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Original article

Synthesis and anti-HIV-1 integrase activity of modified dinucleotides

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ABSTRACT

The synthesis of a series of thirty-eight new modified dinucleotides and dinucleotide conjugate analogues of d-$^{5'}$ApC$^{3'}$ is described. The inhibitory activity of these compounds toward HIV-1 integrase was examined in enzymatic assays using the natural dinucleotide as a reference. Among the compounds, a perylene-dinucleotide conjugate has shown a two micromolar anti-integrase activity due to the presence of both the intercalator and the dinucleotide.

1. Introduction

The efficient replication of retrovirus HIV-1 relies on the virally encoded protein integrase [1–5]. This enzyme is necessary for the first two steps of DNA integration: 3′-processing in which two nucleotides are removed from each 3′-end of the viral DNA, and strand transfer in which each 3′-processed viral DNA end is attached to the DNA host-cell. As a result, HIV-1 integrase has attracted intense effort towards the development of potent inhibitors. Such efforts have led to the development of different classes of compounds, [6–10] with one of them, Raltegravir (Isentress®) a selective strand transfer inhibitor, approved by the FDA [11] and a few currently under clinical trials [12,13]. However, resistance has already been observed in patients treated with Raltegravir, leading to virologic failure. There is therefore an ongoing constant need for the development of new inhibitors [14–17].

Among the different classes of integrase inhibitors, dinucleotide analogs have proven to be potent competitors of the double-stranded oligonucleotide integrase substrate [18]. The monophosphorylated natural dinucleotides p-d-$^{5'}$ApC$^{3'}$, p-d-$^{5'}$ApT$^{3'}$ et p-d-$^{5'}$CpT$^{3'}$ have been shown to be moderate inhibitors of both the processing and strand transfer steps [19]. Modified dinucleotides resistant to nucleases with similar inhibitory effects have also been synthesized [20,21] while others have exhibited more specific inhibition for the strand transfer process [22]. In the quest for new dinucleotides with nuclease stability and improved anti-integrase activities, we have developed different sets of modified dinucleotide $^{5'}$CpA$^{3'}$. This dinucleotide was chosen as a scaffold in order to maintain structural features for recognition since it is preserved at the end of the LTR sequences after the processing step [1]. Modifications in dinucleotides make them more resistant towards nucleases. The sugar ring, the internucleotidic linkage, and the cytosine base have been modified. Intercalating agents and varied ligands were also attached to either the 5′- or the 3′-ends of the dinucleotide and the deoxyribose and phosphodiester groups were replaced by a polymethylene chain to attach the nucleic bases. We report here their synthesis and anti-integrase activity. The different sets of modified dinucleotides synthesized are listed in Fig. 1

2. Chemistry

2.1. The natural dinucleotide d-$^{5'}$CpA$^{3'}$ **1**

The natural dinucleotide D-d-$^{5'}$CpA$^{3'}$ **1** (Figs. 1 and 2), used as a reference for evaluating the anti-integrase properties of the new

Abbreviations: HIV-1, Human Immunodeficiency virus type 1; IN, Integrase; DNA, Deoxyribonucleic Acid; FDA, Food and Drug Administration; IC 50, half maximal inhibitory concentration is a measure of the effectiveness of a compound inhibiting a biochemical or biological function; p-d-$^{5'}$ApC$^{3'}$, 5′-monophosphorylated dinucleotide phosphodiester involving 2′-deoxyadenosine and 2′-deoxycytidine; p-d-$^{5'}$ApT$^{3'}$, 5′-monophosphorylated dinucleotide phosphodiester involving 2′-deoxyadenosine and thymidine; p-d-$^{5'}$CpT$^{3'}$, 5′-monophosphorylated dinucleotide phosphodiester involving 2′-deoxycytidine and thymidine; d-$^{5'}$CpA$^{3'}$, dinucleotide phosphodiester involving 2′-deoxycytidine and 2′-deoxyadenosine.

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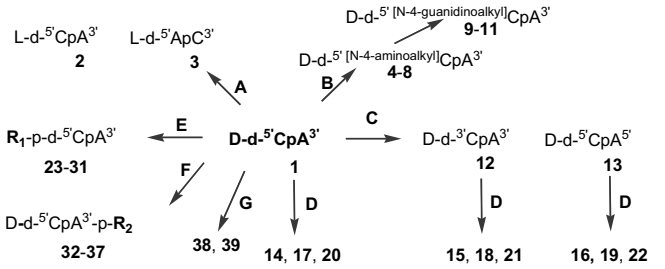


Fig. 1. Sets of modified dinucleotides: A: sugar modification; B: base modification; C: internucleotidic polarity changes; D: internucleotidic linkage modification; E: 5′-conjugates; F: 3′-conjugates; G: interbase linkage.

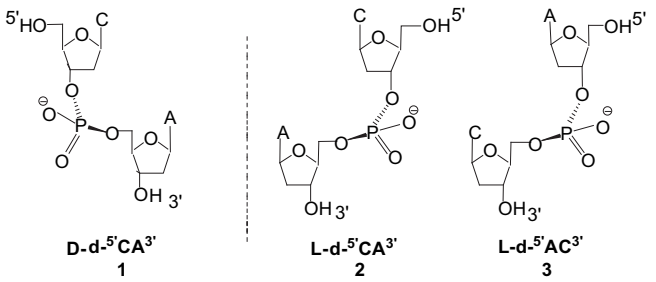


Fig. 2. Structures of dinucleotides **1**–**3.**

compounds reported in this study, was obtained in solution *via* H-phosphonate chemistry [23]. Briefly, the commercially available H-phosphonate derivative of 2′-deoxycytidine, protected at the 5′-hydroxyl function with a dimethoxytrityl group and at the base exocyclic amine function with a benzoyl group, and 2′-deoxyadenosine, protected at the base exocyclic amine and 3′-hydroxyl functions with benzoyl groups, were condensed in the presence of pivaloyl chloride. The fully protected dinucleoside H-phosphonate thus obtained was oxidized by iodine treatment in a pyridine/H_2O mixture and then deprotected using reported procedures [24]. We chose this strategy because the dinucleoside-H-phosphonate derivative can be used as a precursor for the preparation of dinucleotides with varied internucleotidic linkages [25–27], as outlined in section 2.5.

2.2. Dinucleotides **2** and **3** with L-2′-deoxyribose units

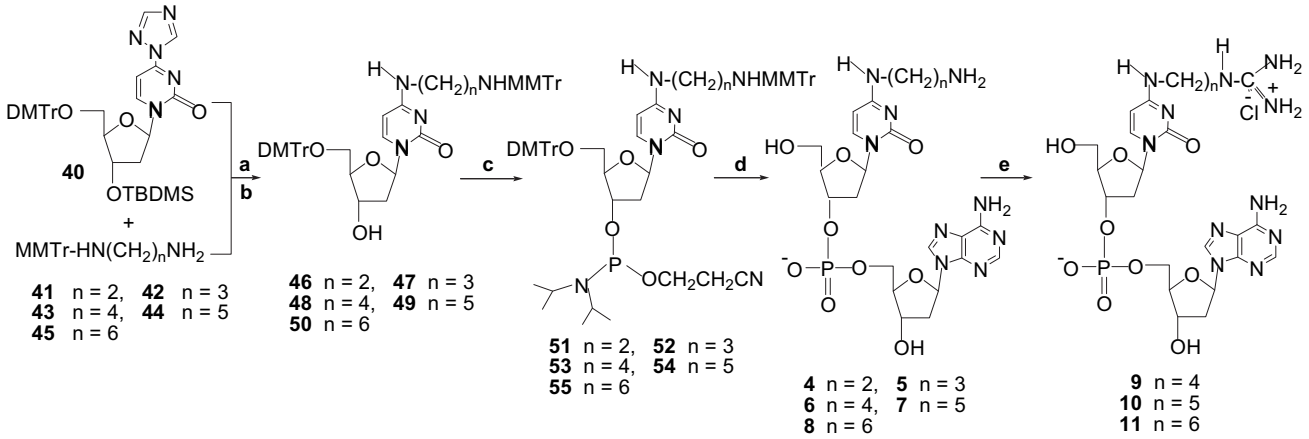
To obtain our first modification, we chose to investigate the influence of the sugar by replacing the natural D-sugar by its corresponding L-enantiomer (Fig. 2). The dinucleotide phosphodiesters L-d-$^{5'}$CpA$^{3'}$ **2** and L-d-$^{5'}$ApC$^{3'}$ **3** were synthesized in solution starting from commercially available unprotected L-dC and L-dA using strategies adapted from those commonly reported in the D-series. Briefly, the exocyclic amino functions of the L-2′-deoxycytidine and the L-2′-deoxyadenosine were benzoylated and the 5′-hydroxyl function dimethoxytritylated [24]. One half of each compound was then transformed into its H-phosphonate derivative using 2-chloro-5, 6-benzo-1, 3, 2-dioxaphosphorin-4-one [28]. The second half of each compound was 3′-benzoylated and then detritylated to afford the L-nucleosides with free 5′-hydroxyl functions. The nucleoside 3′-H-phosphonate derivatives were then reacted with the convenient nucleosides to give the dinucleoside H-phosphonates that were oxidized and then deprotected, as reported for the preparation of compound **1**, to give the L-d-$^{5'}$CpA$^{3'}$ **2** and L-d-$^{5'}$ApC$^{3'}$ **3** dinucleotides. The structures of these deprotected dinucleotide compounds were confirmed by ^{1}H NMR and mass spectrometry analysis.

2.3. N-4-alkylation of the 2′-deoxycytidine

N-4 alkylation of the 2′-deoxycytidine was performed in order to allow additional interactions between the dinucleotide and the integrase (Scheme 1). As substituent groups we chose aliphatic bis-aminated polymethylene linkers with different lengths (from 2 to 6 methylene groups). Alkylation of the 2′-deoxycytidine was performed following previously reported procedures starting from the triazolo derivative of 2′-deoxyuridine **40** [29]. Reaction with the monotritylated linkers **41**–**45**, followed by 3′-desilylation led to mononucleotides **46**–**50**. The latter were transformed into their phosphoramidite derivatives **51**–**55** that were reacted with the conveniently protected 2′-deoxyadenosine. After oxidation, deprotection and purification following classical procedures, the alkylated dinucleotides **4**–**8** were obtained with good yields. The guanidinium derivatives **9**–**11** of the amino-alkylated dinucleotides **6**–**8** involving the tetra, penta and hexamethylene linkers were also obtained following a procedure adapted from our previous work [30].

2.4. Dinucleotides **12** and **13** with modified internucleotidic linkage polarities

In order to evaluate the influence of the internucleotidic polarity, the dinucleotides D-d-$^{3'}$CpA$^{3'}$ **12** and D-d-$^{5'}$CpA$^{5'}$ **13** were



Scheme 1. Synthesis of the dinucleotides **4**–**11**. Reagents and conditions. a: CH_3CN, rt, 15 h. b: 1 M tetrabutylammonium fluoride in THF, 2 h, rt. c: 2-cyanoethyldiisopropyl-chlorophosphoramidite, diisopropylethylamine, dichloromethane. d: 4-N-benzoyl-3′-benzoyl-2′-deoxyadenosine, Te; oxidation step; deprotection and purification steps. e: 1*H*-pyrazole-1-carboxamidine hydrochloride, 1 M aqueous Na_2CO_3; purification.

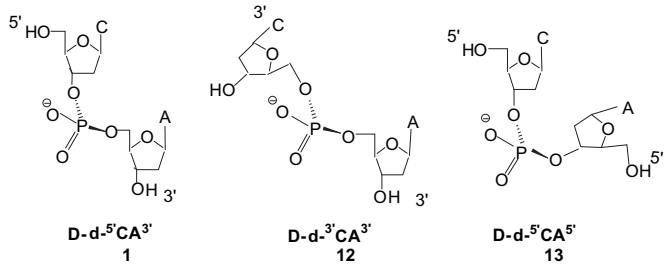
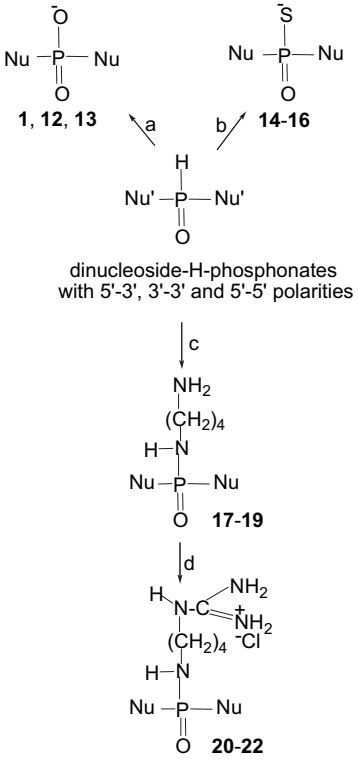


Fig. 3. Structures of dinucleotides **12** and **13** with modified internucleotidic linkage polarities.

synthesized (Fig. 3). As for the preparation of the natural dinucleotide D-d-$^{5'}$CpA$^{3'}$ **1**, the synthesis was performed via H-phosphonate chemistry because of the possibility of the internucleotidic linkage modification when using this strategy (Scheme 2). The conveniently protected monomers were obtained as follows: 2′-deoxycytidine and 2′-deoxyadenosine protected by a dimethoxytrityl group on their 5′-hydroxyl functions and benzoyl groups at both their exocyclic amine and their 3′-hydroxyl functions were detritylated. An H-phosphonate group was incorporated at the 5′-position of the 2′-deoxycytidine as reported above and the latter was reacted with the 2′-deoxyadenosine derivative involving the free 5′-hydroxyl group to give the dinucleotide H-phosphonate derivative D-d-$^{3'}$C^{Bz}p[H]A$^{Bz\ 3'}$. The modified dinucleotide H-phosphonate D-d-$^{5'}$C^{Bz}p[H]A$^{Bz\ 5'}$ was obtained by a reaction between the 3′ H-phosphonate derivative of the 5′-O-dimethoxytrityl-4-N-benzoyl 2′-deoxycytidine and the 3′-hydroxyl function of the 5′-O-dimethoxytrityl-6-N-benzoyl-2′-deoxyadenosine in the presence of pivaloyl chloride. After the oxidation step, deprotection and purification led to the dinucleotides D-d-$^{3'}$CpA$^{3'}$ **12** and D-d-$^{5'}$CpA$^{5'}$**13.**

2.5. Modifications of the internucleotidic linkage

The phosphodiester bond of the natural dinucleotide D-d-$^{5'}$CpA$^{3'}$ **1** and the unnatural dinucleotides D-d-$^{3'}$CpA$^{3'}$ **12** and D-d-$^{5'}$CpA$^{5'}$ **13** (Scheme 2) were replaced by a phosphorothioate group **14**–**16**. An amino-ending linker was also attached to the internucleotidic position by the intermediate of a phosphoramidate linkage **17**–**19**. Finally, a guanidylated linker was also attached to the internucleotidic position **20**–**22.** The syntheses of the dinucleotides **14–22** were performed by modification of the dinucleoside H-phosphonates D-d-$^{5'}$C^{Bz}p[H]A$^{Bz\ 3'}$, D-d-$^{3'}$C^{Bz}[H]pA$^{Bz\ 3'}$ and DD-d-$^{5'}$C^{Bz}p[H]A$^{Bz\ 5'}$ in accordance with literature reports and our previous results. Part of each dinucleoside-H-phosphonate was separately transformed into dinucleoside phosphorothioate by treatment with S_8 in a pyridine/CS_2 mixture to give, after deprotection and purification, the dinucleotide phosphorothioate **14**–**16** [25]. The remaining part of each pure dinucleoside-H-phosphonate was separately transformed into the dinucleoside d-$^{5'}$DMTrC^{Bz}p*[NH(CH$_2$)$_4$NHMMTr]A$^{Bz3'}$ by amidative oxidation with 1,4-diaminobutane, protected at one amino function with a monomethoxytrityl group **43**, in a CCl_4/pyridine mixture, following a procedure adapted from our previously published work [26,27], to give, after deprotection and reversed-phase purification, dinucleosides **17**–**19.** Dinucleosides **20–22** involving the guanidylated linkers were obtained by treatment of dinucleotides **17–19** involving the amino linker with 1H-pyrazole-1-carboxamidine as mentioned above [30]. The replacement of the internucleotidic phosphodiester bond by either a phosphorothioate or a phosphoramidate bond induced the formation of isomer pairs that have been shown to possess different biochemical properties when included



Scheme 2. General scheme for the internucleotidic bond modification. The internucleotidic H-phosphonate linkage can be converted into phosphodiester (route a), phosphorothioate (route b), phosphoramidate (route c). The amino-ending linkers can be guanidylated (route d). Nu′ stands for the protected nucleosides and Nu for the deprotected ones.

