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Nucleic acids and nucleosides containing carboranes

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Abstract

The bioorganic chemistry of carboranes is an important trend in the modern chemistry of boron. The use of carboranyl cluster as a modifying entity for nucleosides and oligonucleotides presents a new concept in the chemistry of nucleic acids and nucleic acids' components, and facilitates studies on new DNA based, carborane-containing materials and pharmaceuticals. Several nucleosides modified with a carboranyl group and a novel class of oligonucleotide analogues bearing a carboranyl cage within an internucleotide linkage {CBMP [(*o*-carboran-1-yl)methylphosphonate] oligonucleotides}, or attached to a nucleic acid base {CDU-[(5-(*o*-carboran-1-yl)-2′-deoxyuridine] oligonucleotides} were developed. Synthesis, physicochemical and biological properties of these new nucleosides and nucleic acids modifications are described.

Keywords: Boron; Carboranes; Nucleosides; Antisense oligonucleotides; Antiviral; BNCT

1. Introduction

The study of electron-deficient boron cage compounds has developed into a major area of inorganic–organometallic chemistry, with considerable overlap into organic and polymer chemistry. The bioorganic chemistry of carboranes is a new trend in this field, which emerged in part as a result of the quest for better boron carriers for boron neutron capture therapy (BNCT) of tumors [1–4]. Among the many low molecular weight compounds synthesized for BNCT [5] are carborane-containing amino acids [6–8], carbohydrates [9], nucleic acid bases and nucleosides [10,11], DNA groove binders [12,13], and porphyrins [14]. A new generation of radiosensitizers for BNCT described recently are biopolymers bearing one or more carboranyl residues. This class of boron trailers includes carboranyl peptides [15] and proteins [16], carboranyl oligophosphates [17,18] and nucleic acids (oligonucleotides) [19–22].

The chemistry of carborane modified nucleic acids has implications beyond BNCT. The carboranyl cluster is a new modifying entity for oligonucleotides potentially useful as antisense agents for antisense oligonucleotide therapy (AOT), and as molecular probes for molecular diagnostics of infectious and genetic disease based on hybridization technology.

There are numerous reviews and books published on all aspects of carborane structure and chemistry [23–25]. Therefore discussion herein of these problems will be limited to fundamentals relevant to the chemistry and biology of carborane oligonucleotides described in the following sections.

2. Icosahedral carboranes

A large part of the research interest in the field of electron-deficient boron cage compounds has centered on boron cage systems in which one or more carbon atoms are present as an integral part of an electron-delocalized borane framework. Compounds of this type have been given the general name 'carboranes'. The term carboranes includes both closed polyhedral and open-cage structures. Polyhedral carboranes of general formula $C_2B_{n-1}H_n$ have been characterized from $n = 2$ to $n = 12$.

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The icosahedral carborane system $C_2B_{10}H_{12}$ (an icosahedron is a polyhedron with 20 faces and 12 vertices), was reported by two different groups in late 1963 [26,27]. For carboranes, it is significant that the carbon atoms participate in the delocalized bonding. As a consequence, the usual empirical rules of valency and coordination in organic compounds may not apply to these structures. For example, for $C_2B_{10}H_{12}$, each carbon atom is hexacoordinated. To account for the existence of stable electron-deficient molecules such as the boranes and the carboranes, delocalization of electrons beyond the two-center bond was postulated in the form of three-center bond formulation.

The majority of preparative methods for 1,2-$C_2B_{10}H_{12}$ *ortho*-carborane synthesis involve the interaction of decaborane and acetylene. Decaborane is attacked by Lewis bases such as alkylamines, alkylsulfides or acetonitrile, forming bis(ligand) derivatives of the general formula $B_{10}H_{12}L_2$, which in turn react with acetylene to give the *o*-carborane in high yield [26]. The use of substituted acetylenes frequently leads to the corresponding C-substituted carborane derivatives.

Below 400°C, 1,2-$C_2B_{10}H_{12}$ is unaffected by heat, but at 400–500°C in an inert atmosphere it rearranges quantitatively to the 1,7 isomer (1,7-$C_2B_{10}H_{12}$, *meta*-carborane) [28]. The latter compound decomposes near 620°C with formation of 1,12-$C_2B_{10}H_{12}$ (*para*-carborane) [29]. Simple substituted derivatives of 1,2-$C_2B_{10}H_{12}$ undergo analogous rearrangements. All three carborane isomers are characterized by an extremely high lipophilicity. The lipophilic character of the neutral carborane molecule is dependent on the positions of the carbon atoms in the cage structure (Figs. 1 and 2) [30,31].

The substantial delocalization of valence electrons is responsible for carborane stability and many properties typical of aromatic systems. At the same time, the presence of a carbon reaction site in the cage makes possible an extremely versatile and extensive derivative chemistry not paralleled by the boron hydrides [25,32]. Exploration of carborane chemical properties for the synthesis of carborane nucleosides, nucleic acids and their components is the subject of this review.

3. *o*-Carborane-containing nucleosides

The rationale for the design and synthesis of boron-containing nucleosides is that such compounds may accumulate selectively in rapidly growing tumor cells, and following their conversion to the corresponding nucleotides, maybe trapped within the cell or, ideally, incorporated into nuclear DNA of tumor cells. In the case of BNCT, such a nuclear localization of the boron carrier, e.g. boronated nucleoside, would be advantageous since the radiobiological effectiveness of the capture reaction occurring in the nucleus is 2–5 times higher than that in the cytoplasm [33,34]. Hence, boronated nucleic acid precursors may be suitable agents for BNCT. Although the first boron-containing nucleoside 5-dihydroxyboryl-2′-deoxyuridine appeared in 1978 [35,36], the first nucleoside containing multiple boron atoms, the *o*-carboranyl cluster, was reported a decade later. In 1990, Soloway and coworkers [37] reported 2′-*O*-(*o*-carboran-1-yl-methyl)uridine (CBU-2′, **1a**), a nucleoside containing a carborane cage connected to the 2′-oxygen through a methylene linker. It was synthesized by a decaboration of the propynyl ether which was formed from the protected uridine. Similarly prepared were 3′-*O*- and 5′-*O*-analogues (CBU-3′, **1b**, and CBU-5′, **1c**), as well as the *nido*-form of CBU-2′ [9]. These were the only sugar-linked carboranyl nucleosides in which the carborane cage was bonded to the sugar component of the nucleosides (Fig. 3).

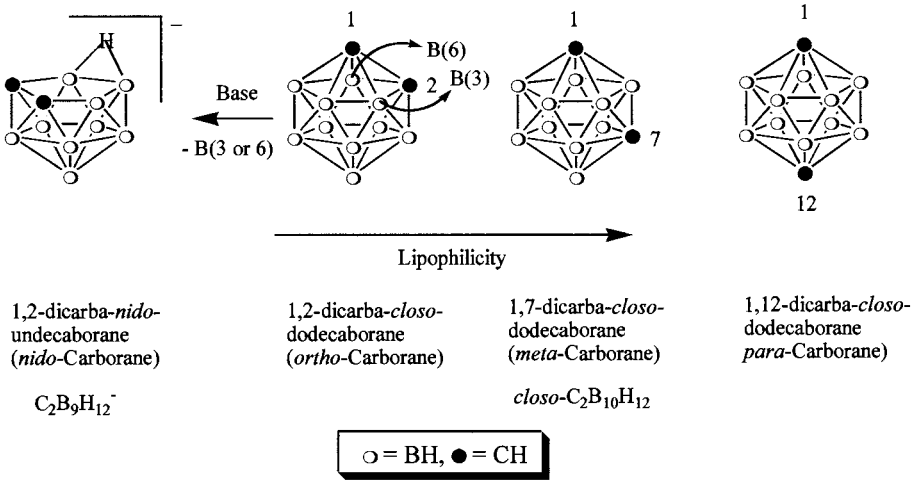


Fig. 1. Structure and numbering of 1,2-, 1,7- and 1,12-dicarba-closo-dodecaboranes and 1,2-dicarba-*nido*-undecaborane ion.

