DOI: 10.1002/cbic.200900232

Potent Triple Helix Stabilization by 5′,3′-Modified Triplex-Forming Oligonucleotides

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Anthraquinone and pyrene analogues attached to the 3′ and/or 5′ termini of triplex-forming oligonucleotides (TFOs) by various linkers increased the stability of parallel triple helices. The modifications are simple to synthesize and can be introduced during standard solid-phase oligonucleotide synthesis. Potent triplex stability was achieved by using doubly modified TFOs, which in the most favourable cases gave an increase in melting temperature of 30 °C over the unmodified counterparts and maintained their selectivity for the correct target duplex. Such TFOs can produce triplexes with melting temperatures of 40 °C at pH 7 even though they do not contain any triplex-stabilizing base analogues. These studies have implications for the design of triplex-forming oligonucleotides for use in biology and nanotechnology.

Introduction

Triplex-forming oligonucleotides (TFOs)[1] have previously been employed in attempts to modulate gene function in vitro and in vivo due to their ability to interfere with transcription and replication by binding to the major groove of double-stranded DNA.[2–9] TFOs composed of pyrimidine bases bind in a parallel orientation to the purine strand of oligopurine–oligopyrimidine duplexes[10] and form T–AT and C^+–GC triplets (Figure 1). To achieve recognition of mixed-sequence DNA, a variety of nucleoside analogues have been introduced in internal positions of TFOs to selectively target AT,[11,12] TA,[13–15] CG[16–20] and GC base pairs.[21]

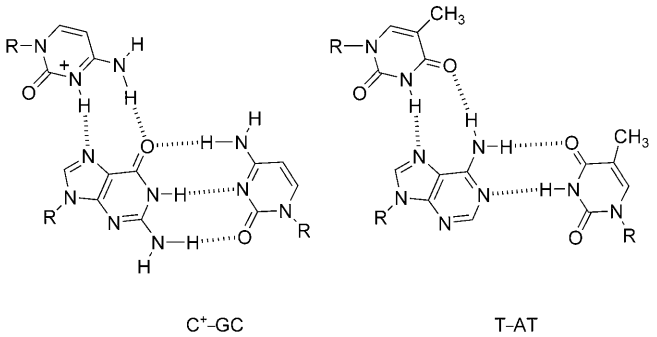

Figure 1. Structure of C^+–GC and T–AT triplets in DNA; R = deoxyribose.

Despite these advances, the use of TFOs is limited by their weak binding to DNA duplexes, particularly above pH 6.[22,23] Triplex stability can be increased by incorporating duplex cross-linking agents, such as psoralen,[24–30] or by employing aromatic analogues to stabilize the triplex by intercalation.[31–34] For in vivo applications it would be valuable to combine thermodynamic stabilization of triplexes with protection of the TFO against exonucleases, which degrade 3′-unmodified oligonucleotides. With this in mind we have designed a variety of simple 3′-oligonucleotide modifications based on pyrene, anthraquinone and tertiary amines. Biophysical studies have been carried out on TFOs containing these modifications in combination with stabilizing base analogues in order to correlate triplex stability with the nature of the 3′ terminus of the TFO. The influence of the duplex sequence at the triplex–duplex junction (T–D junction) has been investigated and the properties of TFOs containing both 5′- and 3′-stabilizing modifications have been studied.

Results and Discussion

Synthesis of pyrene analogues

Pyrene has previously been used in the nucleic acid field as a fluorescent reporter,[35] an intercalating agent at bulges in the middle of triplexes[34,36,37] and as a component of RNA recognition probes.[36] Very recently 3′-pyrene-labelled TFOs have been used to inflict sequence-specific damage on DNA in the form of strand breaks and adducts.[37] In the current study we were interested in evaluating pyrene as a stabilizer at the 3′ terminus of triplexes. Three different precursors were prepared for use in oligonucleotide synthesis, two were based on 2-deoxy-D-ribofuranose and a third one had an L-threoninyl backbone. Precursor **2** was synthesized in 76% yield (Scheme 1) by coupling 1-β-aminohexyl-2-deoxyribose deriva-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900232.

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tive **1**[38] to pyrene-1-butyric acid with DCC and HOBt activation.

In order to produce a 3′-pyrene derivative with a shorter linker, 1-pyrenemethylamine was coupled to 2-deoxy-D-ribofuranose-1-β-ethanoic acid derivative **3**,[39] with HBTU and DMAP activation (Scheme 2) to give precursor **4**.

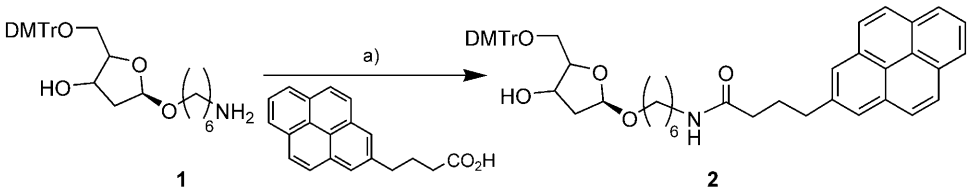
A pyrene-L-threoninyl analogue **6** was synthesized by a simple two-step procedure to provide further structural diversity (Scheme 3). Dimethoxytritylation of the primary hydroxyl group of L-threoninol followed by coupling of its amino function to activated pyrene-1-butyric acid gave precursor **6**. No ester formation was detected during this reaction.

Synthesis of anthraquinone analogues

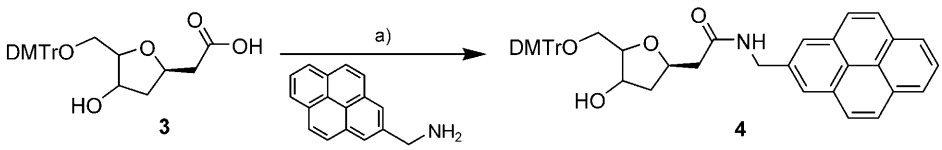
Anthraquinones interact with DNA principally by intercalation. They have been added both to the 5′-end and at internal sites of oligonucleotides,[40] but there are fewer examples of anthraquinones at the 3′-end.[41,42] In the present study two deoxyribose analogues were prepared with and without an aminohexyl chain on the anthraquinone, in addition to an L-threoninol derivative. Anthraquinone deoxyribose derivatives **13** and **14** were prepared in a suitable form for resin functionalization according to Scheme 4. The key step is the amide coupling reaction between the ethanoic acid derivative of deoxyribose analogue **3** and the primary amine on the aminohexyl side chain of anthraquinones **7** and **10**. Anthraquinone **7** was prepared by nucleophilic displacement of the chlorine atom at position 1 of the chloroanthraquinone with hexamethylenediamine. A similar reaction on anthraquinone **9**, which was made by trifluoroacetylation of chloroaminohexylanthraquinone **8**,[43] gave intermediate **10**.

Compound **9** was also used to prepare **11**, which was converted to phosphoramidite **12** for addition to the 5′-end of TFOs (Scheme 4). L-Threoninylanthraquinone derivative **17** was prepared according to Scheme 5. The free amine on DMTr-threoninol **5** was coupled to commercially available anthraquinone-2-carboxylic acid. Initially a mixture of HBTU and DMAP in DMF was used to promote amide bond formation, but the reaction gave two products. After purification by column chromatography the less polar compound was recovered in 75% yield. NMR spectroscopy and mass spectrometry indicated substitution at both the amine and the alcohol (compound **15**) so the ester bond was cleaved selectively by K_2CO_3 in methanol to give the desired compound **16** (71% for the two steps). This double substitution was not observed when HBTU was replaced by EDC. Attempts to functionalize a succinic acid derivatized oligonucleotide synthesis resin with **16** were unsuccessful, presumably due to steric hindrance.

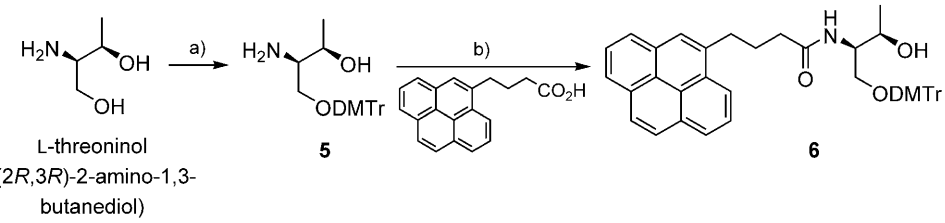
The problem was solved by succinylating the hydroxyl function of **16** to give the unhindered succinic acid derivative **17**, which was then coupled successfully to aminoalkyl-functionalized resin for use in oligonucleotide synthesis. Compound **16** was also used as a precursor to prepare the corresponding phosphoramidite **18** with a very short linker for introduction at the 5′-end of TFOs.



Scheme 1. a) Pyrene-1-butyric acid (1.1 equiv), DCC (2 equiv), HOBt (1.5 equiv), NEt_3, CH_2Cl_2, room temperature, overnight, 76%.



Scheme 2. a) 1-Pyrenemethylamine hydrochloride (1 equiv), HBTU (2 equiv), DMAP (2 equiv), DMF, room temperature, 4 h, 65%.



Scheme 3. a) DMTrCl (1.5 equiv), DMAP (0.5 equiv), pyridine, room temperature, 3 h, 65%; b) pyrene-1-butyric acid (0.9 equiv), HBTU (2 equiv), DMAP (1 equiv), DMF, room temperature, 4 h, 57%.

Synthesis of substituted tertiary amines

When anthraquinone and pyrene analogues are incorporated into single stranded TFOs an increase in triplex stabilization is expected due to intercalation. We were also interested in investigating the stabilizing effect of an amino group at the 3′-end of the TFO, which will be protonated at physiological pH, and should therefore interact with the anionic charge of the phosphodiester backbone in the triplex. To this end, two monomers were prepared by amide bond formation between deoxyribose ethanoic acid derivative **3** and either *N*,*N*-dimethylethylenediamine or procainamide, to yield compounds **19** and **20**, respectively (Scheme 6). Procainamide is of particular interest as it carries both an aromatic phenyl ring and a tertiary amine.

