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SYNTHESIS OF THE 3'-TERMINAL DECARIBONUCLEOSIDE NONAPHOSPHATE OF YEAST ALANINE TRANSFER RIBONUCLEIC ACID

S. S. JONES, B. RAYNER, C. B. REESE*, A. UBASAWA and M. UBASAWA

Department of Chemistry, King's College, Strand,
London WC2R 2LS, England

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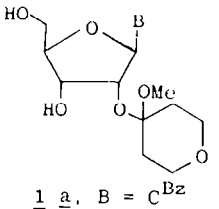
Abstract The preparation, by the phosphotriester approach, of UpCpGpUpCpCpApCpCpA (**23**) from three protected trinucleotide blocks (**19a-c**) is described. The use of the *o*-dibromomethylbenzoyl (DBMB) protecting group in oligoribonucleotide synthesis is described for the first time. Internucleotide linkages are protected by *o*-chlorophenyl groups which are finally removed by treatment with the N^1, N^1, N^3, N^3-tetramethylguanidinium salt of *syn*-4-nitrobenzaldoxime. The first phosphorylation step (leading to phosphodiester intermediates) is carried out by treatment with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 2-ClC_6H_4) and then with water and triethylamine. 1-Mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT, **14**) is used as the activating agent in the second phosphorylation step.

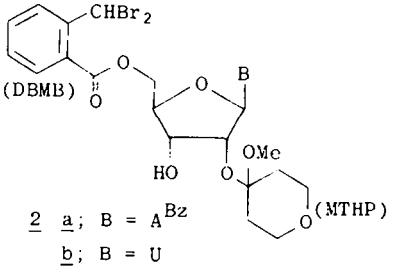
INTRODUCTION AND RESULTS

It now seems clear that the phosphotriester approach[1] to oligonucleotide synthesis, in which the internucleotide linkages remain protected during the steps concerned with the assembly of the desired nucleotide sequence, is to be preferred to the alternative phosphodiester approach. A substantial amount of evidence in support of this conclusion has been obtained by a number of groups of workers engaged in the synthesis of oligodeoxyribonucleotides.[2-6] We believe that this conclusion is, if anything, even more justified in the less thoroughly investigated area of oligoribonucleotide synthesis.[1,7] Indeed, a number of years' ago, we found[8] that even tri-ribonucleoside diphosplates could not be prepared satisfactorily by the phosphodiester approach.

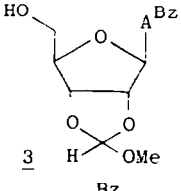
The problems which we encountered[8] in the synthesis of oligoribonucleotides first led us to investigate the possibility of adopting the phosphotriester approach. In the course of our original studies,[9] which were carried out in the deoxy-series, we introduced the use of the phenyl group for the protection of the internucleotide linkages and shortly afterwards showed[10,11] that substituted phenyl (including *o*-chloro-, *o*-fluoro- and *p*-chloro-phenyl) groups could also be used. We were dissatisfied with all of these protecting groups until we recently found[12] that the conjugate base of *syn*-4-nitrobenzaldoxime or of *syn*-pyridine-2-carboxaldoxime could be used to remove aryl (particularly *o*-chlorophenyl) protecting groups from the internucleotide linkages without concomitant cleavage occurring to a singificant extent. We now feel able to recommend without reservation the use of the *o*-chlorophenyl protecting group (and other aryl groups derived from phenols with pK_a's of *ca* 8.5) in the phosphotriester approach to oligonucleotide synthesis. Furthermore, following the recent development of the *o*-dibromomethylbenzoyl protecting group[13] and of an improved phosphorylation procedure[14] (see below), we believe that the block synthesis of oligoribo- and oligodeoxyribo-nucleotides of high quality is now possible. In this paper, we describe the synthesis of UpCpGpUpCpCpApCpCpA, the 3'-terminal decaribonucleoside nonaphosphate of yeast $tRNA^{Ala}$.



1 a, B = C^{Bz}
b; B = G^{Bz}
c; B = U



2 a; B = A^{Bz}
b; B = U



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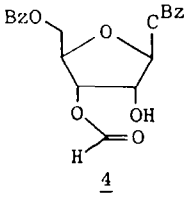
A^{Bz} = 6-*N*-benzoyladenin-9-yl; C^{Bz} = 4-*N*-benzoylcytosin-1-yl;
G^{Bz} = 2-*N*-benzoylguanin-9-yl; U = uracil-1-yl



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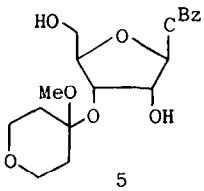

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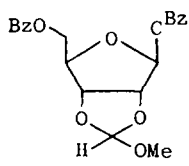

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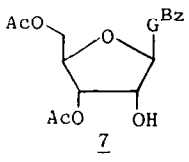

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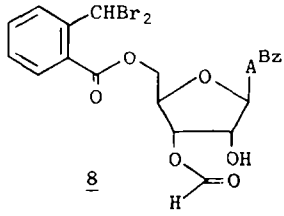

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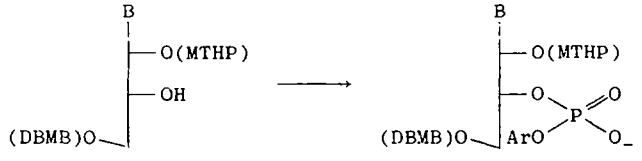
The required decamer was synthesized from five crystalline nucleoside building blocks: 2′-O-methoxytetrahydropyranyl-4-N-benzoylcytidine (**1a**), 2′-O-methoxytetrahydropyranyl-2-N-benzoylguanosine (**1b**), 5′-O-(*o*-dibromomethylbenzoyl)-2′-O-methoxytetrahydropyranyl-6-N-benzoyladenosine (**2a**), 5′-O-(*o*-dibromomethylbenzoyl)-2′-O-methoxytetrahydropyranyluridine (**2b**) and 2′,3′-O-methoxymethylene-6-N-benzoyladenosine (**3**). Building block **1a** was prepared (Experimental) from 5′-O-,4-N-dibenzoyl-3′-O-formylcytidine (**4**). The latter compound (**4**) was obtained together with the isomeric 2′-formate ester [which was converted into 3′-O-methoxytetrahydropyranyl-4-N-benzoylcytidine (**5**)] when 5′-O-4-N-dibenzoyl-2′,3′-O-methoxymethylenecytidine (**6**) was treated with formic acid. We have subsequently found that **1a** may be prepared more conveniently by Markiewicz's procedure.[15] However, building block **1b** was readily prepared in 62% yield from 3′,5′-di-O-acetyl-2-N-benzoylguanosine (**7**).[16] Building block **2a** was prepared (77% yield) from 5′-O-(*o*-dibromomethylbenzoyl-3′-O-formyl-6-N-benzoyladenosine (**8**), which was itself prepared (Experimental) from **3** (see below) and isolated as a crystalline compound in 68% yield. Building block **2b** was prepared from **1c**[17] by treating it directly with *o*-dibromomethylbenzoyl chloride[13] and was isolated as a crystalline compound in 71% yield. Finally, the 3′-terminal building block (**3**) was prepared from 6-N-benzoyladenosine and obtained in 74% yield.

Two separate phosphorylation steps are required in the phosphotriester approach. The first step involves the conversion of a protected nucleoside or oligonucleotide into its 3′-aryl phosphate or an activated form of the latter. In our opinion, it is preferable to isolate the 3′-aryl phosphate itself and thereby ensure that no symmetrical products are obtained and that the desired stoicheiometry is maintained for both phosphorylation steps. The first phosphorylation step is illustrated in Scheme 1 for a 2′,5′-protected ribonucleoside building block **9** (e.g. **2a** or **2b**).*

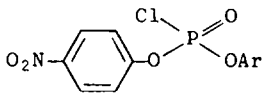
In a search for a monofunctional phosphorylating agent suitable for the first phosphorylation step, we found[18] that protected ribonucleoside and 2′-deoxyribonucleoside building blocks with free hydroxy functions reacted readily with *p*-nitrophenyl phenyl or *o*-chlorophenyl *p*-nitrophenyl phosphorochloridate (**11a** or **11b**) to give the corresponding nucleoside diaryl phosphates which, on subsequent treatment with *p*-thiocresol (**12**) and triethylamine in acetonitrile solution at room temperature, gave the triethylammonium salts of the desired monoaryl phosphates in high yields. While this procedure is very convenient for the preparation of mononucleotide units[18] and has recently been used most successfully[19] in the synthesis of the powerful protein synthesis inhibitor 2-5A, it is unfortunately not suitable for the phosphorylation of protected dinucleoside phosphates or larger oligonucleotide blocks.[20]



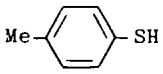
9 10 a; Ar = 2-ClC$_6$H$_4$

Scheme 1.

*In formula **9** and subsequent formulae, *o*-dibromomethylbenzoyl and methoxytetrahydropyranyl are abbreviated (see formula **2**) to DBMB and MTHP, respectively.



