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COMMUNICATION

A FRET Probe for the Detection of Alkylating Agents

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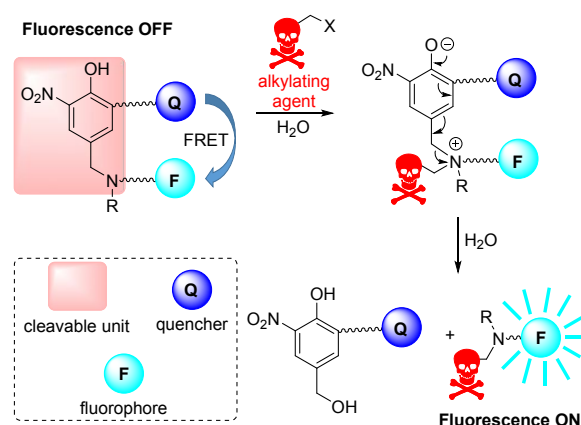
Abstract: Herein, we describe a new strategy for the detection of reactive alkylating agents such as alkyl halides. These toxic compounds react with a FRET-based profluorescent probe, triggering a self-immolative elimination at room temperature, thus leading to a fluorescent signal.

Alkylating agents are usually toxic due to their reactions with essential nucleophiles, in particular DNA bases, present in living organisms.¹ Such compounds are frequently employed in chemical synthesis, and hence in pharmaceutical industry. Sulfur mustard is an alkylating agent notably used during World War I as a chemical warfare agent.² On the other hand, nitrogen mustards are employed as anticancer reagents.³ Because of the inherent toxicity of alkylating agents, several methods have been designed for their detection. Most of these methods rely on their reaction with a nucleophilic agent, leading to an alkylated product that can be detected by color,⁴ luminescence,⁵ fluorescence,⁶ electrical sensing,⁷ or mass spectrometry.^{8,9}

In this report, we investigated a different approach based on a cleavage reaction provoked by the alkylation step to release a fluorescent compound (Scheme 1). For this purpose, we designed a probe equipped with a fluorophore and a quencher connected through a reactive spacer which, upon reaction with an alkylating agent, undergo self-immolation to release the fluorophore. Therefore, the presence of alkylating agents should induce an increase of the fluorescence signal. Very recently, a related approach using a phosphine-containing probe has been described.^{4f} Alkylation of this probe results in the formation of a phosphonium derivative, which upon basic treatment undergoes a β -elimination to release a nitroaniline acting as colored reporter.

To avoid the use of air-sensitive phosphine, we considered using a stable spacer that becomes a self-immolative spacer¹¹ after alkylation. For this purpose, we designed a phenol substituted in para

position with a nucleophilic tertiary amine that would react with the electrophilic alkylating agent, leading to a tetraalkylammonium cation. This intermediate would then undergo an elimination of amine leading to the corresponding quinone methide, which in the presence of water would be converted to a *p*-hydroxybenzyl alcohol.



Scheme 1. Schematic illustration of the FRET probe design.

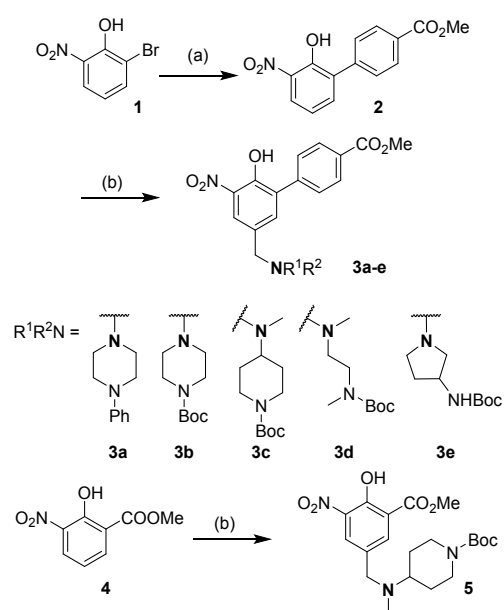
The formation of quinone methides from hydroxybenzyl-trialkylammonium salts has been well documented.¹² It can occur either by heating in an aqueous solution or under flash photolysis. On the other hand, *p*-aminobenzyl quaternary ammonium salts have been successfully used for the release of amine- and heteroaryl-containing drugs at room temperature.¹³ The formation of *o*-hydroxybenzyl alcohol from the corresponding ammonium salt was reported by Freccero, Rokita and colleagues to be much faster from a *p*-nitro substituted starting material, as compared with a substrate having electron-donating or less electron-withdrawing substituents in the para-position.^{12b} Moreover, self-immolative spacers constructed with *o*-nitro phenols, displaying low pKa values, are known to facilitate the 1-6 elimination reaction at room temperature due to the formation of the phenate ion in physiological conditions.¹⁴ Based on these data, we anticipated that it would be

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possible to generate quinone methides from *o*-nitro-*p*-hydroxybenzyl quaternary ammonium salts at room temperature and thus designed a probe containing an *o*-nitro-*p*-hydroxybenzyl amine moiety. The choice of the tertiary amine function of the probe is crucial; it should be sufficiently nucleophilic to allow fast alkylation and the corresponding ammonium group should be a good leaving group to facilitate the 1-6 elimination step of the self-immolation process.