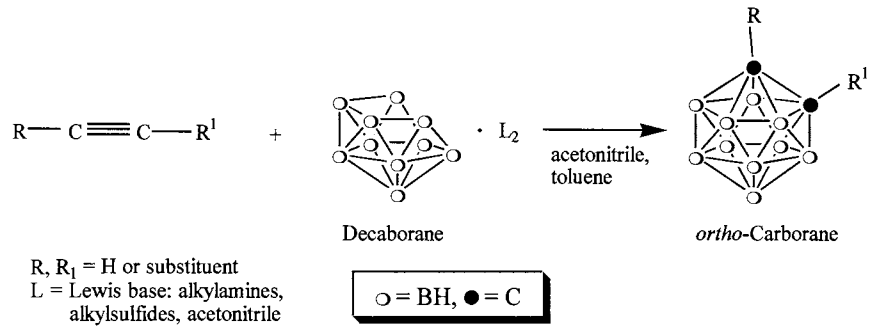


Fig. 2. General method for synthesis of *ortho*-carborane and its derivatives.

In 1992, Yamamoto et al. [38] and Schinazi et al. [39] reported synthesis of 5-*o*-carboranyl-2′-deoxyuridine (CDU, **2a**) and 5-*o*-carboranyluridine (CU, **2b**), the first nucleosides with the nucleic base containing a carboranyl group. For this kind of carboranyl nucleoside, the carboranyl moiety is attached to the 5-position of uracil, the most tolerant position of the nucleic base. This represented a major trend in novel carboranyl nucleoside synthesis. Similar compounds such as 5′-*O*-glucosyl-CU (**2c**) [40], a 5′-*O*-blocked carboranyl nucleoside, and the *nido*-counterpart of 5-*o*-carboranyluridine [41], were also reported. These 5-*o*-carboranyl nucleosides were synthesized by a nucleoside modification method, namely from their 5-iodo precursors by a Heck coupling reaction with trimethylsilylacetylene, followed by decaboration and deprotection [42]. A carboranyl base/sugar coupling method later was developed extensively by Schinazi's group [43]. It included a coupling reaction of the appropriate sugar derivative with silylated 5-*o*-carboranyluracil, followed by deprotection.

Recently, another type of base-attached carboranyl nucleoside, in which the carboranyl moiety and the 5-position of uracil was connected through a linker was reported. The introduction of the linkers was to improve the biochemical phosphorylation and water solubility of the carborane-containing nucleosides. The polyethylene, thioether, vinylcarboxylate, vinylcarboxamide and propiolate linked carborane-containing nucleosides **3a–g** were synthesized by Soloway and coworkers [44–46], whereas the propynyl linked compound **4** was prepared by Kabalka et al. [47]. Most of these compounds were synthesized by the coupling reaction of a substituted carborane part with a 5-substituted uridine (Fig. 4).

To increase the hydrophilic character of these compounds, one or more hydroxyl groups were introduced into the substituent adjacent to the carboranyl moiety in the nucleoside. In 1997, Soloway and coworkers [48] reported the hydrophilic tethered carborane-containing nucleosides **5a–c**. The dihydroxypropyl linker was introduced by an allylation of the mono-substituted carborane followed by an oxidation. The linkers were ester chains which were formed by an esterification reaction. Similar to these kind of hydroxycarboranyl nucleosides were **6a** (HMCU) [42] and **6b** (CDU-DIOL) [48], which were non-tethered hydroxycarboranyl nucleosides (Fig. 5).

A carboranyl nucleoside in which the carboranyl moiety was located at the 6-position of uracil was also made. In 1993, Palmisano and Santagostino [49] reported the synthesis of 6-(phenylcarboranyl)uridine precursor **7**. However, no deprotected nucleoside was reported (Fig. 6).

Synthesis of a carborane-containing purine nucleoside was also attempted. For example, 2-*o*-carboranyl-inosine precursor **8** [42] was reported in 1992. Unfortunately, this nucleoside precursor could not be deprotected to a carboranyl inosine nucleoside.

With all the above mentioned carborane-containing nucleosides consisting of ribose or 2′-deoxyribose, efforts were also made to synthesize carboranyl nucleosides containing other sugars. Encouraged by the superior antiviral profiles of (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC) and (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC), an oxathiolane heterocycle was introduced to the carborane-containing nucleosides by Schinazi and coworkers. In 1993, oxathiolane carboranyl uridines **9a**/**9b** were synthesized as a racemic mixture which contains equal amount of β-D (**9a**) and β-L (**9b**) enantiomers [43,50]. They were prepared by the silylated 5-*o*-carboranyluracil/protected sugar coupling method. The above racemic compounds **9a**/**9b** and their *nido*-forms were also prepared later by Yamamoto et al. [41,51] (Fig. 7).

Since 2′-fluoronucleosides exhibited broad anti-herpesvirus and anti-hepadnavirus activity, and because the 2′-fluoro group imparts greater stability to the glycosidic bond, 2′deoxy-2′-fluoroarabinose nucleosides containing a carboranyl group were synthesized. In 1994, Schinazi's group [52] reported the synthesis of 5-*o*-carboranyl-1-(2-deoxy-2-fluoro-β-D-arabinosyl)uracil (CFAU, **9c**) and its α-isomer **9d**. The β-isomer was prepared either from the 5-iodo-nucleoside by

ethynylation and decaboration, or from the coupling between the protected sugar and silylated 5-*o*-carboranyluracil. The latter method produced both α-D and β-D anomers. The two anomers differ only in the geometry at the 1′-carbon of the carbohydrate. The β-anomer has the 1′-nucleic acid base and the 4′-hydroxymethyl group positioned *cis* to each other, whereas the α-anomer has them positioned *trans* to each other. The presence of the 2′-fluoro moiety in the sugar increased greatly the stability of these compounds towards glycosidic cleavage [53,54].

Demonstrated by β-D-2′,3′-didehydro-3′-deoxythymidine (D4T), and more recently, by β-D-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (D-D4FC) [55], certain 2′,3′-didehydro-2′,3′-dideoxy-nucleosides (d4N) exhibited potent antiviral activity against HIV. Hence, the carborane-containing d4-nucleosides were synthesized by Schinazi and coworkers [56]. Through a protected sugar and the TMS-activated 5-*o*-carboranyluracil coupling reaction, followed by oxidative elimination, the four optical isomers (β-D **9e**, α-D **9f**, β-L **9g**, and α-L **9h**) of 5-*o*-carboranyl-2′,3′-didehydro-2′,3′-dideoxyuridine (D4CU) were synthesized.

Other sugars such as D-xylose were also used in the synthesis of novel carboranyl nucleosides. In 1997, Schinazi's group [57] reported the synthesis of 5-carboranyl-1-(β-D-xylofuranosyl)uracil **9i** by a coupling reaction with silylated 5-*o*-carboranyluracil. Another 2′,3′-dideoxy carboranyl nucleoside precursor, 5-*o*-carboranyl-2′,3′-dideoxy-2′-(phenylthio)uridine **9j**, and its *nido*-form, were reported by Yamamoto and coworkers [41,51].

The synthesis of carborane-containing nucleosides can be reached in two ways: the linear approach [9,37–40,42,44–49] or the convergent approach [41,43,50–52,56,57]. The former approach relies on the construction of the carboranyl component onto a suitable nucleoside, whereas the later approach involves a coupling reaction of a silylated carboranyl-containing nucleic acid base with a suitable carbohydrate. Although the convergent approach may produce α- and β-anomers, its greater versatility has clear advantages for the synthesis of various carborane-containing nucleosides.

