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Improved synthesis of daunomycin conjugates with triplex-forming oligonucleotides. The polypurine tract of HIV-1 as a target

Massimo L. Capobianco,[a,*] Marcella De Champdoré,[a,†] Federico Arcamone,[a] Anna Garbesi,[a] Dominique Guianvarc'h[b] and Paola B. Arimondo[b]

[a]*Istituto di Sintesi Organica e Fotoreattività del Consiglio Nazionale delle Ricerche (ISOF-CNR) Via Gobetti 101, 40129 Bologna, Italy*

[b]*Laboratoire de Biophysique, UMR 5153 CNRS-Muséum National d'Histoire Naturelle, INSERM UR565, 43 rue Cuvier, 75231 Paris cedex 05, France*

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Dedicated to Professor Claude Hélène in memoriam

Abstract—Triple helix-forming oligonucleotides (TFOs) are promising agents for the control of gene expression, as they can selectively bind to a chosen oligopyrimidine·oligopurine region of a gene of interest thus interfering with its expression. The stability of the triplex formed by the TFO and the duplex is often too poor for successful applications of TFOs in vivo and the conjugation of a DNA intercalating moiety to the TFO is a common way to enhance the TFO affinity for its target. In a previous work, we investigated the properties of daunomycin conjugated TFO (dauno-TFO) and found that this class of compounds showed a higher degree of affinity than native oligonucleotides for an oligopyrimidine·oligopurine duplex target and that the presence of the amino sugar increases such stability. Here, we report a significantly improved synthetic procedure for the preparation of the conjugates, based on the protection of the daunosamine moiety by N-trifluoroacetylation. This protecting group is removed as a final step from the conjugation product by mild basic hydrolysis to give the desired dauno-TFO. Compared to the previous synthetic procedure, the improvement is important. The synthesis is now more reproducible and no side products are formed. Moreover, the thus protected daunomycin derivative is very stable, up to at least one year. Two dauno-TFOs, differing by the length of the oligonucleotide moiety, were prepared to target the polypurine tract (PPT) of *HIV-1*. Triplex formation by these compounds with model duplexes was studied by UV spectroscopy, thermal gradient gel electrophoresis (TGGE) and gel electrophoretic mobility shift. The experimental results demonstrate that dauno-TFOs bind to the PPT of *HIV-1* more strongly than the unconjugated TFOs.

1. Introduction

The so-called antigene methodology[1] is an innovative approach for the inhibition of the expression of a selected gene through the specific recognition of an oligopyrimidine·oligopurine sequence on the DNA. The recognition is obtained by using a synthetic triplex-forming oligonucleotide (TFO) able to bind, in the major groove, to the oligopurine strand of the duplex through Hoogsteen or reverse Hoogsteen hydrogen bonds, upon formation of a local triple helix (triplex). The presence of the triplex might eventually interfere with the transcription process, leading ultimately to the inhibition of the protein synthesis coded by the targeted gene. The genome of HIV-1 contains a well conserved polypurine·polypyrimidine tract, called PPT, present both in *nef* and *pol* genes,[2] that could be a valuable target to fight HIV. It has been shown that the PPT sequence is accessible in the chromatin structure to an oligonucleotide to form a triple-helical structure,[3] and the same has been reported for other genes in viable cells that can be modified by reactive TFOs.[4,5] Recently, the antigene strategy has been used to induce mutations at a specific site in mice.[6] However, the triplex formed by natural oligonucleotides is not sufficiently stable as it would be required for efficient gene inhibitor agents.

Keywords: Antigene; Antiviral-agents; DNA-triplex; HIV; Nucleic acids.

* Corresponding author. Tel.: +390516398287; fax: +390516398349; e-mail: capobianco@isof.cnr.it

† Present address: Istituto di Biochimica delle Proteine del Consiglio Nazionale delle Ricerche (IBP-CNR) Via P. Castellino 111, 80131 Napoli, Italy.

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Several modifications have been suggested to enhance the stability of the triple helix;[7–9] such modifications include the conjugation of the TFO to intercalating moieties (e.g., acridines[10,11]) or even involve the use of largely modified oligonucleotides such as PNA[12] and phosphoramidates.[13] Another strategy consists in attaching the TFO to cross-linking molecules (e.g., psoralene[14,15]), alkylating[4] or cleaving agents[16] in order to induce a DNA modification (cross-linkage, alkylation or cleavage) specifically at the duplex target site.[17] It has been shown[18] that the stability of the triple helix is markedly enhanced by conjugation of the TFO with an anthracycline derivative, provided that the binding of the oligonucleotide chain occurs at position 4 of the aglycone moiety in order to allow a correct geometry for the intercalation complex and the required alignment of the TFO in the major groove of the double helix. On the other hand, TFO conjugates containing a carminomycinone-derived chromophore were able to form a triple helix with a polypurine tract (PPT), representing a model of the same sequence present in the human integrated genome of HIV-1 infected cells.[19] The aim of the present study was to prepare daunomycin-TFOs directed against the PPT of *HIV-1* as we expected a higher affinity as compared with the carminomycinone-derived conjugates because of the presence of the amino sugar moiety which provides additional stabilisation of the intercalation complex via specific interactions in the minor groove[18] and determine their affinity for model duplexes containing the said PPT. The conjugates, whose structure is shown in Figure 1, would represent the prototype of novel pharmacological agents addressed against the proliferation of HIV.

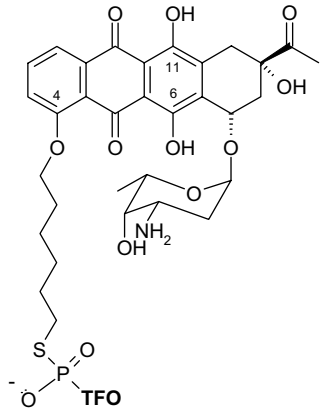


Figure 1. Structure and numbering of the dauno-oligonucleotide conjugates.

