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New Nucleophilic Catalysts for Bright and Fast Peroxyoxalate Chemiluminescence

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Miniaturized detection applications based on chemiluminescence require fast reaction kinetics for optimum performance. In this work, high-intensity light from the analytically useful peroxyoxalate chemiluminescence reaction has been generated at high rates by employing both single-component and dual-component nucleophilic catalysis. 4-(Dimethylamino)pyridine and its derivatives were superior to all other bases in terms of reaction speed and intensity of the generated light and outshone imidazole, which hitherto has been considered as the best catalyst. The light intensity was related to the difference in pK_a between the 4-aminopyridine catalyst and the leaving group of the reagent, and the optimum ΔpK_a was found to be close to 0. Similarly, high light intensities were obtained when mixtures of the imidazole analogue 1,2,4-triazole and the strong, nonnucleophilic base 1,2,2,6,6-pentamethylpiperidine acted as catalysts. The mechanism behind this was concluded to be a "base-induced nucleophilic catalysis", where the ancillary strong base assisted the production of the highly nucleophilic 1,2,4-triazolate anion, which as the actual catalyst then participated in the formation of a more reactive transient reagent. All the investigated catalysts reduced the light yield of the reaction due to base-catalyzed breakdown reactions of the reagents and/or intermediates. The intensity peak maximums of these bright and fast reactions typically appeared after less than 10 ms, whereafter the light decayed to darkness within a few seconds. These reaction characteristics are especially advantageous for sensitive detection applications where the observation volumes and times are limited, e.g., peaks emerging from a capillary-based separation process.

Rapid development in the field of liquid separation sciences toward higher efficiency and miniaturized chromatographic, electrochromatographic, and electrophoretic systems has resulted in the reduction of the bandwidth of the eluted peaks to typically a few nanoliters,[1] which corresponds to a residence time in the detector cell of 1 s or less. This requires an adaptation of the detection systems to stand up to the new and harder demands on sensitivity and bandwidth conservation. If the extracolumn band-broadening is to be kept within acceptable limits, the time constant of the detection system must not exceed one-tenth of the width of the eluted peak.[2] This means that if chemiluminescent (CL) reactions are to be applied as detection schemes with these efficient separation techniques, the light can only be collected during a few milliseconds if the separation efficiency is to be preserved. By only physically reducing the visible volume of the detection cell while employing slow CL reactions, the risk of light-piping from a hidden part of the flow path greatly increases. Equally unacceptable in miniaturized systems are the extra flow elements that are demanded by the CL reactions from which the light intensity slowly climb to its maximum intensity. All these criteria call for an increase in the rate of light production and an increased light intensity. A rapid decay of the light can also be advantageous since the effective cell volume then becomes independent of the physical volume and only relies on the light decay kinetics. In liquid chromatography this phenomenon has been called "chemical band-narrowing"[3] although this description is quite misleading since the effect rather is a reduction of the detection cell related band-broadening.

The reaction between hydrogen peroxide and an activated derivative of oxalic acid can produce chemiluminescent light in the presence of a fluorophore.[4−6] This reaction, commonly referred to as peroxyoxalate chemiluminescence (POCL), has attracted much attention and has successfully been used as a highly sensitive detection technique in several procedures developed for trace level determinations of a large variety of analytes,[4] e.g., fluorophore-labeled amino acids[7] and steroids,[8] polycyclic aromatic hydrocarbons,[9] and hydrogen peroxide.[10] However, while numerous reports concerning the reaction mechanism have been

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published,[11−16] debate still exists as to the actual mechanism of the reaction. It is however fairly well established that the preferred properties of the fluorophore are a low excitation energy and a low oxidation potential[9,13,17] as explained by the chemically initiated electron-exchange luminescence (CIEEL)[18] theory. Because the intensity of the light represents the rate of the light-producing reaction, the most favorable oxalic acid derivatives for sensitive detection are highly activated, and thus reactive, oxalates[19] or oxamides.[20] The function of the catalyst usually present is to promote and speed up the reaction to produce a higher light intensity. Besides its basic properties, not much is known about the preferred nature of the catalyst, and the popularity of imidazole has been based on experimental results[21,22] only. Our study[23] of substituted imidazoles and other good leaving groups as catalyst for this reaction unfortunately did not provide any compound superior to imidazole, but clearly illustrated the importance of the nucleophilicity of the catalyst.

The earlier unexplained catalytic efficiency of imidazole was clarified by the introduction[20] of 1,1′-oxalyldimidazole (ODI) as a stand-alone reagent in POCL. Recent studies of the reaction of imidazole with activated oxalates[24,25] and imidazole-catalyzed POCL[15,16] have confirmed that ODI forms as a transient intermediate during the reaction. The formation of an intermediate reagent, more activated and reactive than the primary reagent, is by definition[26] the result of nucleophilic catalysis. The mechanism is in many aspects similar to the nucleophilic catalyzed hydrolysis of monocarboylic esters (e.g., 4-nitrophenyl acetate).[26,27] The formation of an intermediate seems to be a common feature of efficient catalysts for POCL,[23] corroborating the conclusion that the POCL reaction is predominantly subject to nucleophilic catalysis, rather than general base catalysis. When imidazole is used as catalyst for the POCL reaction of bis(2,4,6-trichlorophenyl) oxalate (TCPO), the formation of ODI is the reaction step limiting the rate of light production[15,16] and, thus, the light intensity. The studies on the reaction between imidazole and TCPO[24,25] have also exposed the cooperative action of two imidazole molecules, where one acts as a general base catalyst for the other, which acts as a nucleophile. From the inferior results of catalysis by 1-methylimidazole,[23] it can be concluded that this cooperation between two imidazole molecules is essential for the catalytic efficiency of imidazole and that the neutral imidazole is too weak a nucleophile to attack a moderately reactive reagent such as TCPO.

