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PHOSPHORYLATION OF NUCLEOSIDE DERIVATIVES WITH ARYL PHOSPHORAMIDOCHLORIDATES

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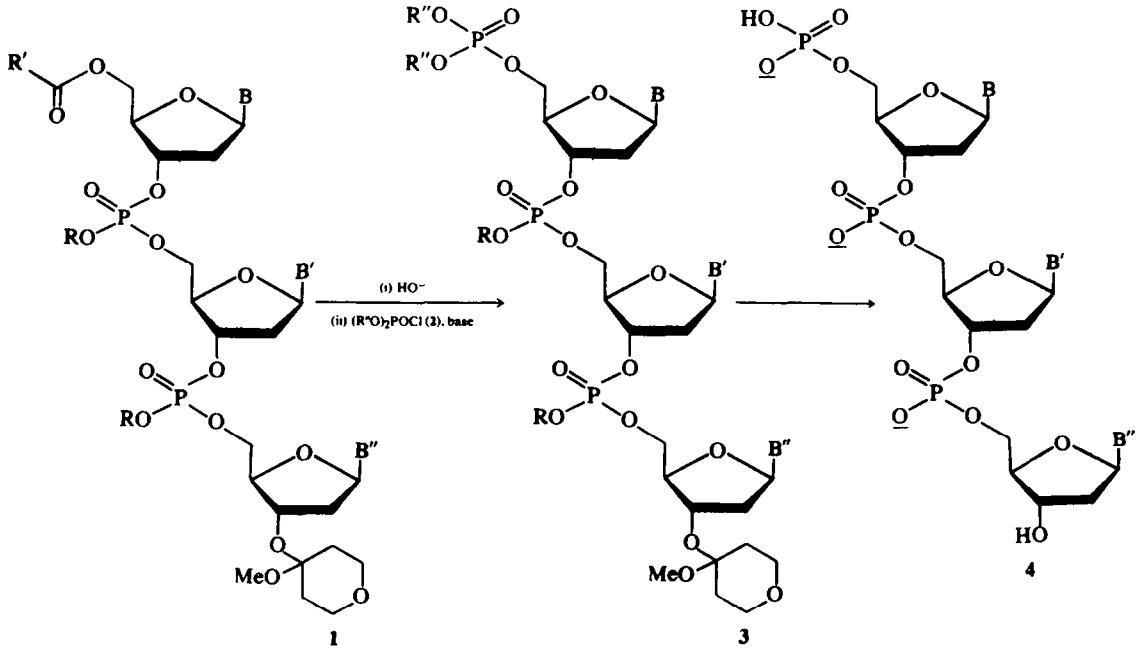
Abstract—The preparation of three aryl phosphorocyclohexylamidochloridates (**7a**, **7b** and **7c**) and an aryl phosphoromorpholidochloridate (**8**) is described. These aryl phosphoramidochloridates react with 2',3'-O-methoxymethylene-uridine, -4-N-anisoylcytidine and -6-N-anisoyladenosine (**9a**, **9b** and **9c**, respectively), in the presence of the 1-ethylimidazole derivative (**11a**) to give high yields of the corresponding fully-protected 5'-phosphoramidates (**10**). Treatment of the latter compounds with aqueous alkali gives the nucleoside 5'-phosphoramidate derivatives (**14**) which, on mild acidic hydrolysis, give the corresponding unprotected 5'-nucleotides (**15**) in virtually quantitative yields. Phosphorylation of 2'-O-methoxytetrahydropyranyluridine (**12**) with **7a** and **8**, under the same conditions, occurs regiospecifically to give the corresponding 5'-phosphoramidate derivatives (**13**). The partially-protected dinucleoside phosphate (**16b**) has been prepared and phosphorylated with **7a** to give, after removal of the protecting groups, the dinucleotide (**18**, pUpU) in high yield.

The starting materials in our phosphotriester approach[1] to oligonucleotide synthesis are suitably-protected deoxyribo- and ribo-nucleoside derivatives and the fully-protected oligomers obtained, such as the trideoxy-ribonucleoside diphosphate (**1**) and corresponding oligoribonucleotide derivatives, all lack terminal 3'- or 5'-monophosphate groups. It has therefore long been our intention to develop a phosphorylation method for the introduction of terminal phosphate groups. A hypothetical example of the application of such a method, involving a dialkyl or diaryl phosphorochloridate (**2**) and leading to a trinucleotide (**4**) with a terminal 5'-phosphate residue, is illustrated in Scheme 1.

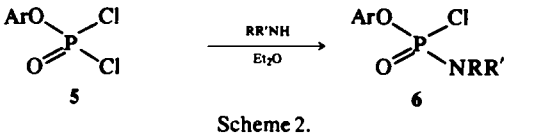
Phosphodiester approaches involving 2-cyanoethyl[2] and 2-arylmercaptoethyl[3] protecting groups and also involving phosphates masked as phosphoramidates[4] or phosphorothioates[5] have been used successfully in the synthesis of oligonucleotides with terminal phosphate groups. However, all of these procedures lack one of the main advantages of the phosphotriester approach: namely, the possibility of purifying the fully-protected intermediates (such as **3**) by adsorption chromatography



1 **3** **4**

Scheme 1.

on alumina or silica gel. In order to design a suitable phosphorylating agent (2) for the triester approach, it is necessary to choose the protecting group (R") in such a way that three main criteria are met: firstly, the reagent (2) must be a stable compound which may be isolated in a pure state; secondly, it must be an effective phosphorylating agent; and thirdly, the protecting groups (R") must remain intact during phosphorylation and during the purification of the intermediates (such as 3), but they must be readily removable in the final steps of the synthesis under conditions which lead to good yields of the desired unblocked oligonucleotides (such as 4). A final desirable but not essential consideration is that the reagent (2) should be readily available. Although it should be possible to find a phosphorylating agent which meets all of these criteria, it is not clear to us that they are indeed met by any of the numerous dialkyl and diaryl phosphorochloridates described in the literature. We now report the initial results of a study involving aryl phosphoramidochloridates (6) as phosphorylating agents and present evidence for the conclusion that a suitably-designed reagent of the latter type would fully meet all of the above criteria. The use of the latter reagents (6) also leads to a very convenient general method for the preparation of nucleoside phosphoramidates.



