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Design and Synthesis of Chemiluminescent Probes for the Detection of Cholinesterase Activity

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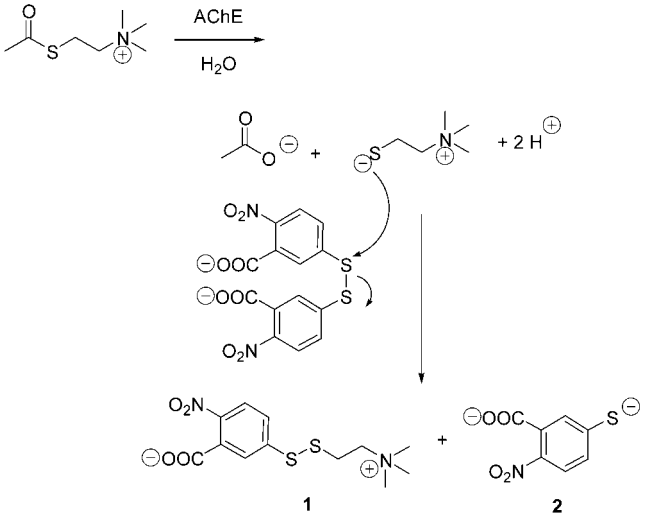
Abstract: Acetylcholinesterase is one of the most widely used and studied enzymes. Not only does this enzyme regulate neurotransmission (and thus play a key role in neurodegenerative processes) but it is also a prime target for pest control agents and warfare agents. Above all, due to its particularly high turnover rate, acetylcholinesterase is among the most efficient reporter enzymes yet described (for use as enzymatic tracer in immunoassays, for instance). However, its activity is detected through a colorimetric reagent, the Ellman reagent, which displays low detection limits and is often subject to background perturbations. In the course of our search for a more sensitive detection assay, we describe here a first-generation 1,2-dioxetane chemiluminescent probe, based on chemically induced electron exchange luminescence, which has an approximately 10 times lower detection limit than the Ellman colorimetric assay (2.5×10^{-19} mol for *Electrophorus electricus* AChE in its tetrameric form).

Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a well-known and thoroughly studied enzyme involved in the regulation of the concentration of neurotransmitter acetylcholine at cholinergic synapses.[1] Its particularly high catalytic potency makes it the ultimate candidate for use as an enzymatic tracer[2] (turnover, 64 000 s^{-1}, three times the turnover of horseradish peroxidase). However, since the first description of the Ellman reagent in the late 1950s, no analytical breakthrough for the detection of its catalytic activity has been described. The Ellman reagent is a buffered mixture of two products. First, acetylthiocholine iodide, a pseudosubstrate of AChE, whose rate of hydrolysis almost reaches that of acetylcholine itself. Second, 5,5′-dithio 2-nitrobenzoic acid, a chromogenic reagent. Once acetylthiocholine iodide has been hydrolyzed into thiocholine iodide and acetic acid, the liberated nucleophilic thiol adds to the disulfide, yielding disulfide **1** and yellow 5-mercapto 2-nitrobenzoic acid **2** ($\epsilon = 13\,600$ at 414 nm) (Scheme 1). Through colorimetric detection, the amount of detectable *Electrophorus electricus* AChE (in its tetrameric form) has been estimated to 1.85×10^{-18} mol.[3]

Scheme 1. Principle of AChE Detection with the Ellman Reagent



In the course of our search for a more sensitive detection assay, which could be used both in 96- and 384-well microtiter plates for immunoassays and for histochemical or immunohistochemical analyses, we focused our attention on a chemiluminescent reagent for the detection of AChE activity. The coupling of enzymatic reactions to chemiluminescence (or

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[||] Laboratoire de Synthèse Bioorganique.

(1) Massoulié, J.; Pezzementi, L.; Bon, S.; Krejci, E.; Vallette, F. M. *Prog. Neurobiol.* **1993**, *41*, 31−91.
(2) (a) Antoine, C.; Lellouche, J.-P.; Maclouf, J.;. Pradelles, P. *Biochim. Biophys. Acta* **1991**, *1075*, 162−168. (b) Pradelles, P.; Grassi, J.; Maclouf, J. *Anal. Chem.* **1985**, *57*, 1170−1173.
(3) Grassi, J. Ph.D. Dissertation, Paris VI University, Paris, France, 1990.

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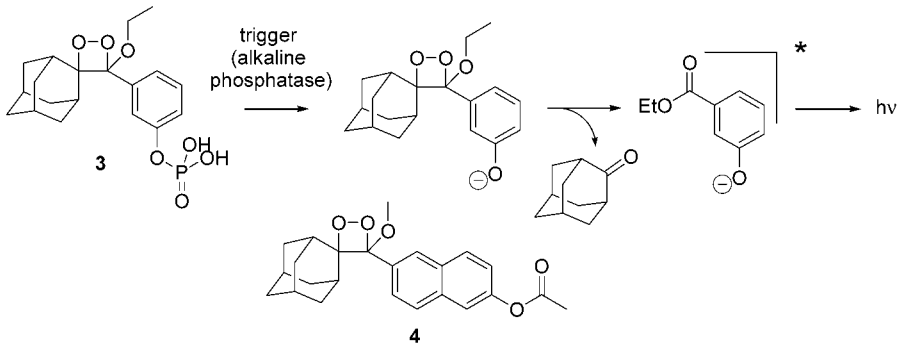
Scheme 2. Structures of the Chemiluminescent Probes

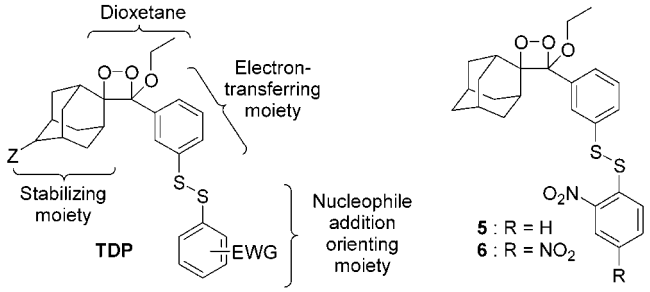


bioluminescence) phenomena allows detection of enzymes (or substrates, or inhibition) at very low concentrations.[4]

Three main chemiluminescent reactions have been used in bioanalytical chemistry:[5] oxidation of luminol catalyzed by enzymes (HRP, horseradish peroxidase, for instance) or metal complexes; peroxyoxalate chemiluminescent reaction;[5d,6] and 1,2-dioxetane or 1,2-dioxetanone ring opening through CIEEL (chemically induced electron exchange luminescence),[5c,7] whose exact mechanism is still the subject of many studies.[8] Luminol oxidation has already been used in two complex multistep multienzymatic reactions to detect AChE activity.[9,10] In our hands, and despite a few technical improvements,[11] these complicated multistep procedures proved unsuitable mainly on two grounds. First, they are very sensitive to enzymatic, metallic, or chemical contaminants. Second, sensitive detection is only possible in a two-step detection: first production of hydrogen peroxide, a relatively unstable and particularly reactive species, then detection of the H_2O_2 produced. Superposition of these two steps dramatically lowers the detection sensitivity.[11] Kinetic analysis is then hardly feasible, and only end-point measurements are of practical use.

