Synthesis and Kinetic Properties of Ribozyme Analogues Prepared Using Phosphoramidite Derivatives of Dysprosium(III) Texaphyrin

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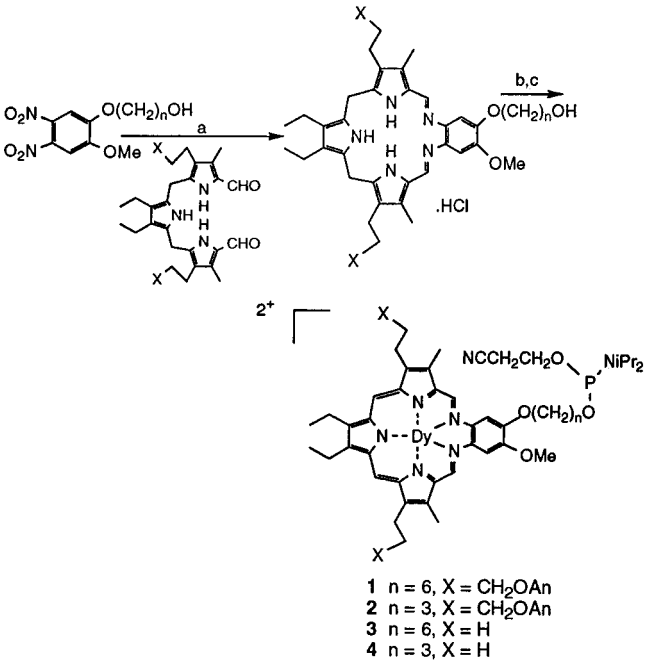
In the last several years considerable progress has been made in terms of using oligonucleotide-appended, metal-based Lewis acidic functional groups to effect the site-directed cleavage of RNA.[1] To date, two generalized strategies have been used to generate the requisite Lewis acid-containing oligonucleotides ("ribozyme analogues"), namely solution-phase conjugation using a preformed metal complex[1b,c] and postsynthetic addition of a metal cation to an oligonucleotide-bound cheland.[1a,d] In both cases, the fully functional cleavage agent is generated after its supporting oligonucleotide is made. This has tended to limit the range of available Lewis acid-bearing conjugates and the extent to which RNA transesterification[2,3] activity can be optimized. In this paper, we describe a new approach to Lewis acid functionality attachment wherein the covalent linkage to the metal complex is made during the course of solid-phase DNA synthesis.[4] This preparative strategy, which is illustrated using a dysprosium(III) texaphyrin Lewis acidic center, allows access to synthetic systems which could not otherwise be obtained.

The application of the standard solid-phase approach[5] to the present problem required the synthesis of the hexamethylene-bridged phosphoramidite derivative of dysprosium(III) texaphyrin (DyTx) (**1**) prepared as shown in Scheme 1. Once in hand, this dysprosium-containing phosphoramidite **1** (as a solution in dichloromethane) could be introduced as an auxiliary reagent on a commercial DNA synthesizer. It was used in this way to prepare conjugate **6** (Figure 1). First, a solid-supported DNA 20-mer was prepared on a 1 μmol scale in the usual way and then the complex-derived reagent **1** was attached as the last "base" of the sequence (i.e., at the 5′-end).[6] Standard oxidation and washing steps, followed by removal from the solid support and deprotection of the blocked bases using aqueous ammonium hydroxide/methylamine, then gave the ribozyme analogue **6** that corresponds in point of attachment to an analogous dysprosium(III) texaphyrin-functionalized oligonucleotide (**5**) prepared using solution-phase methods (Figure 1).[1b] Initial evidence for integrity came from the finding that, like **5** and several earlier-reported oligonucleotide texaphyrin-based systems,[1b,7] conjugate **6** proved capable of effecting the site-directed cleavage of a complementary RNA target (**8**) (Figures 1 and 2).[8,9]

An important advantage of the above protocol is that it is compatible with commercially available phosphoramidites.[10] It

Scheme 1. Synthesis of Phosphoramidites **1**−**4**[a]



[a] Key: (a) Pd(C), HCl, H_2, EtOH; (b) $Dy(OAc)_3$, TEA, MeOH; (c) $(NCCH_2CH_2O)(iPr_2N)_2P$, iPr_2NEt, tetrazole, CH_2Cl_2 (An = COC_6H_4OMe).

