Stereochemical Assignment and Stereoselective Synthesis of 3'-*C*-*P*-*N*-5' R_P-Ethyl Phosphonamidate Modified Nucleic Acid

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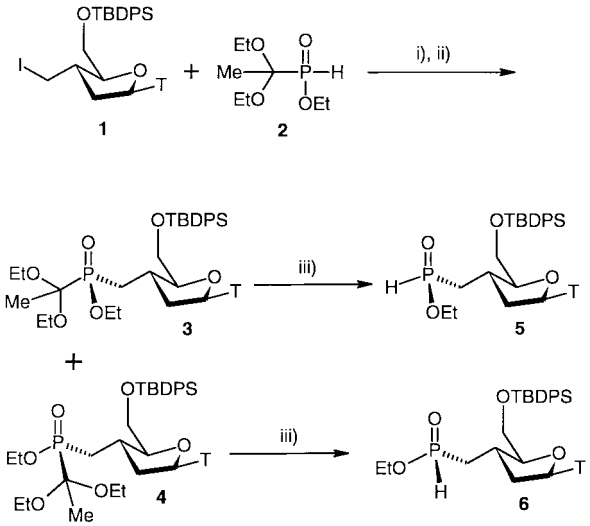
Abstract: The configuration of the duplex stabilising 3'-*C*-*P*-*N*-5' ethyl phosphonamidate nucleic acid backbone modification has been identified as R_P following the preparation of cyclic nucleoside analogues and NOE studies. Complimentary routes for formation of the key phosphonamidate nitrogen to phosphorus bond from stereochemically defined *H*-phosphinate intermediates, involving both a retention and inversion at the phosphorus centre, have been identified.

Key words: antisense, modified oligonucleotides, phosphorus protecting groups, stereoselective

The stereochemistry of modified nucleic acid in which the phosphodiester is replaced with a chiral phosphorus containing linkage has been shown to have a significant impact upon the duplex stabilising properties of such molecules.[2] In the preceding paper[3] a single phosphorus diastereoisomer of 3'-*C*-*P*-*N*-5' ethyl phosphonamidate modified nucleic acid was identified as enhancing binding with complimentary RNA to the extent of ΔT_m+0.9 °C per modification.[3] During this initial exploratory study, individual phosphonamidate diastereoisomers were obtained, after the separation of a 1:1 mixture, at the penultimate stage of the synthesis. To further evaluate the potential of this modification a more efficient route to prepare nucleoside dimers containing the above duplex stabilising phosphonamidate diastereoisomer was desired. Additionally, an assignment of the phosphorus stereochemistry was sought in order to allow a rationalisation of the origins of the enhanced duplex stability.

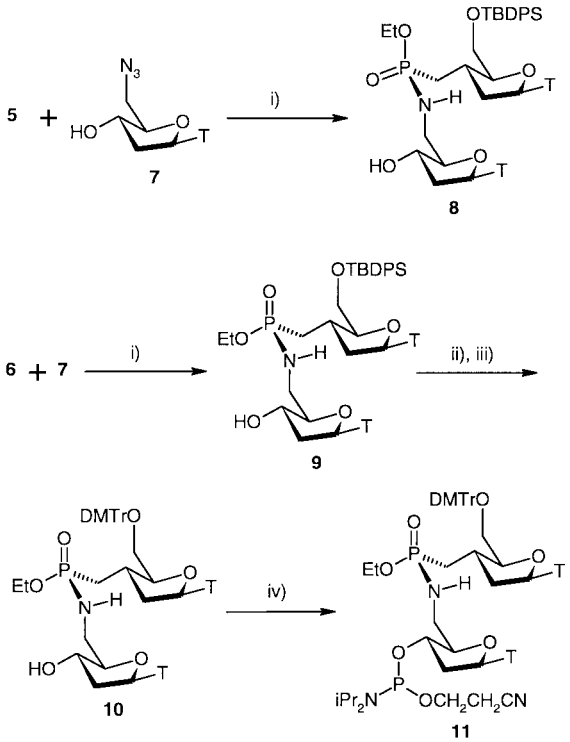
Following the route employed in the initial investigation, thymidine was homologated at the 3'-position to give the 3'-iodomethyl derivative **1**. Alkylation of the potassium anion of the differentially protected hypophosphorous acid synthon **2** with the iodide **1** occurs cleanly to give a 1:1 mixture of the phosphinate diastereoisomers **3** and **4**. In the original preliminary study, these isomers were carried through the synthesis without separation.[3] However, they are readily separable by normal phase medium pressure chromatography, on a multigram scale, to provide the individual diastereoisomer **3** and **4**, assigned as S_P and R_P respectively vide infra, Scheme 1.[4] Our initial interest was to establish if either of the individual *H*-phosphinates **3** or **4** could be carried through the original sequence, whilst retaining the stereochemical integrity at the phosphorus centre to prepare selectively a single diastereoisomeric phosphonamidate dinucleoside. To this end, removal of the ketal protecting group from either **3** or **4** led to significant epimerisation at the phosphorus centre under the standard deprotection conditions of trimethylsilyl chloride, ethanol, chloroform.[5] Reducing the quantity of hydrogen chloride liberated under the above deprotection conditions and performing the reaction in dichloromethane with a minimum amount of ethanol enabled the extent of epimerisation to be maintained consistently below 5%. Following this modified procedure the individual R_P and S_P *H*-phosphinate diastereoisomers **5** and **6** were readily prepared in multigram quantities from **3** and **4** respectively with greater than 95% stereochemical purity.[6]



