Carboranyl Oligonucleotides. 2. Synthesis and Physicochemical Properties of Dodecathymidylate Containing 5-(*o*-Carboran-1-yl)-2′-deoxyuridine†

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Abstract: Base modified oligothymidylates $d(T)_{12}$ containing one or more 5-(*o*-carboran-1-yl)-2′-deoxyuridine (CDU) instead of thymidine were synthesized using an automated β-cyanoethyl phosphoramidite approach. The thermostability of duplexes formed by these new oligonucleotides with natural complementary strand $d(A)_{12}$ and poly(rA) was affected by the location of the carboranyl nucleotide within the chain. $CDUd(T)_{11}$ oligomer modified at the 5′-terminus displayed a melting temperature (T_m = 28 °C) similar to natural $d(T)_{12}$ (T_m = 29 °C), whereas 3′-modified $d(T)_{10}CDUd(T)$ and centrally modified $d(T)_6CDUd(T)_5$ oligonucleotide had lower T_m values (T_m = 20.5 and 15.3 °C, respectively). The T_m studies were supported by molecular modeling of a duplex containing $d(T)_6CDUd(T)_5$, which showed steric interactions between the boron cage and 5′-adjacent base. As an added benefit, CDU modification at the 3′-terminus of the oligomers markedly increased their resistance toward 5′-exonuclease activity, as determined by half-life ($t_{1/2}$) measurements. The $t_{1/2}$ increased in the order $d(T)_{12} < d(T)_{10}CDUd(T) \ll d(T)_9CDU_2d(T)$. The presence of one or more carborane clusters in these new compounds increases their lipophilicity and makes them potential candidates for boron neutron capture therapy (BNCT) and antisense oligonucleotide technology (AOT).

Introduction

The use of boron-containing compounds in the treatment of malignancies is based on the property of nonradioactive boron-10 nuclei to absorb low-energy neutrons. When this stable isotope is irradiated with a neutron, an α particle (helium nuclei) and lithium-7 nuclei are released through a nuclear reaction producing about 100 million times more energy than initially used. The generated radiation, which is mostly confined to the cells containing the boron compound, destroys the target tumor cells, resulting in a therapeutic effect.[1-3] This combined modality, originally proposed by Locher[4] in 1936, is known as boron neutron capture therapy (BNCT).

A major obstacle of BNCT is the low selectivity of known boron-containing compounds for malignant tumors. It is essential to achieve tumor-to-normal-tissue and tumor-to-blood ratios of at least 2.[1-3] The recent synthesis of boron-modified peptides and nucleosides as boron trailer molecules is a new trend in the quest for improved boron carriers.[5,6] Carboranyl-modified oligonucleotides, the new class of oligonucleotide analogues described herein, could complement or provide a more selective approach than first-generation boron carriers. We have recently estimated the minimum number of carboranyl residues necessary on an oligomer for successful BNCT. On the basis of reasonable assumptions, it was demonstrated that no advantage is gained by loading oligomers with more than two to four carboranyl moieties.[7]

In cases where disease (e.g., cancers) originates from expression of genes of known sequence, a genetic target—polydeoxyribonucleic acid (DNA) or polyribonucleic acid (RNA)—may be down-regulated in highly sequence-specific fashion by hybridization of complementary DNA, RNA, or oligonucleotide sequence, forming triple- or double-stranded structures. This phenomenon is the basic advantage of antisense oligonucleotide technology (AOT), which enables more specific targeting of tumors with unique genetic characteristics leading to modulation of gene expression.[8-10] The transport of oligomers into cells remains one of the unresolved problems in delivering these compounds to the desired target in quantities sufficient to reach a biological effect. We anticipate that the lipophilicity of the carboranyl moiety, which consists of a 10 boron atom cluster, will increase their cellular uptake and provide additional advantages over natural, unmodified oligonucleotides.

Recently, we described the synthesis of thymidine(3′,5′)-thymidine (*o*-carboran-1-yl)methylphosphonate, the first oligonucleotide analogue bearing 3′,5′-*O*,*O*-[(*o*-carboran-1-yl)]methylphosphonate internucleotide linkage instead of natural 3′,5′-*O*,*O*-phosphodiester backbone.[11] The increased lipophilicity, resistance to nucleases, and high boron content provide the

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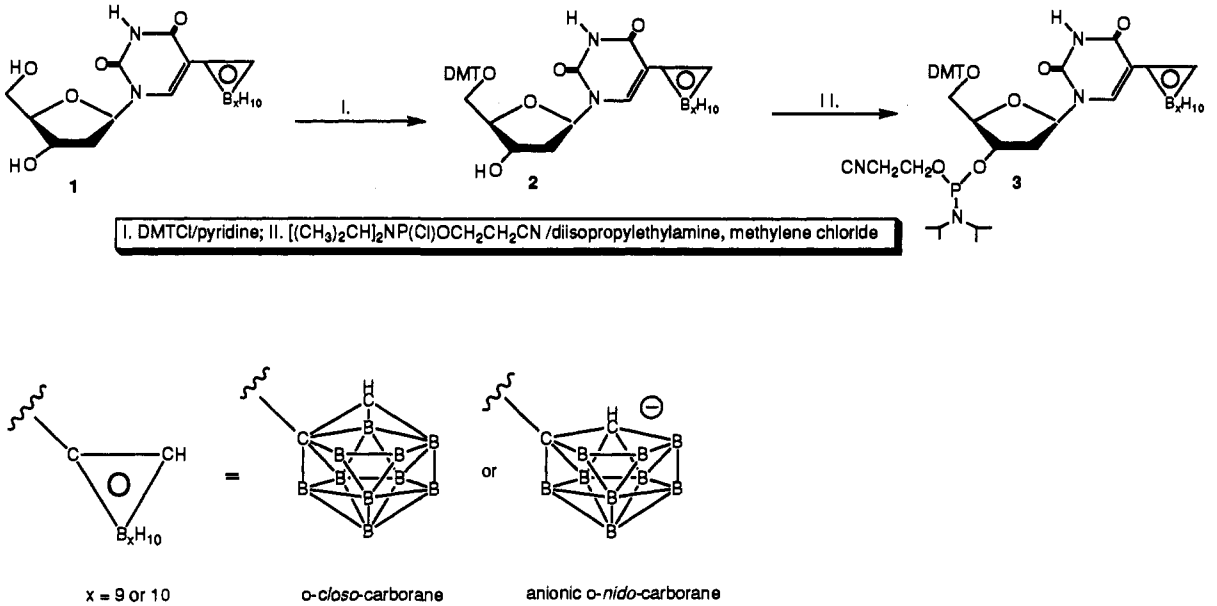

Figure 1. Synthetic route toward monomer for oligonucleotides **6–12**. DMT = dimethoxytrityl.

