Phosphitylation of Guanine or Inosine Bases during the Preparation of Nucleoside Phosphoramidites. Isolation of Model Products as Thiophosphoric Amide Derivatives and Structure Elucidation by ^{15}N NMR Spectroscopy

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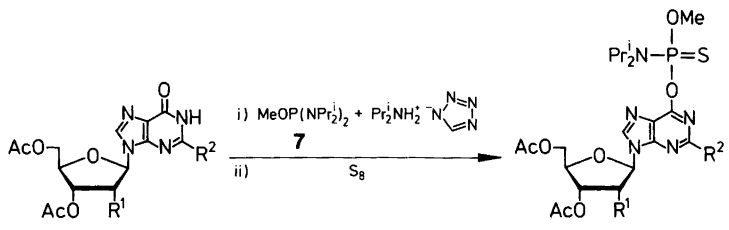
The acid catalyzed reactions of methyl *N,N,N',N'*-tetraisopropylphosphorodiamidite with *N2,O2',O3',O5'*-tetraacetylguanosine, *N2,O3',O5'*-triacetyldeoxyguanosine, or *O2',O3',O5'*-triacetylinosine gave labile O6-phosphitylated products which were converted to stable *P*-sulphides. The site of phosphitylation was shown by ^{15}N NMR spectroscopy to be O6 and not N1. ^{13}C NMR data also supported this conclusions, but UV data were inconclusive.

Side reactions occurring in the lactam function of the guanine and uracil (thymine) moiety of nucleosides during oligonucleotide synthesis are well established when the phosphotriester approach is used.[1–14] Similar side reactions using the phosphoramidite approach have also been reported,[15–19] although isolation and characterization of the resulting by-products have not been possible due to their low stability; until now they have only been characterized in solution by their ^{31}P NMR chemical shifts.

We report here that these unstable by-products can, at least in some cases, be characterized as their *P*-sulfides, which are stable enough to be purified by column chromatography.

The compounds studied here are derived from nucleosides protected at the hydroxy groups, i.e. **1**, **3** and **5** (Scheme 1). They were selected in order to obtain clean reactions at the lactam functions, without concomitant reactions at the hydroxy groups which occur during oligonucleotide synthesis. Reaction of these nucleosides with



1, 3, 5 → **2, 4, 6**

Scheme 1.

1, 2 R^1 = AcO, R^2 = AcNH
3, 4 R^1 = H, R^2 = AcNH
5, 6 R^1 = AcO, R^2 = H

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methyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite and diisopropylammonium tetrazolide gave high yields of phosphitylated products (δ_P 153–148 ppm) which, although containing no hydroxy groups, were still very unstable. However, they could be oxidized with dry sulfur to give stable *P*-sulfides, which after purification were isolated in 28–57 % yield and shown by spectro-