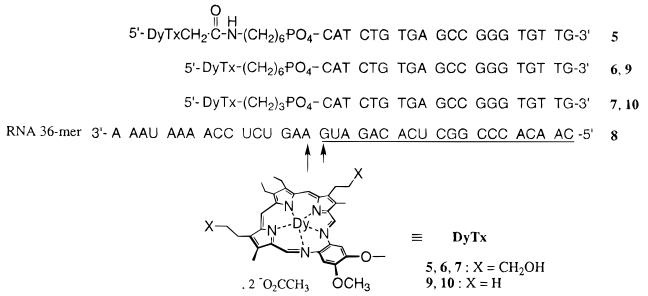


Figure 1. Structures of the five DyTx−DNA conjugates (**5**−**7**, **9**, and **10**) analyzed in this study. Also shown is the sequence of a complementary 5′-^{32}P-radiolabeled RNA target (**8**). The arrows indicate sites of cleavage produced upon incubation of this RNA target with an excess of each of the five complementary DyTx−DNA conjugates **5**−**7**, **9**, and **10** at 37 °C. In all cases, cleavage of the RNA occurred near the junction of the single-stranded and double-helical domains, that is, near the anticipated location of the DyTx catalyst after hybridization.

(1) (a) Bashkin, J. K.; Frolova, E. I.; Sampath, U. *J. Am. Chem. Soc.* **1994**, *116*, 5981−5982. (b) Magda, D.; Miller, R. A.; Sessler, J. L.; Iverson, B. L. *J. Am. Chem. Soc.* **1994**, *116*, 7439−7440. (c) Hall, J.; Husken, D.; Pieles, U.; Moser, H. E.; Haner, R. *Chem. Biol.* **1994**, *1*, 185−190. (d) Matsumura, K.; Endo, M.; Komiyama, M. *J. Chem. Soc., Chem. Commun.* **1994**, 2019−2020.

(2) Metal-promoted cleavage of RNA is found to result initially in an oligomer having a 2′,3′-cyclic phosphate at the 3′-terminus, which can be subsequently hydrolyzed to a mixture of 2′- and 3′-phosphates.[3]

(3) Kazakov, S. A. In *Bioorganic Chemistry: Nucleic Acids*; Hecht, S. M., Ed.; Oxford University Press: New York, 1996; pp 244−287.

(4) Platinum(II)-derivatized oligodeoxynucleotides, prepared via a solid-phase synthesis approach, were recently reported: Manchanda, R.; Dunham, S. U.; Lippard, S. J. *J. Am. Chem. Soc.* **1996**, *118*, 5144−5145.

(5) Brown, T.; Brown, D. J. S. in *Oligonucleotides and Analogues: A Practical Approach*; Eckstein, F., Ed.; IRL Press: New York, 1991; pp 1−24.

(6) Due to the limited solubility of phosphoramidites **1**−**4** (ca. 50 mM in dichloromethane), coupling yields were lower than desired, although ca. 75% conversions were obtained using a modified (triple) coupling protocol.

(7) Magda, D.; Miller, R. A.; Wright, M.; Rao, J.; Sessler, J. L.; Iverson, B. L.; Sansom, P. I. In *DNA and RNA Cleavers and Chemotherapy of Cancer and Viral Diseases*; Meunier, B., Ed.; Kluwer: Boston, MA, 1996; pp 337−353.

(8) All DyTx−DNA conjugates prepared via the phosphoramidite approach were further characterized by digestion with phosphodiesterase I and alkaline phosphatase and compared with authentic complexes and nucleoside components by RP HPLC using standard methods.

(9) Koh, J. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 1470−1478.