Table 1. HPLC, T_m, Half-Life ($t_{1/2}$), and MS Characteristics of CDU-Modified Oligonucleotides **7–12** and Unmodified Dodecathymidylic Acid **6**

oligomer[a]	base composition	R_t (min)	T_m[j] (°C)	T_m[k] (°C)	$t_{1/2}$[l] (min)	ESI-MS[m] (M)
6	$d(T)_{12}$	26.4[d]	29.0	30.0	0.5	
nido-**1.7**[b]	5′CDUd$(T)_{11}$	18.6[e]	28.8	28.0		3706[n]
nido-**2.7**[b]	5′CDUd$(T)_{11}$	20.0[e]	28.4			3706[n]
closo-**7**[b]	5′CDUd$(T)_{11}$	32.0[e]	28.0			3716[o]
nido/closo-**8**[c]	5′d(T)CDUd$(T)_{10}$	9.4, 9.9, 16.8[f]	27.2	25.7		3704[p]
nido-**1.9**[b]	5′d$(T)_6$CDUd$(T)_5$	12.6[g]	15.2	15.2		3704[p]
nido-**2.9**[b]	5′d$(T)_6$CDUd$(T)_5$	13.6[g]	15.3			3704[p]
closo-**9**[b]	5′d$(T)_6$CDUd$(T)_5$	19.8[g]	15.3			3716[o]
nido-**1.10**[b]	5′d$(T)_{10}$CDUd(T)	8.5[h]	20.5	18.5	6.7	3705[o]
nido-**2.10**[b]	5′d$(T)_{10}$CDUd(T)	9.6[h]	20.4			3704[p]
closo-**10**[b]	5′d$(T)_{10}$CDUd(T)	20.5[h]	20.9			3716[o]
nido/closo-**11**[c]	5′d$(T)_9(CDU)_2$d(T)	15.8, 16.0, 16.8, 17.0, 21.3, 21.5, 22.2, 22.6, 28.4[i]	15.3	10.0	76.2	3824[o]
nido/closo-**12**[c]	5′CDUd$(T)_9$CDUd(T)	16.5, 17.0, 17.6, 18.0, 21.1, 21.4, 25.5, 27.7[i]	20.0	17.0	5.1	3824[o]

[a] The nomenclature used for the oligomers reflects the different components existing within the same type of CDU oligonucleotide. For example, nido-1.7 indicates that the carboranyl cage on oligomer **7** is in the nido-1 form, being the diasteroisomer that elutes first by HPLC. [b] *Closo/nido* oligomers were isolated by HPLC and used separately in T_m experiments. [c] *Closo/nido* oligomers were distinguished by HPLC, but used as mixtures in T_m experiments. HPLC conditions: buffer A, 0.05 M triethylammonium acetate (TEAA) (pH = 7.0); buffer B, CH_3CN/H_2O (50/50) containing 0.05 M TEAA. [d] 25 min from 21% B to 23% B. [e] 25 min from 26% B to 40% B, 5 min to 60% B and 5 min with 60% B. [f] 20 min from 30% B to 55% B. [g] 25 min from 26% B to 40% B. [h] 25 min from 30% B to 40% B. [i] 25 min from 30% B to 60% B and 5 min with 60% B. T_m in PIPES buffer at 120 mM NaCl; linear plots of T_m versus $\log_{10}$ (sodium ion activity) gave slopes of 15 ± 1 °C. [j] $d(A)_{12}$ in base molar ratio 1:1 was used as complementary strand for duplex formation. [k] Polyriboadenylic acid in base molar ratio 1:1 was used as target. [l] Half-life of the oligomers in the presence of SVPD was determined as described in the Experimental Section. [m] For oligonucleotides containing one CDU residue, the molecular weight was calculated for the formula $B_{10}C_{121}H_{165}N_{24}O_{82}P_{11}$ for the *closo* form and as $B_9C_{121}H_{165}N_{24}O_{82}P_{11}$ for the *nido* form; for oligomer **8**, only the *nido* form was detected; for oligonucleotides containing two CDU units, the formulas were calculated as $B_{18}C_{122}H_{173}N_{24}O_{82}P_{11}$ for the *nido/nido* derivative. The *nido/closo* compound and *closo/closo* derivative were not detected. [n] [M + 1]. [o] [M]. [p] [M − 1].

chemical foundation for developing boron-containing oligonucleotides as potential candidates for BNCT and AOT.

The aim of this work was to synthesize and determine some of the physicochemical properties of oligonucleotides containing 5-(*o*-carboran-1-yl)-2′-deoxyuridine (CDU) as a new boron-rich class of oligonucleotide analogues, which may allow combination of the advantages of both BNCT and AOT. Boron-containing oligonucleotides could also provide a new generation of diagnostic probes using neutrons to generate the γ radiation, allowing quantitation of the signal which is proportional to the boron content in the sample. New, convenient, and inexpensive sources of neutrons will make this application feasible in the near future.

We describe the synthesis of several modified dodecathymidylates where one or two thymine bases were replaced by 5-(*o*-carboran-1-yl)uracil residues located at different positions on the oligonucleotide chain. The effect of 5-(*o*-carboran-1-yl) modification on conformation of single- and double-stranded structures and duplex stability was examined. Results of preliminary biophysical studies are also reported.

Results

CDU (**1**) was synthesized from 5-iodo-2′-deoxyuridine in a five-step procedure, as described previously.[12,13] 5-(*o*-Carboran-1-yl)-5′-*O*-(dimethoxytrityl)-2′-deoxyuridine 3′-(β-cyanoethyl *N,N*-diisopropylphosphoramidite) (**3**) was obtained in a two-step procedure. First, the 5′-hydroxyl function of CDU was protected with the dimethoxytrityl group; in a second step, the corresponding partially protected CDU (**2**) was reacted with (2-cyanoethyl)(*N,N*-diisopropylamino)chlorophosphine, which yielded the phosphoramidite monomer (Figure 1).

Dodecathymidylic acid analogues bearing one or two 5-(*o*-carboran-1-yl)uracil residues at the 1st (**7**), 2nd (**8**), 7th (**9**), 11th (**10**), and both 10th and 11th (**11**) and 1st and 11th (**12**) locations of the 12-mer **6** (Table 1) (Nomenclature: CDUd$(T)_{11}$

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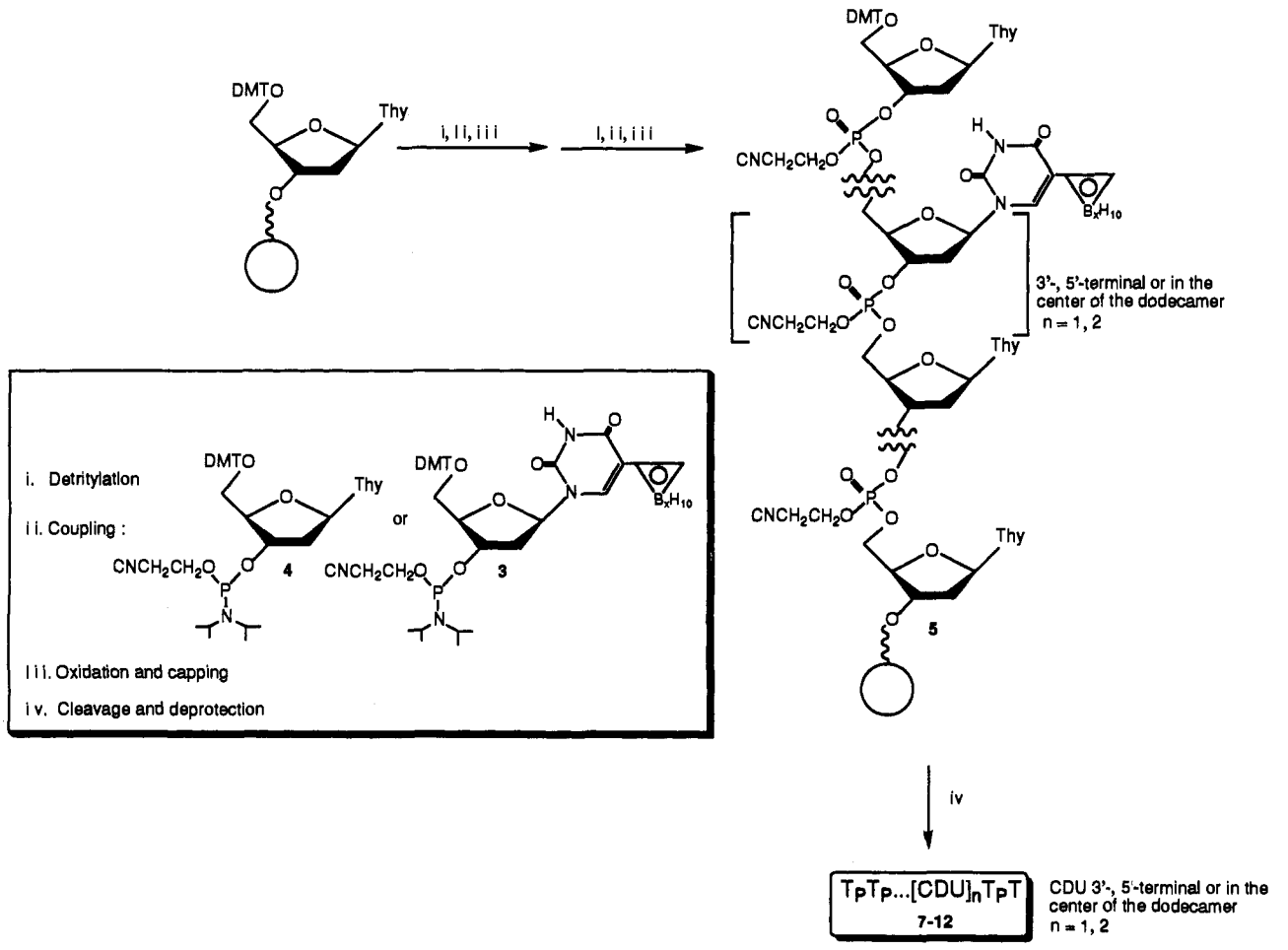

