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Hydrophilically enhanced 3-carboranyl thymidine analogues (3CTAs) for boron neutron capture therapy (BNCT) of cancer☆

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Abstract—Five novel 3-carboranyl thymidine analogues (3CTAs) were designed and synthesized for boron neutron capture therapy (BNCT) of cancer. Phosphorylation of all five 3CTAs was catalyzed by recombinant human thymidine kinase (hTK1) using adenosine triphosphate (ATP) as the phosphate donor. The obtained phosphorylation rates ranged from 4% to 64.5% relative to that of thymidine. The compound with the most favorable hTK1 binding properties had a k_{cat}/K_M value of 57.4% relative to that of thymidine and an IC_{50} of inhibition of thymidine phosphorylation by hTK1 of 92 μM. Among the five synthesized 3CTAs, this agent had also the overall most favorable physicochemical properties. Therefore, it may have the potential to replace N5–2OH, the current lead 3CTA, in preclinical studies. An in silico model for the binding of this compound to hTK1 was developed.

1. Introduction

Boron neutron capture therapy (BNCT) is a binary cancer treatment modality, which relies both on a chemical and a radiation component.[1] In BNCT, a neutron capture reaction occurs when a stable ^{10}B isotope is irradiated with low energy thermal neutrons to produce highly cytotoxic particle radiation in the form of helium (α-particle) and lithium nuclei, both of which mediate cell death primarily by the induction of DNA double strand breaks.[1,2] Thermal neutrons and ^{10}B per se are harmless for human tissue and the helium and lithium nuclei have limited destructive ranges of approximately one cell diameter (<10 μm) in biological tissue, which restricts damage to cells that have been targeted with ^{10}B.[1]

Criteria for successful BNCT agents have been discussed in a recent review.[1] They are: (1) selective targeting of tumor versus normal cells, preferably with intracellular localization; (2) attaining cellular concentrations of $\sim 10^9$ ^{10}B atoms/cell or ~15 μg ^{10}B/g tumor; (3) persisting at a relatively constant concentration in the tumor during neutron radiation in order to deliver a constant radiation dose to the tumor; and (4) being sufficiently non-toxic to attain adequate in vivo tumor boron concentrations.

Boron-containing nucleosides, in particular those that target human thymidine kinase 1 (hTK1), have the potential to fulfill these criteria.[3–6] Human TK1 is a cytosolic deoxynucleoside kinase of the salvage pathways of nucleic acid synthesis and its function is 5′-monophosphorylation of thymidine (Thd) and 2′-deoxyuridine (dUrd).[7] The activity of this enzyme is very low in G_1 and G_0 cells, increases at G_1/early S boundary,

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and reaches maximum values in late S-phase/G_2 of the cell cycle.[4,7] Therefore, hTK1 activity is only found in proliferating cells, and thus, it is widely distributed and expressed in malignant tumors and tumor cells,[4,7] including those of the brain,[5,8] pancreas,[9] and lung.[10] Thus, hTK1-specific boronated nucleosides have the potential to be selectively entrapped in tumor cells due to 5′-monophosphorylation.

Human TK1 plays a pivotal role in anti-HIV chemotherapy.[11] The anti-HIV nucleoside prodrugs zidovudine (AZT) and stavudine (d4T) undergo a stepwise activation via mono-, di-, and triphosphorylation by human nucleoside and nucleotide kinases. While the initial 5′-monophosphorylation by hTK1 is the key step in this activation process, specific toxic effects toward HIV are exerted by the active triphosphate forms of both nucleoside analogues, which block DNA synthesis by inhibition of HIV reverse transcriptase and incorporation at 3′-terminal ends of HIV DNA.

In recent years, hTK1 has also been discovered as a molecular target for several other nucleoside analogues, including 3′-deoxy-3′-fluorothymidine (FLT), 1-(2-fluoro-5-methyl-β-arabinofuranosyl)uracil (FMAU), and 1-(2-fluoro-5-iodo-β-arabinofuranosyl)uracil (FIAU), which have the potential to be used as PET agents in radiolabeled form,[9,12–15] and for 3-carboranyl thymidine analogues (3CTAs), a novel class of nucleoside prodrugs for BNCT,[3–6] which are the subject of the present paper. In a recent review,[6] several shortcomings of existing 3CTAs, including specific enzymatic and physicochemical properties were discussed in detail. The present paper describes the synthesis and evaluation of several novel 3CTAs, which have the potential to overcome these shortcomings. A hypothetical model for the binding of 3CTAs to the active site of hTK1 will be provided.

2. Results and discussion

2.1. Design strategies

Previous efforts in the design, synthesis, and biological evaluation of 3CTAs in our laboratories have focused on compounds that contain a 1,2-disubstituted *o*-carborane cluster with Thd linked via the N3-position through an alkyl spacer to the 1-position-, and a dihydroxypropyl group attached to the 2-position of the carborane cluster to partially compensate for the highly hydrophobic properties of this cage structure.[3,5,6,16] Indeed, the current lead compound of our 3CTA library, N5–2OH (Fig. 1), belongs to this 3CTA subclass.[3,5,6,16] N5–2OH was also a substrate of thymidine kinase from *Bacillus anthracis* and produced efficient growth inhibition of this pathologic bacterium (unpublished results). To overcome several shortcomings of N5–2OH described in the following, we have designed novel *p*-carboranyl 3CTAs that are hydrophilically enhanced with acyclic alcohol functions containing more than two hydroxyl groups. Their design was based on the following considerations.

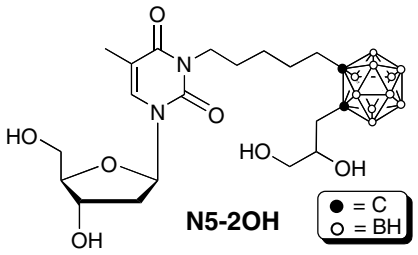


Figure 1. Structure of N5–2OH.

First, CDU (carboranyl deoxyuridine) is a dUrd derivative with an *o*-carborane cluster covalently linked to the 5-position.[17] Under in vitro conditions, approximately 4% of the *closo*-carborane cluster in CDU was degraded to the corresponding negatively charged *nido*-carborane after one hour incubation in S-phase U-251 human glioma cells.[17] Conversion of a *closo*-carborane to a *nido*-carborane may facilitate the intracellular entrapment of a carboranyl nucleoside provided it is already located in the interior of the cell, but it may prevent passive diffusion into a cell in the case of an extracellular location. Based on our current understanding of their membrane transport processes,[18] 3CTAs are entering cells via passive diffusion. *p*-Carborane presumably is stable under in vitro and in vivo conditions,[19] which should alleviate uncertainties related to possible cage degradation in 3CTAs.

Second, N5–2OH was found to be poorly soluble in water and 50% aqueous DMSO was necessary to solubilize this agent for in vivo studies.[5] Hydrophilic attachments with more than two hydroxyl groups may improve the water solubility of 3CTAs. However, Lipinski et al.[20] and others[21] have developed simple rules for physicochemical molecular parameters (molecular weight [M_W], log *P*, polar surface area [PSA], etc.) necessary for effective transcellular passive diffusion of lipophilic molecules. To some extent, these rules may also serve as guidelines for passive cell membrane diffusion of 3CTAs. Overall, 3CTAs should have physicochemical parameters that are in the range of those of AZT, emtricitabine, and abacavir, which are three nucleoside analogue prodrugs that are presumably entering cells largely by passive diffusion (Table 2).[22–24] Therefore, the number of hydroxyl group attached to the *p*-carboranyl 3CTAs described in this paper was limited to four (Schemes 1 and 2).

Third, recent computational docking studies with various geometric isomers of N5–2OH using a homology model of an open or semiopen form of hTK1 have shown that the dihydroxypropyl group attached to the 2-position of the *o*-caborane cage in N5–2OH may fold back onto the Thd moiety thereby interfering with binding to the active site of hTK1 (unpublished results). On the other hand, a linear 1,12-disubstitution pattern in the *p*-carboranyl N5–2OH isomer projected the dihydroxypropyl group away from the nucleoside scaffold (unpublished results). This seemed to decrease interference with the binding to hTK1 (unpublished results).

Table 1. Enzymatic properties of 3CTAs and other nucleoside prodrugs

Compound	rPRs[a]	K_M[b] (μM)	k_{cat} (s^{-1})	rk_{cat}/K_M[c]	IC_{50}[b,d] (μM)
Thd	100/100	3.4 ± 1.0	0.25 ± 0.03	100	—
7	62/67	3.0 ± 1.1	0.13 ± 0.02	57.4	92 ±18
15a	44/50	24.3 ± 7.7	0.11 ± 0.01	6.1	—
15b	61/60	10.7 ± 3.2	0.11 ± 0.01	14.6	—
17a	2/6	—	—	—	—
17b	14/17	—	—	—	—
N5–2OH	41[16]	—	—	35.8[3]	17 ± 3
AZT	52[46]	—	—	43.69[e]	—
FLT	30[46]	—	—	7.63[e]	—

[a] Two rPR (phosphorylation rate relative to Thd) measurements were carried for each Thd, **7**, **15a**, **15b**, **17a**, and **17b** (Section 4.5.1).
[b] k_{cat}, K_M and IC_{50} values ± SDs for Thd, N5–2OH, **7**, **15a**, and **15b** are based on three measurements (Sections 4.5.1 and 4.5.3).
[c] rk_{cat}/K_M: k_{cat}/K_M values relative to that of Thd.
[d] IC_{50}(μM): For details see Section 4.5.1.
[e] rk_{cat}/K_M were calculated from published V_{max} and K_M values of Thd, AZT and FLT.[47] $V_{max} = k_{cat} \cdot Et$ (Et, in molar, concentration of enzyme sites added to the assay).

A similar effect is expected for 3CTAs **15a** and **15b** substituted with even larger hydrophilic groups at the 12-position of *p*-carborane.

Finally, hydrophilic hydroxyl groups attached to the spacer unit between *p*-carborane cage and the Thd scaffold, as in compound **7**, should also be suitable to achieve some of the objectives outlined above.

Compounds **17a** and **17b** (Scheme 3) were not designed with the intention to optimize drug-like properties of 3CTAs. The major objective here was to investigate if these structures will undergo diphosphorylation in the presence of hTK1 having a single phosphate moiety at each of the 5′-positions.