By analogy with the chemiluminescent 1,2-dioxetane substrates **3** used for the detection of alkaline phosphatase,[12] we decided to evaluate this CIEEL with enzymatically triggered hydrolysis of 1,2-dioxetane ester **4** by AChE (Scheme 2). Use of this kind of substrate has already been described for the detection of the catalytic activity of carboxylesterases.[13] Unfortunately, the adamantyl moiety, required for the stability of the dioxetane moiety of **4**, proved too bulky to allow **4** to reach the AChE catalytic site, buried deep in a narrow hydrophobic gorge.[14]

Scheme 3. Structure of the Thiol Detection Chemiluminescent Probe



We thus designed an indirect chemiluminescent disulfide probe, **TDP**, as depicted in Scheme 3. The structure of this reagent is based on the same reaction cascade as for the Ellman reagent. AChE hydrolysis of acetylthiocholine iodide leads to thiocholine iodide. Its nucleophilic thiol moiety should then break the disulfide bond of the chemiluminescent probe to yield an aromatic thiophenolate, whose structure is closely related to that of the phenolate produced by hydrolysis of phosphate **3**. Through cleavage of the 1,2-dioxetane ring, CIEEL should then lead to an excited *m*-mercaptobenzoate anion, whose decay to the ground state should provide light, as for the corresponding *m*-oxobenzoate anion.

Besides the disulfide bond, the structure of the chemiluminescent reagent should be divided into four parts:

(1) the 1,2 dioxetane ring, whose decay will give rise to luminescence;

(2) an adamantyl moiety to stabilize the 1,2-dioxetane ring [if solubility problems are encountered, an additive charged group ($Z = CO_2^-$, SO_3^-) or halogen atom ($Z = Cl$) could be introduced on the adamantyl ring];

(3) an aromatic electron-transferring moiety, which, once the disulfide bond is broken and the thiophenolate formed, will trigger the 1,2-dioxetane ring opening, via electron transfer as in phenolate **3**; and

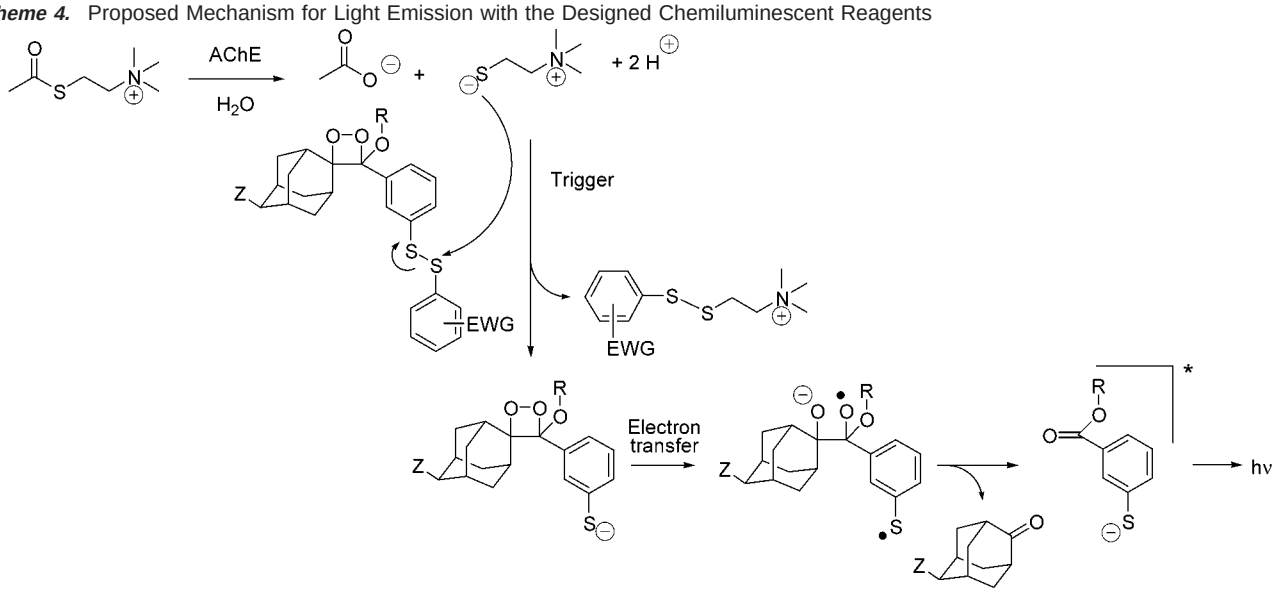
(4) an orienting aromatic moiety, substituted with electron-withdrawing groups (EWG), to remove electrons from its directly attached sulfur atom, and so center the nucleophilic addition of thiocholine iodide on this sulfur atom, as depicted in Scheme 4.

Results and Discussion

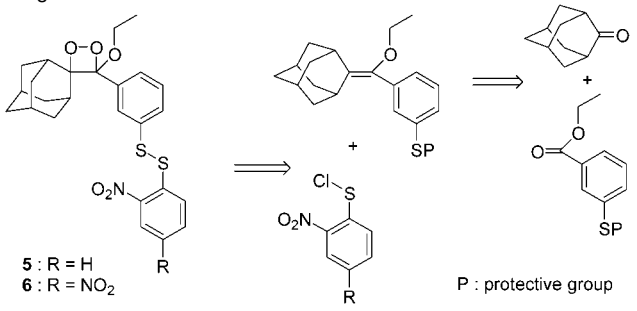
To evaluate this strategy, two simple disulfide reagents, **5** and **6**, were first synthesized. The retrosynthetic scheme for these

