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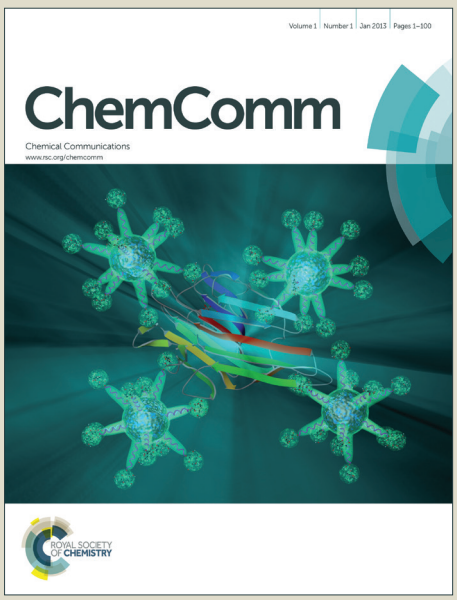

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Methylene blue phosphoramidite for DNA labelling

Gabriel De Crozals, Carole Farre, Monique Sigaud, Philippe Fortgang, Corinne Sanglar and Carole Chaix*

We report the first synthesis of a Methylene blue (MB) phosphoramidite derivative suitable for DNA solid-phase synthesis. The electrochemical and optical properties of the resulting MB modified oligonucleotides were confirmed. This new molecule is an important breakthrough for the design of new probes labelled with MB.

Dimethylaminophenothiazin-5-ium chloride, better known as Methylene blue (MB), has many properties in chemistry and biology. For decades, this compound has been used as a redox indicator,[1] a photosensitizer,[2, 3] a dye for cellular staining procedures,[4] an antiseptic[5, 6] and in medicine against Alzheimer's disease.[7] For sensing applications, many groups have used MB as a redox marker of oligonucleotides (ODN)[8-13] or aptamers.[14-18] For instance, K. Plaxco *et al.* reported on the design of DNA, protein, antibody and drug sensors based on bioreceptor modification using MB as an electrochemical indicator.[19-23] MB proved efficient for electron transfer and had good stability over time in such detection systems, even better than ferrocene.[24] To develop an efficient DNA sensor, the position of MB along the ODN used as a bioreceptor appears to be critical. For instance, internally labelled probes have shown better signal suppression upon target addition than end-labelled probes.[9] Barton's group studied the electrochemical response of MB bound to double stranded DNA and demonstrated both direct reduction of the MB at the electrode through DNA curvature and DNA-mediated reduction through double stranded helix electron transfer depending on π-stacking.[25, 26]

Indeed, until now, the intercalation properties of MB within DNA have been widely investigated but only with free MB molecules in solution.[27-31]

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The standard method for incorporating MB into ODN relies on a post-synthesis functionalization with an NHS-ester derivative.[25, 32-34] This method is limited by the need for NH_2 functions in the oligonucleotidic chain which are not the exocyclic amines of the nucleic bases. Although the introduction of amino groups in ODN is relatively easy by solid-phase synthesis, it is well known that transamidation occurs during post-synthesis deprotection that reduces the coupling efficiency of the amino-linked ODN if used without purification.[35] The MB-NHS ester coupling reaction is long (~20h) and an additional HPLC purification step is required to obtain the expected conjugate.[36] Schubert *et al.* reported a moderate coupling yield of 30% with DNA sequences forming secondary structure in solution.[34] Furthermore, MB tends to interact with DNA *via* electrostatic interaction with the phosphate backbone and π-stacking with nucleic bases (in particular with guanine residues), which also decrease the efficiency of covalent incorporation.[37, 38]

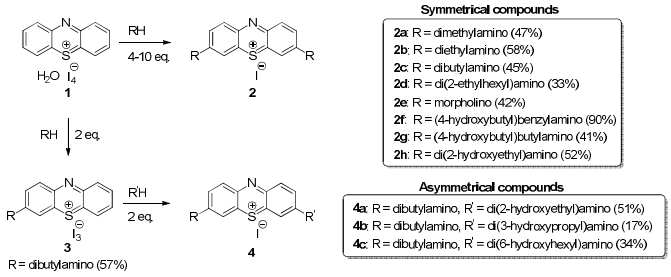
The presence of several markers on the ODN probe has led to a better electrochemical response on sensors.[39-41] However, current methods do not permit easy introduction of multiple labels into an ODN.