4. Pharmacology of *o*-carborane-containing nucleosides

The cellular toxicity, cellular uptake, and subcellular distribution of the carboranyl nucleosides, 2′-*O*-carboranyluridine **1a** (CBU-2′), and its analogues **1b–c** (CBU-3′ and CBU-5′) were studied by Barth and Soloway [9,58]. The cellular toxicity assay showed that CBU-2′ and CBU-5′ were toxic for F98 glioma cells ($IC_{50} = 3–13$ μM). The cellular uptake of CBU-2′ was determined in F98 rat glioma, U-87 MG human glioma, B16 melanoma, SP2/0 myeloma, 3T3 and MDCK fibroblast cells using direct current plasma atomic emission spectroscopy (DCP-AES). After incubation of CBU-2′ for 16 h at a concentration of 6.75 mg boron ml^{-1}, all tumor and non-tumor cells had high uptake of CBU-2′ (46-75 μg boron $(g\ cell)^{-1}$), indicating that uptake was not selective for neoplastic cells and was independent of cell proliferation. CBU-2′, CBU-3′ and CBU-5′ demonstrated minor differences in uptake in F98 rat glioma cells, but the cellular concentration of the *nido*-form of CBU-2′ was 2 orders of magnitude lower than its *closo*-counterpart [9]. The persistence studies showed high cellular retention of CBU-2′ compared to sodium borocaptate (BSH). Subcellular fractionation found that 75.6% of the recoverable boron was distributed in the cell membrane, 15.6% in cytoplasm, and 8.8% in nuclear fraction, but no boron was detected in RNA and DNA fractions. Up-

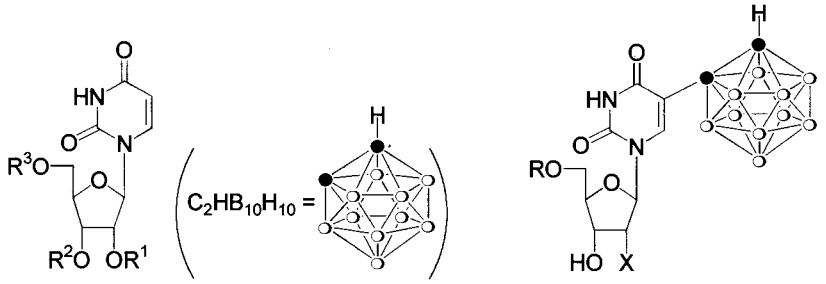


Fig. 3. Sugar-linked (**1a–c**) and nucleic acid base-linked (**2a–c**) carborane-containing nucleosides.

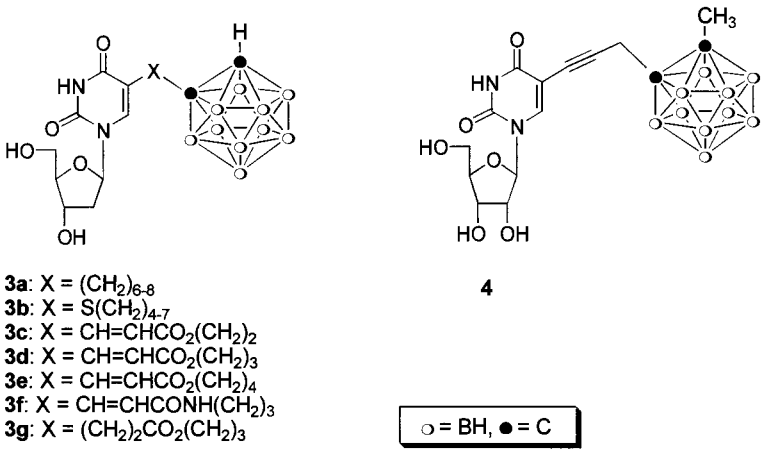


Fig. 4. Tethered nucleic base-linked carboranyl nucleosides.

take of CBU-2′ in F98 cells was not inhibited by metabolic inhibitors such as rotenone, dipyridamole, and *S*-(4-nitrobenzyl)-6-thioinosine, suggesting that uptake was neither energy nor nucleoside transport dependent. In vivo studies in F98 glioma bearing rats showed that the concentration of CBU-2′ in the tumor was 13-fold higher than that in the normal brain of the ipsilateral and contralateral cerebral hemispheres [58], closed to the minimum levels (5–35 μg B g^{-1}) required for BNCT. These assays are not as accurate or sensitive as determinations using radiolabeled material combined with an analytical methodology such as HPLC and/or mass spectroscopy. Nevertheless, it was apparent that such sugar-bonded carboranyl nucleosides were not phosphorylated in various cells to any detectable extent.

5-*o*-Carboranyluracil nucleosides, including CDU (**2a**), 5-CU (**2b**), HMCU (**6a**), CTU (**9a–b**), CFAU (**9c–d**), D4CU (**9e–h**), and 5-*o*-carboranylxylofuranosyluracil (**9i**), were studied extensively for their cytotoxicity, anticancer and antiviral activity, and cellular uptake by Schinazi's group [50,52,56,57,59,60], as well as by Yamamoto's group [38,41,51]. The cytotoxicity studies in PBM, CEM, and Vero cells showed that all these compounds had no significant toxicity in various cells ($IC_{50} > 10$ μM), except for α-D-D4CU which showed modest toxicity ($IC_{50} = 8.8$ μM) in CEM cells [56]. In TIG-1-20 fibroblast cells, CDU, CTU and 5-carboranyl-2′,3′-dideoxy-2′-phenylthiouridine (**9j**) showed high toxicity ($IC_{50} = 0.7–2.5$ μM) [51], whereas the *nido*-counterparts of CU and 5-carboranyl-2′,3′-dideoxy-2′-phenylthiouridine were about 10 times lower in toxicity than those of the corresponding *closo*-compounds [41]. In order to be useful for BNCT, carborane-containing nucleosides should not be highly toxic to normal cells ($IC_{50} > 10$ μM).

The anticancer assays showed that CDU and D4CU had no inhibitory effect in SK-MES-1, MCF-7 and LNCaP cancer cells [56]. Similarly, CDU and CFAU had no significant toxicity to rat 9L and human U-251 glioma cells ($IC_{50} \geq 25$ μM) [52]. In contrast, CDU and CU showed high cytotoxicity ($IC_{50} = 2–4$ μM) towards murine leukemia cells P-388, L1210, MBL-2, melanoma cells B16, and sarcoma cells MethA [38]. HMCU exhibited modest cytotoxicity to P-388 cells ($IC_{50} = 5.6$ μM) [38] and CTU and 5-carboranyl-2′,3′-dideoxy-2′-phenylthiouridine were also toxic towards B16 cells ($IC_{50} = 2.0$ and 0.67 μM, respectively) [51].

The bone marrow toxicity of CDU and CFAU was assessed in human myeloid (CFU-GM) and erythroid (BFU-E) progenitor cells. CDU and CFAU were at least 13-fold less toxic to CFU-GM and 95-fold less to BFU-E cells than 3′-azido-3′-deoxythymidine (AZT) [52].

In vivo cytotoxicity of CDU, CFAU and 5-*o*-carboranylxylofuranosyluracil (**9i**) was studied in mice by administering the compounds at 30 mg kg^{-1} per day for 6 days and monitoring death, weight loss or failure to gain weight for up to 9 weeks [52,57]. Weight loss was observed in the first week for the animals treated with these compounds. Survival rates showed that CFAU (4/5) and 5-*o*-carboranylxylofuranosyluracil (5/6) were more toxic compared to CDU (5/5). All the surviving mice recovered weight by the fourth week. These studies indicated that these compounds were well tolerated in mice.

The antiviral studies showed that except for CFAU [52] and β-L-D4CU [56] which exhibited modest anti-HIV activity ($EC_{50} = 7.3$, and 9.0 μM), all of the above compounds were devoid of anti-HIV and anti-herpes simplex virus activity.

