JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Sequence Specific DNA Cross-Linking Triggered by Visible Light

Marieke Op de Beeck and Annemieke Madder*

Laboratory for Organic and Biomimetic Chemistry, University of Ghent, Krijgslaan 281 S4, B-9000 Ghent, Belgium

Supporting Information

ABSTRACT: A new biocompatible strategy for photoinduced DNA interstrand cross-linking is presented. Methylene blue induced $^1O_2$ formation triggers furan oxidation; the resulting aldehyde then rapidly reacts with complementary A or C with formation of stable adducts. Easily accessible furan modified nucleosides, a commercially available photosensitizer, and visible light irradiation constitute the necessary tools to achieve selective duplex interstrand cross-linking.

Errors in gene expression are an important cause of diseases (e.g., cancer) and methods for selectively modulating transcription and translation are therefore highly desirable. In this respect, the artificial control of gene expression by synthetic oligodeoxynucleotides (ODNs) has been a major research focus with a particular interest in photo activatable ODNs, as their activation can be brought under spatiotemporal control.[1] One of the most irreparable events in nucleic acid based therapies and therefore often exploited in antitumor treatment, is the formation of interstrand cross-links which prevent strand separation during transcription. Photo cross-linking psoralen derivatives which can lead to cross-link formation upon UV irradiation enjoyed major success[2] and were rapidly followed by a myriad of constructs allowing for selective photo controlled nucleic acid targeting.[3] More recently, the benefit of using the less harmful visible light spectrum to trigger cross-link formation has been recognized.[4] In the presence of a suitable sensitizer, visible light irradiation can generate singlet oxygen ($^1O_2$) from ground state triplet oxygen. This process already finds widespread use in photodynamic therapy (PDT) where singlet oxygen's oxidative power is exploited in the treatment of cancer.[5]

Recently, we have developed an oxidation triggered cross-linking method based on N-bromosuccinimide (NBS) induced selective conversion of a furan moiety within ODNs into a reactive aldehyde allowing for fast and efficient formation of interstrand cross-links.[6] Though cross-linking, mediated by NBS induced furan oxidation, could be achieved with exceptionally high yield[6a] and selectivity,[6b] obviously the requirement for NBS treatment severely limits the development of biological applications of this methodology.

In search for a biocompatible oxidation method, $^1O_2$ was identified as a potent and promising alternative oxidant. Though in most cases DNA is not a major target in PDT therapy and rather involves destruction of other cell material, some sensitizers have been shown to accumulate in the cell nucleus and to bind DNA leading to DNA damage upon irradiation.[7] Therefore, the use of $^1O_2$ in an ODN context might thus seem counterintuitive. $^1O_2$ is known to oxidize furan derivatives under various conditions, enals being among the oxidation products formed.[8] Very recently, the use of $^1O_2$ based furan oxidation in water was elegantly illustrated as the ideal green methodology for fast generation of complex molecular natural product skeletons.[9] Inspired thereby, we set out to explore the feasibility of using this procedure to achieve furan oxidation in our inherently aqueous nucleic acid context. Comparison of published rate constants suggests that furan is 3 orders of magnitude more reactive than DNA toward singlet oxygen oxidation.[10] It was further recently shown that $^1O_2$ production can be controlled by nucleic acids[11a] and an isolated example of ODN cross-linking triggered by $^1O_2$ has been reported.[11b] We here describe the successful use of $^1O_2$ for efficient interstrand cross-linking with furan modified ODNs.

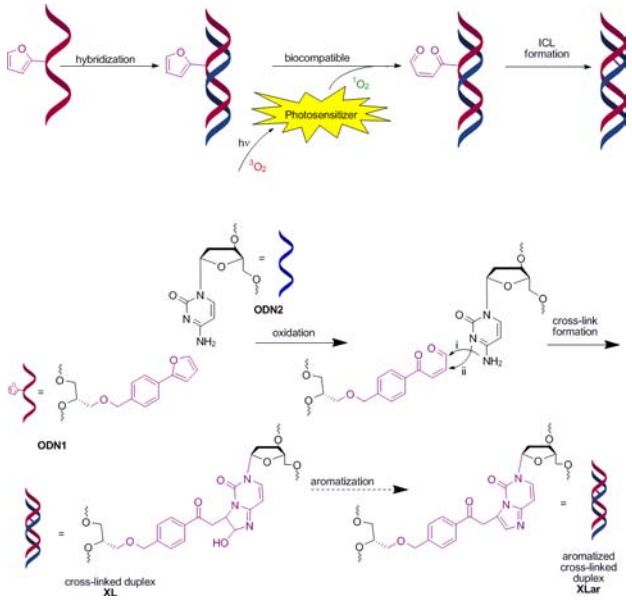


Figure 1. Interstrand cross-link formation of furan modified ODNs triggered by oxidation.