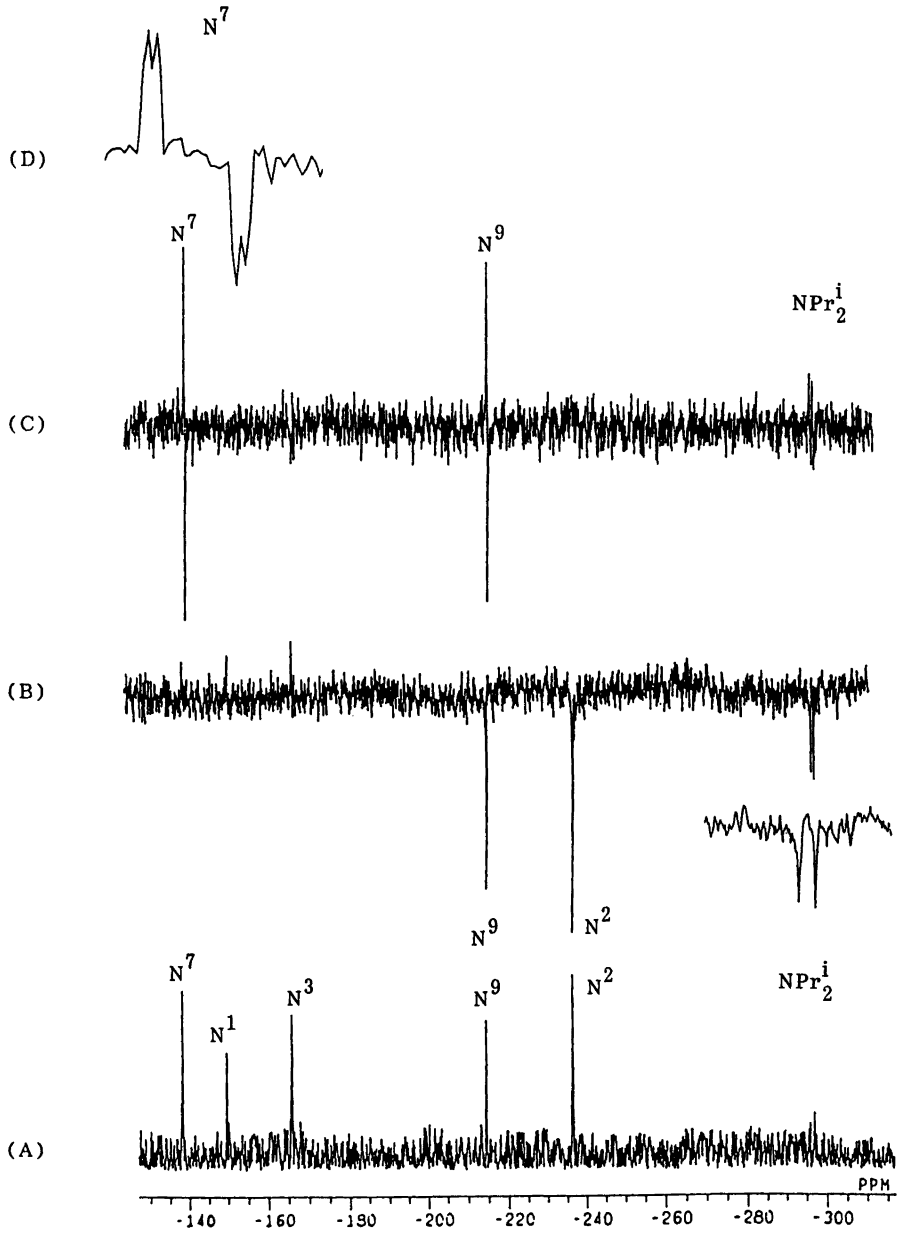

Fig. 1. ^{15}N NMR spectra of compound **2**: (A) ^{1}H-decoupled spectrum without NOE; (B) ^{1}H-decoupled spectrum with NOE with the expansion showing $^1J(^{15}N^{31}P)$ = 18.6 Hz; (C) INEPT spectrum; (D) expansion of N7 resonance, found in INEPT spectrum C, showing two diastereoisomers.

scopic means to be the O6-thiophosphorylated species **2**, **4** and **6** (Scheme 1).

The structural assignment of **2**, **4** and **6** was made primarily on the basis of ^{15}N NMR spectroscopic data, although ^{13}C NMR and UV spectroscopy were also employed. ^{15}N chemical shifts in purine nucleosides are very sensitive to substituent effects,[20] and a putative *N*1-thiophosphoramidate is expected to show a $^{1}J(^{15}NP)$ coupling of ca. 40 Hz.[21] Substituent increments can be used to elucidate the structure of a substituted purine nucleoside quite unequivocally.[20] Thus, a shielding of N3 by ca. 50 ppm due to the NH_2 group at the C2 position was observed in guanosine as compared to inosine.[22] A group such as *para-t*-butylbenzamido at the C2 position shields N3 by ca. 30 ppm with respect to inosine.[23]

