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Transcription Inhibition Using Modified Pentanucleotides

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Abstract—Inhibition of gene expression was recently achieved by targeting the transcriptionally competent open complex using relatively short, pentameric modified oligonucleotides at ~60 μM. Corroborative affinity cleavage experiments using the copper complex of a phenanthroline conjugate provided the impetus to synthesize additional analogues containing substituents at the 2′-position of uridine in a derivative of 5′-GUGGA (−4 to +1), with the purpose of inhibiting transcription at lower concentrations. Conjugates of 5′-GUGGA modified at the 2′-position of uridine were convergently synthesized using a recently reported method. Seven analogues based upon the 5′-GUGGA scaffold were tested for their ability to inhibit transcription of the *lac UV-5* operon. The conjugate containing a tethered pyrene showed 70% inhibition at 20 μM, and modest inhibition at as low as 5 μM. This is a significant improvement over previously tested pentanucleotides and provides direction for the preparation of a next generation of inhibitors.

Inhibitors targeted for gene expression have received considerable attention in the past decades for their potential as therapeutic agents. Strategies available for inhibiting gene expression include either targeting mRNA (antisense) using oligonucleotides or double stranded siRNA, or targeting DNA (antigene), such as triple helix formation.[1–7] Specificity is often achieved by designing molecules that form specific hydrogen bonds with the donors and acceptors presented by the nucleobases of the target. Modified nucleic acids that enhance binding and other properties are often used as tools in these endeavors. Typically, nucleic acid based inhibitors need to be approximately 15 nucleotides long in order to ensure high sequence selectivity. Oligonucleotides of this length can be difficult to transport across cell membranes and are expensive to synthesize on a large scale.

Our laboratory discovered a unique approach for the design of gene specific transcription inhibition by utilizing the formation of an open complex during transcription initiation ('bubble formation').[8,9] Significant inhibition can be achieved by pentanucleotides that are complementary to the template strand of the open complex in the *lac* UV5 and *trp* EDCBA systems at ~60 μM (Fig. 1). These oligonucleotides contained 2′-*O*-methyl groups to enhance stability and 3′-deoxyadenosine at the 3′-termini to prevent their elongation. Gel retardation assays showed that transcription inhibition was not due to the dissociation of RNA polymerase. The specificity of these pentanucleotides as inhibitors was demonstrated by the fact that molecules targeted for *lac* UV5 and *trp* EDCBA systems were only successful for the corresponding promoters, where the transcription start sites differ from each other by only two bases. Additional studies established that pentanucleotide

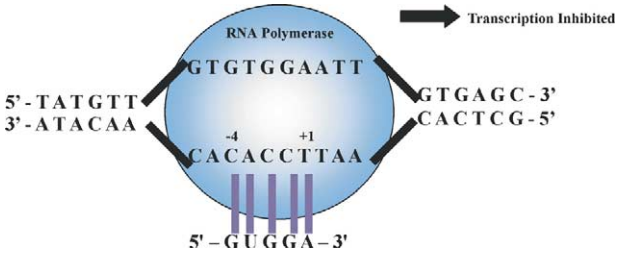

Figure 1. Hybridization of 5′-GUGGA (2′-*O*-methylated, 3′-deoxyadenosine, **2**) (−4 to +1) to *lac* UV5 open complex. This drawing does not depict actual formation of the open complex.

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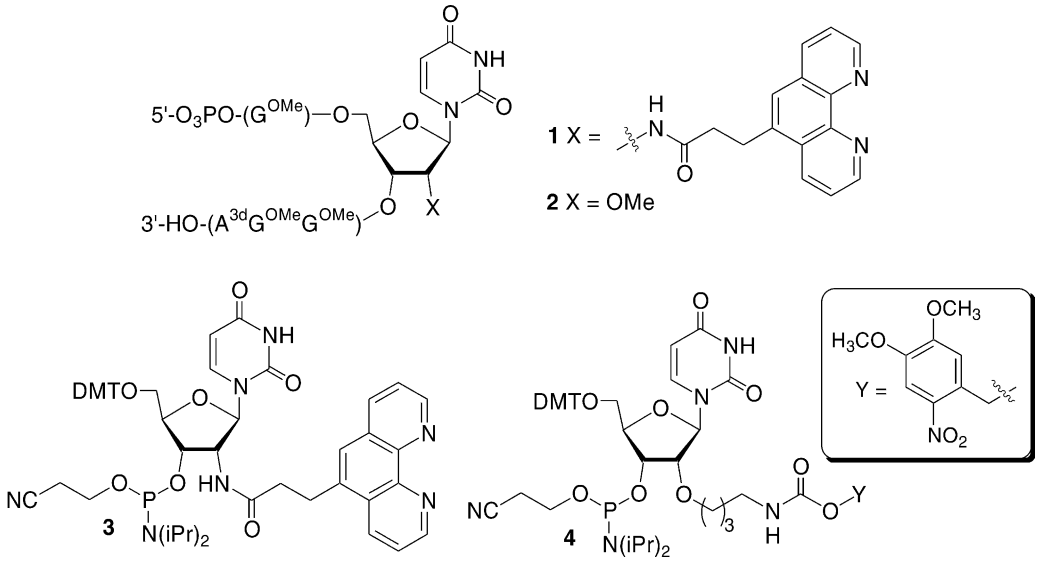


