Synthesis and Characterization of a New Fluorine Substituted Nonionic Dinucleoside Phosphonate Analogue, *P*-Deoxy-*P*-(difluoromethyl)thymidylyl(3′→5′)thymidine

D. E. Bergstrom* and P. W. Shum

Department of Chemistry, University of North Dakota, Grand Forks, North Dakota 58202

Received January 25, 1988

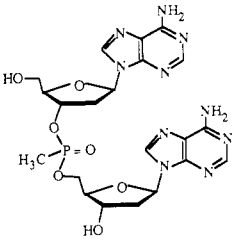
The (difluoromethyl)phosphonate group can be introduced as a 3′→5′ linkage between nucleosides by the sequential reaction of bis(1-benzotriazolyl) (difluoromethyl)phosphonate with 5′-*O*-(4,4′-dimethoxytrityl)thymidine and 3′-*O*-levulinylthymidine. Two diastereomers differing in configuration at phosphorus are formed in nearly equal amounts but can be separated by column chromatography. The protecting groups can be removed by treatment of the protected dinucleoside(difluoromethyl)phosphonate difluoromethylphosphonate with hydrazine in pyridine/acetic acid followed by treatment with 80% acetic acid to give *P*-deoxy-*P*-(difluoromethyl)thymidylyl(3′→5′)thymidine. There are substantial differences in the ^{19}F NMR spectra of the two diastereomers, which suggests that this may be a significant and useful parameter in structure and binding studies on nonionic phosphonate linked oligonucleotide analogues.

Introduction

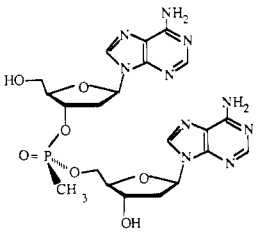
Phosphate-modified oligonucleotide analogues have been extensively investigated for the sequence specific hybridization arrest of mRNA translation.[1-5] and as biochemical probes of nucleic acid–protein interactions.[6-10] The four major types of phosphorus modification include phosphorothioates, phosphoramidates, phosphotriesters, and alkylphosphonates. The latter three linkages are nonionic, thus facilitating transport of the modified oligomers through biological membranes.[11,12] Of the nonionic analogues, the alkylphosphonates appear to be the most stable toward nucleases. Normal phosphodiester linked oligodeoxyribonucleotides have low cell permeability and relatively short half-lives in serum because of degradation by nucleases.[13]

Among the oligodeoxyribonucleotide alkylphosphonates analogues, oligodeoxyribonucleoside methylphosphonates have been most widely studied. Although, originally the methyl group seemed likely be a preferential candidate for nonionic linkages because of its small size, it is now clear that it has a measurable influence on conformation. Hybridization of oligodeoxyribonucleoside methylphosphonates to their natural complementary sequences is enhanced in some cases,[14] but the binding is complicated by a stereoisomer problem. In the case of the binding of $Ap_{Me}A$ (1, p_{Me} = the methylphosphonate linkage) to poly(U) or poly(T) binding differs, but is enhanced for both the *Rp* and *Sp* stereoisomers relative to ApA (2:1 poly(U) to Ap_XA complexes are formed).[15] In the case of the triesters, $Ap_{OR}A$ and $Ap_{OR}Ap_{OR}A$, the size of the substituent does not lessen the ability of these analogues to hybridize. Letsinger et al. reported that these phosphotriester analogues containing bulky, lipophilic groups, such as 2,2,2-trichloro-1,1-dimethylethoxy, had higher melting temperatures in their complexes with poly(U) and poly(T) than did the corresponding ApA and ApApA oligomers.[16] The enhancement in binding is thought to result from decreased charge repulsion between the nonionic analogues and the negatively charged natural complementary oligomers.

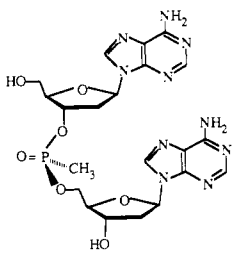
The conformations of some deoxyribonucleoside methylphosphonates in solution have been extensively studied by ^1H NMR.[14] Both the NMR and calorimetric studies[17] indicate that the bases in the two diastereomeric forms of *P*-deoxy-*P*-(methyl)deoxyadenylyl(3′→5′)deoxyadenosine are stacked differently in solution.



1



1 Sp



1 Rp

(1) Miller, P. S. In *Development of Target-Oriented Anticancer Drugs*; Cheng, Y. C., et al., Eds.; Raven: New York, 1983; p 189.
(2) Smith, C. C.; Aurelian, L.; Reddy, M. P.; Miller, P. S.; Ts'o. P. O. P. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2787.
(3) Miller, P. S.; Agris, C. H.; Aurleian, L.; Blake, K. R.; Murakami, A.; Reddy, M. P.; Spitz, S. A.; Ts'o, P. O. P. *Biochimie* **1985**, *67*, 769–776.
(4) Markus-Sekura, C. J.; Woerner, A. M.; Shinozuka, K.; Zon, G.; Quinnan, G. V., Jr. *Nucleic Acids Res.* **1987**, *15*, 5749–5763.
(5) Agris, C. H.; Blake, K. R.; Miller, P. S.; Reddy, M. P.; Ts'o, P. O. P. *Biochemistry* **1986**, *25*, 6268.
(6) Noble, S. A.; Fisher, E. F.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 3387.
(7) Miller, P. S.; Dreon, N.; Pulford, S. M.; McParland, K. B. *J. Biol. Chem.* **1980**, *255*, 9659–9665.
(8) Agarwal, K. L.; and Riftina, F. *Nucleic Acids Res.* **1979**, *6*, 3009–3023.
(9) Koziolkiewicz, M.; Uznanski, B.; Stec, W. J.; Zon, G. *Chem. Scr.* **1986**, *26*, 251–260.
(10) Miller, P. S.; Annan, N. D.; McParland, K. B.; Pulford, S. M. *Biochemistry* **1982**, *21*, 2507–2512.
(11) Miller, P. S.; Barrett, J. C.; Ts'o, P. O. P. *Biochemistry* **1974**, *13*, 4887–4896.
(12) Miller, P. S.; McParland, K. B.; Jayaraman, K.; T'so, P. O. P. *Biochemistry* **1981**, *20*, 1874–1880.
(13) Wickstrom, E. J. *Biochem. Biophys. Methods* **1986**, *13*, 97–102.
(14) Miller, P. S.; Yano, J.; Yano, E.; Carroll, C.; Jayaraman, K.; Ts'o, P. O. P. *Biochemistry* **1979**, *18*, 5134.
(15) Kan, L. S.; Cheng, D. M.; Miller, P. S.; Ts'O, P. O. P. *Biochemistry* **1980**, *19*, 2122.
(16) Letsinger, R. L.; Bach, S. A.; Eadie, J. S. *Nucleic Acids Res.* **1986**, *14*, 3487.
(17) Wierzchowski, K. L.; Zielenkiewicz, A.; Miller, P. S. *Biopolymers* **1984**, *23*, 2361–2382.