Previously, we have shown[23] that O6-substitution causes profound changes in N3, and especially in N1 chemical shifts. Derivatization of O6 leads to a large deshielding of N1 (ca. 70–110 ppm) whose exact size depends on the nature of the substituent. An unambiguous distinction between an N1 and an O6-phosphorylated guanosine derivative is therefore feasible by following the N1 chemical shift. A similar distinction between N3 and O4-substituted pyrimidine nucleosides has also been achieved recently.[24]

We found[23] for a series of *C*2-(*para-t*-butylbenzamido)-C6-substituted purine-*N*9-ribofuranosides that the N3 chemical shifts do not change noticeably with variation of the C6-substituent. Thus, for the *para-t*-butylbenzamido group at the C2-position, N3 resonates at ca. −160 ppm while the N1 chemical shifts are strongly dependent on the nature of the C6-substituent.

The assignment of the ^{15}N chemical shifts and the site of substitution. The ^{1}H-decoupled ^{15}N NMR spectrum of compound **2** without NOE is shown in Fig. 1, in which five nitrogen resonances can be clearly seen; the resonance of the diisopropylamino groups does not appear as a result of an insufficient number of scans. A ^{1}H-decoupled spectrum with NOE of compound **2** clearly shows a negative doublet at high field for the diisopropylamino group. Similarly, N9 and N2 nitrogens have negative NOE's due to the closeness of sugar protons and the proton of the amide function, respectively. The resonance at highest field appears as a doublet of 18.4 Hz, characteristic of $^{1}J(^{15}NP)$ couplings in P(V) compounds with a P=S double bond.[25,26] The absence of $^{1}J(^{15}NP)$ couplings for the other resonances is strong evidence against the thiophosphoramidate being linked to any of these nitrogen atoms. Transfer of magnetization from H8 is used to assign the N9 and N7 resonances by an INEPT pulse sequence. In the imidazole part, an "azine- or pyridine-like" nitrogen absorbs at lower field than the "pyrrole-like" nitrogen. N7 is therefore found at lower field than N9.[22] Such assignments have also been confirmed from the observation (not shown) of the respective $J(^{15}NH)$ coupling constants [$^{2}J(^{15}N7H8)$ is always larger (10–12 Hz) than $^{2}J(^{15}N9H8)$ (7–9 Hz)]. The N2-nitrogen resonates in the usual region for an amide nitrogen, with a $^{1}J(^{15}NH)$ of 88 Hz.[25] The fact that the resonances of N1 and N3 are shifted downfield substantiates our assignment that the thiophosphoramidate residue is located at O6. The substituent effect of the acetamido function at the C2 position on the N3 chemical shift has been estimated to be ca. 35 ppm.[23] Such an estimate has allowed us to assign the resonance at −168.8 ppm to the N3-nitrogen. This means that the resonance at −152.2 ppm should be assigned to the N1-nitrogen.

The ^{15}N NMR spectrum of compound **6** supports the above assignment (Fig. 2). The INEPT spectrum shows both the couplings of N9 and N7 with H8, and those of N1 and N3 with H2. A distinction between the N3 and N1 resonances cannot be made on the basis of $^{2}J(^{15}NH2)$ coupling constants since they are identical. However, the C6 substituent in compound **6** is a particularly good electron-withdrawing group, and therefore N3, which should absorb between 130 and 160 ppm for C6-substituted purines, presumably resonates at −136.2 ppm, while N1 is somewhat deshielded and absorbs at −125.1 ppm. No $^{1}J(^{15}NP)$ coupling is observed. It should be noted that the difference in the ^{15}N chemical shifts between the diastereoisomers of both **2** and **6** is below the limit of the spectrometer resolution (see Experimental), although a double set of signals is just observable in the INEPT spectrum of **2**. The ^{15}N NMR data are listed in Table 1.