Cellular uptake of the two enantiomers D-CDU and L-CDU [61] were studied in human CEM lymphoblastoid cells and human U-251 glioblastoma cells using radiolabeled compounds and measured by HPLC [62]. Accumulation of both D- and L-enantiomers was rapid in both cell lines and reached pseudo-steady state within 3–10 min. Plateau levels following a 1 h incuba-

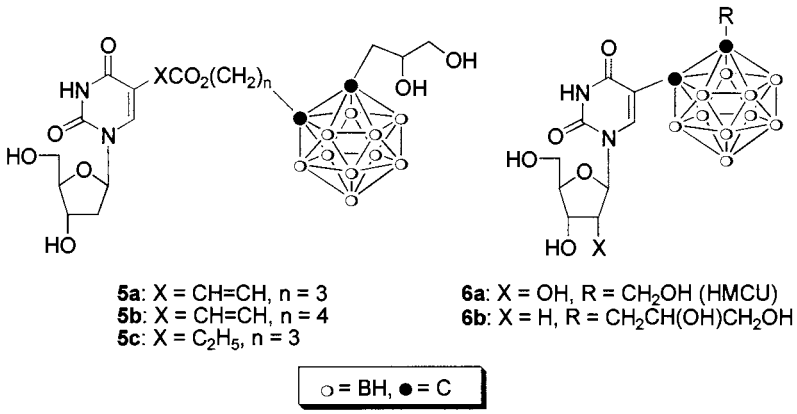


Fig. 5. Tethered (**5a**–**c**) and untethered (**6a**–**b**) nucleic acid base-linked hydroxyl-bearing-carboranyl nucleosides.

tion at 1 μM were 50 pmol/10^6 CEM cells and 250 pmol/10^6 U251 cells, with both cellular concentrations greater than 40-fold of the extracellular level. Uptake curves for D- and L-CDU were indistinguishable, and were not affected by nucleoside uptake inhibitors, and were only inhibited partially by the purine nucleobase inhibitor papaverine. Egress rates in CEM cells following a 2.5 h incubation with 1 μM CDU were biphasic for both enantiomers with ca. 95% of the drug egressing within 0.5 h. After 2 h, 6% of compounds remained in CEM cells, and the similar level of the compounds in U-251 cells maintained even after 24 h. These intracellular levels were at least 2-fold greater than the initial extracellular concentration prior to removal of the CDU from the medium. HPLC analysis of cell extracts after 24 h incubation detected low levels of a hydrophilic metabolite for D-CDU in CEM cells that was susceptible to digestion with alkaline phosphatase, indicating limited phosphorylation of the D-enantiomer. Schinazi et al. [50,59] had demonstrated previously for the first time that D-CDU is phosphorylated in CEM and PBM cells, albeit to low levels. The enantiomers also formed low levels of phosphorylated metabolites in U-251 cells. The similar uptake and egress of the D- and L-CDU suggests that the lipophilic nature of the carboranyl moiety could minimize the importance of stereochemically sensitive uptake processes.

The cellular uptake of CU (**2b**), CTU (**9a-b**), and 5-*o*-carboranylxylofuranosyluracil (**9i**) demonstrated that these compounds had similar levels in B-16 melanoma cells, but the dideoxy carboranylnucleoside **9j** had a higher concentration in TIG-1-20 fibroblast cells [41,51]. In addition, the study also demonstrated no difference between the *closo*- and *nido*-forms of the above compounds in cellular uptake [41], contradicted the previous data obtained from *closo*- and *nido*-CBU-2′ [9].

The pharmacokinetics of CDU after intravenous administration of 25 mg kg^{-1} were studied in rats using HPLC [60]. Plasma concentrations of CDU declined in a biexponential fashion with a terminal half-life of 1.26 h. The plasma protein binding of CDU was linear and the average fraction bound to plasma proteins was 0.95. The brain/total plasma CDU concentration ratios determined in two rats were 0.47 and 0.36, while the brain/unbound plasma CDU concentration ratios were 10.26 and 7.87. The results of the study suggest that it is possible to achieve significant levels of CDU in the brain, and a high degree of plasma protein binding restricted extensive distribution of this lipophilic compound. Recent studies in rats implanted stereotactically with 9L glioma indicate that at certain doses, D-CDU was significantly more effective in increasing survival time than neutron treatment alone [63].

The phosphorylation of the tethered carborane-containing nucleosides **3c**–**g** with thymidine kinase (TK) were studied by Soloway and coworkers [46,48]. After incubation of the compounds with 0.098 units of human TK at room temperature for 2 h using [γ-^{33}P]-ATP as the phosphate donor, the phosphorylation products (corresponding monophosphates) were detected by the radioactivity of the nucleotides and quantitated by β-scanning. The velocity of phosphorylation for these 5-tethered carboranyl nucleosides and non-tethered compound CDU were in the following order: **3g** > **3d** > CDU > **3c** > **3e** > **3f**. A phosphorylation study was also conducted for tethered and untethered carbo-

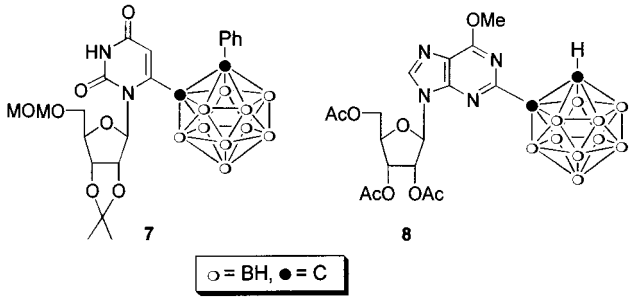


Fig. 6. Protected 6-(phenylcarboranyl)uridine (**7**) and 2-*o*-carboranylinosine (**8**).

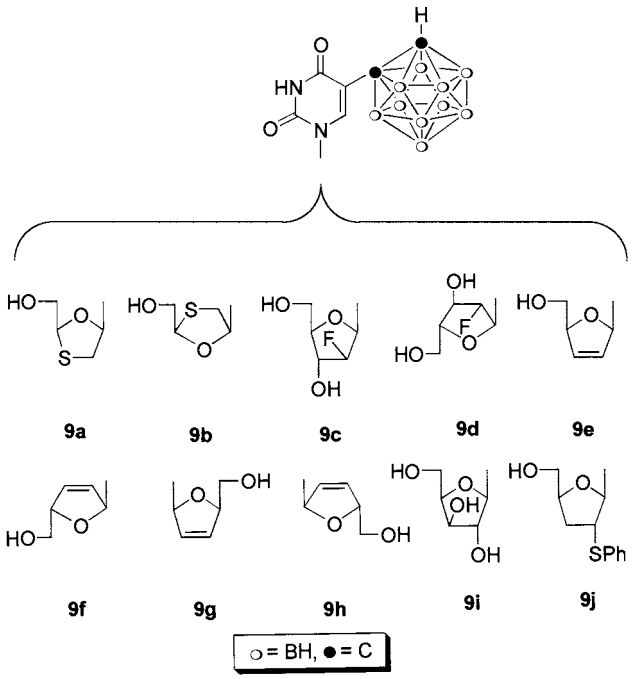


Fig. 7. Sugar-modified 5-o-carboranyluracil nucleosides: β-D/L-5-*o*-carboranyl-2′,3′-dideoxy-3′-thiacytidine (CTU, **9a**/**9b**), 5-*o*-carboranyl-1-(2-deoxy-2-fluoro-arabinofuranosyl)uracil (CFAU, β-D **9c**, α-D **9d**), 5-*o*-carboranyl-2′,3′-didehydro-2′,3′-dideoxyuridine (D4CU, β-D **9e**, α-D-**9f**, β-L **9g**, α-L **9h**), 5-*o*-carboranyl-1-(β-D-xylofuranosyl)uracil (**9i**), and β-D-5-*o*-carboranyl-2′,3′-dideoxy-2′-(phenylthio) uridine (**9j**).

ranyl-containing nucleosides **3g** and CDU, in comparing with the hydroxyl derivatives **5a, 5c** and **6b** [48]. The phosphorylation rates were in the following order: **5c** > **3g** > CDU > **6b** > **5a**. These results showed that **5c** had a higher phosphorylation rate by TK than the other compounds, but it was still far below that of the natural nucleoside 2′-deoxyuridine. Compared to CDU, the effect of the linkers and the hydroxyl groups in these compounds were mixed, depending on the nature of the chains. It seems that a saturated ester linker was better than an unsaturated ester and amide linker. A hydrophilic dihydroxypropyl group attached to the saturated tethered compound (**3g**) increased the phosphorylation rate whereas it decreased the rate when attached to the unsaturated tethered and non-tethered compounds. This suggests that the type of tethered linkers and the hydroxypropyl group used can affect phosphorylation, and probably other biochemical processes. At this time, a simple generalization is not possible and each compound should be evaluated individually. Although these assays were based on the radioactivity calculated from the developed TLC plates, the above phosphorylation study provided some insight into the structure-activity relationship of these compounds. Equally important for these tethered compounds was their potential enzymatic degradation, especially by esterases and amidases, since they contained enzymatic hydrolizable ester or amide linkers. Unfortunately, no enzymatic digestion kinetic studies were conducted for these compounds. Enzymatic degradation of these tethered carboranyl nucleosides would be expected to produce non-carboranyl 5-substituted nucleosides and dialkyl-substituted carboranes.