11 a; Ar = Ph
b; Ar = 2-ClC$_6$H$_4$



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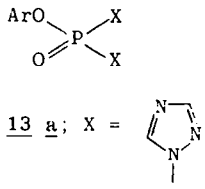
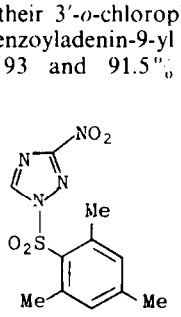


13 a; X =

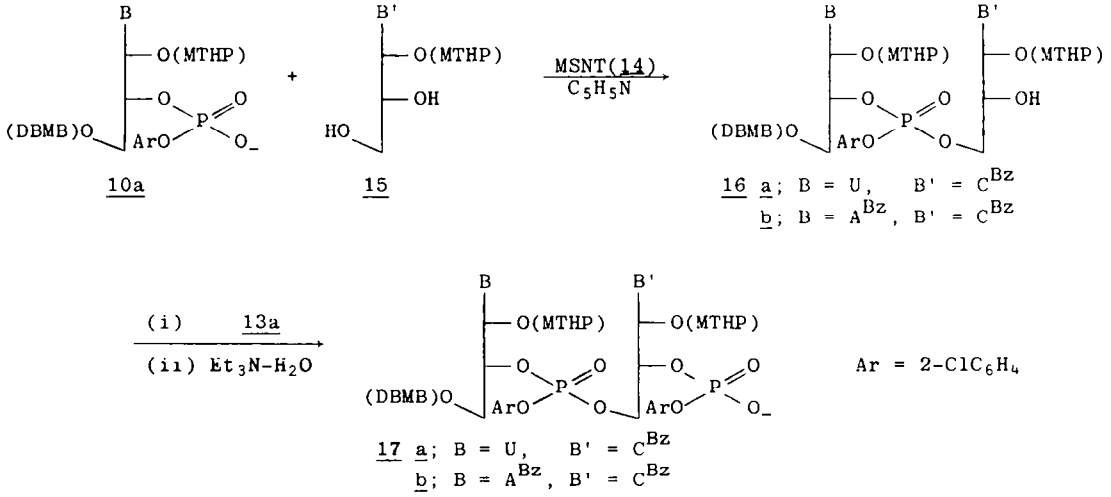
Fortunately, it was possible to find an alternative phosphorylation procedure which could be used both in the stepwise and in the block synthesis of oligonucleotides. *p*-Chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 4-ClC$_6$H$_4$) has been used[21] as a bifunctional phosphorylating agent in the phosphotriester approach. However, if, for example, 2',5'-protected ribonucleoside derivatives (such as **9**) are treated with 2.5-3 molecular equivlents of *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 2-ClC$_6$H$_4$)[14] [prepared from **13** (X = Cl, Ar = 2-ClC$_6$H$_4$), 1,2,4-triazole and triethylamine] in acetonitrile-pyridine solution and the products are then subjected to hydrolysis, the desired 3'-*o*-chlorophenyl phosphates (**10a**) are obtained in very high yields. The latter (**10a**) may readily be obtained free from *o*-chlorophenyl phosphate by chloroform extraction and then isolated as their pure triethylammonium salts by precipitation. Thus an apparently bifunctional phosphorylating agent (**13a**; Ar = 2-ClC$_6$H$_4$) can behave[14] as though it were monofunctional. In this way, the adenosine and uridine building blocks (**2a** and **2b**, respectively) were converted into the triethylammonium salts of their 3'-*o*-chlorophenyl phosphates (**10a**; B = 6-N-benzoyladenin-9-yl and uracil-1-yl, respectively) in 93 and 91.5% isolated yields, respectively.



14 (MSNT)

The second phosphorylation step in the phosphotriester approach involves the reaction between a phosphodiester intermediate (such as **10a**) and the 5'-OH function of a nucleoside or oligonucleotide component in the presence of an activating agent. 2,4,6-Triisopropylbenzenesulphonyl chloride[22] may be used as the activating agent in the second phosphorylation step but condensation reactions then tend to be rather slow. Furthermore, sulphonation appears to compete with phosphorylation and darkening of the reaction medium occurs sometimes. In 1973, Russian workers reported[23] that sulphonation and darkening did not occur when 1-arenesulphonylimidazole derivatives were used as activating agents but that condensation reactions were then very slow indeed. The same advantages (i.e. no sulphonation and darkening) appear to obtain when arenesulphonyl derivatives of tetrazole[24] and 3-nitro-1,2,4-triazole[12,25] are used but the condensation reactions are then comparatively fast. At the present time, we especially favour the use of the mesitylenesulphonyl derivative of 3-nitro-1,2,4-triazole (MSNT, **14**)[12,25] as the activating agent in the second phosphorylation step (Scheme 2). 3-Nitro-1,2,4-triazole may conveniently be prepared in good yield (Experimental) from commercially available 3-amino-1,2,4-triazole; it is readily converted[25] into its crystalline toluene-*p*-sulphonyl, mesitylenesulphonyl (MSNT, **14**) and 2,4,6-tri-isopropylbenzenesulphonyl derivatives by reaction with the appropriate arenesulphonyl chlorides in the presence of triethylamine. MSNT (**14**) and the other arenesulphonyl derivatives of 3-nitro-1,2,4-triazole are stable crystalline solids.[25]

When a solution of the triethylammonium salt of the uridine-derived mononucleotide building block (**10a**; B = uracil-1-yl) and a slight deficiency of 2'-O-methoxytetrahydropyranyl-4-N-benzoylcytidine (**1a**

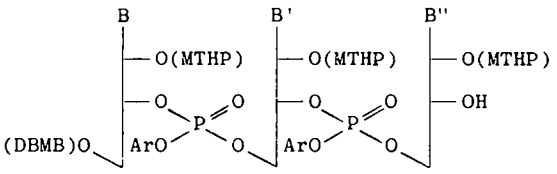


10a 15 16 a; B = U, B' = C^{Bz}
b; B = A^{Bz}, B' = C^{Bz}

17 a; B = U, B' = C^{Bz}
b; B = A^{Bz}, B' = C^{Bz}

Ar = 2-ClC$_6$H$_4$

Scheme 2.



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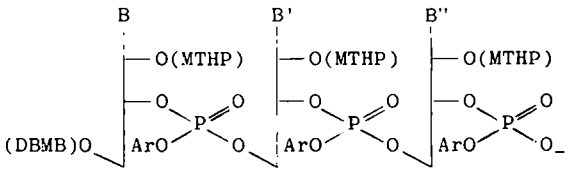

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a; B = U, B' = C^{Bz}, B'' = G^{Bz}
b; B = U, B' = C^{Bz}, B'' = C^{Bz}
c; B = A^{Bz}, B' = C^{Bz}, B'' = C^{Bz}

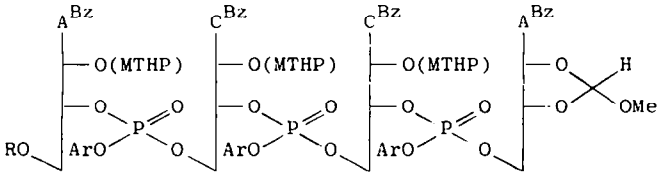
Ar = 2-ClC_6H_4

or **15**; B' = 4-N-benzoylcytosin-1-yl) was treated (Scheme 2) with *ca* 2.5 molecular equivalents of MSNT (**14**) in anhydrous pyridine solution at room temperature and the products worked-up after 20 min, the partially-protected dinucleoside phosphate (**16a**) was obtained and isolated, following short column chromatography of the products, in 79% yield. As far as could be ascertained, the latter material (**16a**) was free from the isomeric 3'→3'-dinucleoside phosphate. Thus phosphorylation of the dihydric alcohol (**15**; B' = 4-N-benzoylcytosin-1-yl) apparently occurred regiospecifically on its 5'-hydroxy function. The partially-protected ApC derivative (**16b**) was prepared in the same way and isolated in 77% yield. Both **16a** and **16b** were treated (Scheme 2) with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 2-ClC_6H_4) and the products worked-up as above to give the corresponding dinucleotides (**17a** and **17b**, respectively) as their triethylammonium salts in 98% isolated yields.

The dinucleotide derivative (**17a**) was then allowed to react in the same way with stoicheiometric quantities of each of the guanosine and cytidine building blocks (**15**; B' = 2-N-benzoylguanin-9-yl and 4-N-benzoylcytosin-1-yl, respectively) in the presence of a threefold excess of MSNT (**14**) in anhydrous pyridine solution at room temperature. After periods of 12 and 16 min, the products were worked-up to give **18a** and **18b** in 78 and 82% isolated yields, respectively. The dinucleotide derivative (**17b**) was similarly condensed with the cytidine building block (**15**; B' = 4-N-benzoylcytosin-1-yl) to give the trinucleoside diphosphate (**18c**) in 77% isolated yield. Each of the three trinucleotide diphosphates (**18a–c**) was then treated with an excess of *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 2-ClC_6H_4). In this way, the corresponding trinucleotide derivatives (**19a–c**) were obtained and isolated as their triethylammonium salts in 96, 96 and 98% yields, respectively.

