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3′-Oligonucleotides conjugation via chemoselective oxime bond formation

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Abstract—Chemoselective oxime ligation of cyclopeptide, fluorescein and mannose derivatives at the 3′-end of an oligonucleotide was achieved. The conjugation was performed by reacting oxyamine containing reporter groups to an oligonucleotide bearing an aldehyde at the 3′-extremity. The aldehyde was generated by mild periodate oxidation of a 1,2-aminoalcohol which was readily incorporated at the 3′-end by automated DNA synthesis using the corresponding commercially available support. The straightforward chemical access, their stability in biological media as well as their unchanged hybridisation properties emphasise the interest of such 3′-conjugates.

1. Introduction

Oligonucleotides have found widespread applications in therapy for gene expression inhibition (antisense, antigene and aptamere strategies) as well as in molecular biology for nucleic acid sequencing. To provide specific properties, a number of molecules including fluorophores, peptides, lipids, and sugars have been conjugated to oligonucleotides.[1–3] Commonly employed methods involve post-solid-phase synthesis modification by preliminary incorporation of a protected nucleophilic group during automated synthesis and subsequent linkage with an electrophilic derivative. Conjugation is generally achieved at the 3′- or 5′-extremities in order to not disturb the hybridisation process between the modified oligonucleotide and the RNA or DNA target. Most of the DNA-conjugates described involve 5′-derivatisation due to its ready chemical access. For instance, primary alkylamine or thiol groups are usually attached for that purpose.[4] However, 5′-modification precludes ^{32}P-labeling by kinase at that position which is by far the widespread technique used for DNA gel analysis. In sharp contrast, 3′-modification has been rarely described possibly because its chemical access is more challenging. Nevertheless, 3′-modified oligonucleotides have the major advantage over 5′-analogues to exhibit a much greater nuclease stability.[5,6] 3′-Conjugates are obtained by either direct reporter incorporation to the solid support prior to oligonucleotide synthesis or by its post-DNA synthesis anchorage to a reactive functionality. This latter approach is usually preferred because it is more compatible with a wide variety of chemical function on the reporter group since the coupling is convergent and the reporter is not subject to DNA synthesis conditions. A number of methods have been described for this purpose whose primary amine incorporation is undoubtedly the most common.[5–10] However, the coupling reaction using a primary amine suffers from drawbacks such as competing reagent hydrolysis (a large excess of the electrophilic reporter molecule is in fact required to achieve complete conjugation) as well as cross reactivity with other functionality present within the oligonucleotide or the reporter group. The lack of chemoselectivity of this approach emphasises the need of a more efficient and selective conjugation reaction. In this context, chemoselective ligation techniques, which have been developed for de novo design of proteins, are of key interest. The reactions proceed essentially in aqueous solvent, and their high selectivity obviates the need for protection of other functional groups on the coupling partners. In particular, the chemoselective ligation through oxime bond formation has been used successfully for the assembly, in a user-defined way, of peptides and glycopeptides.[11–14]

In recent papers, we have reported on the use of the aminooxy-aldehyde coupling reaction that enables one to efficiently synthesise 5′-conjugates without the need of protecting groups throughout the biopolymer and the reporter.[15–17] The reactive moiety, i.e. the aldehyde or the oxyamine, has been introduced at the 5′-end of