Our approach is based upon the elaboration of MB derivatives allowing covalent incorporation at any position of an oligonucleotidic sequence directly during its synthesis. Phosphoramidite chemistry seems the most straightforward method for this purpose. While most fluorescent dyes are now available as phosphoramidites, there is a lack of redox markers which can be incorporated during ODN solid-phase synthesis. Bis-propanol ferrocene phosphoramidite has proved to be efficient in incorporating numerous redox markers in the sequence.[42-45] Since many dyes are unstable under strong basic conditions, labelled DNA has to be deprotected under a mild basic treatment to ensure its integrity. It is worth noting that MB is not stable in these mild basic conditions and the molecule needs to be chemically modified to improve its stability.

Herein, we report the first synthesis of an MB phosphoramidite and its use in labelling synthetic DNA. First, a series of symmetrical MB analogues was synthesized and stability tests were performed under DNA deprotection conditions. Then, after selection of the most stable molecule, unsymmetrical analogues were obtained and their corresponding phosphoramidite synthons were synthesized. Finally, redox oligonucleotidic probes were elaborated by automated solid-phase synthesis using the most efficient MB phosphoramidite.

Since the first synthesis of MB by Heinrich Caro in 1876, few synthetic routes have been explored to produce analogues from the phenothiazinium core. Phenothiazinium derivatives can be obtained either by coupling a *p*-amino-dialkylaniline with a dialkylaniline,[46] or by phenothiazine oxidation followed by regioselective nucleophilic substitution by dialkylamines.[46-49] The second option offers less restrictive reaction conditions, and more importantly, it is the best way to achieve either mono- or di-substitution of the phenothiazinium core.

In the first step, phenothiazine was oxidized by iodine in chloroform to give phenothiazinium tetraiodide hydrate **1** after *in-situ* precipitation. This compound was the starting material for the mono- and di-substituted phenothiazinium salts (Scheme 1). In the second step, **1** was reacted with at least four equivalents of a secondary amine giving symmetrical diaminophenothiazinium salts, **2a-h**. Yields (33-90%) were comparable to those found in the literature.



Scheme 1. Synthesis of symmetrical and asymmetrical analogues of methylene blue.

Different alkyl groups were compared: linear chains, from methyl to butyl **2a-c**, and a ramified chain, 2-ethylhexyl **2d**. It is worth noting that diisopropylamine incorporation was tested but the expected product was not obtained, indicating that this reaction is not possible with α-ramified amines probably due to steric hindrance.

All MB derivatives were dark blue colour in organic solvents and had absorbance properties similar to those of the unmodified methylene blue, **2a**. Absorbance maxima were in the range of 650-675 nm (see Figure 1). Bathochromic effect increased along with the degree of exocyclic amine substitution. This is consistent with previous observations between azure dyes and MB, where a red shift appears as the number of methyl groups increases.[2] Here, maximum absorbance was slightly displaced to higher wavelengths in relation to the length of the alkyl chains. This effect was attributed to the electronic stabilization of the positively charged amine by alkyls in the resonance structures.[50]

Our ultimate aim was to incorporate MB into DNA sequences. This requires a basic treatment at the end of DNA synthesis. Ultra-mild DNA deprotection conditions have been described: 6 hours in K_2CO_3 0.05 M in methanol.[51] To our knowledge, MB degradation has been shown in the literature under strong basic or oxidizing conditions[52] but this is the first study under mild basic conditions.

The λ_{max} absorbance of each symmetrical compound in a K_2CO_3 solution was measured against time. The absorbance decrease was correlated to a degradation process in solution.

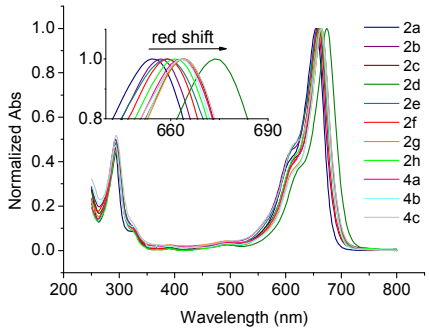
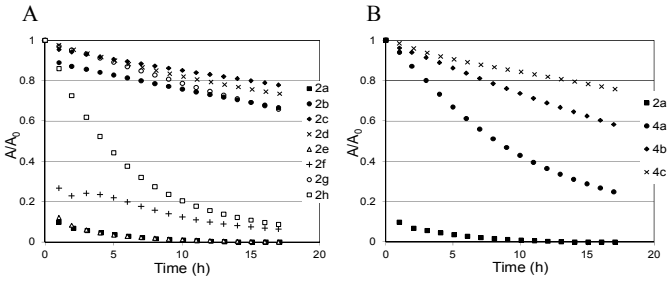


Fig.1. UV/visible spectra of methylene blue derivatives in methanol.