Scheme 1 i) 4 equiv. **2**, 4 equiv. KHMDS, THF, -78 ºC to rt (85-94%); ii) MPLC separation diastereoisomers; iii) 1.1 equiv. Me_3SiCl, 1.25 equiv. EtOH, CH_2Cl_2, 0 ºC to rt (78-92%).

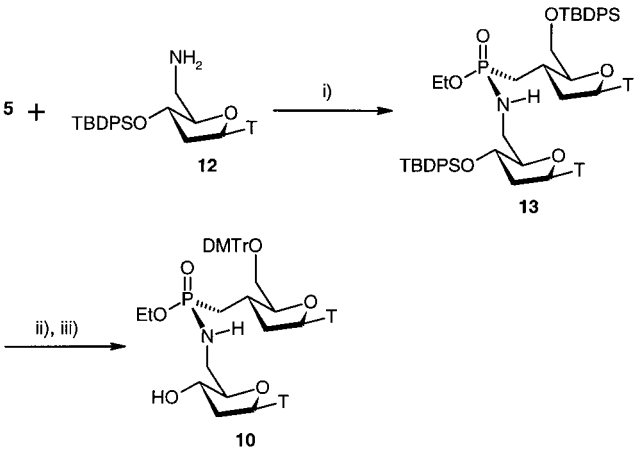
Phosphonamidate formation with either of the separated *H*-phosphinate diastereoisomers **5** or **6** and 5'-azidothymidine **7**, upon activation with bis(trimethylsilyl)trifluoroacetamide, in an analogous manor to that previously described with the mixed diastereoisomers,[3] led to the formation of the corresponding monosilylated dimers **8** and **9** respectively. No significant loss of stereochemical integrity at the phosphorus centre was detected in these reactions.[7] Transformations of this type involving the formation of *O*-silylated *P*(III) intermediates and their subsequent addition to electrophiles are precedented to occur with retention of configuration at the phosphorus

centre.[8] Subsequent removal of the 5'-silyl group from the dimers **8** and **9** followed by selective mono-tritylation at the 5'-position provided intermediates which could be correlated with the original synthesis. This allowed the *H*-phosphinate **6** to be identified as the required diastereoisomer for synthesis, by this route, of the desired DNA-RNA stabilising modification. Further confirmation of this stereochemical assignment was obtained following phosphitylation of the required stereoisomer **10**, derived from **6**, to provide the phosphoramidite **11** as a 1:1 mixture of *P*(III) epimers for oligonucleotide synthesis, Scheme 2.[9]



Scheme 2 i) 6 equiv. $Me_3SiNC(OSiMe_3)CF_3$, pyridine, 0 ºC to rt (64-79%); ii) 2 equiv. $Bu_4N^+F^-$, THF, 0 ºC (94%); iii) 1.2 equiv. DMTrCl, pyidine, rt (71%); iv) 3 equiv. $(^iPr_2N)_2POCH_2CH_2CN$, 5 equiv. Diisopropylammonium tetrazolide, CH_2Cl_2, rt (72%).