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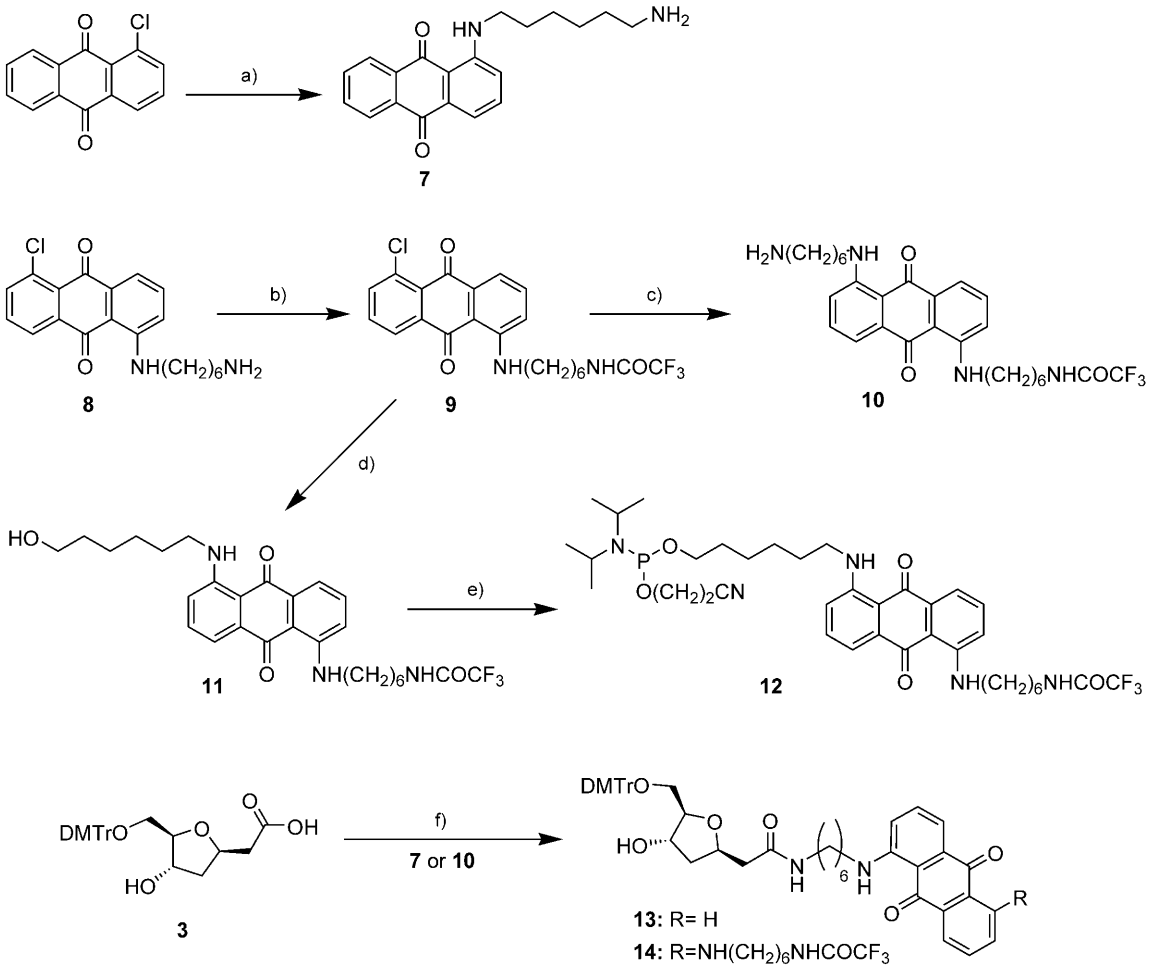
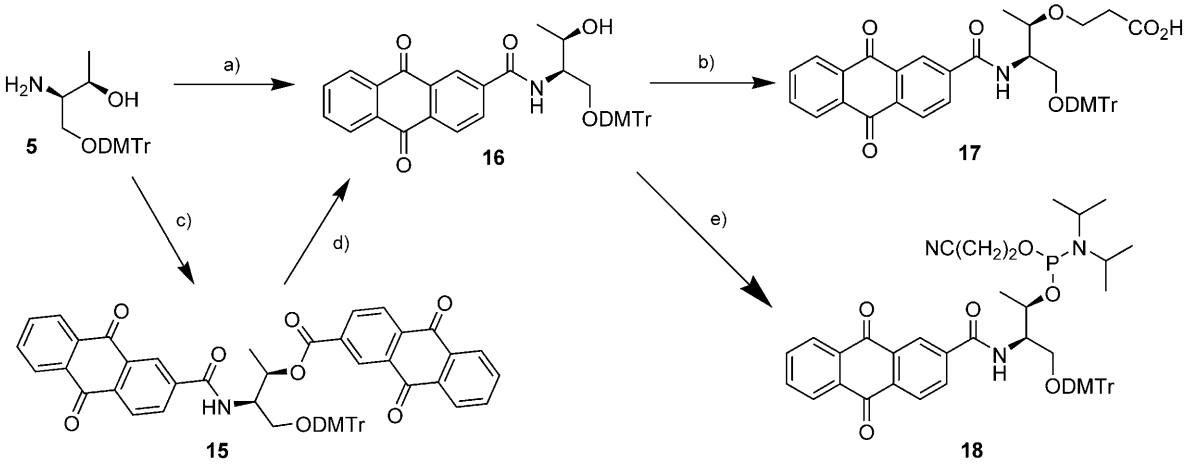


Scheme 4. a) hexamethylenediamine (3 equiv), xylene, reflux, 2 h, 42%; b) ethyl trifluoroacetate (5 equiv), NEt_3 (10 equiv), CH_2Cl_2, room temperature, 90 min, 63%; c) hexamethylenediamine (5 equiv), xylene, reflux, 16 h, 82%; d) $NH_2(CH_2)_6OH$ (5 equiv), xylene, reflux, 18 h, 36%; e) $[N(iPr)_2]_2PO(CH_2)_2CN$ (1 equiv), DIHT (0.5 equiv), CH_3CN, 86%; f) **7** or **10** (1 equiv), HBTU (2 equiv), DMAP (1 equiv), DMF or pyridine, room temperature, 2 h, 67% (**13**) and 26% (**14**).



Scheme 5. a) Anthraquinone-2-carboxylic acid (1 equiv), EDC (1.5 equiv), DMF, room temperature, 2 h, 70%; b) succinic anhydride (1.5 equiv), DMAP (0.5 equiv), pyridine, room temperature, 7 h, 38%; c) anthraquinone-2-carboxylic acid (1 equiv), HBTU (2 equiv), DMAP (1 equiv), DMF, room temperature, 2 h, 75%; d) K_2CO_3 (10 equiv), CH_2Cl_2/MeOH (1:1, *v*/*v*), room temperature, 1 h, 95%; e) $N(iPr)_2P(Cl)O(CH_2)_2CN$ (1.5 equiv), DIPEA (4 equiv), CH_2Cl_2, room temperature, 4 h, 38%.

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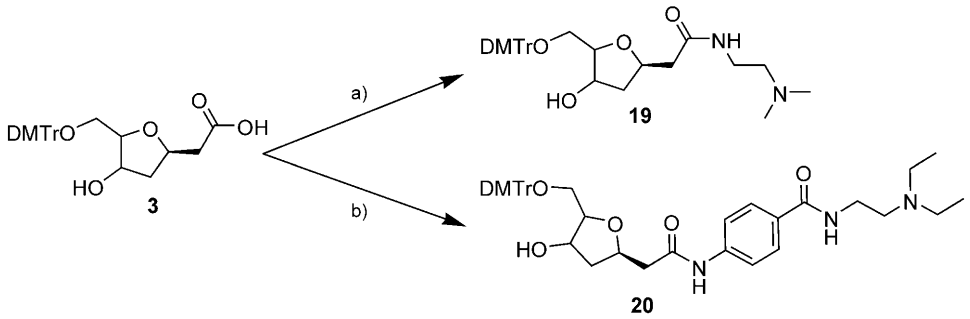

Scheme 6. a) *N*,*N*-Dimethylenediamine (1 equiv), HBTU (2 equiv), DMAP (1 equiv), DMF, room temperature, overnight, 35%; b) procainamide hydrochloride (1 equiv), EDC (2 equiv), DMAP (1 equiv), pyridine, room temperature, overnight, 43%.

The various modifications and abbreviations used in this study are summarized in Figure 2, and the sequences of the corresponding duplexes and TFOs are shown in Table 1.

Ultraviolet melting triplex stability studies

Intercalators placed at the 3′-end of TFOs have been found to be relatively ineffective in stabilizing parallel triplexes[44,45] despite the fact that they are strongly stabilizing when attached to the 5′-end.[31,46–50] This has been attributed to the theory that TFO dissociation involves unzipping from the 5′-end of the TFO.[51] However, an alternative explanation might be that 3′ intercalation is highly sequence dependent. Indeed, most studies have been carried out on duplexes with RpR or RpY base-stacking steps at the junction between the 3′-end of the TFO target and the duplex (3′-T–D junction), which are not the best binding sites for most intercalators. YpR is a better intercalation site,[52] and duplexes with this step at the 3′-T–D junction should be better targets, even though inclusion of a pyrimidine at the 3′-end of the purine-rich target strand would normally reduce the binding affinity. To investigate this hypothesis, and to evaluate the modifications described in this study, we carried out ultraviolet melting studies on parallel triplexes with various dinucleotide steps at the 3′-end of the triplex target site. Hairpin duplexes with a flexible hexa(ethylene glycol) linker between the two strands were used throughout this work (Table 1), as these duplexes have very high stability, which allows clear differentiation between duplex and triplex melting temperatures (T_m). Initial studies were carried out on constructs with RpR and RpY steps at the site of intercalation. Two TC-rich 18-mer TFOs, TFO-1 with 3′-propyl, and TFO-7 with a potentially stabilizing 3′-anthraquinone, were added to hairpin duplexes DAT1 and DAT2 and allowed to form parallel triplexes. These constructs have ApA and ApT steps, respectively, at the 3′-T–D junction and T–AT triplets at the 3′-end of the triplex. In both cases the anthraquinone significantly increased triplex stability across the pH range from 5.5 to 6.6 (Table 2, entries 1 vs. 5, 7 vs. 11). Experiments on the triplexes formed between TFO-2 and TFO-5 and duplexes DGC1 and DGC2, which have 3′-C–GC triplets and GpA and GpT steps at the 3′-T–D junction, showed similar stabilization (Table 2, entries 13 vs. 14 and 16 vs. 17). This is a clear demonstration that triplexes with RpR and RpY base stacking steps at the 3′-T–D junction can be stabilized with suitable 3′ intercalators, and that there is little difference in the degree of stabilization at these two intercalation sites.

The presence of thymine and cytosine bases within the purine strand of polypurine–polypyrimidine duplexes generates TA and CG inversions, which reduce the stability of parallel triplexes. Methods to increase the stability of such constructs are particularly valuable. With this in mind, we investigated triplexes with TA and CG inversions at the 3′-T–D junction. The first series of experiments was conducted on the DTA duplex, which has a TpA step at the 3′-T–D junction and generates an unstable T–TA triplet with TFO-1 and TFO-7. Large increases in T_m relative to the 3′-propyl control were observed when 3′-anthraquinone and pyrene modifications were present in the TFO (Table 2, entries 24, 25 vs. 19). These results were very encouraging, and prompted us to find methods for achieving further stabilization, which is particularly important with in vivo applications in mind. It is known from previous triplex studies that the S-base is a potent stabilizer of TA inversions[15] (Figure 3 A), so we next investigated the stability of triple helices containing 3′-S–TA triplets using TFOs with or without 3′-anthraquinone and pyrene modifications (Table 2, entries 21, 26–33).

As expected, the presence of the S-base had a major stabilizing effect (entry 21 vs. 19) and the added benefit of the 3′ intercalator was observed at higher pH (entries 26–31 vs. 21). The contribution of AnthdR was particularly significant and increased triplex T_m by 6.2 °C relative to S alone, and around 12 °C compared to T. With in vivo applications in mind, studies with 3′-anthraquinone TFOs were also carried out in the presence of spermine (2 mM), a component of cell nuclei. Major increases in stability were observed with melting temperatures close to 50 °C (entries 26 and 27). The stabilizing effect of an extended aromatic system at the 3′-end of the TFO was confirmed by comparing these results with TFOs containing 3′-tertiary amines without such groups. These were only slightly stabilizing at low pH, and were found to be ineffective at pH 6.6 (entries 32 and 33). Although S was originally designed to recognise TA base pairs, it also binds to CG. Therefore, having established the potency of 3′ intercalators in combination with the S-base to stabilize triplexes with 3′-TA inversions, we next evaluated the same TFOs against the DCG duplex with a CG base pair and a CpA step at the 3′-T–D junction. Anthraquinone strongly stabilizes such triplexes (entry 35 vs. 34) but there was very little added benefit from the S-base (generating an S–CG triplet) compared with T (generating T–CG; compare entries 38–43 vs. 35). In this series S-AnthThr and S-MeAcPyr gave the greatest increases in T_m. As previously noted, 3′-tertiary amines with no attached intercalators were ineffective, particularly at pH 6.6 at which triplex stabilisation by protonation

