

1           **Synthesis and Evaluation of Methylene Blue Oligonucleotide**  
2                   **Conjugates for DNA Interstrand Cross-Linking**

3                               Nathalie De Laet<sup>1</sup>, Annemieke Madder \*<sup>1</sup>

4                               1. Organic and Biomimetic Chemistry Research Group

5                               Department of Organic and Macromolecular Chemistry

6                               Ghent University, Krijgslaan 281, S4, 9000 Gent, Belgium

7                               \*Annemieke.madder@ugent.be (Annemieke Madder)

8

## 9 **ABSTRACT**

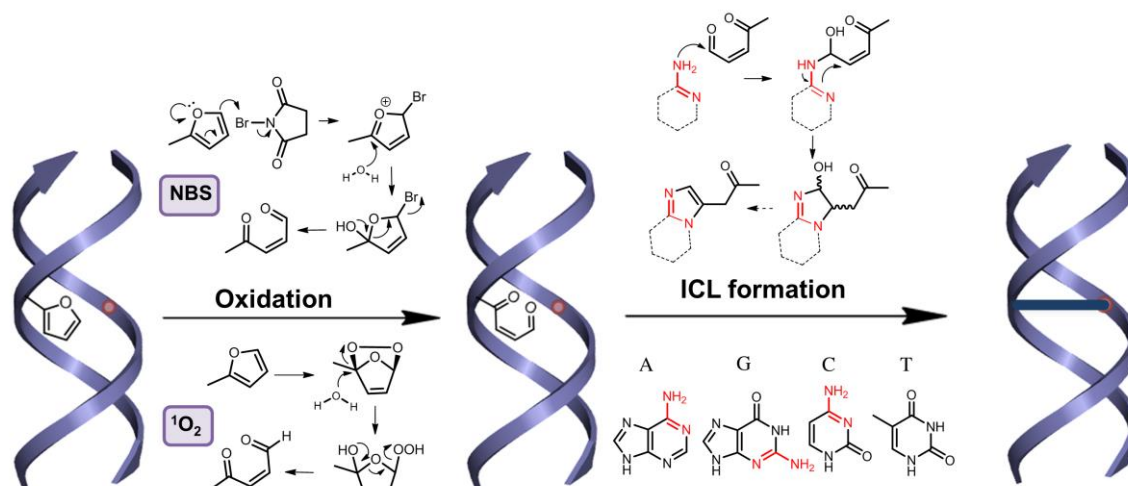
10 Efficient DNA interstrand cross-linking can be achieved with furan containing oligonucleotide  
11 probes upon activation by singlet oxygen ( $^1\text{O}_2$ ). Previously, we have described how this can be  
12 achieved by irradiation of these furan probes with visible light in the presence of  
13 photosensitizers. Now, in an effort to explore cross-linking under conditions that are  
14 representative for experiments in cellular context, the furan mediated oligonucleotide cross-  
15 linking was investigated at low oligonucleotide concentrations, ensuring a sufficiently high  
16 local concentration of singlet oxygen by attaching the sensitizing methylene blue moiety to the  
17 oligonucleotide complementary to the furan modified strand. Four methylene blue-  
18 oligonucleotide conjugates were synthesized, each with a different positioning of methylene  
19 blue with respect to the furan unit present on the complementary strand. The conjugates were  
20 evaluated for singlet oxygen generation and subsequent cross-linking ability. It was observed  
21 that not only the distance of the  $^1\text{O}_2$  source to the furan unit, but also the specific interaction of  
22 methylene blue moiety with the duplex, which is position dependant, influences cross-linking  
23 yields.

24

## 25 1. INTRODUCTION

26 Interstrand cross-linking (ICL) between oligonucleotides has been the subject of interest in  
27 multiple interdisciplinary fields. The possibility to induce these cross-links can be used to gain  
28 insight in DNA repair mechanisms effective in biological systems. Indeed, a DNA abasic site,  
29 which is a well-known form of DNA damage [1,2], can react with a guanine residue of the  
30 opposite strand resulting in cross-link formation [3]. To understand the mechanism whereby  
31 the repair of these defaults occurs, a need for easy accessibility to cross-linked oligonucleotides  
32 is created [4]. In addition to this, the inherent high selectivity of DNA hybridization can be  
33 exploited for gene regulation, were an oligonucleotide can selectively block a complementary  
34 sequence [5, 6]. Sustained blockage and thus more effective regulation could be reached by  
35 introducing a covalent linkage to the oligonucleotide target.

36 Different chemical procedures have been developed to selectively induce ICLs upon  
37 photochemical activation [7, 8, 9, 10 and 11]. Our group previously reported upon the  
38 formation of interstrand cross-linking through the use of furan modified oligonucleotides [12,  
39 13, 14]. Originally, N-bromosuccinimide (NBS) was used to ensure oxidation of the furan unit  
40 leading to the formation of a very reactive 4-oxo-enal (figure 1). This moiety is prone to react  
41 with exocyclic amines present on the bases of the complementary strand with the formation of  
42 an interstrand cross-link.



43  
44  
45  
46  
47  
48  
49

**Figure 1:** Schematic representation of furan mediated DNA cross-linking. The furan group can be oxidized using NBS or singlet oxygen ( $^1\text{O}_2$ ). After oxidation, the formed reactive moiety can react with exocyclic amines of the opposite base on the complementary strand. Both adenine (A) and cytosine (C) contain an exocyclic amine which is located in close proximity of the furan building block, leading to the selective formation of a covalent bond as depicted on the right-hand side of the figure.

50 To increase the biocompatibility of this cross-linking methodology, a more biologically  
51 compatible and sustainable oxidation method was needed. Singlet oxygen ( $^1\text{O}_2$ ) was shown to  
52 also have the capability to selectively oxidize the furan unit leading to the formation of an  
53 interstrand cross-link [15] (figure 1).  $^1\text{O}_2$ -generation can occur in a highly controllable manner,  
54 which constitutes an important step towards the biological applicability of the furan cross-  
55 linking methodology. The furan unit reacts with singlet oxygen through a [4+2] cycloaddition  
56 [16] generating a reactive intermediate. Singlet oxygen is formed by irradiation of a  
57 photosensitizer (PS) with a specific wavelength characteristic for each sensitizer [17]. By  
58 carefully choosing a sensitizer which can be excited by light from the visible region, a cross-  
59 linking method is now available where the use of invasive UV-radiation can be avoided.

