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Bioorganic & Medicinal Chemistry Letters 14 (2004) 2839–2842
Bioorganic & Medicinal Chemistry Letters

The oxime bond formation as an efficient chemical tool for the preparation of 3′,5′-bifunctionalised oligodeoxyribonucleotides

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Received 6 January 2004; accepted 18 March 2004

Abstract—The simultaneous conjugation of peptides or carbohydrates at the 3′- and 5′-end of oligodeoxyribonucleotides was achieved very efficiently through chemoselective oxime bond formation. The method employs bifunctionalised oligonucleotides in single step without the need of protection strategy, under mild acidic conditions. The conjugates were obtained in high yields by reacting an oxyamine containing reporter groups (peptide, mono- and disaccharide) with an oligonucleotide carrying an aldehyde at each extremity.

Modified oligonucleotides represent an attractive tool for biomedical applications. These oligonucleotides offer the potential to rationally design therapeutic agents that can selectively modulate gene expression (antisense, triple helix, siRNA strategies).[1] Many efforts have been devoted to the conjugation of oligonucleotides with a variety of reporter molecules (fluorescent tags, peptides, lipophilic moieties...) to provide specific properties, and a number of methods have been so far reported for the conjugation at the 3′- or at the 5′-extremity.[2] However, a modification at both the 3′- and 5′-terminus of oligonucleotides is desirable as both termini are vulnerable to nuclease attack.[3] But fewer methods have been reported for the synthesis of bifunctionalised oligonucleotides at both extremities. Some strategies to reach this challenging goal have been described by using solid-phase synthesis or by solution-phase fragment coupling.[4,5] The latter method is more attractive as it combines with two favourable properties: (i) it is more compatible with a wide variety of chemical functions on the reporter group since the coupling is convergent and the reporter group is not subject to DNA synthesis, (ii) it involves the separate preparation of reporter groups and the subsequent coupling (through amide or disulfide bond formation) of the two purified (and characterised) moieties. However, the moderate yield of the coupling reaction underlines the need for a more efficient and selective conjugation reaction. In particular, 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.

In this context, chemoselective ligation techniques such as oxime ligation[6] represent a powerful approach for the conjugation of various reporters at the extremity or inside the sequence of an oligonucleotide. The use of the oxyamino-aldehyde coupling reaction, that enables one to anchor a peptide residue at the 5′- or 3′-extremity as well as inside the sequence at the 2′-position of the sugar, have been previously reported.[7–9] The remarkable efficiency of this ligation technique led us to attempt the preparation of 3′,5′-conjugate through oxime bond formation using aldehyde 3′,5′-bifunctionalised oligonucleotide (Fig. 1).

In this paper, we report on the preparation of oligonucleotide sustaining the two aldehyde moieties at each extremity and subsequent reaction with oxyamino containing reporters leading to 3′,5′-conjugates through oxime bond formation. The method was emphasised by using two different peptides: (i) a cyclopentapeptide containing an arginine-glycine-aspartic acid tripeptide motif (RGD), which is well known as a powerful and selective ligand of the $\alpha_v\beta_3$ integrin receptor and that was studied for tumour targeting,[10] (ii) a nuclear localisation signal sequence (NLS) containing peptide, which

Keywords: Conjugation; Chemoselectivity; Oligonucleotide; Oxime.
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doi:10.1016/j.bmcl.2004.03.053