(4) Mayer, A.; Neuenhofer, S. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1044−1072.
(5) (a) Roda, A.; Pasini, P.; Guardigli, M.; Baraldini, M.; Musiani, M.; Mirasoli, M. *Fresenius' J. Anal. Chem.* **2000**, *366(6−7)*, 752−759. (b) for a general review on chemiluminescence mechanisms, see: Mc Capra, F. M. *Methods Enzymol.* **1999**, *305*, 1−47, and the whole vol. 305 for applications in enzymology. (c) Schuster, G. B. *Acc. Chem. Res.* **1979**, *12*, 366. (d) Garcia-Campana, A. M.; Baeyens, W. R. G. *Analysis* **2000**, *28*, 686−698.
(6) (a) Givens, R. S.; Schowen, R. L. *Chemiluminescence and photochemical reaction detection in chromatography*; Birks, J., Ed; VCH: Weinheim, 1989; pp 125−147. (b) Orosz, G.; Givens, R. S.; Schowen, R. L. *Crit. Rev. Anal. Chem.* **1996**, *26*, 1−27.
(7) Dixon, B. G.; Schuster, G. B. *J. Am. Chem. Soc.* **1981**, *103*, 3068.
(8) Adam, W.; Matsumoto, M. Trofimov, A. V. *J. Org. Chem.* **2000**, *65*, 2078−2082 and references therein.
(9) (a) Israel, M.; Lesbats, B. *J. Neurochem.* **1981**, *37*, 1475−1483. (b) Taylor, S. J.; Haggbald, J.; Greenfield, S. A. *Neurochem. Int.* **1989**, *15*, 199−205.
(10) Arakawa, H.; Mae da, M.; Tsuji, A. *Anal. Biochem.* **1991**, *199*, 238−242.
(11) Pecorella, K. Ph.D. Dissertation, Paris V University, Paris, France, **1995**.
(12) (a)Bronstein, I.; Edwards, B.; Voyta, J. C. *J. Biolumin. Chemilumin.* **1988**, *2*, 186. (b) Bronstein, I.; Voyta, J. C.; Thorpe, G. H. G.; Kricka, L. J.; Amstrong, G. *Clin. Chem.* **1989**, *35*, 1441−1146. (c) Edwards, B.; Sparks, A.; Voyta, J. C.; Strong, R.; Murphy, O.; Bronstein, I. *J. Org. Chem.* **1990**, *55*, 6225−6229.
(13) Schaap, P.; Handley, R. S.; Giri, B. P. *Tetrahedron Lett.* **1987**, *28*, 935−938
(14) Harel, M.; Schalk, I.; Ehret-Sabatier, L.; Bouet, F.; Goeldner, M.; Hirth, C.; Axelsen, P.; Silman, I.; Sussman, J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9031.

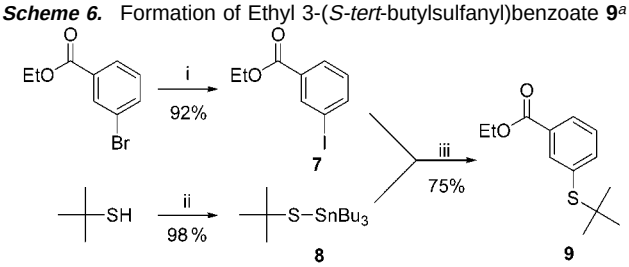
Scheme 4. Proposed Mechanism for Light Emission with the Designed Chemiluminescent Reagents



Scheme 5. Retrosynthetic Scheme for Chemiluminescent Reagents **5** and **6**

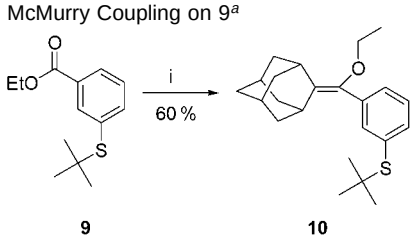


Scheme 6. Formation of Ethyl 3-(*S-tert*-butylsulfanyl)benzoate **9**[a]



[a] (i) KI (15 equiv), CuI (5 equiv), HPMT, 155 °C, 3 days; (ii) Bu_3SnCl, Et_3N, CCl_4, 18 h; (iii) $Pd(PPh_3)_4$, toluene, 110 °C, 48 h.

Scheme 7. McMurry Coupling on 9[a]



[a] (i) $TiCl_3$ (10 equiv), $LiAlH_4$ (5equiv), Et_3N (6 equiv), adamantanone, THF, 70 °C, 20 h.

two reagents is based on two observations. First, the 1,2-dioxetane ring is extremely sensitive and has to be introduced at the very last step of the synthesis, through mild and carefully controlled oxidation, to prevent co-oxidation of the sulfur atoms. Second, the adamantyl enol ether is usually introduced via intermolecular McMurry condensation of adamantanone on the corresponding ethylbenzoate.[12,13] McMurry reaction conditions are harshly reducing and incompatible with a disulfide bond. The following retrosynthetic scheme (Scheme 5) was designed for chemiluminescent probes **5** and **6**.

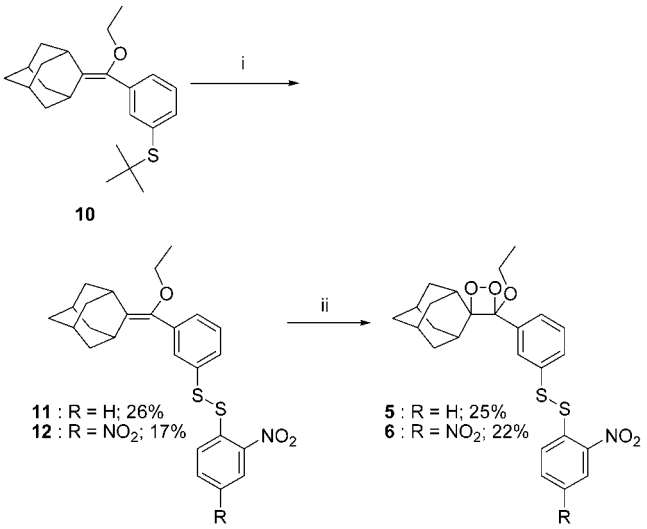
After unfruitful attempts to perform the McMurry coupling with unprotected or conventional *S*-protected thiophenols (as methyl or benzyl thioether, acetic or benzoic thioester), we chose to introduce a *tert*-butyl group on the thiophenol moiety. Ethyl 3-(*S-tert*-butylsulfanyl)benzoate **9** was formed in fairly good yields through a Stille-like coupling[15] of tributyltin *tert*-butylsulfanyl **8** on the aromatic iodide **7** (obtained by transhalogenation of commercially available ethyl 3-bromobenzoate)[16] (Scheme 6).

Unlike our other attempts on thiophenols, thioesters, or disulfides, McMurry coupling of benzoate **9** on adamantanone under standard reaction conditions gave satisfactory yields of enol ether **10** (Scheme 7).

Unsymmetrically substituted disulfide bond formation directly from *S-tert*-butylsulfanyl has already been described using activated sulfenyl chlorides on protected cysteine.[17] Yet coupling of 2-nitrophenyl sulfenyl chloride on *S-tert*-butylsulfanyl **10** using acetic acid as solvent, as described, ended in complete hydrolysis of the enol ether moiety. We thus turned to milder reaction conditions and carefully monitored the reaction. We found that when the substitution was performed in THF and when the course of the reaction was stopped after ca. one-third of the enol ether **10** was consumed, an acceptable yield of 26% of disulfide **1** could be isolated (75% yield based on recovered starting material, which could be recycled) (Scheme 8). If the substitution reaction was performed in CH_2Cl_2, better substitution yields were obtained, but with a mixture of enol ether **11** and corresponding ketone. Altogether, recycling the remaining starting product gave superior final yields of **11**. The same reaction procedure was followed with 2,4-dinitrophenylsulfuryl chloride and allowed isolation of disulfide **12** in 17% yield (57% yield based on recovered starting material).