60 We here aimed at investigating the furan-mediated cross-link reaction at physiologically  
61 relevant concentrations. Indeed, oligonucleotide concentrations above 20  $\mu\text{M}$  have shown to  
62 lead to a loss in sequence specificity [18]. However, as the lifetime of singlet oxygen in

63 aqueous medium is limited [19], the distance between the site where it is generated, i.e. the  
64 photosensitizer, and the furan moiety to be activated can play an important role, rendering the  
65 cross-link reaction inherently concentration dependent. In order to ensure a sufficiently high  
66 local concentration of the photosensitizer, we decided to couple it to the strand complementary  
67 to the furan modified oligonucleotide. By doing so, the influence of the position of the  
68 photosensitizer within the oligonucleotide, on singlet oxygen generation and subsequent cross-  
69 linking can be investigated. To determine which photosensitizer is best suited for attachment to  
70 the oligonucleotide, results of cross-linking experiments with a variety of photosensitizers  
71 added in solution were compared. The PS which showed most promise in these experiments  
72 was then chosen for incorporation. Furan building block **X** (Figure 2), chosen based on  
73 previous research in our lab in which different building blocks were synthesized and evaluated  
74 on their  $^1\text{O}_2$ -cross-linking capacities [15], was incorporated in the oligonucleotide sequence  
75 FON1 (Table 1). Methylene blue, rose bengal, ruthenium tris bipyridinium and zinc  
76 phthalocyanine were selected for screening of their capacity to induce cross-linking. After  
77 optimization of the cross-linking conditions, the chosen photosensitizers were evaluated on the  
78 cross-linking yields (as calculated from HPLC chromatograms) against the observed  
79 competitive degradation of the oligonucleotide strands. Based on these results, methylene blue  
80 was chosen for conjugation to the oligonucleotide. To this end, a reactive form of the sensitizer  
81 was synthesized after which it was coupled to the oligonucleotide and cross-linking at low  
82 oligonucleotide concentration could be executed.

## 83 **2. MATERIALS AND METHODS**

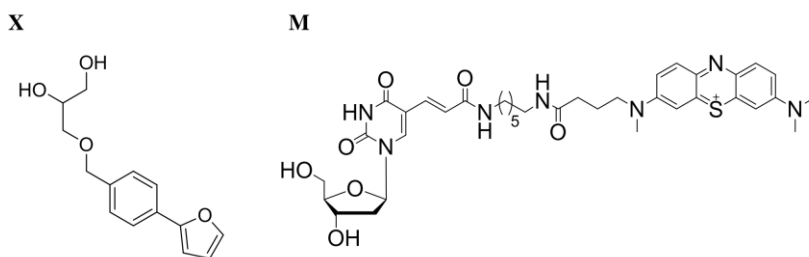
84 **2.1. Chemical synthesis:** All chemical reagents and solvents were purchased from Sigma  
85 Aldrich.

86 **2.1.1. Synthesis of methylene blue succinimide ester s2.** The methylene blue carboxylic acid  
87 **s1** was synthesized (supporting information, figure S5) and subsequently transformed into the  
88 succinimide ester **s2** as described by Barton et al. [20]. The structure of the synthesized  
89 compound was confirmed by ESI-MS (454 g/mol)  $[M+H]^+$  in correspondence to [20]. The  
90 methylene blue succinimide ester **s2** was subsequently coupled to an oligonucleotide,  
91 containing an amino modified thymine base. This methylene blue modified thymine base is  
92 presented in Figure 2 (**M**).

93 **2.1.2. Synthesis of acyclic furan phosphoramidite.** An acyclic furan building block (Figure 2, **X**)  
94 was synthesized containing a furan moiety coupled to a phenyl group. The synthesis was carried  
95 out as previously described [13]. After conversion to the phosphoramidite, the furan building  
96 block was incorporated in the oligonucleotide through automated DNA synthesis.

97 **2.2 DNA synthesis:** DNA reagents and the thymine amino modified phosphoramidite  
98 (aminomodifier C6 dT) were obtained from Glen Research. All the oligonucleotides were  
99 synthesized DMT-on at 1  $\mu$ mol scale on an ABI 394 DNA synthesizer. The synthesis proceeded  
100 through an automated phosphoramidite coupling cycle and was interrupted for a manual  
101 incorporation of the modified phosphoramidites. This coupling involved a repetitive application of  
102 a dry 0,05 M solution of the modified phosphoramidite in acetonitrile and a dry 0,1 M  
103 dicyanoimidazole solution in acetonitrile. The synthesized oligonucleotides were cleaved of the  
104 solid support by treatment with 1 mL of aqueous  $NH_4OH$  while shaking overnight at a temperature  
105 of 55°C. Purification with concurrent DMT removal of the synthesized oligonucleotides was

106 carried out using a Sep-Pak C18 cartridge obtained from Waters. The purity of the  
107 oligonucleotides was evaluated by RP-HPLC, recorded on an Agilent 1200 system equipped with a  
108 Phenomenex Clarity 110 Å C18 column (250 x 4,6 mm, 5 µM) or a Phenomenex Aeris Widepore  
109 column (150 x 4,6 mm, 3,6 µM), both used at 50°C. The mobile phases consisted of A/acetonitrile  
110 and B/0,1 M TEAA buffer containing 5% acetonitrile. Masses of the oligonucleotides were  
111 determined by MALDI-TOF analysis on an ABI Voyager DE-STR MALDI-TOF. The  
112 oligonucleotide samples were mixed with a matrix consisting of 3-hydroxypicolinic acid and  
113 ammonium citrate present in a 9:1 ratio, in a 1:1 sample:matrix ratio and a 1:2 ratio for the  
114 methylene blue conjugated oligonucleotides and the cross-linked species. After mixing with the  
115 matrix, the samples were desalted by treatment with DOWEX beads. The samples were spotted on  
116 a MALDI plate, together with a commercial oligonucleotide sequence with a known mass (5'-  
117 GCA TCT CGT CAG-3'), purchased from Eurogentec, for calibration of the measurement. The  
118 concentrations of the oligonucleotides were measured on the Trinean DropSense96 UV/VIS  
119 droplet reader. The various sequences which were synthesized are presented in table 1 and will be  
120 referred to by their assigned name in what follows.



121

122 **Figure 2:** Structure of the furan and PS modified building blocks incorporated in the oligonucleotide  
123 sequences. (X): furan building block, (M) methylene blue building block

124

125

126

127

128

129 **Table 1:** Overview of the synthesized oligonucleotide sequences. The furan building block incorporated in the  
 130 sequences is presented in Figure 2, **X**. The methylene blue building block is presented in figure 2, **M**. Both the  
 131 non-modified sequences and the methylene blue modified sequences are presented from 5' to 3'. The furan  
 132 modified sequences are presented from 3' to 5' to highlight the complementarity.  
 133

Non-modified sequences (5'-3')	
ON1	5'-GCA CCC CGT CAG-3'
ON2	5'-GTA CCC TGT CTG-3'
Furan ( <b>X</b> ) modified sequences (3'-5')	
FON1	3'-CGT GXG GCA GTC-5'
FON2	3'-CAT GXG ACA GAC-5'
Methylene blue ( <b>M</b> ) modified sequences (5'-3')	
MON1	5'-GTA CCC MGT CTG-3'
MON2	5'-GMA CCC TGT CTG-3'
MON3	5'-GTA CCC TGM CTG-3'
MON4	5'-GTA CCC TGT CMG-3'

134

135 **2.3. General procedure for conjugation of methylene blue to an oligonucleotide:** The  
 136 coupling procedure was based on the work of the group of K. Weisz [21] after which the  
 137 methylene blue conjugated oligonucleotide was obtained by purification via RP-HPLC (linear  
 138 gradient: 0 – 20% ACN in 30 min) (Supporting information, figure S6). The peak belonging to the  
 139 product, which absorbed both at 260 nm and 600 nm, was collected and analyzed by MALDI-MS,  
 140 indicating the correct product: exact mass = 4102,87 Da.; observed mass (MON1) = 4101,76 Da.;  
 141 observed mass (MON2) = 4103,86 Da.; observed mass (MON3) = 4103,73 Da.; observed mass  
 142 (MON4) = 4103,01 Da. HPLC and MALDI TOF-MS data can be found in the supporting  
 143 information (Fig S8, S9, S10 and S11).

