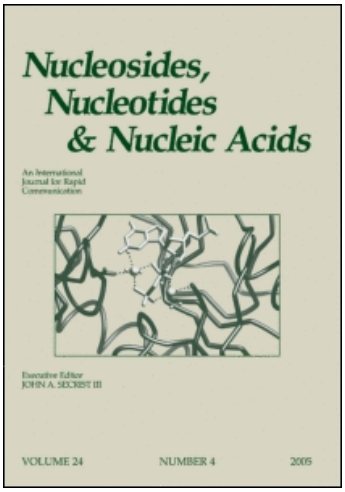

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COVALENT ATTACHMENT OF METHYLENE BLUE TO OLIGONUCLEOTIDES

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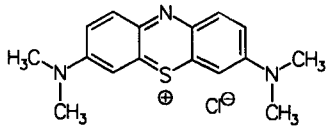
Abstract: The synthesis and application of oligonucleotides derivatized by methylene blue are described. For that, a carboxylated methylene blue derivative was synthesized and transformed into an activated N-hydroxysuccinimidoester. The activated ester was reacted with 5'-aminoalkylated oligonucleotides. The labelled oligonucleotides were isolated and characterized both by reversed phase HPLC and MALDI mass spectrometry. Initial studies on analytical application of these oligonucleotide conjugates are discussed.

Photoactive molecules such as intercalators or photosensitizers which are covalently attached to oligonucleotides are useful tools for biochemistry both in basic and applied science. Considerable efforts have been made to increase the effects of "antisense" oligonucleotides by irreversible photochemical crosslinking of targeted nucleic acids with chromophore/oligonucleotide conjugates[1]. Sequence specific modification and cleavage of DNA strands with oligonucleotides tethered to photosensitizers were reported[2]. Additional to the photochemically induced crosslinking between dyes and DNA strands and the formation of radical ions the generation of singlet oxygen seems to be responsible for damaging and cleavage of DNA[3]. Thus, dyes which are capable to sensitize the formation of $^1O_2$ during irradiation with visible light are of special interest.

Derivatives of various porphyrines[4,5], psoralenes[6] and acridines[7] have been incorporated into oligonucleotides either by phosphoramidite chemistry or by the activated ester approach. The properties of these derivatives were investigated with respect to their capability to stabilize DNA duplexes by intercalation and site directed modification of DNA during irradiation with light. Recently, substituted phenothiazines were shown to be suitable photosensitizers for the covalent attachment to proteins[8-10]. The reported methods have been applied to the synthesis of methylene blue-protein conjugates, but, no efforts are known to introduce methylene blue into oligonucleotides or DNA.

Herein we describe the attachment of a methylene blue derivative to 5'-aminoalkylated oligonucleotides. 3,7-(N,N'-Bis-dimethylamino)-phenazathionium chloride, methylene blue **1**, is frequently used in preparative organic photooxygenations because of its relatively high quantum yield of singlet oxygen formation ($\Phi_{^1O_2} = 0.26$)[11]. Its absorption maximum at 668 nm permits excitation with a low cost diode laser. Moreover, **1** was found to interact with nucleic acids in different ways. The ionic interaction, the intercalation into DNA double strands[12,13] as well as the base specific cleavage of DNA strands on exposure of methylene blue-DNA mixtures to monochromatic light[14] were described. These properties stimulated the idea that methylene blue covalently bound to oligonucleotides will be very useful for both increasing the stability of DNA double strands and for site directed damaging of DNA targets. However, the structural features of **1** and above all the absence of any suitable functional group in the molecule do not allow a direct activation and coupling to biomolecules especially to nucleic acids.



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To overcome this difficulty, we have chosen 3-N-(4-carboxybutyl)-methylamino-7-dimethylaminophenazathionium chloride **4** as methylene blue derivative. The general synthesis of **4** is illustrated in FIGURE 1 and was carried out with some modifications according to the literature[9]. Contrary to the reported synthesis the protection of the carboxylic group was not necessary because the yield in each step of the reaction described in FIGURE 1 are comparable to those reactions using the protected synthons. **4** was then activated by formation of an appropriate N-hydroxysuccinimidoester using N-hydroxysuccinimide in the presence of watersoluble carbodiimide in absolute DMF. Finally, reactions of the activated dye **5** with oligonucleotides bearing an aminoalkyl group at their 5'-ends led to **6**. For that an aminoalkyl terminus was introduced at the 5'-end using the aminolink technique with commercially available phosphoramidites (see experimental part).

