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Synthesis of Dinucleoside Phosphoramidimidates

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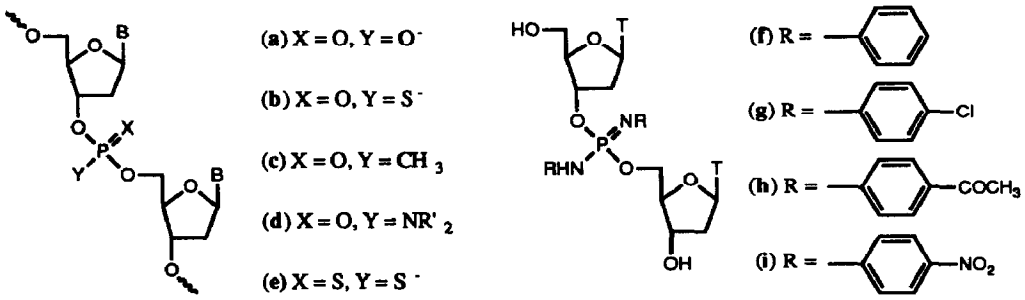
Abstract: The first nucleoside derivatives of phosphoramidimidic acid are described. A series of thymidine dinucleoside phosphoramidimidates **1f-i** with substituted phenyl groups were synthesized and their stability in organic and aqueous media evaluated.

The synthesis of nucleotide analogs has attracted major attention during the past two decades mainly due to their potential uses as antisense oligonucleotides and for studies of enzyme mechanisms.[1] Modifications of the phosphodiester linkages or replacements of the entire backbone have resulted in a wide range of oligonucleotide congeners in attempts to enhance the nuclease stability and cellular uptake compared to the parent phosphodiester linked oligonucleotides.[2]

A simple substitution of one nonbridging oxygen atom in the phosphodiester linkage (*Figure 1*, **1a**) suffers from the introduction of a chiral center at phosphorus (**1b-d**). This is avoided by replacement of both oxygens with identical substituents, e.g. sulfur atoms to give the phosphorodithioates (**1e**).[3]



1a-e **1f-i**

Figure 1. Substitutions of the nonbridging oxygen atoms in the phosphodiester linkage (**1a**), resulting in P-chiral analogs: Phosphorothioates (**1b**)[4], methylphosphonates (**1c**)[5], and phosphoramidates (**1d**)[6]; and P-achiral analogs: Phosphorodithioates (**1e**)[3] and phosphoramidimidates (**1f-i**). B = nucleoside base, T = thymine.

We have now developed a second type of P-achiral nucleotide analog having both the nonbridging oxygen atoms replaced by identically substituted nitrogens (**1f-i**). These dinucleoside phosphoramidimidates have the potential of becoming either protonated at the phosphorimide nitrogen or deprotonated at the phosphoramide nitrogen to give a P-achiral analog. In addition, if a fast exchange of the phosphoramide protons with either the

solvent or neighbouring phosphoramidimidate molecules occurs, it will not be possible to distinguish separate diastereomers.

Non-nucleoside derivatives of phosphoramidimidic acid are known though their chemical and physical properties have not been thoroughly evaluated.[7] The synthesis of a nucleoside phosphoramidimidate with two different alkyl substituents has earlier been claimed but no evidence for the structure of the compound was presented.[8] Phosphoramidimidates carrying basic nitrogen moieties are unstable, probably due to protonation and subsequent cleavage by nucleophiles.[9] In order to stabilize the phosphoramidimidates it was found necessary to put electron attracting substituents on the nitrogen atoms. We synthesized the *N,N'*-diaryl derivatives **1f-i** to elucidate how the para substituents influenced the stability. The Hammett constants σ_p for the substituents on the phenyl units in **1f-i** are 0.000, 0.226, 0.516 and 0.778, resp.[10]

