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EFFICIENT AND EASY SYNTHESIS OF OCTATHYMIDYLATE COVALENTLY LINKED TO INTERCALATING 9-AMINOELLIPTICINE

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Reductive amination of 3'-apurinic octathymidylate with 9-aminoellipticine provides octathymidylate covalently linked to intercalating ellipticine through a 3,4-dihydroxypentamethylene linker. Studies of its binding properties to poly(rA) reveals the formation of two different complexes depending of the temperature (Tm 13°C and 38°C) with dT/rA stoichiometry respectively equal to 2/1 and 1/1. When compared to parent octathymidylate, stability of the latter duplex is enhanced by the interaction energy provided by the dye moiety.

Artificial control of gene expression by single-stranded nucleic acid fragments is now under extensive evaluation. For that purposes synthetic oligodeoxynucleotides has been used as anti-messengers. Due to certains limitations (sensitivity to nucleases, poor cellular uptake) various structural modifications of the oligomers structure have been proposed, i.e. modification of the phosphate backbone (1), of the sugar configuration (2). Furthermore it has been shown that covalent attachment of an intercalating agent, i.e. 2-methoxy-6-chloro-9-aminoacridine, to an oligodeoxynucleotide strongly increases the stability of the complex formed with a complementary sequence (3) and the efficiency of cell penetration (4). However the proposed method for the synthesis of oligonucleotides covalently linked at 3'-end to an acridine derivative (5) was lenghtfull and time consuming. Therefore we would like to describe herewith a new easy method for introducing an intercalating agent on an oligodeoxynucleotide.

MATERIALS AND METHODS

High performance liquid chromatographic analysis were carried out on a Beckman C_{18} (3μm) column for the monitoring of depurination and reductive amination reactions and on a Nucleosil C_{18} (3μm, 4.6 x 100 mm) column (Macherey-Nagel) for the monitoring of enzymatic degradation and periodate oxydation reactions. A Waters U6K injector, two 6000 A pumps, a M720 solvent

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programmer, and a M730 microprocessor-controlled data were employed. UV spectra were recorded using a Pye-Unicam PU 4021 multichannel detector and a Pye-Unicam PU 4850 video chromatography control center. A linear gradient of 0-25% acetonitrile in 0.1 M ammonium acetate (pH 5.9) was applied in 20 min at a flow rate of 1 ml min^{-1}.

Preparative HPLC was run on a Radial-Pak Bondapak C_{18} (10 μm) cartridge in a Waters Z module. The same gradient as above was employed at a flow rate of 2.7 ml min^{-1}.

Calf spleen phosphodiesterase (2 mg, 4 units, in 1 ml) was purchassed from Boehringer Mannheim.

Ultraviolet determinations were obtained using a thermostated Uvikon 810 (Kontron Instruments) equipped with a temperature attachement. The buffer conditions were: Sodium Cacodylate 10 mM, pH7, NaCl 0.1 M.

Depurination of the oligonucleotide d[$(Tp)_8$A]: A solution of d[$(Tp)_8$A] (62 A_{260} units) (6) in 10 mM hydrochloric acid (1.2 ml) was heated at 65°C for 1.5 hour. At that time HPLC analysis indicated complete convertion of the oligonucleotide (R_T 15.73 min, λ_{max} 263 nm) in the apurinic compound d[$(Tp)_8$-r] **1** (R_T 14.98 min, λ_{max} 264 nm) and adenine (R_T 10.07 min, λ_{max} 260 nm).