inhibitors were optimal in length, but there was some latitude with position relative to the transcription start site (Fig. 1). Verification of binding site and orientation was obtained using oligonucleotides (**1**) containing covalently bound mono- and bis-1,10-phenanthroline-copper chelates [$(OP)_2Cu^+$], which cleaved the template strand inside the open complex at positions just upstream from the transcription start site. Phenanthroline modification also indicated that the 2′-position of the uridine was amenable to substitution with components that might further stabilize its binding to the open complex.

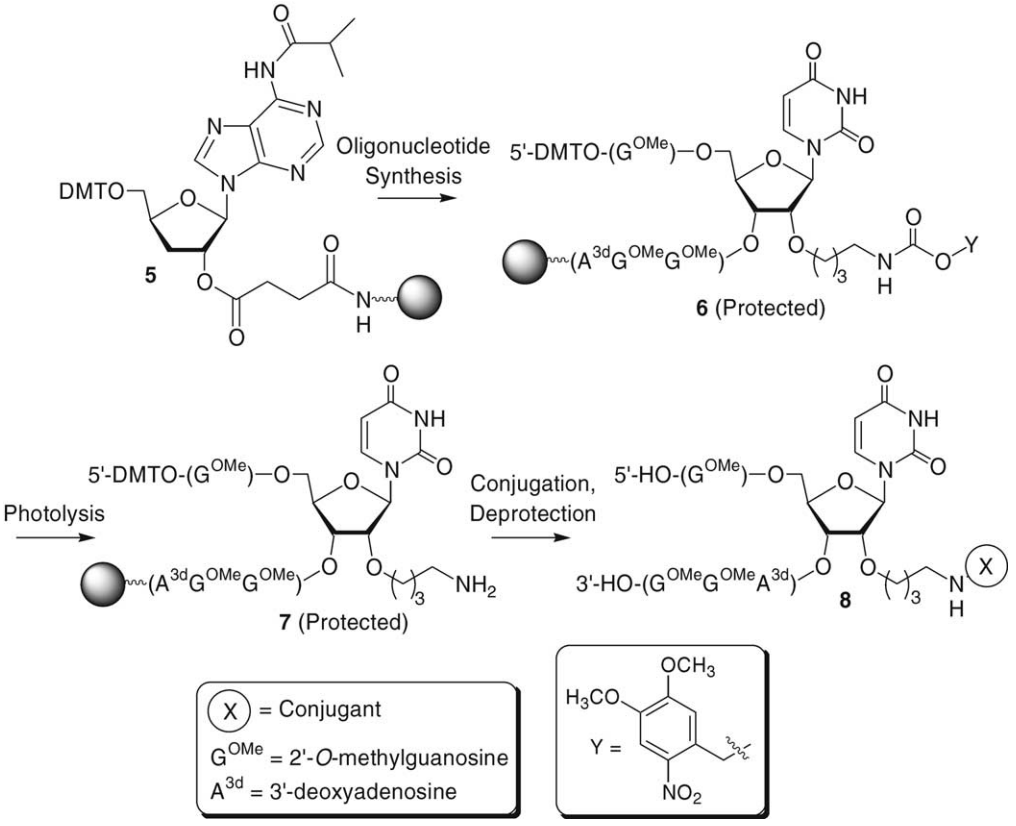
In this study, we wish to report our efforts to discover a more potent transcription inhibitor by improving the binding affinity of this pentanucleotide through altering its potential intercalative ability. We synthesized seven oligonucleotides based on **1** and **2** with a 2′-modified uridine.[9] The substituents varied from alkyl amine to polyaromatics, such as pyrene. The inhibitory effects of these oligonucleotides were assayed, and their abilities to inhibit were compared.