^{13}C NMR and UV spectroscopic results. A comparison of the ^{13}C chemical shifts of the thiophosphorylated nucleosides with those of the original peracylated nucleosides could not clearly differentiate between the two sites. The changes in chemical shift values resulting from thiophos-

phorylation (Δ) were compared with the corresponding changes resulting from methylation[27] (Table 2). For the derivatives **2** and **4** the C6 resonance is almost unchanged by the thiophosphorylation, a behaviour normally seen when reactions occur at the N1 position.[27,28] On the other hand, the effect on the C2 carbon is similar to that seen for the corresponding O6-methylated

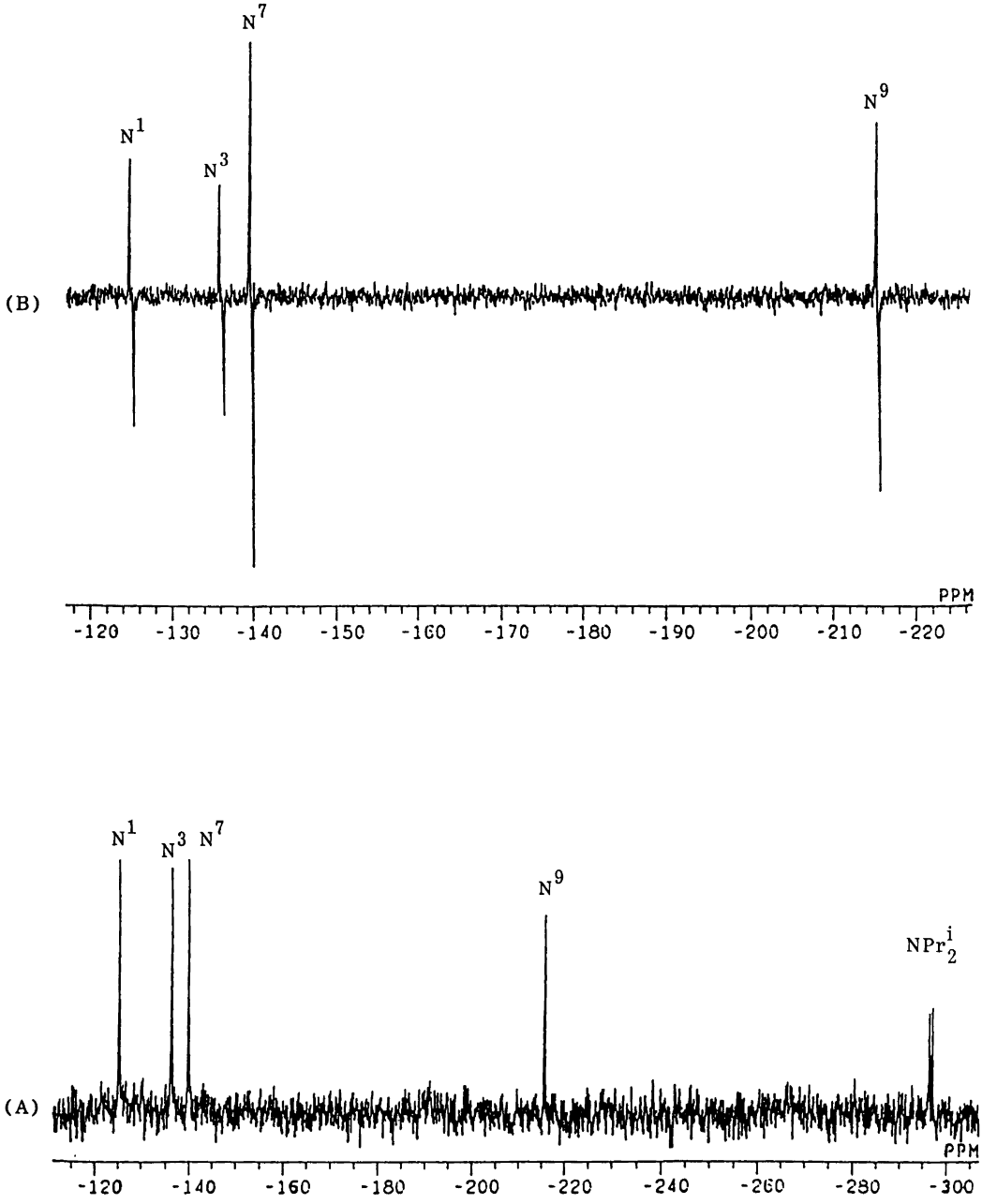

Fig. 2. ^{15}N NMR spectra of compound **6**: (A) ^{1}H-decoupled spectrum without NOE; (B) INEPT spectrum.

Table 1. ^{15}N Chemical shifts[a] for compounds **2** and **6**.

Compound	N1	N3	N7	N9	N2	Pr^i_2N
2	−155.2	−168.8	−139.7	−216.0	−235.3	−296.1
	(0)	(0)	(12.0)	(9.1)	(88.1)	(18.6)[b]
6	−125.1	−136.2	−139.7	−215.5		−296.7
	(15.9)	(15.9)	(12.2)	(9.1)		(18.9)[b]

[a] $CH_3{}^{15}NO_2$ was used as external reference. The values in parentheses are $J(^{15}NH)$ coupling constants. [b] ^1J (^{15}NP) coupling constant.

Table 2. ^{13}C Chemical shift[a] values for selected nucleoside derivatives.

Compound	C2	C4	C5	C6	C8
Tetraacetylguanosine	147.9	147.9	122.3	155.7	138.5