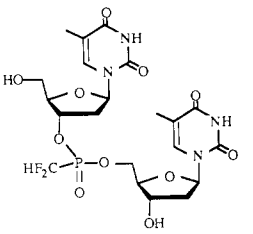
0022-3263/88/1953-3953$01.50/0

Where the methylphosphonate is incorporated at a single site in an 8-mer, the *Sp* isomer gives a less stable duplex. In the *Sp* configuration the methyl of the methylphosphonate points toward the major grove and is relatively close to the 3′ proton.[18] A chirality preference has been observed in a protein–DNA interaction. Caruthers et al. reported that deoxyribonucleoside methylphosphonates could function as a probe in the studies of the interaction between the *lac* operator and *lac* repressor.[19] The stability of the complex formed between the *lac* operator, in which a single linkage was substituted by either a Rp or Sp methylphosphonate, and *lac* repressor was significantly greater for the one of the stereoisomers. However, since absolute configurations were not determined, it was unknown which stereoisomer gave the stable complex.

The structural, biochemical, and biological studies on methylphosphonate linked nucleosides suggest that other isosteric phosphonates may be worth studying. In particular it would be of interest to attach groups that could function as useful probes of the microenvironment surrounding the phosphonate linkage. In addition it would be of interest to attach a group that resembles oxygen in polarity. The CF_2 and CFH groups have been proposed as reasonable isosteric and isopolar replacements for neutral oxygen.[20] These groups could be either incorporated into the phosphate backbone of oligodeoxyribonucleotides in place of the 3′ or 5′ oxygens or incorporated as CF_2H, CFH_2, or CF_3 in place of the methyl in oligodeoxyribonucleotide methylphosphonate analogues. The latter synthetic modification would be simplest to accomplish and would provide nonionic oligodeoxyribonucleoside analogues that could function as convenient ^{19}F NMR spectroscopic probes both in vivo and in vitro. Information about conformational differences assumed by *Sp* and *Rp* stereoisomers and about complementary binding between oligonucleotide strands in which some phosphodiester linkages are replaced by (difluoromethyl)phosphonate could be obtained from ^{19}F NMR signals. Since deoxyribonucleotides are chiral, the fluorines of a CF_2H group introduced a place of a phosphate oxygen would be diastereotopic. Proteins and enzymes that bind oligonucleotides could influence local environment and exacerbate the chemical shift differences between the diastereotopic fluorine atoms.

Our initial research has concentrated on (difluoromethyl)phosphonate analogues for a number of reasons. First, the CF_2H group is likely to be more chemically stable than a CH_2F group, which could potentially function as an alkylating agent. Second, the synthesis of (difluoromethyl)phosphonates is more straightforward than the synthesis of either (trifluoromethyl)- or (monofluoromethyl)phosphonates. Third, the isopolar CF_2H moiety most closely resembles an OH group in structure and in that respect might behave like a nucleic acid at low pH. This could have important implications where binding of a protein to an oligonucleotide involves hydrogen bonding or proton transfer to a phosphodiester group. Of the possible fluoromethyl modifications (CF_2H, CFH_2, or CF_3), the difluoromethyl group is the most likely to be involved in polar interactions with solvent or other molecules that associate with nucleic acids. In a study with diethyl(difluoromethyl)phosphonate **8**, we determined that the proton on the CHF_2 group interacted strongly with the Lewis base pyridine.[21] The dependence of the proton chemical shift on pyridine concentration resembles the data obtained in hydrogen-bonding studies with chloroform. Microwave spectroscopic studies in the gas phase showed that HCF_3 formed a stable hydrogen-bonded complex with ammonia.[22] In addition, Burg reported that phosphines containing the $P–CHF_2$ linkage had a significantly lower volatility than phosphines containing the $P–CH_3$ and $P–CF_3$ linkages.[23] The low volatility is probably due to C–H–F hydrogen bonds, which, although weaker than hydrogen bonds involving oxygen, is stronger than van der Waals attraction.

With this background we set out to synthesize and characterize *P*-deoxy-*P*-(difluoromethyl)thymidylyl(3′→5′)thymidine (**2**) as the first representative of a new class of phosphonate-linked nucleosides having a polar CF_2H group in place of the OH group (O^- at physiological pH) of the natural phosphodiester linkage.