Figure 2. Automatic synthesis of CDU containing dodecathymidylates **7–12**.

represents ar oligomer with CDU as the first nucleotide at the 5′-end, whereas $d(T)_6CDUd(T)_5$ represents an oligomer with CDU as the 7th nucleotide on the 12-mer) were obtained by solid-phase automatic synthesis using a standard β-cyanoethyl cycle[14] (Figure 2). After removal of the 5′-dimethoxytrityl group, the oligonucleotides were then cleaved from the support by incubation with concentrated NH_4OH at room temperature for 1 h. The deprotected oligonucleotides were purified by HPLC and, for selected cases, separated into *nido* [*nido*-7,8-$C_2B_9H_{11}$] and *closo* [*closo*-1,2-$C_2B_{10}H_{12}$] forms (Table 1).[6] The yield for the overall synthesis of CDU-containing oligonucleotides **7–12** was comparable to that of unmodified $d(T)_{12}$ (**6**) according to the quantitation of trityl release during the automated synthetic procedure.

5′-End [^{32}P]-labeled oligonucleotides **6–12** were homogeneous and demonstrated the same electrophoretic mobility when analyzed by acrylamide gel electrophoresis (PAGE) (data not shown). Therefore, in further studies, the oligonucleotides were used as a *closo*/*nido* mixture. It should be pointed out that *closo*-carborane derivatives can be converted easily to the *nido* forms, for example by using pyrrolidine treatment.[15] This approach was used to transform CDU, $CDUd(T)_{11}$ (**7**), and $CDUd(T)_9CDUd(T)$ (**12**) into the corresponding *nido* compounds.

Mixing curves for unmodified $d(T)_{12}$ (**6**) and $CDUd(T)_{11}$ (*nido*-1.7) were obtained by titrating **6** or *nido*-1.7 with $d(A)_{12}$. With both **6** and *nido*-1.7 oligomer, a break in the plot occurred at the ratio of 1:1 (data not shown). No evidence of 1A:2T triplex formation was observed under these conditions.

Melting temperature (T_m; Table 1) measurements of the duplexes between CDU-modified $d(T)_{12}$ (**7–12**) and either $d(A)_{12}$ or poly(rA) as a complementary sequence were performed and compared with those formed between unmodified $d(T)_{12}$ (**6**) and template. Significant effects on T_m were noted, depending on location of the modification within the oligonucleotide chain (Table 1 and Figure 3). Thus, oligonucleotides modified with CDU at either the 1st (**7**), 7th (**9**), or 11th (**12**) position were separated into *closo* and *nido* derivatives by HPLC, and their T_m values were determined as duplexes with $d(A)_{12}$. The T_m values for each kind of oligonucleotide (modified at the 3′-, 5′-end or middle position) were independent of their *closo* and *nido* form; however, the effect of the CDU location was striking. 5′-Modifications at the 1st (**7**) and 2nd (**8**) position did not markedly influence the stability of the duplex compared to unmodified $d(T)_{12}$ (**6**) (Table 1). In contrast, modification in the central position of the oligonucleotide chain (7th position, **9**) resulted in a marked decrease of duplex stability, as noted by a low T_m value of 15.2 °C. A less pronounced effect was generated by the presence of CDU at the 11th position of oligonucleotide **10**. The T_m of the duplex formed by the oligonucleotide **10** decreased to 20.5 °C. Inserting a second CDU nucleoside adjacent to the 3′-end (CDU at the 10th and 11th positions, **11**) caused further destabilization of the duplex, decreasing the T_m value to 15.3 °C. The diverse consequences of 3′- and 5′-end modifications upon the duplex stability were evident from the above data. Insertion of the CDU nucleoside at the 3′-end had a much more unfavorable effect than at the 5′-end. This was well illustrated by comparing T_m values between oligonucleotides **8** and **10**, where the CDU nucleotide is located at the 2nd position from the 5′-end and the 3′-end, respectively. The difference in T_m was 7–8 °C, which

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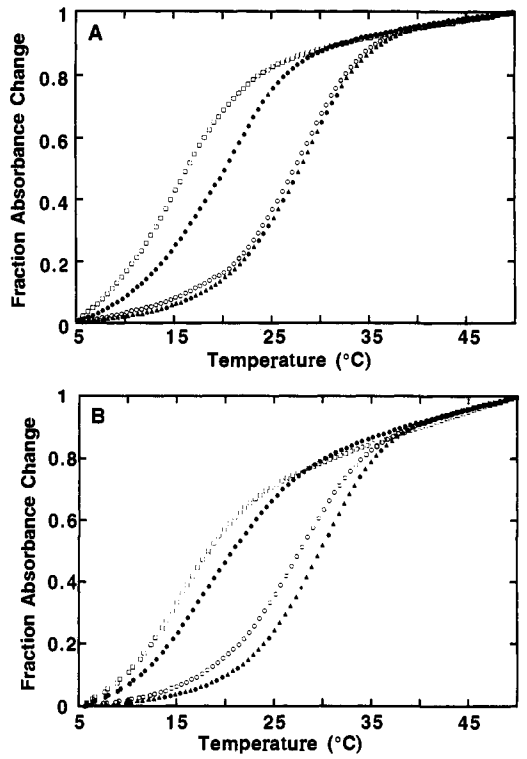

Figure 3. Melting curves for duplexes formed between selected CDU-modified and unmodified $d(T)_{12}$ with $d(A)_{12}$ (A) and poly(rA) (B). The experiments were conducted at 1:1 molar ratio in PIPES buffer at 0.1 M NaCl. $[d(T)_{12}]$:$[d(A)_{12}]$ (▲), $[CDUd(T)_{11}]$:$[d(A)_{12}]$ (○), $[d(T)_6CDUd(T)_5]$:$[d(A)_{12}]$ (□), $[d(T)_{10}CDUdT]$:$[d(A)_{12}]$ (●).

