Synthesis of new riboflavin modified ODNs: effect of riboflavin moiety on the G-quadruplex arrangement and stability

*Valeria Romanucci ^{*a}†, Rosario Oliva ^{a,b}†, Luigi Petraccone ^a, Sandra Claes ^c, Dominique Schols ^c,
Armando Zarrelli ^a and Giovanni Di Fabio ^{*a}*

- a. Department of Chemical Sciences, University of Napoli Federico II, Complesso di Monte Sant'Angelo, Via Cintia 4, I-80126 Napoli, Italy.
- b. Physical Chemistry I - Biophysical Chemistry, TU Dortmund University, Otto-Hahn Strasse 4a, D-44227 Dortmund, Germany.
- c. Department of Microbiology, Immunology and Transplantation, Rega Institute, KU Leuven, Herestraat 49, 3000 Leuven, Belgium.

† These authors contributed equally

***Corresponding authors:**

Valeria Romanucci

e-mail address: valeria.romanucci@unina.it

Giovanni Di Fabio

e-mail address: difabio@unina.it

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ABSTRACT

In the panorama of modified G-quadruplexes (G4s) with interesting proprieties, here, it has been reported the synthesis of new modified d(TGGGAG) sequences forming G-quadruplexes, with the insertion of a riboflavin unit (Rf, vitamin B₂). Exploiting the flavin similarity with the hydrogen bond pattern of guanine and aiming at mimic a typical nucleoside scaffold, the synthesis of the riboflavin building block **3** it has been efficiently carried out. The effect of insertion of riboflavin mimic nucleoside on the G-quadruplex properties has been here, for the first time investigated. A biophysical characterization of Rf-modified sequences (**A-D**) has been carried out by circular dichroism (CD), fluorescence spectroscopy, differential scanning calorimetry (DSC) and native gel electrophoresis. CD and electrophoresis data have suggested that Rf-modified sequences are able to form parallel tetramolecular G4 structures similar to that of the unmodified sequence. Analysis of the DSC thermograms has revealed that all modified G-quadruplexes have a higher thermal stability compared with the natural sequence, particularly the stabilisation is higher when the Rf residue is introduced at the 3'-end. Further, DSC analysis has revealed that the Rf residues introduced at the 3'-end are able to form additional stabilising interactions, energetically almost comparable to the enthalpic contribution of a G-tetrad. Fluorescence measurement are consistent with this result showing that the Rf residues introduced at 3'-end are able to form stacking interactions with the adjacent bases within the G-quadruplex structure. The whole of data suggested that the introduction of Rf unit can stabilize G-quadruplex structures and can be a promising candidate for future theranostic applications.

1. Introduction

G-quadruplexes (G4s) are non-canonical DNA structures which are formed and stabilized by stacked G-tetrads. Each G-tetrad contains four guanines associated through hydrogen bonding between Hoogsteen face (O6 and N7) in order to form a planar structure also called G-quartet stabilized by monovalent cations as K^+ , Na^+ and NH_4^+ which interact with the lone pairs on the O6 atoms surrounding the central core. Over the last years, G-rich oligonucleotides able to form G4, have attracted considerable interest in a large variety of fields including medicinal chemistry, supramolecular chemistry, and nanotechnology [1,2]. Several reports deserved particular attention to the elevated fascinating polymorphism of G-quadruplex structures that seems to be strongly implicated in their biological activity [3,4]. Moreover, the understanding of the marked polymorphism of nucleic acids is essential in the drug design and for the study of complex biological mechanisms [5]. Many reports in the last years have investigated how chemical modifications to the bases or the backbone of oligonucleotides forming G-quadruplexes effect the features of these higher-order structures [6-11]. G-quadruplexes have been recognized as a highly appealing DNA structures implicated in several diseases as cancer [12,13], neurodegeneration [14] and HIV infection [15-16]. In this regard, some G-quadruplex aptamers have been found to be active at different stages of HIV infection [17]. Among G4 discovered to be potent anti-HIV aptamers, many reports described the activity of the Hotoda's sequence. This short d(TGGGAG) sequence forms a very stable parallel tetramolecular G4 and showed significant anti-HIV activity [18]. Taking advantage on our extensive knowledge about d(TGGGAG) sequence [19-21], we synthesized new modified d(TGGGAG) sequences with the insertion of a riboflavin moiety in different position of the sequence.

Riboflavin (Rf) is an essential water-soluble vitamin, precursor of flavin-based redox cofactors FMN and FAD. Rf presents interesting biological and physicochemical properties such as transporter specific cell internalization, implication in redox reactions and photosensitizer [22]. Rf and its derivatives FMN and FAD may serve as highly versatile building blocks for the construction of complex systems [23]. Over the years, many reports described the synthesis of a variety of Rf derivatives including polymers [24], proteins [25] and nucleic acids [26]. Regarding the latter case, the Rf ligand ability has been studied for the selective binding into DNA-duplex aptamers containing abasic site and also as DNA G-quadruplex ligand for biosensor applications [27]. In 1995, Riboflavin was also found to specifically bind RNA aptamer containing G-quartet structure [28]. Very recently, it has been shown that the Rf fluorescence is quenched when bound to G-quadruplex structure respect to single stranded form [29,27].