Previously, several furan modified building blocks have been developed and were evaluated for cross-linking reactions in our lab (Table 1). Detailed selectivity studies have shown the cross-linking reaction to specifically target complementary A and/or C residues. It was further shown via degradation and co-injection studies that the covalent bond in the cross-linked

Received: February 26, 2012
Published: June 14, 2012

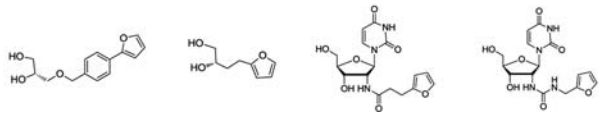
   dx.doi.org/10.1021/ja301901p | J. Am. Chem. Soc. 2012, 134, 10737−10740

Table 1. Cross-Link Selectivity, Structures of Tested Furan Modified Residues and Cross-Linking Yields of NBS versus $^1O_2$ Triggered Reactions

Target strand	Sequence			
ODN2	GCA CCC CGT CAG			
ODN3	GCA CAC CGT CAG			
Modified strand	Sequence	target: ODN2 Yield (%) NBS	target: ODN2 Yield (%) $^1O_2$	target: ODN3 Yield (%) NBS
ODN1	CTG ACG G1G TGC	36	57[a]	29
ODN4	CTG ACG G2G TGC	28	13[b]	49
ODN5	CTG ACG G3G TGC	60	15[b]	<5
ODN6	CTG ACG G4G TGC	55	19[b]	0

Structure of the modified residues

1 = 2 = 3 = 4 =



[a]: Yield obtained under optimized irradiation conditions. [b]: Yield obtained under nonoptimized irradiation conditions.

duplex is formed between the enal generated upon furan oxidation and the base of the nucleotide exactly opposing the furan modified residue. Though cross-link yields vary with the structure of the furan modified building block, no products containing cross-links to flanking bases have been isolated or characterized (see Table S1 for a detailed overview of cross-link selectivity in various sequence contexts).

In general, as for the oxidation of furan using $^1O_2$, when working in organic solvents and in the absence of a subsequent reduction step, mostly structures containing an extra oxygen

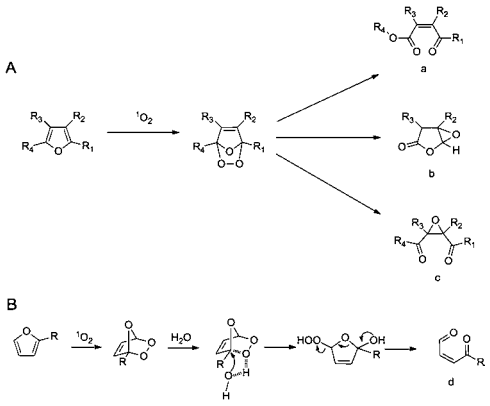


Figure 2. Overview of possible products resulting from singlet oxygen oxidation in MeOH (**A**) and H_2O (**B**).

atom such as **a**, **b**, or **c** (Figure 2) are obtained.[10] In contrast, $^1O_2$ mediated furan oxidation in an aqueous environment has been shown to generate 4-oxo-enal products of type **d** without the need for extra reducing agents. The proposed mechanism for furan oxidation in water is shown in Figure 2 as also postulated very recently by the group of Vassilikogiannakis.[12]

Initial reaction of the reactive 4-oxo-enal, formed upon oxidation of furan, with the exocyclic NH_2 of the nucleoside base is followed by a Michael type addition of the nucleoside N^3 onto the remaining enone system. This results in the formation of stable adducts[13] (see Figure 1) which were identified through extensive enzymatic degradation studies combined with ESI-MS and NMR analysis.[6b] A thorough structural characterization of the cross-linked duplexes revealed that the initially formed cross-linked product (**XL**) can be converted to the aromatized cross-linked duplex (**XLar**) upon prolonged heating.