An efficient nucleophilic catalyst must be an unusually effective nucleophile and the formed intermediate must be unusually susceptible toward nucleophilic attack.[26] The most obvious way to increase the catalytic efficiency is to use a stronger nucleophile, but the concept of nucleophilicity toward esters follows no simple equation[28] and is dependent on several diverse properties such as basicity, polarizability, hydrogen-bonding ability, solvation, resonance stabilization, and steric effects. Another, maybe less palpable approach, is to use a mixture of catalysts, where each compound ideally is dedicated to a specific catalytic task. In the POCL reaction, where the complexity of the catalytic role is high and involves features of nucleophilicity, leaving group ability, and basicity,[23] such a design may very well prove to be successful. In fact, we have recently reported[29] on the combined use of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,2,4-triazole as catalysts for POCL and demonstrated the potential of this system to outshine the imidazole-catalyzed reactions.

4-(Dimethylamino)pyridine (DMAP) and other 4-(dialkylamino)-pyridines[30−32] are strong nucleophiles and have for long been used to catalyze acylation reactions. These compounds act specifically as nucleophilic catalysts, and the mechanism is believed to involve the formation of acylpyridinium ions, which are stabilized by the ability to distribute a positive charge over several resonance structures. These highly reactive ions form loose ion pairs with the leaving groups from the acyl carbon and can thus be easily attacked by another nucleophile. DMAP has once been tested[33] as a catalyst in the POCL reaction with somewhat promising results, but the effect was only briefly studied and no comparison was made with imidazole. Another widely used acylation catalyst is 1-methylimidazole,[34] but its basicity and nucleophilicity is apparently[23] too low for the use as a catalyst in POCL.

In this study, several new compounds have been tested as catalysts for the POCL reaction with the goal of achieving faster reactions and higher light intensities to allow for more sensitive detection applications. Catalysis by strong bases and strong nucleophiles such as DMAP and mixtures containing two catalysts are the different approaches that have been used to realize this. This examination also aims to outline the optimal properties of a catalyst, or combination of catalysts, to allow for a more rational design and selection of reagents and catalysts in the future.

EXPERIMENTAL SECTION

Reagents and Solutions. TCPO was synthesized as previously described[35] or purchased from Aldrich (Steinheim, Germany) or Sigma (St. Louis, MO). ODI was prepared as formerly outlined,[36] under a nitrogen atmosphere and from freshly distilled

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oxalyl chloride. ODI was stored at 5 °C under a moisture-free atmosphere in tight-closing vials protected from light. DMAP was either from Reilly Industries (Indianapolis, IN) or Aldrich. 4-Aminopyridine (98%), 4-pyrrolidinopyridine (PYP; 98%), 1,2,2,6,6-pentamethylpiperidine (PMP; 97%), DBU (98%), 3-aminofluoranthene (3-AFA; 97%), 2,4,6-trichlorophenol (98%), (trimethylsilyl)-imidazole ("derivatization grade"), and oxalyl chloride (98%) were obtained from Aldrich. Fluka (Buchs, Switzerland) supplied triethylamine (p.a.) and morpholine (purum). Hydrogen peroxide (Perhydrol Suprapur, 30% in water) was from Merck (Darmstadt, Germany), HPLC-grade acetonitrile (specified to contain <0.01% water) was from J. T. Baker (Deventer, The Netherlands), and piperazine (anhydrous) was from Sigma. Water was purified and deionized to a conductivity of less than 60 nS·cm^{-1} using Milli-Q equipment (Millipore, Bedford, MA).

Reagent solutions of TCPO (1 mM) in acetonitrile were prepared every second day, while solutions of ODI (1 mM) were prepared every fourth hour in dried acetonitrile (dried using a 3-Å molecular sieve from KeboLab, Stockholm, Sweden). These solutions were stored in poly(tetrafluoroethene) (PTFE) bottles equipped with silica gel-filled drying tubes to enhance the reagent stability[37] and to protect the solutions from ambient humidity during withdrawal. Stock solutions of 4-aminopyridine, DMAP, and PYP were prepared every second week. The mixed hydrogen peroxide/fluorophore/catalyst reagent solutions were made daily from stock solutions of each component and contained 1 mM hydrogen peroxide, 51 μM 3-AFA, and the catalysts (10 mM unless otherwise noted) in acetonitrile. Note that the reaction cell concentrations are half of the concentrations in the reagent solutions.