(15) Terada, M.; Migata, T.; Sano, H.; Ogata, T.; Kosagi, M. *Bull. Chem. Soc. Jpn* **1985**, *58*, 3657.
(16) Suzuki, H.; Kondo, A.; Ogawa, T. *Chem. Lett.* **1985**, 411−412.
(17) Pastuszak, J. J.; Chimiak, A. *J. Org. Chem.* **1981**, *46*, 1868−1873.

Scheme 8. Final Steps of the Synthesis of 1,2-Dioxetanes **5** and **6**[a]



[a] (i) R = H, 2-nitrophenyl sulfuryl chloride, THF, 2 h; R = NO_2, 2,4-dinitrophenyl sulfuryl chloride, THF, 2 h; (ii) $(PhO)_3P$, O_3, −78 to 20 °C, CH_2Cl_2.

The last step of the synthesis of 1,2-dioxetanes **5** and **6** was the careful [2 + 2] cycloaddition of singlet oxygen $^1O_2$ on enol ether. In view of the great sensitivity of the disulfide bond to highly oxidative oxygenated species, oxygen bubbling under UV light irradiation seemed unacceptable. To monitor precisely the amount of $^1O_2$ delivered, we decided to use triphenyl phosphite ozonide. This pentacoordinated phosphorane is formed at −78 °C by the ozone-mediated oxidation of triphenyl phosphite. Upon heating from −78 °C to room temperature, its thermal decomposition leads to the formation of triphenyl phosphate and $^1O_2$.[18] Using this mild and controlled singlet oxygen source, fragile 1,2-dioxetane chemiluminescent probes **5** and **6** could be synthesized in moderate yields. No trace of byproducts displaying oxidation at any of the two sulfur atoms was detected. Interestingly, both **5** and **6** displayed surprisingly high stability and could be isolated, after careful silica gel chromatography, in 25 and 22% yield, respectively, together with recovered starting material (61% for **11** and 67% for **12**), which could be recycled.

Chemiluminescent probes **5** and **6** were poorly soluble in buffered aqueous media. Stock 1.2 mM solutions in either 58/42 acetone/water mixture (**5**) or ethyl alcohol (**6**) were used. Under these conditions, both products proved perfectly stable for over 6 months at −20 °C. First experiments were performed in 0.1 M phosphate buffer, pH 7.4 with dioxetane **6**. A large excess (10 equiv) of thiocholine iodide was added to this first-generation chemiluminescent probe in order to test the rate of the disulfide bond breaking by thiols. The reaction was monitored by measuring the appearance of 2,4-dinitrothiophenol species via their characteristic UV absorption band at ca. 400 nm. Once thiocholine iodide was added, within seconds the UV spectrum dramatically changed. The absorption band at 314 nm disappeared, and a strong absorption band was observed at 402 nm, showing addition of the nucleophilic thiol to the disulfide and release of the 2,4-dinitrothiophenolate ion.

Regioselectivity of this disulfide bond cleavage and the capacity of dioxetanes **6** to emit light when subject to a sulfur atom nucleophilic addition were then tested under chemiluminescence measuring conditions, i.e., in the presence of the chemiluminescence enhancer Sapphire in its appropriate buffer (20 mM Tris-HCl, 1 mM $MgCl_2$, pH 9.5).[19] Increasing amounts (0−2 equiv) of thiocholine iodide (in 10^{-2} M phosphate buffer, pH 7.4) were added to a 40 μM solution of dioxetanes **6** and Sapphire enhancer in the enhancer buffer. UV absorption at 414 nm was measured after overnight reaction at room temperature (Figure 1a). Increase in absorption at 414 nm, reflecting an increase in the amount of 2,4-dinitrothiophenolate, only occurred once 1 equiv of thiocholine iodide had been added. Light emission was then measured. In sharp contrast to the UV results, right after addition of 0.1 equiv of thiocholine iodide to a buffered solution of **6**, flash light emission was observed (Figure 1b). The intensity of the light emission increased with the amount of thiocholine iodide and reached a maximum intensity when 0.8 equiv of thiol was added. The light could be quantified either as the highest intensity reached (usually 3−10 s after addition of thiocholine iodide) or, more reliably, as the total amount of light emitted in a given time lapse (arbitrarily chosen as 800 s). Interestingly, when the same reaction was performed with 1,2-dioxetane **5**, light emission was detected only after addition of over 1 equiv of thiocholine iodide (Figure 1b).

Both experiments are in complete agreement with the following mechanisms described in Scheme 9: for 1,2-dioxetane **5**, thiocholine iodide first adds to the sulfur atom borne by the electron-transferring moiety, yielding mixed disulfide **12** and *o*-nitrothiophenol. Addition of a second thiocholine iodide equivalent yields symmetric disulfide **13** and chemiluminescent species **14**. For 1,2-dioxetane **6**, the additive nitro group sufficiently activates the sulfur atom borne by the nucleophilic addition orienting moiety, so that chemiluminescent species **14** is directly produced by the addition of a first thiocholine iodide molecule. Addition of a second equivalent of thiocholine iodide yields 2,4-dinitrothiophenol (λ_{max} = 402 nm) and symmetric disulfide **13**. Such a surprisingly large difference between the two 1,2-dioxetanes **5** and **6** is striking, pinpointing the influence of the electron-withdrawing groups borne by the nucleophilic addition-orienting moiety. Yet, an expected ordering of the four thio-leaving groups was observed, with 2,4-dinitrothiophenyl ≫ 2-nitrothiophenyl ≫ thiocoline > thiophenyldioxetane.

The luminescence of **6** was then measured in the presence of AChE and acetylthiocholine iodide. Sapphire and its buffer both inhibited AChE, so only endpoint measurements were possible. To estimate the detection limits of this chemiluminescent probe, acetylthiocholine iodide was incubated for 30 min with various amounts of *E. electricus* AChE [in its tetrameric form, in EIA buffer, namely 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 10^{-3} M EDTA, 0.1% bovine serum albumin, and

(18) (a) Murray, R. W.; Kaplan, M. L. *J. Am. Chem. Soc*. **1969**, *91*, 5358−5364. (b) Stephenson, L. M.; Zielinski, M. B. *J. Am. Chem. Soc*. **1982**, *104*, 5819−5820.

(19) Aqueous environments rapidly reduce the chemiluminescent signal intensity of 1,2-dioxetane substrates by water-induced quenching. Chemiluminescence enhancers (quaternary ammonium with fluorescent relay either as micelles or polymers) increase the emission efficiency by partitioning the water from the site of chemiluminescent signal production. Cf. (a) Kohne, D. E. US patent WO9705209, 1997; *Chem. Abstr*. **1997**, *126*, 222603. (b) Akhaven-Tafti, H.; Arghavani, Z.; Eickholt, R.; Rashid, K. US patent WO9615122, 1996; *Chem. Abstr*. **1996**, *125*, 479234. (c) Schaap, A. P.; Akhavan-Tafti, H. US patent WO9007511, 1990; *Chem. Abstr*. **1991**, *114*, 181726.

