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A New Type of Nucleotide Analogue with 4-Pyridylphosphonate Internucleotide Linkage

Annika Kers and Jacek Stawiński*

Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden

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Abstract: Suitably protected dithymidine H-phosphonates were quantitatively converted into the corresponding dinucleoside 4-pyridylphosphonates by treatment with 1,8-diazabicylo[5.4.0]undec-7-ene (DBU) in the presence of trityl chloride in pyridine. The reaction was found to be stereospecific and proceeded, most likely, with retention of configuration at the phosphorus centre. © 1999 Elsevier Science Ltd. All rights reserved.

The implementation of the antisense approach to modulate gene expression as a routine medical treatment still encounters severe problems though its principle, as proposed by Zamecnik and Stephenson,[1] is overwhelmingly simple and of general applicability. Targeting mRNA (antisense approach) or double-stranded DNA (antigene approach) with oligonucleotides, blocks or impairs gene expression and relies on similar mechanisms to those used by nature for gene regulation. Unfortunately, synthetic oligonucleotides bearing natural phosphodiester internucleotide bonds have many shortcomings as potential therapeutics.[2] Recent advances in development of oligonucleotide analogues solved some of the fundamental problems of antisense and antigene techniques (*e.g.* susceptibility of oligonucleotides to enzymatic degradation *in vivo*), but at the same time generated numerous new issues (*e.g.* chirality at the phosphorus center, lower stability of the formed complexes, lack of the RNase H induction ability). Thus, the development of efficient antisense or antigene therapeutics seems to be an exceedingly complex task that must take into account a variety of practical aspects, *e.g.* cellular uptake, nuclease resistance, binding affinity and selectivity, a reasonable pharmacokinetic behaviour in organisms and synthetic availability of a prospective drug.[3] Among these, the last factor, namely, accessibility of compounds to be tested, is of prime importance and may set a practical limit on the structure-activity relationship studies of certain types of compound. For example, although oligonucleotide analogues bearing the P–C bond seem promising from a medical point of view, exploration of this class of oligonucleotide analogues has been limited to methylphosphonates[4,5] and, only recently, to aminoalkylphosphonates,[6] mainly due to problems in the efficient formation of the P–C bond.

Since all synthetically useful methods for the preparation of oligonucleotide analogues bearing phosphorus-carbon linkages utilise reagents with the P–C bond and suffer from the same inherent problems,[7-10] we searched for alternative methodologies where the phosphorus–carbon bond would be formed under mild conditions from readily available precursors.[11,12] For this purpose we have embarked on the exploration of

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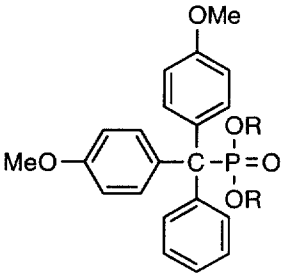
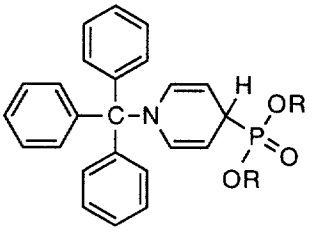
alkylation of H-phosphonate diesters with alkyl halides. During these studies we observed that diphenyl H-phosphonate (DPP) in pyridine reacted slowly (overnight) with dimethoxytrityl chloride (DMT-Cl) to produce the expected diphenyl dimethoxytritylphosphonate **1** (R= phenyl, δ_P = 20.0 ppm, singlet, no J_{PH} coupling constants) as the major product, while trityl chloride (Tr-Cl) under analogous conditions yielded two compounds (in a variable ratio) that resonated at δ_P = 16.2 ppm (J_{PH} = 19.5 Hz, d) and 8.6 ppm (several small J_{PH} coupling constants). Taking into account the ambident reactivity of a trityl cation[13] these results might have suggested that DPP reacted with unsubstituted trityl cations (generated from Tr-Cl) at the *para*-aromatic carbon yielding a semibenzene type of intermediate,[13,14] whereas DMT-Cl reacted at its aliphatic carbon. Additional experiments clarified this point and showed that in the instance of Tr-Cl we had observed the reaction of DPP with N-tritylpyridinium ion,[15] which most likely yielded compound **2** (R = phenyl) resonating at 16.2 ppm in ^{31}P NMR.[16] Attempted isolation of **2** failed and instead compound **3** (R = phenyl, δ_P = 8.6 ppm) was obtained.

