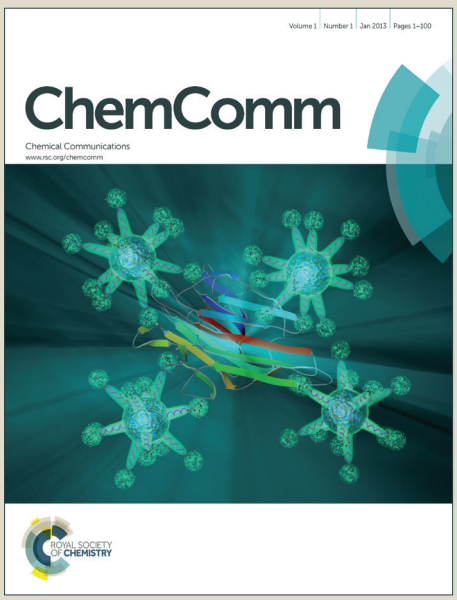
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Development of the Crosslinking Reactions to RNA Triggered by Oxidation

Shuhei Kusano,[a] Takuya Haruyama,[a] Shogo Ishiyama,[a] Shinya Hagihara[b] and Fumi Nagatsugi*[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

The novel crosslink-forming nucleobase, 2-amino-6-(1-ethylthiovinyl)purine (ATVP), which is triggered by the oxidation of sulfide to sulfoxide, has been developed. The oxidation of ATVP within the duplex proceeded with H_2O_2 and $FeCl_2$. We have successfully developed the crosslinking reactions activated by oxidation.

Manipulation of the gene expression is considered as a quite important technology in the biological and medicinal fields.[1] Oligonucleotides (ONs) are often used to control the gene expression *in vitro* and *in vivo* due to their high sequence specificity based on the Watson-Crick base pairing. Gene regulation with ONs is induced by various mechanisms. For example, in antigene methodology, ONs suppress the transcription by preventing the unwinding of the double helical DNA or sterically blocking the complex formation of the transcription factor.[2] In antisense methodology, ONs inhibit the translation by recruiting RNase H[3] or sterically blocking the translation initiation through the binding to the start codon or internal ribosome entry site.[4]

To date, many kinds of chemically modified ONs have been developed to achieve effective gene suppression.[5] One of the approaches toward an effective gene suppression is to form an irreversible bond (crosslink) between the ON and the target gene.[6] We reported that the crosslink formation between the ON and mRNA completely suppresses the translation.[7] The covalently bound ON works as a steric block for the ribosome and hence produces truncated proteins. It was also shown that the crosslink-forming oligonucleotide (CFO) is applicable to the gene specific inhibition of microRNA-mediated silencing by masking the microRNA binding region on the target mRNA.[8] These reports have clearly demonstrated the biological applicability of CFOs.

In order to improve the use of CFOs, development of a stimuli-responsible CFO is effective.[9] In this study, we focused on the development of a CFO activated by oxidation stimulation. In a biological system, reactive oxygen species (ROSs), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (·OH), are produced during the process of electron transport chain and closely related to the oxidative damages of DNA, protein and lipid.[10] The level of ROSs is controlled to maintain cellular function by an enzymatic or non-enzymatic mechanism. In addition, a high level of H_2O_2 is observed in cancer cells rather than normal cells.[11] These facts suggest that a cellular oxidant can be a good candidate for a trigger of the crosslink reaction.

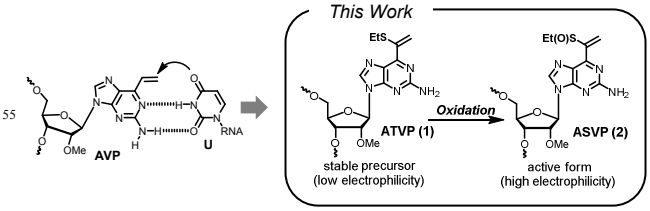
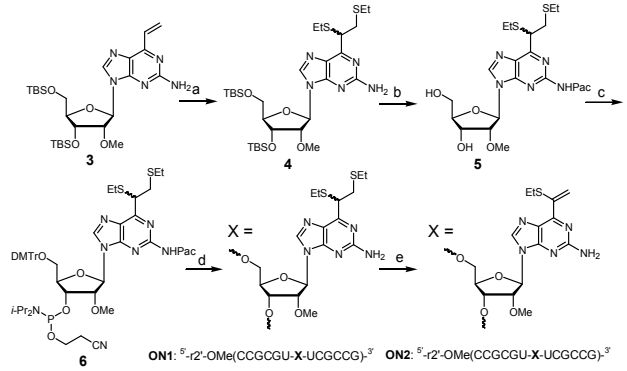