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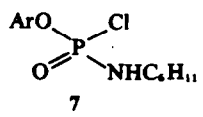
Scheme 2.



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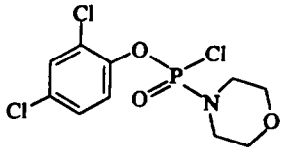
a: Ar = C_6H_5
b: Ar = 2-ClC_6H_4–
c: Ar = 2,4-$Cl_2C_6H_3$–



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Aryl phosphoramidochloridates may readily be prepared, following a procedure of Michaelis[6] (Scheme 2) by treating aryl phosphorodichloridates with two molecular equivalents of the appropriate amines in ether solution; they may usually be isolated as crystalline solids in yields of 80% or more. In the course of the present study, we have prepared three different aryl phosphorocyclohexylamidochloridates (7a, 7b and 7c) and 2,4-dichlorophenyl phosphoromorpholidochloridate (8). Only in the case of the latter compound was the yield of crystalline material below 80%.

All four of the above aryl phosphoramidochloridates were found to be effective phosphorylating agents.† Thus treatment of 2',3'-O-methoxymethyleneuridine[7] (9a) with a twofold excess of phenyl, o-chlorophenyl or 2,4-dichlorophenyl phosphorocyclohexylamidochloridate (7a, 7b or 7c) or of 2,4-dichlorophenyl phosphoromorpholidochloridate (8) in the presence of 5-chloro-1-ethyl-2-methylimidazole[1c] (11a) at 20° gave (Table 1, experiments Nos. 1–4) the corresponding 2',3'-O-methoxymethyleneuridine 5'-aryl phosphoramidate (10a) in good yield. The latter compounds were all isolated as pure colourless glasses following chromatography of the products on silica gel. Phosphorylation also proceeded readily in (i) pyridine solution and (ii) acetonitrile solution in the presence of 1,2-dimethylimidazole (11b). However, when 2',3'-O-methoxymethyleneuridine (9a) was treated with phenyl phosphorocyclohexylamidochloridate (7a) in pyridine solution considerable darkening occurred and an examination of the reaction between (9a) and (7a) in acetonitrile solution in the presence of 1,2-dimethylimidazole (11b) led to the conclusion that the latter base was marginally less effective for the present purpose than 5 - chloro - 1 - ethyl - 2 - methylimidazole (11a).

Table 1. Phosphorylation of nucleoside derivatives with aryl phosphoramidochloridates[a]

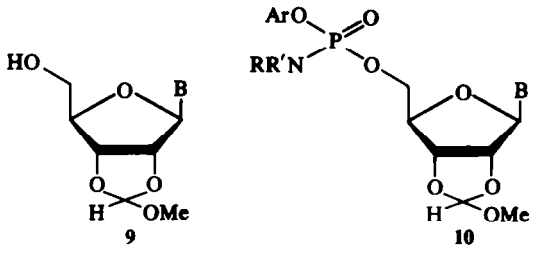
Experiment No.	Nucleoside derivative	Phosphorylating agent	Yield of Product[b] (%)
1	9a	7a	88
2	9a	7b	87
3	9a	7c	88
4	9a	8	80
5	9b	7c	80
6	9c	7c	88
7	12	7a	60[c]
8	12	8	50[c]

[a] Phosphorylation reactions were carried out in acetonitrile solution, in the presence of 5 - chloro - 1 - ethyl - 2 - methylimidazole (11a), at 20°.
[b] 10 in experiments Nos. 1–6 and 13 in experiments Nos. 7 and 8.
[c] Yield of pure crystalline product.

Substrates other than 2',3'-O-methoxymethyleneuridine (9a) were also examined: high yields of products (respectively, 10b and 10c; Ar = 2,4-$Cl_2C_6H_3$, R=C_6H_{11}, R'=H) were obtained (Table 1, experiments Nos. 5 and 6) when 4 - N - anisoyl - 2',3' - O - methoxymethylenecytidine (9b) and 6 - N - anisoyl - 2',3' - O - methoxymethyleneadenosine[7] (9c) were phosphorylated with 2,4-dichlorophenyl phosphorocyclohexylamidochloridate (7c). Finally, the expected regioselectivity was displayed when 2' - O - methoxytetrahydropyranyluridine[8] (12) was phosphorylated with both (7a) and (8) (experiments Nos. 7 and 8) and satisfactory yields of pure crystalline 5'-derivatives (13; Ar=Ph, R=C_6H_{11}, R'=H and Ar=2,4-$Cl_2C_6H_3$, RR'=$(CH_2)_2O(CH_2)_2$, respectively) were isolated from the products.

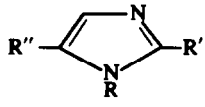
Alkaline hydrolysis of the nucleoside aryl phosphorocyclohexylamidates (10 and 13; R=C_6H_{11}, R'=H) occurred very readily. Thus the half-times of hydrolysis of the phenyl, o-chlorophenyl and 2,4-dichlorophenyl esters of 2',3' - O - methoxymethyleneuridine 5'-phosphorocyclohexylamidates (10a; R=C_6H_{11}, R'=H, Ar=C_6H_5, 2-ClC_6H_4 and 2,4-$Cl_2C_6H_3$, respectively) in 0·1 M sodium hydroxide in aqueous dioxan (4:1, v/v) solution at 20° were found to be 60, 5·8 and 2·9 min, respectively. The alkaline hydrolysis of the aryl phosphoromorpholidates proceeded much less readily: under the same conditions, the half-time of hydrolysis of 2',3' - O - methoxymethyleneuridine - 5' 2,4-dichlorophenyl phosphoromorpholidate (10a; Ar=2,4-$Cl_2C_6H_3$, RR'=$(CH_2)_2O(CH_2)_2$) was found to be 516 min. The faster rate of hydrolysis, by more than two orders of magnitude, of the corresponding phosphorocyclohexylamidate (10a;

†M. E. Wolff and A. Burger (*J. Am. Chem. Soc.* 79, 1970 (1957)) have previously shown that 2',3'-O-isopropylideneadenosine can be phosphorylated with phenyl phosphorodiethylamidochloridate (6; Ar=Ph, R=R'=Et) in t-BuOH solution, in the presence of potassium t-butoxide.

