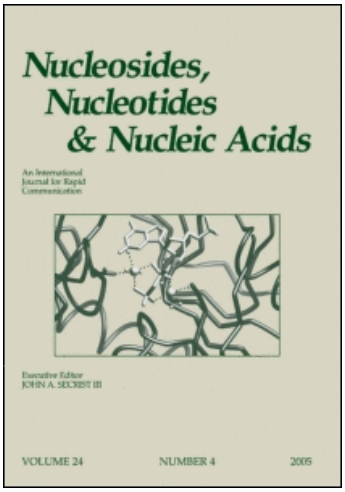


## Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

# Boron Containing Oligonucleotides

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**To cite this Article** Schinazi, Raymond F. and Lesnikowski, Zbigniew J.(1998) 'Boron Containing Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 17: 1, 635 — 647
**To link to this Article: DOI:** 10.1080/07328319808005206
**URL:** http://dx.doi.org/10.1080/07328319808005206

NUCLEOSIDES & NUCLEOTIDES, 17(1-3), 635-647 (1998)

REVIEW

# BORON CONTAINING OLIGONUCLEOTIDES†

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**ABSTRACT**: Synthesis, physicochemical and biological properties of novel class of oligonucleotide analogues, bearing boron modifications are described.



## INTRODUCTION

The pharmacological uses of boron compounds have been known for several decades.[1] Recently, there has been an increased interest in the preparation of boron compounds for their potential medicinal and biochemical applications.[2,3] Boron analogues of aminoacids and their derivatives express hypolipidemic,[4] antineoplastic,[5] antiinflammatory,[6] antiarthitic, antipleurisy, and analgesic properties.[6] Organoboron compounds are being extensively investigated with respect to their possible use in cancer therapy by boron neutron capture therapy (BNCT).[7,8] There is interest in chemically modified oligonucleotides due to their potential application as antisense and antigene probes to block replication, transcription or translation of pathogenic genes.[9,10] Boron containing oligonucleotides are one of the most recently developed type of oligonucleotide analogues. The rationale for their synthesis is potential application as boron carriers for BNCT, and as antisense oligonucleotides for antisense oligonucleotide technology (AOT). Boron containing oligomers used as primers or molecular probes is another important future practical application.

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†Dedicated to the memory of Professor Tsuijaki Hata.

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## OLIGONUCLEOTIDES BEARAING BORON MODIFICATION

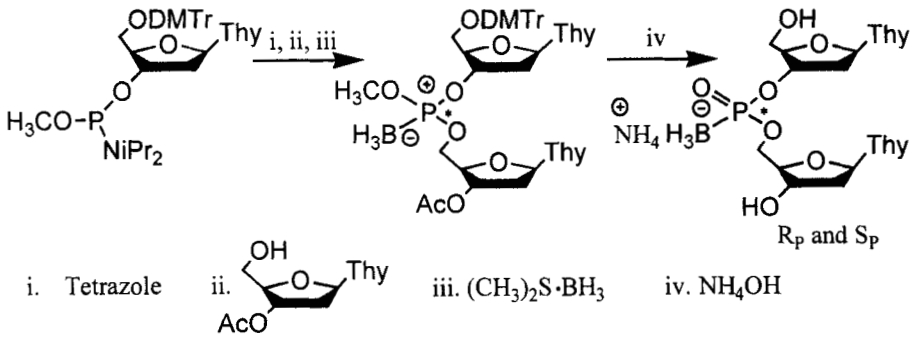
At present there are two major types of boron containing oligonucleotides described in literature. The first type, modified with borane ($-BH_3$)[11,12] or cyanoborane ($-BH_2CN$)[13,14] function, contains one boron atom per modification, the second type, bearing *o*-carboranyl-methylphosphonate [CBMP, $>P(O)CH_2C_2B_{10}H_{12}$],[15-17] or *o*-carboranyl group ($-C_2B_{10}H_{12}$)[18,19] comprises ten boron atoms involved in carboranyl cage structure. The chemistry, biophysical and biological properties of these novel oligonucleotide modifications are subjects of this review.