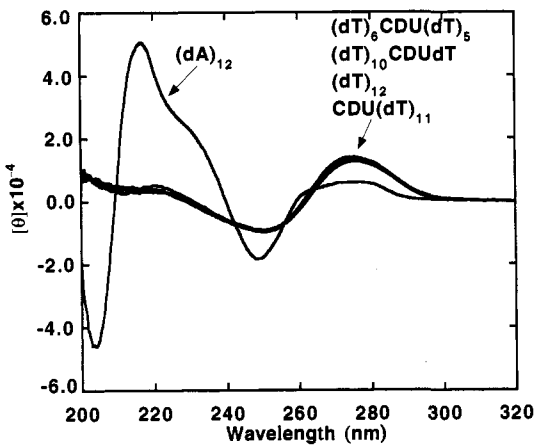

Figure 4. Circular dichroism spectra at 10 °C of single-stranded CDU-modified **7–12** and unmodified $d(T)_{12}$ (**6**) and $d(A)_{12}$.

was significant and revealed the importance of the carboranyl cluster interaction with adjacent bases.

Comparison of circular dichroism (CD) spectra of single-stranded $d(T)_{12}$ (**6**) and CDU modified $d(T)_{12}$ (**7–12**), recorded under analogous conditions, showed them to be almost identical in terms of their shapes and molecular ellipticity values (Figure 4). This suggests identical conformation in solution for the carboranyl oligomers compared to $d(T)_{12}$ (**6**) standard. Interestingly, the CD spectra of duplexes formed between CDU-modified oligothymidylates (**8–12**) and unmodified $d(T)_{12}$ (**6**) and $d(A)_{12}$ showed a reduction in the magnitude of molecular ellipticity at 246 nm, which correlated with increased thermal stability of the duplexes (Figure 5).

The stability of the oligonucleotides toward nucleases is an important factor for their future applications as potential therapeutics. It is generally accepted that 3′-exonuclease activity is responsible for most of the unmodified antisense oligonucleotide

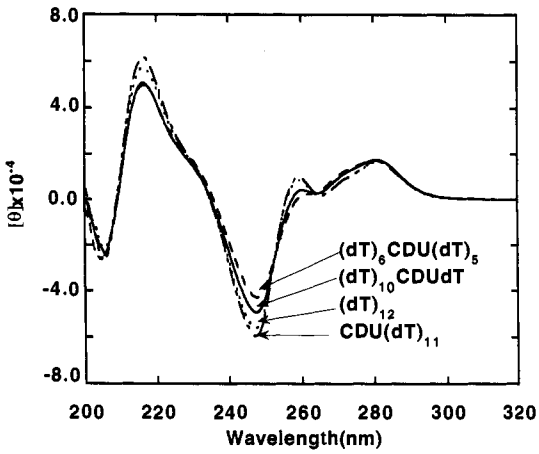

Figure 5. Circular dichroism spectra at 10 °C of duplexes formed between CDU-modified **7–12**, unmodified $d(T)_{12}$ (**6**), and $d(A)_{12}$.

degradation in serum.[16] To test the resistance of CDU-oligonucleotides toward 3′-exonucleolytic activity, snake venom phosphodiesterase (SVPD) from *Crotalus durissus terrificus* was used. The presence of CDU at the 3′-end of oligonucleotides effectively improved their stability toward SVPD. As expected, the oligonucleotide resistance toward nucleolytic activity increased in the order **6** ($t_{1/2}$ = 0.5 min) < **10.1** ($t_{1/2}$ = 6.7 min) ~ **12** ($t_{1/2}$ = 5.1 min) < **11** ($t_{1/2}$ = 76.2 min). The substantial change in half-life caused by CDU modification (150-fold increase for oligonucleotide **11** bearing two CDU molecules at the 3′-end, compared to unmodified oligomer **6**) may have practical implications. Additional stability studies with other enzymes, and in various sera, are ongoing. Preliminary results indicated that some of the CDU-modified oligonucleotides evaluated serve as primers for various polymerases, including human immunodeficiency virus type 1 reverse transcriptase.

Discussion

CDU-modified oligonucleotides were synthesized using standard automated synthetic methods on solid support. The presence of bulky carboranyl substituent at the 5-position of uracil seems to have little effect on the efficiency of the coupling reaction, which for CDU monomer varied from 87% to 92%, as established by trityl release. The availability of CDU may be a limiting factor in the case of the larger scale synthesis, since the literature procedure is tedious and laborious.[12,13] Our laboratory is working on an improved method of synthesis of nucleosides bearing carboranyl-containing purine and pyrimidine bases.[17]

We have found that, during the cleavage of the synthesized oligomer from the support, the transformation of the neutral *closo* form of the boron cage into its *nido* counterparts bearing one negative charge occurred under basic conditions.[6,15,18] This makes it difficult to prepare pure *closo* compounds when alkaline treatment is required for the synthesis. Using a colorimetric assay,[19] HPLC analysis, and mass spectrometry, we determined that even CDU can have traces of *nido* compound when prepared by the method of Yamamoto et al.[12] Molecular ions corresponding to *closo*-CDU (*closo*-**1**) ($B_{10}C_{11}H_{22}N_2O_5$, MW 370.4) and *nido*-CDU (*nido*-**1**) ($B_9C_{11}H_{22}N_2O_5$, MW 359.5) are present in mass spectra in a ratio of ca. 10:1. Conversion to the *nido* isomers occurred most likely during the deblocking of the benzoylated

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(19) TLC spray for the detection of *nido*- and *closo*-carboranyl compounds contained 3 mg of $PdCl_2$ in 50 mL of 1% HCl aqueous solution.

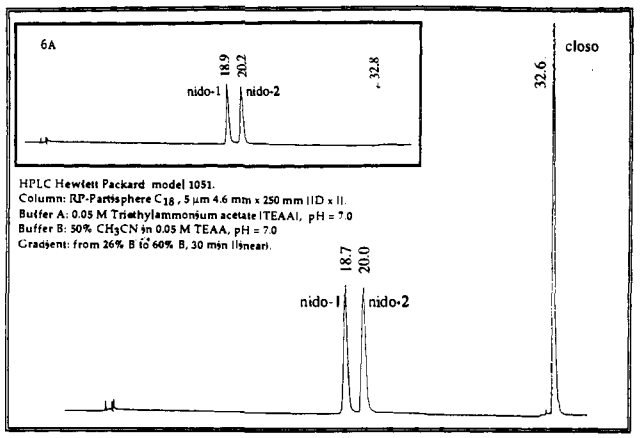

Figure 6. HPLC chromatograms of 5′-CDUd(T)$_{11}$ (**7**) as a mixture of *closo* and *nido* forms before and after pyrrolidine treatment (6A inset).