The next stage of the synthesis involved the preparation of the partially-protected 3'-terminal tetranulceoside triphosphate. The trinucleotide derivative (**19c**) and *ca* 1.3 molecular equivalents of 2',3'-O-methoxymethylene-6-N-benzoyladenosine (**3**) were treated with an excess of MSNT (**14**) in anhydrous pyridine solution. After 30 min, the products were worked-up and any remaining 2',3'-O-methoxymethylene-6-N-benzoyladenosine (**3**) was removed after phosphorylation with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 2-ClC_6H_4). The fully-protected tetramer (**20a**) was then isolated in 75% yield following chromatography of the products. In order to remove the 5'-DBMB protecting group,[13] **20a** was stirred with *ca* 14 molecular equivalents of silver perchlorate (0.33 M) and *ca* 7 molecular equivalents of 2,4,6-collidine (added to prevent the reaction medium from becoming acidic) in acetone-water (98:2 v/v) for 1 hr at room temperature. After the removal of silver ions, the products, containing what was assumed[13] to be the intermediate (**20**; R = *o*-formylbenzoyl), were treated with *ca* 20 molecular equivalents of morpholine at room temperature for 5 min to give the partially-protected tetramer (**20b**). The latter substance (**20b**) was obtained in 94% isolated yield, thereby demonstrating the value of the DBMB protecting group in oligonucleotide synthesis.

The partially-protected tetranucleotide triphosphate (**20b**) and 1.2 molecular equivalents of the trinucleotide derivative (**19b**) were then treated (Scheme 3) with an excess (4.3 molecular equivalents with respect to **19b**) of MSNT (**14**) in anhydrous pyridine solution. After 30 min, the products were worked-up, treated with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 2-ClC_6H_4) and then fractionated by short column chromatography to give the fully-protected heptanucleoside hexaphosphate in 71.5% isolated yield. The 5'-DBMB protecting group was then removed by the procedure[13] described above



20 a; R = 2-$Br_2CHC_6H_4CO$
b; R = H

Ar = 2-ClC_6H_4

$$(DBMB)UpC^{Bz}pC^{Bz}p \quad \underline{19b} \quad + \quad (HO)A^{Bz}pC^{Bz}pC^{Bz}pA^{Bz}(MM) \quad \underline{20b} \quad \xrightarrow{(i),\ (ii)}$$

$$(HO)UpC^{Bz}pC^{Bz}pA^{Bz}pC^{Bz}pC^{Bz}pA^{Bz}(MM) \quad \underline{21}$$

p = $-O-\overset{\overset{O}{\|}}{\underset{\underset{OAr}{|}}{P}}-O-$; Ar = 2-$ClC_6H_4$; MM = 2',3'-O-methoxymethylene; A^{Bz}, C^{Bz} and G^{Bz} = 2'-O-MTHP-*N*-benzoyl protected adenosine, cytidine and guanosine residues, respectively.

Reagents: (i) MSNT(**14**)/C_5H_5N; (ii) (a) $AgClO_4$ - 2,4,6-collidine/Me_2CO-H_2O (98:2 v/v), (b) morpholine.

Scheme 3.

$$(DBMB)UpC^{Bz}pG^{Bz}p\psi pC^{Bz}pC^{Bz}pA^{Bz}pC^{Bz}pC^{Bz}pA^{Bz}(MM) \quad (\underline{22})$$

$$\downarrow \ (i),(ii),(iii)$$

$$UpCpGp\psi pCpCpApCpCpA \quad (\underline{23})$$

Reagents: (i) N^1,N^1,N^3,N^3-Tetramethylguanidinium *syn*-4-nitrobenzaldoximate in dioxan-water (1:1 v/v), 20°C, 18-20 hr; (ii) aqueous ammonia (*d* 0.88), 20°C, 6 days or 50°C, 24 hr; (iii) 0.01 *M*-hydrochloric acid (pH 2), 20°C, 6 hr.

Scheme 4.

to give the partially-protected heptamer (**21**) which was isolated in 78% yield. A solution of the latter substance (**21**) and a twofold excess of the trinucleotide derivative (**19a**) in anhydrous pyridine was then treated with an excess (fivefold with respect to **19a**) of MSNT (**14**). After 1 hr, the products were worked-up and chromatographed to give fully-protected decaribonucleoside nonaphosphate (**22**), of *ca* 80% purity, in an estimated yield of 55%. Some of this material was treated with **13a** (Ar = 2-ClC_6H_4) and the products were worked up and chromatographed to give pure decamer (**22**).

The fully-protected decaribonucleoside nonaphosphate (**22**) can be unblocked at its 5'-end and then extended to give a longer sequence of the yeast $tRNA^{Ala}$ molecule. Indeed, studies directed towards this end are now in progress in this Laboratory. However, small quantities (3.0 and 1.8 μmol) of **22** were completely unblocked by the three step procedure† indicated in Scheme 4 and then chromatographed on DEAE-Sephadex A25. It can be seen from Fig. 1 that one single component (eluted with *ca* 1.0 M-triethylammonium bicarbonate) accounted for nearly all of the nucleotide material eluted from the DEAE-Sephadex column. This main component, i.e. the putative decaribonucleoside nonaphosphate (**23**), was completely digested by treatment with (a) 0.1 M-aqueous sodium hydroxide (16 hr, 37°), (b) ribonuclease A (24 hr, 37°), followed by

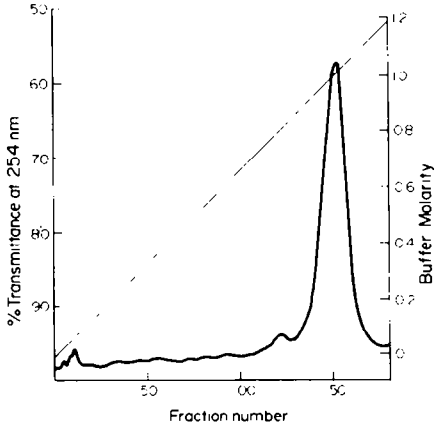


Fig. 1. DEAE-Sephadex chromatography of the products obtained after the complete unblocking [by the procedure indicated in Scheme 4] of the fully-protected UpCpGpUpCpCpApCpCpA. A linear gradient of triethylammonium bicarbonate buffer (pH 7.5) was used.

† An independent study[20] has indicated that unblocking of the internucleotide linkages by the oximate ion procedure leads to the occurrence of only a very small amount (< 0.5%) of terminal phosphoryl migration, due to the partial removal of the 5'-DBMB protecting group.

calf spleen phosphodiesterase (24 hr, 37°) and (c) *crotalus adamanteus* snake venom phosphodiesterase (16 hr, 37°). Hplc analysis of digests (a) and (b) revealed (Experimental) very satisfactory nucleotide-nucleoside ratios. The less satisfactory ratios obtained in the hplc analysis of digest (c) suggested that the enzyme preparation had some phosphomonoesterase activity.

DISCUSSION

The use of the procedures described above led to the synthesis of a decaribonucleoside nonaphosphate (**23**) which appears from its DEAE-Sephadex A25 elution pattern (Fig. 1) and from digestion experiments to be of high quality. Yields were satisfactory in all of the phosphorylation steps except the last [i.e. the condensation between **19a** (Ar = 2-ClC_6H_4) and **21**] and we believe that the yield in this step can be improved (see below). The methods used for the preparation of the nucleoside building blocks are satisfactory but, as indicated above, it may sometimes be advantageous to use Markiewicz's procedure[15] for the preparation of 2'-O-methoxytetrahydropyranyl ribonucleoside derivatives (**1**). The experience which we have gained in the synthesis of the decamer (**23**) prompts us to comment on (a) the protecting groups and (b) the phosphorylation methods which we have used and to suggest where possible improvements might be made.

(a) *Protecting groups.* We have confirmed that the methoxytetrahydropyranyl[17] and methoxymethylene[26] protecting groups, which we introduced in oligoribonucleotide synthesis a number of years ago, are well suited to their specific purposes and we can recommend without reservation that they should be used in the future. In this paper, we have described for the first time the use of the *o*-dibromomethylbenzoyl (DBMB) group[13] for the protection of the 5'-OH functions in oligoribonucleotide synthesis. *o*-Dibromomethylbenzoyl chloride[13] is an easily accessible reagent which reacts readily at primary and secondary hydroxy functions to give DBMB esters; furthermore, it appears to react regioselectively with 2'-O-methoxytetrahydropyranyl ribonucleoside derivatives (**1**) and 2'-deoxyribonucleosides[13] at their 5'-OH functions. The very mild conditions required[13] for the removal of the DBMB group (see above and Experimental) are such that no detectable concomitant N-deacylation or phosphotriester hydrolysis occurs. We believe that the DBMB protecting group is likely to find widespread use in oligonucleotide synthesis in the future.

We have confirmed our observation[2] that phosphotriester intermediates become more polar with increasing molecular weight. This is disadvantageous inasmuch as the intermediates then become less soluble in organic solvents (e.g. chloroform) and hence more difficult to extract from reaction mixtures and purify by short column chromatography. Our initial approach[27] to this problem was to attach lipophilic alkyl resdues to the aryl groups protecting the internucleotide linkages. An alternative approach would be to protect the base residues with more lipophilic acyl groups. In future, we intend to protect adenine and cytosine residues with *p*-t-butylbenzoyl[28] and guanine residues with *p*-t-butylphenylacetyl groups.[28] We anticipate that the use of this combination of protecting groups will lead to a further benefit in that the duration of the ammonia treatment in the final unblocking process [Scheme 4, step (ii)] will be considerably shortened. At present, the slowness of step (ii) is due to the rate of debenzoylation (in aqueous ammonia solution) of the protected guanine residues. The rate of ammonolysis of *p*-t-butylphenylacetyl groups from guanine residues has been found[28] to be much faster. Finally, as stated above, we believe that now that the oximate ion-promoted deblocking procedure[12] is available, *o*-chlorophenyl groups are very suitable indeed for the protection of the internucleotide linkages in the phosphotriester approach.