5. Synthesis of carborane-containing oligonucleotides

An oligonucleotide (ON) is a short piece of synthetic DNA or RNA, which hybridizes to a complementary nucleic acid sequence by Watson–Crick base-pairing in a sequence–specific manner [64]. Specific duplex formation between an oligonucleotide (usually oligodeoxynucleotide (ODN)) and its nucleic acid target is a foundation of nucleic acid hybridization technology, which includes gene-inhibition therapy [65,66], medical molecular diagnostics [67], and newly emerging DNA-chip technology [68].

The rationale for the synthesis of boron containing oligonucleotides was their potential application as boron carriers for BNCT, and as antisense oligonucleotides for antisense oligonucleotide therapy (AOT) [19,69]. Other potential applications of boron oligonucleotides include their use as new probes for the diagnosis of malignancies, viruses, and other pathogens.

Two types of carboranyl modified oligonucleotides have emerged [11,21]. The first type confines (*o*-carboran-1-yl)methylphosphonate (CBMP oligonucleotides, see Fig. 9) [18,69,70] or [*N* - (*o* - carboran - 1 - yl)-alkyl]phosphoramidate [22] modification within the internucleotide linkage. The second type (CDU oligonucleotides, see Fig. 10) contains boron modified nucleic base 5 - (*o* - carboran - 1 - yl) - 2′ - deoxyuridine (CDU) [71], and unmodified phosphodiester backbone [20]. The model oligonucleotides with all 3′,5′-*O*,*O*-{*N*-[(*o*-carboran-1-yl)alkyl]phosphoramidate} linkages were prepared with either a uncharged or charged backbone consisting of a *closo*-carboranyl or *nido*-carboranyl cage, respectively. At this time, the data on the synthesis and characteristics of this interesting oligonucleotide modification are incomplete.

Other types of boron-containing nucleic acids are boranophosphate- and N^7-cyanoborane-2-deoxyguanosine-containing oligonucleotides. These oligomers bearing one boron atom per modification, described first by Sood et al. [72] were reviewed recently [21]. Borane (-BH_3) presents different than carboranyl cluster category of oligonucleotide modifying entity and will not be discussed herein.

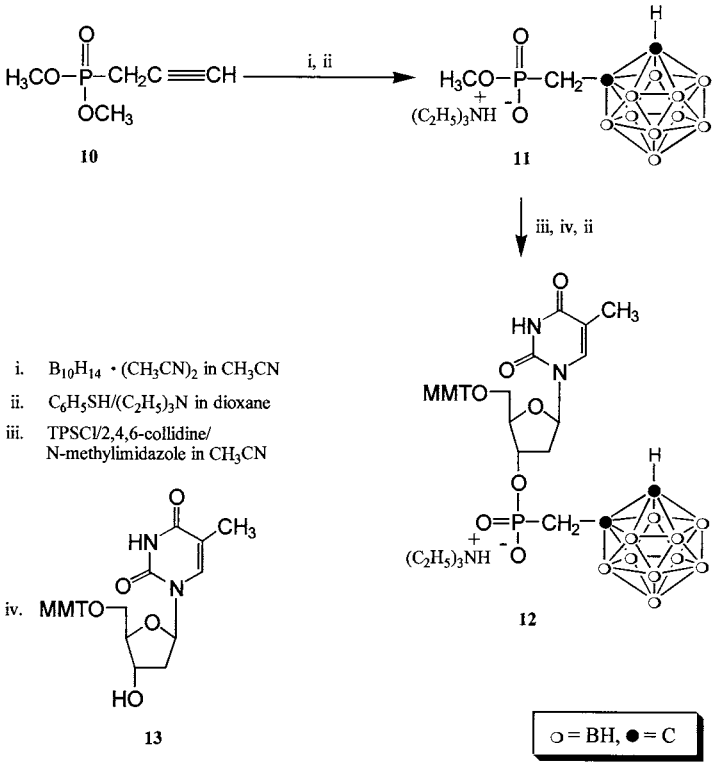
5.1. Carboranylmethylphosphonate oligonucleotides

Thymidine(3′,5′)thymidine (*o*-carboran-1-yl)methylphosphonate is the first oligonucleotide analogue modified with a carboranyl cage [19]. The monomer, 5′-*O*-monomethoxytritylthymidine 3′-*O*-(*o*-carboran-1-yl)methylphosphonate (**12**) for the CBMP dinucleotide and longer CBMP oligonucleotides synthesis was obtained in the reaction of 5′-*O*-monomethoxytritylthymidine (**13**) and borophosphonylating agent *O*-methyl-(*o*-carboran-1-yl)methylphosphonate (**11**), followed by selective demethylation of fully protected intermediate (Fig. 8).

Compound **11** was synthesized in a 3-step procedure [19]. In the first step, trimethylphosphite was allowed to react with propargylbromide, using the Michaelis–Arbuzov type reaction yielding *O*,*O*-dimethylpropargylphosphonate (**10**). Compound **10** was allowed to react with decaborane [26,27] providing *O*,*O*-dimethyl-(*o*-carboran-1-yl)methylphosphonate, which was in turn selectively demethylated yielding **11**. The dimer bearing 3′,5′-*O*,*O*-[(1-*o*-carboran-1-yl)methylphosphonate] internucleotide linkage instead of the natural 3′,5′-*O*,*O*-phosphodiester, was synthesized by phosphotriester method 'in solution' [73], as a mixture of R_P and S_P diastereomers [19,74].

The CBMP oligomers containing a *closo*-carboranyl cage belong to the class of uncharged, nonionic oligonucleotide sugar-phosphate backbone modifications [75]. The CBMP group increases the lipophilicity of the oligonucleotide in two ways: neutralization of the negative charge of the phosphate group (change in the net charge of the natural phosphodiester linkage from −1 to 0 for the modified), and addition of a remarkably lipophilic carboranyl moiety. The high lipophilicity of carboranyl cage is illustrated by the observation that CBMP oligonucleotides lipophilicity is substantially higher not only for the unmodified oligomer, but is also considerably higher than that of methylphosphonate oligonucleotides bearing modification in the same location of the oligonucleotide chain [70]. Furthermore, oligonucleotides bearing the charged, low electron density, *nido*-carboranyl group are more lipophilic than the unmodified counterparts despite the same number of formal negative charges. As anticipated, a prominent effect of carboranyl modification on physicochemical and substrate properties of CBMP oligonucleotides was observed for CBMP oligomers. The similar, though less pronounced effect of carboranyl modification on the oligonucleotide characteristics has also been observed for oligonucleotides bearing modified base CDU instead of CBMP internucleotide group [20,76] (vide infra).

Applying the methodology developed for the synthesis of CBMP dinucleotide, the dodecathymidylates containing CBMP 3′,5′-internucleotide group at the



10 11 12 13

Fig. 8. Synthesis of the monomer 5′-*O*-monomethoxytritylthymidine 3′-*O*-(*o*-carboran-1-yl)-methylphosphonate (**12**) for the CBMP [(*o*-carboran-1-yl)methylphosphonate] oligonucleotide preparation.