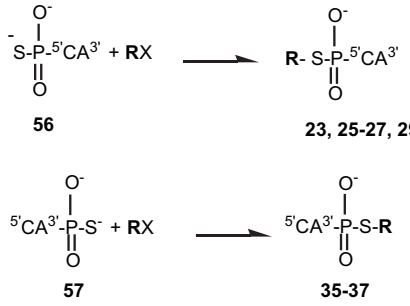
inside oligonucleotides. For these reasons we decided to try obtaining the above-mentioned dinucleotides as pure isomers by reversed-phase separation and were successful for all the dinucleotides except for **16** obtained as an isomer mixture.

2.6. Covalent attachment of varied ligands to either the 5′- or the 3′-end of the dinucleotide D-d-$^{5'}$CpA$^{3'}$ 23–37 (see Table 2 for structures)

In order to induce additional interactions with the integrase, various ligands such as linkers with terminal hydroxyl or amino functions, or intercalating groups were attached to either the 5′- or the 3′-end of the dinucleotide D-d-$^{5'}$CpA$^{3'}$. Two strategies can be used. In the first, the ligands are directly incorporated via the use of modified supports involving either them (3′-conjugates **32**–**34**) or their phosphoramidite (**24**, **28**, **31**) or H-phosphonate (**30**) derivatives. The ligands must withstand the chemical conditions required for both the dinucleotide synthesis and deprotection steps. In the second strategy, a post-synthetic specific reaction is performed between convenient groups, incorporated at preselected positions in both the ligands and the deprotected dinucleotide. In this study, for the preparation of conjugates **23**, **25**–**27**, **29**, and **35**–**37** we chose to use the reaction between halogenoalkyl linkers attached to the ligands and a phosphorothioate group incorporated in either the 5′-end (**56**) or the 3′-end (**57**) of the dinucleotide phosphodiester$^{5'}$CpA$^{3'}$ (Scheme 3). The 5′ and 3′-phosphorothioate incorporations were performed following our previously published procedures [31].

2.6.1. 5′-conjugates 23–31

2.6.1.1. Bis-aminoalkylated-dinucleotide conjugate ***23***. In order to allow possible ionic interactions with integrase, a triamine linker

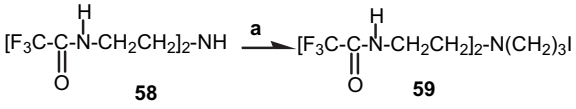


Scheme 3. General scheme for 5′-conjugation (top) and 3′-conjugation (bottom) *via* phosphothiolodiester linkage.

was attached to the 5′-end of the dinucleotide D-d-$^{5'}$CpA$^{3'}$ as follows. Bistrifluoroacetylated diethylenetriamine [32] **58** was reacted with 1,3-diiodopropane to give the 1,7-bis trifluoroacetyl-4-(3-iodopropyl)hepta-1,4,7-triazane **59** (Scheme 4). The latter was then reacted with 5′-thiophosphorylated dinucleotide S-p-$^{5'}$CpA$^{3'}$ **56** to give, after deprotection, the modified dinucleotide **23** involving a linker ending with two amino groups.

2.6.1.2. Intercalator-dinucleotide conjugates ***24–31****.* Intercalating agents are planar molecules with fused aromatic rings [33]. In addition to their intercalating properties these compounds are lipophilic so that they are able to display different interactions with the integrase. We surmised that intercalator-dinucleotide conjugates would be able to interact in two different ways [34]. The dinucleotide would interact specifically with the integrase active site, while the intercalator would be involved in additional interactions nearby with other parts of the enzyme. The different intercalating agents linked to the 5′-end of the natural phosphodiester dinucleotide D-d-$^{5'}$CpA$^{3'}$ were acridine, anthraquinones, thiazole orange and perylene (see Table 1). Depending on their structures, these molecules exhibit different areas and lipophilic properties, thus offering a wide variety of possible interactions with the integrase.

2.6.1.2.1. Acridine derivatives. 6-Chloro-2-methoxy-9-amino-acridine was attached either via a neutral or a positively charged linker. The linkage of the acridine derivative by a hexamethylene tether was performed on solid-phase by using its phosphoramidite derivative, obtained as previously reported [35]. After synthesis, the deprotection step, performed with a sodium hydroxide solution (0.4 N) in a MeOH/H_2O mixture in order to avoid the cleavage of the bond between the acridine ring and the linker, led to the acridine-dinucleotide conjugate **24**. Acridine was also attached to the 5′-end of the dinucleotide via a new linker involving a positively charged group. The presence of this charge precluded the preparation of the phosphoramidite derivative. For this reason the linkage of this new acridine-linker derivative to the dinucleotide was performed by the reaction of the 5′-thiophosphorylated dinucleotide **56** with the bromoalkylated linker attached to the acridine derivative [31]. The synthesis of the new acridine-linker compound was performed following a two-step procedure (Scheme 5). First, 6, 9-dichloro-2-methoxyacridine **60** and 3-dimethylaminopropylamine were reacted in phenol to give the 6-chloro-2-methoxy-9-(3–3–dimethylaminopropyl)-amino acridine **61**. The latter was then reacted



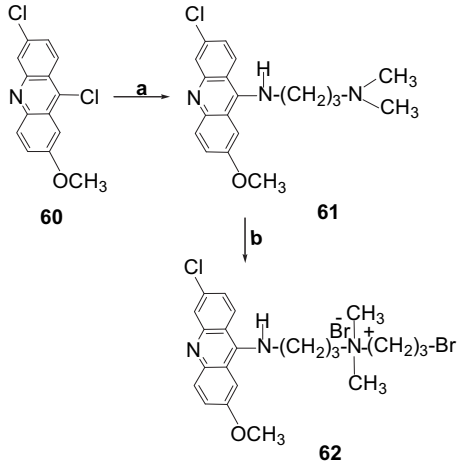
Scheme 4. Synthesis of the protected bis-aminoalkylated linker **59**

Table 1
Structures and activities of the dinucleotides **1–22**.

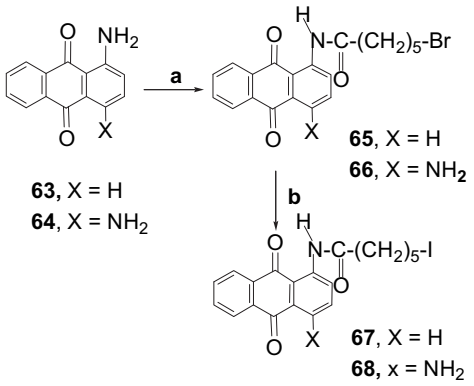
Compounds		IC 50 (μM)
1	D-d-$^{5'}$CpA$^{3'}$	340
2	L-d-$^{5'}$CpA$^{3'}$	≥340
3	L-d-$^{5'}$ApC$^{3'}$	≥340
	D-d-$^{5'}$ [N-4-aminoalkyl]CpA$^{3'}$	
4		>1000
5		>1000
6		>1000
7		>340
8		>340
9	D-d-$^{5'}$ [N-4-guaridiroalkyl]CpA$^{3'}$	>340
10		>340
11		>340
12	D-d-$^{3'}$CpA$^{3'}$	≥340
13	D-d-$^{5'}$CpA$^{5'}$	≥340
14	D-d-$^{5'}$Cp(S)A$^{3'}$	≥340
15	D-d-$^{3'}$Cp(S)A$^{3'}$	≥340
16	D-d-$^{5'}$Cp(S)A$^{5'}$	110
17	D-d-$^{5'}$Cp(amino)A$^{3'}$	≥340
18	D-d-$^{3'}$Cp(amino)A$^{3'}$	102
19	D-d-$^{5'}$Cp(amino)A$^{5'}$	100
20	D-d-$^{5'}$Cp(guanidino)A$^{3'}$	≥340
21	D-d-$^{3'}$Cp(guanidino)A$^{3'}$	100
22	D-d-$^{5'}$Cp(guanidino)A$^{5'}$	100

with 1,3-dibromopropane in acetonitrile to give the new acridine derivative **62**.

2.6.1.2.2. Anthraquinone derivatives. We chose to attach a series of three different anthraquinones to the 5′-end of the dinucleotide in order to display varied interactions between the dinucleotide-intercalator conjugates and the target. The first one, involving an iodopentyl linker attached to the position 1 *via* an amide function **67**, was obtained by reaction of the 1-aminoanthraquinone **63** with 6-bromohexanoyl chloride in the presence of pyridine (Scheme 6). Halide exchange was then performed by NaI treatment to give the anthraquinone with the iodopentyl linker **67**. The attachment of the same linker to position 1 of the 1,4-diaminoanthraquinone **64** was performed following the same strategy except that an excess of anthraquinone was used. The two anthraquinone derivatives **67** and **68** were reacted with the 5′-thiophosphorylated dinucleotide **56** to give the anthraquinone-dinucleotide conjugates **26** and **27**. The third anthraquinone derivative involves two hexamethylene



Scheme 5. Synthesis of the acridine derivative **62** with a positively charged linker Reagents and conditions. a: 3-dimethylaminopropylamine (2 eq), phenol, 100 °C, 2 h. b: 1,3-dibromopropane (2 eq), CH_3CN, 80 °C, 2 h.

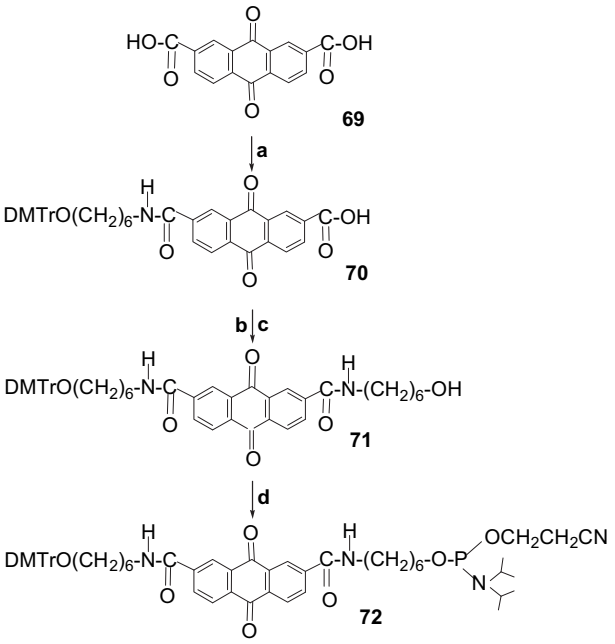


Scheme 6. Synthesis of the anthraquinone-linker derivatives **67** and **68** Reagents and conditions. a: 6-bromohexanoylchloride (1. 2 eq), pyridine (ε), benzene, reflux 2 h for compound **63** (4 h for compound **64**); b: NaI (5 eq), $NaHCO_3$ (5eq), acetone, reflux 8 h.

linkers attached to the 2 and 7 positions via amide linkages (Scheme 7). 2,7-Biscarboxyanthraquinone **69** was obtained as previously reported [36]. At this stage, the bisfunctionalization by two aminohexanol linkers was performed *via* amide bond formation. In order to allow covalent attachment of the anthraquinone derivative to only one dinucleotide, both linkers were selectively protected on their hydroxyl functions by a dimethoxytrityl and a *tert*-butyldiphenylsilyl group, respectively. The dimethoxytritylated linker was attached to the 2,7-biscarboxyanthraquinone derivative after activation with carbonyldiimidazole in the presence of a catalytic amount of hydroxybenzotriazole and triethylamine to give **70**. After purification of the monoderivatized anthraquinone **70**, the silylated linker was attached following the same strategy. The silyl group was removed by tetrabutylammonium fluoride treatment to give the bis substituted anthraquinone **71**. The phosphoramidite derivative **72** was obtained by treatment of **71** with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite in the presence of N,N-diisopropylethylamine. After reaction of the phosphoramidite **72** with the free hydroxyl function of the dinucleotide bound to the support followed by iodine oxidation, the anthraquinone-dinucleotide conjugate **28** was obtained after removal of the protective groups and reversed-phase purification.

2.6.1.2.3. Thiazole orange derivative. Acridine and anthraquinone are intercalating molecules composed of three fused rings. Thiazole orange, composed of a benzothiazole and a quinolinium nuclei linked *via* a monomethine bond, offers many more possibilities of interaction with the integrase. Thiazole orange was attached to the 5′-end of the dinucleotide via a phosphothiolodiester and a heptamethylene linker **29**. The synthesis of the thiazole orange linker derivative was performed as previously reported [37].

2.6.1.2.4. Perylene derivatives. Perylene is composed of five fused rings. It is therefore the most lipophilic among the intercalators used in this study. In order to provide it with different possibilities of interaction with the enzyme, the perylene was attached to the 5′-end of the dinucleotide either via a phosphodiester or to the β-anomeric position of a 2′-deoxyribose following procedures adapted from our previous work [38,39]. The synthesis of the dinucleotide conjugate **30** involving the perylene attached to the ß-anomeric position of a 2′-deoxyribose unit added to the 5′end of the dinucleotide was performed on solid-phase by using the H-phosphonate derivative of the perylene sugar unit [38]. As reported for the acridine-dinucleotide conjugate **24**, the synthesis of the perylene-dinucleotide conjugate **31**, involving an eighth-atom linker, was performed via phosphoramidite chemistry [39].



Scheme 7. Synthesis of the anthraquinone-linker derivative **72** Reagents and conditions. a: Pyr, NEt_3, hydroxybenzotriazole (catalytic amount), 1,1′-carbonyldiimidazole (1. 2 eq), rt, 2 h; 1-O-(4,4′-dimethoxytrityl)-6-hexylamine (0. 9 eq), rt, 20 h. b: Pyr, NEt_3, hydroxybenzotriazole (catalytic amount), 1,1′-carbonyldiimidazole (1. 2 eq), rt, 2 h; 1-O-(*tert*-butyl-diphenylsilanyl)-6-hexylamine (0. 9 eq), rt, 20 h. c: 1 M tetrabutylammonium fluoride in THF (5 eq), rt, 1 h. d: $ClCH_2$-CH_2Cl, DIEA (3.5 eq), 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (1. 4 eq), 75 min.

For both conjugates the deprotection step was achieved by concentrated aqueous ammonia treatment.

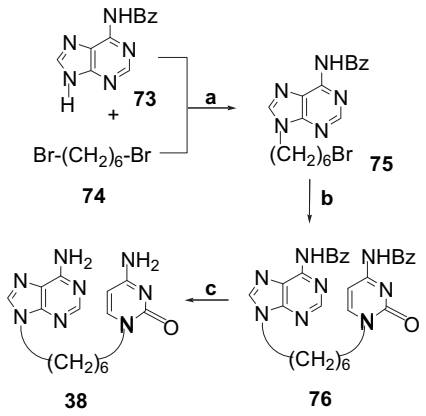
*2.6.2. 3′-conjugates **32–37***

*2.6.2.1. Dinucleotide with hydroxyl- and aminoalkyl linkers **32–34**.* The synthesis of the dinucleotide-linker conjugates **32**–**34** was performed *via* phosphoramidite chemistry on solid-phase using modified supports releasing, after the deprotection step, the linkers with the desired functionalities. The modified support releasing the aminohexyl linker was obtained as previously reported [40]. The modified supports releasing the dinucleotides with hydroxypropyl and the hydroxydodecyl linkers were obtained in a four-step process from 1,3-propanediol and 1,12-dodecanediol following described procedures [41,42].