Fig. 1 Design of the oxidation-induced crosslink-forming nucleobase

Accordingly, we designed a novel oxidation triggered crosslink forming formation with 2-amino-6-(1-ethylthiovinyl)purine (ATVP, **1**) based on 2-amino-6-vinylpurine (AVP) that was developed in our laboratory.[12] AVP is a crosslink forming nucleobase that forms covalent linkage with uracil in complementary RNA (**Fig 1**). Substitution of a hydrogen atom to a sulfide group in the vinyl moiety is expected to decrease the electrophilicity due to the electron donating property of the sulfide group. On the other hand, 2-amino-6-(1-ethylsulfinylviynl) purine (ASVP, **2**) which is the oxidized form of **1**, is considered to enhance the reactivity of the vinyl moiety because of its electron withdrawing property. Altogether, **1** should work as a stable precursor, and once it is oxidized to the sulfinyl vinyl, exhibit crosslink reactivity.



Scheme 1 Synthesis of 2'-OMe RNA containing **1** (a) (i) Bromine water, $CHCl_3$, 58% (ii) DBU then EtSH CH_2Cl_2, 57% (b) (i) Phenoxy acetyl chloride, pyridine, 96% (ii) TBAF, THF, quant. (c) (i) DMTrCl, pyridine 85% (ii) $iPr_2NP(Cl)OC_2H_4CN$, DIPEA, CH_2Cl_2, 79% (d) Automated DNA/RNA synthesizer (e) 1 M NaOH

The synthesis of 2'-OMe ON containing **1** is summarized in **Scheme 1**. 2'-OMe guanosine was converted to the TBS-protected 2-amino-6-vinylpurine (**3**) as previously described.[12]

Bisulfide (**4**) was synthesized by the dibromination of **3,** then subjecting this to the treatment with DBU following the addition of ethanethiol in one pot. The diastereo ratio of **4** was determined as 1:1 by ^{1}H NMR spectroscopy.

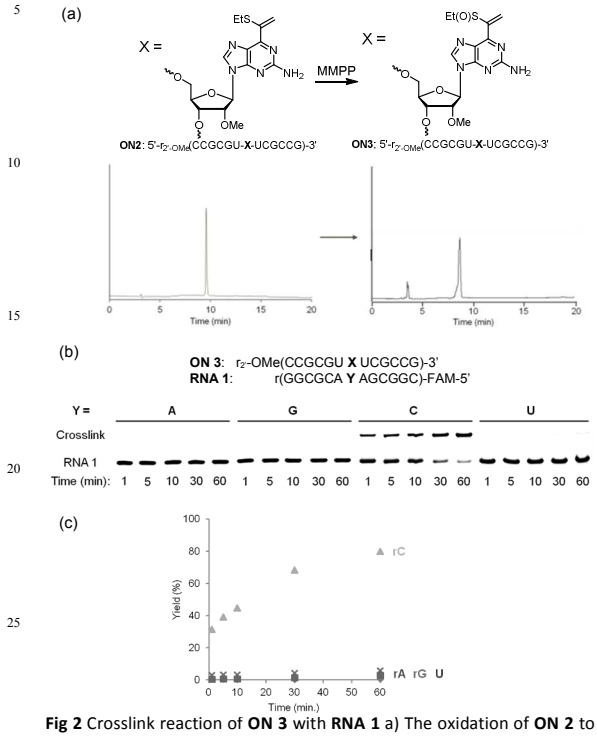


Fig 2 Crosslink reaction of **ON 3** with **RNA 1** a) The oxidation of **ON 2** to **ON 3** with MMPP. This reaction was performed with 5 equivalents of MMPP for 30 min at room temperature. b) The gel electrophoresis for the crosslink reaction. c) The summary for the time course of the crosslink yield, Y = A; ◆, Y = G;■, Y = C;▲, Y = U;●. The reaction was performed using 10 μM **ON 3** and 5 μM **RNA 1** in 100 mM NaCl, 50 mM MES, pH 7 at 37 ºC