**Boranophosphate oligonucleotides.**

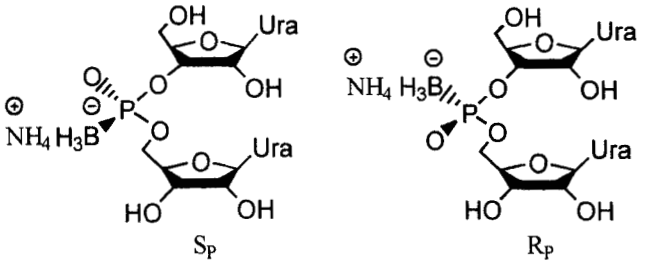
Sood *et al.*[11] and Shaw *et al.*[12] were the first to describe the synthesis of boranophosphate methyl ester and boranophosphate oligonucleotide analogues. This unique, charged oligonucleotide modification represents a link between charged, ionic, hydrophilic phosphodiester oligonucleotides; neutral, lipophilic methylphosphonate modification, and described only recently neutral, hydrophilic hydroxymethylphosphonate oligonucleotide analogues.[20,21] The boranophosphate thymidine dimer and trimer were synthesized (**Scheme 1**).

These oligonucleotides were prepared by the phosphoramidite method in solution, except that the oxidation step with $I_2/H_2O$ was replaced by reaction with dimethylsulfide/borane complex [$(CH_3)_2S/BH_3$]. Thus, reaction of 5'-*O*-dimethoxytritylthymidine 3'-(*O*-methyl-*N*,*N*-diisopropyl)phosphoramidate with 3'-*O*-acetylthymidine in the presence of tetrazole, in $CH_3CN$, followed by $(CH_3)_2S/BH_3$, provided the dimer in 52% yield. Removal of the backbone methoxy group and 3'-terminal acetyl group under treatment with concentrated $NH_4OH$ yielded the boranophosphate derivative. By repeating the coupling and oxidation step the trimer boranophosphate methyl ester was prepared in 22% yield [5% after purification by high pressure liquid chromatography (HPLC)]. By virtue of phosphorus chirality in dincucleoside boranephosphate group, they are formed as a mixture of $S_P$ and $R_P$ diastereomers. In the case of dimer the diastereomers are separable into individual species by of reverse phase (RP) HPLC.[12,22]

A similar method was used by Chen *et al.*[23] for the synthesis of boranophosphate ribodinucleoside, uridine(3',5')uridine boranophosphate (**Scheme 2**). The dimers with 2'- and 3'- acetyl groups at the 3'-terminal uracil, and 2'-*t*-butyldimetylsilyl group at the 5'-terminal uracil residue were obtained in 83% yield, as a mixture of $R_P$ and $S_P$ diastereomers. The diastereomers were separated into individual species, after removal of acetyl and backbone methyl protecting groups under treatment with concentrated $NH_4OH$, by means RP HPLC. The 2'-*t*-butyldimetylsilyl protection was then removed with tetrabutylammonium fluoride.




**Scheme 1**




**Scheme 2**

The absolute configuration at phosphorus atom in the diastereomers of uridine (3',5')uridine boranophosphate was tentatively assigned by their enzymatic digestion with snake venom phosphodiesterase (SVPDE). The resistance of dinucleoside phosphorothioate towards hydrolysis in the presence of SVPDE is indicative of $S_P$ configuration, while susceptibility to hydrolysis indicate the $R_P$ configuration.[24,25] Thus, based on enzymatic criteria, and assuming an isoelectronic structure of boranophosphates with phosphorothioate,[12] the $S_P$ configuration was assigned to the isomer of uridine(3',5')uridine boranophosphate more susceptible to SVPDE digestion. Consequently, the opposite configuration was ascribed to the dimer more resistant to SVPDE cleavage. This correlation is consistent with the tentative absolute configuration assignment done by means of 1D NOE difference experiments with individual diastereomers of thymidine (3',5')thymidine boranophosphate, and independent SVPDE digestion experiments.[26]