Yields of the conjugation reaction varied from 30 to 75 % as estimated by integration of the HPLC-peaks.

In order to investigate the capability of sequence specific cleavage of single stranded DNA under light irradiation and the bioanalytical application of the conjugates we have chosen sequences complementary to M13mp18 (+) DNA. Thus, the 30 mer oligonucleotide 5'-MB-d(TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG) **7** was

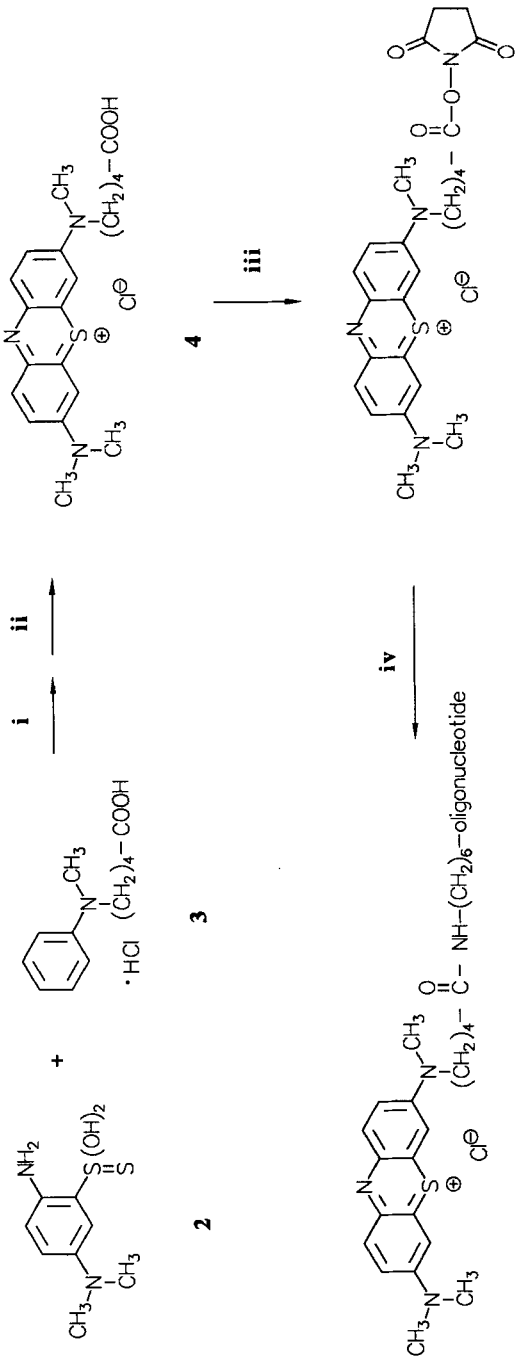

FIGURE 1
Synthesis of the carboxylated methylene blue derivative and coupling to an oligonucleotide
i $K_2Cr_2O_7/H^+$; ii MnO_2/Cu^{2+}; iii N-hydroxysuccinimide, water soluble carbodiimide, DMF; iv aminoalkylated oligonucleotide (pH 8.0)

obtained in a yield of approximately 30%, whereas the 20mer 5'-MB-d(CC AGG GTT TTC CCA GTC ACG) **8** was isolated in yields of almost 75%. We assume that the moderate yield in the case of **7** could be due to differences in the chain length of **7** and **8** and resulting secondary structural effects from that.

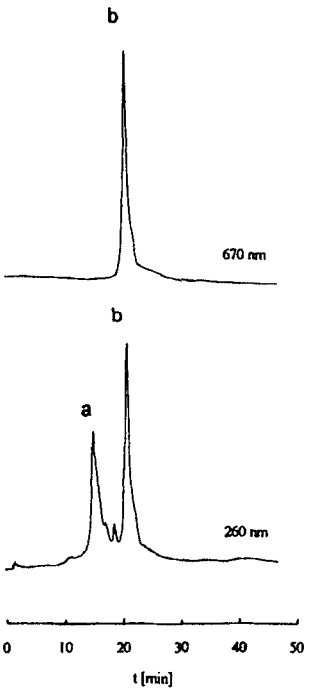

FIGURE 2
HPLC elution profile of the reaction product of **5** with a 20 mer aminoalkylated oligonucleotide, conditions see experimental part.