Thereafter, protection of the amino group with the phenoxy acetyl group, followed by removal of the TBS group provided the corresponding diol **5**. The diol **5** was converted to the phophoroamidite **6** by a conventional method in good yield. The obtained **6** was applied to an automated DNA/RNA synthesizer to obtain the bissulfide **ON1**. **ON1** was purified by RP-HPLC, thereafter **ON1** was treated with 1M NaOH to provide the thiovinyl **ON2** containing **1** as a precursor of **2**. The majority of bissulfide **ON1** remained the un-oxidized form during the oxidation step of oligonucleotide synthesis under standard conditions. We have confirmed the stability of **ON1**and **ON2** by HPLC analysis after 48 h (**Fig S1**).

We first compared the crosslink reactivity of the ONs containing **1** and **2**. **ON3** was prepared from **ON2** using magnesium bis(monoperoxy phthalate)hexahydrate (MMPP) as an oxidant (**Fig 2a**). MMPP has been used in our group for the oxidation of sulifide to sulfoxide in ON without any nucleobase oxidation.[12, 13] As shown in **Fig 2a**, **ON2** was quantitatively oxidized to **ON3** by MMPP within 30 min. The structure of **ON3** was confirmed by MALDI-TOF mass spectroscopy. The crosslink reactions was investigated under neutral conditions using of **ON2**, **ON3** and the complementary **RNA1** (Y = A, G, C and U) labelled with fluorescein at the 5' end and analyzed by 20% denaturing polyacrylamide gel electrophoresis (**Fig 2b)**. The crosslink yields were calculated from the ratio of the crosslinked product to the remaining single-stranded **RNA1**, and were plotted versus the incubation time. **ON3** containing ASVP (**2**) showed a high crosslink reactivity and selectivity to cytosine. The crosslink yield of **ON3** to cytosine reached about 80% in 1 hour (**Fig 2c**) and the reaction rate of **2** was considerably higher than that of AVP.[7] The high reactivity of **2** compared to AVP might be due to the electron withdrawing property of the sulfinyl group. Interestingly, ASVP (**2**) exhibited a highly selective crosslink reactivity to cytosine in RNA, whereas AVP selectively reacted with uracil. We attempted to understand the mechanistic insight for the different base selectivities of ASVP and AVP, however, we could not obtain a specific solution because the analysis was quite difficult due to the high reactivity of ASVP. In contrast, **ON2** did not show any crosslink reactivity even after 12 h incubation at 37 ºC (**Fig. S1**), suggesting that **1** has a sufficient potency as a stable precursor. These results suggest that the reactivity of **1** and **2** would be adjusted by the oxidation state of the sulfur atom. To gain more insight into the crosslink reaction, the HPLC analysis of the reaction between **ON3** and **DNA1** (Y = C) was conducted (**Fig S2**). No significant difference in the crosslink reactivity of the diastereomers, which are derived from the chirality of the sulfinyl group, was observed because the oxygen atom of the sulfoxide is placed in a major groove. Since the activation of ATVP (**1**) by oxidation was ascertained, we subsequently investigated the crosslink reactions triggered by oxidation with H_2O_2 and a metal ion within the duplex (**Fig 3a)**.