It should be pointed out that the chirality at phosphorus is designated as R and S, according to the Cahn-Ingold-Prelog rules, and means opposite space orientation of the ligands

of a phosphorus atom. Because of the priority rules, for different substituents there may be the same spatial orientation, as for instance in boranophosphates (or methylphosphonates) and phosphorothioates, corresponding to different configurational descriptors according to the R/S nomenclature.[22] Comparison of resistance to enzymatic digestion, $^{31}P$ NMR chemical shift, and retention time in RP HPLC of dinucleotides containing boranophosphate, phosphorothioate and methylphosphonate internucleotide linkage, with respect to the absolute configuration at phosphorus atom of modified internucleotide linkage is shown in Table 1.

In addition to chemical method, Tomasz *et al.* described recently an enzymatic incorporation of thymidine boranophosphate into the oligonucleotide chain in the extension of a deoxyribo 17-mer primer by modified T7 DNA polymerase (Sequenase). Thymidine 5'-α-P-boranotriphosphate, 25-mer template containing one deoxyadenosine residue, and Sequenase were used for the primer elongation.[27] Using the same methodology and 2'-deoxyguanosine 5'-α-P-boranotriphosphate, the 14-mer 5'-d(CTATGGCCTCAG*CT)-3' containing one boranophosphate internucleotide linkage located between G12 and C13 at 3'- end was also prepared (**Scheme 3**).[14]

Due to the chirality of the α-phosphorus atom, nucleoside 5'-α-P-boranotriphosphates can exist in $R_P$ or $S_P$ diastereomeric forms. One of the diastereomeric triphosphates is incorporated by Sequenase into oligonucleotide chain more efficiently than the other isomer. Although, the resultant diastereomeric oligonucleotides can be separated into individual species by RP HPLC, the stereoselectivity of the polymerization reaction has not been established unambiguously.[14]

The boronation confers considerable stability to calf spleen phosphodiesterase (BSPDE) and SVPDE.[12] The half time ($t_{1/2}$) for $S_P$ uridine(3',5')uridine boranophosphate at the presence of SVPDE was established as 80 h. The $R_P$ isomer is hydrolyzed only in 5% after 90 h, under the same condition.[23]

**N7-Cyanoborane-2'-deoxyguanosine containing oligonucleotides.**

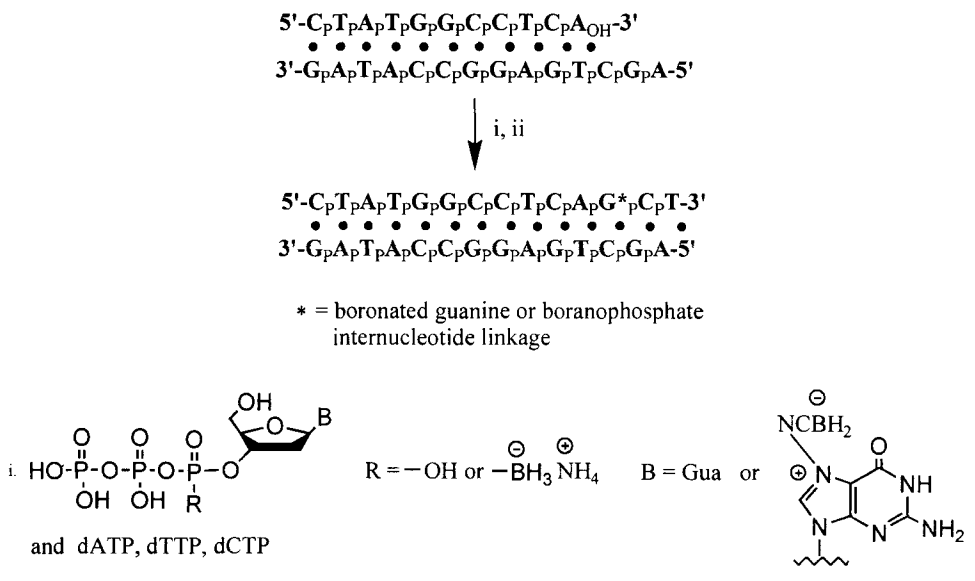
Sood *et al.* reported the synthesis of cyanoborane adducts of pyrimidine and purine 2'-deoxynucleosides.[28] They were used further for the synthesis of boronated oligonuclotides. Boron modified deoxyribonucleotide 14-mer 5'-d(CTATGGCCTCAG*CT)-3' containing N7-cyanoborane-2'-deoxyguanosine at position 12 at the 3'-end were synthesized by template-directed primer extension. N7-Cyanoborane-2'-deoxyguanosine triphosphate, 14-mer template 5'-d(AGCTGAGGCCATAG)-3', and modified T7 DNA polymerase (Sequenase) were used in extension reaction (**Scheme 3**).[14]