144 **2.4. Cross-link protocol:** Cross-link reactions were carried out at 20 µM oligonucleotide  
 145 concentration (standard conditions in all previous experiments) and at an oligonucleotide  
 146 concentration of 2 µM (and 1 µM described in the supporting information, section 5). The



147 oligonucleotides were dissolved in a 10 mM phosphate buffer (pH 7) containing 10 mM of sodium  
148 chloride. All cross-link experiments were repeated three times to ensure reliable and reproducible  
149 results. When cross-linking with the photosensitizer in solution, the sensitizer was added to the  
150 sample just before starting the reaction. With the diluted samples, cross-linking was preceded by  
151 an annealing procedure. This implied heating the samples to 95°C and keeping them at this  
152 temperature for 30 minutes while shaking. After this, the samples were allowed to slowly cool to  
153 room temperature over a time span of 3 hours, ensuring correct duplex hybridization. During the  
154 cross-link reaction the temperature was kept constant at 25°C, in an Eppendorf Thermomixer  
155 comfort with constant shaking at 950 rpm. The cross-link temperature is kept under the melting  
156 temperatures of the used oligonucleotide duplexes ensuring duplex formation at these conditions  
157 (Supporting information fig. S19). Irradiation of the samples was done using a Euromex fiber  
158 optic light source Ek-1 equipped with a color filter dependent on the used photosensitizer and  
159 placed 1 cm of the sample. For methylene blue and zinc phthalocyanine, the samples were  
160 irradiated with red light. For rose bengal, green light and for ruthenium tris bipyridinium blue light  
161 was used.

162 **2.5. Cross-link analysis:** Samples were taken at different sampling times. At every sampling  
163 point a sample was taken for RP-HPLC analysis and gel electrophoresis.

164 *RP-HPLC analysis:* Cross-link samples with the photosensitizer added in solution were measured  
165 with the RP-HPLC equipped with a Clarity C18 (linear gradient: 0 – 20% ACN in 30 min). Cross-  
166 link samples with the photosensitizer conjugated to the oligonucleotide were measured with the  
167 RP-HPLC equipped with an Aeris C18 column (linear gradient: 0-20% ACN in 45 min). Cross-  
168 link yields were determined by integration of the corresponding peaks in the HPLC-chromatogram  
169 and by comparing the area of the peak of the cross-linked species with the peak of the limiting

170 single oligonucleotide strand, both corrected for their extinction coefficient. The extinction  
171 coefficient of the duplex was calculated based on the method described by R. Owczarzy *et al.* [22].  
172 *Gel electrophoresis:* Samples were analyzed by denaturing gel electrophoresis. The gels were  
173 prepared by dissolving 4,2 g urea in 5 mL acrylamide:bisacrylamide (19:1) and 1 mL 10x TBE  
174 buffer. 100  $\mu$ L of a 0,5 M solution of ammonium persulphate was added and the solution was  
175 diluted to 10 mL with milliQ water. After cooling of the solution, N,N,N',N'-  
176 tetramethylethylenediamine was added and the sample was poured between glass plates after  
177 which it was allowed to polymerize for one hour. The gels were subjected to a pre-run in the  
178 consort EV202 at a voltage power of 225V for half an hour. After mixing the samples with  
179 formamide in 9:1 formamide:sample ratio, they were loaded on the gel. During a run of  
180 approximately one hour, the temperature was kept at 25°C with a Julabo F12. The gels were  
181 stained with GelRed<sup>TM</sup> Nucleic acid gel stain (VWR) and photographed using an Autochemi  
182 imaging system.

### 183 **3. RESULTS AND DISCUSSION**

#### 184 **3.1 Selection of the most promising system: cross-linking with the photosensitizer in** 185 **solution**

186 We previously reported on a selective and high-yielding methodology for cross-linking of  
187 oligonucleotides using a furan modified oligonucleotide probe [12,13,14,23]. Various furan  
188 building blocks have been subjected to an extensive evaluation of their cross-linking  
189 properties. The high-yielding furan phenyl acyclic building block (Fig 2, **X**) was chosen out of  
190 this set for incorporation in the oligonucleotides in the current study. The observed cross-link

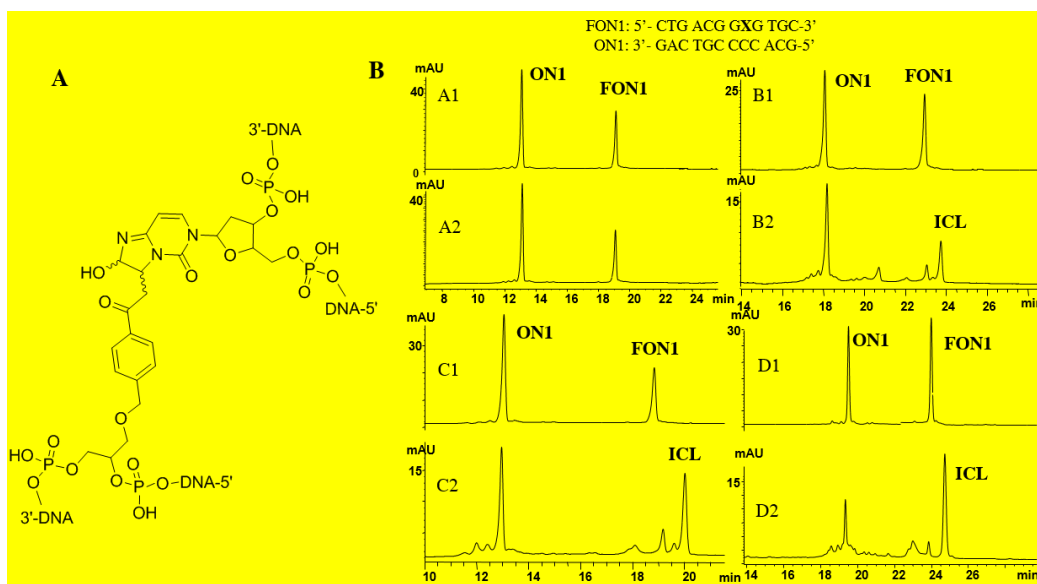
191 yield was not only dependant on the structure of the furan moiety, but also on the exact nature  
192 of the complementary base. As is depicted mechanistically in figure 1, the exocyclic amines  
193 present on cytosine (C) and adenine (A) can react with the oxidized furan moiety with the  
194 formation of an interstrand cross-link (ICL). Due to the lack of an amino group, thymine  
195 cannot act as a reaction partner. Interestingly, it was previously observed that guanine was not  
196 able to form cross-links, which can possibly be attributed to the lack of proximity between its  
197 exocyclic amino group and the furan moiety. Following this information, cytosine was chosen  
198 as complementary base in the study presented in this paper.