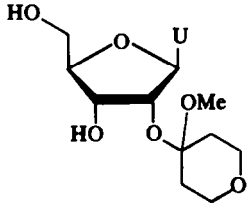


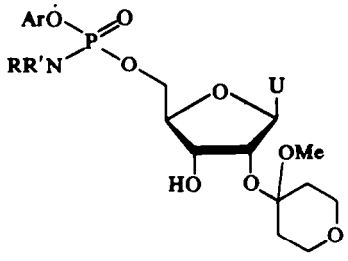
9 10

a: B = uracil-1-yl
b: B = 4-*N*-anisoylcytosin-1-yl
c: B = 6-*N*-anisoyladenin-9-yl



2a: R = Et, R′ = Me, R″ = Cl
b: R = R′ = Me, R″ = H





12 U = uracil-1-yl 13

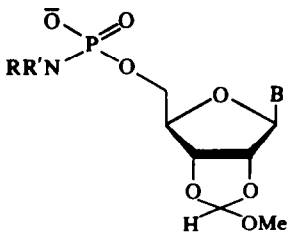
Ar = 2,4-$Cl_2C_6H_3$, R=C_6H_{11}, R′=H) is very likely due to a difference in the reaction mechanism: it seems reasonable to conclude[9] that the latter compound undergoes hydrolysis by an elimination process involving the removal of the phosphoramidate N–H proton rather than by direct attack of hydroxide ion on the phosphorus atom. In all cases, alkaline hydrolysis of the nucleoside-5′ aryl phosphoramidates† (**10** and **13**) led solely to the corresponding 5′-phosphoramidates (such as **14**). The ammonium salts of the latter compounds were isolated as colourless solids in virtually quantitative yields. The scope and applications of this very convenient method for the preparation of nucleoside phosphoramidates, intermediates of considerable importance in synthesis,[10] is currently under investigation.‡

In the present study, we have regarded nucleoside phosphoramidates only as intermediates in the preparation of mononucleotides. Nucleoside phosphoramidates have previously been converted into the corresponding monophosphate esters by (a) acidic hydrolysis[11] and (b) under nitrosating conditions.[12] We have followed procedure (a) as methoxymethylene and methoxytetrahydropyranyl protecting groups may then be removed at the same time. When 2′,3′ - O - methoxymethyleneuridine 5′ - phosphoro - cyclohexylamidate and -morpholidate (**14a**; R=C_6H_{11}, R′=H and RR′=$(CH_2)_2O(CH_2)_2$, respectively) were allowed to stand in dilute hydrochloric acid solution (pH 2) at 20° and the products neutralized and lyophilized, the ammonium salt of uridine 5′-phosphate (**15a**) was obtained as the sole nucleotide product. The half-times of hydrolysis for the two compounds under the latter conditions were found to be 516 and 10 min, respectively. Thus while the phosphoromorpholidate (**14a**; RR′=$(CH_2)_2O(CH_2)_2$) was virtually completely converted into (**15a**) in 1–1·5 hr, the phosphorocyclohexylamidate (**14a**; R=C_6H_{11}, R′=H) required 2–3 days. This result was not anticipated from previously reported[11] studies and the effect of N-substitution on phosphoramidate reactivity (including ease of acidic hydrolysis) merits further investigation. However, the relatively slow acidic hydrolysis rate of nucleoside phosphorocyclohexylamidates was not especially disadvantageous in the present work. Cytidine and adenosine 5′-phosphates (**15b** and **15c**, respectively) were readily obtained in virtually quantitative yields from their respective precursors (**14b** and **14c**) and uridine 5′-phosphate (**15a**) was also obtained in high yield by acidic treatment of the alkaline hydrolysis products of the above described 2′ - O - methoxytetrahydropyranyluridine - 5′ aryl phosphoramidates (**13**).

These preliminary studies were encouraging enough to prompt us to examine whether any of the aryl phosphoramidochloridates (**7a**, **7b**, **7c** and **8**) under consideration would serve the main purpose of this investigation, i.e. whether they would be suitable for the introduction of terminal phosphate groups in the phosphotriester approach to oligonucleotide synthesis. The fully-protected uridylyl - (3′→5′) - uridine derivative (**16a**) was prepared from 5′ - O - *p* - chlorophenoxyacetyl - 2′ - O - methoxytetrahydropyranyluridine,[13] 2,4-dichlorophenyl dihydrogen phosphate,[14] 2′,3′ - O - methoxymethyleneuridine and 2,4,6 - tri - isopropylbenzenesulphonyl chloride[15] in pyridine solution; after removal of the 5′ - O - acyl group, the partially - protected dinucleoside phosphate (**16b**) was isolated as a colourless solid in 70% overall yield. Phosphorylation of the latter material (**16b**) with an excess of phenyl phosphorocyclohexylamidochloridate (**7a**) in acetonitrile solution in the presence of 5 - chloro - 1 - ethyl - 2 - methylimidazole (**11a**) gave the fully-protected dinucleoside phosphate phosphoramidate (**17**), which was isolated as a colourless solid in 85% yield. Treatment of the latter compound (**17**) at 20° with 0·1 M sodium hydroxide solution for 15 hr followed by dilute hydrochloric acid (pH 2) for 3 days gave uridylyl - (5′→3′) - uridine - 5′ phosphate (**18**, pUpU). The ammonium salt of this dinucleotide was isolated as a colourless fluffy solid in virtually quantitative yield; it was found to be homogeneous by cellulose TLC and paper

†In the cases of **16b** and **16c**, alkaline hydrolysis was effected with 20% ammonia (w/v) in aqueous dioxan (4:1, v/v). Under these conditions concomitant removal of the N-anisoyl protecting groups occurred.

