Nucleoside and Oligonucleoside Boranophosphates: Chemistry and Properties

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1. Introduction

In recent years, there has been increasing interest in boron-containing compounds due to their potential medicinal and biochemical applications.[1−5] Although boron compounds have been used for several decades as pharmacological agents in boron neutron capture therapy (BNCT) to treat cancers,[6−8] recent developments in boron chemistry and pharmacology provided significant progress in designing new organoboron compounds which exhibit hypolipidemic,[9−11] anti-neoplastic,[12,13] anti-inflammatory,[13−15] anti-osteoporotic,[15−17] and analgesic[9] properties. This review will be limited to compounds of nucleoside and oligonucleoside boranophosphates, with the focus on their syntheses. Some of their properties and applications will also be discussed. To our knowledge, this review gives the most comprehensive overview of boranophosphate chemistry spanning from its invention in the early 1990s to date.

The discovery of borane-modified phosphate analogues opened a new chapter in nucleotide and nucleic acid chemistry.[18−23] Nucleoside boranophosphates (Figure 1), a new class of boron-containing nucleotide analogues, were first reported by the Shaw lab in the early 1990s.[24,25] These compounds contain a borane (BH_3) moiety in place of one of the nonbridging phosphate oxygens, resulting in a hydrolytically stable analogue of the phosphodiester linkage. This modification imparts unique characteristics to boranophosphate nucleotides and nucleic acids.[18−23] A boranophosphate is isosteric to the neutral methylphosphonate group but, like the phosphate and phosphorothioate groups, retains an identical negative charge. Furthermore, the borane group is isoelectronic with the oxygen occurring in a phosphate and pseudoisoelectronic with the sulfur found in a phosphorothioate. Although boranophosphates possess high water solubility, they are more lipophilic than phosphates.[26−29] In addition, a boranophosphate diester linkage in DNA is very stable to degradation by various nucleases.[25,30−32] Considering these properties, boranophosphates are promising candidates

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Zinaida Sergueeva was born in Novosibirsk, Russia, in 1965. She received a B.S. degree in Chemistry from Novosibirsk State University in 1987 along with the Mendeleev Society Student Award. Then she joined Novosibirsk Institute of Bioorganic Chemistry, Russian Academy of Sciences, and studied chemical synthesis and chemical modification of DNA and RNA under the direction of professor Valentina Zarytova. She was the recipient of the Scholarship for Young Scientists to Study Abroad in 1987 and 1994. Upon completion of her Ph.D. in 1997, she pursued her postdoctoral research in the laboratory of Barbara Ramsay Shaw at Duke University, investigating the synthesis and properties of oligonucleotide boranophosphates. Currently she is a research fellow at Merck.

for exploring new types of modified nucleotide and polynucleotide analogues.

Deoxyribo- and ribonucleoside 5′-(α-*P*-borano)triphosphates (dNTPαBs and rNTPαBs), shown in Figure 2, are good substrates for the DNA and RNA polymerase enzymes that carry out the syntheses of DNA and RNA.[30,32−38] This very important feature finds a number of interesting applications. It makes possible the enzymatic synthesis of oligonucleoside boranophosphates with partial or full substitution of internucleotidic phosphate linkages.[32,34−38] The high stability of a single boranophosphate internucleotidic linkage toward nuclease hydrolysis in such oligomers was successfully exploited in a one-step PCR sequencing method.[32] The presence of an α-*P*-BH_3 group in boranodi- and boranotriphosphate derivatives of 3′-azido-2′,3′-dideoxythymidine (AZT) and d4T (2′,3′-didehydro-2′,3′-dideoxythymidine) was

Mikhail I. Dobrikov was born in 1957 in Barnaul, Russia and currently is a senior research associate scholar at Duke University. He received his M.S. in 1980 and his Ph.D. in 1990 in Bioorganic Chemistry and Chemistry of Natural Compounds from Novosibirsk Institute of Bioorganic Chemistry, Russia, under the supervision of Professor Gennadi Shishkin. His graduate work at the Novosibirsk State University, Russia, focused on the fundamentals of biopolymers' photoimmobilization and enzymatic development of the latent images for registration of optical information. In 1990, he worked as a postdoctoral research scientist with Professor Gennadi Shishkin (Novosibirsk Institute of Bioorganic Chemistry) in the synthesis and investigation of the chemical and biochemical properties of new heterobifunctional cross-linking reagents. After completing his postdoctoral assignment, he continued his research as a senior research scientist in Novosibirsk Institute of Bioorganic Chemistry. His work has involved development of binary systems of oligonucleotide conjugates, sensitive to visible light, for sensitized photomodification of nucleic acids. He joined Professor Barbara Ramsay Shaw's laboratory at Duke University in 1999 and has been involved in the synthesis and biochemical evaluation of nucleoside boranophosphates as a novel type of antiviral drug. He has published several patents and reviews, and numerous journal articles.

Barbara Ramsay Shaw is the William T. Miller distinguished Professor of Chemistry at Duke University. After obtaining her B.S. in Chemistry from Bryn Mawr College, she received her Ph.D. in Chemistry from University of Washington at Seattle in 1973, under the supervision of Professors J. Michael Schurr and Walter Kauzmann (at Princeton). Then she spent two years as a postdoctoral fellow at Oregon State University with Prof. K. E. Van Holde. She joined the Department of Chemistry at Duke University in 1975 and was promoted to a Full Professor in 1992. She is also a member of numerous university programs and centers including the Genetics Program; Integrated Toxicology Program; Developmental, Cell, and Molecular Biology Program; Pharmacology Program; Molecular Biophysics Program; Center for RNA Biology; and Duke Cancer Center. Her current interests mainly focus on the boronated nucleic acids and their applications as antisense and RNAi agents and as antiviral and anticancer drugs. She has published more than 130 scholarly papers.

demonstrated to improve both phosphorylation by nucleoside diphosphate kinase (NDPK) and inhibition of viral reverse transcription,[39,40] while the repair of viral DNA by pyro-

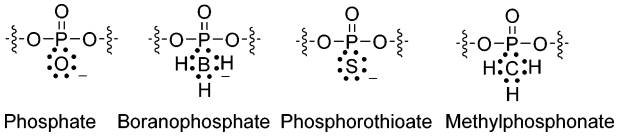


Figure 1. Phosphate and its isolobal analogues

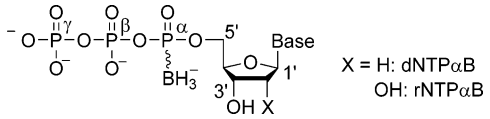


Figure 2. Structures of deoxyribo- and ribonucleoside 5′-(α-*P*-borano)triphosphates.

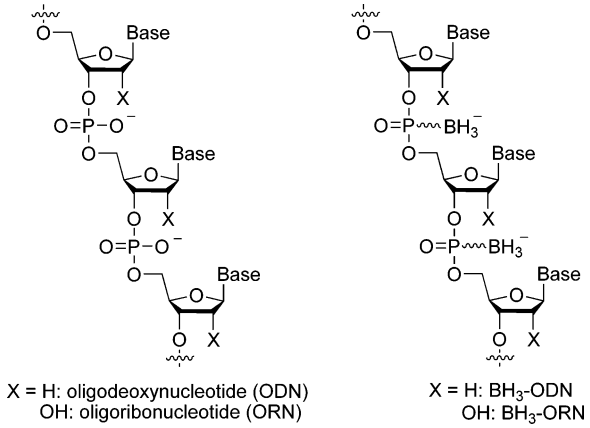


Figure 3. Structures of oligonucleotides and their corresponding boranophosphate DNA and RNA analogues.

phosphorolysis was significantly reduced compared with its parent nucleotide.[40−46] All of these results make α-*P*-BH_3-modified nucleotide analogues promising candidates for targeting viral infections[47−49] and acting as prodrugs.[47,50−55] Moreover, rNTPαBs recently were shown to be compatible with the systematic evolution of ligands by the exponential enrichment (SELEX) procedure and were used for creation of boron-containing aptamers.[36]

Oligonucleoside boranophosphates (BH_3-ODN and BH_3-ORN in Figure 3) are one of the most recently developed types of oligonucleotide analogues designed as potential antisense/antigene agents[37,56−59] and have been used in the RNA interference (RNAi) strategy.[35,38] These DNA and RNA analogues, when compared with their phosphorothioate counterparts, demonstrated an increased ability to activate RNase H-mediated cleavage of complementary RNA[21,60] and to increase the potency of short interfering RNA (siRNA) as gene silencing agents, respectively.[35,38] Therefore, oligonucleoside boranophosphates possess the essential features potentially useful for clinical use.[61−63] Moreover, the presence of boron atoms in boranophosphate oligomers possessing the ability to specifically inhibit gene expression makes them promising agents for BNCT with potentially enhanced therapeutic efficiency.

2. Structural Features and Properties of Boranophosphates (Borane Phosphonates)

2.1. Electronic Structures

Modifications of the natural phosphate analogues will significantly affect their chemical properties and subsequent biological activities. Factors that can change the chemical properties include the electronic charge, polarity, size, hydrophobicity, basicity, and nucleophilicity of the modified group.

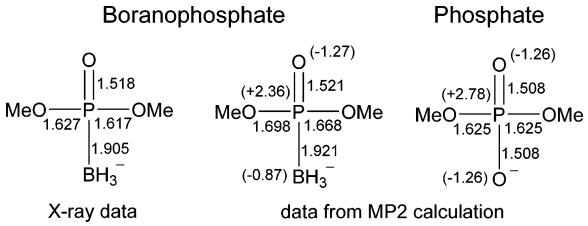


Figure 4. Bond lengths and charge distribution of anionic phosphate and boranophosphate diesters. (Values without parentheses represent bond length in angstroms while those in parentheses represent the atomic charge. Data are taken from refs 64 and 65.)

Compounds that retain the same number of outer shell electrons but are composed of various elements can mimic each other in different ways. For example, the element oxygen has six outer shell valence electrons and is isoelectronic with a BH_3 group, as seen from the Periodic Table. Likewise, the element sulfur is pseudoisoelectronic with oxygen; both have six outer shell valence electrons, but sulfur is in the period below oxygen. Both the boranophosphates and phosphorothioates retain the same negative charge found in natural phosphates. In addition, a methyl group is isosteric with a borane group but results in a neutral phosphate analog. Therefore, boranophosphates can be regarded as mimics of normal phosphodiesters, phosphorothioates, and methylphosphonates.

2.2. Structural Properties

In general, the more a modified phosphate analogue resembles the natural phosphate, the more likely it is to perform like the natural material. Keeping this fact in mind, it is important to examine the following structural properties and their effects on the utility of the compounds.

2.2.1. Bond Length and Charge Distribution

The structure of the boranophosphate diester linkage was studied by *ab initio* calculations on a series of related molecules and ions, including dimethylboranophosphate,[64] and the structures of its diisopropylammonium and potassium salts were determined by single-crystal X-ray diffraction.[64,65] Substitution of diisopropylammonium for potassium has little effect on the uncorrected P−B bond lengths of the anion (1.887 vs 1.895 Å). However, it was found that libration contributed significant systematic error to the crystallographic structure of the diisopropylammonium salt.[64] The P−B bond length predicted by Gaussian 94 calculations (1.921−1.945 Å) for dimethylboranophosphate was somewhat longer than the libration-corrected bond lengths of 1.905 and 1.895 Å found in diisopropylammonium and potassium salt crystals, respectively. The difference between the predicted and observed bond lengths may arise from polarization of the molecule by the cation coordination or hydrogen bonds formed in the crystals; removal of electron density from the phosphoryl oxygen might strengthen the P−B bond at the expense of the P═O bond. This effect was not accounted for in the calculations.[64]

The bonding geometries around phosphorus in crystals of phosphate and boranophosphate diester were found to be tetrahedral, differing mostly in the lengths of the P−O versus P−B bonds (Figure 4). The phosphoryl bond (P═O) in the boranophosphate diester (1.521 Å) is similar to that found in the structures of phosphate diesters (1.47−1.51 Å) and monoesters (1.49−1.53 Å), and it is significantly longer than that seen in phosphate triesters (1.38−1.44 Å).[66]

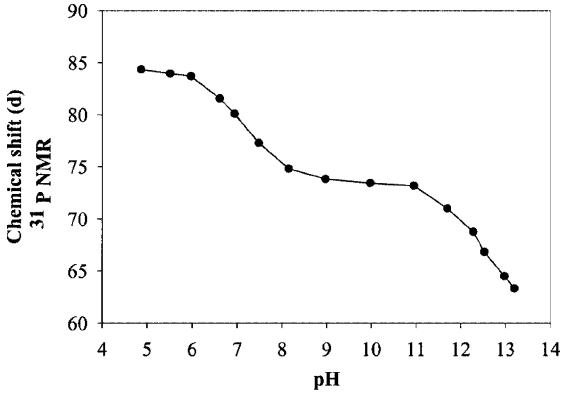


Figure 5. Determination of pK_a values of BPi. (Reprinted with permission from ref 71. Copyright 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.)

Atomic charge data from Gaussian 94 calculations (Figure 4) and NMR data reflect the expected differences in electronegativity of the oxygen and borane moieties (O > BH_3).[64] The phosphorus atomic charge decreases from +2.78 to +2.36 upon replacement of oxygen by a borane, while the magnitude of the substituent charge decreases from −1.26 (oxygen) to −0.87 (BH_3). Consequently, in the boranophosphate, some negative charge is distributed away from the borane and toward the phosphorus, changing the polarity of the phosphate moiety. This charge distribution differs from that of the phosphate, in which the negative charges are distributed equally on and resonate between the two nonbridging oxygen atoms, and in the phosphorothioate, where the negative charge is more localized on the sulfur.[67,68] It is well-known that magnetic resonance frequencies are strongly influenced by the local electron density at observed nuclei. While other factors influence resonance frequencies, the calculated phosphorus atomic charges roughly correlate with ^{31}P NMR data: The 95 ppm resonance of the boranophosphate diester is intermediate between the 140 ppm frequency of the P(III) species, $(MeO)_3P$ (which carries a +1.77 charge on phosphorus), and the −2.4 ppm resonance of the P(V) species $[(MeO)_2PO_2]^-$.[69,70]

2.2.2. Acid and Base Properties

The acid/base character of the inorganic boranophosphate (BPi) has been studied by ^{31}P NMR-monitored pH-titration.[71] Figure 5 shows the ^{31}P NMR chemical shifts of the tributylammonium salt of BPi in H_2O as a function of the pH at room temperature. It was found that the two pK_a values for BPi were 7.12 and 12.54. These values are similar to the values of the second and third protonation equilibria of phosphoric acid (7.21 and 12.67), but they are higher than those for phosphorus acid (1.8 and 6.2).

By comparison, the pK_a values for normal TMP and borano analogue TMPB (nucleoside monophosphates) were 6.63 and 7.06, respectively, as determined by standard pH-titration with NaOH at 22 °C.[29]

2.2.3. Lipophilicity and H-Bonding

The simple replacement of a borane for one of the nonbridging oxygen atoms in a phosphate group imparts a considerable increase in lipophilicity while retaining the overall negative charge on the compound. In partitioning experiments, it was found that negatively charged dithymidine boranophosphate, d(T^{b}pT) (structure **75a** shown in Scheme 32), was 18 times more lipophilic than the parent

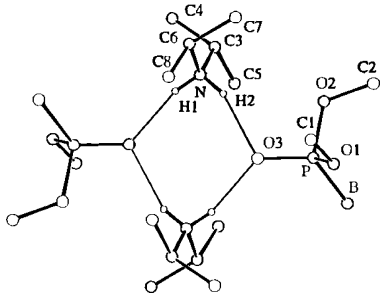


Figure 6. Illustration of the hydrogen-bonding geometry between two $[(MeO)_2P(O)(BH_3)]^-$ diester anions and two $[^iPr_2NH_2]^+$ cations. (Reprinted with permission from ref 64. Copyright 1998 American Chemical Society.)

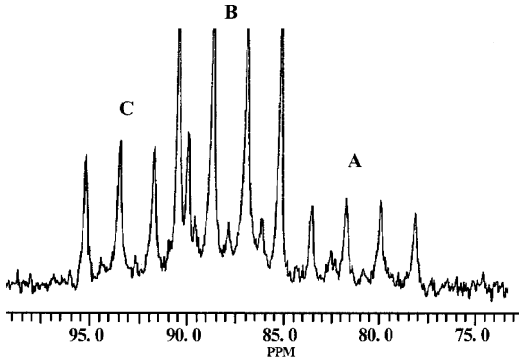


Figure 7. ^{1}H-decoupled ^{31}P NMR spectrum of BPi in MeOH at 81 MHz. (Reprinted with permission from ref 71. Copyright 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.)

dithymidine phosphate, d(TpT).[29,30] Thus, the substitution of a BH_3 group for an oxygen in a d(TpT) molecule (62 atoms) results in a large increase in distribution of this anion from water to the organic phase. Furthermore, boranophosphate dinucleotides consistently have greater retention times on reverse-phase HPLC than do their corresponding phosphorothioate dinucleotides, which in turn have greater retention times than phosphodiester dinucleotides.[28] This suggests that the relative lipophilicity of backbone-modified dinucleotides is boranophosphates > phosphorothioates > phosphates. The same trend should hold for the corresponding DNA and RNA.[27] Thus, boranophosphate analogues may be able to penetrate into cells more readily than do normal phosphates.

X-ray studies, as depicted in Figure 6, demonstrated that the borane group in the diisopropylammonium salt crystal of dimethylboranophosphate is located in a hydrophobic environment and unable to compete with the terminal oxygen to form hydrogen bonds with the acidic protons.[64] This observation is consistent with the observed increase in hydrophobicity of boranophosphate nucleotides relative to normal nucleotides.[27−30] Hence, relative to phosphodiesters and phosphorothioates, the interactions with metal ions, H-bond acceptors, and enzymes, as well as hydration and membrane permeability, are expected to differ for boron-modified nucleotides and polynucleotides.

Recent studies on BPi by ^{31}P NMR have further proved that BH_3 is unable to form hydrogen bonds with acidic protons.[71] Figure 7 shows the ^{31}P NMR spectrum of the tributylammonium salt of BPi in MeOH, which consists of three different but pattern-related signals. Each set of signals has an identical quartet pattern with chemical shifts at δ = 80.0 (A), 86.2 (B), and 90.8 (C) ppm.

Phosphoric acid and its derivatives are known to form H-bonded clusters in solid state,[72] high concentration,[73,74] and

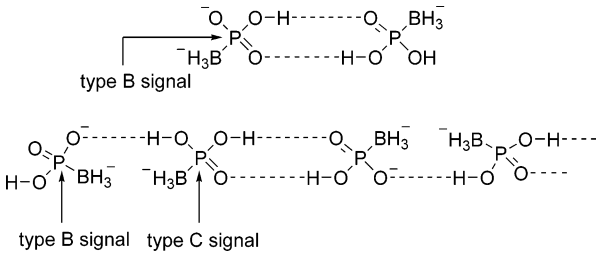


Figure 8. Clusters of BPi formed via hydrogen bonds. (Reprinted with permission from ref 71. Copyright 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.)

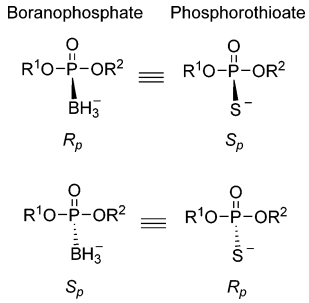


Figure 9. Comparison of the phosphorus configuration assignment between boranophosphates and phosphorothioates.

aprotic organic solvents.[75] Therefore, the assignment of the signals was proposed based on the H-bonding depicted in Figure 8. Signal A, at δ 80.0 ppm, in Figure 7 corresponds to the monomeric BPi; signal B, at δ 86.2 ppm, corresponds to a BPi moiety that has one BPi H-bonded neighbor, which could result both from BPi dimers and from higher clusters; signal C, at δ 90.8 ppm, corresponds to a BPi moiety that has two H-bonded BPi neighbors appearing in trimers and higher clusters. The fact that oxygens in BPi can form hydrogen bonds even in highly polar and H-bonding solvents such as MeOH, whereas the inorganic phosphate forms clusters only in aprotic solvents such as benzene, implies that BH_3 may play a role in the preorganization of BPi clusters. The lipophilic BH_3 moieties possibly form the core of the cluster due to hydrophobic interactions (in MeOH). This core is then further stabilized by P−O$^-$- - -HO−P hydrogen bonds.

2.2.4. Stereochemistry at Phosphorus

Substitution on phosphorus introduces chirality at this position to give R_p and S_p diastereomers. Since the actions of many nucleotide-processing enzymes rely on hydrogen bonding or coordination of metal ions to one of the diastereotopic oxygen atoms, the selective processing of only one isomer of a modified nucleotide by an enzyme is not uncommon.[76−78] For example, R_p and S_p stereoisomers of phosphorothioates differ greatly in their resistance to hydrolysis by phosphodiesterase enzymes.[79] The same is true of boranophosphate-modified nucleotides.[30,31,80,81] Furthermore, the diastereomers of a variety of synthesized nucleoside boranophosphates have been separated by reverse-phase HPLC (RP-HPLC).[26,82−86] It is of note that the R_p isomers of dNTPαB and rNTPαB analogues can be preferentially used as building blocks by a number of DNA and RNA polymerases, resulting in DNA and RNA with S_p boranophosphate oligonucleotide linkages.[27,32,34,35,38,87,88]

It has to be pointed out that the configuration assignment for boranophosphates and phosphorothioates is reversed due to the group priority around the phosphorus atom. As depicted in Figure 9 and assuming that the priority of R^1 is higher than that of R^2, the same spatial orientation of four substituents around phosphorus for boranophosphates and phosphorothioates will result in the opposite configuration assignment, since the priority is increasing in the order of B < O < S. This is important because the relationship between the stereochemistry of phosphorothioates and their biological properties has been well studied.[78,89,90] Thus, it should be possible to derive the P-configurations of boranophosphates by comparison with the corresponding phosphorothioate counterparts based on enzyme−substrate studies.

2.2.5. Chemical Reactivity

Considerable experimental evidence suggests that nucleophilic substitution at phosphorus may proceed via a trigonal bipyramidal pentacoordinate intermediate in which incoming and leaving groups occupy the apical positions.[91,92] The preference of a ligand for an apical position in a pentacoordinate transition state is termed its apicophilicity, which is defined as the difference in energy between two transition states in which the ligand is apical in the first and equatorial in the second.[92] Electronegativity, π-bonding, and steric effects all contribute to apicophilicity. Streitwieser[93] and Thatcher[92] proposed the following relative apicophilicity scale for nucleophilic substitution at phosphorus:

$$CF_3 > CF_2H > CFH_2 > OH > CH_3 > O^- > SH > NH_2 \gg BF_3^- > BH_3^-$$

The relative ordering of empirical apicophilicity largely mirrors the relative electronegativity of the substituent. Due to its lowest electronegativity, the $-BH_3^-$ group has minimal apicophilicity and cannot be easily removed from boranophosphate analogues by nucleophilic substitution. This statement is in accordance with experimental data on the hydrolysis of thymidine boranomonophosphate (see section 3.2),[29,94] which hydrolyzes first into thymidine and BPi (P−OR bond cleavage). The BPi then slowly hydrolyzes to H-phosphonate and boric acid (P−B bond cleavage, Scheme 10).

On the other hand, the $-BH_3^-$ moiety may be seen to resemble the reducing agent borohydride (BH_4^-), but chemically, boranophosphate is very different from borohydride. For example, while hydride transfer from BH_4^- to water occurs readily, the $-BH_3^-$ moiety transfers a hydride only in a highly acidic solution (pH < 2).[71] Similarly, while borane complexes readily reduce nitriles and amides to the corresponding amines,[95−97] the $-BH_3^-$ moiety does not reduce acetonitrile and dimethylformamide (DMF). Moreover, although the P−O$^-$ moiety is a nucleophile, its borane analogue P−BH_3^- is a poor nucleophile.[65] Therefore, as an example, dithymidine boranophosphate, d(T^{b}pT), is stable toward hydrolysis and oxidation, and it does not rapidly react at neutral pH with aldehydes or with hydrogen peroxides.[18,20,29,30]

2.2.6. Low Toxicity

Boron analogues of biomolecules are generally considered to be of low toxicity. The toxicity of boranophosphate oligonucleotides has not yet been determined, but studies of model compounds suggest that it will be low. The deoxynucleoside boranophosphates are minimally toxic to rodents,[98] and the

Scheme 1

$$_5B^{10} + {}_0n^1 \rightarrow [{}_5B^{11}] \rightarrow {}_2He^4\ (\alpha) + {}_3Li^7$$

Table 1. Summary of Hypochromicity Values and Molecular Absorptivities

compd	ϵ_{max} ($\times 10^{-3}$)	λ_{max} (nm)	hypochromicity (%)	stacking interaction	ref
dTMP	9.2	265			23,[a] 100[b]
d(TpT)	9.2	267	8	good	23[a]
d(T^{OMe}pT)	9.1	267	5	good	100[b]
d(T^{OEt}pT)	9.0	267	6	good	100[b]
d(T^{b}pT)	9.1	267	1	fair	23[a]
d($T^{b}p^{OMe}T_{OAc}$)	9.9	267	−7	perturbed	23[a]

[a] In 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5. [b] In H_2O at 28 °C, pH 7.0.

boron degradation products[94] (i.e., borates) of boranophosphate oligonucleotides are minimally toxic to humans.

2.2.7. Boron Neutron Capture Therapy (BNCT)

The boranophosphate offers a unique opportunity to transport boron to tumor cells for selective radiation therapy. BNCT[6,99] is based on the nuclear reaction (Scheme 1) that occurs when ^{10}B, a stable isotope, is irradiated with thermal neutrons to produce ^{11}B in an unstable form, which then undergoes instantaneous nuclear fission to produce a high-energy α particle and a recoiling ^7Li nuclide. These heavily charged particles have path lengths of approximately one cell diameter and deposit most of their energy within the boron-containing cells. If enough low energy thermal neutrons ($_0n^1$) reach the treatment volume and ^{10}B is selectively delivered to tumor cells in amounts higher than in the surrounding normal cells, then the tumor cells can be destroyed as a result of the ^{10}B (n, α) ^7Li capture reaction. In theory, BNCT provides a means for the specific molecular and cellular targeting of high linear energy transfer radiation to tumor cells with the concomitant sparing of normal cells.

2.3. Spectroscopic Properties

Electronic and conformational structures of nucleoside and oligonucleoside boranophosphates can be studied by various spectroscopic techniques, including UV, CD, IR, NMR, and MS spectroscopy.

2.3.1. UV Spectroscopy

In UV studies of d(T^{b}pT) and as shown in Table 1,[23,100] the extinction coefficient (ϵ_{max}) and maximum absorptive wavelength (λ_{max}) were found to be similar to those of the parent compound dithymidine phosphate d(TpT) and its phosphotriester analogues [d(T^{OMe}pT) and d(T^{OEt}pT)]. For all the dimers, the λ_{max} shifted from 265 nm (for the monomer thymidine monophosphate dTMP) to 267 nm; this change might result from the base stacking interactions present in the dimers but not in the monomer dTMP. The values for the hypochromicities of the normal d(TpT) in water and of the two phosphotriester dimers [d(T^{OMe}pT) and d(T^{OEt}pT)] are greater than those for the boranophosphate d(T^{b}pT), which exhibits almost no hypochromicity, and the boranophosphate methyl ester d($T^{b}p^{OMe}T_{OAc}$), which has a negative hypochromicity.

2.3.2. CD Spectroscopy

Experiments on CD spectra of unmodified d(TpT) and diastereomers of modified d(T^{b}pT) showed that their differences were minimal except for some changes in magnitudes.[101] This suggests that borane modification and the chirality of the phosphorus center do not greatly impact the conformations of the dimers. The signs, positions, and magnitudes of the CD bands indicate that both R_p- and S_p-diastereomers of d(T^{b}pT) adopt B-type conformations, the same as that of the unmodified d(TpT) dimer.[102,103] Increasing the temperature led to a reduction in the magnitude of the CD bands, suggesting a decrease in interactions between the two aromatic pyrimidine bases. By comparison, the unmodified d(TpT) showed larger CD intensities (>10%) at a given temperature and a greater change in CD intensity (>7%) between different temperatures than either of the diastereomers of the modified d(T^{b}pT). For the diastereomers of d(T^{b}pT), the S_p-isomer showed an 11% larger intensity than that of the R_p-isomer. All of these results suggest that borane-modified dimers may have different intramolecular interactions than the unmodified dimers and that the configuration of boronated phosphorus exerts a small but noticeable influence on the base stacking interaction. Therefore, the proposed order of increasing ability to adopt the stacked states would be R_p-d(T^{b}pT) < S_p-d(T^{b}pT) < d(TpT).[101]

2.3.3. IR Spectroscopy

Boranophosphates have characteristic bands for P−B and B−H bonds in addition to bands associated with P−OH and P═O bonds. Specifically, three absorptions at 2350, 2381, and 2407 cm^{-1} (s) correspond to B−H stretches,[104] and the absorption at 654 cm^{-1} (m) is the P−B stretch.[71,105] Typical absorptions are observed for P−OH and P═O stretches at 900−1080 cm^{-1} and at 1140−1250 cm^{-1}, respectively.

2.3.4. NMR Spectroscopy

NMR spectroscopy of a boranophosphate analogue is facilitated but also complicated by the presence of the boron that has two stable isotopes, each having an NMR-active nucleus. Naturally abundant boron consists of 19.6% ^{10}B (spin $I = 3$) and 80.4% ^{11}B (spin $I = {}^3/_2$), with the latter possessing superior NMR properties. The ^{11}B NMR chemical shifts cover a broad range of about 250 ppm; their positions depend on the charge, the coordination number, and the substituents at boron. For example, the chemical shift for compounds with the $[O_3P-BH_3]^{3-}$ linkage is ~δ −39.8 ppm when $Et_2O{:}BF_3$ is used as a reference.[94] Thus, ^{11}B NMR spectra can provide important and easily accessible information about the chemical composition of the molecules studied.

When a ^{31}P ($I = {}^1/_2$) atom is bonded to a single ^{11}B atom as in the boranophosphate linkage, four equally spaced and equal intensity lines (a quartet with a 1:1:1:1 pattern) are expected in the ^{31}P NMR spectra. If a ^{31}P atom is coupled to a ^{10}B atom, seven equally spaced and equal intensity lines (a septet with a 1:1:1:1:1:1:1 pattern) are expected. Thus, a P−B bond containing sample with naturally abundant boron can show complicated ^{31}P NMR spectra. The $^{31}P-^{10}B$ coupling constant is about one-third that of the $^{31}P-^{11}B$ coupling constant;[106] hence, the intensity of an individual ^{31}P peak coupled to a ^{10}B is about 14% of the intensity of a ^{31}P peak coupled to a ^{11}B in a naturally abundant boron compound.[107]

As shown in Figure 10A, underlying the quartet (due to ^{31}P coupling with ^{11}B in the ^1H-decoupled ^{31}P NMR spectrum) is a septet due to ^{31}P coupling with ^{10}B at similar chemical shifts. Experimentally, the ^{31}P NMR spectra usually appear as if the boron effects only derive from ^{11}B scalar coupling.[108] For example, ^{31}P spectra have a quartet with a broad 1:1:1:1 pattern at ~δ 80−85 ppm for boranophosphate

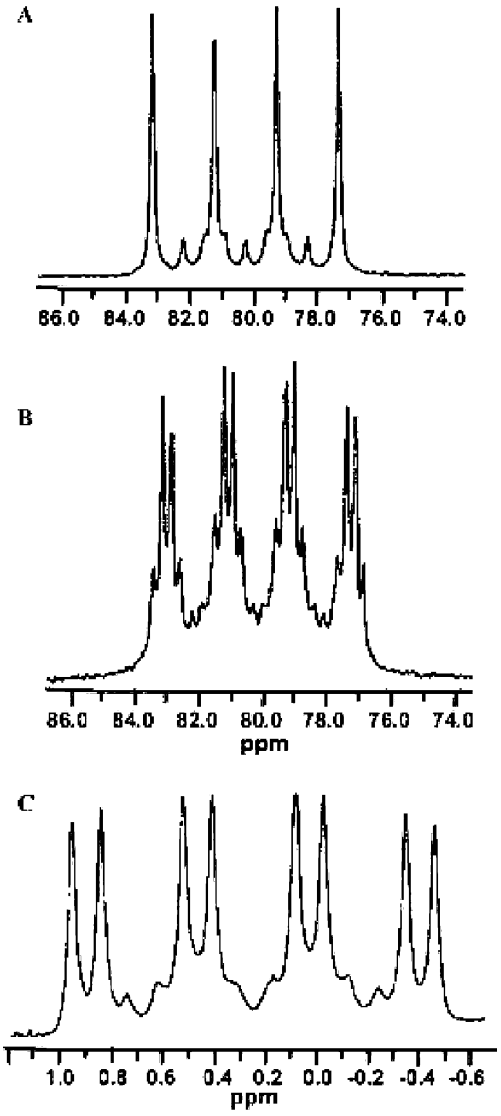


Figure 10. NMR spectra of BPi: (A) ^{1}H-decoupled ^{31}P NMR in D_2O at 81 MHz; (B) ^{1}H-coupled ^{31}P NMR in D_2O at 81 MHz; (C) ^{1}H NMR in D_2O at 200 MHz. (Reprinted with permission from ref 71. Copyright 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.)

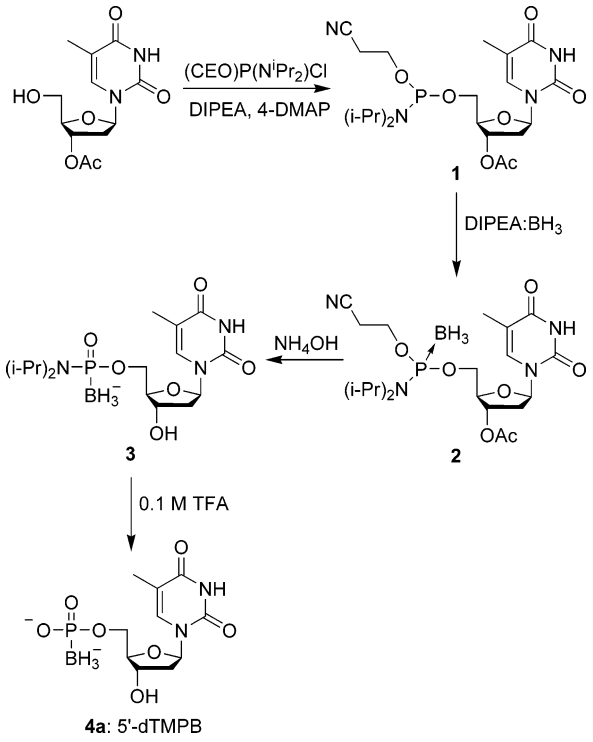
monoesters and at $\sim\delta$ 90−95 ppm for boranophosphate diesters. The ^{1}H-coupled ^{31}P NMR spectrum in Figure 10B shows further splitting of lines into a quartet by three neighboring protons.

The ^{1}H NMR signals from the boranophosphate moiety are located as a very broad 1:1:1:1 quartet, spanning nearly 400 Hz and centered at $\sim\delta$ 0.3 ppm (Figure 10C). The wide breadth of this signal is primarily due to quadrupole relaxation by the ^{11}B nucleus ($I = {}^{3}/_{2}$). In ^{11}B-decoupling experiments, a sharper doublet (due to phosphorus coupling) is observed with a coupling constant $^{2}J_{P-H}$ of 22 Hz.

2.3.5. Mass Spectroscopy

High-resolution mass spectra of all the boranophosphates, due to the approximate 4:1 ratio of ^{11}B:^{10}B, have a characteristic MS signal distribution, where the intensity of the main ^{11}B peak is 4-fold greater than that of the neighboring lower mass ^{10}B isotope peak. One of the abundant characteristic fragment ions is observed from the loss of the borane group,[109] in which the aforementioned signal distribution disappears.