2

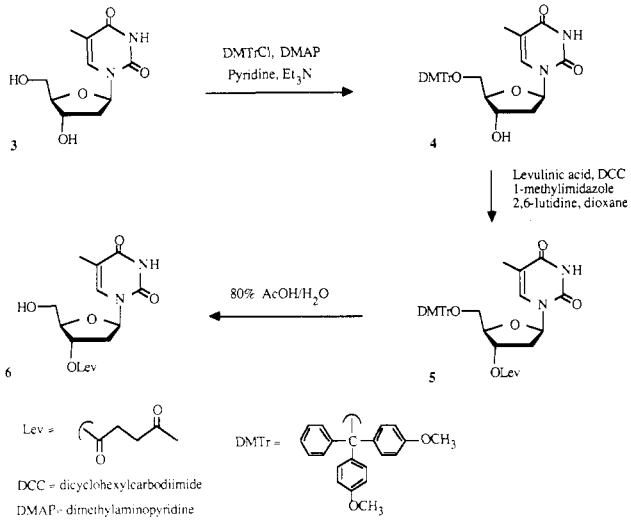
Results and Discussion

Although it would have been attractive to use phosphoramidite type chemistry to incorporate the CHF_2 phosphonate linkage, for a first approach it was most expedient to use a readily accessible difluoromethylphosphonylating reagent and mild reaction conditions. Introduction of fluorine would likely labilize the phosphonate linkage to an even greater extent than observed with methylphosphonate linkages, which are significantly more sensitive to adventitious cleavage by ammonia during deprotection than normal phosphodiester linkages. A preliminary model study with diethyl (difluoromethyl)-phosphonate showed that this functional group would not survive concentrated ammonia, but did remain intact when treated with hydrazine in a pyridine/acetic acid buffer commonly used to cleave reactive esters and amides under nonbasic conditions. As a consequence we chose to model our approach after a scheme devised by van Boom for conventional phosphodiester synthesis via protected hydroxybenzotriazole esters of phosphoric acid.[24] The BTOH phosphotriester approach when applied to dinucleotide synthesis gave a high yield of 3′→5′ linked product and no 3′→3′ linked product.

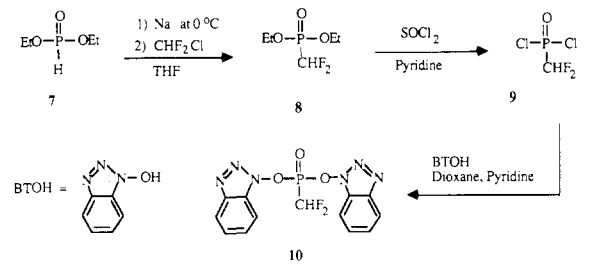
The synthesis of *P*-deoxy-*P*-(difluoromethyl)thymidylyl(3′→5′)thymidine (**2**) is divided into four parts: (1) the synthsis of the 5′-O-protected thymidine **4**, (2) the synthesis of the 3′-O-protected thymidine **6**, (3) the synthesis of the (difluoromethyl)phosphonate moiety, (4) the formation of the required 3′→5′ phosphonodiester linkage by employing the products from (1), (2), and (3).

(18) Bower, M.; Summers, M. F.; Powell, C.; Shinozuka, K.; Regan, J. B.; Zon, G.; Wilson, W. D. *Nucleic Acids Res.* **1987**, *15*, 4915–4930.
(19) Noble, . A.; Fisher, E. F.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 3387.
(20) Blackburn, G. M.; Eckstein, F.; Kent, D. E.; Perree, T. M. *Nucleosides Nucleotides* **1985**, *4*, 165. Blackburn, G. M.; Parratt, M. J. *J. Chem. Soc., Chem. Commun.* **1982**, 1270; **1983**, 886.
(21) Bergstrom, D. E.; Romo, E. H.; Shum, P W. *Nucleosides Nucleotides* **1987**, *6*, 53.
(22) Nelson, D. D., Jr.; Fraser, G. T.; Klemperer, W. *Science (Washington, D.C.)* **1987**, *238*, 1670.
(23) Burg, A. B. *Inorg. Chem.* **1985**, *24*, 3342.
(24) Marugg, J. E.; de Vroom, E.; Tromp, M.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1986**, *14*(5), 2171.

Scheme I



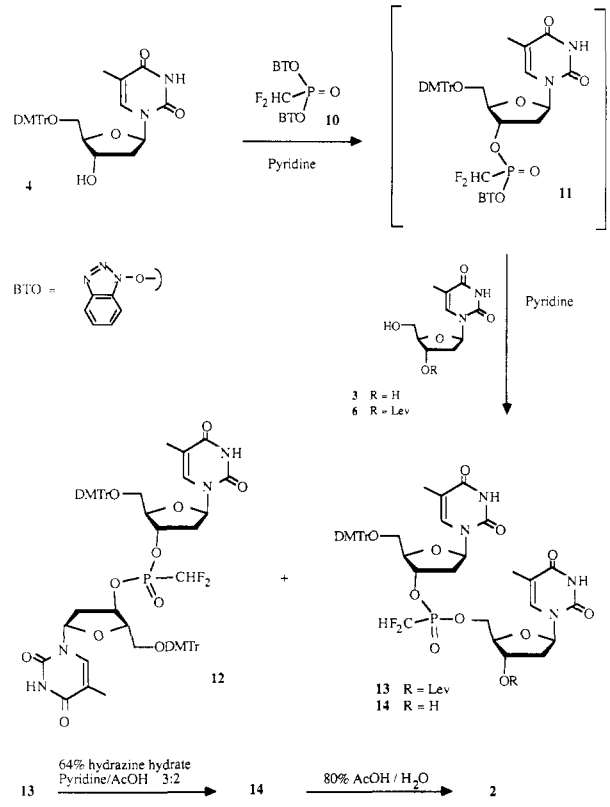
Scheme II



4,4′-Dimethoxytriphenylmethyl, which is currently the most widely used 5′ protecting group in the oligodeoxyribonucleotide synthesis,[25] appeared suitable for the synthesis of the (difluoromethyl)phosphonate-linked nucleosides. As long as precautions were taken to preclude acid, the 4,4′-dimethoxytrityl group would be expected to remain intact during the reactions required to create the (difluoromethyl)phosphonate linkage. It was well established that the group can easily removed under mild acidic conditions such as 80% glacial acetic acid in water or 3% dichloroacetic acid (pK_a = 1.5) in methylene chloride. Compound **4** was obtained in 80% yield by a standard literature procedure.[26]

