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Preparation of Oligonucleotide-Dexamethasone conjugates[1]

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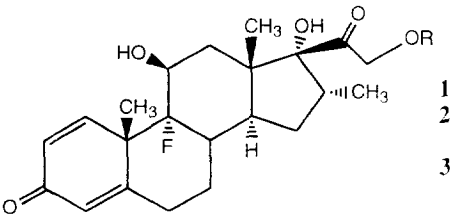
Abstract : Oligonucleotides carrying a dexamethasone molecule at the 5'-end have been prepared. During the preparation of these conjugates an intramolecular side reaction was detected and minimized.

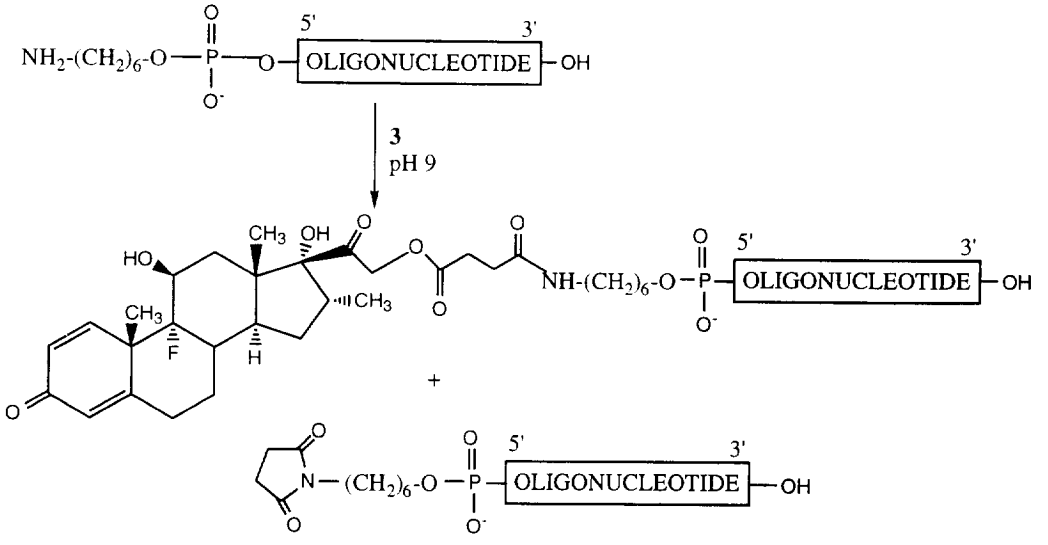
Modified and unmodified oligonucleotides have been widely employed to inhibit gene expression. In order to enhance the activity of oligonucleotides and their analogues, they have been covalently linked to intercalating, alkylating, photocrosslinking and radical generating reagents. Besides increasing the affinity for the target sequence, some of these compounds promoted the uptake of oligonucleotides by cells and improved their resistance to nucleases.[2] Covalent attachment of poly(L-lysine)[3] and lipid molecules such as cholesterol to oligonucleotides enhances the antisense activity of these compounds[4]. In both cases, higher activity has been related with an increased cellular uptake[4,5]. Lipid moieties such as cholesterol may bind to the lipophillic cellular membranes but oligonucleotide conjugates still have to penetrate into the cells. A disulfide bond between oligonucleotides and cholesterol has been proposed to facilitate the release of free oligonucleotides inside the cell[5b, 6]. In the present paper, we would like to describe the preparation of oligonucleotides carrying dexamethasone. Dexamethasone is a glucocorticoid which is lipophillic like cholesterol but it can also be internalized to the cell nucleous by specific glucocorticoid-receptors[7]. For these reasons, it is expected that cellular uptake of oligonucleotides carrying dexamethasone may be better.