Results and Discussion

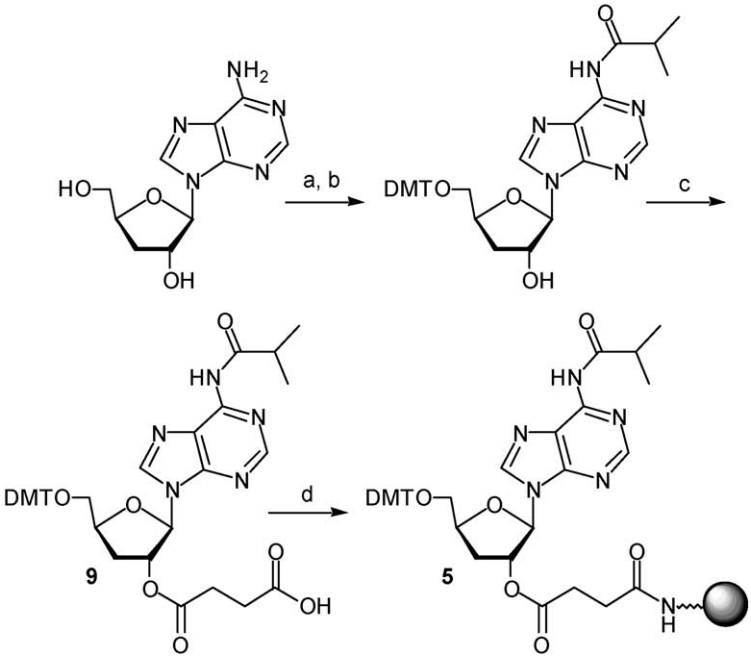
Design and synthesis of inhibitor candidates

The ability of **1** to bind and cleave the target strand of the open complex provided the impetus to design molecules that might have higher affinity for the complex by intercalation into the duplex structure formed between the inhibitor and DNA template. Pentanucleotide **1** was prepared via solid-phase synthesis, and required the corresponding phosphoramidite (**3**), which was obtained from 2′-amino-2′-deoxyuridine. Synthesis of **1** was time consuming and a more efficient process for synthesizing oligonucleotide conjugates based on **1** was desired. We utilized a convergent method for bioconjugate synthesis recently reported in which a single functional group is selectively revealed in an otherwise protected oligonucleotide (Scheme 1).[10–12] This strategy necessitates the synthesis and incorporation of only one modified phosphoramidite, or use of a photolabile solid phase support. Efficient use of the oligonucleotide is achieved by using a single solid-phase synthesis to prepare multiple conjugates. Homogeneous oligonucleotide conjugates are obtained using mild conditions, and a modest excess of reagents relative to oligonucleotide substrate. Although conjugates can be prepared while the oligonucleotide is in solution or bound to its solid phase synthesis support, the latter approach is well suited for preparing analogues of **1** (Scheme 1). Furthermore, we previously utilized **4** to synthesize oligodeoxynucleotide conjugates functionalized at the 2′-position of uridine.[10]

The pentameric scaffold containing **4** was prepared as previously described with two exceptions.[8–10] The 5′-termini of the inhibitors contained hydroxyl groups instead of phosphates. In addition, the pentanucleotide inhibitors require 3′-deoxyadenosine nucleotides at their 3′-termini. Solid-phase support containing *N*-benzoyl protected 3′-deoxyadenosine linked via its 2′-hydroxyl group is commercially available. However, *N*-isobutyryl protected adenines are preferred in the conjugation method due to incompatibility of *N*-benzoyl groups with the *o*-nitrobenzyl photochemistry, and possible inopportune transamidation of phenoxyacetyl protected nucleotides.[13,14] Consequently, solid phase support containing *N*-isobutyryl-3′-deoxy-2′-succinatoadenosine was prepared from 3′-deoxyadenosine.[15] Execution of the standard approach for converting the free nucleoside into needed solid-phase support was hampered by the unexpected lability of the *N*-isobutyryl group (Scheme 2). Regardless of whether the hydroxyl groups were silylated or acylated, we were unable to remove them without cleaving significant amounts of the isobutyryl amide. After extensive experimentation we moved forward using a low yielding procedure for the preparation of the exocyclic amine protected nucleoside based on the knowledge that very small amounts of the 5′-dimethoxytrityl 3′-succinate (**9**) were needed for loading the controlled pore glass (CPG) support.



Scheme 1.



Scheme 2. (a) (i) TMSCI, pyridine, then isobutyryl chloride; (ii) NH_4F, MeOH (8%); (b) DMTCl, pyridine (86%); (c) succhinic anhydride, DMAP, pyridine (65%); (d) PyBOP, Hünig's base, DMAP, CH_3CN, LCAA–CPG.

A number of points regarding the synthesis of oligonucleotides and their conjugates on **5** deserve mention. The photoprotected scaffold (**6**) was prepared using modified ABI 394 RNA synthesis cycles in which the 2′-*O*-methylguanosine phosphoramidite was coupled for 5 min and photolabile phosphoramidite (**4**) was double-coupled (5 min/cycle). We also found that 4,5-dicyanoimidazole was a more effective activating agent than either tetrazole or 5-ethylthiotetrazole.[16,17] Trimethylacetic anhydride was used for capping in place of acetic anhydride in order to prevent transacylation upon photochemical unmasking of the primary amine.[10] We

also found that the photolysis time previously used to unmask **4** after incorporation into oligonucleotides had to be extended to three 40-min cycles. It is unclear what the source of this perturbation was, but previous reports suggest the efficiency of the *o*-nitrobenzyl redox reaction increases when the photochemical substrate is further removed from the surface of the solid-phase synthesis support.[18]