hydroxyl functions of CDU under basic conditions. CDU-modified oligonucleotides bearing boron clusters in the *nido* form were characterized by lower retention times on reverse-phase HPLC (Table 1), which readily allowed the separation of both components. Three peaks were identified in the HPLC chromatograms of oligonucleotides containing one CDU residue and up to nine for oligonucleotides containing two CDU residues for compounds 5′-CDUd(T)$_{11}$ (**7**), 5′-d(T)$_6$CDUd(T)$_5$ (**9**), 5′-d(T)$_{10}$CDUd(T) (**10**), 5′-d(T)$_9$CDU$_2$d(T) (**11**), and 5′-CDUd(T)$_9$-CDUd(T) (**12**) (Table 1). The characteristic shape of HPLC chromatograms was due to the existence of the CDU carboranyl cage in three distinctive forms, one *closo* and two *nido*. Two *nido* forms resulted from subtraction of boron number 3 or 6 from the *closo*-carborane, which led to two *nido*-carboranyl[20] enantiomers. Since the nucleoside units of the oligonucleotide chain are chiral, the CDU oligonucleotide exists as one species for the *closo* form of the carboranyl cage and as two diastereoisomers for the *nido* form of the carboranyl residue. In the case of oligonucleotides containing two CDU residues, the number of possible oligonucleotide components is defined by the formula 3^n, where 3 represents the number of putative forms in which the carboranyl cage can exist (one *closo* and two *nido*) and n is the number of carboranyl cages (CDU residues) per oligonucleotide chain. A similar phenomenon is observed for *P*-chiral oligonucleotide analogues. The number of diastereoisomeric oligomers is defined by the formula 2^n, where 2 is the number of isomers per one *P*-chiral internucleotide linkage and n is the number of *P*-chiral linkages.[22] Comparison of HPLC traces for 5′-CDUd(T)$_{11}$ (**7**) before and after pyrrolidine treatment, leading to selective transformation of the *closo* to the *nido* form of the carboranyl cage, is especially insightful (see for example Figure 6 and inset 6A). The HPLC trace for 5′-CDUd(T)$_{11}$ (**7**) before pyrrolidine treatment showed three peaks: the CDU residue can exist as *nido*-1, *nido*-2, or the *closo* form of the carboranyl cage (three products per CDU residue). After pyrrolidine treatment, the *closo* form of the carboranyl cage was transformed into *nido*-1 and *nido*-2; therefore, the number of CDU species is restricted to two, and the amount of expected isomers decreased to two. In the case of oligonucleotides containing two CDU units, the HPLC chromatogram was more complex, but can be explained following the same reasoning. The HPLC trace of 5′-CDUd(T)$_9$CDUd-(T) (**12**) before pyrrolidine treatment showed nine peaks: the number of CDU units is $n = 2$; therefore, one can expect $3^2 = 9$ derivatives, resulting from a mixture of the different combinations of CDU in the *closo* or *nido* form. This complex mixture should appear as nine peaks on the HPLC chromatogram at maximal resolution. After pyrrolidine treatment, the *closo* form of the carboranyl residue was converted into *nido*-1 and *nido*-2, leading to only two CDU species and $2^2 = 4$ as the amount of expected isomers. Because two of the four isomers, under the eluting condition used, possessed the same R_1, the two peaks overlapped, generating three signals with an integration area ratio of 1:2:1, instead of four peaks with a ratio of 1:1:1:1 (data not shown). The identity of CDU oligomers was additionally confirmed by MS analyses (Table 1).

The T_m measurements using the separated *nido*- and *closo*-CDU oligonucleotides showed no significant differences for both forms of CDU modification (Table 1). This may be due to the high lipophilicity of the boron cage and low density of the negative charge in the *nido* compound.

The T_m values of the duplexes formed between CDU-modified dodecathymidylic acids and d(A)$_{12}$ strongly depended on the location of CDU in the oligonucleotide chain and were not influenced by the *closo*/*nido* form of the carboranyl residue (Figure 3 and Table 1). These results were in agreement with T_m measurements of duplexes formed between CDU-modified oligomers and poly (rA) (Table 1). In effect, the location of the CDU nucleoside within the oligonucleotide chain induced a greater destabilization, which seemed even more pronounced when the modification was closer to the 3′-end. Although, in the case of poly(rA), the length of template was much longer than for the dodecamer d(A)$_{12}$, this unfavorable effect along with the CDU position was amplified compared to the DNA/DNA duplex. To our knowledge, this work represents the first systematic studies on the relationship between the location of pyrimidine nucleoside modified with a bulky cluster at position 5 within an oligonucleotide chain and the duplex stability. However, it is of interest that a similar effect of location was reported within the oligonucleotide chain of base-modified nucleoside bearing a biotinylated linker.[21]

To evaluate the observed differences in duplex stability for different CDU-modified oligonucleotides, molecular mechanics approaches were employed, i.e., the AMBER all-atom force field method and equations.[23] Two sets of experiments were performed. In the first one, weak distance constraints were applied to simply hold the general shape of the carborane ring system, as reported by Holbrey et al.[24] In the second case, the force constants were not used, but the carborane system was held as an aggregate in the SYBYL program during minimization. The conclusions concerning relative stability of the unmodified and CDU-containing duplexes were the same with both methods. Initial models of d(T)$_{12}$·d(A)$_{12}$ DNA duplexes were constructed with carborane substitutions at different positions in the sequence described in the Experimental Section and were energy minimized along with an unsubstituted control. There were significant unfavorable interactions of the carborane substituent with adjacent bases, and the interactions were asymmetric in orientation due to the right-handed twist of DNA (Figure 7).

The refined models clearly showed that a steric clash of the carborane occurred almost exclusively with the base on the 5′-side of the CDU-substituted base. A base pair containing CDU and base pairs above and below it are shown in Figure 7. The top view (along the helix axis) clearly demonstrates that the carborane interacts strongly with the base on the 5′-side of the CDU, but has essentially no interaction with the base on the 3′-side of the carborane-substituted base. In agreement with these observations, energy minimization results with d(T)$_{12}$·d-(A)$_{12}$ showed that substitution on the 5′-end of the d(T)$_{12}$ strand

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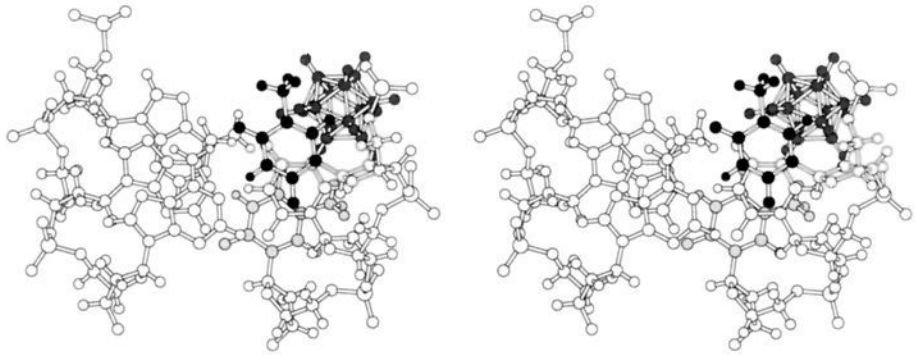

Figure 7. Stereoview of three base pairs from the model for the CDU-substituted duplex described in the text. The backbone and adenine atoms are white, the three pyrimidine bases are shaded. The top base is the 5′T (black), the center base is the CDU (white pyrimidine and dark gray boron cage), and the bottom base is the 3′T (light gray). As can be seen, the carborane cage interacts strongly with the 5′T, but has no significant interactions with the 3′T.

gave overall helix energies that were significantly lower than for the duplex substituted on the 3′-side of $d(T)_{12}$.

Substitution on the interior bases of the duplexes caused even larger helix destabilization. With the 5′-substituted duplex, there is little interaction of the carborane with bases, and the overall helix geometry is similar to the unsubstituted duplex. To relieve some of the strain from the steric interaction at the 3′-end of the duplex, the bases can distort due to end effects and freedom of motion of base pairs at the end of the double helix. CDU-substituted bases in the center of the helix have similar steric interaction, but they do not have the flexibility of base pairs at the 3′-end of the helix.

Thus, duplexes with central CDU substitutions exhibited the highest energy, indicating that this was the most unfavorable position for CDU modification. These observations were in complete agreement with the T_m results, where CDU substitution at the 5′-end of the $d(T)_{12}$ strand caused little change in T_m relative to the unsubstituted duplex. Substitution at the 3′-end destabilized the helix structure, while central substitution caused the largest decrease in T_m (Table 1). Additional molecular modeling studies indicated that steric hindrance between the carboranyl moiety and the 5′-adjacent base is reduced by inserting a three-methylene tether at the 5-position of uracil.

