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Synthesis of an oxyamino-containing phenanthroline derivative for the efficient preparation of phenanthroline oligonucleotide oxime conjugates

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Abstract—A phenanthroline derivative bearing an oxyamino linker was efficiently prepared from commercial 5-nitro-1,10-phenanthroline. The subsequent reaction with an oligonucleotide containing an aldehyde either at the 5′ end or the 3′ end afforded, in good yield, the phenanthroline–oligonucleotide conjugates through oxime bond formation.

1. Introduction

During the last decade, modified oligonucleotides have been of increasing interest as useful tools in molecular biology as well as new therapeutic candidates (antisense, triple helix, siRNA strategies). The conjugation with a variety of reporter molecules including fluorescent tags, peptides, sugars and intercalators in order to provide specific properties to those synthetic oligonucleotides has thus been the subject of great development.[1] Most of the reported methods for the conjugation involve a post-solid-phase synthesis modification by preliminary incorporation of a protected nucleophilic group during automated synthesis and subsequent linkage with a reactive derivative.[2] To avoid disturbing the hybridisation process between the modified oligonucleotide and the RNA or DNA target, conjugation is generally achieved at the 3′- or 5′-ends. The two most popular modified oligonucleotides are amino and thiol functionalised oligonucleotides.[3] 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.

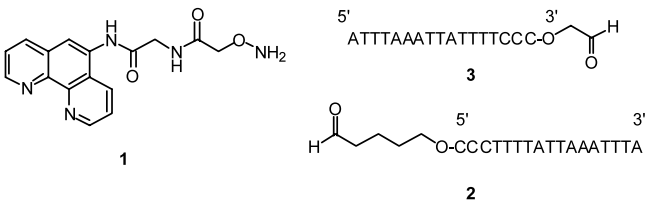

Figure 1. Structure of the phenanthroline derivative **1** and aldehyde-containing oligonucleotides **2** and **3**.

The anchoring of phenanthroline derivatives to oligonucleotides has received particular attention. In fact, the phenanthroline aromatic ring can provide interesting intercalator properties. Furthermore, artificial nucleases have been designed by exploiting the redox properties of coordination complexes bearing one or more phenanthroline ligands.[4] For instance, the bis(1,10-phenanthroline) cuprous chelate represents one of the most used cleaving agents in addition with other complexes containing a ruthenium, osmium or rhodium metallic centre.[5–9] Most of the reported methods for the preparation of phenanthroline–oligonucleotide conjugates employ amino-modified oligonucleotides and an 'activated ester' of the phenanthroline moiety (i.e. phenylcarbamate and iodoacetamido derivatives of the 5-aminophenanthroline).[4,10] Nevertheless, an excess of the phenanthroline derivative has to be used which renders the purification procedure more laborious.

Keywords: chemoselectivity; conjugation; oligonucleotide; oxime; phenanthroline.

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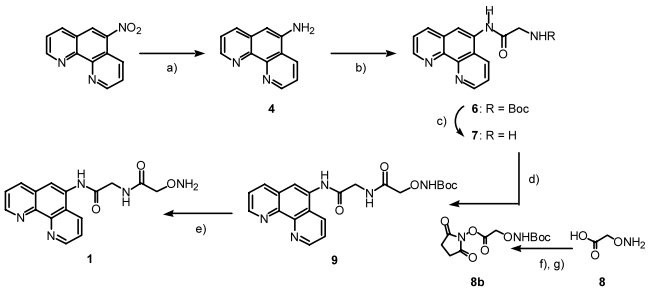


Scheme 1. Preparation of the phenanthroline derivative **1**. *Reagents and conditions*: (a) N_2H_4, Pd/C, EtOH, 70°C, 3 h, 70%; (b) $(BocNHCH_2CO)_2O$ **5**, CH_3CN, overnight, 60%; (c) CH_2Cl_2/TFA (50/50, v/v), 2 h, 90%; (d) **8b**, DMF, 1 h, 80%; (e) CH_2Cl_2/TFA (50/50, v/v), 1 h, 90%; (f) $(Boc)_2O$, NaOH/dioxane, 2 h, 80%; (g) *N*-hydroxysuccinimide, DCC, CH_2Cl_2, 1 h, 90%.

