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Efficient conjugation of oligonucleotides through aromatic oxime formation

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ABSTRACT

The present work reports on the preparation of oligonucleotide conjugates via the formation of aromatic oxime linkage. The conjugation consists in the reaction between the oligonucleotide derivatized at 5′-extremity with a benzaldehyde moiety and an aminooxy reporter group. The conjugation was found highly efficient and was extended for the conjugation of phosphorothioate oligonucleotide. In addition, the stability of the so-formed oxime conjugate was investigated.

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Synthetic oligonucleotides and their analogues have been thoroughly studied in the frame of biochemistry and molecular biology research. These agents are currently used as hybridization probes for the detection of DNA and RNA sequences, as primers for the amplification of DNA and RNA through polymerase chain reaction (PCR), as reagents for accomplishing specific inhibition of gene expression. For this latter case, oligonucleotides can target the mRNA by either antisense or RNAi mechanism, double stranded DNA by triplex (antigene) mechanism, or proteins by aptamer selection.[1] In particular, the antisense oligonucleotides have generated significant interest on account of their potential use as therapeutic agents and tools to investigate gene function. Indeed, these antisense oligonucleotides have shown in vitro and in vivo anti-tumour activity in pre-clinical models. Several antisense agents are currently being evaluated in clinical trials for the treatment of viral diseases such as AIDS.

However, the use of unmodified oligonucleotides in above-mentioned applications is seriously restricted because of different drawbacks such as poor cellular uptake efficiency and targeted delivery, specificity and affinity for the target sequence, non-specific interactions and susceptibility to degradation by nucleases. Further optimizations are therefore required for designing a suitable oligonucleotide candidate. For this purpose, a number of chemical modifications have been introduced into the oligonucleotide structure.

Various tailored oligonucleotides containing unnatural bases, modified carbohydrates or altered phosphate backbones have been prepared and investigated for their influence on intrinsic properties. Among them, the modification of phosphodiester backbone to phosphorothioate has received a great attention and has been proved to enhance significantly the oligonucleotide stability against degradation. In fact, the antisense agent: Vitravene, which has been approved by Food and Drug Administration for the treatment of cytomegalovirus-induced retinitis, is a phosphorothioate-modified oligonucleotide.

The conjugation of oligonucleotides to other molecules or biomolecules (i.e., reporter groups) provides an alternative approach to modulate their biological properties.[2] However, the task of oligonucleotide conjugation is not trivial due to the fact that subtle changes in structure may influence biological properties. The specific sites to be used for the conjugation should therefore be carefully analyzed. Moreover, the chemical reactivity and stability of the oligonucleotide and the molecule to be anchored may not be compatible. Thus, during the last decade, the development of synthetic procedures to prepare oligonucleotide conjugates bearing various linkers, functional groups, fluorescent tags and other modifications was subjected to intensive efforts.[3] Reactions those are highly chemoselective and regioselective under mild conditions are de facto preferred for oligonucleotide conjugation and have resulted in the development of the 'Click Chemistry concept'.[4] In this context, we have developed the oxime tethering procedure, which fulfilled the 'click chemistry' criteria's. The method has been demonstrated highly chemoselective and gives high coupling efficiency. Furthermore, these bonds are stable over a wide pH range. It has been extensively studied for the conjugation of ODNs with peptides,[5] carbohydrates[6] and fluorescent probes[7] as well as for the patterning of glass surfaces[8] or RNA labelling.[9]

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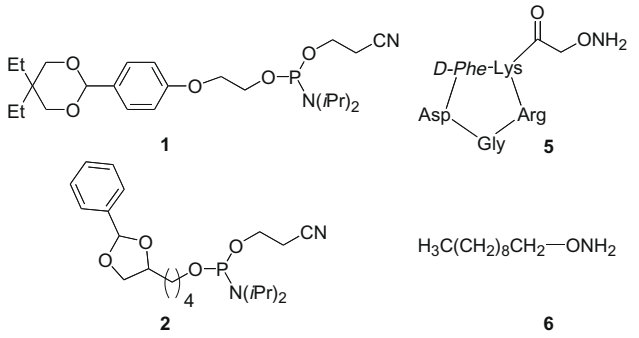


Figure 1. Phosphoramidites **1** and **2** and aminooxy reporters **5** and **6**.