Further, several studies have shown the Rf ability to form non-covalent bonds such as p-p stacking [24], hydrogen [30], or electrostatic interactions [31].

In this work, we report the synthesis, biophysical and biological characterization of new Rf-modified sequences. The d(TGGGAG) sequence has been used as a model of parallel tetramolecular G4 structure. The insertion of riboflavin into lead sequence has been inspired by its similarity with the Hoogsteen hydrogen bond pattern of guanine combined with its excellent fluorescence properties that may be a useful tool for theranostic applications which involve the combination of therapy and diagnostic imaging into an unique system.

To explore how riboflavin unit affects the G quadruplex structure and if is able to additionally stabilize the G-quartet stacking, new Rf-modified sequences (**A-D**) have been investigated through different techniques in a combined approach (CD, DSC, native electrophoresis and fluorescence). Moreover, the anti-HIV activity of all sequences has been evaluated.

2. Results and Discussion

2.1. Synthesis of riboflavin-building blocks and insertion into the lead sequence

The synthetic effort has been devoted for the synthesis of nucleoside-riboflavin building block **3**, in particular aiming to make riboflavin moiety similar to a nucleoside unit, the ribityl chain has been rigidified with 2',4'-*O*-benzylidene protection. This synthetic procedure has been inspired by a previous study described by Schwogler et al. that reported the incorporation of Rf moiety into duplex DNA sequence by H-phosphonate chemistry.[32] Interestingly, even if we followed the same experimental conditions to obtain a selective benzylidene protection, we did not observe similar yield of the desired diastereoisomer **2** (22% respect to 50% previously reported).

After benzylidene protection, the primary hydroxyl group of ribityl chain has been protected with DMT group, indeed the 3'-OH group has been functionalized with phosphoramidite (intermediate **4**).[33] The synthetic approach takes advantage from the different reactivity of primary and secondary hydroxyl groups following the classic nucleoside/tide chemistry. All intermediates were gained in very good yields (Scheme 1), only the intermediate **2** was obtained with a yield not greater than 22%. An efficient post-assembling conjugation to lead sequences provided to the Rf-modified sequences **A** and **B**. Conversely, in order to synthesize the 3'-modified sequences **C** and **D**, it has been again useful the key intermediate **3**. The riboflavin functionalized support **6** has been obtained by succinylation of the free hydroxyl group (3'-OH) of intermediate **3** and subsequent solid phase reaction of **5** with a CPG support. New modified oligonucleotides (ODNs) were purified by RP-HPLC and finally analyzed by MALDI-TOF. The stability of benzylidene protection of the final Rf-modified ODNs was evaluated by HPLC analysis; the results confirmed its high stability, even during the acid treatment carried out in the ODN assembly. None cleavage it was observed.

Scheme 1. Synthetic scheme of riboflavin building blocks **3** and **5** and solid phase synthesis of Rf-modified **A-D** sequences.

2.2. Native gel electrophoresis of Rf-modified sequences defined the molecularity of complexes

In order to determine the stoichiometry of resulting structures formed by Rf-modified ODNs, non-denaturing polyacrylamide gel electrophoresis (PAGE) experiment has been performed. Aiming at observing the mobility of all complexes, $[d(TG_4T)]_4$ and $d(T_6)$ were loaded as G4 and single stranded references, natural $d(TGGGAG)$ and modified sequences (**A–D**) were loaded on the gel in single stranded (ss) conditions (no potassium added, indicated by “–”) and G4 conditions (10 mM phosphate buffer + 100 mM KCl, indicated by “+”, see paragraph 4.5.1). In absence of potassium, as expected, all the sequences show a single well defined band corresponding to the single-stranded form (Fig. 1).

The addition of potassium is central to induce the G-quadruplex folding, in fact in presence of K^+ , this band disappears and a single band at lower mobility was observed for **A**, **C** and **D**. This behavior is similar to the one observed for the $d(TGGGAG)$ and is consistent with the formation of G-quadruplex structures. The variation in the electrophoretic mobility of both ss and G4 forms of modified **A–D** sequences respect to the natural ones, is due to the contribute of Rf moiety and its interaction within the sequence depending on its position in it.[34,21] Only the sequence **B** presents a smearing retarded band (Fig. 1) suggesting the not complete formation of the tetramolecular complex and/or the formation of additional lower molecularity complexes.