(b) *Phosphorylation methods.* The procedure[14] which we have used for the first phosphorylation step (Scheme 1), i.e. treatment with an excess of *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (**13a**; Ar = 2-ClC_6H_4) in acetonitrile–pyridine solution, followed by hydrolysis of the putative intermediate phosphoro-1,2,4-triazolide with water–triethylamine is both rapid and convenient. Phosphorylation of protected nucleosides (e.g. **9**) and oligonucleotides (e.g. **16** and **18**) is generally complete within 30 min and the triethylammonium salts of the corresponding phosphodiesters (e.g. **10**, **17** and **19**, respectively) are usually obtained in high yields. The latter intermediates may be isolated as pure solids, uncontaminated with *o*-chlorophenyl phosphate. Preliminary experiments suggest that side reactions (e.g. phosphorylation of base residues) do not occur to a significant extent.

The procedure which we have used for the second phosphorylation step (e.g. the condensation between **10a** and **15**, Scheme 2), involving MSNT (**14**)[12,25] as the activating agent, also appears to be very satisfactory. If the phosphodiester component (e.g. **10a**) and the component with the free 5'-OH function (e.g. **15**) are both dissolved in anhydrous pyridine before the MSNT (**14**) is added, the condensation reaction is often complete within 15 min. Furthermore, under these conditions, concomitant 5'-O-mesitylenesulphonation apparently does not occur unless the component with the free 5'-OH function is present in excess. As reported above, the yields of condensation products obtained were very satisfactory except in the preparation of the fully-protected decanucleoside nonaphosphate (**22**). We believe[29] that the less satisfactory yield (*ca* 55%) of the latter product (**22**) was due to side-reactions brought about by (a) the presence of a large excess [*ca* twofold with respect to the heptamer component **21**] of the trinucleotide (**19a**), (b) the presence of a very large excess [*ca* tenfold with respect to the heptamer component **21**] of MSNT (**14**) and (c) the relatively long reaction time (1 hr). In the future, we intend to use only a very slight excess of the phosphodiester component, a much smaller excess of MSNT (**14**) and, whenever feasible, shorter reaction times. We hope that, if these modifications are made, the side-rections encountered above will be virtually suppressed and the yields of fully-protected oligonucleotides will be optimized.

EXPERIMENTAL

UV absorption spectra were measured with a Perkin Elmer 402 spectrophotometer. ^1H NMR spectra were measured at

60 MHz with a Perkin–Elmer R12B spectrometer and at 90 MHz with a Bruker HFX 90 spectrometer; tetramethylsilane was used as an internal standard. IR spectra were measured with a Perkin–Elmer 257 spectrometer.

Merck silica gel $60F_{254}$ pre-coated plates, which were usually developed in solvent system A [$CHCl_3$–MeOH (9:1 v/v)] and DC-Alufolien cellulose F_{254} sheets were used for tlc. Paper electrophoresis was carried out in a Savant tank on Whatman No. 1 paper in 0.05 M-sodium phosphate buffer (pH 7.0). Hplc was carried out on a Partisil PXS 10/25 SAX column which was eluted isocratically with 0.05 M-potassium phosphate buffer (pH 3.35). Merck Kieselgel H and Reeve Angel silica gel CT were used for short column chromatography.[30] Anion-exchange chromatography on DEAE-Sephadex A-25 was carried out with linear gradients of triethylammonium hydrogen carbonate buffer (pH 7.5).

Dioxan, acetonitrile and pyridine were dried by heating, under reflux, with CaH_2 for 3–5 hr; these solvents were then distilled at atmospheric pressure and stored over molecular sieves (no. 4A). Dimethylformamide was stirred with CaH_2 at 20° for 16 hr, then distilled under reduced pressure (at *ca* 14 mmHg) and stored over molecular sieves (no. 4A).

Enzymes were purchased from the Sigma Chemical Co.

5'-O-(*o*-*Dibromomethylbenzoyl*)-2-O-*methoxytetrahydropyranyluridine* (**2b**). A soln of *o*-dibromomethylbenzoyl chloride[13] (4.58 g, 14.7 mmol) in acetonitrile (10 ml) was added, dropwise over a period of 1 hr, to a cooled (ice-bath), stirred soln of 2'-O-methoxytetrahydropyranyluridine[17] (5.0 g, 14 mmol) in anhyd pyridine (50 ml). After a further 15 min, the products were allowed to warm up to room temp over a period of 1 hr and then poured into sat $NaHCO_3$ aq (300 ml). The resultant mixture was extracted with $CHCl_3$ (3 × 100 ml). The combined $CHCl_3$ extracts were washed with water (200 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was further dried by evaporation from EtOH–toluene (1:1 v/v) soln and then purified by short column chromatography on silica gel. Elution of the column with $CHCl_3$–EtOH (93:7 v/v), evaporation of the appropriate fractions and crystallization of the residue from CH_2Cl_2–ether gave 5'-O-(*o*-*dibromomethylbenzoyl*)-2'-O-*methoxytetrahydropyranyluridine* (Found: C, 43.5; H, 4.1; N, 4.4. $C_{23}H_{26}Br_2N_2O_9$ requires: C, 43.6; H, 4.1; N, 4.4%) as colourless crystals, m.p. 101–102°; yield, 6.26 g (71%); ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.83 (4 H, m), 3.18 (3 H, s), 3.35–3.9 (4 H, m), 4.29 (2 H, m), 4.50 (3 H, m), 5.61 (1 H, d, J = 8.2 Hz), 5.96 (1 H, d, J = 5.0 Hz), 7.3–8.2 (6 H, m); R_f 0.50 (system A).

2',3'-O-*Methoxymethylene*-6-N-*benzoyladenosine* (**3**). 6-N-Benzoyladenosine (7.427 g, 20 mmol), trimethyl orthoformate (10.6 g, 0.1 mol) and toluene-*p*-sulphonic acid monohydrate (4.185 g, 22 mmol) were stirred together in anhyd acetonitrile (80 ml) soln at 20°. After 2.5 hr, the products were neutralized with methanolic ammonia (half-saturated at 0°, 20 ml) and then concentrated under reduced pressure. The residue obtained was suspended in $CHCl_3$ (200 ml) and the suspension filtered. The filtrate was washed with water (3 × 80 ml), dried ($MgSO_4$) and concentrated to give a glass. Crystallization of this material from EtOAc gave 2',3'-O-*methoxymethylene*-6-N-*benzoyladenosine* (Found: C, 54.9; H, 4.7; N, 16.3. $C_{18}H_{19}N_5O_6$ requires: C, 55.2; H, 4.6; N, 16.9%) as a colourless solid, m.p. 115–116°; yield, 6.147 g (74%); ^{1}H NMR ($CDCl_3$, 90 MHz): δ 3.34 and 3.47 (1 H, s and s), 3.94 (2 H, m), 4.55 (1 H, m), 5.1–5.5 (2 H, m), 5.97 and 6.05 (1 H, s and s), 6.03 and 6.29 (1 H, d and d, J = 3.8 and 3.2 Hz, respectively), 7.35–7.65 (3 H, m), 8.03 (2 H, m), 8.12 (1 H, s), 8.73 and 8.74 (1 H, s and s), 9.32 (1 H, br. s); R_f 0.54 (system A).

5'-O-(*o*-*Dibromomethylbenzoyl*)-3'-O-*formyl*-6-N-*benzoyladenosine* (**8**). A soln of *o*-dibromomethylbenzoyl chloride[13] (0.34 g, 1.05 mmol) in acetonitrile (3 ml) was added dropwise to a cooled (ice-bath), stirred soln of 2',3'-O-methoxymethylene-6-N-benzoyladenosine (0.427 g, 1.0 mmol) in pyridine (5 ml). After 15 min, water (0.8 ml) was added and, after a further period of 10 min, the products were concentrated under reduced pressure to small volume and dissolved in $CHCl_3$ (50 ml). The latter soln was washed with half-sat $NaHCO_3$ aq, dried ($MgSO_4$) and then concentrated under reduced pressure. The residue obtained was dissolved in 95% formic acid (6 ml). After the resulting soln had stood at 20° for 15 min, it was evaporated under reduced pressure and the residue crystallized from EtOH to give 5'-O-(*o*-dibromomethylbenzoyl)-3'-O-formyl-6-N-benzoyladenosine as colourless crystals; yield, 0.403 g (68%); m.p. 125–128°; ^{1}H NMR [$(CD_3)_2SO$–D_2O (containing HCl), 90 MHz]: δ 4.67 (3 H, m), 5.28 (1 H, t, J ~ 5.5 Hz), 5.63 (1 H, m), 6.16 (1 H, d, J = 6.1 Hz), 7.4–8.2 (10 H, m), 8.43 (1 H, s), 8.66 (1 H, s), 8.98 (1 H, s); R_f 0.50 (system A).