2. Results and discussion

The base sequences of the oligonucleotides are reported in Table 1. The choice of the oligonucleotides to target the polypurine region of HIV was based, partially, on the already available literature data. According to Giovannangeli et al.,[2] oligonucleotide **G6** was expected to give a triplex with a T_m of 31 °C, independently of the pH of the medium. However, as oligonucleotide **G6** showed a fierce tendency to self aggregate that severely hampered every purification attempt of the conjugate,[18,19] we decided to synthesise conjugates of oligonucleotides containing only thymidines and cytidines (pyrimidine motif TFO). In this motif, cytosines must be protonated to allow Hoogsteen bonds formation, so the stability of the triplex is pH-dependent and replacement of natural cytidines with 5-methylcytidines is a common way to decrease the pH dependence. At pH 7.0 oligonucleotide **16TC**, with all natural cytidines was reported to melt at 9 °C,[2] whereas oligonucleotide **16TM** in which all cytidines are methylated, was reported to have a T_m of 11 °C. In this case, the methylated cytidines have only a very little effect on the thermal stability of the triplex at pH 7.0 and we tentatively ascribed this lack of stabilisation to the electrostatic repulsion between adjacent protonated cytidines. We tried to overcome this obstacle by synthesising oligonucleotides with alternate 5-methylcytidines (C) and natural cytidines (C), such as **C6** (TTTTCTT TTCCCCCCT), or shortening the cytidine tail as in **C2** (TTTTCTTTTCC). We compared the ability of **C6** and **16TM** to form a triple helix with synthetic duplex (**duplex 1** and **2**), both containing the PPT region, by UV melting and gel electrophoresis experiments (PAGE). As reported in Table 2, the partially substituted **C6** oligonucleotide forms a more stable triplex than the fully substituted **16TM**, showing a 4 °C higher T_m value, at pH 6.4 and 6.0, and a 4-fold increase in the

Table 1. Sequence of the triplex-forming oligonucleotides and of target duplexes cited in this study

TFO	
5′-TTTTCTTTTCC-3′	**C2**
5′-TTTTCTTTTCCCCCCT-3′	**C6**
5′-TTTTCTTTTCCCCCCT-3′	**16TM**
5′-TTTTCTTTTCCCCCCT-3′	**16TC**
5′-TTTTCTTTTGGGGGGT-3′	**G6**
Duplex 1	
5′-CAAGGCAGCTGTAGATCTTAGCCACTTTTT**AAAAGAAAAGGGGGGA**CTG-3′	**49R**
3′-GTTCCGTCGACATCTAGAATCGGTGAAAAA**TTTTCTTTTCCCCCCT**GAC-5′	**49Y**
Duplex 2	
5′-CCACTTTTT**AAAAGAAAAGGGGGGA**CTGG-3′	**29R**
3′-GGTGAAAAA**TTTTCTTTTCCCCCCT**GACC-5′	**29Y**

In bold is represented the oligopurine·oligopyrimidine target site. In the TFO, C stands for 5-methyl-2′-deoxycitidine. We defined as following: Y, the oligopyrimidine-containing strand of the duplex and R, the oligopurine-containing strand.

Table 2. EC_{50} value for the **29R·29Y***TFO triplexes; and T_m values for the **49R·49Y***TFO triplexes

	EC_{50} (μM)[a]		T_m (°C)[b]			
	pH 7	pH 6	pH 7	pH 6.4	pH 6	pH 5.5
16TM	ND[c]	0.16	<10	12 ± 1	23 ± 1	—
C6	2.5	0.04	<10	16 ± 1	27 ± 1	40 ± 2

[a] The EC_{50} values were calculated as the concentration at which 50% of TFO is formed in gel retardation assays and an average value corresponding to three different experiments is reported.

[b] Heating and cooling experiments followed by absorbance measurements at 260 nm were carried out in 10 mM Na cacodylate buffer (at the indicated pH) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 μM of duplex target and 1.2 μM of third strand. An average value corresponding to three different experiments is reported.

[c] ND: not detected.

affinity measured by PAGE (at pH 6.0). Since this stabilisation effect is not strongly pH-dependent, it is probably due to a better interstrand stacking rather than to a lower electrostatic repulsion as initially assumed.

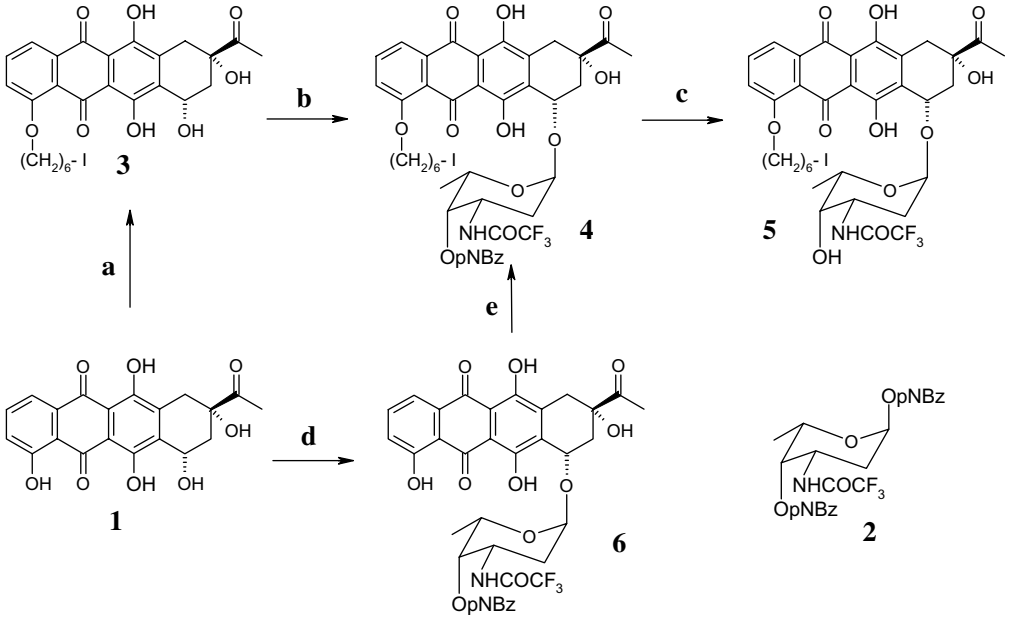
Based on these data and on the fact that the same oligonucleotides have been previously used to study the formation of triplexes upon conjugation with the aglycon moiety of carminomycin,[19] we chose oligonucleotides **C6** and **C2** as the best TFO candidates of new daunomycin conjugates for the formation of triple helices with the *HIV-1* target.