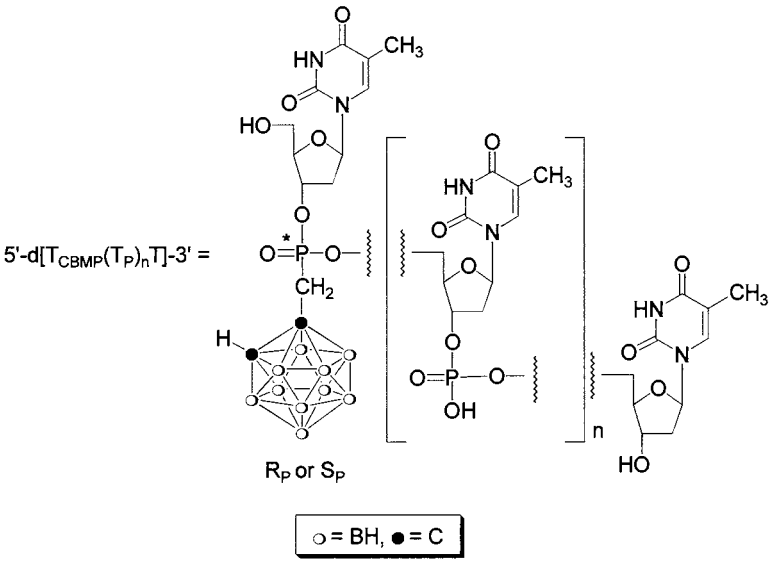


Fig. 9. (*o*-Carboran-1-yl)methylphosphonate (CBMP) containing thymidylic acids. Dodeca-thymidylic acids ($n = 10$) containing CBMP group at 1st, 6th, and 11th location starting from the 5′-end were synthesized.

different locations of the 12-mer 5′-$T_PT_PT_PT_PT_PT_PT_PT_PT_PT_PT_PT_P$-T-3′ {5′-d[$(T_P)_{11}$T-3′], T = thymidine, P = phosphate} were obtained by solid phase automated synthesis [77,78] (Fig. 9). Unmodified phosphodiester linkages were formed using a standard β-cyanoethyl cycle and an automated DNA synthesizer [20,79]. The modified CBMP internucleotide linkage was produced using the phosphotriester method and boronated monomer **12** as for the dimer synthesis [19]. The yield for the coupling reaction involving monomer **12** varied from 15 to 40% during the automated synthetic procedure. The yield for the coupling of unmodified monomer was about 95% and seemed unaffected by the incorporated modification. The oligomers obtained were characterized by reverse phase high pressure liquid chromatography (RP HPLC), ultraviolet spectroscopy (UV), electrospray ionization mass spectrometry (ESI MS), and circular dichroism (CD).

All oligonucleotides bearing carboranyl modification were obtained as mixtures of both *closo*- [*closo*-1,2-$C_2B_{10}H_{12}$], and *nido*- [*nido*-1,2-$C_2B_9H_{11}$] forms of the carboranyl cage. The deprotected oligonucleotides were purified and separated into *nido*- and *closo*-formed by RP HPLC. Replacing one of the anionic oxygen atoms by the (*o*-carboran-1-yl)methyl moiety generated a new center of chirality at the phosphorus and formation of modified oligonucleotide as a mixture of P-diastereomers [74]. Separation of P-diastereomeric oligonucleotides bearing a *nido*-(*o*-carboran-1-yl)-methylphosphonate group at the 11 position {*nido*-5′-d[$(T_P)_{10}T_{CBMP}$T]-3′} was achieved by means of RP HPLC. They were designed as 'fast' and 'slow', according to the order of elution by RP HPLC. The absolute R_P and S_P configuration at phosphorus of these diastereomers has not been established.

An effect of CBMP modification on the stability of the duplexes formed between CBMP containing dodecathymidylates 5′-d[$(T_P)_{11}$T]-3′ and poly r(A) as a complementary sequence, were compared to those formed between unmodified 5′-d[$(T_P)_{11}$T]-3′ and the template by melting temperature (T_m) measurements. 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 the carboranyl cage. The T_m value for all oligonucleotides studied was higher than for unmodified 5′-d[$(T_P)_{11}$T]-3′, indicating higher affinity of modified oligomers to the template than that of unmodified counterpart [77,78]. An exception was oligomer bearing CBMP group in the middle of the oligonucleotide chain at the 7 position {d[$(T_P)_6T_{CBMP}(T_P)_4$T]}, and containing carboranyl residue in *nido*-form, characterized by slightly lower than 5′-d[$(T_P)_{11}$T]-3′ T_m.

The carboranyl cages are characterized by extremely high lipophilicity. The lipophilicity of CBMP oligonucleotides was compared with 5′-d[$(T_P)_{11}$T]-3′ by coinjection experiments 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 and higher significantly than unmodified 5′-d[$(T_P)_{11}$T]-3′.

5.2. 5-o-Carboranyl-2′-deoxyuridine-containing oligonucleotides

Base modified, oligothymidylates 5′-d[$(T_P)_{11}$T]-3′ containing one or more 5-(*o*-carboran-1-yl)-2′-deoxyuridine (CDU oligonucleotides) instead of thymidine at the different locations of the 5′-d[$(T_P)_{11}$T]-3′ were synthesized using an automated β-cyanoethyl phosphoramidite approach and standard coupling cycle [79]. Boronated monomer, 5-(*o*-carboran-1-yl)-5′-*O*-dimethoxytrityl-2′-deoxyuridine 3′-(β-cyanoethyl *N*,*N*-diisopropylphosphoramidite) was used to incorporate the modification (Fig. 10). Monomer was synthesized from CDU nucleoside using standard procedure [20,80].

Dodecathymidylic acid 5′-d[$(T_P)_{11}$T]-3′ analogues bearing one or two CDU residues at different locations of the 5′-d[$(T_P)_{11}$T]-3′ were obtained. They were formed as a mixture of oligomers containing CDU carboranyl caged in both neutral *closo*- and ionic *nido*-form [11,69,81]. 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 5′-d[$(T_P)_{11}$T]-3′. The CDU oligomers were characterized by RP HPLC, UV, ESI-MS and CD (Fig. 10).

The lipophilicity of CDU oligonucleotides as measured by affinity to a RP HPLC column (R_t) was higher than those of unmodified 5′-d[$(T_P)_{11}$T]-3′ and depended upon CDU location within the oligonucleotide chain. It appeared that in all cases CBMP oligonucleotides were more lipophilic than CDU oligonucleotides. It is of interest that the same effect of carboranyl group upon lipophilicity, with respect to location of carboranyl group, was observed for the both types of oligomers.

The thermostabilty of duplexes formed by CDU-oligonucleotides with natural complementary strand 5′-d[$(A_P)_{11}$A]-3′ or poly r(A) was affected by the location of the carboranyl nucleotide within the chain. CDU oligomer modified at the first location {5′-d[$CDU_P(T_P)_{10}$T]-3′}, at the 5′-terminus, displayed a melting temperature similar to natural 5′-d[$(T_P)_{11}$T]-3′, whereas 3′- and modified centrally 5′-d[$(T_P)_{11}$T]-3′ {11th location, 5′-d[$(T_P)_{10}CDU_P$T]-3′}; and 7th, 5′-d[$(T_P)_6CDU_P(T_P)_4$T]-3′, starting from the 5′-end, respectively) had lower T_m values. Similar location dependent effects of carboranyl group upon duplex stability have been observed for oligonucleotides bearing internucleotide CBMP modification. However, the summary effect of CDU modification on heteroduplex was destabilizing or neutral, in contrast to the stabilizing or neutral effect of CBMP modification.