With the aim of developing a new strategy for the conjugation of oligonucleotides, we have investigated the use of the oxime linkage. The high efficiency of this oxime ligation technique for the conjugation of fluorophore, peptides and carbohydrates with oligonucleotides has been largely demonstrated.[11–14] A major advantage of this ligation technique is that it requires neither a coupling reagent nor chemical manipulations except mixing of the two components, namely an oxyamine and an aldehyde derivative.

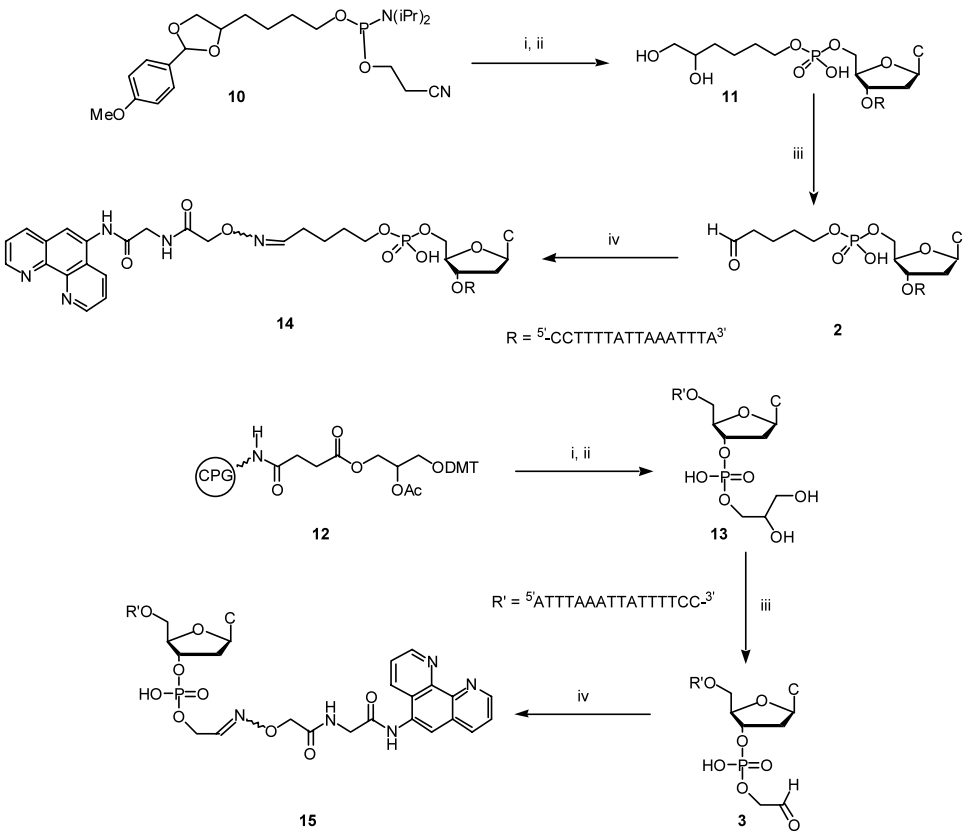
We thus envisioned exploiting these favourable characteristics of the oxime bond formation for the conjugation of a phenanthroline derivative either at the 5′- or the 3′-end of the oligonucleotide by introduction of the reactive oxyamino moiety on the phenanthroline ring and subsequent reaction with the aldehyde-containing oligonucleotide. In the present paper we report the synthesis of the phenanthroline derivative **1**, bearing a glycine linker with a terminal oxyamino group, and its subsequent reaction with the oligonucleotides **2** and **3** containing the aldehyde at the 5′- or at the 3′-end, respectively (Fig. 1). The glycine linker was chosen as it was easily available at low cost.