Levulinyl (4-oxopentanoyl) was chosen as the protecting group for the 3′-OH of thymidine.[27] The levulinyl group can be selectively and rapidly removed under neutral conditions: 64% hydrazine hydrate in pyridine/acetic acid (4:1 v/v) for 10 min. These conditions do not affect the 4,4′-dimethoxytrityl protecting group. On the other hand, the levulinyl group is stable under conditions that remove the 4,4′-dimethoxytrityl group. The reaction of **4** and distilled levulinic acid in the presence of 1-methylimidazole, 2,6-lutidine, dicyclohexylcarbodiimide (DCC), and anhydrous dioxane as solvent yielded 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-levulinylthymidine (**5**) in 83% yield (Scheme I). The crude product was further purified by recrystallization from benzene. Subsequent treatment of **5** with 80% acetic acid in water afforded 3′-*O*-levulinylthymidine (**6**) in 72% yield.

Scheme III



Synthesis of the (difluoromethyl)phosphonate moiety (phosphonylating agent) was achieved by the reaction of diethyl phosphite (**7**) first with metallic sodium and then with chlorodifluoromethane to give diethyl (difluoromethyl)phosphonate (**8**) in 54% yield (Scheme II).[28] The resonance of the hydrogen on the *gem*-difluorocarbon of **8** appears as a doublet of a triplet in the ^{1}H NMR spectrum (J_{HF} = 49 Hz, J_{HP} = 27 Hz). The fluorine atom signals appear as a quartet in the ^{19}F spectrum (J_{PF} = 91 Hz). Compound **8** was refluxed with thionyl chloride for 3 days to give (difluoromethyl)phosphonic dichloride (**9**) in 73% yield.[29] The ^{19}F NMR spectrum of **9** is a doublet of doublet (J_{HF} = 49 Hz, J_{PF} = 115 Hz). The ^{1}H NMR spectrum of **9** appears as a quartet but is actually a doublet of a triplet (J_{PH} = 24 Hz). Dichloride **9** is extremely moisture sensitive; however, it can be stored in a desiccator and fractionally distilled before use.

Compound **9** can function as the phosphonylating agent for 2 equiv of alcohol. Early studies of the synthesis of *P*-deoxy-*P*-methyldeoxyribonucleotidyl(3′→5′)deoxynucleoside using methylphosphonic dichloride as the phosphonylating agent showed that a significant amount of 3′→3′ linked product (10%) was formed and the yields of 3′→5′ linked (required) product was generally low (30–60% depending upon the nucleosides).

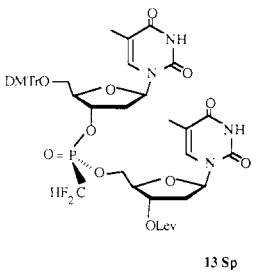
Compound **9** was converted to bis(1-benzotriazolyl) (difluoromethyl)phosphonate (**10**) (Scheme III), which functioned as the phosphonylating agent in the subsequent reactions. Compound **10** is very moisture sensitive and must be generated in situ immediately prior to use.

TLC analysis showed that **4** completely reacted with bis(1-benzotriazolyl) (difluoromethyl)phosphonate (**10**) within 30 min. Two major spots were observed at R_f 0.7 and R_f 0 (silica gel; 9/1 chloroform/methanol). The high

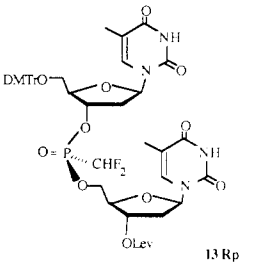
(25) Smith, M.; Rammler, D. H.; Goldberg, I. H.; Khorana, H. G. *J. Am. Chem. Soc.* **1962**, *84*, 430. Gait, M. J. *Oligonucleotide Synthesis: a practical approach*; IRI: Oxford, 1984.
(26) Jones, R. A. In *Oligonucleotide Synthesis: a practical approach*; Gait, M. J., Ed.; IRL: Oxford, 1984; p 27.
(27) van Boom, J. H.; Burgers, P. M. *Recl. Trav. Chim. Pays-Bas* **1978**, *97*(3), 73.
(28) Obayashi, M.; Ito, E.; Matsui, K.; Kondo, K. *Tetrahedron Lett.* **1982**, *23*(22), 2323.
(29) Agarwal, K. L.; Riftino, E. *Nucleic Acids Res.* **1979**, *6*(2), 3009.

R_f spot was identified as the 3′→3′ linked product **12**. Compound **6** was added to the reaction mixture, whereupon TLC showed the appearance of the 3′→5′ linked thymidine dimer **13** (R_f 0.46). No further reaction was observed after 2 h but a substantial amount of baseline material remained which was not identified but which is probably 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-[(difluoromethyl)phosphonate].[30] The yields of dinucleotide analogues **12** and **13** were 35–40% and 10%, respectively. By running the first coupling reaction (formation of **11**) in an ice bath, the yield of the 3′→5′ linked dimer **13** increased to 54%. The enhanced reactivity of phosphonylating agent **10** due to the strongly electron withdrawing difluoromethyl group is undoubtedly influences the formation of 3′→3′ linked dimer **12**. The deprotection of **13** proceeded cleanly under the conditions described above. Cleavage of the phosphonate diester linkage was not observed.

Synthesis of the thymidine dimer by employing the unproected thymidine **3** instead of 3′-*O*-levulinylthymidine **6** in the second coupling reaction has also proven to be feasible. Apparently, the difference in reactivity between the 5′-OH and 3′-OH is substantial enough to ensure the regiospecificity of the reaction. The synthesis of **2** is greatly simplified by using the unprotected thymidine **3** in the second coupling reaction.