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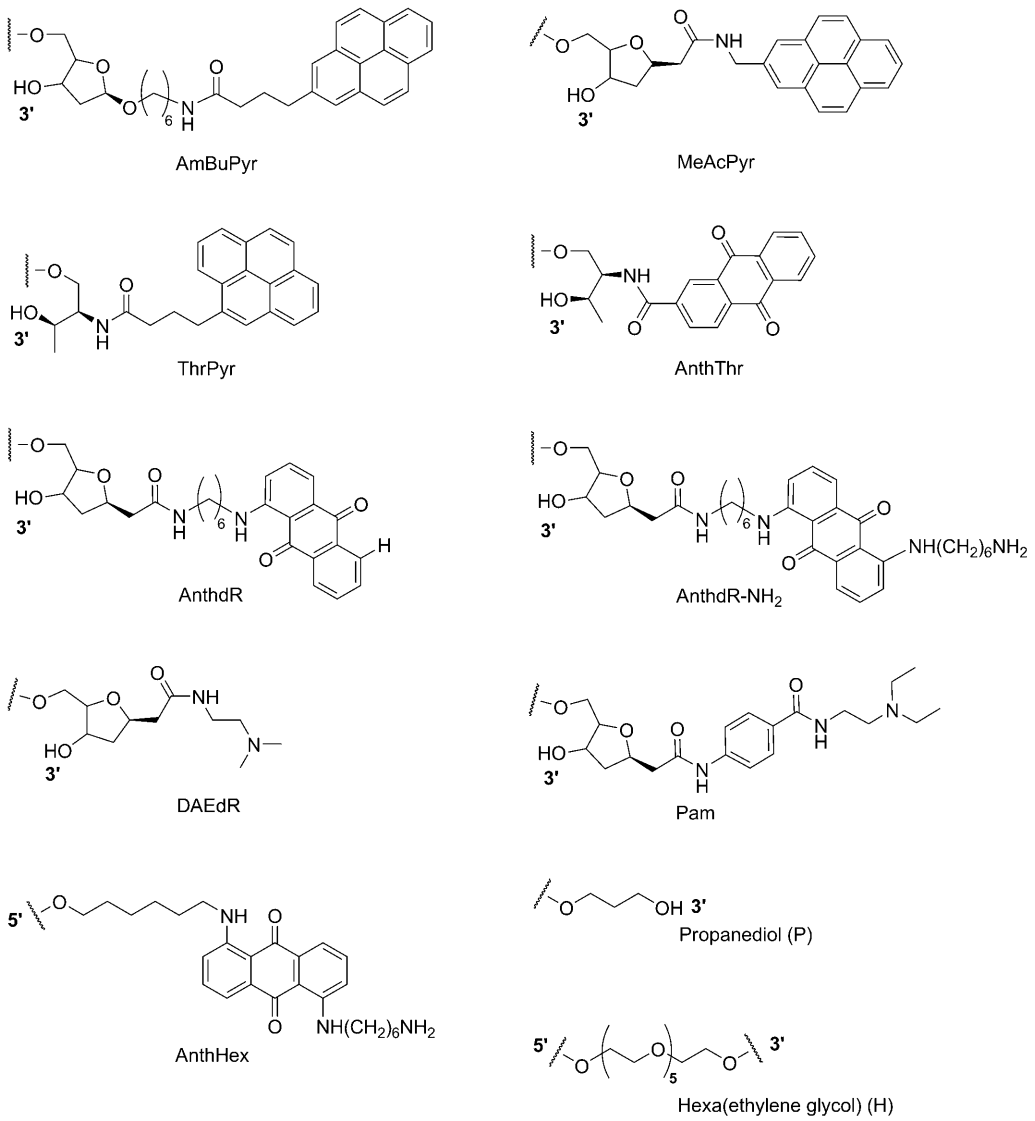

Figure 2. Structure of the oligonucleotide modifications used in this study.

of the amine would be limited (entries 44 and 45). After establishing that anthraquinone stabilizes 3′-T–AT, C^+–GC, T–TA, S–TA and S–CG triplets we carried out similar studies using TFOs containing the CG-stabilizing R^{An} base (Figure 3 B) to target the DCG duplex.[16,53,54] Surprisingly the R^{An} base was less effective than S at targeting CG in this duplex (compare entries 47–50 vs. entries 38–43). No advantage was gained by incorporating R^{An} into the TFO and these complexes were no more stable than those with a 3′-T–TA triplet preceding the anthraquinone. Experiments were then carried out by using the same TFOs containing a 3′-R^{An} in combination with the DTA duplex, to examine TA recognition by R^{An} (entries 51–55). Aminohexylanthraquinone attached to deoxyribose (R^{An}-AnthdR) was by far the best analogue particularly in the presence of spermine, and was as effective as S-AnthdR (entry 52 vs. 26). In all the TFOs modified with 3′-R^{An}, the beneficial contribution of the 3′ intercalator to triplex stability is very clear. It is also noteworthy that when R^{An} is placed at the 3′-end of the TFO adjacent to an intercalator it switches its selectivity to TA rather than CG recognition.

After demonstrating the benefit that can be achieved by the use of 3′ modified TFOs, we next studied TFOs with intercalators at both the 5′- and 3′-ends, and focussed on the most promising 3′ modifications, ThrPyr and AnthdR. The 5′-anthraquinone alone gave increases in triplex stability of around 10 °C (Table 3, entry 10 vs. 13) and this was greatly increased to around 20 °C when modifications were introduced at both the 5′- and 3′ termini of the TFO. This was true for duplex targets with either TA (Table 3, entries 5, 6 vs. 1) or AT base pairs at the 3′-end of the triplex (Table 3, entries 14, 15 vs. 10 and 23, 24 vs. 19). To investigate the influence of linker length between TFO and 5′ modification on its ability to intercalate into the duplex, TFOs with 5′-threoninyl anthraquinone (AnthThr; short linker) were compared to those with 5′-AnthHex (long linker). In general, the 5′-AnthThr was much less effective (Table 3, entry 7 vs. 4, 16 vs. 13, 25 vs. 22); this confirms the

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Table 1. Oligonucleotide sequences used in ultraviolet melting experiments.

Duplexes/triplex

TCCTTCTCTTTTTTCTTYm

5'-GTGTTAGGAAGAGAAAAAAGAA**XX**GGT

3'-CACAATCCTTCTCTTTTTTCTT**ZZ**CCA H

H = hexa(ethylene glycol) linker; XX and ZZ indicate dinucleotides that are varied at the 3′-end of the TFO targets site (underlined); XX is written 5′ to 3′ and ZZ is 3′ to 5′; DTA: XX = TpA, ZZ = ApT; DAT1: XX = ApA, ZZ = TpT; DAT2: XX = ApT, ZZ = TpA; DCG: XX = CpA, ZZ = GpT; DGC1: XX = GpA, ZZ = CpT; DGC2: XX = GpT, ZZ = CpA. The general TFO sequence is shown in italics (Y = T, C, S or R^{An}, m = 3′-modification).

Triplex-forming oligonucleotides (TFOs)

TCCTTCTCTTTTTTCTT**X**-P
TFO-1: X = T, TFO-2: X = C, TFO-3: X = S, TFO-4: X = R^{An}

TCCTTCTCTTTTTTCTTC-**Z**
TFO-5: Z = AnthdR, TFO-6: Z = ThrPyr

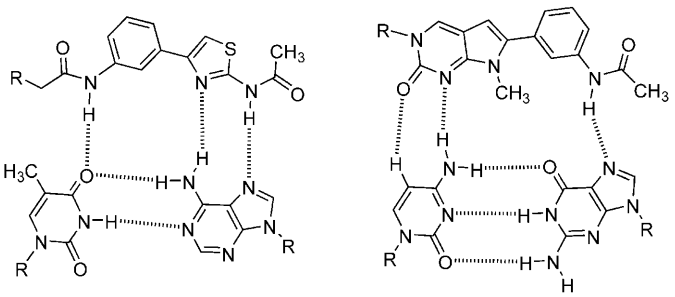
TCCTTCTCTTTTTTCTTT-**Z**
TFO-7: Z = AnthdR, TFO-8: Z = ThrPyr

TCCTTCTCTTTTTTCTTS-Z
TFO-9: Z = AnthdR, TFO-10: Z = AnthdR-NH_2, TFO-11: Z = AnthThr, TFO-12: Z = AmBuPyr, TFO-13: Z = ThrPyr, TFO-14: Z = MeAcPyr, TFO-15: Z = DAEdR, TFO-16: Z = Pam

TCCTTCTCTTTTTTCTTR^{An}-**Z**
TFO-17: Z = AnthdR, TFO-18: Z = AnthdR-NH_2, TFO-19: Z = AmBuPyr, TFO-20: Z = ThrPyr

TFO-21: AnthHex-TCCTTCTCTTTTTTCTTT-P
TFO-22: AnthHex-TCCTTCTCTTTTTTCTTT-AnthdR
TFO-23: AnthHex-TCCTTCTCTTTTTTCTTT-ThrPyr
TFO-24: AnthThr-TCCTTCTCTTTTTTCTTT-P
TFO-25: AnthThr-TCCTTCTCTTTTTTCTTT-AnthdR
TFO-26: AnthThr- TCCTTCTCTTTTTTCTTT-ThrPyr

TFO-27: TCTTTTTTCTTT-P
TFO-28: AnthdR-TCTTTTTTCTTT-ThrPyr



A) S–TA B) R^{An}–CG

Figure 3. A) Structure of S–TA triplet in DNA. The S monomer is able to form three hydrogen bonds with a TA base pair involving O4 on T and N6, N7 on A. B) Structure of R^{An}–CG triplet in DNA. The R^{An} monomer binds to a CG base pair through three hydrogen bonds established with N4, C5 on C and N7 on G; R = β-2-deoxyribose attached at carbon 1.

importance of having a sufficiently long linkage between the anthraquinone moiety and the TFO to permit intercalation into the duplex. Triplex stability at pH 7 is of particular interest for

Table 2. Results of ultraviolet melting experiments (T_m).

	TFO description	pH 5.5[a]	pH 6.2[b]	pH 6.6[b]
Target duplex DAT1; 3′-AT base pair with ApA intercalation site				
1	(TFO-1) T-P	43.2	34.8	27.6
2	(TFO-2) C-P	38.9	30.1	22.5
3	(TFO-5) C-AnthdR	46.9	37.6	29.4
4	(TFO-6) C-ThrPyr	48.0	38.1	29.6
5	(TFO-7) T-AnthdR	51.3	40.5	32.5
6	(TFO-8) T-ThrPyr	52.2	40.0	32.1
Target duplex DAT2; 3′-AT base pair with ApT intercalation site				
7	(TFO-1) T-P	43.5	34.7	27.1
8	(TFO-2) C-P	38.5	30.0	22.4
9	(TFO-5) C-AnthdR	47.2	37.6	29.4
10	(TFO-6) C-ThrPyr	47.3	37.3	29.0
11	(TFO-7) T-Anthdr	51.3	40.7	32.4
12	(TFO-8) T-ThrPyr	53.2	40.1	32.1
Target duplex DGC1; 3′-GC base pair with GpA intercalation site				
13	(TFO-2) C-P	43.8	32.8	24.6
14	(TFO-5) C-AnthdR	50.1	38.8	30.2
15	(TFO-6) C-ThrPyr	52.1	40.4	31.7
Target duplex DGC2; 3′-GC base pair with GpT intercalation site				
16	(TFO-2) C-P	45.1	34.1	25.9
17	(TFO-5) C-AnthdR	51.0	38.6	29.9
18	(TFO-6) C-ThrPyr	51.5	39.6	30.9
Target duplex DTA; 3′-TA base pair with TpA intercalation site				
19	(TFO-1) T-P	38.7	27.3	< 20
20	(TFO-2) C-P	38.0	28.9	< 20
21	(TFO-3) S-P	49.9	34.0	26.2 (39.2)*
22	(TFO-5) C-AnthdR	46.1	37.1	28.7
23	(TFO-6) C-ThrPyr	45.4	36.1	27.8
24	(TFO-7) T-AnthdR	48.1	35.6	28.4
25	(TFO-8) T-ThrPyr	47.9	34.4	28.7
26	(TFO-9) S-AnthdR	51.7	39.2	32.4 (48.8)*
27	(TFO-10) S-AnthdR-NH_2	50.2	38.7	30.8 (48.7)*
28	(TFO-11) S-AnthThr	49.3	36.8	30.2
29	(TFO-12) S-AmBuPyr	49.0	37.7	30.1 (46.3)*
30	(TFO-13) S-ThrPyr	49.1	36.5	29.1 (44.8)*
31	(TFO-14) S-MeAcPyr	50.7	37.6	30.8
32	(TFO-15) S-DAEdR	45.7	34.1	27.6
33	(TFO-16) S-Pam	44.5	33.0	26.7
Target duplex DCG; 3′-CG base pair with CpA intercalation site				
34	(TFO-1) T-P	37.5	26.5	< 20
35	(TFO-7) T-AnthdR	47.3	36.0	28.8
36	(TFO-8) T-ThrPyr	48.2	36.4	28.9
37	(TFO-3) S-P	42.9	33.3	25.9 (38.7)*
38	(TFO-9) S-AnthdR	47.3	35.1	28.1 (42.4)*
39	(TFO-10) S-AnthdR-NH_2	46.9	35.8	28.1 (41.6)*
40	(TFO-11) S-AnthThr	48.9	37.0	30.1
41	(TFO-12) S-AmBuPyr	43.4	34.5	27.0 (40.8)*
42	(TFO-13) S-ThrPyr	47.2	36.1	28.4 (41.7)*
43	(TFO-14) S-MeAcPyr	49.1	37.4	30.2
44	(TFO-15) S-DAEdR	43.9	33.3	< 20
45	(TFO-16) S-Pam	42.2	31.7	24.8
Target duplex DCG; 3′-CG base pair with CpA intercalation site				
46	(TFO-4) R^{An}-P	37.2	26.3	< 20 (33.6)*
47	(TFO-17) R^{An}-AnthdR	47.5	35.1	28.3 (42.8)*
48	(TFO-18) R^{An}-AnthdR-NH_2	45.6	34.8	27.4 (41.8)*
49	(TFO-19) R^{An}-AmBuPyr	43.7	31.8	25.0 (38.8)*
50	(TFO-20) R^{An}-ThrPyr	42.8	33.3	25.9 (39.2)*
Target duplex DTA 3′-TA base pair with TpA intercalation site				
51	(TFO-4) R^{An}-P	37.4	27.1	< 20 (33.3)*

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Table 2. (Continued)

	TFO description	pH 5.5[a]	pH 6.2[b]	pH 6.6[b]
52	(TFO-17) R^{An}-AnthdR	54.5	39.2	32.6 (49.1)*
53	(TFO-18) R^{An}-AnthdR-NH_2	47.0	35.6	28.5 (43.4)*
54	(TFO-19) R^{An}-AmBuPyr	44.6	34.0	26.3 (40.8)*
55	(TFO-20) R^{An}-ThrPyr	42.8	31.7	24.3 (37.5)*

[a] 10 mM acetate buffer (AcOH/AcONa), NaCl (200 mM) adjusted at pH 5.5; [b] 10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4), NaCl (200 mM) adjusted at pH 6.2 or 6.6; melting temperature (T_m)* was performed at pH 6.6 in phosphate buffer (10 mM), NaCl (200 mM) in the presence of spermine (2 mM). Base at 3′-end of TFO indicated by C (cytosine), T (thymidine), S (TA recognition, Figure 3 A), R^{An} (CG recognition, Figure 3 B).