**Table 1.** Characteristics of boranophosphate, phosphorothioate and methylphosphonate internucleotide linkage.

| Internucleotide linkage | Resistance to enzymatic digestion[a] | $^{31}$P NMR δ | RP HPLC[e] | Absolute configuration at phosphorus |
|---|---|---|---|---|
| >P(O)$BH_3$ | lower[b] | higher field[a] | Fast | $S_P$ |
| >P$BH_3$(O) | higher[b] | lower field[a] | Slow | $R_P$ |
| >PS(O) | higher[b] | higher field[a] | Fast | $S_P$ |
| >P(O)S | lower[b] | lower field[a] | Slow | $R_P$ |
| >P(O)$CH_3$ | stable[b,c] | higher field[d] | Slow | $S_P$ |
| >P$CH_3$(O) | stable[b,c] | lower field[d] | Fast | $R_P$ |

[a]Deprotected, [b]SVPDE, [c]BSPDE, [d]3'- and 5'-protected, [e]isomer Slow: characterized by higher $R_f$ on RP HPLC column, isomer Fast: characterized by lower $R_f$ on RP HPLC column.






**Scheme 3**

The circular dichroism (CD) spectra of 5'-d(CTATGGCCTCAG*CT)-3' closely resemble the unmodified parent duplex, forming B-type helix. $T_m$ measurement experiments shown that boron containing hybrid duplexes have similar $T_m$ value relative to unmodified duplexes, indicating that modification does not affect duplex stability. This finding is true as well for N7-cyanoborane-2'-deoxyguanosine as for discussed above internucleotide boranophosphate modification.[14]

**Carboranylmethylphosphonate oligonucleotides.**

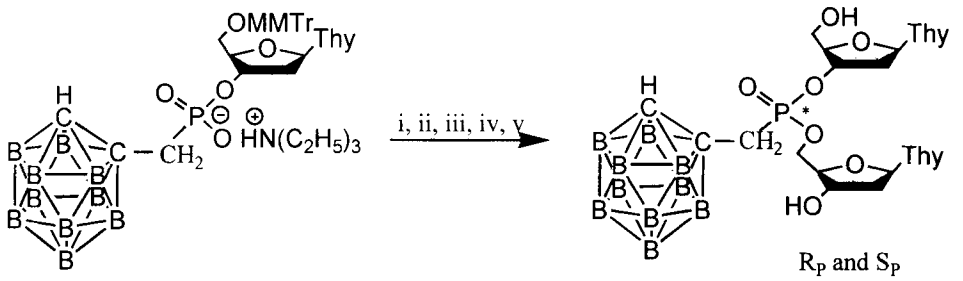
The carboranyl cluster is a new modifying entity for oligonucleotides potentially useful as boron carriers for BNCT, as antisense agents AOT, and as probes for tumor diagnosis and virology.[16] Two types of carboranyl modified oligonucleotides emerged so far.[16] The first type confines *o*-carboranylmethylphosphonate modification within the internucleotide linkage (CBMP oligonucleotides).[15] Another type (CDU oligonucleotides),[18] contains boron rich modified nucleic base, 5-*o*-carboranyl-2'-deoxyuridine (CDU)[29,30] and unmodified phosphodiester backbone.



