

Functionalized adamantylideneadamantane 1,2-dioxetanes: investigations on stable and inherently chemiluminescent compounds as a tool for clinical analysis

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Abstract - Photophysical and thermodynamic properties of adamantylideneadamantane 1,2-dioxetane (1) and some functionalized derivatives, useful as thermochemiluminescent labels, are summarized. The emission of light from these compounds is stimulated simply by thermal activation. The low fluorescence efficiency of S_1 -adamantanone, the emitting product, causes the chemiluminescence efficiency to be moderate ($\phi_{CL} = 10^{-4}$). The quantum efficiency of chemiluminescence ϕ_{CL} can be increased effectively through radiationless energy transfer to a fluorescent acceptor. The donor acceptor pair 1 and 9,10-diphenylanthracene (DPA) is investigated in terms of Förster's theory. Proteins, labeled with derivatives of both 1 and DPA show increased thermochemiluminescent specific activity, in accord with this theory. Upon complexation with cyclodextrins, the evaporation of 1 and its derivatives at 240°C is inhibited. These complexes show a perfect dose-response linearity over a large range of concentration. Complexation also increases the reproducibility of chemiluminescence detection. Association constants for 1 and a derivative with both β - and γ -cyclodextrin are presented.

INTRODUCTION

1,2-Dioxetanes decompose thermally into two carbonyl compounds, of which one can be formed in the first singlet (S_1) or triplet (T_1) excited state (Fig. 1). The yield of T_1 carbonyls from 1,2-dioxetane decomposition can reach values as high as 50% (e.g. from tetramethyl-1,2-dioxetane, TMD), whereas singlet quantum yields vary from 10^{-4} to 0.25. Furthermore, because most ketones, esters, and aldehydes, all being products of the majority of the 1,2-dioxetanes, have low fluorescence efficiencies, the direct chemiluminescence from 1,2-dioxetanes is generally very weak compared to compounds such as luminol, i.e., the chemiluminescence efficiency $\phi_{CL} = 10^{-8} - 10^{-3}$.

Besides the development of synthetic methods (ref. 1-12), investigations on 1,2-dioxetanes have concentrated mainly on three, closely correlated items: what is the mechanism of thermal decomposition of 1,2-dioxetanes, what causes the stability of a 1,2-dioxetane, and what influences the chemiexcitation yields of the carbonyl products.

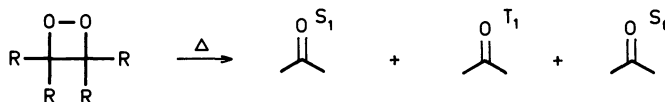


Fig. 1

A series of review articles on this subject has appeared during the last decade (ref. 13-23). The thermal decomposition of 1,2-dioxetanes is a first order process. The energy of activation, E_a , for this process varies over a wide range. Many oxidative chemiluminescent organic reactions are believed to proceed via a 1,2-dioxetane intermediate. In these cases (lophine, lucigenin, peroxyoxalate, acridinium esters, firefly luciferin/luciferase), the 1,2-dioxetane intermediate is very unstable; $E_a < 20$ kcal/mole. Most 1,2-dioxetanes that have been

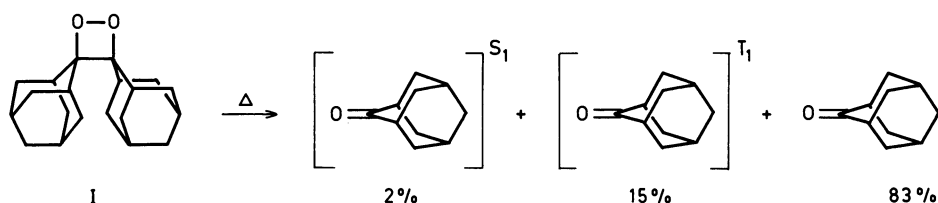


Fig. 2

synthesized and isolated show an E_a between 20 and 26 kcal/mole, i.e. they have a half-life (τ) of a few minutes to a few weeks at room temperature. Therefore these compounds are not useful as inherently chemiluminescent labels for *in vitro* (clinical) analysis.

Adamantylidene adamantane 1,2-dioxetane (ref. 24) (1, Fig. 2) is an exceptionally stable 1,2-dioxetane, with an E_a of 35.2 kcal/mole (ref. 25) and Arrhenius preexponential factor $A = 1.6 \cdot 10^{14}$, or $\Delta G^\ddagger = 32.9$ kcal/mole, $\Delta H^\ddagger = 33.8 \pm 1$ kcal/mole, and $\Delta S^\ddagger = +2.9 \pm 2$ e.u. (ref. 26). This compound is perfectly stable at room temperature ($\tau \approx 10^4$ years). When 1 is heated (as a solid or as a solution in a high boiling solvent, e.g. dodecane, diethylene glycol, dibutyl phthalate, or diphenyl ether) to 200–250°C, the 1,2-dioxetane decomposes rapidly in a first order chemiluminescent process that quantitatively yields adamantanone as the sole product. Of the adamantanone formed, 2% is in the S_1 and 15% in the T_1 state (ref. 26), i.e. from the small family of stable 1,2-dioxetanes (tetra alkyl 1,2-dioxetanes), 1 has the highest singlet efficiency (ϕ_S). A relation between stability of a 1,2-dioxetane and the T_1/S_1 ratio of its carbonyl products has been suggested (ref. 27).