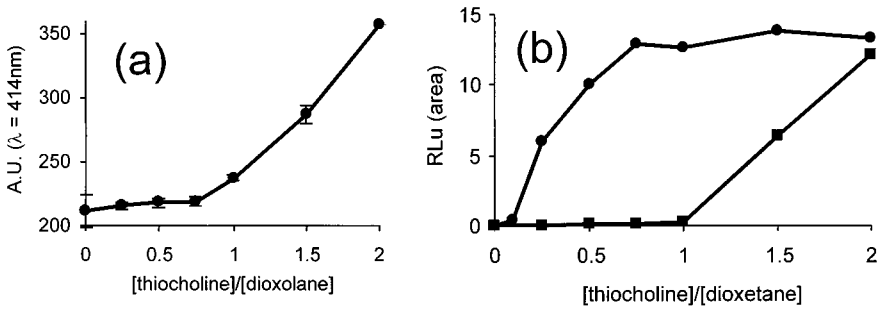
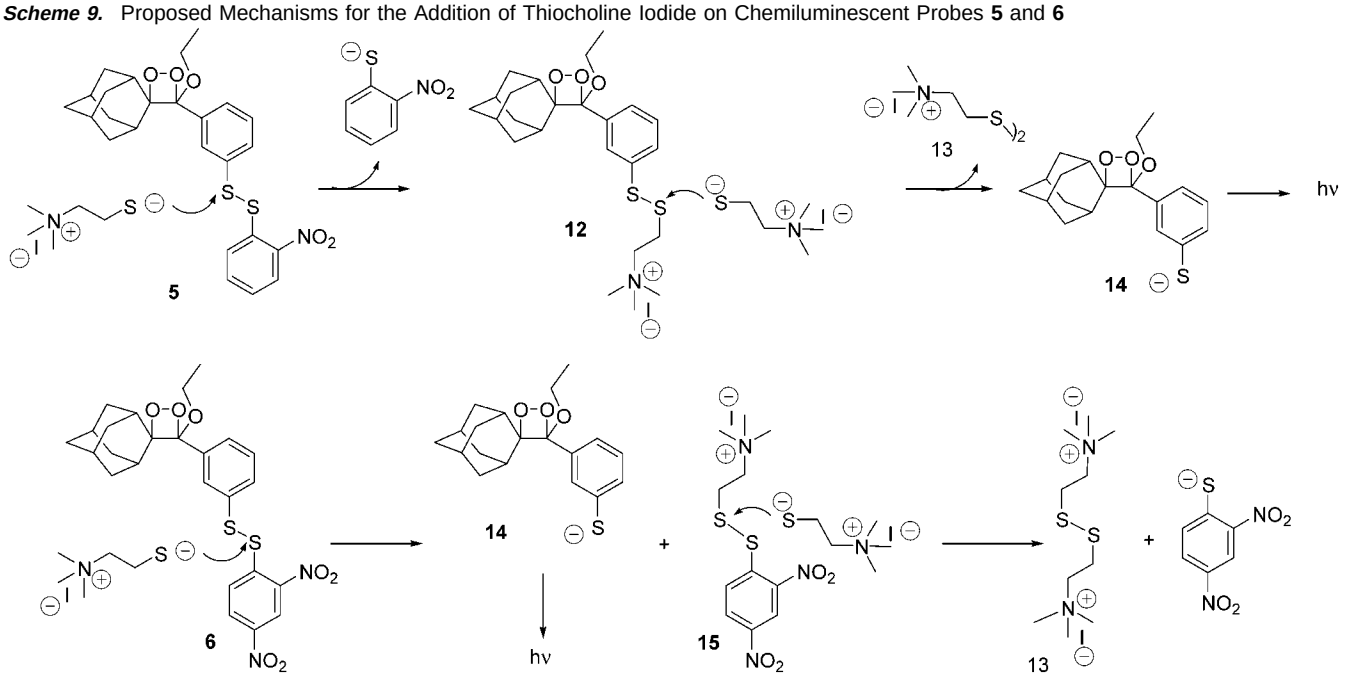


Figure 1. (a) Effect of increasing amounts of thiocholine iodide on the absorbance at 414 nm when incubated overnight with dioxetane **6** (●). The addition of thiocholine iodide (10 μM to 80 μM) on **6** (40 μM) was carried out at 25 °C pH 9.0, in 3.3×10^{-3} M phosphate, 13.3×10^{-3} M Tris-HCl, and in the presence of 6.6×10^{-4} M $MgCl_2$ and 1/3 v/v enhancer Sapphire. (b) Effect of increasing amounts of thiocholine iodide on the light emission in the presence of dioxetane **5** (■) or **6** (●). The reaction of thiocholine iodide (10−80 μM) with **5** or **6** (40 μM) was carried out at 25 °C pH 9.0, in 3.3×10^{-3} M phosphate, 13.3×10^{-3} M Tris-HCl, and in the presence of 6.6×10^{-4} M $MgCl_2$ and 1/3 v/v enhancer Sapphire.

Scheme 9. Proposed Mechanisms for the Addition of Thiocholine Iodide on Chemiluminescent Probes **5** and **6**



0.01% sodium azide], and the amount of light emitted was measured. As depicted in Figure 2, the amount of light emitted can be directly related to the AChE concentration.

The background light emission is due to the uncatalyzed hydrolysis of acetylthiocholine iodide, so a compromise has to be found between (i) an optimal concentration of acetylthiocholine iodide, to be as close as possible to the enzymatic v_{max}, and (ii) the uncatalyzed hydrolysis rate of acetylthiocholine iodide, directly related to its initial concentration. Under optimized enzymatic reaction conditions, the detection limit (defined as the background reaction + 3SD) could be estimated to be 0.1 fM, which, under the reaction conditions used, corresponds to 2.5×10^{-18} mol of enzyme (ca. the same detection limit as for the colorimetric reaction).

