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THE REACTIONS OF FLAVIN ANALOGUES AND OTHER HETEROCYCLES AS MODELS FOR BACTERIAL BIOLUMINESCENCE†

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Abstract - The reactions of 10a-peroxyflavins have been examined to increase understanding of the oxidative and chemiluminescent reactions catalysed by the flavin co-enzymes. A remarkable series of structural requirements in the peroxides used has been uncovered, and in addition to serving as models for the mechanism of bacterial luciferase, they constitute a set of new chemiluminescent reactions.

INTRODUCTION

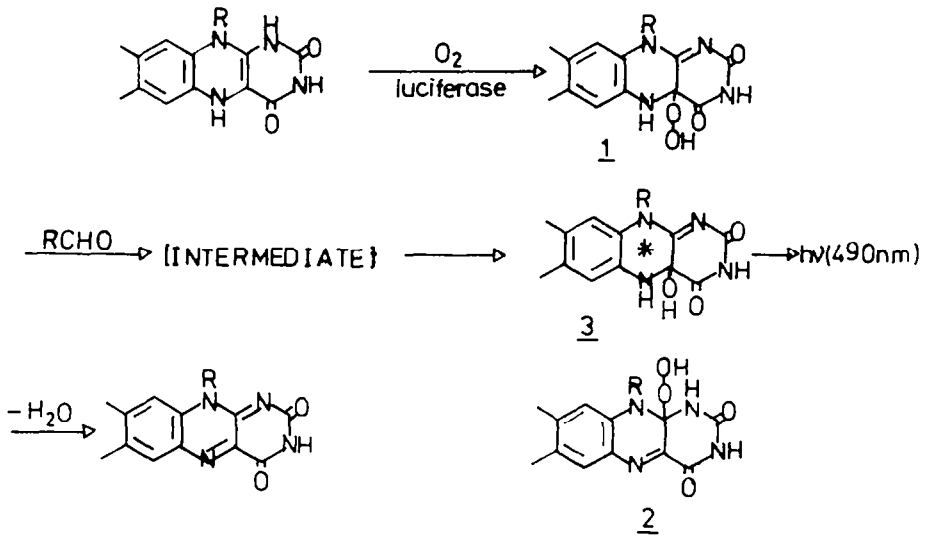
Luminescent bacteria[1] are the most widely distributed of all bioluminescent forms, being found in Arctic and tropical seas as saprophytes and in complex symbiotic relationsnhips with higher organisms. Understanding of the function of light emission is incomplete, nor is there any accepted chemical mechanism for the phenomenon.

The reactions of model heterocyclic compounds have been extremely successful in elucidating the mechanism of other bioluminescent systems.[2-4] However, less success has attended attempts to provide models for the luminescent bacterial reaction. Recent developments[5] even indicate that the accepted biochemical scheme,[6] although well substantiated, may well be an inadequate basis for a chemical mechanism. We wish to describe in detail in this and a succeeding paper[7] our examination of a reasonably efficient chemiluminescent reaction discovered in the course of a search for suitable models. In view of the accumulating evidence[8] for an energy transfer, or sensitised, component of the mechanism, the observations reported may have increased significance.

Choice of a readily accessible model structure is, of course, dictated by the reaction sequence[6] (Scheme 1) on which most work has been based. When we started this work, there was some doubt as to whether a 4a - (1) or 10a-hydroperoxide (2) was involved. It now seems clear that the 4a-peroxide is the intermediate,[9] but that paradoxically it provides little of value as a model.

The principal disadvantage is that 3 is not fluorescent in solution, although a fluorescence spectrum matching the chemiluminescence emission has been obtained for the enzyme-bound species.[9]

† Dedicated to Professor Ralph A. Raphael F.R.S. on the occasion of his 65th birthday, with deep gratitude for the stimulation that his generous spirit and zest for chemistry provided the author (F.McC.) at several points in his career.



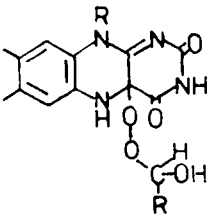
SCHEME 1

We shall show that the reactivities of the two peroxides are very similar, and that the unequivocal fluorescence of the immediate product of the reaction of the key intermediate provides a good basis for study. Such is the difficulty in arriving at a working hypothesis for the decomposition of this key intermediate (its structure can only be assumed at present) that any clean, efficient chemiluminescent reaction such as the present one may provide useful basic information.

Reaction of Flavin Peroxides

Chemiluminescent reactions of organic peroxides in solution, which involve O-O bond rupture, have been shown to proceed by dioxetan and dioxetanone cleavage[10,11] and by electron transfer between radical ions.[12] These mechanisms, and others,[13] have been proposed to account for several *in vivo* bioluminescence reactions.[11] Bacterial bioluminescence,[14] however, appears to be of a distinctly different type from the other well-studied bioluminescent reactions. Bacterial luciferase is a flavoprotein monoxygenase[15] which requires for light emission the oxidation, by a 4a-peroxyflavin 1,[16] of a long-chain aliphatic aldehyde to the corresponding carboxylic acid.[17] Despite much speculation concerning the mechanism[18-24] of the reaction, the precise nature of the chemical events which lead to light emission remains obscure. The emitter (Scheme 1) has not been isolated, but has been proposed as a 4a-substituted flavin, on the basis of a comparison of the bioluminescence emission spectrum with the fluorescence spectra of enzyme-bound 1 and 3.[9,24] Aliphatic aldehyde is the only substrate which is known to be capable of producing *in vivo* light emission.[25] Addition of hydroperoxides to aldehydes is a well established process and led to the suggestion [6] that the intermediate derived from aldehyde and 1 possessed structure 4.

Removal of aldehyde-derived C-H was thought to proceed by a Baeyer-Villiger rearrangement 5, simultaneously yielding an electronically excited state product.[6] As previously indicated, 4a-substituted flavins are not fluorescent in solution and the emitter in the chemiluminescent decomposition of analogues of 2[22,26,27] cannot be identified. Since knowledge of the structure of the emitter is generally essential for the successful study of chemiluminescence in



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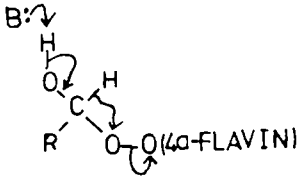

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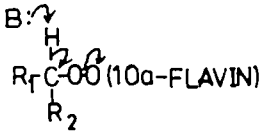
solution,[11-13] we decided to make use of the fluorescent 10-substituted flavins.[28] We report herein that 1) 10a-peroxyflavins 6 readily undergo non-chemiluminescent, eliminative heterolysis (6, arrows) in the presence of base; 2) peroxyalkyl-10a-peroxyflavins 7 can undergo efficient chemiluminescent decomposition, probably as a result of consecutive elimination rections, yielding electronically excited 10a-hydroxyflavin.



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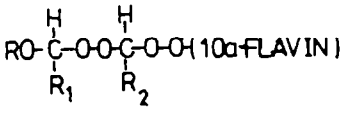

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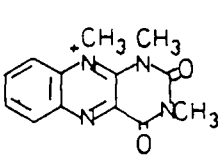
METHODS AND RESULTS

Preparation of 10a-hydroxyflavins

Alkaline hydrolysis of 1,3,10-trialkylflavinium salts, e.g. 8[29,30] and 3,10-dialkylisoalloxazines,[31] e.g. 9 yields spirohydantoins[32] 12 by addition of hydroxyl ion to the 10a-position,[28] followed by rearrangement of the intermediates 10. The spirohydantoin 12 with acid yields[32] ureide 13, a



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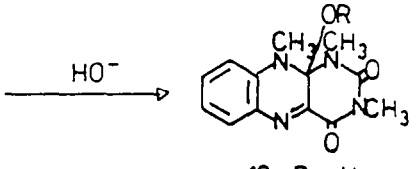

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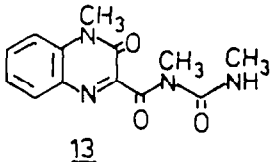
11 R=CH$_3$



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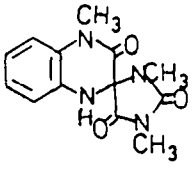

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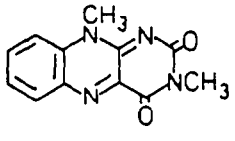

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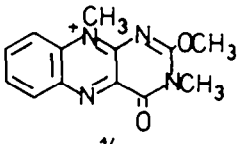
process which can be reversed with base. Reaction of 8 with methoxide ion yields a spectrophotometrically identifiable adduct assigned structure 11 by Müller in 1971. Since this initial observation, Müller and co-workers[33,36] and Hemmerich and Müller[34,35] have proposed that the site of addition of oxygen nucleophiles to 8 and to 2-(O-alkyl)-3,10-dialkylflavinium salts 14 was at

positions 6, 8 and 9a. Nmr evidence presented as proof for 9a-addition[36] to 14 was, as Mager had pointed out,[37] misinterpreted. The assumption that 11 should be blue,[35,38] on the basis of the absorption of the 'model chromophore' 15 at 610 nm, is also at fault. The severely electron-deficient alloxan ring of 15a