The fluorescence efficiency (ϕ_F) of adamantanone is $5.2 \cdot 10^{-3}$ (ref. 28), which is high for an aliphatic ketone ($\phi_F^{\text{acetone}} = 9.4 \cdot 10^{-4}$) (ref. 28, 29). As it is related to ϕ_F , τ for S_1 -adamantanone is also long for an alkanone (τ_{S_1} is $8 \cdot 10^{-9}$ s in CH_3CN (ref. 30)). These properties are likely to be a result of the rigidity of the molecule, together with protection against collisional quenching through steric hindrance.

From the thermoanalytical investigations by Lechtken (ref. 25) it can be concluded that since the chemiluminescence efficiency (ϕ_{CL}) of 1, being the product of ϕ_S and ϕ_F , is independent of the temperature, and since ϕ_F of alkanones is almost independent of the temperature (ref. 29), all three variables ϕ_{CL} , ϕ_S , and ϕ_F remain constant with increasing temperature.

Both 1 and its product adamantanone are colorless compounds ($\lambda_{\text{max}}^{\text{abs}}(\text{1}) = 265$ nm, $\epsilon = 21.5$; and $\lambda_{\text{max}}^{\text{abs}}(\text{adamantanone}) = 280$ nm, $\epsilon = 20$) (ref. 26). Unlike some other 1,2-dioxetanes (ref. 31–33), 1 does not exhibit autocatalytic decomposition modes at higher concentrations. Thus at all concentrations and over a wide range of temperature, the same process takes place with constant efficiency; $\phi_{CL} (= \phi_F \cdot \phi_S)$ of 1 is 1.10^{-4} , i.e. 6.10^{19} photons/mole are emitted during the thermal decomposition. Since no self-quenching or absorption occurs at any concentration, macromolecular substrates can be labeled with many residues of a derivatized 1 without relative loss of specific activity.

We use the term "thermochemiluminescence" (TCL) for the decomposition of 1 because: (i) the 1,2-dioxetane is inherently chemiluminescent, i.e. no additional chemicals are needed for the chemiluminescent process (compounds like luminol and acridinium esters are not inherently chemiluminescent: they are converted to an unstable intermediate, in a bi- or trimolecular reaction, that decomposes with emission of light subsequently); (ii) the chemiluminescent decomposition of 1 requires an energy of activation, such that for the decomposition of half the amount in a time interval (τ) of less than a few minutes, heating to a temperature of more than 200°C is needed (Fig. 3); (iii) the original definition of chemiluminescence (ref. 34), i.e. luminescence from a chemical reaction at ordinary temperatures, is not really applicable to 1.

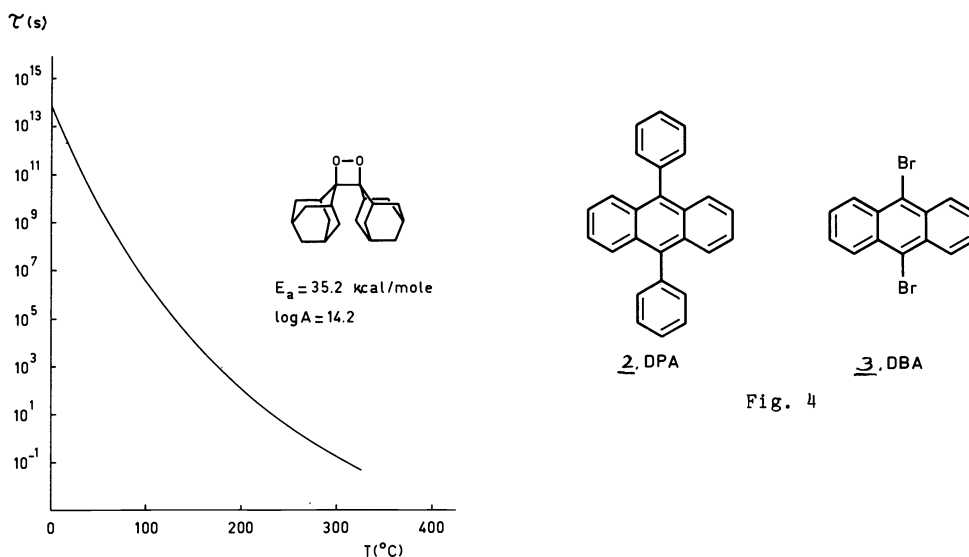


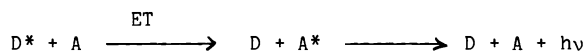
Fig. 3 Thermal stability of 1 as a function of temperature

Of all stable 1,2-dioxetanes, 1 is the most promising candidate as the parent structure for a thermochemiluminescent label (TCL label), because: (i) it is readily accessible (ref. 35); (ii) it can be substituted at a position (ref. 36, 37), where the influence (electronically as well as sterically) of the substituent on the 1,2-dioxetane ring is expected to be small; (iii) of all (tetra-)alkyl 1,2-dioxetanes, 1 shows the highest ϕ_S , and the product S_1 -adamantanone, although not a very efficient one, is a robust fluorescer.

The TCL of 1 resembles radioactivity with respect to unimolecularity and irreversibility; it can serve as a superior alternative in certain applications since the signal can be switched on and off and the method is safer.