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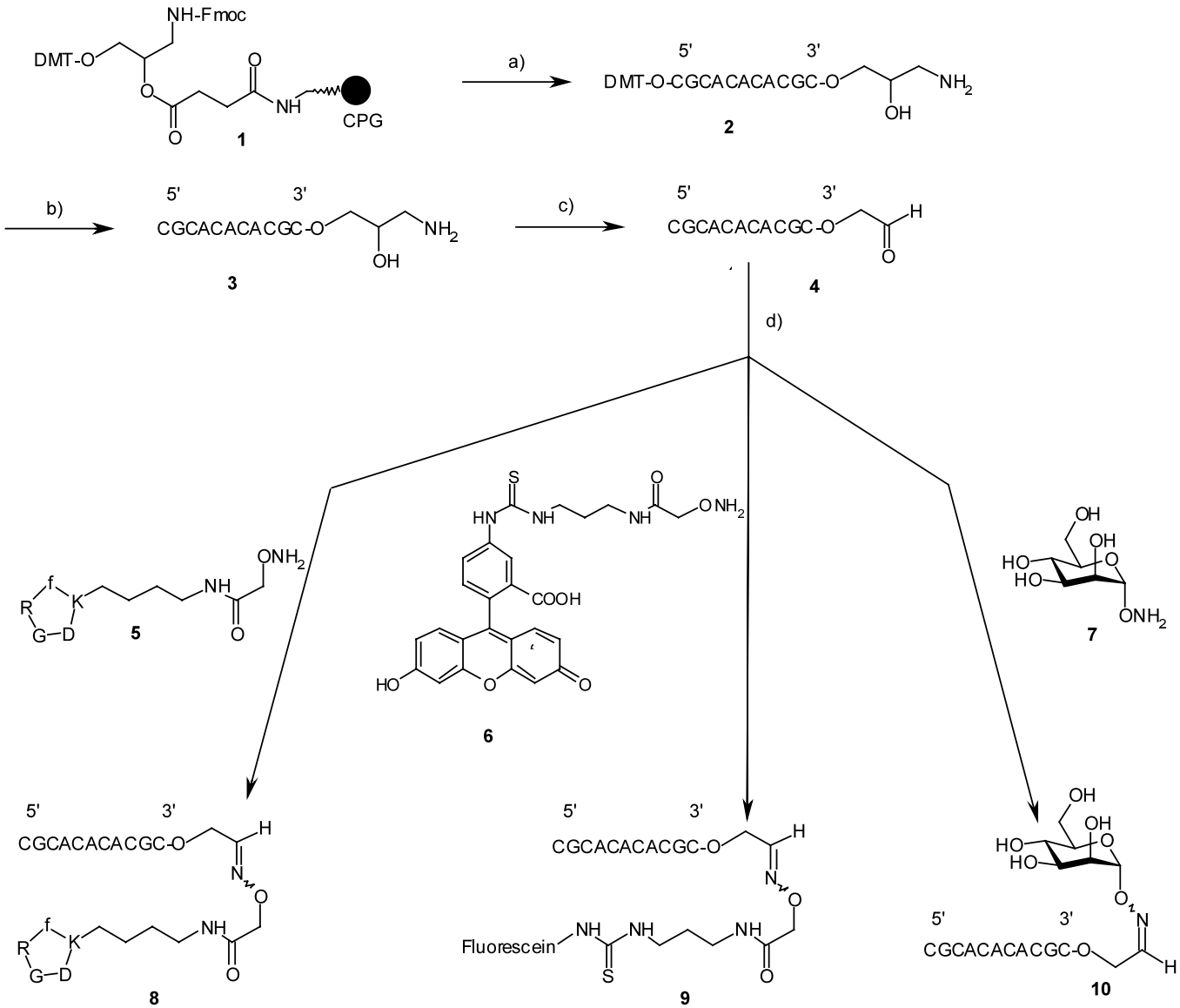
an oligonucleotide by automated DNA synthesis and subsequent conjugation has been performed by using the reporter group bearing the corresponding complementary function. We thus decided to extend our methodology for the 3′-conjugation of oligonucleotide by anchoring an aldehydic precursor at the 3′-end. Aldehyde derivatisation was preferred to oxyamine as it was shown that this modification is more convenient to handle than the oxyamine.[17]

In this paper, we report on the incorporation of an aldehyde at the 3′-end of the oligonucleotide and subsequent reaction with aminooxy containing reporter groups. Three different reporter groups were used to emphasise the interest of this 3′-conjugation strategy: (a) A cyclopenta-peptide containing an arginine–glycine–aspartic acid tripeptide motif (RGD) known as a powerful and selective ligand of the $\alpha_v\beta_3$ integrin receptor.[18] Such peptides including the RGD moiety have been studied for tumour targeting as well as for DNA delivery.[19,20] (b) A fluorescein derivative for fluorescent labelling. (c) A mannose derivative for a model of carbohydrate–oligonucleotide conjugates.[21] Such carbohydrate–oligonucleotide conjugates may serve to target dendritic cells via their sugar-binding receptor (lectins).