Table 3. UV melting study of 5′- and 5′,3′-modified TFOs (T_m).[a]

	TFO description	pH 6.2	pH 6.6	pH 7
Target duplex DTA; 3′-TA base pair with TpA intercalation site				
1	(TFO-1) T-P	27.3	< 20	< 20
2	(TFO-7) T-AnthdR	35.6	28.4	22.0
3	(TFO-8) T-ThrPyr	34.4	27.9	< 20
4	(TFO-21) 5′-AnthHex	39.0	30.7	26.0
5	(TFO-22) 5′-AnthHex-T-3′-AnthdR	48.0	39.9	35.0
6	(TFO-23) 5′-AnthHex-T-3′-ThrPyr	48.7	39.5	35.1
7	(TFO-24) 5′-AnthThr	31.3	23.4	< 20
8	(TFO-25) 5′-AnthThr-T-3′-AnthdR	42.5	36.7	29.1
9	(TFO-26) 5′-AnthThr-T-3′-ThrPyr	40.7	32.9	27.1
Target duplex DAT1; 3′-AT base pair with ApA intercalation site				
10	(TFO-1) T-P	34.8	27.6	< 20
11	(TFO-7) T-AnthdR	40.5	32.5	26.2
12	(TFO-8) T-ThrPyr	40.0	32.1	25.4
13	(TFO-21) 5′-AnthHex	44.1	35.6	31.3
14	(TFO-22) 5′-AnthHex-T-3′-AnthdR	52.3	44.7	40.7
15	(TFO-23) 5′-AnthHex-T-3′-ThrPyr	51.6	43.3	39.5
16	(TFO-24) 5′-AnthThr	37.4	31.9	24.9
17	(TFO-25) 5′-AnthThr-T-3′-AnthdR	47.4	41.3	33.3
18	(TFO-26) 5′-AnthThr-T-3′-ThrPyr	46.5	40.6	32.7
Target duplex DAT2; 3′-AT base pair with ApT intercalation site				
19	(TFO-1) T-P	34.7	27.1	< 20
20	(TFO-7) T-AnthdR	40.7	32.4	25.4
21	(TFO-8) T-ThrPyr	40.1	32.1	25.5
22	(TFO-21) 5′-AnthHex	44.1	35.6	31.2
23	(TFO-22) 5′-AnthHex-T-3′-AnthdR	52.0	44.5	38.4
24	(TFO-23) 5′-AnthHex-T-3′-ThrPyr	51.4	43.7	36.9
25	(TFO-24) 5′-AnthThr	37.3	31.7	24.5
26	(TFO-25) 5′-AnthThr-T-3′-AnthdR	47.3	41.1	33.0
27	(TFO-26) 5′-AnthThr-T-3′-ThrPyr	46.3	40.3	32.7

[a] 10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4), NaCl (200 mM) adjusted at pH 6.2, 6.6 or 7.

in vivo applications and it is noteworthy that stable triplexes were obtained at this pH without recourse to any modified bases in the TFOs (e.g., entry 14).

Short TFOs often have to be used in vivo due to the limited availability of genomic GA-rich sequences, so we investigated the importance of TFO length on triplex stability. A series of shorter TFOs were prepared (Table 4). As expected shortening TFO length from 18 to 12 bases greatly decreased the stability of the unmodified control TFO-27 (T–P), which did not form

Table 4. UV melting study of short (12-mer) TFOs (T_m).

	TFO description	pH 5.5	pH 6.2	pH 6.6
Target duplex DAT1; 3′-AT base pair with ApA intercalation site				
1	(TFO-27) T-P	< 20	< 20	< 20 (22.3)*
2	(TFO-28) 5′-AnthHex-T-3′-ThrPyr	47.1	42.1	38.3 (52.0)*
Target duplex DAT2; 3′-AT base pair with ApT intercalation site				
3	(TFO-27) T-P	< 20	< 20	< 20 (25.3)*
4	(TFO-28) 5′-AnthHex-T-3′-ThrPyr	46.9	41.9	37.6 (51.5)*
Target duplex DTA; 3′-TA base pair with TpA intercalation site				
5	(TFO-27) T-P	< 20	< 20	< 20 (< 20)*
6	(TFO-28) 5′-AnthHex-T-3′-ThrPyr	39.7	35.1	30.7 (42.3)*

Buffer, pH 5.5: 10 mM acetate (AcOH/AcONa) with NaCl (200 mM); buffer, pH 6.2 and 6.6: 10 mM phosphate (Na_2HPO_4/NaH_2PO_4), mixed to the desired pH in the presence NaCl (200 mM); *: melting experiments performed at pH 6.6 in phosphate buffer (10 mM), NaCl (200 mM) and spermine (2 mM).

stable triplexes between pH 5.5 and 6.6 (entries 1, 3, 5). However, when the TFO was modified with 5′-anthraquinone and 3′-threoninylpyrene (Table 4, entries 2, 4, 6 vs. 1, 3, 5) an impressive increase in stability of up to 30 °C was achieved; this illustrates the potent stabilising effect of the 5′,3′ modifications.

We also examined TFO binding to a mismatched triplet sequence by introducing a CG base pair in the centre of the target duplex in place of AT (Table 5). This produces an unstable TCG triplet in place of TAT. The modified TFOs showed excellent discrimination between the correct and incorrect duplexes; a difference in triplex T_m of ~10 °C was observed for both 3′- and 5′,3′-modified TFOs. This is a clear demonstration that terminally modified TFOs are able to discriminate between matched and mismatched sequence, and are therefore highly selective for the correct target duplex.

Table 5. TFO specificity at pH 6.6.[a]

5′-GTGTTAGGAAGAG**X**AAAAAGAA**AA**GGT
H
3′-CACAATCCTTCTC**Y**TTTTTCTT**TT**CCA

	TFO description	XY = AT(DAT1)	XY = CG
1	(TFO-1) T-P	27.6	< 20
2	(TFO-7) T-AnthdR	32.5	22.0
3	(TFO-8) T-ThrPyr	32.1	21.9
4	(TFO-21) 5′-AnthHex	35.6	27.0
5	(TFO-22) 5′-AnthHex-T-3′-AnthdR	44.7	33.5
6	(TFO-23) 5′-AnthHex-T-3′-ThrPyr	43.3	32.1
7	(TFO-27) short T-P	< 20	< 20
8	(TFO-28) short 5′-AnthHex-T-3′-ThrPyr	38.3	< 20

[a] 10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4), NaCl (200 mM).

Conclusions

For the first time we have shown that 3′-modified TFOs can greatly increase the stability of parallel triplexes in a variety of

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base-stacking environments. In addition, a number of other conclusions can be drawn from this study. The 3′ anthraquinones are slightly more stabilizing than pyrenes, although the differences are small. Within the limitations of this study, the nature of the attachment of the 3′ intercalator to the DNA backbone does not have a major influence on triplex stability, although excessively long linkers should probably be avoided. The 3′ intercalators of the kind used in this study can stabilize triplexes containing a wide range of base steps at the T–D junction. Greater stabilization can be achieved by using TFOs with S or R^{An} in combination with 3′ intercalators to target TA inversions rather than CG at the T–D junction, possibly because the 3′ modification can intercalate into TpN steps more readily than CpN steps. This work brings into question, but does not disprove the theory that TFOs dissociate from the duplex by a mechanism that involves fraying exclusively from the 5′-end. The most impressive results were obtained from TFOs with both 5′- and 3′ intercalators, provided that the linker between the 5′ modification and the TFO is sufficiently long to permit intercalation into the duplex. Selectivity for the correct target duplex is excellent, and such TFOs can produce triplexes with melting temperatures above 40 °C at pH 7 even though they do not contain any triplex stabilizing base analogues. These potent triplex-stabilizing modifications have important applications in biology and nanotechnology. In order to fully understand their properties, work is in progress to investigate the effects of these analogues on the kinetics of triplex formation.

Experimental Section

General: Reagents for chemical synthesis were purchased from Sigma–Aldrich or Fluka and were used without further purification. DNA phosphoramidite monomers, solid supports (including phosphate-on synthesis columns for 3′-phosphate addition) and additional reagents were purchased from Link Technologies, Ltd. or Applied Biosystems, Ltd. Dichloromethane, triethylamine, acetonitrile and pyridine were distilled over calcium hydride, and tetrahydrofuran (THF) was distilled over sodium wire and benzophenone shortly before use. All nucleosides and threoninol analogues were dried twice by coevaporation with freshly distilled pyridine just before use, and all reactions were carried out under an atmosphere of argon by using glassware that had been dried at 120 °C, overnight. Flash chromatography was performed on silica (40–63 μm) purchased from Fisher Scientific, and thin layer chromatography was performed on Merck Kieselgel 60 F_{254} coated plates (0.22 mm thickness, aluminium backed). Compounds were visualized by staining with vanillin (2 g of vanillin in 100 mL of EtOH/H_2SO_4 98:2) and by ultraviolet absorbance at 254 nm. The ^{1}H and ^{13}C NMR spectra were recorded by using a Bruker AV300 or a Bruker DPX 400 spectrometer. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. Chemical shifts are given in ppm relative to tetramethylsilane; *J* values are given in Hz and corrected to within 0.5 Hz. Multiplicities of ^{13}C signals were determined by using the DEPT spectral editing technique. High-resolution mass spectra were recorded in acetonitrile, methanol or water (HPLC grade) by using the electrospray technique with a Bruker APEX III FT-ICR mass spectrometer.

5-*O*-(4,4′-Dimethoxytrityl)-1-β-*O*-(hexylamidopropylpyrene-1-yl)-2-deoxy-D-ribofuranose (2): The aminohexyl deoxyribose derivative **1**[38] (1.4 g, 2.7 mmol) and pyrene-1-butyric acid (0.86 g, 3.0 mmol) were coevaporated separately three times with freshly distilled pyridine and dried under high vacuum, overnight. Compound **1** was dissolved in CH_2Cl_2 (20 mL), NEt_3 (1.5 mL) then 1-pyrenebutyric acid and HOBt (0.57 g, 4.2 mmol) were added. To the stirred mixture, a solution of DCC (1.1 g, 5.4 mmol) in CH_2Cl_2 (5 mL) was added dropwise. The reaction was stirred, overnight, at room temperature, filtered and CH_2Cl_2 (100 mL) was added to the filtrate, which was washed with sat. aq. $NaHCO_3$ (3×50 mL) followed by NaOH (2 M; 3×50 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated to give a pale-yellow oil. The crude product was purified by column chromatography on silica by using a gradient of EtOAc in hexane (from 50 to 100% of EtOAc). Compound **2** (1.7 g) was obtained in 76% yield as a white foam. R_f (EtOAc/hexane 60:40) 0.12; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.36–8.34 (d, *J* = 9.0 Hz, 1H; pyrene), 8.21–8.02 (m, 6H; pyrene), 7.92–7.90 (d, *J* = 7.5 Hz, 1H; pyrene), 7.50–7.47 (d, *J* = 7.5 Hz, 1H; pyrene), 7.37–7.24 (m, 9H; DMTr), 6.85–6.84 (m, 4H; DMTr), 5.17–5.15 (dd, J_1 = 2.0 Hz, J_2 = 5.0 Hz, 1H; H1′), 4.47–4.43 (m, 1H; H4′), 4.00–3.96 (m, 1H; H3′), 3.80 (s, 6H; CH_3-O), 3.65–3.6 (m, 2H; H5′), 3.46–3.43 (m, 2H; CH_2), 3.35–3.30 (m, 2H; CH_2), 3.24–3.17 (m, 2H; CH_2), 2.28–2.22 (m, 4H; CH_2), 2.21–2.17 (m, 1H; H2′), 2.10–2.04 (m, 1H; H2′), 1.47–1.40 (m, 4H; CH_2), 1.27–1.26 (m, 4H; CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 181.9 (*C*O), 172.8 (*C*-pyrene), 158.8 (*C*-DMTr), 131.8 (*C*-pyrene), 130.5, 127.9 (*C*H-pyrene, *C*H-DMTr), 127.2, 125.3 (*C*-pyrene, *C*-DMTr), 123.8 (*C*H-DMTr), 113.5 (*C*H-DMTr), 104.2 (*C*-DMTr), 86.6 (C1′), 84.9 (C4′), 73.9 (C3′), 68.0 (CH_2), 65.5 (C5′), 55.6 (CH_3-O), 41.6 (CH_2), 39.8 (C2′), 36.48, 33.17, 29.9, 27.8, 27.0, 26.1 (CH_2); *m/z* (%) LRMS [ES$^+$, MeOH] 828.6 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+Na]$^+$ ($C_{52}H_{55}NO_7Na$): calcd 828.3876; found 828.3871.