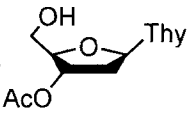
Lesnikowski and Schinazi described thymidine(3',5')thymidine (1-*o*-carboranylmethyl)phosphonate, the first oligonucleotide analogue modified with a carboranyl group.[15] The dimer bearing 3',5'-*O*,*O*-[(1-*o*-carboranylmethyl)phosphonate] internucleotide linkage instead of the natural 3',5'-*O*,*O*-phosphodiester, was synthesized by phosphotriester method in solution, as a mixture of $R_P$ and $S_P$ diastereomers (**Scheme 4**). The 3',5'-*O*,*O*-[(1-*o*-carboranylmethyl)phosphonate] internucleotide linkage was chemically stable at physiological pH, and resistant towards enzymatic digestion with BSPDE and SVPDE.

The dodecathymidylates containing CBMP 3',5'-internucleotide group at the 1st, 6th and 11th locations of the 12-mer $d(T)_{12}$ were obtained by solid phase automated synthesis. Unmodified phosphodiester linkages were formed using a standard $\beta$–cyanoethyl cycle and an automated DNA synthesizer.[31] The modified CBMP internucleotide linkage was produced using the phosphotriester method and boronated monomer, 5'-*O*-monomethoxytritylthymidine 3'-*O*-[(*o*-carboran-1-yl-methyl)phosphonate].[15] The yield for the coupling reaction involving boronated monomer varied from 15% to 40% according to quantitation of trityl release during the automated synthetic procedure. The yield for the coupling of unmodified monomer was about 95% and seemed unaffected by the incorporated modification.[32]

All oligonucleotides bearing carboranyl modification were obtained as mixtures of both *closo*- [*closo*-1,2-$C_2B_{10}H_{12}$], and *nido*- [*nido*-7,8-$C_2B_9H_{11}$] forms of carboranyl cage.[33] The deprotected oligonucleotides were purified by HPLC, and separated into *nido*- and *closo* form. Separation of P-diastereomers of the oligonucleotide bearing *nido*-(*o*-carboran-1-yl-




*o-closo*-carborane

i. 2,4,6-Triisopropylbenzenesulfonyl chloride and 2,4,6-collidine
ii. *N*-Methylimidazole
iii. 

iv. 80% $CH_3COOH$
v. conc. $NH_4OH/CH_3OH$

**Scheme 4**



methyl)phosphonate group at 11th position was achieved. Isomer Fast (characterized by lower value of $R_t$ on RP HPLC column) and Slow (characterized by higher value of $R_t$ on RP HPLC column) isomers were separated into individual species by means RP HPLC.

CD spectra of single-stranded $(dT)_{12}$ and CBMP modified $(dT)_{12}$ recorded at 5 °C were almost identical in term of their shapes and molecular ellipticity values. The CD spectra of duplexes formed between CBMP modified or unmodified $(dT)_{12}$ and poly r(A) at 5 °C showed a substantial decrease in the magnitude of molecular ellipticity of trough and increase of ellipticity value of peaks compared to single stranded oligonucleotides.

$T_m$ measurements of the duplexes formed between CBMP dodecathymidylates and poly r(A) as a complementary sequence, were compared to those formed between unmodified $d(T)_{12}$ and the template. Significant effects of CBMP group on $T_m$ were noted, depending on the location of the modification within the oligonucleotide chain, and the *closo-* or *nido-* status of carboranyl cage. The $T_m$ value for all oligonucleotides studied was in general higher than for unmodified $d(T)_{12}$. The $T_m$ increased in the order *nido*-CBMP at the 6th position < $d(T)_{12}$ < *nido*-CBMP at 11th position, Fast = *closo*-CBMP at the 6th position < *nido*-CBMP at the 1st position < *nido*-CBMP at 11th position, Slow < *closo*-CBMP at the 1st position.