199 Oxidation of furan with singlet oxygen, followed by cross-linking, was already proven  
200 successful and reported earlier [15]. Still, a more extensive optimization of this strategy had to  
201 be undertaken. Therefore, in first instance, a set of different photosensitizers was tested in  
202 solution to select the most promising candidate for conjugation and subsequent cross-linking.  
203 **Previously, we have extensively investigated the potential occurrence of collateral oxidative**  
204 **damage during the cross-link reactions. It was shown that under the currently used carefully**  
205 **fine-tuned conditions, no 8-oxo dG species were formed [15].**

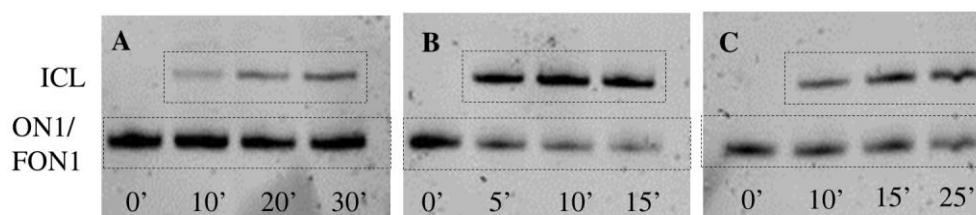
206 Four photosensitizers (zinc phthalocyanine, ruthenium tris bipyridinium, methylene  
207 blue and rose bengal) were tested on their capabilities to induce a cross-link between the furan  
208 modified oligonucleotide FON1 and its non-modified complement ON1 (table 1). Both the  
209 modified and the complementary sequence were synthesized using automated DNA synthesis.  
210 To incorporate the furan phosphoramidite, the synthesis was interrupted by a manual coupling  
211 step as described in the experimental section. To evaluate the cross-linking ability of the  
212 various photosensitizers, the reaction was followed using both RP-HPLC and denaturing

213 polyacrylamide gel electrophoresis. Samples consisted of 20  $\mu\text{M}$  of the duplex, while various  
214 photosensitizer concentrations were tested.

215 It was observed that zinc phthalocyanine could not induce a cross-link reaction between  
216 FON1 and ON1 during irradiation with red light (figure 3B A1/A2). Furthermore, no  
217 degradation of both the furan modified oligonucleotide nor its complement could be observed,  
218 demonstrating the absence of singlet oxygen in the reaction mixture. This behaviour can be  
219 explained by the lack of solubility of zinc phthalocyanine in the aqueous medium. Ruthenium  
220 tris bipyridinium did induce cross-linking between FON1 and ON1 after irradiation with blue  
221 light. Concentrations of 1  $\mu\text{M}$ , 2  $\mu\text{M}$  and 5  $\mu\text{M}$  of the photosensitizer were tested, where the  
222 latter concentration led to cross-link yields of 16% after 30 minute irradiation (HPLC data  
223 presented in figure 3B B1/B2, denaturing PAGE in figure 4A). Rose bengal was examined  
224 in concentrations gradually increasing from 0,5  $\mu\text{M}$  until 5  $\mu\text{M}$ , where it was noted that a  
225 concentration of 0,5  $\mu\text{M}$  under green light irradiation led to cross-link yields up to 40 %. These  
226 high yields were reached after two hours of irradiation and were accompanied by minimal  
227 degradation. Increasing the concentration of the photosensitizer went hand in hand with faster  
228 reactions. By using only 1  $\mu\text{M}$  of rose bengal, yields up to 35 % were reached after 15 minutes  
229 of irradiation (HPLC data presented in figure 3B C1/C2, denaturing PAGE in figure 4B).  
230 When using methylene blue, 25 minutes of red light irradiation resulted in a yield of 53%  
231 (HPLC data presented in figure 3B D1/D2, denaturing PAGE in figure 4C). These high  
232 yields together with its medical relevance, pointed out methylene blue as most promising  
233 sensitizer for furan based oligonucleotide cross-linking.



234  
 235 **Figure 3:** A. Chemical structure of the formed cross-linked species. B. RP-HPLC traces of the cross-link  
 236 reaction mixtures containing 20  $\mu\text{M}$  of the duplex. (A1, B1, C1, D1): Furan modified **FON1** and  
 237 complementary sequence **ON1** before oxidation. (A2): After <sup>1</sup>O<sub>2</sub> oxidation with zinc phthalocyanine (1  
 238  $\mu\text{M}$ , 90 min, ICL yield: 0%), (B2): After <sup>1</sup>O<sub>2</sub> oxidation with ruthenium tris bipyridinium (5  $\mu\text{M}$ , 30  
 239 min, ICL yield: 16%), (C2): After <sup>1</sup>O<sub>2</sub> oxidation with rose bengal (1  $\mu\text{M}$ , 15 min, ICL yield: 35%),  
 240 (D2): After <sup>1</sup>O<sub>2</sub> oxidation with and methylene blue (5  $\mu\text{M}$ , 25 min, ICL yield: 53%). Formation of a  
 241 new peak is observed in B2, C2 and D2 which belongs to the interstrand cross-link (ICL).