2. Results and discussion

The aldehydic group must be masked during the automated DNA synthesis. Since depurination has been observed during the acidic deprotection step of classical acetal protection, a post-oxidation strategy was preferred for the introduction of the aldehyde moiety. The 1,2-aminoalcohol moiety was chosen as a precursor of



Scheme 1. Preparation of 3′-aldehyde containing oligonucleotide **4** and conjugate **8**–**10**. *Reagents and conditions*: (a) automated DNA synthesis then NH_4OH 28%, 55°C for 24 h; (b) AcOH 80%; (c) $NaIO_4$; (d) aqueous solution (adjusted to pH 4.5 with AcOH) of aminooxy derivatives **5**–**7**.

the aldehyde. Oxidative cleavage of the 1,2-aminoalcohol moiety from a serine residue has been successfully used to introduce an aldehyde into peptide.[22] Furthermore, the support **1** containing a 1,2-aminoalcohol has been described and is besides commercially available.[23]

The undecamer d(5′CGCACACACGCX3′) **3**, in which X represents the 3′-linker bearing the 1,2-aminoalcohol, was synthesised according to standard β-cyanoethyl phosphoramidite chemistry using the commercial support **1**[24] (Scheme 1). The coupling efficiency was higher than 98% as determined from trityl responses. After deprotection under the usual conditions (ammonia treatment for 24 h at 55°C), the intermediate oligonucleotide **2** was purified by reverse-phase HPLC (Fig. 1A shows the crude undecamer **2**). Removal of the 5′-trityl protection was then performed in 80% aqueous AcOH at room temperature for 1 h leading to the undecamer **3** (Fig. 1B). The structure of compound **3** was confirmed by ES-MS analysis (Table 1). The aldehydic function was then generated from the 1,2-aminoalcohol by treatment with sodium periodate (50 equiv.) in water.[25] The HPLC analysis (Fig. 1C) showed the exclusive formation of the desired aldehyde **4** in a very short time. Compound **4** was purified by reverse-phase HPLC and obtained in almost 70% isolated yield. The coupling reaction with the aminooxy compound was performed immediately after. The structure of the aldehyde **4** was thus confirmed indirectly by formation of the corresponding oxime ether derivative.

The conjugation reaction was carried out using a slight excess (3 equiv.) of the aminooxy derivatives **5**–**7**[26] in aqueous solution in acidic condition (pH 4.5).[27,28] The course of the reaction was followed by reverse-phase HPLC. In each case, the formation of a major product was observed after 2 h. As an example, the HPLC profile of the crude reaction mixture of oligonucleotide **4** with the RGD peptide **5** is depicted in Fig. 1D. Subsequent purification by HPLC afforded the conjugates **8**–**10** in almost 50% isolated yield. The conjugates **8**–**10** were characterised by ES-MS. In all cases, the experimentally determined molecular weights were in excellent agreement with the calculated values (Table 1).

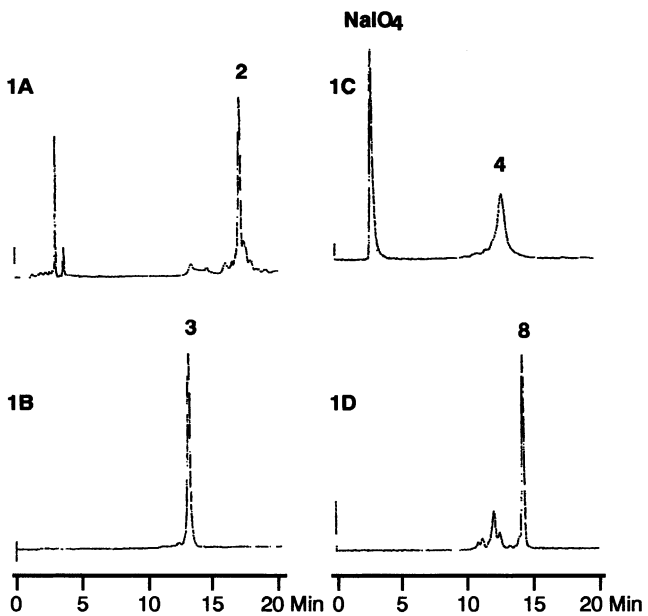


Figure 1. HPLC profiles: (1A) of crude undecamer **2** (conditions I), (1B) of purified undecamer **3** (conditions I), (1C) of crude oxidation mixture of **3** (conditions II), (1D) of crude reaction mixture of oligonucleotide **4** with the RGD peptide **5** (conditions I).[29] Detection at 260 nm.

The chemical stability of the oxime linkage was then studied by incubating the conjugate **8** in a phosphate buffer at pH 4 and pH 7. As previously observed, the oxime bond was found to be very stable.[17] No significant hydrolysis or degradation products (from depurination) were observed even after 48 h of incubation at 37°C.