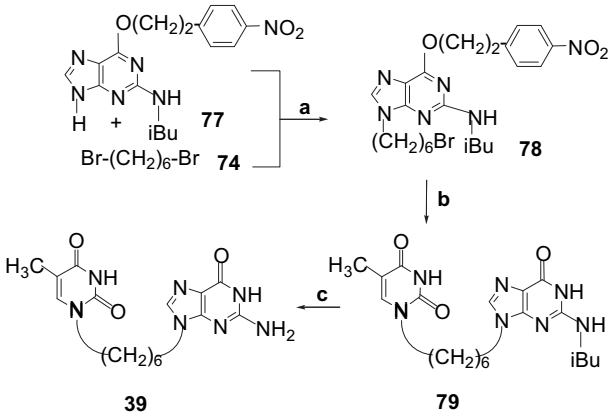
*2.6.2.2. Dinucleotide-acridine conjugates **35–37**.* The acridine was also linked to the 3′-end of the dinucleotide via a phosphothiolodiester linkage and either a tri- or a hexamethylene linker **35**, **36**. These syntheses were performed by the reaction between the dinucleotide bearing a 3′-terminal thiophosphate group **57**, obtained using a previously reported support [31], and acridine derivatives involving bromoalkyl linkers. The latter were obtained using a procedure adapted from one of our previous reports [37]. The acridine derivative **62** involving the quaternary ammonium group was also linked to the 3′-end of the dinucleotide via a phosphothiolodiester linkage **37**.

*2.7. Replacement of the two 2′-deoxyribose units and the phosphodiester linkage of the dinucleotides D-d-$^{5'}$CpA$^{3'}$ and D-d-$^{5'}$TpG$^{3'}$ by a polymethylene linker **38, 39***

As shown on a CPK model, the two 2′-deoxyribose units and the phosphodiester linkage can be replaced by a hexamethylene linker,



Scheme 8. Synthesis of the compound **38** Reagents and conditions. a: K_2CO_3 (1eq); 1,6-dibromohexane (5 eq); 60 °C, 10 h. b: 4-N-benzoylcytosine (1. 2 eq), K_2CO_3 (1.1 eq), DMF, 55 °C, 8 h, then 20 °C, 12 h. c: NH_4OH (28%) (12 mL), MeOH (1.5 mL), 20 °C, 72 h.



Scheme 9. Synthesis of the compound **39**. Reagents and conditions. a: K_2CO_3 (1eq); 1,6-dibromohexane (4 eq); 60 °C, 11 h. b: Thymine (1.15 eq), DBU (1.73 eq), Py, rt, 24 h. c: NH_4OH (28%) (4 mL), 50 °C, 15 h.

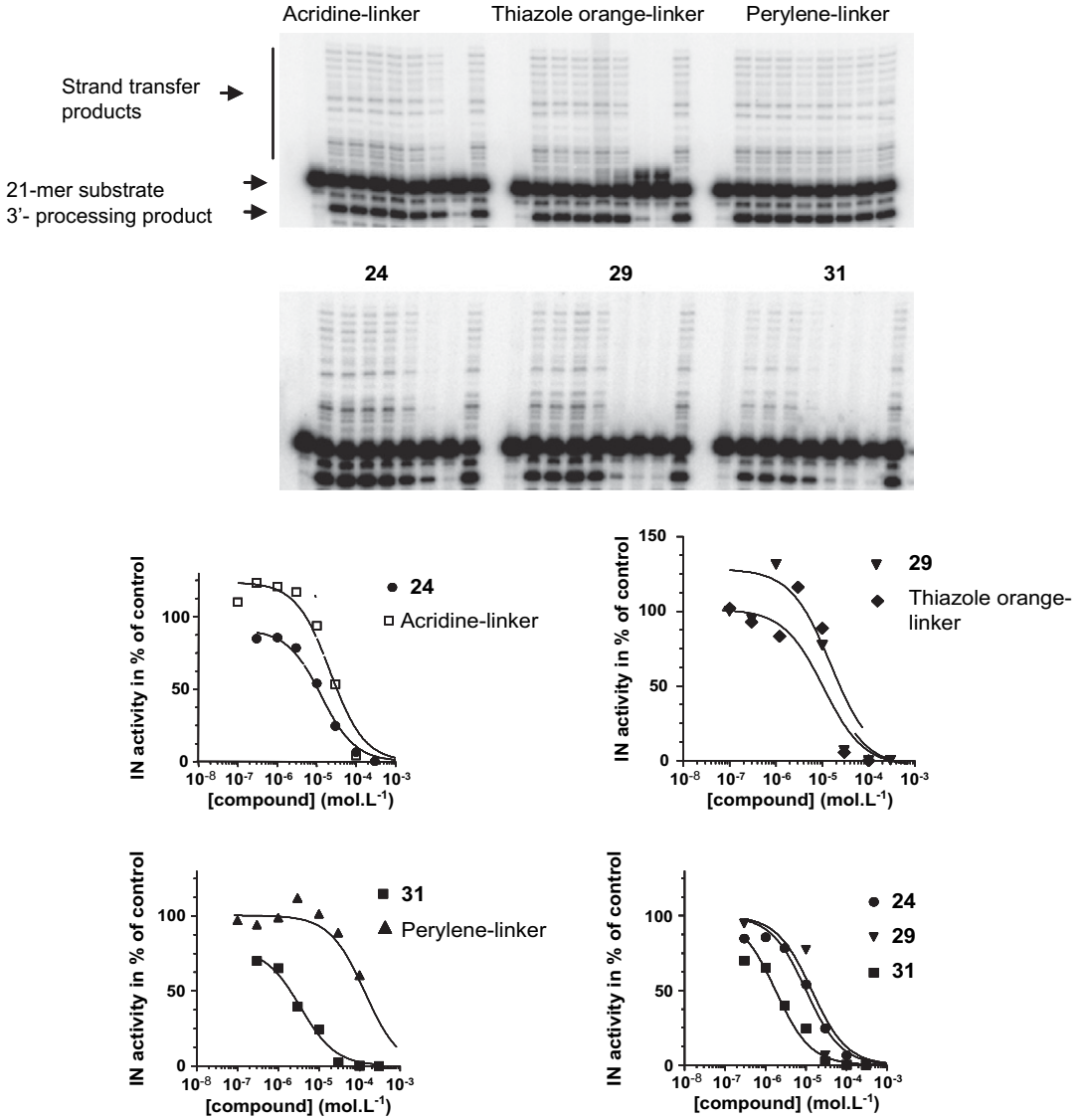
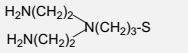
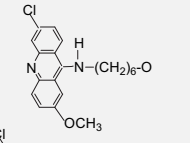
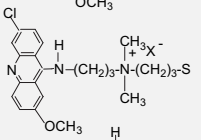
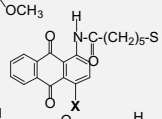
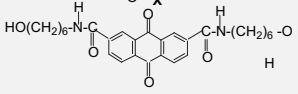
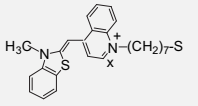
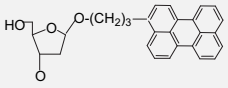
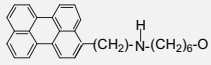
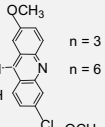
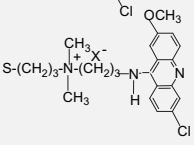


Fig. 4. Anti-integrase activities of conjugates **24**, **29** and **31** compared to those of the corresponding intercalator-linker derivatives. Upper panels: PAGE analysis of integrase overall activity for 1 hr at 37 °C, in the presence of increasing drug concentration (left to right, 100 nM IN + 10 mM EDTA, 300 nM, 1μM, 3μM, 10μM, 30μM, 100μM, 300μM, no drug).

Table 2
Structures and activities of the dinucleotide conjugates **23–37**.

Compounds			IC 50 (μM)
	R_1-p-$^{5'}$CpA$^{3'}$-p-R_2		
	R_1-	R_2	
23		H	786
24		H	13.2
25		H	>100
26	X = H 	H	13.9
27	X = NH_2		13.4
28		H	21
29		H	65.5
30		H	30
31		H	1.8
32	H	$(CH_2)_3$-OH	>100
33	H	$(CH_2)_{12}$-OH	>100
34	H	$(CH_2)_6$-NH_2	>100
35	H	 n = 3	
36	H	n = 6	>100
37	H		>100

while the nucleic bases cytosine and adenine are able to stack together in a manner similar to the one they adopt in the natural dinucleotide D-d-$^{5'}$CpA$^{3'}$. We therefore chose to tether the cytosine and the adenine with a hexamethylene linker attached to position 1 of the cytosine and position 9 of the adenine to give compound **38**. In the same way, the two 2′deoxyribose units and the phosphodiester linkage of the dinucleotide D-d-$^{5'}$TpG$^{3'}$ (complementary to the CA dinucleotide on the double-stranded proviral DNA) were also replaced by a hexamethylene linker to afford compound **39**. The synthesis was performed as reported in Schemes 8 and 9. 6-N-Benzoyladenine **73** was reacted with 1, 6-dibromohexane **74** to give the base-linker **75.** The latter was reacted with 4-N-benzoylcytosine to give the protected compound **76** that was debenzoylated to give **38**. In the same manner, guanine protected at the amino function by an isobutyryl group and a *p*-nitrophenylethyl moiety at position 6 of **77** [43] was reacted with 1, 6- dibromohexane **74** to give the alkylated guanine **78**. The latter was then reacted with thymine to give the protected compound **79** and, after deprotection, compound **39**.

3. Pharmacology

The integration of pro-viral cDNA into host DNA involves two reactions catalyzed by the viral enzyme integrase: 3′end processing of the viral DNA ends and strand transfer [1–10]. In the cytoplasm of the infected cells, the viral RNA genome is reverse transcribed into double stranded viral DNA. Then, integrase binds to both extremities of the viral cDNA, forming with other various viral and cell proteins the pre-integration complex. Inside this complex, the enzyme removes the dinucleotide GT from the two 3′- ends of the linear viral DNA (processing reaction) leading to overhanging CA ends. Then the pre-integration complex is transported from the cytoplasm to the nucleus of the infected cells were the 3′-processed ends of the viral DNA attack phosphodiester bonds on both strands of the target DNA simultaneously resulting in the incorporation of viral DNA into host cell DNA (strand transfer reaction). These two catalytic reactions (3′-terminal processing and strand transfer) can be reproduced in vitro by using recombinant integrase and synthetic DNA duplexes mimicking the terminal sequences of viral DNA as substrate. The anti-integrase activity of potent inhibitors directed towards these two catalytic steps can be evaluated *in vitro* by monitoring the amount of processed and strand–transfer products by polyacrylamide gel electrophoresis analysis (See Fig. 4). Dinucleotide analogues of the preserved d-$^{5'}$-CA-$^{3'}$ at the ends of the viral DNA processed strands can possibly inhibit the integrase activity by competing with the viral duplex target.

4. Results and discussion

Each modified dinucleotide **2**–**39** was screened for inhibitory activity against HIV-1 integrase. Inhibitory activities were studied in a full activity assay monitoring simultaneously both 3′processing and strand transfer activities. The unmodified dinucleotide **1** was also tested for comparative activity. The results can be separated into two parts. Among the modified dinucleotides **2–22**, only five compounds are slightly more efficient (three to four-fold) inhibitors than the unmodified dinucleotide **1** (IC 50 = 340 μM) (Table 1). All these compounds are involving two modifications with respect to the dinucleotide **1** used as a reference: a change of the internucleotide bond polarity and the replacement of the phosphodiester linkage. They are: **16** with a 5′–5′ polarity and a phosphorothioate linkage; **19** and **22** with a 5′–5′ polarity and a phosphoramidate internucleotidic linkage and **18** and **21** with a 3′–3′ polarity and a phosphoramidate internucleotidic linkage. The other changes of the dinucleotide structure such as the replacement of the D-2′-deoxyribose by the L-2′-deoxyribose (compounds **2** and **3**) and the alkylation of the cytidine (compounds **4**–**11**) did not improve the inhibitory effect of the dinucleotide. In the dinucleotide conjugate series **23**–**37** (Table 2) two types of behaviour could be observed. 5′-Conjugation (compounds **23**–**31**) led to an increase in the inhibitory effect, except for compounds **23,** involving at the 5′-end a branched linker with two amino groups, as compared to the activity of the unmodified dinucleotide **1**. On the contrary, no improvement of the dinucleotide potency was observed by 3′-conjugation (compounds **32–37**). The intercalators linked to the 5′-end of the dinucleotide were acridine, anthraquinones, thiazole orange and perylene (compounds **24**–**31**, Table 2) that exhibit different areas and lipophilic properties, thus offering a wide variety of possible interactions with the integrase. Two of them

acridine and perylene were attached to the dinucleotide *via* different linkers. The strongest inhibitory effect was observed with the perylene-dinucleotide conjugate **31** (IC 50 = 1.8 μM), while other conjugates **24** and **26**–**30** exhibited IC 50 values between 13.2 and 66 μM. The linkage of thiazole orange, involving a benzothiazolium and a lepidinium nuclei, (compound **29**) led to a weaker inhibitory effect (IC 50 = 65.5 μM) than that of intercalators with three fused aromatic rings such as acridine (compound **24,** IC 50 = 13.2 μM) and anthraquinones (compound **26**, IC 50 = 13.9 and compound **27**, IC 50 = 13.4 μM). In the anthraquinone-dinucleotide series, the addition of an hydroxyhexyl chain on the intercalator in position opposite to the attachment site of the linker (compound **28**) led to a slightly reduced activity (IC 50 = 21 μM). In these conjugates the intercalators were attached *via* flexible polymethylene chains involving five to seven methyl groups. For a given intercalator the inhibitory effect was dependent on the linker structure. The dinucleotide-acridine conjugate **24** involving a neutral hexamethylene chain (IC 50 = 13.2 μM), was at least 7-fold more efficient than the conjugate **25** with the acridine attached *via* a bulky positively charged linker. The inhibitory activities of the perylene-dinucleotide conjugates **30** and **31** were also dependent on their structures. The attachment of the perylene *via* a phosphodiester bond and a nine atom linker (compound **31)** led to more potent compound (IC 50 = 1.8 μM) than its linkage *via* a three methylene tether to the β-anomeric position of an additional 2′-deoxyribose (compound **30**, IC 50 = 30 μM). The comparison of the inhibitory activities of conjugates **24**, **29** and **31** and those of the corresponding intercalator-linker derivatives (Fig. 4) showed that the activity of conjugates **24** and **29** were similar to those of the corresponding linker-intercalator compounds while that of **31** was due to the presence of both the dinucleotide and the intercalator. These results indicated that for conjugates **24** and **29**, intercalation probably occurred. However, the dinucleotide moiety was not in position favourable to alter efficiently the integrase activity while for the conjugate **31**, both the perylene unit and the dinucleotide were involved in the inhibitory effect. The removal of both the 2′-deoxyribose units and internucleotidic phosphodiester linkage led to inactive compounds **38** and **39**.