ENERGY TRANSFER THERMOCHEMILUMINESCENT LABEL

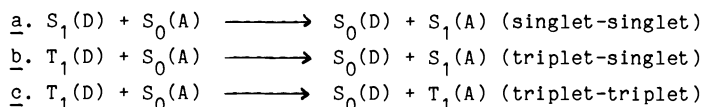
Belyakov and Vassil'ev (ref. 38) introduced the technique of excitation energy-transfer chemiluminescence ("indirect" CL, or ICL) as a way of visualization of poorly luminescent excited carbonyl products from hydrocarbon autoxidation. A strongly fluorescent acceptor molecule (A) adopts the energy of an excited donor (D*) and thereby it is energized to one of its excited states (A*) whereafter it relaxes again to the ground state A with the emission of visible light.



Wilson and Schaap (ref. 32) used 9,10-diphenylanthracene (2, DPA) and 9,10-dibromoanthracene (3, DBA, Fig. 4) as fluorescent acceptors in their study on the thermolysis of *cis*-diethoxy 1,2-dioxetane. DPA is a very efficient fluorescer ($\phi_F = 0.8-1.0$) (ref. 39, 40) and acts as an acceptor of energy of singlet excited carbonyls (ref. 41); DBA on the other hand is a less efficient fluorescer ($\phi_F = 0.1$) than DPA, but it acts as a much better acceptor of energy of triplet excited carbonyls, because of the bromine heavy atom effect on spin-orbit coupling (ref. 38, 42).

Using 2 and 3 together Turro et al. (ref. 4) developed a photochemical titration method for singlet and triplet excited products from 1,2-dioxetane decomposition. The efficiencies of formation of singlet and triplet adamantanone from 1 have been calculated by this popular

method (as well as by chemical counting) (ref. 26). In principle, energy transfer can take place in several distinct ways among which (ref. 43):



The total spin of the system is maintained in a and c, making these fast processes. The processes a and b are more efficient than c because the acceptor spin is conserved. Two mechanisms of energy transfer are involved: Coulomb long-range (≤ 100 Å) interaction (dipole-dipole interaction), predominant in a and b, and an exchange mechanism (ref. 44), predominant in c, which only operates at a distance in which there is considerable overlap of the electron clouds of the donor and the acceptor.

It was proven (ref. 41) that in the case of 1,2-dioxetane decomposition, ET (seen as ICL) from S_1 -ketones to DPA is of the singlet-singlet type and ET from T_1 -ketones to DBA is of the triplet-singlet type. At infinitely high concentration of the acceptor all singlet states and triplet states are trapped: ϕ^{ET} (SS) $\rightarrow 1$ and ϕ^{ET} (TS) goes to a value only dependent on the competition between ET(TS) and ET(TT). The limiting value of ϕ^{ET} (TS) was estimated to be 0.25 (ref. 41, 45).

Hence, taking ϕ_F (DPA) = 1, ϕ_F (DBA) = 0.1, ϕ^{ET} (SS) = 1 and ϕ^{ET} (TS) = 0.25 as the theoretical maximum, upon the addition of DPA or DBA the TCL of 1 is maximally amplified by a factor 192 and 36 respectively. This would make the combination of 1 and DPA a TCL system as efficient as luminol and its analogues. However, since a fast decomposition of 1 requires a temperature of $\sim 230^\circ\text{C}$, and since at high concentrations of DPA fluorescence properties will decrease, these amplification factors will never be reached in practice.

Nevertheless, DPA is an acceptor of choice in the TCL technique since (1) it is thermally stable; (2) its integrated intensity of absorption ($\int \epsilon d\nu$) is independent of temperature and the absorption spectrum shifts only slightly (ref. 46); (3) it has an apolar, rigid, non-basic and non-acidic compound, thus strong influence on ϕ_F and λ_{em} (max) is not observed in the presence of proteins and other biological fluid components (see also ref. 39); (4) the absorption and emission spectra of DPA exhibit only a small overlap at 390-410 nm. Thus DPA shows minimal concentration quenching (ref. 47): the concentration at which it has half its maximal ϕ_F : $C_h \approx 0.5$ M (C_h (DBA) = 0.05 M, C_h (fluorescein) = 0.02 M, C_h (acridone) = 0.025 M); (5) DPA shows no excimer fluorescence (ref. 48); (6) DPA has a very small negative temperature coefficient of ϕ_F ; a value of 1.00 in EPA (ethyl/iso-pentane/ethanol 5:5:2) was found between 77 and 300°K (ref. 49) and for a $5 \cdot 10^{-6}$ M solution in EtOH a value declining from 1.0 at 173°K to 0.80 at 350°K was reported (ref. 50). Extrapolation of the latter results leads to an expected ϕ_F of ≈ 0.5 at 230°K. As was mentioned above, ET(SS) between S_1 -adamantanone and DPA occurs by dipole-dipole interaction of Forster type. According to the theory of Forster (ref. 51) the rate of ET(k_{ET}) and the efficiency (ϕ_{ET}) are given by: (ref. 52)

$$k_{ET} = r^{-6} \cdot K^2 \cdot J \cdot n^{-4} \cdot k_F(D) \cdot 8.71 \cdot 10^{23} \text{ sec}^{-1} \text{ and} \quad (1)$$

$$\phi_{ET} = \frac{r^{-6}}{r^{-6} + R_0^{-6}} \quad (2)$$

$$\text{where } R_0 = (J \cdot K^2 \cdot \phi_F(D) \cdot n^{-4})^{1/6} \cdot 9.7 \cdot 10^3 \text{ Å} \quad (3)$$

wherein r = distance between donor and acceptor; K = orientation factor for dipole-dipole interaction; J = spectral overlap integral; n = refractive index of the medium between donor

