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## Inherently chemiluminescent compounds as new labels in clinical analysis.

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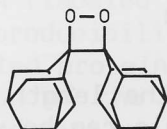
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# summary

This thesis describes the synthesis, the properties, and the use of organic compounds which emit light upon heating to  $\pm 200^{\circ}\text{C}$ , without the need for a chemical additive.

These chemiluminescent compounds belong to the class of relatively very stable 1,2-dioxetanes. 1,2-Dioxetanes are cyclic peroxides which decompose thermally into two carbonyl containing fragments. This chemical decomposition is extraordinary due to the fact that (with a certain efficiency) one of the carbonyl fragments is formed either in its first singlet or in its first triplet excited electronic state. Within a few nanoseconds this excited molecule relaxes to its electronic ground state with emission of a light particle, a photon.

All 1,2-dioxetanes described in this book are derivatives of adamantylideneadamantane 1,2-dioxetane **1**.



**1**

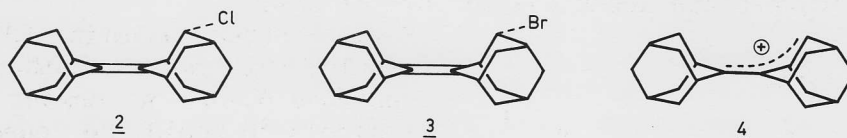
The thermal decomposition of **1** yields adamantanone.

The chemiluminescence efficiency of **1** is  $10^{-4}$ ; i.e. one nanogram of this material emits 200 million photons upon heating. When a sample of **1** is heated to  $\pm 250^{\circ}\text{C}$ , all (blue) light is emitted within a few seconds. Using modern photoncounters, very low level light intensities

(tens of photons per second) can be quantified. Furthermore, because most common materials do not emit (blue) light upon heating to  $200\text{--}250^{\circ}\text{C}$ , compounds based on 1,2-dioxetane **1** can be used as labels in analytical determinations, especially when very low concentrations have to be detected. We introduced the name thermochemiluminescent labels for labels, based on

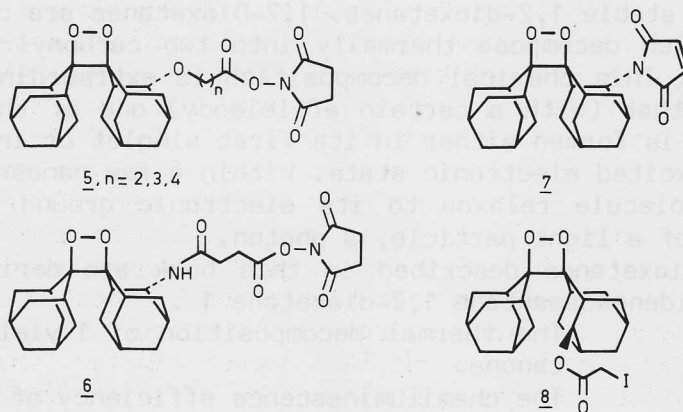
1,2-dioxetane **1**. This term distinguishes compounds based on **1** from the presently known chemiluminescent labels, which are compounds that emit light only upon reaction with an oxidizing agent.

Functionalized adamantylideneadamantanes are the precursors for the thermochemiluminescent labels. Chapter 2 describes the substitution reactions of 4-equatorial chloro- (**2**) and 4-equatorial bromoadamantylideneadamantane (**3**).



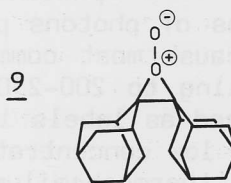
We found that a variety of specifically 4-equatorially substituted adamantylideneadamantanes can be prepared, starting from **2** and **3**. Bromide **3** solvolyses much faster than **2**. In order to facilitate the solvolysis of **2**, the addition of silver salts was necessary. Solvolysis of **2** and **3** yields solely 4-equatorially substituted products. We explain both this stereospecificity and the relatively fast solvolysis of **3** by the existence of anchimeric assistance of the homoallylic double bond. This leads to the carbonium ion structure **4** as an intermediate in these reactions.

Chapter 3 describes the synthesis of a number of thermochemiluminescent labels, starting from the primary solvolysis products. These labels were designed to react with either amino groups (e.g. labels **5** and **6**) or thiol groups (e.g. labels **7** and **8**).



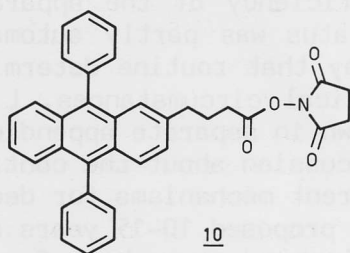
At certain points in the synthesis of **5** and **6**, the length of the spacer between the label and the target molecule can be varied. All labels are stable compounds, emitting light approximately as effectively as **1**.