242  
 243 **Figure 4:** Denaturing PAGE analysis of the cross-link reaction mixtures containing 20  $\mu\text{M}$  of the  
 244 duplex. All samples contain the furan modified **FON1** and complementary sequence **ON1** before  
 245 oxidation. All samples were irradiated with a characteristic wavelength for a period as depicted in the  
 246 figure in presence of the photosensitizer: (A): ruthenium tris bipyridinium (5  $\mu\text{M}$ , blue light, ICL yield:  
 247 16%), (B): rose bengal (1  $\mu\text{M}$ , green light, ICL yield: 35%) and (C): methylene blue (5  $\mu\text{M}$ , red light,  
 248 ICL yield: 53%).  
 249

250 **3.1.1. Towards lower oligonucleotide concentrations with methylene blue in solution.** In a

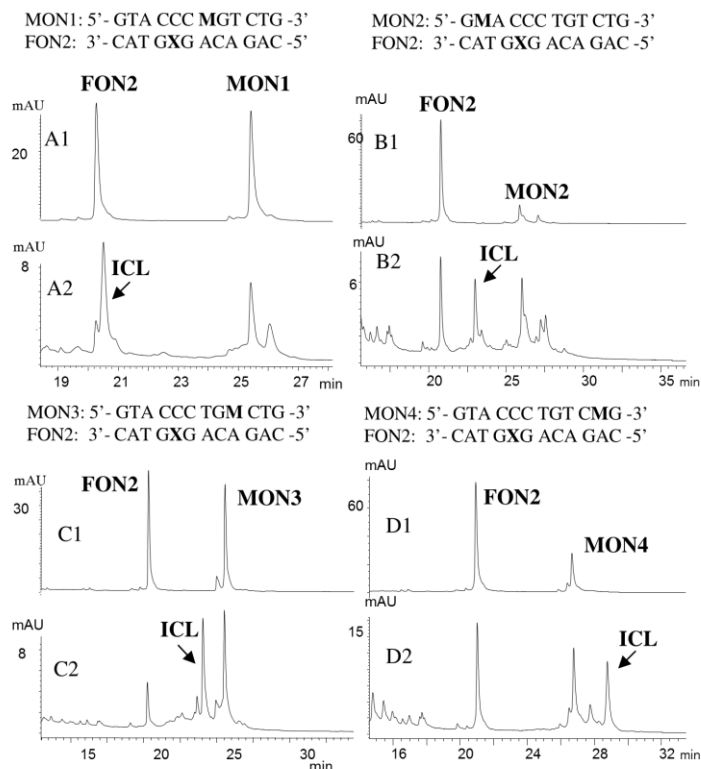
251 cellular context, when using high oligonucleotide concentrations, the selectivity for recognition  
 252 of a specific sequence can substantially decrease (typically, intracellular concentrations above  
 253 20  $\mu\text{M}$  have been shown to lead to non-sequence specific binding of oligonucleotides) [18].  
 254 Therefore, it has to be verified whether our furan-cross-linking system still functions at higher

255 dilution. A sample was prepared with FON2 and its complement ON2 (table 1), with the  
256 oligonucleotide concentration as well as the methylene blue concentration lowered to 2  $\mu$ M. A  
257 new, thymine-rich sequence (ON2) was chosen to allow the later incorporation of the  
258 methylene blue modified thymine at different positions. It was seen that even after 50 minutes  
259 of irradiation of the sample with red light only a minimal amount of cross-link was formed (as  
260 depicted in fig S4 B1/B2/B3 in the supporting information). This result can be explained by the  
261 high dilution, resulting in a low local concentration of singlet oxygen around the furan moiety.  
262 Indeed, when methylene blue moves freely through the solution, distances between furan and  
263 methylene blue can increase in such a way that they become too large for singlet oxygen to  
264 cross. In order to ensure proximity between methylene blue and the furan modified  
265 oligonucleotide, methylene blue was conjugated to the strand complementary to the furan  
266 modified sequence.

### 267 **3.2 Cross-linking experiments with methylene blue immobilized on an** 268 **oligonucleotide strand**

269 Before studying the cross-link formation with diluted oligonucleotide samples, it had to be  
270 evaluated whether methylene blue, when attached to an oligonucleotide, is still able to generate  
271 a sufficient amount of singlet oxygen to induce ICL formation. To evaluate this, methylene  
272 blue was attached to an oligonucleotide sequence by modifying a thymine base. To assess the  
273 influence of the position of methylene blue on duplex formation and on the cross-linking  
274 process, it was incorporated at four different positions so that within each modified duplex a  
275 different distance between the furan building block and methylene blue is ensured. Amino  
276 modified oligonucleotides were obtained through automated DNA synthesis, using a

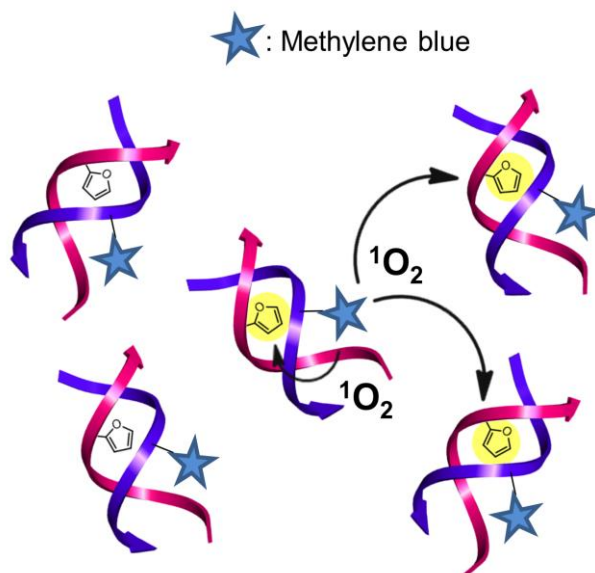
277 commercially available amino modified thymidine phosphoramidite (containing the rather long  
278 CH<sub>2</sub>CONH(CH<sub>2</sub>)<sub>6</sub>-NH<sub>2</sub>-linker, figure 2 (M)) was incorporated by manual coupling.  
279 Conjugation of the methylene blue derivative s2 (supporting information, fig S5) with the  
280 oligonucleotide could be achieved by reacting the amino modified oligonucleotide with the  
281 succinimide ester of methylene blue. Four methylene blue modified sequences were  
282 synthesized, MON1, MON2, MON3 and MON4 (table 1), all complementary to the furan  
283 modified oligonucleotide FON2. Only one nucleotide is positioned between methylene blue  
284 and furan when cross-linking in sequence MON1, two nucleotides in MON2, three in MON3  
285 and five in MON4. The composition of the cross-linking samples was identical to the samples  
286 discussed in previous paragraphs, all containing 20 µM of the oligonucleotide duplex but  
287 lacking the methylene blue added in solution. When comparing the maximum cross-linking  
288 yields of MON1 (figure 5 A1/A2), MON2 (figure 5 B1/B2), MON3 (figure 5 C1/C2) and  
289 MON4 (figure 5 D1/D2) to FON2, it can be seen that a maximum yield of approximately 20%  
290 is reached in all cases at an irradiation of 3,5 minutes to 5 minutes. This result shows that the  
291 conjugated methylene blue is able to promote the formation of an ICL. The minor differences  
292 in the observed cross-link yields do not show a trend with reference to the varying distance  
293 between furan and methylene blue. This lack of correlation can be due to the higher  
294 oligonucleotide concentration of the samples. Both singlet oxygen generated by MB on the  
295 opposite strand and singlet oxygen generated by a nearby duplex can be expected to induce  
296 furan oxidation, as depicted schematically in figure 6.