2	153.9	152.0	120.9	156.1	141.4
			(5.5)	(7.3)	
Δ	6.0	4.1	−1.4	0.4	2.9
Δ(*O*6-methylguanosine)[b]	6.2	2.9	−2.4	4.3	2.8
Δ(*N*1-methylguanosine)[b]	0.6	−2.8	−1.1	0.9	2.0
Triacetyldeoxyguanosine	147.9	147.6	121.7	155.6	138.1
4	153.7	151.7	120.8	155.6	141.1
			(6.1)	(6.8)	
Δ	5.8	4.1	−0.9	0.0	3.0
Δ(*O*6-methyldeoxyguanosine)[b]	6.2	2.9	−2.4	4.3	2.8
Δ(*N*1-methyldeoxyguanosione)[b]	0.6	−2.8	−1.1	0.9	2.0
Triacetylinosine	145.8	148.7	125.3	158.8	138.4
6	152.1	153.2	124.3	156.1	142.1
			(6.4)	(6.7)	
Δ	6.3	4.5	−1.0	−2.7	3.7

[a] Acetone-d_6 (29.2 ppm) was used as internal reference. T = 30 °C. The values in parentheses are J(CP) coupling constants (±1.5 Hz). [b] Ref. 27.

Table 3. UV spectroscopic data for peracylated and thiophosphoryl derivatives.

Compound	λ_{max}/nm (ε/cm^2 mmol^{-1})[a]
Tetraacetylguanosine (**1**)	279 (11.4×10^3), 259 (15.6×10^3)
2	270 (11.4×10^3), 258 (11.7×10^3)
Triacetyldeoxyguanosine (**3**)	280 (10.8×10^3), 259 (14.8×10^3)
4	275 (12.0×10^3), 258 (11.7×10^3)
Triacetylinosine (**5**)	244 (11.1×10^3)
6	252 (9.7×10^3)

[a] In methanol.

compounds. The ^{13}C spectra show J(CP) couplings for the C6 and C5 carbons, as shown in Table 2. No coupling to C2 is observed. This indicates O6- and not N1-substitution, although model compounds which could be used to determine the magnitude of a ^2J(CP) coupling constant for this type of compound are lacking.