Scheme 2



3. Nucleoside Boranomonophosphates

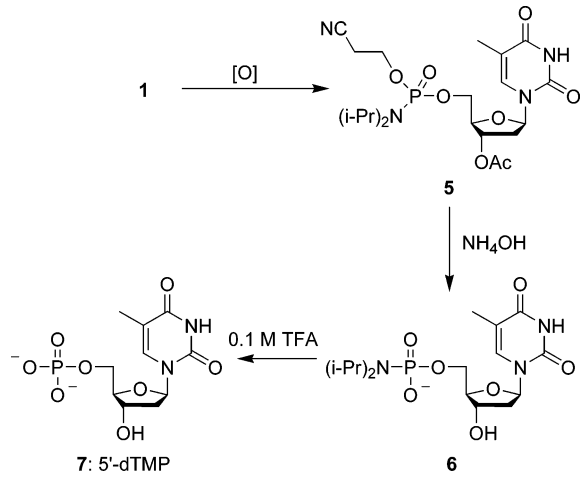
3.1. Synthesis of Nucleoside 2′-, 3′-, and 5′-Boranomonophosphates and Acyclonucleoside Boranomonophosphates

3.1.1. Synthesis via a Phosphoramidite Approach

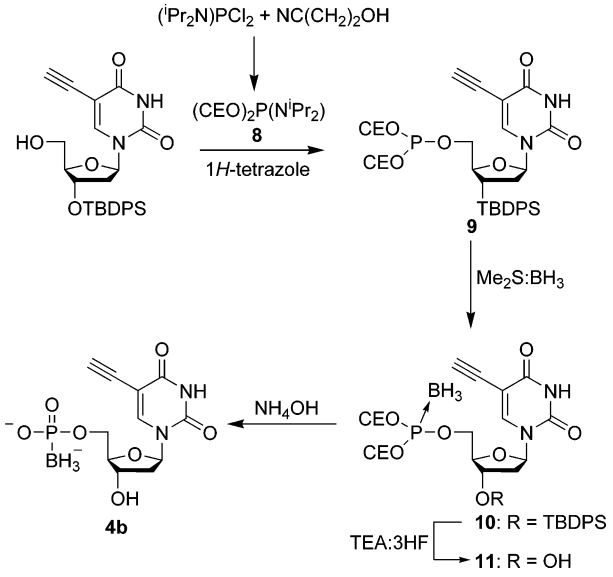
The phosphoramidite approach has been widely used in nucleic acid chemistry, especially in the synthesis of oligonucleotides.[110−112] This approach was applied a decade ago to prepare the first nucleoside 5′-boranomonophosphates (5′-NMPB).[24] The synthesis is described in Scheme 2 and was accomplished through the boronation of thymidine phosphoramidite **1** to form borane complex **2**, which was then converted to boranophosphoramidate **3** via a β-elimination of the cyanoethyl (CE) moiety. Further treatment with trifluoroacetic acid (TFA) resulted in thymidine 5′-boranomonophosphate (5′-dTMPB) **4a**, which was isolated by anion-exchange chromatography on a QA-cellulose $[HCO_3]^-$ column in 52% yield.

A recent study showed that the major byproduct was the parent compound thymidine 5′-monophosphate (5′-dTMP) **7**,[113] which was difficult to remove from 5′-dTMPB by ion-exchange chromatography. The mechanism of its formation is described in Scheme 3. Thymidine 5′-phosphoramidate diester **5** was formed due to dissolved oxygen in the solvents. Sequential treatment of compound **5** with NH_4OH and TFA resulted in the byproduct 5′-dTMP **7**. Thus, careful deoxygenation of solvents could increase the yield of 5′-dTMPB by 20%.[113] It is worth emphasizing that, while separation of phosphoramidate analogues **3** and **6** could be achieved by ion-exchange chromatography, separation of monophosphates 5′-dTMPB **4** and 5′-dTMP **7** had to be carried out by RP-

Scheme 3



Scheme 4



HPLC. The phosphoramidite approach proved to be useful in the preparation of 2′-, 3′-, and 5′-boranomonophosphates and (2-hydroxyethoxy)methyl nucleobase[114] (acyclonucleoside) boranomonophosphates (acycloNMPB).[113]

Recently, we also used the phosphoramidite approach to prepare an anticancer prodrug analogue, 5-ethynyluridine 5′-boranomonophosphate **4b** (Scheme 4),[115] using biscyanoethyldiisopropyl-phosphoramidite **8** produced *in situ* as the phosphitylating reagent. After boronation and removal of the protecting group *tert*-butyldiphenylsilyl (TBDPS), the final compound boranomonophosphate **4b** was obtained in a good yield. This method eliminates the necessity of acid hydrolysis and is advantageous for synthesis of boranophosphates and other modified nucleosides bearing acid-labile groups.

3.1.2. Synthesis via an H-Phosphonate Approach

H-phosphonate chemistry has been well explored in the past decade to develop the protocols for preparation of nucleotide derivatives and oligonucleotides.[116−118] Its applications in the synthesis of boranophosphates serve as an alternative to conveniently obtain this type of compound. It is known that H-phosphonate derivatives in solution exist as an equilibrium mixture of two tautomeric forms, as shown in Figure 11: a tetracoordinate phosphonate form $\lambda^5\sigma^4$ [which resembles P(V) derivatives] and a tricoordinate phosphite form $\lambda^3\sigma^3$ [which is a typical tervalent P(III) derivative].[119]

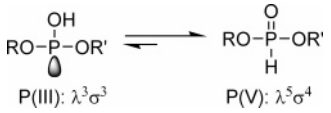


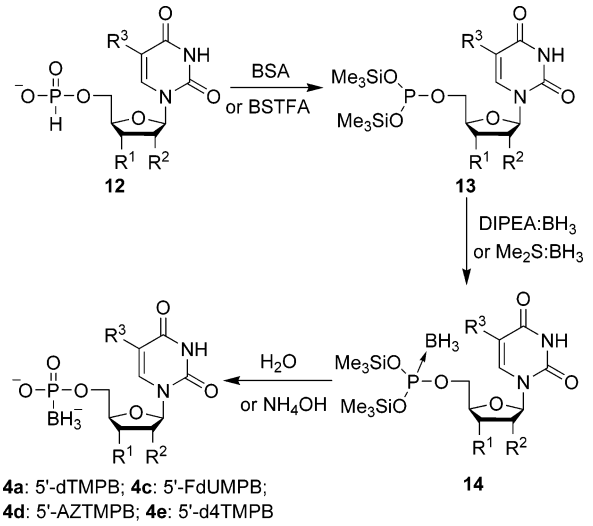
Figure 11. Tautomeric equilibrium of H-phosphonate derivatives.

Since the equilibrium shifts heavily to the phosphonate P(V) form, the phosphorus atom serves as an electrophilic center.[120] However, the equilibrium can be shifted to the P(III) state by fixing the phosphite form with a suitable reagent, thus causing the nucleophilic nature of the phosphorus center to become predominant and act as a electron donor to the BH_3 group. This conversion is readily achieved by several silylating reagents such as *N*,*O*-bis(trimethylsilyl)-acetamide (BSA) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).[37,56,59,121] Therefore, H-phosphonate **12** shown in Scheme 5 was cleanly converted to phosphite **13** in the presence of 5−10 equiv of silylating reagents within 5 min. Subsequent exchange of a borane group between the phosphite triester and boronating reagents produced boranophosphate triester **14**, which, upon water hydrolysis, gave NMPB analogues, specifically, thymidine 5′-boranomonophosphate (5′-dTMPB) **4a**, 5-fluorouridine 5′-boranomonophosphate (5′-FdUMPB) **4c**, 3′-azido-2′,3′-dideoxythymidine 5′-boranomonophosphate (5′-AZTMPB) **4d**, and 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-boranomonophosphate (5′-d4TMPB) **4e** in 65−80% yield.[28,113]

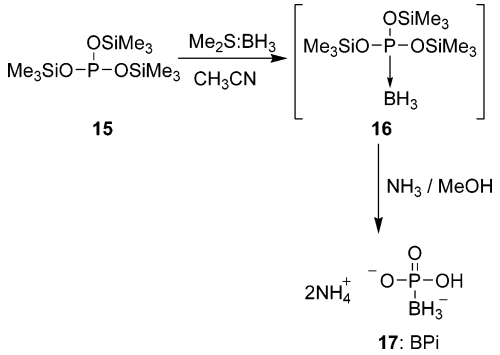
The H-phosphonate approach has served as a protocol for the synthesis of 2′-, 3′-, and 5′-NMPB[28,113] with a variety of nucleosides including 2′,3′-dideoxynucleosides (ddN).[113] Recently, this approach was used to prepare inorganic boranophosphate (BPi)[71] starting from tris(trimethylsilyl) phosphite **15**[122] (Scheme 6). The borane phosphotriester **16** was treated with methanolic ammonia at room temperature for 1 h to give the ammonium salt of BPi as a white solid in 93% yield. No chromatography was required, since all of the impurities could be removed by evaporation. However, it is important to carefully choose the protecting group in the phosphite triester. For example, when the benzyl instead of trimethylsilyl protecting group was used, the removal of the benzyl group by either catalytic hydrogenation or acidic hydrolysis resulted in the cleavage of the P−B bond and formation of phosphorus acid.[71]

However, when the H-phosphonate approach was applied to synthesize acycloNMPB, it was found that the yields of the expected product were extremely low.[113] A mechanism to explain the low yield was proposed as described in Scheme 7.[113] Silylating reagents are typically used in large excess in order to fully convert the H-phosphonate to the phosphite triester. However, in the case of the acyclonucleoside H-phosphonate, a tetrasilylated derivative of acyclothymidine H-phosphonate **18** would form when BSA is in large excess (10 molar equiv). Treatment with a boronating reagent would yield borane complex **19**. Due to the presence of a positive charge on the nitrogen atom at the disilylated nucleobase, the α-carbon in the side chain next to nitrogen is labile to nucleophilic attack. When the nucleophile is water, borane complex **19** would decompose into presumed intermediates **20** and **21**, which are further transformed into thymine and 2-(hydromethoxy)ethoxy boranomonophosphate **22**, respectively. Compound **22** and the desired acycloTMPB are expected to have similar ^{31}P NMR chemical shifts.[113]

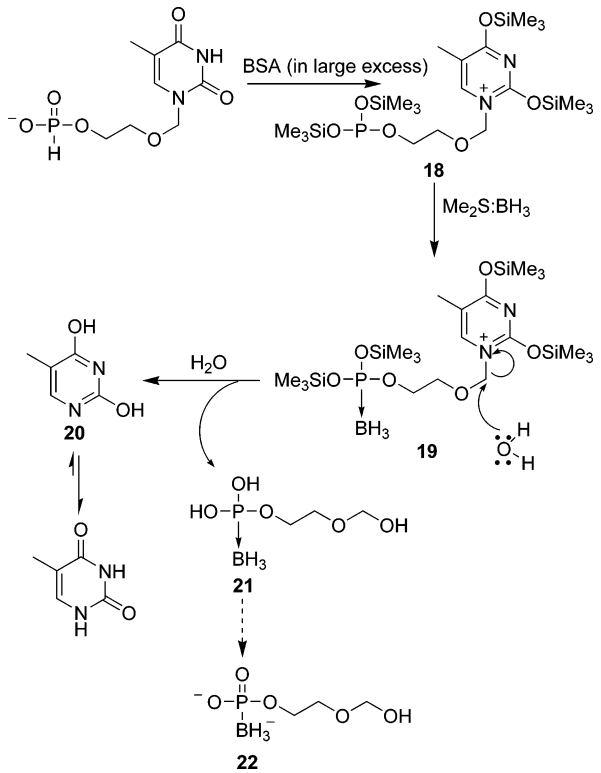
Scheme 5



Scheme 6



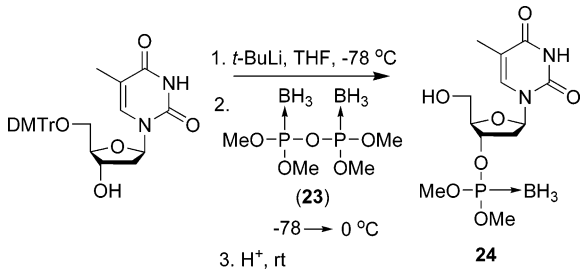
Scheme 7



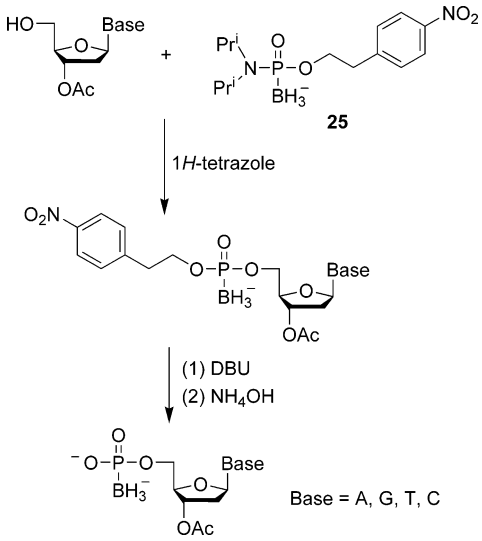
3.1.3. Synthesis via Boranophosphorylating Agents

Synthesis of nucleoside boranophosphates can be achieved not only with the precursor of tricoordinate phosphorus but also with electrophilic boranophosphorylating reagents. Imamoto et al. described a synthesis of thymidine dimethylboranophosphate via a tetramethyl boranopyrophosphate **23** in 1997 as depicted in Scheme 8.[65]

Scheme 8



Scheme 9



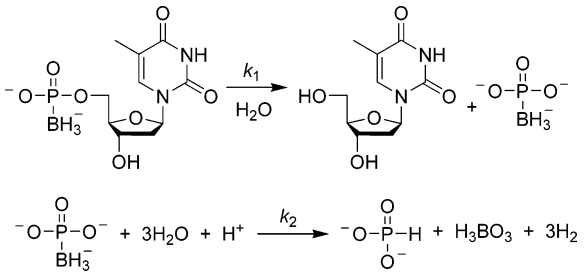
The reaction was carried out through the intermediate activation of 4,4′-dimethoxytrityl (DMTr) protected thymidine with *t*-BuLi in THF at −78 °C. The obtained lithium alkoxide reacted with tetramethyl boranopyrophosphate **23** to give thymidine dimethylborano-phosphate **24** in 70% yield. A similar method has also been used to prepare the nucleoside cyano-, carboxy-, and carbamoyl-substituted boranophosphates.[123] Deprotection of DMT could be accomplished by treatment with acids without the cleavage of the P−B bond, although Caruthers and co-workers reported that the DMTr deprotection procedure was incompatible with the boranophosphate linkage.[56,58] One of disadvantages of this method is that the removal of both methyl groups from **24**, if necessary, cannot be easily achieved.

A new boranophosphorylating reagent, the 2-(4-nitrophenyl)ethyl ester of boranophosphoramidate **25**,[124] was recently developed in the Shaw lab (Scheme 9). It readily reacted with a hydroxyl group on the nucleosides in the presence of 1*H*-tetrazole as a catalyst. The 2-(4-nitrophenyl)ethyl group was removed by 1,4-diazabicyclo[5.4.0]undec-7-ene (DBU) through β-elimination, producing the corresponding NMPB in a good yield.

3.2. Chemical and Enzymatic Stability of 5′-NMPBs

To provide important insights regarding the toxicity of boranophosphate-containing nucleotides as therapeutic agents and their behavior in potential pharmaceutical applications,

Scheme 10



the hydrolysis of 5′-dTMPB in aqueous solution was studied.[94] Unlike many borane-containing compounds, boranophosphate is quite stable in water, and thus, hydrolysis can be followed by ^{1}H, ^{31}P, and ^{11}B NMR spectroscopy and H/D exchange. Shaw et al. demonstrated that 5′-dTMPB slowly decomposes, first by cleaving the P−O bond into thymidine (dT) and inorganic boranophosphate (BPi) ($k_1 = 4 \times 10^{-4}$ s^{-1} at 50 °C in H_2O), followed by an even slower hydrolysis of BPi into phosphonate and boric acid ($k_2 = 2 \times 10^{-5}$ s^{-1} at 50 °C in H_2O) (Scheme 10).[94]

Therefore, the P−B bond in 5′-dTMPB is less susceptible to hydrolysis than the P−O-sugar linkage. This observance is in agreement with *ab initio* SCF theoretical calculations by Thatcher and Campbell.[92] They predicted that the P−O bond would be more labile to hydrolysis than the P−B bond, assuming that phosphate hydrolysis proceeds via a trigonal bipyramidal pentacoordinate intermediate in which incoming and leaving groups occupy the apical positions.[91,125] Although the hydrolysis pathway of 5′-dTMPB is analogous to that of 5′-dTMP, it is significantly more labile than the latter phosphate counterpart. For example, 5′-dTMPB is completely converted to dT and BPi within 22 h at pH 4.8 and 37 °C, while 5′-dTMP is unchanged.[24]

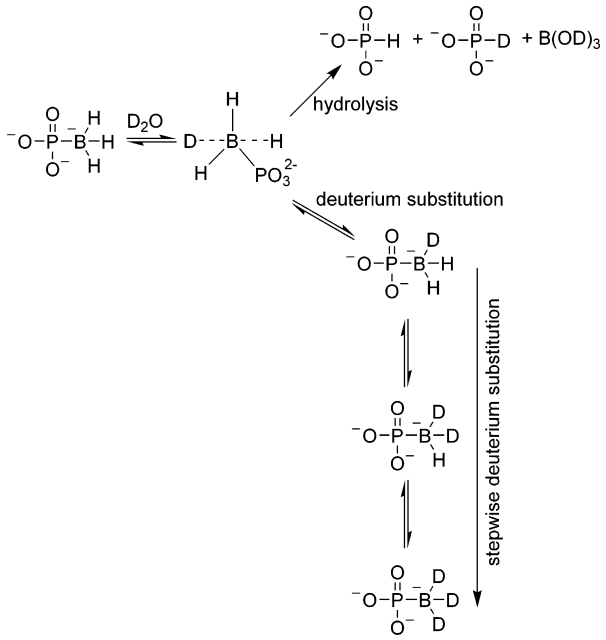
In addition to hydrolysis, the hydrogens in the P−BH_3 linkage of both 5′-dTMPB and BPi exchange with solvent.[94] The exchange process was inferred from the presence of ^{31}P and ^{11}B isotope shifts and changes in coupling patterns involving three-step deuterium substitution of borane hydrogens in D_2O as depicted in Scheme 11. Each deuterium substitution causes a 0.14 ppm downfield shift of the ^{31}P resonance of the P−BH_3 linkage. The deuterium substitution is superimposed upon hydrolysis of partially and fully deuterated boranophosphate intermediates, producing deuterated phosphonate and boric acid in D_2O. At 50 °C, deuterium substitution of $[O_3P-BH_3]^{3-}$ occurs an order of magnitude faster than its hydrolysis.[94]

Acid phosphatase [EC 3.1.3.2] from sweet potato or 5′-nucleotidase [EC 3.1.3.5] from *Crotalus adamanteus* venom completely hydrolyzes 5′-dTMPB to T, but only two times slower than its parent compound 5′-dTMP.[24] On the other hand, for alkaline phosphatase [EC 3.1.3.1] from *Escherichia coli*, 5′-dTMPB is a very poor substrate[24] and may even be an inhibitor like its thio-counterpart thymidine 5′-phosphorothioate.[126]

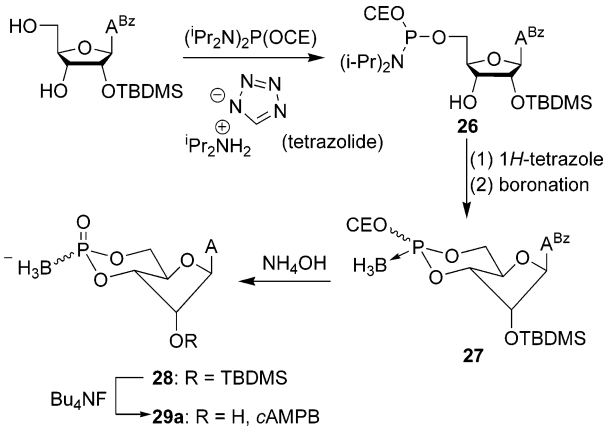
3.3. Synthesis of Nucleoside 2′,3′- and 3′,5′-Cyclic Boranomonophosphates and 3′,5′-Cyclic Boranophosphorothioates

Cyclic phosphate diesters of nucleosides are important intermediates in various biological processes. For example, nucleoside 3′,5′-cyclic monophosphates (*c*NMPs)[127] such as *c*AMP act as mediators of hormone action[128] and modulators

Scheme 11



Scheme 12

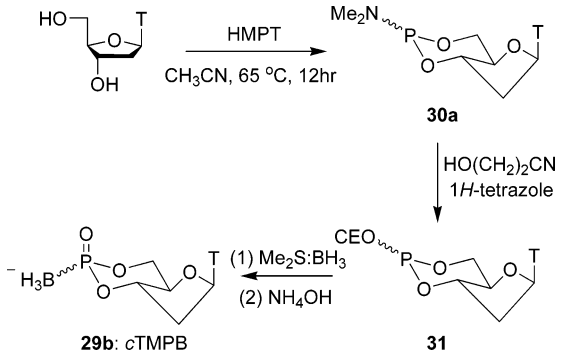


of enzymatic activity;[129] *c*NMPs are key players in coordinated control of glycogen synthesis and breakdown.[130] Nucleoside 2′,3′-cyclic monophosphates (2′,3′-*c*NMPs) are intermediates in the ribonuclease-catalyzed hydrolysis of RNA and are themselves substrates for ribonucleases.[131] To better understand these processes, various modified *c*NMPs[79,132−136] and 2′,3′-*c*NMPs[79] have been prepared to probe the stereochemical and mechanistic aspects of enzymatic reactions and their inhibition. Boranophosphate analogues of nucleoside cyclic monophosphates should provide another useful tool for these purposes. It is noted that 3′,5′-cyclic monophosphate and its borane analogues are abbreviated as *c*NMP and *c*NMPB, respectively, while the 2′,3′-cyclic analogues are abbreviated as 2′,3′-*c*NMP and 2′,3′-*c*NMPB.

3.3.1. Synthesis of Nucleoside 3′,5′-Cyclic Boranomonophosphates (cNMPBs)

Adenosine 3′,5′-cyclic boranomonophosphate (*c*AMPB) **29a** was synthesized via an intramolecular cyclization of phosphoramidite intermediate **26** as depicted in Scheme 12.[86] Phosphitylation of the protected adenosine by 2-cyanoethyl bis(diisopropyl)phosphoramidite ($^{i}Pr_2N)_2P(OCE)$ in the presence of tetrazolide gave phosphoramidite **26**. Addition of

Scheme 13



1*H*-tetrazole triggered cyclization with the 3′-OH group. In this step, a competitive side reaction was an intermolecular coupling to form diadenosine *P*-cyanoethyl phosphite, which could be suppressed by appropriate conditions such as low temperature, low concentration of reactants, and lack of stirring.[86] Without purification, the reaction mixtures were directly treated with the appropriate boronating reagent followed by ammonia hydrolysis. The final compound *c*AMPB **29a** was obtained in 48% yield after the removal of the protecting group. This approach has also been successfully employed to prepare adenosine 3′,5′-cyclic phosphorothioate (*c*AMPS) and *c*AMP.[86]

Thymidine 3′,5′-cyclic boranomonophosphate (*c*TMPB) **29b** was synthesized via a key intermediate cyclophosphoramidite **30a**, which was obtained through direct cyclization between thymidine and hexamethylphosphorus triamide (HMPT) as shown in Scheme 13.[113] The crucial aspects of the experimental procedure that ensured high yields (over 95%) for cyclization were the thorough deoxygenation of the solvent acetonitrile, slow increase of the reaction temperature, and its control below 65 °C.[26,137] Although it has been reported that diphenyl H-phosphonate[138,139] and tri(4-nitrophenyl)phosphite[140] could be used for the 2′,3′-cyclization, neither of these worked for the 3′,5′-cyclization.[113] Because the reaction between cyclophosphoramidite **30a** and 3-hydroxypropionitrile was sluggish with low yield, the intermediate phosphite triester **31** had to be purified before boronation. The final compound *c*TMPB **29b** was obtained after ammonia hydrolysis in an overall 40% yield.

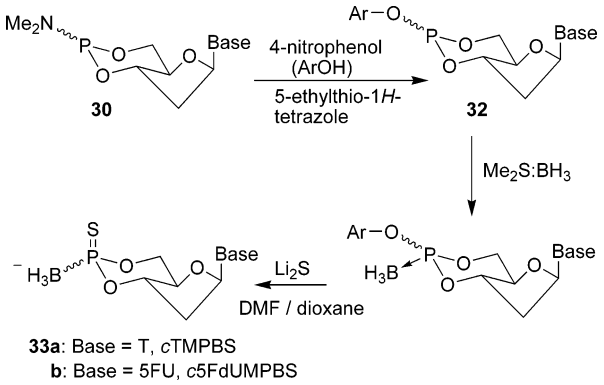
3.3.2. Synthesis of Nucleoside 3′,5′-Cyclic Boranophosphorothioates (cNMPBSs)

Nucleoside 3′,5′-cyclic boranophosphorothioates (*c*NMPBS) **33** were synthesized following the procedures shown in Scheme 14.[26] Unlike the case of *c*TMPB, where the yield for the formation of the phosphite triester was very low,[113] the reaction between cyclophosphoramidite **30** and 4-nitrophenol proceeded cleanly. Without isolation, phosphite triester **32** was treated with $Me_2S{:}BH_3$, and subsequent nucleophilic attack by Li_2S gave *c*NMPBS **33a** and **33b** in good yields.

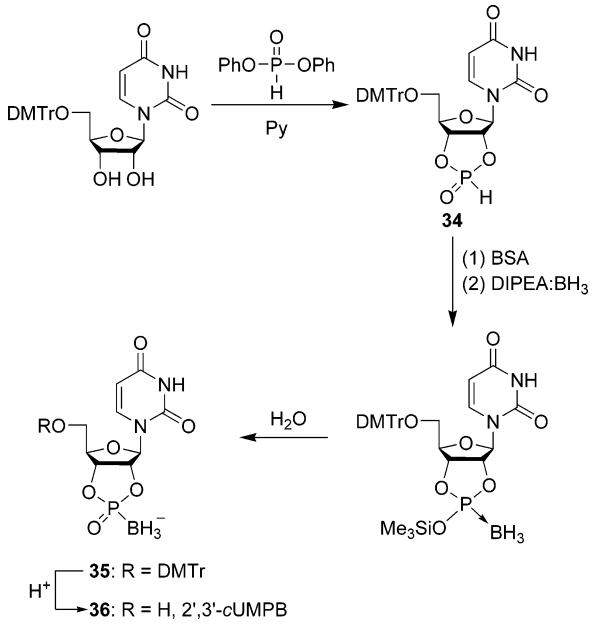
Analogues of *c*NMPBS **33** are the only known *P*-disubstituted chiral cyclic monophosphate with a negative charge. The borano-, thio-disubstitution will change the electron distribution over the phosphate, as well as the interactions between the cyclic monophosphate and metal ions. These properties could make *c*NMPBS **33** useful in elucidating the stereochemical course and role of metal ions in phosphoryl and nucleotidyl transfer reactions.[132,141]

In partitioning experiments, *c*TMPBS **33a** and *c*5FdUMPBS **33b** were found to be 340- and 290-fold more lipophilic than

Scheme 14



Scheme 15



the parent *c*TMP and *c*5FdUMP, respectively.[26] A good lipophilicity in combination with good water solubility could make *c*NMPBS promising as a prodrug candidate.

3.3.3. Synthesis of Nucleoside 2′,3′-Cyclic Boranomonophosphates (2′,3′-cNMPBs)

Synthesis of 2′,3′-*c*NMPB analogues was accomplished via an H-phosphonate approach.[138] As described in Scheme 15, reaction of diphenyl H-phosphonate with 5′-protected uridine gave 2′,3′-cyclic H-phosphonate **34**. Without purification, the intermediate **34** was treated with BSA followed by boronation with DIPEA:BH_3. After removal of the 5′-protecting group, the final compound 2′,3′-*c*UMPB **36** was obtained in 70% yield. Similarly, this approach was used by Kraszewski et al. to synthesize nucleoside 2′,3′-cyclic phosphorothioates.[139]

It has been reported that 2′,3′-cyclization can also be achieved with tri(4-nitrophenyl)phosphite.[140] The resulting 2′,3′-cyclic phosphite triester may serve as a precursor for the preparation of nucleoside 2′,3′-cyclic boranophosphorothioates (2′,3′-*c*NMPBSs) following the same procedure shown for 3′,5′-cyclic boranophosphorothioates **33** in Scheme 14. These analogues could provide valuable information regarding the stereochemistry in ribonuclease-catalyzed reactions.

Table 2. Summary of HPLC Elution Times and ^{31}P NMR Peaks for Diastereomers of Cyclic Monophosphates

		RP-HPLC[a] (min)	$\delta_{^{31}P}$ (ppm)	$^1J_{P-B}$ (Hz)	ref
*c*AMPB	FE	13.8	92.1	186	86
	SE	15.1	96.9	127	
*c*TMPB	FE	21.62	92.1	146	113
	SE	24.12	94.6	146	
*c*TMPBS	FE	9.07	170.7	102	26
	SE	10.23	162.4	115	
*c*5FdUMPBS	FE	7.60	170.6	113	26
	SE	9.20	162.3	130	
*c*AMPS	FE	14.41	56.5		133
	SE	15.55	55.0		
*c*TMPS	FE	R_f 0.54[b]	54.7		136
	SE	R_f 0.51[b]	52.1		
2′,3′-*c*UMPB	FE	9.40	120.8	146	138
	SE	19.11	116.1	130	
2′,3′-*c*UMPS	FE	6.58	77.6		138
	SE	10.24	76.1		

[a] See references for separation conditions. [b] Paper chromatography with a solvent system consisting of ^{i}PrOH:NH_3:H_2O (7:1:2).

3.4. Separation and Spectral Properties of Diastereomers of Borane-Containing Cyclic Monophosphates

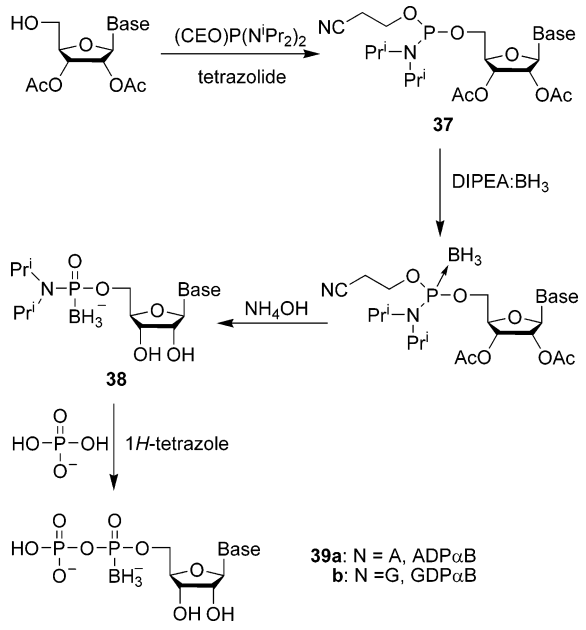
The borane-containing cyclic monophosphates exist as a pair of diastereomers. As seen from the structures of cyclic monophosphates, all of them contain two fused five- and/or six-membered rings. The high rigidity of the ring system and the positions of BH_3 (axial vs equatorial) are likely to cause considerable differences in the properties of the diastereomers, i.e., in retention times of RP-HPLC and in chemical shifts and coupling constants in ^{31}P NMR spectra, which are summarized in Table 2.

The fast- and slow-eluting isomers in RP-HPLC are designated as FE and SE, respectively. The ^{31}P NMR chemical shifts for diastereomers of cyclic phosphorothioates have been well studied and employed to assign the *P*-configurations.[136,142−144] For example, the configurations for the FE and SE isomers of *c*NMPS are R_p and S_p, respectively. For 2′,3′-*c*NMPS, the FE and SE isomers have configurations of S_p and R_p, respectively. In most cases with boranophosphates, information given by ^{31}P NMR cannot be used as a practical way to assign the *P*-configurations due to the broadening effects caused by ^{11}B and significant overlaps between both diastereomers. However, the difference in ^{31}P NMR chemical shifts for the diastereomers of borane-containing cyclic monophosphates is larger than that found for the corresponding cyclic phosphorothioates. In some cases, the difference is as large as $\Delta\delta$ 8.3 ppm for *c*TMPBS and *c*5FdUMPBS, whereas there is only a $\Delta\delta$ 2.6 ppm difference for *c*TMPS.

Although the relationships between ^{31}P NMR chemical shifts and *P*-configurations have not yet been established, use of ^{31}P NMR chemical shifts to assign the *P*-configurations of borane-containing cyclic monophosphates is a practical and reliable approach that needs immediate attention.

Analogues of 2′,3′-*c*NMPs consist of two five-membered rings that are fused together. They are more rigid than the 3′,5′-*c*NMP analogues that include five- and six-membered rings. Therefore, isomers of 2′,3′-*c*NMPs will have a more apparent difference than those of 3′,5′-*c*NMPs in their interaction with the RP-HPLC column, which is well exemplified in the HPLC retention time. As shown in Table 2, the 2′,3′-*c*NMP analogues differ in retention time by 5−10 min while 3′,5′-*c*NMP analogues differ by only 1−3 min.

Scheme 16



4. Nucleoside Boranodiphosphates

4.1. Synthesis of Nucleoside 5′-(α-*P*-Borano)-diphosphates

4.1.1. Synthesis via a Boranophosphoramidate Approach

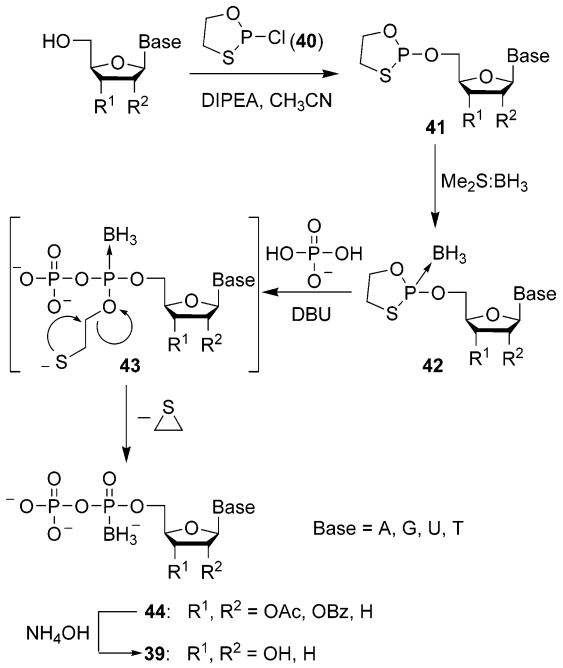
Boronated phosphoramidate intermediate **38** used previously for the synthesis of nucleoside boranomonophosphate is a convenient precursor for the preparation of nucleoside 5′-(α-*P*-borano)diphosphates (NDPαB) **39**, as shown in Scheme 16. This method has been applied for the first preparation of adenosine 5′-(α-*P*-borano)diphosphates (ADPαB) **39a** and guanosine 5′-(α-*P*-borano)diphosphates (GDPαB) **39b** in reasonable yields.[145]

As described in Scheme 16, instead of using 2-cyanoethyl diisopropylphosphoramidochloridite (CEO)P(N^iPr_2)Cl, which might react with an unprotected amino group in the nucleobases, the phosphitylation of base-unprotected nucleosides was accomplished with 2-cyanoethyl tetraisopropylphosphorodiamidite (CEO)P(N^iPr_2)$_2$. With diisopropylammonium 1*H*-tetrazolide as the catalyst for the formation of phosphoramidite **37**, only one of the two diisopropylamino groups of (CEO)P(N^iPr_2)$_2$ was displaced by the 5′-OH group from the nucleoside. By contrast, if 1*H*-tetrazole was used as the catalyst, then both diisopropylamino groups reacted with 5′-OH, resulting in the formation of the byproduct, the dinucleoside phosphite triester.[145] A key step involved intermolecular phosphorylation of boranophosphoramidate **38** with tetrabutylammonium dihydrogen phosphate in the presence of 1*H*-tetrazole. This step was a clean reaction, and byproducts arising from the intramolecular cyclization of diisopropylamino groups with either the 2′- or 3′-OH group were not observed.[145]

4.1.2. Synthesis via an Oxathiaphospholane Approach

Oxathiaphospholanes were first introduced by Stec et al.[146,147] as a method of choice for stereocontrolled synthesis

Scheme 17



of oligo(nucleoside phosphorothioate)s and were recently employed to prepare P-stereodefined phosphoroselenoate analogues of oligodeoxyribonucleotides.[148] The oxathiaphospholane approach involves a tricoordinate phosphorus intermediate and, therefore, is suitable for the introduction of the borane moiety. The detailed synthesis of NDPαB 39 is depicted in Scheme 17.[149]

Reaction of 2-chloro-1,3,2-oxathiaphospholane **40**[147] with base-unprotected nucleoside gave oxathiaphospholane intermediate **41** in the presence of DIPEA. The formation of **41** was clean and confirmed by ^{31}P NMR, in which the singlet at δ 207 ppm for **40** was transformed into two singlets around δ 172 ppm corresponding to the two diastereomers of the P(III) intermediate **41** in equal ratio. The oxathiaphospholane−borane complex **42** has a characteristic chemical shift of δ 160 ppm (broad) in the ^{31}P NMR spectrum. Unlike its thio- and seleno-counterparts, in which oxathiaphospholane−sulfur and oxathiaphospholane−selenium complexes can be purified by silica gel column chromatography,[150,151] the oxathiaphospholane−borane complex **42** was too unstable to be isolated by the same procedure. The crucial step in this approach is the addition of tributylammonium orthophosphate and excessive DBU. The reaction underwent ring-opening condensation followed by spontaneous elimination of ethylene episulfide to generate the ribose-protected NDPαB **44**. It was found that the rate of DBU-assisted ring-opening condensation varied when different quantities of DBU were used.[149] Five equivalents of DBU appeared to be an optimal amount. It also has been observed that the yield of ring-opening condensation would dramatically decrease in the presence of DIPEA from the previous steps, although the exact reason is still unknown.[149] Removal of the protecting groups on the ribose of **44** gave the final desired diphosphate analogues **39** with characteristic peaks at δ −6 ppm for β-P (d) and δ 81 ppm (br) for α-P in the ^{31}P NMR spectrum, as shown in Figure 12.