Fig. 1. Native gel electrophoresis of natural sequence $d(TGGGAG)$ and Rf-modified sequences **A–D** sequences loaded at $50\ \mu\text{M}$ (ss concentration) in two conditions (“–”, no potassium added; “+” 100 mM KCl); 15% polyacrylamide gel supplemented with 10 mM KCl. The gel was run at $26\ ^\circ\text{C}$ at constant voltage (90 V) for 2.0 h.

2.3 CD profiles of Rf-modified sequences confirmed the formation of parallel G quadruplex structures

The sequences **A** and **D** presented an additional Rf unit at 5' and 3'-end of d(TGGGAG) sequence, instead in **B** and **C** sequences, the 5' and 3' terminal bases were replaced with the Rf unit.

All secondary structures formed by Rf-modified sequences (**A-D**) have been characterized by CD in order to evaluate how the insertion of riboflavin moiety into d(TGGGAG) sequence influences its G-quadruplex arrangement. CD experiments have been performed in potassium buffer (10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ + 100 mM KCl, see paragraph 4.5.1). As shown in the CD spectra reported in Fig. 2, the G4 arrangements of **A-D** sequences except **B**, are similar to that adopted by natural d(TGGGAG) sequence with a typical profile of a parallel G4 structure characterized by a positive band at 263 nm and a negative band at 243 nm.[35] On the other hand, the CD spectrum of **B** appears less intense compared to the other sequences. This behavior could be the result of a not complete G-quadruplex formation as already observed by gel electrophoresis. Small differences in the **A-D** CD profiles indicate slight structural or conformational variations in the modified G-quadruplexes due to not identical ODN sequences and also for the contribution of the riboflavin mimic nucleoside.[9]

Fig. 2 CD spectra of natural sequence d(TGGGAG) and Rf-modified **A-D** sequences (1×10^{-4} M ss concentration) registered in 10 mM phosphate buffer (pH = 7.0) containing 100 mM KCl.

2.4 DSC characterization of modified G-quadruplexes unfolding

The thermal unfolding of the studied complexes was followed by means of differential scanning calorimetry. In Fig. 3 the heating and cooling DSC profiles for the modified sequences are shown. For all samples the formation of the G-quadruplexes (an exothermic peak) was not observed in the cooling profiles, this indicates that the formation/dissociation of the complexes is not at thermodynamic equilibrium during the heating and cooling experiments.[36] This result, observed also for the natural sequence (Fig. S1), is in agreement with the well-known slow kinetics of tetramolecular G-quadruplexes folding.[36,37]

On the other hand, successive heating scans of the modified sequences were superimposable indicating that, at ODN concentrations of DSC experiments, few minutes at low temperature (before each temperature scan the sample was equilibrated at 5 °C for 15 minutes) were enough to allow the formation of these G-quadruplexes (Fig. S2). Interestingly, this result reveals that the introduction of the Rf residue increases the G4 formation kinetics in comparison with the unmodified sequence.

Fig 3. DSC first heating (black lines) and cooling (red lines) profiles of (A) Rf-TGGGAG, (B) Rf-GGGAG, (C) TGGGAG-Rf and (D) TGGGA-Rf. All the experiments were carried out at 2×10^{-4} M of ss ODNs in 10 mM potassium phosphate buffer + 100 mM KCl (pH 7.0).

In Table 1, the melting temperatures and the enthalpy changes relative to the G-quadruplex dissociation process were reported. Inspection of Table 1 reveals that all the modified G-quadruplexes have a higher thermal stability (T_m) compared with the natural sequence, particularly the stabilization is higher when the Rf residue is introduced at the 3'-end.

Table 1. Thermodynamic parameters for the G-quadruplex dissociation.

Sequences	ΔH_d	T_m
	(kJ mol ⁻¹ of G-quadruplex)	
TGGGAG	327	67.7
(A)RfTGGGAG	324	85.6
(B) Rf-GGGAG	250	81.7-88
(C) TGGGAG-Rf	365	95.3
(D) TGGGA-Rf	355	99.3

Errors on enthalpy changes are within 5%, errors on T_m are within 0.2 °C

However, differently from the other sequences, the DSC profile of the sequence **B** shows at least two well resolved DSC peaks and an anomalous low total enthalpy change, this result, in agreement with electrophoresis and CD results, suggests by the presence of multiple species and a not complete formation of a single well defined tetramolecular complex. For this reason, it is not possible to assign the corresponding total enthalpy change to a single well defined process (i.e. the G-quadruplex dissociation) and we preferred not to further discuss the enthalpy change for this sequence. Inspection of the enthalpy changes for the other sequences reveals that when the Rf is introduced at the 3'-end (sequences **C** and **D**), there is an enthalpy increase (in comparison with the reference sequence) for the G-quadruplex dissociation of $\Delta\Delta H \sim 40$ kJ/mol and ~ 30 kJ/mol for **C** and **D**, respectively. This enthalpy increase can be assigned to the contribution of the Rf residue in the sequence **C** in which the Rf residue is added to the unmodified d(TGGGAG) sequence. However, in the sequence **D**, the Rf residue replaces a G residue of the reference sequence suggesting that its enthalpic contribution to the G-quadruplex stabilization could be even higher than the observed $\Delta\Delta H$. Taken together these data reveal that the four Rf residues introduced at the 3'-end are able to form additional stabilizing interactions, energetically almost comparable to the enthalpic contribution of a G-tetrad.