6. Substrate properties of carboranyl oligonucleotides

The 3′,5′-*O*,*O*-(1-*o*-carboran-1-yl)methylphosphonate (3′,5′-CBMP) internucleotide linkage was stable chemically at physiological pH, and resistant towards enzymatic digestion with bovine spleen phosphodiesterase (BSPDE) and snake venom phosphodiesterase (SVPDE) [19].

The presence of CBMP at the 5′-end of the oligonucleotide {5′-d[$T_{CBMP}(T_P)_{10}$T]-3′}, protected completely the modified oligomer against digestion by BSPDE (digestion from oligonucleotide 5′-end). The oligonucleotides with other than 5′-end locations of CBMP group were digested, though with a significantly lower rate, until the enzyme reached the CBMP internucleotide

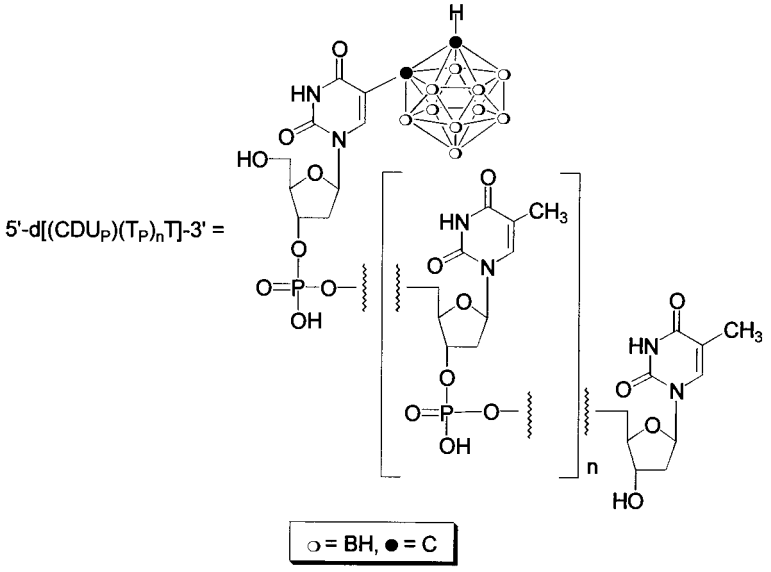


Fig. 10. 5-(*o*-Carboran-1-yl)-2′-deoxyuridine (CDU) containing thymidylic acids. Dodeca-thymidylic acids ($n = 10$) containing CDU nucleoside at 1st, 2nd, 7th, 11th, and both 10th and 11th and 1st and 11th location starting from 5′-end were synthesized.

linkage, when the digestion stopped. The presence of CDU modification resulted in a similar BSPDE resistance profile for CBMP oligonucleotides.

Though no complete protection was achieved, a pronounced effect of the CBMP modification on oligonucleotide resistance towards SVPDE (digestion from oligonucleotide 3′-end) was observed. The resistance was dependent upon CBMP group location, the absolute stereochemistry at phosphorus atom of CBMP modification, and the *closo*-/*nido*-status of the carborane cage. As expected the CBMP 3′-end location provided better protection against SVPDE than middle or 5′-end position of the of the CBMP in the oligomer. The phosphorus stereochemistry favored the slow diastereomer in terms of a higher resistance to SVPDE digestion than the fast diastereomer. It seems that the CBMP group containing the *nido*-carborane cage was less protective than *closo*-CBMP against SVPDE.

The CDU modification located at the 3′-end of the oligomer resulted in marked although not complete protection against SVPDE. The CDU location in the oligomer produced a similar effect as for CBMP oligonucleotides. Two adjacent CDU modifications located at the 3′-end resulted in greater resistance against SVPDE than one CDU modification. Incomplete protection of the oligonucleotides by CBMP and CDU modification can be explained in part by the fact that SVPDE has an endonucleolytic activity in addition to 3′-end exonuclease activity [82,83].

Enzymatic phosphorylation of CBMP-containing oligonucleotides with T4 polynucleotide kinase (T4 PNK, an enzyme catalyzing addition of phosphate to the 5′-terminus of nucleic acids) was observed for all oligonucleotides studied with the exception of the oligonucleotide bearing CBMP group at the 5′-end of the oligomer {5′-d[$T_{CBMP}(T_P)_{10}T$]-3′}. All CDU oligonucleotides, independent of CDU location, were phosphorylated by T4 PNK.

The CDU-containing oligonucleotides were primers for *Escherichia coli* polymerase I and HIV-1 reverse transcriptase, but not for human DNA polymerase α and β. Oligonucleotides bearing CDU modification at the 5′-end were elongated more efficiently than oligonucleotides modified at the 3′-end [76].

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 5′-d[$(T_P)_{11}T$]-3′ [76].

Recently we compared physicochemical and biological properties of CBMP and methylphosphonate oligonucleotides [70]. The CBMP compounds have several advantageous properties compared to the more common methylphosphonate modification [84]. These include the increased lipophilicity (RP HPLC, R_t), thermostability of duplexes formed with poly r(A) (T_m), and resistance against nucleases (SVPDE, $t_{1/2}$).

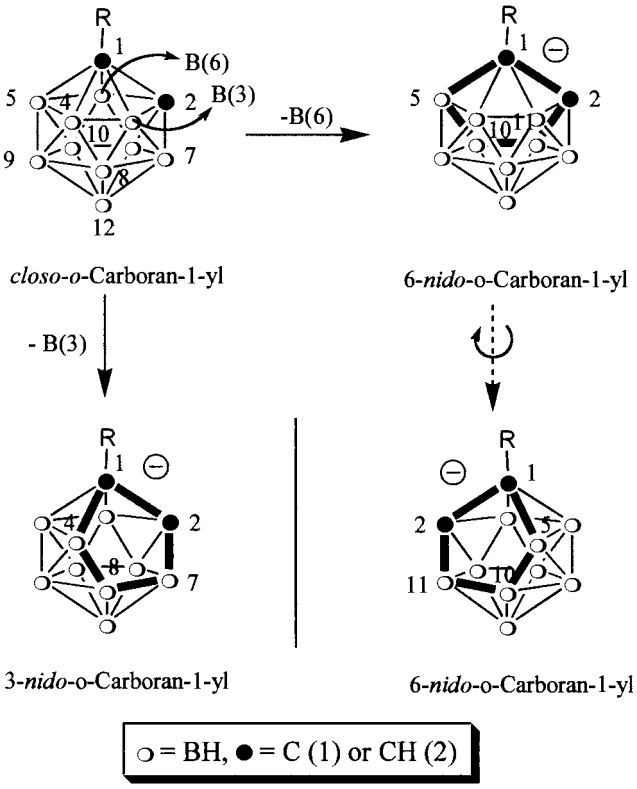


Fig. 11. Chirality of the nido-(*o*-carboran-1-yl) cage.

7. Stereochemical considerations

The stereochemistry of carborane modified biomolecules, with the exception of carborane modified amino acids [31], has received little investigation. The carboranyl cage can exist in three distinctive forms, one *closo*- and two ionic *nido*-forms. The two *nido*-forms result from removal of boron number 3 or 6 from neutral *closo*- cage, which leads to *nido*-*o*-carboran-1-yl enantiomers [81] (Fig. 11).