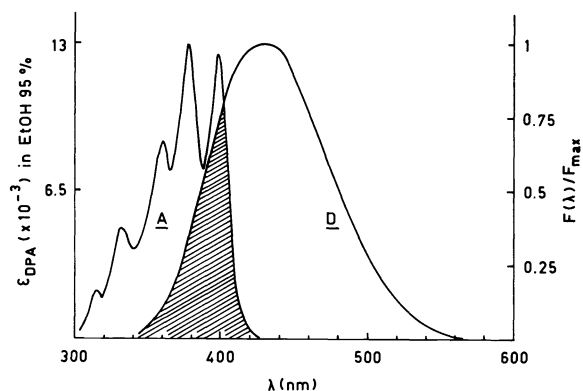


Fig. 5 Spectral overlap of DPA absorbance (A) and thermochemiluminescence of 1 (D).

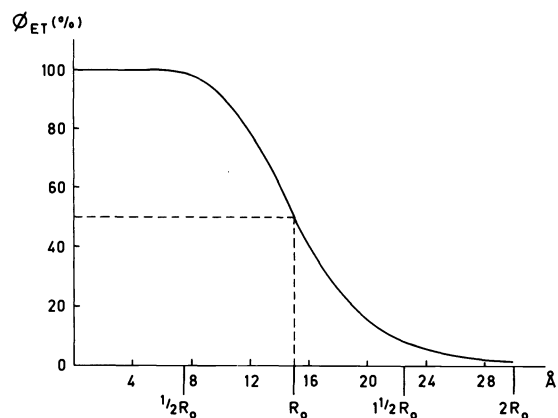


Fig. 6 Efficiency of energy transfer between S_1 -adamantanone and DPA as a function of the distance between the donor and acceptor.

and acceptor; $k_F(D)$ = rate constant for fluorescence emission of the donor; $\phi_F(D)$ = quantum yield of fluorescence of the donor in the absence of an acceptor; R_0 = the distance between donor and acceptor at which $\phi_{ET} = 0.5$. The spectral overlap integral J (in $\text{cm}^3 \text{M}^{-1}$) is the overlap between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor:

$$J = \frac{\int F(\lambda) \cdot \epsilon(\lambda) \cdot \lambda^4 d\lambda}{\int F(\lambda) d\lambda} \quad (4)$$

wherein $F(\lambda)$ = the fluorescence intensity (in arbitrary units) of the donor at wavelength λ (in cm) and $\epsilon(\lambda)$ = the extinction coefficient of the acceptor (in $\text{cm}^{-1} \text{M}^{-1}$). In the case of S_1 -adamantanone (D) and DPA(A) the overlap is as shown in Fig. 5. Taking $K^2 = 2/3$ for free rotating donors and acceptors (ref. 52) and an $\bar{\epsilon}$ (DPA) = $9800 \text{ cm}^{-1} \text{M}^{-1}$ between 350 and 410 nm and $n = 1$ we calculate R_0 to be 15.3 Å. At distance R_0 the $k_{ET} = 3.7 \cdot 10^{10} \text{ s}^{-1}$ according to equation (1).

On the basis of empirical kinetic experiments, using Stern-Volmer analysis, Turro et al. (41) have evaluated $k_{ET}(SS)$ from S_1 -acetone to DPA to be $6.10^{10} \text{ M}^{-1} \text{ s}^{-1}$. Since the shapes of their fluorescence spectra are virtually identical in view of Förster's theory, the only difference between S_1 -acetone and S_1 -adamantanone is their fluorescence life-time: k_F (acetone) = $5.53 \cdot k_F$ (adamantanone) and inversely proportional ϕ_F (adamantanone) = $5.53 \cdot \phi_F$ (acetone) (ref. 28-30). Thus for the donor-acceptor pair acetone-DPA we calculate $R_0 = 8.9 \text{ Å}$ (in benzene) and $k_{ET}(SS)$ at $R_0 = 7.5 \cdot 10^{11} \text{ s}^{-1}$ (τ (acetone) = $2 \cdot 10^{-9} \text{ s}$). When considering a 1 M solution of DPA, Turro's empirical value of k_{ET} (acetone, DPA) can be converted to s^{-1} dimension. Taking the average D-A distance in 10^{-3} M acetone and 1 M DPA as 12 Å, Förster's theory predicts k_{ET} to be $1.2 \cdot 10^{11} \text{ s}^{-1}$, which is in good agreement with the experimental value.

For the system S_1 -adamantanone-DPA with $R_0 = 15 \text{ Å}$, ϕ_{ET} varies with donor acceptor distance r as shown in Fig. 6.

For efficient ET a mean distance of less than 15 Å is clearly needed. This criterion can be met easily when the two components are covalently bound. For TCL-based analytical procedures (immunoassays) we chose to label proteins with both a derivative of 1 and a derivative of DPA (compounds 4 or 5 and 6, respectively as shown in Fig. 7) (ref. 53).