5-*O*-(4,4′-Dimethoxytrityl)-1-β-(acetamidomethylpyrene-1-yl)-2-deoxy-D-ribofuranose (4): 5-Dimethoxytrityl-2-deoxy-D-ribofuranose-1-β-ethanoic acid (**3**;[39] 0.1 g, 0.21 mmol) was dissolved in anhydrous DMF (700 μL) at room temperature in the presence of HBTU (0.16 g, 0.42 mmol). 1-Pyrenemethylamine hydrochloride (56 mg, 0.21 mmol) and DMAP (51 mg, 0.42 mmol) were dissolved in DMF (300 μL) in a separated flask and added dropwise to the activated acid. The reaction was stirred for 4 h (TLC CH_2Cl_2/MeOH 90:10) and diluted by adding CH_2Cl_2 (10 mL) to the mixture. The organic phase was washed with sat. aq. $NaHCO_3$ (5 mL), H_2O (5 mL) and brine (5 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by column chromatography on silica with a gradient of MeOH (from 0 to 5%) in CH_2Cl_2 to give compound **4** (94 mg) in 65% yield as a yellow foam. R_f (CH_2Cl_2/MeOH 90:10) 0.59; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.22–7.86 (m, 9H; pyrene), 7.37–7.11 (m, 9H; DMTr), 6.72–6.68 (m, 4H; DMTr), 5.20–5.06 (m, 2H; CH_2-NH), 4.52–4.46 (m, 1H; H1′), 4.21–4.19 (m, 1H; H3′), 3.91–3.83 (m, 1H; H4′), 3.67 (s, 6H; CH_3-O), 3.06 (ddd, J_1 = 4.7 Hz, J_2 = 5.1 Hz, J_3 = 17.9 Hz, 2H; CH_2-CO), 2.56 (ddd, J_1 = 3.5 Hz, J_2 = 8.2 Hz, J_3 = 17.9 Hz, 2H; H5′), 2.05–1.98 (m, 1H; H2′), 1.89–1.80 (m, 1H; H2′); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 170.4 (*C*O), 158.4 (*C*-DMTr), 144.6 (*C*-DMTr), 135.8 (*C*-pyrene), 126.0 (*C*-pyrene), 125.3 (*C*H-DMTr), 124.9 (*C*-pyrene), 124.8 (*C*-DMTr), 124.7 (*C*-pyrene), 122.8 (*C*H-DMTr), 113.0 (*C*H-DMTr), 86.0 (C1′), 74.9 (C3′), 73.9 (C4′), 64.3 (C5′), 55.1 (CH_3-O), 42.5 (CH_2-N), 41.6 (CH_2-O), 40.7 (C2′); *m/z* (%) LRMS [ES$^+$, MeOH]: 715.0 (100) [*M*+Na$^+$]. *m/z* HRMS [*M*+Na$^+$] ($C_{45}H_{41}NO_6Na$): calcd 714.2832; found 714.2826.

1-*O*-(4,4′-Dimethoxytrityl)-L-threoninol (5): L-Threoninol ((2*R*,3*R*)-2-amino-1,3-butanediol; 1.0 g, 9.6 mmol) was dried by coevaporation three times with freshly distilled pyridine in the presence of

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DMAP (0.6 g, 4.8 mmol) then further dried under high vacuum, overnight. This mixture was then dissolved in dry pyridine (30 mL) and 4,4′-dimethoxytrityl chloride (4.9 g, 14.4 mmol) was added in several portions. The reaction was stirred at room temperature for 3 h and monitored by TLC (CH_2Cl_2/MeOH/NEt_3 94:5:1). Upon completion the reaction was terminated by adding MeOH (5 mL) and NEt_3 (1 mL). The reaction mixture was evaporated to dryness and dissolved in CH_2Cl_2 (50 mL). The organic phase was washed with sat. aq. $NaHCO_3$ (3×50 mL), H_2O (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified on silica by using a gradient of MeOH (0% to 10%) in CH_2Cl_2 with NEt_3 (1%) to give compound **5** (2.5 g) in 65% yield as a yellow oil. R_f (CH_2Cl_2/MeOH/NEt_3 94:5:1) 0.27; ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.12 (s, 2H; NH_2), 7.33–7.11 (m, 9H; DMTr), 6.74–6.72 (m, 4H; DMTr), 3.63 (s, 6H; CH_3-O), 3.58–3.50 (m, 1H; C*H*), 3.16–2.97 (m, 2H; CH_2), 2.57–2.53 (m, 1H; C*H*), 0.98 (s, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ=158.9 (*C*-DMTr), 150.1 (*C*-DMTr), 145.2 (*C*-DMTr), 136.4 (*C*-DMTr), 130.4, 128.7, 128.5, 128.2, 127.2 (*C*H-DMTr), 86.5 (*C*-DMTr), 68.4 (*C*H), 66.3 (CH_2), 57.5 (*C*H), 55.6 (CH_3-O), 20.3 (CH_3); *m/z* (%) LRMS [ES$^+$, MeOH] 430.4 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+Na]$^+$ ($C_{25}H_{29}NO_4Na$): calcd 430.1994; found 430.1994.

1-*O*-(4,4′-Dimethoxytrityl)-L-threoninylamidoprop-1-yl pyrene (6): 1-*O*-(4,4′-Dimethoxytrityl)-L-threoninol (**5**; 2.1 g, 5.1 mmol) and the commercially available pyrene-1-butyric acid (1.4 g, 4.9 mmol) were coevaporated separately with freshly distilled pyridine and dried under high vacuum, overnight. The pyrene-1-butyric acid was dissolved in DMF (10 mL) and HBTU (3.8 g, 10.2 mmol) was added followed by DMAP (0.6 g, 5.1 mmol). The reaction was stirred for 15 min at room temperature then a solution of **5** in DMF (7 mL) was added dropwise. The reaction was stirred for 4 h and followed by TLC (EtOAc/hexane 60:40). CH_2Cl_2 (50 mL) was added and the organic phase was washed with sat. aq. $NaHCO_3$ (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude mixture was purified on silica and eluted with a gradient of EtOAc (40 to 80%) in hexane. Title compound **6** was obtained as a white foam in 57% yield (1.9 g). R_f (EtOAc/hexane 60:40) 0.53; ^{1}H NMR (400 MHz, $CDCl_3$) δ=8.36–7.91 (m, 9H; pyrene), 7.43–7.19 (m, 9H; DMTr), 6.85–6.82 (m, 4H; DMTr), 4.16–4.15 (m, 1H; C*H*), 4.08–4.04 (m, 1H; C*H*), 3.73 (s, 6H; CH_3-O), 3.49–3.36 (m, 4H; CH_2), 2.44–2.40 (m, 2H; CH_2), 2.32–2.10 (m, 2H; CH_2), 1.22 (s, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ=173.4 (*C*O-NH), 159.0 (*C*-DMTr), 150.2 (*C*-pyrene), 144.8, 136.3 (*C*-DMTr), 136.2, 135.8, 135.7, 131.8, 131.4 (*C*-pyrene), 130.4 (*C*H-DMTr), 130.3 (*C*-pyrene), 128.4, 128.3 (*C*H-DMTr), 127.9 (*C*H-pyrene), 127.8, 127.4 (*C*-DMTr), 127.1, 126.2 (*C*H-DMTr), 125.2, 123.8 (*C*H-pyrene), 113.7 (*C*H-DMTr), 87.2 (*C*-DMTr), 69.1 (*C*H), 65.7 (CH_2), 55.6 (*C*H), 53.8 (CH_3-O), 36.7, 33.3, 28.1 (CH_2), 20.4 (CH_3); *m/z* (%) LRMS [ES$^+$, MeOH] 700.5 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+Na]$^+$ ($C_{45}H_{43}NO_5Na$): calcd 700.3039; found 700.3033.

1-(6-Aminohexyl)aminoanthraquinone (7): A mixture of 1-chloroanthraquinone (2.4 g, 10 mmol) and hexamethylenediamine (3.5 g, 30 mmol) in xylene (100 mL) was heated under reflux. The reaction was followed on TLC (CH_2Cl_2/MeOH 9:1) until completion. After 2 h, the solvent was removed under reduced pressure. The crude product was adsorbed into silica prior to purification on silica gel packed with CH_2Cl_2 and NEt_3 (1%). A gradient of MeOH in CH_2Cl_2 (0 to 8%) gave compound **7** (1.3 g) as a red solid with 42% yield. R_f (CH_2Cl_2/MeOH 90:10) 0.10; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ=9.71 (t, J=5.0 Hz, 1H; N*H*), 8.21 (dd, J_1=1.1 Hz, J_2=7.6 Hz, 1H; Ar-*H*), 8.13 (dd, J_1=1.1 Hz, J_2=7.6 Hz, 1H; Ar-*H*), 7.92–7.82 (m, 2H; Ar-*H*), 7.65 (t, J=7.6 Hz, 1H; Ar-*H*), 7.44 (dd, J_1=0.7 Hz, J_2=7.2 Hz, 1H; Ar-*H*), 7.27 (d, J=8.5 Hz, 1H; Ar-*H*), 3.39–3.34 (m, 4H; CH_2), 2.56–2.53 (m, 2H; CH_2), 1.72–1.65 (m, 2H; CH_2), 1.45–1.41 (m, 4H; CH_2); ^{13}C NMR (100 MHz, [D$_6$]DMSO) δ=180.1 (*C*O), 151.4 (*C*-NH$_2$), 135.6 (*C*-Ar), 134.5, 133.4, 126.4, 126.2, 118.6 (*C*H-Ar), 114.9 (*C*-Ar), 42.1, 41.5, 33.1, 28.6, 26.5, 26.1 (CH_2); *m/z* (%) LRMS [ES$^+$, MeOH] 323.3 (100) [*M*+H]$^+$; *m/z* HRMS [*M*+H]$^+$ ($C_{20}H_{23}N_2O_2$): calcd 323.1760; found 323.1754.