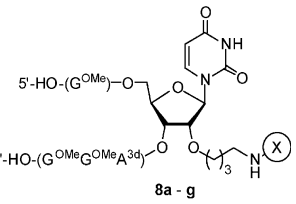
The desired conjugates (**8**) were obtained via either direct condensation of the support bound 5′-dimethoxytritylated oligonucleotide (**7**) with the appropriate aromatic isocyanate (**8c–e**) or PyBOP mediated coupling of a carboxylic acid (**8a**, **8b**). The proximity of the nucleophilic substrate to the surface of the solid-phase support also affected conjugation efficiency necessitating reacting 25–50 equiv of the electrophiles for 3 h at either room temperature or 55 °C. The amide and urea products, as well as the original carbamate in **8f** were stable to the 6 h, 55 °C concentrated aqueous ammonia treatment. The latter was cleaved upon deprotection for 24 h. Short oligonucleotide length, 2′-*O*-methyl groups, and nonpolar conjugants that decreased water solubility made the purification of inhibitor candidates by denaturing gel electrophoresis difficult. Consequently, **8a–f** were purified by anion exchange chromatography and obtained in modest yield compared to those typically prepared using this method (Table 1).[10–12] All seven modified oligonucleotides were characterized by ESI-MS.[19]

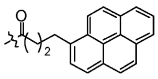
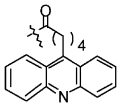
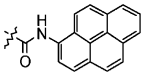
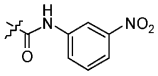
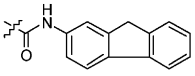
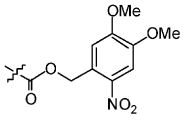
Transcription inhibition by oligonucleotide conjugates

The relative binding affinities and inhibitory abilities of the seven conjugates were conveniently screened by carrying out transcription assays at varying inhibitor concentrations in the *lac* UV5 system. Inhibitor and template were incubated in transcription buffer with RNA polymerase and ribonucleotide triphosphates. α-[^{32}P]-UTP was used in this transcription mixture for the purposes of visualization and quantification of transcript. Transcription was quenched after 1 min. Samples were analyzed on a 10% denaturing polyacrylamide gel and visualized by phosphorimaging. The amount of transcript produced in the presence of each inhibitor was then quantified and compared using **2** as a benchmark (Fig. 2).

Using **1** as a guide, we anticipated that only modified oligonucleotides containing substituents capable of intercalation would enhance inhibition. Oligonucleotide conjugate **8g**, which bears no intercalative substituent, showed no inhibitory capability at 50 μM (data not shown). Evidently, the positive charge does not improve the stability of the complex. The other six conjugates contain one or more aromatic rings tethered to the 2′-*O*-1-(4-aminobutyl) chain of uridine. The conjugates containing aromatic groups are divided into two families, depending upon whether or not the aromatic portion of the molecule is separated from the electrophilic point of attachment (carbonyl group) by a short alkyl chain, as it is in **8a** and **8b**. There was a direct correlation between inhibition and size of the aromatic system amongst the conjugates that did not contain an additional chain. The pyrene conjugate **8c** demonstrated the most significant

Table 1. Transcription inhibitor candidates



Compd	X	% Yield conjugation	HPLC ret. time (min)[a]
8a		60	29.6 (B)
8b		65	36.1 (A)
8c		45	28.3 (B)
8d		58	29.2 (A)
8e		40	44.9 (A)
8f		—	29. (A), 21.0 (B)
8g	H	—	17.4 (A), 16.3 (B)

[a]HPLC solvent system in parentheses. See Experimental for details.

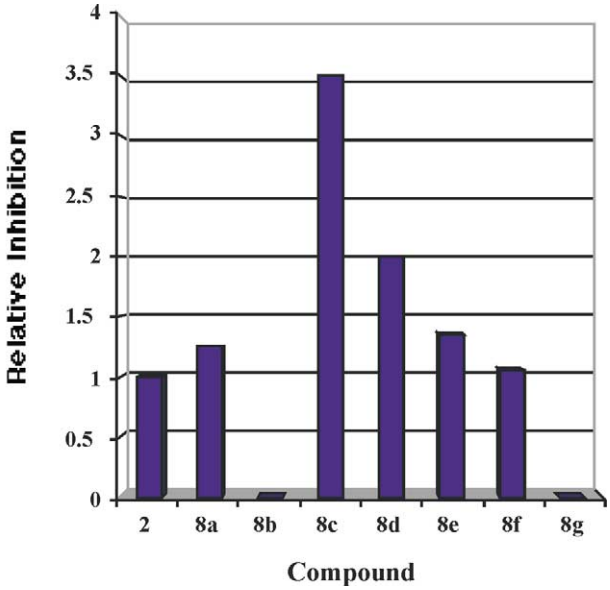


Figure 2. Transcription inhibition of *lac* UV5 by oligonucleotide conjugates **8a–8g** relative to **2** at 20 μM.

inhibitory ability (Fig. 2). Approximately 70% inhibition was achieved at 20 μM. Further transcription assays performed on **8c** showed that inhibition occurred even at 5 μM (Fig. 3). The other pyrene containing conjugate (**8a**) was considerably less effective. Conjugates **8a** and **8c** differ in the length of the tether between the aromatic system and the 2′-oxygen of the uridine in the scaffold and the chemical nature of the linkage. Although a greater number of compounds are necessary to substantiate this proposal, we believe that the former is more significant. This suggestion is based partly on the observation that **8b**, which contains the often used acridine intercalator, was a poor inhibitor. One should also note that the proposed tether length effect is consistent with the observation that **8a** is a stronger inhibitor than **8b**.