5. Conclusion

We report here the synthesis and anti-integrase activity of 38 new dinucleotide $^{5'}CA^{3'}$ analogues. The modified compounds can be classified into two series. The first one concerns the replacement of the D- 2′-deoxyribose by its L enantiomer, the N^4 alkylation of the cytosine, the modification of the internucleotidic linkage polarity and the replacement of the phosphodiester linkage by phosphorothioate and phosphoramidate bonds. Only a few compounds exhibited a moderate increase in the inhibitory effect as compared to that of the parent unmodified dinucleotide. In the second series, different linkers, ending with either hydroxyl or amino functions, and intercalators (acridine *via* neutral and positively charged linkers, three anthraquinone analogs, thiazole orange and two perylene derivatives) were attached to either the 5′- or the 3′-end of the natural dinucleotide. In the dinucleotide conjugate series two types of behaviour can be observed. 5′-Conjugation led to an increase in the inhibitory effect (IC 50 values ranging from 1.8 to 66 μM), except for the compounds involving amino groups or a positively charged linker, as compared to the activity of the unmodified dinucleotide. (IC 50 = 340 μM.). On the contrary, no improvement in the dinucleotide inhibitory effect was observed by 3′-conjugation. The strongest inhibitory effect was observed with the perylene-dinucleotide conjugate involving the longer linker attached to the terminal phosphate (IC 50 = 1.8 μM). A comparison of the inhibitory activities of the conjugates involving acridine, thiazole orange and perylene as well as those of the corresponding intercalator-linker derivatives indicated that only the activity of the conjugate involving the perylene attached to the terminal phosphate was due to the presence of both the dinucleotide and the intercalator. The activities of the other conjugates were similar to those of the corresponding intercalators. Removal of both the 2′-deoxyribose units and internucleotidic phosphodiester linkage led to inactive compounds.

6. Experimental

6.1. General methods

All chemicals were used as obtained unless otherwise stated. 5′-O-(4,4′-Dimethoxytrityl)–4–N-benzoyl-2′-deoxycytidine, 5′-O-(4,4′-Dimethoxytrityl)–6–N-benzoyl-2′-deoxyadenosine, 5′-O-(4,4′-Dimethoxytrityl)–4–N-benzoyl-2′-deoxycytidine-3′-H-phosphonate, 5′-O-(4,4′-Dimethoxytrityl)–6–N-benzoyl-2′-deoxyadenosine-3′-H-phosphonate, β-L-2′-deoxycytidine, β-L-2′-deoxyadenosine, adenine, thymine, cytosine and guanine were purchased from Distribio. 1-Aminoanthraquinone, 1,4-diaminoanthraquinone, 6-bromohexanoyl chloride, sodium iodide, benzoyl anhydride, benzoyl chloride, diethanolamine, *tert*-butyldiphenylsilyl chloride, trimethylsilyl-chloride, isobutyryl chloride, 1,3-diiodopropane, 1,6-dibromohexane, imidazole, phosphorus oxychloride, monomethoxytrityl chloride, dimethoxytrityl chloride, diethylenetriamine, ethyltrifluoroacetate, 3-bromopropanol,1,3-dibromopropane, carbon tetrachloride, ethylenediamine, 1,3-diaminopropane, 1,4-diaminobutane,1,5-diaminopentane, 1,6-diaminohexane, 6-aminohexanol, 1,3-propanediol, 1,12-dodecanediol, sodium iodide, phenol, 2-methoxy-6,9-dichloroacridine, diisopropylethylamine, sodium sulphate, 3-N,N-dimethylaminopropylamine, acetic anhydride, triphenylphosphine, carbon tetrabromide, pivaloyl chloride, 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, aluminium chloride, iodine, sodium hydrogenocarbonate were purchased from Aldrich. Acetic acid, triethylamine and sodium sulfate were purchased from Merck, 2-chloro-5, 6-benzo-1,3,2-dioxaphosphorin-4-one from Fluka, pyridine, dimethylformamide and dichloromethane from SDS, and acetonitrile from Labo-Standa. Analytical thin-layer chromatography (TLC) was performed on precoated alumina plates (Merck silica gel 60F 254 ref. 5554). For flash chromatography, Merck silica gel 60 (70–230 mesh) (ref. 7734) or Merck neutral alumina (Merck 5550) were used. Chelex 100 (100–200 mesh) resin was from Biorad. All 4,4′-dimethoxytrityl-containing substances were identified as orange-colored spots on TLC plates by spraying with a 10% perchloric acid solution. Amino-containing substances were identified as purple-colored spots on TLC plates by spraying with a 0.1% ninhydrin solution in n-butanol. Oligonucleotide syntheses were performed on an Expedite Nucleic Acid Synthesis system 8909 from Perseptive Biosystems. Reversed-phase chromatography analysis was performed on a 600 E (System Controller) equipped with a photodiode array detector (Waters 990) using a Lichrospher 100 RP18 (5 μm) column (125 mm × 4 mm) from Merck with a linear gradient of CH_3CN in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min. Reversed-phase chromatography purifications were performed on a 600 E (System Controller) equipped with a UV detector (VARIAN UV 50) using a Lichrospher 100 RP18 (10 μm) column (250 mm × 10 mm) from Merck with a linear gradient of CH_3CN in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 4 mL/min. Mass analysis ion-molecular weights of the oligonucleotides was performed by Electrospray Mass Spectroscopy using a Quattro II (Micromas) instrument. ^1H NMR spectra were recorded on a Varian Unity 500 Spectrometer. Absorption spectra were recorded with a Uvikon 860 spectrophotometer. The concentrations were determined using the following molar absorption coefficients [44]

$\varepsilon_{260} = 20\ 000\ M^{-1}\ cm^{-1}$ for dinucleotides **1**–**23**, **32**–**34** and for compounds **38** and **39**. For the dinucleotide intercalator conjugates **24**–**31** and **35**–**37** the ε_{260} values were considered to be the sum of the ε values of the dinucleotide and the intercalator molecules as follows: acridine-dinucleotide conjugates: **24**, **25**, and **35**–**37** $\varepsilon_{260} = 65\ 000\ M^{-1}\ cm^{-1}$; anthraquinone-dinucleotide conjugate **26**, $\varepsilon_{260} = 86\ 400\ M^{-1}\ cm^{-1}$; anthraquinone-dinucleotide conjugate **27**, $\varepsilon_{260} = 75\ 400\ M^{-1}\ cm^{-1}$; anthraquinone-dinucleotide conjugate **28**, $\varepsilon_{260} = 87\ 700\ M^{-1}\ cm^{-1}$; thiazole orange-dinucleotide conjugate **29**, $\varepsilon_{260} = 27\ 000\ M^{-1}\ cm^{-1}$; perylene-dinucleotide conjugates **30** and **31**, $\varepsilon_{260} = 60\ 000\ M^{-1}\ cm^{-1}$.

6.2. Chemistry

6.2.1. 2′-Deoxycytidylyl-(3′ → 5′)–2′–deoxyadenosine **1**

5′-O-(4,4′-Dimethoxytrityl)–4–N-benzoyl-2′-deoxycytidine, 3′-H-phosphonate, triethylammonium salt (1 mmol) and 3′-O-benzoyl-6-N-benzoyl-2′-deoxyadenosine (0.98 mmol) were solubilized in anhydrous pyridine (10 mL) and pivaloylchloride (3 mmol) was added dropwise. After a 10 min reaction under stirring, the mixture was diluted with CH_2Cl_2 (50 mL). Then, the organic phase was washed with a 1 M solution of $NaHCO_3$ (50 mL), dried over Na_2SO_4 and concentrated to dryness. The purification and isomer separation were performed by silica gel chromatography (on Merck 60H, 15 μm) using AcOEt/CH_3COOH (99.8:0.2, v/v) then AcOEt/Acetone/CH_3COOH (90:10:0.2, v/v/v) as eluent. Then, an iodine solution (30 mg, 118 μmol) in a pyridine/H_2O (10:1, v/v) mixture (2.54 mL) was added to the fully protected dinucleoside H-phosphonate (80 μmol) in anhydrous pyridine (1.4 mL) under stirring at rt. After 15 min, a solution of Na_2SO_3 (1% in water) was added to the reaction mixture until decoloration. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and the organic phase washed with a 0.2 M aqueous solution of $NaHCO_3$ (10 mL), dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was dissolved with an 80% acetic acid solution in water (2.5 mL). After 10 min, the orange solution was concentrated to dryness and the residue dissolved with a concentrated (28%) aqueous ammonia solution. The suspension was maintained for 6 h at 50 °C. After removal of the ammonia, the water solution was washed with ethyl acetate (3 × 10 mL) and filtered. Purification was performed by reversed-phase chromatography on a Lichrospher Merck 100 RP 18 column (10 μm, 250 mm 10 mm) with a linear gradient of CH_3CN in a 0.1 M triethylammonium buffer, pH 7, and a flow rate of 4 mL/min. ^{1}H NMR (500 MHz, D_2O): δ 1.58 [m, 1H, H-2′(C)], 2.23 [m, 1H, H-2′(C)], 2.60 [m, 1H, H-2′(A)], 2.87 [m, 1H, H-2′(A)], 3.63 (m, 2H, H-5′,5′(C), 4.04 (m, 3H, H-5′, 5′(A) H-4′(C), 4.23 [m, 1H, H-4′(A)], 4.52 [m, 1H, H-3′(C)], 4.78 (m, 1H, H-3′(A), 5.93 [d, $J = 7.55$ Hz, 1H, H-5(C)], 5.99 [dd, $J = 7.71$ Hz, $J = 7.74$ Hz, 1H, H-1′(C)], 6.48 [t, $J = 6.64$ Hz, 1H, H-1′(A)], 7.86 [d, $J = 7.56$ Hz, 1H, H-6(C)], 8.17 [s, 1H, H-2(A)], 8.40 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{19}H_{25}N_8O_9P$ $(M\text{-}H)^-$: 539.1, found: 539.3.

6.2.2. Dinucleotides **2** and **3** involving L-2′-deoxyribose units

Starting from commercial L-dC and L-dA nucleosides, the synthesis of these dinucleotides involving regular internucleotidic phosphodiester linkage was performed in solution *via* the H-phosphonate chemistry. The nucleoside protection (4,4′-dimethoxytrityl for the 5′-hydroxyl function and benzoyl group for 3′-hydroxyl function and exocyclic amine on cytosine and adenine) was achieved as reported for the protection of the corresponding natural D-nucleosides The nucleoside-H-phosphonate derivatives were obtained following a reported procedure using 2-chloro-5, 6-benzo-1, 3, 2-dioxaphosphorin-4-one as a phosphitilating agent [28]. The oxidation, deprotection and purification steps were performed as described above for the preparation of compound **1**.

L-2′-Deoxycytidylyl-(3′ → 5′)–2′–deoxyadenosine **2** ^{1}H NMR (500 MHz, D_2O): δ 1.59 [m, 1H, H-2′(C)], 2.23 [m, 1H, H-2′(C)], 2.60 [m, 1H, H-2′(A)], 2.87 [m, 1H, H-2′(A)], 3.58–3.70 [m, 2 H, H-5′,5′(C)], 4.04 [m, 3H, H-4′(C), H-5′, 5′(A)], 4.23 [m, 1H, H-4′(A)], 4.53 [m, 1H, H-3′(C)], 4.78 [m, 1H, H-3′(A)], 5.92 [d, $J = 7.53$ Hz, 1H, H-5(C)], 5.99 [t, $J = 6.94$ Hz, 1H, H-1′(C)], 6.42 [t, $J = 6.64$ Hz, 1H, H-1′(A)], 7.50 [d, $J = 7.56$ Hz, 1H, H-6(C)], 8.16 (s, 1H, H-2(A)], 8.40 [s, 1H, H-8(A)]. Mass analysis: (ESI) calcd for $C_{19}H_{25}N_8O_9P$ $(M\text{-}H)^-$: 539.1, found 539.1.

L-2′-Deoxyadenosinyl-(3′ → 5′)–2′–deoxycytidine **3** ^{1}H NMR (500 MHz, D_2O): δ 2.23 [m, 1H, H-2′(C)], 2.36 [m, 1H, H-2′(C)], 2.67–2.81 [m, 2H, H-2′,2′(A)], 3.82 [m, 2H, H-5′,5′(C)], 4.05–4.21 [m, 3H, H-5′,5′(A), H-4′(C)], 4.31 [m, 1H, H-4′(A)], 4.52 [m, 1H, H-3′(C)], 4.87 [m, 1H, H-3′(A)], 5.80 [d, $J = 7.56$ Hz,1H, H-5(C)], 6.18 [t, $J = 6.53$ Hz, 1H, H-1′(A)], 6.33 [t, $J = 6.76$ Hz, 1H, H-1′(C)], 7.75 [d, $J = 7.52$ Hz, 1H, H-6(C)], 8.09 [s, 1H, H-2(A)], 8.21 [s, 1H, H-8(A)]. Mass analysis (ESI) for $C_{19}H_{25}N_8O_9P$ (M-H): 539.1, found: 539.1.

6.2.3. Synthesis of the dinucleotides involving the alkylated cytosine (Scheme 1)

*6.2.3.1. General procedure for the monomethoxytritylation of the bis-amine **41**–**45**.* Bis-amines were dried separately by co-evaporation with pyridine, dissolved with pyridine and monomethoxytrityl chloride (0.1 eq) was added. After a 21 h reaction, the mixture was diluted with CH_2Cl_2, washed with a 0.5 M aqueous $NaHCO_3$ solution, dried over Na_2SO_4, filtered and concentrated to dryness. The orange-colored oils were purified by flash chromatography using a $CH_2Cl_2/CH_3OH/NEt_3$ (98:2:1, v/v/v) mixture as eluent to give the monotritylated bisamines **41**–**45** with yields ranging from 90 to 95%.

*6.2.3.2. General procedure for the preparation of the alkylated 2′-deoxycytidine derivatives **46**–**50**.* The triazolo derivative of the 4, 4′-dimethoxytrityl-2′-deoxyuridine protected at the 3′-end with a tertbutyldimethylsilyl group **40** was obtained following a previously reported procedure [29]. The latter (355 mg, 0.51 mmol) and the selected monomethoxytritylated bisamine **41**–**45** (3 eq) were dissolved in CH_3CN (4 mL). After a 15 h reaction, the mixture was concentrated to dryness and the residue dissolved with CH_2Cl_2. The organic phase was washed with water, dried and concentrated to dryness and the residue purified by flash chromatography using a $CH_2Cl_2/CH_3OH/NEt_3$ (98:1:1, v/v/v) mixture as eluent to give the fully protected 4-N-alkylated-2′-deoxyuridine derivatives. The latter (0.44 mmol) were then dissolved with a 1 M tetrabutyl ammonium fluoride solution in THF (5 mL). After a 2 h reaction at rt, the mixture was concentrated to dryness. The oily residue was dissolved with CH_2Cl_2 (50 mL), washed with a 0.5 M aqueous $NaHCO_3$ solution, dried, filtered and concentrated to dryness. The orange-colored oil was purified by flash chromatography using a CH_2Cl_2/CH_3OH (98:2, v/v) mixture as eluent to give the alkylated derivatives **46**–**50** with yields ranging from 42 to 68%.

5′-O-(4,4′-Dimethoxytrityl)-4-N-[2-(4-monomethoxytritylamino)ethyl]-2′-deoxycytidine **46** ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.25 (m, 1H, H-2′), 2.55 (m, 1H, H-2′), 3.39–3.62 (m, 6H, H-5′,5′, $2CH_2$), 3.78 (s, 9H, 3 OCH_3), 4.02 (m, 1H,H-4′), 4.50 (m, 1H, H-3′), 5.21 (m, 1H, OH-3′), 5.36 (d, $J = 6.93$ Hz, 1H, H-5), 6.34 (m, 1H, H-1′), 6.78–6.87 (m, 5H, ArH), 7.16–7.37 (m, 16H, ArH), 7.39–7.47 (m, 6H, ArH), 7.84 (d, $J = 6.58$ Hz, 1H, H-6). Mass analysis (ESI) calcd for $C_{52}H_{52}N_4O_7$ $(M + H)^+$: 845.4, found:845.3.