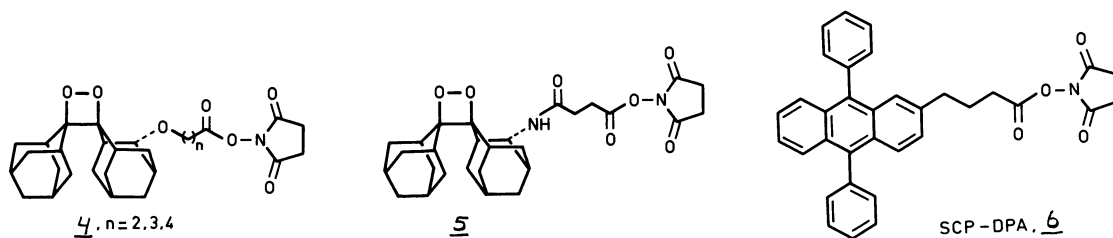


Fig. 7

2-[O-(N-succinimidyl)carboxypropyl], 9,10-diphenylanthracene (SCP-DPA) is a derivative of DPA that mimics the absorption and fluorescence properties to a great extent. As was already found by Cherkasov (ref. 54-56), alkyl substitution of DPA has a marked negative effect on ϕ_F when the alkyl groups are at position 1 and/or 4 only. The spectral distribution of both absorption and fluorescence are not influenced at all. The spectra of the free acid of SCP-DPA (CP-DPA) is shown in Figure 8. The emission maximum of CP-DPA shows a small concentration dependence (Fig. 9). In pure H₂O (curve d), in which CP-DPA is almost insoluble, a red shift of 35 nm was observed. In a basic buffer, CP-DPA is much more soluble and the emission maximum shifts back towards the normal value (curve c). The presence of protein (1% BSA) affected neither the fluorescence spectrum of CP-DPA (curve c) nor its fluorescence intensity. Excimer fluorescence was not observed.

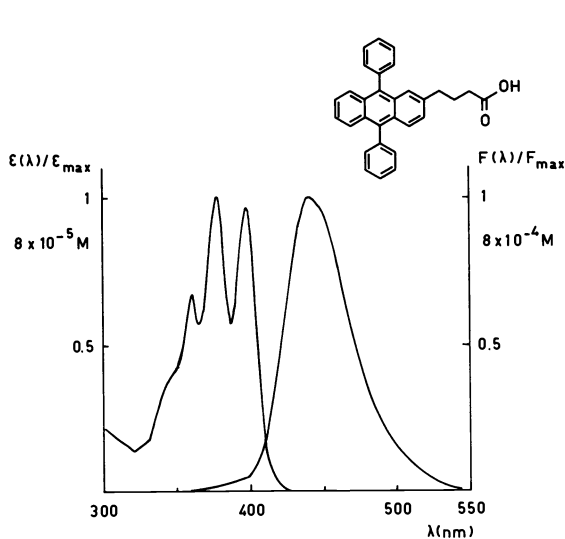


Fig. 8 Absorption and fluorescence spectrum of CP-DPA in 95% EtOH.

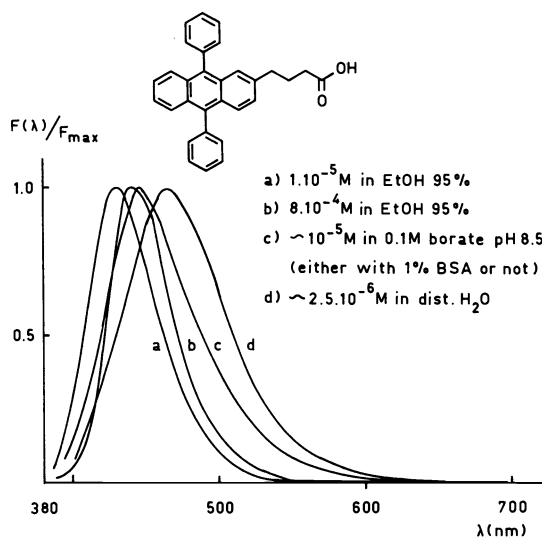


Fig. 9

The extent of amplification of TCL of adamantylideneadamantane 1,2-dioxetane labels by DPA-butuyroyl residues was determined using bovine serum albumin (BSA) as the carrier protein. BSA was labeled at some of its 61 free amino groups with TCL label 4 using a standard coupling procedure (ref. 53). The number of incorporated labels was determined by titration of free aminogroups (ref. 57) as well as by TCL specific activity (i.e. $7 \cdot 10^{19}$ photons/mole label incorporated when measured on a piece of Al₂O₃ thin-layer chromatography material as a carrier).

Subsequently such TCL-BSA was labeled with SCP-DPA in a series of molar ratios (added amounts of SCP-DPA from 5:1 to 300:1) to yield dual conjugates with varying numbers of incorporated SCP-DPA residues (Fig. 10).

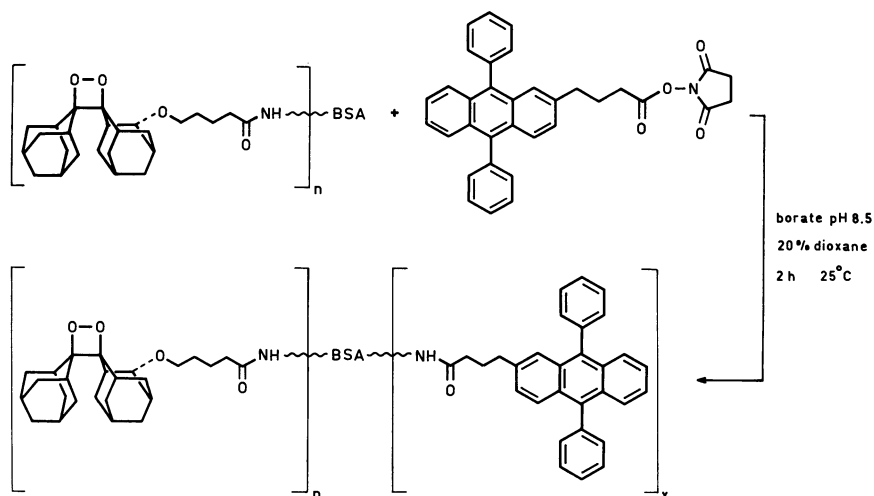


Fig. 10

The brightly blue fluorescent products were purified (dialysis against 20% dioxane/borate pH 9) and the number of DPA residues incorporated was determined spectroscopically. A maximal amplification of TCL by SCP-DPA residues of a factor 40 was observed. In previous experiments, performed in our laboratory, it was found that DPA itself also amplifies the TCL of 1 (in dodecane) maximally with a factor ~ 40. Thus SCP-DPA behaves as powerfully as DPA itself as an amplifier of TCL.