1 R = H Dexamethasone
2 R = $COCH_2CH_2COOH$
3 R = $COCH_2CH_2CO-$

The incorporation of cholesterol to oligonucleotides has been achieved by connecting the hydroxyl group of cholesterol to the 3' and/or 5'-end of oligonucleotides through different linkers [4-6,8]. The addition of dexamethasone at the 5'-end was assayed using the two-step protocol previously used in the

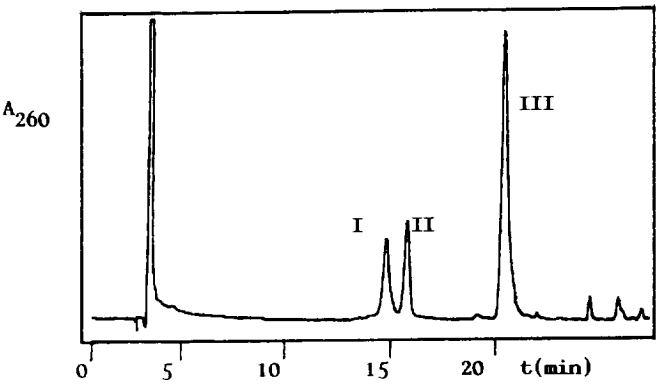
preparation of oligonucleotides with digoxigenin[9]. First, an oligonucleotide sequence is prepared containing a primary amino group at the 5'-end. The purified 5'-amino oligonucleotide is then reacted with a dexamethasone derivative that contains an active ester function. Dexamethasone **1** contains 3 hydroxyl groups : one is a primary alcohol, and the others are one secondary and one tertiary alcohol group that are less reactive. Reaction of dexamethasone with a small excess of succinic anhydride yields hemisuccinate derivative **2** in quantitative yield[10]. N-hydroxysuccinimide ester **3** was prepared by reaction of **2**, with N-hydroxysuccinimide and DCC[11].



Oligonucleotides containing natural phosphodiester linkages A (5' amino-CCGACC 3'), B (5'-amino-CACCGACGGCGC 3') and C (5'-amino- GGCTGCTGGAGCGGGCACAC 3') were prepared on a DNA synthesizer using standard 2-cyanoethyl phosphoramidites. After the assembly of oligonucleotide sequences, a phosphoramidite that contains a monomethoxytrityl protected aliphatic amino group was incorporated at the 5'-end (N-MMT-C_6 amino modifier, Clontech, CA). After ammonia deprotection and purification, the 5'-amino oligonucleotides A-D were reacted with the dexamethasone succinic acid N-hydroxysuccinimide ester **3** in a 1:1 mixture DMF - 0.1M pH 9 sodium carbonate buffer[9]. First, reactions were left overnight at room temperature and the resulting oligonucleotide-dexamethasone conjugates were purified by reversed-phase HPLC[9]. The desired products were retarded from starting amino-oligonucleotides due to the lipophillic properties of dexamethasone[12]. In these conditions, conversion of amino-oligonucleotides to dexamethasone-oligonucleotides was only 10-40 %, while in the case of digoxigenin-oligonucleotides a 70-80% conversion was observed. Increasing the time and the molar excess of **3** did not result in higher yields. On the contrary, yields were lower if time was increased. The explanation of this observation was found during the conjugation of hexamer A with **3**. An extra peak was observed eluting between the starting amino-hexamer and the dexamethasone-hexamer. Mass spectrometry (electrospray) shows that the side-product was the result of the intramolecular attack of the amide to the ester function giving an oligonucleotide with a succinimide function at the 5'-end[12]. The formation of this side product is due to the special lability of the β-keto ester bond used for

connecting the dexamethasone to the oligonucleotide. In order to minimize the formation of this side product, the conjugation of dexamethasone with dodecamer B was studied at room temperature. The optimal time was found to be 1 hour at room temperature obtaining 60-70% conversion of the dexamethasone conjugate[13] (see figure 1). Stability of the dexamethasone-oligonucleotide conjugate B was analysed at pH 5.0, pH 7.0 and pH 9.0[14]. No degradation was observed at pH 5 and pH 7 at room temperature for 12 days. On the contrary, the conjugate was decomposed to the succinyl-oligonucleotide in 24 hr at pH 9.0 if carbonate buffer is used. When the pH 9.0 buffer solution was made with Tris base, decomposition was slower (18% after 24 hr), indicating that carbonate anions catalyse the decomposition. In order to check that the presence of dexamethasone does not change the base pairing properties of the oligonucleotide, melting temperatures of dodecamer duplexes were performed. Melting temperature of the dexamethasone-dodecamer B with its complementary sequence (5' GCGCCGTCGGTG) was 71 °C and without dexamethasone it was 70.5 °C (in 0.1 M NaCl and 50 mM sodium phosphate buffer pH 7.5). This indicates that the presence of dexamethasone at the 5'-end does not affect duplex stability. Finally, phosphorothioate oligonucleotides carrying dexamethasone at the 5'-end have been also prepared following the method described here (data not shown).