2.1. Synthesis of the daunomycin derivative

The new synthetic procedure is fully described in Scheme 1. In respect to that used in the previous work,[18] it is based on the protection of the amino group of 4-*O*-ω-iodohexyl-daunomycin as *N*-trifluoroacetyl derivative **5**. The choice of this protecting group allows us to perform the coupling reaction to the TFO with the amino group of the daunomycin derivative still protected. This grants a higher yield of the coupling reaction, because we previously observed that the unprotected amino group on the daunomycin sugar reacts with the alkyl iodide group to give side products. For the same reason, the protected derivative **5** has a longer shelf life than the unprotected 4-*O*-ω-iodohexyl-daunomycin. Finally, the trifluoroacetyl group allows the recovery of the excess of the daunomycin derivative needed in the coupling reaction. Therefore, this new synthetic procedure is very reproducible and give higher yields. It consists of the following steps.

Hydrolysis of *N*-trifluoroacetyldaunomycin[20] with 0.2 N HCl in refluxing dioxane gave daunomycinone and *N*-trifluoroacetyldaunosamine in quantitative yields. Daunomycinone was then O-demethylated[21] to give carminomycinone **1**, while the amino sugar was treated with *p*-nitrobenzoyl chloride to give **2**, mostly as the α-anomer.[22] Two synthetic strategies were then used to obtain the suitable compound for conjugation **5**.

In the first one, carminomycinone **1** was alkylated with 2 equiv of Ag_2O and 8 equiv of 1,6-diiodohexane in chloroform at reflux temperature to give a mixture of isomers that after chromatographic purification gave **3** with a yield of 15%. Because of the low solubility of **1**, a reasonable reaction rate is obtained only when an excess of reagent is used, and this affects the regioselectivity between the positions 4 and 6. A significant loss of material occurs during the chromatographic separation of the regioisomers, explaining the low yield for **3**. (13% yield of the 6-O-alkylated product was also obtained in average.) Then compound **3** was coupled with the preformed carbocation derived from 0.8 equiv of the amino sugar **2** to give protected glycoside **4** in fair



Scheme 1. The synthetic pathway toward compound **5** that can be conjugated with a terminal thiophosphate oligonucleotide. Reactions and conditions: (**a**) 1,6-diiodohexane, Ag_2O in refluxing chloroform; (**b**) compound **2**, TMSOTf, MS in CH_2Cl_2/Et_2O, from −40 to 0 °C; (**c**) aq. K_2CO_3 in CH_2Cl_2/MeOH at −10 °C; (**d**) like reaction (b); (**e**) like reaction (**a**).

yields (between 50% and 65%). (This procedure minimise the risk of bis-glycosylation on the anthracyclin derivative.) Noteworthy in the case of the *N*-trifluoroacetyl derivative, addition of the anthracyclinone to the preformed sugar carbocation[23] gave better results as compared with the procedure previously used. Although **4** could be used directly for the preparation of the TFO conjugates, its purification is not easy and it is preferable to remove the *p*-nitrobenzoyl group by reaction with aqueous potassium carbonate to give **5**, which is easier to purify on silica gel than **4** and, moreover, still having a protected amino group, can be stored for at least one year without degradation.

Alternatively, **1** was glycosylated with **2** to give protected carminomycin **6** that was then alkylated to give **4**. In this case, the yield of the alkylation step was 40% (higher than in the above strategy), but recovery of the reaction product was hampered by purification problems, even after the removal of the *p*-nitrobenzoyl group as in **5**. Therefore, this strategy was abandoned.

2.2. Synthesis of daunomycin–oligonucleotide conjugates

C2 and **C6** oligonucleotides were synthesised on a Pharmacia Gene Assembler II Plus applying the manufacturer protocols. The oligonucleotide derivatives containing a 5′-thiophosphate group (PS) were prepared using bis-2-cyanoethyl phosphoramidite in the final coupling step, then performing the last oxidation with the Beaucage's reagent. Applying the usual deblocking conditions (16 h with 30% aqueous ammonia solution at 50 °C), both cyanoethyl groups were cleaved, giving the desired oligonucleotide-5′-thiophosphate. The crude mixture containing the deblocked 5′-PS-oligonucleotide was treated overnight with DTT, precipitated by addition of *n*-BuOH, re-dissolved in water, converted to the sodium salt upon ion exchange on a resin and lyophilised. The sodium salt of the oligonucleotide was dissolved in an aqueous solution of DMF containing 6 equiv of **5**, 15-crown-5 and DTT. The reaction mixture was kept at 45 °C for 16 h. The use of **5**, in which the amino group of the daunomycin residue is still protected, together with addition of DTT to prevent the dimerisation of the thiophosphate group, *increased the yield of the conjugation reaction from about 50% to nearly quantitative*. After the extraction of unreacted **5**, the solution was treated with a diluted solution of sodium hydroxide at 0 °C in order to remove the trifluoroacetyl group. The daunomycin–oligonucleotides were then freed from unreacted oligomers and from failed sequences by reversed phase chromatography on a C-18 column using a gradient of acetonitrile. Compound **5** was recovered and purified on silica gel giving a product whose NMR spectra were superimposable to those of the starting material. When compound **4** was used instead of **5** in the conjugation reaction, treatment of the resulting conjugate with sodium hydroxide cleaved both *p*-nitrobenzoyl (first) and trifluoroacetyl groups.

In conclusion this new synthetic strategy, compared to the older one,[18] allowed us to obtain daunomycin derivatives (**4** and **5**) suitable for coupling that can be stored for years without degradation. Furthermore, these compounds, having the amino group still protected, give better yield of conjugation, allowing also the recovery of the excess of daunomycin derivative. Given the several steps necessary to obtain compounds **4** and **5**, the possibility to recover them from the conjugation mixture represents an important advance in the overall synthetic methodology.

2.3. Triple helix formation

To study triplex formation and stability, we used two synthetic duplex targets, respectively 49-bp and 29-bp long, containing the wild-type sequence present in genes *nef* and *pol* of *HIV-1*[2] (Table 1). **Duplex 1** was used in the UV experiments and **duplex 2** in the gel electrophoresis experiments. Triplex formation was followed by UV–vis spectroscopy upon cooling and heating of the samples at a rate of 0.2 °C/min. Dissociation of the third strand from the duplex is associated with an hyperchromism at 260 nm, which was observed with oligonucleotides **C6**, **dauno-C6** and **dauno-C2**.