‡*Note added in proof.* It has recently been found (J. H. van Boom, R. Crea, W. C. Luyten and A. B. Vink, *Tetrahedron Letters* 2779 (1975)) that nucleoside phosphoramidates may also be conveniently prepared in high yields by reduction of their 2,2,2-tribromoethyl esters with Zn–Cu couple.



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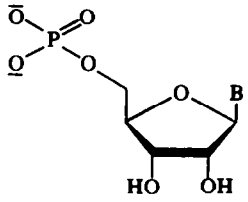
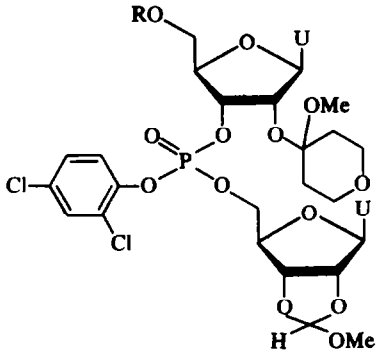
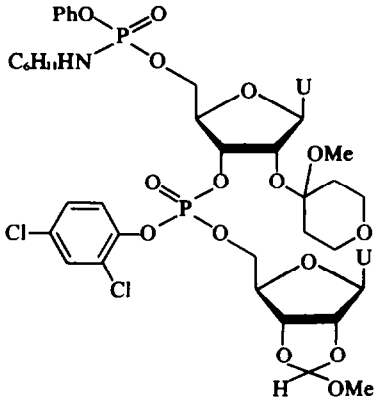


15

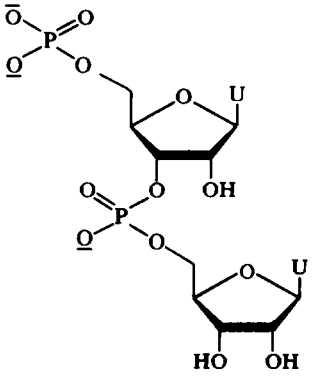
a: B = uracil-1-yl
b: B = cytosin-1-yl
c: B = adenin-9-yl



16a: R = p-ClC$_6$H$_4$OCH$_2$CO
b: R = H



17
U = uracil-1-yl



18

electrophoresis (Table 2) and was quantitatively digested to uridine and uridine 3',5'-diphosphate in the presence of pancreatic ribonuclease. Digestion of (**18**) in the presence of bacterial alkaline phosphatase gave uridylyl - (3'→5') - uridine (UpU).

While it is clear from the above experiment that phenyl phosphorocyclohexylamidochloridate (**7a**) is a useful reagent for the introduction of terminal phosphate residues, it suffers from one disadvantage: namely, that its use makes it necessary for the products to be submitted to acidic hydrolysis (pH 2) for 3 days at 20° in the final step of the synthesis. Such acidic conditions will promote cleavage and migration of the internucleotide linkages in oligoribonucleotides to a small extent[16] and depurination of oligodeoxyribonucleotides to a greater extent[17]. This disadvantage could be overcome by the introduction of a nitrosation step or by using 2,4-dichlorophenyl phosphoromorpholidochloridate (**8**), instead of (**7a**), as the phosphorylating agent. We should prefer to avoid the extra step and do not favour the use of (**8**) which leads to somewhat lower yields of products (Table 1) and necessitates a lengthy alkaline hydrolysis step. It seemed likely to us that the most desirable aryl phosphoramidochloridate (**6**) for the present purpose would be derived from a primary amine but from an amine with a pK_a closer to that of morpholine (8·7)[18] than to that of cyclohexylamine (10·64).[18] Preliminary studies with phenyl phosphoro - (2 - methoxyethylamido) - chloridate (**6**; Ar=Ph, R=MeOCH$_2$CH$_2$, R'=H) support this hypothesis and we now feel convinced that an aryl phosphoramidochloridate can be found which will fully meet all of the above criteria for a phosphorylating agent suitable for the introduction of terminal phosphate residues in the triester approach to oligonucleotide synthesis.

Table 2. TLC and paper electrophoretic data

Compound	R_f[a]	Mobility[b]
U	0·74	0·05
Up	0·37	1·00
pUp	0·10	1·74
UpU	0·57	0·64
pUpU	0·18	1·54

[a]System C [M-aqueous NH$_4$OAc-EtOH (4:6, v/v)] on Merck DC-Alufolien Cellulose F$_{254}$.

[b]Relative to uridine 3'-phosphate (Up) in buffer D [0·05 M-aqueous sodium citrate, pH 3·5] on S+S No. 2403 paper.

EXPERIMENTAL

UV absorption spectra were measured with a Cary C15 recording spectrophotometer. NMR spectra were measured at 100 MHz with a Jeol JNM PS 100 spectrometer. Me$_4$Si was used as internal standard and chemical shifts are expressed in ppm on a δ scale. TLC plates, coated with Merck Kieselgel GF$_{254}$ and Merck DC-Alufolien Cellulose F$_{254}$ were developed in the following solvent systems: A[CHCl$_3$–MeOH (92:8, v/v)] B[M-aqueous NH$_4$OAc-EtOH (3:7, v/v)] and C [M-aqueous NH$_4$OAc-EtOH (4:6, v/v)]. Paper electrophoresis on S+S No. 2403 paper was carried out in a Camag flat-plate apparatus in buffer system D [0·05 M-aqueous sodium citrate, pH 3·5].