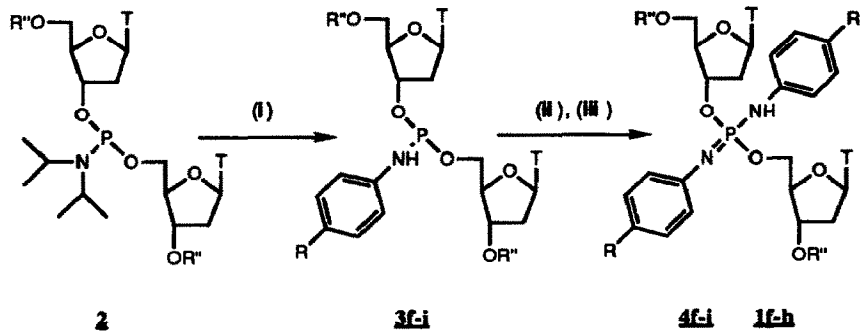


Figure 2. Synthesis of a dinucleoside phosphoramidimidate. (**2-4**) R'' = *tert*-butyldimethylsilyl , (**1**) R'' = H. (i)-(iii) see text.

We chose the dinucleotide *N,N*-diisopropyl phosphoramidite **2** *(Figure 2)*, synthesized as described by Marugg *et al* [11] as starting material since it postponed the choice of amino substituents to the latest possible step in the synthesis. The synthesis of the p-acetylphenyl derivative **1h** was performed as follows: (i) The diisopropylamino group of **2** was substituted with p-aminoacetophenone (2 eq) in an exchange reaction in the presence of tetrazole (2 eq) in CH_3CN (4 ml per mmol phosphoramidite) to give **3h** after 15 min at r.t. in 81 % yield following purification (wash with sat. aq. $NaHCO_3$ and silica gel column chromatography in CH_2Cl_2 / EtOAc / triethylamine 38 : 60 : 2).[12] (ii) Oxidation of **3h** with iodine (1 eq) and p-aminoacetophenone (5 eq) in the presence of triethylamine (3 eq) in CH_3CN (4 ml per mmol amidite) was completed in less than 2 min (r.t.). No Arbuzov-cleavage product was observed (attack on the 5'CH_2 resulting in cleavage of the dinucleotide and formation of a nucleoside phosphorodiamidate) contrary to syntheses involving more basic amino substituents.[9] The crude phosphoramidimidate was purified by silica gel column chromatography (CH_2Cl_2 / MeOH / triethylamine; 98 : 1 : 1) giving an 80 % yield of the bright yellow phosphoramidimidate **4h** (94 % pure, ^{31}P NMR).[13] (iii) Removal of the *O*- protecting groups with tetrabutylammonium fluoride (1.1 M in THF, 3 eq), 1 h at r.t., afforded the *O*-(thymidin-5'-yl) *O*-(thymidin-3'-yl) *N,N'*-di-(p-acetylphenyl)phosphoramidimidate **1h** in 43 % yield (> 99 % pure, by ^{31}P NMR) after purification by silica gel column chromatography in CH_2Cl_2 / MeOH / triethylamine; 94 : 3 : 3). Further elution of the column gave an additional 21 % of **1h** (77 % pure by ^{31}P NMR)[14]

The thymidine dinucleotide phosphoramidimidate **1h** is sensitive to acids, and decomposes in acidic aqueous solutions to give, mainly, dinucleotide phosphoramidate. It is, however, surprisingly stable ($t_{1/2} \approx 100$ h) in a 1 : 4 mixture of CH_3CN / ion exchanged water (pH ≈ 5). In basic solutions (0.1 M NaOH), decomposition is also seen ($t_{1/2} \approx 35$ h), but in conc. aq. NH_3, the compound appears to be stable (for at least 3 weeks). In strongly basic solutions a distinct ^{31}P NMR upfield shift is observed (ion exchanged water /

CH_3CN 4 : 1, δ_P 3.89; 0.01 M NaOH / CH_3CN 4 : 1, δ_P 4.36; conc. aq. NH_3 / CH_3CN 4 : 1, δ_P 13.61; 0.1 M NaOH / CH_3CN 4 : 1, δ_P 17.69). This change in chemical shift reflects the onset of deprotonation of P-NH at pH ≈ 13. The phosphoramidimidate with protected 5'-OH and 3'-OH, **4h**, has a higher relative stability, suggesting that the free hydroxyl groups of **1h** participate in the hydrolysis. This interference will clearly be enhanced in strongly basic solutions where deprotonisation of the sugar hydroxyl groups occurs.