A pronounced effect of the CBMP modification on oligonucleotide resistance towards SVPDE (5'-exonuclease activity; digestion from oligonucleotide 3'-end), was observed. Thus,

resistance increased in the order $d(T)_{12}$ ~ *closo*-CBMP at the 6th position ~ *nido*-CBMP at the 1st position ~ *closo*-CBMP at the 1st position (complete digestion after 5 min) < *nido*-CBMP at the 11th position, Fast < *nido*-CBMP at the 6th position = *nido*-CBMP at the 11th position, Slow < *closo*-CBMP at the 11th position, Fast < *closo*-CBMP at the 11th position, Slow.

The presence of CBMP at the 5'-end of the oligonucleotide completely protected the modified oligomer against digestion by BSPDE (3'-exonuclease activity; digestion from oligonucleotide 5'- end). The oligonucleotide's resistance towards BSPDE activity increased in the order *closo*-CBMP at 11th position, Fast < $d(T)_{12}$ < *closo*-CBMP at 11th position, Slow < *nido*-CBMP at the 6th position < *nido*-CBMP at 11th position, Fast < *nido*- CBMP at 11th position, Slow << *closo*-CBMP at the 6th position ~ *nido*-CBMP at the 1st position ~ *closo*-CBMP at the 1st position (no released thymidine-3'-monophosphate was detected by HPLC after 40 min).



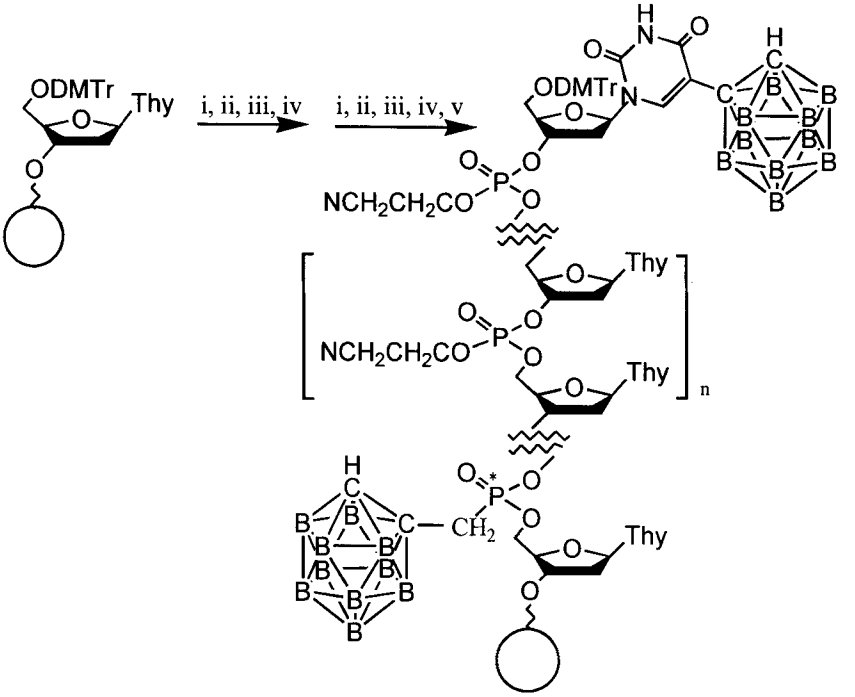
Phosphorylation of CBMP-containing oligonucleotides with T4 polynucleotide kinase was observed for all oligonucleotides with the exception of oligonucleotide bearing CBMP group at the 5'-end of the oligomer (CBMP group at the 1st position).