5'-O-(*o*-*Dibromomethylbenzoyl*)-2'-O-*methoxytetrahydropyranyl*-6-N-*benzoyladenosine* (**2a**). 5,6-Dihydro-4-methoxy-2H-pyran[17] (7.6 g, 67 mmol) was added to a stirred soln of 5'-O-(*o*-dibromomethylbenzoyl)-3'-O-formyl-6-N-benzoyladenosine (4.693 g, 6.95 mmol) and toluene-*p*-sulphonic acid monohydrate (0.38 g, 2.0 mmol) in anhyd dioxan (30 ml) at 20°. After 2 hr, methanolic ammonia (half-saturated at 0°, 10 ml) was added and the products were evaporated under reduced pressure. The colourless solid obtained was suspended in $CHCl_3$ and the suspension was filtered. The filtrate was concentrated under reduced pressure and the resulting glass crystallized from EtOH to give 5'-O-(*o*-*dibromomethylbenzoyl*)-2'-O-*methoxytetrahydropyranyl*-6-N-*benzoyladenosine* (Found: C, 48.9; H, 4.1; N, 9.0. $C_{31}H_{31}Br_2N_5O_8$ requires: C, 48.9; H, 4.1; N, 9.2%) as colourless crystals, m.p. 166°; yield 4.079 g (77%); ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.5–2.0 (4 H, m), 2.94 (3 H, s), 3.1–3.8 (4 H, m), 4.4–4.8 (3 H, m), 5.43 (1 H, m), 6.11 (1 H, d, J = 5.3 Hz), 7.25–8.2 (10 H, m), 8.68 (1 H, s), 9.08 (1 H, s); R_f 0.60 (system A).

2'-O-*Methoxytetrahydropyranyl*-4-N-*benzoylcytidine* (**1a**). Cytidine (11.1 g, 46 mmol), trimethyl orthoformate (54.7 ml), mesitylene-2-sulphonic acid dihydrate (28.36 g, 0.12 mol) and anhyd acetonitrile (200 ml) were stirred together at room temp. After 3 hr, methanolic ammonia (half-saturated at 0°, 40 ml) was added to the clear solution. The products were then concentrated under reduced pressure and the residue extracted with acetone (300 ml). The acetone extract was evaporated and the material obtained was dissolved in anhyd pyridine (100 ml). Benzoyl chloride (28 ml, 0.24 mol) was added dropwise to the cooled (ice water bath), stirred soln obtained and the reactants were then allowed to warm up to room temp. After 1 hr, water (10 ml) was added and, after a further period of 1 hr, the products were concentrated under reduced pressure to small volume, dissolved in $CHCl_3$ and the $CHCl_3$ solution washed with sat $NaHCO_3$ aq. Toluene was added to the dried ($MgSO_4$) $CHCl_3$ layer and the resulting soln was concentrated under reduced pressure. The residue obtained was dissolved in 95% formic acid (200 ml) and the soln was allowed to stand at room temp. After 20 min, the products were filtered and the filtrate was evaporated under reduced pressure. Cytstallization of the material so obtained from EtOH (100 ml) and water (50 ml) gave a mixture of 2'- (and 3'-)-O-formyl-4-N-,5'-O-dibenzoylcytidines; R_f [$CHCl_3$–EtOH (19:1 v/v)] 0.36; yield 13.9 g (63% overall for the three steps, starting from cytidine); ^{1}H NMR [$(CD_3)_2SO$–D_2O containing HCl), 90 MHz], includes the following signals: δ 5.34 (t, J ~ 5 Hz, assigned to H-3' of the 3'-formate), 5.51 (dd, J ~ 2.5 and 5 Hz, assigned to H-2' of the 2'-formate), 5.89 (d, J = 4.4 Hz, assigned to H-1' of the 3'-formate), 5.95 (d, J ~ 2.5 Hz, assigned to H-1' of the 2'-formate). While the combined integrals of the signals at (a) δ 5.31 and 5.51 and (b) δ 5.89 and 5.98 each account for one proton, the integrals of the signals at δ 5.34 and 5.89 are *ca* 3 times as great as those of the signals at δ 5.51 and 5.95.

The above mixture of 2' (and 3')-O-formyl-4-N,5'-O-dibenzoylcytidines (10.0 g, 20.9 mmol), toluene-*p*-sulphonic acid monohydrate (1.094 g, 5.75 mmol), 5,6-dihydro-4-methoxy-2H-pyran[17] (18.7 g, 0.16 mol) and anhyd dioxan (144 ml) were stirred together at room temp. After 30 min, an additional quantity of 5,6-dihydro-4-methoxy-2H-pyran[17] (18.7 g, 0.16 mol) was added and, after a further period of 2 hr, the reaction was quenched by the addition of methanolic

ammonia (half-saturated at 0°, 5 ml). The products were then concentrated under reduced pressure and the residue was extracted with $CHCl_3$. The latter extract was evaporated and the material obtained was dissolved in EtOH (135 ml) and treated with a soln of NaOH (7.78 g) in water (74.8 ml) at room temp. After 20 min, the reaction was quenched by the addition of Dowex 50 × 8 cation-exchange resin (pyridinium form, 400 ml). After the resin had been removed by filtration, the filtrate was concentrated under reduced pressure and the residue was extracted with acetone (300 ml). The acetone-soluble material was fractionated by short column chromatography on silica gel (250 g). Elution of the column with $CHCl_3$–EtOH (15:1 v/v) and concentration of the appropriate fractions gave components (a) and (b) with R_f's 0.20 and 0.14 [$CHCl_3$–EtOH (19:1 v/v)], respectively. Component (a), which crystallized from EtOAc and had m.p. 192-193°, was assigned the structure 3'-O-methoxytetrahydropyranyl-4-N-benzoylcytidine; ^{1}H NMR [$(CD_3)_2SO$–D_2O, 90 MHz] includes the following signals: δ 3.20 (3 H, s), 5.80 (1 H, d, J = 2.1 Hz); yield 0.93 g (9.5%). Component (b), which crystallized from EtOAc was assigned the structure 2'-O-*methoxytetrahydropyranyl*-4-N-*benzoylcytidine* (Found: C, 57.15; H, 6.0; N, 8.9. $C_{22}H_{27}N_3O_8$ requires: C, 57.3; H, 5.9; N, 9.1%), m.p. 195–198°; yield 4.6 g (48%); ^{1}H NMR [$(CD_3)_2SO$–D_2O, 90 MHz]; δ 1.77 (4 H, m), 2.95 (3 H, s), 3.65 (4 H, m), 4.02 (2 H, m), 4.43 (1 H, m), 6.16 (1 H, d, J = 6.7 Hz), 7.39 (1 H, d, J = 7.6 Hz), 7.45–7.7 (3 H, m), 8.02 (2 H, m), 8.46 (1 H, d, J = 7.6 Hz); R_f 0.33 (system A).

2'-O-*Methoxytetrahydropyranyl*-2-N-*benzoylguanosine* (**1c**). 3',5'-Di-O-acetyl-2-N-benzoylguanosine[16] (3.30 g, 6.74 mmol), toluene-*p*-sulphonic acid monohydrate (0.407 g, 2.15 mmol), 5,6-dihydro-4-methoxy-2H-pyran[17] (5.5 g, 48 mmol) and anhydrous dioxan (35 ml) were stirred together at room temp. After 21 hr, an additional quantity (2.5 g, 22 mmol) of 5,6-dihydro-4-methoxy-2H-pyran was added and, after a further period of 1 hr, methanolic ammonia (half-saturated at 0°, 3 ml) was added and the products were concentrated under reduced pressure. The residue obtained was extracted with $CHCl_3$ and the filtered extract was concentrated under reduced pressure. The material obtained was dissolved in EtOH (34 ml) and pyridine (23 ml) and NaOH aq (2 M, 15 ml, 30 mmol) and EtOH (15 ml) were added to the stirred soln at room temp. After 5 min, the products were neutralized by the addition of Dowex 50 × 8 cation-exchange resin (pyridinium form, 100 ml). After the resin had been removed by filtration, the combined filtrate and residue washings (MeOH) were concentrated under reduced pressure. The material obtained was dissolved in $CHCl_3$–EtOH (10:1 v/v) and applied to a column of silica gel (100 g). Elution of the column with $CHCl_3$–EtOH (5:1 v/v), concentration of the appropriate fractions and crystallization of the residue from CH_2Cl_2–EtOAc gave 2'-O-methoxytetrahydropyranyl-2-N-benzoylguanosine (Found: C, 53.7; H, 5.6; N, 13.3. $C_{23}H_{27}N_5O_8$ 0.8 H_2O requires: C, 53.5; H, 5.6; N, 13.6%), m.p. 203° (lit.[31] 203°), yield, 2.098 g (62%); NMR [$(CD_3)_2SO$–D_2O, 90 MHz] includes the following signals: δ 1.45–1.94 (4 H, m), 2.63 (3 H, s), 4.03 (1 H, m), 4.14 (1 H, m), 4.79 (1 H, quart, $J \sim 4.5$ and 8 Hz), 6.10 (1 H, d, J = 7.9 Hz), 7.4–7.8 (3 H, m), 8.07 (2 H, m), 8.35 (1 H, s); R_f 0.17 (system A).