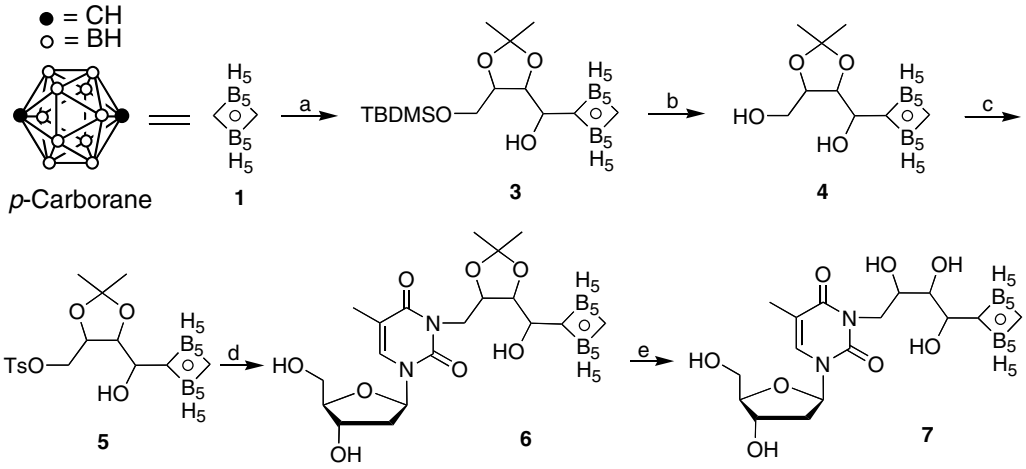
2.2. Synthesis

The synthesis of target compound **7**, with three hydroxyl groups attached to a butylene spacer between Thd and *p*-carborane, is shown in Scheme 1. The mono-lithium salt of *p*-carborane (**1**), prepared by treating *p*-carborane with 1 equiv of *n*-BuLi, was reacted in situ with the (2*S*,3*R*)-4-(*tert*-butyldimethylsilyloxy)-2,3-(isopropylidenedioxy)butanal[25] to obtain compound **3** in 63% yield (Scheme 1). A small amount of disubstituted *p*-carborane (~10%) was obtained as a side-product. Removal of the *tert*-butyldimethylsilyl protective group using TBAF/THF was accomplished at −78 °C to afford compound **4** in 92% yield. Reaction of compound **4** with

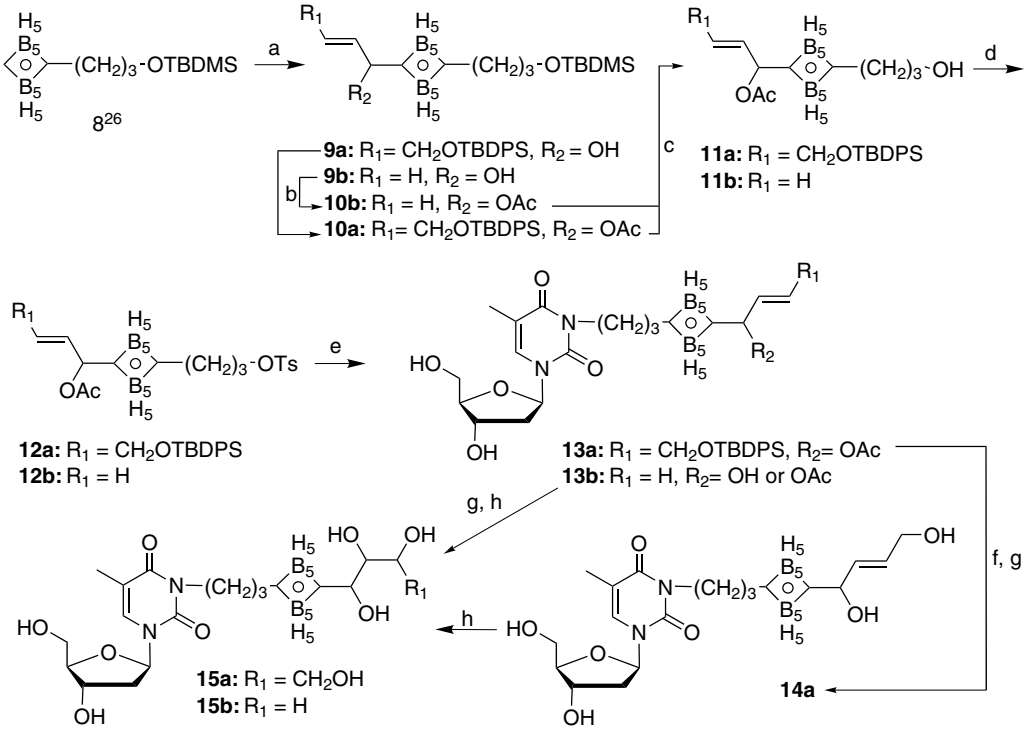
Table 2. Physicochemical properties of 3CTAs and other nucleoside prodrugs

Compound	M_W (Da)	log *P* (calcd)[a]	log *P* (Exp.)	APSA[b] (Å^2)	PSA[b] (Å^2)	Ratio PSA/APSA
Thd	242	−1.11	−1.1[48]	179.2	136.2	0.76
7	488	0.02	—	408.1	168.7	0.41
15a	546	−0.08	—	446.8	216.5	0.48
15b	516	0.45	—	441.7	189.6	0.43
N5–2OH	528	2.17	2.09[3]	499.1	172.4	0.34
AZT	267	—	0.025[49]	174.2	161.7	0.93
Emtricitabine	247	−0.3	−0.43[c]	152.1	141.8	0.93
Abacavir	286	0.22	0.037[d]	269.0	127.5	0.47

[a] See Section 4.4.2 for a detailed description of experimental procedures.
[b] See Section 4.4.1 for a detailed description of experimental procedures.
[c] Gilead Inc., product information (http://www.gilead.com).
[d] Drug bank (http://redpoll.pharmacy.ualberta.ca/drugbank/).



Scheme 1. Reagents and conditions: (a) BuLi/(2*S*,3*R*)-4-(*tert*-butyldimethylsilyloxy)-2,3-(isopropylidenedioxy)butanal (**2**)[25]/THF, 12 h, room temperature; (b) TBAF/THF, 1 h, −78 °C; (c) *p*-toluenesulfonyl chloride, pyridine/CH_2Cl_2 (1:6), 12 h, room temperature; (d) Thd, K_2CO_3, DMF/acetone (1:1), 48 h, 50 °C; (e) 17% HCl in MeOH, 14 h, room temperature.

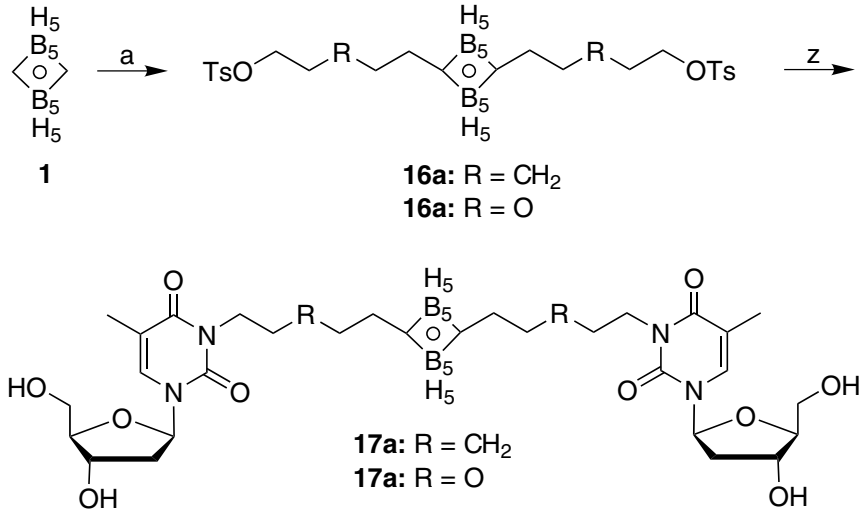


Scheme 2. Reagents and conditions: (a) *n*-BuLi, *trans*-4-(*tert*-butyldiphenylsiloxy)but-2-en-1-al[27] or acrolein, THF, 12 h, room temperature; (b) AcCl, pyridine, dichloromethane, 3 h, room temperature; (c) CCl_4/MeOH (1:1), ultrasonic bath, 10 h, room temperature; (d) *p*-toluenesulfonyl chloride, pyridine/dichloromethane (1:6), 13 h, room temperature; (e) Thd, K_2CO_3, DMF/acetone (1:1), 48 h, 50 °C; (f) TBAF, THF, 2 h, room temperature; (g) MeOH, K_2CO_3, 12 h, room temperature; (h) OsO_4, *N*-methyl morpholine oxide, acetone/H_2O, 12 h, room temperature.

p-toluenesulfonyl chloride in the presence of pyridine at room temperature resulted in selective tosylation of the primary alcohol (compound **5**), although in moderate yield (55%). We hypothesize that tosylation of the hydroxyl function at the 1-position was hampered due to steric hindrance by the *p*-carborane cage. Tosylation at the terminal hydroxyl group was confirmed by ^{1}H NMR, which showed a downfield shift for the CH_2 protons from δ 3.83 ppm in compound **4** to δ 4.02 ppm in compound **5**. Reaction of compound **5** with Thd in the presence of excess of K_2CO_3 in DMF/acetone (1:1) furnished compound **6** (73% yield) and subsequent removal of the acetonide protective group in **6** using HCl in methanol produced compound **7** in 66% yield.