13 Sp



13 Rp

The two stereoisomers of the fully protected 3′→5′ linked thymidine dimer, **13Rp** and **13Sp**, were successfully separated on a silica gel column by eluting with an ethyl acetate/methanol gradient (0–10% MeOH in the presence of 0.5% Et_3N, in 0.25% stepwise increments). The ^1H NMR spectra of the two separated diastereomers were similar, and data on the separated isomers are not presented here. Although the signal of the hydrogen on the *gem*-difluoro carbon (CHF_2) partially overlapped with that of the anomeric hydrogen, the unique signal of the hydrogen on CHF_2 could be clearly identified in the high-field spectra. Two sets of CHF_2 signals were observed in the ^1H spectrum containing a mixture of the diastereomers. The chemical shift of the signals differed by 0.06 ppm (300-MHz spectrometer). The spectrum of each separated diastereomers showed only one set of CHF_2 signals, which matched with the CHF_2 signals in the spectrum containing a mixture of diastereomers.

No unusual features were observed in the ^{13}C NMR spectra. By comparing spectra of compounds **3–6** with dimers **2**, **13**, and **14**, it was relatively simple to make peak assignments for the more complex molecules. Because of splitting by fluorine, the ^{13}C signal due to the CHF_2 group was too weak to be observed.

The ^{19}F signals of the two fluorine atoms are strongly solvent dependent. The ^{19}F spectra of the two diastereomers of **13** were run at 25 °C in $CDCl_3$ and in acetone-d_6. In chloroform the lower R_f diastereomer appeared as eight peaks centered at 27.5 ppm, and the higher R_f diastereomer appeared as six peaks (fewer peaks than predicted are observed because of peak overlap) centered at 27.9 ppm (the relatively weak signals due to fluorine–fluorine splitting were not observed above the background noise; 16 peaks should actually be observed for each isomer under ideal conditions). In acetone-d_6 the low-R_f diastereomer showed four peaks (dd, J_{HF} = 48 Hz and J_{PF} = 94 Hz). The two fluorine atoms had exactly the same chemical shift although they are diastereotopic. The other diastereomer with R_f 0.18 (ethyl acetate) showed 16 peaks as a result of the chemical shift difference between the two diastereotopic fluorine atoms (two ddd, J_{HF} = 48 Hz, J_{PF} = 93 Hz, J_{FF} = 352 Hz). The eight outer peaks were of weak intensity because of the relatively small difference between two fluorine chemical shifts relative to the fluorine–fluorine coupling constant. Burton observed a similar spectrum on a computer-simulated ^{19}F NMR spectrum of ethyl phenyl (bromodifluoromethyl)phosphonate.[31]

The chemical shifts are both solvent and temperature dependent. The ^{19}F NMR signal of the lower R_f diastereomer in acetone-d_6 separated from four into eight peaks when the surrounding temperature fell below −15 °C, while in $CDCl_3$ coalescence was not observed at temperature as high as 50 °C. At low temperature, the two fluorine atoms must reside in chemically distinct environments. The sensitivity to local environment observed here could prove to be very useful where fluorine is used as a probe in hybridization or protein binding studies. More detailed NMR studies are under way and will be presented in a future paper.

The ^{19} NMR (acetone-d_6) spectrum of the fully protected 3′→3′ linkage thymidine dimer **12** showed two sets of four peaks (dd) separated by 5 Hz. Each set represented one of the two fluorine atoms which were diastereotopic to each other, and hence the two fluorine atoms showed up at different chemical shifts in the spectrum. Theoretically, two additional sets of four peaks should be observed at about 350 Hz upfield and downfield from these resonances due to fluorine–fluorine coupling; however, these were not detected because the (difference in chemical shift F–F)/J_{FF} ratio was close to unity.

As expected, the ^{31}P NMR signals for the dimer **13** were observed at a chemical shift significantly downfield from that observed for the phosphorus of the methylphosphonate analogue $DMTrTp_{CH_3}TOAc$. Noble et al. reported the methylphosphonate ^{31}P signals at −31.66 and −32.22 ppm for the two diastereomers,[19] while we observe the $PCHF_2$ phosphorus resonances as triplets centered at 5.39 and 4.97 ppm in the same solvent ($CHCl_3$)9. In comparison the ^{31}P resonance for TpT occurs at −0.36 ppm in D_2O at 30 °C.[32] The ^{31}P signal for dinucleoside phosphotriesters in chloroform solvent is also generally observed near 0 ppm.[33]

Experimental Section

General Methods. All glassware was washed in a base bath and then dried at 110 °C for 15 h. Pyridine was dried by refluxing with BaO for 72 h and distilled. Pyridine was redistilled from over *p*-toluenesulfonyl chloride. Dioxane and tetrahydrofuran (THF) were dried by passing through anhydrous alumina and then distilled over sodium. 4,4′-Dimethoxytrityl chloride was purchased from either Aldrich or Cruachem and used without further purified. Triethylamine (TEA) was refluxed with KOH for 3 h and then distilled. 4-(Dimethylamino)pyridine (DMAP) (from Aldrich) was recrystallized from diethyl ether and then dried in vacuo over

(30) The baseline materials were not isolated. Spraying the TLC plate showed the presence of the di-*p*-methoxytrityl group on the baseline spot.

(31) D. J. Burton, personnel communication.

(32) Cheng, D. M.; Kan, L.-S.; Iuorno, V. L.; Ts'o, P. O. P. *Biopolymers* **1984**, *23*, 575–592.