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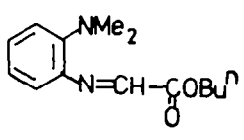

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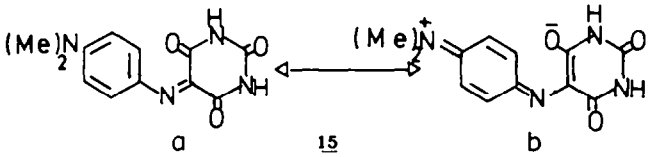


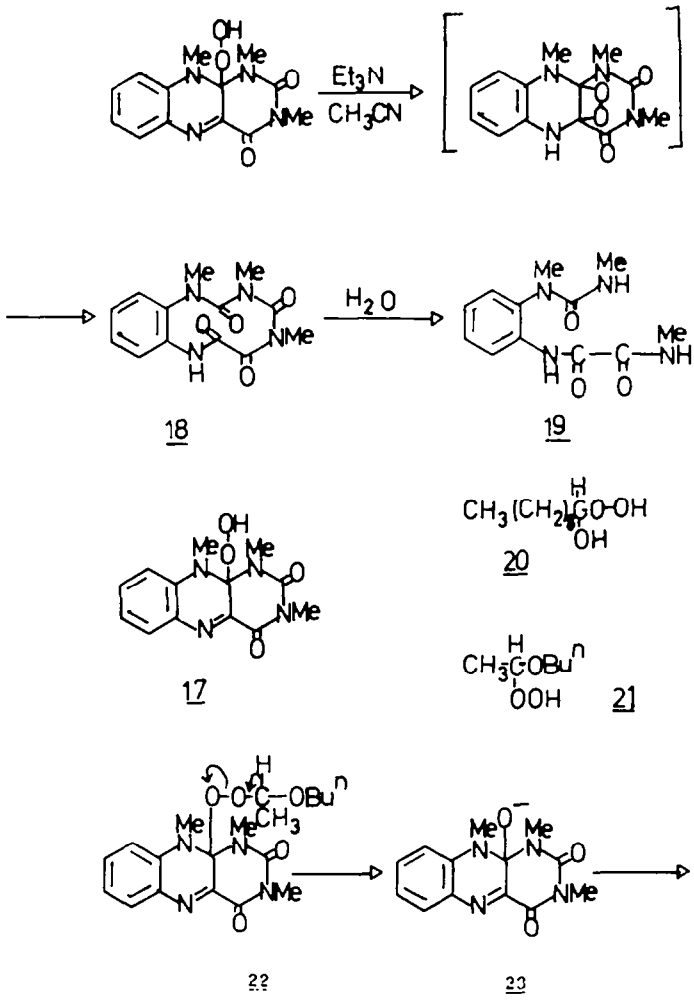
a 15 b

would tend to favour a zwitterionic contribution 15b. The Schiff base 16 has an authentic model chromophore for a 10a-hydroxy flavin and possesses a u.v. spectrum (λ_{max} 286, 410 nm) which is almost identical with that of 11 (λ_{max} 290, 460 nm). This finding, coupled with Mager's isolation of a bis-4a,10a-adduct of 8,[37] confirms the 10a-structure 11 for both monoalkoxy- and monoperoxy-adducts[28] of 8.

Further confirmation of the structure of 10a-adducts has been obtained[37,38] since we started this work, the ring-opened form of the adduct being obtained under carefully controlled conditions. The difference in the energy of the electronic transitions involved in 15 and 16 is remarkable (600 vs 400 nm) although the chromophores are formally very similar. Clearly simple additive rules do not apply to structures such as 15.

Non-chemiluminescent reactions of 10a-peroxyflavins

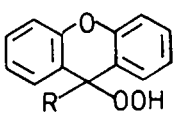
Addition of triethylamine to a solution of 6 and hydrogen peroxide in pure acetonitrile yielded the unstable, but spectrophotometrically identifiable intermediate 17 (λ_{max} ~ 402 nm) which readily decomposed to yield two major products, 12 and an isomer of 17, as well as several minor unidentified products. The structure of the isomer of 17 is interesting in that it is probably formed *via* a dioxetan intermediate. In aqueous conditions 18 is hydrolysed to 19, as confirmed by microanalysis and spectroscopy. Although we have not thought it worthwhile to prove the structure of the unisolable dioxetan intermediate, our experience,[40] backed by detailed ^{18}O labelling studies with other iminoperoxides, makes it seem extremely likely. In any event, this path is not chemiluminescent, and the extreme instability of the presumed dioxetan (the reaction is over in seconds) makes it an unlikely source of the oxidative power of flavin peroxides. Addition of hydroxyalkyl hydroperoxides,[30] e.g. (20), to the 10a-position of 8 would be expected to yield a 10a-substituted analogue of 4. Reaction of 8 with 18 and triethylamine yielded a similar mixture of products to that obtained from reaction of 8 with hydrogen peroxide. Chemiluminescence was observed only when impure 18 (aged samples) was employed. Addition of an alkoxyalkylhydroperoxide,[42] 21 to 6 resulted in a clean conversion to 12, *via* an elimination reaction of 22. The presence of trace impurities in the preparation of 21 was responsible for the accompanying weak chemiluminescence and this is discussed in detail later.

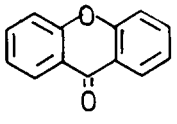


Elimination reactions of the type observed for 22 were found to occur with secondary alkylperoxy adducts of 8, but not readily with primary (or tertiary) alkylperoxy adducts.[43] Thus, addition of xanthyl hydroperoxide[44] 24 to 8 in the presence of triethylamine gave a quantitative yield of 12 and xanthone 26 (via 29) while the n-butylperoxy adduct 27 decomposed only very slowly under the same conditions, being as stable as the t-butylperoxy adduct 28. The elimination reactions of xanthyl peroxides 30 allow the 10a-hydroxy flavin anion 23 to be compared with other leaving groups.

Xanthyl peroxide was chosen for this purpose since xanthone (λ_{max} 340 nm) absorbs in the "window" of the electronic spectra of the other components of the reaction, particularly the strongly absorbing flavins. In addition, the readily accessible excited states of xanthone provided a check for excitation of the carbonyl fragment in the reaction. Such excitation was not observed. The data obtained for the tertiary amine initiated eliminative decomposition of a number of xanthenyl peroxides are recorded in Table 1. The peroxyesters 30, Z = m-ClC_6H_4CO- and $CH_3(CH_2)_8CO$- were prepared by addition of m-chloroperbenzoic acid and perdecanoic acid respectively to the xanthylium perchlorates 31 and 32, and were not isolable, but their rates of decomposition were considerably slower than their formation. The relative rates in Table 1 refer to a comparison of the pseudo first order rate constants (k) for true elimination reactions, monitored spectrophotometrically by the appearance of xanthone 26 (λ_{max} 340 nm). The

rate constants for the disappearance of 29 (λ_{max} 402 nm) and the concurrent appearance of 26 were identical. The kH/kD values refer to a comparison of the pseudo first order rate constants for elimination[43] of 30 R = H and R = D. The results indicate that a 10a-hydroxyflavin anion 23 as a leaving group results in the O-O heterolysis of 30 being 2.3 x 10^2 times faster than with t-butoxy anion, although it is approximately 10-10^2 times less effective than carboxylate anion. A precise correlation of leaving group ability with pK_a is not possible,[45] but inspection of Table 1 indicates that decreasing pK_a of the corresponding conjugate acids does increase leaving group ability. The exception is 24, where the rate is probably affected by hydrogen bonding of the leaving group.





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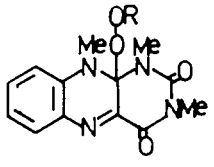
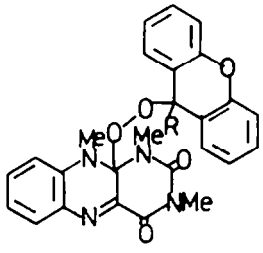
24 R = H

25 R = D





27 R=n-Bu

28 R=t-Bu

29

TABLE 1[a]

^-OZ	k/s^{-1}[b]	k/s^{-1}[c]	k/s^{-1}[d]	k/s^{-1}[e]	Relative Rates	kH/kD
^-OH	6.01±0.05x10^{-3}	9.0±0.4x10^{-5}			1.0	7.9±0.3[b]
$^-O^tBu$	1.85±0.12x10^{-2}				3.1	7.7±0.6[bg]
^-O-(10a-fl.)		6.3±0.3x10^{-2}	1.07±0.1x10^{-2}	3.18±0.11x10^{-2}[f]	7.0x10^2	4.3±0.3[dfg]
^-O-(4a-fl.)				1.32±0.12x10^{-1}[h]	2.9x10^3	
$^-OCOC_9H_{19}$				4.54±0.30x10^{-2}	2.6x10^3	4.0±0.4[e]
-OCO-C_6H_4Cl			2.16±0.13x10^{-1}	2.48±0.12x10^{-1}	1.4x10^4	3.8±0.3[e] 3.0±0.4[d]

[a] k values refer to first order rate constants obtained in acetonitrile at 25°. fl. = flavin.