The fact that in practice a maximum of 40x is found instead of the theoretical 192x can be explained by (1) reduced ϕ_F of DPA at 230° and (2) by (local) concentration quenching of DPA since local concentrations (as densities on the protein surface) of > 1.5 M are needed for a $\phi_{ET} \geq 90\%$. Therefore we consider the 40-fold TCL efficiency as a result of 100% ET. With some simplification of the practical situation an estimation can be made about the relation between the number of acceptors and donors on the surface of a protein and ϕ_{ET} , i.e. TCL amplification.

Consider BSA, thyroglobulin, and IgG's as spherical molecules with amino groups equally available and reactive for coupling and assume a homogeneous distribution of these amino groups on the surface of the protein. Assume furthermore that the donor and acceptor take alternating positions on the surface of the protein and that ET takes place along the surface of the protein. Taking $R_0 = 15$ Å and the radii of BSA, IgG's, and thyroglobulin as 15, 25, and 32 Å, respectively, the expected ϕ_{ET} can be calculated using the relation shown in Fig. 6. The results of the calculations are shown as lines in Fig. 11. The experimental values, found for a series of dually labeled BSA samples and of two dually labeled IgG samples and a thyroglobulin sample are shown as dots in this figure as well.

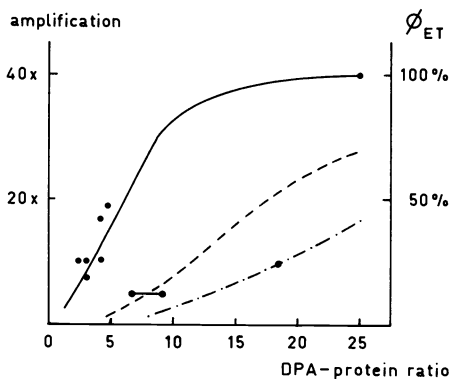


Fig. 11
 The efficiency of energy transfer on proteins labeled with both 4 and SCP-DPA as a function of the number of DPA residues incorporated.

— : on BSA
 --- : on IgG's
 -.- : on thyroglobulin

Notwithstanding the relatively high uncertainty in both the above-mentioned calculation and the extremely simplified theoretical model, we conclude that the observed ϕ_{ET} of S_1^- adamantanone originating from 1,2-dioxetanes, to DPA residues on a protein at -240°C (!) is in agreement with the values predicted by Förster's theory.

BSA, labeled with a large number of residues of both 4 or 5 and 6 is still soluble in most aqueous buffer solutions. In lyophilized form it can be kept for years without decomposition. On Al_2O_3 TLC material it emits $\sim 6 \cdot 10^{22}$ photons/mole, i.e. $\phi_{CL} = 10\%$ at the maximum. The dose response linearity (between 0.1 mg/ml and 0.1 ng/ml) of BSA-dual conjugates is comparable to that of BSA, labeled with 4 or 5 only, i.e. intermolecular (between proteins) ET is not observed. Using dually labeled BSA as a label, coupled to an antibody, a Fluorescence Amplified Thermochemiluminescence Immunoassay, FATIMA, can be performed (ref. 53).

CYCLODEXTRIN COMPLEXES OF ADAMANTYLIDENEADAMANTANE 1,2-DIOXETANE AND ITS DERIVATIVES

Adamantylideneadamantane and many of its simple derivatives such as the 1,2-dioxetane are volatile compounds. Adamantane itself melts at 269°C and sublimates readily at atmospheric pressure and room temperature (ref. 58). 1,2-Dioxetane 1 can be sublimed at a reduced pressure (90°C , 0.01 mmHg) without decomposition. Consequently, the quantitation of TCL from 1 and free labels 4 and 5 as such deposited on a thermally stable carrier material (Teflon, Kapton 500 H) in minute amounts is impossible due to evaporation of the sample before the event of thermochemiluminescent decomposition. TCL of these compounds can be measured accurately on strongly absorbing materials (Aluminum oxide and silicagel thin layer chromatography sheet), but high background values diminish the sensitivity of detection.

From cyclodextrin complexes it is known that evaporation of included volatile substances is inhibited efficiently (ref. 59). Therefore we investigated the effect of α -, β -, and γ -cyclodextrin (CD), on the dose-response linearity, reproducibility of detection, and specific activity of 1,2-dioxetane 1 and TCL label 5 (as the corresponding free acid which is formed through hydrolysis of 5 in aqueous solution).