2. Results and discussion

The preparation of the phenanthroline derivative **1** was accomplished by a straightforward route shown in Scheme 1. Commercial 5-nitro-1,10-phenanthroline was first reduced to the corresponding amino derivative **4** using Pd/C and hydrazine in EtOH at 70°C for 3 h. The glycine linker was then introduced by reaction with the corresponding *N*-Boc anhydride **5**. This compound was easily prepared by mixing *N*-Boc-glycine (2 equiv.) with DCC in acetonitrile for 1 h and subsequent filtration to remove the DCU by-product. Due to the inactivation of the exocyclic amine on the phenanthroline ring, an excess of anhydride has to be used for completion of the reaction. The protected phenanthroline **6** was then purified from the excess of anhydride **5** by column chromatography. The *tert*-butyloxycarbonyl group on compound **6** was then removed by treatment with CH_2Cl_2/TFA solution (50/50, v/v) at room temperature for 2 h to furnish the amino derivative **7** which was used without further purification in the next step. Introduction of the oxyamino group was achieved by coupling the activated ester of *N*-Boc-*O*-(carboxymethyl)-hydroxylamine **8b** with the amino derivative **7**. The oxyamino moiety in **8** was protected with a Boc group using commercial carboxymethoxyamine hemihydrochloride **8** leading to the acid **8a**. Before the coupling reaction, the acid **8a** was reacted with *N*-hydroxysuccinimide in the presence of DCC to give the corresponding activated ester **8b**. Reaction of **8b** with the amino phenanthroline derivative **7** was carried out in dry DMF to afford the protected oxyamino phenanthroline derivative **9**, after purification by column chromatography, as a white powder in 41% overall yield from the 5-aminophenanthroline **4**.[15] The Boc protecting group was then cleaved under acidic conditions (CH_2Cl_2/TFA solution 50/50, v/v) to give the oxyamino phenanthroline derivative **1**. Taking advantage of the high reactivity of the oxyamino moiety with carbonyl derivatives, the structure of **1** was confirmed by formation of the corresponding oxime ether by reacting **1** with acetone.

The preparation of the oligonucleotide **2** bearing an aldehyde moiety at the 5′-end was accomplished using the method that we previously described by incorporating the phosphoramidite **10** at the final step of the automated DNA synthesis (Scheme 2).[12,16] Subsequent oxidative cleavage of the intermediate diol **11** with excess $NaIO_4$ generated the aldehyde function. HPLC analysis (Fig. 2A) showed the exclusive formation of the desired aldehyde-containing oligonucleotide **2** in a very short time. Compound **2** was purified by reverse-phase HPLC and obtained in 70% isolated yield.[17] For the introduction of the masked aldehyde at the 3′-end, the commercial solid supported 3′-glyceryl CPG **12** bearing a 1,2-diol was chosen as starting material (Scheme 2). This support was preferred to the previously reported 1,2-aminoalcohol-containing support[18] as the efficiency of this latter support for the introduction of the 1,2-aminoalcohol decreased dramatically after 3–4 months storage. The oligonucleotide **13** with the 1,2-diol at the 3′-end was synthesised according to standard β-cyanoethyl phosphoramidite chemistry using the support **12**.[16] After the usual deprotection and purification steps, the oligonucleotide **13** was treated with $NaIO_4$ to generate the aldehyde. Compound **3** was obtained after purification by reverse-phase HPLC in almost 50% isolated yield (Fig. 2C shows the HPLC profile of crude oxidation mixture of **13**, and reveals a single major product).

Conjugation reactions were carried out in ammonium acetate buffer at pH 4.5 using the oligonucleotides **2** and **3** containing an aldehyde group at the 5′- and 3′-ends, respectively, and a slight excess (2 equiv.) of the phenanthroline derivative **1**.[19] The course of the reaction was followed by reverse-phase HPLC and the reaction proceeded essentially to completion within 3 h to yield exclusively the corresponding conjugates **14**



Scheme 2. Preparation of aldehyde containing oligonucleotides **2** and **3** and corresponding conjugates **14** and **15**. *Reagents and conditions*: (i) automated DNA synthesis then NH_4OH 28%, 55°C for 16 h; (ii) AcOH 80% for 1 h; (iii) $NaIO_4$ in H_2O; (iv) phenanthroline derivative **1**, ammonium acetate buffer (pH 4.5).

and **15** (HPLC analysis of crude mixture of conjugation with **2** and **3** are depicted in Figure 2B and 2D, respectively).[17] Subsequent purification by reverse-phase HPLC afforded the conjugates **14** and **15** in almost 50% isolated yield. The conjugates **14** and **15** were characterised by ES-MS. In all cases, the experimentally determined molecular weights were in excellent agreement with the calculated values (Table 1).