The syntheses of compounds **15a** and **15b**, having tetrahydroxybutyl and trihydroxypropyl groups, respectively, attached to the 12-position of the *p*-carborane cage, are shown in Scheme 2. Compound **8**[26] was first exposed to 1 equiv of BuLi and was then reacted at room temperature with either *trans*-4-(*tert*-butyldiphenylsiloxy)but-2-en-1-al[27] or acrolein to yield compounds **9a** and **9b** in 57% and 51% yield, respectively. The ^{1}H NMR coupling constant (*J*) measured for the olefinic protons in *trans*-4-(*tert*-butyldiphenylsiloxy)but-2-en-1-al was 15.4 Hz. *trans*-Configuration was retained in **9a** (J_{trans}: 15.22 Hz) and was also observed for **14a** (J_{trans}: 15.1 Hz), the final olefinic compound of this series. Standard acetylation of **9a**/**9b** yielded compounds **10a** and**10b** in 74% and 60% yield, respectively. Selective removal of the TBDMS protective group in **10a** was accomplished by ultrasonication in CCl_4/MeOH (1:1), adapting a procedure described by Lee et al.,[28] to afford **11a** in 67% yield. Compound **11b** was obtained in 71% yield from **10b** using the same method. Compounds **11a** and **11b** were converted to the corresponding tosylates **12a** and **12b** in 80% and 79% yields, respectively, using *p*-toluenesulfonyl chloride in the presence of pyridine at room temperature. Tosylates **12a** and **12b** were reacted with Thd in the presence of excess of K_2CO_3 in DMF/acetone (1:1) to afford compounds **13a** and **13b**. Compound **13a** was obtained in 60% yield and was reacted with TBAF in 'wet' THF and subsequently with potassium carbonate in MeOH to obtain compound **14a** in 53% yield. Osmium tetraoxide/NMMO hydroxylation of **14a** in acetone/H_2O gave target compound **15a** in 53% yield. The reaction did not seem to be hampered notably due to the unfavorable *trans*-configuration at the double bond. Compound **13b** was obtained in 52% yield. HR-ESI analysis of **13b** indicated removal of the acetyl group under the used reaction condition, while acetylated product was clearly identified by HR-ESI analysis of **13a**. However, the ^{1}H NMR of **13b** still indicated the presence of an acetyl group. Due to the uncertainty of the presence of an acetyl group, compound **13b** was exposed to potassium carbonate in MeOH, as described for **13a**, before treatment with osmium tetraoxide/NMMO finally afforded compound **15b** in 42% yield.

The synthesis of target compounds **17a** and **17b**, both with two Thd moieties linked through spacers to the 1- and 12-position of *p*-carborane, is shown in Scheme 3. *p*-Carborane was treated with 2 equiv of *n*-BuLi in THF to generate its dilithium salt, which was further



Scheme 3. Reagents and conditions: (a) *n*-BuLi, 1,5-pentanediol di-*p*-tosylate[18] or di(ethylene glycol)[18] di-*p*-tosylate, THF/4 h, room temperature; (b) Thd, K_2CO_3, DMF/acetone (1:1), 48 h, 60 °C.

reacted with a solution either 1,5-pentanediol di-*p*-tosylate[18] or di(ethylene glycol) di-*p*-tosylate[18] in THF to yield compounds **16a** and **16b** in 40% and 59% yield, respectively. Substantial amounts of undesired side-products produced in these reactions are the results of the bifunctionalities of *p*-carborane, 1,5-pentanediol di-*p*-tosylate, and di(ethylene glycol) di-*p*-tosylate. Compounds **16a** and **16b** were reacted with Thd in the presence of excess K_2CO_3 in DMF/acetone (1:1) for 48 h at 60 °C to afford both target compounds **17a** and **17b** in 70% and 72% yield, respectively.

A detailed analysis of stereochemistries in target compounds **7**, **15a**, and **15b** was not carried out since previous studies in our laboratories with 3CTAs containing multiple hydroxyl groups indicated that these had, if at all, only a minor impact on their binding to the active site of hTK1 (unpublished results). All target compounds were characterized by ^{1}H NMR, ^{13}C NMR, and HR-MS. Samples of target compounds **7**, **15a**, **15b**, **17a**, and **17b** used for biological studies were re-purified by semipreparative reversed phase (C18) HPLC.

2.3. Enzymatic studies

The bisthymidyl compounds **17a** and **17b** showed low (~4%) to moderate (~15.5%) phosphorylation rates relative to that of Thd (rPRs) (Table 1). We hypothesize that this could be due to the bulky substitution pattern of this class of 3CTAs. However, the somewhat higher phosphorylation rate of **17b** indicates that electronegative atoms, such as oxygen, within the spacer between *p*-carborane cage and Thd units may benefit the binding of 3CTAs to the active site of hTK1. Both **17a** and **17b** showed two major phosphorylated products in the autoradiogram (Fig. 2) in a ratio of ~0.8 and ~7. We assume that the upper spots are generated by the MPs of **17a** and **17b**, while the lower spots could be caused either by impurities produced during the PTA or formation of diphosphates (DPs) of **17a** and **17b**. Since the rPRs **17a** and **17b** were relatively low, enzyme kinetically more

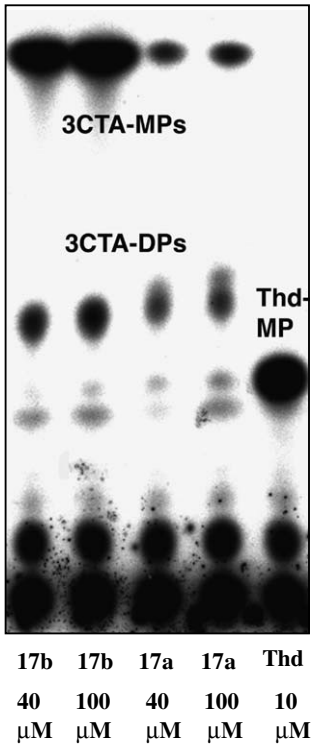


Figure 2. Autoradiogram of Thd, **17a**, and **17b**. The upper spots are the monophosphates (MPs) of the indicated 3CTAs. The spots in the middle could be impurities or diphosphate (DP) forms stemming from monophosphorylation at both Thd moieties. The intensity ratio for both spots of **17b** is ~7, while the intensity ratio of both spots of **17a** is ~0.8 (see Section 4.5.2 for details).

evocative k_{cat}/K_M values relative to that of Thd (rk_{cat}/K_M) were not determined. In contrast to the autoradiogram of the bisthymidyl compounds **17a** and **17b** (Fig. 2), the autoradiogram of compounds **7**, **15a** and **15b** (Fig. 3) showed single phosphorylated products for both compounds reflecting a high degree of purity and stability under synthesis and the assay conditions. Compounds **15a** and **15b** had slightly higher rPRs than

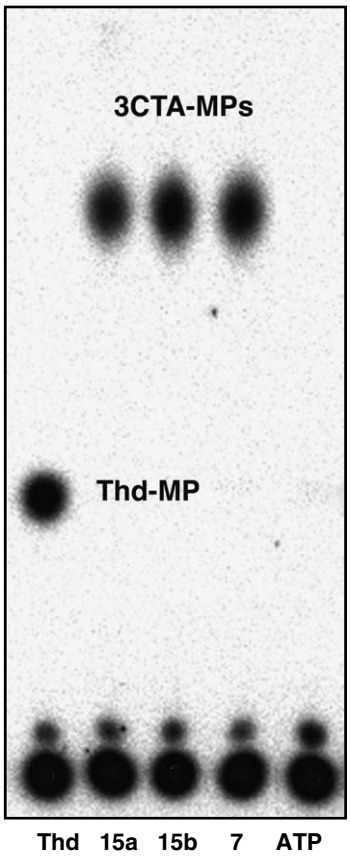


Figure 3. Autoradiogram of Thd, **7**, **15a**, and **15b**. The upper spots are the monophosphates (MPs) of the indicated 3CTAs and Thd (see Section 4.5.2 for details).

N5–2OH but their rk_{cat}/K_M were lower (Table 1). As in the case of compounds **17**, increased size of the substituents at the 12-position of the *p*-carborane cage in **15a** and **15b** may have been the reason for decreased interaction with the active site of hTK1. The overall the highest rk_{cat}/K_M was found for compound **7** (57.4%), which was even superior to those of AZT and FLT, two established hTK1 substrates. Similarly to oxygen in the linker of compound **17b**, the hydrophilic trihydroxybutylene spacer in **7** may have facilitated interaction with the active site of hTK1. The IC_{50} of inhibition of Thd phosphorylation by hTK1 for compound **7** was 92 μM, which is about five times higher than that of N5–2OH (17 μM). It is not unusual among deoxynucleoside kinases that substrates that bind less effectively to the enzymes are more effectively phosphorylated.[29,30] However, the decreased capacity of compound **7** to compete with Thd at the active site of hTK1 is a disadvantage compared with N5–2OH. Compounds **7**, **15a**, and **15b** were also good substrates of TK from *B. anthracis* with rPRs in the range of those found for hTK1 (data not shown).

2.4. In silico studies

Recently, crystal structures of human TK1 (hTK1),[31,32] *Ureaplasma urealyticum* TK (*Uu*TK),[32,33] and *Clostridium acetobutylicum* TK (*Ca*TK) [PDB ID: 1XX6] were determined and are now available for computational structure-based drug design. The crystal structures of human hTK1 and *Uu*TK were resolved as tetramers containing Thd or thymidine triphosphate (TTP), which is a feedback inhibitor of TK. However, *Ca*TK was crystallized as a dimer complexed with adenosine diphosphate (ADP) [PDB ID: 1XX6].

Compound **7** was chosen for docking studies because of its excellent hTK1 substrate characteristics (Section 2.3). The structure of a single arbitrarily chosen stereoisomer of compound **7**, 3-[2(*R*),3(*S*),4(*S*)-trihydroxy-4-(*p*-carboranyl-1-yl)butyl]thymidine, was minimized using the MM+ method in HyperChem 7.51 and its coordinates were transferred into the Sybyl 7.1. The boron atom types in the carborane cluster were changed into C.3 atom types because Sybyl does not provide parameters allowing calculations with structures containing hexavalent boron atoms. This type of manipulation of boron atom types was described previously by us and found to be feasible for docking studies with carborane-containing agents.[34,35] The minimized structure of compound **7** was docked into the active site of the hTK1 crystal structure[31] using the FlexX module of Sybyl

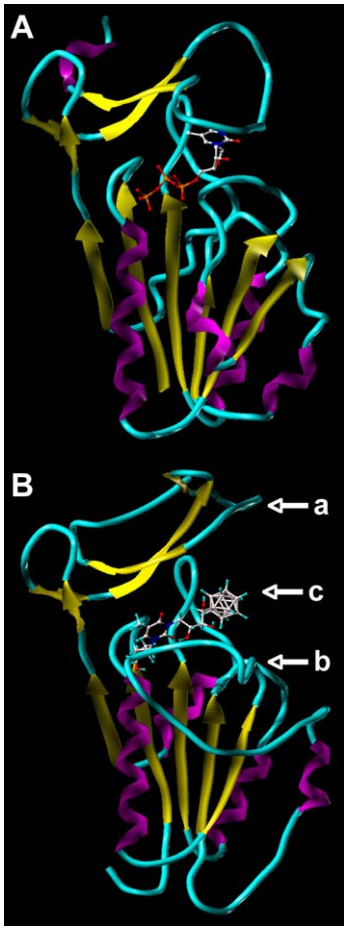


Figure 4. (A) Crystal structure of a hTK1 monomer with TTP (PDB ID: 1W4R). (B) hTK1 homology model, developed using *Clostridium acetobutylicum* TK (*Ca*TK) [PDB ID: 1XX6] as the template, with docked compound **7**. a, lasso domain; b, loop connecting the β2 and β3 strands; and c, extended cleft between the lasso domain and the loop connecting the β2 and β3 strands.