This one-pot approach has been employed to obtain a set of four compounds, including ADPαB **39a**, GDPαB **39b**, UDPαB **39d**, and dTDPαB **39h**, in good yields (30−43%). Since this method does not require the exocyclic amine protection of the nucleobases, it eliminates the risk of possible nucleobase reduction during the boronation step.[96,97]

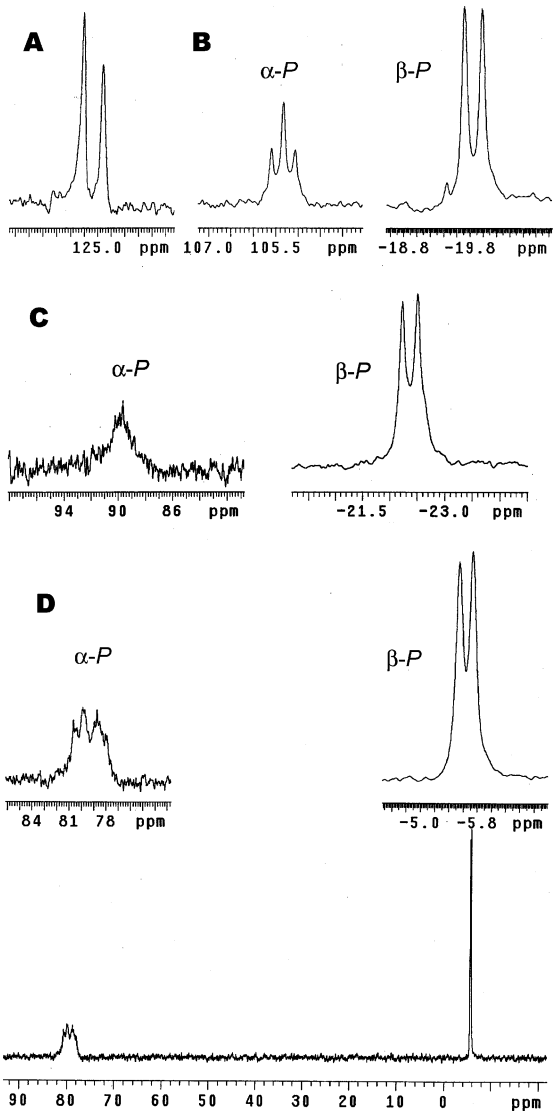


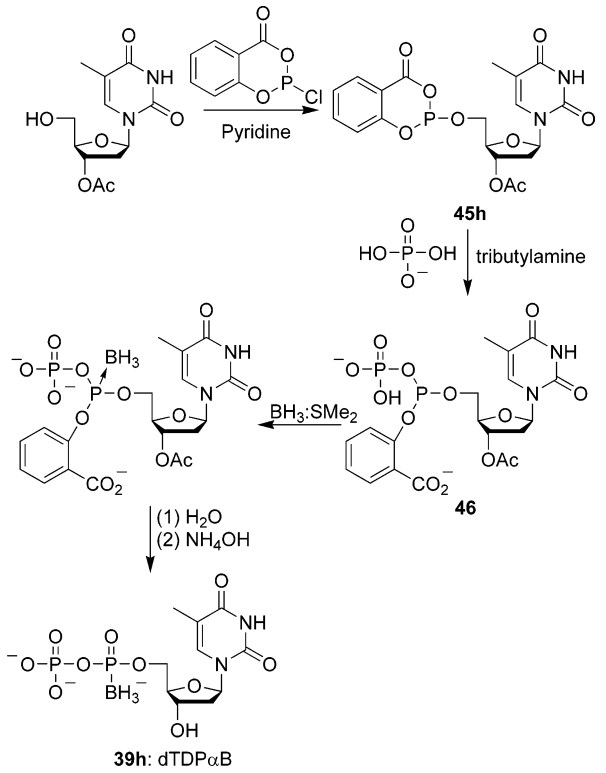
Figure 12. Selected regions of the ^{31}P NMR spectra monitoring the synthesis of ADPαB **39a**. The spectra were recorded at 161.9 MHz and externally referenced to H_3PO_4. (A) Intermediate **45a** in $CDCl_3$; (B) intermediate **48a** in $CDCl_3$; (C) intermediate **49a** in $CDCl_3$; (D) ADPαB **39a** in D_2O.

4.1.3. Synthesis via a Phosphite Approach

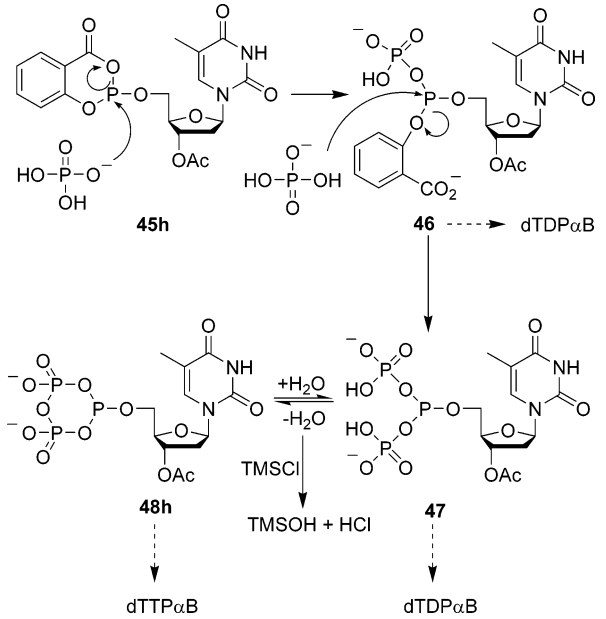
Initial attempts to prepare NDPαB analogues, such as dTDPαB **39h**, involved the use of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (salicyl chlorophosphite) and resulted in a low 12% yield, as depicted in Scheme 18.[149] In addition, thymidine 5′-(α-*P*-borano)triphosphate (dTTPαB) was isolated as one of the major byproducts in 17% yield. This observance was in accordance with the case of its thio-counterpart when salicyl chlorophosphite was employed to prepare thymidine 5′-(α-*P*-thio)diphosphate (dTDPαS).[152]

A mechanism proposed for the formation of dTTPαB is shown in Scheme 19.[149] Due to the bifunctionality of the thymidine phosphite **45h**, it could undergo two subsequent nucleophilic substitutions. With the assumption that the carboxyl group is a better leaving group than the phenolic group, substitution by the first incoming nucleophile, tributylammonium orthophosphate, would yield deoxythymidine

Scheme 18

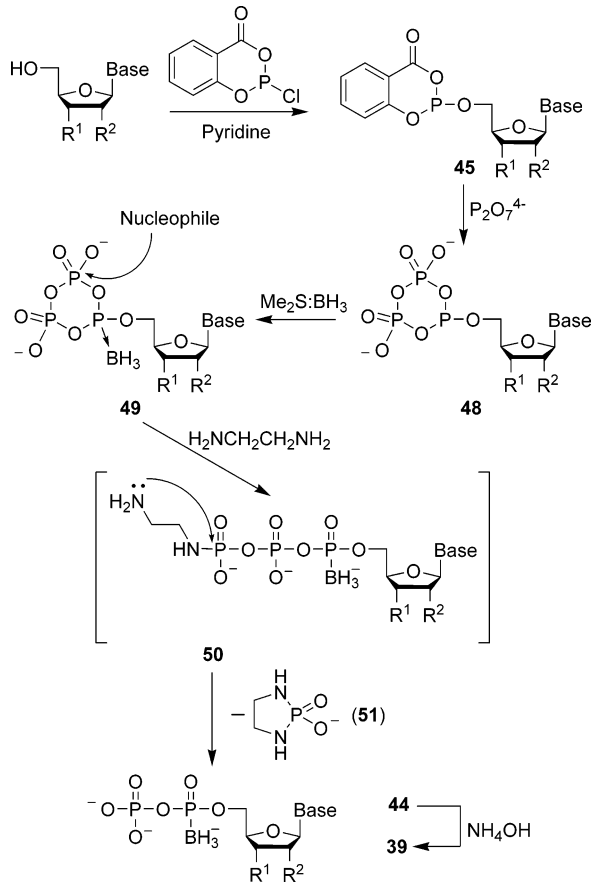


Scheme 19



P-phosphoryl-*P*-(2-carbonylphenolic) phosphite **46**. Subsequently, the phenolic group on intermediate **46** would be replaced with a second incoming nucleophilic tributylammonium orthophosphate to obtain the deoxythymidine *P*-diphosphoryl phosphite **47**, which could then undergo a reversible dehydration reaction (loss of H_2O) to form P^2,P^3-dioxo-P^1-thymidinylcyclotriphosphate **48h**. The intermediates **46**/**47** and **48h**, when subjected to boronation followed by hydrolysis, would yield diphosphate dTDPαB and triphosphate dTTPαB, respectively. Although there was a lack of direct spectral evidence for the formation of intermediates **46**, **47**, and **48h** due to the complexity of ^{31}P NMR for the reaction mixtures, experiments with chlorotrimethylsilane (TMSCl) indirectly supported the proposed mechanism

Scheme 20



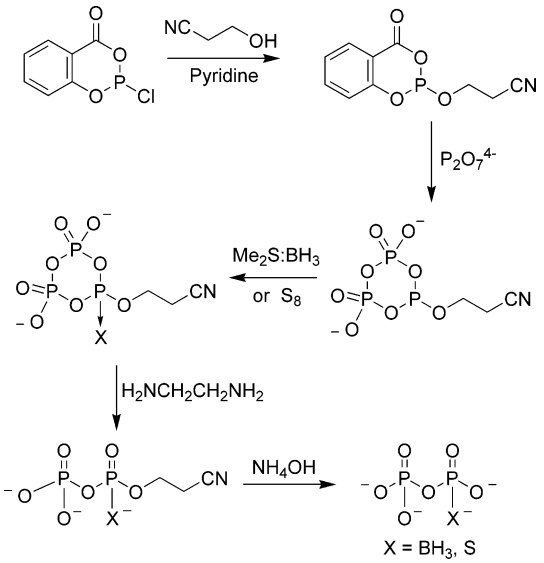
shown in Scheme 19. When TMSCl was introduced into the reaction mixture, the yield of dTTPαB increased while that of dTDPαB decreased.[149]

Until now, the best approach for the preparation of NDPαB analogues involved salicyl chlorophosphite and ethylenediamine.[85] This method is based on modification of the Ludwig−Eckstein synthesis of modified nucleoside triphosphates[82,83,152,153] and the observance that ethylenediamine can act as a dephosphorylating reagent.[154] As illustrated in Scheme 20, nucleoside reacts with salicyl chlorophosphite to give an activated phosphite that is reacted with pyrophosphate to form the cyclic intermediate **48**. An *in situ* boronation of **48** with borane-dimethyl sulfide followed by the addition of ethylenediamine results in the cyclic phosphorodiamidate **51** and the corresponding NDPαB **44** with the ribose ring protected. The formation of these two products can be explained by the ring opening of **49** first to **50**, followed by a second, intramolecular nucleophilic displacement. It is worth mentioning that such a two-step mechanism has suggested a new concept for designing drugs targeting Ras, a major protein responsible for the formation of human tumors.[155]

The reaction course described in Scheme 20 was monitored by ^{31}P NMR as shown in Figure 12. Therefore, although this was a one-pot protocol and the intermediates were not isolated, there was good evidence for the formation of intermediates and the completion of the individual steps.

The ring-opening of the cyclic boranometatriphosphate **49** could occur at a phosphate or at the boranophosphate functionality.[49,156] The final products of NDPαB **39** and cyclic phosphorodiamidate **51** show that the ring-opening occurs exclusively at a phosphate group, which is in

Scheme 21



accordance with the previous results for the synthesis of triphosphate analogues.[49,82,152] This is also rational, since α-boranophosphates are better leaving groups than phosphates. It is also shown that protection of the nucleobase functionality for A, T, U, G, and C is not required, but selectivity for the 5′-OH in the initial phosphitylation step is marginal if the 2′- and 3′-OH are not protected.[157]

This newly developed approach involving salicyl chlorophosphite and ethylenediamine has proven to be useful for the preparation of a wide range of modified nucleotides such as 5′-(α-*P*-thio)diphosphate (NDPαS) and nucleoside 5′-(α-*P*-seleno)diphosphate (NDPαSe).[85] This method is also employed to prepare the thio-[154] and boranopyrophosphate (Liu, H. Y.; Li., P.; and Shaw, B. R. Unpublished data) analogues shown in Scheme 21.

4.2. Separation and Configuration Determination of the Diastereomers of NDPαB

4.2.1. Separation of the Diastereomers of NDPαB

Nucleoside 5′-(α-*P*-borano)diphosphates exist as a pair of diastereomers. For the naturally occurring ribo- and deoxyribonucleosides, the diastereomers of their corresponding NDPαB could be resolved by RP-HPLC and named as fast- (FE) and slow-eluting (SE) isomers according to their elution times. The analytical separations are performed on a reverse-phase column (Waters Delta Pak C18, 300 mm × 3.9 mm, 15 μm, 100 Å). The samples are eluted with a buffer consisting of triethylammonium bicarbonate (TEAB, pH 6.80) and methanol at a flow rate of 1.0 mL/min. Preparative separations are carried out on a preparative column (Waters Delta Pak C18, 100 mm × 25 mm, 15 μm, 100 Å) in a Z-module at a flow rate of 8 mL/min. The eluting conditions and results are summarized in Table 3.[85,149]

4.2.2. Configuration Determination of the Diastereomers of NDPαB

The absolute configurations of the NDPαB diastereomers were determined[40] by cocrystallization with a *Dictyostelium* enzyme having very similar structure to the active sites of nucleoside diphosphate kinase (NDPK).[158,159] Since only one diastereomer could be recognized by *Dictyostelium* NDPK and phosphorylated to a triphosphate via a phospho-histidine intermediate, determination of the stereochemistry of NDPαB

Table 3. HPLC Profile of NDPαB Analogues of Naturally Occurring Nucleosides[a]

			retention time (min) [area (%)]	
entry	compd	MeOH %	FE (R_p)	SE (S_p)
39a	ADPαB	8	8.53 [50.5]	17.24 [49.5]
39b	GDPαB	7	10.73 [51.5]	18.38 [48.5]
39c	CDPαB	5	10.10 [50.4]	13.62 [49.6]
39d	UDPαB	8	6.57 [59.9]	10.19 [40.1]
39e	dADPαB	9	5.58 [45.2]	11.97 [54.8]
39f	dGDPαB	9	10.69 [49.3]	13.29 [50.7]
39g	dCDPαB	6	10.41 [48.0]	17.48 [52.0]
39h	dTDPαB	6	6.72 [48.9]	9.02 [51.1]

[a] Reprinted with permission from ref 85. Copyright 2005 American Chemical Society.

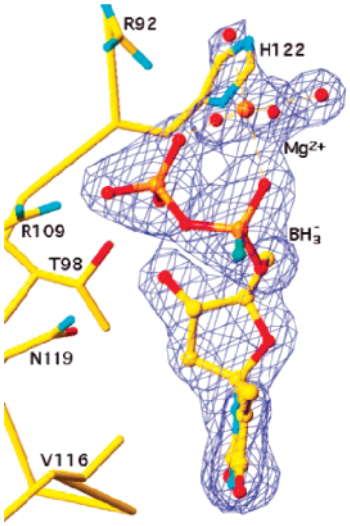


Figure 13. Analogue of R_p-dTDPαB bound to the wild-type *Dictyostelium* enzyme. The BH_3 group (in blue) points toward the reader. An Mg^{2+} ion ligates two nonbridging oxygens on the α- and β-phosphates, and four water molecules (red spheres) complete the octahedral geometry. The thymine base is sandwiched between F64 and V116. The geometry of the BH_3 group was taken from the crystal structure of a dimethyl ester.[64] (Reprinted by permission from Macmillan Publishers Ltd: *The EMBO Journal* (http://embojournal.npgjournals.com) **2000**, *19*, 3520, copyright 2000.)

with NDPK was possible. Experiments showed that only the FE isomer of dTDPαB in the HPLC could be crystallized with *Dictyostelium* NDPK.[40] As represented in Figure 13, the 1.92 Å resolution X-ray structure indicates that the borane group (BH_3 in blue) in the FE isomer points toward the reader with an R_p configuration. Therefore, the SE isomer of dTDPαB has the S_p configuration. Assuming the order of elution in HPLC is irrelevant with the change in nucleosides, the assignments of the configurations for the isomers FE and SE of NDPαB are R_p and S_p, respectively.[40]

The assignments of the absolute configurations for both *P*-diastereomers were further confirmed by analyzing the ^{1}H NMR spectra of a series of ADP analogues.[85] By docking the model developed by Fischer et al.[160] and as depicted in Figure 14, the H-8 signal of the SE isomer should be more shielded than that of the FE isomer due to anisotropy effects from the vicinal X group. Therefore, the chemical shifts of H-8 of the modified ADP analogues in ^{1}H NMR move upfield in the SE isomer relative to the FE isomer. Furthermore, the chemical difference of H-8 between the isomers FE and SE increases in the order of ADPαB < ADPαS < ADPαSe. This observance is rational because the distance from H-8 to the negative charge on X decreases with the

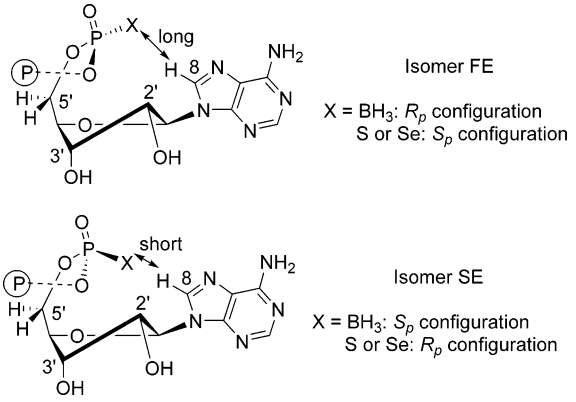


Figure 14. Conformation of *P*-diastereomers of ADP analogues. (Reprinted with permission from ref 85. Copyright 2005 American Chemical Society.)

increased P−X bond lengths in ADPαB (1.91 Å), ADPαS (1.96 Å), and ADPαSe (2.24 Å).[64,161,162]

4.3. Substrate Properties of NDPαB Analogues with NDPK, Pyruvate Kinase, and Creatine Kinase

4.3.1. Substrate Properties of NDPαB Analogues with NDPK

NDPK is involved in the phosphorylation of NDP to NTP. The activation of NDPαB by NDPK has been studied under pre-steady-state kinetic conditions using a fluorescence quenching method.[39,40] Although the phosphorylation of AZT diphosphate (AZTDP) is 10^4-fold slower than that of natural dTDP,[163] the R_p-AZTDPαB isomer is a better substrate for NDPK than AZTDP while S_p-AZTαB is inactive.[39,40] This stereospecificity originates from the requirement that the boranodiphosphate analogue interacts with a Mg^{2+} ion. While the R_p-diastereomer does not prevent metal binding and is active, the S_p-diastereomer loses this interaction.

As shown in Figure 19, the internal H-bond between the 3′-OH and one of the nonbridging oxygens on the β-phosphorus of dNDP, in a similar fashion of dNTP with HIV-RT, appears to be crucial for the optimal nucleotide geometry and efficient phosphotransfer by NDPK.[164] Studies found that the sugar modification in d4T (double bond between the 2′ and 3′ carbons) makes the diphosphate derivative a better substrate for NDPK than AZT or other dideoxy analogues. While all of these analogues lack a 3′-OH group involved in the catalysis by NDPK, the geometry of the C2′−C3′ double bond in d4T allows the formation of an intramolecular CH...O hydrogen bond that partially compensates for the missing 3′-OH...O.[39,40]

The phosphorylation of the R_p-AZTDPαB isomer follows an exponential time course with a rate constant that increases with nucleotide concentration. The affinity of NDPK for R_p-NDPαB analogues is improved by an order of magnitude, increasing the catalytic efficiency over the natural substrate dTDP, and also for AZTDP and d4TDP analogues (Figure 15).[39,40] This 10-fold increase in efficiency, if it were to occur *in vivo*, would be of great value in the case of AZT, which is converted into a triphosphate very poorly by cellular enzymes.

4.3.2. Binding Affinity of NDPαB Analogues with Pyruvate Kinase (PK) and Creatine Kinase (CK)

The rabbit muscle creatine (CK) and pyruvate (PK) kinases are thought to be responsible for the last phosphorylation of some antiviral 2′,3′-dideoxynucleosides.[165] In order to better understand the effect of boranophosphate substitution on biochemical properties, the binding affinity of the R_p- and S_p-ADPαB isomers with CK and PK was investigated.[85,113] An equilibrium titration approach was used to study the binding of NDP analogues with kinases, which was based on fluorescence quenching of the tryptophan residue located in the active sites of both enzymes. The data, presented in Table 4, indicate that both enzymes directly bind two NDP molecules per CK dimer and PK tetramer with strong negative cooperativity ($K_{d1} \ll K_{d2}$). Opposite stereospecificity was observed for CK and PK toward ADPαB diastereomers. Thus, the CK binds with the S_p-isomer more tightly than with the R_p-isomer, whereas the PK binds slightly better with the R_p-isomer than with the S_p-isomer.[85,113]

The steady-state kinetic analysis of phosphorylation indicated that both stereoisomers are very poor substrates for CK.[166] S_p-ADPαB is 500-fold and R_p-ADPαB is 50000-fold less efficient than the native ADP analogue.[166] It has also

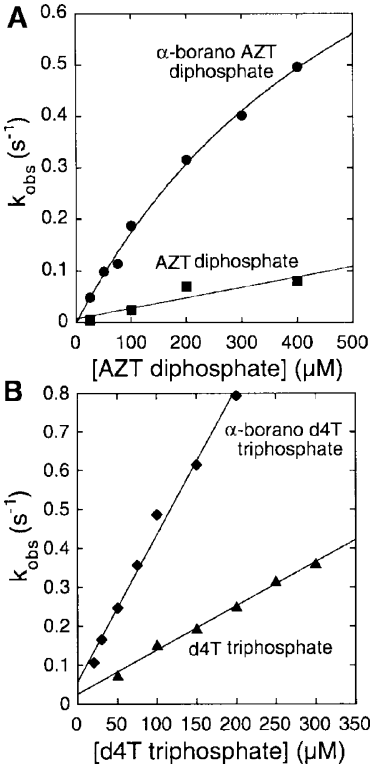


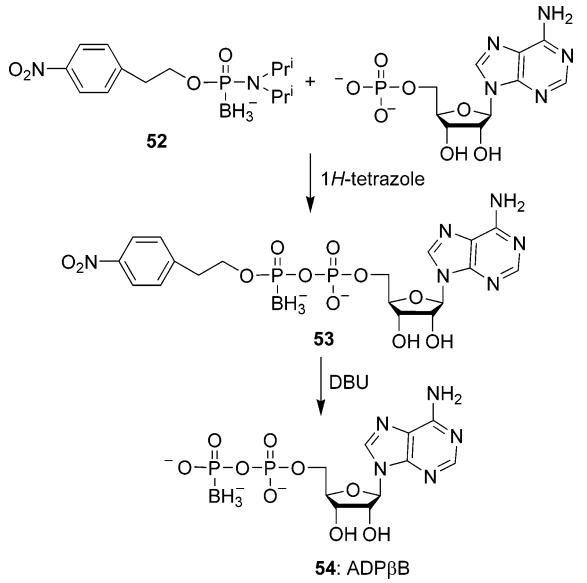
Figure 15. Enhancement of AZTDP and d4TDP activation by NDPK in the presence of R_p-α-*P*-BH_3. (A) Concentration dependence of the pseudo-first-order phosphorylation rate constant k_{obs}. The forward reaction from diphosphates to triphosphates was studied. (B) Concentration dependence of the pseudo-first-order phosphorylation rate constant k_{obs}. The backward reaction from triphosphates to diphosphates was studied. (Reprinted by permission from Macmillan Publishers Ltd: *The EMBO Journal* (http://embojournal.npgjournals.com) **2000**, *19*, 3520, copyright 2000.)

Table 4. Dissociation Constants (K_d, μM Averages ± Standard Deviations) for Binding of NDP Analogues with Rabbit Muscle CK and PK[a]

	CK		PK	
	K_{d1}	K_{d2}	K_{d1}	K_{d2}
ADP	69 ± 37	213 ± 51	5.7 ± 1.2	789 ± 105
R_p-ADPαB	256 ± 45	2241 ± 146	46 ± 11	555 ± 93
S_p-ADPαB	34 ± 2	417 ± 153	68 ± 7	635 ± 102

[a] Data are taken from refs 85 and 113.

Scheme 22



been found that both diastereomers of ADPαB are poor inhibitors for PK, where the R_p- and S_p-isomers are competitive and noncompetitive inhibitors, respectively.[166]

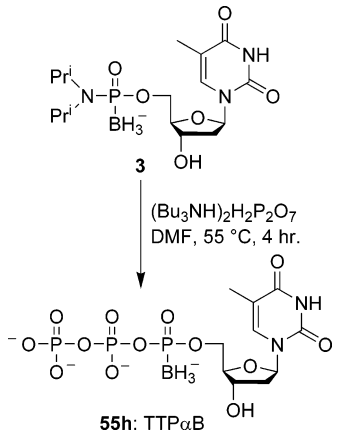
4.4. Synthesis of Nucleoside 5′-(*β*-*P*-Borano)-diphosphates

Nucleoside diphosphate analogues with modifications at the *β*-phosphate group have wide applications in the investigation of various biochemical processes. For example, adenosine 5′-(*β*-*P*-thio)diphosphate has been widely used in stereochemical studies of phosphotransferases and ATP-dependent synthetases.[167,168] It also has been well studied in the signal transmission of P2Y receptors.[169−171] Therefore, nucleoside 5′-(*β*-*P*-borano)diphosphate (NDP*β*B) analogues could serve as a new type of tool to probe these important processes. Although there are a few methods to synthesize nucleoside 5′-(*β*-*P*-thio)diphosphates,[172,173] only one example has been reported for the preparation of NDP*β*B via a boranophosphorylating reagent.[124] As shown in Scheme 22, 2-(4-nitrophenyl)ethoxyldiisopropyl boranophosphoramidate **52** reacted with 5′-AMP to give boranodiphosphate diester **53** in the presence of 1*H*-tetrazole. The protecting group of 4-nitrophenylethyl was removed by DBU to yield the final compound adenosine 5′-(*β*-*P*-borano)diphosphate (ADP*β*B) **54** in an overall 57% yield.

5. Nucleoside Boranotriphosphates

Nucleoside triphosphates are the basic building blocks for enzymatic synthesis of DNA and RNA *in vitro* and *in vivo*. Modified nucleoside triphosphates have important diagnostic and therapeutic applications.[49,174−179] They are indispensable tools for studying numerous biochemical and pharmacological processes. For example, phosphorothioate analogues of ATP have been elegantly used to probe the interaction of metal ions with the phosphate group in nucleotidyl transferase complexes.[79,180−183] Nucleotide analogues have also been used to prepare a versatile set of labeling reagents for molecular biology.[78] Among the α-phosphate modified NTP derivatives, only the nucleoside 5′-(α-*P*-thio)triphosphates (NTPαS)[79] and nucleoside 5′-(α-*P*-borano)triphosphates (NTPαB)[18] can substitute for normal NTP and be readily incorporated into nucleic acids by DNA or RNA polymerases. Further, once

Scheme 23



incorporated, they are good templates for DNA and RNA polymerases in the next round of replication. However, as reviewed by Burgess and Cook,[157] synthesis of α-phosphate substituted NTP analogues in high yields is still a challenge.

5.1. Synthesis of Nucleoside 5′-(α-*P*-Borano)-triphosphates

5.1.1. Synthesis via a Phosphoramidite Approach

The thymidine 5′-(α-*P*-borano)triphosphate (dTTPαB) **55h** was first synthesized by a phosphoramidite approach using boranophosphoramidates as the key intermediate (Scheme 23).[24] Thymidine boranophosphoramidate **3** was obtained following the procedures described in Scheme 2. Reaction of **3** with an excess of $(Bu_3NH)_2H_2P_2O_7$ yielded a diastereomer mixture of dTTPαB **55h** in 30% yield after ion-exchange chromatography. This method can be employed to prepare other dNTPαB analogues. However, it has certain limitations, such as the requirement for exocyclic amino group protection of purine nucleobases and the need for purification of the intermediate boranophosphoramidates such as **3**.

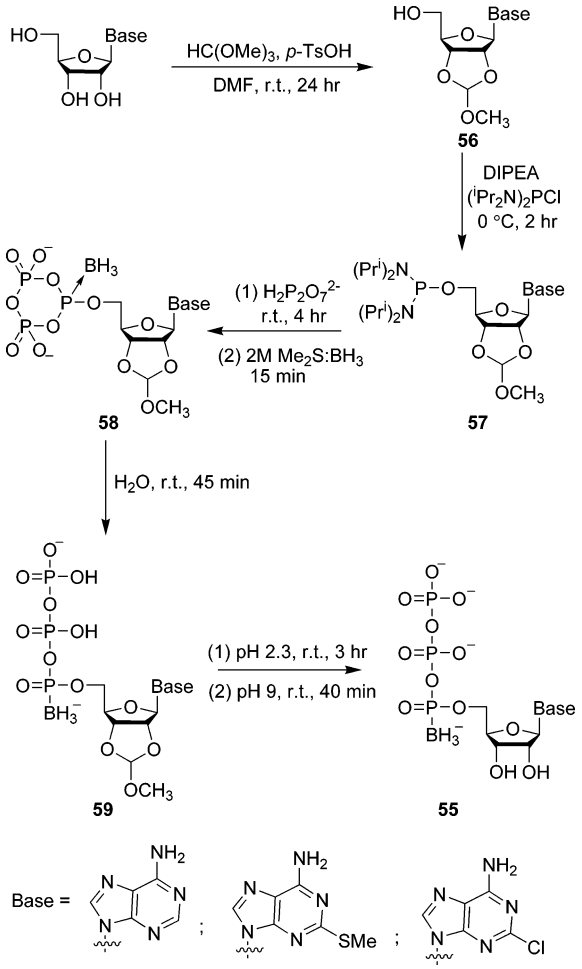
Recently, an improved synthesis via a phosphoramidite approach was developed for the preparation of new analogues of $P2Y_1$ receptor agonists,[184] as shown in Scheme 24. The advantages of the approach were that (1) there was no need to protect and then deprotect the 5′-OH for the preparation of the starting nucleosides; (2) there was no need to protect the N^6 position of the purine nucleobase because the mild phosphitylating reagent bis(diisopropylamino)chlorophosphine was used; (3) steps **56**−**59** were carried out in a one-pot format; (4) mild conditions were applicable to a wide range of nucleoside analogues with overall good yields (31−43%).

A similar method was also reported to prepare the acyclonucleoside triphosphate (acycloNTP) and α-*P*-boranotriphosphate (acycloNTPαB) analogues whose structures are shown in Figure 16.[48] The main modifications to Scheme 24 included the following: (1) a large excess of DIPEA was introduced to accelerate the coupling reaction between acyclonucleosides and phosphitylating reagent; (2) 1*H*-tetrazole was added as the catalyst to significantly reduce the time required for the displacement reaction between acyclonucleoside phosphoramidite and pyrophosphate to occur.

5.1.2. Synthesis via a Phosphite Approach

A convenient one-pot procedure for the preparation of NTPαB **55** was developed by Shaw et al. and is outlined in

Scheme 24



Scheme 25.[82,83,87] The initial three steps—phosphitylation, pyrophosphate exchange reaction, and boronation—are the same as in the synthesis of NDPαB **39** described in Scheme 20. However, instead of using ethylenediamine as the nucleophile to yield NDPαB,[85] water was used to open the cyclic ring followed by ammonia hydrolysis to give the NTPαB **55** as a diastereomeric mixture. The procedure is suitable for preparing both ribo-[82] and deoxyribonucleosides,[83,87] affording α-boranotriphosphates **55** in 25−45% yields after ion-exchange chromatography. Therefore, from a synthetic point of view, the NDPαB and NTPαB can be selectively obtained by controlling the nucleophiles. Furthermore, it may also suggest novel solutions for NTP derivatives with controlled stability. The analogues of NTPαB **55** have characteristic peaks at δ −6 ppm for γ-*P* (d), δ −21 ppm for β-*P* (dd), and δ 83 ppm (br) for α-*P* in the ^{31}P NMR spectrum, as shown in Figure 17.

One of the important features in the synthesis of NTPαB is the opening of the cyclic ring in intermediate **49**. In the case of water and hydroxyl ion (without isotope labeling) as nucleophiles, the nucleophilic attack on any of the three phosphorus atoms of **49** would result in the same product **60**. When other nucleophiles are used, their attack on the different phosphorus atoms would result in the formation of two different α- or γ-substituted products. It was shown by $H_2{}^{18}O$-labeling experiments that the hydrolysis occurs exclusively at β-phosphate to form the γ-product via path B in the synthesis of NTPαS analogues (Scheme 26).[152] Similar studies with borane analogues by Wang et al.[49] and Shaw et al.[85] also proved that the nucleophilic attack by amines occurs exclusively at the β-phosphate to form the γ-product.

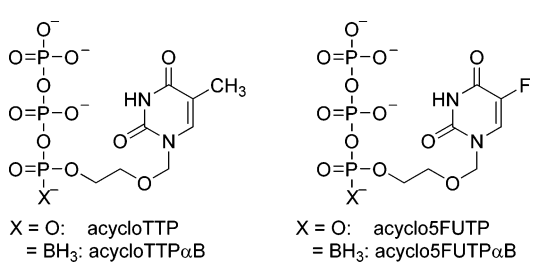
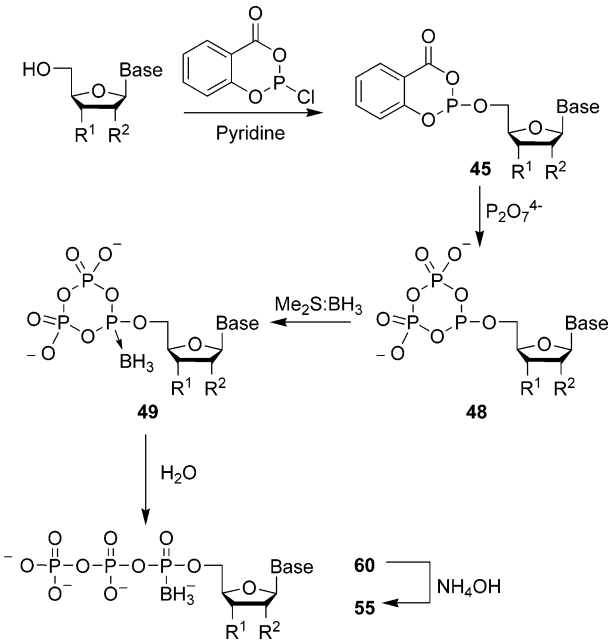


Figure 16. Structures of acycloNTP and acycloNTPαB.