Estimation of Basicity. The pK_a measurements were performed with a HP8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA), which had been blanked against a quartz cuvette with pure acetonitrile. Each base was mixed with 2,4,6-trichlorophenol at approximate concentrations of 2 and 0.5 mM, respectively, and the absorbance of the formed 2,4,6-trichlorophenolate ion was measured at the absorbance maximum with a 10-nm slit window. The absorbtivity of the 2,4,6-trichlorophenolate ion was determined in an acetonitrile solution saturated with potassium hydroxide. With the knowledge of the total concentrations of the two species in the cuvette and the measured concentration of the 2,4,6-trichlorophenolate ion, the relative pK_a values for each base could be calculated. These values were thereafter calibrated against the known pK_a's[38] of morpholine and triethylamine. The reported data are the averages of two or three different measurements and had a relative standard deviation of ~1%.

Stopped-Flow Chemiluminescence. All time-resolved chemiluminescence measurements were performed at 25.0 ± 0.1 °C using a computer-controlled stopped-flow spectrofluorometer (model DX-17MV, Applied Photophysics, Leatherhead, U.K.) with the light source off. The two reagent solutions were simultaneously introduced into the detection cell at a 1:1 mixing ratio from two gastight glass syringes. A total volume of 200 μL was flushed through the cell at a combined flow rate of ~15 mL/s before the flow was abruptly stopped and the light production monitored. The high-sensitivity emission photomuliplier tube of the instrument was operated at 500 V and mounted directly on the backside of the cell assembly without any light or wavelength restrictions. For each reagent composition, three voltage vs time traces were recorded, overlaid, and averaged at frequencies of 4000, 40, and 4 Hz (and if required also at 0.4 Hz). These three (or four) traces of different frequencies were linked together and leveled with the initial 4-kHz trace to allow for evaluation of all parameters of the light emission profile in a spreadsheet computer program. The half-life of the light intensity was always measured from the intensity peak time.

Spectrophotometric Reaction Studies. The studies of reagent decomposition were performed at 22 ± 1 °C, either with the stopped-flow instrument described above operated in the absorbance mode or with the diode array spectrophotometer mentioned earlier in which a pneumatically driven HP89054/55A (Hewlett-Packard) stirring module was mounted. When the stopped-flow instrument was used, equal volumes of a TCPO or ODI reagent solution and a diluted catalyst solution were injected into the detection cell chamber. With the spectrophotometer, appropriate amounts of TCPO or ODI solutions were pipetted into a quartz cuvette and diluted with acetonitrile, after which base was added and the measurement immediately started. The concentrations in the detection chamber of oxalic reagent and catalyst in acetonitrile were in both cases 0.5 and 5 mM, respectively. The decay of TCPO was monitored by the accumulation of the 2,4,6-trichlorophenolate ion (330 nm), whereas the decline of ODI was directly measured at an appropriate wavelength (330 nm) where the absorbtivities of the catalysts were negligible. Absorbance changes were monitored with wavelength slit window of 2−10 nm. Each first-order rate constant was calculated as the slope of the forced-zero-intercept linear least-squares fit (average $R^2 = 0.99$, except for piperazine where $R^2 = 0.86$) of the first part of a plot of the natural logarithm of the inverse of the relative reagent concentration vs time.

WARNING. The degradation product from TCPO, 2,4,6-trichlorophenol, and the fluorophore 3-AFA are suspected carcinogens. All 4-aminopyridines are potentially toxic and should be handled with due care.

RESULTS AND DISCUSSION

Reaction Conditions. Acetonitrile was chosen as the solvent due to its frequent use in analytical applications of POCL and its nearly aprotic properties. The two activated oxalic acid derivatives ODI and TCPO were selected as model reagents to represent very reactive and moderately reactive reagents, respectively. Because of the modest reactivity of TCPO, a base catalyst is required for its reaction with hydrogen peroxide, whereas ODI is sufficiently reactive to be used as a stand-alone reagent.[20] Equal concentrations of the oxalic reagent and hydrogen peroxide were used throughout, and the concentration of catalyst was selected to 10 times higher than the other reagents to obtain a fairly constant catalytic environment. A series of different bases of various strengths and nucleophilicities were tried as catalysts. The strong base and efficient acylation catalyst DMAP and its structural analogues 4-aminopyridine and PYP were tested together with four other strong bases, morpholine, piperazine, triethylamine, and PMP, which all have aqueous pK_a's comparable to DMAP; see Table 1. Triethylamine is known as a base with low nucleophlicity

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Table 1. Dissociation Constants for Different Compounds in Water and in Acetonitrile[a]

no.	compound[b]	$pK_a^{H_2O}$	$pK_a^{CH_3CN}$
1	2,4,6-trichlorophenolate	6.0 [40]	20.4 [44]
2	imidazole	6.99 [41]	14.5 [45]
3	4-aminopyridine	9.29 [42]	16.8
4	DMAP	9.71 [42]	17.1
5	PYP	9.90 [30]	17.6
6	morpholine	8.49 [41]	16.7[c]
7	piperazine	9.78 [41]	18.4
8	triethylamine	10.72 [41]	18.4[c]
9	PMP	11.25 [43]	18.4

[a] The autoprotolysis constant for acetonitrile is 3×10^{-29}.[38] [b] The pK_a values for these bases refer to the release of a proton from the corresponding acid; i.e.; $BH \rightarrow B^- + H^+$. [c] The literature values for these compounds were used to calibrate the relative pK_a scale in this study. Literature values for morpholine and triethylamine are 16.61 and 18.46,[38] respectively.