Figure 3 shows an HPLC diagram of purified **4h**. The small peak (5 %) just ahead of the dinucleoside phosphoramidimidate (42.48 min) is dinucleoside phosphoramidate (39.84 min), an impurity which was not completely eliminated in the purification step. Additionally, an unidentified impurity is seen at 35.26 min (1 %).

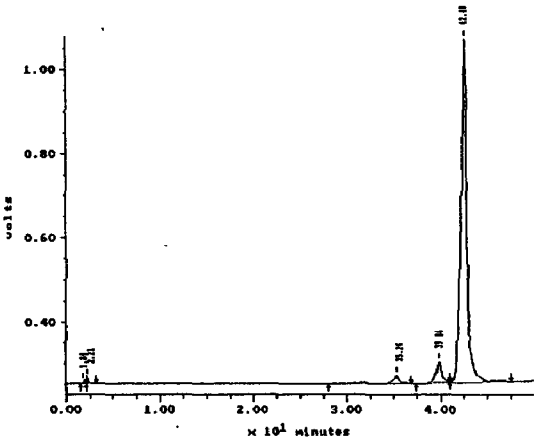


Figure 3. HPLC of **4h** (Hamilton PRP-1 column; elution mixtures: A: 0.1 M NH_4HCO_3 pH 9.0, 5 % CH_3CN, B: 0.1 M NH_4HCO_3 pH 9.0, 80 % CH_3CN; 0-5 min. 100 % A, 5-45 min. gradient 0 ->100 % B, 45-60 min. 100 % B; UV detection at 260 nm).

The phosphoramidimidates **1f,g,i** have been synthesized in an analogous fashion. **1f** and **1g**[15] were more sensitive to acids than **1h**; **1i**[16], however, was unstable even in its 3'-*O*-,5'-*O*-protected form and decomposed during column chromatography. These results indicate, that in order to stabilize a phosphoramidimidate, it is necessary to use substituents on the nitrogen atoms with electron attraction comparable to p-acetylphenyl since para substituents with both a significant higher σ_p (**1i**) and lower σ_p (**1f** and **1g**) have a negative influence. We have not considered enhancing the stability through steric factors, since it is obvious that steric hindrance should be minimized for compounds having prospective antisense applications. The phosphoramidimidates **4f-h** and **1f-h** are stable in CH_3CN solution and to storage at -18° C for at least six months.

We have not been able to distinguish separate diastereomers (^{31}P NMR, TLC, HPLC) of the dinucleoside phosphoramidimidates even though we apparently isolated them in their neutral form (as judged by the assignment of a phosphoramide proton in ^1H NMR and their behaviour in chromatographic procedures where they are slightly less polar than the neutral dinucleoside phosphoramidate). This is presumably due to a fast exchange of the phosphoramide protons, as mentioned earlier.

In summary, we have demonstrated the first synthesis of a stable dinucleoside phosphoramidimidate and characterized it by standard analytical methods. We are currently working on the design of phosphoramidimidates with improved properties in terms of aqueous stability, size of the nitrogen substituents, and on strategies to incorporate them in oligonucleotides in order to examine their biological applications.