The nucleosides of an oligonucleotide chain are themselves chiral. There are three centers of chirality at carbons C1′(R), C3′(R) and C4′(R) of β-D-2′-deoxyriboses in DNA (Fig. 12) and four centers of chirality in β-D-riboses of RNA [carbons C1′(R), C2′(R), C3′(R) and C4′(R)]. The chirality is the same for every nucleotide unit. Consequently unmodified natural DNA or RNA molecule is homochiral (tactic) and can be considered as a single diastereomer. Owing to the stereochemistry of the *o*-carboran-1-yl cage, the CDU oligonucleotides containing a single CDU residue exist as one species for the achiral (though prochiral) *closo*-form of the carboranyl cage, and as two diastereomers for the enantiomeric *nido*-form of the carboranyl

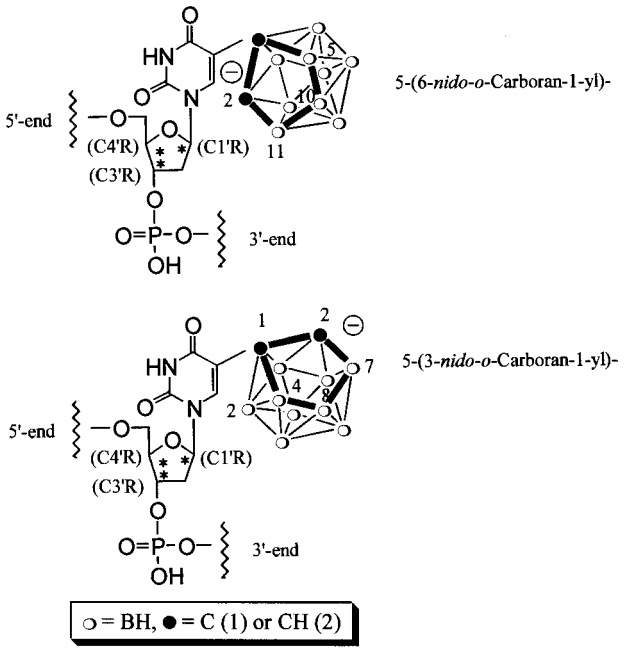


Fig. 12. Diastereomerism of oligonucleotides containing 5-(*o*-carboran-1-yl)-2′-deoxyuridine (CDU).

residue (Fig. 12). In the case of oligonucleotides containing two or more CDU residues, the number of possible oligonucleotide diastereomers is defined by the formula m^n, where m is the number of forms in which the carboranyl cage can exist (*closo*- and/or two *nido*-) and n is the number of carboranyl cages (CDU residues) per oligonucleotide. CDU oligonucleotides bearing one CDU modification, diastereomeric owing to chirality of *nido*-carboranyl cage, were separated into individual species by means of RP-HPLC [20].

The situation is more complicated in the case of oligonucleotides bearing CBMP modification. Replacing one of the anionic oxygen atoms by the *o*-carboran-1-yl-methyl moiety generates a new center of chirality at the phosphorus of modified internucleotide linkage of the oligonucleotide. In the case of CBMP oligonucleotides, the four different groups attached to the phosphorus atom are: phosphoryl oxygen, (*o*-carboran-1-yl)methyl group, nucleoside or oligonucleotide, and oligonucleotide moiety (Fig. 13). Due to this modification and the nonstereoselectivity of the coupling reaction used, the oligonucleotides bearing one CBMP modification with carboranyl cage in *closo*-form were obtained as a mixture of two P-diastereomers. The number of diastereomers arising from phosphorus chirality only is defined by the formula 2^p, where 2 is the number of diastereomeric forms of P-chiral internucleotide linkage can exist, and p is a number of P-chiral internucleotide linkages in the oligonucleotide.

Chirality of the *nido*-carboranyl cage generates another set of two diastereomers for each P-diastereomer. The number of diastereomers of CBMP oligonucleotides with all *o*-carboran-1-yl cages in the *nido*-form can be calculated from the formula $2^p \times m^p$ where 2 is the number of diastereomeric forms of P-chiral internucleotide linkage (CBMP in this case), p is the number of CBMP linkages, m is the number of forms carboranyl cage can exist (*closo*- and/or two *nido*-, for oligonucleotide containing a CBMP group with *o*-carboran-1-yl

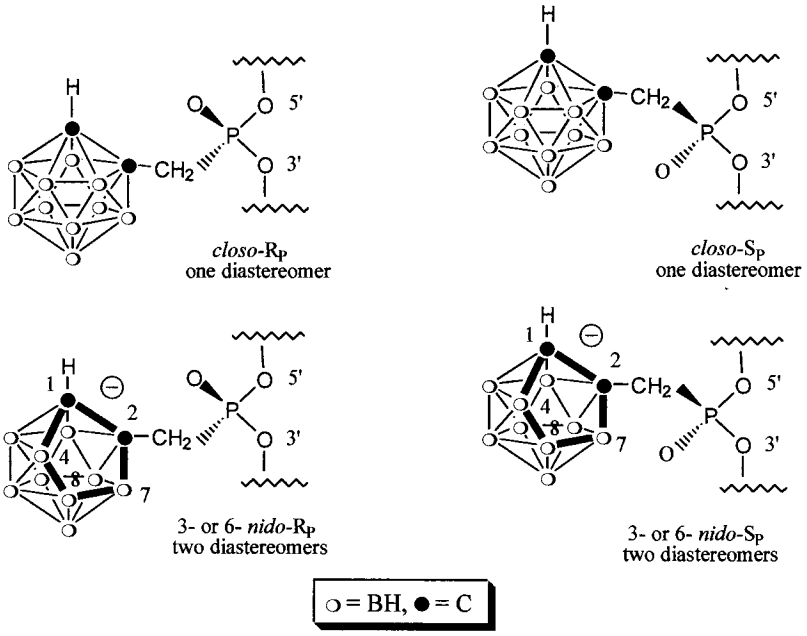


Fig. 13. Diastereomerism of oligonucleotides containing (*o*-carboran-1-yl)methylphosphonate (CBMP) group due to phosphorus and nido-(*o*-carboran-1-yl) cage chirality.

cages in the *nido*-form only $m = 2$), p is the number of CBMP modifications per oligonucleotide. For example, CBM oligonucleotide containing only 3 *nido*-CBMP groups comprises $2^3 \times 2^3 = 2^6$ diastereomers. Diastereomers of CBMP dodecathymidylic acids created by the *nido*-*o*-carboran-1-yl cage chirality were not separable by HPLC under the conditions used. Separation of P-diastereomers was achieved for oligonucleotides bearing *closo*- or *nido*-CBMP modification at the 3′-end {5′-d[$(T_P)_{10}T_{CBMP}T$]-3′}[70].

It has been known for several years that stereochemistry at phosphorus in P-chiral oligonucleotide analogues can affect their physicochemical characteristics and biological properties such as lipophilicity, water solubility, affinity to complementary strand and resistance to enzymatic digestion [74]. Incorporation of the carboranyl cage into the oligonucleotide chain can further complicate these parameters, but at the same time they add a new dimension to the problem of modified oligonucleotide structure–properties relationship. More study is needed to better understand this interdependence.

8. Prospects

Carborane nucleosides are novel class of modified nucleic acid components. They are synthesized as potential boron carriers for BNCT, antivirals and anticancer agents. The number of potential carborane nucleosides for BNCT has increased considerably in recent years. The interest in incorporating a carborane moiety into a nucleoside include: 1) boron clusters may provide a sufficient quantity of fissionable ^{10}B to tumor cells, 2) the enhanced lipophilicity of these compounds compared with standard nucleosides and their derivatives may accentuate membrane permeability, and 3) certain nucleosides such as CDU can be phosphorylated in malignant cells, thus trapping the compound intracellularly.

Oligonucleotides are oligomers built of nucleoside monomer units. The advantages of carborane-containing oligonucleotides as compared to unmodified counterparts include: 1) increased resistance to enzymatic digestion, 2) increased lipophilicity, and 3) formation of stable duplexes with complementary templates if the location of CBMP or CDU modification within the oligonucleotide chain is chosen judiciously.

Owing to the prominent effect of the carboranyl group on the properties of the oligonucleotide molecule, the change of physicochemical and biological characteristics of the oligomer may be achieved with a limited number of carborane modifications, leaving the rest of the molecule natural. This is potentially highly beneficial since unmodified oligonucleotides are virtually non-toxic. Based on the favorable physicochemical and biological properties of certain carborane containing oligonucleotides, oligomers for antisense oligonucleotide technology could be designed and targeted against relevant genes in cancer and in virally infected cells, as well as BNCT. Another possible application of carboranyl oligonucleotides is their use as molecular probes in the molecular diagnostics of cancer and infectious disease.

Acknowledgements

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