The crude products of the reactions were analysed and purified by HPLC. Baseline separation of starting material and the products were possible in analytical and preparative scale, respectively (FIGURE 2). Detection at 260 nm and 670 nm permitted the assignment of the product peak (b) and the peak of unreacted starting material (a).

Purification of the reaction mixture by both gel electrophoresis or size exclusion chromatography (Sephadex G25) was troublesome and led to a considerable loss of product, which is obviously due to strong interactions of modified oligonucleotides with the polymeric matrix. Regardless of these troubles, both products were isolated, purified and subjected to Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS). The expected molecular peaks were found at $[m/z]^+ = 9753.1$ for **7** and $[m/z]^+ = 6614.8$ for **8**, respectively (succinic acid was used as matrix).

The UV-Visible spectrum of the purified and isolated oligonucleotide conjugate **7** (FIGURE 3) displayed the expected absorption maxima at 260 and 670 nm, respectively. Compared to the unbounded methylene blue derivative no remarkable changes in the spectrum of the dye attached to the DNA-fragment could be observed. The ratio of the corresponding absorption coefficients $\varepsilon_{260}/\varepsilon_{670}$ was approximately 4.

The methylene blue oligonucleotide conjugates were used as DNA probes for single stranded M13mp18 DNA in dot blot experiments on nylon membranes. The detection of these probes by chemiluminescence after amplification of dioxetanes *via* photochemical formation singlet oxygen as suggested by McCapra et al.[15] is possible. Until now detection limits of 4 attomol M13mp18 plasmid DNA in dot blots were achieved. Now we are

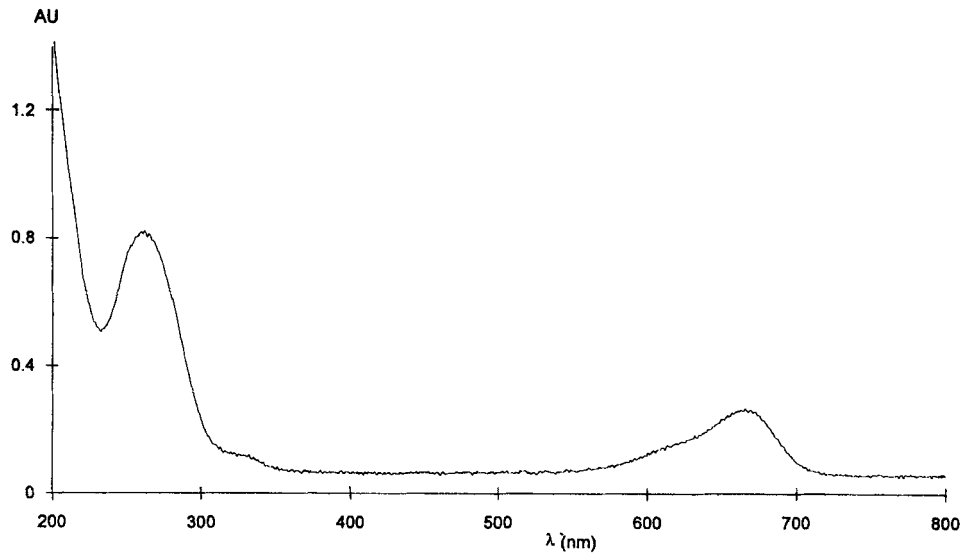

FIGURE 3
UV/Vis spectrum of purified 20mer oligonucleotide conjugate **8** in H_2O

going to investigate the application of the synthesized derivatives with regard to the site directed cleavage of DNA. These results will be published soon in detail.

Taking together results of our work it was shown, that methylene blue derivatives as photoactive dyes with interesting photochemical properties are also suitable for introduction into oligonucleotides. The procedure can be generalised to any sequence of choice.