Figure 2 shows that at pH 7.0 the triplex formed by **C6** melts below 10 °C, while those formed by **dauno-C2** and **dauno-C6** both melt above 20 °C with a broad irreversible transition suggesting, on one hand, that several different structural conformations might be present, and on the other hand, that the enthalpy of the transition is small; probably the triplex formation is entropy-driven as we have already observed for other types of triplexes, formed by (G,A)-oligonucleotides.[24] As expected, the stability of the triplexes was pH-dependent, as shown in Figure 3, confirming the formation of a (T,C)-triplex involving the protonation of the cytosines.

Figure 3 compares the melting profiles of the triplexes formed by **dauno-C6** (left) and by **C6** (right) at pH 6.0 (crosses) at pH 7.0 (circles). This comparison clearly

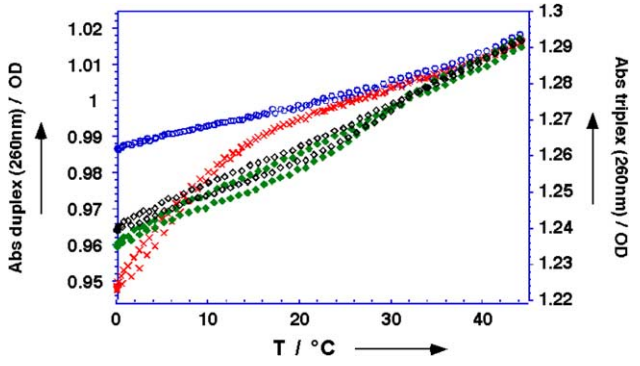


Figure 2. Melting and renaturing profiles recorded at pH 7.0 for the 49R/Y duplex alone (circles), and in the presence of **C6** (crosses), **dauno-C6** (filled diamonds) and **dauno-C2** (open diamonds). Left vertical axis: absorbance of the duplex; right vertical axis: absorbance for the triplexes. An offset of 0.025 and of 0.08 were used for **dauno-C6** and **dauno-C2**, respectively. Samples were dissolved in a solution containing 50 mM NaCl, 10 mM $MgCl_2$ and 10 mM Na cacodylate. Oligonucleotides concentration was: 1 μM **49R**, 1.05 μM **49Y** and 1.2 μM TFO.

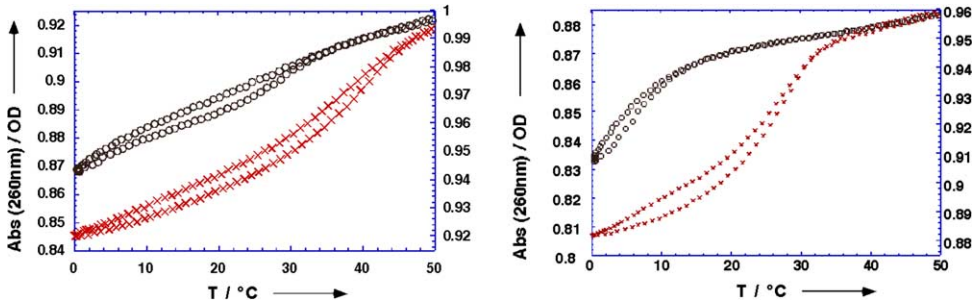


Figure 3. Comparitive analysis of triplex melting in the presence of **dauno-C6** (left) and **C6** (right) with **duplex 1** at pH 7.0 (circles) and at pH 6.0 (crosses). Experimental conditions as in Figure 2. Left vertical axis: absorbance at pH 6.0; right vertical axis: absorbance at pH 7.0.

indicates that the **dauno-C6** forms a triple helical structure more stable and less sensible to pH changes than that formed with the unmodified TFO. It has been previously shown that TFO secondary structures can compete with triplex formation.[25] Further analysis revealed that, *when present alone*, **C6** and **dauno-C6** self-associate at pH 6.0. This association was found to be slightly more stable with the daunomycin conjugate (Fig. 4). The secondary structure formed by the TFOs involves the protonation of the cytosines, as shown by the inverted transition in the denaturing profile recorded at 295 nm (Fig. 4).[26]

This self-association is strongly pH-dependent and concentration-dependent (data not shown). All these data strongly suggest that the structure adopted is probably an intermolecular *i-motif*.[25–27] However, in our case, there is no competition for the formation of the triple helix with the auto-association of the TFOs,[25] because, firstly, the secondary structure melts around 10 °C at pH 6.0 and the triplexes above 20 °C and, secondly, by gel electrophoresis we observed that, at physiological pH, neither **C6** nor **dauno-C6** self-associated at concentrations lower than 5 μM. A first conclusion can be drawn. The triplexes formed by the two conjugates of different length have a similar stability, higher than that measured for the unconjugated triplex, and are less pH-dependent (a ΔT_m of 10 °C is calculated for **dauno-C6** for 1 unit of pH against a ΔT_m of 20 °C for **C6**). However, the melting profiles are difficult to analyse because

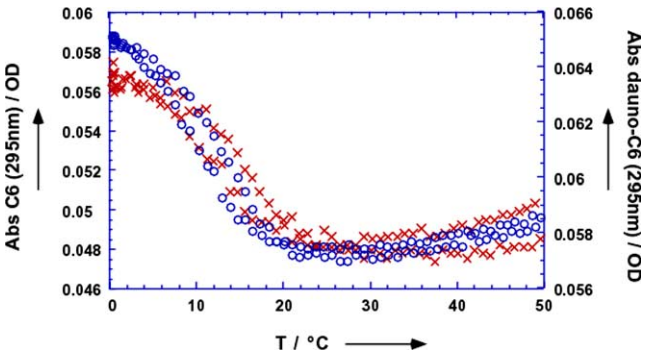


Figure 4. Denaturing and renaturing profiles at 295 nm obtained at pH 6.0 TFO **C6** (circles) and **dauno-C6** (crosses) alone. Left vertical axis, absorbance for **C6**; right vertical axis, absorbance for **dauno-C6**. Experimental conditions as in Figure 2.

very broad; therefore, we used the thermal gradient gel electrophoresis to determine the temperature at which half of the triplexes are dissociated (T_m).

We have previously shown that perpendicular TGGE analysis allows us to follow temperature-dependent structural changes of nucleic acid.[28] Therefore, it is a useful tool to determine the T_m value of triplexes, when it cannot be obtained by other methods.