Conversion of 5'-O-(o-*Dibromomethylbenzoyl*)-2'-O-*methoxytetrahydropyranyluridine* (**2b**) *into the triethylammonium salt of its* 3'-o-*chlorophenyl*) *phosphate* (**10a**; B = uracil-1-yl). A soln of 1,2,4-triazole (0.691 g, 10.0 mmol) and triethylamine (0.926 g, 9.15 mmol) in acetonitrile (10 ml) was added dropwise to a stirred soln of *o*-chlorophenyl phosphorodichloridate (1.12 g, 4.56 mmol) in acetonitrile (10 ml) at room temp. After 20 min, 5'-O-(*o*-dibromomethylbenzoyl)-2'-O-methoxytetrahydropyranyluridine (1.90 g, 3.0 mmol) and, immediately afterwards, pyridine (20 ml) were added. After 45 min, a solution of toluene-*p*-sulphonic acid monohydrate (1.736 g, 9.12 mmol), triethylamine (1.82 g, 18 mmol) and water (0.36 g, 20 mmol) in pyridine (2 ml) was added to the stirred products. After a further period of 15 min, the products were treated with sat $NaHCO_3$ aq (300 ml) and the mixture was extracted with $CHCl_3$ (2 × 50 ml). The combined $CHCl_3$ extracts were evaporated under reduced pressure, the residue redissolved in $CHCl_3$ (30 ml) and toluene (30 ml) and the soln concentrated under reduced pressure. The resultant material was triturated with petroleum ether (b.p. 30–40°, 75 ml). The supernatant was then removed by decantation and the remaining solid was dried in a desiccator; yield 2.59 g (93%); ^{31}P NMR ($CDCl_3$, 36.4 MHz): δ – 6.14.

Conversion of 5'-O-(o-*dibromomethylbenzoyl*)-2'-O-*methoxytetrahydropyranyl*-6-N-*benzoyladenosine* (**2a**) *into the triethylammonium salt of its* 3'-(o-*chlorophenyl*) *phosphate* (**10a**; B = 6-N-benzoyladenin-9-yl). A soln of 5'-O-(*o*-dibromomethylbenzoyl)-2'-O-methoxytetrahydropyranyl-6-N-benzoyladenosine (1.522 g, 2.0 mmol) in pyridine (14 ml) was added to a soln of *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) in acetonitrile (21 ml), prepared as above from 1,2,4-triazole (0.925 g, 13.4 mmol), *o*-chlorophenyl phosphorodichloridate (1.464 g, 5.97 mmol) and triethylamine (1.234 g, 12.2 mmol) at room temp. After 30 min, a soln of triethylamine (1.21 g, 12.0 mmol) and water (*ca* 1 ml) was added to the stirred mixture. After a further period of 10 min, the products were poured into sat $NaHCO_3$ aq (300 ml) and the mixture was extracted with $CHCl_3$ (3 × 100 ml). The combined $CHCl_3$ extracts were washed with sat $NaHCO_3$ aq (3 × 50 ml) and water (50 ml). The dried ($MgSO_4$) organic layer was then concentrated under reduced pressure. The resultant glass was redissolved in $CHCl_3$ (10 ml) and the solution added dropwise to petroleum ether (b.p. 30–40°, 200 ml). The colourless solid ppt obtained was collected by centrifugation and dried in a desiccator; yield 1.959 g (91.5%); ^{31}P NMR ($CDCl_3$, 36.4 MHz): δ – 6.26.

3-*Nitro*-1,2,4-*triazole*. Conc HNO_3 (*d* 1.42, 85 ml) was added dropwise over a period of 3 hr to a mechanically stirred (ice-salt bath) soln of 3-amino-1,2,4-triazole (25.0 g, 0.297 mol) and $NaNO_2$ (100 g, 1.45 mol) in water (150 ml). During this period, the temp of the reaction medium was kept between – 2° and 10°. The mixture was then allowed to warm up (with continuous stirring) to room temp and stirring was continued overnight.

The products were then filtered and the colourless ppt of 3-nitro-1,2,4-triazole[‡] was recrystallized from MeOH; yield, 23.9 g (70%); m.p. 208–210° (lit.[32] 215–216° dec.); λ_{max} (H_2O) 282 nm; ^{1}H NMR [$(CD_3)_2SO$–D_2O, 60 MHz]: δ 8.82(s); R_f 0.2 [$CHCl_3$–EtOH (9:1 v/v)].

Preparation of arenesulphonyl derivatives of 3-*nitro*-1,2,4-*triazole* [carried out by Dr. L. Yau (formerly Y. T. Yan Kui)[25]]

A soln of the appropriate arenesulphonyl chloride (1.0 mol equiv) in dioxan (0.75 ml per mmol) was added to a stirred, cooled (ice-water bath) mixture of 3-nitro-1,2,4-triazole (1.0 mol equiv), triethylamine (1.0 mol equiv) and dioxan (1 ml per mmol). After 1 hr, the products were concentrated under reduced pressure and partitioned between $CHCl_3$ and water. The dried ($MgSO_4$) $CHCl_3$ layer was evaporated and the residue crystallized from an appropriate solvent.

(a) 1-(*Toluene*-p-*sulphonyl*)-3-*nitro*-1,2,4-*triazole* (Found: C, 40.3; H, 3.0; N, 20.9. $C_9H_8N_4O_4S$ requires: C, 40.1; H, 2.95; N, 21.0%) crystallized from benzene (0.5 ml per mmol); m.p. 125–127°; yield, 90%; ^{1}H NMR. ($CDCl_3$, 60 MHz): δ 2.47 (3 H, s), 7.40 (2 H, d, $J \sim 8$ Hz), 7.97 (2 H, d, $J \sim 8$ Hz), 8.74 (1 H, s).

(b) 1-(*Mesitylene*-2-*sulphonyl*)-3-*nitro*-1,2,4-*triazole* (MSNT, **14**) [Found: C, 44.55; H, 4.1; N, 19.2 $C_{11}H_{12}N_4O_4S$ requires: C, 44.6; H, 4.1; N, 18.9%] crystallized from benzene (1.2 ml per mmol); m.p. 130–132°, yield, 92%; ^{1}H NMR ($CDCl_3$, 60 MHz): δ 2.33 (3 H, s), 2.67 (6 H, s), 7.02 (2 H, s), 8.80 (1 H, s).

‡ Note added in proof: We have since found that the weight of 3-nitro-1,2,4-triazole in the precipitate varies but that a high yield may always be isolated if the mother liquors are extracted continuously with ethyl acetate.

(c) 1-(2,4,6-*Tri-isopropylbenzenesulphonyl*)-3-*nitro*-1,2,4-*triazole* (Found: C, 53.1; H, 6.5; N, 14.6. $C_{17}H_{24}N_4O_4S$ requires: C, 53.7; H, 6.4; N, 14.7%) crystallized from benzene–petroleum ether (b.p. 60–80°) [6:1 v/v]; m.p. 132–133.5°; yield, 85%; ^{1}H NMR ($CDCl_3$, 60 MHz): δ 1.26 (18 H, d, $J \sim 7$ Hz), 2.93 (1 H, m), 4.10 (2 H, sept., $J \sim 7$ Hz), 7.22 (2 H, s), 8.77 (1 H, s).

Preparation of partially-protected dinucleoside phosphates

(a) The triethylammonium salt of **10a** (B = uracil-1-yl; 0.803 g, 0.92 mmol) and **1a** (0.415 g, 0.90 mmol) were dissolved in anhyd pyridine (5 ml) and MSNT (**14**; 0.664 g, 2.25 mmol was added to the stirred soln. After 20 min, water (1 ml) was added and after a further period of 20 min, the products were poured into sat $NaHCO_3$ aq (25 ml). The resultant mixture was extracted with $CHCl_3$ (4 × 25 ml). The dried ($MgSO_4$), combined $CHCl_3$ extracts were concentrated under reduced pressure and the residue was fractionated by short column chromatography on silica gel (140 g). Elution of the column with $CHCl_3$–EtOH [96:4–92:8 v/v] and concentration of the appropriate fractions gave the desired product (**16a**; Ar = 2-ClC_6H_4). This material was obtained as a colourless solid by the dropwise addition of its sol in $CHCl_3$ (10 ml) to stirred petroleum ether (b.p. 30–40°, 250 ml); yield, 0.902 g (79%); R_f 0.43 (system A).

(b) In the same way, the corresponding partially-protected ApC derivative (**16b**; Ar = 2-ClC_6H_4) was prepared from the triethylammonium salt of **10a** (B = 6-N-benzoyladenin-9-yl; 1.072 g, 1.00 mmol), **1a**; (0.460 g, 1.00 mmol) and MSNT (0.893 g, 3.03 mmol) in pyridine (5 ml) soln. The condensation reaction was allowed to proceed for 20 min at room temp and was then worked-up as above. The products were fractionated by short column chromatography on silica gel (40 g). Elution of the column with $CHCl_3$–EtOH [95:5 v/v] and concentration of the appropriate fractions gave the desired **16b**; (Ar = 2-ClC_6H_4). The material was isolated as a colourless solid by the precipitation procedure described in (a) above; yield, 1.077 g (76%); R_f 0.56 (system A).