The acyclic furan containing **ODN1** was now chosen for an elaborate evaluation of the singlet oxygen induced cross-linking reaction. Both rose bengal and methylene blue (MB) were initially screened as photosensitizers under different conditions (see Supporting Information). For detailed investigations and in view of its medical relevance, MB was chosen in combination with red light.[14] Parameters such as photosensitizer concentration and irradiation time were carefully investigated and optimized. The reaction mixtures after oxidation with either NBS or $^1O_2$ were compared through HPLC analysis which indicated in both cases the complete consumption of the modified strand **ODN1** and the formation of a seemingly identical cross-linked product (Figure 3B,C). This is further confirmed by ESI-MS and enzymatic digestion studies. Though some additional minor products can be observed in the chromatograms, resulting from degradation of the oxidized furan containing strand, the cross-linked species is clearly the only main product obtained. Actually, this self-destructing behavior of the furan probe when not cross-linking clearly

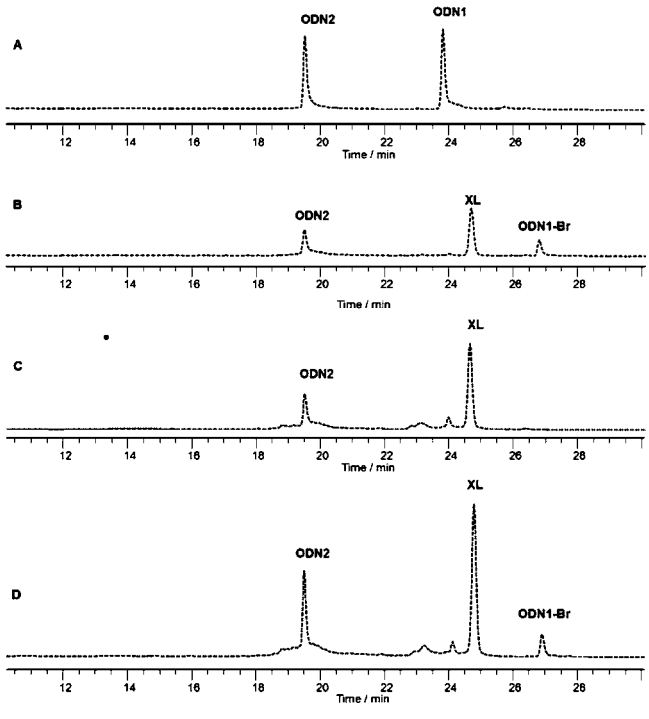


Figure 3. (A) Before reaction: modified **ODN1** and unmodified **ODN2**; (B) after NBS oxidation (4 equiv NBS): **ODN1** has disappeared, cross-linked duplex (**XL**) and brominated furan modified strand (**ODN1-Br**) are observed; (C) after $^1O_2$ oxidation (1 μM MB, 4 h): the modified strand **ODN1** has disappeared, formation of one new peak corresponding to the cross-linked duplex (**XL**) can be observed; (D) coinjection of B and C.

dx.doi.org/10.1021/ja301901p | J. Am. Chem. Soc. 2012, 134, 10737−10740

facilitates final purification of cross-linked duplex from any remaining non-cross-linked modified single strand as both species elute quite closely.

To confirm the structural identity of the cross-linked dinucleoside generated through enzymatic degradation, a synthetic dinucleoside sample was obtained by reaction of protected furan modified building block **1** with 3′, 5′-protected dC (see Supporting Information). This authentic and fully characterized, synthetic dinucleoside was then coinjected with the isolated dinucleoside, obtained through duplex degradation, confirming the postulated structure (see Supporting Information). Upon close inspection of the HPLC profiles, it can be further noticed that a competitive side reaction occurs in case of NBS oxidation (Figure 3B). The resulting species (**ODN1-Br**) was previously characterized as the product of furan bromination and calculations have shown the presence of the phenyl ring conjugated to the furan moiety, to be a major determining factor herein.[6c] As expected, this side product is completely absent in case of $^1O_2$ treatment of the **ODN1**–**ODN2** mixture (Figure 3C).

Having confirmed that clean interstrand cross-linking can be obtained in this way, we set out to identify the exact nature of the oxidizing species. Indeed, the excited state of the sensitized dye can react in one of two ways, defined as either a Type I or a Type II mechanism. A Type I mechanism involves hydrogen-atom abstraction or electron-transfer between the excited sensitizer and a substrate, yielding free radicals. These radicals can react with oxygen to form active oxygen species such as the superoxide radical anion. In a Type II mechanism, singlet oxygen is generated via an energy transfer process during a collision of the excited sensitizer with triplet oxygen.