[b] [24] = [24, Z = ^tBu] = 2.7 x 10^{-4} M, [Et_3N] = 0.65 M.

[c] [24] = [8] = 2.1 x 10^{-4} M, [Et_3N] = 1.0 x 10^{-3} M.

[d] [31] = [m-Cl-C_6H_4-CO_3H] = [Et_3N] = 2.6 x 10^{-4} M.

[e] [31] = [m-Cl-C_6H_4-CO_3H] = [$CH_3(CH_2)_8CO_3H$] = 2.6 x 10^{-4} M, [N-methylmorpholine] = 3.6 x 10^{-4} M.

[f] [8] = 2.5 x 10^{-4} M, [24] = 3.1 x 10^{-4} M, [Et_3N] = 5.8 x 10^{-4} M.

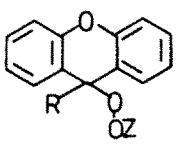
[g] The values were unaltered by changes in base concentration.

[h] [33] = [24] = 1.3 x 10^{-4} M; [Et_3N] = 6.5 x 10^{-4}.

Bruice and co-workers[46] have made an extensive study of the oxidation of iodide ion, alkyl sulphides and amines by 4a-flavin hydroperoxide with remarkably similar results. These workers dismiss the importance of hydrogen bonding[47] to the carbonyl group at C-4 in the enhancement of peroxide reactivity. Our results are in agreement since our elimination reaction does not involve H-bonding.

Although we are aware that the 10a-position does not seem to be involved in natural flavin oxygenase activity, it is still instructive to compare the 4a- and 10a-positions in a search for any special features of the flavin nucleus which lead to enhanced reactivity. The reaction of 5-ethyl-3-methyllumiflavinium perchlorate 33 with xanthyl hydroperoxide was studied as described above, with the result that, although the 4a-position is more reactive, it is so by a factor of only 4.

In our view this confirms the conclusion of Bruice and his co-workers that, as far as model reactions go, only the general electronegativity of the flavin nucleus increases the reaction rate. However, as we suggest later, this conclusion[48] is not at variance with a more specific interaction of the peroxide lone pairs with a polarised double bond in the flavin.



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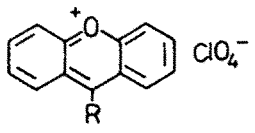
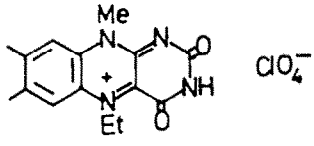


31 R = H

32 R = D



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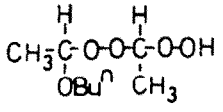
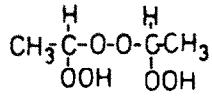
Chemiluminescent reactions of 10a-peroxyflavins

The yield of the chemiluminescent impurities obtained in the preparation of 19 were improved by modification of the original conditions. Four hydroperoxidic products, including 19, were isolated[48] and characterised by ^1H nmr. The diasteroisomers 34 and 35 showed typical chemical shifts for the two



34 , 35



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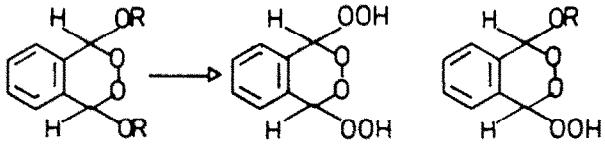
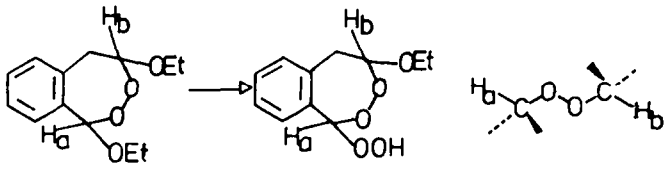
C-H methine protons present in each substance.[49] To help identify the functionality required for chemiluminescence, and to further extend the scope of our studies, two more hydroperoxides, 37 and 38, were prepared. Treatment of 39[50] with hydrogen peroxide led to 37 only, no products of the type 39 being obtained.



39 37 40



41 38 42

Treatment of 41[51] with hydrogen peroxide led to specific introduction of hydroperoxyl at the benzylic position, yielding 38. The ^{1}H nmr spectrum of 38 showed a coupling of 2Hz between Ha and Hb. This coupling through five σ bonds indicates a 'zig-zag' conformation 42 and allows the unequivocal configurational assignments given for 38 to be made.

Figure 1

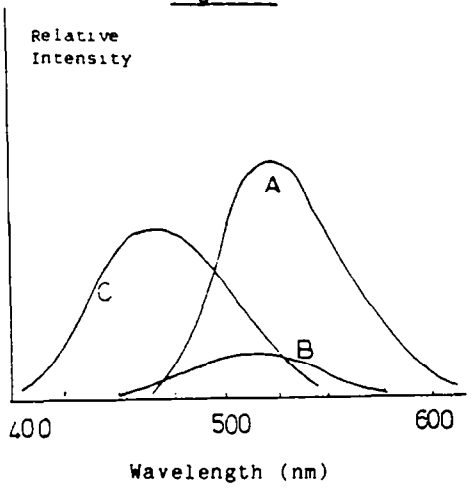


Figure 2

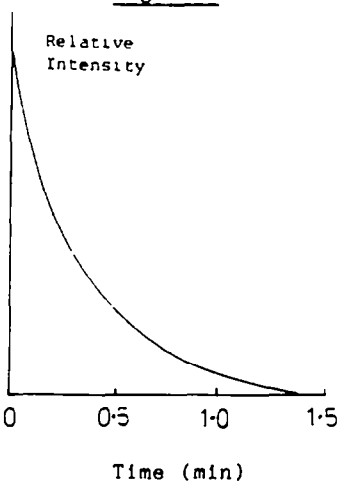


Figure 1 Fluorescence emission spectra in acetonitrile (excitation wavelengths in brackets). Curve A, 11 (406 nm): curve B, 27 (406 nm): curve C, 8 (397 nm). Concentrations 1.94 x 10^{-5} M.

Figure 2 Typical time course of chemiluminescence observed upon addition of Et_3N to an acetonitrile solution of 8 and 34. [8] = 4.6 x 10^{-4} M; [34] = 4.8 x 10^{-4} M; [Et_3N] = 1.0 x 10^{-2} M, 25°C.

TABLE 2

Hydroperoxide	ϕobs[a]	ϕE[b]	K/s^{-1}[c]	[Et_3N]
34	4.2 x 10^{-5}	3.8 x 10^{-4}	3.2 x 10^{-2}	
35	1.0 x 10^{-5}	0.9 x 10^{-4}	6.0 x 10^{-2}	1.4 x 10^{-2}
36	3.4 x 10^{-5}	3.0 x 10^{-4}	6.8 x 10^{-2}	
37	9.6 x 10^{-4}	8.8 x 10^{-3}	~6 x 10^{-2}[d]	1.8 x 10^{-4}
38	1.1 x 10^{-4}	1.0 x 10^{-3}	5.7 x 10^{-2}	5.8 x 10^{-5}

[a] Relative to luminol, corrected for photomultiplier response.

[b] Yield of excited states, based on ϕF for 10 = 0.11.

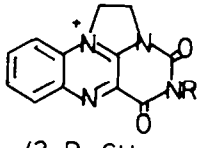
[c] Observed first order rate constants of chemiluminescence decay, 23°.

[d] Not first order.