Merck Kieselgel H was used for adsorption chromatography. Acetonitrile was stirred with CaH$_2$ at room temperature for 48 hr and then distilled from a small quantity of P$_2$O$_5$. 5 - Chloro - 1 - ethyl - 2 - methylimidazole and 1,2-dimethylimidazole (purchased

from the Aldrich Chemical Co.) were stored over No. 4A molecular sieves. Pyridine was heated with CaH_2, under reflux, for 24 hr and then distilled; it was stored in wax-sealed bottles over No. 4A molecular sieves.

Phenyl phosphorocyclohexylamidochloridate (**7a**). A soln of cyclohexylamine (4·95 g, 0·05 mole) in anhyd ether (75 ml) was added in one portion to a stirred, cooled (ice-water) soln of phenyl phosphorodichloridate[14] (5·25 g, 0·025 mole) in anhyd ether (75 ml). After 1 hr, the reactants were allowed to warm up to room temp, filtered (sintered glass funnel) and concentrated. Crystallization of the residue from hot benzene (2 ml) and n-pentane (15 ml) gave *phenyl phosphorocyclohexylamidochloridate* [Found: C, 51·9; H, 6·55; P, 11·2. $C_{12}H_{17}ClNO_2P$ requires: C, 52·75; H, 5·9; P, 11·4%] as colourless crystals, m.p. 94°; yield 5·5 g (80%).

o-Chlorophenyl phosphorocyclohexylamidochloridate (**7b**). Reaction between o-chlorophenyl phosphorodichloridate[14] and two molecular equivs of cyclohexylamine, under the above conditions, and crystallization of the product from benzene-pentane gave *o-chlorophenyl phosphorocyclohexylamidochloridate* [Found: C, 46·55; H, 5·8; P, 9·5. $C_{12}H_{16}Cl_2NO_2P$ requires: C, 46·75; H, 5·2; P, 10·1%], m.p. 115°; yield, 82%.

2,4-Dichlorophenyl phosphorocyclohexylamidochloridate (**7c**). Reaction between 2,4-dichlorophenyl phosphorodichloridate[14] and two molecular equivs of cyclohexylamine, under the above conditions, and crystallization of the product from benzene-pentane gave *2,4-dichlorophenyl phosphorocyclohexylamidochloridate* [Found: C, 42·2; H, 4·5; P, 9·2. $C_{12}H_{15}Cl_3NO_2P$ requires: C, 42·2; H, 4·1; P, 9·1%], m.p. 118°; yield, 80%.

2,4-Dichlorophenyl phosphoromorpholidochloridate (**8**). Reaction between 2,4-dichlorophenyl phosphorodichloridate and two molecular equivs of morpholine, under the above conditions and crystallization of the product from EtOH (−20°) gave *2,4-dichlorophenyl phosphoromorpholidochloridate* [Found: C, 36·0; H, 3·5; P, 9·3. $C_{10}H_{11}Cl_3NO_3P$ requires: C, 36·5; H, 3·3; P, 9·4%] m.p. 46°; yield, 65%.

2',3' - O - Methoxymethyleneuridine - 5' - phenyl phosphorocyclohexylamidate (**10a**; Ar=Ph, R=C_6H_{11}, R'=H)

(a) Phenyl phosphorocyclohexylamidochloridate (0·273 g, 1·0 mmole) was added to a stirred soln of 2',3'-O-methoxymethyleneuridine[7] (0·143 g, 0·5 mmol) and 5 - chloro - 1 - ethyl - 2 - methylimidazole[1c,19] (0·3 ml, 2·0 mmole) in dry acetonitrile (2 ml) at 20°. After 24 hr, the colourless soln was concentrated under reduced pressure, the residual oil dissolved in chloroform (30 ml) and the soln extracted with 10% $NaHCO_3$aq (10 ml) and water (2 × 10 ml). The dried ($MgSO_4$) organic layer was filtered and concentrated to an oil. A soln of the latter material in chloroform (3 ml) was added dropwise with stirring to dry n-pentane (150 ml). The ppt thus obtained was collected by filtration, dissolved in chloroform-methanol (97:3, v/v; 3 ml) and the soln applied to a column (20 cm × 2 cm^2) of Kieselgel H (16 g) suspended in the same solvent mixture. Elution of the column with chloroform-methanol (97:3) and evaporation of the appropriate fractions gave *2',3' - O - methoxymethylene - 5' - phenyl phosphorocyclohexylamidate* as a colourless glass, R_f 0·50 (system A); yield 0·23 g (88%).

(b) Phenyl phosphorocyclohexylamidochloridate (0·273 g, 1·0 mmole), 2',3' - O - methoxymethyleneuridine (0·143 g, 0·5 mmole) and 1,2-dimethylimidazole (0·2 ml, 2·0 mmole) were allowed to react together in dry acetonitrile (2 ml) at 20°. After 24 hr, the products were worked-up as in (a) above to give, following n-pentane precipitation and chromatography, the same product as a colourless glass (0·215 g, 87%).

(c) Phenyl phosphorocyclohexylamidochloridate (0·273 g, 1·0 mmole) and 2',3' - O - methoxymethyleneuridine (0·143 g, 0·5 mmole) were allowed to react together in anhyd pyridine (2 ml) soln at 20°. After 24 hr, the brown-coloured products were worked-up as in (a) above (except that the step involving n-pentane precipitation was omitted) to give, after chromatography, the same product (0·23 g, 88%) as a yellow-coloured glass.

2',3' - O - Methoxymethyleneuridine - 5' - o - chlorophenyl phosphorocyclohexylamidate (**10a**; Ar=2-ClC$_6$H$_4$, R=C_6H_{11}, R'=H). This compound was prepared from 2',3' - O - methoxymethyleneuridine, o - chlorophenyl phosphorocyclohexylamidochloridate and 5 - chloro - 1 - ethyl - 2 - methylimidazole in acetonitrile soln under the conditions described above; it was isolated as a colourless glass, R_f 0·52 (system A); yield, 87%.