and PMP is a very sterically hindered base with essentially no nucleophilicity.[39] For comparison, the rather weak base imidazole, which has a thoroughly documented[21−23] efficiency as POCL catalyst, was also included in the study. Because the basicities of these compounds in acetonitrile most certainly differ from that in water and such data could not be found in the literature, the relative pK_a's of the bases were probed in dilute mixtures of the compounds and 2,4,6-trichlorophenol in pure acetonitrile, using the absorbance of the negatively charged phenoxide ion as indicator. These data are also presented in Table 1 and reveal that the 2,4,6-trichlorophenol released from TCPO during the POCL reaction will be partially deprotonated with strong bases as catalysts. If, for example, triethylamine is used as a catalyst for TCPO at a concentration ratio of 10:1 as in this study, ∼20% of the phenol and 4% of the base is expected to be ionized after the reaction is completed. This proton transfer can cause rather peculiar effects on the light emission profile at low base concentrations.[46]

Single-Component Catalysis. During the very first experiments with strong bases as catalysts for the POCL reaction, it became clear that the light production in many cases was so fast that the stopped-flow instrument[23] failed to detect the peak of the emission profile. An inert zero-dead volume integrated cyclone mixer and fountain detection cell was therefore manufactured and initially utilized[29] for studying the rapid kinetics induced by these catalytic systems. However, with the fastest catalysts it became apparent that this construction suffered from problems in terms of insufficient mixing and development of secondary flow paths within the cell chamber. Consequently, this strategy was abandoned and the equipment described above was employed.

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The data extracted from the chemiluminescence vs time traces obtained with this system and the different catalysts and reagents previously mentioned are summarized in Table 2, and representative emission profiles from some illustrative catalyst/reagent combinations are shown in Figure 1. A quick glance at Figure 1 reveals the profound differences between the two reagents TCPO and ODI. With TCPO, the light intensities were generally lower and the kinetics were slower. The light yields were, on the other hand, much higher with TCPO. For these reasons, TCPO is most appropriate for large-volume detection applications, whereas ODI is better suited for miniaturized systems which require fast kinetics. Shifting part of the attention to Table 2 and the performance of the different catalysts discloses two striking effects common to all the strong bases. First, the peak time of the light production was short, typically a few tens of milliseconds, compared to ∼0.5 s or more with imidazole as catalyst. Second, the total light yields were poor, at least 1 order of magnitude lower than with imidazole. The worst light yields were obtained with morpholine, piperazine, and triethylamine. These reactions emitted less than 1% of the amount of light produced by the imidazole-mediated reactions. When comparing the intensities of the emitted light, the 4-aminopyridines (4-aminopyridine, DMAP, PYP) and the other bases produced very different results. All 4-aminopyridines gave high light intensities, ranging from one-third of up to threefold higher than the imidazole-catalyzed reactions, depending on the reagent. The other bases gave very low light intensities, especially with TCPO. Although the light intensity half-lifes were diverse with the TCPO reagent, and all quite short with ODI, the general tendency was much shorter half-lifes with all strong bases compared to with imidazole.

The large contrast in light intensities and yields between the 4-aminopyridines and the other strong bases implies that there is a difference in their catalytic mechanisms, since the difference in base strength between the compounds is very small. The evident nucleophilic properties of the 4-aminopyridines, the documented sensitivity of the POCL reaction to nucleophilic catalysis, and the high light intensities produced with the 4-aminopyridines compared to with other strong bases together provide significant circumstantial evidence for nucleophilic catalysis of the POCL reaction by these compounds. Thus, new and more reactive derivatives of oxalic acid are expected to be formed during the course of the reaction. In the UV absorption experiments discussed below, we also hoped to be able to observe the formation of such intermediates since this, as previously mentioned, would be evidence for nucleophilic catalysis. Unfortunately, no intermediate formation could be detected with any of the catalysts, but this does not rule out its existence since the bases themselves are strong absorbers in most of this UV region and the concentration of such intermediates most likely is low. Although this investigation does not aim at disclosing the identity of the suspected intermediate, a qualified speculation is that the structure resembles the acylpyridinium ions that have been observed by others.[30]

A closer examination of the 4-aminopyridines and their relative performance reveals that the kinetics as well as the light intensity and yield were dependent on the base strength. The light yield, peak time, and half-life all decreased with increasing base strength regardless of the reagent. Interestingly, the light intensity in-

Table 2. Effect of Base Catalyst and Oxalic Reagent on the Peroxyoxalate Chemiluminescence Reaction Light Emission Profile in Acetonitrile[a]

		TCPO as reagent				ODI as reagent			
no	catalyst	max intens, mV	light yield, mV·s	peak time, ms	half-life, ms	max intens, mV	light yield, mV·s	peak time, ms	half-life, ms
1	none	n/a[b]	n/a	n/a	n/a	75	4900	650	43000
2	imidazole	980	11000	4900	9600	620	4200	250	4000
3	4-aminopyridine	300	1400	3	670	2200	390	8	81
4	DMAP	620	1000	2	83	1900	180	4	27
5	PYP	1400	900	1	43	1800	100	2	11
6	morpholine	4	47	98	2100	37	11	9	120
7	piperazine	8	4	2	46	39	1	2	7
8	triethylamine	38	90	15	610	110	2	2	14
9	PMP	99	500	35	2300	210	9	1	49

[a] The uncertainty of the extracted data in this table was estimated to 2−10% relative standard deviation ($n = 12$), based on four repeated runs (each consisting of averaged repeated injections) on three different preparations of DMAP/hydrogen peroxide/fluorophore mixed reagent solutions reacted with one single ODI reagent solution. The yield and half-life showed the largest deviations. Of course, the relative standard deviations of repeated injections of the same solutions were much smaller, typically below 1%. [b] n/a, no detectable chemiluminescence.