7.1. but its binding pattern did not reflect that of the Thd moiety in TTP, which is shown in Figure 4A. A visual inspection of the *Ca*TK crystal structure indicated structural differences compared with hTK1. Since the amino acid sequence identity of *Ca*TK with hTK1 is sufficiently high (39%) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/), a homology model of hTK1 was generated using the *Ca*TK crystal structure as the template. As shown in Figure 4A and B, there is an extended gap (c) between the loop connecting the β2 and β3 strands (b) and the 'lasso' domain (a) in the homology model of hTK1 compared with the crystal structure of hTK1. In many nucleoside kinases and nucleoside monophosphate kinase, binding of the substrates and ATP is associated with a conformational change from an open unoccupied form, over a partially closed form involving substrate or ATP binding, to a closed form binding both substrate and ATP.[36–40] There may be a similar pattern in TKs, where TTP/Thd binding hTK1s and *Uu*TKs represent closed forms, while APD-binding *Ca*TK may constitute an open or semiopen TK form, which is consequently also reflected by the homology model of hTK1.

We acknowledge the limitations of in silico docking studies involving a homology model.[41] Eventually, only an in-depth X-ray crystallographic analysis can reveal realistic dimensions of open and closed forms of hTK1. Nevertheless, in contrast with the hTK1 crystal structure, docking of compound **7** into the homology model of hTK1 revealed an interesting binding pattern (Fig. 4B). We hypothesize that the extended gap (c) between the loop connecting the β2 and β3 strands (b) and the 'lasso' domain (a) in an open form of hTK1 allows the initial access of compound **7** to the active site of the enzyme. The docking pose of compound **7** in Figure 4B shows binding of the thymine and deoxyribose portions of **7** in the vicinity of the phosphate binding site, while the bulky carborane cage is projected toward the extended gap (c). We hypothesize that partial closure of the lasso domain due to binding of **7** and ATP will eventually result in a relocation and a final orientation of the ribose portion that allows for an effective transfer of the γ-phosphate from ATP to the 5′- or 3′-hydroxyl group of **7**, while its carborane cluster is relocated to the enzyme surface through unfolding and extension of the trihydroxybutylene linker. Unfortunately, this model does not account for the differences in k_{cat}/K_M and IC_{50} values between compound **7** and N5–2OH.

2.5. Physicochemical parameters

Table 2 shows a compilation of calculated and experimental physicochemical parameters of compounds **7**, **15a**, **15b**, Thd, N5–2OH, AZT, emtricitabine, and abacavir. The latter three anti-HIV drugs are believed to pass cellular membranes primarily via passive diffusion.[22–24] The M_Ws of all 3CTAs are about ~2 times higher than those of the three antiviral drugs and are at the borderline for M_Ws set by Lipinski et al.[20] and van de Waterbeemd and co-workers[21] for transcellular passive diffusion of lipophilic molecules through intestinal epithelium and blood–brain barrier (BBB) endothelium, respectively. However, in vitro uptake and retention of N5-2OH[3,5] and also **15a**[42] in various cell lines did not seem to be impaired. Overall, the PSAs of **7**, **15a**, and **15b** are slightly higher than those of AZT, emritcitabine, and abacavir, while their PSA/APSA ratios are comparable with that of abacavir, presumably the most lipophilic of the three antiviral drugs. Also, the log *P*s for the 3CTAs and the antivirals are in the same range. We hypothesize, that the lipophilic character of compounds **7**, **15a**, and **15b** will be more important for effective passive diffusion than their M_Ws. If at all, compound **7** may have slightly superior overall physicochemical properties than **15a** and **15b**.

3. Summary and conclusions

Five novel 3CTAs were synthesized. The hTK1 substrate characteristics of compounds **17a** and **17b** were moderate. Both 3CTAs possess two Thd moieties linked through spacers to the 1- and 12-position of *p*-carborane. They do not have drug-like properties. As anticipated, both compounds showed a phosphorylation pattern that is consistent with mono- and diphosphorylation. Compounds **7**, **15a**, and **15b** have more drug-like properties than compounds **17a/b**. These structures are substituted with multiple hydroxyl groups to enhance hydrophilicity. In this class of 3CTAs, compound **7** appears to have the most favorable hTK1 substrate characteristics (rk_{cat}/K_M = 57.4%) and physicochemical properties. Compared with N5–2OH, however, the hydrophilic properties of compound **7** may have reached the limit for passive cell membrane diffusion also indicating that structural modification of 3CTAs with more than 3–4 hydroxyl groups may not be desirable. In addition, compound **7** does not inhibit Thd phosphorylation by hTK1 as effectively as N5–2OH. Considering the substrate and inhibitor characteristics of both compounds, future 3CTAs may have overall 3–4 hydroxyl groups both in the linker between Thd scaffold and carborane and in a group attached to the second carbon of a carborane cluster. Nevertheless, based on hTK1 substrate characteristics and hydrophilic properties, compound **7** may have the potential to replace N5–2OH as the lead compound of our current 3CTA library. In vitro and in vivo studies with compound **7** are planned in our laboratories to validate this hypothesis. Compound **7** could also be synthesized as the *o*-carborane derivative according to the route shown in Scheme 1, which would be less expensive when ^{10}B-enriched material is used. Replacement of *p*- by *o*-carborane would only result in minor structural changes that should not significantly impact physicochemical properties and binding to hTK1.

4. Materials and methods

4.1. General chemical procedures

Proton and carbon-13 NMR spectra were obtained on Bruker (250 MHz or 400 MHz) FT-NMR instruments at The Ohio State University, College of Pharmacy. Chemical shifts are reported in parts per million (ppm)

from an internal tetramethylsilane standard. The coupling constants are reported in hertz (Hz). High resolution electrospray ionization (HR-ESI) mass spectra were recorded on a Micromass QTOF-Electrospray mass spectrometer and a 3 T Finnigan FTMS-2000 Fourier Transform mass spectrometer at The Ohio State University Campus Chemical Instrumentation Center (OSU-CCIC). Electron-impact (EI) mass spectra obtained with a Kratos MS-25 mass spectrometer using 70 eV ionization conditions were generated at The Ohio State University Department of Chemistry. For compounds **3**, **4**, **6**, **7**, **9a**, **10a**, **11a**, **14a**, **15b**, and **17a** the exact mass of the ^{11}B-monoisotopic peak ($10 \times {}^{11}$B) of the isotope pattern is reported. For compounds **5**, **9b**, **10b**, **11b**, **12a**, **12b**, **13b**, **15a**, **16a**, **16b**, and **17b** the value of the most abundant peak of the isotope pattern is reported. A selection criterion was the lowest parts per million deviation. For compound **13a** low resolution MS data are reported. Compound visualization on silica gel 60 F_{254} precoated TLC plates (0.25 mm layer thickness) (Merck, Darmstadt, Germany) was attained by UV light. Carborane-containing compounds were selectively visualized by spraying a solution of 0.06% $PdCl_2$/1% aqueous HCl on TLC plates and subsequent heating to ~120 °C, which caused the slow formation (15–45 s) of gray spots due to the reduction of Pd^{2+} to Pd^0. Reagent grade chemicals were obtained from commercial vendors and used as such. *p*-Carborane was purchased from Katchem, Prague, Czech Republic. Osmium tetraoxide was purchased from Strem Chemicals (Newburyport, MA). *Caution: osmium tetraoxide is a highly toxic and flammable liquid. A careful study of the MSDS is advisable before usage of this chemical.* Reagent grade solvents were used for column chromatography using silica gel 60, particle size 0.040–0.063 mm (Merck, NJ). Anhydrous benzene, anhydrous dichloromethane, and anhydrous THF were purchased from VWR Scientific Products. THF was dried over sodium metal before use. All chemical syntheses were carried out under argon atmosphere to maintain inert reaction condition unless mentioned otherwise. For biological studies, target compounds **7**, **15a**, **15b**, **17a**, and **17b** were re-purified by semipreparative HPLC on a reversed phase [Discovery@HS C18 [10 μm] (SUPELCO)] column using an Rainin HPLC instrument equipped with a Dynamax DA controller, HPXL pumps, and a Dynamax UV-1 detector (Rainin Instrument Company Inc., Woburn, MA, USA). HPLC grade water and methanol were used as mobile phases. A water/methanol gradient (100:0 to 50:50 over 40 min, from 50:50 to 0:100 over 29 min) with a flow rate of 5 mL/min was applied.

4.2. Synthetic procedures and analytical data

4.2.1. [5-(*tert*-Butyldimethylsilanyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl]-*p*-carboran-1-yl-methanol (3). To a solution of *p*-carborane (**1**) (158 mg, 1.10 mmol) in THF (10 mL) at 0 °C, *n*-butyl lithium (2.5 M in hexanes, 0.48 mL, 1.20 mmol) was added dropwise. After stirring the reaction mixture for 2 h at 0 °C, (2*S*,3*R*)-4-(*tert*-butyldimethylsilyloxy)-2,3-(isopropylidenedioxy)butanal (**2**)[25] (318 mg, 1.20 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 12 h, quenched with water, and extracted with diethyl ether (3× 30 mL). The combined organic layers were washed with brine and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 9:1) to give compound **3** in 63% (320 mg) yield. R_f: 0.45; ^{1}H NMR ($CDCl_3$): δ 0.02 (s, 6H, $Si(CH_3)_2$), 0.84 (s, 9H, $C(CH_3)_3$), 1.31 (s, 6H, $C(CH_3)_2$), 2.61 (d, 1H, OH, J = 6.5 Hz), 2.70 (br s, 1H, $C_{carborane}$–*H*), 3.41–3.60 (m, 2H), 3.69–3.85 (m, 3H): MS (HR-ESI) $C_{15}H_{38}B_{10}O_4SiNa$ $(M+Na)^+$ calcd: 443.3368, found: 443.3406 (8.5 ppm).

