Fine-tuning furan toxicity: fast and quantitative DNA interchain cross-link formation upon selective oxidation of a furan containing oligonucleotide†

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Oligonucleotides containing a furan modified internal nucleoside have been synthesized; selective *in situ* oxidation of the furan moiety to a reactive enal species in the presence of a complementary DNA strand gives rise to fast and efficient formation of an interstrand cross-link.

It has previously been shown that selective control of gene expression is possible *via* artificially created nucleic acids that are complementary to a given RNA (antisense approach) or DNA (anti-gene approach) sequence. To overcome problems associated with reversibility of hybridization, cross-linking strategies have been proposed to ensure the required inhibition of gene expression.[1] Furthermore cross-linked nucleic acids have become important in order to overcome the limitations associated with using short synthetic oligonucleotides (ON) as models of high molecular weight nucleic acids.[2] Since interstrand cross-links caused by environmental and chemotherapeutic agents represent a serious impediment to cell survival, short cross-linked ON duplexes can be of help in the study of natural repair processes, possible causes of resistance to chemotherapeutics. A diverse array of non-natural nucleosides have been developed for cross-linking purposes with natural DNA/RNA targets.[3–9] However most of these methods require the introduction of modified bases and/or extensive additional protection/deprotection schemes.

Inspired by the known toxicity of furan—which is believed to be due to its *in vivo* oxidation into the reactive metabolite 2-butene-1,4-dial[10]—we decided to explore the possibility of using a furan moiety in an ON sequence as a masked reactive aldehyde (Fig. 1). We here report on the preliminary but highly promising results of these studies and show that selective *in situ* oxidation of the furan within the ON is possible and gives rise to a fast and quantitative cross-link formation with complementary strands.

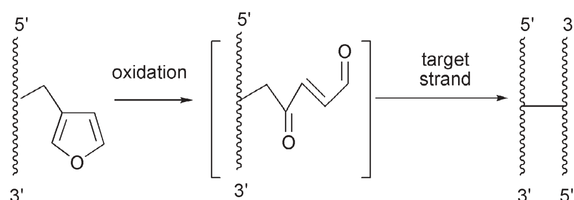
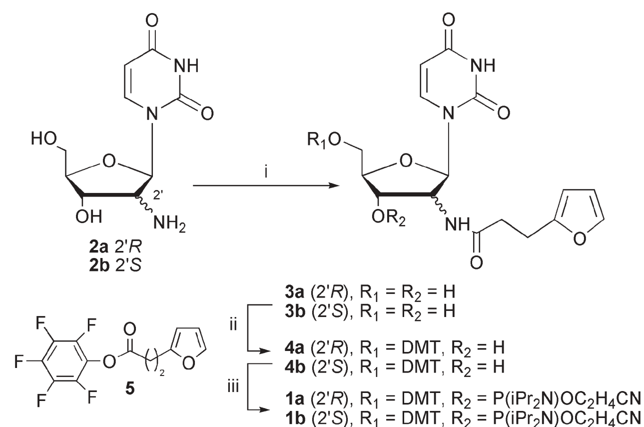


Fig. 1 Schematic representation of the proposed cross-linking strategy.

Nucleosides equipped with a furan moiety have been prepared previously.[11,12] Instead of the introduction of the furan moiety on the base we chose to link the furan *via* a 2′-amino group on the ribose and prepare both the *ribo* (**1a**) and *arabino* (**1b**) configured stereoisomers. Depending on the stereochemistry at 2′ and the sugar pucker of the nucleoside within the ON—which in turn depends on the target ON and the type of duplex formed—this in principle allows for the selective orientation of the furan nucleus towards minor or major groove. Synthesis of the two furan-nucleoside building blocks was performed as shown in Scheme 1.

Nucleoside **3a** was prepared starting from the known 1-(2′-amino-2′-deoxy-β-D-ribofuranosyl)uracil **2a**[13] by treatment with pentafluorophenyl-3-(2-furyl)-propanoate **5** in the presence of DBU. For the synthesis of **3b**, 1-(2′-amino-2′-deoxy-β-D-arabinofuranosyl)uracil hydrochloride[14] was reacted with activated ester **5**. Further conversion into the required DMT-protected phosphoramidites **1a** and **1b** was carried out using standard methodology.