Hydroperoxides 34-37, when treated in pure acetonitrile with 8 in the presence of triethylamine, produced easily visible bright chemiluminescence.[48] A wide variety of bases other than triethylamine initiate the chemiluminescent reactions. These include primary, secondary and tertiary alkyl amines, pyridine, quinoline, phthalimides, alkoxides, carbonates and bicarbonates. Any aprotic organic solvent capable of dissolving traces of 8 gave visible chemiluminescence. The acetonitrile-triethylamine solvent-base combination was used for the study of the reactions reported here and the quantum yields (relative to luminol) obtained under these conditions are given in Table 2. The observed chemiluminescence emission spectrum had λ_{max} = 522 nm and was superimposable upon the fluorescence emission obtained from 11 (Figure 1). The light-emitting moiety is thus identified as containing the chromophore of 11. The major 8-derived product was in all cases 12, which is known to be formed efficiently from 10 or 23.[29] We therefore suggest structures 10 or 23 for the emitter. The emission spectra (Figure 1) of the other fluorescent species which are present in the chemiluminescent reactions, the starting flavinium salt 8, a 10a-peroxyflavin (e.g. chromophore of 28) and the ureide[17] 11, imply that these species are not emitters. The yields of excited states (ϕE values) given in Table 2 are based upon a fluorescence efficiency (ϕF) of 0.11 for 11, assuming that 10 or 23 have the same ϕF. The chemical yield of emitter was taken to be quantitative, based upon the high yields of 10 obtained. The reaction with 37 produced the best quantum yield, the ϕE value corresponding to a yield of excited states of about 1%, a figure which compares very favourably with model reactions for other bioluminescent systems.[2]

The typical time course of chemiluminescence observed in the reaction between 8 and 34 is shown in Figure 2. Maximum intensity was reached rapidly (<1s), the decay then following first order kinetics for several half-lives. The following general observations concerning the reaction were made: a) the observed quantum yield ϕobs, was directly proportional to [8] and independent of [Et_3N]; ϕobs was also independent of [34] providing that [34] >> [8]; b) the maximum intensity of chemiluminescence I_{max} was independent of [34], but displayed approximately linear dependency upon both [Et_3N] (Figure 3) and [8] (Figure 3, inset); c) the observed first order rate constant, K, did not alter with variation in [34] or [8], but showed a roughly linear dependency upon [Et_3N] (Figure 3). Similar results were obtained from the other chemiluminescent hydroperoxides; however 37 and 38 produced chemiluminescence at notably faster rates than 34-36 and required lower [Et_3N] to produce the rates observed for 34-36 (Table 2). Nevertheless all the hydroperoxides showed similar dependence of rate on [Et_3N].

Other flavin substrates were also effective in stimulating light emission. Reaction of 11 with 34 produced a long-lived but low intensity emission (Figure 4), which yielded ~ 90% of the quantum yield observed from 8. In general, all 1,3,10-trialkylflavinium salts gave efficient chemiluminescence with 34-37. The 1,10-ethano-bridged salts 43 and 44 were similarly effective. This result shows that formation of spirohydantoins is not necessary for chemiluminescence, since ring contraction of the pseudo-base 45 does not occur readily. The observation



43 R=CH_3

44 R=H

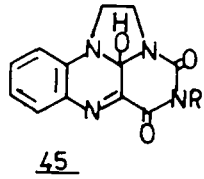


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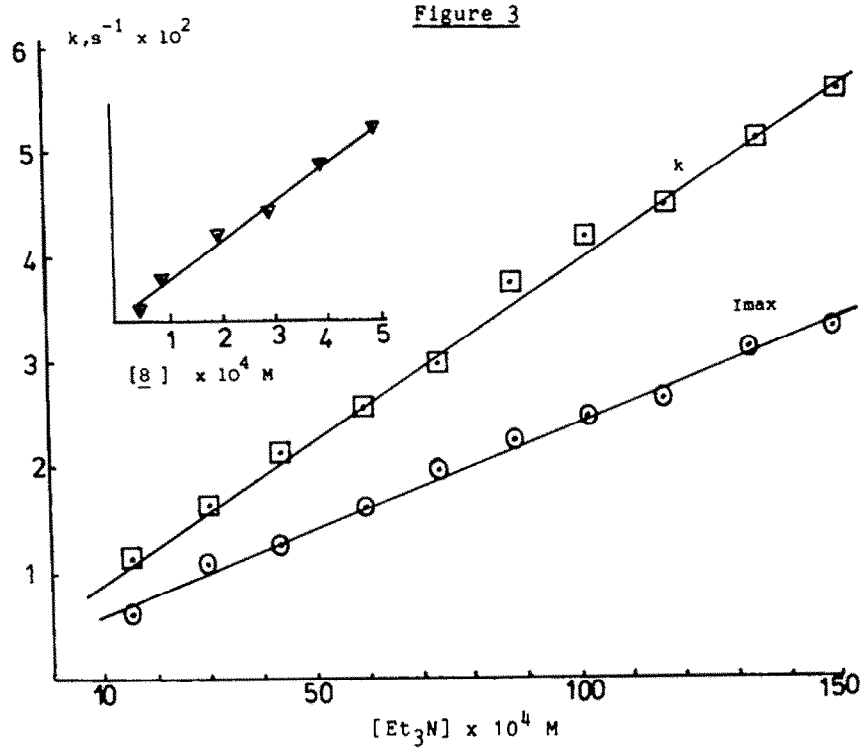
Figure 3



Variation of k (⊡) and I_{max} (⊙) with changes in [Et_3N]; [34] = 3.05 x 10^{-4} M, [8] = 2.55 x 10^{-4} M.
Variation of I_{max} (▽) with [6]; [34] = 6.2 x 10^{-4} M, [Et_3N] = 1.0 x 10^{-2}M

Figure 4

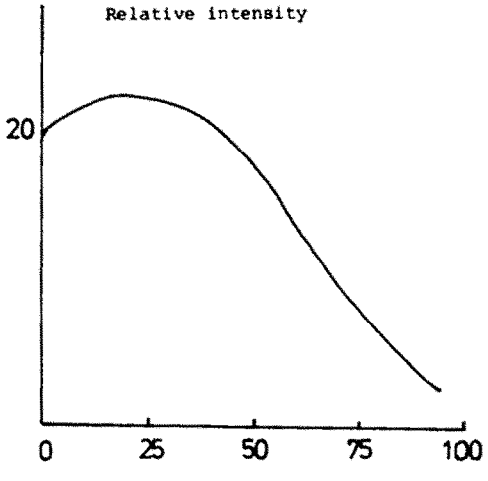


Figure 5

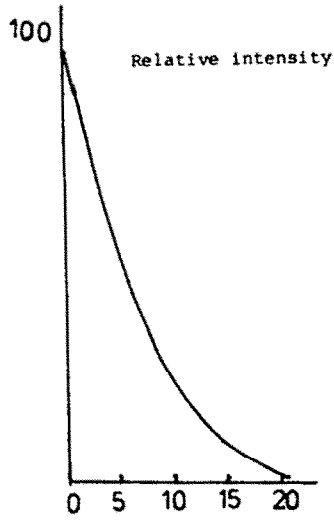
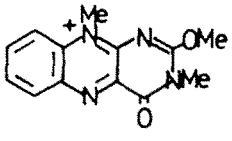


Figure 4 Time course of chemiluminescence observed upon addition of 34 to an acetonitrile solution of 11 and Et_3N. [8] = 4.6 x 10^{-4}M, [MeOH] = 2.0 x 10^{-3} M, [Et_3N] = 1.0 x 10^{-2} M, [34] = 4.8 x 10^{-4} M at 25°.

Figure 5 Time course of chemiluminescence observed upon addition of 34 to an acetonitrile solution of 27 and Et_3N, [8] = 4.6 x 1^{-4} M, [n-BuOOH] = 2.1 x 10^{-3} M, [Et_3N] = 1.0 x 10^{-2} M, [34] = 4.8 x 10^{-4} M at 25°.

of chemiluminescence from 44 shows that 3-alkylation is not a necessary structural feature for the flavinium salts. The 2-(O-alkyl)-3,10-dialkylflavinium salts, e.g. 46, also produced chemiluminescence, but the 2,4-(bis-O-alkyl)-10-alkyl compounds, e.g. 47, did not.

The light yield from the addition of peroxides of any sort to the 4a-position was uniformly low. Quantum yields were lower than those reported by Bruice and co-workers[54] and never exceeded 5 x 10^{-6} einsteins. A particularly significant observation is that the light emission extended (in roughly first order fashion) over many hours (typical k = 2.2 x 10^{-5} s^{-1}). The reaction of the peroxide adduct is, of course, over in seconds and should give the same bright flash as we observe for the 10a-reaction, if an eliminative carbonyl-forming reaction is to have any significance. Crystalline, very pure peroxides such as xanthyl and tetralin hydroperoxides, were weakest of all in light emission, yet gave easily followed reactions in both the 10a- and 4a-substituted cases. The initial reaction of the peroxy-adducts formed from 33 were not detectably chemiluminescent with such simple peroxides.