2.5 Quenching of riboflavin fluorescence depending on G4 or ss arrangements formed by Rf-modified sequences

In order to verify if the Rf residue is able to form stacking interaction with the adjacent bases within the G-quadruplex structures, we exploit the properties of the Rf fluorescence in both ss and G4 forms. In this frame, it has been reported that Rf fluorescence is more quenched when stacked in a G-quadruplex structure than in the unstructured single strand form.[29,27] Hence, the fluorescence emission spectra of all Rf-modified sequences were collected before denaturation of samples and after fast cooling at room temperature. The experiments were conducted at low G-quadruplex concentrations ($\sim 1 \mu\text{M}$). In these conditions, we can assume that, after denaturation, the G-quadruplexes were not formed upon cooling. A significant quenching of riboflavin fluorescence ($\lambda=530 \text{ nm}$) has been found for the sequences **C** and **D** when structured in G4 respect to their dissociated ss form, whereas, a smaller quenching was observed for the G4 of **A** and none for **B** (Fig. 4).

Fig. 4 Fluorescence emission spectra of (A) Rf-TGGGAG, (B) Rf-GGGAG, (C) TGGGAG-Rf and (D) TGGGA-Rf at the temperature of 25 °C (black lines) and after heating at 100 °C and fast re-cooling at 25 °C (red lines). The spectra were recorded after excitation at 450 nm, by using a 1-cm path length

quartz cuvette. All the spectra were recorded at 40×10^{-5} M of ss ODNs in 10 mM potassium phosphate buffer + 100 mM KCl (pH 7.0).

These findings reveal that in the **C** and **D** sequences, the Rf residue is able to establish significant stacking interactions with the adjacent tetrads of G4 structures as confirmed by the higher enthalpy of dissociation measured by DSC experiments.

2.6 Anti-HIV activity of Rf-modified sequences

The anti-HIV activity and cellular cytotoxicity of the Rf-modified oligodeoxynucleotides were evaluated against HIV-1 NL4.3 and HIV-2 ROD replication in a CD4⁺ T cell line MT-4 cell cultures using the 3-(4,5- dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) method as described previously by Pauwels et al.[38] The Rf-modified sequences **A** and **B** exhibited moderate HIV-1 and HIV-2 activity as reported by the IC₅₀ in the Table 2. The sequences **C** and **D** modified at 3'-end did not show anti-HIV activity.

Table 2. IC₅₀ and CC₅₀ values of Rf-modified oligodeoxynucleotides.

Compound	Unit	IC ₅₀ HIV-1 (NL4.3)	IC ₅₀ HIV-2 (ROD)	CC ₅₀
A	μM	2.59	4.47	>20
B	μM	8.15	8.77	>20
C	μM	20	>20	>20
D	μM	>20	>20	>20
AMD3100	ng/ml	12.26	17.92	>1000

IC₅₀: 50% Inhibitory concentration or compound concentration required to inhibit HIV-induced cytopathogenic effect evaluated in the CD4⁺ MT-4 T cell line. CC₅₀: 50% Cytotoxic concentration of the compounds in parallel evaluated in this cell line.

3 Conclusions

In conclusion, we report here, the synthesis of a riboflavin mimic nucleoside and its insertion into a model sequence forming tetramolecular G-quadruplex structure. All secondary structures formed by Rf-modified sequences have been characterized by native PAGE, CD, DSC and fluorescence analyses. Also the anti-HIV activity of all rf-modified sequences has been evaluated. CD and native PAGE data have reported similar behavior to the parallel G-quadruplex structure for almost all modified sequences. The melting temperature (T_m) of all Rf-modified sequences results to be higher than that of natural one, in particular the insertion of Rf unit at 3'-end of the d(TGGGAG) sequence (**C** and **D** sequences) has more effect on the stability of G-quadruplex than modification at the 5'-end (**A** and **B** sequences).

Consistent with these results, DSC and fluorescence analyses have revealed the ability of Rf units inserted at 3'-end of the lead sequence to form additional stabilizing interaction energetically similar to that of a G-tetrad. In particular DSC experiments highlighted the higher enthalpy of G4 dissociation of sequences **C** and **D** respect to the natural d(TGGGAG) one due to the stacking interactions of Rf-moiety with the adjacent bases within the G-quadruplex structure. These preliminary findings reveal the ability of Rf residue to establish significant stacking interactions with the adjacent tetrads of G4 structures.