The relative rates of photooxygenation of a series of 4-equatorially substituted adamantylideneadamantanes to their corresponding 1,2-dioxetanes yielded supporting evidence for a



perepoide intermediate **9** in these reactions.

In chapter 4 we report how the efficiency of chemiluminescence of a 1,2-dioxetane-labeled compound can be increased to a maximum of forty times the original, by means of radiationless energy transfer from the excited adamantanone product to an efficient fluorescer. For this purpose, we prepared a functionalized diphenylanthracene (DPA) derivative as a label for compounds containing an amino group. The amplifying label **10** exhibits fluorescence identical to that of DPA.



Bovine serum albumin (BSA), labeled with both a number of residues **6** and a number of residues **10** could be quantified in concentrations as low as  $10^{-17}$  moles per sample.

Furthermore, chapter 4 describes the results of a study concerning the behaviour of the donor-acceptor pair **6** and **10** in terms of Förster's

theory about the efficiency of energy transfer in relation to the distance between the donor and acceptor, in case both components are bound to the same protein molecule.

This chapter also introduces the concept of FATIMA (this being the acronym for fluorecence amplified thermochemiluminescence immunoassay). FATIMA is an immunoassay, in which the combination of the thermochemiluminescent labels and fluorescent (amplifying) labels is used. Theoretically, such assays can be extremely sensitive.

Chapter 5 describes the first results of some investigations in the field of application of the thermochemiluminescent labels. Iodoacetate **8** reacts fast and selectively with thiol groups of peptides and proteins, as was determined colorometrically. The N-hydroxysuccinimide esters **5** and **6** were found to react with amino groups of proteins, in such way that the labeled proteins remain immunologically active. The specific activity of the labels was unaltered upon conjugation to proteins. Proteins, labeled with **5** or **6** showed good linearity concerning the relation between the concentration of such proteins in a sample and the amount of light emitted by the sample. Dually labeled BSA (labeled with both **6** and **10**) showed similar linearity. The reproducibility of the quantification of small amounts of labeled proteins appeared to be dependent on both the concentration of the protein and the method of determination. A standard deviation of 3% was obtained (as an optimum) when samples were measured on a thin disk of polyimide "Kapton". A series of polymers was investigated for their feasibility to serve as a solid phase for FATIMA. We had to develop new materials for solid phase immunoassay, because for FATIMA, the polymer has to be thermally stable up to 250°C. We found the most promising candidates to be kapton, teflon, Whatmann glassfilter and glassslides. Subsequently, kapton and teflon were tested for their ability to bind proteins in a physical coating procedure

in such a way, that the immunological activity of these proteins is preserved. The feasibility of kapton and teflon as solid phase materials for immunoassay was proven by two enzyme immunoassays performed on these materials. The assay results on kapton surpassed the quality of identical assays, performed on a standard polystyrene matrix. Finally, chapter 5 shows briefly the first results of two types of FATIMA's for carcino embryonic antigen (CEA). These analytical determinations proved that thermochemiluminescent immunoassay is a real possibility.

Chapter 6 describes the thermochemiluminescence counter that we developed in our laboratory. The efficiency of the apparatus was determined to be 0.1%. The apparatus was partly automated by means of a computer, in such a way that routine determinations could be performed under identical circumstances. Listings of the computer programs are shown in separate appendices. Chapter 7, finally, starts with a discussion about the continuing controversy between the two different mechanisms for decomposition of 1,2-dioxetanes, that were proposed 10-15 years ago. Subsequently, we have listed the activation parameters for decomposition of 108 1,2-dioxetanes in four tables. We found a relation between the stability (in terms of  $\Delta G^\ddagger$  and  $\Delta H^\ddagger$ ) of alkyl substituted 1,2-dioxetanes and their molecular weight. Based on this relation, two hypotheses are formulated. According to these hypotheses, 1,2-dioxetanes decompose through a "concerted" mechanism in a torsional mode, in which the solvent plays an important stabilizing role by restricting the motion of torsion, depending on the size of the substituents that are attached to the four membered ring.

Using these hypotheses we can explain the stability of all but one aliphatic 1,2-dioxetanes in a quantitative manner. In this manner we can also rationalize the activation parameters of all other 1,2-dioxetanes in a semi-quantitative way.

Using two distinct methods of calculation we can either calculate or predict the activation parameter  $\Delta G^\ddagger$  for the thermal decomposition of a 1,2-dioxetane. Furthermore, we can now solve a number of problems (that were caused in part by the dominating role of the widely accepted diradical mechanism of decomposition) encountered by a number of workers in the area of 1,2-dioxetane research. In the appropriate stages of the book introductory surveys are given on the areas of chemiluminescence (paragraphs 1.1 and 1.2), applications of chemiluminescence (1.3), 1,2-dioxetanes (1.4), adamantylideneadamantanes (2.1), labels, used in analysis (3.1.1.), labeling techniques (3.1.2), Förster's theory concerning radiationless energy transfer (4.1), and present forms of immunoassay (5.2.1).