The lipophilicity of CBMP oligonucleotides was compared with $d(T)_{12}$ by coinjection experiment under the same HPLC conditions using $C_{18}$ reverse phase column. As anticipated, the lipophilicity, as measured by the retention time ($R_t$), of the *closo*-derivative was found to be higher than that of the *nido*-counterpart. Lipophilicity of both modifications was higher than unmodified dodecathymidylic acid. This is due to replacing of a charged natural phosphodiester internucleotide linkage with the CBMP modification bearing neutral *closo*-carboranyl cage, and by virtue of lipophilic property of carboranyl cage itself.[16, 32]

**5-*o*-Carboranyl-2'-deoxyuridine containing oligonucleotides.**

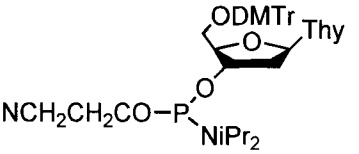
Base modified, oligothymidylates $d(T)_{12}$ containing one or more 5-*o*-carboranyl-2'-deoxyuridine (CDU-oligonucleotides) instead of thymidine were synthesized using an automated β-cyanoethyl phosphoramidite approach and standard coupling cycle. Boronated monomer, 5-(*o*-carboran-1-yl)-5'-*O*-(dimethoxytrityl)-2'-deoxyuridine 3'-(β-cyanoethyl *N*,*N*-diisopropylphosphoramidite) was used to incorporate the modification (**Scheme 5**).[18]

Dodecathymidylic acid analogues bearing one or two CDU-residues at the 1st, $2^{-0Bd}$, 7th, 11th, and both 10th and 11th and 1st and 11th location starting from 5'-end of the 12-mer $d(T)_{12}$ were obtained. They were formed as a mixture of oligomers containing CDU carboranyl cage in both neutral *closo*- and ionic *nido*-form.[16,18,33] The *closo*- and *nido*- CDU-oligomers were separated by RP HPLC. The yield for the overall synthesis of CDU-containing oligonucleotides was comparable to that of unmodified $d(T)_{12}$.



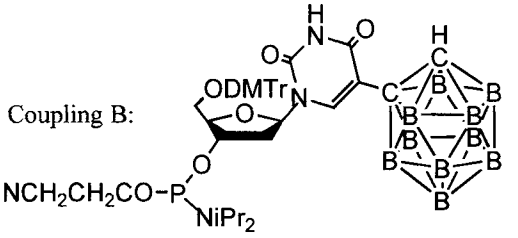

i. Detritylation

ii. Coupling A:

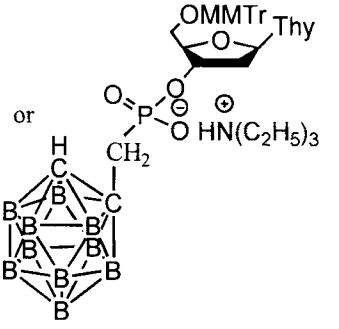



iii. Oxidation and capping

iv. Coupling B:




v. Cleavage from the support and deprotection




**Scheme 5**

Comparison of CD spectra of single stranded $d(T)_{12}$ and CDU modified $d(T)_{12}$ showed them to be almost identical. This suggests identical conformation in solution for the carboranyl oligomers compared to $d(T)_{12}$ standard. The CD spectra of duplexes formed between CDU-modified oligonucleotides or $d(T)_{12}$ and $d(A)_{12}$ showed a reduction of molecular ellipticity related to the thermal stability of the duplexes.

The thermostabilty of duplexes formed by CDU-oligonucleotides with natural complementary strand $d(A)_{12}$ and poly r(A) was affected by the location of the carboranyl nucleotide within the chain. CDU-oligomer modified at the 1st location, at the 5'-terminus, displayed a melting temperature similar to natural $d(T)_{12}$, whereas 3'- and centrally modified $d(T)_{12}$ (location 11th and 7th, respectively) had lower $T_m$ values.