Scheme 2 summarizes the test reactions for oxidation of the furan in nucleoside **3a**. *N*-Bromosuccinimide (NBS) was found to be the reagent of choice for oxidation under aqueous conditions.[15] Upon treatment with 1 equivalent of NBS in pH 7.0 phosphate buffer the corresponding furan oxidized enal derivative **6** (which exists in equilibrium with its hydrated form) was formed next to



Scheme 1 *Reagents and conditions:* (i) **5**, DBU, DMF, 0 °C to room temp., 2.5 h, (75%; **3a/3b**); (ii) DMTCl, pyr, DMAP, room temp., 18 h, (92%, **4a**/96%, **4b**); (iii) *i*$Pr_2NP(Cl)OCH_2CH_2CN$, *i*Pr_2NEt, DCM, room temp., 1 h, (92%, **4a**/97%, **4b**).

† Electronic supplementary information (ESI) available: full experimental details for the preparation and characterization of nucleoside building blocks. RPHPLC profiles and ESI-MS spectra of nucleoside oxidations, oligonucleotides and cross-linked oligonucleotides. See http://www.rsc.org/suppdata/cc/b4/b415092a/

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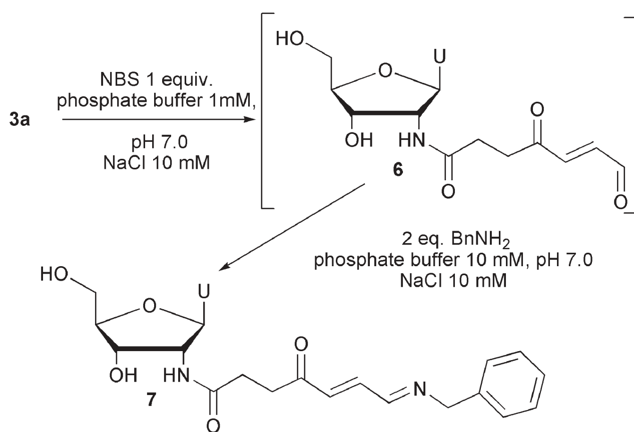

Scheme 2 Oxidation of nucleoside **3a** with NBS.

remaining **3a** as shown by RPHPLC and ESI-MS. Subsequent addition of benzylamine led to the conversion of **6** into an unsaturated imine derivative **7**. These preliminary experiments showed the potential of the furan moiety to act as a masked aldehyde group which is sufficiently reactive to allow for reaction with potential nucleophiles in an oligonucleotide cross-linking context.

Both modified nucleosides were then successfully incorporated into ONs **10** and **11** (Table 1). Replacement of the natural ribonucleoside by a furan containing monomer in the 11-mers resulted in a lowering of the duplex stability with 4 °C and 5 °C respectively when compared to the unmodified DNA duplex **8**:**9**. A similar decrease in duplex stability was observed by Herdewijn *et al.* when incorporating 2′-acetamidonucleosides.[16] The melting temperatures remain sufficiently high to ensure duplex formation at room temperature for the study of the cross-link formation.

We next set out to evaluate the potential of our proposed cross-linking reaction. For this purpose the purified ONs (20 μM) were treated with 1 equivalent of freshly prepared NBS solution in phosphate buffer (pH 7.0, 10 mM, containing 10 mM NaCl) at 25 °C. In a first series of experiments it was shown that whereas ON **8** (Fig. 2 a,b) was stable under these conditions, both ONs **10** and **11** gave a mixture of products (Fig. 2 c,d: example for **11**).

The oxidation was then carried out with **10** and **11** in the presence of the complementary DNA strand. Analysis of the resulting reaction mixture under denaturing RPHPLC conditions revealed after only 15 minutes the consumption of both strands and the concurrent formation of a new slower running species. Leaving the mixtures to stand for longer periods did not lead to further conversion. Gradual addition of extra equivalents of NBS and analysis of the reaction mixtures 15 minutes after each addition revealed that a total amount of 4 equivalents is required for the complete disappearance of the starting ONs (Fig. 2 e–h: example for **9**:**11**). Purification and analysis of the formed material by ESI-MS revealed the complete conversion of the original ON strands to the corresponding cross-linked duplex (**9**–**10XL** and **9**–**11XL**).‡ So in contrast to the complex mixture which is formed when ONs **10** or **11** are oxidized as single strands, NBS treatment of a duplex leads to clean cross-link formation. Further control experiments were performed with **10** in the presence of the non-complementary 5′-d(GCCTGTCAG-C-G)-3′. It was shown that in this case no cross-linking took place and the furan-modified ON **10** was converted into a mixture of products under the reaction conditions while the non-complementary strand could be recovered.