No differences were observed in CD spectra of single-stranded natural and CDU-modified $d(T)_{12}$ (Figure 5). This may suggest a lack of effect of CDU on single-stranded oligonucleotide conformation in solution, at least for oligomers containing one or two CDU molecules. The CD spectra of duplexes formed between CDU-modified oligonucleotides (**7**–**12**) and $d(T)_{12}$ (**6**) and $d(A)_{12}$ showed the reduction of molecular ellipticity at a minimum at 246 nm related to the thermal stability of the duplexes (Table 1, Figures 3 and 5). Thus, the least stable duplex formed by the oligonucleotide modified with CDU at the 7th position (**9**) was characterized by the highest value of molecular ellipticity of the negative CD band at 246 nm, and consequently the most stable unmodified (**6**) and 5′-end modified (**7**) dodecathymidylic acid were characterized by the lowest value of molecular ellipticity of the negative CD band. Both also showed a distinctive peak at 257 nm (Figure 5). The higher Cotton effect observed between 240 and 260 nm for stable duplexes suggests less disturbance of base stacking, which correlates well with a stable, well-arranged double-helix structure.

The stability of oligonucleotides in the presence of nucleolytic enzymes is a crucial factor for their application as potential therapeutic agents. The most effective way to secure the resistance against nucleases is modification of internucleotide linkages. The replacement of all natural phosphodiester linkages within an oligonucleotide chain by methylphosphonates is an example of modifications that ensure complete stability of the oligonucleotide toward exo- and endonucleases.[16,22] However, there are several literature reports on increased enzymatic stability of oligonucleotides modified only at the 3′- or 3′,5′-flanking positions.[25,26] This prompted us to determine the effect of CDU modifications upon the oligonucleotide resistance toward 5′-exonucleolytic activity using SVPD. As expected, the oligonucleotide resistance toward nucleolytic activity was lowest for the unmodified oligomer **6**. The oligonucleotide bearing one CDU residue at the 3′-terminus (*nido*-1.**10**) clearly exhibited greater stability than **6**. The oligonucleotide **11** with two CDU residues was markedly more stable (Table 1).

It seems that the presence of a bulky lipophilic carboranyl substituent at the 5-position of the pyrimidine nucleoside prevented effective interaction of the modified oligonucleotide fragment with the active center of the enzyme. This reasoning is supported by the finding that the stability of the oligonucleotide with two adjacent CDU residues is notably higher than the oligonucleotide with one modification, which suggests further disturbance of oligonucleotide–enzyme interaction. It should be noted that the modified oligonucleotide synthesized contained the CDU residue at the penultimate position of the oligomer, so thymidine is the 3′-terminal nucleoside. We assume that thymidylate is rapidly removed in the first stage of enzymatic digestion, and then the resultant undecamer terminated with CDU is slowly digested by the enzyme. Studies are underway to determine the resistance of CDU-modified oligonucleotides against other nucleolytic enzymes and in various sera.

The lipophilicity of CDU oligonucleotides 5′-$CDUd(T)_{11}$ (mixture of *nido*-1.**7**, *nido*-2.**7**, and *closo*-**7**), 5′-$d(T)_6CDUd(T)_5$ (mixture of *nido*-1.**9**, *nido*-2.**9**, and *closo*-**9**), and 5′-$d(T)_{10}CDUd$-(T) (mixture of *nido*-1.**10**, *nido*-2.**10**, and *closo*-**10**) was compared with $d(T)_{12}$ by the coinjection experiment under the same HPLC conditions using a reverse-phase column (see Experimental Section). In Table 1, optimized HPLC conditions for every oligonucleotide are defined. The HPLC analysis indicated not only different affinities (lipophilicities) of *closo*/*nido* carboranyl forms to C_{18} resin but also a correlation between the CDU location within the oligonucleotide chain and the oligonucleotide R_t. The oligomers' lipophilicity as measured by R_t increased in the order *nido*-1 < *nido*-2 < *closo*- and, with respect to CDU location, in the order $d(T)_{12}$ < central CDU < 5′-terminal CDU < pseudo-3′-terminal CDU.

In summary, we have developed a novel type of oligonucleotide modification which could lead to boron-containing oligonucleotides useful for AOT and BNCT. Because of the γ radiation induction associated with the presence of the boron atoms in the oligomers, the potential exists to use these oligonucleotides as probes for diagnosis of various diseases. We demonstrated that properly designed CDU-modified oligonucleotides formed stable

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duplexes with complementary sequence. The T_m studies were supported by molecular modeling approaches to structural analysis of the effect of CDU modification on duplex stability. As an added benefit, certain 3'- and 3',5'-CDU-modified oligonucleotides exhibited marked resistance toward 5'-nucleolytic enzyme.

Experimental Section

Column chromatography was performed on silica gel 60, 230–400 mesh obtained from Aldrich Co. (Milwaukee, WI). TLC was performed on silica gel F 254 plates obtained from Sigma (St. Louis, MO). C_{18} reverse-phase columns (5 μm, 4.7 × 235 mm) were obtained from Whatman (Hillsboro, OR). Solvents were purchased in the highest available quality. 5'-*O*-Dimethoxytritylthymidine 3'-(β-cyanoethyl *N,N*-diisopropylphosphoramidite) was obtained from Chem-Impex International (Wood Dale, IL, lot no. 105198). Thymidine-loaded 1 μM CPG (500-Å pore size) columns were purchased from Applied Biosystems (Foster City, CA). Phosphodiesterase I (EC 3.1.4.1) type VIII from *Crotalus durissus terrificus* venom (lot 119F0730) was purchased from Sigma Co. (St. Louis, MO). Acrylamide was purchased from International Biotechnologies Inc. (New Haven, CT). *N,N*'-methylenebisacrylamide and urea were bought from Fischer Scientific (Fair Lawn, NJ). ^{31}P NMR spectra were recorded on a Bruker WP-200 spectrometer operating at 81.0 MHz, using 85% H_3PO_4 as an external standard. ^{1}H NMR spectra were recorded on a GE QE Plus spectrometer operating at 300.15 MHz. Tetramethylsilane was used as an external standard. Shifts downfield from the standard were assigned as positive. UV spectra were recorded with a Beckman DU-65 spectrophotometer. Thermal melting curves for the oligomers were determined on a Varian Cary 4 spectrophotometer interfaced to a Dell microcomputer. Poly(rA) (lot 3034110011) was bought from Pharmacia (Piscataway, NJ). Electrospray mass spectra were recorded on a VG 70-S spectrometer and on a PE-Sciex API III LCMS with an electrospray interface (negative mode of ionization). CD spectra were recorded on a Jasco J-600 spectropolarimeter interfaced to an IBM computer. Acrylamide gel electrophoresis was performed using a BRL apparatus (Gaithersburg, MD).