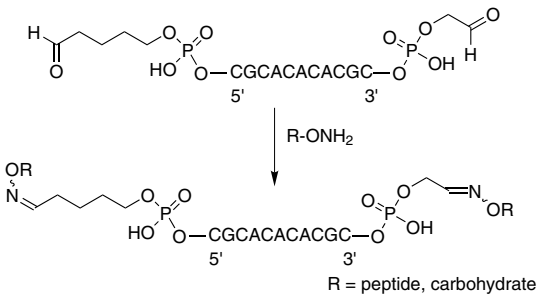


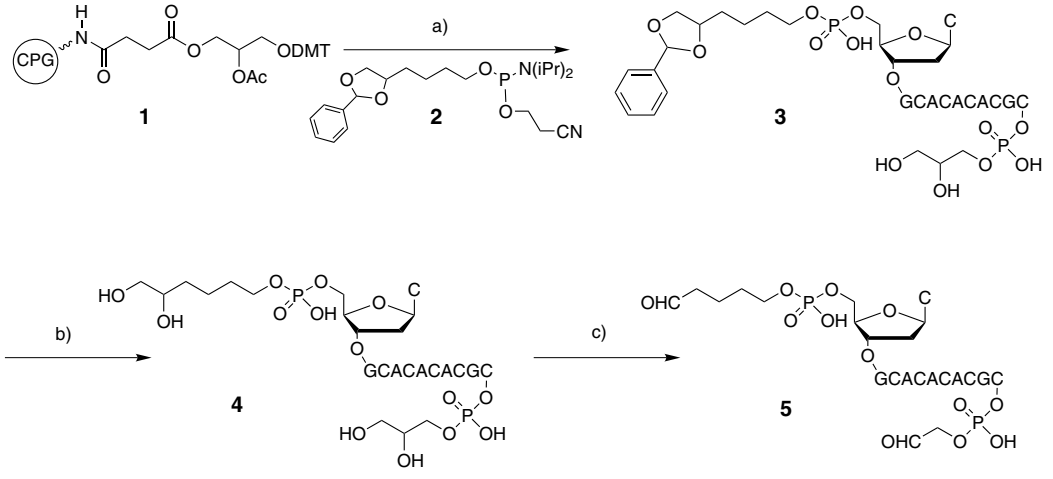
Figure 1. 3′,5′-Bis-conjugation through oxime bond formation.

has been used for cell transfection.[11] Two different carbohydrates were also used as the binding and internalisation properties of glycoconjugates via cell surface sugar-binding receptors (lectins) are well known and may represent a promising strategy for cell or tissues targeting with DNA.[12]

A 1,2-diol functionality was chosen as the precursor of the aldehydic group as it is well known that the aldehyde can be easily generated in mild condition by the oxidative cleavage of the diol. The solid support 3′-glyceryl CPG **1** bearing a 1,2-diol was thus chosen as starting material for the introduction of the masked aldehyde at the 3′-extremity. The 5′-masked aldehyde containing linker was introduced by using the phosphoramidite **2** in which the diol precursor of the aldehyde was protected as a benzylidene acetal. This latter was preferred to the previously reported phosphoramidite in which the 1,2-diol moiety was protected as a 2-methoxybenzylidene acetal.[7] Actually, cleavage of the 2-methoxybenzylidene acetal was occurred during the purification step. The phosphoramidite **2** was prepared in a two-step reaction from commercial 1,2,6-hexanetriol according to the reported procedure.[7,13]

The 3′,5′-bifunctionalised undecamer d(3′-XCGCACACACGCY-5′) **4**, in which X represents the 3′-linker bearing the 1,2-diol and Y the 5′-diol containing linker, was thus prepared using the aforementioned support **1** and incorporation of the phosphoramidite **2** at the final step of the automated DNA synthesis.[14] After cleavage from the solid support and deprotection under the usual conditions (ammonia treatment for 16 h at 55 °C), the 5′-protected intermediate oligonucleotide **3** was purified by reverse-phase HPLC (Fig. 2A shows the HPLC profile of crude bifunctionalised oligonucleotide **3**).[15] The cleavage of the benzylidene acetal protection at 5′-terminal was then performed with 80% aqueous acetic acid for 1 h at room temperature, conditions that are similar to those used for the classical dimethoxytrityl (DMT) deprotection, and afforded the 3′,5′-diol containing oligonucleotide **4**. The structure of **4** was confirmed by MALDI-TOF mass spectrometry (Table 1). Oxidative cleavage of the two diol moieties of the oligonucleotide **4** was then carried out by using a 50-fold excess of $NaIO_4$ in water at room temperature for 1 h, that led to the selective formation of the desired 3′,5′-dialdehyde containing oligonucleotide **5**, which was then purified by simple filtration on reverse-phase C_{18} silicagel (Scheme 1).

The oxyamino containing peptides **6** and **7** were prepared by SPPS using the previously reported protocol.[7] The oxyamino sugars Man-α-ONH_2 **8** and Lac-β-ONH_2 **9** were synthesised using the method we recently described.[16] Conjugation reactions were carried out in ammonium acetate buffer at pH 4.5 using the 3′,5′-dialdehyde oligonucleotide **5** and a slight excess (2 equiv per aldehydic group) of the oxyamino derivatives **6**–**9**. The course of reaction was monitored by reverse-phase HPLC and the reaction proceeded essentially to completion within 5 h to yield the corresponding conjugates **10**–**13** (HPLC analysis of crude mixture of conjugation with **7** and **9** are depicted in Fig. 2C and D, respectively, and show a representative example of conjugation reaction with a peptide and a sugar). Subsequent purification by reverse-phase HPLC afforded the desired conjugates **10**–**13** in almost 40–50% isolated yield (Scheme 2). The conjugates **10**–**13** were characterised by MALDI-TOF MS. In all cases, the experimentally



Scheme 1. Preparation of the 3′,5′-aldehyde containing linkers oligonucleotide **5**. Reagents and conditions: (a) automated DNA synthesis then NH_4OH 28%, 55 °C for 16 h; (b) AcOH 80% for 1 h; (c) $NaIO_4$, H_2O, 1 h.