Reductive amination of d[$(Tp)_8$-r] with 9-aminoellipticine: To the above acidic solution was added water (0.3 ml), sodium cyanoborohydrure (6.2 mg, 0.1 mmole) in 1M ammonium acetate buffer solution pH 5 (0.4 ml) and 9-aminoellipticine diacetate (0.4 mg, 10^{-3} mmole) in 20 mM hydrochloric acid (0.1 ml). The resulting solution was maintained at 37°C for 45 min. HPLC analysis indicated quantitative convertion of the apurinic compound d$(Tp)_8$-r in the adduct $(Tp)_8$-r*-Ell (R_T 17.10 min, λ_{max} 266, 305 nm) which was purified by HPLC (3 injections). The combined appropriate fractions (HPLC purity better than 98%) were evaporated under reduced pressure. The residue was dissolved in 1M triethylammonium bicarbonate buffer solution pH 7.5 (1 ml) and applied on a C_{18} Sep-Pak cartridge (Waters Associates). After washing with water (10 ml), $(Tp)_8$-r*-Ell was eluted with water/acetonitrile (1/1, v/v; 6 ml). After evaporation, the residue was redissolved in water, treated with DOWEX 50W (Na^+ form), filtered on glass wool and lyophilized (20 A_{260} units).

Enzymatic hydrolysis of $(Tp)_8$-r*-Ell by calf spleen phosphodiesterase: To a solution of $(Tp)_8$-r*-Ell (2 A_{260} units) in water (70 μl) was added 0.125 M ammonium acetate pH 7, 0.0025 M EDTA, 0.0625% Tween 80 buffer solution (80 μl) and calf spleen phosphodiesterase (stock solution, 2 μl). The resulting solution was maintained at 37°C. After 30 min, HPLC chromatogram showed the peacks corresponding to $(Tp)_8$-r*-Ell (R_T 18.65 min), $(Tp)_7$-r*-Ell (R_T 18.98 min), $(Tp)_6$-r*-Ell (R_T 19.23 min), $(Tp)_5$-r*-Ell (R_T 19.68 min), $(Tp)_4$-r*-Ell (R_T 20.27 min), $(Tp)_3$-r*-Ell (R_T 21.02 min), $(Tp)_2$-r*-Ell (R_T 21.95 min), Tp-r*-Ell (R_T 23.37 min), r*-Ell (R_T 25.25 min) and Tp (R_T 7.38 min).

Periodate oxydation of $(Tp)_8$-r*-Ell: To a solution of $(Tp)_8$-r*-Ell (2 A_{260} units) in water (20 μl) was added 120 mM $NaIO_4$ dissolved in 20 mM sodium phosphate (pH 4.5) (180 μl). After the reaction was allowed to proceed for 20 min at room temperature, HPLC analysis of the mixture revealed complete degradation of the adduct (R_T 18.73 min) in two compounds, the first one (R_T 16.20 min), λ_{max} 266 nm) excluded the presence of the aminoellipticine chromophore, and showed the chromophore of thymidine. On the contrary the second compound (R_T 26.53 min) did not showed chromophore of thymidine but retains a maximum in the 305-320 nm range.

RESULTS AND DISCUSSION

Synthesis

During the course of our recent work upon the action of aromatic amines on a short synthetic apurinic oligonucleotide (7), we have shown that the

first step of the β-elimination process is the formation of a Schiff base as a transient intermediate. Furthermore reduction of this Schiff base gave the corresponding reduced adduct that can be easily isolated (8).

However, this study was limited to the model d(Tp[AP]pT) (also named d(Tp-r-pT)) bearing an AP site adjacent to dT on both sides, but it could open a new method for introducing an intercalating agent on an oligonucleotide at its 3'-end. Therefore we decided to synthesize the oligomer d[$(Tp)_8$-r], to react it with 9-aminoellipticine upon reducing conditions in order to isolate the corresponding adduct $(Tp)_8$-r*-Ell (Figure I), and to perform some preliminary biophysical studies to evaluate its potentiality to act as a molecular probe.

It is noteworthy that in such condition the intercalating agent will be linked to the oligomer through a 3,4-dihydroxypentamethylene linker. Pentamethylene linker has been shown to exhibit the optimum length for the subsequent binding of acridine derivatized oligothymidylates to their complementary sequences (3).