The experiments were conducted at physiological pH (7.2), so only the triplexes formed by **dauno-C2** and **dauno-C6** were studied. In Figure 5A, melting of the triplex formed by conjugate **dauno-C2** is presented (20 nM of radiolabelled TFO was mixed overnight at 20 °C with 20 μM of **duplex-2** at pH 7.2, 100 mM NaCl and 10 mM $MgCl_2$) and the quantitative analysis of this experiment is reported in Figure 5B. The melting occurs with a transition midpoint of 30 °C (mean value of three independent experiments). A similar behaviour was observed with conjugate **dauno-C6** (Fig. 5C and D) giving a transition midpoint of 34 °C (mean value of three independent experiments).

In summary, the two conjugates form stable triplexes at pH 7.2, and in agreement with the above findings, the triplex formed by the 11-mer TFO conjugate (**dauno-C2**) presents a midpoint of transition (T_m = 30 °C) comparable to that obtained with the longer oligonucleotide (**dauno-C6**) (T_m = 34 °C).

The binding efficiency of the TFO conjugates was also investigated by using gel retardation assays as analytical tool for the detection of triplex formation. For this experiment, the pyrimidine strand **29Y** was radiolabelled with [γ-^{32}P]ATP and a constant amount of radiolabelled **duplex-2**: **29R·29Y*** (20 nM) was incubated with increasing concentration of **C6**, **dauno-C6**, **C2** and **dauno-C2** at pH 7.2 and 20 °C (Fig. 6).

The figure clearly shows retarded species in lanes 4–5 (for **C6**), 7–9 (for **dauno-C6**) and 15–17 (for **dauno-C2**), due to the formation of a complex (the triplex) with slower mobility in the gel in respect to the duplex alone. EC_{50} values, defined as the concentration at which 50% of triplex is formed, were calculated from the percentage of duplex versus triplex. These data confirm that the attachment of daunomycin at the 5′-end of the TFOs

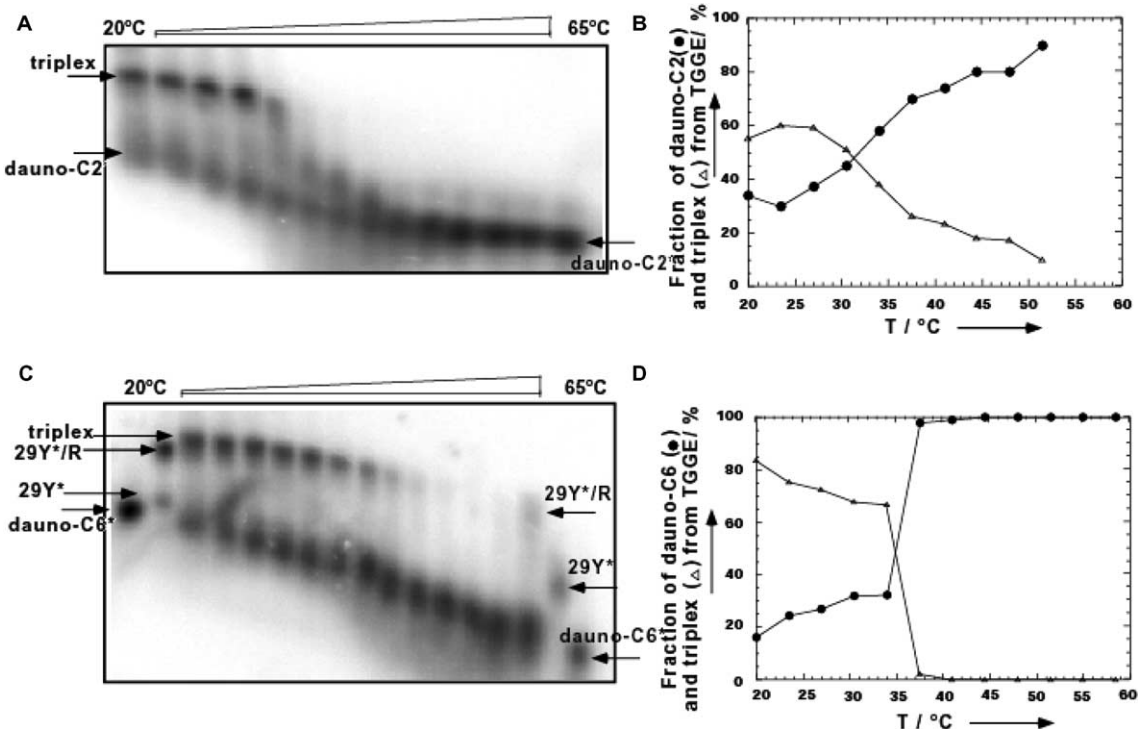


Figure 5. (A) 20 nM radiolabelled **dauno-C2** oligonucleotide was incubated overnight at room temperature in the presence of 20 μM unlabelled **29R/29Y** target duplex in 50 mM HEPES (pH 7.2), 100 mM NaCl and 10 mM $MgCl_2$. The sample was run on a TGGE gel with a linear temperature gradient between 20 and 65 °C. The arrows indicate the corresponding species. The asterisk indicated the radiolabelled species; (B) quantitative analysis of Figure 5A. Triangles: percentage of triplex as a function of the temperature; circles: percentage of single-stranded (mean values of three experiments are reported); (C) 20 nM radiolabelled **dauno-C6** oligonucleotide was incubated overnight at 20 °C in the presence of 20 μM **29R/29Y** target duplex in the same conditions as in Figure 6A. The arrows indicate the corresponding species. The asterisk indicated the radiolabelled species. Radiolabelled single strand **29Y*** and radiolabelled duplex **29Y*/29R** were used as control; (D) quantitative analysis of Figure 5C. Triangles: percentage of triplex as a function of the temperature; circles: percentage of single-stranded (mean values of three experiments are reported).