Gel mobility assay

To characterize further the binding of inhibitor **8c** to the open complex, we performed gel mobility assays (Fig. 4). Inhibitor **8c** (50 μM) was incubated with *lac* UV5 template under conditions suitable for the formation of the open complex, which occurs upon addition of RNA polymerase. Samples were then loaded on a 5% non-denaturing acrylamide gel for analysis. The single band in lane 1 represents movement of template by itself. In lane 2, labeled template was incubated with RNA polymerase prior to loading. The presence of a slower moving band indicates the formation of the open complex (Fig. 4, see arrow). Retarded mobility was due to the binding of RNA polymerase to the template. This slower band is still present in lane 3, when the experiment was carried out in the presence of oligonucleotide, showing that open complex formation was retained, and inhibition was not due to the dissociation of RNA polymerase. We also observed this slower moving band in lane 4, which contained unlabeled template and labeled inhibitor. This signified that **8c** hybridized to the template strand within the open complex. Lanes 5 and 6 show movement of oligonucleotide conjugate with and without RNA polymerase, respectively. The shifted band of interest does not appear in either lane, proving

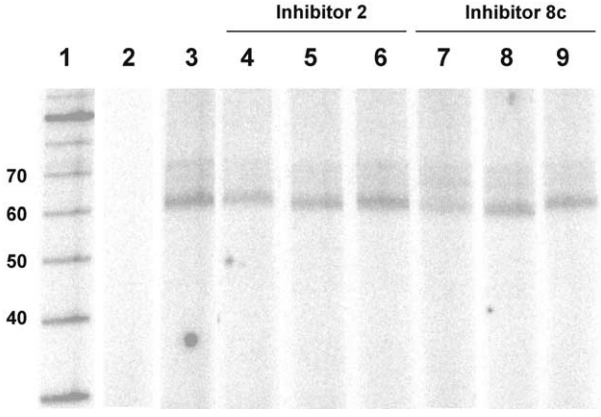


Figure 3. Comparison of transcription inhibition of *lac* UV5 by **2** and **8c** at various concentrations. Lane 1: 10-base DNA marker (Gibco/BRL); lane 2: no template; lane 3: no inhibitor; lane 4: **2** (50 μM); lane 5: **2** (20 μM); lane 6: **2** (10 μM); lane 7: **8c** (20 μM); lane 8: **8c** (10 μM); lane 9: **8c** (5 μM).

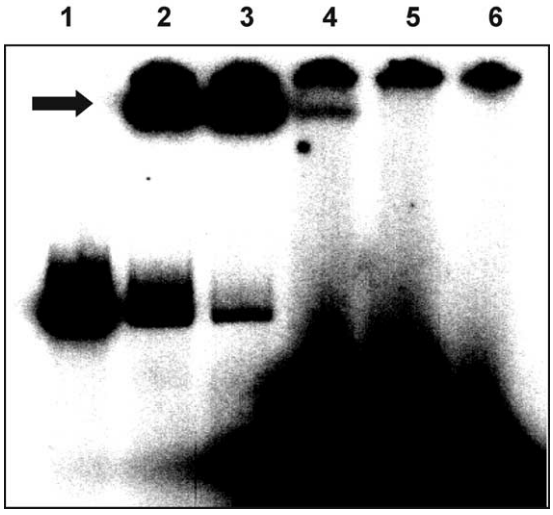


Figure 4. Gel mobility assay of *lac* UV5 open complex. Arrow denotes position of shifted bands. Lane 1: labeled template only; lane 2: labeled template + RNA polymerase; lane 3: labeled template + RNA polymerase + **8c**; lane 4: template + RNA polymerase + labeled **8c**; lane 5: RNA polymerase + labeled **8c**; lane 6: labeled **8c**.

that the retardation in lane 4 was not due to the binding of RNA polymerase directly to inhibitor. Bands appearing at the very top of lanes 3–6 represent sample that has remained in the well. This could be due to either excess radiolabeled sample or non-specific protein binding. The large amount of radiation evident at the bottom of lanes 4–6 is due to unincorporated γ-^{32}P-ATP. The short length of the oligonucleotide conjugates prohibited removal of the excess radionuclide via the typical Sephadex and/or precipitation treatments.