Previously, UV spectroscopy has been used to identify positional isomers.[1,7] For example, it has been stated that all O6-substituted compounds have a λ_{max} of 290–305 nm,[7] i.e. that O6 substitution should result in a bathochromic shift. The UV spectra of **2**, **4** and **6** were compared with those of the parent protected nucleosides (Table 3). As seen from Table 3, no bathochromic shifts were observed. This criterion is therefore misleading and should not be used to distinguish positional isomers.

The present work shows that side reactions take place to a considerable extent when guanosine or inosine derivatives react with alkyl phosphorodiamidites, and that the products are O6-phosphitylated compounds. Similar modifications occur when e.g. DMTdG[ib] and alkyl phosphorodiamidites are used to obtain deoxynucleoside-3′-phosphoramidites for use in DNA synthesis, unless the O6 position is protected. However, the ultimate products in such cases are the desired 3′-phosphoramidites, and O6 protection of 2′-deoxyguanosine derivatives is therefore not necessary.[29] A reaction in the lactam system, being only slowly reversed in the presence of free 3′-OH groups, could be the cause of the low yields sometimes obtained[30] when guanine-containing phosphoramidites are prepared.

Experimental

All ^{15}N NMR spectra were recorded at 27.4 MHz on a Jeol JNM-GX 270 spectrometer using 10 mm o.d. sample tubes. The chemical shifts were measured relative to an external solution of $CH_3{}^{15}NO_2$ in CD_3NO_2 to provide both the reference and the frequency lock. The decoupled spectra with NOE suppressed were recorded with a pulse angle of 45° (1 μs pulse width), 0.85 s acquisition time for 16 K data points, zero filled to 32 K and Fourier transformed with a broadening factor of 2–3 Hz. The pulse delay was 18 s for the ^1H-decoupled spectra without NOE; with NOE the pulse delay was set at 10 s. The following conditions were used for INEPT spectra; ^1H-90° = 58 μs, ^{15}N-90° = 26 μs, τ = 1/4 ($J^{15}NH$) set at 23 ms and a pulse delay of 1 s. The frequency range was 1000 Hz, yielding a digital resolution of 0.6 Hz. A negative value for the chemical shift denotes an upfield shift. The temperature of the probe was ca. 30 °C. The concentration was ca. 0.50 M for compound **2** and 0.40 M for compound **6** in distilled dichloromethane. The ^{13}C chemical shifts were determined at 67.9 MHz and 22.5 MHz on Jeol JNM-GX 270 and Jeol FX 90Q spectrometers, respectively. Acetone-d_6 was used as the internal standard (29.2 ppm). ^1H NMR and ^{31}P NMR spectra were obtained on the JEOL FX 90Q instrument (^1H at 89.5 MHz, chemical shifts relative to tetramethylsilane; ^{31}P NMR at 36.3 MHz, chemical shifts positive in the low field direction, external standard 85 % H_3PO_4). Ultraviolet absorption spectra were recorded on a Shimadzu UV-260 spectrophotometer equipped with a Haake L/Haake D1 temperature controller maintained at 25 °C ± 0.3 °C.

N2,O3′,O5′-triacetyl-O6-[(N,N-diisopropylamino) (methoxy)thiophosphoryl]-2′-deoxyguanosine (**4**). *N2,O3′, O5′*-triacetyl-2′-deoxyguanosine[31] (393 mg, 1.0 mmol) was dried by coevaporation with dry CH_3CN. After addition of diisopropylammonium tetrazolide[15] (85 mg, 0.5 mmol) and dry CH_3CN (5 ml), methyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite[15] (350 mg, 1.3 mmol) was added by syringe with stirring at room temperature to give a clear solution within 1 min. This solution was stirred for 1 h and dry sulfur (128 mg, 4.0 mmol) was then added. The mixture was stirred for 1/2 h, and the solution was filtered to remove excess of sulfur and evaporated to an oil. The latter was dissolved in CH_2Cl_2 (5 ml) and applied to a silica gel column (10 g of silica gel, Merck 60, Art. 9385) wich was eluted with a gradient of from 0 to 10 % MeOH in CH_2Cl_2 (25 ml of each concentration, 2 % steps), collecting fractions of 10 ml. The appropriate fractions were evaporated to give **4** (177 mg, 30 %) as a glass. ^{31}P NMR ($CDCl_3$): δ 68.6 ppm. ^1H NMR ($CDCl_3$): δ 8.17 (s, 1H, NH), 8.01 (s, 1H, H8), 6.32 (t, 1H, J = 6.6 Hz, H1′), 5.39 (m, 1H, H3′), 4.31 (s, 3H, H5′ and H4′), 3.88 (d, 3H, J(PH) = 14.4 Hz, CH_3O-P), 2.51 (s, 3H, CH_3CON-), 2.08 and 2.01 (2×s, 3H + 3H, CH_3CO_2-), 1.28 (d, 12H, J = 6.8 Hz, CH_3-Pr^i). Anal. $C_{23}H_{35}N_6O_8PS$: C, H, N, S.