The amine part of the probe was selected thanks to a series of preliminary assays starting from compounds **3a-e** and **5** (Scheme 2). These compounds contain protected carboxylic acid and amine groups that would make possible attachment of a fluorophore and a quencher.



Scheme 2. Synthesis of amines **3a-e** and **5**. Reagents and conditions: (a) 4-Methoxycarbonylphenylboronic acid, Pd(PPh₃)₄ (10 mol%), Na₂CO₃ (2 equiv), 4:1 THF/H₂O, 80 °C, 6 h, 46%; (b) (CH₂O)_n, R¹R²NH, Et₃Pr₂N, toluene, reflux, 15 h, 57- 86%.

Their synthesis started from commercially available 2-bromo-6-nitrophenol (**1**), which was converted to ester **2** via a Suzuki coupling with 4-methoxycarbonylphenylboronic acid, in 46% yield. Then amines **3a-e** were prepared from **2**, in 57-86% yield, by reaction with the iminium obtained from paraformaldehyde and the starting secondary amine in refluxing toluene, in the presence of diisopropylethylamine. Since both ortho positions of the phenol are occupied, the Mannich reaction leads only to the products alkylated at the para position. The same method allowed the preparation of amine **5** from commercially available methyl 2-hydroxy-3-nitrobenzoate (**4**), in 57% yield.

Preliminary alkylation studies of amines **3a-e**, **5** were conducted in CD₃CN containing 10% D₂O with 20 equiv. of methyl iodide, used as model alkylating agent. NMR spectra recorded after 2 h showed that a small amount of benzylic alcohol was formed from compounds **3a-e** (Fig. S1). On the contrary, no benzylic alcohol was detected from compound **5** (Fig. S2). A further LC-MS study confirmed that there was no benzylic alcohol detected even 4 h after adding methyl iodide

to a solution of **5**. These results indicated that the presence of an ester in the ortho-position relatively to the phenol was detrimental.

After several optimizations, we identified DMSO-d₆ containing 10% D₂O and addition of 1.2 equiv. NaOD as the best reaction conditions for NMR studies (Table S1). Thus, 5 mM solutions of amines **3a-e** were treated accordingly, with methyl iodide (20 equiv.). Their conversions to benzylic alcohol **6** were evaluated by ¹H-NMR after 2 h at room temperature (Table 1 and Fig. S3).

Table 1. Conversion of amines **3a-e** in DMSO-d₆ containing 10% D₂O, 2 h after the addition of methyl iodide (20 equiv.).

Entry	Compound	NR ¹ R ²	Conversion (%) ^a
1	3a		57
2	3b		48
3	3c		79
4	3d		66
5	3e		27

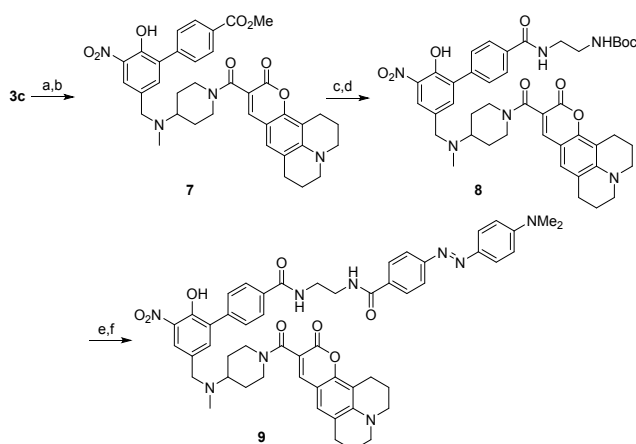
^a Reaction were carried out with [**3a-e**] = 5 mM. Conversion was calculated by NMR using mesitylene as internal standard.

It was found that the most efficient formation of the expected benzylic alcohol was obtained starting from amine **3c**. Kinetic studies indicated a linear reaction in the first 60 min to reach a plateau corresponding to ~80% conversion (Fig. S4 and S5). ¹H NMR analysis of **3c** after treatment for 72 min with methyl iodide (20 equiv.) showed that the reaction was quite clean with only limited amounts of by-products (Fig. S6).