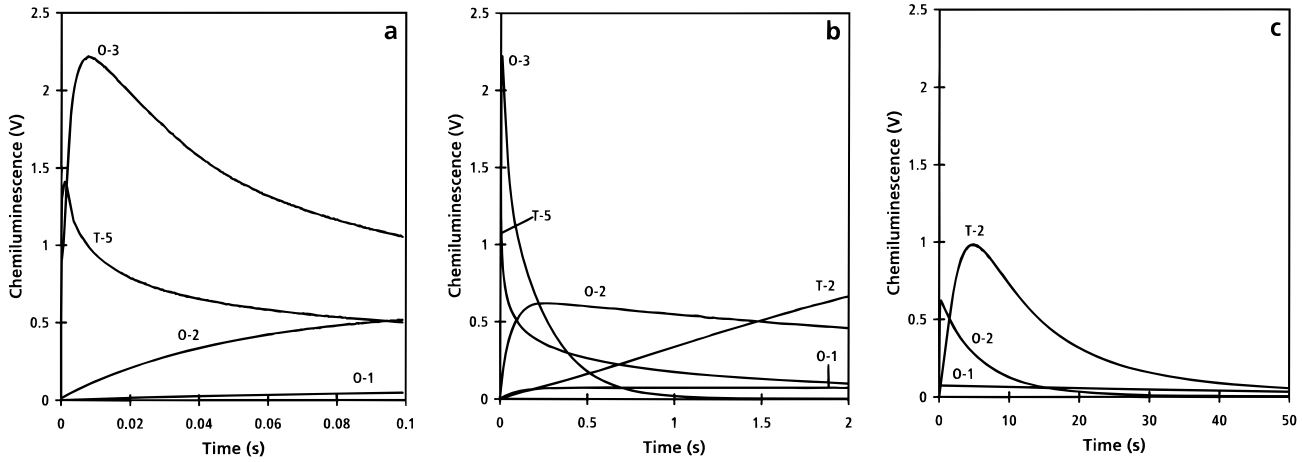

Figure 1. Time-resolved peroxyoxalate chemiluminescence with different base catalysts and oxalic reagents. The figure has been split into three parts with different time resolutions to visualize the substantial impact that the catalysts had on the reaction. The concentrations of the compounds in the reaction vessel were 0.5 mM TCPO or ODI, 0.5 mM hydrogen peroxide, 5 mM catalyst, and 25 μM 3-AFA as fluorophore. T and O mean TCPO and ODI, respectively, and the numbers refer to the position of the catalysts in Table 2. Key: (O-1) ODI with no catalyst, (O-2) ODI with imidazole, (O-3) ODI with 4-aminopyridine, (T-2) TCPO with imidazole, and (T-5) TCPO with PYP.

creased linearly ($R^2 = 0.99$) with pK_a when TCPO was the reagent but decreased ($R^2 = 0.83$) when ODI was used. It is notable that the 4-aminopyridine giving the highest intensity was for both reagents the one with the smallest difference in pK_a relative to the leaving group. By plotting the observed intensities vs the difference in pK_a (see Figure 2), we can conclude that there exists an optimum close to zero; i.e., the 4-aminopyridine catalyst and the leaving group should be of roughly the same basicity to generate the highest possible light intensity. Thus, each reagent has its own dedicated catalyst within the 4-aminopyridine series, which is why it should be beneficial to explore other derivatives than the ones examined here.

Competing Reactions. The low light yields obtained with all strong bases implies that, as we previously suggested[29] for DMAP, there exist competing non-light-producing reactions that are accelerated by these compounds. The negative effect of bases on the quantum yield of the POCL reaction has been observed in several previous studies.[11,12,14,16,24,37,47] Various different explanations have been presented to account for this observation, e.g., formation of a complex between the base and the oxalic reagent,[12] catalysis of reagent[37] or intermediate[16] breakdown and, in semi-aqueous environments, base-catalyzed hydrolysis of the reagent.[24,47] Anyhow, such dark reactions would severely limit the amount of reagents and/or intermediates available for light production and may also rationalize the occurrence of a maximum in light intensity at minimum basicity difference discussed above. To examine this theory and to investigate the stability of the activated oxalic acid derivatives in strongly basic environments, the decomposition rates of the oxalic reagents in the presence of different strong base catalysts in acetonitrile were studied by UV spectroscopy. As evident from Table 3, both TCPO and ODI were unstable in the presence of the bases and decomposed quite rapidly, with one notable exception: TCPO with PMP. Aside from this exception, the decays of the reagents were generally slower with the 4-aminopyridines than with the other strong bases, despite their ability to produce fast and intense light in the corresponding POCL reactions. There was no direct correlation between the basicities of the catalysts (see Table 1) and the reagent decomposition rates. However, among the 4-aminopy-