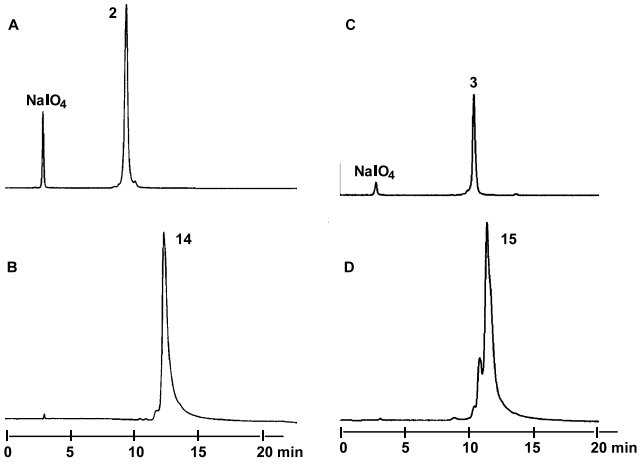


Figure 2. HPLC profiles: (A) crude oxidation mixture of 5′-diol oligonucleotide **11**, (B) crude reaction mixture of oligonucleotide **2** with the phenanthroline derivative **1**, (C) crude oxidation mixture of 3′-diol oligonucleotide **13**, (D) crude reaction mixture of oligonucleotide **3** with the phenanthroline derivative **1**.[17] Detection at 260 nm.

3. Conclusion

In conclusion, we report a straightforward preparation of the oxyamino containing phenanthroline derivative **1** from commercially available 5-nitro-1,10-phenanthroline. The usefulness of this phenanthroline derivative for the efficient and improved preparation of 5′- or 3′-oligonucleotide oxime conjugates was demonstrated. Introduction of the phenanthroline derivative **1** as a ligand in ruthenium complexes may open the way for efficient conjugation to oligonucleotides as well as to peptides through oxime ligation.[21] Work is currently underway to prepare ruthenium complexes using this oxyamino containing phenanthroline ligand for such purposes.

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Table 1. ES-MS analysis*

Oligonucleotides	Calcd mass	Found mass
5′-Diol **11**	5304.50	5304.89
3′-Diol **13**	5262.50	5262.69
5′-Conjugate **14**	5579.70	5579.51
3′-Conjugate **15**	5537.80	5536.81

* 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).

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15. Data for **9**: ^{1}H NMR (200 MHz, d_6-DMSO): δ ppm = 1.39 (s, 9H, *t*Bu), 4.16 (d, J = 5.5 Hz, 2H, CH_2NH), 4.28 (s, 2H, CH_2-O), 7.71–7.82 (m, 2H, H-phen), 8.13 (s, 1H, H-phen), 8.43 (m, 2H, NH+H-phen), 8.61 (d, J = 8.2 Hz, 1H, H-phen), 9.04 (d, J = 4 Hz, 1H, H-phen), 9.12 (d, J = 4 Hz, 1H, H-phen), 10.22 (1H, s, NH), 10.31 (1H, s, NH). MS (DCI): m/e = 425.9. Mp 135–136°C.
16. Automated DNA synthesis was performed on an Expedite DNA synthesiser (Perkin–Elmer) following the manufacturer's protocols for standard β-cyanoethyl nucleoside phosphoramidite chemistry on a 1 μM scale. The support **12** was purchased from Eurogentec.
17. The oligonucleotides and the conjugates were purified on a μ-bondapak C-18 column (Macherey-Nagel Nucleosil: 10×250 mm, 7 μm). The following solvent system was used. 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.
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