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References and notes:

1. (a) J.S. Cohen (1989) "*Oligodeoxynucleotides. Antisense Inhibition of Gene Expression*", Macmillan Press. (b) C.A. Stein, J.L. Tonkinson and L. Yabukov (1991) *Pharmacol Ther* 52 365

2. (a) E. Uhlman and A. Peyman (1990) *Chem Rev* 90 543. (b) F. Eckstein" (1991) *"Oligonucleotides and Analogues. A practical Approach*, Oxford University Press
3. (a) W.K.-D. Brill, J. Nielsen and M.H. Caruthers (1988) *Tetrahedron Lett.* 29 5517, (b) K. Bjergårde and O. Dahl (1991) *Nucleic Acids Res* 19 5843, (c) W.S. Marshall and M.H. Caruthers (1993) *Science* 259 1564
4. F. Eckstein (1985) *Annu. Rev. Biochem.* 54 367
5. P.O.P. T'so, P.S. Miller, L. Aurelian, A. Murakami, C. Agris, K.R. Blake, S.-B. Lin, B.K. Lee and C.C. Smith (1987) *Ann. New York Academy of Sciences* 507 220
6. A. Jager, M.J. Levy and S. Hecht (1988) *Biochemistry* 27 7237
7. (a) V.A. Gilyarov and M.I. Kabachnik (1966) *J. Gen. Chem. USSR* 36 293, (b) E.E. Nifantev, G.F. Bebikh and T.P. Sakodynskaya (1971) *J. Gen. Chem. USSR* 41 2032, (c) G.K. Genkina, B.A. Korolev, V.A. Gilyarov and M.I. Kabachnik (1971) *J. Gen. Chem USSR* 41 76, (d) G.K. Genkina, B.A. Korolev, V.A. Gilyarov, B.I. Stepanov and M.I. Kabachnik (1969) *J. Gen. Chem USSR* 39 306, (e) M.I. Kabachnik, N.A. Tikhonina, V.A. Gilyarov, B.A. Korolev, M.A. Pudovik, L.K. Kibardina and A.N. Pudovik (1982) *J. Gen. Chem USSR* 52 899
8. M.J. Nemer and K.K. Ogilvie (1980) *Tetrahedron Lett* 21 4153
9. K. Bjergårde and M.H. Caruthers, unpublished
10. K.B.Wiberg *"Physical Organic Chemistry"* (1963), J. Wiley & Sons
11. J.E. Marugg, A. Burik, M. Tromp, G.A. van der Marel and J.H. van Boom (1986) *Tetrahedron Lett* 27 2271. *Note*: The synthesis of **2** was virtually identical to this published procedure with the following alterations: 5'-*O*- and 3'-*O*-*tert*-butyldimethylsilyl protected nucleosides were used since phosphoramidimidates do not tolerate the acidic deprotection step required for removal of the standard 5'*O*-dimethoxytrityl group. **2** may be purified by column chromatography, but for the conversion to **3g-i** only an aqueous washing step with sat. aq. $NaHCO_3$ was found necessary. The yield of **2** was quantitative. Analytical data: TLC (CH_2Cl_2 / EtOAc; 40 : 60) R_f 0.16. ^{31}P NMR (CH_3CN) δ 147.65 / 147.49.