Upon acidic treatment (9) the nonamer d[$(Tp)_8$A] gave the putative apurinic d[$(Tp)_8$-r]. Then 9-aminoellipticine and sodium cyanoborohydride were added and the mixture was kept at 37°C. The reaction was monitored by HPLC and after 45 min no more apurinic oligonucleotide was detected.

After purification by HPLC, identification of the final adduct was performed as follow: UV spectra of this oligomer presents a maximum at 315 nm, indicating the presence of the aminoellipticine chromophore. Partial enzymatic degradation with calf spleen phosphodiesterase shows the 9 expected ellipticine derivatized fragments, and periodate oxydation HPLC analysis corroborates the structure as its shows two fragments one presenting only the UV spectrum of the dT chromophore, the other one of the ellipticine moiety.

Interaction of $(Tp)_8$-r*-Ell with poly(rA)

Interaction of $(Tp)_8$-r*-Ell with poly(rA) was investigated using absorption spectroscopy. This interaction gives rise to modifications in the absorption spectrum of the molecule (Fig. 2). At 310 nm, a region in which the ellipticine moiety alone contributes to the absorption, the binding is characterized by an hypochromic shift, together with a bathochrome effect. The binding yields two isobestic points at 335 nm and 355 nm. If the binding

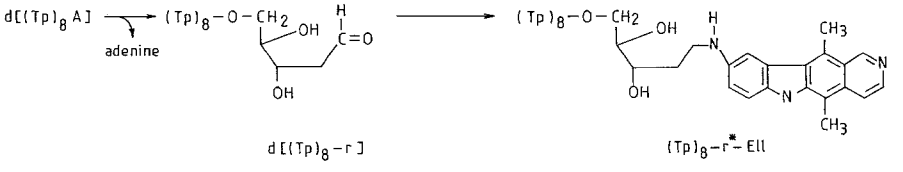

Figure 1 - Chemical synthesis of $(Tp)_8$-r*-Ell.

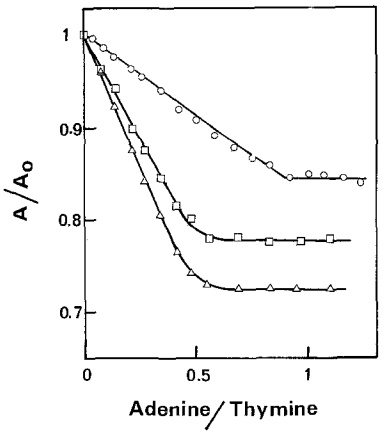

Figure 2 - Relative absorption of $(TP)_8$-r*-Ell: 7.18 10^{-5} M, in the presence of increasing concentrations of poly(rA) in 10 mM sodium cacodylate pH 7, 0.1 M NaCl.
In the ratio Adenine/Thymine, Adenine represents the concentration of poly(rA) expressed in nucleotide and Thymine, the concentration of $(Tp)_8$-r*-Ell expressed in thymine residues. Experiments were performed at 8°C. —Δ— $(Tp)_8$-r*-Ell at 270 nm, —□— $(Tp)_8$-r*-Ell at 300 nm and —○— $(Tp)_7T$ at 270 nm.

is followed at 270 nm, region in which the main contribution to the absorbtion is due to the oligo(dT) residue, it is characterized by an hypochromic effect. The extent of hypochromicity varies with the poly(rA) concentration and reaches a plateau value of about 28% for a ratio of poly(rA) and $(Tp)_8$-r*-Ell equal to 4, stoechiometry which corresponds to the binding of two $(Tp)_8$-r*-Ell molecules per 8 adenine residues of poly(rA) (Fig. 2). In the same conditions, the hypochromicity induced at 270 nm by the association of the unmodified $(Tp)_7T$ to poly(rA) reaches a plateau corresponding to the binding of one $(Tp)_7T$ per 8 adenine residues. These results could correspond to the formation of a triple helix, in the case of the association of $(Tp)_8$-r*-Ell to poly(rA). The composition of this triple helix would then be: $2(Tp)_8$-r*-Ell·$(rAp)_8$. Such triplex formation does not appear when another oligonucleotide modified through an ellipticine derivative (T_4C_5OPC) interacts with poly(rA) (10), but has already been described in the case of the interaction of an azidoproflavine modified α-anomeric octathymidylate with $(dA)_8$ (11).