To validate the cross-link reaction to be mediated by $^1O_2$, some standard tests were performed. In these experiments, disappearance of the furan modified strand was followed as a function of irradiation time. In a first experiment, the furan modified duplex was irradiated with red light in the presence of 5 μM MB during 60 min. The reaction was carried out in triplicate, indicating reproducibility of the process (Figure 4, ○ traces). Next, the same experiment was repeated in D_2O. Typically, it has been observed that for $^1O_2$ mediated processes, reactions occur with higher efficiency in D_2O, this in view of the increased lifetime of $^1O_2$ in D_2O compared to H_2O.[15] A

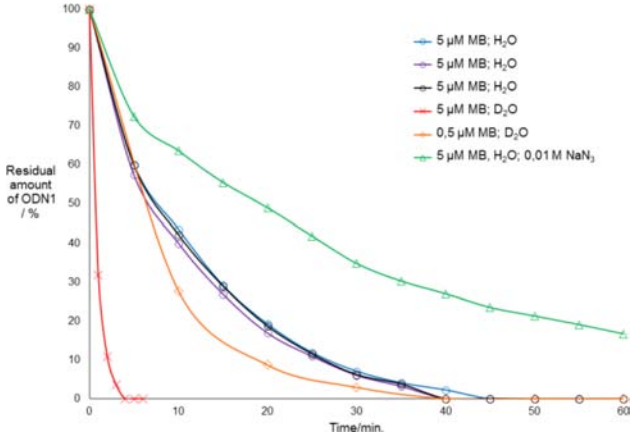


Figure 4. Reaction reproducibility and effect of D_2O and NaN_3 on the consumption of **ODN1** upon irradiation in the presence of MB. The residual amount of furan modified strand is shown as a function of time.

first reaction in D_2O was performed using an identical concentration of MB but shortening the irradiation time to 6 min, after which complete consumption of modified strand was observed (Figure 4, × trace). Second, the concentration of MB was lowered from 5 to 0.5 μM and an irradiation time of 1 h was used. In this case, similar reaction rates as in H_2O were observed (Figure 4, ◊ trace). Both experiments confirmed the oxidation reaction to be $^1O_2$ dependent. Finally, NaN_3, a known $^1O_2$ quencher,[16] was added to the reaction mixture. The significant decline in reaction velocity further evidenced the direct involvement of $^1O_2$ (Figure 4, Δ trace).

To verify the structural integrity of the nucleosides not involved in the cross-linking reaction, the absence of collateral, $^1O_2$ induced DNA damage was carefully checked. Indeed, $^1O_2$ is known to oxidize guanosine residues in ODNs, which would result in the formation of oxidative lesions such as 8-oxodeoxyguanosine (8-oxodG).[17] No additional oxidative DNA damage could, however, be observed in our $^1O_2$ induced cross-linking reactions, as judged by ESI-MS (calculated mass XL, 7290.79 Da; observed mass XL, 7289.83 Da). Furthermore, we applied a procedure, involving treatment of the cross-linked duplex with Na_2IrCl_6 followed by piperidine, known to cause DNA cleavage when dG oxidation products are present.[18] This experiment additionally confirms the absence of further oxidative damage (see Supporting Information).

Under nonoptimized reaction conditions, a moderate maximum cross-linking yield of about 28% was obtained (Figure 5A). However, when performing the cross-linking

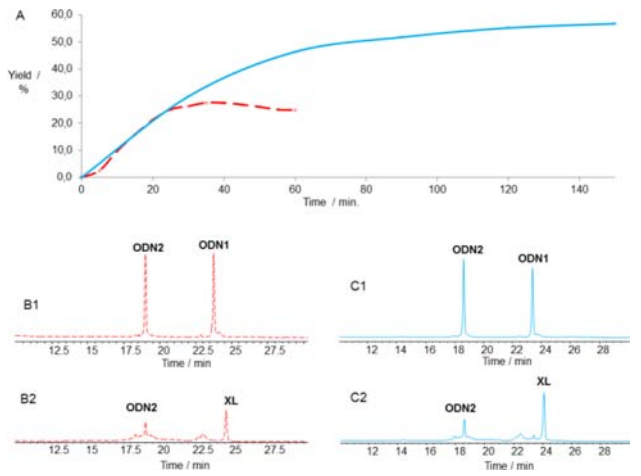


Figure 5. Yield optimization by variation of the concentration of MB and the irradiation time. Dotted red line, 5 μM MB; full blue line, 1 μM MB (A) yield as a function of time; (B) RP-HPLC profiles of the reaction mixture with 5 μM MB; C) RP-HPLC profiles of the reaction mixture with 1 μM MB; B1/C1, before reaction; B2/C2, after irradiation.

reaction in D_2O, hence, increasing the effective concentration of $^1O_2$ and decreasing the reaction time, a significantly lower yield of cross-linked duplex was observed. Likewise, the experiments using NaN_3 as a $^1O_2$ quencher suggest that with a decline in the rate of starting material consumption and hence a longer reaction time, increased amounts of cross-linked duplex could be obtained (see Figure S18B).