A strongly positive influence of β -CD and γ -CD on linearity and reproducibility was observed. Thus when 5 was dissolved in 15 mM γ -CD/borate buffer pH 8.5 in concentrations varying from 10^{-3} to 10^{-9} M and 3 μl aliquots were taken and measured at 240°C on disks of Kapton 500H (i.e. absolute amounts during detection 3×10^{-9} to 3×10^{-15} moles) a dose-response curve, shown in Fig. 12, with a linear regression $r = 0.99990$ was obtained. The CV (as a measure for

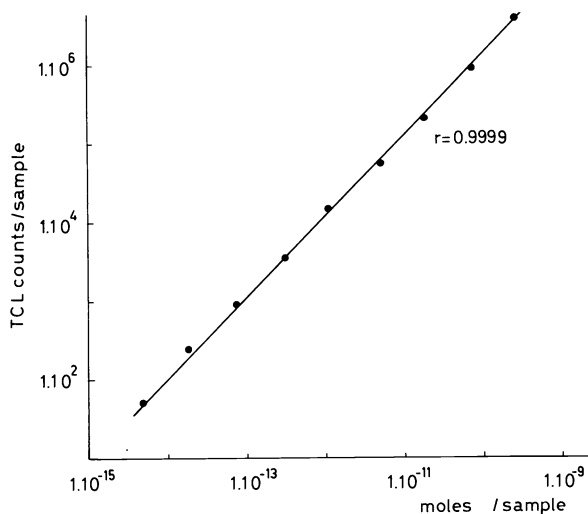


Fig. 12
Dose response curve of
TCL from 5 in 15 mM
 γ -CD/borate buffer pH 8.5

reproducibility) varied from 1.4% at high concentrations to 3.1% at the lowest concentrations. Both the quantum efficiency of TCL from 1 and 5 and the TCL spectral distribution were found to be unaffected by complexation with CD's.

The thermal stability of the 1,2-dioxetanes is only slightly increased, as could be detected by differential scanning calorimetry as well as by analysis of the shape of the TCL curve observed during the standard heating procedure (ref. 55). In order to determine the association constants K for these complexes and to be able to compare these values with those found for complexes of monoadamantane derivatives and cyclodextrins (ref. 60-64), two different methods were used.

First, $K(\gamma\text{-CD}/\underline{5})$ was determined using the fact that free 5 (in acid form) sublimes readily and hence the detected TCL thereof is of very low intensity compared to that of the complexed form. Thus solutions of 5 in EtOH, γ -CD in distilled water, and 100 mM borate buffer pH 8.5 were mixed to obtain solutions with $[\underline{5}]$ ranging from 4×10^{-4} M to 4×10^{-6} M and $[\gamma\text{-CD}]$ ranging from 2.7×10^{-3} M to 2.7×10^{-5} M. From these mixed solutions, 3 μ l aliquots were taken and measured for TCL activity on Kapton 500H as the carrier material ($\text{TCL}_{+\gamma\text{-CD}}$). TCL of solutions of 5 without γ -CD added were measured and these values were subtracted to yield TCL values of the complex, TCL_c . Now using the formula (1):

$$K = \frac{f \cdot [\underline{5}]_0}{([\gamma\text{-CD}]_0 - f \cdot [\underline{5}]_0)([\underline{5}]_0 - f \cdot [\underline{5}]_0)} \quad (1)$$

in which $f = \text{TCL}_c$ divided by the expected TCL with $K = \infty$, $[\underline{5}]_0$ = the initial concentration of 5, and $[\gamma\text{-CD}]_0$ = the initial concentration of γ -CD, from measurements at 7 concentrations a $K(\gamma\text{-CD}/\underline{5}) = 1.2 \pm 0.6 \times 10^4$ was determined.

Analogously, the association constants for 1 and 5 with all three types of cyclodextrins was determined. The results are listed in Table 1.

Second, the spectroscopical method of Selvidge and Eftink (ref. 65) was applied to the competition between phenolphthalein and the 1,2-dioxetane for complexation with β -CD (and γ -CD). The method is based on the fact that the complex of β -CD with phenolphthalein is colorless at pH 10.

It is taken that a 1:1 complex is formed with either guest and that the guests do interact neither in free solution nor during complexation.

Using the formula (2); (ref. 65)

$$K_2 = \frac{[M]_0 - [M] - v \cdot [L_1]_0}{M * ([L_2]_0 - [M]_0 + v \cdot [L_1]_0 + M)} \quad \text{with } [M] = \frac{v}{K_1(1-v)}, \quad \text{in which}$$

K_2 = association constant of 1,2-dioxetane and M; $[M]_0$ = the initial concentration of CD; $[M]$ = the concentration of free cyclodextrin; v = the fraction of phenolphthalein bound to CD (measured as $\Delta A/\Delta A_{\text{max}}$); $[L_1]_0$ = the initial concentration of phenolphthalein; $[L_2]_0$ = the initial concentration of the 1,2-dioxetane; K_1 = association constant of phenolphthalein and CD, the association constants for 1 and 5 (as the free acid) with β -CD could be determined. Subsequently, the method was applied to the pair $\gamma\text{-CD}/\underline{5}$ as well. The value for $K(\gamma\text{-CD}/\underline{5})$ obtained in this manner is less precise due to the relatively low K_1 of phenolphthalein with γ -CD. The results are summarized in Table 2.