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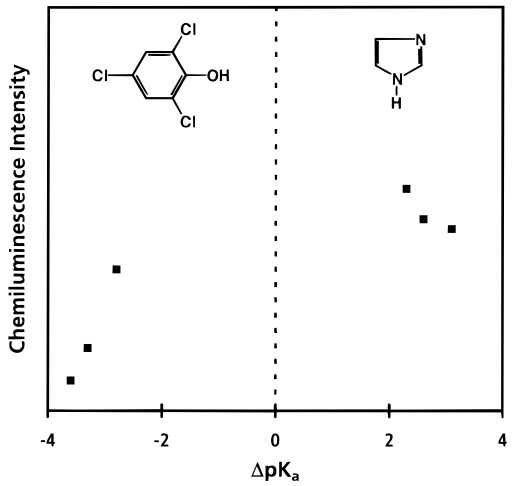

Figure 2. Chemiluminescence light intensity as a function of difference in pK_a in acetonitrile between the 4-aminopyridine catalyst and the leaving group from the reagent. The two insets show the structures of the protonated leaving groups in each set of experiments.

Table 3. The Influence of Different Bases on the Decomposition Rates of Chemiluminescence Reagents in Acetonitrile

base	K_{TCPO}, min^{-1}	K_{ODI}, min^{-1}
none	<0.001	0.012
4-aminopyridine	0.18	0.25
DMAP	0.17	0.34
PYP	0.48	0.95
morpholine	9.7[a]	22
piperazine	450[a]	830
triethylamine	0.33	25
PMP	0.007	6.4

[a] With these bases, the two phenolic groups of TCPO appeared to be released consecutively at two different rates and these decay constants refer to the initial release rate, which should correspond to the disappearance of TCPO. The second decay constants with morpholine and piperazine were 0.89 and 6.5 min^{-1}, respectively.

ridines the decay rates generally increased with base strength. For most catalysts, the consumption rate of the reagent increased by a factor of 2 when changing from TCPO to ODI, but with 4-aminopyridine the rate increase was only ~40% and with triethylamine and PMP the rate increase was as large as 1−2 orders of magnitude. The slow ODI decay induced by 4-aminopyridine promises even higher light intensities in the POCL reaction at higher concentrations of 4-aminopyridine, which is interesting considering that the light was particularly intense with this combination of catalyst and reagent.

The extremely slow TCPO decay that was induced by the strong sterically hindered non-nucleophilic[39] base PMP and the slow decomposition of TCPO observed with triethylamine indicate that this moderately reactive reagent is only marginally affected by basic environments and that the main reason for disappearance of TCPO is nucleophilic attack by the bases. A nucleophilic reaction between the reagent and the base must produce either an activated or a deactivated compound, where the first route should result in high light intensities and the latter low light intensities in the POCL reactions. It thus seems that the attainable light intensities with morpholine and piperazine are limited by dark nucleophilic reagent breakdown reactions, whereas this could

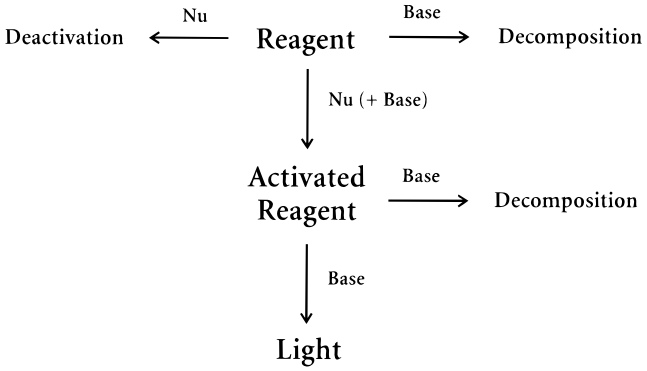

Figure 3. Schematic representation of the catalytic as well as destructive effects of nucleophilic compounds (Nu) and bases (Base) on the peroxyoxalate chemiluminescence reaction. An increase in the rate of the light-producing reaction (vertical path) will increase the light intensity unless the relative rate increase of the quantum yield diminishing trails of reagent deactivation and decomposition (horizontal paths) is greater.