Future studies will be address to the characterization of these interactions and, in turn, to assess the potential formation of Rf-tetrad in a large variety of relevant G-quadruplexes.

4 Materials and Methods

4.1 General methods

Riboflavin (**1**) was purchased from Sigma-Aldrich – Italy. HPLC-grade CH₃CN was purchased from Carlo Erba Reagents and Sigma-Aldrich, respectively. Unless otherwise indicated, other chemicals were obtained from Sigma-Aldrich.

Reactions were monitored by TLC (precoated silica gel plates F254, Merck) and column chromatography (Merck Kieselgel 60, 70-230 mesh). Analysis and purification was performed on a Shimadzu LC-8A PLC system equipped with a Shimadzu SCL-10A VP System control and a Shimadzu SPD-10A VP UV-vis detector. HPLC purifications were carried out on Phenomenex RP18 column (*Gemini, 10 μm C18(2), 250 mm × 21.2 mm i.d.*) using a linear gradient of CH₃CN in 0.1 M TEAA in H₂O (pH 7.0) from 20% to 100% over 30 min at a flow rate of 7 mL/min with detection at 260 and 360 nm. MALDI-TOF mass spectrometric analyses were performed on AB SCIEX TOF/TOF 5800.

Desalting was conducted using gel filtration chromatography on a Sephadex G10 column, eluted with H₂O/EtOH 4:1 (v/v).

4.2 Synthesis of riboflavin building blocks

4.2.1. Synthesis of 2',4'-O-benzylidene-riboflavin (**2**)

Riboflavin (**1**) (2 g, 5.31 mmol) was dissolved in dry DMF (25 mL), (+)-camphor-10- sulfonic acid (CSA) (62 mg, 0.26 mmol) and 796 μL of benzaldehyde dimethyl acetal (5.31 mmol) were added. The reaction was taken at 80 °C in the dark for a night. The reaction was monitored by TLC (dichloromethane-methanol, 9:1, v:v) and quenched at room temperature by addition of triethylamine until neutrality. After evaporation of solvents, the residue concentrated under reduced pressure and subsequently purified on silica gel with dichloromethane-methanol (95:5 to 90:10) to yield compound **2** (537 mg, 1.16 mmol, 22%).

Compound **2**: ¹H NMR (DMSO-*d*₆, 400 MHz, r.t.) 8.02 ppm (1H, *s*, aromatic-Rf); 7.82 ppm (1H, *s*, aromatic-Rf); 5.52 ppm (1H, *s*, H_{6'}); 5.20 and 4.85 ppm (2H, *m*, H_{1'}); 4.18 ppm (1H, *m*, H_{2'}); 3.80 - 3.73 ppm (1H, *m*, H_{5'}); 3.65 ppm (1H, *m*, H_{4'}); 3.60-3.41 ppm (2H, overlapped signals, H_{5'} and H_{3'}); 2.39 ppm (3H, *s*, CH₃-Rf); 2.45 ppm (3H, *s*, CH₃-Rf). ¹³C NMR (DMSO- *d*₆, 100 MHz, r.t.): δ = 160.3; 155.8; 151.0; 146.0; 138.3; 137.5; 136.2; 134.1; 132.4; 130.9; 128.9; 128.1; 126.5; 118.7; 100.0; 82.5; 78.8; 63.8; 61.7; 46.8; 21.0 and 19.2 ppm. 5 Hz, 1H, H-3); 5.33 (*d*, *J* = 6.0 Hz, 1H, H-7"); 4.08 (*m*, 1H, H-8"); 3.78 (*s*, 6H, OCH₃ of 4,4'-dimethoxytriphenylmethyl); 3.60-3.58 (overlapped signals, 4H, OCH₃ of silibinin and H-9"a); 3.54 (*br d*, *J* = 11.0 Hz, 1H, H-9"b); 2.92-2.84 (*m*, 3H, CH of isobutyryl groups in

5, 7 e 4"); 2.80-2.77 (*m*, 1H, CH of isobutyryl group in 3); 1.41-1.25 (*m*, 18H, CH₃ of isobutyryl group in 5, 7 e 4"); 1.15-1.10 (*m*, 6H, CH₃ of isobutyryl group in 3) ppm. ¹³C NMR (CDCl₃, 125 MHz, r.t., mixture of diastereoisomers): δ = 185.2; 175.3; 175.0; 174.5; 169.1; 168.4; 167.7; 162.4; 158.1; 156.2; 151.3; 150.9; 148.9; 148.7; 144.8; 144.6; 143.8; 143.7; 139.9; 136.7; 136.5; 135.7; 135.3; 134.5; 130.0; 129.7; 128.0; 127.7; 127.6; 124.0; 123.8; 122.6; 120.7; 119.7; 113.1; 113.0; 111.3; 111.2; 111.0; 110.8; 110.5; 108.9; 86.0; 80.9; 77.5; 76.8; 76.4; 73.1; 62.6; 55.4; 55.1; 55.0; 21.0; 20.9; 20.6; 20.4; 20.0; 19.6; 19.0; 18.8; 18.7; 18.6 ppm. ESI-MS (positive ions): *m/z* calculated for C₅₄H₄₈O₁₆ = 952.29; found: 976.25 [MNa]⁺.