(33) Herding, W.; Seela, F. *J. Org. Chem.* **1985**, *50*, 5314–5323.

parafilm at room temperature. Levulinic acid and 1-methylimidazole were distilled under reduced pressure and stored over molecular sieves. 2,6-Lutidine was refluxed over calcium hydride, distilled under reduced pressure, and stored over molecular sieves. Dicyclohexylcarbodiimide (DCC) was obtained from Aldrich. Diethyl phosphonate was obtained from Aldrich and distilled under reduced pressure before use. Thionyl chloride (from Aldrich) was purified with triphenyl phosphite and fractionally distilled. 1-Hydroxybenzotriazole (from Sigma) was dried in vacuo over P_2O_5 at 50 °C for 3 days. Triethylammonium bicarbonate (TEAB) solution was prepared by bubbling carbon dioxide into triethylamine solution of required molarity until the pH reached 7.3. Hydrazine hydrate (64%) was purchased from Eastman. Eighty percent acetic acid was prepared from glacial acetic acid and distilled water. Thymidine was obtained from the United States Biochemical Corp. All moisture-sensitive chemicals were weighed out in a dry box filled with dry nitrogen. All moisture sensitive reactions were carried out in an argon or nitrogen atmosphere.

TLC was done on EM Science precoated silica gel F-254 plastic plates and the solvent system was chloroform/methanol (90/10%) unless otherwise stated. Spots containing the DMTr group appeared orange after the TLC plates were sprayed with dilute sulfuric acid. Column chromatography packing was silica gel (EM, 70–230 mesh). The columns were eluted with chloroform/methanol (100/0% to 90/10%) either in a linear gradient or by stepwise 1–2% increments. All ^1H, ^{19}F, ^{13}C, and ^{31}P spectra were run on a Varian VXR 300-MHz spectrometer. The internal reference for ^1H and ^{13}C spectra was TMS. Ten percent hexafluorobenzene in $CDCl_3$ was used as the external reference for ^{19}F spectra while ^{31}P spectra were referenced to external 5% H_3PO_4 in D_2O. UV measurements were done on a Shimadzu Model 260 UV–vis spectrophotometer. The mass spectra of all compounds were run by the Midwest Center for Mass Spectrometry at the University of Nebraska—Lincoln, Department of Chemistry. All compounds were analyzed by fast atom bombardment and the molecular ions of each compounds matched the molecular formula. Melting points are not reported because the compounds undergo slow decomposition on heating and do not give well-defined melting points.

Preparation of 3′-*O*-Levulinyl-5′-*O*-(4,4′-dimethoxytrityl)thymidine (5).[34] 5′-*O*-(4,4′-Dimethoxytrityl)thymidine (4)[26] (16.79 g, 31 mmol), levulinic acid (10.80 g, 93 mmol), 2,6-lutidine (12 mL, 103 mmol), and 1-methylimidazole (3 mL, 38 mmol) were dissolved in anhydrous dioxane (150 mL) in a round-bottom flask (500 mL with 24/40 joint) under argon. DCC (19.19 g, 93 mmol) in ahydrous dioxane (80 mL) was added dropwise to the above solution. The reaction was stirred at 38 °C for 12 h. After the dicyclohexylurea was filtered off, methylene chloride (250 mL) was added to the filtrate. Then, the solution was washed with 2% $NaHCO_3$ (300 mL), and the aqueous layer was extracted repeatedly with methylene chloride (4 × 150 mL). The combined organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo. The resulting gum was dissolved in a minimal amount of chloroform and loaded onto a silica gel column (450 g), which was eluted with chloroform/methanol (100/0 to 90/10% linear gradient). The appropriate fractions were concentrated and dissolved in 150 mL of hot benzene. Cyclohexane was added until turbidity occurred. The product was filtered off and dried in vacuo over KOH and parafilm. Yield: 16.74 g (84%). Anal. (high-resolution MS) calcd for $C_{36}H_{38}O_9N_2$: 642.2578. Found: 642.2578. UV ($CHCl_3$): 267.8 nm ($\epsilon = 1.08 \times 10^4$ M^{-1} cm^{-1}), 241.4 nm ($\epsilon = 1.62 \times 10^4$ M^{-1} cm^{-1}).

Preparation of 3′-*O*-Levulinylthymidine (6).[34] Eighty percent acetic acid (100 mL) was added to a round-bottom flask (300-mL with 24/40 joint) which contained **5** (18.0 g, 28 mmol). The reaction mixture was stirred at room temperature for 2 h. Then, excess acetic acid was removed in vacuo. The residue was dissolved in methylene chloride (300 mL) and washed with 10% sodium bicarbonate solution (300 mL). The aqueous layer was continuously extracted with methylene chloride until no further product remained in the aqueous layer. The combined organic layer was dried over anhydrous sodium sulfate. After the solvent was removed, the residue was loaded onto a silica gel column (250 g), which was eluted with chloroform/methanol (100/0 to 90/10% linearly). The appropriate fractions were combined and the solvent was removed in vacuo. The remaining oil was dissolved in a mixture of ethanol and water. The required product was obtained after lyophilization. Yield: 6.9 g (72%). Anal. (high-resolution MS) calcd for $C_{15}H_{28}O_7N_2$: 340.1271. Found: 340.1271. UV ($CHCl_3$): 265.4 nm ($\epsilon = 0.97 \times 10^4$ M^{-1} cm^{-1}).

Preparation of Diethyl (Difluoromethyl)phosphonate (8). Diethyl phosphite (258.1 g, 1.87 mol) was dissolved in anhydrous THF (1 L) under argon in a three-neck flask (2-L with 24/40 joints) which was connected to a dry ice/acetone condenser. The reaction was cooled in an ice bath and Na wire (45 g, 1.96 mol) was added in portions. After all the sodium dissolved (8–10 h), the argon supply was shut off and anhydrous chlorodifluoromethane (220 g, 2.54 mol) was bubbled into the reaction mixture. The solution turned cloudy within 3 min. After the reaction mixture was stirred overnight, NaCl was filtered off by suction filtration through a sintered glass funnel. The NaCl residue was washed thoroughly with diethyl ether and the solvent was removed from the filtrate in vacuo. The crude product was purified by fractional distillation under reduced pressure (bp 62 °C at 1.5 Torr: lit.[35] bp 85.6–86.5 °C at 12 Torr). Yield: 188.4 g (54%).