Scheme 25



However, earlier work on the synthesis of dithiotriphosphates described in Scheme 27 showed that the nucleophile Li_2S could attack at both α- and β-phosphate positions.[154] Compound **61** was formed via path A by attack on the phosphorothioate P^{α}(=S), and **62** was formed via path B by attack on the phosphate P^{β}(=O). In the case of synthesizing *P*-doubly modified thymidine (α-*P*-borano,α-*P*-thio)triphosphate **71** (Scheme 31), attack of the nucleophile Li_2S occurs

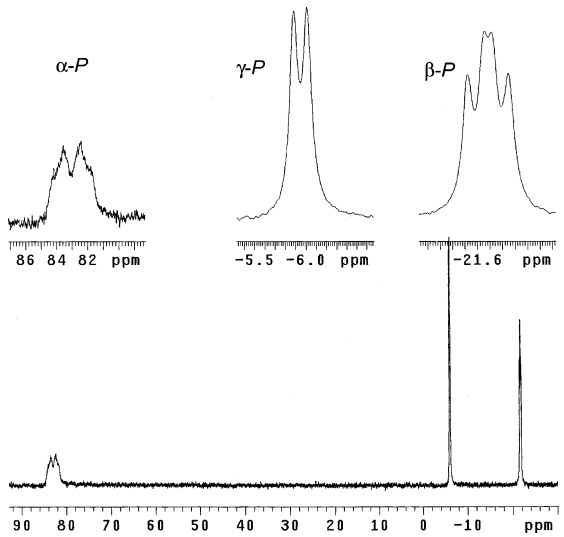
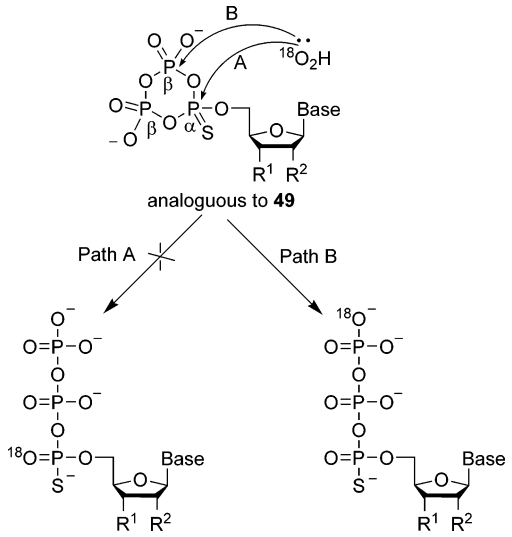
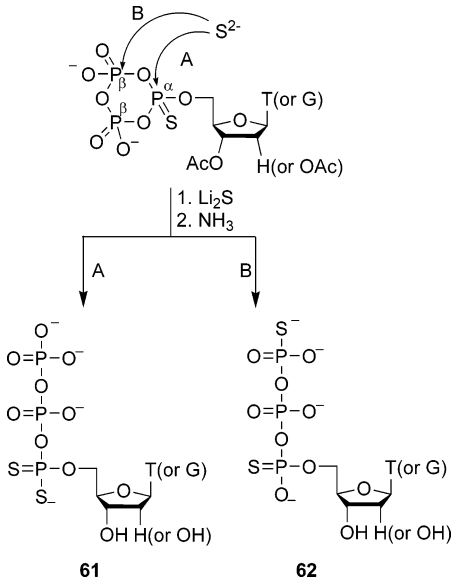


Figure 17. ^{31}P NMR spectrum of ATPαB in D_2O. The spectra were recorded at 161.9 MHz and externally referenced to H_3PO_4.

Scheme 26



Scheme 27



exclusively at the α-boranophosphate to form the α-product **71**.[156,185] The regional selectivity could be tentatively explained by Pearson's hard soft acid base (HSAB) principle. The phosphorus atom in a phosphate group can be regarded as a hard Lewis acid, but the phosphorus atom in boranophosphate and phosphorothioate groups is a soft Lewis acid. Water and hydroxyl ion are hard Lewis bases; therefore, they prefer the hard Lewis acid of a phosphorus atom in a normal phosphate, which results in the formation of only γ-products. Sulfur ion (S^{2-}) is a soft Lewis base; therefore, it prefers the soft Lewis acid of phosphorus atoms in boranophosphates and phosphorothioates, which results in the formation of only α-products (for phosphorothioates, when DMF was used as the solvent). However, it is still difficult to use the HSAB principle to explain the reaction between S^{2-} and phosphate when pyridine and dioxane were used as the solvent mixture.[154]

5.2. Separation and Configuration Determination of the Diastereomers of NTPαB

5.2.1. Separation of the Diastereomers of NTPαB

Nucleoside 5′-(α-*P*-borano)triphosphates exist as a pair of diastereomers. For the naturally occurring nucleosides, the diastereomers of their corresponding NTPαB analogues can be efficiently resolved by reverse-phase HPLC and named as fast- (FE) and slow-eluting (SE) isomers according to their elution times. Typically, analytical and preparative separations are performed on a reverse-phase column (Waters Delta Pak C18, 300 mm × 7.8 mm, 15 μm, 300 Å). Samples are eluted with a buffer consisting of triethylammonium acetate (TEAA, pH 6.80) and methanol at a flow rate of 3.0 mL/min. The eluting conditions and results are summarized in Table 5.[82,83]

Table 5. HPLC Profile of NTPαB Analogues of Naturally Occurring Nucleosides[a]

			retention time (min) [area (%)]	
entry	compd	MeOH %	FE (R_p)	SE (S_p)
55a	ATPαB	10	11.04 [42.7]	17.82 [57.3]
55b	GTPαB	8	9.31 [47.5]	14.56 [52.5]
55c	CTPαB	6	8.55 [51.0]	12.24 [49.0]
55d	UTPαB	8	8.32 [46.4]	11.80 [53.6]
55e	dATPαB	11	13.54 [54.0]	17.37 [46.0]
55f	dGTPαB	8	12.64 [50.0]	15.63 [50.0]
55g	dCTPαB	6	9.61 [49.0]	12.74 [51.0]
55h	dTTPαB	10	10.29 [58.0]	14.78 [42.0]

[a] Data are taken from refs 82 and 83.

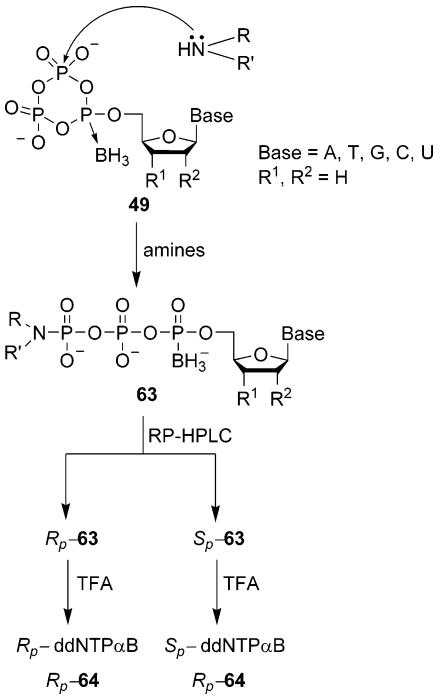
It has to be pointed out that the presence of groups at the 2′- and/or 3′-position in NTPαB is crucial for the separation of *P*-diastereomers. Therefore, as mentioned earlier, diastereomers of NTPαB analogues of naturally occurring nucleosides can be easily resolved by RP-HPLC. The retention time difference between diastereomers could reach 6.78 min, as in the case of ATPαB **55a** (Table 5). However, it is extremely difficult to separate diastereomers of antiviral 2′,3′-dideoxynucleoside 5′-(α-*P*-borano)triphosphates (ddNTPαB, **64**) by RP-HPLC, which prevents one from obtaining the pure diastereomers and delays the studies on these clinically important analogues with viral enzymes.

To circumvent the separation difficulties with the ddNTPαB analogues, Shaw et al. recently developed a strategy involving γ-*P*-substituted phosphoramidate boranotriphosphate **63** described in Scheme 28. This work is currently in progress. The *P*-diastereomers of γ-*P*-substituted compound **63** can be efficiently separated by RP-HPLC. The resulting pure individual isomers were then converted to the corresponding ddNTPαB **64** by removing the amino group from the γ-*P* position in the presence of TFA.

5.2.2. Configuration Determination of the Diastereomers of NTPαB

Although the absolute configurations of the NTPαB diastereomers have not yet been determined by crystallization, they can be established by comparing the enzymatic selectivity with those of the corresponding NTPαS analogues. It is well-known that enzyme catalysis is generally stereospecific. For phosphorothioates, the S_p-dNTPαS form is more active with DNA polymerases[186] and only the diastereomer with the R_p configuration is a substrate for snake venom phosphodiesterase (SVPDE).[187] Assuming that dNTPαB analogues have the same stereospecificity toward DNA polymerases as that of dNTPαS analogues and keeping in mind that the sulfur in phosphorothioates is the largest atom around the phosphorus center while the BH_3 is the smallest group around the phosphorus center in boranophosphates (thus the S_p configuration in dNTPαS corresponds to the R_p one in dNTPαB), it can be inferred that the first eluted peak in the HPLC profile has the R_p configuration, because this

Scheme 28



isomer is incorporated much more effectively into DNA by DNA polymerases than the isomer which eluted second.[32,88] Moreover, the fact that the FE isomer of dNTPαB is completely resistant to SVP is in accordance with its assignment as the R_p configuration.[28] Similarly, the FE isomer of rNTPαB in RP-HPLC is assigned as the R_p configuration because it can be incorporated into RNA by T7 and SP6 RNA polymerases.[27,33] In summary, based on the comparison with NTPαS, the assignments of the configurations for the FE and SE isomers of NTPαB in RP-HPLC are R_p and S_p, respectively.

5.3. Substrate Properties of NTPαB Analogues with Viral RTs and PK

5.3.1. Substrate Properties of dNTPαB Analogues with Viral RTs and DNA Polymerases

To better understand the effect of α-*P*-boranophosphate substitution on the substrate properties of dNTPαB analogues, the steady-state kinetics of single-nucleotide incorporation of natural dNTPs and their α-*P*-borano and α-*P*-thio analogues have been investigated with viral RT and DNA dependent DNA polymerases. The results are summarized in Table 6.[188]

The incorporation efficiency of the R_p-dNTPαB isomer is 1.2-fold higher than that of the corresponding native dNTP with HIV-1 RT while 3- and 5-fold lower than that of the corresponding native dNTP with the bacterial DNA polymerases Klenow and Taq, respectively. Moreover, R_p-dNTPαB and S_p-dNTPαS show opposite effects on the incorporation efficiency with HIV-RT and the Klenow fragment, although they are stereochemically equivalent. The R_p-dNTPαB analogues are better substrates than the S_p-dNTPαS analogues with HIV-1 RT and slightly poorer substrates with Klenow. The R_p-dTTPαB diastereomer is a good substrate for T7 Sequenase ($K_m = 1.6$ μM), and the S_p-dTTPαB is not a substrate, but a poor competitive inhibitor of native triphosphate dTTP ($K_i = 61$ μM). These distinct behaviors make the R_p-dNTPαB analogues promising tools for investigation of the mechanism of phosphoryl transfer reactions catalyzed by viral RT and DNA dependent DNA polymerases.[188]

Table 6. Steady-State Kinetic Constants of Incorporation of Native, Boranophosphate, and Thiophosphate dNTP Analogues by HIV-1 RT, Klenow, Taq, and T7 Sequenase DNA Polymerases[a,b]

enzyme	dNTP	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	SI[c]
HIV-1 RT	dATP	0.05	0.69	13.8	1
	R_p-dATPαB	0.03	0.57	19	1.38
	S_p-dATPαS	0.05	0.63	12.6	0.91
	dTTP	0.05	0.73	14.6	1
	R_p-dTTPαB	0.04	0.67	16.8	1.15
	S_p-dTTPαS	0.10	0.82	8.2	0.56
Klenow	dATP	0.04	13.5	338	1
	R_p-dATPαB	0.04	5.0	125	0.37
	S_p-dATPαS	0.06	7.9	131	0.39
	dTTP	0.027	7.8	289	1
	R_p-dTTPαB	0.03	3.4	113	0.39
	S_p-dTTPαS	0.02	3.2	160	0.55
Taq	dATP	3.5	0.067	0.019	1
	R_p-dATPαB	13.5	0.029	0.0021	0.11
	dTTP	11	0.27	0.025	1
	R_p-dTTPαB	53	0.12	0.0023	0.092
T7 Sequenase	dTTP	2.9	0.34	0.12	1
	R_p-dTTPαB	1.6	0.07	0.044	0.37
	S_p-dATPαB	$K_i = 61$ μM			

[a] The kinetic constants K_m and k_{cat} were determined by a labeled primer-template/polyacrylamide gel assay (Boosalis, M. S.; Petruska, J.; Goodman, M. F. *J. Biol. Chem.* **1987**, *262*, 14689). [b] Data are taken from ref 188. [c] The selectivity index (SI) is determined by $SI = (k_{cat}/K_m)_{dNTP\alpha B}/(k_{cat}/K_m)_{dNTP}$.

The good substrate properties of the R_p-dNTPαB diastereomers to viral RTs allowed us to develop an efficient synthesis of stereoregular *all*-(S_p)-boranophosphate oligodeoxyribonucleotides.[57]

5.3.2. Substrate Properties of ddNTPαB Analogues with Viral RTs and DNA Polymerases

Viral DNA polymerases (i.e., RTs) are responsible for replicating the viral genome and, therefore, for virus propagation.[189] Currently used antiviral agents include 2′,3′-dideoxynucleoside (ddN) analogues, such as AZT or d4T. After cell penetration, the nucleoside analogues undergo a stepwise intracellular phosphorylation to form metabolically active triphosphate ddNTPs that interact at the substrate binding site of polymerases, where they act as competitive inhibitors or chain terminators.[189]

5.3.2.1. Activities of ddNTPαB Analogues with Viral RTs and DNA Polymerases. Recently, nucleoside boranophosphates have received great attention as a new type of nucleotide analogue. Biochemical studies showed that the substitution of a BH_3 group for a nonbridging oxygen yielding an α-R_p-phosphate actually enhances the rate of phosphorylation by NDPK[39,40] and makes the product a more efficient RT inhibitor.[39−46] Examination of crystal structures of nucleotide substrates bound either to NDPK[40] or to DNA polymerases such as HIV-1 RT[190] and bacteriophage T7 DNA polymerase[191] shows that their NDP moieties have similar conformations, which include the conformations of the α- and β-phosphates and the position of one of the bound Mg^{2+} ions. The similarity is especially striking in the case of T7 polymerase, which is a higher resolution (2 Å) structure than that for RT, making the details of the nucleotide

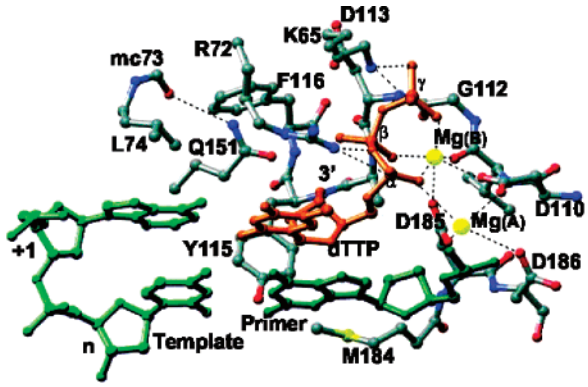


Figure 18. Structure of the HIV-1 RT active site. Template and primer strands, light and dark green, respectively; dTTP, gold; Mg^{2+} ions, yellow spheres; assigned hydrogen bonds and metal ligand interactions, dashed lines. (Reprinted with permission from Huang, H. F.; et al. *Science* **1998**, 282, 1669. Copyright 1998 AAAS (http://www.aaas.org).)

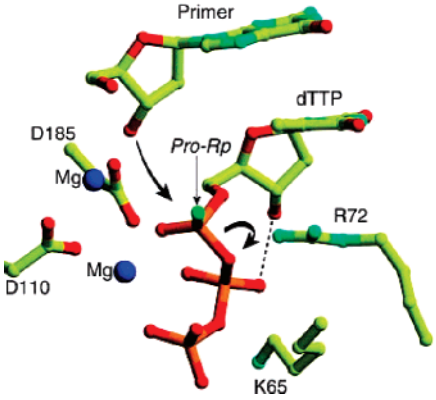


Figure 19. Postulated chemical reaction at the HIV-1 RT active site. Crystal structure of RT in complex with a double-stranded DNA primer/template and a nucleotide. The dTTP molecule and the amino acid side chains are represented in atom-type colors. Magnesium ions are represented as blue spheres. One nonbridging oxygen has been designated pro-R_p (green), as in the case where it is substituted with the BH_3 group, resulting in a chiral phosphorus. The hydrogen bond between the 3′-OH of the incoming dTTP and one oxygen on the β-phosphate is shown as a dashed line. (Reprinted with permission from ref 46. Copyright 2001 The American Society for Biochemistry and Molecular Biology, Inc.)

conformation and the Mg^{2+} binding mode more reliable. Figure 18 illustrates the binding between dTTP and RT. The triphosphate wraps around one of the Mg^{2+} ions (designated as B), for which there is strong density in the map.[190] A nonbridging oxygen from each of the phosphates (pro-R_p for α and β) contributes to the octahedral coordination of this metal. In all three enzymes (NDPK, HIV-1 RT, and bacteriophage T7 DNA polymerase), the same oxygen of the α-phosphate ligates the metal, explaining why the other α-phosphate oxygen can be modified without interfering with activation by NDPK or incorporation into DNA by RT.

Potent inhibition of RT by α-borano analogues of AZT and d4T triphosphates was observed with the R_p-diastereomers, whose apparent inhibition constant K_i, determined in triplicate, was decreased by a factor of 2.2−10 compared with those of their parent triphosphates.[40] In steady-state kinetics, the α-borano analogues could increase the catalytic efficiency of incorporation (k_{cat}/K_m) by 9- and 3-fold for AZTTPαB and d4TTPαB, respectively, with HIV-1 RT[40] and by 28-fold for ddCTPαB with moloney murine leukemia virus (MMLV) RT,[192] yet a slight decrease in the efficiency for ddCTPαB with Taq DNA polymerase was observed (Table 6).[192] In pre-steady-state kinetics, the catalytic incorporation efficiency k_{pol}/K_d was ~50% higher with HIV-RT when the borano group was present.[39,40] Moreover, an increase of 9-fold was observed in k_{pol}/K_d when the diastereomeric mixture of acycloTTPαB was used as the substrate for MMLV-RT.[48,113] Together, these data are presented in Table 7, and collectively, these results support the conclusion that the presence of an α-*P*-BH_3 in triphosphate analogues can increase the incorporation efficiency of the R_p diastereomer by viral RTs[39,40,48,188,192] yet has minimal effect on the incorporation efficiency by bacterial DNA polymerases.[188,192]

Examination of the crystal structure of the RT·DNA·dNTP ternary complex[190] (Figure 19) shows that the 3′-OH of the incoming nucleotide makes an intramolecular hydrogen bond with one nonbridging oxygen of the β-phosphate.[46] In this manner, the nucleotide is held in the active site so as to facilitate the in-line attack of the 3′-OH of the primer onto the α-phosphate of the incoming nucleotide (dTTP). Lysine 65, arginine 72, the main chain nitrogen of alanine 114, and two magnesium ions stabilize the triphosphate moiety.[190] However, the intramolecular hydrogen bond does not exist when a ddNTP is incorporated, and this lack of interaction may explain why ddNTPs are less efficient substrates for incorporation than dNTPs.[46] Interestingly, a similar intramolecular hydrogen bond-activating phosphoryl transfer also exists in NDPK.[40] In this case, the 3′-OH of the phosphorylated nucleoside diphosphate forms a hydrogen bond to the oxygen bridging the β,γ-phosphate and activates it,

Table 7. Steady-State and Pre-steady-state Kinetics of Triphosphate Analogues with Viral (HIV-1 and MMLV) RTs and Bacterial DNA Polymerase (Taq)[a]

			steady-state			pre-steady-state		
	α-*P*-substituent[b]		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_d (μM)	k_{pol} (s^{-1})	k_{pol}/K_d ($\mu M^{-1}\ s^{-1}$)
HIV-1RT	AZTTP	α-O	2.4	0.08	0.033	7.1	12.8	1.8
		α-BH_3	0.2	0.06	0.30	7.6	18.4	2.4
	d4TTP	α-O	1.2	0.055	0.046	21.3	10.8	0.51
		α-BH_3	0.44	0.056	0.127	18.7	16	0.85
MMLV-RT	ddCTP	α-O	93	0.014	0.15			
		α-BH_3	14.6	0.06	4.1			
	acycloTTP	α-O				90	0.013	1.4×10^{-5}
		α-BH_3				36	0.046	12.8×10^{-5}
Taq	ddCTP	α-O	42.6	0.0007	0.017			
		α-BH_3	36.2	0.0005	0.015			

[a] Data are taken from refs 39, 40, 48, 188, and 192. [b] R_p-α-*P*-BH_3 analogues were used.

suggesting why ddNDPs (which lack a 3′-OH) are 10^4-fold less efficient substrates than dNDPs.

5.3.2.2. Drug Resistance of ddNTPαB Analogues to Multidrug-Resistant Viral RTs. In the treatment of AIDS, the prolonged use of nucleoside analogues leads to drug-resistant and often multidrug-resistant viruses. Two types of RT-mediated drug resistance mechanisms have been characterized so far. The first type of resistance mechanism involves excision repair of the nucleotide analogues from the viral DNA chain (unblocking), using PPi or ATP as a cosubstrate for a pyrophosphorolysis reaction.[193,194] A substantial decrease in the pyrophosphorolysis rate was mentioned for removal of an α-thio-AZTMP residue by wild-type HIV-1 RT, compared to the parent AZTMP.[39] Mutations in RT such as D67N, K70R, T215F, or K219Q (referred to as thymidine analogue-associated mutations) increase the unblocking activity in the case of resistance to AZT and d4T.[195,196] When the multidrug-resistant RT carrying the aforementioned mutations was used, a 2-fold increase in the rate of pyrophosphorolysis was observed with AZTMP, whereas a 9-fold decrease of pyrophosphorolysis was observed with the borane analogue of AZTMPαB.[39,40] We propose that less electronegative substituents such as sulfur or BH_3 decrease the positive charge at the α-phosphorus atom and may retard the nucleophilic attack of phosphorus by pyrophosphate. For example, the 3′→5′ borano- and thiophosphodiester bonds are resistant to enzymatic hydrolysis by nucleases.[87,197]

The other type of resistance mechanism involves a selective recognition of the natural nucleotide over the analogue at the RT active site. Discrimination of the resistance mechanism for nucleotide inhibitors can be achieved either through a selective decrease of their binding at the RT active site (reflected by an increase in the binding equilibrium constant K_d) or at the catalytic step of incorporation of the analogues into viral DNA (reflected by a decrease in the k_{pol} value, the catalytic constant of incorporation of nucleotide analogues into DNA). These mechanisms have been typically observed with mutations at K65R, Q151M, and M184V.[46,198,199] As depicted in Figure 20, numerous studies have shown that the presence of α-*P*-BH_3 in AZTTP,[45] ddATP,[46] d4TTP,[43] and 3TCTP[43] can reduce the resistance caused by mutant RTs 3- to 107-fold. This *in vitro* loss of resistance is mainly the result of a conserved efficiency of catalysis (measured with k_{pol}), in which the recovery of sensitivity is independent of the substitutions of amino acids within the active site of RT.

Besides the spectacular improvement in the values of incorporation rate constant provided by the R_p-α-*P*-BH_3 group using drug-resistant RTs, it is quite remarkable that the adjunction of the R_p-α-*P*-BH_3 group to 3TCTP improves the poor incorporation properties of this drug by 7-fold,[43] which is of major clinical relevance even for wild-type (wt) RT. For comparison, for another type of modification of the phosphate group, such as a phosphonate in which an oxygen is replaced by a methylene group between the phosphate and ribose, RT mediated incorporation into DNA proceeds very poorly because of a 1800-fold lower k_{pol}.[200]

The BH_3 group thus shows the capability to compensate for a mutation that induces a defect in the nucleotide incorporation rate k_{pol}. In the mechanistic understanding of the α-boranophosphate rate enhancement effect, these results are important because they show that this effect is not necessarily linked to drug resistance suppression. Rather, the

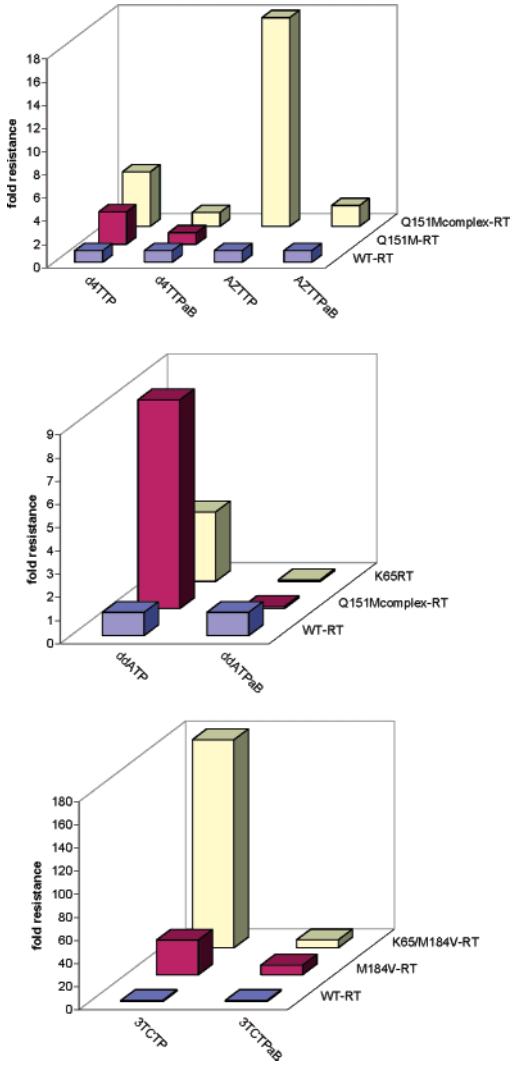


Figure 20. Resistance of wt and mutant HIV-1 RTs to triphosphate and its R_p-α-*P*-BH_3 analogue. The presence of the R_p-ddNTPαB isomers reverses the resistance of mutant HIV-1 reverse transcriptases. Data are taken from refs 43, 45, and 46.

α-boranophosphate group at the RT active site gives the nucleotide properties of incorporation quite independent from the nature of the catalytic amino acid side chains.[43,45] Therefore, one mechanism proposed is that the negative charge introduced by the BH_3 group at the pro-R_p position is the driving force for pyrophosphate (PPi) release.[46] The presence of the BH_3 group, although less electronegative than oxygen, is repulsive for the newly created negative charge on the α,β-phosphate bridging oxygen, and thus promotes departure of the PPi molecule from the pentacoordinated transition state complex. In this manner, bond scission between α- and β-phosphate groups becomes less dependent on interactions with catalytic amino acid residues.

Canard et al. have observed a general increase in the affinity for Mg^{2+} during the pre-steady-state kinetics of incorporation of antiviral nucleoside boranotriphosphates by wt HIV-1 RT (3−8-fold), which means that Mg^{2+} is statistically more present in the active sites with a boranophosphate than in those with its corresponding parent phosphate.[43] In other words, the introduction of the BH_3 group makes the R_p-AZTTPαB and R_p-d4TTPαB isomers considerably better substrates than their parent triphosphates at low Mg^{2+} concentrations. It is reasonable to note that the K_d (0.18 μM) for binding of Mg^{2+} in the presence of

dTTPαB is 8-fold less than that in the presence of native dTTP,[43] and is very close to the intracellular Mg^{2+} concentration (0.1−0.3 μM).[201] This better affinity may account for the observed R_p-BH_3-mediated catalytic rate increase. *Ab initio* calculations indicate that the negative charge located on the nonbridging oxygen in the dimethylboranophosphate anion (−1.27) is slightly higher than that of the dimethylphosphate anion (−1.26).[64] Because of the electron donating effect of the BH_3 group, it is reasonable to propose that, in the presence of a strong electrophile such as Mg^{2+}, an additional increase of the negative charge on the oxygen in the dimethylboranophosphate anion would be observed relative to that in the dimethylphosphate anion. This may explain the higher affinity of Mg^{2+} to the complex of HIV-1 RT and R_p-dNTPαB diastereoisomer. Further mechanistic studies are required to fully understand the functions of boranophosphates.

In summary, due to their potential applications in the suppression of RT-mediated drug resistance, boranophosphate analogues will open a new chapter in antiretroviral drug design.

5.3.3. Binding Affinity of NTPαB Analogues with Rabbit Muscle PK

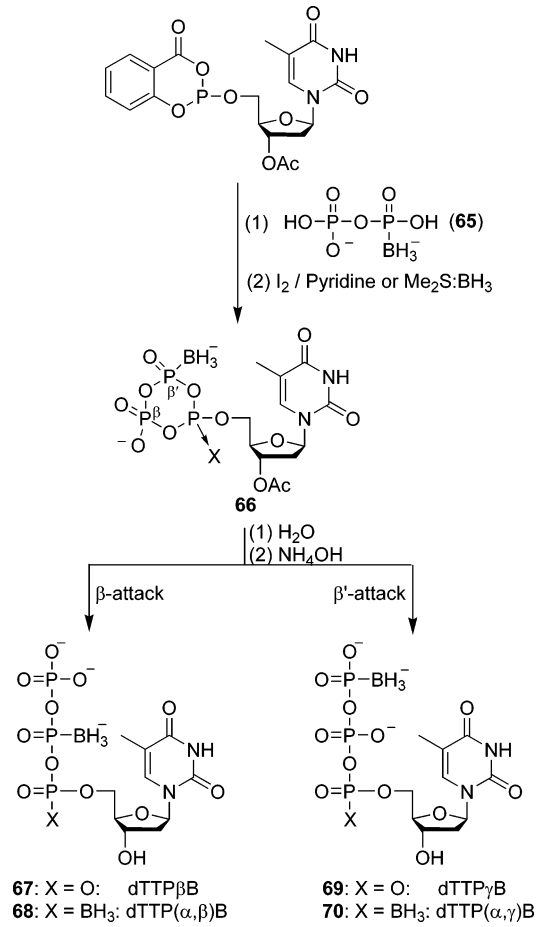
It was recently suggested that PK may be responsible for the last step of phosphorylation of 2′,3′-dideoxynucleoside diphosphates.[165] As described in the above section, ddNTPαB analogues proved to be better chain terminators for viral RTs than their parent ddNTPs. Hence, study of the binding affinity of boranotriphosphates with PK will provide critical information regarding the last phosphorylation of the borano analogues. Shaw et al. used a fluorescence quenching technique to quantitatively characterize each interaction and determine its importance for binding affinity.[202] It has been found that triphosphate analogues bound with tetrameric PK with negative cooperativity.[202] The structure of the NTP analogues strongly affects the first binding mode but has a smaller effect on the second binding mode. The structure−activity relationship has been obtained with different NTP analogues.[202] Replacement of the nucleobase adenine by guanine or cytosine decreases the stability of the substrate−enzyme complex by ∼1.45 kcal/mol. Each 2′- or 3′-OH group contributes only 0.1−0.5 kcal/mol in stabilizing the substrate−enzyme complex, but the absence of both hydroxyls destabilizes the complex by 1.1−1.6 kcal/mol. Replacement of the oxygen between β- and γ-phosphates by a methylene group destabilizes the GDP(CH_2)P−PK complex by 0.8−0.9 kcal/mol relative to the GTP-PK complex. The α-*P*-BH_3 modification decreases the binding affinities of dNTPαB and rNTPαB analogues by 0.2−0.6 kcal/mol relative to those of the naturally occurring nucleoside triphosphates, but it increases the binding affinity of 2′,3′-ddCTPαB compared to the corresponding 2′,3′-ddCTP. In addition, no significant stereospecifity was observed for binding of PK to boranotriphosphate analogues.[202]

5.4. Synthesis of Borane-Containing Novel Triphosphate Analogues

5.4.1. Synthesis of β-*P*-BH_3- and γ-*P*-BH_3-Modified Triphosphates and Diboranotriphosphates

The method developed by Shaw et al.,[82,83] based on modification of the Ludwig−Eckstein procedure,[152] has proven to be powerful in synthesizing a wide range of boranotriphosphate analogues. By replacing the pyrophosphate with boranopyrophosphate **65**, our lab recently obtained a series of boranotriphosphate analogues depending on the position of attack by water in cyclophosphate intermediate **66**. As depicted in Scheme 29, thymidine 5′-(β-*P*-borano)-triphosphate (dTTPβB) **67** and 5′-(α,β-*P*-diborano)triphosphate [NTP(α,β)B] **68** are formed when the water nucleophile attacks the β-position of **66**. Similarly, attacks by water at the β′-position of **66** produce thymidine 5′-(γ-*P*-borano)-triphosphate (NTPγB) **69** and 5′-(α,γ-*P*-diborano)triphosphate [NTP(α,γ)B] **70**. The preparation of boranopyrophosphate **65** is described earlier in Scheme 21.

Scheme 29



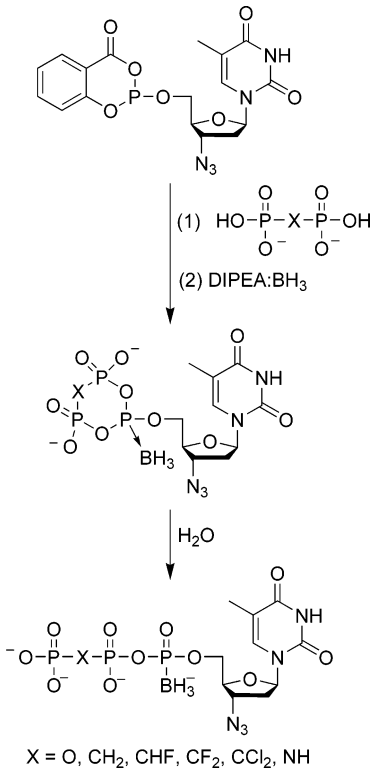
5.4.2. Synthesis of NTPαB Analogues with a Modified β,γ-Bridge

Modifications at the bridging oxygen atom in pyrophosphate will produce boranotriphosphate analogues with a modified β,γ-bridge. This type of compound was specifically developed to have enhanced enzymatic stability toward dephosphorylating enzymes. As shown in Scheme 30, employment of methylenediphosphonate, halomethylenediphosphonates, and imidodiphosphate, instead of pyrophosphate, as the coupling reagent gave the corresponding AZT 5′-α-*P*-boranotriphosphate analogues with β,γ-methylene, β,γ-halomethylene, and β,γ-imido bridges, respectively.[47,49]

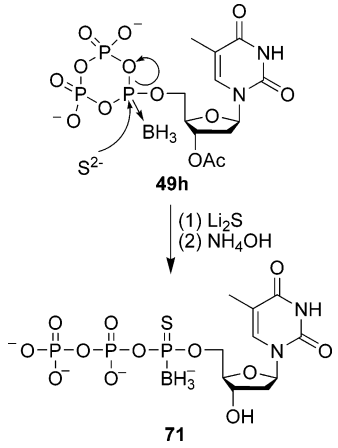
5.4.3. Synthesis of α-*P*-Doubly Modified Boranothiotriphosphate Analogues

Synthesis of *P*-doubly modified nucleoside triphosphate analogues, in which the two nonbridging oxygen atoms of a

Scheme 30

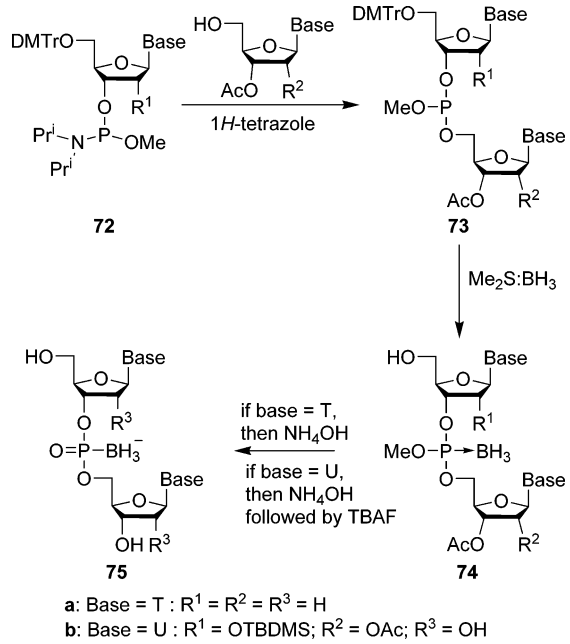


Scheme 31



phosphodiester group are replaced with a borane group and a sulfur atom, was accomplished by treatment of the cyclic boranotriphosphate intermediate **49h** with Li_2S at 55 °C for 1 h.[156,185] As depicted in Scheme 31, instead of attacking the phosphorus atom at the β position as in most cases,[49,82,85,152] the nucleophilic ring opening reaction by the S^{2-} ion occurred exclusively at the P^{α} bearing the borane group. The final compound thymidine 5′-(α-*P*-borano, α-*P*-thio)triphosphate **71** (dTTPαBS) was obtained in 26% yield.[156,185] The ^{31}P NMR spectra in D_2O features three characteristic resonances at around δ 153 ppm (br) for α-*P*, δ −10 ppm (d) for γ-*P*, and δ −22 ppm (dd) for β-*P* (H_3PO_4 as external standard). Preliminary studies of dTTPαBS with Taq DNA polymerase have shown that, while the second eluting HPLC isomer is not a substrate, the first eluting one is a substrate for Taq DNA polymerase, albeit with only 10% efficiency compared with normal dTTP.[185]

Scheme 32



6. Dinucleoside Boranophosphates

A new class of nucleic acid analogues, oligonucleoside boranophosphates bearing internucleotidic boranophosphate linkages, is now the subject of growing interest as promising tools in DNA sequencing,[32] in antisense[60] and therapeutic applications,[18,20] and in BNCT.[18,20] Dinucleoside boranophosphates represent the simplest class of this type of oligonucleotide molecule. Studies on the synthesis, stereochemistry, and biological properties of these simple boranophosphate dimers provide insight into the chemistry of oligonucleoside boranophosphates and help us to better understand them.