1-(6-Trifluoroacetamidohexylamino)-5-chloroanthraquinone (9): Ethyl trifluoroacetate (1.2 mL, 10 mmol) was added to a solution of 1-(6-aminohexylamino)-5-chloroanthraquinone (**8**;[43] 0.71 g, 2 mmol) and dried NEt_3 (2.8 mL, 20 mmol) in CH_2Cl_2 (20 mL). The reaction was stirred at room temperature for 90 min before the solvent was removed under high vacuum. The crude material was purified on silica gel packed with CH_2Cl_2. A gradient of MeOH (0 to 4%) in CH_2Cl_2 gave compound **9** (0.57 g) as a red solid foam in 63% yield. R_f (CH_2Cl_2) 0.66; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ=9.43–9.40 (t, J=5.2 Hz, 1H; N*H*), 9.38 (s, 1H; N*H*COCF$_3$), 8.11 (dd, J_1=1.6 Hz, J_2=7.4 Hz, 1H; Ar-*H*), 7.78 (dd, J_1=1.6 Hz, J_2=8.0 Hz, 1H; Ar-*H*), 7.73 (q, J=7.6 Hz, 1H; Ar-*H*), 7.53 (t, J=8.0 Hz, 1H; Ar-*H*), 7.26 (d, J=6.7 Hz, 1H; Ar-*H*), 7.08 (d, J=8.5 Hz, 1H; Ar-*H*), 3.26 (q, J=6.4 Hz, 2H; CH_2), 3.19 (q, J=6.2 Hz, 2H; CH_2), 1.62–1.33 (m, 8H; CH_2); ^{13}C NMR (100 MHz, [D$_6$]DMSO) δ=182.2 (*C*O), 181.4 (*C*O), 150.8 (*C*-Ar), 136.3, 135.7, 134.9 (*C*H-Ar), 134.2, 132.9 (*C*-Ar), 126.0, 117.6, 114.9 (*C*H-Ar), 114.5, 111.2 (*C*-Ar), 42.5, 32.1, 28.8, 28.6, 26.5, 26.3 (CH_2); *m/z* (%) LRMS [ES$^+$, MeOH] 475.3 (100) [*M*+Na]$^+$, 927.6 (50) [2*M*+Na]$^+$; *m/z* HRMS [*M*+H$^+$] ($C_{22}H_{21}ClF_3N_2O_3$): calcd 453.1193; found 453.1188.

1-(6-Trifluoroacetamidohexylamino)-5-(6-aminohexylamino)-anthraquinone (10): A mixture of 1-(6-trifluoroacetamidohexylamino)-5-chloroanthraquinone (**9**; 0.11 g, 0.24 mmol) and hexamethylenediamine (0.15 g, 1.25 mmol) in xylene (1 mL) was heated under reflux for 16 h. The solvent was removed under reduced pressure before the crude mixture was purified by flash chromatography and eluted with MeOH (1 to 10%) in CH_2Cl_2 containing NEt_3 (0.5%). Compound **10** (0.11 g) was obtained as a red foam in 82% yield. R_f (hexane/EtOAc 50:50) 0.6; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ=9.65 (t, J=4.0 Hz, 2H; N*H*-COCF$_3$), 7.60 (t, J=7.9 Hz, 2H; Ar-*H*), 7.41 (d, J=7.3 Hz, 2H; Ar-*H*), 7.13 (d, J=8.6 Hz, 2H; Ar-*H*), 3.35–3.29 (m, 8H; CH_2), 3.19 (t, J=7.0 Hz, 4H; CH_2), 2.68 (t, J=7.1 Hz, 2H; CH_2), 1.67–1.33 (m, 10H; CH_2); ^{13}C NMR (100 MHz, [D$_6$]DMSO) δ=184.7 (*C*O), 180.6 (*C*OCF$_3$), 151.5 (*C*-NH), 136.0 (*C*-Ar), 135.9 (*C*H-Ar), 117.5, 114.7 (*C*H-Ar), 112.3 (*C*F$_3$), 42.5, 32.1, 28.9, 28.6, 26.6, 26.3 (CH_2); *m/z* (%) LRMS [ES$^+$, MeOH] 533.4 (100) [*M*+H]$^+$; *m/z* HRMS [*M*+H$^+$] ($C_{28}H_{36}F_3N_4O_3$): calcd 533.2746; found 533.2734.

1-(6-Trifluoroacetamidohexylamino)-5-(6-hydroxyhexylamino) anthraquinone (11): A mixture of 1-(6-trifluoroacetamidohexylamino)-5-chloroanthraquinone (**9**; 1.3 g, 2.8 mmol) and 6-amino-1-hexynol (1.6 g, 14.3 mmol) in xylene (100 mL) was refluxed for 18 h. The solvent was removed under vacuum and the residue was dissolved in CH_2Cl_2 (100 mL) washed with sat. aq. $NaHCO_3$ (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by column chromatography on silica gel packed with CH_2Cl_2. A gradient of acetone (0 to 20%) in CH_2Cl_2 gave the desired compound **11** as a red solid (0.54 g) in 36% yield. R_f (CH_2Cl_2/MeOH 90:10) 0.4; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ=9.76 (t, J=5.2 Hz, 2H; N*H*), 9.50 (bs, 1H; N*H*), 7.71 (t, J=8.0 Hz, 2H; Ar-*H*), 7.52 (dd, J_1=1.0 Hz, J_2=7.5 Hz, 2H; Ar-H), 7.23 (d, J=8.5 Hz, 2H; Ar-*H*), 4.45 (dd, J_1=5.5 Hz, J_2=11.0 Hz, 2H; CH_2), 3.51 (q, J=5.5 Hz, 2H; CH_2), 3.46–3.33 (m, 8H; CH_2), 3.28 (q, J=6.1 Hz, 2H; CH_2), 1.77 (q, J=7.0 Hz, 4H; CH_2), 1.65–1.45 (m, 6H; CH_2); ^{13}C NMR (100 MHz, [D$_6$]DMSO) δ=185.2 (*C*O), 181.9 (*C*OCF$_3$), 152.0 (*C*-NH), 136.5 (*C*-Ar), 136.4 (*C*H-Ar), 117.9 (*C*H-Ar), 115.2 (*C*H-Ar), 112.8 (*C*F$_3$), 61.6, 61.5, 33.4, 32.6, 29.6, 29.43,

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29.1, 27.1, 27.0, 26.8, 26.2 (CH_2); *m/z* (%) LRMS [ES$^+$, MeOH] 556.4 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+H]$^+$ ($C_{28}H_{35}F_3N_3O_4$): calcd 534.2574; found 534.2582.

1-(6-Trifluoroacetamidohexylamino)-5-(6-hexylamino) anthraquinone-2-*O*-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite (12): 2-*O*-Cyanoethyl-bis-diisopropylphosphoramidite (51 μL, 0.15 mmol) was added to a solution of 1-(6-trifluoroacetamidohexylamino)-5-(6-hydroxyhexylamino)anthraquinone (**11**; 84 mg, 0.16 mmol) and DIHT (13 mg, 0.08 mmol) in dry acetonitrile (5 mL). The reaction was stirred for 1 h under argon. The reaction mixture was diluted with degassed EtOAc (5 mL) and washed with sat. aq. $NaHCO_3$ (5 mL) and brine (5 mL). The crude product was purified on basic alumina and eluted with EtOAc containing NEt_3 (1%). Compound **12** was recovered as a red solid (0.14 g) in 86% yield. R_f (EtOAc/hexane 80:20) 0.6 and 0.3 on 0.2 mm alumina plates Alugram® alox N/UV_{254} purchased from Macherey–Nagel. ^{1}H NMR (400 MHz, CD_2Cl_2) δ = 9.70 (bs, 2H; N*H*), 7.56–7.49 (m, 4H; Ar-*H*), 7.02–6.98 (m, 2H; Ar-*H*), 3.89–3.71 (m, 4H; C*H*$_2$), 3.68–3.56 (m, 4H; C*H*$_2$), 3.42–3.31 (m, 8H; C*H*$_2$), 2.67–2.63 (m, 2H; C*H*$_2$), 1.81–1.24 (m, 14H; C*H*$_2$), 1.20 (d, *J* = 6.5 Hz, 12H; CH_3); ^{31}P NMR (121 MHz, CD_2Cl_2) δ = 147.3; *m/z* (%) LRMS [ES$^+$, CH_3CN] 756.7 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+H]$^+$ ($C_{37}H_{52}F_3N_5O_5P$): calcd 734.3658; found 734.3644.

***N*-(6-(9,10-Dihydro-9,10-dioxoanthracen-1-ylamino)hexyl)-5-(4,4′-dimethoxytrityl)-2-deoxy-D-ribofuranose-1-β-acetamide (13)**: Compound **3**[39] (0.30 g, 0.62 mmol) and DMAP (76 mg, 0.62 mmol) were combined and dried by coevaporation with freshly distilled pyridine and then left under high vacuum, overnight. Similarly, aminohexyl anthraquinone (**7**) (0.20 g, 0.62 mmol) was coevaporated with pyridine and dried under high vacuum, overnight. Compound **3** and DMAP were then dissolved in anhydrous DMF (1.5 mL) and HBTU (0.47 g, 1.24 mmol) was added. The mixture was left to stir for 15 min at room temperature under argon atmosphere and a solution of the aminohexyl anthraquinone in anhydrous DMF (500 μL) was added under argon. The reaction was followed by TLC (CH_2Cl_2/MeOH/NEt_3 80:19:1) for 90 min. The reaction was then diluted with CH_2Cl_2 (25 mL). The organic phase was washed with sat. aq. $NaHCO_3$ (10 mL), H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified on silica by using a gradient of MeOH (0 to 10%) in CH_2Cl_2 with NEt_3 (1%). This gave compound **13** (0.33 g) as a dark red foam in 67% yield. R_f (CH_2Cl_2/MeOH 90:10) 0.63; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.75 (t, *J* = 4.0 Hz, 1H; N*H*), 8.32–8.26 (m, 2H; Ar-*H*), 7.81–7.71 (m, 1H; Ar-*H*), 7.62 (d, *J* = 8.0 Hz, 1H; Ar-*H*), 7.50 (t, *J* = 8.0 Hz, 1H; Ar-*H*), 7.41–7.23 (m, 9H; DMTr), 7.06 (d, *J* = 8.5 Hz, 1H; Ar-*H*), 6.87–6.85 (m, 4H; DMTr), 6.51 (dd, J_1 = 3.0 Hz, J_2 = 8.5 Hz, 1H; H1′), 4.36–4.34 (m, 1H; H4′), 4.05–4.02 (m, 1H; H3′), 3.80 (s, 6H; C*H*$_3$-O), 3.71–3.17 (m, 8H; C*H*$_2$), 2.51 (ddd, J_1 = 3.5 Hz, J_2 = 8.5 Hz, J_3 = 15.0 Hz, 2H; H5′), 2.09–2.05 (m, 1H; H2′), 1.88–1.81 (m, 1H; H2′), 1.77–1.70 (m, 2H; C*H*$_2$), 1.54–1.45 (m, 2H; C*H*$_2$), 1.43–1.41 (m, 2H; C*H*$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 185.4 (*C*O), 184.2 (*C*O), 171.1 (*C*O-NH), 158.9 (*C*-DMTr), 152.2 (*C*-Ar), 145.1 (*C*-Ar), 136.4 (*C*-DMTr), 135.7 (*C*H-Ar), 135.1 (*C*-DMTr), 135.3, 133.3 (*C*H-Ar), 130.4, 128.7, 128.5, 128.2 (*C*H-DMTr), 127.1, 118.3, 115.9 (*C*H-Ar), 113.6 (*C*H-DMTr), 86.7 (C1′), 75.5 (C4′), 74.3 (C3′), 64.8 (C5′), 55.6 (*C*H$_3$-O), 43.5, 43.0, 41.3 (*C*H$_2$), 39.7 (C2′), 29.9, 29.4, 27.0 (*C*H$_2$); *m/z* (%) LRMS [ES$^+$, MeOH] 805.6 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+Na]$^+$ ($C_{48}H_{50}N_2O_8Na$): calcd 805.3465; found 805.3459.