To test whether or not specificity of **8c** was affected by its increased binding ability, we performed gel mobility assays using the *trp* EDCBA system under the same conditions (data not shown). No retarded band corresponding to that observed in the *lac* UV5 system was found. This study demonstrated that the specificity of **8c** for the *lac* UV5 promoter was retained.

Summary

Using **1** and **2** as a starting point, we convergently synthesized oligonucleotide conjugates that inhibit in vitro transcription. The small library of compounds synthesized provided a molecule that inhibits transcription 3- to 4-fold more strongly than **2**. Moreover, these molecules provide direction for synthesizing a next generation of inhibitors. The next generation of molecules should include conjugates of polycyclic aromatic systems and substituents on these rings that enhance intercalation. Further refinements in the effects of tether length are also warranted.

Experimental

All reactions were carried out in oven-dried glassware under an atmosphere of argon or nitrogen unless

otherwise noted. Diisopropylethylamine, DMF, trimethylchlorosilane, and pyridine were distilled from CaH_2. Acetonitrile was passed through $CuSO_4$ and then distilled from CaH_2.

***N*-Isobutyryl-5′-*O*-dimethoxytrityl-3′-deoxy-2′-succinatoa-denosine (9).** 3′-Deoxyadenosine[15] (0.4 g, 1.6 mmol) was dried three times by azeotroping pyridine (3×10 mL) under vaccuum. Trimethylchlorosilane (1.1 mL, 8 mmol) was added to a solution of the dried nucleoside in pyridine (15 mL) at 0 °C, and then the reaction was warmed to room temperature. After the reaction mixture was stirred for 2 h, isobutyryl chloride (0.16 g, 1.5 mmol) in CH_2Cl_2 (5 mL) was added slowly over 30 min at room temperature. After stirring for 3 h the solvent was removed in vacuo. The crude product (0.18 mg, ~0.45 mmol) was treated with NH_4F (86 mg, 2.3 mmol) in CH_3OH (10 mL) for 2 h. After removing the solvent in vacuo, the crude material was chromatographed on silica gel (CH_2Cl_2/CH_3OH, 10:1) to yield impure compound. The impure material was filtered through dried neutral alumina with $CHCl_3$, followed by CH_3OH to give a pure product (40 mg, 8%). ^{1}H NMR ($CDCl_3$) δ 9.65 (bs, 1H), 8.45 (s, 1H), 8.30 (s, 1H), 5.85 (d, 1H, J=2.8 Hz), 4.96 (m, 1H), 4.68 (m, 1H), 3.96 (d, 1H, J=8.8 Hz), 3.84 (dd, 1H, J=2.2 and 8.8 Hz), 3.02 (m, 1H), 2.45 (m, 1H), 2.20 (m, 1H), 1.25 (d, 6H, J=7.2 Hz).

Dimethoxytrityl chloride (51 mg, 0.15 mmol) was added to a solution of *N*-isobutyryl-3′-deoxyadenosine (40 mg, 0.12 mmol) in pyridine (5 mL). The reaction mixture was stirred overnight at room temperature, and then evaporated to give a crude product, which was purified on silica gel (CH_2Cl_2 to CH_2Cl_2/CH_3OH, 10:1, containing 1% Et_3N) to yield a pale brownish foam (64 mg, 86%). ^{1}H NMR ($CDCl_3$) δ 9.73 (s, 1H), 8.65 (d, 1H, J=3.3 Hz), 8.32 (s, 1H), 7.42–7.20 (m, 9H), 6.82 (d, 4H, J=8.7 Hz), 6.05 (d, 1H, J=2.4 Hz), 5.45 (bs, 1H), 4.74 (bs, 1H), 3.81 (s, 6H), 3.44 (dd, 1H, J=2.7 ad 10.2 Hz), 3.40-3.15 (m, 2H), 2.35 (m, 1H), 2.24 (m, 1H), 1.38 (d, 6H, J=6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 176.1,158.6, 152.3, 150.5, 149.8, 149.4, 144.6, 141.1, 136.2, 135.8, 135.7, 130.1, 128.9, 128.1, 128.0, 127.0, 123.9, 122.7, 113.3, 93.2, 86.6, 80.6, 76.2, 65.0, 55.4, 36.4, 34.4, 19.4; IR (film) 3270, 2924, 2836, 2245, 1725, 1608, 1583, 1510, 1462, 1251, 1178, 1084 cm^{-1}.