4.2.2. Synthesis of 5'-O-DMT-2',4'-O-benzylidene-riboflavin (**3**)

Compound **2** (426 mg, 0.92 mmol) was dissolved in dry pyridine (3 mL), 4,4'-dimethoxytrityl chloride (279 mg, 0.82 mmol) was added at room temperature. After 2 h, the reaction was quenched by addition of methanol (3 mL). Solvents were evaporated, and residual pyridine was coevaporated with toluene. Dichloromethane was added to the residue and washed two times with water. The combined organic extracts were dried over Na₂SO₄, filtered and concentrated under reduced pressure. The residue was purified on silica gel in dichloromethane-methanol (97:30 to 95:50 + 1% triethylamine). The 5'-O-DMT-2',4'-O-benzylidene-riboflavin **3** was obtained in good yield (667 mg, 0.87 mmol, 95%).

Compound **3**: ¹H NMR (CDCl₃, 400 MHz): 8.07 ppm (1H, *s*, aromatic-Rf), 7.95 ppm (1H, *s*, aromatic-Rf); 7.37-7.10 ppm (9H, *m*, aromatic-DMT), 6.82-6.79 ppm (4H, *m*, H_m DMT), 5.68 ppm (1H, *s*, H_{6'}); 5.21-4.83 ppm (2H, *m*, H_{1'}); 4.28 ppm (1H, *m*, H_{2'}); 3.97 ppm (1H, *m*, H_{4'}); 3.80 ppm (6H, *s*, OCH₃-DMT); 3.53 ppm (1H, *m*, H_{3'}); 3.52 ppm (1H, *m*, H_{5'}); 3.41 ppm (1H, *m*, H_{5'}); 2.46 ppm (3H, *s*, CH₃-Rf); 2.43 ppm (3H, *s*, CH₃-Rf). ¹³C NMR (CDCl₃, 100 MHz): δ = 159.2, 158.4, 154.4, 148.1, 144.9, 137.5, 137.4, 136.1, 136.0, 135.5, 135.3, 132.3, 132.2, 130.2, 130.1, 128.8, 128.2, 128.0, 127.7, 126.7, 125.9, 118.2, 113.0, 113.4, 100.4, 86.0, 80.4, 80.2, 63.8, 55.2, 47.5, 21.3 and 19.5 ppm.

4.2.3. Synthesis of 5'-O-DMT-2',4'-O-benzylidene-riboflavin-phosphoramidite (**4**)

To 5'-O-DMT-2',4'-O-benzylidene-riboflavin **3** (145 mg, 0.19 mmol) dissolved in anhydrous dichloromethane (4 mL), DIPEA (131 μL, 0.76 mmol) and 2-cyanoethyl-N,N-diisopropylamino-chlorophosphoramidite (116 μL, 0.28 mmol) were added. After 1 h, the solution was concentrated *in vacuo* and purified on column chromatography with ethyl acetate-hexane (9:1 with 3% trimethylamine) afforded the 5'-O-DMT-2',4'-O-benzylidene-riboflavin-phosphoramidite **4** (146 mg, 0.15 mmol, 80%). The characterization of the intermediate phosphoramidite **4** was carried out by ³¹P-NMR and ESI-MS

analyses. ^{31}P -NMR (161.98 MHz, CDCl_3 , r. t., mixture of diastereoisomers) δ = 149.91; 149.41 ppm; ESI-MS (positive ion) m/z calcd for $\text{C}_{54}\text{H}_{59}\text{N}_6\text{O}_9\text{P}$ = 966,41, found 967,41 $[\text{M}-\text{H}]^+$.

4.2.4. Synthesis of 3'-O-succinyl-5'-O-DMT-2',4'-O-benzylidene-riboflavin (**5**)

5'-O-DMT-2',4'-O-benzylidene-riboflavin **3** (290 mg, 0.37 mmol) was dissolved in dry pyridine (2 mL), triethylamine (TEA) (260 μL , 1.89 mmol) and 150 mg of succinyl anhydride (1.48 mmol) were added. The reaction was stirred at 50 °C for 2 h. After 2 h, the mixture was extracted two times with dichloromethane and water. The combined organic extracts were dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The residue was purified on silica gel in dichloromethane-methanol (97:30 to 95:50 + 1% triethylamine). The 3'-O-succinyl-5'-O-DMT-2',4'-O-benzylidene-riboflavin **5** was obtained in good yield (0.28 mmol, 76%).