Conversion of partially-protected dinucleoside phosphates and trinucleoside diphosphates with free 3'-hydroxy functions into triethylammonium salts of the corresponding 3'-(o-chlorophenyl) phosphates.

The following procedure for the preparation of the partially-protected ApCp derivative **17b** (Ar = 2-ClC_6H_4) is typical. A stirred soln of the above partially-protected ApC derivative **16b** (Ar = 2-ClC_6H_4; 0.577 g, 0.41 mmol) in pyridine (3 ml) was treated, at room temp, with the products of the reaction between *o*-chlorophenyl phosphorodichloridate (0.266 g, 1.08 mmol), triethylamine (0.3 ml, 2.12 mmol) and 1,2,4-triazole (0.163 g, 2.35 mmol) in acetonitrile (2.5 ml).† After 30 min, a soln of triethylamine (0.3 ml, 2.12 mmol) and water (0.1 ml) was added to the mixture and, after a further period of 30 min, the products were poured into sat $NaHCO_3$ aq (50 ml). The mixture obtained was extracted with $CHCl_3$ (3 × 15 ml) and the dried ($MgSO_4$), combined $CHCl_3$ extracts were evaporated under reduced pressure. The residue obtained was redissolved in $CHCl_3$ (2 ml) and the soln was added dropwise with stirring to petroleum ether (b.p. 30–40°, 40 ml). The colourless solid ppt obtained was collected by centrifugation and dried in a desiccator; yield of desired **17b**; (Ar = 2-ClC_6H_4), 0.63 g (91%).

Preparation of partially-protected trinucleoside diphosphates

(a) MSNT (0.373 g, 1.26 mmol) was added to a stirred soln of the triethylammonium salt of the partially-protected ApCp **17b** (Ar = 2-ClC_6H_4; 0.624 g, 0.37 mmol) and **1a** (0.182 g, 0.39 mmol) in anhyd pyridine (2 ml) at room temp. After 15 min, water (0.5 ml) was added and, after a further period of 30 min, the products were poured into sat $NaHCO_3$ aq (25 ml). The products were worked-up according to the procedure described above in the preparation of partially-protected dinucleoside phosphates and chromatographed on a short column of silica gel (18 g). The desired **18c** (Ar = 2-ClC_6H_4) was eluted from the column with $CHCl_3$–EtOH (95:5 v/v) and isolated by precipitation in the usual way (see above); yield 0.639 g (77%); R_f 0.57 (system A).

(b) MSNT (0.445 g, 1.50 mmol) was added to a stirred soln of the triethylammonium salt of the partially-protected UpCp (**17a**; Ar = 2-ClC_6H_4; 0.78 g, 0.50 mmol) and **1a** (0.231 g, 0.50 mmol) in anhyd pyridine (2.5 ml) at room temp. After 16 min, sat $NaHCO_3$ aq (0.5 ml) was added and, after a further period of 15 min, the products were poured into sat $NaHCO_3$ aq (60 ml). The products were worked-up as above and fractionated by short column chromatography on silica gel (40 g). The desired **18b** (Ar = 2-ClC_6H_4) was eluted from the column with $CHCl_3$–EtOH (94:6–93:7 v/v) and isolated by precipitation; yield, 0.78 g (82%); R_f 0.46 (system A).

(c) MSNT (0.222 g, 0.75 mmol) was added to a stirred soln of the triethylammonium salt of the partially-protected UpCp (**17a**; Ar = 2-ClC_6H_4; 0.39 g, 0.25 mmol) and **1b** (0.125 g, 0.25 mmol) in anhyd pyridine (3 ml) at room temp. After 12 min, sat $NaHCO_3$ aq (0.5 ml) was added and, after a further period of 15 min, the products were poured into sat $NaHCO_3$ aq (200 ml). The products were worked-up as above and fractionated by short column chromatography on silica gel (40 g). The desired **18a** (Ar = 2-ClC_6H_4) was eluted from the column with $CHCl_3$–EtOH (93:7–92:8 v/v) and isolated by precipitation; yield 0.378 g (78%); R_f 0.29 (system A).

Preparation of fully-protected ApCpCpA derivative (**2a**)

The partially-protected ApCp (**18c**; Ar = 2-ClC_6H_4), the preparation of which is described under heading (a) above, was converted by the procedure described above into the triethylammonium salt of **19c** (Ar = 2-ClC_6H_4) in 98% isolated yield. A stirred soln of the latter trinucleotide derivative (**19c**; Ar = 2-ClC_6H_4; 0.559 g, 0.24 mmol) and 2',3'-O-methoxymethylene-6-N-benzoyladenosine (0.146 g, 0.35 mmol) in anhyd pyridine (1.4 ml) was treated with MSNT (**3**; 0.416 g, 1.4 mmol) at room temp. After 30 min, water (0.5 ml) was added and, after a further period of 10 min, the products were poured into sat $NaHCO_3$ aq (20 ml). The resultant mixture was extracted with $CHCl_3$ (4 × 15 ml). The dried ($MgSO_4$) combined $CHCl_3$ extracts were concentrated under reduced pressure, redissolved in anhyd pyridine (2 ml) and the soln re-evaporated. After this process had been repeated, the residue was dissolved in anhyd pyridine (1.5 ml) and the soln was treated with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide), prepared from *o*-chlorophenyl phosphorodichloridate (0.074 g, 0.3 mmol), 1,2,4-triazole (0.042 g, 0.6 mmol) and triethylamine (0.08 ml, 0.6 mmole) in acetonitrile (1.5 ml). After 1 hr, the products were poured into sat $NaHCO_3$ aq (50 ml) and the resultant mixture extracted with $CHCl_3$ (4 × 15 ml). The combined $CHCl_3$ extracts were evaporated with toluene and the resulting solid was fractionated by short column chromatography on silica gel (50 g). The desired **20a** was eluted from the column with $CHCl_3$–EtOH (95:5 v/v) and isolated by precipitation; yield, 0.474 g (75%); R_f 0.66 (system A).

Removal of 5'-O-(o-dibromomethylbenzoyl) protecting group from fully-protected ApCpCpA derivative (**20a**)

To a stirred soln of fully-protected ApCpCpA derivative (**20a**; Ar = 0.187 g, 0.0072 mmol) in acetone–water (98.2 v/v; 1.5 ml) at room temp were added sols of 2,4,6-collidine (1 M, 0.5 ml, 0.5 mmol) and silver perchlorate (1 M, 1.0 ml, 1.0 mmol), both in the same solvent [acetone–water (98:2 v/v)]. After 1 hr, a soln of LiBr (1 M, 1.5 ml, 1.5 mmol) in acetone–water

†In this experiment, 0.41 mmol of substrate was phosphorylated with 1.08 mmol of *o*-chlorophenyl phosphorodi-(1,2,4-triazolide). For experiments carried out on a smaller scale it is probably advisable to use a larger excess of phosphorylating agent.

(9:1 v/v) was added to the resulting suspension and, after a further period of 10 min, the products were filtered through hyflo-supercel. Morpholine (0.12 ml, 1.4 mmol) was then added to the filtrate and, after 5 min, the products were concentrated to small volume under reduced pressure, dissolved in pyridine (10 ml) and the resulting soln poured into sat $NaHCO_3$ aq (20 ml). The mixture obtained was extracted with $CHCl_3$ (3 × 15 ml) and the combined $CHCl_3$ extracts were dried ($MgSO_4$) and evaporated under reduced pressure in the presence of toluene (2 × 20 ml) to give a solid residue. The latter material was purified by short column chromatography on silica gel (20 g). The desired **20b** was eluted from the column with $CHCl_3$–EtOH (95:5 v/v) and isolated by precipitation; yield, 0.156 g (94%) R_f 0.57 (system A).

Preparation of fully-protected UpCpCpApCpCpA derivative

MSNT (0.153 g, 0.52 mmol) was added to a stirred anhyd soln of the partially-protected UpCpCp derivative (**19b**; Ar = 2-ClC_6H_4; 0.273 g, 0.12 mmol), prepared as described above, in 96% yield, from **18b** (Ar = 2-ClC_6H_4) and the partially-protected ApCpCpA derivative (**20b**; 0.235 g, 0.10 mmol) in pyridine (1 ml) at room temp. After 30 min, water (0.5 ml) was added and, after a further period of 15 min, the products were poured into sat $NaHCO_3$ aq (30 ml). The resultant mixture was extracted with $CHCl_3$ (8 × 10 ml) and the combined $CHCl_3$ extracts were washed with sat $NaHCO_3$ aq (20 ml), dried ($MgSO_4$) and concentrated under reduced pressure. After the resulting solid residue had been dried by evaporation of its pyridine soln, it was dissolved in pyridine (1 ml) and the soln was treated, at room temp, with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide), prepared from *o*-chlorophenyl phosphorodichloridate (0.143 g, 0.58 mmol), 1,2,4-triazole (0.081 g, 1.17 mmol) and triethylamine (0.17 ml, 1.2 mmol) in acetonitrile (2 ml). After 30 min, the products were poured into sat $NaHCO_3$ aq (30 ml) and the mixture obtained was extracted with $CHCl_3$ (8 × 10 ml). The dried ($MgSO_4$), combined $CHCl_3$ extracts were evaporated in the presence of toluene under reduced pressure to give a solid which was fractionated by short column chromatography on silica gel (50 g). The desired fully-protected heptamer was eluted from the column with $CHCl_3$–EtOH (95:5 v/v) and isolated by precipitation; yield 0.316 g (71.5%); R_f 0.61 (system A).