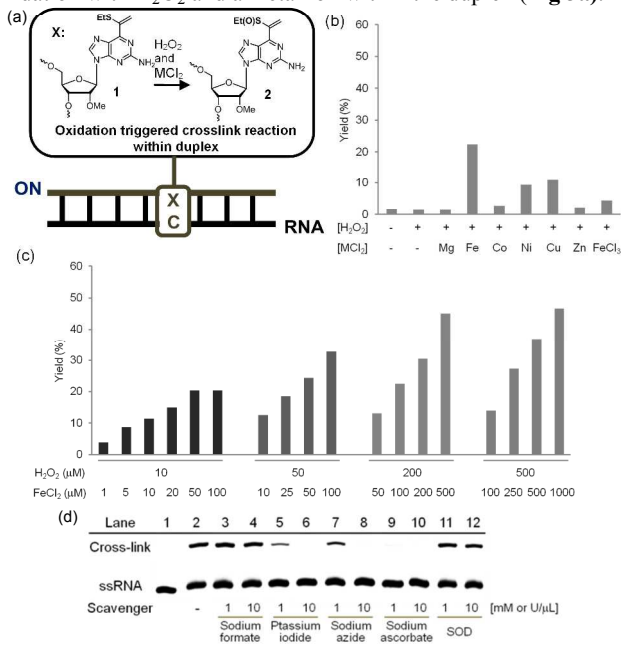


Fig 3 Crosslink reaction of **ON2** with **RNA1** (Y = C) in the presence of H_2O_2 and metal ions. a) Schematic illustration for the oxidation by H_2O_2 and metal ion induced crosslink reaction. b) The effect of metal ion. The reaction was performed with or without 200 μM H_2O_2 and 100 μM MCl_2. c) The concentration dependence of H_2O_2 and $FeCl_2$. d) The gel electrophoresis for the crosslink reaction of **ON2** with or without a scavenger. In this reaction, 50 μM H_2O_2 and 100 μM $FeCl_2$ were used. Lane 1: **RNA1** without **ON2** as a control. Lane 2: without scavenger. Lanes 3 and 4: 1 or 10 mM sodium formate. Lanes 5 and 6: 1 or 10 mM potassium iodide, Lanes 7 and 8: 1 or 10 mM sodium azide. Lanes 9 and 10: 1 or 10 mM sodium ascorbate, Lanes 11 and 12: 1 or 10 U/μL SOD. All crosslink reactions were performed with 10 μM **ON2** and 5 μM **RNA1** in 100 mM NaCl, 50 mM MES, pH 7 at 37 ºC for 1 h.

As an initial investigation, the reaction was performed with 200 μM H_2O_2 in the presence of the 100 μM of each metal ion ($MgCl_2$, $FeCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$ and $FeCl_3$). A

summary of the crosslink yields after 1 h is shown in **Fig 3b**. The crosslink reaction was not accelerated by H_2O_2 alone and in the presence of $MgCl_2$, $CoCl_2$ and $ZnCl_2$. In contrast, the reaction was promoted by $FeCl_2$, $NiCl_2$ and $CuCl_2$, and especially, $FeCl_2$ was the best activator for the reaction. It is noteworthy that $FeCl_3$ in contrast to $FeCl_2$ did not promote the reaction. This result suggested that the presence of Fe(II) under the stated reaction condition is crucial for this oxidation.

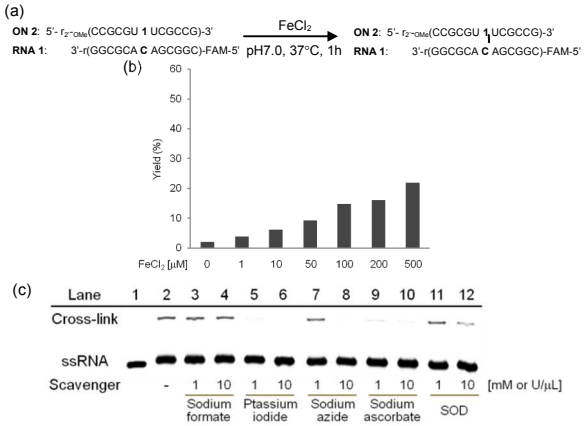


Fig 4 Crosslink reaction of **ON2** with **RNA1** (Y = C) in the presence of $FeCl_2$. a) The reaction equation for the crosslink reaction. b) Concentration dependence of $FeCl_2$ for the crosslink reaction. c) The gel electrophoresis for the crosslink reaction with or without a scavenger. The reaction was performed with 100 μM $FeCl_2$. Lane 1: **RNA1** without **ON2** as a control. Lane 2: without scavenger, Lanes 3 and 4: 1 or 10 mM sodium formate, Lanes 5 and 6: 1 or 10 mM potassium iodide, Lanes 7 and 8: 1 or 10 mM sodium azide, Lanes 9 and 10: 1 or 10 mM sodium ascorbate, Lanes 11 and 12: 1 or 10 U/μL SOD. All crosslink reactions were performed with 10 μM **ON2** and 5 μM **RNA1** in 100 mM NaCl, 50 mM MES, pH 7 at 37 °C for 1 h