To a solution of *N*-Isobutyryl-5′-*O*-dimethoxytrityl-3′-deoxyadenosine (60 mg, 0.1 mmol) in pyridine (5 mL) was added succinic anhydride (15 mg, 0.15 mmol) and DMAP (18 mg, 0.15 mmol). The reaction was stirred overnight, after which additional succinic anhydride (20 mg, 0.3 mmol) was added. After stirring for 3 days, the pyridine was removed, the residue was coevaporated with toluene (3×15 mL). The residue was dissolved in CH_2Cl_2 and washed with 5% Na_2HPO_4. The aqueous layer was extracted with CH_2Cl_2 (3×30 mL). The combined organic layers were washed with brine (20 mL) and dried over Na_2SO_4. The solvent was removed to give a crude product as a foam, which was purified on silica gel (CH_2Cl_2/MeOH, 10:0.1 to 10:1, containing 1% Et_3N) to yield a foam which was dissolved in CH_2Cl_2 (2 mL) and precipitated into rapidly stirred hexanes (30 mL). The mixture was centrifuged and the supernatant was decanted to leave **9** as a white powder (45 mg, 65%). ^{1}H NMR ($CDCl_3$) δ 9.20 (bs, 1H), 8.62 (s, 1H), 8.22 (s, 1H), 7.42–7.05 (m, 9H), 6.78 (d, 4H, J=8.4 Hz), 6.18 (s, 1H), 5.78 (d, 1H, J=3.6 Hz), 4.60 (m, 1H), 3.68 (s, 6H), 3.40 (m, 2H), 3.10 (m, 1H), 3.04 (q, 3H, J=8.4 Hz), 2.64 (m, 5H), 2.22 (dd, 1H, J=4.0 and 12 Hz), 1.25 (t, 6H, J=7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 176.4, 172.3, 158.6, 152.6 150.8, 149.5, 144.6, 142.0, 135.8, 135.7, 130.1, 128.2, 127.9, 127.0, 122.4, 113.2, 90.1, 86.5, 80.6, 78.1, 64.5, 55.4, 45.5, 36.2, 33.1, 30.1, 29.9, 19.5, 8.8; IR (film) 3245, 3197, 2968, 2932, 2874, 2837, 2597, 2553, 2492, 1734, 1718, 1701, 1684, 1608, 1584, 1508, 1457, 1250, 1221, 1177, 1084, 1034, 830, 735 cm^{-1}; HRMS (FAB); calcd for $C_{39}H_{41}N_5O_9$, 724.2983 (M^++H), found 724.2970.

Preparation of *N*-isobutyryl-5′-*O*-dimethoxytrityl-3′-deoxy-2′-succinatoadenosine support (5). LCAA-CPG (0.1 g), PyBOP (2.9 mg, 5.5 μmol), diisopropylethylamine (2 μL, 12 μmol), DMAP (0.7 mg, 5.7 μmol) and **9** (4 mg, 5.4 μmol) were combined in a screw capped vial with acetonitrile (2 mL). The reaction mixture was shaken at room temperature for 20 min. The resin was filtered, washed with MeOH (10 mL), CH_2Cl_2 (10 mL), and then dried under high vacuum. The amount of nucleoside loading on the support (~40 μmol/g) was determined by trityl analysis. Unreacted alkylamines were capped by reacting the resin with trimethylacetyl chloride (21 μL, 113 μmol), DMAP (6.7 mg, 55 μmol), and pyridine (18 μL, 220 μmol) in acetonitrile (2 mL) in a screw capped vial. The reaction mixture was shaken at room temperature for 2 h. The resin was filtered, washed with MeOH (10 mL), followed by CH_2Cl_2 (10 mL), and dried under high vacuum.

Photolytic deprotection

Pentanucleotide bound to support (30 mg, ~0.9 μmol oligonucleotide based upon trityl response) was added to a Pyrex tube containing a stir bar constructed from a standard (white) pipe cleaner and CH_3CN (20 mL). The tube was fitted with a rubber septum and the solution was sparged with Ar for 20 min, after which the needle was raised well above the surface of the solvent. Photolyses were carried out with a VWR Chromato-Vue transilluminator (λ_{max}=365 nm) for 40 min. The resin was filtered, washed with MeOH (10 mL), followed by CH_2Cl_2 (10 mL), collected, and dried under high vacuum. The resin was transferred to a tube in CH_3CN (20 mL) whereupon the sparging and photolysis procedures were repeated two more times. The resin was filtered, washed with MeOH (10 mL), followed by CH_2Cl_2 (10 mL), dried under vacuum, and placed in a screw capped vial for storage. Note: It is important to maintain the temperature during photolysis at ≤25 °C using a fan.

General procedure for conjugation of protected resin bound pentanucleotides

When conjugating to a carboxylic acid a solution (0.15 M) of the coupling reagents [39 mg PyBOP, 16 μL

diisopropylethylamine (2 molar equivalents)] in DMF (500 μL) was prepared in an oven dried 1 dram vial equipped with a septum. A solution (0.15 M) of carboxylic acid was prepared in a second oven dried vial. The resin bound DNA (10 mg, containing ~300 nmol of oligonucleotide, based on trityl response) was treated with 100 μL (25 molar equivalents) of a 1:1 mixture (by volume) of the PyBOP and carboxylic acid solutions. The reaction was capped and shaken at room temperature for 3 h. The resin was washed with CH_3CN (3×2 mL), and dried in vacuo. Isocyanates were conjugated using a DMF solution of isocyanate (0.15 M) prepared in an oven dried vial. The resin bound DNA (10 mg, containing ~300 nmol of DNA, based on trityl response) was treated with 100 μL (50 molar equivalents) of isocyanate solutions. The reaction was capped and shaken at 55 °C for 3 h. The resin was washed with CH_3CN (3×2 mL), and dried in vacuo.