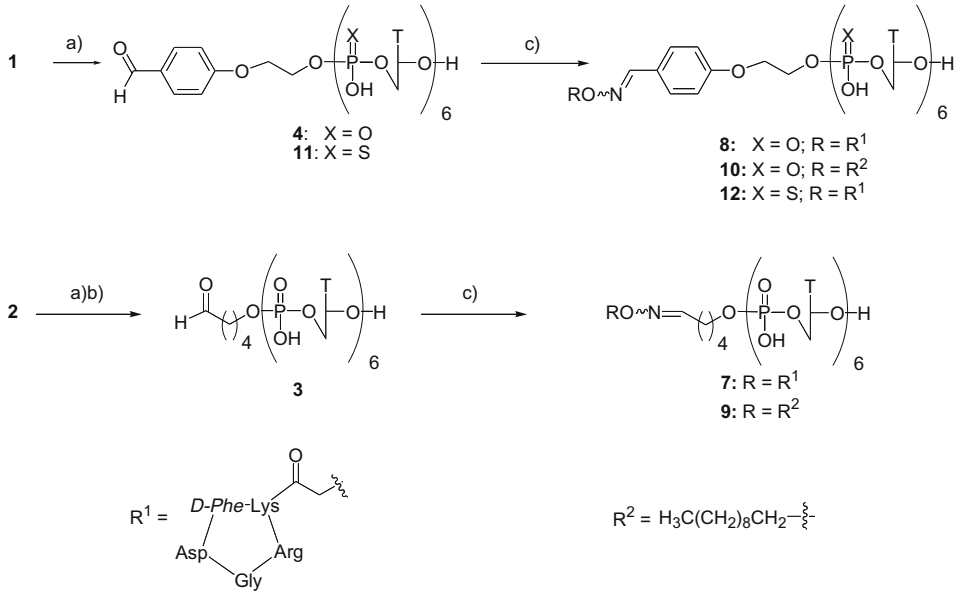
Most of the time, the incorporation of aldehydic group into the oligonucleotides for subsequent coupling reaction with an aminooxy-containing reporter is performed through the oxidation of a diol moiety by using sodium periodate. No side reaction is observed and the aldehyde group can be generally generated in quite quantitatively yield. However, the use of such oxidizing conditions may affect the integrity of oligonucleotide analogues. For example, the sodium periodate is known to be incompatible with the phosphorothioate backbone and results in the formation of phosphodiester backbone.[10]

In another hand, this reagent is very useful for the oxidative cleavage of carbohydrates such as ribose and thus its use is completely unsuitable with RNA. The phosphoramidite **1** was proposed few years ago as an alternative way for incorporating the aldehyde group at 5′-extremity of ODNs (Fig. 1).[11] In our knowledge, no study of the conjugation reaction by oxime formation has been reported by using this aromatic aldehyde. Indeed, due to the aromatic nature of the aldehyde, the reactivity toward nucleophile such as aminooxy moiety might be different as well as the stability of the resulting aromatic oxime ether (Ar-CH=N-OR′).

In this Letter, we demonstrate that the oximation can be achieved efficiently with this aromatic aldehyde by using phosphodiester ODN (PO-ODN) as well as phosphorothioate ODN (PS-ODN). The stability of such oxime conjugate was also studied and compared with the aliphatic oxime linkage (CH_2–CH=N–OR′).