N2,O2′,O3′,O5′-tetraacetyl-O6-[(N,N-diisopropylamino) (methoxy)thiophosphoryl]-guanosine (**2**). *N2,O2′,O3′,O5′*-tetraacetyl-guanosine[32] (451 mg, 1.0 mmol) was treated as described for **4** to give **2** (185 mg, 29 %) as a glass. ^{31}P NMR ($CDCl_3$): δ 68.6 ppm. ^1H NMR ($CDCl_3$): δ 8.15

(s, 1H, NH), 7.98 (s, 1H, H8), 6.05 (d, 1H, J = 4.6 Hz, H1′), 5.85 (m, 1H, H2′), 5.65 (m, 1H, H3′), 4.36 (s, 2H, H5′), 3.88 (d, 3H, J = 14.7 Hz, CH_3O-P), 2.50 (s, 3H, CH_3CON-), 2.08 and 2.02 (s, 3H + 6H, CH_3CO_2-), 1.28 (d, 12H, J = 6.8 Hz, CH_3-Pr^i). Found: C 45.59; H 6.11; N 12.53. Calc. for $C_{25}H_{37}N_6O_{10}PS$: C 46.59; H 5.79; N 13.04.

O2′,O3′,O5′-triacetyl-O6-[(N,N-diisopropylamino) (methoxy)thiophosphoryl]-inosine (**6**). *O2′,O3′,O5′*-triacetylinosine[33] (393 mg, 1.0 mmol) was treated as described for **4** to give **6** (334 mg, 57 %) as a glass. ^{31}P NMR ($CDCl_3$): δ 69.4 ppm. ^1H NMR ($CDCl_3$): δ 8.63 (s, 1H, H2), 8.13 (s, 1H, H8), 6.20 (d, 1H, J = 5.1 Hz, H1′), 5.92 (m, 1H, H2′), 5.62 (m, 1H, H3′), 4.37 (s, 2H, H5′), 3.97 (d, 3H, J = 14.4 Hz, CH_3O-P), 2.06–2.02 (m, 9H, CH_3CO_2-), 1.31 (d, 12H, J = 6.8 Hz, CH_3-Pr^i). Anal. $C_{23}H_{34}N_5O_9PS$: C, H, N, S.