5′-O-(4,4′-Dimethoxytrityl)-4-N-[3-(4-monomethoxytritylamino)propyl]-2′-deoxycytidine **47** ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.73 (m, 2H, CH_2), 2.24 (m, 1H, H-2′), 2.56 (m, 1H, H-2′), 3.38–3.51 (m, 2H, H-5′,5′), 3.54–3.67 (m, 4H, $2CH_2$), 3.76 (s, 9H, 3 OCH_3), 4.02 (m, 1H, H-4′), 4.49 (m, 1H, H-3′), 5.29 (d, $J = 7.29$ Hz, 1H, H-5), 5.75 (m, 1H, OH-3′), 6.34 (m, 1H, H-1′), 6.78–6.86 (m, 5H, ArH),

7.15–7.47 (m, 22H, ArH), 7.77(d, $J = 7.24$ Hz, 1H, H-6). Mass analysis (ESI) calcd for $C_{53}H_{54}N_4O_7$ $(M+H)^+$: 859.4, found: 859.4.

5′-O-(4,4′-Dimethoxytrityl)-4-N-[4-(4-monomethoxytritylamino)butyl]-2′-deoxycytidine **48** ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.58 (m, 4H, $2CH_2$), 2.24 (m, 1H, H-2′), 2.55 (m, 1H, H-2′), 3.37–3.56 (m, 6H, H-5′,5′, $2CH_2$), 3.80 (s, 9H, 3 °OCH_3), 4.01 (m, 1H, H-4′), 4.49 (m,1H, H-3′), 4.92 (m, 1H, OH-3′), 5.25 (d, $J = 7.16$ Hz, 1H, H-5), 6.34 (m, 1H, H-1′), 6.79–6.87 (m, 5H, ArH), 7.15–7.48 (m, 22H, ArH), 7.77 (d, $J = 7.20$ Hz, 1H, H-6). Mass analysis (ESI) calcd for $C_{54}H_{56}N_4O_7$ $(M+H)^+$: 873.4, found: 873.4.

5′-O-(4,4′-Dimethoxytrityl)-4-N-[5-(4-monomethoxytritylamino)pentyl]-2′-deoxycytidine **49** ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.39 (m, 2H, CH_2), 1.47–1.63 (m, 4H, $2CH_2$), 2.25 (m, 1H, H-2′), 2.55 (m, 1H, H-2′), 3.37–3.56 (m, 6H, H-5′,5′, $2CH_2$), 3.80 (s, 9H, 3 °OCH_3), 4.01 (m, 1H, H-4′), 4.49 (m, 1H, H-3′), 4,74 (m, 1H, OH-3′), 5.28 (d, $J = 7.08$ Hz, 1H, H-5), 6.34 (m, 1 H, H-1′, 6.79–6.87 (m, 5H, ArH), 7.15–7.48 (m, 22H, ArH), 7.79 (d, $J = 7.08$ Hz, 1H, H-6). Mass analysis (ESI) calcd for $C_{55}H_{58}N_4O_7$ $(M+H)^+$: 887.4, found: 887.4.

5′-O-(4,4′-Dimethoxytrityl)-4-N-[6-(4-monomethoxytritylamino)hexyl]-2′-deoxycytidine **50** ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.32 (m, 4H, $2CH_2$), 1.56 (m, 4H, $2CH_2$), 2.26 (m, 1H, H-2′), 2.56 (m, 1H, H-2′), 3.38–3.53 (m, 6H, H-5′,5′, $2CH_2$), 3.80 (s, 9H, 3 OCH_3), 4.01 (m, 1H, H-4′), 4.49 (m, 1H, H-3′), 4.73 (m, 1H, OH-3′), 5.29 (d, $J = 7.17$ Hz, 1H, H-5), 6.34 (m, 1H, H-1′), 6.79–6.86 (m, 5H, ArH), 7.15–7.49 (m, 22H, ArH), 7.79 (d, $J = 7.11$ Hz, 1H, H-6). Mass analysis (ESI) calcd for $C_{56}H_{60}N_4O_7$ $(M+H)^+$: 901.5, found: 901.4.

6.2.3.3. General procedure for the preparation of phosphoramidites **51–55**

The corresponding phosphoramidite derivatives were obtained following a classical procedure using the commercially available phosphitilating reagent 2-cyanoethyldiisopropylchloro-phosphoramidite. Compounds **46–50** (0.23 mmol) were dried by co-evaporation with anhydrous pyridine (5 mL) then with anhydrous CH_3CN (5 mL, three times) and left in a dessicator under vacuum overnight. The next day, the dessicator was filled with argon before its opening. The residue was dissolved with 1,2-dichloroethane (5 mL) and diisopropylethylamine (0.16 mL, 0.12 g, 0.92 mmol) was added and then 2-cyanoethyldiisopropylchlorophosphoramidite (0.077 mL, 82 mg, 0.35 mmol) dropwise under stirring at rt. After a 1 h reaction, the mixture was diluted with cold ethyl acetate (30 mL). The organic phase was washed with a 0.5 M aqueous sodium bicarbonate solution (20 mL) and a 3 M aqueous sodium chloride solution (10 mL), dried and concentrated to dryness. The residue was purified on a silica gel column using CH_2Cl_2/AcOEt/NEt_3 (70:30:1, v/v/v) mixture as eluent. Yields 86–93%.

6.2.3.4. General procedure for the preparation of the modified dinucleotides involving the alkylated 2′- deoxycytidine derivatives **4–8**

In a round-stopped flask containing 3′O-benzoyl-4-N-benzoyl-2′-deoxyadenosine (20 mg, 35.5 μmol), a 0.5 M solution of tetrazole in CH_3CN (2 mL) and a 0.059 M solution in CH_3CN of the phosphoramidite derivative of the selected 4-N-alkylated-2′-deoxycytidine (35.4 μmol, 0.6 mL) were simultaneously added. After a 15 min reaction, 2.5 mL of the iodine solution used on the synthesizer were added. Then, after a 5 min reaction, the iodine excess was reduced by adding a 10% aqueous solution of $Na_2S_2O_3$. Next, the mixture was diluted with CH_2Cl_2 (15 mL) and the organic phase washed with a 0.5 M aqueous solution of $NaHCO_3$ (10 mL), filtered, dried and concentrated to dryness. The residue was dissolved with an 80% acetic acid solution in water (1 mL). After 10 min, the orange solution was concentrated to dryness and the residue dissolved with a concentrated (28%) aqueous ammonia solution (6 mL) and MeOH (3 mL). The mixture was maintained under stirring at 50 °C for 6 h. After removal of the ammonia, the residue was dissolved with water (4 mL) and the solution washed with ethyl acetate and filtered. Purification was performed by reversed-phase chromatography on a Lichrospher Merck 100 RP 18 column (10 μm, 250 mm × 10 mm) with a linear gradient of CH_3CN in a 0.1 M triethylammonium buffer, pH 7, and a flow rate of 4 mL/min to give the dinucleotides **4–8**.

4-N-(2-Aminoethyl)–2′–deoxycytidylyl-(3′→ 5′)–2′–deoxyadenosine **4** ^{1}H NMR (500 MHz, D_2O): δ 1.63 [m, 1H, H-2′(C)], 2.27 [m, 1H, H-2′(C)], 2.59 [m, 1H, H-2′(A)], 2.86 [m, 1H, H-2′(A)], 3.27 (m, 4H, $2CH_2$), 3.60–3.75 [m, 2H, H-5′,5′(C), 4.03 (m, 1H, H-4′(C)], 4.07 [m, 2H, H-5′, 5′(A)], 4.24 [m, 1H, H-4′(A)], 4.55 [m,1H, H-3′(C)], 4.75 [m,1H, H-3′(A)], 5.93 [d, $J = 7.55$ Hz, 1H, H-5(C)], 6.01 [dd, $J = 7.80$ Hz, $J = 6.10$ Hz, 1H, H-1′(C)], 6.43 [t, $J = 6.68$ Hz, 1H, H-1′(A)], 7.80 [d, $J = 7.28$ Hz, 1H, H-6(C)], 8.17 [s, 1H, H-2(A)], 8.42 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{21}H_{30}N_9O_9P$ $(M+H)^+$: 584.2, found: 584.3.

4-N-(3-Aminopropyl)–2′–deoxycytidylyl-(3′→ 5′)-2′-deoxyadenosine **5** ^{1}H NMR (500 MHz, D_2O): δ 1.64 [m, 1H, H-2′(C)], 1.96 (m, 2H, CH_2), 2.26 [m, 1H, H-2′(C)], 2.59 [m, 1H, H-2′(A)], 2.85 [m, 1H, H-2′(A)], 3.03 [m, 4H, $2CH_2$), 3.60–3.75 (m, 2H, H-5′,5′(C)], 4.02 [m, 1H, H-4′(C)), 4.07 (m, 2H, H-5′, 5′(A)], 4.23 [m, 1H, H-4′(A)], 4.55 [m,1H, H-3′(C)], 4.75 [m, 1H, H-3′(A)], 5.88 [d, $J = 7.59$ Hz,1H, H-5(C)], 6.01 [dd, $J = 7.62$ Hz, $J = 6.29$ Hz, 1H, H-1′(C)], 6,44 [t, $J = 6.67$ Hz, 1H, H-1′(A)], 7.42 [d, $J = 7.62$ Hz, 1H, H-6(C)], 8.17 [s, 1H, H-2(A)], 8.42 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{22}H_{32}N_9O_9P$ $(M+H)^+$: 598.2, found: 598.3.

4-N-(4-Aminobutyl)-2′-deoxycytidylyl-(3′→ 5′)-2′-deoxyadenosine **6** ^{1}NMR (500 MHz, D_2O): δ 1.59–1.76 [m, 5H, H-2′(C), $2CH_2$)], 2.24 [m, 1H, H-2′(C)], 2.59 [m, 1H, H-2′(A)], 2.85 [m, 1H, H-2′(A)], 3.05 (m, 4H, $2CH_2$), 3.60–3.71 [m, 2H, H-5′,5′(C)], 4.01 [m, 1H, H-4′(C)], 4.07 [m, 2H, H-5′, 5′(A)], 4.23 [m, 1H, H-4′ (A)], 4.54 [m,1H, H-3′(C)], 4.78 [m, 1H, H-3′(A)], 5.83 [d, $J = 7.60$ Hz, 1H, H-5(C)], 6.02 [dd, $J = 7.71$ Hz, $J = 6.18$ Hz, 1H, H-1′(C)], 6.43 [t, $J = 6.64$ Hz, 1H, H-1′(A)], 7.37 [d, $J = 7.62$ Hz, 1H, H-6(C)], 8.15 [s, 1H, H-2(A)], 8.42 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{23}H_{34}N_9O_9P$ $(M+H)^+$: 612.2, found: 612.2.

4-N-(5-Aminopentyl)-2′-deoxycytidylyl-(3′→ 5′)-2′-deoxyadenosine **7** ^{1}H NMR (500 MHz, D_2O): δ 1.44 (m, 2H, CH_2), 1.64 (m, 4H, $2CH_2$), 1.71 [m, 1H, H-2′(C)], 2.23 [m, 1H, H-2′(C)], 2.60 [m, 1H, H-2′(A)], 2.85 [m, 1H, H-2′(A)), 3.0 (m, 4H, $2CH_2$), 3.60–3.72 [m, 2H, H-5′,5′(C)], 4.01 [m, 1H, H-4′(C)], 4.07 [m, 2H, H-5′,5′(A)], 4.23 [m, 1H, H-4′(A)], 4.54 [m, 1H, H-3′(C)], 4.78 [m, 1H, H-3′(A)], 5.82 [d, $J = 7.57$ Hz,1H, H-5(C)], 6.02 [m, 1H, H-1′(C))], 6.43 [t, $J = 6.61$ Hz, 1H, H-1′(A)], 7.36 [d, $J = 7.61$ Hz, 1H, H-6(C)], 8.14 [s, 1H, H-2(A)], 8.41 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{24}H_{36}N_9O_9P$ $(M+H)^+$: 626.2, found: 626.4.

4-N-(6-Aminohexyl)-2′-deoxycytidylyl-(3′→ 5′)-2′-deoxyadenosine **8** ^{1}H NMR (500 MHz, D_2O): δ 1.40 (m, 4H, $2CH_2$), 1.56–170 [m, 5H, $2CH_2$, H-2′(C)], 2.23 [m, 1H, H-2′(C)], 2.59 [m, 1H, H-2′(A)], 2.85 [m, 1H, H-2′(A)], 2.99 (m, 4H, $2CH_2$), 3.60–3.71 [m, 2H, H-5′,5′(C)], 4.01 [m, 1H, H-4′(C)], 4.07 [m, 2H, H-5′,5′(A)], 4.23 [m, 1H, H-4′(A)], 4.54 [m, 1H, H-3′(C)], 4.77 [m, 1H, H-3′(A)], 5.81 [d, $J = 7.60$ Hz, 1H, H-5(C)], 6.02 [dd, $J = 7.66$ Hz, $J = 6.23$ Hz, 1H, H-1′(C)], 6.42 [t, $J = 6.63$ Hz, 1H, H-1′(A)], 7.35 [d, $J = 7.63$ Hz, 1H, H-6(C)], 8.13 [s, 1H, H-2(A)], 8.41 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{25}H_{38}N_9O_9P$ $(M+H)^+$: 640.3, found: 640.3.

6.2.3.5. Preparation of the dinucleotides involving guanidylated cytosine **9–11**

Modified dinucleotides **6–8** (50 DO, 2.5 μmol) and 1*H*-pyrazole-1-carboxamidine hydrochloride (30 mg, 205 μmol) were dissolved in 1 M aqueous Na_2CO_3 (1.8 mL). After an 18 h reaction, the crude guanidylated dinucleotides were purified by reversed-phase

chromatography. The hydrophobicity of the guanidylated dinucleotides **9–11** showed a slight increase as compared to that of the corresponding aminated compounds **6–8**. After reversed-phase purification, the modified dinucleotides were obtained with 64–68% yields and their mass was confirmed by ESI mass spectrometry.

4-N-(4-Guanidinobutyl)-2′-deoxycytidylyl-(3′→ 5′)-2′-deoxyadenosine **9** Mass analysis (ESI) calcd for $C_{24}H_{36}N_{11}O_9P$ (M-H)$^-$: 652.2, found: 652.3.

4-N-(5-Guanidinopentyl)-2′-deoxycytidylyl-(3′→ 5′)-2′-deoxyadenosine **10** Mass analysis (ESI) calcd for $C_{25}H_{38}N_{11}O_9P$ (M-H)$^-$: 666.3, found: 666.3.

4-N-(6-Guanidinohexyl)-2′-deoxycytidylyl-(3′→ 5′)-2′-deoxyadenosine **11** Mass analysis (ESI) calcd for $C_{26}H_{40}N_{11}O_9P$ (M-H)$^-$: 680.3, found: 680.3.

6.2.4. Preparation of the dinucleosides **12** and **13** involving the phosphodiester linkage with modified polarities

The synthesis of the dinucleotide $^{5'}$CpA$^{5'}$ and $^{3'}$CpA$^{3'}$ involving internucleotidic linkages with modified polarities was also performed in solution via H-phosphonate chemistry. The fully protected dinucleotide-H-phosphonate $^{5'}$Cp(H)A$^{5'}$ was obtained by reaction of commercially available 5′-O-(4,4′-dimethoxytrityl)-4-N-benzoyl-2′-deoxycytidine-3′-H-phosphonate, (triethylammonium salt) and 5′-O-(4,4′-dimethoxytrityl-6-N-benzoyl-2′-deoxyadenosine in the presence of pivaloyl chloride. Purification was performed by silica gel chromatography (on Merck 60 H, 15 μm) using AcOEt/Acetone/CH_3COOH (60:40:0.2, v/v/v) as eluent. The fully protected dinucleotide-H-phosphonate $^{3'}$Cp(H)A$^{3'}$ was obtained by using commercially available 4-N-benzoyl- 3′-O-benzoyl-2′-deoxycytidine and 6-N-benzoyl-3′-O-benzoyl-2′-deoxyadenosine and proceeded as follows. The 5′-H-phosphonate derivative of 4-N-benzoyl-3′-O-benzoyl-2′-deoxycytidine was obtained following a reported procedure using 2-chloro-5,6-benzo-1,3,2-dioxaphosphorin-4-one as phosphitilating agent. The 5′-H-phosphonate derivative was then reacted with the 5′-hydroxyl function of the 6-N-benzoyl-3′-O-benzoyl-2′-deoxyadenosine in the presence of pivaloylchloride. Purification was also performed by silica gel chromatography (on Merck 60 H, 15 μm) using AcOEt/CH_3COOH (99.8:0.2, v/v/v) as eluent. Then, the oxidation step was performed as described above for the preparation of compound **1.** The deprotection of the dinucleotide-phosphodiester $^{5'}$CpA$^{5'}$ was achieved as reported for the natural dinucleotide **1** while for the dinucleotide-phosphodiester $^{3'}$CpA$^{3'}$, only the ammonia treatment was required. Purification of the crude dinucleotides **12** and **13** was also achieved as reported for compound **1**.