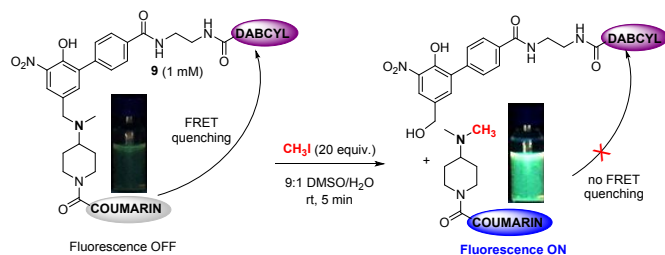
Once the amine was chosen, we synthesized probe **9**, as described in Scheme 5. The probe is derived from coumarin 343 and from the DABCYL quencher. DABCYL has been shown to efficiently quench the fluorescence of several fluorophores, including coumarin derivatives.¹⁵

Thus, the Boc group in compound **3c** was cleaved by treatment with 4 M HCl in dioxane and the obtained deprotected amine was directly converted to compound **7** by peptide coupling with coumarin 343 in 79% over two steps. Saponification of the ester using sodium hydroxide in methanol/water was immediately followed by coupling with *tert*-butyl (2-aminoethyl)carbamate, leading to compound **8** in 82% yield from **7**. Finally, the Boc protecting group of **8** was cleaved by exposure to HCl and the obtained amine was reacted with 4-[4(dimethylamino)phenylazo]benzoic acid *N*-succinimidyl ester in the presence of diisopropylethylamine, to afford the expected probe **9** in 85% yield over two steps (Scheme 4).

The ability of probe **9** to detect an alkylating agent was then evaluated. In the preliminary assay, methyl iodide (20 mM) was added to a solution of **9** (1 mM) in DMSO containing 10% H₂O. A rapid and significant enhancement of the fluorescence response under UV lamp (Ex@365 nm) was observed (Scheme 5).



Scheme 4. Synthesis of probe **9**. Reagents and conditions: (a) 4 M HCl in dioxane, CH₂Cl₂, rt, 6 h; (b) Coumarin 343, DCC, HOBT, Et₃Pr₂N, CH₂Cl₂, rt, 15 h, 79% over two steps; (c) NaOH, MeOH-H₂O, rt, 15 h, then HCl; (d) HBTU, HOBT, Et₃Pr₂N, rt, 45 min, then *tert*-butyl (2-aminoethyl)carbamate, rt, 48 h, 82% over two steps; (e) 4 M HCl in dioxane, CH₂Cl₂, rt, 6 h; (f) DABCYL-OSu, Et₃Pr₂N, CH₂Cl₂, rt, 15 h, 85% over two steps.



Scheme 5. Fluorescence response observed under UV lamp (Ex@365 nm) 5 min after the addition of methyl iodide (20 equiv.) to probe **9** (1 mM) in 9:1 DMSO/H₂O).

Encouraged by this result, we performed a series of experiments using lower concentrations of probe **9** and trying to avoid the presence of water and base. Indeed, it has been shown that highly active alkylating agents such as 2-chloroethyl ethyl sulfide (CEES), a reagent analogue to the warfare agent yperite, can be degraded in the presence of basic water.¹⁶ Fortunately, we found that only trace amounts of water in DMSO were needed for the reaction (Fig. S7) and that no base was required. To evaluate the sensitivity of our method, we added a range of methyl iodide concentrations (0–60 μM) to a solution of **9** (3 μM) in DMSO containing trace water. Fluorescence spectra were recorded after 2 h (Fig. 1 and S8).

The fluorescence intensity was found proportional to the concentration of methyl iodide, which indicates that probe **9** allows a quantitative detection of the alkylating agent. The limit of detection for methyl iodide was calculated as 2.9 μM, based on a literature method.^{6g, 17}

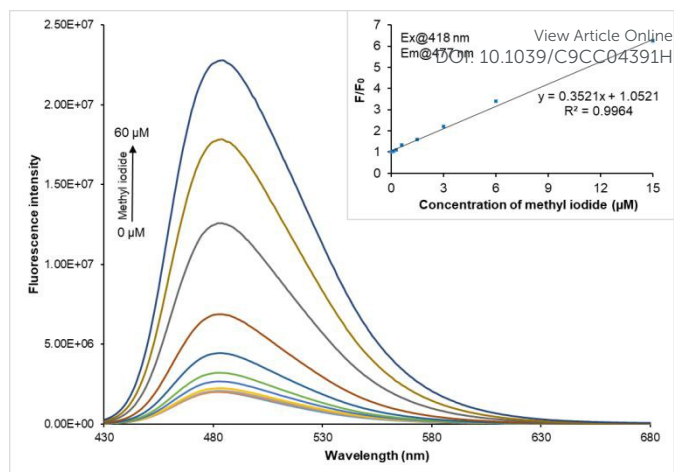


Fig. 1. Fluorescence spectra (Ex@418 nm) recorded 2 h after treatment of probe **9** (3 μM in DMSO containing trace water) with methyl iodide at various concentrations (0–60 μM). Inset: linear calibration curve upon the gradual addition of methyl iodide.