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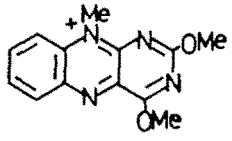

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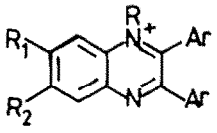
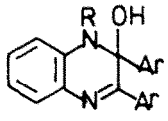




48 $R_1 = R_2 = H$; $R = Me$; $Ar = Ar' = Ph$ 49

Heterocycles other than flavin are also capable of producing light emission. Quinoxalinium salts 48 react with 34-38 to produce bright chemiluminescence;[48] the emitter in this series has been identified as the product pseudo-base 49.

The quantum yields in the quinoxalinium salt series are generally higher than in the corresponding reactions in the flavinium series.[7]

TABLE 3.Comparison of the observed first order rate constants and quantum yields of the deuterated hydroperoxides 55-57 with 38.

Hydroperoxide	ϕH/ϕD	kH/kD
55	1.24 ± 0.14	6.8 ± 0.3
56	1.44 ± 0.12	1.03 ± 0.003
57	2.10 ± 0.13	6.7 ± 0.3

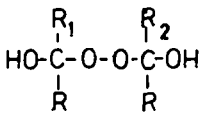
[8]= 4.9 x 10^{-5} M, [55-57, 38] = 3.1 x 10^{-4} M, [Et_3N] = 2.9 x 10^{-4} M at 26.0°; k_H = 0.40 s^{-1}.

TABLE 4. Comparison of the observed first order rate constants for the dark reactions of 32 and 36 in the absence of 8 with the rate constants for the chemiluminescent reactions in the presence of 8.

Hydroperoxide	k/s^{-1}	k/s^{-1} with 6	Rate increase	kHa/kDa
34[a]	3.2×10^{-4}	5.7×10^{-2}	1.8×10^{2}	
38[b]	4.7×10^{-4}	3.8×10^{-1}	8.1×10^{2}	6.4[c]

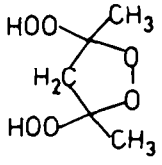
[a] [8] = [34] = 4.5×10^{-4} M, $[Et_3N] = 1.3 \times 10^{-2}$ M.
[b] [8] = [38] = 4.7×10^{-4} M, $[Et_3N] = 5.7 \times 10^{-4}$ M at 25°.
[c] See text.

The chemiluminescent hydroperoxides 34-38 are derivatives of the aldehydo-peroxides 50. In order to determine whether both C-H methine groups



50 $R_1 = R_2 = H$
51 $R_1 = R_2$ = alkyl, aryl
52 R_1 = alkyl, aryl, $R_2 = H$

were a necessary requirement for chemiluminescence, the hydroperoxides 53[55] and 54 were tested in the reaction with 8. The tri-peroxide 54 is derived from a



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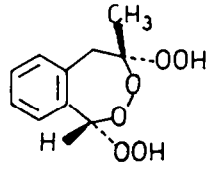


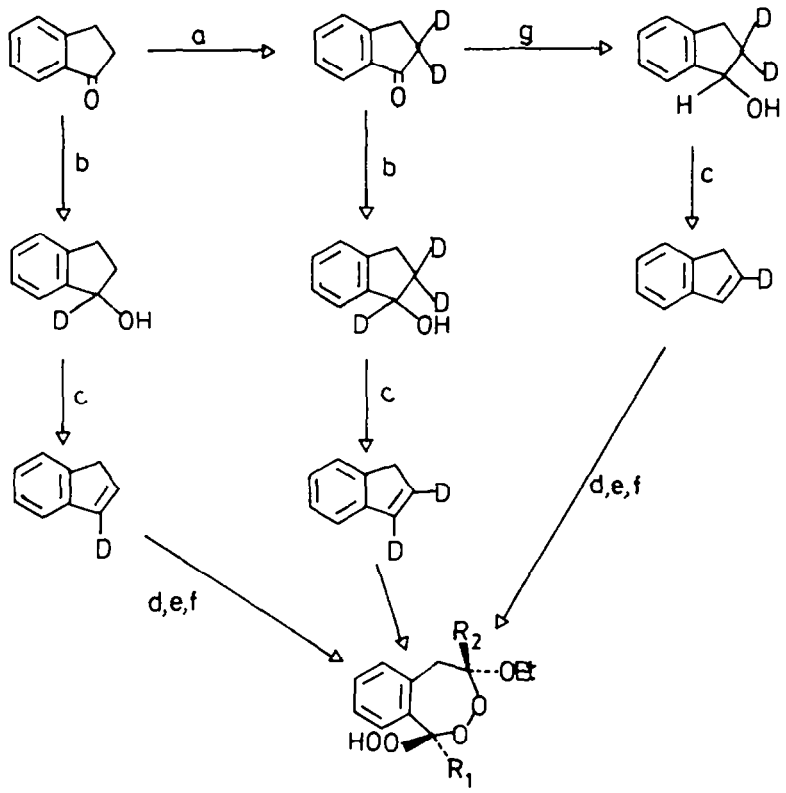
54

bis-keto-peroxide 51, and 53 from a keto-aldehyde-peroxide 52. Both 53 and 54 failed to produce significant chemiluminescence, as did 50 and 39 (R^1 = Me, R = H). The adduct from 53 was as stable as 27 and 28, while the adduct from 54 decomposed in an eliminative manner (see Discussion).

In order to assess the relative importance of Ha and Hb in the chemiluminescent reactions of 38, the deuterated analogues 55-57 were prepared (Scheme 2). ^1H nmr spectra of the products showed quantitative selective deuteration. The deuterium isotope effects which were observed are shown in Table 3. The kH/kD values refer to the ratios of the observed first order rate constants obtained from the decay of the chemiluminescence emissions of the appropriate hydroperoxides with 8. The $\phi H/\phi D$ values are the ratios of the quantum yields obtained from the same reactions. The large kH/kD value for Ha clearly indicates that breakage of the C-Ha bond is the kinetically controlling step. The significant $\phi H/\phi D$ values for Ha and Hb indicate that both C-Ha and C-Hb bonds must be broken to achieve chemiluminescence. The ratio of the first order rate constants obtained from the reaction of 8 with 38 in 1% H_2O/CH_3CN and 1% D_2O/CH_3CN gave a solvent kinetic isotope effect and a product isotope effect of - 1.0. The ratio kH/kD for Ha in the reaction with 48 was 2.3 ± 0.2.

Cleavage of C-Ha in 38 is also rate determining in the reaction of 38 with triethylamine in the absence of 8 (Table 4). Comparison of the rate constants for these 'dark' reactions of 34 and 38 with the corresponding rate constants for

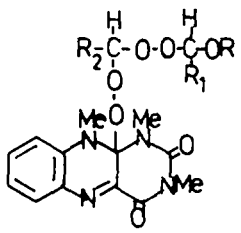
the chemiluminescent reactions in the presence of 8, indicates that the adduct 58 accelerates O-O heterolysis as efficiently as 29.



55 R_1 = D, R_2 = H
56 R_1 = H, R_2 = D
57 R_1 = R_2 = D

SCHEME 2

Reagents: (a) K_2CO_3/D_2O;[56] (b) $LiAlD_4/Et_2O$; (c) p-Me-C_6H_4-SO_3H/benzene; (d) O_3/EtOH; (e) H_2SO_4/EtOH; (f) H_2O_2/H_2O; (g) $LiAlH_4/Et_2O$



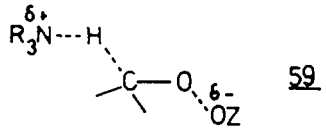
58

10a-Peroxyflavins as Models in Oxygenation

The very thorough and extensive work of Bruice[57] on the use of synthetic 4a-hydroperoxyflavins seems to show that there is no evidence for the transient carbonyl oxide suggested by Hamilton.[58] Although the enhancement of the rate of oxidation is substantial, it seems that it is the result of attachment of the peroxide to a strongly electronegative molecule. If this is so then the phenomenon should be very general.

In oxygenases such as bacterial luciferase and cyclohexanone oxygenase[59] the apparent Baeyer-Villiger-like activity is modelled very well by the eliminative

reaction studied in this work. The results in Table 1 indicate that, in the the case of 29, heterolytic O-O bond cleavage occurs readily in an eliminative process. The decreasing kH/kD values down Table 1 correspond to a shortening of the C-H bond and an increase in the O-O length in the E_2 transition states 59. The better the leaving group (^-OZ), the greater is the extent of O-O bond cleavage in the transition state[60]. The effect of ^-OZ = 23 is reflected in the increased rate of elimination of 23, by a factor of 2.3 x 10^2, in comparison with ^-OZ = $^-OBu^t$. Further inspection of Table 1 reveals that 23 is approximately 10-10^2 times poorer as a leaving group than R-CO_2^-.