Removal of 5'-O-(o-dibromomethylbenzoyl) protecting group from fully-protected UpCpCpApCpCpA derivative

The fully-protected heptamer (0.293 g, 0.066 mmol), silver perchlorate (0.310 g, 1.55 mmol) and 2,4,6-collidine (0.091 g, 0.75 mmol) were stirred together in acetone water (98:2 v/v; 4.5 ml) at room temp. After 2 hr, a soln of LiBr (1M, 1.75 ml, 1.75 mmol) in acetone–water (9:1 v/v) was added and, after a further period of 10 min, the products were filtered through hyflo-supercel. Morpholine (0.12 ml, 1.4 mmol) was then added and, after 5 min, the products were worked-up in the same way as described above in the unblocking of fully-protected ApCpCpA and fractionated by short column chromatography on silica gel (30 g). The desired **21** was eluted from the column with $CHCl_3$–EtOH (9:1 v/v) and isolated by precipitation; yield 0.212 g (78%); R_f 0.47 (system A).

Preparation of fully-protected UpCpGp UpCpCpApCpCpA derivative (**22**)

MSNT (0.149 g, 0.505 mmol) was added to a stirred, anhyd soln of the partially-protected UpCpGp derivative (**19a**; Ar = 2-ClC_6H_4; 0.224 g, 0.101 mmol), prepared as described above, in 96% yield, from **18a** (Ar = 2-ClC_6H_4) and the partially-protected UpCpCpApCpCpA derivative **21** (0.212 g, 0.051 mmol) in pyridine (1 ml). After 1 hr, the products were worked-up as in the above preparation of the fully-protected heptamer but fractionated by short column chromatography on silica gel (40 g) before treatment with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide). The desired **22** was eluted from the column with $CHCl_3$–EtOH (93:7–9:1 v/v) and isolated by precipitation. Tlc [$CHCl_3$–EtOH (9:1 v/v)] revealed that the material obtained (0.221 g) was only *ca* 80% pure. Estimated yield of fully-protected decamer, *ca* 55%.

A stirred soln of the above impure fully-protected decamer (0.177 g) in anhyd pyridine (1 ml) was treated at room temp with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide), prepared from *o*-chlorophenyl phosphorodichloridate (0.501 g, 2.04 mmol), 1,2,4-triazole (0.282 g, 4.08 mmol) and triethylamine (0.6 ml, 4.0 mmol) in acetonitrile (7 ml). After 1 hr, the products were worked up according to the procedure described above in the preparation of the fully-protected heptamer and purified by short column chromatography on silica gel; yield, 0.098 g (55% recovery); R_f 0.52 (system A).

syn-4-Nitrobenzaldoxime [carried out by Dr. L. Yau]: NaOHaq (2.5 M, 22 ml, 55 mmol) was added to a suspension of 4-nitrobenzaldehyde (2.6 g, 17 mmol) and hydroxylamine hydrochloride (6.0 g, 80 mmol) in water (22 ml). The mixture was heated, under reflux, for 20 min and then acidified (to pH 4) by the addition of dil HCl. The cooled products deposited *syn*-4-nitrobenzaldoxime as colourless needles. The product was collected by filtration and recrystallized from water, yield 2.5 g (89%): m.p. 126° (lit.[33] 129, 133°).

Unblocking of fully-protected UpCpGp UpCpCpApCpCpA (**22**)

(a) A soln of the fully-protected decamer (**22**; 0.018 g, 0.003 mmol), *syn*-4-nitrobenzaldoxime (0.046 g, 0.28 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (0.032 g, 0.28 mmol) in dioxan–water (1:1 v/v, 1.0 ml) was stirred at room temp. After 18 hr, the products were concentrated under reduced pressure and the residue was redissolved in aqueous ammonia (*d* 0.88, 5 ml) at room temp. After 6 days, the soln was evaporated under reduced pressure and then acidifed to pH 5 with 0.01 M-HCl. The resulting soln was extracted (i) with $CHCl_3$ (3 × 5 ml) and (ii) with ether (3 × 5 ml); it was then acidified to pH 2 by the addition of a further quantity of 0.01 M-HCl (5 ml) and re-extracted with $CHCl_3$ (3 × 5 ml) and ether (3 × 5 ml). After it had been allowed to stand at room temp for 6 hr, the aqueous soln was neutralized with dilute ammonia and the products were chromatographed on a column (12 cm × 4.5 cm^2) of DEAE-Sephadex A 25. The column was eluted with triethylammonium bicarbonate buffer (pH 7.5, linear gradient from 0.001 - 1.0 M over 1000 ml) and fractions of 6 ml were collected. The appropriate fractions were combined and lyophilized to give the desired decamer (*ca* 100 A_{260} units); λ_{max} 262 nm.

(b) A soln of the fully-protected decamer (**22**; 0.0105 g, 1.8 μmol) was treated with *syn*-4-nitrobenzaldoxime (0.046 g, 0.28 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (0.032 g, 0.28 mmol) in dioxan–water (1:1 v/v, 0.5 ml) at room temp for 20 hr. After evaporation, the products were redissolved in aqueous ammonia (*d* 0.88, 5 ml) and the soln heated at 50° for 24 hr. This led to darkening of the reaction medium. The products were then chromatographed on a column (12 cm × 4.5 cm^2) of DEAE-Sephadex A 25, which was eluted with triethylammonium bicarbonate buffer (pH 7.5, linear gradient from 0.001 1.0 M over 500 ml) and fractions of 6 ml were collected. The appropriate fractions were combined and lyophilized. The residue was dissolved in 0.01 M-HCl (5 ml) and the pH adjusted to 2. After 6 hr at room temp, the products were neutralized with dilute ammonia and rechromatographed as above on DEAE-Sephadex A 25 to give the fully-unblocked decamer (*ca* 50 A_{260} units).

Digestion of unprotected UpCpGp UpCpCpApCpCpA (**23**)

(a) With 0.1 M-*aqueous sodium hydroxide*. 0.1 M-NaOH (0.2 ml) was added to a soln of the decamer (*ca* 5 A_{260} units) in water (0.01 ml) and the resulting soln was maintained at 37° for 16 hr. Hplc analysis of the hydrolysate revealed: (i) adenosine

(R_T 3.5 min), (ii) cytidine 2'(3')-phosphates (R_T 5.2 min), (iii) adenosine 2'(3')-phosphates (R_T's 5.6, 5.9 min), (iv) uridine 2'(3')-phosphates (R_T 7.35) and (v) guanosine 2'(3')-phosphates (R_T's 8.55, 9.55 min). The relative abundances (estimated by adjusting peak integrals in proportion to appropriate extinction coefficients at pH 3.35 and 254 nm) of components (i)–(v) were estimated to be 1.0, 4.9, 1.0, 2.2 and 1.0, respectively.

(b) *With ribonuclease A, followed by calf spleen phosphodiesterase.* 0.1 *M*-Tris hydrochloride buffer (pH 8, 0.1 ml) and a soln of ribonuclease A (20 μg) in 0.1 M-tris hydrochloride buffer (pH 8, 0.02 ml) were added to a soln of the decamer (*ca* 5 A_{260} units) in water (0.01 ml). The resulting soln was maintained at 37° for 24 hr and then concentrated under reduced pressure. The residue was dissolved in 0.1 M-ammonium acetate buffer (pH 7.0, 0.1 ml, 0.002 M with respect to EDTA and containing 0.05% Tween 80) and a soln of calf spleen phosphodiesterase (20 μg) in the same ammonium acetate buffer (0.02 ml) was added. The resulting soln was maintained at 37° for 24 hr. Hplc analysis of the hydrolysate revealed: adenosine (R_T 3.9 min, 1.1 parts), cytidine 3'-phosphate (R_T 5.7 min, 5.1 parts), adenosine 3'-phosphate (R_T 6.5 min, 1.0 part), uridine 3'-phosphate (R_T 7.65 min, 2.1 parts) and guanosine 3'-phosphate (R_T 9.1 min, 0.9 parts).

(c) *With* Crotalus adamanteus *snake venom phosphodiesterase.* 0.1 M-Tris hydrochloride buffer (pH 9, 0.1 ml; 0.01 M with respect to $MgCl_2$) and a soln of *Crotalus adamanteus* snake venom phosphodiesterase (20 μg) in the same buffer (0.02 ml) was added to a soln of the decamer (*ca* 5 A_{260} units) in water (0.01 ml). The resulting soln was maintained at 37° for 16 hr. Hplc analysis of the hydrolysate revealed: uridine and other nucleosides (R_T 3.6 min, 1.8 parts based on ε_{254} = 8,900 for uridine), cytidine 5'-phosphate (R_T 4.45, 5.1 parts), adenosine 5'-phosphate (R_T 5.25, 2.1 parts), uridine 5'-phosphate (R_T 6.4, 1.0 part) and guanosine 5'-phosphate (R_T 9.35, 1.0 part).

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