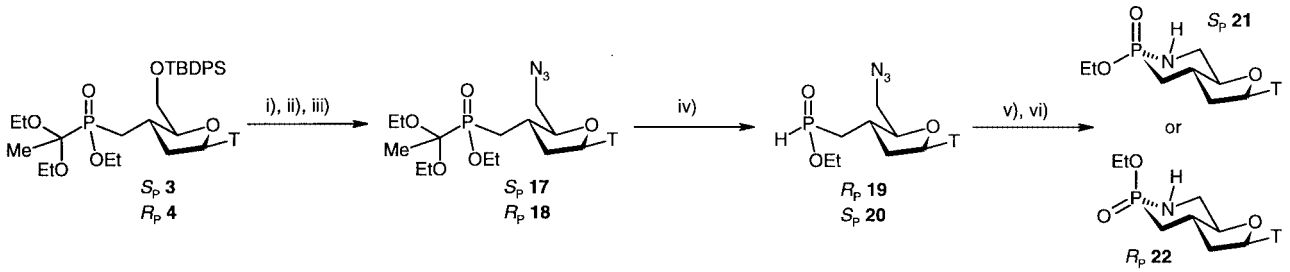
This new sequence described above for the preparation of the desired dinucleoside building block **11** represents an improvement in efficiency over the original route, as an earlier diastereoisomeric separation is made. However, still half the material at the alkylation step remains redundant in the form of the *H*-phosphinate **5**. If conditions could be identified for phosphonamidate formation with **5** that involved an inversion of phosphorus stereochemistry then this material could also be utilised. The Atherton-Todd reaction represents an alternative strategy for *P-N* bond formation from an amine and suitably activated *P-H* species.[10] Stereochemically these reactions involve the formation of an electrophilic *P-Cl* intermediate which would be anticipated to occur with retention of configuration followed by a nucleophilic displacement of chloride ion, which in the majority of examples proceeds with inversion of phosphorus stereochemistry,[11] thus the overall result being an inversion of the phophorus configuration. Applying such conditions with the *H*-phosphinate **5** and the 5'-aminothymidine derivative **12**[12] as the nucleophilic component produced the desired bis-silylated phosphonamidate dimer **13** without significant loss of chiral integrity at the phosphorus centre.[13] Stereochemical correlation with an intermediate of established configuration from the original sequence was made possible after desilylation of **13** followed by selective 5'-monotritylation to again give the R_P phosphonamidate **10**, Scheme 3. Thus, establishing that the anticipated opposite configurational outcome to the azide sequence, of inversion, had occurred at phosphorus.



Scheme 3 i) CCl_4, Et_3N, CH_2Cl_2, pyridine, 3 Å molecular sieves, 0 ºC to rt (75%); ii) 3 equiv. $Bu_4N^+F^-$, THF, 0 ºC (80%); iii) 1.2 equiv. DMTrCl, pyidine, rt (78%).

Methodology has hence been established to improve the overall efficiency by enabling both diastereoisomeric alkylation products **3** and **4** to be employed in the preparation of the required R_P phosphonamidate dinucleoside linkage, via stereochemically complimentary reactions.

To assign the phosphorus stereochemistry in the above reaction sequences cyclic nucleoside analogues originating from **3** and **4** were anticipated to provide derivatives from which the original phosphorus stereochemistry could be deduced by NMR. To this end, intramolecular phosphonamidate bond formation with a 5'-azido nucleoside derivative was investigated, as outlined in Scheme 4.[14] Thus, functional group manipulation at the 5'-position with either **3** or **4**, following a sequence of desilylation, iodination and nucleophilic displacement, produced the 5'-azides **17** and **18** respectively. Subsequent removal of the ortho ester protecting groups, under the improved non-epimerising conditions described above, provided the *H*-phosphinate azides **19** and **20**.[15] Subsequent cyclisation of **19** or **20** upon activation with bis(trimethylsilyl)trifluoroacetamide produced trimethylsilylated cyclic phosphonamidates which could be readily desilylated with tetrabutylammonium flouride to produce **21** and **22** respective-