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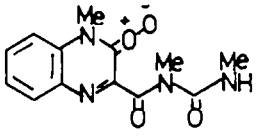
The question arises as to why 23 should be a considerably better leaving group than $^-OBu^t$. Build-up of 23 in the elimination reaction of 29 was not observed: the rapid ring contraction to 12 is clearly an important driving force for disappearance of 23. Hamilton has suggested[58] that ring opening could occur before O-O cleavage, leading in the case of 17 to the carbonyl oxide 60. This idea, applied to 29, requires that formation of 61 activates O-O bond cleavage, and that the leaving group would not be 23, but 13 (or possibly 12). If this were the case, intermediates like 61 would be expected to be formed from any alkyl-4a-peroxyflavin. The observation that stable 10a-peroxyflavins, eg 2, can be formed, shows that the driving force for elimination in 29 cannot be ring-opening, since this would lead to an elimination reaction of 27. Thus, instead of flavin ring-opening, it is the acidity of the C-H bond which is to be broken in the elimination reaction which is the controlling factor which decides whether elimination can take place at all.



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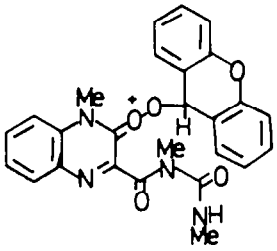

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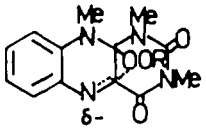
These conclusions are in agreement with those of Bruice, and although different reactions (Baeyer-Villiger oxygenase and O-atom transfer) are being examined, the role of the flavin seems very similar. The fact that 10a- and 4a-substituted flavins are being compared also shows that the detailed structure (such as proximity of a carbonyl group in the 4a-case) is not particularly significant. However, there are indications that other features may play a part. Thus the comparison of 11 with 27 indicates that there is a specific intra-molecular interaction of the 10a-peroxy substituent with the chromophore. The adduct 11 has a fluorescence efficiency, Φ_F = 0.03 (values in acetonitrile). The emission maximum of 28 is also shifted, hypsochromically, by about 8 nm from 11 (figure 1). A similar difference was also observed in the uv spectra of 11 (λ_{max} 406 nm) and 27 (λ_{max} 402 nm). Intramolecular cyclisation of hydroperoxides containing an α-carbonyl or imine is known to be a favourable process[61, 62]; the nature of the interaction of the peroxide moiety with the chromophore in 27-29 is likely to be similar to that previously proposed for dioxetan formation[62] that

is quasi-perepoxide structures of the type 63 or 64. It is interesting in this context that the 10a-hydroperoxide suffers ring cleavage via a presumed dioxetan. Furthermore, the electron-withdrawing inductive effects of N-1, N-10, and C-4a would contrive to make the C-10a site of 27-29 somewhat electrophilic. We suggest that a combination of this inductive effect with the internal non-bonding interactions of the type 53 and 64 would lead to O-O bond polarisation, and hence activation of 10a-peroxyflavins without ring-opening. An exactly comparable description can be applied to the more biologically significant 4a-hydroperoxyflavin.



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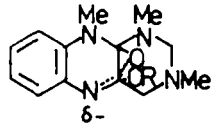

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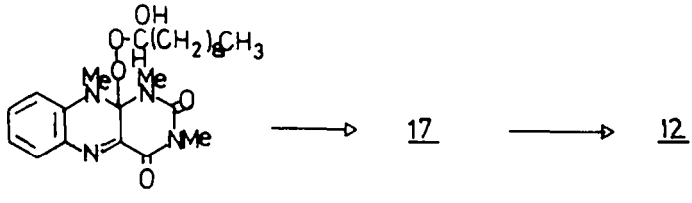
The chemiluminescence of these peroxyflavins is discussed in the next section, but the light reaction has a bearing on the often discussed question of ring-opening of the flavin, to produce a transient carbonyl oxide. On the very reasonable assumption that emission from a singlet state is faster than chemical rearrangement, the first-formed product is the 10a-hydroxy chromophore and no other. This argument will also apply if we take it as extremely unlikely that ring closure to the intact flavin nucleus would allow the preservation of the excitation of a precursor.

Chemiluminescent reactions of 10a-peroxyflavins. Unlike the 4a-substituted analogues[27,54] the adduct from 8 and 18 did not give chemiluminescence. The peroxide 18 rapidly yields decanal and hydrogen peroxide under the basic conditions which were employed; thus the products were the same as those from 8 and H_2O_2 only. Any 65 formed could have reacted by loss of aldehyde to 17, followed by irreversible decomposition.



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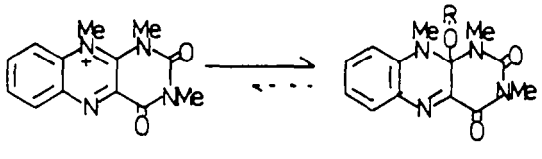
The adducts 11 and 27 reacted at strikingly different rates in the chemiluminescent reaction (figures 4 and 5). The equilibrium of scheme 3 lies well to the right. (No flavinium salt is detectable by u.v. spectroscopy).



8

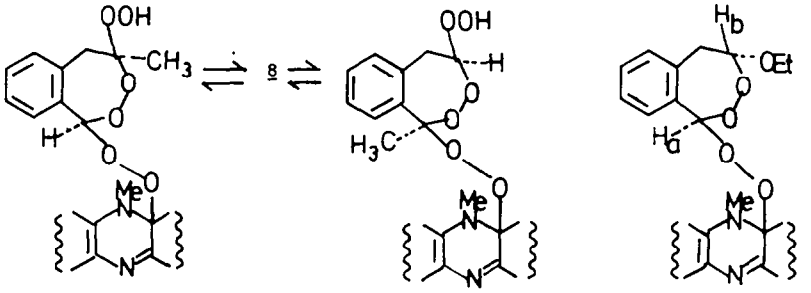
11 R = Me

27 R = ^{n}BuO

Scheme 3

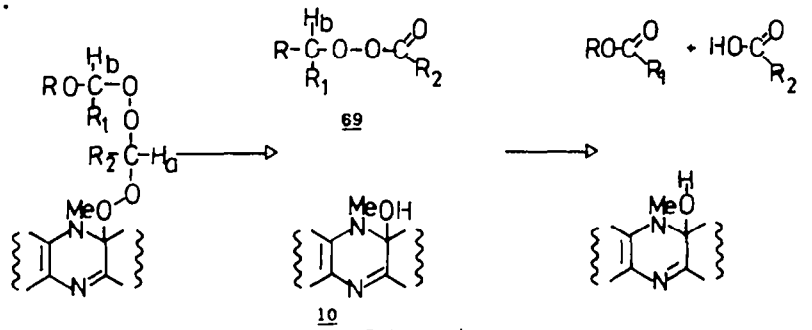
The group ROO^- is expected to be as good a leaving group as it is a nucleophile, and this may account for the observed difference in rates. Thus reaction of 33 - 37 at the 10a-position of 8 leads to the chemiluminescent process.

The primary deuterium kinetic isotope effect observed for Ha in the chemiluminescent reaction of 8 with 38 (k_H/k_D = 6.8 ± 0.3 Table 3) strongly suggests C-Ha bond breakage in an eliminative manner as the rate determining step. The mechanism, by analogy with 29, is assumed to be E2.[60] The alternative Baeyer-Villiger rearrangement of Ha would require k_H/k_D = 1.4-3.0[63] whereas E2 eliminations typically[64] show k_H/k_D = 2.8. Hb in 38 did not exhibit a kinetic isotope effect, but the quantum yield isotope effect (Φ_H/Φ_D = 1.44 ± 0.12) indicates the requirement for C-H_b breakage in the chemi-excitation step. Further evidence for the requirements of C-H_b was obtained from the reaction between 8 and 54, which was non-chemiluminescent. Models suggest that the cis-hydroxyperoxy substituents in 54 are close enough to allow easy interconversion of the adducts 66 and 67. Decomposition proceeds at almost the same rate as for 68, indicating that eliminative reaction of 66, which does not possess Hb, had occurred.



66 67 68

How the products from the rate determining elimination combine to produce chemiluminescence is not immediately apparent. The product peroxyester 69 would certainly be capable of undergoing further elimination, with C-Hb bond breakage[65] (Scheme 4).



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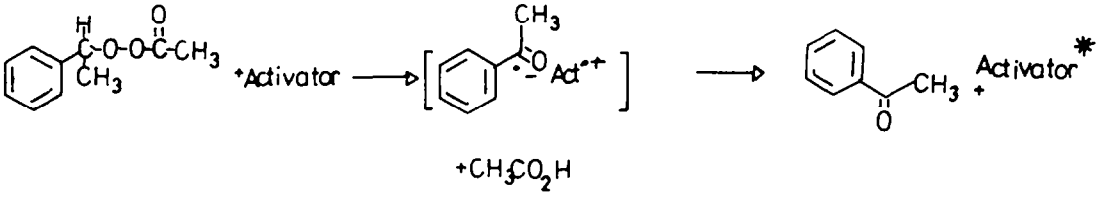
Scheme 4

Schuster[66] has examined the decomposition of secondary peresters and has proposed the scheme, when a fluorescent compound ("activator"), of low ionisation potential is present.