297  
298  
299  
300  
301  
302  
303  
304  
305  
306  
307

**Figure 5:** RP-HPLC of the 20  $\mu$ M cross-link reaction mixtures. (A1): Furan modified **FON2** and the complementary methylene blue modified **MON1** before oxidation, (A2): After  $^1\text{O}_2$  oxidation (3,5 min, ICL yield: 22%) (B1): Furan modified **FON2** and the complementary methylene blue modified **MON2** before oxidation, (B2): After  $^1\text{O}_2$  oxidation (5 min, ICL yield: 26%), (C1): Furan modified **FON2** and the complementary methylene blue modified **MON3** before oxidation, (C2): After  $^1\text{O}_2$  oxidation (3,5 min, ICL yield: 22%) (D1): Furan modified **FON2** and the complementary methylene blue modified **MON4** before oxidation, (D2): After  $^1\text{O}_2$  oxidation (5 min, ICL yield: 23%). A new peak appears in the spectrum which is assigned to the interstrand cross-link (**ICL**). Yields are calculated by comparing the peak area of the ICL with the limiting single strand, both corrected for their extinction factor. **Due to the different structures of the formed ICLs, depending on the position of methylene blue on the cross-linked duplex, different retention times of the cross-linked species are observed.**





308  
309  
310  
311

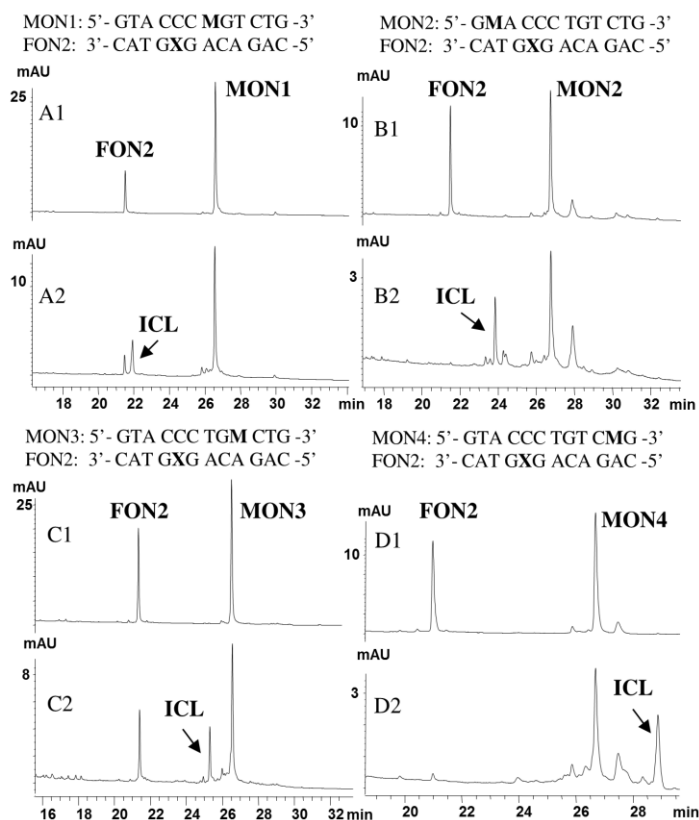
**Figure 6:** Schematic representation of the oxidation of the furan unit by both singlet oxygen generated by methylene blue present on the complementary strand as well as methylene blue present on other duplexes.

312 The cross-link reaction was also analyzed by denaturing PAGE experiments (supporting  
313 information, figure S13). Even though similar yields are obtained as in case of cross-linking  
314 with the photosensitizer in solution, these gels do not show clear bands. Indeed, methylene  
315 blue absorbs light in the same region as where the GelRed™ nucleic acid stain emits light.  
316 Therefore, visualization of methylene blue conjugated oligonucleotides and their  
317 corresponding methylene blue conjugated duplexes required higher sample loading, which  
318 results in bands which are not as delineated and clear as in case of their non-methylene blue  
319 modified analogues.

### 320 3.2.2 *Towards lower oligonucleotide concentrations with a conjugated methylene blue*

321 *moiety.* It was shown that cross-linking of oligonucleotides became very low-yielding when  
322 diluting the samples (vide supra). Since this observation probably can be explained by the low  
323 local photosensitizer concentration resulting from the large distance between methylene blue  
324 and furan, it was expected that by attaching methylene blue to the duplex, higher yields could

325 be obtained. Indeed, methylene blue and furan are now forced to remain in close proximity.  
 326 MON1, MON2, MON3, MON4 and the furan modified FON2 were mixed in a 2  $\mu$ M  
 327 concentration, retaining the same sample composition as previously described. Maximum  
 328 yields were obtained after two to five minutes of irradiation with red light. Here, in contrast to  
 329 the experiments carried out at low concentration with an externally added photosensitizer,  
 330 quite efficient cross-linking could be achieved. Also, different yields were obtained depending  
 331 on the exact position of the conjugated methylene blue. A yield of 33% was reached when one  
 332 base pair is located between MB and furan (figure 7 A1/A2). With two base pairs, yields up to  
 333 24% were observed (figure 7 B1/B2) and with three base pairs only 12% cross-link is formed  
 334 (figure 7 C1/C2). When five base pairs are located between the furan unit and methylene blue,  
 335 again a yield of 28% is reached (figure 7 D1/D2).



336

337 **Figure 7:** RP-HPLC of the diluted 2  $\mu\text{M}$  cross-link reaction mixtures. (A1): Furan modified **FON2** and the  
338 complementary methylene blue modified **MON1** before oxidation, (A2): After  $^1\text{O}_2$  oxidation (5 min, ICL yield:  
339 33%),(B1): Furan modified **FON2** and the complementary **MON2** before oxidation, (B2): After  $^1\text{O}_2$  oxidation (2  
340 min, ICL yield: 24%) (C1): Furan modified **FON2** and the complementary methylene blue modified **MON3**  
341 before oxidation, (C2): After  $^1\text{O}_2$  oxidation (5 min, ICL yield: 12%) (D1): Furan modified **FON2** and the  
342 complementary methylene blue modified **MON4** before oxidation, (D2): After  $^1\text{O}_2$  oxidation (3,5 min, ICL yield:  
343 28%). The new peak appearing in the spectrum belongs to the interstrand cross-link (**ICL**). Yields are calculated  
344 by comparing the peak area of the ICL with the limiting single strand, both corrected for their extinction factor.

345 This fluctuation of cross-link yield shows that not only the distance between methylene blue  
346 and furan influences the yield, but other factors are of importance. When one base pair is  
347 located between furan and methylene blue, the highest cross-link yields are obtained. This can  
348 be accounted to the high proximity between furan and methylene blue and thus the short  
349 distance singlet oxygen has to travel to oxidize the furan unit, leading to ICL formation.