(10) DNA synthesizer reagents and phosphoramidites were purchased from Glen Research, Sterling, VA. Acetyl-protected deoxycytidine phosphoramidite was used as per the manufacturer's protocol for deprotection: Control pore glass-bound oligo-DyTx conjugates were treated by syringe with 1:1 ammonium hydroxide/40% aqueous methylamine at ambient temperature for 90 min. Conjugates **6** and **7** were treated with 40% aqueous methylamine for an additional 9 h at ambient temperature to facilitate anisoyl protective group removal. Conjugates were then purified using preparative reverse-phase HPLC.

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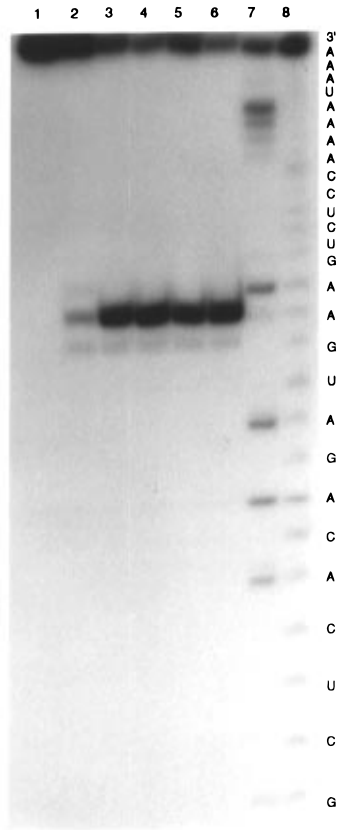

Figure 2. Cleavage of synthetic RNA 36-mer **8** by DyTx−DNA conjugates **5**−**7**, **9**, and **10**. Autoradiograph of a 20% denaturing polyacrylamide gel of the RNA labeled with ^{32}P at the 5′-end. Buffered solutions were prepared of each test species [50 nM DyTx−DNA conjugate, 50 mM HEPES, pH 7.5, 100 mM NaCl, 100 μM EDTA, 2 units/μL RNasin nuclease inhibitor (Promega Corporation, Madison, WI), and 1 mM dithiothreitol (all concentrations final)]. Substrate **8** (ca. 2 nM) was then added to the conjugate solutions. All tubes were incubated at 37 °C for 6 h. Samples were removed from incubation, precipitated with ethanol, resuspended in gel-loading buffer, and electrophoresed on a 20% denaturing polyacrylamide gel. Key: lane 1, conjugate-free control; lane 2, **5**; lane 3, **6**; lane 4, **7**; lane 5, **9**; lane 6, **10**; lanes 7 and 8, ribonuclease U2 (A>G) and bicarbonate sequencing reactions, respectively.

thus allows for the rapid derivatization of oligonucleotides. It also facilitates synthesis of a series of structural variants which can be screened for cleavage activity.

To test this promise, the more tightly tethered conjugate **7** was constructed; it was made using the same methods used to prepare conjugate **6** with the exception that the phosphoramidite **2** was used in lieu of **1**. This new "ribozyme analogue" was designed to reduce the level of conformational freedom available to the appended texaphyrin within the within the heteroduplex formed with substrate RNA **8**. Additionally, as a further test, phosphoramidites **3** and **4** were used to derive analogues **9** and **10**. These latter compounds were designed to allow the consequences incurred by removal of the hydroxymethyl substituents on the DyTx catalyst to be assessed.

Quantitative assessments of cleavage efficiency were made under the same pseudo-first-order conditions used to establish that conjugates **5**−**7**, **9**, and **10** effect the site-selective transesterification of RNA target **8** (Figure 2).[11,12] The results, summarized in Table 1, indicate that shortening the linker increases the rate of RNA cleavage. Specifically, the half-life of RNA **8** was found to decrease from ca. 10 h for conjugate **5** to ca. 2 h for **7**. Interestingly, the completeness of reaction, expressed as P_∞, the percentage of RNA cleaved at $t = \infty$, increased for the conjugates containing shorter linker groups.