4.2.2. (5-Hydroxymethyl-2,2-dimethyl-1,3-dioxolan-4-yl)-*p*-carboran-1-yl-methanol (4). A solution of compound **3** (350 mg, 0.86 mmol) in THF (10 mL) was cooled to −78 °C and TBAF (1.0 M in THF, 1.30 mL) was added dropwise. The reaction mixture was gradually warmed to room temperature, stirred for additional 1 h, poured into ice/water (30 mL), and extracted with diethyl ether (3× 30 mL). The combined organic layers were washed with brine and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 7:3) to give compound **4** in 92% (240 mg) yield. R_f: 0.71; ^{1}H NMR ($CDCl_3$): δ 1.40 (s, 3H, $C(CH_3)_2$), 1.43 (s, 3H, $C(CH_3)_2$), 2.56 (d, 1H, OH, J = 11.7 Hz), 2.78 (br s, 1H, $C_{carborane}$–*H*), 3.26 (d, 1H, C*H*(OH), J = 11.7 Hz), 3.54 (dd, 1H, C*H*, J = 2.92, 3.16 Hz), 3.75 (dd, 1H, C*H*, J = 2.84, 3.12 Hz), 3.85–3.89 (m, 2H, CH_2); MS (HR-ESI) $C_9H_{24}B_{10}O_4Na$ $(M+Na)^+$ calcd: 329.2503, found: 329.2482 (6.4 ppm).

4.2.3. [5-(4-Toluenesulfonyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl]-*p*-carboran-1-yl-methanol (5). A solution of compound **4** (235 mg, 0.80 mmol), *p*-toluenesulfonyl chloride (152 mg, 0.80 mmol), and pyridine (64 mg, 0.80 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature for 12 h. The mixture was diluted with diethyl ether (100 mL) and the organic layer was washed with water (30 mL), cold HCl (0.01 N), and brine, and then dried over $MgSO_4$. After filtration and evaporation, the residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 7:3) to give compound **5** in 54% (200 mg) yield. R_f: 0.38; ^{1}H NMR ($CDCl_3$): δ 1.35 (s, 3H, $C(CH_3)_2$), 1.35 (s, 3H, $C(CH_3)_2$), 2.49 (s, 3H, Ar-CH_3), 2.78 (br s, 1H, $C_{carborane}$–*H*), 3.29 (d, 1H, C*H*(OH), J = 10.2 Hz), 3.78 (d, 1H, C*H*(OH), J = 8.2 Hz), 3.90–3.94 (m, 1H, C*H*(OH)) 4.02–4.11 (m, 2H, CH_2), 7.35 (d, 2H, ArH, J = 9.92 Hz), 7.79 (d, 2H, ArH, J = 8.16 Hz); MS (HR-ESI) $C_{16}H_{30}B_{10}O_6SNa$ $(M+Na)^+$ calcd: 481.2673, found: 481.2664 (1.9 ppm).

4.2.4. 3-{5-[Hydroxy-(*p*-carboran-1-yl)methyl]-2,2-dimethyl-1,3-dioxolan-4-ylmethyl}thymidine (6). A mixture of compound **5** (140 mg, 0.31 mmol), thymidine (91 mg, 0.38 mmol), and K_2CO_3 (138 mg, 1.00 mmol) in acetone/DMF (1:1, v/v, 10 mL) was stirred at 50 °C for 48 h. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (dichloromethane/methanol, 9:1) to give the compound

6 in 73% (120 mg) yield. R_f: 0.40; ^{1}H NMR (CD_3OD): δ 1.24 (s, 3H, $C(CH_3)_2$), 1.25 (s, 3H, $C(CH_3)_2$), 1.84 (d, 3H, CH_3, J = 1.2 Hz), 2.08–2.14 (m, 2H, H-2′), 3.18 (br s, 1H, $C_{carborane}$–*H*), 3.65–3.71 (m, 3H, H-5′ and C*H*(OH)), 3.82–3.84 (m, 1H, H-4′), 3.95–4.05 (m, 4H, CH_2N, C*H*(OH)), 4.29–4.30 (m, 1H, H-3′), 6.21 (t, 1H, H-1′, J = 6.7 Hz), 7.79 (d, 1H, H-6, J = 1.2 Hz); $C_{19}H_{36}B_{10}N_2O_8Na$ $(M+Na)^+$ calcd: 553.3300, found: 553.3314 (2.5 ppm).

4.2.5. 3-[2,3,4-Trihydroxy-4-(*p*-carboranyl-1-yl)butyl]thymidine (7). To a solution of compound **6** (100 mg, 0.19 mmol) in MeOH (5 mL) was added dropwise 17% HCl in MeOH (0.5 mL) and the reaction mixture was stirred for 14 h at room temperature. Excess MeOH was removed by evaporation, the residue was suspended in water and extracted with ethyl acetate (3× 30 mL). The combined organic layers were washed with water and brine, and dried over anhydrous Na_2SO_4. After filtration and evaporation, the residue was purified by silica gel column chromatography (dichloromethane/methanol, 4:1) to give the compound **7** in 66% (60 mg) yield. R_f: 0.50; HPLC retention time (semipreparative): 51.29 min; ^{1}H NMR (CD_3OD): δ 1.84 (d, 3H, CH_3, J = 1.0 Hz), 2.09–2.12 (m, 2H, H-2′), 3.18 (br s, 1H, $C_{carborane}$–*H*), 3.52–3.53 (m, 1H, C*H*(OH)), 3.68–3.76 (m, 3H, H-5′ and C*H*(OH)), 3.81–3.85 (m, 2H, C*H*(OH) and H-4′), 4.08–4.17 (m, 2H, CH_2N), 4.30–4.35 (m, 1H, H-3′), 6.22 (t, 1H, H-1′, J = 6.5 Hz), 7.79 (d, 1H, H-6, J = 1.0 Hz); ^{13}C NMR($CDCl_3$): δ 13.21, 41.46, 44.87, 59.44, 62.73, 72.04, 72.17, 72.92, 73.11, 73.50, 87.27, 88.92, 110.72, 136.55, 152.79, 165.86; MS (HR-ESI) $C_{16}H_{32}B_{10}N_2O_8Na$ $(M+Na)^+$ calcd: 513.2987, found: 513.3016 (5.6 ppm).

4.2.6. 1-{12-[3-(*tert*-Butyldimethylsilanyloxy)-propyl]carboran-1-yl}-4-(*tert*-butyldiphenylsilanyloxy)-but-2-en-1-ol (9a). To a stirred solution of compound **8**[26] (250 mg, 0.75 mmol) in THF (5 mL) was added *n*-butyl lithium (2.5 M in hexanes, 0.36 mL, 0.90 mmol) at 0 °C. The mixture was stirred at 0 °C for 2 h and then *trans*-4-(*tert*-butyldiphenylsiloxy)but-2-en-1-al[27] (233 mg, 0.75 mmol) was added dropwise. The mixture was stirred at room temperature for 12 h, quenched with 10 mL of water, and extracted with diethyl ether (3× 30 mL). The combined organic layers were washed with brine, and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by column chromatography on silica gel (*n*-hexanes/ethyl acetate, 9:1) to obtain the compound **9a** in 57% (270 mg) yield. R_f: 0.35; ^{1}H NMR ($CDCl_3$): δ −0.03 (s, 6H, $Si(CH_3)_2$), 0.83 (s, 9H, $C(CH_3)_3$), 1.04 (s, 9H, $C(CH_3)_3$), 1.28–1.37 (m, 2H, CH_2), 1.65–1.71 (m, 2H), 3.39 (t, 2H, OCH_2, J = 6.0 Hz), 3.97 (t, 1H, C*H*(OH), J = 5.8 Hz), 4.09–4.16 (m, 2H, CH_2), 5.48 (d, 1H, CH, J = 15.22 Hz), 5.55–5.60 (m, 1H, CH), 7.37–7.42 (m, 6H, ArH), 7.63–7.66 (m, 4H, ArH); MS (HR-ESI) $C_{31}H_{56}B_{10}O_3Si_2Na$ $(M+Na)^+$ calcd: 665.4596, found: 665.4607 (1.7 ppm).

4.2.7. 1-{12-[3-(*tert*-Butyldimethylsilanyloxy)-propyl]carboran-1-yl}-prop-2-en-1-ol (9b). Experimental conditions for the synthesis of **9a** were adapted for the synthesis of **9b**. The reaction of the compound **8**[26] (56 mg, 1.00 mmol) with acrolein (305 mg, 0.93 mmol) gave the compound **9b** in 51% (190 mg) yield. R_f: 0.45 (ethyl acetate/hexanes, 1:9); ^{1}H NMR ($CDCl_3$): δ −0.02 (s, 6H, $Si(CH_3)_2$), 0.84 (s, 9H, $C(CH_3)_3$), 1.28–1.37 (m, 2H, CH_2), 1.65–1.71 (m, 2H, CH_2), 3.44 (t, 2H, OCH_2, J = 7.55 Hz), 3.96 (d, 1H, C*H*(OH), J = 5.8 Hz), 5.10–5.17 (m, 2H, allyl), 5.50–5.59 (m, 1H, allyl); MS (HR-EI) $C_{14}H_{36}B_{10}O_2Si$ (M^+) calcd: 372.3482, found: 372.3471 (3.0 ppm).