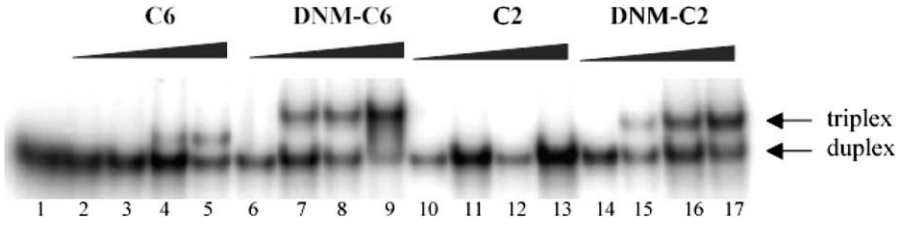


Figure 6. Gel shift mobility assay showing the binding of **C6** (lanes 2–5), **dauno-C6** (lanes 6–9), **C2** (lanes 10–13) and **dauno-C2** (lanes 14–17) to the ^{32}P labelled target **duplex 2** on the pyrimidine strand (20 nM **29R·29Y***, lane 1). Incubation was performed in 50 mM HEPES buffer at pH 7.2 containing 10 mM $MgCl_2$ and 50 mM NaCl at 20 °C with increasing concentration of TFOs equal to 0.1, 0.5, 1, and 5 μM.

significantly increases the binding affinity. Indeed, the **dauno-C6** conjugate exhibits an EC_{50} value of 1.6 μM, which means at least a 3-fold stabilisation of the triplex as compared to the unmodified **C6** ($EC_{50} > 5$ μM). Interestingly, a most pronounced stabilisation was achieved with the shorter conjugate **dauno-C2**: while we were unable to observe triplex formation in the presence of **C2** even if added at high concentrations (40 μM, data not shown), the **dauno-C2** conjugate presents a EC_{50} value comparable to that of the **dauno-C6** conjugates ($EC_{50} = 2.6$ μM).

The appropriate incubation time for the triplex formation was established by a kinetic study by PAGE: equilibrium was attained after 16 h of incubation at 20 °C. This study allowed also to compare the kinetics of triplex formation in the presence of the underivatised **C6** and the **dauno-C6** analog. Preliminary results indicated that the presence of the daunomycin moiety seems not to influence greatly the kinetics of triplex formation (data not shown). Further kinetic analysis with surface plasmon resonance (SPR) is underway.

Interestingly, a comparison of shift mobility assays carried out at 4 and 37 °C confirmed that there is only a 3-fold difference of in the EC_{50} values for the conjugate **dauno-C6** and a 15-fold one for TFO **C6**, confirming that the former triplex is much less temperature-dependent,

as already discussed above after the results of the UV experiments.

3. Conclusion

The new synthetic procedure for the preparation of conjugates between a TFO and a daunomycin derivative described in detail here is a significant improvement over the one used in our previous work, allowing to increase the yield of conjugation from about 50% *to nearly quantitative*, and to easily recover the unreacted reagent whose excess is needed for the complete conversion of the oligonucleotide. The amino protecting group on the daunomycin residue is easily removed by mild basic hydrolysis in the final step and the conjugates can be purified by reverse phase chromatography.

In agreement with previous findings, on the *mdr-1* gene target[18,19] and on the P2 promoter of human *c-myc* gene,[29] the TFO–daunomycin conjugates synthesised herein can find applications as gene inhibitors in the antigene methodology as they showed an enhanced affinity for the oligopyrimidine·oligopurine target when compared to the unconjugated TFOs or to the TFO–carminomycinone conjugates, i.e. lacking the sugar moiety. In fact, the conjugation of both **C2** and **C6** oligonucleotides allowed the formation of a triplex on the *HIV-1* PPT duplex target at physiological pH, while without conjugation the triplex could hardly be detected in our experiments, and **C2** and **C6** conjugated with the aglycons alone formed triplexes that could be only detected at low temperature at pH 7.0. This property can be ascribed, other than to the Hoogsteen recognition of the purine strand, to the simultaneous intercalation of the aglycon within the targeted bases and to a favourable interaction of the amino sugar of the daunomycin moiety in the minor groove of the duplex DNA.

In this work, we performed a detailed study on the targeting of the PPT region of HIV-1 with two short daunomycin-conjugated oligonucletotides. Firstly, we determined, for the TFO alone, that the presence of alternating 5-methylcytidine and natural cytidines increased the triplex stability probably because of a better interstrand stacking. Secondly, for the first time, self-association was characterised for the 16-mer TFO **C6** and its daunomycin conjugate **dauno-C6**. Finally, upon use of gel retardation and TGGE assays, we were able to determine the T_m and K_d values of triplex formation for **C6**, **C2** and their conjugates, and found that the dependence of the triplex stability from both the pH and the temperature is decreased for the daunomycin conjugates. Interestingly, the shorter 11-mer TFO conjugate (**dauno-C2**) resulted almost as stable as the longer 16-mer (**dauno-C6**).

This type of conjugation expands the choices of modifications that can be used to enhance the binding properties of a TFO in the antigene methodology and we have described here a highly improved synthetic procedure. On this same target, the PPT of *HIV-1*, more stable triplexes have been obtained using modified backbone TFOs like LNA[30] and phosphoramidates.[31] In this respect, it would be interesting to prepare conjugates between these classes of modified oligonucleotides and anthracycline derivatives, with the aim to further increase the efficacy of the triple helix to interfere with biological process.

4. Experimental procedures

4.1. General methods

All chemicals were of analytical grade and used as received. Daunomycin hydrochloride was purchased from Biofin Laboratories, Porto Mantovano, Italy. Solvents used for reactions were distilled and stored over molecular sieves. TLC were done on Merck 5719 silica gel (230–400 mesh) plates. NMR spectra were recorded on a Mercury 400 Varian, using TMS and 85% H_3PO_4 as external standards and as references for $^1H/^{13}C$ and ^{31}P, respectively. HPLC analyses and purifications were performed on a Waters 600E Millipore system control equipped with a Waters 484 tuneable absorbance detector. Mass analysis were performed on a APPLEURA QSTAR pulsar I, in ESI mode at 2500 eV, or with a Bruker Esquire 3000 plus.

4.2. 4-*O*-(6-Iodo-*n*-hexyl)-carminomycinone (3)

Synthesis of **3** from carminomycinone **1** (Scheme 1) was performed as already described.[18] Use of a fresh preparation of Ag_2O, obtained by aqueous NaOH treatment of a $AgNO_3$ solution, led to a more reproducible and faster reaction.