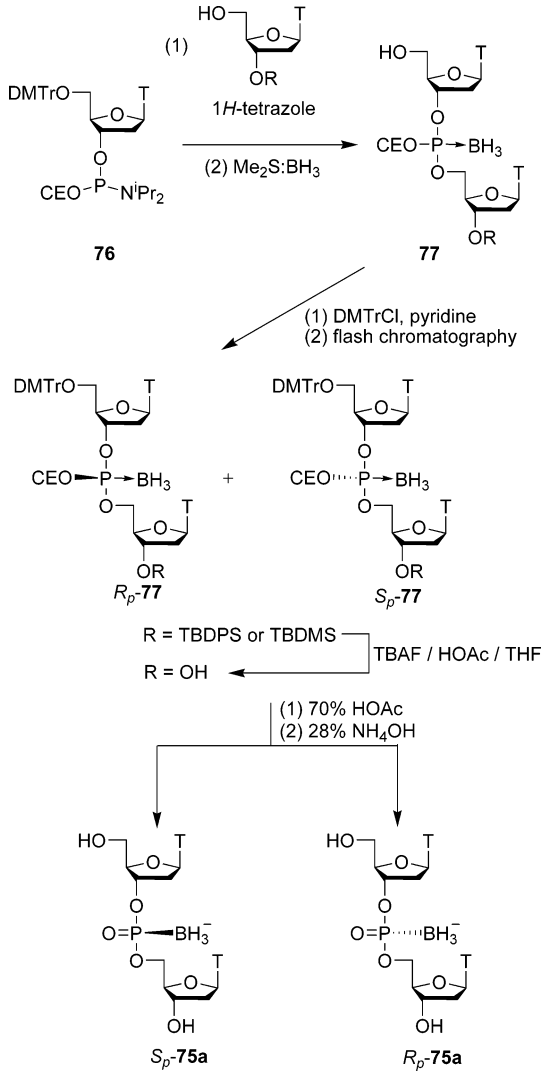
6.1. Synthesis of Dinucleoside Boranophosphates

6.1.1. Synthesis via a Phosphoramidite Approach

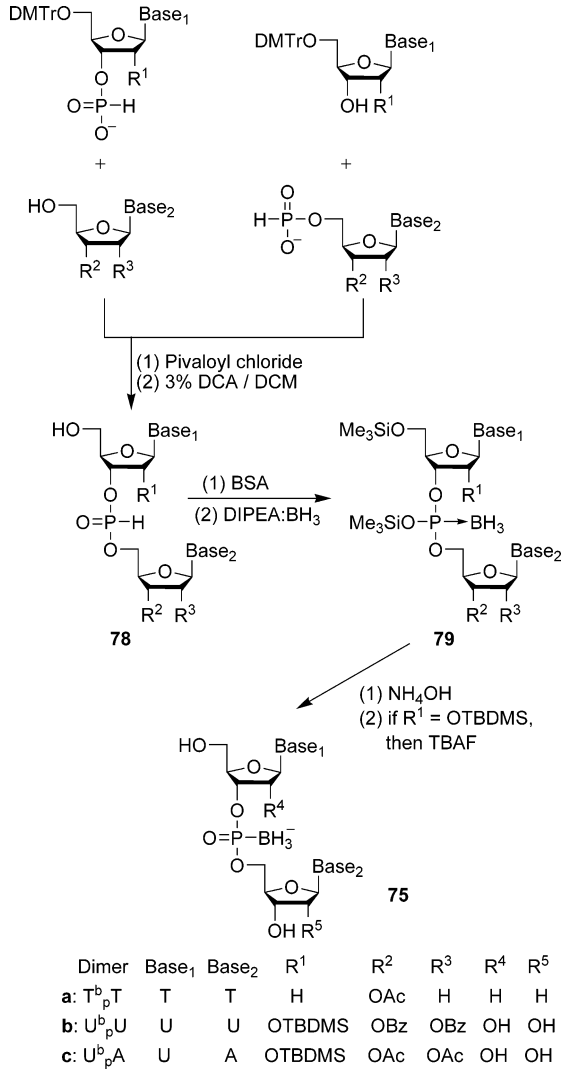
The first synthesis of dithymidine boranophosphate (d(T^{b}pT), the simplest oligodeoxyribonucleotide analogue) **75a** and its methyl ester was accomplished in 1990.[25] The same procedure was later employed to successfully prepare the diuridine boranophosphate (r(U^{b}pU), the simplest oligoribonucleotide analogue) **75b**.[80] The synthesis was performed using the phosphoramidite approach outlined in Scheme 32.

Reaction of 5′-DMTr-nucleoside phosphoramidite **72** with a free 5′-OH nucleoside in the presence of 1*H*-tetrazole resulted in the formation of an intermediate, phosphite triester **73**, which was then converted to the dinucleoside boranophosphate methyl ester **74** (boranophosphotriester) by oxidation with dimethyl sulfide-borane. Both steps could be easily followed by ^{31}P NMR. In the first step, the phosphoramidite peaks at δ 148.8 and δ 148.4 ppm were replaced by new phosphite peaks at δ 140.4 and δ 139.8 ppm for **73**. In the second step, the peaks of phosphite **73** disappeared within 5−10 min and a broad peak at 118.0 ppm for boranophosphotriester **74** was observed. The oxidation step with Me_2S:BH_3 conferred another advantage, in addition to the formation of the desired boranophosphate linkage, it also removed the 5′-DMTr protecting group and hence eliminated the need for an extra 5′-deprotection step that was required for chain

Scheme 33



Scheme 34



Dimer	$Base_1$	$Base_2$	R^1	R^2	R^3	R^4	R^5
a: T^b_pT	T	T	H	OAc	H	H	H
b: U^b_pU	U	U	OTBDMS	OBz	OBz	OH	OH
c: U^b_pA	U	A	OTBDMS	OAc	OAc	OH	OH

elongation.[25] After treatment with concentrated ammonium hydroxide (and tetrabutylammonium fluoride), the dinucleoside boranophosphate **75** was obtained as a mixture of diastereomers, whose ^{31}P NMR had a characteristic broad peak at δ 94.5 ppm.

Jin and Just described an analogous method for the synthesis of d(T^b_pT) **75a** using *P*-cyanoethyl-protected 5′-DMTr-thymidine phosphoramidite **76** as the starting compound (Scheme 33).[203] In this case, the authors made an attempt to separate the diastereomers of **77** immediately after boronation, but it appeared to be possible only after reinstallation of the 5′-DMTr protecting group.[203] Diastereomers R_p-**77** and S_p-**77** were isolated by silica gel flash chromatography in 40−45% yields, and then each diastereomer was separately converted into an unprotected boranophosphate dimer (R_p-**75a** and S_p-**75a**) in 50−60% yields. Since the β-elimination leading from boranophosphate cyanoethyl ester **77** to the free boranophosphate **75** does not involve any change in stereochemistry at the phosphorus atom, all intermediates have stereochemistry as depicted in Scheme 33.

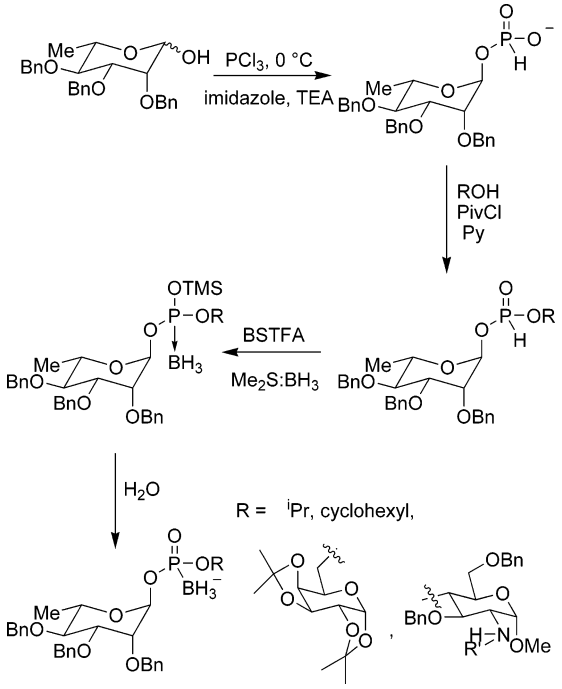
6.1.2. Synthesis via an H-Phosphonate Approach

The H-phosphonate approach has certain advantages over the phosphoramidite method—it does not require additional phosphorus protection and can produce intermediates that are more effective in the borane exchange reaction.[118] Silylation of H-phosphonates results in the formation of reactive silyl phosphite that can be *in situ* converted into boranophosphates using mild boronation agents.[37,56,59]

As described in Scheme 34,[204] the starting compound dinucleoside H-phosphonate diester **78** can be obtained either from 3′-H-phosphonate and 5′-OH nucleoside or from 5′-H-phosphonate and 3′-OH nucleoside. Interestingly, in the preparation of diuridine H-phosphonate, both methods produce one diastereomer (S_p, ^{31}P NMR δ = 10.8 ppm) more favorably over the other one (R_p, ^{31}P NMR δ = 9.8 ppm).[204,205] In the case of the condensation between uridine 3′-H-phosphonate and 5′-OH uridine, only the S_p isomer of diuridine H-phosphonate was observed. When uridine 5′-H-phosphonate was reacted with 3′-OH uridine, the ratio of S_p and R_p for diuridine H-phosphonate was 3:2. Because of the possible incompatibility between DMTr and the BH_3 group,[23,58] DMTr was removed before the reaction proceeded to the next step.

Conversion of the H-phosphonate diester **78** to an activated phosphite triester was achieved by silylation with BSA, whose progress was monitored by ^{31}P NMR with the appearance of peaks at δ 130.7 and 130.8 ppm corresponding to the diastereomers of the phosphite triester. *In situ* boronation with excessive DIPEA:BH_3 resulted in the formation of boranophosphotriester **79**, confirmed by a broad peak at δ 105.0 ppm in ^{31}P NMR.

Scheme 35



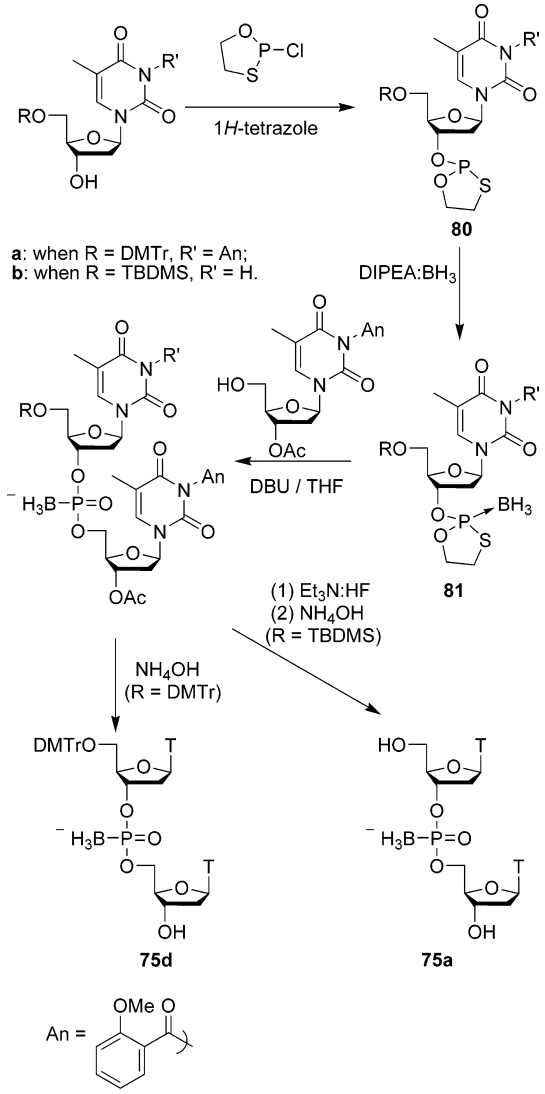
The final compound, dinucleoside boranophosphate diester **75**, was obtained in good yield after the removal of protection groups by NH_4OH (and TBAF). Its formation was characterized by the unique broad peaks at $\sim\delta$ 95 ppm in ^{31}P NMR. The H-phosphonate approach described here has been successfully employed to prepare DNA analogues[81,206] (e.g. d(T^bpT) **75a**) and RNA analogues[31,204] (e.g. r(U^bpU) **75b** and r(U^bpA) **75c**). Further optimization of conditions for silylation and boronation will allow solid-phase synthesis of oligonucleoside boranophosphates.[37,56,58,59]

As shown in Scheme 35, the H-phosphonate approach has also been employed to synthesize the saccharide boranophosphate dimers in good yields as stable analogues of 1-*O*-glycosylphosphates.[207] It was earlier found that the connection of the phosphodiester bridge at the anomeric position[208] gave rise to problems of chemical stability in capsular polysaccharides of bacteria. The instability due to this structural motif makes the polysaccharide-based vaccines containing this linkage labile and thus makes it difficult to store and manipulate related fragments in the formulation of glycoconjugate vaccine.[209,210] Therefore, boranophosphate was introduced as an attractive candidate for obtaining the stable synthetic analogue of the intersaccharidic phosphodiester bridges.[207]

6.1.3. Synthesis via an Oxathiaphospholane Approach

The oxathiaphospholane method, first introduced by Stec et al. for the stereocontrolled synthesis of oligonucleoside phosphorothioates,[146,147] was applied to the synthesis of d(T^bpT) **75a**. Unlike its thio-counterpart, the nucleoside-3′-*O*-oxathiaphospholane−borane complex **81** in Scheme 36 could not be chromatographically separated into diastereomerically pure species due to its instability to moisture. Therefore, complex **81** reacted with the 5′-OH of the nucleoside in the presence of DBU to form an internucleotidic boranophosphate linkage in a nonstereocontrolled fashion.[211] Specifically, the thymidine-3′-*O*-oxathiaphospholane synthon **80** could be prepared and purified by silica gel chromatography in 60−78% yields. After boronation with

Scheme 36

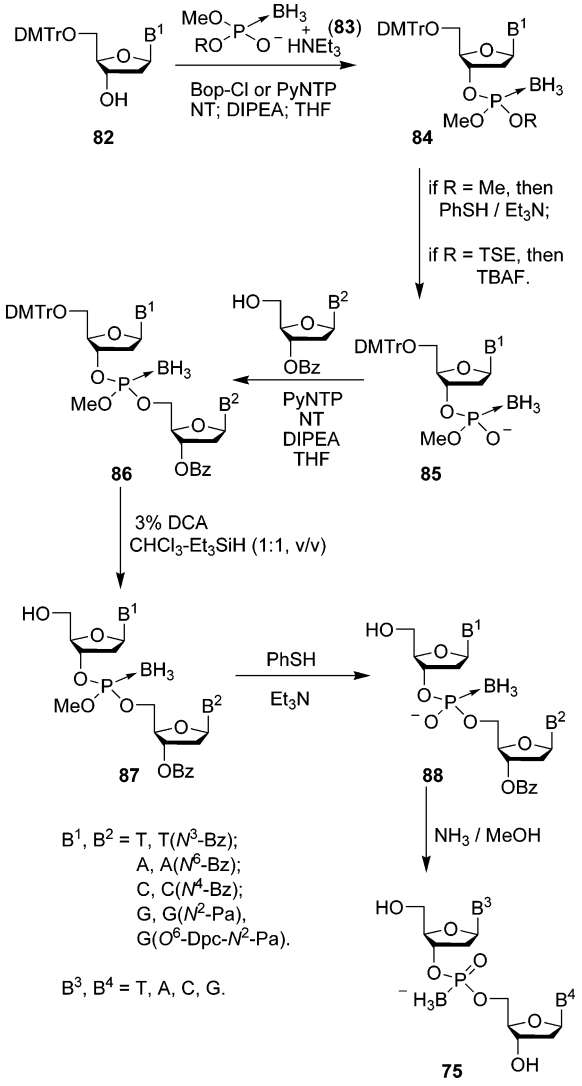


DIPEA:BH_3, the resulting complex **81**, whose signals appeared as a broad peak at δ 164.4 ppm in ^{31}P NMR, was immediately reacted with the 5′-OH of thymidine to form the dithymidine boranophosphate derivative **75** in 60−97% yield.

When the same reaction from **81a** to **75d** was performed under the conditions of solid-phase synthesis,[211] the best yield (68%) of dimer **75d** was obtained when THF was used as a solvent, with a 30-fold molar excess of both **81a** and DBU with respect to the support bound nucleoside, and the reaction time was restricted to 5 min. Further increase in the concentration of the reagents or prolongation of the reaction time did not lead to any yield improvement of condensation. A change of the solvent to acetonitrile resulted in a substantial decrease of the yield of **75d**. Therefore, although the formation of an internucleotidic boranophosphate linkage could be accomplished via an oxathiaphospholane approach both in solution and on the solid support, the yields for the solid support synthesis were far from being satisfactory.[211]

Further attempts to prepare the doubly-*P*-modified dinucleoside boranophosphorothioates $[P(=S)(BH_3)]^-$ by the dithiaphospholane approach, following an analogous procedure to that shown in Scheme 36, were unsuccessful. Although ^{31}P NMR analysis of the reaction mixture revealed some formation of the desired dithymidine boranophospho-

Scheme 37



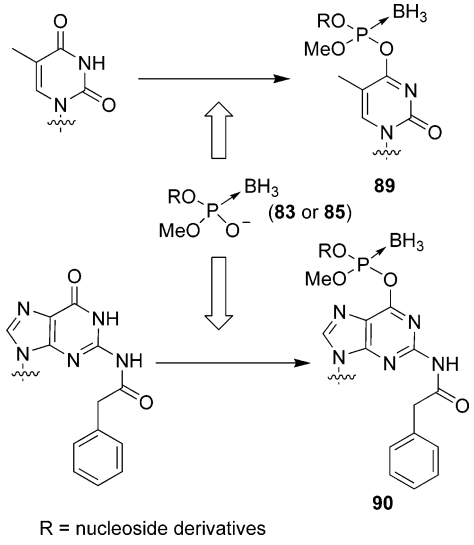
rothioate at δ 166 ppm, the yield was too low to isolate the compound.[211]

6.1.4. Synthesis via a Boranophosphotriester Approach

The aforementioned synthesis of dinucleoside boranophosphates involves trivalent phosphorus atoms that are further oxidized by boronating reagents. However, this boronation step might give rise to undesirable base modifications caused by the reduction of *N*-acyl protecting groups to the corresponding *N*-alkyl derivatives.[96,97] Therefore, in order to avoid the side reactions associated with the conventional synthesis, Wada et al. employed the boranophosphotriester approach shown in Scheme 37 as a new way to introduce the BH_3 group into the internucleotidic phosphate linkage in high yields.[212−214]

The 5′-DMTr-protected 2′-deoxynucleoside **82** with full base protection was boranophosphorylated by **83** in the presence of condensing reagent, 3-nitro-1,2,4-triazole (NT), and DIPEA in THF to yield the boranophosphate monomer **84**. Compounds such as *N*,*N*′-bis(2-oxo-3-oxazolidinyl)-phosphonic chloride (Bop-Cl) and 3-nitro-1,2,4-triazol-1-yltris(pyrrolidinyl)phosphonium hexafluorophosphate (PyNTP)[215] were used as the condensing reagents. Although the coupling yields were similar, the condensing reagent Bop-Cl gave rise to an HCl salt that was hardly soluble in the

Scheme 38



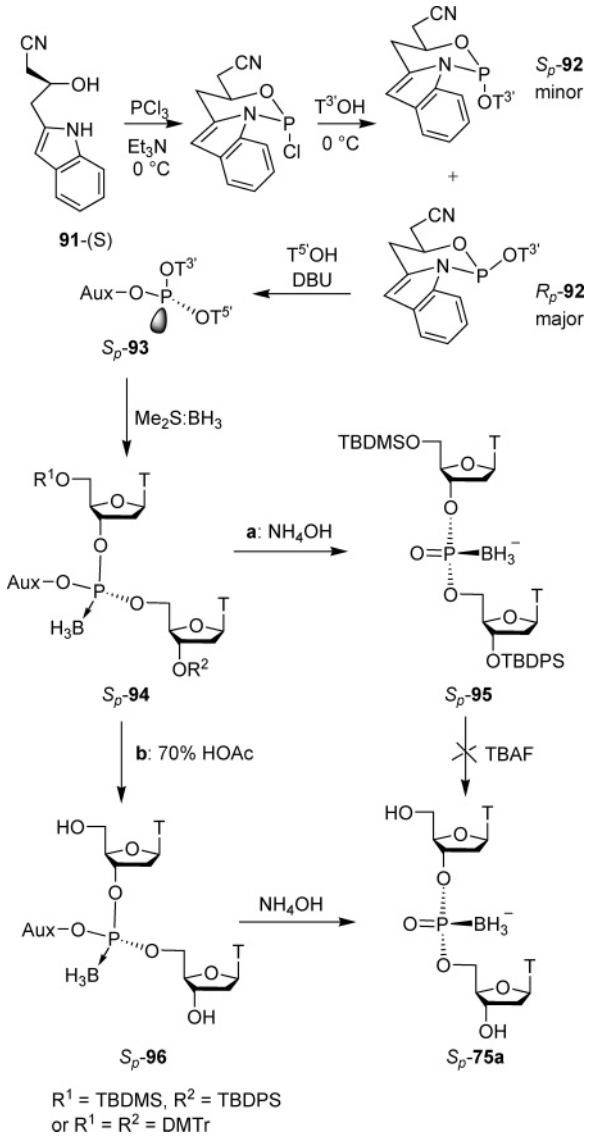
reaction solvent while PyNTP conveniently released the soluble nucleophilic catalyst NT *in situ* and did not form an insoluble HCl salt.[212] One of the phosphorus protecting groups on the purified boranophosphate monomer **84** was cleaved upon treatment with PhSH/Et_3N or TBAF. However, due to side reactions involving the loss of nucleobase protecting groups with PhSH/Et_3N, using a 2-(trimethylsilyl)-ethyl (TSE)[216]-protected boranophosphorylating reagent **83**, whose TSE group could be removed by TBAF without causing any nucleobase side reactions, is recommended. The coupling reaction between boranophosphate monomer **85** and 3′-Bz-2′-deoxynucleoside was accomplished using the same conditions as those for the formation of boranophosphate monomer **84**.[212]

It has been found that boranophosphate monomer **85** can cause side reactions with the nucleobase as shown in Scheme 38, which results in a substantial decrease in coupling yields for purine nucleoside derivatives due to the bulky purine bases in **90**.[212] Although the same side reactions could occur in the formation of boranophosphate monomer **84** between nucleosides **82** and boranophosphorylating reagents **83**, the side products would be quantitatively transformed into the desired compound **85** via hydrolysis of the P−O bond in **89** upon the aqueous workup of the reaction mixture. Removal of the protecting group 5′-DMTr in **86** was quick and successful in the presence of the scavenger triethylsilane, thus eliminating the decomposition of boranophosphates caused by the DMTr cation.[58,97] The final compound, dinucleoside boranophosphate **75**, was obtained in high yields for all four naturally occurring dNs after the sequential demethylation from the phosphorus atom in **87** with PhSH/NEt_3 and the removal of all protecting groups in **88** from the nucleosides with ammonia.[212] This boranophosphotriester approach was recently used for the solid-phase synthesis of unnatural oligonucleotide analogues (see section 7.1.1.2).

6.1.5. Stereocontrolled Synthesis of Dinucleoside Boranophosphates

Stereocontrolled synthesis of backbone-modified dinucleotides and oligonucleotides, for example, oligonucleoside phosphorothioates, is one of the most challenging tasks from the synthetic point of view.[217] This is also true for the boranophosphates, where the difficulties are encountered in

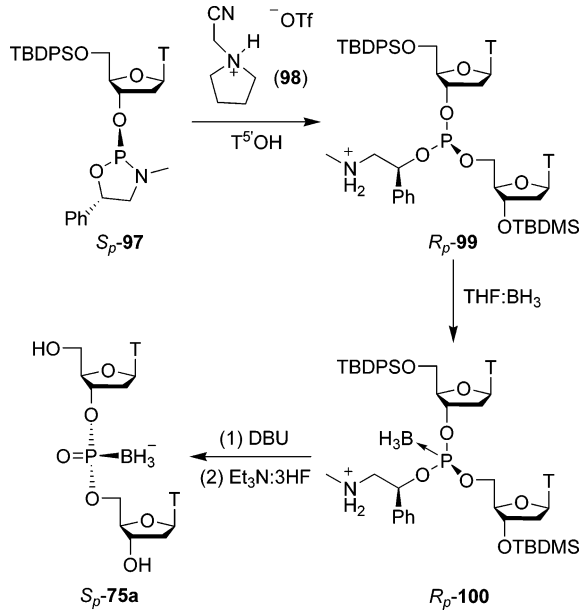
Scheme 39



incorporation of a BH_3 group with the given stereoselective procedures. Although several reports are available on this topic,[81,203,206] the methods all involve the preparation of diastereopure monomer units through time-consuming column separation of mixtures containing an equal amount of diastereomers. Moreover, S_p-stereoregulated fully and partially substituted boranophosphate DNA/RNA can be obtained by an enzymatic method using R_p-dNTPαBs/R_p-rNTPαBs.[34,35,37,38,87,88,218] However, R_p-stereoregulated boranophosphate DNA/RNA is not accessible by an enzymatic method because of the substrate specificity of the enzyme. Under these circumstances, the development of an efficient chemical synthesis of stereodefined boranophosphate DNA/RNA is of great importance. Until now, two approaches have been reported on the stereoregulated synthesis of boranophosphate dimers using the chiral auxiliaries indole-oxazaphosphorine[219] and oxazaphospholidine.[220]

6.1.5.1. Stereocontrolled Synthesis via Chiral Indole-oxazaphosphorine. As shown in Scheme 39, the diastereopure monomer can be obtained from appropriate enantiopure amino alcohols with excellent or complete diastereoselectivity. For example, the equatorial indole-oxazaphosphorine R_p-**92** is a preferred stereoisomer from amino alcohol **91**-(S), and R_p-**92** reacts much faster with the nucleophile

Scheme 40



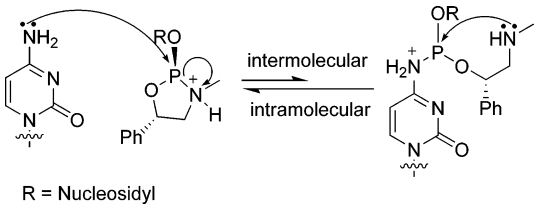
5′-OH-thymidine than does the axial one, S_p-**92**. When 3 equiv of **92** was treated with 1 equiv of a 5′-OH nucleoside, only one diastereomer, S_p-**93**, was obtained. The diastereoselectivity decreased to 10:1 (S_p-**93**:R_p-**93**) if the ratio between phosphite **92** and a 5′-OH nucleoside was 1:1. The diastereopure R_p-**93** could be obtained from **91**-(R) in the same manner. This strategy has also been employed to synthesize oligonucleoside phosphorothioates in a stereocontrolled fashion.[221−223] Extension of its utility in the synthesis of oligonucleoside boranophosphates still needs improvement in yields.

6.1.5.2. Stereocontrolled Synthesis via Chiral Oxazaphospholidine. The method developed by Wada et al. involves nucleoside 3′-*O*-oxazaphospholidine monomer units such as **97** and the less nucleophilic acid activator **98** as shown in Scheme 40.[220] The R_p- or S_p-phosphite triester **99** can be obtained in high yield with excellent diastereoselectivity.[221,222] This method has been successfully employed to stereoselectively prepare phosphorothioate DNA.[221,222] Boronation of phosphite triester **99** with THF:BH_3 complex resulted in boranophosphotriester **100**, which was then treated sequentially with DBU and Et_3N:HF to remove chiral auxiliary and ribose protecting groups, respectively. It is noted that both boronation[224,225] and removal of the chiral auxiliary[221,222] proceed with retention of the *P*-configuration. As an example shown in Scheme 40, boranophosphate dimer d(T^bpT) S_p-**75a** was prepared from S_p-**97** in 66% yield with a diastereomer ratio (dr) of 98:2.[220] Similarly, dimer d(T^bpT) R_p-**75a** was prepared from R_p-**97** in 63% yield with a dr of 96:4.[220]

This method was applied to the solid synthesis of diastereopure d(T^bpT) **75a**. Under the optimized reaction conditions, R_p-**75a** and S_p-**75a** were synthesized with the dr's of 98:2 (94% yield) and 99:1 (92% yield), respectively.[220]

It is noteworthy that, in the case of an unprotected nucleobase amino group, oxazaphospholidine monomers selectively react with a hydroxyl group over an amino group.[220] This is the first example of an *O*-selective phosphitylation, in which the chemoselectivity is independent of the activators. This unique chemoselectivity of the oxazaphospholidine derivatives could be explained by the intramo-

Scheme 41



lecular re-cyclization of the oxazaphospholidine ring, which would be much faster than the intermolecular nucleobase phosphitylations as depicted in Scheme 41.

It also has to be pointed out that a significant loss of diastereoselectivity occurred when using an unprotected nucleobase amino group in the oxazaphospholidine monomer to synthesize the boranophosphate dimer.[220] Although the exact cause of this phenomenon is still unclear, the intramolecular recyclization of the oxazaphospholidine ring could result in the loss of the diastereopurity at the phosphorus center (Scheme 41).

6.2. Studies on the Stereochemistry of the Internucleotidic Boranophosphate Linkage

The stereochemistry at phosphorus in *P*-chiral oligonucleotide analogues significantly affects their physicochemical characteristics and biological properties, such as lipophilicity, affinity to a complementary strand, and resistance to enzymatic digestion.[18] Implicit in the understanding of these properties is the importance of determining the absolute configuration of the boranophosphate linkage.

The two *P*-diastereomers of dithymidine boranophosphate were separated using reverse phase HPLC,[81,101,206] in which the fast- and slow-eluting isomers were designated as FE and SE, respectively. Several studies have been performed in order to establish their absolute configurations.[81,101,206] As seen from Figure 21, in the S_p configuration, the borane group

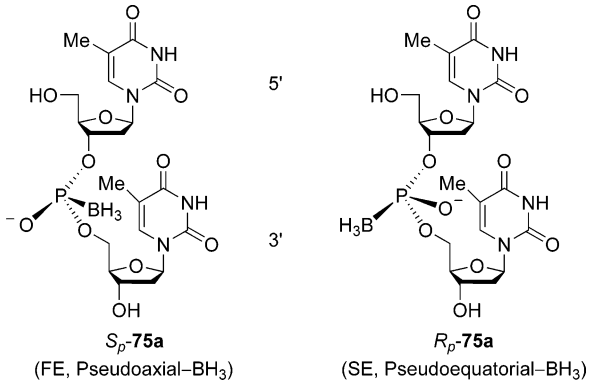


Figure 21. Stereochemical structures of dithymidine boranophosphate **75a**.

is directed toward the base stacking region and therefore is close to the H3′ of the 5′-residue in the dimer. However, in the R_p configuration, the borane group is directed away from the stacked bases and other protons of the dimer.[226] Hence, a large NOE effect between the BH_3 protons and the H-3′ of the 5′-residue is expected in the S_p configuration. This feature has been successfully employed to assign the absolute configurations of dinucleoside methylphosphonates using 1D NOE difference experiments[227] and 2D ROESY experiments.[226,228,229]

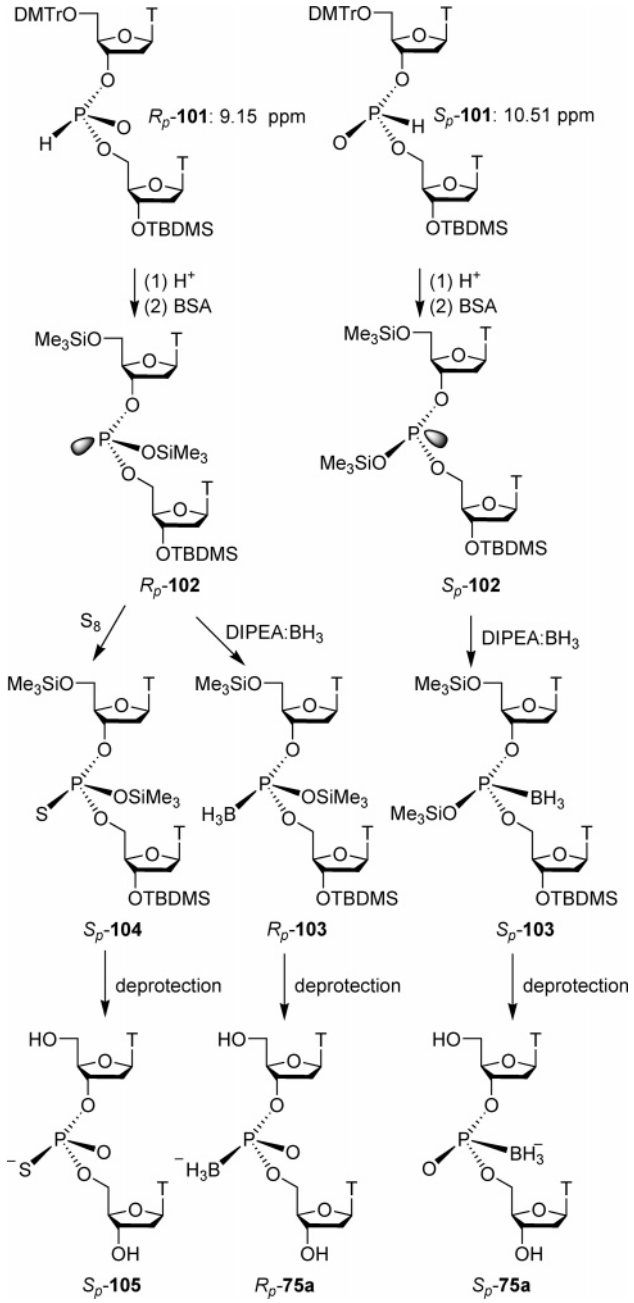
In the study of dithymidine boranophosphate, no conditions were found for ROESY to observe a cross-peak between the BH_3 protons and the H-3′ of the 5′-residue.[101] When 1D ^{1}H NOE difference experiments were carried out, irradiation of the H-3′ of the 5′-residue in FE resulted in a 6% increase in the intensity of the BH_3 proton resonance. Likewise, irradiation of the BH_3 protons resulted in a similar intensity increase of the H-3′ resonance of the 5′-residue in FE. Similar experiments with SE resulted in no apparent signal change. Therefore, the BH_3 group in FE was assigned to the pseudoaxial position or S_p configuration, while the BH_3 group in SE was assigned to a pseudoequatorial position or R_p configuration.[81] Unfortunately, the observed increase in FE 1D ^{1}H NOE difference experiments was not large enough to allow unambiguous assignment of the absolute configurations of the boronated phosphorus in the diastereomers.