350 Increasing the number of base pairs between the furan moiety and the sensitizer gradually  
351 decreases the cross-link yield. Whereas for both the MON1-FON2 and MON2-FON2  
352 duplexes,  $T_m$  analysis shows a clear stabilization of the duplex compared to ON2-FON2,  
353 indicating methylene blue intercalation, this is clearly not the case for the MON3-FON2 and  
354 MON4-FON2 duplexes (supporting information, figure S19, table S3). As both these duplexes  
355 feature a longer distance between the furan and methylene blue moiety, as well as the absence  
356 of methylene blue intercalation, it is surprising to see that the observed ICL yield increases  
357 again (MON4-FON2: 28% yield versus MON3-FON2: 13% yield), a trend which is  
358 consistently observed (as also shown for 1  $\mu\text{M}$  experiments described in the supporting  
359 information section 5, figure S20). The explanation of this unexpected increase in yield can  
360 perhaps be found in an alternative way of binding between the methylene blue unit and the  
361 duplex. Methylene blue is known to also electrostatically interact with oligonucleotide  
362 backbones<sup>24</sup>, which does not influence the duplex stability and thus the  $T_m$  behavior. The  
363 larger non-disturbed sequence between methylene blue and furan in MON4-FON2 potentially

364 promotes electrostatic methylene blue-duplex association, thus increasing again proximity  
365 between furan and the  $^1\text{O}_2$ -generating moiety, potentially explaining the observed trends.

#### 366 **4. CONCLUSIONS**

367 Whereas at 20  $\mu\text{M}$  of oligonucleotides and photosensitizer, furan oxidation based cross-linking  
368 leads to efficient formation of interstrand cross-linked species, for reactions at 2  $\mu\text{M}$   
369 concentration, only a minimal amount of cross-link could be observed with methylene blue in  
370 solution. To further investigate furan mediated cross-linking of oligonucleotides in highly  
371 diluted samples, methylene blue was attached to an oligonucleotide complementary to the  
372 furan modified strand. Irradiation of methylene blue with red light resulted in the formation of  
373 singlet oxygen in near proximity of the furan group to be oxidized. This led to a considerable  
374 increase in the formation of interstrand cross-links in comparison to the system where  
375 methylene blue was present in a 2  $\mu\text{M}$  solution. Apparently, the distance for singlet oxygen to  
376 travel in order to oxidize the furan unit and cause cross-linking became too large when  
377 methylene blue was present in solution in low concentration. Connecting the methylene blue  
378 moiety to the duplex restored the cross-linking capacity. It was further observed that the  
379 position of methylene blue on the oligonucleotide strand had a significant influence on the  
380 cross-link yields.

381 **ACKNOWLEDGMENTS:** The research leading to these results has received funding  
382 from the Agency for Innovation by Science and Technology (IWT) and from the European  
383 Seventh Framework Programme (FP7/2007-2013/) under grant agreement n° 316975.

384 **SUPPLEMENTARY MATERIALS**

385 Figure S1 to S18 can be found at DOI: to be inserted

386 **REFERENCES**

387 [1] T. Lindahl. Instability and decay of the primary structure of DNA. *Nature*. 362 (1993)  
388 709–715.

389 [2] S. Boiteux, M. Guillet. Abasic sites in DNA: repair and biological consequences in  
390 *Saccharomyces cerevisiae*. *DNA Repair*. 3 (2004) 1–12.

391 [3] S. Dutta, G. Chowdhury, K.S. Gates. Interstrand Cross-Links Generated by Abasic  
392 Sites in Duplex DNA. *J. Am. Chem. Soc.* 129 (2007) 1852–1853.

393 [4] D.M. Noll, T.M. Mason, P.S. Miller. Formation and Repair of Interstrand Cross-Links  
394 in DNA. *Chem. Rev.* 160 (2006) 277–301.

395 [5] J. Krützfeldt, N. Rajewsky, R. Braich, K.G. Rajeev, T. Tuschl, M. Manoharan, et al.  
396 Silencing of microRNAs in vivo with “antagomirs”. *Nature*. 438 (2005) 685–689.

397 [6] C. Esau, S. Davis, S.F. Murray, X.X. Yu, S.K. Pandey, M. Pear, et al. miR-122  
398 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* 3 (2006)  
399 87–98.

400 [7] I.S. Hong, M.M. Greenberg. Efficient DNA Interstrand Cross-Link Formation from a  
401 Nucleotide Radical. *J. Am. Chem. Soc.* 127 (2005) 3692–3693.

- 402 [8] K. Fujimoto, A. Yamada, Y. Yoshimura, T. Tsukaguchi, T. Sakamoto. Details of the  
403 ultrafast DNA photo-cross-linking reaction of 3-cyanovinylcarbazole nucleoside: cis-trans  
404 isomeric effect and the application for SNP-based genotyping. *J. Am. Chem. Soc.* 135 (2013)  
405 16161–16167.
- 406 [9] R.S. Cole. Light-induced cross-linking of DNA in the presence of a furocoumarin  
407 (psoralen): Studies with phage  $\lambda$ , *Escherichia coli*, and mouse leukemia cells. *Biochim.*  
408 *Biophys. Acta.* 217 (1970) 30–39.
- 409 [10] S. Schumm, C. Moucheron, P. Dumy, A.K. Mesmaeker. Photocrosslinking in  
410 Ruthenium-Labelled Duplex Oligonucleotides. *Chembiochem.* 4 (2003) 195–202.
- 411 [11] C. Saintomé, P. Clivio, A. Favre, J. Fourrey, P. Laugâa. Site specific photo-  
412 crosslinking of single stranded oligonucleotides by a complementary sequence equipped with  
413 an internal photoactive probe. *Chem. Commun.* (1997) 167–168.
- 414 [12] M. Op de Beeck, A. Madder. Unprecedented C-selective interstrand cross-linking  
415 through in situ oxidation of furan-modified oligodeoxynucleotides. *J. Am. Chem. Soc.* 133  
416 (2011) 796–807.
- 417 [13] K. Stevens, D.D. Claeys, S. Catak, S. Figaroli, M. Hocek, J.M. Tromp, et al. Furan-  
418 oxidation-triggered inducible DNA cross-linking: acyclic versus cyclic furan-containing  
419 building blocks--on the benefit of restoring the cyclic sugar backbone. *Chem. a Eur. J.* 17  
420 (2011) 6940–53.