We observed that the stability of MB analogues under mild basic conditions increased together with the length of alkyl chains (Figure 2). However, degradation seems to follow the same pathways as described before. In our experiment, the colour of MB solutions changed very quickly from dark blue to pink then to colourless. This is consistent with the degradation mechanisms already described where MB is degraded in methylene violet by the loss of its methyl groups.[50] As amine substituents are concerned by the degradation pathway, their modification has a logical impact on stability.



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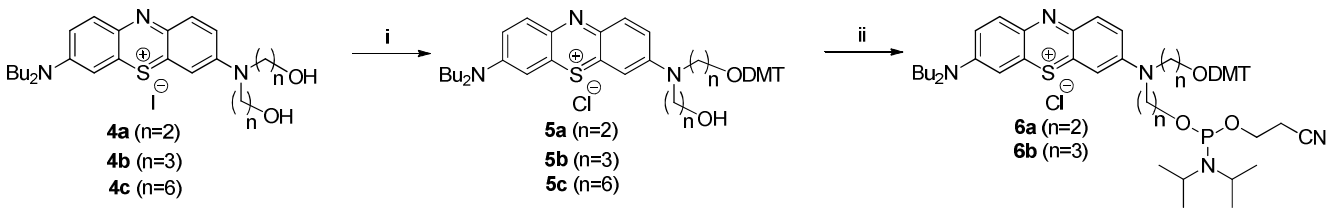
Compound	Stability after 6 hrs	Compound	Stability after 6 hrs
2a	3%	4a	56%
2b	80%	4b	81%
2c	88%	4c	88%
2d	87%		
2e	2%		
2f	18%		
2g	85%		
2h	38%		

Fig.2. MB derivative stabilities in K_2CO_3 0.05 M in methanol. A) Degradation kinetics of symmetrical compounds (**2a-h**) B) Degradation kinetics of asymmetrical compounds (**4a-c**) C) Stability (residual % of MB derivative) after 6 hours.

Considering stability studies on symmetrical compounds **2a-h**, the most stable dibutylamine was selected for the synthesis of asymmetrical compounds. Reaction of **1** with at least two equivalents of dibutylamine led to 3-dibutylaminophenothiazin-5-ium triiodide **3** (57% yield). Then, a second amine bearing two hydroxyls was incorporated, leading to asymmetrical compounds **4a-c** with yields from 17 to 51% (Scheme 1). Stability under mild basic conditions was also studied and confirmed previous observations. In addition, all asymmetrical compounds were more stable than **2a** (Figure 2B).



Scheme 2. Synthesis of methylene blue phosphoramidites i) 4,4′-dimethoxytriphenylmethyl chloride in acetonitrile; ii) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in acetonitrile.

Electrochemical characterization confirmed that all analogues were electroactive. $E_{1/2}$ values measured from -212 mV to -258 mV *vs* Ag/AgCl by cyclic voltammetry (CV) in buffer (20 mM phosphate with 250 mM KCl in water/acetonitrile) were characteristic of MB's electrochemical response (see Table S1 Supporting Information).[27] Compound **2e** gave an $E_{1/2}$ of -90 mV due to the cyclic amine which has a sizeable influence on the molecule, inducing a significant change in the redox system. ΔE values were calculated from 33 to 66 mV for **2a-f** derivatives (except **2d**) and from 25 to 29 mV for **4a-c** derivatives. As the MB transfer is a two-electron process, the ΔE values close to 28.3 mV attest to the reversibility of the redox systems.[53] The poor solubility of **2d** did not enable an interpretable electrochemical response by CV. The low solubility of **2c** probably enlarges its ΔE value.