The tentative assignment made by 1D ^{1}H NOE difference experiments was supported by enzymatic studies[29,30] similar to the work of Burgers and Eckstein.[187] It was shown that the substrate properties of dinucleoside phosphorothioate analogues correlated with their absolute configurations.[187] Enzymes such as SVPDE can differentiate between the two diastereomers of the internucleotidic phosphorothioate linkage by hydrolyzing one diastereoisomer more efficiently than the other. In particular, the hydrolysis reaction of dinucleoside phosphorothioates with SVPDE is highly specific for the R_p diastereomer.[187,230] Similarly, one of the two boranophosphate diastereomers, FE-**75a**, was found to act as a viable substrate for SVPDE.[29,30] Presuming that the enzyme utilizes only substrates in the same absolute configuration at phosphorus, the stereochemistry for dinucleoside boranophosphate diastereomers may be assigned by analogy. Noting that the configuration nomenclature for phosphorothioates is opposite to that for boranophosphates, an R_p configuration in phosphorothioates corresponds to a S_p configuration in boranophosphates. Thus, FE-**75a** was assigned as the S_p configuration and SE-**75a** was assigned as the R_p configuration. The same results were also obtained by treating the RNA analogue, diuridine boranophosphate, with SVPDE; the FE and SE isomers in HPLC were assigned as the S_p and R_p configurations, respectively.[80]

One additional procedure described for the assignment of the absolute configurations of dithymidine boranophosphate used chemical correlation via H-phosphonate intermediates.[206] This method was based on the findings that sulfurization and methylation of dinucleoside H-phosphonates occur stereospecifically and that dinucleoside H-phosphonate diesters can be conveniently separated into R_p and S_p diastereomers using silica gel flash chromatography.[225,231]

As outlined in Scheme 42,[206] the H-phosphonate dimer **101** was separated into diastereopure isomers R_p-**101** and S_p-**101** in the presence of the DMTr group, which was critical for the separation. The configurations of both diastereomers could be unambiguously assigned according to the ^{31}P NMR spectra.[225,231] To avoid the incompatibility between BH_3 and DMTr groups, DMTr was removed before proceeding to silylation followed by a borane exchange reaction, steps which were all expected to retain the configuration around the phosphorus atom.[224,225] The final hydrolysis of the silyl ester would not change the phosphorus configuration. Thus, starting with one diastereopure isomer of H-phosphonate **101** should afford only one diastereomer of the boranophosphate with the same absolute configuration.

Scheme 42



The experimental data were consistent with these predictions.[206] The boranophosphate diastereomer obtained from the fast migrating (R_p) isomer of H-phosphonate **101** had a longer retention time on RP-HPLC and a larger downfield chemical shift in ^{31}P NMR spectra. Therefore, it was assigned as the R_p configuration. As judged from RP-HPLC and ^{31}P NMR spectral analysis of dithymidine phosphorothioate **105**, the same reactions from R_p-**101** produced the S_p-isomer, confirming the assignment of the boranophosphate diastereomer **75a**-SE. The configuration change from R_p (boranophosphate) into S_p (phosphorothioate) is due to the CIP rules.

All three methods described above for assignment of the absolute configuration of boranophosphate dimers—NOE spectroscopic studies, SVPDE substrate specificity, and parallel chemical synthesis—were also used for the configuration assignment of another boranophosphate derivative, N^6-benzyl-2′-deoxy-5′-*O*-(4,4′-DMTr)adenosin-3′-yl 2′-deoxy-4′-*O*-(4-nitrophenyl)uridin-5′-y1 boranophosphate.[81] Such protected dimers can be used as synthons to incorporate the boranophosphate linkage in selected positions of an oligonucleotide chain with a defined stereochemistry.[81]

Stereocontrolled synthesis of boranophosphate dimers provides another way to assign the diastereomer configuration. It has been reported that the R_p-dithymidine phosphorothioate could be synthesized from chiral auxiliary **91**-(S)[223] or monomer S_p-**97**[221,222] following a similar procedure to those described in Schemes 39 and 40, respectively. It is also known that the conversion of a phosphite triester to either phosphorothioate or boranophosphate proceeds with retention of stereochemistry.[206] Therefore, dithymidine boranophosphate and phosphorothioate, prepared from chiral auxiliary **91**-(S) and S_p-**97**, should have the S_p and R_p configurations, respectively, noting that boranophosphates and phosphorothioates have different configuration assignments for the same spatial orientation around the phosphorus atom. Their HPLC profile and NMR spectra can then be compared with the data obtained from other methods to further confirm the configuration assignment. However, unambiguous assignment of absolute configuration for dinucleoside boranophosphates must await X-ray analysis.

6.3. Resistance of the Boranophosphate Internucleotidic Linkage toward Nucleases

The stability of nucleic acid analogues toward phosphatases and nucleases is an important determinant of biological activity. The boranophosphate backbone demonstrated quite a high resistance toward exonucleases.[32,101] Detailed enzymatic hydrolysis studies were performed on diastereomers of deoxyribonucleoside boranophosphate[29,30,81,101] dimers and ribonucleoside boranophosphate[80,204] dimers.

6.3.1. Resistance of Deoxyribonucleoside Boranophosphate Dimers toward Nucleases

It was demonstrated that only the S_p-stereoisomer of d(T^{b}pT) was digestible by SVPDE (3′-exonuclease); the catalytic efficiency decreased 330-fold relative to the phosphodiester d(TpT).[29] Both diastereomers of d(T^{b}pT) were found to be competitive inhibitors of SVPDE. As reflected by K_m and K_i, the enzyme binds more tightly to d(T^{b}pT) than to d(TpT).[30] Another nuclease, bovine spleen phosphodiesterase (BSPDE, 5′-exonuclease), digested the two diastereomers of d(T^{b}pT) with a 30- (S_p-isomer) and 80-fold (R_p-isomer) decrease in catalytic activity relative to the parent phosphodiester congener. Neither diastereomer was a substrate for either nuclease S_1 or P_1.[29] Another dimer, d(A^{b}pC), demonstrated very similar resistance to SVPDE.[81] The R_p-isomer was completely resistant to enzymatic hydrolysis while the S_p-isomer was 500 times more resistant than the natural counterpart.

6.3.2. Resistance of Ribonucleoside Boranophosphate Dimers toward Nucleases

Ribonucleoside boranophosphate dimers also showed quite a high stability toward nucleases.[80,204] One of the diuridine boranophosphate diastereomers with R_p configuration was almost completely resistant toward SVPDE hydrolysis ($t_{5\%}$ = 90 h), while the other one (S_p-isomer) appeared to be quite stable too ($t_{1/2}$ = 80 h).

A detailed comparative study on the substrate properties toward SVPDE, BSPDE, and RNase A was performed on

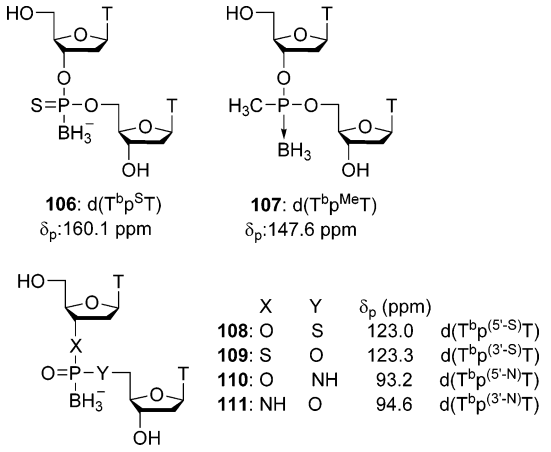


Figure 22. Structures of *P*-doubly modified dinucleoside boranophosphates.

the ribonucleoside dimer r(UpA) and its boranophosphate and phosphorothioate analogues.[27,28] From initial rate comparison, it was found that R_p-r(U^{b}pA) and S_p-r(U^{S}pA) were not digested by SVPDE at all. The S_p-r(U^{b}pA) is hydrolyzed about 2000 times slower than the natural r(UpA) and two times slower than the corresponding R_p-r(U^{S}pA). Additionally, boranophosphate linkages in r(U^{b}pA) appeared to be more resistant to BSPDE than the natural phosphodiester linkages, but less stable than the corresponding phosphorothioate isomers of r(U^{S}pA). For example, R_p-r(U^{b}pA) was hydrolyzed only 4 times slower than the normal r(UpA), and about 35 times faster than the corresponding S_p-r(U^{S}pA). Both isomers of r(U^{S}pA) and r(U^{b}pA) were found to be substrates for RNase A, with a preference seen for R_p-r(U^{S}pA) and S_p-r(U^{b}pA). The results showed that each isomer of r(U^{b}pA) is about 2−3 times more resistant to RNase A than those of r(U^{S}pA) with the same absolute configuration (space orientation).

6.4. Synthesis of *P*-Doubly Modified Dinucleoside Boranophosphates

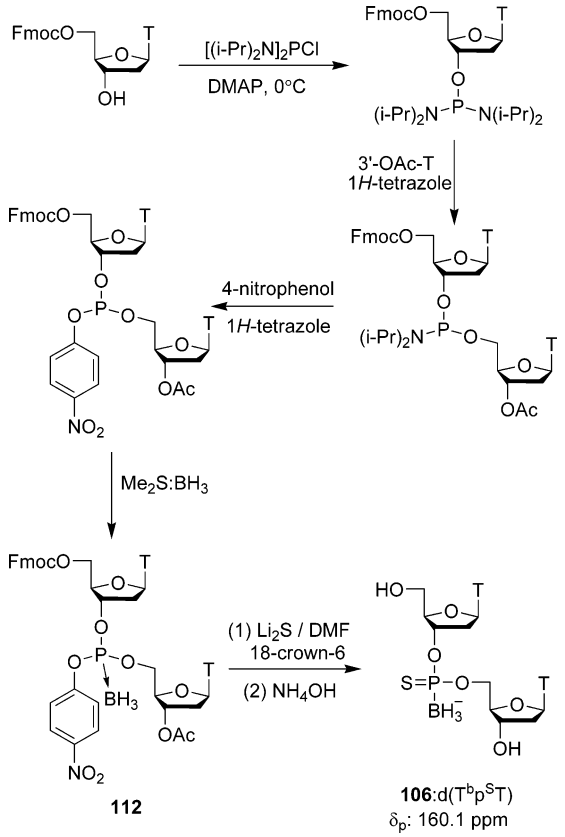
Novel modified nucleotides are currently attracting attention as probes in biochemistry and molecular biology and as possible therapeutic agents against cancer and viral diseases.[79,179,232−237] These studies have highlighted certain challenges that require new analogues to better understand these biological processes. A variety of novel analogues with *P*-doubly modified dinucleoside boranophosphates have been reported[124,238−240] and are summarized in Figure 22.

6.4.1. Modifications at Both Nonbridging Oxygens

6.4.1.1 Synthesis of Dinucleoside Boranophosphorothioates. The general procedure for the synthesis of dinucleoside boranophosphorothioates is outlined in Scheme 43.[238,239] The key step was nucleophilic attack by Li_2S to convert dithymidine boranophosphate triester **112**, whose ^{31}P NMR signal was at δ 116.6 ppm (br), to dithymidine boranophosphorothioate d(T^{b}p^{S}T) **106**, whose ^{31}P NMR had a characteristic peak at δ 160.1 ppm (br). The reaction was carried out in a one-pot procedure starting from 5′-*O*-fluorenylmethoxycarbonyl(Fmoc)-thymidine in 28% yield.

It has been found that the boranophosphorothioate linkage in the dimer was very stable toward acidic and basic hydrolysis as well as enzymatic cleavage by SVPDE and BSPDE.[238] The dimer d(T^{b}p^{S}T) **106** carries a negative charge and is water soluble, yet is intermediate between normal

Scheme 43



phosphate and methylphosphonate in lipophilicity. In partitioning experiments, d(T^{b}p^{S}T) was 320- and 18-fold more lipophilic than natural d(TpT) and boranophosphate d(T^{b}pT), respectively.[238]

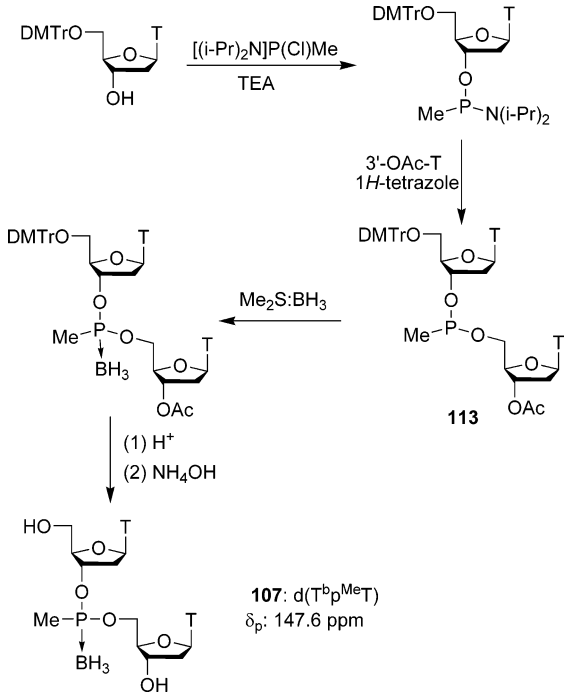
6.4.1.2. Synthesis of Dinucleoside Boranomethylphosphonate. Dinucleoside boranomethylphosphonates were developed as the analogues of nonionic nucleotides and oligonucleotides that had received extensive attention as antiviral and antisense agents.[235,241,242] The synthesis of dithymidine boranomethylphosphonate d(T^{b}p^{Me}T) **107** is depicted in Scheme 44.[240] 5′-O-DMTr-thymidine was phosphitylated with (^{i}Pr$_2$N)P(Cl)Me in the presence of TEA to give the phosphoramidite monomer. After coupling with 3′-*O*-acetylthymidine catalyzed by 1*H*-tetrazole, the phosphite diester **113** was formed, whose ^{31}P NMR signals appeared at δ 189.1 and 188.1 ppm for the *P*-diastereomers. The final compound d(T^{b}p^{Me}T) **107**, after *in situ* boronation and removal of protecting groups, was obtained in 42% yield and had a characteristic peak at ~δ 147.6 ppm in the ^{31}P NMR spectrum.

Studies have shown that the *P*-boranomethylphosphonate internucleotidic linkage[240] is (1) very stable toward neutral and acidic hydrolysis; (2) extremely resistant to cleavage by both SVPDE and BSPDE; and (3) highly lipophilic. The d(T^{b}p^{Me}T) dimer was 6800- and 370-fold more lipophilic than the d(TpT) and d(T^{b}pT) dimers, respectively.

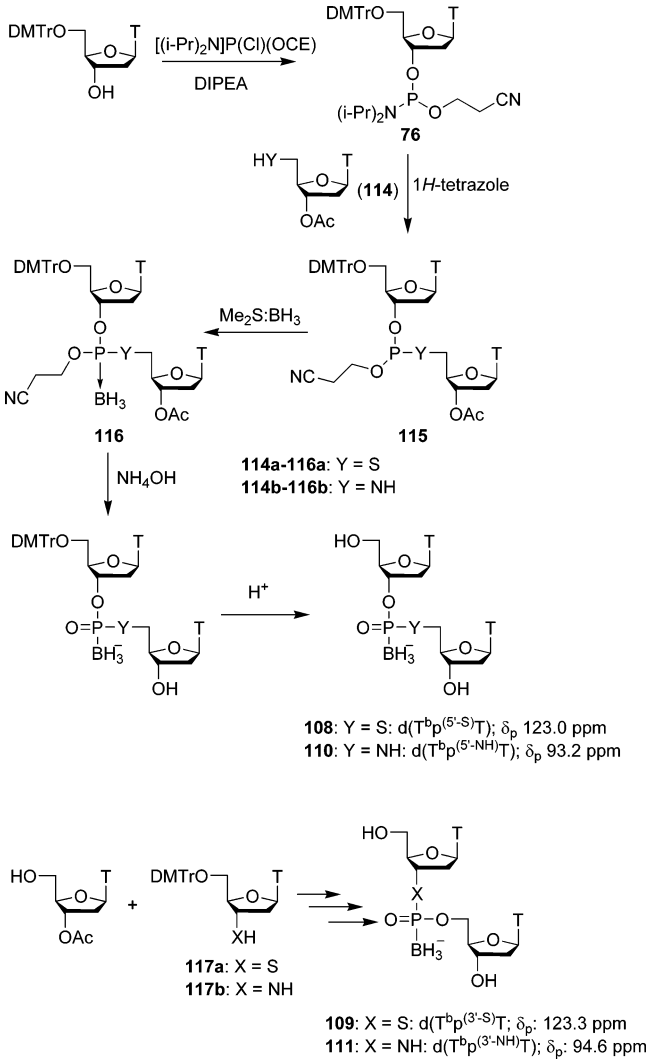
6.4.2. Modifications at Nonbridging and Bridging Oxygens

The dithymidine *O*3′-*S*5′ boranophosphorothioate d(T^{b}p$^{(5'-S)}$T) **108** and *O*3′-*N*5′ boranophosphoramidate d(T^{b}p$^{(5'-N)}$T) **110** were recently synthesized via a phosphoramidite approach outlined in Scheme 45.[124,239] Thymidine phosphoramidite diester **76** was coupled with 5′-mercapto-5′-deoxythymidine **114a** (or 5′-amino-5′-deoxythymidine

Scheme 44



Scheme 45



114b) to form a pair of phosphite diastereomers **115a** (or **115b**), whose signal appeared at δ 192.5 and 193.6 ppm in ^{31}P NMR (δ 139.3 and 139.7 ppm for **115b**). Direct treatment of phosphite **115a** (or **115b**) with $Me_2S{:}BH_3$ resulted in the boranophosphate **116a** (or **116b**), whose formation was confirmed by broad peaks centered at δ 163.0 ppm (δ 117.4 ppm for **116b**) in ^{31}P NMR. The final compound d(T^{b}p$^{(5'-S)}$T) **108** (or d(T^{b}p$^{(5'-N)}$T) **110**) was obtained in 21% yield (or 24% yield for **110**). The dithymidine *O*3′-*S*5′ boranophosphorothioate d(T^{b}p$^{(5'-S)}$T) **108** and *O*3′-*N*5′ boranophosphoramidate d(T^{b}p$^{(5'-N)}$T) **110** have a characteristic peak in ^{31}P NMR spectra at δ 123.0 and 93.2 ppm, respectively. The chemical shift for d(T^{b}p$^{(5'-S)}$T) **108** appears more downfield than that of d(T^{b}p$^{(5'-N)}$T) **110**, which is in agreement with prediction, since the sulfur atom is more electronegative than the nitrogen atom.

The synthesis of dithymidine *S*3′-*O*5′ boranophosphorothioate d(T^{b}p$^{(3'-S)}$T) **109** and *N*3′-*O*5′ boranophosphoramidate d(T^{b}p$^{(3'-N)}$T) **111** could be accomplished in a similar fashion to that of **108** and **110**.[124] In these cases, the 3′-phosphoramidites of 3′-mercapto-3′-deoxythymidine **117a** and 3′-amino-3′-deoxythymidine **117b** were used as the starting materials. The ^{31}P NMR spectra were very similar to those of their 5′-counterparts. Both compounds d(T^{b}p$^{(3'-S)}$T) **109** and d(T^{b}p$^{(3'-N)}$T) **111** were obtained in 24% yields.

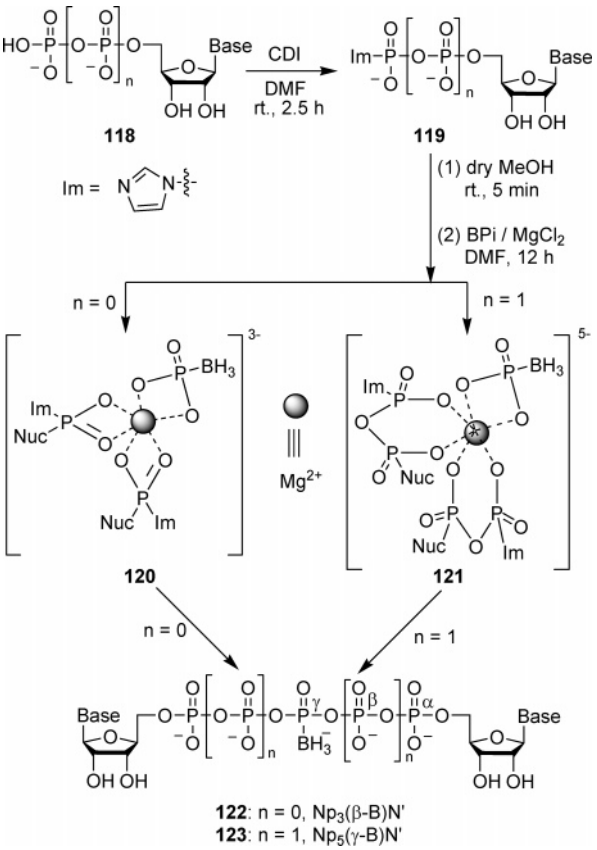
6.5. Synthesis of Dinucleoside Poly(borano)-phosphates

Dinucleoside polyphosphates Np$_n$N′ exert their physiological effects via P2 receptors.[243−245] They are attractive drug candidates, as they offer better stability and specificity compared to nucleotides,[244,246] the most common P2-receptor ligands.[247,248] To date, several Np$_n$N′ analogues have been administered in human clinical trials.[249] The beneficial effect of ATPαB in improving both receptor selectivity and ligand stability[184,250] prompted the studies on dinucleoside poly(borano)phosphates Np$_n$(B)N′.

The synthesis of Np$_n$(B)N′ is outlined in Scheme 46.[251] The nucleotide **118** had to be activated by carbonyl diimidazole (CDI) to form phosphoroimidazolide **119**.[252] Due to the decreased nucleophilicity of inorganic boranophosphate (BPi), which resulted from the extensive H-bonded clustering of BPi in organic solvents,[71] magnesium ions were added to coordinate with BPi and break apart the clusters. Therefore, BPi could act as a P-acceptor (nucleophile) and react with the P-donor phosphoroimidazolide **119** to give dinucleoside poly(borano)phosphates Np$_n$(B)N′. However, the formation of Np$_3$(B)N′ **122** was more efficient than that of Np$_5$(B)N′ **123**.[251] No product was formed when BPi was preactivated by CDI. Thus, BPi cannot be applied as a P-donor in the preparation of Np$_n$(B)N′ analogues. In this reaction, three reactant molecules (two P-donors and one P-acceptor) react concertedly, shown as **120** and **121** in Scheme 46. Various divalent metal ions, such as Zn^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+}, were reported to facilitate dinucleoside polyphosphate synthesis from the corresponding P-acceptor and P-donor.[253] However, the reaction to produce Np$_n$(B)N′ is metal ion specific. Only Mg^{2+} ions have been shown to be able to act as metal ion activators.[251]

Fischer et al. found that Np$_n$(B)N′ analogues exhibited remarkable chemical stability under physiological conditions.[251] Under conditions mimicking gastric juice, the Ap$_3$(β-B)A analogue exhibited a half-life ($t_{1/2}$) of 1.3 h, whereas Ap$_5$(γ-B)A degraded at a much faster rate ($t_{1/2}$ = 18 min). The hydrolysis of Ap$_3$(β-B)A by human nucleotide pyrophosphatase phosphodiesterases (NPP1 and NPP3) was

Scheme 46



slowed by 40% and 59%, respectively, as compared to Ap_3A. However, this effect of the boranophosphate was position-dependent, as $Np_5(\gamma\text{-B})N'$ was degraded at a rate comparable to that of Np_5N'. It was also found that $Ap_{3/5}(\beta/\gamma\text{-B})A$ analogues were potent $P2Y_1$-R agonists.[251] $Ap_5(\gamma\text{-B})A$ was equipotent to 2-MeS-ADP ($EC_{50} = 6.3 \times 10^{-8}$ M), thus making it one of the most potent $P2Y_1$-R agonists currently known.

7. Oligonucleoside Boranophosphates

The past decade has seen an upsurge of interest in nucleic acid-based drug development.[254] This is mainly due to the realization that modified oligomers (oligos), with improved properties, can be used in the sequence specific control of gene expression and have immense potential as therapeutic agents.[236,255−259] A principle mode of action of these modified oligos is through binding to a specific mRNA sequence associated with a disease and thereby (a) terminating the translation of a defective protein (antisense)[236,255,257] or (b) altering splice sites.[260] In addition, transcription can be inhibited by the formation of triplex-forming oligos (antigene).[256,261] Thus, a rational approach for gene therapy is to direct highly specific nucleic acid recognition between a relatively short oligo and a single- or double-stranded nucleic acid. A variety of nucleic acid analogues have been synthesized and evaluated in order to improve their efficacy compared with native DNA and RNA.[179,235,262−264] Desirable properties include low nuclease susceptibility and high cell membrane permeability. For the antisense approach, the ability to activate RNase H, which is capable of irreversibly destroying a specific mRNA, is also an important factor.[265]

While significant advances have been made in the use of modified oligos in the antisense and antigene area, equally as exciting are the more recent developments of modified oligos with alternative and novel modes of action. For example, aptamers, short strands of DNA or RNA generated by *in vitro* selection, can be modified to improve their stability and binding affinity for therapeutically relevant protein targets.[266] Recently uncovered, the mechanism known as RNA-mediated interference (RNAi) is poised to revolutionize the field of nucleic acid-based gene control, raising unprecedented enthusiasm from both academia and industry. Originally discovered in nature as a multistep process triggered by dsRNA, RNAi relies on the *in vivo* generation of so-called short interfering RNAs (siRNA) of 21 nucleotide (nt) length as effectors of targeted gene silencing.[267−270] Characterized by an extremely stringent sequence specificity, and in many cases demonstrating potencies that are orders of magnitude higher than conventional strategies, RNAi is already being seen by many as the ideal method for *in vivo* regulation of gene expression.[271−273]

Although most nucleic acid-based drugs are in the early stages of clinical trials, this class of compounds has emerged in recent years to yield extremely promising candidates for a wide range of diseases, including cancer, AIDS, neurological disorders such as Parkinson's disease and Alzheimer's disease, and cardiovascular disorders.[274,275] Replacing one of the nonbridging oxygens in the phosphate moiety by a BH_3 group results in a new class of backbone-modified oligonucleotides that are stable toward nucleases, display enhanced hydrophobicity, activate RNase H,[18−21] and function as siRNA.[35,38]

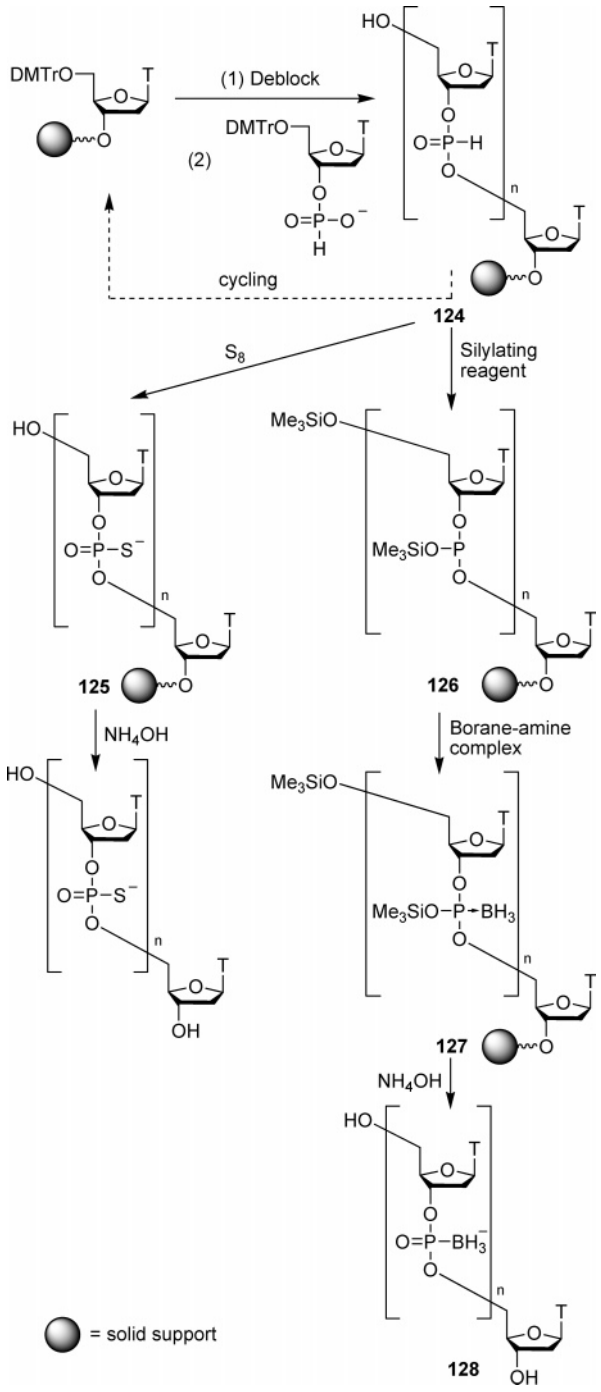
7.1. Synthesis of Oligonucleoside Boranophosphates

7.1.1. Solid Synthesis of Fully Substituted Oligodeoxyribonucleoside Boranophosphates

Fully substituted boranophosphate oligodeoxyribonucleotides (BH_3-ODNs) are a new class of boron-modified nucleic acids and have received considerable attention due to their potential diagnostic and therapeutic applications.[18−21] Although they have been enzymatically synthesized, which will be discussed in section 7.1.3, only the BH_3-ODNs with S_p configuration are obtained because of the substrate specificity of enzymes.[34,35,37,38,87,88,218] Furthermore, the enzymatic method is obviously not suitable for large-scale synthesis. On the other hand, chemical synthesis of BH_3-ODNs can overcome the aforementioned difficulties and warrant further studies on these potentially promising nucleic acid analogues.

7.1.1.1. Synthesis via an H-Phosphonate Approach. Progress has been made toward the synthesis of oligonucleoside boranophosphates in the past decade, with several groups independently reporting the preparation of oligothymidine boranophosphate (up to 15 nt long) via an H-phosphonate approach.[37,58,59] Oligonucleotide synthesis via an H-phosphonate intermediate is amenable to a variety of backbone modifications, including phosphorothioates, phosphoramidates, and normal phosphodiesters.[118,276] The complete oligonucleotide chain can be assembled with each internucleotidic linkage having trivalent phosphorus P(III) in the relatively stable H−phosphonate form.[277] Phosphorus modification can be carried out in a single step, converting all H-phosphonate groups to the pentavalent phosphorus P(V) as shown in Scheme 47 from H-phosphonate oligo **124** to phosphorothioate oligo **125**.

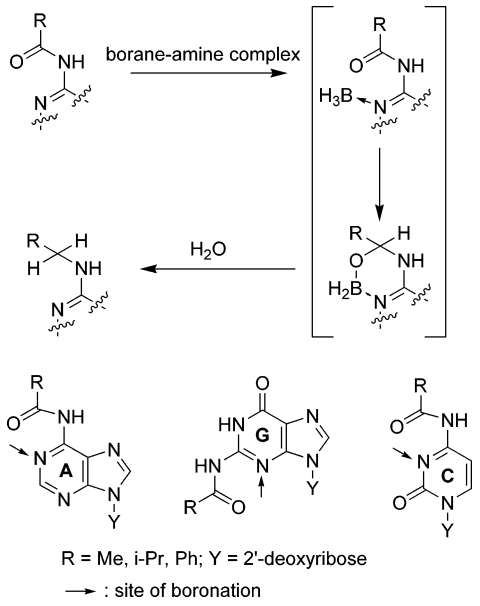
Scheme 47



However, boronation of the H-phosphonate oligo **124** differs from the oxidation, sulfurization, or amidation used to prepare phosphodiester, phosphorothioate, or phosphoramidate oligos. Specifically, boronation requires the intermediate formation of a Lewis base by conversion of the internucleotidic H-phosphonate diester to a phosphite triester. It can be accomplished via silylation of the corresponding H-phosphonate **124** into silyloxyl phosphite **126**. The silyloxyl phosphite can easily exchange with borane−amine complexes, generating boranophosphate triester **127**. The final deprotection with water or ammonia affords the desired boranophosphate oligo **128**.[56]

However, attempts to use this method for synthesizing mixed-nucleobase BH_3-ODNs gave rather complicated mixtures. Significant nucleobase modifications were observed

Scheme 48

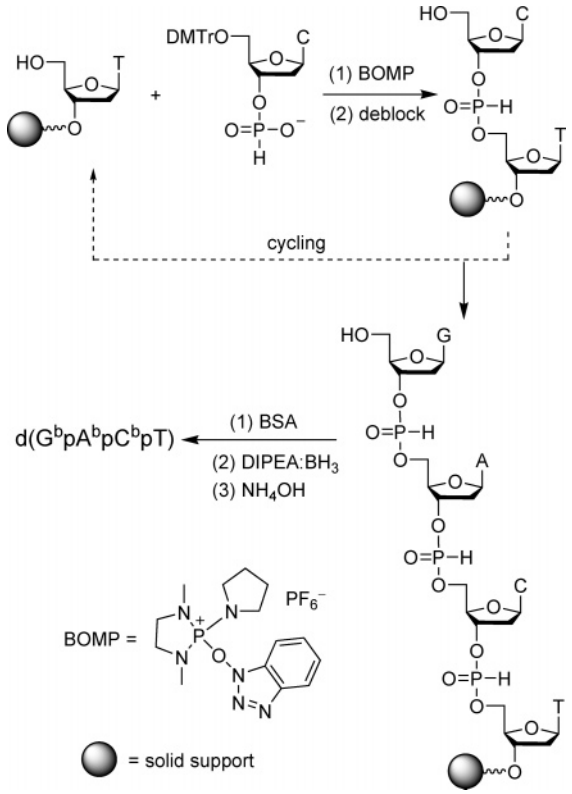


during conversion of the internucleotidic H-phosphonate linkage to boranophosphate by the boronating reagents.[21,37] It was found that conventional *N*-acyl protecting groups on the nucleobases A, G, and C were reduced by borane−amine complexes into the corresponding *N*-alkyl groups following the proposed mechanism described in Scheme 48.[81,96,97] The resulting alkyl groups could not be removed by standard ammonia treatment, yielding nucleobase-modified ODN mixtures.

Concurrently, it was demonstrated that nucleobase-unprotected nucleosides underwent a reversible complexation with borane under the boronation conditions,[278] which suggested that it might be possible to perform the synthesis of mixed-nucleobase BH_3-ODNs without base protection. Indeed, H-phosphonate oligos with unprotected nucleobases could be synthesized via the condensing agent 2-(benzotriazol-1-yloxy)-1,3-dimethyl-2-pyrrolidin-1-yl-1,3,2-diazophospholidinium hexafluorophosphate (BOMP).[278] The model tetramer d($G^bpA^bpC^bpT$) was synthesized following the procedure described in Scheme 49, which resulted in an overall 48% yield for the boranophosphate tetramer after HPLC isolation.