***N*-(6-(9,10-Dihydro-9,10-dioxoanthracen-1-ylamino)-5-yl-(6-aminohexyl)hexyl)-5-(4,4′-dimethoxytrityl)-2-deoxy-D-ribofuranose-1-β-acetamide (14)**: Deoxyribose ethanoic acid derivative **3**[39] (0.17 g, 0.35 mmol), DMAP (43 mg, 0.35 mmol) and HBTU (0.26 g, 0.70 mmol) were combined and dissolved in dry pyridine (5 mL). The mixture was coevaporated three times and dried under high vacuum, overnight. In a separate flask bis-aminohexylanthraquinone (**10**; 0.18 g, 0.35 mmol) was also coevaporated three times with dry pyridine, dried under vacuum, overnight, then dissolved in dry pyridine (5 mL). The mixture of **3**, HBTU and DMAP was dissolved in pyridine (1.2 mL) and added dropwise to the solution of **10** in pyridine under argon at room temperature. After 2 h, the reaction mixture was diluted with CH_2Cl_2 (25 mL). The organic phase was washed with sat. aq. $NaHCO_3$ (10 mL), H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude mixture was adsorbed on silica and purified by column chromatography by using a gradient of acetone (0 to 60%) in cyclohexane to give compound **14** (90 mg) as a purple foam in 26% yield. R_f (cyclohexane/acetone 40:60) 0.66; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.74–9.70 (m, 2H; N*H*), 7.57–7.23 (m, 15H; DMTr, Ar-*H*), 6.98 (t, *J* = 7.5 Hz, 2H; Ar-*H*), 6.87–6.85 (m, 4H; DMTr), 6.49 (t, *J* = 5.5 Hz, 2H; Ar-*H*), 4.52–4.47 (m, 1H; H1′), 4.36–4.35 (m, 1H; H4′), 4.05–4.01 (m, 1H; H3′), 3.83 (s, 6H; C*H*$_3$-O), 3.46–3.17 (m, 10H; C*H*$_2$), 2.50 (ddd, J_1 = 3.2 Hz, J_2 = 8.7 Hz, J_3 = 15.2 Hz, 2H; H5′), 2.08–2.01 (m, 2H; H2′), 1.90–1.35 (m, 14H; C*H*$_2$); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 185.9 (*C*O), 185.8 (*C*O), 171.1 (*C*ONH), 158.9 (*C*OCF$_3$), 151.9 (*C*-NH), 145.2 (*C*-Ar), 136.7 (*C*-DMTr), 136.3 (*C*H-Ar), 135.7 (*C*-DMTr), 135.6 (*C*-DMTr), 130.4, 128.7, 128.5, 128.2, 127.3 (*C*H-DMTr), 116.8, 115.2 (*C*H-Ar), 113.5 (*C*H-DMTr), 113.3 (*C*F$_3$), 86.7 (C1′), 75.5 (C4′), 74.4 (C3′), 64.8 (*C*H$_2$-CONH), 55.6 (*C*H$_3$-O), 43.2 (C5′), 43.1, 43.0, 41.3, 40.3, 39.7, 29.9, 29.4, 29.2, 29.2, 27.2, 27.1, 27.0, 26.7 (*C*H$_2$, C2′); *m/z* (%) LRMS [ES$^+$, MeOH] 1015.7 (100) [*M*+Na]$^+$.

1-(4,4′-Dimethoxytrityl)-L-threoninyl-bis-anthraquinone (15): Anthraquinone-2-carboxylic acid (0.20 g, 0.80 mmol), HBTU (0.60 g, 1.6 mmol) and DMAP (97.7 mg, 0.80 mmol) were dissolved in anhydrous DMF (1 mL) and the mixture was stirred for 15 min before the addition of a solution of 1-*O*-(4,4′-dimethoxytrityl)-L-threoninol (**5**; 0.32 g, 0.80 mmol) in DMF (1 mL). The reaction was stirred at room temperature for 2 h (TLC EtOAc/hexane 60:40) and finally diluted with CH_2Cl_2 (10 mL). The organic phase was washed with sat. aq. $NaHCO_3$ (3×10 mL), H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was adsorbed on silica prior to purification by column chromatography with a gradient of EtOAc (5 to 40%) in hexane. Compound **15** (0.52 g) was obtained in 75% yield as a yellow foam. R_f (EtOAc/hexane 60:40) 0.65; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.82 (s, 1H; N*H*), 8.58 (d, *J* = 1.5 Hz, 1H; Ar-*H*), 8.43–8.29 (m, 8H; Ar-*H*), 8.19 (dd, J_1 = 1.5 Hz, J_2 = 8.0 Hz, 1H; Ar-*H*), 7.87–7.81 (m, 4H; Ar-H), 7.47–7.18 (m, 9H; DMTr), 6.92 (d, *J* = 6.0 Hz, 1H; Ar-*H*), 6.81–6.77 (m, 4H; DMTr), 5.87–5.80 (m, 1H; C*H*), 4.73–4.67 (m, 1H; C*H*), 4.16 (q, *J* = 7.0 Hz, 2H; C*H*$_2$), 3.73 (s, 6H; C*H*$_3$-O), 1.54 (d, *J* = 6.5 Hz, 3H; C*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 182.6 (*C*O), 166.2 (*C*ONH), 165.2 (*C*OO), 158.6 (*C*-DMTr), 144.7, 139.8 (*C*-DMTr), 136.6, 135.8, 135.6, 135.4 (*C*-Ar), 135.0, 134.8 (*C*H-Ar), 134.0, 133.9, 133.8, 133.7 (*C*-Ar), 133.0, 130.4, 129.0, 128.5, 128.4, 128.3, 128.0, 127.8, 127.3 (*C*H-Ar, *C*H-DMTr), 125.7, 113.7 (C-DMTr), 113.6 (*C*H-DMTr), 86.8 (*C*-DMTr), 72.2 (*C*H), 62.5 (*C*H$_2$), 55.5 (*C*H$_3$-O), 54.6 (*C*H), 17.8 (*C*H$_3$); *m/z* (%) LRMS [ES$^+$, MeOH] 898.8 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+Na]$^+$ ($C_{55}H_{41}NO_{10}Na$): calcd 898.2628; found 898.2623.

9,10-Dihydro-*N*-(1-(4,4′-dimethoxytrityl)threoninyl)-9,10-dioxoanthracene-1-carboxamide (16): Two methods were followed to prepare compound **16**, either by cleavage of the ester bond of compound **15** or by direct amide coupling of anthraquinone-2-carboxylic acid to compound **5**.

Method 1: Compound **15** (0.31 g, 0.35 mmol) was dissolved in a mixture of CH_2Cl_2 and MeOH (2.4 mL, 1:1, *v/v*) and K_2CO_3 (0.48 g, 3.5 mmol) was added at room temperature. The reaction was stop-

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ped after 1 h by adding H_2O (5 mL) and CH_2Cl_2 (10 mL; TLC EtOAc/hexane 60:40). The organic phase was washed with water (3× 10 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was adsorbed on silica prior to purification by column chromatography by using a gradient of EtOAc (5 to 50%) in hexane. Compound **16** (0.22 g) was obtained as a pale yellow foam in 95% yield.

Method 2: Anthraquinone-2-carboxylic acid (0.2 g, 0.80 mmol) was dissolved in anhydrous DMF (1.6 mL) before the addition of EDC (0.23 g, 1.20 mmol) at room temperature. The reaction was stirred for 15 min before addition of a solution of compound **5** (0.32 g, 0.80 mmol) in DMF (1 mL). The reaction was followed by TLC (EtOAc/hexane 60:40) then diluted after 2 h with CH_2Cl_2 (10 mL). The organic phase was washed with sat. aq. $NaHCO_3$ (10 mL), H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude mixture was adsorbed on silica prior to purification by column chromatography by using a gradient of EtOAc (5 to 60%) in hexane to give compound **16** (0.35 g) in 70% yield as a yellow foam. R_f (EtOAc/hexane 60:40) 0.51; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.64$ (d, $J = 1.9$ Hz, 1H; N*H*), 8.41 (d, $J = 10.7$ Hz, 1H; Ar-*H*), 8.35–8.32 (m, 2H; Ar-*H*), 8.26 (dd, $J_1 = 1.9$ Hz, $J_2 = 10.7$ Hz, 1H; Ar-*H*), 7.85–7.82 (m, 2H; Ar-H), 7.43–7.21 (m, 9H; DMTr), 7.01 (d, $J = 8.8$ Hz, 1H; Ar-*H*), 6.85–6.81 (m, 4H; DMTr), 4.29–4.20 (m, 2H; C*H*), 3.77 (s, 6H; CH_3-O), 3.53 (ddd, $J_1 = 4.6$ Hz, $J_2 = 3.6$ Hz, $J_3 = 24.9$ Hz, 2H; CH_2), 1.26 (d, $J = 6.0$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) $\delta = 182.5$ (*C*O), 182.3 (*C*O), 166.0 (*C*O-NH), 158.6 (*C*-DMTr), 144.3 (*C*-DMTr), 139.5 (*C*-DMTr), 144.3, 139.5 (*C*-DMTr), 135.4, 135.3 (*C*-Ar), 134.4 (*C*H-Ar), 133.6, 133.4 (*C*-Ar), 132.8, (*C*H-Ar), 129.9, 128.0 (*C*H-DMTr), 127.4, 127.1 (*C*H-Ar), 125.2 (*C*H-DMTr), 87.0 (*C*-DMTr), 68.4 (*C*H), 65.0 (CH_2), 55.2 (CH_3-O), 54.3 (*C*H), 20.2 (CH_3); *m/z* (%) LRMS [ES$^+$, MeOH] 664.5 (100) [*M*+Na$^+$]. *m/z* HRMS [*M*+Na]$^+$ ($C_{40}H_{35}NO_7Na$): calcd 664.2311; found 664.2306.

9,10-Dihydro-*N*-(1-(4,4′-dimethoxytrityl)-3-succinylthreoninyl)-9,10-dioxoanthracene-1-carboxamide (17): DMTr-protected L-threoninylanthraquinone (**16**; 0.16 g, 0.25 mmol) was dissolved in pyridine (1 mL) before the addition of DMAP (15 mg, 0.12 mmol) and succinic anhydride (37 mg, 0.37 mmol) successively. The mixture was stirred at room temperature for 7 h. The reaction was diluted with CH_2Cl_2 (20 mL) and the organic phase was washed with H_2O (3×5 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was adsorbed on silica prior to purification by column chromatography and elution with a gradient of EtOAc (50 to 70%) in hexane to give compound **17** (70.6 mg) as a white foam in 38% yield. R_f (EtOAc/hexane 80:20) 0.51; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.70$ (s, 1H; N*H*), 8.42–8.33 (m, 4H; Ar-*H*), 7.88–7.83 (m, 2H; Ar-*H*), 7.46–7.21 (m, 9H; DMTr), 7.08 (dd, $J = 9.0$ Hz, 1H; Ar-*H*), 6.86–6.83 (m, 4H; DMTr), 5.53–5.47 (m, 1H; C*H*), 4.58–4.52 (m, 1H; C*H*), 3.80 (s, 6H; CH_3-O), 3.37–3.30 (m, 2H; CH_2), 2.68–2.52 (m, 4H; succinyl CH_2), 1.34 (d, $J = 6.0$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 183.7$ (*C*O), 181.6 (*C*O), 172.3 (*C*O-O), 166.1 (*C*O-NH), 158.9 (*C*-DMTr), 144.9 (*C*-DMTr), 139.9 (*C*-DMTr), 136.1 (*C*-Ar), 135.1, 134.8, 134.0 (*C*H-Ar), 133.9, 133.8 (*C*-Ar), 130.4, 128.5, 128.3, 128.3, 127.9, 127.8, 127.2, 126.1 (*C*H-DMTr, *C*H-Ar), 113.6 (*C*H-DMTr), 86.6 (*C*-DMTr), 70.2 (*C*H), 62.6 (CH_2), 55.6 (CH_3-O), 54.4 (*C*H), 31.0 (CH_2), 17.7 (CH_3); *m/z* (%) LRMS [ES$^+$, MeOH] 764.5 (100) [*M*+Na]$^+$; *m/z* HRMS [*M*+Na]$^+$ ($C_{44}H_{39}NO_{10}Na$): calcd 764.2472; found 764.2466.

9,10-Dihydro-*N*-(1-(4,4′-dimethoxytrityl)threoninyl)-9,10-dioxoanthracene-1-carboxamide-2-*O*-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite (18): Compound **17** (0.24 g, 0.37 mmol) was dissolved in CH_2Cl_2 (3 mL) and DIPEA (260 μL, 1.48 mmol) was added. The mixture was stirred for 10 min at room temperature before addition of [N(*i*Pr)$_2$]P(Cl)O(CH$_2$)$_2$CN (124 μL, 0.55 mmol) under argon. The reaction was then stirred for 4 h and stopped by adding aqueous KCl. The organic phase was washed with sat. aq. KCl (20 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified on silica gel packed with CH_2Cl_2 and NEt_3 (1%) by using EtOAc (10%) in CH_2Cl_2. Fractions were collected, evaporated and dried over high vacuum pump for 2 h. The title compound was redissolved in CH_2Cl_2 (3 mL) and added dropwise to cold hexane (100 mL) under argon. Hexane was removed and the formed precipitate was redissolved in CH_3CN, filtered through Millipore® filter and evaporated to dryness. Compound **18** (0.12 g, 0.14 mmol, 38%) was redissolved in benzene and freeze dried, overnight, under high vacuum pump. R_f (CH_2Cl_2/EtOAc 90:10) 0.51; ^{1}H NMR (400 MHz, CD_2Cl_2): $\delta = 8.61$ (s, 1H; Ar-H), 8.37–8.29 (m, 4H; Ar-*H*), 7.90 (m, 2H; Ar-*H*), 7.49–7.24 (m, 9H; DMTr), 7.24–6.84 (m, 4H; DMTr), 4.50–4.46 (m, 1H; C*H*), 4.42–4.34 (m, 1H; C*H*), 3.80 (s, 6H; CH_3-O), 3.78–3.70 (m, 2H; CH_2-O), 3.62–3.54 (m, 2H; CH_2-CN), 3.36–3.26 (m, 2H; CH_2), 2.66 (t, $J = 6.2$ Hz, 1H; C*H*), 2.55 (q, $J = 5.8$ Hz, 1H; C*H*), 1.16–1.14 (m, 12H; CH_3), 1.04 (d, $J = 8$ Hz, 3H; CH_3); ^{31}P NMR (121 MHz, CD_2Cl_2) = 148.38, 147.96. *m/z* (%) LRMS [ES$^+$, CH_3CN] 864.3 (50) [*M*+Na]$^+$, 880.3 (50) [*M*+K]$^+$; *m/z* HRMS [*M*+Na]$^+$ ($C_{49}H_{52}N_3O_8PNa$): calcd 864.3390; found 864.3384.