Asymmetrical MB derivatives **4a-c** were used to prepare the corresponding phosphoramidite synthons (Scheme 2). First, the dimethoxytrityl acido-labile protecting group was coupled to one hydroxyl with yields between 32% and 52%. Then, the two phosphoramidites **6a-b** were successively obtained. We failed to obtain the **6c** compound, because the phosphoramidite group on the MB derivative was not stable enough *in situ* and was immediately transformed into an oxidized form without any possibility of purification. **6a-b** were assessed for incorporation into a DNA sequence by automated synthesis. Poly-thymidine 10 mer was synthesized *via* a standard 1 µmol DNA synthesis cycle. Then, **6a** and **6b** were incorporated using 20 equivalents of synthon per growing DNA chain. Coupling time was increased from 1 min to 3 min, compared with standard phosphoramidite nucleoside synthons. We succeeded in coupling the MB phosphoramidites at the 5' end of DNA with coupling yields of 10% and 50% for **6a** and **6b** respectively. The low coupling yield of **6a** resulted from the lack of phosphoramidite accessibility due to strong hindrance of the (dimethoxytrityl)-oxy-ethyl group. An additional methylene on the di(hydroxyalkyl) chain (from C2 to C3) greatly improved phosphoramidite accessibility and reactivity of the MB analogue. We also succeeded in coupling **6b** on an adenosine at the 5' end of a longer sequence $dT(A)_{20}$ with the same efficiency. The stability of MB derivatives **6a-b** bound to the $d(T)_{10}$ (ODN) was studied under mild basic conditions. At different times (1, 2, 4 and 6 hours), an aliquot of the oligonucleotide (**6a**-ODN and **6b**-ODN, 1 $\mu molmL^{-1}$) solution was analysed by HPLC and the residual full length ODN was quantified (Figure 3). A significant difference of stability was observed between the two oligonucleotides **6a**-ODN and **6b**-ODN. After 6 hours' incubation in K_2CO_3 0.05 M in methanol, **6a**-ODN was almost completely degraded. On the other hand, no degradation was observed with **6b**-ODN after the same treatment. We can compare these results with those shown on Figure 2 for **4a-b** derivatives. It is worth noting that incorporating **6a** into an ODN decreases the stability of the MB derivative compared with **4a**. This lack of stability could be due to a possible rearrangement on the phosphate between **6a** and the ODN caused by the reaction of the second hydroxyl of the di(2-hydroxyethyl)amino group that could form an intracyclic phosphate triester leading to MB cleavage and loss. This intracyclic reaction, which does not occur on **4a** increases the degradation kinetics of **6a**-ODN. Inversely, stability was observed as expected with **6b**-ODN compared with the **4b** monomer. These conditions were sufficient for complete deprotection of the nucleic bases on a synthetic ODN, when ultra-mild protecting groups were used for DNA synthesis.[51] We succeeded in synthesizing a fully deprotected **6b**-$dT(A)_{20}$ as confirmed by HPLC and MS analyses (see Figure S3 Supporting Information)

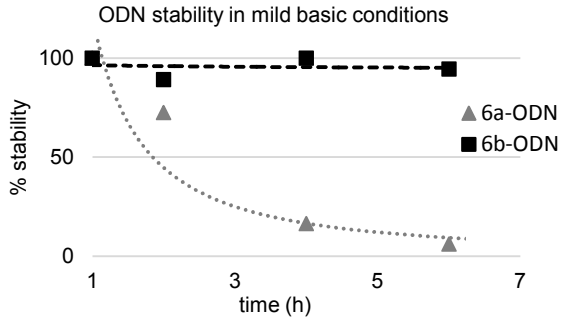


Fig. 3. **6a**-ODN and **6b**-ODN stability in K_2CO_3 0.05M/Methanol.

DMT-ON synthesis of **6a**-ODN could be a solution to avoid the MB cleavage under basic conditions. In this case, the last step of ODN deprotection would be the DMT release under acidic conditions. Nevertheless, **6b** phosphoramidite was preferred because of its higher coupling efficiency than **6a** on the synthesizer.

A redox response of **6b**-ODN was recorded by CV which confirmed the electrochemical properties of the MB derivative **6b** tethered to the ODN (see Figure S4 Supporting Information). The $E_{1/2}$ values were -165 mV *vs.* Ag/AgCl at pH 6.4 and -275 mV at pH 8. These values were similar to the values reported in the literature.[12, 54] The half-peak anodic potential value around 35 mV indicates that the redox process involves 2 electrons (theoretical values of 28.3 mV for a reversible two electrons process and 56.5 mV for one electron process)[53] and the observed negative shift of $E_{1/2}$ value (-110 mV) between pH 6.4 and pH 8 indicates that 2 protons are transferred during the electron transfer according Nernst Equation. The ΔE value was around 51 mV (pH 6.4) and 71 mV (pH 8), which appears to be higher than that of **4b** (see Table S1 Supporting Information). MB bound to ODN affects the reversibility of the electron process,

attesting to a constrained environment. Considering the literature, our approach leads to MB-ODN having a reversible electrochemical response similar to MB-ODN synthesized *via* a standard solution-phase coupling approach. For instance, Abi *et al.* reported a ΔE value close to 80 mV with a MB-C6-DNA obtained by amino coupling on a succinimidyl ester linker.[33]

Conclusions

In conclusion, we succeeded in developing a methylene blue phosphoramidite for direct DNA labelling during synthesis. The new MB derivative demonstrated an improved stability in mild basic environment. After incorporation in DNA, the MB preserves its optical and electrochemical properties. This approach allows easy incorporation of multiple MB labels at any position of an oligonucleotidic sequence. Non-described structures can be achieved by this technique in order to investigate their electrochemical and optical properties.

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