Scheme 4 i) 1.1 equiv. $Bu_4N^+F^-$, THF, 0 ºC (88-87%); ii) 1.2 equiv. $MeP^+(OPh)_3I^-$, 2 equiv. 2,6-lutidine, DMF, rt (53-65%); iii) 5 equiv. NaN_3, DMF, 80 ºC (89-95%); iv) 1.1 equiv. Me_3SiCl, 1.25 equiv. EtOH, CH_2Cl_2, 0 ºC to rt (63-67%); v) 5 equiv. $Me_3SiNC(OSiMe_3)CF_3$, pyridine, 0 ºC to rt (60-66%); vi) 1 equiv. $Bu_4N^+F^-$, THF, 0 ºC (>95%).

ly.[16] Throughout the above sequences the stereochemical integrity at phosphorus was shown to be retained to the extent of >95% by ^{31}P NMR. The stereochemistry at the phosphonamidate centre was then readily assigned in the cyclic nucleoside derivatives following NOE experiments, **21** as S_P and **22** as R_P.[17]

Assuming the same stereochemical outcome for the intermolecular and intramolecular reactions between the *H*-phosphinate and azido functionality's enables the assignment of R_P to be made to the duplex stabilising dinucleoside modification **11** of interest. Extrapolating further allows an assignments of R_P and S_P to be applied to the starting *H*-phosphinates **5** and **6** respectively. This is based upon transformations having occurred with inversion and retention of phosphorus configuration to produce the phosphonamidate dinucleoside intermediates **13** and **9** respectively. Additionally, deprotection of the mixed ortho ester protecting group, to liberate the *H*-phosphinates **5** and **6**, is anticipated to occur with retention of configuration, based upon analogous dealkylation reactions at phophorus.[18] Thus, enabling the assignments of S_P and R_P to be made to the diastereoisomeric alkylation products **3** and **4** respectively.

The above assignment of the duplex stabilising diastereoisomer as R_P is consistent with previously reported examples in which data for stereochemically defined chiral phosphorus containing linkages has been obtained,[2] with the orientation of the ethyl phosphonamidate ester residue within a helical duplex being dependant on the phosphorus chirality. Incorporation of the S_P phosphonamidate diasteroisomer into a helical duplex would be anticipated to induce a pseudoaxial configuration in which the ethyl residue is oriented into the major groove. In contrast, the R_P diastereoisomer is anticipated to adopt a pseudoequitorial conformation orienting the ethyl residue away from the backbone axis and into the surrounding solvent. Rationalisations implicating both steric and entropic factors to account for the greater stability observed with the pseudoequitorial conformation, equating to the R_P configuration in this example, have been proposed.[19]

In summary, two complimentary routes for the synthesis of the duplex stabilising 3'-*C*-*P*-*N*-5' R_P-ethyl phosphonamidate modification have been developed which utilise both diastereoisomeric *H*-phosphinate intermediates. The phosphorus stereochemistry has been assigned as R_P in the modification of interest following NMR experiments after conversion of individual diastereoisomers of the key *H*-phosphinate intermediates to the corresponding cyclic nucleoside analogues. Further studies with this interesting duplex stabilising 3'-*C*-*P*-*N*-5' R_P-ethyl phosphonamidate modification will investigate the incorporation into a broader range of sequences beyond those containing adjacent thymidines.