2',3' - O - Methoxymethyleneuridine - 5' 2,4 - dichlorophenyl phosphorocyclohexylamidate (**10a**; Ar=2,4-$Cl_2C_6H_3$, R=C_6H_{11}, R'=H). This compound was prepared from 2',3' - O - methoxymethyleneuridine, 2,4 - dichlorophenyl phosphorocyclohexylamidochloroidate and 5 - chloro - 1 - ethyl - 2 - methylimidazole in acetonitrile soln under the conditions described above; it was isolated as a colourless glass, R_f 0·52 (system A); yield, 88%.

2',3' - O - Methoxymethyleneuridine - 5' 2,4 - dichlorophenyl phosphoromorpholidate (**10a**; Ar=2,4-$Cl_2C_6H_3$, RR'=$(CH_2)_2$-$O(CH_2)_2$). This compound was prepared from 2',3' - O - methoxymethyleneuridine, 2,4 - dichlorophenyl phosphoromorpholidochloridate and 5 - chloro - 1 - ethyl - 2 - methylimidazole in acetonitrile solution under the conditions described above; it was isolated as a colourless glass, R_f 0·52 (system A); yield, 80%.

4 - N - p - Anisoyl - 2',3' - O - methoxymethylenecytidine - 5' 2,4 - dichlorophenyl phosphorocyclohexylamidate (**10b**; Ar=2,4-$Cl_2C_6H_3$, R = C_6H_{11}, R' = H). This compound was prepared from 4 - N - *p* - anisoyl - 2',3' - O - methoxymethylenecytidine, 2,4 - dichlorophenyl phosphorocyclohexylamidochloridate and 5 - chloro - 1 - ethyl - 2 - methylimidazole in acetonitrile solution under the conditions described above; it was isolated as a colourless glass, R_f 0·63 (system A); yield, 90%.

6 - N - p - Anisoyl - 2',3' - O - methoxymethyleneadenosine - 5' 2, 4 - dichlorophenyl phosphorocyclohexylamidate (**10c**; Ar=2,4-$Cl_2C_6H_3$, R = C_6H_{11}, R' = H). This compound was prepared from 6 - N - *p* - anisoyl - 2',3' - O - methoxymethyleneadenosine,[7] 2,4 - dichlorophenyl phosphorocyclohexylamidochloridate and 5 - chloro - 1 - ethyl - 2 - methylimidazole in acetonitrile soln under the conditions described above; it was isolated as a colourless glass, R_f 0·63 (system A); yield, 88%.

2' - O - Methoxytetrahydropyranyluridine - 5' phenyl phosphorocyclohexylamidate (**13**; Ar=Ph, R=C_6H_{11}, R'=H). Phenyl phosphorocyclohexylamidochloridate (1·64 g, 6·0 mmole) was added to a stirred soln of 2' - O - methoxytetrahydropyranyluridine[8] (1·8 g, 5·0 mmole) and 5 - chloro - 1 ethyl - 2 - methylimidazole (2·0 ml, 14 mmole) in dry acetonitrile (15 ml) at 20°. After 24 hr, when TLC (system A) indicated that the reaction was complete, the products were concentrated under reduced pressure to give an oil which was redissolved in chloroform. The resultant soln was washed with 10% $NaHCO_3$aq (25 ml) and water (4 × 20 ml), then dried ($MgSO_4$) and concentrated to an oil. A soln of the latter material in chloroform (6 ml) was added dropwise with stirring to dry light petroleum (b.p. 40–60°, 200 ml). The ppt obtained was collected by filtration, dried (P_2O_5) *in vacuo* and crystallised from MeOH (−20°) to give *2'-O-methoxytetrahydropyranyluridine - 5' phenyl phosphorocyclohexylamidate* [Found: C, 54·3; H, 6·5; P, 5·4. $C_{27}H_{38}N_3O_{10}P$ requires: C, 54·6; H, 6·4; P, 5·2%] as colourless crystals, m.p. 105°; yield 1·78 g (60%): λ_{max} (95% ethanol) 261 nm (ϵ 11,000); δ($CDCl_3$) includes the following signals: 7·48 (1H, d, J = 8 Hz), 7·24 (5H, m), 6·08 (1H, d, J = 6 Hz), 5·60 (1H, d, J = 8 Hz), 3·09 (3H, s); R_f 0·45 (system A).

2' - O - Methoxytetrahydropyranyluridine - 5' 2,4 - dichlorophenyl phosphoromorpholidate (**13**; Ar=2,4-$Cl_2C_6H_3$, RR' = $(CH_2)_2O(CH_2)_2$). 2,4-Dichlorophenyl phosphoromorpholidochloridate (2·0 g, 6·0 mmole) was added to a stirred soln of 2' - O - methoxytetrahydropyranyluridine (0·71 g, 2·0 mmole) and 5 - chloro - 1 - ethyl - 2 - methylimidazole (0·8 ml, 6·0 mmole) in acetonitrile (7 ml) at 20°. After 30 hr, the products were worked-up in the manner described above in the corresponding reaction with phenyl cyclohexylaminophosphorochloridate and crystallized from MeOH (−20°) to give *2' - O - methoxytetrahydropyranyluridine - 5' 2,4 - dichlorophenyl phosphoromorpholidate* [Found: C, 46·0; H, 5·2; N, 6·5; P, 4·9. $C_{25}H_{32}Cl_2N_3O_{11}P$ requires: C, 46·0; H, 4·9; N, 6·7; P, 4·75%] as colourless crystals, m.p. 105°; yield, 0·65 g (50%); λ_{max} (95% EtOH) 259 nm (ϵ 11,100); δ($CDCl_3$) includes the following signals: 7·32 (4H, m), 5·94 (1H, d, J = 6 Hz), 5·56 (1H, d, J = 8 Hz), 3·08 (3H, s); R_f 0·45 (system A).