Compound **5**: ^1H NMR (CDCl_3 , 400 MHz): 8.03 ppm (1H, *s*, aromatic-Rf); 7.87 (1H, *s*, aromatic-Rf); 7.50-7.11 ppm (9H, *m*, aromatic-DMT), 6.84 ppm (4H, *d*, J = 8.7 Hz, H_m DMT); 5.45 ppm (1H, *s*, $H_{6'}$); 5.37-5.28 ppm (2H, overlapped signals, $H_{3'}$ and $H_{1'}$); 4.58-4.42 ppm (2H, overlapped signals, $H_{2'}$ and $H_{1'}$); 4.07 ppm (1H, *m*, $H_{4'}$); 3.82 ppm (6H, *s*, OCH_3 -DMT); 3.38 ppm (1H, *m*, $H_{5'}$); 3.22 ppm (1H, *m*, $H_{5'}$); 2.83-2.51 ppm (4H, *m*, CH_2 succinyl); 2.46 ppm (6H, *s*, CH_3 -Rf). ^{13}C NMR (CDCl_3 , 100 MHz): δ = 175.3, 172.0, 159.2, 158.4, 154.4, 148.1, 144.9, 137.5, 137.4, 136.1, 136.0, 135.5, 135.3, 132.3, 132.2, 130.2, 130.1, 128.8, 128.2, 128.0, 127.7, 126.7, 125.9, 118.2, 113.0, 113.4, 99.5, 86.0, 78.0, 75.9, 65.5, 62.8, 54.7, 47.9, 29.4, 30.4, 21.2 and 19.4 ppm.

4.3. Synthesis of Rf-functionalized solid support (**6**)

In a typical experiment to 150 mg of HL CPG- NH_2 (108 $\mu\text{mol/g}$, 0.09 mmol) suspended in 1.0 mL of dry pyridine and 50 μL of DIPEA (0.27 mmol), a mixture of **5** (0.27 mmol), DCCI (56 mg, 0.27 mmol), DIPEA (50 μL , 0.27 mmol), and dry pyridine (500 μL) was added and shaken at room temperature for 72 hours. After exhaustive washings with pyridine, DMF, ACN, DCM and Et_2O , the support was dried under reduced pressure. DMT tests performed on dried and weighed samples of resulting support **6** allowed to determine the incorporation yields of derivative **5**, onto the resin, which resulted to be in the range of 0.04-0.05 mmol/g. After treatment with 1.0 mL of acetic anhydride/pyridine (1:1, v/v) at r.t. for 1 hour (*capping*), the resin was washed exhaustively with DCM, CH_3OH and Et_2O , dried under reduced pressure and used for automated syntheses of sequences **C** and **D**.

4.4. Synthesis and HPLC purification of Rf-modified ODN sequences

Oligomers **A** and **B** were synthesised starting with functionalised CPG support with 0.1 meq/g initial loading, on which the sequences d(TGGGAG) and d(GGGAG) were assembled in a standard manner.

An additional coupling with phosphoramidite building block **4** was then performed. Oligomers **C** and **D** were synthesised starting from the support **6** in a standard manner.

Oligomers **A–D** were detached from the solid support and deprotected by treatment with trimethylamine /pyridine (1:1, v/v) at 50 °C for 1 h, followed by treatment with conc. aq. ammonia at 50 °C for 5 h. The combined filtrates and washings were concentrated under reduced pressure, re-dissolved in water, analysed and purified by HPLC. Purification of the crude modified oligonucleotides **A–D** was carried out on Phenomenex RP18 column (*Gemini, 10 μ m C18(2), 250 mm $\times$ 21.2 mm*) using a linear gradient of CH₃CN in 0.1 M TEAA in H₂O, pH 7.0 from 20 to 100% over 30 min at flow rate of 7 mL/min with detection at 260 and 360 nm. The **A–D** ODNs were desalted on a Sephadex G10 column eluted with H₂O/EtOH 4:1 (v/v). The modified ODNs were obtained with an average yield of 25–31%. MS (MALDI-TOF) m/z calcd. for **A** [d(Rf-TGGGAG)] and **C** [d(TGGGAG-Rf)]: [M]_{calc.} = 2398.69 m/z; [M-H]⁻_{exp} = 2397.34 m/z.

MS (MALDI-TOF) m/z calcd. for **B** [d(Rf-GGGAG)]: [M]_{calc.} = 2094.50 m/z; [M-H]⁻_{exp} = 2093.99 m/z.

MS (MALDI-TOF) m/z calcd. for **D** [d(TGGGA-Rf)]: [M]_{calc.} = 2069.49 m/z; [M-H]⁻_{exp} = 2068.73 m/z.