From a comparison of the results thus obtained, it can be concluded that the two methods yield consistent values for K's of β - and γ -CD with both 1 and 5. Based on the fact that one

TABLE 1 Association constants of 1 and 5 with α -, β -, and γ -cyclodextrin determined by TCL

cyclodextrin type	1,2-dioxetane	K
α	<u>1</u>	undetectable [#]
α	<u>5</u>	$1.1 \pm 1.0 \cdot 10^3$
β	<u>1</u>	$2.5 \pm 0.8 \cdot 10^4$
β	<u>5</u>	$1.2 \pm 0.6 \cdot 10^4$
γ	<u>1</u>	undetectable
γ	<u>5</u>	$1.2 \pm 0.6 \cdot 10^4$

#: too small to be measured.

TABLE 2 Association constants of 1 and 5 with cyclodextrins derived from competition with phenolphthalein

CD type	1,2-dioxetane	K_1	K_2
β	-	$1.0 \pm 0.15 \cdot 10^4$	-
β	<u>1</u>	-	$2.3 \pm 0.6 \cdot 10^4$
β	<u>5</u>	-	$2.0 \pm 0.8 \cdot 10^4$
γ	-	$1.5 \pm 0.5 \cdot 10^3$	-
γ	<u>1</u>	-	undetectable [#]
γ	<u>5</u>	-	$2.2 \pm 1.2 \cdot 10^4$

#: too small to be measured

adamantane nucleus fills the inner space of the β -CD molecule and since the K's found for 1 and 5 with β -CD are almost identical within the experimental error, it can be tentatively concluded that in these cases the 1,2-dioxetanes form complexes with β -CD through one adamantane moiety of the molecules.

On the contrary, in the case of complex formation with γ -CD, a completely different way of association must play a role, since a dramatic difference in K is observed for the unsubstituted 1,2-dioxetane 1 and the substituted analogue 5. The inner core of γ -CD has a diameter of 10 Å: it is too wide for accommodating an adamantane moiety.

A space-filling model of γ -CD and 5 and their complex as shown in Fig. 13 indicates that these compounds fit together in such a way that the internal space of the host molecule is filled by a part of the guest greater than one adamantyl fragment: the 1,2-dioxetane moiety is buried in the inner core of γ -CD.

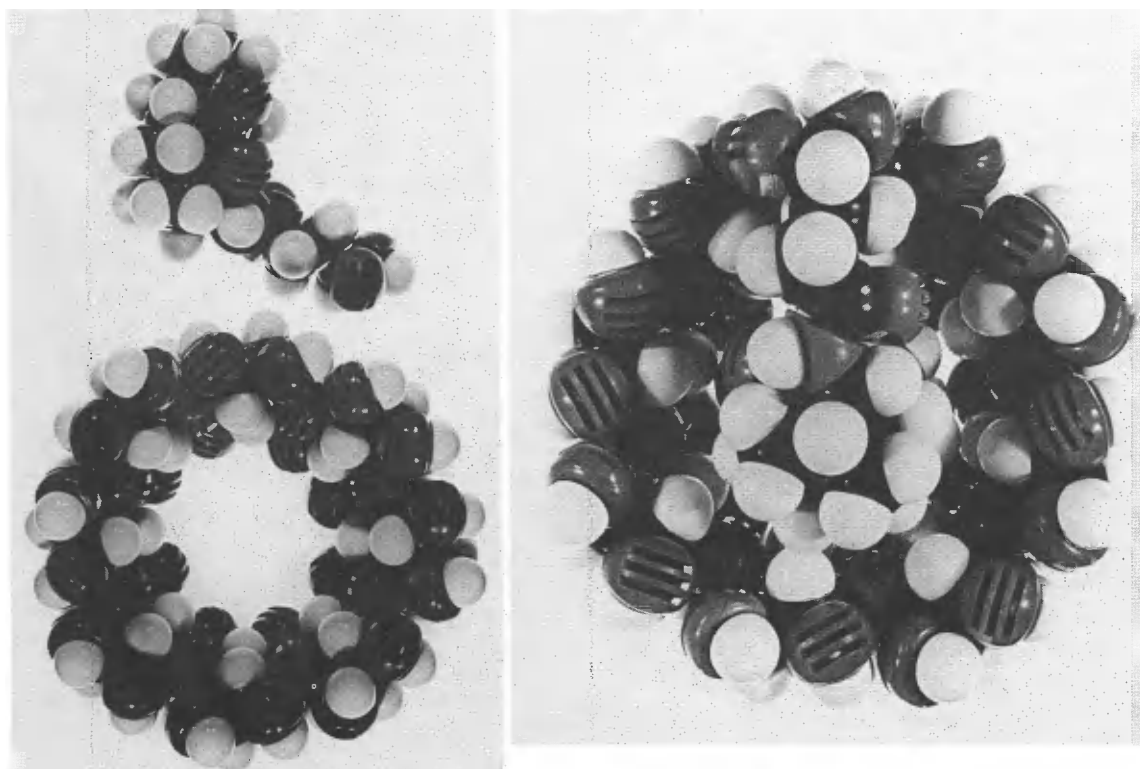


Fig. 13

The spacer moiety of 5 can function as a flexible part of the guest that is able to fill the residual space of the γ -CD inner core in order to expel water molecules disturbing the hydrophobic interaction.

Investigations on the precise conformation of these complexes are under way in our laboratory.

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- Note: The IUPAC-name for adamantane is: tricyclo[3.3.1.1^{3,7'}]-decane. The IUPAC-name for adamantylideneadamantane 1,2-dioxetane is: dispiro[tricyclo[3.3.1.1^{3,7'}]-decane-2,3'-1,2-dioxetane-4',2''-tricyclo[3.3.1.1^{3,7'}]-decane. For sake of simplicity, we will use trivial names.
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