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References

1. Bridson, P. K., Markiewicz, W. T. and Reese, C. B. *J. Chem. Soc., Chem. Commun.* (1977) 447.
2. Bridson, P. K., Markiewicz, W. T. and Reese, C. B. *J. Chem. Soc., Chem. Commun.* (1977) 791.
3. Reese, C. B. and Ubasawa, A. *Tetrahedron Lett. 21* (1980) 2265.
4. Jones, S. S., Rayner, B., Reese, C. B., Ubasawa, A. and Ubasawa, M. *Tetrahedron 36* (1980) 3075.
5. Reese, C. B., Titmus, R. C. and Yau, L. *Tetrahedron Lett. 19* (1978) 2727.
6. Stawinski, J., Hozumi, T. and Narang, S. A. *Can. J. Chem. 54* (1976) 670.
7. Daskalow, H. P., Sekine, M. and Hata, T. *Bull. Chem. Soc. Jpn. 54* (1981) 3076.
8. Daskalow, H. P., Sekine, M. and Hata, T. *Tetrahedron Lett. 21* (1980) 3899.
9. Kamimura, T., Masegi, T. and Hata, T. *Chem. Lett.* (1982) 965.
10. Kamimura, T., Tsuchiya, M., Koura, K., Sekine, M. and Hata, T. *Tetrahedron Lett. 24* (1983) 2775.
11. Sekine, M., Matsuzaki, J.-I., Satoh, M. and Hata, T. *J. Org. Chem. 47* (1982) 571.
12. Adamiak, R. W., Biala, E. and Skalski, B. *Nucleic Acids Res. 13* (1985) 2989.
13. Adamiak, R. W., Biala, E., Gdaniec, Z., Mielewzyk, S. and Skalski, B. *Chem. Scr. 26* (1986) 3; *Ibid.* 7.
14. Adamiak, R. W., Biala, E. and Skalski, B. *Angew. Chem., Int. Ed. Engl. 24* (1985) 1054.
15. Barone, A. D., Tang, J.-Y. and Caruthers, M. H. *Nucleic Acids Res. 12* (1984) 4051.
16. Pon, R. T., Damha, M. J. and Ogilvie, K. K. *Tetrahedron Lett. 26* (1985) 2525.
17. Pon, R. T., Damha, M. J. and Ogilvie, K. K. *Nucleic Acids Res. 13* (1985) 6447.
18. Pon, R. T., Usman, N., Damha, M. and Ogilvie, K. K. *Nucleic Acids Res. 14* (1986) 6453.
19. Nielsen, J., Marugg, J. E., Taagaard, M., van Boom, J. H. and Dahl, O. *Recl. Trav. Chim. Pays-Bas 105* (1986) 33.
20. Remaud, G., Kjellberg, J., Bazin, H., Johannson, N. G. and Chattopadhyaya, J. *Tetrahedron 42* (1986) 5073.
21. Damha, M. J. and Ogilvie, K. K. *J. Org. Chem. 51* (1986) 3559.
22. Markowski, V., Sullivan, G. R. and Roberts, J. D. *J. Am. Chem. Soc. 99* (1977) 714.
23. Remaud, G., Zhou, X.-X., Welch, C. C. J. and Chattopadhyaya, J. *Tetrahedron 42* (1986) 4057.
24. Remaud, G., Welch, C. J., Zhou, X.-X. and Chattopadhyaya, J. *Nucleosides Nucleotides* (1987). *In press.*
25. Martin, G. J., Martin, M. C. and Gousonard, J. P. *NMR Basic Prin. Prog.*, Springer-Verlag, Berlin 1981, Vol. 18.
26. Wrackmeyer, B. *Spectrochim. Acta, Part A 40* (1984) 963.
27. Chang, C., Gomes, J. D. and Byrn, S. R. *Org. Magn. Reson. 22* (1984) 671.
28. Zhou, X., Ugi, I. and Chattopadhyaya, J. *Acta Chem. Scand., Ser. B 39* (1985) 762.
29. Nielsen, J., Taagaard, M., Marugg, J. E., van Boom, J. H. and Dahl, O. *Nucleic Acids Res. 14* (1986) 7391.
30. Schulhof, J. C., Molko, D. and Teoule, R. *Nucleic Acids Res. 15* (1987) 397.
31. Gaffney, B. L., Marky, L. A. and Jones, R. A. *Biochemistry 23* (1984) 5686.
32. van Boom, J. H. and Wreesmann, C. T. J. In: Gait, M. J., Ed., *Oligonucleotide Synthesis, a Practical Approach,* IRL Press, Oxford 1984, Chap. 7.
33. Bredereck, H. *Ber. 80* (1947) 401.

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