4.2.8. 1-{12-[3-(*tert*-Butyldimethylsilanyloxy)-propyl]carboran-1-yl}-4-(*tert*-butyldiphenylsilanyloxy)-but-2-en-1-yl acetate (10a). To a stirred solution of the compound **9a** (250 mg, 0.39 mmol) and pyridine (32 mg, 0.40 mmol) in CH_2Cl_2 (5 mL) was added dropwise a solution of acetylchloride (33 mg, 0.40 mmol) in CH_2Cl_2 (5 mL) and the reaction mixture was stirred at room temperature for 3 h. The solvents were removed under reduced pressure and the residue was extracted in CH_2Cl_2. The organic layer was washed with water and brine and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by column chromatography on silica gel (*n*-hexanes/ethyl acetate, 10:1) to give the compound **10a** in 74% (200 mg) yield. R_f: 0.35; ^{1}H NMR ($CDCl_3$): δ −0.02 (s, 6H, $Si(CH_3)_2$), 0.83 (s, 9H, $C(CH_3)_3$), 1.04 (s, 9H, $C(CH_3)_3$), 1.24–1.32 (m, 2H, CH_2), 1.65–1.71 (m, 2H, CH_2), 2.00 (s, 3H, CH_3), 3.40 (t, 2H, CH_2, J = 6.0 Hz), 4.09–4.14 (m, 2H, CH_2), 5.22 (d, 1H, CH, J = 7.0 Hz), 5.46–5.64 (m, 2H, CH), 7.37–7.42 (m, 6H, ArH), 7.63–7.66 (m, 4H, ArH); MS (HR-ESI) $C_{33}H_{58}B_{10}O_4Si_2Na$ $(M+Na)^+$ calcd: 707.4702, found: 707.4639 (8.9 ppm).

4.2.9. 1-{12-[3-(*tert*-Butyldimethylsilanyloxy)-propyl]carboran-1-yl}-prop-2-en-1-yl acetate (10b). Experimental conditions for the synthesis of **10a** were adapted for the synthesis of **10b**. Treatment of **9b** (160 mg, 0.41 mmol) with pyridine and acetylchloride yielded compound **10b** in 60% (100 mg) yield. R_f: 0.40 (ethyl acetate/hexanes, 1:9); ^{1}H NMR ($CDCl_3$): δ −0.02 (s, 6H, $Si(CH_3)_2$), 0.838 (s, 9H, $C(CH_3)_3$), 1.24–1.38 (m, 2H, CH_2), 1.65–1.71 (m, 2H, CH_2), 2.01 (s, 3H, CH_3), 3.39 (t, 2H, CH_2, J = 6.0 Hz), 5.06–5.16 (m, 3H, allyl and CH), 5.38–5.44 (m, 1H, allyl); MS (HR-ESI) $C_{16}H_{38}B_{10}O_3SiNa$ $(M+Na)^+$ calcd: 437.3501, found: 437.3512 (2.5 ppm).

4.2.10. 1-[12-(3-Hydroxypropyl)carboran-1-yl]-4-(*tert*-butyldiphenylsilanyloxy)-but-2-en-1-yl acetate (11a). A solution of compound **10a** (200 mg, 0.29 mmol) in anhydrous methanol and carbon tetrachloride (1:1, 10 mL) was placed for 10 h in an Aquasonic/Sonics Vibracell VC 130PB sonicator (Model 50 HT/50/60 Hz) at a setting of 0.5. Subsequently, excess solvent was evaporated under reduced pressure and the residue was purified by column chromatography (*n*-hexanes/ethyl acetate, 7:1) to give the compound **11a** in 67% (110 mg) yield. R_f: 0.45; ^{1}H NMR (acetone-d_6): δ 1.04 (s, 9H, $C(CH_3)_3$), 1.37–1.46 (m, 2H, CH_2), 1.69–1.76 (m, 2H, CH_2), 1.99 (s, 3H, CH_3), 3.44–3.53 (m, 3H, CH_2 and OH), 4.10–4.14 (m, 2H, CH_2), 5.18–5.23 (m, 1H, CH), 5.48–5.60 (m, 2H, CH), 7.34–7.45 (m, 6H, ArH), 7.61–7.69 (m, 4H, ArH); MS (HR-ESI) $C_{27}H_{44}B_{10}O_4SiNa$ $(M+Na)^+$ calcd: 593.3837, found: 593.3846 (1.5 ppm).

4.2.11. 1-[12-(3-Hydroxypropyl)carboran-1-yl]-prop-2-en-1-yl acetate (11b). Experimental conditions for the synthesis of **11a** were adapted for the synthesis of **11b**. sonication of **10b** in anhydrous methanol and carbon tetrachloride gave compound **11b** in 71% (62 mg) yield. R_f: 0.40 (ethyl acetate/hexanes, 1:1); ^{1}H NMR ($CDCl_3$): δ 1.28–1.37 (m, 2H, CH_2) 1.65–1.71 (m, 2H, CH_2), 2.01 (s, 3H, CH_3), 3.45 (t, 2H, OCH_2, J = 6.0 Hz), 5.06–5.14 (m, 3H, allyl, CH), 5.38–5.48 (m, 1H, allyl); MS (HR-EI) $C_{10}H_{24}B_{10}O_3$ (M+) calcd: 300.2723, found: 300.2736 (4.3 ppm).

4.2.12. 1-{12-[3-(4-Toluenesulfonyloxy)propyl]carboran-1-yl}-4-(*tert*-butyldiphenylsilanyloxy)-but-2-en-1-yl acetate (12a). To a solution of compound **11a** (130 mg, 0.18 mmol) in CH_2Cl_2 (5 mL) were added *p*-toluenesulfonyl chloride (34 mg, 0.18 mmol) and pyridine (15 mg, 0.18 mmol). The reaction mixture was stirred at room temperature for 3 h. Ice water was added and the product was extracted with CH_2Cl_2 (3× 20 mL). The combined organic layers were washed with 10% aqueous HCl solution, brine, and water, and then dried over $MgSO_4$. After filtration and evaporation, the residue was purified by column chromatography on silica gel (*n*-hexanes/ethyl acetate, 7:1) to give the compound **12a** in 80% (100 mg) yield. R_f: 0.40 ; ^{1}H NMR ($CDCl_3$): δ 1.05 (s, 9H, $C(CH_3)_3$), 1.59–1.65 (m, 2H, CH_2), 1.76–1.82 (m, 2H, CH_2), 2.03 (s, 3H, CH_3), 3.85 (t, 2H, CH_2, J = 6.1 Hz), 4.02 (t, 2H, CH_2, J = 5.6 Hz), 5.21–5.23 (m, 1H, CH), 5.63 (dd, 2H, CH, J = 14.8 Hz, 7.6 Hz), 7.34–7.39 (m, 8H, ArH), 7.63–7.75 (m, 6H, Ar); MS (HR-ESI) $C_{34}H_{50}B_{10}O_6SSiNa$ $(M+Na)^+$ calcd: 746.3990, found: 746.3970 (2.7 ppm).

4.2.13. 1-{12-[3-(4-Toluenesulfonyloxy)propyl]carboran-1-yl}-prop-2-en-1-yl acetate (12b). Experimental conditions for the synthesis of **12a** were adapted for the synthesis of **12b**. Treatment of the compound **11b** with (50 mg, 0.088 mmol) *p*-toluenesulfonyl chloride and pyridine gave the compound **12b** in 79% (30 mg) yield. R_f: 0.35 (ethyl acetate); ^{1}H NMR ($CDCl_3$): δ 1.28–1.37 (m, 2H, CH_2)1.65–1.71 (m, 2H, CH_2), 2.01 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 3.87 (t, 2H, CH_2, J = 6.0 Hz), 5.20–5.28 (m, 3H, allyl and CH), 5.64 (m, 1H, allyl), 7.33 (d, 2H, ArH, J = 13.7 Hz), 7.76 (d, 2H, ArH, J = 13.7 Hz); MS (HR-EI) $C_{17}H_{30}B_{10}O_5S_1$ (M^+) calcd: 454.2812, found: 454.2765 (10.00 ppm).

4.2.14. 3-({1-[12-(2-Acetoxy-4-(*tert*-butyldiphenylsilanyloxy)-but-2-en-1-yl)]carboran-1-yl}-propyl)thymidine (13a). To a solution of the compound **12a** (120 mg, 0.17 mmol) in dimethylformamide/acetone (1:1, v/v, 10 mL) were added K_2CO_3 (69 mg, 0.50 mmol)and thymidine (48 mg, 0.20 mmol) and the reaction mixture was stirred at 50 °C for 48 h. After filtration, excess solvent was removed under reduced pressure. The product was extracted in ethyl acetate, washed with water, and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by column chromatography on silica gel (100% ethyl acetate) to give the compound **13a** in 60% (80 mg) yield. R_f: 0.45 ; ^{1}H NMR (CD_3OD): δ 0.97 (s, 9H, $C(CH_3)_3$), 1.17–1.19 (m, 2H, CH_2), 1.59–1.66 (m, 2H, CH_2), 1.82 (s, 3H, CH_3), 1.93 (s, 3H, CH_3), 2.06–2.25 (m, 2H, H-2′), 3.61–3.77 (m, 4H, H-5′ and OCH_2), 3.83–3.85 (m, 1H, H-4′), 3.97–4.08 (m, 2H, CH_2N), 4.30–4.35 (m, 1H, H-3′), 5.13—5.21 (m, 1H, CH), 5.38—5.53 (m, 2H, CH), 6.21 (t, 1H, H-1′, J = 6.5 Hz), 7.31–7.36 (m, 6H, ArH), 7.57–7.61 (m, 4H, ArH), 7.75 (d, 1H, H-6, J = 1.2 Hz); ^{13}C NMR(CD_3OD): 13.21, 14.48, 27.29, 28.67, 36.28, 41.31, 41.50, 61.48, 62.68, 64.15, 71.99, 74.40, 87.04, 88.78, 110.59, 125.57, 128.83, 130.06, 130.84, 130.88, 130.96, 132.43, 133.10, 134.48, 134.55, 136.52, 152.10, 165.15, 170.09; MS (LR-ESI) $C_{37}H_{56}B_{10}N_2O_8SiNa$ $(M+Na)^+$ calcd: 817.4, found: 817.5.

4.2.15. 3-{1-[12-(1-Hydroxy-prop-2-en-1-yl)carboran-1-yl]-propyl}thymidine (13b). Experimental conditions for the synthesis of **13a** were adapted for the synthesis of **13b**. Treatment of the compound **12b** (58 mg, 0.068 mmol) with K_2CO_3 (69 mg, 0.50 mmol)and thymidine gave compound **13b** in 52% (17 mg) yield. R_f: 0.45 (methanol/dichloromethane, 1:12); ^{1}H NMR (CD_3OD): δ 1.31–1.40 (m, 2H, CH_2), 1.89 (d, 3H, CH_3, J = 1.2 Hz), 2.11–2.31 (m, 4H, H-2′ and CH_2), 3.72–3.98 (m, 5H, H-5′, H-4′ and CH_2N), 4.36–4.41 (m, 1H, H-3′), 5.14–5.25 (m, 3H, allyl and CH), 5.64 (m, 1H, allyl), 6.26–6.31 (t, 1H, H-1′, J = 6.9 Hz), 7.89 (d, 1H, H-6, J = 1.2 Hz); ^{13}C NMR (CD_3OD): δ 13.23, 27.21, 35.62, 40.08, 41.34, 65.56, 62.75, 72.09, 80.03, 81.28, 87.07, 88.85, 110.68, 119.92, 134.31, 136.43, 152.28, 165.34; MS (HR-ESI) $C_{18}H_{34}B_{10}N_2O_6Na$ $(M+Na)^+$ calcd: 505.3327, found: 505.3326 (3.1 ppm).