2′-Deoxycytidylyl-(5′ → 5′)-2′-deoxyadenosine **12** ^{1}H NMR (500 MHz, D_2O):δ 2.04 [m, 1H, H-2′(C)], 2.31 (m, 1H, H-2′(C)), 2.61 [m,1H, H-2′ (A)], 2.83 [m, 1H, H-2′ (A)], 3.89–4.00 [m,2H, H-5′,5′ (C)], 4.07 [m, 3H, H-5′,5′,(A), H-4′ (C)], 4.24 [m,1H, H-4′(A)], 4.37 [m, 1H, H-3′ (C)], 4.72 [m, 1H, 3′(A)], 5.63 [d, J = 7.51 Hz, 1H, H-5(C)], 6.12 [t, J = 6.56 Hz, 1H, H-1′(C)], 6.40 [t, J = 6.64 Hz, 1H, H-1′(A)], 7.52 [d, J = 7.54 Hz, 1H, H-6(C)], 8.14 [s, 1H, H-2(A)], 8.30 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{19}H_{25}N_8O_9P$ (M-H)$^-$: 539.1, found: 539.2.

2′-Deoxycytidylyl-(3′ → 3′)-2′-deoxyadenosine **13** ^{1}H NMR (500 MHz, D_2O): δ 2.37 [m, 1H, H-2′(C)], 2.64 [m, 1H, H-2′(C)], 2.74 [m, 1H, H-2′(A)], 2.91 [m, 1H, H-2′(A)], 3.84 [m, 4H, H-5′,5′(C), H-5′,5′(A)], 4.27 [m, 1H, H-4′(C)], 4.38 [m, 1H, H-4′(A)], 4.83 [m, 1H, H-3′(C)], 4.99 [m, 1H, H-3′(A)], 6.06 [d, J = 7.57 Hz, 1H, H-5(C)], 6.29 [t, J = 6.83 Hz, 1H, H-1′(C)], 6.48 [dd, J = 8.21 Hz, J = 6.01 Hz, 1H, H-1′(A)], 7.86 [d, J = 7.64 Hz, 1H, H-6(C)], 8.20 [s, 1H, H-2(A)], 8.32 [s, 1H, H-8(A)]. Mass analysis (ESI) calcd for $C_{19}H_{25}N_8O_9P$ (M-H)$^-$: 539.1, found: 539.3.

6.2.5. General procedure for the preparation of the modified dinucleosides involving the phosphorothioate linkage **14–16** (Scheme 2)

Elemental sulphur (40 mg) and each fully protected dinucleoside H-phosphonate with 5′–3′, 5′–5′ and 3′–3′ polarities (80 μmol) were dissolved in a pyridine/CS_2 (40:60, v/v) mixture (2.5 mL) at rt. After 2 h, the reaction mixture was concentrated to dryness, deprotected and purified as reported above for dinucleotides **1**, **12** and **13**.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine phosphorothioate **14** Mass analysis (ESI) calcd for $C_{19}H_{25}N_8O_8PS$ (M-H)$^-$: 555.1 found: 555.1.

2′-Deoxycytidylyl-(5′ → 5′)-2′-deoxyadenosine phosphorothioate **15** Mass analysis (ESI) calcd for $C_{19}H_{25}N_8O_8PS$ (M-H)$^-$: 555.1, found: 555.2.

2′-Deoxycytidylyl-(3′ → 3′)-2′-deoxyadenosine phosphorothioate **16** Mass analysis (ESI) calcd for $C_{19}H_{25}N_8O_8PS$ (M-H)$^-$: 555.1, found: 555.0.

6.2.6. General procedure for the preparation of the modified dinucleotides involving the 1, 4-diaminobutane linker attached to the internucleotidic linkage **17–19** (Scheme 2)

A solution of N-monomethoxytritylbutanediamine (0.75 mmol) in anhydrous pyridine was added to each fully protected dinucleoside H-phosphonate with 5′–3′, 5′–5′ and 3′–3′ polarities (0.142 mmol) dissolved in CCl_4 (1.5 mL) at rt. After a 15 h reaction, the mixture was concentrated to dryness, purified by flash chromatography using a CH_2Cl_2/MeOH (95:5, v/v) mixture as eluent. The obtained dinucleotides were deprotected and purified as reported above for dinucleotides **1**, **12** and **13**.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-N-(4-aminobutyl)phosphoramidate **17** (5′ → 3′)-Cp[NH$(CH_2)_4NH_2$]A. Mass analysis (ESI) calcd for $C_{23}H_{35}N_{10}O_8P$ (M + H)$^+$: 611.2. found: 609.3.

2′-Deoxycytidylyl-(5′ → 5′)-2′-deoxyadenosine-N-(4-aminobutyl)phosphoramidate **18** (5′ → 5′)- Cp[NH$(CH_2)_4NH_2$]A. Mass analysis (ESI) calcd for $C_{23}H_{35}N_{10}O_8P$ (M + H)$^+$: 611.2. found: 611.2.

2′-Deoxycytidylyl-(3′ → 3′)-2′-deoxyadenosine-N-(4-aminobutyl)phosphoramidate **19** (3′ → 3′)-Cp[NH$(CH_2)_4NH_2$]A. Mass analysis (ESI) calcd for $C_{23}H_{35}N_{10}O_8P$ (M + H)$^+$: 611.2. found: 611.3.

6.2.7. General procedure for the preparation of the modified dinucleotides involving the guanidylated diaminobutane linker attached to the internucleotidic position **20–22**

Starting from dinucleotides **17–19**, the procedure was identical to the one reported for the preparation of dinucleotides **9–11**.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-N-(4-guanidinobutyl)phosphoramidate **20** (5′ → 3′)-Cp[NH$(CH_2)_4$NH_Gua]A: Mass analysis (ESI) calcd for $C_{24}H_{37}N_{12}O_8P$ (M + H)$^+$: 653.3. found: 653.2.

2′-Deoxycytidylyl-(5′ → 5′)-2′-deoxyadenosine-N-(4-guanidinobutyl)phosphoramidate **21** (5′ → 5′)- Cp[NH$(CH_2)_4$NH_Gua]A: Mass analysis (ESI) calcd for $C_{24}H_{37}N_{12}O_8P$ (M + H)$^+$: 653.3. found: 654.2.

2′-Deoxycytidylyl-(3′ → 3′)-2′-deoxyadenosine-N-(4-guanidinobutyl)phosphoramidate **22** (3′ → 3′)-Cp[NH$(CH_2)_4$NHGua]A: Mass analysis (ESI) calcd for $C_{24}H_{37}N_{12}O_8P$ (M + H)$^+$: 653.3. found: 653.3.

6.2.8. Synthesis of the conjugates

6.2.8.1. Synthesis of the modified dinucleotide **23**

The synthesis of the dinucleotide 5′-phosphorothioate S-p-$^{5'}$CpA$^{3'}$ **56** was performed on solid-phase following a procedure

adapted from our previous reports [31]. 67 DO (21%). Mass analysis (ESI) calcd for $C_{19}H_{26}N_8O_{11}P_2S$ $(M\text{-}H)^-$: 635.1. found: 635.3.

Synthesis of the 1,7-bis-trifluoroacetyl- 4-(3-iodopropyl)hepta-1,4,7-triazane linker **59** (Scheme 4) 1,7-bis-Trifluoroacetyl-hepta-1,4,7-triazane [27] (2 g, 0. 24 mmol), diiodopropane (4.3 g, 0.72 mmol), and NEt_3 (1 mL, 0.36 mmol) were dissolved with CH_3CN and the mixture was heated at 65 °C for 16 h. Then, the mixture was concentrated to dryness and the residue purified by chromatography on a silica gel column using a CH_2Cl_2/acetone/H_2O (95:5:1 to 85:15:1, v/v/v) mixture as eluent. White solid. 0.6 g (27%). ^1H NMR (500 MHz, $CDCl_3$): δ 1.89 (m, 2H, $\underline{CH_2}CH_2I$), 2.60 (t, $J = 6.7$ Hz, 2H, $N\underline{CH_2}(CH_2)_2I$) 2.68 (t, $J = 5.8$ Hz, 4H, NCH_2), 3.18 (t, $J = 6.4$ Hz, 2H, $IC\underline{H_2}$), 3.46 (m, 4H, $HN\underline{CH_2}$), 6.90 (s br, 2H, NH). Mass analysis (ESI) for $C_{11}H_{16}F_6IN_3O_2$. $(M+H)^+$: 464.0, found 464.1.

Synthesis of the modified dinucleotide **23** Compound **59** (35 mg, 75 μmol) was added to the dinucleotide **56** (7.8 μmol, 150 OD) and then 18-crown-6 (30 mg) in MeOH (1.5 mL). After 7 h stirring at 40 °C, aqueous ammonia (28%) solution was added and the mixture was left to stand at rt for 48 h. The mixture was then concentrated to dryness. The residue was dissolved with water (2 ml). The aqueous phase was extracted with CH_2Cl_2 and the crude dinucleotide **23** purified by reversed-phase chromatography. Mass analysis (ESI) calcd for $C_{26}H_{43}N_{11}O_{11}P_2S$ $(M\text{-}H)^-$: 778.2 found: 778.3.

*6.2.8.2. Synthesis of the dinucleotide-acridine conjugates **24**, **25** and **35–37** (Scheme 3)*

*6.2.8.2.1. 2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-5′-[6-(6-chloro-2-methoxy-9-acridinylamino) hexyl] phosphate **24***

The synthesis of the dinucleotide acridine conjugate **24** was performed *via* phosphoramidite chemistry following our previously reported procedure [35]. Mass analysis (ESI) calcd for $C_{39}H_{46}Cl\,N_{10}O_{13}P_2$ $(M\text{-}H)^-$: 959.3. found: 959.1 and 961.2.

*6.2.8.2.2. Synthesis of the dinucleotide-acridine conjugates **35** and **36***

The synthesis of the dinucleotide 3′-phosphorothioate $^{5'}CpA^{3'}$p-S **57** was performed on solid-phase using our previously reported support [31] 94 OD (3 mg, 63%). Mass analysis (ESI) calcd for $C_{19}H_{26}N_8O_{11}P_2S$ $(M\text{-}H)^-$: 635.1, found: 635.3. The synthesis of 6-chloro-2-methoxy-9-(6-bromohexylamino)acridine was obtained as previously reported [37]. The synthesis of the 6-chloro-2-methoxy-9-(3-bromopropylamino)acridine was performed following the same strategy. 6-Aminohexanol was replaced by 3-aminopropanol.

6-Chloro-2-methoxy-9-(3-hydroxypropylamino)acridine ^1H NMR (500 MHz, DMSO-d_6): δ 1.87 (m, 2H, CH_2), 3.29 (s, 1H, NH), 3.53 (m, 2H,CH_2N), 3.84 (m, 2H, CH_2O), 3.93 (s, 3H,OCH_3), 4.65 (t, $J = 4.84$ Hz, 1H, OH), 7.32(dd, $J = 9.30$ Hz, $J = 2.10$ Hz, 1H, H-3), 7.42 (dd, $J = 9.33$ Hz, $J = 2.62$ Hz, 1H, H-7), 7.60 (d, $J = 2.51$ Hz, 1H, H-5), 7.84 (d, $J = 9.32$ Hz, 1H, H-4), 7.87 (d, $J = 2.10$ Hz, 1H,H-1), 8.36 (d, $J = 9.33$ Hz, 1H, H-8). Mass analysis (ESI) calcd for $C_{17}H17ClN_2O_2$ $(M+H)^+$: 317.1, found: 317.1, 319.1 $(M+H{+}2)^+$.

6-Chloro-2-methoxy-9-(3-bromopropylamino)acridine ^1H NMR (500 MHz, $CDCl_3$): δ 2.51 (m, 2H, CH_2), 3.63 (t, $J = 6.06$ Hz, 2H, CH_2Br), 3.89 (s, 1H, NH), 4.02 (s, 3H, OCH_3), 4.08 (t, $J = 6.88$ Hz, 2H, CH_2N), 7.23 (m, 1H, H-3), 7.28 (m, 1H, H-7), 7.38 (d, $J = 2.44$ Hz, 1H, H-5), 7.92 (d, $J = 9.31$ Hz, 1H, H-4), 8.08 d, $J = 2.04$ Hz, 1H, H-1), 8.10 (d, $J = 9.28$ Hz, 1H, H-8). Mass analysis (ESI) calcd for $C_{17}H_{16}BrClN_2O$ $(M+H)^+$: 379.0, found: 379.0, 381.2 $(M+H{+}2)^+$, 383.0 $(M+H{+}4)^+$.

Synthesis of the dinucleotide acridine conjugates **35** and **36** A water solution (1 mM, 1 mL) of the dinucleotide 3′-phosphorothioate was passed over Chelex 100 resin (100–200 mesh, Biorad) and lyophilised. The solid was dissolved with a 15 mM solution of 18-crown-6 in MeOH (1 mL) and a 10 mM solution of 2-methoxy-6-chloro-9-(3-bromopropylamino)acridine [or 2-methoxy-6-chloro-9-(6-bromohexylylamino)acridine] in MeOH (1 mL) was added. The reaction mixture was protected from the light and maintained under stirring for 24 at rt. A 1 M solution of phosphate buffer pH 6 (1.5 mL) was added and the mixture was extracted with CH_2Cl_2. The aqueous solution was concentrated and purified by reversed-phase chromatography.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-3′-[3-(6-chloro-2-methoxy-9-acridinylamino)propyl]thiophosphate **35** 15 OD (23%). Mass analysis (ESI) calcd for $C_{36}H_{41}ClN_{10}O_{12}P_2S$ $(M\text{-}H)^-$: 933,2, found 933.3.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-3′-[6-(6-chloro-2-methoxy-9-acridinylamino)hexyl]thiophosphate **36** 6 OD (9%). Mass analysis (ESI) calcd for $C_{39}H_{47}ClN_{10}O_{12}P_2S$ $(M\text{-}H)^-$: 975.2, found, 975.4.

