA and BMA is difficult, since the positive charge diminishes on the carbon atom of the carbonyl group. In the CMAPE, AMA, and BMA series this is equal to 0.81, 0.67, and 0.42, respectively. Such a cyclic intermediate may be necessary for electronically excited 10-methyl-9-acridinone to be produced. As this is not likely to occur in the case of AMA and BMA, these compounds do not seem to be of much value as fragments of chemiluminescent labels. A literature search seems to bear this out.

Another problem is the elimination, or at least the diminution, of side reactions. This could be achieved by substitutions, mainly in the acridine moiety, which would increase the susceptibility of carbon atom 9 to nucleophilic attack by OOH^- (step I). Our attention is currently focused on the theoretical modeling of derivatives of CMAPE which, according to the above, would exhibit interesting features from the point of view of analytical applications. On the other hand, syntheses of selected compounds and physicochemical investigations concerning their stability and utility in assays of macromolecules in living matter are being carried out.

Acknowledgment. This work was supported by the Polish State Committee for Scientific Research KBN Grants 2 P303 058 06 (Contract No. PB-0593/P3/94/06) and BW/8000-5-0105-8.

JO980566U