We would expect the 10a-hydroxyflavin 8 to be a suitable activator but the rate of reaction is very much higher than in those reactions studied by Schuster where temperatures of 100° are required for a comparable rate. It may be that the RO-group in 69 sufficiently increases the rate of electron transfer (which appears to be rate determining in the reactions studied by Dixon and Schuster). However, as a later study[7] shows, there are objections to these mechanisms.

One very striking feature of the reaction with the various peroxides active in chemiluminescence is the different rates of reaction and different quantum yields with the two diastereoisomers 34 and 35. Although the differences were not large, they were reproducible. We have already shown that both H_a and H_b are involved in the chemiluminescent reaction and this observation is in accord with this. Unfortunately although some concerted processes can be written our present investigation does not lead to a confident prediction of the excitation mechanism. Further examination of model compounds and the luciferase are in progress.

EXPERIMENTAL

Melting points (mp) were determined on a Reichert hot-stage and are not corrected. Nuclear magnetic resonance spectra were recorded on 60 MHz T-60, EM360 and WP80 instruments and the values are given in units relative to tetramethylsilane). Mass spectra were recorded on an SEI MS9 spectrometer. Ultraviolet spectra were recorded on Pye Unicam SP800 and Varian/Cary 210 instruments, fitted with constant wavelength scanning attachments and thermostatically controlled cell holders. Analyses wre determined by the staff of the School of Molecular Sciences, University of Sussex. Acetonitrile used for chemiluminescence and spectrophotomeric studes with peroxides was purified by successive distillations from calcium hydride, EDTA, then phosphorus pentoxide (three times). The flavinium salts 8[67], 43[68], 42[52], 44[69], and 45[69] were prepared by the methods described in the literature. 9-D-t-butyl xanthyl peroxide and 9-D-xanthyl hydroperoxide wre prepared by treatment of 9-D-xanthanol with t-butyl hydroperoxide and hydrogen peroxide[44]. The xanthylium perchlorates 31 and 32 were prepared by treatment of the xanthanols in acetic acid with 60% aqueous perchloric acid.

Schiff base 16 - N,N-dimethyl-o-phenylenediamine (0.136 g, 0.001 mol) and n-butylglyoxalate (0.130 g, 0.0001 mol) were dissolved in n-hexane and the solution refluxed for 4 h in a Soxhlet apparatus containing activated molecular sieves (type 4A). After evaporation of the solvent, the product was obtained as a yellow gum. TLC showed a single major product, uv: λ_{max} 286, 410 nm; nmr (CCl_4): 7.76 (s, 1H, ArN = CH-CO), 6.7 - 7.3 (m, 4H, Ar-H), 4.33 (t, J = 7, 2H, $-CO_2-CH_2-CH_2-$), 2.98 (s, 6H, Ar-N(CH_3) 1.3-2.1 (m, 4H, $-CH_2-$), 1.07 (t,J = 6, 3H, $-CH_2-CH_3$). Further purification was not attempted.

Reaction of 8 with hydrogen peroxide and triethylamine.

A) 1,3,10-Trimethylisalloxazinium perchlorate (1.25 g, 0.0035 mol) was dissolved in dry acetonitrile (200 ml) under nitrogen. The solution was cooled in ice, and when the temperature had fallen to 15°, hydrogen peroxide (32.2 M, 110µl) was added; at 10°, triethylamine (0.49 ml, 0.0035 mol) in acetonitrile (2.0 ml) was added dropwise over a period of 1 min to the stirred solution. The mixture was cooled with stirring to 0-5°. After 1.5 h, the reaction mixture was allowed to warm to room temperature over 0.5 h, then evaporated to dryness in vacuo at <25°. The residue was dissolved in dichloromethane and boiled with decolourising charcoal (1 g), filtered and evaporated to yield 1.42 g product. Separation of the major components by tlc on silica gel (Merck GF254) with ethyl acetate as the eluent yielded two major products. The product (Rf 0.7) was recrystallised from aqueous ethanol, and was identical in all respects with 12. The other product (Rf 0.15) was recrystallised from dichloromethane/petroleum ether (40-60°) (0.13 g), mp ~ 180°, then 194°; this material 16 appears to be isomeric with 17. (C 53.62; H, 5.31; N 19.34%. $C_{13}H_{14}N_2$ requires C 53.79, H 4.86, N 19.30%.) Isolation proved difficult since hydroysis to 19 occurred readily. (C 54.19, H 6.08, N 21.19% $C_{12}H_{16}N_4O_3$ requires C 54.53, H 6.1, N 21.2%) λ_{max} 230(sh.) (Log ε 4.00) 26% (Log ε 3.87), m.p. 165 - 182° (Recryst. petroleum ether). U.v. spectrum unchanged in acid (indicative of an orthophenylene diamide). Nmr ($CDCl_3$) (ppm) 7.25 (multiplet, 4H) 4.2 (broad, 3H) 3.25 (singlet 3H) 3.0 (doublet, J = 5 Hz, 3H) 2.75 (doublet, J = 5 Hz, 3H). Doublets collapse slowly to singlets in the presence of D_2O. Mass spectrum (M/e 264) shows loss of $MeNH_2$(31) HCONMeH (58).

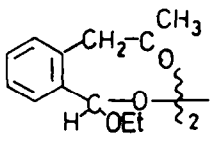
Hydroperoxides 34 - 36 - A stirred solution of hydrogen peroxide (0.53 ml of 35.1 M, 0.0186 mol) and sulphuric acid (0.10 ml, 0.0188 mol.) was cooled to 0° while n-butylvinyl ether (1.57 ml, 0.0124 mol) was added over 3 h. After stirring 1 h more at 0°, saturated ammonium sulphate was added and the mixture extracted several times with ether. The combined ethereal extracts were washed successively with saturated ammonium sulphate, water, sodium bicarbonate solution and saturated sodium chloride solution, then dried with sodium sulphate and evaporated to dryness to yield 0.9 g of a mixture of hydroperoxides. Separation was achieved by the technique of short-column chromatography using silica gel (Merck GF254, 100g) with pure chloroform as the eluent; 2.5 ml fractions were collected, using a fraction collector, over a period of 20 h. This yielded 1-n-butoxy-1'-hydroperoxydienylperoxide (35) [Rf(Merck GF 254; $CHCl_3$) - 0.39], nmr ($CDCl_3$): 10.62 (s, exch. D_2O, 1H, - OOH), 5.40 (q. J - 6.1H, -OO-CH(CH_3)-OO-), 5.03(q, J - 6, 1H, -O-CH(CH_3), 3.73 (m, 2H, -O-CH_2-CH_2), 1.42 (d, J - 6, 3H, -OO-CH(CH_3)-OO-), 1.35(d-J - 6, 3H, -OCHCCH_3)-OO-), ~1.45 (m, 4H, -CH_2-CH_3), 0.92 (ε, J - 6, 3H, -CH_2-CH_3); the diastereoisomers (R.f. 0.34), nmr ($CDCl_3$), nmr ($CDCl_3$): 9.85 (s, exch. D_2O, 1H, OOH), 5.32 (q, J - 6, 1H, -OO-CH(CH_3)-OO-), 5.03 (q, J - 6, 1H -OCH, -OCH(CH_3)-OO-) 3.70 (m, 2H, -O-CH_2-CH_2-), 1.43 (d, J - 6, 3H, -OO-CH(CH_3)-OO-), 1.38 (d, J - 6, 3H, -OCH(CH_3)-OO-, ~1.45 (m, 4H, -CH_2-CH_2-CH_3), 0.93 (t, J - 6, 3H, -CH_2-CH_3); 1 - butoxyethylhydroperoxides 19 (Rf 0.15) nmr ($CDCl_3$): 8.82 (s, exch D_2O, 1H, -OOH), 4.99 (q, J - 6, 1H, -OCH(CH_3)OO-), 3.67 (m, 2H, -O-CH-CH_2-CH_2-, 1.3H (d, J - 6, 3H, -OCH(CH_3)-OO-), ~1.45 (m, 4H, -CH_2-CH_2-CH_3), 0.89 (t, J - 6, 3H, -CH_2-CH_3); 1,1'-bishydroperoxydiethylhydroperoxide (35) (Rf 0.10) nmr ($CDCl_3$): 9.68 (broad s, exch. D_2O, 2H, -OOH), 5.45 (q. J - 6, 2H, -OO-CH(CH_3)-OO-), 1.53 (d, J - 6, 6H, -OO-CH(CH_3)-OO-).