The identity and purity of the tetramer were confirmed by MALDI mass spectroscopy and ^{31}P NMR. In the ^{11}B NMR spectrum, the typical boranophosphate signal at δ −38.3 ppm was detected and no signals at $\sim\delta$ −20 ppm corresponding to borane−nucleobase species were found. Synthesis of longer BH_3-ODNs with BOMP as the condensing agent appeared to be less efficient. For example, the boranophosphate 10-mer d($T^bpC^bpA^bpA^bpA^bpC^bpG^bpT^bpT^bpG^bpA$) was obtained in 20−30% yield. The presence of the truncated sequences together with the high coupling yield assessed from DMT-cation release analysis may reflect partial degradation of the H-phosphonate backbone during the synthetic cycle.[278] A similar problem of inherent instability of the H-phosphonate backbone during chain assembly for natural ODNs was also mentioned by Wada et al.[279] It was concluded that a short (<10 nt) mixed-base sequence BH_3-ODN could be successfully synthesized using *N*-unprotected bases, H-phosphonate chain assembly, and global boronation.[278]

Scheme 49

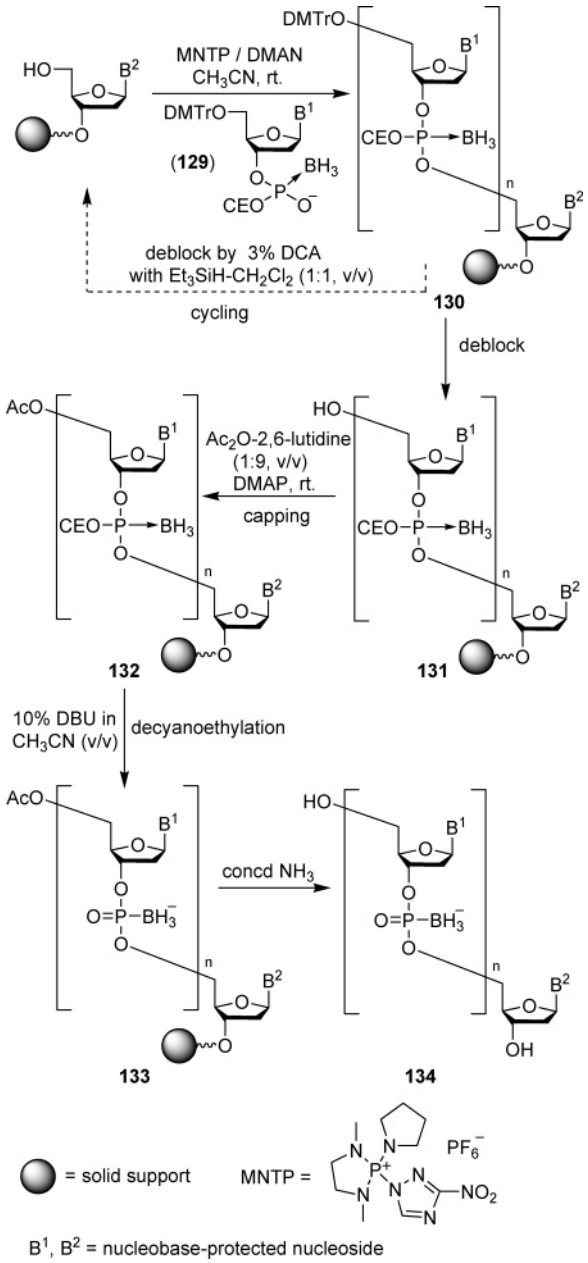


7.1.1.2. Synthesis via a Boranophosphotriester Approach. As shown in Scheme 50, boranophosphate diester **129**[280] is a key monomer and can be prepared from an appropriate protected deoxyribonucleoside and a boranophosphorylating reagent following the same procedure describing the formation of compound **85** in Scheme 37. Formation of boranophosphotriester **130** was accomplished in the presence of the condensing reagent 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP)[281] and bis(*N*,*N*′-dimethylamino)naphthalene (DMAN) as a coexisting base. In order to eliminate the side nucleophilic reaction with the neighboring phosphorus atom involving the end 5′-alkoxide in the presence of DBU, the boranophosphotriester oligo **131** had to be capped with an acetyl group to form oligo **132**.[280] The CE group was removed by treating **132** with 10% DBU in CH_3CN under anhydrous conditions prior to the removal of other base-labile groups. Other bases, such as concentrated NH_3 and NH_3/MeOH, were also tried for the step from oligo **132** to **133**. However, decomposition byproducts from oligo **132** were formed, by the nucleophilic attack of OH^- or MeO^-.[280] The fully deprotected BH_3-ODN **134** was obtained in 92% yield for the dimer, 30% yield for the tetramer, and 8−16% yield for the 12-mer after HPLC purification.[280] This method has also been applied to the preparation of mixed-nucleobase BH_3-ODNs.[280]

7.1.2. Solid Synthesis of ODNs Containing Both Boranophosphate and Phosphate Linkages

ODNs having alternating boranophosphate/phosphate linkages are expected to have useful antisense properties. The duplex stability for such "mixed" oligos with complementary RNA should be enhanced, while properties such as stability toward nucleases and RNase H activity are anticipated to remain similar to those of the all-boronated oligos.

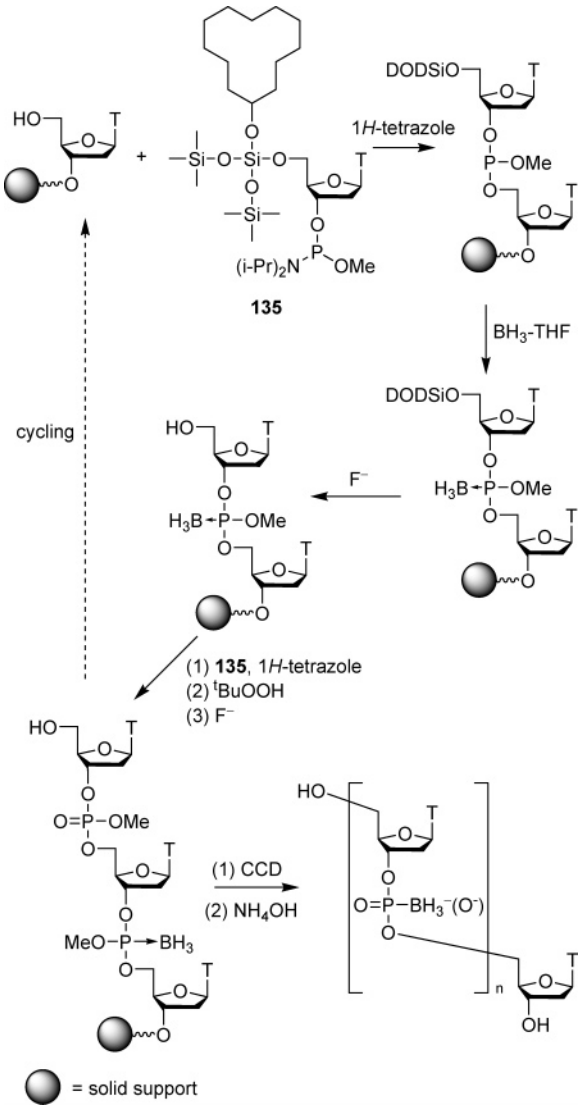
Scheme 50



The H-phosphonate approach based on H-phosphonate chain elongation followed by global boronation cannot be used to prepare ODNs with alternating linkages. A new approach[282] (Scheme 51) for the synthesis of these analogues has been developed by Caruthers et al., using (1) a previously reported procedure for the introduction of a borane group at the trialkylphosphite stage via borane complex exchange and (2) the recently developed bis(trimethylsiloxy)cyclododecyloxysilyl ether (DODSi)[283] as the 5′-*O*-protecting group.

For solid-phase preparation of boranophosphate/phosphate ODNs, polystyrene support was used in order to be compatible with the fluoride ion treatment used to remove the DODSi protecting group. The synthetic cycle began by activation of phosphoramidite monomer **135** with 1*H*-tetrazole followed by coupling and boronation as outlined in Scheme 51. Removal of DODSi with triethylammonium fluoride completed the cycle. Exchange with the borane complex generated boranophosphates; alternatively, oxidation with *tert*-butylperoxide yielded unmodified phosphate triesters. After completion of chain elongation of the mixed

Scheme 51



oligos, methyl groups from phosphate and boranophosphate triesters were removed by treatment with disodium 2-carbamoyl-2-cyanoethylene-1,l-dithiolate (CCD).[284] Following treatment of the support with aqueous ammonia, oligothymidylates containing boranophosphate/phosphate were isolated by RP HPLC in 20−25% overall yield. These chemically synthesized mixed oligothymidylates showed improved binding affinity (compared to all boronated oligothymidylates) and good nuclease stability, and they were capable of activating RNase H.[282]

Very recently, Caruthers et al. developed a new strategy to synthesize mixed oligos with mixed nucleobases in high yield on a solid support.[285] The method uses an appropriate protected phosphoramidite monomer **136**, as depicted in Figure 23.[285] Specifically, the 5′-OH is initially protected with the benzhydroxybis(trimethylsilyloxy)silyl group and later deblocked by Et_3N:HF before the next cycle. The nucleobases are protected with N^6-DMTr, N^2-Fmoc, N^4-trimethoxytrityl (TMTr), and N^3-anisoyl (An) groups for adenine, guanine, cytosine, and thymine, respectively. By following the same procedure described in Scheme 51 and after the chain elongation, protecting groups are removed sequentially. Initially and with an ODN attached to the support, 80% HOAc is used to eliminate DMTr and TMTr groups from adenine and cytosine, respectively. Next, the

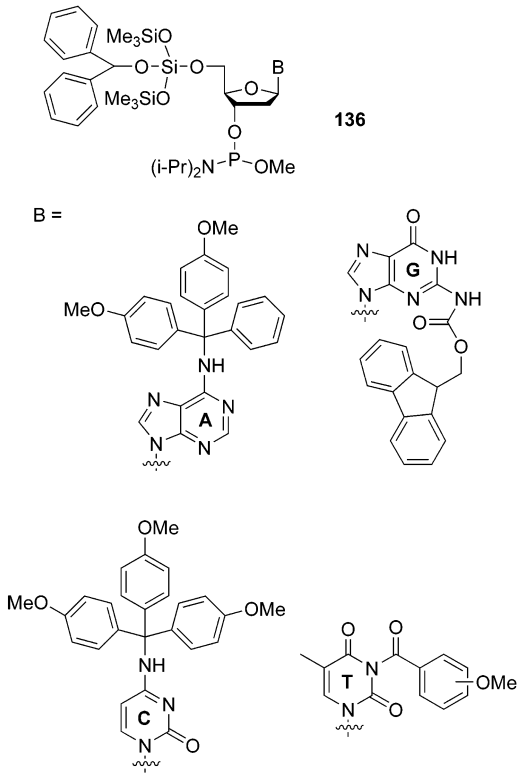


Figure 23. Structures of 5′-silyl-2′-deoxynucleoside-3′-phosphoramidites **136** and nucleobases with protecting groups.

oligos are treated with CCD[284] to remove internucleotide methyl protection. Finally, Fmoc and An groups are eliminated by NH_4OH from guanine and thymine, respectively, to generate fully deprotected oligos. Numerous deoxyoligos were prepared using this method with 99% coupling yield and isolated yields of 70−89%.[285]

7.1.3. Enzymatic Synthesis of Boron-Containing Nucleic Acids

Template-directed enzymatic synthesis of oligonucleoside boranophosphates using dNTPαB and rNTPαB analogues is a powerful alternative to their chemical synthesis and should provide stereoregular oligonucleotides of any sequence.

7.1.3.1. Enzymatic Synthesis of Boranophosphate DNA. Shaw et al. demonstrated that all four R_p-isomers of dNTPαB can substitute for natural dNTP in primer extension or PCR reactions, using a variety of polymerases and templates of different lengths.[32] The steady-state kinetic parameters, K_m and k_{cat}, at 37 °C for incorporation of some R_p-dNTPαB analogues are summarized and compared with those of natural dNTP and S_p-dNTPαS analogues in Table 6.[188] Primer extension reactions were carried out with one or more R_p-dNTPαB analogues (0.5−100 μM) at low (0.1 μM) or high (to at least 25 μM) primer/template concentrations and with primers as small as 11-mers to yield 10 Kb DNA fragments.[32,88] Further adjustment of conditions allowed the synthesis of 50-mers containing 100% boranophosphate groups using the full set of R_p-dNTPαB analogues.[28]

R_p-dNTPαB analogues are stable at high temperature and compatible with both short- and long-range (13 Kb) polymerase chain reactions (PCR) using a variety of thermostable polymerases (Taq, Vent, Taq FS). Under normal PCR conditions, expected amounts of full-length 509 bp DNA were produced with 25−50% boronated linkers.[32] Amplifica-

tion was not impeded by the presence of a base-specific R_p-dNTPαB even with a 13 Kb template.[32] These results indicate that the presence of a boranophosphate in either the primer or the template has minimal, if any, effect on chain elongation and amplification.

To understand the full extent to which boranophosphates can mimic normal nucleic acids, it is important to determine if boranophosphate RNA, and RNA synthesized from boranophosphate DNA, can also be properly decoded during information transfer by the ribosome. In an *in vitro* expression system, RNA synthesized from a 25−50% boranophosphate DNA template is fully capable of expressing equivalent amounts of luciferase as natural DNA.[18] Likewise, we have succeeded in translating RNA containing 25% boranophosphate internucleotidic linkages into active protein.[18]

7.1.3.2. Enzymatic Synthesis of Boranophosphate RNA. It has been reported that boranophosphate RNA could be enzymatically synthesized by T7 RNA polymerase with R_p-rNTPαB analogues.[34] For a short classical RNA (20-nt) and under steady-state kinetic conditions, the yields of full-length transcription products, upon complete substitution of one type of normal rNTP with borano analogues (except at the fourth position of RNA), were nearly the same as with all normal rNTPs. However, substitution at the fourth position caused a substantial decrease in the yield for the full-length transcription product and an increase in abortive termination, which might result from BH_3 interference with the conformational change of the enzyme.[286,287] For the synthesis of borano-modified RNA libraries (80-nt), the yields decreased somewhat along with the increasing percentages of boranophosphate linkages.[27] When substituted with one type of R_p-rNTPαB analogues (N = A/C/U), the yields were 70−86% relative to native rNTPs. The yield decreased to 56% if rGTPs were replaced with R_p-rGTPαB. When substituted with two types of R_p-rNTPαB analogues (N = C and U), the yield was 45%. With three types of R_p-rNTPαB analogues (N = C, U, A/G), the yield was further dropped to 20%. Strikingly, RNA with all boranophosphate internucleotidic linkages was synthesized with a total yield of 15%.

The borano-modified RNA libraries have been used as templates for reverse transcription, and it was found that the yields were comparable or even higher than those of normal RNA libraries if the overextended products were included.[27] With boranophosphate RNA, greater proportions of overextended transcription products were observed relative to normal RNA.[27] RNA libraries with one type of rNTP analogue substituted with the corresponding R_p-rNTPαBs yielded the same DNA product pattern as the normal RNA libraries. When the substitution increased to two types of R_p-rNTPαB anlogues (N = C and U), an overextended DNA product was observed in addition to the full-length one. With the further increasing substitution of R_p-rNTPαB analogues in RNA libraries, the overextended DNA product became the dominant product.

7.2. Properties of Oligonucleoside Boranophosphates

7.2.1. Hybridization Properties of Oligonucleoside Boranophosphates

The observance that boranophosphates can participate in replication and transcription suggests that they mimic DNA and RNA in hybridization properties.[18] For a 14-mer with the sequence d(5′-CTATGGCCTCAG*CT-3′), where asterisk (*) indicates modification, it was found that the ODN containing one stereodefined S_p-boranophosphate linkage (S_p-^{b}ODN) had a decreased melting temperature (T_m) for the DNA duplex (57.5 °C) compared with its parent duplex (58.2 °C).[88] In a similar study and for a chemically synthesized 9-mer with the sequence d(5′-ATGGT*GCTC-3′), the T_m was found to be 38.2, 39.1, and 40.1 °C for DNA duplexes with one R_p-boranophosphate, S_p-boranophosphate, or phosphodiester linkage, respectively.[288] This is the only report on the binding affinity study with R_p- and S_p-stereodefined boranophosphate ODNs. Therefore, it was tentatively concluded that the T_m was increasing in the order of R_p-^{b}ODN < S_p-^{b}ODN ≤ ODN with a temperature difference of ∼0.7−1.0 °C between all duplexes. Although it was speculated that the single borano modification caused only a minor change in the stability of the complementary complex, the binding affinity was thought to increase in the order of R_p-^{b}ODN < S_p-^{b}ODN ≤ ODN according to the T_m.[288] Interestingly, this binding affinity order, R_p-^{b}ODN < S_p-^{b}ODN, was opposite to that observed for uncharged methylphosphonate substitution,[289] where the R_p diastereomer always formed more stable complexes with the complementary DNA than the S_p counterpart. For the phosphorothioate modification, the situation was complicated and their relative stability depended on the sequence rather than on the phosphorus configuration.[290] However, it remains to be determined whether the observed relative stability of the boranophosphate DNA duplex (R_p-^{b}ODN < S_p-^{b}ODN) is also sequence dependent.

For fully substituted BH_3-ODNs, hybridization properties were determined by thermal denaturation studies with complementary DNA and RNA.[56,58,59] It was found that d[$(T^bp)_{13}T$] hybridized with dA_{14} and rA_{14} at low ionic strength (0.1 M KC1), although the binding affinity was less than that of the unmodified phosphodiester control.[58] High ionic strength conditions (1.0 M KC1) increased the T_m of all duplexes by approximately 5 °C. Similar data were obtained in a dT_{12}-dA_{12} model study.[56] At physiologically relevant buffer conditions (0.15 M KC1, 20 mM KH_2PO_4 with pH 7.0, and 10 mM $MgCl_2$), the d[$(T^bp)_{11}T$]:dA_{12} complex exhibited a cooperative melting curve with an estimated T_m of 14 °C.[56,60,280] By comparison, the closest analogues, phosphorothioate d[$(T^Sp)_{11}T$] and methylphosphonate d[$(T^{Me}p)_{11}T$], formed more stable complexes with dA_{12} when the same conditions were used (T_m = 28 and 32 °C, respectively), while the most stable complex was formed by natural dT_{12} with a T_m of 47 °C. The observed hyperchromicity in the 1−65 °C interval was the highest for the unmodified complex dT_{12}:dA_{12} (ΔA_{260} = 0.177 or 36.8%), followed by the phosphorothioate complex d[$(T^Sp)_{11}T$]:dA_{12} (ΔA_{260} = 0.16 or 32.3%), and it was the lowest for methylphosphonate d[$(T^{Me}p)_{11}T$]:dA_{12} and boranophosphate complexes d[$(T^bp)_{11}T$]:dA_{12} (ΔA_{260} = 0.149 or 30.9% and 0.148 or 29.2%, respectively). When the ionic strength was increased, the boranophosphate d[$(T^bp)_{11}T$] formed a more stable complex with dA_{12}, as expected for negatively charged compounds (ΔT_m = 5 °C).

Therefore, dodecathymidine boranophosphate d[$(T^bp)_{11}T$] synthesized as a diastereomeric mixture forms relatively weak complexes with complementary dodecadeoxyadenylate dA_{12}. There could be several reasons for such an effect. The vast majority of phosphate-modified ODNs (such as methylphosphonates, phosphorothioates, phosphorodithioates, or

Table 8. Comparison of the Duplex T_m Values Consisting of BH_3-ODNs and Native DNA and RNA[b]

entry	BH_3−ODNs	counterparts	T_m (°C)	ΔT_m (°C)	$\Delta T_m'$[a] (°C)
	100 mM NaCl, NaH_2PO_4 buffer (pH 7.0)				
1	$d[(T^bp)_{11}T]$	dA_{12}			
2	$d(CAGT)_3$	$d(ACTG)_3$	54.3		
3	$d(C^bpA^bpG^bpT^bp)_3$	$d(ACTG)_3$	39.7	−14.6	−1.2
4	$d(CAGT)_3$	$r(ACUG)_3$	52.4		
5	$d(C^bpA^bpG^bpT^bp)_3$	$r(ACUG)_3$	45.0	−7.4	−0.6
	1 M NaCl, NaH_2PO_4 buffer (pH 7.0)				
6	$d[(T^bp)_{11}T]$	dA_{12}	12.8	−31.3	−2.6
7	$d(CAGT)_3$	$d(ACTG)_3$	58.0		
8	$d(C^bpA^bpG^bpT^bp)_3$	$d(ACTG)_3$	44.7	−13.3	−1.1
9	$d(CAGT)_3$	$r(ACUG)_3$	54.8		
10	$d(C^bpA^bpG^bpT^bp)_3$	$r(ACUG)_3$	50.5	−4.3	−0.4

[a] Change of melting temperature per modification ($\Delta T_m'$). [b] Partially reprinted with permission from ref 280. Copyright 2006 American Chemical Society.

phosphoramidates) perturb the structure of the DNA helix to some extent and form less stable complementary complexes compared to their natural counterparts.[291,292] The same effect is expected for boranophosphate analogues. Since the boranophosphate linkage is isomorphic to the methylphosphonate yet bears a negative charge, the electrostatic repulsion between strands will add to the negative influence of steric distortion on complex stability. Moreover, the dT_n:dA_n family of duplexes adopts several types of helical conformations that differ from the canonical B-form.[293,294] An atypical conformation might accentuate the steric hindrance of the phosphate substituents on the pyrimidine strand. Therefore, boranophosphates would confer far less instability in a mixed sequence environment or when used in a stereoregular form or in combination with normal phosphate linkages.

Hybridization studies on oligonucleotides containing alternating boranophosphate/phosphate linkages were performed by Brummel and Caruthers.[282] In the dT_{14}-dA_{14} and dT_{14}-rA_{14} models, the binding affinity of oligos containing boranophosphate at every other T position ($\Delta T_m = -14.8$ and −15.2 °C for dT_{14}-dA_{14} and dT_{14}-rA_{14}, respectively), and every third T position ($\Delta T_m = -7.9$ and −8.3 °C for dT_{14}-dA_{14} and dT_{14}-rA_{14}, respectively), was found to be improved in comparison to that of the fully modified BH_3-ODN ($\Delta T_m = -29.1$ and −29.9 °C for dT_{14}-dA_{14} and dT_{14}-rA_{14}, respectively).[282] These results suggest that such boranophosphate-containing ODNs may be useful for further research in the antisense area.

Wada et al. recently reported that the binding affinity of BH_3-ODNs with complementary DNA and RNA was increased dramatically by incorporating a full set of nucleobases.[280] As shown in Table 8, a fully substituted BH_3-ODN with mixed nucleobases, $d(C^bpA^bpG^bpT^bp)_3$, showed a high binding affinity with $d(ACTG)_3$ compared with the duplex $d[(T^bp)_{11}T]$:dA_{12}, even at low ionic strength. Furthermore, upon hybridizing with a complementary RNA, $d(C^bpA^bpG^bpT^bp)_3$ formed a more stable complex (DNA:RNA complex, entry 5 and 10) than the corresponding BH_3-ODN:ODN complex (DNA:DNA complex, entry 3 and 8) under the same conditions, while the complex of a control of unmodified natural phosphodiester exhibited a slightly lower T_m (DNA:RNA complex, entry 4 and 9) relative to the DNA:DNA complex (entry 2 and 7).

It should be noted that RNA duplexes with mixed sequence-containing S_p-boranophosphates cause a 0.5−0.8 °C per modification increase in the T_m compared to normal double-stranded (ds) RNA,[27] which was in contrast to the boranophosphate DNA, where modifications reduced the T_m.[56,58,88,280,288] The difference between boranophosphate DNA and RNA might result from the different structures and conformations of the DNA and RNA duplex. For example, the B-form DNA duplex has a wide and deep major groove and a narrow and deep minor groove, while the A-form structure of the RNA duplex has a narrow and deep major groove and a wide and shallow minor groove.[295,296] Also, without the restraining effect of the 2′-OH, DNA has a more flexible backbone but less hydration than RNA.[297] However, the exact reasons for the different behavior of boranophosphate DNA and RNA are still unclear. It was also observed that BH_3 had the greatest effect in increasing the T_m of ds-siRNA with one strand modified.[27] The T_m increased in the order of normal RNA < R_p-phosphorothioate RNA (0.2−0.3 °C per modification) < S_p-boranophosphate RNA (0.5−0.8 °C per modification).[27] Contrary to most phosphorothioate RNA duplexes containing a diastereomeric mixture of each phosphorothioate linkage (where the T_m for phosphorothioate RNA is lower than that for normal RNA),[298] the higher T_m of this phosphorothioate analogue is among the few reported examples[299,300] of stereoregulated R_p-phosphorothioate RNA duplexes having a higher T_m than that of the parent normal RNA duplexes.[27]

7.2.2. Activation of RNase H-Mediated RNA Cleavage by Boranophosphate ODNs

RNase H activation is considered to be one of the most important pathways for modulating gene expression.[265] Among hundreds of oligonucleotide modifications intended for use in antisense applications and capable of forming duplexes with RNA,[301] only three phosphodiester analogues, including phosphorothioate, phosphorodithioate, and boranophosphate, combine hydrolytic stability and resistance to nucleases with the ability to efficiently elicit RNase H hydrolysis in RNA−DNA duplexes.[258] The thioate analogues, however, have nonspecific binding properties and other undesirable effects,[302,303] which may ultimately limit their use.

In an early study, it was reported that the fully modified non-stereoregulated BH_3−ODN, $d[(T^bp)_{13}T]$, was active in stimulating *E. coli* RNase H but was about 10-fold slower in the rate of cleavage compared with the unmodified ODN, dT_{14}.[58]

A more comprehensive study was carried out on fully modified non-stereoregulated dodecathymidine boranophosphate in a $d[(T^bp)_{11}T]$−rA_{12} model.[60] The cleavage kinetics were compared with those of dodecathymidine phosphate T_{12} and phosphorothioate $d[(T^Sp)_{11}T]$. It was shown that the cleavage of poly(A) induced by the boranophosphate dodecamer occurred with remarkably enhanced kinetics in comparison with the cleavage induced by the phosphate or phosphorothioate dodecamers. The relative rates of RNA hydrolysis mediated by RNase H were 76:8:1 at 20 °C and 18:2:1 at 30 °C for borano, thio, and natural backbones, respectively. The hydrolysis rates of the three types of dodecamers for inducing poly(A) cleavage correlate inversely with the melting temperatures of the $d[(T^bp)_{11}T]$−rA_{12}, $d[(T^Sp)_{11}T]$−rA_{12}, and dT_{12}−rA_{12} heteroduplexes. There were also noticeable differences in the composition of the final poly(A) hydrolyzates. The size distribution of poly(A) cleavage products was shifted to longer products for the borano

heteroduplex compared to the thioate and normal heteroduplexes.[60] This effect might reflect a more endonucleolytic and less exonucleolytic type of RNase H hydrolysis.

Another study by Caruthers et al. showed that mixed backbone oligos containing boranophosphate/phosphate linkages have significantly improved ability to direct *E. coli* RNase H activity.[282] Both modified dT_{14} oligos, having a boranophosphate at every other position or every third position, activated RNase H with the same cleavage rate as that for the unmodified oligomer, dT_{14}. This data validates the suggestion that, in order to be recognized by the RNase H catalytic center, any isostere derived by substitution of a nonbridging oxygen(s) should retain a negative charge.[304]

All these data indicate that boranophosphate ODNs can potentially serve as antisense agents based on the RNase H-mediated mechanism. However, in a recent study on a fully modified S_p-boranophosphate ODN with a mixed sequence, it was reported that the rate of induced RNase H hydrolysis was about 10-fold less than that induced by a corresponding normal ODN,[57] which suggests boranophosphate ODNs need further investigation.

7.2.3. Properties of Boranophosphate ODNs as Enzyme Substrates

As was demonstrated previously, boranophosphate ODNs display properties of both neutral methylphosphonate and charged phosphorothioate and phosphodiester oligonucleotides to varying extents.[18,21,29,56] The hybrid character is further revealed in their interactions with enzymes. Oligothymidine boranophosphates, unlike methylphosphonates, could be phosphorylated by T4 polynucleotide kinase.[56] Postsynthetic phosphorylation provides a convenient method for labeling boranophosphate oligos and may allow subsequent derivatization of the 5′-phosphate. Phosphorylation of boranophosphate ODNs supports speculations that a negative charge is an important factor for T4 kinase activity, perhaps contributing to the binding affinity of a substrate.

Enhanced resistance of fully modified boranophosphate ODNs toward nuclease hydrolysis[56] proved to be consistent with the previous data for dimer substrates containing only one boranophosphate linkage.[29] Dodecathymidine boranophosphate $d[(T^bp)_{11}T]$ was more than 2 orders of magnitude more resistant toward SVPDE than the natural congener dT_{12}.[56] The hydrolytic patterns were consistent with the previous observation that only one stereoisomer of the boranophosphate linkage was hydrolyzed,[29] and they were also in good agreement with previous data showing that only one stereoisomer of the dimer $d(T^bpT)$ was a substrate for SVPDE.[29] Boranophosphate oligomers appeared to be much more resistant than the phosphodiesters and phosphorothioates toward P_1 and S_1 nucleases, although hydrolysis might be achieved at very high enzyme concentration and long incubation time.[56]

BSPDE was the only enzyme among those tested that hydrolyzed both stereoisomers of the internucleotidic boranophosphate linkage at an appreciable rate. Though boranophosphate dodecamer $d[(T^bp)_{11}T]$ was hydrolyzed 30 times slower than the natural dodecamer, it was possible to perform complete hydrolysis of dT_{12} within 48 h at 37 °C by 100 μg/mL BSPDE.[56] These conditions were used for developing the procedure for base composition analysis. The complete hydrolysis of mixed-base boranophosphate oligos by BSPDE resulted in a mixture of nucleosides.[278] Concurrently, complete conversion of dA to dI by adenosine deaminase, which is inevitably present as an impurity in commercial preparations of BSPDE, was observed.[278] Nucleoside 3′-boranophosphates were not observed in the final hydrolysis mixture due to their instability ($t_{1/2} \sim 3$ h, 37 °C).[29] BSPDE hydrolysis mixtures were directly analyzed by RP HPLC, and nucleoside ratios were found to be in good agreement with theory.[278]

7.3. Applications of Oligonucleoside Boranophosphates

7.3.1. Application of dNTPαB Analogues for Direct PCR Sequencing

Current methods for PCR sequencing cannot, in fact, directly sequence PCR products. DNA templates must first be amplified by PCR and only subsequently sequenced by a secondary primer extension reaction, which incorporates the dideoxynucleotide chain terminators.[305] The boranophosphate sequencing method, developed in the Shaw laboratory, allows the use of amplified PCR products, after digestion, for direct loading into the sequencing gel.[32]

Because the boranophosphate linkages are quite resistant to Exo III, and by carrying out PCR reactions, with each containing only a small percentage of one base-specific boronated triphosphate, it is possible to determine the position of the boranophosphate by treating the PCR product with Exo III and performing subsequent analysis on a commercially available DNA sequencer. Since the sequencing fragments are generated from full-length PCR products, the distribution of fragments is advantageously skewed toward the longer fragments.

Direct boranophosphate PCR sequencing has been shown to be compatible with a variety of formats.[32] Manual sequencing has been performed with end-labeled primers, as well as with unlabeled primers and radioactive dNTPs. Automatic sequencing has been performed with the Pharmacia and Applied Biosystems 373A sequencers. In all cases, boranophosphate sequencing has been shown to generate accurate and reproducible base calls over extended sequences (450 bases).

For longer sequences, the preference of exonuclease III for cytidine residues produced occasional faint bands in the dC lane. To overcome this problem, a series of 5-substituted analogues of 2′-deoxycytidine (α-*P*-borano)triphosphates (dCTPαB), including 5-Me, 5-Et, 5-Br, and 5-I substitution for cytosine, were synthesized to increase the nuclease resistance of 2′-deoxycytidine residues.[87] The R_p-diastereomer of each dCTP analogue was a substrate for DNA Sequenase and had similar incorporation efficiency to normal dCTP, with 5-I-dCTPαB being the least efficient. As anticipated, substitution at the 5-position of cytosine markedly enhanced the dCTPαB resistance toward exonuclease III (5-Et-dCTPαB > 5-Me-dCTPαB > dCTPαB = 5-Br-dCTPαB > 5-I-dCTPαB). Thereby, replacement of dCTP with 5-Et-dCTPαB or 5-Me-dCTPαB is recommended for direct boranophosphate PCR sequencing.[87]

7.3.2. Application of rNTPαB Analogues for Aptamer Selection

Nucleic acids aptamers recognize specific molecular targets and can serve as highly specific diagnostic or drug delivery agents. Aptamers containing boron may be suitable for BNCT—a therapeutic cancer treatment. Nucleic acid aptamers obtained using the systematic evolution of ligands

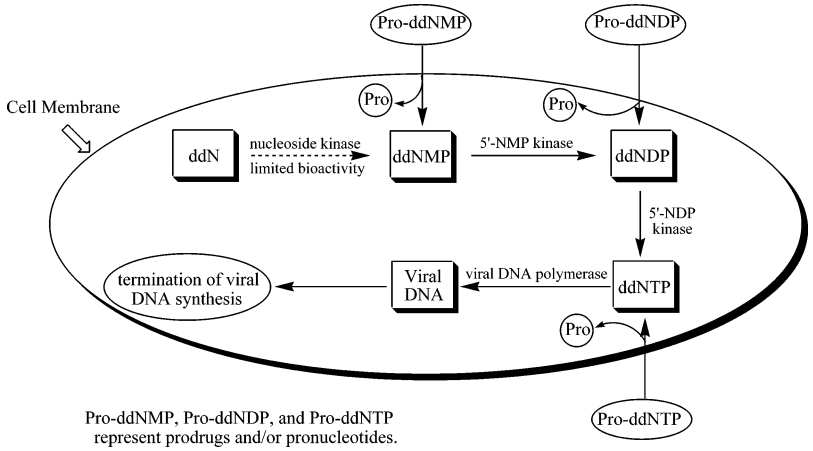


Figure 24. Activation of antiviral 2′,3′-dideoxynucleosides (ddN) and the prodrug concept.

by exponential enrichment (SELEX) protocol can be used to target purified proteins, cell membranes, or whole cells and thereby deliver boron specifically to tumor cells for BNCT while avoiding nontumor cells.

Burke and Shaw et al. investigated the influence of boronated nucleotide analogues on RNA function and on the SELEX process.[36] The study was performed on ATP-binding aptamers in two ways: (1) by retrofitting "normal" aptamers with boranophosphates and (2) by using substitution of guanosine GTPαB for GTP or UTPαB for UTP for *in vitro* selection. Specifically, several previously identified aptamers containing the ζ-fold, which is a common G-rich motif for many adenosine aptamers,[306,307] became inactive when transcribed in the presence of GTPαB but only moderately affected by UTPαB substitution.[36]

De novo selection created new pools of aptamers that tolerate the boranophosphate modification. The selection with UTPαB and normal NTP yielded some aptamers containing the ζ-fold; however, the selection with GTPαB did not. Non-ζ-aptamers tolerated boranophosphate modification, and many required it. Thus, the borane groups appear to play some specific role in binding or structure, as they must be incorporated at specific positions: GMPαB was not tolerated in aptamers selected with UMPαB, and UMPαB could not be used in aptamers that had been selected in the presence of GMPαB.[36] The authors concluded that the rNTPαB analogues were fully compatible with the SELEX method. The resulting aptamers showed no bias against the modified nucleotide, and the observed specificity was comparable with that of aptamers selected with normal nucleotides. Therefore, boronated aptamers could be developed for use in BNCT for anticancer treatment by targeting, for example, a protein, saccharide, or receptor, which is more abundant on tumor cells than on surrounding tissues.