Melting curves of $(Tp)_8$-r*-Ell complexes

Increasing the temperature of the complex formed at low temperature between $(Tp)_8$-r*-Ell and poly(rA) for a ratio rA/dT=1/1 leads to the dissociation of this complex. The hyperchromism at 260 nm induced upon melting, leads to a strongly cooperative melting curve which reaches a maximum of hyperchromicity equal to 37%. The dissociation process is biphasic with two transitions whose temperatures of half dissociation are estimated to

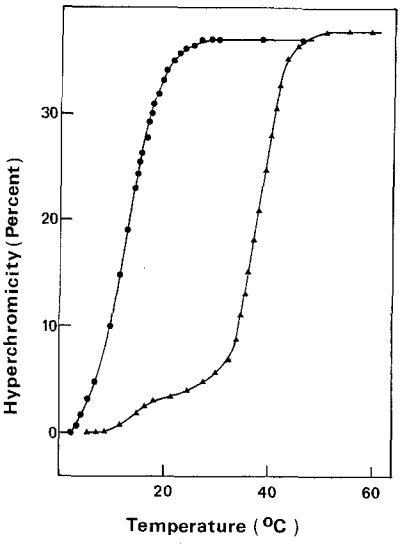

Figure 3 - Melting curves at 260 nm of the complexes formed between $(Tp)_8$-r*-Ell and poly(rA) —▲— or between $(Tp)_7T$ and poly(rA) —●—. The ratio of $(Tp)_8$-r*-Ell or $(Tp)_7T$ versus poly(rA) was 1, with the concentration of each component equal to 10^{-5} M. Buffer conditions were the same than in Fig. 2.

be 13°C and 38°C (Fig. 3). In the same conditions, the complex formed between $(Tp)_7T$ and poly(rA) shows a monophasic transition and reaches a maximum hyperchromicity equal to 37% and a melting temperature equal to 11.8°C. This indicates, as expected, that the amino ellipticine residue which is anchored to the $(Tp)_8$ sequence, strongly stabilizes the complex formed with poly(rA) as already shown for acridine modified oligonucleotides (12-15) or oxazolo pyridocarbazolium modified oligonucleotides (10). As far as the heterogeneity of the transition is concerned, the presence of the ellipticine moiety induces a biphasic transition which can reflect a transition from a triple stranded structure: $2(Tp)_8$-r*-Ell·$(rAp)_8$, to the double stranded $(Tp)_8$-r*-Ell·$(rAp)_8$ followed by a transition from $(Tp)_8$-r*-Ell·$(rAp)_8$ to $(Tp)_8$-r*-Ell + poly(rA). However, we cannot rule out that a second transition would appear at low temperature (< 0°C) in the case of the complex formed between poly(rA) and the unmodified $(Tp)_7T$.

In conclusion, we have developped an original, easy approach to covalently link oligonucleotides with an amino reagent through the highly reactive AP site at its 3'-end. The use of such approach gives directly a C_5 linker coming from the deoxyribose ring. As a first example, we have shown that the formation of such linkage between aminoellipticine and a $(Tp)_8$ oligonucleotide leads to a compound which is able to be associated to its complementary sequence and which strongly enhanced the stability of the formed complex as compared to the unmodified oligonucleotide.

Furthermore numerous extension of this approach can be considered from the use of various primary amines to the site of attachment which could be either at the 3'-end or at any position of the oligonucleotidic chain.

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