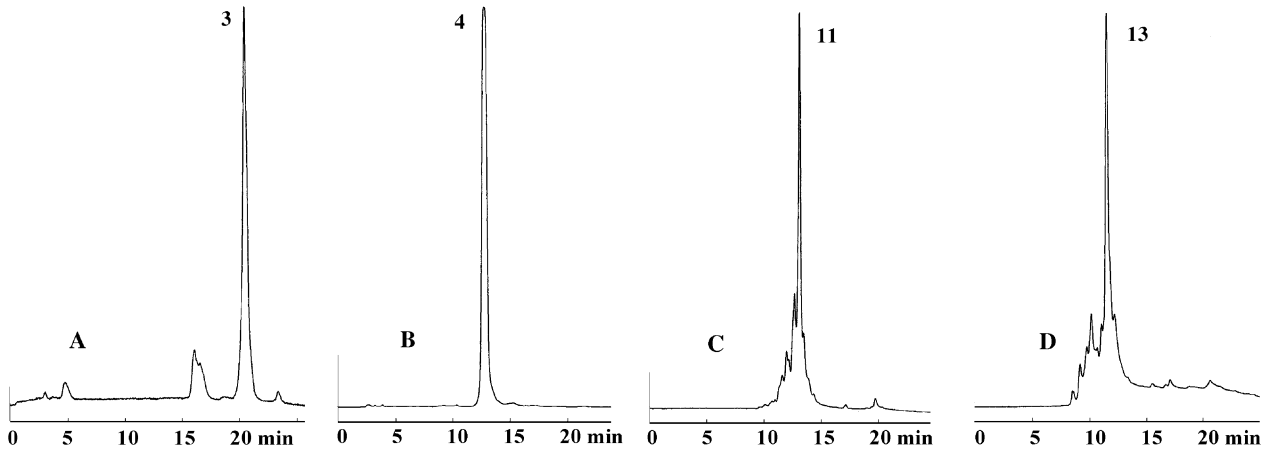
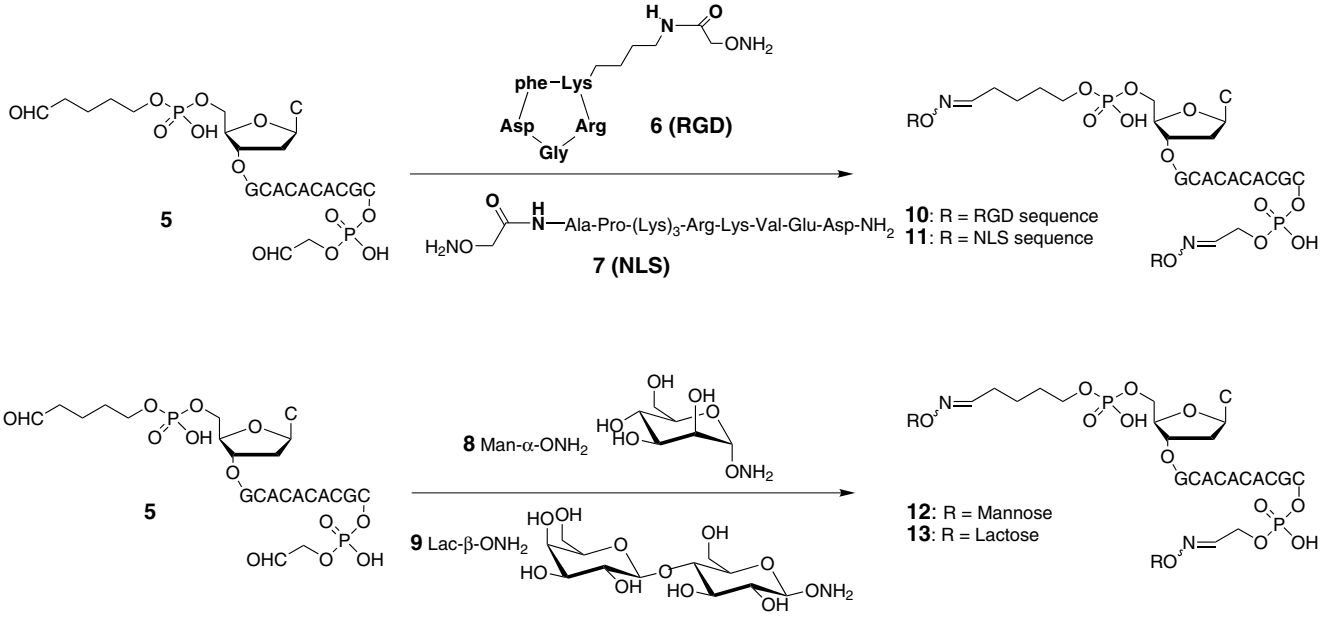