*6.2.8.2.3. Synthesis of the dinucleotide acridine conjugates **25** and **37***

*6.2.8.2.3.1. Synthesis of the acridine linker derivative **62***

6-Chloro-2-methoxy-9-(3-dimethylaminopropylamino)-acridine **61** 6,9-Dichloro-2-methoxyacridine (0.5 g, 1.8 mmol), 3-dimethylaminopropylamine (0.45 mL, 3.58 mmol) and phenol (2 g) were heated at 100 °C for 2 h. After cooling to rt, MeOH (2.5 mL) was added and the resulting mixture poured into a 2 M NaOH water solution (20 mL). The solid obtained was filtered, dried and purified by flash chromatography on a neutral alumina column (63–200 μm, activity 1) using a CH_2Cl_2/MeOH, (98:2, v/v) mixture as eluent. Yellow solid. 368 mg (60%). ^1H NMR (500 MHz, $CDCl_3$): 1.91 (m, 2H, CH_2), 2.41 (s, 6H, $N(CH_3)_2$), 2.65 (t, $J = 5.50$ Hz, 2H, CH_2N), 3.97 (s, 3H, OCH_3), 4.0 (m, 2H, CH_2NH), 7.22 (dd, $J = 9.28$ Hz, $J = 2.03$ Hz, 1H, H-7), 7.30 (d, $J = 2.56$ Hz, 1H, H-1), 7.40 (dd, $J = 9.37$ Hz, $J = 2.62$ Hz, 1H, H-3), 7.97 (d, $J = 9.36$ Hz, 1H, H-4), 8.02 (d, $J = 1.93$ Hz, 1H, H-5), 8.09 (d, $J = 9.29$ Hz, 1H, H-8). Mass analysis (ESI) calcd for $C_{19}H_{22}ClN_3O$ $(M+H)^+$: 344.2, found: 344.3, 346.3 $(M+H{+}2)^+$.

3-Bromopropyl-3-(6-chloro-2-methoxy-9-acridinylamino)propyldimethylammonium bromide **62** (Scheme 5) Compound **61** (100 mg, 0.291 mmol), 1,3-dibromopropane (0.6 mL, 5.79 mmol) and CH_3CN (7 mL) were heated at 80 °C for 2 h. Then, the mixture was concentrated to dryness and the residue dissolved with water (20 mL). The excess of 1, 3-dibromopropane was removed by extraction with CH_2Cl_2 (4 × 15 mL). The aqueous solution was lyophilised and the residue purified by flash chromatography on a neutral alumina column (63–200 μm, activity 1) using a CH_2Cl_2/MeOH, (97:3, v/v) mixture as eluent. Yellow solid. 108 mg (79%). ^1H NMR (500 MHz, D_2O): δ 1.78 (m, 2H, CH_2), 1.88 (m, 2H, CH_2), 2.94 (s, 6H, $N(CH_3)_2$), 3.11 (m, 2H, CH_2), 3.20 (m, 2H, CH_2), 3.42 (t, $J = 5.79$ Hz, 2H, CH_2Br), 3.51 (t, $J = 5.71$ Hz, 2H, CH_2N), 3.85 (s, 3H, OCH_3), 6.75 (m, 1H, ArH), 7.07 (m, 1H, ArH), 7.19 (m, 1H, ArH), 7.45 (m, 2H, ArH), 7.58 (m 1H, ArH). Mass analysis (ESI) calcd for $C_{22}H_{28}BrClN_3O$ M^+: 464.1, found: 464.2, 466.1 $(M+2)^+$, 468.2 $(M+4)^+$.

*6.2.8.2.3.2. Synthesis of the dinucleotide acridine conjugates **25** and **37***

The dinucleotide 5′-phosphorothioate **56** (14 OD, 0.70 μmol) (or dinucleotide 3′-phosphorothioate **57**, 24 OD, 1.2 μmol) was dissolved with an 18.9 mM solution of 18-crown-6 in MeOH (12 eq) and a 21.1 mM solution of **62** in MeOH (7 eq) was added. The reaction mixture was protected from the light and maintained under stirring for 40 h at rt. Then, the solution was concentrated and a 1 M solution of phosphate buffer pH 6 (1.5 mL) was added

and the mixture was extracted with CH_2Cl_2. The aqueous solution was concentrated and purified by reversed-phase chromatography. Compound **25**: 25 OD (59%) λmax = 266, 344, 424 and 446 nm. Mass analysis (ESI) calcd for $C_{41}H_{53}ClN_{11}O_{12}P_2S$, M^+: 1020.3, found 1019.4. Compound **37**: 15 OD (21%) λ_{max} = 266, 345, 423 and 446 nm. Mass analysis (ESI) calcd for $C_{41}H_{53}ClN_{11}O_{12}P_2S$, M^+: 1020.3, found 1020.4.

6.2.8.3. Synthesis of the dinucleotide anthraquinone conjugates ***26 –28***

6.2.8.3.1. Synthesis of the anthraquinone-linker derivatives ***67*** *and* ***68*** *(Scheme 6)*

1-(6-Bromohexanoylamino)anthracene-9,10-dione **65** 6-Bromohexanoylchloride (0.8 mL, 5.4 mmol) was added dropwise, over 15 min to a refluxed mixture of 1-aminoanthraquinone **63** (1 g, 4.5 mmol) and pyridine (catalytic amount) in anhydrous benzene (25 mL). Refluxing conditions were maintained for 2 h. The reaction mixture was cooled to rt and filtered. The residue was washed with CH_2Cl_2 and the organic phase concentrated. The yellow residue was dissolved with CH_2Cl_2 and the organic phase was washed with $NaHCO_3$ and then sat. NaCl, was dried over Na_2SO_4 and concentrated. The residue was purified on a silica gel column using a CH_2Cl_2/MeOH (99:1 to 98.5/1.5, v/v) mixture as eluent. Yellow solid. 1.7 g (94%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.48 (m,2H, CH_2), 1.70 (m, 2H, CH_2), 1.86 (m, 2H, CH_2), 2.53 (t, J = 7 Hz, 2H, CH_2C = O), 3.54 (t, J = 6 Hz, 2H, CH_2Br), 7.81–7.88 (m, 4H, H-3, H-4, H-6, H-7), 8.15 (d, J = 7 Hz, 1H, H-8), 8.21 (d, J = 7 Hz, 1H, H-5), 8.95 (d, J = 9 Hz, 1H, H-2), 12.0 (s, 1H, NH). Mass analysis (ESI) m/z calcd for $C_{20}H_{18}BrNO_3$, $(M+H)^+$: 400.0, found 400.1.

1-(6-Iodohexanoylamino)anthracene-9-10-dione **67** 1-(6-Bromohexanoylamino)anthracene-9-10-dione **65** (0.4 g, 1 mmol), NaI (0.75 g, 5 mmol), $NaHCO_3$ (0.42 g, 5 mmol) and acetone (25 mL) were refluxed for 8 h. Then, the mixture was concentrated to dryness and the residue dissolved with CH_2Cl_2 (60 mL). The organic phase was washed with H_2O, dried over Na_2SO_4 and concentrated. The residue was purified on a silica gel column using a CH_2Cl_2/MeOH (98:2, v/v) mixture as eluent. Yellow solid. 0.42 g (95%).

^{1}H NMR (500 MHz, DMSO-d_6): 1.44 (m, 2H, CH_2), 1.70 (m, 2 H, CH_2), 1.87 (m, 2H, CH_2), 2.53 (t, J = 7 Hz, 2H, CH_2C = O), 3.28 (t, J = 7 Hz, 2H, CH_2I), 7.82–7.89 (m, 4H, H-3, H-4, H-6, H-7), 8.17 (d, J = 7 Hz, 1H, H-8), 8.24 (d, J = 7 Hz, 1H, H-5), 8.97 (d, J = 9 Hz, 1H, H-2), 12.0 (s, 1H, NH). Mass analysis (ESI) m/z calcd for $C_{20}H_{18}INO_3$. $(M+H)^+$: 448.0, found 448.1.

4-Amino-1-(6-bromohexanoylamino)anthracene-9,10-dione **66** 6-Bromohexanoylchloride (0.4 mL, 2.5 mmol) was added dropwise, over 15 min, to a refluxed mixture of 1-4-diaminoanthraquinone (2 g, 8.4 mmol) and pyridine (catalytic amount) in anhydrous benzene (50 mL). Refluxing conditions were maintained for 4 h. The reaction mixture was cooled to rt and filtered. The residue was washed with toluene and the organic phase concentrated. The purple residue was dissolved with CH_2Cl_2 and the organic phase was washed with $NaHCO_3$ and then sat. NaCl, dried over Na_2SO_4 and concentrated. The residue was purified on a silica gel column using a CH_2Cl_2/MeOH (98:2 to 97.5/2.5, v/v) mixture as eluent. Purple solid. 0.8 g (80%). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 1.46 (m, 2 H, CH_2), 1.68 (m, 2H, CH_2), 1.85 (m, 2H, CH_2), 2.46 (t, J = 7 Hz, 2H, CH_2C = O), 3.53 (t, J = 6.5 Hz, 2 H, CH_2Br), 7.28 (dd, J = 9 Hz, J = 1 Hz, 1H, H-3), 7.85 (m, 2H, H-6, H-7), 8.17 (dd, J = 7.5 Hz, J = 1 Hz, 2H, H-5, H-8), 8.69 (dd, J = 10 Hz, J = 0.5 Hz, 1H, H-2) 12.29 (s, 1H, NH). Mass analysis (ESI) m/z calcd for $C_{20}H_{19}BrN_2O_3$, $(M+H)^+$: 415.1, found 415.2.

4-Amino-1-(6-iodohexanoylamino)anthracene-9,10-dione **68** 4-Amino-1-(6-bromohexanoylamino)anthracene-9-10-dione **66** (0.4 g, 1 mmol), NaI (0.75 g, 5 mmol), $NaHCO_3$ (0.42 g, 5 mmol) and acetone (25 mL) were refluxed for 14 h. Then, the mixture was concentrated to dryness and the residue dissolved with CH_2Cl_2 (60 mL). The organic phase was washed with H_2O (3 × 20 mL), dried over Na_2SO_4 and concentrated. The residue was purified on a silica gel column using a CH_2Cl_2/MeOH (99:1 to 98.5:1.5, v/v) mixture as eluent. Purple solid. 0.42 g (70%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.42 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 1.81 (m, 2H, CH_2), 2.42 (t, J = 6.75 Hz, 2H, CH_2C = O), 3.28 (t, J = 6.75 Hz, 2H, CH_2I), 7.23 (d, J = 9 Hz, 1 H, H-3), 7.74–7.88 (m, 2 H, H-6, H-7), 8.11 (m, 2H, H-5, H-8) 8.64 (d, J = 9.5 Hz, 1H, H-2), 12.13 (s, 1H, NH). Mass analysis (ESI) m/z calcd for $C_{20}H_{19}IN_2O_3$. $(M+H)^+$ = 464.0, found 464.1.

6.2.8.3.2. Synthesis of anthraquinone-linker derivative 72; (Scheme 7)

Anthracene- 2,7- dicarboxylic acid -9,10-dione **69** was obtained as previously reported [22]. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.29 (d, J = 8.5 Hz, 2H, H-3, H-6), 8.38 (d, J = 8.5 Hz, 2H, H-4, H-5), 8.65 (s, 2H, H-1, H-8). Mass analysis (ESI) calcd for $C_{16}H_8O_6$. $(M-H)^-$ = 295.0, found 294.6.

1-O-Tertbutyldiphenylsilanyl-6-hexylamine ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.06 [s, 9H, $C(CH_3)_3$], 1.26–1.46 (m, 8H, CH_2, NH_2), 1.58 (m, 2H, CH_2), 2.68 (t, J = 7 Hz, 2H, $\underline{CH_2}NH_2$), 3.67 (t, J = 6.5 Hz, 2H, CH_2OSi), 7.36–7.45 (m, 6H, ArOSi), 7.68 (m,4H, ArOSi). Mass analysis (ESI) for $C_{22}H_{33}NOSi$. $(M+H)^+$ = 357.2, found 356.4.

1-O-(4,4′-Dimethoxytrityl)-6-hexylamine ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.26–1.48 (m, 6H, CH_2), 1.58–1.75 (m, 4H, CH_2, NH_2), 2.68 (t, J = 7.1 Hz, 2H, NCH_2), 3.05 (t, J = 6.6 Hz, 2H, $\underline{CH_2}$ODMTr), 3.80 (s, 6H, OCH_3), 6.81–6.85 (m, 4H, DMTr), 7.18–7.46 (m, 9H, DMTr). Mass analysis (ESI) for $C_{27}H_{33}NO_3$, $(M+H)^+$: 420.6, found 420.4.

2-N-[6-O-(4,4′-Dimethoxytrityl)hexyl] anthracene-2-carboxamide-7-carboxylic acid-9,10-dio-ne **70** To **69** (0.38 g, 1.3 mmol), dried by co-evaporation with anhydrous pyridine (three times), and dissolved with anhydrous pyridine (15 mL) were added NEt_3 (1 mL), hydroxybenzotriazole (10 mg) and carbonyldiimidazole (0.26 g, 1.6 mmol). After 2 h of stirring at rt, 1-O-(4,4′-dimethoxytrityl)-6-hexylamine (0.49 g, 1.17 mmol) was added and the stirring was maintained for 20 additional h. Then, the mixture was concentrated to dryness and the residue was purified on a silica gel column using a CH_2Cl_2/MeOH/NEt_3 (98:2:0.5, v/v/v) mixture as eluent. Yield: 0.36 g (40%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.29–1.40 (m, 4H, CH_2), 1.52–1.60 (m, 4H, CH_2), 2.95 (t, J = 6.4 Hz, 2H, CH_2O DMTr), 3.29 (m, 2H, NCH_2), 3.72 (s, 6H, OMe), 6.87 (d, J = 8.8 Hz, 4H, DMTr), 7.18–7.37 (m, 9H, DMTr), 8.23–8.37 (m, 4H, H-3, H-6, H-4, H-5), 8.68 (dd, J = 6.6 Hz, J = 1.4 Hz, 2 H, H-1, H-8), 8.93 (t, J = 5.5 Hz, 1H, NH). Mass analysis (ESI) m/z calcd for $C_{43}H_{39}NO_8$. $(M-H)^-$ = 696.3, found 696.2.

2-N-[6-O-(4,4′-Dimethoxytrityl)hexyl]-7-N-(6-hydroxyhexyl)anthracene-2,7-dicarboxamide-9,10-dione **71** The synthesis was performed as reported for **70** except that compound **69** was replaced by 2-{[ω-O-(4,4′-dimethoxytrityl)-hexylamido]-7-carboxylic acid}-anthracene-9-10-dione (0.35 g, 0.5 mmol) and 1-O-(4,4′-dimethoxytrityl)-6-hexylamine by 1-O-(tertbutyldiphenylsilanyl)-6-hexylamine. Yellow product. Yield: 0.45 g (85%). The compound (0.40 g, 0.38 mmol) was dissolved in a 1 M tetrabutylammonium fluoride solution in THF (2 mL) and THF (5 mL) and the mixture was stirred for 1 h at rt. The solution was concentrated and the residue dissolved with CH_2Cl_2. The organic phase was washed with H_2O, dried over Na_2SO_4, filtered and concentrated to dryness. The residue was purified on a silica gel column using a CH_2Cl_2/MeOH (97:3 to 93:7, v/v) mixture as eluent. Yellow solid. Yield: 0.27 g, (85%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.40–1.70 (m, 16 H, CH_2), 3.05 (s br, 2H, CH_2O), 3.50 (m, 4H, NCH_2), 3.75 (m, 8H, OCH_3, CH_2OH), 6.50–6.70 (m, 2H, HNCO), 6.80–7.40 (m, 13H,

DMTr), 8.17 (d, $J = 7.5$ Hz, 2H, H-3, H-6), 8.25 (t, $J = 7.5$ Hz, 2H, H-4, H-5), 8.45 (dd, $J = 8$ Hz, $J = 1.5$ Hz, 2H, H-1, H-8). Mass analysis (ESI) m/z calcd for $C_{49}H_{52}N_2O_8$, $(M\text{-}H)^-$: 795.4, found 795.4.