not be true for the 4-aminopyridines because they, as evident from the POCL emission profiles in Table 2, activate the reagent. The ODI decomposition appeared to be related to both the nucleophilicity and the basicity of the bases since the non-nucleophilic bases also induced fast reagent decays. Consequently, the 4-aminopyridines must also induce some basicity-related ODI breakdown apart from the reagent activation. Because the base strength increases in the series 4-aminopyridine, DMAP, PYP, an increase in the decomposition rates within this series can be expected. The low light yields obtained with TCPO and the 4-aminopyridines in the POCL reaction and the decrease in yield with basicity among these compounds indicate the presence of a corresponding base-related decomposition reaction of the activated intermediates. With this evidence, we suggest that the more activated the derivatives of oxalic acid become, the more sensitive they are toward basic environments, and the faster they decompose. Thus, fast nucleophilically catalyzed POCL reactions inevitably lead to reduced light yields. The rate of the catalytic reaction of the 4-aminopyridines with both TCPO and ODI generally increased with base strength according to Tables 1 and 3. However, we suspect that the base-catalyzed breakdown reaction dominates over the catalysis reaction and becomes reaction-limiting with ODI as reagent, whereas the reaction rate of the catalysts themselves are rate-limiting with TCPO as reagent. Hence, the maximum in intensity at minimum basicity difference between the catalyst and the leaving group is caused by a change in the rate-limiting reaction in the multistep POCL process. Figure 3 represents an attempt to summarize the discussion above and provide a generalized diagram to schematically show how the nucleophilic and basic properties of the catalyst affect the reagent and the intermediates involved in the POCL reaction. Furthermore, because the relative increase in light intensity and yield appears to be larger with 4-aminopyridine than with the other acylation catalysts, a stabilization of the intermediate in the catalysis reaction can be suspected. Since 4-aminopyridine has the ability to form hydrogen bonds with other bases via the amino group or expel a proton from this nitrogen, we believe that complete or partial neutralization of the intermediate is advantageous for its stability.

However, complementary POCL experiments with ODI and a low concentrations of the catalyst (both 0.5 mM, data not shown)

showed a dependence of light intensity on basicity opposite to the trend in Table 2, i.e., a slight increase in intensity with base strength of the 4-aminopyridine derivative. At first this result might seem to contradict the conclusion above, but when the nucleophilic catalyst is not in excess over the reagent, most catalyst molecules will be engaged in formation of the transient reagent and only a few are available to degrade the reagent. Thus, this observation actually supports the theory that a change in the process limiting the amount of the transient reagent causes the intensity optimum at $\Delta pK_a = 0$ (cf. Figure 2).

In addition to these experiments, the stability of TCPO was monitored in the presence of DBU but the decomposition was almost instantaneous. This explains the very rapid light decay and inferior light yields that we previously observed[29] when DBU was used as a catalyst for the POCL reaction. This clearly pinpoints the nucleophilicity of DBU, a compound that has been claimed to be a useful non-nucleophilic base.[48] Others have also come to the same conclusion.[49]

Dual-Component Catalysis. As outlined in the introduction, the catalysis by imidazole involves two different modes: general base and nucleophilic catalysis.[24,25] This was verified[25] by the observation that triethylamine catalyzed the reaction between imidazole and TCPO. As we were curious if this could be used to increase the light intensity of imidazole-catalyzed POCL reactions, 4-aminopyridine was tested as an ancillary strong base together with imidazole using TCPO as reagent. However, the light intensity remained essentially unchanged and the only effects were a decreased peak time, a faster light decay rate, and reduced light yield. Alternative nucleophilic catalysts were therefore tested in mixtures with different strong bases. An astonishing effect on TCPO catalysis was observed when the imidazole analogue 1,2,4-triazole was used together with strong bases such as 4-aminopyridine, DMAP, triethylamine, or DBU. The light intensity was quite similar with the different cocatalysts, but the peak time, the half-life, and the yield of the light all decreased with increasing basicity of the ancillary base. The addition of small amounts of a strong base caused a more than 10-fold increase in light intensity from a reaction catalyzed by 1,2,4-triazole. As shown in a preliminary report describing this effect,[29] the light intensity from a reaction catalyzed by 1,2,4-triazole or imidazole was the same when small amounts of DBU were added to 1,2,4-triazole. However, the maximum light intensity was reached ~100 times faster and displayed a peak time of a couple of tens of milliseconds with the 1,2,4-triazole/DBU mixture, compared to several seconds with imidazole.

Supported by the pK_a dependency reported above, the interpretation of the dramatic effect of strong bases on the catalytic efficiency of 1,2,4-triazole must be a simple acid−base equilibrium between the two catalysts. The cocatalyst subtracts a proton from the weak monoacidic base 1,2,4-triazole (aqueous pK_a 2.39 [41]) to produce the 1,2,4-triazolate anion (aqueous pK_a 9.97 [41]), which is a strong enough nucleophile to accomplish an attack on TCPO and effectuate a replacement of the phenolic group(s). However, unlike the mechanism for imidazole catalysis,[24,25] it is not probable that the proton removal from 1,2,4-triazole and the attack on the carbonyl carbon occurs in a concerted mechanism. The reaction between 1,2,4-triazole and TCPO leads to the formation of an intermediate,[23] and it is most likely that this happens also in more basic environments, only much faster. This intermediate can, due to the strong analogy to imidazole-catalyzed POCL, be presumed to be 1,1′-oxalylditriazole (ODT). The intermediate reagent ODT then reacts further with hydrogen peroxide to actualize the formation of the key intermediate responsible for the energy transfer to the fluorophore.

Table 4. Dual-Component Catalysis of TCPO Peroxyoxalate Chemiluminescence with Different Concentrations of 1,2,4-Triazole and 1,2,2,6,6-Pentamethylpiperidine

triazole, mM	PMP, mM	max intens, mV	light yield, mV·s	peak time, ms	half-life, ms
5	0	37[a]	n/a[a]	1100[a]	6400[a]
5	0.5	1700	4100	170	1000
5	5	1500	330	17	110
20	0.5	2000	2500	91	760
20	2	2100	550	29	140
80	0.5	1600	1500	45	500
80	4	1900	150	7	41

[a] This reaction experienced a pulsed emission profile and these figures refer to the first one of these pulses. The second pulse experienced its 16-mV maximum at 280 s and declined so slowly that the whole emission profile could not be integrated; however, the light yield up to 1000 s was 15 V·s and at that time the intensity had decreased by ~20% from the maximum value of the second pulse.

