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FACILE NUCLEOSIDE PHOSPHORYLATION VIA HYDROXYL ACTIVATION

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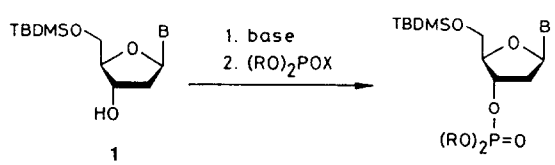
Summary: A rapid preparation of nucleoside phosphates under very mild conditions is described.

A key process in the oligonucleotide synthesis[1] is the condensation of a nucleoside hydroxyl group and a phosphoric acid derivative. The interest in this field has been directed mainly toward the development of highly reactive phosphorylating agents and resulted in elaboration of a variety of powerful reagents including mixed anhydrides of phosphoric and sulfonic acids which readily react with free hydroxyl substrates with added tertiary amines.[1] While phosphorochloridates or p-nitrophenyl phosphates are evidently among the least expensive, these compounds have been employed in very limited cases, particularly in the internucleotide formation, simply because of their poor electrophilicity. This obstacle should be removed by suitable activation of the alcoholic moiety but, to our surprise, no systematic investigations have been recorded in the literature.[2] Described herein is a facile phosphorylation method using some phosphorochloridates or p-nitrophenyl phosphates, which relies on simple basic activation of nucleoside hydroxyl functions.

Treatment of the nucleoside 1 with a strong base followed by exposure of the alkoxide to a phosphorochloridate gave the corresponding 3'-phosphate 2. Typical procedure is as follows.[3] To a THF (30 mL) solution of 5'-O-t-butyldimethylsilyl-2'-deoxyadenosine (1.07 g, 2.94 mmol) was added a 0.59 M solution of t-butyllithium (2.94 mmol) in pentane—hexane (5.0 mL) at -78 °C over 30 min. After 1.5 h, to the resulting heterogeneous mixture was added di-o-chlorophenyl phosphorochloridate (1.03 g, 3.06 mmol) in THF (8.7 mL) and the mixture was



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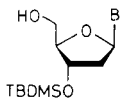
TBDMS = $Si(CH_3)_2$-t-C_4H_9

stirred at the same temperature for 1.25 h. Quenching by brine, extraction with dichloromethane, and chromatography on a silica gel column (1:10:40 methanol/acetone/dichloromethane) afforded the phosphate 2 (B = Ad, R = o-ClC_6H_4, 1.79 g)[4] in 91% isolated yield (95% by NMR assay).

In a similar manner, 5'-phosphates were prepared from nucleosides of types 3 and 4. Some examples of the present method are given in Table I. Nucleosides containing unprotected adenyl, N^3-protected thymidyl or uracil, and N^4-protected cytocyl bases underwent smoothly the phosphorylation using an equimolar amount of the strong base and a small excess of the phosphorochloridate. To achieve the



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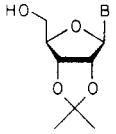

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Table I. Phosphorylation of Nucleosides with Phosphorochloridates[a]

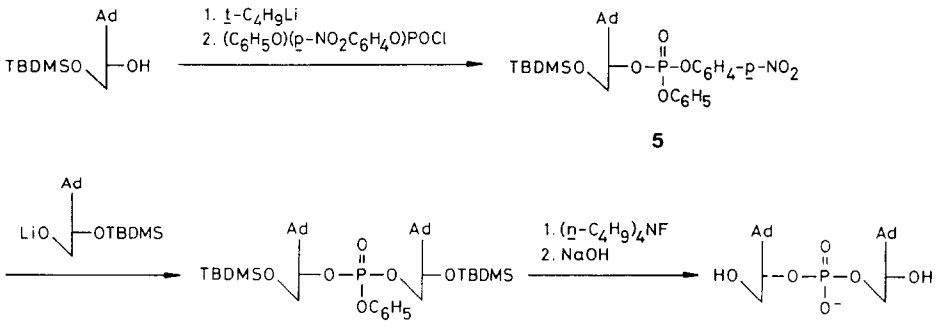
entry	nucleoside (B)	base	R in $(RO)_2POCl$	conditions		% yield of phosphate[b]
				temp, °C	time, h	
1	1 (Ad)	CH_3Li	o-ClC_6H_4	-78	1	90
2	1 (Ad)	n-C_4H_9Li	o-ClC_6H_4	-78	1	93
3	1 (Ad)	t-C_4H_9Li	o-ClC_6H_4	-78	1.25	95, 91[c]
4	1 (Ad)	MesLi[d]	o-ClC_6H_4	-78	0.25	97, 92[c]
5	1 (Ad)	2,6-DMPLi[e]	o-ClC_6H_4	-78	2	88
6	1 (Ad)	NaH	o-ClC_6H_4	-78	1	72
7	1 (Ad)	t-C_4H_9OK	C_2H_5	-78	1	90
8	1 (Th)	t-C_4H_9Li[f]	o-ClC_6H_4	-50	1.5	81[c]
9	1 (Th^{Me})	MesLi	o-ClC_6H_4	-78	1	84[c]
10	1 (Cy^{Bz})	MesLi	o-ClC_6H_4	-78	3	79
11	3 (Ad)	n-C_4H_9Li	o-ClC_6H_4	-78	3	85
12	3 (Ad)	t-C_4H_9Li	o-ClC_6H_4	-78	0.5	92
13	3 (Th)	t-C_4H_9Li[f]	o-ClC_6H_4	-50	1	85[c]
14	4 (Ad)	t-C_4H_9Li	o-ClC_6H_4	-78	0.5	91, 88[c]
15	4 (Ur^{Me})	MesLi	o-ClC_6H_4	-78	1	90[c]
16	4 (Ur^{Me})	t-C_4H_9OK	C_2H_5	-78	1	90[c]