The CDU modification located at the ends of the oligomer markedly increased their resistance toward exonucleases, as determined by half-life ($t_{1/2}$) measurements. The $t_{1/2}$ in the presence of SVPDE increased in order $d(T)_{12}$ < CDU at the 11th position << at both the10th and 11th position. A pronounced effect of CDU modification on the oligonucleotide stability in the presence of BSPDE was observed. Resistance to the BSPDE increased in the order $d(T)_{12}$ < CDU at the 11th position ~ CDU at both 10th and 11th position < CDU at 1st position ~ CDU at 2d position ~ CDU at 1st and 10th position.[19]

The CDU-containing oligonucleotides were primers for *Escherichia coli* polymerase I and human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, but not for human DNA polymerase α and β. Oligonucleotides bearing CDU modification at 5'- end were elongated more efficiently, than oligonucleotides modified at the 3'- end.[19]

All CDU-modified dodecathymidylates formed RNA-DNA complexes with a poly r(A) template which were substrates for *E. coli* RNase H. These heteroduplexes were digested by RNase H in a fashion comparable to the digestion of the unmodified duplex formed by $d(T)_{12}$. Additionally, the efficacy of poly r(A) digestion seemed independent from $T_m$ of the duplex formed with CDU-oligonucleotide.[19]

**Miscellaneous.**

Although several boron containing nucleosides[17] are well characterized biologically, including studies *in vivo,*[34] there is a paucity of information on the biological activity of boron modified oligonucleotides. Most clinical studies on oligonucleotides as potential pharmaceuticals focus on oligomers containing established phosphorothioate or methylphosphonate modified sugar-phosphate backbone. Numerous other modifications, including replacement of the phosphodiester function by another chemical group leading to

oligonucleotides containing dephospho-internucleotide linkages were described.[35] To obtain the more desirable physicochemical properties, usually all the natural internucleotide linkages in these oligomers are modified. This often results in increased toxicity and other adverse effects. Due to a prominent effect of the carboranyl group on the carrier, the desired modulation of physicochemical and biological characteristics of the oligonucleotide molecule may be achieved with a limited number of modified centers. This is highly beneficial since unmodified oligonucleotides are virtually non toxic.

Recently we compared physicochemical and biological properties of CBMP and methylphosphonate oligonucleotides.[36] CBMP compounds have several desirable properties compared to the traditional methylphosphonate modification. These include the increased lipophilicity (RP HPLC, $R_t$), thermostability of duplexes formed with poly r(A) ($T_m$), and resistance against nucleases (SVPDE, $t_{1/2}$). For example, the properties for the 3`-end modified oligonucleotide *closo*-$d(T)_{11}P(O)CH_2(B_{10}H_{11})dT$, $d(T)_{11}P(O)CH_3d(T)$, and unmodified $d(T)_{12}$ are as follows: $R_t$: 17.8 min, 7.6 min, and 5.5 min, respectively; $T_m$: 31°C [*nido*-$d(T)_{11}P(O)CH_2(B_{10}H_{11}dT)$], 31°C and 25°C, respectively; $t_{1/2}$: 1.4 min, 0.8 min, < 0.2 min.



## SUMMARY

Two types of boron modified oligonucleotides have emerged so far. One type contained modification within the oligonucleotide linkage as in boranophosphate or carboranylmethylphosphonate oligomers; another type contained an unmodified phosphodiester backbone and N7-cyanoborane-2'-deoxyguanosine or boron rich modified nucleic base such as CDU. These boron oligonucleotides are now characterized both physicochemically and biochemically. Based on the favorable physicochemical and biological properties of certain boron containing oligonucleotides, oligomers could be designed and targeted against relevant genes in cancer and in virally infected cells,[37] as well as for boron neutron capture therapy.[38,39] These initial studies will serve as a foundation for developing boron-containing therapeutic oligonucleotides for BNCT and AOT. Boron modified oligomers have potential beyond neutron capture and gene therapies, such as for tumor diagnosis and virology.

**ACKNOWLEDGEMENTS**: Supported by the Department of Energy (DE-FG02-96ER62156), NIH grant 2R44-CA6543, State Committee for Scientific Research (KBN), and the U.S. Department of Veterans Affairs.

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