7.3.3. Application of Boranophosphates in siRNA

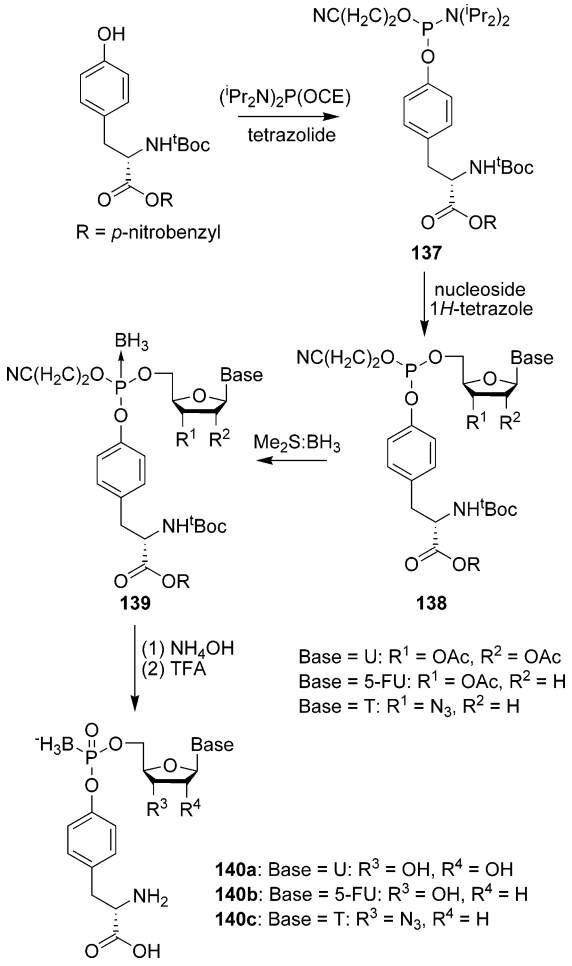
It has been recently reported that boranophosphate-backbone siRNAs were highly active and potent for interference with EGFP expression in HeLa cells.[35,38] Boranophosphate-modified double-stranded (ds)[35] and single-stranded (ss)[38] siRNAs, synthesized with T7 RNA polymerase, were found to be more effective than their phosphorothioate counterparts and often more active than their native siRNAs for gene silencing. The boranophosphate modifications on the antisense strand were more advantageous than those on the sense strand for siRNAs, since the former caused a greater increase in potency than the modifications on the sense strand.[35] The activity of boranophosphate siRNA was determined by the overall degree of modification in the center of the antisense strand rather than by modification at a specific position.[35,38] However, the magnitude of the increased activity of peripheral boranophosphate modification was greater for ss-siRNA than for ds-siRNA.[38] The greater nuclease stability of boranophosphate siRNA might partially account for the increased silencing activity.[35] The boranophosphate siRNA might also increase the stability of the RNA-induced silencing complex (RISC) and thus make the RNAi process more efficient.[38] Results of several different studies suggested that efficient cleavage of the target RNA requires an A-form structure between the guide strand of the ds-siRNA and the target.[308−310] A boranophosphate siRNA might enhance its silencing activity, since its CD spectrum showed that it was more likely to make an A-form helix.[27] It might also be speculated that the increased lipophilicity of boranophosphate nucleotides could facilitate interactions between RNA and the intracellular membrane-associated RISC protein.[35,38] Finally, the absence of toxicity in boranophosphate siRNAs in cell culture was encouraging in the context of possible clinical applications.[35]

8. Nucleoside Boranophosphate Prodrugs

8.1. Boranophosphate Nucleotide Analogues as Potential Antiviral Drugs

2′,3′-Dideoxynucleoside (ddN) antiviral drugs are successfully phosphorylated in cells by different kinases to 2′,3′-dideoxynucleoside 5′-mono- (ddNMPs), 5′-di- (ddNDPs), and 5′-triphosphates (ddNTPs) (Figure 24). The triphosphates of the antiviral nucleosides are the active species that inhibit viral DNA synthesis. The inhibition of viral DNA synthesis primarily involves incorporation of the ddNMP into the elongating DNA chain and subsequent chain termination.[189] Cellular phosphorylation of an antiviral nucleoside by kinases is a crucial process leading to an active ddNTP, but any alternative route that can lead to a sufficient cellular concentration of the active ddNTP can be very useful in the discovery of new antiviral treatments.[189,311−316] A conceivable strategy is to directly use a nucleotide that can bypass cellular phosphorylation partially or entirely as shown in Figure 24. However, due to the high polarity of nucleotides such as ddNMPs, ddNDPs, and ddNTPs, they cannot be efficiently delivered into the cell. Therefore, the so-called prodrug or pronucleotide approach suggests the use of partially or

Scheme 52



entirely neutralized dideoxynucleotide analogues masked with various protecting groups.[189]

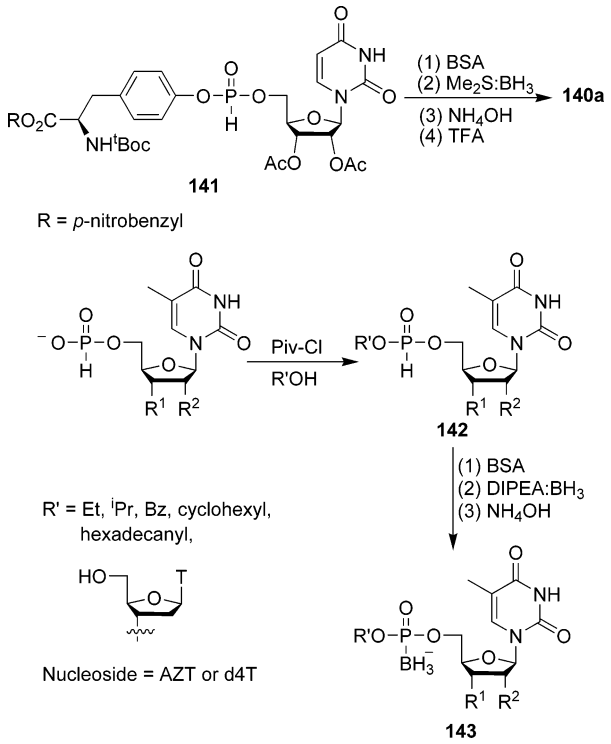
To circumvent the first cellular phosphorylation that is the rate-limiting step for most antiviral dideoxynucleosides, ddNMP prodrugs have been intensively explored,[311−316] and several types of NMP prodrugs have shown promising *in vitro* activities.[311−316] For some dideoxynucleosides such as AZT, the second phosphorylation from ddNMP to ddNDP is the rate-limiting step and thus delivery of ddNDP prodrugs is necessary.[317] Moreover, delivering prodrugs of ddNDPs and ddNTPs can substantially increase the concentration of active triphosphate species within the cell. However, because ddNDPs and ddNTPs have more negative charges than ddNMPs, their prodrugs are more difficult to prepare and thus less studied than the corresponding ddNMP prodrugs.

8.2. Synthesis of Nucleoside Boranomonophosphate Prodrugs

8.2.1. Boranophosphate Prodrugs Conjugated with Amino Acids through a P–O Bond

Nucleotides conjugated with amino acids have been shown to be promising prodrugs.[314−316] Such compounds show good bioavailability and enhanced cellular uptake and are hydrolyzed enzymatically, leaving nontoxic amino acids. Several tyrosine conjugates having boranophosphate through the P−O bond were synthesized via a phosphoramidite approach.[50,318] As outlined in Scheme 52, *N*-protected L-tyrosine was phosphitylated to phosphoramidite **137** and then coupled

Scheme 53



with protected nucleoside. Boronation of the phosphite triester **138** with $Me_2S:BH_3$ proceeded smoothly, yielding boranophosphate triester **139**. The final product **140** was obtained in 32−46% overall yield after the removal of protecting groups.

Although theoretically the amino acid-containing H-phosphonate **141** could be transformed into the corresponding boranophosphate **140a** following the procedures shown in Scheme 53,[50] in practice the low yield for the preparation of H-phosphonate **141** limited its application.[50] Recently, however, Zhao et al. showed that AZT/d4T boranophosphates **143** could be obtained in a reasonable to good yield after purification, using an H-phosphonate precursor **142** similar to **141**.

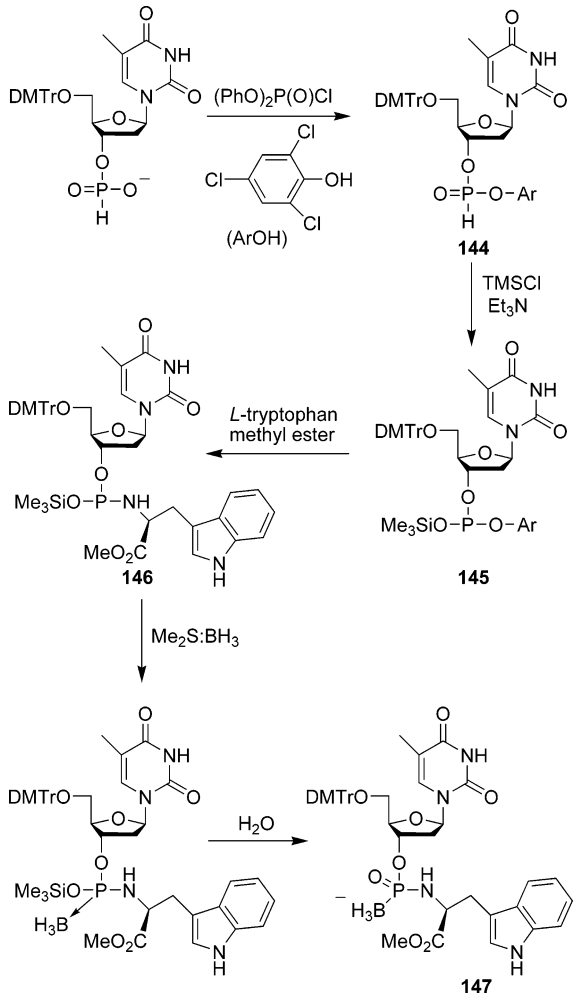
8.2.2. Boranophosphoramidate Prodrugs Conjugated with Amino Acids through the P–N Bond

Nucleoside phosphoramidates conjugated with amino acids have shown promise as a potential pronucleotide strategy.[314,315] Studies by Wagner et al. demonstrated that phosphoramidate monoester prodrugs, such as AZT amino acid phosphoramidate derivatives, are potent antiviral and anticancer agents with enhanced activity but reduced cytotoxicity when compared with the parent AZT.[319,320] These nucleoside phosphoramidate monoesters are thought to exert their biological functions through a P−N bond cleavage by phosphoramidases to yield the corresponding nucleoside monophosphates.[321−325]

Nucleoside amino acid boranophosphoramidates appear to be the perfect structural analogues of phosphoramidate monoester prodrugs. They are expected to have the advantages conferred by both phosphoramidates and boranophosphates. Their synthesis was accomplished via an H-phosphonate approach in a one-pot fashion as shown in Scheme 54.[51,84]

The aryl H-phosphonate **144** was obtained from the 3′-H-phosphonate and 2,4,6-trichlorophenol in the presence of

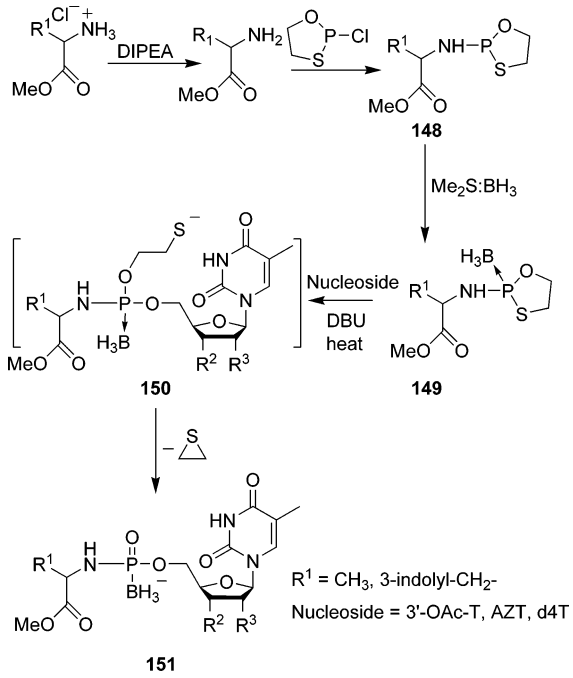
Scheme 54



diphenylchlorophosphate (DPCP) as the condensing reagent. Upon silylation to phosphite triester **145**, the aromatic leaving group was then displaced by the L-tryptophan methyl ester, resulting in the phosphoramidite diester **146**. Treatment of intermediate **146** with borane complex followed by water hydrolysis yielded boranophosphoramidate **147**. Although the overall yield was good for this one-pot reaction, removal of the condensing reagent DPCP could be problematic.[84] When the final compound contained a hydrophobic group such as DMTr, DPCP could be removed by aqueous extraction. However, due to the contamination with hydrolysis products of DPCP, it was virtually impossible to purify the boranophosphoramidate analogues if they did not have a hydrophobic moiety like DMTr.

The nucleoside boranophosphoramidates conjugated with amino acids have also been synthesized in a one-pot procedure via the oxathiaphospholane approach outlined in Scheme 55.[84] Reaction between the amino acid methyl ester and 2-chloro-1,3,2-oxathiaphospholane formed phosphoramidite **148**, which was evidenced in ^{31}P NMR by the disappearance of a singlet at δ 207 ppm for 2-chloro-1,3,2-oxathiaphospholane and the appearance of two singlets at $\sim\delta$ 129.5 ppm for the diastereomers of phosphoramidite **148**. Treatment of intermediate **148** with borane complex resulted in the boranophosphoramidate **149**, whose signal appeared at $\sim\delta$ 136 ppm in the ^{31}P NMR spectrum. Addition of nucleoside and DBU would open the ring to form intermediate **150** and give the desired boranophosphoramidate **151** after elimination of ethylene episulfide. It was found that

Scheme 55



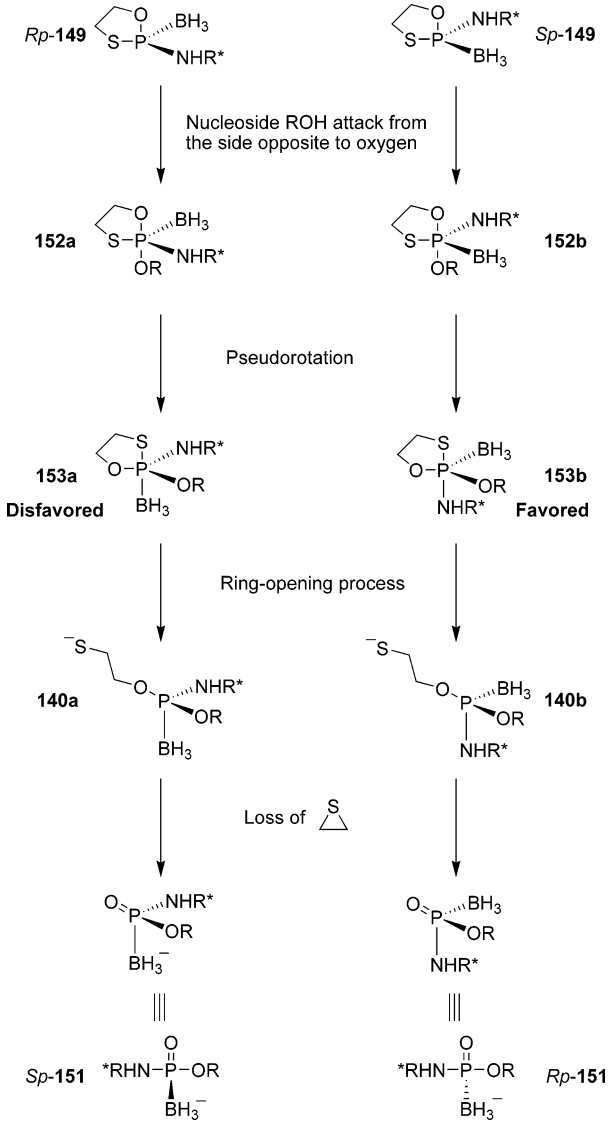
the amount of DBU and the temperature required for the reaction were reagent specific.[84] In other words, the more hindered the amino acid and nucleoside, the greater the amount of DBU that was required and the higher the reaction temperature had to be for completion of the reaction. The boranophosphoramidates **147** and **151** had a characteristic chemical shift at $\sim\delta$ 92 ppm in ^{31}P NMR as reported by Shaw et al.[84] Although Baraniak et al. reported similar compounds with chemical shifts at $\sim\delta$ 105 ppm, insufficient spectral data were provided to characterize the boranophosphoramidate compounds.[326]

It has to be pointed out that diastereoselectivity was involved in the DBU-assisted ring-opening reaction.[84] As shown in Scheme 56, an adjacent-type mechanism[327] is associated with the 1,3,2-oxathiaphospholane ring-opening process. The nucleophilic nucleoside attacks from the side opposite the most apicophilic endocyclic oxygen atom, resulting in a trigonal bipyramidal intermediate **152**. This intermediate, before collapsing, undergoes pseudorotation and places the cleavable P−S bond in the apical position, as shown in structure **153**. Since the borane group has a strong preference for the equatorial position, as we discussed earlier in section 2.2.5, isomer **153b** is favored over **153a**. Thus, after the ring collapse and elimination of ethylene episulfide, the R_p-isomer of boranophosphoramidate **151** is expected to be in excess over S_p-**151**. Experiments have shown that the ratio between the fast- (FE) and slow-eluting (SE) isomers is 3:2. Therefore, the configurations of FE and SE isomers were assigned as R_p and S_p, respectively, which was further supported by molecular modeling and ^{1}H NMR.[84]

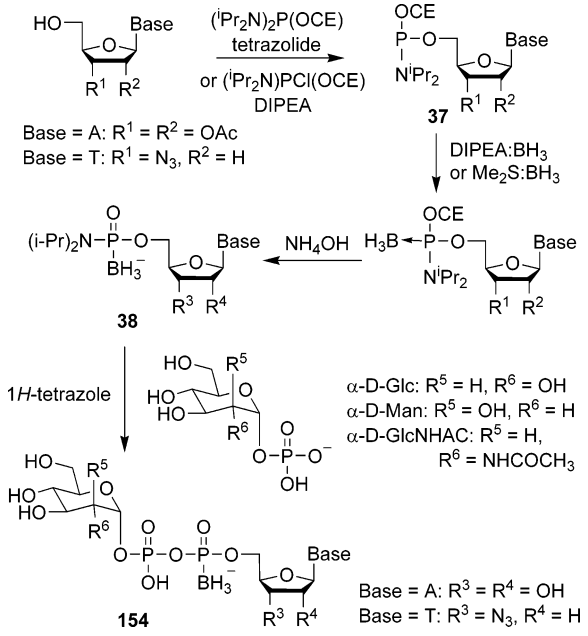
8.3. Synthesis of Nucleoside Boranodiphosphate Prodrugs

For most ddN analogues, the rate-limiting step in the formation of the active triphosphate species is the phosphorylation from the nucleoside to the corresponding monophosphate derivative. However, the conversion from the monophosphate derivative to the diphosphate derivative is

Scheme 56



Scheme 57



the bottleneck reaction for some nucleoside analogues, such as AZT.[328] Therefore, delivery of diphosphates would be more effective than that of monophosphates in these cases. Moreover, due to the low cellular concentration of ddNMP analogues and, in turn, even lower concentration of corresponding ddNDP analogues, direct delivery of ddNDP analogues should significantly increase the concentration of the active triphosphate species in viral cells and thus enhance their antiviral activity.

8.3.1. Synthesis of Nucleoside Boranodiphosphohexose Prodrugs

Nucleoside diphosphohexoses are important cofactors in the biosynthesis of oligosaccharides, glycoproteins, and glycolipids.[329−331] Their analogues containing the boranophosphate linkage could be employed for numerous studies involving nucleoside diphosphate sugars. Moreover, such conjugates containing ddNs such as AZT can be used as potential antiviral prodrugs.[332] Recently, a method for synthesis of hexose-conjugated nucleoside boranodiphosphates was developed as shown in Scheme 57.[53,54] Specifically, phosphoramidite **37**, obtained from the phosphitylation of a nucleoside, was treated *in situ* with excess borane complex followed by ammonium hydrolysis to afford boranophosphoramidate **38**. The nucleoside boranodiphosphohexose **154** was obtained from the coupling reaction between boranophosphoramidate **38** and sugar 1-phosphate in the presence of 1*H*-tetrazole at 55 °C. The diastereomeric mixture of the final product was identified by characteristic peaks at ∼δ −10.8 (d) and δ −10.9 ppm (d) for the β-P and at ∼δ 86.0 ppm (br) for the α-P in ^{31}P NMR.

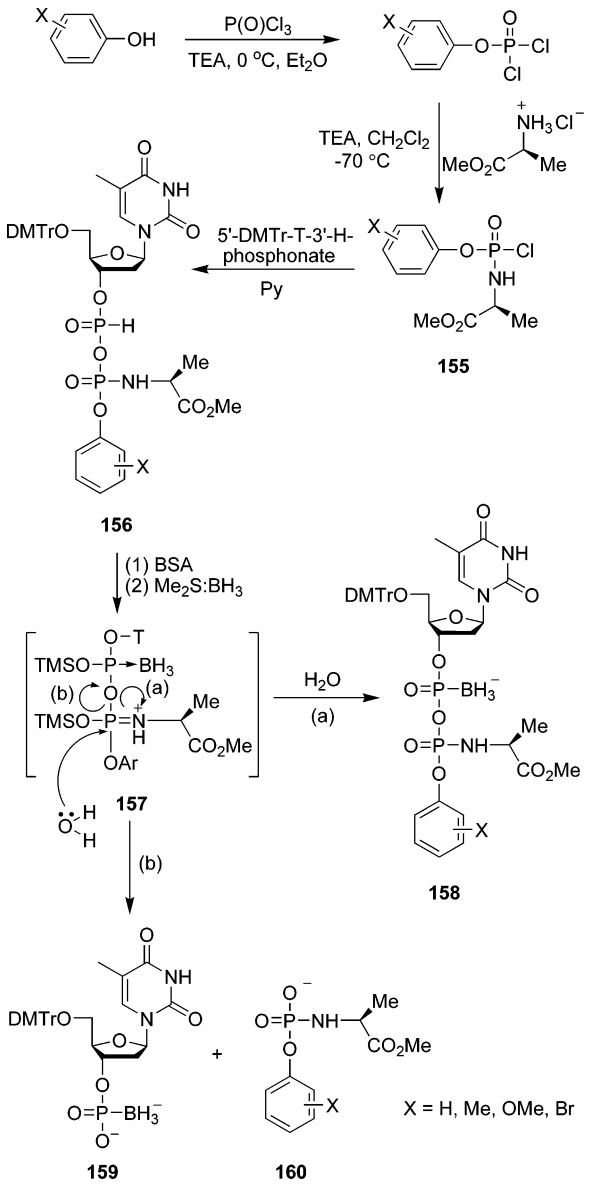
Introduction of BH_3 into the α-position in nucleoside diphosphohexoses may enhance their lipophilicity, assist in their crossing the plasma membrane, and render them more resistant to phosphodiesterases. Interestingly, the AZT boranodiphosphohexose derivative was reported[54] to liberate borano mono- and diphosphates in cells and showed antiviral activity similar to that of the parent AZT, a widely used anti-HIV drug.

8.3.2. Synthesis of Nucleoside α-Boranodiphosphate Phosphoramidate Prodrugs

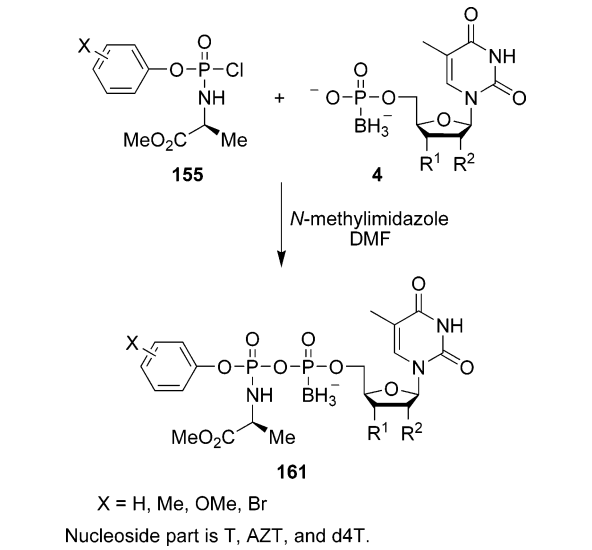
As possible candidates for promising phosphoramidate prodrugs,[314,315] nucleoside α-boranodiphosphate phosphoramidate analogues have been prepared via H-phosphonate and direct coupling approaches.[18,55] As outlined in Scheme 58, the L-methoxyalaninyl phosphorochloridate **155** reacted with 3′-H-phosphonate to give phosphoramidate H-phosphonate **156**. After silylation and boronation, intermediate **157** was obtained. Due to the presence of the labile anhydride linkage, water hydrolysis could form a large quantity of 3′-boranomonophosphate **159** and phosphoramidate **160** (path b) in addition to the desired boranodiphosphate phosphoramidate **158** (path a).

A direct coupling method, as described in Scheme 59, appeared to be more reliable in the preparation of α-boranodiphosphate phosphoramidate **161**.[55] The coupling reaction between L-methoxyalaninyl phosphorochloridate **155** and 5′-NMPB **4** in DMF yielded the title compound **161** in the presence of *N*-methylimidazole. By using different substituents on the phenol ring, control over the rate of releasing diphosphate species is expected. However, preliminary studies on the chemical stability of α-boranodiphosphate phosphoramidate analogues indicated that the boranomono-

Scheme 58



Scheme 59



phosphate was the main species present after hydrolysis.[55] Therefore, the prodrug approach involving boranodiphosphate phosphoramidate analogues needs further improvements.

8.4. Synthesis of Nucleoside Boranotriphosphate Prodrugs

Cellular phosphorylation of an antiviral ddN by kinases is a crucial process leading to an active ddNTP, but any alternate route that can lead to a sufficient cellular concentration of the active ddNTP can be very useful in antiviral research. A conceivable strategy is to directly use a triphosphate entity that can entirely bypass the cellular phosphorylation cascade.[333] However, this strategy is not accepted by the majority of biochemists and medicinal chemists because the natural triphosphate residues are characterized by very rapid dephosphorylation in human blood serum and poor penetration through the cellular membrane. This makes ddNTP analogues problematic for use directly as antiviral drugs.[334−336] Fortunately, recent developments in the design of serum-stable ddNTP analogues modified at the 5′-phosphate[47,49,337−339] and the formulation of a Nanogel drug delivery system[340] make the idea of a ddNTP prodrug approach feasible.

As reported by Cook et al.[47,49] and through personal communication with him, α-*P*-BH_3 triphosphate prodrugs with a substitution at the *β*,*γ*-phosphate (**164**) could be synthesized by direct alkylation of the corresponding modified triphosphate analogue **162** or by the coupling between boranomonophosphate **163** and diphosphate analogues (Scheme 60). Similarly, the α-*P*-BH_3 triphosphate prodrugs with a substitution at the α,*β*-phosphate (**166**) could be prepared from the boranodiphosphate **165** and monophosphate analogues as depicted in Scheme 60. It has been reported that the 5′-α-*P*-BH_3-*β*,*γ*-CF_2-triphosphate analogue was a generic and promising triphosphate prodrug in regards to HIV-1 RT inhibition and serum stability.[47,49] It is thus anticipated that the use of 5′-α-*P*-BH_3-*β*,*γ*-CF_2-triphosphate analogues will lead to the discovery of a new class of anti-HIV agents.

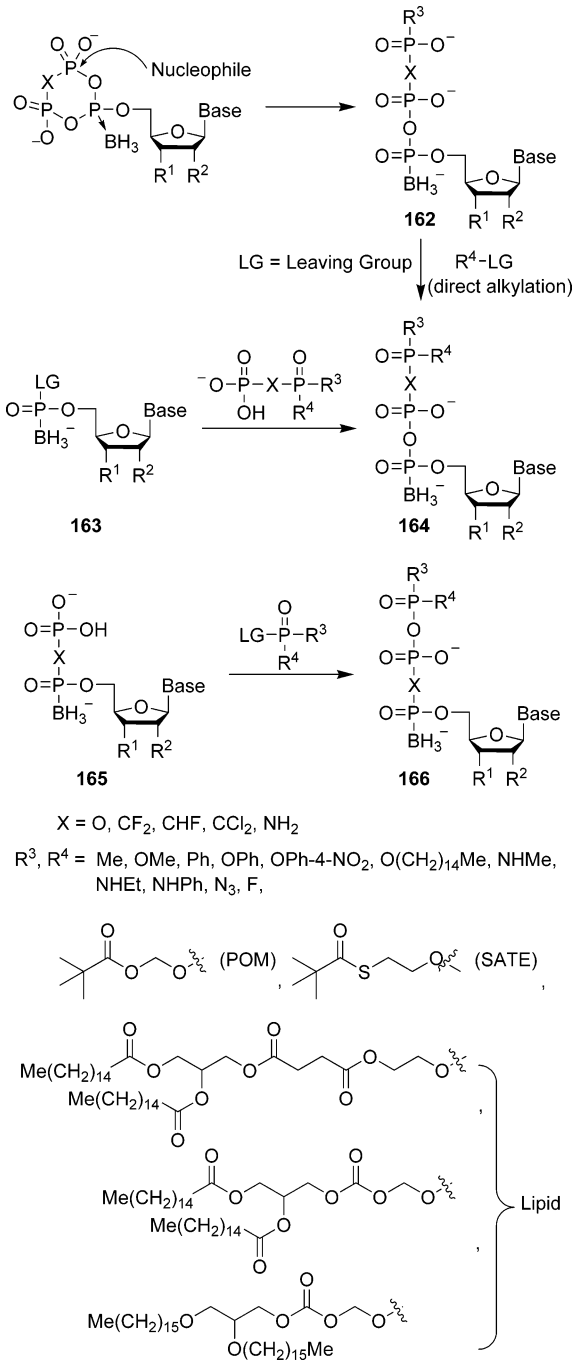
9. Acyclonucleoside Boranophosphonates

The first nucleoside phosphonate was synthesized by Burger five decades ago.[341] Since then, nucleoside phosphonates have received considerable attention as a key class of antiviral agents due to their high stability toward enzymatic cleavage. In particular, acyclonucleoside phosphonates[342,343] have proven to be highly successful as antiviral agents; cidofovir (1996), tenofovir (2001), and adefovir (2002) (Figure 25) have been approved for clinical treatment of herpesvirus infections, AIDS, and hepatitis B, respectively. Like their parent nucleoside monophosphate compounds, the nucleoside phosphonates must be intracellularly phosphorylated in two steps to form the active diphosphorylphosphonates to exert their antiviral function.[189,342,343] However, unlike their parent monophosphate compounds that undergo dephosphorylation, nucleoside phosphonates can resist the cleavage[342,343] due to the replacement of the (O═)P−O bond by an (O═)P−C bond. Analogues of acyclonucleoside boranophosphonates could be interesting and important because of the intriguing substrate properties for viral reverse transcriptase arising from the BH_3 group in NTPαBs (see section 5.3).

9.1. Synthesis of Acyclonucleoside Boranophosphonate

Acyclonucleoside boranophosphonate analogues **170a** and **170b** were recently synthesized via a H-phosphinate ap-

Scheme 60



proach[344] as shown in Scheme 61 that is very similar to the synthesis of boranomonophosphates via an H-phosphonate approach (Scheme 5). However, the ^{31}P NMR chemical shifts of the intermediates and final compounds in Scheme 61 were quite different from those for the corresponding species in Scheme 5. For example, in a model reaction[345] when R was a 9-fluorenemethylene group, silylation of H-phosphinate **167** (δ 24 ppm) resulted in the formation of a peak at δ 160 ppm for phosphonite **168**. Boronation of phosphonite showed a broad peak at δ 125 ppm for the borane complex **169**. The final boranophosphonate **170c** had a broad peak at δ 100 ppm. It is surprising that the ^{31}P NMR chemical shift for acyclonucleoside phosphonate **170a** (δ 83 ppm) reported by Alvarez et al. was quite different from that of its structural analogue **170b** (δ 98 ppm) reported in the same paper[344] and was rather far from our expectation[345] ($\sim\delta$ 100 ppm). Further

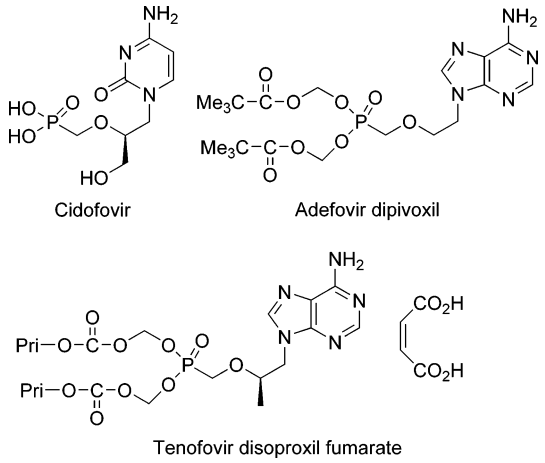
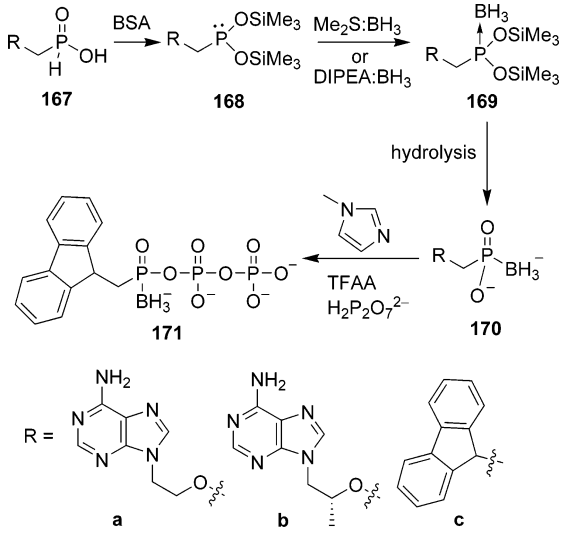


Figure 25. Structures of FDA-approved antiviral acyclonucleoside phosphonates.

Scheme 61



characterization needs to be done to confirm the structure of **170a**. Preliminary studies[344] showed that the monophosphate analogues, i.e., H-phosphonites (**167a** and **167b**) and boranophosphonates (**170a** and **170b**) did not exhibit significant antiviral activity to HIV or cytotoxicity to a broad range of viruses. Further investigation is anticipated.

The triphosphate analogue was made by coupling 9-fluorenemethylene boranophosphonate **170c** with pyrophosphate *N*-methylimidazolide in the presence of trifluoroacetic acid anhydride (TFAA) to form the 9-fluorenemethylene boranophosphonodiphosphate **171c** in 30% yield.[345] Compound **171** has the expected three characteristic phosphorus peaks[345] in the ^{31}P NMR spectrum at δ 116, -10, and -20 ppm, corresponding to α-P, γ-P, and β-P.

9.2. Stability of Acyclonucleoside Boranophosphonates

The stabilities of acyclonucleoside boranophosphonates, specifically, 9-[2-(boranophosphonomethoxy)ethyl]adenine (**170a**) and (*R*)-9-[2-(boranophosphonomethoxy)propyl]-adenine (**170b**), were studied toward chemical and enzymatic hydrolysis.[344] Both compounds were stable for more than 72 h to chemical hydrolysis in buffers with pH values ranging from 1.2 to 11.5. Less than 5% of the material was found to be degraded. While the boranophosphonates **170a** and **170b**

were stable for more than 72 h in nonenzymatic medium (RPMI 1640), the half-lives of **170a** and **170b** in nucleophile-enriched enzymatic medium (culture medium) were approximately 5.3 and 1.3 h, respectively. This represents a 15- and 50-fold drop in half-life for **170a** and **170b**, respectively, when compared with the hydrolysis carried out in the nonenzymatic medium.

In their enzymatic hydrolysis, H-phosphinate **167** was found to be the only metabolite. Moreover, the breakage of the boranophosphonate linkage was not observed in the culture medium. Based on these results, Alvarez suggested[344] that, instead of attacking the phosphorus atom to yield the phosphonate hydrolysis compounds as found for NMPB,[94] the BH_3 group in boranophosphonates was enzymatically reduced to form the P−H bond. However, they did not characterize the fate of the borane group or boron atom or the enzyme responsible for the reduction. Such information may shed light on the mechanism of enzymatic hydrolysis and help in designing stabilized boranophosphonate analogues in the future.

10. Conclusions

Nucleoside and oligonucleoside boranophosphates are new and unique types of compounds in the family of phosphate-modified nucleic acids. They are efficient and near perfect mimics of natural nucleic acids in reading and writing genetic information with high yield and accuracy. Their hybrid nature allows them to combine the most useful features and properties of well-known analogues such as methylphosphonates and phosphorothioates. The hydrolytic stability and nuclease resistance of the boranophosphate linkage can be used for the creation of various biologically active conjugates and new classes of antiviral drugs. The increased lipophilicity of boranophosphate diesters can be employed in the delivery of boranophosphate prodrugs to improve the antiviral activity of known chain terminating nucleoside analogues. The ability of boronated NTPs to be substrates for polymerases has already found applications in direct PCR sequencing, aptamer selection, and RNA interference. The stimulation of RNase H activity by oligonucleoside boranophosphates makes these analogues good candidates for antisense therapy. The highly active and potent silencing effects of boranophosphate siRNA show the promise of its application in RNAi-based therapeutics. Rapidly developing synthetic methods for the preparation of different types of boranophosphates ranging from nucleotides to oligonucleotides, described in this review, raise the hope that soon these analogues will be the compounds of choice for various molecular biology applications and nucleic acid-based diagnostics and therapeutics.

11. Abbreviations

AZT	3′-azido-2′,3′-dideoxythymidine
BNCT	boron neutron capture therapy
BPi	inorganic boranophosphate
BSA	*N*,*O*-bis(trimethylsilyl)acetamide
BSPDE	bovine spleen phosphodiesterase
CE	cyanoethyl
CK	creatine kinase
DIPEA	diisopropylethylamine
DPCP	diphenylchlorophosphate
ds/ss	double-stranded/single-stranded
d4T	2′,3′-didehydro-2′,3′-dideoxythymidine
FE/SE	fast-eluting/slow-eluting
MMLV	moloney murine leukemia virus
NDPK	nucleoside diphosphate kinase
dN	2′-deoxyribonucleoside
rN	ribonucleoside
ddN	2′,3′-dideoxynucleoside
acycloNMPB	acyclonucleoside boranomonophosphate
acycloNTPαB	acyclonucleoside 5′-(α-P-borano)triphosphate
NMPB	nucleoside boranomonophosphate
*c*NMPB	nucleoside 3′,5′-cyclic boranomonophosphate
*c*NMPBS	nucleoside 3′,5′-cyclic boranophosphorothioate
2′,3′-*c*NMPB	nucleoside 2′,3′-cyclic boranomonophosphate
NDPαB	nucleoside 5′-(α-P-borano)diphosphate
NTPαB	nucleoside 5′-(α-P-borano)triphosphate
$Np_n(B)N'$	dinucleoside poly(borano)phosphate
N^bpN'	dinucleoside boranophosphate
ODN	oligodeoxyribonucleotide
^{b}ODN	oligodeoxyribonucleotide with a single boranophosphate internucleotidic linkage
BH^3-ODN	oligodeoxyribonucleotide with all boranophosphate internucleotidic linkages
PK	pyruvate kinase
PPi	pyrophosphate
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	short interfering RNA
RT	reverse transcriptase
SELEX	systematic evolution of ligands by exponential enrichment
SVPDE	snake venom phosphodiesterase
TEAA	triethylammonium acetate
TEAB	triethylammonium bicarbonate

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