Experimental

^{1}H-NMR spectra were recorded on a Bruker AM 300 spectrometer. UV/Vis spectra were done with a Shimadzu UV/Vis spectrometer 160A. HPLC analysis was carried out with a ICI/GAT system using a linear gradient of 10% buffer A (0.1 M triethylammonium acetate, pH 6.5, acetonitrile, 50/50, v/v) to 40% in buffer B (0.1M triethylammonium acetate, pH 6.5, acetonitrile, 98/2, v/v) during 40 minutes on a reversed phase column (LiChrosorb RP 18, 4x125 mm). N-Dimethylaminopropyl-N'-ethylcarbodiimide hydrochloride was obtained from Merck, Darmstadt. Oligonucleotides were synthesized using commercial available phosphoramidites (Pharmacia) on a Gene Assembler® oligonucleotide synthesizer (Pharmacia). Aminoalkylation was performed according to the instruction of the supplier with Aminolink® (Pharmacia). The

synthesized oligonucleotides were deprotected and desalted according to standard procedures. All other reagents and solvents were purchased from Aldrich (Deisenhofen, Germany).

2-Amino-5-dimethylaminophenylthiosulfonic acid (2) was prepared according to ref.16.

5-(N-Methylanilino)-valeric acid (3) was prepared according to ref. 9

3-[N-(4-Carboxybutyl)-N-methylamino]-7-dimethylaminophenazathion chloride (4). 5-(N-Methylanilino)-valeric acid hydrochloride **3** (0.258 g 1.06 mmol) and 2-amino-5-dimethylaminophenylthiosulfonic acid **2** (0.25 g 1.007 mmol) in aqueous solution (14 ml) containing 1 ml 1 N HCl were oxidized by potassium bichromate (0.2 g) that was added in small amounts at 10 °C. After 30 minutes 0.3 ml of glacial acetic acid were added and the solution was stirred for 1 hour. The intermediate product was filtered off and oxidized again in acetonitrile (20 ml) with manganese(IV)oxide (0.25 g) in the presence of catalytic amounts of cupric sulphate during 1 hour under reflux. After evaporation of the solvent the reaction product was purified by silica gel column chromatography (CH_3CN/H_2O/1N HCl; 40/5/1; v/v/v) to yield **4** which appears as a single spot in TLC. Yield: 140 mg, 32,5%; ^{1}H-NMR analysis; 300 MHz (D_2O); δ(ppm)= 7.1-6.4 (m 6H (arom.)); 3.25 (s 3H (CH_3)); 2.99 (s 6H (CH_3)); 2.34 (t 2H (CH_2)); 1.59 (m 6H (CH_2)); UVis (H_2O) λ_{max}= 660 nm, 615 nm; Fp.: 170°C (decomp.).

N-Hydroxysuccinimidoester of 4 (5). **4** (5.3 mg; 13.06 μmol) was transformed to the activated ester **5** by treatment with N-hydroxysuccinimide (NHS) (3.8 mg 33.02 μmol) in the presence of N-dimethylaminopropyl-N'-ethylcarbodiimide hydrochloride (EDC) (6.3 mg 33.02 μmol) in 500 μl absolute DMF for three days. No starting material was detected after that time by TLC (system CH_3CN/H_2O/1 N HCl; 40/5/1; v/v/v). This solution was directly used for introduction of the activated dye into oligonucleotides. Keeping dry, this reagent can be used at least 1 month after its preparation without any observable loss of activity. ^{1}H-NMR analysis; 300 MHz (DMSO-d_6); δ(ppm)= 7.8-7.45 (m 6H (arom.)), 3.75 (broad s 2H (CH_2)); 3.35 (s 6H (CH_3)); 3.3 (s 3H (CH_3)); 2.85 (m 6H (CH_2)); 1.7 (broad s 4H)).

Synthesis of conjugates. Labelling reactions of aminoalkylated oligonucleotides **7** and **8**, respectively, (1.9 O.D. 6.3 nmol) were performed using **5** (63.4 μg 126 nmol) in 0.1 M potassium dihydrogenphosphate (pH 8)/DMF (1:2; v/v; total volume 300 μl) overnight in the dark. After evaporation of the solvent the product was redissolved in water (100 μl) and successively treated with n-butanol (1 ml). The product was collected by centrifugation. N-Butanol extraction was repeated, the dried product was purified by preparative HPLC and stored in the dark to avoid undesired photodestruction of the DNA.

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