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References and Notes

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(4) Stationary phase Kiesegel 60 (80-230 mesh); eluent 5% ethanol in ethyl acetate. First eluting diastereoisomer assigned **3**, second eluting diastereoisomer assigned **4**. NMR spectra were recorded with a Bruker AC400 or Bruker DRX500 instrument. ^{31}P NMR shifts are given as ppm values relative to phosphoric acid.
3; White foam; ^{31}P NMR (MeOH-d_4, 202 MHz) δ 49.58 ppm; ^1H (MeOH-d_4, 500 MHz) δ 7.66-7.58 (m, 4H, *m*-Ph), 7.45 (s, 1H, H6), 7.38-7.27 (m, 6H, *o,p*-Ph), 6.06-6.02 (m, 1H, H1'), 4.17-4.06 (m, 2H, $POCH_2CH_3$), 4.04-3.98 (m, 1H, H5'), 3.87-3.83 (m, 1H, H5'), 3.78-3.73 (m, 1H, H4'), 3.67-3.54 (m, 4H, $COCH_2CH_3$), 2.83-2.72 (m, 1H, H3'), 2.39-2.32 (m, 1H, H2'), 2.28-2.20 (m, 1H, H2'), 2.13-2.04 (m, 1H, H6'), 1.76-1.66 (m, 1H, H6'), 1.40 (d, 3H, *J* = 12 Hz, $PCCH_3$), 1.37 (s, 3H, 5-Me), 1.24 (t, 3H, *J* = 7 Hz, $POCH_2CH_3$), 1.13-1.04 (m, 6H, $COCH_2CH_3$), 0.99 (s, 9H, $SiCMe_3$).
4; White foam; ^{31}P NMR (MeOH-d_4, 202 MHz) δ 49.99 ppm;

^{1}H (MeOH-d_4, 500 MHz) δ 7.65-7.58 (m, 4H, *m*-Ph), 7.47 (s, 1H, H6), 7.38-7.26 (m, 6H, *o*,*p*-Ph), 6.06-6.02 (m, 1H, H1'), 4.16-4.08 (m, 2H, POCH_2CH$_3$), 4.04-3.98 (m, 1H, H5'), 3.85-3.80 (m, 1H, H5'), 3.76-3.73 (m, 1H, H4'), 3.66-3.54 (m, 4H, COCH_2CH$_3$), 2.84-2.73 (m, 1H, H3'), 2.47-2.39 (m, 1H, H2'), 2.28-2.19 (m, 1H, H2'), 2.03-1.95 (m, 1H, H6'), 1.84-1.75 (m, 1H, H6'), 1.42 (d, 3H, *J* = 12 Hz, PCCH_3), 1.38 (s, 3H, 5-Me), 1.20 (t, 3H, *J* = 7 Hz, POCH$_2$CH_3), 1.14-1.05 (m, 6H, COCH$_2$CH_3), 1.00 (s, 9H, SiCMe_3).

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(6) Typical procedure; Trimethylsilyl chloride (1.07 ml, 8.39 mmol) was added dropwise to a solution of the phosphinate **3** (5.24 g, 7.63 mmol) in anhydrous ethanol (0.56 ml, 9.54 mmol) and anhydrous dichloromethane (38 ml) at 0 ºC. The reaction mixture was allowed to warm to room temperature, stirred at that temperature for 36 h at which point it was judged to be complete by ^{31}P NMR (aliquot of reaction mixture with D_2O insert shows consumption of the starting material at 46.59 ppm and formation of a predominant new resonance at 34.61 ppm) and drown-out into 50% saturated $NaHCO_{3(aq)}$ (100 ml). Extraction with dichloromethane (3 X 50 ml), drying of the organic layers over $MgSO_4$ and removal of volatiles gave the crude product which was purified by silica gel flash column chromatography, eluting with 5% methanol in dichloromethane, to give the *H*-phosphinate **5** as an off-white foam (4.02 g, 92%); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 34.20 ppm. Similarly, following the above procedure, the phosphinate **4** could be cleaved to the *H*-phosphinate **6**; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 34.42 ppm. Removal of the mixed orthoester protecting group in both the above examples results in 2-4% epimerisation at the phosphorus centre as judged by ^{31}P NMR analysis of the crude reaction mixtures.

(7) Individual *H*-phosphinates **5** and **6** were reacted with 5'-azido-5'-deoxythymidine **7** following similar reaction conditions to those described in the preceding communication[3], but employing 6 equivalents of bis(trimethylsilyl)trifluoroacetamide to give the phosphonamidate dimers **8** and **9** respectively (64-79%). **8**; Clear colourless glass; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 33.91 ppm. **9**; Clear colourless glass; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 34.38 ppm.
Analysis of the crude reaction mixture indicates <5% epimerisation having occurred in preparation of either diastereoisomer, as judged by ^{31}P NMR.