Preparation of (Difluoromethyl)phosphonic Dichloride (9). Excess thionyl chloride (60 mL) was added dropwise into a round-bottom flask (100 mL) which contained **8** (15.0 g, 79.8 mmol) and pyridine (1 mL). The reaction was protected from moisture and refluxed for 3 days. Thionyl chloride (bp 78–82 °C) was removed by fractional distillation of the mixture. The product was obtained in the fractions that distilled at 128–133 °C (lit.[35] bp 50–52 °C at 50 Torr). Yield: 9.8 g (73%).

Preparation of Bis(1-benzotriazolyl) (Difluoromethyl)-phosphonate (10). 1-Hydroxybenzotriazole (1.35 g, 10 mmol), anhydrous pyridine (1.0 mL, 12.4 mmol), and anhydrous dioxane (20 mL) were combined in a round-bottom flask (100 mL with 14/20 joint) under dry argon. The mixture was stirred at room temperature until all BTOH dissolved. Then, the flask was cooled in an ice bath and **9** (0.85 g, 5 mmol) in anhydrous dioxane (10 mL) was added dropwise. The reaction mixture was stirred at room temperature for 2 h. At the end of the reaction, pyridine hydrochloride was filtered off under anhydrous conditions. The phosphonylating reagent (0.16 M in dioxane) was used immediately for the next step.

Preparation of Fully Protected *P*-Deoxy-*P*-(difluoromethyl)thymidylyl(3′→5′)thymidine (13). Compound **4** (1.09 g, 2 mmol) was dried by coevaporating with anhydrous pyridine (3 × 30 mL) to dryness. The protected nucleosides could also be dried by lyophilizing with anhydrous dioxane overnight. The residue was redissolved in anhydrous pyridine (10 mL) and then was added dropwise into a round-bottom flask (100 mL with 14/20 joint) which contained **10** (12.4 mL, 2 mmol, obtained from the previous experiment) cooled in an ice bath for at least 5 min. The reaction mixture was stirred in the ice bath under anhydrous conditions for 30 min. Compound **6** [0.68 g, 2 mmol, coevaporated with anhydrous pyridine (3 × 30 mL) or lyophilized with anhydrous dioxane] was dissolved in 10 mL of anhydrous pyridine. Then, this solution was added dropwise into the above reaction mixture, and the mixture was stirred at room temperature for 2 h (until no further change was observed by TLC). The reaction mixture was diluted with methylene chloride (100 mL) and washed with 1 M (pH 7.3) TEAB (100 mL), and once with water (100 mL). The organic layer was then dried over anhydrous Na_2SO_4. After filtration of the Na_2SO_4, the solvent was removed in vacuo and the resulting oil triturated with petroleum ether (40–60 °C). The precipitate was dissolved in a minimal amount of chloroform and loaded onto a silica gel column (100 g), which was eluted with chloroform/methanol (100/0 to 90/10% linear gradient). The appropriate fractions were combined, and the solvent was removed. The product was precipitated from petroleum ether and dried in vacuo over KOH and parafilm. Yield: 1.06 g (54%). Anal. (high-resolution MS) calcd for $C_{47}H_{51}F_2O_{15}N_4P$: 980.3058. Found: 980.3058. UV ($CHCl_3$): 266.0 nm ($\epsilon = 2.03 \times 10^4$ M^{-1} cm^{-1}), 241.4 nm ($\epsilon = 2.23 \times 10^4$ M^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.41, 1.42 (2

(34) Kumar, G.; Poonian, M. S. *J. Org. Chem.* **1984**, *49*, 4905–4912.

(35) Soborovskii, L. Z.; Baina, N. F. *J. Gen. Chem. USSR* **1959**, *29*, 1115.

d, 3 H), 1.89 (d, 3 H), 2.17, 2.18 (2 s, 3 H), 2.45 (m, 2 H), 2.59 (t, 2 H), 2.60 (m, 2 H), 2.76, 2.78 (2 t, 2 H), 3.39, 3.48 (2 m, 2 H), 3.79 (s, 6 H), 4.14, 4.18 (2 m, 1 H), 4.25, 4.29 (2 m, 1 H), 4.49 (m, 2 H), 5.21, 5.27 (2 m, 1 H) 5.37 (m, 1 H), 5.92, 6.07 (2 dt, 1 H), 6.29 (m, 1 H), 6.43 (m, 1 H), 6.84 (d, 4 H), 7.2–7.4 (m, 9 H), 7.3–7.4, 7.51, 7.55 (2 H); ^{13}C NMR ($CDCl_3$) δ 11.73, 11.77, 12.24, 12.38, 27.79, 27.82, 29.72, 29.74, 36.71, 36.74, 37.81, 37.83, 39.15, 39.55, 55.29, 63.01, 67.28, 63.08, 67.54, 73.61, 73.72, 80.09, 80.17, 82.29, 82.35, 84.23–85.18, 87.40, 111.81, 111.87, 111.94, 113.27, 113.38, 127.33, 128.06, 128.10, 130.09, 134.99, 135.03, 135.34, 143.99, 144.03, 150.49, 150.57, 150.60, 158.86, 163.77, 163.87, 172.47, 172.65, 206.45, 206.57; NMR coupling constants for the PCF_2H group were J_{HF} = 48.2 Hz, J_{PF} = 90.5 Hz, and J_{HP} = 28 Hz.