4.2.16. 3-{1-[12-(1,4-Dihydroxy-but-2-en-1-yl)carboran-1-yl]-propyl}thymidine (14a). Compound **13a** (70 mg, 0.093 mmol) was dissolved in THF (5 mL) and TBAF (1.0 M in THF, 0.144 mL, 0.093 mmol) was added dropwise at −78 °C. The reaction mixture was allowed to warm up to room temperature and stirred for 2 h, quenched with water, and extracted with diethyl ether (3× 20 mL). The combined organic layers were washed with water, brine and dried over $MgSO_4$. After filtration and evaporation, the residue was dissolved in MeOH (5 mL) and anhydrous K_2CO_3 (13 mg, 0.090 mmol) was added. The reaction mixture was stirred at room temperature for 12 h. The product was extracted in ethyl acetate (2× 20 mL), washed with water, brine, and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by column chromatography on silica gel (dichloromethane/acetone, 4:1) to give the compound **14a** in 53% (25 mg) yield. R_f: 0.35; ^{1}H NMR (CD_3OD): δ 1.35–1.43 (m, 2H, CH_2), 1.56–1.65 (m, 2H, CH_2), 1.81 (s, 3H, CH_3), 1.97–2.17 (m, 1H, H-2′), 3.62–3.71 (m, 4H, H-5′ and CH_2), 3.80–3.85 (m, 1H, H-4′), 3.93–4.10 (m, 3H, CH_2N, CH), 4.30–4.31 (m, 1H, H-3′), 5.32–5.35 (d, 1H, J = 15.1 Hz, CH), 5.47–5.55 (m, 1H, CH), 6.20 (t, 1H, H-1′, J = 6.65 Hz), 7.74 (s, 1H, H-6); ^{13}C NMR ($CDCl_3$): δ 13.14, 28.72, 36.32, 41.31, 41.56, 62.46, 62.75, 72.06, 74.42, 82.35, 85.31, 87.12, 88.88, 110.65, 130.88, 133.12, 136.58, 152.20, 165.29; MS (HR-ESI) $C_{19}H_{36}B_{10}N_2O_7Na$ $(M+Na)^+$ calcd: 537.3351, found: 537.3350 (0.2 ppm).

4.2.17. 3-{1-[12-(1,2,3,4-Tetrahydroxybutyl)carboran-1-yl]-propyl}thymidine (15a). To a solution of the compound **14a** (35 mg, 0.068 mmol) in acetone/water (30:1,

5 mL) were added *N*-methyl morpholine oxide (15 mg, 0.033 mmol) and a solution of OsO_4 in water (0.32 mL, 1 g OsO_4/100 mL water). The reaction mixture was stirred at room temperature for 6 h. An aqueous solution of sodium thiosulfate (20 mg, 0.109 mmol) was used to quench the reaction. The residue was filtered and excess solvent was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (dichloromethane/acetone, 1:1) to give the compound **15a** in 53% (19 mg) yield. R_f: 0.40; HPLC retention time (semipreparative): 54.21 min; ^{1}H NMR (CD_3OD) 1.40–1.50 (m, 2H, CH_2), 1.65–1.71 (m, 2H, CH_2), 1.88 (d, 3H, CH_3, J = 1.2 Hz), 2.12–2.30 (m, 2H, H-2′), 3.24–3.31 (m, 2H, CH), 3.47 (t, 2H, CH_2, J = 6.5 Hz), 3.68–3.76 (m, 3H, CH and H-5′), 3.79–3.90 (H-4′ and CH_2N), 4.21 (m, 1H, H-3′), 6.16 (t, 1H, H-1′, J = 6.82 Hz), 7.81 (d, 1H, H-6, J = 1.2 Hz); ^{13}C NMR (CD_3OD): 13.14, 28.70, 36.29, 41.29, 41.56, 62.73, 63.74, 72.05, 72.41, 72.66, 75.17, 82.35, 85.31, 87.10, 88.87, 110.65, 136.58, 152.20, 165.28; MS (HR-ESI) $C_{19}H_{38}B_{10}N_2O_9Na$ $(M+Na)^+$ calcd: 569.3489, found: 569.3480, $C_{19}H_{37}B_{10}N_2O_9$ $(M-H)^-$ calcd: 545.3518, found: 545.3530 (1.6 ppm).

4.2.18. 3-{1-[12-(1,2,3-Trihydroxypropyl)carboran-1-yl]-propyl}thymidine (15b). Compound **13b** (30 mg, 0.066 mmol) was dissolved in MeOH (5 mL) and anhydrous K_2CO_3 (13 mg, 0.090 mmol) was added. The reaction mixture was stirred at room temperature for 12 h. The residue was extracted in ethyl acetate (2× 20 mL), washed with water and brine, and dried over $MgSO_4$. After filtration and evaporation, the residue was treated with OsO_4 and *N*-methyl morpholine oxide (15 mg, 0.033 mmol) as described above for the reaction with **14a** to yield compound **15b** in 42% (14 mg) yield. R_f: 0.50 (methanol/dichloromethane, 1:5); HPLC retention time (semipreparative): 52.34 min; ^{1}H NMR (CD_3OD) δ 1.40–1.50 (m, 2H, CH_2), 1.68–1.73 (m, 2H, CH_2), 1.90 (d, 3H, CH_3, J = 1.2 Hz), 3.25–3.30 (m, 1H, CH), 3.40 (t, 2H, CH_2, J = 6.9 Hz), 3.71–3.93 (m, 6H, H-4′, H-5′, CH_2N, and CH), 4.36–4.41 (m, 1H, H-3′), 6.30 (t, 1H, H-1′, J = 6.9 Hz), 7.82 (d, 1H, H-6, J = 1.2 Hz); ^{13}C NMR (CD_3OD) δ 12.15, 23.22, 27.71, 41.34, 41.92, 61.74, 64.31, 71.00, 71.05, 71.84, 86.11, 87.86, 109.66, 136.47, 151.20, 164.28; MS (HR-ESI) $C_{18}H_{36}B_{10}N_2O_8Na$ $(M+Na)^+$ calcd: 541.3300, found: 541.3326 (4.8 ppm).

4.2.19. Di-1,12-[5-(4-toluenesulfonyloxy)pentan-1-yl]carborane (16a). To a solution of *p*-carborane (**1**) (170 mg, 1.20 mmol) in THF (10 mL) was added *n*-butyl lithium (1.0 mL, 2.50 mmol, 2.5 M in hexanes) at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then slowly added to a solution of 1,5-pentanediol di-*p*-tosylate[18] (990 mg, 2.4 mmol) in THF (10 mL) at 0 °C. The resulting reaction mixture was stirred for 4 h at room temperature, distilled water (5 mL) was added to quench the reaction. The reaction mixture was extracted with ethyl acetate (30 mL) and the organic layer was washed with brine and water, and was dried over $MgSO_4$, After filtration and evaporation, the residue was purified by column chromatography on silica gel (hexanes/ethyl acetate, 3:1) to give the compound **16a** in 40% (600 mg) yield. R_f 0.30; ^{1}H NMR ($CDCl_3$) δ 0.98–1.07 (m, 4H, CH_2), 1.45–1.50 (m, 12H, CH_2), 2.42 (s, 6H, CH_3), 3.95 (t, 4H, CH_2, J = 6.3 Hz), 7.35 (d 4H, ArH, J = 8.1 Hz), 7.77 (d, 4H, ArH, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$) δ 22.05, 25.23, 28.76, 29.06, 30.11, 37.81, 53.83, 70.48, 128.25, 130.25, 133.50, 145.16; MS (HR-ESI) $C_{26}H_{44}B_{10}O_6S_2Na$ $(M+Na)^+$ calcd: 647.3494, found: 647.3493 (0.2 ppm).

4.2.20. Di-1,12-(ethyloxyethyltosylate)carborane (16b). Experimental conditions for the synthesis of **16a** were adapted for the synthesis of **16b**. Treatment of the *p*-carborane (**1**) with *n*-butyl lithium and di(ethylene glycol) di-*p*-tosylate[18] gave the compound **16b** in 59% (890 mg) yield. R_f: 0.50 (hexanes/ethyl acetate, 1:1); ^{1}H NMR ($CDCl_3$) δ 1.79 (t, 4H, CH_2, J = 7.2 Hz), 2.47 (s, 6H, CH_3), 3.13 (t, 4H, CH_2, J = 7.2 Hz), 3.51 (t, 4H, CH_2, J = 4.8 Hz), 4.11 (t, 4H, CH_2, J = 4.8 Hz), 7.35 (d, 4H, ArH, J = 8.1 Hz), 7.79 (d, 4H, ArH, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$) δ 21.63, 36.69, 68.02, 69.02, 69.39, 76.12, 127.90, 129.78, 132.92, 144.79; MS (HR-ESI) $C_{24}H_{40}B_{10}O_8S_2Na$ $(M+Na)^+$ calcd: 651.3079, found: 651.3051 (4.3 ppm).