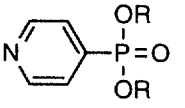
Chart 1



1



2



3

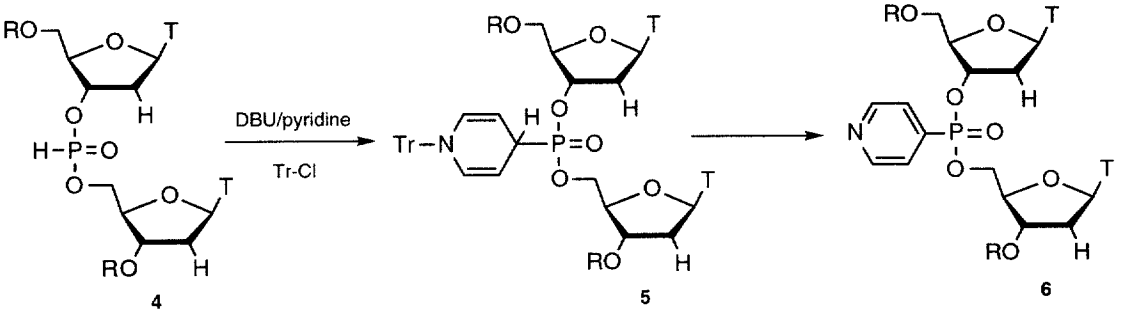
Since the above transformation could provide a convenient access to a new class of nucleotide analogues with a 4-pyridylphosphonate moiety, we investigated the reaction of less reactive H-phosphonate diesters with pyridine promoted by trityl chloride in more detail. We found that diethyl H-phosphonate (δ_P = 7.6 ppm) did not react in pyridine with Tr-Cl nor Tr-Br (overnight, RT), but the addition of 2 equivalent of 1,8-diazabicylo[5.4.0]undec-7-ene (DBU) furnished fast formation of two products, which have tentatively been characterised as 1,4-dihydropyridine derivative **2** (R = Et, δ_P = 22.4 ppm, ca 80%) and its 1,2-dihydropyridine isomer (δ_P = 24.0 ppm, ca 20%). The latter product was not stable under the reaction conditions and underwent gradual isomerisation to **2** (R = Et). Simultaneously, a slow conversion of **2** (R = Et) to the corresponding 4-pyridylphosphonate **3** (R = Et, δ_P = 14.4 ppm) also occurred. After being left overnight, ^{31}P NMR spectroscopy revealed the presence of only two compounds in the reaction mixture, namely, **2** (R = Et, ca 60%) and **3** (R = Et, ca 40%).[17] Also in this instance, we could not isolate the dihydropyridine intermediate **2** (R = Et), which during work-up underwent a complete conversion to the product **3** (R = Et).[18]

The efficacy of this approach in the synthesis of a new type of nucleotide analogue with a 4-pyridylphosphonate internucleotide linkage was assessed by carrying out the reaction depicted in Scheme 1. To this end, a suitably protected dinucleoside H-phosphonate **4** (δ_P = 8.44 and 9.35ppm) was treated in pyridine with Tr-Cl[19] (1.2 equiv.) and DBU (2.4 equiv.). The reaction went to completion within 10 min producing a

diastereomeric mixture of dihydropyridine derivatives **5** (δ_P = 23.99 and 23.19 ppm) and the 4-pyridylphosphonate **6a** and **6b** (δ_P = 16.17 and 15.43 ppm). After work-up (see below), the only nucleotidic material present (^{31}P NMR) was a mixture of 4-pyridylphosphonates **6a** and **6b**. These were isolated by silica gel column chromatography in 82% yield (as a mixture of diastereomers).

Stereochemistry of the formation of 4-pyridylphosphonates **6** was evaluated by carrying out the reaction on the separate diastereomers of dithymidine H-phosphonate **4**.[20] It was found that H-phosphonate diester **4a** was converted to the pyridylphosphonate **6a** with the intermediacy of **5a**, while the diastereomer **4b** afforded as a product **6b** diastereomer (with the intermediacy of **5b**). The transformation was thus stereospecific and, assuming the reaction pathway as in Scheme 1, it seems most likely that it proceeded with overall retention of configuration.