The stability in biological media was also studied by incubating the conjugate **8** in a mixture of synthetic medium RPMI-1640 and calf fetal serum (20%). The 5′-modified undecamer d(5′XCGCACACACGC3′) in which X represents the same RGD moiety and the unmodified undecamer d(5′CGCACACACGC3′) were also incubated in the same conditions for comparison. As anticipated, the 3′-modified oligonucleotide **8** was found much more stable. In fact, degradation up to 50% was observed after 3 h of incubation of the 5′-modified and of the unmodified undecamer, while the conjugate **8** remained stable in these conditions. These results are in agreement with the protecting effect against nucleases observed with 3′-capped oligonucleotides.[5,6]

The consequences on the hybridisation properties of the oligonucleotides introduced by the reporters at the terminal 3′-phosphate were evaluated by melting temperature (T_m) measurements. Undecamers **8** and **10** were hybridised with their complementary strand d(5′GCGTGTGTGCG3′), and the melting temperatures of the resulting duplexes were determined. The 3′-conjugates showed a similar melting temperature in comparison with the unmodified duplexes ($T_m = 62$°C). These results indicate that conjugation via oxime bond formation at the 3′-extremity does not modify the hybridisation properties.

Table 1. ES-MS analysis[a]

Oligonucleotide	Calcd mass	Found mass
Undecamer **3**	3423.3	3423.7
Conjugate **8**	4051.0	4051.7
Conjugate **9**	3910.4	3911.4
Conjugate **10**	3569.3	3569.7

[a] The analysis was performed in the negative mode. The eluent was 50% aqueous acetonitrile and the flow rate was 8 μL/min. The oligonucleotides were dissolved in $H_2O/CH_3CN/NEt_3$, 50/50/2 (v/v/v).

3. Conclusion

In conclusion, derivatisation at the 3′-extremity of oligonucleotides by reaction of an aminooxy reporter group with the aldehyde present on the biopolymer was found to be very convenient. The aldehyde is easily unmasked by oxidative cleavage of a 1,2-aminoalcohol. Moreover, this functionalisation is advantageous as the solid support containing 1,2-aminoalcohol is commercially available. Thanks to the high chemoselectivity of the aminooxy-aldehyde coupling reaction, the conjugation do not required the use of any protecting group on the oligonucleotide or on the reporter moiety. This oxime conjugation also confers a higher resistance to nucleasic activity. 5′- and 3′-oxime conjugation are now demonstrated and represent a versatile tool for post-DNA modification with a wide range of applications.

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24. Automated DNA synthesis was performed on an Expedite DNA synthesiser (Perkin–Elmer) following the manufacturer's protocols for standard β-cyanoethyl nucleoside phosphoramidites chemistry on a 1 μM scale. The support was purchased from ESGS-Groupe Cybergene (Evry, France).
25. *Experimental procedure for oxidation*: To a solution of oligonucleotide **3** (0.140 μmol) in water (500 μL), $NaIO_4$ (50 equiv., 30 μg) was added and the solution was stirred at room temperature for 30 min. The oligonucleotide **4** was then purified by reverse-phase HPLC using system II (0.093 μmol, 66%).
26. (a) Boturyn, D.; Dumy, P. *Tetrahedron Lett*. **2001**, *42*, 2787–2790; (b) Trévisiol, E.; Defrancq, E.; Lhomme, J.; Laayoun, A.; Cros, P. *Tetrahedron* **2000**, *56*, 6501–6510; (c) Renaudet, O.; Dumy, P. *Tetrahedron Lett*. **2001**, *42*, 7575–7578.
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28. *Typical experimental procedure for conjugation*: To a solution of aldehyde containing oligonucleotide **4** (0.093 μmol) in water (300 μL, pH adjusted at 4.5 with AcOH), a solution of RGD peptide **5** (3 equiv., 190 μg) in water (86 μL) was added. The mixture was stirred at room temperature and the progress of the reaction was followed by HPLC. Disappearance of the starting material was achieved after 2 h. Purification by reverse-phase HPLC using system I afforded the conjugate **8** in almost 50% isolated yield (0.040 μmol).
29. The oligonucleotides and the conjugates were purified on a μ-bondapak C-18 column (Macherey-Nagel Nucleosil: 10×250 mm, 7 μm). Two systems of solvent were used. Conditions I: solvent A, 20 mM ammonium acetate buffer/CH_3CN, 95/5 (v/v); solvent B (CH_3CN); flow rate, 4 mL/min; a linear gradient from 0 to 30% B in 20 min was applied. Conditions II: solvent A, 20 mM sodium phosphate buffer/MeOH, 95/5 (v/v); solvent B (MeOH); flow rate, 4 mL/min; a linear gradient from 0 to 35% B in 20 min was applied.