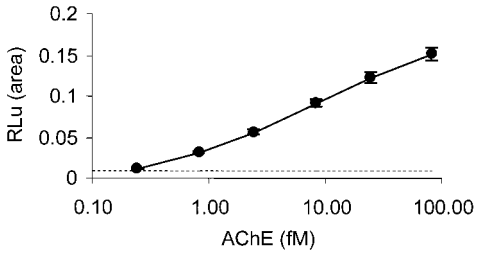


Figure 2. Emission of light after 30-min incubation of acetylthiocholine iodide (0.75 mM) and various amounts of *E. electricus* AChE (0.25−83.3 fM). The enzymatic reaction was performed at 25 °C in 2.5×10^{-2} M phosphate buffer pH 7.4, containing 0.04 M NaCl, 2.5×10^{-4} M EDTA, 0.025% bovine serum albumin, and 0.0025% sodium azide. Light measurement was performed with 40 μM 1,2-dioxetane **6**, at 25 °C pH 9.0, in 3.3×10^{-3} M phosphate, 13.3×10^{-3} M Tris-HCl, and in the presence of 6.6×10^{-4} M $MgCl_2$ and 1/3 v/v enhancer Sapphire. Amount of light emitted was recorded at 800 s. The dotted line corresponds to the background signal observed without enzyme.

The first way to increase the sensitivity of the assay would be to increase the enzymatic reaction time, once we have checked, as depicted in Figure 3, that the amount of light emitted is proportional to the enzymatic reaction time.

Since immunoassays need short staining times, we turned to another parameter which could lower the detection limit: the use of a stronger luminescence enhancer. Interestingly, when Sapphire II enhancer [poly(vinylbenzyltributylammonium chloride)] was used, a new phenomenon was observed: flash luminescence was replaced by glow luminescence, with light production lasting for minutes. Once again, the sensitivity of the assay was directly related to the background acetylthiocholine iodide hydrolysis. To minimize this limitation, the amount of light emitted in a two-minute period was measured once glow

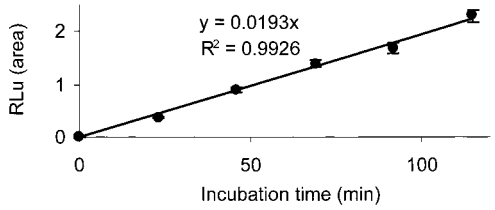


Figure 3. Influence of the time of incubation of acetylthiocholine iodide (0.75 mM) and AChE (0.25 fM) on the luminescence signal. Enzymatic reaction was performed at 25 °C, in 2.5×10^{-2} M phosphate buffer pH 7.4, containing 0.04 M NaCl, 2.5×10^{-4} M EDTA, 0.025% bovine serum albumin, and 0.0025% sodium azide for increasing times (23−115 min) before light recording. Light was measured with 40 μM 1,2-dioxetane **6**, at 25 °C pH 9.0, in 3.3×10^{-3} M phosphate, 13.3×10^{-3} M Tris-HCl, and in the presence of 6.6×10^{-4} M $MgCl_2$ and 1/3 v/v enhancer Sapphire. The light emitted was recorded at 720 s (12 min).

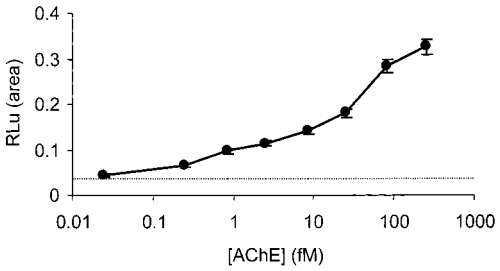


Figure 4. Light emission after 45-min incubation of acetylthiocholine iodide (1.25 mM) and various amounts of *E. electricus* AChE (0.025−250 fM). The enzymatic reaction was performed at 25 °C, in 2.5×10^{-2} M phosphate buffer pH 7.4, containing 0.04 M NaCl, 2.5×10^{-4} M EDTA, 0.025% bovine serum albumin, and 0.0025% sodium azide. Light was measured with 80 μM 1,2-dioxetane **6**, at 25 °C pH 9.0, in 3.3×10^{-3} M phosphate, 13.3×10^{-3} M Tris-HCl, and in the presence of 6.6×10^{-4} M $MgCl_2$ and 1/3 v/v enhancer Sapphire II. The light emitted during 2 min was recorded after a 2-min delay. Dotted line corresponds to the background signal observed without enzyme.

luminescence started. Under optimized conditions (Figure 4), the detection limit was estimated as 0.01 fM, which corresponds, under the reaction conditions, to 2.5×10^{-19} mol.

Conclusion

In conclusion, we have synthesized a new chemiluminescent 1,2-dioxetane probe **6** for the detection of AChE activity. Testing this first-generation probe for use in AChE-based immunoassays, in microtiter plates, we have been able to reach a 0.01 fM detection limit, ca. 10 times lower than with the colorimetric assays. We have shown, for the first time, that not only *m*-oxybenzoate anions but also *m*-mercaptobenzoate anions can be used in intramolecular CIEEL systems. The main drawback of our probe is the required use of a chemiluminescent enhancer that fully inhibits AChE, preventing any continuous light measurements. These encouraging first results have prompted us to synthesize new chemiluminescent probes containing either tethered fluorescers in their very structure, to have an intramolecular fluorescence/luminescence relay,[20] or an O−S bond instead of the disulfide bond, to enhance the luminescence quantum yield.

Experimental Section

Synthesis. General Remarks. Reagents were from Aldrich. All solvents were distilled before use, and reactions were performed under N_2 atmosphere. All chromatography (flash) was performed with Merck silica gel 60 (0.02−0.04 mm). TLC was performed with fluorescent Merck F254 glass plates. NMR spectra were recorded on a Brucker AC-300 (300.15 MHz for ^{1}H NMR and 75.4 MHz for ^{13}C NMR). Chemical shifts (δ) are given in ppm and the coupling constant J is expressed in hertz. MS were obtained with a Finnigan-Mat 4600 quadrupole system.

3-Iodobenzoic Acid Ethyl Ester (7). Ethyl 3-bromobenzoate **(**1.5 mL, 9.37 mmol) was added to a suspension of 23.3 g (140 mmol) of KI and 8.92 g (47 mmol) of CuI in 56 mL of dry HMPA. The reaction mixture was heated to 155 °C for 3 days. After cooling to room temperature, the reaction mixture was added to 200 mL of brine. The aqueous phase was washed thrice with diethyl ether. The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The crude reaction mixture was chromatographed on silica gel (hexane/ethyl acetate 95/5), yielding 2.38 g (92%) iodobenzoate **7** as a colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27 (t, J = 9.5 Hz, 3H), 4.24 (q, J = 9.5 Hz, 2H), 7.01 (t, J = 8.0 Hz, 1H), 7.70 (dt, J = 8.0 and 2.5 Hz, 1H), 7.85 (dt, J = 8.0 and 1.5 Hz, 1H), 8.23 (t, J = 1.5 Hz, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 14.35, 61.26, 93.87, 128.62, 130.05, 132.28, 138.25, 141.51, 164.72. MS (CI, NH_3): m/z 294 (MH^+), 311 (MNH_4^+).