Scheme 1



4a (R_P) ("fast") (δ_P = 8.4 ppm); R = 4,4'-dimethoxytrityl
4b (S_P) ("slow") (δ_P = 9.4 ppm); R = 4,4'-dimethoxytrityl
5a (R_P) (δ_P = 23.2 ppm); R = 4,4'-dimethoxytrityl; Tr = trityl
5b (S_P) (δ_P = 24.0 ppm); R = 4,4'-dimethoxytrityl; Tr = trityl

6a (R_P) ("fast") (δ_P = 15.5 ppm); R = 4,4'-dimethoxytrityl
6b (S_P) ("slow") (δ_P = 16.3 ppm); R = 4,4'-dimethoxytrityl
6c (R_P) ("fast") (δ_P = 15.4 ppm); R = H
6d (S_P) ("slow") (δ_P = 16.1 ppm); R = H

A typical procedure for the preparation of 4-pyridylphosphonates 6

The separate diastereomers **4** (0.3 mmol) were treated in pyridine (10 mL) with trityl chloride or trityl bromide[21] (1.2 equiv.) and DBU (2.4 equiv.). When the starting material **4** disappeared (TLC analysis), the reaction mixture was concentrated, partitioned between aq. $NaHCO_3$ and $CHCl_3$, and the organic layer concentrated. The residue was purified by silica gel column chromatography using toluene-ethyl acetate (1:1, v/v) containing 3-5% MeOH. Yields: **6a**, 76%; **6b**, 87%. To facilitate spectral characterisation of the products, compounds **6a** and **6b** were detritylated using 80% acetic acid : THF (2:1, v/v) and purified by reversed-phase silica gel chromatography [gradient of MeOH in H_2O (0-40%)]. Yields: **6c**, 76%; **6d**, 91%.[22]

In conclusion, we have developed a new type of nucleotide analogue with the 4-pyridylphosphonate internucleotide linkage, accessible in high yield and under mild conditions from the corresponding H-phosphonate diesters. Since the P–C bond is introduced in the last synthetic step, this approach makes full use of the efficiency of the formation of internucleotide bonds *via* the H-phosphonate methodology. Due to the presence of a pyridine moiety (of which the basicity can be controlled by introducing various substituents to the aromatic ring) and the P-C bond (which should confer stability to nucleases) this type of nucleotide analogue can be considered as potential antisense agents. These studies are in progress in this Laboratory.

Acknowledgements
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15. This reaction is thus related to that reported by D. Redmore who observed the formation of dialkyl 4-pyridylphosphonates upon heating at reflux for 2 h of triphenylmethylpyridinium tetrafluoroborate with a sodium salt of dialkyl phosphite in the corresponding dialkyl H-phosphonate as a solvent. Yields: 30-39%. *See*, Redmore, D. *J. Org. Chem.* **1976**, *41*, 2148-2150.
16. The different pathways for the reaction of DMT-Cl *vs* Tr-Cl with diphenyl H-phosphonate in pyridine are probably due to different stability of the corresponding carbocations generated in pyridine. The less stable trityl cation forms apparently stronger complexes with pyridine and facilitates nucleophilic attack by DPP on the 4-position of the pyridine ring. The dimethoxytrityl cation, on the other hand, due to the presence of electron-donating methoxy groups on the aromatic rings is much more stable and thus forms, most likely, rather weak complexes with pyridine. *See also*, Evans, A. G.; Price, A.; Thomas, J. H. *Trans. Faraday Soc.* **1956**, *52*, 332-344.
17. Compounds **1** and **3** (R = Ph and Et) were isolated from the reaction mixtures and characterised by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. The identity of **2** (R = Ph and Et) was corroborated by a detailed ^{31}P NMR analysis (A. Kers, L. Dembkowski, A. Kraszewski, and J. Stawinski, *manuscript in preparation*).
18. The transformation of the dihydropyridine intermediate **2** to the 4-pyridylphosphonate **3** can be effected by the addition of few equivalents of iodine to the reaction mixture.
19. Also other trityl derivatives can be used for this purpose, *e.g.* Tr-Br or Tr-BF_4.
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21. Due to low solubility of Tr-Br in pyridine, the reaction was heterogeneous and required several of hours for its completion.
22. Compounds **6a-d** were of purity >98%. Chemical identity of **6c** and **6d** was confirmed by ^{1}H, ^{13}C, ^{31}P, and ^{1}H- ^{1}H correlated NMR spectroscopy. Some diagnostic spectral data [compound, δ_P; $\delta_{H(py)}$; $\delta_{H1'}$; $\delta_{C(py)}$ (J_{CP})]: **6c**, 15.4 ppm; 7.83-8.83 ppm (4H); 6.29 ppm (2H); 138.79 ppm (190.6 Hz), 137.84 ppm (7.3 Hz), 150.65 ppm (12.8 Hz). **6d**, 16.3 ppm; 7.77-8.79 ppm (4H); 6.19 & 6.28 ppm (2H); 137.80 ppm (188.8 Hz), 137.84 ppm (11.0 Hz), 151.05 ppm (12.8 Hz).