If, as we anticipate, only the basicity of the cocatalyst is important, it would be favorable to reduce its nucleophilicity to minimize the effect of reagent breakdown as previously discussed. Hence, the POCL reaction of TCPO was catalyzed by 1,2,4-triazole in combination with the non-nucleophilic base PMP, which showed the slowest rate of reagent breakdown; see Table 3. The results with this combination of catalysts were very encouraging indeed, as displayed in Table 4. The light intensities were about twice those of the imidazole-catalyzed TCPO reaction, and the peak times were in the range of milliseconds. The total light yield decreased quite dramatically with the addition of PMP compared to the reaction only catalyzed by 1,2,4-triazole, demonstrating the sensitivity of the formed intermediates toward basic environments. The ratio between the two catalysts and their concentration relative to the TCPO reagent influenced the light emission profiles dramatically. Light yield, peak time, and half-life decreased with increasing concentrations of 1,2,4-triazole and PMP. The light intensity was dependent on the concentrations of the two catalysts and first increased but subsequently decreased with catalyst concentrations. The optimum catalyst-to-reagent ratio was in the range of 40:1−160:1 and the ratio between the catalysts (1,2,4-triazole/PMP) giving the highest light intensity was between 10:1 and 1:1. These optimums are probably due to the existence of base-catalyzed breakdown (as outlined above) competing with the light-producing reaction.

Sensitivity to Water. The majority of applications utilizing the POCL reaction as a detection technique involve water as one of the solvent components. Water, or rather the hydroxide ion, is also a nucleophilic competitor to hydrogen peroxide for the available reagent molecules. In addition, the different environment in mixed aqueous/organic solutions will most certainly influence the characteristics of the species involved in the reaction. It is

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Table 5. Effect of Water on the Catalysis of the TCPO Peroxyoxalate Chemiluminescence Reaction

catalyst system	water, %	max intens, mV	light yield, mV·s	peak time, ms	half-life, ms
PYP	0	1400	900	1	43
PYP	5	1400	540	1	87
PYP	20	450	93	3	35
1,2,4-triazole/PMP (10:1)	0	1700	4100	170	1000
1,2,4-triazole/PMP (10:1)	5	1700	4100	70	800
1,2,4-triazole/PMP (10:1)	20	960	330	21	100

therefore highly relevant to investigate to what extent the performance of catalysts subject to testing in this paper are influenced by the presence of water. The PYP catalysis of TCPO and the 1,2,4-triazole/PMP (10:1) catalysis of TCPO were selected as representatives for the single-component and dual-component catalysis, respectively. It is evident from Table 5 that the overall influence of water was greater on PYP catalysis than on 1,2,4-triazole catalysis, but neither of the two catalyst systems were appreciably affected by water concentrations up to 5%. However, in contrast to the 1,2,4-triazole-catalyzed reaction, the light yield with PYP as catalyst decreased in a solution with 5% water. At a water content of 20%, both reactions were markedly negatively influenced and the light intensities were reduced to about 30 and 60% of the original intensities for the PYP and 1,2,4-triazole-catalyzed reactions, respectively. A strange, and at this point unexplainable, divergence was observed for the peak time. It increased with increasing water content with PYP as catalyst but decreased with 1,2,4-triazole and PMP as catalysts. Another notable feature of the 1,2,4-triazole/PMP-catalyzed reactions was the increased half-life but decreased light yield at 5% water compared to with no added water. This apparent discrepancy is simply an illustration of the different light decay kinetics for this reaction. Initially, the decay rate was slower at 5% than at 0% water but this changed to a faster decay rate within 50 ms, which caused the intensity to decline below that of the water free reaction after ~200 ms. The practical consequence is that small amounts of water can be beneficial for the light yield of the 1,2,4-triazole/PMP catalyst system on the short time scale within a small detection cell.

CONCLUSIONS

The POCL reaction is very sensitive to nucleophlic catalysis, not only by imidazole but also by other good nucleophiles that can form unstable and reactive intermediates. By using more potent nucleophilic catalysts such as the 4-aminopyridines, the light intensity can be substantially increased but at the cost of lower total quantum yield. The obvious drawbacks with the use of effective and strongly basic nucleophiles are reagent and intermediate breakdown, which restrict the attainable light intensities. This can be partially circumvented by choosing a catalyst that matches the basicity of the reagent's leaving group or by using a mixture of two catalysts, in which each catalyst is aimed at a specific catalytic task. One targeted for nucleophilic catalysis and one for general base catalysis. Such "base-induced nucleophilic catalysis" demands the use of a strong non-nucleophilic base and a quite poor nucleophile which can be activated by the subtraction of an acidic proton.

However, both these strategies emerge as promising approaches for miniature flow-through POCL detectors, as they combine short peak times with high light intensities. Naturally, this requires the reagents to be merged and efficiently mixed immediately before entering the detector. Instrumental development might thus be necessary before the full potential of these reactions can be utilized.

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