5-*O*-(4,4′-Dimethoxytrityl-1-β-dimethylaminoethylacetamido-2-deoxy-D-ribofuranose (19): 5-*O*-Dimethoxytrityl-2-deoxyribose-1-ethanoic acid (**3**;[39] 0.1 g, 0.21 mmol) was dissolved in anhydrous DMF (800 μL) and HBTU (0.16 g, 0.41 mmol) and DMAP (25 mg, 0.21 mmol) were added. The mixture was stirred for 15 min at room temperature before addition of *N*,*N*-dimethylethylendiamine (18.3 mg, 0.21 mmol) in DMF (1 mL). The reaction was stirred, overnight, at room temperature and monitored by TLC (CH_2Cl_2/MeOH 80:20), then diluted by adding CH_2Cl_2 (5 mL). The organic phase was washed with sat. aq. $NaHCO_3$ (10 mL), H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by column chromatography on silica and eluted with a gradient of MeOH (0.25 to 3%) in CH_2Cl_2 with NEt_3 (1%) to give compound **19** (41 mg) in 35% yield as a yellow foam. R_f (CH_2Cl_2/MeOH 80:20) 0.44; ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 7.45–7.20$ (m, 9H; DMTr), 6.84–6.81 (m, 4H; DMTr), 4.55–4.46 (m, 1H; H1′), 4.30–4.27 (m, 1H; H4′), 3.98–3.94 (m, 1H; H3′), 3.79 (s, 6H; CH_3-O), 3.38 (q, $J = 6.3$ Hz, 2H; CH_2-CO), 3.16 (ddd, $J_1 = 4.7$ Hz, $J_2 = 5.5$ Hz, $J_3 = 21.6$ Hz, 2H; H5′), 2.52–2.45 (m, 4H; CH_2), 2.27 (s, 6H; CH_3), 2.04–1.98 (m, 1H; H2′), 1.84–1.74 (m, 1H; H2′); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 170.8$ (*C*ONH), 158.5 (*C*-DMTr), 144.8 (*C*-DMTr), 136.0 (*C*-DMTr), 130.0, 128.2, 127.8, 126.8, 113.1 (*C*H-DMTr), 86.1 (C1′), 74.9 (C4′), 74.1 (C3′), 64.6 (CH_2-CO), 57.8 (C5′), 55.2 (CH_3-O), 44.9 (CH_3-N), 42.5 (CH_2-NH), 40.7 (C2′), 36.6 (CH_2-N); *m/z* (%) LRMS [ES$^+$, MeOH] 549.5 (100) [*M*+H]$^+$; *m/z* HRMS [*M*+H$^+$] ($C_{32}H_{41}N_2O_6$): calcd: 549.2965; found 549.2959.

5-*O*-(4,4′-Dimethoxytrityl)-1-β-acetamido-*N*-(2-diethylaminoethyl)-benzamido-2-deoxy-D-ribofuranose (20): 5-*O*-Dimethoxytrityl-2-deoxyribose-1-ethanoic acid (**3**;[39] 0.1 g, 0.21 mmol), EDC (80 mg, 0.42 mmol) and DMAP (25 mg, 0.21 mmol) were dissolved in freshly distilled pyridine (500 μL) and the mixture was stirred for 15 min before the addition of a solution of procainamide hydrochloride (57 mg, 0.21 mmol) in pyridine (200 μL). The reaction was stirred, overnight, then diluted by the addition of CH_2Cl_2 (5 mL) and sat. aq. $NaHCO_3$ (5 mL). The organic phase was washed with H_2O (5 mL) and brine (5 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by column chromatography on silica by using a gradient of MeOH (1 to 17%) in CH_2Cl_2 with NEt_3 (1%) to give compound **20** (62 mg) as a yellow foam in 43% yield. R_f (CH_2Cl_2/MeOH 80:20) 0.55; ^{1}H NMR (400 MHz, $CDCl_3$)

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$\delta = 8.72$ (s, 1H; N*H*), 7.77–7.17 (m, 13H; DMTr, Ar-*H*), 6.86–6.83 (m, 4H; DMTr), 4.61–4.58 (m, 1H; H1′), 4.39–4.37 (m, 1H; H4′), 4.13–4.10 (m, 1H; H3′), 3.80 (s, 6H; CH_3-O), 3.57–3.53 (m, 2H; CH_2-NH), 3.30–3.27 (m, 2H; CH_2-CO), 2.79–2.59 (m, 8H; CH_2-N and H5′), 2.13–2.08 (m, 1H; H2′), 1.94–1.87 (m, 1H; H2′), 1.11 (t, $J = 7.0$ Hz, 6H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 181.5$ (*C*ONH), 158.9 (*C*-DMTr), 145.1 (*C*-DMTr), 141.2 (*C*-Ar), 136.1 (*C*-DMTr), 130.4, 128.7, 128.4, 128.3 (*C*H-DMTr), 127.3, 119.6 (*C*H-Ar), 113.6 (*C*H-DMTr), 87.1 (*C*-DMTr), 86.7 (C1′), 75.2 (C4′), 74.0 (C3′), 64.7 (C5′), 55.6 (CH_3-O), 51.9, 47.3, 44.0, 41.4 (CH_2), 37.5 (C2′), 12.0 (CH_3); *m/z* (%) LRMS [ES$^+$, MeOH] 696.5 (100) $[M+H]^+$; *m/z* HRMS $[M+H]^+$ ($C_{41}H_{50}N_3O_7$): calcd 696.3649; found 696.3643.

Resin functionalization: Monomers for 3′-oligonucleotide modification were immobilized on aminolink resin (LCAA-CPG-NH_2, 92 μmol g^{-1} amine loading, Link Technologies, Ltd.). The resin was activated by conversion to the succinic acid derivative either under microwave conditions[55] or by using a nonmicrowave procedure at room temperature.[56] The microwave activation was carried out as follows. LCAA-CPG-NH_2 (0.5 g, 46 μmol) was treated with succinic anhydride (0.4 g, 4 mmol) dissolved in DMF (1 mL) in the presence of DMAP (50 mg, 0.4 mmol). Three cycles of 30 s microwave excitation were performed on a CEM Explorer® 24 positions microwave at 150 °C and 300 W. The resin was then washed with CH_3CN (3×5 mL, 2 min per wash), MeOH (3×5 mL, 15 min per wash) and CH_2Cl_2 (3×5 mL, 2 min per wash) and dried, overnight, under high vacuum pump. The activation efficiency was confirmed by a negative Kaiser test.[57] At this point the succinylated resin was divided into several batches and each batch was functionalized with a different modification as follows. The appropriate 3′ modification (alcohol; 36.8 μmol) was dissolved in DMF (2.5 mL) in the presence of HBTU (147.2 μmol) and DMAP (147.2 μmol) and the mixture was added to the succinylated resin (0.1 g, 9.2 μmol). After 4 h of constant agitation, the resin was washed with CH_3CN (3×5 mL) and CH_2Cl_2 (3×5 mL). Free amino sites on the resin were then converted to unreactive acetamide derivatives by treatment with capping reagents for the ABI DNA synthesiser for 3 h (cap A: 0.1 M *N*-methylimidazole in THF, and cap B: acetic anhydride 0.1 M in pyridine/THF). The resin was then washed with pyridine (3×5 mL), CH_3CN (3×5 mL) and CH_2Cl_2 (3×5 mL) before being dried, overnight, under high vacuum pump. A detritylation step by using a solution of trichloroacetic acid (3%) in CH_2Cl_2 (DNA synthesis grade) was performed to measure the resin loading.[58]

Oligonucleotide synthesis and purification: Oligonucleotide synthesis was carried out by using an Applied Biosystems 394 automated DNA/RNA synthesiser with a standard 0.2 or 1.0 μmol phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping and iodine oxidation. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous CH_3CN to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C and T monomers was 25 s and this was extended to 6 min for modified monomers. In all cases coupling of the first phosphoramidite monomer to the solid support was carried out for 10 min to ensure efficient addition. Stepwise coupling efficiencies and overall yields were determined by automated trityl cation conductivity monitoring, and in all cases were >98.0%. Cleavage of oligonucleotides from the solid support and deprotection were achieved by exposure to concentrated aqueous ammonia for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C. Purification of oligonucleotides was carried out by reversed-phase HPLC by using a Gilson system with a Brownlee Aquapore column (C8, 8 mm×250 mm, 300 Å pore) with a gradient of CH_3CN in NH_4OAc increasing from 0 to 50% buffer B over 30 min with a flow rate of 4 mL min^{-1} (buffer A: 0.1 M NH_4OAc, pH 7.0; buffer B: 0.1 M NH_4OAc with 50% CH_3CN, pH 7.0). Elution of oligonucleotides was monitored by ultraviolet absorption at 295 nm. After HPLC purification, oligonucleotides were desalted by using NAP-10 Sephadex columns (GE Healthcare) according to the manufacturer's instructions.

Oligonucleotide analysis: Oligonucleotide analysis was carried out by capillary gel electrophoresis (CE) and mass spectrometry. Each sample (0.4 OD per 100 μL) was injected on to an ssDNA 100-R gel by using a Tris-borate urea (7 M) buffer (kit N° 477480) on a Beckman coulter P/ACE™ MDQ capillary gel electrophoresis system controlled by 32 Karat software. The injection voltage was 10.0 kV and separation voltage was 9.0 kV. The electropheregram was run for 45.0 min and peaks were monitored by UV absorbance at 254 nm. TFOs were analysed by negative mode electrospray MS by using a Fisons VG platform spectrometer in water with triisopropylamine (2 μL of a 1% solution in MeOH). Hairpin duplexes were analysed by MALDI-TOF by using a ThermoBioAnalysis Dynamo MALDI-TOF spectrometer in positive ion mode.[59]

Ultraviolet melting studies: To determine triplex melting temperatures (T_m), UV melting studies were carried by using a Varian Cary 400 scan UV-visible spectrophotometer with Hellma SUPRASIL synthetic quartz 10 mm path length cuvettes, monitoring at 260 nm with a DNA duplex concentration of 1.0 μM and a volume of 1.2 mL. Samples were prepared as follows. The duplex and the third strand were mixed in a 1:3 ratio (concentrations determined by UV absorbance and extinction coefficients) in Eppendorf tubes (2 mL) and then lyophilized before being resuspended in the appropriate buffer solution (1.2 mL) and filtered through Kinesis cellulose 13 mm diameter 0.45 μM syringe filters. The following buffers were used: sodium phosphate (10 mM) with NaCl (200 mM) adjusted to pH 6.2 or 6.6, and sodium acetate (10 mM) with NaCl (200 mM) adjusted to pH 5.5. The UV melting protocol involved initial denaturation by heating to 80 °C at 10 °C min^{-1} followed by annealing by cooling to 20 °C at 0.5 °C min^{-1}; the temperature was then maintained at 20 °C for 20 min before the start of the melting experiment, which involved heating from 20 °C to 80 °C at 0.5 °C min^{-1}, holding at 80 °C for 2 min then cooling to 20 °C at 0.5 °C min^{-1}. Two successive melting curves were measured before fast annealing from 80 °C to 20 °C at 10 °C min^{-1} to bring the sample to ambient temperature. The T_m values were calculated by using Cary Win UV thermal application software by taking an average of the two melting curves.

Acknowledgements

This work was supported by the European Commission's Sixth framework Programme (project reference ZNIP contract number 037783) and grants from the BBSRC. Oligonucleotide synthesis was carried out by ATD Bio, Ltd.

Keywords: anthraquinone · DNA recognition · DNA · pyrene · triple helix

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Received: April 16, 2009
Published online on June 24, 2009

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