4.3. 4′-*O*-*p*-Nitrobenzoyl-3′-*N*-trifluoroacetyl-4-*O*-(6-iodo-*n*-hexyl)-daunomycin (4)

Compound **2** 926 mg (1.83 mmol) was coevaporated twice with acetonitrile, then dissolved in 135 mL of a 1/1 mixture of CH_2Cl_2/Et_2O. Molecular sieves (4 Å, 5 g) were added and the solution cooled to −40 °C. TMSOTf (661 μL, 3.66 mmol) was added and the reaction mixture was allowed to reach 0 °C in 1 h. The solution was again cooled to −20 °C and a solution of **3** (870 mg, 1.46 mmol) (previously coevaporated with anhydrous acetonitrile) in 250 mL of CH_2Cl_2 was added. The reaction was followed by TLC eluting with 5% acetone in CH_2Cl_2 and judged complete in 2 h. The solution was poured in a separating funnel containing 5% aqueous $NaHCO_3$. The organic phase was separated and washed twice with a saturated NaCl aqueous solution, then dried on $NaSO_4$. Chromatographic purification on silica gel eluting with 1.5% acetone in CH_2Cl_2 gave **4** (884 mg, 0.95 mmol) in 65% yield.

MS-ESI: m/z^+ 990.9 $[M+Na]^+$, m/z^- 966.8 $[M-H]^-$ [exact mass for $C_{41}H_{40}F_3IN_2O_{14}$ = 968].

^1H NMR (400 MHz, $CDCl_3$): δ 14.18 (s, Ar-OH), 13.28 (s, ArOH), 8.35–8.27 (m, 4H, pNBz), 8.02 (d, H-1), 7.75 (t, H-2), 7.36 (d, H-3), 6.28 (d, NH), 5.72 (s, H-1′α-anomer), 5.50 (s, H-4′), 5.35 (s, H-7), 4.47–4.45 (m, 2H,

H-3′, H-5′), 4.24–4.19 (m, 3H, OCH_2–, OH-9), 3.29–3.23 (m, 3H, CH_2I, H-10a), 2.96 (d, $J = 18.8$ Hz, H-10b,), 2.44 (s, 3H, $COCH_3$), 2.28 (m, 2H, H-8), 2.15–2.05 (m, 2H, alkyl chain), 2.00–1.85 (m, 4H, 2H-2′, 2H alkyl chain), 1.70–1.62 (m, 2H alkyl chain), 1.60–1.50 (m, 2H alkyl chain), 1.26 (d, 3H, CH_3 sugar).

^{13}C-APT (100 MHz, $CDCl_3$): δ 197.08, 180.80, 179.85, 166.82, 164.22, 160.76, 157.57 (d, $\underline{C}OCF_3$, $^2J_{C\text{-}F} = 49$ Hz), 138.67*, 138.56, 137.19, 136.34, 134.27, 132.45*, 125.98*, 125.28*, 120.48*, 117.17, 117.16 (q, $CO\underline{C}F_3$, $^1J_{C-F} = 286$ Hz), 111.80, 111.20, 96.55*, 72.04*, 69.64, 66.12*, 64.42*, 45.32*, 33.32, 30.25, 30.13, 29.32, 28.95, 25.25, 24.95, 16.31*, 6.88. (*Figures with asterisk indicate negative peaks: CH and CH_3.)

4.4. 3′-*N*-Trifluoroacetyl-4-*O*-(6-iodo-*n*-hexyl)-daunomycin (5)

A solution of **4** (110 mg, 0.118 mmol) in 40 mL of a 3/1 mixture of MeOH/CH_2Cl_2 was cooled to −10 °C, then 1.0 mL of a 0.5 M aqueous solution of K_2CO_3 was added, obtaining a violet solution. The progression of the reaction was followed by TLC (CH_2Cl_2/MeOH, 95/5 v/v). After 1 h the reaction was quenched with 1 M aqueous HCl till the color turned orange. The phases were separated and the organic phase was dried with Na_2SO_4, then evaporated. Crude **5** was dissolved in CH_2Cl_2, then triturated with cyclohexane in order to remove the *p*-nitrobenzoyl derivatives to give 85 mg of pure **5** (0.106 mmol, 90% yield).

MS-ESI: m/z^+ 841.9 $[M+Na]^+$, m/z^- 817.8 [M−H], [exact mass for $C_{34}H_{37}F_3INO_{11} = 819$].

^{1}H NMR (400 MHz, $CDCl_3$): δ 14.16 (s, ArOH), 13.25 (s, ArOH), 7.98 (d, H-1)), 7.73 (t, H-2), 7.34 (d, H-3), 6.73 (d, NH), 5.53 (d, $J = 3.66$ Hz, H-1′, α-anomer), 5.25 (m, H-7), 4.33 (s, OH-9), 4.27–4.16 (m, 4H, H-3′, H-5′, OCH_2–), 3.68 (m, H-4′), 3.26–3.21 (m, 3H, –CH_2I, H-10a), 2.91 (d, $J = 18.8$ Hz, H-10b), 2.42 (s, 3H, C(O)CH_3), 2.33–2.07 (m, 3H, 2H-8, OH-4′), 2.00–1.85 (m, 6H, 2H-2′, 4H alkyl chain), 1.70–1.61 (m, 2H alkyl chain), 1.58–1.52 (m, 2H alkyl chain), 1.31 (d, 3H CH_3 sugar).

^{13}C-APT (100 MHz, $CDCl_3$): δ 211.55, 186.72, 186.60, 160.37, 156.33, 155.6 (d, $\underline{C}OCF_3$, $^2J_{C-F} = 53$ Hz), 135.40*, 135.30, 133.83, 133.40, 120.84, 119.49*, 119.32*, 115.50 (q, $CO\underline{C}F_3$, $^1J_{C-F} = 287$ Hz), 111.51, 111.38, 100.08*, 70.26*, 69.53, 68.84*, 66.62*, 46.16*, 35.14, 33.54, 33.32, 30.18, 29.75, 28.96, 24.98, 16.77*, 7.21. (*Figures with asterisk indicate negative peaks: CH and CH_3.)