In either instance, detritylation was effected by transferring the resin to a standard oligonucleotide synthesis column and passing the standard trichloroacetic acid solution (3×20 s) through the column, followed by CH_3CN, and drying under vacuum. The free flowing resin was treated with 28% aqueous ammonia (1 mL) for 6 h at 55 °C and concentrated under vacuum. Purification of conjugated pentaribonucleotide was carried out using anion exchange HPLC column (Vydac 301VHP575). Solvent system A: A, 10 mM Tris, pH 8.0, 10% CH_3CN; B, 10 mM Tris, 0.5 M NH_4Cl, pH 8.0, 10% CH_3CN; 0–40% B linearly over 25 min; 40–80% B linearly over 3 min; hold 80% B for 10 min; flow rate, 1 mL/min. Solvent system B: A, 10 mM Tris, pH 8.0, 30% CH_3CN; B, 10 mM Tris, 0.5 M NH_4Cl, pH 8.0, 30% CH_3CN; 0–40% B linearly over 25 min; 40–80% B linearly over 3 min; 80–90% B linearly over 10 min. Solvent system A was used for 3-nitrophenyl isocyanate, 9H-fluoren-2-yl isocyanate, and acridine pentanoic acid conjugated oligonucleotides. Solvent system B was used for pyrenebutyryl conjugated products. Isolated yields were obtained (O.D. 264 nm; 352 nm for pyrenebutyryl conjugated products) by comparing the amount of conjugated oligonucleotide to the amount of starting material, as previously described.[10–12]

Preparation of lac UV5 template strand

The 211 bp *lac* UV5 promoter fragment (−144 to +67) was prepared from a pUC-derived plasmid restricted with EcoRI (GibcoBRL).[20] The fragment was purified on an 8% non-denaturing polyacrylamide gel. Following elution and ethanol precipitation, purified fragment was used directly for transcription studies. A portion of the EcoRI fragment to be used for gel mobility studies was dephosphorylated with calf intestinal alkaline phosphatase (Promega) and then radiolabeled. Radiolabeling proceeded in a kinase buffer with 2 pmol of dephosphorylated *lac* UV5 fragment, γ-[^{32}P]-ATP (3000Ci/mmol), and T4 polynucleotide kinase (GibcoBRL). The reaction was quenched upon phenol/chloroform extraction, and sample was passed through a G-50 spin column to remove salts. The fragment was then subjected to PvuII restriction (GibcoBRL) and purified on an 8% non-denaturing polyacrylamide gel.

In vitro transcription assay

The transcription mixture (10 μL) contained unlabeled *lac* UV5 (EcoRI fragment, 0.1 μM) and 0.3 units/ μL *Escherichia coli* RNA polymerase (Sigma) in a buffer of 40 mM Tris–HCl (pH 7.9), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT, 100 μg/mL BSA, and 5% glycerol. Oligonucleotides were added to give the proper inhibitor concentrations. No inhibitors were added to control samples. The reaction mixture was incubated at 37 °C for 20 min. To initiate transcription, ribonucleotides (final concentration 100 μM each, with 1 μCi of α-[^{32}P]-UTP) were then added. The reaction proceeded for 1 min and was quenched with 10 μL of formamide and dyes. Samples were analyzed on a 10% denaturing polyacrylamide gel. The gel was dried under vacuum at 80 °C. Transcription product was visualized and quantified by phosphorimaging.

Gel mobility assay

Either oligonucleotide inhibitor or template was radiolabeled, depending on the gel mobility assay. Radiolabeled inhibitor was prepared in a kinase buffer with 2 nmol of dephosphorylated oligonucleotide, γ-[^{32}P]-ATP (20 μCi/nmol), and T4 polynucleotide kinase (GibcoBRL). The reaction was quenched upon heat deactivation of the enzyme at 80 °C for 2 min, and radiolabeled inhibitor was then used directly for gel mobility studies.

Inhibitor (50 μM) was incubated with *lac* UV5 template (40 nmol) and RNA polymerase (0.2 units/μL, USB) in transcription buffer for 25 min at 37 °C. No RNA polymerase was added to control samples. After incubation, samples were subjected to heparin challenge (200 μg/mL) for 2 min at 37 °C and immediately loaded on 5% non-denaturing polyacrylamide gel run at 200 volts in the cold (4 °C). The gel was then dried under vacuum at 80 °C. Samples were visualized by phosphorimaging.

Supporting Information

Electrospray ionization mass spectra of oligonucleotides **8a–g**.

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