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(12) 5'-amino-3'-*tert*-butyldiphenylsilyl-5'-deoxythymidine **12** was prepared by catalytic hydrogenation of a methanol solution of the corresponding azide,[3] under 1 atmosphere of hydrogen, over a palladium on carbon catalyst (10% by weight), at room temperature for 18 h (>95%).

(13) Typical procedure; Anhydrous carbon tetrachloride (1 ml) followed by anhydrous triethylamine (0.40 ml) was added dropwise to a suspension of activated 3Å molecular sieves (0.24 g) in a solution of the amine **12** (317 mg, 660 μmol) and *H*-phosphinate **5** (285 mg, 500 μmol) in anhydrous pyridine (0.5 ml) and anhydrous dichloromethane (1 ml) at 0 ºC. The reaction mixture was allowed to warm to room temperature, stirred for 18 h at room temperature, filtered and evaporated to give the crude product which was purified by flash column chromatography, eluent 5% methanol in dichloromethane, to give the phosphonamidate dimer **13** as an off-white foam (395 mg, 75%); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 32.32 ppm.
Analysis of the crude reaction mixture indicates <5% epimerisation having occurred resulting in formation of the diastereoisomeric phosphonamidate dimer; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 31.70 ppm.

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(15) Removal of the mixed orthoester protecting group from **17** and **18**, in an analogous manner to that described above,[6] produced the *H*-phosphinates **19** and **20** respectively with <5% epimerisation as determined by ^{31}P NMR analysis. **19**; Colourless glass; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 33.14 ppm. **20**; Colourless glass; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 33.81 ppm.

(16) The *H*-phosphinate-azides **19** and **20** were cyclised with bis(trimethylsilyl)trifluoroacetamide in pyridine to yield the corresponding *N*-trimethylsilylated cyclic phosphonamidates in an analogous fashion to that described in the previous communication.[3] In contrast to the dimer synthesis, removal of the trimethylsilyl residues was best achieved upon treatment with tetrabutylammonium fluoride (1.1 equiv) in tetrahydrofuran at room temperature for 3 h. Evaporation of volatiles and trituration with ether yielded the respective cyclic phosphonamidate **21** or **22.**
21; White foam; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 28.60 ppm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.42 (s, br, 1H, *H*N3), 7.09 (s, 1H, H6), 6.18 (d, 1H, *J* = 5 Hz, H1'), 4.16-4.04 (m, 2H, CH_2CH$_3$), 3.63-3.58 (m, 1H, H4'), 3.58-3.45 (m, 1H, H5'α), 3.39-3.26 (m, 1H, H5'β), 3.02-2.97 (m, br, PN*H*, 1H), 2.51-2.39 (m, 1H, H3'), 2.37-2.24 (m, 1H, H6'β), 2.23-2.05 (m, 2H, H2'), 1.91 (s, 3H, 5-Me), 1.74-1.60 (m, 1H, H6'α), 1.31 (t, 3H, *J* = 6 Hz, CH$_2$CH_3) ppm.
22; White foam; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 26.28 ppm; ^{1}H nmr ($CDCl_3$, 400 MHz) δ 8.44 (s, br, 1H, *H*N3), 7.09 (s, 1H, H6), 6.10 (d, 1H, *J* = 5 Hz, H1'), 4.18-4.03 (m, 2H, CH_2CH$_3$), 3.60-3.42 (m, 2H, H5'), 3.21-3.12 (m, br, 1H, PN*H*), 3.12-3.02 (m, 1H, H4'), 2.32-2.14 (m, 4H, H2', H3', H6'β), 1.94 (s, 3H, 5-Me), 1.72-1.59 (m, 1H, H6'α), 1.37 (t, 3H, *J* = 6 Hz, CH$_2$CH_3) ppm.

(17) Stereochemical assignments were based upon NOE experiments. The S_P diastereoisomer **21** exhibited interactions between the ethyl phosphonamidate methylene and the 5'α, 6'α and 6'β protons, suggesting multiple contributing conformations. In contrast, the R_P diastereoisomer **22** exhibited a strong interaction between the ethyl phosphonamidate methylene and the 5'β and 6'β proton, consistent with a single preferred conformation.

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