(*tert*-Butylsulfanyl)tributyltin (8). Under argon atmosphere, 4.1 mL (15.1 mmol) of tributyltin chloride was added to a mixture of 1.70 mL (15.1 mmol) of *tert*-butylthiol and 2.53 mL (18.1 mmol) of triethylamine in 100 mL of dry CCl_4. After overnight agitation at room temperature, the reaction mixture was filtered on a Celite pad and washed successively with acetic acid (100 mL of a 5% v/v aqueous solution) and water (100 mL). The organic phase was dried over $MgSO_4$, and the solvents were evaporated under vacuum. The crude reaction mixture (5.7 g, 98% yield of **18** as a white solid) proved sufficiently pure to be used as such for the next step.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.76 (bt, $^3J_{H-H}$ = 7.0 Hz, 9H), 0.97−1.02 (bdd, $^3J_{H-H}$ = 7.0 and 8.0 Hz, 6H), 1.12−1.23 (bq, $^3J_{H-H}$ = 7.0 Hz, 6H), 1.28 (bs, 9H), 1.37−1.49 (m, 6H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 13.51 (3C), 14.31 (1s + 2d, $^1J_{Sn-C}$ = 310 and 330 Hz, 3C), 26.97 (1s + 2d, $^2J_{Sn-C}$ = 60 and 63 Hz, 3C), 28.64 (3C), 36.60 (3C), 43.11.

3-(*tert*-Butylsulfanyl)benzoic Acid Ethyl Ester (9). In a glovebox, purged under N_2, 83.7 mg (0.072 mmol) of $Pd(PPh_3)_4$ was added to a solution of ethyl 3-iodobenzoate **7** (200 mg, 0.72 mmol) in 20 mL of dry toluene. To this bright yellow solution was added a solution of 302 mg of (*tert*-butylsulfanyl)tributyl tin **8** dissolved in 5 mL of toluene. The reaction mixture was then refluxed for 48 h under an argon atmosphere. After cooling to room temperature, 50 mL of diethyl ether and 50 mL of a 10% aqueous KF solution were added. The aqueous phase was extracted thrice with diethyl ether (100 mL). The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The crude reaction mixture was then chromatographed on silica gel (hexane/ethyl acetate 95/5), yielding 128.5 mg (75%) of *tert*-butylsulfanyl **9** as a pale yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.30 (s, 9H), 1.40 (t, J = 9.5 Hz, 3H), 4.39 (q, J = 9.5 Hz, 2H), 7.33 (t, J = 7.5 Hz, 1H), 7.64 (bd, J = 7.5 Hz, 1H), 8.04 (bd, J = 7.5 Hz, 1H), 8.21 (bs, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 14.41, 31.04(3C), 46.11, 61.23, 128.51, 129.86, 131.02, 133.42, 138.25, 141.68, 166.19. MS (CI, NH_3): m/z 256 (MNH_4^+).

2-[1-(3-(*tert*-Butylsulfanyl)phenyl)-1-ethoxymethylene]tricyclo-[3.3.1.1^{3,7}]decane (10). In a glovebox, under N_2 atmosphere, 14.1 mL of $LiAlH_4$ (1 M solution in ether, 14.1 mmol) was added to a cooled (0 °C) suspension of 4.36 g of $TiCl_3$ (28.3 mmol) in 35 mL of dried THF. After 10 min at 0 °C, 2.37 mL of triethylamine (16.9 mmol) was added, and the suspension was refluxed for 1 h. A mixture of 532 mg of adamantanone (3.53 mmol) and 675 μL of ethyl benzoate **9** (2.83 mmol) in 8 mL of THF was then added dropwise for 2.5 h. The reaction mixture was refluxed overnight, cooled to room temperature, and

(20) (a) Schapp, A. P.; Akhavan-Tafti, H. US Patent, US 5616729, 1997; *Chem. Abstr.* **1997**, *126*, 317373. (b) de Silva, R. K.PhD Dissertation, Wayne State University, Detroit, MI, 1989.

partitioned between diethyl ether (200 mL) and water (200 mL). The aqueous phase was extracted thrice with diethyl ether (200 mL), and the combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The crude reaction mixture was chromatographed on silica gel (hexane/ethyl acetate 98/2), yielding 604 mg (60%) of McMurry condensation enol ether **10** as a colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.15 (t, J = 9.0 Hz, 3H), 1.31 (s, 9H), 1.60−1.99 (m, 12H), 2.63 (bs, 1H), 3.29 (bs (1H), 3.47 (q, J = 9.0 Hz, 2H), 7.31−7.37 (m, 2H), 7.44 (bd, J = 7.5 Hz, 1H), 7.51 (bs, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 15.19, 28.48 (2C), 30.46, 31.06 (3C), 32.53, 37.32, 39.06 (2C), 39.32 (2C), 46.02, 64.74, 128.02, 128.20, 129.40, 129.67, 132.31, 135.14, 136.39, 138.53. MS (CI, NH_3): m/z 357 (MH^+).

2-{Ethoxy[3-(2-nitrophenyldisulfanyl)phenyl]methylene}tricyclo-[3.3.1.1^{3,7}]decane (11). 2-Nitrobenzenesulfenyl chloride (65 mg, 0.34 mmol) was added to a solution of 116 mg (0.325 mmol) of enol ether **10** in 3 mL of dry THF. After 2 h at room temperature, the solvent was removed under vacuum. The crude reaction mixture was chromatographed on silica gel (hexane/ethyl acetate/Et_3N 95/5/0.1), yielding 75 mg (65%) recovered starting material **10** and then 39 mg (26%) disulfide **11** as a colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.11 (t, J = 9.0 Hz, 3H), 1.66−1.94 (m, 12H), 2.51 (bs, 1H), 3.23 (bs, 1H), 3.42 (q, J = 9.0 Hz, 2H), 7.21−7.41 (m, 5H), 7.63 (td, J = 1.0 and 7.5 Hz, 1H), 8.15 (dd, J = 1.0 and 8.5 Hz 1H), 8.31 (dd, J = 1.0 and 8.5 Hz, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 15.19, 28.30 (2C), 30.55, 32.44, 37.19, 39.0 (2C), 39.23 (2C), 65.0, 126.21, 126.50, 127.50, 127.93, 128.44, 129.06, 133.58, 134.24, 137.20, 137.40. MS (CI, NH_3): m/z 454 (MH^+), 471 (MNH_4^+).

2-{Ethoxy[3-(2,4-dinitrophenyldisulfanyl)phenyl]methylene}-tricyclo[3.3.1.1^{3,7}]decane (12). 2,4-Dinitrobenzenesulfenyl chloride (625 mg, 2.66 mmol) was added to a solution of 949 mg (2.66 mmol) of enol ether **10** in 7 mL of dry THF. After 2 h at room temperature, the solvent was removed under vacuum. The crude reaction mixture was chromatographed on silica gel (hexane/ethyl acetate/Et_3N 95/5/0.1), yielding 666 mg (70%) of recovered starting material **10** and then 225 mg (17%) disulfide **12** as a pale yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.12 (t, J = 9.0 Hz, 3H), 1.70−1.96 (m, 12H), 2.53 (bs, 1H), 3.24 (bs, 1H), 3.42 (q, J = 9.0 Hz, 2H), 7.24−7.39 (m, 3H), 7.43 (bs, 1H), 8.44 (bs, 2H), 9.13 (t, J = 1.0 Hz, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 15.20, 28.28 (2C), 30.59, 32.50, 37.15, 38.99 (2C), 39.32 (2C), 65.11, 121.65, 127.15, 127.57, 128.69, 128.97, 129.36 (2C), 133.47, 134.05, 137.79, 140.84, 145.90. MS (CI, NH_3): m/z 499 (MH^+), 516 (MNH_4^+).