4.5. Characterization of Rf-modified G-quadruplexes

4.5.1. G-quadruplex preparation

Stock ODNs solutions were prepared at single stranded concentration of 1 mM in 10 mM KH₂PO₄/K₂HPO₄ + 100 mM KCl buffer. Annealing experiments were performed at 80 °C heating for 5 minutes and then a fast cooling to room temperature was carried out. After annealing, the G-rich oligonucleotides arrange in G-quadruplex structure stabilized by potassium coordination. The solutions were stored at 8 °C for 14 days before all experiments. The concentration of the dissolved oligonucleotides **A–D** and natural one was determined by UV measurements.

4.5.2. Native gel electrophoresis

All stock solutions of (**A–D**) were loaded on the gels after dilution to 50 μ M (ss concentration) and with the addition of 25% of glycerol. The oligonucleotide d(T₆) was used as a single-stranded 6-mer marker and d(TG₄T) as a tetramolecular G-quadruplex one, [d(TG₄T)]₄. Native gel electrophoresis was performed on 15% polyacrylamide gel (acrylamide/bi-acrylamide 19:1) and the running buffer (TBE 0.5X) was supported by 10 mM KCl in order to preserve the native conditions. The gel was run at 26 °C at constant voltage (100 V) for 2.5 h. The bands were visualized by UV shadowing after stained with Stains All (Sigma-Aldrich) according to the manufacturer's instructions.

4.5.2. *Circular dichroism (CD)*

CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature control system (model PTC-348WI), using a quartz cuvette with a path length of 0.1 cm. CD parameters for spectra recording were the following: spectral window 220–320 nm, data pitch 1 nm, band width 2 nm, response 4 s, scanning speed 40 nm/min, temperature 10 °C, 3 accumulations. All sequences were analysed at 1×10^{-4} M single stranded concentration in 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ + 100 mM KCl buffer.

4.5.3. *Differential Scanning Calorimetry (DSC)*

DSC measurements were performed by means of NanoDSC from TA Instruments (New Castle, DE, USA). The excess molar heat capacity function ($\langle \Delta C_p \rangle$) was obtained after the subtraction of the baseline. A buffer-buffer scan was subtracted from the sample scan. Briefly a volume of 300 μL of Rf-modified G-quadruplex sample at concentrations ranging from 35 μM to 135 μM (concentration per mol of G-quadruplex) was placed in the calorimetry vessel. Successive heating scans were performed in the range 25–110 °C at scan rate of 1 °C/min to check the reversibility of the melting process. The obtained DSC data were analyzed by means of the NanoAnalyze software supplied with the instrument and plotted using the Origin software package (OriginLab, Northampton, MA, USA). The transition enthalpies, ΔH° , were obtained by integrating the area under the heat capacity vs temperature curves. The enthalpy change values are the averages of at least three different heating experiments.

4.5.4. *Fluorescence spectroscopy*

Fluorescence emission spectra of Rf-modified sequences were acquired by means of Fluoromax-4 fluorimeter from Horiba Scientific (Edison, NJ, USA) by using a 1-cm path length quartz cuvette. The sample concentrations were around 4 μM single strand concentration. The samples were excited at the wavelength of 450 nm, and emission spectra were acquired in the range 470–750 nm. The excitation and emission slits of the monochromators were set to 2 nm and 6 nm, respectively. The temperature was controlled by means of a Peltier system that ensures an accuracy of ± 0.1 °C. During the acquisition, the samples were under gentle stirring. For each sample two spectra were recorded: the first one at the temperature of 25 °C and the second one after heating the sample at 100 °C and fast cooling at 25 °C.

4.5.5. *Anti-HIV assay*

The anti-HIV activity and cellular cytotoxicity of the Rf-modified oligodeoxynucleotides were evaluated against HIV-1 NL4.3 and HIV-2 ROD replication in a CD4^+ T cell line MT-4 cell cultures

using the 3-(4,5- dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) method as described previously by Pauwels et al.[38]

Conflicts of interest

There are no conflicts to declare.

Abbreviations

Rf	Riboflavin
G4	G-quadruplex
CD	Circular dichroism
DSC	Differential scanning calorimetry
PAGE	Polyacrylamide gel electrophoresis
ODNs	Oligodeoxynucleotides
DMT	4,4'-dimethoxytrityl
DMF	Dimethylformamide
DIPEA	N,N-Diisopropylethylamine
DCCI	N,N'-Dicyclohexylcarbodiimide
CPG	Controlled Pore Glass
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography

HIV Human Immunodeficiency Virus

TBE buffer Tris-borate-EDTA buffer

ACN Acetonitrile

DCM Dichloromethane

TEAA Triethylammonium acetate

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Appendix A. Supplementary material: File Romanucci_SI

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