[a] Unless otherwise noted, the reaction was carried out in THF by using 1 equiv of the strong base and 1.1—1.2 equiv of the phosphorylating agent. [b] Determined by ^1H NMR analysis, unless otherwise stated. [c] Isolated yield. [d] Mesityllithium: H. Neumann and D. Seebach, Tetrahedron Lett., 4839 (1976). [e] 2,6-Dimethoxyphenyllithium: G. J. Lambert, R. P. Duffley, H. C. Dalzell, and R. K. Razdan, J. Org. Chem., 47, 3350 (1982). [f] Two equivalents of the reagent were employed.

high-yield reaction of N^3-unprotected thymidine or uridine derivatives, 2 equiv of the strong base[5] and 1.2 equiv of the phosphorochloridate were used. N^4-Unprotected cytidines produced considerable amounts of N^4-mono- and N^4,O-diphosphorylated compounds. Guanyl nucleosides failed the phosphorylation reaction. In the reaction of the protected pyrimidine nucleosides (entries 9, 10, 15, and 16), choice of the base which produces the metal alkoxides is important; mesityllithium and potassium t-butoxide were among the best, but organolithiums having strong nucleophilicity, even t-butyllithium, gave some undesired by-products in the lithiation step. As the phosphorylating agent, p-nitrophenyl phosphates are employable in place of phosphorochloridates.

Thus the present method, complementary to the existing phosphorylation procedures, provides a convenient tool in oligonucleotide synthesis. The advantages include (1) low costs of the reagents, (2) high efficiency of the reaction using an equimolar amount or only slight excess of the phosphorylating agent, (3) mildness of the reaction conditions, (4) operational simplicity (reaction and workup), etc. This recipe is particularly suited for phosphorylation of adenyl nucleosides, since the reaction is effected without any N^6-protection.[6] Phosphorylation of adenyl nucleosides under conventional conditions occurs predominantly at the nitrogen atom rather than hydroxyls,[7] but the O vs N chemoselectivity is thus reversed through the alkoxide formation.

This method is applicable to the formation of some internucleotide linkages. Reaction of the lithium alkoxide, generated from 1 (B = Ad) and t-butyllithium (1 equiv each), and p-nitrophenyl phenyl phosphorochloridate[8] (1.1 equiv) in THF at -78 °C for 1 h, giving 5, was followed by the condensation with the lithium alkoxide of 3 (B = Ad, 1.1 equiv) in the same pot at -50 °C for 5 h to produce the dinucleotide 6[9] in 80% overall yield.[10] Removal of the protective groups was accomplished by treatment with 1 M $(n\text{-}C_4H_9)_4NF$ solution in 5:95 H_2O—THF (20 °C, 8 h)[11] followed by 0.1 N NaOH (20 °C, 4 h) to afford dApdA (7)[12] in 70% yield.