For the purpose of this work, the oligonucleotides **3** and **4** containing only thymidine bases and bearing the aldehyde moiety at the 5′-extremity were employed (Scheme 1). ODN **3** containing the aliphatic aldehyde was prepared from the corresponding phosphoramidite **2** using the previously described protocol.[12] Briefly, the protected diol phosphoramidite **2** was incorporated during the last step of the automated DNA synthesis. After cleavage from the solid support, deprotection of the nucleobases by usual ammonia treatment (28% ammonia, 16 h at 55 °C) and acidic treatment (80% AcOH, 1 h), the oligonucleotide carrying the free 5′-diol was oxidized by using sodium periodate in water affording the desired 5′-aliphatic aldehyde containing oligonucleotide **3**. ODN **4** containing the 5′-aromatic aldehyde linker was prepared using standard procedure and incorporation during the last step of the automated DNA synthesis of the phosphoramidite **1**. After cleavage from the solid support and deprotection of the nucleobases by usual ammonia treatment (28% ammonia, 16 h at 55 °C), the acetal protecting group is readily removed under standard oligonucleotide detritylation conditions with 80% aqueous acetic acid.

The subsequent 5′-conjugation reactions with ODNs **3** and **4** were first performed with the aminooxy compound **5**. Compound **5** is a cyclopenta-peptide consisting of an arginine–glycine–aspartic acid (RGD) motif, thoroughly studied as a ligand towards the $\alpha_v\beta_3$ integrin receptor.[13] Reactions were carried out in ammonium acetate buffer at slightly acidic pH (4.6) and were monitored by RP-HPLC (Figs. 2A and B). Both reactions proceeded to completion with 3–4 h to yield the 5′-RGD conjugates **7** and **8** as major products. Very interestingly, the formation of the conjugate **8** containing the aromatic oxime linkage is almost as efficient than the formation of aliphatic oxime conjugate **7**. In fact, the rate of formation of **8** is quite similar to that for the formation of **7** although the kinetic of formation of the aliphatic oxime linkage was found slightly higher than for aromatic oxime linkage (Fig. 2C). The conjugation was then performed using aminooxy compound **6**. Due to its hydrophobic properties, the conjugation reactions were carried out in 50% aqueous acetonitrile solution.[14] Again, the coupling reaction was shown to be very efficient in both cases affording the conjugates **9** and **10**, containing the aliphatic and the aromatic oxime linkage, respectively. All the conjugates **7–10** were characterized by ESI-MS analysis, which showed experimental molecular



Scheme 1. Synthesis of oligonucleotide conjugates **7**–**12**. Reagents: (a) automated DNA synthesis then 28% ammonia and 80% AcOH treatments; (b) $NaIO_4$, H_2O; (c) **5** or **6** in 0.4 M ammonium acetate buffer (see also Supplementary data).