2' - O - Methoxytetrahydropyranyluridylyl - (3' → 5') - 2',3' - O - methoxymethyleneuridine 2,4 - dichlorophenyl ester (**16b**). 5' - O - *p* - Chlorophenoxyacetyl - 2' - O - methoxytetrahyd-

ropyranyluridine[13] (1·05 g, 2·0 mmole), 2,4 - dichlorophenyl dihydrogen phosphate[14] (0·51 g, 2·1 mmole), 2,4,6 - tri - isopropylbenzenesulphonyl chloride (1·51 g, 5·0 mmole) and anhyd pyridine (10 ml) were stirred together, with the exclusion of moisture, at 20°. After 6 hr, 2',3' - O - methoxymethyleneuridine (0·63 g, 2·2 mmole) was added and, after a further period of 15 hr, the products were concentrated under reduced pressure. A soln of the residual oil in chloroform (60 ml) was washed with 10% $NaHCO_3$aq (30 ml) and water (3 × 30 ml). The dried ($MgSO_4$) organic layer was filtered and concentrated to a glass which was redissolved in chloroform-methanol (95:5, v/v; 6 ml). This solution was applied to a column (20 cm × 4 cm²) of Kieselgel H (100 g) suspended in the same solvent. Elution of the column with chloroform-methanol (95:5, v/v) and evaporation of the appropriate fractions gave a glass (1·57 g).

A soln of the latter material in anhyd MeOH (80 ml) and dioxan (20 ml) was treated with 0·1 M-methanolic K_2CO_3 at 20°. After 3 min, 0·2 M-aqueous sodium dihydrogen phosphate (15 ml) was added and the products concentrated to *ca.* 15 ml under reduced pressure. The residual mixture was shaken with chloroform (50 ml) and 5% $NaHCO_3$aq (20 ml). The organic layer was dried ($MgSO_4$), filtered and the filtrate concentrated under reduced pressure. A soln of the residue in chloroform (8 ml) was added dropwise with stirring to anhydrous n-pentane (200 ml). The precipitated *2,4-dichlorophenyl ester of* 2' - O - *methoxytetrahydropyranyluridylyl* - (3'→5') - 2',3' - O - *methoxymethyleneuridine* was collected by filtration and dried *in vacuo* (KOH) at 20°; yield 1·19 g (70%); R_f 0·19, 0·24 (system A).

Reaction between 2' - O - *methoxytetrahydropyranyluridylyl* - (3'→5') - 2',3' - O - *methoxymethyleneuridine* 2,4 - *dichlorophenyl ester* (**16b**) *and phenyl phosphorocyclohexylamidochloridate.* Phenyl phosphorocyclohexylamidochloridate (0·232 g, 0·85 mmole) was added to a stirred soln of the above partially-protected **16b** (0·405 g, 0·5 mmole) and 5 - chloro - 1 - ethyl - 2 - methylimidazole (0·23 ml, 1·6 mmole) in anhyd acetonitrile (1·5 ml). After 24 hr, the products were concentrated under reduced pressure to give an oil which was dissolved in chloroform (30 ml) and the soln extracted with 10% $NaHCO_3$aq (10 ml) and water (2 × 15 ml). The dried ($MgSO_4$) organic layer was concentrated under reduced pressure. A soln of the glass obtained, in chloroform-methanol (97:3, v/v; 2·5 ml) was applied to a column of Kieselgel H (16 g) suspended in the same solvent. Elution of the column with chloroform-methanol and evaporation of the appropriate fractions gave a glass (0·50 g). A soln of this material in chloroform (2·5 ml) was added dropwise with stirring to anhydrous n-pentane (100 ml) and the precipitated *5'-phenyl phosphorocyclohexylamidate of* 2' - O - *methoxytetrahydropyranyluridylyl* - (3'→5') - 2',3' - O - *methoxymethyleneuridine* 2,4 - *dichlorophenyl ester* (**17**) collected by filtration and dried *in vacuo* over KOH; yield 0·462 g (85%); R_F 0·47 (system A).

Alkaline hydrolysis of 2',3' - O - *methoxymethyleneuridine 5'-aryl phosphoramidate derivatives.* A soln of the substrate (0·05 mmole) in dioxan (5 ml) was added rapidly to 0·125 M NaOH (20 ml) at 20° with thorough mixing. Some of the reaction soln was transferred immediately to a 1 cm cuvette and the change in absorbance (A) at λ_{max} (for the arylate ion) with time was measured with a spectrophotometer. Straight lines were obtained by plotting log ($A_\infty - A_t$) against time. The half-times ($t_{1/2}$) required for the hydrolysis of the phenyl, o-chlorophenyl and 2,4-dichlorophenyl phosphorocyclohexylamidates and for the 2,4-dichlorophenyl phosphoromorpholidate were 60, 5·8, 2·9 and 516 min, respectively.

Acidic hydrolysis of 2',3' - O - *methoxymethyleneuridine 5'-phosphoramidates.* The substrate (0·05 mmole) was dissolved in 0·01 M HCl (10 ml) at 20° and the pH lowered to 2·0 (pH meter) by the addition of 0·1 M HCl. After suitable intervals of time, portions of the reaction soln were neutralized (pH 7–8) and analyzed by TLC (system B) on MN-celluose F_{254} plates. The half-times ($t_{1/2}$) for the conversion of the 5' - phosphorocyclohexylamidate and the 5' - phosphoromorpholidate to 5'-phosphate were found to be 516 and 10 min, respectively.