1,4-Bishydroperoxy-1,4-dihydro-2,3-benzodioxin (37) - 1,4-Bishydroxy-1,4-dihydro-2,3-benzodioxin[70] (0.50 g, 0.00298 mol was dissolved with gentle warming and stirring in hydrogen peroxide (86.2%, 10 ml). The solution was allowed to reach room temperature and stirred for a further 0.5 h before being cooled to 0°. The precipitate was collected and recrystallised from diethyl ether/petroleum ether (60-80°) to yield 37 (0.35, 0.00175 mol. 59%) m.p. 117° (dec.) (Found: C48.07, H 4.05. Calc. for $C_8H_8O_6$: C 48.01, H, 4.03%); nmr (CD_3CN): 10.50 (s, 2H, exch. D_2O, -OOH), 7.28 (s, 4H, Ar-H), 5.89 (s, 2H, Ar-CH-OO-); ms; m/e 167 (M^+ -HO_2, 22%), 151 (6%), 149 (4%), 134 (28%), 133 (39%), 105 (100%).

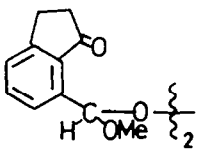
4-Ethoxy-4,5-dihydro-1-hydroperoxy-2,3-benzodioxepin (38) -1,4-Diethoxy-4,5-dihydrobenzodioxepin (41) (1.0 g, 0.0042 mmole) was dissolved with gentle warming in hydrogen peroxide (86.2%, 8.5 ml). The solution was allowed to cool to room temperature with stirring and after 1 h was cooled to 0° and filtered. The precipitate was recrystallised from chloroform/petroleum ether (80-100°) to yield 38 (0.14 g, 0.00062 mol. 15%) m.p. 129-131°. (Found: C 58.26, H 6.17 Calc. for $C_{11}H_{14}O_5$: C 58.40, H 6.24%); nmr ($CDCl_3$): 9.40 (broad s, 1H, exch. D_2O - OOH), 7.0 - 7.6 (m, 4H, Ar-H), 6.57 (broad s, 1H, -OOCH$_a$-OO-) 5.18 (octet J_{pa} - 2, J_{bc} - 4, J_{bd} - 5, 1H, -CH_dH$_b$-CH_c-CH_a-), 2.93 (dd, J_{dp} - 5, J_{cd} - 15, 1H, -CH_cH$_d$-), 1.45 (t, J - 7, 3H, -OCH_2-CH_3). Irradiation at 6.57 caused the signal at 5.18 to convert from octet to dd (J_{bc} - 4, J_{bd} - 5); ms: m/e 193 (M^+ - HO_2).

4,5-Dihydro-1,4-bishydroperoxy-4-methyl-2,3-benzodioxepin (54) - 2-Methylindene (1.0 g, 0.0077 mol) was dissolved in dry ethanol (14 ml) and dry dichloromethane (6 ml) and cooled, with stirring, to -78° (dry ice bath). A stream of ozone in oxygen (12% ozone) was bubbled through the solution until a faint blue colouration was retained. The reaction mixture was purged with N_2 while being allowed to warm to room temperature, then was evaporated to dryness in vacuo, keeping the temperature <20°. The residue was dissolved in dry ethanol (15 ml) and treated with sulphuric acid (0.2 ml of a solution of 1 drop in 1.0 ml of ethanol) and stirred at room temperature for 18 h. The solution was evaporated to dryness in vacuo (temperature <20°), dissolved in diethyl ether and washed successively with saturated sodium bicarbonate, then saturated sodium chloride solution, dried (sodium sulphate) and evaporated to yield crude* 1,4-diethoxy-

*The product contained approximately 40% of the 'dimer' A, as adjudged by nmr.



A



B

Ozonolysis of acenaphthene in methanol gave the dimer B[70], m.p. 70°. (Found: C 68.75; H 5.48%. Calc. for $C_{22}H_{22}O_6$: C 69.10; H 5.80%) The material (m.p. 122-124°, lit. 124-126°) obtained from ozonolysis of 1-methylnaphthalene in

4,5-dihydro-4-methyl-2,3-benzodioxepin (1.55 g, 0.0062 mol; 81%). This product (0.02 g) was warmed with hydrogen peroxide (86.2%, 2.0 ml) until solution occurred. On cooling, a precipitate formed which was collected and recrystallised twice from methylene chloride/petroleum ether (40-60°) to yield pure 49, m.p. 117-120°. (Found: C, 52.5; H, 5.55. Calc. for $C_{10}H_{12}O_6$: C, 52.6; H 5.3%); nmr ($CDCl_3$): 9.30 (broad s, 2H, exch. D_2O, -OOH), 7.0-7.6 (m, 4H, Ar-H), 6.49 (s, 1H, Ar-CH-OO-), 3.66 and 2.60 (d, J = 14, 1H each, Ar-CH_2-C-), 1.31, (s, 3H-CH_3).

Deuterated 4-ethoxy-4,5-dihydro-1-hydroxy-2,3-benzodioxepins 50 - 57 - The routes outlined in Scheme 2 were employed. General directions follow.
A) Deuterated indenes. 1-Indanone or 2,2'-dideutero-1-indanone[53] (0.01 mol) were dissolved in dry detrahydrofuran. Lithium aluminium hydride or deuteride was added in small portions, until no starting material remained, as adjudged by tlc. The mixture was cooled to 0° and excess reagent destroyed by addition of crushed ice, then water. The resulting mixture was thoroughly extracted with diethyl ether, the combined organic extracts dried and evaporated in vacuo to yield the 1-indanols which were recrystallised from petroleum ether (40-60°) (~0.008 mol). The 1-indanols were then dissolved in benzene (60 ml), p-toluenesulphonic acid (0.0011 g) was added, and the solution was refluxed in a Dean-Stark apparatus for 4 h. The cooled reaction mixture was washed with saturated sodium bicarbonate solution, then saturated sodium chloride solution, dried and evaporated in vacuo to yield the crude product. The indenes could be obtained by distillation in vacuo, or, preferably, by column chromatography, using silica gel (Fisons, 200-300 mesh, 60 g, or Merck GF254). Elution with chloroform (petroleum ether (40-60°) (1:1), gave the pure deuterated indenes (usually ~0.001 mol, overall 10%). Nmr ($CDCl_3$) showed the expected absences, as appropriate, of C-2CH) at 6.58 and C-3CH) at 6.90.
B) Hydroperoxides 55 - 56 - The indenes were treated with ozone (at - 78°) and then with acid, as described[48]. Treatment of the products with hydrogen peroxide as described above for 38, gave the desired hydroperoxides, which were recrystallised from chloroform/petroleum ether (80-100°). Nmr ($CDCl_3$) showed complete selective deuteration, in comparison with the data for 38 above. Thus, when Ha = D, Hb appeared as a dd (Jbc = 4, Jbd = 5) at 5.18; and when Hb = D, Ha appeared as an s at 6.57, and Hd as a d (J = 15) at 2.87.

5-Ethyl-3-methyllumiflavinium perchlorate was prepared following Ghisla et al[72] and obtained in 32% overall yield from 3-methyl lumiflavin m.p. 220-225° (Lit.[72] 223-227°). (Found: C,48.0; H, 4.8; N, 13. $C_{16}H_{19}ClN_4$ requires C, 48.2; H, 4.8; N, 14.05%)

Chemiluminescent reactions

Fluorescence and chemiluminescence emission spectra were obtained on an Applied Photophysics spectrofluorometer using DC amplification. The spectra were recorded on a Servoscribe RE542 recorder with coupled disc integrator. Fluorescence efficiencies were determined relative to diphenylanthracene (3 times recrystallised from petroleum ether), with calibration by the method of Melhuish[73].

Chemiluminescence decay rates and efficiencies were obtained in a light-tight box made by Mr. T. Wood and his staff in the School of Chemistry and the light was measured using published[74] electronic circuitry assembled by Mr. P. Sie.

Chemiluminescence quantum yields were measured using the method of Lee and Seliger[75] based on luminol as standard. Chemiluminescence was initiated by placing the peroxide and flavinium salt in CH_3CN in a cuvette and injecting Et_3N as a solution in CH_3CN through a rubber septum.

Acknowledgement

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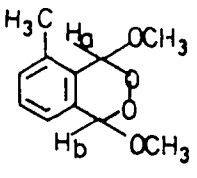
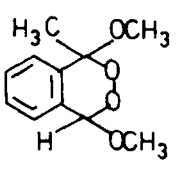
methanol[72] was found to be C [nmr ($CDCl_3$): 5.63 and 5.65 (s, 2H together, H_a and H_b)] and not D as claimed.



C



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