4.5. Oligonucleotide synthesis and purification

Oligonucleotides **29R**, **29Y**, **49R** and **49Y** were purchased from Eurogentec (Belgium) and purified using Micro Bio Spin® 6 Chromatography columns (Bio-Rad, USA). Concentrations were determined spectrophotometrically at 25 °C using molar extinction coefficients at 260 nm calculated from a nearest-neighbour model.[32] Underivatised **C2** and **C6** were synthesised by solid phase synthesis on a Gene Assembler II Plus synthesiser, then deblocked with one night standing at 50 °C with 30% aqueous ammonia. They were purified dissolving the crude compound in 1 mL of water, then precipitating the oligonucleotide by addition of 10 mL of *n*-BuOH. The final purity was in the range 80–90% as judged by HPLC on a ionic exchange column.[18]

4.6. Dauno-conjugates, synthesis and purification

The sodium salt of a 5′-PS-oligonucleotide (10 OD about 75% pure) was dissolved in 125 μL of DMF, 20 μL of water, 11 μL of 15-crown-5, 1 mg of **5** and 0.2 mg of DTT. The reaction mixture was kept in a sealed vial for 16 h in a thermostat at 45 °C. The success of the conjugation can be easily monitored by reverse phase HPLC analysis, being the retention times of the starting oligonucleotides and short-mers up to 11 min, while that of the conjugate is larger than 21 min. The crude mixture was separated from excess **5** by diluting it with 150 μL of water and extracting the unreacted iodo-derivative with a 15/85 v/v mixture of MeOH/CH_2Cl_2. The aqueous phase was cooled to 0 °C, then 2 M aqueous NaOH was added till a final concentration of 0.5 M. The removal of the trifluoroacetyl protecting group, to give a compound whose retention time is about 19 min, was completed in 90 min and was followed by reverse phase HPLC analysis. The pure conjugate was then recovered, by purification on a reverse phase C-18 silica gel column using a gradient of acetonitrile (0–30%) in a 0.1 M TEAA aqueous buffer. Usually 5–6 OD of pure conjugate were obtained.

The retention time of the final **dauno-C6** was the same as that of the compound obtained using the procedure previously described.[18] For both **dauno-C2-** and **-C6** the ^{31}P NMR spectra exhibited the expected ratio between the peak at 22 ppm (substituted PS bond) and that at 0 ppm (other phosphate linkages). The purified conjugate showed the expected UV–vis profile, considering the absorbance at 260 nm as a simple additive contribution from the absorbance values of the oligonucleotides as deduced with Borer's method[32] (87.3 and 123.0 for **C2** and **C6**, respectively) and the value of 20.5 (Merck Index) for the daunomycin contribution, while at 480 nm the mM absorption of daunomycin was 12.1 (Merck Index). In fact the A_{260}/A_{480} ratios were found to be 8.9 (calc. 8.9) for **dauno-C2**, and 11.7 (calc. 11.9) for **dauno-C6**. MS–ES$^-$ (*m/z*): **dauno-C2** calc. 3958.0, found 3957.0 [M−H]; **dauno-C6** calc. 5446.9 found 5446.0 $[M-H]^-$.

4.7. DNA labelling

The pyrimidine-rich strand of the 29-bp duplex was 5′ end-labelled with [γ-^{32}P]ATP (Amersham, France) by T4 polynucleotides kinase (Ozyme, France); triplex-forming oligonucleotides were 3′-end-labelled with [α-^{32}P]ddATP (Amersham, France) by Terminal Transferase (Ozyme, France), according to the manufacturer's instructions.

4.8. Gel retardation assay

Increasing concentrations of the triplex-forming oligonucleotides were added to 20 nM of the **29R/Y** duplex radiolabelled on the oligopyrimidine strand in 10 mM $MgCl_2$, 50 mM NaCl, 50 mM HEPES, pH 7.2, 10% glycerol and 0.5 μg/μL *Escherichia coli* tRNA or in 10 mM $MgCl_2$, 50 mM NaCl, 50 mM MES, pH 6.0, 10% glycerol and 0.5 μg/μL *E. coli* tRNA, and the samples were incubated at 20 °C overnight or as indicated. Electrophoresis was performed on a non-denaturing 15% polyacrylamide gel containing 10 mM $MgCl_2$ and 50 mM HEPES, pH 7.2 or 50 mM MES pH 6.0, at 20 °C. The gels were scanned with a Molecular Dynamics SP Phosphorimager and quantified. The EC_{50} was calculated as the concentration at which 50% of triple helix is formed and a mean value corresponding to three different experiments is reported.

4.9. Thermal gradient gel electrophoresis

TGGE experiments were performed with the TGGE System of Biometra. A non-denaturing 15% polyacrylamide gel, 1 mm thick, containing 10 mM $MgCl_2$ and 50 mM HEPES, pH 7.2, was attached on a Polybond film to avoid deformation at high temperature and it was set on the gradient block so that the temperature gradient was perpendicular to the electric field. Fixed concentrations (20 μM) of the corresponding duplex target were added to 20 nM of the 3′-end radiolabelled TFO, in the presence of 10 mM $MgCl_2$, 100 mM NaCl, 50 mM HEPES, pH 7.2, 10% sucrose and 0.5 μg/μL *E. coli* tRNA. The samples were incubated overnight at 20 °C. The gel was loaded with 30 μL samples. It was then electrophoresed 5 min. at the incubation temperature at 150 V to ensure entry of the samples in the gel matrix. The slots were rinsed, the gel covered for thermal insulation, the linear thermal gradient applied and the samples were electrophoresed for 35 min at 205 V. The gels were scanned with a Molecular Dynamics SP Phosphorimager. The T_m value was calculated as the temperature at which 50% of the triplex is dissociated and a mean value corresponding to three different experiments is reported.

4.10. UV absorption spectrophotometry

A Kontron Uvikon 940 spectrophotometer with 1 cm optical path length quartz cuvettes was used to study thermal denaturation and renaturation of triplex formation. The cell holder was thermoregulated by an 80% water/20% ethylene glycol circulating liquid. Sample temperature was decreased from 60 to 0 °C and increased back to 60 °C at 0.2 °C/min with absorption readings at 245, 260, 295, 520 and 620 nm taken every 1–1.2 °C during 3 cycles. Samples were maintained at each extreme temperature for an additional 10 min. Experimental conditions were as described in the figure and table legends. All samples contained 10 mM Na cacodylate at the indicated pH, 50 mM NaCl, 10 mM $MgCl_2$. Oligonucleotides concentration was: 1 μM **49R**, 1.05 μM **49Y** and 1.2 μM TFO in the triplex experiments; and 1.2 μM TFO in the auto-association experiments.

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