4.2.21. Di-1,12-[5-(*N*-3-thymidyl)pentan-1-yl]carborane (17a). To a solution of the compound **16a** (150 mg, 0.24 mmol) in dimethylformamide/acetone (1:1, v/v, 4 mL) were added thymidine (180 mg, 4.80 mmol) and K_2CO_3 (663 mg, 4.80 mmol). The reaction mixture was stirred at 60 °C for 48 h. Excess solvent was removed under reduced pressure and the resulting residue was purified by column chromatography on silica gel (dichloromethane/MeOH, 12:1.6) to give the compound **17a** in 70% (130 mg) yield. R_f: 0.79; HPLC retention time (semipreparative): 78.85 min; ^{1}H NMR (CD_3OD) δ 1.12–1.16 (m, 8H, CH_2), 1.47–1.55 (m, 4H, CH_2), 1.69–1.79 (m, 4H, CH_2), 1.92 (s, 6H, CH_3), 2.17–2.27 (m, 4H, H-2′), 3.75–3.93 (m, 10H, H-4′, H-5′ and CH_2N), 4.37–4.43 (m, 2H, H-3′), 6.31 (t, 2H, H-1′, J = 6.4 Hz), 7.85 (s, 2H, H-6); ^{13}C NMR (CD_3OD) δ 12.18, 26.33, 27.01, 29.30, 37.69, 40.33, 40.99, 56.47, 61.75, 71.09, 86.09, 87.86, 109.67, 135.44, 151.27, 164.38; MS (HR-ESI) $C_{32}H_{56}B_{10}N_4O_{10}Na$ $(M+Na)^+$ calcd: 789.4825, found: 789.4846 (2.6 ppm).

4.2.22. Di-1,12-[ethyloxyethyl-(*N*-3-thymidyl)]carborane (17b). Experimental conditions for the synthesis of **17a** were adapted for the synthesis of **17b**. Treatment of **16b** (150 mg, 0.24 mmol) with thymidine and K_2CO_3 gave **17b** in 72% (130 mg) yield. R_f: 0.76 (dichloromethane/MeOH, 12:1.6) HPLC retention time (semipreparative): 89.12 min; ^{1}H NMR (CD_3OD) δ 1.82 (t, 4H, CH_2, J = 7.2 Hz), 1.96 (s, 6H, CH_3), 2.25–2.31 (m, 4H, H-2′), 3.18 (t, 4H, CH_2, J = 7.2 Hz), 3.54 (t, 4H, CH_2, J = 4.8 Hz), 3.67–3.96 (m, 6H, H-4′ and H-5′), 4.13 (t, 4H, CH_2, J = 4.8 Hz), 4.39–4.45 (m, 2H, H-3′), 6.34 (t, 2H, H-1′, J = 6.4 Hz), 7.91 (s, 2H, H-6); ^{13}C NMR (CD_3OD) δ 12.38, 37.45, 40.32, 40.54, 61.80, 66.81, 69.00, 71.15, 77.37, 86.17, 87.86, 109.76, 135.69, 151.41, 164.51; MS (HR-ESI) $C_{30}H_{52}B_{10}N_4O_{12}$ Na $(M+Na)^+$ calcd: 792.4471, found: 792.4537 (8.2 ppm).

4.3. Computational studies

4.3.1. Homology modeling. The initial coordinates of the hTK1 homology model were constructed with the SWISS-MODEL (Version 36.0003) using *C. acetobutylicum* thymidine kinase (PDB ID: 1XX6) as the template. Obtained coordinates for the hTK1 homology model were transferred into Sybyl 7.1. (Tripos Inc, St. Louis, Missouri), installed on a Silicon Graphics O_2 computer, and hydrogen atoms were added. The hydrogen atom positions were minimized until an rms of 0.005 kcal mol^{-1} $Å^{-1}$ was reached using the Powell method. The homology model system was solvated with water molecules using solvent/solvate option. The solvated homology model was minimized until the gradient reached 0.05 kcal mol^{-1} $Å^{-1}$ using Tripos force field.

4.3.2. Docking studies. Energy minimizations of a single arbitrarily chosen stereoisomer of compound **7**, 3-[2(*R*),3(*S*),4(*S*)-trihydroxy-4-(*p*-carboranyl-1-yl)butyl]thymidine were performed with a Dell Inspiron 4100 using HyperChem™ 7.51 for windows (Hypercube, Inc, Gainsville, Florida). Because energy minimization (MM2) of compounds containing hexavalent boron cannot be performed with Sybyl,[34,35] compound **7** was constructed with HyperChem and minimized with the MM+ method until an energy gradient of 0.03 was reached (Polak–Ribiere algorithm was used). The structure was saved in mol2 file format and transferred into Sybyl 7.1. Atom and bond types of the compound were manually corrected to adjust their types for docking studies in Sybyl 7.1. Specifically, the atom types of the ten boron atoms in the *p*-carborane cage were modified into C.3 atom types, as described previously by us.[34,35] For docking studies, the active site was generated by selecting the same amino acid residues that are located within a radius of 12.0 Å from TTP in the hTK1 crystal structure (PDB ID: 1W4R). Docking of compound **7** into the active site was performed with the FlexX module in Sybyl 7.1.

4.4. Physicochemical parameters

4.4.1. Calculation of apolar surface areas (APSAs) and polar surface areas (PSAs). Compounds **7**, **15a**, and **15b** were built and minimized with molecular mechanics force field and a gradient of 0.03 kcal mol^{-1} $Å^{-1}$ using HyperChem™ 7.51. The parameters were then transferred to the VEGA ZZ 2.0.4 software package[43] for the calculation of PSAs and APSAs. For reasons indicated in Section 2.4, boron atom types in the carborane clusters of 3CTAs were replaced with C.3 atom types.

4.4.2. Calculation of log *P* values. log *P* calculations were carried out using ChemDraw Ultra7.0.1 (CambridgeSoft Corporation, 100 Cambridgepark Drive, Cambridge, MA). ChemDraw Ultra 7.0.1 is not suitable for calculating log *P* values of compounds containing hexavalent carbon and boron atoms, as they can be found in carborane clusters. Therefore, the carborane clusters in 3CTAs were replaced with adamantane because both are comparable in lipophilicity.[44] In order to mimic the 1,12-substitution pattern of *p*-carborane in compounds **6**, **15a**, and **15b**, the 1,4(6,10)-positions of the adamantane were substituted appropriately. This approach was validated by a comparison of the experimental log *P*s of a series of 12 3CTAs[3] with the calculated log *P*s of their virtual adamantane counterparts (data not shown). The obtained R^2 value of 0.92 indicated that the approximation of using adamantane instead of carborane for the theoretical calculation of the log *P*s of 3CTAs with ChemDraw Ultra 7.0.1 is acceptable.

4.5. Enzymatic studies

4.5.1. Spectrophotometric determination of relative phosphorylation rates (rPRs) and k_{cat}/K_M values. Enzyme assays with purified recombinant hTK1 were carried out adapting a previously described procedure.[45] Briefly, 3CTAs were dissolved in DMSO to produce stock solutions of various concentrations (30–140 μM). Three CTA activities in hTK1 were determined by adenosine diphosphate (ADP) production from adenosine triphosphate (ATP), measured by the change in absorbance at 340 nm that is caused by NADH oxidation in a coupled enzyme system with pyruvate kinase and lactate dehydrogenase. The standard reaction mixture contained 50 mM Tris–HCl, pH 7.6, 2 mM $MgCl_2$, 0.5 mM ATP, 5 mM DTT, 1 mM phosphoenolpyruvate, 0.5 U/mL pyruvate kinase, 0.5 U/mL lactate dehydrogenase, 0.1 mM NADH, and 600 ng of hTK1 in a total volume of 0.25 mL. For the determination of rPRs, a fixed concentration of 40 μM 3CTA was used while for the determination of k_{cat}/K_M values, a concentration range of 2–100 μM 3CTAs was used. The final concentrations of DMSO were ⩽1%. The reaction was performed at 37 °C with a Cary 3 spectrophotometer (Varian Techtron, Mulgrave, Australia) and started by the addition of Thd or 3CTA. The kinetic parameters are calculated from the slope of the absorbance graph and further with the SigmaPot Enzyme Kinetic Module v 2.1. The activity of hTK1 with 20 μM Thd was 640 nmol thymidine monophosphate (TMP) formed per minute and mg hTK1 protein.

4.5.2. Phosphorylation transfer assay (PTA). In order to obtain autoradiograms demonstrating the biosynthesis of 3CTA-5′-monophosphates catalyzed by hTK1, PTAs were carried out as described previously with minor modifications.[16] Briefly, 3CTAs were dissolved in DMSO as described in Section **4.5.1**. The PTA mixtures contained 100 μM (**7**, **15a**, **15b**, **17a**, and **17b**) or 40 μM (**17a** and **17b**) 3CTA 100 μM ATP (with 0.05 μM [γ-^{32}P]-ATP (10 μCi/μL), Amersham Pharmacia Biotech, IL, USA), 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 125 mM KCl, 10 mM DTT, and 0.5 mg/mL bovine serum albumin (BSA). The final concentrations of DMSO were ⩽1%. The reaction mixtures were incubated at 37 °C for 20 min in presence of 50 ng of enzyme. Subsequently, the enzyme was heat inactivated for 2 min at 95 °C. The reaction mixtures were centrifuged and 1μL sample portions were spotted on PEI-cellulose TLC plates (Merck, USA). The TLC plates were placed for 8–12 h in a mixture of isobutyric acid/ammonium hydroxide/water (66:1:33) for development. The products of the kinase reaction were detected by autoradiography during 1–2 h exposure on a phosphor

imaging plate (BAS cassette 2040, FUJIFILM) and visualized by the phospho-imaging system, Fuji BAS 2500/LAS 1000 (FUJIFILM, I&I—imaging and information), with an Image Reader V 1.7E.

4.5.3. Determination of the IC_{50} value of compound 7. This experiment was carried out according to a procedure previously described by us.[3] Briefly, the reaction was performed in an assay mixture containing 50 mmol/L Tris–HCl (pH 7.6), 2 mmol/L DTT, 5 mmol/L $MgCl_2$, 10 mmol/L NaF, 5 mmol/L ATP, 0.5 mg/mL BSA, 5 μmol/L [methyl-^{3}H]dThd, and purified recombinant hTK1 (5 ng). The samples were incubated at 37 °C in the absence and presence of 10, 40, 80, and 160 μM of compound **7**. The DMSO concentrations were kept at 1%. Aliquots of 10 μL of each of the reaction mixtures were spotted onto DE-81 filter paper (Millipore, Billerica, MA) at time intervals of 0, 10, 20, and 30 min. The filters were washed three times with 5 mmol/L ammonium formate, 5 min for each wash, and the radioactivity was determined by γ-scintillation counting. The compound concentrations resulting in 50% inhibition of enzyme activity (IC_{50}) were determined by using the equation $v_I = v_0/(1 + [I]/IC_{50})$.

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