In order to reveal that the oxidation of **ON2** to **ON3** occurred under the stated reaction conditions, **ON2** was incubated with H_2O_2 and $FeCl_2$, and analyzed by HPLC and MALDI-TOF mass spectroscopy. The sulfide of **ON2** was immediately oxidized after the addition of the H_2O_2 and $FeCl_2$, but the oxidation did not proceed if the reaction time was prolonged (**Fig S3**). **Fig 3c** shows that the crosslink yield was improved by increasing the concentration of H_2O_2 and $FeCl_2$, and reached about 50% using 200 μM H_2O_2 and 500 μM $FeCl_2$. To identify the active species for the oxidation using H_2O_2 and $FeCl_2$, the crosslink reactions using H_2O_2 and $FeCl_2$ were performed in the presence of a scavenger for oxidants, such as sodium formate for ·OH,[14] potassium iodide for triplet oxygen ($^3O_2$),[15] sodium azide for singlet oxygen ($^1O_2$),[14] sodium ascorbate for oxidants including free radicals,[16] and superoxide dismutase (SOD)[15] for O_2^-. We confirmed that these scavengers did not restrict the crosslink reactions between **ON3** containing ASVP (**2**) and **RNA1** (data not shown). **Fig 3d** shows that sodium formate (Lanes 3 and 4) did not inhibit the crosslink reaction, meanwhile potassium iodide (Lanes 5 and 6), sodium azide (Lanes 7 and 8) and sodium ascorbate (Lanes 9 and 10) significantly inhibited the reaction. In addition, the crosslink reaction was slightly prohibited by SOD (Lanes 11 and 12). Based on these results, it was deduced that the active species for the reaction is not ·OH generated by the Fenton reaction andO_2^- are not dominant active species for the oxidation reaction, and that $^3O_2$, $^1O_2$ which is known to oxidize the sulfide to sulfoxide,[17] or any types of oxidants generated from $FeCl_2$ participate to the oxidation reaction. Since the strong oxidant ·OH is not an active species for this reaction, a detectable strand scission and oxidation of **RNA1** was not observed during the reaction (data not shown).

To address in more detail the oxidation by H_2O_2 and $FeCl_2$, the crosslink reaction of **ON2** was conducted in the presence of only $FeCl_2$ as an additive. We found that $FeCl_2$ promoted the crosslink reaction without H_2O_2 (**Fig 4b**), though the crosslink yield was less than that with H_2O_2 and $FeCl_2$ as an oxidant. We also revealed that **ON2** was oxidized to **ON3** by $FeCl_2$, even in the absence of H_2O_2 (**Fig S4**). Moreover, the effect of scavengers (sodium formate, potassium iodide, sodium azide, sodium ascorbate and SOD) for the crosslink reaction was similar to the results obtained in **Fig 4c**. The precise mechanism of the oxidation with H_2O_2 and $FeCl_2$ is unclear at this time. However, from the obtained results, we presumed that any types of oxidant generated from $FeCl_2$ such as ferryl ion (**Scheme S1**) or $^1O_2$ is crucial role for the oxidation of **1**

We have developed a novel oxidation triggered crosslink nucleobase ATVP (**1**) and demonstrated that the oxidized form ASVP (**2**) showed a very fast and selective crosslink reaction to cytosine in RNA. Furthermore, **1** in the duplex was oxidized by H_2O_2 and $FeCl_2$ thereby inducing the crosslink reactivity. In tumour cell, H_2O_2 and Fe(II) exist in sub-micro molar and micro molar order respectively.[18] Accordingly, we are considering that the obtained results in this research strongly suggest the oxidation triggered crosslink reaction system is applicable for regulating gene expression in a cell by additionally modifying molecular structure of **1** so as to be oxidized under milder condition.