12. Analytical data on **3h**: TLC (CH_2Cl_2 / EtOAc / triethylamine; 60 : 35 : 5) R_f 0.36. ^{31}P NMR (CH_3CN) δ 132.10 / 131.77 ^1H NMR ($CDCl_3$) δ 8.8-9.5 (NH), 7.87 & 7.85 (2 s, 2 H, H-6), 7.0-7.5 (m, 2 H, arom), 6.0-6.4 (m, 4 H, arom, H-1'), 5.0-5.2 (m, 1 H, H-3'), 3.7-4.5 (m, 7 H, H-3', H-4', H-5'), 2.50 (s, 3 H, $COCH_3$), 2.0-2.6 (m, 4 H, H-2'), 1.91 & 1.86 (2 s, 6 H, 5-CH_3), 0.92 & 0.90 (2 s, 18 H, Si-*tert*-butyl), 0-0.1 (2 s, 12 H, Si-CH_3). FAB^- MS 874.2 (M-1).
13. Analytical data on **4h**: TLC (CH_2Cl_2 / MeOH; 95 : 5) R_f 0.20. ^{31}P NMR (CH_3CN) δ -3.55. ^1H NMR ($CDCl_3$) δ 10.2-10.7 (br, 2 H, 3-NH), 8.9-9.5 (br, 1 H, PNH), 7.8 & 7.9 (2 s, 2 H, H-6), 7.0-7.4 (m, 8 H, arom), 6.3-6.4 (m, 1 H, H-1'), 5.9-6.0 (m, 1 H, H-1'), 5.0-5.1 (m, 1 H-H-3'), 4.0-4.4 (m, 5 H, H-3', H-4', H-5'), 3.4-4.8 (m, 2 H, H-5'), 2.52 & 2.51 (2 s, 6 H, $COCH_3$), 2.4-2.6 (m, 2 H, H-2'), 2.0-2.3 (2 H, H-2'), 1.87 & 1.84 (2 s, 6 H, 5-CH_3), 0.83 & 0.82 (2 s, 18 H, Si-*tert*-butyl). FAB^+ MS 1009.3 (M+1), FAB^- MS 1007.3 (M-1).
14. Analytical data on **1h**: TLC (CH_2Cl_2 / MeOH; 9 : 1) R_f 0.17. ^{31}P NMR (CH_3CN) δ -2.95, (D_2O) δ 3.76. ^1H NMR (DMSO-*d*-6) δ 11.33 & 11.31 (2 s, 2 H, 3-NH), 9.0-9.1 (br, 1 H, PNH), 6.9-8.0 (m, 10 H, H-6, arom), 6.1-6.3 (m 2 H, H-1'), 5.4-5.5 (m, 1 H, H-3'), 5.2-5.3 (m, 1 H, H-3'), 5.0-5.1 (m, 1 H, H-4'), 3.4-4.3 (m, 5 H, H-4', H-5') (H-2' partly hidden by the DMSO signal), 2.46 & 2,45 (2 s, 6 H, $COCH_3$), 2.2-2.3 (m, 2 H, H-2'), 1.75 & 1.68 (2 s, 6 H, 5-CH_3). FAB^+ MS 781.3 (M+1), FAB^- MS 779.1 (M-1)
15. Analytical data on **3f**: TLC (CH_2Cl_2 / EtOAc / triethylamine; 37 : 60 : 3) R_f 0.27. ^{31}P NMR (CH_3CN) δ 133.35 / 133.19. **4f**: TLC (CH_2Cl_2 / MeOH; 95 : 5) R_f 0.33. ^{31}P NMR (CH_3CN) δ -0.53. FAB^- MS 923.2 (M-1). **1f**: TLC (CH_2Cl_2 / MeOH; 9 : 1) R_f 0.25. ^{31}P NMR (CH_3CN) δ -2.95. **3g**: TLC (CH_2Cl_2 / EtOAc / triethylamine; 40 : 58 : 2) R_f 0.23. ^{31}P NMR (CH_3CN) δ 133.51 / 133.27. FAB^- MS 866.2 (M-1). **4g**: TLC (CH_2Cl_2 / MeOH; 5 : 5) Rf 0.30. ^{31}P NMR (CH_3CN) δ -3.27. FAB^- MS 991.0 (M-1). **1g**: TLC (CH_2Cl_2 / MeOH; 9 : 1) R_f 0.33. ^{31}P NMR (CH_3CN) δ -3.35.
16. Analytical data on **3i**: TLC (CH_2Cl_2 / EtOAc / triethylamine; 35 : 60 : 5) R_f 0.31. ^{31}P NMR (CH_3CN) δ 131.53 / 131.05. **4i**: ^{31}P NMR (CH_3CN) δ -11.03.

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