Uridine 5'-phosphate

(a) To a stirred soln of 2',3' - O - methoxymethyleneuridine-5' phenyl phosphorocyclohexylamidate (0·46 g, 1 mmole) in dioxan (20 ml) at 20° was added 0·125 M NaOH (80 ml). After 8 hr, the products were applied to a column of Dowex 50 W cation-exchange resin (NH_4^+ form, 10 cm × 2 cm²) and the column then eluted with water. The eluate (60 ml) was evaporated under reduced pressure, the concentrated soln (*ca.* 20 ml) extracted with ether (3 × 10 ml) and then lyophilized. The fluffy solid obtained, which was homogeneous on TLC [R_f 0·74 (system B)] was dissolved in 0·01 M HCl (100 ml) and the pH lowered to 2·0 (pH meter) by the addition of 0·1 M HCl. After 3 days at 20°, the soln was neutralized with aqueous ammonia and then lyophilized. The residual solid was redissolved in water, treated with Dowex 50 W cation-exchange resin (NH_4^+ form, 2g) and then re-lyophilized. The solid obtained (0·35 g, 95%) was found to be homogeneous by TLC (system B) and paper electrophoresis (buffer D); it had, respectively, the same R_f and mobility as authentic uridine 5'-phosphate; its NMR spectrum was identical to that of diammonium uridine 5'-phosphate.

(b) To a stirred soln of 2',3' - O - methoxymethyleneuridine - 5' 2,4 - dichlorophenyl phosphoromopholidate (0·23 g, 0·4 mmole) in dioxan (5 ml) at 20° was added 0·125 M NaOH (20 ml). After 72 hr, the products were worked-up as above, subjected to acidic hydrolysis (pH 2, 20°, 2 Hr) and the pure diammonium salt of uridine 5'-phosphate isolated, in high yield, as the sole nucleotide product.

(c) 2' - O - Methoxytetrahydropyranyluridine - 5' - phenyl phosphorocyclohexylamidate (0·238 g, 0·4 mmole) was converted, in high yield, into the pure diammonium salt of uridine 5'-phosphate under the conditions described in paragraph (a) above.

(d) 2' - O - Methoxytetrahydropyranyluridine - 5' 2,4 - dichlorophenyl phosphoromorpholidate (0·196 g, 0·3 mmole) was converted, in high yield, into the pure diammonium salt of uridine 5'-phosphate under the conditions described in paragraph (b) above.

Cytidine 5'-phosphate. Aqueous ammonia (25%, w/w; 80 ml) was added portionwise to a stirred soln of 4 - N - *p* - anisoyl - 2',3' - O - methoxymethylenecytidine - 5' 2,4 - dichlorophenyl phosphorocyclohexylamidate (0·67 g, 1·0 mmol) in dioxan (20 ml) at 20°. After 24 hr, the products were concentrated under reduced pressure to *ca.* 30 ml, extracted with chloroform (5 × 10 ml) and acidified to pH 2 (pH meter) with 0·1 M HCl. After 3 days, the products were neutralized with aqueous ammonia and worked-up as described above in preparation (a) of uridine 5'-phosphate to give the diammonium salt of cytidine 5'-phosphate (0·33 g, 90%) as a colourless solid. The latter material was found to be homogeneous by TLC (system B) and paper electrophoresis (buffer D); it had, respectively, the same R_f and mobility as authentic cytidine 5'-phoaphate; its UV absorption spectrum was identical to that of cytidine 5'-phosphate.

Adenosine 5'-phosphate. 6 - N - *p* - Anisoyl - 2',3' - O - methoxymethyleneadenosine - 5' 2,4 - dichlorophenyl phosphorocyclohexylamidate (0·72 g, 1·0 mmole) was converted into diammonium adenosine 5'-phosphate (0·33 g, 90%) by the procedure described above in the preparation of cytidine 5'-phosphate. The product was found to be homogeneous by TLC (system B) and paper electrophoresis (buffer D); it had, respectively, the same R_f and mobility as authentic adenosine 5'-phosphate; its UV absorption spectrum was identical to that of adenosine 5'-phosphate.

Uridylyl - (5'→3') - *uridine 5'-phosphate* (pUpU). To a stirred soln of the 5'-phenyl phosphorocyclohexylamidate of 2' - O - methoxytetrahydropyranyluridylyl - (3'→5') - 2',3' - O - methoxy- in dioxan (8 ml) at 20° was added 0·125 M NaOH (32 ml). After 15 hr, the products were worked-up according to the procedure described above in preparation (a) of uridine 5'-phosphate and then lyophilized to give the ammonium salt of the protected dinucleoside phosphate phosphorocyclohexylamidate as a TLC homogeneous [R_f 0·78 (system C)] fluffy solid (0·35 g).

The latter material (0·27 g) was dissolved in 0·01 M HCl (50 ml) at 20° and the pH lowered to 2 (pH meter) by the addition of 0·1 M HCl. After 3 days, the soln was neutralized (pH 8) with aqueous ammonia and lyophilized. The solid obtained was dissolved in water (8 ml), the soln treated with Dowex 50 W cation-exchange resin (NH_4^+ form, 3 g) and filtered. Lyophilization of the filtrate

gave ammonium uridylyl - (5→3') - uridine 5' - phosphate as a colourless fluffy solid in virtually quantitative yield (estimated spectrophotometrically at 260 nm, assuming $\epsilon = 20{,}000$); the product was found to be homogeneous by TLC (system C) and paper electrophoresis (buffer D).

The product was characterized by NMR spectroscopy (which revealed two distinct H(6) doublets) and by enzymatic digestion with (a) bacterial alkaline phosphatase and (b) pancreatic ribonuclease: (a) The substrate (1·0 mg) was dissolved in 0·05 M-tris hydrochloride buffer (pH 7·5, 0·1 ml) and 0·1% alkaline phosphatase soln (Worthington, 0·1 ml) was added. After the resultant solution had been incubated at 37° for 2 hr, TLC (system C) and paper electrophoresis (buffer D) revealed uridylyl - (3'→5') - uridine and no starting material. (b) A soln of the substrate (1·0 mg) and pancreatic ribonuclease (0·1 mg) in 0·05 M-tris hydrochloride buffer (pH 7·5, 0·1 ml) was incubated at 37°. After 2 hr, TLC (system C) and paper electrophoresis (buffer D) revealed that the substrate had undergone quantitative digestion to uridine 3',5'-diphosphate and uridine.

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