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas ("Functional machinery for non-codingRNAs") and a Grant-in-Aid for Scientific Research (B) and (S) from the Japan Society for the Promotion of Science (JSPS)

Notes and references

[a] *Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai-shi, 980-8577, Japan. Tel: +81-22-217-5633; E-mail: nagatugi@tagen.tohoku.ac.jp*
[b] *Institute of Transformative Bio-Molecules (WPI-ITbM),Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan.*

† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/b000000x/

1 a) M. J. A. Wood, M. J. Gait, H. Yin, *Brain*, 2010, **133**, 957-972. b) C. Wahlestedt, *Nat. Rev. Drug Dicov.*, 2013, **12**, 433-446
2 a) C. Helene, J. J. Toulme, *Biochimica et Biophysica Acta*, 1990, **1049**, 99-125. b) C. Helene, *Eur. J. Cancer*, 1991, **27**, 1466-1471
3 a) F. Eckstein, *Antisense Nucleic Acids Drug Dev.*, 2000, **10**, 117-121. b) B. P. Monia, E. A. Lesnik, C. Gonzalez, W. F. Lima, D. McGee, C. J. Guinosso, A. N. Kawasaki, P. D. Cook, S. M. Freier, *J. Biol. Chem.*, 1993, **268**, 14514-14522.
4 a) E. Zamaratski, P. I. Pradeepkumar, J. Chattopadhyaya, *J. Biochem. Biophys. Methods*, 2001, **48**, 189-208. b) B. F. Baker, S. S. Lot, T. P. Condon, S. Cheng-Flournoy, E. A., Lensik, H. M. Sasmor, C. F. Bennett, *J. Biol. Chem.*, 1997, **272**, 11994-12000 c) C. J. Nulf, D. Corey, *Nucleic Acids Research*, 2004, **32**, 3792-3798
5 a) J. Kurreck, *Eur. J. Biochem.*, 2003, **270,** 1628-1644. b) M. L. Paula, Y. Bruice, I. E. Szabó, T. C. Bruice, *Chem. Rev.*, 2012, **112**, 1284-1309 c) S. Buchini, C. J. Leumann, *Curr. Opni. Chem. Biol.*, 2003, 7, 717-

726
6 a) D. A. Stewart, S. D. Thomas, C. A. Mayfield, D. M. *Nucleic Acids Research*, 2001, **29**, 4052-4061. b) C. Giovannangeil, S. Diviacco, V. Labrousse, S. Gryaznov, P. Charneau, *Proc. Natl. Acad. Sci.*, 1997, **94**, 79-84
7 S. Hagihara, S. Kusano, W.C. Lin, X. G. Chao, T. Hori, S. Imoto, F. Nagatsugi, *Bioorg. Med. Chem.Lett.*, 2012, **22**, 3870-3872
8 S. Hagihara, W.C. Lin, S. Kusano, X. G. Chao, T. Hori, S. Imoto, F. Nagatsugi, *ChemBioChem*, 2013, **14**, 1427-1429
9 a) M. Op de Beeck, A. Madder, *J. Am. Chem. Soc.*, 2012, **134**, 10737-10740. b) A. Shigeno, T. Sakamoto, Y. Yoshimura, K. Fujimoto, *Org. Biomol. Chem.*, 2012, **10**, 7820-7825
10 U. Bandyopadhyay, D. Das, R. K. Banerjee, *Current Science*, 1999, **77**, 658-666
11 T. P. Szatrowski, C. F. Nathan, *Cancer Research*, 1991, **51**, 794-798
12 S. Imoto, T. Hori, S. Hagihara, Y. Taniguchi, S. Sasaki, F. Nagatsugi, *Bioorg. Med. Chem.Lett.*, 2010, **20**, 6121-6124
13 K. Hattori, T. Hirohama, S. Imoto, S. Kusano, F. Nagatsugi, *Chem. Commun.*, 2009, **42**, 6463-6465
14 T. P. A. Devasagayama, S. Steenken, M. S. W. Obendorf, W. A. Schulz, H. Sies, *Biochemistry*, 1991, **30**, 6283-6289
15 A. Jain, K. N. Alvi, H. J. Parish, M. S. Hadi, *Mutat. Res.*, 1995, **357**, 83-88
16 T. C. Tsuo, C. L. Chen, T. Y. Liu, J. L. Yang, *Carcinogenesis*, 1996, **17**, 103-108
17 A. Toutchkine, D. Aebisher, E. L. Clennan, *J. Am. Chem. Soc.*, 2001, **123**, 4966-4973
18 A. A. Qutub, S. A, Popel, *Mol. Cell. Biol.* 2008, **28**, 5106-5119.