Therefore, it was investigated whether combining a reduced $^1O_2$ concentration with longer reaction times would lead to higher yields. Indeed, when lowering the effective concentration of $^1O_2$ through a reduction of the concentration of MB from 5

dx.doi.org/10.1021/ja301901p | J. Am. Chem. Soc. 2012, 134, 10737–10740

to 1 μM, an increased yield of 57% could be obtained (Figure 5). This compares very favorably with yields obtained in previously developed biocompatible, inducible cross-linking methodologies targeting nonmodified DNA.[11b,19]

Generality of the $^1O_2$ triggered furan oxidation cross-linking was further investigated on **ODN4-6** (Table 1 and Supporting Information). It was previously shown that variation of the furan modified building block allows fine-tuning yield and selectivity of the cross-link reaction.[6] For all furan containing duplexes studied, efficient cross-link formation was accomplished, through simple irradiation in the presence of MB. All building blocks are attainable through simple and modular synthetic procedures starting from cheap and easily available starting materials. Furthermore, these building blocks can be incorporated in any desired nucleic acid sequence, allowing the furan unit to be positioned in various regions within a DNA duplex. The current set can thus be regarded as a first and easily expandable toolkit for $^1O_2$ induced cross-linking in various sequence contexts.

Analysis of published rate constants (see Table S2) illustrates that furan derivatives are in general several orders of magnitude more reactive than DNA, fatty acids and thiols toward $^1O_2$ oxidation. Additionally, cross-linking experiments carried out in the presence of biologically relevant concentrations of glutathione show that whereas NBS induced oxidation of the furan modified strands and subsequent cross-linking is completely blocked under these conditions, the singlet oxygen cross-linking pathway is not influenced (Supporting Information, pS32–34). This further underlines the largely improved biocompatibility of the here described method.

In conclusion, the here described simple and versatile strategy for photoinduced ODN interstrand cross-linking complements the limited list of biocompatible cross-linking methods for nucleic acids. Easily accessible furan modified nucleosides in combination with a commercially available photosensitizer and simple visible light irradiation constitute the necessary tools to achieve highly selective duplex interstrand cross-linking. Nonspecific reactions are avoided by allowing duplex hybridization before triggering the reaction. As cross-linking is triggered by controlled $^1O_2$ generation, using low concentrations of methylene blue and red light, collateral oxidative damage to the remainder of the duplex is avoided. As methylene blue is a generally applied in vivo therapeutic drug,[14] furan modified ODNs could become powerful tools in more selective combination therapies.

■ ASSOCIATED CONTENT

Supporting Information

Optimization of the irradiation conditions, experimental data, experimental conditions, enzymatic degradation and chemical synthesis of the cross-linked dinucleoside, verification of the absence of guanosine oxidation and cross-linking experiments with **ODN4–6**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

annemieke.madder@ugent.be

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

M.O.B. is indebted to the Agency for Innovation by Science and Technology in Flanders (IWT) and the Special Research Fund of Ghent University. We further thank the FWO Vlaanderen for financial support.

■ REFERENCES

(1) (a) Fulop, A.; Peng, X. H.; Greenberg, M. M.; Mokhir, A. *Chem. Commun.* **2010**, *46*, 5659–5661. (b) Stafforst, T.; Hilvert, D. *Angew. Chem., Int. Ed.* **2010**, *49*, 9998–10001. (c) Shah, S.; Rangarajan, S.; Friedman, S. H. *Angew. Chem., Int. Ed.* **2005**, *44*, 1328–1332.

(2) (a) Kean, J. M.; Murakami, A.; Blake, K. R.; Cushman, C. D.; Miller, P. S. *Biochemistry* **1988**, *27*, 9113–9121. (b) Higuchi, M.; Yamayoshi, A.; Kato, K.; Kobori, A.; Wake, N.; Murakami, A. *Oligonucleotides* **2010**, *20*, 37–43.