We then looked at the scope of the method and tested solutions of **9** (3 μM) in DMSO in the presence of several alkyl halides. As shown in Fig. 2 and S9, the addition of CEES, methyl bromoacetate, or methyl iodide resulted in more than 10 fold increase in fluorescence intensity. The nitrogen mustard tris(2-chloroethyl)amine showed less reactivity with probe **9** in comparison with CEES, while bis(2-chloroethyl) ether did not trigger any detectable fluorescence increase. The effect of 4-nitrobenzyl bromide on the increase in fluorescence intensity was comparable to the one observed with methyl iodide and slightly higher than the one due to benzyl bromide, whereas no fluorescence enhancement was observed with benzyl chloride. It is worth noting that probe **9** is more sensitive to allyl bromide than to propargyl bromide and that it reacts very weakly with ethyl bromide and (2-bromoethyl)benzene. In addition, epoxides such as epichlorohydrin and ethylene oxide also did not initiate any fluorescence enhancement. As expected, the reactivity of the probe with the alkylating agents is correlated with their electrophilicity. The alkylation of the probe with epoxides and chlorinated electrophiles is too slow to allow efficient release of the fluorophore under the tested conditions.

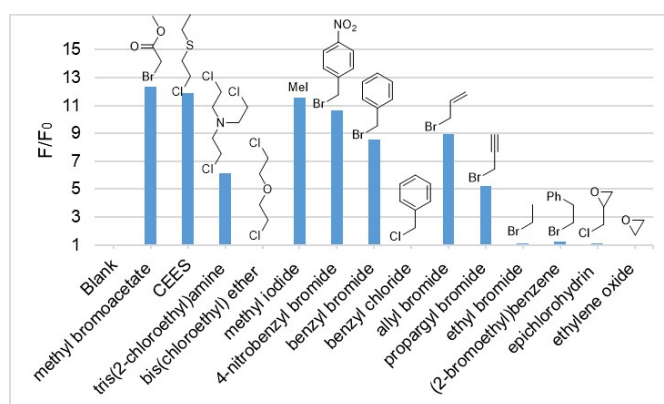


Fig. 2. Fluorescence response (Ex@418 nm, Em@477 nm) of probe **9** (3 μM in DMSO containing trace water) after adding various alkylating agents (60 μM). Data are expressed as F/F₀.

CEES was chosen for further studies. As shown in Fig. 3, fluorescence intensity was enhanced upon the gradual addition of CEES. The limit of detection (LOD) for CEES was calculated as 2.3 μM , which is comparable to the best detection system recently reported for CEES (LOD = 1.2 μM).^{6g}

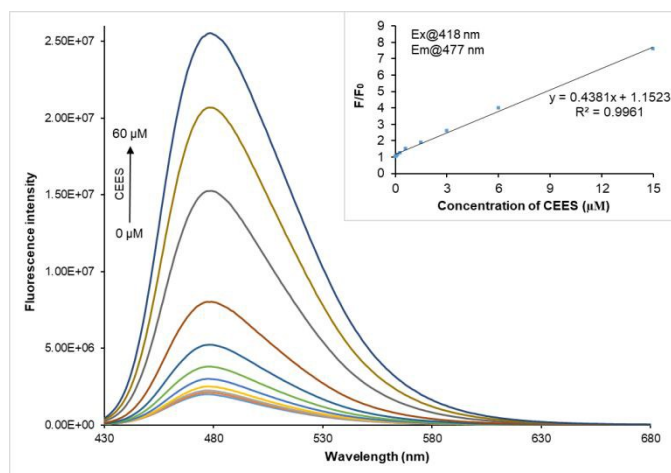


Fig. 3. Fluorescence spectra (Ex@418 nm) recorded 2 h after treatment of probe **9** (3 μM in DMSO containing trace water) with CEES at various concentrations (0–60 μM). Inset: linear calibration curve upon the gradual addition of CEES.

In conclusion, we report a new probe for the detection of alkylating agents relying on FRET. This probe (**9**) is equipped with a fluorophore (coumarin 343) and a quencher (DABCYL) that prevents the fluorescence to occur. Its reaction with alkylating agents at room temperature is followed by the cleavage of the self-immolative unit releasing the coumarin leading to strong fluorescence enhancement. The probe was especially efficient for the detection of reactive alkylating agents, such as methyl iodide, benzyl bromide and 2-chloroethyl ethyl sulfide (CEES), a surrogate of sulfur mustard poison.

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Conflicts of interest

There are no conflicts to declare.

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