Compound **12** was isolated from the same experiment. Compound **14** was prepared by the same procedure except thymidine (**3**) was used instead of 3′-levulinylthymidine (**6**). **12**. Anal. (high-resolution MS) calcd for $C_{63}H_{45}F_2O_{15}N_4P$: 1184.4000. Found: 1184.3996. UV ($CHCl_3$): 266.4 nm ($\epsilon = 2.41 \times 10^4$ M^{-1} cm^{-1}), 241.4 nm ($\epsilon = 3.63 \times 10^4$ M^{-1} cm^{-1}). **14**. Anal. (high-resolution MS) calcd for $C_{42}H_{45}F_2O_{13}N_4P$: 882.2689. Found: 882.2687. UV ($CHCl_3$): 266.2 nm ($\epsilon = 2.00 \times 10^4$ M^{-1} cm^{-1}), 241.6 nm ($\epsilon = 2.01 \times 10^4$ M^{-1} cm^{-1}).

Separation of Diastereomers 13R and 13S. Silica gel (100 g) was suspended in ethyl acetate in the presence of 0.5% TEA. The slurry was packed into a column of 2-cm diameter. Compound **13** (147 mg) was dissolved in minimal amount of ethyl acetate (containing 0.5% TEA) and applied to the column. The column was eluted with ethyl acetate/methanol (100/0 to 90/10%) in the presence of 0.5% TEA. The gradient of elution was 0.25% stepwise in 20-mL increments. The appropriate fractions were combined, and the solvent was removed. The percent recovery was not calculated. ^{19}F NMR: low-R_f isomer, δ 27.5 (ddd, 2 F, J_{HF} = 48.2 Hz, J_{PF} = 90.5 Hz); high-R_f isomer, δ 27.9 (ddd, 2 F, J_{HF} = 48.2 Hz, J_{PF} = 90.5 Hz, J_{FF} = 352 Hz). ^{31}P NMR (proton decoupled): low-R_f isomer, δ 4.97 (t); high-R_f isomer, δ 5.39 (t).

Preparation of *P*-Deoxy-*P*-(difluoromethyl)thymidylyl-(3′→5′)thymidine (2). Compound **13** (0.08 g, 0.08 mmol) was dissolved in a mixture of pyridine (1.5 mL) and glacial acetic acid (1 mL). Then, 64% hydrazine hydrate (0.13 mL) was added and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was cooled in an ice bath and 2,4-pentanedione (0.5 mL) was added. After 10 min, the reaction mixture was diluted with methylene chloride (20 mL) and washed with 1 M TEAB (pH 7.3, 20 mL) and distilled water (20 mL). The organic layer was dried over anhydrous Na_2SO_4. After filtration, the organic layer was concentrated and then triturated with petroleum ether. The precipitate was redissolved in a minimal amount of methylene chloride and then was loaded onto a short silica gel column (8 g). The column was eluted with methylene chloride/methanol (100/0% to 92/8%). The appropriate fractions were combined and solvent was removed in vacuo to yield a viscous oil. No further purification was attempted. The crude product was dissolved into 80% acetic acid (v/v) (2 mL). An orange color appeared immediatedly. The reaction mixture was stirred at room temperature for 3 h or until no starting material was observed on the TLC plate. Then, the excess acetic acid was removed in vacuo and distilled water (10 mL) added. The aqueous mixture was extracted with benzene (2 × 10 mL). The crude product obtained after the removal of water (lyophilization) was further purified by redissolving in water, extracting with benzene, and lyophilizing for 3 days. The yield for this step only was 60%, but the overall yield of the two deprotection steps was approximately 10%. Anal. (high-resolution MS) calcd for $C_{21}H_{27}F_2O_{11}N_4P$: 580.1383. Found: 580.1382. UV (H_2O): 266.8 nm ($\epsilon = 1.71 \times 10^4$ M^{-1} cm^{-1}); ^1H NMR (DMSO-d_6) δ 1.78 (m, 6 H), 2.13 (m, 2 H), 2.37 (m, 2 H), 3.60 (m, 2 H), 3.96 (m, 1 H), 4.08 (m, 1 H), 4.25 (m, 1 H), 4.39 (m, 2 H), 5.20 (m, 1 H), 6.22 (dd, 2 H), 6.64, 6.65 (2 dt, 1 H), 7.43, 7.49 (1 H), 7.68, 7.69 (1 H); ^{13}C NMR (DMSO-d_6) δ 11.88, 11.91, 11.95, 12.19, 37.58, 37.62, 40.21, 60.66, 60.71, 67.51, 69.70, 69.76, 69.79, 79.11, 83.81, 84.08, 85.27, 109.69, 109.74, 109.81, 150.32, 150.35, 163.55, 163.57; ^{31}P NMR (proton decoupled) δ 5.84 (t) 5.93 (t); NMR coupling constants for the PCF_2H group were J_{HF} = 48.9 Hz, J_{PF} = 95.3 Hz, and J_{HP} = 28 Hz.

Acknowledgment. The National Institutes of Health is gratefully acknowledged for support of this research through NIH Grant AI 20480. Mass spectral determinations were performed by the Midwest Center for Mass Spectrometry, a National Science Foundation Regional Instrumentation Facility (Grant No. CHE 8211164). We gratefully acknowledge NSF Instrument Grant CHE-8509872 for contributing to the purchase of the VXR 300 NMR spectrophotometer.

Registry No. **2R**, 115289-03-7; **2S**, 115361-96-1; **3**, 50-89-5; **4**, 40615-39-2; **5**, 93134-37-3; **6**, 78635-98-0; **7**, 762-04-9; **8**, 1478-53-1; **9**, 1554-44-5; **10**, 115288-99-8; **12**, 115289-00-4; **13R**, 115289-01-5; **13S**, 115361-95-0; **14R**, 115289-02-6; **14S**, 115361-97-2; CHF_2Cl, 75-45-6.

Supplementary Material Available: Five tables listing ^1H, ^{13}C, ^{19}F, and ^{31}P NMR assignments and coupling constants for compounds **2–6**, **8**, **9**, **13**, and **14** (5 pages). Ordering information is given on any current masthead page.