(3) (a) Fujimoto, K.; Konishi-Hiratsuka, K.; Sakamoto, T.; Yoshimura, Y. *ChemBioChem* **2010**, *11*, 1661–1664. (b) Kimoto, M.; Endo, M.; Mitsui, T.; Okuni, T.; Hirao, I.; Yokoyama, S. *Chem. Biol.* **2004**, *11*, 47–55. (c) Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483–514.

(4) Le Gac, S.; Rickling, S.; Gerbaux, P.; Defrancq, E.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Angew. Chem., Int. Ed.* **2009**, *48*, 1122–1125.

(5) Plaetzer, K.; Krammer, B.; Berlanda, J.; Berr, F.; Kiesslich, T. *Lasersn Med. Sci.* **2009**, *24*, 259–268.

(6) (a) Stevens, K.; Madder, A. *Nucleic Acids Res.* **2009**, *37*, 1555–1565. (b) Op de Beeck, M.; Madder, A. *J. Am. Chem. Soc.* **2011**, *133*, 796–807. (c) Stevens, K.; Claeys, D. D.; Catak, S.; Figaroli, S.; Hocek, M.; Tromp, J. M.; Schürch, S.; Van Speybroeck, V.; Madder, A. *Chem.—Eur. J.* **2011**, *17*, 6940–6953.

(7) Tada-Oikawa, S.; Oikawa, S.; Hirayama, J.; Hirakawa, K.; Kawanishi, S. *Photochem. Photobiol.* **2009**, *85*, 1391–1399.

(8) (a) Astarita, A.; Cermola, F.; DellaGreca, M.; Iesce, M. R.; Previtera, L.; Rubino, M. *Green Chem.* **2009**, *11*, 2030–2033. (b) Iesce, M. R.; Cermola, F.; Temussi, F. *Curr. Org. Chem.* **2005**, *9*, 109–139. (c) Clennan, E. L.; Pace, A. *Tetrahedron* **2005**, *61*, 6665–6691. (d) Feringa, B. L. *Recl. Trav. Chim. Pays-Bas* **1987**, *106*, 469–488.

(9) Montagnon, T.; Noutsias, D.; Alexopoulou, I.; Tofi, M.; Vassilikogiannakis, G. *Org. Biomol. Chem.* **2011**, *9*, 2031–2039.

(10) (a) Wilkinson, F.; Brummer, J. G. J. *Phys. Chem. Ref. Data* **1981**, *10*, 809–1000. (b) Lee, P. C. C.; Rodgers, M. A. *J. Photochem. Photobiol.* **1987**, *45*, 79–86.

(11) (a) Clo, E.; Snyder, J. W.; Voigt, N. V.; Ogilby, P. R.; Gothelf, K. V. *J. Am. Chem. Soc.* **2006**, *128*, 4200–4201. (b) Hong, I. S.; Greenberg, M. M. *J. Am. Chem. Soc.* **2005**, *127*, 10510–10511.

(12) Noutsias, D.; Alexopoulou, I.; Montagnon, T.; Vassilikogiannakis, G. *Green Chem.* **2012**, *14*, 601–604.

(13) Peterson, L. A. *Drug Metab. Rev.* **2006**, *38*, 615–626.

(14) Schirmer, R. H.; Adler, H.; Pickhardt, M.; Mandelkow, E. *Neurobiol. Aging* **2011**, *32*, 2325.e7–2325.e16.

(15) Schweitzer, C.; Schmidt, R. *Chem. Rev.* **2003**, *103*, 1685–1757.

(16) Li, M. Y.; Cline, C. S.; Koker, E. B.; Carmichael, H. H.; Chignell, C. F.; Bilski, P. *Photochem. Photobiol.* **2001**, *74*, 760–764.

(17) Cadet, J.; Ravanat, J. L.; Martinez, G. R.; Medeiros, M. H. G.; Di Mascio, P. *Photochem. Photobiol.* **2006**, *82*, 1219–1225.

(18) Muller, J. G.; Duarte, V.; Hickerson, R. P.; Burrows, C. J. *Nucleic Acids Res.* **1998**, *26*, 2247–2249.

(19) (a) Hong, I. S.; Greenberg, M. M. *J. Am. Chem. Soc.* **2005**, *127*, 3692–3693. (b) Chatterjee, M.; Rokita, S. E. *J. Am. Chem. Soc.* **1991**, *113*, 5116–5117. (c) Pieles, U.; Englisch, U. *Nucleic Acids Res.* **1989**, *17*, 285–299.