- 421 [14] K. Stevens, A. Madder. Furan-modified oligonucleotides for fast, high-yielding and  
422 site-selective DNA inter-strand cross-linking with non-modified complements. *Nucleic Acids*  
423 *Res.* 37 (2009) 1555–1565.
- 424 [15] M. Op De Beeck, A. Madder. Sequence Specific DNA Cross-Linking Triggered by  
425 Visible Light. *J. Am. Chem. Soc.* 134 (2012) 10737–10740.
- 426 [16] T. Montagnon, D. Kalaitzakis, M. Triantafyllakis, M. Stratakis, G. Vassilikogiannakis.  
427 Furans and singlet oxygen - why there is more to come from this powerful partnership. *Chem*  
428 *Commun.* 50 (2014) 15480-15498.
- 429 [17] M.C. Derosa, R.J. Crutchley. Photosensitized singlet oxygen and its applications.  
430 *Coord. Chem. Rev.* 233 - 234 (2002) 351–371.
- 431 [18] C.A. Stein. The experimental use of antisense oligonucleotides: a guide for the  
432 perplexed. *J. Clin. Invest.* 108 (2001) 641–644.
- 433 [19]. P.B. Merkel, R. Nilsson, D. R. Kearns. Deuterium effects on singlet oxygen lifetimes in  
434 solutions. New test of singlet oxygen reactions. *J. Am. Chem. Soc.* 94 (1972) 1030-2031.
- 435 [20] C.G. Pheaney, J.K. Barton. DNA Electrochemistry with Tethered Methylene Blue.  
436 *Langmuir.* 28 (2013) 7063–7070.
- 437 [21] M. Purwanto, K. Weisz. Synthesis and covalent attachment of a methylene blue  
438 derivative to a triple helix forming oligonucleotide - The way to new anticancer drugs. *Synerg.*  
439 *Netw. Role Fundam. Res. Dev. Asean.* (2011) 163–169.

440 [22] A. V Tataurov, Y. You, R. Owczarzy. Predicting ultraviolet spectrum of single  
441 stranded and double stranded deoxyribonucleic acids. *Biophys. Chem.* 133 (2008) 66–70.

442 [23] A.M. Jawalekar, M. Op de Beeck, F.L. van Delft, A. Madder. Synthesis and  
443 incorporation of a furan-modified adenosine building block for DNA interstrand crosslinking.  
444 *Chem. Commun.* 47 (2011) 2796–2798.

445 [24] P.O. Vardevanyan, A. P. Antonyan, M. A. Parsadanyan, M. A. Shahinyan, L. A.  
446 Hambardzumyan, Mechanisms for Binding between Methylene Blue and DNA, *J. Appl.*  
447 *Spectrosc.* 80 (2013) 595–599.

## 448 **FIGURE CAPTIONS**

449 **Figure 1:** Schematic representation of furan mediated DNA cross-linking. The furan group can  
450 be oxidized using NBS or singlet oxygen ( $^1O_2$ ). After oxidation, the formed reactive moiety  
451 can react with exocyclic amines of the opposite base on the complementary strand. Both  
452 adenine (A) and cytosine (C) contain an exocyclic amine which is located in close proximity of  
453 the furan building block, leading to the formation of a covalent bond as depicted on the right-  
454 hand side of the figure.

455 **Figure 2** Figure 2: Structure of the furan and PS modified building blocks incorporated in the  
456 oligonucleotide sequences. (X): furan building block, (M) methylene blue building block

457 **Figure 3** A. Chemical structure of the formed cross-linked species. B. RP-HPLC traces of the  
458 cross-link reaction mixtures containing 20  $\mu$ M of the duplex. (A1, B1, C1, D1): Furan modified



459 **FON1** and complementary sequence **ON1** before oxidation. (A2): After  $^1\text{O}_2$  oxidation with  
460 zinc phthalocyanine (1  $\mu\text{M}$ , 90 min, ICL yield: 0%), (B2): After  $^1\text{O}_2$  oxidation with ruthenium  
461 tris bipyridinium (5  $\mu\text{M}$ , 30 min, ICL yield: 16%), (C2): After  $^1\text{O}_2$  oxidation with rose bengal  
462 (1  $\mu\text{M}$ , 15 min, ICL yield: 35%), (D2): After  $^1\text{O}_2$  oxidation with and methylene blue (5  $\mu\text{M}$ , 25  
463 min, ICL yield: 53%). Formation of a new peak is observed in B2, C2 and D2 which belongs  
464 to the interstrand cross-link (**ICL**).

465 **Figure 4** Denaturing PAGE analysis of the cross-link reaction mixtures containing 20  $\mu\text{M}$  of the  
466 duplex. All samples contain the furan modified **FON1** and complementary sequence **ON1**  
467 before oxidation. All samples were irradiated with a characteristic wavelength for a period as  
468 depicted in the figure in presence of the photosensitizer: (A): ruthenium tris bipyridinium (5  
469  $\mu\text{M}$ , blue light, ICL yield: 16%), (B): rose bengal (1  $\mu\text{M}$ , green light, ICL yield: 35%) and (C):  
470 methylene blue (5  $\mu\text{M}$ , red light, ICL yield: 53%).

471 **Figure 5.** RP-HPLC of the 20  $\mu\text{M}$  cross-link reaction mixtures. (A1): Furan modified **FON2**  
472 and the complementary methylene blue modified **MON1** before oxidation, (A2): After  $^1\text{O}_2$   
473 oxidation (3,5 min, ICL yield: 22%) (B1): Furan modified **FON2** and the complementary  
474 **MON2** before oxidation, (B2): After  $^1\text{O}_2$  oxidation (5 min, ICL yield: 26%), (C1): Furan  
475 modified **FON2** and the complementary methylene blue modified **MON3** before oxidation,  
476 (C2): After  $^1\text{O}_2$  oxidation (3,5 min, ICL yield: 22%) (D1): Furan modified **FON2** and the  
477 complementary methylene blue modified **MON4** before oxidation, (D2): After  $^1\text{O}_2$  oxidation  
478 (5 min, ICL yield: 23%). A new peak appears in the spectrum which is assigned to the  
479 interstrand cross-link (**ICL**). Yields are calculated by comparing the peak area of the ICL with  
480 the limiting single strand, both corrected for their extinction factor. Due to the different

481 structures of the formed ICLs, depending on the position of methylene blue, different retention  
482 times of the cross-linked species are observed.

483 **Figure 8:** Schematic representation of the oxidation of the furan unit by both singlet oxygen  
484 generated by methylene blue present on the opposite strand as well as methylene blue present  
485 on other duplexes.

486 **Figure 7.** RP- HPLC of the diluted 2  $\mu$ M cross-link reaction mixtures. (A1): Furan modified  
487 **FON2** and the complementary methylene blue modified **MON1** before oxidation, (A2): After  
488  $^1\text{O}_2$  oxidation (5 min, ICL yield: 33%), (B1): Furan modified **FON2** and the complementary  
489 **MON2** before oxidation, (B2): After  $^1\text{O}_2$  oxidation (2 min, ICL yield: 24%) (C1): Furan  
490 modified **FON2** and the complementary methylene blue modified **MON3** before oxidation,  
491 (C2): After  $^1\text{O}_2$  oxidation (5 min, ICL yield: 12%) (D1): Furan modified **FON2** and the  
492 complementary methylene blue modified **MON4** before oxidation, (D2): After  $^1\text{O}_2$  oxidation  
493 (3,5 min, ICL yield: 28%). The new peak appearing in the spectrum belongs to the interstrand  
494 cross-link (**ICL**). Yields are calculated by comparing the peak area of the ICL with the limiting  
495 single strand, both corrected for their extinction factor.