5-(*o*-Carboran-1-yl)-5'-*O*-(dimethoxytrityl)-2'-deoxyuridine (2). After three coevaporations with anhydrous pyridine, CDU (**1**)[12,13] (400 mg, 1.08 mmol) was dissolved in the same solvent (10 mL) under an argon atmosphere. 4,4'-Dimethoxytrityl chloride (457 mg, 1.35 mmol, 1.25 equiv) was added to the stirring solution. After stirring for 6 h at room temperature under argon, the reaction was quenched with MeOH (1 mL) and then diluted with CH_2Cl_2 (30 mL). The mixture was washed with a saturated solution of $NaHCO_3$ (25 mL) and then with water (2 × 25 mL). The organic layer was extracted, dried over Na_2SO_4, filtered, then evaporated under reduced vacuum, and coevaporated with toluene. The residual foam was dissolved in CH_2Cl_2 and applied to a silica gel column, which was eluted using a gradient from 0% to 5% CH_3OH in CH_2Cl_2. The fractions containing the desired product were combined, solvents were evaporated under vacuum, and then the residue was precipitated in *n*-hexane to yield 5'-*O*-(dimethoxytrityl)CDU as a white powder (497 mg, 68% yield). ^{1}H NMR ($CDCl_3$): δ 7.81 (s, 1H, NH); 7.51–7.32 (m, 10H, H-6 and 9H-arom); 6.96 (m, 4H, H in α of OCH_3); 6.23 (t, 1H, H-1'); 5.78 (bs, 1H, H-carboranyl); 4.50 (m, 1H, H-3'); 4.21 (m, 1H, H-4'); 3.90 (s, 6H, 2 × OCH_3); 3.60 (m, 1H, H-5'(H-5")); 3.35 (dd, 1H, H-5"(H-5')); 3.12 (d, 1H exch, OH-3'); 3.2–1.2 (bm, 10H, H of $B_{10}H_{10}$); 2.61 (m, 1H, H-2'(H-2")); 2.15 (m, 1H, H-2"(H-2')).

5-(*O*-Carboran-1-yl)-5'-*O*-(dimethoxytrityl)-2'-deoxyuridine 3'-(β-Cyanoethyl *N,N*-diisopropylphosphoramidite) (3). Compound **2** (200 mg, 0.297 mmol) was dissolved in freshly distilled anhydrous CH_2Cl_2 (1.2 mL). After stirring under an argon atmosphere for 5 min, diisopropylethylamine (DIEA, 207 μL, 1.19 mmol, 4 equiv) was added dropwise under argon followed by the addition of the phosphitylating agent, (2-cyanoethyl)(*N,N*-diisopropylamino)chlorophosphine (100 μL, 0.445 mmol, 1.5 equiv). The reaction was monitored by TLC using *n*-hexane/EtOAc/NEt_3 50:49:1. After stirring for 1 h under argon at room temperature, an excess of phosphitylating reagent was added (20 μL, 0.089 mmol) and the reaction was allowed to continue for 30 min. The mixture was then diluted with EtOAc freshly passed through Al_2O_3 (10 mL) and poured into a brine solution (6 mL). The organic layer was washed two more times with brine (2 × 6 mL), dried over Na_2SO_4, filtered, and evaporated to dryness under vacuum. The residual oil was then dried under high vacuum to remove excess diisopropylethylamine, resulting in a white foam. Purification of the crude material was performed on a silica gel column which was eluted with a gradient of *n*-hexane/EtOAc/NEt_3 from 90:9:1 to 20:79:1. Appropriate fractions containing a mixture of diastereoisomeric 3'-phosphoramidite CDU were combined and evaporated under vacuum. The desired compound **3** was then precipitated in cooled *n*-hexane at –20 °C and the resulting solid dried further under high vacuum for 20 h (218 mg, 84% yield). ^{31}P NMR ($CDCl_3$): δ 149, 90, and 149.61 ppm. ^{1}H NMR ($CDCl_3$): δ 7.99 (s, 1H, NH); 7.74–7.17 (m, 10H, H-6 and H-arom); 6.81 (m, 4H, H in α of OCH_3); 6.11 (m, 1H, H-1'); 5.65 (bs, 1H, H-carboranyl); 4.48 (m, 1H, H-3'); 4.22 (m, 1H, H-4'); 4.13 (m, 1H, H-5'(H-5")); 3.77 (2s, 6H, 2 OCH_3); 3.74–3.19 (m, 5H, H-5', H-2', and H-2", 2 × H of $NCH(CH_3)_2$); 2.72 (t, 1H, H of $POCH_2CH_2CN$); 2.58 (t, 2H, H of $POCH_2CH_2CN$); 2.42 (t, 1H, H of $POCH_2CH_2CN$); 3.2–1 (bm, 10H, H of $B_{10}H_{10}$); 1.2 (sev d, 12H, H of $NCH(CH_3)_2$).

Automated Synthesis of CDU Containing Dodecathymidylate (7–12) and Unmodified d(T)$_{12}$ (6), and d(A)$_{12}$. The natural oligonucleotides as well as the modified material were synthesized using an Applied Biosystems 391 DNA synthesizer. Columns loaded with controlled pore glass functionalized with 5'-*O*-(dimethoxytrityl)thymidine (1 μM) were utilized as a solid support. All 5'-(dimethoxytrityl)-3'-phosphoramidite derivatives were prepared as 0.09 M solutions in anhydrous CH_3CN. Elongation of oligonucleotides was performed using the standard β-cyanoethyl 1 μM DNA synthesis cycle[14] without change in condensation time. Oligonucleotides were then cleaved from the support by incubation in concentrated NH_4OH for 1 h at room temperature. The oligonucleotides were then purified using an HPLC C_{18} reverse-phase column, as described below. The fraction containing the desired product was collected. The buffer was evaporated under vacuum, and the solid was coevaporated several times with 96% EtOH to remove triethylammonium acetate (TEAA). The oligonucleotides were then redissolved in water and lyophilized, and the procedure was repeated twice. All oligonucleotides were stored as lyophilized powder at –20 °C. When needed, they were redissolved in water, stored as frozen solution, and relyophilized as soon as possible.

HPLC Analysis of Dodecathymidylate Containing CDU (7–12). HPLC analysis and purification were performed on the Hewlett-Packard 1050 system, using a Whatman Partisphere C_{18} 5-μm, 4.7 × 235 mm column. All analyses were performed at room temperature, and the oligonucleotides (40–50 ODU) were separated in four fractions for their purification. Typically, a gradient of CH_3CN from 0% to 50% in 0.05 M triethylammonium acetate buffer (TEAA), pH 7.0, was used as eluant at a flow rate of 1.0 mL/min. Coinjection experiments were conducted with 5'-CDUd(T)$_{11}$ (mixture of *nido*-**1.7**, *nido*-**2.7**, and *closo*-**7**), 5'-d(T)$_6$CDUd-(T)$_5$ (mixture of *nido*-**1.9**, *nido*-**2.9**, and *closo*-**9**), 5'-d(T)$_{10}$CDUd(T) (mixture of *nido*-**1.10**, *nido*-**2.10**, and *closo*-**10**), and d(T)$_{12}$ (**6**). The following gradient of CH_3CN in TEAA was used: from 5% to 20% (20 min), 20% (5 min), from 20% to 30% (3 min), 30% (3 min). Under those conditions the following R_t values (min) for the oligomers were recorded: **6**, 15.5; *nido*-**1.9**, 19.9; *nido*-**2.9**, 20.3; *nido*-**1.7**, 21.0; *nido*-**2.7**, 21.5; *closo*-**9**, 23.1; *nido*-**1.10**, 25.0; *nido*-**2.10**, 25.5; *closo*-**7**, 25.8; *closo*-**10**, 32.8. R_t values and the optimized conditions utilized for individual oligonucleotide purification are shown in Table 1.

Acrylamide Gel Electrophoresis (PAGE). Labeled or unlabeled samples of modified oligonucleotides **7–12** and dodecakis(thymidine phosphate) (**6**), prepared as described above, were separated by electrophoresis using a 20% acrylamide denaturing gel containing 7 M urea for 45 min at 50 mA. The samples were visualized using standard autoradiography on X-Omat AR film (Eastman Kodak, Rochester, NY) or, in the case of unlabeled oligonucleotides, by means of UV shadowing.