Table 1. RNA Cleavage Data

conjugate	$T_{1/2}$ (h)[a]	P_∞[a]
5	10.27 ± 0.49	0.565 ± 0.002
6	2.22 ± 0.20	0.640 ± 0.018
7	2.07 ± 0.11	0.741 ± 0.018
9	2.83 ± 0.09	0.687 ± 0.011
10	2.43 ± 0.22	0.819 ± 0.011

[a] Average values from 2 to 4 independent determinations (±standard deviation).

Indeed, this effect, rather than a faster chemical cleavage step, accounts for most of the improved performance observed upon going from hexamethylene-linked conjugates **6** and **9** to trimethylene-linked derivatives **7** and **10**. Apparently, this modification allows the DNA oligomer to more efficiently hybridize with RNA substrate **8**. Finally, derivatives **9** and **10**, which bear truncated substituents on the catalyst, exhibit properties close to those of their corresponding hydroxymethyl-substituted analogues **6** and **7**. This result allows one to rule out a catalytic role for these (hydroxymethyl) substituents.

The results reported here serve to highlight the advantages of the solid-phase synthesis methodology. For instance, it has allowed the preparation of the trimethylene-linked agents **7** and **10** that display enhanced activity relative to congeners **5**, **6**, and **9**. This same approach has also allowed the preparation of conjugates, such as **9** and **10**, that because of the limited aqueous solubility of the catalyst could not normally be obtained using solution-phase methods.

In a more general sense, this new methodology is useful in that it should allow for the generation of a more complete library of texaphyrin-functionalized oligonucleotide conjugates and thus lead directly to further optimizations in terms of their site-directed RNA-cleaving potential. While it is not clear at present the extent to which this synthetic strategy will prove applicable to other, perhaps less stable, metal-containing systems, it is nonetheless important to appreciate that it offers the possibility of producing metal-functionalized oligonucleotides on the scale necessary for kinetic analyses *in vitro* or biological screenings *in vivo* (as, e.g., antisense agents). Current efforts are thus devoted to defining further the range and scope of this conjugate construction methodology.

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Supporting Information Available: Details of synthesis of phosphoramidites **1**−**4**, protocols for solid-phase coupling, kinetic analyses, and enzymatic digestion data (18 pages). See any current masthead page for ordering and Internet access instructions.

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(11) Buffered solutions were prepared of each test species [25 nM DyTx−DNA conjugate, 50 mM HEPES, pH 7.5, 100 mM NaCl, 100 μM EDTA, 2 units/μL RNasin nuclease inhibitor (Promega Corporation, Madison, WI), and 1 mM dithiothreitol (all concentrations final)]. Substrate 5′-^{32}P-radiolabeled RNA **8** (ca. 2 nM) was incubated for 5 min at 60 °C and then added to the conjugate solutions. The resulting solutions were each distributed into individual silanized microcentrifuge tubes, one tube per time point. All tubes except the first, zero time point, were then incubated at 37 °C, using a thermal cycler (Perkin-Elmer Model 2400) to control the temperature. At chosen times, samples were removed from incubation, precipitated with ethanol, and stored at −20 °C, to be subsequently electrophoresed on a 20% denaturing polyacrylamide gel. The ratio of fragments produced by site-specific transesterification to intact substrate RNA was then quantitated using phosphorimaging techniques. Kinetic parameters were obtained following the method of Hendry et al.[12] by fitting the data for percentage of product formed (P_t) at any given time (t) to the equation $P_t = P_\infty - [\exp(-K_{obsd}t)P_\Delta]$, where P_∞ is the amount of product at $t = \infty$, k_{obsd} is the first order rate constant for the reaction, and P_Δ is the difference between the percentage of of products at $t = \infty$ and $t = 0$. P_∞ was typically in the region of 0.6−0.8 (i.e., ca. 60−80% of the substrate was cleaved at the end of the reaction). K_{obsd} was used to calculate RNA half-life ($T_{1/2} = 0.693/k_{obsd}$).

(12) Hendry, P.; McCall, M. *Nucleic Acids Res.* **1996**, *24*, 2679−2684.