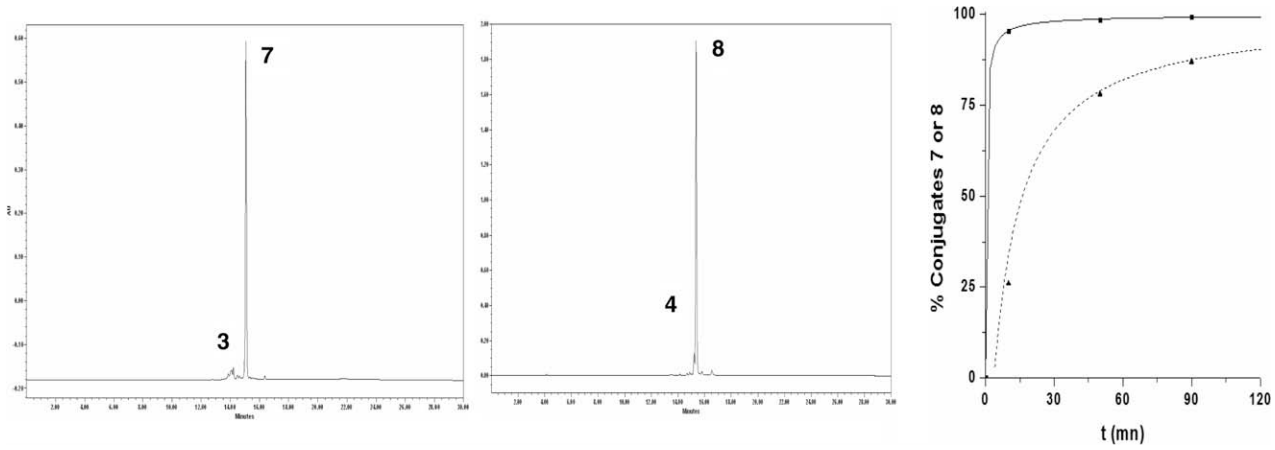


Figure 2. HPLC profiles of (A) crude reaction mixture of aliphatic oxime conjugate **7**, (B) crude reaction mixture of aromatic oxime conjugate **8**, (C) rate of formation of conjugates **7** (straight line) and **8** (dot line). Detection at 260 nm.

weights in excellent agreement with the calculated values (see Supplementary data).

The hydrolytic stability as a function of pH of the aromatic oxime linkage was then studied for comparison with aliphatic oxime linkage. Conjugates **9** and **10** were incubated at 37 °C in a suitable phosphate buffer solution with pH adjusted to 4.0, 7.0 and 9.0. The incubated samples were analyzed by RP-HPLC after periods of time (see Supplementary data).[14] The results obtained from HPLC analysis were further authenticated by ESI-MS analysis. The data show that aromatic oxime is stable over the pH range from 4 to 9 because less than 10% hydrolysis is observed after 24 h of incubation (Table 1). It should be noticed that in acidic conditions (pH 4.0) some hydrolysis is observed after a longer period of time whereas 20% of the conjugate **10** is hydrolysed in 48 h. Interestingly, the data reveal that the aromatic oxime linkage is slightly more stable over the pH range from 4 to 9 than the aliphatic oxime linkage. Nevertheless, both linkages are stable at physiological pH over a 24 h period.

Finally, the conjugation was performed with the phosphorothioate ODN **11**. Indeed, in the case of PS backbone modified ODN, the generation of the aldehyde moiety from a diol moiety was found unsuitable as sodium periodate oxidizes very efficiently the phosphorothioate linkage to a phosphodiester linkage. ODN **11** was prepared using the standard phosphorothioate synthesis procedure and incorporation of the phosphoramidite **1** during the last step. The coupling reaction was performed using peptide **5** in the same conditions as for phosphodiester ODNs **3** and **4** (vide supra). The reaction was monitored by RP-HPLC and showed the exclusive formation of conjugate **12**, which was characterized by ESI-MS analysis (see Supplementary data).

In conclusion, the conjugation by the oxime 'click chemistry' has received a considerable interest during the last decade and the efficiency of such coupling procedure has been proven for DNA conjugation as well as for other biomolecule conjugation.

Table 1
Percentage of hydrolysis of aromatic and aliphatic oxime conjugates at different pH

	% Hydrolysis[a]			
	Aliphatic oxime conjugate **9**		Aromatic oxime conjugate **10**	
	24 h	48 h	24 h	48 h
4.0	15	25	10	20
7.0	<5	10	<2	<5
9.0	<5	15	<2	<5

[a] Percentage hydrolysis was estimated from the area under peak in the HPLC chromatogram.

A prerequisite of such conjugation is the easy access to the aldehyde-containing moiety, that is, the aldehydic oligonucleotide. In this Letter, we demonstrate the efficiency of the oxime ligation by using an aromatic aldehyde. The aromatic moiety is introduced by using the commercially available phosphoramidite **1** and the coupling reaction was found as efficient as the coupling reaction with aliphatic aldehyde. Furthermore, the introduction of **1** is compatible with the phosphorothioate synthesis and thus allows the preparation of PS oligonucleotide conjugate through oxime ligation. Very interestingly, the hydrolytic stability of aromatic oxime linkage is found higher than the corresponding aliphatic oxime linkage. This latter point is obviously of great interest when a higher stability of the linkage between the ODNs and the reporter is required.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.048.

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