Figure 2. HPLC profiles: (A) crude deprotected undecamer **3**, (B) purified undecamer **4**, (C) crude reaction mixture of oligonucleotide **5** with the NLS peptide **7**, (D) crude reaction mixture of oligonucleotide **5** with the Lac-β-ONH_2 carbohydrate **9**.[15] Detection at 260 nm.

Table 1. Mass spectrometry analysis of the oligonucleotides and melting temperatures of duplexes formed by hybridisation of the indicated oligonucleotides with the complementary strand d(GCGTGTGTGCG)

Oligonucleotides	Calcd mass[a]	Found mass[a]	T_m[b] (°C)
3′,5′-Diol **4**	3620.76	3620.09	63.00 ± 1
Bis-RGD conjugate **10**	4874.24	4874.59	64.02 ± 1
Bis-NLS conjugate **11**	6061.56	6060.53	65.00 ± 1
Bis-mannose conjugate **12**	3910.76	3911.26	64.04 ± 1
Bis-lactose conjugate **13**	4234.76	4234.67	65.06 ± 1

[a] The analysis was performed in the positive mode.
[b] Measurements were done in phosphate buffer (sodium phosphate buffer 10 mM, EDTA 1 mM, NaCl 100 mM, pH 7).



Scheme 2. Preparation of the conjugates **10**–**13**. Reagents and conditions: oxyamino derivatives **6**–**9**, ammonium acetate buffer (pH 4.5).

determined molecular weights were in excellent agreement with the calculated values (Table 1).

The hybridisation properties of the different conjugates **10**–**13** were studied by melting temperature (T_m) measurements to evaluate the influence of the reporters at both terminus. The conjugates **10**–**13** containing the RGD peptide, the NLS sequence, the mannose and the lactose reporter groups, respectively, were hybridised with their complementary strand d(5′GCGTGTGTGCG3′) and the melting temperatures of the resulting duplexes were determined (Table 1). The

oligonucleotide **4** containing the 3′,5′-diol linkers was studied for comparison. All the conjugates showed a slightly higher melting temperature in comparison with the unconjugate duplex (Table 1).

In summary, we have described the design, construction, and preliminary analysis of a new class of bis-peptides-DNA and bis-carbohydrates-DNA conjugates. The conjugates are efficiently prepared through oxime bond formation. This strategy of synthesising bifunctional oligonucleotides will provide a platform for increasing the specificity and efficiency of cellular uptake of therapeutic oligonucleotides. Other strategy, in which two different reporters are attached at the 3′- and 5′-extremity of oligonucleotides, respectively, is currently in progress.

Acknowledgements

The Ministère de la Recherche is gratefully acknowledged for post-doctoral fellowship to O.P.E. The 'Institut Universitaire de France' is greatly acknowledged for financial support. We thank Dr. D. Gasparutto (CEA Grenoble) for MALDI-TOF analysis and J. E. Cavendish for careful reading of this manuscript.

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13. Data for the phosphoramidite **2**: ^{31}P NMR (200 MHz, $CDCl_3$): δ ppm = 145.5; MS (FAB, NBA matrix): $(M+H)^+ = 423$.
14. Automated DNA synthesis was carried out on an Expedite DNA synthesiser (Perkin–Elmer) using standard β-cyanoethyl nucleoside phosphoramidites chemistry and the commercial 3′-glyceryl-CPG solid support **1** (14 mg, loading = 71 μmol) on a 1 μM scale. The solid support **1** was purchased from Eurogentec.
15. The oligonucleotides and the conjugates were purified on a μ-bondapak C-18 column (Macherey-Nagel Nucleosil: 10 × 250 mm, 7 μm). The following system of solvent was used: solvent A, 20 mM ammonium acetate/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.
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