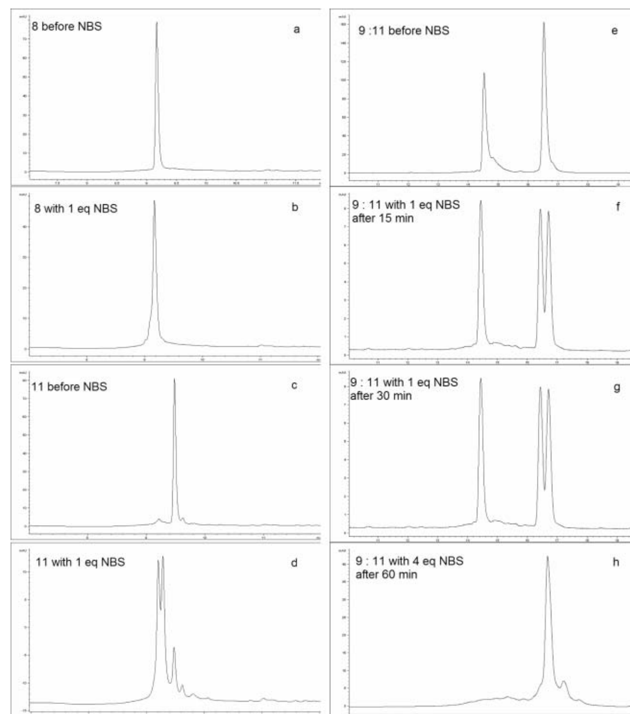

Fig. 2 RPHPLC analysis of oxidation reaction mixtures: a) **8** before NBS addition; b) **8** after 1 equiv. of NBS; c) **11** before NBS addition; d) **11** after 1 equiv. of NBS; e) **9**:**11** before NBS addition, $t = t_0$; f) **9**:**11** after 1 equiv. of NBS, $t = t_0 + 15$ min; g) **9**:**11** after 1 equiv. of NBS, $t = t_0 + 30$ min; h) **9**:**11** after 4 equiv. of NBS, $t = t_0 + 60$ min.

Table 1 T_m values for modified and reference duplexes[a]

Oligonucleotide duplexes		T_m/°C
8:	5′-d(GCCTGTCAG-U-G)-3′	38.1
9:	3′-d(CGGACAGTC-A-C)-5′	
10:	5′-d(GCCTGTCAG-**1a**-G)-3′	33.0
9:	3′-d(CGGACAGTC-A-C)-5′	
11:	5′-d(GCCTGTCAG- **1b**-G)-3′	34.0
9:	3′-d(CGGACAGTC-A-C)-5′	

[a] UV-melting profiles were measured in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl at a ramp rate of 0.5 °C min^{-1} at 260 nm using a Cary 3E UV/VIS spectrophotometer (Varian) containing a Cary Thermoelectrical temperature-controlled sample holder and 10 mm path-length cuvettes. The oligonucleotide concentration was 4 μM for each strand.

As for the site of cross-linking, further studies are necessary to characterize the type and exact position of the covalent bond between both strands. In view of the B-form of the DNA–DNA duplex studied, the furan moiety is most likely located in the major groove in both cases. Inspection of a molecular model reveals the complementary A or its 5′-neighbouring C as the most probable ligation sites. Mass spectrometric data are consistent with a dehydration reaction involving formation of an unsaturated imine upon attack of the adenine or cytosine exocyclic amine on the

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aldehyde functionality. The exact position and nature of the cross-link and a detailed analysis of sequence dependence and importance of linker length are currently being investigated.

The results presented here clearly demonstrate the potential of the *in situ* furan oxidation to trigger cross-linking within an ON duplex as a more convenient and higher yielding alternative to existing methods (*e.g.* acrolein modified ONs).[17] Moreover the described method offers an attractive way for the introduction of aldehyde functionality into DNA for labeling purposes.

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Notes and references

‡ ESI-MS for **9–10XL**: M_{calcd} = 6792.21, M_{exp} = 6791.27; for **9–11XL**: M_{calcd} = 6792.21, M_{exp} = 6792.23.

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