4-Ethoxy-4-[[3-(2-nitrophenyldisulfanyl)phenyl]spiro(1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}]decane) (5). Ozone was gently bubbled in a cooled (−78 °C) solution of triphenyl phosphite (32.7 μL, 0.12 mmol) in 8 mL of dry CH_2Cl_2 until the appearance of a light blue coloration. The reaction medium was then purged with argon until the blue coloration disappeared. Enol ether **11** (37.7 mg; 0.083 mmol), dissolved in 2 mL of dry CH_2Cl_2 was added at −78 °C. The reaction mixture was gently brought to room temperature. Solvents were removed under reduced pressure, and the crude reaction product was chromatographed on silica gel (hexane/ethyl acetate 90/10), yielding first 23 mg (61%) of recovered starting material **11** and then 10 mg (25%) of dioxetane **5** as a pale yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.80−0.89 (m, 2H), 1.08−1.13 (m, 1H), 1.20 (t, J = 9.5 Hz, 3H), 1.38−1.43 (m, 1H), 1.55−1.84 (m, 8H), 1.95 (bs, 1H), 3.03 (bs, 1H), 3.15 (bquint, J = 9.5 Hz, 1H), 3.52 (quint, J = 9.5 Hz, 1H), 7.38 (bt, J = 8.5 Hz, 2H), 7.53 (bd, J = 9.0 Hz, 1H), 7.62 (bt, J = 7.5 Hz, 2H), 7.65−7.80 (m, 1H), 8.16 (bd, J = 7.5 Hz,-1H), 8.31 (bd, J = 8.5 Hz,1H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 15.42, 25.62, 25.77, 29.05, 29.44, 31.35, 31.53, 31.93, 32.65, 32.91, 34.48, 36.11, 39.01, 58.07, 95.05, 110.86, 125.88, 126.33, 127.06, 127.64, 128.47, 128.91, 131.28, 133.81, 135.29, 136.42, 136.81, 145.20. UV [λ, nm (ϵ), in EtOH]: 347 (2686). MS (CI, NH_3): m/z 503 (MNH_4^+).

4-Ethoxy-4-[[3-(2,4-dinitrophenyldisulfanyl)phenyl]spiro(1,2-dioxetane-3,2′-tricyclo[3.3.1.13,7]decane) (6). The same reaction procedure as for the synthesis of dioxetane **5** was performed with 49.3 μL of triphenyl phosphite (0.188 mmol) and 62.5 mg of enol ether **21** (0.126 mmol). Flash chromatography on silica gel (hexane/ethyl acetate 90/10) yielded first 42 mg (67%) of recovered starting material **12** and then 14.5 mg (22%) dioxetane **6** as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.82−0.91 (m, 2H), 1.11−1.15 (m, 1H), 1.23 (t, J = 9.5 Hz, 3H), 1.38−1.43 (m, 1H), 1.50−1.82 (m, 8H), 1.92 (bs, 1H), 3.05 (bs, 1H), 3.15 (bquint, J = 9.5 Hz, 1H), 3.53 (quint, J = 9.5 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H), 7.52 (d, J = 7.5 Hz, 1H), 7.60 (m, 1H), 7.80 (m, 1H), 8.43 (bs, 2H), 9.16 (bs, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 15.30, 25.99, 26.06, 29.78, 31.68, 31.91, 32.20, 33.08, 33.33, 34.86, 36.38, 58.51, 95.40, 111.03, 121.89, 127.54, 128.87, 129.32, 129.60, 134.31, 137.66, 145.27, 145.96. UV [λ, nm (ϵ), in EtOH]: 304 (9010). MS (CI, NH_3): m/z 548 (MNH_4^+).

Luminescence Assays. General. Enhancers and their buffers were from TROPIX (enhancer buffer: 20 mM Tris-HCl, 1 mM $MgCl_2$, pH 9.5). The G4 form of *E. electricus* AChE was purified and stored as described elsewhere.[21] EIA buffer comprised 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 10^{-3} M EDTA, 0.1% bovine serum albumin, and 0.01% sodium azide. Luminescence emission was recorded on a DYNATECH ML 3000 luminometer using Microlite II microtiter plates. UV spectra were recorded on an HP 8452 diode array spectrophotometer. Colorimetric assays were recorded on a LAB-SYSTEM multiscan bichromatic plate reader at 414 nm.

Direct Colorimetric and Luminescence Assay. In a microtiter plate were introduced successively 50 μL of a thiocholine iodide solution in 10^{-2} M phosphate buffer pH 7.4 (4.8×10^{-4} to 6×10^{-5} M), 50 mL of EIA buffer, 100 μL of Sapphire enhancer, and 100 μL of 1.2-dioxetane **5** or **6** (1.2×10^{-4} M in a 1/9 mixture of EtOH and enhancer buffer). For the colorimetric assay, the absorbance at 414 nM of each well was measured after overnight, room-temperature incubation. For the luminescence assay, light emission was measured directly after addition of 1,2-dioxetane **5** or **6**.

Enzymatic Assays. In a microtiter plate were introduced successively 75 μL of a thiocholine iodide solution in 10^{-2} M phosphate buffer pH 7.4 (1.5×10^{-3} M for the assays with enhancer Sapphire, 2×10^{-3} M for the assays with enhancer Sapphire II) and 25 mL of *E. electricus* AChE as its G4 isoform, purified and stored as described elsewhere[21] (dilution range from 10 to 0.001 Ellman units) in EIA buffer. After a 30-min enzymatic reaction, 100 μL of enhancer Sapphire or Sapphire II and 100 μL of 1,2-dioxetane **6** (1.2×10^{-4} M in a 1/9 mixture of EtOH and enhancer buffer for use with enhancer Sapphire, 2.4×10^{-4} M in a 2/8 mixture of EtOH and enhancer buffer for use with enhancer Sapphire II) were added. Light emission was measured directly after addition of 1,2-dioxetane **6**.

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Supporting Information Available: Spectra for compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) (a) Massoulié, J.; Bon, S. *Eur. J. Biochem*. **1976**, *68*, 531−539. (b) Pradelles, P.; Grassi, J.; Maclouf, J. *Anal. Chem.* **1985**, *57*, 1170−1173