2-N-[6-O-(4,4′-Dimethoxytrityl)hexyl]-7-N-{6-O-[(2-cyanoethoxy)-N,N′-diisopropylamino-phosphoramidite]hexyl}anthracene-2,7-dicarboxamide-9,10-dione **72**

Compound **71** (0.26 g, 0.32 mmol) was dried by co-evaporation with anhydrous pyridine (3 × 5 mL) and left in a dessicator under vacuum overnight. The next day, the dessicator was filled with argon before being opened. The residue was dissolved with 1,2-dichloroethane (10 mL) and diisopropylethylamine (0.172 g, 1.33 mmol), then 2-cyanoethyldiisopropylchloro-phosphoramidite (0.11 g, 0.47 mmol) was added dropwise under stirring at rt. After 75 min, silica gel TLC analysis using an EtOAc/acetone/TEA mixture as eluent (75:25:5, v/v/v) showed a nearly complete reaction with the formation of two new spots corresponding to both isomers of the phosphoramidite derivative. The reaction mixture was diluted with cold ethyl acetate (30 mL) previously washed with a cold aqueous 5% sodium carbonate solution. The organic phase was washed with a cold 10% aqueous sodium carbonate solution (10 mL) and with a cold saturated aqueous sodium chloride solution (10 mL), dried over Na_2SO_4 and concentrated to dryness. The residue was purified on a silica gel column using AcOEt/acetone/NEt_3 (75:25:5, v/v/v) mixture as eluent. Yellow oil. Yield: 0.27 g, (83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.18 (d, $J = 6.8$ Hz, 6H, $HC(CH_3)_2$), 1.37–1.50 (m, 8H, CH_2), 1.61–1.75 (m, 8H, CH_2), 2.67 (t, $J = 6.4$ Hz, 2H, $OCH_2\underline{CH_2}CN$), 3.07 (t, $J = 6.6$ Hz, 2H, CH_2ODMTr), 3.52 (m, 4 H, HN-$\underline{CH_2}$), 3.79 (s, 6H, OCH_3), 3.57–3.93 [m, 6H, $\underline{HC}(CH_3)_2$, $O\underline{CH_2}CH_2CN$, $O\underline{CH_2}(CH_2)_5NH$], 6.47 –6.60 (m, 2H, HNCO), 6.82 (m, 4H, DMTr), 7.18–7.46 (m, 9H, DMTr), 8.26–8.31 (m, 2H, H-3, H-6), 8.38 (m,2H, H-4, H-5), 8.58 (dd, $J = 11.3$ Hz, $J = 1.4$ Hz, 2H, H-1, H-8). ^{31}P NMR ($CDCl_3$): 147.27 ppm. Mass analysis (ESI) m/z calcd for $C_{58}H_{69}N_4O_9P$, $(M\text{-}H)^-$ 995.5, found 995.4.

*6.2.8.3.3. Synthesis of the dinucleotide-anthraquinone conjugates **26** and **27***

The dinucleotide 5′-phosphorothioate **56**. (30 OD, 1.5 μmol) was dissolved with a 20 mM solution of 18-crown-6 in MeOH (0.8 mL) and a 20 mM solution of compound **67** or **68** in DMF (0.2 mL, 4 μmol) was added. The reaction mixture was protected from the light and maintained under stirring for 6 h at 40 °C. Then, the solution was concentrated and the residue dissolved with water (3 mL). The mixture was extracted with CH_2Cl_2 (3 × 3 mL). The aqueous solution was concentrated and purified by reversed-phase chromatography.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-5′-[6-(1-anthracenyl-9,10-dione)amino-6-oxohexyl] thiophosphate **26** 17 OD. Mass analysis (ESI) calcd for $C_{39}H_{43}N_9O_{14}P_2S$ $(M + H)^+$: 955.8, found 955.1.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-5′-[6-(4-amino-1-anthracenyl-9-10-dione)amino-6-oxohexyl] thiophosphate **27** 15 OD. Mass analysis (ESI) calcd for $C_{39}H_{44}N_{10}O_{14}P_2S$ $(M + H)^+$: 971.2, found 970.3.

*6.2.8.3.4. Synthesis of the dinucleotide-anthraquinone **28***

Trityl-off dinucleotide $^{5'}$CpA$^{3'}$ (1 μmol) bound to the support was dried in a dessicator under vacuum overnight. The next day, the dessicator was filled with argon before being opened. A 0.5 M solution of tetrazole in CH_3CN (0.8 mL) and a 0.12 M solution of the phosphoramidite derivative **72** (0.3 mL) were added to the dinucleotide and left to react for 1½ h at rt. The support was washed with CH_3CN (3 × 3 mL). A 0.1 M iodine solution (1 mL) (used on the synthesizer) was added. After 2 min, the support was washed with CH_3CN (3 × 1 mL). The support-bound conjugate was treated with aqueous ammonia solution (28%) (4 mL) for 24 h at rt. Then, the solution was concentrated to dryness and the residue dissolved with a 70% acetic acid aqueous solution (3 mL). After a 30 min reaction at rt, the mixture was concentrated to dryness. The residue was dissolved with water (4 mL) and the mixture extracted with CH_2Cl_2 (3 × 5 mL). The crude anthraquinone dinucleotide was purified by reversed-phase chromatography as indicated above. 16 OD. 2′-Deoxycytidylyl-(3′®5′)-2′-deoxyadenosine-5′-{6-[7-N-(6-hydroxyhexyl)-2-anthracenyl-2,7-dicarboxamide-9,10-dione]hexyl} phosphate 28. Mass analysis (ESI) calcd for $C_{47}H_{58}N_{10}O_{17}P_2$ $(M + H)^+$: 1097, found 1096.5.

*6.2.8.4. Synthesis of the dinucleotide-perylene conjugates **30** and **31***

The synthesis was performed as previously reported for the preparation of oligonucleotide-perylene conjugates involving the same perylene-linker units [38,39]. The deprotection step by a concentrated aqueous ammonia solution was adapted to the sequence. The mixture was heated at 50 °C for 8 h. The purification was performed by reversed-phase chromatography.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-5′-{1′-O-[3-(3-perylenyl)propyl]-2′-deoxy-3′-ribosyl}phosphate **30** 19 OD. Mass analysis (ESI) calcd for $C_{47}H_{50}N_8O_{15}P_2$ $(M + H)^+$: 1029.3, found 1029.8.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-5′-[6-(3-perylenylmethylamino)hexyl] phosphate **31** 18 OD. Mass analysis (ESI) calcd for $C_{46}H_{52}N_9O_{12}P_2$ $(M + H)^+$: 984.32, found 982.3.

6.2.8.5. Synthesis of the modified dinucleotide **32–34** The synthesis was performed via phosphoramidite chemistry on modified supports [41,42].

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-3′-(3-hydroxypropyl) phosphate **32** Mass analysis (ESI) calcd for $C_{22}H_{32}N_8O_{13}P_2$ $(M\text{-}H)^-$: 677.2, found: 677.2.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-3′-(12-hydroxydodecyl) phosphate **33** Mass analysis (ESI) calcd for $C_{31}H_{50}N_8O_{13}P_2$ $(M\text{-}H)^-$: 803.3, found: 803.2.

2′-Deoxycytidylyl-(3′ → 5′)-2′-deoxyadenosine-3′-(6-aminohexyl) phosphate **34** Mass analysis (ESI) calcd for $C_{25}H_{39}N_9O_{12}P_2$ $(M\text{-}H)^-$: 718.2, found: 718.4.

*6.2.9. Synthesis of **38** and **39***

*6.2.9.1. 9-[6-(1-Cytosinyl)hexyl]adenine **38** (Scheme 8)*

9-(6-Bromohexyl)-6-N-benzoyladenine**75** 6-N-benzoyladenine **73** (0.6 g, 2.5 mmmol) was dried by co-evaporation with anhydrous CH_3CN (3 × 15 mL), dissolved with anhydrous CH_3CN (25 mL) and potassium carbonate (0.34 g, 2.5 mmol) was added under magnetic stirring at rt. Then 1, 6-dibromohexane **74** (1.6 mL, 10 mmol) was added under an inert atmosphere and the mixture heated at 60 °C for 10 h. The mixture was concentrated to dryness and the residue dissolved with CH_2Cl_2 (50 mL). The organic phase was washed with ice-cold H_2O (15 mL) dried over Na_2SO_4 and concentrated. The residue was dissolved with CH_2Cl_2 (minimum amount) and the solution was added dropwise to pentane under vigorous stirring. The sediment was purified by silica gel chromatography with a CH_2Cl_2/acetone (95:5 to 80:20, v/v) mixture as eluent. White solid. 250 mg (25%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.43 (m, 2H, CH_2), 1.53 (m, 2H, CH_2), 1.86 (m, 2H, CH_2), 1.98 (m, 2H, CH_2), 3.40 (t, $J = 6.66$ Hz, 2H, CH_2Br), 4.30 (t, $J = 7.23$ Hz, 2H, NCH_2), 7.50–7.64 (m, 3H, ArH), 8.03 (m, 3H, H-2, ArH), 8.82 (s, 1H, H-8), 9.02 (s br, 1H, NH). Mass analysis (ESI.) m/z calcd for $C_{18}H_{20}BrN_5O$. $(M + H)^+$: 402.1, found 402.1 and 404.2.

9-[6-(1-Cytosinyl)hexyl]adenine **38** Compound **75** (0.15 g, 0.37 mmol) and 4-N-benzoylcytosine (97 mg, 0.45 mmol) were dried with CH_3CN separately.4-N-benzoylcytosine was dissolved with dry DMF (8 mL), then K_2CO_3 (56 mg, 0.41 mmol) was added. The mixture was stirred at 30 °C for 10 min and compound **75** dissolved in DMF (4 mL) was added. The mixture was heated at 55 °C for 8 h under stirring and the stirring was maintained for 12 h at 20 °C. Then, the mixture was concentrated and the residue was purified on preparative TLC using CH_2Cl_2/EtOH (97:3, v/v) twice and then (95:5, v/v) three times as eluent. Yield: 70 mg (35%). Compound **76** (53 mg, 0.1 mmol) was reacted with concentrated aqueous NH_4OH (12 mL) and MeOH (1.5 mL) for 72 h at 20 °C. The mixture was concentrated to 9 mL and extracted with CH_2Cl_2. The aqueous solution was concentrated and purified by reversed-phase chromatography using a CH_3CN (5 to 27% over 30 min) gradient in a 0.05 M TEAA, pH 7, buffer. Yield: 320 OD, 18%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.25 (m, 4H, CH_2), 1.52 (m, 2H, CH_2), 1.80 (m, 2H, CH_2), 3.58 (t, $J = 7.14$ Hz, 2H, NCH_2), 4.11 (t, $J = 7.06$ Hz, 2H, NCH_2), 5.60 (d, $J = 7.11$ Hz, 1H, H-5′), 7.15 (s, 1H, H-2), 7.52 (d, $J = 7.11$ Hz,1H, H-6′), 8.13 (s, 1H, H-8). Mass analysis (ESI.) m/z calcd for $C_{15}H_{20}N_8O$. $(M + H)^+ = 329.2$, found 329.2. $\lambda_{max} = 263$ nm.

*6.2.9.2. 9-[6-(1-Thymyl)hexyl]guanine **39** (Scheme 9)*

9-(6-Bromohexyl)-2-N-isobutyryl-6-O-[2-(4-nitrophenyl)ethyl] guanine **78** 2-N-Isobutyryl-O-6-[2-(4-nitrophenyl)ethyl]guanine **77** (400 mg, 1.08 mmol) [43], 1,6-dibromohexane **74** (1.38 g, 5.43 mmol), K_2CO_3 (156 mg, 1.13 mmol) and CH_3CN (50 mL) were heated at 60 °C for 11 h. Then, the mixture was concentrated to dryness and the residue dissolved with CH_2Cl_2 (50 mL). The organic phase was washed with H_2O, dried and concentrated to dryness. The residue was purified by silica gel chromatography with increasing concentrations of MeOH in CH_2Cl_2 (0 to 10%). White solid. 231 mg (40%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.19 (m, 2H, CH_2), 1.30 (d, $J = 6.86$ Hz, 6H, $CH(CH_3)_2$, 1.39 (m, 2H, CH_2), 1.70 (m, 2H, CH_2), 1.79 (m, 2H, CH_2), 3.32 (t, $J = 6.59$ Hz, 2H, CH_2Ph), 3.38 (t, $J = 6.56$ Hz, 2H, CH_2Br), 4.14 (t, $J = 7{,}01$ Hz, 2H, CH_2N), 4.93 (t, $J = 6.59$ Hz, 2H, CH_2O), 7.51 (d, $J = 8.64$ Hz, 2H, ArH), 7.90 (s, 1H, H-8), 7.96 (s, 1H, NH), 8.20 (d, $J = 8.67$ Hz, 2H, ArH). Mass analysis (ESI) calcd for $C_{23}H_{29}BrN_6O_4$ $(M + H)^+$: 533.2, found: 533.2, 535.3 $(M + H+2)^+$. 2-N-Isobutyryl-9-[6-(1-thymyl)hexyl]guanine **79**

Compound **78** (110 mg, 0.206 mmol), thymine (30 mg, 0. 238 mmol) and DBU (63 mg, 0.413 mmol) were dissolved in pyridine (2 mL). After a 24 h reaction at rt, water (10 mL) was added and the mixture was neutralized by addition of concentrated acetic acid. The reaction mixture was extracted with CH_2Cl_2 (15 mL). The organic phase was concentrated and the residue purified by silica gel flash chromatography using increasing concentrations of MeOH in CH_2Cl_2 (0 to 8%). White solid. 32 mg (27%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.27 (d, $J = 6.88$ Hz, 6H, $CH(C\underline{H}_3)_2$), 1.34 (m, 4H, $2CH_2$), 1.68 (m, 2H, CH_2), 1.93 (m, 5H, CH_2, CH_3(T)), 2.79 (sp, $J = 6.87$ Hz, 1H, $C\underline{H}(CH_3)_2$), 3.67 (t, $J = 7.26$ Hz, 2H, CH_2N(T)), 4.33 (t, $J = 6.95$ Hz, 2H, $\underline{CH}_2N$(G)), 6.98 (s, 1H, H-6(T)), 7.78 (s, 1H, H-8(G)), 9.50 (bs, 1H, NH), 9.90 (bs, 1H, NH), 12.23 (bs, 1H, NH). Mass analysis (ESI) calcd for $C_{20}H_{27}N_7O_4$ $(M + H)^+$: 430.2, found: 430.3.6.2.9.1.3.3 9-[6-(1-Thymyl)hexyl]guanine **39**

Compound **79** (20 mg, 46.6 μmol) was dissolved in concentrated aqueous ammonia (28%) (4 mL). After a 15 h reaction at 50 °C, the mixture was concentrated to dryness and the crude **39** was purified by recristallization with a H_2O/CH_3CN (9:1, v/v) mixture. White solid, 9 mg (53%).^{1}H NMR (500 MHz, DMSO-d_6): δ 1.23 (m, 4H, $2CH_2$), 1.53 (m, 2H, CH_2), 1.75 (m, 5H, CH_2, CH_3(T)), 3.58 (t, $J = 7.25$ Hz, 2 H, CH_2N(G)), 4.14 (t, $J = 6.94$ Hz, 2H, CH_2N(T)), 6.07 (s, 2H, NH_2), 7.50 s, 1H, H-6(T)), 7.89 (s, 1H, H-8 (G)), 10.68 (s, 1H, NH), 11.16 (s, 1H, NH). Mass analysis (ESI) calcd for $C_{16}H_{21}N_7O_3$ $(M + H)^+$: 360.2, found: 360.3.

6.3. Biological studies

Activity assays were carried out for 1 hr at 37 °C in a buffer containing 10 mM HEPES (pH 7.2), 1 mM DTT, 7.5 mM $MgCl_2$ in the presence of 12.5 nM 21-mer double-stranded DNA substrate and 100 nM recombinant HIV-1 integrase as previously described [45]. Products were separated by electrophoresis in denaturing 18% acrylamide/urea gels. Gels were analysed with a Molecular Dynamics STORM phosphoimager and quantified with Image QuantTM 4.1 software. Inhibition in the presence of drugs was expressed as the fractional product in percent of the control without drug. Inhibition curves were fitted using Graphpad Prism 5 software.

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