Radiolabeling of Dodecakis(thymidine phosphates) containing CDU (7–12). Modified oligonucleotides **7–12** and unmodified dodecakis-(thymidine phosphate) (**6**) (20 pmol of each) were incubated at 37 °C in the presence of 0.5 μL of T4 polynucleotide kinase and 10 μCi [^{32}P-γ]ATP (5000 Ci/mmol) in a 70 mM Tris-HCl buffer (pH 7.6) containing 10 mM $MgCl_2$ and 5 mM dithiothreitol. The final volume of the reaction mixture was 10 μL. After 30 min, the reaction mixtures were incubated at 92 °C for 2 min to heat inactivate the enzyme, and then 10× tracking dye (0.5% bromophenol blue, 0.5% xylene cyanol FF, 30% glycerol in water) (5 μL) was added to the reaction mixture and 5-μL aliquots were analyzed by PAGE.

Mixing Curves. Mixing curves for the oligomers d(T)$_{12}$ (**6**) and 5'-CDUd(T)$_{11}$ (*nido*-**1.7**) were obtained at 20 °C in PIPES buffer [1,4-piperazinebis(ethanesulfonic acid), 10 mM], pH 7.0, containing 1 mM EDTA and 100 mM NaCl, by titration of **6** (50 μM) or *nido*-**1.7** (40 μM) with d(A)$_{12}$ (50 μM). Absorption change at 260 nm was followed as a function of **6** or *nido*-**1.7**:d(A)$_{12}$ ratio.

T_m Measurements. Samples for T_m measurements were prepared by the addition of concentrated stock solutions of d(T)$_{12}$ (**6**) or CDU-modified

$d(T)_{12}$ (**7–12**) and $d(A)_{12}$ or poly(rA) stock solution (from 3 to 5 mM) to 10 mM PIPES buffer (pH 7.0) (1 mL) containing 100 mM NaCl and 1 mM EDTA, in amounts to give a 1:1 base ratio. Strands **7–12** were present at a final concentration of 40 μM. Molar extinction coefficients ϵ_{260} (per base) were calculated as follows: **6**, 8150; $d(A)_{12}$, 12 280; **7–12**, 8200; poly A, 9800.[27] The samples were heated to 85 °C and cooled slowly to room temperature before melting. The insulated cell compartment of a Cary 4 UV spectrophotometer was continuously warmed from 0 to 85 °C at the rate 0.5 °C/min. Samples were heated in quartz cuvettes fitted with a Teflon stopper (1-cm path length). Absorption change at 260 nm was followed as a function of temperature, and T_m values were obtained from first derivative plots after the data were graphed for visualization and analysis.

Circular Dichroism (CD) Measurements. CD spectra were obtained with a JASCO J600 spectrometer at 10 °C in a jacketed cell in which nitrogen gas was flushed to prevent condensation. Samples for CD measurement were prepared by the addition of concentrated stock solutions of the oligomers **7–12** to 2.7 mL of 3.75 mM phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 100 mM NaCl. The cuvette initially contained natural or modified $d(T)_{12}$ **7–12**, and subsequent volumes of $d(A)_{12}$ solution were added in amounts to give a 1:1 ratio. For duplex formation, the samples were heated to 85 °C and cooled slowly to room temperature. CD spectra were obtained from 320 to 200 nm for single strands and duplexes.

Molecular Modeling. Molecular mechanics methods with the AMBER all-atom force field and equations were used to evaluate comparative effects of the carborane cage on the stability of DNA modified at different sequence positions. Effects of the carborane on local DNA conformation when it replaces the 5-methyl group of thymine in a DNA duplex were evaluated. Three new atom types for the AMBER force field[28] were needed to generate the structure and perform energy refinement calculations on the modified duplexes. These were defined as B, CZ and HB, for boron, carbon, and hydrogen attached to boron in the carborane structure. The AMBER HC hydrogen atom type was used for two hydrogen atoms attached to two CZ carbons. Both B and CZ atoms were allowed to have more than four covalent bonds. Force field parameters, except for bond stretch constants, within the cage were set to be 0. The shape of the carborane structure was then determined by the bond relationship among the cage atoms. Bond lengths of 1.79 Å for B—B, 1.72 Å for B—CZ, 1.10 Å for B—HB, and 1.08 Å for CZ—HC were used,[24] and a stretch force constant of 300 kcal/Å^2, similar to other AMBER bond values, was applied. As reported by Holbrey et al.,[24] relatively small distance restraints of 100 kcal/mol were used to maintain the shape of the carborane ring system. The initial structure ($C_2B_{10}H_{12}$) was built by using the crystal atomic coordinates.[24]

The cage structure was refined by energy minimization until a delta rms gradient of 0.01 kcal/mol·Å^2 was reached. This refined boron cage was then manually docked into the major groove of the energy-minimized $d(T)_{12}$·$d(A)_{12}$ DNA duplex with the help of graphics and docking routines in the SYBYL software package. The methyl group of the thymine base to be modified was removed, and the position and orientation of the boron cage were manually adjusted in the DNA major groove to give the best fit with the groove shape. At this point, the carborane structure was linked to the thymine 5-position through a C—C bond. All energy minimizations were performed with the SYBYL 6.0 software package from Tripos on Silicon Graphics INDIGO or IRIS workstations. A modification of the AMBER force field for nucleic acid derivatives and complexes was used as previously described.[29] A set of calculations were also performed with the carborane held as a fixed aggregate in the calculations. The conclusions regarding relative energies of the CDU-substituted duplex were the same for the two sets of calculations.

Resistance of Dodecakis(thymidine phosphates) Containing CDU (*nido*-1.10, 11, and 12) toward Phosphodiesterase I (EC 3.1.4.1). To 100 mM Tris-HCl buffer, pH 8.9 (225 mL), containing 20 mM $MgCl_2$, were added 0.25 A_{260} ODU of oligonucleotide *nido*-1.**10**, **11**, **12** (6.25 μL), 0.3 A_{260} ODU of 2′-deoxycytidine (6.25 μL) used as internal standard, and 3.8×10^{-3} unit (12.5 μL) of phosphodiesterase were added. A blank reaction with no enzyme and a control reaction with $d(T)_{12}$ (**6**) with the enzyme were assayed simultaneously. Reactions were maintained at 37 °C, and then after 5, 10, 20, 40, 80, and 240 min, 40-μL aliquots were withdrawn, heated at 80 °C for 3 min, and immediately frozen at −70 °C prior to analysis by HPLC using the conditions described above. The half-life was calculated using the Dose, Effect, Binding and Kinetics Parameter Program designed by J. Chou and T.-C. Chou, and modified by R. T. Scott, B. S., and R. F. Schinazi, Ph.D., VAMC, Decatur, GA 30033 (originally published and distributed by Elsevier-Biosoft, 68 Hills Road, Cambridge, U.K.).

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Supplementary Material Available: Figures showing HPLC chromatograms of mixtures of the *closo* and *nido* forms of **7** and **9–12**, mass spectra of *nido*-1.7, *nido*-2.7, *closo*-**7**, and *closo*- and *nido*-CDU, circular dichroism spectra of $d(T)_6CDUd(T)_5$, $d(T)_{10}CDUd(T)$, $d(T)_{12}$, and $CDUd(T)_{11}$, stereochemistry of *closo* to *nido* transformation of the carboranyl cluster, PAGE analysis of 5′-$d(T)_{10}CDUd(T)$ oligomers, and absorbance versus $d(T)_{12}/d(A)_{12}$ and *nido*-1.7/$d(A)_{12}$ (17 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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