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2. Only a few reports appeared on the condensation of phosphorylating agents and metal alkoxides of nucleosides. For the phosphotriester approach achieved with sodium metal under forcing conditions (room temperature, 18—28 h): V. Škarić, M. Hohnjec, and Dj. Škarić, Croat. Chem. Acta, 49, 851 (1977). For related phosphodiester methods using potassium t-butoxide: R. v. Tigerstrom and M. Smith, Science, 167, 1266 (1970); R. K. Borden and M. Smith, J. Org. Chem., 31, 3247 (1966). For related phosphorylation of carbohydrates, see: M. Inage, H. Chaki, S. Kusumoto, and T. Shiba, Chem. Lett., 1281 (1982).
3. Caution: Proper stoichiometry of the nucleoside, base, and phosphorylating agent is crucial for obtaining high product yield. Therefore materials of high quality should be used. Purity of t-butyllithium employed here was 90% (organolithium/total base). When an excess of the base was used in the reaction of adenyl derivatives, formation of N,O-diphosphorylated products was observed.
4. Mp 114—115 °C; IR ($CHCl_3$); 3490, 3400 (NH_2), 1290 (P=O) cm^{-1}; UV (CH_3OH) λ_{max} 260 nm (ε 16,700); ^1H NMR ($CDCl_3$): δ 0.08 (s, $Si(CH_3)_2$), 0.89 (s, Si-t-C_4H_9), 2.87 (m, 2 $H_{2'}$), 3.90 (d-like, J = 3.1 Hz, 2 $H_{5'}$), 4.43 (m, $H_{4'}$), 5.55 (m, $H_{3'}$), 5.87 (br s, NH_2), 6.52 (t-like, J = 7.0 Hz, $H_{1'}$), 7.05—7.57 (m, 2 C_6H_4Cl), 8.11 (s, H_2), 8.34 (s, H_8); ^{13}C NMR ($CDCl_3$): δ -6.0, -5.9 ($Si(CH_3)_2$), 17.8 ($SiC(CH_3)_3$), 25.4 ($SiC(CH_3)_3$), 39.2 (d, J = 4 Hz, $C_{2'}$), 62.7 ($C_{5'}$), 80.6 (d, J = 7 Hz, $C_{3'}$), 83.8 ($C_{1'}$), 85.5 (d, J = 7 Hz, $C_{4'}$), 119.4 (C_5), 121.9 (d, J = 2 Hz, C_6H_4Cl), 125.0 (d, J = 7 Hz, C_6H_4Cl), 126.2, 127.6, 130.3 (C_6H_4Cl), 138.0 (C_8), 145.8 (d, J = 7 Hz, C_6H_4Cl), 149.1 (C_4), 152.6 (C_2), 155.6 (C_6).
5. Reaction of these N-unprotected nucleosides with 1 equiv of the strong base produced no corresponding alkoxide but the N^3-metalated substances, because the N^3-protons are the more acidic.
6. Introduction and removal of the protective group often cause a serious loss of the nucleotide. Cf. H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, J. Am. Chem. Soc., 85, 3821 (1963); A. Kume, M. Sekine, and T. Hata, Tetrahedron Lett., 23, 4365 (1982).
7. G. M. Tener, J. Am. Chem. Soc., 83, 159 (1961).
8. I. Dilaris and G. Eliopoulos, J. Org. Chem., 30, 686 (1965).
9. Obtained as a 1:1 mixture of two diastereomers. One of the isomers: IR ($CHCl_3$): 3500, 3410 (NH_2), 1280 (P=O) cm^{-1}; UV (CH_3OH): λ_{max} 260 nm (ε 26,000); ^1H NMR ($CDCl_3$): δ 3.82 (m, 2 $CH_{5'}$,OSi), 4.02—4.48 (m, 2 $H_{4'}$, 2 $CH_{5'}$,OP), 4.65 (m, $CH_{3'}$,OSi), 5.22 (m, $CH_{3'}$,OP), 6.20—6.56 (m, 2 $H_{1'}$, 2 NH_2), 7.97, 8.08 (two s's, 2 H_2), 8.27 (s, 2 H_8); FDMS m/z: 868 (M^+). The other isomer: IR ($CHCl_3$): 3500, 3410 (NH_2), 1290 (P=O) cm^{-1}; UV (CH_3OH): λ_{max} 260 nm (ε 27,000); ^1H NMR ($CDCl_3$): δ 3.74 (m, 2 $CH_{5'}$,OSi), 4.18 (m, 2 $CH_{5'}$,OP), 4.68 (m, $CH_{3'}$,OSi), 5.22 (m, $CH_{3'}$,OP), 6.18 (br s, 2 NH_2), 6.41 (m, 2 $H_{1'}$), 7.98, 8.04 (two s's, 2 H_2), 8.26 (s, 2 H_8); FDMS m/z: 868 (M^+).
10. In this reaction, use of the p-nitrophenyl phosphorochloridate is crucial. Phenyl phosphorodichloridate in the first phosphorylation step formed considerable amounts of by-products.
11. K. K. Ogilvie, S. L. Beaucage, and D. W. Entwistle, Tetrahedron Lett., 1255 (1976).
12. The product was identical with the authentic sample in all respects (HPLC [ODS, 0.1 M KH_2PO_4—0.005 M $(n\text{-}C_4H_9)_4NBr$ in H_2O/CH_3OH (80:20), V_R 15.5 mL], electrophoresis [0.05 M $HCOONH_4$ (pH 3.5)], and enzymatic hydrolysis using snake venom phosphodiesterase).

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