Figure 1 : HPLC profile obtained after 30 minutes of reacting compound **3** with sequence B. (I: sequence B, II: succinyl derivative, III: Dexamethasone derivative (see conditions in note 14).



In conclusion, we have shown that defined oligonucleotides containing dexamethasone at the 5'-end of the oligonucleotide can be synthesized. The study of the biological properties of these oligonucleotide-dexamethasone hybrids is currently in progress.

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References and Notes

1. Abbreviations used are as follows: AcOEt : ethyl acetate; CPG : controlled pore glass; DCC : dicyclohexylcarbodiimide, DCM : dichloromethane; DMAP: *N,N*-dimethylaminopyridine; DMF : N.N-dimethylformamide; DMT: dimethoxytrityl; MMT : monomethoxytrityl; MeOH : methanol; THF : tetrahydrofurane, Tris : tris(hydroxymethyl)aminoethane.

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10. Dexamethasone **1** (0.4 g, 1.0 mmol) was reacted with succinic anhydride (0.15 g, 1.5 mmol) and DMAP (0.13 g, 1 mmol) in pyridine at room temperature for 20 hours. The reaction mixture was concentrated to dryness and the residue was dissolved with 150 ml of DCM / MeOH (8:2) and washed twice with 75 ml of 1N aqueous sulfuric acid and with 75 ml of water. In each extraction 15 ml of methanol were added. The organic layer was dried and concentrated to dryness giving **2** (0.5 g, 100%) as a white solid that was used without further purification. Proton NMR shows the appearance of two multiplets at 2.6 and 2.7 ppm and C-13 NMR shows the appearance of two quaternary carbons at 175.7 and 173.8 ppm and two methylene carbons at 68.8 and 69.7 ppm in agreement with the addition of one succinic acid moiety.

11. Dexamethasone succinic acid **2** (0.25 mg, 0.5 mmol) was reacted with N-hydroxysuccinimide (70 mg, 0.6 mmol) and DCC (125 mg, 0.6 mmol) in 5 ml of THF at room temperature for 20 hours. 15 mg of N-hydroxysuccinimide and 30 mg of DCC were added and the reaction was left at room temperature for 4 more hours. Dicyclohexylurea precipitate was removed by filtration and the solution was concentrated to dryness. The residue was dissolved in AcOEt and filtered again. The solution was washed with water. The organic layer was dried and concentrated to dryness. The residue was dissolved in 1-2 ml of AcOEt and 10 ml of petroleum ether were added. A white precipitate was formed that was collected and dried. Yield 210 mg (72%). Proton NMR in agreement to the expected structure.

12. Mass spectra (electrospray): Succinyl-hexanucleotide A : M 1997.7, M+Na 2019.6, M+K 2035.5, M+Na+K 2041.4. Expected mass : 1998.1. Dexamethasone-hexanucleotide A : M 2389.8. Expected 2390.2. Dexamethasone-dodecamer B : M + 2 Na 4316.0. Expected 4273. Dexamethasone-20 mer C : M 6836.3. Expected 6837.

13. Approx. 5 optical units (0.15 mg) of 5'-amino oligonucleotide were dissolved in 0.5 ml of 50 mM sodium carbonate buffer (pH 9.0) or better 50 mM Tris buffer (pH 9.0), and 5 mg of compound **3** dissolved in 0.5 ml of DMF were added. The mixture was incubated for 1 hr at room temperature and products were purified by HPLC. HPLC conditions were as follows : Column : Nucleosil 120C18 (200 x 4 mm), flow rate 1 ml/min, a 40 min linear gradient from 2 to 45% acetonitrile over 20 mM aqueous triethylammonium acetate. Retention time of 5'-amino dodecamer B 15.4 min, 5'-succinyl derivative of sequence B : 16.5 min and 5'-dexamethasone derivative 21.3 min.

14. The stability assays were run in the following solutions : A) 50 mM citric acid, sodium phosphate buffer pH 5.0; B) 50 mM tris buffer pH 7.0; C) 50 mM Tris buffer pH 9.0; and D) 50 mM sodium carbonate buffer pH 9.0.

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